INVESTIGATING PATHOGENIC MECHANISMS OF THE *HELICOBACTER PYLORI* VIRULENCE FACTOR CAGA USING TRANSGENIC EXPRESSION IN *DROSOPHILA MELANOGASTER*

by

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DISSERTATION ABSTRACT

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Upon colonization of the human stomach, *Helicobacter pylori* establishes intimate interactions with the gastric epithelium, resulting in pathogenic host responses that can lead to gastric cancer. An important component of this interaction is translocation of the CagA effector protein into host cells, where it manipulates several conserved signaling pathways. Experiments in tissue culture cells have shown that CagA activates the phosphatase SHP-2, a component of receptor tyrosine kinase (RTK) pathways whose overactivation is associated with cancer formation. CagA has been proposed to function as a prokaryotic mimic of the eukaryotic Gab adaptor protein, which normally activates SHP-2. We developed a transgenic *Drosophila melanogaster* model to investigate whether CagA can function in a Gab-dependent process: specification of photoreceptor cells in the eye. We demonstrate that CagA expression is sufficient to rescue photoreceptor development in the absence of the Gab homologue through a mechanism that requires *Drosophila* SHP-2, demonstrating that CagA functions as a Gab protein *in vivo* and providing insight into CagA's oncogenic potential.

In addition to its function in RTK signaling, we explore CagA's interactions with other host cell signaling pathways using the transgenic *Drosophila* model. We show that

expressing CagA in the simple model epithelium created during wing development triggers apoptosis through activation of JNK signaling. We demonstrate that loss of several upstream JNK pathway components, including neoplastic tumor suppressors and the homolog of tumor necrosis factor, enhances CagA-induced cell death. Using a *Drosophila* model of metastasis we show that CagA enhances growth and invasion of tumors generated by expression of oncogenic Ras through JNK activation, implicating this pathway as an important driver of human gastric cancer progression.

Finally, we use our transgenic *Drosophila* model to examine a role for CagA in disrupting the gastrointestinal ecosystem. We show that expressing CagA in adult intestinal stem cells is sufficient to significantly enhance epithelial proliferation, increase the production of antimicrobial peptides, and alter the intestinal bacterial community.

This dissertation includes both previously published and unpublished co-authored material.

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I dedicate this dissertation to my mother, whose epic battle with and ultimate triumph over cancer helped me find the strength and determination to achieve this goal.

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CHAPTER I

INTRODUCTION

Modeling *Helicobacter pylori* pathogenesis

The initial identification of *H. pylori* as the only known bacterial carcinogen [1] generated heightened interest in determining the mechanisms through which this pathogen induces gastric cancer formation. Epidemiological information obtained from human patients revealed that infection with strains of *H. pylori* containing a genetic element known as the *cag* pathogenicity island (*cag* PAI) is strongly correlated with more severe clinical outcomes. This region of the *H. pylori* genome was found to encode a type IV secretion system used to inject the CagA effector protein directly into host cells [2]. The effects of this bacterial virulence factor have been difficult to assess in genetically tractable animal models of *H. pylori* infection, due to the observed loss of the *cag* PAI in strains adapted for effective colonization of mice [3].

The challenges of developing animal models to study pathogenic mechanisms of *H. pylori* mediated by CagA have resulted in a strong reliance on various tissue culture models of infection. This system has been very effective in identifying host proteins with which CagA can interact, but less useful for examining the downstream effects of these interactions. This is due in part to the observation that CagA causes varying phenotypes in different tissue culture models including apoptosis, cell elongation, and loss of cell polarity [4,5,6]. Additionally, this system is unable to recapitulate the complex cellular environment in which CagA interacts with host cells during *H. pylori* infection of the human stomach. The inherent limitations of current models have prompted investigation

into alternate methods to determine the function of CagA during *H. pylori* infection of host epithelial cells. Chapter II will discuss previously published work co-authored by Crystal Botham, Karen Guillemin and myself in which we develop a novel transgenic *Drosophila melanogaster* model to study CagA pathogenesis.

Oncogenic mechanisms of the CagA effector protein

Examining both the structure and function of CagA revealed several important characteristics which make this protein an effective virulence factor. CagA shares no sequence similarity to any known protein, and contains only a few recognizable domains which are important for its cellular functions. These include the EPIYA motifs that are phosphorylated by host Src family kinases, and the multimerization sequences which allow CagA to dimerize and interact with specific host cell proteins [7]. The crystal structure of CagA revealed a largely unstructured configuration [8], providing a potential explanation for its versatility in binding to a multitude of proteins within the host cell. Several of CagA's interaction partners have generated special interest due to the established roles of these host cell proteins in tumorigenesis.

The most extensively studied interaction between a host cell protein and CagA is activation of SHP-2, which has been classified as an oncoprotein because gain-of-function mutations have been identified in a number of human diseases [7]. Another protein with proposed importance in CagA-dependent tumorignesis is the polarity determinant Par1, thought to be responsible for CagA's ability to disrupt cell polarity in tissue culture monolayers [9]. However, an inability to observe the effects of these interactions in the epithelial environment where CagA normally resides has prohibited more direct testing of which interactions are responsible for the increased virulence

conferred by CagA. Chapter III will discuss unpublished work co-authored by Karen Guillemin and myself in which we use the transgenic *Drosophila* model to explore effects of CagA expression on epithelial tissue, and uncover novel genetic interactions between CagA and host cell signaling pathways important for pathogenesis.

Helicobacter pylori in the gastrointestinal ecosystem

An important component of the *H.* pylori infection process involves activation of innate immune signaling to induce an inflammatory response characterized by production of cytokines, and previous work has established a role for CagA in certain aspects of this process [10]. Despite established correlations between the context in which inflammation is induced in the stomach and the clinical outcomes of *H. pylori* infection [11], potential effects of the inflammatory state on the gastric environment have not been explored. One known effect of increased intestinal inflammation is disruption of the microbiota, which has been associated with several diseases [12].

The human stomach was initially thought to be uninhabitable by bacteria, but is now known to harbor a relatively complex microbial composition [13,14]. Recent work in the field of *H. pylori* biology has focused on studying this bacterium not only as a pathogen, but also as an important component of the gastric microbiota. Initial studies have shown that the bacterial community associated with *H. pylori* positive individuals differs significantly from the microbiota isolated from individuals not colonized with the gastric pathogen [15]. Despite known functions for CagA in manipulating host cell signaling pathways to promote inflammation, the potential contributions of CagA to observed disruption of the gastric microbiota have not been addressed. Chapter IV will discuss unpublished work co-authored by Allison Banse, David Bauer, Adam Burns,

Karen Guillemin and myself in which we use our transgenic *Drosophila* model to examine the effects of CagA expression on intestinal homeostasis, innate immune signaling and the microbiota.

CHAPTER II

A TRANSGENIC *DROSOPHILA* MODEL DEMONSTRATES THAT THE *HELICOBACTER PYLORI* CAGA PROTEIN FUNCTIONS AS A EUKARYOTIC GAB ADAPTOR

The work described in this chapter was previously published in the May 2008 issue of *PLoS Pathogens*. It was co-authored by Crystal Botham, Karen Guillemin and myself. Crystal Botham and Karen Guillemin conceived and designed the experiments. Crystal Botham and I performed the experiments. Crystal Botham, Karen Guillemin and I analyzed the data. Crystal Botham and Karen Guillemin wrote the paper.

INTRODUCTION

The human pathogen, *Helicobacter pylori*, infects the stomachs of at least half the world's population and chronic infection is associated with the development of diseases such as gastritis, peptic ulcers and gastric cancer [1]. A major virulence determinant of *H. pylori* is the cytotoxin associated gene A (CagA) which is translocated into host cells via a type four secretion system (reviewed in [2]). Inside host cells, CagA is phosphorylated by Src family kinases on tyrosines contained in repeated five-amino acid motifs (EPIYA) in CagA's carboxyl terminus. Phosphorylated CagA disrupts receptor tyrosine kinase (RTK) signaling pathways by directly activating Src homology 2 (SH2) domain containing tyrosine phosphatase (SHP-2) (reviewed in [3]). Normally SHP-2 is activated by the scaffolding adaptor Grb2-associated binder (Gab) proteins, thereby amplifying RTK signaling pathways to control cell growth, differentiation and survival (reviewed in

[4]). The Gab proteins occupy a pivotal position in RTK signaling pathways by interacting directly with RTKs such as the c-Met receptor of the Hepatocyte growth factor/Scatter factor (HGF/SF) as well as downstream cytoplasmic proteins including SHP-2, v-crk sarcoma virus CT10 oncogene homolog (avian)-like (Crk(L)), and Growth factor receptor-bound protein 2 (Grb2) (reviewed in [5,6,7]). Although CagA shares no sequence similarity with Gab proteins, CagA has been shown to activate SHP-2 in tissue culture cells, resulting in cell elongation [8,9]. Similarly, in tissue culture cells CagA has been found to associate with c-Met, Crk(L) and Grb2 [10,11,12]. Based on these interactions, CagA has been hypothesized to mimic Gab proteins and to function as an oncogene by over-activating RTK signaling [13]. The significance of CagA's interactions with RTK signaling pathway proteins, however, has only been explored in tissue culture cells.

We have developed transgenic *Drosophila* with inducible CagA expression as a model to understand CagA's mechanisms of action in complex epithelial tissues. In order to test the hypothesis that CagA can function as a Gab substitute, we investigated CagA activity in a well-characterized Gab-dependent process, the specification of photoreceptors in the *Drosophila* eye [14,15,16]. The *Drosophila* compound eye, whose crystalline array of facets or ommatidia are exquisitely sensitive to perturbations in cell specification, has been used as a powerful system for the discovery and genetic analysis of RTK signaling components [17,18]. *Drosophila* RTK signaling proteins are highly conserved with their mammalian orthologues and oncogenic mutations in these proteins, such as those that constitutively activate RTK receptors or their downstream effectors, function similarly in both *Drosophila* and mammalian cells [19]. The *Drosophila* model

also offers elegant tools for genetic manipulations including the UAS/GAL4 system [20] for expression of transgenes in a tissue specific manner, the FLP/FRT system for the generation of somatic mutant clones [21], and null mutations in most RTK signaling pathway members, which allow us to probe the *in vivo* requirements for CagA's activation of RTK signaling pathways. Finally, *Drosophila* are amenable to forward genetic approaches that will facilitate the discovery of host factors required for CagA function in eukaryotic cells [22].

RTK signaling is required for multiple steps of *Drosophila* photoreceptor development. The *Drosophila* epidermal growth factor receptor (EGFR) is necessary for cell proliferation in the early eye imaginal disc, cell survival in the differentiating region of the disc behind the morphogenetic furrow, and recruitment of all photoreceptors except R8 [23]. A second RTK, Sevenless (SEV) is required exclusively for the R7 photoreceptor to adopt the appropriate fate, as opposed to becoming a nonneuronal cone cell [24] (reviewed in [25]). The *Drosophila* Gab adaptor, Daughter of Sevenless (DOS) is required for full signaling through both the EGFR and SEV pathways [16]. Clones of eye imaginal cells lacking DOS activity fail to proliferate and produce few photoreceptors, similar to clones lacking EGFR [16,26,27]. The EGFR pathway is required additionally for multiple aspects of *Drosophila* development [28].

Here we show that CagA can substitute for the *Drosophila* Gab adaptor, DOS, and rescue phenotypes associated with loss of dos, including larval lethality and photoreceptor differentiation. We further demonstrate that CagA functions through the *Drosophila* SHP-2 homologue, Corkscrew (CSW) similar to Gab. Our work demonstrates the power

of using a genetically tractable system like *Drosophila* to dissect the mechanism of action of a prokaryotic protein that modulates a conserved eukaryotic signaling pathway.

RESULTS

CagA is phosphorylated, cortically-localized in *Drosophila* cells and disrupts eye development

To determine if the *Drosophila* system would be useful for dissecting the molecular mechanism of CagA-induced activation of RTK signaling, we examined whether CagA exhibited similar properties when expressed in *Drosophila* tissue to those previously observed in mammalian tissue culture cells. We used P-element mediated transgenesis to generate *Drosophila* with a transgene encoding an N-terminal hemagglutinin (HA) tagged CagA under control of the yeast GAL4 upstream activating sequence (*UAS-CagA*). Additionally, we generated transgenic flies with a mutated version of CagA lacking the EPIYA tyrosine phosphorylation motifs *(UAS-CagA*^{EPISA}). These transgenic flies were crossed to flies that expressed the GAL4 transcription factor under tissue-specific or inducible promoters to express CagA in specific cells and at specific times during development. In the experiments described here, the *GMR-GAL4* line was used to express CagA in all cells of the developing imaginal eye disc after the morphogenetic furrow.

Western analysis of anti-HA affinity purified proteins from heads of adult *UAS-CagA/GMR-GAL4* flies showed that CagA was expressed (α-HA) and phosphorylated (α-P-Tyr, Figure 1A; all figures are provided in Appendix A). Similar to CagA's distribution in tissue culture cells [8,29], we showed in the *Drosophila* eye disc CagA

was localized predominantly to the cell cortex (Figure 1C). Examination of the cellular morphology of the pupal retina revealed that CagA expression caused disorganization of the epithelium. The wild type retinal epithelium is organized into regular cell clusters, each containing a single R7 and R8 photoreceptor (Figure 1D). In retina expressing CagA, the normal cell shapes and neighbor relationships were perturbed (Figure 1E), similar to CagA-dependent epithelial disorganization observed in mammalian tissue culture monolayers [29,30]. When we examined the eyes of adult flies expressing a single copy of CagA with *GMR-GAL4*, we observed a perturbation of the normal crystalline array of the ommatidia (compare wild type, Figure 1F, with CagA expression, Figure 1G). Expression of two copies of the *UAS-CagA* transgene dramatically enhanced the eye phenotype, indicating that the developmental pathways disrupted were sensitive to the amount of CagA expressed (Figure 1H). Expressing one copy of the CagA mutant lacking the tyrosine phosphorylation sites ($CagA^{EPISA}$) did not perturb the crystalline array of the adult eye to the extent caused by wild type CagA (Figure 1I) even though the $CagA^{EPISA}$ protein was expressed at similar levels as CagA (Figure 1B). Dose dependent perturbations of *Drosophila* eye patterning, as observed with CagA expression, have been used as the basis for genetic screens for modifiers of the rough eye phenotype to elucidate several signaling pathways, including RTK pathways. [17,31]

CagA can substitute for the *Drosophila* Gab

To test the hypothesis that CagA functions as a prokaryotic mimic of eukaryotic Gab proteins, we asked whether CagA expression could rescue phenotypes caused by the loss of the *Drosophila* Gab, DOS. DOS functions downstream of multiple RTKs during development, and homozygous *dos* loss-of-function mutants rarely develop into pupae

and never survive to adulthood [16]. Rescue of *dos* mutants' lethality has been used as an *in vivo* assay to determine the function of specific domains of DOS [26]. We therefore determined the percentage of *dos* homozygous mutants that survived to the pupal stage of development with or without CagA expressed ubiquitously with temporal precision using the heat shock inducible *Hsp-GAL4*. The frequency of *dos* homozygous mutants was scored as a percentage of expected pupae that should develop if the *dos* mutants showed no lethality defect. As expected, a low percentage (33%) of homozygous *dos* mutant pupae expressing only *Hsp-GAL4* were observed (Figure 2A). When CagA was expressed, we observed a significant increase to 89% of the pupae developing that lacked *dos* (Figure 2A). These results indicate that CagA can substitute for essential functions of DOS during *Drosophila* development.

To specifically test whether CagA could substitute for Gab in photoreceptor development, we generated mitotic *dos*/*dos* clones within the eye using the FLP/FRT recombinase system [27,32]. In these experiments the *dos* mutation was recombined onto a chromosome arm containing a centromere proximal FRT recombination site and maintained in trans to a chromosome containing the same FRT site as well as a *GFP* transgene. By expressing FLP recombinase in the developing eye we induced mitotic recombination between FRT sites, which generated clones of homozygous cells (+/+ and *dos*/*dos*) in an otherwise heterozygous background (*dos*/+). The *dos*/*dos* mutant cells were distinguished by their lack of GFP, and the photoreceptors were visualized by staining for the photoreceptor-specific protein ELAV. As previously reported [16,26] the *dos*/*dos* clones rarely contained photoreceptors and were composed of very few cells (Figure 2B–E), due to the dual requirements for EGFR signaling in cell survival and

photoreceptor specification [23]. As expected, expression of DOS with *GMR-GAL4* in *dos*/*dos* cells resulted in much larger clones with increased numbers of photoreceptors (Figure 2B, F–H). Expression of CagA in *dos*/*dos* cells was able to rescue clone size and photoreceptor development similarly to expression of DOS with the same driver (Figure 2B, I–K). Two independent *dos* mutants gave similar results (Figure 2 and data not shown). These data demonstrate that CagA can substitute for DOS during the development of photoreceptors.

