Colonic mucus structure and processing

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ABSTRACT

The mucus layer covering the colonic epithelium creates a crucial first line of defense against the gut residing bacteria. Several lines of evidence suggest that a functional mucus layer is essential for health. For example, it is. suggested that ulcerative colitis is correlated with mucus layer defects. The barrier properties of colonic mucus are partly achieved by creating a dense gel with the MUC2 gel-forming mucin as scaffold. Available MUC2 biochemical and histological data suggest that the mucus is highly structured and organized. Mucus homeostasis is dependent on production, secretion and processing of mucus components. Thus, factors such as goblet cell differentiation, secretory capacity of different cells, and the presence of mucus degrading proteases can affect mucus properties. However, a detailed understanding of mucus structure and processing *in vivo* is lacking.

We have now further developed an existing *ex vivo* system to study the mucus structure at the microscopic level, as well as investigate the involvement of subpopulations of goblet cells in mucus secretion. This *ex vivo* method was also used for studies of mucus proteolytic processing by CLCA1, an abundant protease within the mucus. The results suggest that the colonic mucus gel is heterogeneous due to the presence of different goblet cell populations that secrete mucus with different properties. Furthermore, proteolytic processing of MUC2 by CLCA1 is involved in baseline mucus dynamics.

Increased understanding of mucus structure and processing is important for future development of pharmacological interventions to improve barrier function in ulcerative colitis and prevent mucus stagnation in diseases such as asthma, chronic obstructive lung disease and cystic fibrosis.

Keywords; lectin, CLCA1, SPDEF**,** MUC2, colitis, mucus structure, mucus dynamics, mucus homeostasis

SAMMANFATTNING PÅ SVENSKA

Goblet celler är specialiserade celler i tjocktarmens slemhinna som producerar och utsöndrar proteiner som bygger upp ett gellager, även kallat mukus, närmast slemhinnans ytceller (epitelet). Mukuslagret bildar en skyddande barriär mellan epitelet och de triljoner bakererier som lever i tjocktarmen. Defekter i barriären är kopplat till olika sjukdomar, till exempel inflammatorisk tarmsjukdom. Att bättre förstå vilka faktorer som har betydelse för mukusets egenskaper kan bidra till bättre kunskap om dessa sjukdomar och i förlängningen leda till nya behandlingsmetoder.

MUC2 är det protein som bildar mukusets stomme, och det är visat att MUC2-molekylerna är välorganiserade i mukuset genom specifika interaktioner mellan olika MUC2-molekyler. Däremot vet man mindre om funktionen av andra proteiner som finns i mukuset, t.ex. CLCA1.

I denna avhandling har vi använt en metod som låter oss studera hur mukuset ser ut och beter sig i levande vävnad genom att dissekera ut slemhinnan med mukus och studera den under mikroskop. Eftersom mukuset är transparant har man tidigare förlitat sig på små partiklar som man lägger ovanpå mukuset för att dra slutsatser om mukuskvalitén. Här har vi dock kunnat visa att man genom att använda fluoroscerande molekyler som binder till mukuset kan titta på själva mukusstrukturen. På så sätt har vi kunnat visa att tjocktarmsmukus består av två sammankopplade strukturer; plymer av väldigt tätt mukus som täcker och skyddar känsliga öppningar (krypor) i epitelet, och ”mellankryptsmukus” som länkar samman plymerna. Vi har även kunnat visa att de två olika typerna av mukus utsöndras av olika, tidigare obeskrivna, subpopulationer av goblet celler. Genetiskt modifierade möss som saknar mellankrypsmukus utvecklar inflammation i tjocktarmen och det verkar därför som att mellankryptsmukus är viktigt för mukuslagrets skyddande funktion.

Vidare har vi kunnat visa att CLCA1 i mukus fungerar som ett enzym som kan klyva MUC2 och därmed ändra strukturen på mukuset. Denna process pågår till viss del konstant, men våra resultat tyder även på att det är en noggrant reglerad process. Upptäckten att CLCA1 kan påverka mukuset kan ha stor betydelse för förståelsen av astma och kronisk obstruktiv lungsjukdom (KOL), eftersom uttrycket av CLCA1 korrelerar med dessa.

Sammntaget har vi fört fårståelsen om hur mukuset är uppbyggt och hur det processas framåt, vilket vi hoppas ska stimulera fortsatt forskning på hur mukuset ändras vid sjukdom. Framtida resultat kan därför komma att leda till bättre behandling av sjukdomar som involverar förändringar i mukuset.

LIST OF PAPERS

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

I. Erickson NA *, Nyström EEL *, Mundhenk L, Arike L, Glauben R, Heimestaat MM, Fischer A, Bereswill S, Bircheough GMH, Grüber AD, Johansson MEV
The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice.
PLOS ONE. **2015**; 10(7):e0131991
* Equal contribution

II. Nyström EEL, Birchenough GMH, van der Post S, Arike L, Gruber AD, Hansson GC, Johansson MEV
Calcium-activated Chloride Channel Regulator 1 (CLCA1) Controls Mucus Expansion in Colon by Proteolytic Activity.
EBioMedicine, **2018**, 33:134-143

III. Nyström EEL, Arike L, Recktenwald CV, Hansson GC, Johansson MEV
CLCA1 forms non-covalent oligomers in colonic mucus and has MUC2-processing properties
Manuscript

IV. Nyström EEL, Martinez Abad B, Eklund L, Birchenough GMH, Johansson MEV
Mucus secreted from intercrypt goblet cells is required for proper mucus layer formation in the distal colon and protection against colitis
Manuscript

CONTENT

ABBREVIATIONS

NB: Protein names in capital letters refers to human proteins, whereas protein names in lower case refers to mouse. Gene names are written in italics.

AB-PAS	Alcian blue - Periodic acid-Shiff
ADAM	'A disintegrin and metalloproteinase'
Agr2	Anterio gradient 2
SDS-(UAg)PAGE	Sodium dodecyl sulfate (Urea Agarose) polyacrylamide composite gel electrophoresis
apoMUC	Mucin apoprotein, protein precursor
Arhgap17	Rho GTPase-activating protein 17
Atoh1	Protein atonal homolog 1
BSR	Beta sheet rich
CaCC	Calcium-activated chloride channel
CAT	Catalytic metalloprotease domain of CLCA1
CCh	Carbachol
CD45	Leukocyte common antigen
CF	Cystic fibrosis
Cftr	Cystic fibrosis transmembrane conductance regulator
CK	Cysteine knot
Clca	Calcium-activated chloride channel regulator
COPD	Chronic obstructive pulmonary disorder
Cys	Cysteine rich domain of CLCA1
D1-4	Von Willebrand domain assembly 1-4
DAI	Disease activity index
DIDS	Disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate
DSS	Dextran sodium sulfate
E-domain	Fibronectin type 1-like domain
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
Ern2	Endoplasmic reticulum to nucleus signaling 2
Fcgbp	Fc fragment of IgG binding protein
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
Foxa1/2	Forkhead box A 1/2
Gal	Galactose
GAP	Goblet cell associated passage
Gata4 /6	GATA motif binding proteins 4/6

GF	Germ free
Gfi1	Zinc finger protein Gfi-1
GI	Gastrointestinal
GlcNAc	N-Acetylglucosamine
Hes1	Hairy and enhancer of split-1
IBD	Inflammatory bowel disease
I_{CaCC}	Calcium-activated chloride channel current
icGC	Intercrypt goblet cell
IHC	Immunohistochemistry
IL	Interleukin
IM	Inner mucus
kDa	Kilo Dalton
Klf4/5	Krueppel-like factor 4/5
Klk1	Kallikrein 1
LEL	Lycopersicon esculentium lectin
LPS	Lipopolysaccharide
LTL	Lotus tetragonolobus lectin
MAA	Mackie Amurensis agglutinin
MAMP	Microbial associated molecular patterns
MDA	Mega Dalton
MIDAS	Metal ion dependent adhesion site
MMP	Matrix metalloprotease
MS	Mass specrometry
Muc	Mucin
Munc	Mammalian uncoordinated
Na^+	Sodium ion
NFA	Niflumic acid
Nlrp6	NACHT, LRR and PYD domains-containing protein 6
Notch	Neurogenic locus notch homolog protein
OM	Outer mucus
OVA	Ovalbumin
PAS	Periodic acid-Shiff
PGE_2	Prostaglandin E2
PNA	Peanut agglutinin
PPI	Protein-protein interaction
PTS	Proline, threonine and serine rich
rCLCA	Recombinant CLCA1
RgpB	Arg-gingipain B
senGC	Sentinel goblet cell
SNA	Sambuccus nigra agglutinin
SNARE	Soluble NSF(N-ethylmaleimide-sensitive factor) Attachment protein receptor
Spdef	SAM pointed domain containing ETS transcription factor

T_H	T-helper
TIL-domain	Trypsin inhibitor-like domain
Tmem16A	Transmembrane member 16A
TMPP	N-Succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide
TNBS	2,4,6-Trinitrobenzenesulfonic acid solution
TPEN	N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
UC	Ulcerative colitis
UEA	Ulex europaeus agglutinin
VAMP	Vesicle-associated membrane protein
WGA	Wheat germ agglutinin
VIP	Vasoactive intestinal peptide
Wnt	Wingless/int-1
WT	Wild type
VWA	von Willebrand factor type A domain
VWD	von Willebrand D domain
VWF	von Willebrand factor
Zg16	Zymogen granule protein 16

1 INTRODUCTION

1.1 Intestinal mucus

The number of cells in the human body is equaled by the bacterial cells residing in our gut. These bacteria are beneficial to the host, e.g. by providing essential nutrients such as vitamins, and by degrading complex fibers into host accessible derivatives [1]. However, pathogenic, as well as commensal, bacteria cause harm if they are in direct contact with the epithelium [2,3]. The intestinal tract is thus covered by a gel-like mucus layer that serves as protection of the underlying epithelium by providing a barrier between the host tissue and the microbiota as well as lubricating the tissue.

1.1.1 Mucus properties along the gastrointestinal tract

The mucus layer is continuous throughout the gastrointestinal (GI) tract but has varying properties dependent on location (Figure 1A). For example MUC5AC is the main gel-forming mucin in the stomach, whereas MUC2 dominates in the small and the large intestine [4]. Furthermore, mucus in the small intestine is non-attached to the epithelium and loosely structured. This allows for efficient nutrient uptake while providing a diffusion barrier for anti-microbial peptides which protect the epithelium [5,6]. In contrast, the distal colon has a two-layered ("inner" and "outer") mucus structure [3,5–8]. The dense structure of the attached inner mucus layer has been investigated by size exclusion of μm-sized beads and has been found to exclude beads with the size of bacteria [7]. This is reflected in the ability of the inner mucus layer to separate the bacteria in the fecal content from the epithelium, thus forming a physical barrier between the microbiota and the epithelium in the gut (Figure 1B) [3].

The inner mucus layer is continuously converted to an outer mucus layer. The outer mucus layer is not attached, and has a looser structure due to digestion and expansion mediated by endogenous proteases (discussed further in 1.1.3.3) [3]. In contrast to the mostly sterile inner mucus layer, the outer mucus layer creates a habitat for the microbiota by providing a nutrient source and sites for adhesion.

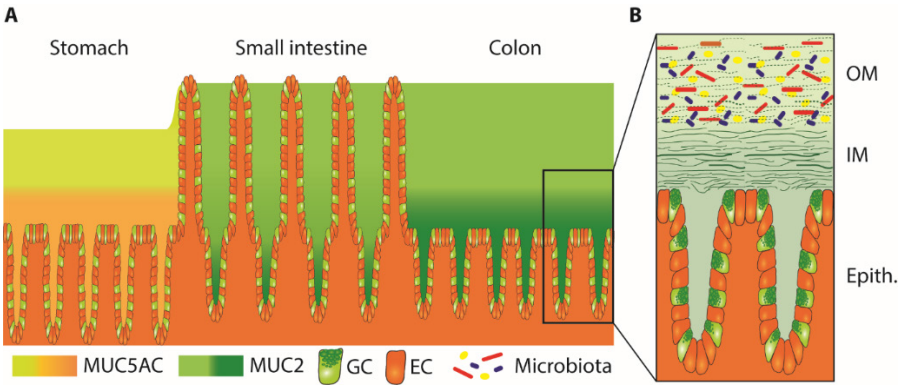

Figure 1: A) Schematic representation of mucus along the gastrointestinal tract. Stomach and colonic epithelia are covered by a two-layered mucus with an attached inner layer (dark color), and a non-attached outer (light color) mucus with MUC5AC (yellow/orange) and MUC2 (green) as main mucin component in the stomach and colon respectively. Villi in the small intestine are covered by a loose, non-adherent mucus with MUC2 as main mucin. B) Schematic representation of the mucus layer organization in colon, with the stratified inner mucus (IM) layer and outer mucus (OM) layer. Microbiota is restricted to the outer mucus layer. Epith. = epithelium, GC = goblet cell, EC = enterocytes

1.1.2 Mucus composition

Proteome analysis of intestinal mucus has identified a core set of proteins that are present in the intestinal mucus along the GI tract, both in human and mouse [3,4] (van der Post *et al.*, submission). The main structural component, the MUC2 mucin, is accompanied by calcium-activated chloride channel regulator 1 (CLCA1) (further introduced in 1.3), Fc-gamma protein binding protein (Fcgbp), zymogen granule protein 16 (Zg16), and kallikrein 1 (Klk1). Zg16 was recently found to bind and aggregate gram-positive bacteria, thus keeping them away from the epithelium [9], but the function of most other proteins found in mucus is still largely unknown.

Mucus also contains material from shed cells. Thus, proteins and DNA normally found intracellularly are found in mucus [4,10]. These compounds might play an active role in the mucus after secretion, and alter the mucus properties [10,11]. However, very little is known about the activity and functional importance of these compounds in the secreted mucus.

1. 1.2.1 Biochemical properties of MUC2

As mentioned, MUC2 is the main structural component of intestinal mucus and provides a scaffolding backbone. MUC2 is a large, > 5000 amino acids, gel-forming mucin. The domain structure in MUC2 (as well as other gel-forming mucins) resembles that of the von Willebrand factor (VWF) [12,13] and are arranged in the following order; von Willebrand D1 assembly (D1), D2, D′D3, a first cysteine domain (CysD), small proline, threonine and serine rich (PTS) domain, second CysD, large PTS domain, C-terminal D4 followed by von Willebrand C assemblies, and a cysteine-knot domain (CK) (Paper III, Figure 3D). The von Willebrand D assemblies can be further subdivided into von Willebrand D-domain (vWD), C8-module, trypsin inhibitor-like (TIL)-domain, and a fibronectin type 1-like (E)-domain, except the D´ which only contains a TIL- and E-domain [14].

The PTS domains, also called mucin domains, are hallmark features of mucins. These domains are heavily *O*-glycosylated in the Golgi which gives the protein a stretched, brush-like arrangement. After secretion, the glycans become hydrated which gives the mucus its gel-like properties and provides lubrication [15]. The attached glycans protect the MUC2 backbone from proteolytic degradation by endogenous or bacterial enzymes [16–18]. However, they also serve as adhesion sites for several bacteria and can thus provide a way for the host to select commensal members from the microbial community [19,20], but can also be exploited by pathogens. Moreover, the MUC2 glycoprotein can serve as a nutrient source for bacteria when their preferred metabolic substrates are lacking, which grants them a niche benefit and might lead to dysbiosis [21,22].

The *O*-glycan pattern is specific for the different GI regions, as well as the host species. For example, human small intestinal mucus is highly fucosylated, whereas small intestinal mucus is only sparsely fucosylated in mice [23,24]. Furthermore, core-3 glycan structures are abundant in human colonic mucus whereas mouse colonic mucus mainly contains core-1 and core-2 structures. Core-4 structures can be found in colonic mucus from both species. However, colonic mucus *O*-glycans from both humans and mice are commonly terminated by fucose, sialic acid and sulphate.

The attachment of *O*-glycans increases the molecular mass of MUC2 by approximately 500%, and the resulting MUC2 monomer thus has a molecular mass of around 2.5 MDa [17]. Additionally, MUC2 undergoes C-terminal

dimerization in the endoplasmic reticulum, and N-terminal trimerization of the D3-assembly in the secretory pathway, generating huge MUC2 oligomers [25–27] (Figure 2). Decreased pH and increased calcium concentration in the secretory pathway aids dense packing of the MUC2 oligomers in secretory vesicles. The D1-D2 assemblies are suggested to facilitate packing by providing non-covalent interaction between the D3-trimers, similar to the function of D1-D2 to stabilize the organized intracellular structure of VWF in Weibel-Palade bodies [28,29].

MUC2 oligomers unfold and expand into huge netlike sheets upon secretion. This process is suggested to be mediated by bicarbonate by precipitating calcium and increasing the pH [28,30,31] (Figure 2). It's further thought that these sheets are continuously pushed towards the intestinal lumen by newly secreted MUC2 sheets, which thus stacks the nets on top of each other [32]. Immunohistochemical (IHC) examination of MUC2 in colonic cross sections from both mouse and human shows a stratified appearance in the inner mucus layer which has supported this notion [3,8]. Formation of non-reducible bonds between separate MUC2 molecules, mediated by transglutaminases, is suggested to keep the different layers of MUC2 sheets associated [11]. The MUC2 three-dimensional mesh thus acts as a sieve which can exclude bacteria.

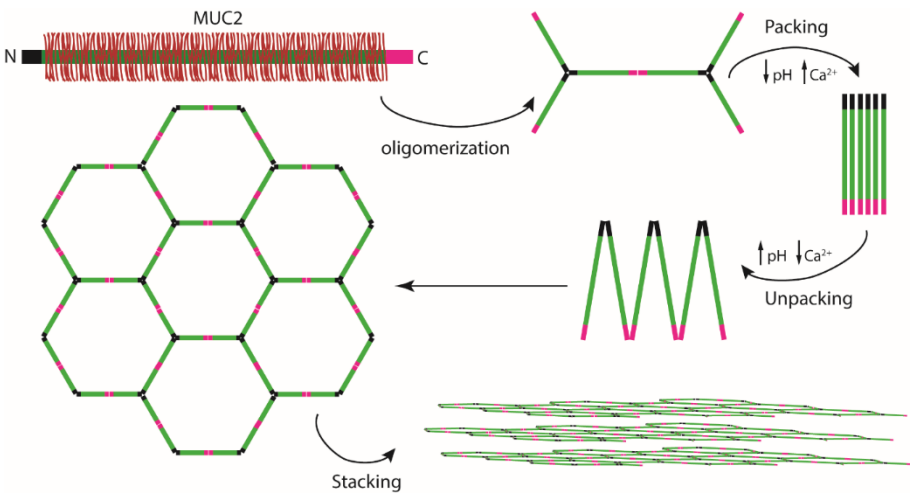

Figure 2: Schematic picture of MUC2 oligomerization by C-terminal dimerization and N-terminal trimerization, intracellular packing, extracellular unpacking and stacking into MUC2 sheets in the IM.

