**H. pylori virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model**

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**SUMMARY**

Infection with *Helicobacter pylori* is a major risk factor for the development of gastric cancer, and infection with strains carrying the virulence factor CagA significantly increases this risk. To investigate the mechanisms by which CagA promotes carcinogenesis, we generated transgenic zebrafish expressing CagA ubiquitously or in the anterior intestine. Transgenic zebrafish expressing either the wild-type or a phosphorylation-resistant form of CagA exhibited significantly increased rates of intestinal epithelial cell proliferation and showed significant upregulation of the Wnt target genes *cyclinD1, axin2* and the zebrafish *c-myc* ortholog *myca*. Coexpression of CagA with a loss-of-function allele encoding the β-catenin destruction complex protein Axin1 resulted in a further increase in intestinal proliferation. Coexpression of CagA with a null allele of the key β-catenin transcriptional cofactor Tcf4 restored intestinal proliferation to wild-type levels. These results provide in vivo evidence of Wnt pathway activation by CagA downstream of or in parallel to the β-catenin destruction complex and upstream of Tcf4. Long-term transgenic expression of wild-type CagA, but not the phosphorylation-resistant form, resulted in significant hyperplasia of the adult intestinal epithelium. We further utilized this model to demonstrate that oncogenic cooperation between CagA and a loss-of-function allele of ps3 is sufficient to induce high rates of intestinal small cell carcinoma and adenocarcinoma, establishing the utility of our transgenic zebrafish model in the study of CagA-associated gastrointestinal cancers.

**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 (Blaser and Atherton, 2004). Although gastric cancer occurs in fewer than 1% of people colonized by *H. pylori* (Amieva and El-Omar, 2008), it is still the second-most common cause of cancer mortality worldwide (Peek and Blaser, 2002), and more than 50% of gastric adenocarcinomas can be attributed to infection with *H. pylori* (Asghar and Parsonnet, 2001). 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 the carcinogenicity of *H. pylori*, 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 (Censini et al., 1996). 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 (Bourzac and Guillemin, 2005).

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 (Selbach et al., 2002; Stein et al., 2002). After 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 (Amieva et al., 2003; Churin et al., 2003; Higashi et al., 2002; Tsutsumi et al., 2003; Mimuro et al., 2002). 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 (Suzuki et al., 2009). 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 the observed oncogenic effects of CagA and a host signaling pathway frequently deregulated in gastrointestinal cancers (Franco et al., 2005).

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 (Barker et al., 2010; Sato et al., 2011; Pinto et al., 2003; Ootani et al., 2009; Sato et al., 2004). On the other hand, activation of Wnt signaling has been shown to result in cancers of the stomach...
Although the mechanisms of interactions of CagA with the Wnt pathway downstream of or in parallel to the β-catenin destruction complex and upstream of the β-catenin transcriptional cofactor Tcf4. Additionally, we demonstrate that long-term expression of wild-type CagA, but not the phosphorylation-resistant form, is sufficient to induce pathologic intestinal hyperplasia in adults and that oncogenic cooperation between the cagA transgene and a loss-of-function allele of p53 results in high rates of intestinal adenocarcinoma and small cell carcinoma.

RESULTS

Generation of CagA-expressing transgenic zebrafish.

In order to generate cagA transgenic animals, we cloned the cagA gene from H. pylori strain G27. Strain G27 was originally isolated from Grosseto Hospital (Tuscany, Italy), and has been used extensively in research on the CagA virulence factor (Amieva et al., 2003; El-Etr et al., 2004; Guillemin et al., 2002; Segal et al., 1999). 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 5.3 kb beta-actin (b-) (Higashijima et al., 1997)
RESEARCH ARTICLE

