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Citrobacter Infection and Wnt signaling

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Abstract

Gut flora generally contributes to a healthy environment while both commensal and pathogenic bacteria that influence the innate and adaptive immune responses, can cause acute and/or chronic mucosal inflammation. *Citrobacter rodentium* (*C. rodentium*) is a member of the family of enteropathogens that provide an excellent *in vivo* model to investigate the host-pathogen interactions in real-time. It is the etiologic agent for transmissible murine colonic hyperplasia (TMCH) while inflammation following *C. rodentium* infection is dependent upon the genetic background. Ongoing and completed studies in this model have so far established that Wnt/ β -catenin, Notch and PI3K pathways regulate colonic crypt hyperplasia while epithelial-stromal cross-talk, mediated by MEK/ERK/NF- κ B signaling, regulates inflammation and/or colitis in susceptible strains. The *C. rodentium*-induced hyperplastic state also increases the susceptibility to either mutagenic insult or in mice heterozygous for *Apc* gene. The ability to modulate the host response to *C. rodentium* infection therefore provides an opportunity to delineate the mechanisms that determine mucosal hyperplasia, intestinal inflammation, and/or neoplasia as disease outcomes.

Keywords

Microbiota; Enteric Infection; *Citrobacter rodentium*; Wnt Signaling; Inflammation; Inflammatory Bowel Disease; Colon Cancer

Introduction

Intestinal Microbiota and Enteric Pathogens

Humans and other multicellular eukaryotic organisms are home to a diverse population of 10^{14} microbes that constitute our microbiota [1]. In mammals, the gastrointestinal tract contains the most numerous and diverse microbial community [2]. Microbial colonization in humans follows a gradient along the length of the gastrointestinal tract, with a lower titre in the stomach and the duodenum (10^1 – 10^3 bacteria/ml) increasing to $\sim 10^7$ bacteria/ml in the jejunum and ileum and peaking to $\sim 10^{12}$ bacteria/ml in the colon [3]. Most of these colonic bacteria are obligate anaerobes while facultative anaerobes are several folds lower. In both human and the murine intestine, the dominant microbiota belongs to the Firmicutes and Bacteroidetes phyla. In addition, Proteobacteria, Actinobacteria, Verrucomicrobia, Cyanobacteria and Deferribacteres have also been detected in humans and mice, and Fusobacteria in humans [4–6].

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Disclosure

No potential conflicts of interest relevant to this article were reported.

Several factors including host age, environmental and genetic factors, diet, and exposure to chemotherapeutic drugs and probiotics influence the composition of the microbiota. In addition, antibiotics can significantly affect the gut microbiota with long-term effects [7] and may result in super infection with antibiotic-resistant bacteria such as *Clostridium difficile* or vancomycin-resistant *Enterococcus* [8]. Microbial ligands on commensal flora are recognized by Toll-like receptors (TLRs) most commonly known as Pattern Recognition Receptors (PRRs); activation of these PRRs by the gut flora serves to protect the gut from injury [9]. Interaction of gut bacteria and TLRs contributes to mucosal homeostasis and controls local inflammation [10]. It is increasingly been recognized that bacterial residents of the large bowel cannot be easily divided as being pathogenic or non-pathogenic. Many indigenous bacteria, e.g. *E. coli*, *Bacteroides*, *Enterococci* and *Clostridium histolyticum*, are known pathogens. Unequivocally however, the host's innate intestinal immune system seems to be heavily involved.