1.1.3 Mucus dynamics

Mucus homeostasis is maintained by a dynamic balance between production, secretion and proteolysis of mucus components (Figure 3). Mucus homeostasis is also dependent on the ionic milieu it is secreted into, as evident in the small intestine of mice that lack a functional CFTR ion channel where mucus remains attached to the epithelium due to lack of bicarbonate secretion [3,13,28,31].

1.1.3.1 MUC2 production

Colonic MUC2 is constitutively expressed in order to constantly renew the mucus layer [32–34]. Production of MUC2 can be enhanced by several stimuli including bacterial components, T_H1-, and T_H2- mediated cytokines, acute phase responses, and viral infection [35–45]. Considering the high rate of MUC2 biosynthesis, especially in surface epithelial goblet cells, altered MUC2 production is likely to affect mucus dynamics within a few hours [32,34].

1.1.3.2 Mucus secretion

The densely packed MUC2 oligomers are stored in secretory vesicles in the goblet cell theca. The content of these vesicles is released either by regulated vesicle secretion, sometimes referred to as baseline secretion, or by stimulated compound exocytosis [46]. Baseline secretion has mainly been studied in airways, and is shown to be dependent on SNARE proteins which are typically involved in vesicle exocytosis [47]. Several VAMP and SNARE-proteins are found in the mucin granules in cultured cells of colonic origin suggesting that similar functions are important also in the intestine [48]. Additionally, mice lacking Munc13-2 or VAMP8, which are involved in baseline exocytosis, have goblet cell mucus accumulation in the intestine and impaired secretion [49,50].

In compound exocytosis, storage vesicles rapidly fuse with each other and the cell membrane, which enhances the secretion as the whole theca contents is secreted from the cell [51]. It is triggered by different stimuli via intracellular calcium signaling. In goblet cells this has been observed after stimulation with the cholinergic agonist carbachol (CCh), which induces almost complete emptying of goblet cells in the intestinal crypts [7,51–53]. The mast cell product histamine, the neuropeptide VIP, and the immune regulator PGE_2 have similar effects, however the goblet cell response varies along the GI tract and also between species as reviewed in Birchenough et al., 2015 [46]. Furthermore, it

was recently described how goblet cells at the crypt opening can endocytose bacterial molecules and, if at high enough concentration, mount a secretory response involving compound exocytosis to clear the crypt opening from intruding bacteria [54].

Pulsed *in vivo* labelling of glycoprotein in mouse colonic tissue has shown that goblet cells throughout the crypt continuously renew their stored material over the time course of a couple of hours to a couple of days and thus continuously secrete and produce MUC2 [32]. However, the rate of production and secretion differs along the crypt axis; Goblet cells at the luminal surface of the epithelium had a markedly higher turnover of their mucin content compared to goblet cells residing in the crypts. In the luminal cells newly produced material was secreted as soon as 3 hours post labelling, and most of the pulse-labelled material was secreted within 7 hours. In contrast, crypt residing cells had both slower production and renewal of their content, and pulse-labelled material was still observed 24 hours post injection. Similar differences in MUC2 turnover were also found in small intestine [34]. However, determinants of mucus turnover rate in different cells are still unknown.

1.1.3.3 Mucus expansion by unfolding and proteolytic processing of MUC2

MUC2 molecules are thought to expand >1000 times upon secretion as the calcium concentration drops and pH increases [28]. The environmental changes induce conformational alterations in MUC2 D1-D2 which breaks the interaction between D1-D2 and D'-D3, allowing the unfolding. The removal of calcium also allows the electrorepulsive forces between glycosylated PTS-domains to drive unfolding. Despite the fact that bicarbonate is secreted from the epithelium, a pH gradient has been noted in rat colonic mucus where the pH increased towards the luminal center [55]. It is thus possible that the unfolding of the densely packed MUC2 molecules is a gradual process. However, the unfolding/expansion of MUC2 has not been investigated in great detail, although the homogenous stratified appearance of the inner mucus layer in IHC indicates that the unfolding is rapid and results in an inner mucus layer that has a homogenous MUC2 organization [3].

The conversion from the attached, dense, inner mucus layer to the unattached, looser, outer mucus layer is at least partly dependent of proteolytic activity in the mucus. This has been inferred from the finding that inhibition of cysteine- and serine proteases decreases the formation of an outer mucus layer both in

mouse and in rat samples, seen as reduced mucus growth *ex vivo* [3]. As germ-free (GF) mice seem to have a similar mucus organization to conventionally raised mice in terms of an inner and outer mucus layer, the responsible proteases are endogenous to the host. The outer mucus layer is penetrable of bacteria sized beads indicating that the outer mucus layer has larger pore size than the inner mucus layer [7]. The proteolytic cleavage thus alters the MUC2 network structure, but without completely breaking it, and is suggested to occur in the terminal domains. It has further been shown that trypsin treatment of the guanidinium chloride insoluble fraction of Muc2 expands but does not dissolve the structure, supporting the notion that proteolysis of MUC2 can lead to volume expansion without complete disruption [3].

We most often consider mucus homeostasis and dynamics in terms of MUC2; however, the mucus and epithelium also contains an array of other components that can possibly greatly influence all the processes described above. These include Agr2 and Ern2, which are important for the production and secretion of Muc2, ion channels for correct ionic milieu in the lumen, and other suggested structural components such as Fcgbp. However, the available functional information on other mucus components are thus far limited [30,31,56–58].

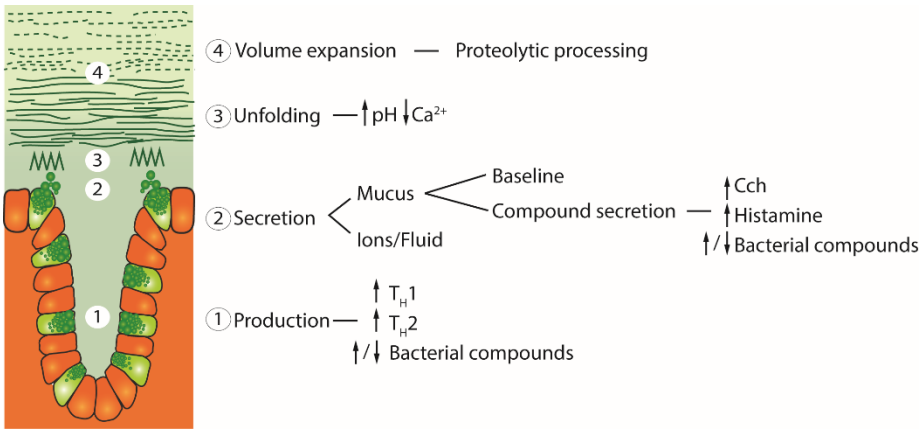

Figure 3: Schematic drawing of the different levels of mucus dynamic control.

1.1.4 Physiological relevance and clinical importance

Although the lubricating properties of GI mucus has long been appreciated, its true involvement in health and symbiosis with our GI-residing microbiota has only recently been explored. Mucus-microbiota symbiosis, or dysbiosis, has received a great deal of interest in relation to the growing problem with inflammatory bowel disease (IBD) e.g. ulcerative colitis (UC), and has also been suggested to be involved in metabolic syndrome [22,59–62].

1. 1.4.1 Colitis

Inflammatory bowel disease can be broadly divided into Crohn's disease and UC. There are several reports correlating mucus defects with UC or animal colitis models [30,63–68]. Furthermore, UC patients have been shown to have abnormal contact between bacteria and the epithelium [64,69], and it is thus reasonable to speculate that the aforementioned mucus defects allow bacterial penetration into the mucus layer. This might drive increased or abnormal immune response, and "wear out" mucus production if the infection is prolonged, and thereby cause a negative feedback loop [70].

The colonic mucus layer is thinner in patients with active UC compared to healthy controls [64,71]. More importantly, colonic mucus from a majority of the patients with active UC was found to be penetrable to bacteria-sized beads *ex vivo,* in contrast to healthy controls which had impenetrable mucus [64]. Interestingly, most patients in remission had impenetrable mucus, but a few were similar to the active UC group. This indicated that the mucus dysfunction recovers during remission. Whether or not mucus dysfunction preceded colitis in humans could not be determined, but in animal models of colitis a correlation between bacteria penetrating the inner mucus layer and inflammation was observed by fluorescent *in situ* hybridization. It has also been shown that bacteria penetrate the inner mucus layer before onset of colitis in DSS-induced colitis suggesting that bacteria dislocation can be a cause of inflammation (discussed further below) [2,64].

Animal models which develop spontaneous colitis that is potentially linked to mucus defects include those with defective ion channel function [64], glycosylation alterations [64,67,68,72], innate immune signaling [64] and ER-stress and protein processing [56,65,73], all of which are implicated in UC pathogenesis [23,74–77]. This suggests that there are multiple possible causes of mucus defects

in UC, but that the result is similar. It also underscores the importance of a wide range of factors in maintaining mucus homeostasis.

Dextran sodium sulphate (DSS)-induced colitis is the most commonly used model to study UC [78]. DSS is a cytotoxic compound that is thought to act by disrupting the intestinal barriers and thereby increase bacterial load at the normally sterile tissue and thus drive inflammation. The precise mechanism of action is however unclear.

Petersson et. al. found that the *in vivo* mucus thickness progressively decreased during DSS-treatment, and that the mucus thickness correlated with the disease activity index (DAI) [79]. The mucus thickness decrease might be an direct effect of DSS as it has been found that direct application of 3% DSS on colonic explants *ex vivo* acutely reduced inner mucus layer thickness [2]. In addition, it has also been found that DSS-treatment increased mucus penetrability to bacteria sized beads within 15 minutes after direct application *ex vivo,* and that bacteria could be detected within the inner mucus layer 12 hours after DSS-treatment *in vivo*. Similarly, increased bacterial contact with the epithelium was observed in Arhgap17-deficient mice, compared to WT controls, after 2 days of DSS administration which correlated with increased DAI scores [80]. Exactly how DSS alter the mucus structure is unknown, but dynamic light scattering microrheology revealed alterations in the polymer properties of intestinal mucus after DSS treatment [81]. In both aforementioned cases, increased bacterial mucus penetration preceded observable colitis, suggesting that a defect mucus barrier and consequent bacteria penetration to the tissue might be one of the factors driving inflammation.

1.1.4.2 Bacterial infection

The importance of the intestinal mucus in maintaining a symbiotic relationship with the gut-residing microbiota is evident from *Muc2*$^{-/-}$ mice. These mice lack a functional mucus layer and bacteria are thus not restricted from the epithelium, which leads to development of spontaneous colitis as early as the weaning period [3,63,82]. The mucus layer is also important for protection against pathogenic bacteria as *Muc2*$^{-/-}$ mice are more severely affected than WT mice by infection with *Citrobacter rodentium* [83]. Mice fed a low-fiber diet were also shown to be more susceptible to *C. rodentium* infection [21]. In light of the recent findings by Schroeder et al, showing that a high-fat/low-fiber diet caused structural changes in the mucus barrier which allowed penetration of bacteria-

sized beads, this increase in susceptibility was probably due to decreased mucus barrier function, yet again highlighting the importance of a functional, protective mucus barrier [22].

Although WT mice normally have a functional mucus barrier, they are also infected upon *C. rodentium* challenge, but to a lesser degree than *Muc2*$^{-/-}$ animals. Similar to other pathogens, *C. rodentium* has evolved means to penetrate and circumvent the inner mucus layer, possibly by producing a mucinolytic serine protease, and can thus gain access to the epithelium [33,83,84]. As a response, the host alters its mucus properties in order to clear the infection [85].

1.1.4.3 Cystic fibrosis

Cystic fibrosis (CF) is a severe disease which affects all mucosal tissues in the body [86,87]. The disease is caused by genetic mutations resulting in a malfunctioning Cystic fibrosis transmembrane conductance regulator (CFTR) protein, which normally function as a chloride and bicarbonate channel. It was long held that defective chloride secretion determined pathogenesis, but recent research indicates that the reduced level of bicarbonate is better correlated with the mucus defects observed in CF, as reviewed in Kunzelmann, Schreiber, and Hadorn, 2017 [87].

CF patients commonly suffer from both airway and intestinal symptoms. Intestinal symptoms includes distal intestinal obstruction syndrome which is characterized by obstruction caused by thick, stagnant mucus [88]. Gustafsson *et al.* showed that mucus stagnation and attachment to the small intestinal epithelium was caused by the lack of bicarbonate, and suggested that this was mediated by insufficient unfolding of the MUC2 oligomers after secretion [31]. Furthermore, increased bacterial load in CF small intestine has been observed, suggesting that stagnation of small intestinal mucus can contribute to bacterial overgrowth [89]. Taken together, CF serves as a clear example of how dysregulated mucus dynamics can cause an imbalance at mucosal surfaces that results in disease.

1.2 The intestinal epithelium

The very proximal parts of the GI tract, the mouth and esophagus, are lined by multiple layers of squamous cells, much like the skin. In contrast, the stomach and the intestines are lined by a single layer of epithelial cells that has to withstand the strain from ingested foods, bacteria, digestive enzymes and harsh acidic environment in the stomach. As discussed above, the mucus layer plays a crucial role in maintaining homeostasis, but the epithelium itself has also evolved specialized features to withstand the challenges. These include the tight junctions between epithelial cells which restrict paracellular passage, the presence of a glycocalyx consisting of transmembrane mucins, and extraordinarily fast turnover.

1.2.1 Epithelial cell differentiation

The turnover time of the intestinal epithelium ranges from 2-7 days [90] as the epithelium is continuously renewed by crypt-residing stem cells which can give rise to all epithelial cell types [91,92]. After initial linage determination, stem daughter cells move up the intestinal crypt into the transit-amplifying zone where they further divide and differentiate into their respective cell type. As the cells migrate up the crypt they continue to differentiate, to finally reach the crypt entrance (and villi in the small intestine) as fully differentiated, mature cells. Finally, the cells are shed from the tissue and undergo anoikis.

The epithelium harbors both absorptive enterocytes and several secretory cell types including goblet, Paneth, enteroendocrine, and Tuft cells [92]. The initial determination of absorptive vs secretory cells depend on the Wnt/Notch pathways [93]. Wnt signaling, via ß-catenin, is crucial for the proliferative properties of intestinal crypt cells, but also drives the initial differentiation into secretory cells via Atoh1. Notch pathway activation instead drives the expression of Hes1, which in turn inhibits Atoh1. By this action, Notch signaling inhibits secretory differentiation and instead drives differentiation into enterocytes.

1.2.1.1 Goblet cell differentiation

Intestinal goblet cells are further differentiated under the influence of several transcription factors including Gfi, Klf4, Gata4/6, Foxa1/Foxa2, Klf5 and Spdef [93]. Spdef acts downstream of Atoh1, Hes1, and Gfi1, and is suggested to

be involved in the terminal differentiation of intestinal, as well as airway, goblet cells [94–97].

In the airways, Spdef was shown to reversibly differentiate epithelial club cells into goblet cells upon stimulation, with such as IL-13, by driving the expression of several genes involved in goblet cell mucus production [96,97]. It was also found that Spdef did not affect proliferation and thus did not regulate the number of secretory cells. Furthermore, *Spdef*$^{-/-}$ animals were claimed to lack submucosal gland goblet cells in naïve conditions and failed to induce goblet cell differentiation from club cells when sensitized with OVA. However, these latter claims were based on the absence of Alcian blue staining, and a recent study showed that although Alcian blue reactivity is reduced in Spdef-deficient submucosal glands, PAS reactivity persists and Muc5b is still expressed at reduced levels [98]. It is thus possible that Spdef increases the production of mucins, but that other factors also contribute to the goblet cell differentiation.

Similarly, Gregorieff et. al. found that Spdef-deficient goblet cells in the intestine store less material, but are not reduced in number [94]. They also described morphological alterations of goblet cells in the intestine, including the presence of an intact brush border (an epithelial cell feature that is normally absent from goblet cells) and decreased theca size. However, this effect was not observed in all goblet cells although the authors did not elaborate further on this finding. In line with the decreased mucin expression upon Spdef-deficiency, induced overexpression of Spdef in transgenic mice was claimed to increase mucin expression both *in vitro* and *in vivo,* further substantiating that Spdef is involved in mucin expression and goblet cell maturation [95].

Induced intestinal overexpression of Spdef was also found to reduce crypt cell proliferation [95]. Subsequent studies have shown this to be mediated by protein-protein interaction between Spdef and ß-catenin, which prevent ß-catenin induced transcription of cell cycle genes [99,100]. However, altered cell proliferation has not been found in Spdef-deficient mice [94,101], or after secretory cell-specific overexpression of Spdef in the airways [96].

1.2.2 Goblet cell subpopulations

Intestinal goblet cells are generally considered a homogenous cell population, differing only in the degree of maturation as they move up the intestinal crypt.

However, previous studies have indicated the presence of distinct functional subpopulations of goblet cells. Firstly, researchers from Washington University showed that goblet cells in the small intestine can form goblet cell associated passages (GAPs) which deliver luminal material to dendritic cells in the lamina propria [102]. These are not present in (proximal) colon from adult mice in steady state conditions but can be induced by antibiotic treatment [103]. As only a subset of the intestinal goblet cells form GAPs, these might comprise their own subpopulation.

Similarly, a small population of goblet cells, referred to as sentinel goblet cells (senGCs), at the crypt entrances in the distal colon can endocytose and react to microbial associated molecular patterns (MAMPs) when these are present at high enough levels [54]. The reaction involves compound exocytosis of mucus from the endocytotic cell and their neighboring goblet cells. This massive secretion is thought to clear the crypt entrance from intruding bacteria, and thus protect the epithelium. The senGCs share endocytotic features with GAPs, however they are investigated in different tissues (distal colon vs small intestine and proximal colon), and differ in their response to cholinergic inhibition. Whether these are distinctive or similar subpopulations of goblet cells is thus hard to conclude with the present data.

