Carcinoembryonic antigen cell adhesion molecule 1: cancer and metabolic regulation

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DEDICATION

This thesis is dedicated to Alain and Marie-Thérèse,
my loving parents.

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CONTRIBUTION OF AUTHORS

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LIST OF ABBREVIATIONS

4E-BP	4E-binding protein
AA	acetaldehyde
aa	amino acid
ACF	aberrant crypt foci
AFAP	attenuated familial adenomatous polyposis
Akt	see PKB
ALK	alkaline phosphatase
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AMPK	AMP kinase
AOM	azoxymethane
AP-1	activating protein-1
AP-2	Activator Protein 2, also transcription factor alpha protein
APC	adenomatous polyposis coli
APS	Adaptor Proteins, Signal Transducing
AR	androgen receptor
ARF	ADP-ribosylation factor
Asef	APC-stimulated guanine nucleotide exchange factor
AST	aspartate aminotransferase
BAD	Bcl-associated death protein
Bax	bcl-2-associated X protein
Bcl	B-Cell Leukemia
Bcl9	B cell CLL/lymphoma 9
Bgp1	biliary glycoprotein1, also known as CEACAM1
BH3	Bcl-2 homology 3
bHLH	helix-loop-helix
Bid	BH3 interacting domain death agonist protein
BIM	also known as BCL2L11 BCL2-like 11 (apoptosis facilitator)
BrdU	bromo-5'-deoxyuridine.
Brg-1	brahma-related gene 1
CABF	concanavalin A-binding fraction
CAM	cell adhesion molecule
Cbl	casitas B-lineage lymphoma protein
CBP	CREB-binding protein
CD66a	cluster of differentiation 66a, also known as CEACAM1
Cdc42	cell division cycle 42
Cdh1	cadherin1 gene
Cdk	cyclin dependent kinase
CDKN	cyclin-dependent kinase inhibitor 2A
Cdx2	caudal type homeobox transcription factor 2
CEA	Carcinoembryonic antigen
CEACAM1	Carcinoembryonic antigen cell adhesion molecule 1
Chr	chromosome
ChREBP	carbohydrate-response-element-binding protein
CIN	chromosomal instability

CK1	cyclin kinase 1
cM	centimorgan
c-Met	Mesenchymal epithelial transition factor
Cos-7	transformed African green monkey kidney fibroblast cell
COX-2	cyclo-oxygenase-2
Cr-1	Cripto-1
CRC	colorectal cancer
Cre	cyclization recombination,
CREB	cAMP response element-binding
CT51	mouse colon carcinoma cell line
CTLA4	cytotoxic T-lymphocyte antigen 4
CYP2E1	cytochrome P4502E1
DC	Dendritic cell
DCC	deleted in colorectal carcinoma
Dkk	dickkopf
DLG	discs large
DMH	Dimethylhydrazine
DNA	deoxyribonucleic acid
Dpc	days postcoitus
DPC4	deleted in pancreatic cancer, locus 4
Dsh	Dishevelled
EB/RP	end binding protein/ retinitis pigmentosa GTPase regulator
EB1	end binding protein 1
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGF-R	epidermal growth factor receptor
eIF4E	Eukaryotic Initiation Factor-4E
ELK1	ETS-like gene 1
EMT	epithelial-mesenchymal transition
EphB	Ephrin B
ErbB2	erythroblastic leukemia viral oncogene homolog 2, also known as neu or Her2 neuro/glioblastoma derived oncogene homolog
ERK	extracellular signal-regulated kinase
Ets	E-twenty-six
FAP	familial adenomatous polyposis
FAS	fatty acid synthase
Fas	TNF receptor superfamily member
FFA	free fatty acid
FGF	fibroblast growth factor
FLNa	filamin A
floxed	flanked by lox P sites
Fos	Finkel" & "Osteogenic Sarcoma
Foxa3	forkhead box A3
FoxO	forkhead box transcription factor O
FRET	Fluorescence resonance energy transfer

Fz	frizzled
Gab-1	grb2 associated binder1 protein
GAP	GTPase-activating protein
GEF	guanine exchange factor
GI	gastrointestinal
GIR	glucose infusion rate
Grg	groucho
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HB-EGF	heparin-binding EGF
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HepG2	liver carcinoma line
HF	high fat diet
HGF	hepatocyte growth factor
HNF-4	hepatocyte nuclear factor 4
HNPCC	hereditary non-polyposis colorectal cancer
HP	hyperplasia
Hsd17b9	hydroxysteroid (17-beta) dehydrogenase 9
HSL	hormone sensitive lipase
HT29	Human colon adenocarcinoma grade II
IEL	intraepithelial lymphocyte
IFNγ	interferon gamma
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IkB	I-kappa B kinase
IL-6	interleukin-6
ILK	integrin-linked kinase
INF- γ	interferon gamma
iNOS	inducible nitrous oxide synthase
IP	intraperitoneal, injection route
IR	insulin receptor
IRF-1	Interferon regulatory factor-1
IRS	insulin receptor substrate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JNK	Jun-N-terminal kinase
Jun	JU-Nana, Japanese for 17 (avian sarcoma virus 17)
Kap3	kinesin-associated protein 3
kDA	kilo Dalton
Lef	lymphoid enhancer binding factor
Lgs	legless
LKB1	also known as STK11
LOH	loss of heterozygosity
LRP	low density lipoprotein receptor-related protein

L-SACC1	liver-specific Ser503Ala CEACAM1, mutant transgenic mouse line
Lyn	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog protein
MAM	methylazoxymethanol
MAP	mitogen-activated protein, see also ERK
MAPKK	MAP-kinase kinase
MDCK	Madin Darby canine kidney cells
MDM2	transformed 3T3 cell double minute 2
MDR	multidrug resistance gene
MEK	MAP kinase or ERK kinase
MEL6	melanocytic cell line
MHV	mouse hepatitis virus
Min	multiple intestinal neoplasia
MIS	Mullerian inhibiting substance
Mknk2	MAP kinase-interacting serine/threonine kinase 2
MMP7	matrix metalloproteinase 7
MMR	mismatch repair
Mom	modifier of MIN
mRNA	messenger ribonucleic acid
MRO	follicular thyroid carcinoma cell line
MSI	microsatellite instability
mTOR	mammalian target of rapamycin
Muc2	mucin gene 2
Myc	MYeloCytomatosis
NEFA	non-essential fatty acid
NES	nuclear export signal
NF-κB	nuclear factor kappa B
NK	natural killer
Nkd	naked
NOC	N-nitroso compounds
NSAID	non-steroidal anti-inflammatory drug
$p16^{INK4a}$	Cyclin-Dependent Kinase Inhibitor p16
PAK-1	p21-activated kinase-1
PAR1	protease activated receptor 1
PBS	phosphate buffered saline
PC-12	pheochromocytoma of the rat adrenal medulla
PCNA	proliferating cell nuclear antigen
PCP	planar cell polarity
PDGF-R	platelet-derived growth factor receptor
PDIP38	DNA polymerase delta-interacting protein 38
PDK1	3-phosphoinositide dependent protein kinase-1
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occuldens-1 protein (zo-1)
PECAM	Platelet endothelial cell adhesion molecule also known as CD31
PGE2	Prostaglandin E2
PGK	phosphoglycerate kinase promoter

PhIP	2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
PI3K	phophatidylinositol 3-kinase
PIN	prostate intraepithelial neoplasia
PIP2	phosphoinositol 4,5-bisphosphate
PIP3	phosphoinositol 3,4,5-trisphosphate
PJS	Peutz-Jeghers syndrome
PKB	protein kinase B, also known as Akt
PKC	protein kinase C
Pla2g2a	secretory phospholipase
PP2a	Protein Phosphatase 2a
PPAR	peroxisome proliferator-activated receptor
PSG	pregnancy-specific glycoproteins
PTEN	phosphatase and tensin homolog deleted on chromosome ten
Pygo	pygopus
Rac	ras-related C3 botulinum toxin substrate
Raf	murine leukemia viral oncogene homolog 1
Ral	Ras like
RALGDS	Ral guanine nucleotide-dissociation stimulator
Ras	retrovirus associated sequences
Rb	retinoblastoma
RCC	renal cell carcinoma
RGD	Arg-Gly-Asp peptide sequence
RhoA	Ras homolog gene family, member A
RNA	ribonucleic acid
ROS	reactive oxygen species
Rsc	chromatic structure remodeling
S	Standard diet
SAMP	Ser-Ala-Met-Pro
SH2	Src-homology
SHBG	sex-hormone-binding globulin
Shc	Src homology 2 domain containing transforming protein C
SHP	Src-homology domain-containing protein tyrosine phosphatase
Sip1	survival of motor neuron protein interacting protein 1
SiRNA	small interfering RNA
SMAD	mothers against decapentaplegic
SNF	sucrose non-fermenting
Socs2	suppressor of cytokine signaling 2
Sp2	Specificity Protein 2 Transcription Factor
SREBP	sterol regulatory element binding protein
Src	SaRComa
STAT	signal transducers and activators of transcription
Stbm	strabismus
STK11	serine/threonine kinase 11
SWI	switch
TAG	triacylglyceride
Tcf4	T cell-specific transcritption factor 4

TFE3	transcription factor E3
TG	triglyceride
TGF-β	transforming growth factor-β
TGN	trans-Golgi network
T_H1	T helper1
TK	thymidine kinase
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
TP53	transformation related protein 53
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRK	tyrosine receptor kinase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
USF	upstream transcription factor
VEGF	vascular endothelial growth factor
Wnt	wingless-type MMTV (mouse mammary tumor virus) integration site
WRO	thyroid carcinoma cell line
WT	wild type

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ABSTRACT

Carcinoembryonic antigen cell adhesion molecule 1, CEACAM1, is a glycoprotein and a member of the CEA (carcinoembryonic antigen) family of genes. It is expressed on the surface of epithelial, endothelial, and hematopoietic cells, within the gastrointestinal tract, reproductive organs, liver, lungs and kidney. CEACAM1 plays a role in inhibiting the immune response, in cell adhesion, in insulin clearance, in cellular apoptosis and proliferation, in angiogenesis, and functions as a tumor suppressor.

In humans, CEACAM1 is dysregulated and often lower in hyperplastic lesions than in normal tissue, especially in cases of colon, prostate, liver, and 30% of breast cancers. The role of CEACAM1 in tumor formation was studied using the *Ceacam1*$^{-/-}$ mouse model, which sustained systemic ablation of CEACAM1. In the absence of CEACAM1, epithelial cells from small intestine and colon undergo less apoptosis than in wild-type cells. Moreover, there is increased proliferation in the *Ceacam1*$^{-/-}$ colonocytes. As CEACAM1 inactivation alone is not sufficient to induce tumors in the mice, the chemical carcinogen, azoxymethane, was used to induce colon tumors. *Ceacam1*$^{-/-}$ mice developed a higher tumor burden than wild-type mice. One drawback to chemical carcinogenesis is that multiple mutations in unidentified genes cause tumors. To understand the contribution of CEACAM1 to tumor development, the *Ceacam1*$^{-/-}$ mice was mated with the genetically modified mouse *Apc*$^{1638N/+}$ that spontaneously forms small intestinal tumors. Compound *Apc*$^{1638N/+}$*: Ceacam1*$^{-/-}$ *mice* developed more tumors than *Apc*$^{1638N/+}$ mice, and these tumors progressed to a more advanced stage. In addition, *Ceacam1*$^{-/-}$ enterocytes showed compromised Wnt signalling.

The *Ceacam1*$^{-/-}$ mouse is also a model for obesity and CEACAM1 is a key factor in insulin clearance. Due to defective insulin clearance, the *Ceacam1*$^{-/-}$ mice suffer from insulin resistance, and altered lipid synthesis. As these mice age, their weight and abdominal fat increase, and they present signs of hepatic steatosis. In this work, the important roles of CEACAM1 in carcinogenesis and metabolism have been elucidated.

RÉSUMÉ

La glycoprotéine CEACAM1 (Carcinoembryonic antigen cell adhesion molecule 1) est une molécule d'adhésion appartenant à la famille des CEA (Antigène carcinoembryonnaire). CEACAM1 se retrouve à la surface des cellules épithéliales, endothéliales, et hématopoétiques, du tube digestif, des organes reproducteurs, du foie, des poumons et des reins. CEACAM1 présente plusieurs fonctions: inhibition de la réponse immunitaire, adhésion cellulaire, clairance de l'insuline, apoptose et prolifération cellulaire, angiogénèse, et rôle de suppresseur de tumeurs.

L'expression de CEACAM1 est souvent diminuée dans plusieurs cas de cancers humains dont le côlon, le foie, la prostate, et 30% des cancers du sein. Le modèle de souris *Ceacam1*$^{-/-}$ a été utilisé afin d'étudier le rôle de CEACAM1 dans la formation de tumeur. En l'absence de CEACAM1, le tissu épithélial de l'intestin grêle et du côlon subit moins d'apoptose. En contrepartie, une augmentation de la prolifération est détectée dans le côlon des souris *Ceacam1*$^{-/-}$. L'absence de CEACAM1 n'étant pas suffisante pour induire l'apparition de tumeurs, nous avons utilisé un cancérogène chimique, l'azoxymethane. Les tumeurs observées dans le côlon des souris *Ceacam1*$^{-/-}$ s'avèrent être plus importantes que celles des souris contrôles. Le désavantage de l'utilisation de l'azoxymethane est que celui-ci provoque des mutations dans plusieurs gènes plus ou moins définis. Afin de comprendre la contribution de CEACAM1 dans le développement tumoral, la souris *Ceacam1*$^{-/-}$ a été croisée avec la souris *Apc*$^{1638N/+}$ qui forme spontanément des tumeurs dans l'intestin grêle. Les souris *Apc*$^{1638N/+}$*: Ceacam1*$^{-/-}$ développent plus de tumeurs présentant un phénotype tumoral plus agressif que celui formé dans les souris *Apc*$^{1638N/+}$. Par ailleurs, les cellules épithéliales de l'intestin grêle *Ceacam1*$^{-/-}$ montrent une dérégulation de la voie de signalisation Wnt.

CEACAM1 étant un facteur clé dans la clairance de l'insuline par le foie, la souris *Ceacam1*$^{-/-}$ est aussi un modèle d'obésité. En raison d'une clairance d'insuline défectueuse, les souris *Ceacam1*$^{-/-}$ souffrent de la résistance à l'insuline avec une synthèse des lipides modifiée. En vieillissant, le poids et les lipides

abdominaux de ces souris augmentent d'une façon significative par rapport aux souris contrôles. De plus, elles développent des signes de stéatose. Nos travaux montrent que CEACAM1 joue un rôle essentiel dans la cancérogenèse et le métabolisme.

Chapter 1 LITERATURE REVIEW

1.1 Colorectal cancer

Cancer is a leading cause of death worldwide. In 2005, cancer accounted for 13% of all deaths (WHO 2006). However, cancer is not a single disease, but rather many variable conditions that have the potential to lead to death. In terms of prevalence, breast (17.9%), colorectal (11.5%), and prostate (9.6%) cancers are the most common (Parkin, Bray et al. 2005). More than 70% of all cancer deaths in 2005 occurred in low and middle income countries. Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030 (WHO 2006). Colon and rectum cancers accounted for about 1 million new cases in 2002 (9.4% of the world total), and unlike most other types of cancers, numbers were not so different in men and women (ratio, 1.2:1). The highest incidence rates of colorectal cancers are in North America, Australia/New Zealand, Western Europe, and in Japanese men. Incidence tends to be low in Africa and Asia and intermediate in southern parts of South America (Parkin, Bray et al. 2005). These large geographic differences for colon and rectal cancers are probably explained by different environmental exposures. The evidence that the risk of colon cancer is quite labile to environmental change is provided by the study of migrants; when populations moved from low-risk to high-risk areas, the incidence of colorectal cancer increases rapidly within the first generation, implying that dietary and other environmental factors constitute a major component of risk. In general, rates of incidence of colorectal cancer are increasing rather rapidly in countries where overall risk was formerly low (especially in Japan, but also elsewhere in Asia), while in high-risk countries, trends are either gradually increasing, stabilizing (North and West Europe), or declining with time (North America). In fact in the U.S., there has been a 7.8% and 4.2% drop in colon cancer-related

deaths for men and women respectively, from 1991 to 2003 (Jemal, Siegel et al. 2007).

Due to poor diet and sedentary lifestyle, people are not only experiencing the increased risk of developing diabetes mellitus but also cancer. Diabetes has frequently been shown to be associated with colorectal cancer (Hu, Manson et al. 1999; Chang and Ulrich 2003; Larsson, Giovannucci et al. 2005; Limburg, Anderson et al. 2005). Diabetes is marked by resistance to insulin, fasting hyperglycemia, and elevated insulin levels. Insulin is an important growth factor for colonic mucosal cells and colonic carcinoma cells in vitro (Koenuma, Yamori et al. 1989; Watkins, Lewis et al. 1990; Bjork, Nilsson et al. 1993). Cohort studies have demonstrated that overweight and obesity increase the risk of colon cancer, more clearly in men than in women (Bergstrom, Pisani et al. 2001; Sturmer, Buring et al. 2006; van den Brandt and Goldbohm 2006; Sedjo, Byers et al. 2007). Other studies have found that waist circumference, which is a measure of visceral obesity, is a better predictor of colon cancer risk than body mass index (BMI) and showed that risk increases linearly with increasing waist size (Giovannucci, Ascherio et al. 1995; Schoen, Tangen et al. 1999; Moore, Bradlee et al. 2004). In addition to the mechanical effects of obesity, such as an increase in intra-abdominal pressure from large amounts of adipose tissue, substances that adipose tissues secrete, such as tumor necrosis factor-α, interleukin-6, leptin, adiponectin, and insulin-like growth factor-1 (IGF-1) have been proposed to be pathogenic links to gastrointestinal diseases. TNF-α , IL-6 and IGF-1 are adipokines that are known to cause insulin resistance syndrome (John, Irukulla et al. 2006). Obesity is strongly associated with the state of insulin resistance, in which levels of insulin and IGF-1 are elevated. IGF-1 inhibits apoptosis and promotes cell cycle progression, leading to the development of cancer (Aaronson 1991; Kaaks and Lukanova 2001). Obesity is also associated with tissue inflammation, which is mediated by adipose tissue. This in turn correlates to the development of colorectal cancer, especially in patients with ulcerative colitis (Seril, Liao et al. 2003).

1.1.1 Environmental factors

Chronic alcohol consumption has been identified as a significant risk factor for upper gastrointestinal cancer and colorectal cancer (Bongaerts, de Goeij et al. 2007; Moskal, Norat et al. 2007; Tsong, Koh et al. 2007), even though alcohol itself is not carcinogenic. The molecular mechanisms behind this risk factor include the generation of acetaldehyde (AA) and reactive oxygen species (ROS), induction of cytochrome P4502E1 (CYP2E1) and local and nutritional factors. Individuals possess genetic polymorphisms of alcohol-metabolizing enzymes which influences the risk of carcinogenesis. The most carcinogenic and mutagenic agent in alcohol-associated cancer is the first and major metabolite of ethanol, AA. Chronic alcohol consumption results in a striking induction of CYP2E1 in the gastrointestinal mucosa of rodents (Shimizu, Lasker et al. 1990). The induction of CYP2E1 also increases the conversion of various xenobiotics, including procarcinogens (nitrosamines, aflatoxin, vinylchloride, polycyclic hydrocarbons, hydrazines) to their ultimate carcinogens (Seitz, Poschl et al. 1998).

Smoking is most often associated with lung cancer, but it also has effects on the rest of the organism. Several studies indicate that cigarette smoking is associated with an increased risk of rectal cancer (Tsong, Koh et al. 2007). Also smokers report symptoms related to colorectal cancer at an earlier mean age than non smokers, with a difference of about 5.5 years (Buc, Kwiatkowski et al. 2006). Tobacco smoking could be a factor of early onset colorectal cancers.

It has been estimated that 30-40% of all tumors can be forestalled with a correct lifestyle and diet. Several studies indicate that a diet high in vegetables and fruits, and low in meat show a significant correlation with a lower risk of colorectal cancer. Protective elements in a cancer-preventive diet include selenium, folic acid, vitamin B_{12}, vitamin D, chlorophyll and antioxidants such as carotenoids (α-carotene, β-carotene, lycopene, lutein, cryptoxanthin)(Divisi, Di Tommaso et al. 2006). Other anticancer dietary measures include supplements of oral digestive enzymes and probiotics. It is theorized that probiotics can prevent cancer by producing short chain fatty acids in the colon that reduce the levels of

procarcinogenic enzymes such as beta-glucuronidase, nitroreductase and azoreductase (Goldin and Gorbach 1984). There are strong correlations between the risk of large bowel cancer and the consumption patterns of meat, fat and fibre. The association between meat and colorectal cancer can be explained by the presence of N-nitroso compounds (NOCs) formed endogenously within the colon by bacterial decarboxylation of amino acids in the presence of a nitrosating agent (Mirvish 1995; Tricker 1997). Meat increases the levels of nitrogenous residues reaching the colon (Silvester and Cummings 1995), so meat increases colonic levels of NOCs. Many classes of NOC have been identified, a number of which are known to cause DNA damage after the formation of alkylating agents during NOC metabolism (Bingham, Hughes et al. 2002). Nitrites and nitrates, used to bind myoglobin in meat to inhibit botulinic exotoxin production, are also powerful cancerous agents and their actions oppose compounds derived from ascorbic acid (Mirvish, Grandjean et al. 1995). Charcoal cooking and smoke-curing of food also produces harmful carbon compounds such as pyrolysates. Even if precautionary measures can limit the negative consequences, high consumption of amino acids has strong pro-cancerous effects (Bingham, Hughes et al. 2002).

One of the oldest hypotheses in the aetiology of colorectal cancer is the fibre hypothesis. It is based on the observations that colorectal cancer is uncommon in developing countries with a high fibre intake and its biological influence. Fibres contribute to reduce colon cancer in two ways. Insoluble fibres soak up liquids causing them to swell and increase the fecal mass, which reduces the concentration of harmful biliary acids and other potential cancerous agents in excrements. Soluble fibres form gelatinous matrix promoting the elimination of biliary acids and other compounds that are potentially harmful for the colon. Case-control studies observed a decreasing risk with increasing fibre intake (Howe, Benito et al. 1992). Therefore, it is important to consume a diet rich in cereals, whole wheat bread, fruits and vegetables (Divisi, Di Tommaso et al. 2006).

1.1.2 Chemoprevention

Many epidemiological studies have been conducted to identify effective compounds that prevent the incidence of colorectal cancer. Since the 1980s, the most widely studied agents for the chemoprevention of colorectal cancer have been aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), and COX-2 inhibitors. COX-2 and enzymes upstream and downstream of the prostaglandin synthesis pathway are overexpressed in various cancer types. Direct interactions of protaglandins with their receptors through autocrine or paracrine pathways to enhance cellular survival or stimulate angiogenesis have been proposed as the molecular mechanisms underlying the pro-carcinogenic functions of COX-2 (Zha, Yegnasubramanian et al. 2004). Tumors that overexpress COX-2 respond favourably to inhibitors of this enzyme, by undergoing reduced proliferation and increased apoptosis (Chan, Ogino et al. 2007). Cohort and case-control studies indicate that long-term aspirin use prevents colorectal cancer mortality, colorectal cancer and adenomas. The benefits of aspirin were only observed at higher doses and longer duration of treatments. However, this also increases the risks for hemorrhagic stroke and gastrointestinal bleeding (Dube, Rostom et al. 2007). Similar results were observed with COX-2 inhibitors. Cohort and case-control studies indicate that long-term NSAID use, such as sulindac, prevented colorectal cancer and adenoma. In fact, sulindac reduces the growth of existing polyps in familial adenomatous polyp patients (Benamouzig, Uzzan et al. 2005). Again, higher doses and longer duration of treatment with NSAIDs, were more beneficial, but not in all studies. However, nonaspirin NSAIDs and COX-2 inhibitors increased the risk for serious cardiovascular events. Nonaspirin NSAIDs increased the risk for peptic ulcers and gastrointestinal bleeding (Rostom, Dube et al. 2007). Unfortunately, the chemopreventive benefits of these drugs are outweighed by the significant long-term risks associated with each agent. The currently recommended and proven strategies of screening for colorectal cancer decrease the risk of colon cancer by 30% to 70%, which greatly marginalizes the benefits of aspirin and other NSAIDs (Lebwohl and Neugut 2007).

Natural COX-2 inhibitors have also been investigated for their potential in colorectal prevention. Curcumin, the yellow pigment in turmeric has been widely used for centuries in the Asian countries without any toxic effects. Epidemiological data also suggest that curcumin may be responsible for the lower rate of colorectal cancer in these countries (Chauhan 2002). Curcumin is a naturally occurring powerful anti-inflammatory medicine (Rao, Rivenson et al. 1995). It inhibits lipooxygenase activity (Huang, Lysz et al. 1991) and is a specific inhibitor of cyclooxygenase-2 expression (Zhang, Altorki et al. 1999). It also reduces the initiation of carcinogenesis by inhibiting the cytochrome P-450 enzyme activity and increases the levels the glutathione-S-transferase (Singh, Hu et al. 1998). The anti-tumor effect of curcumin has been attributed in part to the arrest of cancer cells in S and G2/M cell cycle phase and induction of apoptosis (Hanif, Qiao et al. 1997; Chen, Zhang et al. 1999). However, a recent report indicates that curcumin does not reduce the number of azoxymethane-induced aberrant crypt foci in middle-aged rats. The loss of preventive activity of curcumin due to aging in a rat model could translate into important implications for human intervention (Kwon and Magnuson 2007). Regardless, further studies are being conducted on COX-2 inhibitors, both pharmaceutical (aspirin, sulindac) and natural (curcumin), and their chemopreventive abilities. In fact, novel drugs based on nitric oxide-releasing aspirin and indomethacin are potent inhibitors of colon cancer development without the cardiac effects ascribed to the earlier generation of drugs (Rao, Reddy et al. 2006).

1.2 Structure of small intestine

The epithelium of the small intestine is responsible for the digestion and absorption of nutrients. As such these cells are highly specialized and metabolically active. The small intestine is organized in finger-like villus structures, directly adjacent and above invaginations called crypts of Lieberkühn, see figure1.2.1. The epithelial cells are derived from stem cells located in the middle of the crypt (Nathke 2004). Asymmetric division is essential to ensure maintenance of stem cell numbers and final homeostasis of the intestinal

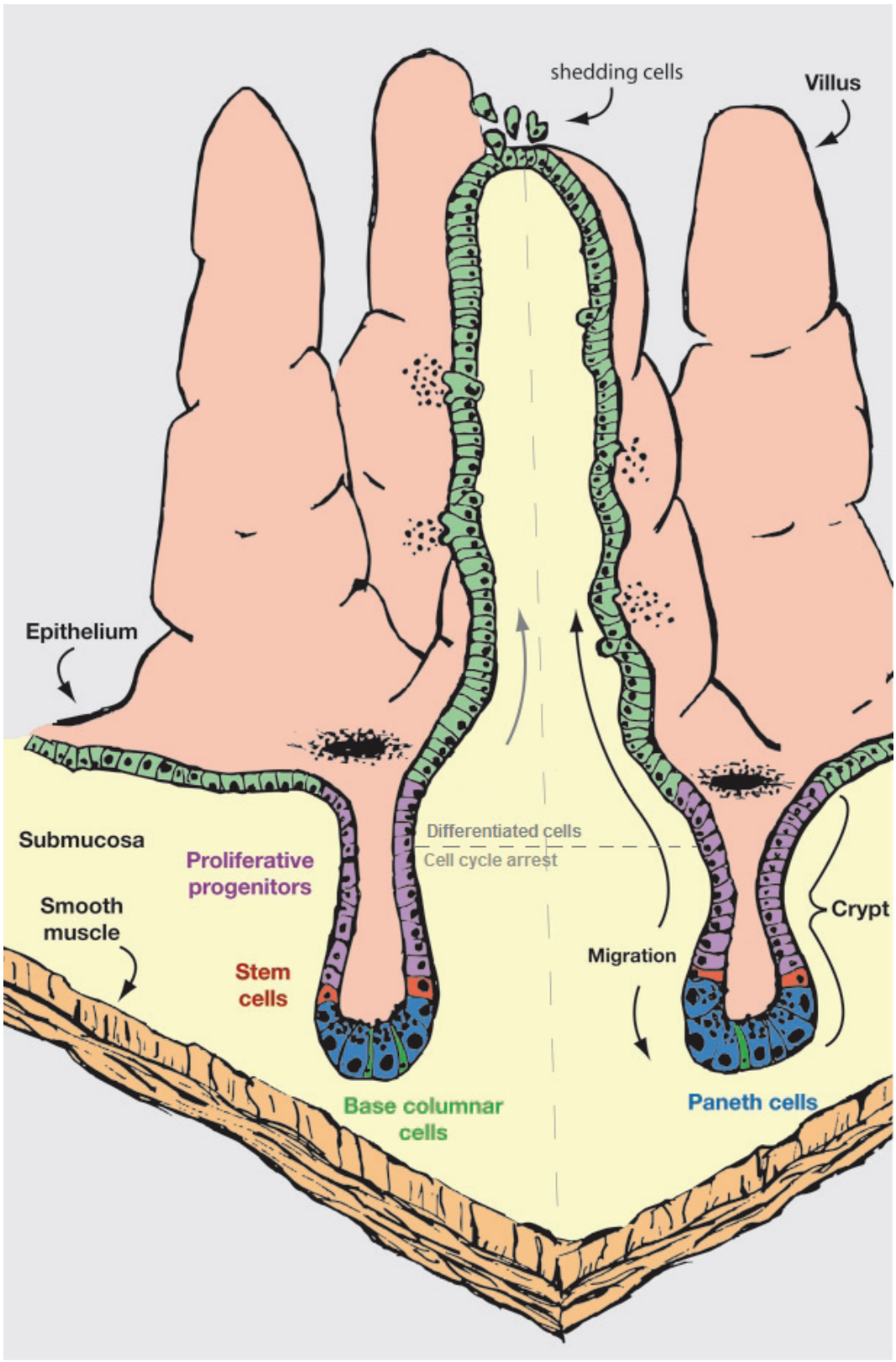
epithelium. There are around 4-6 stem cells per crypt. These cells produce progenitors, which appear undifferentiated in the crypt but eventually produce four cell types: enterocytes, enteroendocrine cells, Paneth cells, and goblet cells (de Santa Barbara, van den Brink et al. 2003). When these cells migrate toward the crypt-villous junction they undergo morphological changes. Once they exit the crypt compartment their differentiation is complete. Enterocytes are the most abundant intestinal epithelial cells (up to 80% of all epithelial cells). Enterocytes are columnar cells exposing apical microvilli, which exponentially increase the absorptive surface; they are also connected to neighbouring cells by lateral junctions. Enterocytes have hydrolytic and absorptive functions and are responsible for degradation of nutrients. Goblet cells are scattered from the middle of the crypt to the tip of the villus and they represent 5% of the small intestine epithelial cells. The turnover of enterocytes and goblet cells is estimated in mouse at around 3 days (de Santa Barbara, van den Brink et al. 2003). Enteroendocrine cells produce numerous hormones that assist in regulating gastrointestinal motility, absorption and digestion. Paneth cells, in contrast to the other intestinal epithelial cell types, have a longer turnover period of about 20 days. Ten Paneth cells are present per crypt; they do not migrate like the rest of the cells and remain at the bottom of crypts. Paneth function is mostly associated with the antimicrobial defence of the intestine (Ayabe, Satchell et al. 2000).

1.3 Structure of the colon

The colon is organized in invaginations representing a compressed version of the crypt-villus architecture, and also undergoes rapid cell turnover. The mature colon epithelium has mainly two differentiated cell types: the enterocyte and goblet cell. The colon also has endocrine cells. The goblet cells are mainly found in the midcrypt, whereas the absorptive enterocytes (or colonocytes) are found at the surface (or top of the crypt), see figure 1.3.1. Endocrine cells are found in highest numbers at the base of the crypt (de Santa Barbara, van den Brink et al. 2003)

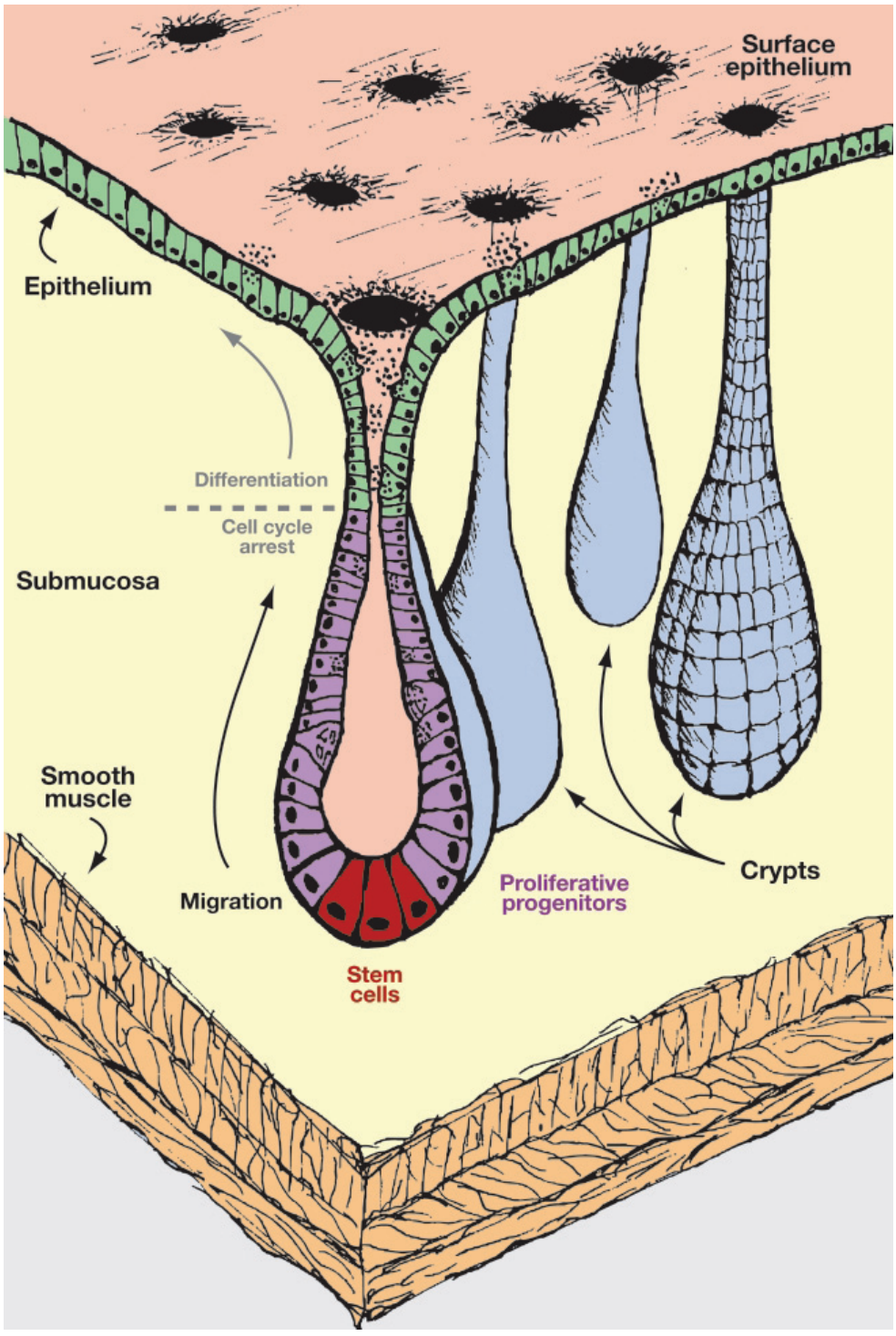
Figure 1.2.1: The structure of the adult small intestine.

Putative stem cells reside immediately above the Paneth cells. Base columnar cells, intermingled between the Paneth cells, may also behave as stem cells. Progenitors stop proliferating at the crypt-villus junction and express differentiation markers. Enteroendocrine, absorptive, and mucus-secreting cells migrate upward, whereas Paneth cells migrate downward and localize at the bottom of the crypts (Sancho, Batlle et al. 2004). Reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 20 (c) 2004 by Annual Reviews www.annualreviews.org


shedding cells
Villus
Epithelium
Submucosa
Proliferative progenitors
Smooth muscle
Stem cells
Differentiated cells
Cell cycle arrest
Migration
Crypt
Base columnar cells
Paneth cells

.

Figure 1.3.1: Structure of the large intestine.
Stem cells reside at the crypt bottom. Progenitors are amplified by constant division along the bottom two thirds of the crypts. Paneth cells are absent in the large intestine. Cell cycle arrest and differentiation occur when progenitors reach the top third of the crypts. (Sancho, Batlle et al. 2004). Reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 20 (c) 2004 by Annual Reviews www.annualreviews.org


Surface epithelium
Epithelium
Differentiation
Cell cycle arrest
Submucosa
Smooth muscle
Migration
Proliferative progenitors
Crypts
Stem cells

1.4 GI epithelia

The digestive tube is regularly exposed to toxins from the foods we eat, and shear stress from the passage of feces, which increases the risks of developing mutations and disease. In normal intestinal physiology there is a rapid turnover of cells from the crypt to the top of the villus, in which a cell starting at the bottom of a crypt will transit and undergo anoikis at the top of the villus in 3-5 days (Sancho, Batlle et al. 2004). This is a way to prevent the accumulation of oncogenic mutations likely to occur in such a hostile environment. This rapid turnover dictates that the accumulation of cells to form a polyp and adenoma requires the slowing of the epithelial cells. It is not known whether initiation of cancer originates in the stem cell or migrating cell compartment. Due to the rapid transit of cells, it is more probable that the initiation of neoplasia occurs in the stem cells.

Many of the same factors important in embryologic pattern formation of the gut are also important in pattern formation of the adult organ. Several Wnt factors are expressed in the adult gut (Gregorieff, Pinto et al. 2005) and significant evidence documents that the Wnt pathway is important in the crypt. β-catenin is present in all membranes along the crypt-villus unit, but nuclear accumulation of β-catenin is specifically found in the epithelial cells located in the bottom third of the small intestine crypt and at the bottom of the colonic crypt (van de Wetering 2002). *Tcf4*, an important transcription factor that associates with β-catenin, is found expressed in the gut epithelium throughout life. *Tcf4* is expressed in a gradient highest in the cells at the base of the crypt (Barker, Huls et al. 1999). Embryonic cells have the capacity to rapidly proliferate indefinitely, a feature that is shared by cancer cells. Therefore many of the factors implicated in embryonic development have been investigated in cancer studies.

1.5 WNT signalling

Wnts are a large family of secreted glycoproteins with at least 19 known human members that are expressed in species ranging from *Drosophila* to man. They play key roles in cell fate specification, CNS patterning, and control of

asymmetric cell division (Miller 2002). Canonical Wnt/β-catenin pathway leads to the stabilization of cytoplasmic β-catenin which subsequently enters the nucleus where, in combination with transcription factors of the TCF/LEF family, it regulates gene expression. This pathway is highly conserved between species and has been described in *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, chicken, and mouse. The genetic program initiated by β-catenin and TCF/LEF transcription factors specifies the transcription of a specific subset of genes, mainly determining cell fate and regulating proliferation. Signalling through this pathway is present during embryogenesis, where it has been shown to regulate many developmental patterning events in organism ranging from worm to man. In the developing vertebrate embryo, the formation of the dorsal-ventral axis depends on the activity of the Wnt signalling pathway. Transcription of Wnt family genes appears to be developmentally regulated in a precise temporal and spatial manner (de Santa Barbara, van den Brink et al. 2003).

Wnt signalling is initiated following Wnt ligand binding to a member of the Frizzled (Fz) family of seven-span transmembrane receptors, together with the co-receptors LRP-5 or LRP-6. Canonical Wnt signalling is only mediated when both Fz and LRP are complexed with Wnt. Most Wnt proteins can bind to multiple Fzs and vice versa, suggesting redundancy in vivo (Giles, van Es et al. 2003). Dickkopf (Dkk) proteins are potent secreted inhibitors of Wnt signalling. Dkk blocks Wnt signalling by binding LRP-6 in a way that sterically hinders Wnt binding (Mao, Wu et al. 2001). Wnt binding to Fz results in hyperphosphorylation of Dishevelled (DSH), which inhibits the activity of GSK-3β (Yanagawa, van Leeuwen et al. 1995). Dsh is at the intersection of Wnt signalling traffic. Two other pathways branch off at the level of Dsh. The non-canonical Wnt pathways transduce Wnt signal to either the JNK pathway or the Ca^{2+}-releasing pathway through Fz receptors, see figure 1.5.1. The cell polarity pathway (PCP) involves the small GTPases rho and cdc42 as well as the Jun-N-terminal kinase (JNK) (Weber, Paricio et al. 2000). Wnt/JNK pathway exists in vertebrates to regulate morphogenetic movements during gastrulation and can be triggered by Wnt-11 and Wnt-5A (Heisenberg, Tada et al. 2000; Yamanaka,

Moriguchi et al. 2002). Three different Ca^{2+}-sensitive enzymes can be activated by Wnts: PKC, CamKII, and calcineurin (Pandur, Maurus et al. 2002). In cell culture JNK can also be activated by calcium signalling (Enslen, Tokumitsu et al. 1996). PKC is part of the Wnt/Ca^{2+} pathway; it is activated by Wnt-5A, which is able to elicit Ca^{2+}-release in zebrafish in a G-protein-dependent manner. PKC may also be a mediator in the signal transduction cascade that leads to the activation of JNK (Su, Jacinto et al. 1994). Proteins that bind Dsh decide its participation in either canonical or non-canonical Wnt signalling. Casein kinase I (CK1) can phosphorylate Dsh and actively promote Dsh function in canonical Wnt signalling (Peters, McKay et al. 1999). Conversely, Naked cuticle (Nkd) is a feedback inhibitor that blocks Dsh function in canonical Wnt signalling but can also activate PCP-like signal in vertebrates (Yan, Wallingford et al. 2001). Dapper is also a negative inhibitor of β-catenin signalling. Five proteins: Stbm, Nkd, CKI, Dapper, and PKC promote activity of one pathway and inhibit the other through their interaction with Dsh (Giles, van Es et al. 2003).

1.5.1 Inactive Wnt signalling

In the absence of canonical Wnt signalling, GSK3β phosphorylates β-catenin at four amino-terminal residues, targeting it for binding to the F-box protein b-TrCP and subsequent ubiquitination and degradation by the proteosome (Hart, Concordet et al. 1999). β-catenin is the key mediator of the Wnt signal. It was originally identified as a component of the adherens junctions, where it links E-cadherin to α-catenin and consequently, the actin microfilament network of the cytoskeleton (Giles, van Es et al. 2003). GSK3β is active in unstimulated resting cells and is a key component in many signalling pathways including Wnt. GSK3β substrates are generally functionally inhibited by GSK3β, as is the case with β-catenin. GSK3β phosphorylates APC at conserved SXXXS motifs in the seven repeated 20 amino acid sequences. This allows binding and serine phosphorylation of free cytoplasmic β-catenin by APC and GSK3β respectively (Rubinfeld, Albert et al. 1996). This alteration in phosphorylation targets β-

catenin for degradation by the ubiquitin/proteasome pathway (Aberle, Bauer et al. 1997). Before phosphorylation of β-catenin by GSK3β can occur, CKI phosphorylates β-catenin at Ser-45 creating a priming site which is necessary and sufficient for GSK3β to subsequently phosphorylate the remaining sites at Thr-41, Ser-37, and Ser-33 (Liu, Li et al. 2002). Diversin has been shown to recruit CKI to the degradation complex and facilitates phosphorylation of β-catenin. Diversin is also involved in JNK activation and has been shown to suppress Wnt signals (Schwarz-Romond, Asbrand et al. 2002). A quaternary complex of Axin, GSK3β, Dsh and Frat1 exists in unstimulated cells, see figure 1.5.1.

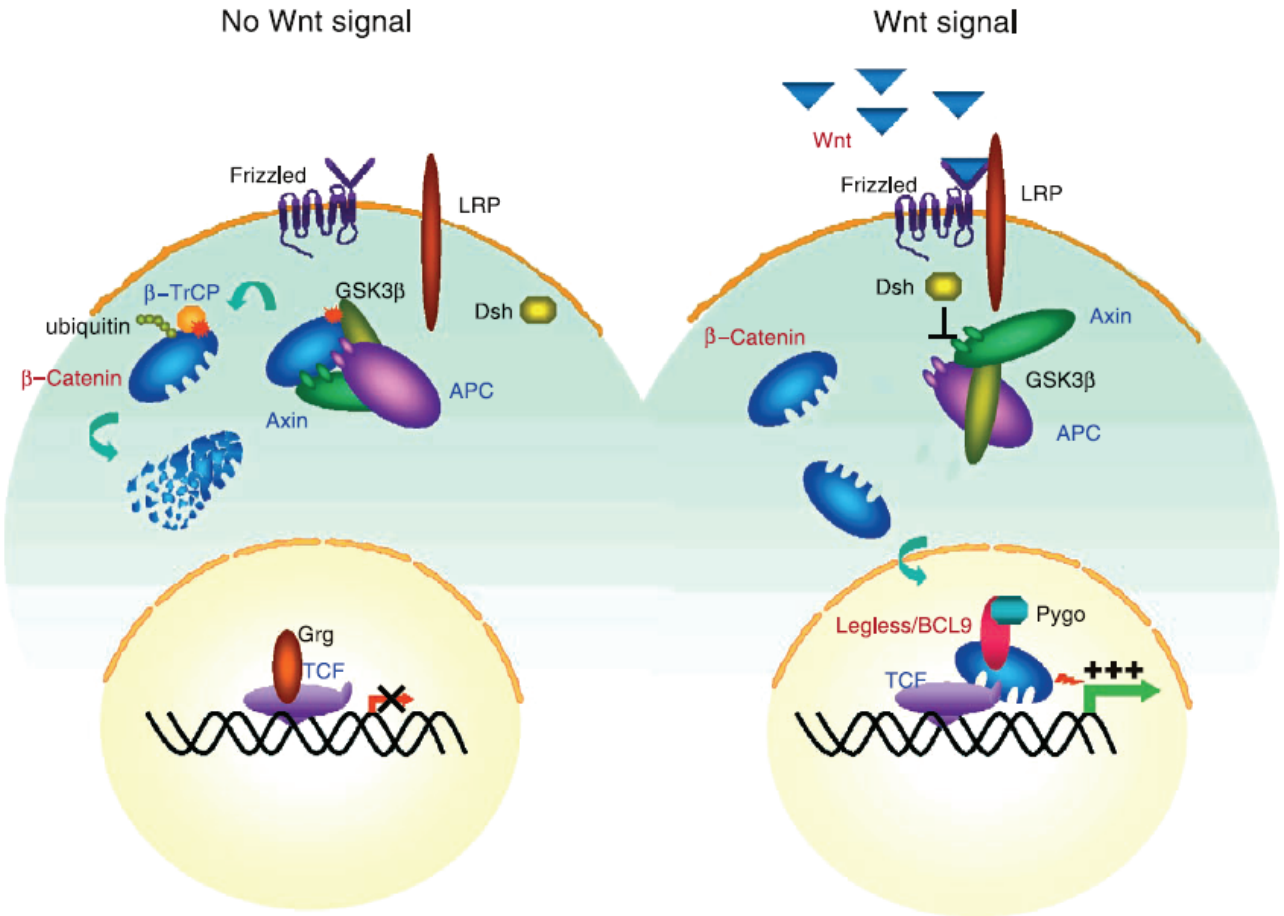
1.5.2 Active Wnt signalling

Following Wnt stimulation, Dsh is recruited to the membrane and phosphorylated which is postulated to cause a conformational change allowing the Frat1-mediated disassociation of GSK3β from axin. With GSK3β no longer bound to axin, phosphorylation of β-catenin does not occur and the cytosolic levels of β-catenin accumulate and translocate to the nucleus. The alternative scenario is that recruitment of Dsh to the membrane triggers dephosphorylation of the multiprotein complex responsible for β-catenin degradation by protein phosphatase 2A (Giles, van Es et al. 2003). Axin and APC form a structural scaffold that allows GSK3β to specifically phosphorylate β-catenin, as well as APC and axin. Phosphorylation of axin by GSK3β is important for its stability (Yamamoto, Kishida et al. 1999), see figure 1.5.1. Axin is an inhibitor of Wnt signalling and its overexpression can compensate for loss of APC which is not absolutely necessary for the proper β-catenin degradation complex (Giles, van Es et al. 2003).

1.5.3 In the nucleus

β-catenin does not bind DNA itself, but is an essential cofactor for TCF/LEF transcription factors. The acetyltransferase CBP (CREB-binding protein) acetylates β-catenin at lysine 49 and this alteration has been reported to

Figure 1.5.1: The Wnt signaling cascade, simplified.
Left: in the absence of Wnt ligand, β-catenin levels are efficiently regulated by a complex containing APC, Axin, and GSK3β. Transcription of TCF target genes is repressed by the presence of Grg co-repressors. Right: Wnt ligand destabilizes the β-catenin degradation complex, allowing transportation to the nucleus. Once there, β-catenin recruits Lgs/BCL9 and pygo and activates TCF target genes. Known oncogenes are lettered in pink; known tumor suppressors are lettered in blue. Reprinted from Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, Vol. 1653, Rachel H. Giles, Johan H. van Es and Hans Clevers, Caught up in a Wnt storm: Wnt signaling in cancer, pp. 1-24., Copyright (2003), with permission from Elsevier.


No Wnt signal
Wnt signal
Frizzled
LRP
β–TrCP
ubiquitin
β–Catenin
GSK3β
Dsh
APC
Axin
Grg
TCF
Wnt
Frizzled
LRP
Dsh
Axin
β–Catenin
GSK3β
APC
Legless/BCL9
Pygo
TCF
+++

improve transactivation of the *c-MYC* locus (Wolf, Rodova et al. 2002). β-catenin recruits Brg-1, a component of the mammalian SWI/SNF and Rsc chromatin-remodeling complexes, to TCF target gene promoters, facilitating chromatin remodelling as a prerequisite for transcriptional activation (Barker, Hurlstone et al. 2001). In the absence of Wnt signal, TCF/LEF binds a family of broadly expressed transcriptional repressors, called Grg or groucho proteins (Roose, Molenaar et al. 1998) which actively repress transcription of TCF target genes by recruiting histone deacetylases which subsequently act to condense chromatin (Chen, Fernandez et al. 1999), see figure 1.5.1.

The TCF (T-cell factor)/Lef (lymphoid enhancer factor) family of DNA binding proteins control transcription of an expanding range of Wnt responsive genes. hTCF-4, is the predominantly expressed family member in the colonic epithelium (Korinek 1997). Genetically engineered Tcf-4 knockout mice show a complete loss of intestinal crypts stem cells and die early (Korinek, Barker et al. 1998). Normally the carboxy terminal of hTCF-4 is bound by its C-terminal binding protein and this interaction represses transcription of downstream target genes. When the amino terminal interacts with β-catenin, the HMG domain is able to alter its conformation and interact with its DNA target sequence. Nuclear β-catenin/TCF activity was recently shown to be regulated by legless and pygopus (Kramps, Peter et al. 2002). Lgs is a nuclear protein orthologous to BCL9, a known oncogene involved in the development of non-Hodgkin's lymphoma. LEF1 is a so-called context-dependent activation domain which instigates transactivation in the presence of the coactivator ALY (Bruhn, Munnerlyn et al. 1997). hTCF-4 consensus sequences have been demonstrated within the promoters of a variety of oncogenes and cell cycle regulators (Bright-Thomas and Hargest 2003). C-myc, cyclin D1, PPARδ, MDR-1 (multidrug resistance gene), MMP7 (matrilysin gene) and gastrin have been identified as downstream targets of activated TCFs (Bright-Thomas and Hargest 2003).

1.5.4 Deregulated Wnt signalling

The common *APC* mutations causing CRC lead to a loss of the majority of β-catenin degradation sites and the associated threonine/serine GSK3β phosphorylation sites. Thus the mutant APC proteins can bind but not down-regulate β-catenin and levels of free cytoplasmic β-catenin are uncontrolled. The free protein associates with hTCF-4 or its homologues, to form a bipartite transcription factor that enters the nucleus (Huber 1999) and promotes abnormal transcription of a variety of Wnt responsive genes. Phosphorylation changes in β-catenin that promote epithelial migration during development reduce the tumor suppressive action of the adherens junction. Other signal pathways also have an effect on Wnt/β-catenin signalling, for example activation of receptor tyrosine kinases and *ras* oncogenes result in tyrosine phosphorylation of β-catenin (Bright-Thomas and Hargest 2003). Signalling via cell surface receptor tyrosine kinases also leads to phosphorylation of GSK3β through the phosphinositide 3-kinase pathway (Eldar-Finkelman, Seger et al. 1995), see figure 1.6.4.2. Thus tyrosine phosphorylation both enhances the release of β-catenin from the adherens junction and stabilizes the free protein within the cell.

1.6 Genetic changes in cancer

Although greatly affected by environmental factors, cancer is essentially a genetic disease. But unlike other genetic diseases, it involves somatic mutations. There is an important correlation between age and the incidence of cancer. Most GI cancers appear in the sixth to seventh decade of life. One hypothesis to explain this correlation is that 3 to 7 genes must be mutated in order to form a malignant tumor (Miller 1980), which requires time to accumulate these mutations. Numerous steps are involved in the progression of normal tissue from dysplasia to malignancy. As long as mutations confer a net growth advantage to cells, these can expand in a clonal fashion, and accumulate more advantageous mutations. There is still some debate as to whether tumors develop in a clonal manner. Work on chimeric mice and a sex chromosome mixoploid patient with

FAP suggested that up to 76% of early adenomas are polyclonal. It is likely that short-range interaction between adjacent initiated crypts is responsible for polyclonality (Leedham, Schier et al. 2005).

In colon cancer aetiology, mutations are often found in three different classes of genes: oncogenes, tumor suppressor genes, and mismatch repair genes (Weinberg 1994; Chung and Rustgi 1995). Oncogenes are normal genes responsible for controlled proliferation. If they are mutated, they result in uncontrolled proliferation and ultimately cancer (Sherr 1996). Tumor suppressor genes were first described by Knudson in his study of the epidemiology of childhood retinoblastoma (Knudson 1971; Knudson 1985). When mutated, these produce cancer in a recessive fashion, meaning that both copies must be inactivated in order to lose gene function. Knudson's two-hit hypothesis based on this definition, explains why inherited disease usually manifests at an earlier age than sporadic disease. Mismatch repair (MMR) genes are associated with the mutator phenotype. Cells with mutations in MMR genes in both alleles accumulate DNA errors throughout the genome, often affecting growth regulatory genes (Markowitz, Wang et al. 1995).

