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Human telomerase reverse transcriptase (*hTERT*) is a target gene of β-catenin in human colorectal tumors

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Abbreviations: hTERT, human telomerase reverse transcriptase; CRC, colorectal cancer; CSC, cancer stem cell; EMT, epithelial-mesenchymal transition; TCF-4, T-cell factor 4; WT, wild-type

The majority of colorectal cancers (CRCs) are characterized by a dysregulated canonical Wnt-signaling pathway leading to the stabilization and subsequent cellular increase and accumulation of β-catenin. After translocation into the nucleus, it acts as a transcription factor resulting in the expression of β-catenin target genes. These resemble most of the hallmarks of cancer except eternal life. The central mediator of this hallmark is hTERT (human telomerase reverse transcriptase). The hTERT gene is regulated, besides others, by the transcription factor c-Myc and, thus, indirectly via β-catenin as c-Myc is a β-catenin target gene. Interestingly, the expression patterns of hTERT and β-catenin, but not c-Myc are overlapping, probably because c-Myc is not only regulated by β-catenin, but also by many other transcription factors and pathways. Therefore, we argued that hTERT might be a direct target gene of β-catenin. In this study, we show evidence that β-catenin directly regulates the expression of the hTERT gene.

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Introduction

The majority of human colorectal carcinomas (CRCs) are characterized by the dysregulation of the canonical Wnt-signaling pathway. This pathway controls the stability of β-catenin, which is a protein with ambivalent functions.[1] On the one hand, it maintains the epithelial phenotype of cells in the context of E-Cadherin as an integral part of the zonula adherens.[2] On the other, it drives together with DNA binding factors of the TCF (T-cell factor)/LEF-1 (lymphocyte enhancing factor-1) family the transcription of a variety of target genes when it has translocated into the nucleus.[3] β-catenin target genes are characterized by consensus binding sites (WWCAAG) for TCFs/LEF-1 in their promoter/enhancer regions, which is known as the TCF-4 binding element (TBE).[4] The expression of β-catenin target genes results in a dramatic change of the cell biology, whereby the target genes sustain the hallmarks of cancer.[5] These hallmarks are epithelial-mesenchymal transition (EMT; *Slug*, *Snail, Vimentin, Fibronectin*),[6] stemness,[7] chemoresistance (*MDR-1*, *multi drug resistance*-gene),[8] proliferation (*c-Myc*,[9] *cyclin D1*,[10,11] *p16*^{INK4A}[12]), angiogenesis (*VEGF*, *vascular endothelial growth factor*),[13] resistance to apoptosis (*Survivin*),[14] as well as invasion and migration (*MMP7*, *matrix metalloproteinase 7,*[8] *uPA, urokinase plasminogen activator*,[15] *Tenascin-C*[16]), besides others. Another essential hallmark for the survival of tumor cells is eternal life, which is usually connected with the expression of the *hTERT* (*human telomerase reverse transcriptase*) gene in the majority of all tumor types or sometimes ALT (alternative telomere lengthening),[17] as the lengthening of telomers is linked to the evasion from replication induced senescence and subsequently cell death.[5,18] Additionally, hTERT is expressed in intestinal stem cells (SCs),[19,20] which are also characterized by active Wnt-signaling what is essential for the maintenance of stemness in adult stem cells,[21] as well as colorectal cancer stem cells (coCSCs).[7,22] A connection between the activity of β-catenin and the regulation of the expression of the *hTERT* gene had already been drawn by c-Myc, which is a target gene of β-catenin[9] and regulates at the same time the transcription of the *hTERT* gene.[23] But, whereas c-Myc is found to be expressed in all tumor cells,[24] hTERT was shown to be expressed only in some tumor cells mostly at the invasive front of CRC[25] and thereby reflects the expression pattern of β-catenin.[26] Additionally, the *c-Myc*-gene is not only regulated by β-catenin, but a plethora of other signaling pathways as well.[27] Thus, it is reasonable that the expression of nuclear β-catenin, with all its consequences, might become decoupled from the hallmark eternal life, as other signaling traits might overdrive the expression of the *c-Myc* gene. Thus,

