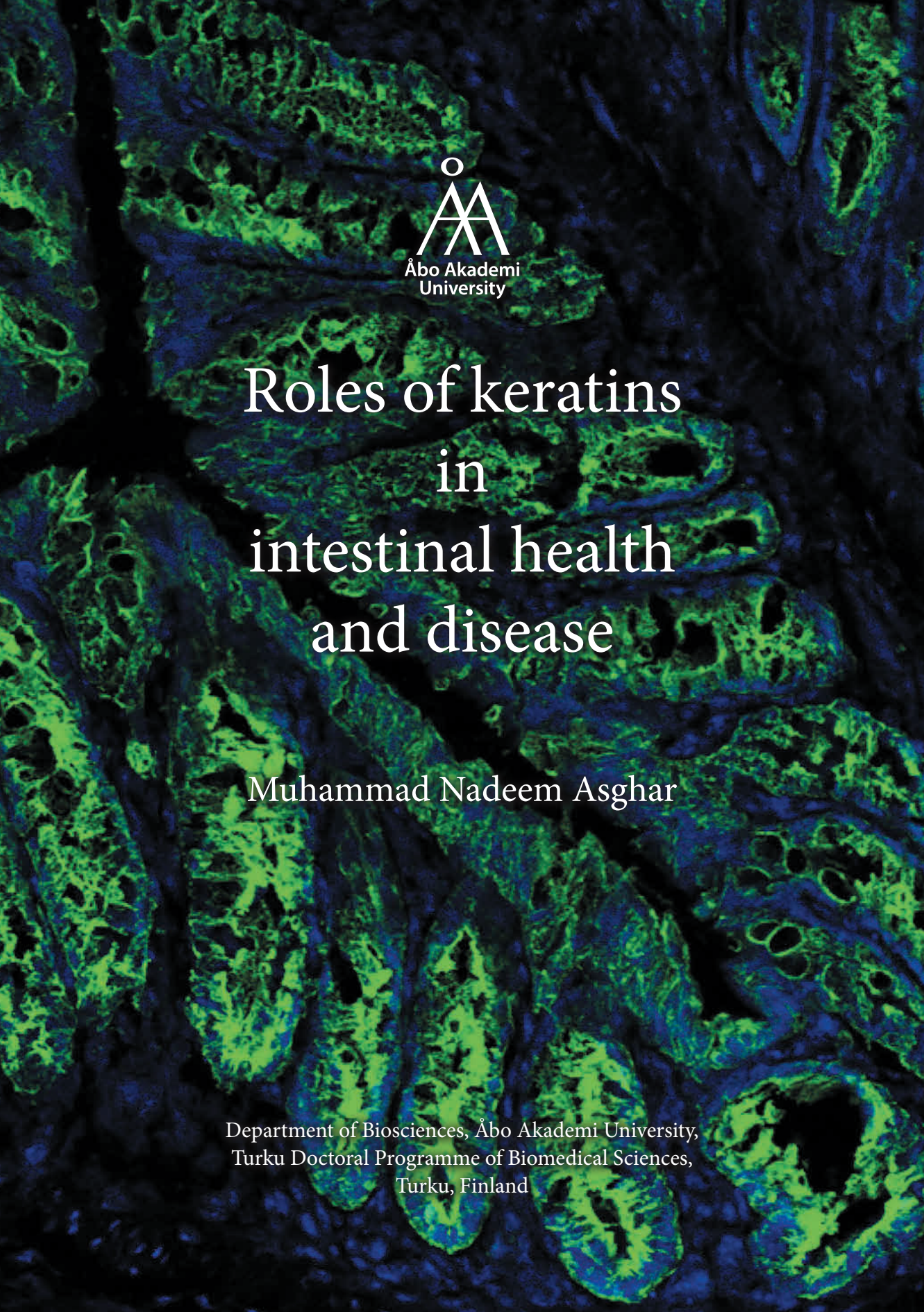

Åbo Akademi University

Roles of keratins in intestinal health and disease

Muhammad Nadeem Asghar

Department of Biosciences, Åbo Akademi University,
Turku Doctoral Programme of Biomedical Sciences,
Turku, Finland

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Muhammad Nadeem Asghar

MSc., M.Phil., Lic.

Department of Biosciences, Åbo Akademi University,
Turku Doctoral Programme of Biomedical Sciences,
Turku, Finland

Supervisor:
Diana Toivola, PhD, Docent
Department of Biosciences,
Åbo Akademi University,
Turku, Finland.

Administrative Supervisor:
Kid Törnquist, PhD, Professor
Department of Biosciences,
Åbo Akademi University,
Turku, Finland.

Reviewers:
Peter Fuchs, PhD, Assistant Professor
Max F. Perutz Laboratories,
University of Vienna,
Centre for Molecular Biology,
Vienna, Austria.

Pekka Taimen, MD, PhD, Docent
Molecular Pathology,
University of Turku and Turku
University Hospital,
Turku, Finland.

Faculty opponent:
Alexander Minin, PhD, Head of Department
Institute of Protein Research,
Russian Academy of Sciences,
Group of Cell Biology,
Moscow, Russia.

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“Truth is stranger than fiction, but it is because fiction is obliged to stick to possibilities; Truth isn't.”

— Mark Twain

Dedicated to my beloved grandmother, Mrs. Maqbool Khatoon (late) and dearest parents, Mrs. Dr. Najma Asghar (late) and Dr. Muhammad Asghar (late).

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ABSTRACT

Intermediate filament keratins (K) play a pivotal role in protein targeting and epithelial cytoprotection from stress as evidenced by keratin mutations predisposing to human liver and skin diseases and possibly inflammatory bowel disease (IBD). The K8-null ($K8^{-/-}$) mice exhibit colonic phenotype similar to IBD and marked spontaneous colitis, epithelial hyperproliferation, decreased apoptosis, mistargeting of proteins leading to defective ion transport and diarrhea. The K8-heterozygote ($K8^{+/-}$) mouse colon appears normal but displays a defective sodium (Na^+) and chloride (Cl^-) transport similar to, but milder than $K8^{-/-}$.

Characterization of $K8^{+/-}$ colon revealed ~50% less keratins (K7, K8, K19, K20) compared to K8 wild type ($K8^{+/+}$). A similar ~50% decrease was seen in $K8^{+/-}$ mRNA levels as compared to $K8^{+/+}$, while the mRNA levels for the other keratins were unaltered. $K8^{+/-}$ keratins were arranged in a normal colonic crypt expression pattern, except K7 which was expressed at the top of crypts in contrast to $K8^{+/+}$. The $K8^{+/-}$ colon showed mild hyperplasia but no signs of inflammation and no resistance to apoptosis. Experimental colitis induced by using different concentrations of dextran sulphate sodium (DSS) showed that $K8^{+/-}$ mice are slightly more sensitive to induced colitis and showed a delayed recovery compared to $K8^{+/+}$. Hence, the $K8^{+/-}$ mouse with less keratins and without inflammation, provided a novel model to study direct molecular mechanisms of keratins in intestinal homeostasis and ion transport.

Different candidate ion transporters for a possible role in altered ion transport seen in the $K8^{-/-}$ and $K8^{+/-}$ mouse colon were evaluated. Besides normal levels of CFTR, PAT-1 and NHE-3, DRA mRNA levels were decreased 3-4-fold and DRA protein nearly entirely lost in $K8^{-/-}$ caecum, distal and proximal colon compared to $K8^{+/+}$. In $K8^{+/-}$ mice, DRA mRNA levels were unaltered while decreased DRA protein level and patchy distribution was detected particularly in the proximal colon and as compared to $K8^{+/+}$. DRA was similarly decreased when K8 was knocked-down in Caco-2 cells, confirming that K8 levels modulate DRA levels in an inflammation-independent manner. The dramatic loss of DRA in colon and caecum of $K8^{-/-}$ mice was responsible for the chloride transport defect. The milder ion transport in $K8^{+/-}$ colon might be related to DRA suggesting a role for K8 in regulation of DRA expression and targeting. The current study demonstrates the importance of keratins in stress protection and cell signaling.

Furthermore, we have also successfully developed a novel, simple, fast, cost effective, non-invasive *in vivo* imaging method for the early diagnosis of murine colitis with specificity for both genetic and experimental colitis. The said modality provides continuous measurements of reactive oxygen and nitrogen species (RONS) and minimizes the use of an increased number of experimental animals by using a luminal derivative chemiluminescent probe, L-012 which provides a cost-effective tool to study the level and longitudinal progression of colitis.

Key words: Intermediate filaments, Keratin 8, inflammatory bowel disease, colon, colitis, stress protection, ion transport, DRA, L-012, *in vivo* imaging, reactive oxygen and nitrogen species.

LIST OF ORIGINAL PUBLICATIONS (THESIS)

This thesis is based on the work from two original publications and one submitted manuscripts, which are referred to in the text by their Roman numbers.

I **Asghar MN,** Silvander JS, Helenius TO, Lähdeniemi IA, Alam C, Fortelius LE, Holmsten RO, Toivola DM. The amount of keratins matters for stress protection of the colonic epithelium. PLoS One. 2015; 10(5):e0127436.

II **Asghar MN[#],** Priyamvada S[#], Nyström JH, Anbazhagan AN, Dudeja PK[*], Toivola DM[*]. Keratin 8 knockdown leads to loss of the chloride transporter DRA in the colon. Manuscript, in revision.

III **Asghar MN,** Emani R, Helenius TO, Alam K, Grönroos TJ, Sareila O, Din M, Holmdahl R, Hänninen A, Toivola DM. *In vivo* imaging of reactive oxygen and nitrogen species in murine colitis. Inflamm Bowel Dis. 2014; 20(8):1435-47.

[#], [*] Equal contribution

LIST OF ORIGINAL PUBLICATIONS (NOT IN THESIS)

i Emani R, **Asghar MN,** Toivonen R, Lauren L, Söderström M, Toivola DM, van Tol EA, Hänninen A. Casein hydrolysate diet controls intestinal T cell activation, free radical production and microbial colonisation in NOD mice. Diabetologia. 2013; 56(8):1781-91.

ii Habtezion A*, Toivola D*, **Asghar MN,** Kronmal G, Brooks J, Butcher E, Omary B. Keratin 8 confers a paradoxical microflora-dependent pro-apoptotic role in the colon. Proc Natl Acad Sci U S A. 2011; 108(4):1445-50.

iii Helenius TO*, Misiorek JO*, Nyström JH*, Fortelius LE, Habtezion A, Liao J, **Asghar MN,** Zhang H, Azhar S, Omary MB, Toivola DM. Keratin 8 absence down regulates colonocyte HMGCS2 and modulates colonic ketogenesis and energy metabolism. Mol Biol Cell. 2015; 26(12):2298-310.

* shared first author

ABBREVIATIONS

AE1/2	anion exchanger 1/2
AIH	autoimmune hepatitis
ALS	amyotrophic lateral sclerosis
AQP	aquaporins
Bfsp	beaded filament structural protein
BSA	bovine serum albumin
CD	Crohn's disease
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CHC	chronic hepatitis C
CLD	congenital chloride diarrhea
CMT	Charcot-Marie-Tooth
CT	computed tomography
DRA	downregulated in adenoma
DSS	dextran sulphate sodium
EBS	epidermolysis bullosa simplex
EDTA	ethylenediamine tetra acetic acid
EGF	epidermal growth factor
ENaC	epithelial Na^+ channel
EPEC	enteropathogenic E. Coli
IBD	inflammatory bowel disease
IECS	intestinal epithelial cells
IF	intermediate filaments
K	keratins
K18	keratin 18
K19	keratin 19
K20	keratin 20
K23	keratin 23
K5	keratin 5
K7	keratin 7

K8	keratin 8
LP	lamina propria
MDB	Mallory-Denk bodies
MF	microfilaments
MPO	myeloperoxidase
MRI	magnetic resonance imaging
MT	microtubules
NaCl	sodium chloride
NASH	nonalcoholic steatohepatitis
NF	neurofilament
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHE	sodium hydrogen (Na^+/H^+) exchangers
NO	nitric oxide
PAT	putative anion transporter
PDGF	platelet derived growth factor
PET	positron emission tomography
Pls 1	plastin 1
RONS	reactive oxygen nitrogen species
SCFAs	short chain fatty acids
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEK	simple epithelial keratins
SPECT	single photon emission computed tomography
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF-α	tumor necrosis factor α
TPA	tissue polypeptide antigen
TPS	tissue polypeptide specific antigen
UC	ulcerative colitis
+/+	wild type
+/-	heterozygote
-/-	knock out

1. INTRODUCTION

Keratins (K) are intermediate filament (IF) cytoskeletal proteins found in all epithelia of the body including the single layer of epithelial cells forming a barrier towards the intestinal lumen and are known for their role in cellular integrity, cell signaling, protein targeting, apoptosis and protection against stress. Simple epithelial keratins (SEK) of type II (K7, K8) and type I (K18, K19, K20, K23) compose the main IF cytoskeleton of intestinal epithelial cells (IECs). Keratins form obligate heteropolymers (one type I and one type II keratin) and share a common structure that consists of a central coiled-coil α-helical rod domain that is flanked by non-α-helical head and tail domains. One of the major challenges in modern biomedicine is to explore the functional significance of keratins in intestine as specific keratin pairs predominate in an epithelial cell and differentiation state selective manner. The usefulness of keratins is obvious by the keratin mutations predisposing to human skin and liver disease and multiple mouse models phenocopying these diseases. Mutations in the genes encoding IF proteins either directly cause or predispose their carriers to more than 80 human diseases. Keratin mutations are found in ~ 10% of patients with liver disease, however, the correlation is still unclear in intestinal epithelia, although a few IBD patients with keratin mutations have been reported. Although the role of keratins in the intestine is not fully understood, it has been shown that K8 null ($K8^{-/-}$) mice have epithelial hyperproliferation, decreased apoptosis and develop a Th2-type ulcerative colitis (UC) which is amendable to antibiotic treatment. The $K8^{-/-}$ mice also display a dramatic alteration in ion transport, leading to chloride secretion (rather than uptake) likely caused by epithelial ion transporter mistargeting, leading to diarrhea. Interestingly, the K8 heterozygote mice ($K8^{+/-}$), which express lower K8 levels as compared to the K8 wild type mice, also display an altered ion transport although no histological abnormalities are observed. This led us to hypothesize that the concentration of colonocyte keratin, or an intact keratin network, is essential to protect from colitis. The first aim of this PhD thesis is to characterize the $K8^{+/-}$ phenotype and to test the susceptibility of the $K8^{+/-}$ mice with lower levels of keratins in response to stress, i.e. experimental dextran sulphate sodium (DSS) colitis.

The colon is responsible for the absorption of sodium (Na^+), chloride (Cl^-), potassium (K^+), short chain fatty acids (SCFAs) and fluid, as well as to secrete bicarbonates (HCO_3^-) and mucus. The osmotic gradient created by salt transport using ion transporters and channels is the driving force for passive water movement in/out of the lumen. Water transport can also occur through specific water channels, the aquaporins. The colonic crypts are responsible for absorptive functions but may perform secretory functions such as in response to cholera toxin. Diarrhea results in electrolyte secretion and loss of fluid into intestinal lumen. An inflamed small

intestine fails to absorb food and results in secretion of water and salts into the alimentary canal. The colon cannot absorb this extra water which leads to diarrhea. Although the exact mechanism is not known, the $K8^{-/-}$ colon shows mistargeting of membrane transporters and altered Na^+ and Cl^- transport leading to diarrhea, which precedes hyperproliferation and inflammation. The $K8^{-/-}$ distal colon has normal tight junctions and paracellular transport besides having a decrease in short circuit current and net Na^+ absorption associated with net Cl^- secretion. The second aim of this thesis is to find out whether keratins are involved in ion transport and how intestinal keratins contribute to the intestinal phenotype and diarrhea.

As use of experimental models for colitis as well as genetic models involves invasive techniques which are skill-requiring, time consuming and laborious, *in vivo* imaging would provide early and noninvasive diagnosis of colitis. Such methods had not been established. L-012 is a promising simple, cost effective chemiluminescent probe with high sensitivity against reactive oxygen and nitrogen species. L-012 is well distributed in the mouse body and mediates a strong ROS/RNS-dependent luminescent signal *in vivo* and is useful for monitoring the development and regulation of inflammation in living organisms.

The thesis work provides an understanding about the roles of keratins, particularly of K8, in normal intestinal homeostasis and during colitis. Our results show that the levels of keratins matter for protection from stress and leads to the reduced levels of chloride transporter, downregulated in adenoma (DRA), in response to K8 ablation. These findings eventually provide a platform for further mechanistic studies to target direct molecular mechanisms of keratins in intestinal homeostasis including ion transport by using a $K8^{+/-}$ mouse as a novel model that lacks the inflammatory component and has lower levels of keratins. This thesis also claims significance for understanding human intestinal diseases which are possibly linked to keratin mutations. The thesis also focuses on the establishment of a novel *in vivo* imaging modality of murine colitis. The outcome of the research may, thus, also be helpful for future development of diagnostics and treatment of colitis.

2. REVIEW OF THE LITERATURE

2.1. Overview of the intermediate filaments

The eukaryotic cytoskeleton is composed of three major protein families, i.e. microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) which can be differentiated from each other on the basis of their size (Fuchs and Coulombe, 1992; Ku et al., 1999; Lazarides, 1980; Steinert and Parry, 1985). Historically the name IFs was given because of the fact that these filaments have an average diameter of 10 nm which is greater than 7 nm of MFs and less than 25 nm MTs (Fuchs and Coulombe, 1992; Omary et al., 2009). MFs and MTs are comprised of highly conserved proteins, actin and tubulin, respectively and are far more widespread in expression profiles (Eriksson et al., 2009; Ku et al., 1999; Moll et al., 1982b). In contrast, the diverse family of IF protein is formed by the transcription of 70 functional genes with alternative splicing and post-transcriptional modifications (Omary et al., 2009; Szeverenyi et al., 2008). Another differentiating feature of IFs is that, unlike MFs (actin) and MTs (tubulin), they are not composed of globular proteins (Table 1).

2.1.1. Classification of intermediate filaments

IF proteins are expressed in a tissue- and cell compartment-specific manner (Fuchs and Coulombe, 1992; Hesse et al., 2001; Lazarides, 1982; Omary et al., 2009) and can be divided into 6 major types based on similarities in central rod domain amino acid sequences, net acidic charge, and secondary structure predictions (Fig. 1). Most IF types are cytoplasmic (I-IV) while one type (V) is nuclear and consists of the lamins. The 6th class of IF proteins, also referred to as orphans, consists of the beaded filament structural protein 1 (Bfsp1; also known as filensin) and Bfsp2 (also known as phakinin and CP49), form highly specialized IFs found only in the lens of the eye. IFs can make homo- or hetero- polymers and can be further subdivided into three assembly groups on the basis of their abilities to copolymerize with each other. The type I cytoskeletal IF proteins are the acidic keratin proteins, and type II cytoskeletal IF proteins are the neutral-basic keratin proteins. At least one type I keratin and one type II keratin are needed to form heteropolymeric keratin IFs. Type III IF proteins, which include vimentin and desmin, can form homopolymeric IFs as well as heteropolymeric IFs in combination with other type III or type IV IFs. The type IV IF proteins include nestin, synemin and neurofilament (NF) triplet proteins with low-, middle-, and high-molecular weight NF subunits i.e. NF-L, NF-M and NF-H. (Eriksson et al., 2009; Perng et al., 2007; Song et al., 2009; Steinert, 1998; Szeverenyi et al., 2008; Toivola et al., 2005).

Table 1. The main aspects of cytoskeletal elements. Adapted from on (Coulombe and Omary, 2002; Ku et al., 1999; Toivola et al., 2005; Vartiainen, 2008).

