

Mouse models of colorectal cancer: Past, present and future perspectives

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Abstract

Colorectal cancer (CRC) is the third most common diagnosed malignancy among both sexes in the United States as well as in the European Union. While the incidence and mortality rates in western, high developed countries are declining, reflecting the success of screening programs and improved treatment regimen, a rise of the overall global CRC burden can be observed due to lifestyle changes paralleling an increasing human development index. Despite a growing insight into the biology of CRC and many therapeutic improvements in the recent decades, preclinical *in vivo* models are still indispensable for the development of new treatment approaches. Since the development of carcinogen-induced rodent models for CRC more than 80 years ago, a plethora of animal models has been established to study colon cancer biology. Despite tenuous invasiveness and metastatic behavior, these models are useful for chemoprevention studies and to evaluate colitis-related carcinogenesis. Genetically engineered mouse models (GEMM) mirror the pathogenesis of sporadic as well as inherited CRC depending on the specific molecular pathways activated or inhibited. Although the vast majority of CRC GEMM lack invasiveness, metastasis and tumor heterogeneity, they still have proven useful for examination of the tumor microenvironment as well as systemic immune responses; thus, supporting development of new therapeutic avenues. Induction of metastatic disease by orthotopic injection of CRC cell lines is possible, but the so generated models lack genetic diversity and the number of suited cell lines is very limited. Patient-derived xenografts, in contrast, maintain the pathological and molecular characteristics of the individual patient's CRC after subcutaneous implantation into immunodeficient mice and

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are therefore most reliable for preclinical drug development – even in comparison to GEMM or cell line-based analyses. However, subcutaneous patient-derived xenograft models are less suitable for studying most aspects of the tumor microenvironment and anti-tumoral immune responses. The authors review the distinct mouse models of CRC with an emphasis on their clinical relevance and shed light on the latest developments in the field of preclinical CRC models.

Key words: Colorectal cancer; Mouse models; Patient-derived xenografts; Carcinogen-induced models; Genetically engineered mouse models; Preclinical drug development

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Core tip: This review highlights the different approaches to model colorectal cancer in the mouse. Carcinogen-induced rodent models, genetically engineered mouse models, heterotopic and orthotopic models as well as patient-derived xenografts are discussed with an emphasis on their specific advantages and disadvantages. Moreover, the historical background of animal models for cancer research and the future perspectives of colorectal cancer research are reviewed as well.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common diagnosed malignancy among both sexes in the United States as well as in the European Union^[1,2]. A decrease in the incidence and overall mortality of CRC in countries with a very high development index over the last decades can be attributed to an interplay of screening programs with detection of precancerous lesions or early stage cancers on the one hand^[3,4], and improved therapeutic concepts leading to an increased stage adjusted survival for all stages of CRC on the other hand^[5]. This development is in sharp contrast to countries with a rapidly growing high development index. Together with an increased CRC incidence and mortality this reflects an adoption of the so-called “western lifestyle” including the risk factors for CRC. While obesity, smoking and red meat consumption are significantly associated with an elevated CRC risk^[6], physical activity, high vegetable, fruit and fiber intake as well as metronomic aspirin therapy, have shown to decrease CRC risk^[7,8]. Besides these modifiable risk factors, a variety of genetic factors influences CRC risk. About 5% of CRC cases are caused by hereditary, highly penetrant cancer syndromes, like familial adenomatous polyposis (FAP) and Lynch syndrome (LS); whereas up to 20%-30% of cases are considered as “familial” due to different germline mutations of varying penetrance^[9]. Since the discovery of the link between APC germline mutations and FAP^[10], followed by the genetic pathology of LS in the early 1990s^[11,12], a myriad of mutations contributing to CRC genesis has been identified, constantly reshaping the genomic landscape of the disease^[13]. The ideal model of CRC should recapitulate the progress from a precancerous adenoma to an invasive carcinoma with metastatic potential and at the same time it has to reflect the inter-individual molecular diversity of the disease. Consequently, a single (mouse) model of CRC simply cannot match all of these requirements. In this review, we discuss the different mouse models of CRC with their distinct advantages and disadvantages with a focus on their translational and clinical relevance.

CARCINOGEN-INDUCED MODELS

Carcinogen-induced models (CIM) in rodents look back on a long tradition but maintained their usefulness for certain applications to the present day. They provide a platform for dietary studies and give insights into the pathways of food-borne carcinogens and colitis-associated carcinogenesis. Administration of the chemical

compounds is possible *via* ad libitum feeding, oral gavage, intraperitoneal/subcutaneous (s.c.) or intramuscular injection, or enema.

In 1915, Yamagiwa *et al*^[14] proofed the carcinogenic properties of coal tar by its repetitive application on the ears of rabbits. At about the same time, first researchers worked on colon carcinogenesis by applying chemical or radioactive substances^[15-17]. In the 1960s, cycasin and its metabolite, methylazoxymethanol, have shown to induce cancers in rodents^[18-20]. In the following years, the more chemically stable substances, azoxymethane (AOM) and its precursor molecule, 1,2-dimethylhydrazine as well as methylazoxymethyl acetate, were extensively used to induce colon carcinogenesis in mice and rats. All three compounds are metabolized to methylazoxyformaldehyde, which is able to alkylate the DNA bases guanine and thymine^[21]. After being processed by Phase-II-reaction, it is secreted to the bile and exceeds its carcinogenic effect to the intestinal mucosa^[22]. Interestingly, these compounds show different carcinogenic potential depending on the mouse strain, housing conditions and the way of administration^[23-25]. Although most authors claim a certain organotropism for AOM and dimethylhydrazine, tumor formation happens mostly in the small intestine and relevant amounts of alkylated DNA adducts can be observed in the liver and the kidneys^[26]. Moreover, intestinal carcinogenesis can be achieved by the oral or rectal application of the direct alkylating topic agents N-methyl-N-nitrosourea (MNU), 3,2'-dimethyl-4-aminobiphenyl and N-Methyl-N'-nitro-N-nitrosoguanidine of which the latter two are traditionally used in rats^[27-29].

Other carcinogens gained attention in connection with the association between meat consumption and increased CRC risk^[30]. Heterocyclic aromatic amines (HAA) form from the reaction between free amino acids, sugars and creatine at high temperatures during the cooking process of meat and fish^[31], whereby 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-Amino-9H-pyrido[2,3-b]indole are the most abundant HAA in various foods^[32]. PhIP is metabolized by the liver enzyme CYP1A2 to N2-Hydroxy-PhIP, which then, after sulfation or acetylation, forms activated esters capable of DNA adduct formation^[33]. Detoxification of PhIP and its metabolites is driven by glutathione conjugation and glucuronidation^[34,35]. Glucuronide conjugates are excreted through urine and bile^[36]. In case of the latter, hydrolyzation by bacterial beta-glucuronidases in the intestines liberate reactive PhIP metabolites, which not only affect the intestinal mucosa, but undergo enterohepatic circulation^[37]. Important to consider is, that the metabolism of PhIP in rodents results in less reactive metabolites than in humans, and its carcinogenic potential measured in animal studies might be even higher in humans^[38]. Nakagama *et al*^[39], by combining a high fat diet with PhIP intake, showed the tumor enhancing properties of this food borne agent simulating the carcinogenic effects of the s.c. "western diet". Moreover, PhIP led to the formation of high-grade dysplasia and adenocarcinomas in a mouse model of chemical induced colitis^[40]. Although other common foodborne HAAs have shown to induce dysplasia and carcinomas in rodents^[41], they are rarely used for modelling colon carcinogenesis. Polycyclic aromatic hydrocarbons, as benzo[a]pyrene, may be used for chemoprevention studies but are insignificant for CRC modeling in general^[42,43]. Dextran sodium sulfate (DSS) must also be mentioned when discussing chemical-induced CRC mouse models. Since the first report of an DSS-induced colitis model nearly 30 years ago^[44], countless studies used DSS to simulate chronic inflammatory bowel diseases and we would recommend the reader to refer to excellent reviews discussing inflammatory bowel diseases and DSS^[45]. As a sulfated polysaccharide, DSS directly damages the colonic epithelium resulting in an impairment of the mucosal barrier with consecutive entry of luminal bacteria and associated antigens into the mucosa, triggering inflammation^[46]. Depending on the animal strain, DSS dosage and administration regimen, mice can develop acute and chronic colitis or even colitis-induced dysplastic lesions^[47-50]. DSS in combination with carcinogenic compounds, primarily AOM, has been proven useful for the research of colitis-induced cancer^[51]. By use of mice with germline *Apc* mutation (APC^{Min} mice) for DSS treatment, the rate of dysplasia and carcinoma formation was further enhanced^[52]. Cooper *et al*^[53] reported an increased CRC incidence of 40% in APC^{Min} mice after two cycles of 4% DSS treatment compared to untreated control animals and identified the loss of heterozygosity (LOH) of *Apc* as underlying cause.

