# BACTERIAL SECRETED PROTEIN GBPA PROMOTES CELL PROLIFERATION IN THE *DROSOPHILA* MIDGUT

by

ZOË WONG

A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2018

## An Abstract of the Thesis of

Zoë Wong for the degree of Bachelor of Arts
in the Department of Biology to be taken June 2018

Title: Bacterial secreted protein GbpA promotes cell proliferation in the *Drosophila* midgut

Approved: ________________________________________

Karen Guillemin

Both commensals and pathogens alike have innovated host-adapted survival strategies throughout the struggle to maintain evolutionary relevance. Successful microbes have found ways to build symbiotic relationships and hosts have similarly been conditioned to develop the means to benefit from, or at the very least tolerate, their associated microbes. In ever-changing environments like the gastrointestinal tract, high selective pressures call for bacterial-host interactions that contribute to homeostasis. In the intestine, maintaining healthy conditions depends on a careful balancing act between cell proliferation and cell death.

Previous work has demonstrated that secretion of bacterial protein GbpA by *Aeromonas veronii* is sufficient to induce epithelial cell proliferation in larval zebrafish[16]. GbpA is already well-studied in its role as a virulence-related colonization factor [43, 44], but far less is understood about its role as a chitin binding protein (CBP). To ask questions about GbpA, we propose to use *Drosophila melanogaster* as a model organism because of their short generation time, ability to be reared germ-free, and established assay for measuring cell proliferation in the midgut epithelium.

I found that GbpA significantly increased cell proliferation in the intestinal epithelium of the *Drosophila* midgut. Furthermore, I showed that domain 1 of the GbpA protein ($GbpA_{D1}$) is both necessary and sufficient to increase cell proliferation. An enzymatically dead mutant of GbpA was unable to elicit the same phenotype, which suggests that GbpA requires catalytic activity for full function. The shared response to GbpA across model organisms provides insight into the regulation of homeostasis and sets the stage for future investigations into the conservation of bacterial-host interactions across systems.

## Acknowledgements

I am beyond grateful for all of the people in the Guillemin lab that have mentored me, collaborated with me, and graciously answered all of my questions. The IMB has been the best learning environment and I am truly appreciative of the synergy between labs, projects, and people. Karen: thank you for being the most supportive PI, for infecting everyone in the lab with your love for microbes, and for inspiring me to know more. Tiffani: thank you for being a phenomenal mentor, a natural fly team leader/manager, and a wonderful person. Elena: thank you so much for sharing your fondness for interesting words, small glassware, strange questions, and satisfying science with me. Maria: thanks for telling me where reagents are, for being encouraging, and for showing me your adorable-dried-up-gel. Ellie: thank you for always brightening my day and encouraging me. Autumn/Deb/Liz/Stu: thank you for being exceedingly helpful no matter how busy you are.

I would also like to thank Dr. Karen Guillemin, Dr. Cristin Hulslander, and Dr. Melissa Graboyes for serving on my Thesis Committee. Dr. Kryn Stankunas from the Biology Honors program and Miriam Jordan from the Clark Honors College also helped me with the thesis process and I appreciate all of their guidance.

And, of course, thank you infinitely to my family. You're the best.

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# Introduction

## The Microbial world

*The microbiota and maintenance of homeostasis*

The microbes that live both in and on us, collectively known as our microbiota, are estimated to include $3.8 \cdot 10^{13}$ cells[1]. The microbial genome, or microbiome, includes over 5 million genes and surpasses the genetic potential of humans by two orders of magnitude [2, 3]. Findings from the NIH's Human Microbiome Project have jumpstarted research on the microbiota and suggested that an individual's microbial community is as unique to them as their fingerprints[4]. The implications of this scale and specificity both broaden our understanding of therapeutic possibilities and reframe our appreciation of physiologic diversity.

In addition to elucidating the sizable magnitude of our microbiota, research has also shed light on the varying effects that microbes have on health outcomes. Studies have linked obesity in mice[5], depression[6], and dietary patterns[7] to changes in microbial fingerprints. As our understanding of the microbiota has improved, however, an appreciation for beneficial microbes has countered the 'disease-causing' narrative of bacterial-host relations that previously dominated the field. Microbes are critical to tissue homeostasis and it is now understood that bacteria are key players in the metabolism of indigestible polysaccharides, production of essential vitamins, and the development of the intestinal epithelium and immune system of the host[8]. Identification of microbial species that are critical to homeostasis has been closely mirrored by the emergence of probiotics in popular culture, as well as a weariness towards the over

prescription of antibiotics. This shift in perception has increased overall excitement in asking experimental questions about the impact of microbial communities of specific organ systems.

*The gut microbiota as a complex community*

The gastrointestinal (GI) tract houses both the highest density and range of diversity of bacteria in the human body. Ideal growth conditions provide the nutrients to sustain an estimated 100 trillion microbes comprised of over 1,000 species[9]. More specifically, the colon consists of up to 1,012 microbes per gram of luminal content, which translates into nearly 60 percent of fecal weight [3, 10]. Though the homeostatic adult intestine is typically stable over time and primarily colonized by the phyla Bacteroidetes and Firmicutes, there is consistent variability between individuals at the species level[11]. The GI tract is a promising candidate for both genetic and environmental microbial research due to its heavily concentrated and wide-ranging assortment of bacterial species.

While this considerable community plays an active role in host health via nutrient absorption, defense against pathogens, and intestinal homeostasis[12] – it also contributes to disease phenotypes including states of inflammation[13-15] and excess cell proliferation[16-18]. This dual nature lays the foundation for many basic research questions about how bacterial-host interactions contribute to homeostasis in the intestine.

*Gnotobiology and germ-free models*

The complexity of microbial communities makes it challenging to parse out nuances of individual bacterial-host interactions. Gnotobiology, or the study of animals

either without or with defined microbial communities, allows us to investigate the impact of resident bacteria on the host. Many studies begin by defining cellular phenotypes in the absence of microbes (germ-free conditions) before investigating the effects of adding an individual strain or a defined community of microbes[19]. The ability to derive animals in the absence of bacteria is key when selecting a model organism for gnotobiotic studies.

The first successful germ-free derivations were reported by Nuttal & Thierfelder[20] in guinea pigs in 1895 and by Schottelius[21] in chickens in 1898. The survival of these animals disproved previously held ideas about host dependence on an essential microbiota, but the first germ-free guinea pigs were only able to survive up to 13 days[22]. Modern gnotobiology studies primarily use the germ-free mouse model, which was established by Pleasants in 1959[23-25]. This model rose in prominence after nutritional requirements were finally met by adequate sterilization techniques. The germ-free mouse model is useful because of its high conservation of mammalian traits and the extensive amount of genome mapping data in the field[26].

