Pathophysiological impact of targeting the ROS-p53 axis

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Gothenburg 2014

Cover illustration: “The Great White Void”

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ISBN 978-91-628-9199-2

Printed by Ineko AB, Gothenburg, Sweden 2014

To boldly go where no one has gone before

ABSTRACT

The goal of this PhD thesis was to define the importance of the interplay between reactive oxygen species (ROS) and their activation of the tumor suppressor p53 in development and disease. We addressed this question using molecular biology and biochemical techniques together with mouse genetics and bioinformatics.

We have made two important discoveries:

First, we show that antioxidant supplementation accelerates lung cancer progression in mice and the growth of human lung cancer cell lines. By reducing the levels of ROS and DNA damage, antioxidants deactivate the p53 protein and help cancer cells to evade growth arrest.

Second, we show that the transcription factor zinc finger protein 148 (Zfp148) is a potent suppressor of p53 activation under oxidative conditions. During lung development, suppression of p53 prevents growth arrest of pulmonary cells and permits prenatal lung maturation. However, in the $Apc^{Min/+}$ model of colorectal cancer and in the $Apoe^{-/-}$ model of atherosclerosis, suppression of p53 promotes tumor development and atherosclerosis, respectively. Thus Zfp148 suppression of p53 plays important roles in both physiological and pathological contexts.

We conclude that:

1) Antioxidant supplementation may stimulate the growth and progression of undiagnosed lung tumors and should be used with caution. The risk of developing lung cancer in patients with chronic obstructive pulmonary disease (COPD) who take the antioxidant acetylcysteine to break down mucus should be carefully evaluated.

2) Therapeutic targeting of Zfp148 may have beneficial effects in cancer and atherosclerosis by increasing p53 activity.

Keywords: ROS, p53, Antioxidants, Zfp148, cancer and atherosclerosis

ISBN: 978-91-628-9199-2

Epub: ISBN: 978-91-628-9201-2

SAMMANFATTNING PÅ SVENSKA

Målet med denna doktors avhandling har vart att definiera betydelsen av reaktiva syreföreningar (ROS) och deras samverkan och aktivering av tumörsuppressor p53 i hälsa och ohälsa. Vi adresserar denna fråga med hjälp av molekylärbiologiska och biokemiska tekniker tillsammans med mus genetik och bioinformatik .

Vi har gjort två viktiga upptäckter :

1) Vi visar att antioxidant tillskott accelererar lungcancer progression hos möss och tillväxten av humana lungcancer cellinjer . Genom att sänka nivåerna av ROS och DNA-skada , inaktiverar antioxidanter p53-protein och hjälper cancerceller att kringgå tillväxtstopp.
2) Vi visar att transkriptionsfaktorn zink finger protein 148 (Zfp148) är en potent suppressor av p53 aktivering under oxidativa förhållanden . Vi viasar även att denna suppressor aktivitet är betydelse full för häming av kolorektal cancer och ateroskleros i mus modeller. För mycket av denna suppressor aktivitet leder dock till en lung utvecklings defekt följt av respiratorisk distress i samband med födsel av möss. Således har vi visat att Zfp148´s hämmning av p53 spelar en viktig roll i både fysiologiska och patologiska sammanhang .

Vi drar slutsatserna att :

1) Antioxidant tillskott stimulerar tillväxt och utveckling av framför allt tidiga icke diagnostiserade tumörer. Därför bör antioxidant tillskott användas med försiktighet . Risken att utveckla utvecklings lungcancer hos patienter med kronisk obstruktiv lungsjukdom (KOL) som tar antioxidanten acetylcystein att bryta ner slem bör utvärderas vården noggrant .

2) Terapeutisk hämmning av Zfp148 kan ha gynnsamma effekter på utveckling av cancer och åderförkalkning genom att ökad p53 –aktivitet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Sayin VI, Nilton A, Ibrahim MX, Agren P, Larsson E, Petit MM, Hulten LM, Stahlman M, Johansson BR, Bergo MO and Lindahl P.

Zfp148 deficiency causes lung maturation defects and lethality in newborn mice that are rescued by deletion of p53 or antioxidant treatment

PLoS One **2013** *8(2):e55720*

II. Nilton A*, Sayin VI*, Bondjers C, Agren P, Bergo MO and Lindahl P

*Equal Contribution

Zfp148 deficiency reduces tumor formation in APCMin/+ mice in a p53-dependent manner

In Manuscript

III. Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P and Bergo MO.

Antioxidants accelerate lung cancer progression in mice

Science Translational Medicine **2014** *6(221):221ra15*

IV. Sayin VI, Khan OM, Pehlivanoglu LE, Staffas A, Ibrahim MX, Asplund A, Agren P, Nilton A, Bergström G, Bergo MO, Borén J and Lindahl P.

Loss of one copy of Zfp148 reduces lesional macrophage proliferation and atherosclerosis in mice by activating p53

Circulation Research **2014** *115(9):781-9*

Reprints were made with approval from respective publisher

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ABBREVIATIONS

ApoE	Apolipoprotein E
APC	Adenomatosis polyposis coli
CMR	Chylomicron remnant
COPD	Chronic obstructive pulmonary disease
CRE	Cyclic recombinase
FAP	Familial adenomatous polyposis
Floxed	Flanked by loxP
FAP	Familial adenomatous polyposis
Floxed	Flanked by loxP
GSH	Glutathione
GT	Gene trap
LDLr	Low-density lipoprotein receptor
LSL	Lox stop Lox
MEF	Mouse embryonic fibroblast
Min	Multiple intestinal neoplasia
NAC	N-acetylcystein
Nrf2	Nuclear factor like 2 also known as NFE2L2
ROS	Reactive oxygen species

VLDL	Very low-density lipoprotein
Zfp148	Zinc finger protein 148
WT	Wild-type
mRNA	Messenger Ribonucleic acid
p53	Protein coded by Trp53 in mice and Tp53 in humans
P1	Postnatal day 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nickend labeling

1 INTRODUCTION

In this thesis I will explore the impact of targeting reactive oxygen species (ROS) and their interplay and activation of p53 (hereafter denoted the ROS-p53 axis) in cancer, vascular disease and development. To achieve this we applied two major strategies. Firstly, we knocked out Zinc finger protein 148 (Zfp148) in mice, a transcription factor that interacts with p53. Secondly, we treated cancer prone mice with ROS scavengers, more widely known as antioxidants. In this section, I will introduce and provide a background to the major concepts of this thesis including ROS, antioxidants, P53 and Zfp148 as well as highlight knowledge gaps coupled to these key factors.

1.1 Reactive Oxygen Species

Our present ecosystem is the product of a series of dramatic events. One such event, termed the great oxidation, started around 2.4 billons years ago as cyanobacteria begun to harness the power of photosynthesis. As a byproduct of photosynthesis, our atmosphere became rich in free oxygen (1, 2). This process nearly wiped out all life on earth, since it was previously obligate anaerobic. As a consequence, the great oxidation sparked the evolutionary adaptation of early life to the presence of oxygen. Whether or not high levels of oxygen were required for higher animals to evolve remains debated. Indeed, recent discoveries of multicellular organisms living their whole lives in an oxygen free environment suggest that this might not be the case (3, 4). Nevertheless, the majority of life forms on earth including all mammals are at present totally dependent on oxygen, a highly toxic agent. Once oxygen was harnessed by respiration in the final step of the electron transport chain, aerobic life became able to generate exponentially more energy molecules per nutrient molecule. However, this boost in energy production came with a

price in the form of byproducts of oxygen metabolism called reactive oxygen species (ROS) which are toxic and cause damage to macromolecules including proteins, lipids and DNA (5-8). In order to sustain long life, an adaptive response was evolved. Nuclear factor like 2 (Nrf2 or NFE2L2), the master transcriptional regulator of the endogenous antioxidant response, maintains homeostasis and redox balance in response to oxidative stress (increased ROS levels) within each cell (9). Naturally, an adaptive antioxidant defense system, which is activated by oxidative stress, will always be one step behind. Hence it is not surprising that ROS are implicated not only in cancer and cardiovascular disease, but also in physiological aging (10).

1.2 Antioxidants

Antioxidants are electron donating molecules that neutralize ROS and other free radicals that may otherwise cause oxidative damage to DNA, proteins and lipids and promote cancer and cardiovascular disease (11, 12). Antioxidants are produced endogenously in cells to balance ROS levels. During oxidative stress, Nrf2 translocates to the nucleus and induces the expression of glutathione (GSH) and other antioxidants (13). Essential antioxidants, including vitamins, carotenes and minerals are found naturally in food. Sufficient intake of antioxidants from food is important, and is underscored by the dramatic symptoms of vitamin deficiency (14-20). Additionally, gene targeting experiments of endogenous antioxidants in mice have led to a range of disease phenotypes including cardiovascular disease and increased susceptibility to cancer development (21-23).

Consequently, popular wisdom—supported by numerous cellular and preclinical studies—holds that antioxidant supplements protect against cancer (24-26) and cardiovascular disease (27-31). However, large randomized

clinical trials have produced inconsistent results related to the effect of supplementation with antioxidants on cancer (32-34) and cardiovascular disease (35-38). Importantly, several studies show that antioxidants may even increase the risk of developing cancer (39-42) and all-cause mortality (43). Nevertheless, there is a big discordance between clinical outcomes on antioxidant supplementation and the use of antioxidant supplements in the population. Indeed, over 10% of random populations take antioxidant supplements on a daily basis (44). One explanation for the misuse of antioxidants is the lack of a mechanistic understanding of how antioxidants may accelerate cancer progression.

1.3 p53

Endogenous antioxidants are the first line of defense against cancer; however, a second more crucial line of defense is kept by tumor suppressors. The tumor suppressor p53 is the most frequent mutated gene in cancer (45, 46) and may be the most widely studied gene overall with over 73 000 publications on the topic. P53 induces DNA repair, senescence, or apoptosis in response to a wide range of stressors including oxidative stress (46, 47). Whether p53 activation leads to DNA repair, senescence or apoptosis depends on a complex interplay between co-factors that control p53 stability, activation and transcriptional outcome (48). The E3 ubiquitin ligase Mdm2 negatively regulates p53 by targeting it for degradation. Mdm2 null mice die during early embryogenesis in a p53-dependent manner, which demonstrates that physiological stress alone triggers p53-dependent senescence or apoptosis in the absence of appropriate control mechanisms (49, 50). In a similar fashion, mice lacking Mdm4, a structural homologue of Mdm2 that lacks ubiquitination activity but binds to the N-terminus of p53 and directly represses transcriptional activity, die during embryogenesis in a p53-dependent manner (51, 52). The interaction between p53 and Mdm2 has been conserved across 2.4 billion years of evolution (53, 54), and is traced back to the beginning of multicellular life. Interestingly, the evolutionary timing of p53 and MDM2 interaction happens to correlate with the early phases of the great oxidation, hinting on a possible evolutionary link.

ROS and p53 have a complex and context dependent relationship, dating back to the great oxidation (55). It is well established that increased levels of ROS (oxidative stress) activates p53, and that p53 in turn can increase levels of ROS by enhancing the transcription of pro-apoptotic genes (56, 57). However, p53 can also reduce levels of ROS by transcriptional induction of antioxidant genes and this function may contribute to the tumor suppressor

properties of p53 (25). Moreover, p53 itself undergoes redox regulation due to redox sensitive cysteine residues (58). There are two clusters of cysteine in the DNA binding domain of p53 which are essential to the specific binding of p53 to target genes (59). Furthermore, exposing p53 to oxidants causes Cysteines-124,141 and 182 on p53 to form disulfide bonds with GSH, effectively changing the DNA binding activity of p53 (60, 61). Notably, the change in DNA binding of p53 after oxidation can be reversed by antioxidants (60, 61). Thus, there is a complex crosstalk between ROS and p53.

When cells are exposed to stressors like increased ROS, the most important function of p53 is to regulate expression of downstream target genes. In response to DNA damage, p53 represses the expression of cell cycle–related genes involved in the G2/M phase transition (62). In tumors lacking functional p53, these genes are expressed at high levels and correlate with increased malignancy and poor clinical outcome (63). Thus, repression of cell cycle–related genes is a crucial tumor suppressor function of p53. The mechanisms underlying p53 regulation of cell cycle genes are not fully understood but involve both direct binding of p53 to regulatory elements and indirect interactions with other transcription factors (63).

1.4 Zinc Finger Protein 148

The transcription factor Zinc finger protein 148 (Zfp148) (also known as: ZBP-89, BFCOL, BERF1) contains four krüppel type zinc finger domains and binds to GC-rich DNA sequences (64-67). The protein is predominantly localized in the nucleus and is expressed ubiquitously in tissues of adult mice (65, 67). Zfp148 harbours putative repressor and transactivation domains in the N- and C-terminal regions respectively (66, 67), and is capable of recruiting co-activators and co-repressors to promoter regions (68-71).

Zfp148 has been linked to a number of target genes, but there are no obvious functional connections between them (65-69, 71-78). However, four arguments suggest that Zfp148 plays a role in cell cycle control. First, Zfp148 interacts physically with p53 (79). Second, overexpressing Zfp148 in cancer cell lines increases p53 levels in the nucleus and induces growth arrest or apoptosis (76, 79, 80). Third, Ataxia telangiectasia mutated (ATM), one of the two central regulators of the DNA damage response (81), phosphorylates Zfp148 at the zinc finger domains (82). Moreover, ATM together with Zfp148 and p300 binds to the promoter of the cyclin dependent kinase inhibitor 1a (p21) (69). And finally, silencing of Zfp148 in the NCI-H460 cell line induces senescence through induction of Ink4a (71). Notably, there is discordance between the outcomes in the cell cycle related studies; however, collectively these studies implicate Zfp148 in cell cycle control.