Secondly, as mentioned in 1.1.3.2, goblet cells along the crypt axis differ in their mucus production and secretion rate [32]. Goblet cells at the luminal surface epithelium have a markedly higher turnover of their secretory cargo, and seem almost exclusively responsible for the renewal of the mucus layer in unstimulated tissue. Additionally, these cells do not seem to store any mature secretory vesicles and does thus not have the typical goblet shape.

Furthermore, goblet cells do not respond to secretagouges uniformly. As mentioned above, apically applied MAMPs only induce secretion from crypt entrance cells in mice, as does the tissue irritant mustard oil in rats [54,104]. The better studied secretory response to acetylcholine is also not uniform along the crypt, with higher responsiveness in the lower segments [7,52,54].

Lastly, several reports have found that intestinal goblet cells have distinct glycosylation patterns both in humans and rodents [105–108]. For example, up to seven distinct populations of goblet cells with different glycosylation profiles were found in human colonic crypts. However, the functional relevance of these differences is yet to be determined.

1.3 Calcium-activated chloride channel regulator 1 (CLCA1)

NB: The murine homolog of human CLCA1 was first named Gob-5, then Clca3, and has recently been renamed to Clca1. This report uses Clca1.

Proteomic studies of both human and mouse mucus have revealed abundant mucus components besides the gel-forming mucin MUC2, including CLCA1, FCGBP, ZG16 and AGR2, of which CLCA1 is one of the most abundant non-mucin proteins [3,4] (van der Post *et al.*, submitted). CLCA1 belongs to a family of CLCAs with four human homologs (CLCA1-4) and eight murine homologs (mClca1-2, 3a, b, c, 4a, b, c) [109]. Orthologues are also found in other species including sheep, pigs and horses and even more distantly related species such as *Xenopus tropicalis* [110]. CLCA1/Clca1 are the only homologs that are secreted, and the other family members are membrane bound proteins. However, parts of these proteins can in some cases be shed from the cell surface [111,112].

1.3.1 Tissue expression

CLCA1 is primarily expressed by goblet cells in the GI tract, including the stomach, small intestine and colon, with the highest expression in the latter [113–116]. This expression pattern follows that of MUC2 along the GI tract and CLCA1 is thus suggested to be a core mucus protein [4]. In addition, CLCA1 has been detected in other mucosal tissues such as uterus, testis, and kidney [113–115]. CLCA1 expression can also be detected in the airways but mainly in correlation with disease, as detailed in 1.3.4.1 [117–120].

1.3.2 Biochemical properties of CLCA1

The 914 amino acid primary translational product of *CLCA1* contain an N-terminal signal sequence that directs CLCA1 to the ER, from which it is sorted to secretory vesicles. Self-cleavage at a conserved site at position 695 or 696 in CLCA1 and Clca1 respectively results in an approximately 85 kDa N-terminal product and an approximately 40 kDa C-terminal product [113,116,117,121–123] (Paper III, Figure 1A). The cleavage takes place intracellularly after which both products are secreted, but *in vitro* studies on constructs with a mutated cleavage site have shown that cleavage is not required for secretion [122,123].

A catalytic zinc-dependent metalloprotease domain (CAT) in the N-terminal part of the protein is responsible for the autocatalytic self-cleavage and the cleavage can be abolished by alterations in the conserved HExxE active site [122–124]. The HExxE motif is commonly found in matrix metalloproteases (MMPs) and in 'a disintegrin and metalloproteinase' (ADAM) proteins which are known to be degraders of extracellular matrix [125]. Thus, a role for CLCA1 in remodeling extracellular mucus is easily conceivable. However, to date the only known substrate for CLCA1 is CLCA1 itself, but the physiological importance of CLCA1 self-cleavage is largely unknown [122–124]. It has been suggested that this cleavage might regulate further CLCA1 activity, as CLCA1 with abolished cleavage was unable to induce calcium-activated chloride currents ($I_{CaCC,}$ 1.3.3.1) [122]. In contrast, a protease null truncated N-terminal CLCA1 protein induced I_{CaCC}, indicating that the proteolytic activity is not necessary for ion channel regulation (discussed further in 1.3.3.1 and 4.3.2).

In addition to the metalloprotease domain, CLCAs also have a cysteine rich domain (Cys) and a highly conserved von Willebrand factor type A (VWA) domain located in the N-terminal part, as well as a predicted fibronectin type III (FnIII) domain in the C-terminus [117,122,124,126]. VWAs are normally involved in protein-protein interactions (PPI), often in multiprotein complexes. These interactions commonly involve divalent cations and the VWA in CLCA1 contains a conserved metal ion-dependent adhesion site (MIDAS) which seem to be important for mediating PPI in CLCA1 [126,127].

The region between the VWA domain and the self-cleavage site, predicted to be rich in β-sheets, is highly conserved between the different CLCAs but no function has been ascribed to this part of the protein [122].

1.3.3 Physiological function of CLCA1

Several functions of CLCA1 has been proposed. In addition to the functions discussed in 1.3.3.1-2, it has also been suggested to be involved in both immune regulation and cell differentiation [128–132]. However, strikingly few reports discuss the role of CLCA1 in the secreted intestinal mucus despite the fact that this is where it is most abundant.

1.3.3.1 Ion channel (regulator)

When discovered, CLCA1 was predicted to be a calcium-activated chloride channel based on early *in silico* transmembrane domain prediction and altered I_{CaCC} in cells overexpressing CLCA1 *in vitro* [116]. However, several subsequent studies have proven that CLCA1 lacks transmembrane domains and that CLCA1 expressing cells secrete the protein both *in vitro* and *in vivo*, which correlates with the abundance of CLCA1 in the intestinal mucus [113,117,121]. This has led to the conclusion that CLCA1 instead functions as an ion channel regulator [117,122,133,134]. Recently, it has been suggested that the ion channel regulated by CLCA1 is Transmembrane member 16A (TMEM16A), also known as Anoctamin-1 [127,134].

1.3.3.2 Mucus properties

Other reports, mainly based on disease states in the lung (see 1.3.4) suggest that CLCA1 is involved in altering mucus properties, secretion and/or expression of mucus proteins [113,117–119,123]. The location of CLCA1 in the mucin granule has led to speculation that it might be involved in the synthesis, packing or secretion of mucins [113]. These hypotheses have however not been tested.

1.3.4 Implication in disease

1.3.4.1 CLCA1 in asthma and COPD

As previously mentioned, CLCA1 has generated interest due to its increased expression in the airways in disease states that involve altered mucus properties and/or expression of mucus proteins, such as asthma and chronic obstructive pulmonary disease (COPD) [118–120,135–137]. Asthma is an immunological disease that is closely associated with T_H2-type cytokines. It has been shown that the T_H2 cytokines IL-9 and IL-13 can affect the expression of CLCA1, thus coupling CLCA1 to the asthma phenotype [135–138]. In turn MUC5AC expression is thought to be induced by the increased levels of CLCA1 [118–120,135,139]. However, it has not been possible to verify these findings in Clca1-deficient mice, which develop asthma and mucin overproduction to the same extent as their WT controls [140–142].

A recent study found Clca1 to be one of the most upregulated proteins in an elastase induced model of COPD [143]. The model also induced formation of an attached stratified mucus layer in the airways, resembling the colonic inner mucus layer. The authors suggest this was a physiological response to increased bacteria burden on the airway to limit contact between the epithelium and bacteria. It is thus possible that Clca1 is induced to transform the mucus organization into a colon mucus-like stratified mucus by yet undefined mechanisms, or to release the mucus as a mechanism to limit mucus accumulation.

1.3.4.2 CLCA1 in cystic fibrosis

CF is another disease that involves altered mucus properties, both in lung and intestine. As previously discussed in 1.1.4.3, it is caused by mutations in the *CFTR*-gene resulting in impaired chloride- and bicarbonate transport in epithelial tissues, which results in altered mucus properties. However, residual chloride transport by other ion channels can be observed in CF tissues, and can ameliorate the disease [144,145]. The hunt for the responsible ion channel led to the discovery of both CLCA1 and TMEM16A, of which TMEM16A appears to be the true CaCC [146–148].

CLCA1 was identified as a modifier of the gastrointestinal phenotype of CF, and a genetic variation of CLCA1 is associated with the development of meconium ileus [149,150]. Furthermore, both lung and intestinal expression of CLCA1 have been shown to be altered in mouse models of CF [151–153], although the nature of the alteration seems to depend on genetic background, and thus must involve interactions with other factors. As CF mice do not have any lung phenotype, a role for Clca1 in the CF lung is not possible to observe in mice. However, increased knowledge of CLCA1 function in healthy colon might shed light on the involvement of CLCA1 in CF.

As discussed in this introduction, the mucus barrier function has a tremendous impact on health, as evident from diseases such as UC, asthma, COPD and CF. However, research on mucus has been limited and we thus lack a detailed understanding of factors affecting mucus barrier properties. Investigation of colonic mucus structure and processing will provide new insight that we hope will ultimately lead to the development of new pharmacological therapies.

2 AIM

The aim of this thesis was to study colonic mucus in regards of its molecular and microscopic structure and processing. This was achieved by testing the following hypotheses:

- Clca1-deficiency results in altered mucus dynamics in regards to mucus attachment or mucus growth, and defective mucus barrier function (Paper I).

- CLCA1 acts as a metalloprotease in intestinal mucus, and the effect of CLCA1 can be blocked by application of metalloprotease inhibitors (Paper II).

- MUC2 is a substrate for CLCA1 metalloprotease activity (Paper III).

- CLCA1 undergoes molecular processing after secretion into the mucus (Paper III).

- Colonic mucus structure can be investigated by *ex vivo* labelling of the mucus using fluorescently labeled lectins (Paper IV).

- Spdef-deficient goblet cells have altered morphology and deficient mucus secretory function, which will result in defective mucus barrier function (Paper IV).

3 METHODOLOGY

3.1 *Ex vivo* investigation of mucus (Paper I, II, IV)

The *ex vivo* method of measuring mucus growth, or mucus penetrability to bacteria-sized beads, used extensively in this work originated from pioneering *in vivo* mucus characterization in anaesthetized rodents [6]. However, the need for a method which required less technical skill, and which allows for easy pharmacological intervention in e.g. human tissue prompted the development of an *ex vivo* method. The method is discussed in detail in Gustafsson *et al.* 2012 [7], but in brief the intestinal segment of interest, either dissected from a sacrificed animal or obtained by colonoscopy from human patients, is mounted in a horizontal Ussing-chamber like system with apical and basolateral physiological buffers (Figure 4). The mucus surface is visualized by application of either charcoal particles or carboxylated beads that sediment on the mucus surface. The mucus thickness can be investigated by measuring the distance between the epithelium and the overlaid particles, either under a stereo microscope with the aid of a micropipette, or using confocal microscopy.

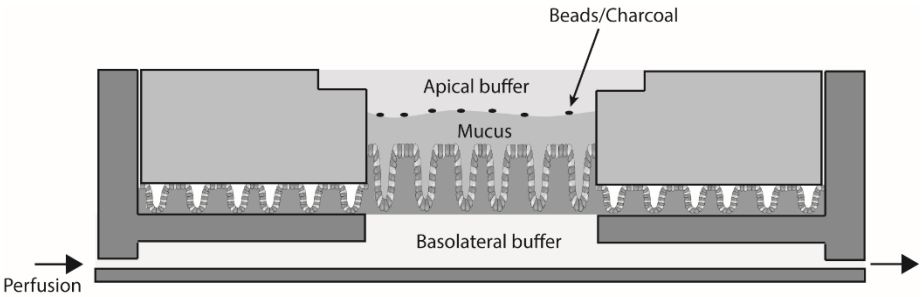

Figure 4: Schematic diagram of the horizontal Ussing-chamber like system for *ex vivo* mucus characterization. Adapted from [7].

In vivo measurements have shown that the colonic mucus is composed of a firmly adherent inner mucus layer and an outer, loosely adherent mucus layer [3,6], and the *ex vivo* method replicates these findings [7]. The *ex vivo* chamber system has been successfully used to assess changes in mucus properties after

in vivo interventions [9,22,64,85,154], and rapid responses to *ex vivo* pharmacological interventions, including ion channel or enzyme inhibitors, or recombinant proteins [2,9,13,31,54] and is thus becoming an established method to study intestinal mucus properties.

3.1.1. Measurement of mucus dynamics (Paper I and II)

Here we have measured mucus thickness over time to investigate mucus growth. Measurements at t = 0 minutes give information regarding the adherent properties of the mucus layer (i.e. remaining mucus layer thickness after tissue flushing), and subsequent growth reveal information concerning mucus dynamics. Mucus dynamics includes production, secretion, unfolding of secreted mucus, and expansion of the mucus structure as discussed in 1.1.3, but it has proven hard to determine the contributing effect of each individual factor to baseline growth, as the processes seem to be intricately interwoven. For example, as we show in Paper II, application of enzyme inhibitors can abolish mucus growth indicating that enzymatically mediated expansion of the mucus structure is a main determinant of mucus growth. However, application of ion channel inhibitors can similarly block mucus growth (discussed further in 3.1.1.1), suggesting that correct ionic milieu is equally important. By combining *ex vivo* mucus measurements with other methods we are now gaining a better understanding of the underlying mechanisms that determine mucus dynamics at steady state.

3.1.1.1 Ex vivo *application of inhibitors and recombinant enzymes (Paper II)*

A major advantage of the *ex vivo* system is the possibility to investigate direct effects of applied inhibitors and recombinant proteins. In Paper II we used this approach to investigate the effect of CLCA1 in colonic mucus. The concentration of applied substances was based on published literature when available. If no literature was available, or if no effect could be detected at the first attempted concentration, the highest concentration without negative effects on the tissue was used. In some cases, the solubility of the substance determined the concentration that was used.

In several graphs we present data as Δ growth rate. This was to simplify the interpretation of recombinant CLCA1 (rCLCA1)-mediated effects as many of the compounds tested in concert with rCLCA1 had an effect on the baseline mucus growth. An example is shown in Figure 5; The CaCC/TMEM16A

inhibitor CaCCinhA01 essentially abolishes mucus growth in WT colon, thus indicating the importance of ion secretion for mucus dynamics. However, simultaneous application of rCLCA1 brings the mucus growth rate back to slightly higher than normal levels, indicating that the effect of CLCA1 is not dependent of TMEM16A in colon. This finding is best represented by the Δ growth rate value as presenting only raw growth rate values could give the misleading impression that CaCCinhA01 inhibits the effect of rCLCA1.

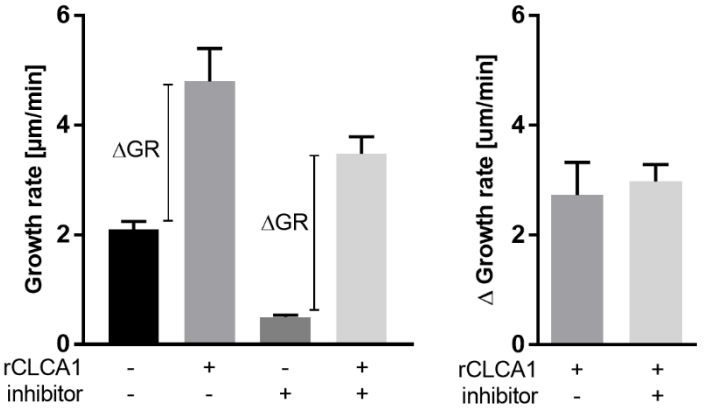

Figure 5: A) Data of mucus dynamic alterations upon treatment with rCLCA1 and an ion channel inhibitor (CaCCinhA01) presented as growth rate. B) The effect of rCLCA1 in (A) plotted as Δ growth rate (ΔGR).

Noticeably, many of the tested compounds had an immediate effect on the mucus growth rate, which could be observed within 15 min. However, tissue *ex vivo* viability is a limiting factor for studying slow acting compounds, as the tissue in general remain stable and viable for only 60 min [7]. It is thus possible that the lack of effect for some compounds is due to the time window of the experiment.

A further confounding factor when interpreting inhibitor based data is specificity. Several inhibitors are claimed to be specific for their target but have commonly only been tested in a rather limited *in vitro* system. For example, the ion channel inhibitor niflumic acid (NFA) has been used as a CaCC inhibitor is several studies, but has also been shown to block volume-regulated ion channels and calcium-dependent potasium channels [155].

3.1.2 Mucus penetrability (Paper I, II and IV)

An important property of the intestinal mucus is its capacity to form a barrier against intestinal bacteria [3]. To investigate this barrier function *ex vivo*, bacteria-sized beads are applied to the mucus surface and their distribution on and in the mucus is investigated by confocal microscopy. This method has been used to detect barrier dysfunction in e.g. inflamed epithelium, after dietary interventions and in GF mice [22,64,154]. In the present work we used this method to show that *Clca1*$^{-/-}$ mice have a functional mucus barrier (Paper I), but that *Spdef*$^{-/-}$ develop a severely dysfunctional mucus barrier phenotype as they age (Paper IV).

Although it has proven useful, several considerations should be kept in mind whilst interpreting mucus penetrability. Firstly, mucus penetration by bacteria is enhanced by their motility and the ability of certain bacteria to degrade the mucus structure [33], whereas the beads penetrate the mucus due to gravity and diffusion alone. Thus, a discrepancy between bead penetration and the localization of bacteria in the mucus layer can sometimes be observed [9].

Secondly, mucus penetration is not solely dependent on the pore size of the MUC2 oligomeric network, but also on interaction filtering, i.e. interactions between the particles and mucus components including lipids and DNA from shed cells, thus complicating the interpretation of results [156]. In this context it should be noted that the beads we use in the presented work are carboxylated, which might affect their interaction with the mucus layer. However, in Paper IV we confirm data obtained with beads by applying fluorescently labelled dextran (FITC-dextran).

Furthermore, the penetrability assay is typically performed on flushed tissue in order to quantify penetrability of the inner mucus layer, which likely gives a better representation of the properties of the mucus protective function. However, we are at this time uncertain whether the flushing of the tissue itself affects mucus properties, although mucus dynamics have been shown to be similar before and after removal of the outer mucus layer *in vivo* [6].