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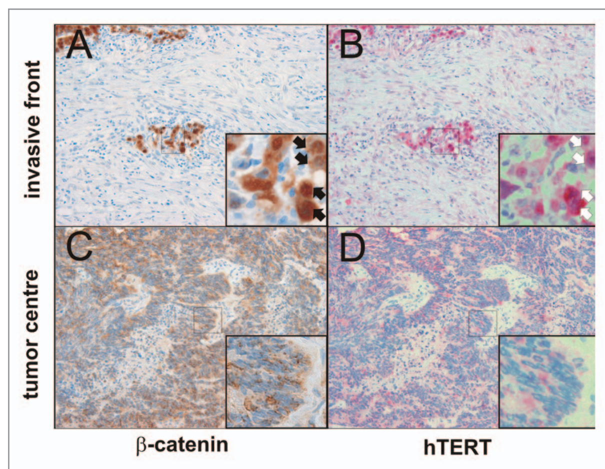

Figure 1. Coexpression of β-catenin and hTERT in tumor cells at the invasive front of colorectal tumors. (A) Tumor cells at the invasive front express nuclear β-catenin (inset, black arrows) and display a mesenchymal differentiation. (B) Corresponding tumor cells also express hTERT (inset, white arrows). (C) Tumor cells in central areas of the tumor display weak or no expression of nuclear β-catenin (inset). These cells are characterized by epithelial differentiation. (D) Corresponding tumor cells display weak or no hTERT expression (inset). At 200x magnification.

we speculated that the *hTERT* gene might be directly regulated by β-catenin. This hypothesis was additionally supported by the finding that the *hTERT* gene contains at least one TCF-4 binding site.[28] Here, we show evidence that *hTERT* is another direct target gene of β-catenin, and that therefore the hallmark eternal life is directly controlled by β-catenin in human colorectal cancer. During the preparation of this manuscript, the relationship of hTERT and β-catenin was also demonstrated in embryonic and adult stem cells as well as in colorectal cancer cell lines.[29]

Results

hTERT and β-catenin are coexpressed at the invasive front of human colorectal cancers. Many CRCs are characterized by an invasive front with tumor cells displaying β-catenin. These tumor cells also express β-catenin target genes.[26,30] Therefore, invasive fronts of such CRCs are a useful screening tool for the identification of β-catenin target genes in human colorectal cancers. Moreover, it had already been shown that hTERT is expressed at the invasive front of CRC.[25] But, no relation with the nuclear localization of β-catenin is known. Therefore, we investigated if nuclear β-catenin and hTERT displayed overlapping expression patterns employing serial histological sections of 24 human CRC with invasive fronts expressing nuclear β-catenin. It turned out that tumor cells with nuclear β-catenin (**Fig. 1A**) also strongly expressed hTERT (**Fig. 1B**). In contrast, tumor cells lacking nuclear β-catenin (**Fig. 1C**) did not display hTERT expression (**Fig. 1D**). Thus, it was reasonable to argue that β-catenin might be involved in the regulation of hTERT.

The promoter/enhancer of the *hTERT* gene contains four TCF-4 binding elements (TBE). A possible link in the regulation of *hTERT* by β-catenin is c-myc as it is on the one hand a target gene of β-catenin[9] and is known to regulate the transcription of the *hTERT* gene.[23] But, as the *hTERT* gene has an outstanding role during the development of cancer[5] and quite a complex transcriptional regulation,[31] a more direct role of β-catenin was envisaged.

Especially, as in a ChIP-on-chip assay using TCF-4 specific antibodies chromatin containing the promoter/enhancer of the *hTERT* gene was detected.[28] To investigate the transcriptional regulation of *hTERT* by β-catenin/TCF-4 in more detail, we analyzed the promoter/enhancer region of the *hTERT* gene (accession number AB016767) for the occurrence of consensus TBEs (WWCAAAG).[28,32] Four TBEs were identified at positions -1,627 (TBE1), -2,149 (TBE2), -2,488 (TBE3) and -2,974 (TBE4), with respect to the transcription start (+1) of the *hTERT* gene. These four TBEs are embedded in binding elements for a variety of other transcription factors like AP2 (activation protein 2), MAD (MAX dimerization protein), c-Myc, MZF2 (myeloid zinc finger protein 2), SP1 (specificity protein 1) or WT1 (Wilms tumor 1-gene) (**Fig. 2A**).[31] Thus, based on the composition of its promoter/enhancer region, the *hTERT* gene might be a putative β-catenin/TCF-4 target gene.