The physical interaction between Zfp148 and p53 suggests a potential role for Zfp148 in tumor suppression beyond cell cycle control (79). Binding studies have shown that the DNA binding zinc finger domains of Zfp148 are required for the binding to p53 (79). Another study shows that mutations in the p53 transactivation domains are dispensable for the interaction between Zfp148 and p53 (83). However, the same study show that hotspot mutations

spanning amino acids 175-281 in the DNA binding domain of p53 abolish the binding between p53 and Zfp148 (83). Furthermore, cells with mutant forms of p53 evade the apoptotic function of Zfp148 (80, 84). Nevertheless, the physiological relevance of the physical interaction between p53 and Zfp148 remains a knowledge gap.

The physiological function of Zfp148 remains unclear. Three gene targeting experiments on Zfp148 in mice have produced inconsistent results (85-87). In the first, Takeuchi et al. showed that Zfp148 heterozygote males suffer from sertoli cell-only syndrome, lacking germline cells, making propagation of the strain impossible. In the second study, Zfp148 deficient mice died during embryogenesis with neural tube defects and anaemia. In the final study, Zfp148 exon 4 knockout mice were generated that showed partial postnatal lethality and dextran sulphate induced colitis. The inconsistency between the gene targeting experiments could be a result of different targeting strategies. Nevertheless, the conflicting gene targeting experiments shows that the physiological role of Zfp148 remains unclear.

"Vision is the art of seeing things invisible." Jonathan Swift

2 AIM

The initial aim of this thesis was to define the impact of *Zfp148* deficiency on development, health and disease.

The specific aims of the four papers included in this thesis were:

I. Zfp148 Deficiency Causes Lung Maturation Defects and Lethality in Newborn Mice That Are Rescued by Deletion of p53 or Antioxidant Treatment

The aim of the first study was to generate, validate and phenotype Zfp148 deficient mice.

II. Zfp148 deficiency reduces tumor formation in $APC^{Min/+}$ mice in a p53-dependent manner

The aim of the second study was to breed the Zfp148 deficient mice on to the $APC^{Min/+}$ model of intestinal cancer to test whether Zfp148 plays a role in colorectal cancer.

III. Antioxidants accelerate lung cancer progression in mice

The aim of the third study was to define the impact and mechanism of antioxidant treatment on lung cancer progression.

IV. Loss of one copy of Zfp148 reduces lesional macrophage proliferation and atherosclerosis in mice by activating p53

The aim of the final paper of this thesis was to define the impact of Zfp148 deficiency on the progression of atherosclerosis.

"Measure what is measurable, and make measurable what is not so." - Galileo

3 EXPERIMENTAL STRATEGIES AND CONSIDERATIONS

In this section I provide a more general description of the genetic strategies and mouse models that are central to the work behind this thesis, why we chose to work with them and discuss their limitations.

Detailed descriptions of methods used in this thesis can be found in the materials and methods section of each enclosed paper.

3.1 The mouse as a model organism

Since experimental research on humans is limited to highly controlled and regulated clinical trials, we depend on model organisms, like the mouse, for gaining insights into mechanisms of health and disease. The most important advantage of using mice as model organisms is their similarity to humans in anatomy, physiology, and genetics. In fact, more than 95% of the mouse genome is similar to our own, making mouse genetic research applicable to human biology and disease (88). Mouse models are cost-effective tools that speed up research and are crucial to the development and validation of targeted drug therapies. Mice are born in large litters, have a small size and a short lifespan, keeping the space, time, and costs required to perform research at reasonable levels.

3.1.1 Transgenic mice

More than 30 years ago the first transgenic mouse was generated. Foreign DNA was introduced into the mouse genome, resulting in expression of the transgene (89-92). Shortly thereafter, through the use of homologous recombination, gain and loss of function experiments on mice became a golden standard for mapping gene function (93-98). The generation of knock-out and knock-in mice is still considered the highest level of evidence when validating gene function.

3.1.2 Modeling human disease in mice

Even though there are striking similarities between men and mice, normal wild-type (WT) mice seldom face our most common diseases, including cancer and cardiovascular disease. Only after generations of back-crossing of laboratory mice into disease prone backgrounds have we been able to generate strains of mice where human disease could be studied (99). The introduction of transgenic techniques revolutionized the field of genetics and opened up for the generation of countless new mouse models of disease (100, 101).

Importantly, a lot of human hereditary or spontaneous genetic disorders manifest in a similar fashion in mice when the underlying genetic events are known and are introduced in the mouse genome (102-105). However, there are limitations. Some conditions are poorly mimicked or overly simplified in mouse models. (105, 106)

3.2 Cre-loxP System

Several of the mouse models used in this thesis utilizes the Cre-loxP System for conditional activation or conditional deletion of target genes, hence an introduction is warranted.

Cyclic recombinase (*Cre*) is an enzyme isolated from the bacteriophage P1 that recognizes and binds to specific *locus of x crossing over* (loxP) sites (107-109). LoxP sites consist of 34 base pair long DNA elements that include two 13 base pair inverted repeats flanking an 8 base pair spacer region. *Cre* cleaves DNA sequences that are flanked by two loxP sites (floxed) oriented in the same direction. The cleaved DNA is excised into a circular loop of DNA(110).

The transgenic introduction of loxP sites into mammalian cells (111) enabled the conditional targeting of genes in a time and cell type dependent manner. The deletion of floxed elements is controlled by the expression of *CRE* recombinase, which is in turn dictated by the experimental setup. The expression of CRE can be controlled in two ways. One way is with the help of exogenous insertion of vectors expressing CRE, like plasmids or virus particles. Another way is with transgenic insertion of CRE behind tissue specific promoters.

In this thesis, expression of *CRE* is (In paper III) *CRE* expression is controlled by adenoviral vectors that are delivered to the lung epithelia of mice through inhalation of a calcium phosphatase precipitate solution (112).

The Cre-loxP system is a powerful tool for conditional gene modifications in animals and has achieved widespread acceptance over the last decade.

However, there are four limitations to consider when interpreting the results from Cre-loxP experiments.

First, endogenous expression of *CRE* is dependent on the promoter region of the inserted transgene which dictates the tissue specificity and temporal control of the *CRE* expression (113). Even though tissue specific promoters are supposed to be fairly specific, depending on the nature of the promoter, it can have leaky expression or just naturally be expressed elsewhere in a developmental stage or context dependent fashion (114-118)

Second, loxP sites have no functionality in mammalian genomes and should therefore not be present. However; a recent genome wide study identified frequent cryptic loxP sites that can promote illegitimate DNA recombination and cause damage in cells and tissues that express *CRE* (119). For example, this is a problem in heart tissue where *CRE* expression alone causes fibrosis (120) and dilated cardiomyopathy (121).

The third potential problem with the Cre-loxP approach is partial recombination, i.e. that some floxed alleles are left undeleted. This becomes evident when more than one floxed allele is targeted for simultaneous deletion, especially if the *CRE* expression is transient (122, 123).

And finally, on rare occasions, the targeted gene may still be expressed from the excided circular DNA fragment. In high proliferative tissues like intestinal epithelium and tumor cells the circular DNA fragment would be diluted over time. However, in non-dividing cells like neurons the presence of this extra chromosomal DNA fragment has been a problem. (124)

3.3 Zfp148 deficient mice

The generation and validation of Zfp148 deficient mouse is well described (paper I) and will be further described in the Results section. The Zfp148 deficient mice were generated with the help of gene-trapping technique. The gene-trap (gt) vector is designed as a false exon with a splice acceptor and a transcriptional stop. Integrating the gt vector between exon 4 and 5 in *Zfp148* renders the major part of the translated region (exon 4 to 9) un-transcribed (exon 5 to 9). Instead a fusion protein containing exon 4 and the gt vecor is obtained containing a reporter element (bgal) and a neomycin resistance element. Of note, the Zfp148 gt allele is not a null allele but rather a hypomorph. The transcriptional machinery occasionally makes mistakes which results in a small degree of leakage of WT transcripts.

3.4 Mouse models of cancer

Cancer is a complex genetic disease, where gain of function mutations of oncogenes and loss of function mutations of tumor suppressor genes result in the transformation of a normal cell into a malignant proliferative tissue, a tumor. The tumor is like an overhealing wound that eventually evolves in to a metastasizing disease. If left unchecked or unsuccessfully treated, the cancer leads to major organ failure and subsequent death (125). In Sweden the number of deaths from cancer related causes was 22 904 in 2012 and in the United States, 585 720 cancer deaths are projected in 2014 (126).

Mouse models have contributed significantly to our understanding of the origin, pathogenesis and biology of cancer. The most common mutations found in tumors from human patients, when introduced into the mouse, indeed led to initiation and progression of cancer. However, the genetically altered mouse models are challenged with being overly simplified, lacking passenger mutations and having a less complex mutational landscape, in some aspects casting doubt over the translational potential between cancer in genetic mouse models and patients. (127-129)

3.4.1 p53 knockout mouse

There are numerous p53-deficient or mutant mouse models. The p53 knockout model used in this thesis was generated in Tyler Jacks laboratory (130). All mice on a p53 knockout background succumb to spontaneous tumors and become moribund on average around 20 weeks of age. The tumor spectrum consists predominantly of lymphomas (>70%), but sarcomas and carcinomas are also common (130).

More relevant for human disease, mice heterozygous for p53 totally recapitulate the Li-Fraumeni syndrome, a hereditary disease where the patients are prone to develop cancers (sarcomas in particular). Similar to the p53 heterozygote mice, Li-Fraumeni patients lack one functional allele of p53 (98, 131, 132). The p53 heterozygote mice are prone to develop cancers (sarcomas in particular) with an average onset of disease around the age of 60 weeks (130, 133, 134). These mice are also more sensitive and prone to develop carcinogen and radiation induced tumors (135-139).

In papers I, II and IV the p53 knockout allele is used for genetic interaction studies, to test if our conclusions that the downstream effects of losing *Zfp148* are dependent on p53 activation. Notably, we don't allow them to age to the point where they develop spontaneous tumors.

3.4.2 *APC*$^{min/+}$ mouse model

The *Apc*$^{min/+}$ mouse used in paper II is an intestinal cancer model generated through a forward genetic screen with an inactivating point mutation in the tumor suppressor gene *APC* (adenomatosis polyposis coli) (140, 141). It was found that the multiple intestinal neoplasia (min) phenotype in these mice co-segregated with a mutation in the *Apc* gene, which had been found mutated in patients with colorectal cancer and in familial adenomatous polyposis (FAP) (142). Studies on the *Apc*$^{min/+}$ model stand as part of the foundation to the Vogelstein model (143), depicting cancer as a disease with linear progression that evolves and becomes progressively more invasive as clones with new mutations emerge within the tumor. Briefly, a transformed cell grows to become a small neoplasia, evolving into an adenoma, which mutates into an adenocarcinoma and finally becomes an invasive metastatic cancer. In this model, loss of the gene APC is an initiating event, mutation of RAS an early event, and loss of p53 a later event (143, 144) (Figure 1).

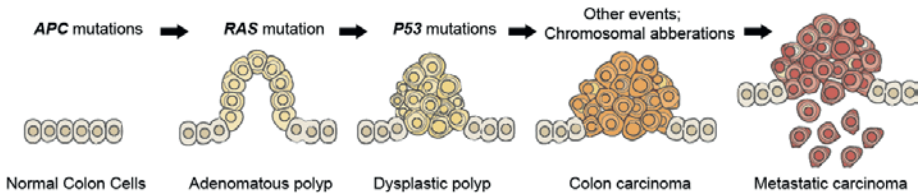


***Figure 1**. Vogelstein model depicting linear progression in colorectal cancer*

One distinct difference between humans and mice concerning the role of *Apc* is that humans with mutant *APC* primarily develop tumors in the colon whereas mice develop tumors in the small intestine.(145)

3.4.3 *Kras*$^{LSL\text{-}G12D}$ mice

The *Kras*$^{LSL\text{-}G12D}$ mouse was generated by the Tyler Jacks laboratory. This mouse carries a latent point-mutant allele, G12D, immediately preceded by a LoxP flanked STOP cassette resulting in a null mutation that renders the mouse heterozygous for *Kras*. Cre-mediated recombination leads to deletion of the lox-stop-lox (LSL) sequence and expression of the constituently active oncogenic protein K-Ras^{G12D}. In lung epithelial cells the expression of K-Ras^{G12D} leads to transformation and initiation of lung tumorigenesis (146). The transformed lung cells proliferate and progress to atypical adenomatous hyperplasia (AAH), which progress to adenomas that in turn progress to invasive adenocarcinomas (147). *CRE*-mediated recombination of *Kras*$^{LSL\text{-}G12D}$ in cells *in vitro* leads to increased proliferation and loss of contact inhibition, which are hallmarks of transformation and cancer (148-150).

3.4.4 *Braf*$^{CA\text{-}V600E}$ mice

The *Braf*$^{CA\text{-}V600E}$ mouse, generated in the Martin McMahon laboratory, carries, similar to the *Kras*$^{LSL\text{-}G12D}$ mouse, a latent point mutation that after *CRE* mediated recombination leads to expression of oncogenic B-Raf^{V600E}. In contrast to the *Kras*$^{LSL\text{-}G12D}$ allele the *Braf*$^{CA\text{-}V600E}$ allele expresses WT B-Raf levels before *CRE* recombinase mediated conditional activation. Expression of oncogenic B-Raf^{V600E} leads to similar transformation of lung cells *in vivo* and cells *in vitro* as expression of K-Ras^{G12D}. However, one important difference in the progression of disease is that B-Raf^{V600E} induced lung tumors do not progress to adenocarcinomas, due to oncogene induced senescence (151).

