

**Mechanisms of intestinal tumor initiation
and progression in *Apc^{MIN}* mouse model of
colorectal cancer**

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Apc^{MIN} mouse model of colorectal cancer**

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I am a slow walker, but I never walk backwards.

Life is good

ABSTRACT

Colorectal cancer (CRC) is a cancer that occurs in the colon or rectum and is one of the most common cancer forms and a leading cause of cancer death in the Western world. The majority of CRCs are caused by inherited or somatic mutations in the *APC* gene. The *Apc^{Min}* model is driven by a truncating mutation in the *Apc* gene and is considered to be one of the best mouse models of CRC. The aim of this thesis is to uncover novel mechanisms behind the initiation and progression of intestinal adenomas in *Apc^{Min/+}* mice.

Clinical studies suggest that the transcription factor Zfp148 may play a role in CRC but the importance of Zfp148 for tumor development has not been properly investigated. We have previously shown that Zfp148 is a potent inhibitor of p53 and hypothesized that Zfp148 deficiency may protect against CRC by increasing p53-activity. In paper I, we show that deletion of one or both copies of Zfp148 markedly reduces tumor formation in *Apc^{Min/+}* mice. The result shows that Zfp148 controls the fate of newly transformed tumor cells by repressing p53, and suggests that targeting Zfp148 might be useful in the treatment of colorectal cancer.

Previous studies show that Zfp148 inhibits activation of p53; however, the underlying mechanism is not understood. We have previously shown that transcription of *Cdkn2a* was increased in *Zfp148* deficient MEFs. ARF is one of two products of *Cdkn2a* and is a major activator of the p53-pathway. Therefore, we hypothesized that Zfp148 inhibits p53 by repressing ARF. In paper II, we tested this hypothesis in mouse embryoblasts (MEFs) and found that Zfp148 regulates cell proliferation and p53 activity by repressing the transcription of ARF.

Finally, we addressed the role of antioxidants. Clinical studies on the ability of dietary antioxidants to prevent CRC show inconsistent results. To understand the effect of antioxidants, we gave two types of dietary antioxidants to *Apc^{Min/+}* mice and investigated tumor development. Our results indicate that dietary antioxidants have no effect on tumor initiation but accelerate progression of existing tumors. The result raises concerns about the widespread use of dietary antioxidants, especially among high risk populations.

This thesis is based on the following three studies.

1. Nilton .A*, Sayin .V.I*, Zou Z.V, Sayin.S. I, Bondjers.C, Gul. N, Agren. P, Fogelstrand. P, Nilsson. O, Bergo, M. O, Lindahl, P.

Targeting Zfp148 activates p53 and reduces tumor initiation in the gut.

Oncotarget. 2016; 7:56183-56192.

2. Zou Z.V, Gul. N, Johansson.J, Ibrahim.M.X, Nilton.A, Tivesten.A, Bergo, M. O, Lindahl, P.

Zfp148 prevents cell cycle arrest by repressing ARF.

In manuscript

3. Zou Z.V, Le Gal. K, Sayin .V.I, Ibrahim.M.X, Gul. N, Bergh.P.-O, Ståhlman.M, Bergo, M. O, Lindahl, P.

Dietary antioxidants accelerate growth of intestinal adenomas in APC^{Min/+} mice.

In manuscript

Abbreviations

| | |
|-------------------------------|--------------------------------------|
| CRC | CRC |
| APC | Adenomatous polyposis coli |
| HNPCC | Hereditary nonpolyposis CRC |
| FAP | Familial adenomatous polyposis |
| MIN | Multiple intestinal neoplasms |
| <i>Cdkn2a</i> | Cyclin-dependent kinase inhibitor 2A |
| MDM2 | Murine double minute 2 |
| ZFP148 | Zinc finger protein 148 |
| GT | Gene trap |
| WT | Wild type |
| EGF | Epidermal growth factor |
| MEF | Mouse embryonic fibroblast |
| ROS | Reactive oxygen species |
| NAC | N-acetylcystein |
| GSK3β | Glycogen synthase kinase 3 β |
| ChIP | Chromatin immunoprecipitation |
| Apoe | Apolipoprotein E |

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1 Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is a cancer that occurs in the colon or rectum and is one of the most common cancer forms and a leading cause of cancer death in the Western world[1]. Studies have shown that the main risk factors of CRC are smoking, high consumption of alcohol, inflammatory bowel disease, and imbalanced dietary habits including high consumption of red meat, high fat diet, and insufficient intake of fibers [2, 3].

Most CRC cases arise sporadically and sporadic cases account for 75% of CRCs [4]. When it comes to the non-sporadic cases of CRC, studies have shown that 5-10% of CRCs are caused by inherited mutations of known oncogenes or tumor suppressor genes, whereas 10-30% of the patients have a family history of CRC suggesting an underlying genetic predisposition, although the genetic or epigenetic factors responsible for the disease may be unknown [5]. Hereditary CRCs are mainly due to germline minor variants and/or single nucleotide polymorphisms in oncogenes or tumor suppressor genes[6].

Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis syndrome (FAP) are the two main hereditary CRC syndromes [5]. HNPCC is caused by inherited deficiency of DNA mismatch repair genes (*MLH1*, *MSH2*, *PMS2* and *MSH6*), whereas FAP is due to inherited mutations in the *APC* gene [7]. The *APC* gene is also the most commonly mutated gene in sporadic cases of CRC (*APC* is mutated in 80% of sporadic cases) suggesting a leading role for the *APC* protein [8]. Other genes that are frequently mutated in sporadic CRCs include *KRAS*, *BRAF*, *TP53*, and, *EGF* [9].

For CRCs to develop, mutations of oncogenes and/or tumor suppressor genes occur in epithelial stem cells in the gastrointestinal mucosae [10], conferring advantages of proliferation and self-renewal on those epithelial cells. As a result, normal epithelium becomes highly proliferative and contributes to benign adenomas, which can with additional genetic alternations transform further into metastatic adenocarcinomas in about 10 years [11]. Typically, mutations in the

APC gene initiate benign polyps, *KRAS* mutations transform the neoplasia into adenomas, followed by *TP53* mutations that result in carcinomas [9] (Fig.1).

1.2 *APC*^{MIN/+} CRC mouse model

Investigations of the molecular pathogenesis of cancers as well as development of novel cancer therapies depend on the availability of relevant animal models that recapitulate critical features of the human disease. The *Apc*^{MIN} mouse is the most commonly used CRC model. Like human CRCs, tumorigenesis in the *Apc*^{MIN} model is driven by truncating mutations in the *Apc* gene. Under the microscope, tumors from *Apc*^{MIN} mice recapitulate many features of early human CRCs, and the *Apc*^{MIN} mouse is considered to be a highly relevant model for early CRC development in human [12].

There are many attractive features with this model: First, the grand majority of the mice only develop tumors in the intestines. Spontaneous tumors may also develop in the mammary glands of female *APC*^{Min/+} mice on certain genetic backgrounds, but at low frequencies and old age [13]. Second, the tumors develop rapidly making research on the model time efficient. And third, the tumor phenotype is affected by multiple signaling pathways or modifier genes, thus making it highly accessible for mechanistic studies.

1.2.1 the history of the *Apc*^{Min} mouse

The *Apc*^{Min} mouse was first reported in 1990 and is a result of an N-ethyl-N-nitrosurea chemical mutagenesis screen [14]. A mutant with circling behaviour and anaemia was identified and found to have multiple adenomas throughout the intestinal tract. This mouse line was therefore given the name Min (Multiple intestinal neoplasia). The circling behaviour was caused by a separate heritable trait and was subsequently lost by outbreeding.

As mentioned before FAP is caused by inherited germ-line mutations in the *APC* gene. Strikingly, sequencing of the *APC* gene revealed a T to A transversion of nucleotide 2549 resulting in a change from leucine to an amber stop codon at position 850 [15]. This mutation is similar to those found in FAP and sporadic CRC. The mouse allele was named *APC*^{Min}.

1.2.2 The APC protein is part of the β -catenin destruction complex

The APC protein plays an essential role in the canonical WNT-pathway [16]. APC is part of the β -catenin destruction complex together with glycogen synthase kinase-3 (GSK3), the scaffolding protein axin, the casein kinase-1, and the ubiquitin ligase β -TrCP. In the absence of extracellular WNT signalling activity, GSK3 phosphorylates beta-catenin thus targeting the protein for destruction at the proteasome. After losing APC, GSK3 β can no longer phosphorylate β -catenin leading to the accumulation of unphosphorylated β -catenin in the cytoplasm, translocation of β -catenin to the nucleus, and activation of Wnt target genes [17]. Activation of the WNT-pathway in colonic epithelium is the key event in the polyp initiation process [18].

1.2.3. the APC^{Min} models is a classical Knudson two-hit model

Apc^{MIN/+} mouse model is a classical Knudson two-hit model; inactivation of both alleles is required for tumor formation [9, 19, 20]. *Apc*^{Min/+} mice carry one germline mutation in the APC gene meaning that all cells have one defective copy of the gene. The remaining copy is sufficient for maintaining a functional β -catenin destruction complex. However, loss of the second APC allele abolishes the function of the destruction complex leading to unauthorized activation of β -catenin and expression of WNT-targeting genes. Loss of the second allele is a stochastic event that preferentially occurs in the intestines by loss-of-heterozygosity. The most common cause of loss-of-heterozygosity of *Apc* is mitotic nondisjunction of chromosome 18 where the *Apc*-gene is located [21, 22].

*1.2.4. Unlike humans, *Apc*^{Min/+} mice develop tumors in the small intestine*

Unlike humans that develop tumors in the colon and rectum, *Apc*^{Min/+} mice mainly develop tumors in the small intestines [23, 24]. The reason for this is not fully understood. On C57BL/6 genetic background, each mouse develop approximately 30 polyps, most of which are located in the distal part of the small intestine (ileum) [25]. With time, tumors may invade the underlying submucosa and muscularis externa, but they don't form lymphnode or distant metastases. One reason for this may be the heavy tumor load in the small intestine which

results in bleedings and anaemia and death at about 30 weeks of age, not giving the mice time to develop more advanced stages of the disease [26].

1.2.5. Modifiers of the APC^{Min} phenotype

As mentioned before, one attractive feature of the Apc^{Min} model is the great number of modifier genes that affect the tumor phenotype thus opening up for studies of underlying mechanisms. The first *modifier of MIN* (*Mom1*) that was identified is secretory phospholipase 2A (*Pla2g2a*) that reduces tumor multiplicity and size when overexpressed in $APC^{Min/+}$ mice [27].

Introduction of somatic mutations typically found in human CRCs like KRASG12D and EGF accelerate tumor development in $APC^{Min/+}$ mice thus recapitulating the effects seen in human, supporting the model's relevance for mechanistic studies [28, 29]. Knockout of the tumor suppressor p53 is an exception. Surprisingly, inactivation of p53 had no or limited impact on tumor multiplicity and size in $APC^{Min/+}$ mice [30-32]. One reason could be that p53 is involved in later stages of tumor development (Fig. 1), but as will be evident in this thesis, our data suggest that p53 may play a role during APC^{Min} -driven tumor initiation.

