CHAPTER 8

APC and Its Modifiers in Colon Cancer

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Abstract

Colon cancer closely follows the paradigm of a single "gatekeeper gene." Mutations inactivating the *APC* (*adenomatous polyposis coli*) gene are found in ~80% of all human colon tumors and heterozygosity for such mutations produces an autosomal dominant colon cancer predisposition in humans and in murine models. However, this tight association between a single genotype and phenotype belies a complex association of genetic and epigenetic factors that together generate the broad phenotypic spectrum of both familial and sporadic colon cancers. In this Chapter, we give a general overview of the structure, function and outstanding issues concerning the role of Apc in human and experimental colon cancer. The availability of increasingly close models for human colon cancer in genetically tractable animal species enables the discovery and eventual molecular identification of genetic modifiers of the *Apc*-mutant phenotypes, connecting the central role of Apc in colon carcinogenesis to the myriad factors that ultimately determine the course of the disease.

Colorectal Cancer

Colorectal cancer is the second leading cause of cancer morbidity and mortality worldwide.[1] Almost half of the population will develop at least one benign adenomatous colonic polyp during life, with less than 3% of those cases going on to develop colorectal cancer. Because symptoms are rare until very late stages, most cases go undetected. Colon cancer manifests itself as polypoid growths that progress to malignancy; metastases to the lymph nodes, liver and lung are the primary cause of death in patients with advanced disease.

In the study of colon cancer, research is divided between sporadic and familial cases. Although hereditary colon cancer predispositions make up less than 5% of all colon cancer cases worldwide, the extensive pedigree information available in such cases has provided statistical power for isolating both the underlying causes and the genetic, environmental and dietary modifiers of the phenotypes. The relationship of sporadic to familial colon cancer is highlighted by the successful use of therapeutics such as nonsteroidal anti-inflammatory drugs (NSAIDs) to treat both diseases.[2] At present, a combination of chemotherapy, radiation treatment and surgery is used to treat colon cancer. The 5-year survival expectation for colon cancer patients ranges from 93% for early stages to 8% in fully advanced stages.[3]

In this chapter, we will introduce and review the genetics and function of the central gatekeeper gene in colon cancer: *Adenomatous Polyposis Coli* (*APC*/*Apc*).[a]

[a]*APC* and *Apc* are the designations for the human and murine genes, respectively; Apc is used herein for the function of the gene, regardless of species.

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Biology of the Human Intestine

The small intestine is composed of interdigitated villi and crypts of Lieberkühn (for a more in-depth discussion, see Sansom, this volume). The villi serve an absorptive function in the processing of food.[4] The colon does not contain villi, but rather is composed of crypts, invaginated into a flat surface that is folded at various intervals called rugae. During human development, the adult intestine expands in part by a process of crypt fission, where entire crypts divide, producing daughter crypts.[5,6] This process "purifies" crypts in that the early polyclonal crypts[7] become monoclonal. Thus, each adult crypt lineage is limited to one somatic genotype. Crypt purification also occurs by stem cell succession, whereby a clone becomes dominant within the crypt. Analysis of methylation patterns in human crypts shows that stem cell succession continues over the life of an adult, as measured by random methylation changes that gradually become fixed in a crypt.[8] An estimated 4-16 adult stem cells reside as a clonal cohort in a niche near the bottom of each crypt. As cells reach the top of the villus in the small intestine or the collar of the crypt in the colon, they undergo apoptosis and are shed into the intestinal lumen. Cells of crypts thus turn over at a high rate (every 3-5 days[9]) owing to the continual flow of newly produced cells up the crypt/villus axis (see Potten and Morris[10] for a review of a classic body of work).

Intestinal epithelial stem cells can differentiate into a number of different cell types.[11,12] Within colonic crypts lie goblet cells that secrete mucus; at the base of small intestinal crypts lie Paneth cells that provide defense and that help to maintain the gut flora. Enterocytes perform an absorptive function for nutrients crossing the epithelium and comprise up to 80% of the small intestine. Finally, rare enteroendocrine cells, comprising ~1% of the intestine, secrete hormones such as serotonin. Below the epithelial layer lies the *lamina propria*, which comprises the stromal connective and endothelial tissue that lends support and circulation to the epithelial cells. The *muscularis mucosa* lies immediately below the epithelial layer and separates it from the submucosa, which is composed of connective tissue. Below that is the *muscularis externa*, the muscle layer along which peristalsis moves food through the intestinal tract. Finally, the serosal layer marks the outermost edge of the intestine and is attached to the mesentery.

Development of Human Intestinal Tumors

Intestinal tumors have been hypothesized to arise from the stem cells near the bottom of crypts, but other interpretations are possible, as discussed below. Accumulating evidence in various fields of cancer research supports the stem cell origin of tumors.[13] Such research began with the study of hematopoietic stem cells, for which the genetics and quantitative biology had been well-established for several decades.[14] It was noticed that the cells of hematopoietic malignancies exhibited similarities to multipotential hematopoietic precursors, particularly the ability to self-renew.[15] Eventually, it was discovered that only a certain subpopulation of hematopoietic cancer cells are capable of transferring cancer to immunocompromised NOD/SCID mice.[16] Recently, solid tumors have been investigated in a similar manner. For example, human breast cancers passaged serially through NOD/SCID mice show that a small number of cancer cells expressing a certain profile of surface markers are sufficient to initiate new tumors, whereas a large number of cancer cells with different profiles are not sufficient.[17] Such "cancer stem cell" profiles have been shown for other cancer types including myeloma, brain and prostate.[18-20] Indeed recent studies have identified CD133 as a marker enriched in a self-renewing, tumor-initiating subpopulation of cells from human colonic tumors.[21,22] Progress in the development of diagnostic cell markers will help to resolve the issue of whether the genetic event that initiates tumorigenesis necessarily occurs in stem cells proper or whether, alternatively, they can also occur in undifferentiated or dedifferentiated daughter cells.[23]

The issue of tumor progenitor cells has led to a debate about whether intestinal tumors form by a "bottom-up" process originating at the stem cell niche, or by a "top-down" process originating in cells in the inter-cryptal space at the top of the crypt/villus axis. Evidence for the "top-down" hypothesis comes from monocryptal human sporadic adenomas in which dysplasia is confined to the top half of the crypt, with normal-appearing cells more basally located in the crypt.[24] The implication is that the dysplasia must have started at the top and grown down towards, rather than emerging from the stem

cell niche. However, it is possible that the dysplasia originated in the middle of the crypt and expanded upwards. Thus, both the "bottom-up" and "top-down" models could be explained by an upwards expansion of stem cell derivatives[25] from the middle of the crypt, or by the transformation of daughter stem cells to becoming tumor-competent. Clearly, the molecular identification of colon cancer stem cells is needed to determine the location of the cell of origin for particular intestinal tumors.