3.4.5 *p53*$^{flox/flox}$ mice

This mouse carries a conditional knockout allele for p53 and was generated by the Tyler Jacks laboratory (152). The conditional allele makes it possible with the help of the Cre-loxP system to knock out p53 in a temporal and spatial manner. We have combined this allele with the Braf and the Kras oncogenic alleles to generate *p53*$^{flox/flox}$*;Kras*$^{LSL\text{-}G12D}$ and *p53*$^{flox/flox}$*; Braf*$^{CA\text{-}V600E}$ mice. These mice combine constitutively activating mutations in Kras or Braf with the loss of two alleles of p53 after inhalation of *CRE* adenovirus, which result in a more aggressive lung tumor model that eventually develops invasive metastasis (153). We used these four lung cancer models described above in paper III.

3.5 Mouse models of atherosclerosis

Atherosclerosis is a complex disease of the vascular wall where cellular changes progress for decades before they manifest in acute cardiovascular events, such as stroke or acute myocardial infarction. Defining the pathogenesis and mechanisms of atherosclerosis in humans is challenging for several reasons including the complexity of the disease, the slow progression in each individual patient, and the lack of noninvasive detection techniques. Hence, we depend on animal models of atherosclerosis to define mechanistic pathways. Over the last two decades mouse models have come to dominate the field of experimental atherosclerosis, providing a tool to uncover the pathogenesis and molecular mechanisms of atherosclerosis.

3.5.1 C57BL/6 strain

As a species the mouse is highly resistant to atherosclerosis. Therefore, normal WT mice, irrespective of diet, do not spontaneously develop atherosclerosis. However, some inbred strains, especially the C57BL/6 mice, can develop atherosclerotic lesions when fed a high-fat and cholesterol rich diet that promotes hyperlipidemia (154). Nevertheless, the atherosclerotic lesions in C57BL/6 mice remain small and confined to the aortic roots and do not progress beyond the earliest phases of the disease, even after prolonged periods on high-fat diet and cholesterol rich diet (155, 156).

3.5.2 Apolipoprotein-E and Low-density lipoprotein receptor knockout mice

To better recapitulate human atherosclerotic development, several mouse models targeting lipoprotein metabolism have been backcrossed into the C57BL/6 genetic background (157). The most well established and widely

used models are the Apolipoprotein E (Apoe E) knockout model (158, 159) and the low-density lipoprotein receptor (LDLr) knockout model (160). In this thesis we choose to work with the ApoE knockout mice on a C57BL/6 N background, even though the LDLr knockout mice has a more human like lipoprotein profile. The ApoE knockout mouse is a more flexible model that progresses faster and also has an inflammatory component (161).

ApoE knockout mice develop spontaneous hypercholesterolemia which subsequently leads to the development of atherosclerosis independent of diet (159). Atherosclerotic lesions are formed throughout the whole aorta but are most profound in the aortic root and the aortic arch. The progression of atherosclerosis in the ApoE knockout mice can be accelerated with the use of high-fat and cholesterol rich diet. The use of high-fat diet shortens the time to develop advanced lesions to around 20 weeks, in contrast to a year on normal chow diet. In ApoE knockout mice, plasma cholesterol is packaged in chylomicron remnant (CMR) particles and very low-density lipoproteins (VLDL), in contrast to LDLr knockout mice and humans in which the cholesterol is packaged in LDL particles (156). However, there are humans with familial ApoE deficiency (162), and their lipid profiles are similar to the profile in ApoE knockout mice.

3.5.3 Bone-marrow transplantations

We used bone-marrow repopulation experiments in paper IV to narrow down the effector cells that caused the atherosclerosis phenotype in *Zfp148* $^{gt/+}$ mice on ApoE knockout background (paper IV). Lethal irradiation followed by bone-marrow transplantation leads to a repopulation of the host (recipient) hematopoietic stem cell pool from the graft (donor) (163). If the transplanted bone marrow cells are from a transgenic background and the recipient is a WT, the recipient after successful engraftment will be a WT mouse with a

transgenic bone-marrow with all the hematopoietic derived cells including macrophages and t-cells becoming transgenic. Bone marrow transplantation is a widely used method to study the role of hematopoietic cells in atherosclerosis. (164)

3.6 Ethical considerations

All animal experiments were approved by the Research Animal Ethics Committee in Gothenburg. The principle of 3R, reduce, replace & refine has been part of the experimental planning.

"Nothing is particularly hard if you divide it into small jobs." –Henry Ford

4 RESULTS & DISCUSSION

In this section I will summarize and discuss some of the key findings in each enclosed paper.

Paper I: Zfp148 Deficiency Causes Lung Maturation Defects and Lethality in Newborn Mice That Are Rescued by Deletion of p53 or Antioxidant Treatment

Our original rationale for knocking out Zfp148 was based on a bioinformatics screen for new transcriptional regulators of the vascular wall (165). However, the biology that unfolded took us elsewhere into cell cycle control, tumor suppression and oxidative stress.

Generation and validation of Zfp148 deficient mice

Zfp148-deficient mice were generated from a gene-trap (gt) ES-cell clone. Analysis confirmed that the gt vector was incorporated between exon 4 and 5 of *Zfp148*, thereby disrupting nearly 90% of the coding sequence including all four zinc finger domains. ES-cells were injected into C57Bl/6 blastocysts to achieve germline transmission of the $Zfp148^{gt}$-allele. Analysis of mRNA levels from tissues of $Zfp148^{gt/gt}$ mice revealed that $Zfp148^{gt/gt}$ mice are hypomorphic, even though the protein is undetectable on western blots. Importantly, we have now established viable *Zfp148*-deficient mice, opening up for studies on the *in vivo* and cellular importance of endogenous Zfp148.

$Zfp148^{gt/gt}$ Mouse embryo fibroblasts (MEF) exhibited increased sensitivity to oxidative stress and slower proliferation at early passages and subsequently senesced prematurely. The sensitivity of $Zfp148^{gt/gt}$ MEFs to oxidative stress was coupled to increased activation of p53 independent of DNA damage. Importantly, reducing oxidative stress by the addition of the

antioxidant n-acetylcystein (NAC) or reducing oxygen pressure to physiological levels (3%) prevented the *Zfp148*$^{gt/gt}$ MEFs from entering premature senescence and reduced the activation of p53. Furthermore, the premature senescence did not manifest in *Zfp148*$^{gt/gt}$ MEFs if one or two alleles of p53 were deleted. As an important control, adding back Zfp148 exogenously with viral vectors to *Zfp148*$^{gt/gt}$ MEF made them escape premature senescence, confirming that phenotypes observed in *Zfp148*$^{gt/gt}$ MEFs and mice are not caused by off-target effects of the initial gene targeting approach **(Figure 2)**.

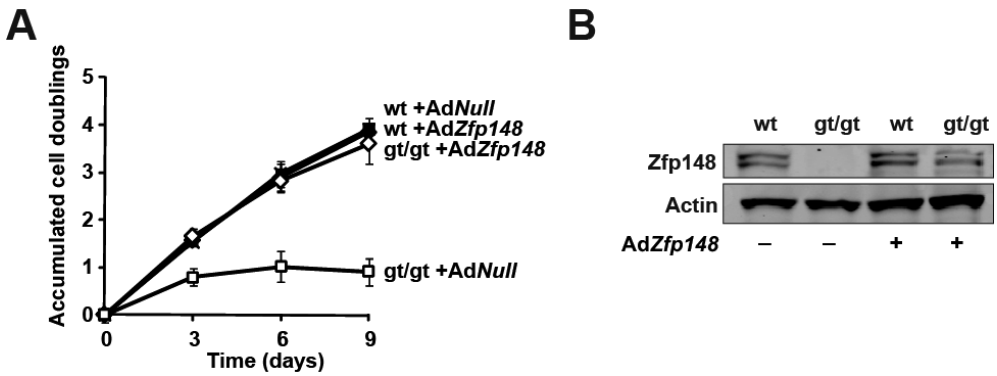


***Figure 2**. Ad back of Zfp148 rescues Zfp148 deficient MEF cells from premature senescence.*

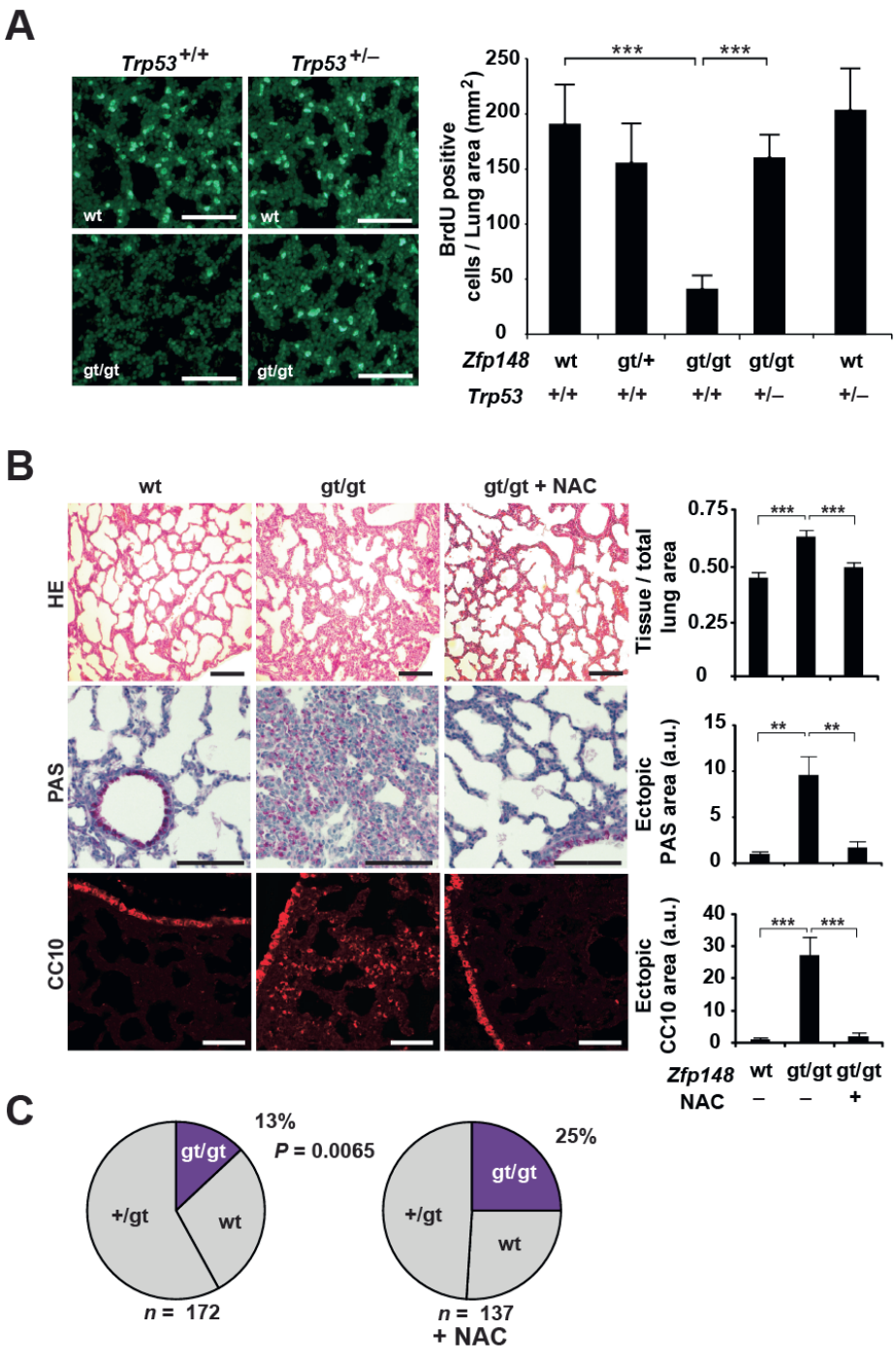
(**A**) MEF cell growth curve. (**B**) Protein levels of Zfp148 in MEF cells from (**A**).

Zfp148$^{gt/gt}$ mice that were conceived through heterozygous intercrosses, develop normally and are found according to the expected Mendelian ratio throughout development. However, the observed ratio was halved postnatal day1 (P1), indicating that half of the *Zfp148*$^{gt/gt}$ mice died shortly after birth.

A closer examination of the P1 *Zfp148*$^{gt/gt}$ mouse revealed a lung maturation defect, suggesting that *Zfp148*$^{gt/gt}$ mice at P1 die of respiratory distress. A closer phenotypic analysis of the embryonic and P1 lungs of *Zfp148*$^{gt/gt}$ mice showed that Zfp148-deficency disrupts cell proliferation at the saccular stage of lung development. Strikingly, loss of one allele of p53 in *Zfp148*$^{gt/gt}$ mice restored cell proliferation in the developing lung and prevented respiratory distress and neonatal lethality. Furthermore, NAC treatment of pregnant females also prevented respiratory distress and neonatal lethality in *Zfp148*$^{gt/gt}$ mice, strongly suggesting that the phenotype is triggered by oxidative stress **(Figure 3)**.

Figure 3. *Deletion of one copy of p53 or antioxidant treatment rescue lung maturation defect in Zfp148-deficient mice.*

*(***A**) Proliferation defect in E.19.5 lungs is restored in *Zfp148*-deficent mice after loss of p53. (**B, C**) Lung maturation defects (**B**) and postnatal day 1 lethality (**C**) is prevented by antioxidant treatment in *Zfp148*$^{gt/gt}$ mice.