CagA's specification of photoreceptors requires SHP-2/CSW

We predicted that if CagA functions similarly to Gab, then CagA would require the downstream signaling molecule SHP-2/CSW to promote photoreceptor development. As a downstream component of RTK pathways, CSW is required for photoreceptor development [17]. In contrast to wild type larval eye discs, in which thousands of photoreceptors are specified (Figure 3A), in larval eye discs of *csw* null mutants only a few photoreceptors develop along the morphogenetic furrow (Figure 3B, E) as described previously [33]. The residual photoreceptors in the *csw* eye discs were mostly R8 cells (data not shown), the only photoreceptor class that does not require RTK signaling for its specification [23]. A significant increase in photoreceptor number could be achieved in the *csw* mutant eye discs by expression of *UAS-CSW* with *GMR-GAL4* (Figure 3C) or *Hsp-GAL4* (data not shown). However, expression of CagA from multiple different transgenic lines using either *GMR-GAL4* or *Hsp-GAL4* failed to increase the number of photoreceptors in two different *csw* null mutants (Figure 3D, E, data not shown). These results argue that CagA, like DOS, requires SHP-2/CSW to promote photoreceptor development.

DISCUSSION

We used a transgenic *Drosophila* system to test the hypothesis that *H. pylori*'s virulence factor CagA can substitute for the Gab adaptor in RTK signaling pathways. This system is ideal for these studies because RTK signaling pathway components can be genetically manipulated, resulting in interpretable phenotypic consequences for tissue development. First, we have demonstrated that CagA in *Drosophila* tissue is phosphorylated, that it associates with the cell cortex, and that its expression causes epithelial disorganization as in mammalian tissue culture cells. Second, we have provided genetic evidence that CagA can substitute for Gab by demonstrating that CagA expression restores larval viability and photoreceptor development in mutants lacking the *Drosophila* Gab, DOS. Our inability to rescue *dos* mutants to adulthood with CagA expression may be due to differences in RTK activation or to non-overlapping functions of Gab and CagA. Indeed too much CagA expression (using an *actin-GAL4* driver) is lethal to flies (unpublished results), which is not the case for ubiquitous expression of DOS [26]. Third, our genetic epistasis analysis with mutants lacking *csw* has shown that CagA functions through the *Drosophila* SHP-2 homologue, similar to results from tissue culture experiments [8,9].

RTK signaling is essential for several fundamental biological processes and erroneous signaling can promote tumor formation [19]. Gain-of-function mutations of SHP-2 have been established as oncogenic in numerous leukemia types as well as other diseases like Noonan's Syndrome [4,34,35]. Over-expression of the Gab scaffolding adaptor proteins is associated with the development of several types of cancers, including breast cancer [6,7] and gastric cancer [36]. The specific cancers that develop as a result of

these mutations reflect tissue sensitivities to increased Gab and SHP-2. In the case of *H. pylori* infection, CagA provides a tissue specific activation of RTK signaling that can precipitate events leading to gastric carcinogenesis [37], as suggested by a recent report of CagA-expressing transgenic mice [38].

Our approach of examining the cellular effects of CagA expression in *Drosophila* tissue takes advantage of the fact that bacterial proteins frequently target essential, highly conserved cell-signaling pathways. *Drosophila* has been employed traditionally as a model organism for dissecting signaling pathways in development, but in recent years it has also proven useful in understanding host-pathogen interactions (reviewed in [39,40]), and in one instance has been used as a heterologous system for expression of the bacterial toxins, anthrax lethal and edema factors [41]. Here we have exploited *Drosophila* eye development to demonstrate CagA's capacity to function as a RTK adaptor. Future studies using this transgenic *Drosophila* model will allow us to better understand the cellular and tissue-wide consequences of CagA's disruption of eukaryotic signaling pathways and to identify candidate host factors through which CagA functions.

MATERIALS AND METHODS

Construction of *UAS-CagA* and *UAS-CagA*^{EPISA}

CagA cDNA was amplified from genomic DNA from *H. pylori* G27. The CagA^{EPISA} (lacking EPIYA tyrosine phosphorylation motifs) cDNA was amplified from a plasmid provided by Manuel Amieva (originally from Markus Stein [42]). CagA^{EPISA} lacks the tyrosines in the four 5-amino acid motifs, EPIYA, which are phosphorylated by host kinases (point mutations at nucleotide 2684 [A→C] and 2740 [A→C] and a deletion

at nucleotide 2878 to 3082). CagA and CagA^{EPISA} were cloned into a modified pUAST vector with an N-terminal hemagglutinin (HA) tag (provided by Chris Q. Doe). Transgenic lines were generated by injecting Qiagen-purified plasmid DNA into *y,w*^{1118} embryos. Several independent transformant lines were established for each construct.

Drosophila strains

Genetic null alleles of *csw* (*csw*^{C114} and *csw*$^{13-87}$) and *dos* (*dos*$^{1.46}$ and *dos*$^{2.46}$) were obtained from Michael Simon. The *UAS-DOS* strain was from Thomas Raabe and the *UAS-CSW* strain (*UAS-flgcsw[WTCIM]*) from Lizabeth Perkins. *UAS-CagA* and *UAS-CagA*^{EPISA} (lacking EPIYA tyrosine phosphorylation motifs) transgenes were expressed in the eye using *P{w[+mC] =GAL4-ninaE.GMR}12* (*GMR-GAL4*, Bloomington Stock Center (BSC) # 1104). *P{GAL4-Hsp70.PB}2* (*Hsp-GAL4*, BSC # 2077) was used for heat-shock inducible expression of transgenes.

Scanning electron microscopy

Fly heads were fixed overnight at 4°C in 2% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90% 95%, three times in absolute ethanol) at room temperature for 10 minutes in each solution. Samples were critically point dried, sputter coated with gold and viewed using a JEOL 6400 SEM.

Larval eye discs

Eye imaginal discs were dissected from third instar wandering larvae, fixed for 30 minutes (4% formaldehyde, 0.1 M PIPES (pH 6.9), 0.3% Triton X-100, 2 mM EGTA, 1 mM $MgSO_4$). Discs were washed (0.3% Triton X-100 in phosphate buffered saline, PBS)

and blocked for one hour (1% BSA, 0.3% Triton X-100 in PBS). Primary antibodies included rat anti-ELAV 1:10 (05HB 7E8A10, from Chris Q. Doe), rat anti-HA 1:100 (Roche) and chicken anti-GFP 1:2,000 (Chemicon). Secondary antibodies included anti-rat conjugated Rhodamine Red 1:200 (Jackson ImmunoResearch), anti-rat conjugated AlexaFluor 488 1:200 (Molecular Probes), anti-mouse conjugated Cy3 1:200 (Jackson ImmunoResearch) and anti-chicken conjugated Cy2 1:100 (Jackson ImmunoResearch). Phalloidin conjugated to Tetramethyl Rhodamine Iso-Thiocyanate (TRITC, Sigma Aldrich, 1:500) was used to stain F-actin. Imaginal discs were visualized using a Nikon TE2000 U with C1 Digital Eclipse confocal microscope.

Pupal retinas

Wandering third instar larvae were placed at 25°C and approximately 50 hours later the pupal retinas were dissected (50% pupal stage). Retinas were dissected in PBS, fixed for 20 minutes (4% paraformaldehyde in PBS) and washed three times in PBT (0.5% Triton X-100 in PBS). Retinas were blocked at least 15 minutes in 10% normal goat serum in PBT. Antibodies were diluted in the blocking solution. Primary antibodies included mouse MAb 24B10 which stains all photoreceptors and their axons [43] (Developmental Studies Hybridoma Bank, 1:200), rabbit anti-SAL, which stains R7 and R8 nuclei (also called SPALT, provide by Reinhard Schuh [44], 1:100), guinea pig anti-SENSELESS, which stains R8 nuclei (proved by Hugo Bellen [45], 1:1000). Secondary antibodies from Molecular Probes included AlexaFluor 555 conjugated anti-mouse, AlexaFluor 488 conjugated anti-rabbit and AlexaFluor 633 conjugated anti-guinea pig, which were all used at 1:250. Pupal retinas were visualized using a Leica TCS SP5 confocal microscope.

Western analysis

Fly heads were collected by flash freezing adult flies in liquid nitrogen, shaking the flies in a conical tube, and then separating the heads from the bodies using a mesh sieve. Heads (~1.5 mL) were homogenized in ice cold lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% Triton X-100 and Complete protease inhibitors [Roche]) and then centrifuged at 16,000 G for 5 minutes. Supernatant from the lysate solution (1.5 mL) was added to 50 μL anti-HA Affinity Matrix (Roche) which was incubated overnight at 4°C with gentle agitation. The anti-HA affinity matrix was washed 4 times with ice-cold lysis buffer. CagA was eluted from the matrix by boiling in 100 μL sample loading buffer and separated using manufactures protocols for 7% NuPAGE® Novex Tris-Acetate gels, transferred to polyvinylidene difluoride membranes, blocked overnight at 4°C (200 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20 and 3% BSA (Fisher)), probed using appropriate antibodies and detected using enhanced chemiluminescene (ECL plus, Amersham Biosciences). Mouse anti-HA was used at 1:1,000 (Babco). Mouse anti-phospho tyrosine was used at 1:2,000 (Cell Signal Technologies). Horseradish peroxidase-conjugated sheep anti-mouse (Amersham Biosciences) was used at 1:5,000.

***dos* assays**

Hsp70-GAL4 balanced over *CyO*, *P{Ubi-GFP}* with $dos^{2.42}$ over *TM3*, *P{Act-GFP}*, *Ser* were crossed to $dos^{1.46}$/*TM3*, *P{Act-GFP}*, *Ser* (negative control) or *UAS-CagA*; *dos1.46*/*TM3*, *P{Act-GFP}*, *Ser*. Progeny were raised at 30°C and pupae were examined for GFP florescence using a Stemi SV 11 Apo Zeiss microscope. The number of non-GFP expressing progeny was scored as a percentage of the total number of pupae

that developed per bottle and averaged across bottles of the same genotype. At least 12 bottles were scored per cross with between 150–450 pupae examined per bottle.

The FLP/FRT recombinase system was used to induce somatic clones in the eye [27]. Males *y w,ey-FLP 3.5/Y*; *GMR-GAL4*; *FRT2*, $dos^{1.46}$/*CyO-TM6B* were crossed to *P{ey-FLP.N}6*, *ry506* (BSC #5577); *P{Ubi-GFP.nls}3L1 P{Ubi-GFP.nls}3L2 P{FRT(whs)}2A* (BSC #5825) (negative control) or *UAS-DOS*; *P{Ubi-GFP.nls}3L1 P{Ubi-GFP.nls}3L2 P{FRT(whs)}2A* (positive control). Male *GMR-GAL4*, *UAS-CagA*; *FRT2*, $dos^{1.46}$/*CyO-TM6B* were crossed to *P{ey-FLP.N}6*, *ry506*; *P{Ubi-GFP.nls}3L1 P{Ubi-GFP.nls}3L2 P{FRT (whs)}2A*. Imaginal eye discs were stained with anti-ELAV and anti-GFP antibodies.

***csw* assay**

Two genetic null alleles of *csw* were used to examine if CagA could rescue loss of *csw*. The csw^{C114} or csw^{13-87} alleles were balanced over *FM7*, *P{Act-GFP}* with *GMR-GAL4* balanced over *CyO*, *P{Ubi-GFP}* on the second chromosome. These females were then crossed to males y^1w^{1118}, *P{Ubi-GFP.nls}X1 P{FRT(whs)}9-2* (BSC # 5832)/*Y* (negative control), y^1w^{1118}, *P{Ubi-GFP.nls}X1 P{FRT(whs)}9-2/Y*; *UAS-CSW* (positive control) or y^1w^{1118}, *P{Ubi-GFP.nls}X1 P{FRT(whs)}*9-2/Y; *UAS-CagA*. Eye imaginal discs were dissected from male larvae.

BRIDGE TO CHAPTER III

Chapter II discussed the development of a novel transgenic *Drosophila* model of CagA pathogenesis and described several experiments which established that expressing this bacterial effector in *Drosophila* cells produced a functional protein with

characteristics similar to those seen during CagA expression in tissue culture cells. We showed that CagA can substitute for the *Drosophila* Gab adaptor during specification of photoreceptor cells, demonstrating the ability of this bacterial protein to function as a host Gab protein *in vivo*. Chapter III will discuss our use of the transgenic *Drosophila* model to explore effects of CagA expression on epithelial tissue, and uncover novel genetic interactions between CagA and host cell signaling pathways important for pathogenesis.

CHAPTER III

TRANSGENIC EXPRESSION OF THE *HELICOBACTER PYLORI* VIRULENCE FACTOR CAGA PROMOTES APOPTOSIS OR TUMORIGENESIS THROUGH JNK ACTIVATION IN *DROSOPHILA*

The work described this chapter was co-authored by Karen Guillemin and myself. Karen Guillemin and I conceived and designed the experiments. I performed the experiments. Karen Guillemin and I analyzed the data. Karen Guillemin and I wrote the paper.

INTRODUCTION

Infection with *Helicobacter pylori* is the strongest risk factor for the development of gastric carcinoma, which is the second most common cause of cancer-related death worldwide [1]. Although approximately half the world's population is infected with *H. pylori*, most of those individuals will develop simple gastritis and remain asymptomatic. However, 10-15% of infected subjects will develop duodenal ulcers and 1% will develop gastric adenocarcinoma [2]. This dramatic variability in clinical outcome of *H. pylori* infection is not well-understood, but likely results from the consequences of long-term interactions between the bacterium and its human host.

Specific bacterial and host genetic factors have been shown to affect *H. pylori* pathogenesis. Strains that possess the *cag* pathogenicity island (*cag* PAI), which encodes a type IV secretion system used to inject the CagA effector protein directly into gastric epithelial cells, are much more virulent [3]. Once inside host cells, CagA is tyrosine

phosphorylated on conserved carboxyl terminal EPIYA motifs by Src family kinases. Variability in the number and composition of these phosphorylation motifs also correlates with differences in the carcinogenic potential of *H. pylori* strains [4]. Host genetic factors that can influence the progression and ultimate disease outcome of *H. pylori* pathogenesis include polymorphisms that enhance expression of certain cytokines [2], and genetic changes that occur during progression from normal mucosa to gastric carcinoma such as loss of tumor suppressors and activation of oncogenes [5]. Although development of a complex disease like gastric cancer requires the cooperation of many bacterial and host genetic factors, it is clear that the CagA effector protein is an important driver of disease progression.

CagA has been shown to interact with a multitude of host cell proteins belonging to several conserved signaling pathways [6], and these interactions are thought to promote carcinogenesis upon *H. pylori* infection. The majority of these interactions were discovered using cell culture models in which CagA expression can disrupt processes such as tight junction formation, motility and cytoskeleton dynamics. However, which interactions between CagA and host cell signaling pathways trigger the processes that lead to gastric cancer remains unclear [4]. Obtaining more specific information about the relative importance of CagA's interactions with host cell proteins will require investigation of their downstream effects on intact epithelial tissue.

In order to examine the effects of both bacterial and host genetic factors, our group has developed a system in which *Drosophila melanogaster* is used to model pathogenesis of the *H. pylori* virulence factor CagA [7]. There are several properties that make this model organism well-suited for studying the pathogenic effects of CagA

expression. First, many canonical cell signaling pathways have been extensively characterized in *Drosophila* and show high conservation with the homologous pathways in humans. Also, genetic tools like the GAL4/UAS system allow expression of CagA in specific cells within an epithelium and examination of how CagA-expressing cells interact with neighboring wild type cells. Finally, we can easily manipulate host genes using resources generated by the rich *Drosophila* research community to assess potential effects on CagA-induced phenotypes. In addition, our model allows us to test whether CagA's interactions are phosphorylation-dependent through expression of a mutant form of CagA known as $CagA^{EPISA}$, in which the EPIYA phosphorylation motifs have been deleted or mutated [8]. Use of this model has already provided insight into CagA's role in manipulating receptor tyrosine kinases, the Rho signaling pathway, and epithelial junctions [7,9,10].

Epithelial polarity is one important feature of host cells known to be perturbed by CagA. Strains of *H. pylori* that encode CagA are exclusively able to cause localized disruption of apicobasal polarity in order to colonize a polarized monolayer of tissue culture cells [11]. CagA-positive strains of *H. pylori* have also been shown to cause apoptosis in both cultured gastric cancer cells and human gastric biopsies [12,13], although the role of CagA-dependent apoptosis in *H. pylori* pathogenesis remains controversial. Loss of epithelial cell polarity has been shown to induce apoptotic cell death or promote tumorigenesis in different cellular and genetic contexts [14]. Cell death resulting from polarity disruption can trigger compensatory proliferation in order to replace lost cells, but this process can become tumorigenic in the presence of genetic alterations that block apoptosis [15]. This mechanism has been proposed to explain how

the ability of CagA to disrupt cell polarity and induce apoptosis may be linked to its tumorigenic potential, but the host cell signaling pathways that could mediate these downstream effects have not been identified [16].

An important host signaling pathway that triggers apoptosis downstream of cell polarity disruption is the c-Jun NH_2-terminal kinase (JNK) pathway. JNK is a stress-activated protein kinase with numerous upstream activators including cytokines, mitogens, osmotic stress, ultraviolet radiation and loss of cell polarity [17]. JNK-mediated apoptosis plays a role in several physiological processes including morphogenetic apoptosis, cell competition and intrinsic tumor suppression. JNK activation performs a cell editing function in each of these processes by removing aberrant cells that arise within an epithelium, thus enhancing the resilience of epithelia to insult. Both expression of the tumor necrosis factor (TNF) homolog Eiger (Egr) and the presence of wild type cells within an epithelium are required for JNK pathway activation downstream of cell polarity disruption, and their absence can lead to tumor formation [18]. Furthermore, JNK signaling has been shown to switch from a proapoptotic to a progrowth role in the presence of oncogenic Ras [19]. These functions of the JNK pathway are well-established in *Drosophila*, and likely also relevant in mammals given the high conservation of this pathway throughout evolution [20].

