BACTERIAL STIMULATION OF INTESTINAL PROLIFERATION VIA THE WNT PATHWAY IN ZEBRAFISH

by

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DISSERTATION ABSTRACT

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This dissertation describes research into microbial influences on host signaling in the zebrafish intestine. Vertebrate organisms are consistently exposed to microbes, especially on epithelial tissues that are exposed to the environment, such as the skin and the gastrointestinal tract. The close association between these tissues and microbes over time has resulted in a symbiotic state, whereby microorganisms have gained the ability to utilize vertebrate epithelia as a niche for replication and the acquisition of nutrients. These associations run the gamut from beneficial to exceedingly pathogenic and often involve complex bidirectional signaling between microbe and host. Microbial signals can interact with host cell pathways involved in a wide range of cellular processes. Here, we describe our investigations into one such pathway, the Wnt signaling pathway, and how microbial activation of Wnt signaling can translate into alterations in cell proliferation and homeostasis in the intestinal epithelium of the teleost fish *Danio rerio*. We report that epithelial cell proliferation in the developing zebrafish intestine is stimulated both by the presence of the resident microbiota and by activation of Wnt signaling and demonstrate that resident intestinal bacteria enhance the stability of β-catenin in intestinal epithelial cells, promoting cell proliferation in the developing vertebrate intestine. We also describe how transgenic expression of the bacterial effector protein CagA from the human gastric

pathogen *Helicobacter pylori* is capable of causing significant overproliferation of the intestinal epithelium and adult intestinal hyperplasia, as well as significant upregulation of the Wnt target genes *cyclinD1* and the zebrafish *c-myc* ortholog *myca*. We show that co-expression of CagA with a mutant allele of the β-catenin destruction complex protein Axin1 resulted in a further increase in intestinal proliferation, while co-expression of CagA with a null allele of the essential β-catenin transcriptional cofactor Tcf4 restored intestinal proliferation to wild-type levels. These results suggest that CagA activates canonical Wnt signaling downstream of the β-catenin destruction complex and upstream of Tcf4. Our studies provide *in vivo* evidence of Wnt pathway activation by CagA and implicate this activation in CagA-induced epithelial overproliferation, an early step in gastrointestinal cancer development.

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

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CHAPTER I

INTRODUCTION

HEALTH AND THE GASTROINTESTINAL MICROBIOTA

Multicellular organisms have coevolved with microbes, and this co-evolution has resulted in the assembly of specific microbial communities within a host, collectively known as the microbiota. Evidence for this co-evolution can be found in the observation that species within the microbiota exhibit a large degree of host adaptivity (1), and also in the observation that microbial communities transplanted from one species to another will be remodeled to resemble the resident microbiota in the transplant recipient (2). As such, the host has developed complicated mechanisms to control the resident microbiota without the concomitant activation of potentially damaging immune responses (3). The vertebrate gastrointestinal tract represents one of the largest niches available for microbial colonization, and becomes robustly colonized shortly after birth. This colonization by the gastrointestinal microbiota is important for the health of the host, and has been linked to several discrete benefits.

The gastrointestinal microbiota serves as a first line of defense against colonization of the intestine by pathogenic microbes. This function is carried out via several mechanisms, including competitive exclusion through absorption of available nutrients and physical occupation of host attachment sites, production of anti-microbial compounds, and by stimulating the host to activate anti-microbial defenses. Additionally, the gastrointestinal microbiota plays a central role in host nutrition, acting to increase nutrient the availability of otherwise indigestible material (4, 5), and by interaction with hormonal pathways

associated with regulation of host energy balance (6). Finally, one of the most interesting of benefits conferred by microbial colonization of the gastrointestinal niche is the signaling between host and microbes to promote proper patterning of the host tissues. The presence of enteric microbes has been shown to be associated with proper spleen and lymph node development (7), as well as with the cell-type census of the intestine itself (8, 9). The effect of the microbial community on intestinal development is of particular relevance to this dissertation, and will be further examined in Chapter II; this chapter contains work co-authored with S. Cheesman, E. Mittge, B. Seredick, and K. Guillemin.

THE GASTROINTESTINAL MICROBIOTA AND DISEASE

Given the variety of roles played by the gastrointestinal microbiota in host development and health, it is unsurprising that disruption of the carefully evolved balance between microbiota and host could be detrimental to both parties. Such an effect can be clearly seen in the case of diarrhea resulting from overgrowth of the normal microbiota member *Clostridium difficile* following perturbation of the normal microbiota with antibiotics (10). Additionally, monoassociation of model organisms with individual microbial species can result in abnormal development and homeostasis of the GI tract, a phenomenon that will be explored further in Chapter II.

It was long believed that despite the rich microbial diversity within the human gastrointestinal tract, the stomach, with its highly acidic lumen, would be free of bacteria. It wasn't until the early 1980's that observations by the gastroenterologists Barry Marshall and Robin Warren identified the first known gastric bacterium, *Helicobacter pylori*, in the stomachs of ulcer patients (11). Since then, *Helicobacter pylori* has been discovered in the

stomachs of nearly half the world's population, has been linked to gastritis, peptic ulcer disease, and gastric cancer, and has been designated a known carcinogenic agent by the World Health Organization, the only bacterium to be so designated. Still, the vast majority of persons colonized by *H. pylori* develop no disease, and gastric cancer rates among *H. pylori*-infected persons are around 1% (12). The mechanisms that underlie this disparity have yet to be fully elucidated, but a combination of bacterial and host-derived factors are likely to be involved. Investigations into the molecular mechanisms of *H. pylori* pathogenesis will be described in Chapter III; this chapter contains work co-authored by T. Peterson, M. Kent, and K. Guillemin.

THE ZEBRAFISH INTESTINE

The teleost fish *Danio rerio* (zebrafish) has several advantages that make it useful for the modeling of host-microbe interactions. First, zebrafish develop rapidly, with a fully functional intestine that allows feeding as early as 5 days post-fertilization (13, 14). Second, zebrafish are transparent throughout early development, allowing observation of the intestine and its associated microbiota as it develops (15). Third, the zebrafish intestine is quite similar to the vertebrate intestine in morphology, organization, and self-renewal. Lastly, due to the fact that zebrafish undergo early development in a sterile environment within the chorion, they can be reared germ-free with relative ease (16). Chapters II and III will describe the use of zebrafish as a model organism for the study of both beneficial and pathogenic interactions between microbes and hosts. Finally, Chapter IV will provide a discussion of some of the implications suggested by my dissertation research.

CHAPTER II

EPITHELIAL CELL PROLIFERATION IN THE DEVELOPING ZEBRAFISH INTESTINE IS REGULATED BY THE WNT PATHWAY AND MICROBIAL SIGNALING VIA MYD88

The work in this chapter was previously published in Volume 108 of PNAS in October 2010. It was co-authored by S. Cheesman, E. Mittge, B. Seredick, K. Guillemin, and myself. S. Cheesman, K. Guillemin, and myself designed research. S. Cheesman, E. Mittge, B. Seredick, and myself performed research. S. Cheesman, E. Mittge, B. Seredick, K. Guillemin, and myself analyzed data. S. Cheesman and K. Guillemin wrote the paper.

INTRODUCTION

The vertebrate intestinal epithelium is one of the most rapidly renewing tissues in the body. Perturbation of normal tissue homeostasis attributable to genetic lesions or environmental insults, such as infection with bacterial pathogens, can lead to hyperproliferative diseases of the intestinal tract (1). The regulation of intestinal epithelial cell proliferation has been studied extensively in the context of colorectal cancer (CRC), which has revealed canonical Wnt signaling as a key regulator of cell division and differentiation (2). Another contributing factor to rates of intestinal epithelial cell proliferation is the associated microbial community, as indicated by the paucity of proliferating cells in the intestines of germ-free (GF) rodents and zebrafish (3–5). However, the mechanisms underlying microbiota-induced cell proliferation are poorly understood.

Evidence for the role of Wnt signaling in intestinal homeostasis comes from mutations that perturb this genetic pathway. Canonical Wnt signaling is modulated by the levels of β-catenin protein; when abundant, β-catenin protein accumulates in the cytoplasm and translocates into the nucleus, where it interacts with co-activators, such as the intestine-specific transcription factor Tcf4, to turn on transcription of pro-proliferative target genes, including *c-myc* and *sox9* (6, 7). In the absence of endogenous Wnt ligands, β-catenin levels are kept low by the activity of the cytoplasmic destruction complex, composed of Apc, Axin, and GSK-3, which target β-catenin for destruction by the proteosome. Constitutive activation of Wnt signaling, such as in the case of genetic loss of the β-catenin destruction complex, results in unchecked intestinal cell proliferation. This is seen in $apc^{Min/+}$ mutant mice in which clonal loss of heterozygosity of the WT *apc* gene results in adenoma formation (8). These animals display a similar phenotype to human patients with familial adenomatous polyposis coli, who develop thousands of colonic polyps as a result of clonal loss of APC function. Conversely, when Wnt signaling is attenuated in transgenic adult mice overexpressing the Wnt receptor inhibitor Dkk-1 (9, 10) or in neonates lacking Tcf4 (7, 11), the small intestine is depleted of proliferating cells that normally replenish the intestinal epithelium.

Similar analyses in zebrafish have shown that Wnt signaling regulates cell proliferation in the adult zebrafish intestine; however its function in the larval intestine during the period of establishment of the gut microbiota has not been determined (12). Zebrafish heterozygous for the apc^{mrc} mutation, which contains a premature stop codon in the *apc* gene, spontaneously develop intestinal neoplasia as adults (13), but $apc^{mrc/mrc}$ homozygotes die before 96 h post-fertilization (hpf), before maturation of the larval gut,

which begins to function in nutrient uptake at 5 d post-fertilization (dpf) (14, 15). Conversely, zebrafish homozygous for the null mutation *tcf4*^{exl} exhibit a loss of proliferative compartments within the intestinal epithelium, but this defect is only reported to be apparent in young adult zebrafish at 5 wk of age (16). An earlier role for *tcf4* in the intestine is suggested by the finding that removal of *tcf4* in the *apc*$^{mrc/mrc}$ mutant rescues expression of the intestinal marker *ifabp* at 72 hpf but not the early larval lethality (17). Collectively, these results demonstrate that appropriate levels of Wnt signaling are crucial for the maintenance of intestinal epithelial renewal in the adult intestine, but they do not explain how intestinal epithelial renewal rates are established during larval development. This is a crucial period in zebrafish development, analogous to the postnatal period in mammals, when the digestive tract is first colonized by microbes that influence the organ's maturation (18).

One mechanism by which animals perceive the presence of microbes is through the innate immune Toll-like receptor (TLR) signaling pathway (19). TLRs were initially studied for their role in perceiving pathogens and activating host protective inflammatory responses. However, there is growing evidence for the critical role that TLR signaling plays in host perception of indigenous beneficial microbes (20), which typically do not elicit a strong inflammatory response. Myd88 functions as a key adaptor protein downstream of the majority of TLRs; when perturbed, it interferes with TLR signaling in mammals. The zebrafish genome has duplicated *tlr* genes but only a single copy of *myd88* (21, 22). We and others have shown that Myd88 functions in zebrafish to modulate innate immune responses to microbes and microbial-associated molecular patterns (MAMPs), such as LPS (23–25). Notably, LPS sensing in zebrafish differs mechanistically from that in mammals

and does not involve a Tlr4–MD2 complex (26).