Properties	Microfilaments	Microtubules	Intermediate filaments
Prototype proteins	Actins	Tubulins	Many, cell, tissue specific
Expression	Eukaryotes	Eukaryotes	Eukaryotes (besides yeast)
Polarity	+	+	-
Solubility	+++	+++	+
Structure	Conserved	Conserved	Diverse (with conserved subdomains)
Diameter (nm)	5-8	25	10-12
Phosphorylation	+	+	++++
Cellular location	Cytoplasm, Nucleus	Cytoplasm	Cytoplasm, Nucleus
Associated diseases	+	-	++++
Stress protection	-	-	+++
Stabilizers	Phalloidin	Taxol	Taxol
Destabilizers	Cytochalasins	Colchicine and others	-
Binding proteins	+++	+++	+
Specific functions	Cell movement, attachment	Cell cycle, intracellular transport	-

2.1.2. Main IF functions and IF-pathies

IFs have been shown to play an important role in cell signaling, protein targeting and to perform cytoprotective functions. They facilitate cells to cope with mechanical and non-mechanical stresses. The mechanical stresses can include shear stress and compression of cells, while non-mechanical stresses are heat, metabolic, oxidative and genetic stresses (Toivola et al., 2005). IFs regulate the enzymatic activity of intracellular kinases by acting as a scaffold (Eriksson et al., 2009). Around 100 human diseases have been related to IFs (Szeverenyi et al., 2008) (Human intermediate filament database; www.interfil.org). IF disorders directly involved in disease manifestation are called IF-pathies including skin and hair diseases, myopathies, neurodegenerative disorders and cataracts (Omary et al., 2009). Missense mutations in conserved amino acid sequences of the α-helical domain either cause or predispose to human disease (Omary et al., 2009). Another leading cause of IF-pathies is formation of cell specific inclusion bodies which are indirectly involved in disease progress and serve as hallmarks for the diseases (Omary et al., 2004). Mutations in IF coding genes result in several human disorders and diseases, such as epidermolysis bullosa simplex (EBS), an inherited skin blistering disorder caused by mutation in K5/K14 (Bolling et al., 2011). Similarly, mutations in the genes encoding glial fibrillary acidic protein (GFAP) and peripherin result in Alexander's disease (Brenner and Messing, 2014) and

several progressive neurological disorders (Corrado et al., 2011) respectively. The majorities of the IF-pathies are rare or orphan diseases with very little prevalence, i.e. less than 200,000 patients are living with this diseases in the US. (Office of Rare Diseases Research, http://rarediseases.info.nih.gov/RareDiseaseList.aspx). However, there are some exceptions, like the mutations in the genes encoding K8, K18, and K19 that predispose individuals to progression of common chronic liver diseases including Hepatitis C (HCV) (Ku et al., 2007). IFs are broadly used in diagnostics and disease activity evaluation besides serving as autoantigens and markers for tumors, tissues and apoptotic and necrotic processes (Omary et al., 2009). The finding that IFs play a role in human diseases has helped in understanding IF functions in homeostasis. There is a need for further studies to explore IF functions by using animal models, *in vitro* models and human specimens.

2.2. Keratins

Keratins are the largest sub group of cytoplasmic IF proteins and form obligate non covalent heteropolymers consisting of type I and type II proteins, exclusively expressed in epithelial cells (Coulombe and Omary, 2002; Moll et al., 2008). However, some non-epithelial neoplasm and mesenchymal tissues such as heart may express low levels of keratins under certain circumstances (Papathanasiou et al., 2015).

2.2.1. Keratin nomenclature and structure

The human genome contains 54 functional keratin genes (*KRT*) at 12q13.13 and 17q21.2 chromosomal sites. The *KRT* gene products are designated as 'K' followed by a number like K8, K18, K20 (Schweizer et al., 2006). Moll et al. (1982) proposed the first nomenclature of keratins based on two-dimensional gel electrophoresis separation and it has been repeatedly updated in recent years (Hesse et al., 2001; Hesse et al., 2004; Schweizer et al., 2006). The novel consensus nomenclature of keratins is based according to the Human and Mouse Genome Nomenclature Committees guidelines (Schweizer et al., 2006) and is an revision of older nomenclatures. Szeverenyi et al. (2008) published a comprehensive catalogue of the human keratins, their amino acid sequence and the nucleotide sequence of the keratin genes in humans as well as in various vertebrate species. Keratins can be divided into acidic type and basic to neutral type on the basis of their isoelectric point which is defined as a pH value at which the net charge of a protein is 0. Based on nucleotide sequence, human keratins are classified into two types: 28 type I keratin genes (17 epithelial and 11 hair keratins) and 26 type II keratin genes (20 epithelial and 6 hair keratins) (Schweizer et al., 2006).

Although IF proteins have quite distinct primary amino acid sequences, they share a common tripartite domain organization (Fig.2) and are major components of the mammalian cytoskeleton (Herrmann et al., 2009; Hesse et al., 2007; Kreplak et al.,

2004; Portet et al., 2009; Smith and Parry, 2007). The keratin polymer central α-helix rod domain contains around 310 amino acid residues and is flanked by a non-helical C-terminal head and an NH2-terminal tail, varying in amino acid number. The molecular weight of keratins varies between 44 and 66 kDa (Herrmann et al., 2009; Moll et al., 2008). It is well known that type I and type II keratins may form a functional polymer (usually tetramer) by combining in 1:1 ratio (Fig. 2).

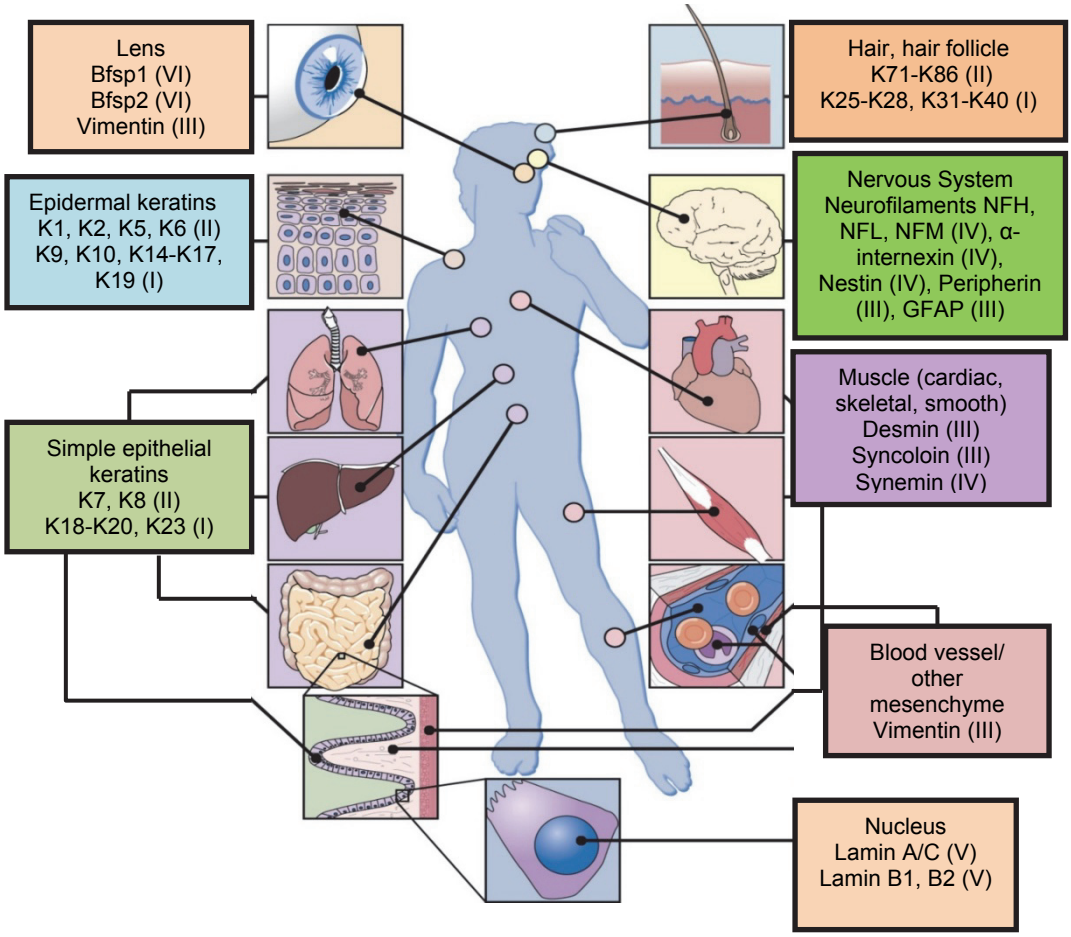

Figure 1. Tissue-and differentiation-dependent expression of intermediate filaments. IFs are expressed in a tissue-and differentiation-dependent manner. Epidermal keratins like K4/K13 are expressed in the esophagus, K20 in suprabasal enterocytes. Type I (K7, K8) and Type 2 (K18, K19, K20) simple epithelial keratins are expressed in simple epithelia such as the liver and intestine, vimentin is highly expressed in the vasculature and other resident mesenchymal cells. Similarly, desmin is expressed in the smooth muscle cells and NFs are expressed in the central and enteric nervous system. Lamins are expressed in the nucleus. Modifed from (Omary et al., 2009; Toivola et al., 2005).

Table 2. IF protein expression in specific cells/tissues and related diseases. [adapted from (Omary et al., 2009; Snider and Omary, 2014; Zupancic et al., 2014)].

IF protein type	Location	Proteins	Expression	Diseases
I, II	Cytoplasm	K1-K28, K31-K40 and K71-K86	Epithelia and epidermal appendages	Several skin, nail, eye, liver, intestine and hair disorders
I, II	Cytoplasm	K7, K15, K20	Epithelia and epidermal appendages	Unknown
III	Cytoplasm	Vimentin GFAP Desmin Syncolin Peripherin	Mesenchymal cells and lens Astroctyes Muscle Muscle Peripheral nervous system	Cataract Alexander disease Myopathies Unknown ALS
IV	Cytoplasm	Neurofilaments A-Internexin Nestin Sysnemin	Central nervous system Central nervous system Neuroepithelial cells Muscle	ALS and CMT Unknown Unknown Unknown
V	Nucleus	Lamins	Nuclear Lamina	Laminopathies
VI	Eye lens	Bfsp1 and Bfsp2	Fiber cells	Cataract

ALS, amytrophic lateral sclerosis; Bfsp, beaded filament structural protein: CMT, Charcot-Marie-Tooth.

Generally the head domain is involved in IF dimerization. It is a 20-amino acid fragment located in the front of coil 1A, which forms the α-helix and coiled-coil structure. Two antiparallel orientated coiled-coil dimers form a tetrameric IF building block which together take part in further assembling of IFs. Eight tetrameric subunits associate laterally to form unit-length filaments (ULFs) which anneal with other ULFs to give rise to a compact keratin filament (Fig. 3, (Herrmann et al., 2009).

2.2.2. Simple Epithelial Keratins (SEK) with unique tissue and cell specific expression

Keratins are highly conserved cytoplasmic IF proteins and expressed both in a cell-lineage and tissue specific manner. Epithelial keratins are disseminated within two different types of epithelium as non-stratified simple epithelia and stratified squamous epithelia. The keratins which are expressed in non-stratified epithelia are loosely distributed within the cytoplasm and are called simple epithelial keratins (SEK). On the

other hand, the epidermis-type keratins or keratinocyte-type keratins are expressed within several layers and are densely bundled filaments (tonofilaments) (Moll et al., 2008). There are clear differences between epidermal, simple-type epithelial and hair keratins in amino acid sequences (Strnad et al., 2012b). Keratins are obligate non-covalent heteropolymers which are composed of one type I and one type II keratin monomer (Moll et al., 2008; Omary et al., 2009). Simple epithelial keratins (SEKs) are found primarily in single-layered simple epithelia and include K7, K8, K18-K20, and K23. All SEK compose the IF cytoskeleton of IECs, where K23 is a recently described keratin the function of which is still unclear (Zhang et al., 2001). K8/K18 and K8/K19 are the most commonly co-expressed pairs and constitute the primary keratin pairs of simple epithelial cells. K8/K18 are the only IFs in hepatocytes while K8/K19 are the major SEKs in the colon. They are frequently found in highly specialized epithelia including hepatocytes, acinar cells of the pancreas, proximal tubular epithelial cells of the kidney and endocrine cells such as pancreatic islet cells along with K7, K19 and K20 (Franke et al., 1981; Moll et al., 1982a; Owens and Lane, 2003). Interestingly, primary and secondary keratins differ considerably in their distribution within distinct subcellular compartments. For example, K8 and K18 are found within cytoplasmic filaments in the pancreas, whereas K7 and K19 are localized in the apico-lateral compartment of the cell (Toivola et al., 2000a).

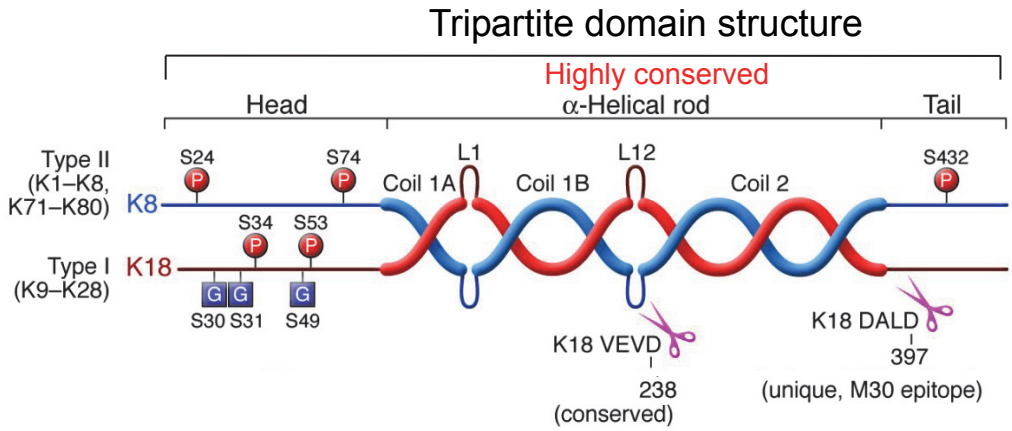

Figure 2. Tripartite domain structure of Ks. Like other IF proteins, Ks are composed of an α-helical central rod flanked by variable N-terminal head and C-terminal tail domains. The head and tail domains are enriched with sites for posttranslational modifications, phosphorylation (P) and glycosylation (G). The rod is formed by coils 1A, 1B and 2A, 2B separated from each other by linkers L1, L12 and L2. The L12 region of rod domain of type I but not type II keratins contains highly conserved caspase cleavage sites. For instance, type II keratin, K18, has two caspase digestion sites, i.e. VEVD and DALD in the L12 region and tail domain respectively. Based on this structure, IF proteins can form either homopolymers, as in desmin and vimentin, or obligate heteropolymers as in type I and II keratins. Modified from (Omary et al., 2009).

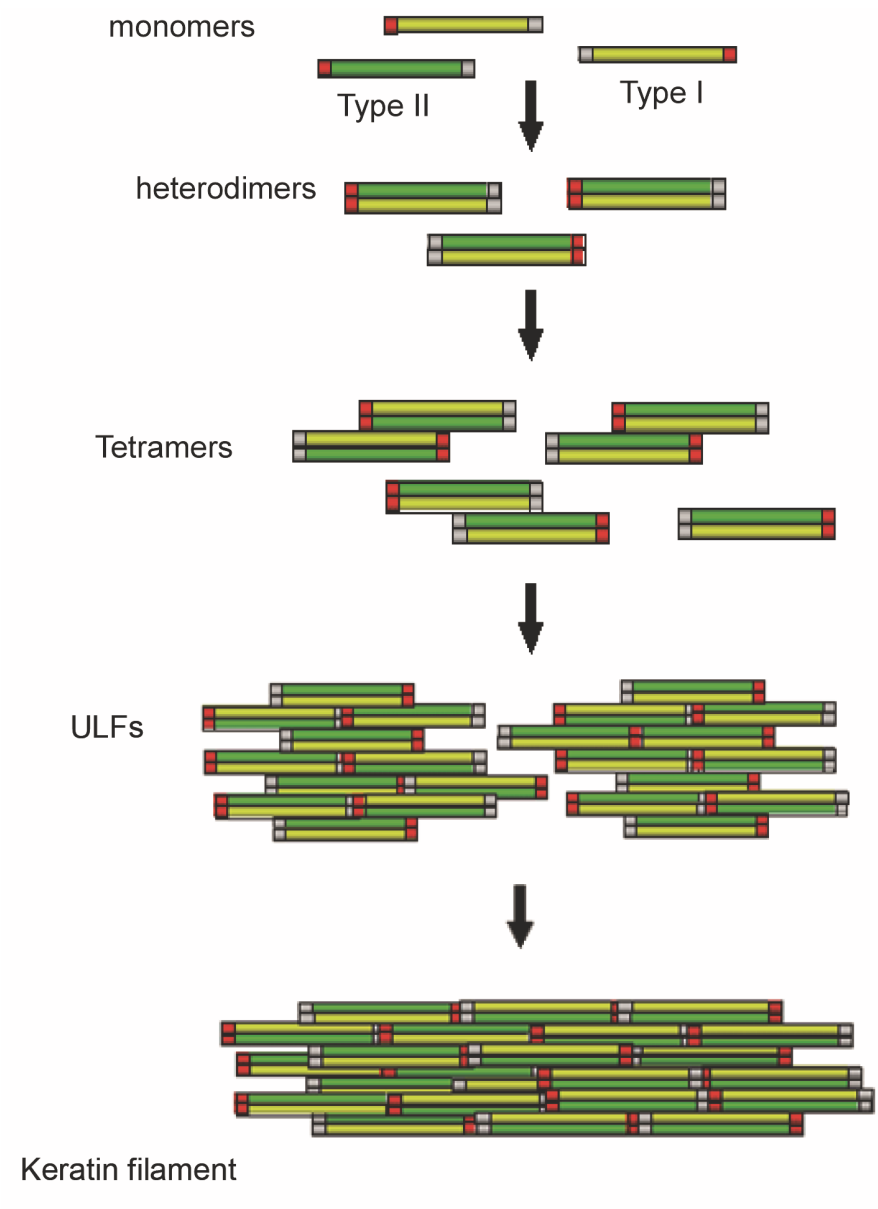

Figure 3. Model summarizing current concepts of keratin intermediate filament assembly. Helix boundary peptides colored red (helix initiation motif) and silver (helix termination motif). A keratin heterodimer is composed of keratin of each type, i.e. I and II. Keratin heterodimers combine with each other to form tetramers. Eight tetramers consisting of two antiparallel coiled-coil dimers form the unit-length filaments (ULFs). Next, ULFs anneal into the filaments (Herrmann et al., 2009). Adapted from (http://www.interfil.org).

However, K8 and K18 may also be found in mesenchymal cells such as smooth muscle cells and fibroblastic reticulum cells of lymph nodes as well as various mesenchymal tumors, where they co-express with vimentin and desmin (Franke and

Moll, 1987; Huitfeldt and Brandtzaeg, 1985; Jahn and Franke, 1989). K19 is important for normal muscle function and its deficiency leads to myopathy (Stone et al., 2007).

K7 and K19 are found in ductal epithelia, mesothelium and urothelium, whereas in the intestine K19 is also co-expressed with K8 which is found abundantly in intestinal epithelium. K20 has the most restricted expression in gastrointestinal epithelium, urothelium and neuroendocrine cells like Merkel cells in skin. K20 is absent from the stem cell compartment and appears to start expressing during terminal differentiation (Moll et al., 2008). K23 is expressed in colonic and pancreatic tumor cells undergoing differentiation by a mechanism encompassing histone hyper-acetylation (Omary et al., 2009; Zhang et al., 2001).

2.2.3. Keratin Regulation

SEKs are regulated by two major mechanisms, i.e. posttranslational modifications (PTMs) and interaction with associated proteins.

2.2.3.1. **Keratins and PTMs**

Keratins are actively regulated by several PTMs, including phosphorylation, glycosylation, transglutamination, sumoylation, acetylation, ubiquitination and prenylation (Omary et al., 2004; Omary et al., 2006). These modifications mostly occur in non-conserved head and tail domains affecting tissue specific functions of SEKs in different organs, as evident from several studies utilizing *in vitro* and *in vivo* models (Omary et al., 2006; Toivola et al., 2004b). Like other IF proteins, PTMs are important for keratin filament assembly and disassembly (Hyder et al., 2008; Izawa and Inagaki, 2006; Omary et al., 2006; Sihag et al., 2007).

The most commonly found and significant PTM in SEKs is phosphorylation. IFs can be phosphorylated by kinases activated during stress. This designates, mainly keratins, to be a phosphate sponge protecting tissues such as liver tissue from potential injuries (Ku et al., 1996; Liao et al., 1996; Omary et al., 2006). There is an adequate knowledge available about IF regulation due to the recent advances in understanding phosphorylation. Phosphorylation plays important roles in regulating filament organization, solubility, and cell-protective functions. A number of kinases and phosphatases modulate phosphorylation to facilitate IF re-organization by targeting one or more Ser/Thr residues, specifically in the N-terminal and C-terminal domains (Omary et al., 1998). Like other IFs, keratin solubility is based on Ser/Thr phosphorylation which guarantees the structural dynamics of filament (Guo et al., 2008; Moritz et al., 2010; Rikova et al., 2007). K8, K19 and vimentin are the known IF targets for tyrosine phosphorylation and K8 phosphorylation at a highly conserved residue in the rod domain (Tyr-267) leads to keratin insolubility (Snider and Omary, 2014; Snider et al., 2013b).