Chemical-induced mouse models are not homogenous and possess specific advantages and disadvantages. They are by far the oldest method inducing CRC in animals and a multitude of studies has been traditionally carried out in rats and other rodents diminishing the comparability of older data with more recent results. Most models reflect very well the progression from aberrant crypt foci to adenomas to carcinomas of the human adenoma-carcinoma sequence^[54]. Therefore, they are still useful to evaluate the influence of diet^[55], dietary supplements^[56,57], chemopreventive interventions^[58,59] and the gut microbiome^[60]. Especially the combination of DSS with carcinogenic agents provided many insights in the link between CRC and

inflammation^[61]. However, chemical-induced carcinomas rarely show invasive properties and local or distant metastases are usually absent. Albeit Yang *et al*^[62] reported lymph node metastases in an intrarectal MNU-model, the use of shrews (phylogenetic unrelated to rodents) as test animals interdicts the comparison to rodent animal models. A further exception is the work of Derry *et al*^[63], who could observe a relevant number of lung metastases in an AOM-induced mouse model, which is to our knowledge the only report of metastatic spread in a chemical-induced mouse model. Besides the lack of invasiveness, a lot of CIM show a high latency from the first application to tumor development. Depending on the carcinogen, dosing protocol and mouse strain, latencies from 24 to 50 wk were reported^[64-66]. By combining the carcinogen with DSS, the time to tumor development can be notably shortened to 10-18 wk^[23,67]. Although the minority of sporadic CRC patients show synchronous adenomas^[68,69], nearly all CIM show a “carpeting” of the colonic mucosa with polyps^[70]. Tumor formation is not restricted to the colon, but can be commonly observed in the whole gastrointestinal tract^[71,72]. Moreover, MNU additionally induces leukemia and lung adenomas^[73] and PhIP leads to formation of mammary and prostate neoplasia^[74]. Another complexing aspect of CIM concerns the genetic aberrations associated with the adenoma-carcinoma sequence, *i.e.*, the accumulation of mutations, predominantly affecting *APC*, *KRAS* and *P53*^[75]. While the AOM model shows frequent *Kras* and β -*catenin* mutations, *Apc* and *P53* are rarely affected^[76-79]. In contrast PhIP, IQ and MNU lead to *Apc* mutations, but show no *P53* or *Kras* mutations^[21]. In general, the vast quantity of different dosing protocols, application forms and animal strains, makes direct comparisons and the selection of the right CIM difficult^[54]. Another aspect, not to be neglected, is the agenda of animal welfare. Quite a few protocols lead to significant weight loss and diarrhea, which is, in combination with often long study durations, detrimental for the animal wellbeing^[80].

GENETICALLY ENGINEERED MOUSE MODELS OF COLORECTAL CANCER

With the knowledge explosion concerning the genetic pathways altered in CRC at the end of the 20th century, the scientific community demanded specific genetic mouse models to focus on certain molecular mechanisms of colorectal carcinogenesis.

In the 1980s, the first genetically engineered mouse models (GEMM) of brain tumors, lymphoma, pancreatic cancer, breast cancer and osteosarcoma emerged^[81-87]. Based on the groundbreaking work of Evans, Smithies and Capecchi^[88] on gene targeting, the first tumor suppressor knock-out mouse models emerged in the early 1990s^[89,90]. To circumvent the obstacle of frequent embryonic lethality caused by germline knock-outs of tumor suppressors, *Cre-loxP* mediated mouse models were designed to allow the tissue specific and conditional knock-out of tumor suppressor genes or activation of oncogenes, respectively^[91-93]. Interestingly, the very first GEMM of CRC, the APC^{Min} mouse, was created without sophisticated methods. Moser *et al*^[94] showed, that the application of N-ethyl-N-nitrosourea leads to nonsense mutations in codon 380 of the *Apc* gene and subsequent breeding of these animals established the first model for multiple intestinal neoplasia. APC^{Min} mice develop a large number of adenomas in the small intestine after 120-140 d due to LOH and show a high mortality with increasing age as a result of intestinal obstruction and anemia without progression to invasive carcinoma^[95]. While these models contributed to the understanding of the early stages of FAP, they do not reflect the majority of spontaneous CRC^[96]. Since a homozygous *Apc* mutation is lethal during embryonic development, breeding of homozygous APC^{Min} mice is impossible^[97]. However, additional treatment of APC^{Min} mice with AOM or other carcinogenic compounds increases malignancy of the resulting tumors and simultaneously shortens the time to tumor development^[98-100]. Till the present day, these models are in use for chemoprevention studies^[101,102] and have enormously contributed to the understanding of the early tumor initiating events^[103]. Interestingly, a change from C57BL/6 to a hybrid genetic background can extend the lifespan of APC^{Min} mice beyond one year, resulting in a high proportion of invasive adenocarcinoma^[104]. Sørdring *et al*^[105] changed the APC^{Min} genetic background from C57BL/6 to A/J mice resulting in increased tumor formation in the colon, with a reasonable number of tumors progressing to carcinomas. Transgenic mice with alternative *Apc* mutations, like the APC^{C+/1638} mouse^[106,107], the APC^{Δ716} mouse^[108] and the APC^{Δ242/+} mouse^[109] vary in tumor count and histopathology. Tumor formation predominantly in the small intestine instead of the colon is the major flaw of most *Apc*-based mouse models. Colnot *et al*^[110] designed the APC^{Δ14/+} mouse, which shows a more severe phenotype with invasion of the muscularis, an increased lethality and a higher colonic tumor

burden compared to APC^{Min} mice but unfavorably still shows relevant tumor development in the small intestine. Early attempts of combining *Apc* mutations with homozygous *P53* knockouts yielded conflicting results, with either no increase^[111,112], or a small increase of gastrointestinal malignancy^[113]. The most likely explanation is that in human cancers *P53* missense mutations frequently act in a dominant negative fashion, overruling the tumor-suppressive function of the wildtype allele^[114,115]. In contrast, targeting *Kras* without tissue specific promoters leads either to embryogenic lethality^[116] or few viable animals succumbing to rapidly developing lung tumors^[117]. To avoid abundant distribution of mutations in the whole organism, transgene expression controlled by a tissue specific gene promoter, most commonly by application of the *Cre-loxP*-system^[91], has proven to be extraordinarily useful. Many workgroups used *Villin-Cre* transgenes to restrict recombination of *loxP*-flanked genes to the epithelial cells of the small and large intestines either with a constitutive expression (*Vil-Cre*) or with a tamoxifen-inducible expression (*Vil-Cre-ER^{T2}*)^[118]. Another option of site-specific *Cre* expression is the fatty acid binding protein liver *Cre* transgene (*Fabpl Cre*), which can be combined with a tetracycline-inducible tet-on system^[119]. Yet, both transgenes' expression is not limited to the large intestine: While *Villin-Cre* is expressed in the epithelial cells of the whole intestines, *Fabpl-Cre* expression can be detected in the distal small intestine, cecum and colon^[119,120]. Also, the AhCre strain, carrying *Cre* under control of the *Cyp1A* promoter, is commonly used for colon cancer models^[121,122]. Here, *Cre*-expression is induced by β -naphthoflavone in the liver and intestines, but constitutive recombination can be observed in other tissues like the renal epithelium^[123]. To achieve a more colon specific expression of *Cre*-recombinase, Hinoi *et al*^[124] constructed a transgene of *Cre* and the promoter region of the *CDX2* homeobox gene. By inserting a guanine repeat tract to this transgene (*CDX2P9.5-G22Cre*), stochastic activation of *Cre*-expression by means of spontaneous frameshift mutations leads to a mosaic-like recombination in the mucosa of the terminal ileum, cecum, and colon^[125]. At last, carbonic anhydrase 1 promoter/enhancer-*Cre* recombinase transgene (*CAC*) facilitates recombination strictly limited to the large intestine^[126]. Paralleling *Cre*-transgene implementation, others achieved spatiotemporal oncogene expression in the large intestine by delivering *Cre* by viral transfection *via* transanal injection, surgery or colonoscopy^[127-129] leading to exquisite models of CRC with metastatic spread^[130]. Supplied with this comprehensive genetic toolbox, a plethora of CRC mouse models were generated and used to evaluate the role of different mutations and their interplay. Among the non-hypermutated tumors, the most frequently mutated genes are *APC*, *P53*, *KRAS*, *PIK3CA*, *FBXW7*, *SMAD4*, and *TCF7L2*^[13]. The role of *Apc* LOH as a driver mutation is highlighted in several mouse models. While generalized deletion of both *Apc* alleles (*Apc^{fllox/fllox}*) leads to rapid death by disorder of cell differentiation^[131], mice with a mosaic-like deletion of both *Apc* genes die rapidly from florid polyposis^[125]. Depending on the type and modality of *Apc* mutation, heterozygotes develop adenomas or invasive adenocarcinomas^[120,132,133]. Whilst mutated *Kras* alone is insufficient to induce colorectal tumorigenesis, it increases the susceptibility of the intestinal mucosa to chemical carcinogenesis^[134] and leads to accelerated tumor formation in combination with *Apc* loss^[122,135]. *Nras* mutation, in contrast, does not alter the effect of *Apc* loss^[136]. In humans, *P53* mutations are often associated with vascular and lymphatic invasion and advanced cancer stages^[137,138]. In fact, the combination of *Apc* mutation with a dominant-negative *P53* mutation leads to increased invasiveness of intestinal tumors with signs of epithelial to mesenchymal transition^[139,140]. Also, loss of *P53* in a constitutively active Notch signaling background leads to intestinal tumor formation and metastasis^[141], whereas Notch signaling does not cooperate with the Wnt-pathway^[142]. Nevertheless, *Apc* deficiency seems to represent a key prerequisite of cancer progression, since *Apc* restoration leads to spontaneous tumor regression of *Kras*-mutated, *P53*-deficient adenocarcinomas^[143]. *FBXW7* codes for the F-box/WD repeat-containing protein 7, the substrate receptor of a ubiquitin ligase, which degrades several proto-oncogenes like *MYC*, *CCNE1*, *NOTCH1* and *JUN* and acts synergistically with *P53* as haploinsufficient tumor suppressor^[144-146]. Intestinal *Fbxw7* deletion enhances tumor development in an *Apc^{Min/+}* background^[147] and a combined deletion of *Fbxw7* and *P53* results in highly aggressive intestinal cancers with metastatic spread to the lymph nodes and liver^[148]. The PI3K/AKT- pathway is well known for its pro-oncogenic and anti-apoptotic signaling and *PI3K* mutations are common in CRC and many other human cancers^[149]. As demonstrated by Leystra *et al*^[150], the intestinal expression of a constitutively active *Pi3k* (*PIK3ca*) is a sufficient driver mutation to induce rapid tumorigenesis with invasion of adjacent organs and addition of *PIK3ca* to a homozygous loss of *Apc*, drives adenoma-to-carcinoma progression with metastatic spread^[129]. Although loss of *Pten*, the counterpart of *Pik3*, does not affect intestinal cell proliferation, in the context of *Apc* deficiency or other mutations, it promotes cancer progression^[151-153]. *SMAD4* is

