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IN VIVO MODELS OF MUCIN BIOSYNTHESIS AND FUNCTION

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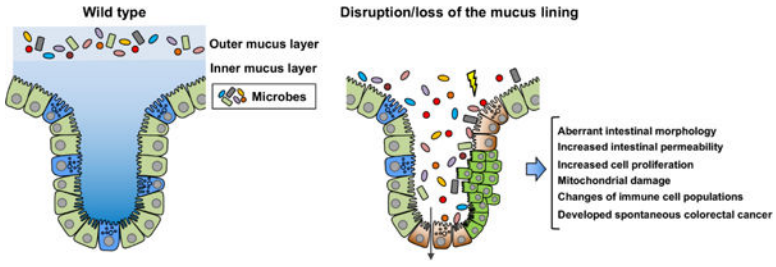
Abstract

The secreted mucus layer that lines and protects epithelial cells is conserved across diverse species. While the exact composition of this protective layer varies between organisms, certain elements are conserved, including proteins that are heavily decorated with N-acetylgalactosamine-based sugars linked to serines or threonines (O-linked glycosylation). These heavily O-glycosylated proteins, known as mucins, exist in many forms and are able to form hydrated gel-like structures that coat epithelial surfaces. In vivo studies in diverse organisms have highlighted the importance of both the mucin proteins as well as their constituent O-glycans in the protection and health of internal epithelia. Here, we summarize in vivo approaches that have shed light on the synthesis and function of these essential components of mucus.

Graphical Abstract



Keywords

mucins; mucus; secretion; secretory granules; O-glycosylation; *Drosophila* ; salivary gland; Muc2; colon; small intestine; Muc5ac; Muc5b

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Competing interests

The authors declare no competing interests.

INTRODUCTION

Mucus and Mucins

Epithelial organs are lined with apical surface matrices, which form a physical barrier and confer protection against external environmental elements. The mucus layer lining the surface of tubular organs forms a continuous physical barrier that protects the underlying epithelial cells from invading pathogens, dehydration, and physical or chemical injury. The mucus layer consists predominately of large, high molecular weight glycoproteins called mucins. Based on biophysical properties, vertebrate mucins have been subdivided into two major subfamilies; transmembrane mucins and gel-forming or secreted mucins (Fig. 1). Transmembrane mucins are found at the apical surface of the mucosal epithelia, as well as on hematopoietic cells. They function in intracellular signaling and as a component of the extracellular glycocalyx (1, 2). Transmembrane mucins have a C-terminal cytoplasmic tail, a transmembrane region, and an extracellular portion characterized by either a SEA (sea urchin sperm protein, enterokinase and agrin) domain or a special variant of the VWD (Von Willebrand factor D) domain along with a repetitive domain composed of tandem repeats rich in the amino acids proline, threonine and serine (the PTS domain) (Fig. 1). The serines and threonines of the extracellular domains are highly O-glycosylated and constitute an essential component of the glycocalyx. The size and number of the tandem repeats vary greatly between mucins (3–6). The PTS domains form scaffolds of dense and heterogenous O-glycosylation and thus form densely arrayed structures envisioned to resemble a bottle brush. The carbohydrate chains comprise up to 80% of the total mass of mucins and contribute to specific physical properties of mucins, such as high viscosity. Several of the human transmembrane mucins are known or predicted to be cleaved in their SEA or VWD domains to yield two subunits that are held together by non-covalent bonding. It has been proposed that dissociation of these two subunits provides a mechanism for mucus shedding to protect the epithelial surface upon shear stress (7, 8). The cytoplasmic tail contains intracellular motifs and putative phosphorylation sites that engage in signal transduction to communicate information about the condition of the external environment to the epithelial cells (9, 10).

Gel-forming mucins are similar in structure to transmembrane mucins but are secreted rather than membrane-bound. A typical gel-forming mucin is composed of N-terminal cysteine-rich VWD domains, followed by a large PTS domain and a C-terminal ‘CK’ domain (Cysteine-knot) (Fig. 1). The N-terminal regions are involved in polymerization through intermolecular disulfide-bonds, while the CK domain at the C-terminus is involved in dimerization of the monomers (11) (Fig. 1). The common characteristic for the gel-forming mucins is the capacity of monomers to form a hygroscopic polymeric branched network which is responsible for the gel-like properties of the mucus lining. Due to their size and polymerization attributes, mucins are thought to be densely packed in a dehydrated state within the secretory granules, mediated by an acidic pH and calcium-based charge shielding. In vitro studies using portions of the intestinal mucin MUC2 or the airway mucin MUC5B have proposed models for packaging mucins into secretory granules, highlighting the importance of pH and calcium (12–15). According to one of the studies, MUC2 is proposed to assemble into a polygonal ring mediated by dimerization at the C-terminus and

calcium-dependent interactions between multiple N-termini, allowing for compaction within a secretory granule. The secretion of the mucins is accompanied by a rise in pH and removal of the calcium ions, which results in rapid unfolding and hydration leading to expansion of mucins in excess of 1000-fold in volume, and formation of higher-order net-like structures that line the epithelial surfaces (16–18).

The proteins and polysaccharides that comprise these protective epithelial barriers vary within the animal kingdom, yet many conserved features exist. The conservation of underlying mechanisms involved in barrier function provides an opportunity to interrogate the molecular role of mucins under normal and disease states in a simpler model system. *Drosophila melanogaster,* with its advanced genetics and molecular tools, is a relevant in vivo model system to study the regulation and biological roles of mucins and O-glycosylation. Many insect epithelia are protected by an apical chitinous cuticle that has an O-glycosylated mucinous component. For example, the fly midgut is lined by a complex structure called the peritrophic membrane (PM), which allows for the selective passage of enzymes and nutrients (19). The PM consists of a scaffold of chitin fibers embedded with highly glycosylated mucins. This composite network is functionally analogous to the vertebrate mucus layer. The *Drosophila* genome encodes several secreted mucin proteins with similar functional regions, including cysteine-rich domains and repetitive PTS domains (Fig. 1). The sites of expression of these mucin-like proteins suggest that they may serve similar functions in barrier formation and protection from environmental insults and microbial invasion (20).