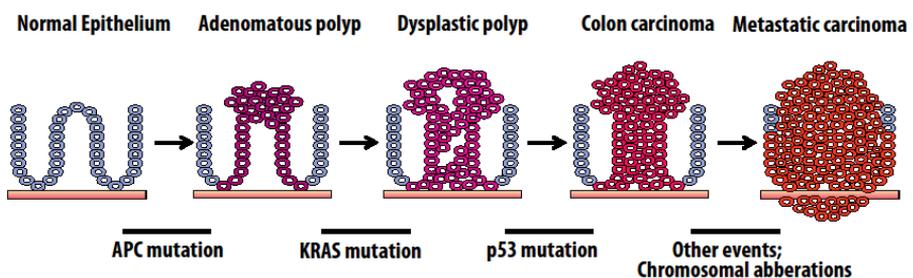


Figure.1. Progression of CRC

1.3 The tumor suppressor p53

p53, also known as cellular tumor antigen p53, phosphoprotein p53, or transformation-related protein 53, is encoded by the *TP53* gene in humans and the *Trp53* gene in mice and contains 12 exons. The p53 protein contains 393 amino acids that includes two N-terminal transactivation domains, one proline rich domain, one central DNA binding domain, and a C-terminal regulatory domain [33]. *TP53* is the most frequently mutated gene in cancers; approximate 50% of cancer patients had somatic p53 mutations [34]. In some cancer subtypes, for instance ovarian serous carcinoma and lung squamous cell carcinoma, mutation of *TP53* can be found in more than 80% of the patients [35].

1.3.1. *TP53* mutations in CRC

TP53 mutations are found in approximately 40-50% of sporadic CRC making this one of the most frequently mutated genes in CRC [36]. Inactivation of p53 is considered to play a critical role in the adenoma-to-carcinoma transition in CRC and *TP53* mutations have been associated with lymphatic and vascular invasion in CRCs and the occurrence of metastases [37]. CRC patients with *TP53* mutations turn out to be more resistant to chemotherapy and have poorer prognosis compared to patients with wildtype *TP53* [37]. Therefore, p53 and the p53 pathway have attracted enormous interest among CRC researchers.

1.3.2. *p53* suppresses tumorigenesis in multiple ways

p53 is mainly considered to be a tumor suppressor – inactivation of p53 causes or accelerates tumor formation. The protein has many functions that are relevant to cancer: It induces DNA repair, cell cycle arrest, apoptosis and senescence, removes damaged cells, and controls metabolism [38-41]. Since p53 is a stress inducible transcription factor, it functions by controlling the transcription of critical regulators of these processes such as p21, PAI-1, and PML for cell cycle arrest and/or senescence, and BAX, PUMA, NOXA, and Killer/DR5 for apoptosis [42, 43]. One key function is to eliminate damaged cells at risk of neoplastic transformation.

1.3.3. p53 is regulated by the E3-ubiquitin ligase MDM2

P53 is mainly regulated at the post-translational level by ubiquitination-induced degradation at the proteasome[44]. The E3-ubiquitin ligase mouse double minute 2 (MDM2) plays a critical role. Mdm2 controls the stability of p53 by adding ubiquitin residues to p53 [45]. Mdm2 is the convergence point for multiple stress sensor pathways that inhibit its activity in response to a broad variety of stressors. One is oxidative stress caused by excessive levels of reactive oxygen species (ROS) typically found in CRCs and other types of cancer. Another is oncogenic stress caused by excessive growth stimulation leading to oncogene induced senescence.

1.3.4. ARF is a major activator of the p53 pathway

The alternative reading frame protein (ARF) is a key regulator of oncogene induced senescence. ARF, which is one of two gene products of the cyclin dependent kinase 2A (Cdkn2a) locus, forms a stable complex with MDM2 and sequesters it away from p53 [46]. ARF is mainly regulated at the transcriptional level by the polycomb repressor complex that attenuates ARF expression by methylating the ARF promoter, thus preventing p53 activation [47]. ARF is also regulated by other types of stressors making this one of the major regulators of the p53 pathway [48]. The importance of ARF is evidenced by frequent somatic mutations of Cdkn2a in various cancers [49]. The other gene product of the Cdkn2a locus, p16, controls the retinoblastoma pathway and is a key regulator of cell cycle progression [50].

1.3.5. The role of p53 in the APC^{Min} model

Interestingly, knock out of *p53* alone does not affect tumor development in *Apc^{Min/+}* mice in a significant manner [30-32]. One possible explanation is that p53 activity is suppressed during early tumor development in mouse intestines thus making p53 less important. Indeed, recent studies provide some evidence to this idea. One study shown that the Wip1 phosphatase regulates apoptosis of stem cells and tumorigenesis in the mouse intestine in a p53 dependent manner [51]. DETAIL... In addition, activated β -catenin have been shown to have capability to silences ARF which is a protein encoded by gene *Cdkn2a*[52] and it's

an activator of p53 [53], these studies suggest a new mechanism of how p53 is involved in CRC. However, there is still a big knowledge gap for understanding the role of p53 in CRC.

1.4 The role of Zfp148 in CRC.

Several studies suggest that Zinc finger protein 148 (ZFP148) is involved in CRC [54, 55]. Increased expression of *ZNF148* (the gene encoding ZFP148 in humans) correlates with decreased survival of CRC patients, and expression levels of *ZNF148* varies dramatically in the different stages of CRC development. Levels of *Zfp148* increased during the transformation from normal mucosa to stage I CRC, followed by a reduction from stage I to stage IV CRC. However, the functional importance of ZFP148 in CRC has not been properly investigated yet.

1.4.1. Zfp148 is a transcription factor

Zinc finger protein 148 (Zfp148), a gene located at chromosome 3q21 in human and chromosome 16 in mouse, also known as ZNF148, ZBP89, BERF-1, and BFCOL1, is a widely expressed krüppel type transcription factor [56]. *Zfp148* binds to GC-rich DNA sequences to activate or inhibit transcription of target genes by recruiting co-activators or co-repressors to the promoter regions [57-60]. Many targets of *Zfp148* have been identified, p21 and p16, suggesting that *Zfp148* plays a role in cell growth arrest and senescence [58, 61, 62]. Since *Zfp148* participates in cell growth arrest and senescence, *Zfp148* may play a role in tumorigenesis. Indeed, studies have demonstrated that *Zfp148* expression level was elevated in several kinds of tumors including gastric cancer [63], CRC [54], breast cancer [64] and melanoma [65].

1.4.2. Zfp148 is required for intestinal integrity in mice

Studies in mice suggest that *Zfp148* is required for the integrity of the normal intestinal epithelium [66-69]. Deletion of the N-terminal domain of *Zfp148* predisposes mice to dextran dosium sulfate induced colitis, a phenotype that has been associated with increased formation of APC-driven intestinal tumors in other mouse models. Moreover, conditional deletion of *Zfp148* in the intestinal epithelium sensitized the mice to colitis and sepsis after infection with

Salmonella typhimurium and blunted the epithelial cell response to butyrate – a metabolite produced by the microbiota and associated with CRC risk.

1.4.3. *Zfp148* is a potent suppressor of p53 activity

Zfp148 binds physically to the tumor suppressor p53 suggesting that it may play a role in the p53 pathway [61]. Pull-down assays demonstrated that Zfp148 binds to p53 via the zinc finger domains, and overexpression of Zfp148 AGS gastric carcinoma stabilized the p53 protein. However, the significance of this interaction has not been clear.

Insertion of a gene-trap into the fourth exon of Zfp148 (designated Zfp148^{gt}), thus interrupting transcription of the major part of the coding region including the zinc finger domains, reduced mRNA levels by 97% in mice (liver, skeletal muscle, adipose tissue) compared to controls and abolished protein expression on western blots in mouse embryonic fibroblasts (MEFs) [70]. These mice reveal a strong genetic interaction with p53 demonstrating that Zfp148 deficiency activates p53 in certain contexts.

First, Zfp148 deficiency increased levels of total or phosphorylated p53 in MEFs, intraperitoneal macrophages, and aortic roots compared to controls [70, 71]. And second, Zfp148 deficiency arrested cell proliferation *in vitro* and *in vivo*, in a p53-dependent manner: In MEFs, Zfp148 deficiency induced proliferation arrest and premature senescence; in prenatal mouse lungs, Zfp148 deficiency induces proliferation arrest leading to lung maturation defects and neonatal lethality [70]; and in *Apoe*^{-/-} knockout mice, Zfp148 deficiency reduced lesional macrophage proliferation [71]. All these phenotypes were rescued by deletion of one or both copies of Trp53. The results suggest that Zfp148 represses p53 activation but the mechanism remains to be solved.

1.4.4. What about Zfp148 and the APC^{Min} model

Collectively, the epidemiological data, Zfp148's role in intestinal integrity and the interaction between Zfp148 and p53 raises the possibility that Zfp148 could play an important role in CRC. One preliminary study suggested that Zfp148 may protect against CRC, but the methodology was problematic: Overexpression of Zfp148 in intestinal epithelial cells increased apoptosis and reduced the numbers of intestinal polyps in *Apc^{Min/+}* mice [69]. However, the protein was heavily overexpressed and the relevance of this finding is questionable. Thus, there is a strong need to investigate the role of Zfp148 in CRC using proper strategies. We hypothesized that Zfp148 deficiency may protect against CRC by unleashing p53 activity, and tested this hypothesis in the *Apc^{Min}* model (Paper I).

1.5 The effect of antioxidants in CRC.

Diet habits have a significantly impact on the risk of developing CRC, and antioxidant rich food has been considered to protest against CRC [72]. Two studies showed that diets rich in antioxidants such as selenium and Vitamin C and E were associated with lower risk of CRC [73, 74]. However, according to a comprehensive review published by the World Cancer Research Fund (WCRF) there is limited evidence to suggest that consumption of fruits and non-starchy vegetables, which are rich sources of antioxidants and other potential cancer preventive agents, have protective effect against CRC [75]. Indeed, clinical trials of antioxidant supplements did not show significant effect against CRC [76-78]. Overall, the role of dietary antioxidants in CRC remains unclear.

1.5.1. Antioxidants counteract oxidative stress by neutralizing reactive oxygen species

Antioxidants is a group of molecules that neutralizes reactive oxygen species (ROS) by serving as radical scavengers, hydrogen or electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, or synergist and metal chelating agents [79]. ROS are reactive molecules containing oxygen. Imbalance between the production of ROS and antioxidant defence induces oxidative stress [80]. Oxidative stress is considered to play a critical role in multiple chronic diseases including cancer, age related degeneration,

cardiovascular disease, diabetes and others [81], therefore, antioxidants have been long considered to have protecting effect to those diseases.

1.5.2 Inconsistent results of antioxidants in preventing oxidative stress relevant diseases

Clinical trials of dietary antioxidants against chronic diseases have produced inconsistent results. Some trials showed that antioxidants have beneficial effects, but most studies showed no effects or even adverse effects. In a women's health study (>45 years of age), vitamin E did not reduce the risk of heart attack, stroke, cancer, or age-related macular degeneration, and in a study of 14000 male physicians (>50 years of age), vitamin E or vitamin C did not reduce the risk of prostate or total cancer [82, 83]. Similarly, dietary antioxidants did not show significant effect against CRC [76-78]. More dramatically, one large trial showed that vitamin E increased the risk of prostate cancer among elderly men [83] and one trial found that beta-carotene increased the incidence of lung cancer in male smokers [84]. Since antioxidant supplements are widely used, a better understanding of the effect of antioxidants in cancer is urgently needed.