1.6.1 Multistep model

Fearon and Vogelstein have described the molecular basis for sporadic colon cancer as a multistep model of carcinogenesis (Fearon and Vogelstein 1990). The model describes that each accumulated genetic event or mutation confers a selective growth advantage to an affected colon cell. The cause of sporadic colon cancer is the cumulative effect of somatic mutations. According to this model, the accumulation of multiple genetic mutations rather than the sequence of mutations would determine the biological behaviour of the tumor, although *APC* mutations usually occur early in the process and mutations of the *p53* suppressor gene usually happen later, see figure 1.6.1. Aberrant crypt foci (ACF) are tiny lesions at the earliest stage of colorectal carcinogenesis, which consist of large, thick crypts identified by dense, methylene blue staining. The number of ACF, especially dysplastic ACF, increases significantly from normal

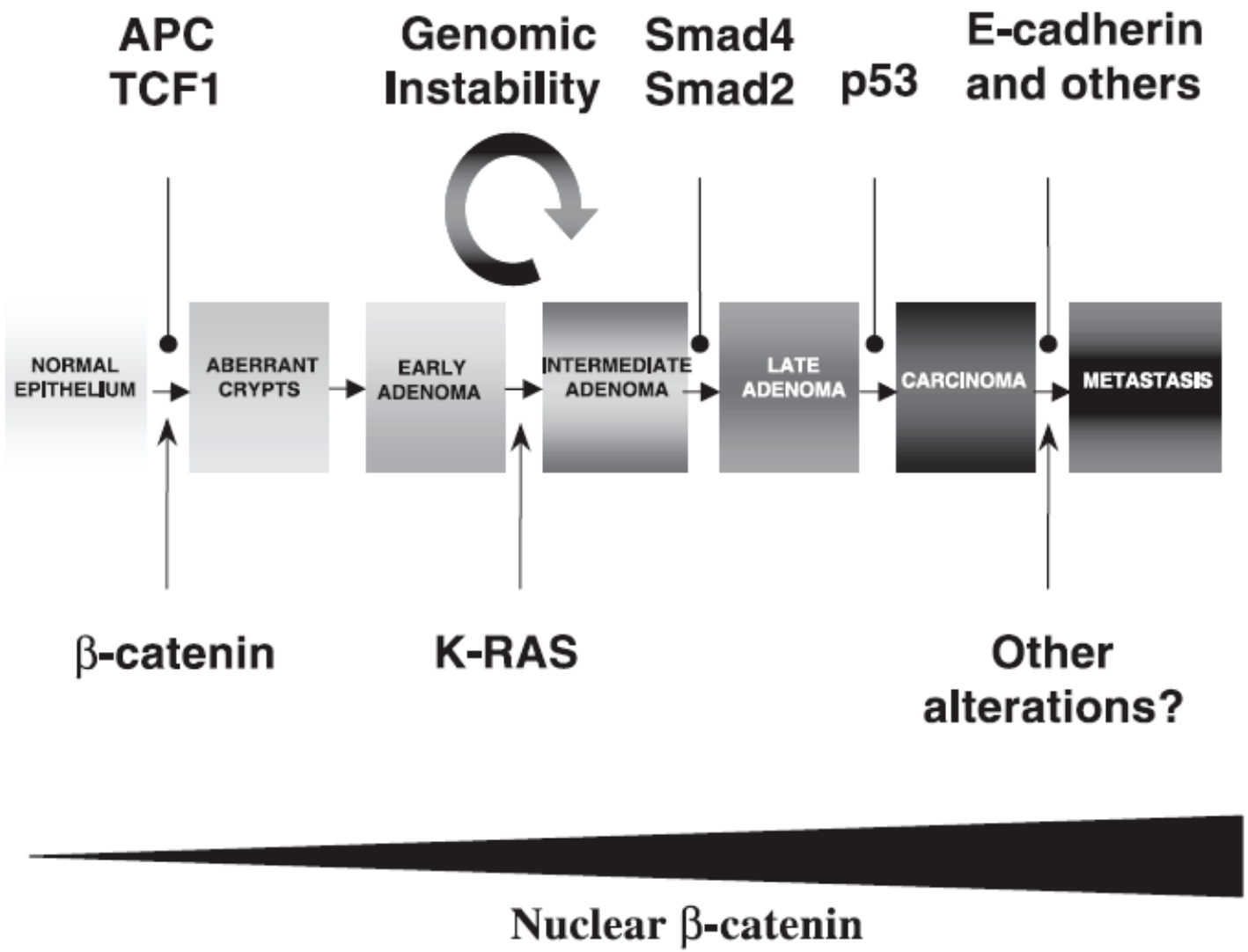
subjects to adenoma patients and then to cancer patients. The number, size and dysplastic features of ACF are significantly correlated with the number of adenomas in patients. Thus, it was surmised that ACF are precursor lesions of the adenoma-carcinoma sequence in humans (Niitsu, Takayama et al. 2004).

Sporadic colon cases have very heterogeneous genetic mutations. However, mutations in *APC* are detected in >80% of tumors, also >50% have *Ras* mutations. *P53* is known as the "guardian of the genome" and as such, it is the most commonly mutated gene in human cancer, with up to 75% of sporadic colorectal tumors exhibiting *p53* inactivation (Calvert and Frucht 2002). In 1989, the gene *deleted in colorectal carcinoma* (*DCC*) was identified. It is located on the long arm of chromosome 18, and 70% of colorectal carcinomas and 50% of cases of advanced adenomas have been shown to have *DCC* mutations. The *DCC* gene encodes a transmembrane receptor for netrins, key factors in axon guidance in the developing nervous system (Keino-Masu, Masu et al. 1996; Leonardo, Hinck et al. 1997). The lack of tumor predisposition in mice heterozygous for *DCC* inactivating mutations (Fazeli, Dickinson et al. 1997), and the presence of other known candidates of tumor suppressor genes on chromosome 18q raised questions about *DCC*'s role as a tumor suppressor gene (Thiagalingam, Lengauer et al. 1996). However, interest in the contribution of *DCC* inactivation to cancer has not completely waned. *DCC* may activate downstream signalling pathways, when engaged by netrins. In the absence or low levels of netrin, *DCC* can promote apoptosis (Mehlen and Fearon 2004). The other candidate tumor suppressor gene on chromosome 18q includes DPC4/Smad4. The SMAD family of proteins are intracellular proteins that mediate the effects of signalling from extracellular transforming growth factor (TGF)-β and TGF-β-related factors. Of these, Smad4 is essential for signalling by TGF-β (Zhou, Buckhaults et al. 1998) and BMP factors (Candia, Watabe et al. 1997).

1.6.2 Hereditary colorectal cancers

Approximately 3-5% of colorectal cancer cases are hereditary, indicating genetic modifications in these individuals (Kaz and Brentnall 2006). The clustering of

Figure 1.6.1: The adenoma–carcinoma sequence for colorectal cancer.
A mutation in *APC* or β-catenin results in the activation of the Wnt signalling pathway, triggering tumor formation. Subsequent progression towards malignancy is accompanied by sequential mutations in KRAS, deletion of chromosome 18q affecting genes encoding SMAD2 and SMAD4, p53, and genes involved in tumor invasiveness such as E-cadherin. Tumor suppressor proteins are represented above the adenoma– carcinoma sequence, whereas oncogenes are depicted below. Increasing levels of nuclear β-catenin accompany tumor progression. Reprinted from Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, Vol. 1653, Rachel H. Giles, Johan H. van Es and Hans Clevers, Caught up in a Wnt storm: Wnt signaling in cancer, pp. 1-24., Copyright (2003), with permission from Elsevier.


APC
TCF1
Genomic
Instability
Smad4
Smad2
p53
E-cadherin
and others
NORMAL EPITHELIUM
ABERRANT CRYPTS
EARLY ADENOMA
INTERMEDIATE ADENOMA
LATE ADENOMA
CARCINOMA
METASTASIS
β-catenin
K-RAS
Other alterations?
Nuclear β-catenin

Familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC) and Peutz-Jeghers syndrome (PJS) has helped identify the genes involved in cancer development. In the case of HNPCC, the mismatch repair genes are mutated, in PJS it is a loss of function of the Ser/Thr kinase LKB1 and in FAP, the gene *APC* (*Adenomatous polyposis coli*) is mutated.

1.6.2.1 Hereditary non-polyposis colorectal cancer

HPNCC in characterized by early onset, colorectal, endometrial, gastric, and genitourinary cancers. The cause of HNPCC was discovered through the observation that HNPCC-associated cancer exhibited microsatellite instability (MSI), also known as the replication error phenotype (Calvert and Frucht 2002). Microsatellites consist of multplite di- and trinucleotide repeats throughout the genome. DNA mismatch repair (MMR) genes are responsible for recognizing and repairing single base and large strand slippage mismatches during DNA replication, and are often referred as "caretaker" genes (Leedham, Thliveris et al. 2005). Mutations in all six MMR genes have been found in HNPCC patients, but the most common germline mutations are in *hMLH1*, *hMSH2* and *hPMS2* genes (Kinzler and Vogelstein 1996). Mutations in MMR genes result in abnormal sequences of parts of the DNA, which leads to MSI and accelerated tumorigenesis. Patients who suffer from HNPCC have few polyps, but these are very likely to progress to cancer, especially once they acquire *APC* mutations.

1.6.2.2 Peutz-Jeghers syndrome

Peutz-Jeghers syndrome is an autosomal dominant disease, associated with germline mutations in *LKB1* (also known as *STK11*). It is a disorder with predisposition to gastrointestinal polyposis and cancer. The *LKB1* gene is very rarely mutated or epigenetically silenced in sporadic tumors (Avizienyte, Loukola et al. 1999). Heterozygosity for PJS is characterized by gastrointestinal polyps, but these polyps (harmatomas) possess low malignant potential, comprised of disorganized non-dysplastic gastrointestinal mucosa with prominent branching smooth muscle component. LKB1 encodes a Ser/Thr protein kinase that is widely

expressed in all tissues. No downstream targets of LKB1 kinase activity have been identified, but LKB1 can direct the phosphorylation and activity of the serine-threonine kinase PAR1A (Spicer, Rayter et al. 2003). PAR1A is implicated as a positive regulator of the Wnt/β-catenin signalling pathway. LKB1 can modify transcription driven by the Wnt-regulated TCF response element.

Lkb1 deficiency prevents culture-induced senescence without loss of *Ink4a/Arf* or *p53*. This suggests that loss of Lkb1 functions as an early neoplastic event and renders cells resistant to subsequent oncogene-induced transformation (Bardeesy, Sinha et al. 2002). There is some evidence that LKB1 physically associates with p53 and regulates specific p53-dependent apoptosis pathways. It is present in the cytoplasm and nucleus of cells and translocates to mitochondria during apoptosis (Karuman, Gozani et al. 2001). The order of mutational events may be an important parameter in dictating the type of cooperating mutations and the malignant potential of the initiated neoplasm: early loss of LKB1 may promote strictly benign neoplasia, whereas loss of LKB1 in a later stage lesion could facilitate malignant progression (Bardeesy, Sinha et al. 2002). LKB1 affects proliferation and cell fate through the Wnt pathway, cell survival by modulating the action of p53, and can alter the microenvironment.

1.6.2.3 Familial Adenomatous polyposis

FAP is an autosomal dominant condition which is characterized by multiple benign adenomatous polyps in the colon and rectum. Among hundreds to thousands of adenomatous polyps in an affected individual, some progress into invasive tumor and metastasis. The polyps usually appear by adolescence or third decade of life. The risk of cancer is generally considered to be related to the polyp number (Debinski, Love et al. 1996). The incidence of FAP in the population is approximately 1 in 8000 (Bisgaard, Fenger et al. 1994). Colorectal tumors from FAP patients harbor either additional somatic *APC* mutations or loss of heterozygosity at *APC* locus in addition to the germ-line mutation, following Knudsen's two-hit hypothesis (Lamlum, Ilyas et al. 1999).

Attenuated FAP (AFAP) is characterized by the presence of less than 100 adenomatous polyps but still carrying a significantly increased risk of the development of colorectal cancer (Jones, Jagelman et al. 1986). A variant of FAP, known as Gardner syndrome, refers to the association of colonic polyps with rectoepidermoid skin cysts and benign-osteoid tumors of the mandible and long bones (Lotfi, Dozois et al. 1989). Desmoid tumors, which usually arise in abdominal wall or bowel mesentery, are a cause of significant morbidity and mortality in FAP patients (Jones, Jagelman et al. 1986; Lotfi, Dozois et al. 1989). Hereditary desmoid disease, also attributed to mutations in the *APC* gene (Lotfi, Dozois et al. 1989; Eccles, van der Luijt et al. 1996), is characterized by autosomal dominant inheritance of multiple desmoid tumors in absence of colonic polyposis. The other manifestation of FAP is Turcot's syndrome, which refers to the association between multiple colorectal polyps and medulloblastoma, a primary brain tumor found in the cerebellum of children (Hamilton, Liu et al. 1995). Mutations in the *APC* gene also lead to various other clinical conditions such as papillary carcinoma of the thyroid and adrenocortical adenoma (Naylor and Gardner 1981; Kartheuser, Walon et al. 1999).

1.6.3 Apc

Mutations in the adenomatous polyposis coli (APC) gene are responsible for familial adenomatous polyposis (FAP), and also play a rate-limiting role in the majority of sporadic colorectal cancers. Loss of APC function triggers the chain of molecular and histological changes involved in the adenoma-carcinoma sequence (Fodde 2002).

1.6.3.1 Structure of APC

Inactivation of APC function is recognized as the initiating event in tumorigenesis. The gene *APC* encodes a large (2843 amino acids) multifunctional protein that is highly conserved with homologues in a number of species including *Drosophila*, *Caenahabditis elegans*, mouse, rat, *Xenopus*, zebrafish, and human (Nathke 2004). The structure of APC is described in figure 1.6.3.1. The

N-terminus of APC contains multiple regions of heptad repeats that are predicted to form coiled-coil domains that are involved in oligomerization. APC has two groups of nuclear export signals (NES) and both are required for shuttling of APC between nucleus and cytoplasm (Henderson 2000; Neufeld, Nix et al. 2000).

The heptad repeats in the N-terminal region of APC span a set of armadillo repeats, and this region is part of the stable, well-ordered portion of APC. Proteins that bind to this region of APC include the regulatory subunit of phosphatase 2a (PP2a) (Seeling, Miller et al. 1999), APC-stimulated guanine nucleotide exchange factor (GEF) for Rho family proteins (Asef) (Kawasaki, Senda et al. 2000), and Kap3, a linker protein for kinesins (Jimbo, Kawasaki et al. 2002). Isolated N-terminal fragments of APC stimulate the exchange activity of Asef more strongly than the full-length APC protein (Kawasaki, Sato et al. 2003).

The middle of the APC protein contains the domains important for interactions with proteins in the Wnt signalling pathway and is sufficient to function on its own in this pathway (Rubinfeld, Albert et al. 1996). It contains three to four 15-amino acid repeats that can constitutively bind to β-catenin (Rubinfeld, Souza et al. 1993; Rubinfeld, Albert et al. 1996). Following these domains are seven 20-amino acid repeats that also bind to β-catenin but are regulated by phosphorylation (Rubinfeld, Albert et al. 1996). Interspersed are SAMP sequences that are involved in binding to axin. All wild-type APC proteins identified so far can participate in the Wnt signalling pathway and interact with β-catenin, axin, and GSK3β (Nakagawa, Murata et al. 1998; van Es, Kirkpatrick et al. 1999; Nakagawa, Koyama et al. 2000).

The C-terminal region of APC contains motifs that mediate interactions with a number of structural proteins. APC can bind to EB1, a small microtubule end binding protein (Su, Burrell et al. 1995; Askham, Moncur et al. 2000). The last 15 amino acids of APC constitute a binding side for PDZ domains (Matsumine, Ogai et al. 1996). The C-terminal third of APC is the least well conserved and lacks significant stretches of well-ordered structure.

Figure 1.6.3.1: The adenomatous polyposis coli (APC) protein

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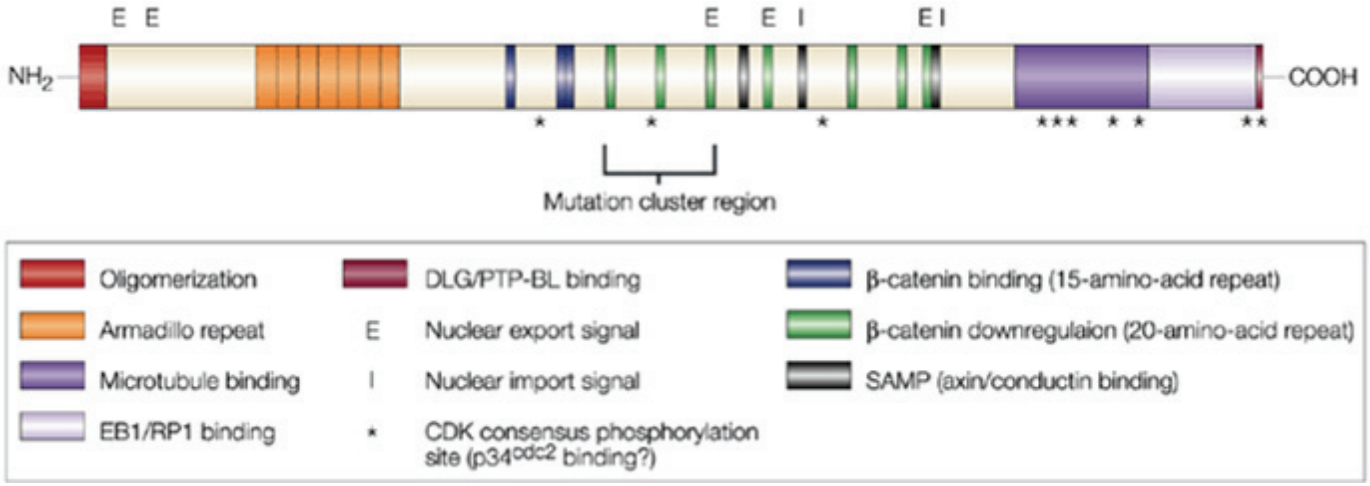
Conserved regions, such as the Armadillo repeats, and regions that interact with other proteins, including tubulin, the microtubule-associated protein EB1, discs large (DLG), β-catenin and axin/conductin, are shown. APC also contains several consensus sites for phosphorylation, five nuclear export signals (E) and two nuclear import signals (I). Most somatic mutations occur in the mutation cluster region. Most of these mutations lead to truncated proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (1: 55-67), copyright (2001)


E E
E E I
E I
NH2
COOH
Mutation cluster region
Oligomerization
Armadillo repeat
Microtubule binding
EB1/RP1 binding
DLG/PTP-BL binding
E Nuclear export signal
I Nuclear import signal
* CDK consensus phosphorylation site (p34cdc2 binding?)
β-catenin binding (15-amino-acid repeat)
β-catenin downregulaion (20-amino-acid repeat)
SAMP (axin/conductin binding)

1.6.3.2 Functions of APC

As previously described, APC is best characterized as a scaffolding protein in a multi-protein complex whose activity is modulated by Wnt-signalling (Bienz and Clevers 2000; Polakis 2000; Fodde 2002). This complex regulates the phosphorylation of β-catenin and therefore controls the amount of β-catenin available for transcriptional activation. The complex includes GSK3β, β-catenin, axin, and numerous kinases and phosphatases. In the absence of extracellular Wnt signals, GSK3β in the APC/β-catenin/axin complex is able to phosphorylate all 3 proteins, which increases their interaction. The phosphorylation of β-catenin by GSK3β creates a recognition site for ubiquitin ligase and leads to the destruction of β-catenin by the proteasome. In the presence of Wnt signal, the protein Dishevelled (Dsh) inactivates GSK3β so that kinase activity in the complex drops.

APC is also involved in regulating cytoskeletal functions. Truncated forms of APC expressed in colonic tumors lack most regions involved in the direct interactions with the cytoskeleton, in particular microtubules (Polakis 1995). APC gathers in the migrating edges of cells and this has led to the hypothesis that APC is involved in directed cell migration. Binding of APC to microtubules is decreased in response to phosphorylation (Zumbrunn, Kinoshita et al. 2001), suggesting that its activity as a scaffolding protein in the Wnt pathway and as a microtubule stabilizing protein are inversely related. Due to the many roles it plays in key intracellular pathways that control cell growth, cell adhesion, and migration, APC is often referred as a "gatekeeper" gene and often found as the initial mutation involved in tumor progression. Mutations in *APC* are especially penetrant, as can be attested by the various animal models (Taketo 2006). Mice with *APC* mutations on a single allele display a similar phenotype to that found in FAP patients. Mice missing both wild type allele are embryonic lethal (Shibata, Toyama et al. 1997).

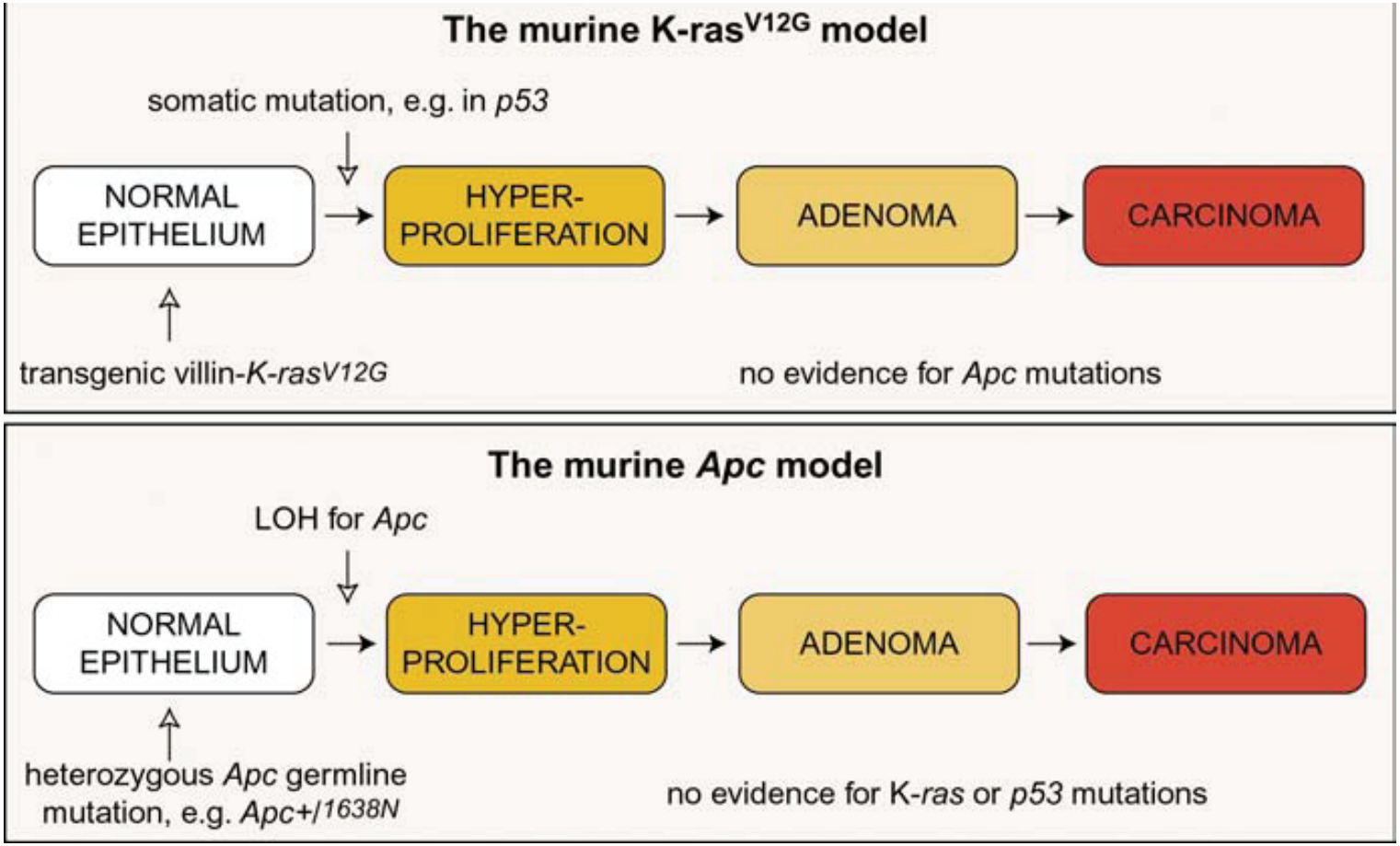
1.6.3.3 Mutations in *APC*

The most common mutations in *APC* result in truncation of the molecule. The axin/conductin-binding motifs and a variable number of the 20-amino-acid repeats that are associated with the downregulation of the intracellular β-catenin levels are often missing. In FAP patients, germ-line mutations are scattered throughout the 5' half of the *APC* gene; however, most somatic mutations are clustered between codons 1286 and 1513, the so-called mutation cluster region (Miyoshi, Nagase et al. 1992). There is a balance of functions between the N and C-terminal portions of APC. When C-terminal regions are missing as a result of tumor-associated *APC* truncation mutations, this balance is disrupted which results in a dominant effect of the remaining N-terminal domain that is expressed in tumor cells (Nathke 2004). The location of the *APC* mutation seems to predict the FAP phenotype to some extent. Mutations in exon 15 usually lead to the development of hundreds to thousands of colonic polyps, while mutations in either the 5' or 3' end of the gene or in exon 9, result in attenuated FAP (Spirio, Olschwang et al. 1993; Gardner, Kool et al. 1997).

1.6.4 Ras: initiator, and promoter of cancer

As mentioned above, in human primary carcinomas of the **small intestine**, *RAS* is frequently mutated (53%) and p53 to a lesser extent (27%), whereas APC mutations occur at a very low frequency (6%) (Arai, Shimizu et al. 1997). A certain proportion of colorectal cancers may not be initiated by mutation of *APC*, as is generally hypothesized, but by other mechanisms, by example activating *Ras* mutations. Studies of the transgenic mice that express K-ras^{V12G} controlled by the villin promoter, revealed a role for ras in tumor initiation. More than 80% of the transgenic animals displayed single or multiple intestinal lesions, ranging from ACF to invasive adenocarcinomas. There was no evidence of inactivating mutations of the *Apc* gene. Spontaneous mutation of the tumor-suppressor gene *p53* was found in almost 50% of the tumors tested. The multistep model of tumorigenesis is thus redrawn in mice as seen in figure 1.6.4.1. The mutant K-ras^{V12G} did not activate Akt/PKB in the normal mucosa of the transgenic animals

Figure 1.6.4.1: The murine villin-K-rasV12G model of intestinal tumorigenesis compared with *Apc* models.
Transgenic villin-K-rasV12G animals express oncogenic K-ras in the intestinal epithelium; spontaneous, somatic genetic alterations (e. g. inactivation of *p53*) are required for tumorigenesis. Murine models that recapitulate the role of *Apc* (Apc^{min}, Apc^{1638N}) have demonstrated that a somatic inactivation of the wildtype allele of *Apc* is sufficient to induce tumorigenesis, even in the absence of further mutations in other oncogenes or tumor suppressor genes. Springer, Cellular and Molecular Life Sciences, vol. 60, 2003, p. 502, Murine models of colorectal cancer: studying the role of oncogenic K-ras, K.-P. Janssen, fig. 2, © Birkhäuser Verlag, Basel, 2003; with kind permission from Springer Science and Business Media


The murine K-ras^{V12G} model
somatic mutation, e.g. in *p53*
NORMAL EPITHELIUM
HYPER-PROLIFERATION
ADENOMA
CARCINOMA
transgenic villin-*K-rasV12G*
no evidence for *Apc* mutations
The murine *Apc* model
LOH for *Apc*
NORMAL EPITHELIUM
HYPER-PROLIFERATION
ADENOMA
CARCINOMA
heterozygous *Apc* germline mutation, e.g. *Apc+/1638N*
no evidence for K-*ras* or *p53* mutations

and no anti-apoptotic effect of oncogenic Ras was observed (Janssen 2002). Recent study found frequent *K-ras* mutations in ACF, but no evidence for *APC* or *β-catenin* mutations was obtained (Takayama, Ohi et al. 2001). Although the role of Ras in tumor initiation is still under scrutiny its role in subsequent steps of tumorigenesis is well characterized.

Oncogenic *Kras* accelerates intestinal tumorigenesis in mice that also carry a mutant allele of the *Apc* (Schubbert, Shannon et al. 2007). *Kras* expression can initiate lung, pancreatic and haematological malignancies *in vivo*, and functions as a secondary mutation in the multistep pathogenesis of colon cancer. Ras proteins are small GTPases that regulate cell fates by coupling receptor activation to downstream effector pathways that control diverse cellular responses including proliferation, differentiation and survival (Repasky, Chenette et al. 2004; Mitin, Rossman et al. 2005). Many extracellular stimuli, including growth factors, are regulated by Ras proteins which act downstream of cell surface receptors. The active GTP-bound Ras can interact productively with more than 20 effectors, including Raf, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS) (Schubbert, Shannon et al. 2007). The best characterized Ras effector pathway is the Raf-MEK-ERK cascade. There are three Raf serine/threonine kinases (ARAF, BRAF and RAF1) involved in the MEK-ERK cascade. The primary function of Raf is to phosphorylate and activate MEK (also MAP-kinase kinase, MAPKK) (Cobb and Goldsmith 1995). MEK, in turn, catalyzes the phosphorylation of ERK1 and ERK2. Phospho-ERK then dimerizes and translocates to the nucleus where it leads to transcription-factor activation and cell proliferation, differentiation, survival, invasion and metastasis (Sebolt-Leopold and Herrera 2004), see figure 1.6.4.2. ERK kinases can phosphorylate both cytosolic and nuclear substrates, which include transcription factors such as JUN and ELK1, an ETS family member that regulates FOS expression (Schubbert, Shannon et al. 2007). JUN and FOS proteins form the activator protein 1 (AP1) transcription factor. Activation of these transcriptional regulators leads to the expression of proteins that control cell-cycle progression, such as cyclin D1 (Rodriguez-Viciana, Warne

et al. 1994; Arber, Sutter et al. 1996). Increased cellular levels of cyclin D1 promote cells through the G1 checkpoint, thus increasing proliferation.

1.6.4.1 Ras and apoptosis

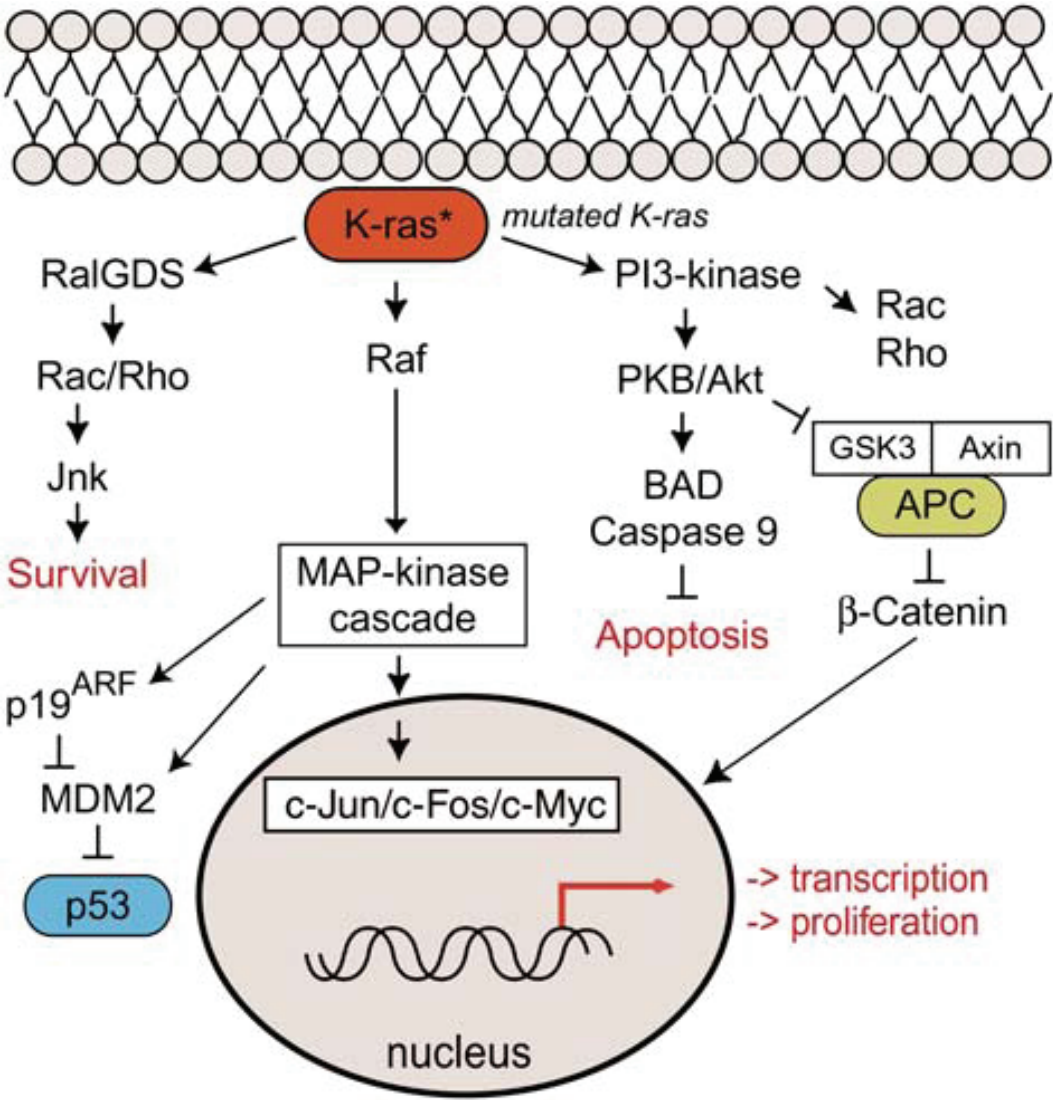
Mutant H-Ras suppresses apoptosis by preferentially activating Akt (Yan, Roy et al. 1998; Liebmann 2001), which phosphorylates caspase-9 (Cardone, Roy et al. 1998) and Bad (Datta, Dudek et al. 1997), a member of the Bcl-2 family. Upon phosphorylation, these pro-apoptotic proteins are inhibited. The Fas receptor is also downregulated by Ras, and protects cells from apoptosis (Peli, Schroter et al. 1999). K-ras-dependent hyperactivation of the Raf/MAPK pathway cooperates with TGF-β-receptor signalling, which is required for epithelial-mesenchymal transition (EMT) *in vitro* (Janda, Lehmann et al. 2002). Ras-activated PI3K signalling causes cells to scatter and is found to be responsible for protection of TGF-β-induced apoptosis (Janda, Lehmann et al. 2002). The protein GSK3 is not only a component of the Wnt/wingless pathway, but also a substrate of Ras-activated PKB that is capable of downregulating its activity (McCormick 1999). The Myc protein is stabilized by Ras, and the transcription of *Myc* is induced by the β-catenin pathway. Moreover, oncogenic β-catenin induces the accumulation and activation of the p53 protein (Damalas, Kahan et al. 2001). Metastastic tumors often express lower levels of the extracellular matrix component thrombospondin, and the expression of thrombospondin has been shown to be negatively regulated by oncogenic K-ras (Zabrenetzky, Harris et al. 1994).

1.6.4.2 Downstream of Ras

BRAF mutations are also found frequently in thyroid (30-50%), colorectal (5-20%) and ovarian cancers (30%) (Davies, Bignell et al. 2002). Most of the cancer-associated mutations are in the kinase domain, which result in gain of function mutants that constitutively activate the kinase and the MEK-ERK pathway. *MEK1* and *MEK2* mutations have not been reported in cancer or in other human disease. In most cases, the somatic missense *Ras* mutations found in

Figure 1.6.4.2: Schematic representation of the signalling cascades downstream of *K-ras.*

Oncogenic K-*ras* is a potent activator of several downstream effectors, the most prominent being the MAP-kinase cascade, the GTPases Rac/Rho and the PI 3-kinase. Activation of these pathways leads to suppressed apoptosis and transcriptional activation, resulting in increased cellular proliferation. There are several interconnections with other signalling cascades, such as the Wnt/APC pathway, and the tumor suppressor *p53*. Springer, Cellular and Molecular Life Sciences, vol. 60, 2003, p. 497, Murine models of colorectal cancer: studying the role of oncogenic K-ras, K.-P. Janssen, fig. 1, © Birkhäuser Verlag, Basel, 2003; with kind permission from Springer Science and Business Media.


K-ras*
mutated K-ras
RalGDS
Raf
PI3-kinase
Rac
Rho
Rac/Rho
PKB/Akt
GSK3
Axin
Jnk
BAD
Caspase 9
APC
Survival
MAP-kinase
cascade
Apoptosis
β-Catenin
p19ARF
MDM2
c-Jun/c-Fos/c-Myc
p53
-> transcription
-> proliferation
nucleus

cancer cells impair the intrinsic GTPase activity and confer resistance to GAPs, thereby causing cancer-associated mutant Ras proteins to accumulate in the active, GTP-bound conformation (Trahey and McCormick 1987). Studies on oncogenic Ras proteins with impaired activation support the conclusion that PI3K, Raf-MEK-ERK and RALGDS activation contribute to Ras-induced transformation (Schubbert, Shannon et al. 2007). The overexpression of oncogenic Ras in primary cells can induce senescence or growth arrest, unless accompanied by other genetic lesions such as loss of *CDKN2A* ($p16^{INK4a}$), *CDKN2D* ($p19^{INK4d}$) or *TP53* (Serrano, Lin et al. 1997).

1.6.4.3 Mutagenesis by Ras

A strong correlation between *KRAS* mutations and aneuploidy has been reported (Giaretti, Pujic et al. 1995). KRAS may induce genomic instability via the mitogen-activated protein kinase (MAPK) pathway and/or by affecting G1 and G2/M cell-cycle transit times and apoptosis (Orecchia, Infusini et al. 2000). Moreover, KRAS activation also affects *MDM2* transcriptional regulation. This accounts for the observation that KRAS-transformed cells are more resistant to DNA-damage-induced p53-dependent apoptosis (Ries, Biederer et al. 2000).

In contrast to human colorectal cancer, the number of mutations required for tumorigenesis in murine models seems relatively small (Janssen 2003). Ras is found at the intersection of several signalling pathways. Mutations in this important regulator of cellular functions can promote proliferation and survival, as well as contribute to the evolution of tumors into the more dangerous entity of cancer.

1.6.5 Accumulation of genetic mutations

The mechanisms by which cancer cells acquire new mutations are through MSI or chromosomal instability (CIN) that is thought to arise as a consequence of improper mitosis and spindle checkpoint activity. As reviewed above MSI occurs in patients with HNPCC. FAP patients accumulate mutations through CIN. One

of the inherent functions of APC is to interact with the protein EB1 and microtubules. EB1 has been shown to play an essential role in the proper assembly and positioning of the mitotic spindle in *Drosophila* tissue culture cells (Rogers, Rogers et al. 2002). APC has also been observed at the plus ends of kinetochore microtubules and in association with the mitotic checkpoint proteins Bub1p and Bub3p (Fodde, Kuipers et al. 2001; Kaplan, Burds et al. 2001). The mitotic abnormalities associated with tumor cells containing CIN correlate with *APC* mutations. Truncating mutations in *APC* act dominantly to interfere with microtubule plus-end attachments and cause a dramatic increase in mitotic abnormalities (Green and Kaplan 2003). Thus a single mutated allele of the *APC* gene could be sufficient to initiate the genetic evolution of tumor development. Moreover, the inactivation of APC causes the upregulation of the WNT/β-catenin signal. One of the downstream targets is conductin, which is up-regulated during mitosis, and localizes along mitotic spindles of colon cancer cells, and binds to polo-like kinase1. High conductin expression compromises the spindle checkpoint, and contributes to CIN (Hadjihannas, Bruckner et al. 2006).

Mutations in other genes that are not involved in the WNT pathway also affect signal transduction. For example, loss of E-cadherin is often observed in epithelial tumors and is generally correlated with cell adhesion defects (Christofori and Semb 1999). A great number of clinical and experimental studies have revealed that E-cadherin adhesion function is frequently lost during the development of most, if not all, human epithelial cancers, including carcinomas of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney and lung (Bracke, Van Roy et al. 1996; Behrens 1999). E-cadherin loss could result in an increase of the cytoplasmic pool of β-catenin that is available for nuclear signalling. The growth-suppressor activity of E-cadherin is adhesion-independent and results from inhibition of β-catenin/TCF signalling pathway (Gottardi, Wong et al. 2001), and loss of E-cadherin expression can contribute to upregulation of this pathway in human cancer (Smits, Ruiz et al. 2000).

1.6.5.1 p53

One important discovery of the 1990s was that virtually all DNA tumor viruses that cause tumors in experimental animals or humans encode proteins that inactivate both Rb and p53. These genes are involved in the balance of cell growth and cell death (apoptosis). Any dysregulation in proliferation or apoptosis has the potential of causing neoplasms. Several cancer genes directly control transitions from a resting stage (G0 or G1) to a replicating phase (S) of the cell cycle. Some well known tumor-suppressor genes include Rb (a transcription factor mutated in retinoblastoma) and p16 is an inhibitor of cdk4 and cdk6, and oncogenes such as cdk4 (a kinase) and cyclin D1 (which interacts with cdk4 and cdk6).

Loss or mutation of p53 is strongly associated with an increased susceptibility to cancer. Most functions of p53 have been considered in the light of how p53 might protect from malignant progression. Some p53-null mice can develop normally (Donehower 1996), which rules out major functions of p53 in normal physiology. However, females of some strains show neural-tube closure defects that reveal a role for p53 in normal development (Choi and Donehower 1999). The lack of p53 leads to a failure in progenitor cell apoptosis and an overproduction of neural tissue (Vousden and Lane 2007).

1.6.5.1.1 Functions of p53

Other functions of p53 that might be profoundly important during normal life are being uncovered. These include roles for p53 in regulating longevity and ageing, glycolytic pathways that might determine endurance and overall fitness, and apoptotic responses during ischemic and other types of stress (Vousden and Lane 2007). There is also evidence that p53 plays a role in the regulation of glycolysis, autophagy, the repair of genotoxic damage, cell survival and regulation of oxidative stress, invasion, motility, cellular senescence, angiogenesis, differentiation and bone remodelling (Vousden and Lane 2007). The ability of p53 to regulate gene expression is important for activation of the responses listed above. However, transcriptionally independent activities of p53

that can potentiate the apoptotic response have also been described (Yee and Vousden 2005). These functions involve a direct interaction of p53 with members of the Bcl2 family of proteins allowing p53 to function as a so-called BCL2-homology domain-3 (BH3)-only protein. The transcriptionally-independent apoptotic activity of p53 complements its ability to activate the expression of pro-apoptotic BH3-only proteins at the transcriptional level, as interfering with either of these functions can severely impair the death response. p53, through its cell-cycle-arrest and apoptotic activities, can have a strong inhibitory effect on cell growth. Proteins that regulate the activities of p53 include ubiquitin ligases that control its protein stability, enzymes involved in post-translational modifications of p53 (such as kinases and acetylases), transcriptional co-activators that can modulate the transcriptional activity of p53, and many more (Vousden and Lane 2007). MDM2 is one of the key ubiquitin ligases responsible for limiting the levels of p53. Deletion of *Mdm2* results in a failure to restrain p53-mediated apoptosis, and as a consequence mice die at the preimplantation stage (Marine, Francoz et al. 2006).

1.6.5.1.2 Regulation of p53

Many signals can activate p53, including inappropriate cell proliferation driven by oncogene activation, telomere erosion, nutrient deprivation and hypoxia, as well as low levels of DNA damage (Levine, Hu et al. 2006). ARF, a small protein that binds and inhibits MDM2, has an important role in signalling to p53 in response to some oncogenes but not DNA damage (Kamijo, van de Kamp et al. 1999). Ribosomal protein L11 has a role in activating p53 in response to ribosomal stress without a requirement for ARF (Lohrum, Ludwig et al. 2003). Metabolic stress that results in low glucose levels has been shown to activate p53, through a pathway that involves AMP kinase (AMPK), and has been proposed to contribute to the short-term survival of cells suffering temporary starvation (Jones, Plas et al. 2005). The loss of this response in tumors contributes to the capability of these cells to continue to proliferate in nutrient-poor conditions and so provide a proliferation advantage to tumor cells.

Low levels of DNA damage that are encountered during normal life are dealt with, through p53, by lowering ROS (Reactive Oxygen species) levels (and so reducing damage) and by promoting the survival of the slightly damaged cell to allow repair (a process to which p53 can also contribute). In response to more severe, sustained stress, such as oncogene activation or exposure to high doses of radiation, p53 switches from promoting survival and repair to the induction of apoptosis (Bensaad and Vousden 2005). Whether p53 helps or hinders the ageing process is not yet clear. Slight constitutive hyperactivation of p53 results in alarming premature ageing phenotype in mice (Tyner, Venkatachalam et al. 2002). In humans, a polymorphism in p53 that results in a slight reduction in its activity is associated with an enhanced cancer risk, but also with increased longevity (van Heemst, Mooijaart et al. 2005). Many cancers retain expression of a p53 protein with only a single amino-acid change, which most often occurs in the core DNA-binding domain, leading to both the disruption of normal p53 function and the accumulation of high levels of mutant p53 (Soussi and Lozano 2005). Mutant forms of p53 acquire novel transcriptional activities that are also likely to contribute to the enhanced malignant potential of cells that express these proteins (Kim and Deppert 2004). Polymorphisms in p53 itself can also result in subtle, but potentially extremely important, differences in activity (Pietsch, Humbey et al. 2006). It seems that the p53 pathway needs only to be slightly tweaked for an effect to be seen.

1.6.5.2 Myc

The *MYC* gene is one of the classic cancer promoting "oncogenes". It is overexpressed in many types of tumor, and the MYC protein it encodes causes a surge in the proliferation of cells. But it has another effect as it enhances programmed cell death. In MYC-associated tumors, there is usually a mutation in an ancillary protein that disrupts the apoptosis pathway. Hemann *et al*. reports that MYC can be mutated in a way that interferes with its apoptotic function (Hemann, Bric et al. 2005). Translocated *MYC* genes often harbour specific point mutations that tend to result in altered amino acids in a specific part of the MYC

protein that augments the tumorigenic potential of MYC. However, *in vitro* assays failed to show either enhanced cancer-promoting activity or reduced apoptosis (Chang, Claassen et al. 2000). The apoptosis protein BIM is activated only by wild-type MYC. The pro-apoptotic BH3-only protein Bim has been shown to inhibit MYC-induced lymphomagenesis. Bim was initially identified by virtue of its ability to bind Bcl2 and it acts as a potent inhibitor of the pro-survival members of the Bcl2 family. In mouse models, loss of one copy of the *BIM* gene confers strong resistance to apoptosis (Egle, Harris et al. 2004). Failure of mutant MYC to induce Bim results in a defective ability to suppress Bcl2 activity and seems to contribute to their enhanced tumorigenicity. MYC mutants retain ability to activate the $p19^{ARF}$-p53 pathway and efficiently promote proliferation (Hemann, Bric et al. 2005). Therefore, activation of BIM by wildtype MYC does not require p53 signalling.

MYC inhibits p21 production by binding to the *p21* gene promoter in a complex with the protein Miz-1 after which it recruits further repressor proteins. Overexpression of wild-type MYC reduces p21 levels (Seoane 2002). p21 acts as a cyclin-dependent kinase inhibitor or acts upstream of BIM, serves as a switch to determine whether a cell will stop dividing or undergo apoptosis. Impairment of either the p53 or the BIM signalling route is enough to render wild-type MYC as oncogenic as the MYC mutants (Egle, Harris et al. 2004).

1.7 Signalling pathways involved in tumor growth

As hinted above, cells respond to multiple stimuli to coordinate responses via complex signalling pathways. Several of these pathways overlap and central protein malfunctions can lead to inappropriate cell growth, and contribute to tumor progression. Upon activation of receptor tyrosine kinases such as IGF-R, PDGF-R, EGF-R or the HGF receptor c-Met, PI3K associates with the receptor which leads to allosteric activation of the catalytic subunit. Active PI3K converts the lipid substrate phosphoinositol 4,5-bisphosphate (PIP2) into the second messenger phosphoinositol 3,4,5-trisphosphate (PIP3). Proteins having a pleckstrin homology domain, such as Akt, interact with PIP3 and become

activated. The importance of elements downstream of receptor tyrosine kinases is illustrated in the observation that the p110α catalytic subunit of PI3K is activated in 32% of colon cancers (Samuels, Wang et al. 2004). In a genomic screen of human colorectal tumors, 40% had alterations in one of eight PI3K-pathway genes (Parsons, Wang et al. 2005). IGF-1/IGF-1R and the EGF receptor family members ErbB2 as well as Src activate PI3K/Akt in colon cancer cells. In addition, CD44 is found activated in colon cancers and can also induce PI3K/Akt activity via the Src-family tyrosine kinase Lyn (Michl and Downward 2005). Periostin is also a potent activator of the PI3K/Akt signalling pathway through interaction with αvβ3 integrin (Bao, Ouyang et al. 2004).

PKB/Akt is also the cellular homologue of the viral oncogene v-Akt which induces leukemia in mice (Michl and Downward 2005). There are three different isoforms, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Akt contains a central kinase domain with a threonine residue (T308) whose phosphorylation by PDK1, another serine/threonine kinase located at the plasma membrane, is essential for Akt activation. The C-terminal tail of Akt contains a second regulatory phosphorylation site at serine 473. In response to growth factors and other extracellular stimuli both Thr308 and Ser473 are phosphorylated and essential for maximal Akt activation, see figure 1.7.1. The majority of the known Akt substrates are involved in the regulation of cell survival, cell cycle progression, metastasis, protein synthesis and metabolism, see figure 1.7.2. All three Akt isoforms are expressed in both normal colon and colorectal tumor tissues (Zinda, Johnson et al. 2001). Akt expression is stronger in 57% of colorectal adenomas and lesser in hyperplastic polyps. Another PI3K isoform, PI3Kγ shows anti-tumor effects when overexpressed and mice lacking the catalytic subunit of PI3Kγ develop colorectal carcinomas (Sasaki, Irie-Sasaki et al. 2000).

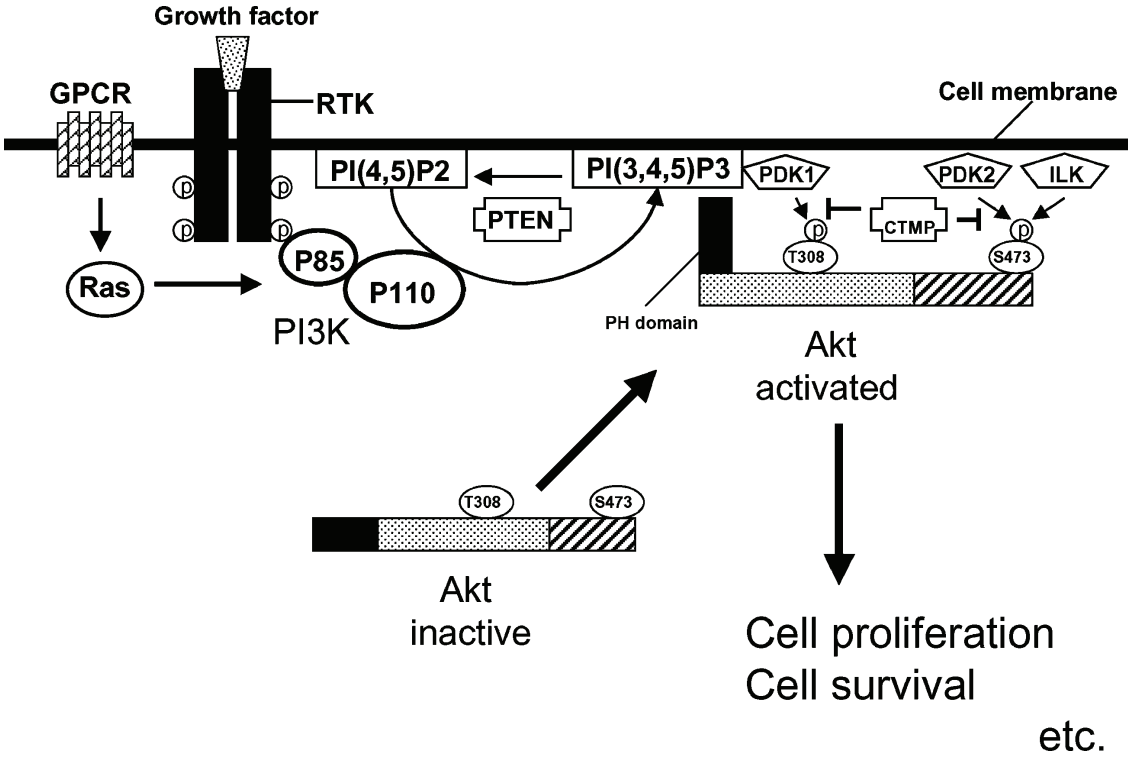
There are two important factors in the evolution of cancer, proliferation and survival. Proliferation can be achieved by skipping through cell cycle checkpoints. Even if cells are able to grow uncontrollably they must avoid cell death in order to form a tumor. In normal cellular function, differentiation eventually leads to apoptosis; cancer cells escape death by staying

undifferentiated. Activated AKT exerts anti-apoptotic activity at many different levels: it prevents release of cytochrome *c* from mitochondria, inactivates forkhead transcription factors known to induce expression of pro-apoptotic factors such as Fas ligand, or inactivates the pro-apoptotic factor BAD and pro-caspase-9 (figure 1.7.2), and it activates the IkB kinase, a positive regulator of nuclear factor kappa B (NF-κB), which results in transcription of anti-apoptotic genes (Cross, Alessi et al. 1995). FoxO forkhead transcription factors represent well-known downstream targets of Akt signalling (Medema, Kops et al. 2000). FoxOs have been shown to enhance expression of the cyclin-dependent kinase inhibitor p27kip1, and reduce expression of the cyclin D1 and D2 (Medema, Kops et al. 2000). Inactivation of FoxO by Akt-mediated phosphorylation results in enhanced cell cycle progression (Schmidt, Fernandez de Mattos et al. 2002). AKT also affects regulation of mRNA translation via control of phosphorylation of 4E-BP1 and its dissociation from the mRNA cap binding protein eIF4E (Cohen 2005). AKT mediates the activation of endothelial nitric oxide synthase (an important modulator of angiogenesis), promotes tumor invasiveness, and enhances telomerase activity (Cohen 2005). AKT is a major modulator of enterocyte differentiation. Inhibition of PI3K by chemical inhibitors or overexpression of PTEN enhanced enterocyte-like differentiation in a human colon cancer cell model of intestinal differentiation (Wang, Wang et al. 2001). AKT was shown to regulate levels of E-cadherin mRNA and protein (Grille, Bellacosa et al. 2003), which affects cellular adhesion and the behaviour of tumors.

P21-acitvated kinase-1 (PAK-1) is a downstream target of Akt that promotes cell migration. It has been used as a predictor of invasive behaviour (Michl and Downward 2005). mTOR is another downstream target of Akt. Rapamycin, an mTOR inhibitor, can reduce angiogenesis and tumor growth in an orthotopic colon tumor mouse model (Seeliger, Guba et al. 2004). Therefore, the Akt pathway plays a crucial role in tumorigenesis and tumor progression in a variety of malignancies including gastrointestinal adenocarcinomas. Growth factors and cytokines mediate their effects through the Akt pathway which in turn

Figure 1.7.1: Activation of Akt

The binding of growth factors to their receptor tyrosine kinase (RTK) or G protein-coupled receptors (GPCR) stimulates the phosphorylation of phosphatidylinositol 3-kinase (PI3K) comprised of P85 and P110 subunits. PI3K converts phosphatidylinositole-4,5 bisphosphonate (PI(4,5)P2) to PI(3,4,5)P3, whereas PTEN (phosphatase and tensin homologue deleted on chromosome 10) reverses this reaction. Akt translocates to the cell membrane and interacts with PI(3,4,5)P3 via its PH domain, being phosphorylated at two residues (Thr308 and Ser473) by phosphoinositide-dependent kinase (PDK) 1, PDK2 and integrin-linked kinase (ILK). Carboxy terminal modulating protein (CTMP) inhibits the phosphorylation. Once active, Akt controls fundamental cellular processes such as the cell cycle and cell survival. Springer and Apoptosis, vol. 9, 2004, 667-676, PI3K-Akt pathway: Its functions and alterations in human cancer, M. Osaki, figure 1, © 2004 Kluwer Academic Publishers with kind permission from Springer Science and Business Media


Growth factor
GPCR
RTK
Cell membrane
PI(4,5)P2
PI(3,4,5)P3
PDK1
PDK2
ILK
PTEN
CTMP
T308
S473
P85
P110
Ras
PI3K
PH domain
Akt
activated
T308
S473
Akt
inactive
Cell proliferation
Cell survival
etc.

activates a multitude of downstream signalling effectors. The central role of Akt in complex networks regulates numerous effects in both normal and malignant cells.