O-glycosylation of mucins

The unique structural and rheological properties of mucins are conferred by the abundant O-linked glycans present within the PTS domains. These glycans can bind water, forming the hydrated, gel-like properties that are characteristics of mucins. Additionally, regions that are densely glycosylated can form rigid rod-like structures that are maximally extended (21, 22). The initiation of O-linked glycosylation begins with the addition of the sugar *N*-acetylgalactosamine (GalNAc) through an α *O*-glycosidic linkage to the hydroxyl group of threonines and serines to form the Tn antigen (GalNAcα-S/T) (Fig. 2). This initiation event is controlled by a large, evolutionarily-conserved family of enzymes known as the UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts or GalNAc-Ts or Galnts in mammals; PGANTs in *Drosophila*; EC 2.4.1.41) (23, 24) (Fig. 2). These enzymes are type II transmembrane proteins that reside within the Golgi apparatus and consist of a catalytic domain and a ricin-like lectin domain. Family members display unique tissue and cell-specific expression patterns (25–27) and unique preferences for particular protein substrates (28–31). There are 19 family members in mice, 20 in humans, and 10 in *Drosophila*. After the initial transfer of GalNAc, additional sugar extensions may occur, including the addition of galactose by the core 1 β1,3-N-acetylgalactosyltransferase (C1Galt1 or T-synthase in mammals; (32)) to form the core 1 or T antigen structure (Galβ1,3GalNAcα-S/T) (Fig. 2). It is worth noting that in mammals (but not *Drosophila*), the C1Galt1 enzyme requires a specific chaperone, known as Cosmc, to have functional activity (33). The core 3 structure (GlcNAcβ1,3GalNAcα-S/T) is formed by the addition of *N*-acetylglucosamine (GlcNAc) by the core 3 β1,3-N-acetylglucosaminyltransferase

(C3GnT) (34) (Fig. 2). In *Drosophila*, O-linked glycans tend to be less extended than their mammalian counterparts and consist predominantly of unextended GalNAc (Tn antigen), the core 1 structure and the glucuronylated core 1 structure (35–40). In mammals, core 1 and core 3 O-glycans can be elaborated by the addition of other sugars to form the branched core 2 or core 4 glycans, respectively (Fig. 2). In mammals, each of these core structures can be further elongated by the addition of other sugars. Finally, the addition of charged sugars (GlcA in *Drosophila* and sialic acid in mammals) or sulfate at the termini can further change the properties of these glycans by virtue of the negative charge they impart (Fig. 2).

Genetic ablation of individual *Galnt/pgant* family members in model organisms has highlighted the importance of O-glycosylation in both organismal viability as well as organ function. Because *Drosophila* has fewer genes encoding PGANTs than mammals (and therefore less functional redundancy within this family), it has been easier to dissect the specific roles of members of this family (40). For example, studies in the fly were the first to identify that a member of the *Galnt/pgant* family was essential for viability (41, 42). Subsequently, at least 4 additional family members have been found to be essential or to substantially influence viability (43). In addition, loss of certain *pgant* family members has resulted in defects in the production and secretion of extracellular matrix components, including mucins, which has had a variety of effects in different cells and tissues (40, 44, 45).

The loss of specific *Galnt*s in mammals has been more challenging to characterize, given the degree of functional redundancy built into the large mammalian family. While the loss of individual *Galnt*s in mice has not yet resulted in lethality, many tissue-specific defects have been characterized (46). For example, loss of *Galnt1* in mice results in defects in lymphocyte homing (47), defects in salivary gland development (due to defects in the secretion of extracellular matrix components; (48)) as well as abnormal cardiac function and heart valve development (49). Loss of *GALNT2* in humans and a variety of model species results in developmental disorders and altered lipid chemistry (50–52). Loss of *Galnt11* in mice results in kidney defects due to the effects of O-glycosylation on the endocytic receptor megalin (53), providing insight into the genome-wide association study that linked polymorphisms near *GALNT11* to chronic kidney disease in humans (54). In mammals, there is a single *C1Galt1* that is ubiquitously expressed and essential for viability (55, 56). Tissue-specific ablation of *C1Galt1* results in defects in the separation of lymphatic and blood vessels during development (55), defective platelet formation and defects in kidney function (57, 58). Acting as a unique molecular chaperone, Cosmc is essential for the formation of active C1Galt1 (33), and deletion of *Cosmc* in mice results in the loss of C1Galt1 activity and phenotypes similar to those seen upon ablation of *C1Galt1*, including the expression of Tn antigen and embryonic lethality associated with hemorrhaging (59). Interestingly, *Cosmc* deficient animals die between E10.5 to E12.5, while *C1GalT* deficient animals die at E13.5 (59). Additional studies in murine endothelial/hematopoietic cells (EHC) lacking *Cosmc* indicate that these extended O-glycans play important roles in platelet biogenesis and function, shedding light on the nature of the hemolytic anemia seen in Tn syndrome patients (58). Recent studies have also found that *Cosmc*-deficient B cells showed cell migration defects associated with impaired chemokine signaling (60), similar to homing defects seen upon the loss of *Galnt1* (47) and further underscoring the roles of O-glycans

in lymphocyte function. In summary, these studies highlight the multifaceted and complex roles of O-glycosylation across many tissues within many organisms. Given the complexity of this post-translational modification that is characteristic of mucins and mucus, many groups have employed in vivo models to study the biosynthesis and biological function of mucus. Highlights from some of the in vivo studies specifically examining mucins or the enzymes that affect their glycosylation, synthesis or secretion are summarized below.

IN VIVO MODELS TO ASSESS MUCUS COMPOSITION, SECRETION AND FUNCTION

Drosophila studies on mucin formation and function

Drosophila tissues produce a variety of mucins that are, like their mammalian counterparts, heavily O-glycosylated by members of the PGANT family. While *Drosophila* mucins are typically smaller in length and size than mammalian mucins, they do contain cysteine-rich regions that allow for the formation of multimeric structures and PTS domains that are heavily O-glycosylated, as mentioned above (20) (Fig. 1).

