Structure in stress management – Keratins in intestinal stress protection

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To my beloved family

Now so much I know
that things just don't grow
If you don't
bless them with your patience

"Emmylou"
First Aid Kit

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ABSTRACT

Intermediate filaments (IFs), a major constituent of the cellular cytoskeleton, contribute mainly to the mechanical stability of the cell. Simple epithelial keratins (SEKs) are IF proteins expressed in the single-layered and glandular epithelia of organs that involve secretion and absorption. In the colon, the main keratins (K) expressed are K7, K8, K18, K19 and K20. A new emerging role of SEKs in stress protection has been described for epithelial organs such as the liver, pancreas and kidney, where also the phosphorylation of K8 has been used as a marker of stress.

The role of K8 in intestinal stress protection was examined by studying the stress response of the K8 wild type ($K8^{+/+}$), K8 heterozygous ($K8^{+/-}$) and K8 knock out ($K8^{-/-}$) mice. $K8^{+/+}$ mice were subjected to human disease-related stresses, while $K8^{+/-}$ mice were examined in regards of stress protection by investigating their stress susceptibility to colitis. The stress response in $K8^{-/-}$ mice was studied by investigating their impaired energy metabolism in response to ketogenic situations.

As demonstrated by murine intestinal stress models, colonic keratins act as stress-responsive proteins, which are upregulated in stress and recovery. Depletion of the *in vivo* colonic microbiota upregulated protein levels of the main colonic keratin-pair, K8-K19, confirmed by the lipopolysaccharide (LPS)-mediated *in vitro* inflammational stress model. Another inflammational stress-induced keratin pair identified was the K7-K20 pair, upregulated by the experimental dextran sulphate sodium (DSS) colitis model. Except for the K7-K20 upregulation on protein level, this stress model also affected the keratin distribution by showing a wider crypt distribution of both K7 and K20 in response to stress. The role of keratins in colonic stress protection was confirmed by examining the stress susceptibility of the $K8^{+/-}$ mouse. The $K8^{+/-}$ mouse, which has 50 % less K8 compared to the $K8^{+/+}$ mouse, displays hyperproliferation, longer crypts and a higher susceptibility to DSS-induced colitis when compared to $K8^{+/+}$ mice, suggesting a protective role for K8 in colonic stress. In addition, by examining the $K8^{-/-}$ mouse in regards of stress protection, the role of K8 in colonic energy homeostasis was investigated. K8 was found to modulate the energy metabolism in colonocytes by affecting the amount of the ketogenic control enzyme, 3-hydroxy-3-methylglutaryl-CoA-synthase (HMGCS2). As a consequence of the deletion of K8 filaments, murine HMGCS2 levels were downregulted in the $K8^{-/-}$ mouse, together with the downregulation of the upstream short chain fatty acid (SCFA) transporter, monocarboxylate transporter isoform 1 (MCT1). Therefore, elevated $K8^{-/-}$ colonocyte luminal SCFAs that feed into the ketogenic pathway cannot enter the $K8^{-/-}$ colonocytes in sufficient amounts, leading to a decreased ketogenesis and overall ketogenic energy metabolism, contributing to the inflammatory $K8^{-/-}$ phenotype. When the impaired $K8^{-/-}$ ketogenesis was challenged with ketogenic conditions, the $K8^{-/-}$ mouse showed a blunted response, suggesting a protective role of K8 in the overall decreased $K8^{-/-}$ energy metabolism.

In conclusion, these data, as shown by the $K8^{+/+}$ stress-responsive behavior, the increased DSS-susceptibility of the $K8^{+/-}$ mice and the blunted $K8^{-/-}$ ketogenesis, indicate that colonic keratins likely play an important role in the protection from intestinal stress.

SWEDISH SUMMARY/SVENSK SAMMANFATTNING

Keratin (K) intermediärfilament (IF) är cytoskelettstrukturer i epitelceller som skyddar cellen från mekanisk och icke-mekanisk stress. Mutationer i gener som kodar för keratiner leder till flera svåra sjukdomar i bland annat huden, levern och möjligtvis tarmen (Omary 2009). K7, K8, K18, K19 och K20 är enkla epitelkeratiner (eng. simple spithelial keratins, SEK) och de är de vanligast förekommande keratinerna i tjocktarmens enkla epitelvävnad. De indelas i typ I och typ II keratiner som bildar heteropolymera filament med varandra (Moll, Divo & Langbein 2008). Möss utan K8 ($K8^{-/-}$) utvecklar diarré, ökad proliferation av tarmepitelceller samt en kronisk inflammation som liknar den som ses vid sjukdomarna ulcerativ kolit (eng. Ulcerative colitis, UC) och Crohns sjukdom (eng. Crohn's disease, CD) (Baribault et al. 1994, Habtezion et al. 2005). Det är känt sedan tidigare att keratin- och intermediärfilamentnivåer ökar vid stress (Toivola et al. 2010) i lever (Guldiken et al. 2015a), bukspottskörtel (Zhong et al. 2004), njure (Djudjaj et al. 2016), lunga (Sivaramakrishnan et al. 2009) och hud (Jin, Wang 2014), samt att keratinerna är reglerade av posttranslationella modifikationer vid stress (Snider 2016). Regleringen av gentranskriptionen av keratinerna i tjocktarmen är relativt okänd, även om man vet att transkriptionsfaktorn p53 deltar i transkriptionen för K8 (Mukhopadhyay, Roth 1996) och att transkriptionsfaktorn AP1 deltar i transkriptionen för K18 (Gunther et al. 1995).

Hypotesen för detta arbete var att tjocktarmens keratiner har en skyddande roll mot tarmpåfrestningar samt att de skyddar mot inflammatoriska tarmsjukdomar. De specifika målsättningarna var (1) att undersöka tjocktarmens keratinnivåer och -modifikationer under olika stress- och återhämtningstillstånd i tjocktarmen hos K8 vildtyps ($K8^{+/+}$) möss, (2) att undersöka med hjälp av K8 heterozygot ($K8^{+/-}$) musen om keratinerna bidrar till skyddandet mot tarmstress och (3) att undersöka keratinernas roll i tjocktarmens inflammatoriska energimetabolism med hjälp av $K8^{-/-}$ musen.

I det första delprojektet undersöktes tjocktarmens keratiner i fråga om deras nivåer och modifikationer under olika stresstillstånd i tarmen. I många andra organ såsom levern, bukspottskörteln, njurarna, lungorna och huden har keratinerna visats vara uppreglerade till följd av stress på samma sätt som andra kända stressproteiner. Denna uppreglering till följd av stress kunde också påvisas i tjocktarmen, där specifika keratinpar uppreglerades av olika stressmodeller. Keratinparet K8-K19 visade en uppreglering i möss då de skyddande tarmbakterierna avlägsnades med hjälp av en antibiotikabehandling, vilket även kunde konfirmeras med hjälp av aktiveringen av inflammatoriska signalleringsräckor med en lipopolysackarid (LPS) behandling på humana tjocktarmscancerceller. Det andra stress-känsliga keratinparet, K7-K20, uppreglerades till följd av dextran sulfat natrium (eng. Dextran sulphate sodium, DSS)-inducerad experimentell tarminflammation. K7, som basalt befinner sig i

bottnet på tarmkryptorna, och K20, som basalt befinner sig i toppen på tarmkryptorna, uppvisade till följd av en kronisk DSS-behandling förutom en uppreglering, även en bredare distribution, med det stress-responsiva området i mittersta delen av tarmkryptan. Förutom denna uppreglering av tarmkeratinerna till följd av olika stressmodeller, sågs en ökning i fosforyleringen av K8 vid aminosyran Serin 74 (S74), vilken kunde användas som en markör för tarmstress i fortsatta studier (Publikation I).

I det andra delprojektet undersöktes hur keratinerna bidrar till skyddandet mot tarmstress och upprätthållandet av tarmhälsan genom att analysera keratinnivåerna hos $K8^{+/-}$ musen. Resultaten visade att $K8^{+/-}$ musen uppvisar en mellanform av fenotyperna $K8^{-/-}$ och $K8^{+/+}$ gällande keratin-mängder, krypt-längd och Na/Cl transport. Dessutom hade $K8^{+/-}$ mössen ett högre sjukdomsaktivitetsindex samt en långsammare återhämtning efter en DSS-behandling jämfört med $K8^{+/+}$ musen, vilket antyder att $K8^{+/-}$ mössen är mera känsliga för DSS-inducerad kolit och att mängden keratiner är avgörande vid skyddandet mot tarmens påfrestningar (Publikation II).

I det sista delprojektet undersöktes keratinernas roll i regleringen av energimetabolismen och ketogenesen i utvecklingen av det inflammatoriska tillståndet i tjocktarmens epitelceller hos $K8^{-/-}$ musen. De keratin-specifika skillnaderna undersöktes genom att kartlägga $K8^{-/-}$ musens största proteinskillnader i tjocktarmen jämfört med $K8^{+/+}$ musen. Den största upptäckten var att enzymet 3-hydroxy-metylglutatyl CoA syntas 2 (eng. 3-hydroxy-methylglutatyl CoA synthase 2, HMGCS2), som reglerar ketogenesen, var kraftigt nedreglerat i tjocktarmen hos möss som saknar K8. Förutom en minskad mängd HMGCS2, uppmättes även en minskad aktivitet hos ifrågavarande enzym. Energimetabolismen och bakterieförhållanderna i tjocktarmarna på dessa möss studerades också för att få en större klarhet i det inflammatoriska tillståndet. Förutom en nedreglering av enzymet HMGCS2, visades även att butyrattransportören monokarboxylat transportör 1 (eng. monocarboxylate transporter 1, MCT1) var nedreglerad. Butyrat, en kortkedjad fettsyra (eng. short chain fatty acid, SCFA) som produceras av de godartade bakterierna i tarmen, är den huvudsakliga energikällan för epitelcellerna i tjocktarmen. Hypotesen testades genom att utsätta $K8^{-/-}$ möss för ketogena förhållanden, varvid resultaten visade att HMGCS2 inte uppregleras i samma proportion hos $K8^{-/-}$ möss som hos $K8^{+/+}$ möss, vilket sannolikt bidrar till den inflammatoriska $K8^{-/-}$ fenotypen och antyder att K8 har en skyddande roll även i mekanismer relaterade till energimetabolismen (Publikation III).

Sammanfattningsvis påvisar denna avhandling keratinernas skyddande roll i tjocktarmen genom att keratinerna uppregleras till följd av inducerad stress, vilket stöds av att nedsatta K8 nivåer förorsakar en ökad känslighet till tarmsjukdom.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by the Roman numerals (I-III). The original publications have been reproduced with the permission of the copyright holders. In addition, this thesis includes unpublished data.

I. Helenius, T. O., Antman, C. A., Asghar, M. N., Nyström, J. H., Toivola, D. M. Keratins are altered in intestinal disease-related stress responses. *Cells*. 2016; 5(3), 35

II. Asghar, M. N., Silvander, J. S. G., Helenius, T. O., Lähdeniemi, I. A., K., Alam, C., Fortelius, L. E., Holmsten R. O., Toivola D. M. The amount of keratins matters for stress protection of the colonic epithelium. *PLoS One*. 2015; 10(5): e0127436

III. Helenius, T. O.*, Misiorek, J. O.*, Nyström, J. H.*, Fortelius, L. E., Habtezion, A., Liao, J., Asghar, M. N., Zhang H., Azhar, S., Omary, M. B., Toivola, D. M. Keratin 8 modulates the ketogenic enzyme HMGCS2 in colonocyte energy metabolism. *Mol Biol Cell.* 2015; 26(12): 2298-2310

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PUBLICATIONS NOT INCLUDED IN THESIS

IV. Strnad P., Guldiken N., Helenius T. O., Misiorek J. O., Nyström J. H., Lähdeniemi I. A., Silvander J. S., Kuscuoglu D., Toivola D. M. Simple Epithelial Keratins. *Methods Enzymol.* 2016; 568: 351-388.

V. Asghar M. N., Emani R., Alam C., Helenius T. O., Grönroos T. J., Sareila O., Din M. U., Holmdahl R., Hänninen A., Toivola D. M. In vivo imaging of reactive oxygen and nitrogen species in murine colitis. *Inflamm Bowel Dis.* 2014 (8): 1435-1447.

ABBREVIATIONS

ADP	Adenosine diphosphate
ALD	Alcoholic liver disease
ALS	Amyotrophic lateral sclerosis
AP1	Activator protein 1
APC	Adenomatous polypsis coli
ATP	Adenosine triphosphate
Bfsp	Beaded filament structural protein
BMP	Bone morphogenetic protein
BrDU	5-bromo-2'-deoxyuridine
C	Cysteine
Caco-2	Human epithelial colorectal adenocarcinoma
CAM	Ca^{2+}/calmodulin-dependent protein
CD	Crohn's disease
Cdc2	Cell division cycle 2 protein
CoA	Coenzyme A
CBP/p300	CREB-binding protein/p300
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl	Chloride
CMT	Charcot-Marie-Tooth disease
c-RAF	RAF proto-oncogene serine/threonine-protein kinase
CRC	Colorectal cancer
CREB	cAMP regulatory element binding protein
DAI	Disease activity index
DC	Distal colon
Dhh	Desert hedgehog
DP	Desmoplakin
DRA	Downregulated in adenoma
DSS	Dextran sulphate sodium
EBS	Epidermolysis bullosa simplex
ECL	Enhanced chemiluminescence
ETS	E26 transformation-specific
FMT	Faecal microbiota transplant
G	Glycine
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
H	Histidine
H^+	Proton

HAT	Histone acetyl transferase
HCV	Hepatitis C infection
HDAC	Histone deacetylation
HGPS	Hutchinson-Gilford progeria syndrome
HMG CoA	3-hydroxy-3-methylglutaryl-CoA
HMGCS1	Cytocolic 3-hydroxy-3-methylglutaryl-CoA synthase 1
HMGCS2	Mitochondrial 3-hydroxy-3-methylglutaryl-CoA-synthase 2
HRP	Horseradish peroxidase
HSF2	Heat shock factor 2
HSP	Heat shock protein
HT29	Human colorectal adenocarcinoma cell line
IBD	Inflammatory bowel disease
IF	Intermediate filament
IFNγ	Interferon-γ
Ihh	Indian hedgehog
IκBα	Inhibitor of kappa B alfa
IL	Interleukin
JNK	c-Jun N-terminal kinase
K	Keratin
KLF4	Kruppel-like factor 4
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MCT1	Monocarboxylate transporter isoform 1
MDB	Mallory Denk bodies
Min6	Murine insulinoma cell line
N	Asparagine
Na	Natrium
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor κB
O.C.T.	Optimum cutting temperature
PC	Proximal colon
PCR	Polymerase chain reaction
PKCδ	Protein kinase C δ
PPARα	Peroxisome proliferator-activated receptor α
PPRE	Peroxisome proliferator regulatory element
PVDF	Polyvinylidene fluoride
p38 MAPK	p38 mitogen-activated protein kinase
p53	Tumor protein p53
R	Arginine

RAF-1	Rapidly accelerated fibrosarcoma-1
RONS	Reactive oxygen and nitrogen species
RXR	*Cis*-retinoid receptor
S	Serine
SCFA	Short chain fatty acids
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEK	Simple epithelial keratins
Shh	Sonic hegdehog
siRNA	Small interfering RNA
SLC5A8	Solute carrier family 5 member 8
SMCT	Sodium-coupled monocarboxylate transporter 1
SP1	Specificity protein 1
Src	Proto-oncogene tyrosine-protein
SUMO	Small ubiquitin-like modifier
S6	Ribosomal s6 kinase
T	Threonine
TEM	Transmission electron microscope
TG	Transgenic
TGF-β	Transforming growth factor-β
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
UC	Ulcerative colitis
ULF	Unit length filament
WB	Western blot
WT	Wild type
Y	Tyrosine
YAP	Yes-associated protein
ZO-1	Zonula occludens-1
2D-DIGE	Two-dimensional fluorescence difference gel electrophoresis
$^{+/+}$	Wild type
$^{+/-}$	Heterozygote
$^{-/-}$	Knockout

INTRODUCTION

In all living cells of the human body, a cytoskeleton contributes to the molecular structure of the cells. The main functions of the cytoskeleton, which consists of actin filaments, microtubules and IFs, are to provide mechanical strength and shape to the cell, separate chromosomes in cell division, provide a scaffold in cellular organization and take part in signaling cascades inside the cells. Many functions have likely not been discovered yet, especially regarding the IFs of the cytoskeleton although many emerging roles of IFs are described by the disease phenotypes linked to them. So far, no diseases coupled to mutations in genes coding for actin filaments or microtubules have been described, in contrary to IFs, which display a connection to more than 80 human diseases.

SEKs are IFs expressed in the single-layered and glandular epithelia, in organs that involve secretion and absorption. SEKs are present in many different cell types in the colon, from nutrient-absorptive enterocytes and hormone-secreting enteroendocrine cells to cells with specialized tasks such as mucus-secreting goblet cells. In these cells different cellular processes are likely dependent on the SEKs present.

The inner layers of the colon facing the intestinal lumen are highly exposed to outer influences, giving the single-layered epithelium of the colon extra challenges besides of the constant functions in nutrient processing. A newly emerging role of the intestinal microbiota has been suggested for intestinal health.

For analyzing the specific roles of colonic SEKs, specific transgenic mouse models and cell lines were used. In this thesis, the colonic model systems were challenged with different stress conditions, where after the outcome was studied in regards keratins and their colonic functions.

REVIEW OF THE LITERATURE

1. Cytoskeleton and intermediate filaments

The cytoskeleton, which contributes to the mechanical integrity of the cell, can be found all over the cell. Apart from mechanical functions, the cytoskeleton has functions in the regulation, migration, division, transport and signaling of cells (Kim, Coulombe 2010). Besides microtubules and actin filaments, the cytoskeleton consists of IFs with an intermediate diameter of 10 nm compared to the small 7 nm actin filaments and the larger 25 nm microtubules. Except for the distinct role of IFs in cell structure and as stress mediators, the role of IF proteins has recently been extended to function as signaling and regulatory proteins (Herrmann et al. 2007, Pallari, Eriksson 2006, Toivola et al. 2005). Another difference between these three cytoskeletal elements is in the polymerization of the proteins. In contrast to microtubules and actin filaments, IFs are non-polar filaments where the assembly and the depolymerization is not restricted to a specific end of the filament (Koster et al. 2015).

While microtubules and actin filaments mainly consist of tubulin and actin respectively, the family of IFs consists of proteins encoded by more than 70 different genes (Hesse, Magin & Weber 2001, Herrmann et al. 2007). IFs are involved in the cause or in the predisposition to disease in more than 80 human diseases, by a predisposition, a mutation or by inclusion-formation associated to IF overexpression (Omary 2009). The classification, tissue distribution and related diseases of IF proteins can be seen in Table 1.

Table 1. IF-proteins, their tissue-distribution and related human diseases. Table created based on (Eriksson et al. 2009, Omary 2009, Szeverenyi et al. 2008, Omary, Coulombe & McLean 2004).

