Diet, cell signalling, and intestinal tumourigenesis in multiple intestinal neoplasia mice

ACADEMIC DISSERTATION

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Yliopistopaino
Helsinki 2008
To my family
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Abstract

Colon cancer development is a multistep event that in many cases, especially in sporadic colon cancer, is initiated by a mutation in the \textit{APC} tumoursuppressor gene. The cell gradually acquires other mutations in oncogenes and tumoursuppressors and as a consequence, the epithelial cell starts to proliferate. This leads to the formation of tumours in the intestine. Environmental factors, including diet, affect colon cancer development. During the last few years, a vast amount of new, functional, foods have been introduced to the consumers. Several products are already available that are marketed as promoting intestinal health. To be able to reliably call a dietary compound a chemopreventive substance it is of fundamental importance to understand the mechanism by which it affects tumour formation and the integrity of the epithelial cells.

The focus of this thesis was to confirm the chemopreventive effects of three different dietary compounds, inulin, conjugated linoleic acid, and white currant, on tumour formation in an experimental model for colon cancer. The multiple intestinal neoplasia (Min) mouse carries an inherited mutation in the \textit{Apc} gene that causes adenomas to form in the intestine. Inulin is a non-digestible fibre found naturally in chicory roots, artichokes and onions, amongst others. Nowadays it is widely used as an added ‘dietary fibre’ in several food products. Conjugated linoleic acid (CLA) is a conjugated form of the fatty acid linoleic acid. CLA is formed by bacterial fermentation of linoleic acid in the rumen of cows and other ruminants. Concomitantly, it can naturally be found in milk and meat of ruminants. White currant is a colourless berry low in phenolic compounds. To further study the mechanism involved in tumorigenesis, we elucidated three different signalling pathways pivotal in colon cancer formation; the Wnt-, nuclear factor \( \kappa \)B-, and p53-pathways.
Contrary to what was expected, inulin and the conjugated linoleic acid isomer trans-10, cis-12, were tumour growth promoting dietary constituents when fed to Min mice. Both diets decreased the NF-κB levels in the mucosa, but physiological adenoma development did not affect NF-κB. Diet altered β-catenin and p53 signalling in the adenomas, confirming their involvement in adenoma growth. White currant, on the other hand, was chemopreventive. The chemopreventive effect was accompanied by increased p53 levels in the mucosa, and decreased β-catenin and NF-κB levels in the adenoma. This could explain the reduced adenoma number and size. A clear pattern for the behaviour of β-catenin, NF-κB and p53 was not found in this study.

In conclusion, this thesis cannot confirm the chemopreventive effects of inulin and CLA. Despite white currant only containing low amounts of phenolic compounds, it was still able to act as an anticarcinogen. This underlines the importance of carefully testing new dietary compounds in different settings to reliably confirm their health benefits. To our knowledge, this is the first study to investigate the behaviour of β-catenin, NF-κB, and p53 in such detail in the Min mouse.
List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I  Rajakangas J, Basu S, Salminen I, Mutanen M. Adenoma growth stimulation by the trans-10, cis-12 isomer of conjugated linoleic acid (CLA) is associated with changes in mucosal NF-κB and cyclin D1 protein levels in the Min mouse. J Nutr 2003;133:1943-1948.


* These authors contributed equally to this work

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Contribution of the author to papers I-IV

I The author planned the study together with the other authors. The experimental study, including the empirical work and preparation of the manuscript, was carried out by the author.

II The author planned the study together with the other authors. The author participated in the empirical work and was responsible for most of the laboratory analyses. The author participated in writing the manuscript together with A-M. Pajari and M.Mutanen.

III The author planned the study together with the other authors. The experimental work, including the empirical work and preparation of the manuscript, was carried out by the author.

IV The author planned the study together with the other authors. The experimental work, including the empirical work and preparation of the manuscript, was carried out together with M. Misikangas.
Abbreviations

AIN American Institute of Nutrition
AP-1 activator protein 1
APC human adenomatous polyposis coli gene
Apc murine adenomatous polyposis coli gene
APC adenomatous polyposis coli protein
β-TrCP β-transducin repeat-containing protein
CDK cyclin-dependent kinase
COX cyclooxygenase
CLA conjugated linoleic acid
FAP familial adenomatous polyposis coli
GSK-3β glycogen synthase kinase 3β
HDAC histone deacetylases
HNNPC hereditary non-polyposis colorectal cancer
IkB inhibitor of κB
IKK inhibitor of κB kinase
JNK cJun NH₂ terminal kinase
LOH loss of heterozygosity
MDM murine double minute
Min multiple intestinal neoplasia
MMP matrix metalloproteinase
NF-κB nuclear factor κB
NLS nuclear localization sequence
NSAID non-steroidal anti-inflammatory drug
PCNA proliferating cell nuclear antigen
RB retinoblastoma
SDS sodium dodecyl sulphate
TNF tumour necrosis factor
UV ultraviolet
VEGF vascular endothelial growth factor
Colon cancer and the \textit{APC} gene

Colorectal cancer is the second most common type of cancer, both in terms of incidence and mortality, in the developed countries (Stewart & Kleihues 2003). Worldwide, it is the third most common cancer type. Each year 945 000 new cases are diagnosed and approximately 492 000 people die of the disease. In Finland, colon cancer ranks second in females, after breast cancer. In males, colon cancer ranks third, after prostate and lung cancers. In Finland, the incidence of colon cancer has increased dramatically since the 1950’s. In females, the incidence has increased from 7.4 to 12.9 cases per 100 000 and in males from 6.4 to 16.3 cases per 100 000 from 1959 to 2005 (Finnish Cancer Registry 2007).

Approximately 95% of colon cancers develop sporadically, and only 5% are caused by heredity genetic predisposition. The two most common forms of hereditary colon cancer are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). Common for these are the susceptibility associated with inherited mutations; in the case of FAP, a mutation in the \textit{APC} tumorsupressor gene, and in HNPCC mutations in mismatch repair genes (Bodmer \textit{et al.} 1987; de la Chapelle 2004). These mutations cause the onset of cancer as early as in the teens. The development of sporadic colorectal cancer is a slow process and usually it is a matter of decades before cancer can be detected. The initiation of malignancy in sporadic cancer cases is also caused by mutations in the human genome. The mutations can be caused by different factors, including diet, tobacco smoke, UV-irradiation, different chemical compounds, etc. The cell has many ways of repairing the damage that is caused by mutagens, but if the cell adopts too many mutations that cannot be repaired, the formation towards malignancy begins.

The formation of sporadic colorectal cancer is a multistep event, in which the Adenomatous Polyposis Coli (\textit{APC}) tumorsuppressor gene plays a pivotal role, and it has been called a “gatekeeper” of the genome (Kinzler & Vogelstein 1996). Mutation of \textit{APC} is the first event leading to the formation of sporadic cancer and a mutation in this gene is found in a majority of sporadic colorectal cancer patients (Powell \textit{et al.} 1992). Mutation of \textit{APC} follows the Knudsen “two-hit model”, in which mutation of a tumorsuppressor requires both alleles of the gene to be mutatated for
cancer to develop (reviewed in Fodde et al. 2001). In the case of hereditary colon cancer one allele is mutated in the germline and second allele lost by somatic mutation. In sporadic colon cancer both alleles are lost due to somatic mutations. The emergence of mutations in other oncogenes and tumoursuppressors leads to disturbance in the homeostasis of epithelial cells, uncontrolled proliferation, and finally the formation of intestinal tumours (Figure 1). According to Hanahan & Weinberg, the six hallmarks of cancer are: self-sufficiency in growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg 2000). These factors together are responsible for the development of malignant tumours.

![Figure 1. The development of colon cancer through the adenoma-carcinoma sequence. A mutation in APC tumour suppressor gene is followed by several other mutations. Consequently, adenomas and finally carcinomas develop in the intestine. LOH=loss of heterozygosity. (Modified from Fearon & Vogelstein 1990 and Fodde et al. 2001).](image)

The APC gene has several functions in maintaining the integrity of the epithelial cell; it participates in cellular migration, cell division, and adhesion. For an extensive review on the various functions of the APC gene, interested readers are referred to an article by Näthke (Näthke 2006).
Diet and colon cancer

Lifestyle factors, such as diet, exercise, tobacco smoking and alcohol intake, have a major role in the development of colon cancer. Especially diet is a key component in the case of cancer in the intestine. It has been estimated that diet could account for as much as 70% of colorectal cancer deaths, so the impact of a healthy diet could be enormous (Doll & Peto 1981). Some evidence suggests that consumption of fruit and vegetables is associated with a reduced risk of colon cancer. The European Prospective Investigation into Cancer and Nutrition (EPIC) has shown that dietary fibre protects against colon cancer (Bingham et al. 2003), but the results on this issue are not consistent and it remains unclear if fibre, as such, has a protective effect (Park et al. 2005). Consumption of red- and processed meat increases the risk of colorectal cancer, while fish decreases it (Norat et al. 2005).

During the last few years, a vast amount of new, functional foods, have been introduced to the consumers. For a list of products available on the Finnish market see http://www.mm.helsinki.fi/MMKEM/Funktionaaliset/funketuotteet.htm (only in Finnish). These products often contain added compounds that are believed to prevent disease and promote health. The studies on the benefits of functional foods and the compounds they contain are often focused on a specific aspect of health and disease. Several products are already available that are marketed as promoting intestinal health, among them are products that contain different bacteria and added fibre. In this thesis, three different compounds, namely inulin, conjugated linoleic acid, and white currant, were studied to evaluate their chemopreventive effect in colon cancer.

The fibre inulin, is widely used as an added ‘dietary fibre’ in several food products. Inulin is a linear fructan polymer with fructans linked by β2 → 1 glycosidic bonds to form a chain. This non-digestible fibre can naturally be found in plant foods such as asparagus, garlic, leek, onion, and chicory root and its daily intake in Europe has been estimated to be 3-11g (Van Loo et al. 1995). Inulin is also produced industrially, either by extraction from chicory root, which may contain up to 80% inulin, or by synthetic methods (Gupta et al. 1985). Inulin has been shown to possess several beneficiary effects on health (Kaur & Gupta 2002), one of which is the prevention of colon tumour formation (reviewed in Pool-Zobel 2005). One of mechanisms by which
inulin is suggested to decrease cancer formation is by increasing the production of gut fermentation products produced in the colon by the microbial flora. Indeed, the beneficial effects of inulin has been shown to increase when appropriate bacteria is simultaneously introduced (Roberfroid 1998; Gallagher & Khil 1999). Recently, the first human study on inulin effects in colon cancer was published (Rafter et al. 2007). The study used human colon cancer and polypectomised patients who received a mixture of oligofructose-enriched inulin and the probiotics *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12. The result showed that the intestinal flora changed, colorectal proliferation was reduced, and several other biomarkers showed favourable alterations. Some markers, such as immunological parameters in the fecal water showed no change, however, or even an increase in production in cancer patients.

Another group of compounds that the food industry has been interested in, because of their health promoting effects are different fatty acids, especially ω−3 and ω-6 fatty acids. There has also been interest in conjugated linoleic acid (CLA). CLA is a common name for the different conjugated forms of linoleic acid (C18:2) where the double bonds are conjugated doublebonds instead of the normal doublebonds seen in linoleic acid. In food, nine different forms of CLA have been found, the most common form is *cis*-9, *trans*-11 CLA (MacDonald 2000, Fritsche & Steinhart 1998). CLA is formed in the stomach of ruminants where bacteria fermentate linoleic acid and produce different conjugated forms of that fatty acid. CLA can also be formed endogenously by the Δ-9 desaturase enzyme from vaccenic acid (C18:1) that is formed during incomplete oxidation of polyunsaturated fattyacids (Griiniari et al. 2000). Good natural sources of CLA are milk and milkproducts and the meat of ruminants such as cow and lamb, in which CLA can account for approximately 1% of the total fat (Fritsche & Steinhart 1998). The two most common CLA isomers are the *cis*-9, *trans*-11 and *trans*-10, *cis*-12, and most research is done using these two isomers, or a mixture of them. CLA has been shown to affect body composition and to reduce body fat (reviewed in Wang & Jones 2004). For this reason, CLA is sold at health stores as a dietary supplement. CLA, however, has also been found to be chemopreventive, especially in breast cancer, but also in colon cancer (reviewed in Lee et al. 2005). In a Swedish study, women who consumed more than 4 servings of
fatty milk products daily had a decreased risk of colorectal cancer compared to women who consumed less than one serving daily. When adjusted for CLA intake, the inverse correlation remained but was no longer significant (Larsson et al. 2005). So far, the experimental data on CLA in colon cancer prevention is limited to animal and cell culture studies, no studies on human subjects are available. In rat and mice CLA has been found to reduce the incidence of tumours (Park et al. 2001), suppress development of aberrant crypt foci (ACF) (Suzuki et al. 2006) decrease metastasis (Soel et al. 2007), and increase apoptosis (Park et al. 2001). In colon cancer cell lines, the mechanisms of cancer prevention have been studied, but the exact mechanism whereby CLA acts is still inconclusive.