The *Drosophila* larval salivary glands (SGs) are one of the major secretory organs in the fly and produce abundant amounts of mucins known as salivary gland secretory (Sgs) proteins (61). The SGs synthesize, package and secrete mucins in a highly organized program that is developmentally and hormonally controlled (Fig. 3A). Using a variety of fluorescently-labeled proteins, including mucins (Sgs3-GFP; (62)), real-time confocal and lattice light-sheet imaging techniques have allowed detailed visualization of the entire process of mucin synthesis, packaging and secretion in vivo (62). During the third larval instar, a small pulse of the hormone 20-hydroxyecdysone (20E) initiates the transcription of the *Sgs* genes to begin synthesis of the mucins they encode (Fig. 3B). Sgs proteins are then packaged into secretory granules that remain in the cytoplasm until a second large pulse of 20E during the late third instar, which triggers the fusion of the secretory granules with the plasma membrane and the secretion of the mucins from the SG (Fig. 3B). These Sgs or “glue” proteins are responsible for allowing the larvae to adhere to a substrate in preparation for metamorphosis. Thus the regulated synthesis and expulsion of these mucins is a crucial part of the fly life cycle. This system lends itself to identification and characterization of various factors in mucin biosynthesis given the powerful genetic tools available in *Drosophila*, combined with the fact that mature secretory granules are 3–8 μM in diameter (10–100X larger than those found in mammalian cells), thus providing a great deal of spatial resolution when examining these subcellular structures. Genetic ablation experiments have detailed the essential role of the actomyosin machinery for proper expulsion and secretion of the mucins (63–65). More recent studies revealed that the cargo receptor Tango1 is essential for mucin packaging and secretion in both SGs and the digestive tract (66, 67). High resolution imaging has demonstrated that Tango1 forms a docking site between the ER and Golgi, where cargo proteins such as mucins move from the ER to the Golgi without the need for vesicular trafficking between these compartments (Fig. 3C). Once in the Golgi, mucins are glycosylated by the PGANT family members, and mucin-containing secretory granules bud from the trans-Golgi in a clathrin and AP-1 dependent fashion (68). These small, immature granules undergo homotypic fusion until mature granules are

formed (65, 66). Granule maturation steps are dependent on several endosomally derived proteins, including Rab GTPases (69–71). Additionally, the restructuring of these mucins occurs during this maturation phase so that multiple mucins are packaged within the same granule but are spatially segregated by virtue of their unique intragranular structures (Syed et al., 2021, under review). Interestingly, the loss of certain PGANTs results in aberrant O-glycosylation of the mucins and secretory granules that are abnormal in size and shape, highlighting a crucial role for O-glycosylation of mucins in proper secretory granule structure (72) (Fig. 3D). Future work in this system will focus on the dynamics of mucin hydration and expansion upon secretion as well as the functional role of O-glycosylation in adhesive and antimicrobial properties of mucins. Recent studies performing adhesion force measurements of the endogenous secreted mucins have highlighted their adhesive properties across a variety of substrates and will be informative for assessing the specific impact of O-glycosylation on adhesion (73).

Studies within the larval and adult digestive systems of *Drosophila* have shed further light on the biological roles of mucus and mucins. As mentioned above, the *Drosophila* digestive system, like its mammalian counterpart, contains a protective mucus layer known as the PM (Fig. 4A). Like the mammalian mucous membrane, the PM consists of highly O-glycosylated mucins, antimicrobial peptides, and other components (74, 75). The mucins and other components are embedded in a scaffold of chitin, which is an insect-specific polymeric sugar composed of long linear chains of β1,4 linked N-acetylglucosamine. Genetic studies ablating some components of the PM have revealed that the loss of the chitin-binding glycoprotein known as Drosocrystallin (Dcy) results in a reduction in the thickness of the PM and upregulation of the production of antibacterial peptides upon oral infection with *Pseudomonas entomophila*. In addition, this study also showed that *dcy* mutants showed similar induction of antimicrobial peptides when exposed to the nonlethal strain *Erwinia carotovora 15* (76). Similarly, another study showed that *Drosophila* transglutaminase (TG) is required to crosslink Dcy and that loss of TG also resulted in lethality in the presence of *P. entomophila* (77). Taken together, these studies demonstrate the importance of the PM structure and the crosslinking of Dcy fibers as a physical protective barrier from exotoxins secreted by pathogenic microorganisms.

Ablation of members of the *pgant* family, specifically in the digestive system, leads to severe defects in the formation of the PM and results in lethality, highlighting a crucial role for these family members in this organ system. Loss of *pgant5* results in decreased PM biosynthesis and abnormal gut acidification (43). Interestingly, *pgant4* is specifically expressed in the PR cells of the proventriculus, which are responsible for synthesizing and secreting components of the PM (67). Loss of *pgant4* in these cells results in the complete loss of the PM and lethality (Fig. 4A). Mechanistically, it was determined that *pgant4* is responsible for glycosylating Tango1 (the protein responsible for forming docking sites between the ER and Golgi) in PR cells. Loss of *pgant4* results in furin-mediated proteolysis of Tango1, disruption of secretory apparatus structure, defects in the secretion of mucins and lethality (45, 67), indicating that *pgant4* plays an essential role in mucin biosynthesis and secretion within the digestive system.

Mutations in *pgant4*, which result in the complete absence of the protective PM, represent a novel in vivo system to examine the effects of the loss of this membrane within the larval digestive system, which is under a constant barrage of mechanical and microbial insults (45). It is worth noting that the larval stage of development is characterized by continuous feeding on solid food consisting of fermented fruit, in contrast to the adult stage, where animals primarily consume liquids. Loss of the PM during the larval stage resulted in dramatic epithelial cell damage and apoptosis (Fig. 4A). In addition, damaged epithelial cells were found to secrete the IL-6-like cytokine Upd3. Interestingly, the upregulation of Upd3 activated JAK/STAT signaling within the progenitor cell niche, a normally quiescent group of cells that will later form the adult digestive system during metamorphosis. This niche disruption resulted in aberrant proliferation of the adult midgut progenitor cells and trans-differentiation of the niche cells. Increased antimicrobial peptide production upon loss of the PM was also noted (45). Niche disruption could be induced by overexpressing *upd3* or rescued by deleting *upd3*, highlighting the essential role of this cytokine produced by damaged epithelial cells. Recently, studies in mammalian systems have also shown that damaged mammalian epithelial cells similarly upregulate IL-6, suggesting conserved signaling mechanisms in tissue damage responses (78). In the *Drosophila* system, *upd3* expression from damaged cells could be partially rescued by treatment with antibiotics, highlighting a role for microbes in this damage-induced signaling cascade (45) (Fig. 4A). Taken together, this study highlights the importance of PM/mucous membrane for epithelial protection and stability of the progenitor cell niche and identifies the specific signaling cascades that are activated in response to tissue damage. This in vivo system has the potential to be used as a screening platform for mucin mimetics or other therapeutics directed at mucin production, secretion, stability, and function.

