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DISSERTATION

Colitis-associated colon tumorigenesis is suppressed

in transgenic mice rich in endogenous n-3 fatty acids

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Abkürzungen /　Abbreviations

5-ASA	5-aminosalicylic acid		**LT**	Leukotriene
AA	Arachidonic acid		**LX**	Lipoxin
AOM	Azoxymethane		**MAPK**	Mitogen activated protein kinase
APC	Adenomatous polyposis coli		**MSI**	Microsatellite instability
CAC	Colitis-associated colorectal cancer		**N-3**	Omega-3
			N-6	Omega-6
CD	Crohn´s disease		**NEMO**	= IKKγ
CIN	Chromosomal instability		**NF-kB**	Nuclear factor kappa B
COX	Cyclooxygenase		**NO**	Nitric oxide
CRC	Colorectal cancer		**NOS**	Nitric oxide synthase
DALM	Dysplasia-associated lesion or mass		**NSAID**	Non-steroidal antiinflammatory drugs
DCC	Deleted in colorectal carcinoma		**PCR**	Polymerase chain reaction
			PG	Prostaglandin
DHA	Docosahexaenoic acid		**PPAR**	Peroxysome proliferator-activated receptor
DMH	Dimethylhydrazine			
DPC4	Deleted in pancreatic cancer (= Smad4)		**PUFA**	Polyunsaturated fatty acids
			RONS	Reactive oxygen and nitrogen species
DSS	Dextran sodium sulfate			
ELISA	Enzyme-linked immunosorbent assay		**RvE1**	Resolvin E1
			Smad	Protein homolog of both the SMA (*C.elegans*) and MAD (*drosophila*) protein
EPA	Eicosapentaenoic acid			
FAP	Familial adenomatous polyposis (coli)		**TGF-β**	Transforming growth factor beta
HNPCC	Hereditary nonpolyposis colorectal cancer		**TLR**	Toll like receptor
			TNBS	2,4,6-trinitrobenzene sulfonic acid
IBD	Inflammatory bowel disease			
			TNF-α	Tumor necrosis factor alpha
IkB	Inhibitor kappa B		**UC**	Ulcerative colitis
IKK	IkB kinase		**VEGF**	Vascular endothelial growth factor
IL-1	Interleukin 1			
LPS	Lipopolysaccharide		**Wt**	Wild type

1. Einleitung / Introduction

The work presented here concentrates on the effects of omega-3 polyunsaturated fatty acids (n-3 PUFA) in colorectal cancer that evolves from chronic colitis (see 1.2). Besides that and with respect to the complexity and multiple interactions of the involved events, this scientific paper aims to provide a detailed view on colonic inflammation and carcinogenesis in general, and on the role of a distinct tissue fatty acid status. To investigate n-3 PUFA action in this setting, we used an experimental animal model of colitis-associated colorectal cancer (CAC), applied to a recently engineered transgenic mouse high in endogenous n-3 PUFA.

1.1   Colorectal cancer

1.1.1 Epidemiology and etiology

Colorectal cancer (CRC) is the second most common cancer in Germany. The number of newly diagnosed cases per year for men and women is estimated at more than 35.000 each and the average age of diagnosis is 69 years in men and 75 years in women[1]. Ninety percent of CRC occur after age 50, due to accumulation of random somatic mutations[2]. Moreover, colorectal cancer is the second leading cause of cancer-related deaths in the German population **(Figure 1.1)**. The cumulated survival rate is reported at 60% among both men and women[1].
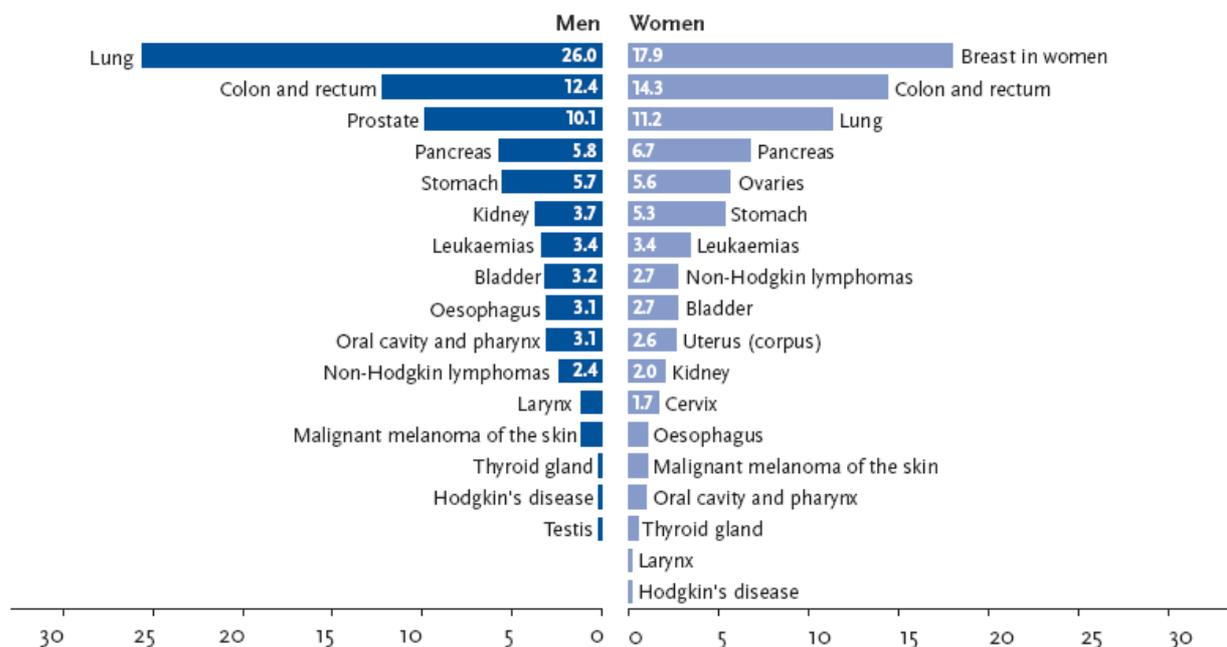
Figure 1.1: *Sites of cancer-related deaths in Germany in 2004* (in percent)[1].

Worldwide, about 1 million people are diagnosed with CRC every year[3]. The frequency of CRC shows a considerable geographic variation with high incidences in industrialized countries, whereas South America and China have a low incidence. This is predominantly attributed to national differences in diet and environmental factors[4]. A diet high in saturated fat, a high intake of red meat and a low intake of vegetables increase the risk of sporadic colorectal cancer, as do regular alcohol consumption, overweight and lack of exercise[1]. In accordance with our present work, multiple studies indicate that n-3 PUFA might have beneficial effects in CRC prevention[5,6]. Furthermore, it has been proposed that dietary fibres reduce the risk for CRC[7], however, this effect remains controversal[8]. Long term cigarette smoking increases CRC risk and mortality[9,10]. Conditions such as personal history of adenomas (the precursor lesion) or colorectal cancer, a first-degree relative with adenomas or with CRC also increase the individual risk of colorectal cancer[11].

Human cancer is assumed to be a genetic disease due to sequential accumulation of mutations in oncogenes and tumor suppressor genes[12]. In CRC, carcinogenesis is a multistep process from normal epithelium to dysplastic precursor lesions to carcinoma, regardless of the underlying etiology[13,14]. In fact, colon cancer can evolve from sporadic mutations, from an inherited predisposition or from a chronic inflammation (inflammatory bowel disease, IBD) in the colon (colitis-associated colorectal cancer, CAC).

1.1.2 Sporadic colorectal cancer

At least two major molecular events can account for the transition to sporadic colorectal cancer: Chromosomal instability (CIN, 85%) and microsatellite instability (MSI, 15%)[14-16]. CIN leads to abnormal segregation of chromosomes and aneuploidy. The CIN pathway is characterized by multistep LOH (loss of heterozygosity) on chromosome 5q (adenomatous polyposis coli, APC), 7p (p53) and 18q (deleted in colorectal carcinoma = DCC)[17]. Loss of APC function is among the earliest events in colorectal carcinogenesis, leading to activation of the Wnt / beta-catenin signalling pathway and subsequent transcription of target genes[18,19]. Furthermore, mutations of the K-ras oncogene have been detected in 15-68% of sporadic colorectal adenomas and in 40-65% of CRC[20]. Loss of the p53 tumor suppressor is a late event in tumor development and is believed to facilitate the progress from adenoma to carcinoma[17]. Having largely intact chromosomes, the MSI pathway of sporadic CRC results from defects in the DNA mismatch repair system[20]. These errors affect target genes such as

transforming growth factor beta (TGF-β), insulin-like growth factor II receptor (IGFIIR) and Bcl2-associated X protein (BAX). The resulting failure in colonocyte homoeostasis leads to malignant growth[21].

It is widely accepted that colorectal tumors manifest either through the CIN or the MSI phenotype, however, a subset of CRC is associated with both MSI and APC or p53 mutations[20]. Furthermore, it has been shown that epigenetic factors contribute to colon carcinogenesis. Molecular alterations such as the CpG island methylator phenotype (CIMP) involve promoter silencing and loss of gene expression[22,23].

The spectrum of somatic mutations in colon carcinogenesis seems to be even more extensive. A systematic sequencing analysis of colorectal cancers found that tumors harbored approximately 90 mutant genes, of which 69 genes were considered relevant to the pathogenesis of CRC[24].

1.1.3 Morphological aspects

The multistep transition to CRC involves a variety of precursor lesions (neoplastic polyps) with distinct histology, reflecting different malignant potential. There are two histologic types of polyps: hyperplastic and adenomatous. The vast majority of hyperplastic polyps seem to have no association with colon cancer[25,26], however, large polyp size (>1cm), high number (>20) and a family history of hyperplastic polyps or CRC are linked with cancer development[27]. Most CRC develop from adenomatous polyps (adenomas)[28,29], which are classified as tubular, villous or tubovillous. While villous adenomas account for only 10% of colorectal adenomas, they show the highest potential for malignant growth (up to 50%)[30]. While most of the diagnosed adenomas are polypoid, studies emphasize flat and depressed lesions to be more prevalent than recognized[31]. Recent findings furthermore suggest that discrete serrated polyps, some of them previously classified as hyperplastic polyps, may be genetically and morphologically distinct and caused by microsatellite instability, linking flat and dysplastic lesions directly to the pathogenesis of colorectal cancer[32]. The long held paradigm that all sporadic CRCs arise from the traditional *adenoma-carcinoma sequence*, has been challenged[33]. Also in the setting of chronic colitis, morphology of precursor lesions is distinct (see 1.2.3).

1.1.4 Inherited colorectal cancer

The hereditary familial colorectal cancer syndromes, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome), are rare. However, they are instructive for the understanding of genetic abnormalities leading to CRC and were instrumental in the elucidation of the CIN and MSI pathways. Both are characterized by an early age for CRC manifestation and predominant involvement of the right colon. They are inherited in an autosomal dominant manner. FAP is linked to mutations of the Adenomatous Polyposis Coli (APC) tumor suppressor gene and accounts for 1% of all CRC cases[30]. Hence, FAP can be attributed to the CIN pathway, where dysregulated beta-catenin signaling results from two hits in the APC gene[20,34]. Hundreds to thousands of adenomatous polyps (adenomas) can be found in the large bowel of patients with FAP, representing precancerous lesions with a 100% risk for developing CRC[30]. Affected patients with HNPCC show mutations of distinct DNA repair genes and microsatellite instability (MSI pathway)[35,36]. Among all CRC cases, 5% are related to HNPCC [30]. The medium age for CRC in HPNCC patients is 45 years.

Together with the hereditary syndromes of FAP and HNPCC, inflammatory bowel diseases (IBD) are among the top three high-risk conditions for CRC. This entity is described in greater detail in 1.2.

1.1.5 Therapy

Clinically, symptoms of CRC are rather unspecific. Bloody stool, meteorism, anemia, fatigue, ileus and fever can be indicators for colorectal cancer[30]. It has been shown that flat and polypoid lesions are precursors for the majority of colorectal carcinomas (1.1.3)[31,37]. Detection and removal of these pre-malignant lesions can decrease CRC incidence and prevalence in the population, indicating an important role for screening measures in CRC prevention. Stage 0 CRC (Carcinoma in situ) is limited to the mucosa and can be cured by local excision in most of the cases. In other stages, CRC treatment can include primary surgery, adjuvant chemotherapy and neoadjuvant radiation therapy (rectal cancer), according to the TNM classification of tumors. The 5-year-survival is strongly dependent on the surgeon´s experience and on CRC tumor stage, ranking from >95% (stage I) to 5% (stage IV)[30].

1.2   Colitis-associated colorectal cancer

1.2.1   Inflammation and cancer

In 1863, the German pathologist Rudolf Virchow first described a link between inflammation and cancer[38]. It is estimated that 15-20% of all cancer deaths are linked to underlying infections and inflammatory reactions[39].

The transition from normal to invasive neoplastic tissue progresses in two stages. First, somatic cells accumulate irreversible DNA sequence alterations (*initiation stage*). Second, continuous or repeated stimuli subsequently lead to induction of cellular proliferation, involving changes in the cellular microenvironment that promote tumor formation (*promotion stage*)[40]. The initiation stage of tumor growth is well defined by mutation of oncogenes, tumor suppressor genes, and other key regulators of cellular proliferation. The promotion stage, in which a multiplicity of cell types interact via secreted factors, seems to be more complex. Chronic inflammation is widely assumed to be instrumental in this promotion stage of tumor development[41,42]. Inflammation is a biological response that involves immune cell infiltration (macrophages, neutrophils, dendritic cells, T-cells), and the expression of soluble mediators such as cytokines, chemokines, growth factors, lipid mediators and matrix-degrading enzymes[39,43]. Subsequent resolution of inflammation and healing are associated with release of anti-inflammatory cytokines and chemokines. Chronic inflammation in most cases is a response to persistent injury and / or infection[40].

1.2.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) such as Crohn´s disease (CD) and ulcerative colitis (UC) is characterized by chronic relapsing inflammation predominantly in the large bowel. In addition to genetic and environmental factors, commensal bacteria and the innate immune system seem to be central contributors in IBD pathogenesis. There is growing evidence that chronic intestinal inflammation results from dysregulated immune responses to gut bacteria in the (genetically) susceptible host[44].

First described in 1925, colorectal cancer in IBD patients has long been recognized[45]. Unlike conditions such as FAP and HPNCC with well defined genetic predispositions, colitis-associated colorectal cancer (CAC) is related to chronic inflammation of the mucosa, a

hallmark of IBD[46]. A recent meta-analysis estimated the overall risk for CAC in UC patients at 3.7%, and at 5.4% in those with pancolitis[47]. The risk for CD patients to develop CAC remains controversial due to methodological biases in published studies. Hence, in patients with comparable duration and extent of disease, the risk of CAC is probably similar between CD and UC[48,49]. The risk is directly related to the *duration* of colitis. After seven years, CAC risk increases at a rate of 0.5-1% per year[46]. Moreover, there is a close correlation between *extent* of inflammation (colitis severeness) and CAC risk[46,50]. Although they represent only about 1% of all CRC cases, risk for CAC is approaching 20% with prolonged disease course (>20 years) and extensive colitis[51].

1.2.3 Morphological aspects

While sporadic CRC predominantly evolves from polypoid lesions, IBD-associated cancers typically arise from (non polypoid) elevated or flat dysplastic mucosa[52]. Elevated lesions, macroscopically referred to by the term DALM (dysplasia associated lesion or mass), can be subdivided into adenoma-like or non-adenoma-like (irregular, broad based or strictured), based on their endoscopic appearance[53,54]. In IBD-associated cancers, there is a higher rate of multifocal neoplasms, related to the distribution of mucosal inflammation[52].
Microscopically, dysplasia in IBD is defined analogous to neoplastic tissue in general: low-grade dysplasia (LGD, nuclei confined to basal) and high-grade dysplasia (HGD, nuclei appear more apically)[55]. In most of the cases, inflamed mucosa progresses to cancer from indefinite dysplasia (dysplasia not yet detectable) to LGD, HGD, and finally to CAC[52]. IBD-associated CRC (CAC) thus serves as an excellent model of inflammation-induced cancer and might even contribute to a better understanding of sporadic and hereditary CRC.

1.2.4 From IBD to CAC

The role of inflammation in colorectal carcinogenesis is supported by a recent cohort study including more than 30,000 IBD cases, where first-degree relatives did not show significantly increased risks for CRC[56]. However, family history of CRC was associated with a more than 2-fold risk for IBD patients to develop CAC[57]. These findings indicate that, as for sporadic cancer, both genetic and acquired factors are also relevant for CAC pathogenesis.

As in sporadic CRC, CAC arises from sequential episodes of somatic mutation and clonal expansion[17]. The occurrence of CIN and MSI cancers is estimated in analogy to sporadic CRC with 85% and 15%, respectively[58]. However, timing and frequency of alterations is different in CAC, compared with sporadic CRC[17] (**Figure 1.2.4.1**). APC loss is rare (less than 33%) and usually occurs late in the transition to CAC[59-61]. Indeed, allelic loss of the p53 tumor suppressor is more frequent in CAC (50-85%), suggesting an important role in carcinogenesis[62,63]. P53 mutations have been shown to occur early and can even be detected in mucosa that is non-dysplastic or indefinite for dysplasia, implicating ineffective apoptosis[62,64]. As in sporadic CRC, epigenetic changes (methylation of CpG islands) have been demonstrated throughout the mucosa of UC patients and may contribute to genetic alterations[65].
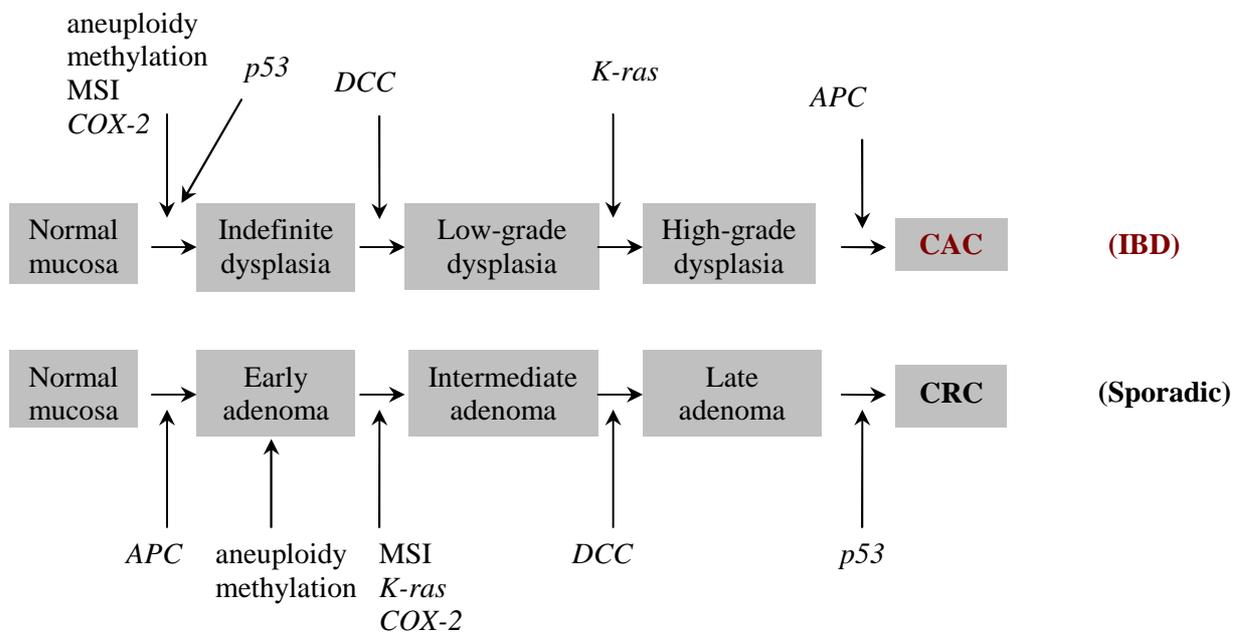
Figure 1.2.4.1: *Carcinogenesis of IBD-associated (CAC) and sporadic CRC compared* (adapted from [52]).

