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Journal
Oncogene, 28(47)

ISSN
0950-9232

Authors
Najdi, R
Syed, A
Arce, L
et al.

Publication Date
2009-11-26

DOI
10.1038/onc.2009.271

Peer reviewed

HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2010 May 26.

Published in final edited form as:

Oncogene. 2009 November 26; 28(47): 4133–4146. doi:10.1038/onc.2009.271.

A Wnt-kinase network alters nuclear localization of TCF-1 in colon cancer

Rani Najdi, B.S.[1], **Adeela Syed, Ph.D.**[2], **Laura Arce, Ph.D.**[1], **Heidi Theisen, Ph.D.**[2], **Ju-Hui T. Ting, Ph.D.**[1], **Fawzia Atcha, Ph.D.**[1], **Anthony V. Nguyen, M.D.**[3], **Micaela Martinez, B.S.**[3], **Randall F. Holcombe, M.D.**[3], **Robert A. Edwards, M.D.**[4], **J. Lawrence Marsh, Ph.D.**[2,4,*], and **Marian L. Waterman, Ph.D.**[1,*]

[1] Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA 92697, USA

[2] Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA 92697, USA

[3] Division of Hematology/Oncology, Department of Medicine, University of California, Irvine, Irvine, CA 92697, USA

[4] Department of Pathology, University of California, Irvine, Irvine, CA 92697, USA

Abstract

Constitutive activation of the Wnt/β-catenin pathway has been implicated as the primary cause of colon cancer. However, the major transducers of Wnt signaling in the intestine, TCF-1 and TCF-4, have opposing functions. Knock-out of TCF-4 suppresses growth and maintenance of crypt stem cells, while knock-out of TCF-1 leads to adenomas. These phenotypes suggest that TCF-4 is Wnt-promoting while TCF-1 acts like a tumor suppressor. Our study of TCF expression in human colon crypts reveals a mechanistic basis for this paradox. In normal colon cells, a dominant negative isoform of TCF-1 (dnTCF-1) is expressed that is equally distributed between nuclear and cytoplasmic compartments. In colon cancer cells, TCF-1 is predominantly cytoplasmic. Localization is due to active nuclear export and is directed by an autocrine-acting Wnt ligand that requires CaMKII activity for secretion and a downstream step in the export pathway. TCF-4 remains nuclear; its unopposed activity is accompanied by downregulation of dnTCF-1 and increased expression of full-length isoforms. Thus, the dnTCF-1, TCF-4 balance is corrupted in cancer by two mechanisms, a Wnt/CaMKII kinase signal for nuclear export, and decreased dnTCF-1 expression. We propose that dnTCF-1 provides homeostatic regulation of Wnt signaling

*Co-correspondence: Marian L. Waterman, Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA 92697, USA, Phone: 19498243096, Fax: 19498248598, mlwaterm@uci.edu, J. Lawrence Marsh, Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA 92697, USA, Phone: 19498246677, Fax: 19498244709, jlmarsh@uci.edu.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at Oncogene's website.

and growth in normal colon and alterations in nuclear export and promoter usage contribute to aberrant Wnt activity in colon cancer.

Keywords

CaMKII; colon cancer; export; TCF-1; Wnt

Introduction

Over 80% of sporadic colon tumors contain mutations that result in the stabilization of β-catenin and constitutive activation of canonical Wnt signaling (Bienz and Clevers, 2000; Kinzler and Vogelstein, 1996; Miyoshi *et al.*, 1992). This aberrant activation results in accumulation of β-catenin in the nucleus where it interacts with transcription factors from the LEF/TCF (Lymphoid Enhancer Factor/T-Cell Factor) family to regulate transcription of Wnt target genes and promote growth (Clevers, 2006; Klaus and Birchmeier, 2008). However, three observations indicate that the role of TCFs in tissues such as the colon is more complex than simple transducers of Wnt signaling. Firstly, several of the key genes, *TCF1*, *TCF4* and *LEF1* (*TCF1* and *TCF4* are also known as *TCF7* and *TCF7L2* by HUGO nomenclature) can produce different protein isoforms with very different and sometimes opposing activities through the use of alternative promoters and/or alternative splicing (Arce *et al.*, 2006; van Noort and Clevers, 2002). Secondly, inactivation of *TCF1* and *TCF4* results in opposite phenotypes in the mouse intestine. Loss of *TCF4* suppresses growth and survival of intestinal stem cells in embryos, but loss of *TCF1* causes adenomatous polyps in adult mice (Korinek et al., 1998; Roose et al., 1999). Thirdly, Wnt pathway-activating mutations found in colon cancer can alter the expression of at least some LEF/TCF proteins (Hovanes *et al.*, 2001). The opposing phenotypes seen in knockouts and the complex pattern of gene regulation that may be altered in colon cancer suggest that not all LEF/TCFs are oncogenic mediators of β-catenin action. However, the molecular basis for the paradoxical observation of an apparent tumor suppressor action of *TCF1* remains unclear.

LEF/TCF loci can produce different protein isoforms through the use of alternative promoters and/or alternative splicing (Arce et al., 2006; van Noort and Clevers, 2002). An alternative splicing event leads to the inclusion or absence of a carboxy terminal domain called the E-tail, which has recently been shown to contain a novel, auxiliary DNA interaction motif. This motif can augment DNA binding allowing this isoform to bind a broader range of Wnt response elements including proliferation genes (Atcha *et al.*, 2007; Chang *et al.*, 2008). In total, alternative splicing can result in 4 different C-terminal tails (A, B, C and E). In addition, the use of alternative promoters provides further functional diversity. Specifically, the *TCF4* gene uses a single promoter that produces a full-length activating TCF-4 that can bind β-catenin and activate Wnt target genes (i.e. FL-TCF-4; full length-FL, Figure 2c). In contrast, the *TCF-1* gene can be transcribed from two different promoters: one promoter produces mRNA encoding a full-length activating form of TCF-1, while a second intronic promoter generates a truncated, dominant-negative isoform that is missing the β-catenin binding domain (dnTCF-1; Figure 2c) (Van de Wetering et al., 1996). The dominant-negative TCF-1 isoform suppresses Wnt target gene expression by recruiting

repressors instead of the co-activator β-catenin (Arce et al., 2006). The tumor-suppressing capacity of the dnTCF-1 isoform is evident in its ability to suppress growth of colon cancer cells (van de Wetering et al., 2002). These different isoforms suggest a hypothesis for the *TCF1* knock-out study in mice, which is the idea that TCF-1 functions as a tumor suppressor. This model was recently challenged by a study showing that siRNA knock-down of TCF-1 in a human colon cancer cell line actually slowed the growth rate, prompting the opposite hypothesis that TCF-1 action is oncogenic (Tang et al., 2008). These opposing hypotheses are reconciled by our study of *TCF1* regulation.

TCF-4 and TCF-1 isoform expression and action have not been studied in normal human intestinal epithelia or colon tumors. Therefore, to better understand the role of TCFs in colon cancer and to address the opposing models of TCF-1 function, we have examined the distribution and regulation of TCF-1 and TCF-4 in human colon tissues and cells. We find that TCF-1 is often, but not always, excluded from the nucleus in human colon tumors, while TCF-4 always remains in the nucleus. We have discovered TCF-1 exclusion to be an active process controlled by CaMKII signaling and a secreted Wnt signal, demonstrating that an extracellular Wnt ligand can influence the balance between TCF-1 and TCF-4 in nuclei. Remarkably, we find that dnTCF-1 is the most abundant TCF-1 isoform in normal colon crypts, while a switch to FL-TCF-1 predominates in colon cancer. Thus, regulation of nuclear export and expression alter the balance between dominant-negative and full-length TCFs.

Results

The tumor suppressor phenotype of *TCF1* in knockout mice suggests that this gene could be damaged in human tumors, but to date, no mutations in the *TCF1* locus have been identified. In fact, TCF-1 mRNA levels appear to increase during carcinogenesis (Van der Flier *et al.*, 2007). We therefore hypothesized that TCF-1 function might be down-regulated by other mechanisms; hence, we investigated the distribution of TCF-1 protein in primary human colon cancer specimens and adjacent normal colon crypts (Figure 1 and Supplementary Figure S1). Tissues were probed with antibodies specific for all isoforms of either TCF-1 or TCF-4. Staining with β-catenin antibody showed elevated levels in cells, indicating that Wnt signaling was increased (Figure 1a and b). Staining for TCF-4 revealed that it was predominantly nuclear in both normal (Figure 1c and Supplementary Figure S1a, green) and colon cancer tissues (Figure 1d and Supplementary Figure S1b, green). In contrast, TCF-1 was detected in both the nucleus and the cytoplasm of normal colonic tissues (Figure 1e and Supplementary Figure S1c, green), and it was often absent from the nuclei of adjacent colon cancer tissues (Figure 1f and Supplementary Figure S1d, green). Scoring of subcellular localization patterns of TCF-1 and TCF-4 in multiple fields of 9 different primary tumor specimens showed that none of the tumors display the balanced nuclear/cytoplasmic TCF-1 distribution of matched normal cells in the surrounding tissue. Eight of the nine tumors had TCF-1 predominantly in the cytoplasm while 8 out of 9 specimens showed TCF-4 either predominantly or exclusively in the nucleus of tumor cells (see Supplementary Table 1).