considered as a tumor suppressor, similar to other constituents of the TGF β -pathway^[154,155]. Since the genes *Apc*, *Smad2* and *Smad4* are all located on chromosome 18 in the mouse, they are suited to generate distinct cis- and trans compound heterozygotes by meiotic recombination. Compared to their single mutation littermates, mice with combined *Apc* and *Smad4* mutations, show accelerated tumor development^[156] and increased malignancy^[157,158]. In contrast, compound heterozygotes of *Apc* and *Smad2* mutations show no increased tumor development compared to littermates with a single *Apc* mutation^[159]. Notably, homozygous *Smad3* mutation leads to aggressive CRC with lymphatic spread and, upon *Apc* deficiency, drastically reduced life span^[160,161]; but *SMAD3* is rarely mutated in human CRC^[162]. Findings from several CRC-GEMM highlight the role of TGF β -signaling as a strong tumor suppressor, since *Tgfb1*^[163-165], as well as *Tgfb*-receptor 2^[140,166,167] knockout, induce local invasion and metastatic spread. Regarding the role of *TCF7L2* mutations in CRC, so far, no GEMM of CRC addressing this topic have been published. Besides the conventional adenoma-carcinoma sequence, the serrated pathway represents an alternative route of CRC development with distinct molecular and clinical features. The underlying *BRAFV600E* mutation occurs in 15%-20% of sporadic CRC, causes a constitutive activation of the MAPK/ERK pathway and is strongly associated with the CpG Island methylator phenotype and microsatellite instability (MSI) due to *MLH-1* methylation^[168-170]. *BrafV600E* causes crypt hyperplasia and combined with *Apc* or *P53* mutations, as well as mutations affecting *Ink4A/Arf*, gives rise to invasive carcinomas^[171-174]. Although not common in human CRC, mutation of the GSK3 β phosphorylation site causes degradation-resistant β -catenin^[175,176], and has been remodeled in the mouse. GSK3 β -resistant β -catenin increases proliferation of the intestinal epithelium and causes adenoma formation, but does not mediate malignant progression^[177-179]. Paralleling the research on canonical cancer pathways, there has been reasonable effort to reproduce MSI, a hypermutable phenotype caused by malfunction of DNA mismatch repair (MMR) enzymes^[180,181]. MSI-CRC can occur in the context of hereditary MMR gene mutations (LS) or can be detected in up to 15% of spontaneous CRC, caused by hypermethylation of MMR genes (spMSI)^[182,183]. The constituents of the MMR machinery have been extensively studied by somatic knockout models. Since the knockout of the MMR genes *MLH1*^[184], *MSH2*^[185,186], *MSH6*^[187] and *PMS2*^[188] predominantly cause hematopoietic malignancies with consecutive reduced lifespan in the mouse and consequently only a minor fraction of homozygotes develop intestinal neoplasia^[189,190], these knockouts were frequently put in an APC deficient setting, to increase intestinal carcinogenesis^[184,191,192]. Kucherlapati et al^[193,194] demonstrated, that mutations of *Fen1* and/ or *Exo1*, both cooperation partners of MMR enzymes, lead to similar patterns of MSI tumor development. However, MSI-high tumors rarely show activation of the Wnt-pathway and are typically chromosomal stable, whereas *APC* mutations are typically associated with a chromosomal unstable phenotype^[195,196]. Therefore, these models do not adequately recapitulate Lynch-type or spMSI tumors. To overcome the aforementioned obstacles, a floxed *Msh2* allele was combined with either a LS related missense mutation (*Msh2*^{G674D}) or an *Msh2* ^{$\Delta 7$} null mutation in mice carrying a *Villin-Cre* transgene, leading to intestinal carcinogenesis and chemoresistance typical for MSI-high tumors^[197]. An overview of the above addressed mutations can be found in **Figure 1** and are summarized in **Table 1**.

In summary, GEMM have contributed enormously to the understanding of the molecular processes of CRC initiation, progression and crosstalk of common cancer-associated pathways. Besides the models for spontaneous CRC and common cancer syndromes, like Familial Adenomatous Polyposis and LS, several models recapitulate metastatic disease, either as "classic" GEMM^[141,148,166,198] or upon viral *Cre* delivery^[129,130]. Yet, these models have several limitations. First of all, cancer development is a stepwise process with an initial driver mutation and subsequent acquisition of further mutations^[199], and thus can only be partly reflected in tumor mouse models by the combination of a constitutively active with an inducible mutation^[172]. Roper et al^[128] recently demonstrated *in vivo* genome editing of *Apc* and *P53* by viral delivery of the correspondent sgRNA in mice expressing CRISPR-Cas9 under the control of a *Villin-Cre* transgene. Second, the number of combined mutations is limited in the mouse, since the resulting phenotype shows often a drastically reduced lifespan^[152]. Triple mutant (*Car1*^{CreER/+}; *Apc*^{fl/fl}; *Kras*^{L^{SL}-G12D/+}) and quadruple mutant mice (*Car1*^{CreER/+}; *Apc*^{fl/fl}; *Kras*^{L^{SL}-G12D/+}; *P53*KO; *Smad4*^{fl/fl}) showed a reduced lifespan of merely one month. Moreover, GEMM are time consuming and expensive, since breeding of the transgenic mice often takes multiple generations and requires careful interbreeding to yield the desired alterations. In terms of animal welfare, it should be noted that the breeding process yields many "reject" mice, which are neither used for further breeding nor for research. Also, the construct of the transgene, or the viral vector, respectively, is complicated. While GEMM represent a valuable tool for basic