A
Trp53+/+
Trp53+/–
wt
wt
gt/gt
gt/gt
BrdU positive cells / Lung area (mm2)
250
200
150
100
50
0
***
***
Zfp148 wt gt/+ gt/gt gt/gt wt
Trp53 +/+ +/+ +/+ +/– +/–
B
wt
gt/gt
gt/gt + NAC
HE
PAS
CC10
Tissue / total lung area
0.75
0.50
0.25
0
Ectopic PAS area (a.u.)
15
10
5
0
**
**
Ectopic CC10 area (a.u.)
40
30
20
10
0
Zfp148 wt gt/gt gt/gt
NAC – – +
C
13%
gt/gt
+/gt
wt
n = 172
P = 0.0065
25%
gt/gt
+/gt
wt
n = 137
+ NAC

The results from paper I clear up some of the discrepancies that emerged from earlier gene-targeting attempts of Zfp148. Takeuchi et al suggested that *Zfp148* heterozygous mice developed a phenotype similar to the sertoli only-cell syndrome (87). However, this phenotype was not reproduced in our study or in any of the three other studies that inactive Zfp148 in mice (not counting conditional inactivation in specific tissues) (85, 86, 166). Moreover, there has been no follow up since the original publication dating 11 years back (87). Altogether, this suggests that the phenotype observed by Takeuchi et al. was not caused by deletion of *Zfp148*.

Another publication is in direct conflict with our findings. Woo et al. showed that *Zfp148*-deficiency caused death at embryonic day 8.5-10.5 with unclosed neural tubes and anemia (86). Interestingly, these *Zfp148*-deficient mice were generated by the same gene-trap ES-cell clone as we used to generate our *Zfp148*-deficient mice. Importantly, we observed the same phenotype (unclosed neural tubes and anemia) in embryos of the first generation (F1) intercrosses. However, we showed that this phenotype was caused by a second gene-trap that was inserted in another locus. After the second gene-trap was bred out in subsequent intercrosses, the embryonic lethal phenotype with unclosed neural tubes in $Zfp148^{gt/gt}$ mice disappeared. Thus, the early embryonic death was not caused by Zfp148 deficiency (Figure 4).

A

Litter #	Embryo #	Phenotype	LacZ PCR	X-Gal staining	Zfp148^{wt} PCR
A	1	-	+	++	-
A	2	-	+	++	-
A	3	-	+	+	+
A	4	-	-	-	+
A	5	-	+	+	+
B	6	-	+	-	+
B	7	-	+	-	+
B	8	-	-	-	+
B	9	pale	-	-	+
B	10	unclosed neural tube, ectoderm defect, somite defect, unturned	+	N.D.	+
B	11	-	+	-	+
B	12	-	+	-	+
B	13	resorbed	N.D.	N.D.	N.D.
C	14	dead, very small, pale	+	+	+
C	15	-	+	+	+
C	16	-	+	+	+
C	17	abnormal, posterior defect, pericardial edema	+	N.D.	+
C	18	unclosed neural tube	+	+	+
C	19	-	+	+	+
C	20	-	+	-	+
C	21	unclosed neural tube, somite defect, posterior defect, pericardial edema	+	-	+

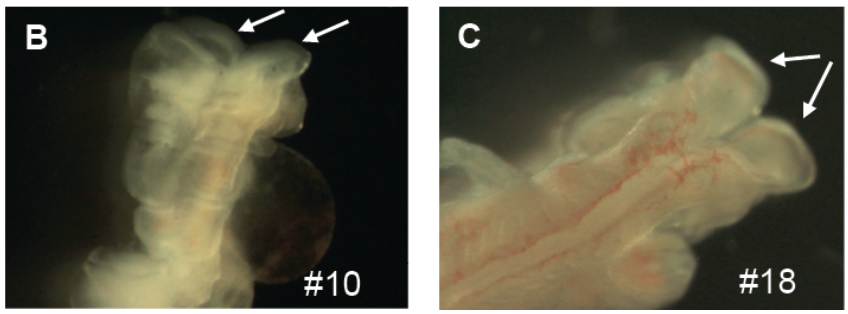


***Figure 4**. Integration of second gene-trap caused unclosed neural tubes and lethality in embryos from the F1 generation*

(A) Dissection of 21 E9.5 embryos of F1 generation intercrosses identified a proportion of embryos with unclosed neural tubes. Importantly, these mice had at least one intact Zfp148 allele. Moreover, the gene-trap vector was propagated to 79% of the brown offspring (F1 mice) of crosses between chimeric mice and C57Bl/6 mice, which is consistent with the presence of two gene-trap alleles in the injected ES-cells. (**B**, **C**) Dorsal view of E9.5 embryos exhibiting unclosed neural tubes (arrows).

Paper II: *Zfp148* deficiency reduces tumor formation in $Apc^{Min/+}$ mice in a p53-dependent manner

In paper II, we show that *Zfp148*-deficiency reduces tumor formation in $Apc^{Min/+}$ mice. We hypothesized that *Zfp148*-deficiency would increase p53 activation and thus reduce intestinal tumor development. Indeed, the loss of either one or two alleles of *Zfp148* reduced the numbers of tumors in $Apc^{Min/+}$ mice. Additionally, *Zfp148*-deficient mice survived markedly longer in $Apc^{Min/+}$ background compared to $Apc^{Min/+}$ controls **(Figure 5)**.

Mechanistically, we were able to show that the reduced tumor formation in $Zfp148^{gt/+}$ $Apc^{Min/+}$ mice depended on p53. However, we did not observe any differences in proliferation or apoptosis in tumors from $Zfp148^{gt/+}$ $Apc^{Min/+}$ mice compared to $Apc^{Min/+}$ controls, suggesting that the tumor protective effect of losing *Zfp148* occurs during tumor initiation and not during tumor progression. In support of this argument, tumors from $Zfp148^{gt/+}$; $ApcMin^{/+}$mice were equally invasive and showed similar histopathological grade at the moribund stage as tumors from $Apc^{Min/+}$ controls.

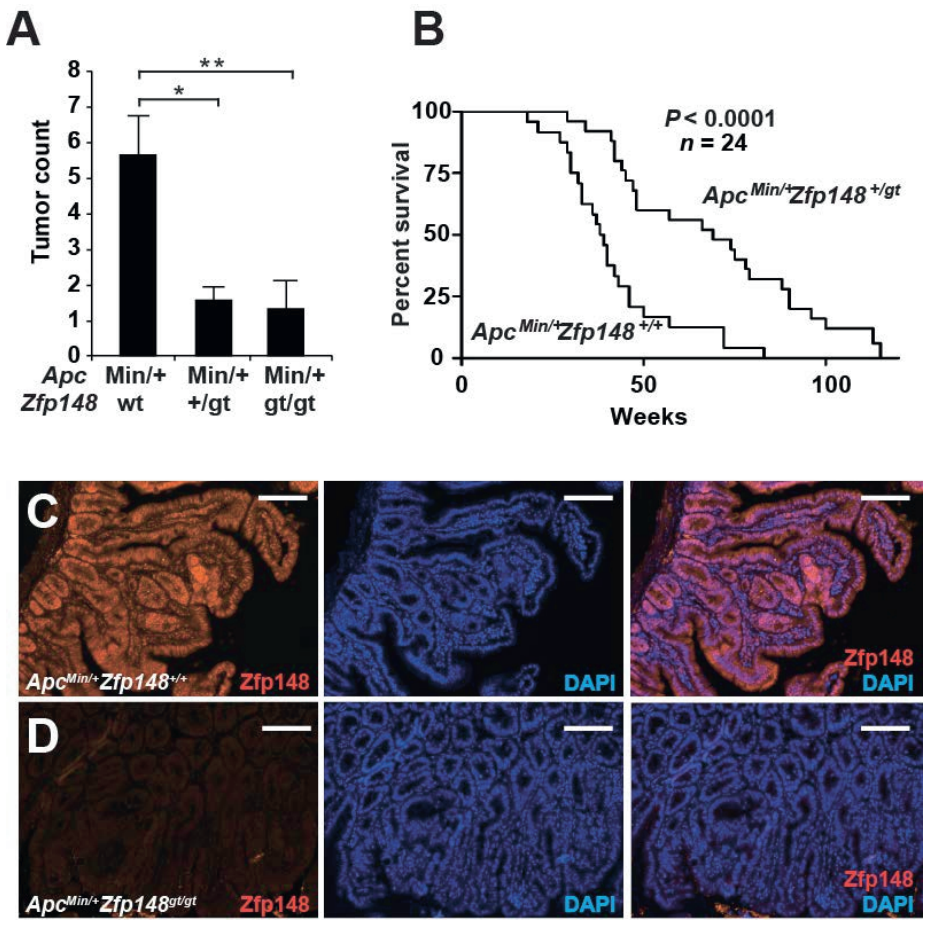


Figure 5*. : *Zfp148 deficiency reduces tumor formation in $Apc^{Min/+}$ mice

(A) Tumor counts (**B**) Two year survival study. (**C**, **D**) Intestinal tumor stained for Zfp148 from (**C**) $Zfp148^{+/+}$ $Apc^{Min/+}$ mice and (**D**) $Zfp148^{gt/gt}$ $Apc^{Min/+}$ mice

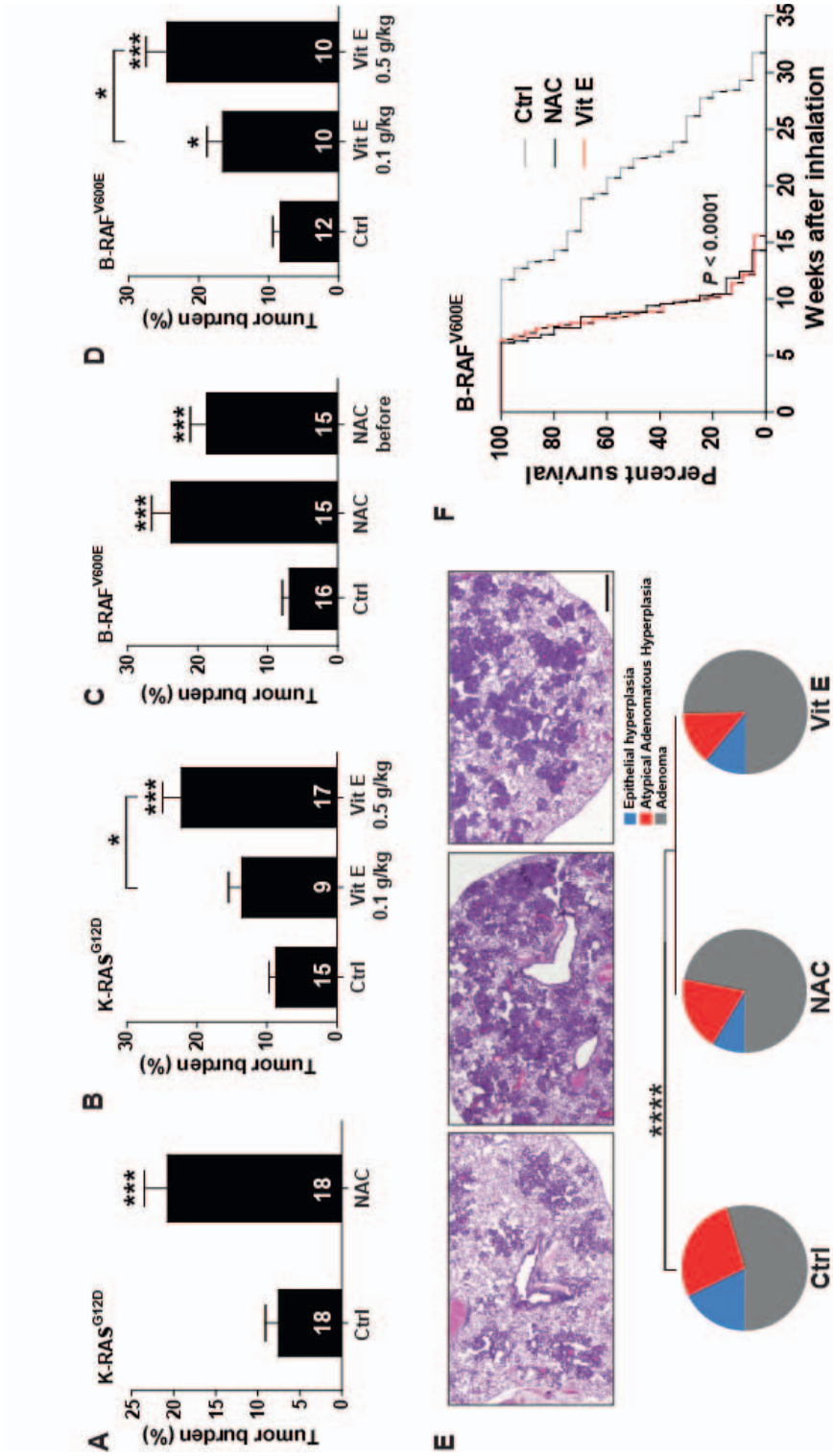
Paper III: Antioxidants accelerate lung cancer progression in mice

This study was initiated serendipitously. In a natural continuation of Paper I, where the role of Zfp148 was studied in lung cancer progression, the antioxidant NAC was given with the hope that it might lower tumor progression or rather have no effect. To our great surprise we saw an acceleration of tumor progression.

We show that, supplementing the diet with the antioxidants NAC and vitamin E markedly increases tumor progression and reduces survival in mouse models of B-RAF– and K-RAS–induced lung cancer (Figure 7). Moreover, the increase in tumor burden after antioxidant treatment was dose dependent (Figure 7). Extensive mechanistic analysis revealed that antioxidants reduced levels of ROS, DNA damage and p53 activation in both mouse and human tumor cells resulting in increased tumor cell proliferation and progression of disease. Additionally, the presence of WT p53 was required for the pro-tumorigenic effect of antioxidants. Because p53 inactivation occurs late in tumor progression, antioxidants may accelerate the growth of early tumors or precancerous lesions.

