A BACTERIAL LYTIC POLYSACCHARIDE MONOOXYGENASE GBPA PROMOTES EPITHELIAL PROLIFERATION IN *DROSOPHILA MELANOGASTER*

By

STEPHANIE LORRAINE VANBEUGE

A DISSERTATION

Presented to the Department of Biology

and the University of Oregon Division of Graduate Studies

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

September 2022

DISSERTATION APPROVAL PAGE

Student: Stephanie VanBeuge

Title: A Bacterial Lytic Polysaccharide Monooxygenase GbpA Promotes Epithelial Proliferation in *Drosophila melanogaster*

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Victoria Herman	Chairperson
Karen Guillemin	Advisor
Judith Eisen	Core Member
Annie Zemper	Core Member
Michael Harms	Institutional Representative

and

Krista Chronister	Vice Provost for Graduate Studies

Original approval signatures are on file with the University of Oregon Division of Graduate Studies.

Degree awarded September 2022

© 2022 Stephanie Lorraine VanBeuge

DISSERTATION ABSTRACT

Stephanie Lorraine VanBeuge

Doctor of Philosophy

Department of Biology

September 2022

Title: A Bacterial Lytic Polysaccharide Monooxygenase Promotes Epithelial Proliferation in *Drosophila melanogaster* Through Innate Immune Signaling

Animals are colonized by a consortium of microbes that sense and respond to their immediate environments. These microbes, collectively called the gut microbiota, promote epithelial proliferation in a diversity of animal hosts. While the effect of this relationship is well established, the mechanism underlying this response is less understood. In this work, we establish a molecular connection between colonization by the microbiota and the resulting increase in gut epithelial proliferation. We show that different homologs of a highly conserved chitin degrading enzyme promote epithelial proliferation in both zebrafish and fruit flies. Probing the mechanism of this conserved relationship in flies, we show that other enzymes that compromise the chitin lining of the gut will also stimulate epithelial proliferation. Finally, we find that proliferation is a result of innate immune sensing of increased concentration of luminal GlcNAc monomers which are the product of chitin-cleaving enzymes. The comparative work presented in this dissertation explores a new way of thinking of host-microbiome relationships that focuses on microbial function over identity or abundance of specific species.

CURRICULUM VITAE

NAME OF AUTHOR: Stephanie Lorraine VanBeuge

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene

University of Utah, Salt Lake City

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2022, University of Oregon

Bachelor of Science, Cell and Molecular Biology, 2017, University of Utah

AREAS OF SPECIAL INTEREST:

Cell and Molecular Biology

Molecular Genetics

Host/Microbiome Interactions

PROFESSIONAL EXPERIENCE:

Graduate Teaching Assistant, University of Oregon, AY 2017-18

Research Associate, Nitin Phadnis lab, Department of Biology, University of Utah, 2015-2017

Research Associate, Yingbin Fu lab, Department of Ophthalmology, University of Utah, 2014-2015

GRANTS, AWARDS, AND HONORS:

Raymond-Stevens Fellowship, University of Oregon, 2022

Genetics Training Program Trainee, National Institutes of Health, 2018-2020

Undergraduate Research Opportunities Program Trainee, University of Utah, 2016-2017

PUBLICATIONS:

Kumar, S., Nakashizuka, H., Jones, A., Lambert, A., Zhao, X., Shen, M., Parker, M., Wang, S., Berriochoa, Z., Fnu, A., **VanBeuge, S.**, Chevez-Barrios, P., Tso, M., Rainier, J., Fu, Y. (2017) Proteolytic Degradation and Inflammation Play Critical Roles in Polypoidal Choroidal Vasculopathy. The American Journal of Pathology, 187(12), 2841-2857. DOI: https://doi.org/10.1016/j.ajpath.2017.08.025

A. Banse, **S. VanBeuge**, T. J. Smith, S. L. Logan, K. Guillemin, "Secreted *Aeromonas* GlcNAc binding protein GbpA stimulates epithelial cell proliferation in the zebrafish intestine." bioRxiv 2022.06.27.497793; doi: https://doi.org/10.1101/2022.06.27.497793

VanBeuge, S., Wong, Z. C., Sweeney, E. G., Guillemin, K., "A lytic polysaccharide monooxygenase GbpA stimulates intestinal epithelial cell proliferation in *Drosophila melanogaster*." In preparation.

ACKNOWLEDGEMENTS

They say it takes a village to raise a child. I would argue it also takes a village to get a PhD. Although I've been the one to do the work of getting the degree, I would not have been able to succeed without my village there to help me.

To my mentor Dr. Karen Guillemin, I owe the deepest thanks for your patience, your kindness, and most importantly your willingness to take me on as a student and teach me. I count myself as incredibly lucky to have been your student. I will keep in mind the important lessons you've taught me about being the best scientist I can be, thinking outside of the box but within the realm of possibility, and never being afraid to ask questions. Thank you for everything.

Thank you to Dr. Emily Sweeney for taking me in as a new grad student and showing me what it takes to be an excellent scientist. Thank you as well to my friends and mentors in lab Elena Wall, Drs. Jarrod Smith, Michelle Massaquoi, and Kristi Hamilton for all their support and advice and for helping me learn and succeed as a young scientist. I would also like to thank the members of my dissertation committee, Drs. Tory Herman, Judith Eisen, Mike Harms, and Annie Zemper, for their guidance and expertise in helping me make decisions about my thesis project.

I've found a lifelong friend in Grace, and I'm grateful I've been able to experience graduate school with her and with the amazing group of friends I've made in school. I've loved spending our summers together floating down the Willamette and our Friday nights exploring what Eugene has to offer as well as always having someone to sit next to at seminars. Thank you all so much for your friendship and support through school.

I would like to acknowledge my mom and stepdad, who have been unwavering pillars of support and have always made me feel like a boat with the wind in my sails, gliding over obstacles and continuing forward. Thank you for everything Mom and Larry. I would also like to thank my dear friend, Connor, who helped me make the decision to attend University of Oregon in the first place. Without you, I would not have had the same journey.

I would like to extend a special thank you to my care team, including Drs. Randy Jensen, Howard Colman, Donald Cannon, and their teams of talented and caring medical professionals. Thank you for taking care of me when I needed it most.

To the Wolbach family, thank you for kindness and encouragement through this process. Finally, to my partner Taylor, thank you for your support through everything, for listening to me talk about every thought that enters my head, for always being there to make me laugh at the end of a rough day or week, and for your unending patience and kindness.

To my village

TABLE OF CONTENTS

TABLE OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Bacteria and chitin: same concepts, different animals

Bacteria are one of the most abundant life forms on Earth. In addition to inhabiting biotic and abiotic surfaces, the ocean, and soil, bacteria also colonize animals (Douglas, 2018; Flemming and Wuertz, 2019; Milligan-Myhre et al., 2011). The collective term given to the diverse consortia of microbes that colonize animal guts is the gut microbiota. While this collection of bacteria, viruses, and fungi can often be overlooked when thinking about the organs that comprise the human and other animal bodies, these resident microbes play a key role in host development and maintaining homeostasis (Al-Asmakh and Zadjali, 2015; Leulier et al., 2017; McFall-Ngai et al., 2013; Rawls et al., 2004; Sommer and Bäckhed, 2013; Walsh and Guillemin, 2022). Studies focusing on the effect of the gut microbiome have shown that exposure to specific bacteria and bacterial products can even influence animal behaviors (Cryan and Mazmanian, 2022; Lyte et al., 1998). While the gut microbiota is required for host animals to meet many important developmental milestones and maintain cellular and systemic homeostasis (Hill et al., 2016; Rawls et al., 2004), this consortium is constantly being surveilled by the host immune system for colonization by pathogenic bacteria and general gut dysbiosis (Pickard et al.; Pott and Hornef, 2012; Round and Mazmanian, 2009). Even in mutualistic relationships, the host immune system senses bacterial activities to maintain cellular homeostasis and execute critical functions (McFall-Ngai et al., 2013).

Resident gut bacteria also surveil their hosts to make decisions such as what tissues to colonize. A key class of host molecules bacteria sense are glycans, such as the chitin-rich lining of the arthropod intestine or the heavily glycosylated mucin lining of the mammalian digestive tract (Bergstrom and Xia, 2013; McFall-Ngai et al., 2013; Varki, 2017). An example of this kind of glycan-mediated relationship comes from bobtail squid, which is colonization by luminescent symbiont *Vibrio fischerii* that navigates to it residence in the light organ along chitin gradients (Pan et al., 2015).

In addition to surveying host-derived glycan landscapes, the gut microbiome is constantly receiving additional stimuli as the host ingests glycan-containing food which then travels through the digestive tract. Food consumed by host animals is often varied and can bring with it differing types of bacteria or different nutrients that can be used by both bacteria and host as energy sources. Some of the components of these ingested foods, such as large polysaccharide polymers, are preferentially broken down by bacterial enzymes.

One of the most abundant such polymers, chitin, is widely represented in nature including in crab shells, arthropod exoskeletons, and fungi cell walls (Tang et al., 2015; Yang and Zhang, 2019; Zhang et al., 2021). Chitin is a complex polymer composed of β-(1→4)-linked N-acetylglucosamine (GlcNAc) monomers (Islam et al., 2017). This biomolecule is both highly diverse and modular, capable of taking the form of smaller fragments of polymers or larger crystalline sheets (Alvarez, 2014; Islam et al., 2017). Chitin degradation is a highly conserved cellular function, given the high abundance of this carbon source in nature (Madland et al., 2021; Sabbadin et al., 2018; Vaaje-Kolstad et al., 2017; Vandhana et al., 2022). Organisms as distantly related as mammals and

bacteria endogenously produce their own forms of chitinases (Meibom et al., 2004; Zhao et al., 2020). In vertebrates like humans, mice, and zebrafish, these chitinases are often expressed in response to infection by a pathogen (Teng et al., 2014; Zhao et al., 2020). In arthropods like *Drosophila*, chitinases can be expressed for a variety of other reasons, including cuticle molting at critical life stages (Pesch et al., 2016).

Like the endogenous production of chitinases, innate immune sensing of chitin is also highly conserved through the tree of life, with Toll Like Receptor 2 (TLR-2) predicted to be one of the receptors sensing and surveying chitin fragments in the gut lumen (Alvarez, 2014; Bueter et al., 2013; da Silva et al., 2008; Elieh Ali Komi et al., 2018; Fuchs et al., 2018; Lee et al., 2008; Reese et al., 2007). Previous work in mammalian cell lineages has shown that increasingly small sizes of chitin particles (<70 μm) stimulate a pro-inflammatory immune response (Alvarez, 2014; da Silva et al., 2008; Fuchs et al., 2018). Although arthropods and mammals are separated by millions of years of evolutionary time, they both appear to sense and respond to chitin and its enzymatic breakdown products. In the work presented here, we analyze the mechanism behind a highly conserved relationship that follows this pattern of mutual surveillance: intestinal epithelial proliferation in response to colonization by the gut microbiota (Cheesman et al., 2011; Jones and Guillemin, 2018; Jones et al., 2013a).

Lytic polysaccharide monooxygenases are highly conserved microbial modifiers of the chitin landscape

Because microbes are constantly encountering chitin, an abundant source of carbon, it is unsurprising that they have developed multiple ways to degrade this biomolecule and use the monomers for cellular metabolism. Ancestral microbes evolved

a way to effectively degrade chitin through use of an enzyme that was not restricted to binding to free ends of chitin (Vandhana et al., 2022). This type of enzyme, called an endochitinase, can bind to crystalline chitin anywhere along the length of the polymer (Wong et al., 2012). In this body of work, we will look at how a class of endochitinases, lytic polysaccharide monooxygenases (LPMOs), interact with host cell signaling. LPMOs can be further split into families based on the kinds of biopolymers they can degrade, with the focus of this work being on the AA10 family, which degrades chitin (Book et al., 2014; Vandhana et al., 2022). An important characteristic of this protein family is a Cu(II) binding site involving two Histidines, which are required for the protein to be enzymatically active (Book et al., 2014; Vaaje-Kolstad et al., 2017; Vandhana et al., 2022; Wong et al., 2012).

While this work focuses on the host response elicited by this class of enzyme on the host gut epithelium, it is important to keep in mind that bacteria's primary purpose for producing this enzyme is not communication with the host. Rather, this protein likely evolved as a consequence of the high abundance of chitin in the environment of many bacteria, allowing them to use this as an energy source.

Drosophila as a comparative model for conserved host-microbe associations

The *Drosophila* midgut is a comparatively simpler model than vertebrate guts like the zebrafish and mouse, making it an excellent model for learning about conserved mechanisms that define host-microbiota relationships. Whereas vertebrates are typically colonized by large numbers and variable assemblages of microbial genera, fruit flies are primarily colonized by simple communities of *Acetobacter* and *Lactobacillus* species. The identity of the microbes colonizing arthropod and vertebrate guts can be quite

different, but the impact of microbial colonization on the rate of intestinal epithelial proliferation is conserved across these organisms. This highly conserved relationship that is dependent on the colonization event rather than the specific identity of the bacteria colonizing the gut makes looking at evolutionarily conserved host-microbiome relationships in *Drosophila* especially appealing. This observation illustrates a key change to how we might approach future host-microbiome relationship studies because it suggests that the activities of the gut microbiota, such as catabolism of host-derived biomolecules, are more important than the specific identity of the microbes. Because chitin is a very common host-produced or ingested polymer, many bacteria are capable of chitin degradation, including bacteria that natively colonize vertebrates.

Another advantage of exploring the conserved mechanism of host-microbiome interactions in flies is that we can effectively look at different genetic responses to perturbations in vivo (Charroux and Royet, 2012; Douglas, 2018). This allows us to look more precisely at the principles and conserved themes of how the gut microbiota interacts with the host innate immune system and other cellular mechanisms like proliferative signaling. In *Drosophila*, there is also a wide array of well-established tools and lineages available for public use that are easily customized for studying individual questions.

Finally, *Drosophila* are advantageous for studying genetic questions because of their large brood size and quick generation time, which is matched by few other model organisms suitable for studying host-microbiome relationships. In addition, fly embryos are sterile until they hatch from their eggs, making it possible to easily rear axenic flies through sterilizing eggs before the larvae hatch and placing the sterilized eggs on sterile laboratory fly food. Because the *Drosophila* microbiome is so simple, checking for

axenic flies after sterilization consists of culturing 1-2 representative larvae or pupae on appropriate media. Considering these factors, *Drosophila* presents an ideal model for studying conserved mechanisms of the relationship between host animals and the gut microbiota.

Barriers: setting and sensing boundaries

The digestive tract is one of few organ systems that are open to environment and colonized by bacteria (Huttenhower et al., 2012; Miguel-Aliaga et al., 2018). Colonization by the microbiota is normally tolerated by the host immune system without mounting an inflammatory response. Arthropod and vertebrate guts share many features of their structure and development (Takashima and Hartenstein, 2012). Both organisms possess a gut epithelium composed of a single, heterogenous layer of cells containing stem cells, absorptive cells, and secretory cells (Takashima and Hartenstein, 2012). The stem cells continuously self-renew along with sustaining the absorptive and secretory cell populations throughout the lifetime of the host, which are extruded from the gut epithelium as they die (Takashima and Hartenstein, 2012). In addition to this cellular barrier there is an additional extracellular barrier in the gut lumen containing sugars like GlcNAc and proteins (Nakashima et al., 2018). This additional barrier serves multiple purposes: first, it provides an extended physical barrier between host cells and bacteria in the gut lumen. Second, this barrier is composed of sugars and proteins secreted by the host. This nutrient rich substance provides a means of selection for species of bacteria better equipped to survive on the sugars and proteins present in the mucosal layer compared to others that cannot metabolize the sugars locally available (Xu et al., 2020). One sugar in particular, GlcNAc, is present in digestive tracts of many divergent animals

ranging from arthropods, where it is the primary building block of the chitin lining, to mammals, where it is a common sugar decorating the mucin lining (Nakashima et al., 2018). Equally highly conserved is the innate immune system, which can mount an immune response to small particles of GlcNAc (da Silva et al., 2008). From this, we can surmise that the host, through immune surveillance, can sense damage to the mucosal barrier through detection of liberated sugar fragments.

The highly conserved nature of gut mucus makes this component of the gut an excellent subject for analyzing evolutionarily conserved interactions between host and microbiome. Microbes that colonize animal guts must contend with their mucosal barriers, regardless of the identity of the microbe or the host they're colonizing (Alberdi et al., 2021; Broderick et al., 2014). In this body of work, we seek to establish a more complete functional and molecular mechanism of the conserved relationship between animals and their resident gut microbiotas, focusing on the conserved metabolic activities of bacteria living on host-derived polysaccharides, and the conserved strategies of animals to detect damage to their polysaccharide-based barriers.

CHAPTER II

SECRETED *AEROMONAS* GLCNAC BINDING PROTEIN GBPA STIMULATES EPITHELIAL CELL PROLIFERATION IN THE ZEBRAFISH INTESTINE

This section includes previously unpublished co-authored material written by myself and Dr. Karen Guillemin, with editorial assistance from Dr. Alison Banse, Dr. Jarrod Smith, and Dr. Savannah Logan. Experiments were executed by Drs. Alison Banse, Savannah Logan and Jarrod Smith. Reproduced with permission.

Introduction

Host-associated microbes, collectively called the microbiota, are critical to the development and physiological function of their host animals (Bosch and McFall-Ngai, 2021; Walsh and Guillemin, 2022). This complex assemblage of microorganisms contributes to host health in ways that range from stimulating host metabolism to promoting immune system maturation (Massaquoi et al., 2022). One way in which microbial communities influence host health is by stimulating cell proliferation of the mucosal epithelia on which they reside. This impact of the microbiota is apparent when comparing epithelial cell proliferation rates of animals raised in the presence (conventionally reared, CV) or absence (germ free, GF) of microbes. For example, GF mice have reduced rates of skin epithelial cell renewal (Wang et al., 2021). The animal digestive tract houses the most abundant microbial population and correspondingly the intestinal epithelium shows marked increases in epithelial cell proliferation in CV relative to GF animals, as has been reported in young and adult mice (Abo et al., 2020; ABRAMS et al., 1963), larval zebrafish (Cheesman et al., 2011; Rawls et al., 2004; Rawls et al., 2016), and larval and adult fruit flies (Broderick et al., 2014; Jones et al., 2013b).

However, the mechanisms underlying microbiota-induced intestinal epithelial cell proliferation are incompletely understood (Jones and Guillemin, 2018).

Previously, we showed that *Aeromonas veronii*, a common member of the zebrafish intestinal microbiota, secretes an unknown factor(s) that is sufficient to promote epithelial proliferation in the developing intestine of GF zebrafish. The gnotobiotic zebrafish model offers the ability to manipulate the presence (Melancon et al., 2015) and genetics (Wiles et al., 2018) of resident microbes in the larval zebrafish, which, combined with the optical transparency and sophisticated genetic tools of the zebrafish model, make it a powerful system to identify bacterial factors that influence aspects of animal tissue development and homeostasis. Our group has used the gnotobiotic zebrafish model to discover specific secreted bacterial proteins that modulate the abundance of intestinal neutrophils(Rolig et al., 2018) and the expansion of pancreatic beta-cells (Hill et al., 2016). Here, we use gnotobiotic zebrafish to identify a secreted *Aeromonas* factor that stimulates intestinal epithelial proliferation, which we show is a homologue of the *Vibrio cholerae* N-acetylglucosamine-binding protein A (GbpA) (Kirn et al., 2005a).