Table 1 Overview of genetically engineered mouse models

| Link to Figure 1 | Ref. | Methods | Results |
|------------------|-------|--|---|
| 1 | [127] | <i>loxP</i> flanked <i>Apc</i> exon 14 (<i>Apc</i> ^{580S}) Colorectal tumor induction by rectal infection with <i>Cre</i> -delivering Adenovirus (<i>AxSRaCre</i>) | Adenoma formation in the distal rectum in most of the <i>Apc</i> ^{580S} homozygotes. 50% of animals show invasive adenocarcinoma after 1 yr without lymphatic or distant metastases |
| 2 | [109] | <i>Apc</i> ^{Δ242/+} | Adenomas: Higher in numbers but smaller in size and no differences in histology compared to <i>Apc</i> ^{Min/+} mice |
| 3 | [110] | <i>Apc</i> ^{Δ14/+} | Shift of tumor distribution, more severe phenotype, invasion of muscularis propria, 50% dead after 12 mo |
| 4 | [120] | <i>Fabpl-Cre; Apc</i> ^{15lox/+} | Increased survival due to lower number of tumors, but larger tumors predominantly in the colon, 91% at least low-grade adenoma, 50% carcinoma; invasiveness and metastases not reported |
| 5 | [108] | <i>Apc</i> ^{+/D716} (C57BL/6J background) | Intestinal polyposis with emphasis on the small intestine |
| 6 | [126] | <i>Cac; Apc</i> ^{580S/+} | Transgene expression limited to the large intestine. Adenoma formation without malignancy |
| 7 | [125] | <i>CDX2P9.5-G22Cre</i> <i>Apc</i> ^{flax/flax} | Frameshifted <i>Cre</i> -recombinase with a long guanine nucleotide tract under control of the homeobox promotor <i>CDX2P9.5</i> leads to limited activation of <i>Cre</i> by spontaneous somatic mutations in the large intestine: <i>Apc</i> ^{flax/flax} homozygotes dye rapidly from florid polyposis of proximal colon and cecum |
| 8 | [374] | <i>Apc</i> ^{+/Δ716} <i>Cdx2</i> ^{+/-} | Increased adenoma formation in the colon, reduced number of polyps in the small intestine |
| 9 | [132] | <i>Apc</i> ^{+/flx1-15} ; Villin- <i>Cre</i> mice (conditional) and <i>Apc</i> ^{Δe1-15} constitutive null allele | More severe polyposis compared to <i>Apc</i> ^{Min/+} mice |
| 10 | [375] | <i>BubR1</i> ^{+/-} <i>Apc</i> ^{Min/+} | Increased tumor formation in the large intestine and higher malignancy through increased chromosomal instability (invasiveness and metastases not reported). Note, that <i>BUBR1</i> mutations are uncommon in CRC ^[376] |
| 11 | [377] | <i>Δ³⁷Ephb2; Apc</i> ^{Min/+} <i>Ephb3</i> ^{+/-} ; <i>Apc</i> ^{Min/+} <i>Ephb3</i> ^{-/-} ; <i>Apc</i> ^{Min/+} | Reduced tumor formation in the small intestine, but large adenocarcinomas of the colorectum |
| 12 | [133] | <i>CDX2P-CreER^{T2} Apc</i> ^{flax/flax} | Tamoxifen inducible <i>Apc</i> -knockout in the distal intestine |
| 13 | [378] | long living <i>Apc</i> ^{Min/+} mice | Some adenomas progress to adenocarcinomas |
| 14 | [104] | C57BL/6J <i>Apc</i> ^{Min/+} × SWR/J or C57BR/cdcJ | Hybrid <i>Apc</i> ^{Min/+} mice survive longer due to decreased adenoma frequency. After one-year high amount of invasive adenocarcinomas. 3% metastasis to lymph nodes |
| 15 | [105] | Change of the <i>Apc</i> ^{Min/+} genetic background from C57Bl6/J to A/J mice | Increased tumor formation in the intestine. 50% adenocarcinomas in the small intestine and 20% in the colon |
| 16 | [131] | <i>AhCre</i> ⁺ ; <i>Apc</i> ^{flax/flax} | β-naphthoflavone-inducible Cyp1A promoter <i>Cre</i> -transgene (<i>AhCre</i>). Rapid death upon induction due to disruption of intestinal architecture |
| 17 | [98] | <i>Apc</i> ^{Min/+} + AOM | 6-fold increase of colonic tumor formation compared to <i>Apc</i> ^{Min/+} mice |
| 18 | [379] | <i>Apc</i> ^{Min/+} + AOM | Increased incidence of colonic adenocarcinomas |
| 19 | [52] | <i>Apc</i> ^{Min/+} + DSS | High incidence of well differentiated colonic carcinomas |
| 20 | [380] | <i>Apc</i> ^{Min/+} + PhIP | 2- to 3-fold increase of tumor formation compared to <i>Apc</i> ^{Min/+} mice |

| | | | |
|----|-----------|--|--|
| 21 | [99] | $Apc^{Min/+}$ + AOM + DSS | Mainly small intestinal tumor formation |
| 22 | [111] | $Apc^{Min/+} P53^{-/-}$ | No increased adenoma formation or malignancy compared to $Apc^{Min/+} 53^{+/+}$ - and $Apc^{Min/+} P53^{+/+}$ -mice |
| 23 | [112] | $Apc^{Min/+} P53^{-/-}$ | No increased malignancy or adenoma formation compared to $Apc^{Min/+}$ mice |
| 24 | [381] | $Apc^{Min/+} P53^{-/-}$ | Slight, but not significant, increase in malignancy |
| 25 | [113] | $Apc^{Min/+} Mom1^{R/R} P53^{-/-}$ $Apc^{Min/+} Mom1^{R/S} P53^{-/-}$ | P53 deficiency increases intestinal adenoma multiplicity and malignancy |
| 26 | [139] | $Apc^{\Delta716} Trp53^{+/LSL \cdot R270H} Villin-CreER$ $Apc^{\Delta716} Trp53^{LSL \cdot R270H/LSL \cdot R270H} Villin-CreER$ | Homozygotes die rapidly from lymphoma while heterozygous $P53^{R270H}$ leads to invasive adenocarcinomas with features of EMT |
| 27 | [128] | Deletion of <i>Apc</i> and <i>P53</i> by viral delivery of corresponding sgRNA into $Rosa26^{LSL-Cas9-GFP/+}$; $Villin^{CreER}$ | <i>In vivo</i> editing of <i>Apc</i> alone or in combination with P53 via Cre mediated Cas9-expression and provision of sgRNA by viral infection of the colonic epithelium leads to tumor formation without metastatic properties |
| 28 | [382] | $Fabpl:Cre^{+/0} Tdg^{flx/-} Apc^{Min/+}$ | TDG knockout increases adenoma formation, no carcinomas |
| 29 | [122] | $AhCre^{+/T}; Kras^{+/LSLV12}$ $AhCre^{+/T}; Kras^{+/LSLV12}, Apc^{+/fl}$ | Cytochrome p450 mediated Cre expression in the liver and intestine induced by β -naphthoflavone (<i>AhCre</i>). $Kras^{V12}$ mutation does not alter the intestinal epithelium, but combined with APC-loss, accelerates tumorigenesis in the intestine. 17% of the tumors are invasive adenocarcinomas |
| 30 | [383] | $CDX2P9,5-G22Cre; Apc^{flx/flx}; LSL-Kras^{G12D}$ | Severe debilities in mice with reduced weight and lifespan and anal bulging. <i>Kras</i> mutation does not increase malignancy |
| 31 | [136] | $Fabpl-Cre; Apc^{2lox14/+}; Kras^{LSL-G12D/+}$ $Fabpl-Cre; Apc^{2lox14/+}; Nras^{LSL-G12D/+}$ | $Kras^{G12D}$, but not $Nras^{G12D}$ drives colon cancer progression. <i>Nras</i> indistinct from $Apc^{Min/+}$ mice |
| 32 | [143] | $shApc/Kras^{G12D}/P53^{fl/fl}/Lgr5$ | Mice with inducible and reversible <i>Apc</i> deletion via short hairpin RNA show duodenal and colonic tumor formation. Additional, conditional mutations drive cancer progression, but upon <i>Apc</i> restoration by withdrawal of doxycycline rapid tumor regression can be induced |
| 33 | [130,384] | $Apc^{CKO}/LSL-Kras$ | Cre-mediated knockout of <i>Apc</i> and $Kras^{G12D}$ activation by surgical application of AdenoCre to the colonic epithelium leads to adenocarcinomas with 20% liver metastases after 20 weeks |
| 34 | [385] | $Apc^{+/580S}, Kras^{+/LSL}, CAC^{+}$ | Only adenomas |
| 35 | [116] | $CMV-cre \times LSL-Kras^{G12D}$ $LSL-Kras^{G12D}; Fabpl-Cre$ | Germline embryonic expression of an endogenous $Kras^{G12D}$ allele is uniformly lethal. Diffuse colonic hyper- and dysplasia |
| 36 | [117] | $Kras^{+/N12} \times CMV-Cre^{+/T}$ | High embryonic lethality; adult animals succumb to pulmonary neoplasia, no phenotypic changes in the intestine |
| 37 | [134] | $LSL-Kras^{G12D}/Villin-cre +AOM$ | Increased number of microadenomas in the proximal colon |
| 38 | [198] | $Villin-Cre/K-ras^{G12Dint}/Ink4a/Arf^{-/-}$ | Within 12 wk progression to invasive adenocarcinomas (79%) with 60% lung metastases |
| 39 | [165] | $Apc^{\Delta716} Tgfb\beta 2^{flx/flx}; villin-CreER$ | Disruption of TGF β -signaling leads to locally invasive adenocarcinomas |