β-catenin and TCF-4 bind to TBEs in the promoter/enhancer region of the *hTERT* gene. Next, it was investigated if TCF-4 and β-catenin bound to the TBEs in the promoter/enhancer of the *hTERT* gene. In a first step, it was tested if the DNA binding domain (DBD) of a recombinant TCF-4/GST (glutathione-S-transferase) fusion protein bound specifically to TBE3 containing fragments (**Fig. 2A**, EMSA TBE3) of the *hTERT* gene, which was chosen randomly from the four TBEs (**Table 1**) applying electric mobility shift assays (EMSAs). Therefore, a radioactively end-labeled TBE3-DNA probe was incubated with TCF-4/GST, resulting in the retardation of the migration of the probe, thus, indicating binding of TCF-4/GST to the probe (**Fig. 2C**, lane 1). This binding was competed by adding unlabeled TBE3- (**Fig. 2C**, lane 2) or TBE4-DNA probes (**Fig. 2A and C**, lane 3), or by adding the second TBE of the *c-Myc* promoter/enhancer (**Fig. 2C**, lane 6). No competition was seen when mutant variants of the probes TBE3- (MUT3, **Fig. 2C**, lane 3) or TBE4-DNA probes (MUT4, **Fig. 2C**, lane 5) were taken instead. Binding was due to TCF-4, as GST alone did not bind to the radioactively end-labeled TBE3-DNA probe (**Fig. 2C**, lane 7). In a second step, it was investigated if β-catenin was associated with the *hTERT* promoter/enhancer in the context of the native chromatin employing chromatin immunoprecipitations (ChIPs). Therefore, two different β-catenin specific antibodies (β-CATI, β-CATII) were incubated together with chromatin of the cultivated colorectal cell line DLD-1. Both antibodies bound chromatin of the *hTERT* promoter/enhancer containing TBE3 and TBE4 (**Fig. 2A**, ChIP-PCR, **Fig. 2D**, hTERT) as well as the *c-Myc* promoter/enhancer (**Fig. 2D**, *c-Myc*), which was used as a positive control as their precipitates resulted in PCR products specific for the respective regions of the genes containing the TBEs. The antibody driven interaction with the chromatin was specific for TBEs as no TATA-box containing fragments of the *glycerol-aldehyde-phosphate-dehydrogenase* (*GAPDH*) gene was precipitated with β-CATI or –II (**Fig. 2D**, *GAPDH*). This absence of signals was not due

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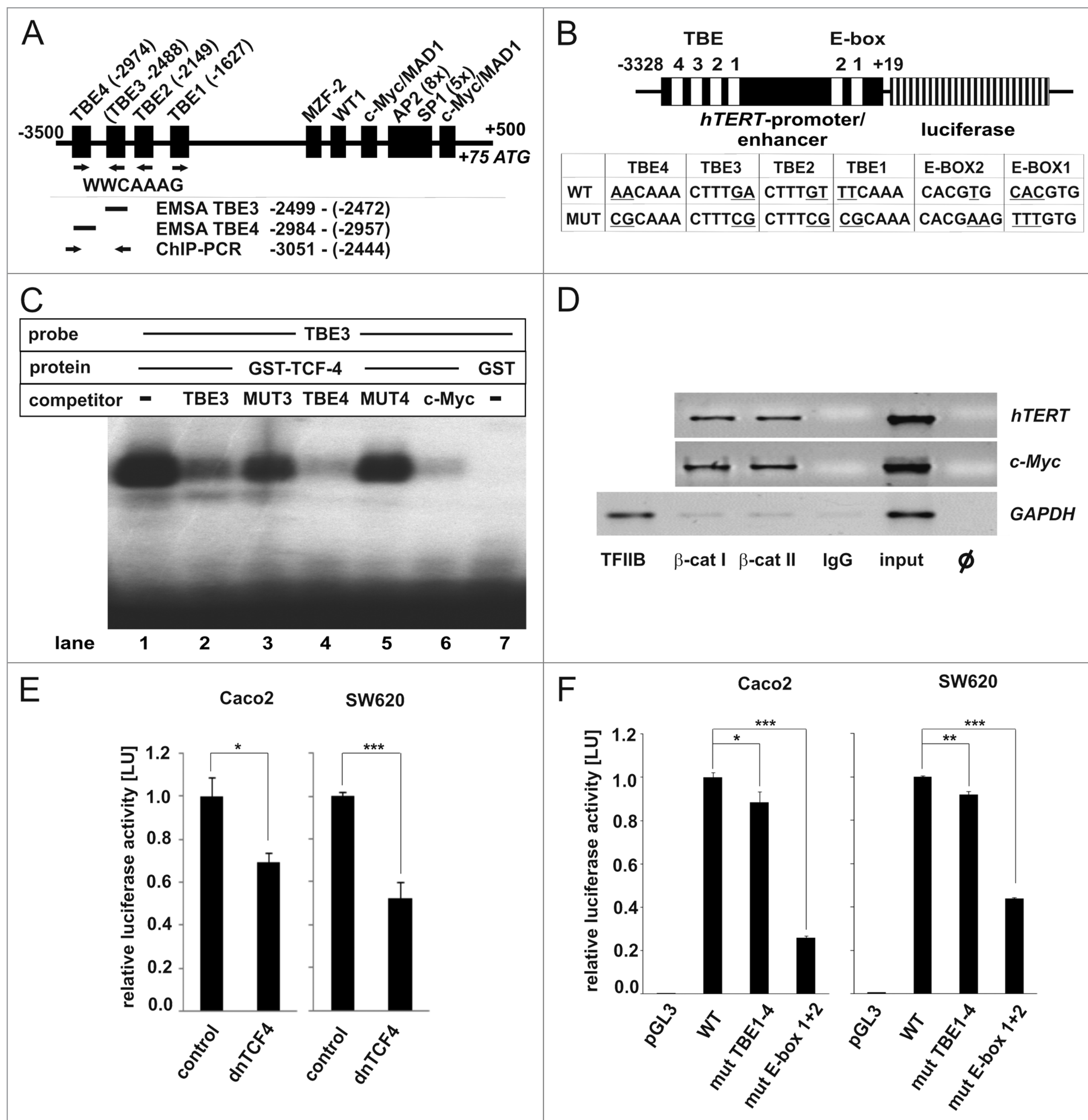