Combined, the aforementioned factors will in most cases likely result in an underestimation of actual mucus penetrability using the carboxylated beads. Thus, findings indicating increased bead penetrability suggest that the mucus layer is severely affected and most likely will result in increased bacteria contact with the epithelium *in vivo.*

A complementary approach for investigating bacteria penetrability is to directly stain bacteria in unflushed mucus samples *ex vivo* [9]. This can be achieved by applying nucleic acid stain to the mucus and imaging bacterial and tissue cells by confocal microscopy. However, presence or absence of a fecal pellet in the tissue at the time of sacrifice greatly affect the results and mounting unflushed tissue without disturbing the mucus layer is challenging, which make this method more useful as a complementary method rather than for screening.

3.1.3 *Ex vivo* lectin staining of mucus (Paper II and IV)

One of the major drawbacks with the *ex vivo* methods discussed above is that they rely on application of visible particles to draw conclusion about the naturally transparent mucus layer. We thus wanted to develop a technique to directly visualize the mucus. Lectins are carbohydrate binding proteins which are commonly used for visualization of the mucus in histology [68,107,157]. They are typically smaller than antibodies, thus more likely to be able to penetrate the mucus *ex vivo,* and fluorophore-conjugated lectins are commercially available. Successful application of fluorescently labeled lectins to visualize airway mucus bundles led us to develop this technique for intestinal mucus [158].

The lectins were chosen based on the high abundance of fucose, sialic acid and N-actylglucoseamine (GlcNAc) in colonic MUC2 oligosaccharides [24]. Both UEA1 (α-1,2 linked fucose) and WGA (sialic acid, GlcNAc) gave strong reproducible signals from the mucus as discussed in Paper IV. Other lectins were tested, including Sambucus Nigra Lectin (SNA, α-2,6 galactose (Gal) linked sialic acid), Maackia Amurensis (MAAI and II, Gal (β-1,4) GlcNAc and α-2,3 linked sialic acid) and Jacalin and Peanut agglutinin (PNA) which bind T-antigen with (Jacalin) or without (PNA) sialic acid. SNA and MAA stained structures in the mucus, but these structures were less defined than the staining obtained with UEA1 and WGA, and were thus not used further. Both Jacalin and PNA gave poor mucus staining *ex vivo*, likely due to terminal residues masking the T-antigen and thus blocking the binding.

As discussed for different inhibitors (3.1.1.1), the specificity for different lectins is less well defined as it is largely influenced by more than the suggested simple glycan structures *in* and *ex vivo* [159]. Thus, we have not investigated the exact lectin binding properties or drawn any strict conclusions concerning how glycosylation in the different mucus structures differs. Instead, we have only

used the lectins as a way to generate informative visualizations of different structures in the mucus. However, loss of lectin reactivity should be interpreted with caution, as this might be due to differences in glycosylation, or attachment of other modifiers rather than lack of the mucus structure *per se*. In paper IV we found that Spdef-deficient mice lacked UEA1 stained intercrypt mucus. As these mice also had a strong phenotype in the goblet cells that produced this mucus, we are confident in our interpretation that these mice lack the intercrypt mucus structure (further discussed in 4.2.2).

Although not further tested here, we believe that the visualization of the mucus will provide useful information regarding how different factors change mucus properties. This thus might provide a crucial new tool for future mucus research.

3.1.4 Phenotypic effects of the microbiota (Paper I-IV)

The profound role of the microbiota in defining important factors in the host has become increasingly evident over the past decade. This has also been shown for the intestinal mucus layer, where differences in the microbiota correlate with different mucus phenotypes observed in mice of genetically identical background [160].

A good example demonstrating how microbiota-mediated mucus phenotypes can confound interpretation of data is the case of the inflammasome component Nlrp6. It was suggested that Nlrp6-deficieny led to the development of a dominant colitogenic microbiota (dysbiosis) which correlated with a defective mucus layer, though the causative relationship was not investigated in detail [161,162]. However, the mucus phenotype reported in *Nlrp6*$^{-/-}$ mice could not be reproduced when investigated in our animal facility [54], and dysbiosis was not observed in *Nlrp6*$^{-/-}$ mice when littermate controls were used [163]. This indicated that neither mucus defects, nor the observed dysbiosis were inherent to the Nlrp6-deficiency. The latter study instead showed that maternal and cage variables were a stronger determinant of microbiota composition than genotype. Thus, differences in microbiota composition can strongly influence the mucus phenotype independently of host genotype, and proper animal controls are crucial for correct interpretation of data.

Microbiota composition is dependent on several factors including age, gender, mother, and diet [164], which ideally need to be accounted for in experimental

design. For this, littermate controls are the gold standard, although cage co-housing animals of different origin can provide an alternative [164,165]. In the work presented here, we have used both littermate and cage co-housed mice and mice from homozygote breeding programs according to Table 1.

Table 1: Animal study design for comparison of *Clca1*$^{-/-}$ *and Spdef*$^{-/-}$ to WT in the different experiments presented in Paper I-IV.

Paper	Experiment	Animal set-up*
Paper I	Proteome analysis	1
	Ex vivo	1
	IHC and FISH	1
	DSS	2
	Microbiota analysis	2
Paper II	Effects of rCLCA1	1
	Clca1$^{-/-}$ phenotypes	1, 3
Paper III	All	1
Paper IV	Electron microscopy	1
	Ex vivo	2
	Biochemical analysis	1
	Timeline	1, 3
	PCR and trancriptomics	1
	DSS	2
	Colitis	1, 3

* 1= Separate breeding programs, genotypes separately caged, 2= co-housed littermates, 3 = phenotypes confirmed in co-housed animals.

At the time of designing and implementing several of the studies that are presented in this thesis, the impact of the microbiota was not fully understood. As this understanding has emerged, our group is increasingly using littermate controls, either to confirm findings from separately housed mice from homozygote breeding programs, or for initial phenotype screening.

In the case of our *Clca1*$^{-/-}$ to WT comparison, the littermate controlled microbiota analysis in Paper I did not reveal any differences between the genotypes. Furthermore, we were not able to detect any mucus phenotype in

Clca1$^{-/-}$ mice with the other methods used in this paper. Although an altered microbiota can potentially mask a phenotype, we have not repeated penetrability, proteomic analysis or immunohistological analysis in samples from littermate controls. However, baseline mucus growth, in addition to treatment with enzyme inhibitors, has been investigated in co-housed controls in order to confirm that the microbiota did not affect this phenotype. Lectin staining of the mucus structures were also controlled under co-housed conditions. Mass spectrometric analysis of Muc2 absolute quantity and peptide abundance in Paper III were performed on samples from separate breeding programs which thus might have skewed the data. However, we have mechanistic *in vitro* data that strongly support our *in vivo* findings, and we thus think that our conclusions are valid, although this should be verified in future experiments.

In paper IV we present data from both littermate, co-housed, and separately bred and housed mice. As the heterozygote breeding was recently set up we are still in the process of acquiring data from littermate controls for all parts of the manuscript. So far there has been a very good agreement between the data acquired from littermates, co-housed and separately housed mice, thus arguing that the effects we describe are true phenotypes caused by the lack of Spdef and not by genotype-independent variables.

3.2 Histological examination of fixed tissue (Paper I, II, IV)

Due to the hydrated properties of the mucus, it is poorly preserved in traditional fixatives such as formalin. Thus, the common practice in the field is tissue fixation in Methacarn (methanol-Carnoy) [166]. Furthermore, a fecal pellet is required in order to preserve the mucus structure, and no attached mucus can usually be observed in tissue sections lacking a pellet. Whether or not this is due to poor mucus preservation or actual absence of mucus between pellets is under debate [167]. However, we consistently detect mucus in interpellet areas *ex vivo* in both flushed and unflushed tissue specimens, and we therefore strongly believe that the absence of an attached mucus layer in tissue sections that lack a pellet is an artefact.

Although it has not been systematically investigated, mucus preservation in fixed samples is affected by many factors apart from the choice of fixative, such as stool consistency and the actual cutting. Thus, quantifying mucus thickness from histological sections generates large variation that is not reproduced when mucus thickness is quantified *ex vivo* (Birchenough, unpublished data).

3.3 Proteomics (Paper I, II, III)

Detailed investigation of sample protein composition, termed proteomics, has been made possible by the tremendous development in mass spectrometry. This method can provide large scale identification of proteins in a sample, be used for protein quantification, investigate post-translational modifications and protein interactions [168]. Whereas a bottom-up approach is most often used for investigation of sample proteome, functional proteomics uses targeted methods to study single proteins. The most commonly used proteomic approach includes enzymatic digestion of the sample proteins into peptides which are separated with liquid chromatography and injected into a mass spectrometer. Ionization of the peptides allows determination of the peptide mass, and further fragmentation of the peptides yields peptide sequence information, after which both are matched against *in silico* digested proteins from sequence databases [169].

3.3.1 Absolute MUC2 quantification (Paper III)

Although the intensity of MS^1 peaks in a mass spectra roughly correlate with the abundance of the peptide, mass spectrometry data is not inherently quantitative since the ionization and detection of the peptides depends on their chemical properties [169]. Instead, absolute quantification is based on spiking samples with known amounts of labelled peptides from the protein of interest, and comparing peak intensity of the unlabeled native peptide to that of the labelled [170]. Furthermore, selective reaction monitoring is used to improve the sensitivity of the peptide quantification.

Quantification of MUC2 in tissue has traditionally relied on histological examination of Alcian blue-Periodic acid Shiff (AB-PAS) reactivity, MUC2 immunofluorescent intensity, or PCR-based methods [94,95,171]. MUC2

concentration in mucus has been less investigated, but has been compared between samples by semi-quantitative gel-electrophoresis and blotting [3,39,172]. However, absolute quantification of MUC2 in mucus has not been performed. In Paper III we thus set up a method for this purpose.

The absolute quantification of Muc2 in mucus generated data with large variability. This variability was also observed between technical replicates from the same mouse which would be expected to be consistent. We were able to reduce variability between technical replicates by introducing 10 µm beads for mucus surface visualization, instead of the charcoal particles which were initially used (Figure 6A). However, median mucus Muc2 concentration from WT samples still varies considerably between experiments (Figure 6B), but we believe that samples prepared and analyzed in parallel can be confidently compared, as samples from different groups analyzed at the same time are relatively consistent (Figure 6C).

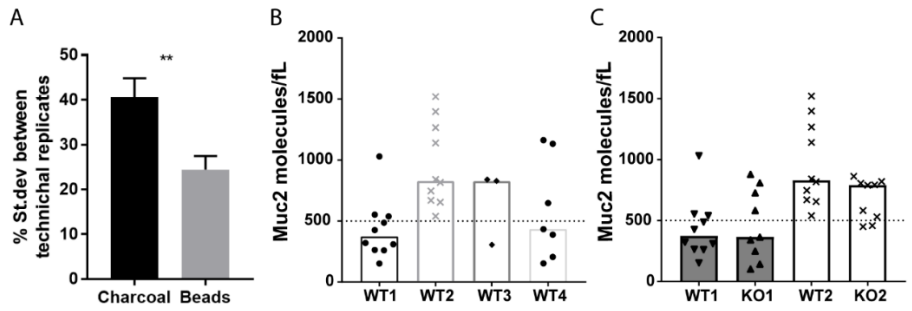

Figure 6: A) Standard deviation (St. dev) between technical replicates using either charcoal or beads for mucus surface visualization. B) Absolute quantification of Muc2 from WT mucus in different experiments (1-4). C) Comparison of Muc2 concentration between WT and a knockout mouse strain (KO) at two different occasions (1-2). Data presented as mean ± SEM (A) or median (B-C).

Apart from the indication that *Clca1*$^{-/-}$ animals have slightly higher Muc2 concentration (Paper III), this method has also been used to compare the effect of fiber-free diet on mucus barrier function [22]. This study found a correlation between mucus barrier defects and reduced Muc2 concentration in mucus, further indicating that this method might prove useful in determining underlying causes of mucus defects.

3.3.2 Cleavage site determination (Paper III)

Several mass spectrometry based methods have been developed to aid cleavage site identification for different proteases, as reviewed by Van den Berg and Tholey [173]. N-terminal labelling using (N-Succinimidyloxycarbonylmethyl) tris(2,4,6-trimethoxyphenyl)phosphoniumbromide (TMPP) enhances ionization efficiency and also shifts the retention time of the labelled peptides which enhance detection of the labelled peptides. This approach has been used to identify the cleavage site of Arg-gingipain B (RgpB) from *Porphyromonas gingivalis* in the MUC2 C-terminus [16]. In Paper III we similarly used TMPP-labelling to identify the CLCA1 cleavage sites in MUC2 N-terminus *in vitro*. Although the identified cleavages were in agreement with *in vivo* data, they should ideally be confirmed using full length MUC2 *in vitro* or by identifying the cleaved peptides *in vivo*. Proteomes from WT and Clca1-deficient mucus were searched using a non-tryptic search algorithm in order to investigate if the proposed cleavage peptides could be found in *in vivo* samples, without success. This does however not prove that the peptides are not present, but might be due to poor performance of the unlabeled peptides or the higher complexity of full proteome samples.

3.3.3 Mucus proteome analysis (Paper I and II)

Mass spectrometry can efficiently be used to investigate the full (within the limit of detection) proteome of a sample, and can be used as a method to compare the relative abundance of large number of proteins [170]. However, efficient analysis of proteomic data relies on either well defined targets, such as known mucus proteins in our case, or well annotated protein databases. Unfortunately, the function of most proteins in mucus is unknown, and it is thus difficult to perform bioinformatic analyses, or even understand the possible physiological implication of variations in single proteins. Furthermore, a vast number of the proteins found in the secreted mucus are intracellular proteins that likely, but not necessarily, originate from shed cells. Although a core mucus proteome of approximately 50 proteins (based on the presence of a signal sequence, membrane spanning domain or lipidation) has been suggested [174], we cannot exclude that intracellular proteins from shed cells have a functional role in the mucus.

3.4 Animal models of colitis (Paper I and IV)

Animal models of colitis has greatly enhanced the understanding of factors contributing to IBD, although no single animal model fully recapitulates the heterogeneous nature of IBD [175,176]. To date approximately 70 models exist which can be broadly divided into chemically induced, cell-transfer, congenital mutant, and genetically engineered models [177]. Of the chemically induced (by e.g. DSS, TNBS, acetic acid, and iodoacetamide) colitis models, DSS is one of the most frequently used based on its simplicity and reproducibility [78]. As mentioned in 1.1.4.1, the precise mechanism of DSS action is unknown. However, DSS-induced colitis is independent of the adaptive immune system, and is thus useful for studying the contribution of the mucus layer and innate immunity in colitis.

3.4.1 Ethical considerations

In Paper I and IV WT and Clca1- or Spdef-deficient mice were treated with DSS in the drinking water in order to investigate altered colitis susceptibility. The DSS-experiment on *Clca1*$^{-/-}$ animals and their controls was performed at Freie Universität in Berlin. During DSS-treatment of *Spdef*$^{-/-}$ and their controls, the mice were monitored daily for development of disease by recording weight, presence of blood in stool, and overall behavior. In line with our ethical permission (74-15), animals were sacrificed upon weight loss >10%, or reduced general health indicated by lethargy, kyphosis or inactivity.

3.5 Human biopsy collection (Paper II, III and IV)

Human colon biopsies or material from these are used in Paper II and IV. The biopsies were taken from patients with known or suspected colonic disease, admitted to the gastroenterological department at the Sahlgrenska hospital. Up to 40 biopsies (out of which up to 20, normally 8, were taken for research) were collected during colonoscopy of sedated patients under pain relief. Sampling of biopsies for research extended the time for colonoscopy approximately 5-10 min but sampling was otherwise not believed to cause any extra discomfort to the patient, and if it was the examination was discontinued.

Patients in the study were given an extra symptom questionnaire to be filled in before the colonoscopy. Patient identity was blinded to the researchers.

Patients were included in compliance with human research ethical committee in Gothenburg, Sweden (040-08), and the Declaration of Helsinki. The patients received written information about the study in advance and oral information and an opportunity to ask questions before being included. Participation in the study did not directly benefit the patients. Only adults who were able to give consent were included in the research.

3.6 Biochemical investigation of MUC2 (Paper III and IV)

The molecular mass of the unprocessed, non-glycosylated MUC2 polypeptide is approximately 500 kDa, which is increased to approximately 2.5 MDa after *O*-glycosylation [17]. Thus, conventional biochemical separation techniques such as SDS-PAGE is unsuitable for studies of MUC2 [178]. MUC2 from reduced mucus samples can however be separated by SDS-UAgPAGE, a technique requiring technical skills and expertise [179]. This can be used to investigate MUC2 monomers, and also lower order oligomeric states of MUC2, as shown in Paper IV.

To aid biochemical analysis of MUC2, truncated constructs of either N- or C-terminal MUC2 have been generated [26,27]. The molecular masses of these truncated proteins are within the working limit of conventional biochemical techniques. These constructs have aided the understanding of MUC2 oligomerization, proteolytic processing of MUC2 by meprin ß, intracellular packing of MUC2, and in Paper III we were able show that CLCA1 is able to cleave the MUC2 N-terminus by using these constructs [13,26–28,180].

4 RESULTS AND DISCUSSION

4.1 Colonic mucus structure (Paper IV)

Functional studies of mucus structure alterations have been performed by investigating the penetrability of fluorescent beads into the mucus layer *ex vivo* [9,22,64]. However, this method investigates the mucus by secondary read outs and does not allow for direct visualization of mucus itself. In Paper IV we took advantage of the high density of *O*-glycans present on MUC2 to visualize the mucus layer by fluorescently-conjugated lectins *ex vivo*. By doing so we were able to show that, in contrast to previous beliefs, the inner mucus layer has a heterogeneous structure. Mucus secreted from the crypts was highly reactive to WGA lectin and formed dense plumes which prevented diffusion of particles smaller than 0.2 μm, even down to approximately 25 nm. Conversely, mucus secreted from luminal surface goblet cells (intercrypt mucus) was highly reactive to UEA1 and had different properties in terms of pore size. The identification of crypt plumes and intercrypt mucus was primarily based on their different reactivity with WGA and UEA1 lectin respectively. However, whether the different glycosylation pattern plays a role in determining the exclusion properties is currently unknown.

The presence of dense mucus plugs over the crypt openings make sense in terms of preventing bacteria reaching the stem cells, as disruption of the stem cell niche would have detrimental effects. It is also possible that the presence of only crypt covering plumes would result in a stagnant mucus layer and that the intercrypt mucus has developed to flush and lubricate the surface.