V. cholerae GbpA was discovered in a screen for bacterial mutants with impaired adhesion to cultured intestinal epithelial cells (Kirn et al., 2005a) and the *gbpA* mutant was also shown to be defective for binding to chitin-rich zooplankton and chitin-coated beads. Chitin is a complex polymer composed of β-(1→4)-linked N-acetylglucosamine (GlcNAc) monomers. GlcNAs is also a major O-linked glycan component of intestinal mucins, providing a biochemical basis for the parallel binding of *V. cholerae* to chitin- and mucin-rich surfaces. In a neonatal mouse model of infection, the *gbpA* mutant *V. cholerae* were recovered from intestines at lower levels and correspondingly caused less

pathology (Bhowmick et al., 2008; Kirn et al., 2005a). The defective colonization of the *gbpA* mutant was interpreted to be a consequence of its defective adhesion to intestinal epithelia, although such an adhesion defect has not been demonstrated for the mutant during intestinal infection. Unexpectedly for an adhesin, the GbpA protein was found to be a secreted protein (Kirn et al., 2005a). Further structural and biochemical analysis showed that a middle region of the protein (domains 2 and 3) can confer binding of GbpA to *Vibrio* cells (Wong et al., 2012), although most of the protein is found in the cell free supernatant. The amino terminal domain of GbpA (domain 1) shares homology with the AA10 family of chitin-degrading lytic polysaccharide monooxygenases (LPMOs) (Book et al., 2014). The C terminal domain (domain 4) resembles a chitin binding domain from *Serratia marcescens* chitinase B and both domains are sufficient to bind to chitin (Wong et al., 2012). GbpA's domain 1 was subsequently shown to be a functional LPMO (Loose et al., 2014). The importance of this enzymatic activity of GbpA for *V. cholerae* colonization or pathogenesis has not been explored.

Here we describe the impact of secreted GbpA from *Aeromonas* on intestinal epithelial cells in the context of normal colonization of the microbiota. Using *gbpA* deficient *Aeromonas*, we show that this gene plays no role in bacterial colonization or epithelial adhesion in the zebrafish intestine. We show both *Aeromonas* and *Vibrio* GbpA can stimulate the proliferative response in zebrafish and further demonstrate that the LPMO-containing domain 1 of *Aeromonas* GbpA is sufficient for this activity. Our results demonstrate that the capacity of GbpA to stimulate intestinal epithelial proliferation is intrinsic to this secreted bacteria protein independent of bacterial adhesion

or colonization. These finding contribute to a growing appreciation for the role of LPMOs in microbial-host interactions (Vandhana et al., 2022).

Results

A. veronii requires the Type II Secretion System for production of a secreted factor that induces intestinal epithelial proliferation

Earlier work from our group demonstrated that *A. veronii* strain HM21 produced an unknown secreted factor(s) that was sufficient to promote cell proliferation in 8 dpf GF larvae, as measured by the number of cells labeled during a 16 hour period of exposure to the nucleotide analogue EdU within a defined 210 μm region of the anterior intestine immediately caudal to the esophageal-intestinal junction (Cheesman et al., 2011) (Figure 2.1A, B). This factor(s) was present in cell free supernatant (CFS) from *A. veronii* HM21 grown overnight in TSB and fractionated through a spin column to remove small molecular weight material, suggesting the factor(s) was unlikely to be a metabolite and could be a secreted protein (Cheesman et al., 2011). Many Gram-negative bacteria employ the Type II Secretion System (T2SS) to secrete biologically active proteins into the extracellular environment. To test whether the pro-proliferative factor(s) were substrates of the T2SS, we added CFS from an *A. veronii* mutant lacking a functional T2SS (Δ*T2SS*) (Maltz and Graf, 2011) or the complement of this mutant with restored T2SS function (Δ*T2SS* + *T2SS*) (Maltz and Graf, 2011) to the aquatic environment of 6 dpf GF larval zebrafish and assayed cell proliferation at 8 dpf. We observed that while the CFS from *A. veronii* with a functional T2SS promoted cell proliferation in GF fish similarly to fish with a conventional microbiota, the CFS from the mutant strain lacking T2SS activity was unable to promote cell proliferation above GF levels (Figure 2.1C).

This observation suggests that the pro-proliferative factor(s) is a protein secreted by the T2SS.

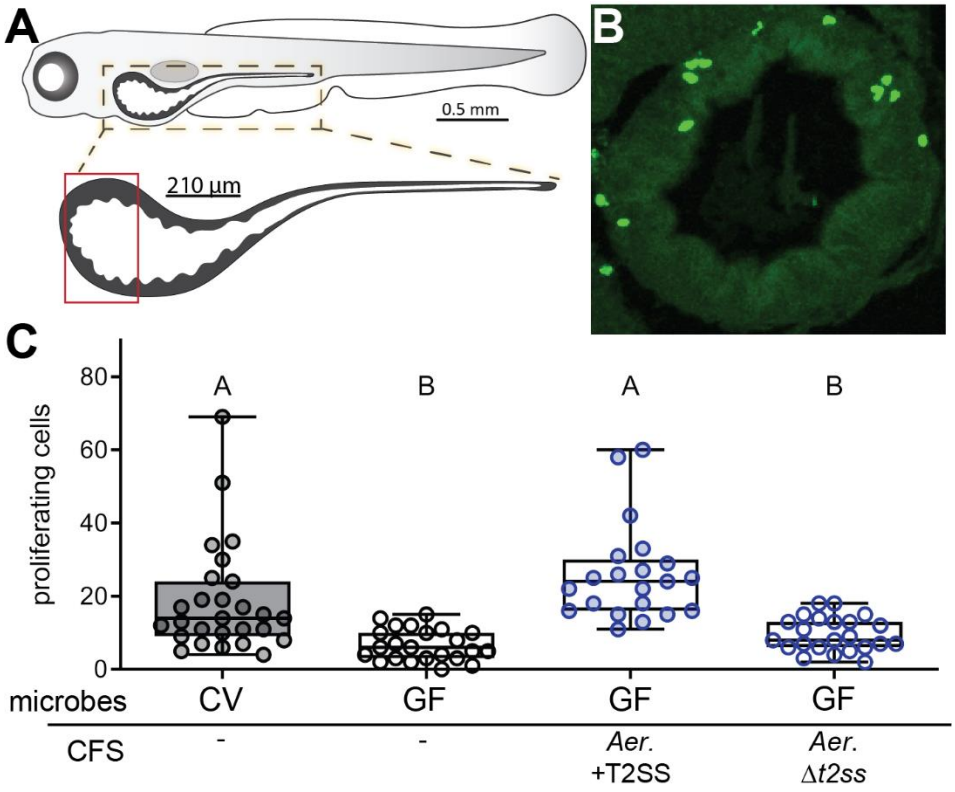


Figure 2.1 The T2SS of A. veronii is required for secretion of a pro-proliferative factor that stimulates intestinal epithelial cell proliferation. A. Schematic of the larval zebrafish intestine, highlighting the proximal 210 µm region in which proliferative epithelial cells were quantified, as marked by incorporation of the nucleotide analog EdU. **B.** Representative transverse section of the proximal zebrafish intestine stained to reveal cells that incorporated EdU. **C.** Quantification of proximal intestinal epithelial cell proliferation in 8 dpf CV larvae and 8 dpf GF larvae untreated or exposed from 6 dpf to CFS from A. veronii with a functional or mutated T2SS.

The *A. veronii* pro-proliferative factor is encoded by *gbpA*

To identify candidate pro-proliferative proteins secreted by *A. veronii*, we analyzed a mass spectrometry dataset we had generated of the abundant proteins in the CFS from the T2SS mutant and the complementation strains (Rolig et al., 2018), focusing

on proteins present in the complementation strain and absent in the mutant strain. To further narrow the number of candidate proteins, we fractionated the CFS using ammonium sulfate precipitation, tested these fractions for pro-proliferative activity, and analyzed their composition on Coomassie stained protein gels. We found that the pro-proliferative activity was concentrated in a fraction that appeared to contain a single dominant protein species of approximately 55 kd (Figure 2.2A). Only two of our candidate proteins identified by mass spectrometry were close to this molecular weight: a hemolysin and a homologue of the *V. cholerae* GbpA secreted protein (Kirn et al., 2005a). We determined that hemolytic activity, assayed on blood agar plates, was concentrated in a fraction lacking pro-proliferative activity (Figure 2.2A). We therefore turned our attention to GbpA.

GbpA from *A. veronii* has a similar predicted domain architecture to *V. cholerae* GbpA, with low identity putative cell surface binding domains [domain 2 (aa 201-300) and 3 (aa 309-403)] sandwiched between higher identity N-terminal LPMO domain [domain 1 (aa 25-188)] and C-terminal carbohydrate-binding module (CBM) domain [domain 4 (aa 427-468)]. (Figure 2.2B). The LPMO and CBM domains share 60% and 51% sequence similarity respectively, while domain 3 and 4 are 48% and 34% similar. The LPMO domains of both *Vibrio* and *Aeromonas* GbpA contain two copper-coordinating histidine residues that are necessary for the oxidation reaction which characterizes LPMOs.

To test whether GbpA was necessary for the pro-proliferative activity in the CFS from *A. veronii*, we generated an isogenetic strain of HM21 *A. veronii* in which the *gbpA* open reading frame was replaced by a kanamycin resistance cassette (*gbpA::kan,* Δ*gbpA*).

CFS was collected from the WT and Δ*gbpA* strains and added to the aquatic environment of 6 dpf GF larvae. Whereas the WT CFS elicited a robust proliferative response, the CFS from the Δ*gbpA* mutant failed to induce proliferation above the level observed in GF larvae (Figure 2.2C), demonstrating that *gbpA* is required for the proliferative response elicited by *A. veronii* CFS.

Colonization of the zebrafish intestine by *Aeromonas* or *Vibrio* does not require *gbpA*

The *V. cholerae gbpA* mutant was found to exhibit reduced binding to chitin beads following 30 minutes of incubation, as assayed by immunofluorescent microscopy, and reported as number of bacterial cells per bead (Kirn et al., 2005a). We performed a similar chitin bead binding assay with both WT and Δ*gbpA* strains, assessing the fraction of bacteria recovered from chitin beads after both 30 minutes and 1 hour of incubation, using dilution plating. Only about 5% of the population of WT *A. veronii* bound chitin bead at 30 minutes, and this fraction reduced to about 3% by 1 hour (Figure 2.3A). The Δ*gbpA* population exhibited a lower fraction of chitin bead binding, approximately 0.8%, at both time points (Figure 2.3A). GbpA was suggested to confer binding of *V. cholerae* cells by a mechanism whereby the protein is first secreted into the extracellular environment, unassociated with the bacterial cell surface, and then subsequently binds to both the bacterial cell and GlcNAc through different protein domains (Loose et al., 2014; Wong et al., 2012). A prediction of this model is that co-incubation of WT and Δ*gbpA* cells strains should rescue chitin-binding defects associated with the Δ*gbpA* mutant, since the predominant form of GbpA from the WT strain is in solution in the culture medium

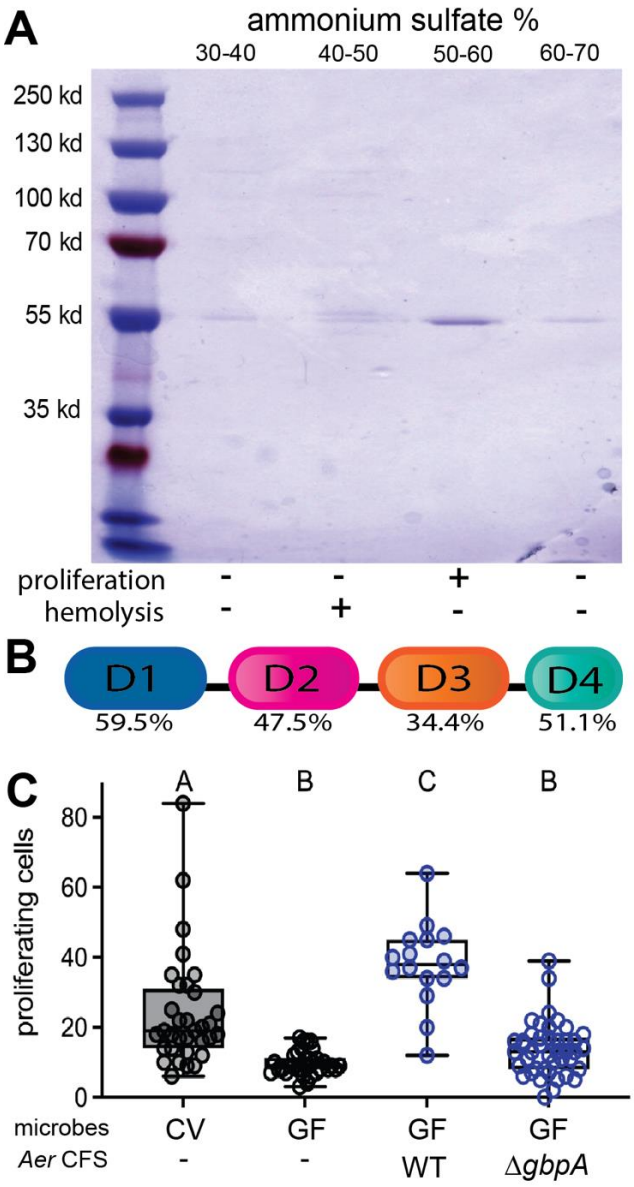


Figure 2.2 *A. veronii gbpA* encodes the secreted pro-proliferative factor that stimulates intestinal epithelial cell proliferation. A. The major protein constituents of ammonium sulfate fractions of *A. veronii* CFS separated by SDS-PAGE and visualized with Coomassie Briliiant Blue, with the corresponding proliferation and hemolysis activity of each fraction indicated below. **B.** Schematic of the shared domain architecture of *A. veronii* and *V. cholerae* GbpA proteins, with the amino acid identity indicated for each of the four protein domains. **C.** Quantification of proximal intestinal epithelial cell proliferation in 8 dpf CV larvae and 8 dpf GF larvae untreated or exposed from 6 dpf to CFS from WT or *ΔgbpA A. veronii*.

and equivalently accessible to WT or Δ*gbpA* cells. Arguing against the mechanism, we found no change in the percentage of the Δ*gbpA* mutant population that bound to chitin beads when co-incubated with WT cells (Figure 3A). This failure of the WT cells to complement the mutant cells' chitin-binding defect was also reflected in the competitive indices, calculated as the ratio of the Δ*gbpA* to WT cells recovered from the chitin beads in the mixed incubations, normalized to the ratio of the two strains added initially (Figure 2.3B).

In human pathogenic *V. cholerea* strains of both the classic and El Tor biotypes, *gbpA* is required for colonization of the neonatal mouse intestine (Bhowmick et al., 2008; Kirn et al., 2005a). To test whether *gbpA* was required for *Aeromonas* colonization of the zebrafish intestine, we inoculated the aquatic environment of GF zebrafish on 4dpf, and on 6dpf we assessed the bacterial colony forming units (CFU) per intestine. We observed that both WT and Δ*gbpA* strains were able to colonize the intestine to similar levels, demonstrating that *gbpA* does not play a crucial role in zebrafish gut colonization (Figure 2.3C).

Certain colonization factors are required only under circumstances of bacterial competition. To test whether *A. veronii gbpA* was required for competitive colonization of the zebrafish intestinal, we co-inoculated WT and Δ*gbpA* strains at equal concentrations to the aquatic environment of GF zebrafish at 4dpf and assayed the CFU/gut of each strain at 6dpf. We calculated the competitive index as the ratio of mutant to WT strains recovered normalized to the ratio inoculated. We observed a modest competitive disadvantage of the Δ*gbpA* mutant when competing against the WT strain (Figure 2.3D).

Vibrio species are normal residents of the zebrafish intestine and human-derived *V. cholerae* can colonize larval zebrafish (Logan et al., 2018). We therefore tested a *gbpA* mutant strain of *V. cholerae* that exhibited a mouse colonization defect (Kirn et al., 2005a) in our gnotobiotic zebrafish assay. As with the *A. veronii* strains, we found that both the *V. cholerae* Δ*gbpA* and WT strains colonized GF zebrafish larvae to similar levels (Figure 2.3E). When the two *V. cholerae* strains were co-inoculated into GF larvae, we noted a modest competitive disadvantage of the Δ*gbpA* mutant when competing against the WT strain (Figure 2.3F).

GbpA has been shown to promote the adhesion of *V. cholerae* to cultured intestinal epithelial cells and intestinal tissue explants(Bhowmick et al., 2008; Kirn et al., 2005a). To test whether *gbpA* was important for *A. veronii* colonization and distribution in the zebrafish intestine, we generated fluorescently labeled strains of both WT (*tn7::GFP*) and the Δ*gbpA* mutant (*gbpA::kan, tn7::dTomato*) and imaged these strains in the intestines of live 6 dpf larval zebrafish using light sheet microscopy (Taormina et al., 2012). We observed no significant difference in the distribution of the two strains relative to each other or along the zebrafish intestine (Figure 2.3G). Neither strain exhibited an epithelial proximal distribution but instead were found in bacterial aggregates of a range of sizes within the intestinal lumen, consistent with other imaging we have performed on *Aeromonas* strains in the larval zebrafish intestine (Jemielita et al., 2014; Taormina et al., 2012; Wiles et al., 2016). To exclude the possibility that any of the genetically manipulated *A. veronii* strains had a growth disadvantage under nutrient rich conditions, we compared their growth curves in TSB relative to the WT strain and found that neither the *gbpA::kan* insertion nor the fluorescent protein expression insertions produced any

growth defects (Figure 2.3H). Collectively these results show the Δ*gbpA* mutant has a slight defect in chitin binding and a slight competitive disadvantage in larval zebrafish intestinal colonization, which is not complemented by the presence of GbpA-producing WT cells. Our findings argue against a role for GbpA in *Aeromonas* epithelial adhesion in the larval zebrafish intestine.