The serine-specific phosphorylation for K8, K18 and K19 is widely studied (Strnad et al., 2012a; Toivola et al., 2002; Zhou et al., 1999). Keratin phosphorylation helps to guard tissues and cells such as hepatocytes from injury (Omary and Ku, 2006; Omary et al., 2006). Knock-in mice with phosphorylation site mutations in the head domain of K8 (Ser74) or K18 (Ser54) show increased apoptosis and liver damage (Ku et al., 1998a; Ku et al., 1998b; Ku and Omary, 2006). Studies with transgenic mice expressing either K8 Gly62Cys or K8 Ser74Ala showed that the carriers of the human K8 variant (Gly62Cys) are predisposed to liver disease by inhibition of K8 *in vivo* phosphorylation by the stress-activated p38 kinase at the adjacent K8 Ser74 (Omary, 2009). Similarly, K18 Ser-52 and 33 are two phosphorylation sites which are important during mitosis and many stress conditions (Ku et al., 1998a).

Previous studies have shown that other PTMs, such as glycosylation, can affect phosphorylation by blocking the access of kinases to serine (S) for kinases (Omary et al., 1998; Snider and Omary, 2014). Dephosphorylation also regulates SEK phosphorylation state and serine/threonine (S/T) phosphatase inhibitors calyculin-A (Cl-A), microcystin-LR and okadaic acid (OA) cause hyper-phosphorylation and disassembly of keratin filaments (Eriksson et al., 1998; Toivola et al., 2002). The filament organization and solubility can be disturbed by attached phosphates (Omary et al., 1998; Zhou et al., 1999). Disassembly of mainly insoluble filaments redistributes them to the soluble pools (Omary et al., 1998) and many studies have revealed the correlation between the phosphorylation and functions of SEKs in different *in vivo* and *in vitro* models (Omary et al., 2006).

Increased acetylation of K23, K8 and K18 has been reported in pancreatic cancer cells and colonic epithelia in response to butyrate (short chain fatty acid) treatment (Zhang et al., 2001). The increased acetylation is thought to be a regulatory mechanism of keratin stabilization. Keratins with different levels of acetylation most likely are able to regulate the homeostasis of epithelial cells by their loss or presence in the colon or pancreas (Zhang et al., 2001) (Leech et al., 2008)**.** A recent study shows that keratin filaments can also be regulated by a novel mechanism involving K8 acetylation via glucose and SIRT 2 (Snider et al., 2013a).

The process by which IFs are modified by small ubiquitin-like modifier (SUMO) proteins in a covalent and reversible manner is termed sumoylation (Geiss-Friedlander and Melchior, 2007). Sumoylation of keratin IFs is markedly increased by phosphorylation but the exact mechanism is not known (Snider and Omary, 2014). Mutation of the highly conserved phosphotyrosine, Tyr-267, in the K8 rod domain and the paralogous residue on GFAP (Tyr-242) leads to defective filament formation (Snider et al., 2013b). The use of PTMs in diagnostics and therapeutics for human diseases is an important avenue for future studies (Snider and Omary, 2014).

2.2.3.2. Keratin binding proteins

Regulation of SEKs is mediated by their interaction with different proteins. In contrast to MTs and MFs, relatively few keratin-associated proteins, i.e. hsp70 and some cell signaling proteins, including 14-3-3 proteins, desmoplakins, Raf-1 kinase, p38 and Jun kinases, PKC, and protein phosphatase-2A, are known (Liao et al., 1995; Omary and Ku, 2006; Yin and Green, 2004). It is well established in cell culture and *in vivo* studies that K18 binds to 14-3-3 protein during mitosis and that it is regulated by K18 S33 phosphorylation. A mutation in the K18 S33 phosphorylation site results in collapsed keratin filaments, in a tissue- and context-dependent fashion (Omary et al., 2006),(Ku et al., 1998a). The 14-3-3 protein serves as a molecular scaffold for different signaling molecules, e.g. Raf kinase (Yaffe et al., 1997). A few studies have revealed that K8 bound to 14-3-3 is a possible regulatory protein for Raf, which in turn might phosphorylate K18 (Ku et al., 2004).

It has been shown earlier that keratins also play an important role in wound healing by a mechanism involving K17 phosphorylation and binding to 14-3-3, leading to activation of the mTOR signaling pathway (Kim et al., 2006; Omary and Ku, 2006). K8/K19 may bind to γ-tubulin complex protein (GCP6) that joins MTs with IFs. Previous studies using transgenic $K8^{-/-}$ mice and Caco-2 cell line have revealed that this process is upregulated by the cyclin-dependent kinase 1 (Cdk-1), which phosphorylates the GCP6 protein. The attached phosphate helps GCP6 o bind to K8/K19. This is evident by keratin involvement in MTs distribution within epithelia (Oriolo et al., 2007a).

Keratins are connected with MFs through plastin 1 (Pls1) which is one of the major actin-bundling proteins in the microvilli and binds to K19. Pls1 knock out ($^{-/-}$) mice have shorter villi and fragile microvilli brush border due to the absence of Pls1. These findings indicate that the mechanical strength of the intestinal epithelium is dependent on actin-Pls1-K19 interactions (Grimm-Gunter et al., 2009). Trichoplein is another keratin-binding protein which binds to K8/K18 and is also found concentrated in desmosomes. This protein is thought to be involved in the organization of the apical network of keratin filaments and desmosomes in simple epithelial cells (Nishizawa et al., 2005). The regulatory role of SEKs in small intestine and colon is evident by the presence of many keratin-associated proteins in these organs.

2.2.4. Functions of SEKs

Keratins play important roles in cellular integrity, cell signaling, protein targeting, apoptosis and in protection against stress (Ku and Omary, 2006; Pallari and Eriksson, 2006; Pan et al., 2013; Toivola et al., 2010; Toivola et al., 2005).

2.2.4.1. Scaffolding and cytoprotective functions of SEK

Like other IFs, keratins also play a chief role in scaffolding the epithelial cells and stabilizing the cellular architecture in protection against mechanical and non-mechanical stresses (Coulombe and Omary, 2002; Marceau et al., 2001; Omary and Ku, 2006; Omary et al., 2002). The cytoprotective functions of K8 and K18 are well supported by transgenic animal models including over expression of K18 or by $^{-/-}$ mice for K8 and K18 involving epithelial cells of liver, skin, cornea and oral cavity. The liver phenotype of K8$^{-/-}$ and K18$^{-/-}$ mice is obvious as hepatocytes from these mice lack keratin filaments and are therefore more susceptible to liver injury (Baribault et al., 1994; Caulin et al., 2000; Gilbert et al., 2001; Loranger et al., 1997; Magin, 1998; Magin et al., 1998; Marceau et al., 2001; Omary et al., 2002; Toivola et al., 1998; Zatloukal et al., 2000). The functions of SEK may vary depending on the tissues. For example, K8$^{-/-}$ hepatocytes and pancreatic acinar cells are devoid of all keratins, but K8$^{-/-}$ pancreata showed similar susceptibility to injury as K8$^{+/+}$ mice (Toivola et al., 2000b). In contrast to K8/18 expression in the liver and K8/19 expression in the colon, the mouse gall bladder expresses K8, K18 and K19 with low levels of K7. Previous studies showed that different keratin null mice show different compensatory keratin expression in the absence of type I or type II keratins. For instance increased K20 expression is seen in K19$^{-/-}$ gallbladders in contrast to K18$^{-/-}$ gallbladders (Tao et al., 2003). K8$^{-/-}$ gallbladder shows marked increase in K7 levels while the liver does not show such an increase. It is important to mention here that the gallbladder has several keratins to start with (Tao et al., 2003) but the liver has only one type II (Omary et al., 2002) and K7 loss in K7$^{-/-}$ mice is not compensated by other keratins (Sandilands et al., 2014).

Cells and tissues regularly face many intrinsic and extrinsic stressful conditions. These intrinsic conditions include genetic and endoplasmic reticulum stress, whereas extrinsic or environmental stresses include heat, toxin, radiation, mechanical and infection. These extrinsic stresses can be of osmotic, oxidative and metabolic nature such as hypoxia and autophagy (Fig. 5). The cells respond to stress situations by heat stress response and upregulation of proteins which protect them from being damaged (Toivola et al., 2010). Previous studies have shown that mRNA and protein levels of resident or transient keratins are upregulated under various types of stress (Toivola et al., 2010).

Like other IFs, keratin filaments have a unique feature that enables them to be stretched 3 times longer than their original size before breaking (Gu and Coulombe, 2007; Haines and Lane, 2012; Kim et al., 2010; Pekny and Lane, 2007). The identification of K5 and K14 mutations in the patients suffering from epidermolysis bullosa simplex (EBS) was proved as first connection between IFs and mechanical stress (Coulombe et al., 2009). Similarly, fragile liver phenotype was seen in K8$^{-/-}$, K18$^{-/-}$ and K18 R90C mutant mice (Omary, 2009). As SEKs are expressed in an epithelial cell- and differentiation state-selective manner, their upregulation has been

shown, for example after griseofulvin-induced injury in mouse liver (Cadrin et al., 2000), caerulein- and choline/methionine-deficient diet (CDD), ethionine-supplemented diet (CDE), CDD-induced injuries in pancreas (Zhong and Omary, 2004) and high-fat lithogenic diet (LD) induced gallbladder injury (Tao et al., 2003). Stress is also often accompanied by increased keratin phosphorylation and other post-translational modifications (Omary et al., 2006; Toivola et al., 2005) and in patients with liver cirrhosis, keratin phosphorylation correlates with disease prognosis (Toivola et al., 2004b). In lungs, shear stress results in increased keratin solubility, degradation, aggregation and K8/18 phosphorylation (Flitney et al., 2009; Ridge et al., 2005; Sivaramakrishnan et al., 2008). K8/18 hyper-phosphorylation is detected in liver, lungs and skin under stress conditions like hepatotoxicity, heat and drugs (Liao et al., 1997). The upregulation of IFs has also been seen during tissue repair processes. The mRNA levels of K6, K16 and K17 are increased during skin injury (DePianto and Coulombe, 2004; Omary and Ku, 2006). Upregulation of K19 and K20 has also been reported during recovery from pancreatitis. Nestin and Vimentin have also been upregulated during repair from damaged muscle (DePianto and Coulombe, 2004). It was recently shown that K8 plays an important role in β-cell intracellular organization and systemic blood glucose control in normal homeostasis and diabetes (Alam et al., 2013).

2.2.4.2. Protein targeting functions of SEK

Unlike actin and microtubules, the role of IFs in membrane transport could not be widely studied until the 21st century due to the absence of IF polarity and specific motors and due to the non-availability of pharmacological agents. However, recent studies have provided significant evidence for the role of IFs in membrane protein targeting and organelle positioning (Styers et al., 2005). IFs may overcome their limitations by binding with other cytoskeletal elements, i.e. actin and microtubules and their specific motor proteins to perform their protein targeting and transport functions (Chang and Goldman, 2004; Helfand et al., 2003; Helfand et al., 2004; Strelkov et al., 2003). Previous studies have suggested that the golgi apparatus and autophagosome functions may regulate IFs. This is evidenced by the bundling of vimentin filaments in response to the overexpression of vimentin binding proteins and defective accumulation of phagosomes (Blankson et al., 1995; Gao and Sztul, 2001; Gao et al., 2002; Holen et al., 1992).

Many studies using K8$^{-/-}$ intestine and liver cells and K18 R90C-expressing cultured cells helped in revealing the non-mechanical roles of SEKs in crucial cellular processes like protein localization and targeting, protein synthesis and epithelial cell polarity (Oriolo et al., 2007b; Toivola et al., 2005). The K8$^{-/-}$ small intestine and colon show mistargeting of ion transporters resulting in diarrhea (Ameen et al., 2001; Toivola et al., 2004a). The K8$^{-/-}$ liver has increased basolateral distribution of the bile canaliculus Ecto-ATPase (Ameen et al., 2001), increased apical distribution of the Fas receptor (Gilbert et al., 2001), and reduced desmoplakin deposition at desmosomes (Loranger et

al., 2006). Similarly, K18 R90C expressing cells show redistribution of E-cadherin (Hanada et al., 2005).

2.2.5. Human diseases linked with SEK IFs

SEKs are involved in human diseases with altered epithelial morphology and physiology. The cytoprotective functions of SEK are well established by keratin mutations predisposing to skin and liver diseases in humans. Many studies using mouse models have also reported the multiple disease associations with keratin mutations (Coulombe and Omary, 2002; Omary et al., 2009), (Usachov et al., 2012). In contrast to epidermal keratins, which when mutated mostly have a dominant-negative effect, the simple epithelia keratins appear to act as susceptibility factors. Most mutations are missense mutations in conserved amino acid sequences of the α-helical domain in the keratin protein. These mutations can influence directly or indirectly by predisposing their carriers to disease in many tissues (Fig. 6) (Omary et al., 2004), http://www.interfil.org, (Owens et al., 2004).

Previous studies clearly showed that the liver is the primary target of these mutations. Since 12 % of patients suffering from liver diseases have K8/K18 mutations(Omary et al., 2009). Keratin variants are found in approximately 4% of the white population and predispose to development and adverse outcome of multiple liver diseases like Hepatitis C (HCV) (Strnad et al., 2012a). The transgenic $K8^{-/-}$ and $K18^{-/-}$ mice have a fragile liver (Omary 2009) and are susceptible to apoptosis and liver injuries (Ku and Omary 2006) partly due to changes in phosphorylation of K8 at S74 which is responsible for cytoprotection of liver cells (Ku 2007). Formation of cell specific inclusion bodies, i.e. Mallory-Denk bodies (MDBs), are also a hallmark for chronic liver diseases such as alcoholic and nonalcoholic steatohepatitis (NASH) or copper storage diseases (Ku et al., 2007; Zatloukal et al., 2007) and may be found in hepatocellular carcinoma and apoptotic hepatocytes (French et al., 2013). MDBs are made up of K8/K18 that have been ubiquitinated, or bound by other proteins such as heat shock proteins, or p62. MDBs also play an important role in protection against apoptosis and are targets of autoantibodies. MDBs are also sometimes observed in additional diseases such as chronic hepatitis C (CHC) (Rakoski et al., 2011). However, the exact function of MDBs is still unknown (Ku et al., 2007; Zatloukal et al., 2007).

Several human diseases have been linked to mutations in SEKs and epidermal keratins. For instance, mutations in K5/K14 leads to epidermis bullosa simplex (EBS), an inheritable skin blistering disorder in which the epidermis is detached from the connective tissue due to a tissue breakdown in the epidermis (Bolling et al., 2011). K8 mutations have also been thought to predispose humans to pancreatitis but a clear correlation is still lacking (Omary et al., 2009).

There is no clear evidence for the relevance of SEK mutations to intestinal diseases. The involvement of K8 mutations in IBD is hypothetically based on the $K8^{-/-}$ mouse model. The ablation of K8 seems to develop colonic epithelial hyperproliferation and IBD, which was observed in some patients with K8 mutations, but this correlation needs further investigations (Omary et al., 2009). A recent study using the *in vitro* colonocyte model clearly showed that the K8 mutations (K8 G62C, K8 K464N) and the K18 mutation (K18 S230T) affect the barrier function in intestinal epithelium. Therefore, these mutations may be considered as susceptibility factors for IBD (Zupancic et al., 2014).

IFs are broadly used in diagnostics and disease activity evaluation while serving as autoantigens and markers for tumors and tissues due to their tissue specificity and abundance in cells (Omary et al., 2009; Strnad et al., 2012a). For instance, many adenocarcinomas have been characterized by the high expression of K7, K8, K18, K19 and K20 (Moll et al., 2008; Omary et al., 2009). Recent studies have shown that K14 and K17 expression may be used as a biomarker for breast and gastric cancer progression respectively (Cheung et al., 2013; Ide et al., 2012).

Epithelial and non-epithelial tumors can be differentiated on the basis of pan-keratin staining (Moll et al., 2008). Similarly, specific keratin expression patterns may help predict the origin of primary tumors along with the probability of tumor growth (Moll et al., 2008). K8 and K18 are highly expressed in hepatocellular carcinoma, whereas cholangiocarcinomas are associated with specific expression of K7, K8 and K18. Similarly, K20 is highly expressed in colorectal cancer (Chu and Weiss, 2002; Moll et al., 2008). The presence of SEKs or SEK fragments in serum can be used as markers for apoptosis because of caspase mediated cleavage of K8/18 during apoptosis (Linder, 2007; Moll et al., 2008; Omary et al., 2009). Tissue polypeptide antigen (TPA) which is a mixture of K8, K18, and K19, tissue polypeptide-specific antigen (TPS, derived from K18), cytokeratin fragment 21-1 (CYFRA 21-1; derived from K19) and a caspase-generated fragment of K18, can be used as a potential serum marker in the diagnostics of epithelial cell associated carcinomas such as colorectal, breast, lung and bladder cancer. These markers can also be used to monitor treatment responses and tumor prognosis (Duffy, 2006; Nisman et al., 1998). Auto-antibodies are not normally present in serum but are produced as a result of cell death leading to exposure to the immune system. Necrosis and apoptosis-generated keratin serum fragments can also be used as noninvasive markers of multiple liver diseases, particularly NASH (Strnad et al., 2012a). Like SEK serum markers, SEK specific autoantibodies can be used for evaluating the disease prognosis and treatment response. Many studies have shown the development of auto-antibodies specific for SEKs during autoimmune and malignant liver diseases. SEK targeted auto-antibodies like K8/K18 antibodies have been detected in acute liver failure (Bernal et al., 2007; Le Naour et al., 2002; Li et al., 2008). It has been previously found that patients with autoimmune hepatitis (AIH)

exhibit high titers of antibodies specific for K8, K18 and K19 which are decreased after corticosteroid treatment (Bernal et al., 2007).

2.2.6. The role of SEKs in the small intestine and colon

Simple intestinal epithelia express K7, K8 (type II) and K18, K19, K20 (type I) (Fig. 1, Fig. 4). A novel keratin, K23, is expressed insignificantly within normal colon epithelial cells, but strongly in the phosphorylated form in colorectal adenocarcinomas and has a potential role as a tumor suppressor in colorectal cancers (Birkenkamp-Demtroder et al., 2007). SEKs in the intestine are responsible for protecting colonocytes from stresses by maintaining cellular integrity and stability. They also provide liver cells with protection against apoptosis; however, colonocytes show resistance to apoptosis in the absence of SEKs (Gilbert et al., 2001; Habtezion et al., 2011; Habtezion et al., 2005; Herrmann et al., 2009; Marceau et al., 2001). The role of keratins in the intestine is ambiguous. The $^{-/-}$ mouse models have helped tremendously in understanding keratin functions *in vivo* and indicate the frequency of functional redundancy of SEK. The first breakthrough came in 1993, when Helen Baribault successfully created the K8$^{-/-}$ mouse. The deletion of the K8 gene possesses a strong effect on embryogenesis and gut functional integrity depending on the genetic background of the mouse. Fifty percent of the K8$^{-/-}$ mice with FVB/N background are embryonically lethal and develop ano-rectal prolapse. The female K8$^{-/-}$ mice are sterile (Baribault et al., 1994; Baribault et al., 1993). These K8$^{-/-}$ mice develop a Th2-type UC similar to humans (Habtezion et al., 2005), with epithelial hyperproliferation (Baribault et al., 1994; Toivola et al., 2004a). The K8$^{-/-}$mice also display a dramatic alteration in ion transport, leading to chloride secretion (rather than uptake) caused by a distinct epithelial ion transporter mistargeting, resulting in diarrhea (Toivola et al., 2004a).