	TBE4	TBE3	TBE2	TBE1	E-BOX2	E-BOX1
WT	AACAAA	CTTTGA	CTTTGT	TTCAAA	CACGTG	CACGTG
MUT	CGCAAA	CTTTCG	CTTTCG	CGCAAA	CACGAAG	TTTGTG

Figure 2. For figure legend, see page 3334.

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to a systemic error as the DNA worked when it was used as the template (**Fig. 2D**, input), but most importantly, also when an antibody specific for the TATA-box binding protein B was used instead (**Fig. 2D**, TFIIB). Additionally, binding was not due to unspecific binding of the antibodies as isotype-specific controls also did not result in the precipitation of gene-specific template DNA fragments (**Fig. 2D**, IgG). Taken together, both TCF-4 and β-catenin bound specifically to TBEs of the human *hTERT*-gene, which is a prerequisite for a transcriptional regulation.

β-catenin/TCF-4 regulates the transcriptional activation of the *hTERT* promoter/enhancer. In a next step, the functional relevance of β-catenin/TCF-4 for the transcriptional regulation of the *hTERT* gene was investigated by performing transient *hTERT* promoter/enhancer *luciferase*-reporter gene assays in cultivated colorectal Caco2 and SW620 cells. For the experiment, a fragment of the *hTERT* promoter enhancer (–3,328/+19) driving the *firefly luciferase* gene[23] was transiently transfected together with increasing an expression plasmid encoding a dominant-negative form of the *TCF-4* gene (*dnTCF-4*), which inhibits β-catenin/TCF-4 transcriptional activity.[33] dnTCF-4 suppressed the activity of the *hTERT* promoter/enhancer luciferase reporter-gene construct to residual amounts of about 70% (Caco2) or

