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Telomere protection and TRF2 expression are enhanced by the canonical Wnt signalling pathway

Irmina Diala[1,2], Nicole Wagner[1], Frédérique Magdinier[2,3], Marina Shkreli[1], Maria Sirakov[4†], Serge Bauwens[2], Caroline Schluth-Bolard[2], Thomas Simonet[2], Valérie M. Renault[1], Jing Ye[1,2,5], Abdelnnadir Djerbi[1], Pascal Pineau[6], Jinkuk Choi[7], Steven Artandi[7], Anne Dejean[6], Michelina Plateroti[4‡] & Eric Gilson[1,2,8+]

[1]Faculté de Médecine de Nice, Institut for Research on Cancer and Aging, Nice (IRCAN), Nice University, Nice, France, [2]Laboratory of Molecular Biology of the Cell, Lyon, France, [3]Laboratory of Medical Genetics and Functional Genomics, Aix-Marseille University, La Timone Faculty of Medicine, Marseille, France, [4]Institut de Génomique Fonctionnelle de Lyon, Lyon University, Lyon, France, [5]Shanghai Jiatong University, Shanghai Ruijin Hospital, Shanghai, China, [6]Nuclear Organisation and Oncogenesis Unit/INSERM U993, Institut Pasteur, Paris, France, [7]Department of Medicine, Stanford University School of Medicine, Stanford, California, USA, and [8]Department of Medical Genetics, Archet 2 Hospital, CHU of Nice, Nice, France

The DNA-binding protein TRF2 is essential for telomere protection and chromosome stability in mammals. We show here that TRF2 expression is activated by the Wnt/β-catenin signalling pathway in human cancer and normal cells as well as in mouse intestinal tissues. Furthermore, β-catenin binds to TRF2 gene regulatory regions that are functional in a luciferase transactivating assay. Reduced β-catenin expression in cancer cells triggers a marked increase in telomere dysfunction, which can be reversed by TRF2 overexpression. We conclude that the Wnt/β-catenin signalling pathway maintains a level of TRF2 critical for telomere protection. This is expected to have an important role during development, adult stem cell function and oncogenesis.

[1]Faculté de Médecine de Nice, Institut for Research on Cancer and Aging, Nice (IRCAN), Nice University CNRS UMR7284/INSERM U1081, 28 Avenue de Valombrose, Nice F-06107, France
[2]Laboratory of Molecular Biology of the Cell, CNRS UMR5239, Ecole Normale Supérieure de Lyon, Lyon F-69364, France
[3]Laboratory of Medical Genetics and Functional Genomics, INSERM UMR_S 910, Aix-Marseille University, La Timone Faculty of Medicine, Marseille F-13385, France
[4]Institut de Génomique Fonctionnelle de Lyon, Lyon University, CNRS, INRA, Ecole Normale Supérieure de Lyon, Lyon F-75015, France
[5]Shanghai Jiatong University, Shanghai Ruijin Hospital, Shanghai 200025, China
[6]Nuclear Organisation and Oncogenesis Unit/INSERM U993, Institut Pasteur, Paris F-75015, France
[7]Department of Medicine, Stanford University School of Medicine, Stanford, California 94305, USA
[8]Department of Medical Genetics, Archet 2 Hospital, CHU of Nice, Nice F-06202, France
[†]Present address: Institut de Biologie et de Médecine Moléculaires, Université libre de Bruxelles, Bruxelles B-1050, Belgium.
[‡]Present address: Centre de Génétique et de Physiologie Moléculaire et Cellulaire, Université Lyon 1, Lyon F-69100, France.
[+]Corresponding author. Tel: +33 4 93 377 793; +33 4 93 377 786;
Fax: +33 0 4 93 37 70 92;
E-mail: eric.gilson@ens-lyon.fr

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INTRODUCTION

Telomeres are of paramount importance in determining cell fate, as they link replicative history to a great diversity of functions regulating tissue homeostasis. If the telomere length maintenance depends on telomerase, which can compensate for replicative erosion [1,2], telomere capping relies on the organization of chromatin in a way that protects the chromosome ends from aberrant signallization and repair [3,4].

The shelterin complex is a key protein component of telomeric chromatin in mammals and consists of six polypeptides: TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 [5]. This complex appears to act as a protein hub that protects telomeres from being recognized as a double-strand break, thereby avoiding inappropriate DNA damage response activation and aberrant recombinational repair. Among the shelterin components, TRF2 has a crucial role in regulating the molecular events that maintain telomere integrity [3]. Suppression of TRF2 activates an ATM-dependent DNA damage response pathway that induces apoptosis or senescence, depending on the cell type [6].

Expression of telomerase is tightly regulated during normal development and aging, and telomerase is upregulated in the vast majority of human cancers [7]. However, very little is known on the regulation of the shelterin components expression. The current view is that shelterin is ubiquitously expressed to protect telomeres in all tissues in which the telomeres are not too short to preclude DNA binding [5,8]. Recent studies challenge this prevailing paradigm by showing that the expression of shelterin subunits, notably TRF2, is increased during malignant transformation, suggesting that shelterin expression might be the target of oncogenic signalling pathways [9–14].