The length of the gastrointestinal tract is inhabited by healthy intestinal microbiota from week one after birth. It exerts a homeostatic influence on the gut through direct inhibition of other microbes that lead to 'colonization resistance' to nonindigenous strains of bacteria, such as *C. difficile*, *Salmonella* spp. and *Campylobacter jejuni*. However, breakdown of the resistance predisposes humans to infection by these enteropathogens. Strikingly, the part of the intestine with the highest bacterial colonization, the colon, is also most affected by cancer, with ~150,000 annual cases in the United States [11]. *Helicobacter pylori* highlighted the potential for bacteria to cause cancer. It is becoming increasingly clear that chronic infection with other bacteria, notably *Salmonella typhi*, can also facilitate tumor development [12]. Infections caused by several bacteria (e.g. *Bartonella* spp., *Lawsonia intracellularis* and *Citrobacter rodentium*, [13–15]) can induce cellular proliferation and prime the colonic mucosa towards neoplasia while chronic bacterial infections can inhibit apoptosis. However, the underlying cellular mechanisms are far from clear. Conversely, bacterial toxins such as PMT (*Pasteurella multocida* toxin) [16] or *Escherichia coli* cytotoxic necrotizing factor [17] interfere with cellular signaling mechanisms and could play a direct role in cancer causation and progression. Finally in colorectal cancer (CRC) patients, the fecal levels of an opportunistic pathogen *Streptococcus gallolyticus* are increased from 10% to about 50% [18]. This bacterium is associated with infectious endocarditis and approximately 60% of patients assessed in this group also had premalignant/malignant colonic lesions [19, 20] which largely exceeds the rates reported in the general population (~25%) [21]. Thus, it is not unprecedented to associate bacterial infection with either onset or progression of colon carcinogenesis.

Citrobacter rodentium

Enteric pathogens such as enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* (*C. rodentium*), all of which use attaching and effacing (A/E) lesion formation as a major mechanism of tissue targeting and infection, represent the classic genome organization [22]. Both EPEC and EHEC are poorly pathogenic in mice but infect humans and domestic animals. In contrast, *C. rodentium* is a natural mouse pathogen that is related to *E. coli* and provides an excellent *in vivo* model for A/E lesion forming pathogens [23]. *C. rodentium* also provides a model of infections that are mainly restricted to the lumen of the intestine. The mechanism by which the host responds to such infections has become a topic of great interest in recent years. In this review however due to space constraints, we will focus primarily on *C. rodentium*'s involvement in the activation of the Wnt signaling pathway and whether the changes accrued broaden our understanding of *C. rodentium*-induced pathogenesis.

Citrobacter rodentium, formerly *Citrobacter freundii* biotype 4280, is the etiologic agent of transmissible murine colonic hyperplasia (TMCH) while *C. freundii* has been reclassified as a human pathogen based on the biochemical testing that distinguishes *C. rodentium* from *C. freundii* as indole negative and positive for ornithine decarboxylase. *C. rodentium* is a gram-negative, facultatively anaerobic rod and is generally considered an opportunistic pathogen that is host adapted to laboratory mice. The ability to generate the A/E phenotype by these organisms requires the locus of enterocyte effacement (LEE), a pathogenicity island that encodes a type III secretion system (TTSS) and several translocated effectors [24, 25]. Translocated intimin receptor (Tir), a TTSS effector critical for intimate bacterial attachment and actin pedestal formation, becomes localized in the host plasma membrane [26, 27, 28]. The extracellular domain of Tir is recognized by the outer membrane adhesin intimin, encoded by the *eae* gene [29, 30]. Following oral infection of immunocompetent mice with *C. rodentium*, there is transient colonization and inflammation that peaks after 1 week and is cleared within 2 to 3 weeks [31]. Bacterial colonization is limited to the intestinal mucosa with little dissemination to peripheral organs. Most of the clinical signs are mild while microscopically, the infection is associated with crypt hyperplasia, goblet cell loss, and mucosal infiltration of immune cells, including T cells, macrophages, and neutrophils. A Th1 host immune response, mediated by infiltrating CD4+ T cells and macrophages, is required for efficient bacterial clearance [32]. Thus, *C. rodentium* infection is an excellent model for the investigation of host-pathogen immune interactions in the gut.