Possible combinatorial effects of microbial and Wnt signaling on intestinal epithelial homeostasis are suggested by the observation that *apc*$^{Min/+}$ mice develop 50% fewer small intestinal adenomas when reared GF than when reared under conventional conditions (27). Similarly, deletion of Myd88 in *apc*$^{Min/+}$ mice results in fewer adenomas than in *apc*$^{Min/+}$ controls (28). We set out to investigate how the microbiota and Wnt signaling affect proliferation of the developing vertebrate intestinal epithelium. We used a gnotobiotic zebrafish model, which allowed us to manipulate readily both the presence and composition of the microbiota and the genetic makeup of the host. The overall development, tissue organization, and physiology of the teleost and mammalian intestines are highly similar (12, 14, 15). In this study, we made use of the *axin1*^{tm213} mutant that contains a missense mutation in the Gsk3-binding domain of Axin1 (29, 30), which disrupts the function of the β-catenin destruction complex. Homozygous *axin1* mutants are viable through 8 dpf, allowing analysis of the effects of excessive Wnt signaling on the larval intestine. We report that cell proliferation in the larval zebrafish intestine is increased both by the presence of the microbiota and by up-regulation of Wnt signaling in an *axin1* mutant. We demonstrate that *myd88* is required for perception of the microbial signals that promote intestinal cell proliferation. We show that a dominant member of zebrafish gut microbiota, *Aeromonas veronii*, secretes pro-proliferative signals and that zebrafish with a mutation in the β-catenin co-activator Tcf4 have a decreased proliferative response to monoassociation with *A. veronii*. Finally, we show that GF larvae have reduced numbers of intestinal epithelial cells with cytoplasmic β-catenin and that *A. veronii* monoassociation is sufficient to promote the accumulation of cytoplasmic β-catenin in the intestinal

epithelium, demonstrating that resident intestinal bacteria enhance Wnt pathway activity and elevate rates of epithelial renewal in the developing vertebrate intestine.

RESULTS

Zebrafish Intestinal Epithelial Cell Proliferation Is Stimulated by the Microbiota

To investigate the influence of microbes on zebrafish intestinal cell proliferation, we exposed larvae to the nucleotide analogues BrdU and 5-ethynyl-2′-deoxyuridine (EdU) and quantified S-phase nuclei in 30 serial sections in the intestinal bulb (Fig. 1 A and B: see Appendix for all figures). The absolute numbers of labeled cells varied with the different nucleotide analogues and even between trials, but the relative levels of cell proliferation between treatment groups were reproducible between trials. The spatial distribution of S-phase cells within the larval intestinal epithelium was sporadic, with some bias toward the bases of the emerging epithelial folds (Fig. 1C), as described (14, 15). We observed enrichment of proliferating cell nuclear antigen (PCNA) in the same cells that were labeled with EdU (Fig. 1 C–E), confirming that these were proliferating cells. We observed no PCNA staining in terminally differentiated goblet cells, which were visualized with Alcian blue staining (Fig. 1F), suggesting that zebrafish intestinal epithelial cells undergo proliferation before committing to a particular cell fate, similar to mammalian intestinal epithelial cells. In conventionally reared (CV) larvae with normal microbiota, we observed a decrease in the number of dividing cells between 6 and 8 dpf (Fig. 1G), similar to other reports of proliferation patterns in the developing zebrafish intestine (14, 15, 31). In larvae reared GF, the intestinal epithelia exhibited significantly fewer dividing cells than in CV controls at both 6 and 8 dpf (Fig. 1G). This observation was consistent with previous

analyses of cell proliferation in the 6-dpf GF zebrafish intestine (4, 32).

To determine whether these differences in cell proliferation resulted in an increase in intestinal cells in CV vs. GF animals, we scored the total number of epithelial cells in single H&E-stained tissue sections from three defined locations along the intestine (Fig. 1 H–J). We observed no significant differences in the total numbers of intestinal epithelial cells in CV and GF 8-dpf intestines at any of the three anatomical locations (Fig. 1K). The larval intestinal epithelium contains very few apoptotic cells (14), and there is no difference in the number of these cells between GV vs. CV larvae (4). Because fewer cells are dividing in GF vs. CV animals but the total numbers of cells in the two are similar, we infer that intestinal epithelial cells must undergo a slower rate of turnover in the absence of microbes.

Myd88 Is Required for Intestinal Epithelial Cell Proliferation in Response to Microbial Signals

The TLRs are key sensors of MAMPs produced by both pathogenic and beneficial microbes. In mammals, Myd88 functions as a common downstream adaptor of the TLRs, and thus is a good target for disrupting TLR signaling. To test whether Myd88 functions in reception of the microbial cell proliferation-promoting signal in the zebrafish intestine, we examined cell proliferation levels in the intestinal epithelia of *myd88* morpholino (MO)-injected animals. We observed significantly fewer proliferating cells in the *myd88* MO-treated animals as compared with their WT siblings (Fig. 1L). Next, we compared intestinal epithelial cell proliferation in *myd88* MO-injected larvae that were reared in the absence of the microbiota. If Myd88 function were required for the perception of the microbial signals

that promote intestinal cell proliferation, in the absence of Myd88, the rate of cell proliferation should be the same irrespective of the presence or absence of the microbiota. Consistent with this prediction, we observed that the levels of cell proliferation in CV and GF *myd88* MO-injected larvae were indistinguishable (Fig. 1L).

We have shown that on colonization of the zebrafish intestine, the microbiota elicit an influx of neutrophils into the intestinal epithelium by means of a Myd88-dependent mechanism (23). We wondered whether a mild inflammatory response to the microbiota, similar in mechanism but smaller in scale to the hypertrophic response of the epithelium to pathogen infection or injury (1), could produce the increased intestinal cell proliferation observed in the colonized larval intestine. Inhibition of TNF receptor with a pair of *tnfr* MOs efficiently blocked neutrophil influx in CV larvae (23). However, this treatment had no effect on the number of proliferating cells in the intestinal epithelium (Fig. 1M), suggesting that the microbiota-induced cell proliferation in the developing intestine is mechanistically distinct from pathological hypertrophy, which is known to require Myd88 function (33–35).

Wnt Signaling Promotes Cell Proliferation in the Zebrafish Larval Intestine

Canonical Wnt signaling is a key pathway regulating the balance between proliferating and differentiated cells in the mammalian intestine (2). We sought to establish whether Wnt signaling functions in the larval zebrafish intestine to establish rates of epithelial proliferation during the period when the gut is first colonized by microbes. We therefore examined whether components of the Wnt signaling pathway are expressed in the larval intestine and whether they respond to known inducers of Wnt signaling.

In situ hybridization against the β-catenin cofactor gene, tcf4, revealed strong expression within the brain and modest expression in the intestinal bulb in 6-dpf larvae (Fig. 2A). Low-level intestinal and brain expression of the Wnt target gene *sox9b* was also detected in larvae (Fig. 2B). We were unable to detect expression of the other Wnt target genes *c-myc*, *axin2*, *cdx1a*, or *nt-1* by in situ hybridization in the larval intestine or the GFP product of the Wnt reporter topD line (36) by immunohistochemistry, but we were able to amplify these mRNAs from dissected larval intestines by RT-PCR (Fig. 3 A and B). In adult intestines, tcf4 was expressed throughout the villi (Fig. 2C) and sox9b was localized to the base of the villi (Fig. 2D) in the region where cell proliferation becomes restricted in adults. We also examined expression of the Wnt pathway transducer β-catenin in the larval intestine. The zebrafish genome contains two β-catenin genes (37), which encode highly similar proteins that are recognized by polyclonal antisera raised against the human protein (38). Using these antisera, we detected strong cytoplasmic β-catenin expression in a subset of pharyngeal and esophageal cells (Fig. 2 F and G) and in a few scattered cells along the length of the intestine (Fig. 2 H and I). The antisera also cross-reacted with luminal intestinal microbes (Fig. 2 H and I), but these were easily distinguished from intestinal epithelial cells by their shape and location.

We next asked whether we could detect changes in levels of Wnt pathway components in response to modulators of Wnt signaling. First, we treated zebrafish larvae with LiCl, an inhibitor of β-catenin destruction complex member GSK-3 (39). Larvae exposed to 75 mM LiCl from 3 dpf exhibited stronger pharyngeal β-catenin staining at 6 dpf than untreated controls (Fig. 2 F and G). Second, we analyzed β-catenin distribution in the intestines of homozygous 6-dpf *axin1*^{tm213} mutants. We noted that relative to their WT

siblings, the *axin1* mutants had more cells with cytoplasmic β-catenin distributed along the length of their intestines (Fig. 2 H and I). We were also able to measure a modest 2-fold increase in transcript level of the Wnt target gene *c-myc* in intestines of *axin1* 8-dpf mutant larvae relative to WT siblings (Fig. 3C). Collectively, these data provide support for a functional Wnt pathway in the larval intestinal epithelium that is up-regulated in *axin1*^{tm213} mutants, but they suggest that the level of Wnt signaling in this tissue is low compared with that in other tissues, such as the brain.

We next performed functional studies in the *axin1* mutant to test whether Wnt signaling regulates cell proliferation in the larval intestine. We noted that in live 6-dpf *axin1* animals, the intestines appeared larger and the tissue thicker and more convoluted (Fig. 3 D and E). H&E-stained sections of 8-dpf *axin1* intestines revealed epithelial hypertrophy and regions of disordered epithelia (Fig. 4B), in contrast to the orderly alignment of cells in the intestinal epithelia of WT siblings (Fig. 4A). On quantification, we found that at both 6 and 8 dpf, *axin1* mutant larvae had significantly more proliferating cells than WT siblings (Fig. 4C). We quantified an increase in the total number of intestinal epithelial cells in the *axin1* mutant 8-dpf larvae, most notably in the intestinal bulb (Fig. 4D). The elevated intestinal epithelial cells in the *axin1* mutant (1.8-fold more than in WT in the intestinal bulb) could not account for the increased number of S-phase cells in these animals (7-fold more than in WT in the intestinal bulb), confirming that rates of intestinal epithelial cell proliferation were elevated in the *axin1* mutant.

Microbial Signals and Axin1 Loss Promote Cell Proliferation Combinatorially

We had so far shown that both the presence of microbes and activation of Wnt

signaling through impairment of the β-catenin destruction complex increased cell proliferation in the intestinal epithelium. We next asked whether the microbiota induced cell proliferation by promoting Wnt signaling upstream of the β-catenin destruction complex. If this were the case, we would expect *axin1*-deficient animals, which experience constitutive Wnt signaling, to be insensitive to microbial signals, and thus to exhibit similar levels of intestinal epithelial cell proliferation in the presence or absence of microbes. However, when we quantified cell proliferation in 6-dpf GF *axin1* larvae, we observed significantly fewer dividing nuclei than in CV *axin1* siblings (Fig. 5A). These observations suggest that the microbial signals promoting cell proliferation do so through a pathway that functions in parallel to or downstream of Axin1. If Myd88 functions to transduce the signals from the microbiota that promote intestinal epithelial cell proliferation, we would predict that an *axin1* mutant lacking *myd88* would resemble an axin1 mutant reared under GF conditions. Similar to our observations in GF *axin1* mutants, we observed a significant decrease of cell proliferation in the intestinal epithelia of *axin1* mutants injected with the *myd88* MO, with levels that resembled those of CV WT controls (Fig. 5B). These data are consistent with the model that Myd88 transduces the microbial signals that promote intestinal epithelial cell proliferation in parallel to or downstream of Axin1.

Secreted Bacterial Signals Modulate Levels of Intestinal Epithelial Cell Proliferation

We next wanted to explore the nature of the microbial signals that promote intestinal cell proliferation. Because we had shown that Myd88 is required for this response, we wondered whether any TLR ligand could induce this response. We have shown previously that LPS induces certain Myd88-mediated responses when administered

to zebrafish larvae in their water, including toxicity (at 50 μg/mL or higher) and induction of intestinal alkaline phosphatase activity (at 3 and 30 μg/mL) (23). We tested whether purified LPS that was competent to elicit these responses (Fig. 6 A and B) was sufficient to stimulate intestinal proliferation when added to GF larvae at the sublethal dose of 30 μg/mL. We found that exposure to 30 μg/mL LPS caused no increase in the number of proliferating cells in the intestinal epithelia of GF larvae (Fig. 5C). Having ruled out a generic MAMP as the intestinal epithelial cell proliferation-promoting factor, we sought to establish whether specific members of the zebrafish gut microbiota possessed this activity.

The complexity of the zebrafish microbiota is similar to that of mammals, but the community is dominated by Gram-negative Gamma-Proteobacteria (4, 32). Previously, we had found that monoassociation with a strain of *A. veronii* biovar sobria, a member of the dominant genera of the zebrafish microbiota, was sufficient to reverse multiple GF traits (40). We therefore tested whether *A. veronii* monoassociation could induce cell proliferation in GF animals, using two different experimental time courses: inoculation of GF larvae at 4 dpf and assaying at 6 dpf or inoculation at 6 dpf and assaying at 8 dpf. At both end points, monoassociation with *A. veronii* was sufficient to stimulate intestinal epithelial cell proliferation significantly (Fig. 5 D and F).