Adaptations of germ-free techniques to *Danio rerio* (zebrafish) and *Drosophila melanogaster* (fruit flies) present the ability to increase the scale of experiments in an inexpensive and less labor-intensive manner. Zebrafish have emerged as a gnotobiotic model over the last three decades and have the added benefit of optical transparency and vertebrate homology [27, 28]. Fruit flies boast simple microbial communities and short generation times, which make them ideal candidates for high throughput gnotobiotic assays. Both zebrafish and fruit flies can be easily made germ-free before their larval stages and germ-free status can be confirmed throughout development.

*Microbial influence on rates of intestinal epithelial proliferation*

The establishment of germ-free mice, zebrafish, and fruit flies opens the door for comparative studies across model organisms. Findings that are corroborated throughout model systems can elucidate conserved functions of the microbiota on animal development and point to microbial mechanisms that are relevant to humans. Notably, germ-free mice, zebrafish, and fruit flies all display decreased rates of intestinal epithelial cell proliferation in comparison to their conventional counterparts [16, 29-34]. Research in the Guillemin lab supports the aforementioned pattern and shows decreased levels of intestinal epithelial cell proliferation in germ-free models of both zebrafish (Figure 1A-C) and fruit flies (Figure 1D-E).

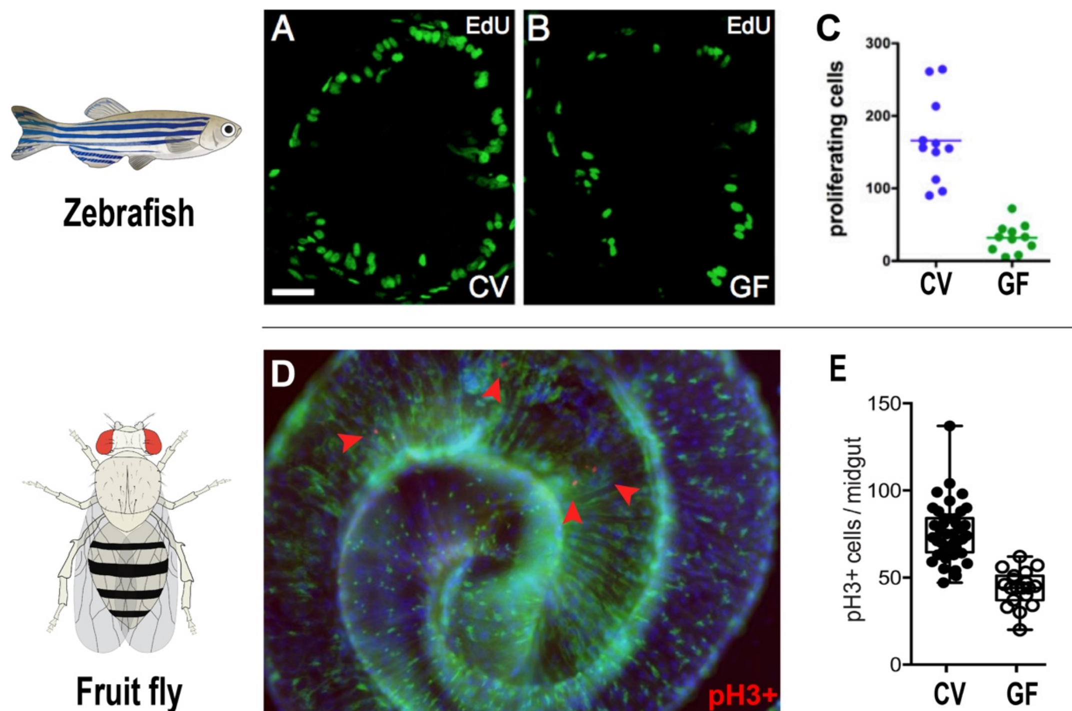

Figure 1: Intestinal epithelial cell proliferation decreases in germ-free models of zebrafish and fruit flies

(**1A-C** adapted from Cheesman et al. 2011[16]) Germ-free zebrafish (6-days post fertilization larvae) have lower levels of cell proliferation in the intestinal bulb in comparison to conventionally raised counterparts. Proliferating cells are stained with GFP and labeled with EdU in transverse cross-sections of the intestinal bulb of 6-dpf larvae reared conventionally (CV – A) or germ-free (GF – B).

(**1D-E** adapted from Tiffani Jones unpublished image and Jones et al. 2017[35]) Germ-free flies (7 day old adult females) have lower levels of cell proliferation in the epithelial midgut in comparison to conventionally raised counterparts. Proliferating cells in the epithelial midgut (D) are marked with phosphohistone H3+ (red and marked with arrowheads). Nuclei are stained blue with DAPI and intestinal stem cells are marked with GFP.

The intestinal epithelium is one of the most active self-renewing tissues in the human body[16] and it follows that homeostasis of intestinal epithelial cell proliferation is directly impacted by both the host and resident microbes.

In the newly colonized host, rapid turnover of intestinal epithelial cells can decrease the proportion of cells that have been stimulated by microbial exposure[36]. Upregulating proliferation of the intestinal epithelium could also ameliorate host immune responses, facilitate repair of epithelial damage, and lower pathogen-induced intestinal permeability[37]. Constant self-renewal helps maintain epithelial integrity by preventing persistent bacterial colonization, confining the spread of bacterial migration, and localizing inflammation [38-40]. As a protective barrier that separates the host from the external environment of the intestinal lumen, the intestinal epithelium faces incredible pressures to digest food, absorb nutrients, and maintain tight junctions[38]. These pressures may have promoted turnover of intestinal epithelial cells throughout the co-evolution of host organisms and their associated microbes.

In addition, it's likely that resident microbes also benefit from heightened intestinal epithelial turnover. While host systems attempt to balance rates of self-renewal with levels of apoptosis and cell exfoliation, microbes often subvert host functions and disrupt homeostasis in the process. Tipping the scale in favor of the self-renewal of intestinal epithelial cells is a way for microbes to increase access to cellular material that can be utilized as nutrients[36]. Additionally, bacteria can take advantage of transient gaps that form during cell shedding of the intestinal epithelium. Whether these gaps are formed under physiological or pathophysiological conditions, bacteria can adhere to or enter the basolateral side of adjacent cells[38]. Spontaneous epithelial cell shedding also leaves multicellular junctions vulnerable for bacterial internalization, which can provide lasting benefits for microbial species[41].