1.8 Final stage: Cancer

In order for a tumor to be categorized as cancer it must become invasive. The steps involved in cancer evolution include the transformation of epithelial cells into mesenchymal cells, also called epithelial-mesenchymal transition (EMT). Mesenchymal architecture has no interactions among cells, no apicobasal polarized orientation and cells are motile with some invasive properties, which facilitate metastasis. Multiple oncogenic pathways mediated by growth factors, Src, Ras, Ets, integrin, Wnt/β-catenin and Notch signalling, induce EMT. A molecular feature of EMT is the down-regulation of E-cadherin.

E-cadherin acts as a tumor suppressor inhibiting invasion and metastasis, and it is frequently repressed or degraded during transformation. AKT is shown to repress transcription of the E-cadherin gene (Larue and Bellacosa 2005). Constitutively active Akt in cells produces a transcription factor, Snail, which is known to repress expression of the E-cadherin gene (Grille, Bellacosa et al. 2003).

1.8.1 E-cadherin

E-cadherin is considered to act as a tumor suppressor for two main reasons: transcription of its gene is silenced in various carcinomas, and re-expression of a native form of E-cadherin in carcinomas *in vitro* is sufficient to reduce the aggressiveness of tumor cells (Vleminckx, Vakaet et al. 1991). Germline mutations of the *Cdh1* gene are associated with a syndrome of hereditary gastric and colorectal cancer (Guilford, Hopkins et al. 1998). Functional loss of E-cadherin may result from the production of a defective protein or transcriptional silencing due to promoter hypermethylation. E-cadherin is transcriptionally repressed by Snail, Slug, Sip1 and Ets. Snail expression is inversely correlated with E-cadherin transcription. The overproduction of Snail or Slug induces EMT *in vitro*, presumably through down-regulation of E-cadherin (Batlle, Sancho et al.

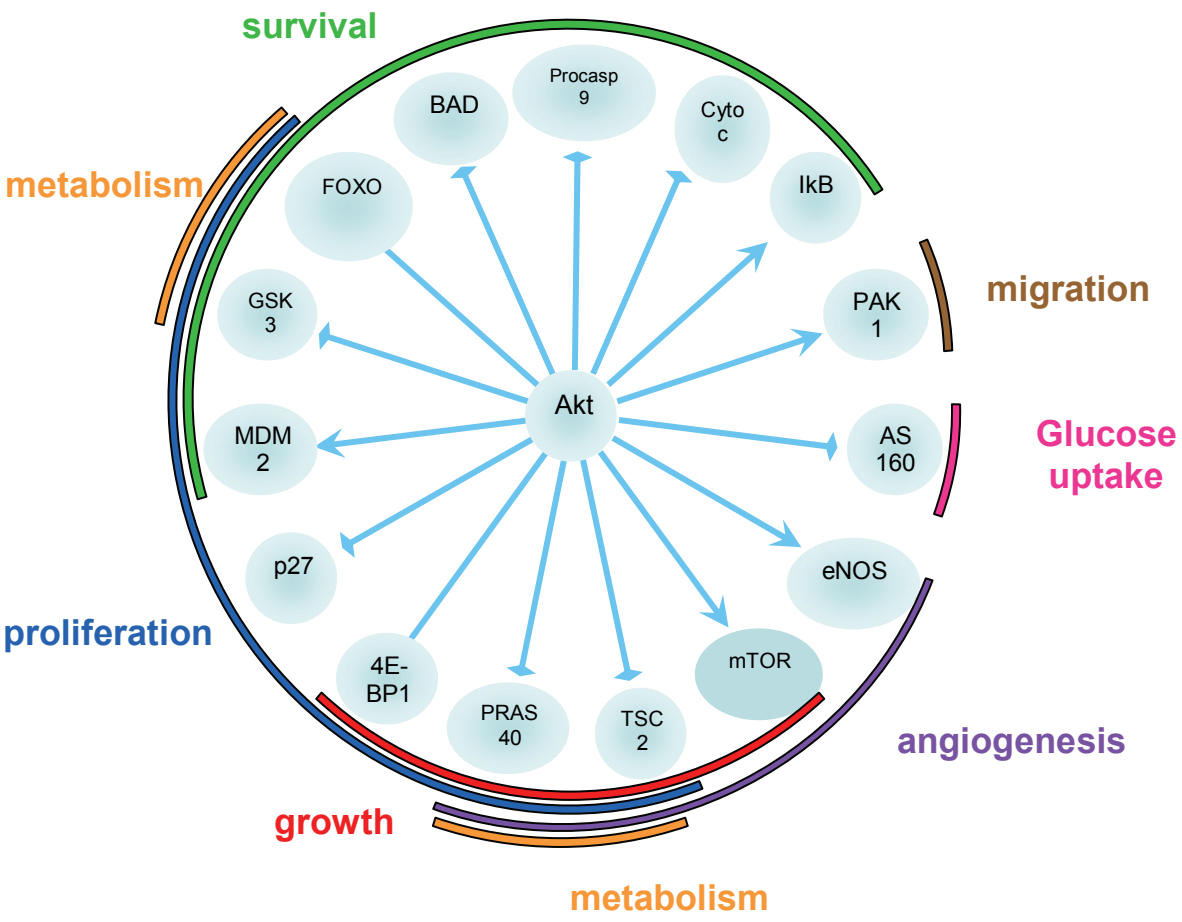
2000). Snail and Slug are zinc-finger proteins originally shown to be involved in mesoderm formation, together with Twist. The basic helix-loop-helix transcription factor Twist plays a critical role in the early steps of metastasis. Twist overexpression caused EMT of human immortalized epithelial cells (Yang, Mani et al. 2004). E-cadherin is positively regulated by the vitamin $1,25(OH)_2D3$ via the vitamin D receptor and Snail can repress E-cadherin and the vitamin D receptor, so the balance between vitamin D receptors and Snail may regulate E-cadherin levels (Palmer, Larriba et al. 2004). Phosphorylation by the p21-activated kinase PAK1 promotes Snail nuclear retention and repressor activity (Yang, Rayala et al. 2005). EMT induced by activated Akt involves loss of cell-cell adhesion, morphological changes, loss of apicobasolateral cell polarization, induction of cell motility, decrease in cell-matrix adhesion, and changes in the production or distribution of specific proteins. Akt also induces the production of metalloproteinases important for cell invasion (Kim, Kim et al. 2001). The sequestration of E-cadherin may be associated with the AKT-mediated activation of the Rab5 protein, while the transcriptional repression of E-cadherin expression may be associated with the activation of Snail gene expression (Batlle, Sancho et al. 2000).

Other factors such as the growth factor Cripto-1 (Cr-1), hyaluronan and M-Ras can activate Akt (Larue and Bellacosa 2005). Cr-1 belongs to a family of extracellular proteins in intercellular signalling pathways during mesoderm formation. Hyaluronan is a high-molecular-weight glycosaminoglycan involved in EMT of endothelial cells during development. Stimulation of hyaluronan synthesis in epithelial cells induces a transition to mesenchymal morphology, through stimulation of the PIK3/Akt pathway (Zoltan-Jones, Huang et al. 2003). M-Ras, a 27 kDa protein, is a member of the RAS superfamily of GTP-binding proteins.

1.8.3 Growth factors

FGF, EGF, TGFα and IGF2 can also induce EMT (Larue and Bellacosa 2005). ILK is activated in a PI3K-dependent manner by cell attachment to

Figure 1.7.2: Targets of Akt signalling


survival
metabolism
migration
Glucose uptake
proliferation
angiogenesis
growth
metabolism
Akt
BAD
Procasp 9
Cyto c
FOXO
IkB
GSK 3
PAK 1
MDM 2
AS 160
p27
eNOS
4E-BP1
PRAS 40
TSC 2
mTOR

fibronectin or insulin stimulation and its targets include AKT. Overexpression of ILK leads to nuclear translocation of β-catenin, increased invasiveness and repression of E-cadherin via upregulation of Snail transcription (Larue and Bellacosa 2005). Wnt/β-catenin induction of EMT is due to the activation of Slug, a direct transcriptional target of β-catenin/TCF (Conacci-Sorrell, Simcha et al. 2003) and stabilization of Snail, via inhibition of its phosphorylation and degradation (Yook, Li et al. 2005). Consistent with observations, inhibition of GSK3 activity leads to Snail transcription, E-cadherin repression and EMT (Bachelder, Yoon et al. 2005). Wnt and PI3/AKT signalling could converge at the level of inhibition of GSK3, although there are indications that the two pathways may affect and phosphorylate different pools of GSK3 (Larue and Bellacosa 2005).

1.8.3.1 TGFβ

TGFβ has a double role in tumorigenesis, as a tumor suppressor and a tumor promoter (Muraoka-Cook, Shin et al. 2006). The TGFβ pathway is known to prevent epithelial cell transformation by inhibiting proliferation and inducing senescence or apoptosis. But during late tumorigenesis, enhanced TGFβ signalling via autocrine or paracrine stimulation is associated with cancer progression, via increased motility, invasiveness and ultimately metastasis (Muraoka-Cook, Shin et al. 2006). TGFβ stimulates AKT phosphorylation via PI3K (Bakin, Tomlinson et al. 2000). TGFβ-mediated EMT is independent of SMAD signalling. The small GTPase RhoA, upstream of PI3K/AKT, is activated in response to TGFβ stimulation and is required for EMT (Bhowmick, Ghiassi et al. 2001).

1.8.3.2 PDGF

PDGF activates the Akt signaling (Lokker, Sullivan et al. 2002). PDGF is expressed in 83% of colon cancers and is associated with increased microvessel density. Further, stimulation of PDGF increases growth of colon cancer cells *in*

vivo (Kitadai, Sasaki et al. 2006). Inhibition of PDGF-β receptors also reduces interstitial hypertension and increases transcapillary transport in colonic carcinomas (Cohen 2005).

1.8.4 Src

Src links a number of critical signalling pathways. Src is a non-receptor tyrosine kinase localized to the intracellular membrane, which acts as an intermediary between growth factor binding to the receptor and downstream signalling important for a number of cellular processes, including proliferation, differentiation, and survival. c-Src promotes tumor growth and invasion both through activation of downstream STATs (Cao, Tay et al. 1996), and through modulation of the actin cytoskeleton focal adhesions (Brunton, Avizienyte et al. 2005) and integrins (Courter, Lomas et al. 2005). Binding of ligand to the EGFR results in c-src association with EGFR (Muthuswamy and Muller 1995) and phosphorylation of downstream targets. c-Src can phosphorylate the EGFR itself, following activation by EGFR ligand binding, and through lateral activation via other growth factor receptor pathways (Biscardi, Ishizawar et al. 2000), see figure 1.8.1.

1.8.5 Cdx2

Cdx2 encodes a transcription factor that plays a crucial role in the determination of intestinal identity during embryogenesis (Beck, Chawengsaksophak et al. 1999). Its expression is specifically retained in the intestinal epithelium throughout adulthood, and becomes heterogeneous and reduced in CRC (Kaimaktchiev, Terracciano et al. 2004; Subtil, Guerin et al. 2007). A reduced level of Cdx2 in Cdx2+/- mice facilitates colon tumor progression, demonstrating the importance of this protein in carcinogenesis (Aoki, Tamai et al. 2003; Bonhomme, Duluc et al. 2003). Futhermore, transcription factors involved in EMT such as Snail and Slug are upregulated during wound healing and are able to repress Cdx2 transcription (Gross, Duluc et al. 2008). *In vitro*, forced expression of Cdx2 in human colon cancer cell lines delayed wound

repair and reduced migration, whereas inhibition of Cdx2 expression by RNA interference enhanced migration (Mallo, Soubeyran et al. 1998). *In vivo*, forced expression of Cdx2 inhibited tumor cells spreading in nude mice xenografted at three different sites: the cecum wall to investigate local invasion, the tail vein to follow-up dissemination through the lymphatic system, and the spleen to address liver homing. These data provide evidence that Cdx2 antagonizes the process of tumor cell dissemination (Gross, Duluc et al. 2007).

1.9 APC mouse models

Several mouse models have been developed to mimic the conditions found in FAP (familial adenomatous polyposis) patients. Inactivation of p53 is not required in the case of Apc-induced tumorigenesis in mice and *p53* mutations are very rare in the case of several murine models that recapitulate the role of inactivated Apc (Janssen 2003).

1.9.1 *Apc*^{Min}

Chemical mutagenesis produced a mouse model for FAP carrying a nonsense mutation at codon 850, but still stably expressing truncated Apc protein. Mice heterozygous for this mutation develop multiple intestinal neoplasia (Min), bearing 50-100 polyps, and seldom live longer than 3 months (Moser 1990). The Min adenomas resemble the human disease quite well; however they arise in the small intestine, not the colon. Female *Min* mice, that live long enough, may also develop mammary tumors. Mice homozygous for the *Min* mutation die *in utero*, at approximately 8 days postcoitus (Moser, Shoemaker et al. 1995). Depending on the inbred mouse strain harbouring this mutation, the number of polyps varies significantly.

1.9.1.1 Modifier of Min, Mom1

Linkage analysis has demonstrated that a single locus *MOM1* (for modifier of MIN) on mouse chromosome 4 accounts for much of the difference between strains (Dietrich, Lander et al. 1993). *Mom1* (modifier of Min1) modifies tumor

number and size by about 50% in *Min*/+ animals. A transgene overexpressing the protein encoded by *Mom1*, secretory phospholipase Pla2g2a, drastically decreased the number of tumors in the *Min* mice (MacPhee, Chepenik et al. 1995).

1.9.1.2 Mom2, Mom3, Mom7

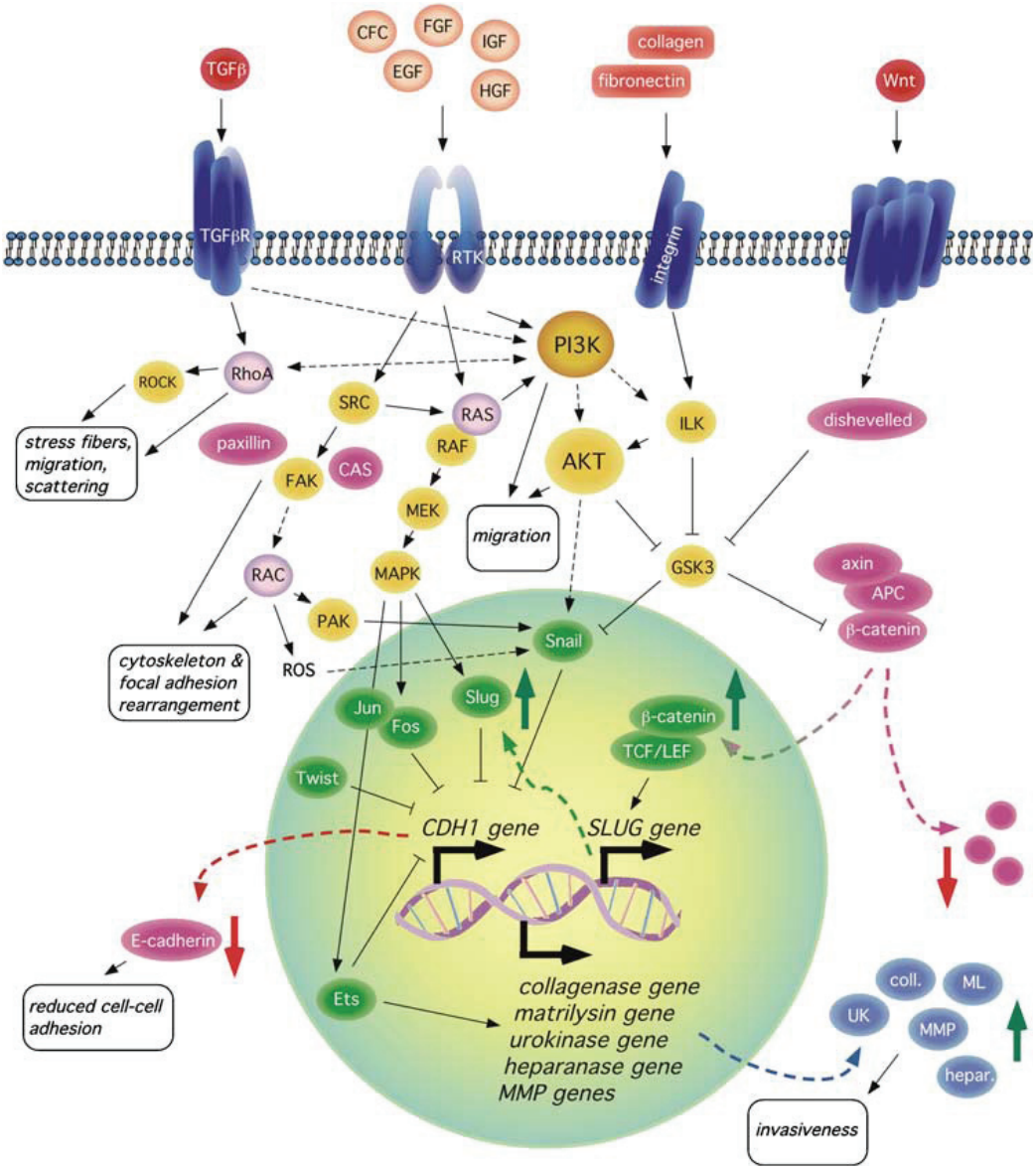
A second spontaneous modifier was described, *Mom2*, which can suppress up to 95% of tumor formation in Min mice (Silverman, Koratkar et al. 2002). The polyp-resistant $Mom2^{R}$ phenotype resulted from a spontaneous mutation and linkage analysis localized *Mom2* to distal chromosome 18. An intercross of congenic lines revealed that $Mom2^{R}$ encodes a recessive embryonic lethal mutation. Expression and sequence analyses of candidate genes identified a duplication of four nucleotides within exon 3 of the α subunit of the ATP synthase (*Atp5a1*) gene (Baran, Silverman et al. 2007).

Haines *et al.* identified another polymorphic modifier locus linked to Apc, *Mom3* (Haines, Johnson et al. 2005), the phenotype of which is affected by pregnancy (Suraweera, Haines et al. 2006). *Mom3* arose from an outbred stock, and the lack of known polymorphic markers has prevented its resolution beyond 25 cM. Numerous other genetic modifiers have been described upon breeding known mutations onto the $Apc^{Min/+}$ background. A subset of modifiers has been shown to affect the pathway leading to loss of heterozygosity (LOH) at the Apc locus. Kwong et al. describe the localization of a new Modifier of Min, *Mom7*, to within the first 7.4 Mb of chromosome 18. Noting its centromere proximal position relative to *Apc*, they speculate that the modifier may directly regulate the loss of heterozygosity of distal elements (Kwong, Shedlovsky et al. 2007).

1.9.2 $Apc^{\Delta 716}$

Conventional gene targeting has generated mice with truncated *Apc* alleles at codons 474 (Sasai, Masaki et al. 2000) and 1309 (Quesada, Kimata et al. 1998). Both mice share phenotypes with the Min mouse. Homologous recombination was used to generate mice expressing Apc truncated at residue 716 (Oshima 1995). Like the *Min* mice, the $Apc^{\Delta 716}$ heterozygotes developed about 300 polyps

Figure 1.8.3.1: Schematic of the signal transduction pathways associated with epithelial-mesenchymal transition.
End points of EMT are boxed. RTK: receptor tyrosine kinase; ROS: reactive oxygen species. Reprinted by permission from Macmillan Publishers Ltd: Oncogene ("Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways." 24(50): 7443-54), copyright (2005)


CFC
FGF
IGF
EGF
HGF
collagen
fibronectin
Wnt
TGFβ
TGFβR
RTK
integrin
PI3K
ROCK
RhoA
SRC
RAS
ILK
dishevelled
stress fibers, migration, scattering
paxillin
RAF
AKT
FAK
CAS
MEK
migration
RAC
MAPK
GSK3
axin
APC
β-catenin
PAK
Snail
cytoskeleton & focal adhesion rearrangement
ROS
Slug
Jun
Fos
β-catenin
TCF/LEF
Twist
CDH1 gene
SLUG gene
E-cadherin
reduced cell-cell adhesion
Ets
collagenase gene
matrilysin gene
urokinase gene
heparanase gene
MMP genes
coll.
ML
UK
MMP
hepar.
invasiveness

throughout the intestinal tract, mostly in the small intestine. Shibata et al. (Shibata, Toyama et al. 1997) created a conditional Apc model in which the *Apc* exon 14 is deleted upon Cre recombinase expression in the colon resulting in Apc truncated at codon 580. The mice developed adenomas within 4 weeks.

1.9.3 *Apc*^{1638N}, *Apc*^{1638T}

Two Apc mouse models make use of an introduced termination mutation at residue 1638. The first, *Apc*^{1638N}, introduces the PGK-neomycin gene at residue 1638 in the opposite transcriptional orientation of *Apc*, resulting in greatly reduced levels of the truncated polypeptide (Fodde 1994). Approximately 2% of the 182 kDa Apc^{1638N} protein is present and functional (Fodde 1994). Apc$^{1638N/+}$ mice develop considerably fewer (about 3) intestinal tumors than Min mice and live considerably longer, but their tumors tend to develop higher up in the gastrointestinal tract, at the transition from stomach to small intestine (Smits, Kartheuser et al. 1997). The *Apc*$^{1638N/+}$ mice also develop high numbers of extracolonic lesions, particularly cutaneous follicular cysts and benign desmoid growths (Fodde 1994). Human FAP patients with APC mutations located between codons 1445 and 1548 are also often associated with severe desmoids as well as osteomas, epidermoid cysts, and polyps of the upper gastrointestinal tract (Caspari, Olschwang et al. 1995). Moreover, gastric tumors occur in the *Apc*$^{1638N/+}$ mice (Smits, Ruiz et al. 2000). Homozygotes are also embryonic lethal (Fodde 1994).

When the same PGK-neomycin gene at residue 1638 is introduced in alignment with the transcriptional orientation of Apc, it results in a truncated allele, called 1638T. The truncated protein lacks the C-terminal domains binding to tubulin, EB/RP proteins, and DLG, but can regulate β-catenin levels efficiently. Heterozygous as well as homozygous Apc^{1638T} mice are not only viable and fertile, but they remain tumor-free (Smits, Kielman et al. 1999). Embryonic stem cells homozygous for *Apc*^{1638T} exhibit chromosome instability underscoring the role of the C-terminus in chromosome segregation (Fodde 2001). The

predisposition to chromosomal instability is not sufficient to drive tumor formation in these mice.

1.9.4 Polyp formation in APC mouse models

Histologically, all these *Apc* mutants form polyp adenomas indistinguishable from each other. The major factor that affects the polyp multiplicity among these mutants appears to be the extent of Wnt signal activation. It is estimated that the threshold of APC protein levels needed to form one polyp per mouse is 15% of the wild type (Li, Ishikawa et al. 2005). Introduction of cycloxygenase (COX)-2 gene mutation dramatically decreases the polyp number in the *Apc* mice (Oshima, Dinchuk et al. 1996). The overexpression of the COX-2 protein in intestinal polyps of various sizes is found at a very early stage of polyp formation. Prostaglandin E2 (PGE2) indirectly transactivates PPARδ through phophoinositide (PI)3K/Akt signalling. Treatment of Apc^{Min} mice with PGE2 increased intestinal adenoma burden, which was negated in Apc^{Min} mice lacking PPARδ, suggesting a crosstalk between the two pathways (Wang, Wang et al. 2004). Introduction of a homozygous mutation in p21, a cyclin-dependent kinase inhibitor, into Apc^{1638N} mice increases tumor multiplicity about twice, and affects goblet cell differentiation (Yang, Mathew et al. 2001).

Promoter regions of many cancer-related genes are silenced by methylation of the cytidine residues (Laird, Jackson-Grusby et al. 1995). Additional mutation in the Cdx2 gene in $Apc^{\Delta 716}$ mice reverses the polyp localization, shifting most polyps to the colon as in human FAP (Aoki, Tamai et al. 2003). The transcription factor SOX9 is regulated by the Wnt pathway, and represses the *Cdx2* and *Muc2* genes (Blache, van de Wetering et al. 2004). Math1 is a bHLH transcription factor downstream of the Notch signalling pathway, necessary for cell fate determination that is repressed by the Wnt signalling pathway (Leow, Romero et al. 2004). When Apc^{1638N} mice were bred with animals carrying an E-cadherin gene knockout mutation, the intestinal and gastric tumor multiplicity increased nine and five times respectively (Smits, Ruiz et al. 2000). Activation of PPARγ in

the *Apc*^{Min} mice increases the number and size of colon tumors (Lefebvre, Chen et al. 1998).

Loss of p27 in tumors is correlated with tumor agressiveness, depth of tumor cell invasion and poor state of differentiation (Mori, Mimori et al. 1997; Yasui, Kudo et al. 1997; Singh, Lipman et al. 1998; Kim, Lee et al. 2000). Experiments in p27 deficient mice have established p27 as a haploinsufficient tumor suppressor. *p27*-null mice are predisposed to spontaneous pituitary adenomas (Fero, Rivkin et al. 1996; Kiyokawa, Kineman et al. 1996; Nakayama, Ishida et al. 1996), as well as radiation and ENU-induced tumors in multiple epithelial tissues, especially in colon adenomas and adenocarcinomas (Fero, Randel et al. 1998). Compound mutant mice carrying the *Apc*^{Min} and *p27* gene deletions, have shortened latency period to develop intestinal tumors. p27 deficiency also leads to increased adenoma to adenocarcinoma progression, indicating that reduction of p27 cooperates with mutations in *Apc* during GI tumorigenesis (Philipp-Staheli, Kim et al. 2002).

SMAD4 and *APC* are both on mouse Chr 18. As polyps are initiated by *Apc* LOH in Apc$^{\Delta 716}$ intestines, and because this LOH is caused by loss of the entire Chr 18 due to somatic recombination at the ribosomal DNA locus near the centromere, LOH of *Apc* also results in LOH at *Smad4*. The loss of both *Apc* and *Smad4* loci results in intestinal polyps that progress rapidly into very invasive adenocarcinomas but not metastasis (Takaku, Oshima et al. 1998). Loss of Ephrin B (EphB) expression strongly correlates with degree of malignancy and reduction of EphB activity accelerates tumorigenesis in the colon and rectum of *Apc*^{Min} mice (Batlle, Bacani et al. 2005).

1.10 Chemically-induced carcinogenesis models

Rodents do not usually develop spontaneous colon cancer, but colon tumors can be induced with chemical carcinogens. The most commonly used are dimethylhydrazine derivatives. First investigated by Druckrey, the synthetic compounds 1,2-dimethylhydrazine and azoxymethane, both chemically related to the naturally occurring carcinogen cyasin, have proven to be of great value in

their reliable and specific ability to produce colon tumors in several rodent species (Druckrey 1970). The potency and organospecificity of procarcinogens, such as 1,2-dimethylhydrazine and azoxymethane, are to a great extent determined by the chemical reactivity and the availability of sufficient amounts of their ultimate carcinogenic forms in the cells of the target tissues. The amount of the carcinogen, in turn, is a function of the activities of the metabolic pathways leading to its formation, the activities of detoxification pathways and also of the biological half-lives of all of the metabolic species involved (Fiala 1977). Dimethylhydrazine (DMH) is metabolized to azoxymethane (AOM) and methylazoxymethanol (MAM) in rodents. AOM-induced tumors share many histopathologic characteristics with human tumors (Corpet and Pierre 2003). They have mutations of *K-ras* and *β-catenin* genes (Dashwood, Suzui et al. 1998) and show microsatellite instability, but, unlike human tumors, they are seldom mutated at the *Apc* gene (15%) are never mutated at the *p53* gene (De Filippo, Caderni et al. 1998), and have a low tendency to metastasize in rats. Maltzman *et al.* demonstrated loss of the wild-type APC protein is AOM-induced **mouse** colon tumors and suggests that alterations in expression of this tumor suppressor gene, is involved in animal model of cancer (Maltzman 1997). Mutations of the *Apc* gene are associated with the transition from ACF (aberrant crypt foci) to adenoma and adenocarcinoma and not from normal mucosa to ACF (De Filippo, Caderni et al. 1998). Modulation of certain genes is also observed in AOM treated animals. TGF-β1 and TGFβR-II mRNA levels in tumor samples are increased, when compared with control mouse colon tissue (Wang, Guda et al. 2000). Colon tumors exhibit an eightfold increase in $p16^{INK4a}$ mRNA level when compared with control colon tissue. A heterogeneous Rb immunostaining is observed in preneoplastic lesions and adenomas. Immunohistochemical analysis also shows a reciprocal relationship between $p16^{INK4a}$ and Rb protein expression (Wang 2000). AOM has become the carcinogen of choice to induce colon tumors in rodents because it offers a number of advantages as compared with the parent compound, including its enhanced potency and chemical stability (Druckrey 1970). Marked strain specificity has been described in mice (Evans, Hauschka et al. 1974).

Other colon carcinogens used less frequently include specific nitrosamines and heterocyclic amines like PhIP. Cooked food contains a variety of mutagenic heterocyclic amines (HCAs) for which mutagenic as well as carcinogenic activities have been demonstrated (Sugimura 1992). Among them, 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) is the most abundant and causes lymphomas in mice (Esumi, Ohgaki et al. 1989) and tumors of the intestine and mammary gland in rats (Ito, Hasegawa et al. 1991). PhIP induces *Apc* mutations frequently (40-60%) and microsatellite instability (Canzian, Ushijima et al. 1994), but not K-ras or p53 mutations (Corpet and Pierre 2003).

The difference in mutated genes may be carcinogen-specific rather than species-specific. Frequent mutations and altered cellular localization of β-catenin are found in AOM-induced rat colon tumors (Takahashi, Fukuda et al. 1998). β-catenin mutations have also been found in PhIP-induced rat colon tumors without Apc mutations (Dashwood, Suzui et al. 1998). These results indicate that Wnt/Apc/β-catenin signalling indeed plays important roles in chemically-induced rat colon carcinogenesis, as it does in human cancers. Markedly elevated expression of iNOS and COX-2 in AOM-induced rat colorectal cancers was observed by immunoblotting and immunohistochemical staining (Takahashi, Fukuda et al. 1997).

Many cell adhesion molecules are tumor suppressors. Their genes may not be tumor suppressor genes in the traditional sense; the gene is not necessarily disabled in tumor cells as is the case with the classical tumor suppressors such as Rb or p53 genes; rather, the function of the gene is compromised through downregulated gene expression or protein function (Ruoslahti and Obrink 1996).

1.11 CEACAM family

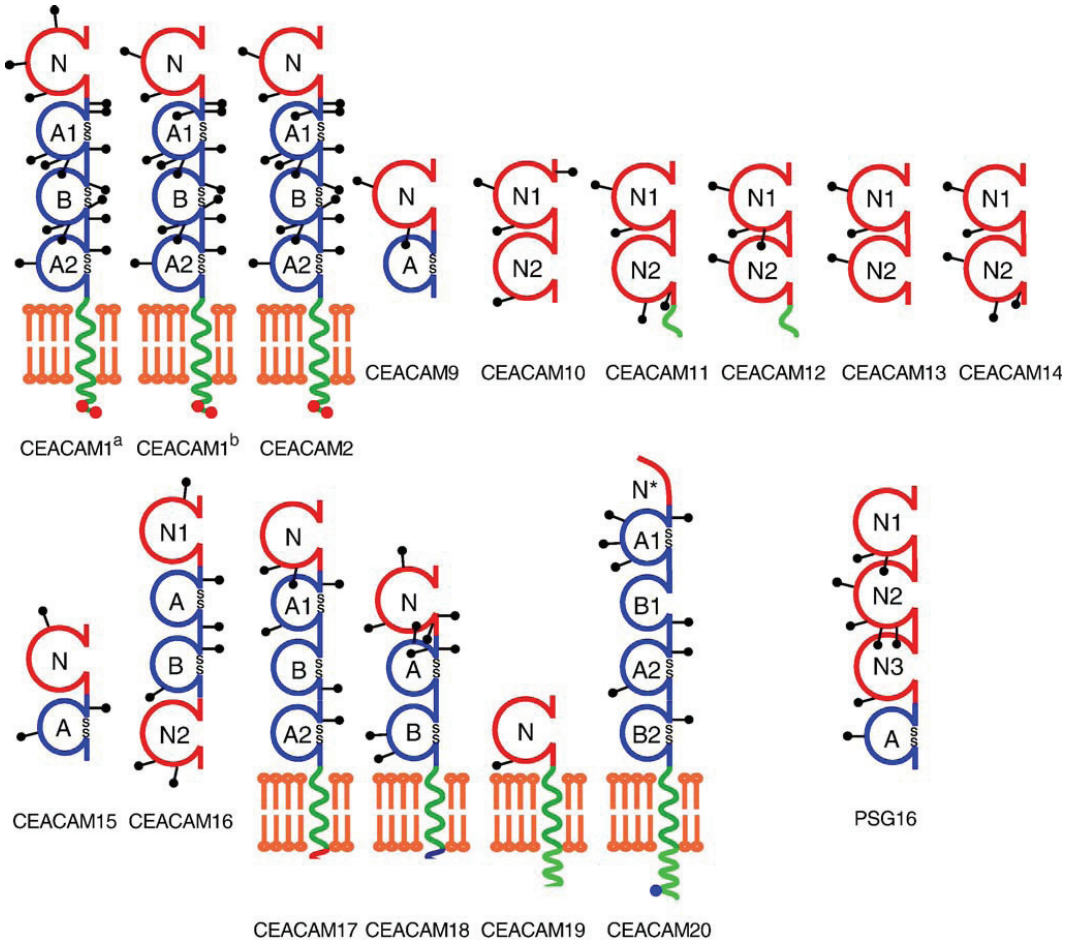
Carcinoembryonic antigen (CEA) was first described in 1965 by Gold and Freedman as an antigen associated with intestinal malignancies (Gold and Freedman 1965). Since its first description, several members have been added to this family of evolutionarily conserved proteins. The CEA family, a subgroup of

the immunoglobulin (Ig) superfamily, consists of diverse glycoproteins that vary greatly between mammalian orders. In humans, the CEA family is encoded by 18 genes and 11 pseudogenes on chromosome 19q13.1-3 (Thompson 1991). They are subdivided into the CEA-related cell-cell adhesion molecules (CEACAM) and the pregnancy-specific glycoproteins (PSG), which are exclusively expressed in the trophoblast (McLellan, Fischer et al. 2005). In mouse, CEACAM molecules are composed of either Ig variable (IgV)-like N domains only or one to eight IgV-like domains and one to three Ig constant (IgC)-like domains of subtype A or B. Two murine CEA-related molecules are transmembrane bound (CEACAM1, CEACAM2), whereas all other so-far identified members are secreted (Kataoka, Takata et al. 2000). In contrast, the human CEACAM family members are composed of just one IgV-like and zero to six IgC-like domains. CEACAM1, CEACAM3, and CEACAM4 are transmembrane-bound molecules, while CEA/CEACAM5, CEACAM6, CEACAM7 and CEACAM8 are linked to the cell membrane via glycosyl phosphatidyl inositol anchors. In the long splice variant cytoplasmic tail of CEACAM1 and human CEACAM3 as well as CEACAM4, functional, immunoreceptor tyrosine-based inhibitory motifs (ITIM) and immunoreceptor tyrosine-based activation motifs (ITAM) respectively have been identified (Chen 2001; Schmitter, Agerer et al. 2004), see figure 1.11.1. So far, only CEACAM1 has an orthologue identified in rodents and primates. Humans share 5 of 23 CEA-related genes with mice. Most of the rodent-specific genes are exclusively expressed during development in trophoblast cells of the placenta (Zebhauser, Kammerer et al. 2005). PSGs have been suggested to modulate the maternal immune system by shifting it toward a T_H2 bias, which is characteristic of pregnancy. PSGs were found to bind to receptors on macrophages and monocytes, which stimulate these cells to produce anti inflammatory cytokines (Wessells, Wessner et al. 2000; Snyder, Wessner et al. 2001).

1.12 CEACAM1

The focus of my research has been on CEACAM1 formerly known as Bgp1, CD66a, and pp120.

Figure 1.11.1: Domain organization of the murine CEA family.
The members of the murine CEA family are composed of IgV-like N domains (red) and IgC-like domains of subtype A or B (blue). Potential N-glycosylation sites are marked by lollipops. Highly conserved disulfide bridges in the IgC-like domains are indicated by two S's. In each case, the longest known splice variant is shown. Red and blue dots in the cytoplasmic domains symbolize the presence of ITIM and ITAM consensus motifs, respectively. Reprinted from Genomics, Vol 86(5), Roland Zebhauser, Robert Kammerer, Andreas Eisenried, Andrew McLellan, Tom Moore and Wolfgang Zimmermann, Identification of a novel group of evolutionarily conserved members within the rapidly diverging murine Cea family, pp. 566-80., Copyright (2005), with permission from Elsevier.


N
A1
B
A2
CEACAM1a
CEACAM1b
CEACAM2
N
A
CEACAM9
N1
N2
CEACAM10
CEACAM11
CEACAM12
CEACAM13
CEACAM14
CEACAM15
CEACAM16
CEACAM17
CEACAM18
CEACAM19
N*
B1
B2
CEACAM20
N3
PSG16

1.12.1 Structure

CEACAM1 is expressed with one to four extracellular Ig domains and either a long or short cytoplasmic tail. The nomenclature was standardized so that CEACAM1-4L would represent the molecule that has 4 Ig domains and a long cytoplasmic tail and CEACAM1-S would represent all variants with a short tail, see figure 1.12.1. In humans 11 different CEACAM1 splice variants have been detected. They differ with respect to the number of extracellular immunoglobulin-like domains, membrane anchorage and length of their cytoplasmic tail. In mice, CEACAM1-2L, CEACAM1-2S, CEACAM1-4L, and CEACAM1-4S are the major product (Beauchemin, Draber et al. 1999) although some soluble forms have recently been identified (Budt, Michely et al. 2002). The long cytoplasmic splice variant of CEACAM1-L contains 73 aa in mice and 71 aa in humans, whereas the short cytoplasmic splice variant CEACAM1-S has only 10 aa in both species (Barnett 1989). Both the long and short cytoplasmic domains contain several residues that are phosphorylation sites for several kinases and can be dephosphorylated by phosphatase, see figure 1.12.2. The long cytoplasmic domain of CEACAM1 is responsible for most of the signal transduction properties. It contains two Tyr residues within ITIMs, Tyr 488, and Tyr515, as well as 17 Ser/Thr residues, many of which are located in consensus sites for a variety of Ser/Thr protein kinases (Formisano 1995). The extracellular domains of all CEACAMs are heavily glycosylated and generally more than half of the receptor's molecular weight consists of carbohydrate (Lucka, Fernando et al. 2005). The rat liver CEACAM1 is highly sialylated and mostly of the complex type and to a much lesser extent, of the high-mannose type, with up to 16 potential glycosylation sites (fig. 1.11.1). It has been postulated that the glycan structure of CEACAM1 mediates the binding of granulocytes to endothelial cells (Stocks, Kerr et al. 1995). In granulocytes, CEACAM1 is the major membrane glycoprotein that binds the Lewis X antibodies, and in addition carries high mannose residues. The Lewis X groups of CEACAM1 may bind to lectins, such as C-type lectins (Lucka, Fernando et al. 2005). CEACAM1 variants are

subjected to different post-translational modifications or sugar additions in the kidney and seminal vesicle (Baum, Troll et al. 1996).

In PC12-cells, the half-life of the highly sialylated CEACAM1 is 26 hours, and replacement of the *N*-acetylneuraminic acid by a novel, engineered *N*-propanolyneraminic acids can increase the half-life to 40 hours (Horstkorte, Lee et al. 2001). This highlights the importance of the type of glycosylation on CEACAM1 which can modulate its expression on the cell surface. A non-glycosylated β-pleated sheet in the amino-terminal domain mediates homophilic intercellular binding (Watt, Teixeira et al. 2001; Tan 2002) and is the target for various bacterial and viral adhesions. At least two Ceacam1 alleles exist in the mouse, which encode proteins differing considerably in their IgV-like domain (N domain) sequence and their ability to support mouse hepatitis virus entry (Dveksler 1993). In humans, CEACAM1 is also used as a receptor by various bacterial pathogens, e.i., *Neisseria meningitidis* and *Neisseria gonorrhoeae, Haemophilus influenzae*, and *Moraxella catarrhalis* (Gray-Owen, Dehio et al. 1997; Virji, Evans et al. 2000; Hill 2003).

1.12.2 Expression pattern

CEACAM1 is the most widely expressed member of the murine CEA family. Transcripts are detected in all tissues tested (Prall, Nollau et al. 1996; Han 2001). CEACAM1 is not expressed in early post-implantation embryos (7.5 dpc), but is found in the placenta and extra-embryonic tissues at this time. *Ceacam1* mRNA transcription is most prevalent between 10.5-12.5 dpc and 16.5-18.5 dpc of mouse embryonic development (Daniels 1996).The primitive gut epithelium and surface ectoderm were the first embryonic tissues to express CEACAM1. Significant CEACAM1 expression was also observed later during epithelial-mesenchymal interactions. There is also prominent expression during myogenesis (Daniels 1996). CEACAM1 was originally shown to be expressed in normal human gallbladder mucosa and at the bile canalicular surface of hepatocytes (Svenberg 1976). Surface expression of CEACAM1 is found on certain

epithelial, endothelial, lymphoid and myeloid cells (Odin 1986; Prall, Nollau et al. 1996; Kammerer 1998).

1.12.3 Cellular expression

The cellular localization of CEACAM1 is dictated by the cytoplasmic domain, and by its extracellular binding. In polarized epithelial MDCK cells CEACAM1-S is expressed exclusively at the apical surface whereas CEACAM1-L occurs both in apical and lateral cell surfaces. Tyr515, but not Tyr488, constitute a prominent lateral targeting signal. Laterally localized CEACAM1-L is present in adherens junctions but not in tight junctions or desmosomes. Overexpression of CEACAM1-L does not affect the organization of tight junction or adherens junction proteins. However, upon overexpression of CEACAM1, the abundance of desmosomes in the lateral cell surfaces decreases significantly and the submembraneous cytokeratin filaments become disorganized. The effect of CEACAM1-L on desmosomes depends on the presence of amino acids 484-518 in the C-terminal part of the cytoplasmic domain, among which an intact Tyr515 is indispensable (Sundberg, Beauchemin et al. 2004).

The retention at intercellular contacts critically depends on homophilic CEACAM1-CEACAM1 interactions and association with the actin cytoskeleton (Sadekova, Lamarche-Vane et al. 2000). Consequently, in Swiss 3T3 cells, the targeting of CEACAM1 at cell-cell boundaries is regulated by the Rho GTPases. Activated Cdc42 and Rac1 or their downstream effector PAK1 can target CEACAM1 to sites of cell-cell contacts, but activated RhoA and Rho were unable to do so, resulting in a concentration of CEACAM1 at the cell surface. Inhibition of this pathway results in CEACAM1 intercellular localization suggesting that a tightly regulated balance of Rho GTPase activities is necessary to target CEACAM1 at cell-cell boundaries. The transmembrane domain of CEACAM1 is responsible for the Cdc42-induced targeting at cell-cell contacts (Fournes 2003).

During its intracellular assembly, CEACAM1 is transported from the trans-Golgi network to the sinusoidal domain of the plasma membrane before its final transcytosis to the bile canalicular domain. Most of the newly synthesized

CEACAM1-L isoform reaches complete maturation in about 60 minutes. However, only 40% of the CEACAM1-S isoform undergoes complete maturation and a significant portion appears to be targeted to lysosomes (Choice 1998).

1.12.4 CEACAM1 ligands

Other than itself, CEACAM1 has very few known ligands. It can bind to itself in homophilic interactions both in cis and in trans. Only the CEACAM1-4L and CEACAM1-4S transfectants are able to bind significantly to immobilized CEACAM1-4Fc molecules. The Val39 and Asp40 residues within the CC' loop of the GFCC'C" face of the N-terminal domain play a crucial role in homophilic interactions. The A2 domain and long cytoplasmic tail are not essential for homophilic interaction, although they appear to stabilize or increase avidity of binding (Watt, Teixeira et al. 2001). CEACAM1 also participates in heterophilic interactions with other members of the CEA family (CEA/CEACAM5) (Oikawa, Inuzuka et al. 1991). Certain pathogens use CEACAM1 as their receptor, and in line with this, Opa proteins from *Neisseria meningitidis* and *Neisseria gonorrhoeae* bind to the extracellular domain of CEACAM1 (Gray-Owen, Dehio et al. 1997; Hill 2003). CEACAM1 is also the receptor for the MHV (mouse Hepatitis virus), and their spike proteins bind to the N-domain of CEACAM1 through protein-protein interactions (Hemmila 2004). Tan et al. solved the crystal structure of the soluble murine sCEACAM1a[1,4], composed of two Ig-like domains which has MHV neutralizing activity. The CEACAM1 N-terminal domain has a uniquely folded CC' loop, among the Ig superfamily members, that encompasses key virus binding residues (Tan 2002). Many investigators use various specific anti-CEACAM1 antibodies or soluble CEACAM1 to decipher the various signal transduction pathways of CEACAM1.

1.13 CEACAM1 signalling

CEACAM1 mediates signalling through association with kinases and phosphatases, and is thus subjected to various states of phosphorylation. Several signalling pathways have been mapped out and most involve the long cytoplasmic

Figure 1.12.1: Nomenclature of the CEACAM1 molecules.

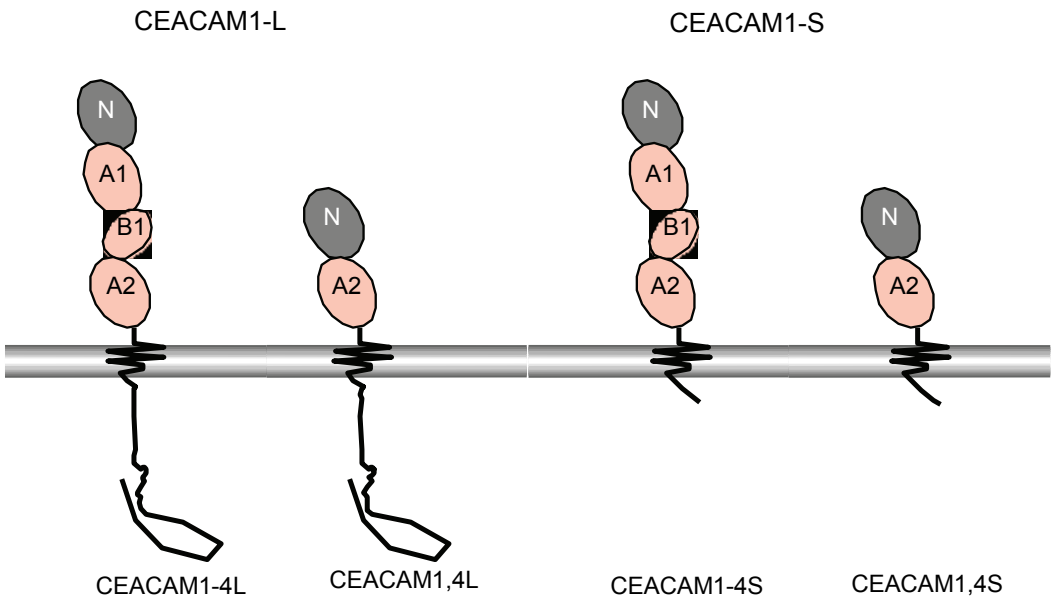

CEACAM1-L
CEACAM1-S
N
A1
B1
A2
N
A2
N
A1
B1
A2
N
A2
CEACAM1-4L
CEACAM1,4L
CEACAM1-4S
CEACAM1,4S

Figure 1.12.2: Sequence of the cytoplasmic domains of CEACAM1.
Single letter amino acid designation is used. Residues involved in signalling are in bold


CEACAM1-S LYSRKSGGSGSF
450
460
470
480
490
500
510
520
CEACAM1-L LYSRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVAYTVLNFNSQQPNRPTSAPSSPRATETVYSEVKKK
Proximal calmodulin binding site
ITIM
Site of priming phosphoserine
ITIM

domain of CEACAM1-L, which contains two tyrosine residues nested within ITIMs (see Figure 1.12.2). These tyrosine residues interact with protein tyrosine kinases of the Src family. From membrane fractions of granulocytes and the colonic cell line HT29, phosphokinase activity was co-immunoprecipitated with CEACAM1 (Brümmer 1995). *In vitro* experiments indicate that the long cytoplasmic domain of CEACAM1-L is a substrate and binding partner of pp60c-Src. Furthermore, the catalytic activity of c-Src is stimulated after binding to CEACAM1. The down regulation of CEACAM1 in about 80% of colorectal carcinomas may contribute to a dysregulation of c-Src in colorectal cancer (Brümmer 1995). Tyrosine phosphorylated CEACAM1, expressed in mouse colon carcinoma CT51 cells, can reversibly associate with protein tyrosine phosphatase SHP-1. Association of CEACAM1-L and SHP-2 is mediated by both tyrosine residues. Either of the two SH2 domains of SHP-2 can bind tyrosine phosphorylated CEACAM1 *in vitro* (Beauchemin 1997). Other residues are also implicated in the association of CEACAM1 and its binding partners as seen by deletion of the Lys residues located at the C-terminal end can significantly diminished SHP-2 binding (Huber 1999). CEACAM1 also associates with SHP-2-Tyr phosphatase, but not with an unrelated Tyr phosphatase PTP-PEST. CEACAM1 and SHP-2 interaction involves the second Tyr (515) and the SHP-2 N-terminal SH2 domain (Huber 1999).

The Ser503 has been shown to play a crucial role in CEACAM1-L-mediated processes such as insulin receptor internalization (Formisano 1995) or bile salt extrusion (Sippel, Fallon et al. 1994). CEACAM1 is basally phosphorylated on Ser 503 by cyclic adenosine monophosphate (cAMP)-dependent serine kinase in the absence of insulin (Najjar 1995). Protein kinase C phosphorylates the short isoforms of CEACAM1 (Edlund, Wikstrom et al. 1998) and other unknown kinases are also responsible for CEACAM1 serine/threonine phosphorylation. Both Tyr488 and Ser503 are needed for insulin receptor internalization (Formisano 1995). Moverover, mitogenic responses to insulin were enhanced in cells transfected with either the Tyr488 or Ser503 mutants (Formisano 1995). Ser503 also participates in CEACAM1-L-mediated tumor

inhibition as its mutation to an Ala led to *in vivo* tumor development, contrary to the tumor inhibitory phenotype observed with the wild-type CEACAM1-L protein (Fournes 2001). PKC had no obvious effect on *in vivo* phosphorylation of CEACAM1-L. Basal phosphorylation of Ser503 also regulates the intracellular trafficking of CEACAM1 (Choice 1998). The Ser503 mutation to alanine correlates with incomplete maturation of full length CEACAM1 in NIH 3T3 cells and hepatocytes. The intracellular domain contains significant information that regulates vectorial transport from TGN (Trans-Golgi network) to the plasma membrane (Choice 1998).

The role of CEACAM1 in regulation of proliferation has been studied in several *in vitro* systems. CEACAM1 is constitutively phosphorylated in adherent growing cells as well as in cells growing in suspension. CEACAM1-L-mediated cell aggregation is accompanied by a 40% reduction in CEACAM1-L phosphorylation compared with non-aggregated cells. The phosphorylation status indicates a CEACAM1-mediated outside-in signalling induced by cell-cell adhesion (Edlund, Wikstrom et al. 1998).

The expression levels and the S:L isoforms ratios differ in proliferating and quiescent rat epithelial cells. In confluent cells, CEACAM1 exerts inhibitory effects on cell proliferation. Addition of anti-CEACAM1 antibodies to quiescent, confluent cells causes decreased expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, stimulated growth factor-dependent DNA synthesis, and alters the S:L isoforms ratio toward the ratio characteristic of proliferating cells. CEACAM1 contributes to contact inhibition of cell proliferation in confluent cells but allows proliferation when expressed at different isoforms ratios. Endogenous CEACAM1 can regulate $p27^{Kip1}$ levels and DNA synthesis (Singer, Scheffrahn et al. 2000). CEACAM1 exerted its effects by regulating the activity of the Erk 1/2 MAP kinase pathway and the expression levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. CEACAM1 mediates contact inhibition of proliferation in cells that have been starved of growth factors (Scheffrahn, Singer et al. 2005). In the context of cancer, loss of CEACAM1 removes the block on cells that are in a nutrient poor environment from proliferating. This in part explains the correlation

between loss of CEACAM1 expression and cancer development, which results in loss of contact inhibition.

In PC12 cells, formation of large CEACAM1 clusters and rapid and transient CEACAM1 tyrosine dephosphorylation can be induced by treatment with CEACAM1-specific antibodies. Clustering also stimulates binding of CEACAM1 to the actin cytoskeleton. CEACAM1-L tyrosine dephosphorylation and interaction with the cytoskeleton are sensitive to neuronal differentiation of PC12 cells. Downstream activation of the mitogen-activated protein kinases Erk1 and Erk2, but not of JNK or p38 is how CEACAM1 mediates signalling (Budt, Cichocka et al. 2002).

1.13.1 Other binding partners of CEACAM1

Apart from kinases and phosphatases, other proteins have been shown to interact with both the short and long cytoplasmic domains of CEACAM1. The short cytoplasmic tail contains sequences that can bind calmodulin (Edlund, Blikstad et al. 1996), tropomyosin and globular actin (Schumann, Chen et al. 2001), indicating a regulated interaction with the cytoskeleton. F454 and K456 are key residues that interact with actin orchestrating the cytoskeletal reorganization. Residues T457 and / or S459 are phosphorylated in CEACAM1 transfected cells grown in 3D culture and mutation analysis of these residues T457A/S459A or F454A has shown that lumen formation is blocked. This establishes that the short cytoplasmic domain membrane receptor can directly mediate substantial intracellular signalling (Chen, Kirshner et al. 2007). Yeast two-hybrid experiments indicate direct binding between filamin A (FLNa) and CEACAM1 (Klaile 2005). FLNa mediates at least three functions that are crucial for cell shape modulation and motility (Stossel, Condeelis et al. 2001; van der Flier and Sonnenberg 2001). (1) FLNa homodimers crosslink filamentous actin to three-dimensional, orthogonal networks. (2) It anchors actin filaments to cell extracellular matrix adhesion sites by binding to β-integrin subunits. (3) FLNa provides a scaffold for small GTPases of the Ras and Rho families. FLNa has been implicated in migration (Cunningham, Gorlin et al. 1992). In migration

studies the interaction of CEACAM1-L and filamin A drastically reduced migration and cell scattering. CEACAM1-L binding to filamin A reduced the interaction of the latter with RalA, a member of the Ras-family of GTPases. Co-expression of CEACAM1-L and filamin A leads to a reduced focal adhesion turnover (Klaile 2005). The interaction between CEACAM1 and FLNa could be one of the mechanisms through which CEACAM1 regulates cell migration.

Recently, DNA polymerase delta-interacting protein 38 (PDIP38) has been identified as a novel binding partner of CEACAM1-L and CEACAM1-S (Klaile, Muller et al. 2007). PDIP38 can occur in the nucleus, in the cytoplasm and at the plasma membrane in a variety of cells. Interaction of CEACAM1 and PDIP38 is of functional importance in NBT-II (rat bladder) cells. Perturbation of CEACAM1 by antibody clustering induces increased binding to PDIP38 and results in rapid recruitment of PDIP38 to the plasma membrane. Subcellular localization of PDIP38 is regulated by CEACAM1 (Klaile, Muller et al. 2007). PDIP38 was originally identified as a PCNA- and DNA polymerase δ-interacting protein, implicating a function in the regulation of gene expression, DNA duplication or DNA repair (Liu, Rodriguez-Belmonte et al. 2003). PDIP38 thus represents an interesting interaction partner for CEACAM1, since it could link the transmembrane adhesion receptor signalling directly to regulatory processes inside the nucleus.