Secreted GbpA from *Aeromonas* and *Vibrio* promote intestinal cell proliferation

Having ruled out a role for GbpA promoting intestinal epithelial cell proliferation through facilitating *Aeromonas* adhesion to the intestinal epithelium, we next explored whether secreted GbpA protein was sufficient to promote intestinal cell proliferation in larval zebrafish. We cloned the *A. veronii gbpA* gene and introduced it on an inducible high copy plasmid into *E. coli*, which lacks any *gbpA* homologues in its genome. Upon induction, a 55 kd protein was the major protein species in the *E. coli + pGbpA* CFS, which was absent in the CFS of *E. coli* containing just the empty expression vector. To test whether this recombinant *A. veronii* GbpA had similar chitin binding activity as the *V. cholerea* GbpA protein (Kirn et al., 2005a), we performed chitin binding assays with both proteins. We used CFS from *E. coli* expressing high levels of *A. veronii* GbpA and applied this material to chitin beads. We then collected the flow through (FT) as well as the eluate (E). We observed that *A. veronii* GbpA was present only in the eluate (Figure 2.4A), indicating that it bound efficiently to the chitin beads. We observed a similar binding to chitin beads when we used CFS from the complementation strain of *V. cholerae ΔgbpA*, *pGbpA–His* that expresses *V. cholerae* GbpA at high levels from a plasmid pGbpA-His (Kirn et al., 2005a). Consistent with their similar domain

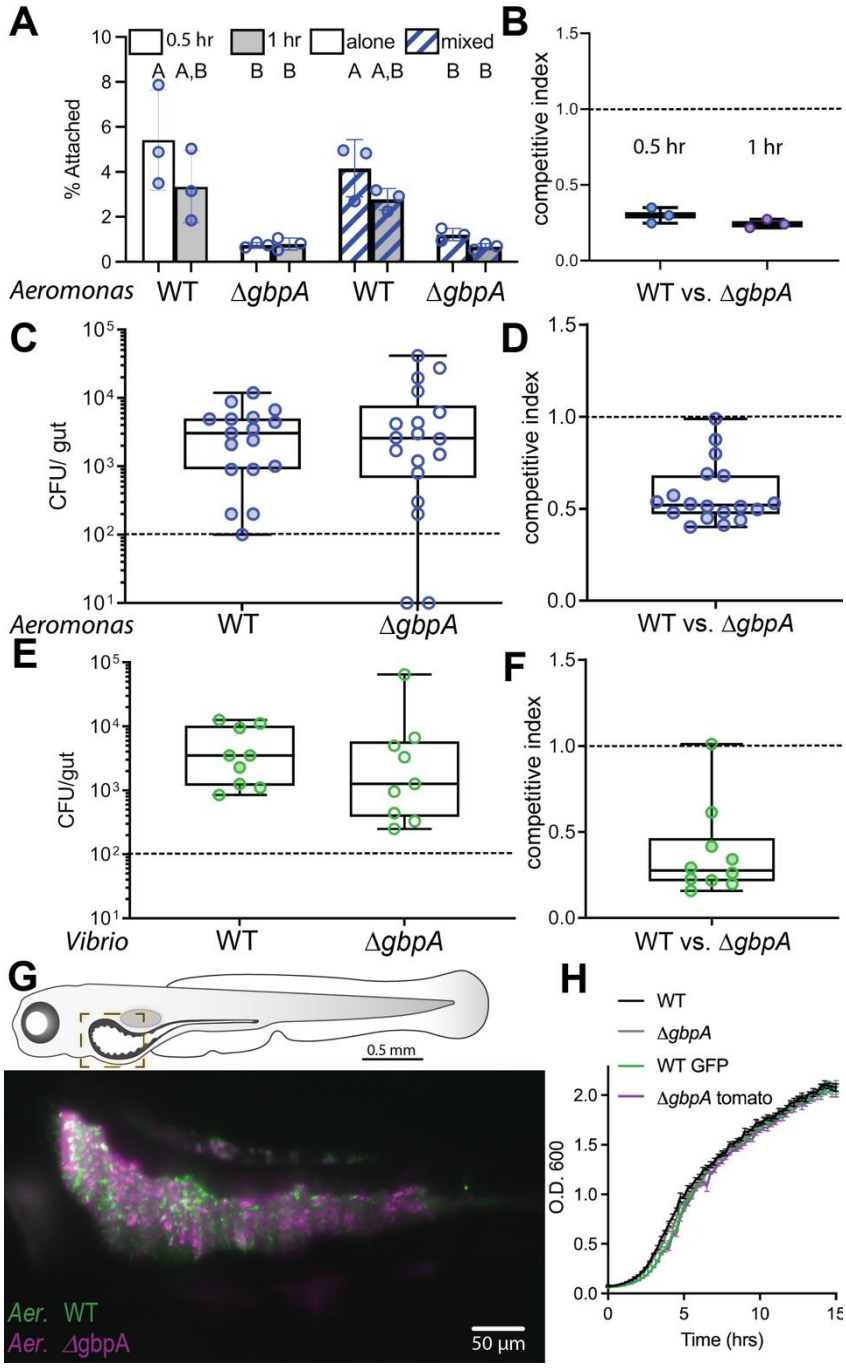


Figure 2.3 Colonization of the zebrafish intestine by *A. veronii and V. cholerae* does not require *gbpA*. **A.** Binding of WT and *ΔgbpA A. veronii* to chitin beads, quantified as the percent of total bacteria, after either 0.5 hr (white bars) or 1 hour (grey bars) of incubation. Bacterial strains were added to chitin beads alone (solid bars) or mixed with the other strain (striped bars). **B.** Competitive index of *ΔgbpA* versus WT *A. veronii* recovered from chitin beads. **C.** *A. veronii* CFUs recovered at 6 dpf following inoculation of GF zebrafish with individual strains at 4 dpf. **D.** Competitive index of *ΔgbpA* versus WT *A. veronii* recovered at 6 dpf following co-inoculation of GF zebrafish with the two strains at 4 dpf. **E.** *V. cholerae* CFUs recovered at 6 dpf following inoculation of GF zebrafish with individual strains at 4 dpf. **F.** Competitive index of *ΔgbpA* versus WT *V. cholerae* strains recovered at 6 dpf fish following co-inoculation of GF zebrafish with the two strains at 4 dpf. **G.** Light sheet micrograph of zebrafish intestine colonized with WT (green) and *ΔgbpA* (purple) *A. veronii* in the proximal intestinal region indicated in the schematic. **H.** Growth curves measuring OD_{600} for each *A. veronii* strain grown in TSB.

architectures, these results indicate the *A. veronii* and *V. cholerae* GbpA proteins share the biochemical property of chitin binding.

We next tested the capacity of recombinant *A. veronii* GbpA to induce intestinal epithelial proliferation in GF zebrafish larvae. We collected the CFS from the *E. coli* strains expressing *A. veronii* GbpA or the empty vector and added each to the aquatic environment of 6 dpf GF larvae. We observed that whereas the control CFS had no effect on cell proliferation of GF larvae, the GbpA-enriched CFS promoted cell proliferation to levels like those observed in CV larvae (Figure 2.4B).

We next explored whether the LPMO-containing domain 1 of *A. veronii* GbpA was sufficient to induce intestinal epithelial cell proliferation. We cloned domain 1 (D1) (aa 25-188) of *A. veronii* GbpA into the inducible expression construct and used a similar strategy to collect CFS from *E. coli* enriched for this protein domain. This CFS containing D1 induced cell proliferation to a similar extent as the full length GbpA from *A. veronii*, indicating that this domain is sufficient to induce the proliferative response in the intestinal epithelium.

To verify that the pro-proliferative activity detected in the *E. coli* CFS was indeed GbpA, we purified a recombinant version of the protein from *E. coli* CFS using a GST tag, which was then removed by proteolysis. When we applied purified GbpA to 6 dpf GF larvae, we observed high levels of cell proliferation at 8 dpf (Figure 2.4B).

Given the similarity between *A. veronii* and *V. cholerae* GbpA, we next tested whether *V. cholerae* GbpA could also induce a proliferative response in the GF larval zebrafish intestine. We collected CFS from WT, Δ*gbpA* mutant, as well as the

complement *(*Δ*gbpA, pGbpA-His*) *V. cholerae* strains (Kirn et al., 2005a) and applied these each to 6 dpf GF fish. Similar to our observations with *A. veronii*, we observed that the WT and complementation CFS promoted CV-like levels of cell proliferation at 8 dpf, while the mutant CFS did not promote cell proliferation (Figure 2.4C). This observation suggests that the pro-proliferative activity observed for secreted *A. veronii* GbpA is shared across other GbpA-like proteins produced by resident intestinal bacteria.

Discussion

Since early descriptions of epithelial cell renewal in the intestines of GF mice (ABRAMS et al., 1963), the microbiota has been appreciated as a source of pro-proliferative stimuli that elevates homeostatic rates of intestinal epithelial cell proliferation in many animals. The nature of the microbiota-derived molecules with this activity is incompletely understood. Several bacterial metabolites have been shown to elevate intestinal epithelial proliferations rates in mice and fruit flies, including reactive oxygen species (Reedy et al., 2019), indoles (Powell et al., 2020), and polyamines (Nakamura et al., 2021). Our previous characterization of intestinal epithelial proliferation in gnotobiotic larval zebrafish indicated that *A. veronii*, a prominent bacterial colonizer of the zebrafish intestine, stimulated a proliferative response through secreted factors of greater molecular weight than these metabolites (Cheesman et al., 2011). Here we show that a pro-proliferative factor secreted by *A. veronii* is a homologue of the *V. cholerae* protein GbpA.

The best characterized bacterial proteins that elicit intestinal epithelial cell proliferation are protein toxins of bacterial pathogens. For example, the *Helicobacter pylori* oncogenic virulence factor CagA induces expansion of Lgr5+ stem cells during

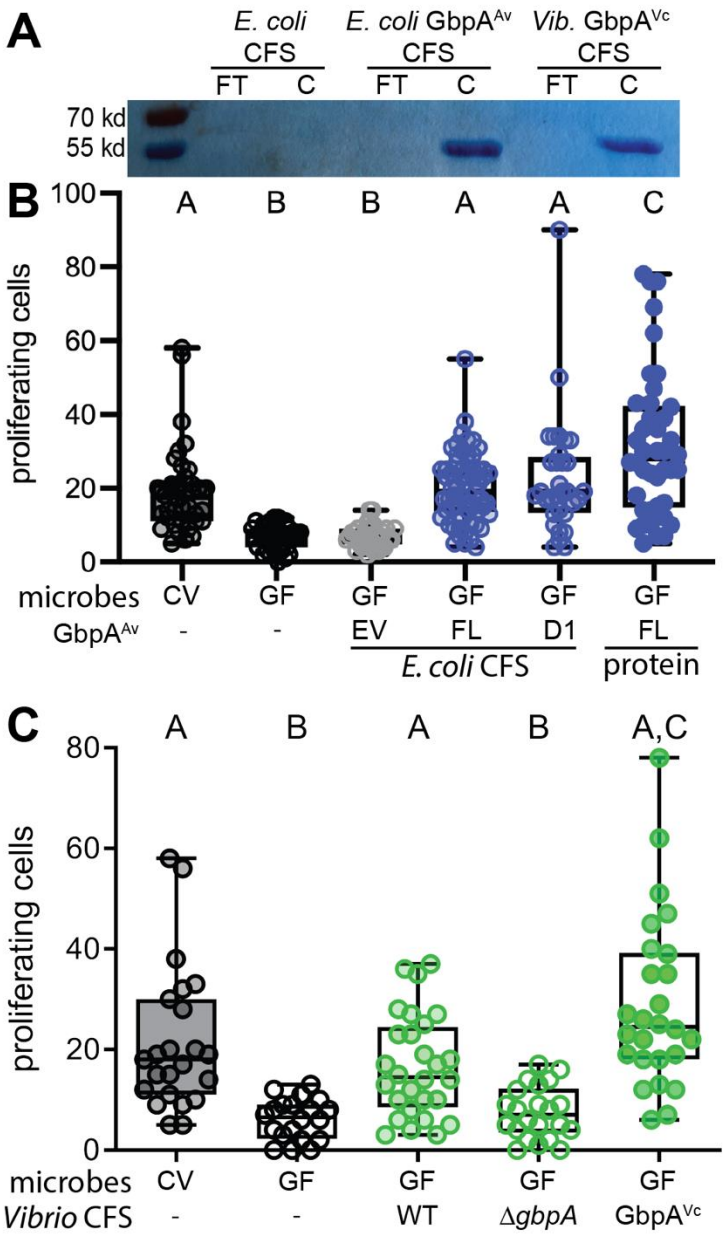


Figure 2.4 Secreted GbpA proteins are sufficient to increase intestinal epithelial proliferation in GF zebrafish. A. CSF from engineered *E. coli* expressing no recombinant protein or IPTG-inducible GbpA^{Av} and from the *V. cholerae gbpA* complementation strain expressing arabinose-inducible GbpA^{Vc} were incubated with chitin beads, rinsed, and the protein content of the flow through (FT) and chitin bead (C) fractions were separated by SDS-PAGE and visualized with Coomassie Brilliant Blue. **B.** Quantification of proximal intestinal epithelial cell proliferation in 8 dpf CV larvae and 8 dpf GF larvae untreated or exposed from 6 dpf to CFS of *E. coli* expressing full length GbpA^{Av}, domain 1 of GbpA^{Av}, or purified full length GbpA^{Av} protein. **C.** Quantification of proximal intestinal epithelial cell proliferation in 8 dpf CV larvae and 8 dpf GF larvae untreated or exposed from 6 dpf to CFS of WT *V. cholerae*, the *gbpA* deletion strain, and the *gbpA* complementation strain expressing arabinose-inducible GbpA^{Vc}.

infection of the gastric epithelium (Sigal et al., 2015) and transgenic expression of CagA is sufficient to increase epithelial cell proliferation in both zebrafish (Neal et al., 2013) and fruit fly (Jones et al., 2017) intestines. These pathogen-associated cell proliferative responses, however, are distinct from responses to microbiota colonization in that they are typically associated with inflammation and hypertrophic expansion of the tissue. The proliferative response to the microbiota in the larval zebrafish intestinal epithelium occurs even when tumor necrosis factor (TNF) signaling is blocked (Cheesman et al., 2011), in contrast to the epithelial proliferation in a zebrafish model of spontaneous intestinal inflammation and dysbiosis, which is prevented by interfering with TNF signaling (Rolig et al., 2017).

GbpA was previously characterized as a virulence factor of human pathogenic *V. cholerae* strains and implicated in disease by a proposed adhesion mechanism of the secreted protein acting to crosslink bacterial cells to GlcNAc moieties on intestinal mucins (Bhowmick et al., 2008; Kirn et al., 2005a; Wong et al., 2012). We generated *gbpA* deficient *A. veronii* to explore the function of this gene in bacterial-host interactions in the larval zebrafish intestine. We found that the *A. veronii* Δ*gbpA* mutant exhibited reduced binding to chitin beads, as reported for *V. cholerae*, but when we further explored this phenotype in a co-incubation assay with our WT and Δ*gbpA A. veronii* strains, we found that this defect was not rescued in trans by GbpA from the WT cells. This unexpected result challenges the current model that GbpA functions primarily as an adhesin since the predominantly soluble GbpA in the extracellular environment should be readily accessed by both the WT and Δ*gbpA* mutant cells. Additional evidence challenging the idea that GbpA's major function is as a chitin adhesin comes from a

previous study showing that a *V. cholerae fliA* mutant upregulates *gbpA* expression but is defective for chitin binding compared to the WT strain due to downregulation of the cell surface associated adhesin FrhA (Kitts et al., 2019; Syed et al., 2009). We also found no evidence for GbpA conferring a colonization advantage to either *A. veronii* or *V. cholerae* in the larval zebrafish intestine when introduced in mono-associations, but a slight competitive disadvantage in the presence of GbpA-secreting WT strains. Finally, we found no evidence that GpbA expression altered the intestinal biogeography of *A. veronii*, which normally colonizes the intestinal lumen in cellular aggregates. Our observations argue against the model that *A. veronii* utilizes GpbA as an adhesion to colonize GlcNAc-rich surfaces. Instead, we hypothesize that GbpA is part of a GlcNAc utilization program that *A. veronii* deploys when GlcNAc is an advantageous carbon source. In support of this idea, *V. cholerae* has been shown to upregulate *gbpA* in the presence of GlcNAc and chitin oligosaccharides (Meibom et al., 2004).

We predict the binding defects observed on chitin resin reflect physiological differences between the WT and Δ*gbpA Aeromonas* due to alterations in nutrient processing and acquisition in the Δ*gbpA* mutant. This idea is consistent with a recent characterization of *Pseudomonas aeruginosa* mutants lacking the gene for Chitin binding protein D (CbpD), a LPMO with similar N and C terminal chitin binding domains as GbpA (Askarian et al., 2021). The authors showed that *cbpD* deficient *P. aeruginosa* have markedly altered transcriptomes and proteomes as compared with WT cells grown in various media, and the altered metabolic state of the cells correlated with reduced survival in blood and pathogenicity in mouse tissues. This new model for GbpA function helps resolve the longstanding question of how a protein found predominantly in the

extracellular environment, unattached to the bacterial cell surface, contributes to *V. cholerae* colonization and pathogenesis.

Independent of possible fitness advantages conferred by GbpA to *A. veronii* during host colonization, we show that the secreted protein stimulates epithelial proliferation in the GF larval intestine. This pro-proliferative activity is recapitulated by the LPMO-containing domain 1 of the protein and by the related *V. cholerae* GbpA. We hypothesize that a consequence of *A. veronii* secreting GbpA in the intestine as part of a GlcNAc utilization program is that the enzymatic activity or byproducts are sense by the host through innate immune pathways that monitor intestinal mucosal integrity or glycan composition. We have shown that the host proliferative response to the microbiota requires the innate immune adaptor Myd88, but that activation of innate immune signaling with bacterial lipopolysaccharide (LPS) is not sufficient to elicit intestinal epithelial cell proliferation (Cheesman et al., 2011). In the zebrafish intestine, GbpA would have access to chitin as a component of the zebrafish intestinal lining (Tang et al., 2015). Chitin is a feature of all invertebrate intestines and many vertebrate intestines, where it coexists in varied proportions with a meshwork of glycan-rich mucins (Nakashima et al., 2018). Even in mammalian intestines that lack a chitin layer, the intestinal mucus contains ample GlcNAc polysaccharides that could be cleaved by bacterial secreted LPMOs.

As a bacterial protein that can target the intestinal lining, GbpA represents an example of a Microbial Associated Competitive Activity (MACA), a term we coined to describe microbial activities important for competitive fitness in multi-species communities that are sensed by host tissues as sources of information for regulating

programs of development and repair (Wiles and Guillemin, 2020). In this regard, the intestinal epithelial proliferation observed upon microbiota colonization is not an outcome that host-associated bacteria LPMOs evolved to evoke, but rather an adaptation of the host tissue to bolster epithelial renewal programs in the face of degrative enzymes secreted by specific constituents of its intestinal microbiota. The MACA framework explains how specific secreted proteins from microbiota members can have profound impacts on host tissue development and physiology and how different nonhomologous proteins can elicit similar effects by executing similar activities. Future studies of GbpA will test whether it elicits host epithelial proliferative response via its LPMO activity and whether specific byproducts of its enzymatic reaction or depletion of its co-substrates dioxygen or hydrogen peroxide are sensed by the host to induce epithelial renewal programs.

Materials and Methods

Animals

All experiments with zebrafish were performed using protocols approved by the University of Oregon Institutional Animal Care and Use Committee and following standard protocols (The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. University of Oregon Press, Eugene). WT (Ab/Tu) zebrafish were reared at 28 °C. GF embryos were derived by surface sterilization of the chorions and maintained as previously described(Bates et al., 2007). No exogenous food was provided during the duration of the experiments. CV controls were clutch mates of the GF derived embryos that were not subjected to surface sterilization and were reared in parallel.

Experimental bacterial strains

To create the *A. veronii ΔgbpA* mutant strain, a vector containing a kanamycin resistance cassette was transformed into SM10 *E. coli*. Conjugation between wild-type *Aeromonas veronii* HM21RS and the vector carrying SM10 *E. coli* strain was carried out, allowing the kanamycin resistance gene to replace the *gbpA* locus in *A. veronii* via allelic exchange. Candidate mutants were selected for loss of the plasmid and maintenance of kanamycin resistance. Insertion of the kanamycin cassette into the *gbpA* locus was verified in these candidates by PCR. Fluorescently marked derivatives of these strains were engineered with an established Tn7 transposon-based approach(Choi et al.). Briefly, a cassette containing the constitutively active synthetic promoter Ptac cloned upstream of genes encoding dTomato or superfolder GFP was chromosomally inserted at the *attTn7* locus to generate *A. veronii attTn7*::*Ptac-sfGFP and A. veronii ΔgbpA attTn7*::*Ptac-dTomato*. Joerg Graf provided the *A. veronii Δt2ss* mutant and isogenic complementation strain *A. veronii Δt2ss+T2SS*(Graf, 1999; Maltz and Graf, 2011). Ron Taylor provided *V. cholerae* classical O1 isolate CG842 (strain ATCC 39315 / El Tor Inaba N16961) and isogenic mutant *V. cholerae ΔgbpA* and complementation strain *V. cholerae ΔgbpA+pGbpA*(Kirn et al., 2005a).

GbpA expression constructs and protein purification

The UniProt ID for GbpA from *A. veronii* strain HM21 is: A0A7Z3TUS8 and the UniProt ID for GbpA from *V. cholerae* strain ATCC 39315 is: Q9KLD5. To generate a plasmid for the expression of unmodified *A. veronii* GbpA, PCR product of the *gbpA* ORF inclusive of the stop codon were generated by using the primers CBPf (gcatcatatggcagcaaaaatccatc)/CBPr1(gcatctcgagtcacttcagctcaatccaggctt). The PCR

product was cloned into the NdeI and XhoI sites of plasmid pET21b (Novagen). To generate a plasmid for the expression of a cleavable GST tagged GbpA, PCR product of the *gbpA* ORF lacking the secretion signal and inclusive of the stop codon were generated by using the primers GSTCBPf (gcatgaattccacggctacatcagccagccc)/ GSTCBPr (gcatctcgagtcatcacttcagctcaatccagg) and cloned into the EcoR1 and Xho1 sites of pGEX6p1. The plasmids were then transformed into *E. coli* BL21(DE3) RIL-CodonPlus cells (Stratagene).