1.5.3 3 Antioxidants accelerate tumor progression in mouse models of lung cancer and melanoma

Cancer development can be divided into tumor initiation and progression, so our group and our collaborator hypothesized that antioxidants inhibit the initiation of new tumors but accelerate progression of existing tumors (which could explain the inconsistent results). We found that antioxidants increased proliferation of KRAS or BRAF-driven lung adenocarcinomas by decreasing levels of ROS and p53 activity in tumors, and dramatically shortened the survival of mice carrying BRAF-driven lung tumors [85]. Moreover, NAC treatment increased lymph node metastasis and distant metastasis of BRAF-driven melanomas by increasing Rho-GTP mediated cell migration and invasion [86]. These two findings suggest that antioxidants accelerate progression of existing tumors and increase the incidence of cancer in high risk populations. However, whether dietary antioxidants inhibit the initiation of new tumors is not known. Another question is whether the accelerated tumor progression by antioxidant supplementation is a general phenomenon, or confined to certain tumor types.

To address these two questions, we decided to give dietary antioxidants to *APC^{Min/+}* mice and investigate the effects on tumor initiation and progression in the gut.

2 Rationale and aims

The overall aim of this thesis is to uncover novel mechanisms behind the initiation and progression of intestinal adenomas in *Apc^{Min/+}* mice. We address this aim in three sub-studies.

1. Several lines of evidence suggest that Zfp148 plays a role in CRC, but the functional importance of Zfp148 for intestinal tumor development has not been properly investigated. Since Zfp148 deficiency increases p53 activity and induces cell proliferation arrest, we hypothesize that *Zfp148* deficiency protects *APC^{MIN/+}* mice against tumors by increasing p53 activity.

Aim of paper I:

To test whether Zfp148 deficiency protects against tumor development in *Apc^{Min/+}* mice.

2. The mechanism by which Zfp148 deficiency activates p53 is not well understood. Because the *Cdkn2a* gene product ARF is a potent activator of p53 and recent studies implicate ARF in the pathogenesis of *APC^{Min/+}* mice, we hypothesize that *Cdkn2a* may be required for activation of p53 in Zfp148-deficient cells.

Aim of paper II:

To test whether *Cdkn2a* is required for activation of p53 in *Zfp148* deficient MEFs.

3. Studies on the effect of dietary antioxidants in CRC show inconsistent results. One possible explanation is that antioxidants have different effects on tumor initiation and progression. We test this idea in the *APC^{Min}* model. The concept that dietary antioxidants prevent cancer is widely accepted by the public, so there is an urgent need to understand the effects and mechanisms of antioxidants in CRC.

Aim of paper III:

To test the effect of dietary antioxidant in the *Apc^{Min/+}* mouse model.

3 Methods

In this section, essential methods will be described. Detailed information of unmentioned methods in this part can be found in the materials and methods section from each enclosed paper.

Experimental mice. *Cdkn2atm1rdb* (*Cdkn2a*^{-/-}) and *Trp53tm1Tyj* (*Trp53*^{-/-}) mice were obtained from The Jackson Laboratory and *Zfp148gt/+* mice were produced by us. The mice were kept on a 129/Bl6 mixed genetic background and all experiments were performed with littermate controls. Genotyping was performed by PCR amplification of genomic DNA from mouse-tail biopsies. Mice were fed on a regular diet and had unlimited supply of food and water. *Apc*^{Min/+} mice were obtained from The Jackson Laboratory and kept on a Bl6 genetic background. NAC (616-91-1, ≥99% purity, Sigma) was administered in the drinking water (1 g/litre). Vitamin E (dl- α -tocopheryl acetate; 7695-91-2, Zhejiang Medicine Co.) was administered in chow pellets (Lantmännen) at 0.5 g/kg chow (61.5 mg/kg body weight), calculated on the basis of observed daily food intake. Mice had unlimited supply of food and water. All animal procedures used in this study were approved by the Animal Research Ethics Committee in Gothenburg.

Tumor multiplicity and area. Mice were dissected; the intestines were removed and separated into three segments; colon and two segments of the small intestines. The segments were rinsed in PBS and prepared using a modified Swiss roll technique [87]. Tissues were fixed in 4% formaldehyde, imbedded in paraffin and 4 μ m thin sections were stained with Haematoxylin and Eosin. Tumor counts and area calculations were performed on one single sagittal section from each segment by investigator blinded to different group.

Cell culture. Primary MEFs were isolated from E13.5–15.5 embryos as described [70]. The H460 cancer cell line was from the American Type Culture Collection. The H460 cancer cell line was from the American Type Culture Collection. The cells were confirmed mycoplasma free. Cells were cultured in

DMEM low glucose medium with 10% fetal bovine serum, 100ug/ml penicillin and streptomycin, 1% non-essential amino acids, 1mM glutamine, and 0.1 mM β -mercaptoethanol (β -mercaptoethanol was not used for H460 cells). Growth curves were established using a modified 3T3 protocol (50000 cells/well in 6-well plates). Human colon cancer cell lines (American Type Culture Collection, ATCC) were cultured in Mc Coy's medium supplemented with 10% FBS (HT-29, HCT-116), L-15 medium supplemented with 10% FBS and dishes sealed with parafilm (SW620), RPMI medium supplemented with 10% FBS (Colo320, CCL-252), or F12K medium supplemented with 10% FBS (LoVo)(Cat.no. 26600023, 21127022, 21875059 and 11415049, Thermo Fisher). NAC ($\geq 99\%$ purity; A7250) and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane- 2-carboxylic acid; 53101-49-8, Sigma) were used at concentrations of 1 mM and 200 μ M in the case of NAC, and 100 μ M and 20 μ M for Trolox.

Cell proliferation assay. Cell proliferation assay of both MEFs and H460 were done by using the classical cell doubling analysis protocol. Cell proliferation assay of human CRC cell lines were assessed by seeding 10^4 cells per well in 96-well Image Lock plates (Cat.no. 4379, Essence Bioscience) with control medium or medium supplemented with NAC or Trolox; the plates were then monitored every 1-2 hours with the Incucyte System (Essence Bioscience) for a total of 91-106 hours.

Cell cycle analysis. Cells were synchronized at G1 phase by using 72 hours serum starvation, and released from quiescence by adding serum back into culture medium for various time points. And then trypsinized, fixed in 70% ethanol overnight followed by incubation with 7-aminoactinomycin D (7-AAD, Life Technology, A1310) or propidium iodide (PI) and RNase A (SIGMA, R6513) at 37°C for 2 hours. Samples were analysed in a FACScan flow cytometer with CellQuest Pro software (version 4.0.2, Becton Dickinson).

Adenoviral Transduction of MEFs. Cells were incubated with 15 multiplicities of infection of empty control adenoviruses (*AdNull*) or adenoviruses encoding Zfp148 (*AdZfp148*) or Zfp148FLAG (*AdZfp148FLAG*) (Vector Biolabs) for 36-48 h before analyses.

Intestinal explant experiments

One 2 cm segment of the distal ileum was collected, as previously described [88]. Intestinal contents from the segment were removed through flushing with cold PBS and the segment was divided into four equal longitudinal parts. Each part was placed in cell culture plates containing growth medium (Dulbecco's modified Eagle's medium with glutamine and pyruvate, 4.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) with or without 2µM GSK3IX inhibitor(Santa Cruz). The plates were then incubated 6-20h at 37°C in 5% CO₂. The mixture containing the tissue was transferred to Eppendorf tubes and centrifuged at 14,000rpm for 5 min. The tissue was collected after removal of the supernatant and used for analysis of mRNA or protein expression. All explant experiments were performed on *APC^{MIN/+}* background.

Gene expression analysis for paper I. Total RNA was extracted from crypt-enriched tissues that were isolated by laser microdissection (PALM) from 10µm thick cryosections of OCT-embedded and snap frozen Swiss roll preparations of small intestines (proximal). RNA concentration was measured with ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA). 250 nanograms of total RNA from each sample were used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the GeneChip® WT PLUS Reagent Kit User Manual (P/N 703174 Rev 1 Affymetrix Inc., Santa Clara, CA). GeneChip® ST Arrays (GeneChip®XXX Gene 2.0 ST Array) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev 3, Affymetrix Inc., Santa Clara, CA) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip®Scanner 3000 7G. The gene expression data set has been deposited in the GEO repository with accession number GSE77773.

Gene expression analysis for paper II. Total RNA from MEFs, lungs, brains and macrophages was isolated using the Gene Elute kit (Sigma) and hybridized to Affymetrix Mouse Gene 1.0 ST chips. Data were normalized with the Robust

Multi-Array Analysis (RMA) method [89]. The heat-map was constructed using the Hierarchical Clustering Explorer 3.0 software. Gene ontology statistics was calculated using the DAVID software [90, 91].

Real-time quantitative PCR. TaqMan assays were performed as described [92] using TaqMan universal PCR mastermix (Applied Biosystems) and pre-designed TaqMan assays for p16 and ARF (Applied Biosystems).

ChIP-on-chip analysis. MEFs (1×10^8) transduced with AdZfp148FLAG were fixed in 1% formaldehyde. 200–500-bp DNA fragments were precipitated using the anti-FLAG M2 antibody (Sigma) conjugated to sheep anti-mouse IgG Dynabeads (Invitrogen), purified (QIAquick, Qiagen), amplified (WGA2 kit, Sigma), and hybridized to MM8_Deluxe_Promoter_HX1 chips at NimbleGen (www.nimblegen.com). We used Zfp148^{gt/gt} cells to avoid competition with endogenous Zfp148 and p53-null background to avoid co-precipitation of p53-binding DNA elements.

ChIP. MEFs (4×10^6) were fixed in 1% formaldehyde for 10 minutes, harvested by trypsin. ChIP was performed by the Pierce Magnetic ChIP Kit (26157, Thermo Scientific) with anti-Zfp148 (HPA001656, Sigma Aldrich Atlas) used for precipitation. Real-time PCR performed by using SYBR Green Master Mix (A25741, Applied-biosystem) and pre-designed primers:

P1F: CCGATCAACTCGGCCCTC, P1R: TCGGTTTTAAGCGCGAGCA.

P2F: GCGTACCGCTAAGGGTTCAA, P2R: AACGTCTCCTGCGCCGAT.

P3F: GTCACCGCGATGGGTGG, P3R: CATCTTTGCTCCACGCCCA.

Western blotting. Cells were washed three times with ice-cold PBS, and then lysed in 9 M UREA buffer (SIGMA U0631-1KG) contains complete cocktail inhibitors. Lysates were sonicated and centrifuged at 4°C (12,000g, 10 MIN). Discard pellet, and measuring the protein concentration with the Bio-Rad DC protein assay (5000112). Samples were diluted with the same UREA buffer in to the same final concentration and incubated with LDS sample buffer (Invitrogen, NP0008) at 70°C for 10 min, resolved on 4-12% bis-Tris gel (Invitrogen, NP0336BOX), and then transferred to PVDF membrane. Membranes incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibody at room temperature for 1 hour. Blocking buffer was fluorescent western blocking buffer from Rockland (MB-070). Immunoreactivities were

visualized by using LI-Cor Odyssey Imager (Odyssey software version 3.0, Li-Cor). Primary antibodies were: Zfp148 (HPA001656, Sigma Aldrich Atlas), ACTIN (A2066) (Sigma Aldrich Atlas), p16 (sc-1207, Santa cruz) and p19^{arf} (sc-3278, Santa cruz), BAX (SC-493, 1:500, Santa Cruz), c-PARP (9542 S 1:500, Cell Signal), and β -actin (2228, 1:20000, Sigma Aldrich), p21 (SC-6246, 1:1000, Santa Cruz). Secondary antibodies were anti-mouse IRDye 680RD (926-68072), anti-rabbit 680RD (926-68071) and anti-goat 680RD (926-68074).