An early stage of colonic tumorigenesis is the benign adenoma that progresses to adenocarcinoma in situ—tumors that have developed high-grade dysplasia but are confined to the region above the submucosa. Progression to adenocarcinomas with invasion into or beyond the submucosa can be classified using different systems. The Dukes staging system (Dukes A, B, C, D, or E) is a measure of how far the invasive front of the cancer penetrates the intestinal wall.[26] In the AJCC/TNM system, numbers identifying T (tumor), N (metastasis to the nodes) and M (metastasis to distant sites) provide a comprehensive view of tumor progression.[27] For example, a $T_4N_1M_0$ cancer indicates an adenocarcinoma that has invaded through the wall of the intestine and spread to 1-3 regional lymph nodes, but not yet to distant sites. Finally, the histological classification of polyps can be villous, tubulovillous, tubular, hyperplastic, or serrated. The rare villous adenoma class is believed to have the greatest potential for malignancy.[28] Hyperplastic and serrated polyps have traditionally been viewed as benign; however, recent evidence points to a possible hyperplastic-serrated-adenocarcinoma progression sequence that involves somatic hyperactivation of the BRAF oncogene.[29] The combination of these classification systems allows for a standardization of terminology among physicians. However, not all tumors fall into only one class and even tumors in the same nominal class can behave differently between and within patients.

Discovery of *APC* Mutations in Human Colon Cancer

Familial adenomatous polyposis (FAP) was first described as Gardner's syndrome[30] and included extracolonic manifestations such as osteomas and congenital hypertrophy of the retinal pigment epithelium (CHRPE). Over time, it became clear that different classes of FAP existed with different symptoms, of which Gardner's syndrome was only one. For example, "classical" FAP manifests as one hundred or more polyps in the colon, usually developing by twelve years of age, whereas patients with fewer than a hundred polyps are classified as attenuated FAP (AFAP). Many extracolonic symptoms further subdivide FAP.[31]

Linkage studies and the FAP-associated interstitial 5q Herrera deletion narrowed the genetic region underlying FAP to the 5q21 subchromosomal region (Fig.1).[32,33] The *APC* gene was then linked to FAP concurrently by Kinzler et al,[34] Nishisho et al,[35] Joslyn et al[36] and Groden et al.[37] *APC* mutations were subsequently found in ~80% of sporadic colorectal tumors,[38] confirming that Apc acts as a central gatekeeper protein in colorectal tumorigenesis. *APC* mutations and hypermethylation have also been found in various other cancer types, including pancreatic and gastric cancers.[39,40]

Function of Apc

Soon after the discovery of the *APC* gene, the function of the gene product came under intense scrutiny. The crucial understanding of its function came concurrently from Su et al[41] and Rubinfeld et al[42] who identified the relationship between Apc and the regulation of β-catenin. We now know that the central lesions in both hereditary and sporadic colon tumors result in activation of the Wnt signaling pathway (see Kennell and Cadigan, this volume). In nearly all tumors, deactivating *APC* or *GSK3*β mutations or stabilizing *CTNNB1* (encoding β-catenin) mutations are present.[43] More specifically, the canonical tumor suppressor function of Apc is to form a "destruction complex" with Axin/Axin2 and GSK-3β that promotes the ubiquitination and subsequent proteasomal degradation of the oncogene β-catenin in the absence of Wnt signaling. Loss of Apc function results in an accumulation of β-catenin, which translocates to the nucleus and engages the Tcf/Lef transcription factor complex to activate transcription of a large number of target genes including cyclinD1, c-myc and CRD-BP.[44] The tumorigenic consequences

of unregulated β-catenin activity may be related to both the direct stimulation of cellular growth and proliferation and to the disruption of differentiation programs.

In addition to its role in the Wnt signaling pathway, Apc also functions to promote microtubule stability in a number of cellular contexts. The impact of the disruption of this function on tumorigenesis is not well understood (see Caldwell and Kaplan, Morrison and Bahmanyar et al, this volume). However, it is worth noting that two groups have reported that stabilized β-catenin, expressed either from a conditionally activatable allele exposed to Cre or from a transgene, is sufficient to induce intestinal polyposis in mice,[45,46] suggesting that loss of the microtubule-binding functions of Apc is not absolutely required for early tumor formation. Furthermore, as discussed below, mice homozygous for the 1638T *Apc* allele lacking the microtubule- and EB1-binding domains of Apc, but not the β-catenin binding domains, do not develop tumors. Despite these findings, an attractive speculation is that the disruption of microtubule functions contributes to tumor progression rather than to tumor initiation. Investigation of this idea awaits analysis of the progression stages of colonic neoplasia and the construction of mouse lines in which only the C-terminus of *Apc* can be conditionally deleted.

Structure of *APC*

The human *APC* gene spans 58 kb, with a 15-exon coding region of 8529 bp encoding a 2843 amino acid (aa), 310 kD protein. Several exons exist 5′ of exon 1: 0.1, 0.2, 0.3,[47] BS[48] and possibly more. The extent to which these isoforms play a role, if any, in colon cancer is unknown; many appear to be neuron-specific.[49]

The canonical Apc transcript initiates at exon 1 and produces a protein with eight known functional sub-domains (Fig. 2). The majority of truncating mutations with severe phenotypes remove most of the β-catenin-binding "20 amino acid" (20aa) repeats (1256-2031aa).[50] Interestingly, more C-terminal truncations that remove only the Axin-binding SAMP repeats (1568-2053aa),[51] microtubule binding repeats (2220-2597aa),[52] EB1-binding domain (2670-2843aa) and/or PDZ domain (the C-terminal 73aa that mediates anchoring to the cytoskeleton)[53] generally have an attenuated phenotype. N-terminal truncations that apparently affect only the homodimerization domain (6-57aa), owing to bypass through the use of an internal translation restart site, likewise generally give attenuated phenotypes (see Fig. 1).[54] Mutations that truncate within the armadillo repeats (453-767aa)—which bind several proteins including Asef and KAP3, both involved with different aspects of cytoskeletal function[55,56]—or within the β-catenin-binding "15 amino acid" (15aa) repeats (1021-1187aa) tend to be somewhat milder than the 20aa repeat truncations. An interesting molecular correlation in tumors was observed that may explain these findings: germline *APC* mutations in the mutation cluster region (MCR) spanning most of the 20aa repeats generally exhibit acquired loss of the wildtype allele, while *APC* mutations outside of this region generally exhibit acquired truncating mutations in the wildtype allele.[57] Several hypotheses have been put forth: the "just-right"[58] and "loose fit"[59] hypotheses, each of which proposes that an optimal number of 15aa repeats must remain after biallelic *APC* inactivation to produce a severe FAP phenotype. These hypotheses remain to be rigorously tested.