Figure 7. **:** *Antioxidants accelerate lung cancer progression in mice*

NAC and vitamin E increase tumor growth (**A**–**E**; size, number, and stage) and reduce survival (**F**) in mice with K-RAS^{G12D}- and B-RAF^{V600E}-induced lung cancer


A
K-RAS^{G12D}
Tumor burden (%)
Ctrl
NAC
B
K-RAS^{G12D}
Tumor burden (%)
Ctrl
Vit E 0.1 g/kg
Vit E 0.5 g/kg
C
B-RAF^{V600E}
Tumor burden (%)
Ctrl
NAC
NAC before
D
B-RAF^{V600E}
Tumor burden (%)
Ctrl
Vit E 0.1 g/kg
Vit E 0.5 g/kg
E
Epithelial hyperplasia
Atypical Adenomatous Hyperplasia
Adenoma
Ctrl
NAC
Vit E
F
B-RAF^{V600E}
Percent survival
Weeks after inhalation
Ctrl
NAC
Vit E
P < 0.0001

One important question is if the doses administered to the mice are relevant for humans. It is well established that extrapolation of doses used in mice to a human equivalent dose by a simple conversion based on body weight is not accurate (167). We calculated the appropriate NAC dose for our mouse study with the help of body surface area conversion (167) so it would match prescription to chronic obstructive pulmonary disease (COPD) patients. For our vitamin E studies, we adjusted the dose in our mice studies to match the relative fold increase found in vitamin supplements compared to recommended daily intake (RDI). The standard vitamin E supplement exceeds RDI 20 times and can range up to 80 times RDI. Our vitamin E doses were adjusted to be 5 (low dose) and 50 (high dose) times higher than RDI for mice found in normal laboratory mouse chow. Hence, doses used in our study are relevant in a human context.

Another important question is whether the effects of NAC and vitamin E on lung cancer progression can be generalized to other antioxidants? NAC and Vitamin E have distinct molecular properties. Vitamin E is fat-soluble, regulates enzymatic activities, and is used as a dietary supplement, whereas NAC is water-soluble, participates in glutathione metabolism, and is used as a mucolytic agent (168, 169). Despite distinct molecular properties, unbiased whole-transcriptome sequencing of K-RAS^{G12D} tumors revealed that the transcriptional changes induced by NAC and vitamin E were highly coordinated, indicating that they have a common mechanism of action. Moreover, the mechanism we propose is coupled to the drugs antioxidant properties. By lowering the levels of ROS and DNA damage, the extra antioxidants help cancer cells evade p53 activation. Collectively, these arguments indicate that antioxidants in general accelerate lung cancer progression.

Recent genomic analyses of cancers show a high frequency of gain of function mutations in the antioxidant master regulator Nrf2 (170-173), suggesting that decreasing levels of ROS promotes tumor growth. Consistent with this notion, targeting Nrf2 signaling and subsequently lowering endogenous antioxidants inhibits tumor progression (174-176). Furthermore, a recently published study shows that overexpression of Peroxiredoxin 6, an endogenous antioxidant enzyme, accelerates the progression of pre-existing skin tumors through reduction of ROS (177). In addition to confirming our data, this study shows that the pro-tumorigenic effects of antioxidants are not confined to lung tumors. Collectively, these studies are in line with our, showing that tumors thrive in the presence of increased antioxidants.

Antioxidants accelerate tumor progression. Our findings from Paper III published earlier this year have sparked a worldwide debate among scientists and physicians (178-182) about whether it can still be claimed that there is no harm in use of antioxidant supplements. A recent review in the New England Journal of Medicine (183) translates our findings to the clinical setting, highlighting it to primary care physicians.

Collectively, our data suggests that antioxidants may stimulate the growth and progression of undiagnosed lung tumors and should be used with caution, especially in individuals with increased risk of developing lung cancer including smokers and COPD patients.

Paper IV: Loss of one copy of Zfp148 reduces lesional macrophage proliferation and atherosclerosis in mice by activating p53

It has been previously established that loss of p53 and other tumor suppressors accelerates the progression of atherosclerosis (184-186). However, whether increased activation of p53 can protect against atherosclerosis remains unknown. Here we test the hypothesis that targeting Zfp148 would reduce the progression of atherosclerosis in mice by activating p53.

We show that loss of one copy of *Zfp148* reduces atherosclerosis in *ApoE*-deficient mice independent of diet and without any apparent effects on lipid metabolism. With bone marrow transplantation experiments, we could pin down the effector cell responsible for reduced atherosclerosis in $Zfp148^{gt/+}$ $ApoE^{-/-}$ mice to the hematopoietic system. Mechanistic experiments show that lesional macrophages from $Zfp148^{gt/+}$ $ApoE^{-/-}$ mice exhibited reduced proliferation compared to controls, and that plaques from $Zfp148^{gt/+}$ $ApoE^{-/-}$ mice had less proliferating cells, increased apoptosis and increased p53 activation compared to controls. Importantly, there was no difference in atherosclerosis between $Zfp148^{gt/+}$ $ApoE^{-/-}$ mice and controls on a p53 heterozygote background, and there was no difference in p53 activity or cell proliferation. These results show that loss of *Zfp148* reduces atherosclerosis through p53 activation (Figure 8)

Whether the reduced atherosclerosis in *Zfp148*-deficient mice is mediated by oxidative stress is currently unknown. Interestingly, two studies revealed that deleting Nrf2, the master regulator of the endogenous antioxidant response, in $ApoE^{-/-}$ mice reduced atherosclerosis (187, 188). As shown in paper I and III, increased ROS levels can activate p53. It is therefore possible that increased p53 activity and decreased macrophage proliferation are part of the

underlying mechanism in the Nrf2 studies, suggesting a potential link to our study.

Drug therapies against atherosclerosis in clinical use or in late-stage clinical trials target inflammation or lipid metabolism. However, despite the success of cholesterol lowering drugs, disease progression is seen in many patients, emphasizing the need for alternative therapies that target other pathways (189). Paper IV suggests that therapeutic targeting of macrophage proliferation through the actions of Zfp148 and p53 might be one such alternative approach. Since Zfp148 reduced atherosclerosis independently of lipid metabolism, drugs targeting Zfp148 could have synergistic effects with lipid lowering drugs.

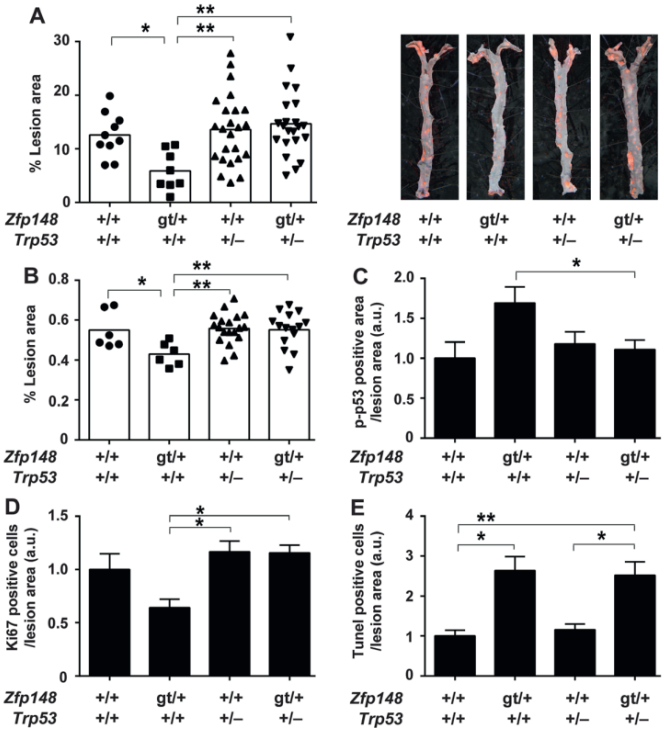


***Figure 8*. :** *Activation of p53 reduces atherosclerosis and lesional cell proliferation in Zfp148$^{gt/+}$ ApoE$^{-/-}$ mice.*

A, Quantification of subendothelial lipid accumulation in aorta as percentage of Sudan IV–stained area per total area. (**B** to **E**) Quantification at the level of the aortic root for subendothelial lipid accumulation (**B**), p-p53^{Ser18} positive area/lesion area (**C**), proliferating Ki67-positive cells/lesion area (**D**), and terminal deoxynucleotidyl transferase dUTP nickend labeling (TUNEL) positive cells/lesion area (**E**).

"The ancient Oracle said that I was the wisest of all the Greeks. It is because I alone, of all the Greeks, know that I know nothing." – Socrates

5 CONCLUSIONS

We conclude that:

Paper I

- Zfp148 is required for structural maturation of the prenatal lung by preventing oxidative stress–dependent p53 activity during the saccular stage of lung development. The result demonstrates for the first time that Zfp148 plays a critical role for cell cycle progression in vivo, and establishes Zfp148 as a novel factor in mammalian lung development.

Paper II

- Zfp148 is a modifier of the APC locus and that Zfp148 deficiency protects against intestinal adenomas by unleashing p53 activity. The results raise the possibility that therapeutic targeting of Zfp148 may protect against colorectal cancer by increasing p53 activity.

Paper III

- Antioxidants NAC and vitamin E markedly increase tumors and reduce survival in mouse models of B-RAF- and K-RAS-induced lung cancer. NAC and vitamin E, which are structurally unrelated, produce highly coordinated changes in tumor transcriptome profiles, and increase cell proliferation by reducing ROS, DNA damage, and p53 expression in mouse and human lung tumor cells. Inactivation of p53 increases tumor growth to a similar degree as antioxidants and abolishes the antioxidant effect. Thus, antioxidants accelerate tumor growth by inactivating the ROS-p53 axis.

Paper IV

- *Zfp148* deficiency reduces atherosclerosis in the *Apoe*$^{-/-}$ model without affecting lipid metabolism. We further show that the effector cell is of hematopoietic origin and that *Zfp148*deficiency confers protection against atherosclerosis by increasing p53 activation, thus reducing proliferation of lesional macrophages.

6 GENERAL DISCUSSION

Here I will discuss general findings that span beyond the individual papers.

As we have seen throughout the thesis (paper I-IV), adjustments of the ROS-p53 axis can have major impact on both physiological and pathological processes. In some contexts, the adjustments of the ROS-p53 axis can be beneficial to the host organism (paper II + IV) and in others it can be harmful (paper I and III). There is a huge body of preclinical evidence which suggests that targeting the ROS-p53 axis can have beneficial effects on disease progression. However, it remains to be seen if we will be able to therapeutically manipulate the ROS-p53 axis to successfully treat human malignancies, including cancer and atherosclerosis.

In this thesis we establish Zfp148 as a potential target for increasing p53 activity.

Zfp148-deficent MEFs, lung cells and macrophages display a lowered threshold to p53 activation in response to oxidative stress. Although indirect mechanisms might be underlying the activation of p53, three lines of evidence support the possibility that Zfp148 primarily regulates p53. Firstly, there is a physical interaction between Zfp148 and p53, which is dependent on the zinc finger domains of Zfp148 and the C terminal parts of the DNA binding domain of p53 (79, 83). Secondly, in response to oxidative stress, Zfp148 deficient cells display increased p53 activation independent of DNA damage (paper I and IV). Finally, there is clear evidence of genetic interaction between Zfp148 and p53 (paper I, II and IV). However, to confirm the proposed mechanism of regulation, further biochemical analysis of Zfp148 is required.

Briefly, our data suggests that targeting Zfp148 and its downstream network might be beneficial in preventing intestinal cancer and atherosclerosis through activation of the ROS-p53 axis. However, our data also show that excessive activation of the ROS-p53 axis can lead to defects in lung development and shorten lifespan. Finally, we show that decreasing the ROS-p53 axis through antioxidant supplements can lead to increased tumor progression.

A graphical abstract depicting the ROS-p53 axis (blue boxes) and the consequences of our interventions throughout the thesis (grey boxes) is provided (Figure 9).

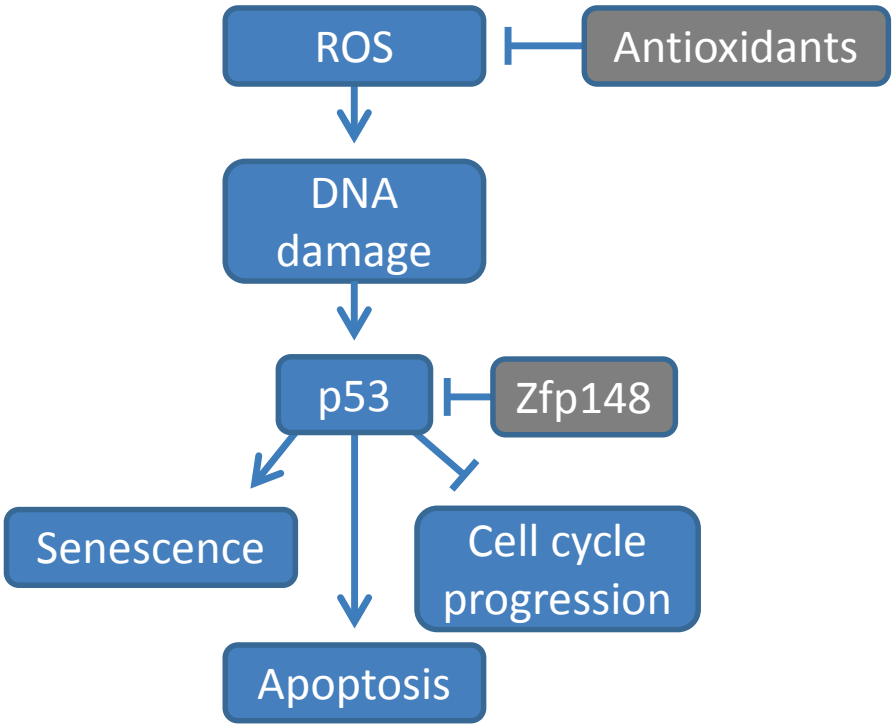


Figure 9. *Graphical abstract of ROS-p53 axis, summerizing our findings from papers I-IV.*

"I never think about the future - it comes soon enough." –Albert Einstein

7 FUTURE PERSPECTIVES

We are following up our findings in the papers included in this thesis.