The hallmark pathologic lesion of *C. rodentium* infection is colonic hyperplasia while inflammation is dependent upon the genetic strain under investigation. Resistant strains such as NIH:Swiss and C57BL/6J (B6) experience a self-limiting disease that peaks between one and two weeks post infection, followed by a clearing of the infection and complete recovery. However, the inbred mouse strains C3H/HeJ (C3), C3H/HeOuJ (C3Ou) and FVB/N (FVB) are highly susceptible to *C. rodentium* infection and develop more severe symptoms leading to high rates of mortality. Khan et al. [33] first identified a role for toll-like receptor 4 (TLR4) signaling in *C. rodentium* pathogenesis when they found delayed mortality in TLR4-deficient C3 mice compared with C3Ou mice that express functional TLR4 when mice were inoculated with 10^4 – 10^6 instead of 10^8 CFU, which also resulted in variable onset of infection [33]. TLR4-deficient C57BL/10ScNJ mice develop less morbidity and mortality than C57BL/10J mice that express functional TLR4 when infected with *C. rodentium* [33]. Lebeis et al. [34] however, found no difference in mortality between C57BL/6J mice and TLR4-deficient mice infected with *C. rodentium* S1116 strain which apparently causes less severe colitis [35] due to a truncated lipopolysaccharide structure [36]. Intriguingly, compared with wild type C57BL/6 mice, the same strain caused a severe necrotizing colitis in MyD88-deficient mice with no crypt elongation, indicating lack of a protective regenerative response [34]. Thus, innate immune signaling is critical to protect the host while an adaptive immune response develops resulting in bacterial clearance. Finally, Diez E. et al. [37] recently showed that a single locus on proximal chromosome 15 called *C. rodentium* infection 1 (Cri1) is responsible for the susceptibility of C3, C3Ou and FVB mice to *C. rodentium* infection suggesting a common genetic cause of fatal infectious colitis in these mice following infection with *C. rodentium*.

More recently, a fully annotated genome of a virulent strain of *C. rodentium*, ICC168 has been sequenced. The genome is 5,346,659 bp in length, with an average G+C content of 54.72 % and is deposited in the EMBL/GenBank databases with accession number FN543502. This exciting development is expected to lead to mapping of all the virulence factors in its genome, in addition to identification of novel factors that bear resemblance to not only known A/E pathogens but to other related pathogens. Indeed, recent studies utilizing the 'stable isotope labeling with amino acids in cell culture' (SILAC), a quantitative proteomic technique [38], have analyzed the bacterial type three secretome of

EPEC and *C. rodentium* [39, 40•]. In the future, similar approaches may help unravel the basis of host specificities of *C. rodentium*, EPEC and EHEC.

Wnt Signaling Pathway and Colon Cancer

Most mammalian genomes, including human, contain 19 Wnt genes that fall into 12 conserved Wnt subfamilies. Wnt proteins are ~40 kDa in size, harbor many conserved cysteines [41] and are lipid modified [42]. They bind a heterodimeric receptor complex, consisting of a Frizzled (Fzd) and an LRP5/6 protein (Figure 1). The ten mammalian Fzd proteins are seven-transmembrane receptors and have large extracellular N-terminal cysteine-rich domains [43] that bind Wnt ligands [44, 45]. Interestingly, a single Wnt can bind multiple Fzd proteins [43] and vice versa [45]. Signaling by dimeric Wnt receptors includes a ligand-induced conformational change of the receptors followed by phosphorylation of key target proteins. A crucial step in signaling is binding of Axin to the cytoplasmic tail of LRP6 when Axin is phosphorylated by at least two separate kinases, GSK3 and CK1 γ in the PPPSP motif [46–49••]. Wnt/ β -catenin signaling pathway is regulated at many levels that includes secreted proteins that antagonize the ligand.

In the context of gastro-intestinal tract, Wnt/ β -catenin signaling is critical for the maintenance of intestinal crypt cell proliferation. Wnt ligands bind intestinal stem cells and crypt epithelial progenitor cells and prevent glycogen synthase kinase 3 β (GSK3 β)-dependent N-terminal phosphorylation and proteosomal degradation of β -catenin [50–53]. Upon receptor activation by Wnt ligands, Axin is recruited to the phosphorylated tail of LRP leading to inhibition of β -catenin ubiquitination that normally occurs within the complex. This leads to saturation of the complex by phosphorylated form of β -catenin, leading newly synthesized β -catenin to accumulate and translocate to the nucleus to initiate the transcription of Wnt target genes such as c-Myc and cyclin D1, in association with transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family [54, 51, 55] (Figure 1). Mutations in mouse TCF4 lead to loss of intestinal stem cells and almost complete lack of intestinal crypts [56] which further implicate Wnts as critical regulators of stem cell signals. It is therefore not surprising that Wnt pathway mutations leading to constitutively active Wnt signaling, are frequently observed in cancer. Germline mutations in the APC gene cause familial adenomatous polyposis [57, 58]. In most cases of sporadic colorectal cancer, loss of both APC alleles leads to inappropriate stabilization of β -catenin and the formation of constitutive complexes between β -catenin and TCF4 [59, 60]. In colorectal cancers that harbor wild-type APC, either Axin2 is mutated [61], or activating point mutations in β -catenin remove the regulatory N-terminal Ser/Thr residues [62]. Interestingly, while the aberrant activation of the Wnt pathway is required during the initiation of colorectal cancers, a recent study has shown that sustained Wnt pathway activation is also needed for colorectal tumor maintenance [63]. Finally, since Wnt signaling activity can designate colon cancer stem cells [64], it is an attractive target for new therapeutics.