Protein purification was achieved using a glutathione Sepharose 4B column (GE Healthcare) following the recommended protocol. Glutathione *S*-transferase (GST) cleavage was achieved after elution of GbpA from the column by adding 1 unit of PreScission protease enzyme and incubation overnight at 4°C per the instructions of the manufacturer (GE Healthcare).

CFS preparation

Cultures of *A. veronii* strain HM21(Graf, 1999) in tryptic soy broth (TSB) and *V. cholerae*(Kirn et al., 2005a) in Luria broth (LB) with 0.02% arabinose, pH 6.5, were grown at 30 °C for 17 h on a rotary shaker at 170 rpm. Overnight cultures of *E. coli* BL21(DE3) were grown at 37°C in LB supplemented with 100 μg/ml ampicillin for plasmid maintenance, diluted 1:50 into 50 ml fresh LB/ampicillin, and grown at 37°C until OD_{600} reached ~0.5. IPTG was then added to a final concentration of 1 mM to induce expression of GbpA. The culture was grown with IPTG for 2-3 hours at 30°C. This resulted in a CFS dominated by GbpA, as confirmed via sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue

staining, which revealed a dark band of the expected size for GbpA. This band was absent from BL21 CFS carrying an empty pET-21b vector.

Cultures prepared as above were spun at 5,600 × g for 10 min at 4 °C to pellet cells, and the supernatant was passed through a 0.22-μM filter (Corning) on ice. CFS was concentrated through an Amicon Ultra-15 spin concentrator to remove small products, which were toxic to the zebrafish larvae. Protein concentration was determined by Bradford assay. All CFS exposures were performed using ~500 ng/mL total protein.

Chitin binding of *Aeromonas* cells and GbpA protein

To assay *Aeromonas* binding to chitin, magnetic chitin resin (NEB #E8036S) was prepared by washing three times in PBS. Input bacterial suspensions of WT *A. veronii* or *A. veronii ΔgbpA dTomato* (10^9 colony forming units (CFU)/mL) were applied to the resin and incubated for 30 min or 1 hr at 30˚C with gentle rotation. The resin was washed three times with PBS to remove unbound bacteria and finally resuspended in PBS supplemented with 0.4% GlcNAc (Chem-Impex #01427) to release bacteria attached to the resin. Samples were plated on tryptic soy agar (TSA) to calculate the output CFUs and the percent of bacteria attached calculated ($[CFUs^{output}/CFUs^{input}]*100$). The competitive binding assay was conducted similarly except equal amounts of WT *A. veronii* and *A. veronii ΔgbpA dTomato* were mixed prior to chitin resin exposure and the CFUs were determined for each strain by visualizing the dTomato expressing colonies using a fluorescent microscope.

To assay GbpA protein binding to chitin, cell free supernatants (CFS) were collected from *E. coli* BL21(DE3) or *V. cholera* following induction of *gbpA* expression

as outlined above in CFS preparation with *E. coli* carrying the empty vector (pET-21b) serving as a control. Chitin binding was assayed as described previously(Kirn et al., 2005a). Briefly, normalized CFS was applied to chitin resin and incubated for 1 hr. and the flow through (FT) collected. The resin was washed 5X in PBS, resuspended in 2X protein loading buffer, and boiled for 5 minutes. Proteins in the FT and C fractions were separated via SDS-PAGE and visualized using Coomassie Brilliant Blue.

Ammonium sulfate fractionation

Ammonium sulfate fractionation was performed on un-concentrated, sterile CFS from 50 mL overnight cultures by slowly adding 100% ammonium sulfate until desired concentration was achieved. These solutions were prepared at 4°C. Precipitated proteins were collected from the 30-40%, 40-50%, 50-60% and 60-70% ammonium sulfate fractions. Precipitated proteins were collected from each fraction by centrifugation at 4°C and 14,000 g for 15 min. The proteins were resuspended in cold embryo medium (EM) and dialyzed for 2–3 hr at 4°C before adding them to 6 days post fertilization (dpf) GF larvae at a final concentration of 500 ng/mL. Pro-proliferative activity was observed in the 50-60% fraction. Hemolysis was assessed by spotting the fractions on blood agar plates.

Labeling and quantification of proliferating cells

Larvae were immersed in 100 μg/mL 5-ethynyl-2′-deoxyuridine (EdU) solution (A10044; Invitrogen) for 16 h before termination of the experiment. Larvae were fixed in 4% paraformaldehyde for 24 hr at 4 °C, processed for paraffin embedding, and cut into 7-μm sections. For EdU detection, slides were processed according to the Click-iT EdU

Cell Proliferation Assay Kit (C35002; Molecular Probes). Samples were imaged on a Nikon Eclipse TE 2000-V inverted microscope equipped with a Photometrics Coolsnap camera. EdU-labeled nuclei within the intestinal epithelium were counted over 30 serial 7-μm sections beginning at the esophageal-intestinal junction and proceeding caudally into the bulb. Analysis of this extended region was necessary because of the stochastic patterns of cell proliferation. The absolute numbers of labeled cells varied between trials. Despite these differences in the absolute numbers of labeled cells, the proportional trends of proliferating cells between treatments were consistent and reproducible between trials.

Colonization assay

Bacteria were added to GF flasks at 4 dpf at a final concentration of 10^6 CFUs/mL and incubated with the larvae for 48 hr at 28°C. Larvae were sacrificed at 6 dpf, immediately before the gut was removed and homogenized in a small sample of sterile EM. Dilutions of this gut slurry were plated onto tryptic soy agar and allowed to incubate overnight at 30°C. Colonies from each gut were quantified. A minimum of 10 guts per mono-association or di-association were analyzed.

Light sheet microscopy

Gnotobiotic larval zebrafish were prepared for light sheet imaging as described by Jemielita et al(Jemielita et al., 2014). Briefly, larvae are anesthetized in dishes filled with sterile EM and tricaine methanesulfonate (MS-222) at 120μg/ml. They were then moved to melted agarose gel and pulled into glass capillaries where the gel was allowed to cool. The capillaries were mounted on a sample holder and a plug of gel containing each live

larval zebrafish was extruded into a sample chamber filled with sterile EM and MS-222. Fluid in the sample chamber was maintained at 28^{o}C.

Light sheet fluorescence microscopy was carried out using a home-built light sheet microscope based on the design of Keller et al(Keller et al., 2008). Two Coherent sapphire lasers (488nm and 561nm) were rapidly scanned using a galvanometer to create a thin sheet at the focus of an imaging objective. This thin sheet was used to excite fluorescence in specimens. The excitation light was captured as the sample was moved through the sheet, creating a three-dimensional image. To image the entire larval zebrafish gut, four sub-regions were imaged and subsequently registered. A complete image of the gut can be captured in two minutes in two colors with single-micron spacing between planes. All exposure times were 30ms and laser power in both colors was set to 5mW.

Statistical analysis

Statistical analysis was performed in Prism 9. For comparison of two treatment groups, t-tests were performed. For comparisons across multiple treatment groups, one- and two-way ANOVA analyses were performed as appropriate.

Bridge

The results from this chapter provide a framework to explore the mechanisms by which GbpA is promoting epithelial proliferation in host animals. In this study we describe it as a pro-proliferative factor and begin to analyze the conserved nature of this proteins effect on host epithelial proliferation. The results described in this chapter provide evidence for a conserved relationship between LPMO activity and host epithelial

proliferation. In the next chapter, we will further explore how LPMO activity elicits host epithelial proliferation. Because we are interested in understanding the evolutionarily conserved features of this relationship, we will continue this investigation using *Drosophila melanogaster*.

CHAPTER III

A BACTERIAL LYTIC POLYSACCHARIDE MONOXYGENASE GBPA PROMOTES EPITHELIAL PROLIFERATION IN *DROSOPHILA MELANOGASTER* THROUGH INNATE IMMUNE SIGNALING

This chapter contains previously unpublished co-authored data and was written by me with editorial assistance from Drs. Karen Guillemin and Emily Sweeney. Experiments were performed by me, except for the feeding assay comparing the proliferative effect of different GbpA domains, which was performed by Zoë Wong.

Introduction

Animals are colonized along their digestive tract by microorganisms collectively called the gut microbiota. To examine the systemic effects of the gut microbiota, germ free (GF) animals can be reared under axenic conditions, where the microbiota has been removed (Walsh and Guillemin, 2022). Studies using GF animals have demonstrated a highly conserved relationship between the gut microbiota and host gut epithelial proliferation, with an increase in epithelial proliferation in colonized mice (Hörmann et al., 2014), fish (Cheesman et al., 2011), and fruit flies (Broderick et al., 2014). Epithelial renewal is a highly conserved process that occurs throughout the lifetime of the host (Takashima and Hartenstein, 2012). Stem cells proliferate and give rise to progenitor cells which differentiate into absorptive enterocytes, which comprise the bulk of the gut epithelium, and secretory cells (Takashima and Hartenstein, 2012). These differentiated cells eventually die and are sloughed off from the epithelium and excreted from the host as food moves through the gut. To maintain the integrity of the gut epithelium, these cells

must continuously be replaced for the hosts entire lifetime (Blander, 2016; Williams et al., 2015). However, the rate of epithelial proliferation must be controlled to avoid over-proliferation, which could compromise the integrity of the gut epithelium and lead to the formation of tumors (Takashima and Hartenstein, 2012). While the proliferative effect of the gut microbiota has been established, the mechanisms behind this remains unclear.

Investigation in GF zebrafish has recently demonstrated that a bacterial secreted protein, GlcNAc binding protein A (GbpA) from the zebrafish intestinal commensal *Aeromonas veronii hm21* can increase the rate of gut epithelial proliferation in GF zebrafish (Banse et al., 2022). The homologous protein has been extensively described as a virulence factor for *Vibrio cholerae* (Bhowmick et al., 2008; Kirn et al., 2005b), but homologs are present in many bacteria. All homologs of GbpA belong to the AA10 family of lytic polysaccharide monooxygenases (LPMOs), which use an oxidative mechanism to cleave polysaccharides such as chitin, a polymer of GlcNAc subunits (Loose et al., 2014). Structural analysis of *Vibrio cholerae* GbpA reveals 4 distinct domains separated by short, disordered regions. Domain 1, our primary focus in this paper, displays chitin binding and LPMO activity belonging to the AA10 family (Loose et al., 2014). Domains 2 and 3 are predicted to bind to bacterial cell surfaces based on sequence, and domain 4 is another chitin binding domain that binds with somewhat less affinity than domain 1 and does not display any detectable enzymatic activity (Wong et al., 2012). Because this protein is a demonstrated virulence factor, this structure fits handsomely into a model where *Vibrio* secretes GbpA as a strategy to bring itself into closer contact with chitin-rich surfaces for both protection and increased proximity to a carbon source.

Serratia marcescens Chitin Binding Protein 21 (CBP21) is a naturally occurring form of GbpA D1, along with many of the other related forms of GbpA found in other bacteria and fungi (Book et al., 2014; Wong et al., 2012). Both proteins are well-characterized members of a larger, highly conserved class of enzymes called lytic polysaccharide monooxygenases (LPMOs). Notably, *S. mar* CBP21 has both sequence and structural homology to GbpA D1 from both *Vibrio* and *Aeromonas*. Extensive comparative work between *Vibrio* and *Serratia* proteins has revealed conserved chitin binding sites along the surface of the proteins (Wong et al., 2012). These enzymes are generally classified as biomass degraders and can generally degrade cellulose, chitin, or both. A characteristic feature of this family of enzymes is a Histidine brace near the N-terminus that coordinates cooper binding, which is required for enzyme activity (Wong et al., 2012). The first Histidine is on the N-terminus of the fully matured protein, exposed upon final processing and secretion of the enzyme. This His along with the other critical His, which occurs approximately 100 amino acids later, bind to copper to render the protein enzymatically active.

The gut epithelium is separated from the gut microbiota by a conserved gelatinous barrier containing GlcNAc polymers. In mammals this barrier is composed of glycan-decorated mucin proteins, while in arthropods this is a chitin-based barrier called the peritrophic matrix (PM) (Nakashima et al., 2018). The intestinal lining of non-mammalian vertebrates, such as fishes, contain both mucin- and chitin-based components (Nakashima et al., 2018; Tang et al., 2015). In addition to its presence in the guts of many animals, chitin is a highly abundant biopolymer that composes structures such as crab shells, insects, and fungal cell walls. Given the high level of conservation of this sugar in

host gut barriers along with the conserved proliferative response to colonization by the microbiota, we began testing the effect of GbpA on *Drosophila melanogaster* because of its tractability as a gnotobiotic model (Douglas, 2018).

To gain more mechanistic insights into how GbpA and its homologs are acting to increase epithelial proliferation, the fly presents an ideal model system because this protein is not produced by any known native members of the symbiotic gut microbiota, except for a homolog produced by *Serratia marcescens*, a natural fly pathogen. Flies are also easily reared under axenic conditions and genetically tractable, making it easy to test specific mechanisms by which GbpA might be promoting epithelial proliferation. This tractability is particularly advantageous when studying the innate immune system sensing pathways mediated by transcription factors Relish/NFkB, which is conserved in flies. Although flies are not colonized by the same bacteria as mice and fish, the bacteria that are colonizing them still contain AA10 family LPMOs, which we hypothesize are responsible for promoting intestinal epithelial proliferation in a conserved manner.

Here we report that *Aeromonas*-derived GbpA increases gut epithelial proliferation in GF flies as previously shown in GF zebrafish. We present evidence that the LPMO-containing Domain 1 (GbpA D1) is sufficient to increase epithelial cell proliferation in GF flies. Furthermore, we found that a mutated version of this domain (GbpA D1AA), predicted to be enzymatically inactive, does not induce gut epithelial cell proliferation. We explore potential mechanisms GbpA may be using to promote epithelial proliferation in GF flies by testing the proliferative effect of homologous proteins, non-homologous chitin degrading proteins, and GlcNAc monomers. We hypothesize that bacterial enzymatic action degrades host barriers and liberates increasingly small GlcNAc

particles, which is sensed by the host innate immune system (Round and Mazmanian, 2009), prompting a pro-proliferative damage response.

Results

Colonization by the gut microbiota stimulates epithelial proliferation

We sought to investigate the effect of GbpA in *Drosophila melanogaster* because of the high degree of conservation in eliciting epithelial proliferation in other studies using highly diverged organisms (Banse et al., 2022; Bhowmick et al., 2008; Kirn et al., 2005a). For this study we used flies expressing GFP from the *escargot* promoter to visualize intestinal stem cells and enteroblasts (referred to as WT throughout; Figure 3.1A*).* We determined the proliferative response of intestinal epithelial cells to colonization by the gut microbiota by staining dissected intestines from GF and CV derived adults for expression of phosphorylated histone, a marker of mitotic cells. Similar studies from our group and others had shown increased numbers of mitotic intestinal epithelial cells in the midgut is responsive to presence of the gut microbiome (Broderick et al., 2014; Jones et al., 2017). Congruent with these results, we demonstrated that the gut microbiome promotes increased epithelial proliferation in the midgut (Figure 3.1B). Research exploring the effects of GbpA on host organisms has shown that this protein promotes epithelial proliferation in zebrafish (Banse et al., 2022). Although *Danio rerio* and *Drosophila melanogaster* have substantially different digestive systems and structures, they both contain chitin (Nakashima et al., 2018) and display a significant proliferative response to colonization by bacteria (Figure 3.1C) (Bhowmick et al., 2008; Cheesman et al., 2011). Because GbpA and its homologs are chitin-degrading AA10 family LPMOs that are expressed in symbiotic bacteria present in both systems (Book et

al., 2014; Vaaje-Kolstad et al., 2017), we wanted to test if this protein could also be attributed to increased proliferation in flies.

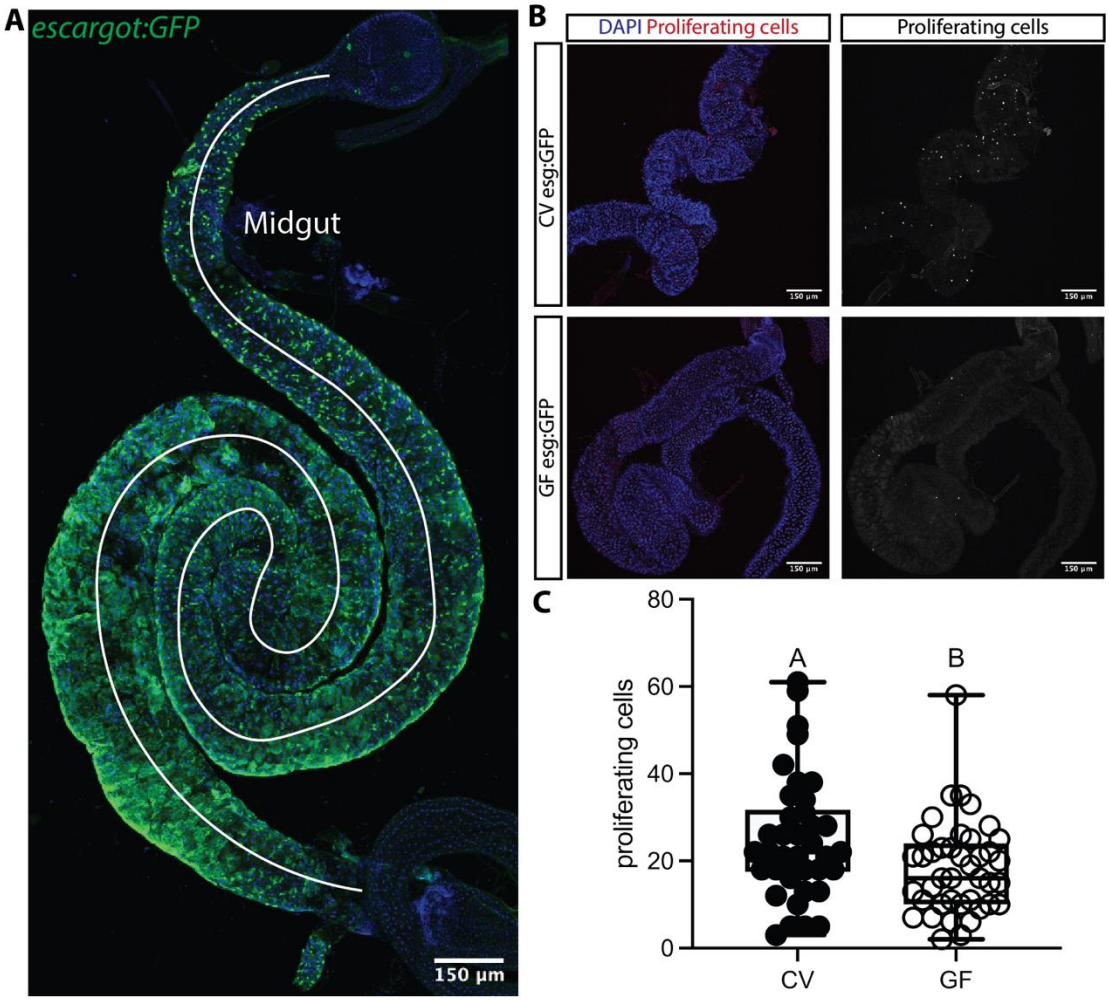


Figure 3.1 Colonization by the gut microbiota promotes intestinal epithelial proliferation. **A.** Fluorescence confocal micrograph of an escargot:GFP fly gut highlighting the length of the midgut from base of the proventriculus, denoted by the domed structure lacking GFP+ cells to the midgut-hindgut junction, denoted by a dense region of nuclei and branching of malphigian tubes. Green: escargot driven GFP; blue: DAPI stained cell nuclei. Image is composed of two images taken with overlapping edges and stitched together using FIJI. Scale bar is 150 microns. **B.** Fluorescent confocal images showing proliferating cells in CV and GF flies. Red: Phosphohistone 3, marking cells in S phase; blue: DAPI, cell nuclei. Scale bar, 150 microns. **C.** Quantification of intestinal epithelial proliferation in CV and GF flies. T-test analysis displayed, p-value = 0.0215.