CRISPR-CAS9 genome editing. MEFs were infected with lenti-CRISPR-v2. After 24 hours infection, targeted cells were selected by incubating with puromycin for 72 hours to obtain batch clones. The guide-RNA sequences were:

| gRNA | Target sequence (coding strand) |
|---------|---------------------------------|
| ARF A | TGGTGAAGTTCGTGCGATCC |
| ARF B | AGAGGGCCGCACCGGAATCC |
| p16 A | GGGCCGTGTGCATGACGTGC |
| p16 B | AACGCCCCGAACCTTTTCGG |
| control | GACCGAACGATCTCGCGTA |

4 Results

4.1 Paper I Targeting Zfp148 activates p53 and reduces tumor initiation in the gut.

The facts that Zfp148 is able to suppress p53 activity and *Zfp148^{gt/+}* mice have normal life span and health conditions bring up the potential possibility that Zfp148 could be a potential target in cancer therapy. Previous studies have shown that p53 suppresses the development of CRC. We hypothesized that deletion of *Zfp148* could suppress the development of CRC by elevating p53 activity.

***Zfp148* deficiency reduces tumor growth in *Apc^{MIN/+}* mice.**

To investigate whether Zfp148 is involved in intestinal adenoma formation, we crossed *Zfp148* deficient mice onto *Apc^{MIN/+}* mice. Intestines were harvest at age of 12 weeks, and followed by histological analysis. First we found that deletion of both one and two copies of *Zfp148* significantly reduced tumor numbers compared to *Apc^{MIN/+}* mice (**Fig 2.A**), and no difference in anatomical distribution was observed (**Fig 2.b**). Furthermore, *Zfp148^{gt/gt}Apc^{MIN/+}* mice shown lower histopathological grade compared to *Apc^{MIN/+}* mice (**Fig 2.C**). Therefore, Zfp148 is required for the formation of intestinal adenomas. Zfp148 deficiency also prolonged the median survival of *Apc^{MIN/+}* mice (**Fig 2.D**).

***Zfp148* deficiency doesn't affect tumor growth rate in *Apc^{MIN/+}* mice.**

To understand the mechanism that *Zfp148* deficiency prolonged survival and lower tumor burden in *Apc^{MIN/+}* mice, we investigated cell proliferation and apoptosis in tumor cells. However, we did not observe any differences in cell proliferation or apoptosis of tumor cells or normal epithelial cells between 12 weeks old *Zfp148^{gt/+}Apc^{MIN/+}* and *Apc^{MIN/+}* controls(**Fig.3.**). Therefore, the lower tumor burden in *Zfp148^{gt/+}Apc^{MIN/+}* mice is not induced by reduced growth rate of tumor cells.

As mentioned above, the cancer development includes two steps: initiation and progression. Since there was no difference of growth rates between tumor cells

and normal epithelial cells observed, we hypothesized that *Zfp148* deficiency may affect the tumor initiation. Briefly, tumor initiation is driven by β -catenin and suppresses by p53. However, no difference between *Zfp148^{gt/+}Apc^{MIN/+}* and *Apc^{MIN/+}* controls was observed from gene expression profiling of β -catenin and p53 signaling in normal crypts and immunohistological analysis of intestines (**Fig.4**). Therefore, *Zfp148* deficiency does not have effect on the activation of β -catenin and p53 in normal crypts. Since the loss of second *APC* allele leads to constitutive activation of β -catenin, *Zfp148* may play a role in downstream of β -catenin. To test this, we treated intestinal explants *Zfp148^{gt/+}Apc^{MIN/+}* and *Apc^{MIN/+}* controls with pharmacological inhibitor of glycogen synthase kinase 3 β (GSK3 β) for 6 and 20 hours. Inhibition of GSK3 β activates β -catenin since phosphorylation of β -catenin by GSK3 β is required for proteasomal destruction of β -catenin. Activation of β -catenin was confirmed by the induction of 3 β -catenin targeted genes at 6 hours after adding GSK3 β inhibitor (**Fig.5.A**); meanwhile *Zfp148* deficiency didn't affect expression level of those three β -catenin targeted genes (**Fig.5.A**). Significant induction of four p53-targeted genes appeared at explants from *Zfp148^{gt/+}Apc^{MIN/+}* compared to explants from *Apc^{MIN/+}* controls with 20 hours of GSK3 β inhibitor treatment (**Fig.5.B**). Western blots shown that protein levels of p53, p53 targeted genes and apoptosis marker cleaved-Parp1 were significantly elevated in explants from *Zfp148^{gt/+}Apc^{MIN/+}* compared to explants from *Apc^{MIN/+}* controls after 20 hours of GSK3 β inhibitor treatment (**Fig.5.C**). Those results suggest that *Zfp148* deficiency eliminated newly transformed tumor cells by increasing p53 activity and thus induced apoptosis. To test this idea, we bred *Zfp148^{gt/+}Apc^{MIN/+}* onto *Trp53^{+/-}* and *Trp53^{-/-}* genetic backgrounds and analyzed the tumor development at age of 12 weeks. We found that there was no difference in tumor counts, hemoglobin and hematocrit between *Zfp148^{gt/+}Apc^{MIN/+}* and *Apc^{MIN/+}* controls on *Trp53^{+/-}* and *Trp53^{-/-}* genetic backgrounds (**Fig.6**). Therefore, we conclude that *Zfp148* deficiency inhibits tumor formation in *Apc^{MIN}* mouse model through elevating p53 activity.

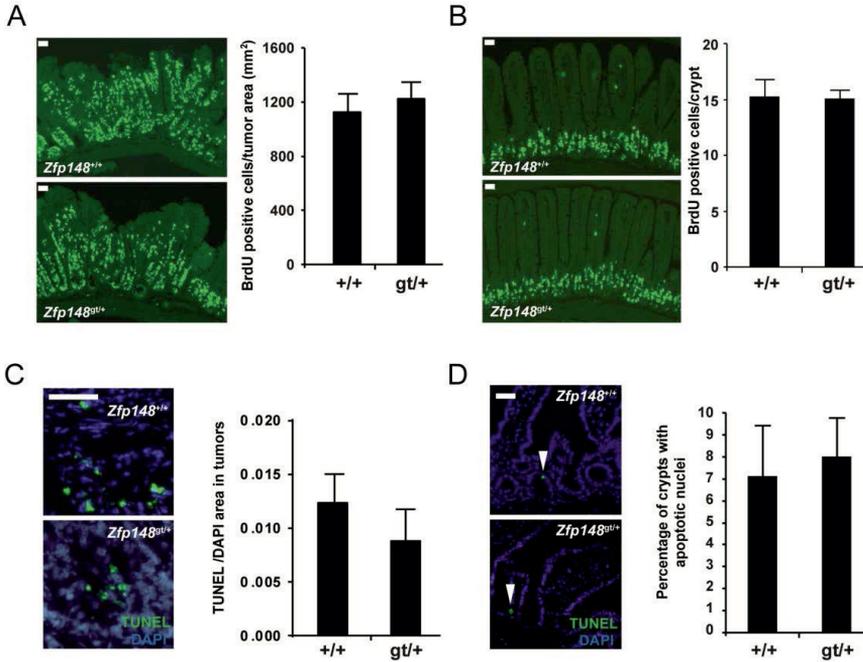


Fig.3. Tumor growth rate is not affected in *Apc^{Min/+}Zfp148^{gt/+}* mice. A.-B.

Left: Representative immunofluorescence micrographs showing BrdU-positive cells in tumor tissue **A**. and normal intestinal epithelium **B**. in *Apc^{Min/+}* and *Apc^{Min/+}Zfp148^{gt/+}* mice. Right: Quantification of BrdU-positive cells in tumortissue (A; BrdU-positive cells per tumor area; n = 11-18 tumors in intestines from 5-8 mice per group) and normal intestinal epithelium (B; average number of BrdU-positive cells per crypt; n = 60 crypts per mouse in 5-7 mice per group). C.-D. Left: Immunofluorescence micrograph showing TUNEL-positive cells (green) in tumor tissue **C**. and normal intestinal epithelium **D**. in *Apc^{Min/+}* and *Apc^{Min/+}Zfp148^{gt/+}* mice. Nuclei are stained blue with DAPI (4',6-diamidino-2-phenylindole). Right: Quantification of TUNEL-positive cells in tumor tissue (C; TUNEL-positive area per DAPI-positive area; n = 8-9 tumors per group) and normal intestinal epithelium (D; percentage of crypts with TUNEL-positive cells; n = 6-7 mice per group).

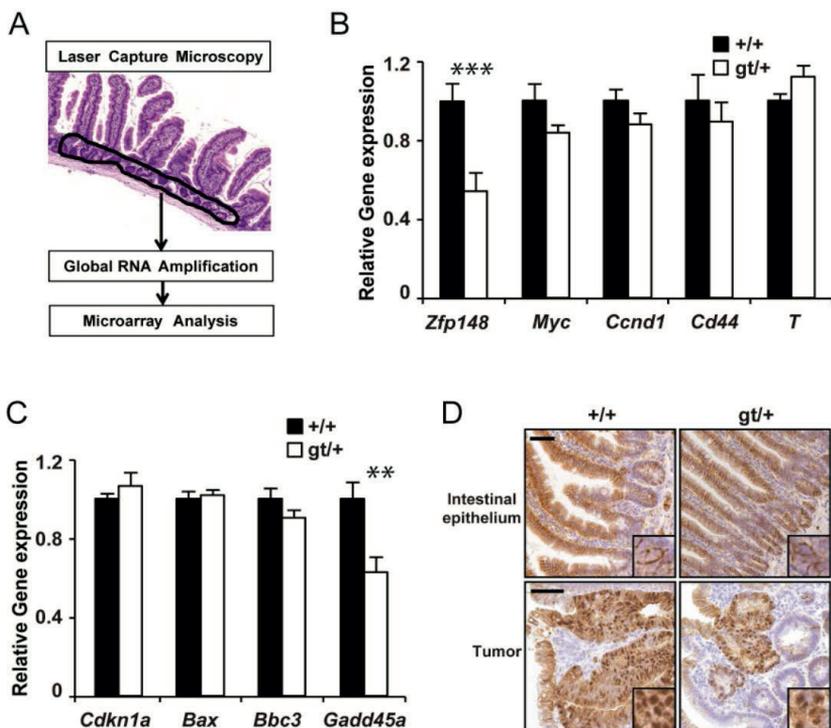


Fig.4. *Zfp148* deficiency does not affect basal expression of p53 or β -catenin target genes in crypt epithelium.

A. Schematic representation of the gene expression analysis procedure. **B.-C.** mRNA levels of *Zfp148* and four p53-target genes **B.** and four β -catenin target genes **C.** In laser microdissected crypt epithelium from *Apc^{Min/+}* and *Apc^{Min/+}*Zfp148*^{gt/+}* mice at 12 weeks of age ($n = 6$). **D.** Representative Immunohistochemistry micrographs showing β -catenin (brown) in normal intestinal epithelium (top) and in tumors (bottom) in *Apc^{Min/+}* and *Apc^{Min/+}*Zfp148*^{gt/+}* mice at 12 weeks of age. Inserts show subcellular localization of β -catenin. Sections are counter stained with Mayer's hematoxylin. Data are represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$. Scale bars, 50 μ m.