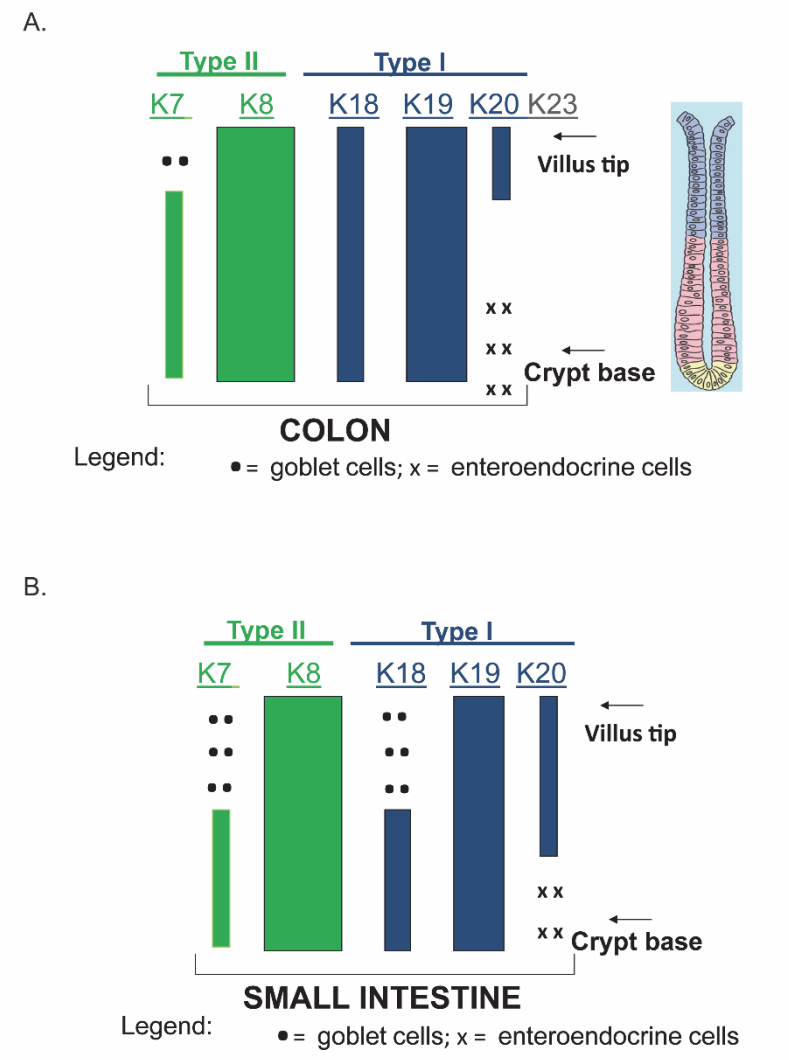

Figure 4. SEK distribution in small intestine and colon. K8 is abundantly found in the colon in contrast to the scarce amount of K7 which is found in the crypt base and goblet cells. K18 and K19 are distributed uniformly throughout the villus-crypt axis in the colon. K20 is found in the upper parts of colonic crypts. On the other hand, the small intestine contains K8 and K19 as abundant and uniformly distributed keratins, but K7 and K18 are preferentially located in the crypt base with suprabasally located goblet cells. K20 is also found suprbasally in the villus. Modified from (Zhou et al., 2003).

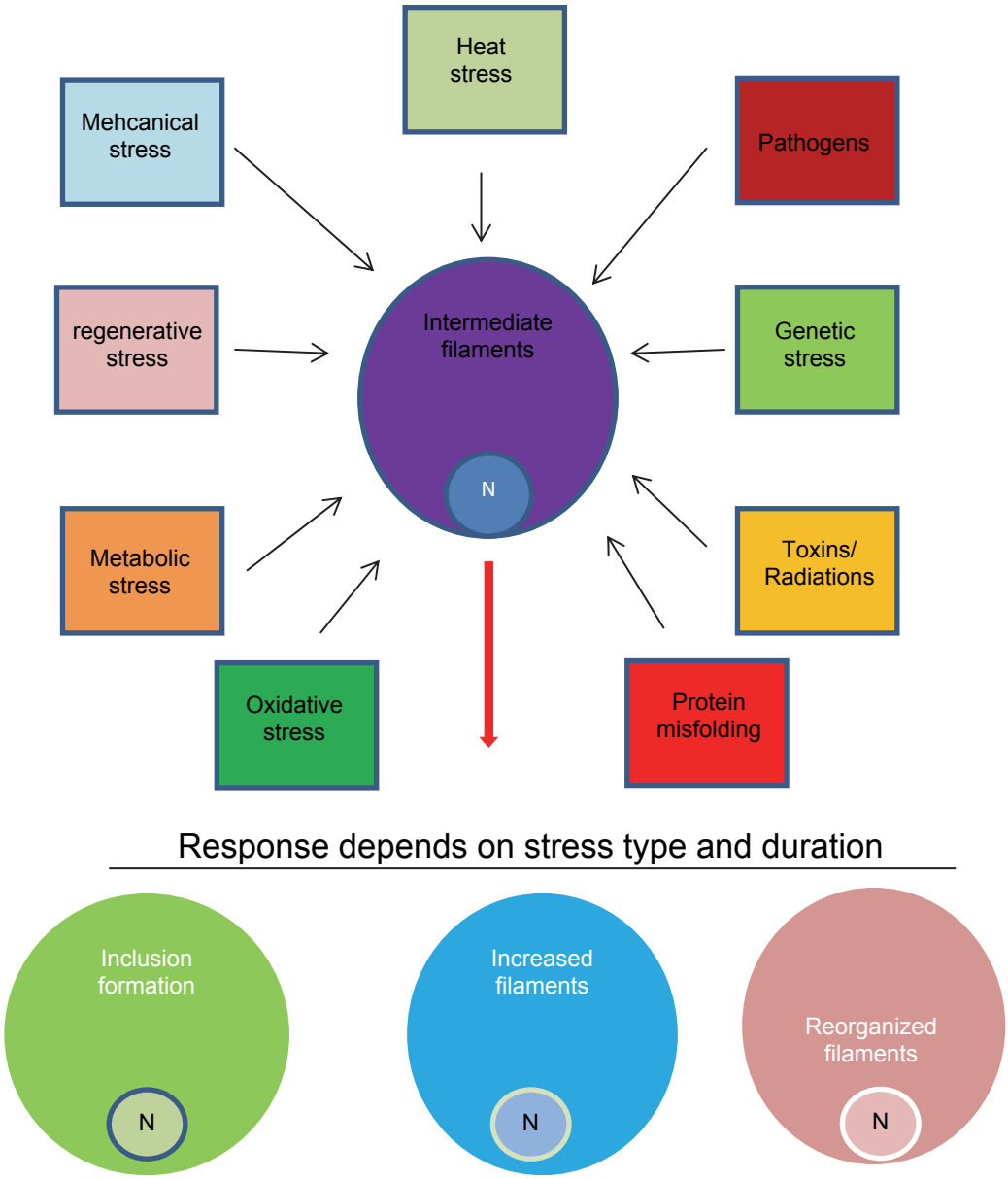

Figure 5. Different forms of stress modulate IFs. Cells experience different kinds of stresses and respond according to form and duration of stress such as formation of inclusion bodies, up-regulating or reorganizing the IFs. Modified from (Toivola et al., 2010).

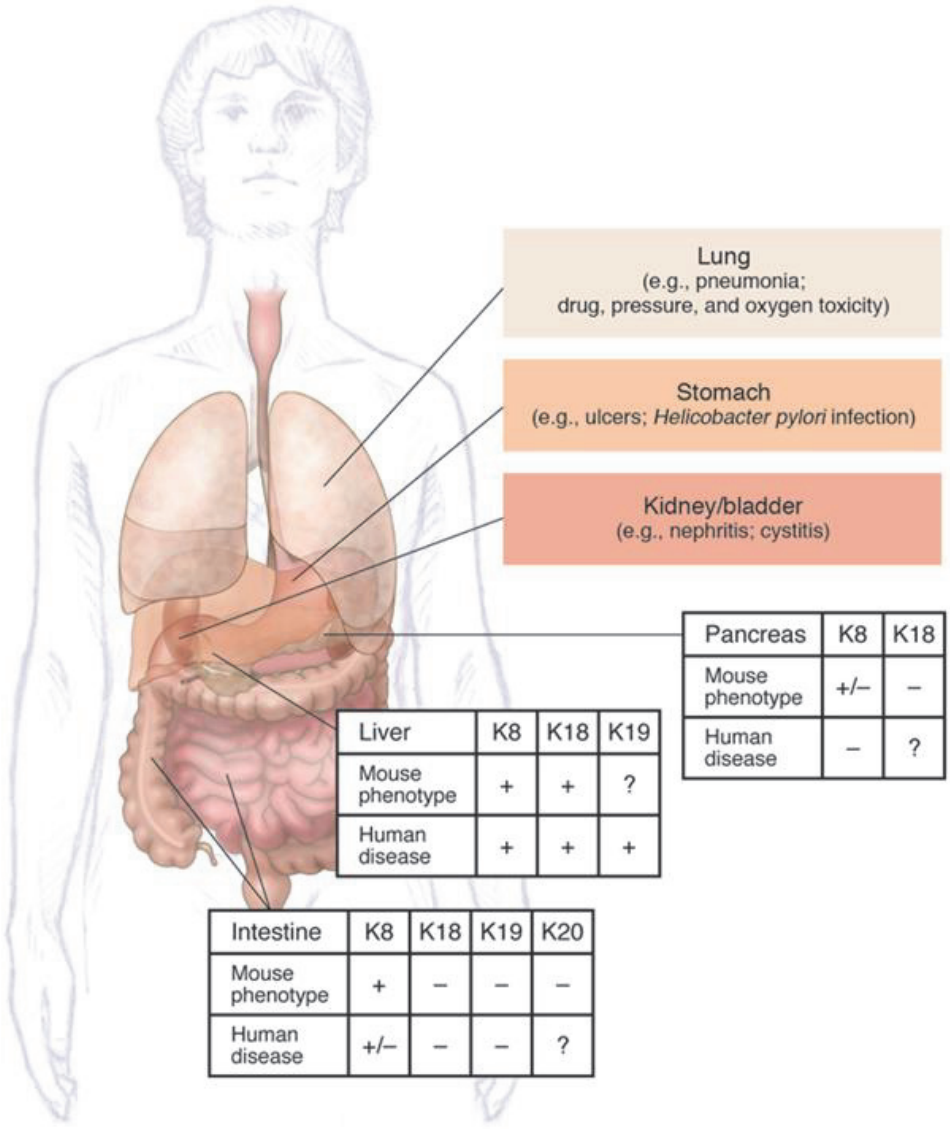

Figure 6. SEK mutations predispose carriers to different diseases. Studies utilizing animal models and human patients show that mutations in keratins lead to the phenotypes in liver, pancreas, and intestine. +, positive phenotype; -, absent phenotype; +/-, borderline phenotype; ?, untested and therefore unknown. Adapted from (Omary et al., 2009).

The intestinal phenotype of $K8^{-/-}$ mimics the UC phenotype of IBS in humans. The K8-null inflammation and ion transporter mistargeting can be ameliorated by using antibiotics. This suggests the involvement of microflora in $K8^{-/-}$ colitis (Habtezion et al., 2011; Habtezion et al., 2005). SEK's hallmark of cell and tissue specific expression is highlighted by $K8^{-/-}$ mice showing susceptibility to apoptosis in the liver but, on the other hand, resistance to apoptotic cell death in the intestine (Habtezion et al., 2011).

Interestingly, the $K8^{+/-}$ mice, which express less K8 levels as compared to $K8^{+/+}$ mice, display a similar but milder ion transport-defect with reduced short circuit current, decreased Na^+ and Cl^- absorption, although no major inflammation is observed under baseline conditions (Toivola et al., 2004a). On the basis of these findings we hypothesized that the concentration of colonocyte keratins, or an intact keratin network, is indispensable to protect colonocytes from stress (colitis). $K18^{-/-}/K19^{-/-}$ (double $^{-/-}$) mice show early embryonic lethality (Hesse et al., 2000). $K7^{-/-}$ and $K19^{-/-}$ mice do not have intestinal phenotype (Sandilands et al., 2014; Tamai et al., 2000). *In vitro* studies also revealed that K8 ablation leads to development of shorter microvilli in Caco-2 cells silenced for K8 (Salas et al., 1997). K8 plays an important role in the early scaffold organization in the apical domain of polarized epithelial cells (Wald et al., 2005). Recently, it has been shown that K8 plays a role in colonocyte energy metabolism and homeostasis. Mitochondria of $K8^{-/-}$ colonocytes have decreased levels of ketogenic enzyme, HMGCS2, leading to aberrant ketogenesis in $K8^{-/-}$ colon. The $K8^{-/-}$ colon also shows downregulation of PPARα and the SCFA (butyrate) transporter MCT1 (Helenius et al., 2015).

2.3. Epithelial ion transport

The main function of the adult colon is to absorb Na^+, Cl^-, K^+, short chain fatty acids (SCFAs) and fluid, including water, as well as to secrete HCO_3^- and mucus (Field, 2003). The osmotic gradient created by salt transport using ion transporters and channels is the driving force for passive water movement in/out of the lumen.

2.3.1. Ion transporters

The electroneutral sodium chloride (NaCl) absorption is the key process which enables Na^+, Cl^- and fluid absorption in the intestine. NaCl absorption in the gastrointestinal tract involves coupling of Na^+/H^+ and Cl^-/ HCO_3^- exchangers (Kato and Romero, 2011) (Fig.7).

2.3.2. Sodium transporters

Active Na absorption takes place either through electrogenic transport (e.g. the epithelial Na^+ channel ENaC) or through electroneutral NaCl absorption, generally a result of coupled Na^+/H^+ and Cl^-/HCO_3^- exchangers. The luminal-membrane sodium hydrogen exchange is mediated through SLC9A2 [solute carrier family 9 member A2; also called NHE2 (sodium hydrogen exchanger 2)] and SLC9A3 (also called NHE3) of the SLC9 gene family.

2.3.2.1. ENaC

The epithelial Na^+ channel (ENaC) is responsible for transporting Na^+ across epithelia of many organs including the distal colon. ENaC comprises three subunits, α, β, and γ, each subunit ranges between 85 to 95 kD in size. PTMs are important in the regulation of ENaC activity and expression (Canessa et al., 1993; Canessa et al., 1994). Disturbances in Na^+ and Cl^- absorption in the colon are associated with diarrheal disorders like cystic fibrosis (CF) and UC and a decreased expression of ENaC subunits is seen in human biopsy samples from patients with colitis (Greig et al., 2004; Kunzelmann and Mall, 2002).

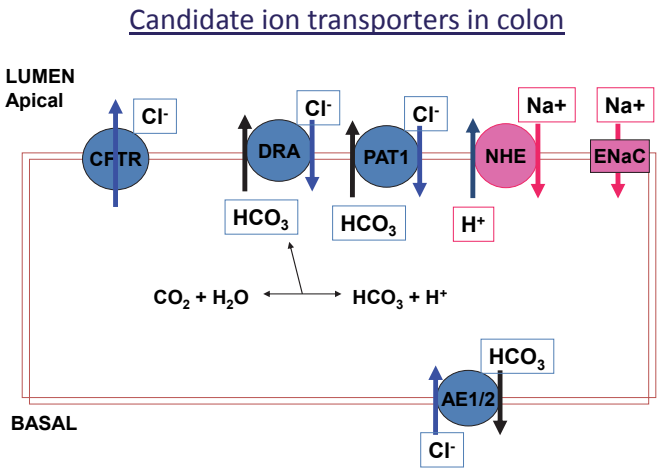

Figure 7. Candidate ion transporters for electroneutral NaCl absorption. Based on (Kunzelmann and Mall, 2002; Malakooti et al., 2011).

2.3.2.2. NHE

Sodium hydrogen exchangers (NHE) belong to the SLC9 gene family and are the main Na^+ transporters in the ileum and proximal colon. Out of 9 members of this gene family, NHE1, NHE2, NHE3 and NHE8 are expressed in mammalian intestine (Dudeja et al., 1996; Xu et al., 2005). NHE1 is localized in the basolateral membrane of IECs and performs housekeeping functions (Sardet et al., 1989). NHE2 and NHE3 are situated in the apical membrane and serve in intestinal Na^+ absorption (Wormmeester et al., 1998). NHE2 and NHE3 are predominantly expressed in the colon and ileum, respectively (Bachmann et al., 2004; Dudeja et al., 1996). Although $NHE3^{-/-}$ mice have an intestinal phenotype with mild diarrhea, low blood pressure and mild metabolic acidosis, $NHE2^{-/-}$ mice seem to have normal intestinal Na^+ absorption (Gawenis et al., 2002). NHE8 is differentially expressed along the human gastrointestinal tract and important in early life (Bobulescu and Moe, 2009), but its functional role in Na^+ absorption in the adult intestine is not clear. Several studies have correlated the

impaired Na^+ absorption with NHE3 down-regulation during diarrheal disorders like IBD (Greig and Sandle, 2000).

Butyrate has been shown to enhance Na^+ absorption by increasing NHE2 and NHE3 levels in rat distal colon (Binder and Mehta, 1989; Krishnan et al., 2003). Diarrhea is a characteristic feature of IBD and has been shown to be contributed by inflammation and pro-inflammatory cytokines like IFNγ, TNFα, and IL-1β in the intestine. Colonic inflammation and treatment of mice with TNFα and IFNγ have been shown to result in decreased expression and activity of NHE3 (Amin et al., 2006; Barmeyer et al., 2004; Rohlff et al., 1997).

Reduced NHE3 function has been observed in UC, but NHE3 mRNA and protein levels were not altered indicating the importance of its transcriptional regulation (Sullivan et al., 2009; Yeruva et al., 2009). TNBS and LPS treatment results in reduced NHE8 mRNA and protein levels in rats (Xu et al., 2009). Glucocorticoids, epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate **(**PMA**)** have also been found to increase NHE3 activity in the mammalian intestine (Malakooti et al., 2005; Yun et al., 1993).

2.3.3. Chloride transporters

The luminal membrane $Cl^-/HCO3^-$ exchange is mediated by SLC26A3 [also called DRA (down-regulated in adenoma)] and SLC26A6 [also called PAT-1 (putative anion transporter 1)] of the SLC26 gene family (Kato and Romero, 2011; Mount and Romero, 2004). DRA and PAT-1 are expressed ontogenically and variably along the length of the intestine (Farkas et al., 2010; Walker et al., 2008) and the crypt-villus axis. The specific expression of these transporters depends on transcriptional regulation (Farkas et al., 2010; Walker et al., 2009). The basolateral chloride transport is mediated by the anion exchanger AE1/2.

2.3.3.1. AE1/2

The AE1/2 is expressed basolaterally in erythrocytes, heart, distal colon and kidney epithelial cells (Alper et al., 1997; Holappa et al., 2001). Distal renal tubular acidosis is caused by mutations in the gene encoding AE1. The $AE1/2^{-/-}$ mice suffer from achlorhydria and osteoporosis leading to their death. A hypomorphic $AE2^{-/-}$ mice with partial loss of AE2 gene are viable but develop defective spermatogenesis leading to male infertility and a syndrome resembling primary biliary cirrhosis (Alper, 2009).

2.3.3.2. PAT-1

The PAT-1 (SLC26A6) gene is located on chromosome 3p21.3 and consists of 21 exons and 20 introns. The PAT-1 gene encodes an integral membrane protein of 738 amino acid with a predicted topology of 11 transmembrane helices and an intracellular -NH2

and -COOH terminus (Lohi et al., 2003; Waldegger et al., 2001). The apical Cl^-/HCO_3^- exchange is controlled by PAT-1 in the small intestine, as it is predominantly expressed in the small intestine and in very low levels in the colonic crypts (Wang et al., 2002). PAT-1 expression was found to be decreased in Caco-2 cells by the pro-inflammatory cytokine IFNγ albeit via a distinct cis element/transcription factor (Saksena et al., 2008). PAT-1 plays an important role in Cl^- absorption without being coupled to water movement as evidenced by decreased Cl^-/HCO_3^- exchange activity in PAT-$1^{-/-}$ mice without any diarrheal phenotype (Wang et al., 2005).

2.3.3.3. DRA

SLC26A3 belongs to a solute carrier family and is normally expressed in the colon but not found in colonic adenomas/adenocarcinomas. The SLC26A3 gene encodes a glycoprotein of 764 amino acids (Byeon et al., 1996). DRA has been identified as the major Cl^-/HCO_3^- exchanger involved in electroneutral NaCl absorption in the intestine (Musch et al., 2009; Walker et al., 2008). Mutations in human DRA (hDRA) gene have been associated with the development of congenital chloride diarrhea (CLD). CLD is an autosomal recessive disorder and results in watery stool with high chloride concentration and metabolic alkalosis (de la Chapelle, 1993; Hoglund et al., 1996; Makela et al., 2002). Unlike PAT-1, DRA activity is coupled to water movement. DRA$^{-/-}$ mice exhibit a diarrheal phenotype, including chloride losing diarrhea, colonic hyperplasia and upregulation of ion transporters such as NHE3 and H,K-ATPase in the colon. This phenotype resembles CLD in humans (Schweinfest et al., 2006). Unlike PAT-1, DRA is mostly expressed in the colon and to a lesser amount in the small intestine and is localized to apical membranes of IECs (Farkas et al., 2010; Kato and Romero, 2011). DRA is expressed ontogenically in the surface epithelium and upper colonic crypts (Yang et al., 1998). DRA is upregulated in the colon of NHE3$^{-/-}$ mice, indicating its role in chloride absorption (Melvin et al., 1999). Some of the previous studies have also shown downregulation of DRA under *Citrobacter rodentium* driven intestinal inflammation (Borenshtein et al., 2008a; Farkas et al., 2010; Saksena et al., 2010; Yang et al., 1998), while an increased DRA expression has been observed in response to anti-inflammatory/pro-absorptive agents such as probiotics, i.e. lactobacillus acidophilus and butyrate (Alrefai et al., 2007; Raheja et al., 2010).