We also monitored TCF-1 expression through membrane array analysis of primary patient biopsies. Consistent with previous reports, we detected an overall increase in *TCF1*

expression compared to normal colon tissue and the Wnt target gene Axin II (Supplementary Figure S2a). Primary samples from human tubular (low-malignant potential) and villous polyps (high-malignant potential) showed that *TCF1* expression is increased significantly in the villous adenomas. These changes in expression level suggest a correlation between the malignancy potential of the tumor and amount of TCF-1 expressed (Supplementary Figure S2b). Thus, TCF-1 and TCF-4 protein and mRNA are expressed in both normal and colon cancer cells, but the subcellular distribution of TCF-1 protein is dramatically altered such that it is absent from the nucleus in cancerous cells. However, the immuno-fluorescence and membrane array analysis do not distinguish between the various isoforms of TCF-1. We therefore performed qRT-PCR analysis of *TCF1* in normal colonic mucosa and colon cancer. In cancer samples, FL-TCF-1 mRNA expression increases, while dn-TCF-1 mRNA levels drop (Supplementary Figures S2c and d). To precisely define TCF-1 isoform expression in colon cells, we performed western and cDNA sequence analysis. A 48-KDa doublet of TCF-1 proteins were the major polypeptides detected when western blots of SW480, Colo320, DLD1 colon cancer cell extracts were probed with antibodies that detect all forms of TCF-1 (Figure 2a and d, lanes 1–3). There are two different isoforms of TCF-1 that migrate as 48kD in SDS-PAGE gels (Figure 2c): FL-TCF-1 with a B tail and dn-TCF-1 with an E tail. To distinguish between these two isoforms western blots were simultaneously probed with a fluorescently-tagged antibody that detects all forms of TCF-1 (Figure 2d; red) and an antibody that specifically detects E tail containing isoforms of TCF-1 (Figure 2d; green). Co-staining showed that the most abundant polypeptides in colon cancer cells did not have an E tail and were therefore likely to be full-length forms with a B tail (FL-TCF-1B; Figure 2d, lanes 1–3; white arrowheads). For comparison, extracts from freshly isolated normal colon crypts from three different patient samples were prepared for western blotting. In contrast to colon cancer cells, the 48-KDa TCF-1 polypeptides were co-stained by both the pan-specific and E-tail specific antibodies, indicating that the most abundant forms of TCF-1 in normal human crypts are dominant-negative isoforms with an E-tail (dnTCF-1E; Figure 2d, lanes 4–6; white arrowheads). Conversely, TCF-4 has no reported dominant negative isoform, and we detected only full-length TCF-4 with an E-tail (Figure 2b and Supplementary Figure S3c). To confirm the western blot analysis, RT-PCR was performed with primers that detect different mRNA isoforms of TCF-1 (Supplementary Figure S3a). Both full-length and dominant-negative mRNA were detected in four of four cell lines tested (Colo320, DLD-1, HT29 and SW480; Supplementary Figure S3b, 1st and 4th panels), indicating that both promoters are active. However, primers that detect E tail isoforms indicated that only dominant-negative dnTCF-1E was detected in all four cell lines; full-length TCF-1E was not detected (Supplementary Figure S3b, 2nd and 5th panels). By cloning and sequencing the cDNAs generated by the RT-PCR analysis, we found that 43 out of 47 cDNAs encode FL-TCF-1B (data not shown). Thus, TCF-1 exists primarily as a full-length isoform (FL-TCF-1B) in colon cancer cells and as a dominant-negative isoform (dnTCF-1E) in normal human crypts.

Despite the fact that TCF-1 expression increases and the pattern switches to a full-length isoform, TCF-1 protein is excluded from the nucleus in cancer tissues. To test whether a dynamic trafficking process controls TCF-1 protein selectively, we surveyed six colon cancer cell lines (DLD-1, Colo320, HT29, HCT116, RKO and SW480) for subcellular

localization patterns of TCF-1 and TCF-4. In DLD-1 and HT29 cells, TCF-1 showed a cytoplasmic and nuclear distribution (Figure 3a and Supplementary Figure S4a), while in Colo320 cells, TCF-1 was cytoplasmic (Figure 3c). In the HCT116 and RKO cell lines, TCF-1 localization varied between the nucleus and the cytoplasm (data not shown), while TCF-1 was nuclear in SW480 cells (Figure 3e). The variable patterns of localization suggest that TCF-1 might be an actively shuttling protein.

To determine whether TCF-1 is actively cycling in and out of the nucleus, colon cancer cell lines were treated with Leptomycin B (LMB), an inhibitor of the exportin CRM1, which directs proteins through nuclear pores into the cytoplasm (Fornerod et al., 1997; Fukuda et al., 1997). When the DLD-1 and Colo320 cell lines were treated with Leptomycin B for 6 hours, TCF-1 accumulated in the nuclei (Figure 3b and d). Therefore, TCF-1 is actively shuttling in colon cells and is dependent on the CRM1 export pathway for nuclear exit.

To investigate the signals that trigger TCF-1 nuclear export, we used SW480 cells, which have predominantly nuclear TCF-1 (Figure 3e). The export pathway is inactive in SW480 cells and TCF-1 remained nuclear when the cells were treated with Leptomycin B (Supplementary Figure S5c). A TAK1-NLK pathway has been implicated in TCF (also known as POP-1) localization in *C. elegans* (Lo et al., 2004), and a CaMKII-TAK1-NLK pathway in negative regulation of the DNA binding activity of LEF/TCF family members LEF-1 and TCF-4 in mammals (Ishitani et al., 2003a; Ishitani et al., 2003b; Ishitani et al., 1999). To test whether TCF-1 export is regulated by a similar pathway, a constitutively active CaMKII (T286D) expression plasmid was transiently introduced into SW480 cells and nuclear localization patterns monitored (Figure 3e). Overexpression of CaMKII (T286D) triggered TCF-1 export and cytoplasmic accumulation in 79% of SW480 cells (Figures 3f and Supplementary Figure S6a). Since full length TCF-1B is the predominant form of TCF-1 in colon cancer cells, a trigger of its nuclear export would be predicted to have a negative effect on Wnt signaling. To test for this prediction, SW480 cells were co-transfected with the expression vector for activated CaMKII (T286D) and Super8XTopflash, a luciferase reporter plasmid with 8 multimerized Wnt response elements. Compared to mock-transfected cells, Super8XTopflash activity was reduced as much as 50–70% (Figure 3h). Thus activation of the CaMKII pathway has a negative, Wnt-opposing effect in colon cancer nuclei. Co-expression of dominant-negative TAK1 [TAK1 (K63W)] and NLK [NLK (K155M)] prevented active CaMKII (T286D) from triggering TCF-1 export from 84% of nuclei, suggesting that TAK-1-NLK might act downstream of CaMKII to trigger export (Figures 3g and Supplementary Figure S6a). Because of the high levels of TCF-1 export in DLD-1 and Colo320 cells, one might expect that these cell lines also express high levels of activated CaMKII. On the other hand, the absence of TCF-1 export in SW480 cells suggests a low level of activated CaMKII. To test whether there are differences in endogenous CaMKII activity and whether these differences correlate with TCF-1 export, we probed for phosphorylated, active CaMKII in western and immuno-fluorescence assays. When CaMKII is activated by Ca^{2+} and Calmodulin, CaMKII auto-phosphorylates at Threonine 286 and remains in the active state independent of the levels of Ca^{2+} and Calmodulin (Griffith, 2008). Using antisera specific for the phospho-T286 modification, we detected active CaMKII in DLD-1 and Colo320 cells (Figures 4a, b and d, 1st and 2nd panels), while SW480

cells had no detectable amounts of activated CaMKII (Figures 4c and d, 3rd panel). These data suggest that there is a correlation between the activity levels of endogenous CaMKII and the level of TCF-1 export in colon cancer cells.

The ability of CaMKII signaling to drive TCF-1 out of the nucleus is striking, but even more interesting is the observation that nuclear export occurs in approximately 80% of the cells despite the fact that the transfection efficiency is at most 21% (Supplementary Figures S6b and c). This suggests that CaMKII (T286D) is inducing TCF-1 export non-autonomously by upregulating the secretion of a soluble factor into the culture medium. To test this hypothesis, SW480 cells were transfected with CaMKII (T286D), and after 48 hours, the conditioned media was added to a new culture of untransfected SW480 cells. Adding conditioned media to untransfected SW480 cells drove TCF-1 into the cytoplasm in ~45% of cells (Figure 5b and Supplementary Figure S6a). In contrast, adding media from mock-transfected SW480 cells had no detectable effect on TCF-1 localization (Figure 5a). To test whether a secreted factor is responsible for the TCF-1 export activity we observed in other colon cancer cell lines, media from cultured DLD-1 cells was added to untransfected SW480 cells. The DLD-1 conditioned media alone was sufficient to trigger TCF-1 export in 50% of SW480 cells (Figure 5d and Supplementary Figure S6a). In contrast, adding fresh RPMI media, which is the media used to culture DLD-1 cells, had no effect on TCF-1 localization (Figure 5c). We therefore conclude that nuclear export of TCF-1 is regulated by a secreted factor whose production and/or release is directed by CaMKII signaling.