**Discovery of GbpA in *Aeromonas veronii cell free supernatant (CFS)***

*A. veronii increases proliferation in larval zebrafish*

Previous work in the Guillemin lab noted that a mono-association of resident microbe *Aeromonas veronii* is sufficient to restoring cell proliferation levels of germ-free larval zebrafish back to CV levels[16]. Furthermore, treatment with cell free supernatant (CFS) of *A. veronii* rescues the germ-free phenotype, which suggests that *A. veronii* mediates cell proliferation by secreting a product that is capable of interacting with the host. Most Gram-negative γ-proteobacteria, like *A. veronii*, utilize the highly conserved type II secretion system[42] (T2SS) and follow-up research by Allison Banse revealed that *A. veronii* requires the T2SS for full function. Banse used mass spectrometry analysis to screen proteins that were present in the CFS of a T2SS

complementation strain and absent in the T2SS mutant strain. After using ammonium sulfate precipitation to narrow down candidate proteins, Banse identified this protein as GlcNAc-binding protein A, or GbpA (unpublished work).

GbpA is a 55kDa protein that has been described as a virulence-related colonization factor in *Vibrio cholerae* [43-44]. The protein mediates adhesion of *V. cholerae* to host cells in its environment, which ranges from plankton in an aquatic environment to human intestinal cells [45-46]. Though initially recognized for its ability to confer colonization and persistence advantages to *V. cholerae*, GbpA also binds to carbohydrates that contain *N*-acetylglucosamine and has been shown to bind to mucin and chitin [44, 47]. Chitin is the second most abundant natural polysaccharide and serves as an important Carbon and Nitrogen source for organisms that can breakdown colloidal chitin[48]. GbpA is found in microbes that are not capable of utilizing chitin as a nutrient source, which suggests that bacteria have an ulterior motive for GbpA translation.

*GbpA is sufficient to promote intestinal cell proliferation in larval zebrafish*

To answer questions about the sufficiency of GbpA to increase intestinal cell proliferation, Banse cloned the *A. veronii gbpA* gene on an inducible high copy plasmid into *E. coli*. Induction with IPTG resulted in CFS that was dominated by the 55kDa GbpA protein. In comparison to an empty expression vector, the GbpA-enriched CFS was sufficient to increase levels of cell proliferation to those initially observed in CV larval zebrafish.

## Further investigation into GbpA

### *Conservation in Drosophila*

This work asks questions about the effect of GbpA on cell proliferation in the intestinal epithelium of *Drosophila*. Previous research shows sufficiency of GbpA to induce intestinal cell proliferation in larval zebrafish and overlapping characteristics between germ-free systems suggest that a conserved host response to GbpA could be reasonable. Tight control of intestinal epithelial cell proliferation is part of a fragile balancing act and improving our understanding of conserved bacterial-host interactions will contribute to our appreciation of homeostasis across systems.

# Hypothesis

**GbpA is sufficient to increase cell proliferation in *Drosophila***

GbpA is a highly conserved Chitin-Binding protein (CBP) and it follows that the bacterial-host response to GbpA could be conserved across zebrafish and fruit flies. Structural homologs of GbpA are expressed by *Enterobacter* and *Serratia* species, which are both microbes that naturally colonize *Drosophila.* Additionally, fruit flies express Chitinase 2 (Cht2) in the matrix of the epidermis, trachea, and digestive system in order to promote cuticle formation[49]. The cuticle is the tough, outer body wall that protects arthropods against invading pathogens[49]. It's possible that GbpA could elicit a response in fruit flies because of cuticle-forming pathways that evolved separately.

**Domain 1 of GbpA (GbpA $_{D1}$) is sufficient for increased cell proliferation**

Common domains of CBPs include the catalytic domain, fibronetctin type III domain (Fn3)[50], chitin-binding domain type III (ChtBD3)[51], cadherin-like domains[52], and cysteine-rich domains (CRD) [53, 54]. Understanding which domains of GbpA are required for increased intestinal epithelial cell proliferation will elucidate the mechanisms that GbpA employs to act on the host.

Uniquely, GbpA includes four domains. Its first domain, GbpA $_{D1}$, is primarily made up of a four-stranded and three-stranded β-sheet[43]. A 65-residue pseudo-domain resides between β-strands 1 and 3 and is comprised of short α-helices, a β-strand, and loops[43]. This allows two disulphide bonds to form in the loop/helical region (Cys-42 and Cys-56) and in-between β-strands 4 and 5 (Cys-152 and Cys-169), which could be targets of future experiments[43]. In *Vibrio*, GbpA is important for persistence in the

intestine and GbpA $_{D1}$ has been shown to be required for binding to intestinal mucin[43]. This suggests that GbpA $_{D1}$ contains necessary machinery and is required for proper bacterial-host interaction.

The second and third domains of GbpA are distantly related to bacterial surface proteins and are not as highly conserved[43]. Domain 4 shows 26% sequence identity to the chitin-binding domain of the C-terminal of *S. marcescens* chitinase B. Though the potential for shared chitinase sequence might be interesting, domain 4 is also not highly conserved. Only one of two aromatic amino acids that is thought to be important in *Sm*ChiB-chitin interactions is conserved in GbpA sequence. More broadly, less is understood about domain 4 as the crystal structure of GbpA is missing the C-terminal domain 4[43].

Based on the determined structure of GbpA, we expect that the GbpA$_{D1}$ will be able to increase cell proliferation levels and will be significantly similar to results from the full GbpA construct.

**GbpA $_{D1}$ is required for increased cell proliferation**

Conversely, we would also expect that GbpA$_{D2-4}$ would not be sufficient to increase cell proliferation levels and would model results from the empty GbpA construct. Necessity of GbpA$_{D2-4}$ in GbpA's overall function has yet to be demonstrated. This would imply that GbpA$_{D1}$ is required for increased intestinal epithelial cell proliferation and that GbpA$_{D2-4}$ is not sufficient to induce increased cell proliferation.

**The enzymatic activity of GbpA is required for increased cell proliferation**

$GbpA_{D1}$ is also GbpA's catalytic domain and must recognize and bind copper ($Cu^{2+}$) in order to have proper enzyme function. We will construct a $GbpA_{H25A,\ H111A}$ mutant by converting the Histidines at position 25 and position 111 into Alanine. Histidine contains an imidazole group and typically has a negative charge when found at $pH > 4$. Alanine, on the other hand, does not have a sidechain. We expect the $GbpA_{H25A,\ H111A}$ mutant to be enzymatically dead because the Alanines in the 25 and 111 positions will be unable to attract and bind $Cu^{2+}$.