Genotype-Phenotype Correlation in FAP

One difficulty in understanding the genotype-phenotype correlation is the current lack of a comprehensive public database of FAP patients. For research on mouse models, this lack of data makes it difficult to contextualize observations in terms of the human disease. So far, a literature search has found only one large-scale attempt to compile such information, although it presents only the results of the analysis and does not make the raw data available.[60] Compounding this difficulty is that most reports on human cases do not count the multiplicity of tumors, but rather give only an estimate. Further difficulties come from differences in phenotype that may relate to whether the patient has received surgery or chemotherapeutics and to the age of diagnosis. To address this gap temporarily, we have compiled data on 441 cases from 37 reports (see http://mcardle.oncology.

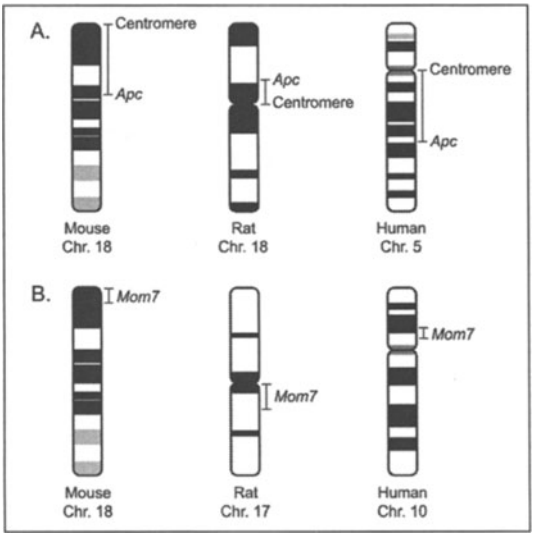

Figure 1. Organization of the mouse, rat and human chromosomes bearing the *APC/Apc* and *Mom7* orthologs. A) The *Apc* locus of the mouse lies on a telocentric chromosome, in contrast to its orthologs in the rat and human, each of which lies on a metacentric chromosome. A metacentric character enables a facile discrimination between whole chromosome loss versus somatic recombination. B) The *APC/Apc* locus of the mouse is linked to the *Mom7* locus on Chr 18, while the orthologs of these two loci are not linked in the rat and human karyotypes.

wisc.edu/dove/Data/FAP.htm). We suggest that a curated public database be generated under the aegis of a society for gastroenterology, for easy access to vetted information of this sort.

These data lead to a conclusion different from that of Crabtree et al,[60] who claim that "mutations between codons 1020 and 1169 hav[e] the mildest disease" and that the most N-terminal truncations (i.e., prior to codon 248) do not lead to an attenuated phenotype. Instead, it seems that N-terminal truncations produce the mildest disease, although mutations between codons 1020

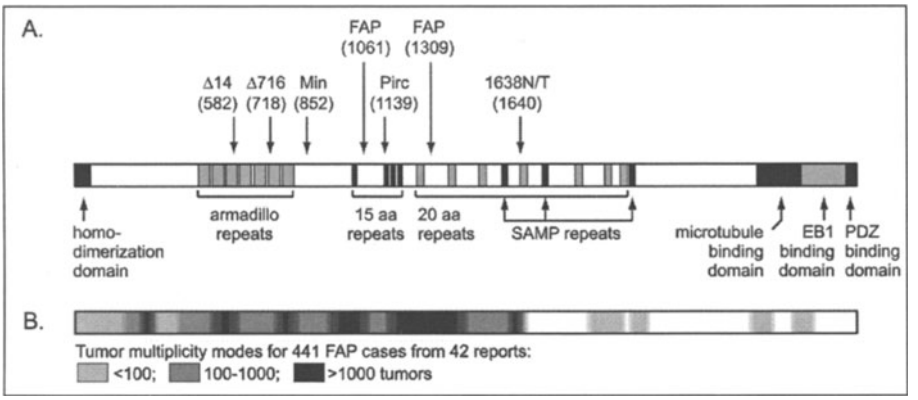

Figure 2. The structure of the Apc protein. A) Arrows on top indicate orthologous locations of mouse model mutations and the two most common FAP mutation sites. B) The genotype-phenotype correlation of sites of protein truncation to disease severity. The data used to generate this bar can be found at http://www.mcardle.wisc.edu/dove/Data/Apc.htm. aa, amino acid. Adapted from Amos-Landgraf and Kwong et al, Proc Natl Acad Sci USA 2007; 104:4036-4041.

and 1169 tend to generate fewer tumors than mutations in the classic MCR (codons 1250-1450; cf. the "loose fit" hypothesis mentioned above, which predicts that MCR mutations leave behind a more optimal number of β-catenin-binding 15aa repeats). These discrepancies could be explained by geographic ancestry, as most of the patients of Crabtree et al's come only from the UK, whereas our compiled data are based on reports from around the world. In this regard, it is interesting to note the significant differences in presentation of colonic cancer in patients from the Middle East compared to those from the United States,[61] possibly indicative of segregating modifier alleles (see below).

Biology of the Murine Intestine: An Introduction to Murine Models of Colon Cancer

The mouse has long been used as a model for various human diseases, due to its experimental tractability and frequently significant reflection of the human phenotype. For colon cancer, mice readily form polyps after certain chemical treatments or genetic modifications and have been an invaluable tool for drug and modifier locus discovery, among other benefits. In the following sections, we introduce numerous well-used mouse models, as well as a novel rat model. We also discuss other animal models involving *Apc* inactivation.

One caveat in using animal models is the deviation from human biology. The murine intestine—both mouse and rat—generally resembles that of the human in both development and structure, particularly in the formation of crypts and villi in the small intestine and in the crypt architecture of the colon. However, a few major differences exist: (i) the murine colon and small intestine are intermingled within the peritoneum, rather than separated, (ii) the rugae of the proximal murine colon have a diagonal rather than perpendicular pattern and (iii) the murine cecum is proportionately much larger. The extent to which these differences affect tumorigenesis is unknown, but must be taken into consideration when extrapolating from model animals to humans.