Mucus formation and function in mammals

MUC5AC and MUC5B—MUC5AC and MUC5B are the major mucin components of respiratory mucus (79), which forms a barrier to trap and remove pathogens via mucociliary clearance (MCC) (80). Diseases of the respiratory tract, such as chronic obstructive pulmonary disease and asthma, are associated with mucus overproduction and impaired MCC (79). To assess the role of these mucins in the protection of the respiratory system in vivo, mice were created where either *Muc5b* or *Muc5ac* were ablated (81). Loss of *Muc5b* resulted in severe disruptions in MCC. *Muc5b*−/− mice had upper airway obstructions and failed to clear debris from upper and lower respiratory tracts. Additionally, *Muc5b*−/− mice displayed chronic infections by multiple bacterial species, including *Staphylococcus aureus*, and had inflammation that failed to resolve (81). Spontaneous lethality could be rescued by antibiotic treatment, indicating that the cause of death was due to infection. Similar debris accumulation and chronic infections were also present in the middle ear, suggesting a role for Muc5b there as well. Various immune system defects were also seen in the *Muc5b*−/− mice, including decreased levels of the cytokine IL-23, decreased phagocytosis, and accumulation of apoptotic macrophages, suggesting that Muc5b is required for proper phagocyte clearance (81). Interestingly, loss of Muc5ac did not result in any of these phenotypes, suggesting that Muc5b is the essential mucin for particulate and bacterial clearance in the airways of mice (81). In humans, genetic variants that result in increased expression of MUC5B are a risk factor for developing idiopathic pulmonary fibrosis (82).

In support of a functional link between lung fibrosis and *Muc5b* overexpression, mice designed to have increased expression of *Muc5b* in the respiratory system showed evidence of aberrant MCC and enhanced lung fibrosis (83).

Muc5ac is also one of the major mucins present within the stomach. Recent studies using *Muc5ac*-deficient mice have demonstrated that they suffer from increased inflammation and epithelial defects within the gastric system (84). Additionally, these mice developed spontaneous antropyloric proliferation and adenomas, along with displaying higher colonization densities when exposed to *Helicobacter pylori.* Taken together, this study highlights the protective role of Muc5ac within the gastric system.

MUC2—As mentioned above, the major intestinal mucin in mammals is Muc2 (MUC2 in humans). Detailed studies of the in vivo composition and structure of Muc2 have revealed its complexity within the digestive system. Studies in mice have shown that Muc2 exists as a single, loosely-organized, permeable layer within the small intestine (SI), which is not attached to the underlying epithelia (1, 16, 85–87). This layer is rich in bacteria and also contains antibacterial peptides/proteins that limit bacterial contact with the epithelia (88). Indeed, genetic ablation of one such antibacterial protein (RegIIIγ) in mice resulted in bacteria reaching the SI epithelium (88). Interestingly, this study showed a sophisticated feedback system where enterocytes of the SI sense the presence of bacteria and up-regulate RegIIIγ levels within the mucus layer to protect the epithelium, highlighting the dynamic nature of mucus and how its composition can be altered in response to environmental signals in vivo (88). In vivo labeling of mucins within the SI using N-acetylgalactosamine (which labels O-linked glycans on mucins) demonstrated different rates of mucin biosynthesis by different subsets of goblet cells, with those in the villi area undergoing more rapid synthesis than those in the crypt (5).

In contrast to the SI, Muc2 in the colon is organized into 2 distinct layers (85–88); an outer layer that is rich in commensal bacteria and a tightly packed inner layer that is attached to the epithelium and devoid of bacteria (85–88) (Fig. 4B). Studies in mice have demonstrated the unique relationship between the mucus layer, diet and the intestinal microbiota. Mice fed a Western style diet displayed altered colonic microbial composition and changes in the penetrability and growth of the inner mucus layer (89). The mucus layer defects could be rescued by dietary supplementation with prebiotic fiber inulin or by administering the probiotic *Bifidobacterium longum*. The authors concluded that the presence of a distinct bacteria is critical for proper mucus barrier formation and function (89). Recent studies in mice have revealed additional details of mucus within the colonic system and its dependence on the microbiota (90). Detailed imaging of Muc2 throughout the colon revealed that the inner mucus layer actually consists of two biochemically distinct layers based on glycosylation status, one being produced by the proximal colon goblet cells and the other produced by distal colon goblet cells. The layer produced by the proximal colon goblet cells is responsible for encapsulating the fecal pellet and microbiota. Moreover, this study further demonstrated that microbiota induced *Muc2* expression in the proximal colon goblet cells and that O-glycans on the proximal colon-derived Muc2 influenced the composition of the microbiota, indicating a complex and dynamic interplay between the cells that produced

and secrete the mucus lining and the microbial communities that reside within the intestinal system (90).

Other in vivo studies have identified key factors unique to the formation of the nonattached mucus layer of the SI in mice (91). This detached layer was found to be dependent on the metalloendopeptidase meprin, which is expressed in the enterocytes of the SI. Mice deficient for *meprin* displayed a much more dense mucus layer that was more firmly attached within the SI. This study also identified the importance of proper Muc2 unfolding in the presence of bicarbonate upon secretion, as mice that were deficient in the cystic fibrosis transmembrane conductance regulator (CFTR, which regulates transport of Cl^- and HCO_3^-) channel had attached mucus which could not be cleaved by the addition of exogenous meprin. These in vivo studies highlight the integrated role of ions and enzymes in the proper unfolding, release and formation of the mucus layer of the SI (91).

The in vivo importance of Muc2 within the mammalian digestive system was elucidated upon ablation of *Muc2* in mice. *Muc2*−/− mice displayed aberrant intestinal morphology, increased cell proliferation, decreased apoptosis and developed spontaneous colorectal cancer (92) (Fig. 4B). Further characterization demonstrated that the absence of Muc2 also resulted in the spontaneous development of colitis (93–95) as well as increased intestinal permeability and mitochondrial damage (96) (Fig. 4B). Interestingly, the phenotypes present in the *Muc2*−/− mice, such as bacteria in close contact with the epithelial cells ((97, 98)) and changes to immune cell populations (elevated neutrophils, T cells, and macrophages), mimicked those seen in biopsies from patients with ulcerative colitis (99, 100), highlighting the importance of this mucin in intestinal health.