IF type	Protein	Distribution	Related disease
Type I	Acidic keratins	Epithelial cells	Skin, nail, hair, liver disorders
Type II	Basic and neutral keratins	Epithelial cells	Skin, nail, hair, liver disorders
Type III	Vimentin	Mesenchymal cells, lens	Cataract
	Desmin	Muscle cells	Cardiomyopathies
	Syncoilin	Muscle cells	Muscular dystrophy
	Peripherin	Peripheral nervous system	ALS
	GFAP	Astrocytes	Alexander disease
Type IV	Neurofilaments	Central nervous system	ALS, CMT, Parkinson's disease
	Internexin	Central nervous system	-
	Nestin	Neuroepithelial cells	-
	Synemin	Muscle cells	-
Type V	Lamins	Nuclear lamina, all cells	HGPS
Type VI	Bfsp1	Fiber cells, lens	Cataract
	Bfsp2	Fiber cells, lens	Cataract

Besides other types of post-translational modifications (PTM), such as glycosylation, acetylation, farnesylation and ubiquitylation, IFs are highly phosphorylated in response to apoptosis, mitosis and stress, leading to a non-filamentous and soluble form (Snider, Omary 2014, Snider, Omary 2016). For example in liver (Fickert et al. 2003, Toivola et al. 2004b) and the central nervous system (Ackerley et al. 2004), IF hyperphosphorylation serves as a marker and protector of tissue injury.

1.1 Simple epithelial keratins

The keratin subfamily of IFs consists of proteins coded by 54 functional genes clustered on two chromosomal sites; type I keratins on chromosome 17q21.2 and type II keratins on chromosome 12q13.13 (Schweizer et al. 2006). Keratins are divided into type I and type II keratins, which form obligate heteropolymers with each other and they are expressed in a tissue and/or cell-specific manner (Moll, Divo & Langbein 2008).

K7, K8, K18, K19 and K20 are SEKs, which are expressed in polarized (Oriolo et al. 2007), single-layered and glandular epithelia, in organs involving secretion and absorption. The primary and most studied SEK pair is K8/K18. In many cells, such as in hepatocytes and in renal tubular cells, K8/K18 is the only existing keratin-pair, while in intestinal epithelial cells, duct-lining cells, mesothelial cells and in urothelial cells other SEKs are also expressed (Moll, Divo & Langbein 2008, Omary et al. 2009). Other described SEKs are K7, K19, K20 and K23. K7 and K19 are commonly expressed in ductal epithelia, such as in bile ducts and in pancreatic ducts. K20, expressed in intestinal, gastric foveolar and urothelial epithelium (Moll, Divo & Langbein 2008), is used as an immunohistochemical marker of tumorigenesis, since it is expressed in primary and metastatic carcinomas (Moll et al. 1992). The less abundant and not yet well-characterized SEK, K23, is basally expressed in gallbladder and bile ducts, and in smaller amounts in liver and colon (Guldiken et al. 2016). It is also expressed during the differentiation of pancreatic (Zhang et al. 2001) and colorectal (Liffers et al. 2011) cancer cells. Beyond SEK, keratins in stratified epithelium are expressed in *e.g* skin (K2, K5-K6, K9, K14-K16) (Wang, Zieman & Coulombe 2016) and hair (K31-K40, K81-K86) (Schweizer et al. 2007).

1.1.1 Simple epithelial keratin function

The proteins in the keratin family are highly cell type-specifically expressed, and inside the cell they can be found all the way from the nucleus, passing through the cytoplasm, to the cell membrane where they contribute to the cell-cell junctions in desmosomes. This suggests that SEKs play a central role in the mechanical stability and integrity of the cell. Apart from the structural stability, SEKs function as stress protectors (Toivola et al. 2010). SEKs share similar features with other known stress proteins, such as for example heat shock proteins (HSPs), in regards of upregulation of mRNA and/or protein levels in stress and in recovery from stress. The absence or lowered levels of SEKs cause injury or susceptibility to injury,

further confirming the role of SEKs as stress protectors. How this is done is likely SEK- and tissue-specific, but evidence of providing mechanical support, acting as protein scaffolds, serving as anti-apoptotic proteins and/or providing a phosphate buffer has been proposed (Toivola et al. 2010). SEKs are mostly anti-apoptotic and protect from Fas-mediated apoptosis. In mice, $K8^{-/-}$ hepatocytes are more sensitive to apoptosis (Tao et al. 2009, Gilbert et al. 2001), while $K8^{-/-}$ colonocytes show a microbiota-dependent resistance to apoptosis (Habtezion et al. 2011). The role of SEKs in the regulation of cellular organelles has been demonstrated by models where SEK networks have been modified, *e.g.* mitochondria in liver hepatocytes are smaller and irregularly distributed in mice that lack K8 (Tao et al. 2009). The role of SEKs in targeting cellular proteins to their aimed positions has been described both for colonocyte ENaC (Toivola et al. 2004a) and hepatocyte ecto-ATPase (Satoh, Hovington & Cadrin 1999). The chloride transporter downregulated in adenoma (DRA) is dramatically downregulated in $K8^{-/-}$ mice when compared to $K8^{+/+}$ mice, even if it was not mistargeted (Asghar et al. 2016). Disturbances in protein localization due to SEK modifications have also been reported using the K18 Arginine (R) 90 Cysteine (C) mutant cell line, where the proteins desmoplakin and zonula occludens-1 were relocated to keratin inclusions (Hanada et al. 2007). SEKs also display an important role in functioning as signaling platforms, for example by acting as scaffolds and binding the adapter protein 14-3-3, which regulates the activity of many downstream signaling proteins (Ku et al. 2002, Pallari, Eriksson 2006). Recently, the existence of nuclear keratins and their direct role in cell proliferation and gene expression of cancer cells has been suggested (Hobbs, Jacob & Coulombe 2016). Studies focused on nuclei of skin and cervical tumor cells indicate active roles of K17 in gene expression and cell cycle regulation (Hobbs, Jacob & Coulombe 2016) .

1.1.2 Simple epithelial keratin structure

SEKs are obligate heteropolymers, meaning that one type I and one type II keratin form pairs with each other. The common structure of all SEKs is a central coiled-coil α-helical rod domain with a non-α-helical N-terminal head and C-terminal tail domain (Fig. 1) (Omary et al. 2009). The rod domain is highly conserved between all SEKs and it is divided into three subdomains, 1A, 1B and 2, which are linked together by two linkers, L1 and L12 (Omary et al. 2009). The tail domains of the different SEKs are somewhat different in length, for example K19 has a 13-amino acid long tail while the tail of K8 is 85-amino acid long. In spite of the varying length, the head and tail domains include the most commonly known sites for PTMs (Omary et al. 2006). For example, K8 phosphorylation in the head domain occurs mainly on the amino acid Serine (S) 24 (Ku, Omary 1997) and S74 (Liao, Ku & Omary 1997) and K8 phosphorylation on the tail domain occurs on S432 (Ku, Omary 1997). All known phosphorylation sites of SEKs are described in Figure 1.

SEK type I-type II heterodimers form tetramers that are assembled to form unit length filaments (ULF)s. Eight ULFs are further assembled longitudinally, forming mature keratin filaments with a diameter of 10 nm (Herrmann et al. 2009).

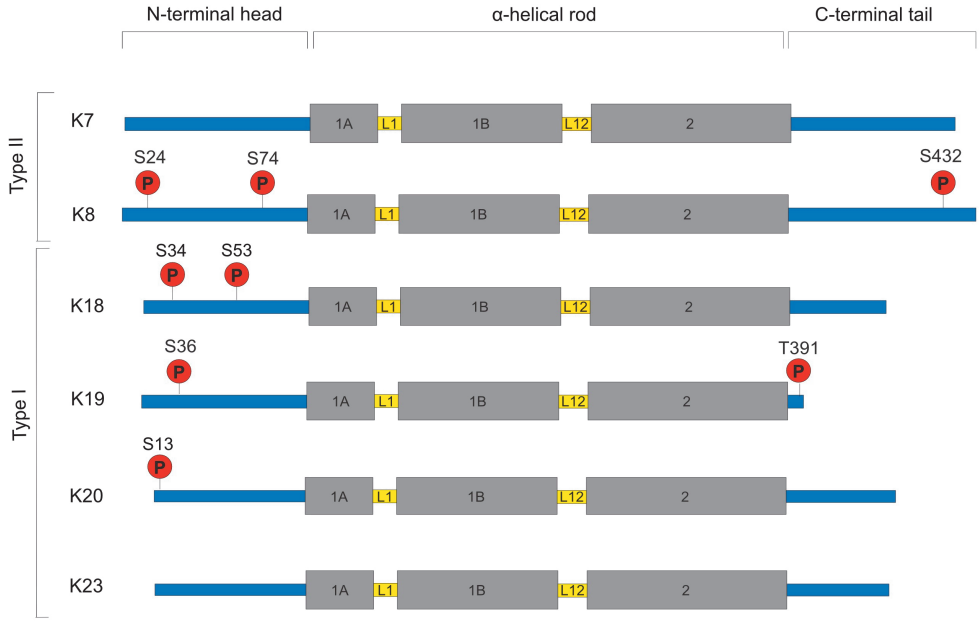

Figure 1. Structure and known phosphorylation sites of SEKs. SEKs consist of a central coiled-coil α-helical rod domain (consisting of subdomains 1A, 1B and 2, linked together by two linkers, L1 and L12) with a non-α-helical N-terminal head domain and C-terminal tail domain. Known SEK phosphorylation sites are described for K8 (Liao, Ku & Omary 1997, Ku, Omary 1997), K18 (Ku, Omary 1994, Ku, Liao & Omary 1998), K19 (Zhou et al. 2010, Zhou et al. 1999) and K20 (Zhou et al. 2006).

1.1.3 Simple epithelial keratin regulation

1.1.3.1 Transcriptional regulation

Although it is known that keratins are extensively regulated at transcriptional level, the exact mechanism of how this is done is not well understood. No universal regulators of all SEKs have been found, and therefore it is thought that the expression of all SEKs have their own specific transcription mechanism including their own set of transcription factors and promoter regions.

The mechanisms on transcriptional regulation of K18 are the most extensively studied of all SEKs. The promoter, the regulatory sites and the enhancer of transcription have been mapped for the K18 gene (Prochasson et al. 1999). Transcription factors shown to be involved in the regulation of K18 transcription are Specificity protein 1 (SP1) (Gunther et al. 1995), Activator protein-1 (AP-1)(Rhodes, Oshima 1998), CREB-binding protein/p300 (CBP/p300) (Prochasson et al. 1999) and E26 transformation-specific (ETS) (Pankov et al. 1994). However, other mechanisms have also been shown to influence the gene transcription of K18, e.g. the acetylation state of the transcription factors binding the promoter enhances K18 gene transcription (Gunther et al. 1995), while DNA methylation represses the transcription of the K18 gene (Umezawa et al. 1997).

The transcriptional regulation of K8 is not as extensively studied as the gene transcription of K18. K8 expression is regulated by the binding of tumor suppressor protein p53 (p53) to the 5' region of the K8 gene. Deletion of this p53-binding region abolished K8 activation, suggesting that the expression of K8 is regulated by p53 binding (Mukhopadhyay, Roth 1996). Similarly, transcription factors ETS1 and ETS2 have been shown to be involved in the regulation of K8 gene expression by binding to the enhancer of the K8 gene at the 3' region, which contains binding sites for ETS (Mukhopadhyay, Roth 1996, Takemoto et al. 1991). Except for K8 and K18, the transcriptional regulation of K19 has been studied to some extent. Regulatory elements on the K19 gene have been identified (Hu, Gudas 1994), and transcription factors Kruppel-like factor 4 (KLF4) and SP1 are known to bind these sites (Brembeck, Rustgi 2000).

1.1.3.2 Post-translational modifications

PTMs occurring on the highly dynamic SEKs in response to stress, mitosis or apoptosis are phosphorylation, glycosylation, sumoylation and acetylation. The best-studied PTM of SEKs is phosphorylation, which maintains the structural keratin dynamics by regulating the assembly and disassembly of keratin filaments, and therefore regulating the solubility (Omary et al. 2006, Izawa, Inagaki 2006, Snider, Omary 2014). During baseline conditions, 95% of all SEKs are in an insoluble filamentous form, meaning that only 5% of the soluble SEKs are continuously hyperphosphorylated. In response to stress, mitosis or apoptosis, SEKs are phosphorylated. This phosphorylation serves as a protecting phosphate "sponge", binding phosphate from other phosphate-activated proteins (Omary et al. 2006). The SEK phosphorylation sites are found in the head and tail domains, mostly at serine residues (Fig. 1). Phophorylation of K8 is modulated by stress-activated protein kinases (Table 2), for example by protein kinase C (PKC)(Omary et al. 1998) c-Jun N-terminal kinase (JNK) (He et al. 2002), p38 mitogen-activated protein kinase (p38 MAPK) (Ku, Azhar & Omary 2002) and PKCδ (Ridge et al. 2005). Rapidly accelerated fibrosarcoma-1 (Raf-1) has been described as a K18 phosphorylation kinase (Ku, Fu & Omary 2004) Protein phosphatase 2A (PP2A) has been described as a phosphatase for K8 (Tao et al. 2006).

Table 2. Selected SEK kinases. Description of SEK kinases, their site of action and associated event indicate that SEKs are mostly phosphorylated in stress, mitosis and/or apoptosis.

SEK	Site	Kinase	When	Ref.
K8	S74	JNK	Stress, mitosis, apoptosis	(He et al. 2002)
K8	S74	p38	Stress, mitosis, apoptosis	(Ku, Azhar & Omary 2002)
K8	S432	MAPK, Cdc2	EGF stimulation, mitosis	(Ku, Omary 1997)
K8	S24	MAPK	Stess, mitosis	(Ku, Omary 1997)
K18	S53	CAM, S6, PKCδ	Stress, mitosis	(Ku, Omary 1994)
K19	ST391	Src	Stress	(Zhou et al. 2010)
K20	S13	PKCδ	Stress, apoptosis	(Zhou et al. 2006)

In regards of glycosylation, K8 and K18 are the most studied keratins. They are glycosylated by O-linked glycosylation, where β-N-acetylglucosamine is added to serine/threonine residues. K18 is known to be glycosylated at S30, S31 and S49 in the head domain, and the major role of this PTM is to facilitate the phosphorylation kinases in response to cellular stress and injury (Ku et al. 2010, Chou, Smith & Omary 1992). K8, K18 and K19 can also be post-translationally modified by sumoylation, where a small ubiquitin-like modifier (SUMO) is covalently attached to lysine residues. During baseline conditions, SEKs are not sumoylated, but an increase in sumoylation can be seen in response to oxidative and apoptotic stress or during hyperphosphorylation seen in human liver disease (Snider et al. 2011). Another PTM regulating SEK function is acetylation. Except for modifying histones as a transcriptional control site, post-translational acetylation is known to regulate K8 function by reducing the solubility of K8 and forming filamentous keratin networks. Basally K8 is acetylated at several identified lysine residues (Leech et al. 2008), which promotes K8 filament stabilization. Increased K8 acetylation at Lysine 208 has been observed during hyperglycemia (Snider et al. 2013) and in response to butyrate treatment (Drake et al. 2009).

1.2 Genetic mouse models of simple epithelial keratins

Genetic mouse models have been proven very important in determining the roles of SEKs. A selection of generated mouse models are described here, divided in knockout (KO) mouse models (Table 2) and transgenic (TG) mouse models (Table 3), which are further classified into WT overexpressor and SEK variant overexpressor mice.

1.2.1 Knock out mouse models

The functions of SEKs are demonstrated by the SEK knock out mouse models (Table 3). The first described SEK knock out mouse was the $K8^{-/-}$ mouse generated in the C57Bl/6 background (Baribault et al. 1993). Displaying a 95% embryonic lethality, an extensively studied knock out was created in the FVB/n background with only 50% embryonic lethality (Baribault et al. 1994). This model displays a hepatic, colonic and pancreatic phenotype, including mild hepatitis, increased susceptibility to liver injury (Ku et al. 2007), colorectal hyperproliferation, a TH2-type colitis (Habtezion et al. 2005), impaired protein targeting (Toivola et al. 2004a, Asghar et al. 2016) and abnormal insulin and glucose metabolism (Alam et al. 2013).

Contrary to the $K8^{-/-}$ mouse, the $K18^{-/-}$ mouse does not develop embryonic lethality or a major intestinal phenotype, likely due to the compensatory role of the other co-existing type I keratin K19 (Magin et al. 1998). When both K18 and K19 are deleted, early embryonic lethality is observed, supporting the role of K19 as a compensatory keratin for K18 (Hesse et al. 2000). Other knock out models that display high embryonic lethality are the K type $I^{-/-}$ and K type $II^{-/-}$ mice, which lack all type I and type II keratins respectively. K type $I^{-/-}$ mice die at birth due to barrier

defects and fragile skin (Kumar et al. 2016), whereas K type II$^{-/-}$ die at embryonic day 9.5 due to growth retardation (Vijayaraj et al. 2009, Bar et al. 2014). However, the deletion of K7 demonstrates only a mild phenotype (Sandilands et al. 2013), with hyperprolifereation of the bladder urothelium. Similarily, the K19$^{-/-}$ mouse displays a mild phenotype with a mild myopathy (Stone et al. 2007) and a cholestatic liver injury (Chen et al. 2015).

Table 3. SEK knock out mouse models. Knock out mouse models of SEKs generated in C57Bl/6 or FVB/n strain and their described phenotypes. Table created based on (Strnad et al. 2016, Omary et al. 2009).

Keratin modification in mouse strain	Phenotype	Reference
K7$^{-/-}$ in C57Bl/6	Increased proliferation of bladder uroth	(Sandilands et al. 2013)
K8$^{-/-}$ in C57Bl/6	Gestational lethality by liver failure	(Baribault et al. 1993)
K8$^{-/-}$ in FVB/n	Colorectal hyperplasia in cecum, colon and rectum with colitis and mild hepatitis	(Baribault et al. 1994)
K18$^{-/-}$ in C57Bl/6	No clear phenotype in young mice, old mice develop MDB, susceptibility to Fas- and TNFα-induced liver injury	(Magin et al. 1998)
K19$^{-/-}$ in FVB/n	Mild myopathy, compensatory overexpression of K18 and K20 in gallbladder, attenuated ductal reaction in liver injury	(Stone et al. 2007)
K8$^{-/-}$/K19$^{-/-}$ in FVB/n	Embryonic lethality due to defects in plancenta	(Tamai et al. 2000)
K18$^{-/-}$/K19$^{-/-}$ in FVB/n	Embryonic lethality due to defects in placental trophoblast giant cells	(Hesse et al. 2000)
K type I$^{-/-}$ in FVB/n	Embryonic lethality due to barrier defects and fragile skin	(Kumar et al. 2016)
K type II$^{-/-}$ in FVB/n	Embryonic lethality due to severe growth retardation	(Vijayaraj et al. 2009, Bar et al. 2014)

1.2.2 Transgenic mouse models

In order to study how increased levels of SEKs affect the phenotype and for the generation of controls for many overexpressor mutations, both human and mouse K8 overexpressing mice have been generated (Table 4). Mice overexpressing human K8 show a phenotype of pancreatitis (Casanova et al. 1999) and epidermal abnormalities (Casanova et al. 2004), but also increased liver injury (Ku, Omary 2006) and Mallory Denk body (MDB)-formation in response to high-fat diet (Kucukoglu et al. 2014, Ku, Omary 2006). When murine K8 is overexpressed, mice are susceptible to develop increased amounts of MDBs (Nakamichi et al. 2005) and a exocrine pancreatic phenotype including spontaneous chronic pancreatitis and increased levels of type I keratins K18, K19 and K20 (Toivola et al. 2008). Overexpression of human WT K18 protects mice from MDB formation (Harada et al. 2007), while WT K19 (Bader, Franke 1990) and K20 (Zhou et al. 2003) overexpressing mice show no phenotype.