Mutagenic assault and sustained DNA damage due to factors produced by an inflammatory micro-environment may support the carcinogenic process, such as "wounds that fail to heal"[41]. Reactive oxygen and nitrogen species (RONS) cause oxidative and nitrosative stress that is associated with cellular damage in chronic inflammation and appear to potentiate neoplastic transformation[66]. Here, these mechanisms are linked to p53 mutations and inactivation of the DNA mismatch repair system[67,68]. Inflammation-associated genes such as cyclooxygenase-2 (Cox-2) are high in colitis and remain elevated in neoplastic colon tissue[69].

Increased Cox-2 expression has been detected in inflamed and dysplastic mucosa of UC patients and in sporadic CRC, linking lipid mediators to inflammation and cancer[70,71]. Consequently, non steroidal anti-inflammatory drugs (NSAID) have been shown to reduce the development of polyps even in patients with FAP[72-74]. Furthermore, activated nuclear factor kappa B (NF-kB), a central transcription factor for inflammation and proliferation, can be detected in inflamed mucosa of IBD patients[75].

The proposed model of how colitis promotes CAC development thus involves genetic and epigenetic aspects, oxidative stress, DNA repair and apoptosis, and the adaptive immune system and its mediators **(Figure 1.2.4.2)**[17].
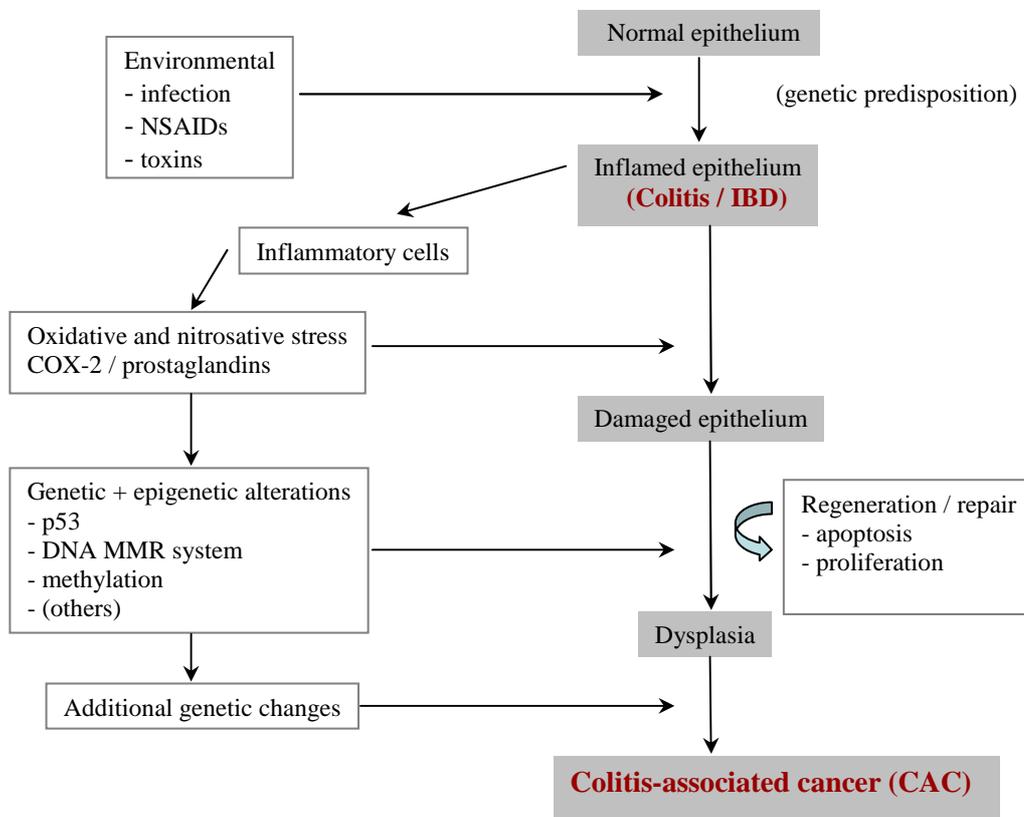
Figure 1.2.4.2: *Model of intestinal inflammation and CAC development* (adapted from [17]).

Most of the events mentioned above are rather descriptive, underlining the obvious link between IBD and CAC development. Whereas the exact causes of IBD remain unknown, innate immune signaling seems to play a crucial role in IBD pathogenesis and thus in contributing to CAC **(Figure 2.1)**. It is well known that the human body requires the polymorphic intestinal flora in order to develop a regular adaptive immune response. The intestinal mucosa acts as a barrier and protects the host against potentially pathogenic

bacterial invasion. At the same time, it tolerates commensal enteric bacteria, permitting them to act in nutrient metabolism. This intestinal homoeostasis can be maintained by interactions between microbiota and mucosa through Toll like receptors (TLRs). Thereby, intestinal epithelial cells and lamina propria mononuclear cells respond to defects in the mucosal barrier by activating TLR dependent pathways, leading to increased epithelial proliferation, wound healing and activation of acute inflammatory cells and mediators[44,76]. In the setting of chronic colitis seen in IBD, this process of repair might result in cancer development. The link between luminal bacteria and epithelial repair is supported by observations in germ-free animals, which showed diminished intestinal epithelial cell proliferation compared with colonized mice[77,78]. Interestingly, germ-free animals were more susceptible to chemically induced colitis[79]. Suggested pathways that link TLRs to inflammation and gastrointestinal cancer include Cox-2 and NF-kB[44]. However, there are also mediators that promote anti-inflammatory effects and tissue restitution in the setting of acute inflammation, such as transforming growth factor beta (TGF-β)[80].

1.3   Experimental CAC in mice

Animal models are essential tools for preclinical testing of new preventive and therapeutic options in humans. To mimic non-hereditary tumor development, tumor models based on chemical carcinogens have been developed. Reliable and reproducible induction of colitis-associated tumors is needed to investigate molecular events that might lead from chronic inflammation to CAC.

1.3.1 Azoxymethane

The mutagenic agent azoxymethane (AOM) is also used as a tumor model for non-hereditary tumor development. When administered intraperitoneally, AOM alone initiates cancer in the colorectum within 30 weeks[81]. AOM exerts its effects by DNA alkylation, thereby facilitating base misparings[82]. It is a direct metabolite of dimethylhydrazine (DMH), which was frequently used in former studies[83,84]. Compared to DMH, AOM shows higher potency and enhanced stability in dosing solution[81].

AOM is suggested to predominantly cause tumor initiation, although some activity in tumor promotion has been reported[85]. Tumors have been considered chromosomally stable and

showed low-level microsatellite instability, suggesting mechanisms independent of the classical CIN or MSI pathways[86]. After application into the peritoneum, AOM needs further stepwise activation to exert its colonotropic mutagenicity **(Figure 1.3.1)**. This includes an essential hydroxylation step to methylazoxymethanol in the liver by CYP2E1[87] and subsequent metabolization involving the intestinal microflora[81].

Colonotropic tumor induction by stepwise activation   **Chemical formula**

Intraperitoneal injection of azoxymethane

via bloodstream to the liver

Hydroxylation to methylazoxymethanol by cytochromes P450

via bile to the intestine

Activation to methyl-diazonium promoted by factors of the bacterial flora
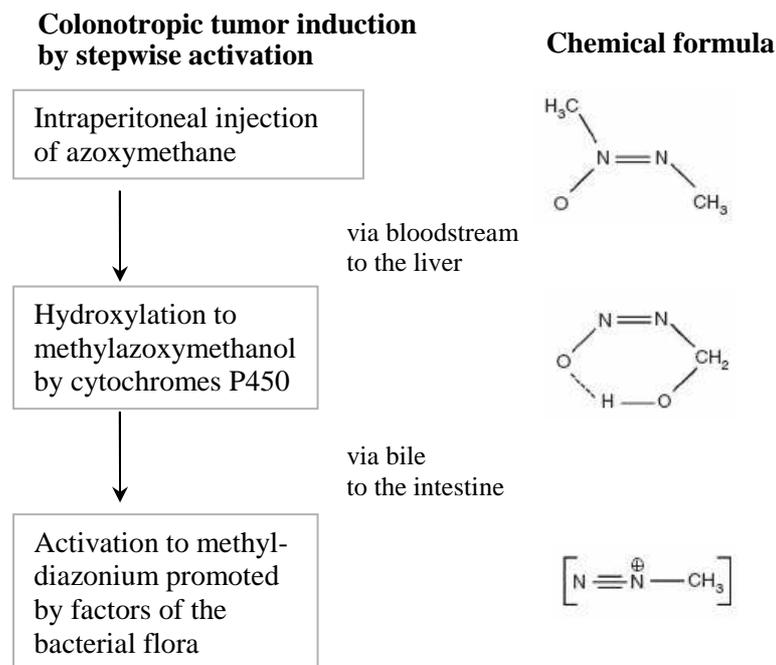
Figure 1.3.1: *Stepwise activation of azoxymethane in rodents* (adapted from [81]).

The AOM model has been shown to be a valuable model for investigations of tumor initiation, progression and of factors involved[88-90]. A meta-analysis concludes that the AOM model of carcinogenesis exceeds other models regarding prediction of chemopreventive efficacy[91]. Advantages include high reproducibility, simple application mode and low price[81]. Tumor numbers are high in the distal part of the colon, resembling the predominant site of UC in humans. They frequently exhibit histopathological features similar to human colorectal neoplasia, while lacking mucosal invasiveness and metastasis[92-94]. On a molecular level, AOM-induced tumors show aberrant expression for APC as well as mutations and dysregulated cellular localization of beta-catenin, suggesting an important role of the Wnt pathway in this model[95]. Furthermore, target genes such as cyclin D1 and cyclin dependent kinase 4 have been shown to be altered[96]. Mutations of the K-ras oncogene and levels of enzymes such as Cox-2 and iNOS-2 have been reported to correspond with findings in human CRC[97,98].

Genetic background of animals seems to be an important modifier of AOM-induced tumors, reflected by a broad array of susceptibility to this model[85,99]. Strains of high and moderate susceptibility are A/J, SWR/J or FVB/N mice. The recommended regimen includes six injections of 10 mg AOM per kg body weight, which is tolerated well by most strains[88,93].

However, exclusively carcinogen-induced colorectal tumors need a long term experimental setup and are predominantly useful for studies of factors that drive sporadic CRC.

1.3.2 Dextran sodium sulfate

Dextran sodium sulfate (DSS) is the most widely used chemical to induce experimental colitis in rodents, being toxic to the epithelial lining and resulting in bloody diarrhea[100,101]. Due to disruption of the mucosal barrier, macrophages in the lamina propria get exposed to luminal enteric bacteria[100]. Ohkusa *et al.* first described a DSS hamster model that has subsequently been adapted to mice[100,102]. After 3-7 days of DSS administration in the drinking water, loss of crypts and ulceration can be observed[103]. In following cycles, the mucosa shows regenerative changes, glandular disarray, separation, shortening of crypts and crypt branching such as in human UC[103]. The treatment regimen must be strain specific, and DSS concentration can range from 1-5%[104-106]. Balb/c, C3H/HeJ and C57BL/6J mice are clearly susceptible to DSS[107,108].

Sequential observation studies revealed higher incidence of dysplastic crypts and colonic neoplasms in APC[Min] mice treated with DSS, compared to wild type mice[106]. Moreover, administration of DSS increased inflammation scores and showed high intensity staining of beta-catenin, Cox-2, inducible nitric oxide synthase (iNOS) and nitrotyrosine (a marker for nitrosative stress). Strong nuclear staining for p53 has been observed in APC[Min] mice treated with DSS but not in wild type mice under the same treatment regimen[106]. It is widely accepted that there is a link between murine DSS-induced chronic inflammation and colorectal carcinogenesis which resembles human IBD-associated dysplasia and cancer (CAC)[103]. As in humans, duration of disease is associated with a higher incidence of neoplasia in the large bowel[103], and mice with neoplasia show significantly higher inflammation scores than those without dysplasia or cancers[104]. Studies suggested that chemically induced CAC is caused by

genetic mutations, increased cryptal cell proliferation, changes in crypt cell metabolism, changes in bile acid enterohepatic circulation, and alterations in bacterial flora[101,109].

However, DSS administration alone requires a long treatment period and repeated administration to induce CAC[104,110,111], with a relatively low incidence and multiplicity of tumors[112].

1.3.3 AOM / DSS model

To investigate colitis-associated tumor development, a two stage model that combines single injection of azoxymethane (see 1.3.1) with administration of proinflammatory DSS (see 1.3.2) has been proposed[113,114]. In this model, different mouse strains showed multiple colonic tumors after a period of 20 weeks (concentration of DSS 1%), and chemoprevention studies have been successfully performed[114-116]. Sequential observations with AOM and DSS 2% in ICR mice found 40% incidence of adenoma in week 3 and 40% of carcinoma in week 4. The incidence of carcinoma gradually increased to 100% in week 6. Dysplasia (low grade and high grade) was widespread in the colon during the whole time-course. Strain differences regarding susceptibility for the AOM / DSS model have been reported: Incidence of colonic carcinoma was 100% in Balb/c mice and 50% in C57BL/6N, whereas only adenomas developed in C3H/HeN mice (29%) and DBA/2N mice (20%)[115]. Thus, this model enables carcinogenesis even in mouse strains with lower susceptibility to AOM (but moderate to high susceptibility to DSS), such as C57BL/6N[81]. Compared with studies using AOM alone, DSS demonstrates a powerful tumor promoting activity in this model[114,117].

Expression of iNOS and nitrotyrosine staining indicate that mice treated with AOM and DSS generate neoplasms via dysplastic lesions induced by nitrosative stress[117]. All large neoplasms were tested positive for beta-catenin, Cox-2 and iNOS, but not for p53[114]. TGF-β and peroxisome proliferator-activated receptor γ (PPARγ) are down-regulated in colonic mucosa after treatment with AOM and DSS[118]. Low-dose 5-aminosalicylic acid (5-ASA) was recently shown to be beneficial in chemoprevention of AOM / DSS-associated dysplasia[119]. Furthermore, blocking tumor necrosis factor alpha (TNF-α) resulted in reduced number and size of tumors as well as decreased colonic infiltration by neutrophils and macrophages, supporting the strong role of inflammation in this model of colitis-associated colorectal cancer and the link to human CAC[120].

1.4   Lipid mediators in IBD and CAC

1.4.1 Lipid mediator system and diet

Beyond protein mediators such as cytokines and chemokines, fatty acid derived endogenous mediators are well known to play an important role in inflammation, pain and proliferation[121,122]. The most important polyunsaturated fatty acids (PUFA) are arachidonic acid (AA, C20:4) of the omega-6 (n-6) series, and eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) of the omega-3 (n-3) series **(Figure 1.4.1)**. Since humans lack the ability to desaturate the n-3 and n-6 bond, both types of fatty acids cannot be interconverted and must be obtained from the diet[123].

The vast majority of studies investigating the lipid mediator system have focused on arachidonic acid and n-6 derived mediators, such as prostaglandins, leukotrienes, lipoxins and thromboxane A2 **(Figure 1.4.1)**. Essential for the formation of prostaglandins (PG) from AA are the cyclooxygenases, of which two isoforms (Cox-1 and Cox-2) exist. PGG_2 is generated from AA and subsequently metabolized via PGH_2 to PGD_2, PGE_2, PGF_2, PGI_2 and thromboxane A_2, depending on specific synthases, cell and tissue type[122]. They bind to distinct G-protein coupled prostanoid receptors and thus exert a variety of effects, ranging from inflammatory responses to blood clotting and nociception[122,124,125]. The AA derived mediator group of leukotrienes (LT) and lipoxins (LX) are formed by enzymes named lipoxygenases. Leukotrienes, such as LTB_4, LTC_4, LTD_4 and LTE_4, are known as highly potent proinflammatory mediators[122,126]. Lipoxins, however, were found to contribute to resolution of inflammation[127]. They were shown to inhibit neutrophil function and to induce phagocytosis of apoptotic neutrophils by macrophages[128,129]. It is accepted that the Cox-2 enzyme is involved in IBD and CAC pathogenesis, however, many of the involved mediators remain to be investigated (see 1.4.2).

Although it has been deduced from epidemiological studies that fish oil rich in n-3 PUFA is generally associated with a reduced incidence of cardiovascular disease[130], for a long time little was known about potential bioactive products derived from n-3 EPA and DHA. Recently, several studies examined the role of newly identified n-3 derived mediators as bioactive agents in their own right, such as resolvins (resolution phase interaction products) and protectins[131]. Resolvin E1 (RvE1) and E2 (RvE2) are derived from EPA, while DHA is the precursor PUFA for resolvins from the D series (RvD1 to RvD6) and for protectins[132]

(Figure 1.4.1). For RvE1, a distinct orphan receptor (ChemR23) has been identified[133]. These families of endogenous n-3 derived mediators have anti-inflammatory and pro-resolution effects in order to restore tissue homoeostasis[132].
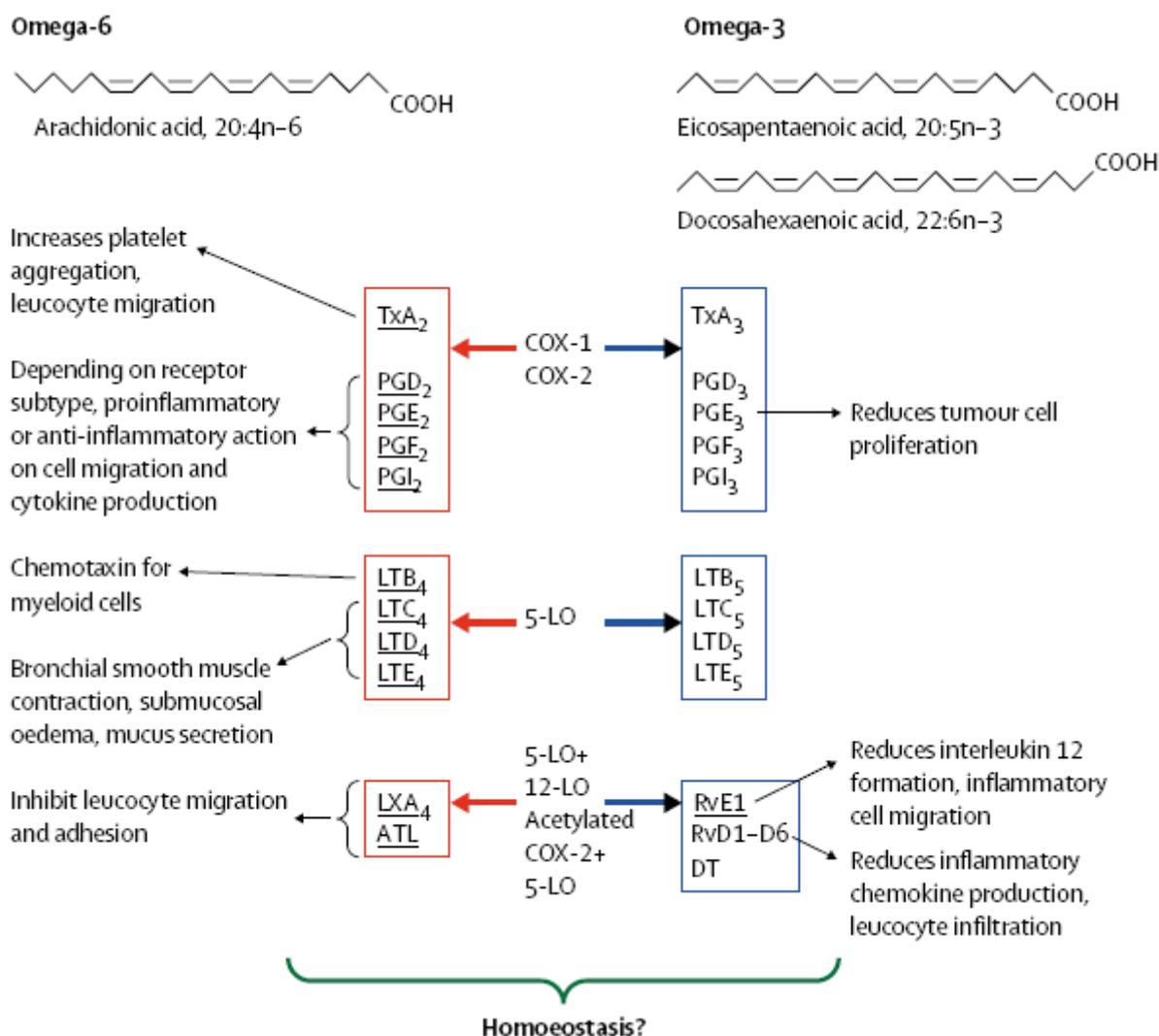
Figure 1.4.1: *N-6 / n-3 polyunsaturated fatty acids and derived lipid mediators*[122].