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|----|-------|--|--|
| 40 | [164] | AOM-treatment of <i>Fabp1^{45nt-132} Cre; Tgfbβ2^{flx/flx}</i> mice | Higher incidence of colonic adenomas and adenocarcinomas |
| 41 | [167] | <i>Villin-Cre; Apc^{1638N/nt}; Tgfbβ2^{flx/flx}</i> | Compared to <i>Apc^{1638N/nt}</i> similar tumor incidence but increased progression to locally invasive adenocarcinoma |
| 42 | [163] | <i>Tgfb1^{-/-} Rag2^{-/-}</i> | Rapid formation of adenomas and adenocarcinomas |
| 43 | [166] | <i>LSL-Kras^{G12D/nt}; Villin-Cre; Tgfbβ2^{E2flx/E2flx}</i> | Wnt-independent induction of invasive carcinomas in the intestine with 15% distant metastases |
| 44 | [153] | <i>Villin-Cre; Pten^{flx/flx}; Tgfbβ2^{flx/flx}</i> | Mice with inactivation of TGF β R2 combined with loss of PTEN show high number of mucinous adenocarcinomas throughout the intestine. 8% show distant metastases (not Wnt, but deregulation of CDK inhibitor expression). <i>Pten</i> loss without mutation has no effect |
| 45 | [152] | <i>Villin-CreERT^T; Apc^{flx}; Pten^{fl/fl}; Kras^{LSL/+}</i> | <i>Villin-CreERT; Apcf/+; Ptenf/fl; KrasLSL/+</i> mice show rapid morbidity due to invasive small intestinal tumors |
| 46 | [151] | <i>AhCre; Pten^{fl/fl}</i> | PTEN is dispensable in the intestinal epithelium, but increases tumorigenesis in the context of APC deficiency |
| 47 | [386] | <i>Apc^{Min/+} Pld1^{-/-} vs Apc^{Min/+} Pld1^{+/+} +AOM/DSS</i> | <i>Pld1</i> -knockout/suppression leads to decreased tumor burden |
| 48 | [157] | <i>Dpc4^{+/-}; Apc^{+/Δ716}</i> | <i>Dpc4</i> and <i>Apc^{Δ716}</i> cis-compound heterozygote mice show adenoma to carcinoma progression in the small intestine and colon with submucosal infiltration |
| 49 | [159] | <i>Smad2^{+/-}; Apc^{+/Δ716}</i> | Combination of <i>Apc</i> mutation and loss of <i>Smad2</i> leads to no changes in tumor size or properties compared to <i>Apc^{+/Δ716}</i> mice |
| 50 | [161] | <i>Apc^{Min/+}; Smad3^{-/-}</i> | Reduced lifespan of 2 months due to rapid tumor development in the distal colon with mixed histology but no metastases |
| 51 | [156] | <i>Apc^{+/Δ1638N}/Smad4^{+/ΔE6sal} (cis and trans)</i> | <i>Smad4</i> mutation leads to intestinal tumors without malignant properties. Both mutations lead to high tumor burden in the upper GI (cis>trans); some show invasion of the submucosa |
| 52 | [387] | <i>cis-Apc^{+/-}/Smad4^{+/-} Mmp7^{-/-}</i> | <i>Mmp7</i> knockout reduces tumor size but does not affect invasiveness |
| 53 | [388] | <i>Smad2^{+/-}; Apc^{580D/+} (cis)</i> | Larger tumors, higher incidence of malignant phenotype (compared to <i>Apc^{580D/+}</i> mice) |
| 54 | [160] | <i>Smad3^{-/-} (129/Sv genetic background)</i> | Adenocarcinomas of the intestine with penetration of the whole intestinal wall and lymphatic spread. Lower tumor burden in C57/BL6 \times 129/Sv <i>Smad3^{-/-}</i> hybrids. Note, that <i>Smad3</i> mutations occur only in 2% of CRC (Fleming <i>et al</i> ^[62] , 2013) |
| 55 | [179] | <i>Smad4^{fl/fl}; Catnb^{lox(ex3)/+}; Lgr5-Cre^{ERT2}-IRES-GFP</i> | Mosaic <i>Cre</i> -expression leads to adenoma formation |
| 56 | [178] | <i>Catnb^{+/Δlox(ex3)}; Krt1-19^{+/Δcre}</i> <i>Catnb^{+/Δlox(ex3)}; Tg-Fabp1^{Cre}</i> | Constitutional <i>Cre</i> -mediated excision of β -catenin phosphorylation site leads to a plethora of small intestine adenomas |
| 57 | [177] | <i>Villin-creER^{T2}/Catnb^{loxEx3/WNT}</i> | Expression of GSK3 β -resistant β -catenin leads to substitution of enterocytes by highly proliferative crypt stem cells (rapid death) |
| 58 | [142] | <i>Nicd/Apc^{+/Δ1638N}</i> | NOTCH-signaling does not influence adenoma formation |
| 59 | [141] | <i>Nicd/P53^{-/-}</i> | <i>Villin-CreER^{T2}</i> tamoxifen-dependent <i>P53</i> deletion in constitutively active NOTCH-signaling background leads to intestinal tumor formation and metastasis |
| 60 | [158] | <i>Car1^{CreER/+}; Apc^{fl/fl}; Kras^{LSL-G12D/+}; P53^{KO}; Smad4^{fl/fl}</i> | Rapid tumor formation in cecum and proximal colon, but high mortality in triple and quadruple mutants |
| 61 | [192] | <i>Msh2^{Δ7N/Δ7N}/Apc^{+/Δ1638N}. Msh2^{Δ7N/Δ7N}/Apc^{Min/+}</i> | Rapid tumor formation in the small intestine, early death (2-3 months), more tumors in <i>Msh2^{Δ7N/Δ7N}/Apc^{Min/+}</i> |
| 62 | [191] | <i>Apc^{Min/+}/Msh2^{+/-}; Apc^{Min/+}/Msh2^{+/-}; Apc^{Min/+}/Msh2^{-/-}</i> | Accelerated tumor formation in the small intestine in MSH2-deficient mice. Mice homozygous for <i>Msh2^{-/-}</i> dye rapidly from lymphomas |

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|----|-----------|---|---|
| 63 | [389] | $Kras^{V12}/Cre/Msh2^{-/-}$ | β -naphthoflavone inducible <i>Kras</i> mutation ($AhCre^{+/T}$, $Kras^{+/LSL.V12}$) combined with homozygous <i>Msh2</i> -knockout leads to increased number of intestinal adenomas. No carcinomas, relevant number of thymic lymphomas |
| 64 | [197] | VCMsh2 ^{LoxP/LoxP} VCMsh2 ^{LoxP/K674D} VCMsh2 ^{LoxP/null} | Villin-controlled <i>Cre</i> -expression leads to intestinal MMR-deficiency, similar to Lynch syndrome. 50% of tumors in the small intestine are malignant. A high proportion of carcinomas in VCMsh2 ^{LoxP/null} mice are resistant to cisplatin and FOLFOX |
| 65 | [185,186] | $Msh2^{-/-}$ | Death due to lymphoma |
| 66 | [194] | $Apc^{1638N/+} Exo1^{+/-} Fen1^{+/-}$ | Increased tumor multiplicity and incidence, higher progression to malignancy, high incidence of hematopoietic cancers |
| 67 | [193] | $Fen1^{null}/Apc^{1638N}$ | Increased malignancy of intestinal tumors compared to Apc^{1638N} mice through MSI |
| 68 | [184] | $Mlh1^{-}/Apc^{1638N}$ | Increased tumor incidence and multiplicity, 30% adenocarcinomas, reduced lifespan of 3.3 mo. High amount of extraintestinal tumors |
| 69 | [187] | $Msh6^{-/+}$ $Msh6^{-/-}$ | Reduced life span in hetero- and homozygotes due to lymphomas and gastrointestinal tumors. Tumors show no signs of MSI |
| 70 | [390] | $Msh3^{-/-}; Msh6^{-/-}$ | Decreased life span, death due to intestinal adenocarcinomas or lymphomas |
| 71 | [190] | $Msh6^{TD/TD}; Msh6^{TD/+}$ | B or T cell non-Hodgkin lymphomas, adenomas of the small intestine, basal cell carcinomas |
| 72 | [388] | $Pms2^{-/-}; Pms1^{-/-}$ | <i>Pms2</i> -deficient mice develop lymphomas and sarcomas, but no intestinal tumors; <i>Pms1</i> deficiency does not cause tumor development |
| 73 | [150] | $Fc^{\pm}; Pik3ca^{*+} (FVB/N-Tg(Fabp1-Cre))1Jig;$ $Gt(ROSA)26Sor^{tm7(Pik3ca^*,EGFP)Rskv}$ | Constitutively active PI3K causes mucinous adenocarcinomas of the proximal colon with infiltration of the whole intestinal wall |
| 74 | [129] | $Apc^{fl/fl} Kras^{G12D/+} Pik3ca^{p110^*} + Cre$ -Adenovirus via coloscopic injection | Additional driver mutations do not increase tumor proliferation, but cause progression to adenocarcinoma and metastatic disease |
| 75 | [172] | $Apc^{CKO/CKO}; Braj^{cA/+}$, AdenoCre delivery via colonoscopy | <i>Cre</i> -mediated <i>Apc</i> -knockout combined with latent $Braj^{600E}$ cause neoplasia of the distal colon (50% adenocarcinomas) |
| 76 | [174] | Villin-Cre; $Braj^{\Delta SL-V637E/+}$ Villin-Cre; $Braj^{\Delta SL-V637E/+}; P53^{LSL-R172H/+}$ Villin-Cre; $Braj^{\Delta SL-V637E/+}; p16^{Ink4^*}$ | Some invasive adenocarcinomas (13%), dominant negative P53 mutation leads to 60% cancers with 2% metastases. Also, $p16^{Ink4^*}$ mutation causes carcinomas in a <i>Braj</i> mutational background |
| 77 | [171] | $Braj^{f/LSL-V600E}; AhCreER^{T+/6} x$ $Cdkn2a (Ink4a/Arf)^{\Delta Ex2,3} = VE; Cdkn2a^{\Delta Ex2,3/\Delta Ex2,3}$ | <i>CypA1</i> -promotor-driven, tamoxifen-inducible <i>Cre</i> -recombinase facilitates $Braj^{600E}$ expression in the small intestine with consecutive crypt hyperplasia. Repression of $p16^{Ink4^*}$ leads to tumor formation in various tissues and decreased survival (6 wk) |
| 78 | [140] | $Apc^{\Delta 716} (A), Kras^{+/LSL-G12D} (K), Tgfb2^{lox/lox} (T),$ $Trp53^{+/LSL-R270H} (P), Fbxw7^{lox/lox} (F),$ and Villin-CreER | <i>Kras</i> mutation increases multiplicity of tumors, whilst P53 gain-of-function mutation and <i>Tgfb2</i> -knockout leads to invasiveness, no spontaneous metastases |
| 79 | [147] | $Apc^{Min/+}; Fbw7^{\Delta G}$ | Reduced survival for <i>Fbw7</i> deficient mice, also in heterozygous setting |
| 80 | [148] | $Fbw7^{lox/lox}, P53^{lox/lox}, Villin-Cre$ | Aggressive carcinomas with metastatic spread to lymph nodes and liver |
| 81 | [391] | $Mutyh^{-/-}$ | Spontaneous adenoma and adenocarcinoma development in the intestine; predominantly in the upper small intestine. Tumorigenesis increased by oxidative stress (KBrO ₃) |