GbpA Domain 1 is sufficient to increase intestinal epithelial proliferation when introduced orally

For this study, we raised flies axenically from egg laying to adulthood. 14 days after egg laying and approximately 4 days after eclosing from their pupal cases, adult GF females were fed one of a variety of forms of protein. The following day, guts were dissected, fixed, and stained using immunohistochemistry then quantified using fluorescence confocal microscopy (Figure 3.2A). To examine the effect of GbpA on gut epithelial proliferation in *Drosophila*, we first used enriched preparations of GpbA full length protein and truncated derivatives harvested from *E. coli* cells engineered to overexpress the recombinant proteins. Cell free supernatant (CFS) was collected from *E. coli* cultures induced to express high amounts of GbpA protein variants from expression vectors or containing an empty vector as a control. This filter sterilized CFS was fed to GF flies in a sucrose mixture containing bromophenol blue to select for adults that had ingested the solution (Figure 3.2B). We tested three variants of *Aeromonas* GbpA: the full-length protein, GbpA Domain 1 containing the LPMO domain (GbpA D1; amino acids 25-193) and GbpA Domains 2-4 (GbpA D2-4; amino acids 199-473) (Figure 3.2C). Both the full-length protein and GbpA D1 were sufficient to increase epithelial proliferation above the level measured in GF fed CFS from the empty vector control or GbpA D2-4 (Figure 3.2D). These results indicate that GbpA D1 is necessary and sufficient to increase epithelial proliferation.

Aeromonas veronii GbpA D1 has 57% sequence identity to the analogous protein *Vibrio cholerae* GbpA Domain 1 and 43% sequence identity to *Serratia marcescens* Chitin Binding Protein 21 (CBP21) (Figure 3.2E). In addition to sequence similarity, a

predicted structure of GbpA D1, using I-TASSER, confirmed high structural homology to *Vibrio* GbpA and *Serratia* CBP21 (RMSD across 167 residues = 0.68 Å) (Yang and Zhang, 2015). To test if the conserved enzymatic activity was necessary for this type of protein to elicit proliferation, we generated a mutant form of GbpA D1, where both His involved in copper binding (Figure 3.2E) were mutated to Ala to prohibit copper binding (GbpA D1AA), rendering the protein enzymatically inactive. When GF flies were fed GbpA D1AA-enriched CFS, there was no increase in the number of proliferating cells compared to GF flies fed a vehicle control from *E. coli* cells expressing an empty vector on the same plasmid backbone (Figure 3.2F). These results confirm that GbpA D1 is sufficient to increase gut epithelial proliferation and demonstrate that the domain's LPMO activity is necessary to elicit the pro-proliferative effect.

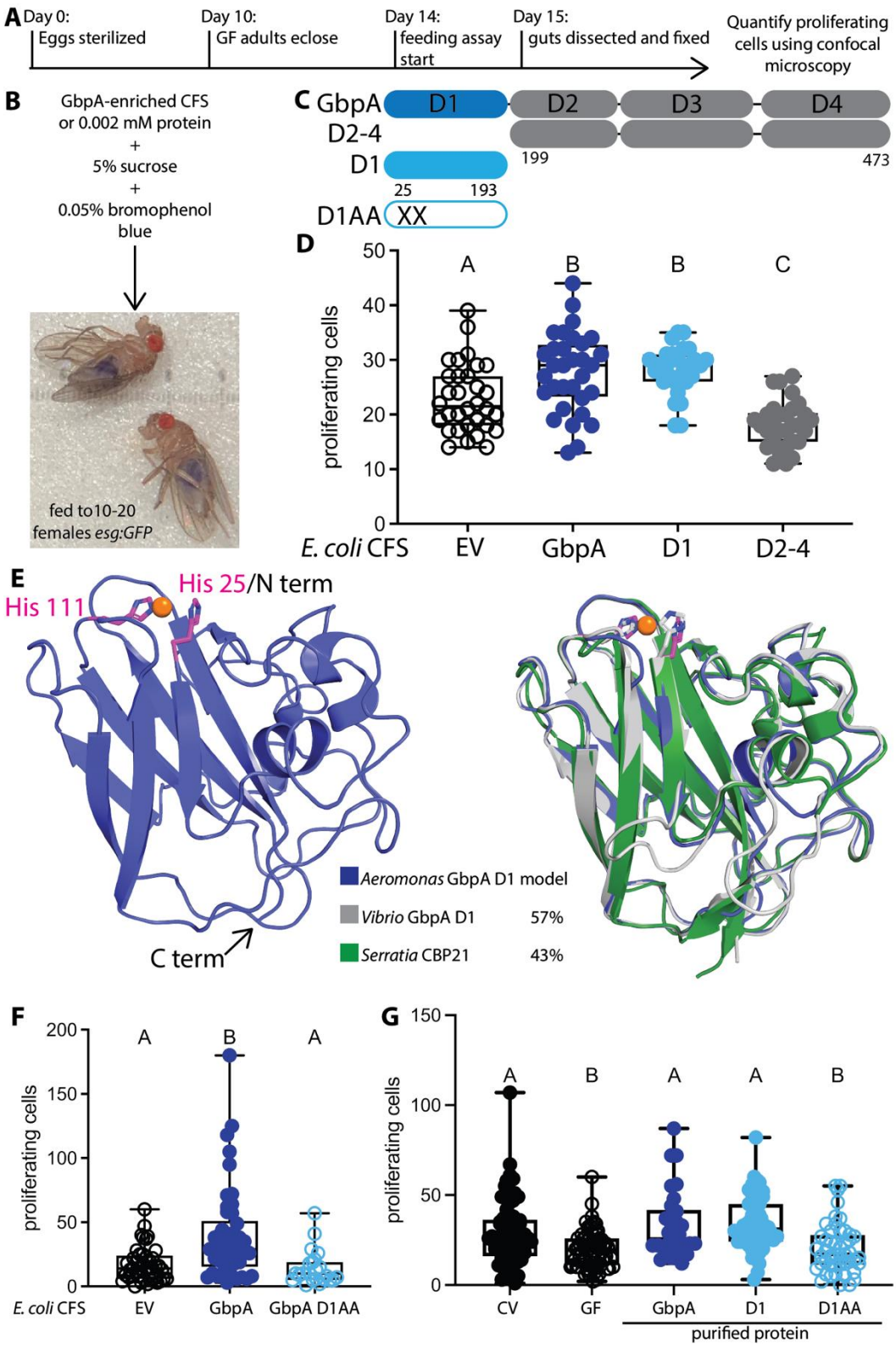
To establish if GbpA was increasing epithelial proliferation through synergistic action with another secreted factor or nutrients in the media used to grow *E. coli* used for expression, we purified the protein and fed it to GF flies (Figure 3.2B). GbpA, GbpA D1, and GbpA D1AA were purified from *E. coli* and fed to 4dpe GF female flies at a concentration of 0.002 mM in sucrose water containing bromophenol blue. GF flies fed purified GbpA and GbpA D1 showed a significant increase in the number of proliferating cells compared to GF control flies fed control protein buffer (Figure 3.2G), demonstrating that enzymatically active GbpA is sufficient to increase epithelial proliferation in GF *Drosophila melanogaster*.

GbpA is sufficient to increase epithelial proliferation when introduced via ectopic expression

We next explored whether GbpA expression in host cells would be sufficient to elicit the proliferative response observed upon feeding enriched or purified preparations of GbpA protein. We used the Gal4/UAS expression system to drive expression of GbpA within different cells of the intestinal epithelium (Figure 3.3A, 3.3B). The open reading frame (ORF) for *Aeromonas veronii hm21* GbpA was inserted into flies downstream of both a UAS promoter and a pelB leader sequence, which is a bacteria-derived sequence that directs proteins to the periplasm. Using signal peptide predictor SignalP 6.0 (Teufel et al., 2022), we believe that eukaryotic cells are capable of faithfully interpreting this signal to cleave GbpA at the appropriate site so the N-terminal histidine is exposed to help coordinate copper binding.

We used the *esg* driver to express transgenic *gbpa* specifically in intestinal stem cells and the *myo1A* driver to express *gbpa* in mature enterocytes. We hypothesize that

Figure 3.2 GbpA must be enzymatically active to increase epithelial proliferation in flies. A. Timeline for feeding assay experiments. **B.** Composition of experimental food fed to flies. Two representative females with blue abdomens are shown as an example of what flies who have eaten look like. **C.** Simplified representation of each GbpA domain, showing constructs for domain 1 (D1; aa 25-193) in blue and domains 2-4 (D2-4; aa 199-473) in gray. **D.** Feeding assay comparing *E. coli* CFS enriched with forms of GbpA shown in C. One-way ANOVA analysis displayed, p-value = <0.0001. **E.** Structural alignment of *Aeromonas* GbpA D1model (blue), *Vibrio* GbpA D1 (gray, PDB ID 2XWX), and *Serratia* CBP21 (green, PDB ID 2BEM). Characteristic Histidines (pink) involved in copper (Cu, orange) binding are mutated to Alanines in subsequent experiments. For illustrative purposes the Sodium bound in CBP21 has been replaced with a Cu. **F.** Feeding assay comparing *E. coli* CFS enriched with WT (GbpA) and mutated (GbpA D1AA) forms of GbpA. One-way ANOVA analysis displayed, p-value = <0.0001. **G.** Feeding assay comparing proliferative effect of purified proteins. GbpA: full-length, wild-type GbpA; GbpA D1: GbpA WT Domain 1 only; GbpA D1AA: GbpA mutant Domain 1 only. One-way ANOVA analysis displayed, p-value = <0.0001.


A
Day 0:
Eggs sterilized
Day 10:
GF adults eclose
Day 14:
feeding assay start
Day 15:
guts dissected and fixed
Quantify proliferating cells using confocal microscopy
B
GbpA-enriched CFS or 0.002 mM protein
+
5% sucrose
+
0.05% bromophenol blue
fed to10-20 females esg:GFP
C
GbpA
D1
D2
D3
D4
D2-4
199
473
D1
25
193
D1AA
XX
D
proliferating cells
50
40
30
20
10
0
A
B
B
C
E. coli CFS
EV
GbpA
D1
D2-4
E
His 111
His 25/N term
C term
Aeromonas GbpA D1 model
Vibrio GbpA D1
57%
Serratia CBP21
43%
F
proliferating cells
200
150
100
50
0
A
B
A
E. coli CFS
EV
GbpA
GbpA D1AA
G
proliferating cells
150
100
50
0
A
B
A
A
B
CV
GF
GbpA
D1
D1AA
purified protein

transgenically expressed GbpA exerts its action as a protein when secreted into the intestinal lumen, but it was not possible to visualize the secreted protein. We believe the N-terminal HA epitope tag is cleaved as this protein is being expressed because this tag is followed by a pelB leader sequence to ensure secretion of this protein from the cell. To ensure both drivers were functioning properly, we drove expression of green fluorescent protein (GFP) in both stem cells and enterocytes. We found that both drivers were successfully stimulating expression of GFP (Figure 3.3C, 3.3D). This gives us reason to believe that the same drivers can express GbpA using the same expression system, even if we cannot visually resolve the HA tag because of it being degraded by host cell machinery during the post-translational processing. We found that driving expression of GbpA in both cell types increased epithelial proliferation in GF flies above the levels measured in control flies with the driver but without the *gbpA* transgene (Figure 3.3E, 3.3F). These results demonstrate the host-produced GbpA as well as bacterially derived GbpA can stimulate intestinal epithelial proliferation.

Chitin degradation activity compromises host barrier integrity

Based on our findings that GbpA stimulates intestinal epithelial proliferation in an enzymatic activity-dependent manner, we sought to test the hypothesis that GbpA promotes cell proliferation by compromising the chitin-based intestinal barrier and stimulating a host proliferative repair response. To quantify the integrity of the fly peritrophic matrix (PM), we developed an assay testing flies' susceptibility to Avermectin B1 (AVM), a potent pesticide capable of killing *Drosophila melanogaster* (Ishaaya et al., 1986). Previous research has shown that flies resistant to AVM treatment

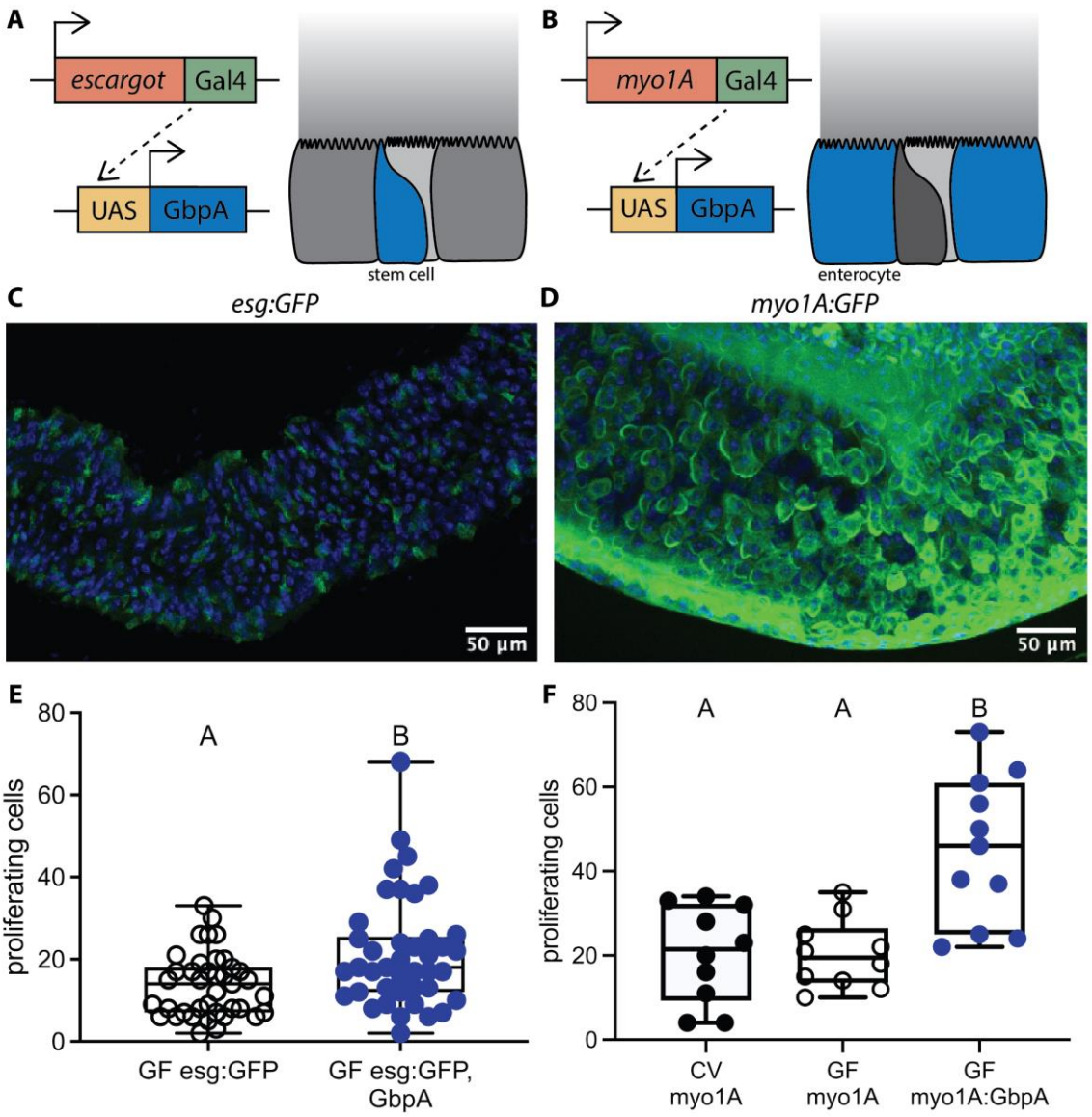


Figure 3.3 Transgenic expression of GbpA promotes epithelial proliferation. A. Cartoon representation of *escargot-Gal4* driving expression of *UAS-gbpA* in epithelial stem cells. **B.** Representation of *myo1A-Gal4* driving expression of *UAS-gbpA* in enterocytes in the gut epithelium. **C.** Representative max projection of fly gut epithelium from an individual driving expression of green fluorescent protein (GFP) under direction of *escargot*. Scale bar 50 µm. **D.** Representative max projection of fly gut epithelium from an individual driving expression of GFP under direction of *myo1A*. Scale bar 50 µm. **E.** Proliferation counts comparing GF flies expressing GbpA under the control of escargot compared to control flies not containing the transgene. T-test analysis displayed, p-value = 0.0023. **F.** Proliferation counts comparing GF flies expressing GbpA under the control of Myo1A compared to control flies not containing the transgene. One-way ANOVA analysis displayed, p-value = 0.0001.

have a thicker chitin layer of their PM (Chen et al., 2016), and thus we reasoned that reducing the PM would increase susceptibility to AVM. In previous studies, 87% of flies were dead after 5 days of feeding on grapes dipped in 0.057 mM AVM compared to flies fed grapes dipped in distilled water (Ishaaya et al., 1986). When we fed GF flies GbpA followed by AVM, we saw that they perished at a significantly faster rate than GF flies fed a protein buffer control (Figure 3.4A). To test if LPMO activity was necessary for this increased susceptibility to AVM, we compared death rates of flies fed GbpA D1 and GbpA D1AA. In this experiment, we saw GF flies fed GbpA D1 died at a significantly faster rate than GF flies fed Gbpa D1AA, and that flies treated with GbpA D1AA were indistinguishable from flies fed (Figure 3.4A). These results are consistent with the model that GbpA's LPMO activity compromises the peritrophic matrix appreciably, resulting in the increased susceptibility of the GbpA treated flies to AVM.

Although LPMOs are a large family of chitin-degrading enzymes that are highly conserved across many bacteria and fungi, there are other enzymes capable of degrading chitin through different mechanisms. AA10 LPMOS like GbpA can attach to crystalline chitin in the middle of lattices as opposed to free ends (Madland et al., 2021), making them an endochitinase. Another class of chitin-degrading enzymes, chitinases, are also able to bind chitin, however these are restricted to the free ends, making them exochitinases. While the mechanisms by which each of these enzymes degrades chitin is different, they could both cause PM degradation and compromised barrier integrity. To test whether a different chitinase could compromise the PM, we fed flies 1mg/mL commercially available purified chitinase from *Streptomyces gresius* and then exposed them to AVM. GF flies fed this chitinase preparation died at a significantly faster rate

than water-fed flies and a similar rate to GF flies fed GbpA D1 (Figure 3.4B). This result indicates that multiple classes of chitin-degrading enzymes can compromise the PM and raised the possibility that other types of PM perturbation than those imposed by LPMOs could elicit an increase in epithelial proliferation as a more general response to chitin degradation of host barrier structures.