That a secreted factor controls TCF-1 export raised the possibility that Wnts, which are extracellular ligands, could be involved. In fact, Wnts have been shown to trigger POP-1 export in *C. elegans* and activate CaMKII signaling in human 293 cells (Ishitani *et al.*, 2003a; Kohn and Moon, 2005; Lo *et al.*, 2004). If CaMKII (T286D) expression triggers the secretion of a Wnt ligand, then co-expression of Wnt ligand inhibitors and competitors should prevent TCF-1 export. To test for that, SW480 cells were co-transfected with expression plasmids for CaMKII (T286D) and SFRPs (Secreted Frizzled-Related Proteins), secreted inhibitors that specifically bind to Wnt ligands (Bovolenta *et al.*, 2008). Indeed, expression of SFRP1, 3 or 4 reduced CaMKII-induced export as TCF-1 was restricted to the nucleus in 73–95% of cells (Figure 6c-e and Supplementary Figure S6a). Overexpression of SFRP4 also blocked export of TCF-1 in DLD-1 cells, suggesting that the endogenously secreted factor in DLD-1 cells is also a Wnt (Figure 6g). In addition to SFRPs, we also tested whether Dkk-1 was able to block TCF-1 export. Dkk-1 is a secreted inhibitor that binds the Wnt co-receptor LRP5/6 on the plasma membrane and prevents Wnt-receptor interactions (Glinka et al., 1998). SW480 cells were transfected with Dkk-1 for 24 hours and then supplemented with conditioned media from CaMKII-transfected cells. Introducing Dkk-1 into the cells blocked the secreted signal's ability to trigger TCF-1 export in 88% of SW480 cells (Figure 6i and Supplementary Figure S6a). These data show that CaMKII directs TCF-1 nuclear export via the secretion of a Wnt ligand.

The ability of Wnt inhibitors such as SFRPs and Dkk-1 to block CaMKII action on TCF-1 suggests that Wnt ligands act downstream of CaMKII (Figures 6 and Supplementary Figure S6a). However, current models place Wnt signaling upstream of CaMKII signaling (Ishitani *et al.*, 2003a; Kohn and Moon, 2005; Lo *et al.*, 2004). Specifically, Wnt5a and Wnt 11 have

been proposed to activate CaMKII in HEK293 cells, *Xenopus* embryos and Zebrafish embryos, while MOM-2, a *C. elegans* Wnt, has been shown to activate the TAK1-NLK pathway. To test for the possibility that CaMKII is also required downstream of the secreted Wnt, SW480 cells were simultaneously treated with conditioned media from CaMKII-transfected cells (which contains the secreted Wnt ligand) and a CaMKII-specific inhibitor, KN-93 (Sumi *et al.*, 1991). Inhibition of CaMKII activity with KN-93 blocked the ability of the secreted Wnt to trigger TCF-1 export and restricted TCF-1 to the nucleus in 90% of cells (Figure 7 and Supplementary Figure S6a). Thus, Wnt ligand signaling requires a downstream CaMKII activity to stimulate TCF-1 export. However, CaMKII signaling itself requires downstream Wnt action because Wnt-specific inhibitors SFRP and DKK-1 can block the ability of CaMKII (T286) to trigger TCF-1 export. Taken together, these data suggest that the CaMKII signaling pathway functions as an auto-activating Wnt signaling loop (Figure 8).

Discussion

TCF1 function in normal intestinal epithelia

Here we report a conserved biological process that establishes a balance between Wnt-promoting and Wnt-opposing actions of TCFs in the intestine. Several TCF-1 isoforms are expressed in normal colon crypts with the most abundant form being a growth suppressive dominant-negative called dnTCF-1E (Figure 2 and Supplementary Figure S3). The alternative E-tail C-terminus contributes a second DNA binding domain that converts this dominant negative form to one that can bind with high affinity to a broad spectrum of Wnt response elements, including genes important for cell growth (Atcha *et al.*, 2007; Chang *et al.*, 2008). Since the most abundant form of TCF-4 in the intestine is a full-length E-tail-containing form (FL-TCF-4E; Figure 2b and Supplementary Figure S3c), dnTCF-1E is an equal and effective competitor for regulation of Wnt target genes. DnTCF-1E localization is equally nuclear and cytoplasmic in normal colon crypt cells (Figure 1 and Supplementary Figure S1), and we report the discovery of a signaling mechanism that controls TCF-1 nuclear export. We propose that this export signal sets up an important balance between FL-TCF-4E and dnTCF-1E to establish proper patterns of cell growth and differentiation. Distortion of the balance between Wnt-promoting and Wnt-opposing TCFs would be predicted to cause aberrant growth and cell behavior. In fact, disruption of the TCF-4/TCF-1 balance by deletion of the *TCF1* locus does indeed lead to adenoma formation by 3 months of age in mice (Roose et al., 1999). Clearly, regulation of *TCF1* gene expression is important for limiting the level of Wnt signaling in intestinal epithelia.

TCF1 function in colon cancer

We report a striking switch in TCF-1 expression that occurs during colon carcinogenesis such that the growth-suppressing dnTCF-1E isoform is under-expressed and the growth-promoting full-length FL-TCF-1B is over-expressed to become the most abundant isoform (Figure 2a and d, Supplementary Figures S2 and S3). This switch to expression of FL-TCF-1B protein in cancer is strikingly similar to the activation of FL-LEF-1 in most human colon cancers (Hovanes et al., 2001). FL-LEF-1 production is activated by β-catenin/TCF-4 complexes binding to Wnt response elements in the *LEF1* promoter. The *TCF1* locus has

also been proposed to be a Wnt target gene and recent genome-wide chromatin immuno-precipitation studies in colon cancer cells confirm this prediction. Antibodies specific for TCF-4 detect complexes binding directly to the *TCF1* promoter that produces mRNA for full-length isoforms (Hatzis et al., 2008). Interestingly, the internal second promoter that produces dnTCF-1 isoforms is not occupied by TCF-4 and is therefore unlikely to be regulated by β-catenin complexes. These observations are consistent with a Wnt-driven switch from dominant-negative to full-length TCF-1 protein in the transformation of normal to cancerous colon. Thus, in addition to full-length TCF-4 and LEF-1, FL-TCF-1B contributes to increased Wnt signaling through recruitment of the growing pool of stabilized β-catenin to Wnt target genes although not the E-tail-dependent growth genes. Our observations predict that loss of TCF-1 in colon cancer would result in decreased Wnt signaling and cell growth, and indeed, over-expression of activated CaMKII in SW480 cells lowers the activity of Wnt signaling in Luciferase assays (Figure 3h). Consistent with our predictions, Tang et al. report that knockdown of *TCF1* in the DLD-1 colon cancer cell line decreases cell growth (Tang et al., 2008). Thus, loss of TCF1 in cancer has opposite consequences from its loss in normal colon epithelia; in colon cancer, it contributes an oncogenic function.

Despite the switch from growth-suppressing to growth-promoting TCF-1 isoforms, there is significant relocation of FL-TCF-1B to the cytoplasm in primary human colon tumors (Figures 1f, 3a and c). The cytoplasmic localization pattern is not universal or uniform. We observe that regions or fields of the tumors show predominantly cytoplasmic or a mixed subcellular localization like normal colon epithelia (Atcha and Edwards, unpublished observation). In colon cancer cell lines where the environment is uniform, FL-TCF-1B localization varies among the cell lines where patterns of mostly cytoplasmic (Colo320) contrast with mostly nuclear localization (SW480). The nuclear localization of TCF-1 in SW480 cells is most likely due to the unusually high levels of β-catenin and low levels of activated CaMKII compared to other colon cancer cell lines (Figure 4 and Supplementary Figure S5d) (Sievers et al., 2006). Nuclear β-catenin can bind to full-length LEF/TCFs and trap them in the nucleus, but stimulation of the latent export pathway by overexpression of activated CaMKII overrides any nuclear entrapment and TCF-1 exits to the cytoplasm (Figure 3f). Nuclear exclusion is unique only to TCF-1 as TCF-4 remains nuclear in colon cancer tissues and colon cancer cell lines (Figure 1d and Supplementary Figures S1b, S4 and S5b). Therefore, in the normal colon, dnTCF-1E and TCF-4 are both located in nuclei to counteract each other's actions and maintain an organized balance between "Stop" and "Go" growth signals (Figure 8a). As colon cells undergo transformation and become more cancerous, TCF-1 expression and localization changes (Figure 8b). When the CaMKII pathway is activated, dnTCF-1E is exported to the cytoplasm. Export is followed by a decrease in the levels of growth suppressing dnTCF-1E and an increase in the expression of FL-TCF-1B. The net effect is to eliminate all dnTCF-1E action in the nucleus. There are two distinct scenarios that explain how the switch in expression and export phenomena could both occur in colon cancer. It is possible that aberrant levels of TCF-1 export precede the switch in expression. In this scenario, export of dnTCF-1E would leave FL-TCF-4E unopposed in the nuclei of colon cells, predisposing cells to tumorigenesis and elevated Wnt signaling. The increase in Wnt levels might in turn upregulate the expression of FL-TCF-1B,

a predicted target of Wnt signaling (Figure 8) (Hatzis *et al.*, 2008). Alternatively, it is possible that the switch in expression takes place first. In this case, the Wnt/CaMKII pathway, which results in the export of a full-length activating isoform of TCF-1 and a drop in Wnt signaling levels (Figure 3h), might correlate with good prognosis for colon cancer.