Bacterial infection and Wnt signaling

As mentioned earlier, there has been a trend towards studies that are focusing more on the role of bacterial infection in carcinogenesis. There is also evidence of studies that directly implicate bacterial infection in the activation of Wnt signaling. The *H. pylori*'s cytotoxin-associated gene secretion system activates β -catenin along with p120 and PPAR δ , which promote gastric epithelial cell proliferation via activation of cyclin E1 [65]. The *H. pylori*-induced dysregulation of β -catenin dependent pathways may explain in part the augmentation in the risk of gastric cancer conferred by this pathogen [66•]. Similarly, *C. rodentium*-induced pathogenesis offers an excellent opportunity to identify changes associated with epithelial cell proliferation and mucosal priming for neoplasia. The cellular

and molecular events that occur in response to *C. rodentium* infection are similar to those that occur in colon adenomas and carcinomas [67]. Furthermore, *C. rodentium*-induced colonic crypt hyperplasia increases the susceptibility of the mouse colon to neoplastic transformation [15, 68•]. We showed for the first time that colonic crypt hyperplasia in response to *C. rodentium* infection was associated with NF- κ B activation [69] and alterations in casein kinase I ϵ (CKI ϵ) that influence β -catenin signaling [70•]. This was a major first step towards delineating the mechanistic basis of *C. rodentium*-induced pathogenesis. Since TMCH is a self-limiting disease, we have further shown that both TLR4-induced NF- κ B activation and CKI ϵ mediated phosphorylation of β -catenin at Ser-45 (β -cat⁴⁵), resulting in stabilization/nuclear translocation of β -cat⁴⁵, play important roles in the regulation of proliferative and the regression phases of hyperplasia [71•, 72].

Recent studies in the TMCH model also implicate phosphatidylinositol-3-kinase (PI3K)/Akt signaling in the activation of β -catenin in intestinal stem and progenitor cells through phosphorylation at Ser-552 (p- β -catenin⁵⁵²). In response to *C. rodentium* infection, increased numbers of p- β -catenin⁵⁵²-stained epithelial cells were found throughout expanded crypts [73]. Furthermore, inhibition of PI3K signaling attenuated epithelial Akt activation, the Ser-552 phosphorylation and activation of β -catenin, and epithelial cell proliferative responses following *C. rodentium* infection. PI3K inhibition also impaired bacterial clearance despite having no impact on mucosal cytokines (IFN- γ , TNF, IL-17, and IL-1 α) or chemokines (CXCL1, CXCL5, CXCL9, and CXCL10) induction [73]. Thus, while adaptive immunity including B cells and IgG antibodies is required for bacterial clearance [74], results of this study add new dimension to our understanding of how PI3K/Akt-induced activation of Wnt/ β -catenin pathway can also play a role in host defense against *C. rodentium* independently of adaptive immune responses.