Bacterial activation of JNK signaling has also demonstrated importance in enhancing epithelial robustness. During oral infection of *Drosophila* with the human pathogen *Pseudomonas aeruginosa*, the bacterium activates JNK signaling in the intestinal epithelium to trigger apoptosis and subsequent compensatory proliferation, thereby stimulating epithelial renewal. The same effect was not seen during infection

with an avirulent strain of *P. aeruginosa* that does not secrete the virulence factor pyocyanin, suggesting a role for this effector protein in activating JNK signaling in response to damage induced by the bacterium [21]. Similar to the adult *Drosophila* intestine, the larval imaginal disc epithelia are particularly resistant to the effects of stress-induced apoptosis and can recover after losing over 50% of their cells during development to produce normal adult structures [22]. This inherent epithelial resilience makes the imaginal discs a relevant tissue in which to examine potential effects of JNK-dependent apoptosis mediated by a bacterial virulence factor.

In this study, we discovered a role for the CagA virulence factor in activating JNK signaling. We used transgenic *Drosophila* to express CagA in the developing wing imaginal disc, a simple polarized epithelial structure formed during larval stages of development. We found that CagA expression caused a distinct pattern of cell death in which apoptotic cells are basally extruded from the epithelium. In addition we showed that this apoptosis phenotype is enhanced by coexpression with Basket (Bsk), the *Drosophila* homolog of JNK, and suppressed by coexpression with a dominant-negative form of Bsk. From these results, we conclude that expression of CagA triggers JNK pathway activation which causes apoptosis in an intact epithelium. Furthermore, we used a *Drosophila* model of metastasis to show that CagA expression can enhance the growth and invasion of tumors generated by expression of activated Ras. This increase in tumorigenic capacity is suppressed by coexpression with dominant-negative Bsk, leading us to conclude that CagA promotes tumor growth and invasion through JNK pathway activation.

RESULTS

CagA expression in the *Drosophila* wing causes apoptosis and epithelial disruption

In order to examine the effects of expressing the *H. pylori* effector protein CagA on an intact epithelium, we used the GAL4/UAS system to drive its expression in the wing imaginal disc. The *Drosophila* wing begins to form during early larval life when it exists as a primordial sac which contains both a simple columnar epithelium and the squamous epithelium of the peripodial membrane [23]. Cells within the wing imaginal disc proliferate extensively in larval stages followed by disc evagination during pupation, resulting in the adult wing structure. This developmental process is distinct from that of the eye imaginal disc used to model CagA pathogenesis previously [7,9,10], which undergoes systematic differentiation during larval stages. In addition, the fate of imaginal disc cells is specified early in development [24] which allowed us to express CagA in distinct regions of the wing disc (Figure 1A; all figures are provided in Appendix B).

We used two different GAL4 drivers to express CagA either in a subset of wing cells or throughout the wing imaginal disc: beadex-GAL4 (bx-GAL4) is expressed specifically in cells of the columnar epithelium that give rise to the dorsal surface of the wing blade (Figure 1B), and 765-GAL4 is expressed ubiquitously throughout the wing. A membrane-localized GFP construct (mGFP) was used to visualize the expression domain. Expressing CagA with the 765-GAL4 ubiquitous wing driver did not cause any observable phenotype (Figure 1C). However, expressing CagA with the bx-GAL4 dorsal wing driver caused clusters of apoptotic cells to form near the center of the expression domain in wing imaginal discs from third instar larvae (Figure 1D). This phenotype was dose-dependent, since expressing two copies of CagA increased both the size and number

of apoptotic clusters formed (Figure 1E). A similar phenotype has been shown to result from localized JNK pathway activation in the wing imaginal disc epithelium but does not occur upon more ubiquitous activation [25].

Interestingly, although expressing one copy of $CagA^{EPISA}$ with the bx-GAL4 driver did not cause a phenotype (Figure 1F), expressing two copies induced formation of small apoptotic clusters within the expression domain (Figure 1G). This reduction in apoptosis induction suggests that the phenomenon does not require phosphorylated CagA, but that $CagA^{EPISA}$ is a less potent activator of cell death. This observation is consistent with data obtained from transgenic expression of $CagA^{EPISA}$ in the eye imaginal disc epithelium, where less severe phenotypes were shown to result from differential cellular localization of the phosphorylation-resistant form of CagA [9].

Cells within the apoptotic clusters generated by CagA expression were extruded from the basal surface of the wing imaginal disc epithelium. Further examination of this tissue revealed an enrichment of matrix metalloproteinases, which break down basement membrane, specifically in cells located directly apical to the apoptotic clusters (Figure 1H). This observation indicates that apoptotic cells generated by CagA expression are actively removed from the wing epithelium and not passively lost during development of the imaginal disc.

Many complex cellular interactions are required during wing disc development to ensure proper formation of the adult wing structure (Figure 1I). While this process did not appear to be disrupted by ubiquitous expression of CagA in the wing (Figure 1J), CagA expression specifically in the dorsal wing caused a dose-dependent disruption of the imaginal disc epithelium which affected the overall appearance of the adult wing

(Figure 1K and 1L). This phenomenon also did not require phosphorylated CagA since expression of $CagA^{EPISA}$ caused a less severe dose-dependent disruption of the adult wing (Figure 1M and 1N). The observation that ubiquitous expression of CagA in the wing does not cause apoptosis or epithelial disruption suggests that wild type cells surrounding those which express CagA are required to produce both phenotypes. This is consistent with the previous observation that JNK-dependent apoptosis is only triggered when aberrant cells within an epithelium are surrounded by wild type cells [26]. Taken together, these data prompted us to examine a potential role for JNK signaling in the apoptosis and epithelial disruption phenotypes resulting from localized expression of CagA in the wing imaginal disc.

CagA-induced apoptosis occurs through activation of the JNK signaling pathway

Several aspects of the apoptosis phenotype caused by CagA expression in the wing imaginal disc suggested an interaction between CagA and the JNK pathway. In order to determine the nature of this potential interaction, we examined the effects of expressing several forms of Bsk, the *Drosophila* homolog of JNK, on the CagA-induced wing phenotype. Ectopic overexpression of wild type Bsk with the bx-GAL4 dorsal wing driver generated small apoptotic clusters (Figure 2A), indicating that the presence of excess JNK in the wing can phenocopy CagA expression. Furthermore, the cell death phenotype caused by CagA expression in the wing was dramatically enhanced by coexpression with wild type Bsk (Figure 2B). Coexpression of Bsk with $CagA^{EPISA}$ also caused a substantial amount of apoptosis in the wing imaginal disc, suggesting that this interaction is not dependent on phosphorylated CagA (Figure 2C). As expected, expression of a dominant-negative form of Bsk (Bsk^{DN}) alone did not cause apoptosis in

the wing imaginal disc (Figure 2D). Significantly, coexpression of Bsk^{DN} with CagA almost completely suppressed the apoptosis phenotype caused by CagA expression (Figure 2E), indicating that blocking JNK signaling suppresses CagA-dependent cell death in the wing. These data suggest that CagA expression triggers wing imaginal disc apoptosis through JNK pathway activation.

We also examined the effects of JNK pathway modulation on the epithelial disruption phenotype caused by CagA expression. Although ectopic overexpression of wild type Bsk with bx-GAL4 caused only a minor adult wing phenotype in the form of extra vein material (Figure 2F), coexpression of Bsk with CagA dramatically enhanced the epithelial disruption phenotype (Figure 2G). Ectopic overexpression of Bsk with $CagA^{EPISA}$ was not sufficient to induce epithelial disruption (Figure 2H). Expression of Bsk^{DN} also gave rise to only subtle vein defects in an otherwise normal adult wing (Figure 2I). Interestingly, Bsk^{DN} expression was not able to rescue but instead enhanced the epithelial disruption caused by CagA expression (Figure 2J). One explanation for this apparent contradiction is that blocking JNK signaling prevents the induction of apoptosis that is required to remove aberrant CagA-expressing cells from within the epithelium, which are then allowed to accumulate and lead to a more severe disruption of the adult structure. We tested this hypothesis using the apoptosis inhibitor p35, a baculovirus-derived suicide substrate for effector caspases. Overexpressing p35 alone with bx-GAL4 did not produce a phenotype (Figure S1A; all supporting information is provided in Appendix D), while coexpressing p35 with CagA effectively blocked apoptosis but enhanced disruption of the adult wing epithelium (Figure S1B). This observation is consistent with the inhibition of apoptosis resulting in more severe CagA-dependent adult

phenotypes. Enhancement and suppression of CagA-induced apoptosis in the wing imaginal disc was quantified using a method we developed to measure the percentage of the expression domain that is caspase-positive. These quantitative data showed that both the enhancement of CagA-induced apoptosis seen with coexpression of ectopic Bsk, and its suppression upon expression of Bsk^{DN} were statistically significant (Figure 2K).

In order to further examine the genetic interaction between CagA and JNK signaling, we used a *lacZ* reporter allele of *puckered* (*puc*), the main component of a negative feedback loop in the JNK pathway. This construct has been used extensively as a readout for JNK pathway activation in *Drosophila* tissue using antibody staining for β-galactosidase (β-gal). Expressing CagA in combination with *puc-lacZ* in the dorsal wing imaginal disc demonstrated that cells adjacent to those undergoing apoptosis are activating JNK signaling (Figure 2L). These data provide additional evidence that CagA expression activates JNK signaling in the wing imaginal disc epithelium.

Loss of neoplastic tumor suppressors and the TNF homolog Eiger enhances CagA-induced apoptosis

JNK signaling is activated by a complex set of signals including TNF and loss of epithelial polarity. To examine the mechanism through which CagA activates JNK signaling, we used the bx-GAL4 driver to express CagA in combination with RNAi-mediated knockdown of known epithelial polarity determinants and examined wing imaginal discs for enhancement of the apoptosis phenotype (Figure 3A). We tested a panel of polarity proteins, many of which caused apoptosis when knocked down in the absence of CagA expression (Table S1). We chose to target a protein from each of the previously described complexes whose localization and function establish epithelial cell

polarity [27], and to simplify our analysis we selected polarity proteins that did not cause an apoptosis phenotype when knocked down on their own (Figure S2A-S2C). When tested in combination with CagA expression, we found that RNAi-mediated knockdown of neither the junctional protein Bazooka (Baz), nor the apical protein Crumbs (Crb) enhanced apoptosis (Figure S2D and S2E). In addition, knockdown of Par1, which has been shown to interact with CagA in tissue culture cells [28], did not enhance the apoptosis phenotype caused by CagA expression in this context (Figure S2F).

Interestingly, RNAi-mediated knockdown of the basolateral protein Discs Large (Dlg) did not cause a significant phenotype (Figure 3B) but markedly enhanced the apoptosis caused by CagA expression (Figure 3C). The same effect was seen with knockdown of Lethal Giant Larvae (Lgl), another basolateral protein (Figure S2G and S2H). The genes encoding these polarity proteins are known as neoplastic tumor suppressor genes (nTSGs) because their loss causes tumor formation in *Drosophila* [29], and generating clones of cells which lack this specific class of polarity determinants has been shown to trigger JNK-dependent apoptosis in imaginal discs [30]. Our data suggest that nTSGs normally suppress CagA-mediated JNK pathway activation and subsequent apoptosis in the wing imaginal disc.

Disruption of the nTSGs activates JNK signaling through endocytosis of the TNF homolog Egr [31]. Homozygous *egr* mutant animals are viable and, as expected, no apoptosis was observed in their wing imaginal discs (Figure S2I). Conversely, ectopic overexpression of wild type Egr in the dorsal wing imaginal disc caused a severe apoptosis phenotype (Figure S2J), consistent with previous data showing Egr to be a potent activator of cell death in *Drosophila* epithelia [32]. We made the unexpected

observation that expression of CagA in the dorsal wing disc of an *egr* mutant animal enhanced the apoptosis phenotype (Figure 3D). Interestingly, RNAi-mediated knockdown of Egr alone in the dorsal wing with bx-GAL4 did not cause a phenotype (Figure S2K) or enhance apoptosis when coexpressed with CagA (Figure S2L). This observation suggests that loss of Egr in wild type cells surrounding the CagA expression domain is responsible for the enhanced apoptosis phenotype seen in the wing imaginal discs of *egr* mutant animals expressing CagA.

Recent data has demonstrated that loss of nTSGs in clones of imaginal disc cells causes Egr-dependent activation of nonapoptotic JNK signaling in their wild type neighbors. JNK activation in surrounding wild type cells leads to induction of a phagocytic pathway which triggers engulfment of polarity-deficient cells within the clone [30]. A similar mechanism can be invoked to explain the enhancement of CagA-induced apoptosis seen in *egr* mutant wing imaginal discs. Loss of Egr in the wild type cells surrounding the expression domain may prevent engulfment of CagA-expressing cells. This would increase the number of aberrant cells available to undergo apoptosis upon CagA-mediated activation of JNK signaling via another parallel upstream pathway. We hypothesize that multiple cellular consequences of CagA expression can activate JNK signaling combinatorially. Supporting this view, we demonstrated that CagA-induced apoptosis was enhanced by ectopic overexpression with a wild type form of the small GTPase Rho1 (Figure 3E), another upstream activator of the JNK pathway that did not cause a phenotype when overexpressed alone (Figure S2M), and which our group has shown is activated by CagA [9].

Enhancement of CagA-induced apoptosis in the wing imaginal disc was quantified using the previously described method. These data showed significant enhancement of apoptosis with coexpression of CagA and knockdown of nTSGs, ubiquitous loss of Egr or overexpression of Rho1. Knockdown of several other polarity proteins or Egr in CagA-expressing cells did not enhance the apoptosis phenotype (Figure 3F). These observations suggest that specific polarity protein complexes within the cell, as well as other upstream activators are responsible for transducing the signals that lead to JNK pathway activation upon CagA expression in the wing imaginal disc (Figure 3G).

CagA expression enhances the growth and invasion of tumors generated by expression of oncogenic Ras through JNK pathway activation

The finding that CagA activates the JNK pathway is intriguing in light of recent evidence indicating that activation of JNK signaling can switch from proapoptotic to progrowth in the presence of oncogenic Ras [19]. In order to examine a potential role for CagA-mediated JNK pathway activation in promoting tumorigenesis, we used a slight variation of a previously established *Drosophila* metastasis model to create whole eye clones expressing an activated form of the Ras oncogene (Ras^{V12}) in epithelial cells of the eye imaginal disc using the eyeless (ey) driver with the FLP/FRT system to generate primary tumors [33]. We then evaluated the size of GFP-marked tumors in whole larvae (Figure 4A) and dissected cephalic complexes (Figure 4B) in order to determine whether coexpression of CagA could enhance the growth and invasive potential of these tumor cells through activation of the JNK signaling pathway.

Expression of Ras^{V12} alone in whole eye clones caused overgrowth of eye imaginal disc cells which resulted in tumor formation (Figure 4C), as previously

described [34]. Although generating whole eye clones expressing either GFP alone (Figure S3A) or with CagA (Figure S3B) was not tumorigenic, coexpression of CagA enhanced the growth of tumors generated by Ras^{V12} expression (Figure 4D). Whole eye clones expressing $CagA^{EPISA}$ were also not tumorigenic (Figure S3C), and when combined with Ras^{V12} expression caused only a minor enhancement of tumor growth (Figure 4E). As expected, coexpression of Bsk^{DN} did not affect the growth of tumors generated by Ras^{V12} expression alone (Figure 4F). However, Bsk^{DN} expression caused a severe reduction in the growth of tumors expressing both Ras^{V12} and CagA (Figure 4G). Quantification of these data was accomplished by determining the size of dissected cephalic complexes of each genotype and showed a significant growth enhancement with combined expression of Ras^{V12} and CagA, which was suppressed by coexpression of Bsk^{DN} (Figure 4H). These data demonstrate that expression of CagA can enhance the growth of tumors generated by expression of Ras^{V12} in a JNK-dependent manner.

Generating whole eye clones that express Ras^{V12} alone most commonly caused either a mildly invasive phenotype characterized by the migration of a small number of GFP-positive cells along one edge of the ventral nerve cord (VNC), or a noninvasive phenotype in which cells within the optic lobe approached but did not migrate into the VNC (Figure 5A). Whole eye clones expressing either GFP alone (Figure S4A) or with CagA (Figure S4B) were not invasive, but coexpression of CagA with Ras^{V12} resulted in a much larger number of GFP-positive tumor cells migrating from both optic lobes into the VNC. These cells were not terminally differentiated, as indicated by a lack of staining with the neuron-specific ElaV antibody, and phalloidin staining showed a morphology distinct from other cells in the VNC (Figure 5B). Expressing $CagA^{EPISA}$ in whole eye

clones also did not produce an invasive phenotype (Figure S4C), and coexpression of $CagA^{EPISA}$ with Ras^{V12} caused a less pronounced enhancement of the mild invasion caused by expression of Ras^{V12} alone (Figure 5C), suggesting that the phosphorylation-resistant form of CagA is less effective at promoting tumor progression. Coexpression of Bsk^{DN} did not affect the invasive phenotype generated by Ras^{V12} expression alone (Figure 5D), but Bsk^{DN} expression caused a dramatic reduction in the invasive capacity of tumors expressing both Ras^{V12} and CagA (Figure 5E). These data show that CagA expression can enhance the invasion of Ras^{V12}-expressing tumor cells through JNK activation.