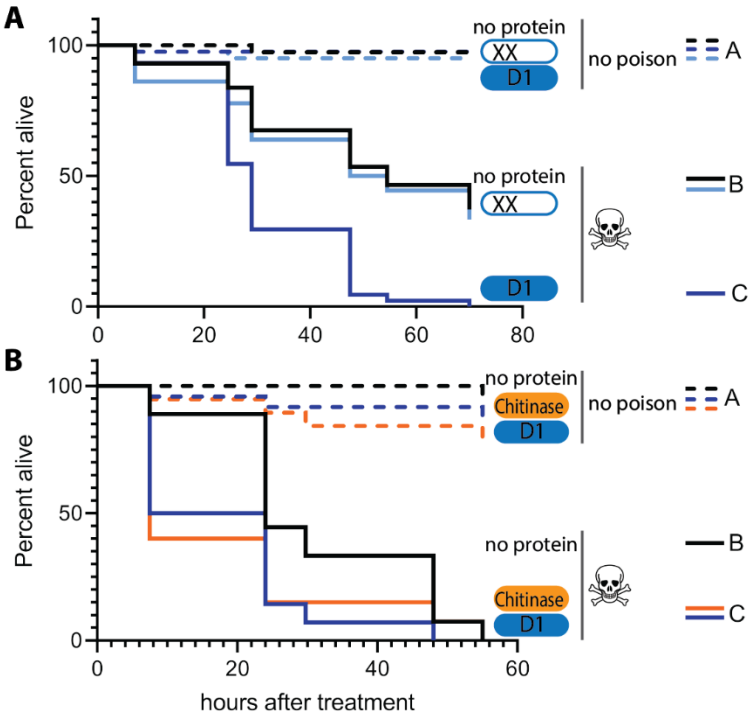


Figure 3.4 GbpA degrades host barrier structures like known chitinases. A. Survival of GF female flies fed purified protein or protein buffer followed by treatment with Avermectin B1 (skull and crossbones). Flies fed the functional form of the protein (GbpA D1, dark blue) die at a significantly faster rate than flies fed an inactive mutant of the protein (GbpA D1AA, light blue) and buffer after exposure to AVM. Regardless of treatment group, flies do not succumb to acetone, the buffer control for AVM. Mantel-Cox analysis displayed, p-value = <0.0001. **B.** Survival of GF female flies fed protein or protein buffer followed by treatment with AVM (skull and crossbones). Flies fed GbpA D1 (dark blue) and chitinase (orange) die at a significantly faster rate than flied fed protein buffer after exposure to AVM. Mantel-Cox analysis displayed, p-value = <0.0001.

Chitin degradation activity is sufficient to increase epithelial proliferation in *Drosophila melanogaster*

To explore the generality of PM perturbations as stimulants of protective proliferative response, we next tested the capacity of other LPMOs or chitinases to induce epithelial proliferative in the GF intestine. While LPMOs can cleave within large three-dimensional chitin networks, chitinases typically act on the free ends of GlcNAc polymers (Yang et al., 2017) (Figure 3.5A, 3.5B).

Thus far we have used structural and biochemical information from *Vibrio* GbpA to inform hypotheses about the *Aeromonas* protein. To test the capacity of another related LPMO to stimulate intestinal epithelial proliferation, we purified recombinant *Serratia marcescens* chitin binding protein 21 (CBP21) expressed in *E. coli.* This protein is of particular interest to this study because it is produced by *Serratia*, a native *Drosophila* pathogen. CBP21 is homologous to GbpA D1 and has been extensively characterized for its AA10 family LPMO activity (Vaaje-Kolstad et al., 2005; Wong et al., 2012). When we fed purified CBP21 to GF flies as in previously described feeding assays, we measured a significant increase in the number of proliferating cells compared to GF flies fed a protein buffer control (Figure 3.5C). When we fed GF flies a mutant form of CBP21 where both conserved copper binding Histidines have been mutated to Alanine (CBP21AA), we did not see an increase in epithelial proliferation compared to GF flies (Figure 3.5C). This result demonstrates that LPMOs as class of enzymes can increase GF fly gut epithelial proliferation and that the enzymatic activity is required for this response.

To test whether any chitin-degrading enzyme could increase epithelial proliferation in GF flies, we used the *Streptomyces griseus* chitinase that increased AVM

susceptibility (Figure 3.4B). When fed chitinase at a concentration of 0.1 mg/mL, GF flies displayed a significant increase in the number of proliferating epithelial cells relative to the control-fed GF flies (Figure 3.5D). This result demonstrates that multiple chitin-degrading activities that compromise the PM are sufficient to increase intestinal epithelial proliferation.

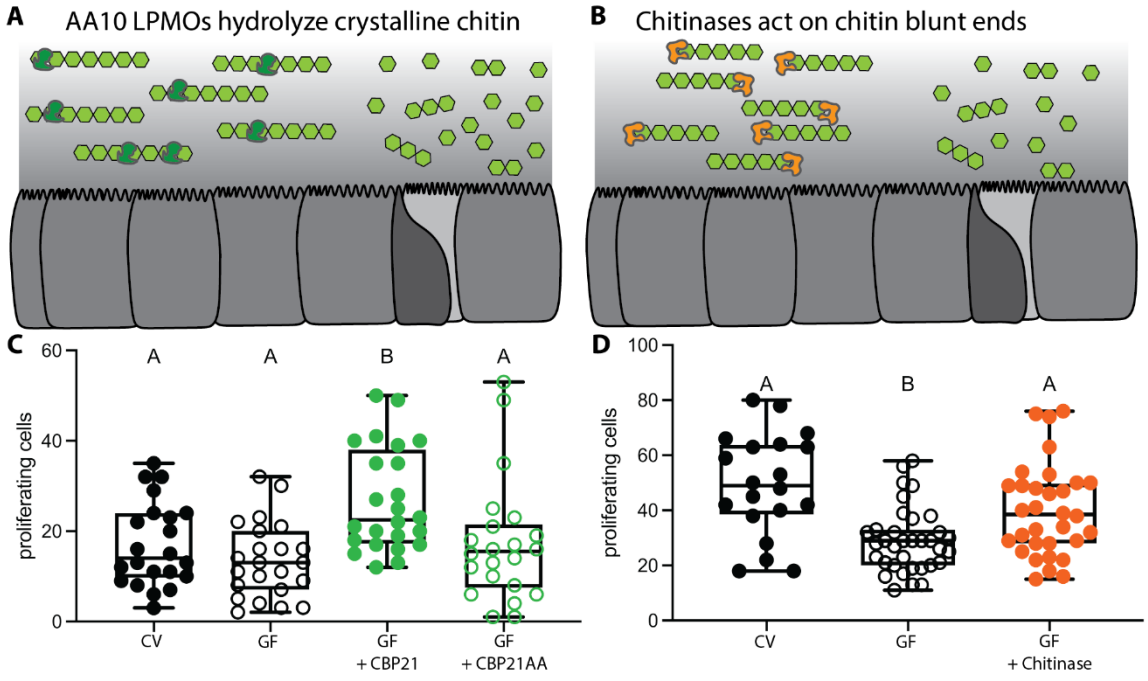


Figure 3.5 Chitin degradation activity is sufficient to increase intestinal epithelial proliferation. A. Cartoon representation of LPMOs interacting with chitin in the host gut lumen. AA10 LPMOs can bind and cleave bonds along the length of chitin. **B.** Representation of chitinases interacting with chitin in the host gut lumen. Chitinases bind to chitin free ends and cleave bonds starting from the edges. **C.** Proliferation counts from feeding assay comparing GF flies fed functional, WT CBP21 and the inactive mutant (CBP21AA). Flies fed the active form of the protein have a higher rate of epithelial proliferation. One-way ANOVA analysis displayed, p-value = 0.0005. **D.** Proliferation counts from feeding assay comparing GF flies to those fed chitinase from Streptomyces griseus. Flies fed chitinase displayed a higher rate of proliferation than GF flies fed water. One-way ANOVA analysis displayed, p-value = <0.0001.

GbpA's enzymatic products are sensed through Relish signaling

Previous research in zebrafish has shown that impairment of the innate immune system by knock down of the adaptor protein Myd88 blocked the intestinal epithelial proliferative response to the gut microbiota (Cheesman et al., 2011). We sought to test whether impairment of the immune system in flies would similarly block the intestinal epithelial response to GbpA. To perform this experiment, we used Relish mutant flies (*rel[E38]*) lacking all isoforms of the Relish homologue of NFkB. When these GF flies were fed GbpA in the same fashion as previous experiments, their gut epithelium did not exhibit increased intestinal epithelial proliferation (Figure 3.6A), indicating that Relish signaling is required for GbpA responsiveness. We noted that CV *rel[E38]* flies had a higher rate of epithelial proliferation than GF *rel[E38]* flies, indicating that pathways other than Relish are involved in intestinal epithelial proliferative responses to the gut microbiota.

The enzymatic activity of LPMOs and chitinases will breakdown chitin and increase the concentration of GlcNAc monomers, which can be sensed by innate immune receptors including TLR2 in mammals (Alvarez, 2014; Elieh Ali Komi et al., 2018; Fuchs et al., 2018). To test the hypothesis that chitin degradation products are sensed through Relish-mediated immune signaling, we fed GF flies increasing concentrations GlcNAc monomers. We found that when GF WT flies were fed food containing 1 M GlcNAc, they displayed a significant increase in the number of proliferating cells (Figure 3.6B). In contrast, when the same concentration of GlcNAc was introduced to GF *rel[E38]* flies, they did not exhibit any proliferative response (Figure 3.6B). These results

demonstrate that Relish signaling is required to increase epithelial proliferation in response to increased concentration of luminal GlcNAc monomers.

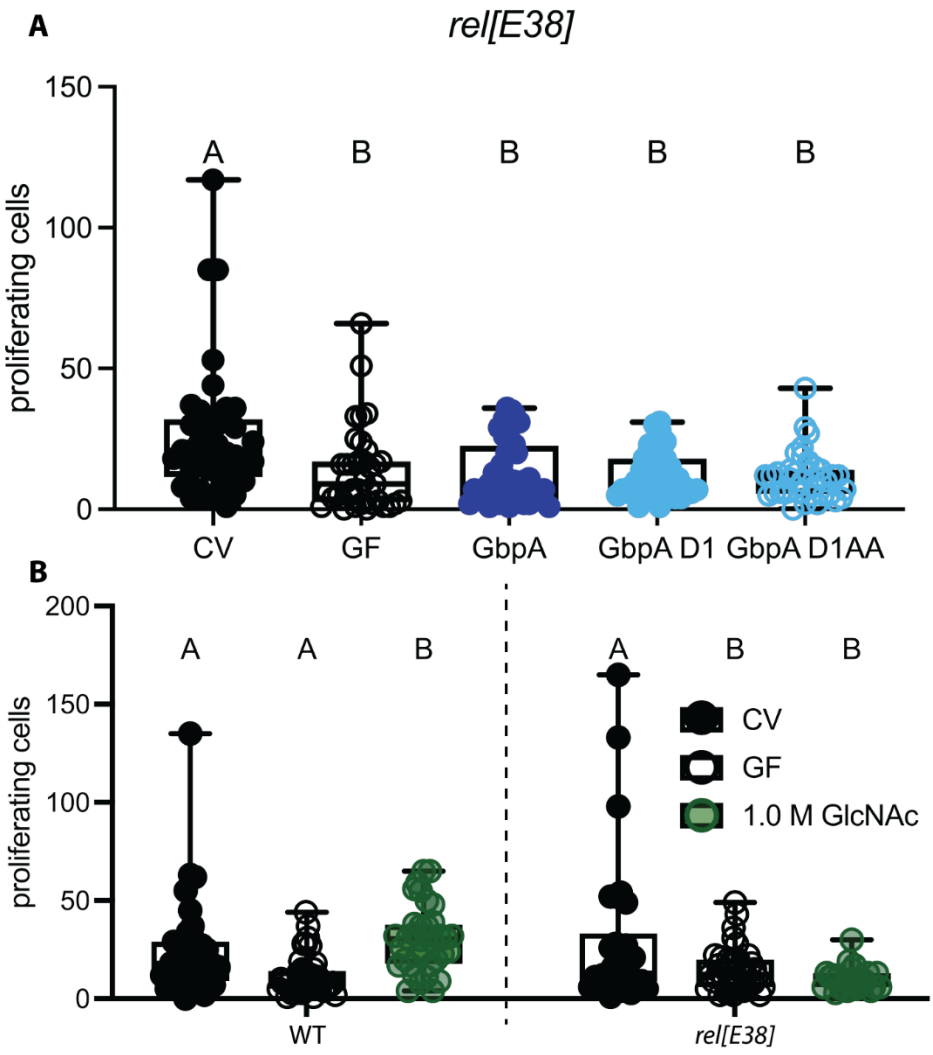


Figure 3.6 Increased abundance of luminal GlcNAc monomers promotes epithelial proliferation through Relish signaling. A. Proliferation counts from *rel[E38]* flies. When fed purified variants of GbpA, these flies do not display a proliferative response. One-way ANOVA analysis displayed, p-value = <0.0001. **B.** Proliferative counts from WT and *rel[E38]* fed 1.0 M GlcNAc monomers. In WT flies, there is a significant proliferative response to this treatment. In *rel[E38]* flies, there is no significant response to the same treatment. Two-way ANOVA analysis displayed, p-value = 0.0005.

Discussion

In this body of work, we've shown that chitin degrading enzymes as a class can increase the rate of epithelial proliferation in *Drosophila melanogaster*. This result is consistent

with other fly studies demonstrating colonization by the gut microbiota promotes epithelial proliferation (Broderick et al., 2014). Previously, the relationship between colonization by the gut microbiota and epithelial proliferation had been described as a causative relationship. While identification of this causal link was important, it did not describe any mechanisms. The work done here and by others in zebrafish has begun to shed light on the specific relationship we're seeing between host and microbiota. Our results suggest that colonization by a specific bacteria or presence of a specific protein is largely unimportant for microbiota-induced gut epithelial proliferation. Instead, our results suggest bacteria in the gut degrade chitin, leading to an increase in abundance of free GlcNAc monomers in the gut lumen which stimulates the innate immune system through Relish/NFkB signaling, which then results in gut epithelial proliferation (Figure 3.7). In the *Drosophila* midgut, Relish activation is involved in the signaling response to bacterial and fungal cell wall components (Anderson, 2000; Fuchs et al., 2018; Myllymäki et al., 2014; Tauszig et al., 2002), which contain GlcNAc monomers. In mammalian cells, chitin is a potent activator of the innate immune system (da Silva et al., 2008). Our results support a model where Relish signaling activation is necessary to elicit epithelial proliferation in response to changes in luminal GlcNAc monomer concentration, but not colonization by the gut microbiota. This study illuminates how powerful a simple model can be to explain highly conserved relationships in a vast evolutionary landscape involving many different pairings of hosts with their individual microbiomes. These results begin to explain how, mechanistically, two animals as highly diverged in both microbiome profile and evolutionary distance as a zebrafish and a fruit fly can have the same cellular response to the same bacteria-derived enzyme.

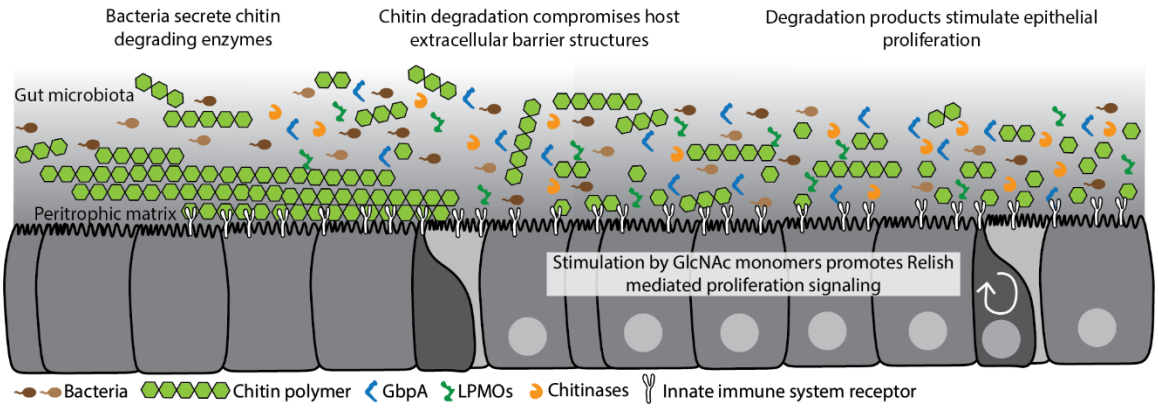


Figure 3.7 Host barrier degradation stimulates epithelial proliferation through Relish-mediated innate immune signaling.

While our work addresses what this protein does in a host system, finding what this protein does for the bacteria that secrete it remains more elusive. Previous work comparing Δ*gbpa* and WT *Aeromonas* and *Vibrio* strains have not found any growth, competitive index, or zebrafish colonization defects (Banse et al., 2022). Although the results from this work have indicated that GbpA is not specifically required for any one bacterial function, this is unsurprising when one considers bacteria are sensing and responding to many different carbon sources and signals in their immediate environments. GbpA and other LPMOs that can break down large chitin networks are advantageous for bacteria to make, since chitin is a highly abundant carbon source (Islam et al., 2017; Tang et al., 2015; Yang and Zhang, 2019; Zhang et al., 2021). However, it is unlikely that bacteria are exclusively using one method to break down environmental chitin. Research exploring the synergism between chitinases and LPMOs have found that bacteria use a combination of secreted LPMOs and chitinases to break down extracellular chitin more readily (Fennell et al., 2021; Yang et al., 2017b). To better understand what this specific LPMO is doing for the bacteria that secretes it, understanding chitin

metabolism may be more informative given the widespread conservation of LPMOs and abundance of chitinases in bacteria.

Perhaps our most interesting discovery presented here was that LPMO enzymes are not required to elicit their characteristic proliferative response in GF flies. Instead, we provide evidence that demonstrates innate immune signaling is responding to an increase in the concentration of luminal GlcNAc monomers. This result is striking because it suggests that the innate immune system is not only sensing and responding to bacteria and microbial signals, but also responding to concentrations of building units related to barrier structures like the PM. A similar relationship is seen in mammals, where small chitin fragments activate TLR signaling in macrophages (da Silva et al., 2008). It is of note that chitin fragments are sensed through TLR signaling, as this is a highly conserved signaling pathway in animals (Fuchs et al., 2018). The high degree of conservation of both binding substrate and receptor along with the proliferative response in host animals supports the hypothesis that epithelial proliferation in response to colonization by the microbiota is a conserved response to bacterial degradation of host barrier structures.

While this study focuses on the effect of microbial chitin degradation, animals also produce their own chitinase enzymes (Teng et al., 2014; Zhao et al., 2020; Zhu et al., 2008). In vertebrates, chitinase expression is primarily induced by infection with bacterial and fungal pathogens (Bueter et al., 2013; Elieh Ali Komi et al., 2018). Future studies comparing the proliferative effect of these chitinases would be ideal for testing if chitinase activity from any source is sufficient to increase epithelial proliferation or if microbe-derived chitinase activity is uniquely required for this effect. This line of questioning could also be used to examine functional differences between animal derived

chitinases and microbially derived chitinases. Another interesting line of questioning using animal derived chitinases would be looking at synergistic reactions between them and microbial chitinases.

To get large sheets of chitin, GlcNAc monomers must first be synthesized and linked together. Vertebrate fish like zebrafish and salmon can synthesize chitin along with many invertebrates, like arthropods (Tang et al., 2015). However, mammals cannot, and must consume chitin for it to be in their gut, but they do synthesize GlcNAc, which is present on gut mucins (Bergstrom and Xia, 2013; Trastoy et al., 2020). This research illuminates the importance of concentration of GlcNAc monomers in the gut, making chitin synthesis genes an appealing target for future studies probing this aspect of host-microbiome relationships. On one hand, one could imagine an increase in GlcNAc synthesis would lead to increased size or integrity of the mucosal barrier, resulting in a decreased proliferative reaction in response to the microbiota. On the other hand, increased GlcNAc synthesis could result in an increased concentration of GlcNAc monomers and itself could promote epithelial proliferation regardless of colonization status.