Figure 2 (See previous page). β-catenin interacts with TBEs in the *hTERT* promoter/enhancer and confers transcriptional activation. (A) Schematic representation of the *hTERT* promoter/enhancer with four located consensus motif TBEs (TCF binding elements) and other known responsive elements: MZF2 (myeloid zinc finger protein 2), WT1 (Wilms tumor 1 gene), c-Myc/MAD1 (MAX dimerization protein 1), AP2 (activation protein 2) and SP1 (specificity protein 1). The arrows below the TBEs represent the orientation of the consensus sequence WWCAAAG. The sites of the EMSA-DNA probes and competitors as well as the binding site of *hTERT* primer used in the ChIP-assay are indicated. Figure is not drawn to scale. (B) The *hTERT* promoter/enhancer *luciferase* reporter gene construct consists of a 3,347 bp fragment of the *hTERT* promoter/enhancer harboring the four TBEs (1, 2, 3, 4) and the two E-boxes driving the firefly *luciferase* reporter gene. Figure not drawn to scale. The table shows the mutated consensus sequences of the TBEs and E-boxes. (C) Specific binding of recombinant GST-TCF-4 (glutathion-S-transferase-T-cell-factor-4) fusion protein to a radioactively endlabeled DNA probe containing the TBE3 sequence of the *hTERT*- promoter/enhancer using electric mobility shift assays (EMSA). Binding was competed by adding unlabeled TBE3 or TBE4 sequence containing fragments of the *hTERT* promoter/enhancer or the TBE2 sequence of the *c-Myc* promoter/enhancer. (D) β-catenin specifically binds the genomic region of the *hTERT* gene containing TBE3 and TBE4 in the context of native chromatin of cultured colorectal DLD-1 cells applying ChIP (chromatin immunoprecipitation). Two different antibodies specific for β-catenin, an antibody specific for TFIIB and immunoglobulins (IgG) as controls were used. Specific binding of β-catenin was analyzed by PCRs using primer pairs spanning the genomic regions of the *hTERT* and *c-Myc* promoter/enhancer region containing the gene specific TBE3 and TBE4 or TBE2, respectively. PCRs using primer-pairs for GAPDH promoter/enhancer region served as a negative control. Antibody for TFIIB was used as systemic control for *GAPDH* promoter/enhancer. (E) *dnTCF-4* expression plasmid suppresses the transcriptional activity of *hTERT* promoter/enhancer *luciferase* reporter gene constructs. As control pcDNA-CAT plasmid was used. (F) SW620 and Caco2 cells were transfected with constitutive active β-catenin expression plasmids to enhance Wnt-signaling. Mutation of the four TBEs or of the two E-boxes in the *hTERT*-promoter/enhancer results in a significant downregulation of the *hTERT* promoter/enhancer activity compared with the activity of the WT hTERT promoter/enhancer. pGL3-basic plasmids were used as negative control. Data are represented as mean ± SD (n = 3). Error bars represent standard deviations. Student's t test (two-sided) was used for comparisons. For the analyses, $p < 0.05$ considered significant. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

50% (SW620), respectively (**Fig. 2E**). Thus, β-catenin/TCF-4 might play a direct role in the transcriptional regulation of the *hTERT* gene.

As the hTERT-gene is also transcriptionally regulated by c-Myc, and both the c-Myc mRNA as well as protein are characterized by short half-lives of about 20 min,[34] it might be possible that the observed effects were induced by a negative effect of dnTCF-4 on the *c-Myc*-gene.[9] To discriminate the β-catenin/TCF-4 effects from those of c-Myc, we mutated the four TBEs and the two E-boxes (Enhancer Box, c-Myc binding site) in the *hTERT* promoter region (**Fig. 2B**). These were cotransfected together with expression plasmids encoding constitutive active β-catenin into Caco2 and SW620 cells. Here, TBE-mutated *hTERT* promoter/enhancer-driven luciferase reporter plasmid showed in both cell lines a significant reduction of the *hTERT* promoter activity compared with the WT *hTERT* promoter/enhancer constructs. Expectedly, the mutation of both E-boxes also resulted in a significant reduction of the *hTERT* promoter activity, because hTERT is a well-known target gene of c-Myc (**Fig. 2F**).

The transcription of the *hTERT* gene is directly regulated via β-catenin, but independently of c-Myc. To demonstrate the direct regulation of the *hTERT* gene via β-catenin, a knockdown approach applying β-*catenin*-specific siRNA (small interfering RNA) was chosen. To overcome the concomitant downregulation of *c-Myc* in this approach *c-Myc*-expression plasmids were cotransfected additionally, 24 h before knocking down β-*catenin*. Therefore, β-*catenin*,[35] or as a control *GFP*-specific siRNAs, were transiently transfected into cultivated colorectal SW480 or DLD-1 cells (**Fig. 3**, GFP-■, β-catenin-□) in the absence (0 ng *c-Myc*) or presence (600 or 2,000 ng *c-Myc*) of *c-Myc* expression plasmids. The knockdown of β-*catenin* resulted in a substantial reduction of both β-*catenin* as well as *c-Myc*-specific mRNA levels, thus proving the function of the experimental approach. Concentrations of the *c-Myc* expression plasmid were used, which resulted in comparable amounts of *c-Myc* that were not further influenced by the knockdown of β-catenin (**Fig. 3**, c-*Myc*). Twenty-four hours after the knockdown, cells were harvested and investigated for the expression of β-*catenin, c-Myc* and *hTERT* on the level of mRNA using RT-qPCR, normalized to the reference gene *HPRT1*. In SW480 cells, a trend ($p < 0.08$) in the loss of *hTERT* expression could be shown, whereas in DLD-1 cells, there is a significant downregulation of *hTERT*. In spite of the reconstitution of *c-Myc*, the β-*catenin* knockdown resulted in a robust loss of the expression of *hTERT* (**Fig. 3**, *hTERT*).