In order to determine the significance of CagA's enhancement of invasion, we used a previously described method [35] to categorize invasive phenotypes into four distinct classes which represent a progression from non-invasive to severe invasion of the VNC (Figure 5F). Quantitation of the percentage of cephalic complexes exhibiting each class of VNC invasion showed a significant difference between expression of Ras^{V12} alone and in combination with CagA, which was suppressed by coexpression of Bsk^{DN} (Figure 5G).

DISCUSSION

In the current study, we used transgenic expression of the CagA virulence factor in *Drosophila* to demonstrate a role for JNK pathway activation in *H. pylori* pathogenesis. When CagA was expressed in a subset of wing imaginal disc cells juxtaposed to non-expressing cells, the epithelium underwent apoptosis and proper formation of adult wing structure was disrupted. We showed that the apoptosis phenotype occurs through activation of the JNK signaling pathway. CagA-induced apoptosis was

enhanced by loss of nTSGs or ectopic expression of the small GTPase Rho1 in the CagA-expressing cells and loss of the TNF homolog Egr in non-expressing cells (Figure 6A). We next showed that CagA-mediated JNK pathway activation can enhance the growth and invasion of tumors generated by expression of oncogenic Ras. Our data uncover a novel genetic interaction between CagA and JNK signaling and demonstrate its potential importance in promoting tumor progression.

Distribution of CagA within an epithelium can affect manipulation of host proteins and intercellular interactions

Infection of tissue culture cells with *H. pylori* has been shown to activate JNK signaling, but a role for CagA in this process remains controversial [36-38]. Additionally, these experiments were performed in nonpolar AGS cells, so if polarity disruption plays a role in JNK pathway activation downstream of CagA, as our data suggest, these cell culture models may not reveal this interaction. JNK pathway activation has also been shown to result from infection with several pathogenic bacteria in epithelial cell culture models of infection [39]. Interestingly, the enteroinvasive bacterium *Shigella flexneri* was shown to activate JNK and upregulate TNFα expression in both infected and adjacent uninfected epithelial cells in culture [40], similar to our data showing that JNK-mediated tissue responses to CagA expression involve a cell-nonautonomous requirement for TNF/Egr. The distribution of *H. pylori* during infection of the gastric epithelium is known to be heterogeneous [2]. We therefore hypothesize that interactions between cells containing CagA protein and uninfected neighboring cells could also be important for pathogenesis in *H. pylori*.

Our data suggest that CagA is an important mediator of JNK pathway activation during *H. pylori* infection, and identify several host proteins involved in this process. We observe genetic interaction between CagA and nTSGs, but not junctional proteins involved in polarity. This is consistent with recent data from tissue culture cells which demonstrated that CagA-positive strains of *H. pylori* specifically disrupt apicobasal polarity in a polarized monolayer prior to affecting the integrity of cellular junctions [11]. Our data also suggest that CagA can activate JNK-dependent apoptosis through multiple upstream pathways. The observation that overexpression of Rho1 enhances CagA-dependent apoptosis in the wing imaginal disc epithelium is consistent with previous data from our group demonstrating a role for CagA in activating the Rho pathway to disrupt epithelial patterning [9].

Use of the unique genetic tools available in *Drosophila* has provided important insight into potential interactions between CagA-expressing cells and neighboring wild type cells. Our observation that loss of TNF/Egr in wild type cells surrounding those expressing CagA can enhance apoptosis, presumably by reducing engulfment of CagA-expressing cells, indicates that the genetic state of uninfected cells may also play a role in *H. pylori* pathogenesis. This finding is interesting with respect to data from human patient samples showing a positive correlation between Toll-like Receptor 4 (*TLR4*) polymorphisms, conferring decreased expression of cytokines including TNF, and *H. pylori*-associated gastric cancer [41]. Our data suggest that in addition to effects on gastric inflammation in response to *H. pylori* infection, host genetic polymorphisms in *TLR4* and other innate immune signaling components may also have more direct effects on CagA-mediated disruption of host cell signaling pathways.

Genetic changes in host cells can alter the downstream effects of CagA signaling during long-term association with *H. pylori*

Since it was first discovered, JNK has been demonstrated to have both pro-tumorigenic and tumor suppressor functions in different cell types and organs. Studies in *Drosophila* have helped shed light on the genetic contexts in which JNK activation functions to promote tumor progression, namely in the presence of oncogenic Ras [42]. Recently, JNK was shown to be required for activated KRas-induced lung tumor formation in mice [43], suggesting a conserved role of JNK activation in cooperating with activated Ras to promote tumorigenesis in mammals. A potential role for JNK pathway activation has also been explored in mammalian gastric cancer. Activation of JNK signaling has been detected in human gastric cancer samples, and mice lacking JNK1 exhibit a decrease in gastric apoptosis and an attenuation of gastric tumor development induced by the chemical carcinogen *N*-methyl-*N*-nitrosourea [44]. A role for *H. pylori* in the context of mammalian gastric cancers induced by cooperation between JNK and Ras signaling has not been explored.

Our finding that CagA expression can induce JNK-dependent apoptosis in a polarized epithelium is interesting with respect to data suggesting that JNK signaling has evolved as a cell editing mechanism to remove aberrant cells from within an epithelium [18]. Activation of JNK signaling could represent a host response aimed at removing cells containing CagA protein from the gastric epithelium. Similarly, *P. aeruginosa*-mediated activation of JNK signaling in the intestinal epithelium of *Drosophila* can trigger epithelial renewal as a host defense mechanism. However, this process can become pathogenic and lead to dramatic overproliferation of intestinal cells in animals

harboring oncogenic Ras mutations [21]. In *H. pylori* infection, which can persist for many years before the development of gastric cancer, JNK-mediated apoptosis could be an effective mechanism to limit pathogenic effects on the gastric epithelium. However, this process of tissue editing can also increase cell turnover, contributing to accumulation of genetic mutations in host cells. Our data show that acquisition of an oncogenic mutation in host epithelial cells experiencing CagA-mediated JNK pathway activation can promote tumor progression, suggesting that this potential host defense strategy can become tumorigenic in certain genetic contexts (Figure 6B).

Although half the world's population is thought to be infected with *H. pylori*, a small percentage of those individuals will develop gastric cancer [2]. This observation indicates that, in addition to the presence of the *cag* PAI in more virulent strains, host genetics must also play a crucial role in determining the outcome of *H. pylori* infection. Our results suggest that a change in host genetics during long-term association with *H. pylori* could cause JNK activation to switch from conferring a protective function against CagA-induced cellular changes to enabling tumor progression. Data collected from tissue biopsies indicate that Ras mutation may play a role in the development of gastric cancer in human patients [45], and our data put forward the idea that enhanced tumorigenic potential created by cooperation between JNK pathway activation via the bacterial genetic factor CagA and sporadic activation of Ras in host cells could drive gastric cancer formation in a subset of *H. pylori* infections.

MATERIALS AND METHODS

Fly strains and generation of whole eye clones

The following fly stocks were used: UAS-CagA, UAS-CagA^{EPISA} [7]; bx-GAL4, UAS-mCD8::GFP (mGFP), UAS-bsk.B (Bsk^{1}), UAS-bsk.A-Y (Bsk^{2}), UAS-bsk.K53R (Bsk^{DN}), *egr*^{MB06803} (*egr*$^{-/-}$), UAS-Dlg-RNAi, UASp-FLAG.Rho1 (Rho1), UAS-Ras85D.V12 (Ras^{V12}), UAS-Crb-RNAi, UAS-Patj-RNAi, UAS-Cora-RNAi, UAS-Cdc42.N17 (Cdc42^{DN}) (from Bloomington Stock Center); 765-GAL4 (provided by Ross Cagan, Mount Sinai School of Medicine); *puc*^{E69} (*puc-lacZ*), Regg1^{GS9830} (UAS-Egr) (provided by Michael Galko, MD Anderson Cancer Center); UAS-Lgl-RNAi, UAS-Baz-RNAi, UAS-Par1-RNAi, UAS-Scrib-RNAi, UAS-Par6-RNAi, UAS-aPKC-RNAi, UAS-Mir-RNAi (provided by Chris Doe, University of Oregon); UAS-Egr-RNAi (from Vienna *Drosophila* Resource Center); eyFLP; Act>y^{+}>GAL4, UAS-GFP (provided by Tory Herman, University of Oregon). Flies were raised at 25°C using standard methods. Whole eye clones were generated as previously described [33] without the GAL80 repressor to express transgenes in all cells that give rise to the eye-antennal disc.

Histology

Larval tissues were fixed and stained using standard protocols. The following primary antibodies were used: rabbit anti-active caspase-3 (1:200; BD Pharmingen), mouse anti-Mmp1 (1:50; Developmental Studies Hybridoma Bank), mouse anti-β-galatosidase (1:500; Sigma) and rat anti-ElaV (1:10; Developmental Studies Hybridoma Bank). Both Cy3 and Cy5-conjugated secondary antibodies were used (1:200; Jackson ImmunoResearch), as well as Alexa Fluor 546 phalloidin (1:40; Molecular Probes). Intact adult wings were mounted in a 1:1 mixture of lactic acid and ethanol.

Image analysis and quantitation

Adult wings, intact larvae and whole cephalic complexes were visualized using light microscopy or GFP fluorescence on a Zeiss dissecting microscope. Wing imaginal discs, ventral nerve cords and cephalic complexes were visualized on a Nikon confocal microscope. Images were processed using Adobe Photoshop, where levels were adjusted to optimize the signal-to-noise ratio in each color channel while maintaining similar levels of background noise and desired signal between channels and images. Adult wing images were removed from their background using the Extract filter in Adobe Photoshop. XZ confocal planes were created using the Reslice function in Image J. Projections of confocal cross sections were created using the Merge to HDR command in Adobe Photoshop. Apoptosis was quantified by selecting the single confocal cross section of each wing imaginal disc exhibiting the highest level of active caspase-3 staining and manually tracing the expression domain, then determining the percentage of this domain showing active caspase-3 staining using the Threshold function in Image J. Cephalic complex size was quantified using the Threshold function in Image J to determine the area of the tissue in μm^2. Graphs were created with GraphPad Prism software, which was also used to calculate two-tailed p values using the unpaired t test with Welch's correction.

BRIDGE TO CHAPTER IV

Chapter III discussed our use of the transgenic *Drosophila* model to uncover a novel role for CagA in activating the JNK pathway to cause different downstream effects depending on the cellular and genetic context of host cells. We showed that non-uniform

expression of CagA in a simple model epithelium triggers apoptosis through activation of JNK signaling. We further explored the effects of host genetic context on CagA-induced JNK activation using a *Drosophila* model of tumorigenesis and metastasis, and established that CagA enhances the growth and invasion of tumors when coexpressed with activated Ras. This work demonstrated the importance of host cell context on CagA's functions, and prompted us to explore a role for CagA in the gastrointestinal ecosystem. Chapter IV will discuss our use of the transgenic *Drosophila* model to examine effects of CagA expression on intestinal homeostasis, innate immune signaling and the microbiota.

CHAPTER IV

THE *HELICOBACTER PYLORI* CAGA EFFECTOR PROTEIN ALTERS INTESTINAL HOMEOSTASIS, INNATE IMMUNE SIGNALING AND THE MICROBIOTA IN A TRANSGENIC *DROSOPHILA* MODEL

The work described in this chapter was co-authored by Allison Banse, David Bauer, Adam Burns, Karen Guillemin and myself. Karen Guillemin and I conceived and designed the experiments. Allison Banse, David Bauer, Adam Burns and I performed the experiments. Allison Banse, David Bauer, Adam Burns, Karen Guillemin and I analyzed the data. Karen Guillemin and I wrote the paper.

INTRODUCTION

The bacterial pathogen *Helicobacter pylori* infects approximately half the world's population, and is strongly correlated with development of gastric cancer. Upon infection of the human stomach, *H. pylori* establishes intimate interactions with the gastric epithelium which play an important role in pathogenesis [1]. A crucial component of the *H. pylori*-host epithelium interaction is translocation of the CagA virulence factor directly into host cells. This effector protein is known to disrupt various host signaling pathways, and strains of *H. pylori* which encode CagA show enhanced virulence [2]. Recent work has demonstrated the importance of CagA in several host responses to *H. pylori* infection. First, transgenic expression of CagA in the mouse stomach was shown to enhance proliferation of the gastric epithelium [3]. Also, *H. pylori* is known to trigger a variety of host innate immune responses, some of which were found to be CagA-dependent [4]. In

addition, *H. pylori* infection has demonstrated an ability to alter the gastric microbiota in both mice and humans [5,6], but a role for CagA in this process has not been explored.

In recent years, *Drosophila melanogaster* has emerged as a model for studying host-pathogen interactions and their effects on the intestinal epithelium. The general organization of the *Drosophila* gastrointestinal (GI) tract is similar to that of its mammalian counterpart, with specific regions corresponding to organs including the esophagus, stomach, and small and large intestines [7]. Further interest in the *Drosophila* GI tract was generated when a distinct population of adult stem cells was discovered in the intestinal epithelium [8,9], demonstrating another level of homology to the mammalian intestine. In addition to intestinal stem cells (ISCs), the *Drosophila* intestinal epithelium exhibits a cellular composition with established similarities to that of the mammalian intestine. Also present in the *Drosophila* intestinal epithelium are enteroblasts which, like transit-amplifying cells in the mammalian intestine, differentiate into mature enterocytes. The *Drosophila* intestinal epithelium also contains enteroendocrine cells which are similar to mammalian Paneth cells. Importantly, the molecular mechanisms that govern ISC proliferation and differentiation are also well-conserved between *Drosophila* and mammals. In both cases Wnt/Wingless signaling controls ISC self-renewal [10], and Delta/Notch signaling is responsible for directing cell fate specification [11]. All of these similarities make the *Drosophila* intestine a useful model system in which to study the effects of a bacterial pathogen.

The high conservation of *Drosophila* host cell signaling pathways and the ease with which they can be genetically manipulated has provided important insights into the molecular mechanisms involved in host responses to oral infection with a pathogen. One

important host response which has been shown to occur upon oral infection of *Drosophila* with pathogenic strains of both *Erwinia carotovora* (*Ecc15)* and the human pathogen *Pseudomonas aeruginosa* (PA14) is enhanced proliferation in the intestinal epithelium [12,13]. Infection with *Ecc15* was also shown to upregulate expression of several antimicrobial peptides (AMPs) in the intestine, demonstrating an innate immune response to this pathogen. Several studies examining more global expression patterns revealed induction of genes encoding components of the JAK-STAT pathway upon infection with *Ecc15* [12], and activation of this pathway was also shown to occur during infection with the pathogen *Pseudomonas entomophila* [14]. Further analysis demonstrated activation of JNK signaling upon infection with both *Ecc15* and PA14, implicating this pathway in host response to bacterial infection in the intestinal epithelium [12,13]. Thus in addition to stimulating canonical innate immune signaling, oral infection with a pathogen activates other host cell signaling components that could mediate important downstream effects on the intestinal epithelium.

Several studies have examined the microbial composition of the *Drosophila* intestine, and established the presence of relatively few species from only two predominant genera: *Acetobacter* and *Lactobacillus* [15,16]. This simple and cultureable bacterial community is contrasted by the exceedingly complex and largely unculturable microbiota present in mammals [17], and facilitates examination of potential changes in microbial composition. Derivation of germ-free *Drosophila* has revealed several important contributions of the microbiota to age-related deterioration of the intestinal epithelium. *Drosophila* reared germ-free were shown not to exhibit the characteristic change in stem cell morphology, increase in stem cell number, and enhanced intestinal

proliferation normally observed in older *Drosophila*. In addition, germ-free *Drosophila* do not show the increase in expression of AMPs, or JAK-STAT and JNK pathway components known to occur in older conventionally-reared *Drosophila* [12]. These data demonstrate the importance of the microbiota in intestinal homeostasis and suggest that an altered microbial community may disrupt such processes in *Drosophila*.

In the current study, we discovered a role for the CagA virulence factor in disrupting intestinal homeostasis, innate immune signaling and the microbiota. We used our transgenic *Drosophila* model [18] to express CagA in ISCs, and observed an increase in intestinal epithelial proliferation. We analyzed AMP expression, and found that CagA enhanced production of this innate immune signaling molecule in the intestine. In addition we showed that CagA expression in ISCs caused a reduction in the overall abundance of the intestinal bacterial community, while maintaining a similar composition at the genus level. From these results, we conclude that expression of CagA profoundly affects the *Drosophila* intestine through various mechanisms that are relevant to potential effects of *H. pylori* infection in the human stomach.

RESULTS

CagA expression enhances intestinal stem cell proliferation

In order to examine the effects of expressing the *H. pylori* effector protein CagA on the intestinal epithelium, we used the GAL4/UAS system to drive its expression in ISCs. The adult *Drosophila* GI tract exhibits a simple organization of several distinct components [19], some with proposed homology to human organs (Figure 1A; all figures

are provided in Appendix C). ISCs are located along the length of the midgut, and give rise to all cell types within this region of the digestive system [7].