The cytoplasmic domains of rat and mouse CEACAM1-L are substrates for tissue transglutaminase, and lend support to the notion that higher molecular weight forms of CEACAM1-L are formed by transglutaminase modification (Hunter, Sigmundsson et al. 1998). Transglutaminases are a group of enzymes found in all cells studied, which catalyse formation of proteolytically-resistant bonds within or between proteins. They are implicated in diverse cellular processes, including covalent cross-linking of fibrin clots, formation of the cornified envelope of keratinocytes, stabilisation of extracellular matrices, formation of a detergent-insoluble protein scaffold in cells undergoing apoptosis, and regulation of cellular growth (Facchiano, Facchiano et al. 2006).

The long cytoplasmic domain of CEACAM1-4L contains a highly conserved sequence that has ~ 50% sequence homology with Tcf-3 and -4, transcription factors that bind β-catenin. The association of CEACAM1 and β-catenin was tested by quantitative yeast two-hybrid, BIAcore, GST-pull down, and confocal analyses. The interaction with the armadillo repeats of β-catenin depends on residues H469 and K470. In breast cancer cells that do not express CEACAM1 or E-cadherin, transfection with CEACAM1 causes redistribution of β-catenin from the nucleus to the cytoplasm (Jin 2007).

1.13.2 CEACAM1 dimers: a mode of signal transduction

CEACAM1 exists as non-covalently linked dimers both in solution and on the cell surface. Dimer formation is the result of cis-interactions within the membranes of individual cells. Dimerization is affected by intracellular calcium ion concentrations. At high Ca^{2+} concentrations the binding of Ca^{2+}-loaded calmodulin to membrane-proximal sequences in the cytoplasmic domain of both the short and long isoforms of CEACAM1 causes dissolution of cis-dimers *in vitro* (Hunter, Sawa et al. 1996). Potential serine and threonine phosphorylation sites that overlap the calmodulin-binding sites indicate that protein kinase C (PKC) and / or other kinases might also affect dimerization (Obrink 1997). Homophilic binding seems higher when multiple IgC2 domains are present (Watt, Teixeira et al. 2001). CEACAM1 can have both positive and negative effects on cell signalling. The equilibrium between monomeric, and dimeric forms undoubtedly affects cellular responses to CEACAM1 binding. CEACAM1 can exert its signal-regulating activities by discriminating between binding of Src kinases and SHP phosphatases. Major factors that regulate this discrimination are the expression levels and expression ratios of the transmembrane forms of CEACAM1-L and CEACAM1-S, the concentration of secreted CEACAM1, and homophilic binding of CEACAM1 presented by neighbouring cells (Obrink 2002). According to computer generated models, the CEACAM1 dimers would preferentially bind to Src kinases exclusively, while CEACAM1 monomers could

bind both SH2 domains of phosphatases SHP-1 and -2 and Src kinases (Obrink 2002).

1.14 Functions of CEACAM1

1.14.1 Cell Adhesion Molecules

Adhesive interactions are thought to play a major role in the construction of the body plan of multicellular organisms during development. Adhesion is also important in the maintenance of the body plan; tumor cells are able to loosen their attachment to leave their original location and become lodged at distant sites. In addition to mediating adhesive interactions, adhesion molecules also serve as signalling molecules (Ruoslahti and Obrink 1996). This is the case for CEACAM1 as seen above.

The majority of Ig CAMs are involved in cellular recognition phenomena. The common building block is the immunoglobulin domain of about 100 aa residues that is arranged as a sandwich of two sheets of anti-parallel β-strands. They generally mediate adhesion in a calcium-independent manner (Obrink 1991). Initially CEACAM1 was identified as a cell-cell adhesion molecule mediating Ca^{2+}-independent homophilic binding between the N-terminal Ig domains presented by the surfaces of adjacent cells (Ocklind 1982). In addition, it has been reported that CEACAM1 mediates heterophilic binding to other CEA family members (Oikawa 1992). Specific hydrophobic amino acid residues, on the non-glycosylated GFCC'C" face of CEACAM1 N-terminal domain, are involved in heterophilic interactions with *Neisseria gonorrhoeae* Opa proteins and *H. influenzae* and are also critical for protein-protein interactions between 2 CEACAM1 molecules on opposing cells (Watt, Teixeira et al. 2001).

Analyses of whole liver membrane fractions demonstrated that CEACAM1 is one of five major proteins that bind calmodulin in a calcium-dependent manner (Blikstad, Wikstrom et al. 1992). Binding of calmodulin by the cytosolic domains causes a down-regulation of the homophilic self-association of the CEACAM1. This suggests that calmodulin can regulate the functional activity of CEACAM1.

Calmodulin in the presence of calcium greatly reduces CEACAM1 binding to itself. No obvious effect of calmodulin was seen in the absence of calcium (Edlund, Blikstad et al. 1996). The co-localization and coordinate reorganization of both CEACAM1 and actin by anti-CEACAM1 antibodies indicated that these two proteins were associated and suggested that interactions with the cytoskeleton may be important for the regulation of CEACAM1 function. The specific up regulation of CEACAM1 in cells induced to undergo epithelial to mesenchymal-like transitions (EMT) by the serum substitute Ultroser G suggested that CEACAM1 isoforms are important modulators of the adhesive properties of NBT-II cells (rat bladder cells) (Hunter, Lindh et al. 1994).

1.14.2 Cholesterol crystallization

CEACAM1 is secreted into bile, hence its historic name of biliary glycoprotein (Bgp). Its concentration in hepatic bile was estimated to be about 10 mg/L (Svenberg, Wahren et al. 1979). CEACAM1 is considered to be a bile-specific protein. The 85kd glycoprotein appears to be the most effective promoter of cholesterol crystallization (de Bruijn, Mok et al. 1996). CEACAM1 exhibits potent cholesterol crystallization promoting activity *in vitro* and accounts for most of the activity in CABF (concanavalin A-binding fraction). The protein component of the cholesterol crystallization promoting LDP (low-density protein-lipd complex) is CEACAM1 (Jirsa, Muchova et al. 2001). CEACAM1 accounts for most of the activity present in CABF purified from rapidly nucleating gallbladder bile. (Jirsa, Muchova et al. 2001).

In addition to its cell adhesion activities, CEACAM1 proteins play crucial roles in immune response regulation (Gray-Owen 2006), vascular neogenesis (Wagener and Ergun 2000), insulin metabolism (Najjar 2002), apoptosis (Kirshner, Chen et al. 2003; Nittka, Gunther et al. 2004), tumor development (Fournes 2001) and they function as receptors for pathogenic bacteria and viruses (Gray-Owen, Dehio et al. 1997; Hemmila 2004).

1.14.3 CEACAM1 as a pathogen receptor

Two *Ceacam1* alleles have been identified in mice. The Ceacam1a allele confers susceptibility to MHV infection and is present in most inbred mouse strains, and outbred mice. CEACAM1a is the sole receptor for MHV-A59 in both liver and brain and its deletion from the mouse renders the mouse completely resistant to infection by this virus (Hemmila 2004). In humans, carbohydrate moieties in CEACAM1 appear to be recognized by enterobacteria such as *E. coli* and *Salmonella* strains (Leusch, Drzeniek et al. 1991; Sauter, Rutherfurd et al. 1993), and the N-terminal domain of CEACAM1 is the main target of several bacterial pathogens specialized to colonize the human mucosa. *Neisseria gonorrhoeae* and *N. meningitides*, *Heamophilus influenzae* and *Moraxella catarrhalis* express specific surface proteins that bind to CEACAM1, and stimulate the internalization of the micro-organisms (Gray-Owen, Lorenzen et al. 1997).

1.14.4 CEACAM1 in the immune system

CEACAM1 is expressed at low levels on resting T and B cells, but is undetectable in resting NK (natural killer) cells. Human IELs (Morales, Christ et al. 1999) and peripheral blood T cells (Kammerer 1998) express only CEACAM1, indicating a unique role for this receptor in T-cell function. Activation by cytokines dramatically increases the relative mRNA and protein levels of CEACAM1 in all these cell types. CEACAM1 mobilization to the cell surface precedes the activation-induced expression of the co-inhibitor receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) (Nakajima 2002), and the long cytoplasmic domain of human CEACAM1 can replace the well-described inhibitory function of the cytoplasmic domain of the low-affinity Fc receptor for IgG, FcγRIIB in B cells (Chen 2001). Homophilic trans-ligation of CEACAM1 on NK cells causes inhibition of their cytotoxic activity (Markel 2002). CEACAM1-mediated inhibition requires the tyrosine residues in the membrane-proximal end, less so the membrane-distal ITIM of CEACAM1, and is mediated by SHP-1 and SHP-2 (Chen 2001). This indicates that the Tyr488 residue is crucial for NK cell

activity. T cells from CEACAM1-deficient mice hyperproliferate and show an increase in IL-2 and IFNγ secretion in response to various *in vitro* and *in vivo* stimuli (Gray-Owen 2006; Nagaishi, Pao et al. 2006). Therefore, the CEACAM1 function is predominantly inhibitory and depends on the signalling function of the long cytoplasmic domain.

Neutrophils express CEACAM3, CEACAM4, CEACAM6 and CEACAM8, in addition to CEACAM1. CEACAMs are rapidly mobilized to the cell surface in response to various activating signals (Stocks and Kerr 1993). CEACAM1-dependent intercellular binding confers survival signals that prevent neutrophil apoptosis, which might allow persistence of these important phagocytes at a site of infection (Singer, Klaile et al. 2005). Dendritic cells (DCs) express both long and short cytoplasmic variants of CEACAM1, but no other CEACAMs (Gray-Owen 2006). CEACAM1 is functionally important during inflammation and also mediates inhibitory signals *in vivo* (Iijima 2003).

Pathogens exploit the immunosuppressive capacity of CEACAM1. CEACAM1-specific MHV spike glycoproteins have also recently been shown to have an immunosuppressive effect. Purified spike protein inhibited in vitro differentiation of naïve cells into T_H1 cells without a similar effect on T_H2-cell differentiation, and prevented cytokine expression by differentiated T_H1 cells (Iijima 2003). Trans-ligation of CEACAM1 by Opa proteins from *N. gonorrhoeae* suppresses the activation and proliferation of primary human CD4+ T cells. CEACAM1 bound Opa variants is associated with SHP-1 and SHP-2 (Boulton and Gray-Owen 2002) which function in the CEACAM1-dependent inhibition of epithelial-cell growth and CD4+ T-cell activation (Chen 2004). Opa binding to CEACAM1 on B cells reduces their production of immunoglobulin by killing the B cells (Pantelic, Kim et al. 2005). DC function is also impaired by Opa-CEACAM1 binding. CEACAM1-binding Opa proteins cause a significant reduction in the capacity of DCs to present either allotypic or recall antigen (Gray-Owen 2006), and also drives DCs to an incompletely mature state.

1.14.5 Immunosuppression and cancer

CEACAM1 might also be involved in suppression of tumor immunosurveillance, because upregulation of CEACAM1 on melanomas allows homophilic binding with CEACAM1 on NK cells, thereby inhibiting NK-cell-mediated killing (Markel 2002). CEACAM1 expression is induced in some epithelial cell malignancies and high CEACAM1 expression has a significant association with poor prognosis (Thies, Moll et al. 2002; Gray-Owen 2006). This is consistent with immunosuppressive activities of CEACAM1. CEACAM1 expression by a cell line protects it from targeted killing by NK cells in a dose-dependent manner (Markel 2002). This explains the poor prognosis for survival if CEACAM1 is expressed by the primary melanoma lesions (Thies, Moll et al. 2002), although CEACAM1 expression also increases the invasive behaviour of the melanoma (Ebrahimnejad 2004). Antibody ligation of CEACAM1 effectively inhibits the activation of cytotoxic T cells in vitro (Morales, Christ et al. 1999). Intercellular binding of CEACAM1 to adjacent cells thereby functions to inhibit cellular growth, whereas CEACAM1-dependent interactions with an immune cell inhibits target-cell killing. Therefore, the aberrant expression of CEACAM1 during proliferation of a cell that has lost the ability to control its cell cycle would generate a tumor that resists immune-cell-mediated killing (Thies, Moll et al. 2002) (Laack 2002).

1.14.6 Vascularization

The finding that vascular endothelial growth factor (VEGF) increases CEACAM1 expression in endothelial cells and that VEGF-induced endothelial tube formation is blocked by a monoclonal CEACAM1 antibody leads to the conclusion that CEACAM1 is a major effector of VEGF in early microvessel formation (Ergün 2000). Moreover, soluble CEACAM1 exhibits pro-angiogenic effects by stimulating the proliferation, chemotaxis and capillary-like tube formation of human microvascular endothelial cells in vitro, as well as increasing the vascularization of the chorioallantoic membrane of chicken embryos in vivo

(Ergün 2000). CEACAM1 is expressed in the microvessels of areas of active tumor maturation among differentiating neuroblastic / ganglion cells, whereas it is completely absent in the vessels of poorly differentiated / undifferentiated as well as in entirely mature Schwannian-rich areas. VEGF expression has been found in differentiating neuroblastic/ganglion cells adjacent to CEACAM1-positive microvessels. These results point to a role for CEACAM1 / VEGF cross-talk during the maturation phase of neuroblastic tumors (Poliani 2007). In a matrigel plug assay *Ceacam1*$^{-/-}$ mice failed to establish new capillaries whereas in transgenic mice overexpressing CEACAM1-L in endothelial cells, the implants were vascularized extensively (Horst 2006). Induction of hind limb ischemia by femoral artery ligation in *Ceacam1*$^{-/-}$ mice showed significantly reduced growth of arterioles and collateral blood flow compared with their WT littermates. Conversely transgenic mice overexpressing CEACAM1-L in endothelial cells exhibited an increase in revascularization and collateral blood flow after arterial occlusion. CEACAM1 expression is important for the establishment of newly formed vessels *in vivo* (Horst 2006). Analyses with cDNA arrays revealed that CEACAM1 is part of the hypoxia-induced genetic program, as it is prominently induced on the microvessels of the left ventricle of chronically hypoxic rats as well as upon myocardial infarction of mice (Chen, Chen et al. 2005).

In figure 1.14.1, a model of the angiogenic action of CEACAM1 is shown. VEGF activated endothelial cells produce and secrete CEACAM1, which stimulates proliferation and migration of endothelial cells and functions as an autocrine factor. In a further step of blood vessel formation, transmembrane forms of CEACAM1 act as membrane-bound cell adhesion molecules in endothelial tube formation. Finally, through binding to components of the basal lamina, CEACAM1 is also involved in the deposition of the vascular basement membrane and in the recruitment of accessory cells and, in this way, may contribute to the maturation of newly formed blood vessels (Wagener and Ergun 2000).

CEACAM1 is expressed on tumor-associated small endothelia but not in large and quiescent blood vessels (Kilic 2005). The association of CEACAM1 and tumor angiogenesis is further demonstrated by the observation that

CEACAM1 downregulation on prostate intraepithelial neoplasia (PIN) is inversely correlated with its upregulation in adjacent blood vessels. CEACAM1 silencing increases expression of angiogenic / lymphangiogenic factors such as vascular endothelial growth factor (VEGF)-A, -C, -D and angiogenic inhibition collagen 18/endostatin. Disappearance of epithelial CEACAM1 in PIN is accompanied by its upregulation in adjacent vasculature that apparently correlates with vascular destabilization and increased vascularization of prostate cancer (Tilki 2006). However, the CEACAM1-mediated signalling mechanisms remain to be fully elucidated.

1.14.7 CEACAM1 and cancer

The expression of CEACAM1 is significantly downregulated in premalignant adenomas (Nollau 1997) and 90% of colon tumors (Neumaier 1993), prostate (Hsieh 1995; Busch 2002), 30% of breast carcinomas (Luo 1997), liver (Hixson 1985; Tanaka 1997), endometrial (Bamberger 1998) and bladder cancers (Kleinerman 1996). Loss of CEACAM1 expression reflects aggressive tumor biology and thus indicates a poor prognosis for patients with HCC (Hepatocellular carcinoma) (Cruz, Wakai et al. 2005). Loss of CEACAM1 expression is discerned in 80% of poorly differentiated or undifferentiated HCCs (Tanaka 1997). Another study revealed high frequency of loss of CEACAM1 expression in poorly differentiated tumors (Cruz, Wakai et al. 2005). This suggests that loss of CEACAM1 expression in cells from a variety of tumors indicates a high metastatic potential. However CEACAM1 is not always downregulated in cancer. Overexpression of CEACAM1 is found in melanomas (Thies, Moll et al. 2002), lung adenocarcinomas (Laack 2002) and gastric carcinomas (Kinugasa 1998). CEACAM1 is expressed in pancreatic adenocarcinoma, and serum levels of CEACAM1 serve as a useful indicator for the presence of pancreatic cancer (Simeone, Ji et al. 2007). CEACAM1 expression in tumor cells has been demonstrated to have prognostic significance in lung carcinoma (Laack 2002; Sienel, Dango et al. 2003) and malignant melanoma (Thies, Moll et al. 2002).

Figure 1.14.1: Model of the role of CEACAM1 in the formation of blood vessels.

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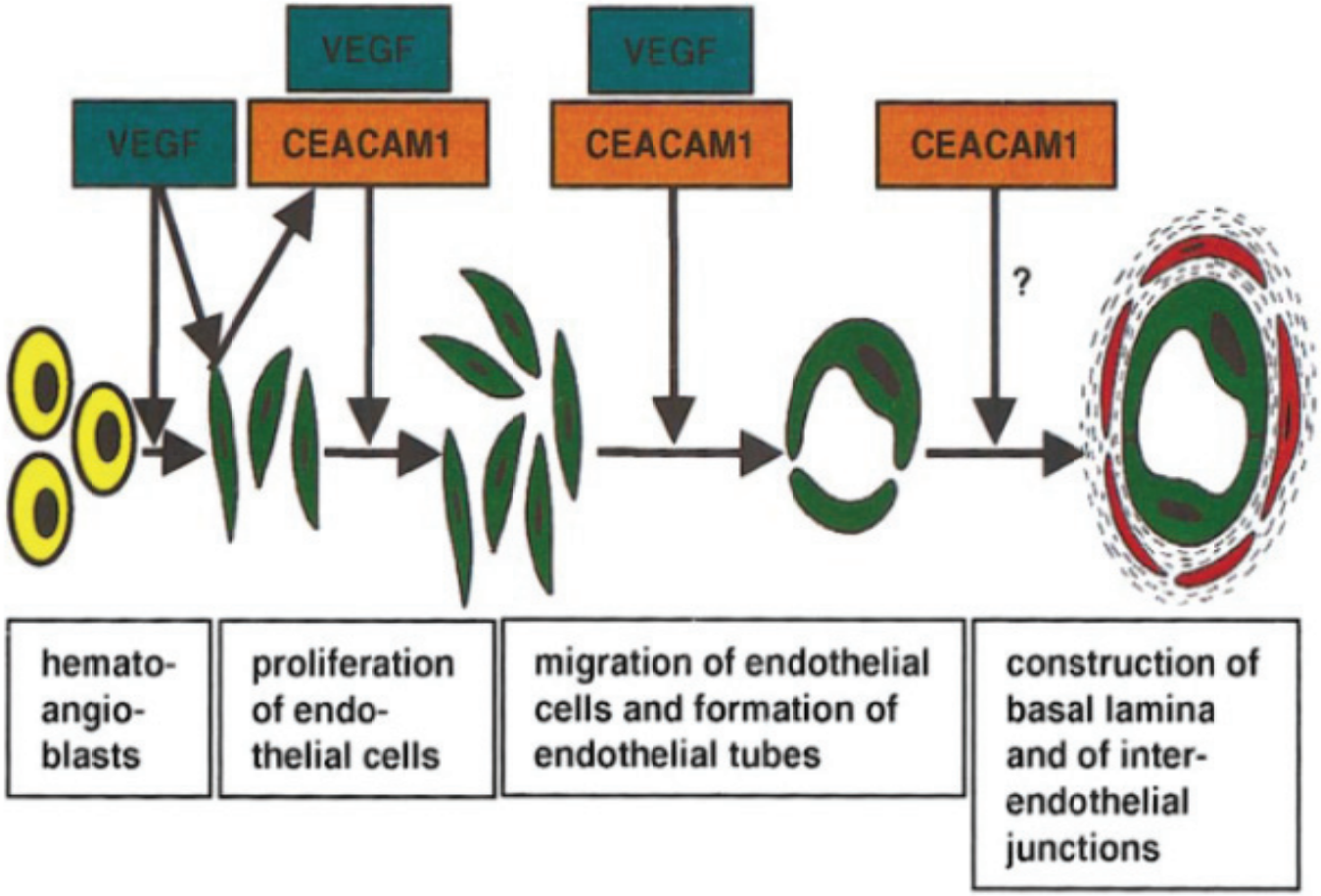

VEGF
VEGF
VEGF
CEACAM1
CEACAM1
CEACAM1
?
hemato-angio-blasts
proliferation of endo-thelial cells
migration of endothelial cells and formation of endothelial tubes
construction of basal lamina and of inter-endothelial junctions

1.14.7.1 Regulation of CEACAM1 expression in cancer

So far no mutations in CEACAM1 have been identified in the various cancer settings in which it is implicated (Rosenberg 1993). This indicates that the down regulation of CEACAM1 is either under transcriptional control, or post-translational modification targeting it for degradation. Very little research has been conducted on the latter case.

1.14.7.1.1 Sp2

In normal prostate epithelial cells Sp2 binds to the CEACAM1 promoter *in vitro* and *in vivo*, and transient overexpression of Sp2 down-regulates endogenous CEACAM1 expression (Phan 2004). Sp2 is a member of the Sp family of transcription factors, which consists of Sp1 through Sp5 (Suske 1999). Sp1 is a general transcription factor that regulates many ubiquitously expressed genes by binding to GC boxes at their promoters. Sp2 has been shown to bind to a GT-box promoter element within the T-cell receptor alpha promoter *in vitro* (Kingsley and Winoto 1992). Sp2 appears to repress CEACAM1 gene expression by recruiting histone deacetylase activity to the CEACAM1 promoter (Phan 2004). This indicates that down regulation of CEACAM1 expression in prostate tumors occurs mainly at the transcriptional level. Androgen is the most important factor that regulates prostate growth and differentiation.

1.14.7.1.2 Androgenous Receptor

It has been reported that the androgen receptor (AR) can enhance the (rat) *Ceacam1* promoter activity in a ligand-dependent manner *in vitro*. This androgen regulation is through direct AR-promoter binding because a mutant AR defective in DNA binding fails to upregulate reporter gene expression (Phan, Sui et al. 2001). Regulation of CEACAM1 expression by androgen *in vivo* is a complex process. In the rat, expression of CEACAM1 in the ventral prostate is negatively regulated by androgen *in vivo* (Hsieh, Luo et al. 1995). In mouse prostate, increased CEACAM1 expression is detected in the dorsal prostate lobe following

castration ((Pu, Luo et al. 1999). Whether AR can upregulate the expression of CEACAM1 or not, depends on the status of the cells, and the context in which they reside.

1.14.7.1.3 Estrogen

CEACAM1 is present in the apical zones of the luminal and glandular epithelium cells of the rat uterus in a stage-specific and hormone-dependent manner. Steroid hormones are essential for the expression of CEACAM1 in the uterine epithelial cells. Progesterone induces CEACAM1 expression in the glandular epithelium, and estrogen induces CEACAM1 expression in the luminal epithelium, however, progesterone induces down-regulation of CEACAM1 from the surface of the uterine luminal epithelium of juvenile rats. CEACAM1 is absent in the luminal epithelial cells but present in the glandular epithelial cells of the rat uterus at the time of blastocyst implantation. These promoter studies indicate that the *Ceacam1* gene contains elements that are under estrogen regulation (Svalander, Odin et al. 1990).

1.14.7.1.4 Calcium

CEACAM1 expression in Hec1B, an endometrial tumor cell line that does not normally express CEACAM1, can be induced at the mRNA and protein levels when treated with TPA and calcium ionophore A23187. Skut1B are endometrial mixed mesenchymal cells and did not induce CEACAM1 expression. Induction of CEACAM1 is dependent on protein kinase C protein activity and luciferase reporter assays with CEACAM1 promoter constructs demonstrate that the re-expression of CEACAM1 is regulated at the transcriptional level (Bamberger, Briese et al. 2006).

1.14.7.1.5 IFN- γ

Treatment of breast cancer cells with Mullerian inhibiting substance (MIS) and interferon γ (IFN- γ) co-stimulated IRF-1 and CEACAM1 expression and synergistic induction of CEACAM1 by a combination of MIS and IFN- γ was

impaired by antisense IRF-1 expression (Hoshiya, Gupta et al. 2003). MIS is a member of the TGFβ family, it has an important role in innate and adaptive immunity, and IRF-1 plays a role in regulating the growth of different mammalian cell lines. Different aspects of the tumor suppressor function of IRF-1 may be explained, at least in part, by the observation that it induces several growth regulatory genes including those with anti-proliferative activity such as IFNα/β, p21, and the cell adhesion molecule CEACAM1 (Hoshiya, Gupta et al. 2003).

The human colonic cancer cell line HT29, that expresses low levels of CEACAM1 undergoes apoptosis when treated with INF-γ and anti-CEACAM1 antibodies. IFN-γ induces CEACAM1 expression in human colon carcinoma cells (Chen, Lin et al. 1996). Exposure to chokeberry juice (rich in polyphenols) inhibited Caco-2 cell proliferation by causing G(2)/M cell cycle arrest. CEACAM1 expression was up-regulated by this treatment both at the mRNA and protein levels (Bermudez-Soto, Larrosa et al. 2007). Indicating a further link between CEACAM1 and proliferation control.

1.14.7.1.6 Promoter elements of the Ceacam1 gene

Analysis and footprinting experiments of the promoter region of *CEACAM1* identified two cis-acting elements. These elements bind several transcription factors, among them, USF, HNF-4 and an AP-2-like factor. In cotransfection experiments, both the USF and HNF-4 transcription factors transactivate the *CEACAM1* gene promoter and compete for the same regulatory element (Hauck 1994).

1.14.7.2 Kidney cancer

In normal kidney, CEACAM1 was found in epithelial cells of proximal tubules and in endothelial cells. In contrast, tumor cells of 30 clear cell, 3 chromophobic, and 2 chromophilic renal cell carcinomas (RCCs) were completely devoid of CEACAM1. Transient expression of the tumor suppressor CEACAM1 by tumor cells and subsequent homophilic interaction with CEACAM1 on tumor-

infiltrating lymphocytes could represent a novel immune escape mechanism in RCC (Kammerer 2004).

1.14.7.3 Bladder cancer

CEACAM1 also suppresses bladder cancer progression. In an orthotopic tumor model, recombinant adenovirus expressing CEACAM1 were used to infect the 253J B-V cell line, a tumorigenic human bladder carcinoma subline. When these cells were injected orthotopically into nude mice, the increased expression of CEACAM1 in the 253J B-V cells repressed the growth of 253J B-V-induced tumors. Taken together, these data indicate that CEACAM1 is a potent tumor suppressor in human bladder cancer (Kleinerman 1996).

1.14.7.4 Thyroid cancer

CEACAM1 is not appreciably expressed in normal thyroid tissue or benign thyroid tumors. In a human thyroid tissue array, CEACAM1 reactivity is associated with metastatic spread but not with increased tumor size. Introduction of CEACAM1 into endogenously deficient WRO cells (less aggressive thyroid cancer cell) results in reduced cell cycle progression associated with p21 upregulation and diminished Rb phosphorylation. Forced CEACAM1 expression enhances cell-matrix adhesion and migration and promotes tumor invasiveness. Conversely downregulation of CEACAM1 expression in MRO cells (follicular) accelerated cell cycle progression and significantly enhanced tumor size in xenografted mice. CEACAM1 is a unique mediator that restricts tumor growth whereas increasing metastatic potential (Liu 2006).

1.14.7.5 Melanoma

In melanocytic MEL6 cells, CEACAM1 expression markedly enhances invasion and migration, which is dependent on the presence of Tyr488. Treatment with antibodies blocks CEACAM1-enhanced cell invasion and cell

migration. Expression of integrin β(3) induces the up-regulation of CEACAM1 in MEL6 cells. Invasion and migration are blocked by integrin-antagonizing RGD peptides (Ebrahimnejad 2004). These results indicate that CEACAM1 acts downstream of integrin, and affects integrin-mediated migration and invasion of melanomas.

1.14.7.6 Breast cancer

In normal breast, CEACAM1 expression is confined to the apical surface of ductal and lobular epithelial cells, while in invasive carcinoma of the breast, CEACAM1 is expressed throughout the cytoplasm (Huang 1998). The same report indicates the down regulated expression of CEACAM1-4L in 30% of breast carcinoma, but not the other isoforms. However, another study indicates that 23% of invasive breast carcinomas do not express total CEACAM1 (Riethdorf, Lisboa et al. 1997). Both the pattern of surface expression and total CEACAM1 expression vary during the progression of mammary tumors. Even though CEACAM1 expression is not altered in all breast cancers, it can suppress the tumorigenicity of breast cancer cells. The cytoplasmic domain, but not the extracellular adhesion domain, of CEACAM1 is critical for growth suppression. Thus, the adhesion and the growth suppression functions of CEACAM1 are independent of each other. Furthermore, mutation at the tyrosine phosphorylation site in the cytoplasmic domain of CEACAM1 did not obliterate CEACAM1's growth suppression function, suggesting that tyrosine phosphorylation is not involved in the signal transduction pathway leading to cell growth suppression (Luo 1997). A similar effect is observed in prostate cells where mutation of Tyr488 to phenylalanine did not abolish the tumor-suppressive activity of CEACAM1; however, mutation of Ser503 to alanine did abolish the growth-inhibitory activity (Estrera, Chen et al. 2001). A mutation of Ser 503 to aspartic acid (to mimic a phosphorylated site) produced tumor–suppressive activity similar to that of the wild-type CEACAM1. Therefore, phosphorylation at Ser503 is essential for CEACAM1's growth-inhibitory function in vivo (Estrera, Chen et al. 2001).

1.14.7.7 Colon cancer

One of the major differences between colon and prostate / breast tumors lies in the requirement for Tyr488 phosphorylation. A single point mutation of Tyr488, conforming to the ITIM, was sufficient to reverse the *in vivo* tumor cell growth inhibition of CT51 mouse colon carcinoma cells (Izzi, Turbide et al. 1999). However, the Ser503 residue remains of critical importance as Ser503 mutation to an Ala leads to *in vivo* tumor development, contrary to the tumor inhibitory phenotype observed with the wild-type CEACAM1-L protein in CT51 cells (Fournes 2001). Substitution or deletion of residues in the C-terminal region of the CEACAM1 cytoplasmic domain also leads to reversal of tumor cell growth inhibition. Removal of the N-terminal domain of CEACAM1, essential for intercellular adhesion, does not impair the tumor inhibitory effect (Izzi, Turbide et al. 1999). Therefore, the one difference in CEACAM1's ability as a tumor suppressor in breast/prostate cancer and colon cancer seems to be the dependence on Tyr 488 phosphorylation. On one hand, breast and prostate tissues possess many hormone responsive elements, whereas colon cells participate in nutrient absorption and have a high proliferative rate. The contexts of these tissues are very different, which could account for the different requirements in CEACAM1 phosphorylation.

Four members of the family that are expressed in normal colon are CEA, CEACAM1, CEACAM6, and CEACAM7. They exhibit an exclusively apical expression pattern in the colon, and other organs (Shively 2004). CEACAM1 is a major player in the process termed anoikis that states that cells not in contact with basement membrane are doomed to die. Apoptotic figures are decreased in ACF and HP (hyperplasia) that lack CEACAM1 expression (Nittka 2004).

Loss of CEACAM1 expression is more common in neoplastic tumors than APC mutations. APC functions were normal in hyperplastic aberrant crypt foci and hyperplastic polyps, whereas loss of CEACAM1 expression in the hyperplastic tumors correlates with normal or reduced apoptosis, respectively. CEACAM1 acts as a regulator of apoptosis in CEACAM1-transfected Jurkat

cells, as well as HT29 colon cancer cells *in vitro*. In both hyperplasia and neoplasia, the loss of CEACAM1 expression starts in the transition zone between normal mucosa and the tumor lesions (Nittka 2004). In colorectal adenocarcinoma, it has been reported that reduced CEACAM1 expression of tumor cells was associated with regional lymph node metastasis (Neumaier 1993).

1.14.7.8 Control of cancer growth

The CEACAM1-S and CEACAM1-L proteins are co-expressed in most cells in various ratios depending on the cell type. In submandibular gland and seminal vesicle a significant increase of the S-isoform was found, whereas in kidney the L-isoform was overexpressed (Baum, Troll et al. 1996). In T cells CEACAM1-L predominates over CEACAM1-S isoforms with a relative ratio of 3:1 (Gray-Owen 2006), whilst in mouse B cells express both CEACAM1-2 and CEACAM1-4, with approximately equal ratios of the long- and short-cytoplasmic-tail-containing isoforms (Coutelier 1994; Greicius 2003). The amount of longer variant in the normal colonic epithelial cells constitutes 15-20% of total CEACAM1 expressed in this tissue. To mimic the *in vivo* situation double transfectant lines were created in tumorigenic CT51 mouse colonic carcinoma cells. CEACAM1-L isoforms exhibits a dominant tumor growth inhibition phenotype over that of the shorter variant within physiological levels of expression CEACAM1. Significant overexpression of CEACAM1-L isoforms alone leads to reversal of the tumor inhibition phenotype (Turbide 1997). The difference in expression of the short and long cytoplasmic domain isoform has a considerable effect on the signalling abilities of CEACAM1.

CEACAM1-L confers survival signals. CEACAM1-specific antibodies and soluble CEACAM1 protect peripheral blood mononuclear cells from apoptosis. Monocyte survival correlates with a CEACAM1-dependent up-regulation of the cellular inhibitor of apoptosis Bcl-2 and the abrogation of caspase-3 activation. CEACAM1 binding triggers a PI3K dependent activation of the protein kinase Akt without influencing the activity of extracellular signal-related kinase ERK; the PIK3-specific inhibitor LY294002 blocked the protective effect of

CEACAM1. In another system, CEACAM1 confers a PI3K- and Akt-dependent survival signal that inhibits mitochondrion-dependent apoptosis of monocytes (Yu, Chow et al. 2006).

The apoptotic function of CEACAM1 has been mainly studied in mammary cell lines and lumen formation. In a 3D model of breast morphogenesis, CEACAM1 plays an essential role in lumen formation in a subline of the non-malignant human breast cell line. Lumena are formed by apoptosis as opposed to necrosis and apoptotic cells within the lumena express CEACAM1-S. Apoptosis is mediated by Bax translocation to the mitochondria and release of cytochrome *c* into the cytoplasm (Kirshner, Chen et al. 2003). MCF-7 cells expressing CEACAM1-L isoforms grown in humanized mammary mouse fat pads formed glands with lumen. Phosphorylation mimics of Thr and Ser residues to Asp induce gland formation with a central lumen-containing apoptotic cells. Null mutations did not. Phosphorylation mimic mutants of CEACAM1-S can induce downregulation of β1-integrin, overexpression of β2-integrin, inhibits phosphorylation of focal adhesion kinase and results in myofibroblast differentiation, as well as production of abundant extracellular matrix (Yokoyama, Chen et al. 2007).

CEACAM1 is expressed in HepG2 cells (liver carcinoma line). When HepG2 cells are treated with small interfering RNA targeted against CEACAM1, the growth rate in monolayer culture is increased. Inhibition of CEACAM1 expression significantly increases the growth rate of these cells. Cell-cell attachment is a requisite for anchorage-independent growth. CEACAM1 acts as a tumor suppressor in HepG2 cells in anchorage-dependent growth conditions, while in anchorage-independent growth conditions, it augments proliferation by potentiating cell-cell attachment (Hokari, Matsuda et al. 2007).

1.14.8 Metabolism

The common pathology associated with insulin signal malfunction is insulin resistance which often leads to diabetes. The hormone insulin is released during the fed state and stimulates a number of responses differing according to the organ

involved. Insulin maintains glucose homeostasis by increasing glucose uptake in fat and muscle and inhibiting hepatic glucose production. Insulin also stimulates cell growth and differentiation, and promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breadown. Insulin resistance or deficiency results in dysregulation of glucose and lipid homeostasis, and produces elevations in fasting and post-prandial glucose and lipid levels (Saltiel 2001). The insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R) have at least nine identified intracellular substrates. These include four insulin-receptor substrate (IRS) proteins (White 1998), Gab-1, $p60^{dok}$, Cbl, APS and isoforms of Shc (Pessin and Saltiel 2000). The IR and its substrates can be serine phosphorylated, to inhibit their action (Hotamisligil, Peraldi et al. 1996) and provide negative feedback to insulin signalling and serve as a mechanism of cross-talk from other pathways that produce insulin resistance. These serine kinases include Akt, GSK3, and mTOR. Insulin action is also attenuated by tyrosine phosphatases that rapidly dephosphorylate the receptor and its substrates. Like other growth factors, insulin stimulates ERK through phosphorylation of IRS proteins, and the MAPK signalling cascade to initiate transcriptional programme that leads to cellular proliferation or differentiation (Saltiel 2001).

Glucose and lipid homeostasis is a complex interplay of signal networks, in which insulin plays a central role. As such insulin and its numerous signalling mediators are also implicated in cellular proliferation and differentiation.

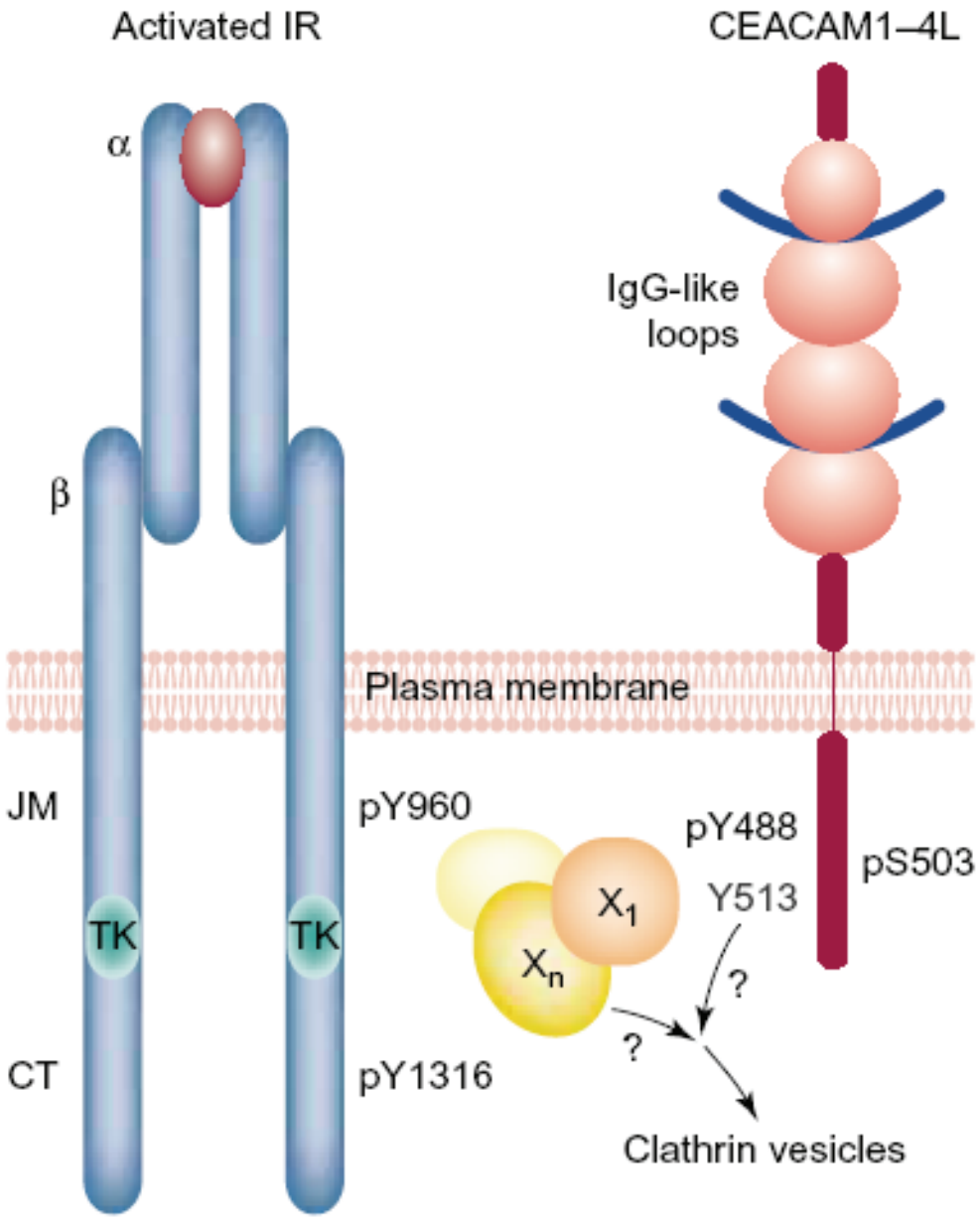
1.14.8.1 Insulin Clearance

CEACAM1 plays a role in the regulation of insulin action. Insulin binding to the insulin receptor (IR) promotes the phosphorylation of specific cellular target molecules, including members of the insulin receptor substrate (IRS) family, Shc, and CEACAM1. CEACAM1-L is a direct substrate of the IR, which phosphorylates CEACAM1 at Tyr 488, and this modification requires an intact Ser 503 residue in the cytoplasmic tail of CEACAM1 (Najjar 2002). CEACAM1

phosphorylation by the IR negatively regulates the mitogenic action of insulin. This down-regulation of insulin signalling can be attributed to the fact that CEACAM1 promotes endocytosis of the IR-insulin complex, leading to insulin degradation in lysosomes via clathrin-coated pits, see figure 1.14.2. The N-terminal domain is not necessary for this function (Soni, Al-Hosaini et al. 1999). As mentioned above CEACAM1 associates with the tyrosine phosphatase SHP-1. One of the characteristic of mice bearing a functionally deficient SHP-1 protein (Ptpn6 me-v/me-v also known as viable motheaten) is that they are markedly glucose tolerant and insulin sensitive as a result of enhanced insulin receptor signalling to IRS-PI3K-Akt in liver and muscle. In these mice CEACAM1 is not tyrosine dephosphorylated, and both increased tyrosine phosphorylation of CEACAM1, and clearance of serum insulin are markedly increased (Dubois 2006). This further indicates the importance of CEACAM1 as a signalling

Figure 1.14.2: Proposed model of receptor-mediated insulin endocytosis.

Activation of the tyrosine kinase of the IR by insulin binding phosphorylates the receptor at many sites, including Y960 in the JM and Y1316 in the CT of the β-subunit of the IR. Phosphorylation of Y1316 regulates phosphorylation of CEACAM1 on Y488. This causes CEACAM1 binding to an intracellular molecule (X1), which mediates its indirect interaction with phosphorylated Y960 in the receptor. Molecule X1 might function either alone or as part of a complex of proteins (Xn). CEACAM1, through Y513 and/or the mediator proteins, might then target the insulin endocytosis complex to AP2 adaptin proteins in clathrin vesicles to target insulin for degradation. Abbreviations: CEACAM1, carcinoembryonic antigen-related cell adhesion molecule; CT, C-terminus; JM, juxtamembrane domain; IR, insulin receptor; TK, tyrosine kinase. Reprinted from Trends in Endocrinology & Metabolism, Vol. 13, Sonia M. Najjar, Regulation of insulin action by CEACAM1, pp. 240-245, Copyright (2002), with permission from Elsevier.


Activated IR
CEACAM1–4L
α
β
IgG-like
loops
Plasma membrane
JM
CT
TK
TK
pY960
pY1316
pY488
Y513
pS503
X1
Xn
?
?
Clathrin vesicles

molecules whose response can be highly regulated by phosphorylation of its key residues.

The *in vivo* investigation on the role of CEACAM1 on hepatic insulin metabolism was conducted on transgenic mice over-expressing in liver a dominant-negative, phosphorylation-defective S503A-CEACAM1 mutant (L-SACC1) (Poy 2002). L-SACC1 transgenic mice develop hyperinsulinemia resulting from impaired insulin clearance. Chronic hyperinsulinemia acts as a cause of insulin resistance. Inactivation of CEACAM1 causes insulin resistance in liver that is mostly due to increased expression of fatty acid synthase and lipid metabolism, resulting in elevated intrahepatic levels of triglyceride and long-chain acyl-CoAs. Insulin resistance in L-SACC1 mice is attributed to defects in insulin-stimulated glucose uptake in skeletal muscle and adipose tissue (Park 2006). Mice develop visceral adiposity with increased amounts of plasma free fatty acids and plasma and hepatic triglycerides. Insulin acutely reduces hepatic FAS activity by inducing phosphorylation of CEACAM1 and its interaction with FAS. Ceacam1 -/- mice show loss of insulin's ability to acutely decrease hepatic FAS activity (Najjar 2005). This contributes to the increased levels of plasma FFA and increased triglyceride released from the liver (Poy 2002). Insulin resistance in peripheral tissues was associated with significantly elevated intramuscular fat contents that may be secondary to increased whole body adiposity. This highlights the importance of CEACAM1 in hepatic insulin clearance in the pathogenesis of obesity and insulin resistance (Park 2006).

1.14.8.2 EGF signalling

Upon phosphorylation by the insulin receptor, CEACAM1-L binds to and sequesters Shc. This downregulates the Ras/MAPK mitogenic pathway and by enhancement of the ability of Shc to compete with insulin receptor-stubstrate-1 for phosphorylation, downregulates the PI-3 kinase/Akt pathway that mediates cell proliferation and survival (Poy 2002). In addition, CEACAM1 is a substrate of the EGFR. CEACAM1 reduces EGFR-mediated growth of transfected Cos-7 cells in response to EGF (Poy 2002). The effect of CEACAM1 on EGF-

dependent cell proliferation is mediated by its ability to bind to and sequester Shc, uncoupling EGFR signalling from the Ras/MAPK pathway. Impaired CEACAM1 phosphorylation leads to ligand-independent increase of EGF-mediated cell proliferation. Indirect phosphorylation of CEACAM1-L by EGFR occurs on Tyr488, not Tyr513, and it requires prior phosphorylation of Ser503, much like the IR. EGFR is constitutively activated in L-SACC1 hepatocytes but is inactive in WT hepatocytes. CEACAM1-L inactivation raises the level of insulin, which in turn causes visceral obesity with increased FFAs and HB-EGF output. This in turn activates EGFR in the hepatocyte, independently of EGF and increases cell proliferation (Abou-Rjaily 2004). By activating the EGFR-dependent mitogenesis pathway, FFAs link altered metabolism with aberrant cell growth (Dulin, Sorokin et al. 1998).

1.15 CEACAM1 knockout mouse:

The *Ceacam1*$^{-/-}$ *mouse* was developed in Dr. Beauchemin's lab by replacing the first and second exons with a TK-neomycin selection cassette. This resulted in effective elimination of CEACAM1 expression at the mRNA and protein level (presented in chapter 1 of this thesis)(Leung, Turbide et al. 2006). The abrogation of CEACAM1 in the mouse results in a complex phenotype. Eventhough *in vitro* studies have shown that CEACAM1 is involved in numerous cellular functions, the knockout mouse is a viable organism. This is probably due to compensatory mechanisms. However, this genetically modified mouse can be used to study several disease models. As previously mentioned CEACAM1 is a tumor suppressor and its role in colon tumorigenesis was investigated using the *Ceacam1*$^{-/-}$ *mouse*. Phenotypically, a few weeks after birth, *Ceacam1*$^{-/-}$ mice accumulate more weight than their wild-type siblings. This continues throughout life. The excess weight is due to hyperphagia. The focus of my work has been the *Ceacam1*$^{-/-}$ mouse as a model for tumor development and insulin resistance correlated with obesity.

1.16 Objectives

The objective of this thesis work is to understand the role of CEACAM1 in tumor progression in the colon. The normal colon epithelium of the *Ceacam1*$^{-/-}$ mouse was characterized for proliferation and apoptosis. These two processes are the most important factors in tumor growth. To determine how CEACAM1 manifested changes in proliferation components of cell cycle checkpoints were investigated. The continuation of this investigation expanded into research of tumor progression in the small intestine using the *APC*$^{1638N/+}$ mouse model. Characterization of the small intestinal epithelium was necessary to determine how CEACAM1 loss affected this tissue. Several downstream components of signalling pathways were investigated to determine CEACAM1's influence in the progression of small intestinal tumors.

In addition to investigating the tumor suppressive functions of CEACAM1, the metabolic irregularities of the knockout mice were also examined. Previously published studies on the transgenic L-SACC1 mice, provided direction for the research on the metabolic aberrancies of the *Ceacam1*$^{-/-}$ mouse. Through DNA array experiments interesting candidate genes were identified to elucidate the role of CEACAM1 in metabolic regulation.

Chapters 2 and 3 of this thesis address the function of CEACAM1 in the development of colon, and small intestinal cancer. The fourth chapter covers the metabolic instabilities of the *Ceacam1*$^{-/-}$ mouse.

Chapter 2 Loss of CEACAM1 increases colon tumor progression when induced by chemical carcinogen

2.1 Preface to Manuscript I

Numerous reports provide evidence that CEACAM1 is a tumor suppressor (Estrera, Chen et al. 2001; Fournes 2001; Phan, Sui et al. 2001; Bamberger, Kappes et al. 2002; Volpert 2002; Nittka 2004; Nittka, Gunther et al. 2004; Cruz, Wakai et al. 2005; Scheffrahn, Singer et al. 2005; Bermudez-Soto, Larrosa et al. 2007; Hokari, Matsuda et al. 2007; Liu, Wei et al. 2007). Pathological observations of tumor sections indicate that CEACAM1 is downregulated in the earliest precursor of colon tumors, the hyperplasia (Nittka, Gunther et al. 2004). In parallel, reduced apoptosis is observed. *In vivo* studies indicate that expression of CEACAM1 can hinder the growth of carcinoma cell lines injected in mice (Fournes 2001). Certain residues in the long cytoplasmic domain have been identified as necessary for CEACAM1's tumor growth inhibitory activity (Izzi, Turbide et al. 1999; Fournes 2001). This indicates that downstream elements of CEACAM1 signalling mediate its effect and this was independent of the cell adhesive properties, since the extracellular domain did not inhibit tumor growth (Izzi, Turbide et al. 1999). Several *in vitro* studies indicate that CEACAM1 can affect cell growth through the cyclin-dependent kinase inhibitor p27 (Singer, Scheffrahn et al. 2000). This still needs to be proven *in vivo* in the context of colon tumorigenesis.

The following is a study investigating colon tumor formation in the *Ceacam1*$^{-/-}$ mouse model. In the normal colon of mice, loss of *Ceacam1* affected both proliferation and apoptosis. Complete ablation of *Ceacam1* in mice does not induce spontaneous tumor formation in any of the tissues of these animals. In order to induce colon tumors in mice, the commonly used chemical carcinogen azoxymethane was used. The results of these studies were published in Oncogene (Leung, Turbide et al. 2006).

2.2 Deletion of the Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (*Ceacam1*) Gene Contributes to Colon Tumor Progression in a Murine Model of Carcinogenesis.

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Running Title: CEACAM1 in colon cancer progression.

2.2.1 Abstract

CEACAM1 is a glycoprotein that is part of the carcinoembryonic antigen and the immunoglobulin superfamilies. We have shown that it functions as a tumor suppressor and that this function depends upon the presence of the longer CEACAM1 cytoplasmic domain. In this report, we describe the generation of a *Ceacam1*$^{-/-}$ mouse. The *Ceacam1*$^{-/-}$ colon exhibits increased *in vivo* proliferation relative to the wild-type counterpart with a corresponding decreased expression of the $p21^{Cip1}$ and $p27^{Kip1}$ Cyclin D kinase inhibitors. The colonic villi undergo decreased apoptosis. Out of 35 litters of mice, no spontaneous tumors in any tissues normally expressing CEACAM1 were found over the lifespan of the animals, suggesting that CEACAM1 may not be involved in initiation of tumor development. However, when mice are treated with azoxymethane to induce colonic tumors, we find that *Ceacam1*$^{-/-}$ mice developed a significantly greater number of tumors than their littermate controls. Moreover, the tumor size was greater in the knockout mice relative to that in the wild-type mice. These results indicate that deletion of CEACAM1 favours progression of colon tumorigenesis.

Key Words: CEACAM1, colon cancer, azoxymethane, knockout, CEA.

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2.2.2 Introduction

Carcinoembryonic antigen (CEA) is recognized as an important tumor marker used clinically to follow the progression of colorectal cancer (Gold and Freedman 1965). CEA is part of the large immunoglobulin-like CEA gene family comprising as many as 31 genes (Beauchemin, Draber et al. 1999), all located on chromosome 19q13.2 in humans (Olsen 1994). Twenty of these genes are expressed in a variety of normal and tumor epithelial, hematopoietic cells and in placenta (Beauchemin, Draber et al. 1999). Orthologs have been found in the mouse and rat genomes (Beauchemin, Draber et al. 1999). Contrary to CEA that is widely overexpressed in human colorectal tumors (Cournoyer 1988), the carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1)* gene is markedly downregulated in human (Neumaier 1993) and mouse (Rosenberg 1993) colon cancer. CEACAM1 expression is also considerably diminished in human prostate (Hsieh 1995; Busch 2002), breast (Luo 1997; Huang 1998), liver (Hixson 1985; Tanaka 1997), endometrial (Bamberger 1998) and bladder cancers (Kleinerman 1996). CEACAM1 decreases early in the development of cancer, being absent from hyperplastic colonic aberrant crypt foci and hyperplastic polyps (Nittka 2004), microadenomas (Ilantzis 1997) and adenomas (Nollau 1997), and in hyperplastic prostate (Kleinerman 1995). These results have also been confirmed using gene chip techniques on patient samples (Zhang 1997; Buckhaults 2001; Notterman 2001). One mechanism regulating the tumor suppressive activity of CEACAM1 in prostate cancer is attributed to the enhanced expression of the Sp2 transcriptional repressor, a member of the Sp family of transcription factors. This factor binds to the *CEACAM1* promoter *in vitro* and *in vivo* where it recruits histone deacetylase; consequently, the *CEACAM1* promoter is hypoacetylated in prostate cancer cells and the CEACAM1 protein is significantly decreased (Phan 2004). However, CEACAM1 is not always down-regulated in cancer. Overexpression of CEACAM1 is indicative of disease progression and metastasis in melanomas (Thies, Moll et al. 2002), lung

adenocarcinomas (Muenzner, Dehio et al. 2000; Laack 2002) and gastric carcinomas (Kinugasa 1998).

The CEACAM1 tumor suppressor function has been studied by modulating the expression of this glycoprotein in colonic and prostatic cancer cell lines. Expression of the CEACAM1-L protein (with a long 73 amino acid cytoplasmic domain) in the mouse CT51 colonic carcinoma cell line revealed that expression of the longer CEACAM1 isoform reduced the number of colonies in a clonogenic assay, whereas the CEACAM1-S-expressing cells (with a short 10 amino acid cytoplasmic domain) behaved as the wild-type cells. This result suggested that CEACAM1-L played a role in inhibition of tumor cell growth (Kunath, Ordonez-Garcia et al. 1995). The ability of CEACAM1-L to function as a tumor suppressor was confirmed *in vivo* as CEACAM1-L-transfected CT51 cells significantly diminished the number and size of tumors formed in syngeneic BALB/c mice (Kunath, Ordonez-Garcia et al. 1995). Similarly, human PC-3 prostatic cells transfected with CEACAM1-L abolished tumor development in nude mice (Hsieh 1995).