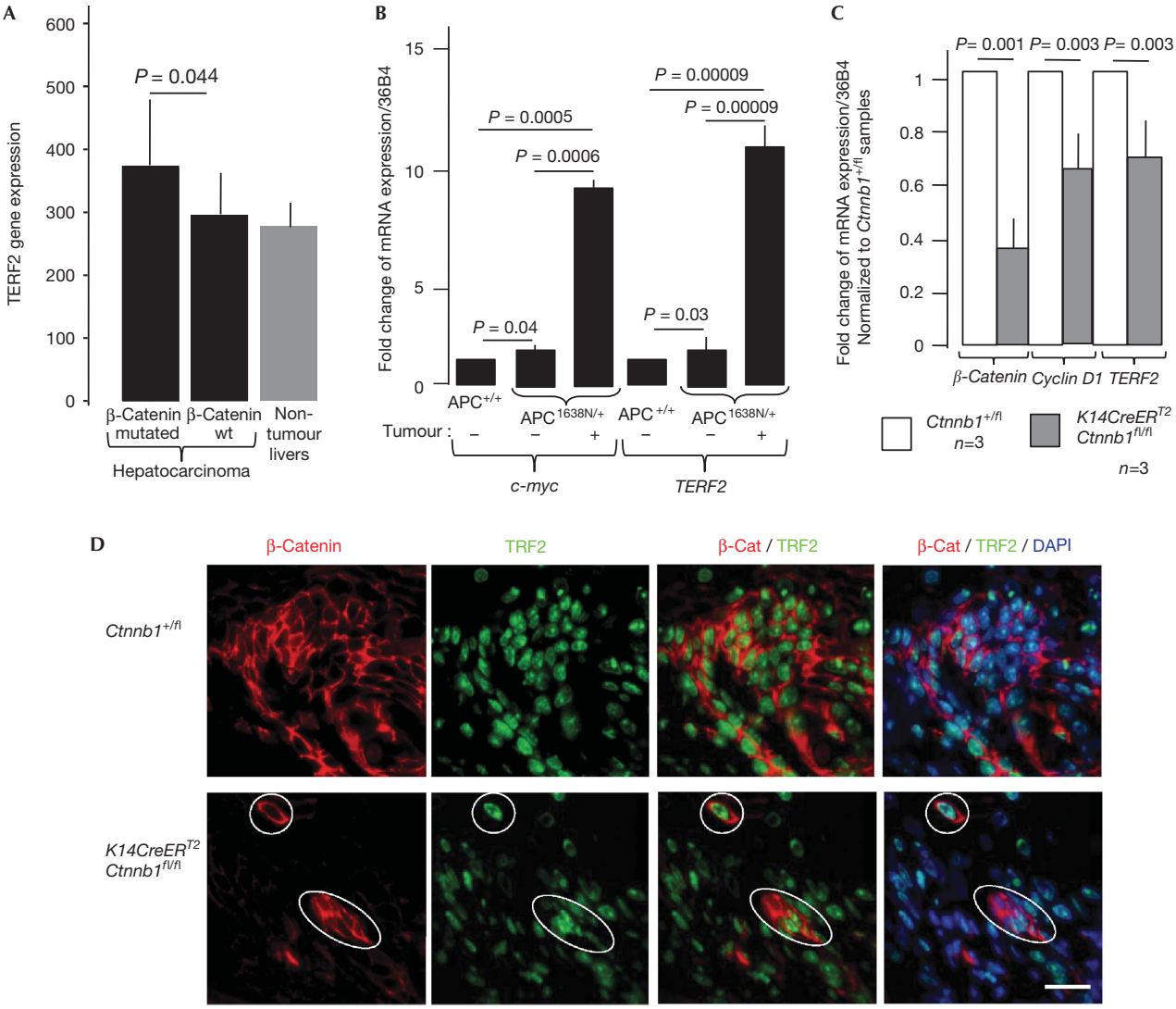

Fig 1 | Upregulation of *TERF2* mRNA when activation of the canonical Wnt/β-catenin signalling pathway is more pronounced. (**A**) *TERF2* mRNA expression in 56 hepatocellular carcinomas with known β-catenin mutations. *P*-value determined according to a Mann–Whitney *U*-test. Error bar represents mean ± s.e.m. (**B**) Relative *TERF2* mRNA levels in normal intestine and adenocarcinoma samples from $APC^{+/+}$ and $APC^{1638N/+}$ mice ($n = 6$ for each type of mouse). The values are normalized to tissues of $APC^{+/+}$ mice. The *P*-value was determined using Bonferroni multiple comparison test. Error bar represents mean ± s.e.m. for six independent experiments. (**C**) Reverse transcription–quantitative PCR analysis of the expression of *Ctnnb1*, *c-Myc* and *TERF2* genes in skin samples from the indicated newborn mice treated topically with Tamoxifen between days 0.5 and 1.5 and euthanized at day 3. The *P*-value was determined using Wilcoxon matched-pairs signed rank test. Error bar represents mean ± s.e.m. (**D**) Representative images of immunofluorescence analysis using antibodies directed against TRF2 or β-catenin of skin samples of the indicated mice treated with Tamoxifen. The white circles show examples of cells that still express a high level of β-catenin and of TRF2. The bar represents 15 µm. DAPI, 4′-6-diamidino-2-phenylindole.

RESULTS AND DISCUSSION

To determine whether *TERF2* mRNA expression correlates with particular features of hepatocellular carcinomas (HCCs), we analysed an expression profile done using an Affimetrix microarray on 56 human HCCs with a known mutational status of the *Ctnnb1* gene encoding β-catenin (Fig 1A). An increase of *TERF2* gene expression, but not of the other shelterin genes, was noticed in HCC with *Ctnnb1* mutation when compared to HCC with intact *Ctnnb1* gene ($P < 0.05$) or to a set of five non-tumour liver samples (Fig 1A).