I. Loss of Zfp148 activates p53 and causes a lung maturation defect. We are identifying the downstream targets of Zfp148 by combining genome-wide expression analysis and with genome-wide transcription factor occupancy analysis. We are also genetically dissecting the molecular pathways causing the lung defect in *Zfp148*-deficient mice.

II. Zfp148-deficiency reduces tumor formation in $Apc^{Min/+}$ mice. We are cross breeding the Zfp148-deficent mice into several tumor models, including lymphoma, sarcoma and lung cancer models. We will evaluate if targeting Zfp148 in other tumor models will have similar effects as in $Apc^{Min/+}$ mice.

III. We are evaluating the effect of Antioxidants on several cancer models, including colorectal cancer, melanoma and leukaemia. Also we are studying the effect of NAC on lung cancer incidence in COPD patients in a Swedish COPD registry with 7209 patients.

IV. Experiments on LDLr knockout mice are ongoing to validate our findings in the ApoE knockout model. We are also working on translating our findings to the clinic by analysing Zfp148 in the Biobank of Karolinska Endarterectomies (BiKE) containing more than 800 human atherosclerotic plaques and plasma samples along with clinical data from individual patients.

ACKNOWLEDGEMENT

Through the course of my life, I have come across a lot of people that directly or indirectly have contributed to the making of this thesis. Without you this particular thesis wouldn't have existed.

I want to extend my gratitude to:

Per Lindahl, my thesis supervisor, for being the greatest teacher and a mentor. You have provided the perfect atmosphere to foster scientific traditions and creative thinking! I have enjoyed our countless coffee meetings about projects, science, life, and everything in between. Thank you for having patience and faith in me and letting me loose to pursue every little crazy idea from NAC-ing MEFs to "ett svagt samband…" ! It has been a dramatic journey with an epic ending!

Martin Bergö, my co-supervisor, for letting me in on all the benefits of the Bergö lab! Your work ethics is an inspiration to us all. "Small but significant" investments made by you have had a great overall impact. Thank you for taking those risks, and believing in me!

All the members of the **Lindahl lab** past & present. **Erik Larsson**, bet you didn't see this one coming. "Kryptonit i Fickan" – from your first email to me, you really shared the spirits of the lab and showed me the way! **Henrik Lindskog**, for teaching me how to "quick-culture-cells" and taking me over the bridge in SF. **Zhiyoun Zou- Tony**, for bringing a lot of joy into our lab. **Nadia Gul**, with you came slightly more order to our lab, what a difference it makes, looking forward to CRISP-ing with you! **Lara**, the sky is the limit, keep up the good work. **Annika Asplund**, you should have stayed longer. **Anna Nilton**, for being a friend, a colleague and a big sister, in every sense and purpose, that "mountain peak up high"!

The **Bergö Lab**, members past and present. **Meng**, for leading the way. **Omar**, looking forward to seeing you again brother – good science takes time. **Bjarni**, you were a blast, **Anna-Karin** for inspiration, **Annika W** for always smiling, **Frida** you a lot of fun, **Murali** keep up the good work, **Ella** your star is shining bright, **Jaroslaw** for inspiring talks about oncogene induced senescence, **Kristell** keep the torch burning-but don't burn yourself-it's getting hot, A **Staffas**, for fun and a lot of FACSing, **Martin D**, we shared a memorable talk in a canoe, remember that, see you on the other side! **Mohamed**, for help with mirax scanning - I really need to acknowledge that! **Christin**, for being "så fiiiin". Thank you all for being the extended academic family - You guys are the best!

Jan Borén, for your contributions to the atherosclerosis paper, it wouldn't have been published in Circ. Res. if you hadn't provided us with guidance in the complex world of the atheroma. Your weekend round at Wlab is a hallmark of a great laboratory.

Fredrik Bäckhed, for making sure Wlab is a progressive place to be. Thank you for your guidance, will definitely need some more!

Jonas Nilsson, for being an inspiring collaborator, mentor and friend! I look forward to our future collaborations. **Lisa Nilsson**, I don't know where to begin? The history of oncogenes over a full-necropsy? You are just awesome!

Levent Akyürek, for stimulating discussions, and Mentoring. Have really enjoyed our lunch sessions.

Stefano Romeo, for bringing a lot of young blood to Wlab and adding to the spirit of productivity. Your advice has been valuable.

Solinas Giovanni, you have only just joined Wlab, but we have already had several inspiring talks! Thank you for sharing your valuable advice.

Ola Hammarsten, for sharing your time, knowledge and reagents with us. I hope we can collaborate even more in the future.

"**Alla på wlab**" My friends and colleagues for making our workplace something to long for! **Anna W, Tulika, Ying-ing, Siavash, Antonio, Annelie, Carlo, Rosa, Bennedetta, Sofie, Louise O, Yangzhen, Felix, Rosita, Kristina S, Valentina T** and everyone else!

Lisa Nguy Bowwgy, for the occasional road block in our doorway, and that 1,2,3 1,2,3 –thing. **Linnea Schmidt** for being the schmiiididididiliiiididit, you are a little ball of energy! Bzzzzt. **Mohamed Ibrahim**, thank you for not spoiling the latest Batman movie "The Dark Knight Rises" and for being so pragmatic.

People in my writing room: **Thomas, M Rutberg, A Lundqvist, Martina, Mattias** BEAR in mind**, Nadia, Christina**! Thank you for providing that zone of comfort and peace along with endless scientific discussions. My side of the room just scored 1-0! Just *Sayin* ©™!

Åsa Tivesten and her group, **Anna W**, **Annelie**, **Marta** and the rest for sharing lab 10 with us. We have had a lot of fun doing what we do, sharing space, time, reagents, tools and valuable advice!

It-Sven and **Magnus**, the heart of Wlab? You guys make sure the clock ticks 24/7. There is not a problem that you cannot fix!

SCC people, **Joy, Lydia, Mariam, Andre, Berglind, Shawn, Nina, Marta, Camilla, Som** and everyone else! It has been a blast!

Friends and collaborators within GUnit and associates: **Dinko-TheDinK**, **Ali-SirRoc**, **Erik**, **Mange-Keeper of the groove**, **Martin *Hu* Tamté-Dessan**, **Kaveh-Cavedog**, **Milad-Money-M**, **Musse Matlak**, **MartinMC**, **Mohamed-Xavier**! You are all uniquely inspiring! Keep that up and the world is your oyster.

Friends outside of the University for remaining my friends. My dissertation will be a good time to catch up!

Mohammad Russell Bohlooly, for placing that seed of inspiration half a life ago! This is *NOT* the end product!

Baba, for being that never ending fountain of youth! I can only dream of growing "young" like you! Everyone should strive to achieve the things you have done.

Lille-Bro, the end of one journey marks the beginning of another! Make sure to enjoy the ride. Big brother will be watching.

Annecim, you provided me the tools for a happy and prosperous life, you don't ever have to worry about a thing. Thank you for believing in me when I had doubts, for always praying for me and being the best mom anyone can have.

Last I want to extend my deepest gratitude to Sama, my wife, the light of my life, my tranquility base, my beginning and my end! Thank you for making me better in every sense and purpose and filling my life with love.

REFERENCES

1. Dole M. The natural history of oxygen. *The Journal of general physiology*. 1965;49(1):Suppl:5-27.
2. Holland HD. The oxygenation of the atmosphere and oceans. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2006;361(1470):903-15.
3. Danovaro R, Dell'Anno A, Pusceddu A, Gambi C, Heiner I, and Kristensen RM. The first metazoa living in permanently anoxic conditions. *BMC biology*. 2010;8(30.
4. Levin LA. Anaerobic metazoans: no longer an oxymoron. *BMC biology*. 2010;8(31.
5. Shibutani S, Takeshita M, and Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*. 1991;349(6308):431-4.
6. Kamiya H. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary. *Nucleic Acids Res*. 2003;31(2):517-31.
7. Sakai A, Nakanishi M, Yoshiyama K, and Maki H. Impact of reactive oxygen species on spontaneous mutagenesis in Escherichia coli. *Genes Cells*. 2006;11(7):767-78.
8. Cabiscol E, Piulats E, Echave P, Herrero E, and Ros J. Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. *J Biol Chem*. 2000;275(35):27393-8.
9. Maher J, and Yamamoto M. The rise of antioxidant signaling--the evolution and hormetic actions of Nrf2. *Toxicology and applied pharmacology*. 2010;244(1):4-15.
10. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, and Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*. 2007;39(1):44-84.
11. Willcox JK, Ash SL, and Catignani GL. Antioxidants and prevention of chronic disease. *Critical reviews in food science and nutrition*. 2004;44(4):275-95.
12. Dhalla NS, Temsah RM, and Netticadan T. Role of oxidative stress in cardiovascular diseases. *Journal of hypertension*. 2000;18(6):655-73.

13. Kang KW, Lee SJ, and Kim SG. Molecular mechanism of nrf2 activation by oxidative stress. *Antioxidants & redox signaling.* 2005;7(11-12):1664-73.
14. Thomson AD. Mechanisms of vitamin deficiency in chronic alcohol misusers and the development of the Wernicke-Korsakoff syndrome. *Alcohol and alcoholism (Oxford, Oxfordshire) Supplement.* 2000;35(1):2-7.
15. Dror DK, and Allen LH. Vitamin E deficiency in developing countries. *Food and nutrition bulletin.* 2011;32(2):124-43.
16. Hodges RE, Hood J, Canham JE, Sauberlich HE, and Baker EM. Clinical manifestations of ascorbic acid deficiency in man. *The American journal of clinical nutrition.* 1971;24(4):432-43.
17. Reuler JB, Broudy VC, and Cooney TG. Adult scurvy. *JAMA : the journal of the American Medical Association.* 1985;253(6):805-7.
18. Maronn M, Allen DM, and Esterly NB. Phrynoderma: a manifestation of vitamin A deficiency?... The rest of the story. *Pediatric dermatology.* 2005;22(1):60-3.
19. Silverberg SJ. Vitamin D deficiency and primary hyperparathyroidism. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2007;22 Suppl 2(V100-4.
20. Puntambekar P, Basha MM, Zak IT, and Madhavan R. Rare sensory and autonomic disturbances associated with vitamin B12 deficiency. *Journal of the neurological sciences.* 2009;287(1-2):285-7.
21. Becks L, Prince M, Burson H, Christophe C, Broadway M, Itoh K, Yamamoto M, Mathis M, Orchard E, Shi R, et al. Aggressive mammary carcinoma progression in Nrf2 knockout mice treated with 7,12-dimethylbenz[a]anthracene. *BMC cancer.* 2010;10(540.
22. Lee JM, Chan K, Kan YW, and Johnson JA. Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. *Proc Natl Acad Sci U S A.* 2004;101(26):9751-6.
23. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet.* 1995;11(4):376-81.
24. Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, and Goldschmidt-Clermont PJ. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science.* 1997;275(5306):1649-52.
25. Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, and Chumakov PM. The antioxidant function of the p53 tumor suppressor. *Nature medicine.* 2005;11(12):1306-13.
26. Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, and Wahl GM. c-Myc can induce DNA damage, increase reactive oxygen

species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell.* 2002;9(5):1031-44.
27. Shimada K, Murayama T, Yokode M, Kita T, Uzui H, Ueda T, Lee JD, and Kishimoto C. N-acetylcysteine reduces the severity of atherosclerosis in apolipoprotein E-deficient mice by reducing superoxide production. *Circulation journal : official journal of the Japanese Circulation Society.* 2009;73(7):1337-41.
28. Ivanovski O, Szumilak D, Nguyen-Khoa T, Ruellan N, Phan O, Lacour B, Descamps-Latscha B, Drueke TB, and Massy ZA. The antioxidant N-acetylcysteine prevents accelerated atherosclerosis in uremic apolipoprotein E knockout mice. *Kidney international.* 2005;67(6):2288-94.
29. Fuhrman B, and Aviram M. Anti-atherogenicity of nutritional antioxidants. *IDrugs : the investigational drugs journal.* 2001;4(1):82-92.
30. Mercer JR, Yu E, Figg N, Cheng KK, Prime TA, Griffin JL, Masoodi M, Vidal-Puig A, Murphy MP, and Bennett MR. The mitochondria-targeted antioxidant MitoQ decreases features of the metabolic syndrome in ATM+/-/ApoE-/- mice. *Free radical biology & medicine.* 2012;52(5):841-9.
31. Galis ZS, Asanuma K, Godin D, and Meng X. N-acetyl-cysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy? *Circulation.* 1998;97(24):2445-53.
32. Blot WJ, Li JY, Taylor PR, and Li B. Lung cancer and vitamin supplementation. *N Engl J Med.* 1994;331(9):614.
33. van Zandwijk N, Dalesio O, Pastorino U, de Vries N, and van Tinteren H. EUROSCAN, a randomized trial of vitamin A and N-acetylcysteine in patients with head and neck cancer or lung cancer. For the EUropean Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. *J Natl Cancer Inst.* 2000;92(12):977-86.
34. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M, Li GY, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *Journal of the National Cancer Institute.* 1993;85(18):1483-92.
35. Gaziano JM. Antioxidants in cardiovascular disease: randomized trials. *Nutrition.* 1996;12(9):583-8.
36. Rimm EB, and Stampfer MJ. The role of antioxidants in preventive cardiology. *Current opinion in cardiology.* 1997;12(2):188-94.
37. Cherubini A, Vigna GB, Zuliani G, Ruggiero C, Senin U, and Fellin R. Role of antioxidants in atherosclerosis: epidemiological and