# Results

## GbpA is sufficient to increase cell proliferation in *Drosophila*

Earlier work from the Guillemin lab investigated the secreted products in the cell free supernatant (CFS) of *A. veronii*, a resident microbe of the zebrafish microbiota known to increase proliferation in the intestinal epithelium. CFS from an *A. veronii* deletion mutant of the T2SS was not capable of increasing cell proliferation, suggesting that the pro-proliferative secreted product was part of the T2 secretome. Ammonium sulfate precipitation revealed that GbpA, a chitin-binding protein, was in the correct fraction and of the right molecular weight (55 kDa). The *A. veronii gbp* gene was cloned into an inducible, high copy plasmid in *E. coli* to determine whether or not GbpA was sufficient to increase cell proliferation. The CFS of the GbpA-enriched CFS of the *E. coli* was sufficient to elevate intestinal epithelial cell proliferation to levels similar to the conventionally raised counterpart, while the CFS from the empty vector *E. coli* did not have an effect.

To determine if GbpA was sufficient to raise cell proliferation in the *Drosophila* midgut, the same *E. coli* constructs were used as previously described (Figure 3A, 3B). Adult flies were cleared of their microbes through manual transfer and CFS was orally administered to 6-day old flies via a CFS-sucrose solution that was applied to whatman filter paper (Figure 9). Adult females were dissected on day seven and midguts were stained for pH3+, which marks proliferating cells by staining cells in late G2 and M phase. I observed that whereas the empty vector control had no effect on levels of cell

proliferation of germ-free flies, the GbpA-enriched CFS significantly increased cell proliferation.

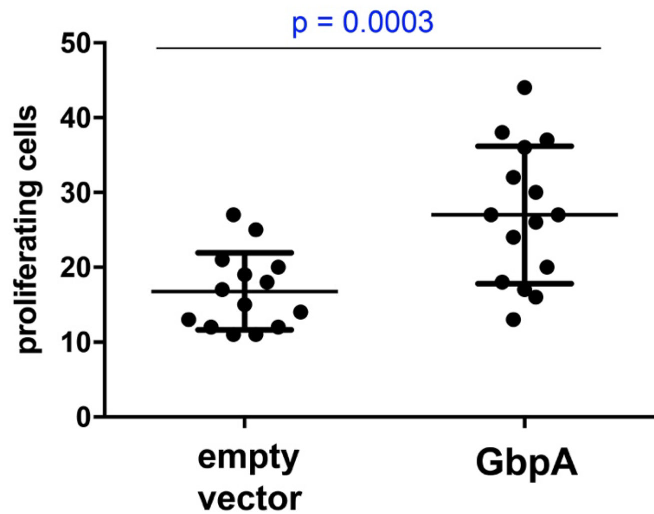


Figure 2: GbpA significantly increases intestinal epithelial cell proliferation in *Drosophila*

Quantification of proliferating cells by pH3+ staining reveals a significant increase in cells undergoing mitosis in adult flies that have been treated with GbpA-enriched CFS in comparison to empty vector CFS ($p=0.0003$, $n=15$ per condition from three independent experiments).

**GbpA $_{D1}$ is both sufficient and necessary for increased cell proliferation**

Next, I investigated the domain-specificity of GbpA. To do this, I used constructs that were previously generated by Allison Banse. This GbpA has four distinct protein domains and we created a construct that only contained domain 1 ($GbpA_{D1}$) to allow us to test sufficiency of this protein domain. To test necessity of $GbpA_{D1}$, we constructed a vector that was only missing domain 1 from the construct. The empty vector served as our negative control and the full vector acted as our positive control, as we had previously tested both vectors.

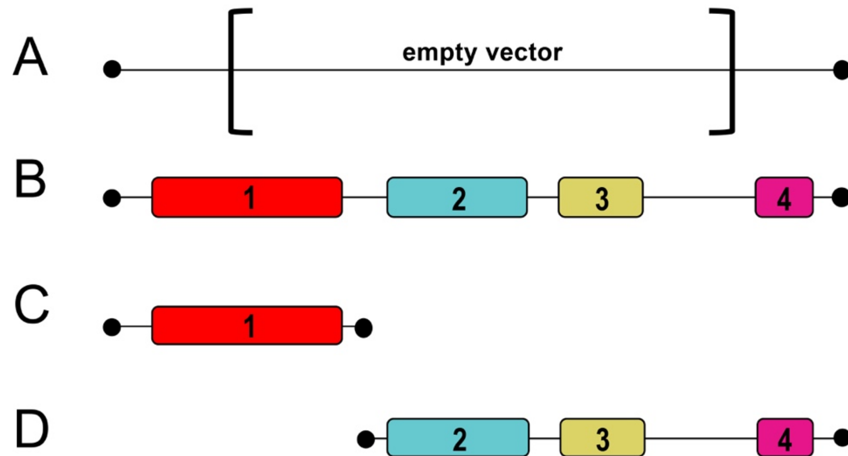


Figure 3: GbpA constructs in *E. coli*

**(A)** The empty vector served as our negative control and internally controlled for other protein species that may have been present in the CFS.

**(B)** The *A. veronii gbpA* gene was introduced into a high copy plasmid in pET-21b *E. coli*. The full GbpA construct was experimentally tested (Figure 2) before serving as a positive control for later experiments (Figure 3).

**(C)** $GbpA_{D1}$ encodes for domain 1 (amino acids 1-193).

**(D)** $GbpA_{D2-4}$ encodes for domains 2-4 (amino acids 199-473).

We compared the effect of treatment with $GbpA_{D1}$ (Figure 3C) to the effect produced by the full construct (Figure 3B) and found that $GbpA_{D1}$ alone is sufficient to increase proliferation in the intestinal epithelium (Figure 4). $GbpA_{D2-4}$ (Figure 3D), on the other hand, produced levels of cell proliferation that were statistically similar to the empty vector construct (Figure 4). This suggests that $GbpA_{D1}$ was necessary for increasing cell proliferation in the midgut.