We proceeded to characterize the *A. veronii*-associated cell proliferation promoting activity by testing whether it was secreted or required the presence of the bacteria. We generated *A. veronii* cell-free supernatant (CFS) by centrifugation and filtration and further purified it using a concentrator with a molecular mass cutoff of 10 kDa, which reduced the toxicity of the solution when administered to fish [likely by removing toxic LPS (23)]. *A. veronii* CFS, when added back to GF larvae at 6 dpf, was sufficient to increase intestinal

cell proliferation at 8 dpf (Fig. 5D). As with the *A. veronii* monoassociations, the levels of intestinal epithelium cell proliferation in CFS-treated larvae were sometimes slightly higher than those found in CV larvae (Fig. 5D).

We used the simplified *A. veronii* monoassociation model to investigate the relation between bacterial and Wnt signaling further. We reasoned that if *A. veronii* monoassociation stimulated cell proliferation independent of inhibition of Axin1, the effects of bacterial inoculation and axin1 deficiency should be combinatorial. Indeed, when we monoassociated axin1 mutants with *A. veronii*, we observed levels of intestinal epithelial cell proliferation that significantly exceeded those of the CV *axin1* mutants (Fig. 5E), consistent with our other evidence suggesting that microbiota signals promote cell proliferation in parallel to or downstream of Axin1.

To address whether bacterial signals promote proliferation through components of the Wnt pathway downstream of Axin1, we made use of the zebrafish *tcf4*^{exl} null mutant (16). Previous characterization of this mutant had revealed no impairment in intestinal epithelial cell proliferation before 5 wk post-fertilization. When we carefully analyzed cell proliferation in these mutants at 6 dpf, we observed a slight but statistically significant decrease relative to WT siblings (Fig. 5F), demonstrating that canonical Wnt signaling is required for normal levels of cell proliferation in the larval intestine. We reasoned that if *A. veronii* monoassociation promotes intestinal cell proliferation upstream of Tcf4, the proliferative response should be blocked in *A. veronii*-monoassociated *tcf4* mutants. We observed that the proliferative response to *A. veronii* was partly decreased in the *tcf4* mutants (Fig. 4F; 1.8-fold increase over GF) as compared with the WT response (2.4-fold increase over GF), suggesting that Tcf4 function is partially required for transduction of the

A. veronii proliferation-promoting signal.

Resident Bacteria Promote β-Catenin Stability in Intestinal Epithelial Cells

To look for direct evidence of Wnt signaling regulation by the microbiota, we quantified intestinal epithelial cells with cytoplasmic β-catenin in the same region of the intestinal bulb in which we had quantified S-phase nuclei. Consistent with the role of Axin1 in the β-catenin destruction complex, we observed significantly more cells with cytoplasmic β-catenin in *axin1* mutant vs. WT intestinal epithelia (Fig. 5G). When WT animals were reared GF, their intestinal epithelia contained significantly fewer cells with cytoplasmic β-catenin than CV siblings (Fig. 5 G and H), indicating that the presence of the microbiota stabilized β-catenin in the intestinal epithelium. This observation also held true in *axin1* mutants, in which the number of cells with cytoplasmic β-catenin was significantly reduced in the absence vs. presence of the microbiota (Fig. 5G). Finally, we quantified β-catenin-positive cells in the intestinal epithelia of larvae colonized solely with *A. veronii*. We found that monoassociation with *A. veronii* was sufficient to increase numbers of intestinal epithelial cells with cytoplasmic β-catenin to levels significantly higher than those observed in GF or CV animals (Fig. 5H), demonstrating that a resident intestinal bacterium is capable of stabilizing β-catenin in intestinal epithelial cells.

DISCUSSION

When the vertebrate intestine is first colonized by microbes at birth or hatching, this tissue is still undergoing maturation and establishment of adult patterns of tissue renewal. Studies in GF zebrafish clearly demonstrated a role for the microbiota in stimulating rates

of intestinal cell proliferation during this period of development (4). We can speculate on why microbes elicit an increase in intestinal epithelial renewal. From the host's perspective, increased cell turnover may be beneficial as a mechanism to purge epithelial cells exposed to increased concentrations of harmful chemicals, such as reactive oxygen species, associated with microbial colonization. From the microbes' perspective, stimulating cell turnover would increase the numbers of host cells shed into the lumen and the availability of host-derived glycans that can serve as nutrient sources for the microbial community (41).

We report here that Myd88, an adaptor for the TLR family of innate immune receptors, is required for the normal proliferative response to the microbiota in the developing zebrafish intestine. In mice, adult Myd88 mutants have normal or slightly elevated numbers of proliferating intestinal epithelial cells in the absence of injury (33, 34), but the effect of Myd88 signaling on rates of intestinal epithelial proliferation has not yet been characterized in neonates (42). The mechanisms underlying intestinal epithelial cell proliferation during normal development are likely to be different from those operating in the proliferative response to injury in the adult intestine. Intestinal epithelial cell proliferation in adult mice in response to the toxin dextran sodium sulfate (DSS) or *Citrobacter rodentia* infection occurs with a robust induction of proinflammatory cytokines, which is blunted in the absence of Myd88 (35, 43). Furthermore, blocking TNF signaling attenuates tumor formation in a murine model of DSS-induced colitis-associated CRC (44).

In contrast to the described role of Myd88 in promoting inflammation-associated hypertrophy in the intestines of adult mice, we found no correlation between signals that induce inflammation and cell proliferation in the developing zebrafish intestine. LPS,

which is sufficient to protect against DSS toxicity associated with a defective proliferative response in the sterilized mouse intestine (35), and which induces inflammatory responses in the zebrafish intestine (23), did not induce epithelial cell proliferation. Furthermore, we found that inhibition of inflammatory responses with TNF receptor MOs had no effect on cell proliferation in the larval intestine.

We provide evidence that Wnt signaling is both necessary and sufficient to promote cell proliferation in the larval zebrafish intestine, using the *tcf4* and *axin1* mutants, respectively. Given the importance of Wnt signaling in regulating cell renewal in this tissue, we asked whether microbial signals could increase cell proliferation by up-regulating Wnt signaling. We addressed the interactions between microbial and Wnt signaling in intestinal epithelial renewal by manipulating both host genetics and microbial associations. These tests were imperfect because of the complexity of the microbial signals and the limitations of Wnt pathway mutants (the $axin1^{tm213}$ allele may have some residual activity because the phenotype is temperature-sensitive, and the $tcf4^{exl}$ mutant larvae may have some maternal gene product that could explain the mild proliferation defect at this stage). Nonetheless, our data support the model that the microbiota promote intestinal epithelial cell proliferation, in part, by up-regulating Wnt signaling downstream of Axin1 and upstream of Tcf4. The proliferative effects of the axin1 mutation were combinatorial with the presence or absence and the composition of the microbiota, suggesting that the proliferative signals from the microbiota act in parallel to or downstream of Axin1. The proliferative response to *A. veronii* monoassociation was considerably reduced in the *tcf4* mutant, indicating that the signals from microbiota may act partly through Tcf4 to promote intestinal epithelial proliferation.

To gain mechanistic insight into the interaction between the microbiota and the Wnt signaling pathway, we quantified intestinal epithelial cells with cytoplasmic β-catenin. Cytoplasmic accumulation of β-catenin is indicative of active Wnt signaling. Loss of the β-catenin destruction complex component, Axin1, resulted in elevated numbers of intestinal epithelial cells with cytoplasmic β-catenin. Most intestinal epithelial cells in the axin1 mutant still displayed low levels of β-catenin, indicating that other factors prevent the accumulation of β-catenin in these cells. Consistent with our observation that the presence of the microbiota and the loss of axin1 acted combinatorially to promote intestinal epithelial cell proliferation, we observed that these factors both contribute to β-catenin levels in the intestine, with significantly fewer β-catenin-positive cells in GF vs. CV *axin1* mutants. Furthermore, we demonstrated that colonization of the GF intestine with *A. veronii* was sufficient to stabilize β-catenin to higher than CV levels. Our observation that loss of the β-catenin co-activator Tcf4 partially blocks the pro-proliferative activity of *A. veronii* is consistent with the model that *A. veronii* induces intestinal epithelial cell proliferation by increasing the stability of β-catenin in these cells.

One possible mechanism for microbial signaling through the Wnt pathway could involve a Myd88-dependent up-regulation of prostaglandin E_2, a molecule that has been shown to stimulate Wnt signaling and promote stem cell regeneration in a number of larval zebrafish tissues through phosphorylation-based regulation of β-catenin stability (45). Another possible mechanism could involve microbial induction of reactive oxygen species, which have been shown to promote Wnt signaling through inhibition of β-catenin ubiquitination (46). Additionally, gastrointestinal bacteria may produce specific effector molecules that activate Wnt signaling, as has been shown for the CagA protein of

Helicobacter pylori (47). All these mechanisms stimulate Wnt signaling downstream of Axin1 and upstream of Tcf4, similar to the zebrafish microbiota-associated signals we describe here.

We do not know the chemical nature of the microbial signals that promote cell proliferation in the zebrafish larval intestine, but our experiments shed some light on their properties. We showed that LPS exposure is not sufficient to stimulate intestinal cell proliferation and that *A. veronii*-secreted factors greater than 10 kDa stimulate proliferation in the intestine. We hypothesize that members of the microbiota produce multiple pro-proliferative factors at various concentrations and with various potencies. Indeed, Rawls *et al*. (32) observed differences in the extent to which individual bacteria could induce intestinal cell proliferation in monoassociations. Different assemblages of gut microbes would therefore have different proliferative capacities. Notably, colonization of *axin1* mutants with a monoculture of *A. veronii* elicited significantly more cell proliferation than colonization with complex microbiota. In humans, the majority of spontaneous and hereditary mutations associated with CRC impair the β-catenin destruction complex (48). Our data suggest that although an individual's genetic Wnt status clearly plays an important role in determining CRC risk, the individual's microbiota and innate immune system function will likely contribute to the risk for intestinal epithelial hyperproliferation. Proinflammatory microbes are likely to contribute to the development of colitis-associated CRC (49). Our data suggest that members of the microbiota also influence rates of intestinal epithelial cell proliferation independent of inflammation via direct modulation of β-catenin signaling.

MATERIALS AND METHODS

All experiments with zebrafish were performed using protocols approved by the University of Oregon Institutional Animal Care and Use Committee and following standard protocols (50). WT (Ab/Tu) zebrafish were reared at 28 °C. The *axin1*^{tm213} mutant line was reared at 30 °C; at that temperature, the eye development defect was fully penetrant among the homozygotes (29). The *tcf4*^{exl} mutant line (16) was kindly provided by Tatyana Piotrowski (University of Utah, Salt Lake City, Utah). Progeny from *tcf4*$^{exl/+}$ parents were euthanized at 6 dpf, and tail tissue posterior to the vent was removed for genotyping. Genotyping was carried out by PCR as described (16). GF embryos were derived and maintained as previously described (40). All larvae were starved through the duration of the experiments.

Larvae were immersed in 200 μg/mL BrdU (B-5002; Sigma) and 20 μg/mL 5′-fluoro-2′-deoxyuridine (F-0503; Sigma) or 100 μg/mL EdU (A10044; Invitrogen) for 16 h before termination of the experiment. Larvae were fixed in BT fixative (4% paraformaldehyde, 0.15 mM $CaCl_2$, 4% sucrose in 0.1 M PO_4 buffer) (50) for 4 h at room temperature with gentle shaking, immediately processed for paraffin embedding, and cut into 7-μm sections. To detect BrdU, sections were deparaffinized and tissue-denatured in 2 M warm HCl for 20 min, neutralized in 0.1 mM sodium borate for 10 min, and rinsed in PBS/0.5% Triton X-100 (PBSt). Tissue was blocked for 1 h at room temperature in 10% (vol/vol) normal goat serum in PBSt and soaked in anti-BrdU (1:150, 11170376001 mouse monoclonal; Roche) overnight at 4 °C. Slides were then rinsed in PBSt, incubated in secondary goat-anti-mouse 488 (1:500; Molecular Probes) for 2 h, rinsed again, and mounted in Vectashield (Vector). For EdU detection, slides were processed according to

the Click-iT EdU Cell Proliferation Assay Kit (C35002; Molecular Probes).