Discussion

Taken together, we show evidence that β-catenin directly regulates the expression of the *hTERT* gene in human colorectal cancer in addition to its already known indirect effect via c-Myc.[23] This finding adds the cancer hallmark eternal life[5] to the many other well-known functions and hallmarks that are governed by β-catenin. Thus, β-catenin can be considered more as the master switch of colorectal carcinogenesis[3] as well as colorectal cancer stemness.[7]

Our results described here are supported by experimental work done in embryonic and adult stem cells as well as in colorectal cancer cell lines.[29] Here, we add proof that this regulation might also take place in human colorectal cancers. We showed that the expression pattern of hTERT at the invasive front of CRC[25] is reflected by the expression patterns of β-catenin but not of c-Myc, as the latter is expressed in all tumor cells.[24] Additionally, this tiny fraction of nuclear β-catenin-positive tumor cells generally shows a mesenchymal differentiation thus EMT[26] together with the expression of mesenchymal indicator genes like fibronectin or vimentin, which are also β-catenin target genes.[17,36] These points characterize these cells as colorectal cancer stem cells (coCSCs).[37,38] This is additionally supported by the fact that these cells also express hTERT as coCSC express also β-catenin target genes like CD44[39] or CD166.[28] Therefore, it is not surprising that the number of these cells correlates with low survival,[40] as does high hTERT activity.[41] Because for CSC, the parallel upregulation of stemness and eternal life is relevant,[5] the direct

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Table 1. Sequences of oligonucleotides

Name	Sequence	Length[1] [bp]	Amount/ concentrations	UPL[2] number
	Electric Mobility Shift Assay (EMSA)			
TBE3	ATT ATT TCA AAA CAA AGG TTT ACA GAA A			
TBE4	GAG TTA CCC TCC TTT GAT ATT TTC TGT A			
MUT3	ATT ATT TCA ACG CAG AGG TTT ACA GAA A			
MUT4	GAG TTA CCC TCC TGT GCG ATT TTC TGT A			
c-Myc	CTA GCG CAC CTT TGA TTT CTG CAC CTT TGA TTT CTG			
	Chromatin immunoprecipitation (ChIP)			
hTERT	ACT CGC GCT GCC CTT CTA GC ACG GTG TAT CCC CAG TCT ACG AAG	617	400 nM 400 nM	
c-Myc	ACA GAC GCC TCC CGC ACG GG CCA CAC CGA GAA CGC ACT GC	451	400 nM 400 nM	
GAPDH	TAC TAG CGG TTT TAC GGG CG TCG AAC AGG AGG AGC AGA GAG CGA	165	400 nM 400 nM	
	RNA interference (RNAi)			
β-catenin	CAG UUG UGG UUA AGC UCU UdT dT		60 nM	
GFP	AAG CUA CCU GUU CCA UGG CCA dTT		60 nM	
	RT-qPCR			
β-catenin	AGC TGA CCA GCT CTC TCT TCA CCA ATA TCA AGT CCA AGA TCA GC	73	900 nM 900 nM	21
c-Myc	CAC CAG CAG CGA CTC TGA GAT CCA GAC TCT GAC CTT TTG C	102	300 nM 300 nM	34
hTERT	CAC GCG AAA CCT TCC TC ACC ACT GTC TTC CGC AAG TT	80	300 nM 900 nM	46
HPRT1	TGA CCT TGA TTT ATT TTG CAT ACC CGA GCA AGA CGT TCA GTC CT	102	900 nM 900 nM	73

[1]Length of amplicons resulting from gene specific PCRs. [2]UPL (universal probe library) given are the numbers of the probes of the UPL (Roche). In the EMSA probes TBE sequences are underlined.

transcriptional regulation of hTERT via β-catenin leads to a coupling of stemness and eternal life.