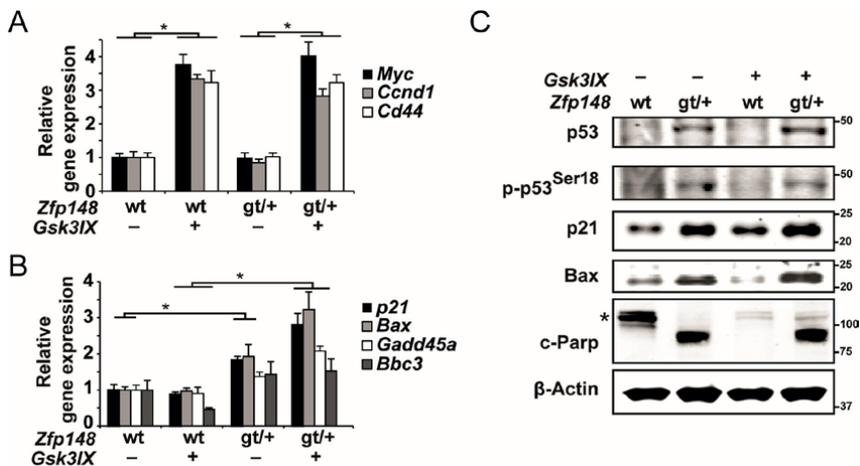


Fig.5 Constitutive activation of β -catenin induces p53-activation and apoptosis in small intestine explants from $Apc^{Min/+}Zfp148^{gt/+}$ mice.

A-B. Taqman RT-PCR assessment of mRNA levels of three β -catenin target genes **A.** and four p53-target genes **B.** in small intestine explants that were dissected from $Apc^{Min/+}$ and $Apc^{Min/+}Zfp148^{gt/+}$ and treated with $2\mu M$ of the GSK3 β -inhibitor GSK3IX or DMSO. The explants in **A.** were treated for 6 hours and those in **B.** for 20 hours ($n = 6$). **C.** Western blots of phosphorylated and total p53, the p53 targets p21 and Bax, and the apoptosis marker cleaved Parp1 (89-kD fragment) in small intestine explants that were dissected from $Apc^{Min/+}$ and $Apc^{Min/+}Zfp148^{gt/+}$ and treated with $2\mu M$ GSK3IX or DMSO for 20 hours. Asterisk indicates uncleaved Parp1 (116-kD fragment). β -Actin was used as loading control. Data are represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$.

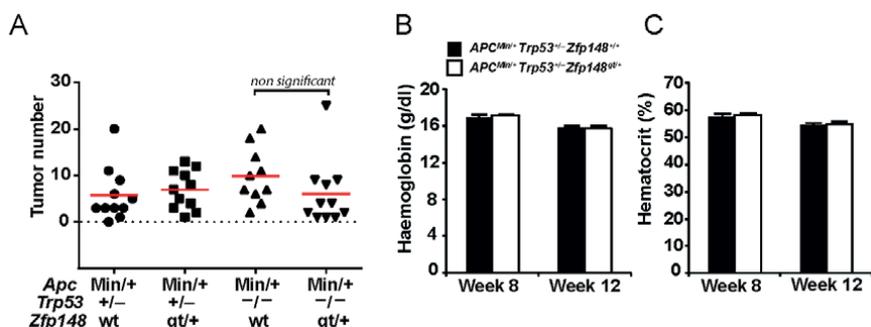


Fig.6 Deletion of one or two copies of *Trp53* restores tumor growth in $Apc^{Min/+}Zfp148^{gt/+}$ mice.

A. Tumor count in small intestine of $Apc^{Min/+}Trp53^{+/-}$, $Apc^{Min/+}Zfp148^{gt/+}Trp53^{+/-}$, $Apc^{Min/+}Trp53^{-/-}$ and $Apc^{Min/+}Zfp148^{gt/+}Trp53^{-/-}$ mice at 12 weeks of age ($n = 11$). **B.-C.** Levels of haemoglobin **B.** and hematocrit **C.** in blood from $Apc^{Min/+}Trp53^{+/-}$ and $Apc^{Min/+}Zfp148^{gt/+}Trp53^{+/-}$ mice at 8 and 12 weeks of age ($n = 11$). Data are represented as mean \pm SEM.

4.2 Paper II Zfp148 prevents cell cycle arrest by repressing ARF.

In our previous study, we found that deletion of *Trp53* rescued *Zfp148* deficiency induced cell proliferation arrest in MEFs and expression level of *p14* is increased in *Zfp148* deficient MEFs [70]. In paper II, we tested new hypothesis that *Zfp148* regulates cell cycle progression through inhibiting expression of *Cdkn2a* in primary MEFs.

ZFP148 expression is dynamically regulated during the cell cycle with peak expression during S-phase.

Cell cycle regulation proteins are normally regulated during the cell cycle progression. To investigate whether expression of *Zfp148* is regulated during the cell cycle progression, we cultured human lung cancer cell line H460 and MEFs in culture medium containing 0.2% serum for 72 hours to induce quiescence, and then release cells from quiescence by serum addition. Flow cytometry and western blotting were performed on samples that harvested at different time points after adding serum. Our data shows that *Zfp148* protein levels are regulated during the cell cycle progression in both H460 cell lines and MEFs, the peak expression was observed in S-phase (**Fig.7.**).

***Zfp148* deficiency down regulates expression of E2F-responsive cell cycle genes.**

Genes that differentially expressed between *Zfp148* proficient and deficient MEFs were identified through transcript profiling (**Fig.8.A**). Our data revealed a set of 300 genes was down regulated in *Zfp148* deficient MEFs and *Trp53* deficiency abolished down regulation of this gene set suggesting that the down regulation of this gene set in *Zfp148* deficient MEFs is p53 dependent. In addition, majority of known E2F targeting genes were clustered at the top of the list of the down-regulated genes (**Fig.8.C**). Interestingly, the regulation of cell cycle related genes was not abolished on *Trp53* null background. Gene ontology analysis on the data archived from *Trp53* deficient MEFs shown that cell cycle related genes were still the most down regulated genes (**Fig.8.B.D**). Therefore, *Zfp148* deficiency down regulates cell cycle related genes in both p53 dependent and independent manners. We performed microarrays on RNA harvest from lungs,

brains and bone marrow derived macrophages from *Zfp148^{gt/gt}* mice to identify whether the set of 300 genes was down regulated in vivo. The set of 300 genes was down regulated in lungs and to a lesser extent in brains and macrophages from *Zfp148^{gt/gt}* mice. Cell cycle related genes were only down regulated in lungs, but not in brains or macrophages (**Fig.9.**). This result matches our previous finding that Zfp148 deficiency induced lung maturation defect and proliferation arrest in neonatal lungs.

ARF is required for cell proliferation arrest of *Zfp148^{gt/gt}* MEFs.

One potential explanation for the down regulation of cell cycle genes by p53 dependent and independent mechanism is that Zfp148 regulates expression of *cdkn2a* which encodes ARF and p16 (**Fig.10.A**), and both of them regulates a separate signaling pathway in cell cycle regulation. First, we found that mRNA levels of ARF and p16 were higher in *Zfp148^{gt/gt}* than WT MEFs (**Fig.10.B**). Then, to test whether elevated expression of *Cdkn2a* is involved in the Zfp148 deficiency induced cell proliferation arrest, we bred *Cdkn2a^{+/-}* mice onto *Zfp148^{gt/+}* mice. Data shown that deletion of one copy of *Cdkn2a* was sufficient to prevent senescence and restore the proliferation in *Zfp148^{gt/gt}* MEFs (**Fig.10.C**). As mentioned above, *Cdkn2a* gene encodes two proteins, ARF and p16. To determine whether ARF or p16 mediates the effect of *Cdkn2a* deficiency on *Zfp148^{gt/gt}* MEFs, CRISPR and lentiviral-guide RNAs were applied here to knock out ARF or p16 separately in *Zfp148^{gt/gt}* MEFs. Western blotting shown that both targets were efficiently deactivated (**Fig.10.E**); however, one of the p16 targeted guide RNA reduced ARF expression level as well (**Fig.10.E**). Cell proliferation assay shows that inactivation of ARF restored proliferation of the *Zfp148^{gt/gt}* MEFs. Meanwhile; inactivation of p16 had no effect (**Fig.10.F**).

ZFP148 interacts physically with the ARF promoter.

Since both mRNA level of p16 and ARF were increased in *Zfp148^{gt/gt}* MEFs, we hypothesized that Zfp148 is regulating the transcription of *cdkn2a* gene by binding to the promoter of *Cdkn2a*. We used ChIP-chip and ChIP-qPCR to test this hypothesis. By ChIP-chip, we found that Zfp148 binds to the ARF promoter with binding regions centered at 800 base pairs upstream of the transcription start

site (**Fig.11.A**), a weaker binding was observed for p16. We used the CONSITE motif prediction tool to map potential binding sites in the ARF promoter. CONSITE identified numerous SP1-motifs which could bind ZFP148 concentrated to the ~800bp region and thus overlapped with ChIP-chip data (**Fig.11.A**). Data from ChIP-qPCR with antibody against endogenous ZFP148 showed that Zfp148 binds to the ARF promoter at ~400bp and a trend towards binding at ~800 bp(**Fig.11.B**).

Accumulating studies show that Zfp148 induced cell cycle arrest by suppressing activation of p53 [70, 93, 94], but the mechanism is not clear. In paper II, we found that, first; Zfp148 is dynamically regulated during the cell cycle with peak expression during S-phase, together with the fact that *Zfp148* deficiency down regulates E2f dependent cell cycle genes that are mainly expressed during the S-phase. This finding suggests that Zfp148 deficiency induces cell proliferation arrest though down regulation of cell cycle genes. Second, cell cycle genes are down regulated in *Zfp148* deficient MEFs in both p53 dependent and independent manners suggesting that *Cdkn2a* is potentially involved in this mechanism. We have several lines of data to support this idea. First, we observed increased expression level of p16 and ARF in *Zfp148* deficiency MEFs compare to WT controls. Second, knock out *Cdkn2a* abrogated proliferation arrest in *Zfp148* deficiency MEFs. In addition, we revealed that Zfp148 interacts physically with ARF promoter and knock out ARF but not p16 was responsible for restored proliferation. These results suggest that Zfp148 maintains cell proliferation by repressing ARF expression. However, there are still some questions remained. We reported that *Zfp148* deficiency caused lung maturation defect and thus induced neonatal lethality in mice, these phenotypes were rescued by deletion of *Trp53*. Whether deletion of *Cdkn2a* has the same effect in vivo need to be further investigated.