BrdU- or EdU-labeled nuclei within the intestinal epithelium were counted over 30 serial 7-μm sections beginning at the esophageal-intestinal junction and proceeding caudally into the bulb. Analysis of this extended region was necessary because of the stochastic patterns of cell proliferation. The absolute numbers of labeled cells varied between trials and the type and batches of nucleotide analogues. For example, fewer labeled cells were observed with BrdU vs. EdU treatment, likely because of less efficient incorporation of the nucleotide into cells and less efficient detection of the antigen. Despite these differences in the absolute numbers of labeled cells, the proportional trends of proliferating cells between treatments and genotypes were consistent and reproducible between trials.

Filter-sterilized LPS (*Escherichia coli* serotype 0111:B4, product no. 62325; Sigma) solution was injected into flasks of 3-dpf GF larvae to a final concentration of 30 μg/mL, and cell proliferation was quantified at 6 dpf by labeling S-phase nuclei with EdU as above.

Larvae were fixed overnight in Dietrich's fixative, processed for paraffin sectioning, cut into 7-μM sections, and stained according to standard protocols (51). The total number of epithelial cells was quantified in a single section at the location of the esophageal-intestinal junction, 30 sections caudal in the bulb, and 60 sections caudal in the distal intestine.

Larvae were fixed overnight in BT, processed for paraffin embedding, and cut into 7-μM sections. Following deparaffinization, antigen retrieval was performed by boiling in 0.1 M sodium citrate for 1 or 20 min (for detection of PCNA and β-catenin, respectively)

and cooling to room temperature for 30 min. Slides were blocked in 10% (vol/vol) normal goat serum in PBSt for 1 h and then exposed to anti-β-catenin (1:1,000, C2206 rabbit polyclonal; Sigma) or anti-PCNA (1:5,000 for immunofluorescence or 1:20,000 for colorimetric detection, P8825 mouse monoclonal; Sigma) overnight at 4 °C. Antibodies were detected with appropriate fluorophore-conjugated secondary antibodies (Molecular Probes) as above. To visualize proliferating and goblet cells, anti-PCNA was detected with HRP-conjugated secondary using a Vectastain ABC kit (Vector Laboratories) and subsequently stained with Alcian blue solution (pH 2.5) for 90 min, counterstained with nuclear fast red (Vector Laboratories) for 10 min, dehydrated in 95% (vol/vol) alcohol, cleared in xylene, and mounted in Permount (Fisher Scientific). Intestinal epithelial cells with cytoplasmic β-catenin were quantified in 30 serial sections caudal from the esophageal-intestinal junction.

In situ hybridization on larvae was performed as described (50), with the addition of a 20-min soak in proteinase K (2 μg/mL) followed by a 20-min postfixation. In situ hybridization on adult cryosectioned tissue was performed as described (52). RNA probes included sox9b (53) and tcf4 (54).

Samples were imaged on a Nikon Eclipse TE 2000-V inverted microscope equipped with a Photometrics Coolsnap camera. Confocal images were captured using a Nikon D-Eclipse C1 microscope. Images were manipulated in Adobe Photoshop.

Cultures of *A. veronii* biovar sobria strain HM21R (55) were grown at 30 °C for 17 h on a rotary shaker at 170 rpm. Flasks of GF larvae were inoculated with 10^5 to 10^6 cfu/mL culture on 4 or 6 dpf, and the experiment was terminated on 6 or 8 dpf, respectively. Monoassociation experiments were deemed successful when dissected

homogenized intestines yielded an average of 10^3 cfu on tryptic soy agar plates.

A. veronii cultures prepared as above were spun at 5,600 × g for 10 min at 4 °C, and the supernatant was passed through a 0.22-μM filter (Corning) on ice. CFS was concentrated through an Amicon Ultra-15 spin concentrator to remove small products, which were toxic to the fish. Protein concentration was determined by Bradford assay. All CFS exposures were performed using 500 ng/mL total protein, corresponding to ≈10^8 *A. veronii* cells/mL.

Splice-blocking MOs targeting *myd88* and *tnfr* were used as described (23). MO-injected larvae exhibited good survival, developed a swim bladder, and were grossly similar to their control siblings. Efficacy of the MO was assessed by RT-PCR and, in the case of the *tnfr* MO, by the ability to inhibit influx of myeloid peroxidase-positive neutrophils into the intestines (23).

Student's unpaired t tests were performed with GraphPad Prism software.

CHAPTER III

HELICOBACTER PYLORI VIRULENCE FACTOR CAGA INCREASES INTESTINAL EPITHELIAL CELL PROLIFERATION BY ACTIVATION OF WNT SIGNALING IN A TRANSGENIC ZEBRAFISH MODEL

The work described in this chapter was co-authored by T. Peterson, M. Kent, K. Guillemin, and myself. K. Guillemin, and myself designed research. I performed research. T. Peterson, M. Kent, K. Guillemin, and myself analyzed data. K. Guillemin and myself wrote the paper.

INTRODUCTION

Helicobacter pylori is a pathogenic Gram-negative bacterium that colonizes over 50% of the world's human population. Colonization with *H. pylori* is linked to numerous gastric disorders including gastritis, peptic ulcer disease, and gastric adenocarcinoma (17). Although gastric cancer occurs in fewer than 1% of people colonized by *H. pylori* (18), it is still the second most common cause of cancer mortality worldwide (19), and more than 50% of gastric adenocarcinomas can be attributed to infection with *H. pylori* (20). Most people infected with *H. pylori*, however, do not develop gastric cancer, and the molecular mechanisms underlying this disparity have yet to be fully elucidated.

Although there are many factors that appear to contribute to *H. pylori*'s carcinogenicity, strains that translocate the CagA protein into host cells are significantly more likely to cause gastric cancer than strains lacking this ability. CagA is one of 28 gene products encoded by the *cag* pathogenicity island (cag PAI), a 40 kb stretch of DNA

shown to be present in most strains isolated from patients with severe gastric pathology (21). During infection with *H. pylori*, CagA is translocated into host cells via a type IV secretion system (TFSS), where it interacts with a multitude of host cell proteins. These interactions have been shown to affect signal transduction pathways, the cytoskeleton, and cell junctions (22).

After translocation into host cells by the *H. pylori* TFSS, CagA can be phosphorylated by Src family kinases on tyrosine residues within conserved Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (23). Upon phosphorylation, CagA has been shown to induce morphological changes in cultured epithelial cells through interaction with a variety of host-cell proteins such as SHP-2, Met, Csk, Grb2, and ZO-1 (23-27). In addition to its phosphorylation-dependent effects, CagA has also been shown to interact in a phosphorylation-independent manner with pathways associated with proliferation and inflammation (28). Although it is not yet clear which of these myriad interactions are required for the development of gastric cancer in persons colonized by *H. pylori*, the ability of CagA to interact with components of the canonical Wnt signaling pathway provides a potential link between CagA's observed oncogenic effects and a host signaling pathway frequently deregulated in gastrointestinal cancers (29).

In addition to its role in early embryogenesis, the canonical Wnt signaling pathway plays a crucial role in regulating the proliferation and homeostasis of gastrointestinal epithelia. In normal stomach and intestinal epithelia, Wnt signaling has been shown to be important for proliferation, stem cell maintenance, and tissue renewal (30-34). On the other hand, perturbation of Wnt signaling has been shown to result in cancers of the stomach and colon (35-37). Activation of the Wnt pathway is tightly

controlled via regulation of the primary Wnt effector protein β-catenin. β-catenin complexes with E-cadherin to form adherens junctions between epithelial cells, and in the absence of Wnt ligand, is also bound by Axin/APC/Gsk3β in the so-called 'β-catenin destruction complex' where it is targeted for proteosomal degradation. Upon binding of Wnt by the co-receptors Frizzled and LRP, Axin1 is sequestered at the membrane, preventing assembly of the β-catenin destruction complex. This results in cytoplasmic accumulation of β-catenin, and subsequent translocation of β-catenin into the nucleus. Upon nuclear translocation, β-catenin binds the essential transcriptional cofactor TCF, and initiates transcription of Wnt target genes.

Non-phosphorylated CagA has been previously shown to disrupt the β-catenin/E-cadherin complex in cultured epithelial cells, causing cytoplasmic and nuclear accumulation of β-catenin, and subsequent activation of the Wnt pathway (38-40). Additionally, CagA has been shown to increase signaling through β-catenin via activation of phosphatidylinositol 3-kinase/Akt (28). Although the mechanisms of CagA's interactions with the Wnt pathway have yet to be fully elucidated, it is clear both that CagA is capable of activating Wnt signaling through β-catenin, and that inappropriate activation of Wnt signaling is potentially oncogenic.

Understanding the wide variety of host cell interactions required for *H. pylori*-induced pathogenesis has necessitated the use of animal models, and to date numerous primate and rodent models have been developed (41-44). Although previously unexploited in the study of *H. pylori* pathogenesis, the teleost fish *Danio rerio* (zebrafish) has emerged as a model organism for the study of various human diseases, including leukemia (45), melanoma (46, 47), and intestinal neoplasia (48). In lieu of a stomach,

zebrafish possess an anterior digestive compartment known as the intestinal bulb. The zebrafish intestinal bulb is a columnar, non-ciliated epithelium like the mammalian stomach, and expresses *sox2* and *barx1* (49), two mammalian stomach markers (50-52). Unlike the mammalian stomach, however, it is non-acidic and lacks the chief and parietal cell types. Nonetheless, the zebrafish intestinal bulb has been proposed to share a common ontogeny with the mammalian stomach and its renewal is regulated by similar molecular pathways, including the Wnt pathway (53). Finally, the rapid development of the zebrafish intestinal tract makes it an ideal model for the study of gastrointestinal development and disease (54).

CagA has been shown to interact with a plethora of host cell pathways, yet it is not yet clear which of these interactions is essential for the development of gastric cancer in humans. Here, we describe the development of a novel transgenic model system that simplifies the complexity of *H. pylori* infection to study the effects of a single bacterial protein on host cell biology in the zebrafish intestine. We report that proliferation in the zebrafish intestinal epithelium is increased by transgenic expression of CagA and that this increase occurs independently of CagA phosphorylation. We demonstrate that expression of CagA induces cytoplasmic and nuclear accumulation of the Wnt effector β-catenin, as well as activation of known Wnt target genes. Finally, we show that CagA causes overproliferation of the zebrafish intestinal epithelium via interaction with the canonical Wnt signaling pathway downstream of the β-catenin destruction complex and upstream of the essential β-catenin transcriptional cofactor Tcf4.

RESULTS

Generation of CagA-expressing Transgenic Zebrafish

In order to generate *cagA* transgenic animals, we cloned the *cagA* gene out of *H. pylori* strain G27. Strain G27 was originally isolated from Grossetto Hospital (Tuscany, Italy), and has been used extensively in research on the CagA virulence factor (24, 38, 56, 57). The cloned gene was then 3'-tagged with EGFP to facilitate *in vivo* visualization of CagA expression. To express CagA ubiquitously in zebrafish, the *cagA/EGFP* fusion construct was connected downstream of the 5.3kb *beta-actin* (*b*-) (58) promoter (Fig. 7A). To facilitate intestine-specific expression of the fusion construct, we connected *cagA/EGFP* downstream of a 1.6kb fragment of the zebrafish *intestinal fatty acid binding protein* (*i*-)(59) promoter (Fig. 7B). By 6 days post-fertilization *b-cagA/EGFP* transgenic zebrafish exhibited ubiquitous fluorescence, while *i-cagA:EGFP* transgenics exhibited fluorescence in the distal esophagus and anterior intestine (Figure 8 A and B). CagA's phosphorylation state has been previously shown to have significant effects on the type and severity of CagA-induced pathologies, so in order to determine the role of CagA phosphorylation in the intestinal epithelium, we fused the previously described phosphorylation-resistant $cagA^{EPISA}$ allele (60) (Fig. 9) to EGFP and connected it downstream of the *b-actin* promoter (Fig. 1C). *b-*$cagA^{EPISA}$*/EGFP* transgenics exhibited ubiquitous fluorescence and were indistinct from *b-cagA/EGFP* fish (Fig. 8C). Expression of *cagA* mRNAs was verified in transgenic animals by RT-PCR (Figure 8D), and analysis of relative intestinal *cagA* transcript level in the transgenic lines was performed using quantitative real-time PCR (Fig. 8E). CagA-expressing zebrafish

showed normal intestinal development (Fig. 8F and G), and were histologically indiscernible from wild-type clutch-mates (Fig. 8H and I).