The discovery of two distinct mucus types in the colonic tissue is similar to what has also been observed in pig airways where MUC5B from submucosal glands forms bundles that rolls over the tissue and becomes coated in MUC5AC from goblet cells at the epithelial surface [158]. MUC5B and MUC5AC are not only expressed at distinct places but also have different glycosylation, as observed by their different lectin reactivity. Although the system in the airway relies on two different, but very similar mucins, there appears to be many homologies between the airways and the intestine.

However, the rationale for having mucus structures with different properties in the intestine needs to be further investigated.

The homogenous appearance of the mucus layer in fixed tissue does contradict our finding of a heterogeneous mucus structure *ex vivo* [3,8]. One reason for this discrepancy might be compression of the mucus gel in histological sections, as these have to contain a fecal pellet in order to preserve the mucus layer. The presence of a pellet might compress the plumes to the level of the epithelial surface, and/or induce increased formation of the intermixed UEA1/WGA material seen as a fine network at the mucus surface in our *ex vivo* experiments, which we believe corresponds to the stratified mucus observed in fixed sections. However, we have so far not found an *ex vivo* method to study the mucus structure under altered luminal pressure. It is also possible that the handling of the tissue for *ex vivo* investigation in of itself induces artefacts, (e.g. increased secretion from the crypt goblet cells) that result in increased plume volume. However, as the architecture with crypt plumes and intercrypt mucus can be observed in unflushed tissue which has been subjected to minimal handling we believe that what we have described here represents the physiological state

4.2 Intercrypt goblet cells (Paper IV)

4.2.1 Identification of intercrypt goblet cells (Paper IV)

The UEA1-reactive intercrypt mucus was secreted from goblet cells at the luminal epithelial surface. Based on four lines of evidence, we suggest that these cells, here called intercrypt goblet cells (icGCs), represent a novel subtype of goblet cell. Firstly, icCGs are morphologically distinct and do not have the typical goblet shape due to reduced theca size since they do not store their secretory cargo [32]. They are thus mostly overlooked in investigations of goblet cells, especially with AB-PAS staining. They can however be easily identified by staining for apo-MUC2, due to their high rate of MUC2 synthesis (discussed further below).

Secondly, icGCs seem to have a different glycosylation machinery since they produce predominantly UEA1 reactive (presumably fucosylated) material, and very little WGA reactive material. Whether this is due to a transcriptional shift

in the glycosylation machinery as part of GC terminal differentiation is yet to be determined. Furthermore, we have not examined how the different glycosylation relates to mucus properties.

Thirdly, mucus secreted from icGCs has different penetrability properties compared to the crypt covering plumes, and is more penetrable to 0.2 µm beads. This seem to represent a weak point of the mucus structure as we have noted that bacteria almost exclusively penetrate intercrypt mucus in mice that have increased bacterial penetration (Figure 7, unpublished). In steady state condition, however, bacterial access to the intercrypt mucus is limited by the dense intermixed UEA1/WGA network.

Fourthly, these cells have a higher Muc2 production and secretion rate compared to other goblet cells which might provide a flushing effect that can prevent the tissue from infection under normal conditions [32].

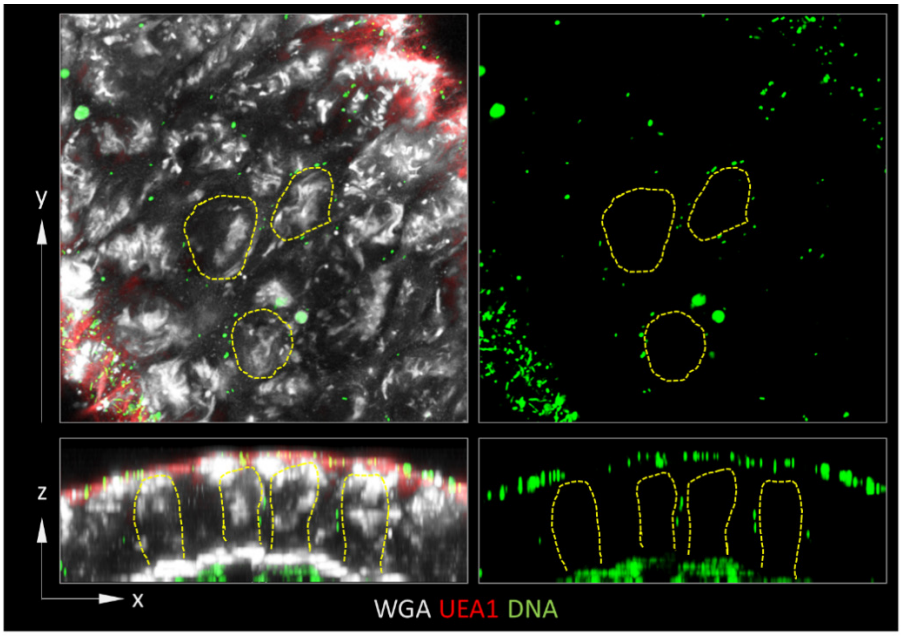

Figure 7: x/y, and x/z-projections of colonic explant *ex vivo*. Bacteria can be detected in intercrypt mucus in unflushed colonic mucus of a mouse model with increased mucus penetrability. Mucus was stained with WGA (grey) and UEA (red). Bacteria and host cells are stained with Syto9 (green). Yellow dashed lines outline examples of crypt plumes.

4.2.2 Loss of intercrypt goblet cell function in *Spdef* $^{-/-}$ mice results in a dysfunctional mucus barrier (Paper IV)

Spdef-deficient mice were found to have a loss-of-function phenotype in icGCs. In line with a previous report, the icGC phenotype included an increased number of microvilli on the apical surface, ER-enlargement and reduced theca size compared to WT icGCs [94]. We further found that these alterations correlated with a secretory defect. Thus, although we did identify a difference in the transcription of some glycosylation pathway proteins in *Spdef* $^{-/-}$ goblet cells, we think that the lack of UEA1-intense intercrypt mucus was due to an actual loss of this mucus, rather than altered glycosylation. Lack of functional icGCs strongly correlated with a defective mucus barrier, with decreased mucus thickness and an increased penetrability to beads, indicating the importance of the intercrypt mucus for the formation of a functional mucus barrier.

As the abnormal mucus phenotype in Spdef-deficient mice developed with age, we do not believe that Spdef is directly regulating icGCs, but that the effects observed in those cells are secondary to the loss of Spdef. Furthermore, our transcriptional data from 6-week-old *Spdef*$^{-/-}$ and WT animals did not reveal any difference in the transcription of core mucus genes such as *Muc2* and *Agr2,* although these genes were altered in 11-week-old mice. This suggests that Spdef, despite earlier reports, is not directly regulating *Muc2* expression [94,95,181]. Unfortunately, due to poor knowledge of many of the goblet cell and mucus-specific proteins, gene ontology annotation enrichment analysis of altered gene expression in the goblet cell fraction did not reveal any specific pathways that are regulated by Spdef, apart from *O*-glycan processing.

Mucus quality defects have been noted in UC but it has remained unknown whether these defects are a result of or a driving factor in inflammation. As Spdef-deficient mice have a slow progression of colitis (compared to chemically induced colitis models) we were hoping to resolve this issue. Indeed, a strong mucus phenotype developed before overt signs of inflammation, such as crypt elongation or immune cell infiltration [182]. However, the number of $CD45^+$ cells in the tissue increased before we noted a drastic change in mucus penetrability. It is thus possible that subtle mucus changes occur at earlier time points, which increase bacterial contact with the tissue and drives immune activity. Since we can see both mucus and immune changes before the onset of colitis, we were unable to resolve the

aforementioned question. This shows how interconnected the mucus, epithelium and immune system are in maintaining homeostasis. We do however believe that a more detailed investigation of the temporal aspects of the phenotypes observed in Spdef-deficient mice will help to shed light on the relation between mucus defects and colitis development.

4.3 Effects of CLCA1 in mucus (Paper I and II)

4.3.1 Mucus phenotype in Clca1-deficient mice (Paper I and II)

Due to the abundance of CLCA1 in colonic mucus we first hypothesized that it has a structural role in the mucus and that loss of CLCA1 would weaken mucus barrier function. Alternatively, we hypothesized that the proposed protease effect of CLC1 would have profound effect on the mucus structure, that would affect mucus dynamics. Thus we investigated mucus barrier function and dynamics in Clca1-deficient mice. However, no altered mucus phenotype could be found in Clca1-deficient mice by using IHC to study the mucus layer stratification or goblet cell appearance. Furthermore, *Clca1*$^{-/-}$ mice did not have a defective mucus barrier as they had an impenetrable mucus layer *ex vivo* and had a good separation between the tissue and bacteria as shown by FISH of intestinal sections. Additionally, WT and Clca1-deficient mice had similar mucus dynamics in naïve and CCh stimulated tissue.

A previous study investigating *Clca1*$^{-/-}$ airways suggested that their lack of abnormal phenotype was due to increased expression of Clca2 (previously mClca5) [142]. This led us to think that the lack of an intestinal mucus phenotype in C*lca1*$^{-/-}$ animals could similarly be due to increased expression of other Clca homologs in the intestine. However, we could not find elevated protein levels of any other Clca-family member in the mucus by proteomics.

Conversely, as we investigated CLCA1 function using protease inhibitors (discussed further in 4.3.2), we found a striking difference between WT and C*lca1*$^{-/-}$ mice. Whereas WT baseline mucus growth was inhibited by the metalloprotease inhibitor EDTA, similar to what we found in GF mice and human *ex vivo* explants, the *Clca1*$^{-/-}$ mucus growth rate was not significantly altered. Instead, mucus growth in *Clca1*$^{-/-}$ explants was completely inhibited

by cysteine-protease inhibitors, whereas these only had minor effects in WT tissue. It thus appears that lack of Clca1 induces cysteine protease activity, although the responsible protease and the regulatory mechanisms so far remain unknown.

Despite the masking of growth phenotype in *Clca1*$^{-/-}$ by a cysteine protease, there are differences in the processing of the mucus between WT and *Clca1*$^{-/-}$ mice, evident by lectin staining of the mucus. Alterations in the mucus structure were primarily observed at the mucus surface, whereas the tissue proximate mucus appeared normal (not shown). The surface structure of flushed intestinal mucus in WT animals is, as we also describe in Paper IV (see 4.1), relatively smooth, whereas it was more condensed in *Clca1*$^{-/-}$. This effect was even more pronounced when unflushed mucus was investigated (Figure 8). We thus suggest that CLCA1 is involved in the transition from inner to outer mucus. The mechanism for this is further discussed in 4.4.

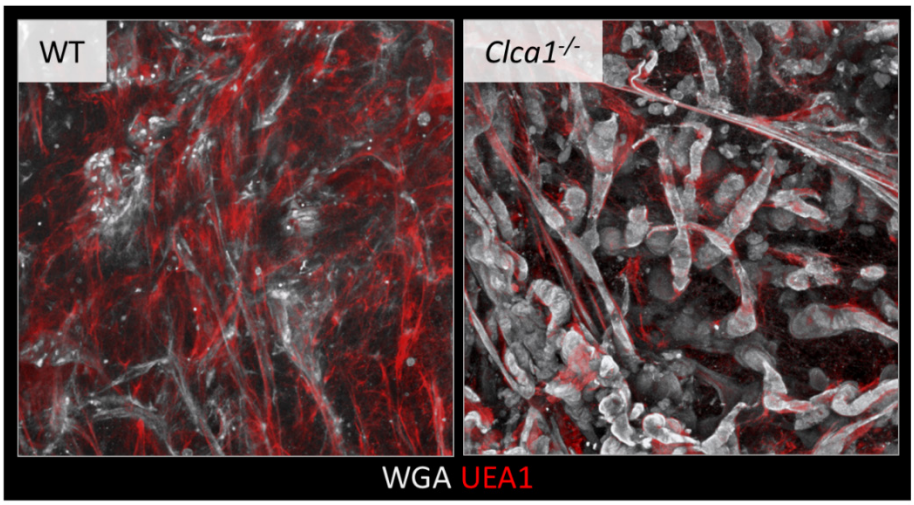

Figure 8: Representative x/y-projections of the mucus surface from unflushed WT and *Clca1*$^{-/-}$ explants stained with WGA (grey) and UEA (red).

4.3.2 Effects of recombinant CLCA1 in mucus (Paper II)

To further characterize the function of CLCA1 in mucus we investigated the effect of exogenously applied rCLCA1 *ex vivo*. *Ex vivo* mucus application of recombinant proteins has previously been successful in both rescuing mucus phenotypes, and providing functional evidence for proteins [9,13]. Application of

rCLCA1 resulted in a marked increase of the mucus growth rate, as well as increased penetrability of the mucus. Importantly, the innermost mucus remained impenetrable, suggesting that CLCA1 enhance the conversion between the inner and outer mucus layer. rCLCA1 application also slowed the movement of beads above the mucus surface which we interpreted as increased viscosity at the mucus/liquid border due to excess processing of the mucus structure.

The effect on the mucus of rCLCA1 was shown to be mediated by the metalloprotease activity in the N-terminus of CLCA1, as the effect could be blocked by the metalloprotease inhibitor EDTA but not the cysteine- and serine protease inhibitor cOmplete protease inhibitor (EDTA-free), and was abolished by a protease null CLCA1 mutation. Similar inhibition as with EDTA was found with EGTA and TPEN, which preferably chelate calcium and zinc respectively. Although the protein sequence suggest binding of both calcium and zinc, the requirement for different ions needs further testing. We did not note any inhibition by Batimastat, a commercial MMP inhibitor previously reported to have inhibitory effect on CLCA1 *in vitro* [122]. Another study also failed to detect inhibition of CLCA1 with Marimastat, an inhibitor similar to Batimastat [123]. Although CLCA1 has similarities with MMPs and ADAMS, it lacks several of their common features and we therefore suggest that CLCA1 constitutes a novel class of proteases.

Importantly, the CLCA1-induced mucus growth increase was not mediated by ion secretion. CLCA1 has repeatedly been reported to act as an ion channel regulator of CaCCs, potentially by stabilizing TMEM16A at the cell membrane [117,122,127,133,134]. The interaction between CLCA1 and TMEM16A is suggested to be mediated by the VWA domain in CLCA1, and the VWA domain alone was sufficient to induce I_{CaCC} *in vitro* [127]. However, the specificity of the interaction, i.e. whether or not other proteins containing a VWA, or isolated VWA domains from other proteins, could elicit the same response was not tested. The investigators further claimed that CLCA1 stabilized TMEM16A at the apical membrane by inhibiting internalization of TMEM16A, evident by membrane localization of both CLCA1 and TMEM16A when co-expressed. However, the investigators did not investigate the co-localization in detail, and although CLCA1 has also previously has been suggested to be membrane associated *in vitro*, we have not observed any membrane staining of CLCA1 in intestinal sections [116,117,133,134]. Regarding CLCA1-induced mucus growth, if

TMEM16A modulation was involved in this process then it should be blocked by the potent TMEM16A inhibitor CaCCinhA01 [155,183]. Although less specific, DIDS and NFA has also previously been shown to inhibit CLCA1- mediated ion currents *in vitro* [116,133]. However, none of these inhibitors prevented the CLCA1-mediated growth rate increase, thus suggesting that the proposed ion channel regulatory effect of CLCA1 does not play a role under these circumstances.

Research concerning the ion channel regulatory properties of CLCA1 has primarily focused on airway tissue, and it is possible that CLCA1 expressed in the airways in asthma and COPD has another function than constitutively expressed intestinal CLCA1. However, investigation of airways from *Clca1*$^{-/-}$ mice did not reveal alterations of calcium-activated chloride currents in either naïve or IL-13 treated conditions [141], and *Clca1*$^{-/-}$ mice did not respond differently to WT mice in a virus induced airway model of hyperplasia [142].

The involvement of CLCA1 in the inner to outer mucus layer conversion might be a key determinant for smooth distal transport of mucus and thus prevent stagnation. This could pose an explanation of the result from a previous report showing that restoration of reduced Clca1 expression ameliorated CF mucus obstruction in small intestine [152]. This was not due to altered electrostatic ion transport, however other possible mechanisms were not investigated. Furthermore, this possibly sheds new light on the role of CLCA1 in diseases which have mucus stagnation as a hallmark pathological feature. As discussed in 1.3.4.1, there has long been a belief that the overexpression of CLCA1 noted in T_H2-type asthma and COPD is the cause of the goblet cell hyperplasia and mucin overproduction typical for these diseases (reviewed in Patel *et. al.*, 2009 [110]). It is however possible that the overexpression of CLCA1 is a response induced to facilitate removal of increased mucus, and also that there are unknown feed-back loops that make it challenging to dissect whether CLCA1 expression drives mucin expression or vice versa. This could also account for the contradictions in the literature regarding this matter [119,135,137,140,141]. The recent finding that airway mucus becomes more similar to colonic mucus in a model of COPD further argues that the role of CLCA1 in diseased airway is similar to its normal function in the intestine, which we suggest is to facilitate removal of mucus [143]. We therefore propose that the role of CLCA1 in these diseases should be reconsidered and that CLCA1 may be considered a friend rather than a foe.

4.4 MUC2 cleavage by CLCA1 (Paper III)

Our data in Paper III strongly suggest that CLCA1 cleaves MUC2. This was shown using both an *in vitro* assay and by identifying differences in the Muc2 peptide distribution in *Clca1*$^{-/-}$ mice compared to WT.

Recombinantly expressed and purified CLCA1 and MUC2-terminal proteins were used in an *in vitro* assay which showed that CLCA1 was able to degrade the MUC2-N, but not -C, terminus. However, the purification fraction containing the highest amount of 85 kDa N-terminal rCLCA1 was only partly able to process the MUC2 construct, whereas a fraction containing a smaller N-terminal fragment harboring the CAT/Cys+VWA domains of CLCA1 completely degraded MUC2 into 4 cleavage products. Thus we believe that the CAT/Cys+ VWA secondary N-terminal product of CLCA1 is more enzymatically active (further discussed in 4.5.1). We do not fully understand how this product is generated in our *in vitro* system, but speculate that it is due to low-grade spontaneous degradation of the full length N-terminus, or alternatively due to the presence of an activating protease in the *in vitro* expression system. Preferably, we would aim to express the CAT/Cys+VWA domains as a truncated protein in order to further characterize it; however, our attempts thus far have been unsuccessful, as this protein seems to be retained in the ER of the expressing cells. Whether or not this is an inherent problem or can be resolved by using another cell line is a subject for future investigation.