clinical update. *Current pharmaceutical design.* 2005;11(16):2017-32.
38. Blomhoff R. Dietary antioxidants and cardiovascular disease. *Current opinion in lipidology.* 2005;16(1):47-54.
39. Watson J. Oxidants, antioxidants and the current incurability of metastatic cancers. *Open biology.* 2013;3(1):120144.
40. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, Hartline JA, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA : the journal of the American Medical Association.* 2009;301(1):39-51.
41. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA : the journal of the American Medical Association.* 2011;306(14):1549-56.
42. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med.* 1994;330(15):1029-35.
43. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, and Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA : the journal of the American Medical Association.* 2007;297(8):842-57.
44. Ford ES, Ajani UA, and Mokdad AH. Brief communication: The prevalence of high intake of vitamin E from the use of supplements among U.S. adults. *Annals of internal medicine.* 2005;143(2):116-20.
45. Levine AJ, Momand J, and Finlay CA. The p53 tumour suppressor gene. *Nature.* 1991;351(6326):453-6.
46. Vogelstein B, Lane D, and Levine AJ. Surfing the p53 network. *Nature.* 2000;408(6810):307-10.
47. Vousden KH, and Lane DP. p53 in health and disease. *Nature reviews Molecular cell biology.* 2007;8(4):275-83.
48. Kruse JP, and Gu W. Modes of p53 regulation. *Cell.* 2009;137(4):609-22.
49. Jones SN, Roe AE, Donehower LA, and Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature.* 1995;378(6553):206-8.
50. Montes de Oca Luna R, Wagner DS, and Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature.* 1995;378(6553):203-6.
51. Parant J, Chavez-Reyes A, Little NA, Yan W, Reinke V, Jochemsen AG, and Lozano G. Rescue of embryonic lethality in Mdm4-null

mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat Genet.* 2001;29(1):92-5.
52. Migliorini D, Lazzerini Denchi E, Danovi D, Jochemsen A, Capillo M, Gobbi A, Helin K, Pelicci PG, and Marine JC. Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol.* 2002;22(15):5527-38.
53. Lane DP, and Verma C. Mdm2 in evolution. *Genes & cancer.* 2012;3(3-4):320-4.
54. Khoo KH, Verma CS, and Lane DP. Drugging the p53 pathway: understanding the route to clinical efficacy. *Nature reviews Drug discovery.* 2014;13(3):217-36.
55. Liu B, Chen Y, and St Clair DK. ROS and p53: a versatile partnership. *Free radical biology & medicine.* 2008;44(8):1529-35.
56. Macip S, Igarashi M, Berggren P, Yu J, Lee SW, and Aaronson SA. Influence of induced reactive oxygen species in p53-mediated cell fate decisions. *Mol Cell Biol.* 2003;23(23):8576-85.
57. Polyak K, Xia Y, Zweier JL, Kinzler KW, and Vogelstein B. A model for p53-induced apoptosis. *Nature.* 1997;389(6648):300-5.
58. Hainaut P, and Mann K. Zinc binding and redox control of p53 structure and function. *Antioxidants & redox signaling.* 2001;3(4):611-23.
59. Cho Y, Gorina S, Jeffrey PD, and Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science.* 1994;265(5170):346-55.
60. D'Autreaux B, and Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews Molecular cell biology.* 2007;8(10):813-24.
61. Velu CS, Niture SK, Doneanu CE, Pattabiraman N, and Srivenugopal KS. Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry.* 2007;46(26):7765-80.
62. Taylor WR, and Stark GR. Regulation of the G2/M transition by p53. *Oncogene.* 2001;20(15):1803-15.
63. Brosh R, and Rotter V. Transcriptional control of the proliferation cluster by the tumor suppressor p53. *Mol Biosyst.* 2010;6(1):17-29.
64. Merchant JL, Iyer GR, Taylor BR, Kitchen JR, Mortensen ER, Wang Z, Flintoft RJ, Michel JB, and Bassel-Duby R. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. *Mol Cell Biol.* 1996;16(12):6644-53.
65. Wang Y, Kobori JA, and Hood L. The ht beta gene encodes a novel CACCC box-binding protein that regulates T-cell receptor gene expression. *Mol Cell Biol.* 1993;13(9):5691-701.
66. Hasegawa T, Takeuchi A, Miyaishi O, Isobe K, and de Crombrugghe B. Cloning and characterization of a transcription factor that binds to

the proximal promoters of the two mouse type I collagen genes. *J Biol Chem.* 1997;272(8):4915-23.
67. Passantino R, Antona V, Barbieri G, Rubino P, Melchionna R, Cossu G, Feo S, and Giallongo A. Negative regulation of beta enolase gene transcription in embryonic muscle is dependent upon a zinc finger factor that binds to the G-rich box within the muscle-specific enhancer. *J Biol Chem.* 1998;273(1):484-94.
68. Bai L, and Merchant JL. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. *J Biol Chem.* 2000;275(39):30725-33.
69. Bai L, Kao JY, Law DJ, and Merchant JL. Recruitment of ataxia-telangiectasia mutated to the p21(waf1) promoter by ZBP-89 plays a role in mucosal protection. *Gastroenterology.* 2006;131(3):841-52.
70. Wu Y, Zhang X, Salmon M, and Zehner ZE. The zinc finger repressor, ZBP-89, recruits histone deacetylase 1 to repress vimentin gene expression. *Genes Cells.* 2007;12(8):905-18.
71. Feng Y, Wang X, Xu L, Pan H, Zhu S, Liang Q, Huang B, and Lu J. The transcription factor ZBP-89 suppresses p16 expression through a histone modification mechanism to affect cell senescence. *FEBS J.* 2009;276(15):4197-206.
72. Hasegawa T, Xiao H, and Isobe K. Cloning of a GADD34-like gene that interacts with the zinc-finger transcription factor which binds to the p21(WAF) promoter. *Biochem Biophys Res Commun.* 1999;256(1):249-54.
73. Borghaei RC, Rawlings PL, Jr., Javadi M, and Woloshin J. NF-kappaB binds to a polymorphic repressor element in the MMP-3 promoter. *Biochem Biophys Res Commun.* 2004;316(1):182-8.
74. Bai L, and Merchant JL. Transcription factor ZBP-89 is required for STAT1 constitutive expression. *Nucleic Acids Res.* 2003;31(24):7264-70.
75. Keates AC, Keates S, Kwon JH, Arseneau KO, Law DJ, Bai L, Merchant JL, Wang TC, and Kelly CP. ZBP-89, Sp1, and nuclear factor-kappa B regulate epithelial neutrophil-activating peptide-78 gene expression in Caco-2 human colonic epithelial cells. *J Biol Chem.* 2001;276(47):43713-22.
76. Remington MC, Tarle SA, Simon B, and Merchant JL. ZBP-89, a Kruppel-type zinc finger protein, inhibits cell proliferation. *Biochem Biophys Res Commun.* 1997;237(2):230-4.
77. Zhang X, Diab IH, and Zehner ZE. ZBP-89 represses vimentin gene transcription by interacting with the transcriptional activator, Sp1. *Nucleic Acids Res.* 2003;31(11):2900-14.
78. Malo MS, Mozumder M, Zhang XB, Biswas S, Chen A, Bai LC, Merchant JL, and Hodin RA. Intestinal alkaline phosphatase gene

expression is activated by ZBP-89. *American journal of physiology Gastrointestinal and liver physiology.* 2006;290(4):G737-46.
79. Bai L, and Merchant JL. ZBP-89 promotes growth arrest through stabilization of p53. *Mol Cell Biol.* 2001;21(14):4670-83.
80. Chen GG, Chan UP, Bai LC, Fung KY, Tessier A, To AK, Merchant JL, and Lai PB. ZBP-89 reduces the cell death threshold in hepatocellular carcinoma cells by increasing caspase-6 and S phase cell cycle arrest. *Cancer letters.* 2009;283(1):52-8.
81. Marechal A, and Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harbor perspectives in biology.* 2013;5(9).
82. Bai L, and Merchant JL. ATM phosphorylates ZBP-89 at Ser202 to potentiate p21waf1 induction by butyrate. *Biochem Biophys Res Commun.* 2007;359(3):817-21.
83. Chen GG, Merchant JL, Lai PB, Ho RL, Hu X, Okada M, Huang SF, Chui AK, Law DJ, Li YG, et al. Mutation of p53 in recurrent hepatocellular carcinoma and its association with the expression of ZBP-89. *The American journal of pathology.* 2003;162(6):1823-9.
84. Okada M, Tessier A, Bai L, and Merchant JL. P53 mutants suppress ZBP-89 function. *Anticancer research.* 2006;26(3A):2023-8.
85. Law DJ, Labut EM, Adams RD, and Merchant JL. An isoform of ZBP-89 predisposes the colon to colitis. *Nucleic Acids Res.* 2006;34(5):1342-50.
86. Woo AJ, Moran TB, Schindler YL, Choe SK, Langer NB, Sullivan MR, Fujiwara Y, Paw BH, and Cantor AB. Identification of ZBP-89 as a novel GATA-1-associated transcription factor involved in megakaryocytic and erythroid development. *Mol Cell Biol.* 2008;28(8):2675-89.
87. Takeuchi A, Mishina Y, Miyaishi O, Kojima E, Hasegawa T, and Isobe K. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. *Nat Genet.* 2003;33(2):172-6.
88. Oliver PL, Bitoun E, and Davies KE. Comparative genetic analysis: the utility of mouse genetic systems for studying human monogenic disease. *Mammalian genome : official journal of the International Mammalian Genome Society.* 2007;18(6-7):412-24.
89. Gordon JW, and Ruddle FH. Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science.* 1981;214(4526):1244-6.
90. Jaenisch R. Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci U S A.* 1976;73(4):1260-4.
91. Harbers K, Jahner D, and Jaenisch R. Microinjection of cloned retroviral genomes into mouse zygotes: integration and expression in the animal. *Nature.* 1981;293(5833):540-2.

92. Jaenisch R, Jahner D, Nobis P, Simon I, Lohler J, Harbers K, and Grotkopp D. Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. *Cell.* 1981;24(2):519-29.
93. Thomas KR, and Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell.* 1987;51(3):503-12.
94. Misra RP, and Duncan SA. Gene targeting in the mouse: advances in introduction of transgenes into the genome by homologous recombination. *Endocrine.* 2002;19(3):229-38.
95. Capecchi MR. The new mouse genetics: altering the genome by gene targeting. *Trends in genetics : TIG.* 1989;5(3):70-6.
96. Schwartzberg PL, Goff SP, and Robertson EJ. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. *Science.* 1989;246(4931):799-803.
97. Zijlstra M, Li E, Sajjadi F, Subramani S, and Jaenisch R. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature.* 1989;342(6248):435-8.
98. Srivastava S, Zou ZQ, Pirollo K, Blattner W, and Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature.* 1990;348(6303):747-9.
99. Crow JF. C. C. Little, cancer and inbred mice. *Genetics.* 2002;161(4):1357-61.
100. Bedell MA, Jenkins NA, and Copeland NG. Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. *Genes & development.* 1997;11(1):1-10.
101. Bedell MA, Largaespada DA, Jenkins NA, and Copeland NG. Mouse models of human disease. Part II: recent progress and future directions. *Genes & development.* 1997;11(1):11-43.
102. Erickson RP. Mouse models of human genetic disease: which mouse is more like a man? *BioEssays : news and reviews in molecular, cellular and developmental biology.* 1996;18(12):993-8.
103. Hardouin SN, and Nagy A. Mouse models for human disease. *Clinical genetics.* 2000;57(4):237-44.
104. Herman GE. Mouse models of human disease: lessons learned and promises to come. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources.* 2002;43(2):55-6.
105. Semsarian C. Use of mouse models for the analysis of human disease. *Current protocols in human genetics / editorial board, Jonathan L Haines [et al].* 2009;Chapter 15(Unit 15 2.
106. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, et al. Genomic responses in mouse models poorly mimic human

inflammatory diseases. *Proc Natl Acad Sci U S A.* 2013;110(9):3507-12.
107. Iida S, Meyer J, Kennedy KE, and Arber W. A site-specific, conservative recombination system carried by bacteriophage P1. Mapping the recombinase gene cin and the cross-over sites cix for the inversion of the C segment. *The EMBO journal.* 1982;1(11):1445-53.
108. Sternberg N, Hamilton D, Austin S, Yarmolinsky M, and Hoess R. Site-specific recombination and its role in the life cycle of bacteriophage P1. *Cold Spring Harbor symposia on quantitative biology.* 1981;45 Pt 1(297-309.
109. Abremski K, and Hoess R. Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J Biol Chem.* 1984;259(3):1509-14.
110. Nagy A. Cre recombinase: the universal reagent for genome tailoring. *Genesis.* 2000;26(2):99-109.
111. Sauer B, and Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A.* 1988;85(14):5166-70.
112. Fasbender A, Lee JH, Walters RW, Moninger TO, Zabner J, and Welsh MJ. Incorporation of adenovirus in calcium phosphate precipitates enhances gene transfer to airway epithelia in vitro and in vivo. *The Journal of clinical investigation.* 1998;102(1):184-93.
113. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, and Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circulation research.* 2001;89(1):20-5.
114. Kucherlapati MH, Nguyen AA, Bronson RT, and Kucherlapati RS. Inactivation of conditional Rb by Villin-Cre leads to aggressive tumors outside the gastrointestinal tract. *Cancer research.* 2006;66(7):3576-83.
115. Clausen BE, Burkhardt C, Reith W, Renkawitz R, and Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic research.* 1999;8(4):265-77.
116. Kuhn R, Schwenk F, Aguet M, and Rajewsky K. Inducible gene targeting in mice. *Science.* 1995;269(5229):1427-9.
117. Sauer B. Inducible gene targeting in mice using the Cre/lox system. *Methods.* 1998;14(4):381-92.
118. Wicksteed B, Brissova M, Yan W, Opland DM, Plank JL, Reinert RB, Dickson LM, Tamarina NA, Philipson LH, Shostak A, et al. Conditional gene targeting in mouse pancreatic ss-Cells: analysis of ectopic Cre transgene expression in the brain. *Diabetes.* 2010;59(12):3090-8.