Colourful berries, such as blueberry, black currant, etc., contain high amounts of flavonoids and other phenolic compounds (reviewed in Heinonen 2007). These bioactive compounds have been shown to have beneficial effects on health, including the prevention of cancer formation (reviewed in Duthie 2007) and flavonoids are at present being added to functional foods. White currant (*Ribes x pallidum*) is an edible berry that has evolved due to genetic mutation of red currant. White currant only contains low levels of these phenolic compounds compared to other berries. Most of the phenolic content is hydroxybenzoic acid derivates and proantocyanidins, whereas red and black currants, for example, mostly contain anthocyanins (Määttä et al. 2001). Because of the low content of phenolic compounds, white currant has not been studied in detail as a chemopreventive food. One study, however, found that white currant juice inhibits the growth of an intestinal cell line and this was not correlated to the antioxidant properties of the berries (Boivin et al. 2007).

To understand how diet affects cancer formation, we should investigate the response of diet on the health of epithelial cells. In the cells, cell signalling pathways are responsible for mediating information from outside the cell, so the cell can respond to stimulus in the intestine. In the following sections, three fundamental signalling pathways in colon cancer formation are presented.
The Wnt pathway

During organ development and remodelling, the wnt pathway is the major signalling pathway responsible for proliferation of cells (reviewed in Clevers 2006). It is also responsible for the development of new cells in the crypts of the epithelial villi. To activate the pathway, the wnt ligand binds to its Frizzled receptor at the surface of the cell, phosphorylation of β-catenin is inhibited, and β-catenin can transfer to the nucleus where it binds to the transcription factor Tcf-4/Lef and activates transcription (Behrens et al. 1996) (Figure 2). In epithelial cells, β-catenin is also present at the cell membranes where it together with E-cadherin forms adherens junctions and is involved in cellular polarity and cell migration (Gumbiner 2005).

When cell proliferation is not needed, β-catenin is degraded in the cytosol to prevent it from entering the nucleus. Absence of wnt-signaling causes β-catenin to be phosphorylated by a huge multiprotein complex, consisting of APC, glycogen synthase kinase 3β (GSK-3β), and axin (Behrens et al. 1998). Phosphorylation of β-catenin at serines 33 and 37 causes ubiquitination and degradation by the 26S proteasome (Aberle et al. 1997).

A mutation in the APC gene can cause a change in the APC protein, which inhibits the assembly of the APC-GSK-3β-axin complex. It is still not completely understood how a mutation in APC affects the assembly of the complex. If axin levels in APC mutated cells is increased, β-catenin can be phosphorylated (Lee et al. 2003), indicating that APC is not always necessary for phosphorylation. If the complex responsible for β-catenin phosphorylation is unable to function, β-catenin is not degraded by the proteasome and accumulates in the cytosol (Rubinfeld et al. 1996). Finally, it translocates to the nucleus and binds to the transcription factor Tcf-4/Lef and activates the transcription of its targets (Figure 2). It seems that β-catenin translocates to the nucleus independently, without the co-operation of importins or the use of a Nuclear Localisation Signal (NLS) (Fagotto et al. 1998). Likewise, β-catenin can be exported out of the nucleus independently, or with the help of APC or axin (Eleftheriou et al. 2001; Neufeld et al. 2000). A resent study, however, shows that truncated APC can control the activity of β-catenin transcription (Schneikert et al. 2006).
This inhibition of β-catenin activity is cell-cycle dependent, since increase of APC levels near the G1-S boundary is able to inhibit β-catenin in a concentration dependent manner.

The list of β-catenin targets increases constantly, there are now about 100 targets that have been recognised, and most probably more remain to be identified. The two first genes to have been identified as β-catenin targets were the cyclin D1 and c-myc genes (Tetsu & McCormick 1999; He et al. 1998). It is also likely that these two targets are responsible for the proliferative response to β-catenin signalling. It has, however, been argued that cyclin D1 in fact is not a direct target of wnt signalling in vivo, but is upregulated as a secondary event (Sansom et al. 2005). Recently, the same authors showed that deletion of c-myc from Apc deficient mice completely restores the effects of Apc deficiency (Sansom et al. 2007). This effect was independent of high levels of

**Figure 2.** The function of the wnt pathway during normal cellular conditions (A and B) and during cancer development (C). A) The wnt-pathway is activated as the wnt ligand binds to the Frizzled receptor; β-catenin enters the nucleus to activate the transcription of its targets. B) In the absence of wnt signalling, β-catenin is phosphorylated and degraded. C) Mutation of APC leads to changes in the APC protein, and the complex responsible for β-catenin phosphorylation is out of action. This leads to accumulation of β-catenin in the cytosol, translocation to the nucleus and transcription of targets genes. (Modified from Fodde et al. 2001).
nuclear β-catenin, indicating c-myc is an essential target that mediates the neoplastic change in cells with Apc mutations. Other genes that have been identified as wnt/β-catenin targets include PPARδ (He et al. 1999), c-jun, fra-1, and uPAR, which are all part of the Activator protein-1 (AP-1) transcription factor (Mann et al. 1999), Matrix metalloproteinase-7 (MMP-7) which degrades extracellular matrix components, (Brablez et al. 1999), and Vascular endothelial growth factor (VEGF) that is involved in angiogenesis (Zang et al. 2001). A complete list of wnt targets can be found at the wnt homepage at http://www.stanford.edu/%7ernusse/pathways/targets.html. The targets of β-catenin have different functions, involving all aspects of cancer development, including cell migration, angiogenesis, and metastasis.

Because of the high frequency of APC mutations in colon cancer, the wnt pathway is a key regulator of cancer development especially in this tissue. The intestinal epithelium also differs from many other tissues because of the constant renewal of epithelial cells. In humans, as well as in mice, the lifespan of an epithelial cell is 3-5 days. New cells are produced at the crypt of the villi from stem cells that reside near the bottom of the crypt. In mice lacking Tcf4, the crypt progenitor department is absent, indicating that wnt is needed for the establishment of progenitor cells (Korinek et al. 1998). The wnt proteins needed for the activation of the pathway are expressed by the epithelial cells, and the surrounding mesenchyme (Greogrieff et al. 2005).

Several observations in human colorectal cancer tissue, animal models, and cell culture systems have shown that accumulation of β-catenin is detrimental for epithelial cells and is essential in cancer development. In human colorectal cancer tissue, β-catenin expression is significantly higher in adenomas and carcinomas compared to normal epithelia (Chen et al. 2007). Also in aberrant crypts, nuclear and cytosolic β-catenin levels are increased (Sena et al. 2006). In addition, it has been shown that nuclear accumulation of β-catenin strongly correlates with tumour size and dysplasia (Brablez et al. 2000). The wnt target cyclin D1 is also frequently upregulated in colon cancer (Arber et al. 1996). In tumours, β-catenin expression is strongest at the invasive front of the carcinoma, where it is also localised in the nucleus, but in the centre of the carcinoma, β-catenin is localised in the cytosol and at
the membranes (Brablez et al. 2001). Increased expression of β-catenin and nuclear translocation also causes hyperproliferation of epithelial cells in the crypt (Sellin et al. 2001).

Mutations in the β-catenin gene (CTNNB1) have also been found in colorectal tumors, proving the importance of the wnt pathway in tumorigenesis (Morin et al. 1997). Mutations in β-catenin are more common in small adenomas than in large ones, indicating that this mutation is an early event in cancer formation (Samowitz et al. 1999). Several studies, using mice, have shown the importance of Apc mutations and β-catenin in tumour formation. When β-catenin was mutated so that it could not be phosphorylated, intestinal adenomas developed in the mice carrying that mutation (Harada et al. 1999). This showed that stabilised β-catenin could cause the formation of adenomas. Also several knockout mice have been generated that have mutations in the Apc gene (for review see Taketo 2006). These mice all have truncating mutations of Apc, and only the length of the Apc protein differs. Interestingly, the Apc$^{\Delta 716}$ mouse that has the shortest remaining Apc protein develops approximately 300 adenomas in the intestine, whereas the Apc$^{1638N}$ mouse with a longer Apc protein only develops approximately 3 adenomas (Fodde et al. 1994; Oshima et al. 1995).
The nuclear factor κB pathway

NF-κB function

Nuclear factor κB is a collective name for a family of transcription factors. It was first described in 1986 by Sen & Baltimore (Sen & Baltimore 1986) as a nuclear factor needed for immunoglobulin kappa light chain transcription in B-cells, hence the name NF-κB. The NF-κB pathway has been studied for its role in immunology, and many of the activators and targets are important mediators of infection, stress and injury. During the last few years it has become apparent that the pathway also has a role in the development of cancer. Compared to many other signalling pathways, NF-κB has a wide variety of activators and a myriad of targets. Because NF-κB is ubiquitously expressed and resides in the cells as an inactive form just waiting for activation, the response is quick compared to many other pathways.

NF-κB is a dimer that consists of members of the rel protein family, which include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (reviewed in Ghosh et al. 1998 and Karin & Ben Neriah 2000). These proteins have a common conserved region of 300 amino acids at the N-terminal end known as the rel homology domain (RHD). At this region of the protein, DNA-binding, dimerisation, and interaction with Inhibitor of κBs (IκB) takes place, it also contains the nuclear localisation sequence (NLS). The first NF-κB molecule described was a p50/p65 heterodimer, this is the most common of the NF-κB dimers (Nolan et al. 1991). There are currently three different types of NF-κB pathways: the canonical or classical pathway, the non-canonical or alternative pathway and pathway 3. The best understood, and in the future referred to in the text as the NF-κB pathway, is the canonical pathway. Readers interested in the non-canonical pathway and pathway 3 are referred to a review by Scheidereit (Scheidereit 2006).

In the cytosol, NF-κB is bound to its inhibitor, IκB (Figure 3). IκB binds NF-κB and masks its NLS, thus inhibiting its translocation into the nucleus. The IκB family of proteins includes IκBα, IκBβ, IκBε, IκBϕ, and Bcl-3 as well as NFκB1 (p105) and NFκB2 (p100) (Reviewed in Baldwin 1996). Recent studies also suggest that histone
deacetylases can bind to NF-κB in an IκB manner (Campbell et al. 2004). These inhibitors typically have ankyrin-repeat-motifs, regions of protein/protein interaction, which interact with the rel domain of NF-κB. The degradation of the IκB-NFκB complex requires the complex to be phosphorylated by the Inhibitor of κB kinases, IKKs. IKK is a multi-component complex that consists of equal amounts of IKKα and IKKβ and two molecules of NEMO (also called IKKφ, IKKAP1 and FIP-3). IKK simultaneously phosphorylates IκBα at serines 32 and 36, a great specificity for phosphorylation at these sites exists.

Phosphorylation of the IκB-NF-κB complex signals for the degradation of IκB by the ubiquitin proteolysis system. As a consequence NF-κB is liberated from the complex and the NLS is unmasked. NF-κB binds to the karyopherins and translocates to the nucleus. In the nucleus, NF-κB dimers bind to DNA at κB sites, which are highly conserved regions in the DNA, and activate the transcription of its targets (reviewed in Ghosh et al. 1998 and Karin & Ben Neriah 2000).

**Figure 3.** The canonical NF-κB pathway. Activation of the pathway leads to phosphorylation of IκB by IKK and degradation of IκB by the proteasome. The IκB-NF-κB complex is liberated and translocates to the nucleus where it binds to κB sites of the DNA and activates transcription of its targets. (Modified from Gilmore 2006).
Over 200 activators of the NF-κB pathway exist (Pahl 1999). These include bacteria, viruses, and their products, inflammatory cytokines, physiological-, physical-, and oxidative stresses such as hyperoxia, UV irradiation, and hydrogen peroxide. Environmental hazards, including cigarette smoke also activate NF-κB. Other activators are a wide variety of drugs, receptor ligands, apoptotic mediators, physiological mediators, and several chemical agents. The target genes of NF-κB include cytokines, immunoreceptors, cell adhesion molecules, stress response genes, regulators of apoptosis, growth factors, and transcription factors (Pahl 1999).

**NF-κB in cancer**

The first indication that NF-κB might be involved in cancer came when the p50 subunit was cloned. Sequence analysis revealed that p50 and p65 have a homology to v-Rel, an oncogene of the avian reticuloendotheliosis virus, and the proto-oncogene c-rel, its cellular counterpart (Kieran et al. 1990; Ruben et al. 1991). Today, NF-κB in the context of cancer is probably the most studied and fastest growing area in NF-κB research. It seems that inflammation, as a component in cancer formation, is considered one of the main reasons in activating the NF-κB pathway (Karin & Greten 2005).

NF-κB, through its target genes, has an opportunity to affect most of the different aspects in cancer formation (Reviewed in Bassères & Baldwin 2006 and Karin et al. 2002). It seems, however, that the central role of NF-κB in cancer development is due to its ability to activate the transcription of antiapoptotic genes. One of the most important antiapoptotic proteins is Bcl-XL, which is a member of the Bcl-2 family and a target of NF-κB (Chen et al. 2000). Also the caspase inhibitors cIAP1 and cIAP2, as well as the specific caspase-8 inhibitor c-FLIP, are targets of NF-κB (Wang et al. 1998; Micheau et al. 2001). Another group of NF-κB targets are those that enhance cell proliferation. Of these, cyclin D1 and c-myc have gained the most attention (Guttridge et al. 1999; Duyao et al. 1992). In light of colon cancer development, cyclooxygenase-2 (COX-2) is a target that has clearly been shown to affect tumourigenesis (Yamamoto 1995). NF-κB also activates targets necessary for
angiogenesis and metastasis, including matrix metalloproteinases (MMP) and vascular endothelial growth factor (VEGF) (Vincenti et al. 1998; He 1996; Chilov et al. 1997). All the aforementioned targets promote tumour formation and growth, enabling NF-κB to affect cancer promotion at several levels. Some targets of the pathway, such as p53, Fas, and Fas ligand (FasL) also induce apoptosis, however (Wu & Lozano 1994; Chan et al. 1999; Matsui et al. 1998). The fact that pro apoptotic targets of NF-κB exist has raised questions on the role of NF-κB in apoptosis.