Analysis of the CLCA1-mediated MUC2 cleavage products revealed three possible cleavage sites in MUC2-N; IRL^{754}-^{755}IGQ, TAL^{774}-^{775}ATS and VIQ^{940}-^{941}RDE, in the E2-TIL´interface, TIL´-domain, and VWD3 domain respectively. The compact organization of the D-assembly subdomains argues that IRL^{754}-^{755}IGQ is the actual CLCA1 cleavage site as this site is more exposed [14]. This would indicate that CLCA1 is able to cleave off the D1-D2 assemblies from MUC2.

Altered Muc2 processing as determined by peptide abundance was found in *Clca1*$^{-/-}$ intestinal mucus compared to WT. Although absolute quantification of total Muc2 only trended towards being increased in the knock-out animals, the absolute amounts of the peptide located in the D1 assembly was significantly higher in Clca1-deficient mice. However, the ratio of the D1 peptide to the reference peptide is still less than one in these mice, which indicates that D1 is

additionally processed. Furthermore, comparison of the relative abundance of Muc2 peptides in mucus from WT and *Clca1*$^{-/-}$ again revealed differences in the D1 assembly region, where Clca1-deficient mice have comparatively higher abundance of peptides.

The effect on the mucus structure by the cleavage of the D1-D2 assemblies proposed herein is still not understood, although it seems to have a large impact on the transition from inner to outer mucus layer as *Clca1*$^{-/-}$ animals have a markedly different outer mucus layer organization (see 4.3.1). Based on the similarity between MUC2 and VWF, we suggest that D1-D2 stabilize the oligomeric bundles of MUC2 which are formed intracellularly [28], and that CLCA1 either actively aids the unfolding of MUC2 or stabilizes the unfolded state by removing or altering D1-D2 conformation. Although the unfolding of MUC2 has previously been assumed to be spontaneous upon increased pH and reduced concentration of calcium, our results suggest that other factors such as CLCA1 might be crucial for the process.

4.5 Biochemical properties of CLCA1 (Paper II and III)

4.5.1 Regulation of CLCA1 activity (Paper II and III)

As shown and discussed in both Paper II and III, CLCA1 is very abundant in colonic mucus. Thus, its enzymatic activity is probably very tightly controlled. Enzymatic activity can be regulated by a broad range of factors including pH, endogenous inhibitors, presence of a pro-peptide and allosteric hindrance. As CLCA1 is similar to MMPs and ADAMS which are expressed as zymogens, it has been suggested that removal of a pro-peptide is involved in CLCA1 activation. However, Yurtsever et al. claimed this not to be the case as they could not detect loss of an N-terminal tag after *in vitro* expression of CLCA1 [122]. However, in our opinion the design of the experiment cannot exclude the existence of a pro-peptide, and this thus need further testing.

In the same paper, it was also shown that autocatalytic cleavage is required for the ability of CLCA1 to act as an ion channel regulator, and this was thus suggested as a regulatory mechanism. However, we could only detect cleaved CLCA1 in intestinal mucus samples, which contradicts the hypothesis of self-

cleavage as a fine-tuning regulation. Furthermore, a CLCA1 mutant with abrogated cleavage was able to induce mucus growth to the same extent as WT CLCA1, adding to the evidence that self-cleavage is not required for CLCA1 activity in the intestine.

We were able to detect a previously uncharacterized N-terminal product of CLCA1 in fresh intestinal mucus samples by Western blot. Based on the molecular mass and identification with antibodies against different epitopes in CLCA1, we suggest that this product contain the catalytic CAT/Cys domain together with the VWA domain. The *in vitro* MUC2 cleavage assay (discussed in 4.4) further suggests that this product is more enzymatically active, and we thus propose that a second cleavage of the CLCA1 N-terminus regulates enzyme activity. This cleavage would be between the VWA and the β-sheet rich (BSR) domain, but the exact cleavage site and the responsible enzyme for the cleavage remain unknown. Furthermore, although the CAT/Cys+VWA product was resistant to limited proteolysis with trypsin, it appears to be more unstable than the full N-terminus as it was lost upon freezing and thawing of samples. Thus, the activity after activation might be limited by a decreased half-life. However, this concept needs further investigation.

4.5.2 CLCA1 interactions and domain structure (Paper III)

In Paper III we show evidence that CLCA1 forms large oligomers consisting of both N- and C-terminal CLCA1. These oligomers are held together by non-covalent association between the N- and C-termini, and by C-terminal dimerization via disulfide bonds. The non-covalent interaction between the N- and the C-termini is possibly mediated by interaction between the VWA and FnIII domain, although this was not investigated. Oligomerization often serves to stabilize a protein structure, but might also confer regulatory functions if it is dependent on environmental factors such as pH. Thus, further investigation of CLCA1 oligomerization might provide useful input regarding CLCA1 function and regulation.

Clca1 was also identified in bands corresponding to different Muc2 oligomeric states in SDS-AgPAGE analysis of colonic mucus, indicating an interaction between Clca1 and Muc2. As we discuss in Paper III, it can be envisioned that Clca1 has to bind its target, Muc2, in order to maintain proximity to its substrate after secretion. However, other functions of this interaction are possible and Clca1 might have yet undiscovered structural roles in the mucus.

Limited proteolysis of CLCA1 indicated that the CAT/Cys, VWA and FnIII form structural domains that are protected against proteolysis. Furthermore, the combined CAT/Cys+VWA domains also seems largely resistant to proteolysis, indicating that together these form a functional truncated product. Unfortunately, we do not have any antibody directed against the BSR domain and could thus not investigate this region in further detail.

Secretion of a truncated CLCA1 protein encompassing the full N-terminus failed due to ER retention. Two shorter truncated N-terminal proteins (1-300/ 1-477) were also poorly secreted although ER retention was not proven in these cases. This indicates that the full CLCA1, including the C-terminal part, is needed for correct protein folding and processing. However, we cannot exclude that the ER-retention is cell line specific, and therefore this needs further investigation.

4.6 Human mucus (Paper II, III and IV)

The objective for our studies is to better understand the physiology of the human intestinal mucus and epithelium. Thus, investigation of human samples has been crucial to confirm that our results using mice as a model organism are translational. By collaboration with the gastroenterology unit at the Sahlgrenska University hospital we have been fortunate to obtain fresh intestinal biopsies from colonoscopy examinations. We have thus been able to investigate human mucus structure and properties *ex vivo* by lectin staining, bead penetrability, mucus growth rate dynamics in response to CLCA1 and protease inhibitors, and have also collected mucus samples for biochemical investigation of CLCA1.

Lectin staining of human sigmoid colon revealed a similar pattern to that observed in mice, with crypt-covering plumes surrounded by intercrypt mucus that merged at the mucus surface (Paper IV). However, the lectin reactivity was different in human samples compared to mice. Whereas GlcNAc and sialic acid-binding WGA primarily stained crypt mucus in mice, it seemed to preferentially bind intercrypt mucus in human samples. LEL, which like WGA binds different GlcNAc containing structures, gave a similar but not identical staining pattern. In contrast, fucose-recognizing LTL gave stronger staining of

the crypt plumes in humans. LTL has so far not been tested in mouse tissue, but UEA1 which recognizes α1-2-linked fucose gives strong staining of intercrypt mucus in mouse. Investigation of the glycosylation patterns is of interest, especially in relation to its changes during inflammation [184]. However, here we have not aimed to investigate glycosylation *per se* but merely use it as a tool to visualize the intestinal mucus. That said, future studies of how the glycosylation and concurrent lectin reactivity, as well as the overall mucus structure, change during inflammation are both feasible and highly interesting.

Human intercrypt mucus was penetrable to 0.2, but not 1 μm carboxylated beads similar to what was found in mouse mucus. Mucus from healthy human subjects has previously been shown to be impenetrable to 0.5 – 2 μm beads [64]. It thus seems as there is a size exclusion limit in the intercrypt mucus between 0.2-0.5 μm.

As we show in Paper II and in agreement with previous findings [7], human baseline mucus growth rate is in a similar range to that of mouse mucus. Furthermore, human mucus growth was inhibited by addition of EDTA, and was increased by rCLCA1 treatment. These results correlate with those from mouse samples, suggesting that metalloproteases are involved in the mucus dynamics in both species and that CLCA1 is a likely candidate for this effect.

Investigation of CLCA1 from collected human mucus and epithelium by Western blot identified similar biochemical processing of CLCA1 in human as to mouse (Paper III). We thus only identified fully cleaved N- and C-terminal CLCA1 in human mucus samples, which were found in large oligomers when investigated under native conditions. Similar to murine Clca1, human C-terminal CLCA1 dimerization was also observed. Importantly, we were able to detect the truncated second N-terminal product we have suggested is the fully active CLCA1 in fresh human mucus, implying that this processing event is conserved in both species.

Lastly, the *in vitro* MUC2 cleavage assay used both CLCA1 and MUC2-terminal proteins that are based on the human sequences. Thus, any effect observed here should recapitulate human conditions. However, we were able to find evidence for the same Muc2 cleavage event in mucus from mice, again suggesting good translatability between human and mouse CLCA1 function.

5 CONCLUSIONS

By using an *ex vivo* set up to measure colonic mucus properties such as mucus growth in native conditions or in response to exogenous application of proteins or inhibitors, penetrability to bacteria sized beads, and investigation of lectin reactivity of the mucus, in combination with mass spectrometry and biochemical methods we were able to conclude that:

- Deficiency of Clca1 in colonic mucus does not result in altered mucus dynamics *ex vivo* due to compensatory activity by a cysteine protease. Neither does Clca1-loss results in a defective mucus barrier in terms of mucus penetrability, but alters the outer mucus layer structure.
- CLCA1 acts as a metalloprotease in intestinal mucus.
- MUC2 is a substrate for CLCA1 metalloprotease activity, which cleaves off the D1-D2 assemblies of MUC2. This transformation is important for the transition from inner to outer mucus layer.
- CLCA1 forms oligomeric assemblies in mucus, mediated by C-terminal disulfide-bonds and non-covalent interaction between CLCA1 N- and C-termini.
- A novel N-terminal product of CLCA1, consisting of the CAT/Cys and VWA domains, is detected in mucus and is more proteolytically active than the full length N-terminal CLCA1.
- Microscopic mucus structures can be visualized and investigated by application of fluorescently labelled lectins *ex vivo.*
- The inner mucus layer is not a homogenous structure, but consists of dense mucus plumes covering the crypt openings, interconnected by intercrypt mucus that has a less dense structure. Crypt plumes and intercrypt mucus merge to form dense nets. The organization of different mucus structures can be visually distinguished *ex vivo* due to their different lectin reactivity.
- Intercrypt goblet cells are a functional subtype of goblet cell. Loss of intercrypt goblet cell function in *Spdef*$^{-/-}$ results in a defective mucus barrier and subsequent development of colitis.

6 FUTURE PERSPECTIVE

Our observations describing mucus structure and processing have shed new light on determinants of intestinal mucus barrier function. However, this field is far from fully explored. We hope that the development of an *ex vivo* mucus visualization method using fluorescent lectins will provide more detailed information of how mucus is structured, not only in colon, but also in the small intestine and proximal colon. Furthermore, investigation of mucus structural alterations in disease, and different mouse genetic models, is likely to give useful information of how barrier defects arise and how it relates to disease development.

Our identification of icGCs as a functional subpopulation of goblet cells, together with the previous identification of senGCs, strongly indicates that the goblet cells are less homogenous as a cell type than previously thought. Development of single cell transcriptomics can be envisioned to further define and characterize different goblet cell subpopulations, which might provide new insight into goblet cell functions apart from mucus secretion.

Our finding that CLCA1 is able to process MUC2 in order to loosen the mucus structure presents a novel function for CLCA1. However, further characterization of the interaction between CLCA1 and MUC2, as well as of the regulation of CLCA1 activity is needed to fully understand the function of CLCA1 *in vivo.*

Increasing our understanding of the mucus processing activities of CLCA1 will further decipher the involvement of CLCA1 in diseases such as asthma and COPD. The long held belief has been that CLCA1 is responsible for the mucus hypersecretion and plugging in these diseases. However, our results suggest that CLCA1 is rather increased in order to facilitate removal of the mucus. A recent study observed that airway mucus is stratified and anchored to the epithelium in COPD and CF, thus resembling colonic mucus [143]. As Clca1 was found to be one of the most upregulated proteins in the mouse-model employed by this study, it can be speculated that Clca1 is part of the transition to a colon-like mucus phenotype, possibly by stabilizing the stratified organization. This hypothesis requires further studies in suitable models. In addition, investigation of CLCA1 in lung disease, e.g. if it is found in the more proteolytically active form or not, and whether or not CLCA1 can process the airway mucins MUC5AC and MUC5B should help to clarify the role of Clca1 in pulmonary diseases.

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8 REFERENCES

1. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol Hepatol.* 2012;9(10):577-589. doi:10.1038/nrgastro.2012.156.

2. Johansson MEV, Gustafsson JK, Sjöberg KE, et al. Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. *PLoS One*. 2010;5(8): e12238. doi:10.1371/journal.pone.0012238.

3. Johansson MEV, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA*. 2008;105(39):15064-15069. doi:10.1073/pnas.0803124105.

4. Rodríguez-Piñeiro AM, Bergström JH, Ermund A, et al. Studies of mucus in mouse stomach, small intestine, and colon. II. Gastrointestinal mucus proteome reveals Muc2 and Muc5ac accompanied by a set of core proteins. *Am J Physiol Gastrointest Liver Physiol.* 2013;305(5):G348-56. doi:10.1152/ajpgi.00047.2013.

5. Ermund A, Schütte A, Johansson MEV, Gustafsson JK, Hansson GC. Gastrointestinal mucus layers have different properties depending on location. I. Studies of mucus in mouse stomach, small intestine, Peyer's patches and colon. *Am J Physiol Gastrointest Liver Physiol.* 2013;305(5): G341-G347. doi:10.1152/ajpgi.00046.2013.

6. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Liver Physiol.* 2001;280(5):G922-G929. doi:10.1152/ajpgi.2001.280.5.G922.

7. Gustafsson JK, Ermund A, Johansson MEV., Schütte A, Hansson GC, Sjövall H. An ex vivo method for studying mucus formation, properties, and thickness in human colonic biopsies and mouse small and large intestinal explants. *AJP Gastrointest Liver Physiol.* 2012;302(4):G430-G438. doi:10.1152/ajpgi.00405.2011.

8. Matsuo K, Ota H, Akamatsu T, Sugiyama A, Katsuyama T. *Histochemistry* of the surface mucous gel layer of the human colon. *Gut*. 1997;40(6):782-789. doi:10.1136/gut.40.6.782.

9. Bergström JH, Birchenough GMH, Katona G, et al. Gram-positive bacteria are held at a distance in the colon mucus by the lectin-like protein ZG16. *Proc Natl Acad Sci U S A*. 2016;113(48):13833-13838. doi:10.1073/pnas.1611400113.

10. Macierzanka A, Mackie AR, Bajka BH, Rigby NM, Nau F, Dupont D. Transport of particles in intestinal mucus under simulated infant and adult physiological conditions: impact of mucus structure and extracellular DNA. *PLoS One*. 2014;9(4):e95274. doi:10.1371/journal.pone.0095274.

11. Recktenwald C V, Hansson GC. The Reduction-insensitive Bonds of the MUC2 Mucin Are Isopeptide Bonds. *J Biol Chem*. 2016;291(26):13580-13590. doi:10.1074/jbc.M116.726406.

12. Gum JR, Hicks JW, Toribara NW, Siddiki B, Kim YS. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. *J Biol Chem*. 1994;269(4):2440-2446.

13. Schütte A, Ermund A, Becker-Pauly C, et al. Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus. *Proc Natl Acad Sci U S A*. 2014;111(34):12396-12401. doi:10.1073/pnas.1407597111.

14. Zhou Y-F, Eng ET, Zhu J, Lu C, Walz T, Springer TA. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120(2):449-458. doi:10.1182/blood-2012-01-405134.

15. Crouzier T, Boettcher K, Geonnotti AR, et al. Modulating Mucin Hydration and Lubrication by Deglycosylation and Polyethylene Glycol Binding. *Adv Mater Interfaces*. 2015;2(18):1500308. doi:10.1002/admi.201500308.

16. van der Post S, Subramani DB, Bäckström M, et al. Site-specific O-glycosylation on the MUC2 mucin protein inhibits cleavage by the Porphyromonas gingivalis secreted cysteine protease (RgpB). *J Biol Chem*. 2013;288(20):14636-14646. doi:10.1074/jbc.M113.459479.

17. Carlstedt I, Herrmann A, Karlsson H, Sheehan J, Fransson LA, Hansson GC. Characterization of two different glycosylated domains from the insoluble mucin complex of rat small intestine. *J Biol Chem*. 1993;268(25):18771-18781. doi:10.1007/BF01210191

18. Stanley RA, Ram SP, Wilkinson RK, Roberton AM. Degradation of pig gastric and colonic mucins by bacteria isolated from the pig colon. *Appl Environ Microbiol*. 1986;51(5):1104-1109.

19. Sommer F, Adam N, Johansson MEV, Xia L, Hansson GC, Bäckhed F. Altered Mucus Glycosylation in Core 1 O-Glycan-Deficient Mice Affects Microbiota Composition and Intestinal Architecture. *PLos One*. 2014;9(1):e85254. doi:10.1371/journal.pone.0085254.

20. Juge N. Microbial adhesins to gastrointestinal mucus. *Trends Microbiol.* 2012;20(1):30-39. doi:10.1016/J.TIM.2011.10.001.

21. Desai MS, Seekatz AM, Koropatkin NM, et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell*. 2016;167(5):1339-1353.e21. doi:10.1016/j.cell.2016.10.043.

22. Schroeder BO, Birchenough GMH, Ståhlman M, et al. Bifidobacteria or Fiber Protects against Diet-Induced Microbiota-Mediated Colonic Mucus Deterioration. *Cell Host Microbe*. 2018;23(1):27-40.e7. doi:10.1016/j.chom.2017.11.004.

23. Holmén Larsson JM, Karlsson H, Sjövall H, Hansson GC. A complex, but uniform O-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn. *Glycobiology*. 2009;19(7):756-766. doi:10.1093/glycob/cwp048.

24. Holmén Larsson JM, Thomsson KA, Rodríguez-Piñeiro AM, Karlsson H, Hansson GC. Studies of mucus in mouse stomach, small intestine, and colon. III. Gastrointestinal Muc5ac and Muc2 mucin O-glycan patterns reveal a regiospecific distribution. *Am J Physiol Gastrointest Liver Physiol*. 2013;305(5):G357-63. doi:10.1152/ajpgi.00048.2013.