Studies in the mouse digestive system have identified novel cells that synthesize and secrete Muc2, in addition to the canonical goblet cells at the base of the crypt. For example, sentinel goblet cells, which lie at the entrance of the crypt and sense bacteria, secrete massive amounts of Muc2 in response to bacteria, allowing for bacterial expulsion and protection of the crypt (101). These cells are released from the epithelium once they have secreted their Muc2, highlighting another important cell type in vivo that is responsible for the production of the mucus and protection of the intestinal epithelia (101). More recent studies performing single-cell transcriptomic and proteomic analyses have identified novel intercrypt goblet cells (icGC) that lie outside of the crypt and secrete mucus with unique properties. Using live tissue explants, Nystrom et al., (102) demonstrated that this mucus-filled the area between the canonical mucus secreted from the crypt regions. Unlike the crypt mucus, this mucus is impenetrable to bacteria and more penetrable to smaller molecules. The importance of these novel goblet cells and the mucus they secrete in vivo was highlighted using a mouse model with dysfunctional igGCs, which had a compromised mucus barrier, and increased susceptibility to induced and spontaneous colitis (102). Taken together, these in vivo studies highlight the complexity of mucus formation, composition and function, and the dynamic interplay between mucus and the many components that make up the intestinal environment.

Mucin-type O-glycosylation in mammalian mucus

As mentioned earlier, one of the key features of mucus and mucins is the abundance of O-linked glycans present, which confer many of the structural, rheological, adhesive and anti-

microbial features that have been documented in diverse in vitro and cell culture systems (1, 85, 103, 104). While genetic ablation of individual mucins informs how the loss of the entire entity (protein core and all associated glycans) affects the intestinal system, it does not address the specific roles of specific glycans on Muc2 that are unique to the digestive system. Detailed characterization of the glycans present on mouse Muc2 has shown that within the colon, core 1 and core 2 (that can be neutral or sialylated) are predominant (105) (Fig. 2). Moreover, there are region-specific differences along the intestinal tract in terms of specific extensions of O-glycans (90, 106), highlighting the complexity of these modifications. The glycans on human colonic MUC2 are predominantly core 3 and contain the charged sialic acid sugar (NeuAc) (107), which differ substantially from those seen in mice (105), highlighting key differences in the structure/composition of mucus between rodents and humans. The abundance of O-glycans, along with studies demonstrating altered O-glycosylation profiles of MUC2 in ulcerative colitis, suggest that O-glycosylation plays an important role in digestive system health (108).

GalNts/GalNAc-Ts

The roles of the specific glycosyltransferase that initiate mucin-type O-glycosylation in the formation and function of the mucus layer in mammals are just beginning to be investigated. However, association studies have identified somatic and germ-line mutations in *GALNT12* in individuals with colon cancer (109). *GALNT12* is one of the most abundantly expressed family members in the colon, and studies in mouse models are underway to assess its influence on mucus within the intestinal tract.

The human disease hyperphosphatemic familial tumoral calcinosis, which is characterized by proteolysis of the phosphate regulating hormone FGF23 and dysregulated blood phosphate levels, is caused by mutations in *GALNT3*(110). Loss of *Galnt3* in mice results in similar hyperphosphatemia (111) and influences the glycosylation of a major secreted mucin (Muc10) of the oral cavity (112). Interestingly, the composition of the oral microbiome in *Galnt3*−/− mice is altered and less stable over time relative to WT, suggesting that O-glycans present on secreted mucins that comprise the oral mucus modulate stability and diversity of the microbiome (112).

Core 1- and Core 3-O-glycans in mucus of the digestive system

Initiation of the synthesis of the core 1 structure in mammals is catalyzed by the core 1 glycosyltransferase C1Galt1 in combination with its specific chaperone Cosmc (Fig. 2). Targeted ablation of *C1Galt1* using an intestinal epithelium-specific Cre recombinase (IEC *C1Galt1*−/−) results in a compromised mucus barrier and spontaneous colitis in mice, mimicking human ulcerative colitis (113) (114). Targeted ablation of *C1Galt1* also resulted in changing the nature of the O-glycans found on Muc2, with a loss of core 1 and core 2 O-glycans (that could be neutral or sialylated) and the presence of core 3 and core 4 O-glycans that were highly sialylated (105). However, other studies using this mouse model did not find evidence of spontaneous colitis but rather increased susceptibility to induced colitis (115). The authors attributed the differences in colitis severity to differences in animal housing. In terms of the interplay between the mucus layer and the microbiota, Sommer et al. (115) also found evidence of altered microbial composition and an altered intestinal

architecture in these mice, suggesting that the content of the mucus layer plays a role in regulating microbiota and intestinal homeostasis.

Recent studies in mice lacking *C1Galt1* in gastric epithelial cells (GEC *C1Galt1*$^{-/-}$) provided insight into the role of core 1 O-glycans in proper gastric mucus function (116). Loss of *C1Galt1* in mouse gastric epithelial cells resulted in aberrant expression of *Muc5AC* and *Muc1*, loss of the gastric mucus layer, and spontaneous gastritis and gastric cancer, driven by caspase 1 and caspase 11-dependent mucosal inflammasome (116). Interestingly, it was found the gastric microbiota did not play an essential role in caspase activation. Additionally, the basal gastric pH of GEC *C1Galt1*$^{-/-}$ mice was higher than wild type mice, suggesting that the mucus diffusion capacity is disrupted upon loss of proper O-glycans (116).

A previous genome-wide association study identified the C1Galt1 chaperone Cosmc as a risk factor for intestinal bowel disease (IBD) (117), and subsequent mouse studies confirmed its involvement in intestinal mucus formation (118). Furthermore, mice deficient for *Cosmc* within intestinal cells displayed compromised mucus, bacteria-dependent inflammation and increased susceptibility to experimental colitis (118). Thus, this in vivo study offered additional support for the role of Cosmc and core1 O-glycans in the proper formation and function of the mucus layer in mammals.

While C1Galt1 is ubiquitously expressed, C3GnT that initiates the synthesis of core 3 O-glycans is most highly expressed in the proximal region of the mouse colon (119). Mice lacking *C3GnT* within intestinal epithelial cells had reduced Muc2 mucus barrier in the colon and increased permeability. These mice were also more susceptible to induced colitis and colorectal tumors (120). In line with this, IEC *C3GnT*−/− mice also displayed increased colonic epithelial cell proliferation and increased Wnt signaling (120).