An endogenous function of hTERT in Wnt-signaling is additionally discussed as being involved as a cofactor in a β-catenin transcriptional complex,[42-44] hence there might be a positive feedback loop between hTERT and the Wnt pathway regarding the maintenance of the stemness. But this finding is challenged by other studies, which did not confirm this function of hTERT.[45,46]

Our results show that *hTERT* is a direct target gene of β-catenin and indicate, thereby, that the Wnt signaling pathway controls self-renewal and telomerase activity at the same time,[47] thereby linking two essential components in the biology of colorectal cancers.

Materials and Methods

Immunohistochemistry. Immunohistochemical staining of serial sections of 24 well-differentiated colorectal adenocarcinomas with an invasive front displaying nuclear β-catenin was done as described.[30] Briefly, 5 μm sections were subjected to antigen retrieval using citrate buffer (DAKO), employing microwave treatment. Subsequently, the sections were incubated with antibodies specific for hTERT (Calbiochem; 1:100 - PC563T) or β-catenin (Sigma-Aldrich; 1:750 - C2206) overnight at 4°C, followed by washing. Next, binding was visualized using Envision AP (alkaline phosphatase) (DAKO) for hTERT or Envision HRP (horseradish peroxidase) (DAKO) for β-catenin staining. Finally, sections were counterstained using methylene blue according to the user's manual. Pictures were taken employing an AnalySis photo system. The usage of the tissue blocks for research purpose was allowed by the local ethical committee of the university.

Electric mobility shift assay (EMSA). Unlabeled competitor, or water in the case of controls, was incubated with 1 μl of crudely purified recombinant glutathione S-transferase (GST)-TCF-4 (amino acid 265–496), comprising its DNA binding domain or GST as a control in binding buffer (10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT and 4% (w/v) Ficoll, 12.5 ng/μl poly-*dIdC* (*deoxy-inosinic-deoxy-cytidylic*) acid, 62.5 μg/ml BSA) in a volume of 16 μl. After incubating for 5 min at room temperature, 0.5 ng of [^{32}P]-ATP end-labeled double-stranded oligonucleotides (**Table 1**) with a specific activity of

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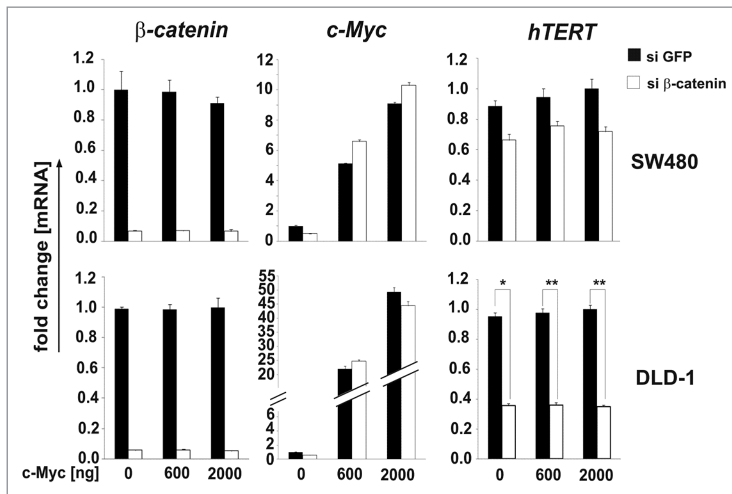

Figure 3. Knockdown of β-*catenin* leads to a loss of *hTERT* expression independently of c-Myc. Specific siRNA mediated knockdown of β-*catenin* leads to a substantial downregulation of *hTERT*- mRNA in DLD-1 and SW480 cells both in the absence or presence of *c-Myc* expression plasmid. Expression levels are normalized to those of the *HPRT1* gene. Data are represented as mean ± SD. The experiment was done in duplicates and repeated at least twice. Representative examples are shown. Student's t test (two-sided) was used for comparisons. For the analyses, p values < 0.05 considered significant. *p < 0.05, **p < 0.01.

3×10^8 dpm/μg (Hartmann Analytik) were added and the mixture incubated for a further 20 min, also at room temperature. Where mentioned, a 30-fold molar amount of unlabeled oligonucleotides (15 ng, **Table 1**) was used for competition. Reaction products were separated with the help of 5% (w/v) 0.25x TBE (1x TBE: 89 mM TRIS-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) polyacrylamide slab gels.

Cell lines, cell culture. Cultivated colorectal cell lines DLD-1, SW480, SW620 and Caco2 cells (American Type Culture Collection) were maintained in DMEM with 4,500 mg/l glucose and 10% (v/v) fetal bovine serum (Invitrogen) at 5% CO_2. The authenticity of the cell lines was tested by the DSMZ (German Collection of Microorganisms and Cell Lines) applying DNA typing.[48] Testing for all cell lines was less than half a year ago.