25. Asker N, Axelsson MA, Olofsson SO, Hansson GC. Dimerization of the human MUC2 mucin in the endoplasmic reticulum is followed by a N-glycosylation-dependent transfer of the mono- and dimers to the Golgi apparatus. *J Biol Chem*. 1998;273(30):18857-18863. doi:10.1074/jbc.273.30.18857.

26. Lidell ME, Johansson MEV, Mörgelin M, et al. The recombinant C-terminus of the human MUC2 mucin forms dimers in Chinese-hamster ovary cells and heterodimers with full-length MUC2 in LS 174T cells. *Biochem J*. 2003;372(Pt 2):335-345. doi:10.1042/BJ20030003.

27. Godl K, Johansson MEV, Lidell ME, et al. The N terminus of the MUC2 mucin forms trimers that are held together within a trypsin-resistant core fragment. *J Biol Chem*. 2002;277(49):47248-47256. doi:10.1074/jbc.M208483200.

28. Ambort D, Johansson MEV, Gustafsson JK, et al. Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin. *Proc Natl Acad Sci U S A*. 2012;109(15):5645-5650. doi:10.1073/pnas.1120269109.

29. Huang R-H, Wang Y, Roth R, et al. Assembly of Weibel-Palade body-like tubules from N-terminal domains of von Willebrand factor. *Proc Natl Acad Sci U S A*. 2008;105(2):482-487. doi:10.1073/pnas.0710079105.

30. Xiao F, Yu Q, Li J, et al. Slc26a3 deficiency is associated with loss of colonic HCO 3 − secretion, absence of a firm mucus layer and barrier impairment in mice. *Acta Physiol*. 2014;211(1):161-175. doi:10.1111/apha.12220.

31. Gustafsson JK, Ermund A, Ambort D, et al. Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *J Exp Med*. 2012;209(7):1263-1272. doi:10.1084/jem.20120562.

32. Johansson MEV. Fast renewal of the distal colonic mucus layers by the surface goblet cells as measured by in vivo labeling of mucin glycoproteins. *PLos One*. 2012;7(7):e41009. doi:10.1371/journal.pone.0041009.

33. McGuckin MA, Lindén SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol*. 2011;9(4):265-278. doi:10.1038/nrmicro2538.

34. Schneider H, Pelaseyed T, Svensson F, Johansson MEV. Study of mucin turnover in the small intestine by in vivo labeling. *Sci Rep*. 2018;8(1):5760. doi:10.1038/s41598-018-24148-x.

35. Enss ML, Schmidt-Wittig U, Müller H, Mai UE, Coenen M, Hedrich HJ. Response of germfree rat colonic mucous cells to peroral endotoxin application. *Eur J Cell Biol*. 1996;71(1):99-104.

36. Li JD, Dohrman a F, Gallup M, et al. Transcriptional activation of mucin by Pseudomonas aeruginosa lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. *Proc Natl Acad Sci USA*. 1997;94(3):967-972. doi:10.1073/pnas.94.3.967.

37. Shigemura H, Ishiguro N, Inoshima Y. Up-regulation of MUC2 mucin expression by serum amyloid A3 protein in mouse colonic epithelial cells. *J Vet Med Sci.* 2014;76(7):985-991. doi:10.1292/jvms.14-0007.

38. Burger-van Paassen N, Vincent A, Puiman PJ, et al. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. *Biochem J*. 2009;420(2):211-219. doi:10.1042/BJ20082222.

39. Hasnain SZ, Wang H, Ghia JE, et al. Mucin Gene Deficiency in Mice Impairs Host Resistance to an Enteric Parasitic Infection. *Gastroenterology*. 2010;138(5):1763-1771.e5. doi:10.1053/j.gastro.2010.01.045.

40. McKenzie GJ, Bancroft A, Grencis RK, McKenzie ANJ. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol*. 1998;8(6):339-342. doi:10.1016/S0960-9822(98)70134-4.

41. Turner J, Stockinger B, Helmby H. IL-22 Mediates Goblet Cell Hyperplasia and Worm Expulsion in Intestinal Helminth Infection. *PLOS Path.* 2013;9(10): e1003698. doi:10.1371/journal.ppat.1003698.

42. Ahn D, Crawley SC, Hokari R, et al. TNF-alpha activates MUC2 transcription via NF-kappaB but inhibits via JNK activation. *Cell Physiol Biochem*. 2005;15(1-4):29-40. doi:10.1159/000083636.

43. Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am J Physiol Liver Physiol*. 2007;292(1):G315-G322. doi:10.1152/ajpgi.00265.2006.

44. Boshuizen JA, Reimerink JHJ, Korteland-van Male AM, et al. Homeostasis and function of goblet cells during rotavirus infection in mice. *Virology*. 2005;337(2):210-221. doi:10.1016/j.virol.2005.03.039.

45. Enss M-L, Cornberg M, Wagner S, et al. Proinflammatory cytokines trigger MUC gene expression and mucin release in the intestinal cancer cell line LS180. *Inflamm Res*. 2000;49(4):162-169. doi:10.1007/s000110050576.

46. Birchenough GMH, Johansson ME V, Gustafsson JK, Bergström JH, Hansson GC. New developments in goblet cell mucus secretion and function. *Mucosal Immunol*. 2015;8(4):712-719. doi:10.1038/mi.2015.32.

47. Adler KB, Tuvim MJ, Dickey BF. Regulated mucin secretion from airway epithelial cells. *Front Endocrinol*. 2013;4:129. doi:10.3389/fendo.2013.00129.

48. Rodríguez-Piñeiro AM, van der Post S, Johansson ME V, Thomsson KA, Nesvizhskii AI, Hansson GC. Proteomic study of the mucin granulae in an intestinal goblet cell model. *J Proteome Res*. 2012;11(3):1879-1890. doi:10.1021/pr2010988.

49. Cornick S, Moreau F, Gaisano HY, Chadee K. Entamoeba histolytica-Induced Mucin Exocytosis Is Mediated by VAMP8 and Is Critical in Mucosal Innate Host Defense. *MBio*. 2017;8(5):e01323-17. doi:10.1128/mBio.01323-17.

50. Zhu Y, Ehre C, Abdullah LH, et al. Munc13-2 −/− baseline secretion defect reveals source of oligomeric mucins in mouse airways. *J Physiol*. 2008;586(7):1977-1992. doi:10.1113/jphysiol.2007.149310.

51. Specian RD, Neutra MR. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. *J Cell Biol*. 1980;85(3):626-640. doi:10.1083/jcb.85.3.626.

52. Phillips TE, Phillips TH, Neutra MR. Regulation of intestinal goblet cell secretion. III. Isolated intestinal epithelium. *Am J Physiol*. 1984;247(6 Pt 1):G674-81. doi:10.1152/ajpgi.1984.247.6.G674.

53. Halm DR, Halm ST, Hoda RM, et al. Secretagogue response of goblet cells and columnar cells in human colonic crypts. *Am J Physiol Cell Physiol.* 2000;278(1):C212-33. doi:10.1152/ajpcell.2000.278.1.C212.

54. Birchenough GMH, Nyström EEL, Johansson MEV, et al. A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. *Science*. 2016;352(6293):1535-1542. doi:10.1126/science.aaf7419.

55. Mcneil NI, Ling KLE, Wager J. Mucosal surface pH of the large intestine of the rat and of normal and inflamed large intestine in man. *Gut*. 1987;28:707-713. doi:10.1136/gut.28.6.707.

56. Park S-W, Zhen G, Verhaeghe C, et al. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. *Proc Natl Acad Sci U S A*. 2009;106(17):6950-6955. doi:10.1073/pnas.0808722106.

57. Johansson MEV, Thomsson KA, Hansson GC. Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J Proteome Res*. 2009;8(7):3549-3557. doi:10.1021/pr9002504.

58. Tsuru A. Negative feedback by IRE1b optimizes mucin production in goblet cells. *Proc Natl Acad Sci USA*. 2013;110(8):2864-2869. doi:10.1073/pnas.1212484110.

59. Johansson MEV, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. *Nat Rev Gastroenterol Hepatol*. 2013;10(6):352-361. doi:10.1038/nrgastro.2013.35.

60. McGuckin MA, Eri R, Simms LA, Florin THJ, Radford-Smith G. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis.* 2009;15(1):100-113. doi:10.1002/ibd.20539.

61. Chassaing B, Koren O, Goodrich JK, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature.* 2015;519(7541):92-96. doi:10.1038/nature14232.

62. Chassaing B, Raja SM, Lewis JD, Srinivasan S, Gewirtz AT. Colonic Microbiota Encroachment Correlates With Dysglycemia in Humans. *Cell Mol Gastroenterol Hepatol.* 2017;4(2):205-221. doi:10.1016/J.JCMGH.2017.04.001.

63. Van der Sluis M, De Koning BAE, De Bruijn ACJM, et al. Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating That MUC2 Is Critical for Colonic Protection. *Gastroenterology*. 2006;131(1):117-129. doi:10.1053/J.GASTRO.2006.04.020.

64. Johansson MEV, Gustafsson JK, Holmén-Larsson J, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut.* 2014;63(2):281-291. doi:10.1136/gutjnl-2012-303207.

65. Heazlewood CK, Cook MC, Eri R, et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med.* 2008;5(3):0440-0460. doi:10.1371/journal.pmed.0050054.

66. Dawson PA, Huxley S, Gardiner B, et al. Reduced mucin sulfonation and impaired intestinal barrier function in the hyposulfataemic NaS1 null mouse. *Gut*. 2009;58(7):910-919. doi:10.1136/gut.2007.147595.

67. Fu J, Wei B, Wen T, et al. Loss of intestinal core 1-derived O-glycans causes spontaneous colitis in mice. *J Clin Invest.* 2011;121(4):1657-1666. doi:10.1172/JCI45538.

68. Bergstrom K, Fu J, Johansson MEV, et al. Core 1– and 3–derived O-glycans collectively maintain the colonic mucus barrier and protect against spontaneous colitis in mice. *Mucosal Immunol*. 2017;10(1):91-103. doi:10.1038/mi.2016.45.

69. Swidsinski A, Loening-Baucke V, Theissig F, et al. Comparative study of the intestinal mucus barrier in normal and inflamed colon. *Gut.* 2007;56(3):343-350. doi:10.1136/gut.2006.098160.

70. Johansson MEV. Mucus Layers in Inflammatory Bowel Disease. *Inflamm Bowel Dis*. 2014;20(11):2124-2131. doi:10.1097/MIB.0000000000000117.

71. Pullan RD, Rhodes M, Newcombe RG, et al. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut*. 1994;35(3):353-359. doi:10.1136/gut.35.3.353.

72. An G, Wei B, Xia B, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med*. 2007;204(6):1417-1429. doi:10.1084/jem.20061929.

73. Zhao F, Edwards R, Dizon D, et al. Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice. *Dev Biol.* 2010;338(2):270-279. doi:10.1016/j.ydbio.2009.12.008.

74. Corfield AP, Myerscough N, Bradfield N, et al. Colonic mucins in ulcerative colitis: evidence for loss of sulfation. *Glycoconj J*. 1996;13(5):809-822. doi:10.1007/BF00702345

75. Rhodes JM. Unifying hypothesis for inflammatory bowel disease and associated colon cancer: sticking the pieces together with sugar. *Lancet*. 1996;347(8993):40-44. doi:10.1016/S0140-6736(96)91563-9.

76. Hasnain SZ, Tauro S, Das I, et al. IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. *Gastroenterology*. 2013;144(2):357-368.e9. doi:10.1053/j.gastro.2012.10.043.

77. Franke A, Balschun T, Karlsen TH, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet*. 2008;40(11):1319-1323. doi:10.1038/ng.221.

78. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol.* 2014;104:Unit 15.25. doi:10.1002/0471142735.im1525s104.

79. Petersson J, Schreiber O, Hansson GC, et al. Importance and regulation of the colonic mucus barrier in a mouse model of colitis. *Am J Physiol Liver Physiol*. 2011;300(2):G327-G333. doi:10.1152/ajpgi.00422.2010.

80. Lee S, Kim H, Kim K, Lee H, Lee S, Lee D. Arhgap17, a RhoGTPase activating protein, regulates mucosal and epithelial barrier function in the mouse colon. *Sci Rep*. 2016;6(1):26923. doi:10.1038/srep26923.

81. Krajina BA, Tropini C, Zhu A, et al. Dynamic Light Scattering Microrheology Reveals Multiscale Viscoelasticity of Polymer Gels and Precious Biological Materials. *ACS Cent Sci.* 2017;3(12):1294-1303. doi:10.1021/acscentsci.7b00449.

82. Burger-van Paassen N, van der Sluis M, Bouma J, et al. Colitis development during the suckling-weaning transition in mucin Muc2-deficient mice. *Am J Physiol Liver Physiol*. 2011;301(4):G667-G678. doi:10.1152/ajpgi.00199.2010.

83. Bergstrom KSB, Kissoon-Singh V, Gibson DL, et al. Muc2 Protects against Lethal Infectious Colitis by Disassociating Pathogenic and Commensal Bacteria from the Colonic Mucosa. *PLoS Pathog*. 2010;6(5):e1000902. doi:10.1371/journal.ppat.1000902.

84. Bhullar K, Zarepour M, Yu H, et al. The Serine Protease Autotransporter Pic Modulates Citrobacter rodentium Pathogenesis and Its Innate Recognition by the Host. *Infect Immun*. 2015;83(7):2636-2650. doi:10.1128/IAI.00025-15.

85. Gustafsson JK, Navabi N, Rodriguez-Piñeiro AM, et al. Dynamic Changes in Mucus Thickness and Ion Secretion during Citrobacter rodentium Infection and Clearance. *PLos One*. 2013;8(12):e84430. doi:10.1371/journal.pone.0084430.

86. O'Sullivan BP, Freedman SD. Cystic fibrosis. *Lancet*. 2009;373(9678):1891-1904. doi:10.1016/S0140-6736(09)60327-5.

87. Kunzelmann K, Schreiber R, Hadorn HB. Bicarbonate in cystic fibrosis. *J Cyst Fibros*. 2017;16(6):653-662. doi:10.1016/J.JCF.2017.06.005.

88. Houwen RH, van der Doef HP, Sermet I, et al. Defining DIOS and constipation in cystic fibrosis with a multicentre study on the incidence, characteristics, and treatment of DIOS. *J Pediatr Gastroenterol Nutr*. 2010;50(1):38-42. doi:10.1097/MPG.0b013e3181a6e01d.

89. De Lisle RC, Roach E, Jansson K. Effects of laxative and N-acetylcysteine on mucus accumulation, bacterial load, transit, and inflammation in the cystic fibrosis mouse small intestine. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(3):G577-584. doi:10.1152/ajpgi.00195.2007.

90. Darwich AS, Aslam U, Ashcroft DM, Rostami-Hodjegan A. Meta-Analysis of the Turnover of Intestinal Epithelia in Preclinical Animal Species and Humans. *Drug Metab Dispos*. 2014;42(12):2016-2022. doi:10.1124/dmd.114.058404.

91. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol.* 2009;71:241-260. doi:10.1146/annurev.physiol.010908.163145.

92. Barker N. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol*. 2014;15(1):19-33. doi:10.1038/nrm3721.

93. McCauley HA, Guasch G. Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. *Trends Mol Med*. 2015;21(8):492-503. doi:10.1016/j.molmed.2015.06.003.

94. Gregorieff A, Stange DE, Kujala P, et al. The Ets-Domain Transcription Factor Spdef Promotes Maturation of Goblet and Paneth Cells in the Intestinal Epithelium. *Gastroenterology*. 2009;137(4):1333-1345.e3. doi:10.1053/j.gastro.2009.06.044.

95. Noah TK, Kazanjian A, Whitsett J, Shroyer NF. SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. *Exp Cell Res*. 2010;316(3):452-465. doi:10.1016/j.yexcr.2009.09.020.

96. Chen G, Korfhagen TR, Xu Y, et al. SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. *J Clin Invest.* 2009;119(10):2914-2924. doi:10.1172/JCI39731.

97. Park K-S, Korfhagen TR, Bruno MD, et al. SPDEF regulates goblet cell hyperplasia in the airway epithelium. *J Clin Invest.* 2007;117(4):978-988. doi:10.1172/JCI29176.

98. Chen G, Volmer AS, Wilkinson KJ, et al. Role of Spdef in the Regulation of Muc5b Expression in the Airways of Naïve and Muco-obstructed Mice. *Am J Respir Cell Mol Biol.* 2018;59(3):383-396. doi:10.1165/rcmb.2017-0127OC.

99. Lo Y-H, Noah TK, Chen M-S, et al. SPDEF Induces Quiescence of Colorectal Cancer Cells by Changing the Transcriptional Targets of β-catenin. *Gastroenterology*. 2017;153(1):205-218.e8. doi:10.1053/J.GASTRO.2017.03.048.

100. Noah TK, Lo Y, Price A, et al. SPDEF Functions as a Colorectal Tumor Suppressor by Inhibiting β-Catenin Activity. *Gastroenterology*. 2013;144(5):1012-1023.e6. doi:10.1053/j.gastro.2013.01.043.

101. Aronson BE, Stapleton KA, Vissers LA, Stokhuijzen E, Bruijnzeel H, Krasinski SD. Spdef deletion rescues the crypt cell proliferation defect in conditional Gata6 null mouse small intestine. *BMC Mol Biol*. 2014;15(1):3. doi:10.1186/1471-2199-15-3.

102. McDole JR, Wheeler LW, McDonald KG, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*. 2012;483(7389):345-349. doi:10.1038/nature10863.

103. Knoop KA, McDonald KG, McCrate S, McDole JR, Newberry RD. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol*. 2015;8(1):198-210. doi:10.1038/mi.2014.58.

104. Neutra MR, O'Malley LJ, Specian RD. Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. *Am J Physiol.* 1982;242(4):G380-G387. doi: 10.1152/ajpgi.1982.242.4.G380

105. Podolsky DK, Fournier DA, Lynch KE. Human colonic goblet cells. Demonstration of distinct subpopulations defined by mucin-specific monoclonal antibodies. *J Clin Invest.* 1986;77(4):1263-1271. doi:10.1172/JCI112429.

106. Oliver MG, Specian RD. Intracellular variation of rat intestinal mucin granules localized by monoclonal antibodies. *Anat Rec.* 1991;230(4):513-518. doi:10.1002/ar.1092300410.