Substantial evidence shows that NF-κB inhibits apoptosis (Reviewed in Barkett & Gilmore 1999 and Dutta et al. 2006). One of the first indications for a role in anti-apoptosis came from RelA knockout mice that died in utero due to massive liver apoptosis (Beg & Baltimore 1996). It has recently been shown that Bcl-2 overexpression in RelA knockout mice is not able to rescue them from apoptosis, showing that RelA does not inhibit apoptosis through Bcl-2 alone (Gugasyan et al. 2006). As mentioned earlier, NF-κB has several targets that inhibit apoptosis. Activation of anti-apoptotic targets is, however, only one of the means in which it can inhibit apoptosis. NF-κB can activate the transcription of Murine double minute 2 (MDM2), which inhibits the action of p53 (Tergaonkar et al. 2002). It can also repress the transcription of pro-apoptotic molecules, such as caspase-8 (Chen et al. 2003). On the other hand, evidence also shows a pro-apoptotic role for NF-κB. The pathway has several pro-apoptotic targets, including p53, Fas and FasL, and Bax (Wu & Lozano 1994; Chan et al. 1999; Matsui et al. 1998; Grimm et al. 2005). Also, it can repress the transcription of the anti-apoptotic genes Bcl-XL and XIAP (Campbell et al. 2004).

It is still not very well understood what determines the fate of the cell in response to the NF-κB pathway. It seems that it is the nature of the stimulus and the context of the cell that determines if pro- or anti-apoptotic responses are activated (Dutta et al. 2006). Different effectors of the pathway have been shown to have different ways of regulating the response, for example UV-C irradiation, daunorubicin and doxorubicin drugs convert RelA to a transcriptional repressor (Campbell et al. 2004). Aspirin can affect the pathway by sequestering RelA in the nucleolus and thus inhibit its transcriptional activity (Stark & Dunlop 2005). Perkins has suggested a mechanism for the pro- or anti-apoptotic effect of NF-κB (Perkins 2004). In this model, NF-κB
could in the early stages of cancer act as a tumour suppressor. Here, activation of p53 and Arf by oncogenic stimulus would facilitate the binding of NF-κB to HDAC corepressor complexes. This would then inhibit the expression of anti-apoptotic genes. When the cell requires mutations and the loss of function of p53 and Arf, NF-κB would bind to co-activators Bcl-3 and p300. This activating stimulus would cause NF-κB to act as a tumour promoter. Another mechanism that at least in the case of TNFα can determine if the response is pro- or anti-apoptotic is the interaction between the NF-κB and the JNK pathways (Reviewed in Papa et al. 2006). Here, it is the composition of the TNF-R1 receptor complex that determines if caspases, through TRADD, or the JNK pathway through RIP1, activates apoptosis, or if NF-κB, through TRADD and RIP simultaneously, activates anti-apoptosis and inhibits the two other pathways. As described above, several factors exist that determine the outcome of NF-κB, and a clear picture on how the pathway behaves in a specific cancer type, such as colon cancer, is still to be elucidated.

In human colon cancer, NF-κB has been found to be upregulated in the tumours compared to normal epithelia and also to bind to DNA in 8 of 10 tumours (Maihöfner et al. 2003; Lind et al. 2001). In the tumours p65 is also found to be nuclear (Evertsson & Sun 2002; Maihöfner et al. 2003). The expression of p65 has also been shown to increase when tumour dysplasia increases (Yu et al. 2003; Aranha et al. 2007). In this study, p65 also correlated positively with Bcl-2, Bcl-XL, and proliferation, but negatively with apoptosis. Another study showed NF-κB in colon adenomas to be active in stromal macrophages and that the staining of NF-κB coincides with COX-2 (Hardwick et al. 2001) Interestingly, JNK was also found to be upregulated in colon adenomas indicating that inflammatory signals were activated. In concordance with these results, p65 has been found to be activated in inflamed intestinal mucosa (Rogler et al. 1998). An interesting observation is the effect of non-steroidal inflammatory drugs (NSAID) on the NF-κB pathway. NSAIDS have been shown to reduce the risk of colon cancer in humans (Thun et al. 2002), and many of them also seem to inhibit NF-κB. At least aspirin, sulindac and sulindac sulfone, as well as curcumin have been shown to inhibit NF-κB activation in vitro (Kopp & Ghosh 1994; Yamamoto et al. 1999; Plummer et al. 1999). On the other hand, aspirin has also been shown to induce nuclear localization of NF-κB, where it is moved to the
nucleolus to inhibit the transcription of anti-apoptitic genes (Stark et al. 2001; Stark & Dunlop 2005). A distinct mechanism for the role of NF-κB in colon cancer has not yet been identified and it is most probable that the pathway has several mechanisms that affect colon cancer formation.
The p53 pathway

p53 function

The key regulator of cell fate is the p53 transcription factor. The actions of p53 are described in detail in several excellent reviews (Voudsen & Lu 2002; Kastan 2007; Shu et al. 2007; Stiewe 2007). A diverse multitude of cellular stress, such as DNA damage, hypoxia, mitogens, etc. activates p53. The response of the cell to it is either cell cycle arrest, apoptosis, senescence, or differentiation. It is not well known what determines how the cell will respond to different p53-activating signals, but it appears to depend on the activating signal itself, the duration of the signal, and the cellular context. The response to p53 is largely determined by which targets of p53 are activated. This is believed to be controlled by the co-activators that bind to DNA together with p53 (Voudsen 2006).

The interaction of p53 with its inhibitors, MDM2 and MDM4, is of fundamental importance in the activation of p53 (Toledo & Wahl 2006). Under normal cellular conditions, p53 levels are kept low due to MDM2 (Figure 4). In the cytosol, MDM2 ubiquitinates p53 and causes its degradation. The activity of p53 is kept low due to MDM4 (also called MDMX), which interacts with the transactivating domain of p53. During stress, MDM2 starts to ubiquitinate itself and MDM4, causing their degradation and activation of p53. Activated p53 causes transcription of more MDM2, allowing p53 to activate the transcription of its other targets at full activity. When p53 is no longer needed, MDM2 again starts to ubiquitinate p53 and MDM4 levels increase and inhibits transcriptional activity.

During stress, the two main functions of p53 are to either stop the cell cycle and give the cell an opportunity to repair defects, or activate programmed cell death. P53 promotes cell cycle arrest at a checkpoint in G1 phase. p53 enters the nucleus and the transcription of p21 is activated. P21 inhibits the function of the cyclin-dependent kinases (CDK) and phosphorylation of the retinoblastoma (RB) protein is blocked (reviewed in Kasten & Giordano 1998). Therefore, RB remains bound to E2F and the cell cycle stops at the G1 phase. Once the DNA has been repaired, the cell enters the S-phase and proliferates.
Figure 4. Activation of p53 (Modified from Toledo & Wahl 2006). A) p53 levels are kept low and inactive due to MDM2 and MDM4. B) When p53 activity is needed, MDM2 ubiquitinates itself and MDM4, allowing p53 to enter the nucleus and activate its targets. C) p53 activates MDM2, which causes more degradation of MDM4, and MDM2, and so p53 activity reaches its maximum capacity. D) When p53 is no longer needed, MDM2 again starts to ubiquitinate p53 and MDM4 can again inhibit p53.

In cases where the cell is damaged to the point that it cannot be repaired, it is eliminated by programmed cell death, *i.e.* apoptosis. Again p53 is activated and in the nucleus activates the transcription of genes that encode for apoptotic proteins. P53 can induce apoptosis by different mechanisms, involving both the intrinsic and extrinsic pathways (Figure 5). Also, p53 can inhibit the action of anti-apoptotic proteins thus adding to the chance of apoptosis occurring. Activation of the extrinsic apoptotic pathways by p53 involves transcription of death receptors, such as Fas. Fas is a membrane receptor that on the cell surface is activated by the Fas ligands (FasL) of neighbouring cells. Activation of Fas, or other death receptors, causes the activation of downstream caspase-8.

Activation of the intrinsic pathway by p53 is possible by two mechanisms; p53 can either induce the transcription of apoptosis activators, such as bax, or directly inhibit the action of anti-apoptotic proteins in the cytosol. The activation of bax, or permeabilization of the mitochondrial membrane by inhibition of Bcl-2 or Bcl-X\textsubscript{L} causes the release of cytochrome c (cyt c) from the mitochondrion. This activates caspase-9. Activation of both caspase-8, from the extrinsic pathway, and caspase-9,
by cyt c, has the same effect: caspase-3 is activated leading to the activation of the effector caspases -6 and -7. Caspase-6 and -7 then enter the nucleus and start to cleave proteins in the nucleus and finally the cell dies. Interested readers are referred to reviews describing the general mechanisms of apoptosis in more detail (Jin & El-Deiry 2005; Israelis & Israelis 1999).

**Figure 5.** The involvement of p53 in apoptosis. P53 can affect the extrinsic apoptotic pathway (indicated in blue) by activating the transcription of death receptors such as Fas. Activation of pro-apoptotic molecules, such as bax, activates the intrinsic apoptotic pathway (indicated in green) and release of cytochrome c from the mitochondria. Alternatively, p53 can activate the intrinsic pathway by inhibiting anti-apoptotic proteins such as Bcl-2. (Modified from Chipuk & Green 2006).
P53 in cancer

The tumour suppressor and transcription factor p53 is a key component in cancer development, and has also been called the guardian of the genome. As illustrated in Figure 1, page 12, mutations in the p53 gene (TP53 in humans and Trp53 in mice) are one of the steps that lead to formation of malignant tumours, and a mutation in p53 is found in approximately 50% of all cancers (Hollstein et al. 1991). The frequency estimates of p53 mutations in colon cancer tumours vary between approximately 40% to over 80% (Baker et al. 1990; Soong et al. 1997). Most mutations in human colorectal tumours are missense mutations and are located in exons 5-8 (Greenblatt et al. 1994). When p53 function is inhibited by p53 knock-out in mice, they develop tumours throughout the body (Donehower et al. 1992). Introduction of wildtype p53 to p53 mutant colon cancer cells activates apoptosis and induces regression of tumours in nude mice (Shaw et al. 1992). Mutation of p53 in the Min mouse does not, however, affect the number or size of tumours developed in the intestine, indicating that p53 mutation is not essential for the development of intestinal tumours (Clarke et al. 1995; Fazeli et al. 1997). In human colon cancer, p53 is mutated late in the process of cancer formation, and mutations in p53 increase dramatically in the transition from low-grade dysplasia to high-grade dysplasia (Boland et al. 1995). This indicates that it is not crucial for the cell to eliminate p53 function in order for colon cancer to develop, but it is the final event that causes the development of malignant tumours. If the wild type p53 could be activated early in tumourigenesis, this could protect the cell from mutations and activate apoptosis to remove damaged cells.
**Interactions between β-catenin, NF-κB, and p53 pathways**

Each of the pathways described above, the wnt, NF-κB and p53 pathways have their own important function both in normal cellular conditions and in cancer formation and progression. But in addition to acting as separate, individual pathways that signal for different actions needed in the cell, they also interact with each other, adding to the complexity and variety of cell signalling. One of the simplest means by which β-catenin, NF-κB, and p53 could influence one another’s function is to activate the transcription of each other. Not to make things too simple, however, only NF-κB activates the direct transcription of p53 (Wu & Lozano 1994), although all three pathways have a vast amount of targets.

**β-catenin and NF-κB**

The β-catenin and NF-κB pathways have several similarities; both are activated by signals from outside the cell, their action is regulated by phosphorylation and degradation, and they both enter the nucleus where they activate transcription. The ubiquitin-proteasome pathway that degrades both β-catenin and the IκB-NF-κB complex is described in more detail later in the chapter. Some reports show a specific interaction between β-catenin and NF-κB. The work of Deng et al. has shown that in colon cancer cells, β-catenin can physically interact with NF-κB and bind to it (Deng et al. 2002). This interaction downregulated NF-κB transcriptional activity and reduced the expression of the target Fas. The same authors have shown that alteration in GSK-3β and APC could also alter NF-κB activity through β-catenin (Deng et al. 2004), an observation that could prove important in APC mutated colon cancers. Interestingly, it has also been reported that the NF-κB subunit RelA (p65) can inhibit the transcriptional activity of β-catenin in vitro (Masui et al. 2002). This inhibition was not dependent on the transcriptional activity of RelA, but was probably due to interference of RelA on the β-catenin/Tcf-4 complex. Diclofenac, a non-steroidal anti-inflammatory drug (NASAID), was suggested to inhibit β-catenin dependent...
transcription by this mechanism, i.e. activation of NF-κB that then inhibits β-catenin (Cho et al. 2005).