Interestingly, mice deficient for both core 1- and core 3-derived O-glycans in intestinal epithelial cells (IEC *C1Galt1*−/−;*C3GnT*−/− double knockouts) resulted in spontaneous colitis that was bacterially independent (121). In addition, moderate inflammation was observed in the duodenum (121), in contrast to the severe inflammation seen in the colon (122). Within the duodenum, there was also a difference in the mucus lining relative to WT, with very little Muc2 seen in the lumen, indicating that the loss of O-glycosylation resulted in a decrease in either the stability or secretion of Muc2 (121). The increased proliferation in the crypt in these double KO mice was observed along with a predisposition to duodenal tumorigenesis in aged adult mice (121). Taken together, these results indicate that the O-glycans normally abundant within the mucus lining play an essential role in duodenal health (121).

The colonic mucus barrier of the same IEC *C1Galt1*−/−;*C3GnT*−/− double knockout animals was examined and compared to WT and each single-gene knockout (IEC *C1Galt1*−/− or IEC *C3GnT*−/−) (122). IEC *C1Galt1*−/−;*C3GnT*−/− double knockouts displayed spontaneous colitis that was more severe and developed earlier than seen in the IEC *C1Galt1*−/− single knockouts. Moreover, spontaneous colitis was seen in both the proximal and distal colon of IEC *C1Galt1*−/−;*C3GnT*−/− double knockout animals, whereas it was

only present in the distal colon of IEC *C1Galt1*−/− animals. While a reduction in the thickness of the mucus barrier was present in the IEC *C1Galt1*−/− single knockouts, the barrier was absent in the IEC *C1Galt1*−/−;*C3GnT*−/− double knockouts, indicating that the degree of O-glycan loss correlated with the thickness/integrity of the mucus barrier, as well as the severity of inflammation. Interestingly, antibiotic treatment of the IEC *C1Galt1*−/−;*C3GnT*−/− double knockouts could partially restore the mucus barrier and attenuate the colitis, suggesting that O-glycans normally present protect mucins from degradation by microbes. In support of this, it was found that Muc2 from the IEC *C1Galt1*−/−;*C3GnT*−/− double knockout animals was more sensitive to proteolysis than that from WT mice (122). An additional study from the same group also reported that IEC *C1Galt1*−/−;*C3GnT*−/− double knockout animals developed spontaneous colorectal tumors earlier than those seen in IEC *C1Galt1*−/− (119). Tumor development was also reduced with antibiotic treatment (119). Taken together, these in vivo studies highlight both the crucial role of the mucus layer in the protection of the colonic epithelia as well as the essential role of O-glycans in the protection of the mucus layer from bacterially-induced degradation (Fig. 4B).

CONCLUSIONS

The complexity of mucin/mucus biosynthesis, modification, and secretion highlight the need for systems that can interrogate biological functions in a native, in vivo setting. Powerful imaging platforms, combined with fluorescently-labeled mucins, have enabled unprecedented visualization of the mucus layer in vivo. In addition, new molecular and bioinformatic tools allow the identification of novel cell populations involved in the synthesis of mucus and mucins in vivo. Genetically tractable model organisms that allow one to study the in vivo consequences of disruption of components of the mucus lining can also be used as platforms to test potential therapeutics, mucin mimetics, or factors that modify the structure or composition of mucus. Taken together, these in vivo approaches are vital to attaining a comprehensive understanding of the multifaceted roles of mucus in both health and disease.

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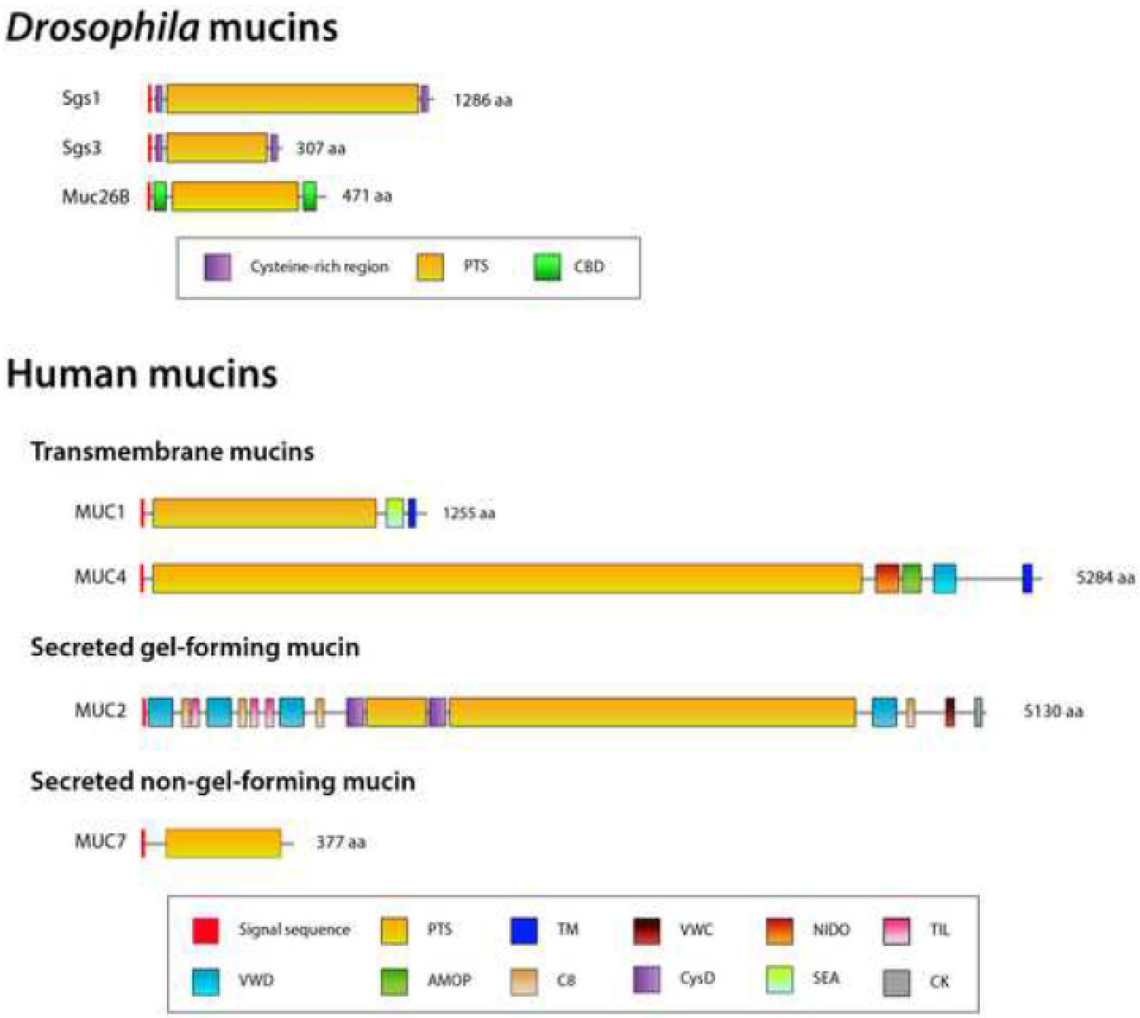

Figure 1. *Drosophila* and human mucins.