Mouse Models of Intestinal Cancer

The first hereditary mouse model of colon cancer was described in 1990. Efficient ENU mutagenesis of the germline of C57BL/6J (B6) mice and subsequent outcrossing to AKR/J mice identified a phenodeviant with both a circling behavior and anemia.[62] After continually backcrossing to B6, it was noted that the anemia trait segregated separately from the circling phenotype. Dissection of the anemic mice revealed multiple lesions throughout the intestinal tract, the majority in the small intestine. Histological preparations confirmed these lesions to be adenomas. This line of mice was therefore given the name Min (Multiple intestinal neoplasia). Su and colleagues[63] used the link between *Apc* mutations and FAP to narrow the search for the gene underlying the Min phenotype. Sequencing of the *Apc* gene of Min mice revealed a single change—from leucine to an amber stop codon at position 850. This mutation segregated perfectly with the small intestinal phenotype of Min mice; the mutant allele was thus termed Apc^{Min}. Min mice have since been extensively characterized in the literature and are currently the fourth best-selling line at the Jackson Laboratory. Its popularity can be attributed in part to several properties: (i) Along with more recent targeted *Apc* mutants, Min is the only mouse cancer model with a single genetic change that produces a fully penetrant, organ-specific, consistent and discrete tumor phenotype. (ii) Adenomas in Min mice develop rapidly, with lesions visible as early as two months. Tumor multiplicities are on the order of 100 per intestinal tract, providing strong statistical power. (iii) The multiple pathways impacting tumorigenesis enable many entry points for basic or applied study (see section below on modifiers).

Many other lines of mice with targeted genetic modifications of *Apc* have since been produced. Table 1 provides a summary of mice generated with these disruptions. When heterozygous, the Δ474, Δ14, Δ716, lacZ and Δ1309 models all give phenotypes similar to that of Min.[64-68] In contrast, heterozygosity for the 1638N allele results in 0-2 tumors (none in the colon)[69] while the 1638T model is tumor-free and, unlike any other truncating allele, is homozygous viable.[70] Each of these two alleles truncates the protein at amino acid 1638; however, 1638N has only approximately 2%

the transcript expression level of wild type Apc while 1638T has the full expression level. The latter observation implies that the C-terminus of Apc containing the direct microtubule and PDZ binding domains is nonessential, either for normal embryonic development or for preventing tumor initiation. However, it is important to note that the 1638T allele is not completely wildtype, since animals doubly heterozygous for 1638T and Min are embryonic lethal (as discussed by Sansom, this volume). Nonetheless, the two observations suggest that it is the reduction in Apc protein, not the codon 1638 truncation itself, which results in the 1638N tumor phenotypes. That a reduction in functional Apc protein levels leads to tumor initiation was confirmed by Li and colleagues,[71] who inserted a neomycin cassette in either orientation (reverse, neoR, or forward, neoF, see Table 1) into the 13th intron of *Apc* to generate full-length hypomorphic alleles. These heterozygous mice developed fewer than two adenomas per mouse, with Apc protein levels and activity (as measured by β-catenin transcriptional activity) inversely correlating with tumor multiplicity. However, it is unclear whether the neomycin/hygromycin cassette in these insertion alleles of Fodde et al and Li et al exerts a regional position effect on a neighboring gene(s) that may also contribute to the phenotype.[72] In this context, a clear demonstration of modification of the Min phenotype by a cis-linked recessive lethal factor has been provided in the analysis of the modifier locus *Mom2*.[73]

Recent advances in molecular cloning have enabled the construction of three independent conditional alleles of *Apc* in which specific exons are flanked by *loxP* sites (see Table 1): one allele that removes exon 11 upon the administration of Cre recombinase, resulting in truncation at codon 468[74] and two alleles that remove exon 14, resulting in truncation at codon 580.[65,75] The homozygous ablation of *Apc* in various organs has broadened the understanding of the known functions of *Apc* in maintaining homoeostasis in the liver, kidney, thymus and intestine.[74,76-79] Indeed, carcinomas are induced in the liver and kidney upon tissue-specific deletion of *Apc*. The ability to temporally control *Apc* loss, combined with a titration of Cre, opens up novel avenues for understanding the sufficiency of Apc loss for tumorigenesis. The recent finding that somatic c-*Myc* deletion abrogates the phenotype of concomitant *Apc* loss in the intestine confirms the power of such conditional alleles for pathway analysis.[80]

Finally, chemical carcinogens such as AOM[81] and ENU[82] have been shown to induce intestinal cancer in wild type mice and have been used as models of colon cancer.[83]

Biology of Mouse Intestinal Tumors

Tumors in the small intestine of the Min mouse are composed of dysplastic crypts surrounded and supported by hyperplastic villi and crypts, displaying a characteristic "rose" shape. By contrast, colonic tumors are peduncular, forming a spherical mass of dysplastic cells supported by a stromal stalk.[84] Tumors have a higher mitotic index than adjacent normal tissue[85] and crypt fission indices of Min intestines are also higher than wild type.[5] In contrast to the top-down/bottom-up controversy in human tumorigenesis,[86,24] reviewed by Leedham and Wright,[87] there is little controversy over the directionality of tumor development in the Min or Δ716 mouse models: tumors begin as an outpocketing in the crypt and the dysplastic cell population expands in both directions along the crypt/villus axis.[84]

Rat Models of Intestinal Cancer

Wild type rats develop colon cancer at a very low incidence (<0.1%)[88] with the exception of the Wistar-Furth/Osaka line that spontaneously develops adenocarcinomas at a rate of 30-40%.[89] However, the genetic factors underlying this predisposition are unknown and no recent studies have been reported. The majority of current rat models of colon cancer rely on the induction of tumors via treatment with the carcinogens AOM, DMH, or PhIP.[90] The advantages of carcinogen-treated rat models are that tumors often progress to adenocarcinomas and that tumors have not been reported in the small intestine; the disadvantages are low polyp multiplicities (<2 in F344), long tumor latencies (>10 months) and laborious carcinogen administration regimens with the potential for inconsistent dosage. Carcinogen treatments have been required in the past, owing to the lack of rat embryonic stem cells required for generating genetically engineered rats. However, the ability