107. Gouyer V, Gottrand F, Desseyn J-L. The Extraordinarily Complex but Highly Structured Organization of Intestinal Mucus-Gel Unveiled in Multicolor Images. *PLos One*. 2011;6(4):e18761. doi:10.1371/journal.pone.0018761.

108. Akiyama S, Mochizuki W, Nibe Y, et al. CCN3 Expression Marks a Sulfomucin-nonproducing Unique Subset of Colonic Goblet Cells in Mice. *Acta Histochem Cytochem*. 2017;50(6):159-168. doi:10.1267/ahc.17027.

109. Mundhenk L, Erickson NA, Klymiuk N, Gruber AD. Interspecies diversity of chloride channel regulators, calcium-activated 3 genes. *PLos One*. 2018;13(1):e0191512. doi:10.1371/journal.pone.0191512.

110. Patel AC, Brett TJ, Holtzman MJ. The role of CLCA proteins in inflammatory airway disease. *Annu Rev Physiol.* 2009;71:425-449. doi:10.1146/annurev.physiol.010908.163253.

111. Bothe MK, Braun J, Mundhenk L, Gruber AD. Murine mCLCA6 is an integral apical membrane protein of non-goblet cell enterocytes and co-localizes with the cystic fibrosis transmembrane conductance regulator. *J Histochem Cytochem.* 2008;56(5):495-509. doi:10.1369/jhc.2008.950592.

112. Elble RC, Walia V, Cheng H, et al. The Putative Chloride Channel hCLCA2 Has a Single C-terminal Transmembrane Segment. *J Biol Chem*. 2006;281(40): 29448-29454. doi:10.1074/jbc.M605919200.

113. Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. *J Histochem Cytochem.* 2002;50(6):829-838. doi:10.1177/002215540205000609.

114. Komiya T, Tanigawa Y, Hirohashi S. Cloning and identification of the gene gob-5, which is expressed in intestinal goblet cells in mice. *Biochem Biophys Res Commun*. 1999;255(2):347-351. doi:10.1006/bbrc.1999.0168.

115. Agnel M, Vermat T, Culouscou JM. Identification of three novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea. *FEBS Lett.* 1999;455(3):295-301. doi:10.1016/S0014-5793(99)00891-1.

116. Gruber AD, Elble RC, Ji H-L, Schreur KD, Fuller CM, Pauli BU. Genomic Cloning, Molecular Characterization, and Functional Analysis of Human CLCA1, the First Human Member of the Family of Ca2+-Activated Cl−Channel *Proteins. Genomics.* 1998;54(2):200-214. doi:10.1006/geno.1998.5562.

117. Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. HCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. *J Biol Chem*. 2005;280(29):27205-27212. doi:10.1074/jbc.M504654200.

118. Hoshino M, Morita S, Iwashita H, et al. Increased Expression of the Human Ca 2 + -activated Cl- Channel 1 (CaCC1) Gene in the Asthmatic Airway. *Am J Respir Crit Care Med.* 2002;165:1132-1136. doi:10.1164/rccm.2107068.

119. Nakanishi A, Morita S, Iwashita H, et al. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci U S A*. 2001;98(9):5175-5180. doi:10.1073/pnas.081510898.

120. Song L, Liu D, Wu C, et al. Antibody to mCLCA3 Suppresses Symptoms in a Mouse Model of Asthma. *PLos One*. 2013;8(12):e82367. doi:10.1371/journal.pone.0082367.

121. Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD. Both cleavage products of the mCLCA3 protein are secreted soluble proteins. *J Biol Chem*. 2006;281(40):30072-30080. doi:10.1074/jbc.M606489200.

122. Yurtsever Z, Sala-Rabanal M, Randolph DT, et al. Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation. *J Biol Chem*. 2012;287(50):42138-42149. doi:10.1074/jbc.M112.410282.

123. Bothe MK, Mundhenk L, Kaup M, Weise C, Gruber AD. The murine goblet cell protein mCLCA3 is a zinc-dependent metalloprotease with autoproteolytic activity. *Mol Cells*. 2011;32(6):535-541. doi:10.1007/s10059-011-0158-8.

124. Pawłowski K, Lepistö M, Meinander N, Sivars U, Varga M, Wieslander E. Novel conserved hydrolase domain in the CLCA family of alleged calcium-activated chloride channels. *Proteins Struct Funct Genet*. 2006;63(3):424-439. doi:10.1002/prot.20887.

125. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol*. 2003;200(4):448-464. doi:10.1002/path.1400.

126. Whittaker CA, Hynes RO. Distribution and Evolution of von Willebrand/Integrin A Domains: Widely Dispersed Domains with Roles in Cell Adhesion and Elsewhere. *Mol Biol Cell*. 2002;13(10):3369-3387. doi:10.1091/mbc.e02-05-0259.

127. Sala-Rabanal M, Yurtsever Z, Berry KN, Nichols CG, Brett TJ. Modulation of TMEM16A channel activity by the von Willebrand factor type A (VWA) domain of the calcium-activated chloride channel regulator 1 (CLCA1). *J Biol Chem*. 2017;292(22):9164-9174. doi:10.1074/jbc.M117.788232.

128. Long AJ, Sypek JP, Askew R, et al. Gob-5 contributes to goblet cell hyperplasia and modulates pulmonary tissue inflammation. *Am J Respir Cell Mol Biol*. 2006;35(3):357-365. doi:10.1165/rcmb.2005-0451OC.

129. Dietert K, Reppe K, Mundhenk L, Witzenrath M, Gruber AD. mCLCA3 Modulates IL-17 and CXCL-1 Induction and Leukocyte Recruitment in Murine Staphylococcus aureus Pneumonia. *PLos One*. 2014;9(7):e102606. doi:10.1371/journal.pone.0102606.

130. Erickson NA, Mundhenk L, Giovannini S, Glauben R, Heimesaat MM, Gruber AD. Role of goblet cell protein CLCA1 in murine DSS colitis. *J Inflamm.* 2016;13(1):5. doi:10.1186/s12950-016-0113-8.

131. Bustin SA, Li S-R, Dorudi S. Expression of the Ca 2+ -Activated Chloride Channel Genes CLCA1 and CLCA2 Is Downregulated in Human Colorectal Cancer. *DNA Cell Biol*. 2001;20(6):331-338. doi:10.1089/10445490152122442.

132. Yang B, Cao L, Liu B, McCaig CD, Pu J. The Transition from Proliferation to Differentiation in Colorectal Cancer Is Regulated by the Calcium Activated Chloride Channel A1. *PLos One*. 2013;8(4): e60861. doi:10.1371/journal.pone.0060861.

133. Hamann M, Gibson A, Davies N, et al. Human ClCa1 modulates anionic conduction of calcium-dependent chloride currents. *J Physiol.* 2009;587(10): 2255-2274. doi:10.1113/jphysiol.2009.170159.

134. Sala-Rabanal M, Yurtsever Z, Nichols CG, Brett TJ. Secreted CLCA1 modulates TMEM16A to activate Ca2+-dependent chloride currents in human cells. *Elife*. 2015;4:e05875. doi:10.7554/eLife.05875.

135. Alevy YG, Patel AC, Romero AG, et al. IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. *J Clin Invest*. 2012;122(12):4555-4568. doi:10.1172/JCI64896.

136. Toda M, Tulic MK, Levitt RC, Hamid Q. A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma. *J Allergy Clin Immunol*. 2002;109(2):246-250. doi:10.1067/mai.2002.121555.

137. Thai P, Chen Y, Dolganov G, Wu R. Differential regulation of MUC5AC/Muc5ac and hCLCA-1/mGob-5 expression in airway epithelium. Am *J Respir Cell Mol Biol.* 2005;33(6):523-530. doi:10.1165/rcmb.2004-0220RC.

138. Zhou Y, Dong Q, Louahed J, et al. Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. *Am J Respir Cell Mol Biol.* 2001;25(4):486-491. doi:10.1165/ajrcmb.25.4.4578.

139. Hui-lan Z, Li M, Li H, et al. Murine calcium-activated chloride channel family member 3 induces asthmatic airway inflammation independently of allergen exposure. *Chinese Med J.* 2013;126(17):3283-3288. doi:10.3760/cma.j. issn.0366-6999.20130620.

140. Robichaud A, Tuck SA, Kargman S, et al. Gob-5 is not essential for mucus overproduction in preclinical murine models of allergic asthma. *Am J Respir Cell Mol Biol.* 2005;33(3):303-314. doi:10.1165/rcmb.2004-0372OC.

141. Mundhenk L, Johannesson B, Anagnostopoulou P, et al. mCLCA3 does not contribute to calcium-activated chloride conductance in murine airways. *Am J Respir Cell Mol Biol*. 2012;47(1):87-93. doi:10.1165/rcmb.2010-0508OC.

142. Patel AC, Morton JD, Kim EY, et al. Genetic segregation of airway disease traits despite redundancy of calcium-activated chloride channel family members. *Physiol Genomics.* 2006;25(3):502-513. doi:10.1152/physiolgenomics. 00321.2005.

143. Fernández-Blanco JA, Fakih D, Arike L, et al. Attached stratified mucus separates bacteria from the epithelial cells in COPD lungs. *JCI Insight*. 2018;3(17)e120994. doi:10.1172/jci.insight.120994.

144. Gray MA, Winpenny JP, Porteous DJ, Dorin JR, Argent BE. CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. *Am J Physiol*. 1994;266(1):C213-21. doi:10.1152/ajpcell.1994.266.1.C213.

145. Rozmahe R, Wilschanski M, Matin A, et al. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet*. 1996;12(3):280-287. doi:10.1038/ng0396-280.

146. Caputo A, Caci E, Ferrera L, et al. TMEM16A, A Membrane Protein Associated with Calcium-Dependent Chloride Channel Activity. *Science* . 2008;322(5901): 590-594. doi:10.1126/science.1163518.

147. Schroeder BC, Cheng T, Jan YN, Jan LY. Expression Cloning of TMEM16A as a Calcium-Activated Chloride Channel Subunit. *Cell*. 2008;134(6):1019-1029. doi:10.1016/j.cell.2008.09.003.

148. Yang YD, Cho H, Koo JY, et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature*. 2008;455(7217):1210-1215. doi:10.1038/nature07313.

149. Ritzka M, Stanke F, Jansen S, et al. The CLCA gene locus as a modulator of the gastrointestinal basic defect in cystic fibrosis. *Hum Genet*. 2004;115(6):483-491. doi:10.1007/s00439-004-1190-y.

150. van der Doef HPJ, Slieker MG, Staab D, et al. Association of the CLCA1 p.S357N variant with meconium ileus in European patients with cystic fibrosis. *J Pediatr Gastroenterol Nutr.* 2010;50(3):347-349. doi:10.1097/MPG.0b013e3181afce6c.

151. Brouillard F, Bensalem N, Hinzpeter A, et al. Blue native/SDS-PAGE analysis reveals reduced expression of the mClCA3 protein in cystic fibrosis knock-out mice. *Mol Cell Proteomics.* 2005;4(11):1762-1775. doi:10.1074/mcp.M500098-MCP200.

152. Young FD, Newbigging S, Choi C, Keet M, Kent G, Rozmahel RF. Amelioration of Cystic Fibrosis Intestinal Mucous Disease in Mice by Restoration of mCLCA3. *Gastroenterology*. 2007;133(6):1928-1937. doi:10.1053/j.gastro.2007.10.007.

153. Leverkoehne I, Holle H, Anton F, Gruber AD. Differential expression of calcium-activated chloride channels (CLCA) gene family members in the small intestine of cystic fibrosis mouse models. *Histochem Cell Biol*. 2006;126(2):239-250. doi:10.1007/s00418-006-0164-7.

154. Johansson MEV, Jakobsson HE, Holmén-Larsson J, et al. Normalization of Host Intestinal Mucus Layers Requires Long-Term Microbial Colonization. *Cell Host Microbe*. 2015;18(5):582-592. doi:10.1016/j.chom.2015.10.007.

155. Liu Y, Zhang H, Huang D, et al. Characterization of the effects of Cl- channel modulators on TMEM16A and bestrophin-1 Ca2+ activated Cl- channels. *Pflügers Arch - Eur J Physiol*. 2015;467(7):1417-1430. doi:10.1007/s00424-014-1572-5.

156. Witten J, Samad T, Ribbeck K. Selective permeability of mucus barriers. *Curr Opin Biotechnol*. 2018;52:124-133. doi:10.1016/j.copbio.2018.03.010.

157. Amat CB, Motta J-P, Fekete E, Moreau F, Chadee K, Buret AG. Cysteine protease–dependent mucus disruptions and differential mucin gene expression in Giardia duodenalis infection. *Am J Pathol.* 2017; 187(11):2486-2498. doi:10.1016/j.ajpath.2017.07.009.

158. Ermund A, Meiss LN, Rodriguez-Pineiro AM, et al. The normal trachea is cleaned by MUC5B mucin bundles from the submucosal glands coated with the MUC5AC mucin. *Biochem Biophys Res Commun*. 2017;492(3):331-337. doi:10.1016/j.bbrc.2017.08.113.

159. Verbert A. *Methods on Glycoconjugates*. Harwood Academic Publishers; 1995.

160. Jakobsson HE, Rodríguez-Piñeiro AM, Schütte A, et al. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Rep*. 2015;16(2):164-177. doi:10.15252/embr.201439263.

161. Wlodarska M, Thaiss CA, Nowarski R, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell*. 2014;156(5):1045-1059. doi:10.1016/j.cell.2014.01.026.

162. Elinav E, Strowig T, Kau AL, et al. NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. *Cell*. 2011;145(5):745-757. doi:10.1016/j.cell.2011.04.022.

163. Mamantopoulos M, Ronchi F, Van Hauwermeiren F, et al. Nlrp6- and ASC-Dependent Inflammasomes Do Not Shape the Commensal Gut Microbiota Composition. *Immunity*. 2017;47(2):339-348.e4. doi:10.1016/J.IMMUNI.2017.07.011.

164. Stappenbeck TS, Virgin HW. Accounting for reciprocal host–microbiome interactions in experimental science. *Nature.* 2016;534(7606):191-199. doi:10.1038/nature18285.

165. Moore RJ, Stanley D. Experimental design considerations in microbiota/inflammation studies. *Clin Transl Immunol*. 2016;5(7):e92. doi:10.1038/cti.2016.41.

166. Johansson MEV, Hansson GC. Preservation of Mucus in Histological Sections, Immunostaining of Mucins in Fixed Tissue, and Localization of Bacteria with FISH. In: *Methods in Molecular Biology*. 2012;842:229-35. doi:10.1007/978-1-61779-513-8_13.

167. Kamphuis JBJ, Mercier-Bonin M, Eutamène H, Theodorou V. Mucus organisation is shaped by colonic content; a new view. *Sci Rep*. 2017;7(1):8527. doi:10.1038/s41598-017-08938-3.

168. Aebersold R, Mann M. Mass-spectrometric exploration of proteome structure and function. *Nature*. 2016;537(7620):347-355. doi:10.1038/nature19949.

169. Steen H, Mann M. The abc's (and xyz's) of peptide sequencing. *Nat Rev Mol Cell Biol.* 2004;5(9):699-711. doi:10.1038/nrm1468.

170. Bantscheff M, Lemeer S, Savitski MM, Kuster B. Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal Bioanal Chem*. 2012;404(4):939-965. doi:10.1007/s00216-012-6203-4.

171. Tytgat KMAJ, van der Wal J-WG, Einerhand AWC, Büller HA, Dekker J. Quantitative Analysis of MUC2 Synthesis in Ulcerative Colitis. *Biochem Biophys Res Commun.* 1996;224(2):397-405. doi:10.1006/BBRC.1996.1039.

172. Tawiah A, Cornick S, Moreau F, et al. High MUC2 Mucin Expression and Misfolding Induce Cellular Stress, Reactive Oxygen Production, and Apoptosis in Goblet Cells. *Am J Pathol.* 2018:188(6):1354-1373 doi:10.1016/j.ajpath. 2018.02.007.

173. Van den Berg BHJ, Tholey A. Mass spectrometry-based proteomics strategies for protease cleavage site identification. *Proteomics.* 2012;12(4-5):516-529. doi:10.1002/pmic.201100379.

174. van der Post S. *Study of the Colonic Mucus Layer by Mass Spectrometry*. Institue of Biomedicine, Department of Medical Biochemistry, Sahlgrenska Academy, University of Gothenburg; 2014. https://gupea.ub.gu.se/handle/2077/36909?locale=sv.

175. Jurjus AR, Khoury NN, Reimund J-M. Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Methods*. 2004;50(2):81-92. doi:10.1016 /j.vascn.2003.12.002.

176. Wirtz S, Neurath MF. Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev*. 2007;59(11):1073-1083. doi:10.1016/j.addr.2007.07.003.

177. Mizoguchi A. Animal models of inflammatory bowel disease. *Prog Mol Biol Transl Sci*. 2012;105:263-320. doi:10.1016/B978-0-12-394596-9.00009-3.

178. Corfield AP. *Glycoprotein Methods and Protocols*: The Mucins. Humana Press; 2000.

179. Issa SMA, Schulz BL, Packer NH, Karlsson NG. Analysis of mucosal mucins separated by SDS-urea agarose polyacrylamide composite gel electrophoresis. *Electrophoresis*. 2011;32(24):3554-3563. doi:10.1002/elps.201100374.

180. Nilsson HE, Ambort D, Bäckström M, et al. Intestinal MUC2 Mucin Supramolecular Topology by Packing and Release Resting on D3 Domain Assembly. *J Mol Biol.* 2014;426(14):2567-2579. doi:10.1016/J.JMB. 2014.04.027.

181. Lo Y-H, Chung E, Li Z, et al. Transcriptional regulation by ATOH1 and its target SPDEF in the intestine. *C Cell Mol Gastroenterol Hepatol*. 2016;3(1):51-71. doi:10.1016/j.jcmgh.2016.10.001.

182. Erben U, Loddenkemper C, Doerfel K, et al. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol*. 2014;7(8).

183. Bradley E, Fedigan S, Webb T, et al. Pharmacological characterization of TMEM16A currents. *Channels.* 2014;8(4):308-320. doi:10.4161/CHAN.28065.

184. Larsson JMH, Karlsson H, Crespo JG, et al. Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation. *Inflamm Bowel Dis.* 2011;17(11):2299-2307. doi:10.1002/ ibd.21625.