The CEACAM1-L tumor suppressor function is independent of its function as an intercellular adhesion molecule since deletion of the first Ig domain of the rat (Luo 1997) and mouse (Izzi, Turbide et al. 1999) CEACAM1, responsible for its cell adhesion activity (Cheung 1993), did not affect tumor development *in vivo*. The long cytoplasmic domain contains several binding motifs including 17 Ser/Thr residues and two Tyr residues phosphorylated by a variety of kinases in different cells. Indeed, the presence of a phosphorylated Tyr488, located within an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) (Ravetch 2000), is critical for inhibition of tumor development *in vivo* (Luo 1997; Izzi, Turbide et al. 1999), as is the presence of Ser503 (Estrera, Chen et al. 2001; Fournes 2001) and that of the last three Lys residues (Izzi, Turbide et al. 1999). When both CEACAM1-S and CEACAM1-L are introduced tandemly into the CT51 colonic carcinoma cells, the tumor inhibitory function then depends on the ratio of these two isoforms (Turbide 1997).

In this manuscript, we provide complete information on the generation of the *Ceacam1*$^{-/-}$ mouse. Deletion of CEACAM1 produced an increased rate of proliferation in the colonic crypt with a decreased rate of apoptosis. Correspondingly, the p21^{Cip1} and p27^{Kip1} CDK inhibitors were decreased in *Ceacam1*$^{-/-}$ colonic cells. Clinical pathology of tissues in older *Ceacam1*$^{-/-}$ mice did not reveal any tumor, suggesting that CEACAM1 is likely not involved in initiation of tumorigenesis. Azoxymethane carcinogen treatment induced not only a greater number of tumors, but also larger tumors, in the colon of CEACAM1-deficient mice relative to their wild-type counterparts. The size of the tumors in the *Ceacam1*$^{-/-}$ mice was also increased relative to those found in the WT controls. These results therefore suggest that CEACAM1 inhibits the progression of colon tumors *in vivo*.

2.2.3 Results

2.2.3.1 Generation of the *Ceacam1*$^{-/-}$ mice

The strategy leading to complete abrogation of CEACAM1 expression in mice was based on the removal of the first two exons of the *Ceacam1* gene (Figure 2.2.3.1 *a*, *b* and *c*). The initiator ATG codon is positioned in the first exon and most functions associated with the CEACAM1 protein depend on the presence of the first Ig-like domain encompassed within the second exon. For this purpose, an *Xba*1-*Xho*1 restriction fragment encoding these two exons was removed from the gene and a cassette encoding the *TK* (thymidine kinase) promoter and the *neo*^{r} gene was inserted (Fig. 2.2.3.1*b*). The targeting vector was electroporated into mouse R1 ES stem cells and 33 positive clones out of 830 were isolated after selection in G418-containing medium. A Southern blot was hybridized with a ^{32}P-labelled probe specific to the *neo*^{r} gene (Fig. 2.2.3.1 *c*, probe 3) to confirm the targeting event and the number of integration sites in the +/- ES cell clones. The two selected ES cell clones (2D2 and 11H11) both produced a single 9.4 kb fragment positive for the *neo* probe (Fig. 2.2.3.1 *g*). Four chimeric male mice were obtained by microinjection of the ES cell lines

(2D2 and 11H11) into C57Bl/6 mouse blastocysts and transmitted the *Ceacam1* targeted allele through the germline. The heterozygous *Ceacam1*+/- progeny mice were mated to produce homozygous mice. Mice (+/- and -/-) derived from these two ES cell lines were positive for the *neo* probe, although the 11H11-derived mice appeared to have two neo-positive fragments relative to a single fragment observed in the originating ES cell line and the 2D2-derived mice (Fig. 2.2.3.1 *g*). The targeting event was also confirmed by hybridizing the genomic blots with a *Ceacam1*-specific probe (Fig. 2.2.3.1*a*, probe 2). In this case, the 12.3 kb *EcoR1* restriction fragment was positive for this probe in the +/+ and +/- mice, but was negative in the -/- mice. Interestingly, the *Ceacam1*-targeted mice exhibited a 7.0 kb 129/Sv *Ceacam2* fragment after breeding instead of the approximate 9.0 kb fragment in C57Bl/6 mice (Fig. 2.2.3.1*f*). Since the *EcoR1* sites of the *Ceacam2* fragments detected by the probe used are located within the promoter region and intron 2, the size of the gene product is not modified. The targeting event was also confirmed with other characteristic restriction digests of the 2D2 and 11H11 ES cell genomic DNA (data not shown). The frequency of germline transmission was calculated to be 22% on a mixed background (C57Bl/6, BALB/c, 129Sv). Mating of heterozygous mice produced expected Mendelian ratios of *Ceacam1*$^{-/-}$ offspring (1.0 +/+: 1.8 +/-: 0.9 -/-). CEACAM1 is expressed in ovary and prostate, yet its abrogation had no incidence on gender ratios (53% males: 48% females).

Figure 2.2.3.1: Gene Targeting of the mouse *Ceacam1* gene.
The *Ceacam1* gene contains 9 exons represented by the white boxes. The ATG initiator codon is located in the first exon of the gene. Two alternate stop codons are present in exon 8 and 9 of the gene; their respective usage gives rise to two different *Ceacam1* mRNAs. The protein domains encoded by the various exons are indicated underneath the gene structure (***a***). A targeting vector was engineered by replacing a Xba1-Xho1 fragment encompassing the first two exons, the first intron and part of the second intron of the *Ceacam1* gene by a *Tk-neo*^{r} cassette (***b***). Cleavage of the wild-type 129/Sv *Ceacam1* gene with *EcoR1* produces a 12 kb restriction fragment. Insertion of the *Tk-neo*^{r} cassette introduces a novel *EcoR1* site in the recombinant targeted allele, thereby producing a 1.7 kb *EcoR1* restriction fragment, as detected using a *BamH1-Hind111* 93 bp probe (probe 1) located in the *Ceacam1* promoter outside of the targeting vector (***c*** and ***d***). This same probe binds to the promoters of the 129/Sv and C57Bl/6 *Ceacam2* and *Ceacam10* genes (***d***). *Ceacam1*- (***e***) or *Ceacam2*-specific probes (***f***) (probe 2) located in the first Ig domains (Nédellec 1994) were used to hybridize to the same Southern blot. A *neo*-specific probe (Blau, Turbide et al. 2001) (probe 3) was hybridized to the same blot. 5'UT: 5' untranslated region; L: leader sequence; D1-D4: Ig domains; TM: transmembrane domain; C: cytoplasmic domain; 3'UT: 3' untranslated region; *neo*^{r}: neomycin resistance gene; *Tk*: thymidine kinase promoter. N.S.: non-specific. *Ceacam1* rec.: *Ceacam1* recombinant allele.

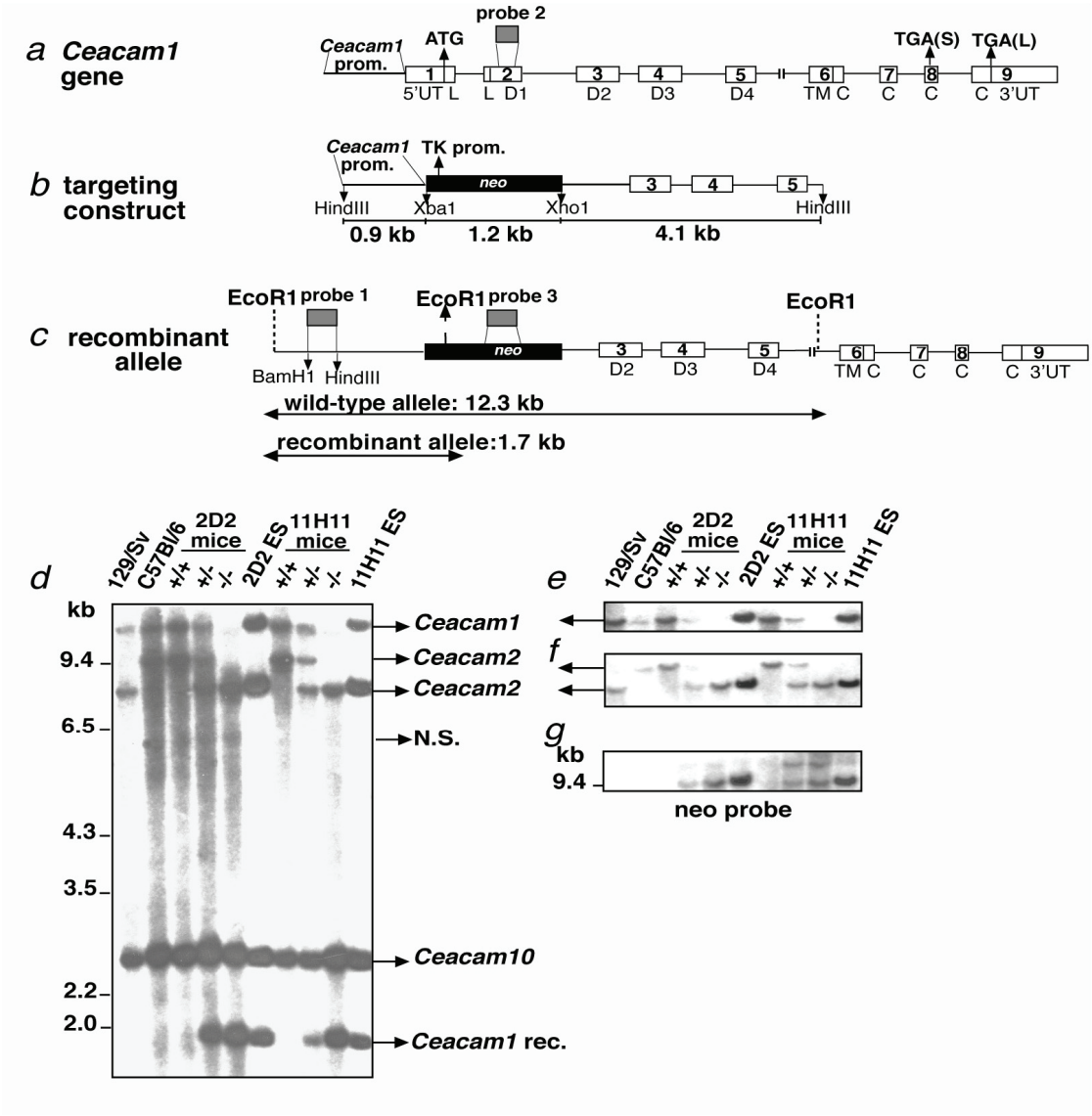
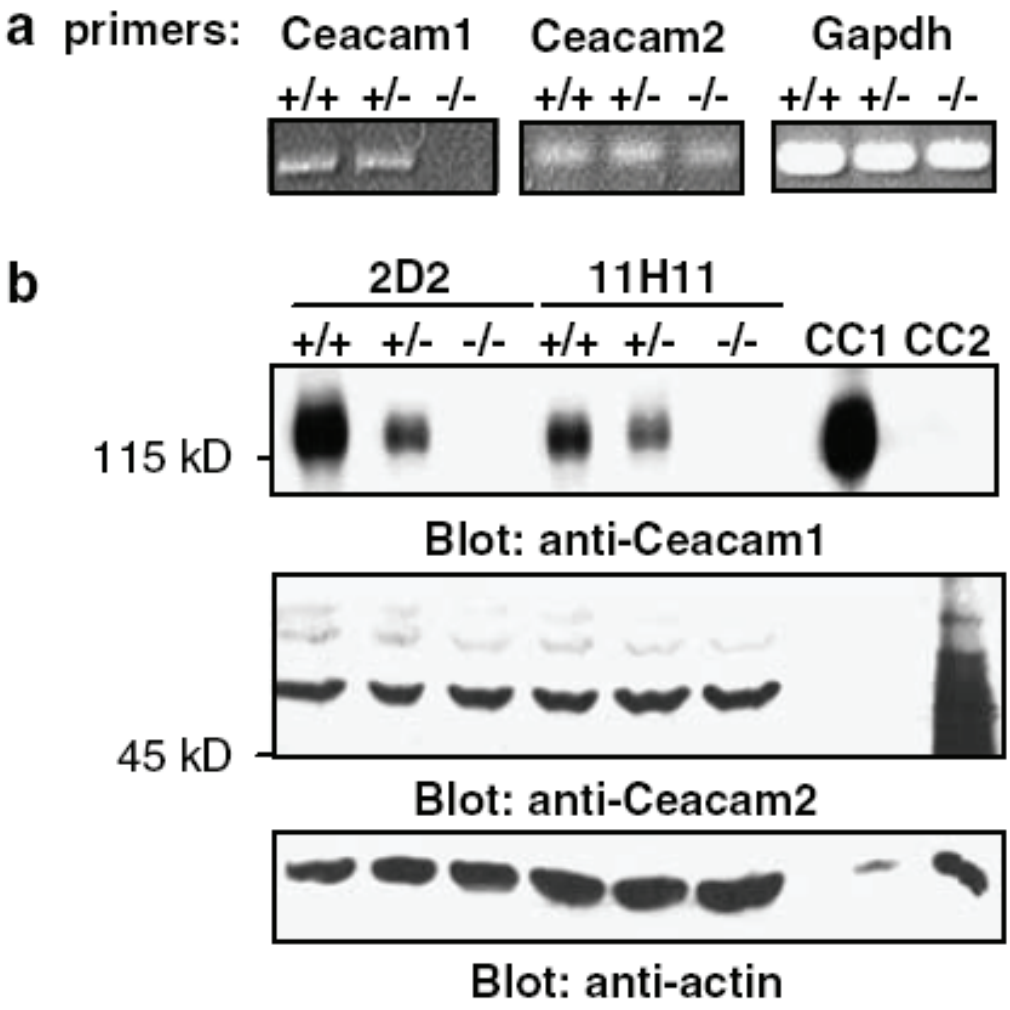

a Ceacam1 gene
Ceacam1 prom.
ATG
probe 2
TGA(S)
TGA(L)
1 2 3 4 5 6 7 8 9
5'UT L L D1 D2 D3 D4 TM C C C C 3'UT
b targeting construct
Ceacam1 prom.
TK prom.
neo
HindIII Xba1 Xho1 HindIII
0.9 kb 1.2 kb 4.1 kb
c recombinant allele
EcoR1 probe 1
EcoR1 probe 3
EcoR1
BamH1 HindIII
wild-type allele: 12.3 kb
recombinant allele:1.7 kb
d
129/Sv C57Bl/6 2D2 mice +/+ +/- -/- 2D2 ES 11H11 mice +/+ +/- -/- 11H11 ES
kb
9.4
6.5
4.3
3.5
2.2
2.0
Ceacam1
Ceacam2
Ceacam2
N.S.
Ceacam10
Ceacam1 rec.
e
f
g
kb
9.4
neo probe

Figure 2.2.3.2: Expression of *Ceacam1* and *Ceacam2* transcripts and proteins.
Total RNA was prepared from colon and kidney of mice from each mouse lines (2D2 and 11H11) and used to generate cDNAs via RT-PCR using *Ceacam1*, *Ceacam2* or *Gapdh* primers as described in Materials and Methods. After 40 (*Ceacam1* and *Ceacam2*) or 30 (*Gapdh*) rounds of amplification respectively, samples were loaded on a 1.0% agarose gel and subjected to electrophoresis. The gels were photographed after staining the gels with ethidium bromide (***a***). Protein lysates were prepared from mouse colon (***b***, top panel) or kidney (***b***, middle panel) and equal amounts of protein were separated on 8.0% SDS-PAGE gels. The proteins were transferred to Immobilon membranes and immunoblotted with polyclonal anti-Ceacam1 (***b***, top panel) or anti-Ceacam2 (***b***, middle panel) antibodies. Specificity of the antibodies was confirmed using purified CEACAM1 (CC1) or CEACAM2 (CC2) protein samples. Equal loading of proteins was confirmed by immunoblotting with an anti-actin antibody (***b***, bottom panel).


a
primers:
Ceacam1
Ceacam2
Gapdh
+/+ +/- -/-
+/+ +/- -/-
+/+ +/- -/-
b
2D2
11H11
+/+ +/- -/- +/+ +/- -/- CC1 CC2
115 kD
Blot: anti-Ceacam1
45 kD
Blot: anti-Ceacam2
Blot: anti-actin

2.2.3.2 Abrogation of CEACAM1 expression in Ceacam1-targeted mice

The complete abrogation of CEACAM1 expression was verified by Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting. Total RNA prepared from colon and kidney of several mice from each litter was subjected to RT-PCR. The +/+ and +/- mice were positive for the *Ceacam1* mRNA whereas no *Ceacam1* mRNA was present in the *Ceacam1*$^{-/-}$ colon of mice (Fig. 2.2.3.2 *a)*. No changes were detected in the amount of *Ceacam2* cDNA amplified from kidney mRNA, suggesting that this second *Ceacam*-like gene remained intact in the *Ceacam1* knockout mice. *Gapdh* mRNA was used as an internal control. These results indicated that the gene inactivation strategy completely abrogated the transcription of *Ceacam1* (Fig. 2.2.3.2*a*). The expression of the CEACAM proteins in mouse colon and kidney tissues was examined by immunoblotting total proteins from several different mice with polyclonal anti-CEACAM1 and anti-CEACAM2 antibodies (Fig. 2.2.3.2*b*). In both these tissues and others (data not shown), expression of all CEACAM1 isoforms in the homozygous mice was completely eliminated relative to expression in the wild type or heterozygous mice (Fig. 2.2.3.2*b*). Actin levels, as controls for loading, were constant in these tissues (Fig. 2.2.3.2*b*). In kidney extracts, the amount of the CEACAM2 protein revealed with a specific anti-CEACAM2 polyclonal antibody (Robitaille 1999) remained constant in all genotypes, in line with the actin control.

2.2.3.3 CEACAM1-deficient mice show increased proliferation and decreased apoptosis in their colon

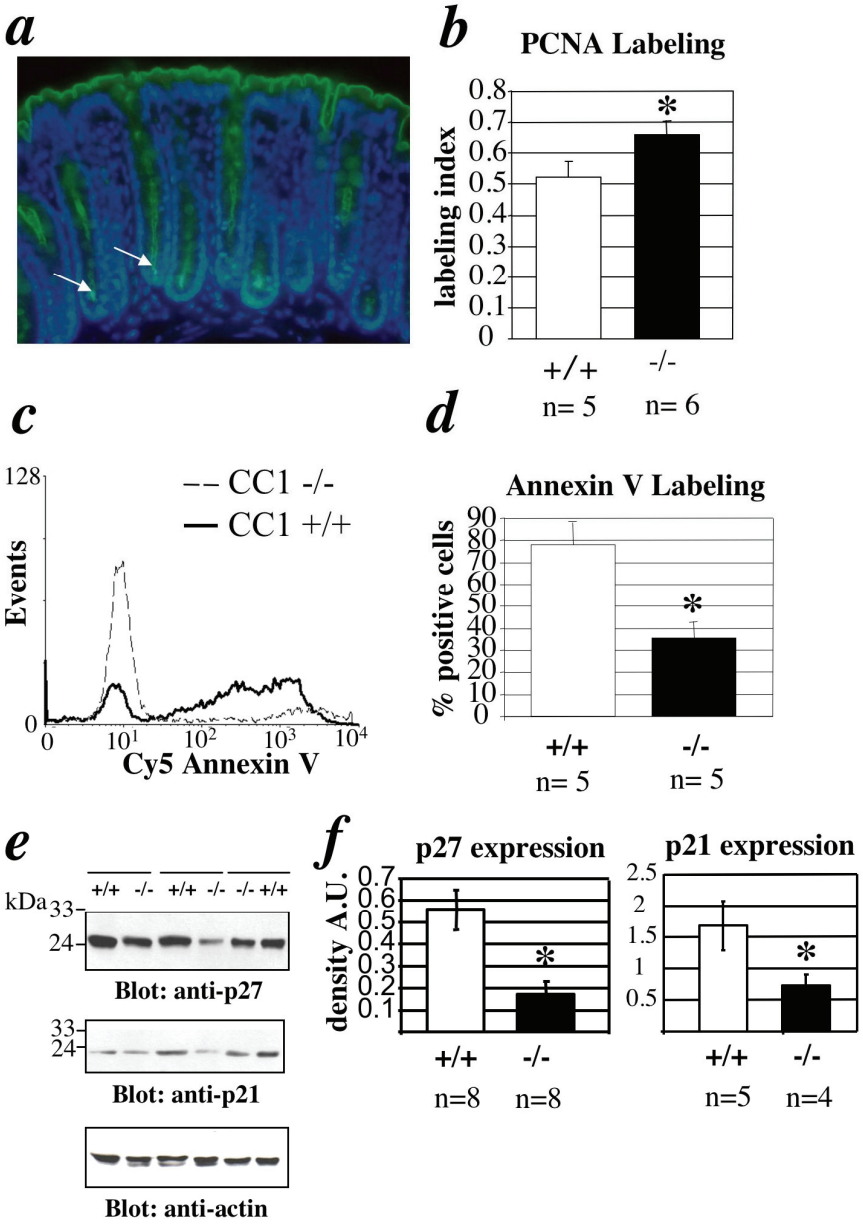
Wild-type mice express the two major CEACAM1 isoforms in their colon albeit in different amounts. We have previously shown that the CEACAM1-L isoform is only expressed at approximately one-seventh the level of CEACAM1-S in colon tissue and that it reduces the proliferation of mouse CT51 colonic carcinoma cells. The CEACAM1-S isoform, although predominantly expressed in normal colon, does not influence cell proliferation but it has been shown to participate in apoptosis in a breast 3D lumen formation assay and in HT29 colon

carcinoma cells. Since both isoforms are tandemly expressed in this tissue and were both eliminated by the genetic ablation procedure, we examined whether elimination of the CEACAM1 isoforms affected proliferation or apoptosis of colonic epithelial cells. Staining of colon sections from wild-type mice with a polyclonal antibody specific to CEACAM1 (Ab 2457) demonstrated that, contrary to what has been observed in human colon (Frangsmyr 1999), CEACAM1-positive cells were found in the bottom of the colonic crypt which is the proliferative zone (Fig. 2.2.3.3*a*). Similar results were obtained with the CC1 monoclonal antibody. Colon sections of 3 month-old +/+ and *Ceacam1*$^{-/-}$ littermates (5 and 6 mice respectively) were stained with an antibody to Proliferating Cell Nuclear Antigen (PCNA). A proliferation index was calculated by counting PCNA-positive cells of 5 different microscope fields over the total number of cells in the villi. *Ceacam1*$^{-/-}$ colon revealed a 30% increase in the number of proliferative cells as compared to those of the wild-type mice ($p<0.05$, Fig. 2.2.3.3*a*). Staining of colon sections for BrdU after a 2 or 6 h *in vivo* labeling period produced similar results (data not shown). Similarly, examination of BrdU-positive colonic cells migrating from the bottom of the crypt to the top after a 24 h BrdU incorporation indicated that the rate of migration was identical in the wild-type and CEACAM1-null mice.

In the colon, proliferation occurs in stem cells and progenitor cells located at the bottom of the colonic crypts (Potten 1990). As differentiation proceeds, these cells move upwards finally undergoing apoptosis at the top of the crypt and are then extruded into the gut lumen. We studied whether elimination of the CEACAM1 isoforms affected the rate of early apoptosis in this tissue by measuring annexin V labeling of dissociated colonic cells. Labeling was decreased by approximately 50% in knockout colonic cells compared to those of the wild-type mice in 2 separate experiments comprising five animals of each genotype ($p< 0.001$, Fig. 2.2.3.3*b* and *c*).

Figure 2.2.3.3: Increased proliferation and decreased apoptosis in normal *Ceacam1*$^{-/-}$ colon.

(a) Sections of wild-type colons were immunostained with anti-CEACAM1 pAb 2457 and detected with a FITC-conjugated secondary antibody. Slides were counterstained with DAPI and images were overlaid. CEACAM1 staining is detected at the bottom of the colon crypt in the proliferating area (indicated by white arrows). (***b***) WT (white bars) and *Ceacam1*$^{-/-}$ (black bars) littermates were sacrificed, colons were retrieved, embedded in paraffin, sectioned and stained with an anti-Proliferating Cell Nuclar Antigen (PCNA) antibody. A labeling index was measured by counting the number of stained cells and dividing by the number of cells in each microscope field. Five microscope fields were assessed for every pair of animals (n=5 or 6) studied. Values are plotted as mean ± standard error. * $p<0.05$. (***c***) Colon cells from +/+ (heavy line) and *Ceacam1*$^{-/-}$ (dashed line) mice were prepared as described in Materials and Methods and subjected to labeling with a Cy5-labeled anti-Annexin V antibody. The positive cells were detected using a BD FACscan. (***d***) The percentages of colonic cells obtained from 5 +/+ and -/- mice labeled with the anti-Annexin V antibody obtained from the FACS profiles were calculated and plotted as mean ± standard error. The *Ceacam1*$^{-/-}$ mice showed a significant decrease in apoptotic cells ($P < 0.001$). (***e***) Nuclear extracts were prepared from colons of WT and *Ceacam1*$^{-/-}$ siblings and equal amounts of proteins were separated on 8.0% SDS-PAGE gels. The proteins were transferred to Immobilon membranes and immunoblotted with an anti-p27 (top panel), an anti-p21 (middle panel) or anti-actin antibody. (***f***) Blots were scanned using an ImageJ program and values were normalized using the anti-actin scans.


a
b
PCNA Labeling
labeling index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
+/+
-/-
n= 5
n= 6
c
d
128
Events
0
-- CC1 -/-
— CC1 +/+
0
10^1
10^2
10^3
10^4
Cy5 Annexin V
Annexin V Labeling
% positive cells
90
80
70
60
50
40
30
20
10
0
*
+/+
-/-
n= 5
n= 5
e
f
kDa
33
24
+/+ -/- +/+ -/- -/- +/+
Blot: anti-p27
33
24
Blot: anti-p21
Blot: anti-actin
p27 expression
p21 expression
density A.U.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2.5
2
1.5
1
0.5
*
*
+/+
-/-
+/+
-/-
n=8
n=8
n=5
n=4

Tissue sections were also processed using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays as a measure of late stage apoptosis. Calculations of the labeling index, in five different microscope fields for all mice, indicated that apoptosis was weaker in the CEACAM1 knockout animals (WT, 0.120 ± 0.01 *versus* KO, 0.109 ± 0.01, P=0.065) (data not shown). These combined results suggest that disruption of the *Ceacam1* gene, leading to the elimination of all isoforms, produces an overall increased rate of colonic proliferation in tandem with a decreased rate of apoptosis *in vivo*. However, early apoptosis was decreased to a greater extent, possibly due to tissue processing to obtain single cell suspensions for flow cytometry.

We then evaluated whether the increased rate of proliferation in the CEACAM1-null colon influenced the expression of Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Nuclear fractions were prepared from 8 pairs of wild-type and $Ceacam1^{-/-}$ colonic cells and proteins were immunoblotted using antibodies specific for these two proteins (Fig. 2.2.3.3*e*). Quantification of both the $p21^{Cip1}$ and $p27^{Kip1}$ proteins demonstrated that expression in the CEACAM1-deficient mice was reduced by 56% for $p21^{Cip1}$ (p<0.05) and 66% for $p27^{Kip1}$ (p<0.005) relative to the +/+ controls (Fig. 2.2.3.3*f*). This suggests that elimination of CEACAM1 modulates cell cycle regulation in these mice.

2.2.3.4 CEACAM1-deficient mice do not develop tumors spontaneously

CEACAM1 is down-regulated and generally absent in human colorectal tumors. We questioned whether $Ceacam1^{-/-}$ mice might therefore develop colon tumors as they aged. We examined 35 litters comprising 111 mice of $Ceacam1^{-/-}$ and +/+ mice of the 2nd and 3rd backcrosses in C57Bl/6 and 129Sv backgrounds at regular intervals of 6, 12, 18 and 22 months and all tissues normally expressing CEACAM1 were retrieved and processed for histological analyses. No tumors were detected macroscopically in any of these tissues. Microscopically, no changes were seen in the structure of the colon, intestine, prostate, breast, ovary, uterus, kidney, thymus or brain of the $Ceacam1^{-/-}$ mice relative to the same structures observed in the wild-type mice. Thus, although the number of mice

examined is still too low to detect a rare tumor event, it appears that CEACAM1 does not play a role in initiation of tumor development.

2.2.3.5 Azoxymethane-treated CEACAM1-deficient mice exhibit enhanced tumorigenesis.

Carcinogen induction is a widely used procedure for studying changes in the molecular and pathological process of tumor development. The methylating agent azoxymethane (AOM) specifically targets the colon (Papanikolaou 1998). Genetic modifications found in human colon cancer (Fearon 1990) have been identified in carcinogen-treated animals including mutations in K-*ras* (Vivona 1993), *p53* (Okamotu 1995; Singh 1997), *Adenomatous polyposis coli* (APC) (Maltzman 1997), *cyclin D1* and *cyclin-dependent kinase 4* (Wang 1998), $p16^{INK4a}$ (Wang 2000) and the □-catenin protein (Takahashi 2000). To gauge whether CEACAM1 contributes to colon tumor progression *in vivo*, mice were injected repeatedly with AOM. Less than 5% of the mice had to be sacrificed prior to the experimental end-point. After 19-20 weeks, mice were euthanized, their colons removed and all other organs were thoroughly examined, in particular the liver and lung which are common sites of metastases in human colon cancer. No aberrant growths were found in any tissue other than the colon.

In backcross 3 mice, $Ceacam1^{-/-}$ mice demonstrated an overall 3-fold increase in the number of colon tumors relative to those found in C57Bl/6 control mice (6.00 ± 0.09 *versus* 2.38 ± 0.07, $p< 0.01$) (Fig. 2.2.3.4*a*, panel A). The average size of these tumors was also greater in $Ceacam1^{-/-}$ mice than in wild-type mice (Fig. 2.2.3.4*a*, panel B). $Ceacam1^{-/-}$ mice showed a trend towards an increased number of smaller tumors (< $2mm^2$) (WT, 1.34 ± 0.39 *versus* KO, 2.26 ± 1.04, P= 0.21), although not statistically significant.

Figure 2.2.3.4: Number and size of azoxymethane-induced tumors in CEACAM1-deficient mice.
(a) Backcross 3 WT and *Ceacam1*$^{-/-}$ mice were treated with AOM for 8 weeks and sacrificed after 19 weeks. Colons were retrieved and analyzed for the number of lesions that developed (A) and the size of these lesions (B and C). The numbers of animals analyzed are indicated under each column. Values were plotted as mean ± standard error. * $p<0.05$. (***b***) A similar experiment was repeated with backcross 8 WT and *Ceacam1*$^{-/-}$ mice. Values were plotted as mean ± standard error. * $p<0.05$.

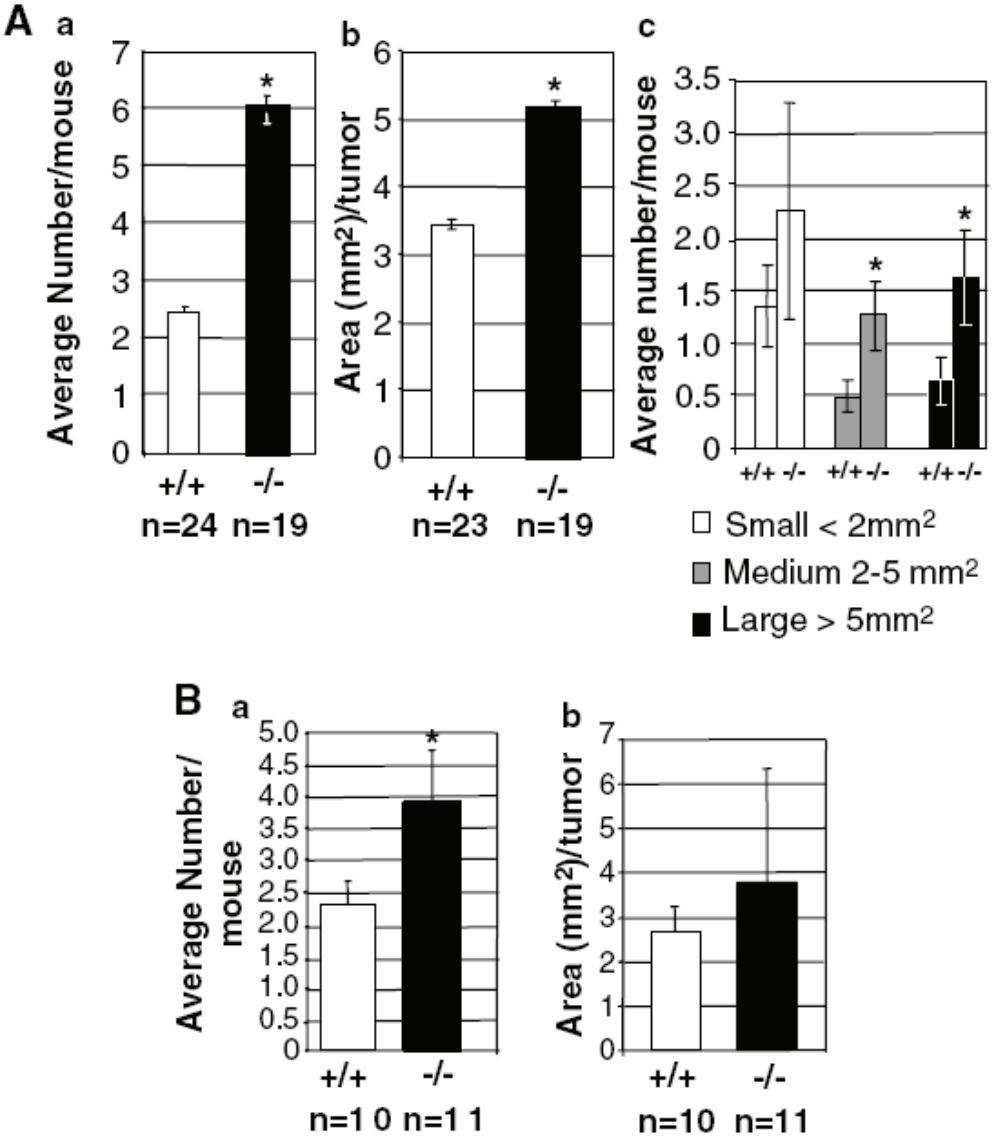
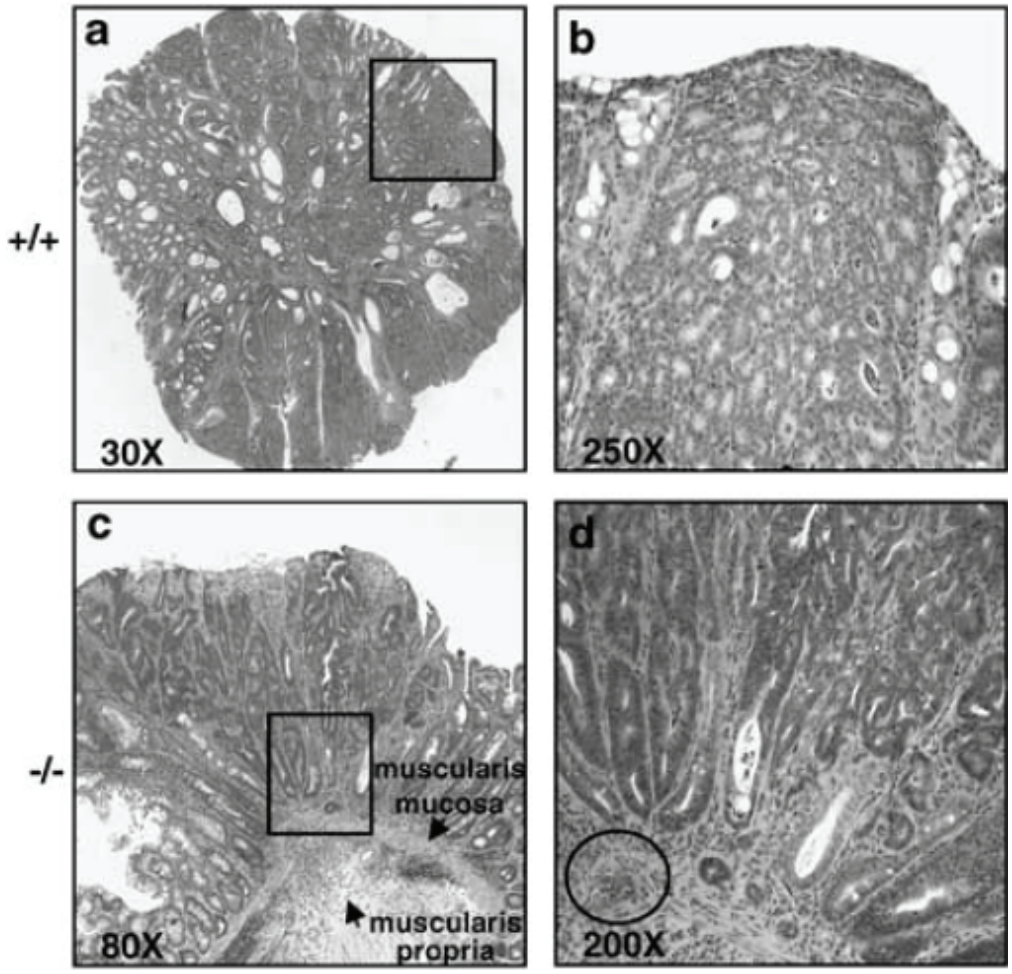

A
a
Average Number/mouse
7
6
5
4
3
2
1
0
*
+/+
-/-
n=24 n=19
b
Area (mm²)/tumor
6
5
4
3
2
1
0
*
+/+
-/-
n=23 n=19
c
Average number/mouse
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
+/+ -/- +/+ -/- +/+ -/-
Small < 2mm²
Medium 2-5 mm²
Large > 5mm²
B
a
Average Number/ mouse
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
+/+
-/-
n=1 0 n=1 1
b
Area (mm²)/tumor
7
6
5
4
3
2
1
0
+/+
-/-
n=10 n=11

Figure 2.2.3.5: Representative tumors from the WT and *Ceacam1*$^{-/-}$ mice.
Tumors retrieved from either WT or *Ceacam1*$^{-/-}$ mouse colon were embedded in paraffin, sectioned and stained with H/E. They were classified according to clinical standards. **(a)** Representative adenoma with high-grade dysplasia and focal intramucosal carcinoma (squared off). (**b**) represents a higher magnification of the intramucosal carcinoma in (**a**). (**c**) Representative of focal invasive adenocarcinoma (the area squared off) from a *Ceacam1*$^{-/-}$ mice. (**d**) represents a higher magnification of the invasive carcinoma in the submucosa, having traversed the muscularis mucosa, as highlighted in the circled area.


a
b
+/+
30X
250X
c
d
-/-
muscularis
mucosa
muscularis
propria
80X
200X

However, the knockout mice carried a greater number of tumors larger than 2 mm^2 (average number of medium tumors (2-5 mm^2)/mouse: WT, 0.48 ± 0.15 *versus* KO, 1.26 ± 0.33, *P*= 0.02; average number of large tumors (5 mm^2) /mouse: WT, 0.65 ± 0.23 *versus* KO, 1.63 ± 0.44 *P*= 0.03) (Fig. 2.2.3.4*a*, panel C) indicating a more rapid progression in the development of these tumors than those found in the wild-type control mice.

In backcross 8 *Ceacam1*$^{-/-}$ mice and their wild-type siblings, the *Ceacam1*$^{-/-}$ mice again developed a significant increase in the number of tumors relative to those found in the WT mice (WT, 2.3 ± 0.36 *versus* KO, 3.9 ± 0.81, $p<$ 0.05) (Fig. 2.2.3.4*b*). The *Ceacam1*$^{-/-}$ mice also had larger tumor sizes (WT, 2.67 mm^2 ± 0.57 *versus* KO, 3.79 mm^2 ± 2.56, *P*=0.13).

The large tumors were examined and classified according to clinical standards. Adenomas were defined as proliferations of the colonic epithelium with at least low-grade dysplasia. A diagnosis of high-grade dysplasia required increased cytological atypia and architectural complexity. The term intramucosal carcinoma was used when there was evidence of invasion into the lamina propria, characterized by prominent back-to-back glands and a cribriform architecture, but without invasion into the submucosa. In humans, intramucosal carcinoma is categorized as “in situ” carcinoma, as it is considered to have no potential for metastasis. A diagnosis of invasive adenocarcinoma was made when invasion into the submucosa was identified. Most samples contained adenomas with at least high-grade dysplasia (Fig. 2.2.3.5, panel a). Tumors from the CEACAM1-deficient mice were generally classified as intramucosal carcinomas (panel b) with a minimal (< 2%) number of tumors having invaded the submucosa (panel c, d). Tumors from both the +/+ and *Ceacam1*$^{-/-}$ mice were analyzed for expression of E-cadherin and □-catenin. There were essentially no differences in localization of E-cadherin between colon tumors of +/+ and -/- mice. 15-20% of tumor cells exhibited nuclear expression of □-catenin, as is often seen in colon tumors (Brabletz 2002; Wong 2004). In summary, these results confirm that deletion of CEACAM1 contributes to the progression of colon cancer.

CEACAM1-L is normally expressed in the endothelium. We questioned whether abrogation of its expression modified angiogenesis in the colonic tumors. To investigate this, we quantified CD31 staining, a measure of vascular density, on tumor sections from wild-type, heterozygous and homozygous *Ceacam1*-deficient mice. No significant differences in vascular density were measured between these tumors. This suggests that the loss of CEACAM1 in endothelium does not alter colon tumor angiogenesis and that the progression of tumors is due to elimination of CEACAM1 in epithelial cells.

2.2.4 Discussion

CEA is widely used clinically as a measure of colorectal tumor recurrence. Recent studies have also suggested that CEACAM1 and CEACAM6 also play a significant role in colonic tumor development (Neumaier 1993; Nollau 1997; Nollau, Prall et al. 1997; Scholzel 2000; Jantscheff 2003). Since the mouse neither expresses CEA nor CEACAM6 and relies for most of its CEA-related functions on the expression of CEACAM1, studies investigating the role of these proteins depend on the generation of adequate transgenic mouse models (Chan 2004) or the availability of mice deficient for CEACAM1. In this manuscript we describe the generation of the *Ceacam1*$^{-/-}$ mouse. In order to gain some understanding into the function of CEACAM1 in colonic homeostasis, we have examined the proliferation and apoptotic responses in *Ceacam1*$^{-/-}$ mouse colon as well as gauged the sensitivity of these animals to colon cancer development.

Elimination of the CEACAM1 isoforms has changed the growth properties of colonic crypts. Crypts are lined with epithelial cells formed by the proliferation of stem cells located at the bottom. As these epithelial cells mature, they migrate up the length of the crypt and differentiate until they reach the apex that constitutes the area of extrusion into the lumen and anoikis (Potten 1990). We have shown that in mouse colon, CEACAM1 is indeed expressed at the bottom of the crypt and therefore its elimination accelerates proliferation, confirming the function of CEACAM1-L defined in a number of epithelial cell

lines as a regulator of cell proliferation (Hsieh 1995; Kunath, Ordonez-Garcia et al. 1995; Singer, Scheffrahn et al. 2000). In addition, CEACAM1 is also abundantly expressed in the upper third of the colonic crypt that undergoes differentiation. The $p21^{Cip1}$ and $p27^{Kip1}$ proteins are responsible for the induction of differentiation in intestinal epithelium (Quaroni 1997; Deschenes 2001). The significant decrease in expression of CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ observed in *Ceacam1*$^{-/-}$ colon suggests that the induction of differentiation programs may be slowed with colonic cells consequently undergoing a higher proliferation rate. In support of this, one of the genes demonstrating increased expression in dnTCF-induced LS174T cells was CEACAM1. Downregulation of TCF activity by expression of the dnTCF construct in colonic cell lines resulted in induction of $p21^{Cip1}$, G1 arrest and differentiation (van de Wetering 2002). The *Ceacam1*$^{-/-}$ mice also show a reduced rate of apoptosis. This was more apparent in the early phases of apoptosis as measured by Annexin V labeling.

Downregulation of CEACAM1 in tumorigenesis is a very early phenomenom as CEACAM1 is considerably decreased or absent in colorectal hyperplastic polyps (Nittka 2004) or adenomas (Nollau 1997) and in hyperplastic prostate (Kleinerman 1995). Although the number of mice examined was too small to detect a rare genetic event, no tumors developed without carcinogen induction, demonstrating that CEACAM1 is apparently not involved in the initiation of tumors. However, its downregulation in benign lesions suggests that it is likely a gene modified early in the progression of the disease once the tissue has accumulated defined genetic mutations. In this context, we have treated mice with azoxymethane, a carcinogen that specifically induces the development of colon cancer by mutating a significant number of genes (Vivona 1993). *Adenomatous polyposis coli* (*APC*) is thought to be the first gene altered in the colon cancer genetic cascade resulting in truncation of the protein and consequently, modification of the □-catenin transcriptional activity (Kinzler 1996; Fodde 2001). Experiments are currently under way to determine whether introducing an *Apc* truncating mutation (Fodde 1994) on the *Ceacam1*$^{-/-}$ background will lead to enhanced intestinal tumor development.

A number of studies have shown that CEACAM1-L is expressed in newly-forming immature blood vessels of tumors and those resulting from physiological angiogenesis as seen in wound healing and endometrial proliferation (Ergün 2000; Volpert 2002; Oliveira-Ferrer 2004; Muller 2005). Since the targeting of *Ceacam1* also eliminated the expression of CEACAM1 in endothelial cells, we examined whether the angiogenic properties of the carcinogen-induced tumors was in any way affected by the lack of CEACAM1 expression. The vascular density from all 3 genotypes did not show statistically significant differences in these conditions (data not shown). But, vascular density may not be a faithful readout of the angiogenic status in colon cancer, as discussed by Eberhardt (Eberhard 2000). We have investigated the angiogenic status of *Ceacam1*$^{-/-}$ mice, and blood vessels show enhanced vascular structural defects leading to leakage in neo-angiogenic conditions and therefore failed to establish new capillaries in Matrigel plug assays. Furthermore, *Ceacam1*$^{-/-}$ mice showed reduced growth of arterioles and collateral blood flow was reduced after induction of hindlimb ischemia by femoral artery ligation (Horst 2006). Thus, endothelial growth may also be affected in *Ceacam1*$^{-/-}$ tumoral angiogenesis.

The results presented in this manuscript highlight the importance of CEACAM1 in regulating proliferation in the colon and to that effect, its elimination renders the colon more sensitive to carcinogen-induced tumor development. The contribution of epigenetic and genetic changes to the development of CEACAM1-deficient tumors is currently being addressed.

2.2.5 Materials and Methods

2.2.5.1 Generation of the Ceacam1 knockout mice

The strategy leading to complete abrogation of CEACAM1 expression in mice was based on the removal of the first two exons of the *Ceacam1* gene (Figure 2.2.3.1*a*, *b* and *c*). The strategy used was to eliminate the initiator ATG codon and the second exon since many CEACAM1 functions depend on the presence of the first Ig-like domain encompassed by the second exon. A *Xba1*-

Xho1 restriction fragment encoding the two exons was removed from the gene and replaced by a cassette encoding the *TK* (thymidine kinase) promoter and the *neo*^{r} gene (Fig. 2.2.3.1*b*). The targeting vector was electroporated into mouse R1 ES stem cells and ES cell clones were isolated after selection for 8 days in the G418-containing medium. The targeting event was evaluated by Southern analyses of *EcoR1*-digested genomic ES cell DNA using a ^{32}P-labelled *BamH1-HindIII* probe (Fig. 2.2.3.1*c*, probe 1). The Southern blot was then hybridized with a ^{32}P-labelled probe specific to the *neo*^{r} gene (Fig. 2.2.3.1*c*, probe 3) to confirm the targeting event and the number of integration sites in the +/- ES cell clones. Chimeric mice were generated by microinjection of the ES cell lines (2D2 and 11H11) into C57Bl/6 mouse blastocysts. The heterozygous *Ceacam1*+/- progeny mice were mated to produce homozygous mice. The targeting event was also confirmed by hybridizing the genomic blots with a *Ceacam1*-specific probe (Fig. 2.2.3.1*a*, probe 2) and confirmed with other characteristic restriction digests of the 2D2 and 11H11 ES cell genomic DNA (data not shown). Experiments were performed in the C57Bl/6 background on backcrosses # 2, 3 and 8 for both the 2D2 and 11H11 *Ceacam1*$^{-/-}$ mice. Mice were handled according to the standards defined by the Canadian Council on Animal Care.

2.2.5.2 Genotyping

Genotyping was performed using <1 cm of tails clipped from 3-week old pups. Genomic DNA was prepared using a "DNeasy Tissue" kit (Qiagen, Mississauga, ON). Approximately 5 µg of genomic DNA was cleaved with *EcoR1* and separated on 0.75% agarose gels. The DNA was transferred to Hybond XL membranes (Amersham Biosciences, England) and hybridized at 42˚C for 18 h with 2-4 X 10^{6} dpm of random-primed 93 bp □-^{32}P-dATP-labeled *BamH1-HindIII* fragment cleaved from within the *Ceacam1* promoter in a region located outside of the targeting vector was used as a probe (Nédellec 1995). Membranes were washed at a final stringency of 65˚C in a 0.1 X SSC and 0.1% SDS solution. Alternatively, the mice were genotyped by PCR amplification of their genomic DNA in a final volume of 15 □l containing a 3X dilution of the

Vent polymerase buffer supplied by the manufacturer, 500 □M of dNTPs, 5 ng/□l of the *Ceacam1*-specific oligonucleotides (PN8, 5'CTGCCCCTGGCGCTTGGA and PN5, 5'TACATGAAATCGCACAGTCGC) and 5 ng/□l of the *neo*^{r}-specific oligos (neoforward, 5'CGGTGCCCTGAATGAACTGC and neoreverse, 5'GCCGCCAAGCTCTTCAGCAA) and 0.4 U of Vent polymerase (New England Biolabs, Beverly, MA). Amplifications proceeded through 30 cycles of 20 sec at 94°C, 30 sec at 57°C and 30 sec at 72°C. The wild-type +/+ mice produced a *Ceacam1* fragment of 250 bp upon agarose gel electrophoresis, whereas the homozygous -/- mice had a 550 bp *neo* fragment and the heterozygous +/- mice showed both fragments.

2.2.5.3 RT-PCR

RNA was isolated from colon or kidney tissue following Invitrogen's TRIzol Reagent protocol. Synthesis for amplification of the *Ceacam1*, *Ceacam2* and *Gapdh* cDNAs was performed at 42ºC for 1 h using 200 U of Superscript II reverse-transcriptase (Invitrogen) according to manufacturer's protocol. 5 □g of total RNA were primed with primer 5'TTGATACCTCACTCTCAGCCA for *Ceacam1* cDNA; 20 □g of RNA with primer 5' GGCTCCAGGATCCACCTTTTCTTC for the *Ceacam2* cDNA; and 4 □g of RNA with primer 5'GACTCGAGTCGACGGTACCCT$_{17}$ were used for the *Gapdh* cDNA. One tenth of the cDNAs were then used for PCR amplification with Vent polymerase (New England Biolabs) with 50 ng/□l of the following primers: *Ceacam1* forward, 5'CCAAATGATCACCATGAAG, *Ceacam1* reverse, 5'GGCTCCAGGATCCACCTTTTCTTC; *Ceacam2* forward, 5'ATGGCTTTTCCACTCCACGC, *Ceacam2* reverse, 5'GTCTTATTAGTGCCTGTTAC; *Gapdh* forward, 5'CCATGGAGAAGGCTGGGG, *Gapdh* reverse, 5'CAAAGTTGTCATGGATGACC.

2.2.5.4 Nuclear extract preparations

Epithelial cells were collected from the colonic mucosa by scraping with a scalpel blade and rinsed with PBS containing protease inhibitors. Cells were resuspended in 2 packed-cell volumes of buffer A (10 mM HEPES, pH 7.9 containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and NP-40 0.05%). After 20 min of incubation on ice, cells were mechanically disrupted by 5 passages through 21G needles and centrifuged at 5000g for 5 min at 4°C. Cells were washed once with buffer A and resuspended in buffer C (20mM HEPES pH 7.9 containing 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and 1mM PMSF) by rotating for 30 min at 4°C. Nuclear pellets were collected by centrifugation at 13,000 rpm for 30 min at 4°C and protein concentration was evaluated using a Bio-Rad protein assay kit.

2.2.5.5 Western Analyses

Protein samples were prepared from cell (Huber 1999) or nuclear lysates. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat milk in 0.1% Tween-TBS for 30 min and incubated with primary and secondary antibodies for 2 h. CEACAM1 and CEACAM2 were detected using rabbit polyclonal Abs 2457 and 2052, respectively. Antibodies to $p27^{Kip1}$ and to $p21^{Cip1}$ were from Transduction labs, and antibodies to actin were from Sigma. ECL Western Blotting Reagents and secondary antibodies (α-mouse-HRP and α-rabbit-HRP antibodies, Amersham Biosciences, England) were used for detection.

2.2.5.6 Preparation of Tissues, Histological Analyses and Antibodies Used

The mice were sacrificed by cervical dislocation and tissues were removed, washed in PBS and fixed in 4% paraformaldehyde/PBS or 10% phosphate-buffered formalin and processed for immunohistochemistry. Fixed tissues were dehydrated in ethanol and paraffin-embedded. Tissue sections, 4 μm in thickness, were deparaffinized according to standard protocol. Slides were microwaved in citric acid buffer to improve antigen availability. Sections were blocked in 1%

BSA and 5% normal serum. Primary antibody was applied for 18 h at 4°C. Appropriate secondary and tertiary antibodies (DAKO) were applied and DAB was used for colour development. Slides were counterstained with hematoxylin, following standard histological procedures. The following antibodies were used according to manufacturer's instructions: anti-BrdU (Sigma), PCNA (Santa Cruz), TUNEL (Roche), E-cadherin (Transduction labs), □-catenin (Transduction labs). The anti-CEACAM1 (2457) (Hemmila 2004) and anti-CEACAM2 (2052) (Robitaille 1999) antibodies are polyclonal rabbit antibodies raised to purified proteins.

To define vascular density in tumors, paraffin sections were deparaffinized and treated for 10 min with trypsin (0.01g trypsin, 0.01g $CaCl_2$/ml H_2O). Sections were blocked in 1%BSA and 5% normal goat serum. Primary antibodies, anti-CD31 from Pharmingen and anti CEACAM1 pAb 2457, were applied for 2 h at 20°C. Appropriate FITC-conjugated secondary antibodies with were incubated for 50 min and slides were mounted with medium containing DAPI.

2.2.5.7 Preparation of colonic cells and staining with annexin V.

Primary cells were isolated from flushed colons with an enzyme mix of dispase I and collagenase I (Roche). Tissue pieces of 2 mm^2 were kept in enzyme mix suspension for 1 h at 37°C. Tissue slurry was then centrifuged, and single cell supernatant was passed through 70□m cell strainer. Cells were prepared for annexin V labeling according to manufacturer's specifications (Pharmingen). Cells were analyzed by FACScan (BD Biosciences).

2.2.5.8 In vivo BrdU-labeled proliferation assays.

Analyses of *in vivo* colonic cell proliferation was performed by intraperitoneal injection of a solution of 120 mg/kg of 5'-bromo-2'-deoxyuridine (BrdU, Sigma, St-Louis, MO) dissolved in normal saline (Velcich, Yang et al. 2002) into 6 sibling pairs of 6-month old *Ceacam1*$^{-/-}$ and +/+ mice. Mice were

sacrificed 2-24 h post-BrdU injections, colons were retrieved and paraformaldehyde-fixed sections were prepared for immunohistochemistry.