Table 1. **Apc *mutant mouse lines***

Allele	Truncation Codon	Conditional?	Genetic Background	Intestinal Tumor #	% of wt Protein per Allele	Initial Reference of Phenotype
Δ468	468 (armadillo repeats)	Yes	N/Av	N/Av	N/Av	Gounari et al, 2005[74]
Δ474	474 (armadillo repeats)	No	B6	>100	100	Sasai et al, 2000[64]
Δ14	580 (armadillo repeats)	Yes	B6	>100	100	Colnot et al, 2004[65]
580S	580 (armadillo repeats)	Yes	Mixed	N/Av	N/Av	Shibata et al, 1997[75]
Δ716	716 (armadillo repeats)	No	B6	>100	0*	Oshima et al, 1995[149]
lacZ	716 (armadillo repeats)	No	Mixed	>100	100	Ishikawa et al, 2006[150]
Min	850 (armadillo repeats)	No	B6	>100	100	Moser et al, 1990[62]
Δ1309	1309 (15aa repeats)	No	B6	40	100	Niho et al, 2003[68]
1638N	1638 (SAMP repeats)	No	B6	1	2	Fodde et al, 1994[69]
1638T	1638 (SAMP repeats)	No	B6	0	100	Smits et al, 1999[70]
Ex13 NeoR	Full-length	No	B6	1	20	Li et al, 2005[71]
Ex13 NeoF	Full-length	No	B6	0.3	10	Li et al, 2005[71]

*This is suggested, but not proven.[71] N/Av = not available.

to generate target-selected mutations, including nonsense alleles, has recently been implemented by several laboratories.[91,92] This capacity has been drawn upon to generate a rat strain carrying a nonsense allele in codon 1137 of *Apc*. F344 rats heterozygous for this allele develop multiple intestinal neoplasms by three months of age, predominantly in the colon and survive in the range of one year.[93] The important colonic predisposition of tumorigenesis in this strain has led to its designation as Pirc: polyposis in the rat colon.

The size of the laboratory rat confers certain advantages to the Pirc model; for one, classical endoscopy can be used to monitor and biopsy colonic tumors.[93] In addition, microCT and micro-PET imaging can strengthen the annotation of each of the tumors, whose sizes—often exceeding 1 cm in diameter—greatly facilitate visualization and biopsy sampling. It can significantly enhance the molecular and morphological analysis of tumor progression to annotate individual neoplasms while keeping the animal alive. While these methods are also feasible in mouse models of colon cancer, the colonic predisposition, size and longevity of the tumor-bearing Pirc rat can provide significant advantages in developing these experimental avenues. Thus, the rat's promising utility for genetics combined with its size and feasibility for longitudinal studies of therapeutic regimes poises the Pirc kindred as a model for colon cancer that is complementary to the genetically powerful Min mouse model.

Coincidentally, the rat and mouse *Apc* loci each lie on Chromosome 18 of their respective genomes. The synteny over Chromosome (Chr) 18 is remarkably conserved between the mouse and the rat. The only difference in synteny is the most proximal 10 Mb of the mouse chromosome, the homologous region of which is located on rat Chr 17. However, a more important difference between these two versions of Chr 18 is the placement of the centromere. *Apc* lies ~30 Mb distal of the acrocentric mouse centromere but ~11 Mb proximal of the metacentric rat centromere (Fig. 1). By contrast, in the metacentric human Chr 5, *Apc* is ~65 Mb distal of the centromere.

Apc Mutations in Other Organisms

To date, *Apc* mutants have been isolated in three other experimental organisms. The $Apc^{MCR/+}$ zebrafish (*Danio rerio*) develops intestinal, hepatic and pancreatic neoplasms, demonstrating the conservation of organ-specific gene functions between vertebrate phyla.[94] *Drosophila melanogaster* lines heterozygous for mutations in either of the two *Apc* homologs, *dApc1* or *dApc2*, develop with a completely normal phenotype despite the evolutionary conservation of Wnt signaling function.[95] It is interesting to note in this context that *dApc1* can complement the function of human *Apc* in suppressing β-catenin-mediated transcription in colon cancer cell lines.[96] Finally, RNAi-induced reduction of *Caenorhabditis elegans Apr-1*, a gene homologous to the N-terminal half of human *APC*, results in aberrations in blastomere development and endoderm specification.[97] Recent studies have linked Wnt signaling and the regulation of WRM-1, a nematode homolog of β-catenin, to Apr-1 function during critical asymmetric cell divisions in development.[98]

Mechanisms of Loss of Heterozygosity at the *Apc* Locus

Biallelic loss of *Apc* function appears to be required for tumorigenesis, but it remains open whether a heterozygous phenotype (also see below) is a necessary preliminary step to the complete loss of Apc function in tumors. In principle, loss of function of the wild type allele from the heterozygote can occur through any of several mechanisms, including: somatic recombination, nondisjunction with or without reduplication, coding or regulatory mutations, epigenetic silencing, or partial or full gene deletion. Early studies in Min mice demonstrated whole-chromosome loss of heterozygosity (LOH),[99] narrowing the possibilities to somatic recombination or nondisjunction. However, the acrocentric nature of mouse chromosomes makes it difficult to distinguish between somatic recombination, which results in the homozygosis of all alleles distal to the recombination site and mitotic nondisjunction, which results in the loss of an entire homolog. Unless the centromere can be marked, each of these processes gives identical results for acrocentric, but not for metacentric chromosomes. Subsequent studies in Min mice harboring an abnormal Robertsonian metacentric Chromosome 18,[100] in Pirc rats with a naturally metacentric Chromosome 18 (Fig. 1),[93] and in FAP patients with

Apc truncations past codon 1286,[101] are consistent with somatic recombination; the majority of these intestinal tumors exhibit LOH limited to a single chromosome arm. Further, the genomes of the early mouse tumors appear to be stable, as assessed by FISH and karyotypic analysis.[102] Somatic recombination has also been shown to be involved in LOH of other tumor suppressors in humans, such as the retinoblastoma gene *Rb1*.[103,104]

By contrast, analysis of sporadic rather than familial human colon tumors suggests that the loss event may occur via a karyotypically unstable pathway. For example, Thiagalingam and colleagues[105] demonstrated that the observed single *p*-arm loss seen in 36% of tumors involved complex translocations rather than conservative somatic recombination. However, it is unclear whether the translocations were the cause of LOH, or instead were acquired during tumor progression. A study by Shih and colleagues,[106] showed allelic imbalance across the genome by digital SNP analysis; however, this finding will require confirmation using more current technology such as Pyrosequencing.[107] Another study has shown that 1638N tumors exhibit significant genomic copy number changes by comparative genomic hybridization;[108] this highlights differences between the 1638N and the genomically stable Min models since the 1638N phenotype may be influenced by regional position effects from the neomycin cassette.[72] In these investigations, another open issue is whether the earliest stage in tumorigenesis is being analyzed. Thus, the debate over the role of genomic instability in colorectal tumorigenesis remains divided into two hypotheses: that instability is a prerequisite for initiation and will be observed at the "birth" of the neoplasm, or that it is acquired during dysplastic growth along the neoplastic pathway and necessary only for progression.