Long-term regulation of gene expression is associated with physiological or pathophysiological changes in the intestine such as in response to luminal SCFA butyrate, or IBD (Malakooti 2011). Reduced DRA expression has been shown in patients with UC, as well as in the IL-$10^{-/-}$ mouse (Kuhn *et al.* 1993) and the HLA-B27/2m transgenic rat, which are two known *in vivo* models of colitis (Farkas et al., 2010; Yang et al., 1998). Cell culture studies showed decreased DRA expression in response to pro-inflammatory cytokines IL1β and IFNγ (Saksena et al., 2008; Saksena et al., 2010; Yang et al., 1998). The mechanism of DRA inhibition by IFNγ involves

JAK1 and JAK2 and STAT1. JAK1 and JAK2 are activated by the binding of IFNγ to its surface receptor, which results in phosphorylation and activation of the latent cytosolic STAT1. STAT1 is further dimerized and translocates to the nucleus (Decker et al., 1991; Levy, 1998; Liu et al., 1998) where it binds to the GAS element of the DRA promoter. This, in turn, leads to a decrease in the DRA mRNA, protein expression and function (Fig. 8) (Saksena 2010).

Enteropathogenic E. coli (EPEC) infection decreases DRA surface expression via disruption of microtubules in the mouse colon (Gill et al., 2007). A recent study showed that $DRA^{-/-}$ mice are susceptible to DSS colitis. The study showed that the absence of DRA caused a reduced colonic $HCO3^-$ secretory rate, a loss of colonic fluid absorption, a lack of a firmly adherent mucus layer and a severely reduced colonic mucosal resistance to DSS damage (Xiao et al., 2014).

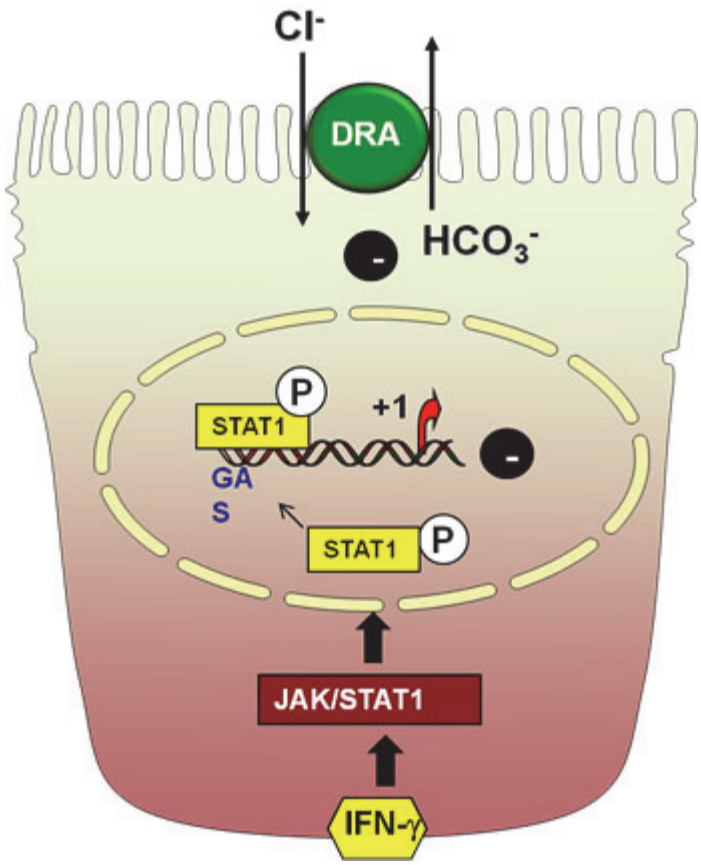

Figure 8. Proposed model for inhibition of DRA by IFNγ. Binding of IFNγ to its receptor activates JAK-1/2. Activated JAK 1and 2 then phosphorylate and activate cytoplasmic STAT1, which dimerizes and is translocated to the nucleus. In the nucleus, phosphorylated STAT1 binds to the GAS element of the DRA promoter which in turn leads to a decrease in DRA mRNA, protein expression and function. Adapted from (Saksena et al., 2010).

2.3.3.4. CFTR

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated Cl^- channel. CFTR is expressed in the apical membrane of epithelia and mediates salt and liquid movement (Sheppard and Welsh, 1999). Dysfunction of CFTR causes a genetic disease called cystic fibrosis (CF), whereas a defect in the activity of this chloride channel is seen in diarrhea and in polycystic kidney and lung diseases (Linsdell, 2014; Molina et al., 2015). Mutations in the CFTR gene result in both impaired Cl^- secretion and enhanced Na^+ absorption in the colon among CF patients (Kunzelmann and Mall, 2002).

2.3.4. Keratins in intestinal ion transport

Lack of K8 leads to the development of Th2-colitis and abnormal intestinal ion transport in transgenic mice. In ussing chamber experiments $K8^{-/-}$ mice show a dramatic decrease in short circuit current and net sodium absorption in the distal colon but have normal tight junction permeability and paracellular transport when compared to $K8^{+/+}$ mice (Toivola et al., 2004a). Interestingly, the $K8^{+/-}$ distal colon that expresses approximately 50% less keratins compared to $K8^{+/+}$, has an intermediate ion transport, short circuit current and proliferation phenotype, but no inflammation (Toivola et al., 2004a). The $K8^{-/-}$ small intestine appears normal compared to the $K8^{-/-}$ colon, but mistargeting of epithelial ion transporters has been reported (Ameen et al., 2001). As mistargeting of membrane proteins in the intestine of K8 null mice is relatively widespread (Ameen et al., 2001), the direct or indirect consequences result in modulation of Na^+ and Cl^- transport and diarrhea, which precede hyperproliferation and inflammation (Toivola et al., 2004a). K19 is expressed in the apical submembrane cytoskeleton in different epithelia including non-brush border cells and it plays an important role in the organization of the apical pole in epithelial cells (Salas et al., 1997). Studies using $K8^{-/-}$ mice have revealed that K19 is required for the apical localization of γ-tubulin complex protein (GCP) 6 and γ-tubulin (Salas, 1999). K18 has been shown to interact with the C-terminal hydrophobic patch of CFTR leading to high expression of the latter. *In vivo* studies utilizing $K18^{-/-}$ mice revealed low surface expression of CFTR in duodenal and gallbladder epithelia. Therefore, K18 plays an important role in the regulation of CFTR and K8/K18 may be modifier genes in cystic fibrosis (Duan et al., 2012).

2.4. Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions of the colon and small intestine caused by multi-factorial conditions in a genetically predisposed host (Baumgart and Carding, 2007; Kaser et al., 2010; Rocchi et al., 2012; Xavier and Podolsky, 2007). This may be well supported by the hygiene hypothesis,

suggesting that the lack of exposure to infectious agents due to high-quality living standards and medical conditions, increases the risk of inflammatory and autoimmune disorders like IBD (Feillet and Bach, 2004; Garn and Renz, 2007). The most prevalent IBDs include UC and Crohn's disease (CD).

The annual incidence of UC in Europe ranges from 0.6-24.3 per 100,000 persons per year in comparison to Asia and Middle East with an incidence of 0.1-6.3 per 100,000 persons per year (Logan, 1998). The annual incidence of CD in Europe ranges from 0.3-12.7 per 100,000 persons per year in contrast to lower incidence ranging from 0.04-5.0 per 1000 persons per year in Asia and the Middle East (Molodecky et al., 2011). The highest incidence of IBD has been reported in Northern Europe and North America (Lakatos, 2006). In Finland, an increasing incidence of IBD has been reported. In 1993, IBD prevalence was recorded as 216 per 100,000 inhabitants and in 2013 the number of patients reached 595 per 100,000 inhabitants. The prevalence of IBD and especially UC was much more increased in northern Finland when compared to the southern part of the country (Jussila et al., 2012). IBD causes a considerable financial burden to the health care system in the European society. Around 2.5-3 million Europeans are affected by IBD and 4.6-5.6 billion Euros/year are spent for disease management (Burisch et al., 2013).

Mouse models of colitis provide vital tools facilitating the study of the molecular and pathophysiological mechanisms which are responsible for IBD initiation and progression (Mizoguchi and Mizoguchi, 2010; Wirtz and Neurath, 2007; Xavier and Podolsky, 2007). Genetic and chemical colitis mouse models provide the means for examining early and late inflammatory processes as well as for evaluation of new therapies still needed in the field (Hoffmann et al., 2002; Mizoguchi, 2012; Mizoguchi and Mizoguchi, 2010). To date, numerous animal models have been developed to study IBD. These models include spontaneous colitis models, inducible colitis models, genetically modified models, and adoptive transfer models. Chemically induced murine models of colitis are widely used because they are simple, easy to induce and have quick onset and duration and the severity of inflammation is controllable. These models include DSS and trinitrobenzene sulfonic acid (TNBS) induced colitis which are commonly used animal models of mucosal inflammation in numerous studies focusing on IBD (Neurath et al., 2000; Wirtz et al., 2007; Wirtz and Neurath, 2007). In addition, several $^{-/-}$ and transgenic mice, such as the NOD2^{2939insC} mutant, NOD2$^{-/-}$, IL-12p35$^{-/-}$ and IL-10$^{-/-}$, spontaneously develop intestinal inflammation (Wirtz and Neurath, 2007).

Citrobacter rodentium serves as a bacterial model to study mucosal immunology, including the deregulation of intestinal inflammatory responses during bacteria-induced colitis (Borenshtein et al., 2008b; Collins et al., 2014). Previous studies have successfully demonstrated that TLR4 and MyD88$^{-/-}$ mice have less pathology in response to DSS colitis, although bacterial translocation to mesenteric lymph nodes was more commonly detected (Fukata et al., 2005). Studies have shown that alterations

in TLR4 and TLR5 function has an effect on intestinal homeostasis and may lead to the development of metabolic syndrome (TLR5) (Rhee et al., 2005; Vijay-Kumar et al., 2010).

2.4.1. IBD diagnostics

Conventional means to assess the severity of murine colitis longitudinally *in vivo* are either indirect, (such as body weight loss, stool hydration fecal blood content and disease activity index), or labor intensive (such as *post mortem* histopathology analysis) (Mizoguchi, 2012; Mizoguchi and Mizoguchi, 2010; Talapka et al., 2014; Wirtz et al., 2007; Wirtz and Neurath, 2000). *In vivo* imaging has revolutionized the research by providing non-invasive tools and recent developments in preclinical imaging modalities have improved the analysis of longitudinal studies of disease models (Beckmann et al., 2007).

Studies in the recent past have shown that a few techniques have been developed for imaging the human or murine gastrointestinal tract, but these techniques have some technical limitations such as excessive motion artifacts due to peristalsis, cardiovascular pulsation and respiration (Jelicks, 2010). Current *in vivo* modalities include high-frequency micro-ultrasound, magnetic resonance imaging (MRI), computed topography (CT), optical imaging such as fluorescence and bioluminescence, positron emission tomography (PET) and single photon emission computed tomography (SPECT) (Willmann et al., 2008). MRI, CT and ultrasound are used for anatomical imaging, whereas optical imaging and PET are preferred for molecular levels studies. The use of radiations in CT, the high cost of MRI, the low sensitivity of ultrasound or invasive specific endoscopic techniques are the limitations in the cost effective and accurate diagnostics of IBD (Becker et al., 2005; Mitsunaga et al., 2012; Moussata et al., 2010). There is a need to invent simple, non-invasive, cost effective and radiation-free imaging techniques both for human colitis diagnosis and murine model analysis (Fredin et al., 2008; Melgar et al., 2007; Strobel et al., 2011).

2.4.2. Reactive oxygen and nitrogen species (RONS) in IBD

Reactive oxygen species (ROS) are a heterogeneous group of oxygen radicals and other strongly oxidizing molecules and have similar characteristics as reactive nitrogen species (RNS). Reactive oxygen and nitrogen species (RONS) are well known for their roles in modulation of gene expression, cellular function, cellular damage and signaling. However, during the last decade there has been an increasing interest in revealing the mechanisms by which RONS act as signals or mediators of changes in cell function, proliferation and differentiation (Jackson et al., 2002; Sen and Packer, 1996; Zhu et al., 2012). RONS are produced from both extracellular and intracellular sources. The extracellular RONS play a pivotal role in initiating cellular signaling by

activating growth factor and cytokine receptors without receptor ligands or via generation of lipid peroxides within cell membranes (Coffer et al., 1995; Hayes and Lockwood, 1987; Suzuki et al., 1997). On the other hand, intracellular production of RONS is governed by a number of stimuli like tumor necrosis factor-α (TNF-α) or other RONS via induction of NADPH oxidase (Suzuki et al., 1997). Interaction of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) with their receptors causes a transient increase in hydrogen peroxide (Garcia-Ruiz et al., 1997; Lo and Cruz, 1995). However, excessive production of RONS may have deleterious effects such as damage to macromolecules and interruption of cellular signaling. Increased levels of RONS are a hallmark of inflammation and have also been found elevated in human and murine colitis (Kaser et al., 2010; Keshavarzian et al., 1992a; Keshavarzian et al., 1992b; Simmonds et al., 1992). RONS play an important role in immune response during inflammation but may also become deleterious by propagating an inappropriate inflammatory response in diseases like rheumatoid arthritis (Winrow et al., 1993). Increased levels of nitric oxide (NO) were found in the serum, colonic tissues and cultured mucosal explant from IBD patients (Ljung et al., 2002; Lundberg et al., 1994; Oudkerk Pool et al., 1995). RONS are commonly detected indirectly in colitis using microscopic or colorimetric assays for myeloperoxidase (MPO), which is an enzyme producing RONS in neutrophils that are present at the site of inflammation (Araki et al., 2006; Sedghi et al., 1993).

2.4.3. Measurement of RONS and *in vivo* imaging of murine colitis

There are many methods available, including biomarkers and probes, for the detection of RONS (Halliwell and Whiteman, 2004). A vast majority of these methods involve detection of antioxidant levels and oxidation metabolites in tissue and blood samples (Halliwell and Whiteman, 2004). In comparison to these indirect methods, genetically encoded fluorescent RONS indicators or injected, mostly luminescent RONS sensitive probes, such as luminol, lucigenin, and dihydroethidium (DHE), provide real time measurement of RONS (Lucas and Solano, 1992; Videla et al., 1993).

Due to the short life span of RONS the conventional imaging methods are not effective enough. The use of different *in vivo* imaging techniques enables us to visualize the location of RONS production. These techniques use either genetically encoded fluorescent or chemiluminescent RONS sensitive probes. *In vivo* imaging may provide early and direct detection of RONS. The RONS levels in biopsies and cells have been detected by using luminol-enhanced chemiluminescence (Keshavarzian et al., 1992a; Keshavarzian et al., 1992b; Simmonds et al., 1992). 8-amio-5-chloro-7-phenylpyrido[3,4-d] pyridazine-1,4-(2H, 3H) dione (L-012) is a luminol analog and chemiluminescent probe with high sensitivity for RONS and has been used in detection of RONS in cell culture (Daiber et al., 2004; Imada et al., 1999; Nishinaka et al., 1993). The L-012 probe distributes widely in the mouse body and produces

chemiluminescence in a spatial and temporal manner corresponding to inflammation. This compound, thus, provides a simple and cost effective method to study regulation of RONS (Kielland et al., 2009). The same probe has also been used for *in vivo* imaging of lipopolysaccharide (LPS)-induced inflammation in animal models of arthritis (Kelkka et al., 2012a; Kielland et al., 2009). However, this probe has not yet been used for *in vivo* imaging of colitis. The short half-life of this probe makes its use limited and challenging.

3. AIMS OF THE STUDY

The main aim of this study is to unravel the roles of keratins in intestinal homeostasis. Specific aims are as follows:

A. To describe the baseline colonic phenotype of $K8^{+/-}$ mice as a possible model to address the roles of keratins in the intestine.

B. To test if $K8^{+/-}$ mice have an increased susceptibility to experimental colitis.

C. To understand how keratins may regulate ion transport or protein targeting in intestinal epithelia.

D. To evaluate the possible use of L-012 for imaging colitis *in vivo*.

E. To evaluate the difference of gene expression between $K8^{+/+}$ and $K8^{+/-}$ mice to fully elucidate the function of K8.

4. MATERIALS AND METHODS

The details of the methods used during the PhD studies are thoroughly described in the separate publications/manuscript (I-III). The following section describes briefly the methods used and those techniques not included in I-III.

4.1. Mice (I, II, III)

The $K8^{-/-}$, $K8^{+/-}$ and $K8^{+/+}$ mice in the FVB/n background were generated by interbreeding of $K8^{+/-}$ mice and genotyped as previously described (Baribault et al., 1994; Toivola et al., 2004a). Pre-diabetic 4-5 week old female NOD mice (Alam et al., 2010) and BALB/c control mice were bred and maintained in Turku University Central Animal Laboratory under specific pathogen free conditions. All animal experiments were approved by the Animal Experimental Board in Finland and conformed to the legal acts, regulations and requirements set by the European Union concerning protection of animals used for research.

4.2. Microarray

The colonic epithelial cells were isolated by scraping the whole colonic epithelium by the following method: The colon was excised and placed on an ice-cold glass surface. The colon was cut open longitudinally and rinsed with ice-cold phosphate-buffered saline. The colonic epithelium was collected by scraping with a glass slide . Total RNAs were isolated with NucleoSpin® RNA II kit (Macherey-Nagel, Germany). RNAs were reverse transcripted, labeled and hybridized to Illumina Sentrix Bead Arrays (Illumina, San Diego, CA) where the Illumina Mouse WG-6 v.2.0 Expression BeadChip was used (>45 000 transcript-specific probe sequences/array). RNA quality was controlled prior the amplification and labeling. The microarray assay was conducted by The Finnish Microarray and Sequencing Centre at Turku Centre for Biotechnology. After GO, KEGG and REACTOME annotations and analysis, genes with more than 1.5 fold change in the expression level were selected for further analysis.

4.3. Real time PCR (I, II)

Taqman RT-PCR (I): Total colon RNA was isolated from colon epithelial scrapings obtained by scratching the mucosa with a microscope slide. RNA was isolated using an RNeasy mini kit (Qiagen, Netherlands). Contaminating DNA was removed with DNase I enzymes (Promega, WI, USA) and the RNA quality was analyzed in a 1 %

agarose gel. 1 µg of each RNA sample was synthesized by reverse transcription to cDNA using a transcription kit (Promega, WI, USA). Target genes were amplified using specific primers (Supplemental Table 1, I) and KAPA probe Fast ABI Prism qPCR mix (Kapa Biosystems, MA, USA). qPCR was performed with Step One Plus Real-Time PCR system (Applied Biosystems, CA, USA). Gene expression levels were normalized to the housekeeping gene β-actin. Each cDNA was tested in a triplicate and the amplification was analyzed using a 1 % agarose gel.

SYBER Green (II): Total RNA was extracted from epithelial scrapings of ileum, proximal and distal colon with a microscope slide. RNeasy Mini Kit (Qiagen; Netherlands) was used to isolate RNA according to the manufacturer's instructions. Equal amount of RNA from $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ was reverse transcribed and amplified in one step reaction using Brilliant SYBR Green qRT-PCR Master Mix Kit (Agilent Tech., Santa Clara, CA). Gene expression levels were normalized to GAPDH (Kumar et al., 2014).