We next investigated whether *TERF2* gene expression is upregulated in $APC^{1638N/+}$ mice, a model of familial adenomatous polyposis, which is caused by a germline mutation in the *APC* gene, whose product targets β-catenin for degradation. We found a modest but significant increase in *c-Myc* mRNA level, a direct target of β-catenin, in the normal intestine mucosa of $APC^{1638N/+}$ mice, and a marked overexpression of *c-Myc* in $APC^{1638N/+}$ tumours (Fig 1B). Notably, *TERF2* mRNA mirrored *c-Myc* expression pattern. These results are in agreement with

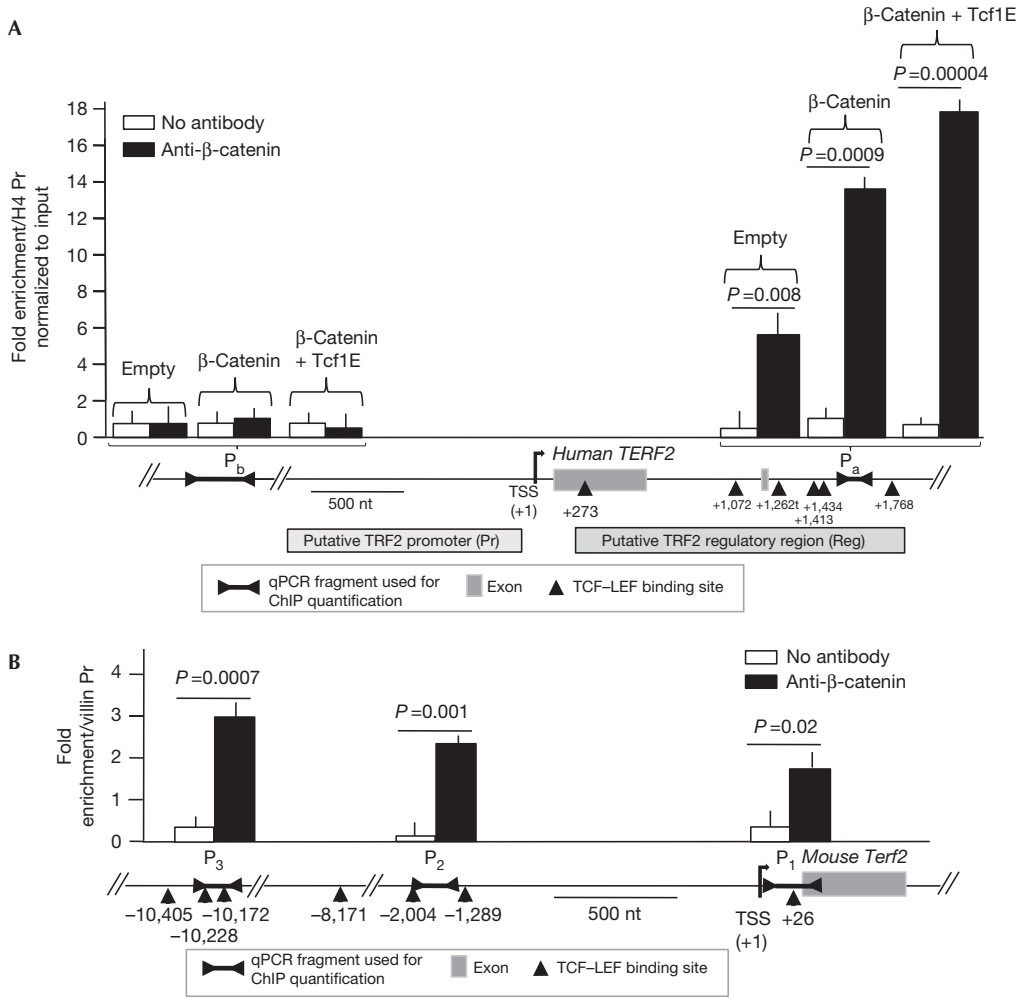

Fig 2 | β-Catenin binds to *TERF2* regulatory regions containing TCF–LEF sites (marked as triangles). The coordinates of the centres of the DNA sequences of these sites are given relative to the transcription start site (+1). Each quantitative PCR (qPCR) value represents the average of at least three independent chromatin immunoprecipitation (ChIP) experiments. *P*-value was determined using Mann–Whitney test. (**A**) Genomic map of the human *TERF2* gene and quantification by qPCR of the enrichment of the P_a and P_b DNA fragments relative to a sequence of the histone H4 promoter (H4 Pr). Empty, β-catenin and Tcf1E indicate that the HCT116 cells were transfected with an empty plasmid or with a plasmid expressing a mutated form of β-catenin or with a plasmid expressing Tcf1E. (**B**) Genomic map of the mouse *TERF2* gene and quantification of the enrichment of the P_1, P_2 and P_3 DNA fragments relative to a sequence of the Villin promoter in immunoprecipitates from ChIP experiments of normal intestinal tissues from $APC^{1638N/+}$ mice using a β-catenin antibody. For both panels, error bar represents the mean ± s.e.m. for four independent experiments.

the overexpression of TRF2 observed in colorectal carcinoma [15] (J.Y. Scoazec and E.G., unpublished observations).