CagA transgenic zebrafish model

To determine the effects of CagA expression on the larval zebrafish intestine, we examined wild-type and CagA transgenic animals at 6 dpf, by which time autonomous feeding has begun, and at 15 dpf, by which time intestinal folding is complete (Ng et al., 2005). CagA-expressing zebrafish larvae showed normal intestinal development (Fig. 2A,B) and were histologically indiscernible from wild-type clutch-mates (Fig. 2C,D). In addition, the CagA-expressing larvae exhibited no gross abnormalities in cell junctions, as assessed by staining with a pan-cadherin antibody (supplementary material Fig. S2). We next sought to establish the effects of CagA on larval intestinal proliferation, because CagA had been previously shown to increase epithelial cell proliferation in vitro and in vivo (Mimuro et al., 2002; Nagy et al., 2011). To determine the proliferation state of CagA-expressing intestines, we analyzed animals at 6 and 15 dpf that had been exposed to the nucleotide analog 5-ethyl-2′-deoxuryridine (EdU) for ~10 hours and counted 5-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 dpf (Fig. 2E,F). To determine whether this increase in proliferation had an effect on the cell census, we quantified total epithelial cell number in single hematoxylin and eosin (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 (Fig. 2G,H), indicating that expression of CagA caused increased turnover of intestinal epithelial cells. Increased intestinal cell turnover would require an increase in cell death; however, consistent with previous reports and due to the transient nature of extruded apoptotic cells (Crosnier et al., 2005), we observed very few TUNEL-positive cells in the intestines of wild-type and CagA-expressing animals (Fig. 2I), with no significant difference observed between the two groups. Finally, the intestinal epithelia of b-cagA animals did not display an increased number of local neutrophils at 8 dpf, indicating a lack of CagA-induced intestinal inflammation at this stage (supplementary material Fig. S3).

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 (Cheesman et al., 2011). 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 (Franco et al., 2005; Suzuki et al., 2009; Nagy et al., 2011). Accordingly, we examined whether CagA expression was capable of activating the Wnt signaling pathway in the zebrafish intestine at different developmental stages. 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 Wnt target genes c-myc (myca) (He et al., 1998), axin2 (Yan et al., 2001) and cyclinD1 (Tetsu and McCormick, 1999) were modestly increased in all CagA-expressing lines relative to the wild-type strain (Fig. 3A–C). We next asked whether CagA was capable
of inducing β-catenin accumulation in epithelial cells of the larval intestine, 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 with wild-type animals (Fig. 3D,E). The fact that EdU labeling was not usually coincident with cytoplasmic and nuclear accumulation of β-catenin is probably due to the fact that, whereas relocalization of β-catenin is a transient event, the EdU-labeled cells had undergone S-phase any time during the 12-hour labeling period.

In order to assess the significance of CagA-induced β-catenin accumulation, we next compared the intestinal β-catenin accumulation observed in CagA-expressing animals to that of a known Wnt signaling mutant, axin1tm213. The axin1tm213 homozygotes 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 until 8 dpf, allowing study of the juvenile intestine (Heisenberg et al., 2001; van de Water et al., 2001). As expected, we observed 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 axin1tm213 mutants, consistent with constitutively activated Wnt signaling (Fig. 3E).

We reasoned that if CagA were capable of activating Wnt signaling upstream of the β-catenin destruction complex, then axin1tm213 homozygotes should be refractory to CagA-induced accumulation of β-catenin and levels of β-catenin accumulation in b-cagA; axin1tm213(tm213) double mutants should resemble those of axin1tm213 homozygotes. Instead, when we generated b-cagA, axin1tm213(tm213) fish, we found that expression of CagA in axin1 homozygous mutants resulted in a dramatic increase in cell proliferation and β-catenin accumulation (Fig. 3F). 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 was strongly correlated with increased epithelial proliferation (Fig. 3G,H), suggesting that CagA stimulates proliferation through activation of the Wnt pathway.

**CagA-dependent overproliferation of the intestinal epithelium requires tcf4**

To determine whether CagA-induced overproliferation of the intestinal epithelium is 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 (Muncan et al., 2007). We reasoned that if the pro-proliferative effects of CagA were acting upstream of Tcf4, rates of intestinal proliferation in i-cagA; tcf4flox/flox double mutants should be identical to those observed in tcf4flox/flox animals. As previously observed, i-cagA animals showed a significant increase in proliferation over the wild type, whereas tcf4flox/flox mutants showed levels of intestinal proliferation similar to wild-type animals (Fig. 4). Rates of intestinal proliferation in i-cagA; tcf4flox/flox larvae were statistically indistinguishable from wild-type and tcf4flox/flox mutants, indicating that CagA requires Tcf4 function to increase intestinal epithelial proliferation. This result places activation of the Wnt signaling pathway by CagA downstream of or in parallel to Axin1 and upstream of Tcf4 (supplementary material Fig. S4).