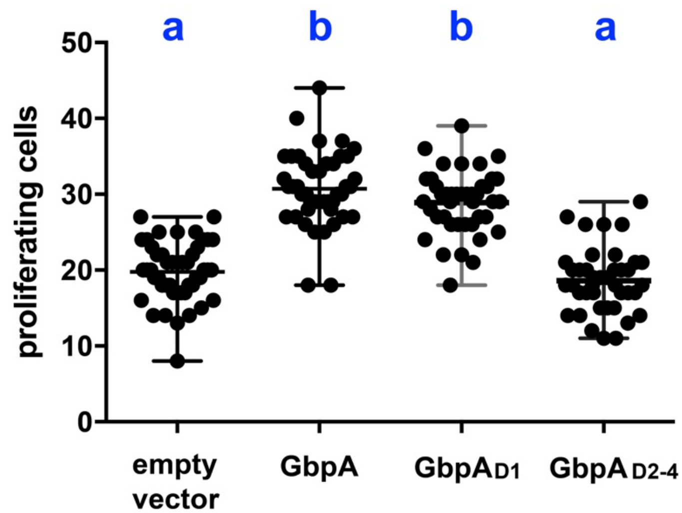


Figure 4: $GbpA_{D1}$ is both necessary and sufficient to increase intestinal epithelial cell proliferation

Quantification of proliferating cells by pH3+ staining revealed a significant increase in cell proliferation in adult flies that have been treated with either the full construct GbpA or $GbpA_{D1}$ in comparison to both empty vector CFS and $GbpA_{D2-4}$. (Conditions that share the same letter are not statistically different from each other, as assessed by ANOVA, $n \geq 40$ per condition from at least four independent experiments).

**The enzymatic activity of GbpA is required for increased cell proliferation**

Converting Histidines in positions 25 and 111 into Alanines allowed for investigation into the necessity of enzymatic activity. These mutations removed the possibility for GbpA to interact with copper in the same position and rendered the protein enzymatically dead. Treatment with $GbpA_{H25A,\ H111A}$ CFS on adult flies did not have an effect and levels of cell proliferation were similar to that of the empty vector control (Figure 5A). A chitin-binding assay was performed (Figure 5B) to test whether or not the $GbpA_{H25A,\ H111A}$ mutant was still capable of binding chitin. The presence of a dark band on the SDS-page around 55 kDa suggests that both GbpA and $GbpA_{H25A,\ H111A}$ are still correctly folded and functional. The absence of a band for $GbpA_{H25A,\ H111A}$

would suggest that the measured level of cell proliferation could be attributed to a misfolded state, rather than to the absence of the two Histidines.

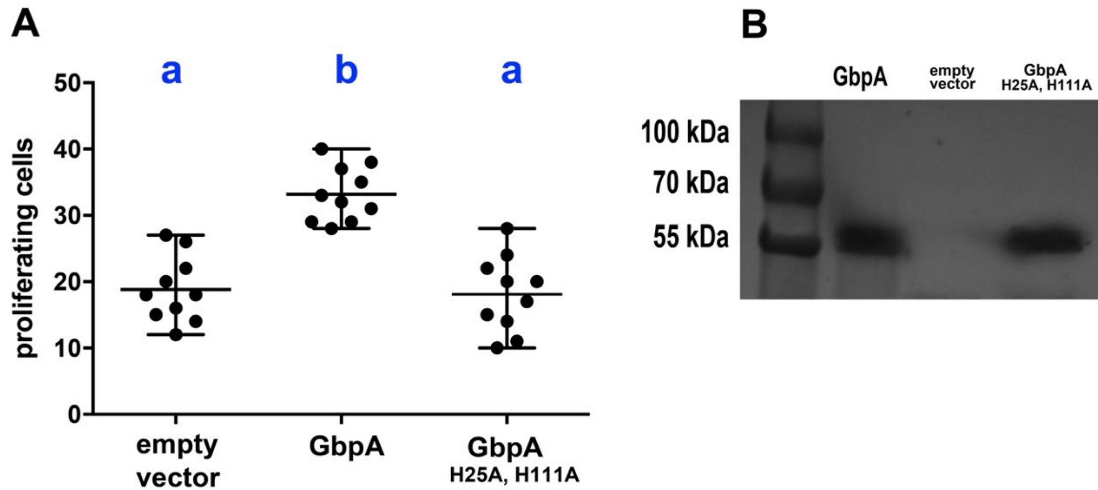


Figure 5: GbpA-mediated cell proliferation requires enzymatic activity

**(A)** Quantification of proliferating cells by pH3+ staining revealed that adult flies that have been treated with $GbpA_{H25A,\ H111A}$ have levels of cell proliferation that mirror the empty vector. (Conditions that share the same letter are not statistically different from each other, as assessed by ANOVA, $n > 10$ per condition from three independent experiments).

**(B)** Chitin binding assay – SDS-page with a pageruler plus pre-stained ladder. Dark bands in the GbpA and $GbpA_{H25A,\ H111A}$ lanes around 55kDa suggest that GbpA protein is still folded and able to bind chitin. The absence of this band for the empty vector suggests the absence of GbpA or the lack of functional GbpA.

# Discussion

## GbpA increases intestinal epithelial cell proliferation in *Drosophila*

Adult flies treated with GbpA-enriched sucrose solution exhibited a significant increase in levels of intestinal epithelial cell proliferation. Flies were cleared of their microbial community prior to dissection and immunostaining, suggesting that the change in proliferation can be attributed to the presence of GbpA. Overnight treatment was sufficient to elicit a response, which positions this assay as an effective and efficient approach to quantifying bacterial-host response.

Though proliferation levels of adult flies treated with GbpA were statistically different from that of the empty vector control group, cell proliferation across treatment groups was lower than expected. Past work in the lab has consistently observed levels of cell proliferation ranging from 60-100 cells in CV-raised flies and around 40-60 cells in GF flies (Figure 1E). In comparison, all flies in this experiment had cell proliferation levels that were substantially lower and no higher than 50 ph3+ cells/midgut.

While this difference can be attributed to changes in fly genotype across experiments, adaptation of the germ-free derivation protocol, and natural variation that occurs over time – it also highlights issues with reproducibility in gnotobiotic studies. Changes in laboratory environments due to building construction, altered air flow, water quality, and more can pose experimental design challenges that go outside of a researcher's control. Reproducibility is gaining more attention in cancer biology and translational research [55, 56], but the existence of these confounding variables

necessitates vigilance when planning the proper positive and negative controls for an experiment.

**GbpA$_{D1}$ is both necessary and sufficient for increased cell proliferation**

Treatment with GbpA $_{D1}$ was both necessary and sufficient to increase intestinal epithelial cell proliferation. This suggests that domain 1 is highly conserved and functionally important. In fact, GbpA $_{D1}$ is structurally homologous to known chitin binding protein CBM33[43] and includes highly conserved residues Y54, E55, E60, H114, D182 and N185 that are important for chitin binding by CBP21[57]. CBP21 is a chitin-binding protein that is expressed in *Serratia marcescens* and has been shown to facilitate the degradation of chitin[58]. The fact that GbpA $_{D1}$ alone is structurally similar to fully functional CBPs suggests that GbpA $_{D1}$ may be functionally important across CBPs. It would be interesting to continue investigation into GbpA $_{D1}$ by tracking its evolution across bacterial species.