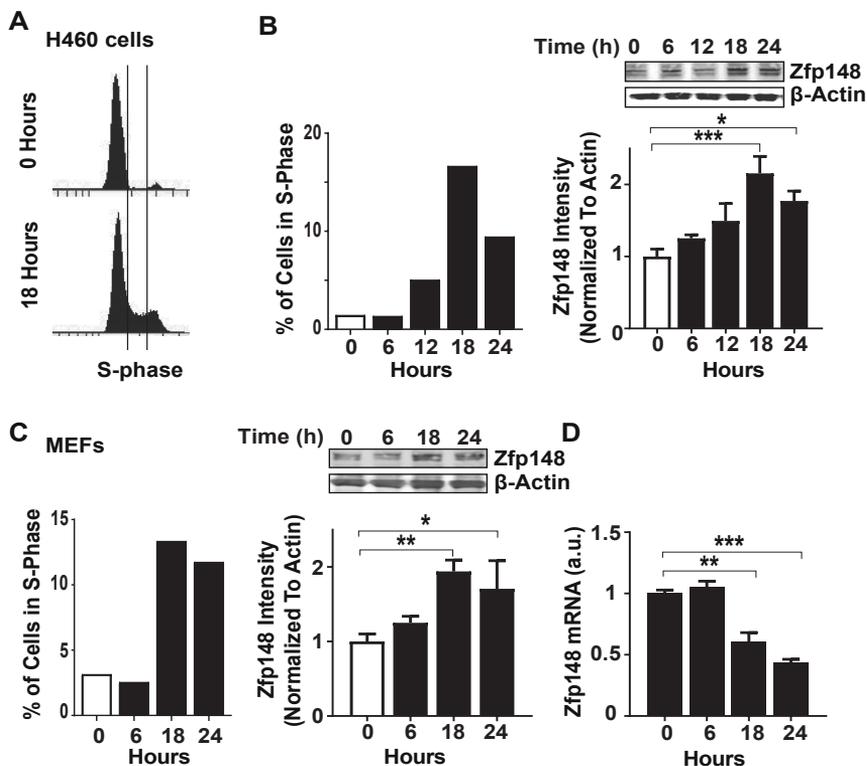


Fig. 7. Expression of *Zfp148* is regulated during the cell cycle.

(A) FACS plots showing 7-AAD distribution of serum starved H460 cells at 0 (top) and 18 (bottom) hours after media was supplemented with serum. Lines indicate gating of cells in S-phase. **(B, C)** Left: Cell cycle distribution of H460 cells **(B)** or MEFs **(C)** at indicated time points after addition of serum (n=4-5). Right: (top) Representative Western blots showing *Zfp148* expression at different phases of the cell cycle. β -Actin was used as loading control. (Bottom) Quantification of *Zfp148* protein levels at different phases of the cell cycle (n=4-5). *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$

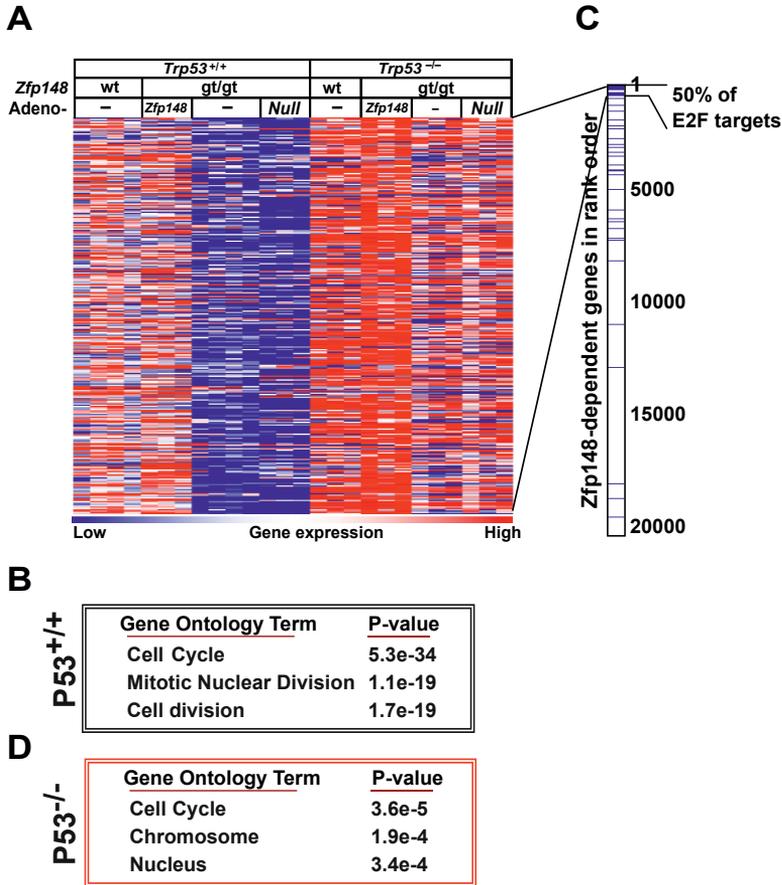


Fig.8. E2F-responsive cell cycle genes are down-regulated in Zfp148-deficient MEFs.

(A) Heat map showing expression profiles of the top 300 genes that were downregulated in the absence of Zfp148 (n is indicated by the number of columns for each genotype, every column is an independent MEF line isolated from an independent mouse).

(B) Table showing GO-terms in rank order.

(C) Rank order of 59 validated E2F target genes according to the heat-map in (A).

(D) Table showing GO-terms on p53-null background in rank order.

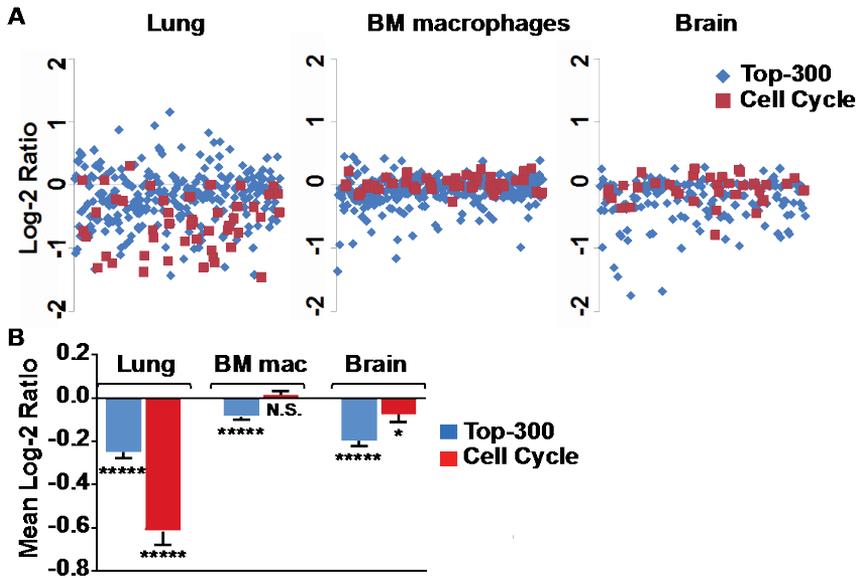


Fig.9 Cell cycle genes are downregulated in lungs of newborn *Zfp148^{gt/gt}* mice.

(A) Data are log₂ expression ratios of the top 300-gene set shown in Figure 2 (n = 3).

Red color indicates top 300-genes that are linked to the GO-term "Cell cycle".

(B) Graphs showing mean log₂ expression ratios of all top 300-genes in blue and cell cycle linked top 300-genes in red. ****P<0.00001

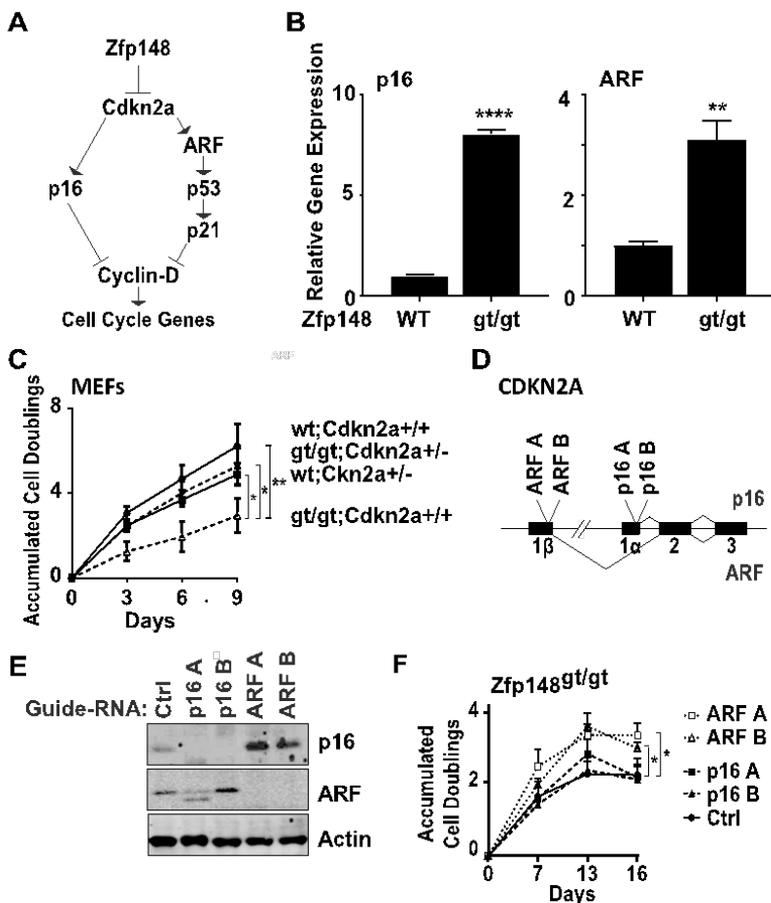


Fig.10. The *Cdkn2a* gene product ARF is required for proliferation arrest of *Zfp148*^{gt/gt} MEFs.

(A) Schematic of *Cdkn2a* regulation of cell cycle genes. (B) Graphs showing p16 and ARF mRNA levels in wildtype and *Zfp148*^{gt/gt} MEFs (n=6). (C) Growth curves (accumulated cell doublings) of wildtype MEFs and *Zfp148*^{gt/gt} MEFs on *Cdkn2a*^{+/+} and *Cdkn2a*^{-/-} background (n=5). (D) Schematic of the mouse *Cdkn2a* locus showing genome locations of guide-RNA target sites for ARF A, ARF B, p16 A, and p16B. (E) Western blots showing expression of ARF and p16 in *Zfp148*^{gt/gt} MEFs infected with guide-RNAs targeting ARF, p16, or scrambled control. Actin was used as loading control. (F) Graphs showing growth (accumulated cell doublings) of *Zfp148*^{gt/gt} MEFs infected with the indicated guide-RNAs (n=5, data are mean ± SEM). *=*P*<0.05, **=*P*<0.01, ***=*P*<0.001

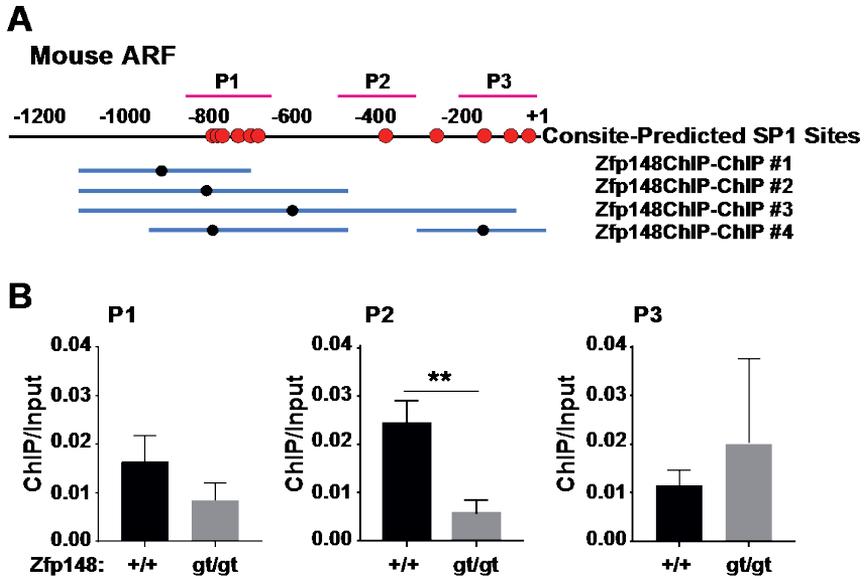


Fig.11. Zfp148 interacts physically with the ARF promoter.