4.4. Tissue lysates and western blotting (I, II)

For western blotting, total lysates of colonic tissues were prepared by homogenization in a sample buffer containing 0.187M Tris-HCl pH 6.8, 3% sodium dodecyl sulphate (SDS) and 5m Methylene diamine tetra acetic acid (EDTA). Protein concentrations were measured using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) and an equal amounts of protein were loaded and separated by SDS polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and immunoblotted with indicated antibodies. Proteins were visualized using Western Lightning Chemiluminescence (Perkin-Elmer, Waltham, MA, USA). Protein signals were quantified from scanned films using ImageJ (National Institutes of Health, USA) by normalizing to Hsc70 (I). The western blot for II in collaboration with Prof. Dudeja at University of Chicago was performed using the following method: Tissue lysates were prepared from colonic scrapings and total protein was extracted using RIPA lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). A bullet blender was used to homogenize mucosa followed by sonication (three pulses for 20 s each). Cell debris was removed by centrifuging the lysates at 13000 rpm for 7 min at 4°C. The Bradford method was used to determine the protein concentration in the samples. Equal amounts (75 µg/sample) of tissue lysates were solubilized in SDS-gel loading buffer and boiled for 5 min. Lysates were run on a 7.5% SDS-polyacrylamide gel and transblotted to nitrocellulose membrane after electrophoretic separation. After one hour of incubation in blocking buffer (1X-PBS and 5% non-fat dry milk) the membrane was probed with affinity purified anti-DRA antibody (1:100 dilution; Dudeja lab) or anti GAPDH antibody (Sigma; 1:3,000 dilution) in 1X-PBS and 2.5% non-fat dry milk overnight at 4°C. After washing the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit

IgG antibody (1:2,000 dilution) for 1 h at room temperature and washed for 30 min with agitation, during which the wash buffer was changed every 5 min followed by ECL (enhanced chemiluminescence, from Bio-Rad, Hercules, CA) detection (Kumar et al., 2014).

4.5. Immunofluorescence staining (I, II)

Mice were euthanized by CO_2 inhalation and colons were excised. Colon samples were fresh frozen and embedded in Optimal Cutting Temperature compound (OCT) (Sakura Finetek, Netherlands). Sections of colonic tissue from $K8^{-/-}$, $K8^{+/-}$ and $K8^{+/+}$ mice were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature and permeabilized using 0.5% NP-40 in PBS for 5 minutes, followed by blocking in 2.5% NGS (Normal Goat Serum) for 120 minutes at room temperature. Sections were then incubated with 1% NGS containing anti-DRA (1:100) and anti-villin (1:100, Abcam) antibody for 60 min. After washing with 1% NGS, sections were incubated with secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG for 60 minutes and then mounted with Slowfade Gold antifade with DAPI reagent (Invitrogen) using cover slips. Edges of the coverslip were sealed using quick dry nail enamel. Sections were imaged using a Carl Zeiss LSM 510 laser-scanning confocal microscope at 20x magnification (Kumar et al., 2014). Images for MPO and DHE stainings were obtained by using Leica TCS SP5 Matrix confocal and DM IRBE (inverted) immunofluorescence microscopes respectively. Histological sections stained by H&E were imaged by using AxioVert 200M microscopes.

4.6. RT-PCR Primers

Table. 3. List of primers used in RT/PCR. (I, II)

Protein	Forward	Reverse
β-Actin	5'-TGG CTC CTA GCA CCA TGA AGA-3'	5'-GTG GAC AGT GAG GCC AGG AT- 3'
K7	5'-GGA GAT GGC CAA CCA CAG-3'	5'-GGC CTG GAG TGT CTC AAA CTT-3'
K8	5'-TGA ATT TGT CCT CAT CAA GAA GG-3'	5'-GGA TCT CCT CTT CAT GGA TCT G-3'
K18	5'-AGA TGA CAC CAA CAT CAT CAC AAG G-3'	5'-CTT CCA GAC CTT GGA CTTCCT-3'
K19	5'-TGA CCT GGA GAT GCA GAT TG-3'	5'-CCT CAG GGC AGT AAT TTCCTC-3'
K20	5'-AGC TGA GAC GCA CCT ACC AG-3	5'-TGC GCT CCA GAG ACT CTT TC-3'
K23	5'-TCA TGA AGA AAC GCC ATG AG-3'	5'-CCT TGA AGT CAC TCG GCA AG-3'
DRA	5′ -TGGTGGGAGTTGTCGTTACA-3′	5′ -CCCAGGAGCAACTGAATGAT-3′
PAT-1	5′ -GAAATGGAGCTGCAGAGGA-3′	5′ -GCT GGAGCAGAAGAGAATGG-3′
NHE-3	5′ -GGCCTTCATTCGCTCCCCAAG-3′	5′ -ATGCTTGTACTCCTGCCGAGG-3′
GAPDH	5′ -TGTGTCCGTCGTGGATCTGA-3′	5′ -CCTGCTTCACCACCTTCTTGAT-3′

4.7. Antibodies

4.7.1. Primary Antibodies

Table. 4. List of primary antibodies used for immunostaining and western blotting. The table provides information regarding antibody concentration, supplier information and their use in different methods.

Antigen	Antibody	Conc. WB/IHC	Supplier	Application	Used in
K8	Troma I	1:100000 /1:1500	Hybridoma bank, USA	WB, IHC	I, II
K19	Troma III	1:1000 /1:100	Hybridoma bank, USA	WB, IHC	I
K18	Troma II	1:5000 /1:100	Hybridoma bank, USA	WB, IHC	I
K7	RCK 105	1:300 /1:10	Progen, Germany	WB, IHC	I
K20	IT-Ks 20.10	1:500 /1:30	Progen, Germany	WB, IHC	I
Desmoplakin	Anti-desmoplakin 1&2	Ready to use	Progen, Germany	IHC	I
Tubulin	mouse anti-tubulin	1:200	Sigma, Germany	WB	I
Caspase	rabbit anti-caspase-7	1:1000	Cell signallling, USA	WB	I
Caspase	anti-cleaved caspase-7	1:1000	Cell signallling, USA	WB	I
Hsc70	Anti Hsc70	1:1000	Stressgen, Canada	WB	I
MPO	anti-MPO Rabbit polyclonal Ab-1	1:50	Thermo Scientific, USA	IHC	I, III
DRA	Anti-DRA Rabbit affinity purified	1:100	Dudeja lab, USA	IHC	II
Villin	Anti-Villin mouse monoclonal	1:100	Abcam, USA	IHC	II
NHE3	Anti-NHE3 mouse monoclonal	1:100	Abcam, USA	IHC	II

* WB: western blot; IHC: immunohistochemistry

4.7.2. Secondary Antibodies

Table. 5. List of secondary antibodies used for immunostaining and western blotting. The table provides information regarding antibody concentration, supplier information and their use in different methods.

Antibody	Conc.	Supplier	Application	Used in
Alexa 488 goat anti-mouse	1:200	Invitrogen, USA	IHC	I, II
Alexa 488 goat anti-rat	1:200	Invitrogen, USA	IHC	I
Alexa 488 goat anti-rabbit	1:200	Invitrogen, USA	IHC	I
Alexa 488 donkey anti-rabbit	1:200	Invitrogen, USA	IHC	III
Alexa Flour 568 goat anti-mouse	1:500	Invitrogen, USA	IHC	I, II
Rabbit anti-mouse HRP	1:5000	GE Healthcare, UK	WB	I
Horse anti-rabbit HRP	1:1000	Cell signaling, USA	WB	I
Donkey anti-rat HRP	1:25000	GE Healthcare, UK	WB	I

4.8. Methods

Table. 6. List of different methods used in PhD thesis.

Name	Publication
Immunoflourosence staining	I, II, III
Bright field microscopy	I, II, III
Immunoflouroscence microscopy	I, II, III
Confocal microscopy	I, II, III
In vivo imaging	I, III
In situ imaging	III
Ex vivo imaging	III
Histology	I, III
PCR	I, II, III
RT-PCR	I, II
Microarray, *Ex-vivo* colon culture	Thesis
SDS-PAGE	I, II
Western blot	I, II
High salt extraction, BrdU labeling	I
Isolation of lamina propria cells	II
Isolation of peritoneal cells	I
Induction of DSS colitis, Flow cytometry	I, III

4.9. Reagents

Table. 7. List of different reagents used in this PhD thesis showing supplier information and their usage in different methods.

Reagent	Supplier	Application	Used in
DAPI	Invitrogen, USA	Immunohistochemistry	I
DRAQ-5	Cell signaling, USA	Immunohistochemistry	I, III
TOTO-3	Invitrogen, USA	Immunohistochemistry	I, III
Hematoxylin and Eosin	Sigma-Aldrich, USA	Tissue staining	I, III
Triton X-100	Sigma-Aldrich, USA	High salt extraction	I, III
1X protease inhibitor mix	Roche, Germany	High salt extraction	I
EDTA	Sigma-Aldrich, USA	High salt extraction	I
DNase I enzymes	Promega, USA	RT-PCR	I, II
RNA later	Qiagen, Netherlands	RT-PCR	I, II
transcription kit	Promega, USA	RT-PCR	I, II
KAPA probe Fast ABI Prism qPCR mix	Kapa Biosystems, USA	RT-PCR	I, II
L-012	Wako Chemical, Germany	In vivo, in situ and ex vivo imaging	I, III
Optimal Cutting Temperature compound	Sakura Finetek, Netherlands	Immunohistochemistry	I, III
Hank's buffered saline solution	Sigma-Aldrich, USA	Lamina propria cells isolation	I, III
BrdU	BD Pharmingen	Immunohistochemistry	I
Borax solution	Sigma-Aldrich, USA	Immunohistochemistry	I
H2O2	Sigma-Aldrich, USA	Lamina propria cells isolation	I
Dextran sulphate sodium	TdB consultancy, Sweden	Chemical models for colitis	I, III
Collagenase VIII	Sigma-Aldrich, USA	Flow cytometry	I
CD-FITC	Immunotools, Germany	Flow cytometry	I
Anti-CD49d-PE	Immunotools, Germany	Flow cytometry	I
Anti-L-selectin	Immunotools, Germany	Flow cytometry	I

4.10. Statistical analysis (I, II, III)

Mann-Whitney U (non-parametric data or data without normal distribution) and Student's T test (parametric and data with normal distribution) were used for two-group comparisons, whereas Bonferronis post hoc test (non-parametric data or data without normal distribution) or One-way ANOVA (parametric and data with normal distribution) was used for comparisons of three or more groups. Survival data was analyzed by using the Kaplan-Meier test. The average ± SEM is shown in all figures and all p values < 0.05 were considered as significant. Graph Pad Prism and Excel software were used for statistical analysis.

5. RESULTS AND DISCUSSION

5.1. Characterization of the baseline colonic phenotype of $K8^{+/-}$ mice as a possible model to address the roles of keratins in the intestine. (I)

5.1.1. $K8^{+/-}$ mice have lower levels of keratins which are normally distributed in colonic epithelium with the exception of K7.

In order to elucidate the effect of the absence of one *KRT8* allele in $K8^{+/-}$ mice in terms of amount of keratins, the mRNA and protein levels of colonic keratins, i.e. type II (K7, K8) and type I (K18, K19, K20), were evaluated. In contrast to other colonic keratins, K8 mRNA levels were found to be decreased up to 50% as compared to the $K8^{+/+}$ colon. Expectedly, the K8 protein levels were also decreased by 50% in these mice. Interestingly, the protein levels of other colonic keratins, i.e. K7 and K18, were significantly decreased. K19 and K20 protein expression was decreased to less extent (Fig.1, I) while mRNA levels of K7, K18, K19, K20 and K23 were found to be unaltered. This finding implies that colonic keratins are regulated at the post-transcriptional level and filament assembly and stabilization requires K8 type I protein for making a dimer with its type II keratin protein partner (Coulombe and Omary, 2002; Omary et al., 2009). Microarray assay was performed to identify the colonocyte genes that are differentially expressed in response to lower levels of keratins in $K8^{+/-}$ mice. These experiments did not show any upregulation or downregulation of K type II mRNA in $K8^{+/-}$ mice as compared to $K8^{+/+}$ colonic crypt scrapings (not shown). Type II K8 and Type I K19 were the main colonic Ks found in high salt extraction. Ks have unique features of compensating each other in some instances (Coulombe and Omary, 2002). However, no compensatory K protein was detected in high salt extracts.

Immunofluoroscence staining did not only confirm the decreased amount of keratins in $K8^{+/-}$ colon but also revealed a normal keratin-distribution on the crypt-brush border axis with the exception of K7 (Fig. 2, I). Small amount of K7 was expressed in the apical compartments of $K8^{-/-}$ colonic crypts, which is quite predictable as type I keratins (K18, K19, K20), which are present at low levels in the $K8^{-/-}$ colonic crypts required a type II partner to make the filament. This indicates that K7 compensates for the absence of K8 in the cells close to the luminal region. The apical membrane is of great importance in maintaining the simple epithelia of the small intestine (Ameen et al., 2001). Like $K8^{+/-}$ mice, $K7^{+/-}$ mice have been shown to have 50% decreased K7 mRNA and protein levels in bladder epithelium. Like $K8^{-/-}$ colon, mRNA levels of other keratins in the $K7^{-/-}$ bladder were found at the same levels as in $K7^{+/+}$ (Sandilands et al., 2014). Moreover, slightly increased K20 mRNA has been detected in the $K7^{-/-}$ bladder. The $K8^{-/-}$ colon has slightly higher but not significant K20 mRNA levels as

compared to $K8^{+/+}$. Our novel data based on colon studies indicate that the amount of K8 is crucial in regulating the protein levels of the other SEKs in the intestine. There is a clear dose dependent effect of keratin levels seen in transgenic mice, i.e. $K8^{-/-} < K8^{+/-} < K8^{+/+}$, which can be used for further mechanistic studies to reveal the role of intestinal keratins.

5.1.2. $K8^{+/-}$ mice exhibit a colonic phenotype.

To characterize the phenotype of $K8^{+/-}$ mice, the $K8^{+/-}$ baseline morphology was studied by hematoxylin and eosin staining of colon sections and by measuring the crypt length (Fig. 3, I). It was observed that $K8^{+/-}$ mice have significantly thicker epithelium, as compared to $K8^{+/+}$ mice. This increased thickness may be caused by increased epithelial proliferation. An increased BrdU staining in $K8^{+/-}$ colonic crypts confirmed a mild hyperproliferation of colonocytes in these mice (Fig. 4, I). The $K8^{-/-}$ mice develop colonic hyperplasia (Baribault et al., 1994; Baribault et al., 1993). However, $K8^{+/-}$ crypts were not very long as in $K8^{-/-}$ mice but still significantly longer than $K8^{+/+}$ crypts, indicating an intermediate colonic phenotype. Unlike $K8^{+/-}$, $K7^{+/-}$ mice do not show any kind of hyperplasia in the bladder or any other epithelium, although $K7^{-/-}$ mice show hyperproliferation in the bladder epithelium (Sandilands et al., 2014). In contrast to $K8^{-/-}$ colon which show decreased apoptosis (Habtezion et al., 2011), $K8^{+/-}$ colon show normal apoptotic rate. To our knowledge, the $K8^{+/-}$ mouse could be used as model which has epithelial hyperproliferation but normal rate of apoptosis suggesting the importance of the amount of K8 in the colonic crypts for the normal intestinal epithelial cell proliferation.

It is obligatory for tissue homeostasis to keep a balance between cell proliferation and apoptosis. Keratin function has been associated with proliferation and cell cycle (Pan et al., 2013). Increased proliferation has been seen in breast cancer cells after keratin knockdown and keratin over expression leads to decreased proliferation (Iyer et al., 2014). K17 has been shown to regulate the protein synthesis and cell size during wound healing after phosphorylation and binding with 14-3-3 to initiate mTOR activity in keratinocytes (Kim et al., 2006). In terms of cell type context, the 14-3-3 may impact cell cycle associated regulators, i.e. Cdc25, in Xenopus eggs (Margolis et al., 2006). K5 and K14 also perform the key role of maintenance of cell proliferation via phosphatidylinositol 3-kinase (PI3)/Akt-mediated cell proliferation and/or Notch1-dependent cell differentiation (Alam et al., 2011). In contrast to decreased Akt kinase activity in the $K17^{-/-}$ mouse and K14 silenced keratinocytes, K8 and K18 ablation leads to increased Akt kinase activity in hepatocytes (Alam et al., 2011; Galarneau et al., 2007; Kim et al., 2006). The $KtyII^{-/-}$ mouse model lacking all type II keratins shows depressed mTORG1 activity and reduced protein synthesis besides having embryonic lethality and mistargeting of GLUT-1 and GLUT-3 transporters (Vijayaraj et al., 2009). Epithelial-to-mesenchymal transition (EMT) is known to lack keratin expression but

has high vimentin expression. K8 and K18 silencing leads to increased migration and invasiveness of epithelial cancer cells (Fortier et al., 2013). In contrast, a recent study using a novel lung cancer model has revealed that EMT does not directly depend on the keratin cytoskeleton but does rely on the changes in signaling pathways that results in alterations in keratins expression and organization (Konig et al., 2013). The colon cancer cells have decreased expression of K8 and K20, which suggests that EMT of cancer cells with higher metastatic potential eventually results in a decrease in the patient survival (Knosel et al., 2006). The exact mechanism of hyperproliferation in $K8^{+/-}$ mice is currently unknown and there is a need for future mechanistic studies to understand the possible link between hyperproliferation and the low levels of keratins in intestine.

5.1.3. In contrast to $K8^{-/-}$ mice which show spontaneous colitis, $K8^{+/-}$ mice have no signs of intestinal inflammation.

In contrast to the spontaneous Th2 mediated-colitis observed in $K8^{-/-}$ mice (Baribault et al., 1994; Habtezion et al., 2005), $K8^{+/-}$ mice do not show any obvious signs of colitis. However, the presence of hyperplasia led us to evaluate the inflammatory status of $K8^{+/-}$ mice. Myeloperoxidase (MPO) is an enzyme which is secreted by neutrophils at the site of inflammation and MPO staining on tissue sections is a widely used marker of inflammation (Loria et al., 2008). MPO staining of colon samples revealed no differences in the inflammation status between the $K8^{+/-}$ and $K8^{+/+}$ mice. In addition, we used a novel *in vivo* imaging method to monitor oxidative stress in $K8^{+/-}$ mice. This method uses a chemiluminescent probe, L-012, with a high sensitivity for RONS (Asghar et al., 2014; Kielland et al., 2009). $K8^{+/-}$ mice showed similar amount of L-012 signals as $K8^{+/+}$ mice, which was in clear contrast to the high signals found in $K8^{-/-}$ mice (Fig.5, I). These findings were correlated by histological evaluations showing no infiltrating leukocytes or neutrophils in $K8^{+/-}$ and $K8^{+/+}$ colon tissue (Fig. 3, I). We also performed FACS analysis to further determine the inflammatory status of $K8^{+/-}$ colon. We found that in contrast to colonic T-cells of $K8^{-/-}$ mice, those of $K8^{+/-}$ mice were comparable to cells present in $K8^{+/+}$ colon in terms of their number (Fig. 9), percentage of naïve (L-selectin positive) to effector T-cells (L-selectin negative) and their integrin α4 expression (Fig. 5, I). In $K8^{-/-}$ mice the number of colonic lamina propria (LP) T-cells was dramatically increased and they displayed a Th2 type inflammatory cytokine profile. Similar to several other murine colitis models (Connor et al., 1999), the amount of the gut-homing molecule alpha4beta7 integrin was increased in $K8^{-/-}$ LP T-cells and $K8^{-/-}$ cells aberrantly expressed the receptor for L-selectin, which is important in the initial activation and trafficking of lymphocytes to the LP. Other colitis models, such as G (alpha) i2-deficient mice which display a Th1-type UC-like disease, similarly expressed increased levels of alpha4beta7 integrin and showed a large increase in memory (L-selectin-low) CD4+ T-cells in the LP (Hornquist et al., 1997). In contrast to the shortened $K8^{-/-}$ colon (Baribault et al., 1994), the $K8^{+/-}$ colon length was not

different from that of $K8^{+/+}$ mice (Supplemental Table 2, I). Thus, from this data we concluded that $K8^{+/-}$ mice display a mild but significant colonic phenotype characterized by increased crypt length and thicker epithelium, but no inflammation.

Interestingly, our microarray assays comparing gene expression patterns of $K8^{+/-}$ and $K8^{+/+}$ colonocytes revealed that Egln3 which acts as a negative regulator of the NF-κB pathway (Fu and Taubman, 2013) was slightly upregulated in $K8^{+/-}$ mice. Similarly, the expression of Lgals 9 and Psmb9, which are known to be positive regulators of the NF-κB pathway (Hensley, 2010; Kempe, 2005) was found downregulated. The expression of none of these genes was altered in $K8^{-/-}$ mice which exhibit colonic inflammation (not shown). RT-PCR experiments confirmed the decreased levels of Psmb9 mRNA in the $K8^{+/-}$ mice as compared to $K8^{+/+}$ mice which were detected by microarray assays (Fig. 10). Based on these findings, we hypothesize that a defect in the NF-κB pathway may serve as the reason for the colonic hyperproliferation but lack of colonic inflammation in $K8^{+/-}$ mice. Further mechanistic studies are needed to explore these findings in more detail and to reveal the exact mechanism causing the phenotypes observed.