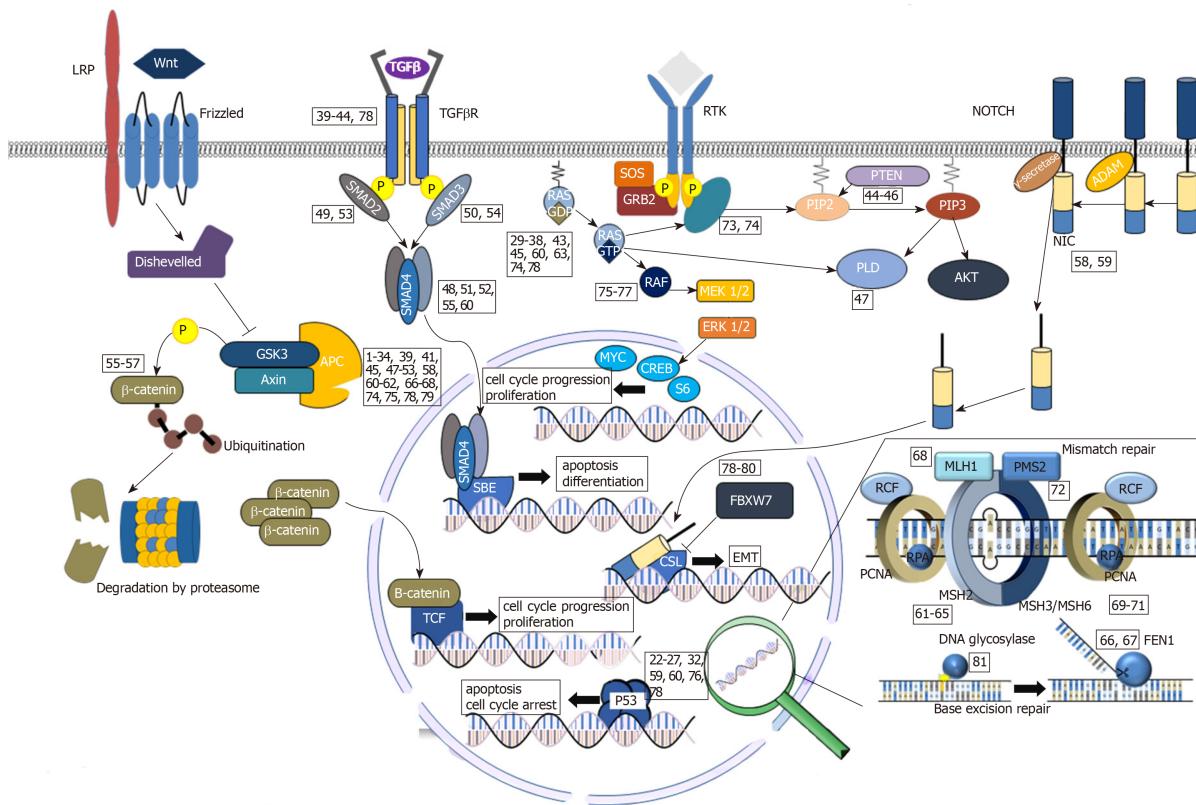


Figure 1 Overview of the frequently altered pathways in colorectal cancer. The numbers in square brackets label the corresponding model descriptions as given in **Table 1**.

research, their use for preclinical studies is limited due to a lack of genetic heterogeneity on the one hand, and discrepancies to the human tumor development on the other hand. On a final note, it should be added, that GEMM of CRC can be applied the other way round: Mice harboring mutagenic *SB* transposons were crossed to mice expressing *SB* transposase under control of a *Villin-Cre*-transgene, to generate mice, that develop intestinal lesions due to random insertional mutagenesis and can be screened for unknown CRC driver mutations^[200]. Moreover, by combining this approach with well-known driver mutations, new pathway-associated mutations could be identified^[201,202].

TRANSPLANT MODELS FOR COLORECTAL CANCER

Transplant mouse models can be classified in various ways: Syngeneic tumor transplantation is characterized by tumor tissue or cancer cell line engraftment within the same mouse strain; whereas xenogeneic grafts are derived from a different mouse strain or human donors. Additionally, it can be distinguished between heterotopic and orthotopic models. As cancer grafts, tumor cells, organoids and tumor tissue pieces can be employed. In 1876, Novinsky successfully transferred tumors (likely canine venereal sarcomas) from one dog to another in two independent trials^[203]. Shortly thereafter, Hanau^[204] and Morau (reviewed in^[205]) reported independently the successful passaging of epithelial rodent tumors. Around the turn of the century, several transplantable rodent tumors, mostly of a sarcomatoid phenotype, were established^[206]. Later, the discovery of the human leukocyte antigens and oncogenic viruses elucidated the results of the earlier transplantation studies^[207,208]. Toolan pioneered in the xenograft field by attenuating graft rejection through X-radiation and cortisone treatment^[209,210].

Animals for transplant models

A fundamental prerequisite for the successful engraftment of xenogeneic tissue in mice is the impairment of the host immune system. A detailed explanation of the development and sophistication of immunocompromised mice would fill several pages and is exquisitely reviewed elsewhere^[211]. In short, with the discovery of “nude” mice (recessive mutation of *FOXP1* leading to hairlessness and athymia)^[212] and the

subsequent breeding of NMRI^{nu/nu} mice, xenotransplantation was possible for the first time without additional immunoablative treatment^[213]. A more immunodeficient animal strain, C:B-17 scid, carrying a homozygous mutation of the *Prkdc* gene resulting in a lack of functional B and T lymphocytes, was established by Bosma *et al*^[214] in 1983. To overcome NK cell function, SCID mice were crossed with non-obese diabetic mice (NOD)^[215] by several workgroups generating NOD/LtSzscid^[216], NOD/LtSz-scid $\beta 2m^{null}$ ^[217] and NOD/Shi-SCID mice^[218]. However, the life span of NOD/SCID mice is limited by the development of thymic lymphomas^[219] and they show relevant “immune leakiness” caused by spontaneous rearrangement of T and B cell receptors^[220,221]. Lastly, to abolish NK cell activity completely, deficiency of the IL-2 receptor subunit gamma (IL2R γ) and the Janus kinase 3 (Jak3) were introduced to NOD/SCID mice generating the commonly used strains NOG (NOD/SCID/IL2R $\gamma^{tm1.5ug}$)^[222], NSG (NOD/SCID/IL2R γ^{tm1Wjl})^[223] and NOJ (NOD/SCID/Jak3^{null})^[224]. Since the DNA-dependent protein kinase catalytic subunit, encoded by the *Prkdc* gene, is also responsible for DNA repair, SCID mice are very sensitive for radiation and DNA-damaging agents. Therefore, Shultz *et al*^[225] developed the more robust, but equal immunodeficient NOD/LtSz-Rag1^{null}Pfp^{null} strain. Since then, many more immunodeficient strains, with in part different genetic backgrounds, have been developed^[211,226]. In general, it can be noted, that the more severe immunodeficient the host, the higher are the engraftment rates. Nevertheless the NMRI^{nu/nu} strain is still of high relevance for xenografting: The strain is less prone to opportunistic infections^[227], more tolerant for chemotherapeutic agents and shows reasonable engraftment rates for primary patient-derived xenografts (PDX) and good engraftment rates for subsequent mouse to mouse passaging^[228,229]. An economical plus is that this mouse strain is the least expensive immunodeficient one. It is mandatory to house immunodeficient mice in a specific pathogen free environment, using sterile techniques and microisolator caging. For syngeneic mouse models, viz. transplantation of cell or organoids from mice, exact strain conformity of donor and recipient mouse must be guaranteed, since even closely related substrains can differ genetically^[230].