(A) Schematic showing the ARF promoter with CONSITE predicted SP1 motifs (red dots), ChIP-chip binding regions (blue) with peak positions in black, and position of ChIP-qPCR primers (P1-3). (B) Graphs showing quantity of qPCR amplified DNA precipitated from wildtype or Zfp148gt/gt MEFs, as fraction of input (n=5, data is mean \pm SEM). **=P<0.01.

4.3 Paper III Dietary antioxidants accelerate growth of intestinal adenomas in *Apc^{Min/+}* mice.

Antioxidants have been considered to have positive effect on protecting CRC. But, studies shown inconsistent results. Two studies indicated that diet with fluent antioxidants such as selenium, Vitamin C and E is associated with lower risk of CRC [73, 74]. However, clinical trials of supplementary antioxidants in diet did not show significant effect[76]. One potential explanation for those conflict findings could be that antioxidants suppress the tumor initiation, but accelerate the tumor progression. To test this hypothesis, we gave extra antioxidants to the diet of *Apc^{Min/+}* mice. *Apc^{Min/+}* mouse model is a classical Knudson two-hit model; the tumor initiation in this model is driven by the mitotic nondisjunction caused the loss of second APC allele .

Antioxidants have no effect on tumor initiation but accelerated tumor progression.

To investigate the effect of antioxidants on intestinal tumorigenesis, we supplied NAC to the drinking water or Vitamin-E to the chow of *Apc^{MIN/+}* mice (mothers received supplements also during pregnancy) started from 2 weeks before birth until age of 12(**Fig.12.A**). Control mice were given pure water and chow containing recommended daily intake of Vitamin-E. Tumor numbers and size were analyzed under microscope from histological section running through the length of small intestines, the tumors colon was scored during dissection.

At age of 12 weeks, there was no difference in tumor counts between antioxidant treated mice and control mice (**Fig.12.B.C.D**), gender difference was not observed as well. However, the mean tumor areas in both NAC and Vitamin-E treated mice were increased compared to control mice (**Fig.12.E**). These results suggested that antioxidants treatment have no effect on suppressing tumor initiation but accelerate the tumor progression in *Apc^{MIN/+}* mice. Previous studies indicate that tumor initiation in *Apc^{MIN/+}* mice mainly occurs at young age. For addressing the effect of antioxidants on tumor progression, second antioxidants treatment was performed at age of 12 weeks and terminated at 19 weeks. As expected, the tumor counts between antioxidants treated mice and control mice shown no difference, as well as the mean tumor area (**Fig.12.G.H**). However, the

tumor cell proliferation was higher in both NAC and Vitamin-E treated mice compare to control mice (**Fig.12.I**). Therefore, we conclude that antioxidants don't suppress tumor initiation but accelerate tumor progression in *Apc^{MIN/+}* mice.

Antioxidants increased inflammation response in tumors.

To understand the mechanisms of increased cell proliferation in antioxidants treated tumors, total RNA was isolated from tumors harvested from mice at age of 19 weeks. Data from massive parallel sequencing shows that, first, the transcript profiles of NAC and Vitamin-E treated tumors overlapped and both were distinct from controls (**Fig.13.A.B**), which suggests that intestines received antioxidants from supplementary antioxidants in drinking water or chow. Second, we tested genes those were significantly regulated in response to one or two antioxidants treatment for enrichment of *à priori* defined gene set by using hypergeometric distribution and the DAVID software. Data shows an enrichment of inflammatory response genes in both antioxidants treated samples (**Fig.13.C.D**). Together with the fact that inflammation is one of the strongest risk factors for CRC, we assume that the up regulation of inflammatory response genes is responsible for the accelerated tumor progression. However, we still need to do further investigation before the conclusion could be made.

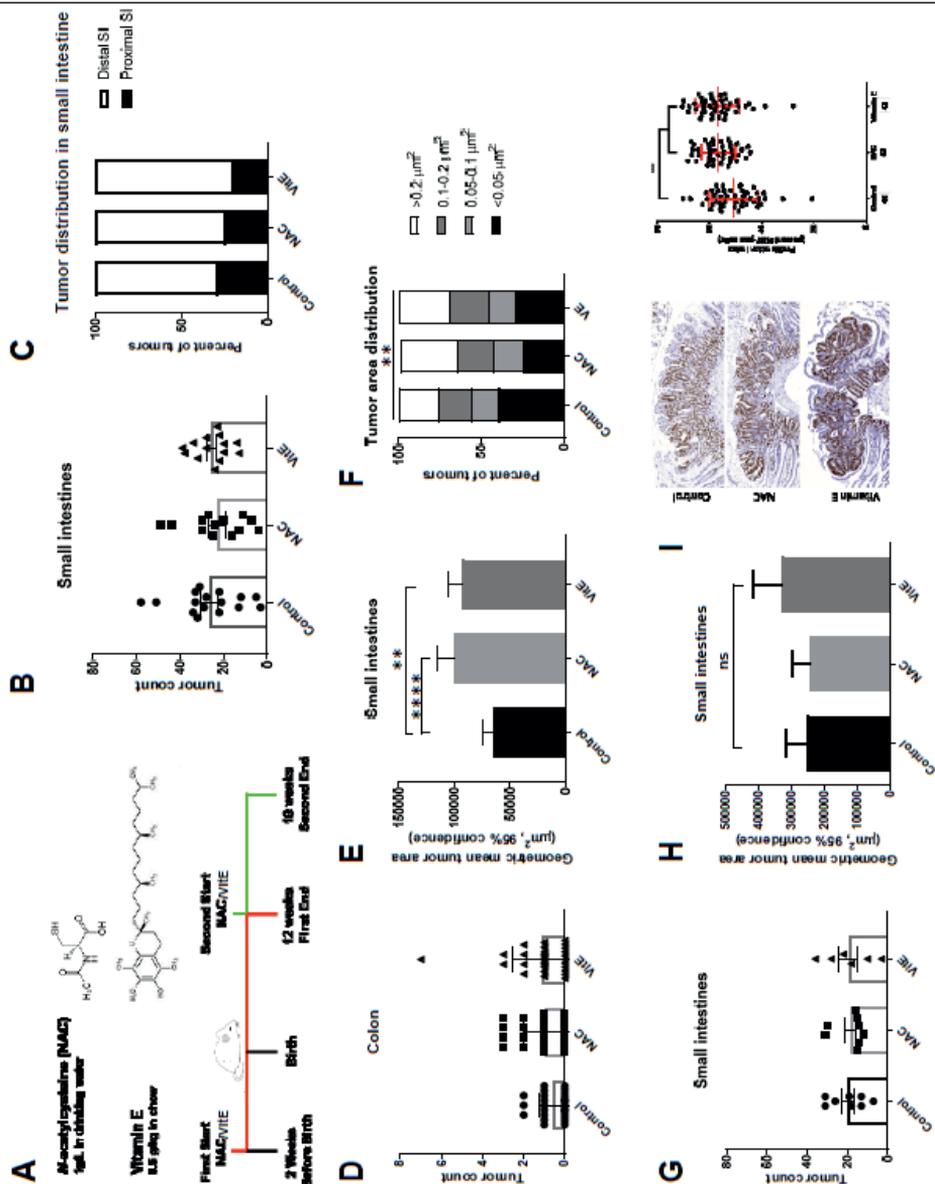
Human CRC cells didn't response to extra antioxidants in the same way as in *Apc^{Min/+}* mice.

To test if the some effect of extra antioxidants could be observed in human colorectal tumors, we exposed human CRC cell lines carry the most common somatic mutations in human CRCs to NAC or Trolox (a water soluble Vitamin-E analogue) and analyzed the cell proliferation. In summary, we did not observe consistent phenotypes in antioxidants treated human CRC cell lines (**Fig.14**). This result suggests that antioxidants accelerate proliferation of tumor cells indirectly.

In paper III, we conclude that dietary antioxidants don't have effect on suppressing tumor initiation but accelerate tumor progression in *Apc^{Min/+}* mice models, and the acceleration could due to the elevated inflammatory response in gut. However, this is not the final answer for understanding the effect of

antioxidants on cancer development. First, the effect of elevated inflammatory response in the increased growth of tumor cells needs to be further investigated. Second, as mentioned above, the tumor initiation in *Apc^{Min/+}* mouse is driven by the driven by the mitotic nondisjunction caused the loss of second APC allele. In contrast, human cancer genomes have more complicated genomic alternations including point mutations, deletions, and insertions than APC mice. Therefore, for achieving the fully understanding of the effect of antioxidants in cancer, investigations on cancer models driven by different types of genetic alternations would be required. Overall, results from paper III suggest the potential risk for diagnosed CRC patients and high risk of CRC population to have supplementary antioxidants in diet.

Fig.12 Supplementation with NAC or Vitamin-E did not affect tumor initiation but accelerated progression of existing tumors. (A) Study design with administration of two structurally unrelated antioxidants, NAC and Vitamin-E, to the drinking water or chow of *Apc^{Min/+}* mice, from 2 weeks before birth to 12 weeks of age (early treatment; panels B-F), or from 12 to 19 weeks of age (late treatment; panels G-I). (B) Tumor count in one sagittal section of the small intestine at 12 weeks of age (n=13 NAC, 14 Vitamin-E, and 18 controls). (C) Tumor count in colon at 12 weeks of age (n=26-32). (D) Distribution of tumors between proximal (P1) and distal (D1) small intestines at 12 weeks of age. (E, F) Geometric mean area (E) and area distribution (F) of small intestine tumors at 12 weeks of age (n=303-357 tumors, error bars indicate 95% confidence interval). (G) Tumor count in one sagittal section of the small intestine of *Apc^{Min/+}* mice treated from 12 to 19 weeks of age (n=8 NAC, 6 Vitamin-E, 8 controls). (H) Geometric mean area of small intestine tumors from