A novel nuclear export pathway

Nuclear export of TCF-1 is the first example of altered trafficking of any LEF/TCF in response to growth regulating signals in mammals and is particularly relevant to cancers linked to misregulated Wnt signaling. The only other example of signal-induced nuclear export of a TCF protein is that of POP-1 during asymmetric cell division in *C. elegans* (Lo et al., 2004). In this event, POP-1 export is induced by TAK1-NLK signals in a CRM1 dependent fashion (Lo et al., 2004). Consistent with that, our findings for mammalian TCF-1 suggest that nuclear exclusion is an active export process mediated by the exportin CRM1 (Figure 3c and d). Although TCF-1 has no detectable Nuclear Export Signal (NES) or Leucine-rich domain that CRM1 could bind to, it is possible that its interaction with CRM1 could be mediated indirectly. In fact, the interaction of POP-1 with CRM1 in *C. elegans* is mediated by a 14-3-3 protein (Lo *et al.*, 2004). Thus, similar to the *C. elegans* system, the nuclear activities of TCF-1 can be down-regulated by a TAK1-NLK signal through triggering its export. This is notably different from CaMKII/TAK1/NLK action on LEF/TCFs in a kidney cell line (HEK293). In these cells, Wnt/CaMKII signals down-regulate LEF-1 and TCF-4 function by promoting protein degradation rather than nuclear exclusion. In this case, the non-canonical Wnt5a pathway signals via CaMKII and TAK1 to stimulate NLK phosphorylation and subsequent degradation of LEF-1 and TCF-4 (Ishitani et al., 2003a; Yamada et al., 2006). Our discovery of CaMKII action in colon cancer must be different because neither TCF-1 nor TCF-4 is degraded (data not shown). Instead, the CaMKII pathway induces the nuclear export of TCF-1 (Figure 3e–h). This is the first demonstration of the importance of the CaMKII as a key modulator of Wnt signaling and growth in the human colon.

A distinguishing feature of CaMKII action in colon cancer is the presence of an intermediate soluble signal that induces TCF-1 export (Figure 5b). CaMKII triggers the production and/or release of this signal and either dominant negative TAK1 and NLK1 expression, Dkk-1 expression or SFRP expression can block this action (Figures 3g and 6). The secreted soluble factor is a Wnt since our data shows that the inhibitors Dkk-1 and SFRPs each prevent CaMKII from inducing TCF-1 export (Figure 6). Wnt ligands can either function through a Frizzled/LRP/*β*-catenin-dependent pathway, or through LRP/β-catenin-independent pathways such as the Ca^{2+}/CaMKII pathway. The ability of Dkk-1, an inhibitor of LRP/*β*-catenin-dependent Wnt signaling to block TCF-1 export suggests that this arm of the Wnt pathway is required for export. Conversely, the ability of the CaMKII inhibitor KN-93 to block Wnt action from the conditioned media suggests that a *β*-catenin-independent, Ca^{+2}/CaMKII signal is also required. This is a unique aspect of TCF-1 export where two distinct Wnt signals appear to function together. Adding more complexity to the export pathway is our finding that, in addition to its action downstream of the Wnt ligand, CaMKII acts upstream to trigger Wnt production or secretion (Figure 7). Thus, overexpression of CaMKII in SW480 cells establishes an auto-activating loop in which

secretion of the Wnt ligand activates CaMKII in adjacent, non-transfected cells to produce more secreted Wnt ligand and to also direct TCF-1 export.

Inappropriate over-activation of this signal would skew the balance of TCFs in normal crypt epithelia and disrupt Wnt functions. Such changes would remove the Wnt-suppressing actions of dnTCF-1E from the nucleus and this could lead to dysplasia or other growth problems even when there are no activating mutations of the Wnt pathway that cause stabilized β-catenin to accumulate. It will be important to determine how many of the 19 different Wnt ligands can trigger this pathway, and which are influential in diseases such as cancer.

Materials and Methods

RT-PCR Analysis

Total RNA was isolated from Jurkat or colon cancer cell lines using TRIZOL Reagent (Life Technologies). One microgram of total RNA was reverse transcribed using random hexamer primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies). For PCR, 1μL of the reverse transcription (RT) reaction mixture was amplified with 30 cycles using primers for full length TCF-1: P1 sense primer in exon Ia' is 5′-AGCTCAAGTCGTCGCTCGTG-3′. TCF-1 antisense primer in exon III is 5′-GTGGGATGTGGGCTGTTGAAATG-3′. Melting, annealing and extension temperatures for this pair were 95°C, 62°C and 72°C. Expected sizes are 366bp and 459bp. TCF1-E tail-specific antisense primer in exon 9 is 5′- GGTTGAGGCCAAAGCGAGCA-3′. Annealing temperature for P1 sense and E-tail antisense was 62°C. Expected sizes are 973bp and 1066bp. Primers used to detect dominant negative TCF-1 isoforms: P2 sense primer in exon I is 5′-CGCCTTCAGGAGACAGAATTG -3′. Annealing temperature for P2 sense and exon III antisense primer pair as well as the P2 sense and E-tail antisense primer pair was 62°C. Expected sizes for the P2/exon III primer pair are 226bp and 319bp, and expected sizes for the P2/E-tail primer pair are 831bp and 924bp. Primers for the GAPDH PCR were: 5′-ACTGCCAACGTGTCAGTGGTG-3′ (sense), 5′-TTACTCCTTGGAGGCCATGTG- 3′ (antisense). Annealing temperature for the GAPDH primer pair was 50°C, and expected size for the product is 300bp. A Full length TCF-1E expression plasmid was used as a positive control.

Western Analysis

Cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). Blots were blocked in 5% milk for 30 min and hybridized with primary antibodies against TCF-1 (1:200 dilution; Upstate 7H3), TCF-4 (1:200 dilution, Upstate 6H5-3), TCF-1E (1:1000 dilution), β-catenin (1:500 dilution, Santa Cruz C-18), phospho-CaMKII (Thr286) (1:200 dilution, Santa Cruz sc-12886), or Actin (1:500 dilution, Santa Cruz I-19) overnight at 4°C. After hybridization, the blots were washed and hybridized with anti-mouse IgG-HRP, anti-rabbit IgG-HRP (1:5000 dilution, Amersham Biosciences), or Bovine anti-goat IgG-HRP (1:15000 dilution, Santa Cruz). ECL reaction was performed according to manufacturer's protocol and exposed to film (Amersham Biosciences). For fluorescent westerns, blots were hybridized with Alexa Fluor

680 goat anti-mouse IgG (1:1000 dilution, Invitrogen) and IRDye 800CW goat anti-rabbit IgG (1:5000 dilution, LI-COR Biosciences). Images were analyzed using the Odyssey Infrared imaging system (LI-COR Biosciences).

TCF-1E antibody—A fragment of the E-tail from human TCF-1E (aa436–561) was expressed in bacteria as a fusion to GST. Recombinant GST-E tail was injected into rabbits and polyclonal anti-sera tested for specificity by Western and immunofluorescence analyses using Cos-1 cells transfected with TCF-1E, TCF-3E or TCF-4E expression vectors (Supplementary Figure S3d). Specificity was further tested with immuno-histochemistry of human tissues in the presence of excess recombinant GST (staining remained) or GST-E tail fusion protein (staining absent).

Isolation of human colonic crypt epithelial cells

Normal colon tissue, obtained under an IRB-approved, exempt protocol, was rinsed 6X in Ca/Mg-free Hanks' Balanced Salt Solution (HBSS) and cut into 5mm strips. Strips were washed (10mM DTT in HBSS) and shaken at 37°C for 30 minutes, then incubated in 1mM EDTA in HBSS for 3X 15-minute washes at 37°C. EDTA-detached crypts were then collected and lysed. A five-minute incubation with ACK lysis buffer was used to eliminate red blood cells (0.15M NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA). Phase-contrast microscopy confirmed that intact crypts were isolated (Figure 2d).

Immuno-histochemistry of primary tumor specimens

Immunofluorescent detection of TCF-1, TCF-4 and β-catenin was performed on paraffin-embedded normal and tumor colon tissue. Patient tissues were obtained under an IRB-approved, exempt protocol from the UCI Department of Pathology and Lab Medicines' tissue bank and procurement service. Tissue sections were de-paraffinized with 3 × 4′ xylene washes, re-hydrated with 2 × 2′ 100% ethanol washes followed by 2′ washes with 95%, 80%, 70% ethanol and a final 5′ wash with water. Antigen retrieval was performed in 1000ml of 10mM Sodium Citrate (pH 6) microwaved on high for 10′. Sections were cooled for 20′ in a water bath, then 3 x 2′water washes and 3 × 2′ in 1X phosphate-buffered saline (PBS) washes. Sections were blocked with 5% normal goat serum (Vector Labs)/1X PBS for 20' at room temperature (RT) and hybridized with an antibody specific for all TCF-1 isoforms (1:100, Upstate 7H3), TCF-4 polyclonal antibody (1:250, Santa Cruz H125), or β-catenin polyclonal antibody (1:250, Santa Cruz H102) overnight at 4°C. Hybridization with secondary goat-anti-rabbit antibody conjugated to Alexa Fluor 568 (1:200 dilution; Molecular Probes) was for 1 hour at RT. Sections were washed with PBS, exposed to DAPI (1:1000) for 1 second, washed with PBS and mounted with gel/mount (Biomeda). Confocal images were obtained using a Zeiss LSM 510 Meta confocal microscope and analyzed with LSM5 Image Browser.