CagA Expression Causes Overproliferation of the Intestinal Epithelium

CagA has been previously shown to increase proliferation *in vitro* and *in vivo* (27, 61) To determine the proliferation state of CagA-expressing intestines, we exposed animals at 6 and 15 dpf to the nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU) and counted S-phase nuclei in 30 serial sections of the intestinal bulb. Expression of CagA resulted in a significant increase in EdU labeled cells in all transgenic lines at 6 and 15 days post-fertilization (Fig. 10A & B). We observed enrichment of Proliferating cell nuclear antigen (PCNA) in the same cells that were labeled with EdU, verifying that these cells were proliferative (Fig. 10C). To determine if this increase in proliferation had an effect on the cell census, we quantified total epithelial cell number in single H&E-stained sagittal sections along the length of the intestine. We did not observe any significant difference in total cell counts between CagA transgenics and wild-type animals at 6 and 15 dpf (Figure 10D & E), indicating that expression of CagA caused increased turnover of intestinal epithelial cells. Increased intestinal cell turnover would require an increase in cell death, however, we observed very few TUNEL-positive cells in the intestines of wild-type and CagA-expressing animals (Fig 10F & G), consistent with previous reports of low numbers of apoptotic cells in the developing zebrafish intestine (62), with no significant difference observed between the two groups.

CagA Expression Activates the Wnt Pathway Downstream of the β-catenin Destruction Complex

We had previously shown that epithelial cell proliferation in the zebrafish intestine is regulated by the Wnt pathway (53). In addition, previous studies had shown that CagA can induce cytoplasmic and nuclear accumulation of the Wnt effector protein β-catenin, and can activate transcription of canonical Wnt target genes (28, 29, 61). Accordingly, we examined whether CagA was capable of activating the Wnt signaling pathway in the zebrafish intestine. We first utilized quantitative real-time PCR to assess the relative expression levels of known Wnt target genes in dissected adult intestines. Transcript levels of the canonical Wnt target genes *c-myc* (*myca*) (63) and *cyclinD1* (64) were modestly increased in all CagA-expressing lines relative to the wild-type strain (Fig. 11A & B). We next asked whether CagA was capable of inducing β-catenin accumulation in intestinal epithelial cells, indicating activation of the Wnt pathway. CagA expression caused a significant increase in the number of intestinal epithelial cells with cytoplasmic and nuclear accumulation of β-catenin as compared to wild-type animals (Fig. 11C & D).

We next compared the intestinal β-catenin accumulation observed in CagA-expressing animals to that of a known Wnt signaling mutant, *axin1*^{tm213}. *axin1*^{tm213} homozyotes exhibit deregulated Wnt signaling as a result of a missense mutation in the Gsk3β binding domain of Axin1, which prevents assembly of the β-catenin destruction complex. These mutants die as a result of craniofacial defects, but are viable through 8 dpf, allowing study of the juvenile intestine (65) (66). We observed significant increases over wild-type and CagA-expressing animals in both the number of proliferating cells and the number of cells featuring cytoplasmic and/or nuclear accumulation of β-catenin

in the intestinal epithelia of *axin1*$^{tm213/tm213}$ mutants (Fig. 11E). Finally, we crossed CagA-expressing animals to *axin1*$^{tm213/tm213}$ mutants to obtain CagA-expressing, *axin1*^{tm213} homozygotes. We reasoned that if CagA were capable of activating Wnt signaling upstream of the β-catenin destruction complex, then *axin1*^{tm213} homozygotes should be refractory to CagA-induced accumulation of β-catenin, and levels of β-catenin accumulation in *b-cagA*; *axin1*$^{tm213/tm213}$ double mutants should resemble those of *axin1*^{tm213} homozygotes. Instead, co-expression of CagA with *axin1*$^{tm213/tm213}$ resulted in a dramatic increase in cell proliferation and β-catenin accumulation (Fig. 11F). Taken together, these data indicate that CagA is capable of causing sustained activation of canonical Wnt signaling in the intestinal epithelium, and that it does so either downstream of, or in parallel to the β-catenin destruction complex. Furthermore, CagA-induced accumulation of β-catenin correlated strongly with increased epithelial proliferation (Fig. 11G & H), suggesting that CagA may stimulate proliferation through activation of the Wnt pathway.

CagA-dependent Overproliferation of the Intestinal Epithelium Requires *tcf4*

To determine if CagA-induced overproliferation of the intestinal epithelium was dependent on canonical Wnt signaling downstream of the β-catenin destruction complex, we utilized a null allele of the essential β-catenin transcriptional cofactor, Tcf4 (49). We reasoned that if CagA's pro-proliferative effects were acting upstream of Tcf4, rates of intestinal proliferation in *i-cagA; tcf4*^{null} double mutants should be identical to those observed in *tcf*^{null} animals. As previously observed, *i-cagA* animals showed a significant

increase in proliferation over wild-type, while *tcf4*^{null} mutants showed a slight but insignificant decrease in intestinal proliferation relative to wild-type animals (Fig. 12A). Rates of intestinal proliferation in the *i-cagA; tcf4*^{null} double mutants were statistically indistinguishable from wild-type and *tcf4* null mutants, indicating that CagA interacts with the canonical Wnt signaling pathway to increase intestinal epithelial proliferation downstream of Axin1 and upstream of Tcf4 (Fig. 12B).

DISCUSSION

H. pylori related disease involves numerous factors, both microbe- and host-derived. Here, we have used transgenic expression of CagA to describe how the *H. pylori* virulence factor CagA is able to disrupt normal programs of gastrointestinal epithelial renewal by overactivation of an important host signaling pathway, the Wnt pathway, to cause significant overproliferation of an intact epithelium *in vivo*. Further, we show that despite the myriad signaling effects CagA is purported to have on cultured epithelial cells, activation of canonical Wnt signaling upstream of the essential β-catenin cofactor Tcf4 and downstream of the β-catenin destruction complex is required for CagA's effects on intestinal epithelial proliferation. Our data also corroborate previous studies, which indicate that CagA's effects on Wnt pathway components are not dependent on the phosphorylation state of CagA (28).

It has been previously reported that phosphorylation of CagA is required for the development of gastric neoplasia in mouse (35), so it is not entirely clear why expression of the phosphorylation-resistant *b-cagA*^{EPISA} allele induces significant overproliferation of the zebrafish intestine coupled with increased Wnt activation. It is possible that CagA's

activation of Wnt signaling and subsequent induction of proliferation is capable of sensitizing the intestinal epithelium to further oncogenic stimulus, perhaps in the form of the previously observed phosphorylation-dependent epithelial depolarization by CagA (24) or in the form of pro-oncogenic polymorphisms in host genes like IL-1β or IL-10 as previously suggested (67).

MATERIALS AND METHODS

Transgenic zebrafish were developed using the Tol2kit as previously described (68). All zebrafish experiments were performed using protocols approved by the University of Oregon Institutional Care and Use Committee, and following standard protocols (69).

Zebrafish larvae were immersed in 100 μg/mL EdU (A10044; Invitrogen) with .5% DMSO for 8-12 hours, fixed overnight at 4° C (4% paraformaldehyde in PBS) with gentle shaking, processed for paraffin embedding, and cut into 7μM sections. Slides were then processed using the Click-iT EdU Imaging Kit (C10337, Invitrogen). EdU labeled nuclei within the intestinal epithelium were counted over 30 serial sections beginning at the intestinal-esophageal junction and proceeding caudally into the intestinal bulb.

TUNEL staining was carried out using the Click-iT TUNEL Imaging Assay (C10245, Invitrogen). TUNEL-positive cells within the intestinal epithelium were counted over 30 serial sections beginning at the intestinal-esophageal junction and proceeding caudally into the intestinal bulb.

Immunohistochemistry was carried out of paraffin sections as previously described using anti-β-catenin (1:1000, C2206 rabbit polyclonal, Sigma) or anti-PCNA (1:5000, P8825 mouse monoclonal, Sigma) (53).

Reference gene testing was performed using the geNorm reference gene selection kit (Primerdesign) and qBase^{PLUS} software (Biogazelle). Baseline, threshold, and efficiency calculations were performed using LinRegPCR software (70) Quantitative RT-PCR reactions were performed using the SYBR FAST qPCR kit (Kapa Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) using primers listed in Table 1 (See Appendix).

All statistical analyses were performed with Graph-Pad Prism software.

CHAPTER IV

CONCLUSION

MICROBES AND THE DEVELOPING INTESTINE

The gastrointestinal microbiota plays a large and multifaceted role in the development and proper function of the vertebrate GI tract. Here, we demonstrate that the colonization of the zebrafish intestine by microbes is essential for the proper development and homeostasis of the intestinal epithelium. We show that secreted signals from the microbiota stimulate epithelial proliferation via the TLR adaptor protein Myd88. We also show that the microbial signals act partially through the canonical Wnt signaling pathway to stimulate proliferation. Finally, we have shown that intestinal proliferation is significantly altered when we reduce the microbial community down to a single member.

Although we have shown that secreted signals from the microbiota affect rates of proliferation, we have not yet determined what these signals may be. Additionally, although we have established that the Wnt pathway is partially involved in determining the proliferative response to the microbiota, we do not know what other factors may be acting downstream of Myd88 to control proliferation. Nonetheless, our data indicate that host genetic factors cooperate with microbial factors to determine rates of proliferation in the intestinal epithelium, and that careful regulation of the microbial community is required for proper maintenance of intestinal homeostasis. Finally, these data suggest that an individual's microbial complement may play a significant role in determining susceptibility to diseases, such as cancer, that affect intestinal proliferation.

HELICOBACTER PYLORI AND CANCER

H. pylori is a major risk factor for gastric cancer, but the mechanisms involved are not entirely clear. Here, we describe the development of a novel *in vivo* model of CagA-induced pathology that accurately recapitulates the major hallmarks of CagA pathogenesis in vertebrate models, and use this model to gain novel insight into the molecular mechanisms of *H. pylori* pathogenesis. We demonstrate that the *H. pylori* virulence factor CagA is capable of hijacking the Wnt pathway within the intestinal epithelium to drastically enhance levels of proliferation, disturbing the careful homeostatic balance between cell proliferation and cell death, and resulting in severe intestinal hyperplasia. These data show that despite the large number of reported *in vitro* interactions between CagA and host proteins, CagA's ability to interact with the Wnt pathway is the most significant with regards to epithelial overproliferation. This is not entirely surprising, as Wnt signaling has been shown to be crucial for maintaining the gastric and intestinal stem cell niches (1, 2) What remains to be seen, however, is how *H. pylori* is interacting with the gastric stem cells to induce proliferation. Previous studies have shown that all cell populations in the intestine other than the stem cells are refractory to oncogenic transformation (3) yet the stem cells reside deep within the crypts of the epithelium seemingly out of reach. Additionally, it is unclear what selective advantage (if any) would be gained by *H. pylori* through the deregulation of homeostasis in the gastric epithelium. As *H. pylori* has colonized humans throughout our evolution, and gastric cancer does not develop until late in life, it is possible instead that *H. pylori*'s carcinogenicity is simply an accidental side-effect of us living too long.