The evidence presented here along with evidence from another recent study looking at the same protein in zebrafish (Banse et al., 2022) suggests that chitin degradation generally promotes epithelial proliferation. Future studies looking at the effect of a similar protein in mice would also be necessary to verify if this relationship is indeed conserved in mammals. If this class of enzyme has a similar proliferative effect in mammals, this enzyme could potentially be used as a treatment for patients to promote epithelial proliferation and repair without use of harsh drugs or antibiotics. Because

treatment with LPMOs restores epithelial proliferation, we predict this class of enzyme may be acting as a homeostasis factor.

The work presented here takes a simple, conserved reaction to the gut microbiota and looks at what this relationship can tell us about the evolution of host-microbiome relationships. We've found using *Drosophila*, a simpler and more ancestral representation of the host-microbiota relationship, that the highly conserved proliferative reaction to colonization by the microbiota is not actually caused by the microbiota at all, but rather is caused by a sensed increase in the amount of luminal GlcNAc monomers. This body of work illustrates the importance of looking past broad organismal differences and instead focusing on common elements between different systems when considering evolutionary questions.

Materials and Methods

Drosophila

Drosophila melanogaster were reared at 25°C, 12h:12h light:dark cycle in a humidified chamber on standard cornmeal agar medium. All assays were performed on mated adult females. Fly lines used for this study were: *esg-Gal4/CyO; UAS-GFP/TM6B* (Micchelli and Perrimon, 2006), *rel[E38]* (BDSC 9458), *myo1A-Gal4* (BDSC 67088), and *UAS-GbpA/CyO*.

Axenic fly preparation

Newly laid eggs (<18hr) were collected from apple juice caps into a sterile mesh bottom basket and briefly rinsed with ddH_2O. Eggs were then bathed in a 1:1 bleach:water bath for 5 minutes followed by two 70% EtOH baths and two sterile ddH_2O

baths. After washing, the mesh containing sterilized eggs was removed from the rest of the basket and placed in a sterile culture bottle containing standard cornmeal agar and reared under standard conditions.

To confirm if flies were successfully reared without colonization by bacteria, 1-2 larvae were selected from each bottle. These larvae were ground in sterile 200 μL 1X PBS using a pipette tip and 150 μL of this homogenate was plated on MRS + Agar plates. Plates were incubated at 30℃ for 48 hours. At the conclusion, test plates were checked for any visible bacterial growth. Flies were considered GF only if there were no bacterial colonies present at the end of 48 hr. Flies considered CV did not endure a sham axenic preparation and were not tested for bacterial growth on media.

Cloning

The open reading frame for GbpA was amplified from a pET-11a plasmid using primers 5'-atggcagcaaaaatccaactcaatcacatc-3' and 5'-ggcaagaagcctggattgagctgaagtgactcgag-3'. Amplified DNA was run on a 1% agarose gel and excised and cleaned using the Zymo Clean and Concentrate 5 Kit (D4014).

To generate transgenic flies, the ORF was transferred into a pUAST-attB plasmid. The pUAST-attB plasmid was extracted from *E. coli* DH5α using a Qiagen Plasmid Miniprep Kit (27106X4), then the historic ORF removed by performing a restriction enzyme double digest with restriction enzymes BglII and XhoI in NEBuffer 3.1 (B7203). Linearized plasmid was extracted and cleaned from a 1% agarose gel using the Zymo GelDNA Recovery Kit (D4007). Amplified GbpA ORF and the linearized plasmid backbone were combined in a ligation reaction using T4 Ligase enzyme (NEB, M0202)

and buffer (NEB, B0202) and incubated overnight in a thermocycler at 16°C. Plasmid concentration was assessed on a Nanodrop before transforming into *E. coli* DH5α. Successful transformation was confirmed via PCR amplification of the target insertion and DNA sequencing. After confirming faithful inclusion of GbpA into pUAST-attB, this plasmid purified for *Drosophila* embryo injection by BestGene using a Qiagen Plasmid Maxi Kit (12162).

Protein purification

GbpA, GbpA D1, and GbpA D1AA carrying C-terminal TEV cleavage sites and 6x-His tags were generated on pET-11a by GenScript. Each of these plasmids was transformed into E. coli BL21 DE3. Protein expression was induced at $OD_{600} = \sim 0.6$ using 1 mM IPTG for 18 hours at 18°C, shaking. Proteins were purified from the periplasm using a Nickel bead affinity column. Isolated periplasm was flowed over the prepared column in a 1:1 ratio with lysis buffer (10mM Imidazole). Approximately 25 mL Wash buffer (10 mM Imidazole) was applied to the column to elute loosely bound protein from the column, until $OD_{280} < 0.01$. To elute other loosely bound proteins, we applied approximately 25 mL Wash buffer (40 mM Imidazole) over the column, until $OD_{280} < 0.01$. Target protein was eluted from the column using 300 mM Imidazole in 1 mL fractions. Samples from fractions with the highest OD_{280} were run on an SDS-PAGE gel to confirm if the desired product was present and to identify any impurities then dialyzed overnight at 4°C.

CBP21 and CBP21AA carrying C-terminal TEV cleavage sites and 6x-His tags were generated on pET-22b by GenScript. Both plasmids were transformed into E. coli BL21 DE3. Protein expression was induced at $OD_{600} = \sim 0.5$ using 1 mM IPTG for 4

hours at 30℃, shaking. Proteins were purified from the periplasm using a Nickel bead affinity column. Isolated periplasm was flowed over the prepared column in a 1:1 ratio with lysis buffer (10mM Imidazole). Approximately 25 mL Wash buffer (10 mM Imidazole) was flowed over the column to elute loosely bound protein from the column, until $OD_{480} < 0.01$. To elute other loosely bound proteins, we flowed approximately 25 mL Wash buffer (40 mM Imidazole) over the column, until $OD_{280} < 0.01$. Target protein was eluted from the column using 300 mM Imidazole in 1 mL fractions. Samples from fractions with the highest OD_{280} were run on an SDS-PAGE gel to confirm if the desired product was present and to identify any impurities then dialyzed overnight at 4℃.

Concentrations of each protein were determined using OD_{280} absorbance measured on a spectrophotometer and Beer's Law $(A = \varepsilon bC)$. Observed concentrations were used along with molecular weights of each protein to match dosage administered by molarity.

Feeding assays

For assays using GbpA enriched CFS, 15-20 females aged 4 dpe were fed CFS from E. coli BL21 DE3 carrying pET-11a containing the ORF for GbpA. E. coli containing the plasmid of interest were grown at 37℃ until $OD_{600} = 0.6$ at which point expression of GbpA was induced using 1 mM IPTG and bacteria were incubated at 30C for 4-6 hr to allow for enrichment of GbpA in the supernatant. At the end of this expression period, bacterial cultures were pelleted to separate bacteria from supernatant. Isolated supernatant was passed through a 0.2 um filter to remove bacteria and CFS containing GbpA concentrated to 1-2 mL using a 20,000 MWCO filter in a swinging bucket centrifuge. Concentrated CFS was fed to GF flies by applying 800 uL to Whatman

filter paper and allowing flies to feed freely for 24 hr at 25°C with a 12h:12h light:dark cycle.

For assays using purified protein, 15-20 Female flies aged 4 dpe were fed purified GbpA, GbpA D1, GbpA D1AA, CBP21, and CBP21AA at a concentration of 0.002 mM in liquid food containing 5% sucrose and 0.05% bromophenol blue in sterile ddH_2O. Chitinase from *Streptomyces griseus* (referred to as chitinase, Millipore Sigma, C6137) was introduced at a concentration of 0.1 mg/mL and GlcNAc monomers (Chem-Impex, 01427) were introduced at a concentration of 1 M. Vehicle control samples were given sterile ddH2O or dialyzing buffer where appropriate. 800 μL of liquid food was applied to Whatman filter paper and placed with flies in an empty, sterile narrow vial then sealed. Flies were allowed to feed freely for 24 hr at 25°C with a 12h:12h light:dark cycle.

GbpA transgenic expression

Transgenic flies capable of expressing GbpA were generated by BestGene using the FlyC31 system (Bateman et al., 2006). Purified pUAST-attB containing HA-GbpA was injected into embryos containing an attP site on chromosome II (BDSC 24482). Incorporation of the gene was confirmed by phenotype, with flies containing UAS-GbpA expressing a light orange eye color.

To drive expression of GbpA in stem cells, we used flies carrying *escargot-Gal4* (Micchelli and Perrimon, 2006) and crossed them to flies carrying *UAS-GbpA*. To drive expression of GbpA in enterocytes, we used flies carrying *myo1A-Gal4* (Jiang et al., 2009). Expression of GbpA was confirmed phenotypically via proliferative response in GF individuals.

Gut dissections and immunohistochemistry

At the conclusion of the feeding assay, flies were anesthetized using CO_2 then briefly fixed in 95% EtOH and carcasses washed in cold, sterile 1X PBS. Guts were then dissected using sharp, fine-tipped forceps and micro dissecting scissors under a dissection microscope in a 1 mL drop of cold, sterile 1X PBS and stored in fresh cold, sterile 1X PBS up to 20 minutes, while guts in one experimental group were dissected. Guts were then fixed in 4% Paraformaldehyde/1X PBS for 20 minutes at room temperature, shaking. Guts were permeabilized by washing 3x 15 min with PBS containing 0.1% Triton X-100 (Fisher Scientific, BP151-500; PBS-T) on shaker then blocked in PBS containing 0.1% Triton X-100 and 1% Bovine serum albumin (RPI, 9048-46-8; PBS-TB) approximately 2 hr at room temperature on shaker. Primary antibodies diluted in PBS-TB and applied overnight at 4°C on shaker, covered: chicken anti-GFP (1:500, AVES, GFP-1010), rabbit anti-PH3 (1:500, Cell Signaling Technology, 9710). Unbound primary antibody removed by washing 3x 15 min with PBS-TB. Secondary antibodies diluted in PBS-TB and applied either 2 hr at room temp or overnight at 4°C on shaker, covered: goat anti-chicken AF488 (1:1000, Invitrogen, A-11001), goat anti-rabbit AF594 (1:1000, Invitrogen, A-11012). Unbound secondary antibody removed by washing 3 times 15 min with PBS-TB. Vectashield with DAPI (Vector Laboratories, H-1200) applied and guts stored at 4°C covered and shaking prior to mounting and imaging guts.

Confocal microscopy

Prepared guts in Vectashield were mounted to 25 x 75 x 1 mm Fisherbrand frosted tab slides (Fisherbrand, 22-034-486) sealed with 22 x 60 mm Fisherbrand No. 1 coverslips (Fisherbrand, 12-545-JP). Slides were stored at 4°C protected from light prior

to imaging. Optical sections were captured of each gut in panels using a Leica TCS confocal with a 10X lens. AF488 secondary antibody signal was captured using 485 nm excitation with 506-564 nm absorbance range, AF594 secondary antibody (pseudocolored to magenta using ImageJ) was captured using 561 nm excitation with 573-781 nm absorbance range, and DAPI nuclear stain was captured using 405 nm excitation laser with 410-493 nm absorbance range.

Avermectin (AVM) pharmacokinetic toxicity assays

Approximately 50 adult axenic female flies were fed GbpA D1, GbpA D1AA, CBP21, or chitinase according to feeding assay methods above. At the conclusion of the feeding assay, flies were placed in prepared AVM bottles. These bottles were prepared by applying 1 mL 0.057 mM AVM (Millipore Sigma, 31732) in acetone or 1 mL acetone for controls. Acetone was allowed to evaporate from bottles for 24 hr in a chemical hood prior to the addition of flies. At the end of the 24 hr feeding period, the number of surviving adults was counted and set as the starting number for the survival assay. Flies were kept at 25℃ with a 12 on/12 off light cycle for the duration of the experiment. Survivors were checked in the mornings and evenings for up to 6 days.

CHAPTER IV

CONCLUDING REMARKS

Introduction

This dissertation explores a highly conserved interaction between animals and their gut microbiota. We show that the activity of a class of LPMO endochitinases is sufficient to promote epithelial proliferation in zebrafish and fruit flies. In flies, we further show that epithelial proliferation is not a unique response to bacterial LPMOs, but rather a general response to increased GlcNAc monomers in the gut generated by diverse chitinases. We also provide evidence that the activities of chitin degrading proteins are sensed through Relish/NFkB signaling via the ezymatic byproducts of GlcNAc polymers. Taken together, our results support a model where bacteria colonize the gut and degrade host barriers, releasing GlcNAc monomers, which activates Relish/NFkB-mediated innate immune signaling and promotes epithelial proliferation. This model represents a new way of thinking about host-microbe relationships, emphasizing microbial function over presence of a specific gene or species.

Evolutionary Implications

When describing new evolutionary or ecological models, the Occam's razor states that the simplest answer is the correct one. It is certainly easy in biology to come up with complex answers to complex questions, but these, as parsimony would dictate, often involve assumptions that are incorrect. In this body of work, we've presented an open-ended question: how does the gut microbiota promote epithelial proliferation? While the proliferative relationship is ultimately one between individual hosts and their associated consortium of microbes, this question also carries with it a larger implication of how

evolutionarily distant animals like flies and fishes retain the same response to colonization by populations of microbes that are ultimately very different from each other because of the hosts they colonize. When comparing any two animal microbiomes or digestive tracts, the differences are often much easier to identify than the commonalities. However, when we begin to look for these commonalities, we can start to understand the evolutionary context behind the shared outcomes we see in colonized animals today.

The model we present here is an excellent representation of how executing comparative studies can inform what we know about evolutionarily conserved relationships. By looking at the common elements of this relationship between two highly divergent animals and their associated microbiomes, we have been able to take a seemingly complex biological and evolutionary question and show that the answer is quite simple. This result also speaks to the power of using model organisms to study cellular mechanisms. Because processes like proliferation, innate immune signaling, and microbial colonization are so highly conserved, the use of divergent organisms presents an advantage for examining the basis of these processes. Our use of *Drosophila* in this study revealed the relationship between the innate immune system and sensing of luminal GlcNAc monomers.

Host Contributions to Chitin Synthesis and Degradation

Our studies considered conserved chitinases in diverse bacterial species. Given the amount of contact animals have with chitin, it is unsurprising that they also endogenously produce chitinases (Teng et al., 2014; Zhao et al., 2020; Zhu et al., 2008). In invertebrates like arthropods that are primarily composed of chitin, chitinases can be expressed as part of body patterning events (Pesch et al., 2016; Tiklová et al., 2013; Zhu

et al., 2008). In vertebrate organisms like fish and mice, chitinases are often expressed in response to colonization by pathogenic bacteria or fungi (Teng et al., 2014; Zhao et al., 2020). Future studies looking at these chitinases and their ability to increases epithelial proliferation would present an opportunity to further probe the relationship between chitin degradation and epithelial proliferation.

While chitinases are conserved in mammalian lineages, chitin synthesis genes are not (Tabata et al., 2018; Tang et al., 2015). However, chitin synthesis genes are conserved in earlier diverged animals and have been reported in organisms like bony fishes and arthropods (Tang et al., 2015). Future studies looking at the influence of these chitin synthesizing genes could explore how different amounts and orientations of chitin across epithelial tissues could shape the proliferative responses of these tissues when treated with chitin degrading enzymes. It may be the case that animals can distinguish self-produced chitin-degradation products from those produced by exogenous chitinases to inform protective proliferative responses of their tissues.

REFERENCES CITED

Abo, H., Chassaing, B., Harusato, A., Quiros, M., Brazil, J. C., Ngo, V. L., Viennois, E., Merlin, D., Gewirtz, A. T., Nusrat, A., et al. (2020). Erythroid differentiation regulator-1 induced by microbiota in early life drives intestinal stem cell proliferation and regeneration. *Nat Commun* **11**, 513.

ABRAMS, G. D., BAUER, H. and SPRINZ, H. (1963). Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. *Lab Invest* **12**, 355–64.

Al-Asmakh, M. and Zadjali, F. (2015). Use of germ-free animal models in microbiota-related research. *Journal of Microbiology and Biotechnology* **25**, 1583–1588.

Alberdi, A., Martin Bideguren, G. and Aizpurua, O. (2021). Diversity and compositional changes in the gut microbiota of wild and captive vertebrates: a meta-analysis. *Scientific Reports* **11**, 22660.

Alvarez, F. J. (2014). The Effect of chitin size, shape, source and purification method on immune recognition. *Molecules* **19**, 4433–4451.

Anderson, K. v. (2000). Toll signaling pathways in the innate immune response. *Current Opinion in Immunology* **12**, 13–19.

Askarian, F., Uchiyama, S., Masson, H., Sørensen, H. V., Golten, O., Bunæs, A. C., Mekasha, S., Røhr, Å. K., Kommedal, E., Ludviksen, J. A., et al. (2021). The lytic polysaccharide monooxygenase CbpD promotes Pseudomonas aeruginosa virulence in systemic infection. *Nature Communications* **12**,.

Banse, A. v., VanBeuge, S., Smith, T. J., Logan, S. L. and Guillemin, K. (2022). Secreted Aeromonas GlcNAc binding protein GbpA stimulates epithelial cell proliferation in the zebrafish intestine. *bioRxiv*.

Bateman, J. R., Lee, A. M. and Wu, C. (2006). Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. *Genetics* **173**, 769–77.

Bates, J. M., Akerlund, J., Mittge, E. and Guillemin, K. (2007). Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* **2**, 371–82.

Bergstrom, K. S. B. and Xia, L. (2013). Mucin-type O-glycans and their roles in intestinal homeostasis. *Glycobiology* **23**, 1026–1037.

Bhowmick, R., Ghosal, A., Das, B., Koley, H., Saha, D. R., Ganguly, S., Nandy, R. K., Bhadra, R. K. and Chatterjee, N. S. (2008). Intestinal adherence of Vibrio cholerae involves a coordinated interaction between colonization factor GbpA and mucin. *Infection and Immunity* **76**, 4968–4977.

Blander, J. M. (2016). Death in the intestinal epithelium - Basic biology and implications for inflammatory bowel disease. *FEBS J.* **283**, 2720–2730.

Book, A. J., Yennamalli, R. M., Takasuka, T. E., Currie, C. R., Phillips, G. N. and Fox, B. G. (2014). Evolution of substrate specificity in bacterial AA10 lytic polysaccharide monooxygenases. *Biotechnology for Biofuels* **7**, 109.

Bosch, T. C. G. and McFall-Ngai, M. (2021). Animal development in the microbial world: Re-thinking the conceptual framework. *Curr Top Dev Biol* **141**, 399–427.

Broderick, N. A., Buchon, N. and Lemaitre, B. (2014). Microbiota-Induced Changes in Drosophila melanogaster Host Gene Expression and Gut Morphology. *mBio* **5**, 1–13.

Bueter, C. L., Specht, C. A. and Levitz, S. M. (2013). Innate Sensing of Chitin and Chitosan. *PLoS Pathogens* **9**, 1–3.

Charroux, B. and Royet, J. (2012). Gut-microbiota interactions in non-mammals: What can we learn from Drosophila? *Seminars in Immunology* **24**, 17–24.

Cheesman, S. E., Neal, J. T., Mittge, E., Seredick, B. M. and Guillemin, K. (2011). Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. *Proc Natl Acad Sci U S A* **108**, 4570–4577.

Chen, L. P., Wang, P., Sun, Y. J. and Wu, Y. J. (2016). Direct interaction of avermectin with epidermal growth factor receptor mediates the penetration resistance in drosophila larvae. *Open Biology* **6**,.

Choi, K.-H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R. and Schweizer, H. P. A Tn7-based broad-range bacterial cloning and expression system.