β-catenin and p53

As was described above for interaction between β-catenin and NF-κB, the interaction of β-catenin and p53 also act in two-ways. Increased cellular β-catenin can increase the levels of transcriptionally active p53 (Damalas et al. 1999). This is achieved by inhibiting the action of MDM2 that is responsible for the degradation of p53 in the cytosol (see Figure 4, p.26) (Damalas et al. 2001). Similarly, increased p53 is able to downregulate β-catenin by increasing its degradation by the ubiquitin pathway (Sadot et al. 2001). The mechanism by which p53 increases β-catenin degradation is not completely understood yet. Some studies indicate that it is the increase in the p53 targets, Siah-1 and PTEN that is responsible for reducing the level of β-catenin (Liu et al. 2001; Matsuzawa & Reed 2001). Others suggest that p53 induces a faster mobilisation of Axin to the β-catenin phosphorylation complex and thus increases the phosphorylation and degradation of β-catenin (Levina et al. 2004). The increased activity of p53 by β-catenin, followed by an increase in β-catenin degradation forms a feedback loop in which increased β-catenin increases p53 transcription, which in turn down regulates β-catenin (Figure 6). This loop could prove to be an important factor in the inhibition of cancer formation due to the high levels of β-catenin in the developing tumours.

![Figure 6](image_url)

*Figure 6. β-catenin and p53 regulate each others action and form a negative feedback loop.*
NF-κB and p53

Of the interactions between β-catenin, NF-κB, and p53, the simplest one is the activation of p53 transcription by NF-κB (Wu & Lozano 1994). P53 is one of the targets of NF-κB that activates apoptosis. A lot of discussion on the role of NF-κB in apoptosis focuses on the fact that p53 is a target of the pathway. It has been shown that NF-κB is in fact required for p53 dependent apoptosis (Ryan et al. 2000). Induction of p53 caused activation of NF-κB that correlated with the ability of p53 to induce apoptosis. Benoit et al. also showed that p53 and NF-κB have additive effect for the activation of the p53 promoter. NF-κB activity also maintains or weakly induces p53 gene transcription (Benoit et al. 2000). The interactions between NF-κB and p53 described above seem to require an intact p53 gene and might therefore be relevant only in the early stages of colon cancer formation since p53 is often mutated later in the process. Interestingly, mutated p53 also enhance NF-κB activity in melanoma cells (Gulati et al. 2006). It has also been shown that mutant p53 activates the transcription of the NFκB2 gene, which encodes p100, a precursor of the p52 subunit (Sciani et al. 2005). Also p52 regulates p53 target gene expression, but this is not due to DNA-binding of p52 (Schumm et al. 2006).

The ubiquitin-proteasome pathway

A shared feature of both the wnt and NF-κB pathways, as well as p53, is the ubiquitin-proteasome pathway, a machinery that is used in the cells to degrade proteins. For the wnt-pathway the ubiquitin-proteasome pathway is a way of deactivating cell signalling, as β-catenin does not enter the nucleus. For NF-κB, on the other hand, ubiquitination and degradation leads to activation. In the case of p53, ubiquitination keeps p53 levels low when it is not needed, but is also responsible for its activation as MDM2 is ubiquitinated. The function of the ubiquitin-proteasome system in the different signalling pathways is described in detail in the reviews by Karin & Ben Neriah 2000, Maniatis 1999, Laney & Hochstrasser 1999, and Watson & Irwin 2006.
The first event that leads to the degradation of a protein is the phosphorylation of the unwanted protein. In the case of β-catenin, the first step is the interaction between β-catenin and axin. Axin recruits a kinase, casein kinase I (CKI) to phosphorylate β-catenin at serine 45. This makes β-catenin a substrate for glycogen synthase kinase 3β (GSK3β) that phosphorylates β-catenin at threonine 41 and serines 37 and 33. The parallel step in the NF-κB pathway is the phosphorylation of the IκB-NF-κB complex by IKK at serines 32 and 36 on IκBα. After phosphorylation, a specific receptor subunit of an E3 ubiquitin ligase (β-TrCP) recognises the phosphorylated protein. β-TrCP contains a consensus motif that specially recognises phosphorylated IκB and the same motif also recognises phosphorylated β-catenin. β-TrCP then recruits other components of E3 and the ubiquitin ligase covalently attaches ubiquitin polypeptides, small proteins that are found in all eukaryots, to lysine residues on IκB or β-catenin and forms a ubiquitin chain. Interestingly, it has been shown that β-catenin upregulates the expression of β-TrCP, which results in acceleration of β-catenin degradation and activation of NF-κB (Spiegelman et al. 2000).

When IκB or β-catenin have been ubiquitinated, they are degraded by the 26S proteasome. The effect of degradation has the opposite effects on the wnt and NF-κB pathways; degradation of the IκB-NF-κB complex unmasks the nuclear localisation signal on NF-κB and it is transferred to the nucleus. β-catenin, on the other hand, is degraded and thus its level is kept low in the cytosol. It is noteworthy that no loss of function mutations in β-TrCP have been found in human cancers (Sparks et al. 1998). It is thought that this might be due to the important role of NF-κB in cancer, or the fact that β-catenin could be degraded by other means.

The involvement of ubiquitination in the activity of p53 differs from that of β-catenin and NF-κB. P53 is ubiquitinated at several lysine residues due to its specific E3 ligase, MDM2. Also other p53-specific E3 ligases, Pirh2, COP1 and ARF-BP1, have been described to ubiquitinate p53. Ubiquitination of p53 causes its degradation by the proteasome as was the case with β-catenin and NF-κB. It has also been shown that MDM2-dependent monoubiquitination of p53 causes its nuclear export. Ubiquitination of p53 causes its degradation and deactivation. Ubiquitination also
activates p53, as MDM2 causes its own ubiquitination and p53 is free to enter the nucleus. This shows the multitude of functions that can occur in cells by simple biochemical reactions.

In summary, there are several ways in which the wnt, NF-κB and p53 pathways interact and regulate each other. β-catenin and NF-κB, as well as β-catenin and p53, interactions work both ways and they can regulate one another’s function. The interaction between NF-κB and p53 focuses on the ability of NF-κB to regulate apoptosis. The interactions described above are only a part of those that have been reported in the literature and concentrates on direct interaction between the key proteins in the pathways. To make any conclusions on how the pathways interact in the formation of colon cancer is impossible.
Dietary effects on wnt, NF-κB, and p53 pathways

The data available on the mechanisms of diet on colon cancer formation are contradictory and epidemiological studies have not been able to give convincing conclusions. Using experimental models for cancer formation it is possible to study the changes in the intestinal epithelia, caused by diet and possible mechanisms by which diet also affects tumour development. From this kind of information it is possible to better understand the role of diet in colon tumourigenesis. In the intestine diet is probably the most important regulator of mucosal cell environment.

The studies on the effect of diet or different dietary constituents on wnt-signalling have not been numerous. Retinoic acid did not affect cellular β-catenin in the mucosa of Min mice even though adenoma formation on growth was increased (Mollersen et al. 2004b). Carnasol, a constituent of rosemary, decreased the tumour number in the Min mouse, and restored β-catenin at the membrane in Min mouse enterocytes ex vivo (Moran et al. 2005). Curcumin, an active constituent of turmeric, decreased the tumour number in Min mice with concomitant reduction of β-catenin (Mahmoud et al. 2000). In colon cancer cells, curcumin has also been shown to decrease nuclear β-catenin and the transactivation of β-catenin /Tcf-4 (Park et al. 2005; Jaiswal et al. 2002). The effect of CLA on β-catenin levels in Caco-2 cells was investigated in one study, and in that study, CLA increased cellular levels of β-catenin (Bozzo et al. 2007). Taken together these studies do not give a convincing explanation for the behaviour of wnt signalling during carcinogenesis.

The action of dietary constituents on the NF-κB pathway in colon cancer is an area that is gaining more attention, but at the moment the data is scarce. In vitro, curcumin, the active ingredient in turmeric, has been shown to inhibit NF-κB activation by inhibiting the action of IKK (Plummer et al. 1999). β-carotene, on the other hand, was found to inhibit cell growth while at the same time increasing the DNA-binding activity of NF-κB (Palozza et al. 2003). Interestingly, there are several hundred inhibitors of NF-κB that are known, all of which potentially could affect colon cancer formation. These include a vast amount of compounds that are derived from natural
products such as fruits, berries, vegetables, etc. A complete list of NF-κB inhibitors can be found in supplementary tables S1-S8 in the review by Gilmore & Herscovitch (Gilmore & Herscovitch 2006).

Diet has great potential in acting on the p53 pathway, especially in the epithelial cell. Diet that were high in sugar, red meat, fast food, and trans-fatty acids were more likely in colon cancer patients with $p53$ mutations than in those with wild type $p53$ (Slattery et al. 2002). No associations, however, between energy intake, folate, calcium, fibre, or specific foods and $p53$ mutations existed. Little data is available on the action of specific dietary components on p53 in colon cancer. Gallotannin, a plant polyphenol, was found to induce p53-dependent cell cycle arrest in colon cancer cells, but it did not induce apoptosis (Al-Ayyoubi & Gali-Muhtasib 2007).
The Min mouse

Familial Adenomatous Polyposis (FAP) is a hereditary form of colorectal cancer syndrome in which the APC gene is mutated, causing the formation of hundreds of tumours in the intestine as early as in the teens. An animal model for this condition is the multiple intestinal neoplasia (Min) mouse. It was discovered in 1989 by exposing B6 (C57BL/6J) mice to ethylnitrosurea. Some of the mice developed anaemia and further investigation showed them to have several tumours in their small intestine, this trait was found to be hereditary (Moser et al. 1990). The mutation caused by ethylnitrosurea was later localised to codon 850 of the mouse Apc gene. The mutation is a point mutation that changes a thymine to an adenine, causing leucine to become a stop-codon (Su et al. 1992). This causes the Apc protein to shorten from 2843 aminoacids to 850 aminoacids (Schoemaker et al. 1997a). The Min mouse is heterozygous for the Apc mutation, i.e. they have one mutated allele and one healthy allele. Homozygous Min mice die at gestation (Moser et al. 1995). The heterozygous mutation of Apc lies in the germline, which means that all cells carry that mutation. For an adenoma to develop, loss of both Apc alleles is required. The loss of the other, healthy allele is a random event that is caused by a sporadic mutation of the gene. It is not until this loss of heterozygosity (LOH) that adenomas start to develop (Andreassen et al. 2002; Mollersen et al. 2004a).

The Min mice develop the major part of the benign adenomas in the distal small intestine with only a few in the colon, a characteristic that is believed to be due to LOH being the mechanism of inactivation of the second Apc allele (Haigis et al. 2004). Altogether approximately 30-50 adenomas develop throughout the intestine. The mice live to approximately 120 days, the cause of death being severe anaemia or intestinal obstruction (Moser et al. 1990). As described earlier, a mutation in Apc causes disturbance in Apc protein function, accumulation of β-catenin in the cytosol, translocation to the nucleus and activation of target genes. In contrast to humans, Min mice develop tumours in the entire intestine, not only the colon. The human and murine APC/Apc genes, however, are 90% identical at the aminoacid levels (Su et al. 1992) and the phenotype and underlying mechanism of tumour formation is similar for human FAP and Min mice (Shoemaker et al. 1997b).
The Min mouse has been used for several years to study intestinal carcinogenesis \textit{in vivo}. A comprehensive list of agents that have been tested for cancer promotion or prevention in the Min mouse can be found on Corpets website at http://www.inra.fr/reseau-nacre/sci-memb/corpet/Data/table.php?file=Min-mice.txt. The Min mouse was chosen as an experimental model in this thesis because of its close genetic background to humans, but also for practical reasons; the animals are commercially available, they do not need chemical exposure to carcinogens, and they are possible to breed and can thus be fed experimental diets from weaning. Also, the number and size of adenomas that develop in the intestine are in a range that is easy to calculate and measure.

Another option to study dietary effects on colon cancer formation would be to use mouse models with chemically induced tumours. In chemically induced colon cancer models, tumors are most commonly produced by azomethane treatment, which causes $\beta$-catenin and $K$-ras mutations (Takahashi & Wakabayashi 2004). These mutations are usually found in the later stages of the adenoma-carcinoma sequence, whereas the $Apc$ mutation is the initiating step in tumour formation. It should emphasized, however that the results presented in this thesis can only be compared to human colon cancer with an $APC$ mutated background, and not all colon cancers show that mutation. Also, the Min mice have a heterozygous mutation that lies in the germline, and all cells of the animal carry that mutation. Adenomas only develop in the intestine and sometimes stomach and breast, but it cannot be ruled out that the mutation has additional effects on the growth and development of the mouse. Therefore, the use of a conditional knockout mouse that would only have the $Apc$ gene mutated in the intestine would be more appropriate to study sporadic colon cancer. This would ensure that the effects in the mouse are specific for intestinal $Apc$-loss and not due to the lack of Apc in other organs. An additional advantage is the longer life span of conditional knockouts that would allow longer feeding periods.
Aims of the study

Colon cancer is one of the leading causes of cancer death in the developed countries, and lifestyle factors such as diet greatly affect cancer development. The underlying mechanism of colon cancer formation is fairly well understood, most often starting with a mutation in the APC tumour suppressor. Several signalling pathways mediate information between the cell surface and nucleus, as well as within the cell, mediating different functions in the cell. Of these pathways, wnt, NF-κB and p53, are pivotal in the development of colon cancer. The mechanism by which diet modulates cancer formation is less understood and is of fundamental importance as new dietary constituents are developed and added to foods, especially with regarded to the formation of colon cancer. These new compounds include inulin, a non-digestible fibre, conjugated linoleic acid and its different isoforms, as well as different phenolic compounds. In this study, the function of β-catenin, which is the mediator of wnt-signalling, NF-κB, and p53 were investigated in the multiple intestinal neoplasia mouse after dietary treatment with inulin, two conjugated linoleic acid isomers, or white currant. This dissertation specially aims at answering the following questions:

How do β-catenin, NF-κB, and p53 signalling behave during tumour development and growth in the Min mouse compared to wild type littermates?