**CagA expression causes phosphorylation-dependent intestinal hyperplasia in adult zebrafish**

*M. pylori*-associated gastric adenocarcinoma occurs as a result of lifelong exposure to the bacterium, with CagA+ strains posing a

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**Fig. 2. CagA expression causes overproliferation of the intestinal epithelium.** (A,B) H&E stained sagittal sections of wild-type (A) and b-cagA transgenic (B) zebrafish intestine at 6 dpf. (C,D) H&E stained sagittal sections of wild-type (C) and b-cagA transgenic (D) zebrafish intestine at 15 dpf. (E,F) Intestinal epithelial cell proliferation at 6 dpf (E) and 15 dpf (F). Bars represent proliferation (mean ± s.e.m.) as a percentage of wild-type; n=10, *P<0.05 using one-way ANOVA with Tukey’s test. (G,H) Total intestinal epithelial cell counts of single H&E stained midline sagittal sections at 6 dpf (G) and 15 dpf (H). (I,J) TUNEL-positive cells in the intestinal epithelium at 6 dpf (I) and 15 dpf (J). Scale bars: 10 μm.
CagA transgenic zebrafish model

RESEARCH ARTICLE

significantly greater cancer risk (Asghar and Parsonnet, 2001). In order to study the long-term effects of CagA exposure in our model, we performed histological analysis of adult \( b\)-cagA, \( i\)-caga and \( b\)-cagaEPISA animals at 1 year of age. Wild-type adults (18 months post-fertilization) served as controls. Upon examination, no hyperplastic or neoplastic lesions were found in any of the wild-type controls (Fig. 5A,G; supplementary material Table S1). A proportion of the \( b\)-cagA and \( i\)-caga individuals exhibited significant intestinal epithelial hyperplasia at 1 year of age (Fig. 5B,C). Surprisingly, despite the significant increases in proliferation and Wnt activation observed in younger \( b\)-cagaEPISA animals, no hyperplasia was observed in age-matched adults of this genotype (Fig. 5D). These data suggest that although the phosphorylation-independent activation of Wnt signaling by CagA is sufficient to induce sustained overproliferation of the larval intestinal epithelium, it is not sufficient to induce significant hyperplastic changes in the adult intestinal epithelium, as seen in the groups expressing the non-mutant CagA, either ubiquitously or in an intestine-specific manner.

Coexpression of the \( cagA \) transgene with a \( p53 \) loss-of-function allele results in high rates of intestinal adenocarcinoma

The tumor suppressor gene \( p53 \) is frequently mutated in diffuse- and intestinal-type gastric cancers (Nobili et al., 2011; Ranzani et al., 1995), and gastric adenocarcinomas isolated from CagA+ \( H. pylori \)-infected patients exhibit frequent mutation in \( p53 \) (Shibata et al., 2002). Additionally, CagA has been shown to subvert the tumor suppressor function of the apoptosis-stimulating protein ASPP2 in cultured cells, leading to enhanced degradation of \( p53 \) (Buti et al., 2011). In order to examine the potential for oncogenic cooperation between the \( cagA \) transgene and \( p53 \) we bred \( b\)-cagA and \( i\)-caga animals to animals homozygous for a loss-of-function allele of \( p53 \) (\( tp53^{M214K} \)) to obtain \( b\)-cagA; \( tp53^{M214K/M214K} \) or \( i\)-caga; \( tp53^{M214K/M214K} \) animals. The zebrafish ortholog of \( p53 \) is highly conserved in both structure and function and the \( tp53^{M214K} \) DNA-binding domain mutation is orthologous to methionine 246 missense mutations previously identified in human tumors (Storer and Zon, 2010). At 1 year post-fertilization, all of the \( tp53^{M214K/M214K} \) fish failed to thrive and exhibited high rates of ocular malignant peripheral nerve sheath tumors, recapitulating previous studies using this \( p53 \) allele (Berghmans et al., 2005). An insufficient number of \( b\)-cagA; \( tp53^{M214K/M214K} \) individuals survived to this time point for analysis, but we were able to examine small numbers of both \( tp53^{M214K/M214K} \) and \( i\)-caga; \( tp53^{M214K/M214K} \) (Fig. 5E,F) lines. In both lines, we observed examples of intestinal epithelial hyperplasia and definitive neoplasia (Fig. 5G).