Diet has been changing over the last centuries, and modern Western diet is deficient in n-3 PUFA, with an n-6 / n-3 fatty acid ratio of 15-20:1[134]. While man probably evolved on a 1:1 ratio of n-6 / n-3 fatty acids, modern diet reflects a strong deviation from this balanced ratio[123]. The shift of the fatty acid ratio may contribute to an increased risk of characteristic diseases of modern Western civilizations such as cardiovascular disease and cancer[134,135]. In turn, supplementation with n-3 fatty acids has been shown to mediate anti-inflammatory, anti-cancer and neuroprotective effects[136,137]. Consequently, n-3 PUFA and derived mediators might modulate IBD and CAC development (see 1.4.3).

1.4.2 Cyclooxygenase-2

The cyclooxygenases are the critical enzymes for the formation of prostaglandins and other mediators, which play a major role in many physiological processes such as blood clotting, wound healing and immune responses[138]. In 1991, the discovery of the second isoform of cyclooxygenases, Cox-2, resulted in an enormous burst of research activity[139].

In inflamed colon tissue of IBD patients, Cox-2 is predominant (compared to Cox-1)[140,141]. Linking lipid mediators to colonic inflammation and cancer, high Cox-2 expression has been detected in colitis-associated neoplasia as well as in colorectal adenomas and in CRC[69,142]. In a landmark study, Oshima *et al.* crossed Ptgs2 (the gene encoding for Cox-2) knockout mice with APC knockout mice[143]. They found a significantly decreased number of intestinal adenomas (652 to 95 per animal), thus giving direct genetic evidence for the link between Cox-2 expression/activity, and polyp formation. High Cox-2 expression correlates with a more advanced stage, larger tumor size and reduced survival in patients with colorectal cancer[144]. In addition to Cox-2 overexpression, 15-PGDH, the key rate limiting enzyme of PG catabolism, is downregulated in CRC[145]. This indicates an important role for Cox-2 even in CRC that is not primarily associated with chronic inflammation.

Extensive studies of PGE_2 (formed by Cox-2), which is the most abundant PG in CRC, identified several mechanisms that could be involved in the promotion of tumorigenesis and tumor growth[146]. PGE_2 promotes proliferation by stimulating cellular proliferation and angiogenesis, inhibiting apoptosis, enhancing cellular invasiveness, and by modulation of immunosuppression[147]. In greater detail, the Wnt signalling pathway is directly activated by PGE_2 via its EP2 receptor, resulting in induction of cell proliferation through beta-catenin[148]. Cell survival and migration is influenced by transactivation of epidermal growth factor receptor (EGFR) [149,150]. Utilizing the same pathway, PGE_2 transactivates PPARδ (which is also a transcriptional target of the Wnt pathway) and thus promotes tumor cell survival[151,152]. PGE_2 induces anti-apoptotic proteins such as Bcl-2 and increases activity of NF-kB, a key mediator in apoptosis[148,153]. There is a correlation of Cox-2 and vascular endothelial growth factor (VEGF) expression, suggesting a link between Cox-2 and angiogenesis[154]. Interestingly, mutant Ras oncogene upregulates Cox-2 expression, and PGE_2 has been shown to activate the Ras-MAPK pathway, demonstrating a mechanism of Cox-2 auto-upregulation by PGE_2[155,156]. PGE_2 furthermore contributes to the shift from a Th1 (TNF-α, interferon

gamma = INF-γ, IL-2) to a Th2 dominant (IL-4, IL-10, IL-6) immune response, thus mediating immuno-suppressive effects[157-159]. These data demonstrate the impressive number of different actions of prostaglandin signalling in cancer development.

To date, anti-tumor strategies targeting this system mainly focused on prevention of inflammation and proliferation by inhibiting major enzymes (cyclooxygenases), thereby reducing mediators such as prostaglandin E_2. Since it has been shown that non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin are able to reduce the incidence of colorectal adenomas[73], the main emphasis of this research was to study the effectiveness of NSAIDs for colon cancer prevention. However, this approach was questioned by a study demonstrating that the NSAID drug sulindac failed in preventing colorectal tumor development in patients with familial adenomatous polyposis coli[160]. Three major trials then investigated the effect of selective Cox-2 inhibitors (celecoxib and rofecoxib) in patients at high risk for adenomas and CRC due to a past history of adenomas[72,161,162]. They reported a striking efficacy of celecoxib and rofecoxib in preventing high risk adenomas. However, all three trials were stopped early, as they showed increased rates of side effects such as myocardial infarction and stroke[163-165]. These observations were (at least partly) attributed to suppressed Cox-2 derived PGI_2 levels after treatment with selective Cox-2 inhibitors[166]. Subsequently, rofecoxib has been withdrawn from the market. These results highlight the role for Cox-2 in CRC chemoprevention and development, but also place emphasis on the complexity of the Cox-2 downstream signalling. Thus, there is a strong need for alternative options in CRC chemoprevention.

1.4.3 N-3 PUFA: Epidemiology and mechanisms

Referring to the concept that IBD development comprises both genetic and environmental components (see above), high intake of dietary n-6 PUFA has been associated with an increased UC and CD risk[167,168]. In contrast, n-3 PUFA supplementation showed beneficial effects on symptoms and progression of IBD[169]. In a study performed by Belluzzi *et al.*, fish oil capsules were effective in reducing the relapse rate of CD patients[170]. Stenson *et al.* found improvement of histological findings, weight gain and reduction of leukotriene B_4 levels in a multicenter study that investigated the effect of fish oil supplementation in UC[171]. It was furthermore shown that fish oil intake indeed results in incorporation of EPA and DHA into the intestinal mucosa and might accelerate remission in UC patients[172,173].

In 1969, Wynder *et al.* first described in Japanese case-control data that there might be a relationship between patients with colon cancer and high caloric fat intake[174]. Since then, several epidemiological studies suggested a correlation between dietary fats and the pathogenesis of colon cancer. This hypothesis was supported by the fact that Mediterranean countries with their different fat consumption, in comparison to other Western countries, have a lower incidence of colorectal cancer: A report by an expert panel assembled by the American Institute for Cancer Research and World Cancer Research Fund revealed evidence for a strong correlation between diets high in saturated fat and the risk for the development of colorectal cancer[175]. In comparison, diets rich in fish or fish oil might decrease this risk, maybe due to their high n-3 PUFA levels. Anti *et al.* investigated the effect of n-3 PUFA in 20 patients with sporadic adenomatous colorectal polyps for 12 weeks[176]. They found a significant reduction in abnormal mucosal cell proliferation, as well as decreased arachidonic acid levels. In a second trial, the same researchers demonstrated similar findings within a long term administration (6 months) of fish oil, while showing increased mucosal levels of EPA and DHA[177]. These results indicate that low-dose fish oil administration shows beneficial effects in patients at risk for colorectal cancer. The association of n-3 PUFA and colorectal cancer risk has been recently reviewed in greater detail[5,6].

To explain the effects in IBD and CRC caused by n-3 supplementation, different mechanisms have been proposed: 1) AA is replaced by EPA at the 2-position of membrane phospholipids. 2) n-3 PUFAs may compete with n-6 fatty acids for lipoxygenase and cyclooxygenase enzymes; with EPA being metabolized to 3-series prostaglandins, which are less bioactive, as well as to 5-series leukotrienes and thromboxanes. Thus, the biosynthesis of proinflammatory mediators derived from AA might be inhibited by n-3 fatty acids. 3) direct interactions of long chain PUFAs with cell proteins have been taken into consideration[122]. Recently, a distinct class of lipid mediators (referred to as resolvins and protectins) was discovered (see 1.4.1)[133,178], which is implicated in resolution of inflammation and tissue protection[179].

Regarding colon cancer, modulation of Cox-2 activity by n-3 PUFA has been reported in feeding studies and in colon cancer cell lines[180,181]. Administration of fish oil or EPA led to decreased formation of PGE_2, while it increased levels of prostaglandin E_3 (PGE_3), an EPA derivate[182,183]. While the 2-series PGE_2 is considered to be mitogenic and pro-inflammatory, PGE_3 has been shown to inhibit tumor cell proliferation[183]. Interestingly, a study performed by Reddy *et al.* showed that celecoxib administered in diets high in fish oil led to significantly

lower AOM-induced colon tumor incidence and multiplicity in F344 rats, implicating synergistic effects between celecoxib and n-3 PUFAs in the prevention of colon tumors[184]. This finding was supported by a study that tested low dose celecoxib administration and DHA in human HCA-7 colon cancer cells[185]. Beyond modulation of cyclooxygenase activity, Singh *et al.* reported antitumor effects of a HFFO (high fat fish oil) diet in colon carcinogenesis through modulation of Ras-p21[186]. A study performed by Calviello *et al.* found that both DHA and EPA led to decreased VEGF (vascular endothelial growth factor) expression in human HT-29 colon cancer cells, correlating with reduced tumor growth due to diminished neoangiogenesis[187]. DHA has been shown to down-regulate iNOS expression and NF-kB activity in human Caco-2 colon cancer cells[188]. Furthermore, DHA appears to have pro-apoptotic effects through alteration of beta-catenin levels and related target genes[189]. Beneficial effects of n-3 PUFA on liver metastasis of colon cancer cells have been described[190].

In summary, these findings indicate that n-3 PUFA might play a beneficial role in the prevention of CRC development. However, research data in this field are sometimes inconsistent and controversial, most likely due to confounding factors of dietary supplementation with n-3 PUFA[191].

1.5 N-3 supplementation: the transgenic approach

Mammals lack the ability to naturally produce n-3 PUFA from n-6 PUFA, and are thus dependent on dietary (essential) n-3 sources such as fish oil, nuts and certain vegetables. The high content of n-3 fatty acids in marine vertebrates stems from the ability of phytoplankton and algae to convert linoleic acid (n-6) to α-linolenic acid (n-3), which enters the food chain of marine life and is further elongated and desaturated to EPA and DHA[135].

The *fat-1* gene of the roundworm *Caenorhabditis elegans* encodes an n-3 fatty acid desaturase that can convert n-6 PUFA to n-3 PUFA by introducing a double bond at the n-3 position of their hydrocarbon chain[192] (**Figure 1.4.4**). Transgenic *fat-1* mice were engineered to express the *fat-1* gene, resulting in a characteristic shift of the fatty acid composition in all tested tissues and organs. In *fat-1* expressing cells, n-6 fatty acids can be converted to their n-3 counterparts, namely 18:2n-6 (linoleic acid) to 18:3n-3 (α linolenic acid), 20:2n-6 (eicosadienoic acid) to 20:3n-3 (eicosatrienoic acid), 20:3n-6 (dihomogammalinolenic acid) to

20:4n-3 (eicosatetraenoic acid), 20:4n-6 (AA) to 20:5n-3 (EPA), 22:4n-6 (docosatetraenoic acid) to 22:5n-3 (docosapentaenoic acid, DPA) and 22:5n-6 (DPA) to 22:6n-3 (DHA).

n-6 FA: $CH_3-CH_2-CH_2-CH_2-CH_2-CH=CH-\ldots-COOH$

$\downarrow$ *n-3 desaturase*

n-3 FA: $CH_3-CH_2-CH=CH-CH_2-CH=CH-\ldots-COOH$
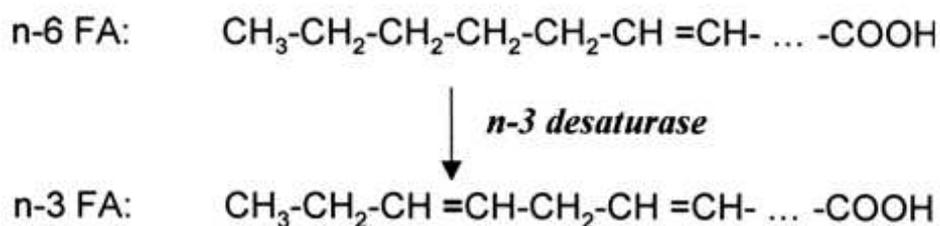
Figure 1.4.4: *The n-3 desaturase catalyzes the conversion of n-6 to n-3 fatty acids (FA)*[193].

Accordingly, the concentrations of n-6 fatty acids are significantly reduced in *fat-1* mice, causing the n-6 / n-3 ratio to drop from 30/1 (wild type) to almost 1-5/1, without the need of dietary n-3 PUFA supply[193]. The absolute fat content is not affected, and the high n-3 fatty acid profile remains stable during the whole life cycle of mice. Confounding factors of conventional feeding studies that lead to unreliable or inconsistent results, such as amount of dietary intake (feeding procedure), flavor, impurity, sensitivity to oxidation, duration of diet change etc. can be eliminated with this model, indicating that the *fat-1* transgenic mouse can serve as a new and unique model to elucidate the significance of the n-6 / n-3 ratio regarding development, gene expression or physical activity. A recent study using female C57bl *fat-1* mice in DSS-induced colitis revealed significantly decreased colonic inflammation and enhanced mucoprotection, compared to wild type mice[194].

In the study presented here, *fat-1* mice are used to investigate the effect of endogenously high n-3 fatty acids in colitis-associated colorectal tumorigenesis induced by azoxymethane and dextran sodium sulfate.

Considering the epidemiology and the vast variety of effects that are demonstrated in intestinal inflammation and carcinogenesis (see 1.4.3), it can be proposed that n-3 PUFA and derived mediators might modulate key players that account for the transition from chronic colitis to colon cancer. In our study, we focused on NF-kB, iNOS (nitrosative stress) and TGF-β.

1.6   Between IBD and CAC

1.6.1   Nuclear factor kappa B

Nuclear factor kappa B (NF-kB) was discovered and first published in 1986 by Sen and Baltimore as a protein bound to the kappa immunoglobin light chain gene enhancer in B cell nuclei[195]. Since then, the role of NF-kB as a transcription factor in innate and adaptive immune responses has been intensively studied and its presence has been demonstrated in most cell types[196]. NF-kB is considered to be a key player in inflammatory processes and basically comprises five homologous, dimerized subunits: RelA (p65), c-Rel, RelB, p50 (NF-kB1), and p52 (NF-kB2)[197,198]. NF-kB dimers are retained in the cytoplasm by specific inhibitors (IkBs) in unstimulated cells[199].
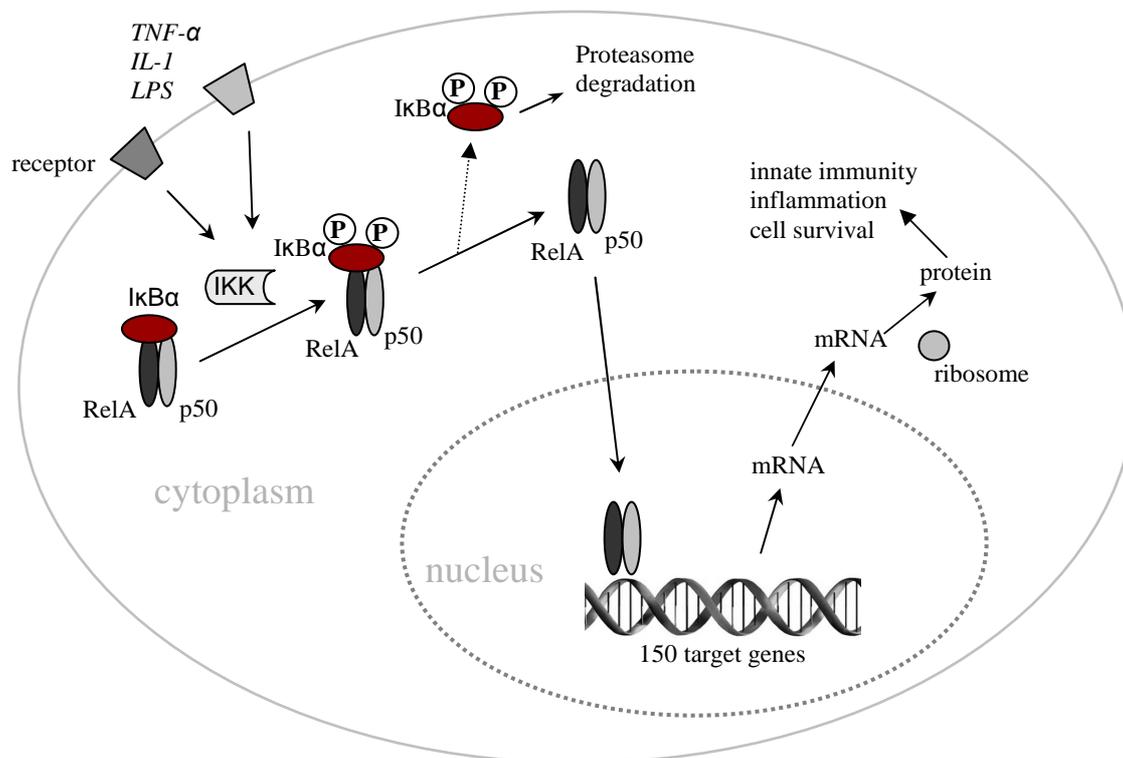
Figure 1.6.1: *The classical (canonical) pathway of NF-kB translocation.*

Cell stimulation activates the upstream IkB kinase complex (IKK), which consists of two catalytic (IKK-α and IKK-β) and one regulatory subunit (IKKγ/NEMO)[199]. The IKK complex subsequently phosphorylates the IkBs, thus targeting them for polyubiquitination and degradation by the proteosome[200]. NF-kB dimers can then translocate to the nucleus, coordinating the transcription of more than 150 target genes involved in inflammation and malignant conversion[196]. Two major pathways account for this translocation of NF-kB: the

classical (canonical) pathway involves dimers composed of RelA, c-Rel and p50, phosphorylated and activated by the IKKβ subunit (**Figure 1.6.1**)[201,202]. Linking NF-kB to inflammation, this pathway is predominantly triggered by proinflammatory stimuli such as TNF-α, interleukin 1 (IL-1) and toll like receptor (TLR) agonists (lipopolysaccharide, LPS)[203]. The alternative (non-canonical) pathway depends on IKKα activation and results in release of RelB/p52 dimers[201], playing an important role in cancers of lymphoid origin[204].