Next, we assessed whether the Wnt/β-catenin signalling pathway regulates the expression of *TERF2 in vivo* by the means of a conditional knockout mouse model that allows acute deletion of β-catenin encoding gene, *Ctnnb1*. The skin of $K14CreER^{T2}$;$Ctnnb1^{fl/fl}$ newborn mice were topically treated with Tamoxifen at postnatal days 0.5 and 1.5 and killed at day 3. By this time, as expected, the expression of *Ctnnb1* and *Cyclin D1*, a target of the Wnt/β-catenin signalling pathway, were decreased in skin samples from $K14CreER^{T2}$;$Ctnnb1^{fl/fl}$ mice when compared with similarly treated samples from $Ctnnb1^{+/fl}$ control mice (Fig 1C,D). Concomitantly, *TERF2* expression decreased, both at the mRNA and protein levels (Fig 1C,D). It is worth noting that the level of TRF2 remains high in the few keratinocytes from $K14CreER^{T2}$;$Ctnnb1^{fl/fl}$ Tamoxifen-treated mice that were still expressing β-catenin after Tamoxifen treatment (encircled in Fig 1D), ruling out a problem of TRF2 immunostaining. Together, those results show that β-catenin regulates TRF2 expression in both human and mouse cells.

Analysis of the human *TERF2* gene using the Ensembl genome database (http://www.ensembl.org) revealed a potential regulatory region located downstream of the transcription start site (named Reg in Fig 2A). Activation of the canonical Wnt signalling pathway triggers the stabilization and translocation of β-catenin to the nucleus that subsequently binds to members of the TCF–LEF family of transcription factors to activate gene transcription [16]. Notably, the *TERF2* Reg sequence contains six putative TCF–LEF