Shown are *Drosophila* and human mucins. Human mucins fall into 3 categories: transmembrane, secreted gel-forming and secreted non-gel forming. Both *Drosophila* and human mucins have highly O-glycosylated PTS domains as well as domains that are involved in multimer formation. Sgs1, salivary gland secretion protein 1; Sgs3, salivary gland secretion protein 3; Muc26B, mucin 26B; PTS, proline-threonine-serine domain; CBD, chitin-binding domain. TM, transmembrane domain; VWC, Von Willebrand C-domain; NIDO, nidogen homology region; TIL, trypsin inhibitor-like cysteine-rich domain; VWD, Von Willebrand D-domain; AMOP, the adhesion-associated domain; C8, conserved cysteine-rich domain; CysD, domain rich in cysteine residues; SEA, sea urchin sperm protein, enterokinase and agrin domain; CK, cysteine knot domain.

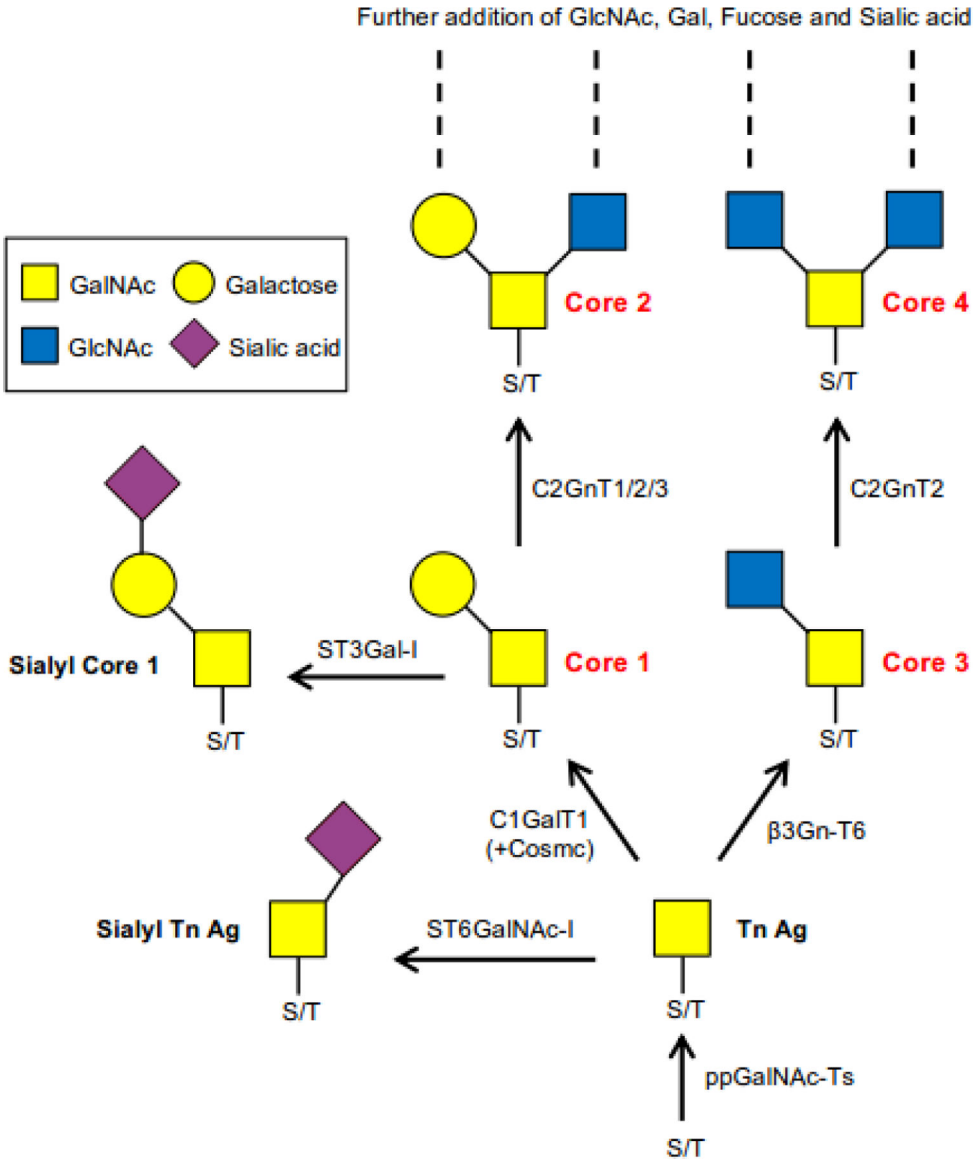

Figure 2. Mucin-type O-glycosylation.

The enzymes responsible for the synthesis of mucin-type O-glycans and the common core structures are shown. S/T=serine or threonine.