Mathematical models have been invoked to support each hypothesis. Nowak and colleagues[109] showed theoretically that chromosomal instability (CIN) can drive the majority of sporadic LOH events: a hypothesized efficient statistical "tunneling" effect of CIN could drive cells towards an equilibrated LOH population. By contrast, Komarova and Wodarz[110] suggested that CIN would not be efficient, owing to the lag time required for the initial genomic hit to create CIN. Furthermore, Tomlinson and colleagues[111] used an evolutionary approach to stem cell statistics to show that any instability associated with colonic tumors could be explained by a selective, exponential accumulation of aberrations, rather than by a pre-existing state of instability. Such mathematical models may prove to be valuable frameworks for the design of new quantitative experimental tests.

Are Some Apc Truncation Peptides Dominant Negative?

Several lines of evidence suggest that certain truncated Apc proteins might act in a dominant negative manner, either by homodimerizing to wild type Apc or by competing for binding to β-catenin. For example, transfection of constructs encoding the N-terminal 750aa, 1309aa, 1450aa, or 1807aa of human Apc into colorectal cancer cell lines induced chromosome segregation dysfunctions, even in diploid cell lines.[112,113] Another example is that endogenous N-terminal Apc fragments bind to exogenous C-terminal fragments, altering the former's ability to bind to its partner Kap3.[114] Thus, truncated Apc proteins could dominantly interfere with the function of the remaining allele's product. Less direct lines of evidence come from analysis of normal tissue in Min mice. For example, differences have been observed between the intestines of Min and wild type mice in enterocyte migration,[115] E-cadherin localization[116] and Egfr expression.[117] It is not yet resolved whether these effects are autonomous to the heterozygous normal tissue, or are caused by a systemic effect of the tumors carried in the Min mouse.

By contrast, a line of mice transgenic for a Δ716 or Δ1287 fragment of the *Apc* gene failed to develop intestinal tumors.[118] Here, it is unclear whether the transgene expression levels reached a tumorigenic threshold, especially in the presence of two copies of the wild type allele. The question of whether Min is dominant negative has important implications for the study of LOH. If normal heterozygous tissue from Min animals has a phenotype that predisposes to tumorigenesis, then the familial case may differ from the sporadic case, where normal tissue is homozygous wild type for Apc. A full understanding of Apc action must also account for the full-blown polyposis

phenotype of locus-wide deletions including the classical Herrera deletion by which the *APC* locus was first mapped.[33,119,120] It is also worth noting that similar C-terminal truncations of APC2 in Drosophila do not exhibit dominant negative effects on Wnt signaling or viability, but in some cases do have dominant effects on cytoskeletal organization in the embryo.[95] Thus, the question of predisposing haploinsufficiency or dominant negativity requires resolution.

Modifiers of Murine Intestinal Cancer

Many different pathways have an impact on the initiation and/or progression of intestinal adenomas: karyotypic stability, DNA mutation rates, stem cell turnover, cellular growth and proliferation, cellular differentiation, environmental factors, diet, exercise, therapeutic drugs and others. In this chapter we address only genetic modifying factors (for a review of diet and therapeutic drugs, see ref. 93).[90] In experimental genetics, a modifying locus has no phenotypic consequence in the absence of mutation at the primary locus of interest, in this case *Apc*. In epidemiology, however, the factors controlled by modifying loci may be found to have an impact, since the functional state of the primary locus may vary covertly or overtly in the population being studied.

The phenotypic variation of Min among different inbred strains highlights the importance of modifier alleles. Historically, B6-Min mice develop approximately 100 tumors in the intestinal tract. Other inbred backgrounds on which the Apc^{Min} allele has been introgressed show a broad spectrum of tumor multiplicities (Table 2). For example, BTBR is a strongly enhancing background, with mice becoming moribund by 60 days of age due to the presence of more than 600 tumors.[121] At the other extreme lie AKR mice, which develop only one to four tumors per animal and can survive for up to a year of age.[122] C3H and 129S6 have milder suppressive phenotypes compared to AKR. General strain effects have led the way for the identification of polymorphic modifier loci by quantitative trait locus analysis of the phenotypes of *Min* carriers in outcrossed progeny.[123]

Perhaps the most well-known modifier is *Mom1* (Modifier of Min 1). A quantitative trait locus (QTL) analysis using SSLP markers in crosses involving 4 inbred strains found a QTL on chromosome 4 that was shared among all mapping crosses.[123] It was apparent that at least two alleles of *Mom1* existed: a resistance allele found in AKR/J, MA/MyJ and CAST/EiJ and a sensitivity

Table 2. The genetic background dependence of the Min phenotype

Strain	Mom1	Age (Days)	Small Intestine	Colon	N	Reference
129S6	S/S	103-163	45	1	23	L.N. Kwong, unpublished
BTBR/Pas	S/S	54-82	625	12	74	Kwong et al, 2007[121]
C3H/HeJ	S/S	100-120	16	0.4	89	Koratkar et al, 2004[151]
C57BL/6J	S/S	90-120	128	3	48	Kwong et al, 2007[121]
AKR/J	R/R	146-336	4	0	42	Kwong et al, 2007[121]
129 x B6 F1	S/S	92-164	82	0.2	35	L.N. Kwong, unpublished
AKR x B6 F1	R/S	104-143	25	0.1	15	Kwong et al, 2007[121]
BTBR x B6 F1*	S/S	80-93	117	1.6	16	A. Shedlovsky, unpublished
BTBR x B6 F1**	S/S	84-89	215	1.4	19	A. Shedlovsky, unpublished
C3H x B6 F1	R/S	130-150	8	0	10	Koratkar et al, 2004[151]
CAST x B6 F1	R/S	100-120	3	0	14	Koratkar et al, 2002[152]
CAST x B6 F1	R/S	185-215	7	0	11	Koratkar et al, 2002[152]

*Min from B6 parent. **Min from BTBR parent.