2.2.5.9 Carcinogen treatment and colon tumor preparations.

Twelve-week old age-matched or sibling *Ceacam1*$^{-/-}$ and +/+ mice were injected intraperitoneally with azoxymethane (AOM, Sigma, St-Louis, MO) dissolved in saline at a dose of 10 mg/kg of body weight, once per week, for 8 weeks. The animals were systematically examined twice per week for appearance of clinical symptoms. Animals displaying discomfort were immediately sacrificed. End-points were defined as 19-20 weeks after the first injection. At least 10 mice of each genotype were tested per experiment and experiments were repeated three times. After sacrificing the mice, the entire colon was removed, flushed with cold phosphate-buffered saline, opened longitudinally and fixed flat on strips of 4% paraformaldehyde-soaked Whatman filter paper. Lesions of different sizes were identified under a dissection microscope and categorized by area as either > 5mm^2, 2-5 mm^2 or < 2 mm^2 using a millimeter grid. After measurements were taken on the whole tissue, lesioned areas were embedded in paraffin for histological examination.

2.2.5.10 Quantification and Statistical analysis

Exposed films of Western blots were scanned, and images were analyzed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2005). p21 and p27 immunoblots were normalized using actin as loading control. The ImageJ program was also used to calculate vascular density by establishing the amount of positive fluorescein signal on 6 microscope fields per tumor section using image thresholds and analysis of particles larger than 20 pixels. Positive signals were expressed as the square pixel area. Data was analyzed using the Student *t* test for statistical significance. *P* values were considered significant if <0.05. Standard error was calculated and represented in the bar graphs.

2.2.6 Concluding remarks

CEACAM1 is a cell adhesion molecule that has significant effects on the homeostasis of the colon. In the absence of CEACAM1, mouse colon epithelium hyperproliferates and undergoes apoptosis at a reduced rate. On such a dysregulated organ, carcinogen-induced tumor formation was increased, and the development of tumors was accelerated. The mechanism by which CEACAM1 controls proliferation is through the cyclin kinase inhibitors p27 and p21. It still undetermined how CEACAM1 controls apoptosis in the colon, but in mammary tissues, it involves Bax translocation to the mitochondria and release of cytochrome *c* into the cytoplasm (Kirshner, Chen et al. 2003). Many interacting players need to be identified in order to define CEACAM1's signalling pathway.

The chemical carcinogen azoxymethane is an alkylating agent that creates DNA adducts. It is a powerful agent to induce colon tumors and treatment with AOM potentially mutates a large variety of genes, which may not recapitulate the sequential multistep mutagenesis found in human colorectal cancers.

Chapter 3 Intestinal tumor progression in the *Ceacam1*$^{-/-}$ mice

3.1 Preface to Manuscript II

Tumor formation in the colon has been described as a multistep pathway of tumorigenesis, involving sequential mutations paralleled to pathologic staging (Fearon 1990). In the previous model of tumor induction, numerous genes were mutated by the chemical carcinogen azoxymethane (Vivona 1993). The contribution of CEACAM1 to tumor progression was imprecise by the unspecific effect of AOM. Pathologic observations indicate that downregulation of CEACAM1 precedes Apc mutational inactivation. Mutation of the tumor suppressor, *Apc*, induces spontaneous polyp formation in the small intestine of mice (Taketo 2006). Of the various Apc models available the mouse *Apc*$^{1638N/+}$ was selected because it has the potential to form polyps in both the small intestine and the colon (Fodde, Smits et al. 1999). The *Apc*$^{1638N/+}$ mouse model develops mostly intestinal polyps with a latency of 5-7 months, along with desmoid tumors (Eccles, van der Luijt et al. 1996).

To recapitulate the multistep pathway described by Fearon *et al*. and the histological observation of colon tumors, the *Ceacam1*$^{-/-}$ mouse model was crossed with the *Apc*$^{1638N/+}$ mouse model. The small intestine is a distinct organ from the colon, therefore several parameters had to be researched in the small intestine of the *Ceacam1*$^{-/-}$ mice vs. the wild type mice. The results of these experiments were submitted to Oncogene in Aug. 2007.

3.2 Intestinal Tumor Progression Is Promoted by Decreased Apoptosis and Dysregulated Wnt Signaling in *Ceacam1*$^{-/-}$ Mice.

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3.2.1 Abstract

The Carcinoembryonic Antigen Cell Adhesion Molecule 1 (CEACAM1) is downregulated in colonic and intestinal hyperplastic lesions as well as in other cancers, where it functions as a tumor suppressor. We have previously shown that deletion of CEACAM1 contributed to the development of colon tumors induced by the carcinogen azoxymethane. To investigate the functions of CEACAM1 in the normal intestine and in intestinal tumors, we generated a compound knockout mouse model and examined the intestinal phenotype in both the *Ceacam1*$^{-/-}$ and *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ compound mice. *Ceacam1*$^{-/-}$ intestinal cells exhibited a significant decrease in apoptosis, with no change in proliferation, however. *Ceacam1*$^{-/-}$ enterocytes also displayed decreased Glycogen Synthase Kinase 3-ß activity with corresponding nuclear localization of □-catenin and increased TCF/Lef transcriptional activity in CEACAM1-null CT51 colonic cells. A significant increase in C-Myc and cyclin D1 expression, known targets of the Wnt signaling pathway, was also observed. Compound *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice demonstrated an increase in intestinal tumor multiplicity, the effect on tumor progression being more important. Increases in intussusceptions and desmoid lesions were also observed. CEACAM1 therefore actively participates in Wnt signaling in intestinal cells and its down-regulation in intestinal tissue contributes to malignancy by augmenting tumor multiplicity and progression.

Submitted to Oncogene Aug. 2007

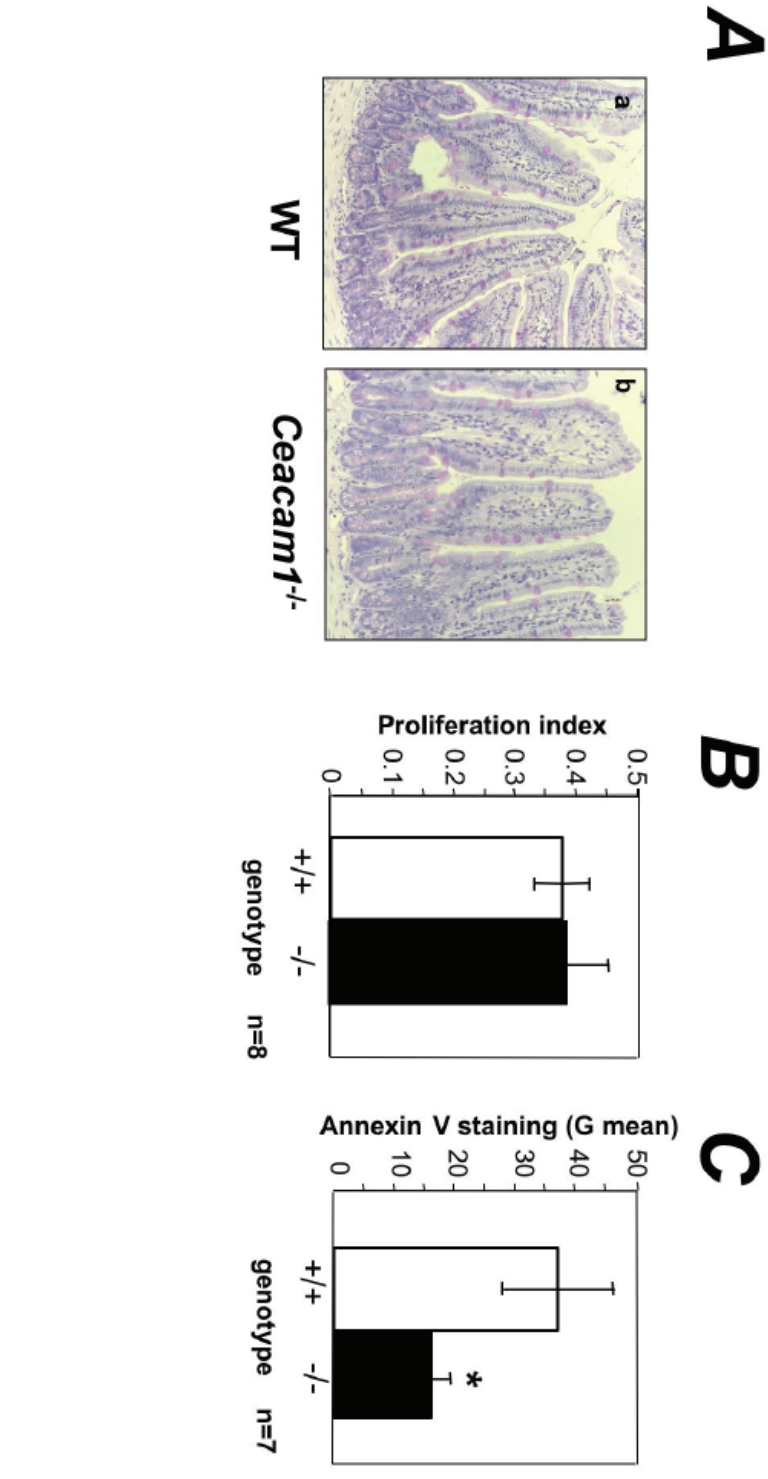
3.2.2 Introduction

Colorectal cancer (CRC) arises from the dysregulation of the balance between proliferation, differentiation and apoptosis of normal intestinal cells that become progressively disrupted by accumulation of mutations in several signaling pathways namely the Wnt, Ras, p53, Dcc and TGF-β pathways (Kinzler 1996). The earliest manifestation of colorectal neoplasia is the appearance of aberrant crypt foci (ACF) that progress to hyperplastic polyps, adenomas and finally carcinomas. This cascade is initiated with mutations in the *Adenomatous*

Polyposis coli gene (*APC)* gene. A small proportion (<10%) of CRC cases are hereditary and familial adenomatous polyposis (FAP) patients have germline mutations in the *APC* gene (de la Chapelle 2004). However, more than 80% of sporadic CRCs also demonstrate *APC* mutations (Rowan, Lamlum et al. 2000; Fodde 2001; Demant 2003). Mutations in *APC* cause its loss of functions and constitutive activation of Wnt signaling which stabilizes ß-catenin followed by its nuclear translocation (Korinek 1997; Morin 1997). Nuclear ß-catenin then associates with various members of the T-cell factor (TCF) transcriptional activators that regulate Wnt target genes involved in cell proliferation, differentiation, migration and apoptosis (Korinek 1997; Morin 1997).

CEACAM1 is a member of the carcinoembryonic antigen (CEA) family (Beauchemin, Draber et al. 1999). CEACAM1 is a cell adhesion molecule mediating trans-homophilic association between its first immunoglobulin (Ig) domains expressed on opposing cells (Gray-Owen 2006). CEACAM1 is expressed in epithelia, hemopoietic and endothelial cells (Gray-Owen 2006). The *CEACAM1* gene produces alternatively spliced isoforms containing Ig-like domains linked to either a short 10 aa (CEACAM1-S) or long 71-73 aa (CEACAM1-L) cytoplasmic domain (Gray-Owen 2006). CEACAM1 expression is down-regulated in colon tumors (Neumaier 1993; Kunath, Ordonez-Garcia et al. 1995) and several other cancers (Horst 2004). CEACAM1 down-regulation is an early event in the genetic progression of colon cancer. SAGE comparisons and DNA chip analyses show its absence in hyperplastic lesions, intestinal microadenomas and adenomas (Nollau 1997; Buckhaults 2001; Notterman 2001; Nittka 2004). CEACAM1 tumor suppressor activities are dependent upon the presence of the L domain (Kunath, Ordonez-Garcia et al. 1995). In support of the role of CEACAM1-L in inhibition of tumor growth, we have recently shown that *Ceacam1*$^{-/-}$ normal colon exhibits increased proliferation and decreased apoptosis. Upon azoxymethane carcinogenic induction, a greater number of colon tumors form in CEACAM1-deficient mice relative to littermate controls (Leung 2006). However, since both *Ceacam1* and *Apc* loss of function represent early events in intestinal cancer development, we questioned whether deletion of the *Ceacam1*

Figure 3.2.3.1: Structure, proliferation and apoptosis in intestines of WT and *Ceacam1*$^{-/-}$ mice.
*(*A) PAS staining of 3 month-old WT and *Ceacam1*$^{-/-}$ intestines demonstrate that CEACAM1 deletion does not alter the structure of this tissue nor goblet cell differentiation. (*B*) Sections of 3 month-old intestines labelled with an anti-Ki67 antibody. Positive cells were counted in 5 microscope fields to calculate proliferation indices , which were similar in all groups (n=8 mice/group). (*C*) Intestinal cells of WT and *Ceacam1*$^{-/-}$ mice were labelled for Annexin V and evaluated by flow cytometry. Bar graph represents average geometric mean of flow cytometry curves (n=7 mice/group; *, $p<0.05$).


A
a
b
WT
Ceacam1-/-
B
Proliferation index
0
0.1
0.2
0.3
0.4
0.5
+/+
-/-
genotype
n=8
C
Annexin V staining (G mean)
0
10
20
30
40
50
+/+
-/-
*
genotype
n=7

gene influenced intestinal tumor progression driven by the *Apc* loss of function and whether both genes impacted on the same signaling pathways. The *Ceacam1*$^{-/-}$ enterocytes displayed decreased apoptosis, with dysregulation of Wnt signaling, β-catenin nuclear accumulation and enhanced TCF/Lef transcriptional activity. Compound *Apc*$^{1638N/+}$: *Ceacam1*$^{-/-}$ mice demonstrated an increase in intestinal tumor multiplicity and progression relative to controls. These results suggest that CEACAM1 can in part regulate the localization of β-catenin and hence the activation of genes activated by the Wnt signaling pathway to reduce progression of intestinal cancer.

3.2.3 Results

3.2.3.1 Intestinal apoptosis is decreased in CEACAM1-deficient mice

We have previously shown that structural characteristics of the *Ceacam1*$^{-/-}$ normal colonic crypts were not altered relative to wild-type controls but that proliferation was significantly increased and apoptosis decreased (Leung 2006). To verify whether intestinal villi exhibited similar characteristics, sections of 3 month-old *Ceacam1*$^{+/+}$ and $^{-/-}$ jejunum (n=8) were stained (Figure 3.2.3.1*A*). *Ceacam1*$^{-/-}$ jejunum exhibited the same length of intestinal villi and the same number of crypt cells relative to wild-type controls. Goblet cell differentiation was similar as judged by the PAS staining (Figure 1*A*). The size of *Ceacam1*$^{-/-}$ enterocytes was similar to that of the wild-type littermates. The migration of the differentiating enterocytes was not affected in the *Ceacam1*$^{-/-}$ mice.

Stainings with an antibody to BrdU after a 6 h *in vivo* labeling period (data not shown) or with an antibody to Ki67 (Figure 3.2.3.1*B*) were used to gauge proliferation of jejunal and ileal enterocytes. The *Ceacam1*$^{-/-}$ intestinal proliferation index was similar to that of the WT mice (Figure 3.2.3.1*B*). CEACAM1-L regulates hepatocyte proliferation through its insulin receptor- (Poy 2002) and EGFR-mediated- (Abou-Rjaily 2004) Tyr phosphorylation with consequent downregulation of the Ras-MAPK signaling. Hence, we monitored

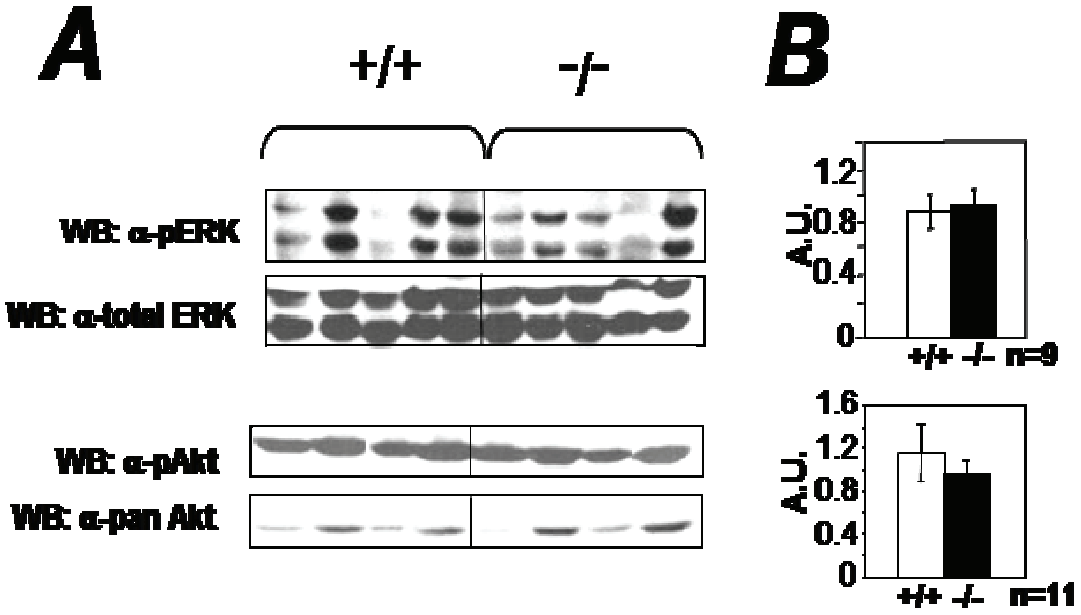
Erk activity by determining phospho-ERK expression in the *Ceacam1*$^{-/-}$ normal jejunum and ileum (Figure 3.2.3.2*A* and *B*) and found no significant differences between the WT and CEACAM1-null mice.

Apoptosis was measured by TUNEL assays and Annexin V staining. *Ceacam1*$^{-/-}$ mice showed a 2-3 fold decrease in early and late apoptosis (Figure 3.2.3.1*C*), as observed in CEACAM1-deficient colon (Leung 2006). We examined the status of the survival factor Akt by immunoblotting *Ceacam1*$^{-/-}$ intestinal proteins with an anti-phospho-Akt antibody. There was no significant change in activated Akt in this tissue (Figure 3.2.3.2*A* and *B*). Collectively, these results indicate that a similar number of enterocytes are produced in *Ceacam1*$^{-/-}$ crypts. However, these enterocytes do not undergo programmed cell death in a timely fashion.

3.2.3.2 Intestinal tumor multiplicity and progression are increased in *Apc*$^{1638N/+}$*: Ceacam1*$^{-/-}$ Mice

Systemic deletion of the *Ceacam1* gene does not lead to the development of any tumor unless a carcinogenic or genetic insult is initiated (Leung 2006). We questioned whether lack of CEACAM1 expression influenced *Apc*-driven intestinal tumor progression. We mated heterozygous C57Bl/6 *Apc*$^{1638N/+}$ mice with a mutation in the endogenous *Apc* gene (Fodde 1994) with two C57Bl/6 *Ceacam1*-deleted mouse lines (2D2 and 11H11) (Leung 2006), thereby producing cohorts of doubly heterozygous and *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice. Results from both lines were pooled as they showed similar results. All animals were sacrificed between 6-7 months of age. *Apc*$^{1638N/+}$ mice produced on average 1.5 ± 0.1 tumors/animal (n=94), whereas the *Apc*$^{1638N/+}$:*Ceacam1*$^{+/-}$ had approximately 2.0 ± 0.2 tumors/animal (P=0.11 relative to *Apc*$^{1638N/+}$ mice, n=136) and *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice showed a slight increase with 2.3 ± 0.2 tumors/animal (p<0.01 relative to *Apc*$^{1638N/+}$, n=112) (Figure 3.2.3.3*A*). A similar increase in hyperplastic lesions was observed between the cohorts (7.5 ± 0.2 in *Apc*$^{1638N/+}$ to

Figure 3.2.3.2: Immunoblotting of WT and *Ceacam1*$^{-/-}$ jejunal and ileal cell lysates.
(*A*) Cell lysate proteins prepared from the indicated number (n) of WT and *Ceacam1*$^{-/-}$ mice were subjected to SDS-PAGE and immunoblotted with relevant antibodies. (*B*) Protein expression was computed from scanned immunoblots relative to either actin, pan-Erk or pan-Akt levels. A.U. represent arbitrary units.


A
+/+
-/-
WB: α-pERK
WB: α-total ERK
WB: α-pAkt
WB: α-pan Akt
B
A.U.
1.2
0.8
0.4
0
+/+ -/- n=9
A.U.
1.6
1.2
0.8
0.4
0
+/+ -/- n=11

8.2 ± 0.3 lesions in *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice). Importantly, the *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ lesions were larger by >30% (p<0.001) relative to those found in the *Apc*$^{1638N/+}$ or doubly heterozygous mice (Figure 3.2.3.3*B*), indicating that loss of CEACAM1 expression contributed more significantly to tumor growth. Clinical pathology revealed a broad spectrum of tumors ranging from hyperplasia and adenomas (Figure 3.2.3.3*C*), to intramucosal carcinomas and finally invasive adenocarcinomas (Figure 3.2.3.3*D*). Tumors of approximately the same size from *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice relative to those of *Apc*$^{1638N/+}$ mice had reached a more advanced stage and were often invasive carcinomas relative to high-grade dysplastic adenomas in *Apc*$^{1638N/+}$ mice. This finding suggests that absence of CEACAM1 impacted on tumor progression. No metastasis was seen by gross observation of the 6-7 month old lymph nodes, liver and lungs of *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice carrying locally invasive tumors. The *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice also demonstrated a significant increase in the number of intestinal intussusceptions (15.5%, n=112) relative to double heterozygotes (4.9%) and *Apc*$^{1638N/+}$mice (3.6%). Intussusceptions result from the presence of large intestinal tumors causing complete obstruction of the proximal segment and peristaltic seizure with intestinal propulsion forcing the telescoping of this segment onto the lower one (Yang, Bancroft et al. 2005). Indeed, the size of the tumors present in *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ intussusceptions (17.2 ± 2.5 mm^2, p<0.05) was 21% larger than that found in the *Apc*$^{1638N/+}$ mice (14.1 ± 0.6 mm^2). The increase in intestinal obstruction in the compound mice also attests to the importance of CEACAM1 in tumor progression.

3.2.3.3 Extra-intestinal lesions are increased in *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice

The *Apc*$^{1638N/+}$mice also sustain tumor induction at extra-intestinal sites. For instance, colonic polyps were initially noticed in this model (Fodde 1994). Correspondingly, we found a significant increase in the number and grade of colon hyperplasias and adenomas in double heterozygotes (p<0.05) with a similar tendency in the *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice (data not shown). In addition, the *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice demonstrated a significant increase of approximately

2-fold in the number of desmoid tumors ($p<0.01$ relative to $Apc^{1638N/+}$ and double heterozygotes, n=24) (Figure 3.2.3.3*E*). Desmoids represent a major cause of morbidity in the FAP patients due to local invasion (Smits, van der Houven van Oordt et al. 1998); these were characterized pathologically as fibromatosis. These results suggest that CEACAM1 plays a role in dermal tumor progression initiated by *APC* mutations.

3.2.3.4 The Wnt signaling pathway is enhanced in *Ceacam1*$^{-/-}$ mice

The phenotypes observed in *Ceacam1*$^{-/-}$ jejunum and ileum or in the intestinal tumor progression in $Apc^{1638N/+}$:*Ceacam1*$^{-/-}$ mice suggest that absence of CEACAM1 created disruptions in major signaling pathways. We therefore examined expression and activity levels of key intestinal proteins in the Wnt signaling pathway as it sustains major alterations in intestinal cancer. Neither E-cadherin, total β-catenin nor the Snail repressor displayed altered expression levels (Figure 3.2.3.4*A* and *B* and data not shown). CEACAM-L has been shown to bind directly to β-catenin in breast tumor cells and hepatocytes (Jin 2007; Fiset *et al*., submitted). To confirm that CEACAM1-L indeed interacted with β-catenin in gastrointestinal cells, we stained mouse CT51 colon carcinoma cells stably expressing CEACAM1-L with relevant antibodies. CEACAM1-L staining (Figure 3.2.3.5, panel b) overlapped with that of β-catenin cell surface expression (Figure 3.2.3.5, panel a), as judged by the yellow overlay (Figure 3.2.3.5, panel c). Moreover, increased β-catenin nuclear expression was confirmed in immunoblots of jejunal and ileal nuclear fractions of *Ceacam1*$^{-/-}$ intestines relative to WT intestines (Figure 3.2.3.4*A* and *B*). In line with this finding, a significant increase in GSK3-β phosphorylation was observed in *Ceacam1*$^{-/-}$ intestines (Figure 3.2.3.4*A* and *B*), indicating that this enzyme is mostly inactive (Patel, Doble et al. 2004) favouring the shuttle of cytosolic β-catenin to the nucleus. To examine whether deletion of *Ceacam1* impacted on β-catenin transcriptional activity, we co-transfected mouse CT51 colon carcinoma cells either devoid of CEACAM1

Figure 3.2.3.3: Development of neoplasia in *Apc*$^{1638N/+}$:*Ceacam1*$^{+/-}$ and $^{-/-}$ mice.

(*A*) Mice were sacrificed 6-7 months after birth and tumors were counted. *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ (n=112) mice produced more tumors than *Apc*$^{1638N/+}$ (n=94; *, $p<0.01$) or *Apc*$^{1638N/+}$:*Ceacam1*$^{+/-}$ (n=136) mice. (*B*) *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ tumors were larger than those found in *Apc*$^{1638N/+}$ or *Apc*$^{1638N/+}$:*Ceacam1*$^{+/-}$ mice (*, $p<0.001$). (*C*) Left panel, adenoma of an *Apc*$^{1638N/+}$ mouse demonstrating high grade dysplasia. (*D*) Left panel, invasive carcinoma of the submucosa from an *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mouse. The boxed areas (right panels) represent higher magnifications. (E) *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice (n=24) develop more desmoid lesions than *Apc*$^{1638N/+}$ (n=27) or *Apc*$^{1638N/+}$:*Ceacam1*$^{+/-}$ mice (n=11, *, $p<0.01$).

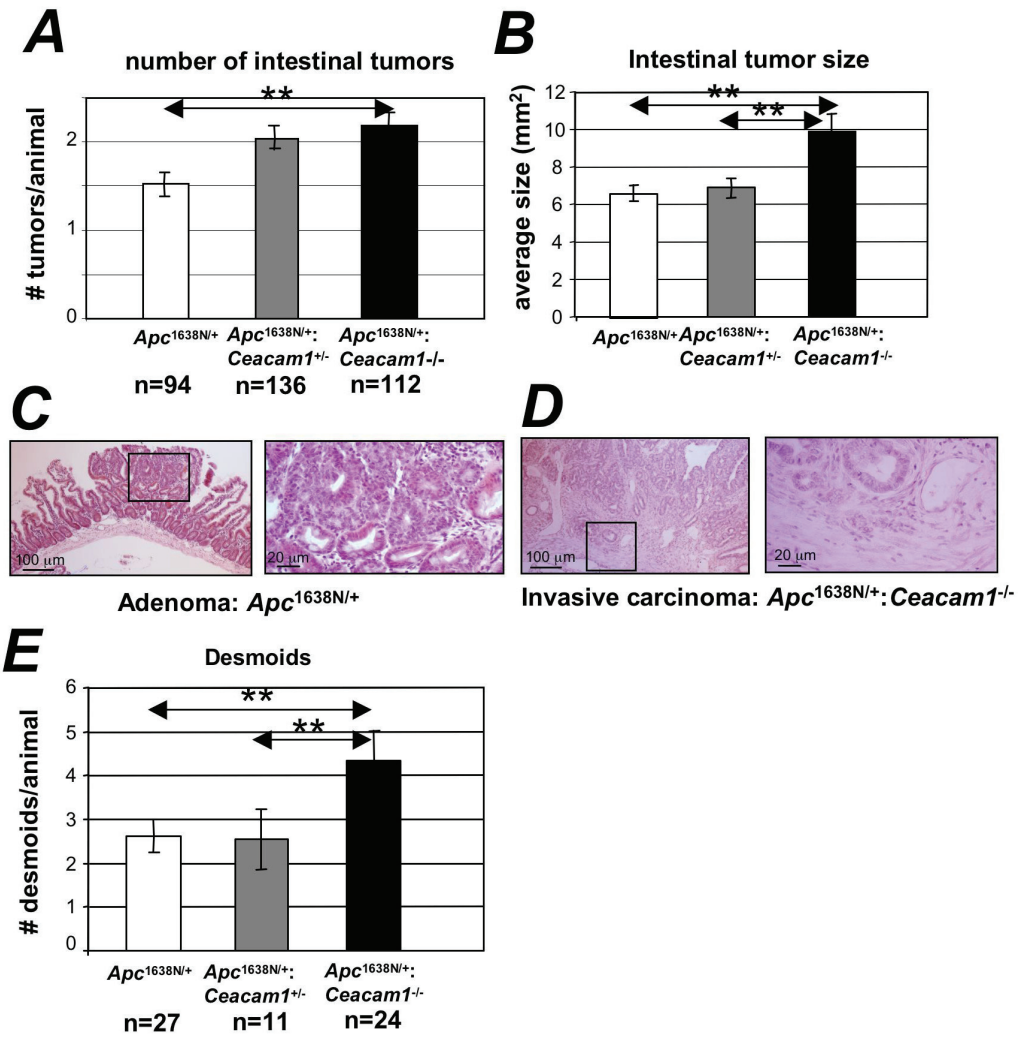

A
number of intestinal tumors
tumors/animal
0
1
2
**
Apc1638N/+
Apc1638N/+: Ceacam1+/-
Apc1638N/+: Ceacam1-/-
n=94
n=136
n=112
B
Intestinal tumor size
average size (mm2)
0
2
4
6
8
10
12
**
**
Apc1638N/+
Apc1638N/+: Ceacam1+/-
Apc1638N/+: Ceacam1-/-
C
100 µm
20 µm
Adenoma: Apc1638N/+
D
100 µm
20 µm
Invasive carcinoma: Apc1638N/+:Ceacam1-/-
E
Desmoids
desmoids/animal
0
1
2
3
4
5
6
**
**
Apc1638N/+
Apc1638N/+: Ceacam1+/-
Apc1638N/+: Ceacam1-/-
n=27
n=11
n=24

Figure 3.2.3.4: : Immunoblotting of WT and *Ceacam1*$^{-/-}$ jejunal and ileal cell lysates.

(*A*) Cell lysate proteins prepared from the indicated number (n) of WT and *Ceacam1*$^{-/-}$ mice were subjected to SDS-PAGE and immunoblotted with relevant antibodies. Nuclear fraction lysates were prepared to detect β-catenin, p21 and cyclin D1 proteins. (*B*) Protein expression was computed from scanned immunoblots relative to either acti. A.U. represent arbitrary units; *, $p<0.05$ for GSK-3βc-Myc and Cyclin D1.

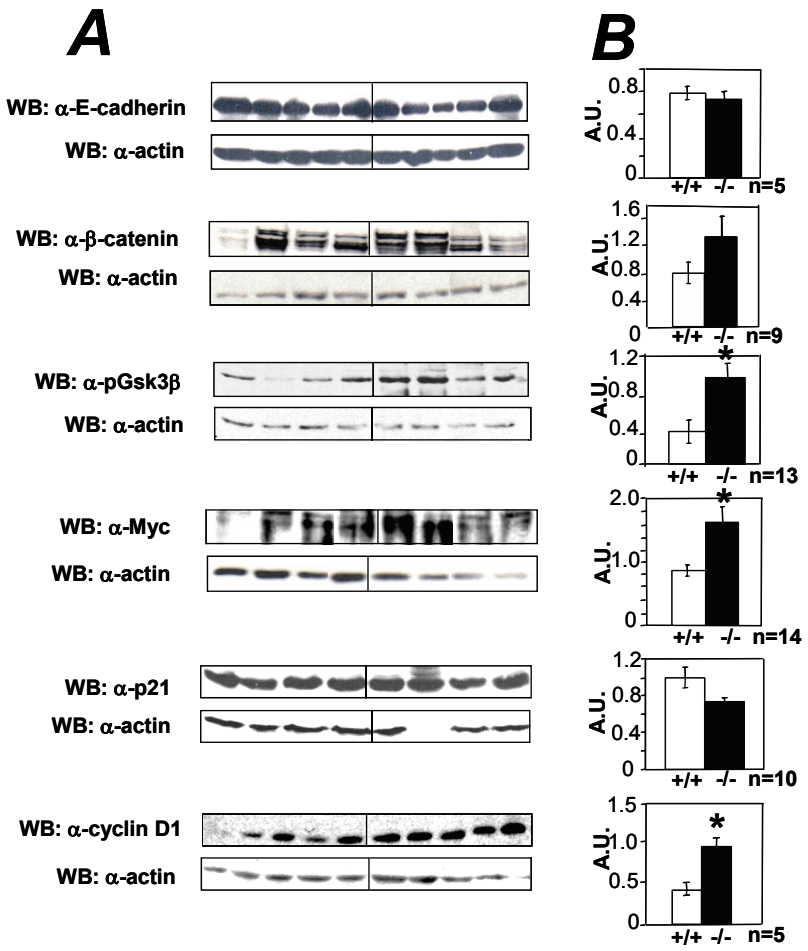

A
B
WB: α-E-cadherin
WB: α-actin
WB: α-β-catenin
WB: α-actin
WB: α-pGsk3β
WB: α-actin
WB: α-Myc
WB: α-actin
WB: α-p21
WB: α-actin
WB: α-cyclin D1
WB: α-actin
A.U.
0.8
0.4
0
+/+ -/- n=5
1.6
1.2
0.8
0.4
0
+/+ -/- n=9
1.2
0.8
0.4
0
*
+/+ -/- n=13
2.0
1.0
0
*
+/+ -/- n=14
1.2
0.8
0.4
0
+/+ -/- n=10
1.5
1.0
0.5
0
*
+/+ -/- n=5

Figure 3.2.3.5: Confocal images of co-localization of CEACAM1 and β-catenin in mouse CT51 colon carcinoma cells.

Mouse CT51 colon carcinoma cells expressing the CEACAM1-L protein were stained for (a) β-catenin (red) or for (b) CEACAM1 (green) and labelled with Cy3- and FITC-conjugated antibodies, respectively. (c) Co-localized signal in the overlay (yellow).

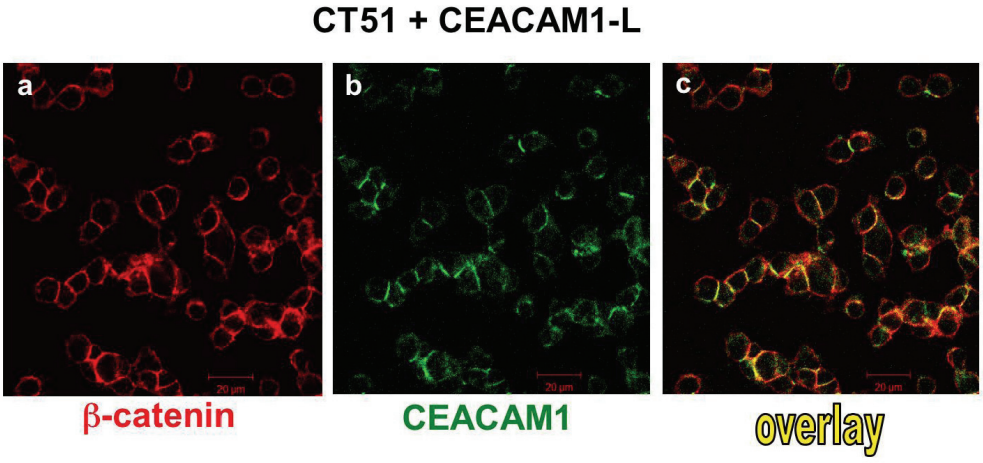
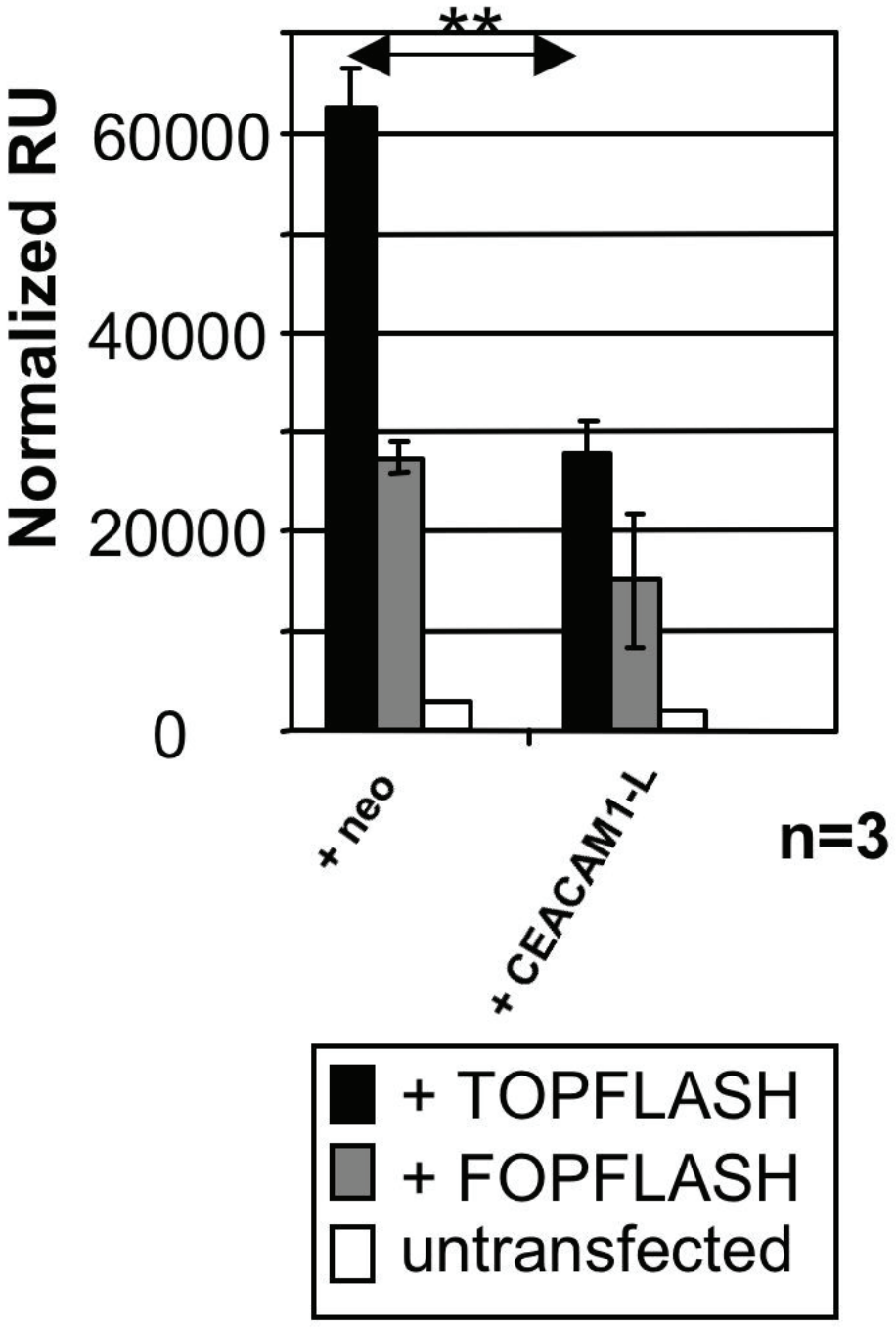

CT51 + CEACAM1-L
a
b
c
β-catenin
CEACAM1
overlay

Figure 3.2.3.6: Effect of CEACAM1-L on β-catenin transcriptional activity.

Mouse CT51 colon carcinoma cells either devoid of CEACAM1 (CT51*neo* control cells) or stably expressing CEACAM1-L were transfected with a pRSVLacZ construct and the TOPFLASH reporter containing the TCF/Lef transcription factor consensus elements. Transfections were normalized for β-galactosidase activity in CT51 cells. Controls included the FOPFLASH construct with TCF/Lef consensus elements unable to bind ß-catenin. Untransfected cells had minimal transcriptional activity. FOPFLASH activity was not statistically different between the cell lines. **, $p<0.01$. R.U., relative units.


**
Normalized RU
60000
40000
20000
0
+ neo
+ CEACAM1-L
n=3
+ TOPFLASH
+ FOPFLASH
untransfected

(CT51 or *neo* control cells) or stably expressing CEACAM1-L with the TOPFLASH reporter consisting of *Luciferase* gene controlled by TCF/Lef transcription factor consensus elements and pRSVLacZ constructs. Controls included the FOPFLASH construct with mutations in the TCF/Lef consensus sequence and in the reverse orientation rendering it unable to bind β-catenin. The signal was normalized with β-galactosidase activity. Untransfected cells had minimal transcriptional activity (Figure 3.2.3.5). FOPFLASH activity was not statistically different between the cell lines, whereas CT51 (data not shown) or *neo* control cells demonstrated a significant enhancement of β-catenin/TCF transcriptional (TOPFLASH) activity relative to those expressing CEACAM1-L (**, $p<0.01$, Figure 3.2.3.6). c-Myc is one of the downstream targets of the Wnt signaling pathway and of the MAP kinase pathway (Hanahan and Weinberg 2000) in intestinal epithelial cells (He, Sparks et al. 1998; van de Wetering 2002; Sansom, Meniel et al. 2007). Higher c-Myc expression was noticed in the *Ceacam1*$^{-/-}$ jejunum and ileum in comparison to their control littermates ($p<0.05$, Figure 3.2.3.4*A* and *B*). This was paralleled by a decrease in $p21^{Cip1}$ cyclin kinase inhibitor (Figure 3.2.3.4*A* and *B*). Similarly, cyclin D1, another Wnt target, also showed significant enhanced expression ($P=0.03$, Figure 3.2.3.4*A* and *B*). These results indicate that expression of CEACAM1-L in colon carcinoma cells significantly decreases β-catenin transcriptional activity while absence of CEACAM1 produces the reverse effect.

3.2.4 Discussion

Analyses of CEACAM1-deficient mouse models confirmed CEACAM1's roles as a cell adhesion molecule (Obrink 1997; Gray-Owen 2006), an inhibitory co-receptor in T lymphocytes (Nagaishi, Iijima et al. 2006), an angiogenic factor (Horst 2006) and a signaling molecule affecting insulin clearance via the insulin receptor (Najjar 2002). CEACAM1 also functions as a tumor suppressor in a number of cancers derived from the epithelium (Hsieh 1995; Kunath, Ordonez-Garcia et al. 1995; Nittka 2004; Leung 2006). Unless triggered by a colon-

specific carcinogen, *Ceacam1*$^{-/-}$ mice do not develop colon tumors during their lifespan (Leung 2006). Since CEACAM1 is down-regulated early in the development of human colon tumors (Nollau 1997; Nittka 2004) and *APC* is considered one of the first genes mutated in intestinal cancer (Fodde 2001), we questioned whether CEACAM1 impacted on the same signaling pathways as APC and enhanced the progression of intestinal tumors developing on the *Apc*$^{1638N/+}$ mutant background. We first examined changes in the normal adult *Ceacam1*$^{-/-}$ intestine relative to proliferation and apoptosis. Contrary to results observed with the *Ceacam1*$^{-/-}$ colon, *Ceacam1*$^{-/-}$ and WT intestinal crypts proliferated at the same rate. This was confirmed by similar Erk phosphorylation activity. This result suggests that CEACAM1 is regulating other functions in intestine. However, *Ceacam1*$^{-/-}$ enterocytes, in parallel with colonocytes (Leung 2006), demonstrated a 2-3-fold decrease in apoptosis. This re-emphasizes the role of CEACAM1 as a positive regulator of this process, as was previously shown in human HT-29 cells and CRCs (Nittka 2004). However, levels of phosphorylated Akt were not significantly different in CEACAM1-null intestines relative to controls, implicating suppression of apoptosis through other signaling pathways.

The different results obtained from the chemically-induced carcinogenesis, and the present report, suggests that CEACAM1 and APC signal through the same pathway. When tumor initiation involves a gene other than APC we can observe a substantial increase in tumor multiplicity. One cannot discount the differences in the microenvironment of the small intestine and the colon. Despite this, the inhibition of apoptosis in the absence of CEACAM1 is probably the greatest factor in the tumor growth we observe.

Although no increase in normal intestinal cell proliferation was observed, compound *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice showed a slight but significant enhancement of intestinal tumor multiplicity relative to the *Apc*$^{1638N/+}$ littermates. The effect is more muted than that observed in carcinogen-induced colon tumors (Leung 2006), but azoxymethane-elicited tumorigenesis produces mutations in a significant number of genes (Vivona 1993). Additionally, we showed that the second *Apc* allele was mostly lost in tumors whereas expression from the

remaining *Ceacam1* allele appears unaffected (see table 3.2.1). Importantly however, the *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$-derived tumors progressed to a more advanced stage as defined by the larger tumor sizes (>30%) and a higher ratio of carcinomas:adenomas in the compound mice *versus* their *Apc*$^{1638N/+}$ littermates, suggesting that both proliferation and migration of tumorigenic intestinal cells were influenced by the CEACAM1 deletion. In addition to intestinal tumor development, CEACAM1 loss also increased the formation of intussusceptions (5 fold enhancement) and desmoids lesions. Intussusceptions are uncommon in human patients and generally cause intestinal blockage due to a pathologic lesion that is most often malignant (Azar and Berger 1997). The development of intussusceptions in *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice may be linked to adipose deposits surrounding large tumors within the intussusceptions.

Table 3.2.1: Average copy number of the Apc and Ceacam1 RNAs expressed in normal and tumor tissues.

		Apc			***Ceacam1***		
Genotype	n	Tumor	Normal	*P* value	Tumor	Normal	*P* value
Apc$^{+/-}$	6	10.6 ± 5.9	55.5 ± 24.6	0.06	3.2 ± 1.3	14.0 ± 6.9	0.09
Apc$^{+/-}$/*CC1*$^{+/-}$	6	17.6 ± 7.3	49.8 ± 39.1	0.21	3.0 ± 1.1	2.9 ± 1.2	0.40
Apc$^{+/-}$/*CC1*$^{-/-}$	6	1.6 ± 0.4	176.2 ± 139.3	0.13	2.3 ± 1.1	4.4 ± 2.1	0.20

Average copy numbers, calculated from $2^{-\Delta Ct}$ ± SE, are shown in the table. P values compare tumor and normal cDNAs

Remarkably, loss of CEACAM1 expression affected the Wnt signaling pathway in the intestines. β-catenin nuclear expression and transcriptional activity are significantly enhanced in CT51 cells negative for CEACAM1 expression and in CEACAM1-negative intestinal tissue, *versus* those expressing the CEACAM1-L isoform, suggesting that CEACAM1 and APC are both influencing the Wnt signaling pathway. This is further supported by a significant increase in the GSK3-β phosphorylation in the *Ceacam1*$^{-/-}$ intestines, rendering this kinase inactive. Dephosphorylated β-catenin cannot be appropriately targeted for degradation in the destruction complex. Notably, GSK3-β inhibition through its phosphorylation *in vivo* is dependent upon insulin-mediated activation of

PI3K/Akt pathways (Patel, Doble et al. 2004). CEACAM1 regulates insulin receptor endocytosis and insulin clearance from the liver and inactivation of GSK3-β activity is likely a consequence of dysregulated insulin receptor signaling in these mice (Poy 2002). Furthermore, *Ceacam1*$^{-/-}$ intestines demonstrated a significant increase of c-Myc and cyclin D1 and a decrease p21^{Cip1} expression, all well-known Tcf-LEF targets (van de Wetering 2002). Recently, Jin *et al*. have shown that the CEACAM1-L directly associates with β-catenin armadillo repeats (Jin 2007). Similarly, Fiset *et al.* demonstrated that CEACAM1 forms a complex with β-catenin, SHP-1 and Cdk2 at the plasma membrane. Upon β-catenin phosphorylation by Cdk2, it dissociates from CEACAM1 and is degraded, consequently loosening the density of the actin network at internalization sites (Fiset *et al.*, submitted). The results presented herein highlight that *in vivo* CEACAM1 downregulation in intestinal tissue results in decreased apoptosis, rendering these cells sensitive to accumulation of genetic mutations of relevant genes including *APC*. It has recently been shown that increased Wnt signaling contributes to chromosomal instability in APC or β-catenin mutant cells (Aoki, Aoki et al. 2007). Ultimately, dysregulation of Wnt signaling in *Ceacam1*$^{-/-}$ intestines may also enhance genomic instability and contribute to tumor progression.

3.2.5 Materials and Methods

3.2.5.1 Animal models

C57Bl6/129 *Ceacam1*$^{-/-}$ lines 2D2 and 11H11 (Leung 2006) (backcross 6) were mated to C57Bl6/129P (backcross 8) *Apc*$^{1638N/+}$ mice (Fodde 1994) (NCI Frederick Mouse Repository) to obtain *Apc*$^{1638N/+}$:*Ceacam1*$^{-/-}$ mice. *Ceacam1*$^{-/-}$ and *Apc*$^{1638N/+}$ mice were genotyped as described (Fodde 1994; Leung 2006). Experiments were performed on animals having reached a minimum of backcross 10. Skin, small intestine and colon of 6-7 month-old mice were examined at necropsy and lesions/tumors were counted, measured, fixed in 10% buffered

formalin or frozen in OCT. Tumors were independently graded by two clinical pathologists. All animal experiments were carried out according to protocols approved by the Animal Ethics Committee of McGill University.

3.2.5.2 In vivo BrdU-labelled proliferation assays

In vivo intestinal cell proliferation assays were performed with 5'-bromo-2'-deoxyuridine labelling (BrdU, Sigma, St-Louis, MO) (Velcich, Yang et al. 2002) on 6 sibling pairs of 6-month old *Ceacam1*$^{-/-}$ and WT mice. Mice were sacrificed 2-24 h post-BrdU injections and tissues processed as described (Leung 2006).

3.2.5.3 Immunohistochemistry, Immunofluorescence and Flow cytometry

Tissue sections were stained using anti-BrdU (1:1000, Sigma) and anti-Ki67 (1:50, DAKO) antibodies (Leung 2006). Periodic Acid Schiff (PAS) staining was used for mucin detection. Mouse CT51 colon carcinoma cells stably expressing CEACAM1-L have previously been described (Kunath, Ordonez-Garcia et al. 1995). For immunofluorescence, sections were treated with antibodies to β-catenin (1:500) and CEACAM1 (CC1, 1:1000) (Dveksler 1993) and labelled with FITC- and Cy3-conjugated antibodies (Jackson Labs, West Grove, PA). Sections were mounted in DAKO Cytomation mounting medium and analyzed on a Zeiss LSM 5 Pascal using Axiovert 200 confocal microscope. Jejunal and ileal single cell suspensions were obtained by incubating the tissue in 0.5 mM DTT, 1 mM EDTA in PBS for 15 minutes at 37^{o}C with occasional shaking. Recovered cells were stained with Annexin V antibodies as described (Leung 2006).

3.2.5.4 Immunoblotting analysis

Jejunal and ileal epithelial cells were collected by scraping of the intestinal mucosa and homogenized in lysis buffer (10 mM TrisCl pH 8.0, 2 mM EDTA, 1% NP-40, 1 X Roche protease inhibitors). Nuclear extracts were obtained as previously described (Leung 2006). 50-100 μg of protein lysate was loaded on

10-12% SDS polyacrylamide gels. Immunoblots were incubated with antibodies to $p21^{Cip1}$ and c-Myc (1:1000, Upstate, Charlottesville, VA), E-cadherin (1:500, Transduction Laboratories, Lexington, KN), p-Akt (1:1000, Thr308 and Ser473) and pan Akt, p-Erk and pan Erk, β-catenin, pGSK3-β (1:1000, all from Cell Signaling, Danvers, MA), cyclin D1 (1:1000, Santa Cruz). Signals were detected using the Western Lightning Chemiluminescent reagent plus (Perkin Elmer, Boston, MA).

3.2.5.5 Luciferase assays

Mouse CT51 colon carcinoma cells (5×10^4), expressing or not, CEACAM1-L (Kunath, Ordonez-Garcia et al. 1995) were transfected with 2 μg of either the TOPFLASH or FOPFLASH plasmids (Upstate Biotech) and 200 ng of the pRSV-LacZ plasmid using Lipofectamine 2000 (InVitrogen). 48 hours after transfection, cells were lyzed and analyzed for light emission on a Lumat LB9507 (Berthold Technology) luminometer (Kobrossy, Rastegar et al. 2006). β-galactosidase assays were performed as described (Kobrossy, Rastegar et al. 2006).

3.2.5.6 Expression detected by Real-time PCR

cDNA was used for real-time PCR to detect expression levels of *Ceacam1* and *Apc* genes. For the *Ceacam1* cDNA, the oligos CC1F (5'AGGTCACCTAAGTGCTTACGCCAA) and CC1R (5'GGCCATTTCCTGCTTCTGGTTTGT) were used in a master mix containing SYBR green (Qiagen) with cycling identical to standard PCR techniques using a Roche Lightcycler. The *Apc* gene was amplified using forward oligo 5'-AAACGGCATGATATTGCACGCTCC and reverse oligo 5'-TGTTTGCTGTGTTCACGCTTCCAG with annealing temperature of 67°C and elongation at 72°C for 15 seconds. The gene *PSMB* was used as a control with the forward oligo 5'-AGGAATCATCATTGCAGGCTGGGA, and the reverse oligo 5'- AAAGCGAGAGCATTGGCAGTGAAC. Cycle treshold (Ct) was calculated

from second point derivative. The levels of the target genes were normalized on the basis of PSMB expression using the Δ Ct method, where Ct(target)-Ct(PSMB) = Δ Ct. Copy number was calculated as follows: $2^{-\Delta Ct}$.

3.2.5.7 Statistical analysis

Data was analyzed using the Student's t-test. *P* values were considered significant if <0.05. Standard errors (S.E.) are represented in the bar graphs.

3.2.6 Concluding Remarks

The model to reconstruct the conditions described in the multistep sequence of carcinogenesis has revealed that CEACAM1 promotes tumor progression and not initiation on a background of Apc deficiency. CEACAM1 independently of Apc regulates Wnt signaling. There are distinct differences between the colon and small intestine, and the induction of tumors via chemical carcinogen or genetic manipulation. Only apoptosis is affected by the absence of CEACAM1 in the small intestine, whereas in the colon both proliferation and apoptosis are altered. The role of CEACAM1 in contact inhibition is perhaps more relevant in the colon than in the small intestine.

It must not be forgotten that the studies were conducted on systemic knockouts of *Ceacam1*. An underlying phenotype of the *Ceacam1-/-* mice is that they are slightly obese and have impaired insulin clearance. These abnormalities have the potentially to contribute to tumorigenesis and are further investigated in the next section.

Chapter 4 Lipid metabolism and insulin resistance in the *Ceacam1*-/- mouse

4.1 Preface to Manuscript III

CEACAM1 is abundantly expressed in the liver. CEACAM1 is tyrosine phosphorylated by the activated insulin receptor, which induces internalization of the two receptors in clathrin-coated pits by an unknown mechanism. In this way CEACAM1 plays a significant role in insulin clearance. Experiments with the transgenic mouse overexpressing a dominant-negative CEACAM1 specifically in the liver, established that CEACAM1 activity is necessary for insulin clearance. L-SACC1 mice developed secondary insulin resistance, which does not affect muscular and adipose tissue response.

The *Ceacam1-/-* mouse is slightly obese, which prompted studies in metabolism. The glucose and lipid homeostasis were altered in the *Ceacam1-/-* mouse. Furthermore, gene array analysis of liver RNA identified many enzymes involved in lipid biosynthesis. Increased lipid production in the liver resulted in steatohepatitis in older mice. The following sections describe the results of experimentation on the *Ceacam1-/-* mouse in the context of lipid and insulin homeostasis, to be submitted for publication.

4.2 *Ceacam1*-/- mice develop liver insulin resistance and lipid dysfunctions.

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Running Title: CEACAM1 and insulin homeostasis.