To understand the effects of mutations in human disease and the roles of PTM sites, mice overexpressing SEK variants (Table 4) were generated. They have mostly been studied in regards of the liver phenotype, explaining the liver-dominant phenotypes described here. Overexpression of the most common human mutation of K8 in mice, the Glycine (G) 62C mutation, does not show any basal phenotype, but exhibits a stress-induced liver injury in response to a secondary stress (Ku, Omary 2006, Guldiken et al. 2015b). Many mutations of SEK phosphorylation and glycosylation in overexpressing mouse lines are used for studying PTMs. For example, the human K8 S74A (Ku, Omary 2006) and the human K8 R341C/H (Guldiken et al. 2015b) variants predispose mice to liver injury. When all known K18 glycosylation sites are mutated (K18 S30/31/49A), mice do not show any basal phenotype, only susceptibility to liver and pancreatic injury (Ku et al. 2010).

Table 4. Selection of transgenic mice overexpressing SEK variants. Mice overexpressing SEK variants and their described phenotype. Table created based on (Strnad et al. 2016, Omary et al. 2009).

Keratin modification	Phenotype	Reference
K8 human WT overexpression	Pancreatitis and epidermal and hair follicle dysplasia	(Casanova et al. 1999, Casanova et al. 2004)
K8 human WT overexpression	Increased liver injury and MDB formation after feeding with high fat diet	(Ku, Omary 2006, Kucukoglu et al. 2014)
K8 mouse WT overexpression	MDB formation during aging	(Nakamichi et al. 2005, Toivola et al. 2008)
K18 human WT overexpression	Protected from MDB formation	(Harada et al. 2007)
K19 human WT overexpression	None	(Bader, Franke 1990)
K20 human WT overexpression	None	(Zhou et al. 2003)
K8 human G62C	Predisposition to selected liver stress situations (Fas, microcystin LR, acetaminophen) and inhibits phosphorylation at S74, reduced ability to form MDBs	(Guldiken et al. 2015b, Ku, Omary 2006)
K8 human S74A	Predisposition to liver injury, reduced ability to form MDBs	(Ku, Omary 2006)
K8 human R341C/H	Predisposition to hepatotoxicity	(Guldiken et al. 2015b)
K18 human S30/31/49 overexpression	Susceptible to induced apoptosis, liver and pancreas injury	(Ku et al. 2010)
K type II	Embryonic lethality due to severe growth retardation	(Vijayaraj et al. 2009)

1.3 Intermediate filaments and simple epithelial keratins in stress protection

Except for roles in mechanical stability, the involvement of IF proteins in the cellular stress response has recently emerged, which has been reviewed in (Toivola et al. 2010). For example, in response to shear stress, vimentin is overexpressed in endothelial cells (Tsuruta, Jones 2003) and lamin A in nuclei (Philip, Dahl 2008), while skin keratins K6, K16 and K17 are overexpressed in response to wound healing and UV-stress (Freedberg et al. 2001, DePianto, Coulombe 2004).

Regarding SEKs, their dynamic nature is reflected by the fast assembly and disassembly of the filamentous network during cell growth and differentiation, but also in response to stress. Together with HSPs, the keratin protein family was identified as the most differentially expressed protein family when proteomics data was collected and meta-analyzed from 188 earlier published articles (Petrak et al. 2008). K8 was found on place 10, displaying the first piece of evidence for the role of SEKs in stress protection. In a similar way as HSPs, SEKs are upregulated both on mRNA and protein levels upon stress and recovery (Fig. 2). In the liver, stress-induced overexpression of hepatic keratins has been shown in human liver disease, in murine stress models and in *in vitro* stress-studies (Guldiken et al. 2015a, Zatloukal et al. 2007, Guldiken et al. 2016). Also in other organs of the digestive track, as in pancreas, caerulein- and choline/methionine-deficient diet-induced murine pancreatitis causes an overexpression of pancreatic SEKs (Zhong et al. 2004, Wogenstein et al. 2014). Similar organ-specific overexpression of SEKs has been reported in kidneys during murine fibrosis, toxin- and injury-related models, as well as in human kidney disease (Djudjaj et al. 2016). Similarly, *in vitro* alveolar epithelial keratins are upregulated in response to shear stress (Sivaramakrishnan et al. 2009).

SEK phosphorylation has previously been described during stress, mitosis and apoptosis. In response to stress, K8 (Toivola et al. 2002, Djudjaj et al. 2016, Ridge et al. 2005, Fausther, Villeneuve & Cadrin 2004, Burcham, Raso & Henry 2014, Liao, Ku & Omary 1997, Toivola et al. 2004b), K18 (Sivaramakrishnan et al. 2009, Djudjaj et al. 2016, Guldiken et al. 2015a, Toivola et al. 2004b) and K20 (Zhou et al. 2006) are known to be phosphorylated. K8/K18 phosphorylation in response to liver and kidney stress are used as markers of disease (Toivola et al. 2004b, Djudjaj et al. 2016). Shown by K8 phosphomutant mice, hyperphosphorylated K8 acts as a phosphate buffer/sponge, where stress-activated protein kinase-mediated apoptosis is prevented(Ku, Omary 2006). Therefore K8 and especially overexpression of K8 in stress protects the tissue from injury by serving as a phosphate sponge.

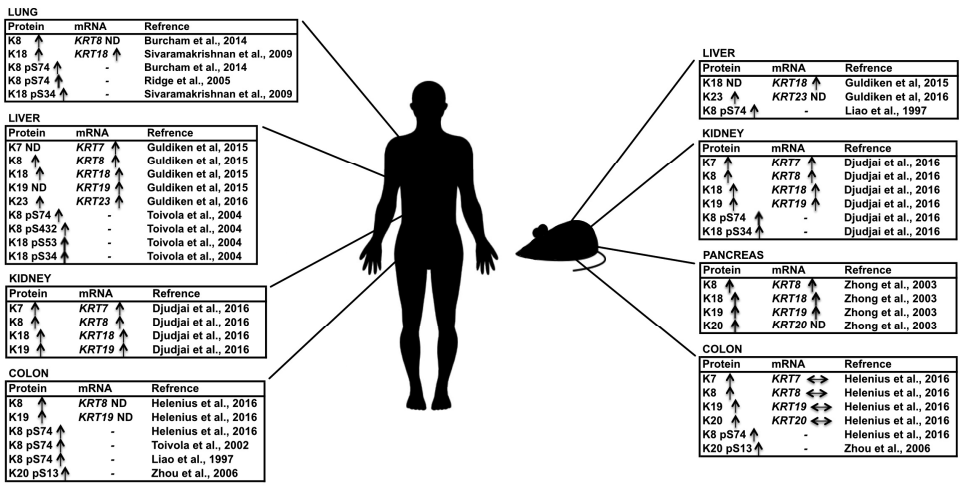

Figure 2. SEKs in stress protection. Human and mouse SEKs show significant upregulations and increased phosphorylations in response to different stress models. ↑ = upregulation, ↓ = downregulation, ↔ = no change, ND = not determined.

1.4 Gene mutations of simple epithelial keratins

Mutations in K5 and K14 in the keratinocytes of the skin are known to cause epidemolysis bullosa simplex (EBS), a skin blistering disease (Lane, McLean 2004). In contrary to this clear connection of disease and keratin mutations in stratified squamous epithelium, the role of keratin mutations in simple epithelial disease is still not clear, even if some evidence for hepatic, intestinal and pancreatic disease exist. About 5% of the human population has mutations in *KRT8* and *KRT18* genes, including mostly patients with liver disorders (Toivola et al. 2015).

In hepatocytes, it has been shown that mutations in *KRT8* and *KRT18* genes predispose to human liver diseases (Ku et al. 2003, Ku et al. 2007, Omary et al. 2009), meaning that a second hit, *e.g.* toxins or alcohol, is needed for the outbreak of the disease. K8 variants found in humans with liver disease are G62C, Tyrosine (Y) 54 Histidine (H), R341H and G434S. The mutations predisposing to liver diseases, K8 G62C and K8 G434S, are known to inhibit phosphorylation of nearby K8 at S74 and S432, respectively, shown by mouse models that overexpress these human mutants (Ku, Omary 2006, Omary et al. 2009).

Assessment of the role of K8 mutant variants in inflammatory bowel disease (IBD) pathogenesis failed to show any major correlation in two major studies (Buning et al. 2004, Tao et al. 2007), even if heterozygous missense mutations on *KRT8* gene have been identified in a few patients (Owens et al. 2004, Owens, Lane 2004). The *KRT8* and *KRT18* gene has been localized to the *IBD2* locus on chromosome 12 (de Jong, Drenth 2004, Waseem et al. 1990), suggesting a link between IBD and K8/K18 mutations. So far, K8 mutations are only considered as predisposing factors for IBD. Similarly as in the intestine, SEK variants have not been associated with pancreatic disease in neither human patients (Treiber et al. 2006, Schneider et al. 2006) nor mouse models (Toivola et al. 2008). Disease associations for

mutations in the minor SEKs K7, K19, K20 and K23 have not been identified in detail so far.

1.5 Associated diseases of simple epithelial keratins

Animal models have contributed to this knowledge by providing insights about SEK function by knock out, knock in and overexpressor models. Keratins are reorganized in disease, for example by upregulation, aggregation or by postranslational modifications (Toivola et al. 2015, Omary et al. 2009, Omary 2009, Haines, Lane 2012).

In liver disease, such as alcoholic liver disease (ALD), nonalcoholic steatohepatitis (NASH) and hepatitis C infection (HCV) increased K8 expression partly due to the formation of Mallory Denk bodies (MDBs) is seen (Zatloukal et al. 2007). MDBs are protein aggregates that constitute of K8, K18, ubiquitin and ubiquitin-binding protein p62, and they serve as a marker of liver stress. MDBs are characterized by an unbalanced K8/K18 ratio, where excess K8 is predominant (Zatloukal et al. 2007).

Some evidence of SEK associated diseases in the intestine has been reported. In multifactorial diseases, such as in intestinal diseases, SEKs are proposed to play a role in the overall intestinal health. The first evidence of SEK involvement in IBD comes from the $K8^{-/-}$ mouse model (Baribault et al. 1994), which suffers from an early colitis-like phenotype, which is amendable to antibiotic treatment (Habtezion et al. 2005). Colonic keratin levels are altered in human colitis (Corfe et al. 2015), providing further evidence on the role of colonic keratins in disease development and progression. The $K8^{-/-}$ mouse does not develop colorectal adenocarcinomas spontaneously, but when predisposed to a chemical or a genetic tumor model, colorectal adenocarcinomas were formed in the colon due to an increase in the inflammasome activity. This provides further evidence on the modulating role of keratins on colonic inflammation and tumorigenesis (Misiorek et al. 2016).

Pancreatic SEKs have been shown to increase in caerulein-induced and choline/methionine-deficient diet-induced pancreatitis of the exocrine pancreas (Zhong et al. 2004, Toivola et al. 2000). Regarding endocrine pancreas, K8 deletion causes an increase in glucose tolerance together with insulin sensitivity in diabetes-induced stress (Alam et al. 2013). The newly emerging role of keratins in the kidney has been demonstrated by a study showing keratin upregulation in human kidney injury due to myeloma cast nephropathy, diabetic nephropathy and systemic lupus erythematosus, as well as in mouse models for kidney tubulointerstitial fibrosis, adenine nephropathy, ischemia-reperfusion injury, folic acid nephropathy and glomerulonephritis (Djudjaj et al. 2016). Therefore, renal SEKs should be considered as potential markers of tubular cell stress in diseased murine and human kidneys. It is still unclear if keratin mutations are found in patients with kidney disease (Snider 2016). In other organs with simple epithelial linings, such as the thymus (Odaka et al. 2013), lung (Sivaramakrishnan et al. 2009)

and gallbladder (Kasprzak et al. 2011, Tao et al. 2003), SEKs are likely required mainly in the mechanical stress protection, but no clear connections between SEKs, diseases and mutations have been established or studied.

SEKs are also used as markers of disease. Keratins can be used as markers of tumors with epithelial origin, while the origin of the primary tumor can be distinguished by determining the exact keratin expression pattern of the metastasis (Omary et al. 2009). For example hepatocellular carcinomas express K8, K18 and K19 (Kim et al. 2011, Govaere et al. 2014), while cholangiocarcinomas express K7, K8 and K18 and colorectal carcinomas only K20 (Omary et al. 2009, Chu, Weiss 2002, Tot 2002).

The treatment of SEK related diseases are still limited and mostly palliative, meaning that the symptoms of the disease are treated rather than the actual cause. Gene therapy is considered as another future possible treatment of SEK-related diseases, even if this direction is still uncharted (Omary 2009, Haines, Lane 2012).

2. Colon

The digestive tract, which extends from the mouth to the anus, consists of the oral cavity, esophagus, stomach, pancreas, liver, small intestine and colon. In humans, the colon is approximately 1.5m long, and it is divided from the proximal to distal end into the ascending colon, transverse colon, descending colon, sigmoid colon and rectum. The inner part of the colon, i.e. the lumen, is lined with a single-layer of epithelial cells that form invaginations, called colonic crypts (Fig. 3). The lamina propria is situated underneath the epithelial crypts, where immune cells, blood vessels, lymphatics and nerves are found. Beneath the lamina propria is a thin muscular layer that contributes to the local peristaltic movements of the colon. In the submucosa, blood vessels and collagen fibers can be found, whereas the next muscular layer consists of smooth muscles responsible for the continued peristaltic movements. The outermost layer of the colon is the serosa, which consists of connective tissue, protecting the colon from outer damage in the abdominal cavity (Ross 2015).

The main function of the intestinal epithelium is to absorb excess water and salts from food digested in the small intestine. Another important function is to absorb vitamin K, vitamin B12, riboflavin and thiamin as fermentation products from intestinal bacteria. The colonic epithelium forms the foremost protection against outer stresses, such as microbes and toxins ingested with the food (Ross 2015).

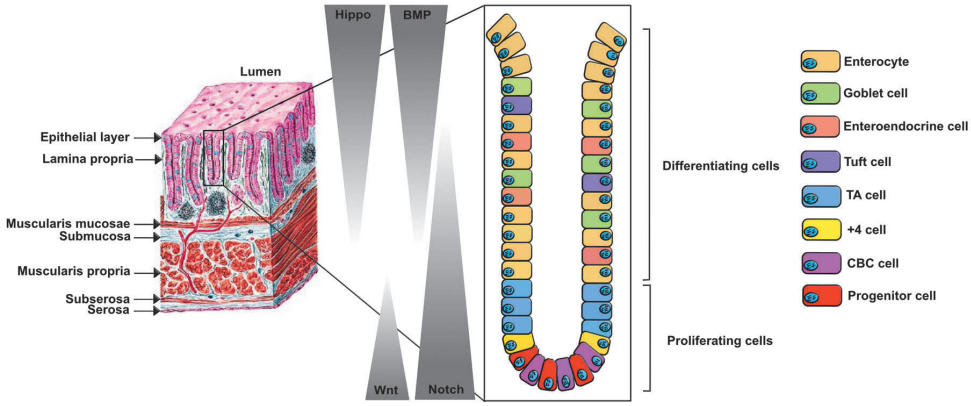

Fig. 3. Colon layers, cell types and regulatory signaling pathways. Serosa, muscular layers, mucosal layers, lamina propria and epithelial layers make up the colonic structure. The single-layered epithelium consists of colonic crypts, where proliferating epithelial progenitor cells are regulated by Wnt signaling based at the bottom of the crypts and Notch-signaling which regulates the differentiation of the cells at the top of the crypts. Hippo and BMP signaling inhibits proliferation and induces maturation of all secretory cell types. Figure based on (Jeon et al. 2013).

2.1 Colon epithelial cells

The colonic crypts consist of a single layer of epithelial cells. Undifferentiated colonic stem cells are found at the bottom of the crypts, from where they proliferate further upwards in the crypt and differentiate into different kinds of colonic cells (Fig. 3). Eventually, these cells die of apoptosis at the top of the crypt after 3-5 days (Clevers 2013, Ratanasirintrawoot, Israsena 2016).

At the bottom of the crypts, crypt base columnar (CBC) cells and +4 cells have been identified as intestinal stem cells, which express Lgr5, widely used as an intestinal stem cell marker (Cheng, Leblond 1974, Ratanasirintrawoot, Israsena 2016, Clevers 2013). These intestinal stem cells have been shown to be able to differentiate to all intestinal cell types both *in vivo* (Barker et al. 2007) and *in vitro* (Sato et al. 2009). As the colonic stem cells move towards the intestinal lumen, they lose their progenitor cell identity and become transit-amplifying (TA) cells. TA cells can differentiate into two cell lineages, absorptive cells (enterocytes) and secretory cells (goblet cells, tuft cells and enteroendocrine cells) (Clevers 2013).

Cell types present at the whole differentiating crypt-axis are enterocytes, enteroendocrine cells, goblet cells and tuft cells. The main intestinal cells are the absorptive enterocytes, whose main function is absorption of water and nutrients. Enterocytes are polarized cells with a luminal brush border. Enteroendocrine cells are specialized cells that secrete their own cell-specific hormone (Gunawardene, Corfe & Staton 2011), as for example somatostatin that inhibits other gastrointestinal hormones. The role of enteroendocrine cells in colorectal cancer (CRC) is still unclear, but it has been suggested that enteroendocrine cell perturbation may have a role in the pathology of CRC (Hamada et al. 1992) or even the other way around, that colorectal tumors themselves cause perturbations in the

balance of enteroendocrine cells (Nitta et al. 2001). Mucus-secreting goblet cells are distributed all over the crypt length. Recent studies have shown interactions between colonic microbiota and goblet cells by showing that an IBD-like phenotype contributes to intact mucus layers in mice (Johansson, Hansson 2013).