Is diet able to affect the cell signalling pathways, especially wnt, NF-κB and p53 in the Min mouse?

Can the chemopreventive effects of conjugated linoleic acid and inulin be confirmed in the Min mouse?

Can white currant, a berry with low phenolic content, be considered a negative control for studies using other berries or does it affect tumourigenesis in the Min mouse?
Materials and methods

This section gives a brief overview of the materials and methods used in studies I-IV. Detailed descriptions can be found in the original publications (I-IV).

Animals

The Laboratory Animal Ethics Committee of the University of Helsinki approved the study protocols for all studies that included feeding mice (I, II, and IV). For study I, the mice were obtained from the Jackson Laboratory, Bar Harbor, ME, USA. For study II and IV, the mice were bred at the Laboratory Animal Center, University of Helsinki, from mice originally obtained from the Jackson Laboratory by mating wild type C57BL/6J females with Min males. The DNA of the offspring was isolated using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) and the genotype was determined using allele specific PCR (Dietrich et al. 1993). For studies I and IV, only the Min genotype was used, but in study II the wild littermates were also included. After weaning, at approximately 5 weeks of age, the animals were randomly divided into treatment groups, with 10-12 animals/group. The mice were housed in plastic cages in a temperature and humidity controlled room with 12 hour light-dark cycles. During the treatment period, mice had free access to food and water and their weight and physical condition were controlled weekly.

Diets

The basis for the diets used in all studies was the AIN-93G diet (Reeves et al. 1993). This is a semi-synthetic diet designed to meet the nutritional requirements of growing rodents. In study I, the AIN-93G diet was used as a control with soybean oil replaced by sunflower and rapeseed oils, and the experimental diets were prepared by adding 1% (w/w) of either cis-9, trans-11 conjugated linoleic acid or trans-10, cis-12 conjugated linoleic acid (Natural Lipids, Hovdebygda, Norway) to the control diet, and decreasing the amount of other fats (sunflower and rapeseed oils). For studies II and IV, the control diet was a modified version of the AIN-93G diet, were fibre was
excluded, and the amount of fat was increased to a Western-type diet with 40% of energy from fat. The proportion of saturated, monosaturated and polysaturated fat also mimicked a Western-type diet being approximately 3:2:1. Before the animals were included in the study they were fed a commercial rodent chow (Altromin, Ringsted, Denmark). For study II, 10% inulin (Orafti, Tienen, Belgium) was added to the control diet and a similar amount of energy was decreased from all components of the diet. For study IV, 10% freeze-dried white currant (Marja Carelia, Kiihtelysvaara, Finland) was added to the control diet and a similar amount of energy was decreased from all components of the diet. The assumption was that animals on the experimental diet would eat the same amount of energy as the controls, and thus receive equal amounts of nutrients, excluding those supplied by inulin of white currant.

**Tumour scoring and sample collection**

At the end of the feeding period, mice were sacrificed by CO₂ asphyxiation. The abdomen was opened and a blood sample was taken from the abdominal aorta. The entire intestine was removed, and the small intestine divided into five parts of equal length. The colon and caecum were kept together. The intestines were opened longitudinally, rinsed with ice-cold saline and put flat on an objective glass. Tumours were scored under a microscope connected to a camera and TV screen by two observers blinded to the dietary treatment. A piece of tissue, approximately 0.5 cm in length was taken for immunohistochemical analysis and fixed in paraformaldehyde and mounted in paraffin (study IV). From the remaining intestine, adenomas were cut out from the surrounding mucosa, and the reminder of the mucosa scraped off using an objective glass. Samples were snap frozen in liquid nitrogen and stored at -70°C.

**Sample preparation**

From the adenoma and mucosa tissues proteins were extracted for Western-blot analyses. Tissues were homogenised in ice-cold buffer and nuclear, cytosolic, and membrane proteins were separated by centrifugation as described in the original publications. Samples were concentrated using the Ultrafree 4 centrifugation devices
(Millipore, Bedford, MA, USA). The protein content of the samples was measured using the Bradford assay (Protein Assay, Bio Rad, Hercules, CA, USA). A denaturating buffer was added to all samples and they were stored at -70°C.

**Western blotting**

Proteins were separated according to size in sodium dodecyl sulphate (SDS) gels after which they were transferred to either nitrocellulose or polyvinyl membranes using an electric current (Western blotting). Membranes were blocked to minimise unspecific binding of primary antibody. After blocking, membranes were incubated with a specific primary antibody as indicated in the original publications, washed and incubated with a secondary antibody. Proteins were visualised by the enhanced chemiluminescense system (Amersham, Little Chalfont, UK) and transferred to an x-ray film. Films were scanned and bands analysed using the Quantity One software (Bio Rad). Results were calculated by dividing each sample value with the value of an internal standard and the result of each sample was the mean of two separate runs. Equal loading of samples was ensured by incubation with actin or lamin antibodies.

**Immunohistochemical analysis**

In study IV, paraffine was removed from the samples by xylene and rehydrated in a descending ethanol series. Samples were boiled in a citric acid buffer to unmask proteins. Staining of proteins was done using commercial kits (PowerVision Homo-mouse IHC Detection Kit, ImmunoVision Technologies Company, Brisbane, CA or UltraVision Anti-Rabbit Detection System, Lab Vision UK, Suffolk, UK). Two observers blind to the dietary treatment evaluated the IHC staining with a scale ranging between 0 (no staining) and 3 (very strong staining). Staining was performed on triplicates for each sample, and sections with incomplete staining were excluded from the analysis.
Statistical analysis

The non-parametric Mann-Whitney test was used to compare groups. Correlations were tested by Pearson’s correlations. In study II, Kruskall-Wallis was used for testing differences between time points. Statistical analysis was done using the SPSS software (versions 7.0-11.0, SPSS Inc. Chicago, IL, USA) or StatView software (version 5.0.1, SAS Institute Inc., Cary, NC, USA). Differences were considered significant when p<0.05.
Results

Methods used and general observations

Adenoma number and size were measured from the entire intestine using a camera attached to a lightmicroscope, thereby showing the picture on a TV-screen. This allows two people to observe the intestine simultaneously and decisions on whether or not an adenoma is present can be done in consensus. In study I and study II/III, the smallest measured adenomas were 0.5 mm in diameter. In study IV, which was done when the researchers had gained more experience in detecting adenomas, the smallest measured adenomas were 0.3 mm. Also in study IV the measurements were done at two microscopes by two pairs, shortening the time required for measurements and thus probably also decreasing the proteolysis of the samples. In the studies of this thesis, the groups consisted of 9-12 mice, an amount that is enough to show clear effects of the diet. It can, however, not be ruled out that that some results could have been statistically significant if more mice had been used. This is particularly the case when the deviation has been considerable.

The β-catenin, NF-κB, and p53 levels in the cell have been analysed by Western blotting, and thus protein levels are measured. This gives a reliable result on how much of the protein is present in the cell, in most cases even divided into specific cell compartments. Elevated β-catenin levels in the cytosol and nucleus are clear indicators that the degradation of β-catenin is disturbed. To confirm β-catenin results immunohistochemistry was used in study IV. P53 levels in the nucleus rise as p53 is liberated from its inhibitory complex in the cytosol and therefore nuclear p53 is probably a better indicator of activation, although p53 also has direct functions in the cytosol. As for the NF-κB pathway, the nuclear levels indicate that NF-κB has translocated to the nucleus and gives an estimate on how much NF-κB is available for binding DNA. NF-κB was measured as the p65 monomer, as this is the subunit that is transcriptionally active (Schmitz & Bauerle 1991). Nuclear accumulation of p65 does, however, not necessarily mean that all the NF-κB in the nucleus is transcriptionally active, as it can also accumulate in the nucleolus where it is transcriptionally inactive (Stark & Dunlop 2005). Another method for measuring the amount of NF-κB
available for DNA binding would be the electron mobility shift assay (EMSA). There were several attempts, with different kits and methods to set up EMSA measurements for NF-κB from intestinal tissue of Min mice. Unfortunately, the attempts were unsuccessful and therefore only protein levels of NF-κB can be referred to in the studies. To confirm that the NF-κB pathway is active, cytosolic degradation of IκB should be measured from the cytosolic fractions.

In all studies, the mice generally grew well during the feeding periods. In studies II/III and IV some mice were sacrificed in advance due to excess weight loss and/or other signs of illness such as bleeding from the anus. These mice were excluded from the studies. There were no significant differences in body weights between the dietary groups. The number of adenomas that developed in the small intestine and colon of the Min mice was similar to those reported earlier, ranging from approximately 45 to 75 in the small intestine of 15 week-old mice. In study I, only male mice were used, but in studies II/III and IV, both male and female mice were included in the study. No statistical differences in any of the parameters, except for body weight, were observed between males and females and therefore all results from studies II/III and IV include both genders.

β-catenin, NF-κB, and p53 in wild type mice and in Min mice during tumour development and growth (study II/III)

In study II/III, mice were sacrificed at different ages and wild type mice were also included in the study. To investigate the timeline of adenoma development and the behaviour of cell signalling during that time, this section of the results concentrates on the development and growth of adenomas in Min mice that were fed a control diet. β-catenin, NF-κB and p53 protein levels, measured by Western-blotting, are compared in this section between different aged Min mice as well as to wild type mice at the corresponding age.
Adenomas develop between 6 and 9 weeks, and grow between 9 and 15 weeks:

At 6 weeks of age, and at this time fed only a standard laboratory chow, the Min mice had an average of 5.4 adenomas in their small intestine, ranging from 1 to 14. The medium size of the adenomas was 0.64 mm (Figure 7). The 6-week-old Min mice had no adenomas in the colon. Between 6 and 9 weeks the number of adenomas in the small intestine increased dramatically, and the 9-week-old mice had an average of 40 adenomas in their small intestines (p<0.001). Also adenomas in the colon occurred in 5 of 11 mice. The number of adenomas in the small intestine did not increase more than slightly between 9 and 15 weeks, as the 15-week-old Min mice had an average of 54 adenomas in their small intestines, nor did the incidence of colonic adenomas increase. The size of the adenomas in the small intestine, on the other hand, did not differ between the 6- and 9 week old mice, but increased between 9- and 15-weeks to an average of 1.2 mm (p<0.001).

Figure 7. A) Adenoma number and B) adenoma size in Min mice at different ages.
The 6-week-old mice (n=10) were fed a standard laboratory chow, the 9-and (n=11) 15-week (n=9) old mice were fed a control diet for 3 or 9 weeks, respectively. At the end of the feeding periods, adenoma number and sizes were measured under a light microscope as described in the Materials and Methods section. Results are presented as box-plots, where the box represents the interquartile range, containing 50% of the values. The whiskers extend from the box to the maximum and minimum values. The median is indicated by a line across the box. The nonparametric Mann-Whitney U-test was used to test for differences between different ages.
Nuclear levels of β-catenin drop in wild type mice between 6 and 9 weeks but show no difference in control-fed Min mice:

β-catenin levels in the nuclear fraction of the mucosa tissue of wild type mice were measured using Western-blotting. The levels decreased significantly between 6 and 9 weeks (p=0.013) (Figure 8), but did not differ between 9- and 15-weeks. In the Min mice, β-catenin in the nucleus of the mucosa was low at 6 weeks and differed significantly from the wild type at the same age (p=0.004). Nuclear levels of β-catenin in the Min mice showed a slight increase between 6-, 9-, and 15-week –old mice, but the change was not statistically significant. As can be seen in Figure 8, the results show different amounts of deviation and this could affect the overall results. As the statistical analyses were done using non-parametric testing that compares medians instead of means, results are presented showing the median value.

Cytosolic levels of β-catenin show the same pattern as the nuclear fraction:

β-catenin levels in the cytosol of the mucosa, measured by Western-blotting, in wild type mice behaved similar to the nuclear fraction. The levels dropped between 6 and 9 weeks (p=0.021) and stayed at the same level at 15 weeks. In the Min mice, similar to the nuclear fraction, the levels were lower in the Min mice compared to the wild at 6 weeks (p=0.03). Also in the cytosol, the levels did not change statistically significantly between 9 and 15 weeks. It is, however, noteworthy that the levels of cytosolic β-catenin were significantly higher (p=0.018) in the Min mice compared to the wild at 15 weeks. No significant change in levels in either genotype between 9 and 15 weeks, however, occurred.
Figure 8. A) nuclear β-catenin (n=7-11) B) NF-κB (n=8-11) C) p53 (n=7-11) in the mucosa tissue of wild type and Min mice at different ages. Proteins were extracted from the mucosa tissue and fractioned into different cellular compartments. Proteins were separated according to size by SDS gelelectrophoresis, blotted using Western-blotting and visualised using ECL as described in the Materials and methods section. Results are expressed as relative units that have been calculated by dividing the band intensity of each sample with that of an internal standard. The protein sample for each mouse was run as a duplicate and the result for each mouse calculated as the average of these. Results are presented as box-plots, where the box represents the interquartile range, which contains 50% of the values. The whiskers extend from the box to the maximum and minimum values. The median is indicated by a line across the box.