4.2.1 Abstract

Carcinoembryonic Cell Adhesion Molecule 1 (CEACAM1) is a cell adhesion molecule within the Ig superfamily. The Tyr-phosphorylated isoform of CEACAM1 has also been shown to play an important role in insulin clearance from the liver and insulin receptor signaling. In this report, we demonstrate that mice having sustained the complete ablation of the *Ceacam1* gene develop a slight obesity due to the increase of their visceral fat. This phenotype results in a sexual dimorphism as it is more apparent in female than male *Ceacam1*-/- mice. As of 3 months of age, the mice exhibit a significantly altered gene expression profile where most enzymes involved in lipidogenesis, under the transcriptional control of the SREBP1c transcription factor, are significantly elevated in their expression. The *Ceacam1*-/- mice also have significantly elevated total and esterified hepatic cholesterol as well as elevated hepatic triglyceride levels at 12 mo, which is also apparent in lipid inclusions in their steatotic livers. Although no hyperglycemia was noticed in these mice, a mild hyperinsulinemia was detected in fasted *Ceacam1*-/- female mice, with a 14-fold enhancement in the post-prandial state. In spite of the mild fasted hyperinsulinemia, the *Ceacam1*-/- mice demonstrated whole body and hepatic insulin resistance when tested with the euglycemic clamp assay. In conclusion, the *Ceacam1*-/- mice, and in particular the female mice, present a metabolic phenotype with significant disruption of lipidogenic enzyme levels as of 3 months of age, and with concomitant whole body and hepatic insulin resistance.

4.2.2 Introduction

The regulation of glucose and lipid metabolism depends on proper signaling by insulin receptor-activated pathways (White 1998),(Taniguchi, Emanuelli et al. 2006). It is now well understood that binding of insulin to the insulin receptor results in the latter's dimerization and autophosphorylation, but also in the phosphorylation and activation of a number of downstream effectors (Saltiel 2001). Phosphorylation of the Insulin Receptor Substrate proteins (IRS1-4) constitutes a major signaling event activating either the Ras/MAP kinase cascade through binding of the Grb2-SOS-Shc complex to both Ras and IRS1 (Boulton 1991) or the PI3-Kinase cascade through its direct binding to IRS1 (Shepherd 1995). In turn, activation of PI3-kinase regulates stimulation of glucose transport as well as glycogen and lipid biosynthesis. The mechanisms by which this activity is exerted have not yet been completely deciphered but both the Ser/Thr kinase AKT (Cho 2001) as well as the atypical protein kinase C isozymes (Standaert 1997) play a role in insulin metabolism.

CEACAM1-4L (herein named CEACAM1-L) is an Ig-like membrane-anchored glycoprotein and a substrate of the insulin receptor in hepatocytes (Najjar 1995). Upon phosphorylation of Tyr488 within its cytoplasmic domain, CEACAM1-L regulates receptor-mediated insulin endocytosis via clathrin-coated pits and targets it for degradation (Formisano 1995; Choice 1998). However, as demonstrated by mutagenesis studies, CEACAM1-L Tyr488 phosphorylation also requires the presence and most likely the phosphorylation of Ser503 by an unknown Ser kinase (Najjar 1995). The importance of this residue in insulin trafficking was recently highlighted by the generation of a hepatocyte-specific transgenic mouse expressing the CEACAM1-L S503A mutant in a dominant-negative approach (L-SACC1 mice). The mice developed chronic hyperinsulinemia which caused secondary insulin resistance and defects in insulin clearance (Poy 2002),(Park 2006). These mice also developed elevated levels of free fatty acids that contributed to insulin resistance (Dai 2004). The Tyr phosphatase SHP-1, a known partner of CEACAM1-L (Beauchemin 1997),

contributes to the regulation of insulin clearance as well as to glucose homeostasis through modulation of insulin signaling in liver and muscle (Dubois 2006).

Mouse CEACAM1 exhibits a number of alternatively spliced isoforms containing either two or four Ig-like domains linked via a transmembrane domain to either a short 10 aa (CEACAM1-S) or long 73 aa (CEACAM1-L) cytoplasmic domain (McCuaig 1993). Only the CEACAM1-L isoform sustains Tyr phosphorylation by the activated insulin receptor. To clarify the functions of the mouse CEACAM1 proteins, we have generated a genetically-targeted mouse model demonstrating complete ablation of all CEACAM1 isoforms and have examined the phenotypes associated with liver functions. We report here that, as shown with the liver-specific L-SACC1 mice (Poy 2002), the female *Ceacam1*-/- mice are slightly obese and develop insulin resistance. Their livers demonstrate signs of steatosis and dysfunction at 6 and 12 months of age. These mice also exhibit increased transcriptional activation of a number of enzymes involved in lipidogenesis and modulation of genes involved in inflammation. These results emphasize that CEACAM1 plays an important role in maintenance of insulin-mediated signaling and that its absence most likely contributes to the development of the metabolic syndrome.

4.2.3 Results

4.2.3.1 Generation of the *Ceacam1* knockout mice

Complete deletion of the mouse *Ceacam1* gene has been reported (Hemmila 2004). Two independent mouse lines, named 2D2 and 11H11, were derived from independent ES cell clones and both were used in the experiments described herein. Complete abrogation of CEACAM1 expression was confirmed by Northern and Western blotting (Leung 2006). The mice are healthy and viable in a pathogen-free environment.

4.2.3.2 The *Ceacam1* knockout mice exhibit elevated body weight

To examine whether the complete elimination of the CEACAM1 isoforms produced a phenotype related to insulin signaling, *Ceacam1*-/- mice bred on a C57Bl/6 background were evaluated for several parameters. All parameters were verified at 3 mo of age, but not statistical differences were noted in any of them (data not shown). Because the phenotype might become apparent at a later age, cohorts of mice were examined at 6 and 12 mo of age. As shown in Fig. 1A panel c, when tested over a 7 mo period, both males and females displayed a 10-15% increase in overall body weight relative to their +/+ siblings. The same phenotype was observed on the 129Sv background. At 6 mo of age (Fig. 4.2.3.1A panel a), female and males showed a trend towards increased weight (P = 0.06 and P = 0.1), and combined results were statistically significant (P = 0.03). At 12 mo of age (Fig. 4.2.3.1A panel b), *Ceacam1*-/- females were clearly heavier (P= 0.003) than their wild-type littermates. No significant differences were noticed in the males, however. The liver of all animals were weighed and only a slight non-statistically significant difference was noted when sexes were combined (Fig. 4.2.3.1B panel a, P= 0.07). The increased weight particularly in *Ceacam1*-/- females was attributed to the increased weight of fat at 6 mo of age (Fig. 1C panel a) or 12 mo of age (Fig. 4.2.3.1C panel b). Six month-old *Ceacam1*-/- males also showed a trend towards increased fat (Fig. 4.2.3.1C panel a). We then questioned whether the *Ceacam1*-/- mice were slightly obese because of elevated food intake. The food consumption of siblings was measured over a 2 mo period and both male and female *Ceacam1*-/- mice showed an increase of 10-20% of their food ingestion, confirming that they are hyperphagic (Fig. 4.2.3.1D). As obese mice often demonstrate altered leptin receptor signaling (Fantuzzi 2005), we verified whether the obese phenotype demonstrated by the *Ceacam1*-/- mice was due to such an alteration. Female *Ceacam1*-/- mice showed a significant elevation in their fasting leptin levels relative to the wild-type mice (p<0.05), whereas male mice did not reveal the same trend (Fig. 4.2.3.1E) and exhibited values that were very similar to those of the +/+ mice. These results therefore indicate that *Ceacam1*-/- female mice exhibit apparent signs of slight obesity which is due to a

significant increase of the visceral fat, and that consequently, leptin levels are increased.

4.2.3.2 Lipid Profiles in *Ceacam1*-/- mice

Since *Ceacam1*-/- mice exhibited increased visceral adiposity, we examined the *Ceacam1*-/- mice for lipid parameters in plasma and liver. No differences were noticed at 3 (data not shown) and 6 mo in the levels of serum FFAs, triglycerides (TG), high density lipoproteins and cholesterol (Fig. 4.2.3.2A-D panels a). However, female *Ceacam1*-/- mice exhibited significant decreases in serum TGs, high-density lipoproteins and cholesterol (Fig. 4.2.3.2B-D, panels b) at 12 mo of age. Since both the serum HDL and cholesterol decreased, the ratio HDL/cholesterol remained the same. At 3 mo of age and after a 4 h fast followed by a 2 g/kg glucose gavage, no significant differences were seen between the *Ceacam1*-/- and +/+ liver TGs, cholesterol and phospholipids levels (data not shown). However, *Ceacam1*-/- mice of 12 mo of age demonstrated significant differences relative to wild-type littermates in non-fasted total and esterified liver cholesterol (P = 0.02 respectively) (Fig. 4.2.3.3A panel a) and liver TGs (P= 0.005) (Fig. 4.2.3.3A panel b), suggesting that hepatocytes represent a major organ for lipid dysfunction in the *Ceacam1* knockout mice. Indeed, at 12-14 mo, the livers of the *Ceacam1*-/- mice demonstrated a trend towards increased fat vesicles (Fig. 4.2.3.3B and C), indicative of fatty livers and steatosis. This was confirmed with sudan-black staining specific for lipid vesicles (data not shown).

Figure 4.2.3.1: *Ceacam1*$^{-/-}$ mice are overweight.

On all graphs data is presented with F: females, M: males, F+M: both genders, the number of animals, n, is indicated below the graphs. A) Mouse weight at (a) 6 and (b) 12 months. Panel c: growth curves from 2 weeks to 28 weeks age. B) Livers were weighed at time of necropsy, when mice were (a) 6 or (b) 12 months old and represented as a percentage over total bodyweight. C) Visceral fat consisting mostly of gonadal fat pad were weighed from (a) 6 and (b) 12 month-old mice. Data represented as a percentage over total body weight. D) Food consumption of 4 month old mice was monitored over 56 days. E) Leptin adipokine was measured in serum of mice. $\Delta\, p<0.1$, * $p<0.05$, ** $p<0.01$.

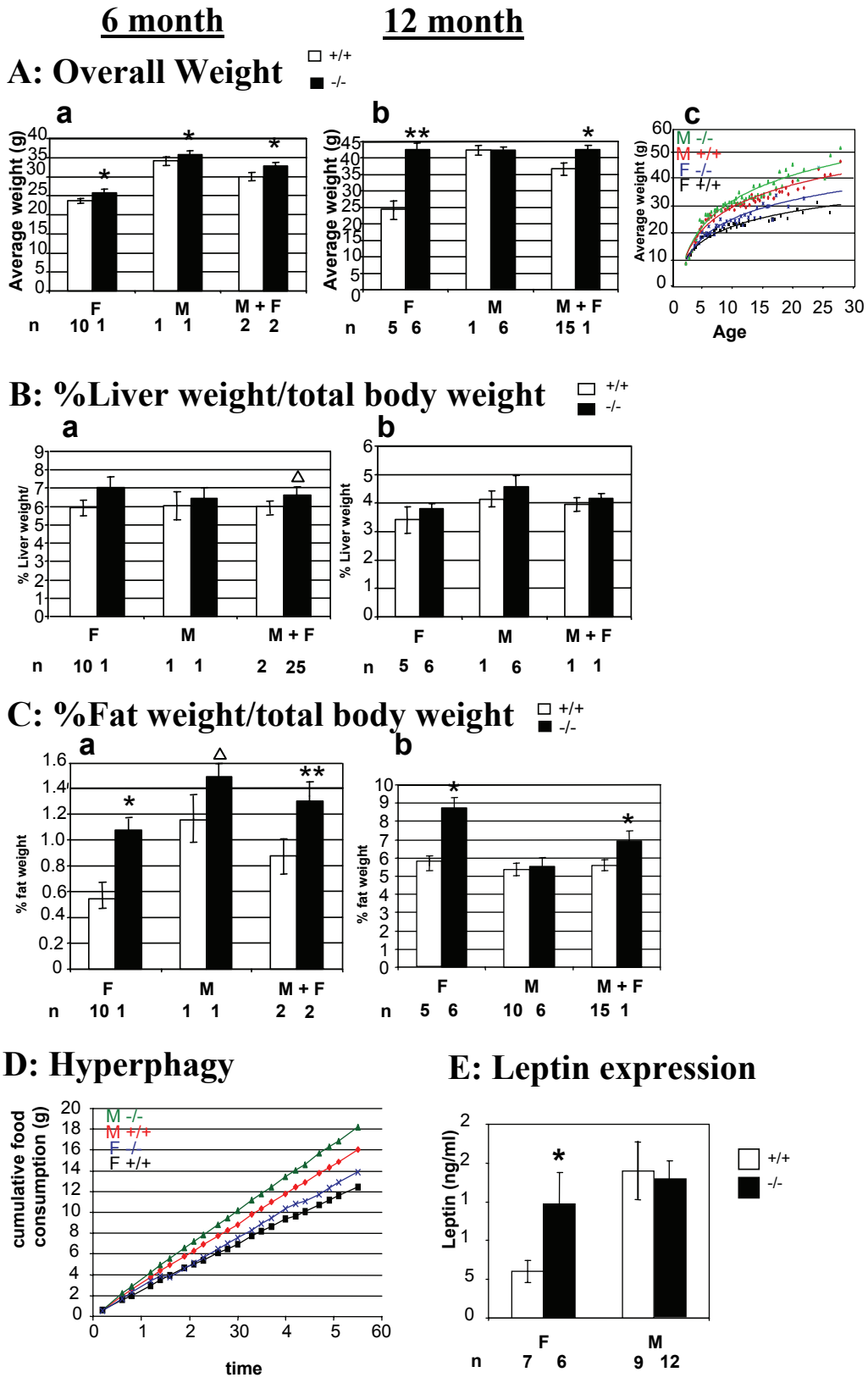

6 month
12 month
A: Overall Weight
+/+
-/-
a
b
c
Average weight (g)
F
M
M + F
n 10 1 1 1 2 2
n 5 6 1 6 15 1
M -/-
M +/+
F -/-
F +/+
Age
B: %Liver weight/total body weight
% Liver weight/
% Liver weight
n 10 1 1 1 2 25
n 5 6 1 6 1 1
C: %Fat weight/total body weight
% fat weight
n 10 1 1 1 2 2
n 5 6 10 6 15 1
D: Hyperphagy
cumulative food consumption (g)
time
E: Leptin expression
Leptin (ng/ml)
n 7 6 9 12

Figure 4.2.3.2: Serum lipid profiles in (a) 6 month and (b) 12 month old mice.

Mice were fasted for 16h. At necropsy, serum and organs were collected, the number of animals, n, is indicated below the graphs. A) Non-essential free fatty acids were measured. B) Serum triglycerides. C) Serum high density lipoproteins. D) Serum cholesterol. *p<0.05, **p<0.01

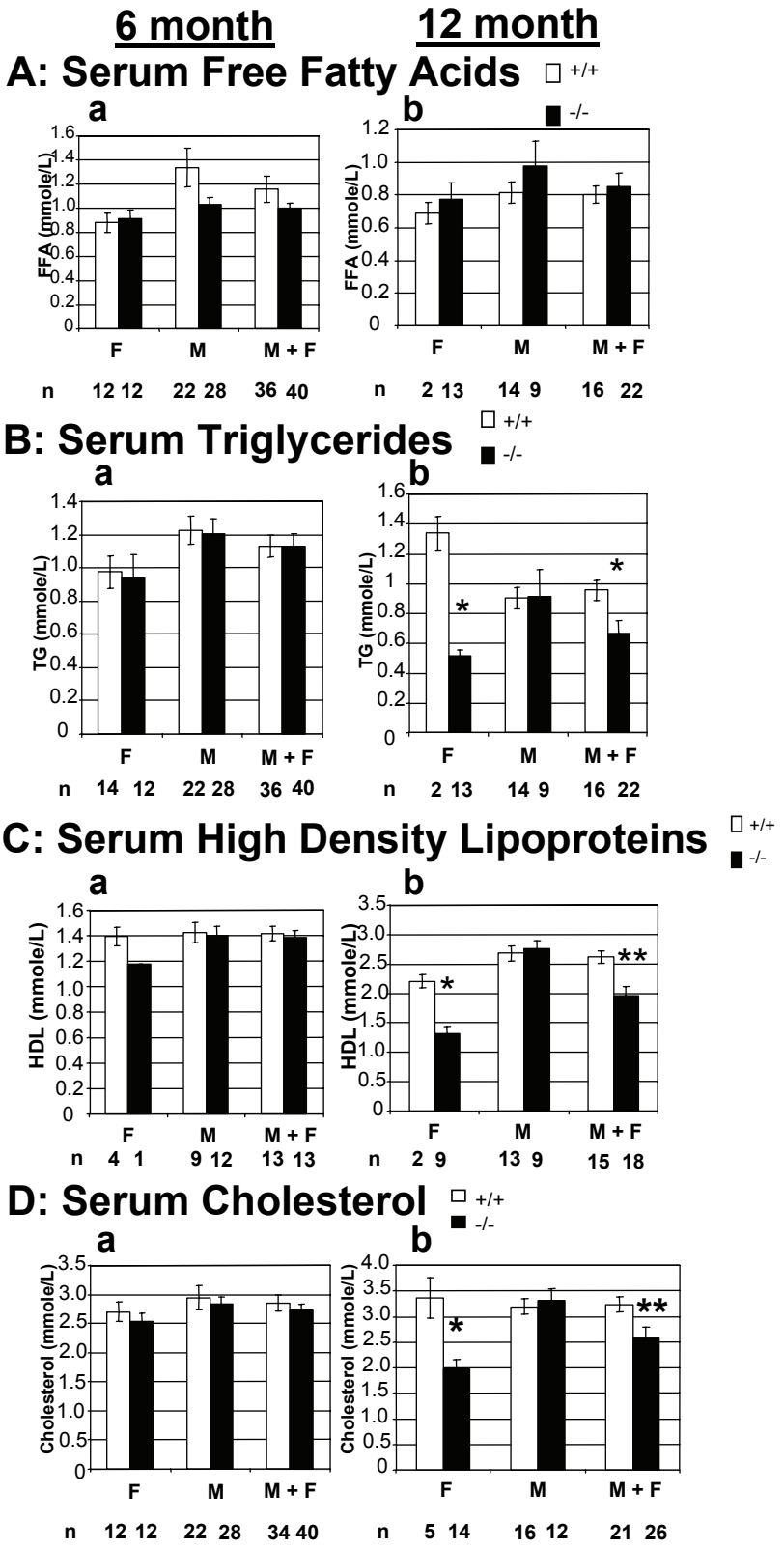
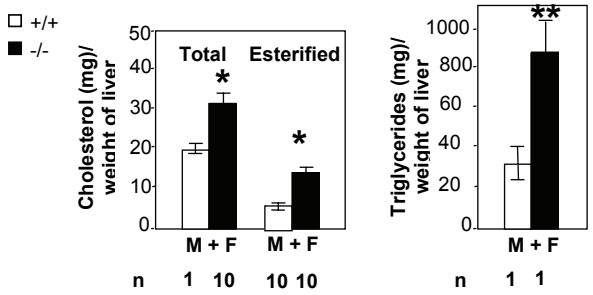

6 month
12 month
A: Serum Free Fatty Acids
+/+
-/-
a
b
FFA (mmole/L)
F
M
M + F
n 12 12 22 28 36 40
n 2 13 14 9 16 22
B: Serum Triglycerides
TG (mmole/L)
n 14 12 22 28 36 40
n 2 13 14 9 16 22
C: Serum High Density Lipoproteins
HDL (mmole/L)
n 4 1 9 12 13 13
n 2 9 13 9 15 18
D: Serum Cholesterol
Cholesterol (mmole/L)
n 12 12 22 28 34 40
n 5 14 16 12 21 26

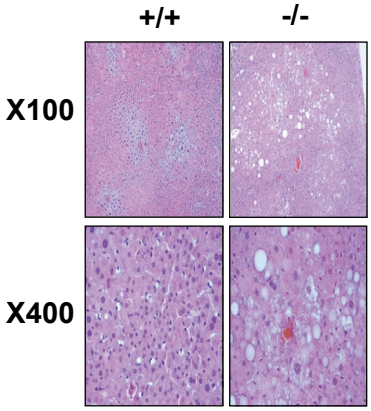
Figure 4.2.3.3: Lipids in livers of *Ceacam1*$^{-/-}$ mice.

A) (a) Total and esterified cholesterol and (b)triglycerides measured in the liver. B) Paraffin sections of liver sections stained with hematoxylin and eosin, seen at 100x and 400x magnification. C) Pathological grading of the degree of steatosis, where 0 indicates normal liver, 1 ballooning cells are present, 2 ballooning cells and small lipid inclusions, 3 ballooning cells and large lipid inclusions.

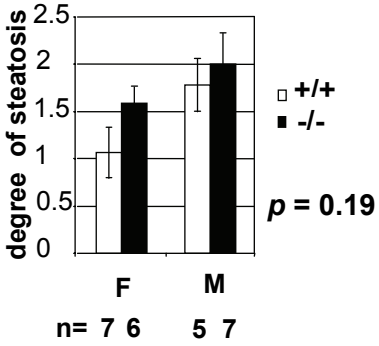
A: Liver Total/Esterified Cholesterol and



B:



C:



Biochemistry profiles were also evaluated on 3-5 mo old mice to gauge their liver and kidney functions. Assays were performed in duplicates on either blood collected from 16 h-fasted mice for analyses of liver performance or 24 h-urine samples collected in metabolic cages. As determined in Table 4.2.1, at 3 mo (urine) or 5 mo (blood), there were no significant differences in any of these parameters (Table 4.2.1) or in glucose, sodium, potassium, chloride, calcium, phosphorus and magnesium levels in the urine of the CEACAM1-deficient mice and their wild-type counterparts (data not shown). Liver enzyme measurements were repeated on a larger cohort of C57Bl/6 *Ceacam1*-/- and WT male and female mice at 6 and 12 mo of age. At 6 mo, male *Ceacam1*-/- mice had significantly higher levels of serum aspartate aminotransferase (ASP) and alkaline phosphatase (ALK) Fig. 4.2.3.4B and C, panel a), whereas alanine aminotransferase (ALT) levels were statistically similar (Fig. 4.2.3.4A, panel a). However, at 12 mo of age, all enzyme levels (ALT, ASP and ALK) were more elevated in *Ceacam1*-/- mice relative to their littermate controls (Fig. 4.2.3.4A-c, panel b), suggesting that the ablation of CEACAM1 leads to liver damage in the long term. Liver and kidney tissue sections were also examined and no important structural differences were noticeable up to 5 mo. As mentioned above, the livers of 12-14 mo *Ceacam1*$^{-/-}$ showed signs of fatty livers with lipid inclusions.

Table 4.2.1: Biochemistry of liver and kidney of WT and Ceacam1 -/- mice.

	WT	*Ceacam1-/-*
Liver function		
Alanine aminotransferase (U/L)	34.3±3.0	37.4±3.1
Aspartate aminotransferase (U/L)	129.4±15.2	140.6±14.6
Alkaline phosphatase (U/L)	64.1±5.2	70.4±3.4
Kidney function		
Urea (mmole/L)	767.4±453.1	853.2±676.0
Creatinine (□mole/L)	3561±1593	4360±2878

The levels of several enzymes were determined in duplicate experiments from fasting wild-type (WT) and CEACAM1-deficient (*Ceacam1*-/-) mice (n=5) at 5 mo. To evaluate kidney functions, the urine of 3 mo old mice was collected for

24 h in metabolic cages in duplicate experiments, and urea and creatinine were determined. There were no significant differences between the WT and *Ceacam1*-/- mice (P>0.05).

4.2.3.3 *Ceacam1*-/- mice are nor-insulinemic, but develop secondary insulin resistance.

CEACAM1 plays an important role in insulin metabolism and in insulin clearance from the liver (Poy 2002). We verified whether the *Ceacam1*-/- mice also showed deficiencies in insulin and glucose parameters by evaluating levels in plasma taken from 4-5 h fasted siblings at various time points. At 3, 6 or 12 mo of age, the fasting blood glucose levels and the plasma insulin levels were not statistically different between *Ceacam1*-/- and +/+ mice when blood was retrieved by either retro-orbital or tail-vein samplings (Fig. 4.2.3.5A and B panels a and b). Importantly however, preliminary data obtained in different conditions demonstrated that female *Ceacam1*-/- mice verified after a 6 h fast showed a slight but significant hyperinsulinemia (p<0.05, Fig. 4.2.3.5B, panel c) whereas the males did not (Fig. 4.2.3.5B, panel d). Even more important, the female *Ceacam1*-/- insulin levels sustained a 14 fold enhancement following a 20 min glucose gavage (p<0.005, Fig. 4.2.3.5B, panel c), in spite of the fact that only two individuals have so far been tested. The male *Ceacam1*-/- mice also showed increased plasma insulin levels post-prandially, but these values were not significant when compared to those of the WT mice examined in the same conditions (Fig. 4.2.3.5B, panel d). These new results emphasize that the phenotype is more predominant in female *Ceacam1*-/- mice under post-prandial conditions.

We also evaluated their glucose tolerance and insulin sensitivity. In glucose tolerance tests performed on 3 mo old male mice, there were no statistical differences in the levels of glucose (Fig. 4.2.3.5C panel a) or insulin concentrations (Fig. 4.2.3.5C panel b) measured after a 4 h fast and post-glucose IP delivery between the CEACAM1-deficient mice and their wild-type siblings. The only difference noticed was that glucose concentrations peaked for both *Ceacam1*-/- and +/+ mice at 15 min versus 60 min when blood was sampled via

Figure 4.2.3.4: Liver function enzyme measurements.

Liver enzymes were measured from serum of 6 month and 12 month-old mice. A) Serum Alanine aminotransferase, a greater difference between +/+ and -/- is exhibited in 12 month old mice. B) Serum Aspartate aminotransferase, at 6 and 12 months a tendency is detected in which the -/- mice have levels more elevated than +/+ mice. C) Serum alkaline phosphatase levels have a tendency to be more elevated in -/- mice than +/+ mice. $\Delta\, p<0.1$, * $p<0.05$, ** $p<0.01$.

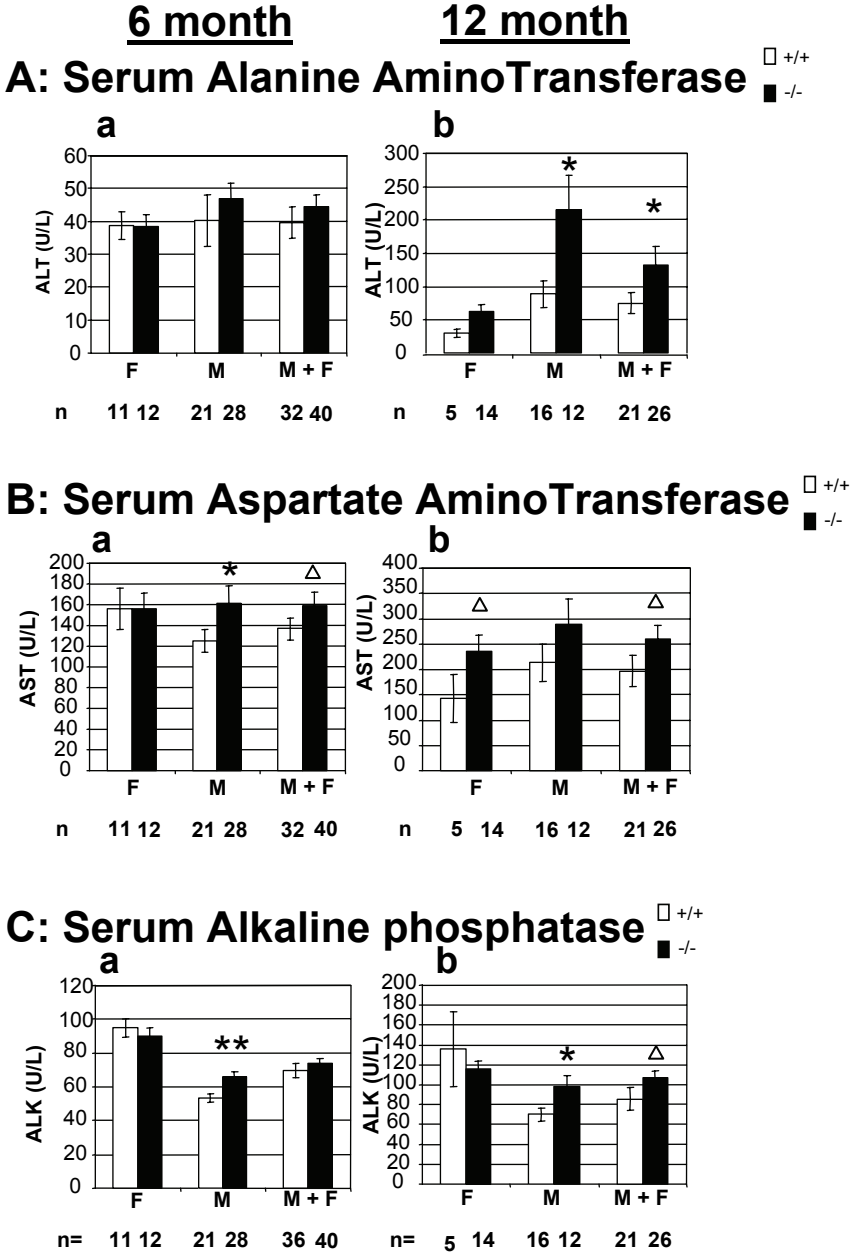
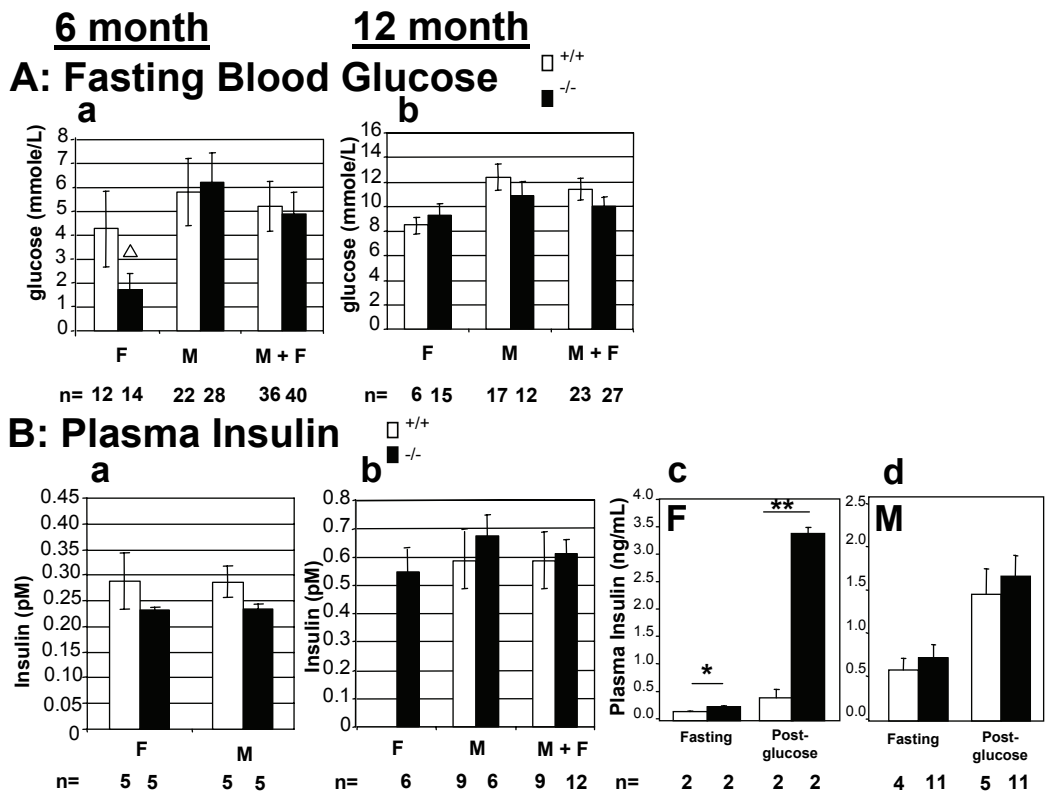
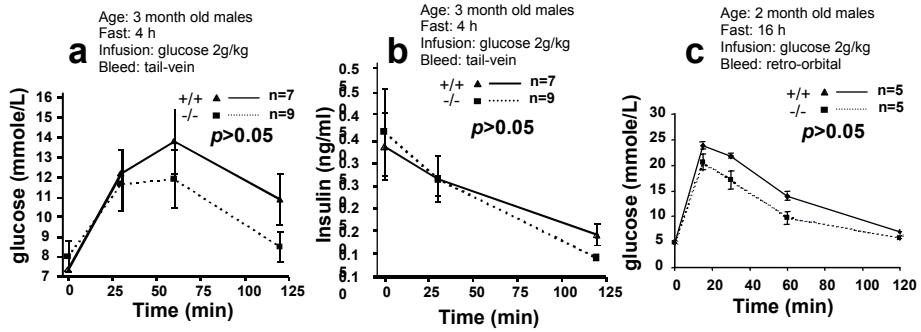

6 month
12 month
A: Serum Alanine AminoTransferase
+/+
-/-
a
b
ALT (U/L)
F
M
M + F
n 11 12 21 28 32 40
n 5 14 16 12 21 26
B: Serum Aspartate AminoTransferase
AST (U/L)
C: Serum Alkaline phosphatase
ALK (U/L)
n= 11 12 21 28 36 40
n= 5 14 16 12 21 26

Figure 4.2.3.5: Glucose homeostasis.

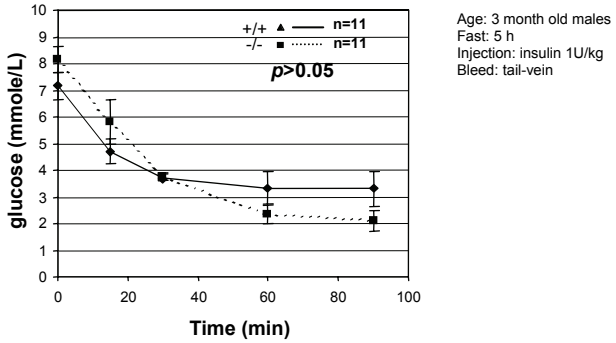
A) Blood glucose was measured after mice were fasted for 16 h. No significant differences were found between the +/+ and -/- mice. B) Plasma insulin levels were also measured. No significant differences were found between the +/+ and -/- fasted mice. Insulin levels post-prandial in (c) and (d). Significant differences are found in the females +/+ vs -/-, but no differences were found in the males. C) Glucose tolerance curves. Mice were fasted for 4h and given a dose of glucose either by IP injection or gavage. Panel (a): glucose tolerance curve. Panel (b): insulin levels of the same mice used in the glucose tolerance test. Panel (c): glucose tolerance test on mice fasted for 16 h and sampled by retro-orbital bleed. D) For the insulin tolerance test, male mice were fasted 5 h and injected with 1U/kg of insulin right after time 0, blood sampling was from the tail vein.
Data in bar graphs are mean ± SEM. $\Delta\ p< 0.1$, * $p<0.05$, *** $p<0.001$



C: Glucose Tolerance Tests



D: Insulin Tolerance Tests



Age: 3 month old males
Fast: 5 h
Injection: insulin 1U/kg
Bleed: tail-vein

the retro-orbital sinus (Fig. 4.2.3.5C panel c) as compared to bleeding from the tail vein (Fig. 4.2.3.5C panel a). Route of glucose delivery (gavage versus IP injection) may also be an important factor for differences. Insulin sensitivity was measured in 3 mo old male mice by intraperitoneal insulin tolerance tests after injection of 1U/kg of insulin (Fig. 4.2.3.5D). In these conditions, the male CEACAM1-null mice were as insulin sensitive as their wild-type siblings. These measurements will soon be followed up in CEACAM1-negative females.

As no significant differences were noticed between the *Ceacam1* gene targeted mice and their wild-type littermates at various ages under fasting conditions, we fed a small number of male and female 3 mo old mice (n=5) with a high fat high-sucrose diet for 2 mo and compared their weights, lipid profiles and glucose and insulin concentrations after this time period. Both *Ceacam1*-/- and WT male and female mice fed on a high fat diet were significantly heavier than mice fed a standard chow (P=0.001-0.008, Fig. 4.2.3.6A, panel a). However, both male and female *Ceacam1*-/- mice also showed a trend towards increased weight on the high fat diet relative to the wild-type mice (Fig. 4.2.3.6A panel a, P = 0.08 for females and P = 0.1 for males). The increase in weight between mice fed on a high-fat/high-sucrose diet versus those on a regular diet was not due to augmentation of liver weight (Fig. 4.2.3.6A, panel b), but more so to the significantly elevated fat weight (P=0.001-0.04, Fig. 4.2.3.6A, panel c). *Ceacam1*-/- males showed a significant reduction in the levels of serum FFAs when fed a high fat diet (Fig. 4.2.3.6B, panel a), whereas *Ceacam1*-/- females on a regular diet demonstrated a trend towards lower serum TGs and total cholesterol (Fig. 4.2.3.6B, panels b and c), as had been noticed in *Ceacam1*-/- females at 12 mo of age (Fig. 4.2.3.2B and D, panel c). Interestingly, *Ceacam1*-/- females fed a high fat diet exhibited a trend towards higher total serum cholesterol (Fig. 4.2.3.6B, panel c). In this small cohort of mice (n=5) as compared to a larger one (Fig. 4.2.3.5A, panel b), *Ceacam1*-/- males fed a regular diet were significantly hyperglycemic but had lower levels of plasma insulin (Fig. 4.2.3.6C, panels a and b). A similar trend in lowered insulin levels was observed in *Ceacam1*-/- female mice fed a regular diet. There were no significant differences however, in fasting

glucose and plasma insulin levels in WT vs *Ceacam1*-/- mice fed on a high fat diet (Fig. 4.2.3.6C, panels a and b). As had been noticed previously (Fig. 4.2.3.4), *Ceacam1*-/- female mice fed a regular diet exhibited signs of liver damage as measured by the significantly increased levels of ALT, AST and ALK (Fig. 4.2.3.6D, panels a-c). Strikingly, neither male nor female *Ceacam1*-/- mice fed the higher fat diet showed significant differences with their littermate controls in the levels of these enzymes, although overall levels remained high for ALT and AST (Fig. 4.2.3.6D, panels a and b). Overall, these results demonstrate that, in this small cohort of mice (n=5), *Ceacam1*-/- mice fed with a high fat diet exhibited only minor changes in weight, fat weight and cholesterol as compared to those evaluated in mice fed a regular chow. These results will be confirmed using a larger cohort of mice placed under various fasting and post-prandial conditions at a later time point.

The gold standard to define insulin resistance is a technique known as euglycemic clamps studies. This consists in the measurements of the amount of glucose needed to be infused through a canula placed in the carotid artery and jugular vein of the mice to maintain euglycemia of 6-7 mM upon an infusion of by 4 mU /kg /min of human insulin. As observed on Fig. 4.2.3.7 and Table 4.2.2, *Ceacam1*$^{-/-}$ males fed on a standard diet require less glucose (Fig. 4.2.3.7A, Glucose Infusion Rate (GIR) = 49.9 ± 2.6 mg/kg/min) than their wild-type littermates (Fig. 4.2.3.7A: GIR = 58.7 ± 3.5 mg/kg/min)($p<0.05$) to maintain euglycemia, and are therefore resistant to the actions of insulin. This was also confirmed with mice fed on a high fat/high sucrose (HF) diet for 8 weeks (Fig. 4.2.3.7A). In this case, both the WT and *Ceacam1*$^{-/-}$ mice required less glucose than those fed a normal diet, but the *Ceacam1*$^{-/-}$ mice on this diet exhibited an even lower amount (23.6 ± 2.1mg/kg/min) than their WT counterparts (36.8 ± 3.1 mg/kg/min, $p<0.05$). Glucose Infusion Rates in the *Ceacam1*$^{-/-}$ mice on HF were lowered by approximately 50% compared to those fed a normal diet ($p<0.05$). These results therefore indicate that *Ceacam1*$^{-/-}$ mice do exhibit whole body insulin resistance in spite of their lack of hyperinsulinemia in the fasting state.

Figure 4.2.3.6: Parameters on mice fed a high fat diet.

Mice were fed standard diet (S) or a high fat diet (HF) for 2 months. A) At the end of regimen, total body, liver and fat was weighed. Animals fed the HF diet gained significant amount of weight compared to animals on S chow, and this correlates with increases in the percentage of fat. B) Serum FFA, TG, and total cholesterol were measured. (a): -/- males show a significant reduction in the levels of serum FFAs on HF diet. (b): -/- females on S chow show a trend towards lower serum TGs. (c): -/- females on S chow show a trend toward lower total cholesterol, -/- females on HF diet exhibit a trend towards higher total serum cholesterol. C) (a): fasting glucose levels. HF diet induces hyperglycemia in male mice. (b): fasting plasma insulin levels. D) Enzymes indicators of hepatic function were tested. (a): ALT. (b): AST. (c): ALK. Δ $p<0.1$, * $p<0.05$, ** $p<0.01$.

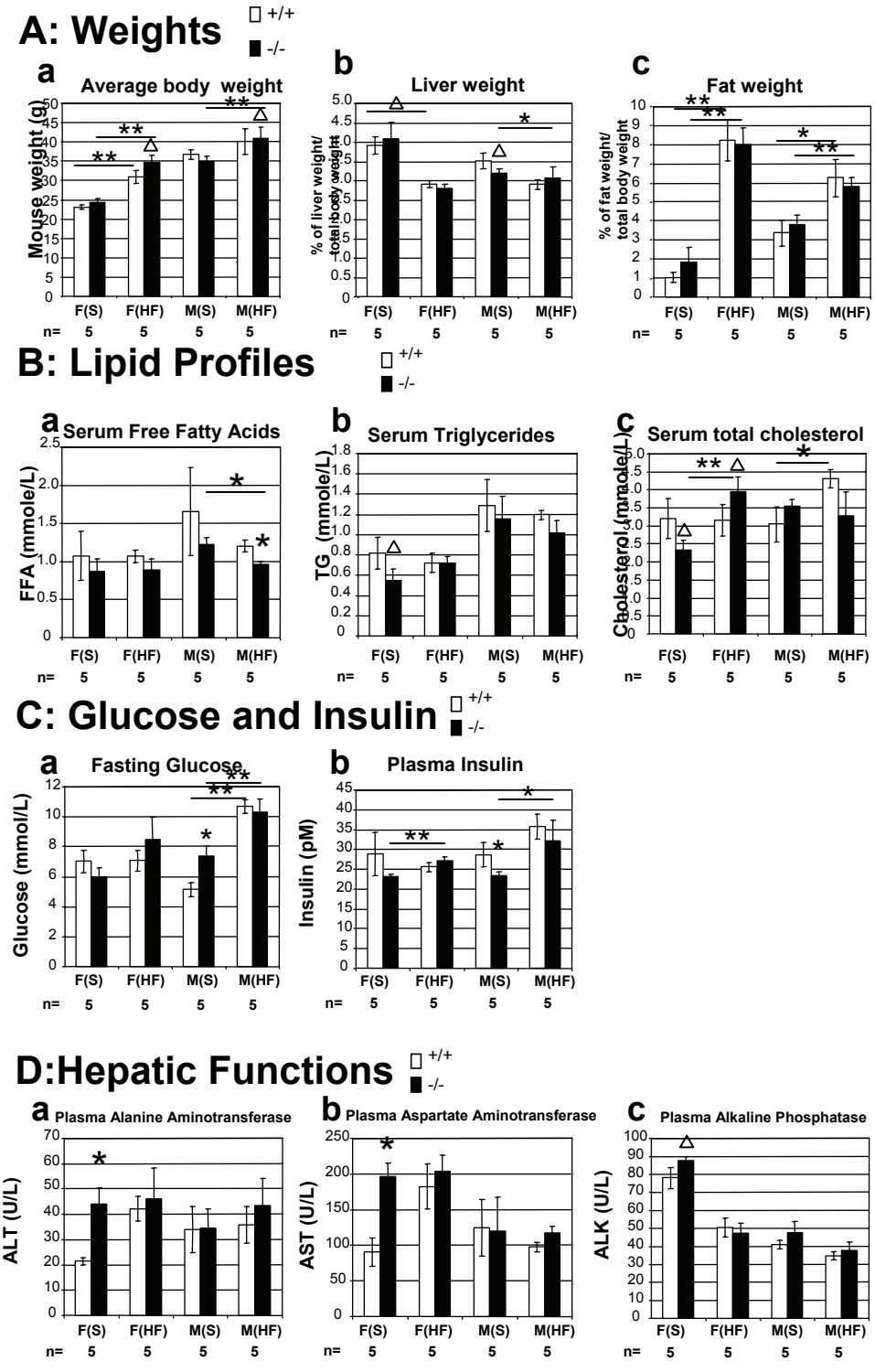

A: Weights
+/+
-/-
a
Average body weight
Mouse weight (g)
b
Liver weight
% of liver weight/ total body weight
c
Fat weight
% of fat weight/ total body weight
F(S) F(HF) M(S) M(HF)
n= 5 5 5 5
B: Lipid Profiles
a
Serum Free Fatty Acids
FFA (mmole/L)
b
Serum Triglycerides
TG (mmole/L)
c
Serum total cholesterol
Cholesterol (mmole/L)
C: Glucose and Insulin
a
Fasting Glucose
Glucose (mmol/L)
b
Plasma Insulin
Insulin (pM)
D:Hepatic Functions
a
Plasma Alanine Aminotransferase
ALT (U/L)
b
Plasma Aspartate Aminotransferase
AST (U/L)
c
Plasma Alkaline Phosphatase
ALK (U/L)

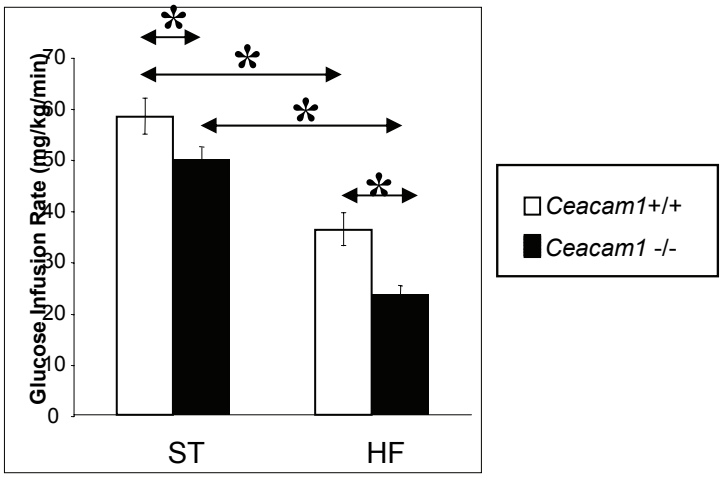
Table 4.2.2: Basal and clamp characteristics in *Ceacam1*$^{+/+}$ and *Ceacam1*$^{-/-}$ mice fed with either a standard or a high-fat high-sucrose diet.
Values are for 5h-fasted, conscious, unrestrained mice at least 5 days following surgical catheterization. Data are mean ± SEM. Δ $p< 0.05$ vs standard-fed counter-part ^{o} $p < 0.05$ vs Ceacam1 +/+

	Standard Diet		*High Fat Diet*	
	Ceacam1$^{+/+}$	*Ceacam1*$^{-/-}$	*Ceacam1*$^{+/+}$	*Ceacam1*$^{-/-}$
n	6	5	5	6
Body weight (g)	39 ± 2	43 ± 1	47 ± 2 $^{\Delta}$	50 ± 1 $^{\Delta}$
Glucose (mg/dl)				
Basal	6.4 ± 0.4	6.8 ± 0.2	6.4 ± 0.4	6.3 ± 0.3
Clamp	6.6 ± 0.1	6.7 ± 0.1	6.8 ± 0.2	6.7 ± 0.1
Insulin (uU/ml)				
Basal	5.4 ± 0.1	6.6 ± 0.2	7.3 ± 0.4	12.4 ± 1.1 $^{\Delta, o}$
Clamp	54.7 ± 4.3	58.1 ± 5.5	57.3 ± 4.3	60.7 ± 10.4
GIR (mg/kg/min)	58.7 ± 3.5	49.9 ± 2.6 ^{o}	36.8 ± 3.1 $^{\Delta}$	23.6 ± 2.1 $^{\Delta, o}$
Hepatic glucose production (mg/kg/min)				
Basal	18 ± 2	18 ± 1	21 ± 1	22 ± 1
Clamp	0 ± 1	3 ± 1 ^{o}	2 ± 1 $^{\Delta}$	5 ± 1 $^{\Delta, o}$
Hepatic Insulin Action (% Suppression of Basal HGP)	98 ± 1	84 ± 1 ^{o}	89 ± 2 $^{\Delta}$	77 ± 1 $^{\Delta, o}$
Glucose Uptake (mg/kg/min)				
Basal	17 ± 2	17 ± 1	18 ± 1	18 ± 1
Clamp	40 ± 2	34 ± 3	26 ± 2 $^{\Delta}$	25 ± 1 $^{\Delta}$

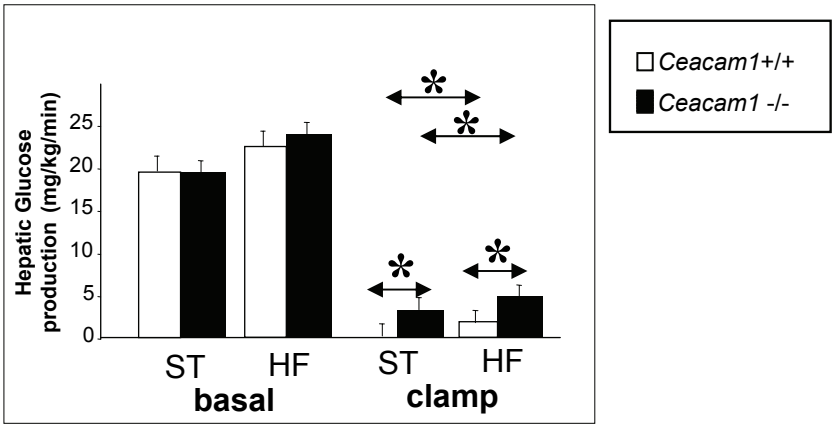
Figure 4.2.3.7: Euglycemic clamp measurements.

A) Clamp measurements were done on mice fed a standard diet (ST) of a high fat diet (HF). Mice are injected with a low dose of insulin and continuously infused with glucose to maintain euglycemia. -/- mice require less glucose infusion than +/+ mice both on ST or HF diet. B) Hepatic glucose production at basal state is not different in -/- and +/+ mice. During the clamp measurement, low dose insulin is injected, -/- mice have an elevated hepatic glucose production. C) Glucose uptake by muscle tissue. Under clamp conditions, animals on HF diet have lower glucose uptake. Data are mean ± SEM. * $p<0.05$.

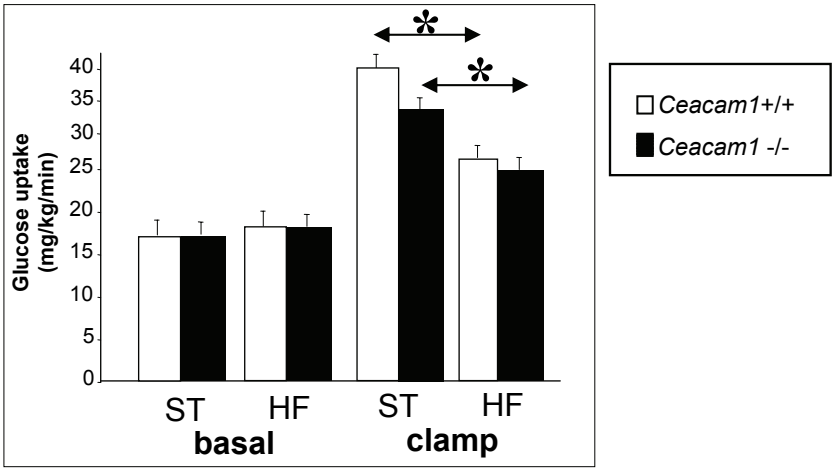
A. Whole body euglycemic clamp measurements



B. Hepatic glucose production



C. Glucose Uptake



Using the clamp technique also gives indications as to hepatic and muscle insulin resistance. No differences between the *Ceacam1*+/+ and -/- mice were noticed in hepatic glucose production in basal conditions when fed either a standard or high fat diet (Fig. 4.2.3.7B). However, there was a slight increase in *Ceacam1*$^{-/-}$ males as compared to *Ceacam1*+/+ males in hepatic glucose output under clamp conditions whether fed a standard or high fat diet (p<0.05, Fig. 4.2.3.7B). These results indicate that the *Ceacam1*$^{-/-}$ males also demonstrate hepatic insulin resistance. However, no such changes were noticed in glucose uptake by the muscles under clamp conditions (Fig. 4.2.3.7C), suggesting that liver insulin resistance is mainly responsible for the whole body insulin resistance measured.

4.2.3.4 Disruption of gene expression patterns in *Ceacam1*$^{-/-}$ liver

The metabolic effects of obesity and insulin resistance of *Ceacam1*$^{-/-}$mice fed a standard chow only became overtly apparent as the mice aged (> 6 months). However, as it was likely that a number of genes might be responding to this metabolic imbalance at an earlier time point, we examine a gene expression array and compared the expression of mRNAs modulated in the liver of WT and *Ceacam1*$^{-/-}$ 3 month old mice. For this experiment, the livers of male mice on a homogeneous 129Sv background were retrieved and processed for RNA extractions. After proceeding with reverse transcription, the cDNA targets were hybridized to the Affymetrix Mouse MOE430A GeneChips. Genes either downregulated (Table 4.2.3) or overexpressed (Table 4.2.4) by more than fourfold were selected for pathway analyses. Thirteen genes were decreased in expression of which the two first were *Ceacam1* and *Ceacam2* showing a decrease of at least 6 fold in expression. Other than *Ceacam1*, pathway analyses indicated that 6 of these genes were major contributors to metabolism (yellow shading in Table 4.2.3). In particular, *Hsd17b9*, *Socs2* and *Foxa3* are either involved as a quantitative trait loci for diabesity (*Hsd17b9*) (Brown, Olver et al. 2005), as a gene that limits growth hormone action on body and organ growth (*Socs2*)

(Michaylira, Simmons et al. 2006), or as an important transcription factor (*Foxa3*) that regulates the pro-glucagon gene and consequently gluconeogenesis as well as the development of steatosis (Liu, Shen et al. 2002; Cui, Hosui et al. 2007). Other genes were involved in detoxification and proteolysis.

Strikingly, 28 genes were overexpressed in *Ceacam1*$^{-/-}$ livers. In this case, 7 of these genes (orange shading in Table 4.2.4) were all part of the same pathway, shown in Fig. 4.2.3.8, as either participating in the biosynthesis of TG/phospholipids or cholesterol. These genes are all transcriptionally activated by either the sterol regulatory element binding proteins (SREBP) 1c or 2 (Brown and Goldstein 1997; Edwards 2000). We therefore proceeded to the validation of the genes expressed in this pathway. mRNAs from 4 or 6 month old, 5 h-fasted C57Bl/6 WT or *Ceacam1*$^{-/-}$ sibling livers (backcross 16, n = 2-3) were extracted and evaluated for quantitative expression of the 7 highlighted "orange" genes using qRT-PCR techniques. As seen in Table 4.2.5, many of the mRNAs for these genes are over-expressed at least 2 fold in the *Ceacam1*$^{-/-}$ livers, although statistical significance has not yet been reached in the small cohort of mice analyzed. In particular, the acetyl CoA-carboxylase that had not been detected in the chip assay was overexpressed either 3.6 or 6.8 fold in the 4 or 6 month-old *Ceacam1*$^{-/-}$ liver samples. Additionnally, the glycerol-3-phosphate acyltransferase and the fatty acid synthase mRNAs were overexpressed 2.6-4.7 and 1.6 fold in these samples (Table 4.2.5). These results demonstrate that the systemic elimination of the *Ceacam1* gene from the mouse results in severe disruption in lipidogenic gene expression profiles, consequent with increased adiposity and insulin resistance.