Transcriptional activity of NF-kB can be categorized in four functional groups: cell cycle regulating genes, anti-apoptotic genes, inflammatory and immunoregulatory genes, and genes that operate the negative autoinhibitory feedback of NF-kB[205].

The involvement of the IKKβ dependent canonical pathway in inflammation and cell survival is widely accepted[206,207]. Levels of activated p65 were reported to significantly correlate with inflammation severity in IBD[75]. Here, activation of NF-kB in macrophages and epithelial cells is a frequently observed event[75,208]. Lamina propria fibroblasts were also linked with pro-inflammatory activity[209]. NF-kB is upregulated in colorectal cancer, and increased RelA protein correlates with colon tumorigenesis[210,211]. NSAIDs have been shown to inhibit the canonical NF-kB activation pathway, suggesting preventive effects beyond inhibition of cyclooxygenases and prostaglandin synthesis[212].

In the AOM / DSS mouse model of colitis-associated cancer, enterocyte-specific ablation of IKKβ (leading to decreased NF-kB activity) resulted in significantly decreased tumor incidence, caused by increased epithelial apoptosis. At the same time, induction of oncogenic mutations (initiation stage) as well as size and composition of tumors (progression) were not affected, indicating a role for IKKβ dependent NF-kB activation during early tumor promotion[90]. Tumor size, however, was significantly decreased when IKKb was deleted in myeloid cells, without affecting apoptosis. This observation is attributed to suppressed expression of pro-inflammatory cytokines (after IKKb knockout), which may serve as growth factors during tumor progression. Thus, inflammation-induced tumorigenesis can be affected cell type specificly through inactivation of the IKK/NF-kB pathway in this model.

1.6.2 Nitric oxide and nitrosative stress

It is well recognized that reactive oxygen and nitrogen species (RONS) accompany chronic intestinal inflammation and contribute to neoplastic transformation[17]. Nitric oxide (NO) appears to be a key player in this setting, creating highly reactive intermediates with pleiotropic effects. There are three main isoforms of nitric oxide synthase (NOS) that catalyze the production of NO from L-arginine: endothelial (eNOS), neuronal (nNOS) and inducible nitric oxide synthase (iNOS). Regulated by intracellular Ca^{2+} concentration, eNOS and nNOS are constitutively expressed. However, the inducible isoform (iNOS) is predominantly under transcriptional control of a) NF-kB related pro-inflammatory cytokines (e.g. TNF-α, IL-1b) and b) INF-γ (activates interferon regulatory protein-1, IRF-1)[213,214]. A crosstalk between NF-kB and IRF-1 is suggested while bound to the NOS promoter, thus mediating NO cytotoxicity in case of microbial infection[214]. The iNOS protein was indeed first characterized in cytokine stimulated macrophages, while iNOS activity can now be detected in various cell types, including epithelial and cancer cells[215]. NO production via iNOS is downregulated by steroids, TGF-β, p53, and NO itself[216]. Generally, it has been shown that iNOS derived NO is involved in numerous physiological and pathophysiological conditions, such as blood pressure regulation, infection, inflammation, and cancer development[216].

IBD is considered one of the main "radical overload" diseases, with chronic intestinal inflammation resulting in a cancer-prone phenotype[17]. Studies demonstrated significantly increased iNOS expression and nitrotyrosine accumulation (marker of peroxynitride, the reactive product of superoxide and NO) in patients with Crohn´s disease and ulcerative colitis, supporting an important role of NO in IBD[217-219]. Neutrophils and macrophages have been shown to generate free radicals and other pro-oxidant molecules during acute inflammation[17]. Interventional studies in animals give direct evidence for the involvement of RONS in colonic inflammation. It has been shown that intrarectal administration of (NO derived) peroxynitride in rats resulted in colonic inflammation[220]. Radical scavengers such as superoxide dismutase (SOD), catalase and NOS inhibitors significantly attenuated colonic inflammation in chemically induced colitis, including the DSS model[101]. Hence, mice genetically deficient in iNOS were less susceptible to DSS colitis, compared to wild type mice[221].

Interestingly, beneficial physiological effects and detrimental consequences of NO generation in intestinal inflammation seem to be related to the magnitude and persistence of NO

22

exposure, as well as to the cell types involved. Whereas NO exerts salutary effects during acute colitis, serving as an autocrine and paracrine activation signal, sustained generation of NO is associated with production of reactive nitrogen species and direct effects on cellular constituents. By this, NO and derived mediators might play an important role in colitis-associated colorectal cancer development[216,222]. Several studies demonstrated elevated iNOS expression and activity in human colorectal adenomas[223,224]. These findings were supported by studies in AOM-induced colon tumors in rats[225]. Crossing APC (Min/+) with iNOS knockout mice attenuated adenoma development in both the small and large intestine, compared to APC (Min/+) mice without iNOS deficiency[226].
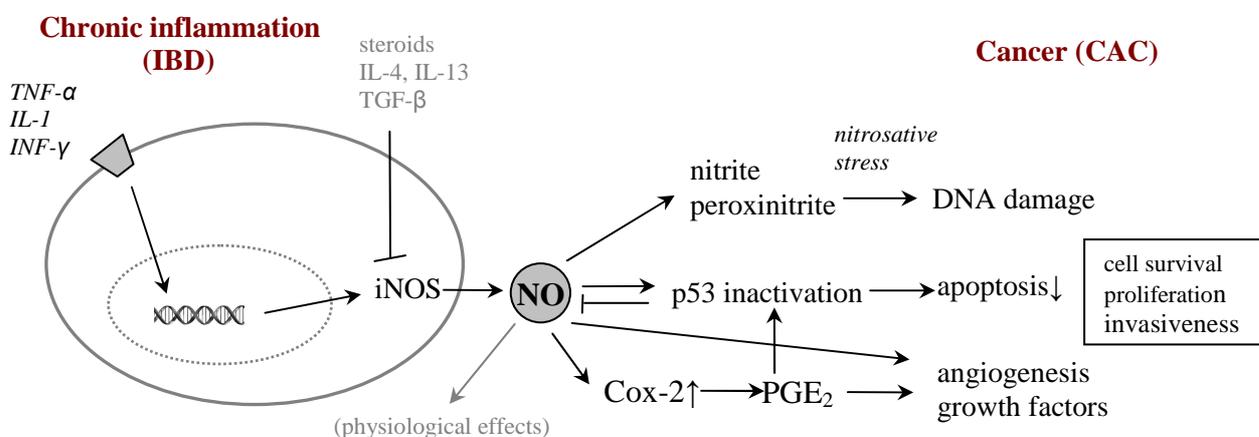
Figure 1.6.2.2: *Role of NO in inflammation and carcinogenesis.*

The role of NO and its derivatives in carcinogenesis is complex and remains not fully understood **(Figure 1.6.2.2)**. Oxidation of NO with superoxide generates peroxynitrite and nitrosating species, such as nitrates, nitrites and N_2O_3. Peroxynitrite has been shown to directly react with DNA, causing mutations and strand breaks. N_2O_3 can form carcinogenic N-nitroso compounds and is also linked with DNA cross-linking and strand breaks. NO nitrosates purines and pyrimidines (leading to DNA damage) as well as thiol residues on proteins (resulting in loss of their catalytic activity). In cells exposed to NO, transitions (GC→AT, GC→CG) are among the most frequently observed mutations[216]. Several studies revealed a connection between Cox-2 and iNOS expression and/or activity in mouse macrophages and colonic epithelial cells[227,228]. In colon tumorigenesis, NO is suggested to stimulate Cox-2 activity, leading to increased levels of tumor promoting prostaglandins, such as PGE_2[216]. Enhanced activity of iNOS and Cox-2 has been observed in the AOM model of chemically induced colon tumors, both being decreased when suppressed by iNOS inhibitors fed in the diet[229,230]. There is some evidence that NO directly interacts with p53 and caspases

by nitrosylation, thus leading to suppressed apoptosis and promoting malignant transformation[231]. Furthermore, a non genotoxic mechanism has been proposed: Cox-2 derived PGs were shown to accumulate p53 dose dependently in the cytosol, where it is inactive. Treatment with selective Cox-2 inhibitors reversed this phenomenon, thus protecting p53 tumor suppressor function[232].

The modulation of NO activity and nitrosative stress might have an impact in the prevention of colitis and colitis-associated colorectal cancer. However, the exact mechanisms by which NO proceeds from "physiological and salutary" to "harmful and carcinogenic" remain unclear[222].

1.6.3 Transforming growth factor beta

Transforming growth factor beta (TGF-β) is known as a potent regulator of cell proliferation and differentiation, embryonic development, wound healing and angiogenesis. Virtually every cell in the body can produce TGF-β (there are three isoforms: TGF-β1, TGF-β2, TGF-β3) and express three cell surface receptors (Type I, II and III) for it. Type I and II can phosphorylate transcription factors known as Smads that form Smad complexes which translocate into the nucleus in order to regulate gene transcription. Ten Smad proteins have been identified to date, of which Smad6 and Smad7 were shown to inhibit TGF-β signalling. TGF-β also acts through pathways beyond Smad, such as mitogen-activated (MAPK) and stress-activated protein kinase pathways[80].

The implication of TGF-β in inflammatory processes is based on its function in maintaining immune homoeostasis and promoting epithelial restitution. Mice deficient in TGF-β1 showed a dramatic phenotype with multifocal inflammatory disease, leading to organ failure and death within 2 weeks[233]. As described above, pathogenesis of IBD is characterized by disruption of the intestinal mucosal barrier and the development of adaptive and innate immune disturbances. Several studies support an important role for TGF-β in the control of gut inflammation and in the induction of tolerance. In sites with high exposure to antigens (intestine, lung), blocked TGF-β signalling led to a markedly increased inflammatory response[234,235]. Interestingly, it has been shown that TGF-β activated Smad signalling inhibits TLR-induced (and NF-kB-dependent) pro-inflammatory gene expression at the receptor (TLR-2 degradation) and nuclear level (blocked histone phosphorylation, **Figure 1.6.3.1**)[236].
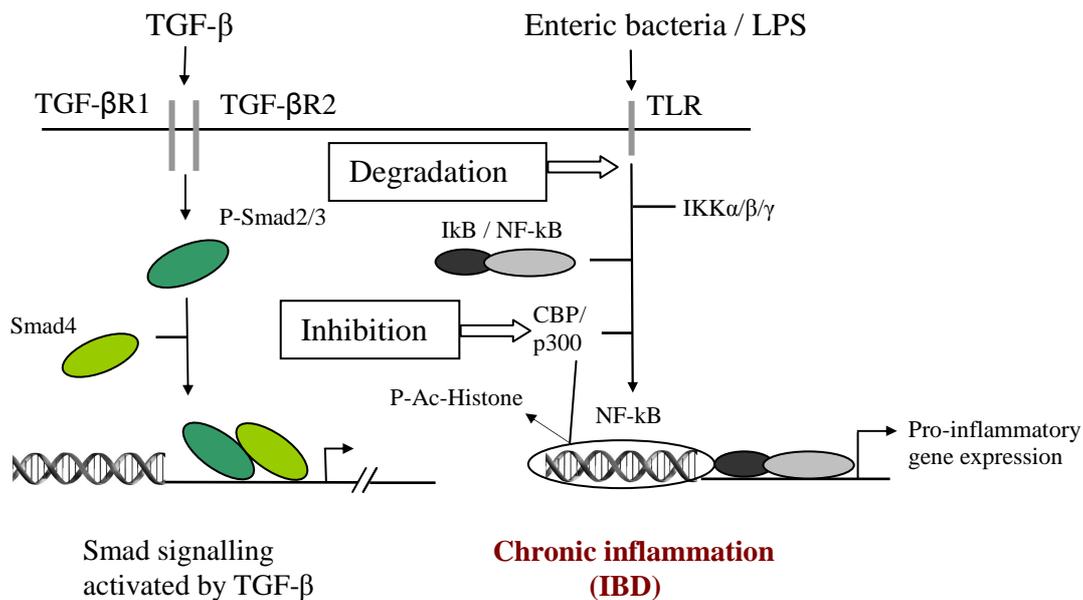
Figure 1.6.3.1: *TGF-β activated Smad signalling inhibits NF-kB-dependent pro-inflammatory gene expression induced by TLR at the receptor (TLR2 degradation) and nuclear (blocked CBP/p300-mediated histone phosphorylation) level* (adapted from [237]).

TGF-β, released from cells in Peyer´s patches or mesenteric lymph nodes, is furthermore suggested to induce regulatory T cells (Tregs) which then migrate to the mucosal effector sites in order to induce tolerance against distinct antigens[238]. It is widely accepted that TGF-β is critical for the CD4^{+}CD25^{+} Treg-mediated control of colitis[239]. A direct role of TGF-β in suppression of colonic T cells has been proposed[240]. These findings indicate that increased levels of TGF-β regulate the immune response in the state of colonic inflammation. Indeed, there is evidence that dysregulated TGF-β signalling contributes to experimental and human IBD. Smad3 knockout mice died from defects in mucosal immunity, and colon samples from IBD patients showed reduced phosphorylated Smad3 levels and high levels of inhibitory Smad7, compared to control patients[241,242]. Overexpression of TGF-β1 in lamina propria immune cells inhibited TNBS-induced experimental colitis[243], on the other hand, increased susceptibility to DSS-induced colitis was demonstrated in transgenic mice engineered to express a dominant negative TGF-β receptorII[244].

In addition to its immuno-suppressive and immuno-regulatory effects on T cells, TGF-β plays an important role as a tumor suppressor in human cancer, regulating proliferation, differentiation and apoptosis. Predominantly in the G1 phase, TGF-β was shown to arrest the cell cycle by inhibiting essential regulators (cyclin dependent kinases 2 and 4; cyclins A and E) via Smad activation. This leads to decreased Rb phosphorylation and subsequently decreased expression of c-myc, which is known to regulate cell progression[80]. Mutations in the TGF-β pathway that confer resistance to these effects can thus result in uncontrolled proliferation of cancer cells: Blobe *et al.* state that mutations affecting at least one component of the TGF-β pathway occur in 83 percent of colon cancers[80]. For instance, mutations of the type II receptor and of Smad4 (initially identified as DPC4) are frequently observed events in colon cancer[245,246]. Other mediators that promote increased resistance to TGF-β signalling are p53[247], and the oncogene products of c-myc[248] and Ras[249].

TGF-β

**Chronic inflammation
(IBD)** ──────────▶ (benign lesion)

*TGF-β signalling
perturbation + mutation*
──────────────▶ **Cancer
(CAC)**

cell cycle arrest (G1)
T cell suppression (Treg)
TLR signalling↓
extracellular matrix↑

growth↑
migration / invasion↑
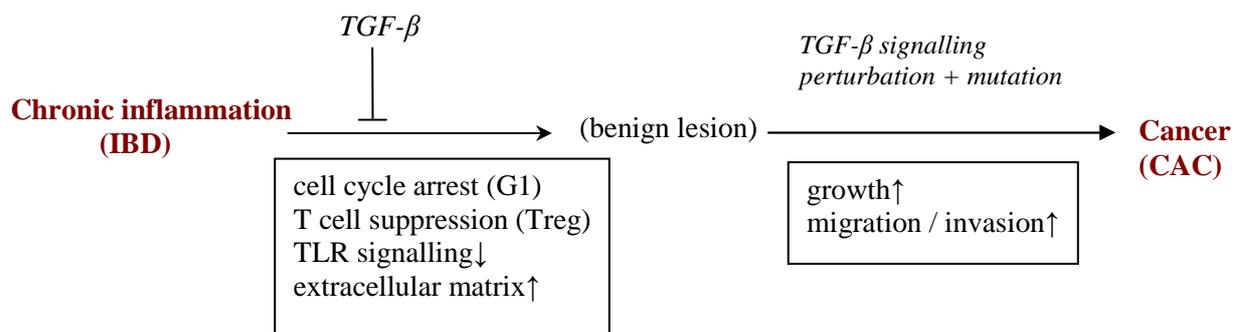
Figure 1.6.3.2: *Biphasic role of TGF-β as a tumor suppressor and promoter.*

TGF-β stimulates fibroblasts to produce extracellular matrix proteins and cell-adhesion proteins, and it decreases enzymes that degrade extracellular matrix, thus enhancing extracellular matrix production. Together with cell cycle arrest, anti-inflammatory properties and apoptosis, this effect contributes to an overall tissue protective effect in non-cancerous tissue.

In cancer cells, TGF-β promotes invasiveness by increasing their proteolytic activity and their binding to cell adhesion molecules. Moreover, TGF-β directly stimulates angiogenesis, thus contributing to tumor progression. Suppression of the immune system (which is rather beneficial in normal cells or in inflammation) leads to hyporesponsiveness of the immune system to the tumor. Considering secondary resistance to growth inhibition by distinct mutations (see above), TGF-β is suggested to play a biphasic role in carcinogenesis (**Figure 1.6.3.2**)[80]. Whereas it promotes healing and tissue restitution, and inhibits growth in normal or

inflamed tissues, mutations or excessive TGF-β activity seem to be important in late stages of cancer development. The TGF-β signalling pathway is thus considered as both a tumor suppressor pathway and a promoter of tumor progression and invasion. It is likely that TGF-β is involved in the pathogenesis from IBD to colitis-associated colorectal cancer (CAC).

2. Fragestellung / Research aims

2.1   Establishment of the AOM / DSS model in transgenic C57bl *fat-1* mice

The *fat-1* transgenic mouse is a new and unique approach to investigate the effects of endogenously elevated n-3 PUFA tissue levels in inflammation and cancer development. In *fat-1* mice, it has been shown that the alteration of the lipid mediator system ameliorates DSS-induced colitis, compared to wild type (wt) mice. The AOM / DSS model has been demonstrated to reliably cause DNA damage and chronic colitis in several mice strains, subsequently leading to the development of multiple colon tumors. It was the primary objective of this study to establish the AOM / DSS model in transgenic C57bl *fat-1* mice, in order to test the influence of an enhanced n-3 PUFA status on colitis-associated tumorigenesis in the large bowel.

2.2   Evaluation of inflammation severity (tissue status) in the AOM / DSS model in *fat-1* versus wild type mice

In inflammatory bowel diseases such as Crohn´s disease and ulcerative colitis, n-3 PUFA are recognized to have beneficial effects on inflammation severity and disease course. Hudert *et al.* recently demonstrated that *fat-1* mice high in endogenous n-3 PUFA are protected from DSS-induced colitis, very likely due to alteration of lipid mediators such as eicosanoids, and the newly discovered resolvins and protectins. They found lower levels of pro-inflammatory parameters (IL-1β, TNF-α, iNOS and NF-κB), which are considered pathognomonic for IBD, as well as beneficial effects of n-3 PUFA on intestinal mucosal integrity, compared to wild type mice.