119. Semprini S, Troup TJ, Kotelevtseva N, King K, Davis JR, Mullins LJ, Chapman KE, Dunbar DR, and Mullins JJ. Cryptic loxP sites in mammalian genomes: genome-wide distribution and relevance for the efficiency of BAC/PAC recombineering techniques. *Nucleic Acids Res.* 2007;35(5):1402-10.
120. Lexow J, Poggioli T, Sarathchandra P, Santini MP, and Rosenthal N. Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver. *Disease models & mechanisms.* 2013;6(6):1470-6.
121. Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, and Jay PY. Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. *Journal of cardiac failure.* 2006;12(5):392-8.
122. Kumar MS, Hancock DC, Molina-Arcas M, Steckel M, East P, Diefenbacher M, Armenteros-Monterroso E, Lassailly F, Matthews N, Nye E, et al. The GATA2 transcriptional network is requisite for RAS oncogene-driven non-small cell lung cancer. *Cell.* 2012;149(3):642-55.
123. Liu M, Sjogren AK, Karlsson C, Ibrahim MX, Andersson KM, Olofsson FJ, Wahlstrom AM, Dalin M, Yu H, Chen Z, et al. Targeting the protein prenyltransferases efficiently reduces tumor development in mice with K-RAS-induced lung cancer. *Proc Natl Acad Sci U S A.* 2010;107(14):6471-6.
124. Turlo KA, Gallaher SD, Vora R, Laski FA, and Iruela-Arispe ML. When Cre-mediated recombination in mice does not result in protein loss. *Genetics.* 2010;186(3):959-67.
125. Schafer M, and Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nature reviews Molecular cell biology.* 2008;9(8):628-38.
126. Siegel R, Ma J, Zou Z, and Jemal A. Cancer statistics, 2014. *CA: a cancer journal for clinicians.* 2014;64(1):9-29.
127. DuPage M, Mazumdar C, Schmidt LM, Cheung AF, and Jacks T. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature.* 2012;482(7385):405-9.
128. McFadden DG, Papagiannakopoulos T, Taylor-Weiner A, Stewart C, Carter SL, Cibulskis K, Bhutkar A, McKenna A, Dooley A, Vernon A, et al. Genetic and clonal dissection of murine small cell lung carcinoma progression by genome sequencing. *Cell.* 2014;156(6):1298-311.
129. Cheon DJ, and Orsulic S. Mouse models of cancer. *Annual review of pathology.* 2011;6(95-119.
130. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, and Weinberg RA. Tumor spectrum analysis in p53-mutant mice. *Current biology : CB.* 1994;4(1):1-7.
131. Santibanez-Koref MF, Birch JM, Hartley AL, Jones PH, Craft AW, Eden T, Crowther D, Kelsey AM, and Harris M. p53 germline

mutations in Li-Fraumeni syndrome. *Lancet.* 1991;338(8781):1490-1.
132. Malkin D. p53 and the Li-Fraumeni syndrome. *Cancer genetics and cytogenetics.* 1993;66(2):83-92.
133. Cheung AM, Elia A, Tsao MS, Done S, Wagner KU, Hennighausen L, Hakem R, and Mak TW. Brca2 deficiency does not impair mammary epithelium development but promotes mammary adenocarcinoma formation in p53(+/-) mutant mice. *Cancer research.* 2004;64(6):1959-65.
134. Purdie CA, Harrison DJ, Peter A, Dobbie L, White S, Howie SE, Salter DM, Bird CC, Wyllie AH, Hooper ML, et al. Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene.* 1994;9(2):603-9.
135. Mahler JF, Flagler ND, Malarkey DE, Mann PC, Haseman JK, and Eastin W. Spontaneous and chemically induced proliferative lesions in Tg.AC transgenic and p53-heterozygous mice. *Toxicologic pathology.* 1998;26(4):501-11.
136. Blanchard KT, Barthel C, French JE, Holden HE, Moretz R, Pack FD, Tennant RW, and Stoll RE. Transponder-induced sarcoma in the heterozygous p53+/- mouse. *Toxicologic pathology.* 1999;27(5):519-27.
137. Carmichael NG, Debruyne EL, and Bigot-Lasserre D. The p53 heterozygous knockout mouse as a model for chemical carcinogenesis in vascular tissue. *Environmental health perspectives.* 2000;108(1):61-5.
138. Bigot-Lasserre D, Chuzel F, Debruyne EL, Bars R, and Carmichael NG. Tumorigenic potential of carbaryl in the heterozygous p53 knockout mouse model. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association.* 2003;41(1):99-106.
139. Mao JH, Li J, Jiang T, Li Q, Wu D, Perez-Losada J, DelRosario R, Peterson L, Balmain A, and Cai WW. Genomic instability in radiation-induced mouse lymphoma from p53 heterozygous mice. *Oncogene.* 2005;24(53):7924-34.
140. Moser AR, Pitot HC, and Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science.* 1990;247(4940):322-4.
141. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, and Dove WF. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science.* 1992;256(5057):668-70.
142. Nakamura Y, Nishisho I, Kinzler KW, Vogelstein B, Miyoshi Y, Miki Y, Ando H, and Horii A. Mutations of the APC (adenomatous polyposis coli) gene in FAP (familial polyposis coli) patients and in

sporadic colorectal tumors. *The Tohoku journal of experimental medicine.* 1992;168(2):141-7.
143. Fearon ER, and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990;61(5):759-67.
144. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, and Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med.* 1988;319(9):525-32.
145. Yamada Y, and Mori H. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer science.* 2007;98(1):6-10.
146. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, and Tuveson DA. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes & development.* 2001;15(24):3243-8.
147. Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, Fraire AE, Gabrielson EW, Gunning WT, Haines DC, et al. Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer research.* 2004;64(7):2307-16.
148. Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer cell.* 2004;5(4):375-87.
149. Hanahan D, and Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-74.
150. Hanahan D, and Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100(1):57-70.
151. Dankort D, Filenova E, Collado M, Serrano M, Jones K, and McMahon M. A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors. *Genes & development.* 2007;21(4):379-84.
152. Lowe SW, Schmitt EM, Smith SW, Osborne BA, and Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature.* 1993;362(6423):847-9.
153. DuPage M, Dooley AL, and Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nature protocols.* 2009;4(7):1064-72.
154. Smith JD. Mouse models of atherosclerosis. *Laboratory animal science.* 1998;48(6):573-9.
155. Reardon CA, and Getz GS. Mouse models of atherosclerosis. *Current opinion in lipidology.* 2001;12(2):167-73.
156. Daugherty A. Mouse models of atherosclerosis. *The American journal of the medical sciences.* 2002;323(1):3-10.

157. Mukhopadhyay R. Mouse models of atherosclerosis: explaining critical roles of lipid metabolism and inflammation. *Journal of applied genetics.* 2013;54(2):185-92.
158. Meir KS, and Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arteriosclerosis, thrombosis, and vascular biology.* 2004;24(6):1006-14.
159. Nakashima Y, Plump AS, Raines EW, Breslow JL, and Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association.* 1994;14(1):133-40.
160. Ishibashi S, Goldstein JL, Brown MS, Herz J, and Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *The Journal of clinical investigation.* 1994;93(5):1885-93.
161. Baitsch D, Bock HH, Engel T, Telgmann R, Muller-Tidow C, Varga G, Bot M, Herz J, Robenek H, von Eckardstein A, et al. Apolipoprotein E induces antiinflammatory phenotype in macrophages. *Arteriosclerosis, thrombosis, and vascular biology.* 2011;31(5):1160-8.
162. Schaefer EJ, Gregg RE, Ghiselli G, Forte TM, Ordovas JM, Zech LA, and Brewer HB, Jr. Familial apolipoprotein E deficiency. *The Journal of clinical investigation.* 1986;78(5):1206-19.
163. Weiden PL, Sullivan KM, Flournoy N, Storb R, and Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. *N Engl J Med.* 1981;304(25):1529-33.
164. Aparicio-Vergara M, Shiri-Sverdlov R, de Haan G, and Hofker MH. Bone marrow transplantation in mice as a tool for studying the role of hematopoietic cells in metabolic and cardiovascular diseases. *Atherosclerosis.* 2010;213(2):335-44.
165. Nelander S, Larsson E, Kristiansson E, Mansson R, Nerman O, Sigvardsson M, Mostad P, and Lindahl P. Predictive screening for regulators of conserved functional gene modules (gene batteries) in mammals. *BMC genomics.* 2005;6(68.
166. Sayin VI, Nilton A, Ibrahim MX, Agren P, Larsson E, Petit MM, Hulten LM, Stahlman M, Johansson BR, Bergo MO, et al. Zfp148 deficiency causes lung maturation defects and lethality in newborn mice that are rescued by deletion of p53 or antioxidant treatment. *PloS one.* 2013;8(2):e55720.
167. Reagan-Shaw S, Nihal M, and Ahmad N. Dose translation from animal to human studies revisited. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2008;22(3):659-61.

168. Bell SJ, and Grochoski GT. How safe is vitamin E supplementation? *Critical reviews in food science and nutrition.* 2008;48(8):760-74.
169. Sadowska AM. N-Acetylcysteine mucolysis in the management of chronic obstructive pulmonary disease. *Therapeutic advances in respiratory disease.* 2012;6(3):127-35.
170. Kim YR, Oh JE, Kim MS, Kang MR, Park SW, Han JY, Eom HS, Yoo NJ, and Lee SH. Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *The Journal of pathology.* 2010;220(4):446-51.
171. Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, and Hirohashi S. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A.* 2008;105(36):13568-73.
172. Hayes JD, and McMahon M. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends in biochemical sciences.* 2009;34(4):176-88.
173. Cancer Genome Atlas Research N. Comprehensive genomic characterization of squamous cell lung cancers. *Nature.* 2012;489(7417):519-25.
174. Kwak MK, and Kensler TW. Targeting NRF2 signaling for cancer chemoprevention. *Toxicology and applied pharmacology.* 2010;244(1):66-76.
175. DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES, et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature.* 2011;475(7354):106-9.
176. Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, Kikuchi N, Satoh H, Sakamoto T, Hizawa N, et al. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2009;15(10):3423-32.
177. Rolfs F, Huber M, Gruber F, Bohm F, Pfister HJ, Bochkov VN, Tschachler E, Dummer R, Hohl D, Schafer M, et al. Dual role of the antioxidant enzyme peroxiredoxin 6 in skin carcinogenesis. *Cancer research.* 2013;73(11):3460-9.
178. Glasauer A, and Chandel NS. Targeting antioxidants for cancer therapy. *Biochemical pharmacology.* 2014.
179. Mendelsohn AR, and Larrick JW. Paradoxical effects of antioxidants on cancer. *Rejuvenation research.* 2014;17(3):306-11.
180. Shaheen SO. Antioxidants and respiratory disease: the uric acid paradox. *Thorax.* 2014.
181. Potter JD. The failure of cancer chemoprevention. *Carcinogenesis.* 2014;35(5):974-82.

182. Sanchis-Gomar F, Pareja-Galeano H, and Lucia A. The hMTH1 paradox: antioxidants recommended in cancer? *DNA repair.* 2014;21(163-4.
183. Chandel NS, and Tuveson DA. The promise and perils of antioxidants for cancer patients. *N Engl J Med.* 2014;371(2):177-8.
184. Mercer J, and Bennett M. The role of p53 in atherosclerosis. *Cell cycle.* 2006;5(17):1907-9.
185. Wu D, Yang H, Xiang W, Zhou L, Shi M, Julies G, Laplante JM, Ballard BR, and Guo Z. Heterozygous mutation of ataxia-telangiectasia mutated gene aggravates hypercholesterolemia in apoE-deficient mice. *Journal of lipid research.* 2005;46(7):1380-7.
186. Suzuki M, Minami A, Nakanishi A, Kobayashi K, Matsuda S, Ogura Y, and Kitagishi Y. Atherosclerosis and tumor suppressor molecules (Review). *International journal of molecular medicine.* 2014;34(4):934-40.
187. Sussan TE, Jun J, Thimmulappa R, Bedja D, Antero M, Gabrielson KL, Polotsky VY, and Biswal S. Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PloS one.* 2008;3(11):e3791.
188. Barajas B, Che N, Yin F, Rowshanrad A, Orozco LD, Gong KW, Wang X, Castellani LW, Reue K, Lusis AJ, et al. NF-E2-related factor 2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow antioxidant protection. *Arteriosclerosis, thrombosis, and vascular biology.* 2011;31(1):58-66.
189. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA, Skene AM, et al. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N Engl J Med.* 2004;350(15):1495-504.