Heterotopic tumor models

The advantages of s.c. tumor engraftments are glaring: They require nominal surgical skills, allow high throughput of samples due to time efficacy and tumor growth can be monitored by the naked eye. Early s.c. models of solid tumors were mostly carried out by injection of a tumor cell suspension into the mouse flank. Although some of these models correctly predicted clinical response for specific cancer entities and therapeutics^[231] large drug screens revealed that these models are of rather modest value for the prediction of clinical response in humans^[232-235]. Moreover, the resulting tumors from a homogenous cell suspension do not reflect the intratumor-heterogeneity and an adequate tumor microenvironment is also absent^[236]. Especially established CRC cell lines show low genetic diversity due to high passage and selective pressure^[237]. On a site note, one of the most cited CRC cell lines, HCT116, was established almost 40 years ago; enough time for mislabeling, cross-contaminations and high passage selection^[238,239]. While tumor cell lines in general remain a cornerstone of cancer research^[240,241], their heterotopic *in vivo* application creates no scientific added value. The creation of a s.c. tumor graft as intermediate step for subsequent orthotopic transplantation can be considered as an exception. Even though these cell-derived grafts are frequently referred to as PDX in the literature, we believe that this term should be avoided and “cell line-derived xenograft - CDX” is more reasonable. The implantation of tumor cells or tumor pieces under the renal capsule follows the rationale that the high vascularized environment propagates better engraftment^[242,243] and was historically used for the subrenal capsule assay^[244]. While this model might be advantageous for some cancers^[245], we see no advantage for CRC engraftment over the s.c. PDX model, a fortiori comparing practical effort, monitoring of tumor growth and animal welfare. Intravenous injection of cell suspension is often used to simulate hematogenous dissemination of tumor cells^[246,247], but circumvents crucial steps of metastasis, namely degradation of the surrounding tissue and lymphatic and/or vascular invasion^[248]. Thus, the same applies to the splenic injection of tumor cells to generate liver metastasis^[249,250] or the intraperitoneal injection to simulate peritoneal carcinosis^[251].

PDX models

PDX differ greatly from the aforementioned heterotopic tumor models. They are established by the s.c. implantation of a tumor piece from a surgical resection or biopsy into the flanks of immunocompromised mice; with lower tumor take rates for biopsy samples^[237]. Tissue can either be implanted directly after resection or cryopreserved in fetal calf serum containing 10% DMSO for implantation at a later

time^[229]. Incubation of the tissue in Matrigel® prior to tumor implantation, significantly increases engraftment rates^[228]. In order to obtain sufficient tumor tissue for larger scale studies, the resulting tumor can be fragmented and re-grafted subsequently. In recent years, our workgroup focused on the build-up of a large CRC biobank consisting of more than 140 PDX-models (general procedure is presented in Figure 2). This PDX panel reflects adequately the clinical and molecular heterogeneity of the patient population undergoing surgical resection of primary or metastatic CRC^[252]. It is well accepted that PDX closely recapitulate the histology of the original “donor” tumor over several passages^[253-255] and are also genetically stable^[256,257]. However, Ben-David *et al*^[258] demonstrated recently that changes in copy number alterations occurred in early passages of PDX tumors compared with both the P0 PDX and the donor tumor. A common criticism in heterotopic mouse models is related to the absence of tumor-stroma interaction or the “tumor microenvironment”^[259]. While this is true for immune cells, the stromal component remains intact in PDX^[255]. Although the human stromal compounds (fibroblasts, blood vessels *etc.*) are quickly and steadily replaced by their murine counterparts, the overall architecture of the tumor remains unaffected in the majority of cases^[260,261]. Moreover, these murine stroma cells adopt and maintain a human-like metabolic phenotype^[262]. These features indicate that PDX are indeed good and valuable models for preclinical testing of conventional and novel anticancer agents in the era of personalized cancer therapy^[263,264]. A proof of concept study with advanced refractory cancers demonstrated that drug responses measured in a PDX model, can be used for successful clinical decision making^[265]. A PDX model of CRC metastases closely resembled the efficacy of cetuximab and identified druggable targets in resistant tumors^[266]. Moreover, a PDX clinical trial approach (one animal per model per treatment) reflects well the heterogeneity of the patient population and allows testing of new drugs and combinatorial regimen^[267]. A large study with over 1000 PDX models confirmed the consistency between clinical and PDX clinical trial drug response^[268]. The ultimate goal of precision medicine would comprise of the profound genetic and functional characterization of a given tumor to identify relevant drug targets and subsequent validation of potential therapies with aid of a PDX bearing “avatar” mouse to provide the most efficient treatment for the individual patient^[264,269]. Currently, several clinical trials following this approach for colorectal^[270,271] and pancreatic cancer are recruiting^[272]. Yet, a median duration of 12.2 mo until PDX model establishment^[237] remains an unsolved issue for patients urgently in need for treatment. When it comes to drug testing, it should be considered, that immunotherapy approaches can only be restrictedly evaluated in immunodeficient host mice. A further disadvantage of PDX models is a potential selection for more aggressive tumors. The data concerning the association between successful PDX engraftment and clinical or molecular features is in part conflicting. While we and others could not find significant associations between tumor grading and PDX engraftment^[237,252], a Korean study observed significant correlations with tumor staging and grading^[273]. A smaller study with a high PDX establishment rate found a significant correlation of PDX success with positive nodal status and grading^[274], while Julien *et al*^[275] only found significances for the combination of a positive nodal status with an elevated carcinoembryonic antigen level. Moreover, we observed a significant correlation between PDX engraftment and molecular features like *KRAS* and *BRAF* mutations as well as MSI^[252]. Apart from the tumor biology, choice of the host mouse strain, repeated attempts of engraftment, quantity and quality of the resected tissue as well as previous treatment of the patient are additional factors influencing the success of model establishment. Collins *et al*^[276] recently reported PDX engraftment rates varying from 14 to 100% with a median PDX establishment rate of 68% reviewing 14 CRC-PDX studies. Compared to establishment rates for primary CRC cell lines of about 10%, the PDX is clearly superior^[252,277]. A selection bias relating to cancer biology can be diminished by increasing the number of enrolled patients and molecular characterization of the individual PDX. In fact, a very recent PDX study found an underrepresentation of the consensus molecular subtype number 2, due to worse engraftment^[278]. Another crucial pitfall of the PDX model is the development of EBV-associated lymphomas at the implantation site, which can mimic successful engraftment^[279]. Depending on the mouse strain and cancer entity, between 2.3% (colorectal) and 75% (prostate cancer) of primary engrafted bona fide xenografts turn out to be human de-novo lymphomas^[280-284]. Since this condition is more frequently reported in NSG and comparable strains, development of de-novo lymphoma in NMRI^{nu/nu} mice might be hindered by high NK cell activity^[285]. Thus, after successful engraftment of a PDX in NSG mice, we conduct the subsequent passaging in NMRI^{nu/nu} mice and xenograft histology is frequently evaluated by an experienced pathologist. Interestingly, Butler *et al*^[286] significantly reduced the frequency of lymphomas in an ovaria cancer PDX model by a unique dosage of rituximab during implantation. Additionally, PDX serve not only in the

field of therapy development; they are also a vital tool for the maintenance of a healthy biobank, allowing the establishment of secondary cell lines and supply sufficient tumor samples for the exchange with other work groups^[252].

In summary, we consider the PDX model as keystone of cancer research, holding great potential in the developing field of precision medicine for CRC.

Orthotopic models

Orthotopic CRC models are implemented to overcome the drawbacks of heterotopic models, videlicet lack of an adequate tumor microenvironment and metastatic behavior.

At the beginning of the 1980s, the first orthotopic engraftments of CRC cell lines was reported by Snipes^[287], demonstrating the feasibility of an intramural cell injection causing locally invasive cancer growth. A few years later, Bresalier *et al*^[288] demonstrated that the orthotopic injection of human CRC cells causes metastases in the liver. In 1991, Fu *et al*^[289] successfully engrafted 13 patient-derived tumors by removing the serosa and sewing the tumor pieces to the cecal wall. Beside the local tumor outgrowth, lymph node and liver metastases were reported for a few mice. Since the murine cecum is relatively large compared to its human counterpart and readily accessible by laparotomy, it rapidly became the favored site for orthotopic engraftment by different approaches. Many authors generated a s.c. cell graft in a donor mouse, of which a small piece was sewed to the cecum after damaging the serosa^[290-292]. Several varieties of this technique can be found in the literature: While some authors removed the mucosa and sewed the tumor pieces onto the cecal wall^[293,294], others formed a subserosal tunnel for tumor inoculation, which was afterwards closed by a suture^[295] or surrounded the tumor piece with a “pouch” formed by a serosal duplication^[296]. Others reported the technically more challenging subserosal injection of a cell suspension into the cecal wall^[297]. The cell injection method is often referred to as subserosal^[298], while other work groups describe a submucosal injection^[299]; both frequently with Matrigel® addition to the cell suspension to avoid cell spillage^[300]. Considering the gauntness of the cecal wall, we will here refer to both techniques as “intracecal” injection. The possibility of a “preconditioning” *via* s.c. PDX has also been described for the cell injection approach^[301]. Beside the cecum, other colonic sites like the descending colon can be accessed for cell injection^[302,303]. Despite frequent metastases, some aspects of these techniques may be viewed critically. First, given the fact that CRC arise from the mucosa, these models cannot be considered as genuine orthotopic and also the injection approach might mimic an advanced CRC. Second, they allow the contact of cancerous tissue with the abdominal cavity, hence it cannot be ruled out that some of the metastases are the result of intraabdominal cell spillage. Moreover, these approaches require the opening of the abdomen and can cause inflammation and morbidity. Nevertheless, the cecal orthotopic model is frequently applied, especially for basic research to identify the underlying mechanisms of metastatic progression, since it can render metastatic, end stage disease within a few weeks^[304]. The upregulation of genes associated with advanced CRC, could also be observed in liver metastasis from an orthotopic model^[290,305]. The orthotopic approach allows to elucidate the role of certain molecular pathways by direct comparison of the metastatic properties of a given cell with their genetically engineered counterpart^[306-310]. The additional transfection of these cells with a reporter, like GFP, DsRed or luciferase, allows monitoring of tumor progress by *in vivo* imaging^[311-313]. The orthotopic approach can be used in transgenic mice to clarify the role of distinct molecules^[314] or certain cell types^[315]. To further stress the functionality of stromal components, a co-injection of tumor cells and stromal cells is feasible^[316-318]. By using NSG mice, the efficiency of an immune cell-based therapy can be tested in the context of such an orthotopic model^[319]. Furthermore, circulating tumor cells (CTC) can be isolated from murine blood^[320]. In addition to the above discussed surgical approaches, there are less invasive concepts that do not require surgery and diminish the risk of intraabdominal cell spillage. Kashtan *et al*^[321] demonstrated the successful engraftment of murine tumor cells by submucosal injection in the distal rectum in 1992 and this approach was adapted by several work groups^[322-324]. To reach more proximal parts of the rectum or the descending colon, submucosal cell application can be performed *via* small endoscopic instruments^[325,326]. Depending on the cell line, liver metastases have been observed^[327]. Lastly, tumor cells can be inoculated in the colon mucosa by the acid enema approach described by Kishimoto *et al*^[328]; a true orthotopic model with intramucosal tumor development and liver metastases. In brief, the mucosa is damaged by 4% acetic acid enema and after neutralization with PBS, a tumor cell suspension is instilled and the anus temporarily sealed. Hite *et al*^[329] subjected all three orthotopic models (intracecal injection, transanal submucosal injection and acid enema) to a direct comparison. They found the submucosal