**GbpA increases cell proliferation through enzymatic activity in Domain 1**

Treating adult flies with GbpA $_{H25A,\ H111A}$ did not have an effect on cell proliferation in comparison to the empty vector control. Since GbpA $_{H25A,\ H111A}$ is a specific point mutant, it appears that these Histidines are necessary for full function. The Histidines in positions 25 and 111 are both important for making contact with copper and follow-up experiments could confirm this by testing the mutant in a copper-poor and copper-rich background. Future work should also aim to identify the substrate that this catalytic domain is acting on. It's possible that GbpA is involved in

upregulating a host signaling pathway and determining its substrate would be the first step to untangling these bacterial-host interactions.

**LPMO enzyme activity and identification of GbpA's natural substrate**

Lytic polysaccharide monooxygenase (LPMO) activity cleaves β-1,4 glycosidic bonds and was first identified in *Serratia marcescens* (CBP21)[59]. A 'histidine brace' in the active site coordinates oxygen activation by binding to a single copper ion and structural analysis reveals an immunoglobulin-like β-sandwich core with an active site positioned at the center of a flat surface [60, 61]. Proteins with LPMO activity typically play a role in biomass degradation, which made characterization of GbpA as an LPMO surprising[62]. Our findings show that GbpA's LPMO activity is required for an increase in intestinal epithelial cell proliferation (Figure 5A), which could be explained by LPMO-driven cleavage of mucins. Gastrointestinal mucins are glycoproteins that can be found in membrane-bound and secreted forms[63]. The ectopic expression of mucins has been shown to have a pro-inflammatory response through TLR-4 in humans and it's possible that an increase in secreted gastrointestinal mucins could activate Toll-like receptors[64]. The LPMO-driven cleavage of membrane-bound mucins could cause an imbalance of luminal mucins, which could cause Toll-like receptors to initiate an innate immune response.

LPMO's active site and the insoluble nature of polysaccharide substances makes it challenging to understand how substrates bind to LPMO. Recent evidence suggests that LPMOs bind crystalline chitin by engaging the targeted polysaccharide chain and connecting the bulk solvent to the copper site via a tunnel that only allows for the diffusion of small molecules[65]. This model highlights the importance of the constrained

active site and supports our finding that functional GbpA requires H25 and H111 in its active site. Future work should focus on identification of GbpA's natural substrate and should investigate how the substrate subsequently interacts with host processes.

**Conservation of chitin-binding proteins**

The emergence of reliable gnotobiotic models allows for cross-comparison and identification of microbial functions and relationships that are conserved across animals. Identification of the host response to GbpA in zebrafish and *Drosophila* is an example of a microbial function that has been conserved across animals. The response to GbpA appears to be conserved in both zebrafish and flies, suggesting that both animals have evolved in similar manners. This conserved response also suggests that this research could be extended to more complex model organisms.

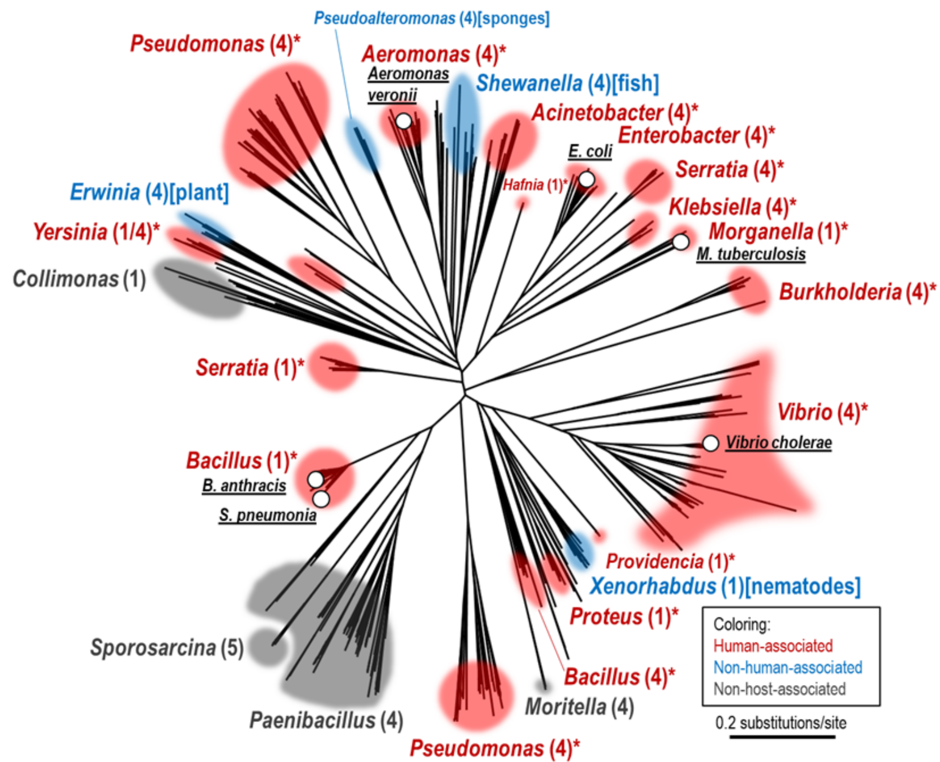


Figure 6: Phylogenetic analysis of sequences related to GbpA

Human-associated microbial species are noted by red coloring and have the potential to modulate immune response, promote persistence, and increase cell proliferation as a chitin-binding protein. (Phylogenetic analysis and image courtesy of Arden Perkins)

Chitin-binding proteins are widely conserved and phylogenetic analysis reveals many CBPs that are expressed by human-associated microbes (Figure 6). These microbes are promising candidates for investigating the impact of bacterial-host interactions on the maintenance of homeostasis. Though humans are incapable of both synthesizing and metabolizing chitin, recent studies have observed the presence of chitinolytic enzymes, chitin-binding proteins, and chitinases in gastric juice and immune cells [66-68]. Additionally, chitinase family proteins can be constitutively expressed in cells that are considered the body's first line of defense including macrophages and epithelial cells of the lung and GI tract [69-70]. Chitinases could also be

therapeutic targets as they have been implicated in allergies and the inflammatory response[71].

**GbpA homologues and impact on human health**

Phylogenetic analysis reveals high conservation of GbpA and GbpA production in many human-associated microbes including *Listeria monocytogenes*, *Bacillus cereus* and *Yersinia pestis* [62] (Figure 6). The strong bias for the representation of GbpA in host-associated species suggests that this protein family may have potent effects on eukaryotic cells that could be exploited for therapeutic modalities. For example, GbpA could be harnessed to increase proliferation in patients with uncontrolled caspase activity, intestinal ischemia, or short bowel syndrome [72, 73]. Conversely an excess of GbpA could be detrimental in the context of excessive epithelial cell proliferation. Metagenomic analyses could inform antibiotic treatments that target GbpA-producing classes of bacteria in patients with a predisposition for gastrointestinal cancers.