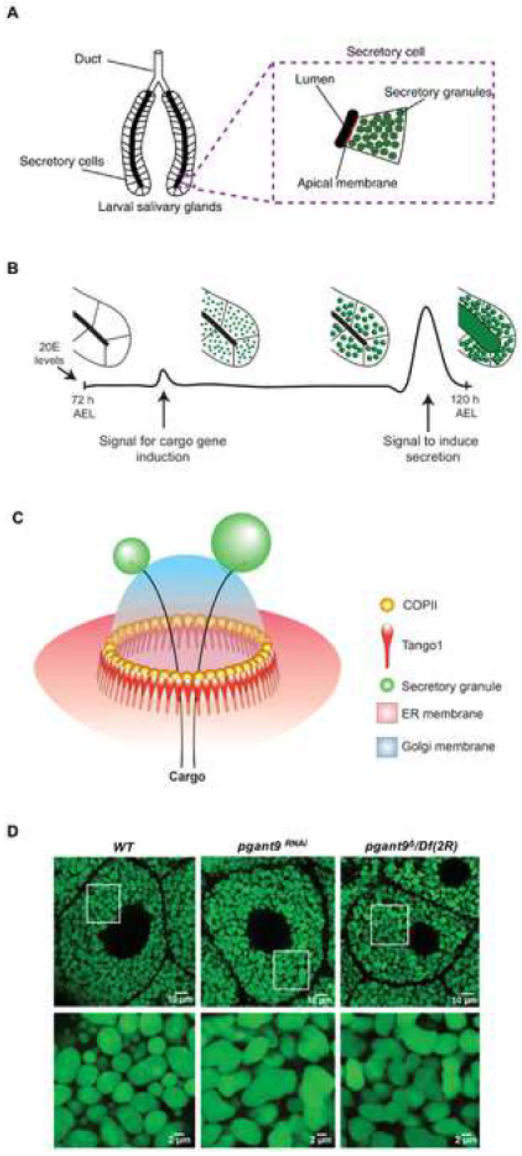

Figure 3. Biosynthesis and packaging of mucins within the *Drosophila* salivary glands.

(A) A *Drosophila* third instar larval salivary gland (SG) is shown. An enlarged single secretory cell with secretory granules (green) containing mucins is shown. **(B)** Secretory mucin formation and secretion are regulated by the hormone 20-hydroxyecdysone (20E) during 3rd instar larval development. Mucin gene expression and protein synthesis are induced by a small 20E pulse during the early 3rd instar larval stage. Mucins are then packaged into small secretory granules (green dots), which fuse to each other to form mature granules. During the late 3rd instar larval stage, a large pulse of 20E induces the secretion of secretory granule contents. AEL, after egg lay. **(C)** Model of Tango1 forming a docking site between the ER and Golgi to facilitate mucin movement between compartments and secretory granule formation. Tango1 forms ring structures that mediate the formation of COPII rings, which serve as docking sites for the cis Golgi to assist secretory mucin movement from the ER to the Golgi. Secretory granules bud from the trans-Golgi side

of these structures. **(D)** Loss of PGANT9 results in abnormal secretory granules with an irregular, shard-like appearance, suggesting that O-glycosylation of mucin influences the maturation and morphology of secretory granules.

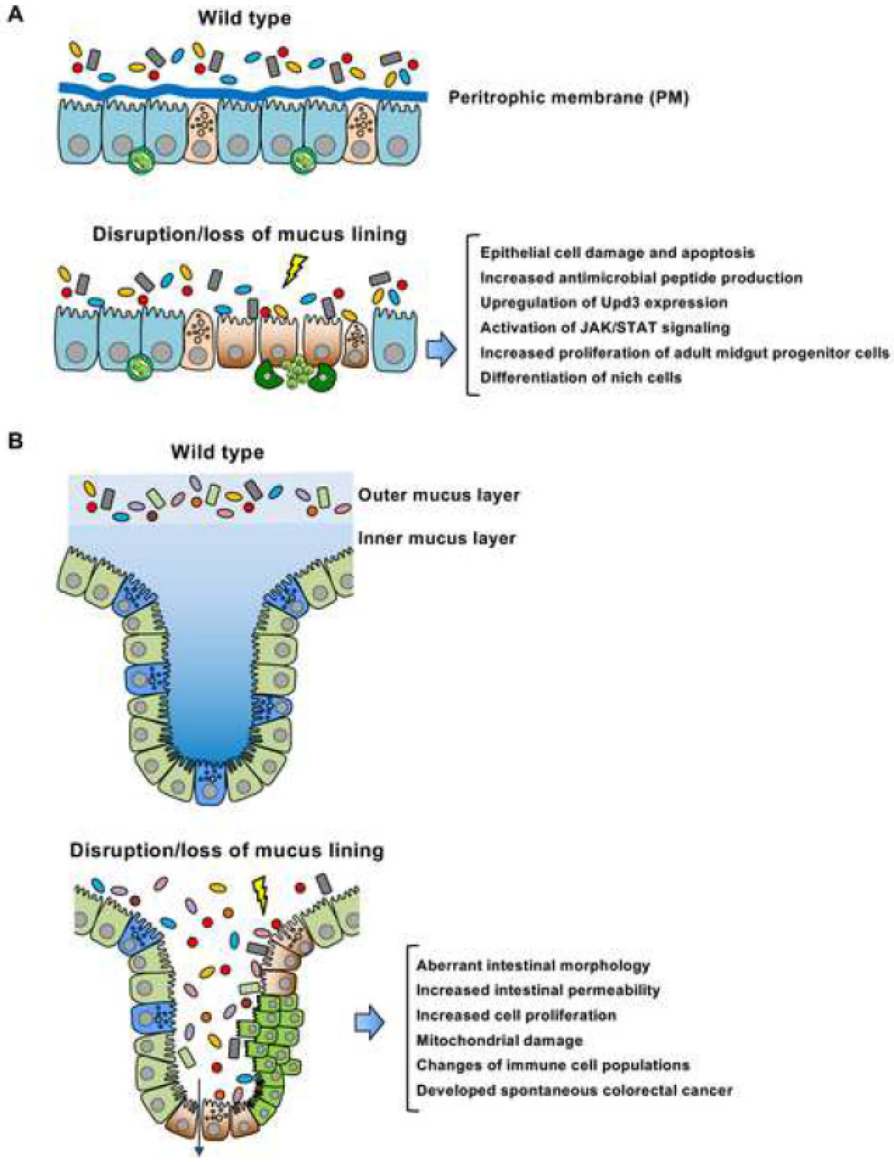

Figure 4. The protective mucus layer plays essential roles in health and homeostasis in the *Drosophila* and mammalian digestive systems.

(A). The peritrophic membrane (PM) of the *Drosophila* larval midgut contains highly O-glycosylated mucins and protects the epithelium from mechanical and microbial damage. Disruption/loss of this protective mucus layer results in epithelial cell damage, activation of the host defense response, and induction of specific signaling cascades that lead to increased cell proliferation and disruption of progenitor cell niche. B. The mucus lining of the mammalian colon, which is comprised of the highly O-glycosylated mucin Muc2, exists as 2 layers: an outer layer that is rich in commensal bacteria and a tightly-packed inner layer that is attached to the epithelium and devoid of bacteria. Disruption/loss of this mucus lining in the mammalian colon results in aberrant intestinal morphology, increased intestinal permeability, intestinal inflammation, increased cell proliferation and spontaneous colorectal cancer.