Different junction proteins mediate the cell-cell contact between the epithelial cells of the colonic epithelium. Desmosomal proteins from the cadherin-family tie the epithelial cells together by connecting the cytoskeletal SEK networks of two cells together, forming an intact epithelium in between cells (Nekrasova, Green 2013). Gap junction proteins, proteins from the connexin-family, form pores between cells, allowing cytosolic matter to be transported from one cell to another (Bennett et al. 1991). The most important proteins in regards of the intestinal barrier are the tight junction proteins from claudin and occuldin protein-families, which form junctions around the top of the intestinal epithelium (Groschwitz, Hogan 2009). Other junction proteins found in the intestine are catenin in adherens junctions at the membranes between cells and hemidesmosomes that attach the epithelial cells to the basal lamina (Groschwitz, Hogan 2009).

2.1.1 Colonocyte proliferation and differentiation

The colonic epithelium is constantly in a renewal state, and the whole epithelial layer is renewed in 3-5 days. The homeostasis of the intestinal epithelium is maintained by a number of signaling pathways. The Hedgehog, TFG-β and BMP pathways maintain the crypt structure, while the Wnt, Notch, and Hippo signaling pathways together control the cell proliferation and differentiation from intestinal stem cells (Fig. 3) (Jeon et al. 2013).

The proliferation of colonic epithelial stem cells is highly regulated by the Wnt-signaling pathway and its key regulator β-catenin (Clevers, Nusse 2012, Pinto et al. 2003, Haegebarth, Clevers 2009). The repressing of this pathway by the tumor suppressor Adenomatous polypsis coli (APC), which is a negative regulator of the Wnt-signaling pathway, drives the colon epithelia towards CRC (Krausova, Korinek 2014, Jeon et al. 2013). As the Wnt-signalling pathway controls the proliferation of the intestinal epithelium, the differentiation of CBC stem cells to differentiated cells is regulated by the Notch-signaling pathway (Clevers 2013, Sancho, Cremona & Behrens 2015) . Active Notch signaling leads to high differentiation levels of progenitor cells to absorptive enterocytes, while the inhibition of Notch signaling shifts the progenitor cell differentiation towards secretory cells such as goblet cells, enteroendocrine cells and tuft cells (Sancho, Cremona & Behrens 2015).

In contrast to the Wnt- and Notch signaling pathway, the Hippo signaling pathway regulates intestinal regeneration and tumorigenesis. This tumor-suppressing pathway inhibits proliferation and the deletion of the downstream protein Yes-associated protein (YAP), results in undifferentiated stem cells and in the absence of all secretory cell types (Cai et al. 2010, Karpowicz, Perez & Perrimon 2010). Another proliferation inhibitory pathway is the BMP pathway, which antagonizes

the Wnt-pathway and induces maturation of the secretory cell types (Jeon et al. 2013, Radtke, Clevers & Riccio 2006). A signaling pathway that regulates intestinal repair is the hedgehog-signaling pathway. Hedgehog signaling decreases during the injury phase and increases during the repair phase. This pathway activates transcription factors from the glioblastoma (GLI)-family, which in turn activate transcription of target genes. The three known hedgehog-proteins regulating the hedgehog-pathway in the intestine are sonic hedgehog (Shh), indian hedgehog (Ihh), and desert hedgehog (Dhh) (van den Brink 2007, Liang et al. 2012) . The transforming growth factor β (TGF-β) signaling pathway regulates embryonic development, repair, proliferation, and cell differentiation of the intestine (Shi, Massague 2003).

2.2 Colonic microbiota

The microbiota of the gastrointestinal tract consists except for archea, viruses and fungi, mostly of bacteria, which include non-pathogenic commensal bacteria (bacteria that do not cause disease) and pathogenic bacteria (bacteria that cause disease) that inhabit the intestine in symbiosis. The intestinal bacteria can shift from non-pathogenic to pathogenic state in response to stress situations, such as in disease (Chow, Tang & Mazmanian 2011, Sekirov et al. 2010). Perturbations in the intestinal microbiota have been implied to be a major cause in the development of IBD, which has been verified partly by the fact that the areas of active IBD are areas with high numbers of bacteria (Quigley 2013), and partly by studies showing a positive outcome for IBD patients by modifying their microbiota by probiotics (Rachmilewitz et al. 2004, Isolauri, Salminen 2005). Furthermore, *Faecalibacterium prausnitzii*, which is a bacterium with anti-inflammatory properties, has been found to be less abundant in patients with IBD compared to healthy controls (Sokol et al. 2009). Also, many recent studies have shown successful outcomes in treating *Clostridium difficile* diarrhea with faecal microbiota transplants (FMT) (Leszczyszyn, Radomski & Leszczyszyn 2016).

The role of microbiota in the development of obesity (Ridaura et al. 2013, Turnbaugh et al. 2006, Ley 2010), cancer (Wu et al. 2009) and autoimmune diseases (Kim, Yoo & Kim 2016) such as diabetes, rheumatois arthritis and multiple sclerosis has been reported. The presumed protective role of the commensal microbiota comes from inhibiting proliferation of pathogenic bacteria, competing for nutrients and occupying the mucosa of pathogens (Nagao-Kitamoto et al. 2016). The intestinal microbiota has also been proposed to play an important role in the energy homeostasis of the colonic epithelium by maintaining the cell barrier and anti-inflammatory immunity with the help of short chain fatty acids (SCFA). SCFA are produced by intestinal microbiota, which fermentate dietary fiber into SCFA (Furusawa et al. 2013, Lewis, Heaton 1997, Quigley 2013). Another important function of colonic microbiota is the synthesis of vitamins B and K (Quigley 2013).

The intestinal microbiota consists of 300-500 different bacterial species and 2 million different bacterial genes (Quigley 2013). In the adult intestine, the most prominent phyla are *Firmicutes* and *Bacteroidetes* (Fig. 4), which are developed by the age of three years (Qin et al. 2010, Sekirov et al. 2010). The composition of the adult microbiome is thought to be individual, even though the genera *Enterococcus*, *Clostridium*, *Bifidobacterium* and *Bacteroides* are found dominant in all adult intestines regardless of ethnicity, culture or sex (Arumugam et al. 2011). Before the age of three years, *Proteobacteria* and *Actinobacteria* dominate the intestine of the infant (Koenig et al. 2011), and the colonization of the newborns sterile intestine by these species is influenced by different factors such as mode of delivery, sanitation and exposure to antibiotics (Marques et al. 2010). During the aging of the intestine, the same phyla *Firmicutes* and *Bacteroidetes* are found dominant, even if the ratio between these radically shifts towards *Bacteroidetes* (Mariat et al. 2009).

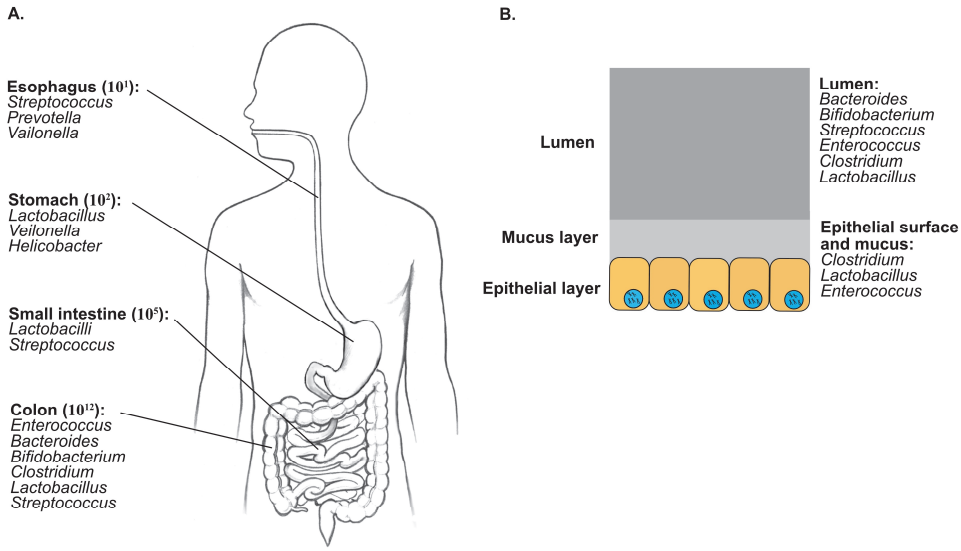

Figure 4. The bacterial genera present in the adult gastrointestinal tract. A. The amount of bacteria (bacteria/gram) in the digestive tract increases towards the colon, where the phyla *Firmicutes* and *Bacteriodetes* are prominent. B. Longitudinal variations in bacteria and bacterial phyla found in the colon. Figure based on (Sekirov et al. 2010, Pei et al. 2004).

2.3 Colon in stress and recovery

The colon is exposed to various stresses through the colonic lumen and the intact single-layered epithelium that lines the inner part of the colon acts as the first line of response against outer stress. The fast renewal of the colonic epithelium enables regeneration in response to stress and injury. The most common stress models related to human diseases and the treatment of human diseases are described below.

2.3.1 Inflammational stress and model systems

The intestinal inflammational stress response is characterized by the loss of intestinal barrier function and increased epithelial permeability, mediated by cytokines and immune cells (Groschwitz, Hogan 2009). The most common mediators of intestinal inflammation in IBD are pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) (Ye, Ma & Ma 2006, MacDonald et al. 1990), interferon-γ (IFNγ) (MacDonald et al. 1990) and interleukin-22 (IL-22), together with the pro- and anti-inflammatory cytokines TGFβ and interleukin-10 (IL-10) (Madsen et al. 1999). They are produced by different T cell differentiation patterns, so that cytokines secreted in Crohn's disease (CD) are derived by TH1 and TH17 T cell differentiation while ulcerative colitis (UC) cytokines are derived from TH2 T cell differentiation (Strober, Fuss 2011). Anti-inflammatory cytokines are involved in maintaining intestinal homeostasis, shown for example by the IL-10 KO mouse which develops colitis, while pro-inflammatory cytokines are known to be increased in diseased intestinal tissues such as in IBD and CRC (Sanjabi et al. 2009).

The most commonly used model for colonic inflammation is the chemically induced dextran sulphate sodium (DSS)-colitis, administered in drinking water. DSS-treatment causes weight loss, occult blood in stool, piloerection and anemia. Histologically, mucin depletion, neutrophil infiltration, cryptitis, crypt abscess and inflammation in mucosa and submucosa can be seen. DSS is directly toxic to gut epithelial cells by affecting the integrity of the mucosal and epithelial barrier. This loss of barrier function enables entry of luminal microbiota and their products to the underlying lamina propria, which stimulates the innate immune system and causes an immune response. DSS-colitis is duration-, concentration-, molecular size- and strain-dependent, meaning that the severity of the experimental colitis varies with different experimental setups (Perse, Cerar 2012). For example, acute DSS colitis is achieved by a 2% DSS-treatment for one week, while chronic colitis requires a cyclic treatment with recovery periods in between (Breynaert et al. 2013). The molecular size of the used DSS also determines the outcome of the treatment. 54 kDa DSS is carcinogenic due to the induction of colitis-induced dysplastic regions, while larger or smaller molecular sizes of DSS do not induce carcinogenic activity in the colon. The molecular size of DSS also affects the location of the colitis; for example 40 kDa DSS induces colitis in distal colon (DC) while 5 kDa DSS induces colitis in proximal colon (PC) and cecum (Perse, Cerar 2012, Rose, Sakamoto & Leifer 2012).

Another murine inflammational model that causes colonic hyperplasia, diarrhea and colonic inflammation is the *Citrobacter rodentium*-induced colitis model. *Citrobacter rodentium* is a gram-negative bacterium that is administered by oral gavage, after which it colonizes the gastrointestinal system and acts as a rodent equivalent to the human bacterial infection *Eschericia Coli* (*E. Coli*). The mechanism of action is not fully understood, but indications of the involvement of

the bacteria in attaching and effecting intestinal lesions have been shown (Mundy et al. 2005, Collins et al. 2014).

A model used for both *in vivo* and *in vitro* inflammation is LPS treatment. LPS is a major component in the cell membrane of gram-negative bacteria. LPS activates different signaling events, for example the activation of nuclear factor κB (NF-κB) (Medzhitov, Preston-Hurlburt & Janeway 1997), which in turn lead to the release of a number of proinflammatory cytokines, such as different interleukins and TNF-α (Chow et al. 1999, Cario et al. 2000). By treating intestinal cell lines, such as Caco-2 and HT29 cells, with LPS, an activation of colonic *in vitro* stress-mediated inflammatory pathways can be achieved (Cario et al. 2000). *In vivo* activation of stress-mediated inflammatory pathways can be achieved by *intra peritoneal* (*i.p.*) injection of LPS on mice.

Genetic mouse models displaying an inflammatory phenotype (Mizoguchi et al. 2016) are for example the $K8^{-/-}$ (Baribault et al. 1994) and $Il\text{-}10^{-/-}$ (Kuhn et al. 1993) mouse models. The $K8^{-/-}$ mouse model displays a spontaneous TH2-type colitis, resembling the IBD phenotype. The $Il\text{-}10^{-/-}$ colitis is ameliorated by germ-free conditions (Matharu et al. 2009), and both the $K8^{-/-}$ (Habtezion et al. 2005) colitis and the $Il\text{-}10^{-/-}$ colitis (Madsen et al. 2000) are amendable to a broad-spectrum antibiotic treatment.

2.3.2 Removal of microbiota

The most commonly known model for removal of microbiota is the antibiotic treatment-induced diarrhea, where broad-spectrum antibiotics not only depletes the pathogens, but also depletes most of the normal flora so that other pathogenic organisms can colonize the colon after the antibiotic treatment (Sekirov et al. 2010). Severe diseases caused by antibiotic treatment are for example colitis caused by *Clostridium difficile* and necrotizing enterocolitis in infants (Quigley 2013). Except for eliminating the normal microflora, an antibiotic treatment may cause a mild gut inflammation, an enlarged cecum, fewer Peyer's patches and an increased expression of several toll-like receptors (TLRs) (Cerf-Bensussan, Gaboriau-Routhiau 2010, Grasa et al. 2015). Antibiotic therapy is also known to prevent and treat genetic colitis models such as those seen in the $K8^{-/-}$ (Habtezion et al. 2005) and $Il\text{-}10^{-/-}$ (Madsen et al. 2000) mice.

2.4 Colon epithelial cell energy homeostasis

2.4.1 Short chain fatty acids in colonic energy homeostasis

Under basal conditions, the main energy source for colonic epithelial cells is SCFA, derived from the fermentation of dietary fiber by the colonic microbiota. The most commonly found SCFAs in the colon are the unbranched SCFAs acetate, propionate and butyrate, which account for 90% of all present SCFAs in the colon. Other branched SCFAs found in the colon are isobutyrate, valerate, isovalerate and hexanoate. SCFAs are primarily formed in in the proximal colon where access to

dietary fibers and colonic microbiota is higher. Also, the environment is more favorable for SCFA formation in the proximal colon compared to the DC where toxic metabolites such as ammonia and sulphur-containing compounds are formed in a larger extent (Hamer et al. 2008). The SCFAs acetic acid, propionic acid and butyric acid are mostly found in acid form in the acidic stomach, while they form salts (sodium acetate, sodium propionate and sodium butyrate) in the alkaline environment of the colon. Except for free diffusion of lipid-soluble protonated SCFAs across the cell membrane, water-soluble ionized SCFAs are transported into colonocytes by two different active transporters: MCT1(Cuff et al. 2005) and solute carrier family 5 member 8 (SLC5A8) also called sodium-coupled monocarboxylate transporter 1 (SMCT) (Gupta et al. 2006). MCT1 is a proton (H^+)-coupled active transmembrane transporter, whereas SLC5A8 is a sodium (Na^+)-coupled active transmembrane transporter. The most potent SCFA is butyrate, and it is mostly transported into the colonocytes via active transport.

Butyrate acts as an inhibitor of the protein histone deacetylase (HDAC), which in contrary to the protein histone acetyltranferase (HAT) removes acetyl-groups from histones. Therefore the HDAC inhibitor, *i.e.* butyrate, hyperacetylates histones, which in turn binds transcription factors more easily and facilitates transcription (Lazarova, Bordonaro 2016). Besides for butyrate's role as an energy source for the colonocytes, it has lately emerged as a protector of both CRC and IBD. The butyrate-mediated tumor-suppression is thought to rely partly on the fact that cancerous colonocytes produce energy by fermentation of lactic acid via glycolysis in the cytosol (Fig. 5B) as its primary energy source (Warburg effect) instead of oxidizing SCFAs in the mitochondria (Fig. 5A) (Donohoe et al. 2012). Although Warburg effect is seen in normal stem cell metabolism (Kaiko et al. 2016), it is more prominent during CRC in the whole crypt-axis. Due to the active glycolysis of the cancerous colonocyte, butyrate is accumulated inside the nucleus where it functions as an HDAC inhibitor, which regulates genes involved in colonocyte proliferation, diffentiation and apoptosis (Bultman 2016). The cancer-protective role of butyrate has also been shown by the ability of butyrate to inhibit excess stem cell proliferation (Kaiko et al. 2016). The protective mechanism in IBD also relies on the fact that butyrate acts as an HDAC inhibitor, leading to increased apoptosis and reduction of pro-inflammatory cytokines (Felice et al. 2015).

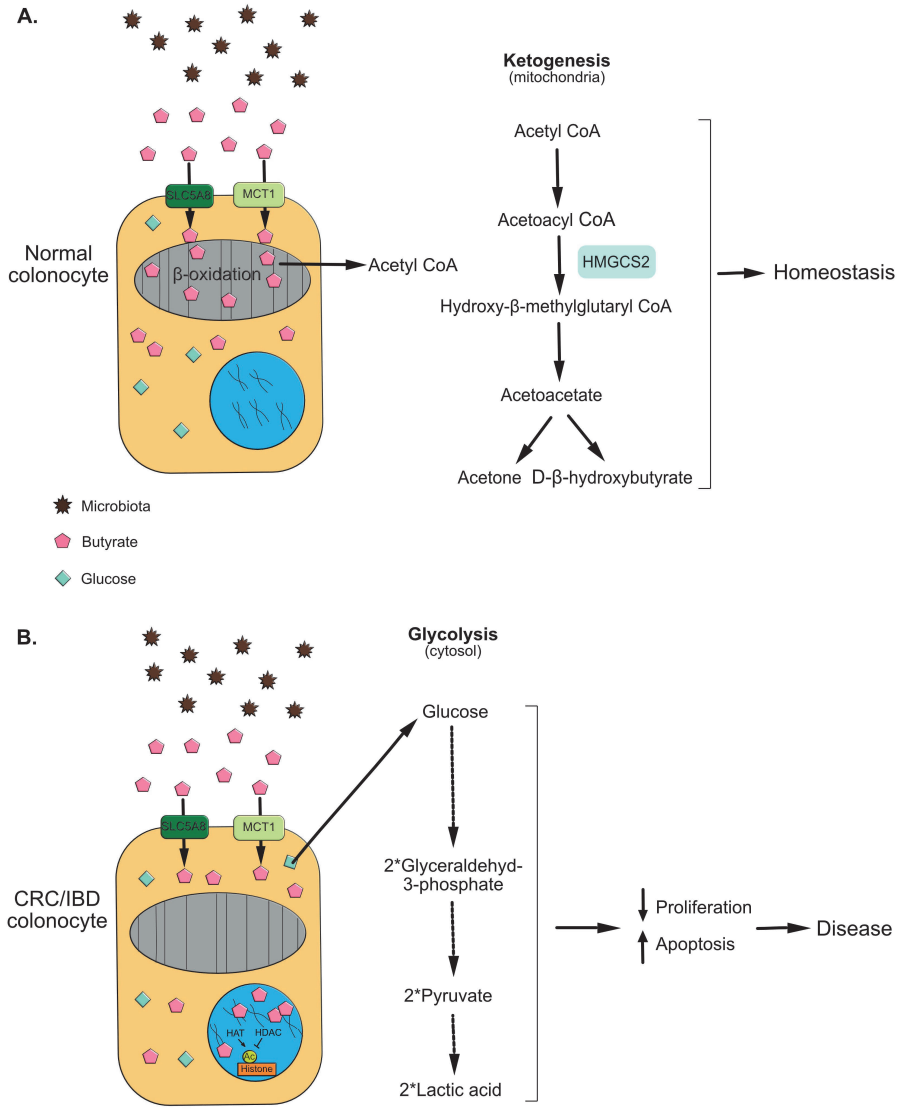

Figure 5. Energy metabolism of normal and diseased colon. A. During normal conditions, butyrate produced by colonic microbiota is transported into the colonocyte by SLC5A8 or MCT1, where after it is primarily β-oxidized to acetyl CoA in the mitochondria. Acetyl CoA enters ketogenesis, which produces the three ketone bodies acetoacetate, acetone and β-hydroxybutyrate, controlled by HMGCS2. B. In disease, such as CRC or IBD, the colonocyte uses glucose for energy due to the Warburg effect where cytosolic glucose is fermentated to lactic acid via glycolysis (dotted arrows indicate hidden steps). Butyrate is accumulated in the nucleus and acts as an inhibitor of histone deacetylation, leading to decreased proliferation and increased apoptosis via histone hyperacetylation that promotes transcription of controlled genes. Figure based on (Bultman 2016).