Immuno-fluorescence assay

Cells were cultured in either RPMI media (DLD-1 and Colo320) or DMEM media (SW480) with 10% FBS (Cellgro) and plated at a density of 100,000 cells per well in 6-well plates 24 hours prior to transfection when they were transiently transfected using FuGENE 6 Transfection Reagent. Cells were transfected with plasmids containing FLAG-CaMKII

(T286D) (pCMV; 0.5μg), HA-TAK1 (K63W) (pCMV; 0.5μg), FLAG-NLK (K155M) (pCMV; 0.5μg) (Kind gift from Dr. T. Ishitani), Dkk-1 (pcDNA3; 0.4μg) (Kind gift from Dr. B. Hoang), SFRP1 (pcDNA3.1; 0.5 μg), SFRP3 (pcDNA3.1; 0.5 μg), SFRP4 (pCMV-Sport2; 0.5 μg), or a plasmid containing GFP (EVR2; 1μg). For the export assay, cells were treated with either 75 nM Leptomycin B (LC Laboratories) or 20–60 μM KN-93 (Calbiochem) for 6 hours prior to fixation at 37°C. Cells were fixed with 3.7% formaldehyde (Fisher Scientific) in PBS for 15 minutes at room temperature, washed 3 times, and then permeabilized with 5% NP-40 (Nonidet P-40, Sigma). Cells were blocked with 1.5% Normal Donkey serum (Vector Labs) for 1 hour at RT and probed with either anti-TCF-1 (1:50, Upstate 7H3), anti-TCF-4 (1:50, Upstate 6H5) or anti-phospho CaMKII (Thr286) (1:50, Santa Cruz sc-12886) antibodies overnight at 4°C. Hybridization with secondary antibody (Cy5 or FITC-conjugated AffiniPure Donkey Anti-mouse IgG; 1:1500; Jackson Immuno-Research) and the F-actin stain (BODIPY FL phallacidin, 0.5units/ml, Invitrogen) was for 1 hour at RT. Nuclei were then stained with DAPI (50μg/ml, Invitrogen) for 5 seconds followed by three washes. Confocal images were obtained using a Zeiss LSM 510 Meta confocal microscope and analyzed with LSM5 Image Browser.

Luciferase assays

SW480 cells were cultured in DMEM media with 10% FBS and plated at a density of 200,000 cells per well in 6-well plates 24 hours prior to transfection when they were transiently transfected with 0.1μg of Super8xTopflash reporter plasmid (kind gift from Dr. R.T. Moon), 0.1μg of thymidine kinase β-galactosidase plasmid and 0.4μg of the expression vector for CaMKII (T286D) using FuGENE6 Transfection Reagent. Cells were harvested 48 hours post transfection when luciferase activity was measured and normalized using β-galactosidase levels. Duplicate samples were assayed for each condition, and three experiments were independently performed to calculate the standard deviation values for error bars.

Tissue acquisition

Patients with suspected or documented colorectal cancer or colonic polyps were identified through clinics at the H.H. Chao Digestive Diseases Center and the Chao Family Comprehensive Cancer Center at the University of California, Irvine. All patients completed written informed consent and enrolled onto an IRB-approved clinical trial. The clinical trial was registered with clinicaltrials.gov prior to study initiation (NCT00256334). Charts for over 250 patients scheduled for colonoscopic examination were screened. More than 50 individuals were enrolled onto the tissue acquisition component of this study. All tissues were histologically reviewed to confirm diagnosis. Biopsies of normal mucosa, colon cancer and colon adenomas were transported to the pathology department where a small sample of tissue was allocated for research testing and immediately placed into RNAlater (Applied Biosystems, Foster City, CA). RNA was later isolated with a Trizol reagent.

Membrane Array analysis

Specimen cDNA was analyzed with a GEArray Q Series Human Wnt Signaling Pathway Gene Array (SuperArray Bioscience, Frederick, MD) which profiles the expression of genes

involved in and downstream of Wnt signaling. GEArray expression analysis software was utilized for background normalization and normalization with multiple housekeeping gene controls on each membrane.

qRT-PCR analysis

Isolated RNA was reverse transcribed into cDNA using the High Capacity cDNA kit (Applied Biosystems). cDNA was amplified for 45 cycles with the QuantiTect SYBR Green PCR Kit (Qiagen) using primers for full-length or dominant-negative TCF-1. Primers for the FL-TCF-1 PCR were P1 sense primer and exon III antisense primer (see above for sequence). Primers for the dn-TCF-1 PCR were P2 sense primer and exon III antisense primer. Melting, annealing and extension temperatures for both pairs were 95°C, 57°C and 72°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Special thanks to Didi Mwengela, Amanda Chamberlain, Candice Solomon and David Hernandez for help in cloning and sequencing *TCF-1* cDNA. We would also like to thank Eric. J Stanbridge for critical reading of the manuscript, Michelle Digman for help with confocal imaging, Tohru Ishitani, Bang Hoang and Randall T. Moon for reagents and advice. This work was supported by NIH grants RO1 HD36081 and RO1 HD36049 to JLM, NIH CA096878 and CA108697 to MLW, R21 DK071591 to RAE and NIH grant CA-82450 to RFH. This work was made possible in part, through access to the confocal facility of the Optical Biology Shared Resource of the Cancer Center Support Grant (CA-62203) at the University of California, Irvine.

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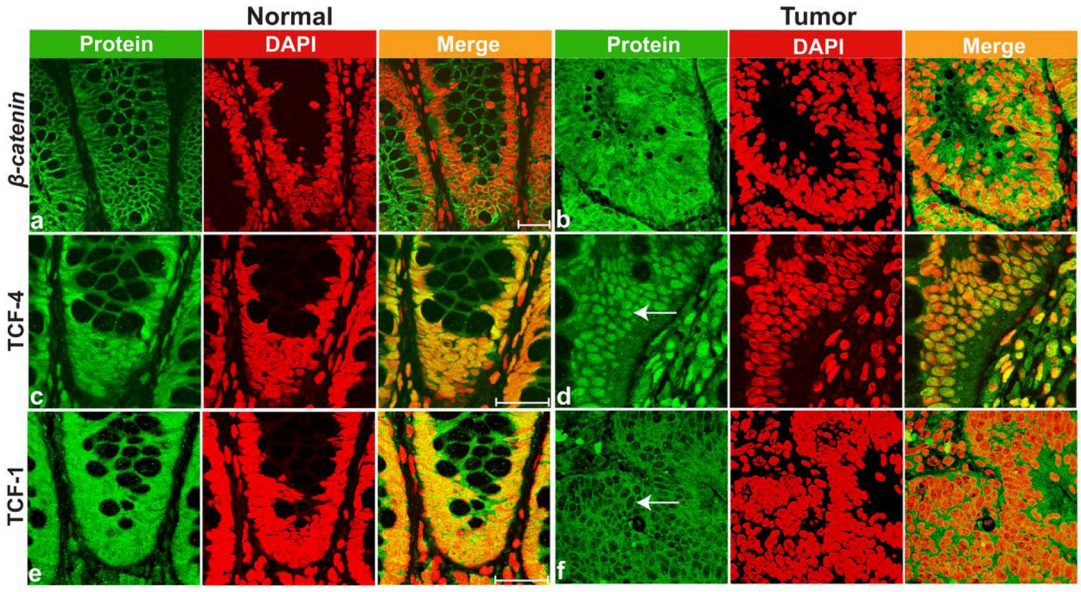

Figure 1.

TCF-1 is localized in the cytoplasm in colon cancer. Immuno-fluorescent detection of TCF-1 and TCF-4 in paraffin-embedded sections of tumor and normal matched human colon tissue. Antibody staining is green. Nuclei are stained with DAPI (false colored here as red to allow visualization). In the merged images, co-localization of antibody and DAPI is yellow. Control experiments show that in normal colon tissues, (**a**) β-catenin is cytoplasmic while in colon cancer, (**b**) β-catenin is nuclear and cytoplasmic thus validating the tissue integrity for these studies and confirming elevated Wnt signaling in the tumor. TCF-4 is predominantly nuclear in both normal and colon cancer tissue (**c** and **d**). TCF-1 is both nuclear and cytoplasmic in normal colon tissue (**e**), but TCF-1 is cytoplasmic and absent from nuclei in colon cancer (**f**). Scale bar is 20 μm.