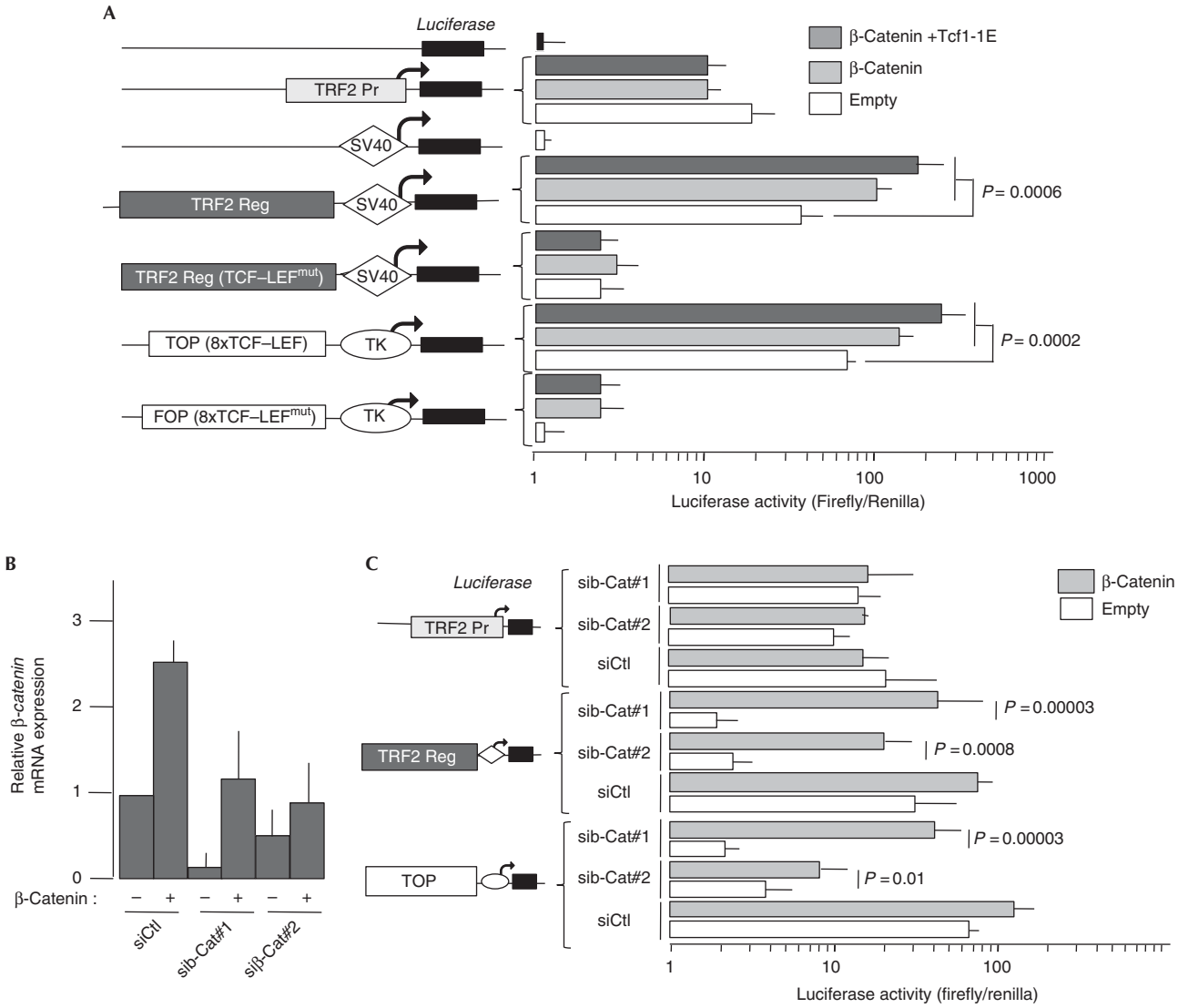

Fig 3 | The TCF–LEF sites of the human *TERF2* gene are capable of transactivating a luciferase reporter gene in a β-catenin-dependent manner. (**A**) The various reporter constructs derived from the pLG-3/Prom plasmid (for the *TERF2* Reg sequence) and pLG-3/basic (for the *TERF2* Prom sequence). TCF–LEF^{mut}: all the TCF–LEF sites are inactivated by mutation. TOP-Flash and FOP-Flash are positive and negative controls, respectively, for β-catenin/TCF transactivation. Data show average relative luciferase activity in HCT116 cells and represent the mean ± s.d. ($n = 6$). Co-transfected plasmids overexpressing activated β-catenin and Tcf1E are identified by black/white scales. (**B**) Efficiency of inhibition of *Ctnnb1* mRNA expression on transfection of two different short interfering RNAs (siRNAs) directed against *Ctnn1b* mRNA (#1 and #2). SiCtl is a scramble control siRNA. (**C**) HCT116 cells transfected with various siRNAs as in **A**. Statistical significance of data was assessed using Bonferroni multiple comparison test. For all panels, error bar represents the mean ± s.e.m. for six independent experiments.

transcription factor binding sites between position +273 and +1768 (Fig 2A). Three clusters of TCF–LEF binding sites were also found in the promoter region of the mouse *Terf2* gene (Fig 2B). Chromatin immunoprecipitation (ChIP) experiments with antibodies raised against β-catenin in colorectal cancer cells with a mutant *Ctnnb1* gene (HCT116) revealed an enrichment of a DNA fragment localized inside the TCF–LEF site cluster (P_a) relative to a fragment of the histone H4 promoter (H4 Pr), which does not contain TCF–LEF sites (Fig 2A; Supplementary Table S1 online). Importantly, this enrichment was further increased on β-catenin and Tcf1E overexpression and not observed with a DNA fragment located roughly 18-kb upstream of the Reg sequence (P_b in Fig 2A). Similar ChIP experiments performed on normal intestinal tissue from APC$^{1638N/+}$ mice revealed an enrichment of DNA sequences corresponding to the three clusters of TCF–LEF binding sites of the mouse *Terf2* gene relative to a DNA sequence of the Villin promoter, which does not contain TCF–LEF sites (P_1, P_2 and P_3 in Fig. 2B).

The Reg sequence boosts the transactivating activity of the weak SV40 promoter in a luciferase assay. Importantly, mutations of the TCF–LEF binding sites abrogated the ability of the Reg region to drive transcription of the reporter gene suggesting that those

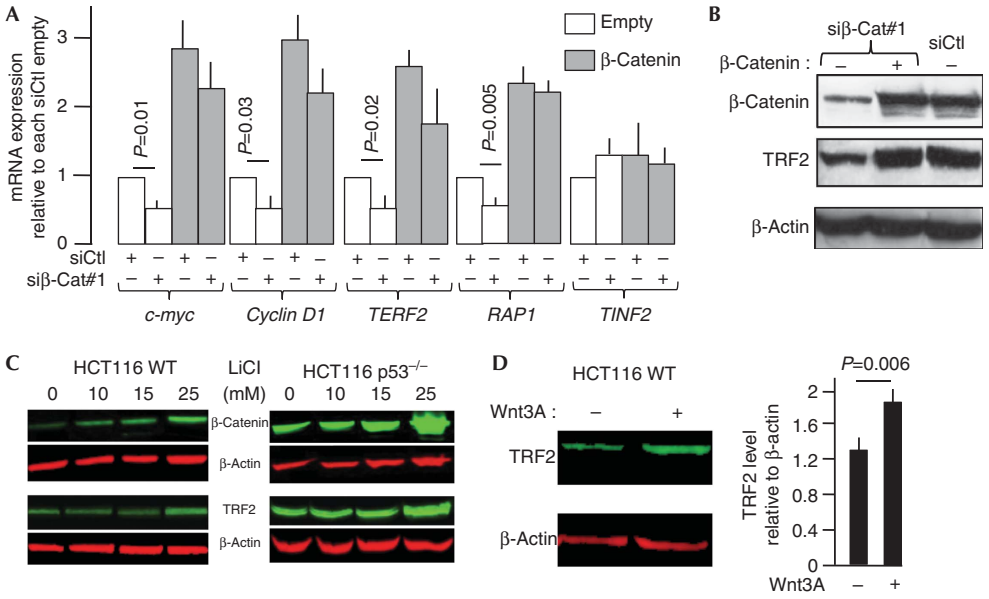

Fig 4 | β-Catenin regulates the expression of TRF2. (**A**) mRNA expression of *β-catenin*, two known targets of the canonical Wnt signalling (*cyclin D1* and *c-Myc*), *TERF2*, *RAP1* and *TINF2*. The *P*-value was determined using Wilcoxon matched-pairs signed rank test. (**B**) Western blotting of lysates from HCT116 cells under the indicated conditions. (**C**) Image and quantitative analysis of β-catenin, TRF2 and β-actin expression in HCT116 and HCT116 $p53^{-/-}$ cells treated with increasing amounts of LiCl. (**D**) Image and quantitative analysis of TRF2 expression in HCT116 cells treated with the Wnt3A peptide. For all panels, error bar represents the mean ± s.e.m. for three independent experiments.