2.4.2 Colonic ketogenesis

Ketogenesis is the process where fatty acids are broken down to ketone bodies and energy. Fatty acids are β-oxidized to acetyl CoA in the mitochondria, from where acetyl CoA normally enters the citric acid cycle and further to the electron

transport chain to produce energy in the form of ATP. Alternatively, due to over production of acetyl CoA or in response to low carbohydrate-levels, acetyl CoA can enter the ketogenic pathway, which produces the three ketone bodies acetoacetate, acetone and β-hydroxybutyrate. If too much ketone bodies are produced, as for example during fasting, high-fat diet or during untreated diabetes, ketoacidosis occurs in response to insufficient levels of carbohydrates. In this state fatty acids alone are used for energy, resulting in an accumulation of H^+-binding ketone bodies that is a life-threatening situation. Ketogenesis occurs mainly in liver and colon, but in smaller extents also in skeletal muscles, heart, kidneys, testes and pancreas (VanItallie, Nufert 2003, Hegardt 1999).

2.4.3 Regulation of ketogenesis

The main rate-limiting enzyme of ketogenesis is 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2), which catalyses the condensation of acetoacetyl CoA and acetyl CoA to form 3-hydroxy-3-methylglutaryl CoA (HMG CoA) and free CoA (Fig. 5A). HMG CoA is active at two sites and produced by two different proteins encoded by two different genes: in the cytosol HMG CoA is produced by the cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) and in the mitochondria HMG CoA is produced by the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2). The cytosolic enzyme produces several important factors related to the isoprenoid biosynthesis pathway, such as cholesterol. The mitochondrial enzyme acts as the most important control site of the ketogenic pathway (Hegardt 1999, Fukao, Lopaschuk & Mitchell 2004).

The transcriptional regulation of HMGCS2 is mainly controlled by the transcription factor peroxisome proliferator-activated receptor α (PPARα), which activation is regulated by the amount of fatty acids present (Rodriguez et al. 1994). Other transcription factors that are known to regulate HMGCS2 transcription are *cis*-retinoid receptor (RXR), specificity protein 1 (Sp1) and cAMP regulatory element binding protein (CREB). Fatty acids activate the heterodimerizing of PPARα to RXR, which activates the binding to a specific DNA sequence called peroxisome proliferator regulatory element (PPRE) that initiates the transcription of the gene coding for HMGCS2 (Meertens et al. 1998, Hegardt 1998, Hegardt 1999). Another mechanism of HMGCS2 regulation is HMGCS2 succinylation and acetylation. These posttranslational modifications both reduce the activity of HMGCS2, whereas the desuccinylation and deacetylation increases HMGCS2 activation (Quant, Tubbs & Brand 1990, Newman, Verdin 2014).

2.4.4 Starvation and ketogenic diet

Both starvation and ketogenic diets causes a switch in the energy metabolism from normal carbohydrate-enforced citric acid cycle to the fatty acid-enforced ketogenesis that produces ketone bodies used for energy (Newman, Verdin 2014, Cullingford, Eagles & Sato 2002) . The ketogenic diet is a diet that contains high levels of fat and low levels of carbohydrates and protein (in a 4:1:1 ratio), stimulating the ketogenic pathway to produce ketone bodies for energy. Earlier

studies have clearly shown, that the ketogenic diet significantly increases blood ketone bodies, liver HMGCS2 levels (Cullingford, Eagles & Sato 2002) and protects against pentylenetetrazole-induced epileptic seizures (Bough, Eagles 1999). The ketogenic diet is known to significantly increase lifespan by increasing resistance to neurological diseases such as epilepsy and Alzheimer's, Parkinson's and Huntington's disease. The mechanism of action for this is still unclear, but the neuronal changes in ATP production, altered neuronal pH, inhibitory effects of ketone bodies on ion channels or synthesis of the inhibitory neurotransmitter gamma aminobutyric acid (GABA) have been proposed (Freeman et al. 2006, Cullingford, Eagles & Sato 2002).

2.5 Diseases of the colon

2.5.1 Inflammatory bowel diseases

IBD is for now an incurable disease, where the active disease phase is followed by remission. IBD comprises the two main intestinal inflammatory diseases, CD and UC, and they are both characterized by chronic intestinal epithelial damage. CD differs from UD by affecting the whole GI tract rather than only the colon and rectum as UC does. At the molecular level, UC is restricted to the epithelial layer of the colon, whereas CD can affect all layers of the colon (Baumgart, Carding 2007). Although the areas affected are different in CD and UC, the symptoms including diarrhea, abdominal pain, rectal bleeding and weight loss are the same for both diseases (Baumgart, Carding 2007). Other inflammatory intestinal diseases that are not classified as IBD are microscopic colitis (collagenous and lymphocytic colitis) and diverticular disease.

The development of IBD is thought to be due to a combination of genetic risk factors, reduced and altered levels of gut microbiota (Nagao-Kitamoto et al. 2016) and environmental factors that ultimately lead to an inflammatory response in the intestine (Blander 2016, Nagao-Kitamoto et al. 2016, Baumgart, Carding 2007). IBD is characterized by an increased amount of apoptosis, where intestinal epithelial cells die faster compared to healthy controls (Chen et al. 2010, Nunes, Bernardazzi & de Souza 2014, Hagiwara, Tanaka & Kudo 2002, Sartor 2006). Except for increased apoptosis, increased permeability of intestinal epithelial cells is also observed in IBD, which together with a defective mucosal barrier allows a close contact of the luminal content with underlying layers and endothelial cells. Microbial factors and epithelial cell abnormalities might facilitate the immunological response seen in IBD. IBD is an immune-mediated disease, characterized by an abnormal mucosal immune response. In response to the initiated immune response, pro-inflammatory and anti-inflammatory cytokines are produced, and a perturbation in the balance between these cytokines is thought to be a contributing factor to the outbreak of IBD(Neurath 2014). Currently it is not known if the increased apoptosis, increased permeability of the intestinal epithelial cells or the imbalance in cytokines is a cause or a consequence of the disease (Baumgart, Carding 2007, Michielan, D'Inca 2015).

To date, there is no known treatment for CD or UC. Immunosuppressive medication can be used to control the inflammatory response, and steroids are being used for their anti-inflammatory properties. Anti-inflammatory drugs can also be combined with drugs that keep the disease in remission. Sometimes surgery, like colostomy or ileostomy, is necessary to remove the most inflamed areas. TNF antagonists have traditionally been used as medical therapy for IBD (Beigel et al. 2014), even if many patients fail to respond to the treatment (Dulai, Sandborn 2016). A novel treatment that has recently gained substantial interest is FMT, which has shown positive results regarding successful IBD therapy (Pigneur, Sokol 2016).

The epidemiology of IBD shows that the incidence is higher in northern Europe and North America (M'Koma 2013). This could partly be explained by the genetic risk factors, such as susceptibility alleles (Khor, Gardet & Xavier 2011), but other predisposing factors such as diet, sanitation and other environmental factors are needed for the development of IBD. The highest prevalence of UC is found in Europe, where 505 persons of 100 000 inhabitants are affected, most of whom are diagnosed during the first two decades of their life (Ye et al. 2015).

2.5.2 Colorectal cancer

CRC originates from abnormal growth of epithelial cells in colon and rectum. Genetic mutations in the Wnt signaling pathway are known to cause CRC by mutations in the APC gene. APC inhibits β-catenin accumulation, leading to the inhibition of oncogene transcription. The tumor suppressor protein, tumor protein p53 (p53), regulates pathways associated to cell differentiation and proliferation such as the Wnt signaling pathway. Mutations in the p53 gene, as well as in the apoptosis activator Transforming growth factor-β (TGF-β), often lead to CRC. Also mutations in other proto-oncogenes such as Kirsten rat sarcoma viral oncogene homolog (KRAS) and RAF proto-oncogene serine/threonine-protein kinase (c-Raf) are crucial in the development of CRC by over-activating cell proliferation (Markowitz, Bertagnolli 2009). Except for spontaneously developing CRC, chronic inflammation in IBD-patients is a major risk factor for developing colitis-associated CRC (Axelrad, Lichtiger & Yajnik 2016, Markowitz, Bertagnolli 2009).

If CRC is detected at earlier stages, the prognosis is good due to surgical removal of the tumors. During later stages, when liver and lung metastases are usually seen, neither surgery, chemotherapy or radiation therapy has been shown to be successful (Cunningham et al. 2010). CRC epidemiology is similar to the epidemiology of IBD: found mostly in industrialized countries in Europe and North America with an annual diagnosis rate on 1 000 000 people. It is among the five most commonly found cancers causing death (Cunningham et al. 2010).

3. Simple epithelial keratins in colon

3.1 Simple epithelial keratin expression and regulation in colon

K7, K8, K18, K19, K20 and K23 are classified as SEKs since they are all expressed in single-layered epithelia (Omary et al. 2009). Even if specific keratin-pairs are tissue-specifically expressed in different epithelial organs, the intestine is the only known organ where all SEKs are expressed basally. Of type II keratins, only K8 is expressed in the whole colonic crypt. The presumable type I pair to K8 are K18 or K19, both expressed throughout the crypt. Therefore K8, K18 and K19 are the most prominently found keratins in the colonic epithelial cells. Type II K7 and type I K20 are expressed in lesser extent, and K7 can be found at the bottom of the crypts while K20 is found only at the top of the crypts close to the intestinal lumen (Fig. 6) (Zhou et al. 2003) . The most recently described SEK, type I K23, is likely expressed only in small amounts in the colon (Guldiken et al. 2016).

The fast renewal rate of the colonic epithelium requires all SEKs to be in a dynamic state. Colonic SEKs are present in soluble and insoluble filamentous states. During normal conditions, 95% of the filaments are in the insoluble filamentous form. In response to stress, keratins reorganize mostly in response to PTMs (Toivola et al. 2010). The main PTMs are S/T/Y phosphorylation, lysine acetylation, S/T glycosylation and sumoylation. Keratin phosphorylation shifts the keratin pool against a more soluble pool where keratins appear as granules (Woll, Windoffer & Leube 2007, Snider, Omary 2014). The insoluble filamentous keratin pool can be solubilized by high concentrations of urea (9M) or guanidine hydrocloride (4-6M) (Snider, Omary 2014, Majumdar et al. 2012). The role of *in vivo* sumoylation in maintaining intestinal stem cells and proliferation has been shown, where K8 has been identified as a substrate for SUMO(Demarque et al. 2011).

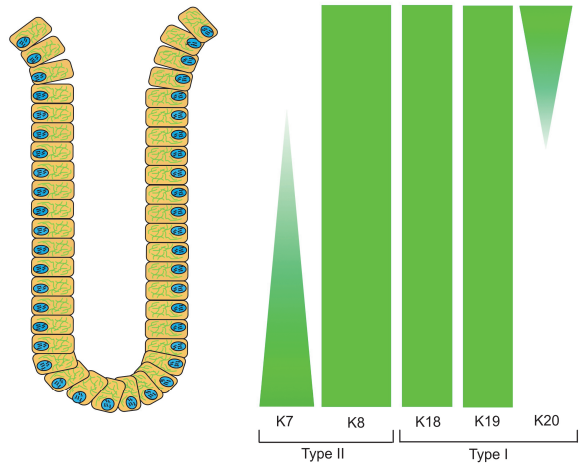

Figure 6. SEKs expressed in the colon during baseline conditions. K8 (type II), K18 (type I) and K19 (type I) are the main keratins expressed in the single-layered epithelium of the colon. K7 (type II) and K20 (type I) are expressed in a lesser extent mainly so that K7 is expressed at the bottom of the crypts and K20 at the top of the crypts. The distribution of the colonic type I K23 has not been described yet. Bar width indicates SEK amount and height indicates SEK distribution. Figure based on (Zhou et al. 2003).

3.2 Simple epithelial keratin functions in colon

The function of all SEKs present in the colon has not been clarified in detail yet. SEK functions in other organs, such as the liver and pancreas, show similarities with known intestinal functions of SEK, although some functions are organ-specific and dependent on other secondary factors, such as the impact of *e.g.* the intestinal microflora. The intestine is, to present knowledge, the only organ where all SEKs are present (Majumdar et al. 2012, Pastuszak et al. 2015, Coch, Leube 2016), thus the compensatory role of the intestinal keratins might be significant in the outcome of the different phenotypes. This is seen for example in the $K18^{-/-}$ mouse model, which does not display any specific phenotype likely due to the compensatory role of type I K19 (Magin et al. 1998).

The most significant evidence of the role of intestinal K8 comes from the $K8^{-/-}$ mouse phenotype. This mouse lacks almost all type II keratins, leading to no functional keratin-filaments (Baribault et al. 1994). As the $K8^{-/-}$ mouse develops a TH2-type colitis, diarrhea and hyperplasia (Habtezion et al. 2005), a function for colonic keratins in the development of IBD has been suggested. Unlike in liver (Ku et al. 2016), where the lack of K8 is known to induce apoptosis, lack of colonic K8 leads to a microbiota-dependent resistance to apoptosis (Habtezion et al. 2011), while lack of small intestinal K8 does not show any changes in the rate of apoptosis (Ameen, Figueroa & Salas 2001). In the $K8^{-/-}$ mouse, the anion exchanger AE1/2 and the Na-transporter ENaC-γ are mislocalized (Toivola et al. 2004a) and there is a complete loss of the Cl-transporter DRA (Asghar et al. 2016). Disturbances in the ezrin-actin scaffold have been reported in intestinal epithelial cells with altered levels of K8 (Wald et al. 2005), showing the significance of keratins in protein targeting. Type I K18 has been identified to interact with the chloride transporter cystic fibrosis transmembrane conductance regulator (CFTR) in small intestine and gall bladder (Duan et al. 2012), suggesting a role for K18 and its partner in the regulation of CFTR distribution in small intestine. However, CFTR levels were not altered in the colon of $K8^{-/-}$ mice (Asghar et al. 2016). K8 has also been proposed a role in the regulation of innate immunity. When toll-like receptor (TLR)-signaling is activated, K8 inhibits TRAF6-polyubiquitination, which is necessary for the transcription of proinflammatory genes such as NF-κB. Shown by both *in vivo* and *in vitro* models, this further confirms the protective role of K8 in the colonic epithelium (Dong et al. 2016).

In the intestine, desmosomal and tight junction proteins attach colonocytes to each other and hemidesmosomal proteins attach the single-layered epithelium to the basal membrane, essential for maintaining the intestinal barrier function. Keratins are anchored to these structures and have been proposed a role in the formation of the IBD phenotype (Baumgart, Carding 2007, Sartor 2006). Surprisingly, when the small intestinal desmosomal protein desmoplakin (DP) is missing, keratin filament localization was not affected, even if keratin filaments were no longer anchored to the desmosomes. However, the microvilli in DP-lacking enterocytes were shorter, indicating a role for keratin-bound desmosomes in the organization of the

intestinal brush border (Sumigray, Lechler 2012). The intestinal permeability of the $K8^{-/-}$ colonocytes was increased in one study when compared to $K8^{+/+}$ colonocytes (Misiorek et al. 2016) but unchanged in another (Toivola et al. 2004a). Small intestinal $K8^{-/-}$enterocytes also showed an increased permeability (Mashukova et al. 2009). In colonocytes, colonic cancer cells expressing K8/K18 mutations (K8 G62C, K8 K464N, K18 S230T) displayed increased permeability and diffuse distribution of tight junction proteins zonula occludens-1 (ZO-1) and claudin-4, suggesting that K8/K18 play a role in colonic barrier function (Zupancic et al. 2014). Similarly, when the colonic barrier function was compromised by DSS-treatment, $Il\text{-}6^{-/-}$ mice showed an increased colonic permeability mediated by K8/K18 (Wang et al. 2007).

K8 has also been suggested to play a role in the polarization and the organization of the small intestinal apical cell membrane, shown by the loss of apical membrane proteins in $K8^{-/-}$ mice (Ameen, Figueroa & Salas 2001). Similarly, downregulation of K19 showed abnormally distributed actin filaments and other membrane proteins in the apical cell membrane, together with a decreased amount of small intestinal microvilli, suggesting a role for K19 in the organization of the apical cell membrane (Salas et al. 1997). Smaller microvilli in the small intestine were also observed in the $plastin\text{-}1^{-/-}$ mouse, where K19 was shown to interact with plastin-1 for anchoring microvilli to the keratin network (Grimm-Gunter et al. 2009). A function for SEKs in colonic stem cells has been shown by K19, where K19-positive cells above the crypt base generate radioresistant Lgr5-positive CBC cells (Asfaha et al. 2015). An emerging role for colonic keratins in the protection from CRC has been established by inflammation-induced tumorigenesis (Misiorek et al. 2016), providing one more piece of evidence on the function of intestinal keratins as stress modulators and protectors of health.

OUTLINE AND AIMS OF THE THESIS

In hepatocytes, K8 has a defined protective role whereas in colonocytes, the role of K8 is not yet fully understood. Lack of K8 in mouse colon causes a phenotype similar to human IBD. Hepatic, renal, pulmonary and pancreatic keratins protect the organ from disease-related stress, while keratin dynamics in intestinal diseases are poorly studied. For a better understanding of the roles of colonic keratins, colons from $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice were studied with respect to keratins in stress protection and energy metabolism.