NF-κB levels drop in both genotypes between 6 and 9 weeks, and rise again at 15 weeks only in the Min mice:

**NF-κB levels in the nuclear fraction of the mucosa** was measured by Western blotting. In wild type mice the level was high in 6-week-old mice, and dropped significantly (p=0.003) at 9 weeks. The levels stayed low, and no difference was seen between 9- and 15-week-old wild type mice, but it should be noted that the deviation in the 15-week-old mice is larger than in the 9-week-old. In Min mice, the same drop in NF-κB levels between 6 and 9 weeks was seen (p=0.016), but contrary to the wild type mice, NF-κB levels rose again and were significantly (p=0.032) higher at 15 weeks compared to 9 weeks. NF-κB was measured by Western-blotting from the nuclear fraction. This gives a measurement of the nuclear translocation of NF-κB, but does not give indication of transcriptional activity.
P53 levels drop between 6 and 9 weeks in wild type, while it rises in Min mice between 9 and 15 weeks:

P53 levels in the nuclear fraction of the mucosa tissue was measured by Western-blotting. The levels in wild type mice behaved similar to β-catenin and NF-κB; p53 levels dropped between 6 and 9 weeks (p=0.069), and stayed low at 15 weeks. In Min mice mucosa, p53 levels behaved similar to NF-κB and were significantly lower compared to the wild type at 6 weeks (p=0.012). The levels stayed low at 9 weeks, but rouse at 15 weeks (p=0.026).

In summary, both nuclear and cytosolic β-catenin, as well as NF-κB and p53 behaved similarly in the mucosa of wild type mice. All parameters were high in 6-week-old mice, dropped at 9 weeks and stayed low at 15 weeks. In the Min mice mucosa tissue, β-catenin in both the nuclear and cytosolic fractions were significantly lower at 6 weeks compared to the wild type, but no statistically significant change was seen between Min mice at different ages. NF-κB levels, on the other hand, were high in 6-week-old mice, dropped at 9 weeks but rouse again at 15 weeks. This pattern was similar to wild type mice at 6 and 9 weeks but the rise at 15 weeks was not seen in the wild type. P53 levels in the Min mice were low at 6 and 9 weeks, similarly to β-catenin but rouse at 15 weeks, similarly to NF-κB.

β-catenin and p53 levels rise when adenomas grow:

The protein measurements from the adenomas could only be done from 9- and 15-week-old mice, as so few adenomas could be collected from 6-week-old mice. In the Min mice, both nuclear and cytosolic β-catenin in the adenomas, measured by Western-blotting, increased between 9 and 15 weeks (p=0.002 and p=0.006). NF-κB levels in the adenomas showed no difference between different ages. Similarly to β-catenin, p53 levels in the adenomas of Min mice rose between 9 and 15 weeks (p=0.026).
Effects of diet on adenoma formation in Min mice (studies I-IV)

The results on dietary affects on adenoma formation are summarised in Table 1.

_Inulin increases the size of the adenomas and also the number in the distal small intestine:_
Inulin affected the number and size of adenomas differently according to the age of the mice and concomitantly the length of the feeding period. At 9 weeks of age, and a feeding period of 3 weeks, no differences in either adenoma number or size was found. In the 15-week-old mice, after a feeding period of 9 weeks, an increase in adenoma size occurred in the inulin group. A difference in adenoma number was apparent in the distal part of the small intestine (p=0.043). The difference in adenoma size was apparent in the entire small intestine (p=0.004), but was even more significant in the distal small intestine (p=0.0005).

_Trans-10, cis-12 CLA increases adenoma size:_
Conjugated linoleic acid isomers cis-9, trans-11 and trans-10, cis-12 had different effects on adenoma formation. Cis-9, trans-11 CLA had no effect on either adenoma number or size in Min mice that were 14 weeks old and were fed experimental diets for 8 weeks. Trans-10, cis-12 CLA, on the other hand, significantly increased the size of the adenomas (p=0.008), but did not increase the number of adenomas.

_White currant reduces the number and size of the adenomas:_
White currant reduced the number of adenomas in the small intestine (p=0.016) of 15-week-old Min mice that were fed white currant for 10 weeks. In the distal part of the small intestine, white currant also reduced the size of the adenomas (p=0.019).
<table>
<thead>
<tr>
<th>Study II/III</th>
<th>n</th>
<th>Feeding period (wk)</th>
<th>Adenoma number in small intestine</th>
<th>Adenoma size in small intestine (mm)</th>
<th>Adenoma size in distal small intestine (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 wk Standard chow</td>
<td>10</td>
<td></td>
<td>4 (1-14)</td>
<td>0.65 (0.4-1.0)</td>
<td>0.54 (0.4-1.2)</td>
</tr>
<tr>
<td>9 wk Control</td>
<td>11</td>
<td>3</td>
<td>43 (11-114)</td>
<td>0.71 (0.51-0.83)</td>
<td>0.65 (0.48-0.78)</td>
</tr>
<tr>
<td>9 wk Inulin</td>
<td>9</td>
<td>3</td>
<td>44 (23-128)</td>
<td>0.75 (0.66-1.0)</td>
<td>0.7 (0.63-0.87)</td>
</tr>
<tr>
<td>15 wk Control</td>
<td>9</td>
<td>9</td>
<td>54 (35-84)</td>
<td>1.13 (1.03-1.44)</td>
<td>1.03 (0.83-1.3)</td>
</tr>
<tr>
<td>15 wk Inulin</td>
<td>9</td>
<td>9</td>
<td>69 (47-90)</td>
<td>1.47 (1.34-1.6) (p=0.004)</td>
<td>1.42 (1.29-1.61) (p&lt;0.001)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study I</th>
<th>n</th>
<th>Feeding period (wk)</th>
<th>Adenoma number in small intestine</th>
<th>Adenoma size in small intestine (mm)</th>
<th>Adenoma size in distal small intestine (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>8</td>
<td>33 (15-99)</td>
<td>1.15 (0.81-1.49)</td>
<td>0.93 (0.53-1.33)</td>
</tr>
<tr>
<td>(c-9, t-11) CLA</td>
<td>10</td>
<td>8</td>
<td>46 (13-94)</td>
<td>1.26 (1.11-1.38)</td>
<td>1.01 (0.71-1.31)</td>
</tr>
<tr>
<td>(t-10, c-12) CLA</td>
<td>10</td>
<td>8</td>
<td>48 (14-88)</td>
<td>1.32 (1.10-1.67)</td>
<td>1.21 (0.95-1.46) (p=0.008)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study IV</th>
<th>n</th>
<th>Feeding period (wk)</th>
<th>Adenoma number in small intestine</th>
<th>Adenoma size in small intestine (mm)</th>
<th>Adenoma size in distal small intestine (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>10</td>
<td>81 (47-114)</td>
<td>1.0 (0.71-1.22)</td>
<td>0.88 (0.57-1.20)</td>
</tr>
<tr>
<td>White currant</td>
<td>12</td>
<td>10</td>
<td>51 (36-84) (p=0.016)</td>
<td>0.95 (0.78-1.19)</td>
<td>0.67 (0.58-0.90) (p=0.019)</td>
</tr>
</tbody>
</table>

\(1\) Studies I and IV include the two most distal parts of the small intestine, when the small intestine was divided into five sections of equal length. Study II/III include the three most distal parts of the small intestine when the small intestine was divided into five sections of equal length. Study II/III include the three most distal parts of the small intestine when the small intestine was divided into five sections of equal length.

\(2\) Compared to the control diet

\(3\) Compared to the control diet at 15 weeks
Effects of diet on β-catenin, NF-κB, and p53 levels in Min mice (studies I-IV)

The effects of inulin on β-catenin, NF-κB, and p53 are presented in Figure 9. The results presented here on how inulin affects β-catenin have slightly different p-values that those presented in study II. When separating proteins using SDS gelelectrophoresis, β-catenin produces several different bands that represent full-length β-catenin, but also different sized degradation products (for detail see Figure 3 in study II). In the original publication of study II, β-catenin results have been obtained by statistical comparisons of only the uppermost bands. In study III, and in this thesis, the results have been obtained by statistically comparing the sum of the three largest β-catenin bands. The use of different β-catenin bands to generate the results only slightly changed the p-values and had no effect on the overall result.

**Inulin** did not affect β-catenin levels in the nuclear fraction of the mucosa of Min mice, at either 9 or 15 weeks. In the cytosol, however, β-catenin levels were lower in the inulin group compared to the control at 15 weeks (p=0.028). In the adenoma tissue, no effect was seen in the nucleus, but opposite to what was seen in the mucosa, inulin significantly increase the levels of cytosolic β-catenin at 15 weeks (p=0.034).

**Inulin** had no effect on NF-κB in the mucosa of Min mice at 9 weeks, and decreased the level at 15 weeks with borderline significance (p=0.064). In the adenoma, no difference was seen between the control and inulin groups.

In the mucosa of Min mice, **inulin** had no affect on p53 at either timepoint. In the adenoma, inulin decreased p53 levels with borderline significance (p=0.068).
Figure 9. Inulin-effects on cell signalling proteins in the mucosa and adenoma of 9- and 15-week-old Min mice. Proteins were extracted from the mucosa tissue and fractioned into different cellular compartments. Proteins were separated according to size by SDS gelelectrophoresis, blotted using Western-blotting, and visualised using ECL as described in the Materials and methods section. Results are expressed as relative units that have been calculated by dividing the band intensity of each sample with that of an internal standard. The protein sample for each mouse was run as a duplicate and the result for each mouse calculated as the average of these. Results are presented as box-plots, where the box represents the interquartile range, which contains 50% of the values. The whiskers extend from the box to the maximum and minimum values. The median is indicated by a line across the box.
To summarise the effects of inulin on β-catenin, NF-κB, and p53: At 9 weeks, inulin had no effect on any of the parameters in either mucosa or adenoma. At 15 weeks, in the mucosa, inulin caused a decrease in cytosolic β-catenin, and NF-κB without affecting p53. In the adenoma tissue at 15 weeks, inulin increased the level of β-catenin in the cytosol, had no affect on NF-κB, and decreased the levels of p53.

**Conjugated linoleic acid** had no effect, when fed to Min mice for 8 weeks, on β-catenin levels in the adenomas, and it was not measured from the mucosa. In the mucosa, NF-κB levels were significantly lower in the trans-10, cis-12 group compared to the control. In fact, nuclear NF-κB was below the detection limit in all mucosa samples from the trans-10, cis-12 fed mice. In the control and cis-9, trans-11 groups, nuclear NF-κB was found in approximately half of the animals, but in these groups also some mice had levels below detection. No differences in mucosal p53 levels existed between the groups.

**White currant,** when fed to Min mice for 10 weeks, had no effect on β-catenin or NF-κB in the mucosa. P53 levels, on the other hand, were slightly increased in the mucosa of the white currant group (p=0.087). In the adenoma tissue, white currant affected both the β-catenin and NF-κB levels. Nuclear β-catenin was significantly lower in the white currant group (p=0.035) compared to the control. The same as seen in NF-κB levels: white currant significantly reduced nuclear NF-κB levels (p=0.014). P53 levels in the adenoma tissue were not affected by white currant feeding.

The results on the action of different dietary compounds on cell signalling in the mucosa and adenoma tissues are summarised in Tables 2a and 2b.
### Table 2a. Summary of the results on cell signalling in the mucosa tissue of Min mice

(\(\downarrow\) indicates decrease, \(\uparrow\) indicates increase, and \(\leftrightarrow\) indicates no change)

<table>
<thead>
<tr>
<th></th>
<th>Adenoma no</th>
<th>Adenoma size</th>
<th>(\beta)-catenin(^1)</th>
<th>NF-(\kappa)B</th>
<th>P53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>(\leftrightarrow)</td>
<td>(\uparrow)</td>
<td>(\leftrightarrow)(n) (\downarrow)(c)</td>
<td>(\downarrow)</td>
<td>(\leftrightarrow)</td>
</tr>
<tr>
<td>(T10, , c12) CLA</td>
<td>(\leftrightarrow)</td>
<td>(\uparrow)</td>
<td>n.a.(^2)</td>
<td>(\downarrow)</td>
<td>(\leftrightarrow)</td>
</tr>
<tr>
<td>White currant</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\leftrightarrow)</td>
<td>(\leftrightarrow)</td>
<td>(\uparrow)</td>
</tr>
</tbody>
</table>

\(^1\)(n) indicates result from nucleus, (c) cytosol

\(^2\)\(\beta\)-catenin was not measured from the mucosa in study I

### Table 2b. Summary of the results on cell signalling in the adenoma tissue of Min mice

(\(\downarrow\) indicates decrease, \(\uparrow\) indicates increase, and \(\leftrightarrow\) indicates no change)

<table>
<thead>
<tr>
<th></th>
<th>Adenoma no</th>
<th>Adenoma size</th>
<th>(\beta)-catenin(^1)</th>
<th>NF-(\kappa)B</th>
<th>P53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>(\leftrightarrow)</td>
<td>(\uparrow)</td>
<td>(\leftrightarrow)(n) (\uparrow)(c)</td>
<td>(\leftrightarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>(T10, , c12) CLA</td>
<td>(\leftrightarrow)</td>
<td>(\uparrow)</td>
<td>(\leftrightarrow) (t)</td>
<td>n.a.(^2)</td>
<td>n.a.(^2)</td>
</tr>
<tr>
<td>White currant</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)(n)</td>
<td>(\downarrow)</td>
<td>(\leftrightarrow)</td>
</tr>
</tbody>
</table>

\(^1\)(n) indicates result from nucleus, (c) cytosol, and (t) total fraction

\(^2\)NF-\(\kappa\)B and p53 were not measured from the adenomas in study I
Discussion

Timing of adenoma formation and cell signalling in wild type vs. Min mice

To better be able to interpret the effects of diet on cell signalling, we should first understand how the signalling pathways behave during physiological tumour formation in the Min mouse. This was done by comparing cell signalling in wild type and Min mice at different stages of tumour formation and growth. In the Min mouse mucosa, one of the Apc alleles is mutated, whereas the wild type mice have both alleles intact. The loss of one Apc allele could affect the function of the cell and disturb its normal cell signalling.