TCF–LEF binding sites mediated the responsiveness of *TERF2* expression to Wnt/β-catenin signalling (Fig 3A). Moreover, overexpression of β-catenin alone and overexpression of both β-catenin and Tcf1E together induced a specific activation of the native Reg region (Fig 3A). This is in contrast to the basal activity conferred by the *TERF2* Pr promoter fragment (Fig 3A). Finally, the ability of Reg region to induce firefly expression was inhibited by two independent short interfering RNA (siRNA) sequences, which efficiently target *Ctnnb1* mRNA (Fig 3B,C). Importantly, the effect of these siRNA on the ability of the Reg region to drive transcription was rescued by β-catenin overexpression (Fig 3C). The use of both a Wnt-positive reporter containing eight known TCF–LEF sites (TOP) and a Wnt-negative reporter (FOP) validated these assays (Fig 3A,C). The ability of the Reg element to respond to β-catenin was further confirmed by using LiCl treatment, a component that is known to stabilize β-catenin [17] (Supplementary Fig S1 online).

We next investigated whether the Wnt/β-catenin signalling pathway controls expression of the endogenous *TERF2* gene. In HCT116 cells, which show constitutive β-catenin-mediated transcriptional activity, the knockdown of *Ctnnb1* led to a nearly twofold decrease in the *TERF2* mRNA level (Fig 4A). A similar decrease of expression was observed for the known β-catenin targets *c-Myc* and *cyclin D1* (Fig 4A). Conversely, β-catenin overexpression increased *c-Myc*, *cyclin D1* and *TERF2* mRNA levels and reversed the downregulation caused by siRNA transfection (Fig 4A). The β-catenin level also determined the expression of the *RAP1* gene encoding a TRF2-interacting protein, but did not influence expression of the *TINF2* gene, which encodes another shelterin component. The ability of β-catenin to regulate *TERF2* and *RAP1* expressions appears to be a general phenomenon, as very similar results were obtained using HepG2 cells harbouring a *Ctnnb1* mutation (Supplementary Fig S2 online). The fact that no RAP1 overexpression was found associated with mutated *Ctnnb1* in the HCCs (Fig 1A) might be due to a threshold effect of microarray data or to cell type-specific differences in RAP1 regulation.

The ability of β-catenin to control endogenous TRF2 expression was confirmed at the protein level, HCT116 cells transfected with an *Ctnnb1* siRNA showing a marked decrease of TRF2 protein by western blot (Fig 4B). This effect was rescued by overexpression of β-catenin (Fig 4B). The impact of β-catenin on TRF2 expression in HCT116 cells was further supported by the progressive accumulation of both β-catenin and TRF2 in HCT116 cells treated with increasing amounts of LiCl (Fig 4C). It was recently shown a p53-dependent degradation of TRF2 by Siah1 [18]. Indeed, we detected larger amounts of TRF2 in HCT116 $p53^{-/-}$ cells compared with HCT116 cells, with the amount of TRF2 being further upregulated by LiCl (Fig 4C).

In agreement with the view that TRF2 is a target of the Wnt canonical pathway, addition of purified Wnt3A to the culture medium stimulated the expression of TRF2 in HCT116 cells (Fig 4D), in a variety of hepatoma cell lines, both mutated and non-mutated for *Ctnnb1*, and in IMR90 cells, a normal fibroblast line (Supplementary Fig S3 online).

The increase in the level of TRF2 due to β-catenin/Tcf1E overexpression led to an increase in its binding to telomeres, as revealed by slot blotting with a telomeric DNA probe (Fig 5A,B) and several interstitial telomeric sequences [19], as revealed by quantitative PCR (qPCR; Fig 5C). These results indicate that β-catenin regulates telomere protection by modulating the TRF2 level. We therefore tested whether changes in the expression of