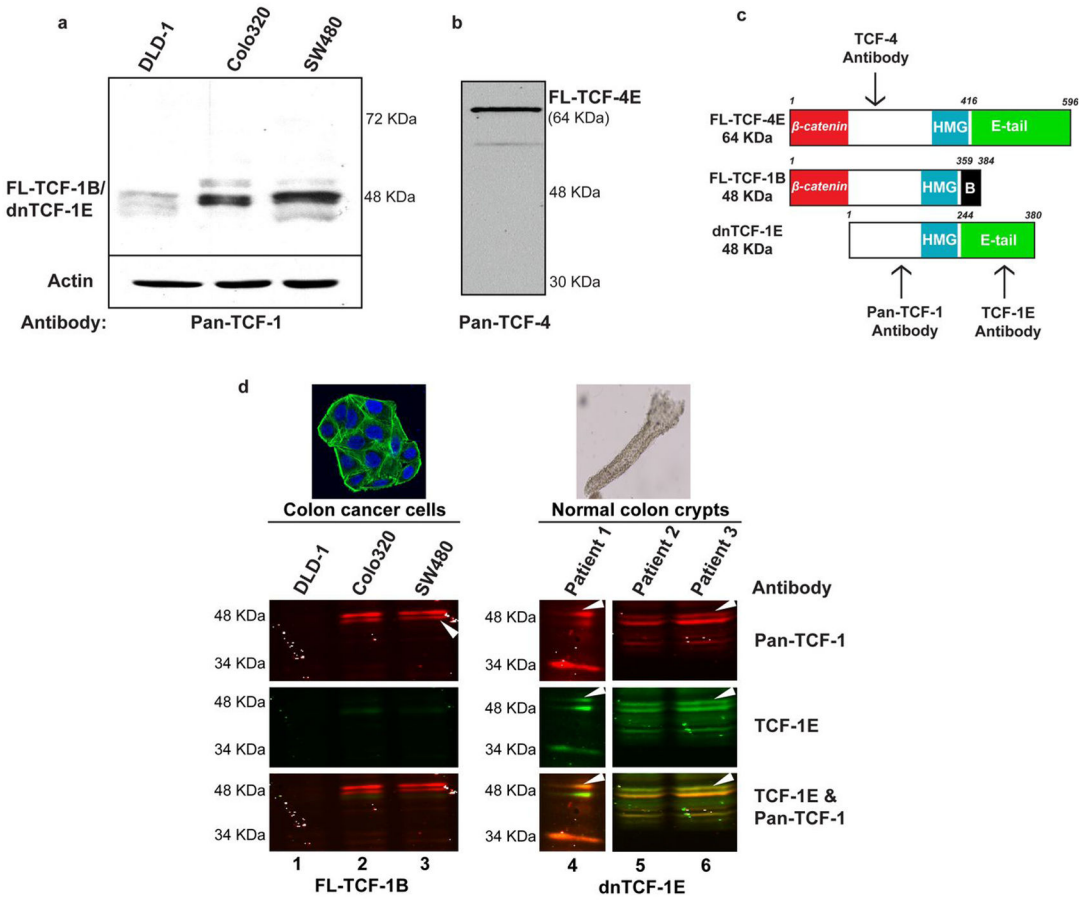

Figure 2.

TCF-1 isoforms in normal colon and colon cancer. (**a**) Western blots of DLD-1, Colo320 and SW480 colon cancer cell lines probed with antibodies that detect all forms of TCF-1 reveal FL-TCF-1B as the major form in colon cancer. (**b**) Western blots of DLD-1 colon cancer cells probed with an antibody that detects all forms of TCF-4 confirm that TCF-4 is expressed as a full-length E-tail isoform. (**c**) Schematic showing the predicted sizes of FL-TCF-4E (64 KDa), FL-TCF-1B (48 KDa) and dnTCF-1E (48 KDa) and the antibody locations on TCF-1 and TCF-4 (**d**) Crypt epithelial cells were isolated and purified from normal colon tissue of three different patients. Western blots of colon cancer cell line extracts and the crypt epithelial cell extracts were probed with two antibodies. One antibody detects all forms of TCF-1 and another detects E tail-containing isoforms of TCF-1. The merged signals from the two antibodies confirm that FL-TCF-1B is the major form in colon cancer and that dnTCF-1E is the major form in normal colon.

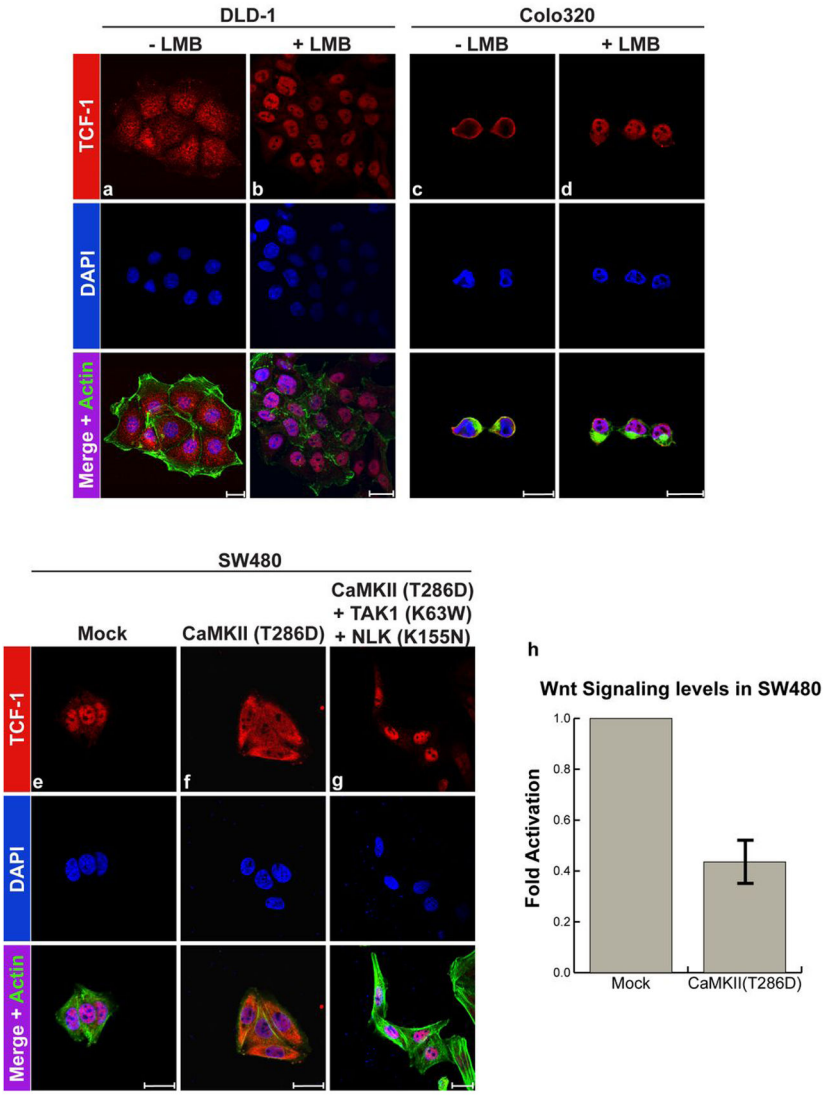

Figure 3.

TCF-1 is exported from the nucleus in response to CaMKII via a CRM1 dependent mechanism. The colon cancer cell lines DLD-1, Colo320 and SW480 were stained for endogenous TCF-1 (red). F-actin was stained with Phalloidin (green) to illuminate the cytoplasm and nuclei were stained with DAPI (blue). (**a**) TCF-1 is nuclear and cytoplasmic in DLD-1 cells. (**c**) TCF-1 is cytoplasmic in Colo320 cells. (**b**) and (**d**) TCF-1 localizes to the nucleus of both DLD-1 and Colo320 cells after a 6-hour treatment with the CRM1 inhibitor Leptomycin B. (**e**) TCF-1 is predominantly nuclear in mock transfections of SW480 cells. (**f**) TCF-1 is exported to the cytoplasm in SW480 cells expressing CaMKII (T286D). (**g**) When CaMKII (T286D) is co-expressed with kinase dead TAK1 (K63W) and NLK (K155M), TCF-1 remains nuclear. Scale bar is 20 μm. (**h**) Transfection of activated CaMKII (T286D) in SW480 cells lowers Wnt signaling levels by 50–70% as measured by luciferase reporters.

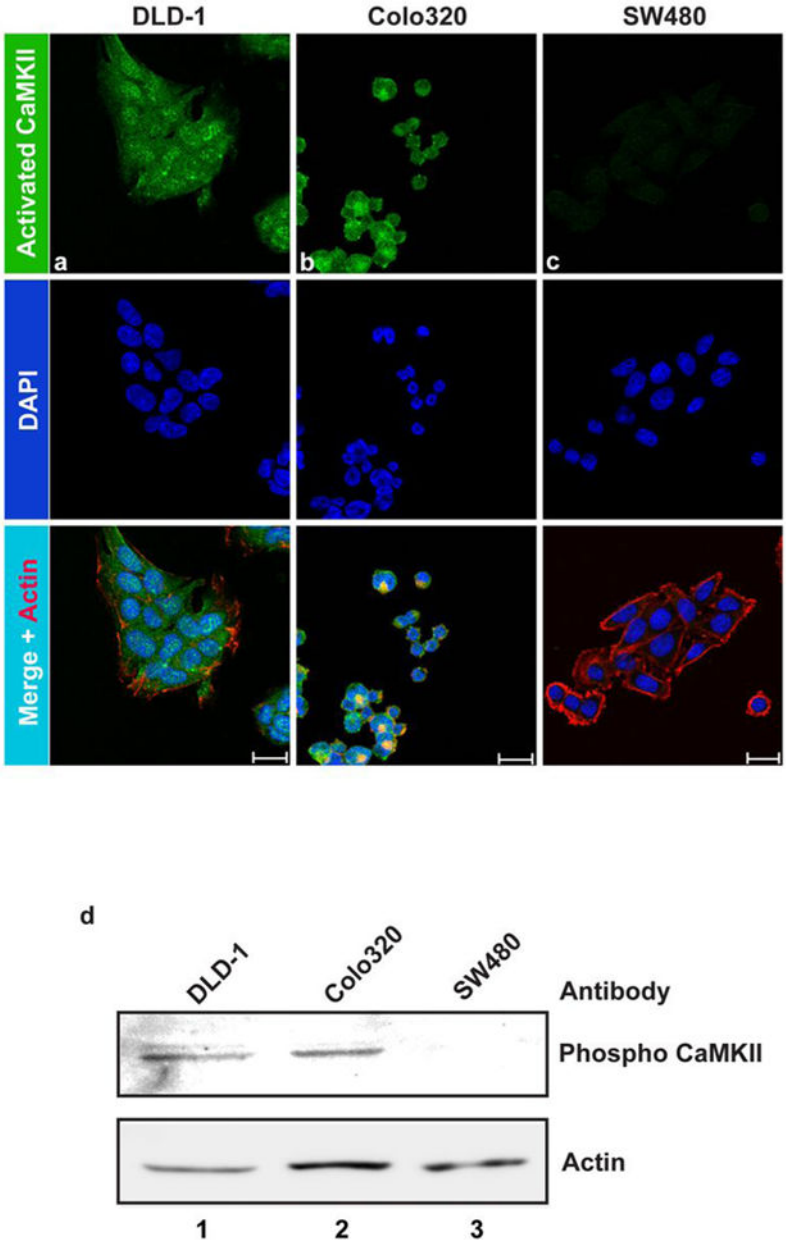

Figure 4.