Chromatin immunoprecipitation (ChIP). ChIPs were done using ChIP-IT kits (Active Motif), following essentially the instruction manual. Briefly, chromatin of cultivated colorectal DLD-1 cells was fragmented to a size of about approximately 400 bp using ultra-sound sonification applying alternating 20 sec pulse–30 sec pause five times (G. Heinemann; HTU SONI130) on ice. Immunoprecipitation was done with the help of 2 μg of two different β-catenin specific antibodies (Sigma; β-CATI: C2,206; βCATII: clone 14; BD) or TFIIB (TATA-binding protein B of the transcription factor II)-specific antibodies (Active Motif) as well as mouse IgG (Santa Cruz Biotechnology) as isotype controls. Analysis of the reverse-crosslinked chromatin precipitates was done employing PCRs using 10% of the precipitates as template together with 400 nM of each primer spanning the region of the *hTERT* enhancer containing TBE3 and TBE4 (**Table 1** and **Fig. 2A**). One percent of the chromatin (input) or water (no template) were used as the positive or negative control, respectively.

Mutation of TBE and E-box sequences. The mutations in the four TBEs and two E-boxes in the hTERT-promoter were done using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to user's instructions. The mutated consensus sequences of the TBEs and E-boxes are listed in **Figure 2B**.

***Luciferase* gene reporter assay.** For the functional investigation of the relevance of β-catenin/TCF-4 for the transcriptional regulation of the *hTERT* gene, cultivated colorectal Caco2 and SW620 cells were transiently transfected 24 h after seeding in 24 cluster well plates. 120 ng pGL3-hTERT promoter (3328), pGL3-hTERT promoter (mut TBE1–4), pGL3-hTERT promoter (mut E-box1+2) or pGL3-basic (Promega; E1751) as negative control were transfected using Fugene 6 Transfection Reagent (Promega; E2691) in combination with 160 ng pEGFP-dnTCF-4 or pcl-neo-β-catenin-D45 encoding expression plasmid as well as 40 ng of *pCMV-Renilla* (Promega) for the normalization of results. After 24 h, Dual Luciferase Reporter Assay (Promega; E1960) was done according to user's instructions. Fluorescence intensities were measured with an Orion II luminometer (Berthold) in a 96-well format and analyzed with the SIMPLICITY software package (DLR). All experiments were done in triplicates and repeated at least twice.

RNA interference, RNA isolation and quantitative reverse transcriptase PCR (RT-qPCR). To specifically knockdown the β-*catenin* mRNA in cultivated DLD-1 and SW480 cells, the cells were transfected with 60 nM siRNA specific for either β-*catenin* or *GFP* (Qiagen) as the control (**Table 1**) using 3 μl Lipofectamine RNAiMAX (Invitrogen; 13778–150), following essentially the user's instructions (forward transfection in the manual). To prevent a decrease of *c-Myc* levels in these cells, they were transiently transfected 24 h before the application of siRNA with 600 ng or 2,000 ng *pUHD10–1-c-Myc* - or *pcDNA3-CAT*-expression plasmid as the control and to fill up to a constant DNA-amount in 6 cluster well plates with the help of Fugene 6 Transfection Reagent (Roche), following the instruction manual. Another 24 h after, siRNA transfection, cells were harvested and total RNA was isolated using RNeasy kits (Qiagen; 74104). The concentrations of RNA were measured by UV-photometry. Four hundred–1,000 ng of RNA isolates were reverse transcribed in the presence of 100 μM random hexamer primers together with 200 U RevertAid Reverse Transcriptase (both Fermentas; S0142, EP0441), following the user's recommendations. Two μl of the crude RT-reaction were used as the template in RT-qPCRs employing Light Cycler 480 Probes Master (Roche; 04902343001) together with specific primer-pairs (**Table 1**) and Universal ProbeLibrary Probes (Roche; **Table 1**), following the manufacturer's instructions. C_p (critical point) values of RT-qPCRs specific for β-*catenin*, *c-Myc*, *hTERT* and the reference gene *HPRT1* (*hypo-xanthin phosphoribosyl-transferase*) were determined employing a LightCycler 480 device (Roche). All concentrations of β-*catenin, c-Myc* and *hTERT*-specific RNAs were normalized to those of the *HPRT1*

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gene. All experiments were done in duplicates and repeated at least twice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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