Adenomas in the Min mouse seemed to develop between the ages of 6 and 9 weeks, while no difference in adenoma number was apparent at a later age. This observation might be misleading as only adenomas 0.5 mm in diameter or more were measured. It is likely that small microadenomas were already present at 6 weeks. Indeed, Schoemaker et al. (1995) showed that the initiation of tumour formation in Min mice takes place during the first few weeks of life. The growth of the adenomas took place between 9 and 15 weeks, when adenomas had already developed. It seems that initiation of tumour formation and adenoma growth mostly occur independently of each other and this might be due to different cell signals being involved in the initiation and promotion stages.

Nuclear β-catenin levels in the mucosa dropped between 6- and 9-week-old wild type mice, but was low in the Min mice at the same time. β-catenin is physiologically needed in the cell during tissue development, and this could explain the high levels in young wild type mice (Clovers 2006). In Immorto-Min colonic epithelium (IMCE) it was shown in vitro that β-catenin levels and localisation was similar to that in the young adult mouse colon (YAMC) cells (Husoy et al. 2003). Therefore, it seems that mutation of one of the Apc alleles is insufficient to affect cellular β-catenin levels. Immunohistological staining of Min mouse mucosa has also shown that β-catenin is present in the membranes and in the nucleus in only a few cells in the crypts (Kongkanuntn et al. 1999). It is therefore possible that the β-catenin in Min mice is
located on the membrane and levels in the nucleus are low. It should, however, be remembered that only protein levels of β-catenin were measured and not the transcriptional activity. Also, the Results section only describes the results from 9- and 15-week old mice, it is plausible is that β-catenin levels peaked between the timepoints in study II/III. We also included 12-week-old Min mice were included in the study (data not shown), however, and no difference appeared compared to 9- and 15-week-old Min mice. The high level of cytosolic β-catenin in young wild type mouse strengthens the hypothesis that β-catenin at this stage is required for growth of the wild type animal. Contrary to the nucleic levels, the levels of cytosolic β-catenin were higher in the Min mice than the wild at 15 weeks, possibly showing an effect of the Apc mutation and an influence of adenoma growth.

Mucosal NF-κB levels in both wild type and Min mice dropped from the age of 6 weeks to 9 weeks. Between these ages, the number of adenomas increased from 5 to 40 in the small intestine of the Min mice. This seems to indicate that the NF-κB pathway plays a normal physiological role in the epithelial cell during this time, and is not responsible for formation of new adenomas. It also seems that a mutation of one Apc allele is not sufficient to affect NF-κB levels. Between 9 and 15 weeks, NF-κB levels rose in the Min mice and at the same time, an increase in adenoma size occurred. Thus, NF-κB seems to be involved in the growth of the adenomas, as has also been suggested by others (Greten et al. 2004). It should be remembered that these results are from the mucosa and not the adenoma tissue. In the adenoma tissue no difference in NF-κB levels between 9- and 15-weeks emerged. A plausible explanation for this is that activated NF-κB in the mucosa activates the transcription of pro-inflammatory cytokines that then acts on the tumour tissue. Indeed, it has been shown that NF-κB is essential in the inflammatory response during inflammation-associated cancer (Li et al. 2005). Another option is that the growth of the adenomas is caused by other factors and that the rise in mucosal NF-κB is a response to protect the cell from signals that could cause it to start the transformation towards malignancy.

P53 levels in the mucosa followed the same pattern as NF-κB. In both wild type and Min mice the levels dropped between 6 and 9 weeks, but stayed low at 15 weeks in
wild type mice while rising in Min mice. As most adenomas develop between 6 and 9 weeks it appears that no protection in the mucosa is given by p53 against the hyperproliferation and arise of new adenomas. The rise in p53 in 15-week-old Min mice could reflect the changes in cell signalling during tumour growth. A rise in cytosolic β-catenin at the same time could also have increased the levels of transcriptionally active p53 by inhibiting its degradation in the cytosol (Damalas et al. 1999, Damalas et al. 2001).

Taken together, the results indicate that β-catenin, NF-κB, or p53 signalling is not affected by a mutation in one allele of the Apc gene, at least in the early stages of cancer formation. As cytosolic β-catenin, NF-κB, and p53 levels all were high in Min mice at 15 weeks this is probably an effect of the changed cell signalling due to the increased area of adenomas and malignancy in the intestine. Recent data, however, emphasises that truncated APC is still able to decrease the transcriptional activity of β-catenin, without affecting the degradation of the protein (Schneikert et al. 2007). Therefore, measuring mere protein levels probably does not give an accurate picture of transcription.

Diet affects adenoma formation and growth

In study II/III, inulin increased the number of adenomas in the distal small intestine, but had no affect when the entire small intestine was compared to control fed mice. Most of the adenomas in the Min mouse, however, develop in the distal part (Haigis et al. 2004) and therefore an increase in this area should be considered significant. Inulin also increased the size of the adenomas in the small intestine and this phenomenon was even more pronounced in the distal part. Thus, inulin cannot be considered chemopreventive in the Min mouse. Previously, Mutanen et al. (2000) showed that inulin increased the number of tumours developed in the intestine of Min mice compared to ryebran. No difference, however, existed between inulin and the AIN-93G control diet. Others have shown oligofructose to reduce the number of colon tumours without affecting small intestinal adenomas in Min mice (Pierre et al. 1997) Also feeding oligofructose enriched inulin to Min mice seems to reduce tumour
incidence (reference Lipkin in Pool-Zobel 2005). The vast majority of animal studies showing chemopreventive effects of inulin have been made using chemically induced rat models (reviewed in Pool-Zobel 2005). It is possible that the difference in model systems, the timing of inulin administration, the type of fructan used, etc. cause the difference in effects, but an increase in adenoma size in the Min mouse should be remembered when health promoting effects of inulin are postulated.

In Study I of this thesis, the cis-9, trans-11 isomer of CLA had no effect on either adenoma number or size. The trans-10, cis-12 isomer, on the other hand increased the size of the adenomas, but had no effect on the number of adenomas. This is the first study that has investigated the effects of the two different CLA isomers separately in colon cancer. Previously a mixture of CLA isomers with equal amounts of cis-9, trans-11 and trans-10, cis-12 CLA has been shown to increase the size of the adenomas in Min mice, but not to affect on the number of adenomas (Hansen-Petrik et al. 2000; Rajakangas et al. 2002). Most likely the growth promoting effect of the mixture has been due to the trans-10, cis-12 isomer. As it is the trans-10, cis-12 isomer that has been shown to affect body composition, this is probably the isomer that the food industry and consumers are more interested in. The CLA that today is sold in health stores is a mixture of isomers, but as harmful effects on intestinal health can be also seen with a mixture, caution should be taken before introducing pure isomers to the consumers.

Previously, in 1,2-dimethylhydrazine (DMH) treated rats, a mixture of CLA isomers decreased the incidence of colon tumours (Park et al. 2001). In colon cancer cells, most studies that have investigated the effect of CLA isomers, have gained favourable results. In one study, the cis-9, trans-11 isomer reduced the metastasis of cancer cells, but trans-10, cis-12 had no effect (Soel et al. 2007). Another study showed CLA isomers to inhibit cell proliferation in three different colon cancer cell lines (Beppu et al. 2006). The results from study I, however, cannot confirm the anti-carcinogenic properties reported for CLA-isomers. As the results showing no change or increase in tumour size have been obtained using the Min mouse and the favourable results on CLA and colon cancer have been obtained using carcinogen-induced rat models or cell lines, the model system could be of essence. Therefore, more research that
focuses on the properties of the different isomers in various models are urgently warranted.

Previously, we have shown blueberry, lingonberry, and cloudberry to reduce tumour formation in Min mice (Miskangas et al. 2007). As the phenolic compounds are thought to produce the chemopreventive effects by berries, white currant could be seen as a negative control for many high-phenolic berries. Opposite to what was expected, white currant decreased the number of tumours in the small intestine and also reduced the size in the distal small intestine. This is the first study to show white currant to be anticarcinogenic in vivo. Interestingly, white currant juice has been found to inhibit the proliferation of five cancer cell lines, including an intestinal cell line and this was not correlated to the antioxidant properties of the berries, in fact, white currant had the lowest antioxidant capacity of the berries tested (Boivin et al. 2007). The results from Study IV show that chemoprevention can be also achieved with a berry with low phenolic compound contents. Berries naturally contain high levels of vitamins, minerals, and fibre, and may contain some yet unidentified bioactive compounds. Interestingly, it was shown that cloudberry divided into pulp or seeds did not inhibit tumourigenesis in the Min mouse. Neither did ellagic acid, its main phenolic compound, although whole cloudberries have been shown to be highly chemopreventive (Päivärinta et al. 2006, Misikangas et al. 2007). It is plausible that berries as a whole have beneficial effects and that the chemoprevention cannot be attributed to a single compound. This theory is strengthened by the fact that white currant juice, that was able to inhibit proliferation, was extracted from the whole berry and was shown to be more potent than red or black currant juices (Boivin et al. 2007).

The conclusion that can be drawn from study IV is that white currant cannot be considered as a negative control for other berries as it clearly affected adenoma formation and growth. Future studies need to clarify what properties in berries cause the anticarcinogenic effects and whether they can be attributed to single compounds or if several compounds act in synergy.

The results on adenoma formation and growth in studies I and II/III seem to contradict the general view on the effects of inulin and CLA on colon cancer formation. The result on inulin has, however been reproduced by our laboratory (Misikangas et al. 2005) and in that study inulin also affected the mucosa of wild type mice by decreasing membrane β-catenin levels. The tumour size promoting effect of trans-10,
cis-12 CLA has not been confirmed, but a mixture of cis-9, trans-11 and trans-10, cis-12 isomers has been shown to increase the size of the adenomas (Rajakangas et al. 2002). When the results of studies I and II/III are put in this context they seem reliable and correct. The results from study IV have not been reproduced, and are the first to show chemopreventive effects of white currant in vivo.

Changes in cell signalling due to diet

An interesting observation is that both diets that increased the size of the adenomas, inulin and trans-10, cis-12 CLA, reduced nuclear NF-κB levels in the mucosa. In the control fed Min mice on the other hand, mucosal NF-κB increased between 9 and 15 weeks and seemed to be associated with the growth of the adenomas. This could show a direct effect of diet on cell signalling as dietary induced growth of the adenomas clearly causes a different response in NF-κB than the physiological growth of the adenomas. The reduction in nuclear NF-κB due to diet could be an attempt to activate apoptosis in the mucosa, by repressing NF-κB antiapoptotic responses. Another option is that in the mucosa, the NF-κB pathway would activate apoptosis, and as NF-κB is absent from the nucleus, apoptosis is inhibited and cells are free to proliferate. No difference in p53 was seen in the mucosa in either inulin or CLA treated mucosa, however, but a positive correlation existed between NF-κB and p53 in the mucosa in study II/III (see original publication). Some studies support the theory of NF-κB activity evoking a positive response in the cell. In colon cancer cells, the NSAIDs aspirin, sulindac, and sulindac sulphone, all of which are inhibitors of cancer growth, actually increase the nuclear translocation of NF-κB (Stark et al. 2001; Loveridge et al. 2003). Also transfection of colon cancer cells with oncogenes decreases NF-κB activity (Cadoret et al. 1997) and SCID mice developed tumours when injected with embryonic mouse fibroblasts that lack p65 (Gazputan et al. 2002). The fact that NF-κB also is required for p53 transcription (Benoit et al. 2000) and that NF-κB is an essential component of p53 dependent apoptosis (Ryan et al. 2000) shows that nuclear presence NF-κB can be considered positive and that inhibition of NF-κB nuclear translocation, as seen by inulin and CLA, is not beneficial from cancer point of view. Study II/III and I are the first to investigate the effects of inulin and CLA on NF-κB in
colon cancer background. Previously, inulin has been shown to induce the activation of NF-κB in RAW 264.7 macrophages. NF-κB induced the expression of inducible NO synthase (iNOS) that produces nitric oxide (NO), a compound that fights bacteria and viruses (Koo et al. 2003). Both CLA isomers have been found to inhibit bile acid activated NF-κB in colon cancer cells (Shah et al. 2006), similar to what was seen in study I. In a prostate cancer cell line, cis-9, trans-11, but not trans-10, cis-12 CLA was found to induce apoptosis and to reduce NF-κB transcriptional activity and phosphorylation of IκBα (Song et al. 2006). Also in skin cancer, the cis-9, trans-11 isomer has been found to inhibit NF-κB nuclear translocation by reducing IKK activity and thus IκB phosphorylation and degradation (Hwang et al. 2007), but that study did not include trans-10, cis-12 CLA. In study I, cis-9, trans-11 CLA had no affect on NF-κB, but trans-10, cis-12 CLA showed a reduction in nuclear NF-κB. The studies of this thesis show that a diet that increases the size of the adenomas in Min mice reduces nuclear NF-κB in the mucosa. The cause and consequence of this observation demands further studies, but show that diet can affect cell signalling and that the NF-κB pathway is a potent modulator of colon cancer development in response to diet. This effect, however, seems to be limited to the increased growth of the adenomas as white currant did not affect NF-κB levels in the mucosa.