Cryan, J. F. and Mazmanian, S. K. (2022). Microbiota-brain axis: Context and causality. *Science* **376**, 938–939.

da Silva, C. A., Hartl, D., Liu, W., Lee, C. G. and Elias, J. A. (2008). TLR-2 and IL-17A in Chitin-Induced Macrophage Activation and Acute Inflammation. *The Journal of Immunology* **181**, 4279–4286.

Douglas, A. E. (2018). The Drosophila model for microbiome research. *Lab Animal*.

Elieh Ali Komi, D., Sharma, L. and dela Cruz, C. S. (2018). Chitin and Its Effects on Inflammatory and Immune Responses. *Clinical Reviews in Allergy and Immunology* **54**, 213–223.

Fennell, T. G., Blackwell, G. A., Thomson, N. R. and Dorman, M. J. (2021). gbpA and chiA genes are not uniformly distributed amongst diverse Vibrio cholerae. *Microb Genom* **7**,.

Flemming, H.-C. and Wuertz, S. (2019). Bacteria and archaea on Earth and their abundance in biofilms. *Nature Reviews Microbiology* **17**, 247–260.

Fuchs, K., Gloria, Y. C., Wolz, O., Herster, F., Sharma, L., Dillen, C. A., Täumer, C., Dickhöfer, S., Bittner, Z., Dang, T., et al. (2018). The fungal ligand chitin directly binds TLR2 and triggers inflammation dependent on oligomer size. *EMBO Rep* e201846065.

Graf, J. (1999). Symbiosis of Aeromonas veronii biovar sobria and Hirudo medicinalis, the medicinal leech: a novel model for digestive tract associations. *Infect Immun* **67**, 1–7.

Hill, J. H., Franzosa, E. A., Huttenhower, C. and Guillemin, K. (2016). A conserved bacterial protein induces pancreatic beta cell expansion during zebrafish development. *Elife* **5**, 18.

Hörmann, N., Brandão, I., Jäckel, S., Ens, N., Lillich, M., Walter, U. and Reinhardt, C. (2014). Gut microbial colonization orchestrates TLR2 expression, signaling and epithelial proliferation in the small intestinal mucosa. *PLoS ONE* **9**, 2–12.

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., FitzGerald, M. G., Fulton, R. S., et al. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214.

Ishaaya, I., Yablonski, S., Gurevitz, E. and Renneh, S. (1986). Toxicity of Avermectin B1 to Drosophila melanogaster (Diptera: Drosophilidae) and Lobesia botrana (Lepidoptera: Tortricidae). *Journal of Economic Entomology* **79**, 1621–1623.

Islam, S., Bhuiyan, M. A. R. and Islam, M. N. (2017). Chitin and Chitosan: Structure, Properties and Applications in Biomedical Engineering. *Journal of Polymers and the Environment* **25**, 854–866.

Jemielita, M., Taormina, M. J., Burns, A. R., Hampton, J. S., Rolig, A. S., Guillemin, K. and Parthasarathy, R. (2014). Spatial and Temporal Features of the Growth of a Bacterial Species Colonizing the Zebrafish Gut. *mBio* **5**,.

Jiang, H., Patel, P. H., Kohlmaier, A., Grenley, M. O., Donald, G. and Edgar, B. A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. *Cell* **137**, 1343–1355.

Jones, T. A. and Guillemin, K. (2018). Racing to Stay Put: How Resident Microbiota Stimulate Intestinal Epithelial Cell Proliferation. *Current Pathobiology Reports*.

Jones, R. M., Luo, L., Ardita, C. S., Richardson, A. N., Kwon, Y. M., Mercante, J. W., Alam, A., Gates, C. L., Wu, H., Swanson, P. A., et al. (2013a). Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO Journal* **32**, 3017–3028.

Jones, R. M., Luo, L., Ardita, C. S., Richardson, A. N., Kwon, Y. M., Mercante, J. W., Alam, A., Gates, C. L., Wu, H., Swanson, P. A., et al. (2013b). Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO J* **32**, 3017–28.

Jones, T. A., Hernandez, D. Z., Wong, Z. C., Wandler, A. M. and Guillemin, K. (2017). The bacterial virulence factor CagA induces microbial dysbiosis that contributes to excessive epithelial cell proliferation in the Drosophila gut. *PLoS Pathogens* **13**, 1–20.

Keller, P. J., Schmidt, A. D., Wittbrodt, J. and Stelzer, E. H. K. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* **322**, 1065–9.

Kirn, T. J., Jude, B. A. and Taylor, R. K. (2005a). A colonization factor links Vibrio cholerae environmental survival and human infection. *Nature* **438**, 863–866.

Kirn, T. J., Jude, B. A. and Taylor, R. K. (2005b). A colonization factor links Vibrio cholerae environmental survival and human infection. *Nature Letter* **438**, 6–9.

Kitts, G., Giglio, K. M., Zamorano-Sánchez, D., Park, J. H., Townsley, L., Cooley, R. B., Wucher, B. R., Klose, K. E., Nadell, C. D., Yildiz, F. H., et al. (2019). A Conserved Regulatory Circuit Controls Large Adhesins in Vibrio cholerae. *mBio* **10**,.

Lee, C. G., da Silva, C. A., Lee, J.-Y., Hartl, D. and Elias, J. A. (2008). Chitin regulation of immune responses: an old molecule with new roles. *Current Opinion Immunology* **20**, 684–689.

Leulier, F., MacNeil, L. T., Lee, W.-J., Rawls, J. F., Cani, P. D., Schwarzer, M., Zhao, L. and Simpson, S. J. (2017). Integrative Physiology: At the Crossroads of Nutrition, Microbiota, Animal Physiology, and Human Health. *Cell Metab* **25**, 522–534.

Logan, S. L., Thomas, J., Yan, J., Baker, R. P., Shields, D. S., Xavier, J. B., Hammer, B. K. and Parthasarathy, R. (2018). The Vibrio cholerae type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. *Proc Natl Acad Sci U S A* **115**, E3779–E3787.

Loose, J. S. M., Forsberg, Z., Fraaije, M. W., Eijsink, V. G. H. and Vaaje-Kolstad, G. (2014). A rapid quantitative activity assay shows that the Vibrio cholerae colonization factor GbpA is an active lytic polysaccharide monooxygenase. *FEBS Letters* **588**, 3435–3440.

Lyte, M., Varcoe, J. J. and Bailey, M. T. (1998). Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. *Physiology & Behavior* **65**, 63–68.

Madland, E., Forsberg, Z., Wang, Y., Lindorff-Larsen, K., Niebisch, A., Modregger, J., Eijsink, V. G. H., Aachmann, F. L. and Courtade, G. (2021). Structural and functional variation of chitin-binding domains of a lytic polysaccharide monooxygenase from Cellvibrio japonicus. *Journal of Biological Chemistry* **297**, 101084.

Maltz, M. and Graf, J. (2011). The Type II Secretion System Is Essential for Erythrocyte Lysis and Gut Colonization by the Leech Digestive Tract Symbiont *Aeromonas veronii*. *Applied and Environmental Microbiology* **77**, 597–603.

Massaquoi, M. S., Kong, G., Chilin, D., Hamilton, M. K., Melancon, E., Eisen, J. S. and Guillemin, K. (2022). Global Host Responses to the Microbiota at Single Cell Resolution in Gnotobiotic Zebrafish. *BioRXiv*.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. v., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences* **110**, 3229–3236.

Meibom, K. L., Li, X. B., Nielsen, A. T., Wu, C.-Y., Roseman, S. and Schoolnik, G. K. (2004). The *Vibrio cholerae* chitin utilization program. *Proceedings of the National Academy of Sciences* **101**, 2524–2529.

Melancon, E., Gomez De La Torre Canny, S., Sichel, S., Kelly, M., Wiles, T., Rawls, J., Eisen, J. S. and Guillemin, K. (2015). Best practices for germ-free derivation and gnotobiotic zebrafish husbrandry. *Methods Cell Biology* **155**, 1683–1695.

Micchelli, C. A. and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. *Nature* **439**, 475–479.

Miguel-Aliaga, I., Jasper, H. and Lemaitre, B. (2018). Anatomy and Physiology of the Digestive Tract of Drosophila melanogaster. *Genetics* **210**, 357–396.

Milligan-Myhre, K., Charette, J. R., Phennicie, R. T., Stephens, W. Z., Rawls, J. F., Guillemin, K. and Kim, C. H. (2011). Study of host-microbe interactions in zebrafish. *Methods Cell Biol* **105**, 87–116.

Myllymäki, H., Valanne, S. and Rämet, M. (2014). The *Drosophila* Imd Signaling Pathway. *The Journal of Immunology* **192**, 3455–3462.

Nakamura, A., Kurihara, S., Takahashi, D., Ohashi, W., Nakamura, Y., Kimura, S., Onuki, M., Kume, A., Sasazawa, Y., Furusawa, Y., et al. (2021). Symbiotic polyamine metabolism regulates epithelial proliferation and macrophage differentiation in the colon. *Nature Communications* **12**,.

Nakashima, K., Kimura, S., Ogawa, Y., Watanabe, S., Soma, S., Kaneko, T., Yamada, L., Sawada, H., Tung, C.-H., Lu, T.-M., et al. (2018). Chitin-based barrier immunity and its loss predated mucus-colonization by indigenous gut microbiota. *Nature Communications* **9**, 3402.

Neal, J. T., Peterson, T. S., Kent, M. L. and Guillemin, K. (2013). H. pylori virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model. *Dis Model Mech* **6**, 802–10.

Pan, M., Schwartzman, J. A., Dunn, A. K., Lu, Z. and Ruby, E. G. (2015). A Single Host-Derived Glycan Impacts Key Regulatory Nodes of Symbiont Metabolism in a Coevolved Mutualism. *mBio* **6**, e00811.

Pesch, Y.-Y., Riedel, D., Patil, K. R., Loch, G. and Behr, M. (2016). Chitinases and Imaginal disc growth factors organize the extracellular matrix formation at barrier tissues in insects. *Scientific Reports* **6**, 18340.

Pickard, J. M., Zeng, M. Y., Caruso, R. and Núñez, G. Gut Microbiota: Role in Pathogen Colonization, Immune Responses and Inflammatory Disease.

Pott, J. and Hornef, M. (2012). Innate immune signalling at the intestinal epithelium in homeostasis and disease. *EMBO Reports* **13**, 684–698.

Powell, D. N., Swimm, A., Sonowal, R., Bretin, A., Gewirtz, A. T., Jones, R. M. and Kalman, D. (2020). Indoles from the commensal microbiota act via the AHR and IL-10 to tune the cellular composition of the colonic epithelium during aging. *Proc Natl Acad Sci U S A* **117**, 21519–21526.

Rawls, J. F., Samuel, B. S. and Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS* **101**, 4596–4601.

Rawls, J. F., Mahowald, M. A., Ley, R. E. and Gordon, J. I. (2016). Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection. *Cell* **127**, 423–433.

Reedy, A. R., Luo, L., Neish, A. S. and Jones, R. M. (2019). Commensal microbiota-induced redox signaling activates proliferative signals in the intestinal stem cell microenvironment. *Development* **146**, dev171520.

Reese, T. A., Tager, A. M., Luster, A. D., van Rooijen, N., Voehringer, D. and Locksley, R. M. (2007). Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* **447**, 92–96.

Rolig, A. S., Mittge, E. K., Ganz, J., Troll, J. v, Melancon, E., Wiles, T. J., Alligood, K., Stephens, W. Z., Eisen, J. S. and Guillemin, K. (2017). The enteric nervous system promotes intestinal health by constraining microbiota composition. *PLoS Biol* **15**, e2000689.

Rolig, A. S., Sweeney, E. G., Kaye, L. E., DeSantis, M. D., Perkins, A., Banse, A. v, Hamilton, M. K. and Guillemin, K. (2018). A bacterial immunomodulatory protein with lipocalin-like domains facilitates host–bacteria mutualism in larval zebrafish. *Elife* **7**,.

Round, J. L. and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology* **9**, 313–323.

Sabbadin, F., Hemsworth, G. R., Ciano, L., Henrissat, B., Dupree, P., Tryfona, T., Marques, R. D. S., Sweeney, S. T., Besser, K., Elias, L., et al. (2018). An ancient family of lytic polysaccharide monooxygenases with roles in arthropod development and biomass digestion. *Nature Communications* **9**,.

Sigal, M., Rothenberg, M. E., Logan, C. Y., Lee, J. Y., Honaker, R. W., Cooper, R. L., Passarelli, B., Camorlinga, M., Bouley, D. M., Alvarez, G., et al. (2015). Helicobacter pylori Activates and Expands Lgr5(+) Stem Cells Through Direct Colonization of the Gastric Glands. *Gastroenterology* **148**, 1392–404.e21.

Sommer, F. and Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology* **11**, 227–238.

Syed, K. A., Beyhan, S., Correa, N., Queen, J., Liu, J., Peng, F., Satchell, K. J. F., Yildiz, F. and Klose, K. E. (2009). The *Vibrio cholerae* Flagellar Regulatory Hierarchy Controls Expression of Virulence Factors. *Journal of Bacteriology* **191**, 6555–6570.

Tabata, E., Kashimura, A., Kikuchi, A., Masuda, H., Miyahara, R., Hiruma, Y., Wakita, S., Ohno, M., Sakaguchi, M., Sugahara, Y., et al. (2018). Chitin digestibility is dependent on feeding behaviors, which determine acidic chitinase mRNA levels in mammalian and poultry stomachs. *Scientific Reports* **8**, 1–11.

Takashima, S. and Hartenstein, V. (2012). Genetic Control of Intestinal Stem Cell Specification and Development: A Comparative View. *Stem Cell Reviews and Reports* **8**, 597–608.

Tang, J. W., Fernandez, J., Sohn, J. J. and Amemiya, C. T. (2015). Chitin is endogenously produced in vertebrates. *Current Biology* **25**, 897–900.

Taormina, M. J., Jemielita, M., Stephens, W. Z., Burns, A. R., Troll, J. v, Parthasarathy, R. and Guillemin, K. (2012). Investigating bacterial-animal symbioses with light sheet microscopy. *Biol Bull* **223**, 7–20.

Tauszig, S., Jouanguy, E., Hoffmann, J. A. and Imler, J.-L. (2002). Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *Proceedings of the National Academy of Sciences* **97**, 10520–10525.

Teng, Z., Sun, C., Liu, S., Wang, H. and Zhang, S. (2014). Functional characterization of chitinase-3 reveals involvement of chitinases in early embryo immunity in zebrafish. *Dev Comp Immunol* **46**, 489–98.

Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G. and Nielsen, H. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology*.

The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. University of Oregon Press, Eugene.

Tiklová, K., Tsarouhas, V. and Samakovlis, C. (2013). Control of Airway Tube Diameter and Integrity by Secreted Chitin-Binding Proteins in Drosophila. *PLoS ONE* **8**, 67415.

Trastoy, B., Naegeli, A., Anso, I., Sjögren, J. and Guerin, M. E. (2020). Structural basis of mammalian mucin processing by the human gut O-glycopeptidase OgpA from Akkermansia muciniphila. *Nature Communications* **11**, 4844.

Vaaje-Kolstad, G., Houston, D. R., Riemen, A. H. K., Eijsink, V. G. H. and van Aalten, D. M. F. (2005). Crystal structure and binding properties of the Serratia marcescens chitin-binding protein CBP21. *Journal of Biological Chemistry* **280**, 11313–11319.

Vaaje-Kolstad, G., Forsberg, Z., Loose, J. S., Bissaro, B. and Eijsink, V. G. (2017). Structural diversity of lytic polysaccharide monooxygenases. *Current Opinion in Structural Biology* **44**, 67–76.

Vandhana, T. M., Reyre, J.-L., Sushmaa, D., Berrin, J.-G., Bissaro, B. and Madhuprakash, J. (2022). On the expansion of biological functions of lytic polysaccharide monooxygenases. *New Phytol* **233**, 2380–2396.

Varki, A. (2017). Biological roles of glycans. *Glycobiology* **27**, 3–49.

Walsh, K. T. and Guillemin, K. (2022). The Impacts of Microbiota on Animal Development and Physiology. In *Evolution, Biodiversity, and a Reassessment of the Hygiene Hypothesis* (ed. Rook, G. A. W.) and Lowry, C. A.), pp. 177–196.

Wang, G., Sweren, E., Liu, H., Wier, E., Alphonse, M. P., Chen, R., Islam, N., Li, A., Xue, Y., Chen, J., et al. (2021). Bacteria induce skin regeneration via IL-1β signaling. *Cell Host Microbe* **29**, 777-791.e6.

Wiles, T. J. and Guillemin, K. (2020). Patterns of partnership: surveillance and mimicry in host-microbiota mutualisms. *Current Opinion in Microbiology* **54**, 87–94.

Wiles, T. J., Jemielita, M., Baker, R. P., Schlomann, B. H., Logan, S. L., Ganz, J., Melancon, E., Eisen, J. S., Guillemin, K. and Parthasarathy, R. (2016). Host Gut Motility Promotes Competitive Exclusion within a Model Intestinal Microbiota. *PLOS Biology* **14**, e1002517.

Wiles, T. J., Wall, E. S., Schlomann, B. H., Hay, E. A., Parthasarathy, R. and Guillemin, K. (2018). Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages. *mBio* **9**,.

Williams, J. M., Duckworth, C. A., Burkitt, M. D., Watson, A. J. M., Campbell, B. J. and Pritchard, D. M. (2015). Epithelial Cell Shedding and Barrier Function: A Matter of Life and Death at the Small Intestinal Villus Tip. *Veterinary Pathology* **52**, 445–455.

Wong, E., Vaaje-Kolstad, G., Ghosh, A., Hurtado-Guerrero, R., Konarev, P. v., Ibrahim, A. F. M., Svergun, D. I., Eijsink, V. G. H., Chatterjee, N. S. and van Aalten, D. M. F. (2012). The Vibrio cholerae colonization factor GbpA possesses a modular structure that governs binding to different host surfaces. *PLoS Pathogens* **8**,.

Xu, Z., Takizawa, F., Casadei, E., Shibasaki, Y., Ding, Y., Sauters, T. J. C., Yu, Y., Salinas, I. and Sunyer, J. O. (2020). Specialization of mucosal immunoglobulins in pathogen control and microbiota homeostasis occurred early in vertebrate evolution. *Sci Immunol* **5**,.

Yang, J. and Zhang, Y. (2015). I-TASSER server: new development for protein structure and function predictions. *Nucleic Acids Res* **43**, W174-81.

Yang, J. and Zhang, K.-Q. (2019). Chitin Synthesis and Degradation in Fungi: Biology and Enzymes.pp. 153–167.

Yang, Y., Li, J., Liu, X., Pan, X., Hou, J., Ran, C. and Zhou, Z. (2017a). Improving extracellular production of Serratia marcescens lytic polysaccharide monooxygenase CBP21 and Aeromonas veronii B565 chitinase Chi92 in Escherichia coli and their synergism. **7**, 170.

Yang, Y., Li, J., Liu, X., Pan, X., Hou, J., Ran, C. and Zhou, Z. (2017b). Improving extracellular production of Serratia marcescens lytic polysaccharide monooxygenase CBP21 and Aeromonas veronii B565 chitinase Chi92 in Escherichia coli and their synergism. *AMB Express* **7**,.

Zhang, X., Yuan, J., Li, F. and Xiang, J. (2021). Chitin Synthesis and Degradation in Crustaceans: A Genomic View and Application. *Marine Drugs* **19**, 153.

Zhao, T., Su, Z., Li, Y., Zhang, X. and You, Q. (2020). Chitinase-3 like-protein-1 function and its role in diseases. *Signal Transduction and Targeted Therapy* **5**, 201.

Zhu, Q., Arakane, Y., Beeman, R. W., Kramer, K. J. and Muthukrishnan, S. (2008). Characterization of recombinant chitinase-like proteins of Drosophila melanogaster and Tribolium castaneum. *Insect Biochem Mol Biol* **38**, 467–77.