Here, it was our intention to investigate colonic inflammation induced by AOM / DSS in *fat-1* versus wt mice. First, we analyzed histomorphological changes and length of colon tissue (a marker for inflammation). Second, we measured gene expression / protein content of important markers involved in chronic intestinal inflammation: iNOS (nitrosative stress), NF-kB (transcription factor for pro-inflammatory cytokines) and TGF-β (wound healing and tissue restitution). We thereby intended to investigate whether deviations in key players of inflammatory activity might account for differences in colitis-associated colon tumorigenesis caused by elevated n-3 PUFA levels, comparing transgenic *fat-1* and wt mice.

2.3   Effects of endogenously high n-3 PUFA levels on occurrence and morphology of colorectal tumors

Epidemiological data indicate beneficial effects of n-3 PUFA in the prevention of human colorectal cancer. However, feeding studies to investigate mechanisms and pathways of n-3 PUFA action lack reliability due to confounding factors of dietary supplementation. The *fat-1* transgenic mouse overcomes this issue and thereby provides a unique model to evaluate the effects of an endogenously elevated n-3 PUFA tissue status in colorectal tumorigenesis. A landmark study performed by Karin *et al.* demonstrated that tumorigenesis induced by AOM / DSS can be affected through inactivation of the IKK/NF-kB pathway in mice, resulting in decreased colon tumor incidence. We wanted to apply the AOM / DSS model to transgenic *fat-1* mice in order to investigate tumor histomorphology, incidence and size, compared to wt mice. Furthermore, we analyzed the role of nitrosative stress and NF-kB activity in the setting of chronic colitis and colon tumor promotion. Our hypothesis was that tumor development might be suppressed in *fat-1* mice, probably due to attenuated inflammation severity and alteration of characteristic parameters involved in colitis-associated colon tumorigenesis.

3. Methodik / Materials and methods

3.1   Mice

3.1.1 Transgenic *fat-1* mice

Transgenic *fat-1* mice were generated and maintained as described previously[193]. Transgenic mice were backcrossed onto a C57bl background at least four times. Heterozygous *fat-1* mice were then mated with wt mice to obtain wt and heterozygous *fat-1* offspring. In this study, all transgenic *fat-1* mice were heterozygous and female. C57bl is a widely used inbred mouse strain which shows a high degree of genetic and phenotypic uniformity.

3.1.2 Housing conditions and animal care

Animals were kept under specific pathogen-free conditions in standard cages and were fed a modified AIN-76A diet (energy composition: protein 20%, carbohydrate 58% and fat 22%) in which 5% corn oil was substituted with 10% safflower oil. This diet is thus high in n-6 and low in n-3 PUFA. Sterile drinking water was given ad libitum. The animal facility provided an air-conditioned atmosphere with a controlled 12 hour light-dark cycle. Each cage housed two weight-matched female mice, combining wt and *fat-1* transgenic mice. Mice were on daily surveillance regarding general health status and body weight check.

Animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC), Protocol 2004N000101 (PI Jing X Kang).

3.2   Fatty acid profiling

Fatty acid profiles were analyzed using gas chromatography as described previously[250]. Briefly, 1cm of mice tails (in order to perform the phenotyping of mice) or blocks of colon tissue (5x5 mm) were grounded to powder under liquid nitrogen. Samples were then subjected to extraction of total lipids and fatty acid methylation by heating at 100°C for 1 h under 14% boron trifluoride (BF3)–methanol reagent (Sigma, St. Louis, MO) and hexane (Sigma). Fatty acid methyl esters were analyzed by gas chromatography using a fully automated 6890N Network GC System (Agilent Technologies, Santa Clara, CA) equipped with a flame-

ionization detector. Peaks of resolved fatty acids were identified by comparison with fatty acid standards (Nu-chek-Prep), and area percentage for all resolved peaks was analyzed using GC ChemStation Software (Agilent Technologies, Santa Clara, CA).

3.3　AOM / DSS model

3.3.1　Experimental setup

For tumor induction, female mice were injected intraperitoneally with the genotoxic carcinogen AOM (10 mg/kg, single dose, Wako Chemicals, Richmond, VA), followed by DSS (1.5%, for one week, MP Biomedicals, Solon, OH) in sterile, non-acidified drinking water. A body weight of 19 g was considered the threshold for starting the treatment. The treatment scheme is summarized in **Figure 3.3.1**. Previous studies have shown that similar treatment schemes result in colonic adenocarcinomas developing within 4 weeks and a 100% tumor incidence at week 6 in the ICR mouse strain, whereas C57BL6 mice showed a 50% adenocarcinoma incidence 18 weeks after treatment (1% DSS for 4 days)[115,117,251]. Preliminary studies in our laboratory showed colon tumor development within 9 weeks (data not shown). Mice were anesthetized with isoflurane (IsoFlo, Abbott Laboratories, Abbott Pak, IL) after 9 weeks and sacrificed for biochemical and pathological analyses.
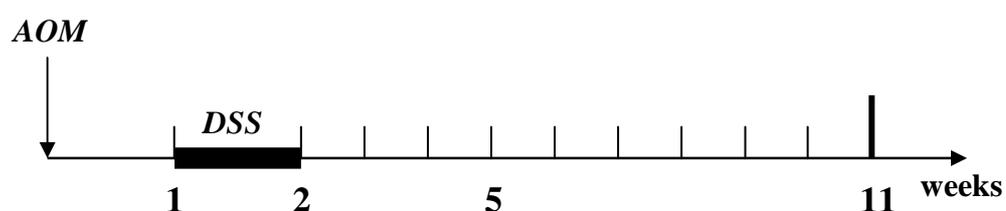
Figure 3.3.1: *Treatment scheme.* Single intraperitoneal AOM injection, after one week followed by DSS for another 7 days in the drinking water. Mice were sacrificed 9 weeks after treatment.

3.3.2　Surveillance of mice during treatment and disease

Clinical assessment of all AOM / DSS-treated animals for body weight, stool consistency, rectal bleeding and general appearance was performed daily. To evaluate rectal bleeding, stool samples were tested using Hemoccult paper (Beckman Coulter, Fullerton, CA).

3.3.3 Assessment of colitis and neoplasia

The colon was excised from the ileocecal junction to the anal verge, flushed several times with phosphate-buffered saline (Gibco, Carlsbad, Ca) and opened longitudinally. Gross examination was performed to measure colon length and to evaluate the pattern of tumor development, including quantity, size and position of tumors within the large bowel. According to their localization, tumors were assigned to either the intermediate or distal third of the colon sample. In addition, the incidence (defined as number of mice with tumors / total mice in the group), the mean number of tumors/mouse ± standard deviation, as well as the mean tumor volume in the group ± standard deviation was calculated for each group. For tumor volume, we used a common approximated formula: $V = 0.5 \times (\text{length})^2 \times \text{width}$. Tumors were excised separately for evaluation by real time Polymerase Chain Reaction (PCR) and part of the colon was used for fatty acid profiling by gas chromatography. Additionally, (inflamed) colonic tissue, along with colon tumors, was processed for histopathological evaluation and further biochemical analyses. All procedures were performed in a blinded manner.

3.4 Histomorphology

3.4.1 Hematoxylin & Eosin stain

Colon tissue was stained with Hematoxylin & Eosin, and stainings were scored for inflammatory activity by an experienced pathologist in a blinded manner, according to the scoring system used previously[105,194]: (i) *severity* of inflammation (0 no inflammation, 1 mild, 2 moderate and 3 severe) and (ii) *thickness* of inflammatory involvement (0 no inflammation, 1 mucosa, 2 mucosa plus submucosa and 3 transmural); *epithelial damage* (0 intact epithelium, 1 disruption of architectural structure, 2 erosion and 3 ulceration) and *extent* of lesions (0 no lesions, 1 punctuate, 2 multifocal and 3 diffuse). Colonic neoplasms were evaluated according to the criteria used by Suzuki *et al.*[115]. In order to perform the histological evaluation, we used an Olympus BX51 (Olympus, Center Valley, PA) microscope with UPlanFI lenses and obtained representative photos with an Olympus QColor5 camera using QCapturePro Software.

3.4.2 Immunohistochemistry

Fresh colon specimens of inflamed tissues, tumors and of untreated wt control colons were fixed in 10% neutral-buffered formalin overnight, followed by automated processing and embedding in paraffin. For staining, rabbit antinitrotyrosine polyclonal antibody (Chemicon, Temecula, CA, 1:500) was used as primary antibody. The secondary antibody was BGAR (biotinylated goat anti-rabbit, Vector Labs, Burlingame, CA) at a concentration of 1:250. Antigen retrieval was performed with Na Citrate (pH 6) for 10 min in a microwave. Normal human cerebellum served as positive control tissue, negative controls were performed without the primary antibody.

3.5   Semiquantitative Real-time PCR

3.5.1 RNA extraction

After removal and initial assessment of the colon, tissue specimens were shock frozen in liquid nitrogen and stored at –80°C until further processing. Total RNA was isolated from whole colon tissue using the RNeasy mini kit (Quiagen, Valencia, CA) following the manufacturer's instructions: Briefly, a high-salt buffer system allows up to 100 μg of RNA to bind to a silica membrane. Colon tissue was lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which inactivates RNases to ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to a spin column, where the total RNA became bound to the membrane and contaminants were efficiently washed away. RNA was then eluted in 30–100 μl of RNAse free water and stored at -20°C. RNA concentration and purity was determined spectrophotometrically by their absorbance (optical density, OD) at 260 nm in relation to the absorbance at 280 nm (OD_{260} / OD_{280}) with a GeneQuant pro analyser (Amersham Biosciences, Piscataway, NJ).

2 μl of sample RNA were diluted in 500 μl NaOH (= 250x dilution), and total RNA concentration was calculated with the following formula:

$$RNA\ concentration\ (\mu g/\mu l)\ =\ \frac{Absorbance\ at\ 260\ nm\ (OD_{260})\ x\ Dilution\ x\ 40}{1000}$$

3.5.2 cDNA (reverse transcription)

A Reverse Transcription System (Promega, Madison, WI) was used to perform reverse transcription of mRNA.

For cDNA (complementary DNA) generation, we used random primers. The mastermix for each sample was prepared as follows: 2 µl reverse transcription buffer, 4 µl 25mM $MgCl_2$, 2 µl dNTP mixture, 0.5 µl recombinant RNasin ribonuclease inhibitor, 1 µl random primers and 0.7 µl AMV (Avian Myeloblastosis Virus) reverse transcriptase enzyme were mixed in separate tubes. Template RNA (1µg) and nuclease-free water (dH_2O) were then incubated in a microcentrifuge tube at 70°C for 10 minutes. The amount of dH_2O was calculated with the following formula:

Amount of dH_2O $=$ 20 µl (total amount per tube) – 10.2 µl (mastermix) – template RNA

(containing 1 µg mRNA)

Subsequently, template RNA / dH_2O mix was added to the mastermix tubes, followed by incubation for 10 min at room temperature. Reverse transcription was then performed with a programmable thermal controller (PTC-100, MJ Research, Ramsey, MN). The thermal cycler program was set at 42 °C for 45 min and 95 °C for 5 min.

In the next step of the protocol, DNA concentration was analyzed spectrometrically. Therefore, 10 µl of reverse transcription product was added to 50µM NaOH (diluted in 500µl of sterile water). Final DNA concentration was determined as follows:

DNA concentration (µg/ml) $= \dfrac{\text{Absorbance at 260 nm } (OD_{260}) \text{ x Dilution x 50}}{0.020}$

3.5.3 Primers

Primers were designed with Primer Select 5.00 Software (DNAstar, Madison, WI) and purchased from Invitrogen (Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) was the housekeeping gene used in order to standardize interindividual values. Primer sequences are shown in **Table 3.5.4.**

Primer	Full name	Sequence	Product length
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Fw 5´CTGACGTGCCGCCTGGAGAAA Rev 5´CGGCATCGAAGGTGGAAGAGTG	160 bp
iNOS	Inducible nitric oxide synthase	Fw 5´CCTCAGTTCTGCGCCTTTGCTCAT Rev 5´CAGGCTGCCCGGAAGGTTTGTA	149 bp
TGF- β	Transforming growth factor beta	Fw 5´GGAAGGACCTGGGTTGGAAGTGGA Rev 5´AAGCGCCCGGGTTGTGTTGG	144 bp

Table 3.5.4 *Primer sequences used in this study.*Fw forward primer; Rev reverse primer; bp base pairs.

3.5.4 Real-time PCR Protocol

Real-time PCR was performed using Absolute QPCR SYBR Green Mix (ABgene, Rochester, NY) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's protocol.

The PCR mastermix used contained (per sample) 2.5 µl Taq buffer, 0.25 µl Taq polymerase, 1 µl dNTP, 2.5 µl 25mM $MgCl_2$, 4 µl 50% Glycerol, 0.75 µl DMSO, 12.875 µl nuclease-free water, and 0.125 µl SYBR Green® dye. The mastermix was prepared for each gene to be investigated (adding 0.25 µl of forward and 0.25 µl of backward primers x amount of tubes) and subsequently aliquoted into reaction tube strips. Then, 0.5 µl of template DNA was added to the respective tube, resulting in a total volume of 25 µl (per tube).

All samples were processed in triplicates and means were standardized to GAPDH values. Semiquantitative mRNA expression was expressed as a fold induction to wt control animals, using the 2ΔCt method. For calculation, values of the housekeeping gene (GAPDH) were substracted from Ct values of the target genes resulting in $\Delta Ct_{target\ gene}$ ($Ct_{target\ gene}$ - $Ct_{housekeeping\ gene}$ = $\Delta Ct_{target\ gene}$). Then, ΔCt values of wt (AOM / DSS), *fat-1* (AOM / DSS) and wt control mice (experimental groups) were set into relation to wt control mice ($\Delta Ct_{experimental\ group}$ - mean $\Delta Ct_{wt\ control}$ = ΔΔCt) for each target gene. The exponential ΔΔCt values obtained were then normalized with the following formula:

n-fold expression (experimental group to wt control) = $2^{-\Delta\Delta Ct}$

3.6   NF-kB activation assay (ELISA)

3.6.1 Protein extraction

Whole-cell extracts from frozen colon tissues (blocks of 1x1 mm) were collected using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, samples were disrupted and homogenized in 3 ml (per gram of colon tissue) ice-cold lysis buffer with a Tissuemiser Homogenizer (Fisher Scientific, Pittsburgh, PA). Samples were then centrifuged at 10.000g for 10 min (4°C), followed by a second centrifugation after transfer to new, pre-chilled tubes. The supernatant fluids obtained represent the whole-cell lysates.

3.6.2 Protein concentration

Protein concentrations were determined using the Coomassie Protein Assay Reagent Kit (PIERCE, Rockford, IL) following the manufacturer´s instructions. When the Coomassie dye binds to protein, an absorbance shift from 465 nm to 595 nm and thus a simultaneously color shift of the reagent occurs. The Coomassie color response curve is non-linear. To address this issue, we have run a standard curve with 2.0 mg/ml BSA stock standard in different dilutions. After subtraction of the average 595 nm reading for the blanks from the 595 nm reading of the standard / unknown sample (colon), the standard curve was prepared and protein concentrations were determined according to the standard curve. To analyse the absorbance of the samples, we used a Victor 1420 microplate reader (Perkin Elmer, Waltham, MA).

3.6.3 ELISA protocol

To quantify the activated p65/RelA protein, TransAM NF-kB p65 Activation Assay (Active Motif, Carlsbad, CA) was performed according to the manufacturer's instructions. Lysates (5 µg of total protein) were incubated at room temperature for 1 h in 96-well dishes containing immobilized oligonucleotides that comprise the NF-kB consensus DNA-binding site (5´-GGGACTTTCC-3´). Consecutively, the primary antibody against activated p65 and the horseradish peroxidase-conjugated secondary antibody were incubated in the same manner, separated by washing steps. The reaction was developed for 5 min at room temperature and its intensity measured immediately at 450 nm using a microplate reader (Victor 1420, Perkin Elmer, Waltham, MA).

3.7   Statistical methods

All results are presented as mean ± standard deviation (SD), except where stated otherwise. Student's T-test was used to evaluate the difference between two groups. Real-time PCR was analyzed using the 2ΔCt method. For comparison of tumor incidences in the distal colon, Chi Square test was used. Statistical significance was accepted at the level of $P<0.05$ and Prism 4 for Windows Software (Graph Pad) was used for all calculations.