We used the escargot-GAL4 (esg-GAL4) driver to express CagA in ISCs. Expressing GFP alone with esg-GAL4 produced an adult intestine with small ISCs dispersed throughout the midgut epithelium and few proliferating cells (Figure 1B), as previously reported [12,13]. CagA expression caused a dramatic change in the overall morphology of the intestinal epithelium characterized by an apparent increase in both the size and number of esg^+ cells, and also enhanced epithelial proliferation (Figure 1C). Quantitation of proliferation was achieved by determining the number of cells positive for the mitosis marker phospho-Histone H3 (PH3) in the adult midgut and showed a significant increase in proliferating cells upon expression of CagA with esg-GAL4 (Figure 1D). These data demonstrate that CagA expression in ISCs is sufficient to disrupt epithelial morphology and stimulate proliferation of the intestinal epithelium.

CagA expression increases antimicrobial peptide production in the intestinal epithelium

In order to determine whether expressing CagA in the intestinal epithelium affects innate immune signaling, we assessed the expression levels of AMPs in the intestine. *Drosophila* sense and respond to microbes through two main innate immune signaling pathways: Toll and Imd. Activation of either pathway results in binding of the NFκB transcription factor homolog Relish (Rel) to promoters of antimicrobial peptide genes, and their subsequent expression [20]. Expressing GFP alone in ISCs leads to low expression of the AMPs *Drosomycin* and *Diptericin*. Expressing CagA with esg-GAL4 induces dramatic upregulation of both AMPs (Figure 2), indicating that CagA triggers an

innate immune response that results in NFκB-mediated activation of AMP production. Interestingly, *Drosomycin* is known to be upregulated upon activation of the Toll pathway, while *Diptericin* expression occurs downstream of Imd pathway activation [20]. Thus our data suggest that CagA triggers expression of AMPs through activation of both innate immune signaling pathways in *Drosophila*.

CagA expression alters the intestinal bacterial community

The observation that CagA expression increases AMP production in the *Drosophila* intestine prompted us to examine potential effects of this innate immune response on the microbial composition of the gastrointestinal tract. Our culture-based analysis of the microbiota in intestines of *Drosophila* expressing GFP alone in ISCs revealed the presence of bacteria from two genera: *Acetobacter* and *Lactobacillus*, consistent with previous reports [15,16]. Further examination revealed the presence of three distinct strains of *Acetobacter* and two strains of *Lactobacilli*, each with unique colony morphology and exhibiting 96-98% identity based on 16S rRNA sequence data. Upon expression of CagA with esg-GAL4, we observed a dramatic reduction in the overall abundance of intestinal bacteria (Figure 3). Interestingly, we determined that the proportion of each genera present in CagA-expressing intestines was maintained, as compared to the GFP-expressing control. This observation implies that CagA expression reduces bacterial abundance through a mechanism that does not target specific genera. Our analysis of intestinal AMP expression also suggests a non-specific mechanism of bacterial targeting since downstream targets of both the Toll and Imd pathways are upregulated upon CagA expression in ISCs. However, preliminary data indicate a greater reduction in the abundance of certain strains of bacteria within each genus upon CagA

expression, which suggests an ability to target specific bacterial strains present in the microbiota.

DISCUSSION

In this study, we used transgenic expression of the CagA virulence factor in *Drosophila* to demonstrate its role in manipulating intestinal homeostasis. When CagA was expressed in ISCs, the intestinal epithelium exhibited enhanced proliferation and its overall morphology was disrupted. We also showed that ISC-specific CagA expression increased AMP production, indicating an altered host innate immune response. We next showed that CagA expression in ISCs was sufficient to cause a significant reduction in abundance of the intestinal microbiota. Our data reveal novel effects of the CagA virulence factor on the intestine (Figure 4), and suggest potential mechanisms through which CagA could disrupt homeostasis of the human gastric epithelium during *H. pylori* pathogenesis.

Expression of the CagA effector protein was shown to enhance gastric epithelial proliferation in a transgenic mouse model, although the potential mechanisms responsible for this effect were not explored [3]. Infection studies in *Drosophila* have demonstrated bacterial stimulation of intestinal epithelial proliferation, and implicated several important host cell signaling pathways in this response [12-14]. In addition, increased proliferation upon age-related deterioration of *Drosophila* intestinal epithelia was shown to occur only in the presence of commensal microbes, suggesting a role for the intestinal microbiota or specific species therein during this process [12]. We observed a significant enhancement of intestinal epithelial proliferation upon expression of CagA in *Drosophila*

ISCs. This increase in proliferation could result from direct manipulation of host cell signaling involved in maintaining intestinal homeostasis, possibly via the JAK-STAT or JNK pathway. Alternatively, CagA expression could stimulate proliferation indirectly by altering the intestinal microbiota to create a more pro-proliferative bacterial composition. Effects of the microbiota can be tested directly using our *Drosophila* model by generating germ-free animals that express CagA in ISCs and determining whether enhancement of intestinal epithelial proliferation is observed. As these two proposed mechanisms are not mutually exclusive, it is possible that both manipulation of host cell signaling and alteration of the intestinal microbiota contribute to CagA-mediated stimulation of intestinal epithelial proliferation. Interestingly, recent data demonstrated that *H. pylori* infection of INS-GAS mice, which exhibit increased gastrin production, resulted in delayed development of gastrointestinal intraepithelial neoplasia in the absence of an intestinal microbiota [5]. This observation suggests a role for the intestinal bacterial community in promoting gastric cancer, and our *Drosophila* model could provide insight into the potential function of CagA in this process.

H. pylori infection has demonstrated induction of CagA-dependent innate immune responses which occur through several host cell signaling pathways and converge on NFκB to activate transcription of inflammatory molecules [4]. In *Drosophila*, bacterial infection is known to upregulate expression of AMPs through the NFκB homolog Rel as part of the innate immune response [12]. Our data demonstrate that expression of CagA in ISCs increases production of AMPs in the *Drosophila* intestine. There are several conserved host cell signaling pathways through which CagA could stimulate AMP expression, and the tools available in *Drosophila* to manipulate host genetics will

facilitate examination of potential mechanisms. Specifically, determining if enhancement of AMP expression is maintained in *rel* mutant *Drosophila* expressing CagA in ISCs will reveal whether this innate immune response is mediated through the NFκB homolog. Additionally, CagA expression could affect AMP production indirectly by altering the microbial composition of the intestine to generate a more pro-inflammatory community. This hypothesis can also be tested by generating germ-free animals that express CagA in ISCs and determining whether enhancement of AMP production is observed.

Recent studies have demonstrated changes in the gastric microbiota upon infection with *H. pylori* in mammals [5,6]. We show that expression of CagA in ISCs is sufficient to induce a significant reduction in abundance of the intestinal microbiota in *Drosophila*. Although the relative proportion of each of the two predominant genera appears to be maintained upon CagA expression, we observe instances in which specific strains within each genus are more dramatically reduced than others. One possible mechanism to reduce bacterial abundance could involve the upregulation of AMPs that we observed, and more extensive analysis of which AMPs are targeted by CagA may provide insight into the specificity of bacterial targets of CagA. Additionally, analysis of the microbiota in CagA-expressing *rel* mutant *Drosophila*, or those in which expression of specific AMPs has been knocked down will determine the importance of enhanced AMP production on altering the microbiota. Our data suggest that CagA expression reduces overall abundance of the intestinal bacterial community through both a general antimicrobial mechanism, and through a more precise mechanism that targets specific bacterial strains.

MATERIALS AND METHODS

Fly strains and rearing

The following fly stocks were used: UAS-CagA [18]; UAS-mCD8::GFP (mGFP) (from Bloomington Stock Center); esg-GAL4, UAS-GFP (from Norbert Perrimon, Harvard Medical School). Flies were raised at 25°C using standard methods. Adult flies of each genotype were collected upon eclosion, then housed in the same vial for one week before dissection and subsequent analysis.

Histology

Female adult intestines were fixed and stained using standard protocols. The following primary antibodies were used: chicken anti-GFP (1:1000; Millipore), rabbit anti-phospho-Histone H3 (1:4000; Santa Cruz Biotechnology). Both Cy2 and Cy3-conjugated secondary antibodies were used (1:2000; Jackson ImmunoResearch).

Image analysis and quantitation

Adult intestines were visualized on a Nikon confocal microscope. Images were processed using Adobe Photoshop, where levels were adjusted to optimize the signal-to-noise ratio in each color channel while maintaining similar levels of background noise and desired signal between channels and images. Graphs were created with GraphPad Prism software, which was also used to calculate two-tailed p values using the unpaired t test with Welch's correction.

RT-PCR

Total RNA was extracted from 10 dissected female guts using Trizol (Invitrogen), then subjected to reverse transcription using Superscript III (Life Technologies). The

amount of cDNA was normalized between samples, then analyzed after 35 rounds of PCR with primers for *Drosomycin* (sense, 5'-GCA GAT CAA GTA CTT GTT CGC CC-3'; antisense, 5'-CTT CGC ACC AGC ACT TCA GAC TGG-3', *Diptericin* (sense, 5'-GGC TTA TCC GAT GCC CGA CG-3'; antisense, 5'-TCT GTA GGT GTA GGT GCT TCC C-3'), and the control, *Rp49* (sense, 5'-AGA TCG TGA AGA AGC GCA CCA AG-3'; antisense, 5'-CAC CAG GAA CTT CTT GAA TCC GG-3').

Bacterial isolation and identification

Adult female flies were surface-sterilized with 70% ethanol and rinsed in sterile water, then intestines were dissected and homogenized in sterile PBS, and cultured on both MRS agar and mannitol agar. Each colony type with a distinct morphology was subjected to PCR with 16S rRNA primers (27F, 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R, 5'-GGT TAC CTT GTT ACG ACT T-3'), then the sequence of each product was obtained and used to determine the genus with BLAST. 16S rRNA sequences of different isolates from each genus were aligned to determine percent identity in order to define the number of unique bacterial strains.

CHAPTER V

CONCLUSION

Effects of CagA-mediated JNK pathway activation on epithelial tissue

Several studies in tissue culture models have demonstrated activation of JNK signaling upon infection with *H. pylori* [1,2,3], but a role for CagA in this process remains controversial [4]. Additionally, this system does not allow examination of the effects of cell polarity on JNK pathway activation, or its potential downstream effects on an epithelium. Development of our transgenic *Drosophila* model allowed us to demonstrate a genetic interaction between CagA and the JNK signaling pathway, and show that this interaction results in apoptosis and disruption of intact epithelial tissue. We also discovered that manipulation of several upstream activators of JNK signaling, including loss of neoplastic tumor suppressors or TNF/Eiger, enhances CagA-induced cell death. Our data demonstrate a novel interaction between CagA and an important host cell signaling pathway with observable effects on epithelial integrity *in vivo*.

Although our work established a genetic interaction between CagA and JNK signaling, the molecular mechanism responsible for activation of this pathway remains unclear. Our finding that CagA can activate JNK signaling through multiple upstream pathways, and its observed ability to interact promiscuously with numerous proteins suggests that multiple host cell factors could mediate activation of the JNK pathway. Our work implicates JNK signaling as an important target of CagA which should be explored during *H. pylori* pathogenesis in mammals. With the recent development of transgenic mice expressing CagA [5] and strains of *H. pylori* capable of delivering CagA into the

mouse gastric epithelium [6], it will be of interest to examine whether the JNK pathway is also activated in these murine models in a CagA-dependent manner. Despite the fact that activation of JNK signaling during *H. pylori* infection of the human stomach likely occurs through a combination of bacterial factors, our data suggest that CagA is an important mediator of this process.

Effects of host cell context on CagA's functions in an epithelium

Tissue culture models of *H. pylori* infection have allowed for identification of a multitude of host cell proteins that interact with CagA [7], but the limitations of this system have prevented examination of CagA's effects in the type of complex environment encountered during infection of the human stomach. Our transgenic *Drosophila* model demonstrates that CagA-mediated JNK pathway activation can cause different downstream effects depending on the cellular and genetic context of host cells. We show that non-uniform expression of CagA in an epithelium triggers apoptosis through activation of JNK signaling. We also demonstrate that CagA enhances the growth and invasion of tumors when coexpressed with activated Ras in a JNK-dependent manner, providing an important example of how altering host genetics can cause a dramatic shift in the function of JNK signaling from proapoptotic to protumorigenic.

Although our work has demonstrated cooperation between CagA-induced JNK pathway activation and oncogenic Ras in promoting tumor progression, the specificity of this interaction has not been explored. Given the observation that a number of tumor-promoting mutations have been identified in gastric cancer patients [8], we suspect that CagA's activation of JNK signaling in the presence of other tumorigenic mutations will also drive tumor progression. This hypothesis can be explored by examining genetic

changes in host gastric epithelial cells over the course of infection with *H. pylori*. Our finding that changes in host cell context can profoundly affect CagA's functions also merits investigation into a role for CagA in disruption of the gastrointestinal ecosystem upon infection with *H. pylori*, which has been observed in both human patients and mice [9,10]. We have begun exploring this phenomenon using our transgenic *Drosophila* model to demonstrate that CagA expression perturbs intestinal homeostasis through various mechanisms. Our work suggests that interactions between CagA and host cell proteins are dynamic and dramatically influenced by cellular and genetic changes in host cells during long-term association with *H. pylori*.

APPENDIX A

FIGURES FOR CHAPTER II

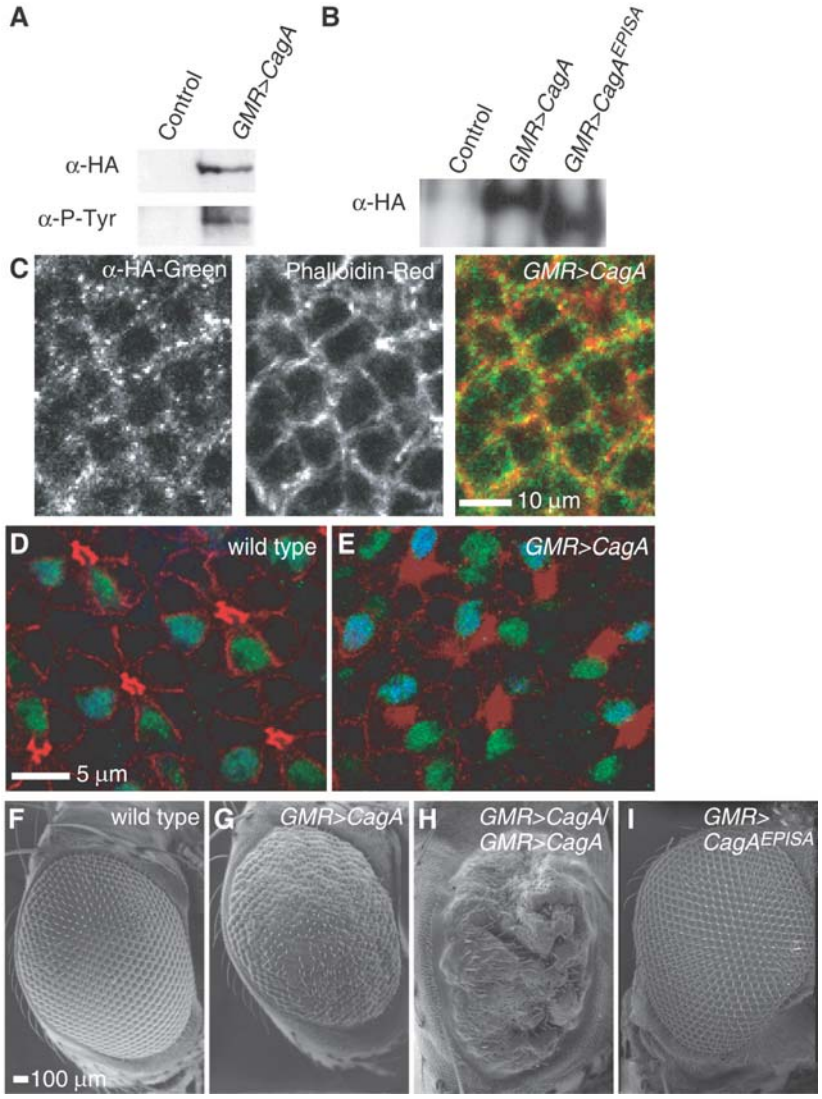


Figure 1. CagA is phosphorylated, associates with the cortex in *Drosophila* cells and disrupts eye development. (A) *UAS-CagA* was expressed in the *Drosophila* eye with *GMR-GAL4*. CagA protein (α-HA) was tyrosine phosphorylated (α-P-Tyr). Controls expressed only *GMR-GAL4*. (B) The CagA and CagA^{EPISA} proteins were expressed in the eye to similar levels. (C) CagA (α-HA, green) localized to the cortex (phalloidin, red) of cells in the larval eye disc. Wild type (D) and *UAS-CagA*/*GMR-GAL4* (E) pupal retinas were stained with MAb 24B10 (red) to outline the photoreceptors, and antibodies to visualize the R7 (green) and R8 (cyan) photoreceptors. Scanning electron microscope micrographs of adult eyes from flies with (F) one copy of *GMR-GAL4* and no *UAS* transgene, (G) one copy of *UAS-CagA*, (H) two copies of *UAS-CagA* and (I) one copy of *UAS-CagA*^{EPISA}.