Wnt/Notch Cross-talk and Colon Cancer

Notch signaling has been shown to play an important role in cell-fate determination, as well as in cell survival and proliferation [75–78]. Notch signaling is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Delta or Jagged) leading to the release of notch intracellular domain (NICD) that heterodimerizes with the DNA binding protein RBP-J (recombination signal sequence-binding protein J κ , also called CSL, CBF1, Su (H) and LAG-1) and activates transcription of target genes such as Hes 1. There is increasing evidence that Notch signals are oncogenic in many cellular contexts, for example in T cell leukemia (T-ALL), breast and colon cancer [79–83]. In addition, the cross-talk between Notch and Wnt pathways including genetic interactions in *Drosophila* [84], the physical binding of Notch to β -catenin [85] or their association to common cofactors [85] have been described. Moreover, GSK3 β directly phosphorylates the Notch protein thus modulating its transcriptional activity [86] and β -catenin activates Jagged1 transcription thus leading to Notch activation during murine hair follicle differentiation [87]. Conversely, in different types of tumor cells, Notch activates the Wnt pathway stabilizing β -catenin by unknown mechanisms [88] or by transcriptional activation of slug [89]. More recently, Notch signaling has also been implicated in the maintenance of intestinal barrier function. Thus, accurate coordination of the Notch and Wnt signals is indispensable for maintenance of intestinal homeostasis and that aberrant regulation of the cross-talk may result in a disease state. Despite these advances, little is known regarding the mechanism that coordinates the complex cross-talk between the Notch and Wnt/ β -catenin pathways as they relate to bacterial infection-induced colitis or colon carcinogenesis.

We have recently attempted to examine the interplay between the Notch and Wnt/ β -catenin pathways in various genetic strains *in vivo*. Results of our study provide circumstantial evidence that in addition to Wnt/ β -catenin pathway, Notch signaling is also activated during

C. rodentium-induced TMCH and that blocking Notch signaling with γ -secretase inhibitor, dibenzazepine for 10 days results in almost complete inhibition of both Notch and Wnt/ β -catenin signaling concomitant with the disruption of the intestinal barrier and onset of colitis [90•]. The chronic inhibition of the Wnt/Notch pathway cripples the ability of the colonic mucosa to regenerate itself which is not necessarily due to loss of colonic stem cells [90•]. Whether colitis in these mice predisposes them to mutagen-induced carcinogenesis is currently being investigated. Thus, the balancing act between cell proliferation and restoration of barrier integrity following *C. rodentium* infection seems to depend upon the interplay between the Notch and Wnt/ β -catenin pathways. Since Notch signaling is constitutively activated in colorectal cancer and its inhibition suppresses the cell growth and sensitizes cancer cells to treatment-induced apoptosis, extensive studies on the potential use of γ -secretase inhibitors in the treatment of colon cancer is being carried out. Our findings present a caveat however, particularly for patients with colitis who are undergoing treatment for colorectal cancer that possible exacerbation of colitis in the aftermath of chronic Notch inhibition could jeopardize the outcome.

Effect of CR-induced inflammation on Wnt signaling

Ulcerative colitis (UC) and Crohn's disease (CD), two components of the inflammatory bowel disease (IBD) are both characterized by an exaggerated immune response directed against luminal and/or enteric bacterial antigens while infections by murine pathogens *C. rodentium* [23] and *Helicobacter hepaticus* [91] provide surrogate examples of a similar phenomenon. It has been reported previously that ileal CD was associated with a reduced expression of TCF4, a known regulator of Paneth cell differentiation and alpha-defensin expression [92]. The levels of TCF4 mRNA were decreased in patients with ileal disease irrespective of degree of inflammation [92]. Similarly, compromised expression of TCF4 in mice heterozygous for *tcf4* gene led to a significant decrease in both Paneth cell alpha-defensin levels and bacterial killing activity [92]. Thus, even though no etiologic agent has so far been identified in IBD, the inflammatory state in these patients is associated with significant alterations in the components of the Wnt/ β -catenin pathway. Since the *C. rodentium*-induced TMCH model represents the murine model of IBD, we have recently started delineating changes in the components of both NF- κ B and Wnt/ β -catenin pathways during *C. rodentium*-induced acute and/or chronic inflammatory state.