4. Ergebnisse / Results

4.1   Fatty acid profiles of colon tissue in transgenic *fat-1* and wt mice

Colon samples of mice were analyzed using gas chromatography. Both *fat-1* and wt littermates were maintained on the same dietary regime (10% safflower oil) high in n-6 and low in n-3 PUFA (n-6 / n-3 = 20). Hence, the data demonstrate that *fat-1* transgenic mice used in our study showed significantly higher levels of important n-3 PUFA (eicosapentaenoic acid C20:5, docosapentaenoic acid C22:5 and docosahexaenoic acid C22:6) in the colon (**Figure 4.1.1**), compared to wt mice. Accordingly, wild type mice showed high levels of n-6 PUFA, whereas n-3 levels were low (leinolenic acid C18:3(3), docosahexaenoic acid C22:6(3)) or not detectable (eicospentaenoic acid C20:5(3) and docosapentaenoic acid C22:5(3)) (**Figure 4.1.2**).
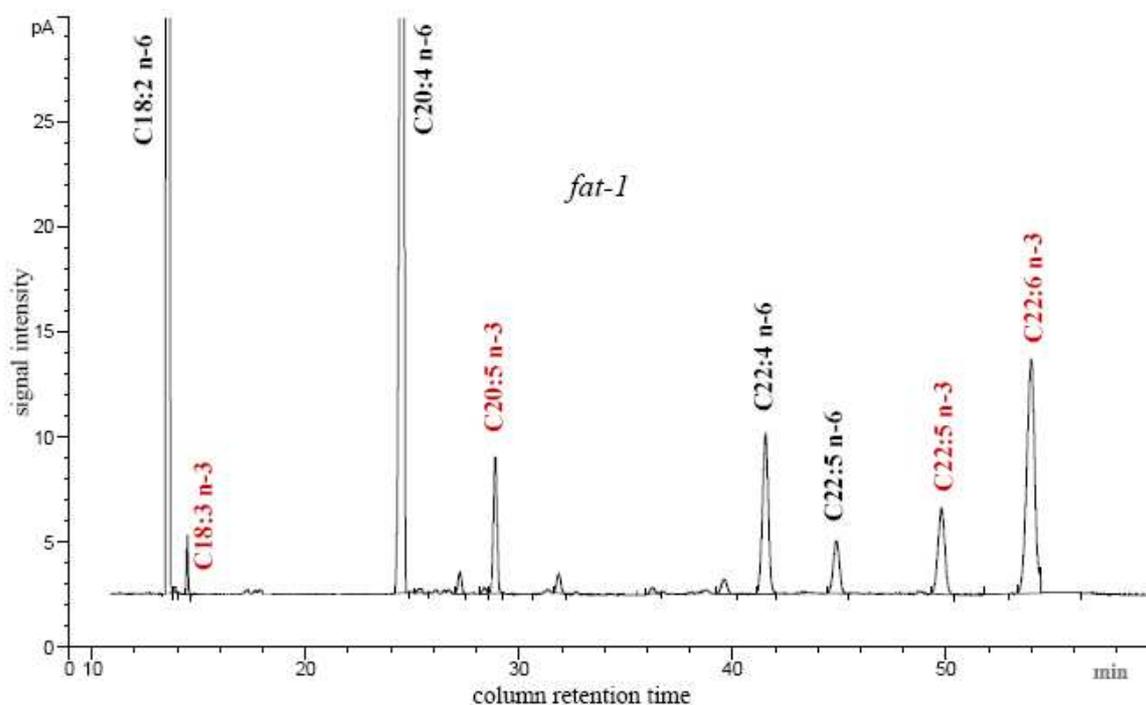
Figure 4.1.1: *Gas chromatography of colon tissue. The figure shows a characteristic fatty acid profile of fat-1 transgenic mice.* C18:2(6) linoleate, C20:4(6) arachidonic acid, C22:4(6) docosatetraenoic acid, C22:5(6) docosapentaenoic acid (6); C18:3(3) leinolenate, C20:5(3) eicosapentaenoic acid, C22:5(3) docosapentaenoic acid (3), C22:6(3) docosahexaenoic acid. N-3 PUFA are highlighted.
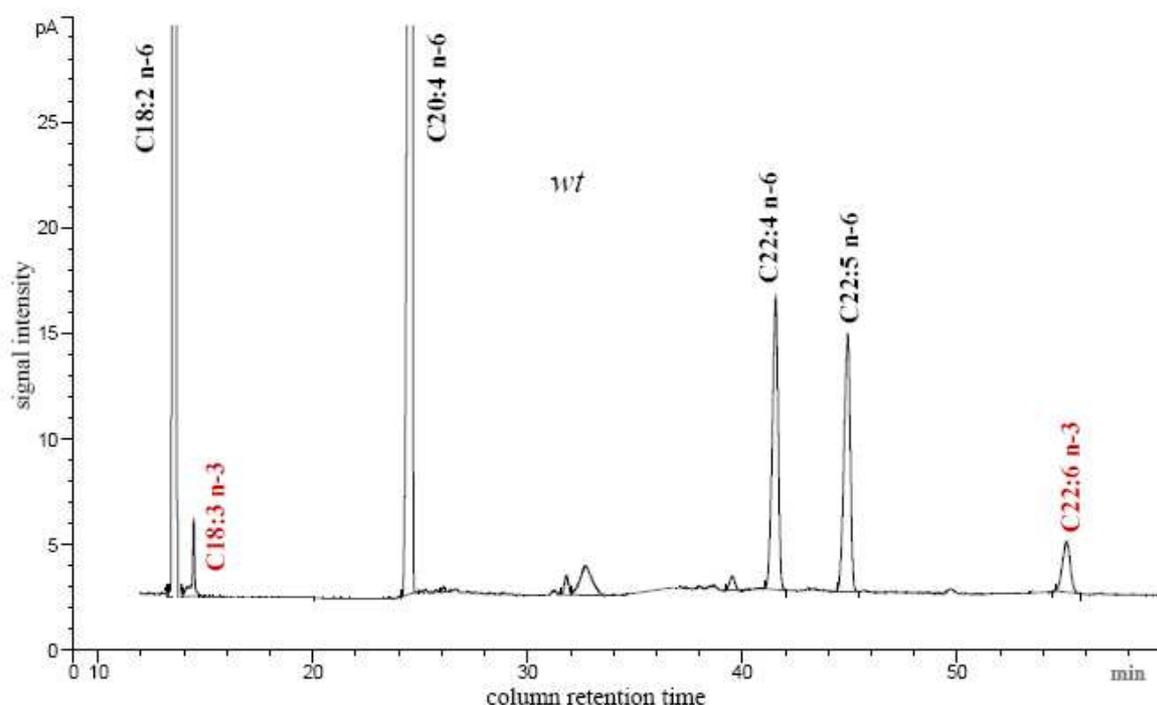
Figure 4.1.2: *Gas chromatography of colon tissue. The figure shows a characteristic fatty acid profile of wild type mice.* C18:2(6) linoleate, C20:4(6) arachidonic acid, C22:4(6) docosatetraenoic acid, C22:5(6) docosapentaenoic acid (6); C18:3(3) leinolenate, C22:6(3) docosahexaenoic acid. C20:5(3) eicosapentaenoic acid and C22:5(3) docosapentaenoic acid(3) were not detectable in the sample. N-3 PUFA are highlighted.

Fatty acids	wt (n=11)	*fat-1* (n=15)
	Percentage of total fatty acids	
n-6 PUFA		
LA (C18:2 n-6)	$18,70 \pm 5,86$	$16,65 \pm 2,98$
AA (C20:4 n-6)	$12,17 \pm 2,90$	$10,69 \pm 1,28$
DTA (C22:4 n-6)	$2,59 \pm 0,62$	$1,70 \pm 0,24^{+}$
DPA (C22:5 n-6)	$2,30 \pm 0,53$	$0,60 \pm 0,12^{+}$
Total n-6	$36,11 \pm 3,73$	$29,64 \pm 1,82^{+}$
n-3 PUFA		
ALA (C18:3 n-3)	$0,17 \pm 0,04$	$0,17 \pm 0,09$
EPA (C20:5 n-3)	n.d.	$0,79 \pm 0,25^{+}$
DPA (C22:5 n-3)	n.d.	$1,01 \pm 0,12^{+}$
DHA (C22:6 n-3)	$0,55 \pm 0,21$	$2,53 \pm 1,12^{+}$
Total n-3	$0,72 \pm 0,22$	$4,50 \pm 1,27^{+}$
n-6 / n-3 ratio	**$49,83 \pm 17,56$**	**$6,59 \pm 2,86^{+}$**

Table 4.1: *Colon fatty acid profiles in wt versus fat-1 mice.* LA linoleic acid, AA arachidonic acid, DTA docosatetraenoic acid, DPA docosapentaenoic acid, ALA alpha linolenic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid; n.d. fatty acid not detectable in the sample; $^{+}$ P<0.01

Transgenic *fat-1* mice had a ratio of long-chain n-6 PUFA (C18:2, C20:4, C22:4, C22:5) to n-3 PUFA (C18:3, C20:5, C22:5, C22:6) of 6.6 in the colon, whereas the wild type littermates had a ratio of 49.8 (**Table 4.1**). Thus, although both wt and *fat-1* mice consume the same diet high in n-6 PUFA, their tissue fatty acid profiles are distinct.

4.2   Suppressed experimental colitis in *fat-1* mice

4.2.1 Body weight course

Treatment was started when mice reached a body weight of 19g. Both wt and transgenic *fat-1* mice showed a similar course of body weight development. AOM treatment and DSS administration resulted in significant body weight loss, whereas weight increase occurred after stopping DSS treatment. In the following weeks, the body weight steadily increased in both groups. Body weight means appeared different between both groups at the point of sacrificing, not reaching statistical significance (P=0.48) (**Figure 4.2.1**).
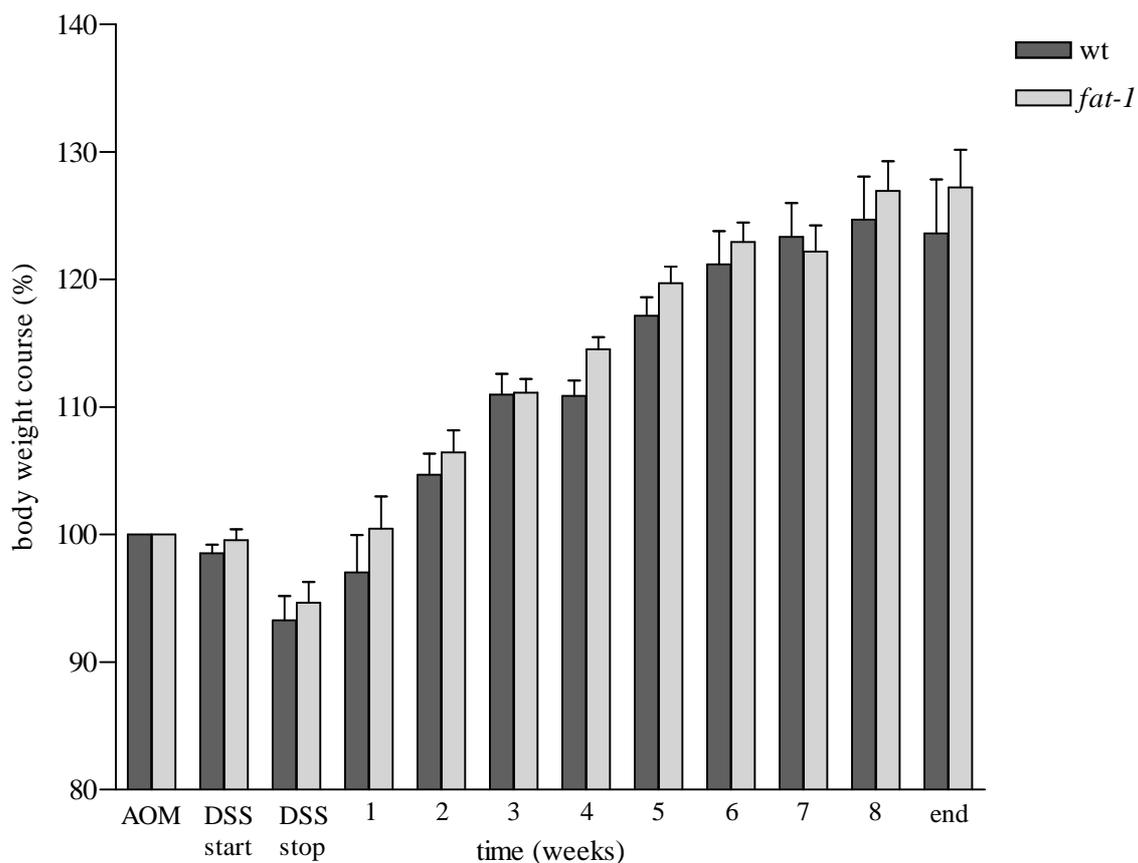
Figure 4.2.1: *Body weight course of fat-1 (grey) versus wt mice (black).* Both groups showed a comparable pattern of body weight change. At the end of the observation period, however, wt mice showed attenuated weight gain as compared to transgenic *fat-1* mice.

4.2.2 Colon length
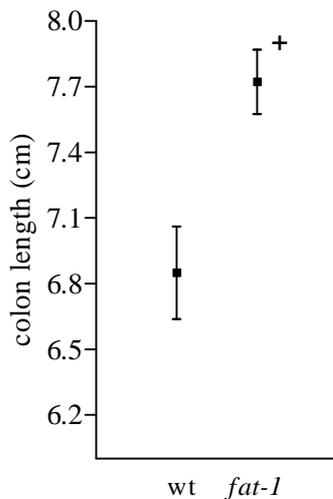
Figure 4.2.2: *Significantly reduced colon shortening in transgenic fat-1 mice.* [+] P<0.05.

Colon shortening is a hallmark of DSS-induced colonic damage. Increased colon shortening is attributed to more severe inflammatory activity and swelling of the large bowel. In our study, colons of wt mice were significantly shorter, compared to *fat-1* transgenic mice **(Figure 4.2.2)**.

4.2.3 Disease activity during observation

Both groups showed intermittent rectal bleeding (confirmed with hemoccult paper) after treatment with AOM and DSS (during the whole period of observation). While *fat-1* and wt mice were matched together in the same cages, stool samples could not safely be attributed to one group. However, the frequency of rectal bleeding decreased with the time of recovery from initial treatment in both groups (data not shown). Interestingly, four wt mice developed anal prolapses, whereas only one transgenic *fat-1* mouse presented with this finding **(Figure 4.2.3)**.
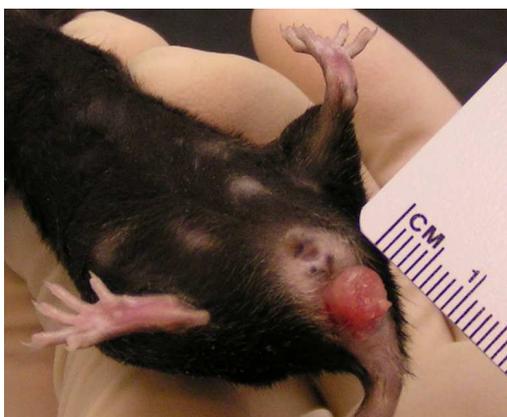
Figure 4.2.3: *Anal prolapse in a wild type mouse after treatment with AOM and DSS.*

4.2.4 Histopathological grading of inflammation

In addition to colon length, the microscopic evaluation (Hematoxylin & Eosin stained slides, *fat-1* n=19, wt n=11) of the distal part of the colon revealed significantly decreased inflammation severity and mucosal thickness in colon specimens of *fat-1* animals **(Figure 4.2.4.1).** Moreover, the grade of mucosal damage and the extent of inflammation in the mucosa were attenuated in transgenic *fat-1* mice, compared to wild type controls. Characteristic histomorphology of inflammatory activity in both groups is demonstrated in **Figure 4.2.4.2.**
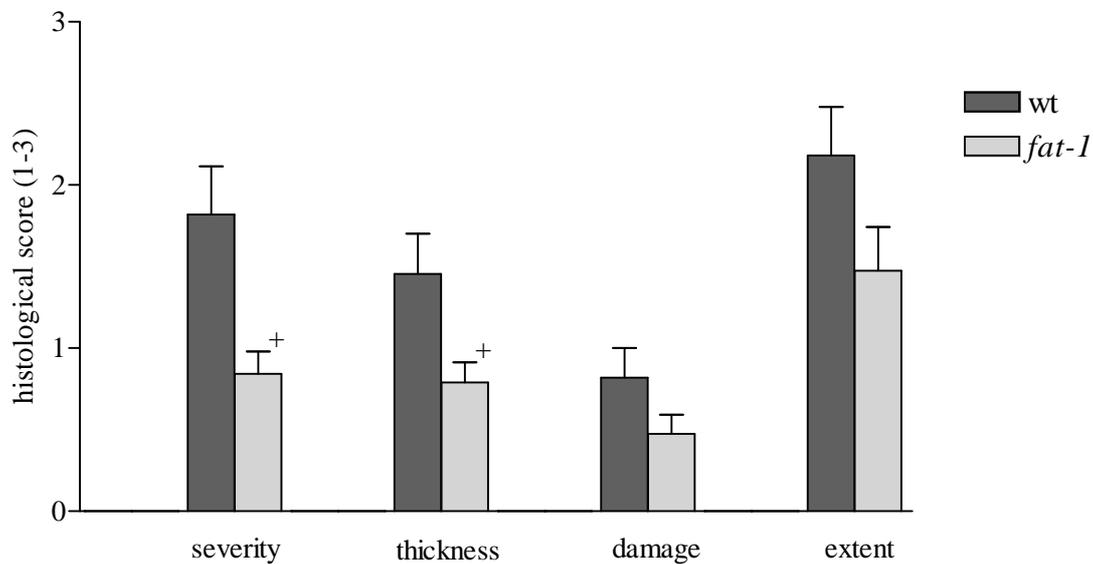
Figure 4.2.4.1: *Histopathological grading of colon mucosa samples.* Analysis showed markedly attenuated inflammatory activity in *fat-1* mice in all analyzed categories. Hematoxylin & Eosin stains. [+]P<0.05

normal mucosa   wt   *fat-1*
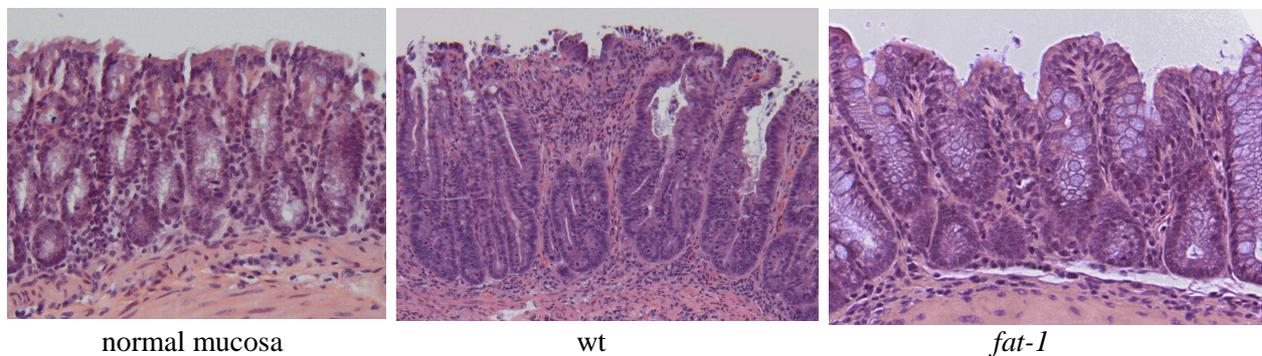
Figure 4.2.4.2: *Hematoxylin & Eosin stains of colitis in transgenic fat-1 and wt animals.* Example slides, indicating more severe colitis in wt animals. 20x magnification.

4.3   Suppressed experimental colon tumorigenesis in *fat-1* mice

4.3.1 Tumor incidence and tumor count

Treatment with AOM and DSS resulted in a tumor incidence of 56.3% (9 out of 16) in the distal part of the colon (where the DSS-induced inflammation is most severe, **Figure 4.3.1.1, 4.3.1.2**) in *fat-1* mice as compared with 90.9% (10 out of 11) in their wt littermates (P= 0.053). In the intermediate part of the colon, total tumor incidence was similar between the two groups of animals (75% in *fat-1* versus 72.7% in wt animals). Overall tumor incidence was 87.5% in *fat-1* mice as compared with 100% in wt animals. Interestingly, there were significantly fewer tumors in the *fat-1* animals (2.4 ± 0.47) than in wt animals (4.2 ± 0.71, P<0.05, **Figure 4.3.1.3**). No tumors were found in the proximal part of the colon in either group of animals.

Figure 4.3.1.1: *Photographs showing colons with tumors from a wt control mouse (upper) and from a fat-1 animal (lower).*
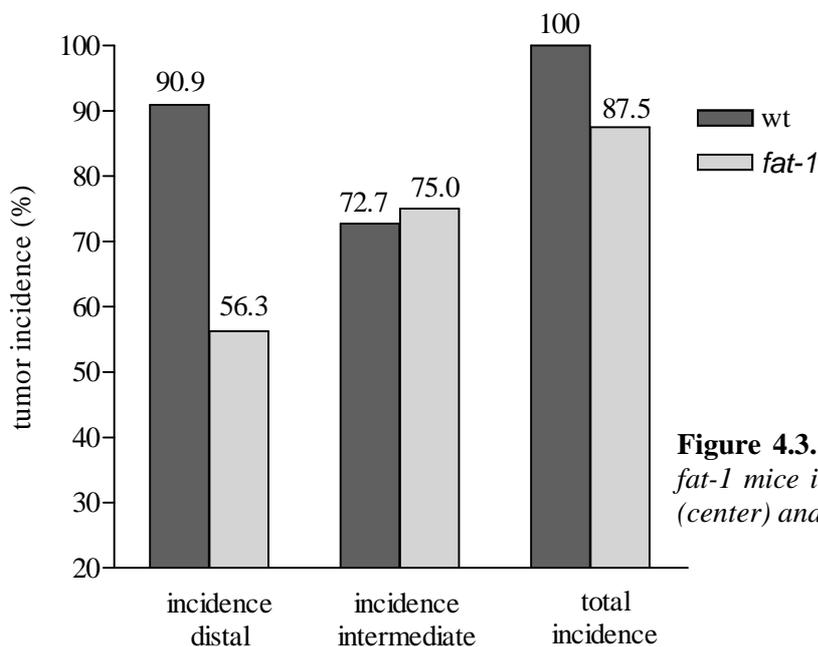
Figure 4.3.1.2: *Tumor incidence in wt versus fat-1 mice in the distal (left), the intermediate (center) and the whole colon (right).*
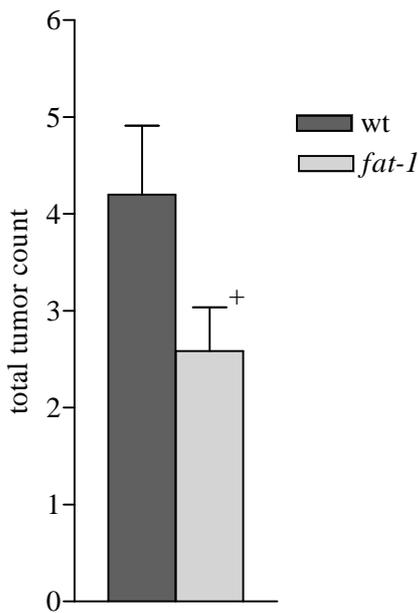
Figure 4.3.1.3: *Average number of tumors per mouse in wt versus fat-1 group.* [+] P<0.05.