Our understanding of bacterial-host interactions can be improved by investigating responses that are conserved across systems. GbpA's conserved ability to increase intestinal epithelial cell proliferation in the *Drosophila* and zebrafish midgut presents a promising mechanism for modulation of intestinal epithelial homeostasis. Maintaining homeostasis relies on a careful balancing act between cell proliferation and cell death. Ultimately, the dance between host and associated microbes must reconcile competing cellular processes in order to achieve this fragile balance.

# Methods

## ***Drosophila*** **cultivation and experimental timeline**

*Drosophila melanogaster* (*Wolbachia*-free) were reared at 25°C in a humidified chamber with a 12h:12h light:dark cycle and fed a standardized cornmeal agar medium (11.6g liter$^{-1}$ agar, 116g liter$^{-1}$ Brewer's yeast, 69g liter$^{-1}$ cornmeal, 116g liter$^{-1}$ dextrose, 0.48% propionic acid, and 0.045% phosphoric acid).

*Drosophila* were derived germ-free by manually transferring into new food bottles every day or after approximately 24 hours for 6 days (Figure 5). Recent studies show that both the establishment and maintenance of the *Drosophila* microbiome require constant access to a microbe-associated food source, which supports the below model as an alternative method[74].

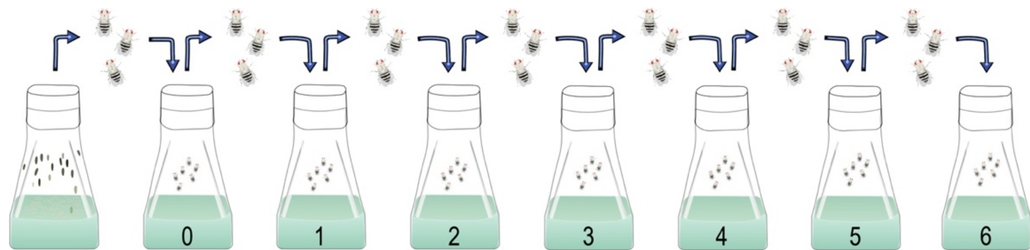


Figure 7: Experimental timeline and protocol for alternative germ-free derivation

Newly eclosed adult *Drosophila* (0 days) were transferred into new food < 24 hours after eclosing, which marked the 0-day time point. *Drosophila* were transferred daily into new food bottles for 6 days until they had undetectable levels of microbes. The numbers on the food bottles represent days since the 0-day time point. Presence of microbes was tested by plating serial dilutions of manually homogenized whole flies on MRS agar plates. Plates were incubated at 30°C for two days and examined after two days and seven days. (Experimental timeline is continued in figure 7).

The above does not illustrate the traditional method of germ-free derivation, which was used in Figure 1 and in past studies. This alternative germ-free derivation method was

chosen as an effective and labor un-intensive way to clear *Drosophila* of their conventional communities and was confirmed to produce germ-free flies before the 7-day time point (Figure 6).

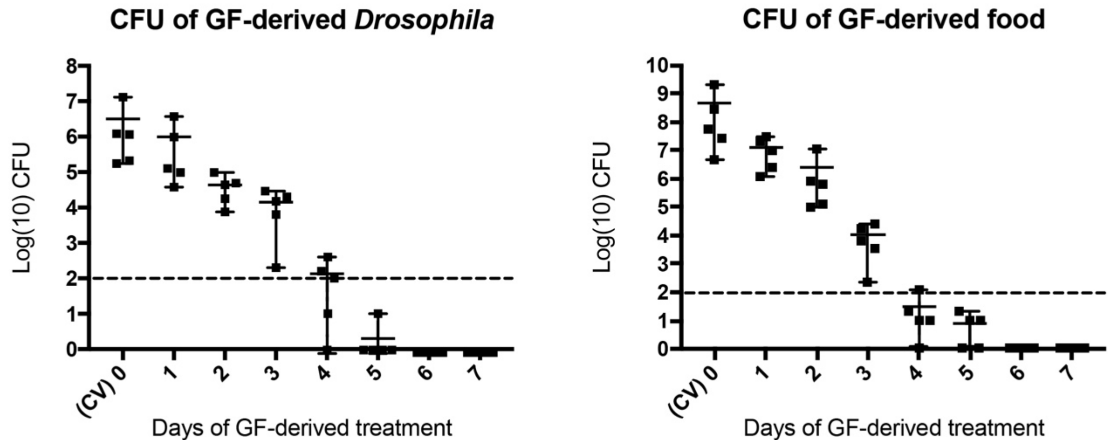


Figure 8: Colony Forming Units (CFUs) of adult *Drosophila* fall below the Limit of Quantification (LOQ) by day 5 of the GF-derived treatment

(Left) Adult flies are 'derived germ-free' after 5 days of being manually transferred to new, sterile food bottles every 24 hours. CFU of whole flies were below the LOQ (as indicated by the dashed line) after day 5 and had 0 detectable CFUs on days 6 and 7.

(Right) CFUs of the food of adult flies were below the LOQ (as indicated by the dashed line) after day 5 and had 0 detectable CFUs on days 6 and 7.

On day 7 of the experimental timeline, CFS was prepared (as described below) and added to filter paper in a 5% sucrose solution to force consumption of the CFS.

### CFS preparation and confirmation of GbpA as major species

Overnight 5mL cultures of *E. coli* BL21 (DE3) were grown shaking at 37°C in LB with 100 µg/mL ampicillin to maintain the pET-21b plasmid (Figure 9A). Cultures were diluted 1:10 into 50 mL fresh LB (100 µg/mL ampicillin) and grown at 37°C until $OD_{600}$ 0.5 (Figure 9B). To induce GbpA expression, IPTG was added at a final

concentration of 1mM and grown at 30°C for 2.5 hours (Figure 9B). Cultures were subjected to centrifugation at 7,000rpm for 10 minutes and the supernatant was filter-sterilized on ice with a 0.22µM filter (Corning). The membranes of filter concentrators (10kDa MWCO, VWR 89131-980) were filled with $ddH_2O$ and hydrated via centrifugation for 10 minutes at 4°C. Filter concentrators were emptied, filled with 20mL of supernatant, and spun at 3,2000rpm for 2.5 hours at 4°C (Figure 9C).