In the affected genotypes displaying hyperplastic changes, the intestinal mucosa was thrown into irregular and haphazard folds lined by a ragged and thickened epithelium often 2-6 cells deep with pseudostratification of nuclei, which was most prominent within invaginations between the mucosal villi (mucosal sulci). Infolding of the hyperplastic epithelium frequently resulted in formation of mucosal pseudocrypts, with the most severely affected intestines also displaying frequent epithelial fusion between adjacent mucosal folds. In addition, numerous apecitic intestinal epithelial cells were observed and directly reflected rapid epithelial cell proliferation and turnover. Small numbers of a chronic inflammatory cell infiltrate, composed mostly of...
lymphocytes and few eosinophilic granule cells, were seen percolating through the hyperplastic epithelium in many areas. Foci of dysplastic intestinal epithelial cells were often identified in hyperplastic areas, usually within mucosal sulci. Dysplastic cells demonstrated progressive disorganization including ‘piling-up’ of cells and loss of nuclear polarity, nuclear and cytoplasmic pleomorphism, hyperchromatic elongated nuclei and inconspicuous nucleoli with sparse cytoplasm (increased nuclear to cytoplasm ratio) and occasional bizarre mitotic figures. In all cases where dysplastic cells were observed, there was no invasion through the basement membrane (i.e. carcinoma in situ) except for one fish in the \( tps3^{M214K/M214K} \) group, which had a solitary maxillary (upper jaw) focus of carcinoma in situ within the oropharyngeal cavity. When definitive intestinal neoplasia was seen, adenocarcinoma was most often found in the anterior intestine and small cell carcinoma in the anterior or mid-intestine.

Adenocarcinomas displayed variable degrees of differentiation, ranging from well to poorly differentiated, with a tendency to form disorganized and cribriform acinar-like pseudocysts that penetrated deep into the lamina propria, in the absence of an interceding basement membrane. Individual tumor cells had hyperchromatic, ovoid to elongated nuclei with granular chromatin, multiple small nucleoli and sparse basophilic cytoplasm. In less differentiated adenocarcinomas, bizarre mitotic figures were occasionally seen. Locally extensive fibrogenesis within the lamina propria (intraproprial desmoplasia) and variable numbers of chronic inflammatory cell infiltrates, comprised of intermingled lymphocytes and eosinophilic granule cells, were often associated with the adenocarcinomas. The two small cell carcinomas identified in the \( i-cagA; tps3^{M214K/M214K} \) group were composed of densely cellular nests of polygonal to fusiform cells, lacking an organoid pattern, which infiltrated deep into the lamina propria and were not associated with pseudocysts. Individual neoplastic cells within nests had pleomorphic, deeply basophilic nuclei with dense granular chromatin, inconspicuous nucleoli and minimal cytoplasm. Solitary necrotic tumor cells were seen in some of the nests, accompanied by small aggregates of lymphocytes. Lymphovascular invasion and distant metastasis was not observed in either of the tumor types. Incidence and overall severity of lesions within the expression domain of the \( cagA \) transgene were higher in \( i-cagA; tps3^{M214K/M214K} \) animals than in the corresponding anatomical region of \( tps3^{M214K/M214K} \) animals (Fig. 5G; supplementary material Table S1). These data indicate that expression of \( CagA \) with concomitant \( pS3 \) loss is sufficient to induce high rates of adenocarcinoma and small cell carcinoma in the zebrafish intestine and demonstrate the utility of our model for the study of \( CagA \)-associated gastrointestinal cancers.