4.3.2 Tumor size and morphology

Tumors in the intermediate part of *fat-1* colons were smaller (7.8 ± 1.9 mm^3 in *fat-1* versus 25.6 ± 7.0 mm^3 in wt animals, P<0.01, **Figure 4.3.2.1**). In the distal part, we could not observe significant differences, which was complicated by merging tumor masses in this colon section in both groups. Whereas tumor size within wt mice was different between intermediate and distal part, *fat-1* mice showed similar tumor volumes at all affected colon sites.
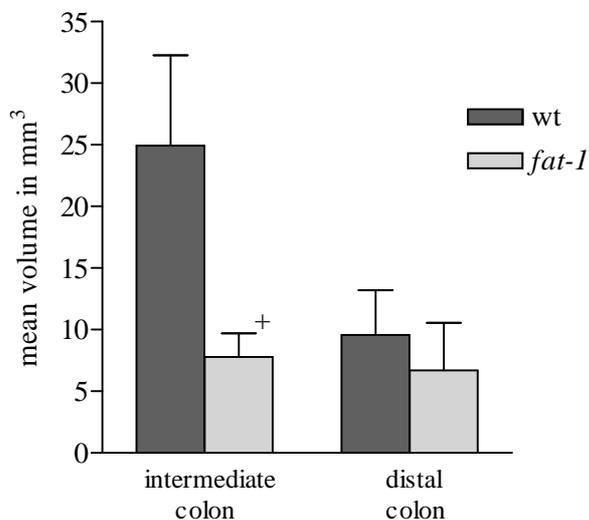
Figure 4.3.2.1: *Comparison of tumor volume between wt and fat-1 animals with colon tumors.* [+] P<0.01.

Morphologically, dysplasia and adenomatous polyps **(Figure 4.3.2.2.)** were the most abundant findings beyond inflamed colonic mucosa in both wt and *fat-1* transgenic animals. We could demonstrate the development of several adenocarcinomas in randomly selected tumors of both groups, demonstrating the potential to mimic invasiveness in our model of tumorigenesis **(Figure 4.3.2.3)**.
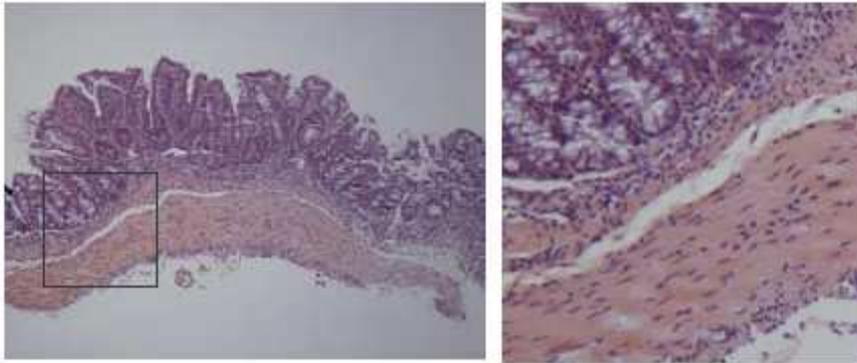
Figure 4.3.2.2 *Representative Hematoxylin & Eosin stain of inflamed colon and adenoma from a fat-1 mouse*. The panel on the right is a higher magnification (2.5x) of the area indicated by the square frame.
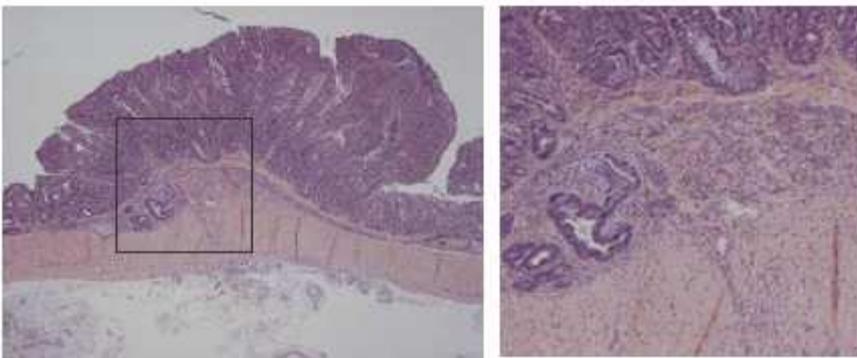
Figure 4.3.2.3 *Representative Hematoxylin & Eosin stain of inflamed colon and adenocarcinoma from a wt mouse*. The panel on the right is a higher magnification (2.5x) of the area indicated by the square frame.

4.4   Important mediators between IBD and CAC

4.4.1 Nuclear factor kappa B

NF-kB activity was assessed by measuring activated p65 in cell extracts from the colons of *fat-1* and wt animals. There was significantly lower activity of NF-kB in the transgenic *fat-1*

mice, as compared with the wt group **(Figure 4.4.1)**. These results are consistent with the findings in acute DSS colitis in *fat-1* mice (Figure 4A in [194]).
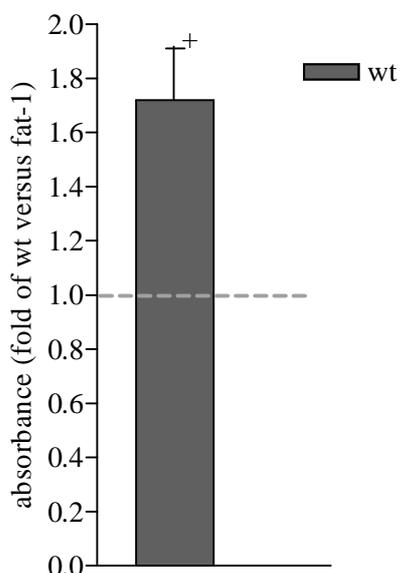
Figure 4.4.1 *NF-kB-activity in the colon, measured by p65 enzyme-linked immunosorbent assay of tissue extracts* (1 denoting levels in *fat-1* animals, [+] P<0.05). wt n=6, *fat-1* n=6.

4.4.2 Inducible nitric oxide synthase and nitrosative stress

Nitrotyrosine as a marker for nitrosative stress was detectable in colon tumors **(Figure 4.4.2.1)**, with increased mRNA expression of inducible nitric oxide synthase (iNOS) in the colon tumors of wt animals **(Figure 4.4.2.2 A/B)**. Expression of iNOS in the tumors of *fat-1* animals was significantly lower. These results were consistent with the results in acute DSS colitis, where iNOS expression was lower in the *fat-1* mice that were protected from colitis[194]. However, in the non-tumorous colon, there was no significant difference of iNOS mRNA expression between the *fat-1* and wt animals in this study **(Figure 4.4.2.2)**.

wt   *fat-1*
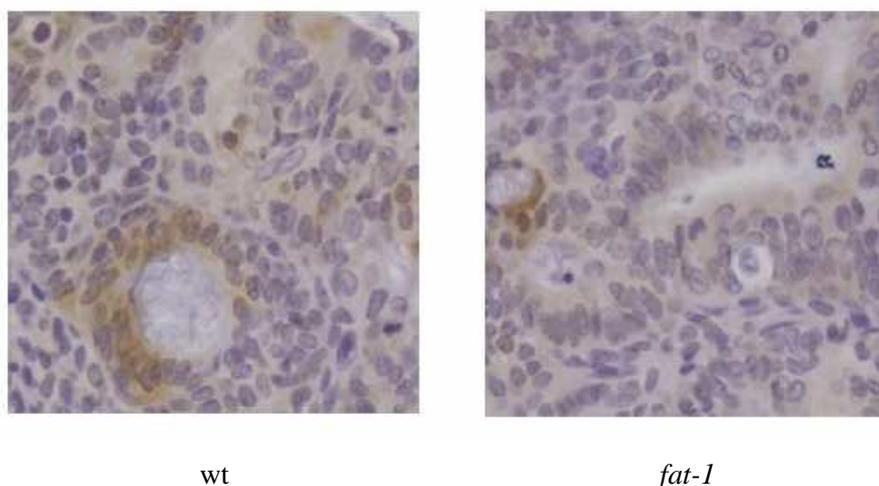
Figure 4.4.2.1 *Representative nitrotyrosine stainings in a colon tumor from a wt mouse (left panel) as compared with a fat-1 animal* (right panel). 40x magnification.

A

B
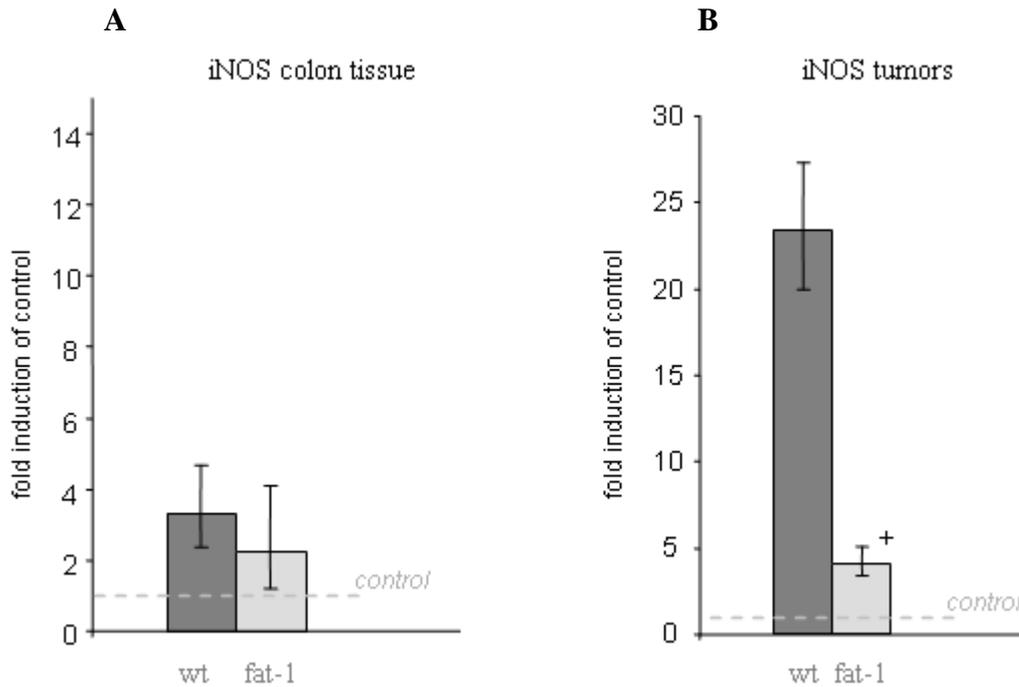
Figure 4.4.2.2 A/B *mRNA levels of iNOS in the normal colon tissues (A, no significant difference) and colon tumors (B, [+] P< 0.05) of wt and fat-1 animals.* Untreated animals served as controls. A wt n=5, *fat-1* n=5. B wt n=3, *fat-1* n=4.

4.4.3 Transforming growth factor beta

mRNA expression of the anti-proliferative transforming growth factor beta (TGF-β) was increased in the colons of *fat-1* mice **(Figure 4.4.3)**, which could contribute to the suppression of tumorigenesis in the *fat-1* animals.
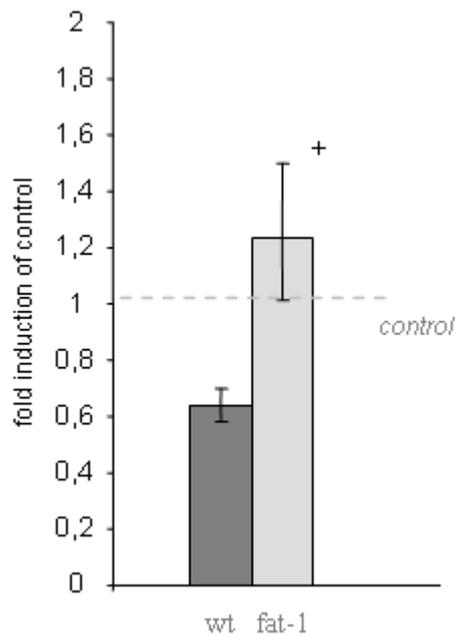
Figure 4.4.3 *mRNA of TGF-β in distal colon tissue.* TGF-beta expression is significantly higher in *fat-1* animals ([+] P<0.05). Untreated animals served as controls. wt n=5, *fat-1* n=4.

5. Diskussion / Discussion

5.1   Summary of the study

The results presented here show that an endogenously increased n-3 PUFA tissue status in the colon decreases inflammation-triggered colon tumorigenesis. In the recently engineered *fat-1* mice, lower amounts of n-6 PUFA were detected, compared to their wild-type littermates. Hence, we could demonstrate significantly higher tissue levels of the n-3 PUFA DPA, DHA and EPA in lipid profiles of *fat-1* mice.

The suppressed tumorigenesis in the *fat-1* mouse was evidenced by a lower incidence of colon neoplasia in the *fat-1* mice as well as a smaller size of the tumors formed. Inflammation was less severe in the distal colon of *fat-1* mice with high endogenous n-3 PUFA tissue levels, confirming our previous findings of protection from colitis in *fat-1* mice. The lower degree of inflammatory changes was associated with a decreased tumor incidence in the distal colon. These changes were, on a molecular level, accompanied by a lower NF-kB activity in the transgenic *fat-1* mice together with a significantly lower mRNA expression of iNOS in the tumors and an increased expression of anti-proliferative TGF-β in colonic tissue.

In summary, these findings indicate that an increased tissue status of n-3 fatty acids due to *fat-1* gene expression can suppress colitis-associated colon carcinogenesis. This might be attributed to the generation of n-3 derived lipid mediators as well as to decreased inflammatory and proliferative factors (iNOS, NF-kB) and promotion of tissue restitution (TGF-β).

5.2   Evaluation of advantages and limitations
5.2.1 Mice and fatty acid composition

There is epidemiological evidence that n-3 PUFA may play a beneficial role in cancer prevention and / or development. However, research data in this field are inconsistent and controversial, most likely due to confounding factors of diet or n-3 fatty acid supplements[191]. To examine the effects of two different n-6 / n-3 fatty acid ratios, two diets must be utilized to feed the animals in order to establish the different fatty acid profiles in both groups. At

present, no pure n-3 and n-6 fatty acids are available for animal diet. In fact, many variables can arise from diet composition and feeding procedure. Differences regarding the effective amount of dietary intake, impurity and contamination, sensitivity of nutrients to oxidation, duration of diet change etc. are characteristic limitations of feeding studies and affect the validity of the results obtained. To date, fish oils and plant seed / vegetable oils are widely used to provide dietary n-3 and n-6 fatty acids, respectively. Patient compliance in clinical trials might be negatively influenced by the unpleasant flavor of fish oil and by irritating side effects (fishy aftertaste, indigestion, laxative effect, heartburn, flatulence).

The transgenic approach using the *fat-1* gene, as presented here, only affects the ratio of n-6 to n-3 PUFA by endogenous desaturation of n-6 PUFA to their n-3 counterparts. Differences in general behaviour between *fat-1* and wild type mice are not yet recognized. In contrast to feeding studies, *fat-1* and wild type animals (born to the same mother) can be fed with an identical diet, in which the total amount of fatty acids is not altered. In this study, the concentrations of n-6 fatty acids were significantly reduced in *fat-1* mice, causing the n-6 / n-3 ratio to drop from 49.8 (wild type) to 6.6, thus indicating fatty acid conversion. The ratio in colons of wt mice thereby reflects the composition of the Western diet (high in n-6, low in n-3 PUFA), whereas *fat-1* mice appear to be a promising and unique model for the well-controlled investigation of consistently high n-3 PUFA levels in human health and disease. Finally, the transgenic approach can save time and costs that are usually involved in dietary manipulation.

However, it can be argued that (not genetically modified) mammals lack the *fat-1* gene, questioning whether and how the methodic advantages can be translated to humans. It is the aim of studies that utilize the *fat-1* gene to reliably elucidate the underlying mechanisms of n-3 PUFA action. To date, n-3 supplementation remains the therapeutic strategy, however, new options might be available by administration of n-3 derived mediators (such as RvE1) in the future. Moreover, the *fat-1* gene is successfully used for the generation of transgenic domestic animals (e.g. pigs), in order to provide n-3 rich food (meat) and to subsequently balance the n-6 / n-3 ratio in humans[252].

5.2.2 AOM / DSS model

Experimental animal models such as the AOM / DSS model resemble human diseases, in order to investigate their pathology and to develop new therapeutic concepts. In contrast to genetically modified animals, chemical agents such as AOM and DSS have been utilized to mimic non-hereditary (colitis-associated) tumor development in the colon. In the protocol used here, a single intraperitoneal injection of AOM was followed by DSS administration in the drinking water, causing genetic damage and severe colitis within a short term of treatment. Human colitis-associated cancer results from chronic relapsing and remitting inflammation (characteristic for IBD such as Crohn´s disease and ulcerative colitis) and involves genetic susceptibility and complex interactions of the innate and adaptive immune system. While DSS colitis shows features of human IBD, it is not clear at which point and to which degree its mechanism is effective in the complex causal chain of initiating and maintaining colonic inflammation. Thus, the relevance of the murine AOM / DSS model to human colitis-associated cancer is limited, and observations cannot be translated to the human condition in an unrestricted manner. However, AOM-induced tumors show aberrant protein expression for APC and beta-catenin, K-ras mutations, and elevated levels of Cox-2 and iNOS, which are typical findings in human CRC[81]. Interventions that work in human IBD also reduce colitis-associated tumor formation in the AOM / DSS model, such as treatment with 5-ASA or inhibition of TNF-α[120,253]. In the setting of IBD and colorectal cancer, this animal model can thus provide the basis for important basic research trials, which might subsequently and successfully be translated to humans.

In preliminary studies we found that age, body weight and gender are important contributors to the disease course in the AOM / DSS model. Moreover, the genetic background (strain) of mice determines the susceptibility to this model in many respects, up to the point of different colonic microflora. To standardize these potentially confounding factors, we only used female C57bl mice, a common inbred mouse strain that shows a high degree of genetic and phenotypic uniformity. Furthermore, treatment was not started before an age of 9-10 weeks and a body weight of 19 gram. In order to avoid investigator-related biases, all experimental procedures were performed in a blinded manner. Regarding the intraperitoneal AOM injection, however, individual metabolisation and activation of AOM in each mouse must be taken into consideration. Here, it would be of interest whether n-3 PUFA probably influence the initial activation of AOM in mice.