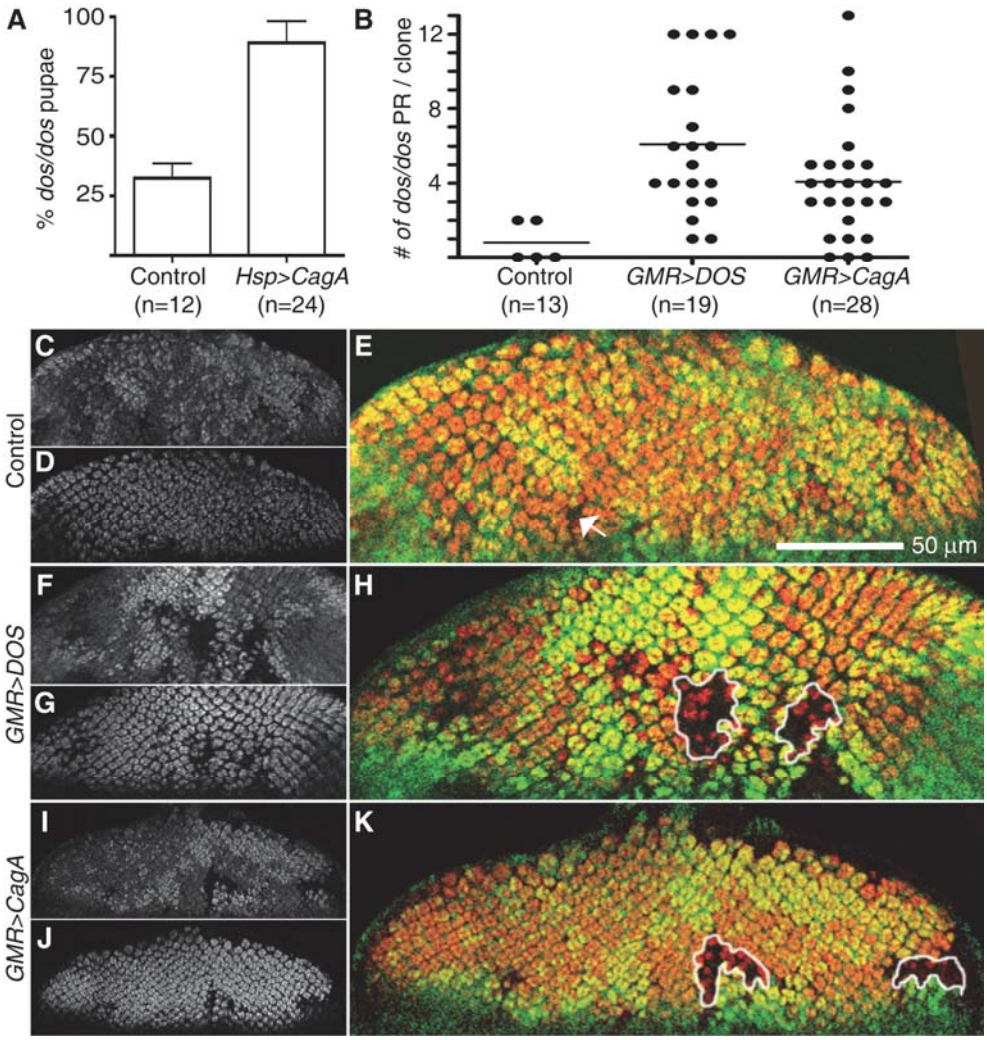


Figure 2. CagA can substitute for the *Drosophila* Gab. (A) Few homozygous *Hsp-GAL4*/+; *dos*/*dos* individuals survived to the pupal stage (control) measured as *dos*/*dos* pupae that developed as a percentage (%) of expected pupae that should develop if *dos* mutants were fully viable. Significantly more *UAS-CagA*/*Hsp-GAL4*; *dos*/*dos* pupae were recovered (Chi squared, p value<0.02). n = number experiments completed, with between 150-450 pupae examined in each experiment. Error bars indicate standard error. The *dos*/*dos* mitotic clones (marked by the absence of GFP (C, F, I, and green staining in E, H, K)), were induced using the FLP/FRT system in different genetic backgrounds, and photoreceptors were visualized with ELAV staining (D, G, J, and red staining in E, H, K). (B) The number of photoreceptors (PR) per *dos*/*dos* clones is shown for a representative experiment (n = number of eye discs examined). An area lacking GFP was counted as a clone if it was large enough to normally contain at least one ommatidium. (C–E) In control flies expressing *GMR-GAL4*, few *dos*/*dos* clones were observed. Any clones were miniscule (arrow in E) and contained few photoreceptors. Photoreceptors development was rescued by expression of either *GMR-GAL4*; *UAS-DOS* (F–H) or *GMR-GAL4*; *UAS-CagA* (I–K) as indicated by the formation of larger *dos*/*dos* clones containing several photoreceptors (clones are outlined in H and K).

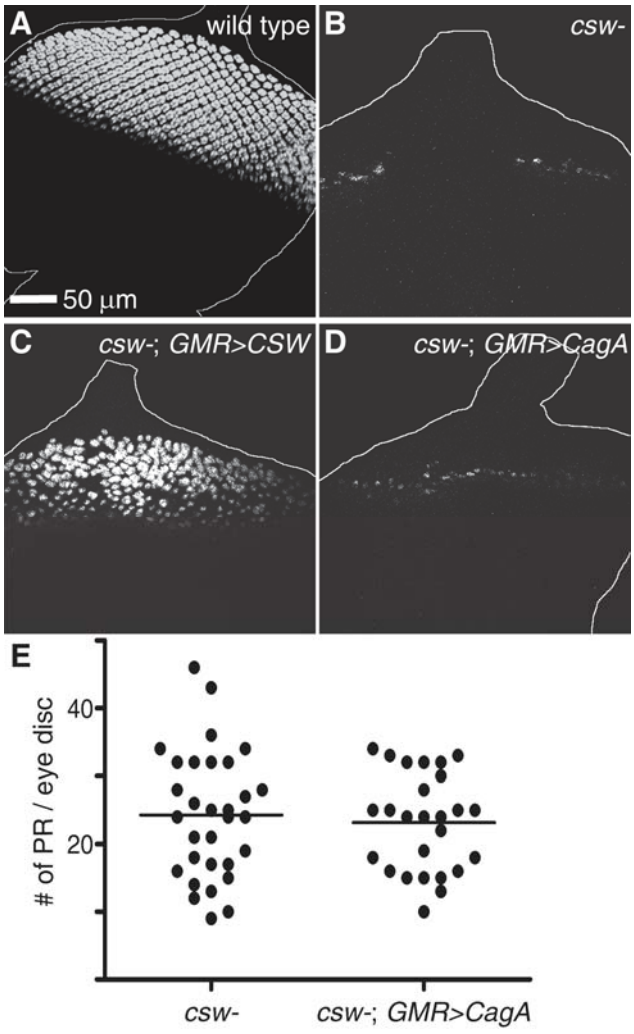
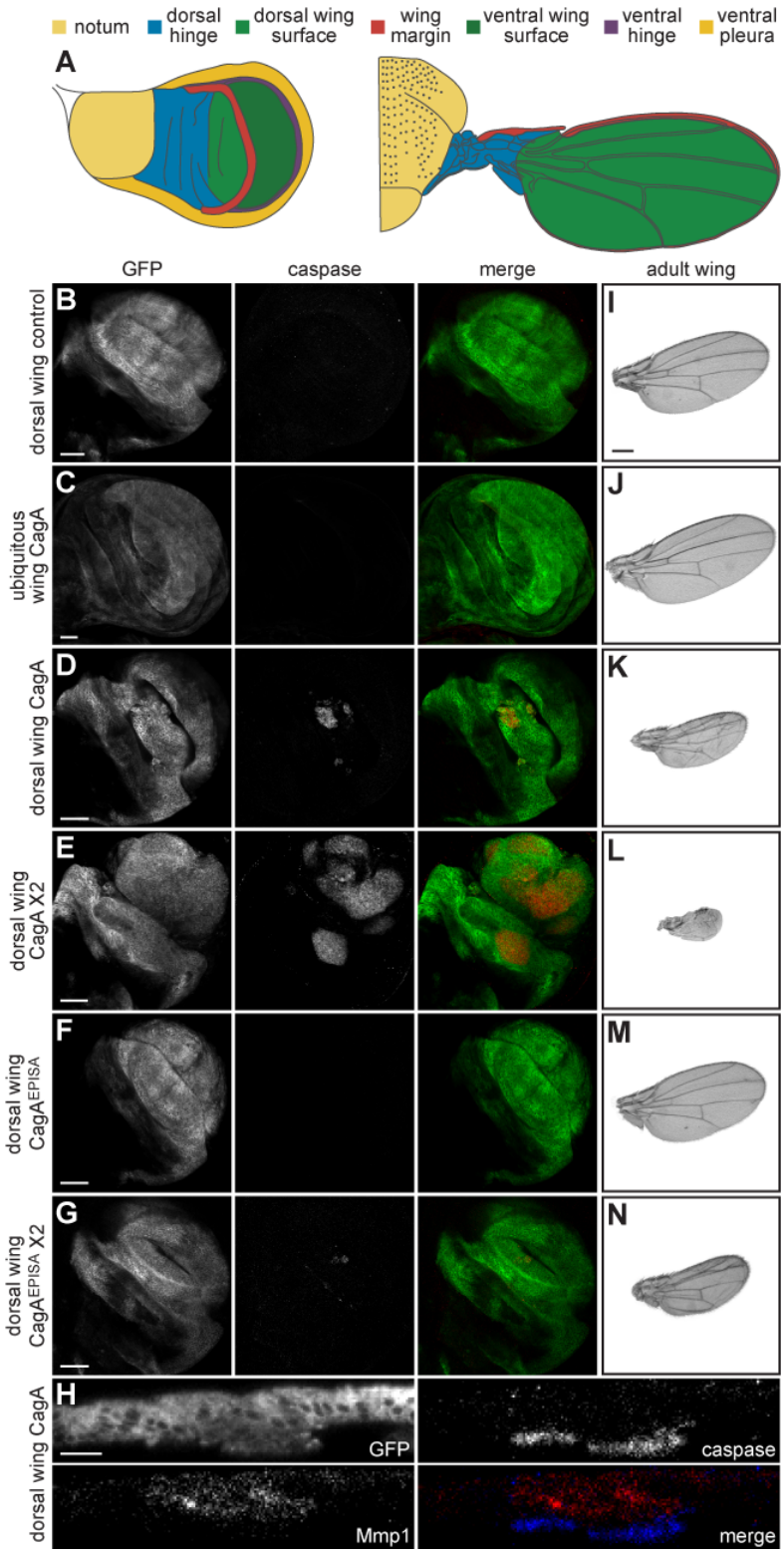


Figure 3. CagA's specification of photoreceptors requires SHP-2/CSW. (A) Wild type larval eye discs contain thousands of photoreceptors, which were visualized by anti-ELAV staining. (B) Few photoreceptors develop in *csw* mutant larval eye discs. (C) Expression of *UAS-CSW* using *GMR-GAL4* in the *csw* mutant partially rescued the lack of photoreceptor development but expression of *UAS-CagA* did not (D). (E) There was no significant difference (T-test, p value>0.2) in the number of photoreceptors (PR) that developed in *csw* mutants with or without CagA expression.

APPENDIX B

FIGURES FOR CHAPTER III

Figure 1 (next page). CagA expression causes apoptosis and epithelial disruption. (A) Schematic illustrating the fate of different domains within the wing imaginal disc. Each color-coded region of the larval structure on the left corresponds to the specified region of the adult wing on the right (modified from [24]). (B-G) Confocal cross sections of male third instar larval wing imaginal discs showing mGFP expression and stained with an antibody against active caspase-3 to mark apoptotic cells. A control wing disc epithelium expressing only mGFP with the bx-GAL4 dorsal wing driver (B) lacks apoptotic cells. Ubiquitous expression of CagA in the wing disc with the 765-GAL4 driver (C) does not cause apoptosis, while expressing CagA with bx-GAL4 (D) triggers formation of apoptotic clusters within the expression domain. Expressing two copies of CagA with bx-GAL4 (E) causes a dose-dependent enhancement of the apoptosis phenotype. Expressing $CagA^{EPISA}$ with bx-GAL4 (F) does not cause a phenotype, while expressing two copies of $CagA^{EPISA}$ (G) produces small apoptotic clusters. Scale bars, 50 μm. (H) XZ confocal plane of a male wing imaginal disc epithelium expressing mGFP and CagA with bx-GAL4 stained with antibodies against active caspase-3 to show basal extrusion of apoptotic cells and matrix metalloproteinase 1 (Mmp1) to show evidence of basement membrane breakdown. Scale bar, 20 μm. (I-N) Adult wing images from male flies of each indicated genotype. Neither expression of mGFP alone with bx-GAL4 (I) nor expression of CagA with 765-GAL4 (J) causes a phenotype in the adult wing. Dorsal wing expression of CagA with bx-GAL4 (K) disrupts epithelial integrity in a dose-dependent manner (L). Expressing $CagA^{EPISA}$ with bx-GAL4 (M) does not cause an adult wing phenotype, while expressing two copies of $CagA^{EPISA}$ (N) causes epithelial disruption. Scale bar, 500 μm.


notum
dorsal hinge
dorsal wing surface
wing margin
ventral wing surface
ventral hinge
ventral pleura
A
GFP
caspase
merge
adult wing
dorsal wing control
ubiquitous wing CagA
dorsal wing CagA
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dorsal wing CagA EPISA
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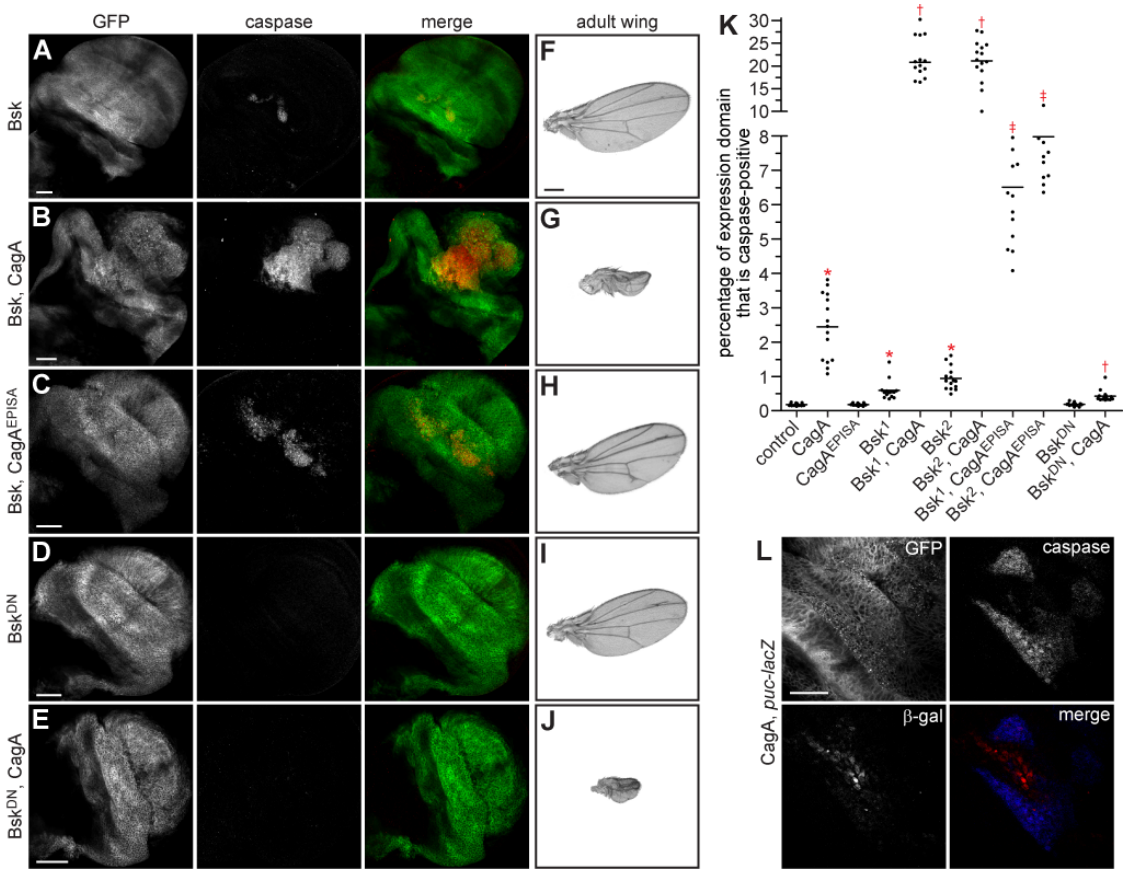
GFP
caspase
Mmp1
merge

Figure 2 (next page). CagA-induced apoptosis occurs through JNK pathway activation. (A-E) Confocal cross sections of male third instar larval wing imaginal discs showing mGFP expression with bx-GAL4 and stained with an anti-active caspase-3 antibody to mark apoptotic cells. Ectopic overexpression of wild type Bsk in the dorsal wing disc (A) causes a mild apoptosis phenotype that is strongly enhanced by coexpression with CagA (B). Coexpression of Bsk with $CagA^{EPISA}$ (C) also enhances the apoptosis phenotype. Expression of Bsk^{DN} alone (D) does not cause apoptosis, and coexpression with CagA (E) strongly suppresses apoptosis induced by CagA expression. Scale bars, 50 μm. (F-J) Adult wing images from male flies expressing different forms of Bsk alone or in combination with CagA. Ectopic overexpression of Bsk with bx-GAL4 (F) causes only subtle vein defects in the adult wing, while coexpression with CagA (G) enhances epithelial disruption. Coexpression of Bsk with $CagA^{EPISA}$ (H) does not significantly affect formation of the adult wing structure. Expression of Bsk^{DN} with bx-GAL4 (I) also causes only subtle vein defects in the adult wing, while coexpression with CagA (J) enhances epithelial disruption. Scale bar, 500 μm. (K) Quantitation of apoptosis as a percentage of the expression domain showing active caspase-3 staining, n = 15 wing discs per genotype; bar indicates average value for each group. * indicates values that differ significantly from the control with expression of a single transgene; † indicates values that show significant enhancement or suppression compared to CagA; ‡ indicates values that show significant enhancement compared to $CagA^{EPISA}$; $p < 0.0001$. (L) Confocal cross section of a male wing imaginal disc epithelium carrying the *puc-lacZ* reporter allele and expressing mGFP and CagA with bx-GAL4. Staining with antibodies against active caspase-3 and β-galactosidase (β-gal) shows that apoptotic cells lie adjacent to those in which JNK signaling has been activated. Scale bar, 50 μm.