allele in C57B/6J (B6). *Mom1* is semidominant where each copy affects tumor number by a factor of about 2. MacPhee and colleagues[124] suggested that the *Pla2g2a* gene (encoding secretory phospholipase 2A) might explain the *Mom1* effect. This hypothesis was confirmed in a line of B6 Min mice transgenic for a cosmid containing the resistance allele *Pla2g2a*,[125] which showed reduced polyp number. Subsequent higher resolution genetic analysis showed that the *Mom1* locus consists of both *Pla2g2a* and at least one other distal factor.[126] The effect of *Mom1* explains a significant proportion of the variance in tumor multiplicity seen in crosses between B6-Min and AKR or C3H mice (Table 2). Interestingly, the *Pla2g2a* gene seems to act in a cell non-autonomous fashion: it is expressed from postmitotic Paneth and goblet cells within the micro-environment, affecting the net growth rate of adjacent tumors.[85] (Evidence has been reported that the secretory phopholipase A2 can instead stimulate colonic tumor growth when expressed autonomously within the tumor lineage.[127]) This apparent non-autonomous action of *Pla2g2a* illustrates the necessity of investigations in the whole animal, as such effects would be lost in cell culture or non-orthotopic xenograft models.[128] The exact mechanism by which *Pla2g2a* exerts its effects on colon tumorigenesis remains unresolved,[129] highlighting the challenges of cancer modifier genetics. Furthermore, its relevance to the human disease is unresolved. Three studies have failed to find significant cancer-associated germline or somatic variation in the human *PLA2G2A* gene.[130-132] One sporadic colon cancer patient has been reported with a constitutional frameshift mutation in this gene.[133] Finally, a correlation has been reported between PLA2G2A expression and gastric adenocarcinoma patient survival.[134] Overall, the identification of *Mom1* has had a long-lasting impact on modifier genetics, as it was an important proof of principle that such studies could identify at the molecular level genetic determinants modifying a cancer phenotype.

By utilizing similar mapping methods, additional polymorphic Modifiers of Min have been discovered: *Mom2*, *Mom3* and *Mom7*, each of which resides on Chromosome 18. *Mom2* arose spontaneously in a stock of $Apc^{Min/+}$ mice on the C57BL/6J background and mapped distal to the *Apc* locus.[135] Congenic line, expression and sequencing analyses pinpointed a recessive embryonic lethal 4 bp duplication in the ATP synthase *Atp5a1* gene.[73] When in cis with the mutant *Min* allele, this mutant *Mom2* allele confers an ~12-fold resistance to tumor multiplicity, but has no effect when in trans. Along with a decreased LOH incidence, these results indicated that somatic recombination proximal to both the *Apc* and *Atp5a1* loci would generate homozygous *Atp5a1* segregants that would be cell- and therefore tumor-lethal.

The *Mom3* locus was discovered in a line of Min mice that had become strain-contaminated,[136] resulting in an increase in tumor multiplicity compared to control B6-Min mice. It mapped to within the first 25cM of chromosome 18, proximal to *Apc*. However, the lack of additional polymorphic markers, along with the unknown contaminating strain background, prevented further positional refinement. In a separate study, the *Mom7* locus mapped to a similar region as *Mom3*, but came from defined crosses of the B6.$Apc^{Min/+}$ line to the AKR, BTBR and A/J strains.[121] Congenic line and in silico mapping analyses reduced the *Mom7* interval to the first 4.4 Mb of chromosome 18, including the complex sequence of the centromere. Unlike *Mom2*, *Mom7* is homozygous viable for all alleles and the B6 allele shows a dominant resistance phenotype in both the trans and cis configurations. Whether *Mom7* and *Mom3* represent the same underlying modifier must be resolved by complementation testing. Interestingly, the Rb(7.18)9Lub Robertsonian translocation (Rb9), also at pericentromeric Chromosome 18, lowers tumor multiplicity in $Apc^{Min/+}$ mice.[100] FISH analysis showed that the Chromosome 18 homologs were mispaired in the nucleolar organizing region, leading to the hypothesis that the opportunity for somatic recombination at *Apc* is decreased by this centric fusion. Although *Mom7* and Rb9 map to the same location, it is important to note that Rb9 involves a gross physical chromosome abnormality, while *Mom7* involves a normal chromosome; furthermore they have qualitatively different effects, with *Mom7* resistance fully dominant and Rb9 semidominant, making it unlikely that they represent the same modifier. Furthermore, none of these modifiers shows the "overdominant effect" predicted for sequence heterozygosity, which would suppress somatic recombination in heterozygotes but not in homozygotes.[137] Thus, the *Mom7* and *Mom3* are modifiers distinct from Rb9.

As illustrated by the growing set of modifiers of the Min phenotype, it is clear from Table 3 that strategies for cancer prevention and therapy have many points of entry, providing both a wealth of candidate therapeutic targets and the challenge of converting any of them into potential human therapies. However, the benefit of such modifier studies extends beyond clinical relevance; each dataset informs both the functions of the modifier and of *Apc*. In turn, each modifier has a role in processes other than tumorigenesis. For example, the increases in both karyotypic instability and tumor multiplicity in *BubR1*$^{+/-}$;*Apc*$^{Min/+}$ mice provide insight into the normal checkpoint functions of both BubR1 and *Apc*.[138] Another interesting example is that deletion of *H19* induces the biallelic expression of *Igf2*, increasing Min tumor multiplicities.[139] This genetic model of loss-of-imprinting (LOI) highlights the functional importance of genomic imprinting. In human sporadic colorectal cancer patients, LOI at *Igf2* is often elevated in peripheral blood lymphocytes compared to healthy controls,[140] implying that LOI can precede the loss of Apc function and become a risk factor for otherwise normal individuals.

Probing deeper into the modifiers organized in Table 3, several interesting patterns are noted. First, mutations in either of the mitotic stability genes *BubR1*[138] or *Cdx2*[141] generate a complex modifying phenotype, whereby the multiplicities of tumors of the small intestine decrease, while multiplicities of colonic tumors increase. This striking disparity between the effects of the same mutation in two different regions of the gut suggests that the small intestine and colon have different abilities to respond to CIN. Perhaps the small intestine expresses a senescence and/or apoptosis response that efficiently blocks CIN-induced tumor formation. By contrast, the hyper-recombination phenotypes of *Blm*[142,143] or *Reql*[144] mutations affect the entire intestinal tract.

The contrast between the regionally diverse response to mitotic instability and the uniform response to hyperrecombinational instability suggests that different responses to different types of instability exist in different regions of the intestinal tract. In the same vein, the Mbd2 and Mbd4 methyl-binding proteins have opposite effects on intestinal tumor multiplicity,[145,146] indicating that the epigenetic machinery has both positive and negative indirect regulators of methylation-associated DNA mutation and/or silencing. Indeed, the potency of mutations in mismatch repair genes to generate tumors in the ascending colon illustrates both the centrality of sequence stability to tumor suppression and the regionality of these effects. Next, mutations in the Ephrin family of genes[147] demonstrate that differentiation is key to tumorigenesis, mirroring the dysregulation of ephrin receptors in mice conditionally inactivated for *Apc*.[78] Finally, many "classic" regulators of numerous tumor pathways—including p53, p27, p21, c-Jun and cyclin D1—modify the Min phenotype, raising the possibility that therapies directed towards other classes of cancer could also have an effect on colonic tumors.