Levels of activated CaMKII correlate with TCF-1 export level in colon cancer. DLD-1, Colo320 and SW480 cells were stained for the phospho-T286 modification of CaMKII (Green). F- actin staining is red and nuclear staining is blue. (**a**) and (**b**) DLD-1 and Colo320 cells express high levels of activated CaMKII while SW480 cells do not (**c**). Scale bar is 20 μm. (**d**) Western blots of colon cancer cell extracts probed for phosphorylated, active CaMKII show detectable levels in DLD-1 and Colo320 cells, but not in SW480 cells.

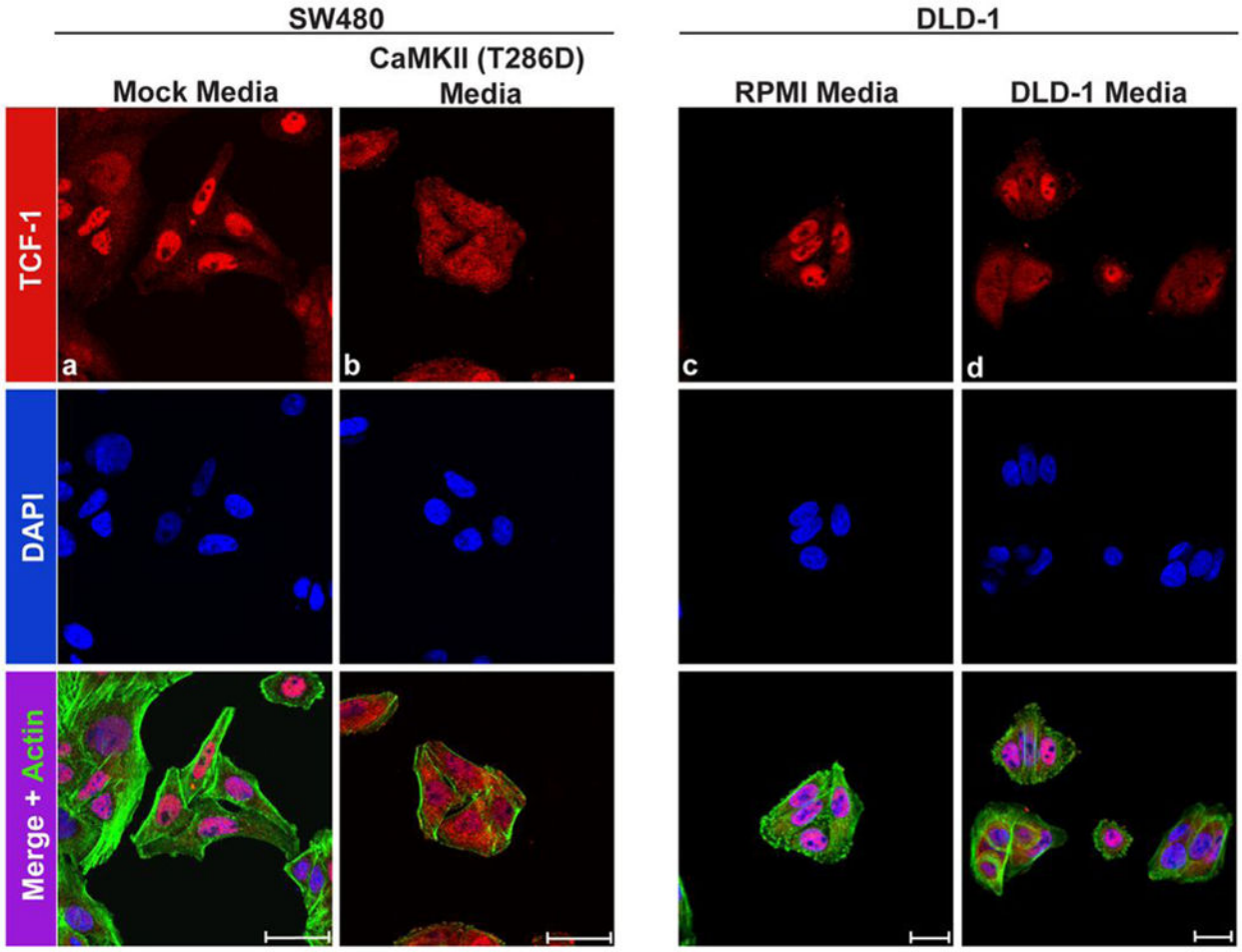

Figure 5.

TCF-1 export is regulated by secreted ligand. Media from SW480 cells that had been mock-transfected or transfected with a plasmid containing constitutively active CaMKII (T286D) for 48 hours, or RPMI media, or media from DLD-1 cells was transferred to newly seeded SW480 cells and these cells were stained for endogenous TCF-1 (red). F-Actin staining is green and nuclear staining is blue. (**a**) In cells with mock-transfected media, TCF-1 is nuclear. (**b**) Cytoplasmic TCF-1 is observed in cells treated with conditioned media from CaMKII (T286D)-transfected cells. (**c**) TCF-1 is nuclear in cells grown in RPMI media, but cytoplasmic in cells treated with conditioned media from DLD-1 cells (**d**).

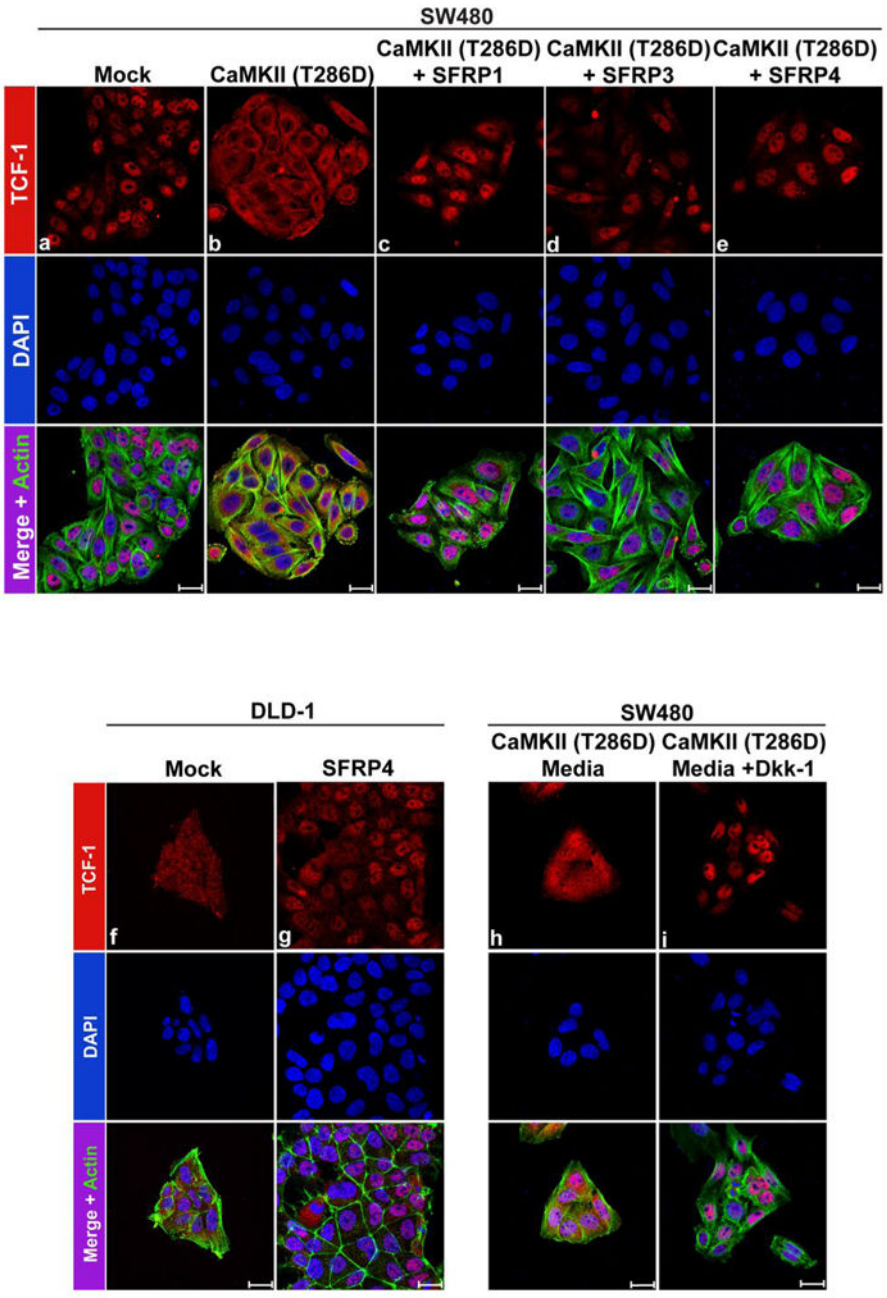

Figure 6.