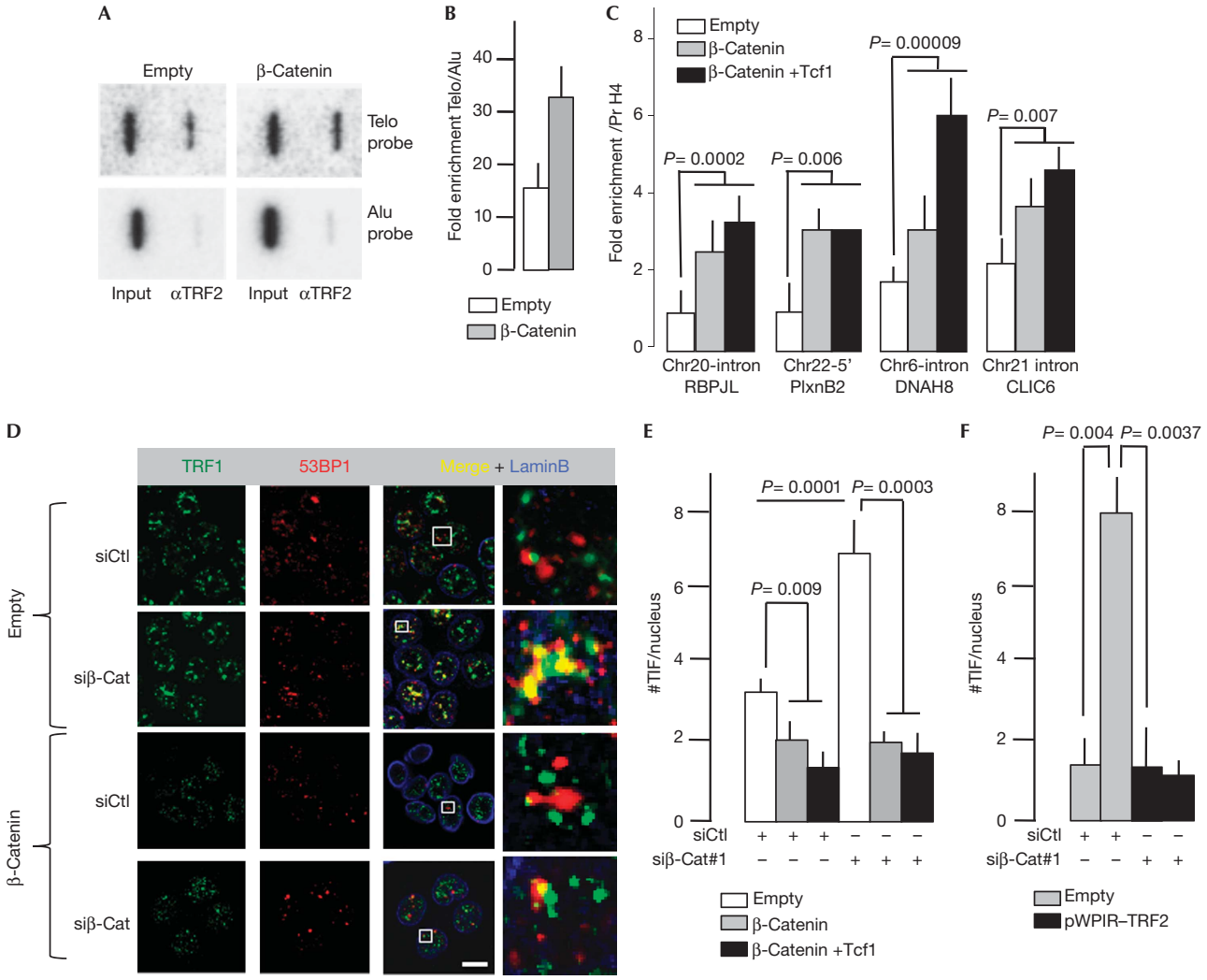

Fig 5 | Telomere changes caused by modulation in β-catenin levels. Images (**A**) and quantification (**B**) of chromatin immunoprecipitation (ChIP) analysis of HCT116 cells overexpressing β-catenin and Tcf1E ($n = 3$), performed using TRF2 antibodies and revealed by slot blotting with a telomeric DNA probe. Error bar represents the mean ± s.e.m. (**C**) The ChIP-immunoprecipitated DNA from **A** and **B** was subjected to PCR with primers specific for different interstitial telomeric sequences (ITSs). The name of the ITS-proximal gene is shown. *P*-value was determined using Mann–Whitney test. Error bar represents the mean ± s.e.m. for six independent experiments. (**D**) Representative images from immunofluorescence experiments used to analyse TIF. (**E**) Number of TIF in HCT116 cells on *Ctnnb1* knockdown. At least 40 nuclei per condition were analysed. *P*-value was determined using Student's *t*-test. (**F**) Number of TIF in HCT116 cells combining *Ctnnb1* knockdown and TRF2 overexpression. At least 40 nuclei per condition were analysed. *P*-value was determined using Student's *t*-test. For panels E and F, error bar represents the mean ± s.e.m. for three independent experiments. H4 Pr, histone H4 promoter.

β-catenin could affect telomere protection by scoring the number of telomere dysfunction-induced foci (TIF), that is, the number of telomere foci (detected by TRF1 antibodies) colocalizing with 53BP1 immuno-signals (Fig 5D). While the overexpression of β-catenin or β-catenin and Tcf1E together reduced both the mean number of TIF per nucleus (Fig 5D,E), *Ctnnb1* knockdown caused a marked increase in the TIF number in the control condition (empty). Ruling out an off-target effect, overexpression of β-catenin or β-catenin plus Tcf1E reverse the effect of *Ctnnb1* knockdown (Fig 5D,E). The increase of TIF triggered by the reduction of *Ctnnb1* expression is accompanied by a decrease in cell viability and an increased percentage of cell-expressing β-galactosidase, a marker of cellular senescence (Supplementary Fig S4 online). These results indicate that β-catenin is required for protection against telomere dysfunction and senescence in HCT116 colon cancer cells.

On the basis of our results showing that TRF2 expression is regulated by β-catenin (Figs 2–5), we tested whether the mechanism by which β-catenin protects telomeres involves TRF2. The overexpression of TRF2 in β-catenin-compromised HCT116 cells triggers the appearance of significantly less TIFs than in cells transduced with the empty vector (Fig 5F). This shows

that an overdosage of TRF2 rescues the telomere dysfunction triggered by *Ctnnb1* knockdown. We conclude that β-catenin maintains a level of TRF2 critical for telomere protection in HCT116 cells.

Overall, our findings show that in normal and cancer cells, both from mouse and human, the activation of Wnt/β-catenin signalling leads to an increased TRF2 level as well as an enhanced telomere protection. Previously, TERT was shown to enhance the transcriptional output of the Wnt pathway [20,21]. Therefore, it would be interesting to determine whether TERT cooperates with β-catenin to induce TRF2 overexpression during tumour progression. Along this line, three groups recently showed that the expression of the protein component of telomerase TERT is also regulated by the Wnt/β-catenin signalling pathway [22–24]. Altogether, these findings indicate that an activation of the Wnt signalling pathway can reinforce telomere stability by coupling and enhancing the two main telomere maintenance pathways that are the telomerase activity and the shelterin capping complex. This Wnt-mediated telomere protective effect is expected to have an important role during development, adult stem cell function and oncogenesis.

In conclusion, TRF2 should not merely be viewed as a 'housekeeping' protein required for telomere protection, but as a regulated factor that couples the functional state of telomeres to the long-range organization of chromosomes and gene regulation networks.