APPENDIX

FIGURES AND TABLE

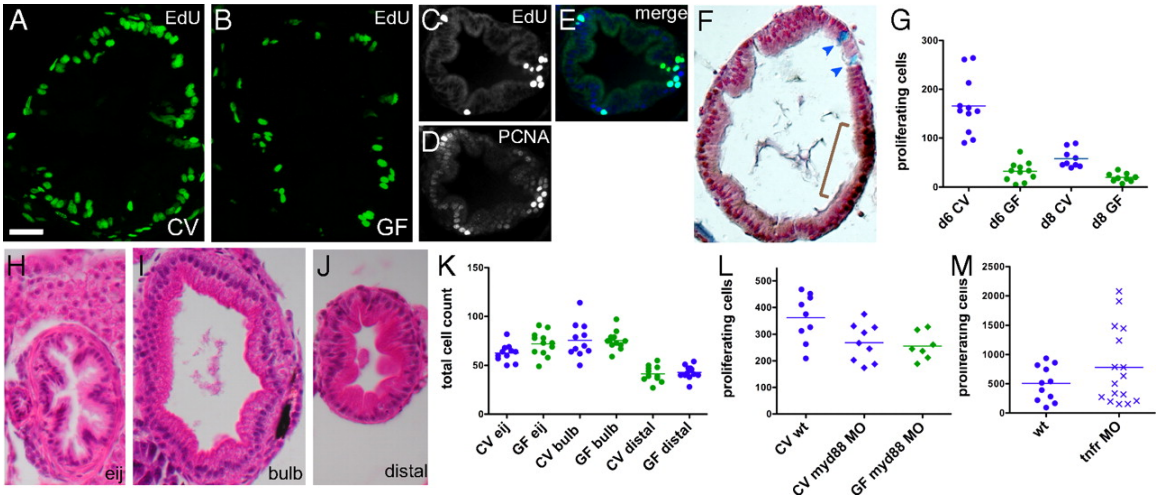

Fig. 1. Microbes induce epithelial cell proliferation in the zebrafish larval intestine via Myd88 and not inflammation. Transverse sections of the intestinal bulb of 6-dpf larvae reared CV (A) or GF (B) labeled with EdU to reveal S-phase cells are shown. A transverse section of the distal intestine shows colocalization of EdU-labeled (C) and PCNA-labeled (D) cells (E, merge). (F) Distinct cell populations are stained with Alcian blue, marking differentiated goblet cells (arrowheads), and anti-PCNA, marking mitotic cells (brown bracket). (G) Total numbers of S-phase cells over 30 serial sections in the intestinal bulb of individual 6-dpf larvae are represented for the treatment groups and genotypes indicated. Here and in subsequent figures, the genotype and microbial exposure of each larva are indicated by the shape and color of the data point, respectively. Significantly fewer BrdU-labeled cells were found in 6- and 8-dpf larvae reared GF vs. CV ($P < 0.001$). H&E sections of 8-dpf GF intestines at three locations within the intestinal tract are shown: the esophageal intestinal junction (H, eij), defined as position 0; the bulb, 30 sections caudal to the junction (I); and the distal intestine, 60 sections caudal to the junction (J). (K) There was no significant difference between total intestinal epithelial cell counts at the three positions described above in 8-dpf CV vs. GF larvae. (L) Significantly fewer EdU-labeled cells were found in CV or GF myd88 MO vs. CV WT (wt) ($P < 0.05$). (M) There was no significant difference in the numbers of EdU-labeled cells between CV vs. GF myd88 MO or between wt vs. tnfr MO. (Scale bars: A and B, 15 μM; C–F and H–J, 25 μM.)

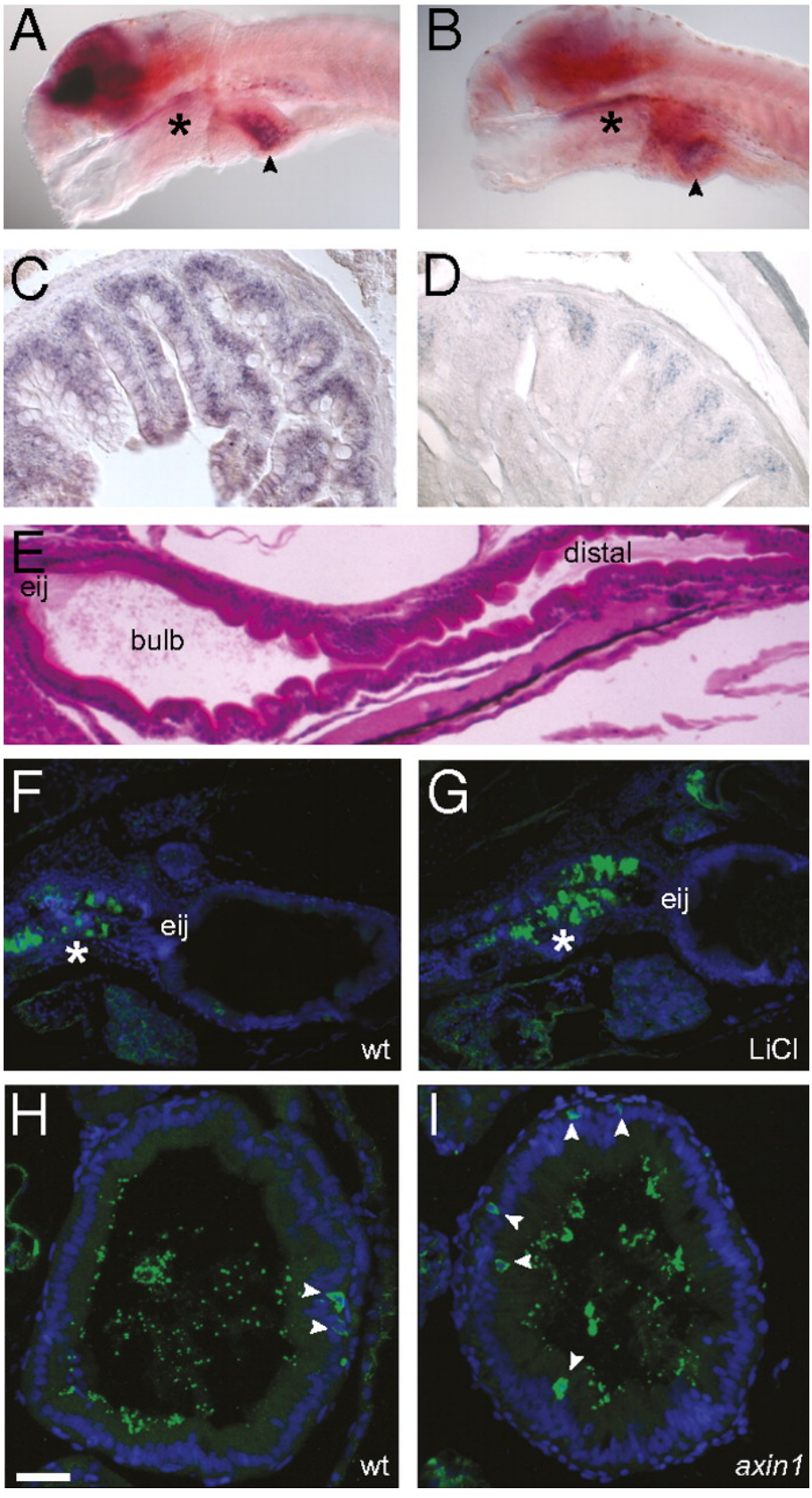
A
B
C
D
E
eij
bulb
distal
F
eij
wt
G
eij
LiCl
H
wt
I
axin1

Fig. 2. Wnt signaling occurs in the larval zebrafish intestine. (A) In situ hybridization with a tcf4 probe reveals strong brain expression as well as signal in the pharynx (*) and intestinal bulb (arrowhead) in a 6-dpf larva. (B) In situ hybridization with a sox9b probe reveals expression in the brain, pharynx (*), and intestinal bulb (arrowhead) of a 6-dpf larva. Sections of adult proximal intestine displaying expression of tcf4 (C) and sox9b (D) are shown. (E) H&E sagittal section of a 5-dpf larva showing the location of the esophageal intestinal junction (eij), bulb, and distal intestine. Sagittal sections of 6-dpf untreated (F) and LiCl-treated (G) larvae show β-catenin expression (green) in the pharynx and esophagus (*). Nuclei are stained with DAPI (blue). Transverse sections through the intestinal bulb of 6-dpf WT (wt) (H) and axin1 mutant (I) larvae show scattered cells with cytoplasmic β-catenin staining (green, arrowheads). Luminal microbes cross-react with the anti-β-catenin antisera. Nuclei are stained with Torpo (blue). (Scale bars: A and B, 100 μM; C and D, 25 μM; E–G, 50 μM; H and I, 15 μM.)

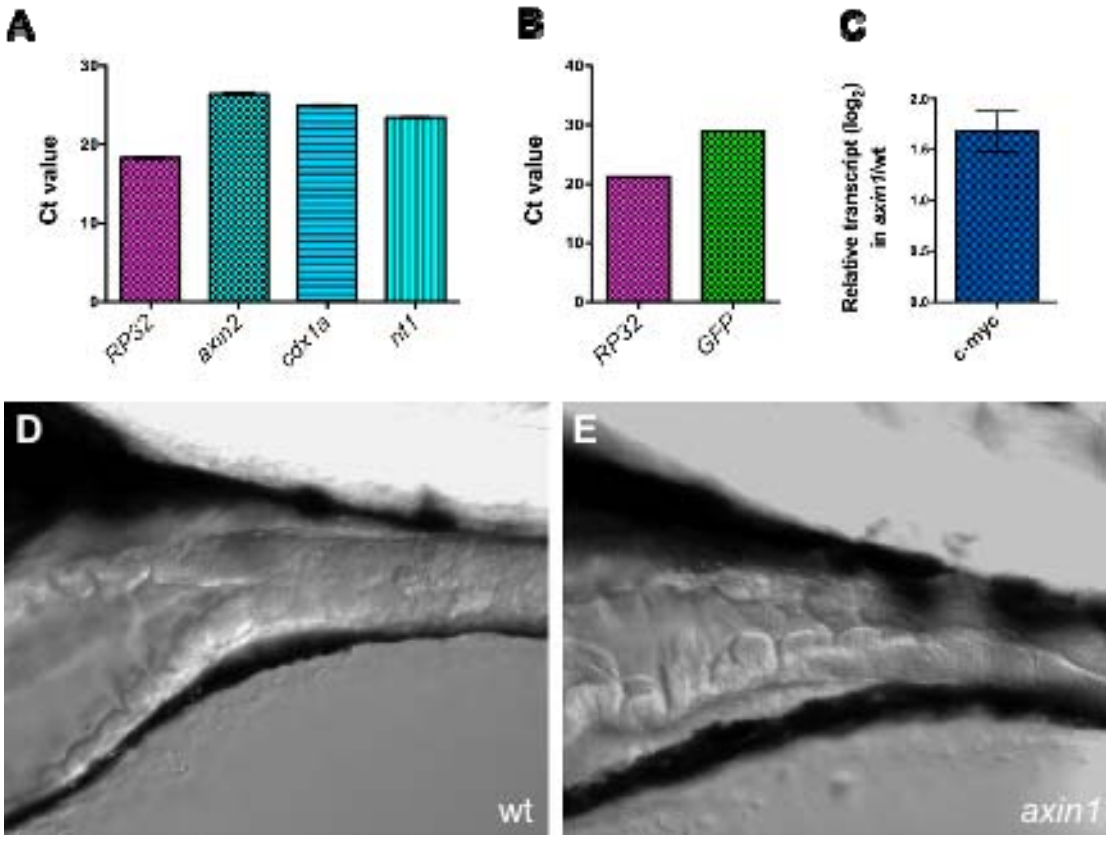

Fig. 3. Wnt signaling in the larval zebrafish intestine. Amplification of Wnt target genes and reporter from 6 dpf WT (A) and topD (B) larvae. The relative cycle threshold (Ct) values for a housekeeping gene (rp32) and the indicated Wnt target genes axin1, cdx1a, and nt1 (A) or the gfp reporter (B) are shown. The mean and SD of three triplicate reactions are shown. For all primer pairs, a no-template control reaction yielded no detectable product (i.e., Ct = 40). (C) Relative abundance (log2) of c-myc amplified from axin1 vs. WT (wt) 6-dpf larval intestines is shown. Levels of c-myc were normalized to levels of rp32 for each sample. Error bars represent upper and lower limits, based on the SD of the ΔCt values. (D and E) Intestines of live 6-dpf wt and axin1 larvae. The axin1 intestine appeared thicker and more convoluted.