In addition to the genes involved in this pathway, many other genes active in metabolism (yellow shading in Table 4.2.4) or in sulfate and/or methione metabolism (green shading in Table 4.2.4) were significantly overexpressed. Of note, the Mknk2 kinase that is crucial for phosphorylation of the eIF4e translation initiation factor (Ueda, Watanabe-Fukunaga et al. 2004) is also abundant in this mouse model.

Table 4.2.3: list of genes decreased by 4-6 fold in *Ceacam1*$^{-/-}$ mouse liver.

Fold Change	*Name of Gene*	*Symbol*	*Accession*	*Function*
-6	hydroxysteroid (17-beta) dehydrogenase 9	Hsd17b9	NM_013786	metabolism,
-4	vanin 1	Vnn1	NM_011704	nitrogen metabolism, response to oxidative stress
-4	aminolevulinic acid synthase 1	Alas1	NM_020559	metabolism
-4	suppressor of cytokine signaling 2	Socs2	NM_007706	regulation of body size, metabolism
-4	cysteine sulfinic acid decarboxylase	Csad	NM_144942	cysteine catabolism to taurine, metabolism
-4	forkhead box A3	Foxa3	NM_008260	liver-specific transcription factor, glucose homeostasis
-6	CEA-related cell adhesion molecule 2	Ceacam2	NM_007543	cell adhesion
-6	CEA-related cell adhesion molecule 1	Ceacam1	NM_001039185	cell adhesion/immunomodulatorinsulin clearance/angiogenesis
-5	highly similar to Cytochrome P450 4A3		AK098088	
-5	cathepsin C	Ctsc	NM_009982	proteolysis and peptidolysis
-4	heat shock protein 1B	Hspa1b	NM_010478	response to heat, cell survival
-4	cytochrome P450, family 4	Cyp4a10	NM_010011	detoxification, electron transport, obesity
-4	poliovirus receptor-related 3, nectin	Pvrl3	NM_021495	cell adhesion molecule

Table 4.2.4: List of genes increased 4-6 fold in *Ceacam1*$^{-/-}$ mouse liver

Fold Change	*Name of Gene*	*Symbol*	*Accession*	*Function*
6	thyroid hormone responsive SPOT14	Thrsp	NM_009381	nuclear protein, lipd synthesis
5	7-dehydrocholesterol reductase	Dhcr7	NM_007856	cholesterol biosynthesis
4	acetyl-Coenzyme A synthetase 2 (ADP forming)	Acas2	NM_019811	fatty acid biosynthesis
4	stearoyl-Coenzyme A desaturase 1	Scd1	NM_009127	fatty acid biosynthesis, insulin sensitivity
4	fatty acid synthase	Fasn	NM_007988	fatty acid biosynthesis
4	long chain fatty acyl elongase	Elovl	NM_130450	fatty acid biosynthesis
4	glycerol-3-phosphate acyltransferase	Gpam	NM_008149	fatty acid biosynthesis
6	proline dehydrogenase	Prodh	NM_011172	metabolism
5	farnesyl diphosphate synthetase	Fdps	NM_134469	isoprenoid biosynthesis, fatty liver dystrophy
5	carboxylesterase 2	Ces2	NM_145603	metabolism, HNF-4 responding gene
4	aldehyde dehydrogenase 1 family, member B1	Aldh1b1	NM_028270	metabolism
4	histocompatibility 2, class II antigen A, alpha	H2-Aa	NM_010378	antigen presentation, linked to obesity
4	dicarbonyl L-xylulose reductase	Dcxr	NM_026428	NADPH metabolism, xylulose metabolism
4	sulfotransferase family 1A, phenol-preferring	Sult1a1	NM_133670	steroid metabolism, obesity
4	3'-phosphoadenosine 5'-phosphosulfate synthase 2	Papss2	NM_011864	metabolism, sulfate assimilation
4	thiosulfate sulfurtransferase	Tst	NM_009437	metabolism, sulfate transport
4	betaine-homocysteine methyltransferase	Bhmt	NM_016668	metabolism, methionine biosynthesis, diabesity
4	N-sulfotransferase	Sultn	NM_016771	metabolism, sulfate assimilation
4	MAP kinase-interacting serine/threonine kinase 2	Mknk2	NM_021462	regulation of protein biosynthesis
5	orosomucoid 1	Orm1	NM_008768	acute-phase response, transport
5	ESTs, promyelocyte leukemia Zn finger protein			
4	cytochrome P450, family 2, subfamily a, peptide 5	Cyp2a5	NM_007812	electron transport, USF responding gene
4	B-cell leukemia/lymphoma 6	Bcl6	NM_007528	transcriptional repressor
4	hemoglobin alpha, adult chain 1	Hba-a1	NM_008218	oxygen transport
4	peroxisomal membrane protein 2	Pxmp2	NM_008993	peroxisome organization and biogenesis
4	hemoglobin, beta adult major chain	Hbb-b1	NM_008220	oxygen transport
4	agmatine ureohydrolase (agmatinase)	Agmat	NM_001081408	polyamine biosynthesis
4	leukocyte cell-derived chemotaxin 2	Lect2	NM_010702	chemotaxis, inflammation

4.2.4 Discussion

In this report, we have examined how the deletion of the *Ceacam1* gene impacts on the metabolic parameters relating to insulin action. A previous report (Poy 2002) confirmed the role of CEACAM1 in insulin clearance; the genetically modified mouse (L-SACC) in that case resulted from the significant over-expression of a hepatocyte-specific mutant of the CEACAM1-L isoform, thus creating a dominant negative effect on the endogenous gene. In this case, the functional elimination of CEACAM1 was strictly in the liver, although phenotypic effects were also observed in other insulin-sensitive organs such as the muscle and most possibly the adipocytes (Poy 2002). The mutation converted the conserved Ser503 residue to a non-phosphorylatable Ala, thus preventing the insulin-induced Tyr phosphorylation of Tyr488 within the cytoplasmic domain. As the Tyr488 phosphorylation is key to internalization of the insulin receptor (Choice 1998), preventing this phosphorylation resulted in mice deficient in recycling of the insulin receptor and showing hyperinsulinemia, random hyperglycemia, secondary insulin resistance, visceral adiposity and increased plasma free fatty acids and plasma and hepatic triglycerides (Poy 2002). The phenotypes of the L-SACC mice were mostly studied between the ages of 2-6 months of age. However, CEACAM1 is also expressed in many other epithelial compartments as well as in hematopoietic and endothelial cells. Because inflammation and endothelial dysfunctions influence metabolic parameters, we questioned whether the effects were the same as those described above in a mouse model having sustained the full deletion of the *Ceacam1* gene.

We report that the *Ceacam1*$^{-/-}$ mice also have a slight tendency to obesity, mostly apparent at 6 and 12 months of age and this phenotype is enhanced in females. The weight of their livers is enhanced, as is their visceral adiposity. The CEACAM1-null mice are hyperphagic and the female mice in particular secrete more leptin into the circulation. Contrary to the L-SACC mice, the *Ceacam1*$^{-/-}$ mice did not produce more plasma FFAs. However, the 12-month old female mice had a significant reduction of plasma triglycerides, high-density lipoproteins and serum cholesterol whereas the males did not exhibit this difference. This

Figure 4.2.3.8: Pathway of mono-unsaturated fatty acid and cholesterol synthesis.

SREBP1-c controls fatty acid synthesis. Genes that were upregulated in the *Ceacam1*$^{-/-}$ mice were part of this synthesis pathway. Fold overexpression indicated beside the enzyme name. SREBP2 controls cholesterol synthesis. Only the last gene in the production chain was found to be overexpressed in the *Ceacam1*$^{-/-}$ mouse livers.

GENES REGULATED BY

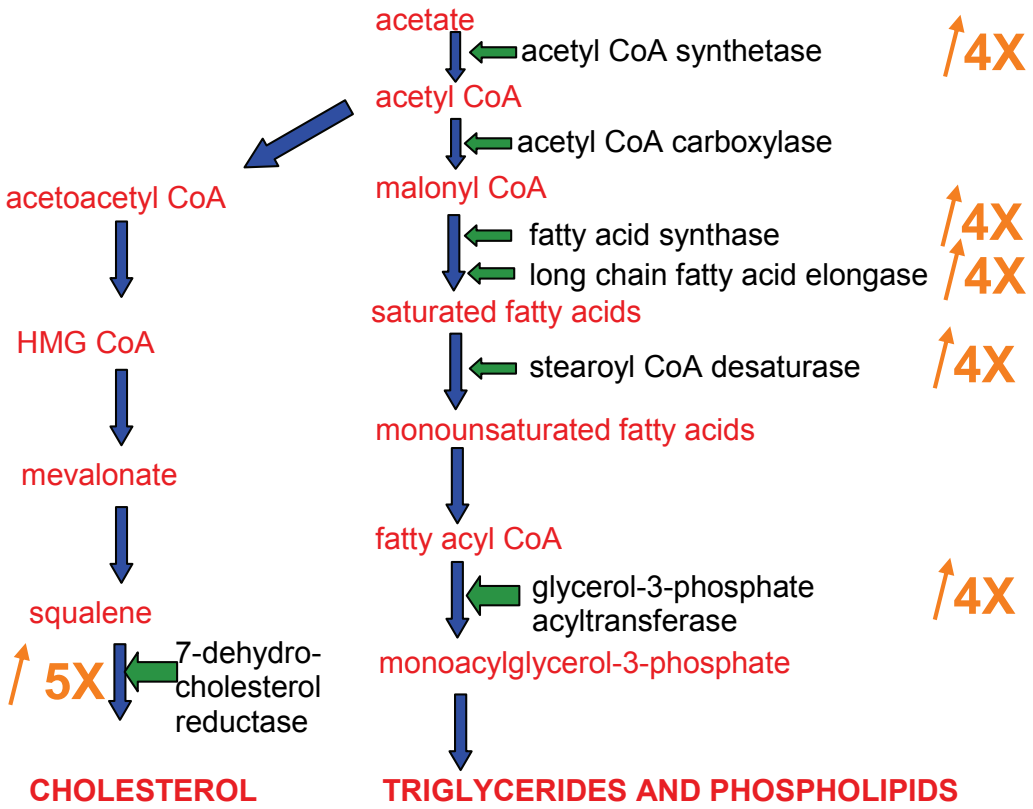


Table 4.2.5: Validation of Chip Analysis in WT and *Ceacam1*$^{-/-}$ Mice.

4 month-old mice. Liver taken after a 5 h fast.

Enzymes	*WT*	*standard error*	*CC1 KO*	*standard error*	*p-value*	*fold change*
CoAs	6.73	1.21	21.6	14.78	0.19	3.2
CoAC	0.96	0.12	3.44	1.39	0.11	3.6
FAS	1.84	0.80	2.98	0.65	0.19	1.6
FAE	0.95	0.11	2.63	0.59	0.05	2.8
StCoA	18.5	7.12	23.1	2.60	0.30	1.2
GPAT	0.71	0.12	3.35	2.56	0.19	4.7
7DHCR	1.92	0.14	3.18	0.53	0.07	1.6

6 month-old mice. Liver taken after a 5 h fast.

Enzymes	*WT*	*standard error*	*CC1 KO*	*standard error*	*p-value*	*fold change*
CoAs	2.29	0.81	3.12	0.62	0.23	1.4
CoAC	0.72	0.29	4.88	2.61	0.18	6.8
FAS	6.61	1.19	2.22	0.30	0.03	-3.0
FAE	0.38	0.18	0.58	0.25	0.27	1.5
StCoA	5.76	1.32	42.2	27.56	0.21	7.3
GPAT	0.54	0.40	1.39	0.40	0.12	2.6
7DHCR	2.96	1.20	3.81	0.87	0.30	1.3

implies a sexual dimorphism in the phenotype, which had previously been noted in the L-SACC mice where females also developed hyperinsulinenia, increased visceral adiposity and elevated FFAs and TGs (Dai 2004).

In comparison to plasma values, in *Ceacam1*$^{-/-}$ mice of 12 months of age, total and esterified liver cholesterol and TGs were elevated suggesting a possible defect in cholesterol transport, confined to the liver of these mice and unable to shuttle to the serum. These increased lipids become apparent in liver sections with increased lipid vacuoles and steatosis as seen in Fig. 4.2.3.3. This was only seen in liver sections of one-year old *Ceacam1*$^{-/-}$ mice though, suggesting a late onset on appearance. However, dysregulation of lipidogenesis enzyme expression was evident at 3 months of age as revealed by the expression arrays (Tables 4.2.3 & 4.2.4) and the chip validation data (Table 4.2.5). Seven of the enzymes controlled by either the SREBP-1c or SREBP2 transcriptional modulators had significantly enhanced expression in 3 month-old liver samples. Importantly, in addition to increased fatty acid synthase (FAS) expression described in this report, CEACAM1 has been shown to associate directly with FAS and regulate its activity upon insulin receptor activation. In fact, *Ceacam1*$^{-/-}$ mice exhibit very

high levels of FAS activity (Najjar 2005), thereby compounding the effect of increased FAS expression and activity. Besides the over-expressed genes, a number of interesting genes relating to metabolism and inflammation are also decreased (Table 4.2.3). Of note, the gene most decreased, *Hsd17b9*, is considered a quantitative trait loci for diabesity (Brown, Olver et al. 2005). As well, *Socs2* (Suppressor Of Cytokine Signaling 2), a target gene of the *Stat5* transcription factor, is significantly decreased and likely involved in hepatosteatosis and the development of fatty livers in *Stat5*-/- mice (Cui, Hosui et al. 2007). There is also a decrease in the liver-specific *Foxa3* transcription factor that has been described as interacting with the proglucagon gene while not being essential for its expression; *Foxa3*-/- mice show mild hypoglycemia during a prolonged fast (Liu, Shen et al. 2002). The *vanin 1* (*Vnn1*) gene has been associated with response to oxidative stress and inflammatory process (Martin, Penet et al. 2004). Therefore, lipidogenic and inflammatory transcriptional programs are significantly dysregulated in *Ceacam1*$^{-/-}$ 3 mo-old mice. These alterations are important enough that, by 6 or 12 months of age, the alanine- and aspartate-aminotransferase and alkaline phosphatase markers, indicative of liver dysfunction, are elevated in serum.

One of the major differences between the *Ceacam1*$^{-/-}$ and L-SACC mouse models is in their fasting insulin and glucose levels. L-SACC mice are hyperinsulinemic and exhibit random hyperglycemia, whereas the fasted insulin levels are only very minimally different in female *Ceacam1*$^{-/-}$ mice. Glucose levels were not altered at all. As well, the *Ceacam1*$^{-/-}$ mice are not glucose or insulin intolerant after either a 4-5 or 16 h fast, contrary to the L-SACC mice (Poy 2002). Intriguingly, Najjar's laboratory has seen the contrary in the fasting and refed insulin levels in *Ceacam1*$^{-/-}$ males (Najjar 2005). There is one potential explanation for these disparities; the L-SACC or *Ceacam1*$^{-/-}$ mice studied in Najjar's laboratory are subjected to a long 16 h fast and blood collection is strictly via the retro-orbital vessel. Many investigators consider that a prolonged 16 h fast is grossly exaggerated for mice and close to starvation; this does not constitute a physiological measurement corresponding to a normal fast in between food

intake. In spite of this, three separate investigators collaborating with our laboratory have not found differences between a 4 h or 16 h fast of *Ceacam1*$^{-/-}$ males, whether blood was collected via the retro-orbital or tail vein (Fig. 4.2.3.5), and despite the fact that mice were fed a normal or a high-fat diet. The only significant difference noted is in the hyperinsulinemic state of the *Ceacam1*$^{-/-}$ females in a post-prandial state. More experiments will be required to explore these differences.

Regardless of these discrepancies, the most notable finding relative to glucose homeostasis lies in the fact that the *Ceacam1*$^{-/-}$ mice clearly demonstrate hepatic and whole body insulin resistance, as shown by the increased levels hepatic glucose production and lowered glucose infusion rates in euglycemic clamp study shown in Fig. 4.2.3.7 and Table 4.2.2. Whether on a normal or high-fat diet, the *Ceacam1*$^{-/-}$ mice clearly do not show hyperglycemia and basal insulin levels are only significantly increased on a high fat diet (Table 4.2.2). Muscle glucose uptake measurements were only decreased in the high fat diet conditions relative to the normal diet under clamp conditions.

In summary, increased lipogenesis as revealed by the gene expression profiles in the liver and high levels of hepatic cholesterol and TG expression are a consequence of hepatic insulin resistance in *Ceacam1*$^{-/-}$ mice. The future challenge will be to link increased visceral adiposity and lipids to the progression of colon cancer in *Ceacam1*$^{-/-}$ mice.

4.2.5 Materials and Methods

4.2.5.1 Generation of the *Ceacam1*$^{-/-}$ mice and Animal Husbandry.

The strategy leading to complete abrogation of CEACAM1 expression in mice was based on the replacement of the first two exons of the *Ceacam1* gene by a *neo* cassette (Leung 2006). Genotyping of the mice was performed as described (Leung 2006). Experiments were performed in the C57Bl/6 background on backcrosses 8-16 for both the 2D2 and 11H11 *Ceacam1*$^{-/-}$ mice. Mice were kept on a sterilized rodent diet (Harlan Teklad LM-485, #7912; Harlan Teklad,

Madison, WI) or transferred to a 45% kcal%fat (high-fat high-carbohydrate diet, # D12451, Research Diets Inc., New Brunswick, NJ) for 8 weeks. Animals were housed under controlled temperature (23°C) and 12 h light/dark cycle with water and food *ad libitum*. Mice were handled according to the standards defined by the Canadian Council on Animal Care.

4.2.5.2 Phenotypic Analyses.

Prior to sacrifice, the mice were fasted for 5 h and were anesthetized with an overdose of ketamine-based rodent cocktail. Whole venous blood was retrieved from either the retro-orbital sinuses to measure plasma insulin and leptin levels by radioimmunoassay (Linco Research), or from the tail vein or cardiac puncture to measure fasting glucose levels with a glucometer (Accu-chek), plasma FFAs using the NEFA kit (Wako), triglycerides using Roche and cholesterol using Roche. To measure liver functions, serum Alanine aminoTransferase (ALT), Aspartate AminoTransferase (AST) and alkaline phosphatase (ALK) levels were evaluated. Kidney functions were evaluated from 24 h-urine samples collected in metabolic cages. Visceral adipose tissues were weighed and visceral adiposity was computed over the total body weight.

4.2.5.3 Insulin and Glucose Tolerance Tests.

Male mice were fasted for 4-5 or 16 h and insulin (0.125mU/g body weight) was injected intraperitoneally (I.P.) prior to blood retrieval from either retro-orbital or tail vein bleeding at 0, 30, 60, 120 min for glucose determination. For glucose tolerance tests, 5 or 16 h fasted male mice were injected I.P. with glucose (2 g/kg body weight) and blood drawn from the tail vein at 0, 15, 30, 60, 120 min after injections to determine glucose levels.

4.2.5.4 Hyperinsulinemic-euglycemic clamps.

All clamp procedures were approved and carried out at the Center for Lipid Research of Laval University (Quebec, Canada). 24-week-old *Ceacam1*$^{-/-}$ male mice were catheterized at least 5 days before the experiments after being

anesthetized with isoflurane. The left common carotid artery and the right jugular vein were catheterized. The free catheter ends were tunneled under the skin to the back of the neck and attached with stainless steel connectors. The tubing was externalized and sealed with a heated forceps. Lines were flushed daily with ~50 µl saline containing 200 units/ml heparin and 5 mg/ml ampicillin. Animals were individually housed after surgery, and their body weight was recorded daily. Animals within 90% of their presurgical weight by postsurgery day 5 were included in the study. After a 5h fast, conscious mice were studied in unrestrained conditions in individual containers (7x7x4 cm) with bedding. The protocol consisted of a 90-min tracer equilibration period (t = -90 to 0 min) beginning at 1:00 P.M. followed by a 120-min experimental period (t = 0 to 120 min) beginning at 2:30 P.M. A blood sample (~5 µl) was obtained at t = -90 min to determine initial glucose levels (Precision PCx; MediSense, Abbott Laboratories, MA). A 5 µCi bolus of [3-^{3}H]-glucose purified by high-performance liquid chromatography was given at t = -90 min followed by a 0.05 µCi/ min infusion for 90 min. At t = -20, -10 and 0 min, blood samples (50 µl) were drawn for the assessment of basal glucose, insulin and glucose turnover levels. The insulin clamp was begun at t = 0 min with a primed-continuous infusion of human insulin (16 mU/kg bolus followed by 4 mU /kg /min; Humulin R; Eli Lilly, Indianapolis, IN). The [3-^{3}H]-glucose infusion was increased to 0.2 µCi/ min for the remainder of the experiment to minimize changes in specific activity from the equilibration period. Specific activity for individual time points did not vary by more than 15% from the average specific activity during the last 40 min of the clamp, and the slope of specific activity over time was not significantly different from zero. Euglycemia (6.0-7.0 mM) was maintained during clamps by measuring blood glucose every 10 min starting at t = 0 min and infusing 50% dextrose as necessary. Blood samples (60–200 µl) were taken every 10 min from t = 80 to 120 min and processed to determine glucose specific activity. Clamp insulin levels were determined from samples obtained at t = 100 and 120 min. Mice received saline-washed erythrocytes from donors throughout

the experimental period (5–6 µl/min) to prevent a decrease of greater than 5% hematocrit.

4.2.5.5 Preparation of Tissues and Histological Analyses.

The mice were sacrificed by cervical dislocation and tissues were removed, washed in PBS and fixed in 4% paraformaldehyde/PBS or 10% phosphate-buffered formalin and processed for immunohistochemistry. Fixed tissues were dehydrated in ethanol and paraffin-embedded. Tissue sections, 4 µm in thickness, were deparaffinized according to standard protocol. Slides were counterstained with hematoxylin or sudan black, following standard histological procedures.

4.2.5.6 Extraction of RNA.

Tissues were retrieved from the 129Sv or C57Bl/6 WT and *Ceacam1*$^{-/-}$ mice and snap-frozen on dry ice. The tissues were then powdered using a mortar and pestle kept at -80oC and the RNA was extracted using materials provided in the RNAqueous kit (Ambion) following manufacturer's recommendation.

4.2.5.7 Microarray analyses.

For all experiments Affymetrix Mouse MOE430A GeneChips consisting of 22690 probe sets were used. The analysis was carried out on biological independent replicates. The targets for Affymetrix DNA microarray analysis were prepared as described by the manufacturer; the amount of total RNA used for the cDNA-synthesis was 10 µg for each reaction. The IVT-ENZO-Kit was used for the *in vitro* transcription. GeneChip microarrays were hybridized with the targets for 16 h at 45°C, washed and stained using the Affymetrix Fluidics Station according to the GeneChip Expression Analysis Technical Manual. Microarrays were scanned with the Hewlett-Packard-Agilent GeneChip scanner, and the signals were processed using the GeneChip expression analysis algorithm (v.2; Affymetrix). To compare samples and experiments, the trimmed mean signal of each array was scaled to a target intensity of 100. Absolute and comparison analyses were performed with Affymetrix MAS 5.0 and DMT software using default parameters.

To assist in the identification of genes that were positively or negatively regulated in the experiment, we selected genes that were increased or decreased at least fourfold compared to the baseline (wild type) (Rajagopalan 2003). Annotations were further analyzed with interactive query analysis at www.affymetrix.com. Pathways and other functional groupings of genes were evaluated for differential regulation using the visualisation tool GenMAPP (UCSF) as described previously (Bonner, Lemon et al. 2003; Doniger, Salomonis et al. 2003).

4.2.5.8 Quantification and Statistical analysis.

Data was analyzed using the Student *t* test for statistical significance. *P* values were considered significant if $p<0.05$. Standard error was calculated and represented in the bar graphs.

Chapter 5 Conclusion

5.1 Summary

5.1.1 Manuscript I

The *Ceacam1*$^{-/-}$ mouse was generated by removing the first two exons of the *Ceacam1* gene and inserting a *neo* gene cassette under the TK promoter. This genetic manipulation resulted in efficiently abrogating CEACAM1 expression in all mouse tissues. One of the organs that highly expresses CEACAM1 is the colon. In the absence of CEACAM1, the colon epithelium exhibits increased proliferation, as tested by BrdU incorporation and PCNA staining of paraffin sections. In correlation with increased proliferation, colonocytes expressed more nuclear cyclin-dependent kinase inhibitors p21 and 27. These proteins can regulate the cell cycle by interacting with all G1/S Cdks and their cognate cyclins (Sherr and Roberts 1999), but particularly inhibiting the activities of Cdk2/cyclin E. Active E-type cyclin/Cdk2 complexes phosphorylate pRb, leading to a wave of transcriptional activity essential to proceed through the G1/S transition (Barbacid, Ortega et al. 2005).

Significantly less apoptosis was detected in the colon epithelium of *Ceacam1*$^{-/-}$ mice. Both early signs of apoptosis, measured by Annexin V labeling, and late signs of apoptosis, measured by TUNEL staining, were weaker in the *Ceacam1*$^{-/-}$ mice than in their wild type counterparts. Even though *Ceacam1*$^{-/-}$ mice exhibit deregulated proliferation and apoptosis, no tumors developed spontaneously. The induction of tumors with the chemical carcinogen, azoxymethane, resulted in a higher tumor burden in the *Ceacam1*$^{-/-}$ mice. Tumors were greater both in numbers and in size in the CEACAM1-deficient mice. The vascularization of tumors from the *Ceacam1*$^{-/-}$ mice was not affected as tested by staining with CD31, an endothelial marker also known as PECAM1. Thus, downregulation of CEACAM1 prior to tumor development promotes tumor formation and growth, through increased proliferation and survival.

5.1.2 Manuscript II

A second model of tumorigenesis was used to study tumor formation in the *Ceacam1*$^{-/-}$ mouse. The genetically modified mouse *Apc*$^{1638N/+}$, produces a truncated APC protein that is very poorly expressed, with only 2% of the protein present (Fodde 1994). Unlike the colon, the small intestinal epithelium of the *Ceacam1*$^{-/-}$ mouse does not hyperproliferate. Signalling via tyrosine receptor kinases, such as IR and EGFR have mitogenic effects that were found to be regulated by CEACAM1-L in hepatocytes. The activity of the downstream effectors of TRK was analyzed. There was normal Erk and Akt activity in the *Ceacam1*$^{-/-}$ intestinal epithelium. The level of p21 in the *Ceacam1*$^{-/-}$ mouse small intestine was not significantly lower. Although the intestinal epithelium of the *Ceacam1*$^{-/-}$ mouse does not hyperproliferate, it does not undergo apoptosis at a normal rate. Staining by TUNEL and Annexin V labelling indicate that intestinal epithelium lacking CEACAM1 undergo less apoptosis. Compound *Apc*$^{1638N/+}$: *Ceacam1*$^{-/-}$ mice developed more tumors than *Apc*$^{1638N/+}$ control mice. The compound mice also developed more intussusceptions and desmoid tumors. Tumors that were devoid of CEACAM1 expression were also larger. These results are similar to ones obtained with the AOM model of tumorigenesis. However tumor multiplicity was inferior. Importantly, the staging of the tumors in the compound mice was more advanced; emphasizing that tumor burden was significantly more advanced in *Apc*$^{1638N/+}$: *Ceacam1*$^{-/-}$ mice. Recently, CEACAM1 has been shown to bind directly to β-catenin (Jin 2007). The activity of Wnt signalling was therefore measured in CT51 colon carcinoma cell lines that were transfected with CEACAM1-L, with a TOPFLASH reporter. Cells that did not express CEACAM1-L had higher nuclear β-catenin levels than those that did. Immunofluorescence staining also revealed that CEACAM1-L and β-catenin co-localized at the membrane. Concomitantly with more nuclear β-catenin, the targets downstream of TCF/Lef transcriptional program, c-Myc and cyclin D1, were overexpressed in the small intestine of *Ceacam1*$^{-/-}$ mice. In summary, these results indicate that down-regulation of the *Ceacam1* gene, even before any tumor

mutations are imposed on the intestinal epithelium, changes cellular programs and create a situation favourable to tumor development.

5.1.3 Manuscript III

One of the visible phenotypes of the *Ceacam1*$^{-/-}$ mouse is its slight obesity. Growth curves of mice indicate that they start accumulating weight soon after weaning. Concomitantly with increasing body mass mice consumed more food. The increased body weight was due to increased percentage of visceral fat and liver. A sexual dimorphism was observed, where the *Ceacam1*$^{-/-}$ females showed a more significant increase in body weight and fat percentage. Serum profiles of lipids revealed that the *Ceacam1*$^{-/-}$ females had significantly lower serum triglyceride, HDL, and cholesterol at 12 months. However, hepatic lipid content was higher in the *Ceacam1*$^{-/-}$ mice. In particular, cholesterol and triglyceride levels were significantly elevated in 12-month old mice. Concomitantly, paraffin sections of livers from 12-month old mice revealed a tendency toward steatosis, a condition used to describe fatty livers. The liver does not normally store lipids and as a consequence suffers some inflammation. Indicators of liver dysfunction include ALT, AST, and ALK. Older *Ceacam1*$^{-/-}$ mice had elevated levels of AST and ALK. The role of CEACAM1 in insulin clearance has been supported in several experiments including an animal model, the L-SACC1 mouse. Disruption of CEACAM1 function in the liver resulted in mice developing hyperinsulinemia and insulin resistance. Therefore the glucose homeostasis of the *Ceacam1*$^{-/-}$ mouse was tested. Fasting glucose and insulin levels were normal, but post-prandial insulin levels were dramatically elevated in female mice only, indicating an exaggerated response to food intake, and possibly insulin resistance. Glucose and insulin tolerance tests were conducted on male mice with no significant differences in the response of the *Ceacam1*$^{-/-}$ animals. The female *Ceacam1*$^{-/-}$ mice will be tested, as they appear to have a more perceptible phenotype.

Mice were also fed a high fat diet in order to exacerbate their glucose homeostasis and further study the insulin signalling in these animals. As expected, all animals on the HF diet increased in weight and adiposity. No

significant differences were found between the control and the *Ceacam1*$^{-/-}$ mice, in their weights, or serum lipid profiles, as well as fasting glucose and insulin levels. The liver enzymes AST, ALT and ALK were already elevated in female mice on standard diet, but all mice demonstrated elevated liver enzyme levels on HF diet. To ascertain if the *Ceacam1*$^{-/-}$ mice were insulin resistant euglycemic clamp measurements were performed. The euglycemic clamp conditions revealed that indeed the *Ceacam1*$^{-/-}$ mice were insulin resistant, both on standard and HF diet. Futhermore, hepatic glucose production was not completely inhibited by insulin, also indicating hepatic insulin resistance. Peripheral organs, such as the muscle, were not significantly affected by the expression of CEACAM1; however, the HF diet clearly induces symptoms of insulin resistance both in the liver and periphery.

Gene array analysis of liver cDNA revealed that several genes involved in metabolism and inflammation were overexpressed in the *Ceacam1*$^{-/-}$ mice. Seven of the enzymes controlled by either the SREBP-1c or SREBP-2 transcriptional modulators had significantly enhanced expression, which would indicate an increased production of monounsaturated fatty acids and cholesterol. Several genes relating to metabolism and inflammation are also decreased (Table 4.2.3). The gene most decreased, *Hsd17b9*, is considered a quantitative trait loci for diabesity (Brown, Olver et al. 2005). As well, *Socs2*, a target gene of the *Stat5* transcription factor, is significantly decreased and likely involved in hepatosteatosis. There is also a decrease in the liver-specific *Foxa3* transcription factor that has been described as interacting with the proglucagon gene, which might be responsible for the *Foxa3*-/- mouse's mild hypoglycemia after a prolonged fast (Liu, Shen et al. 2002). Finally, the *vanin 1* (*Vnn1*) gene has been associated with response to oxidative stress and inflammatory process (Martin, Penet et al. 2004). Therefore, lipidogenic and inflammatory transcriptional programs are significantly dysregulated in *Ceacam1*$^{-/-}$ mice. Importantly, this dysregulation occurs as of 3 months of age, strongly suggesting that even though no overt signs are detected in serum metabolic parameters at this age, changes in the lipidogenic program has already been altered in CEACAM1-deficient livers.

5.2 Tumor development

Using an *in vivo* model, the *Ceacam1*$^{-/-}$ mouse, we have demonstrated that CEACAM1 functions as a tumor suppressor. Lack of CEACAM1 expression results in increased susceptibility of developing aggressive tumors in the colon and small intestine. Tumor development depends of several factors. Once initiated by the common initiating mutations in Apc or Ras, survival and growth of a tumor relies in part on aberrant hyperproliferation and cell cycle checks, as well as inhibition of apoptosis. Although CEACAM1 deficiency does not participate in initiation of tumors, it contributes significantly to the processes of proliferation and apoptosis.

Several *in vivo* tumor assays have demonstrated that CEACAM1 is a tumor suppressor. Transfection of various mutants of CEACAM1 in cells revealed that the long cytoplasmic domain alone could confer tumor suppression. Several key residues were also identified, of which the Tyr488 and Ser503 are critical to the tumor inhibitory effect in CT51 cells. These two residues are binding sites or requirements for the interaction of several kinases and tyrosine phosphatases. Once phosphorylated, CEACAM1 can interact with the phosphatases SHP-1 and -2, as well as Src family kinases and mediate signalling events. In T-cells the interaction of CEACAM1 with SHP-1 is crucial for its inhibitory function (Donda 2000; Nagaishi, Pao et al. 2006).

It was demonstrated *in vitro* that CEACAM1 participates in contact inhibition (Singer, Scheffrahn et al. 2000; Scheffrahn, Singer et al. 2005). Due to the fact that CEACAM1 is highly expressed on the surface of the gastrointestinal tract, it is reasonable to think that it would be involved in the control of proliferation. Another clue that points to the fact that CEACAM1 participates in contact inhibition is its mode of signalling which involves clustering of CEACAM1 molecules. It was shown in PC12 cells that clustering of CEACAM1 with antibodies stimulates binding of CEACAM1 to the actin cytoskeleton, and a transient tyrosine dephosphorylation, which also activates the MAPK Erk-1 and -2 (Budt, Cichocka et al. 2002). In NBTII epithelial cells, CEACAM1 regulates

the activity of the Erk1/2 MAP kinase pathway and the expression of p27, in a cell density-dependent manner. Moreover, disruption of clusters by antibodies obstructs CEACAM1 signalling, by de-activating Erk, restoring levels of p27, and inhibiting DNA synthesis (Scheffrahn, Singer et al. 2005). In the absence of CEACAM1, increased proliferation is observed in mouse colons. This is in part due to a decreased expression of the cyclin-dependent kinase inhibitors p21 and p27 (Leung 2006). These proteins interact with Cdk2/cyclinE to inhibit cells from proceeding through the cell cycle at the G1/S phase.

In conjunction with increased proliferation in the colon, decreased apoptosis was consistently detected in the small intestine and the colon. The formation of lumen in breast acini requires the expression of CEACAM1-S (Kirshner, Chen et al. 2003; Chen, Kirshner et al. 2007). Apoptosis is mediated by Bax translocation to the mitochondria and release of cytochrome *c* into the cytoplasm (Kirshner, Chen et al. 2003). The apoptotic effect of CEACAM1 requires the phosphorylation of Thr457 and Ser459 residues (Chen, Kirshner et al. 2007). So far, the identification of which CEACAM1 downstream effectors are responsible for apoptosis in the colon or small intestine is still unknown. It is possible that the situation found in mammary tissue is similar to that found in the colon. A model of colon cells grown in matrigel or extracellular matrix could address this issue.

Although most of the signal transduction events occur through the long cytoplasmic domain of CEACAM1, the contribution of CEACAM1-S cannot be completely ignored. Whether CEACAM1-S functions in the oligomerization of CEACAM1 molecules or tethers it to the extracellular matrix (Sadekova, Lamarche-Vane et al. 2000; Chen, Kirshner et al. 2007), its expression remains important. Observations that both CEACAM1-S and CEACAM1-L are often co-expressed and studies of co-transfected CT51 cells with different ratios of CEACAM1-S and –L indicate that both isoforms are necessary to mediate the tumor suppressive function of CEACAM1 (Turbide 1997). In the colon normal expression of CEACAM1 ratios are 1:7 long to short cytoplasmic domain. Thus, the short isoform is expressed more abundantly. Using double transfectant cell

lines of CT51 cells, it was discovered that when the ratio of CEACAM1-L: CEACAM1-S expression stayed within normal physiological levels (≤ 1.0) CEACAM1-L acted as a dominant growth inhibitor. Reversing the ratio of CEACAM1-L: CEACAM1-S expression, to values ≥1.5, had an adverse effect on the inhibition of *in vivo* tumor development (Turbide 1997). Hence, CEACAM1 signal can be modulated depending on the ratio of isoforms expressed.

One of the highlights of this work was the demonstration that CEACAM1 also modulates the Wnt signalling pathway. As described before, Wnts control many morphological processes during development. If Wnt activation is dysregulated in normal tissues, inappropriate growth and survival signals promote tumor development. One mechanism for CEACAM1's modulation of the Wnt/β-catenin signal transduction may be similar to that described for E-cadherin. E-cadherin sequesters β-catenin at the cell-membrane, making it less available for translocation to the nucleus. In the absence of E-cadherin at the membrane, β-catenin is frequently found in the nucleus (Gottardi, Wong et al. 2001). The interaction of CEACAM1 and β-catenin was demonstrated by Jin *et al*. In addition, I have shown that in CT51 colonic carcinoma cells, CEACAM1 and β-catenin do co-localize. In the same fashion as E-cadherin, CEACAM1 could sequester β-catenin at the plasma membrane. I propose that in the absence of CEACAM1, β-catenin is more accessible to translocate to the nucleus. Once in the nucleus, TCF-Lef target genes can be activated, such as the c-Myc oncogene and Cyclin D1. Recently, c-Myc has been identified as the critical mediator of the early stages of neoplasia following loss of Apc. Simultaneous loss of *Apc* and *Myc* in the adult murine small intestine, rescues the phenotypes of perturbed differentiation, migration, proliferation, and apoptosis, which occur upon deletion of *Apc* alone (Sansom, Meniel et al. 2007). The elevated levels of Myc detected in the intestine of the *Ceacam1*$^{-/-}$ mice reinforce the tumor-prone phenotype of these animals.

CEACAM1 expression is lost during the earliest stage of tumorigenesis, the hyperplastic lesion (Nittka 2004), but its expression is sometimes up-regulated in more advanced tumors (Hauck 1994). Introduction of CEACAM1 into

endogenously deficient WRO thyroid cancer cells results in reduced cell cycle progression associated with p21 upregulation and diminished Rb phosphorylation. Forced CEACAM1 expression also enhances cell–matrix adhesion and migration and promotes tumor invasiveness. Conversely, downregulation of CEACAM1 by small interfering RNA (siRNA) in MRO (aggressive thyroid cancer) cells accelerated cell cycle progression and significantly enhanced tumor size in xenografted mice (Liu, Wei et al. 2007). Therefore in advanced tumors CEACAM1 promotes migration and invasion. It would appear CEACAM1 inhibits tumor growth only at the early phase of tumor development. In advanced tumors, CEACAM1 could also ensure tumor survival by inhibiting the immune response (Kammerer 2004). Upregulation of CEACAM1 on melanomas allows homophilic binding with CEACAM1 on NK cells, thereby inhibiting NK-cell-mediated killing. In addition, CEACAM1 expression by a cell line protects it from targeted killing by NK cells in a dose-dependent manner (Markel 2002). Therefore, the aberrant expression of CEACAM1 during proliferation of a cell that has lost the ability to control its cell cycle would generate a tumor that resists immune-cell-mediated killing (Thies, Moll et al. 2002) (Laack 2002). It would be interesting to see if re-expression of CEACAM1 in tumors could inhibit their elimination by the immune response. This could be done with an inducible/conditional floxed *Ceacam1* knockout mouse model.

5.3 Metabolic response to insulin

The *Ceacam1*$^{-/-}$ mouse exhibits a complex phenotype, because CEACAM1 is involved in many functions. The *Ceacam1*$^{-/-}$ mouse is slightly obese due to increased visceral adiposity and hyperphagia. It has been established that CEACAM1-L is responsible for the internalization of the activated IR and thus insulin clearance (Najjar 1995; Li Calzi, Choice et al. 1997; Poy 2002; Dubois 2006). Over time, the dysregulation of insulin clearance becomes more apparent in mice fed a high fat diet or in older mice. The *Ceacam1*$^{-/-}$ mouse is insulin resistant and shows signs of steatohepatitis. In humans non-alcoholic steatohepatitis is often associated with abdominal obesity, hypertension, and

diabetes. Insulin resistance has been implicated as a key mechanism in the pathogenesis of steatohepatitis. Adipokines such as IL-6, TNF-α, leptin, resistin, and adiponectin are thought to contribute to peripheral insulin resistance (Kaser, Moschen et al. 2005). In insulin-resistant states, insulin does not fully suppress the activity of hormone-sensitive lipase (HSL) in adipose tissues, which results in enhanced lipolysis and release of fatty acids (Machado and Cortez-Pinto 2006). As a consequence, the liver becomes infiltrated with lipids. There is no evidence that hepatic uptake of fatty acids is regulated and, as a result, plasma fatty acid concentration is directly related to the influx of fatty acids to the liver (Tamura and Shimomura 2005). This would be in line with the concept that the liver acts as a buffer for the incoming flux of fatty acids (Frayn 2002; Gauthier, Favier et al. 2006). Studies by Ravikumar *et al.* (Ravikumar, Carey et al. 2005) recently demonstrated faster incorporation of dietary fatty acid into liver triglyceride in the type 2 diabetic subjects (4 vs 6 h) and almost 50% higher accumulation compared with control subjects without any differences in plasma NEFAs. Thus, insulin resistance could augment the rate at which the liver captures circulating NEFAs. This might be another mechanism that could explain the hepatic steatosis in the *Ceacam1*$^{-/-}$ mouse. The rate of incorporation of fatty acids into liver triglyceride should be compared between wild type and *Ceacam1*$^{-/-}$ mice, to determine if CEACAM1 could be involved in the transport of hepatic lipids.

The hepatic lipid accumulation in the knockout animals is due in part to increased fatty acid synthesis, as the *Ceacam1*$^{-/-}$ mouse liver overexpresses many of the genes involved in lipid synthesis under the control of SREBP1-c. Alternatively, the reduction of β-oxidation of fatty acids could also result in accumulation of fat in the liver. This possibility has not yet been investigated. The majority of fatty acids are metabolized through β-oxidation occurring principally in the mitochondria (Kunau, Dommes et al. 1995). β-Oxidation also occurs in peroxisomes (Kunau, Dommes et al. 1995), where a variety of fatty acids, particularly very long chain fatty acids (VLCFAs) and branched-chain fatty acids (Reddy 2001) are metabolized. Other types of fatty acid oxidation include α-oxidation and ω-oxidation by members of the cytochrome P450 4A family in the

endoplasmic reticulum (microsomes) (Waxman 1999; Reddy 2001). The P450 cytochromes are a superfamily of hemoproteins that catalyze the oxidation of endogenous and exogenous compounds, including fatty acids (Enriquez, Leclercq et al. 1999). The dicarboxylic acids formed are further degraded by the classical peroxisomal β-oxidation system (Reddy and Mannaerts 1994). The extra-mitochondrial fatty acid oxidation systems become more important during periods of increased influx of fatty acids into the liver (Rao and Reddy 2001). The activity of β-oxidation will be investigated in the future.

Moreover, increased adiposity secretes more adipokines, which all contribute to further dysregulate glucose and lipid homeostasis. Leptin and adiponectin, the two major fat-derived hormones, have been shown to increase insulin sensitivity and concomitantly reduce hepatic triglyceride content (probably by promoting fatty acid oxidation) in an animal model of insulin resistance or in humans with lipodystrophic diabetes (Kakuma, Lee et al. 2000; Yamauchi, Kamon et al. 2001). It has been suggested that the metabolic advantage conferred by the hyperleptinemia of obesity is the prevention of triacylglyceride (TAG) accumulation in non-adipose tissue (Unger, Zhou et al. 1999). Interestingly, in obesity and states of insulin resistance, the protective effects of hyperleptinaemia seem to be limited due to leptin resistance (Flier 2004). Tumor necrosis factor alpha (TNF-α) is an important proinflammatory cytokine that impairs insulin signaling through several proposed mechanisms. First, TNF-α represses genes involved in uptake and storage of non-esterified fatty acids in the adipose tissue (Ruan, Miles et al. 2002). Those fatty acids are thus readily available for the liver, and increase the pool of intrahepatic FFA. Second, TNF-α activates JNK and IκKβ. This results in serine phosphorylation of IRS and inhibition of insulin signaling (Hotamisligil 2006; Taniguchi, Emanuelli et al. 2006). Additionally, JNK activation induces TNF-α, therefore representing an autocrine/paracrine loop potentiating insulin resistance. Third, TNF-α is a potent proinflammatory cytokine, which favours IL-6 and related SOCS-3 production (Emanuelli, Peraldi et al. 2000; Senn, Klover et al. 2003; Ueki, Kondo et al. 2004). The latter blocks the activity of the insulin receptor. Finally, adiponectin and TNF-α suppress each

other's production and activity (Kershaw and Flier 2004; Kadowaki, Yamauchi et al. 2006). Circulating IL-6 is strongly associated with obesity and is a predictor of development of type II diabetes. IL-6 is an inhibitor of insulin signaling in isolated hepatocytes and in the liver of experimental animals (Senn, Klover et al. 2003); (Klover, Zimmers et al. 2003). In models of genetic or diet-induced obesity, injections of IL-6 neutralizing antibodies allow normalisation of IR phosphorylation in response to insulin and increase insulin-mediated suppression of hepatic glucose output (Klover, Clementi et al. 2005). This improvement of insulin signaling is restricted to the liver. Although there is a good association between hepatic fat accumulation and hepatic insulin resistance, it is still not clear if hepatic insulin resistance is due to systemic factors such as, circulating lipids, or to elevated intrahepatic lipids.

Gene array analysis of liver RNA revealed that almost all the genes involved in mono-unsaturated fatty acids synthesis were upregulated. Whether this effect was a direct effect of CEACAM1's absence or an indirect result of the insulin resistance developed in the *Ceacam1*$^{-/-}$ mouse, will still need to be determined. The expression of genes involved in the lipid synthesis should be gauged in animals fed a high fat diet, which induces insulin resistance. Comparison of the wild type mice on high fat diet and *Ceacam1*$^{-/-}$ mice on standard diet should tell us if the upregulated lipid synthesis genes were due to the absence of CEACAM1 or the result of insulin resistance. Insulin stimulates the SREBP1-c, which is responsible for most of insulin's effects on lipogenesis (Horton 2002). Interestingly, SREBP1-c was not upregulated in the liver of the *Ceacam1*$^{-/-}$ mouse. This could indicate an insulin-independent regulation of fatty acid synthesis enzymes in the *Ceacam1*$^{-/-}$ mouse or perhaps CEACAM1 controls the activation of SREBP1-c. Other elements that could control the synthesis of fatty acids include carbohydrate-response-element-binding protein (ChREBP), which senses carbohydrate availability for lipid synthesis. Glucose activates ChREBP, which stimulates gene expression of most enzymes involved in lipogenesis (Iizuka, Bruick et al. 2004). TFE3 (transcription factor E3) is a novel bHLH transcription factor that strongly activates various insulin signaling molecules,

protecting against the development of insulin resistance and the metabolic syndrome. Regulation of IRS-2 is the primary site where TFE3 in synergy with Foxo1, and SREBP-1c converge. Taken together, TFE3/Foxo1 and SREBP-1c reciprocally regulate IRS-2 expression and insulin sensitivity in the liver. (Shimano 2007)

CEACAM1 expression could be an indicator of cardiovascular risk (Wisloff, Najjar et al. 2005). In humans, there is strong association between fitness and survival which suggests a link between impaired oxygen metabolism and disease. Rats were selected based on low and high intrinsic exercise capacity, to yield models that contrast for disease risk. An interesting correlation was found between aerobic capacity and expression of hepatic CEACAM1. Individuals who could sustain exercise for a longer period expressed more hepatic CEACAM1 than those that exercise little. The number of *Ceacam1* mRNA molecules in the liver of high capacity runners was nearly double of those detected in low capacity runners (Wisloff, Najjar et al. 2005). This suggests that CEACAM1 upregulation corresponds to fitness level.

5.4 Insulin resistance a risk factor for cancer

Obesity and diabetes are now recognized as risk factors of cancer. Chronically increased insulin levels have been associated with colon cancer pathogenesis (Giovannucci, Ascherio et al. 1995; Schoen, Tangen et al. 1999). The tumorigenic effects of insulin could be directly mediated by insulin receptors in the neoplastic target cells, or might be due to related changes in endogenous hormone metabolism, secondary to hyperinsulinaemia. Insulin has effects on the synthesis and biological availability of insulin-like growth factor 1 (IGF1) and the male and female sex hormones, including androgens, progesterone and estrogen (Calle and Kaaks 2004). Both insulin and IGF1 act as growth factors that promote cell proliferation and inhibit apoptosis (Ish-Shalom, Christoffersen et al. 1997; Prisco, Romano et al. 1999; Le Roith 2000; Lawlor and Alessi 2001). Experiments with insulin-deficient animals have shown that insulin promotes tumour growth and development in xenograft models and in chemical models of

carcinogenesis (Heuson and Legros 1970; Shafie and Grantham 1981; Cocca, Martin et al. 1998). Similarly, animal experiments have also shown reduced tumour growth after inactivation of the IGF1 receptor, or after manipulations to reduce circulating or tissue IGF1 levels (Khandwala, McCutcheon et al. 2000; LeRoith and Roberts 2003).

Over 80% of IGF1 in circulation is bound to IGF-binding proteins (IGFBPs). The IGFBPs functions include the stabilization of a large pool of IGF1 in the circulation, the regulation of the efflux of IGF1 from this pool towards target tissues and at the tissue level, the regulation of the availability of the IGF1 for binding to its receptor (Calle and Kaaks 2004). In addition, IGFBP3 has shown to exert pro-apoptotic effects independently of IGF1 (Clemmons 1998). The major source of IGF1 is the liver, which is stimulated to synthesize IGF1 by the growth hormone (GH) (Kaaks and Lukanova 2001). Epidemiological studies indicate that increased serum levels of IGF1 are directly related to risk of different forms of cancer. Increased levels of serum IGF1 have been found to be related to increased risks of breast cancer, especially among pre-menopausal women, and prostate and colorectal cancers (Calle and Kaaks 2004).

Adiposity influences the synthesis and bioavailability of endogenous sex steroids - the oestrogens, the androgens and progesterone. Adipose cells increase the circulating levels of insulin and IGF1 bioactivity. This results in reduced hepatic synthesis and blood concentrations of sex-hormone-binding globulin, a plasmatic binding protein with high specific affinity for testosterone and estradiol (Pugeat, Crave et al. 1991). Adiposity-related decreases in SHBG levels generally increase the fraction of bioavailable estradiol. In women, a decrease in SHBG generally leads to increased levels of bioavailable testosterone (Pugeat, Crave et al. 1991). In men, decreases in SHBG leads to reduction in total testicular testosterone production (Kaaks, Lukanova et al. 2000). High insulin levels can increase ovarian and possibly also adrenal, androgen synthesis and in some pre-menopausal women can cause the development of the polycystic ovary syndrome (Dunaif 1997). Epidemiological evidence imply that adiposity-induced alterations in circulating levels of sex steroids could explain the association

between excess weight and risks of cancers of the breast and endometrium in women (Flototto, Djahansouzi et al. 2001). Among men, prostate cancer development is also thought to be related to endogenous hormone metabolism, such as by androgen production (Kaaks, Lukanova et al. 2000).

Chapter 6 Future perspectives

The role of CEACAM1 in metastasis still needs to be investigated. Several similarities are found between the two adhesion molecules CEACAM1 and E-cadherin when it concerns expression pattern in cancer. E-cadherin is absent at the invasive front of tumors, but its expression returns at the site of metastasis (Elzagheid, Algars et al. 2006). It is speculated that the loss of E-cadherin, and thus adhesion, is necessary for motility, but once cells find a hospitable environment adhesion is crucial for seeding. It is still unclear if CEACAM1 could operate in the same manner or if its function in advanced tumors is to escape immune surveillance. Some evidence indicates that CEACAM1's expression in aggressive thyroid cancers promotes migration and invasion (Liu, Wei et al. 2007). Several models of metastasis could help clarify the purpose of CEACAM1 in advanced cancers. The orthotopic transplantation of colon cancer cells into the cecum of syngeneic animals or intrasplenic inoculation appears to resemble the human metastatic disease most closely, providing a model for study of the treatment of metastases (Kobaek-Larsen, Thorup et al. 2000).

Many of the downstream elements of CEACAM1 still need to be identified. Much of the signalling cascade stemming from CEACAM1 depends on other receptor tyrosine kinases. Although CEACAM1 interacts with Src kinase (Brümmer 1995) and the tyrosine phosphatases SHP-1 (Beauchemin 1997) and -2 (Huber 1999), it is still unknown how these participate in the CEACAM1 signalling cascade, and if CEACAM1 itself is able to activate downstream effectors. CEACAM1 downstream signalling partners could be identified by

using Fluorescence resonance energy transfer (FRET) studies, where proteins not necessarily interacting, but in close proximity could present a positive signal.

CEACAM1 stimulates formation of new blood vessels and its upregulation is observed in growing prostate tumors (Tilki 2006). It is interesting to note that in the *Ceacam1*$^{-/-}$ mice, which lack this angiogenic factor tumors grew to a larger size. Moreover, B16F10 melanoma cells expressing high levels of CEACAM1, form larger tumors in the *Ceacam1*$^{-/-}$ mice, but no significant changes in vascular density were noticed in either of the tumor growth settings (Nouvion and Beauchemin, personal communication). The explanation might lie with the activation of the enzyme COX-2. This enzyme is the target for many pharmaceutical agents. COX-2 is responsible for the synthesis of prostaglandins. Activated prostaglandin receptors in turn enhance cell survival and stimulate angiogenesis (Zha, Yegnasubramanian et al. 2004; Onguru, Kurt et al. 2006; Qualtrough, Kaidi et al. 2007). Levels of PGE2 and activity of COX-2 should be gauged in *Ceacam1*$^{-/-}$ mice and tumors, to elucidate if this pathway is implicated in the tumor development in *Ceacam1*$^{-/-}$ mice.

The above mentioned experiments were conducted on an animal model with systemic deletion of *Ceacam1*. However CEACAM1 is expressed not only on epithelial cells, but also endothelial and hematopoietic cells. It is conceivable that these other cell types were contributing factors in the phenotypes and experimental results obtained. The *Ceacam1*$^{-/-}$ mouse exhibits alterations in immune response. Stimulated T-cells from these animals hyperproliferate and produce more cytokines (Gray-Owen 2006). Such cytokines could either stimulate a chronic inflammation leading to a damaged epithelium prone to mutations, or a hyperactive immune response that would target cancers more efficiently. Moreover, CEACAM1 participates in the formation of new blood vessels. In order to separate the functions of CEACAM1 in the different cellular types and their contribution to cancer development, the mouse model needs to be refined. This will be made possible through the generation of a *Ceacam1* conditional knockout mouse, where the *Ceacam1* gene flanked by loxP sites (floxed), can be excised when mice express the Cre recombinase under the control

of a selected promoter. Transgenic mice expressing the cre recombinase under an inducible promoter such as the Tet-on or Tet-off could present an interesting model as well, where expression of CEACAM1 can be controlled at a certain time of the experiment by the addition of tamoxifen in the water of animals.

Therefore, CEACAM1 represents an interesting target for future therapeutic use in patients afflicted by cancer, particularly colon cancer and that its implication in metabolism may also open new avenues for patients suffering from insulin resistance.

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