GFP
caspase
merge
adult wing
A
B
C
D
E
F
G
H
I
J
Bsk
Bsk, CagA
Bsk, CagA^EPISA
Bsk^DN
Bsk^DN, CagA
K
percentage of expression domain that is caspase-positive
control
CagA
CagA^EPISA
Bsk^1
Bsk^1, CagA
Bsk^2
Bsk^2, CagA
Bsk^1, CagA^EPISA
Bsk^2, CagA^EPISA
Bsk^DN
Bsk^DN, CagA
L
GFP
caspase
CagA, puc-lacZ
β-gal
merge

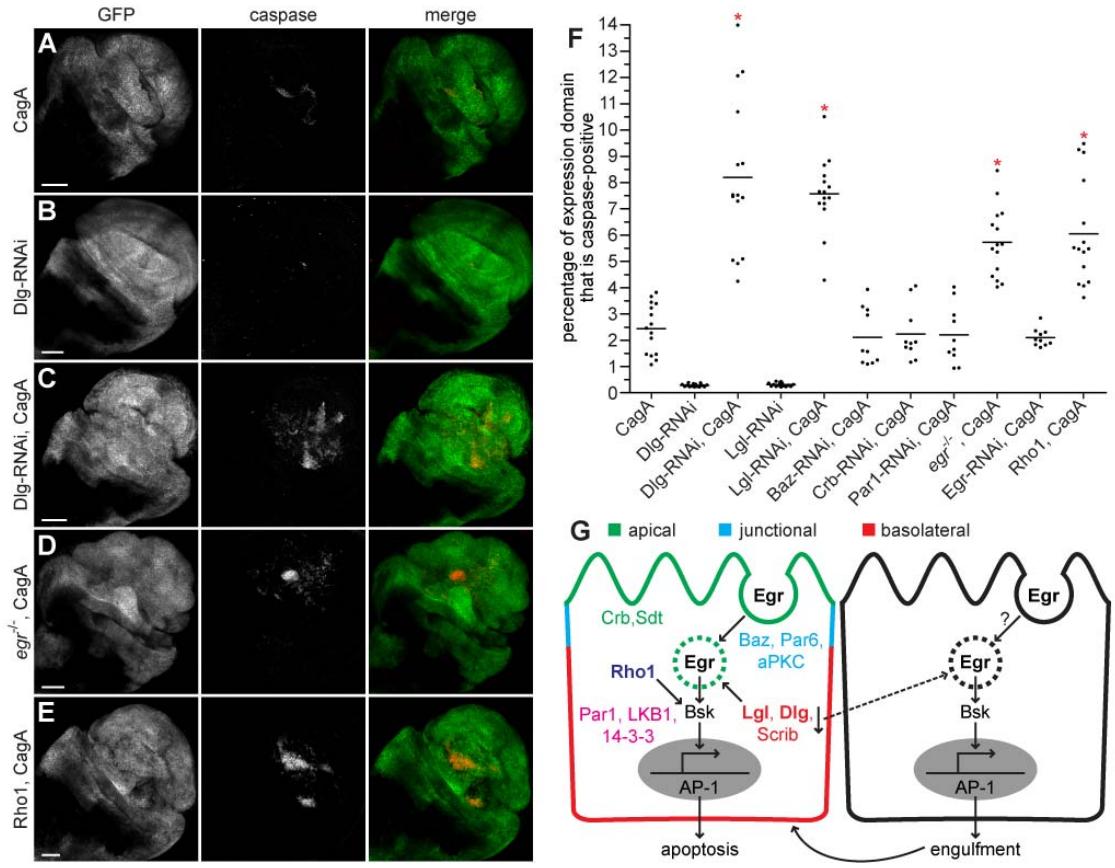


Figure 3. CagA genetically interacts with nTSGs, Eiger and Rho1. (A-E) Confocal cross sections of male third instar larval wing imaginal discs showing mGFP expression with bx-GAL4 and stained with anti-active caspase-3 antibody to mark apoptotic cells. Dorsal wing expression of CagA with bx-GAL4 causes formation of apoptotic clusters (A). RNAi-mediated knockdown of the nTSG Dlg alone (B) does not cause significant apoptosis, but enhances apoptosis induced by CagA expression (C). The apoptosis phenotype is enhanced when CagA is expressed in an *egr* mutant background (D). Coexpression of Rho1 with CagA (E) also enhances apoptosis. Scale bars, 50 µm. (F) Quantitation of apoptosis as a percentage of the expression domain showing active caspase-3 staining, n = 10 or 15 wing discs per genotype; bar indicates average value for each group. * indicates values that show significant enhancement compared to CagA, whose quantitation (from Figure 2) is provided for comparison; $p < 0.0001$. (G) A model showing the localization of polarity protein complexes in an epithelial cell, their known interactions with other upstream activators of JNK signaling in *Drosophila*, and the downstream effects of these interactions.

Figure 4 (next page). CagA enhances tumor growth through JNK activation. (A-B) Schematics depicting GFP-marked whole eye clones in third instar larvae (A) and a dissected cephalic complex (B), which includes the eye discs (ed), brain lobes (bl) and ventral nerve cord (vnc). (C-G) Images of female third instar larvae and dissected cephalic complexes with GFP-marked tumors. Expression of Ras^{V12} in whole eye clones (C) causes overgrowth which results in tumor formation. Coexpression of CagA with Ras^{V12} (D) markedly enhances the size of tumors, while coexpression of $CagA^{EPISA}$ with Ras^{V12} (E) causes only a minor enhancement of tumor growth. Whole eye clone expression of Bsk^{DN} with Ras^{V12} (F) does not significantly alter tumor size, while coexpression of Bsk^{DN} with Ras^{V12} and CagA (G) suppresses the growth advantage conferred by CagA expression. Scale bar for whole larvae images, 1 mm; scale bar for dissected cephalic complex images, 250 μm. (H) Quantitation of cephalic complex size as a measure of area in μm^2, n = at least 30 cephalic complexes per genotype; bar indicates average value for each group. * indicates significant enhancement compared to Ras^{V12}; † indicates significant suppression compared to Ras^{V12}, CagA; $p < 0.05$.

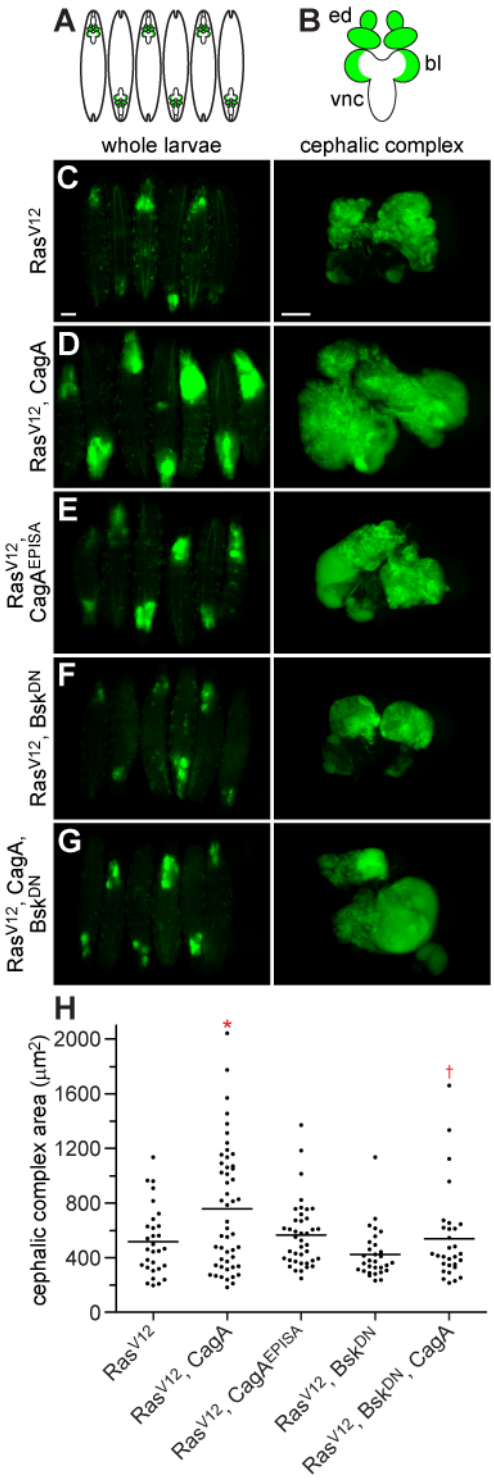
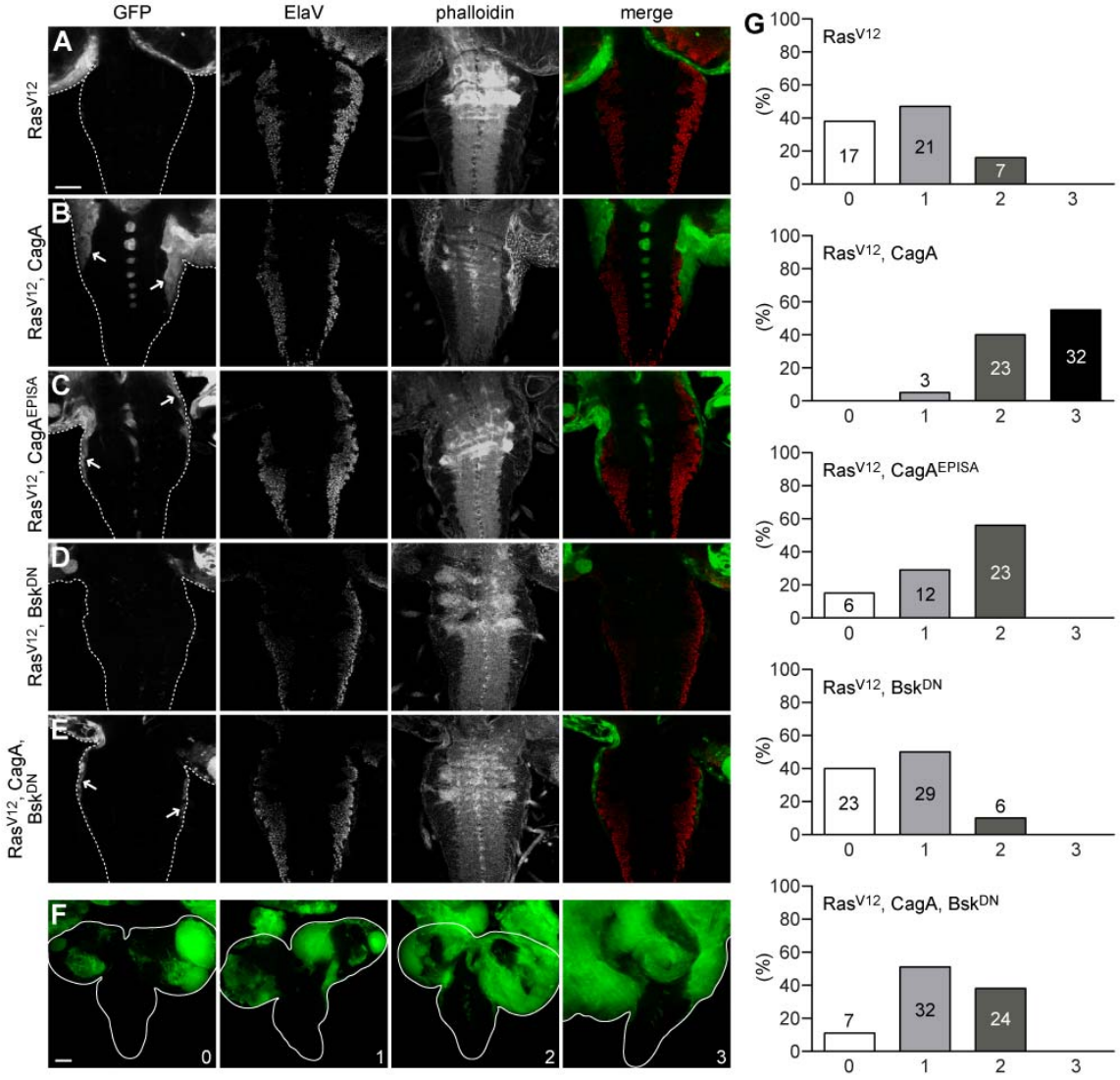

A
B
ed
bl
vnc
whole larvae
cephalic complex
C
RasV12
D
RasV12, CagA
E
RasV12, CagAEPISA
F
RasV12, BskDN
G
RasV12, CagA, BskDN
H
cephalic complex area (µm2)
2000
1600
1200
800
400
0
*
†
RasV12
RasV12, CagA
RasV12, CagAEPISA
RasV12, BskDN
RasV12, BskDN, CagA

Figure 5 (next page). CagA enhances tumor invasion through JNK activation. (A-E) Confocal cross sections of cephalic complexes from third instar larvae with GFP-marked tumors stained with an antibody against ElaV to mark terminally differentiated cells and phalloidin to reveal f-actin structure. VNCs are outlined in panels showing GFP expression, and arrows highlight invading tumor tissue. Expressing Ras^{V12} alone in whole eye clones (A) causes a mild invasive phenotype characterized by either no invasion or migration of tumor cells from one optic lobe. Coexpression of CagA with Ras^{V12} (B) dramatically enhances the extent of VNC invasion from both optic lobes, while coexpression of $CagA^{EPISA}$ with Ras^{V12} (C) shows a milder enhancement of invasion. Coexpression of Bsk^{DN} with Ras^{V12} (D) does not significantly affect the invasive capacity of tumor cells, while coexpression of Bsk^{DN} with Ras^{V12} and CagA (E) suppresses the VNC invasion phenotype. Scale bar, 50 μm. (F) Projections of several confocal cross sections from third instar larval cephalic complexes with GFP-marked tumors showing different classes of invasiveness: (0) noninvasive, (1) invasion from one optic lobe, (2) invasion from both optic lobes, (3) significant invasion of the VNC. Brain lobes and ventral nerve cords are outlined. Scale bar, 50 μm. (G) Histograms showing the percentage of cephalic complexes classified into each category, and indicating the number of samples analyzed in each column.


GFP
ElaV
phalloidin
merge
A
RasV12
B
RasV12, CagA
C
RasV12, CagAEPISA
D
RasV12, BskDN
E
RasV12, CagA, BskDN
F
0
1
2
3
G
RasV12
17
21
7
RasV12, CagA
3
23
32
RasV12, CagAEPISA
6
12
23
RasV12, BskDN
23
29
6
RasV12, CagA, BskDN
7
32
24
(%)
0
20
40
60
80
100

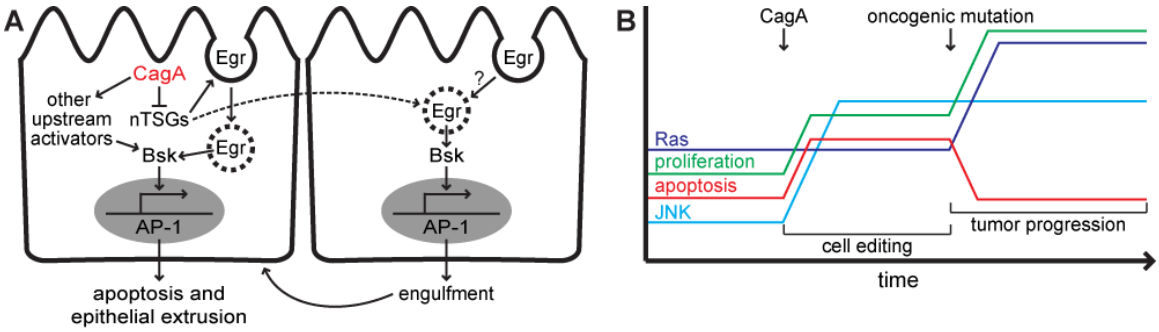


Figure 6. Models illustrating short-term effects of CagA on an epithelium and long-term effects resulting from a change in host genetic background. (A) Once inside the host epithelial cell, CagA effector protein downregulates the neoplastic tumor suppressors (nTSGs) which induces endocytic activation of the TNF homolog Eiger (Egr) leading to activation of JNK (Bsk). CagA also triggers Egr-dependent JNK pathway activation in neighboring wild type cells. In the absence of this pathway, CagA activates JNK signaling through other upstream pathway components including the small GTPase Rho1. In a wild type host genetic background, CagA-mediated JNK pathway activation causes apoptosis and subsequent extrusion from the epithelium, or engulfment by neighboring cells. (B) Introduction of CagA into host cells causes upregulation of JNK signaling which triggers apoptosis and compensatory proliferation within the epithelium as part of the cell editing process. When the host genetic background is perturbed by expression of an oncogenic mutation which blocks apoptosis, CagA-mediated JNK pathway activation drives tumor progression.