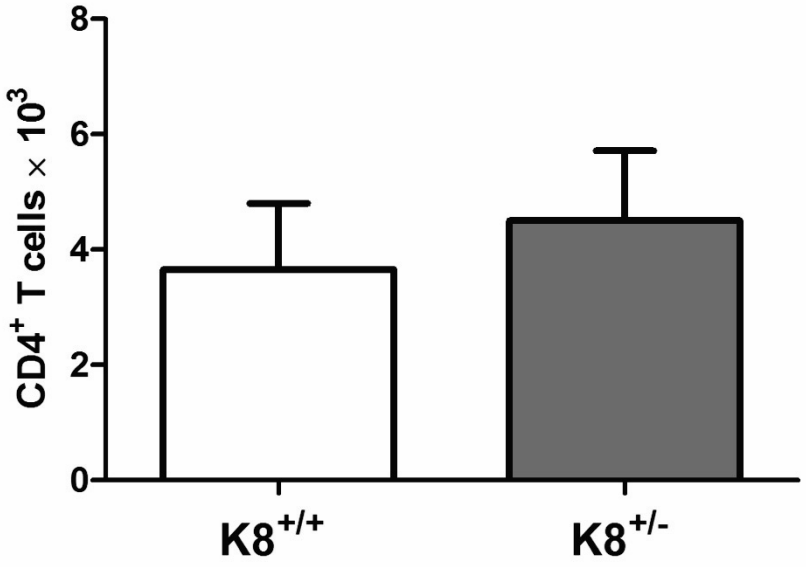

Figure 9. $K8^{+/-}$ and $K8^{+/+}$ lamina propria exhibit a similar number of T cells.

Table 8. Microarray analysis reveals that certain genes involved in the NF-KB pathway are downregulated in $K8^{+/-}$ colon.

Symbol	Fold change	P-Value	Protein	Function	Reference
Egln3	1.592	0.0002	Egl nine homolog 3	negative regulator of the NF-κB pathway	(Fu, 2013)
Lgals9	-2.074	0.0086	galectin-9	positive regulator of the NF-κB pathway	(Kempe, 2005)
Psmb9	-1.812	0.0091	proteasome subunit beta type-9	Antigen processing and presentation / activation of NF-κB pathway	(Hensley, 2010)

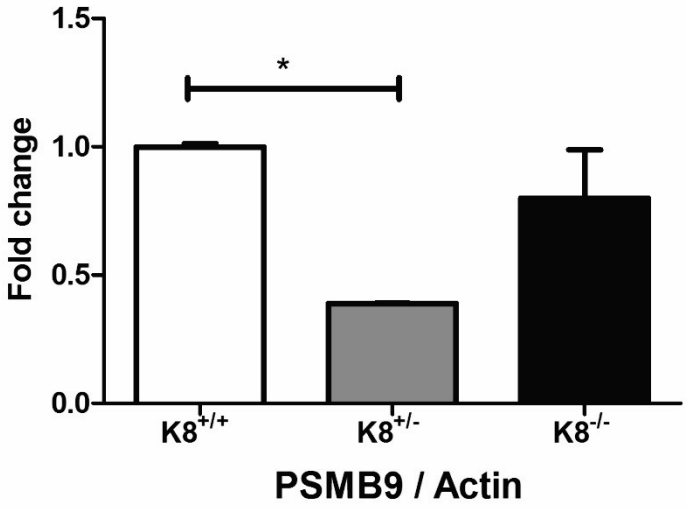

Figure 10. RT-PCR confirms decreased mRNA levels of PSMB9 in the $K8^{+/-}$ colon found in the microarray analysis. Total colon RNA was isolated from colon epithelia obtained by scraping the mucosa with a microscope slide. β-actin was used as housing keeping gene.

5.2. $K8^{+/-}$ mice have an increased susceptibility to experimental colitis. (I)

To test our hypothesis that the amount of keratins matters for protection during colonic stress, we evaluated the susceptibility of $K8^{+/-}$ mice, which have reduced levels of keratins, to experimental colitis. We used an established chemical model of colitis, DSS to induce acute and chronic colitis in $K8^{+/-}$ mice by using different doses of DSS, i.e. 5%, 3%, 2.5% and 2% in autoclaved drinking water followed by different recovery periods, i.e 7 days for acute and 14 days per cycle for chronic inflammation. DSS causes epithelial damage and results in a robust inflammatory response (Breynaert et al., 2013; Burisch et al., 2013; Wirtz et al., 2007). Five percent DSS in drinking water

for 7 days followed by regular water for 3 days resulted in significant body weight loss in $K8^{+/-}$ mice along with development of bloody diarrhea and low survival rate as compared to $K8^{+/+}$ mice (Fig. 6, I). Similarly, 3% DSS treatment for 5 days followed by drinking water up to day 12, resulted in no difference in body weight loss, however, $K8^{+/-}$ mice showed more signs of diarrhea and a higher disease activity index (DAI) score as compared to $K8^{+/+}$ mice (not shown). In another experiment, colitis was induced in $K8^{+/+}$ and $K8^{+/-}$ mice by 2% DSS for 7 days followed by regular water applied for 5 days. Treated mice were analyzed for body weight change, stool consistency, L-012 signals, and histological alterations after 12 days. $K8^{+/-}$ mice showed a trend of higher DAI score on day 8 (Fig.11 A). Histology scoring revealed that $K8^{+/-}$ mice showed a trend towards loss of epithelium and crypts as compared to $K8^{+/+}$ mice (Fig.11 C, E). $K8^{+/-}$ mice showed peak L-012 signals (Fig.11 B) indicating DSS induced inflammation on day 2, day 4 and day 8, while $K8^{+/+}$ mice showed their peak signals on day 5 showing an early onset of DSS induced colonic inflammation in $K8^{+/-}$ mice. However, there was no significant difference in colon length between $K8^{+/+}$ and $K8^{+/-}$ mice (Fig. 11 D). In order to investigate the recovery period in more detail, we induced chronic colitis with two cycles of one week 2.5% DSS administration followed by two weeks of autoclaved drinking water. In this setup $K8^{+/-}$ mice showed higher DAI score with higher histological damage than $K8^{+/+}$ mice (Supp. Fig. 3, I). Therefore, it is concluded that $K8^{+/-}$ mice are more susceptible to DSS-induced colitis and are slower in recovery than $K8^{+/+}$ mice. These findings support the significance of the colonic keratin amount for stress protection.

Cytoprotection from stress is a characteristic feature of keratins (Pan et al., 2013; Pekny and Lane, 2007; Toivola et al., 2010). Our finding of increased susceptibility of $K8^{+/-}$ mice to DSS induced colitis further strengthens the model of a cytoprotective role of keratins in response to colonic stress. $K8^{+/-}$ mice showed delayed recovery from DSS damage, which indicates the importance of keratins not only in protection from stress but also in recovery from stress. It is well known that keratins are upregulated up to many folds during different kinds of stress and that constitutive and de novo keratin RNA and protein synthesis are the reasons for keratin upregulation. However, hyperproliferation of a particular cell type also leads to keratin upregulation in response to injury. For instance, bile duct proliferation leads to increases in the K19 levels (Toivola et al., 2010). The increased sensitivity of $K8^{+/-}$ mice to colonic stress may be linked to the colonic hyperproliferation in these mice. However, the exact mechanism by which keratins play this critical role is not known yet and this link has not been comprehensively studied in other models. Although $DRA^{-/-}$ mice have been shown to develop hyperplasia (Schweinfest et al., 2006), further studies are needed to find out the keratin-ion transport link. On the other hand, Wnt antagonist $DKK^{-/-}$ mice showed hyperplasia due to increased proliferation which, in turn, was thought to protect mice from DSS damage and help in recovery (Koch et al., 2011). This indicates the ambiguity of the pathways. It is not clear whether keratin mutations predispose individuals to IBD (Owens et al., 2004; Tao et al., 2007), although keratin mutations in

some patients have been reported to predispose the carriers to liver disease. A recent study showed disruption of the intestinal barrier function by K8 and 18 mutations in colonic cell cultures (Zupancic et al., 2014). It has been shown that an additional stress, such as alcohol or drugs, in patients carrying keratin mutations caused potential damage to liver leading to liver diseases (Omary, 2009). Therefore, the $K8^{+/-}$ mouse with its mild colonic phenotype but increased susceptibility to the additional stress of DSS, supports a similar predisposition to stress. In contrast to the $K8^{-/-}$ model which exhibits inflammation interfering with many other processes, and to models overexpressing keratin mutations which cannot be used to study protective roles of keratins due to their significantly increased amounts of colonic keratins (Ku and Omary, 2006), the $K8^{+/-}$ mouse should be a valuable tool for studying the role of keratins in the intestine.

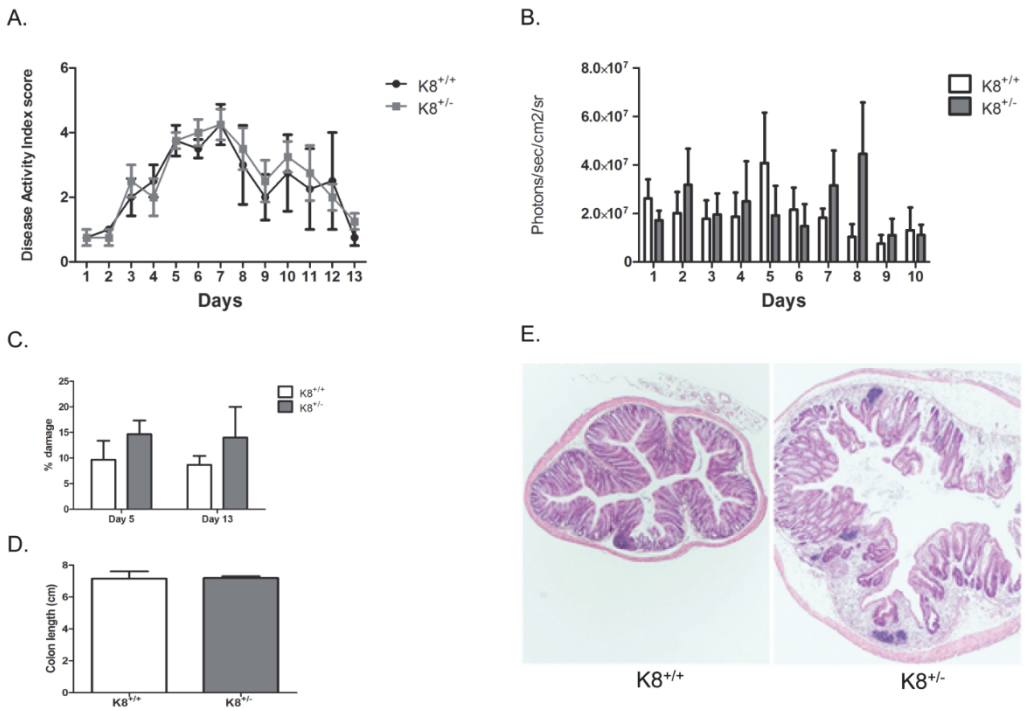

Figure 11. $K8^{+/-}$ mice display a non-significant trend of increased susceptibility to 2% DSS experimental colitis. Colitis was induced in $K8^{+/+}$ and $K8^{+/-}$ (n=8) mice by 2% DSS for 7 days followed by regular water for 5 days. Treated mice were analyzed for body weight change, bleeding and stool consistency as disease activity index (A), L-012 signals (B), colon length (D), and histology on day 13 (C,E). The early onset of colitis in $K8^{+/-}$ mice was shown by their peak L/012 signals on day 2, day 4 and day 8. On the other hand $K8^{+/+}$ mice showed their peak signals on day 5. Increased crypt length and inflammation was seen in $K8^{+/-}$ mice compared to $K8^{+/+}$ counterparts.

5.3. Keratins in ion transport in the intestinal epithelia. (II)

5.3.1. $K8^{-/-}$ mice display downregulation of DRA mRNA in the colon.

The diarrheal phenotype of $K8^{-/-}$ mice is characterized by decreased short circuit current and net sodium absorption associated with net chloride secretion in the distal colon (Toivola et al., 2004a). However, paracellular transport and tight junction permeability are as normal in the $K8^{-/-}$ as in the $K8^{+/+}$ distal colon (Toivola et al., 2004a). The membrane-proximal markers H, K-ATPase-and F-actin are patchy while AE1/2 protein is redistributed and its levels are increased. ENaC protein is redistributed (Toivola et al., 2004a). $K8^{-/-}$ enterocytes have been shown earlier to exhibit anomalous apical membrane proteins including alkaline phosphatase, sucrase isomaltase and CFTR, while the $K8^{-/-}$ colon was shown to have normal polarized expression of CFTR (Ameen et al., 2001). A recent study has shown that K18 binding to CFTR increases its surface expression by accelerating CFTR apical recycling (Duan et al., 2012). The same study also suggested K8, K18 and K19 as modifier genes for CF (Duan et al., 2012). The $K8^{+/-}$ colon, which expresses 50% less keratins compared to the $K8^{+/+}$ colon shows an intermediate ion transport, short circuit current and a proliferation phenotype, but lacks inflammation (Asghar et al., 2015; Toivola et al., 2004a). The $K8^{-/-}$ small intestine appears normal although mistargeting of epithelial ion transporters have been reported (Ameen et al., 2001). The effectiveness of ion transport as a function of keratin levels was assessed in $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice by examining the distribution and expression levels of the chloride channel DRA, the anion exchanger AE1/2, the Na-transporter ENaC gamma and the Na^{+}/H^{+} exchangers NHE1 and NHE3, as these potential candidate transporters could account for the abnormality in Na^{+} and Cl^{-} transport. No differences in mRNA levels of CFTR (Fig. 12), NHE3 (Fig. 13) and PAT-1 (Fig. 14) were observed in cecum and colon scrapings from $K8^{-/-}$ or $K8^{+/-}$ mice compared to $K8^{+/+}$ mice. However, the mRNA levels of the chloride transporter DRA were decreased 3-4 fold in the $K8^{-/-}$ caecum, proximal and distal colon compared to $K8^{+/+}$. Interestingly, DRA mRNA levels were unaltered throughout the $K8^{+/-}$ and $K8^{-/-}$ small intestine (Fig. 1. II). This finding matches with previous study showing that $K8^{+/-}$ enterocytes have a normal distribution of membrane markers (Ameen et al., 2001). This is possibly due to the fact that PAT-1 is the main chloride transporter in the small intestine, while DRA predominantly controls chloride transport in colon (Farkas et al., 2010; Musch et al., 2009; Walker et al., 2008; Wang et al., 2002). DRA levels may be associated with K8 via microflora and short chain fatty acids (SCFA). $K8^{-/-}$ mice have less bacteria (Habtezion et al., 2011) but have increased levels of SCFA in the colon (Helenius et al., 2015). The SCFA transporter, MCT1 is upregulated by butyrates. Therefore, DRA levels may be regulated by K8 indirectly.

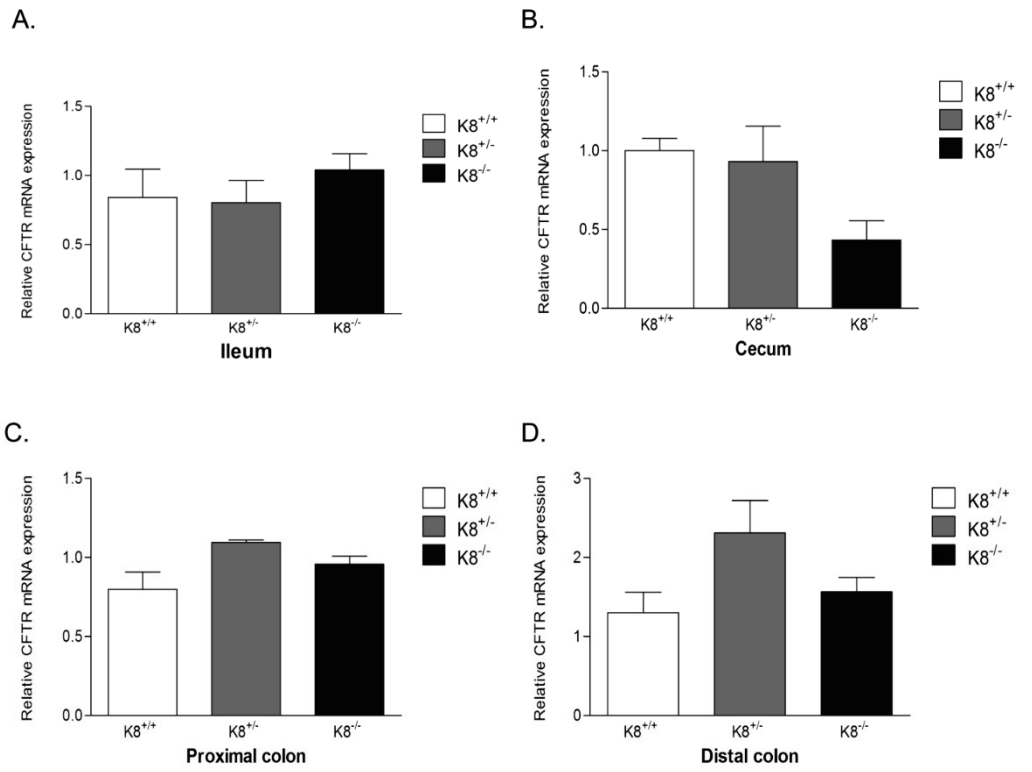

Figure 12. Unaltered mRNA levels of CFTR in the ileum, cecum, proximal and distal colon. The mRNA levels of CFTR were determined from epithelial scrapings isolated from ileum (A), cecum (B), proximal (C) and distal colon (D) of the $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice (n=4). One-way ANOVA with Tukey's test was used for statistical analysis. $P < 0.05$ or less was considered statistically significant. P values for ileum, cecum, proximal and distal colon were 0.5, 0.06, 0.05 and 0.10 respectively.

5.3.2. Reduced levels of DRA protein in $K8^{-/-}$ mice.

Western blot analysis showed that DRA protein levels were significantly reduced in the $K8^{+/-}$ proximal colon, while almost abolished in $K8^{-/-}$ proximal and distal colon, suggesting an involvement of K8 in DRA regulation. $K8^{+/-}$ mice showed normal DRA mRNA levels; however, the DRA protein was significantly reduced in proximal colon whereas cecum and distal colon showed a trend of non-significant decrease protein levels (Fig. 2, II). Together these findings suggest an involvement of K8 in DRA mRNA and protein expression. This finding led us to hypothesize that the aberrant intestinal ion transport found in $K8^{+/-}$ mice may be due to lower amount of keratins.

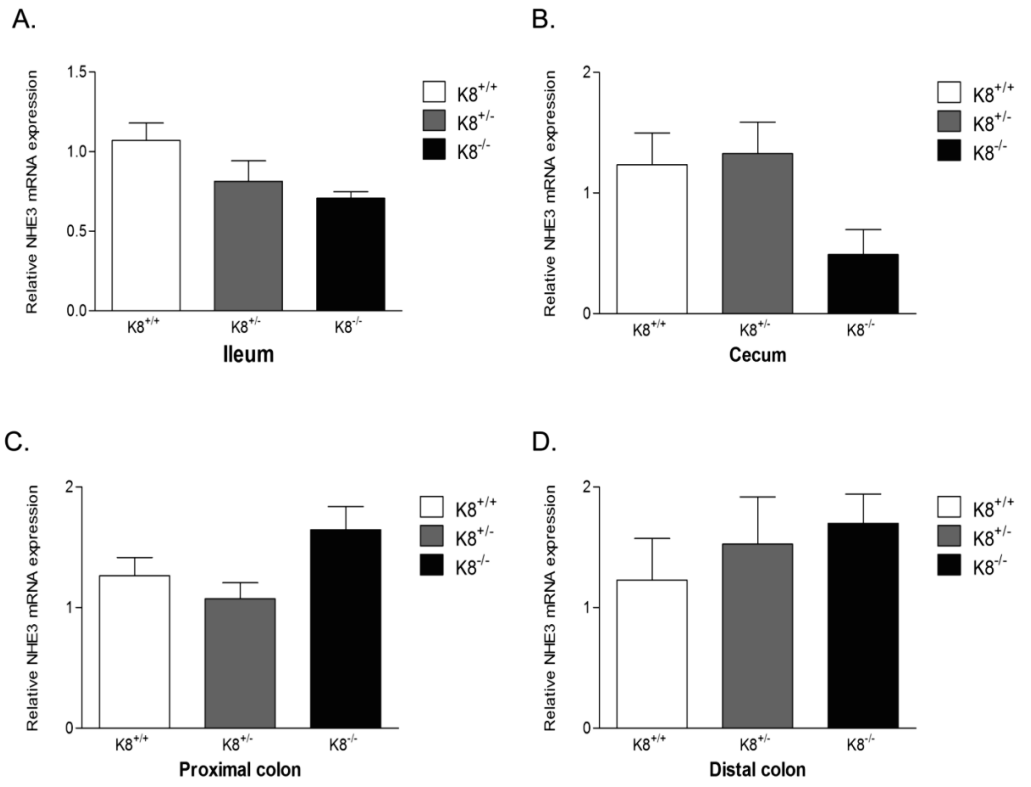

Figure 13. Unaltered mRNA levels of NHE3 in the ileum, cecum, proximal and distal colon. The mRNA levels of NHE3 were determined from epithelial scrapings isolated from ileum (A), cecum (B), proximal (C) and distal colon (D) of the $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice (n=4). One-way ANOVA with Tukey's test was used for statistical analysis. $P < 0.05$ or less was considered statistically significant. P values for ileum, cecum, proximal and distal colon were 0.08, 0.07, 0.08 and 0.6 respectively.