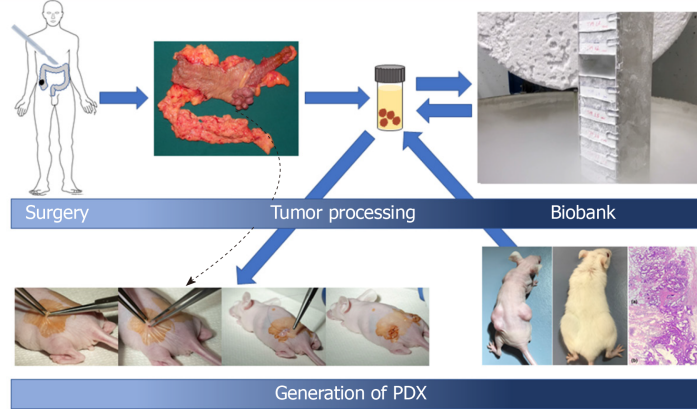


Figure 2 Establishment of PDX. After surgery, a small sample of the tumor, which is not needed for pathological diagnosis, is obtained and cut into pieces of 27 mm³. These can be either implanted immediately in recipient mice or vitally cryopreserved in liquid nitrogen. The resulting patient-derived xenograft (b) closely reflects the histology of the donor tumor (a) (Previously published in^[229]). Patient-derived xenograft can be further processed for subsequent implantation or cryopreservation.

injection to be the most efficient in tumor formation and metastatic behavior and at the same time well tolerated by the animals. In contrast, the acid enema approach showed the lowest tumor formation frequency but a considerable mortality of 15%. Enquist *et al*^[312] pursued a different concept by sewing a tumor piece directly to the mucosa by creating an artificial rectal prolapse. They were able to engraft pieces from transgenic *ApcMin/+;KrasLSLG12D/+;Villin-Cre* adenomas in the colon of syngeneic animals and a small subset of these tumors progressed to carcinomas. More strikingly, they managed also to transfer s.c. human PDX to the colons of NSG mice reflecting stage-dependent biological behavior as lymph node metastases could be observed for stage III PDX^[312].

Today, orthotopic models are copiously used for the *in vivo* validation of new therapeutic compounds and as a proof-of-principle approach^[330-333]. However, their clinical relevance is limited by the common use of similar, poorly differentiated cell lines. The orthotopic PDX model is promising, but its tumor take rate is not higher as in the s.c. PDX model. A very recently published, “crossover” concept comprising the s.c. engraftment of a PDX, followed by enzymatic disintegration of the PDX to a cell suspension and subsequent orthotopic injection into the rectal submucosa yielded an engraftment rate of 70% for s.c. PDX and 46% for the orthotopic model. Moreover, a metastatic spread was observed for 60% of the tumors successfully engrafted orthotopically^[334].

RECENT DEVELOPMENTS AND FUTURE PERSPECTIVES

The increasing field of precision medicine has a growing need for highly translational cancer models. Conversely, the increasingly negative public perception of animal studies constrains the scientific community to further stress the 3R-principle (replacement, reduction and refinement) in cancer research^[335]. Aside from the improvement of *in vivo* models, this implies the refinement of *in vitro* methods as well.

Humanized mice are severely immunocompromised mice, which can be reconstituted with various types of human bone marrow-derived cells or CD34⁺ hematopoietic stem cells^[336,337]. Since human stem and progenitor cells can be attained from umbilical cord blood or from peripheral drawn blood samples after GM-CSF treatment and cultured *in vitro*^[338], these cells can be transferred to sub-lethally irradiated NSG mice. Morton *et al*^[339] observed that PDX of head and neck cancer engrafted into these, so called “Xact mice”, are infiltrated with human B and T lymphocytes. Many transgenic mice further support engraftment with CD34⁺ stem cells by overexpression of human interleukins and signaling molecules. Thus NBSGW^[340], hIL2-NOG^[341], NSG-SGM3^[342] and SRG-15 mice^[343] have been introduced recently and antitumor effects against PDX of different cancers could be observed^[341,343]. These models hold great promise for the research of CRC immunotherapy, especially for highly immunogenic hypermutated CRC. Capasso *et al*^[344] showed very recently that check point inhibition with nivolumab leads to growth inhibition of human MSI-H PDX thereby accurately reflecting the clinical response of this CRC subtype. In contrast, no sustainable growth inhibition was observed in MSS

tumors or MSI-H tumors in “standard” NSG mice^[344,345].

Although patient-derived cell cultures are a valuable tool for high-throughput drug screenings, they exhibit considerable shortcomings^[346]. First, the establishment rate of primary patient-derived CRC cells with conventional 2D culturing methods approximates some 10%^[252,275], although higher success rates of 40% and more can be found in the literature^[347,348]. Second, conventional 2D cultures change the biological properties of cells, possibly altering drug response *in vitro*^[349]. Cell polarization, lack of stroma and abundance of growth factors, nutrients and oxygen are factors that might change the behavior of tumor cells^[350]. In recent years, more complex cell culturing methods have emerged. CRC cells, cultured in an extracellular matrix form three dimensional spheres, so called spheroids, that differ in their biological properties from 2D cultured cells^[351]. In contrast, organoids are three dimensional structures, derived from intact tumor pieces or tumor stem cells cultured in an extracellular matrix scaffold^[352]. Patient-derived organoids (PDO), quite similar to PDX, recapitulate closely the histological and genetic properties of their parental tumors. Moreover, high rates of successful establishment have been reported^[353] and reliable drug response prediction seems possible^[354-356]. PDOs can be implanted s.c. or orthotopically into mice, resulting in PDO xenografts (PDOX)^[264,354,357]. Furthermore, the Clevers group pioneered in the inauguration of non-malignant intestinal organoids exploiting the stem cell niche *in vitro*^[358]. These organoids can be modified *in vitro* to exhibit malignant properties and used to enlighten the role of cancer-driving pathways by *in vivo* engraftment^[359,360]. The available data strongly suggests, that PDOs reflect more faithfully the biological virtue and drug response of the parental tumor compared to conventional 2D cell cultures^[352]. The circumstance, that organoids can be derived from CTC, could render them an excellent tool for the preclinical testing of patients with advanced stage cancer that do not undergo surgery^[361]. Additionally, CTC reflect genetic changes associated with acquired drug resistance during chemotherapy^[362]. Further steps to a reduction of animal experiments imply the faithful remodeling of the host organism *in vitro*. Several research groups created a “cancer on a chip” model that combines the advances of 3D cell culture connected with artificial organs that resemble the most common organs of metastatic spread^[363]. Miller and Shuler introduced a “body on a chip model” with 14 artificial organs, which could be modified for cancer research^[364]. At last, the widely acknowledged work of Guinney *et al.*^[365] regarding the consensus molecular subtypes of CRC draw great attention to the value of computed models in cancer science and the capabilities of bioinformatic research. Retrospective analysis of clinical trial samples partly demonstrated the association of drug response with molecular subtypes^[366]. The constantly growing knowledge of cancer pathways and their crosstalk on the one hand, and the increased inter-individual complexity of tumors on the other hand, call for a method to integrate and interpret the overwhelming amount of data^[367]. *In silico* methods, like data mining, pattern recognition, machine learning and network approaches, are able to predict the behavior of “virtual” HCT116 cells^[368], can reveal genetic patterns associated with survival^[369], can be used to detect new biomarkers^[370], allow the identification of unknown driver mutations^[371] and potential preclinical compounds^[372]. Yet, *in silico* models often lack explanatory power and need careful interpretation by bioinformaticians. Their ability to correctly predict treatment response for an individual patient to a new compound is still a long way off^[373].

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