APPENDIX C

FIGURES FOR CHAPTER IV

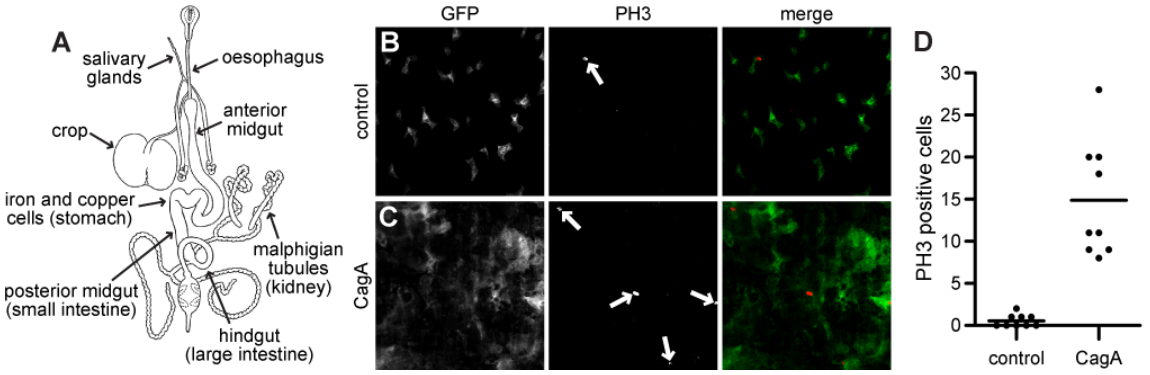


Figure 1. CagA expression enhances intestinal stem cell proliferation. (A) Diagram illustrating the *Drosophila* gastrointestinal tract (modified from [19]), including components with proposed homology to the human digestive system (B) Adult intestinal epithelia expressing GFP alone (control) with esg-GAL4 contained small and distinct stem cells distributed throughout the tissue, and few proliferating cells which were visualized with anti-phospho-Histone H3 (PH3) staining (marked by arrows). (C) Expression of CagA caused a change in esg^{+} cell morphology characterized by an apparent increase in both the size and number of cells, and also enhanced proliferation in the epithelium. (D) Quantitation of PH3 positive cells in the adult midgut showed a significant ($p < 0.0001$) increase in proliferating cells upon expression of CagA with esg-GAL4.

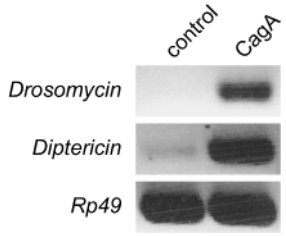


Figure 2. CagA expression increases antimicrobial peptide production in the intestinal epithelium. Adult intestines expressing CagA in stem cells using esg-GAL4 showed enhanced expression of the antimicrobial peptides *Drosomycin* and *Diptericin* compared to expressing GFP alone (control), demonstrated by RT-PCR analysis. Expression levels of the ribosomal protein *Rp49* did not change upon expression of CagA with esg-GAL4.

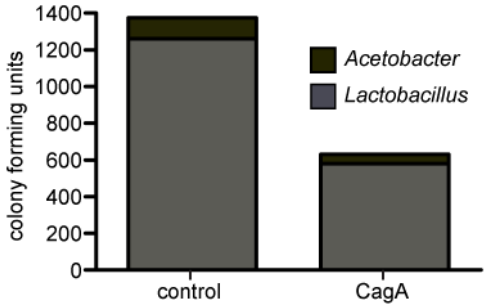


Figure 3. CagA expression alters the intestinal bacterial community. (A) The microbiota of adult intestines expressing CagA in stem cells using esg-GAL4 was different from those expressing GFP alone (control). CagA-expressing intestines showed a marked reduction in the overall abundance of bacteria, while the relative proportion of both the *Lactobacillus* and *Acetobacter* genera was similar to the control intestines.

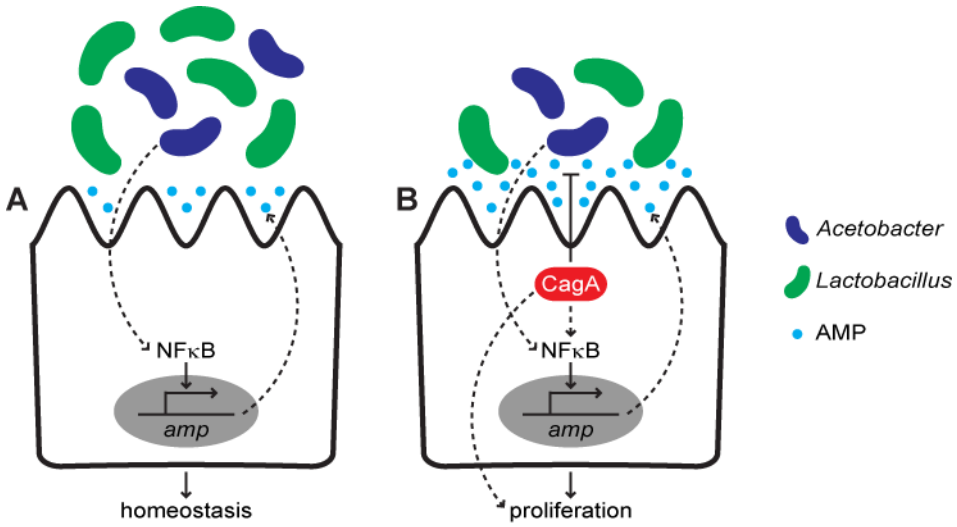


Figure 4. A Model illustrating the effects of CagA on the gastrointestinal ecosystem. (A) Wild type intestinal epithelial cells are stimulated by the microbiota to produce low levels of AMPs and induce sufficient proliferation to maintain homeostasis. (B) CagA expression in ISCs triggers a proliferative response in the intestinal epithelium, enhances AMP production and causes a reduction in the abundance of intestinal bacteria.

APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER III

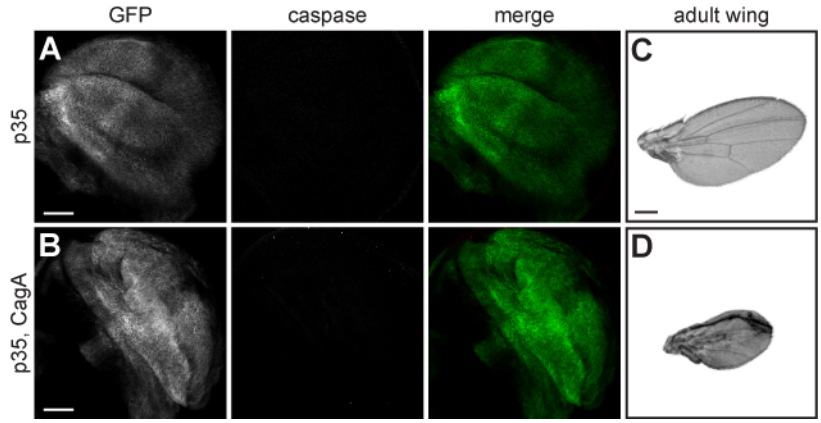
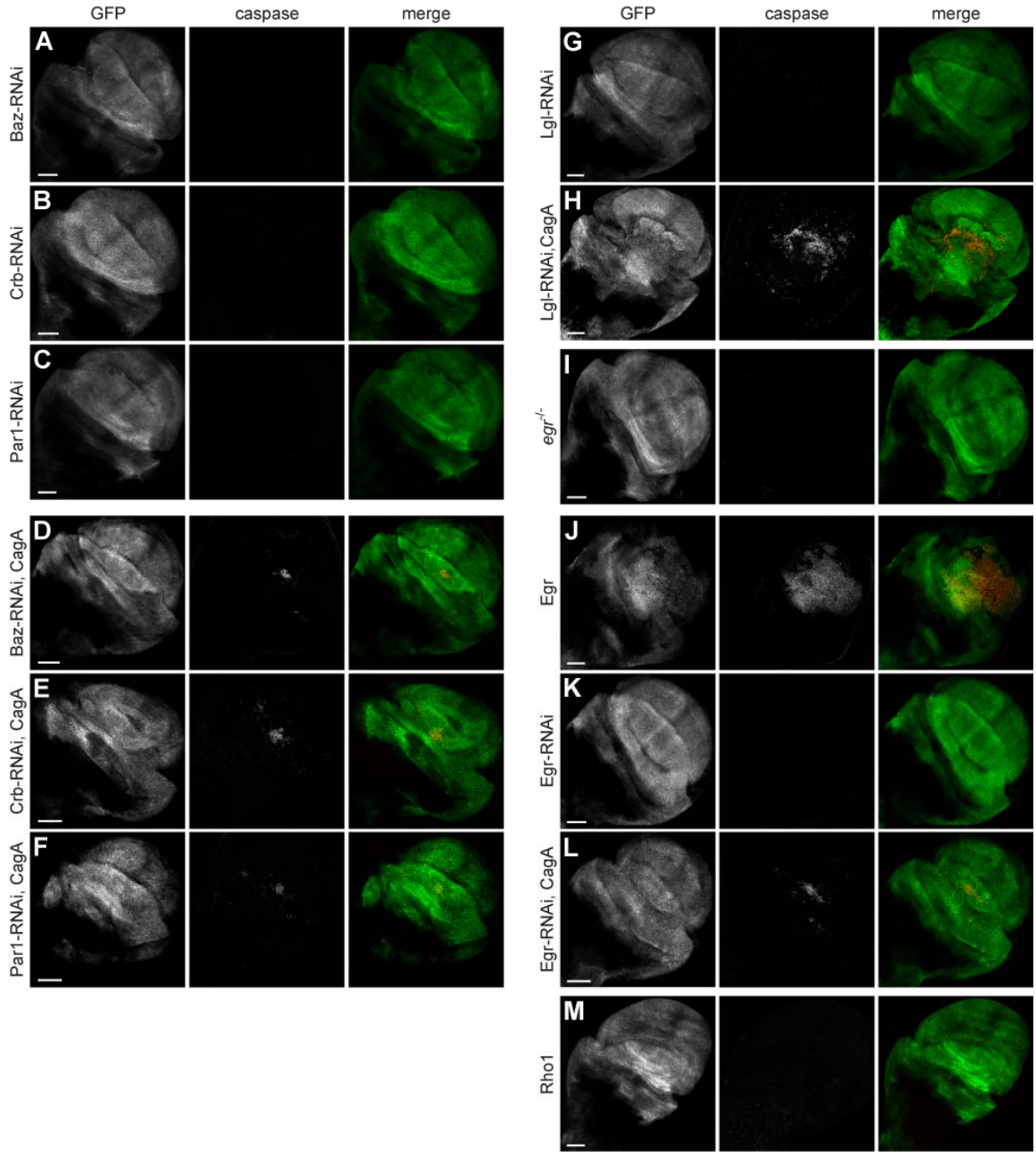


Figure S1. Apoptosis inhibition enhances CagA-dependent epithelial disruption. (A-B) Confocal cross sections of male third instar larval wing imaginal discs showing mGFP expression with bx-GAL4 and stained with anti-active caspase-3 antibody to mark apoptotic cells. Ectopic overexpression of p35 in the dorsal wing disc (A) does not cause a phenotype, and coexpression with CagA suppresses the apoptosis normally caused by CagA expression (B). Scale bars, 50 μm. (C-D) Adult wing images from male flies expressing the apoptosis inhibitor p35 alone or in combination with CagA. Ectopic expression of p35 with bx-GAL4 (C) does not cause a phenotype, while coexpression with CagA (D) enhances epithelial disruption. Scale bar, 500 μm.

Figure S2 (next page). Manipulation of specific polarity determinants and upstream activators of JNK signaling enhances CagA-induced apoptosis. (A-M) Confocal cross sections of male third instar larval wing imaginal discs showing mGFP expression with bx-GAL4 and stained with anti-active caspase-3 antibody to mark apoptotic cells. RNAi-mediated knockdown of polarity determinants Baz (A), Crb (B) or Par1 (C) alone in the dorsal wing does not induce apoptosis. Coexpression of CagA with knockdown of Baz (D), Crb (E) or Par1 (F) does not enhance the apoptosis phenotype. Knockdown of the neoplastic tumor suppressor Lgl alone (G) also does not cause significant apoptosis, but when combined with CagA expression (H) markedly enhances apoptosis. Wing imaginal discs of *egr* mutant animals do not exhibit apoptosis (I). Ectopic expression of Egr alone in the dorsal wing causes a significant apoptosis phenotype (J). RNAi-mediated knockdown of Egr alone does not cause apoptosis (K), and does not enhance the apoptosis phenotype when combined with CagA expression (L). Ectopic expression in the dorsal wing of the small GTPase Rho1 alone does not cause apoptosis (M). Scale bars, 50 μm.


GFP
caspase
merge
A
Baz-RNAi
B
Crb-RNAi
C
Par1-RNAi
D
Baz-RNAi, CagA
E
Crb-RNAi, CagA
F
Par1-RNAi, CagA
G
Lgl-RNAi
H
Lgl-RNAi,CagA
I
egr-/-
J
Egr
K
Egr-RNAi
L
Egr-RNAi, CagA
M
Rho1

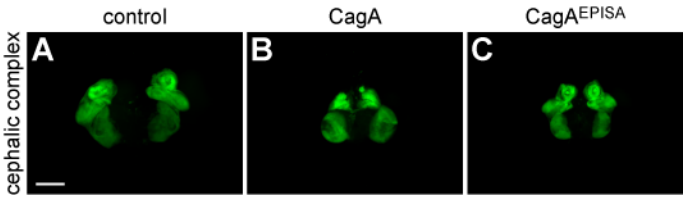


Figure S3. Expression of CagA alone does not induce cephalic complex overgrowth. (A-C) Images of dissected cephalic complexes with GFP-marked tumors. Expression of GFP alone (A), with CagA (B) or with $CagA^{EPISA}$ (C) in whole eye clones does not cause overgrowth or result in tumor formation. Scale bar, 250 μm.

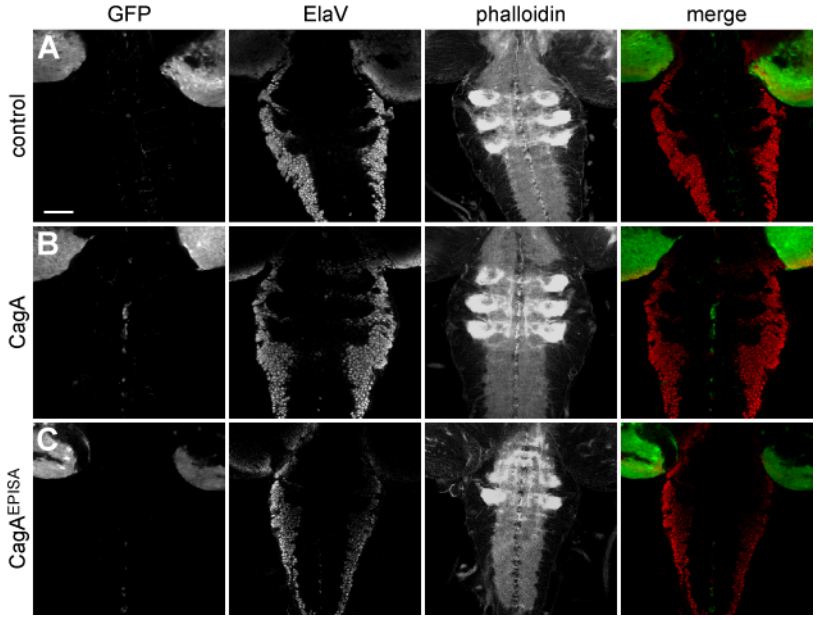


Figure S4. Expression of CagA alone does not induce ventral nerve cord invasion. (A-C) Confocal cross sections of cephalic complexes from third instar larvae with GFP-marked tumors stained with an antibody against ElaV to mark terminally differentiated cells and phalloidin to reveal f-actin structure. Expression of GFP alone (A), with CagA (B) or with $CagA^{EPISA}$ (C) in whole eye clones does not cause an invasive phenotype. Scale bar, 50 μm.

	control		CagA	
	apoptosis	**epithelial disruption**	**apoptosis**	**epithelial disruption**
Scrib-RNAi	severe	severe	n/a	n/a
Dlg-RNAi	none	mild	enhanced	enhanced
Lgl-RNAi	none	mild	enhanced	enhanced
Crb-RNAi	none	mild	not enhanced	not enhanced
Patj-RNAi	none	mild	not enhanced	enhanced
Cora-RNAi	moderate	mild	n/a	n/a
Par1-RNAi	none	none	not enhanced	not enhanced
Baz-RNAi	none	none	not enhanced	enhanced
Par6-RNAi	severe	severe	n/a	n/a
aPKC-RNAi	severe	severe	n/a	n/a
Cdc42^{DN}	severe	severe	n/a	n/a
Mir-RNAi	none	none	not enhanced	enhanced

Table S1. Knockdown of specific polarity determinants in the wing causes apoptosis and epithelial disruption, and enhances CagA-dependent phenotypes. Expression of each polarity determinant was subject to knockdown using the bx-GAL4 driver. Effects on both apoptosis in the wing imaginal disc and epithelial disruption in the adult wing were determined. Those proteins whose knockdown alone did not produce a significant phenotype were tested for their ability to enhance or suppress CagA-dependent phenotypes in the larval and adult wing.

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