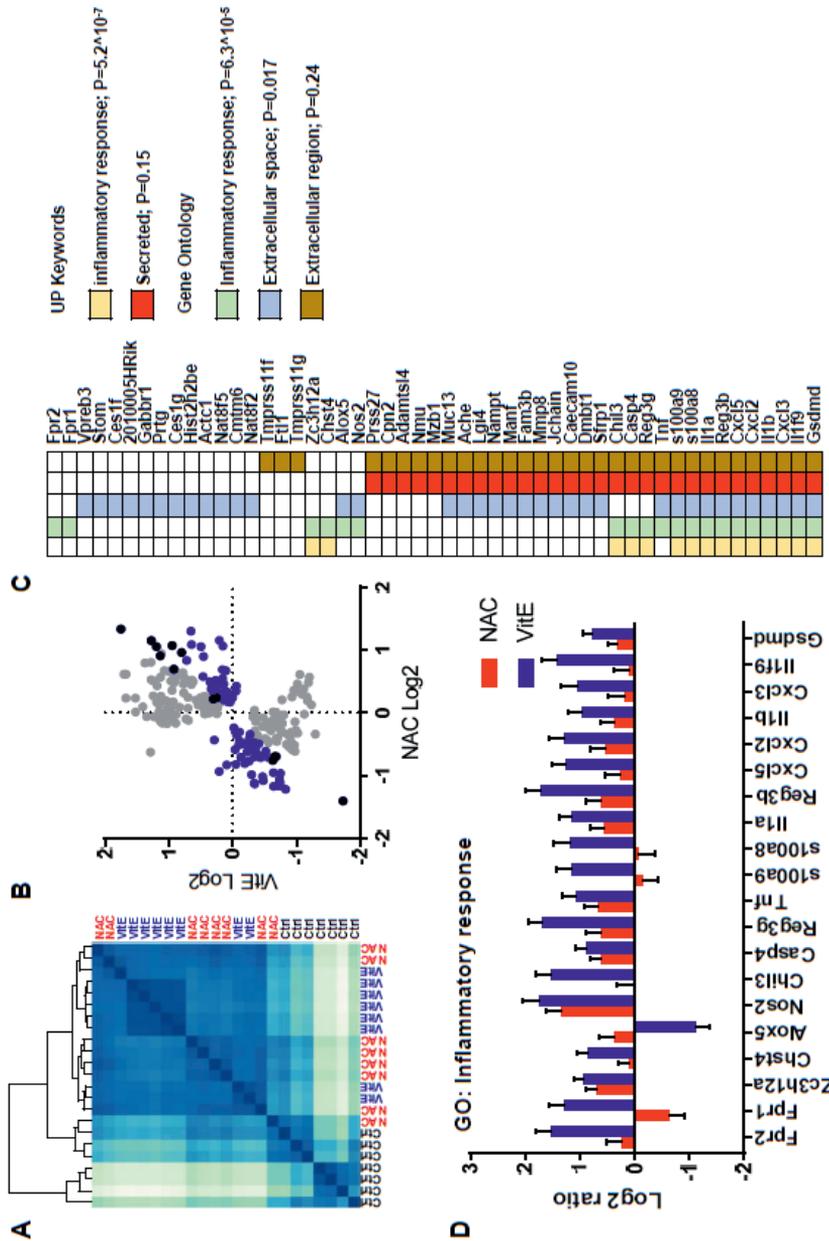


Fig.13. NAC and Vitamin-E increased expression of inflammatory response genes.

(A) Sample to sample distance of transcription profiles of tumors from NAC and Vitamin-E treated mice, or untreated controls, at 19 weeks of age (n=7-8). (B) Ratio-ratio plot showing Log2-expression of genes that were differentially expressed (adjusted p-value <0.05) in NAC (blue) or Vitamin-E (gray) treated tumors, or both (black). (C) Table showing enriched pathways and genes in NAC or Vitamin-E treated tumors. P-values were adjusted for multiple testing. (D) Mean Log2-expression ratios of inflammatory response genes in NAC (red) and Vitamin-E (blue) treated tumors compared to controls (n=7-8).

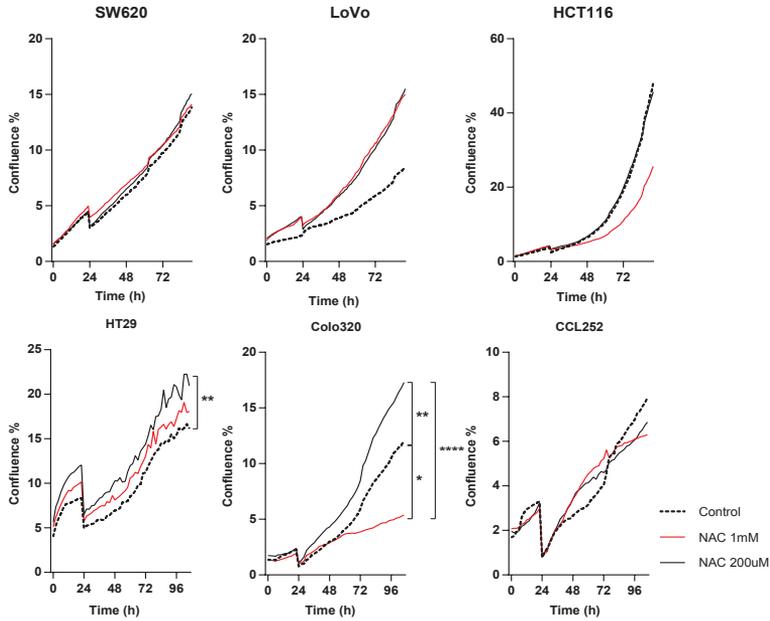
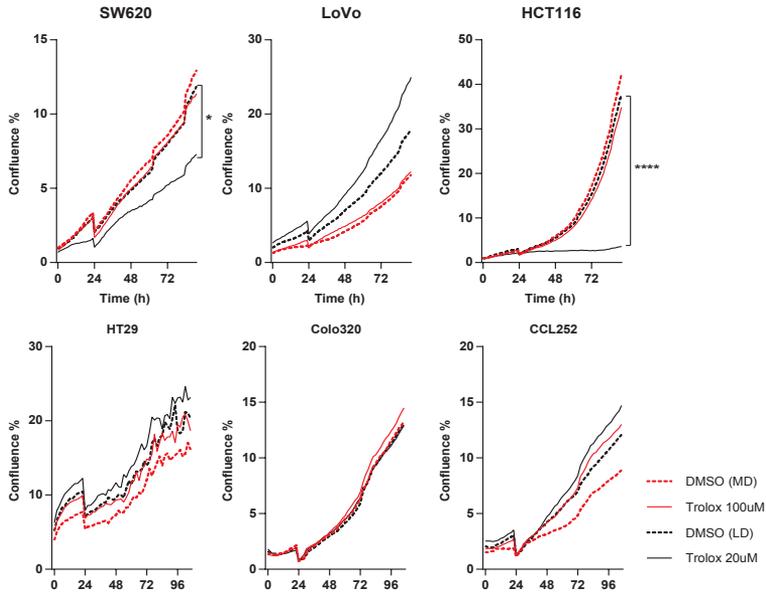
A**B**

Fig.14 NAC and Trolox exhibit inconsistent effects on proliferation of human colon cancer cell lines.

Real-time analyses of proliferation of a panel of six human colon cancer cell lines cultured in medium supplemented with NAC (A) or Trolox (B); data are means of six replicates per cell line.

5 Discussion

In paper I, we found that loss of one copy of *Zfp148* dramatically decreased the tumor frequency, the secondary manifestations of colorectal tumors, and extended the survival of *Apc^{Min/+}* mice. This effect was entirely dependent on functional p53 suggesting that *Zfp148* deficiency retards tumor development by elevating p53 activity. Since there was no difference in cell proliferation or apoptosis between *Zfp148^{gt/+} Apc^{Min/+}* and *Apc^{Min/+}* controls, we hypothesized that *Zfp148* deficiency inhibits tumor initiation by setting a threshold for p53 activation in response to constitutively activated β -catenin. Indeed, we obtained two lines of evidence to support this hypothesis. First, in experiments with GSK3 β inhibition of intestinal explants, we found that levels of total and phosphorylated p53, p53 target genes, and the apoptosis marker cleaved parp-1 were upregulated in explants from *Zfp148^{gt/+} Apc^{Min/+}* mice compared *Apc^{Min/+}* controls. Second, deletion of one or two copies of *Trp53* abolished the effect of *Zfp148* deficiency on tumor development in *Apc^{Min/+}* mice.

Our finding that inactivation of p53 restores tumor growth in *Zfp148^{gt/+} Apc^{Min/+}* mice provides additional evidence for *Zfp148* being a potent inhibitor of p53 activity [61, 70, 71]. In paper II, we hypothesized that *Zfp148* inhibits p53 activity by suppressing *Cdkn2a*. We found that *Zfp148* is dynamically regulated during the cell cycle with peak expression during S-phase, and that *Zfp148* suppresses ARF through binding to its promoter, thus suppressing the p53 pathway and preventing proliferation arrest of MEFs. Although this paper significantly improves our understanding of the mechanism by which *Zfp148* regulates p53 activity in MEFs, the role of ARF for suppressing *APC^{Min}*-driven intestinal polyposis in *Zfp148* deficient mice remains to be investigated.

Investigations of the Wip1 phosphatase provide interesting suggestions: Wip1 is an established negative regulator of the p53 pathway. Since *Wip1* and *Zfp148* knockout mice exhibit a similar phenotype on *Apc^{Min/+}* background, it is possible that *Zfp148* and Wip1 functions in the same pathway: Deletion of *Wip1* suppresses *Apc^{Min}* induced polyposis through increased p53 activity in response to constitutively activated β -catenin [51]. Interestingly, suppression of *Apc^{Min}* induced polyposis in *Wip1* knockout mice requires *Cdkn2a* [95].

From our previous studies, we found that mice lacking of one copy of *Zfp148* were healthy and had a normal life span suggesting that targeting of *Zfp148* could reduce incidence of p53 mediated cancers without side effects. Our finding from paper II provides a new potential strategy of targeting *Zfp148* for the therapy of p53-mediated cancers.

Based on the findings in paper I, we believed that *Apc^{MIN/+}* mice is a proper model for investigating tumor initiation and progression. Therefore, we tested the effect of dietary antioxidants on tumor initiation and progression in *Apc^{MIN/+}* mice. We found that dietary antioxidants did not affect tumor multiplicity in *Apc^{MIN/+}* mice but increased tumor cell proliferation and tumor size. Therefore we conclude that dietary antioxidants do not suppress tumor initiation in *Apc^{MIN/+}* mice, but accelerate the progression of existing ones. Several clinical trials of antioxidant effects in CRC have produced results in line with our finding. One study with 270 thousands participants showed that antioxidant supplements had no significant effect on CRC incidence or colorectal adenoma recurrence [78]. Another recent study showed that elevated total antioxidant capacity increased the risks of rectal cancer but decreased the risk of developing colon cancer [77]. Overall, they didn't find any significant association between dietary antioxidants and CRC incidence.

We observed elevated expression of inflammatory response genes in tumors from mice treated with dietary antioxidants. Since inflammation and expression of inflammatory cytokines are strong risk factors for CRC [3], upregulated inflammatory response genes in antioxidant treated tumors could potentially explain the accelerated tumor progression [3]. Interestingly, this finding is in conflict with some old understandings of antioxidants in inflammatory bowel disease. One study showed that Crohn's disease (one type of inflammatory bowel disease) patients had reduced plasma antioxidants and reduced total intestinal antioxidant capacity [96]. Another study showed that antioxidant nutrient deficiencies can be observed in ulcerative colitis (another type of inflammatory bowel disease [97]) suggesting that oxidative stress is elevated in ulcerative colitis. Therefore oxidative stress is clearly involved in inflammatory bowel disease and antioxidants are considered to have positive effect in the therapy of

inflammatory bowel disease [98]. In addition, no studies have shown that antioxidants are related to cytokine production or immune response in CRC. Our result from paper III brings up the possibility that dietary antioxidants could elevate the inflammatory response in the gastrointestinal tract, but additional investigation is required before this issue will be resolved.

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