5.3.3. NHE3 has normal distribution and DRA is lost in in $K8^{-/-}$ colonic crypts.

Immunofluorescence staining and confocal microscopy were performed to find out the cellular location of the colonic DRA as a function of K8. Our results showed that DRA was abolished in the apical membrane of the $K8^{-/-}$ distal colon as compared to $K8^{+/-}$ and $K8^{+/+}$ mice, while villin which is a marker for microvilli was normally distributed (Fig. 15; Fig. 3, II). Interestingly, $K8^{+/-}$ distal colon showed patchy apical staining for DRA (Fig. 3, II). This finding led us to speculate the role of K8 in controlling DRA levels. The NHE3 protein was also found to be normally distributed in colonic crypts of $K8^{-/-}$ mice (Fig. 4, II).

It was concluded that the dramatic loss of DRA in colon and cecum of $K8^{-/-}$ mice was likely responsible for the chloride transport defect. The milder ion transport phenotype in $K8^{+/-}$ colon might be related to DRA, suggesting a role for K8 in the regulation of

DRA expression and targeting. The patchy DRA staining in the $K8^{+/-}$ distal colon is interesting and future studies may provide a clue about how the amount of keratins regulates DRA levels in the colon.

5.3.4. K8 knock down by siRNA leads to reduced DRA expression in Caco-2 cells.

Since the $K8^{-/-}$ mouse has inflammation (Habtezion et al., 2005) and DRA expression has also been shown to be reduced during inflammation (Gill et al., 2007; Saksena et al., 2010), it was not clear whether the reduced DRA expression in K8-/- colon was either due to K8 deficiency or inflammation. To study this, we first performed immunostaining on sections from distal colon of 1-2 day old mice as $K8^{-/-}$ mice develop inflammation at the age of 2 weeks. To our surprise, we found that DRA was not expressed in these mice (not shown). We then used in vitro approaches to elucidate if the reduced DRA levels in the $K8^{-/-}$ mice were secondary to inflammation or, loss of K8 directly. K8 was silenced by using siRNA in Caco-2 cells and the DRA expression was evaluated. Western blot analysis showed that the DRA expression was reduced 3-4-fold in K.8 silenced Caco-2 cells. Interestingly, K8 levels were also decreased 3-4 folds in response to K8 siRNA treatment in Caco-2 cells. This suggests that DRA expression is modulated by keratins directly in an inflammation-dependent manner. (Fig. 5, II). This is the first evidence to our knowledge showing a direct effect of reduced keratin levels on DRA expression. The outcome of this study provides insight for future studies to explore the mechanism by which K8 regulates DRA expression**.**

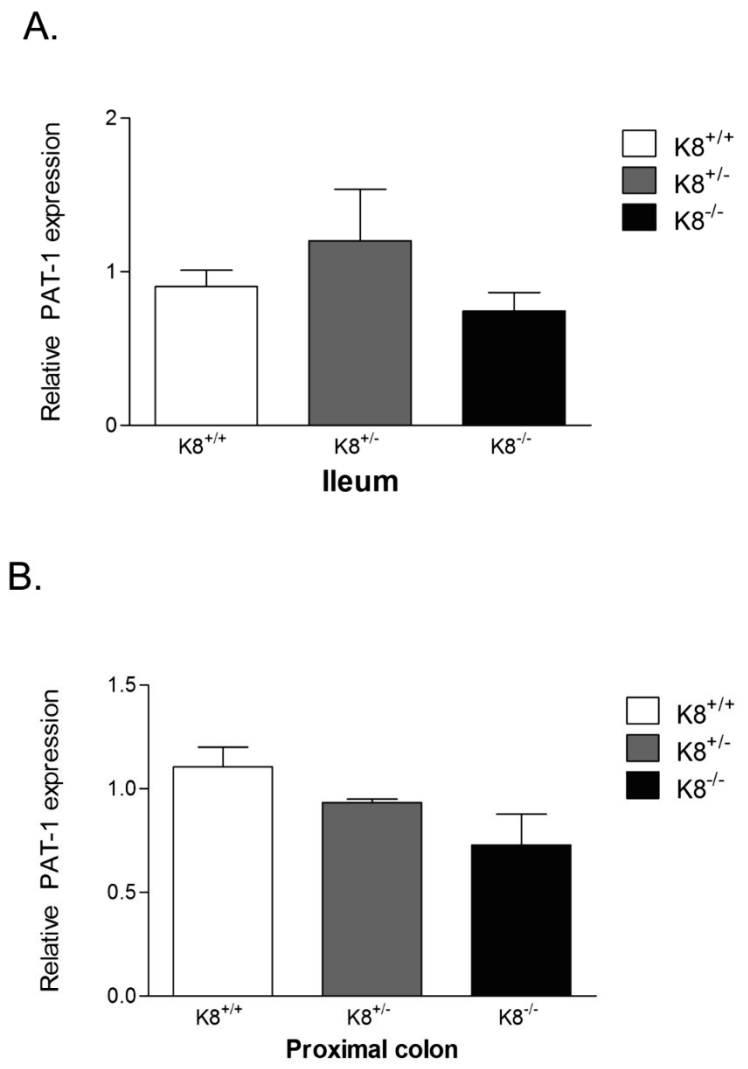

Figure 14. Unaltered mRNA levels of PAT-1 in ileum and proximal colon. The mRNA levels of PAT-1 were determined from epithelial scrapings isolated from ileum (A) and proximal colon (B) of the $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ mice (n=4). One-way ANOVA with Tukey's test was used for statistical analysis. $P < 0.05$ or less was considered statistically significant. P values for ileum, cecum and proximal colon were 0.3 and 0.08 respectively.

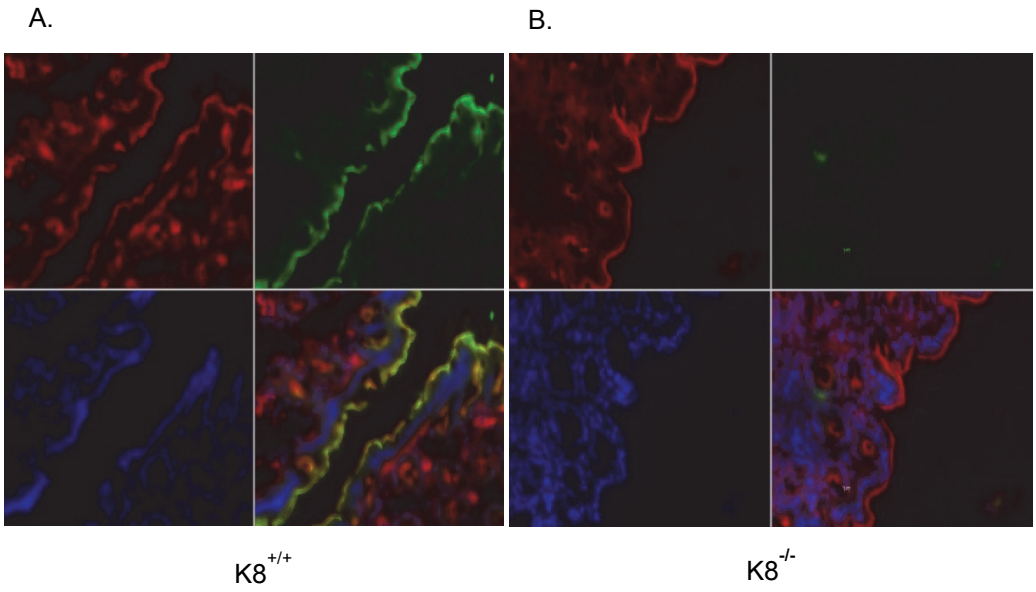

Figure 15. Immunofluorescence staining of distal colon shows the dramatic loss of DRA in K8$^{-/-}$ mice. (modified from Fig.3, III). Distal colon cryosections fixed with acetone at -20C were stained by using DRA Ab. The results show absence of DRA (green) in K8$^{-/-}$ distal colon apical membrane (B) as compared to normal staining in K8$^{+/+}$ mice (A). Apical villin (red) was not altered and DAPI (blue) was used to stain the nuclei.

DRA plays an important role in maintaining intestinal homeostasis in humans and mice. Mutations in the hDRA gene lead to the autosomal recessive disorder CLD (Hoglund et al., 1996; Makela et al., 2002). Like K8$^{-/-}$ mice, DRA$^{-/-}$ mice also have a diarrheal phenotype resembling CLD with upregulation of ion transporters (Schweinfest et al., 2006). NHE3$^{-/-}$ mice exhibit high DRA expression (Melvin et al., 1999). Similarly, it has been shown in previous studies involving human UC patients and IL-10$^{-/-}$ mice that DRA expression is reduced in inflammation (Farkas et al., 2010; Ku et al., 1999; Kuhn et al., 1993; Yang et al., 1998). Cell culture studies have shown that DRA was downregulated in response to IL1β and IFN γ (Saksena et al., 2010; Yang et al., 1998). Reduced DRA levels have also been found in EPEC infection and DSS colitis (Gill et al., 2007; Xiao et al., 2014). It is speculated that K8 may regulate DRA levels via STAT-1 pathway.

Our studies reveal a novel link between K8 and DRA expression in the mammalian colon. K8$^{-/-}$ mice have decreased NaCl absorption but no change in paracellular permeability and ENaC mistargeting (Toivola et al., 2004a). In contrast, ENaC knockout mice do not develop diarrhea (Hummler and Vallon, 2005). Our studies also showed that NHE3 expression or targeting was not altered. Therefore, it is concluded that K8 inactivation leads to dramatic downregulation of DRA which is the main cause of diarrheal phenotype observed in K8$^{-/-}$ mice.

5.4. Establishment of a novel *in vivo* imaging of colitis. (III)

IBD is a complex disease and the early diagnosis may be challenging. There is a need to develop an easy and direct method for the specific and early diagnosis of colitis which could also be used for monitoring disease progression and/or treatment effectiveness, as current techniques analyzing mouse colitis have their own limitations (Becker et al., 2005; Mitsunaga et al., 2012; Mizoguchi, 2012; Mizoguchi and Mizoguchi, 2010; Moussata et al., 2010; Wirtz et al., 2007). The fourth aim of my PhD thesis was to establish a novel *in vivo* imaging method for colitis diagnosis. We evaluated the use of a luminol derivative, the chemiluminescent probe L-012, for *in vivo* imaging. L-012 has high sensitivity for detection of RONS (Daiber et al., 2004; Imada et al., 1999; Kielland et al., 2009).

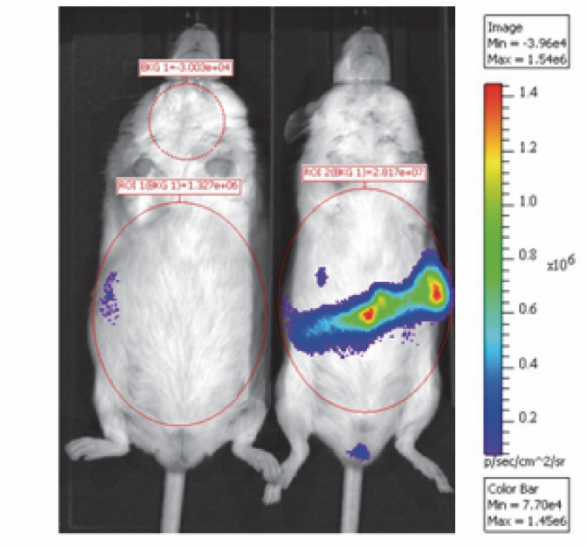

Figure 16. ***In vivo*** **imaging of murine colitis using L-012.**

Our data showed that *in vivo* imaging of RONS is able to detect the previously described chronic colonic inflammation in two genetic and one chemical model of colitis, i.e. $K8^{-/-}$, NOD mice and DSS (Fig.1, 3, 4, III, Fig. 16). The L-012 signals correlated with *ex-vivo* imaging and histological evaluations (Fig. 2, III). MPO and CellRox staining of colon sections confirmed the inflammation $K8^{-/-}$ mice and L-012 signals corresponding to the $K8^{-/-}$ inflammation correlated with a high MPO score (Fig.2, III). Due to its high specificity for RONS, L-012 is the only chemiluminescent probe that has been used for *in vivo* imaging of skin and arthritis disease models (Kelkka et al., 2012a; Kelkka et al., 2012b; Kielland et al., 2009; Zhou et al., 2012), which do not require deeper tissue penetration. However, organs like colon requires deep tissue imaging due their anatomical position. This bottle neck was solved by lipopolysaccharide manipulation of RONS (Kielland et al., 2009) besides correlating

with abdominal location of an NF-kB luciferin reporter in a systemic lupus model (Zangani et al., 2009). Unlike $K8^{-/-}$ mice, many IBD mouse models, e.g. IL-10 knockout mice do not develop early spontaneous colitis but these mice develop colitis at the age of few months. L-012 imaging may be used to monitor the onset and progression of colitis in these models or to see the effect of a treatment (Mizoguchi, 2012; Mizoguchi and Mizoguchi, 2010).

We found that L-012 may be used as a probe to monitor the progression of experimental colitis and possibly for early detection of colitis in individual mice in the DSS model (Fig. 4, III). This was evidenced by the peak L-012 signals already on day 4 in the 2% DSS model, while signs of colitis such as bloody stool or body weight loss were observed from day 6 and 7 onwards. The L-012 signals were down to baseline levels on day 9 when the severity of colitis was highest, as indicated by histology (Fig. 4, III). The lower L-012 signals are due to the damaged lamina propria and colonic epithelium in response to DSS treatment leading to the loss of RONS producing neutrophils (Murphy et al.; Ohtsuka and Sanderson, 2003; Pravda, 2005). In contrast to the genetic model of colitis, high variations in L-012 signals were seen among individual mice after receiving DSS treatment and in young untreated mice. These variations may be due to age dependent individual mouse immune system development and individual mouse response to DSS. As a matter of fact, quantitative imaging of RONS is challenging as they are also present in healthy colon and bursts are short-lived (VanderVen et al., 2009). Although previous studies using e*x vivo* imaging of human biopsies showed increased luminol signals correlating with the colitis disease-state (Sedghi et al., 1993).

Ex vivo and *in situ* imaging revealed that the main source of abdominal signals in $K8^{-/-}$ mice was distal colon (Fig. 5, III) as further validated by PET imaging using [^{18}F] FDG (Fig.6, III). *In vivo* imaging also showed that the $K8^{-/-}$ rectal prolapses (Baribault et al., 1994) had strong L-012 derived signals, further confirming the location of RONS in the colon of $K8^{-/-}$ mice, while $K8^{-/-}$ peritoneal cells had no L-012 chemiluminescence indicating that the abdominal signals are not derived from the peritoneum (Fig. 7, III). Careful analysis of L-012 data revealed that $K8^{-/-}$ mice had an ontogenic increase in L-012 chemiluminescent signals corresponding to colonic inflammation. This correlates well with previous studies showing that 6-month-old $K8^{-/-}$ mice had higher colonic cytokine levels (Habtezion et al., 2005), increased risk for rectal prolapse (Baribault et al., 1994) and more auto-antibodies than 3-month-old $K8^{-/-}$ mice (Toivola et al., 2015).

L-012 has no apparent toxic or adverse effects (Kelkka et al., 2012a; Kelkka et al., 2012b; Kielland et al., 2009) as monitored by mouse health and appearance, even upon multiple consecutive administrations as seen in the control mice used during DSS colitis imaging experiment. The data suggests that *in vivo* imaging by using L-012 is as a novel method to study colitis in genetic and induced colitis models. Moreover, this probe may also be useful in studying the susceptibility of genetic mice to DSS.

Recently, L-012 has been used to study the spatial and temporal aspects of inflammation in response to different diet in NOD mice. The whole gut imaging revealed increased RONS production in ileum of NOD mice highlighting the mechanistic towards diet-dependent regulation of diabetogenesis in type 1 diabetes (Emani et al., 2013). The use of L-012 should also aid in diminishing the number of mice used in experiments following colitis. Future development of chemiluminescent probes such as luminol derivatives with longer half-life or other RONS sensitive probes in combination with 3-dimensional imaging, or probes coupled with nanoparticles, would be useful in improving the quality of *in vivo* imaging and would make it possible to provide a better *in vivo* imaging modality for human use.

6. CONCLUDING REMARKS

This work was initiated in November 2009 and at that time, the role of keratins in the simple epithelium of the colon had not been extensively studied. This thesis work is primarily focused on the role of the IFs of epithelial cells, the keratins, in intestinal health and diseases. We have investigated the intestinal phenotype of $K8^{+/-}$ mice, which express 50% less keratin as compared to $K8^{+/+}$ mice and, thus, provide a novel model to study the functional significance of keratins in the intestine. Our findings are consistent with the fact that keratins are stress-protective proteins. In analogue, human individuals carrying keratin mutations are predisposed to several diseases. By using different colitis models and multiple imaging modalities, we have shown that decreased keratin levels in mice lead to altered ion transport, increased epithelial proliferation and susceptibility to experimental colitis, proposing that keratin amounts matter in intestinal health. We further investigated the role of keratins in intestinal ion transport and showed that the intestinal ion transport phenotype in $K8^{-/-}$ and possibly in $K8^{+/-}$ mice is due to dramatic loss of the main chloride transporter DRA. We also successfully established a novel *in vivo* imaging method for the early detection of colitis, which proved useful for genetic colitis models. The current thesis work provides new information about the structural and functional roles of keratins, particularly K8, in colitis and in the normal colon. This work is also significant for understanding the human diseases which are linked to keratin mutations. The outcome of this thesis work may also be helpful towards future development of diagnostics and treatment of colitis. In conclusion, this thesis does not only highlight the significance of K8 in the colon but also opens the door for future studies in order to find out the mechanism by which keratins regulate the intestinal homeostasis and ion transport in epithelia.

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Muhammad Nadeem Asghar
From my desk
Turku, October 27, 2015

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Roles of keratins in intestinal health and disease

The thesis provides advance understanding of keratin function and the cell biology of intermediate filaments in the colon. This thesis consists of three main projects. The first study shows that sufficient amount of keratins are needed to protect intestinal epithelial cells from stress and decreased keratin levels lead to altered ion transport, increased epithelial proliferation and higher susceptibility for experimental colitis. The second study has clearly identified the reduced amount of a main chloride transporter, DRA as the main reason for the intestinal ion transport phenotypes in keratin 8 heterozygote and keratin 8 deficient mice. The third study demonstrates the successful development of a novel *in vivo* imaging method for the early detection of colitis in mice using the luminal chemiluminescent probe, L-012, which detects reactive oxygen and nitrogen species. The outcome of this thesis work may also be helpful towards future development of diagnostics and treatment of colitis.

M. Nadeem Asghar graduated from University of Punjab, Lahore in the field of Medical Laboratory Technology in 2001. He received his M.Sc. degree in Biochemistry from Arid Agriculture University Rawalpindi in 2004. He also specialized in Vaccine Quality Control Technology from BIKEN, Osaka University, Japan in 2006. Furthermore, he obtained his M.Phil./Lic. degree in Biochemistry/Molecular Biology from Quaid-i-Azam University Islamabad in 2007. He has worked at National Institute of Health, Islamabad from 2002-2009. He joined the Epithelial Biology research group at Department of Biosciences, Åbo Akademi University in November 2009 to pursue his doctorate degree. He has also been teaching at College of Medical Technology, NIH, Islamabad and Department of Biosciences, Åbo Akademi University. His research work has been highly appreciated enabling him to win 4 best poster presentation awards in international conferences at USA and Europe.

Åbo Akademi University

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