Hypothesis: Keratins play a protective role in the intestinal stress response and in the protection from intestinal diseases.

The specific aims for this thesis were to:

Aim I. Analyze colonic keratin levels, localization and phosphorylation using colonic disease-related stress and recovery models.

Aim II. Investigate the role of keratin levels for colon health by studying the susceptibility of $K8^{+/-}$ mice to colitis.

Aim III. Investigate changes in the $K8^{-/-}$ colon energy metabolism to understand the role of K8 in the colon.

EXPERIMENTAL PROCEDURES

1. Methods

Selected experimental procedures are described below. Detailed descriptions of all the methods and reagents are described in the original publications (I-III). All experimental methods used in publications I-III are listed in Table 5.

Table 5. Methods used in publications I-III

Method	Publication
ADP/ATP assay	III
Antibiotic treatment (in vivo)	I
BrDU labelling	II
Confocal microscopy	I, II, III
Coomassie staining	II, III
Cristae quantification	III
Disease activity index (DAI) calculations	I
In vivo DSS colitis	I, II, III
High salt extraction	II
HMGCS2 activity assay	III
Image quantification	I, II, III
Immunofluorescence	I, II, III
Immunohistochemistry	II
In vivo imaging of RONS	II
In vivo ketogenic diet	III
Isolation of lamina propria cells	II
Mitochondria isolation	III
NAD/NADH assay	III
Profiling of fecal bacterial levels	III
Profiling of fecal SCFA levels	III
Quantitative PCR	I, II, III
Screening of $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice	I, II, III
SDS-PAGE and Western blot	I, II, III
Starvation (in vivo)	III
Statistical analysis	I, II, III
Transfection	PhD Thesis
Transmission electron microscopy	III
Western blot quantification	I, II, III
2D-DIGE	III

1.1 SDS-PAGE, Western Blot and quantification

Samples for protein analysis of DC/PC, liver, small intestine were suspended in 24 µl homogenization buffer (0.187 M Tris-HCl pH 6.8, 3% SDS, 5 mM EDTA) per 1 mg of tissue and homogenized by 75 strokes in a Potter-Elvenhjelm tissue homogenizer in order to obtain total tissue lysates. HT-29 or Caco2 cells were lysed in homogenization buffer (0.187 M Tris-HCl pH 6.8, 3% SDS, 5 mM EDTA). Tissue/cell lysates were incubated at 95°C for 5 minutes and sheared with a 27G needle. Protein concentrations were measured with a Pierce BCA protein assay kit

(Thermo scientific, Waltham, MA, USA), samples were normalized and 20 µg protein per sample was loaded and separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane (PVDF) and further analyzed by Western Blot.

Membranes were blocked with 5% fat-free milk in PBS-Tween, washed and incubated with primary antibody against the protein of interest (Table 6). After washing, membranes were incubated with a horseradish peroxidase (HRP)-linked secondary antibody (Table 7) raised in the animal corresponding to the primary antibody. Chemiluminescence detection of the HRP-conjugated secondary antibody was done by enhanced chemiluminescence (ECL). Protein bands were quantified with Image J software (National Institutes of Health) and normalized to loading control (Hsc70/Actin/Tubulin).

Table 6. Primary antibodies used in publications I-III. All antibodies, their applications and manufacturers used in publications I-III listed.

Name, clone (if monoclonal) (Manufacturer)	Application	Publication
ACC (Cell Signaling technology)	WB	III
AMPK-α (Cell Signaling technology)	WB	III
AMPK-β1 (Cell Signaling technology)	WB	III
Caspase-7 (Cell Signaling technology)	WB	II
Cleaved caspase-7 (Cell Signaling technology)	WB	II
COX IV (Cell Signaling technology)	WB	III
Cytochrome c (Cell Signaling technology)	WB	III
Glut4 (Cell Signaling technology)	WB	III
HMGCS2 (AVIVA)	WB, ICC	III
HMGCS2 (Genway)	WB, ICC	III
Hsc70 (Stressgen)	WB	I, II, III
HSF2 (Abcam)	WB	I
IκB-α (Santa Cruz biotecnology)	WB	I
K7, RCK-105 (Abcam)	ICC, WB	I, II
K8, Troma I (Hybridoma bank)	ICC, WB	I, II, III
K8, 273 (J. Eriksson)	WB, IP	I
K18, Troma II (Hybridoma bank)	WB, ICC	II
K18, 275 (J. Eriksson)	WB, ICC	I
K19, Troma III (Hybridoma bank)	WB, ICC	I, II
K20, IT-Ks 20.10 (Progen)	WB, ICC	I, II
K20 (Epitomics)	WB, ICC	I, II
MCT1 (Chemicon)	WB, ICC	III
MCT1 (Santa Cruz biotechnology)	WB, ICC	III
MPO (Thermo Scientific)	ICC	II
P-ACC (Cell Signaling)	WB	III
P-AMPK-α (Cell Signaling technology)	WB	III
P-AMPK-β (Cell Signaling technology)	WB	III
P-K8 S74 (M. B. Omary)	WB, ICC	I
PPARα (Santa Cruz biotechnology)	WB	III
Prohibitin (Abcam)	WB	III
α-smooth muscle actin (Abcam)	WB	III
β-Tubulin (Sigma)	WB	II, III

Table 7. Secondary antibodies used in publications I-III. All antibodies, their applications and manufacturers used in publications I-III listed.

Name (Manufacturer)	Application	Publication
Alexa Fluor 488 (Invitrogen)	ICC	I, II, III
Alexa Fluor 546 (Invitrogen)	ICC	I, II, III
Alexa Fluor 568 (Invitrogen)	ICC	I, II, III
Anti-chicken HRP (Genway)	WB	III
Anti-goat HRP (Cell Signaling)	WB	III
Anti-mouse HRP (GE healthcare)	WB	I, II, III
Anti-rabbit HRP (Cell Signaling)	WB	I, II, III
Anti-rat HRP (GE healthcare)	WB	I, II, III

1.2 Immunofluorescence staining

Fresh frozen colon samples in Optimum cutting temperature (O.C.T.) compound were cryosectioned (6 µm), fixed in -20°C acetone for 10 minutes and immunostained with antibodies (Table 6 and Table 7) as described in (Ku et al. 2004). HT-29 or Caco2 cells were grown on microscope cover slips and fixed in -20°C acetone for 10 minutes or 1 % PFA in RT for 20 minutes prior to immunofluorescence staining as described in (Ku et al. 2004). Samples were analyzed with Leica TCS SP5 matrix confocal microscope (Leica, Mannheim, Germany).

1.3 Experimental *in vivo* DSS-induced colitis

1.3.1 Acute DSS colitis

2-2.5% dextran sulphate sodium (40 kDa, TdB Consultancy AB, Uppsala, Sweden) was administered in autoclaved drinking water to 2-3 months old Balb/c or FVB/n mice for 7-8 days with or without recovery (7 days) to achieve a model for acute colitis (Perse, Cerar 2012, Rose, Sakamoto & Leifer 2012) . Control mice for each experiment were age-, sex- and strain-matched littermates, and were treated equally as DSS-treated mice, except that they received autoclaved drinking water without DSS.

1.3.2 Chronic DSS colitis

For mimicking human chronic colitis with alternating relapse and remission, 2-month-old FVB/n mice were treated one week with 2.5% DSS, followed by a two-week recovery period after which this cycle was repeated once and the animals were sacrificed at day 45 (Breynaert et al. 2013). Control mice for each experiment were age-, sex- and strain-matched littermates, and were treated equally as DSS-treated mice, except that they received autoclaved drinking water without DSS.

1.3.3 Disease activity index

A disease activity index (DAI) was used to determine colitis severity. DAI was calculated by grading daily measurements starting on day 0 on body weight loss (1

point per each 5% of body weight loss), presence of occult blood in stool (0 = none; 1 = blood in stool pellets; 2 = clotted or fresh blood at anus) and stool consistency (0 = normal; 1 = formed but very soft; 2 = slightly loose; 3 = liquid)(Breynaert et al. 2013).

1.4 Induction of *in vivo* ketogenic conditions

1.4.1 Ketogenic diet

3-6 months old $K8^{+/+}$ and $K8^{-/-}$ mice were subjected to ketogenic diet (TD.96355, Harlan Teklad Laboratories, Madison, USA) for 3 and 14 days. Corresponding age-, sex- and strain-matched littermates of $K8^{+/+}$ and $K8^{-/-}$ mice were subjected to a control diet (TD.00606, Harlan Teklad Laboratories, Madison, USA). The macronutrient composition of the ketogenic diet (by weight) was: 15.3% protein, 0.5% carbohydrate and 67.4% fat with energy content of 6.7 kcal/g. The corresponding composition of the control diet was: 9.2% protein, 70.9% carbohydrate and 5.1% fat with an energy content of 3.7 kcal/g. Since the ketogenic diet contains twice as much energy, mice eat a smaller amount of the ketogenic diet than the control diet, whereas the intake of protein, minerals and vitamins is halved in the ketogenic diet (manufacturer's suggestion). Cages, water and control diet were changed on day 7, and the ketogenic diet was changed on days 1, 2, 4, 6, 7, 9 and 11. Daily measurements of mouse and food weight as well as blood glucose and β-hydroxybutyrate levels were done. Blood glucose and β-hydroxybutyrate levels were measured from fresh blood obtained from the submandibular vein using goldenrod lancets (Medipoint Inc. NY, USA) using a glucose/β-hydroxybutyrate meter (Precision Xceed by Abbott Diabetes Care Ltd, Witney, UK) on days 0, 3, 7 and 14. The mice were sacrificed by CO_2 inhalation on day 14 and samples from PC, DC, liver and small intestine were collected.

1.4.2 Starvation

$K8^{+/+}$ and $K8^{-/-}$ mice 3 months of age were subjected to 24 hours of starvation with unlimited access to water. Weight as well as blood glucose and β-hydroxybutyrate levels were measured at 0, 12 and 24 hours after the beginning of starvation. Blood glucose and β-hydroxybutyrate levels were measured from fresh blood obtained from the submandibular vein using goldenrod lancets (Medipoint Inc. NY, USA) using a glucose/β-hydroxybutyrate meter (Precision Xceed by Abbott Diabetes Care Ltd, Witney, UK) on days 0, 3, 7 and 14. The mice were sacrificed by CO_2 inhalation on day 14 and samples from PC, DC, liver and small intestine were collected.

1.5 Transfection of Min6-cells

Murine insulinoma (Min6) cells were grown to 80% confluency, trypsinized, washed and diluted in 400µl Optimem. Cells were transfected with or without 0.6 µg/µl human K8 plasmid + 0.8 µg/µl human K18 plasmid and 5.7 µg/µl MCT1-GFP (kind gift from P. Dudeja, University of Chicago) at 220V and 975W. After

transfection, cells were plated, grown to 80% confluency on sterile microscope cover glasses and fixed in 1% PFA prior to immunofluorescence staining with antibodies of interest (Table 5 and Table 6), as described in (Ku et al. 2004).

2. Mice

The mice were housed at the Central Animal Laboratory of University of Turku (Koe-eläin keskus). The mice were treated according to the approved animal study protocol issued by the state Provincial Office of South Finland (licence numbers: 2007-07005, 2893/04.10.03/2011, 197/04.10.07/2013 and 3956/04.10.07/2016). The mice were sacrificed by CO_2 inhalation. All mouse strains used in publications I-III are listed in Table 8.

Table 8. Mouse strains used in publications I-III. All mouse strains and their backgrounds used in Publications I-III

Name (background)	Publication
BALB/c	I, III
Human K8 overexpressing mice (FVB/n)	PhD Thesis
$K8^{+/+}$, $K8^{+/-}$, $K8^{-/-}$ (FVB/n)	I, II, III

2.1 Screening of $K8^{+/+}$, $K8^{-/-}$ and $K8^{+/-}$ mice

$K8^{+/+}$, $K8^{-/-}$ and $K8^{+/-}$ mice in the FVB/n background were generated by interbreeding $K8^{+/-}$ mice or $K8^{+/-}$ females with $K8^{-/-}$ males (Baribault et al. 1994). DNA was extracted by lysing the ear piece in 2 mg Proteinase K (Sigma-Aldrich, St. Louis, USA)/5 ml Direct PCR lysis reagent (Viagen, Cedar Park, USA) by incubation on a 55°C water bath, followed by a 45 min incubation at 85°C. Polymerase chain reaction (PCR), consisting of one cycle at 94°C for 10 min, 40 cycles of 94°C for 45 sec, 70°C for 30 sec, 72°C for 45 sec and one cycle of 72°C for 10 min, was performed on 2 µl DNA and a mastermix of 2.5µl 5x Phusion HF buffer (Thermo Scientific, Waltham, MA, USA), 2µl 10mM dNTPs mix (Thermo Scientific, Waltham, MA, USA), 0.3125µl 2U/µl Phusion DNA polymerase (Thermo Scientific, Waltham, MA, USA), 1µl 10 ng/µl of each primer ($K8^{-/-}$/$K8^{+/+}$/$K8^{+/-}$ or $K8^{+/+}$/$K8^{+/-}$ and $K8^{-/-}$/$K8^{+/-}$, Table 9; Metabion, Steinkirchen, Germany) and 15.2 µl sterile water. The samples were separated on a 1% agarose gel (1% agarose in 0.5xTBE and Midori DNA stain (Nippon genetics, Tokyo, Japan) and exposed to UV-light, showing the $K8^{-/-}$ allele ($K8^{-/-}$ and $K8^{+/-}$ primers) at 800 bp and the $K8^{+/+}$ allele ($K8^{+/+}$ and $K8^{+/-}$ primers) at 500 bp (Fig. 7).

Table 9. Primers used for PCR of $K8^{-/-}$, $K8^{+/+}$ and $K8^{+/-}$ genotyping. Used primers and their sequences for genotyping of $K8^{-/-}$, $K8^{+/+}$ and $K8^{+/-}$ mice.

Primer	Sequence
$K8^{+/+}$	5' CGG TTA GTC GGG AAG AGA GGG GTC 3'
$K8^{-/-}$	5' CCT GTC ATC TCA CCT TGC TCC TGC C 3'
$K8^{+/-}$	5' TGG GGT TAG GCC CTG CCT CTA GTG TCT 3'

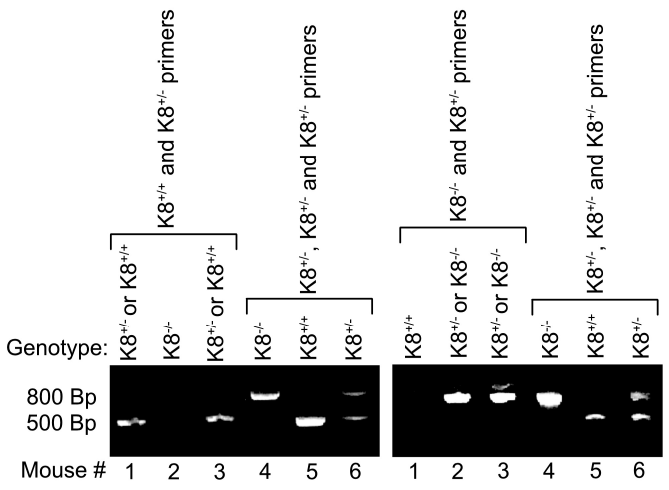

Figure 7. Genotyping of $K8^{+/+}$, $K8^{-/-}$ and $K8^{+/-}$ mice. PCR products from extracted mouse DNA was run on a 1% agarose gel and mouse genotypes were determined according to primers indicated. Mice # 1, 2 and 3 were determined by 2 simultaneous sets of pcr with primers for $K8^{+/+}$/$K8^{+/-}$ and $K8^{-/-}$/$K8^{+/-}$ and while mice number 4, 5, and 6 were determined by one pcr with primers for $K8^{-/-}$/$K8^{+/-}$ /$K8^{+/+}$. Final genotypes are: Mouse #1= $K8^{+/+}$, mouse #2 = $K8^{-/-}$ and mouse #3 = $K8^{+/-}$

3. Cell lines

All cell lines and their origins used in publications I-III are listed in Table 10.

Table 10. Cell lines used in publications I-III. Cell lines and their origins used in Publication I-III.

Name, origin	Publication
HT29, human colorectal adenocarcinoma	I, III
Caco-2, human colorectal adenocarcinoma	III
Min6, murine insulinoma	PhD Thesis

4. Selected reagents

Selected reagents and their manufacturers used in publications I-III are listed in Table 11.

Table 11. Reagents used in publications I-III. Reagents and their applications used in Publications I-III.

Name (Manufacturer)	Application	Publication
Acrylaminde (Sigma)	SDS-PAGE	I, II, III
ADP/ATP assay kit (Abnova)	ADP/ATP ratio assay	III
5-bromo-2'-deoxyuridine (BD biosciences)	Proliferation staining	II
Dextran sulphate sodium 40kDa (TdB)	Colitis induction	I, II, III
DMEM (Sigma)	Cell culture, Caco2	III
Draq5 (Cell Signaling technology)	DNA staining	I, II
Imipenem (Hospira)	Antibiotic treatment	I
KAPA Fast ABI qPCR mix (Kapa biosystems)	qPCR reaction	I, II, III
Ketogenic diet (Harlan)	Ketogenesis induction	III
L-012 (Wako)	In vivo imaging	II
MatriSperse cell recovery (BD biosciences)	Crypt isolation	III
NAD/NADH assay kit (Abnova)	NAD/NADH ratio assay	III
Pierce BCA protein assay kit (Thermo scientific)	Protein assay	I, II, III
RNA later (Qiagen)	Sample storage	I, II, III
RNeasy mini kit (Qiagen)	RNA isolation	II, III
RPMI-1640 (Sigma)	Cell culture, HT-29	I, II, III
Toto-3 (Life Technologies)	DNA staining	III
Vancomycin (Hospira)	Antibiotic treatment	I

RESULTS AND DISCUSSION

1. Intestinal keratins display a pairwise and context-depending upregulation in response to colonic stress and recovery (Publication I)

1.1 Main colonic keratins K8 and K19 are stress-responsive proteins that are upregulated in colonic stress

The most abundantly found keratins in the colon are K8 and K19, which are distributed all over the crypt. The upregulation of type II K8 and its presumable type I pair K19 was shown by depleting the colonic pathogenic and non-pathogenic microbiota by *in vivo* murine antibiotic treatment (Publication I, Fig. 4). Interestingly, *in vivo* antibiotic treatment upregulated HSF2 in a similar manner as in human ulcerative colitis patients (Miao et al. 2014). The stress-induced upregulation of these main colonic keratins was confirmed by *in vitro* analyses on K8/K19 levels in bacterial LPS-stressed HT29 cells (Publication I, Fig. 6). The *in vitro* upregulation of K8 is supported by earlier studies on *Bifidobacterium Breve*-induced stress on HT29 cells (Sanchez et al. 2015), by IL-6 treatment on Caco-2 cells (Wang et al. 2007) and by *in vivo* LPS-treatment (Dong et al. 2016). By treating colorectal cancer cells with LPS, inflammatory TLR4 signaling pathways were activated, as confirmed by the decreased levels of the inhibitor of kappa B alfa (IκBα) in LPS treated cells (Publication I, Fig. 6). Consequently, a significant 2-fold upregulation of K8 on protein level was observed (Publication I, Fig. 6). Colonic keratins were mostly upregulated in response to stress, but a slight decrease in colonic K8, K18 and K19 was observed in the acute DSS stress models (Publication I, Fig 1-2). A similar decrease in K8, K18 and K19 has been observed in human ulcerative colitis patients (Corfe et al. 2015), which might be due to a local destruction of the colonic single-layered epithelium by DSS.