Wnt signaling regulates TCF-1 export. SW480 cells were transfected with a plasmid containing constitutively active CaMKII (T286D) alone or with expression plasmids for SFRP and stained for TCF-1 (red). (**a**) TCF-1 is predominantly nuclear in mock transfections of SW480 cells. (**b**) TCF-1 is exported to the cytoplasm in SW480 cells expressing CaMKII (T286D). Expression of SFRP1 (**c**), SFRP3 (**d**), or SFRP4 (**e**) block CaMKII's ability to trigger TCF-1 export. DLD-1 cells were either mock-transfected or transfected with a plasmid containing SFRP4 and stained for TCF-1 (red). (**f**) TCF-1 is nuclear and cytoplasmic in mock-transfected DLD-1 cells, but is restricted to the nucleus in

SFRP4-transfected DLD-1 cells (**g**). Media from SW480 cells that had been transfected with a plasmid containing constitutively active CaMKII (T286D) for 48 hours was transferred to newly seeded SW480 cells or Dkk-1 transfected SW480 cells and these cells were stained for TCF-1 (red). (**h**) Cytoplasmic TCF-1 is observed in cells treated with conditioned media from CaMKII (T286D)-transfected cells. (**i**) Dkk-1 blocks the conditioned media's potential to trigger TCF-1 export. Scale bar is 20 μm.

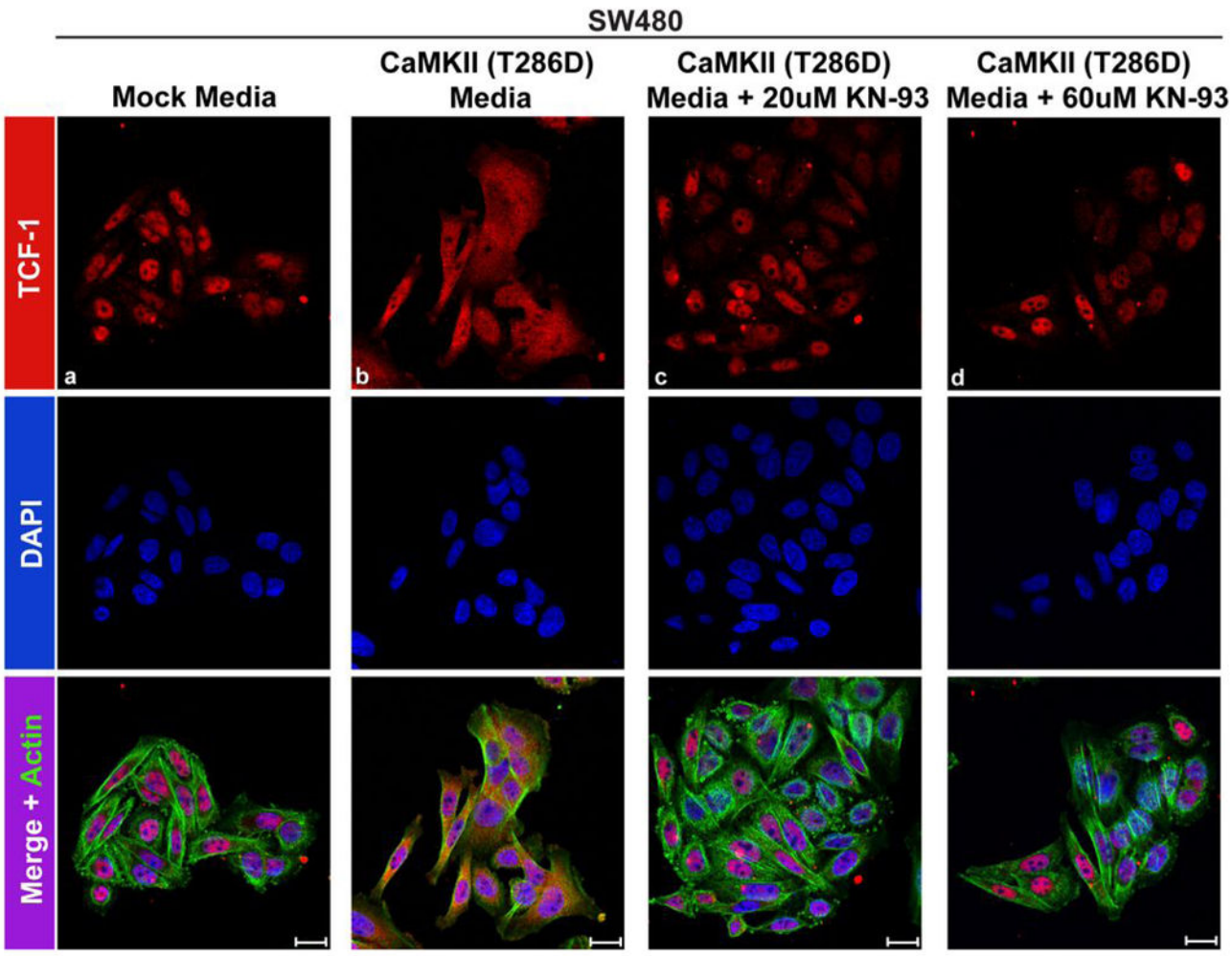

Figure 7.

TCF-1 export is an auto-activating loop. Media from SW480 cells that had been mock-transfected (**a**) or transfected with a plasmid containing constitutively active CaMKII (T286D) for 48 hours (**b**) was transferred to newly seeded SW480 cells and treated with 20μM (**c**) or 60μM (**d**) of the CaMKII inhibitor, KN-93 for 6 hour. (**c**) and (**d**) Varying KN-93 concentrations block the conditioned media from triggering TCF-1 export.

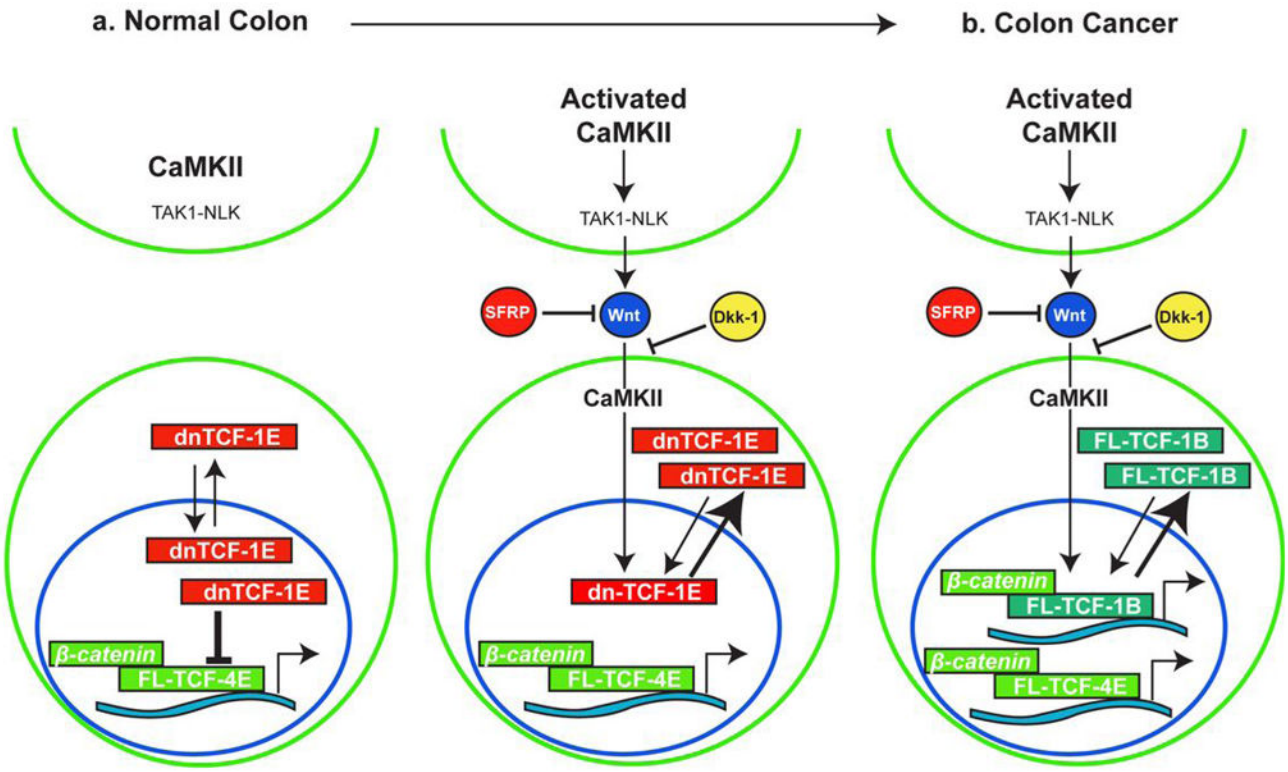

Figure 8.

Working Model. (**a**) In normal colon, dnTCF-1E is expressed in the nuclei of cells where it counteracts the actions of FL-TCF-4E and preserves a balance between the signals that promote growth and those that promote differentiation. (**b**) In colon cancer, export of dnTCF-1E protein predominates. Export is mediated by CRM1 in response to activated Ca^{2+}/Calmodulin dependent Kinase II (CaMKII) and TAK-1-NLK kinases. Export requires Wnt ligand action, a soluble factor secreted in response to activated CaMKII since SFRP proteins and Dkk-1 can block the export pathway. CaMKII actions is also required downstream of the Wnt ligand, suggesting that the export pathway functions as an auto-activating loop. (**c**) FL-TCF-1B is expressed and dnTCF-1E is under-expressed to further skew the balance towards the growth-promoting signals in colon cancer.