Conclusion

The complexity of both morphological and molecular pathways in colon cancer presents a challenge to clinical therapies, which are already multifaceted. For example, the FOLFOX regimen combines fluorouracil, leucovorin and oxaliplatin, which can be used in addition to standard surgery and radiation treatments. Despite the complexity, the many different animal models now available—mouse, rat, zebrafish and invertebrates—expand our ability to identify and validate different therapeutic targets. Indeed, the convenience of these animal models simplifies many aspects of colon cancer research that would otherwise be difficult to control from a highly heterogeneous human population. The effectiveness of such models emerged from the discovery of Apc as the central molecule negatively regulating colon cancer. This discovery, a result of Herculean efforts by several centers of human genetics[33,34,37,148] allowed for both the identification of the molecular basis of the Min phenotype and the characterization and construction of single-gene mutants with profound cancer phenotypes. Overall, the study of colon cancer radiates out from our understanding of the mechanisms of action of the Apc protein, a central node regulating multiple cancer pathways.

Table 3. Molecular genetic modifiers of Apc *knockout mouse models*

Modifier Affects	Modifier Gene(s)	Modifier Allele(s)	Allele Property	Apc Model	Effect of Mutant Allele on Intestinal Tumor Multiplicity	Factor of Effect	Reference
Karyotypic stability	BubR1	$Bub1b^{Gt(neo\text{-}btk)1Dai}$	Knockout (het)	Min	Decrease/Increase[a]	2/10[a]	Rao et al, 2005[138]
	Cdx2	$Cdx2^{tm1Mmt}$	Knockout (het)	Δ716	Decrease/Increase[a]	9/6[a]	Aoki et al, 2003[141]
	Terc	$Terc^{tm1Rdp}$	Knockout	Min	Decrease (at G4)	10	Rudolph et al, 2001[153]
DNA mutation rate	Pms2	$Pms2^{tm1Lisk}$	Knockout	Min	Increase	3	Baker et al, 1998[154]
	Mlh1	$Mlh1^{tm1Lisk}$	Knockout	Min	Increase	3	Shoemaker et al, 2000[155]
	Msh2	$Msh2^{tm1Mak}$	Knockout	Min	Increase	7	Reitmair et al, 1996[156]
	Msh3/Msh6	$Msh3^{tm1Rak}$ $Msh6^{tm1Rak}$	Knockout	1638N	Increase	12	Kuraguchi et al, 2001[157]
	Fen1	$Fen1^{tm1Rak}$	Knockout (het)	1638N	Increase	1.5	Kucherlapati et al, 2002[158]
	Myh	$Mutyh^{tm1Jhmi}$	Knockout	Min	Increase	1.5	Sieber et al, 2004[159]
Recombination rates	Rb9	Rb(7.18)9Lub	Translocation	Min	Decrease	19	Haigis and Dove, 2003[100]
	Recql4	$Recql4^{tm1Glu}$	Knockout	Min	Increase	2	Mann et al, 2005[144]
	Blm	Blm^{tm3Brd}	Hypomorph	Min	Increase	3	Luo et al, 2000[143]
Differentiation	EphB2	Δ^{cy}EphB2	Dom neg Tg	Min	Decrease	3	Batlle et al, 2005[147]
	EphB3	$EphB3^{tm1Kln}$	Knockout	Min	Increase	2	Batlle et al, 2005[147]
DNA methylation	Mbd2	$Mbd2^{tm1Bh}$	Knockout	Min	Decrease	10	Sansom et al, 2003[145]
	Mbd4	$Mbd4^{tm1Bird}$	Knockout	Min	Increase	2	Millar et al, 2002[146]
	Dnmt1	$Dnmt1^{tm1Jae}$	Knockout (het)	Min	Decrease	2	Cormier and Dove, 2000[85]

continued on next page

Table 3. Continued

Modifier Affects	Modifier Gene(s)	Modifier Allele(s)	Allele Property	Apc Model	Effect of Mutant Allele on Intestinal Tumor Multiplicity	Factor of Effect	Reference
Stromal regulation	Foxl1	Foxl1^{tm1Khk}	Knockout	Min	Increase	8	Perrault et al, 2005[160]
	TSP1	Thbs1^{tm1Hyn}	Knockout	Min	Increase	2	Gutierrez et al, 2003[161]
Cell growth and proliferation	c-Jun	Jun$^{tm2.1Wag}$	Hypomorph	Min	Decrease	2	Nateri et al, 2005[162]
	Cyclin D1	Ccnd1^{tm1Wbg}	Knockout	Min	Decrease	6	Hulit et al, 2004[163]
	Egfr	Egfr^{wa2}	Hypomorph	Min	Decrease	10	Roberts et al, 2002[164]
	p21	Cdkn1a^{tm1Led}	Knockout	1638N	Increase	2	Yang et al, 2001[165]
	p27	Cdkn1b^{tm1Mlf}	Knockout	Min	Increase	5	Philippp-Staheli et al, 2002[166]
	p53	Trp53^{tm1Ldo}	Knockout	Min	Increase	2	Halberg et al, 2000[167]
	Igf2	H19^{tm1Tilg}	Activates Ifg2	Min	Increase	2	Sakatani et al, 2005[139]
Pleiotropic	Matrilysin	Mmp7^{tm1Lmm}	Knockout	Min	Decrease	2	Wilson et al, 1997[168]
	Pla2g2a	Pla2g2a^{AKR}	Tg	Min	Decrease	2	Cormier et al, 1997[125]
	BAH	Asph^{tm1Jed}	Knockout	Min	Increase	2	Dinchuk et al, 2002[169]
	E-cadherin	Cdh1^{tm1Cbm}	Knockout (het)	1638N	Increase	9	Smits et al, 2000[170]
	PPAR-δ	Ppard^{tm1Jps}	Knockout	Min	Increase	1.5	Harman et al, 2004[171]
	Netrin-1	Tg-netrin-1	Tg	1638N	Enhances progression	N/A	Mazelin et al, 2004[172]
	Smad4	Smad4^{tm1Mmt}	Knockout	Δ716	Enhances progression	N/A	Takaku et al, 1999[173]

[a]Effects on the small intestine and colon, respectively; [b]The Robertsonian translocation is centromeric fusion of chromosomes 7 and 18. Note: The *Mom* (Modifier of Min) and *Scc* (Susceptibility to colon cancer)[174] loci are in general not yet fully defined in molecular detail (see text) and are therefore not included in Table 3.

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