A major criticism towards DSS-induced colitis in mice regards the comparability of individual DSS intake. Since DSS is administrated by drinking water, it is essential that every mouse has unrestricted access to the feeding bottle. In our experiments, cages housed only two animals, matching *fat-1* and wt littermates. The drinking habits of animals can also be influenced by discontinuity of night cycles / exposition to light and room temperature / humidity. Our animal facility addressed this concern by providing automated light / dark cycles (12h / 12h) and permanent air-conditioning. It is indeed not possible to exactly monitor the individual intake of drinking water for each animal, and inter-individual variations in DSS ingestion cannot be excluded. However, the homogenic results we obtained within the transgenic *fat-1* and wt groups indicate that these arguments may not be a principal limitation of the results presented here. We found considerable signs of inflammation even 9 weeks after treatment with AOM / DSS, suggesting that the colons were chronically inflamed in our experimental setup. This is in contrast to other investigators, who link chronic colitis only with repeated cycles of DSS[81]. Probably, this regimen would better mimic the pathognomonic disease course seen in human IBD. However, multiple cycles of toxic DSS cause more aggressive and severe colitis in the vast majority of mice, which might have disguised some significant differences between our two test groups (*fat-1* and wt).

Due to the highly reliable experimental protocol and relevant links to human IBD and colorectal cancer, the AOM / DSS animal model is one of the most widely used experimental regimens to investigate colitis-associated tumorigenesis in the colon.

5.3   Determination of inflammation and proliferation

5.3.1 Tissue morphology

Our histomorphological investigation (H&E stains) supports attenuated colitis in *fat-1* mice. However, images of tissue samples always represent snap-shots of current events in the body. To improve the validity of our findings, all procedures (sample preparation, staining, grading) were performed in a blinded manner and with the help of an experienced pathologist. The grading system used is widely accepted and has been validated before. We obtained colonic stainings of all mice that were included in the study in order to calculate differences according to the grading system.

5.3.2 Nuclear factor kappa B

It is widely accepted that NF-kB plays a central role in IBD-associated colorectal cancer development: Levels of activated p65 were reported to significantly correlate with inflammation severity in IBD[75]. Furthermore, NF-kB is upregulated in colorectal cancer, and increased RelA protein correlates with colon tumorigenesis[210,211]. Accordingly, a landmark study performed by Karin *et al.* demonstrated that tumorigenesis induced by AOM / DSS can be affected through inactivation of the IKK / NF-kB pathway in mice, resulting in decreased colon tumor incidence. Our findings of decreased p65 in *fat-1* mice and attenuated colon tumor development, compared to their wt littermates, partially confirm the results of this study. Furthermore, they are consistent with previous results in *fat-1* animals[194,254].

However, recent research delineates a rather complex role of NF-kB that is predominantly dependent on the cell type involved. Whereas NF-kB in macrophages is considered to mediate explicit pro-inflammatory effects in IBD[75,255], controversial findings were reported regarding its role in epithelial cells. Fukata *et al.* first described a link between TLR4 and MyD88 (its adapter protein) signalling and Cox-2 expression in the inflamed intestine[256]. After acute DSS injury, there were increased signs of colitis in mice deficient in TLR4 or MyD88, suggesting a direct link between NF-kB and Cox-2 and supporting a role for Cox-2 derived PGE_2 in TLR4-related mucosal repair. Compared to beneficial findings in the short term, persistent TLR4 signalling was thought to contribute to colitis-associated carcinogenesis. In contrast to this study, Nenci *et al.* found spontaneous severe pancolitis in mice lacking NEMO (IKKγ) or both IKKα and IKKβ in intestinal epithelial cells[257]. NEMO deficient cells cannot activate NF-kB, which led to apoptosis, impaired expression of antimicrobial factors and translocation of luminal bacteria. Interestingly, NEMO deficient mice revealed no signs of colitis after being crossed with mice lacking MyD88, indicating that TLR-mediated bacterial recognition is essential for disease pathogenesis in this model. This study demonstrates an IBD-like phenotype in mice, suggesting that NF-kB signalling defects in intestinal epithelial cells might be an important early event in IBD pathogenesis. Although NF-kB inhibition by antisense oligonucleotides against RelA/p65 or small-molecule inhibitors of IKKb decreased intestinal inflammation in mice, the above mentioned studies raise concerns about the potential use of IKKβ inhibitors in IBD[258-260]. In this context, a recent study highlights the cell specific dependence of the anti-apoptotic and pro-inflammatory effects of NF-kB, indicating that NF-kB inhibition might lead to apoptotic loss of epithelium with subsequent ulceration in acute

disease phase of intestinal inflammation. When targeting myeloid cells in the chronic phase, inhibitors of IKKb and NF-kB might show beneficial effects[261].

N-3 PUFA appear as an additional approach for NF-kB modulation. In a recent study performed by Hudert *et al.*, transgenic *fat-1* mice high in endogenous n-3 PUFA were protected from DSS-induced colitis. This effect was partly attributed to alteration of NF-kB activity and derived pro-inflammatory mediators, most likely due to the distinct PUFA status (high in n-3 PUFA) of *fat-1* mice[194]. Interestingly, another study demonstrated suppressed LPS-induced NF-kB activation in *fat-1* mice on a calorie-restricted diet, suggesting direct effects of n-3 PUFA on inflammatory gene expression as well as beneficial effects through combination of high n-3 PUFA and calorie restriction[254]. In our study presented here, we link lower NF-kB activity in *fat-1* mice with suppressed colitis-associated tumorigenesis for the first time. The exact pathways of NF-kB alteration by n-3 PUFA remain unknown, however, newly discovered n-3 derived mediators are potential participants in this setting. In summary, NF-kB is a central player in IBD and colorectal cancer, and compounds such as n-3 PUFA that can regulate NF-kB activity might have an impact in the reduction of disease burden.

5.3.3 Inducible nitric oxide synthase and nitrosative stress

Several studies demonstrate increased iNOS expression and nitrotyrosine accumulation in patients with Crohn´s disease and ulcerative colitis[217-219]. Hence, mice genetically deficient in iNOS were less susceptible to DSS colitis, compared to wild type mice[221]. Reducing NO activity and nitrosative / oxidative stress might be a promising approach to reduce colonic inflammation and thereby the potential for malignant progression. Seril *et al.* found that radical scavengers such as superoxide dismutase, catalase and NOS inhibitors significantly attenuated DSS-induced colonic inflammation[101]. Here, we were able to demonstrate that *fat-1* mice show significantly decreased iNOS expression in colitis-associated colon tumors, compared to wt mice.

One might argue that NO production in the body is not limited to the iNOS isoform, suggesting a role for nNOS and eNOS in the above mentioned pathology. While these two enzymes are constitutively expressed, iNOS is induced by NF-kB related cytokines and INF-γ in the state of inflammation. Beck *et al.* showed that iNOS expression is mainly upregulated within inflammatory cell infiltrates of the lamina propria and the submucosa in areas of

experimental DSS-induced colonic inflammation and ulceration. Interestingly, iNOS knock out mice showed less severe features of DSS-induced colitis, whereas mice deficient in nNOS or eNOS were not protected[262]. This identifies the iNOS isoform to be the critical source for exacerbated NO production during colonic inflammation. However, extrapolation of NO data from experimental animal colitis models to human IBD is questionable. Trying to address contradictory findings that occurred during the past 10 years, it must be taken into account that studies differed in species, strains, housing conditions, chemical models and execution. Moreover, the effects of pharmacological interventions in NO production during the course of colitis strongly depend on the time-points of intervention, the combination of agents used, as well as on the bioavailability of these agents[222].

This again underlines the advantages of the *fat-1* model applied in our study, with persistently elevated levels of n-3 PUFA in the colon.

The exact mechanisms by which the universal messenger NO switches from its homoeostatic role in acute inflammatory events to detrimental effects in sustained inflammation and malignant transformation remain unknown. However, the modulation of iNOS and NO production seems to clearly alter the disease course of colitis and neoplasia development in the large bowel. In our study, endogenously high levels of n-3 PUFA might contribute to ameliorated oxidative damage and nitrosative stress in experimental colitis-associated tumorigenesis.

5.3.4 Transforming growth factor beta

Disruption of the mucosal barrier and dysregulation of the immune response are hallmarks of IBD etiology. In the setting of acute inflammation, TGF-β is mainly implicated in tissue restitution after mucosal injury and in maintenance of immune homoeostasis. It has been demonstrated that targeted disruption of the TGF-β gene results in severe multifocal inflammatory disease[233]. Accordingly, Hahm *et al.* found that loss of TGF-β augments inflammation severity in IBD[244]. In our study presented here, we demonstrate higher levels of TGF-β mRNA in inflamed colon samples of transgenic *fat-1* mice, compared to their wt littermates. Enhanced mucosal protection may thus account for significantly attenuated colonic inflammation in *fat-1* animals. Interestingly, TGF-β is suggested to downregulate NO production[216]. This might explain our present finding of decreased iNOS expression in colitis-associated tumors of *fat-1* mice.

In contrast, altered TGF-β signalling is linked with the development of colorectal cancer: Blobe *et al.* reported that mutations affecting at least one component of the TGF-β pathway can be detected in up to 83 percent of colon cancers[80]. The constellation of beneficial effects and malignant potential thus creates a discrepancy in the relevance of TGF-β levels. While high levels of TGF-β can be protective in acute and moderate chronic inflammation, the same condition may promote progression and invasion during tumorigenesis due to mutations in TGF-β signalling. Regarding our study presented here, the content of TGF-β in colon tumors and the analysis of potential mutations remain for further investigation in order to evaluate the effect of endogenously high n-3 PUFA levels in colitis-associated tumorigenesis.

5.4   The n-6 / n-3 PUFA ratio and Western civilization: a disparity

During evolution and civilization, human beings underwent dramatic changes in lifestyle and diet. The "ancient" food was rich in n-3 fatty acids, with a rather balanced ratio of n-6 to n-3 PUFA (≈1:1)[135]. Based on that condition, the human body could consequently establish and adopt its genetic pattern regarding fatty acid metabolism over a long time period. With the emergence of agriculture and adoption of animal husbandry, a deviation in diet consumption was initiated, resulting in a relatively higher n-6 PUFA intake. This development was strikingly enhanced in the past century (industrialized food production), creating the modern Western diet that is characterized by its high content of grains and processed food. This diet is rich in n-6 PUFA such as linoleic and arachidonic acid, and deficient in n-3 PUFA, with an n-6 / n-3 ratio of 15-20:1[134]. While man probably evolved on a 1:1 ratio of n-6 / n-3 fatty acids, modern diet thus reflects a strong deviation from this balanced ratio, and the human body was challenged to adapt to the new ratio (on the genetic level) in a short period of time. The shift of the fatty acid ratio is suggested to contribute to an increased risk of certain diseases of modern Western civilizations, such as cardiovascular disease and cancer[134,135]. Supplementation with n-3 fatty acids might overcome this disparity and has been shown to mediate anti-inflammatory, anti-cancer and neuroprotective effects[136,137].

In fact, the impact of the n-6 / n-3 ratio in the complex setting of immunoregulation, inflammation and cancer and the elucidation of distinct biochemical pathways of n-3 PUFA action remain for further investigation. While n-3 supplementation in feeding studies experiences limitations regarding reliability and consistency, our transgenic *fat-1* model with

high endogenous n-3 PUFA levels evolves as a promising tool to address these research issues.

5.5   N-3 PUFA and chemoprevention of colorectal cancer

With regard to mounting epidemiological data that indicate beneficial effects of n-3 PUFA in CRC prevention, research trials that mainly applied dietary n-3 supplementation have been performed. However, these data are sometimes inconsistent, due to confounding factors of n-3 supplementation. While the amount of data on the molecular basis of n-3 PUFA for colon cancer prevention is limited to date, the recent discovery of n-3 derived lipid mediators (linked with anti-inflammatory properties) as well as the introduction of the *fat-1* mouse model led to important progress in this field[121,123,132,193]. It would be of interest to quantify the required amounts of DHA and EPA in n-3 supplementation as well as to delineate underlying pathways of potential effects in the prevention of colonic inflammation and carcinogenesis. The central concept of a dominant role of Cox-2 in colon cancer prevention may have significant limitations that necessitate its reappraisal[165]. Whereas long term medication with NSAIDs and selective Cox-2 inhibitors seems too toxic to recommend, n-3 PUFAs have been an essential part of human nutrition throughout evolution. Consequently, there might be a place for an increased uptake of n-3 PUFA, as well as for a general recommendation to rise the n-3/n-6 ratio in the diet, in order to prevent colonic inflammation and carcinogenesis[5,6].

5.6   Conclusion and impact of the study

The results presented here support a protective role of n-3 PUFA in colitis-associated tumorigenesis induced by azoxymethane and dextran sodium sulfate, evidenced by suppressed colitis and reduced tumor development. In contrast to feeding studies, we used an innovative transgenic approach with high endogenous n-3 PUFA in the colons of *fat-1* mice. Hence, a significantly higher status of n-3 PUFA could be demonstrated in colons of transgenic *fat-1* mice, compared to wt animals. We propose that the observed anti-tumorigenic effect of n-3 PUFA may be mediated partially by modulation of central mediators that link chronic inflammation with cancer, such as NF-kB (inflammatory and proliferative cytokines), iNOS (oxidative and nitrosative stress) and TGF-β (tissue protection). The results presented here might support our recent findings of suppressed inflammation in acute DSS colitis[194].

Our study thus offers an excellent basis for an attempt to further elucidate the molecular interactions and underlying pathways of n-3 PUFA action in the setting of chronic colitis and colon cancer. More chemopreventive trials to address the relationship between the formation of n-3 derived lipid mediators and carcinogenesis using approaches of lipidomics and proteomics are warranted. In fact, a recently published study performed by Jia *et al.*, using the same experimental setup with AOM and DSS, confirmed our results of suppressed colitis-associated tumorigenesis in *fat-1* mice[263]. They furthermore demonstrated decreased CD3^{+}, CD4^{+} T helper, and macrophage cell numbers in *fat-1* mice as well as significantly decreased n-6 derived eicosanoids by mass spectrometry.

In this context, dietary supplementation with n-3 PUFA may be an effective and safe means of CRC prevention and it may be an alternative to the use of anti-inflammatory cyclooxygenase inhibitors, particularly selective Cox-2 inhibitors, which exhibit significant side effects when used long term. Because n-3 PUFA have further beneficial effects, such as cardioprotective effects, supplementation with n-3 PUFA to prevent colon cancer could be a strategy worth pursuing now.

6. Auszug / Abstract

Colorectal cancer (CRC) is the second leading cause of cancer deaths in USA. Anti-inflammatory drugs were shown to be effective in the prevention of CRC, supporting a link between inflammation and tumorigenesis in the colon. However, due to their side effects, long-term administration of these drugs for CRC prevention is not feasible. An increased tissue content of omega-3 polyunsaturated fatty acids (n-3 PUFA) can dampen colon inflammation in animals as well as in humans. Whether increasing colon tissue n-3 PUFA alone is effective in preventing colon tumorigenesis remained to be investigated. Here we show that endogenously increased tissue levels of n-3 PUFA in the *fat-1* transgenic mouse model lower incidence and growth rate of colon tumors induced by inflammation (dextran sodium sulfate) plus treatment with carcinogen (azoxymethane). This was accompanied by lower activity of nuclear factor kappa B (NF-kB), higher expression of transforming growth factor beta in the colons and lower expression of inducible nitric oxide synthase in the tumors of *fat-1* animals. Our data provide new insight into the mechanism by which n-3 PUFA suppresses tumorigenesis through dampening of inflammation and NF-kB activity. These results support a protective role of n-3 PUFA supplementation in the prevention of CRC[264].

7. Zusammenfassung / Summary (German)

In der vorliegenden Studie haben wir in transgenen *fat-1* Mäusen den Effekt von Omega-3 Fettsäuren auf die Entstehung von chemisch induzierten Kolontumoren untersucht. Zur Induktion von Tumoren benutzten wir das genotoxische Karzinogen Azoxymethan (AOM), kombiniert mit Dextran Sodium Sulfat (DSS), welches eine chronische Entzündung (Kolitis) im Kolon hervorruft. Die transgenen *fat-1* Mäuse exprimieren eine Konvertase, welche Omega-6 in Omega-3 Fettsäuren umwandeln kann. Dadurch findet sich in diesen Mäusen ein endogen erhöhter Omega-3 Fettsäuregehalt. Unsere Hypothese war, dass die erhöhten Omega-3 Fettsäuren in diesem transgenen Tiermodell die Entstehung von Kolontumoren durch eine Hemmung der entzündlichen Gewebsreaktion vermindern.

Im Kolongewebe der transgenen *fat-1* Mäuse konnten wir (verglichen mit Mäusen vom Wildtyp) niedrigere Spiegel von Omega-6 Fettsäuren messen. Zusätzlich haben wir signifikant höhere Spiegel der Omega-3 Fettsäuren DPA, DHA und EPA in den Fettsäureprofilen der *fat-1* Mäuse nachweisen können. Die Ergebnisse unserer Studie zeigen, dass sich ein endogen erhöhter Gehalt an Omega-3 Fettsäuren protektiv auf die durch AOM und DSS induzierte Entzündung und Tumorgenese im Kolon auswirkt. Die Hemmung der Tumorgenese wurde belegt durch eine niedrigere Tumorinzidenz in *fat-1* Mäusen sowie durch eine geringere Größe der entstandenen Kolontumoren. Im distalen Kolon zeigte die histopathologische Untersuchung am Ende der Versuche zudem einen geringeren Schweregrad der chronischen Kolitis in den *fat-1* Mäusen. Dieses Ergebnis bestätigte frühere Versuche mit chemisch induzierter akuter Kolitis im *fat-1* Mausmodell. Auf molekularer Ebene zeigten sich eine erniedrigte Aktivität des Transkriptionsfaktors NF-kB, sowie signifikant niedrigere mRNA Expressionen von iNOS in den Tumoren und von TGF-β im Kolongewebe der *fat-1* Mäuse (verglichen mit den Mäusen vom Wildtyp). Wir vermuten, dass der protektive Effekt der Omega-3 Fettsäuren möglicherweise durch die Modulation dieser zentralen Mediatoren von Entzündung und Proliferation bewirkt wird. Eine kürzlich publizierte Studie von Jia et al. mit gleichem experimentellem Aufbau konnte unsere Ergebnisse abgeschwächter Entzündung und Tumorgenese in *fat-1* Mäusen reproduzieren[263].

Unsere Studie bietet eine gute Basis zur Erforschung weiterer molekularer Zusammenhänge sowie des ursächlichen Metabolismus von Omega-3 Fettsäuren im Zusammenhang mit chronischer Entzündung und Kolonkarzinom. Zusätzliche Studien sind nun notwendig, um

die Beziehungen und Einflüsse von Omega-3 Lipidmediatoren (Resolvine, Protektine) auf die Karzinogenese aufzudecken.

Bisherige Versuche der Prävention des Kolonkarzinoms, v.a. durch entzündungshemmende selektive Cox-2 Inhibitoren, zeigten erhebliche Nebenwirkungen mit erhöhtem Risiko kardiovaskulärer Ereignisse im Langzeitversuch. Omega-3 Fettsäuren sind seit jeher essentieller Bestandteil der menschlichen Nahrung. In diesem Kontext könnte die Ergänzung mit Omega-3 Fettsäuren bzw. die generelle Empfehlung zur Erhöhung des Omega-3 / Omega-6 Ratio in der Ernährung eine effektive und sichere Alternative mit dem Ziel der Prävention der Kolontumorigenese darstellen.

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9. Selbständigkeitserklärung / Declaration of originality

„Ich, Johannes Nowak, erkläre an Eides statt, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Colitis-associated colon tumorigenesis is suppressed in transgenic mice rich in endogenous n-3 fatty acids" selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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11. Lebenslauf / Curriculum vitae

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.