**DISCUSSION**

Here, we describe the development of a novel \( in vivo \) model of \( CagA \)-induced intestinal pathology in zebrafish that recapitulates major hallmarks of \( CagA \) pathogenesis observed in cell culture and murine models such as increased epithelial proliferation, cellular accumulation of \( \beta \)-catenin and intestinal hyperplasia (Ohnishi et al., 2008; Mimuro et al., 2002; El-Etr et al., 2004; Suzuki et al., 2005; Murata-Kamiya et al., 2007; Nagy et al., 2011). We utilized transgenic expression of \( CagA \) to investigate how the \( H. pylori \) virulence factor \( CagA \) is able to disrupt normal programs of intestinal epithelial renewal via activation of an important host signaling pathway, the Wnt pathway, to cause significant overproliferation of an intact epithelium \( in vivo \). We show that activation of canonical Wnt signaling upstream of the essential \( \beta \)-catenin cofactor Tcf4 and downstream of the \( \beta \)-catenin destruction complex is required for the early effects of \( CagA \) on intestinal epithelial proliferation.

We further utilized our novel transgenic zebrafish system to demonstrate that long-term expression of \( CagA \) is sufficient to cause intestinal hyperplasia in adult zebrafish. Notably, although expression of the phosphorylation-resistant \( b-cagA^{EPSSA} \) allele is capable of inducing significant sustained overproliferation of the larval intestinal epithelium coupled with increased Wnt activation, it failed to induce significant intestinal hyperplasia in adult animals. These data corroborate a previous study using a \( CagA \) transgenic mouse model, which demonstrated that the ability of \( CagA \) to induce severe epithelial hyperplasia \( in vivo \) is correlated with its capacity to be phosphorylated by host kinases (Ohnishi et al., 2008). It is possible that the activation of Wnt signaling by \( CagA \) and subsequent induction of proliferation act in concert with further oncogenic stimuli, which might occur in the form of previously observed phosphorylation-dependent events such as epithelial depolarization (Arnieva et al., 2003) or ERK activation by \( CagA \) (Higashi et al., 2004). These data illustrate the utility of long-term \( in vivo \) modeling of \( CagA \) pathogenesis because the cumulative effects of \( CagA \) expression cannot be predicted from the transient cellular responses it elicits.

Host genetics play a significant role in the development of \( H. pylori \)-associated gastric cancer. For example, certain alleles of the host genes \( p53 \), \( IL-1\beta \) and \( IL-10 \) are strongly correlated with the development of gastric adenocarcinoma in \( H. pylori \)-infected humans (Shibata et al., 2002; El-Omar et al., 2003). Transgenic expression of \( CagA \) in mice was sufficient to cause gastric and intestinal carcinomas, but these only developed in less than 5% of the animals (Ohnishi et al., 2008). We observed high rates of intestinal neoplasia in our \( CagA \) transgenic zebrafish model when expressed with a mutant allele of the tumor suppressor \( p53 \). These data provide the first direct \( in vivo \) evidence for oncogenic cooperation between \( CagA \) and \( p53 \) and provide a robust model of \( CagA \)-induced carcinoma. Our results are consistent with previous findings of increased \( p53 \) mutational frequency in \( H. pylori \)-infected humans (Shibata et al., 2002; El-Omar et al., 2003). Transgenic expression of \( CagA \) in mice was sufficient to cause gastric and intestinal carcinomas, but these only developed in less than 5% of the animals (Ohnishi et al., 2008). We observed high rates of intestinal neoplasia in our \( CagA \) transgenic zebrafish model when expressed with a mutant allele of the tumor suppressor \( p53 \). These data provide the first direct \( in vivo \) evidence for oncogenic cooperation between \( CagA \) and \( p53 \) and provide a robust model of \( CagA \)-induced carcinoma. Our results are consistent with previous findings of increased \( p53 \) mutational frequency in \( H. pylori \)-infected humans (Shibata et al., 2002; El-Omar et al., 2003). Transgenic expression of \( CagA \) in mice was sufficient to cause gastric and intestinal carcinomas, but these only developed in less than 5% of the animals (Ohnishi et al., 2008). We observed high rates of intestinal neoplasia in our \( CagA \) transgenic zebrafish model when expressed with a mutant allele of the tumor suppressor \( p53 \). These data provide the first direct \( in vivo \) evidence for oncogenic cooperation between \( CagA \) and \( p53 \) and provide a robust model of \( CagA \)-induced carcinoma. Our results are consistent with previous findings of increased \( p53 \) mutational frequency in \( H.
CagA transgenic zebrafish model

RESEARCH ARTICLE

pylori-associated gastric cancer cases (Shibata et al., 2002) and corroborate a previous study establishing CagA as a bona-fide oncprotein (Ohnishi et al., 2008). More importantly, these data support the use of our model in the screening of putative gastric cancer susceptibility loci for oncogenic cooperation with CagA.