The role of K8 and K19 in the tumorigenesis of the colonic epithelium has also contributed to the emerging role of SEKs as colonic stress proteins. Several studies on human biopsies from colorectal neoplasia suggest that keratin levels are altered in carcinogenesis (Evans et al. 2015). A significant downregulation of keratins and other cytoskeletal components in tumor tissue together with overexpression of K8 in the normal mucosa of cancerous tissue was seen when compared to healthy controls (Polley et al. 2006). Also, $K8^{-/-}$ mice are known to be more susceptible to CRC in response to a chemical and genetic CRC model (Misiorek et al. 2016).

In other organs where SEKs are present, as for example in the liver, the main hepatic keratins K8, K18 (Guldiken et al. 2015a, Szabo et al. 2015) and the newly described K23 (Guldiken et al. 2016) were also upregulated in response to induced stress both *in vivo* and *in vitro*. Renal keratins, K7, K8, K18 and K19 have been shown to be stress-responsive in kidney injury models (Djudjaj et al. 2016). The

pancreatic keratins, K8, K18, K19 and K20, were similarly overexpressed in response to stress and recovery (Zhong et al. 2004, Wogenstein et al. 2014). Regarding keratin overexpression beyond SEKs, K6, K16 and K17 have been shown to be overexpressed in induced and disease-mediated stress in skin (Zhussupbekova et al. 2016, Jin, Wang 2014, Depianto et al. 2010, Freedberg et al. 2001).

1.1.1 Colonic K8 S74 phosphorylation is increased in the acute colonic stress response

In a similar way as in liver (Toivola et al. 2004b), kidney (Djudjaj et al. 2016) and lung (Burcham, Raso & Henry 2014) , colonic K8 was hyperphosphorylated at S74 in response to stress. This was shown for the *in vivo* acute colitis model (Publication I, Fig. 1-2), the microbiota-depleting stress model (Publication I, Fig. 4) and the *in vitro* LPS model (Publication I, Fig. 6). Hepatic K8 pS74 has been studied in more detail, where this phosphorylation mediates changes in keratin solubility, shifting the predominant non-soluble filament state of keratins to a more soluble non-filamentous state where keratins appear as dots instead of filaments (Ku, Azhar & Omary 2002, Snider, Omary 2014) . K8 S74 phosphorylation occurs in mitosis, apoptosis or stress, and it has been suggested to protect the tissue from stress by serving as a phosphate "sponge" for stress-activated kinases (Ku, Omary 2006). The significantly increased K8 pS74 levels observed in the *in vitro* LPS-treated HT29 cells consisted of equal amounts of mitotic and non-mitotic cells (Publication I, Fig. 6C-D), while *in vivo* antibiotic treatment caused significantly more K8 proliferating pS74-positive cells compared to non-proliferating pS74-positive cells (Publication I, SFig. 2). Taken together, main intestinal keratins K8 and K19 were shown to act as colonic stress proteins, where K8 pS74 serves as a marker of the acute stress response.

1.2 Type II K7 and type I K20 are increased and differentially distributed in response to chronic stress

1.2.1 Protein levels of K7 and K20 are increased in the colitis stress response

K7 and K20 were found to be increased in several experimental colitis-stress models. The biggest increase in K7 and K20 protein levels was seen after a cyclic DSS treatment (chronic DSS), where mice were subjected to DSS for one week, followed by a 2-week recovery time. Mice sacrificed at the end of the second recovery period displayed a 2-2.5-fold increase in colonic K7 and K20, although no changes in mRNA levels were observed at that time (Publication I, Fig. 3). Other acute DSS-induced stress models also displayed small, but significant, upregulations of K7 and K20 (Publication I, Fig. 1-2). In human colitis, a positive correlation between K7 expression and UC has been shown (Stenling et al. 2007, Tatsumi et al. 2006). Control patients and patients with inactive colitis lacked completely colonic K7, while 90 % of patients with active UC displayed a patchy K7 expression in the inflamed areas of the colonic epithelium.

Altered K7 and K20 expression in colorectal carcinomas has been reported in the literature. Increased K20 expression in neoplasia-associated lesions has been observed in human colon (Stenling et al. 2007). The present knowledge suggests that low or no expression of K7 together with high expression of K20 indicate colorectal adenocarcinoma (Chu, Wu & Weiss 2000) . Low K7/high K20 levels are also used in the prognosis of Merkel cells carcinomas in skin, while high K7/low K20 indicates ovarian-, lung- and breast adenocarcinomas (Chu, Wu & Weiss 2000). Nevertheless, together this data implies that these highly disease-responsive keratins, K7 and K20, should be considered as stress-responsive proteins.

1.2.2 K7 and K20 serve as stress-responsive keratins by altering their distribution in the colonic stress response

Colonic K7 and K20 were both significantly increased during chronic stress and in the recovery from stress. Their baseline distribution, where K7 is found at the base-mid crypt axis and K20 at the top brush border (Zhou et al. 2003), was found widened after stress conditions. This increased distribution of K7 was seen in response to a cyclic chronic DSS-treatment, where K7 was expressed all over the crypt and K20 in the top-mid crypt compartment (Publication I, 3D). The same was observed in the $K8^{+/-}$ and $K8^{-/-}$ mouse, where a decrease in the overall keratin amounts leads to the wider crypt-expression of the remaining K7 and K20 (Publication II, Fig. 2B). These studies also indicate that the presumable main type I partner for K7 in stress situations is K20, since they are both stress-responsive keratins upregulated in a similar manner both during chronic DSS treatment and in response to the deletion of one K8 allele. The presumable K7-K20 pairing during stress in the mid-crypt region was observed by this overlapping *de novo* expression of these keratins, supported by the observation of increased K20 mRNA levels in the $K7^{-/-}$ mouse (Sandilands et al. 2013). Since K7 and K20 are not expressed in the same cells on the crypt axes during baseline conditions, they are not thought to form pairs during normal conditions. In fact, when K18 is knocked out, the levels of K7 also dramatically decrease in uterus, bile ducts and kidneys, suggesting that K7 is required for K18 stabilization during normal conditions (Magin et al. 1998).

Except in the chronic colitis-model, K7 and K20 levels also increased in murine *in vivo* senescence (Publication I, Fig. 3D-E and 5D-E). A wider distribution pattern of K20 with single-cell K20 positive cells was found in the base-mid crypt compartment (Publication I, Fig. 5D-E). In human biopsies from neoplastic areas, K20 has been reported to distribute lower down in the crypts when compared to biopsies from healthy colon (Stenling et al. 2007). Another piece of evidence pointing at K20 serving as a stress-responsive protein is the fact that the $K8^{-/-}$ mouse shows a moderate, but not significant, increase in the transcription of the K20 gene (Publication II, Fig. 1C), in a similar way as the $K7^{-/-}$ mouse (Sandilands et al. 2013). The $K7^{-/-}$ mouse does not display any colonic phenotype, only increased proliferation of the bladder urothelium. By challenging the $K7^{-/-}$ mice with *e.g.* DSS, the colonic susceptibility could be addressed in regards of colonic K7 functions.

Stress-induced changes in keratin distribution have also been seen in murine pancreatitis, where cytoplasmic K19 and K20 filaments were increased in contrast to baseline apical and lateral keratin distribution in acinar cells of the exocrine pancreas (Zhong et al. 2004). Similarly, keratins of the renal collecting ducts redistributed to a cytoplasmic pattern in response to stress (Djudjaj et al. 2016).

With all evidence collected from different colonic stress models presented in Publication I together with previous publications of SEKs in stress protection, intestinal keratins are suggested to be differentially and dynamically upregulated and post-translationally modified by phosphorylation in stress and recovery (Fig. 8).

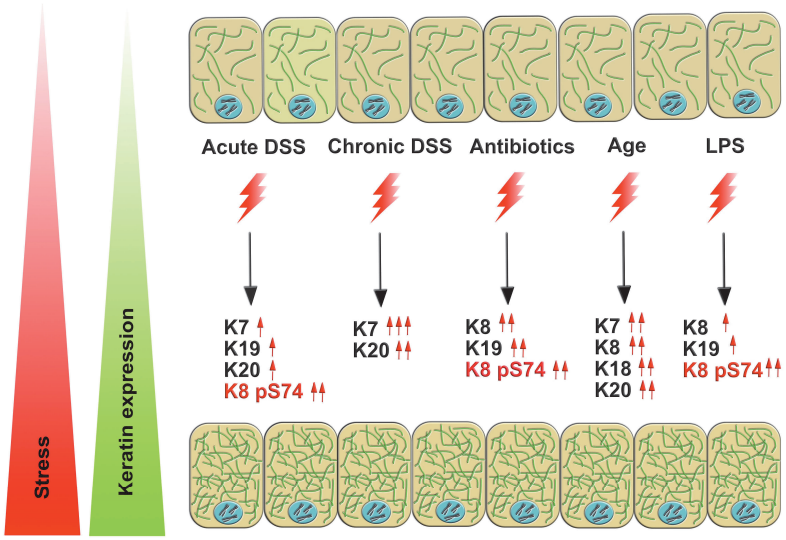

Fig. 8. Intestinal keratins display a pairwise and context-depending upregulation in response to colonic stress and recovery. A summary of Publication I show that during DSS-stress, the K7-K20 pair is upregulated, while the K8-K19 pair is upregulated in response to the deletion of colonic microbiota. Arrows indicate fold-change in keratin expression on protein level.

2. Decreased K8 levels compromise intestinal stress protection (Publication II)

2.1 50% decrease in K8 render mice more susceptible to colonic stress

The $K8^{-/-}$ mouse lacks all cytoplasmic K8 and the $K8^{+/-}$ mouse showed an intermediate phenotype in regards of keratin levels (Publication II, Fig.1A-B, 2A-B), crypt length (Publication II, Fig. 3A-B) and abnormal Na/Cl transport (Toivola et al. 2004a). In contrast to the $K8^{-/-}$ mouse (Habtezion et al. 2005), the $K8^{+/-}$ mouse did not show any signs of spontaneous colitis (Publication II, Fig. 6A-B), defects in apoptosis/anoikis rate (Habtezion et al. 2011) or differences in colon length (Publication II, Table 1; (Habtezion et al. 2005, Baribault et al. 1994) (Table 12).

The $K8^{+/-}$ phenotype with one active allele for K8 reduced K8 levels in colon approximately by 50% on both protein and mRNA levels, confirmed by immunofluorescence staining. A similar 50% decrease was seen for K7 and K18, but only on protein levels (Publication II, Fig. 1). The $K8^{+/-}$ mice showed slightly longer crypts (Publication II, Fig. 3) and a wider area of proliferative cells with an increased number of 5-bromo-2'-deoxyuridine (BrdU)-positive cells/crypt compared to the $K8^{+/+}$ mouse, even if apoptosis rates were not affected (Publication II, Fig. 4).

When the $K8^{+/-}$ mouse was subjected to acute stress by experimental DSS colitis, they were more susceptible to DSS than corresponding $K8^{+/+}$ mice by displaying a higher bleeding score, a higher stool consistency score, a lower body weight % and a lower survival score compared to $K8^{+/+}$ mice (Publication II, Fig. 6A-F). The high DAI and slower recovery of $K8^{+/-}$ mice subjected to chronic DSS compared to the $K8^{+/+}$ mice indicate that the $K8^{+/-}$ mouse is more sensitive to DSS-induced colitis (Publication II, Suppl. Fig. 3A), supporting the hypothesis of the protective role of K8 in intestinal stress and in the recovery from stress. Likewise, the $K8^{+/-}$ mouse showed intermediate levels of the Na/Cl transporters AE1/2, ENaC-γ (Toivola et al. 2004a) and DRA (Asghar et al. 2016) when compared to $K8^{+/+}$ and $K8^{-/-}$ colons, implicating that lower levels of K8 in the colon are linked to impaired protein targeting and function. The permeability of the $K8^{+/-}$ colonocytes did not display any changes compared to the $K8^{+/+}$ phenotype (Misiorek et al. 2016), suggesting that the lower keratin levels in the $K8^{+/-}$ colon are sufficient for intestinal barrier function. Similarly, the 50 % decrease in K8 levels in the $K8^{+/-}$ mouse is likely sufficient for protecting from CRC, as the $K8^{+/-}$ mouse did not develop colonic tumors in response to a chemical or a genetic tumor model as the $K8^{-/-}$ mouse did. However, the levels of the STAT3 regulating protein IL-22BP of $K8^{+/-}$ colonocytes showed an intermediate phenotype (Misiorek et al. 2016). For future studies, the $K8^{+/-}$ mouse, which does not display a baseline colitis phenotype like the $K8^{-/-}$ mouse does, might be a more reliable model for studying the protective role of keratins in the colon.

Table 12. Summary on the $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ colonic phenotypes. The $K8^{+/-}$ mouse displays an intermediate phenotype in regards of keratin protein levels, crypt length, abnormal Na/Cl transport, DRA protein levels and IL-22BP protein levels. In some cases, sufficient protection by the intermediate levels of K8 in the $K8^{+/-}$ mice is established (*e.g.* susceptibility to CRC), whereas this is not always achieved (*e.g.* susceptibility to experimental colitis). ↑ = Increased, ↓ = Decreased, ↔ = Unchanged, ND=Not determined. Red arrows indicate intermediate phenotypes of the $K8^{+/-}$ mouse.

Phenotype	$K8^{+/+}$	$K8^{+/-}$	$K8^{-/-}$	Reference
Keratin protein levels	↔	↓	↓↓	(Baribault et al. 1994), Publication II
Colon length	↔	↔	↓	Publication II
Crypt length/hyperproliferation	↔	↑	↑↑	Publication II
Epithelial permeability/barrier	↔	↔	↑/↓	(Misiorek et al. 2016, Toivola et al. 2004a)
Colonocyte apoptosis/anoikis	↔	↔	↓	(Habtezion et al. 2011)
Spontaneous Th2-type colitis	↔	↔	↑	(Baribault et al. 1994, Habtezion et al. 2005)
Susceptibility to DSS colitis	↔	↑	ND	Publication II
Susceptibility to CRC	↔	↔	↑	(Misiorek et al. 2016)
Ketogenesis	↔	↔	↓	(Nyström 2013) Publication III
Luminal microbiota	↔	ND	↓	(Habtezion et al. 2011) Publication III
Luminal SCFA levels	↔	ND	↑	Publication III
MCT1 levels	↔	↔	↓	Publication III(Nyström 2013)
Abnormal Na/Cl transport	↔	↑	↑↑	(Toivola et al. 2004a)
AE1/2 protein levels	↔	↔	↑	(Toivola et al. 2004a)
Cl-transporter DRA levels	↔	↓	↓↓	(Asghar et al. 2016)
IL-22 mRNA levels	↔	↔	↑	(Misiorek et al. 2016)
IL-22BP protein levels	↔	↓	↓↓	(Misiorek et al. 2016)
P-STAT3	↔	↔	↑	(Misiorek et al. 2016)

2.2 SEKs can act as partially compensatory keratins during partial K8 deletion

As a consequence of the deletion of one of the two alleles of K8, K7 and K20 mRNA levels were not decreased in the $K8^{+/-}$ mouse, although K8 levels were 50 % lower at both protein and mRNA levels (Publication II, Fig. 1). This indicates that colonic keratins are transcribed independently from each other, and that keratins could compensate for each other in the sudden absence of another keratin.

When K7 was removed, like in the $K7^{-/-}$ mouse, K20 mRNA expression in the bladder urothelium was increased, as an expected type I partner (Sandilands et al. 2013). A small, but not significant, increase in K20 mRNA was also seen in the $K8^{+/-}$ mouse colon (Publication II, Fig. 1). The $K7^{-/-}$ mouse did not show an obvious phenotype, likely due to the fact that the closely related other type II keratin, K8, is present and compensates for K7 (Sandilands et al. 2013). Interestingly, K7 immunofluorence staining showed a wider distribution in the base-midcrypt staining in the $K8^{+/+}$ to a fainter, but whole crypt staining in the $K8^{+/-}$ and $K8^{-/-}$ mouse. This indicates that K7, as the only type II keratin present in the $K8^{-/-}$ mouse, compensates for the loss of type II K8 in topmost cells of crypts.

3. K8 deletion leads to a perturbed energy homeostasis and a decreased overall energy metabolism in the colon (Publication III)

3.1 K8 modulates colonic energy metabolism shown by the decreased expression of the ketogenic enzyme HMGCS2 in the blunted K8$^{-/-}$ response

A two-dimensional differential in-gel electrophoresis (2D DIGE) coupled with mass spectrometry identified HMGCS2 as one of the major downregulated proteins in the K8$^{-/-}$ colonocytes (Publication III, Fig. 1A-B). The most differentially expressed proteins in the K8$^{-/-}$ colonocytes were mitochondrial, cytoplasmic and endoplasmic reticulum proteins involved in the energy metabolism and oxidative stress of the colon. HMGCS2 is the rate-limiting control enzyme of ketogenesis, and the downregultion in murine K8$^{-/-}$ colonocytes compared to K8$^{+/+}$ colonocytes was confirmed by (1) protein analysis of *in vivo* colonic crypt lysates (Publication III, Fig. 1C), (2) *in vivo* total colon lysates (Publication III, Fig. 2A) and (3) in two different *in vitro* small interfering RNA (siRNA)-treated cell lines (Publication III, SFig. 1). The enzyme activity of HMGCS2 was not affected (Publication III, Fig. 1D-F). Overall mitochondrial health was assessed by analyzing proteins involved in energy metabolism, suggesting that the mitochondria of K8$^{-/-}$ mice are unaffected by the altered phenotype (Publication II, SFig. 6). In addition to that, ultrastructural analyses of mitochondria were done, showing, opposite to K8$^{-/-}$ liver mitochondria (Tao et al. 2009), no changes in colonocyte mitochondrial size but instead fewer cristae per mitochondrial section area in K8$^{-/-}$ mice (Publication III, SFig. 5).

By challenging the colonic ketogenesis by inducing ketogenic conditions in K8$^{-/-}$ and K8$^{+/+}$ mice, the role of K8 was examined. As expected, ketogenic conditions, such as starvation and ketogenic diet, upregulated HMGCS2 levels in K8$^{+/+}$ mice (Publication III, Fig. 4A-F). Interestingly, this response was blunted in the K8$^{-/-}$ mice, as the K8$^{-/-}$ mouse was not able to significantly increase the HMGCS2 levels in response to ketogenic conditions, suggesting a role for K8 in the overall colonic energy metabolism.