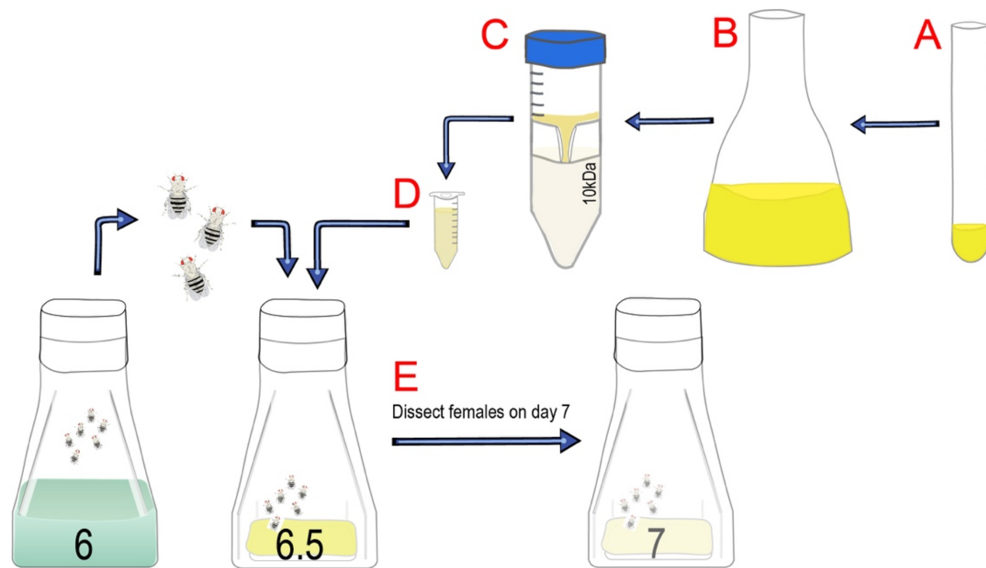


Figure 9: CFS preparation and protein concentration

**(A)** Overnight 5 mL cultures of *E. coli* carrying a plasmid of interest were grown at shaking at 37°C in LB with 100 µg/mL ampicillin. **(B)** Cultures were diluted 1:10 into 50 mL LB with 100 µg/mL ampicillin and later inducted with IPTG. **(C)** Filter-sterilized CFS was transferred to a 10 kDa protein concentrator and spun for 2.5 hours at 3,200 rpm 4°C. **(D)** Concentrated CFS was added 1:1 to a 10% sucrose solution and vortexed. Solution was added to folded whatman filter paper (size 4) in an empty bottle. 6-day adult flies were transferred into this bottle and left overnight. **(E)** 7-day adult female flies were dissected.

CFS was mixed with 10% sucrose at a 1:1 ratio to create a 5% sucrose solution (Figure 9D). Whatman filter paper (size 4) was folded into the bottom of an empty fly bottle and 1000 µL of the 5% sucrose-CFS solution was added to the filter paper. The

filter paper was allowed to dry for 30 minutes before 6 day old germ-free derived flies were transferred into the new experimental bottle (Figure 9E).

Protein expression was initially verified with $OD_{280}$ levels and GbpA expression was later confirmed by SDS-page gel electrophoresis. The presence of a dark band at the expected 55kDa size for the *E.coli* BL21 (DE3) with the pET-21b vector indicated that GbpA was the major species in the concentrated CFS. The absence of a 55kDa band from the *E.coli* BL21 empty pET-21b vector species confirmed the lack of GbpA.

**Midgut dissections**

All assays were performed on mated, 7 day old adult females (esg-Gal4 UAS-GFP;Dr/TM3, Sb). Females were anaesthetized with $CO_2$ before removal of the head, wings, and legs. Guts were dissected in Grace's unsupplemented insect medium (ThermoFisher) with a compound microscope and microdissection forceps and scissors. The crop and Malpighian tubules were removed from midgut samples before staining.

**Antibody staining and imaging**

Dissected guts were fixed in 30 minutes in fresh 4% Paraformaldehyde/1X Grace's. Guts were washed 3 times for 15 minutes in 1X PBS + 0.1% Triton X-100 (PBST) before being blocked with PBST + 0.02% BSA (PBST-B) for 30 minutes at room temperature. Primary antibodies were applied for 2 hours, rocking at room temperature (Rabbit anti-phospho histone H3 1:1000 in PBST-B; Millipore and Chicken anti-GFP 1:1000 in PBST-B; AVES labs). Guts were washed 3 times for 15 minutes with PBST-B before secondary antibodies were applied (AlexaFluor 594 Goat anti-Rabbit 1:1000 in PBST-B and AlexaFluor 488 Goat anti-Chicken 1:1000 in PBST-B).

Guts were covered in foil and rocked overnight at 4°C. Next, guts were washed 3 times for 15 minutes with PBST-B and mounted on glass microscope slides with ProLong Diamond with DAPI anti-fade mountant (Life Technologies). Guts were arranged parallel to each other and secured with a coverslip. Proliferating cells, as marked by the presence of pH3+ stain, were manually counted on a Nikon compound microscope and confocal microscope. Total proliferating cell count included pH3+ cells from the anterior base of the proventriculus to the posterior junction of the midgut and hindgut.

**Generation of GbpA Constructs**

Allison Banse generated all of the GbpA expression constructs for this study. Primers CBPf (gcatcatatggcagcaaaaatccatc) and CBPr1 (gcatctcgagtcacttcagctcaatccaggctt) were used to generate PCR product of *cbp* ORF (inclusive of the stop codon), which was cloned into NdeI and XhoI sites of pET21b (Novagen). Primers GSTCBPf (gcatgaattccacggctacatcagccagccc) and GSTCBPr (gcatctcgagtcatcacttcagctcaatccagg) were used to generate PCR product of the *cbp* ORF (inclusive of the stop codon and exclusive of the secretion signal). This PCR product was cloned into EcoR1 and Xho1 sites of pGEX6p1 and both plasmids were transformed into *E. coli* BL21(DE3) RIL-CodonPlus cells (Stratagene).

**Chitin-binding assay**

Chitin resin beads (NE BioLabs #S6651S) were suspended in 500µL of CBD Column Binding buffer (NaCl 500 mM, Tris-HCI 20 mM, EDTA 1 mM, Tween-20 0.1%, pH 8 at 25°C). 500µL of concentrated CFS was added to beads before vortexing and incubating at 4°C on a rocker for 1 hour. Supernatant was collected after

centrifugation at 5000rpm for 2 minutes. Beads were washed three times with 500µL of CBD Column Binding buffer and proteins were disassociated from chitin beads by boiling for 2 minutes in SDS-page buffer. Samples were run on SDS-page and Coomassie protein-stain and methanol/acetic acid de-stain allowed for band visualization.

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