The two other pathways investigated in this thesis, wnt and p53, were not affected by diet to the same extent as the NF-κB pathway in the mucosa. Inulin decreased β-catenin levels in the cytosol, but no such effect was seen in the nucleus. No data on β-catenin levels from the mucosa of CLA fed mice is available. A decrease in cytosolic β-catenin could be due to increased turnover of the protein, but at this time conclusions on the significance of this result are difficult to draw. One study has suggested that β-catenin levels are lower in more aggressive malignancies and is only needed in early tumour development (Takayama et al. 1996), a hypothesis that is supported by the results from study II/III. As a white currant diet that reduced the number and size of adenomas did not affect β-catenin levels in the mucosa, it seems that reduced wnt signalling in the mucosa is not responsible for the chemoprevention.
P53 levels in the mucosa were only affected by white currant, where mucosal levels were increased. If only looking at p53 results, it seems that white currant has been able to activate p53 dependent apoptosis and thus prevented the formation of new adenomas in the intestine. When looking at the complex regulation of apoptosis and the cross talk between signalling pathways this conclusion might be too simple. For example, increased p53 is able to downregulate β-catenin by increasing its degradation by the ubiquitin pathway (Sadot et al. 2001). This could have happened in the white currant group and not in the inulin and CLA groups. Also the regulation of apoptosis by NF-κB might affect p53 levels as discussed above. CLA has previously been shown to induce p53 dependent cell cycle arrest (Kemp et al. 2003). On the other hand, cell cycle arrest was shown to occur in p53 mutant colon cancer cells, showing that functional p53 is not required for CLA induced cell cycle arrest (Lim et al. 2005). Also the trans-10, cis-12 isomer has been shown to inhibit cell cycle progression by induction of p21 (Cho et al. 2006). No such result was obvious in the mucosa, however, and p53 was not measured from the adenoma tissue of CLA treated mice.

It has been demonstrated that cellular β-catenin is associated with the development of intestinal tumours (Romagnolo et al. 1999). In aberrant crypts nuclear and cytosolic β-catenin levels are increased (Sena et al. 2006), and nuclear accumulation of β-catenin strongly correlates with tumour size and dysplasia (Brablez et al. 2000). It has also been shown that increased expression of β-catenin and nuclear translocation causes hyperproliferation of epithelial cells in the crypt (Sellin et al. 2001). In the adenoma tissue, inulin that increased the size of the adenomas also increased cytosolic β-catenin. In Min mice, inulin has, in other studies also, been shown to increase β-catenin levels (Mutanen et al. 2000, Misikangas et al. 2005). Trans-10, cis-12 CLA that also increased the size of adenomas, did not change total cellular pools of β-catenin in the adenoma. Previously, the cis-9, trans-11 isomer of CLA had been found to reduce β-catenin expression in colon cancer cells (Lampen et al. 2005), but in study I this isomer had no affect. Another study showed a mixture of cis-9, trans-11 and trans-10, cis-12 isomers not to affect β-catenin protein levels in caco-2 cells, but to shift the protein from the cytosol to the membrane (Bozzo et al. 2007). In study I total cellular β-catenin was measured and thus it is possible that such a shift has happened.
During the physiological growth of the adenomas, both nuclear and cytosolic β-catenin increased in the adenomas. The effect of inulin on β-catenin could therefore be seen as an effect of adenoma growth and may not be attributable to the diet.

NF-κB levels were unaffected by inulin, and this result is comparable to that seen between 9- and 15-week-old Min mice. It seems therefore that NF-κB levels in the adenoma are not involved in either the physiological or diet induced growth of the adenomas, contrary to what was seen in the mucosa. NF-κB levels were not measured from the adenoma tissue of CLA fed mice.

Contrary to inulin and CLA, white currant decreased both β-catenin and NF-κB levels, proving the strong chemopreventive effect of white currant. In various cell cultures, it has been reported that dokosahexaonic acid together with a synthetic organoselenium, NO-donating aspirin, and β-lapachone, affects both pathways simultaneously (Naranayan et al. 2004, Williams et al. 2003, Choi et al. 2003). Curcumin, the active ingredient of turmeric, can also target both the β-catenin and NF-κB pathways (Thangapazhan et al. 2006). In the adenoma tissue, with a chemopreventive diet, NF-κB seems to also respond also by decreasing nuclear translocation as seen in the mucosa with inulin and CLA. In fact, it has been shown that it is the nature of the stimulus that determines if the response to NF-κB is pro or antiapoptotic (Dutta et al. 2006). In the adenoma it could therefore be that NF-κB activates antiapoptotic responses and inhibition of NF-κB by white currant induces apoptosis. No difference in p53 levels was seen, however. It has also been suggested that in the early stages of cancer NF-κB could act as a tumour suppressor, changing to a tumour promoter as mutations in the cell increase (Perkins 2004). As the lesions in Min mice are adenomas, and carcinomas are rarely found, this hypothesis could be valid in white currant treated mice.

The strong effect of white currant on both β-catenin and NF-κB signalling in adenoma tissue could indicate a possible interaction between these two pathways. It has been shown in colon cancer cells that β-catenin can physically bind to NF-κB, and that this inhibits NF-κB target gene expression (Deng et al. 2002). It has also been reported
that the NF-κB subunit can *in vitro* inhibit β-catenin dependent transcription (Masui *et al.* 2002). None of these interactions have taken place in this study as both pathways were attenuated. Also, an activating or inhibiting effect on the shared ubiquitin-proteasome pathway can be ruled out as the effects are opposite in the β-catenin and NF-κB pathway. It could be possible, however, that white currant activates the degradation of β-catenin to such an extent that proteins of the ubiquitin-proteasome pathway cannot be spared for the NF-κB pathway so it is not activated. To summarise, the known interactions between the wnt and NF-κB pathways do not seem to explain the results of this study. It is thus more likely that white currant affects these pathways independently. Nevertheless, simultaneous inhibition of NF-κB and β-catenin in the adenoma tissue produced by dietary means shows the greatest potential of natural compounds to affect cell signalling.

P53 levels were slightly decreased by inulin in the adenoma, but were unfortunately not measured from the *trans*-10, *cis*-12 CLA fed mice. The opposite effect on p53 was seen as the adenomas grew between 9 and 15 weeks and a decrease in p53 could explain the growth of the adenomas by inulin. White currant decreased both β-catenin and NF-κB levels in the adenoma, but had no effect on p53. These results indicate that β-catenin is a modulator of adenoma growth, both by inducing and reducing the growth of adenomas, but it might be that diet is not responsible for the effect. The role of NF-κB in adenoma growth seems to be restricted to the inhibition of NF-κB and a reduction in growth. P53, on the other hand, might be involved in diet induced growth of adenomas.

Recently, attention has been drawn to the tumour microenvironment as an essential factor in the development of cancer, and the tumour microenvironment is an important target for chemoprevention (Aritztia *et al.* 2006; Albini & Sporn 2007). The tumour microenvironment is composed of a mixture of different cell types, including endothelial cells, fibroblasts, lymphocytes and macrophages that effect, and are themselves affected by, the tumour. The cells in the microenvironment are activated by the inflammation caused by the lack of homeostasis in the tumour tissue, and the process of tumour formation has been compared to acute inflammation (Huang & Ingber 2007). This leads to a cycle where immunological cells activate proliferation
that in turn accelerates the immunological response. Inflammation has been considered one of the key activators of cancer formation and NF-κB is also involved in the immune responses of cells (Karin & Greten 2005). A mechanism whereby diet can modulate cancer formation is by changing the dynamics of this microenvironment. Diet can, for example, cause changes in the bacterial community of the intestine, it can fight oxidative stress by its antioxidant properties or contain antimicrobial compounds.

In studies I-IV, the different dietary compounds could have changed the microenvironment differently. In fact, trans-10, cis-12 CLA increased the oxidative stress of the mice. Oxidative stress has been shown to activate the NF-κB pathway; on the other hand antioxidants have been shown to decrease the activity (Gloire et al. 2006). From study I, it seems clear that the oxidative stress did not activate NF-κB. It is, however, plausible that the antioxidant properties of white currant decreased the NF-κB levels. White currant probably contains various different compounds that act on the microenvironment and this could have been the reason for the chemoprevention. Inulin changed the microbial content of the intestine in study II/III (Apajalahti et al. 2002), and this is also one of the mechanisms by which it has been proposed to inhibit cancer formation (Reddy 1999). Bacteria mainly mainly in the colon and caecum of mice, and the adenoma results are evident in the small intestine. It is therefore more likely that inulin has additional ways of affecting the epithelial cells and may influence the microenvironment by other mechanisms than just through the bacterial community. Recently a role of the microenvironment in wnt activation was proposed, in which autocrine and paracrine effectors could activate wnt signalling (Fodde & Brablez 2007). This may explain the results of the different dietary factors on β-catenin behaviour and again suggests that diet could act on the microenvironment. Taken together, the three different diets investigated in this thesis could all have caused their effects by changing the microenvironment in the intestine and by specifically target the tumour microenvironment. In in vivo studies it might be difficult to show precise mechanisms of action, but instead they have the advantage of being able to affect diverse environments, such as the microenvironment
Summary and conclusions

New dietary components that aim at increasing health and preventing disease such as cancer are rapidly increasing in popularity. To convincingly show that these compounds really are chemopreventive, their mechanism of action in cancer should first be understood. We studied the actions of three dietary constituents in the Min mouse. Both inulin and the trans-10, cis-12 isomer of conjugated linoleic acid increased the size of adenomas in the Min mouse. The cis-9, trans-11 isomer of CLA on the other hand had no effect on tumour formation. This underlines the importance of carefully studying the actions of new dietary compounds in various model systems before health claims are made.

At the moment, there is a huge interest in finding new compounds and molecules that could be utilised in functional foods. The ‘health promoting’ compounds are believed to make foods more attractive to the consumers. It seems, that eating a balanced, healthy basic diet that potentially includes all these compounds in their natural form, is considered inadequate and even boring. As shown in study IV, white currant, a berry with low levels of phenolic compounds was still able to reduce both the number and size of adenomas in the Min mouse. The conclusions that can be drawn from this are that fruit, vegetables and berries, despite low content of known chemopreventive compounds, are still able to prevent adenoma formation. It is possible, that white currant is a good source of an agent not yet identified, or more likely the health benefit is a sum of the compounds that the berry itself consists of.

Cell signalling plays a pivotal role in the formation of colon cancer. We studied the wnt, NF-κB, and p53 pathways to get a better understanding on how diet can affect cancer formation. First, their behaviour was elucidated during normal physiological conditions in wild type mice, as well as during the physiological development and growth of adenomas in Min mice. This showed that cell signalling is active during the rapid growth of the wild type mice. In the Min mouse mucosa, levels of β-catenin, NF-κB, and p53 were low during tumour formation. Therefore, it seems that the mutation of one Apc allele is insufficient to increase cellular levels of the investigated proteins.
The inulin diet increased both the number and size of the adenomas and the trans-10, cis-12 CLA increased the adenoma size. NF-κB levels decreased in the mucosa with both diets, and as physiological adenoma development did not affect NF-κB, this might be a result of diet. Diet altered β-catenin and p53 signalling in the adenomas, confirming their involvement in adenoma growth. White currant was chemopreventive in the Min mouse. The chemopreventive effect was accompanied by increased p53 levels in the mucosa, and decreased β-catenin and NF-κB levels in the adenoma. These observations could explain the reduced number and size of adenomas and again shows that β-catenin and NF-κB are not involved in the formation of adenomas.

We did not find a clear pattern for the behaviour of β-catenin, NF-κB, and p53. The complexity of cell signalling and the response in the cell to different stimuli is in constant flux and the results seen in the different proteins are mere averages of one particular moment in the life of the cells. As the result is also obtained from tissues, not single cells, the complexity of cell signalling seems overwhelming when considering the interactions between pathways. The results from in vivo experiments do, however, give an opportunity to see how a specific component behaves in physiological settings, where digestive fluids, bacteria, immunological parameters, etc. influence the action of that compound.

In conclusion, this thesis cannot confirm the chemopreventive effects of inulin and CLA. Despite white currant only containing low amounts of phenolic compounds, it was still able to act as an anticarcinogen. This underlines the importance of carefully testing new dietary compounds in different settings to reliably confirm their health benefits. To our knowledge, this is the first study to investigate the behaviour of β-catenin, NF-κB, and p53 in such detail in the Min mouse. Further studies are warranted to get a more detailed picture on how these pathways are involved in adenoma formation and growth and how diet may modulate them.
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