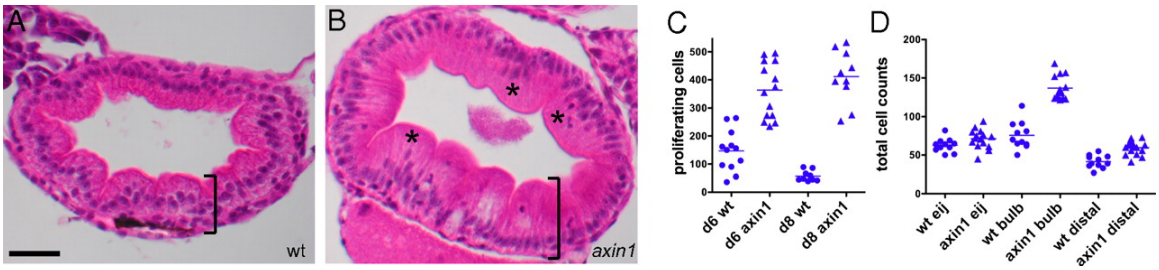

Fig. 4. Up-regulation of Wnt signaling causes intestinal hyperplasia. (A) H&E-stained section of an 8-dpf WT (wt) intestinal bulb shows an orderly array of cells in the intestinal epithelium. (B) An 8-dpf axin1 mutant intestine is thicker (bracket) and has more disorganized epithelial cells (*). (C) At both 6 and 8 dpf, axin1 mutants have significantly more BrdU-labeled cells within the intestinal epithelium than wt ($P < 0.0001$). (D) At 8 dpf, axin1 mutant intestines have significantly more epithelial cells in the bulb and distal intestine vs. wt ($P < 0.0001$). (Scale bar: A and B, 25 μM.)

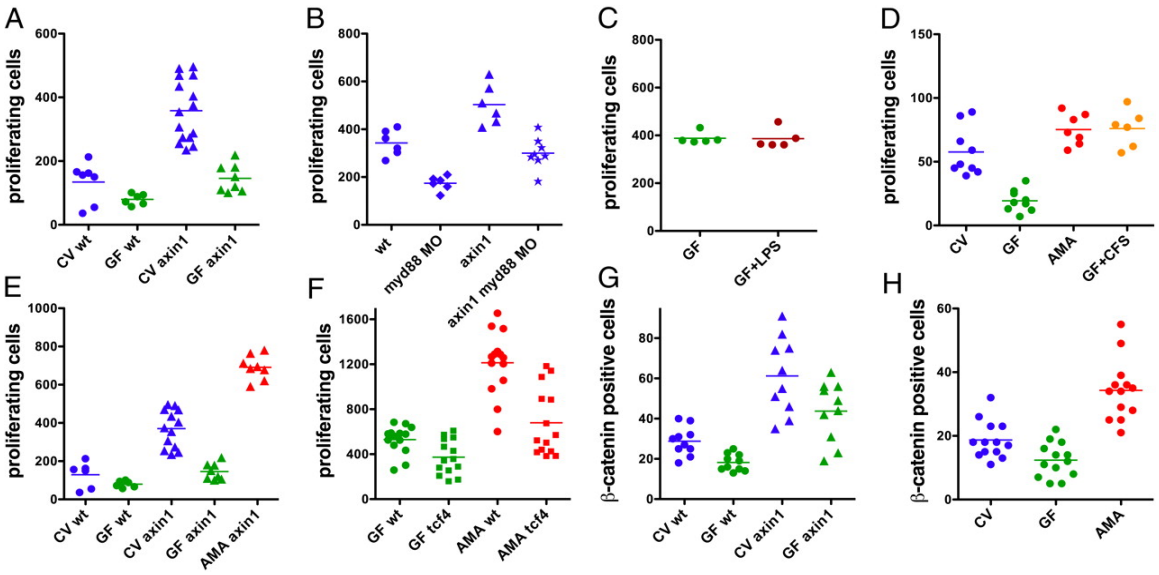

Fig. 5. Combinatorial effects of microbial and Wnt signaling on intestinal epithelial cell proliferation. (A–F) S-phase intestinal epithelial cells were quantified in 30 serial sections in the intestinal bulb of individual larvae as in Fig. 1. To label S-phase nuclei, the experiments in A and D used BrdU and the experiments in B, C, E, and F used EdU. All experiments were performed with 6-dpf larvae except the experiment in D, which was performed with 8-dpf larvae. (A) CV axin1 had significantly more proliferating intestinal cells than CV WT (wt), GF wt, and GF axin1 ($P < 0.0001$). GF axin1 had significantly more proliferating cells than GF wt ($P < 0.01$). (B) axin1 had significantly more proliferating cells than wt, myd88 MO, and axin1 myd88 MO ($P < 0.001$). axin1 myd88 MO had significantly more proliferating cells than myd88 MO ($P < 0.001$). (C) Exposure of GF larvae from 3 to 6 dpf to 30 μg/mL LPS caused no change in cell proliferation relative to untreated GF larvae. (D) Monoassociation of GF larvae with *A. veronii* (AMA) or treatment with 500 ng/mL *A. veronii* CFS induced a significant increase in intestinal cell proliferation relative to GF ($P < 0.0001$). (E) axin1 mutants monoassociated with *A. veronii* had significantly more proliferating cells than CV axin1 ($P < 0.0001$). (F) tcf4 mutants had significantly fewer dividing intestinal cells than wt siblings when reared GF ($P < 0.01$) and when monoassociated with *A. veronii* ($P < 0.0001$). Intestinal epithelial cells with cytoplasmic β-catenin were quantified in 30 serial sections in the intestinal bulb of 6-dpf larvae. (G and H) CV wt larvae had significantly more β-catenin-positive cells than GF wt larvae ($P < 0.05$). (G) CV axin1 had significantly more β-catenin-positive cells than GF axin1 ($P < 0.05$). (H) *A. veronii* monoassociated larvae had significantly more β-catenin-positive cells than CV animals ($P < 0.0001$).

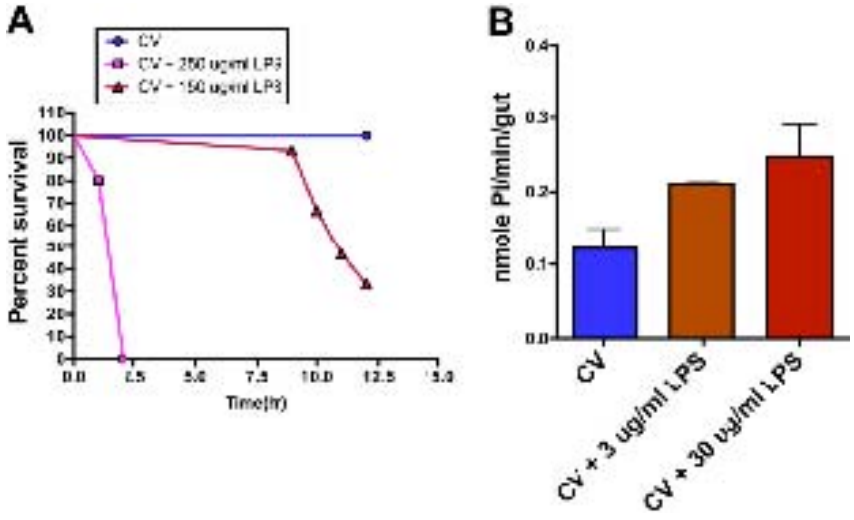

Fig. 6. LPS does not induce intestinal epithelial cell proliferation. (A) Exposure of CV 5-dpf larvae to LPS (E. coli serotype 0111:B4, product no. 62325, lot no. 0001418664; Sigma) at 150 and 250 µg/mL caused dose-dependent killing (n = 45 larvae per treatment). (B) CV larvae exposed to 3 and 30 µg/mL LPS from 6 to 8 dpf exhibited elevated levels of intestinal alkaline phosphatase activity (two replicate groups of ≈10 larvae were measured for each treatment; error bars indicate SD).

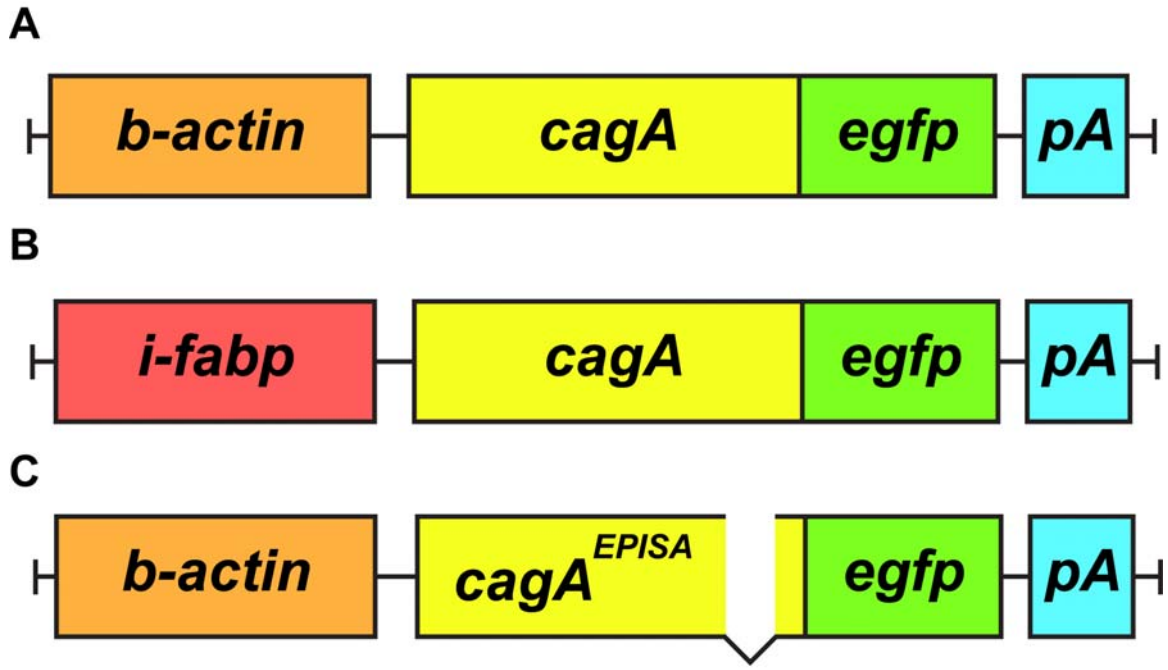

Fig. 7. Transgenic constructs (A) The *cagA:egfp* fusion cassette was cloned downstream of the 5.3kb *b-actin* promoter fragment. (B) The *cagA:egfp* fusion cassette was cloned downstream of the 1.6kb *i-fabp* promoter fragment. (C) The *cagA*^{EPISA}*:egfp* fusion cassette was cloned downstream of the 5.3kb *b-actin* promoter fragment.

Fig. 8. ***cagA***^{EPISA} (A) The phosphorylation resistant *cagA*^{EPISA} allele lacks EPIYA motifs for phosphorylation by Src family kinases

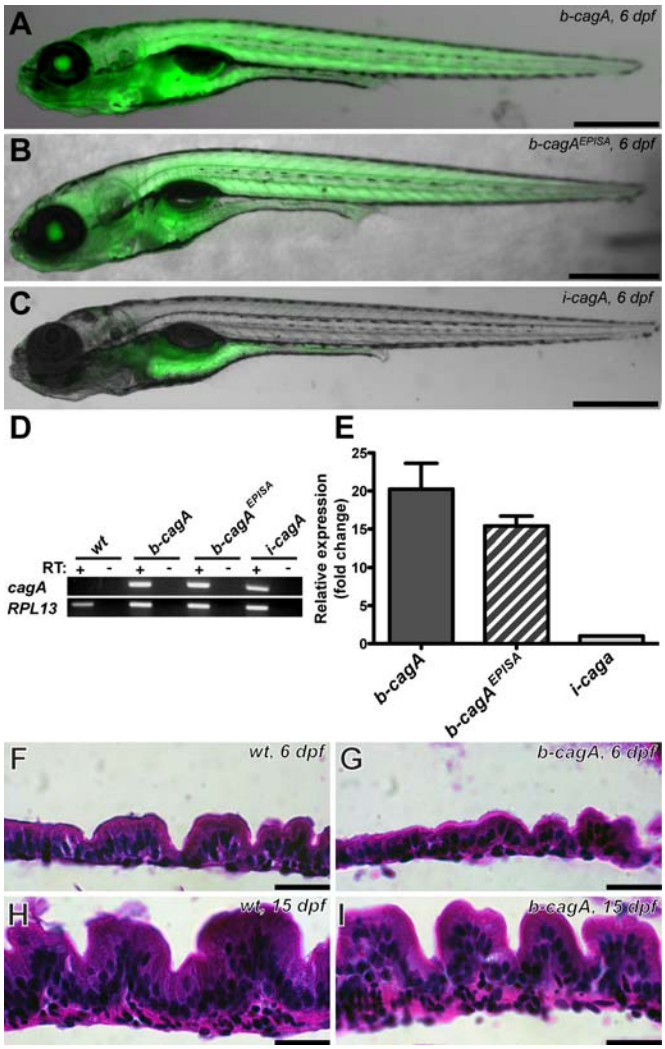

Fig. 9. Development of CagA+ transgenic zebrafish (A) ubiquitous CagA:EGFP fusion protein expression driven by the *b-actin* promoter. (B) ubiquitous CagA^{EPISA}:EGFP fusion protein expression driven by the *b-actin* promoter. (C) intestinal CagA:EGFP fusion protein expression driven by the *i-fabp* promoter. (D) RT-PCR of dissected larval intestine showing expression of *cagA* and *RPL13* housekeeping control gene at 6 dpf. (E) quantitative RT-PCR of dissected adult intestines showing relative expression levels of *cagA* transcript in transgenic lines at 1 year of age. (normalized to SDHA and β-actin) (F) H&E stained sagittal section of wild-type zebrafish intestine at 6 dpf. (G) H&E stained sagittal section of *b-cagA* transgenic zebrafish intestine at 6 dpf. (H) H&E stained sagittal section of wild-type transgenic zebrafish intestine at 15 dpf. (I) H&E stained sagittal section of *b-cagA* transgenic zebrafish intestine at 15 dpf.