METHODS

Patients, tumour samples and RNA extraction procedure. The tumour specimens were collected from four different surgical centres (Paris, Milan, Rennes and Shanghaï). Adjacent non-neoplastic liver tissues were obtained at the time of surgery. After resection, tumour specimens were immediately frozen in liquid nitrogen and continuously stored at $-80\,^{\circ}C$ until processed for array profiling. Tissue specimens were obtained retrospectively under strict anonymity from historical collections established in surgery or pathology units of the participating hospitals. This study, respectful of the declaration of Helsinki, was approved by Institutional Human Research review boards. Only samples with a ribosomal RNA ratio 28S/18S >1.8 were included in the analysis.

Gene expression arrays. Microarray analysis was carried out using Affymetrix U133A GeneChips according to the manufacturer's instructions. Biotin-labelled cRNA, produced by *in vitro* transcription, was fragmented and hybridized to the Affymetrix U133A GeneChip arrays (www.affymetrix.com_products_arrays_specific_Hu133A.affx) at 45 °C for 16 h and then washed and stained using the GeneChip Fluidics. The arrays were scanned by a GeneArray Scanner and patterns of hybridization detected as light emitted from the fluorescent reporter groups incorporated into the target and hybridized to oligonucleotide probes. All analyses were performed in a MIAME (minimal information about a microarray experiment)-compliant fashion, as defined in the guidelines established by MGED (www.mged.org).

Cell treatment and protein analysis. For knockdown studies, the cells were transfected with two validated human β-catenin (*Ctnnb1*) siRNAs (named siβ-Cat#1 and siβ-Cat#2) or negative control siRNA (named siCtl; Ambion). We used as expression vectors pCI-NEO-β-CATENINXL for the S33Y mutant β-catenin construct and Evr2-Tcf1E for Tcf1E. Telomere dysfunction-induced foci assay by immunofluorescence was performed as previously published [25]. Primary antibodies against TRF2 (Imgenex), β-catenin (BD Transduction Laboratories) and β-actin (Abcam) were used. For ECL detection, horseradish peroxidase-conjugated secondary antibodies were used; for fluorescence western blotting, we used DyLight-conjugated secondary antibodies (Thermo Scientific) and an Odyssey Infrared Imaging system (LI-COR).

Generation of conditional knockout mice, skin sample collection and analysis. *Ctnnb1*$^{fl/+}$ strain was described previously [26]. For acute deletion of β-catenin, *K14CreER*^{T2} mice were intercrossed with *Ctnnb1*$^{fl/fl}$ alleles. Entire litters of newborn pups derived from *Ctnnb1*$^{fl/+}$ or *Ctnnb1*$^{fl/fl}$ females crossed with *K14CreER*^{T2}*; Ctnnb1*$^{fl/+}$ or *K14CreER*^{T2}*;Ctnnb1*$^{fl/fl}$ males were topically treated with 40 µl of saturated (200 mg/ml) Tamoxifen solution (Sigma) dissolved in 9:1 dimethylsulphoxide/ethanol at postnatal days 0.5 and 1.5. All mice were treated in accordance with AAALAC approved guidelines at Stanford University. Dorsal skin samples were obtained from mice post mortem.

Immunohistochemistry. Two-µm paraffin sections were used for immunohistological investigations. An indirect immunofluorescence double-labelling technique was used to mark TRF2 (antibodies orf2 [8]) and β-catenin (antibodies cat.# 610154, BD Transduction Laboratories, Le-Pont-de-Claix, France) expressing cells. The reaction products were visualized by incubation (1.5 h at room temperature) with Cy3- and Cy2-conjugated secondary antibodies. Counterstaining of the nuclei was done with 4′-6-diamidino-2-phenylindole.

Luciferase transcription reporter assay. The *TERF2* gene fragments cloned in the luciferase plasmids have the following coordinate (human genome hg19 release): Pr = 69,420,372–69,419,875 and Reg = 69,419,615–69,417,951.

To normalize transfection efficiency in reporter assays, the HCT116 cells were co-transfected with 0.1–0.2 µg of a plasmid carrying the internal control reporter *Renilla reniformis* luciferase driven by a TK promoter (pRL-TK; Promega). Then 72 h after transfection, a luciferase assay was performed using a Dual Luciferase Assay System kit (Promega) in accordance with the manufacturer's protocols. Statistical significance was determined using Student's *t*-test. $P<0.05$ was considered as significant.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was performed as previously published [19]. We used the following antibodies: TRF2m (Imgenex 124A; mouse monoclonal) and β-catenin (BD Transduction Laboratories; mouse monoclonal). After DNA precipitation, purified samples for β-catenin ChIP were used for qPCR. TRF2 ChIP was analysed by slot blotting with telomere versus Alu probes for telomeric localization and by qPCR for interstitial telomeric sequence enrichment [19]. The primers used for qPCR are given in Supplementary Table S2 online.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: I.D. designed and performed most of the experiments; N.W. designed, performed and analysed the mouse skin microscopy; M.S. and S.A. conceived the mouse skin experiments; J.C. performed the mouse skin experiments; M.S. performed the APC mouse experiments; M.P. conceived and analysed APC mouse experiments; S.B., J.Y. and A.Dj contributed to TIF analysis; C.S.-B., T.S., J.Y. and F.M. contributed to ChIP experiments; V.M.R. designed molecular and cellular experiments; P.P. and A.D. conceived, performed and analysed the microarray experiments; and E.G. conceived, coordinated and analysed most of the experiments, as well as wrote the manuscript with the input from the co-authors. M.Si and M.P. conducted the experiments with AMP mice. M.P. contributed to the design of the experiments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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