Basally ketogenesis and active HMGCS2 are most prominent in the liver, but it is also found in the colon, where it is regulated by the availability of microbiota-produced SCFAs. In the colon, HMGCS2 levels were considerably higher in PC compared to DC, likely explained by the higher amounts of microbiota (Publication III, Fig. 2A,C). HMGCS2 was not detected in the adult small intestine (Hegardt 1999), supporting the hypothesis that K8 plays a role in the energy metabolism since the K8$^{-/-}$ mouse does not display any major inflammational phenotype in the small intestine (Baribault et al. 1994). The colonic inflammation of the K8$^{-/-}$ mouse itself is not the cause of the perturbed ketogenesis, since HMGCS2 levels were not decreased in the experimental DSS-colitis setting (Publication III, Fig. 2D). The ketogenic conditions clearly affected the liver, since blood glucose was decreased and β-hydroxybutyrate was increased in response to

the ketogenic conditions. However, no differences in these parameters were seen between the genotypes (Publication III, Fig. 3A-D). In addition to no observed changes in liver HMGCS2 levels during baseline conditions (Publication III, Fig. 2C), the earlier established roles of K8 in cytoprotection (Omary et al. 2009) and altered liver mitochondrial ultrastructure (Tao et al. 2009) in the $K8^{-/-}$ mouse, suggests disturbances in liver energy metabolism upon K8 deletion without affecting the ketogenic pathway.

The main transcriptional regulator of HMGCS2 in baseline and ketogenic conditions is PPARα. In liver, it has been shown that PPARα manages energy stores (Kersten et al. 1999) and increases hepatic HMGCS2 (Rodriguez et al. 1994), which is supported by our *in vivo* colonic data where we showed that PPARα levels are mostly following the expression pattern of HMGCS2 (Publication II; Fig 2, 4).

3.2 Downregulation of the butyrate transporter MCT1 leads to increased amounts of luminal SCFAs

When the decrease of $K8^{-/-}$ colonocyte HMGCS2 was investigated in detail, the butyrate transporter MCT1 was also found downregulated. As $K8^{+/+}$ HMGCS2 levels were upregulated after starvation, MCT1 levels also increased (Publication III, Fig. 5C), suggesting the involvement of MCT1 in the blunted $K8^{-/-}$ ketogenic response. HMGCS2 expression is known to be dependent on the colonic microbiota and the availability of SCFAs (Cherbuy et al. 2004), and $K8^{-/-}$ mice colon is known to be inhabited by fewer microbes than the $K8^{+/+}$ mice colon (Habtezion et al. 2011). Further analyses of the luminal SCFAs and colonic microbiota by fecal gas chromatography profiling showed that luminal butyrate and isobutyrate levels, the two most prominent of all colonic SCFAs, were increased in the $K8^{-/-}$ mice colon although there were no differences in the bacterial content between the two genotypes (Publication III, Fig. 6). The main hypothesis for the mechanistic role of K8 in the decreased levels of HMGCS2 is that keratins contribute to the proper targeting of MCT1 to the cell membrane, supported by the fact that the $K8^{-/-}$ mouse exhibits mistargeting and loss of other membrane proteins as well (Toivola et al. 2004a, Asghar et al. 2016).

To analyze the localization of MCT1 in $K8^{-/-}$ conditions a double immunofluorescent staining with K8 and MCT1 was done. Although the $K8^{-/-}$ colon is hyperproliferative and difficult to analyze, MCT1 was found similarly located in the cell membrane of $K8^{+/+}$ and $K8^{-/-}$ colonocytes, suggesting that MCT1 localizes to the cell membrane without the help of K8 (Publication II, SFig. 7A). However, it is known that *in vivo* fiber feeding triggers the relocalization of MCT1 to the apical cell membrane (Borthakur et al. 2012), which was not analyzed here. The *in vivo* double immunofluorescent staining showed a clear lateral staining of MCT1 in both $K8^{+/+}$ and $K8^{-/-}$ colonocytes, supported by several studies showing lateral distribution in baseline and starved situations. This *in vivo* data was confirmed by two different *in vitro* models. First, in K8 siRNA-treated Caco-2 cells, MCT1 was also found localized to the cell membrane in a similar way as in control

cells (Publication II, SFig. 7B). Similarly, when Min6 cells, which basally lack all keratins, were transfected with a plasmid for MCT1-GFP +/- K8/K18, MCT1 similarly localized to the cell membrane independent of the presence of K8/K18 filaments (Fig. 9). In both of these $K8^{-/-}$ *in vitro* models, the decrease in MCT1 levels was obvious.

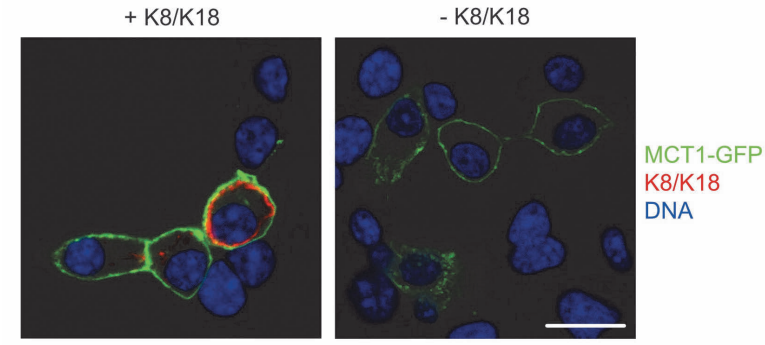

Figure 9. MCT1 localization remains unchanged regardless of K8/K18 presence. Confocal analyses of Min6-cells transfected with MCT1-GFP plasmid +/- K8/K18 showed no changes in MCT1 localization, even if MCT1 levels were decreased in the absence of K8. Green staining indicates MCT1, red staining K8/K18 filaments and blue staining cell nuclei. Scale bar = 20 µm.

Apart from the primary role of MCT1 as an SCFA transporter in colonocytes, MCT1 is also involved in the export of the toxic metabolite lactate (Halestrap 2012). Lactate is produced by glycolysis during the anaerobic breakdown of glucose in colonocytes (especially during Warburg metabolism), from where it is transported to the liver where it enters gluconeogenesis for glucose production. With less MCT1 present in the $K8^{-/-}$ colonocytes, lactate might be accumulated inside the cell and influence the overall energy metabolism and acidic content of the colonocyte. As a consequence to a blunted pH regulation in the $K8^{-/-}$ colonocytes, acidic intracellular content of $K8^{-/-}$ colonocytes has been observed (Toivola et al. 2004a).

MCT1 downregulation as a function of K8 was further confirmed by rescuing the $K8^{-/-}$ phenotype. MCT1 levels in mice overexpressing human K8 (hK8) showed increased levels of MCT1 together with increased K8 and K19 (Fig. 10). The transcriptional regulator of HMGCS2, PPARα, was also found upregulated in this model system. Earlier studies have shown that PPARα can directly regulate MCT1 expression (Konig et al. 2008), therefore also serving as a direct transcriptional regulator of MCT1.

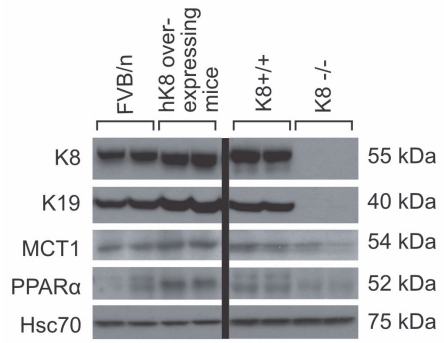

Figure 10. *In vivo* overexpression of human K8 upregulates colonic MCT1, PPARα, K8 and K19 in a similar matter. Protein analysis showed that by overexpressing human K8 in FVB/n mice, the $K8^{-/-}$ phenotype with decreased MCT1, K8 and K19 levels was rescued. Hsc70 was used as a loading control.

In conclusion, the results from Publication III show that the decreased amounts of the butyrate transporter MCT1 in the $K8^{-/-}$ mouse leads to elevated luminal levels of microbiota-produced butyrate. The lower levels of intracellular butyrate feeding into the ketogenic pathway leads to decreased levels of HMGCS2, causing an overall impaired ketogenesis and energy metabolism in the $K8^{-/-}$ colon, suggesting a protective role of colonic K8 in energy homeostasis (Fig. 11).

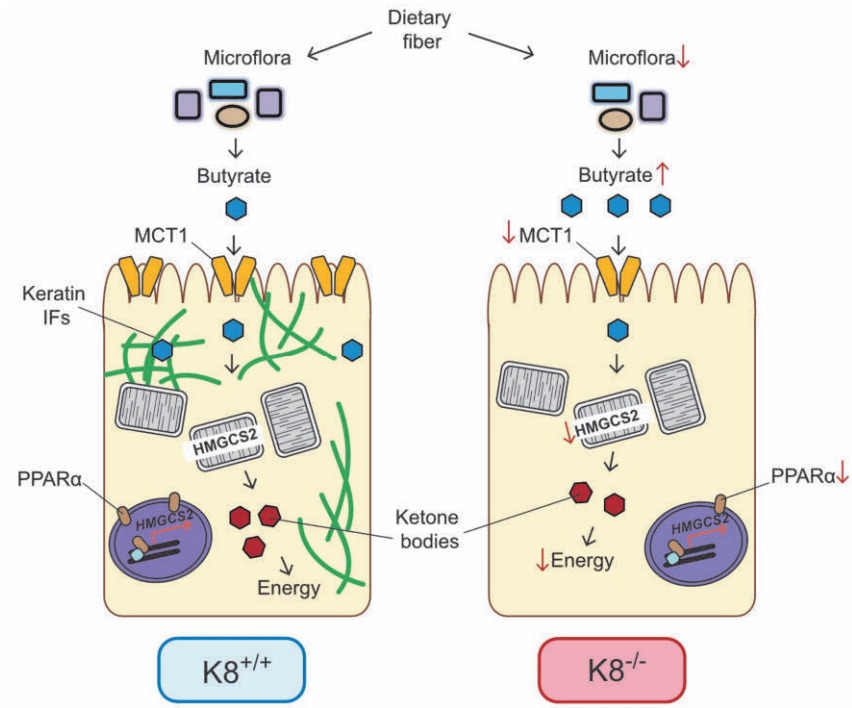

Figure 11. The K8-/- energy metabolism is decrased in the colon. The ketogenic enzyme HMGCS2 is downregulated in the K8-/- colon during baseline conditions, and shows a blunted response during ketogenic conditions. When studying upstream regulation of HMGCS2, the transcriptional regulator PPARα and the butyrate transporter MCT1 were also found down-regulated in the K8-/- colon. Therefore butyrate, which was found elevated in the K8-/- lumen, cannot enter the colonocytes properly, leading to lower levels of HMGCS2 and a lower amount of ketone bodies which are normally used as energy by the colonocytes.

4. Colonic keratins act as stress proteins which are primarily regulated on protein level (Publication I, II and III)

Keratins are primarily upregulated in stress at protein levels, since no increase in mRNA levels of stress-induced keratins were seen in any of the stress models used in Publication I (Publication I, Fig 1F, 3F and 4D). Similarly, as seen in the $K8^{+/-}$ mouse, only K8 mRNA levels were significantly decreased in response to the deletion of one K8 allele (Publication II, Fig. 1). mRNA levels of the remaining keratins, K7, K18, K19 and K20, were unaltered in this genotype, even if the protein levels of K7, K18, K19 and K20 (not significant) were decreased (Publication II, Fig. 1). The same observation was made in the $K7^{-/-}$ mouse, where only K7 mRNA was significantly decreased in the $K7^{+/-}$ mouse (Sandilands et al. 2013), yet another piece of evidence indicating that keratin mRNAs are transcribed independently from each other.

No major changes in keratin mRNA levels were observed in any of the used stress models in Publication I (Fig. 1, 3, 4) or most of the ketogenic models in Publication III (Fig. 4G). In the rapidly renewed colonic epithelium, the overall rate of mRNA-protein turnover is fast and the time point when protein changes can be detected may not overlap with the peak time point for mRNA levels. The same has been reported for the downregulation of the Cl-transporter DRA in the $K8^{-/-}$ colon, where a total loss of this protein was seen although mRNA levels were unaltered (Asghar et al. 2016). Therefore, the situation in the colon is somewhat different from that in the liver (Guldiken et al. 2015a) and exocrine pancreas (Zhong et al. 2004) where keratin overexpression in induced stress has been detected on both protein and mRNA levels. Increased mRNA levels were also noted in murine diseased kidneys (Djudjaj et al. 2016), although the increase in mRNA levels was not proportional to the even higher increase in protein levels in response to stress. This suggests the involvement of translational regulation of keratin expression in the kidney. However, it is possible that the observed changes in keratin levels could be a consequence of protein stability and/or protein degradation, as seen for pulmonary K8 and K18 in stress-mediated ubiquitination by the proteasome (Jaitovich et al. 2008, Rogel, Jaitovich & Ridge 2010) . In addition, it is known that mRNA and protein levels do not always correlate due to post-transcriptional mechanisms and different *in vivo* half-lives of proteins (Greenbaum et al. 2003, Chen et al. 2002). More detailed studies about the rather unknown keratin transcription factors p53, SP1 and AP1 could also shed some light over the exact mechanism for keratin upregulation in stress and recovery (Rhodes, Oshima 1998).

CONCLUDING REMARKS

This thesis shows, by three different murine models, how keratins are modulated in stress and in recovery from stress and disease (Fig. 12). Keratins play a role in colonic stress protection, shown by: (1) their upregulation after induced stress and (2) by the increased susceptibility to experimental colitis when 50 % of K8 is removed ($K8^{+/-}$). (3) When all K8 is removed, the colonic energy homeostasis is impaired and blunted, contributing to a disease phenotype. Decreasing keratin protein levels cause increased stress sensitivity; two K8 alleles protect from stress, one K8 allele causes disease susceptibility and the loss of both K8 alleles causes disease (Figure 12 and Table 12).

Colonic keratins are generally upregulated in response to disease-related stresses in a context-dependent matter, demonstrating the stress-responsive nature of keratins. During the acute stress response, hyperphosphorylation of K8 occurs, which could be used as a marker of acute intestinal disease. The total deletion of K8 (and the consequent lack of most colonocyte keratins) in mice leads to colitis, and mice with 50% less K8 show increased susceptibility to DSS-induced colitis. This shows that keratins protect from intestinal stress by being upregulated in response to stress.

The milder $K8^{+/-}$ phenotype is useful for future studies regarding keratins in intestinal stress protection, instead of the $K8^{-/-}$ mouse model that exhibits baseline colitis or the K8 overexpressing mutant mouse models where the impact of the increased keratin levels may be protective.

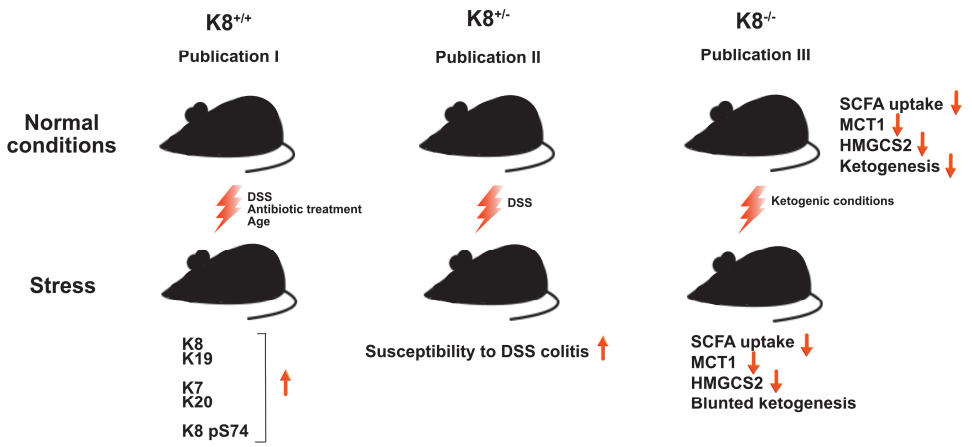

Figure. 12. $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$: Three models that support the importance of keratins in colonic stress protection. The $K8^{+/+}$ mouse shows a stress-responsive behavior and the increased DSS-susceptibility of the $K8^{+/-}$ mice indicates that colonic keratins play an important role in protection from intestinal stress. This is further verified by the blunted $K8^{-/-}$ ketogenesis. These three mouse models with different amounts of K8 indicate that colonic keratins likely play an important role in the protection from intestinal stress.

The implication of the results in this thesis on human health reveals the potential role of keratins as intestinal disease protectors. The protective role of SEKs in other human and murine organs has already been established, whereas the role of intestinal SEKs in response to stress has only recently been described. Due to this, increased and reorganized intestinal keratins could be considered, similarly as in other organs, as markers for stress.

In conclusion, when normal colonocytes are subjected to stress, keratins are upregulated in a context-dependent manner. However, when colonocytes with decreased or non-existing keratins are subjected to stress, they develop a more severe disease, indicating that colonic keratins are required for intestinal stress protection and overall health.

FUTURE PROSPECTS

In regards of intestinal keratins and their role in intestinal stress protection, the research focus lies on further analyses on the behavior of colonic keratins in the intestinal stress response. Since several keratins respond to murine disease models, a specific research direction would be to validate these results in human disease. Samples from human intestinal diseases, such as UC, CD and CRC, would be of high importance to analyze for keratin expression. Results related to demographic information, histologic/endoscopic findings, disease status and medications would significantly improve our understanding on how keratins contribute to colonic health. Also human colon biopsies from colon tissue subjected to external stresses, such as microbiota-depletion by antibiotics or irradiation-mediated intestinal damage could be analyzed in regards of a changed keratin profile.

The role of K8 mutant variants in the pathogenesis of IBD has been identified in a few patients, even if a general conclusion about K8 mutations in IBD has not been established yet. The current knowledge points to the fact that K8 mutations are possibly predisposing factors for the development of IBD. Large studies would be needed for further validation of the correlation between keratin mutations and IBD in humans. Mouse knock in models expressing human K8 mutations, for example the commonly found human mutation G62C, could be used for further analyses on human K8 mutations in IBD development.

Future experiments for investigating the mechanistic and molecular role of keratins in stress protection could be done by investigating protein stability by different *in vitro* methods. Another direction for demonstrating a mechanism for keratin upregulation in stress would be to collect *in vivo* samples at different time points during stress-experiments, obtaining a time-dependent set of samples which could be further analyzed for specific components in affected signaling pathways. Studies regarding keratin transcription factors and their regulation could also contribute to the understanding of how intestinal keratins protect from stress.

Another interesting research focus would be to analyze the K8 deletion-mediated impaired colonic energy metabolism in regards of keratin levels using the $K8^{+/-}$ mouse. Ketogenic conditions could be used for determining if the remaining keratin levels in the $K8^{+/-}$ phenotype are able to maintain energy homeostasis in the simple-layered epithelia of the colon.

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