MATERIALS AND METHODS

Ethics
All zebrafish experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of Oregon Animal Care Service is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and complies with all United States Department of Agriculture, Public Health Service, Oregon State and local area animal welfare regulations. All activities were approved by the University of Oregon Institutional Animal Care and Use Committee (Animal Welfare Assurance number A-3009-01).

Animals
Transgenic zebrafish were developed using the Tol2kit as previously described (Kwan et al., 2007). tp53M214K (Berghmans et al., 2005), and axin1tm213 (Heisenberg et al., 2001) animals were obtained from Monte Westerfield (University of Oregon) and tcf4exI (Muncan et al., 2007) from Tatjana Piotrowski (University of Utah). All zebrafish experiments were performed using protocols approved by the University of Oregon Institutional Care and Use Committee, and following standard protocols (Westerfield, 2007). CagA transgenics can be obtained by contacting the corresponding author.

EdU labeling and detection
Zebrafish larvae were immersed in 100 μg/ml EdU (A10044; Invitrogen) with 0.5% DMSO for 8-12 hours, fixed overnight at 4°C (4% paraformaldehyde in PBS) with gentle shaking, processed for paraffin embedding and cut into 7-μM 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
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
Immunohistochemistry was carried out on paraffin sections as previously described using anti-β-catenin (1:1000; C2206 rabbit polyclonal, Sigma) (Cheesman et al., 2011).
Histopathology
Histopathological analysis of H&E stained sections was performed by pathologists with expertise in laboratory fish (T.S.P. and M.L.K.) in a blinded manner. For each adult zebrafish genotype, four consecutive sagittal serial sections of the entire intestinal tract, anterior to posterior, were evaluated for epithelial hyperplasia, dysplasia and the presence of neoplasia. Classification of intestinal epithelial hyperplasia included two or more of the following criteria: epithelial cell nuclear pseudostratification, multi-layering of mucosal fold epithelial cells and formation of pseudocrypts, which indicated extensive infolding of hyperplastic epithelium lining the intestinal mucosal folds. Dysplastic changes of the intestinal epithelial cells, observed in several fish within the hyperplastic intestinal epithelium, were classified as an increased nuclear to cytoplasm ratio, nuclear hyperchromatism with indiscernible nucleoli, ‘piling-up’ of epithelial cells, loss of nuclear polarity (i.e. loss of basally oriented epithelial cell nuclei) and abnormal mitotic figures. Classification of intestinal adenocarcinoma included the following criteria: invasive cribriform pseudocysts that interfaced directly with the lamina propria in the absence of an interceding basement membrane, disorganized histoarchitectural patterns of the pseudocysts, loss of differentiation from well-defined pseudocysts to complete absence of acinar-like structures and a desmoplastic response to the neoplastic cells. Small cell carcinoma was classified as densely cellular and discrete small sheets and nests of tumor cells within the lamina propria, with minimal cytoplasm, that lacked an organoid growth pattern. Intratumoral inflammatory infiltrates were also accounted for and classified by chronicity and cell type. Other proliferative lesions, which occurred in only one fish, are described in the Results section.

Quantitative RT-PCR
Reference gene testing was performed using the geNorm reference gene selection kit (Primerdesign) and qBase® software (Biogazelle). Baseline, threshold and efficiency calculations were performed using LinRegPCR software (Ruijter et al., 2009). 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 supplementary material Table S2. Expression data were normalized to the geometric mean of the reference genes using StepOne (ABI) software.

Myeloperoxidase staining
Myeloperoxidase (Mpo) staining was carried out using the Leukocyte Peroxidase (Myeloperoxidase) Staining Kit (Sigma-Aldrich). Mpo-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.

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

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
J.T.N. and K.G. designed experiments. J.T.N., T.S.P., M.L.K. and K.G. analyzed data. J.T.N., T.S.P., M.L.K. and K.G. wrote the paper.

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SUPPLEMENTAL MATERIAL
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