In contrast to outbred mice, genetically susceptible inbred [e.g., C3H/HeNHsd (C3H)] mice exhibit significant inflammation that are superimposed upon the hyperplastic response of the colonic mucosa. Factors that determine inflammatory response are not understood as yet although variations in microbial composition between mouse strains have been predicted to contribute to differences in "host" susceptibility [93]. Since cross-talk between epithelial and mono-nuclear cells of the lamina propria is critical for sustained inflammatory axis, we systematically analyzed distribution of NF- κ B activity in the epithelium and cells of the lamina propria constituting the stroma following CR infection and investigated how signaling via ERK and p38 MAPKs modulate functional NF- κ B activity in various cell types in the distal colons of C3H mice. In response to *C. rodentium* infection, we observed distinct compartmentalization of NF- κ B activity in the crypts and the crypt-denuded lamina propria in association with distinct and robust expression of pro-inflammatory cytokines and chemokines [94••]. These changes were associated with crypt hyperplasia that preceded the onset of colitis [94••]. In addition to NF- κ B, in an ongoing study, we have observed significant alterations in β -catenin expression which coincides with the dual phases of hyperplasia preceding active colitis. Interestingly, β -catenin undergoes degradation at time point that coincides with the onset of colitis [manuscript under preparation]. These results clearly suggest that *C. rodentium* induces complex immune-mediated responses in the inbred mice which are distinct from the outbred counterparts.

Conclusions

The idea that bacterial infection could cause cancer has only recently become accepted following the discovery of *Helicobacter pylori*'s role in gastric carcinogenesis. Since then, a trend is emerging towards identifying other potential bacteria with pro-carcinogenic properties. As mentioned earlier, *Pasteurella multocida* toxin (PMT), a highly potent mitogen modifies and activates several members of the heterotrimeric G-proteins leading to upregulation of the downstream proto-oncogenes including the Rho GTPase, focal adhesion kinase, cyclooxygenase-2, β -catenin and calcium signaling components [16]. Although the etiologic role for PMT in human cancer is not yet established, it serves as a novel paradigm for a bacterial link to cancer. Similarly, infectious agents such as Human Papilloma Virus, Hepatitis B and C virus, and *Helicobacter pylori*, alone are responsible for an estimated 15% of the global cancer burden [95]. Finally, a recent study found a marked over-representation of *Fusobacterium nucleatum* in patients suffering from colorectal cancer [96]. In the future, metagenomic studies will hopefully provide a useful approach to identifying microbial sequence signatures in diseases with infectious etiology.

As far as *C. rodentium* is concerned, while the infection by itself doesn't cause cellular transformation or cancer, CR-induced activation of the Wnt, Notch and NF- κ B pathways causes hyperplasia of the colonic crypts that mimics adenomas or adenocarcinomas in humans. Since hyper-activation of Wnt signaling is a hallmark of colon cancer and of many other human cancers, our findings clearly suggest that the TMCH model can be used as an excellent template to study the early stages of colon carcinogenesis. Indeed, we have shown recently that following CR infection, aberrant overexpression of a calcium channel TRPV6 (transient receptor potential vanilloid-type) contributes to colonic crypt hyperplasia in mice and to colon cancer cell proliferation in humans [97]. Blocking TRPV6 with high calcium diet (1%) causes almost complete abrogation of hyperplasia. As proof of principle, we also investigated TRPV6 expression in adenomatous polyps (stage I) and advanced human cancers (stages III/IV) to determine if similarities exist with regard to TMCH model. A significant increase in TRPV6 expression, compared to normal mucosa, was observed in stage I tumor while stages III and IV cancers were negative for TRPV6 thus linking TRPV6 to early stages of colon cancer [97]. The remarkable parallel with TMCH also suggests that TMCH is not unique but shares a common final pathway in response to diverse initiating events. In conclusion, *C. rodentium*-induced TMCH associated with alterations in Wnt/ β -catenin, Notch, PI3K and NF- κ B pathways, can be used as an animal model linking bacterial infection, IBD and colorectal cancer.

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Figure 1. Wnt signaling pathway

When Wnt is absent, the destruction complex comprising of Axin, APC and the dual kinases, CK1 α and GSK-3 β phosphorylate β -catenin and present it to β -TrCP for ubiquitination followed by proteasomal degradation. Wnt target genes remain repressed due to binding of repressors such as Groucho and HDAC. In the presence of Wnt, the entire degradation complex can associate with the phosphorylated LRP5/6 wherein, β -catenin can still undergo phosphorylation at the consensus sites but its ubiquitination by β -TrCP is inhibited. Newly synthesized β -catenin which escapes phosphorylation translocates to the nucleus to activate downstream targets in association with transcription factor TCF.