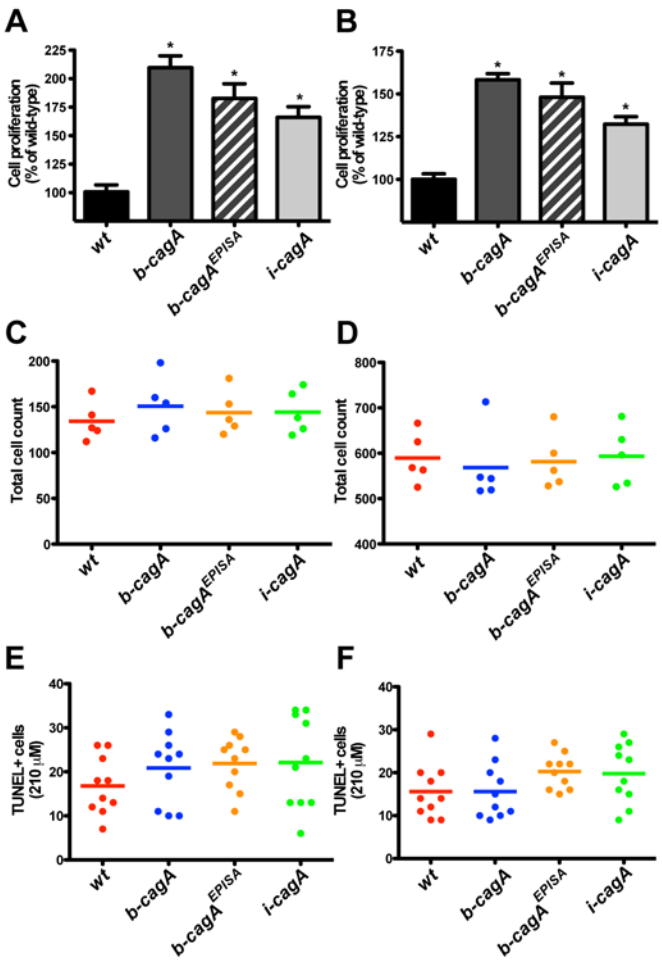

Fig. 10. CagA expression causes overproliferation of the intestinal epithelium (A & B) Intestinal epithelial cell proliferation at 6 dpf and 15 dpf, respectively. Bars represent proliferation as a percentage of wild-type. (* = p<.05, One-way ANOVA with Tukey's test)

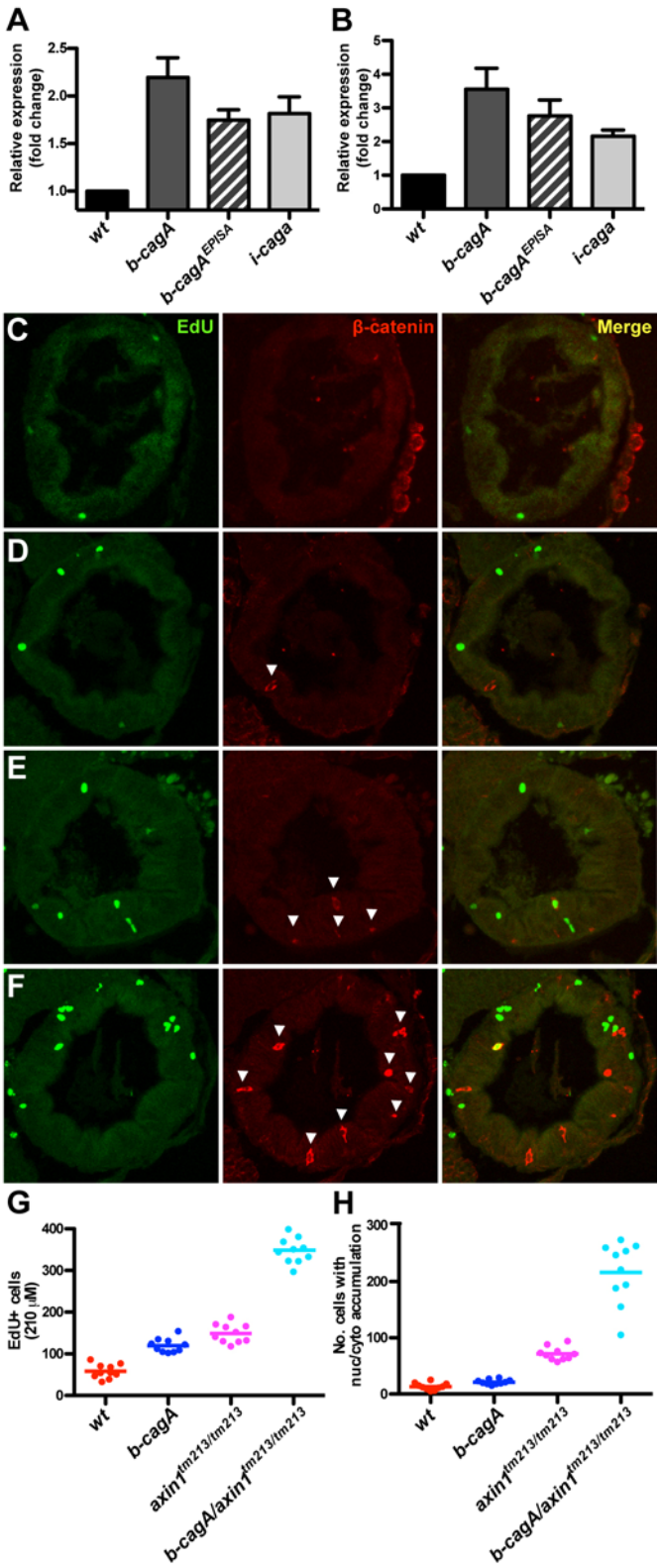
A
Relative expression (fold change)
2.5
2.0
1.5
1.0
wt
b-cagA
b-cagA^EPISA
i-caga
B
Relative expression (fold change)
5
4
3
2
1
0
wt
b-cagA
b-cagA^EPISA
i-caga
C
EdU
β-catenin
Merge
D
E
F
G
EdU+ cells (210 μM)
400
300
200
100
0
wt
b-cagA
axin1^tm213/tm213
b-cagA/axin1^tm213/tm213
H
No. cells with nuc/cyto accumulation
300
200
100
0
wt
b-cagA
axin1^tm213/tm213
b-cagA/axin1^tm213/tm213

Fig. 11. CagA activates canonical Wnt signaling in the intestinal epithelium (A) Quantitative RT-PCR data showing relative expression levels of the Wnt target gene *mycA*. (normalized to SDHA and β-actin) (B) Quantitative RT-PCR data showing relative expression levels of the Wnt target gene *cyclinD1*. (normalized to SDHA and β-actin) (C-F) Immunofluorescence micrograph showing number of proliferating cells (EdU, green) and cells with nuclear/cytoplasmic accumulation of β-catenin (red staining & white arrowheads) in intestinal epithelium of wild-type (C), *b-cagA* (D), *axin1*^{tm213} (E), and *b-cagA/axin1*^{tm213} (F) animals at 6 dpf. (G) Quantification of proliferating (EdU+) cells. (H) Quantification of cells with nuclear/cytoplasmic accumulation of β-catenin.

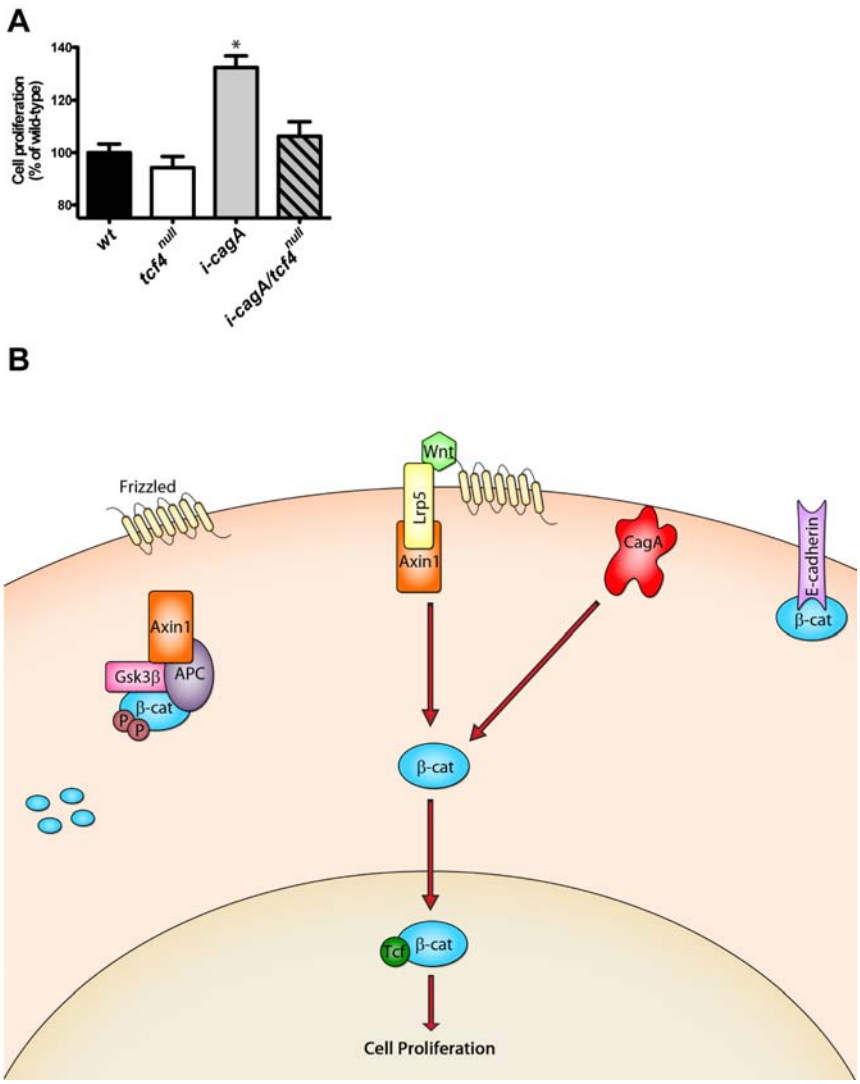

Fig. 12. CagA-dependent Overproliferation of the Intestinal Epithelium Requires *tcf4*. (A) Intestinal epithelial cell proliferation at 15 dpf. Bars represent proliferation as a percentage of wild-type. (* = p<.05, One-way ANOVA with Tukey's test) (B) Proposed mechanism for CagA-dependent overproliferation of the intestinal epithelium.

Gene	Forward (5'-3')	Reverse (5'-3')
Succinate dehydrogenase (SDHA)	GeNorm zebrafish reference gene kit	GeNorm zebrafish reference gene kit
β-actin	GeNorm zebrafish reference gene kit	GeNorm zebrafish reference gene kit
cagA	tggagggcctactggtgggga	tcaggcggtaagccttgtatgtcgg
myca	ccagcagcagtggcagcgat	ggggactggggtacctcgactct
cyclinD1	aggcttttgaaacgtaagcctgcgg	aggtacacttgggcatccgtgca

Table 1. Primers used for quantitative real-time PCR

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Chapter IV

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