


The CCCTC-binding factor (CTCF)–forkhead box protein M1 axis regulates tumour growth and metastasis in hepatocellular carcinoma

Bin Zhang^{1†}, Yajing Zhang^{2,3,4†}, Xiaoping Zou¹, Anthony WH Chan⁵, Rui Zhang^{3,4}, Terence Kin-Wah Lee^{3,4}, Hang Liu³, Eunice Yuen-Ting Lau³, Nicole Pui-Yu Ho³, Paul BS Lai⁶, Yue-Sun Cheung⁶, Ka-Fai To⁵, Hoi Kin Wong⁷, Kwong Wai Choy⁷, Vincent W Keng³, Larry MC Chow^{3,4}, Kenrick KY Chan³, Alfred S Cheng⁸ and Ben CB Ko^{2,3,4*} 

¹ Department of Gastroenterology, Nanjing Drum Tower Hospital, Nanjing University, Nanjing, PR China

² The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, PR China

³ Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, SAR, PR China

⁴ State Key Laboratory of Chirosciences, The Hong Kong Polytechnic University, Hong Kong, SAR, PR China

⁵ Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, SAR, PR China

⁶ Department of Surgery, The Chinese University of Hong Kong, Hong Kong, SAR, PR China

⁷ Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong, SAR, PR China

⁸ School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, SAR, PR China

*Correspondence to: Ben CB Ko, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, PR China. E-mail: ben.ko@polyu.edu.hk

†Equal contributions.

Abstract

CCCTC-binding factor (CTCF) is a DNA-binding protein that interacts with a large number of highly divergent target sequences throughout the genome. It is implicated in a variety of functions, including chromatin organization and transcriptional control. The functional role of CTCF in tumour pathogenesis remains elusive. We showed that CTCF is frequently upregulated in a subset of primary hepatocellular carcinomas (HCCs) as compared with non-tumoural liver. Overexpression of CTCF was associated with shorter disease-free survival of patients. Short hairpin RNA (shRNA)-mediated suppression of CTCF inhibited cell proliferation, motility and invasiveness in HCC cell lines; these effects were correlated with prominent reductions in the expression of telomerase reverse transcriptase (TERT), the shelterin complex member telomerase repeat-binding factor 1, and forkhead box protein M1 (FOXM1). In contrast, upregulation of CTCF was positively correlated with FOXM1 and TERT expression in clinical HCC biopsies. Depletion of CTCF resulted in reduced motility and invasiveness in HCC cells that could be reversed by ectopic expression of FOXM1, suggesting that FOXM1 is one of the important downstream effectors of CTCF in HCC. Reporter gene analysis suggested that depletion of CTCF is associated with reduced FOXM1 and TERT promoter activity. Chromatin immunoprecipitation (ChIP)–polymerase chain reaction (PCR) analysis further revealed occupancy of the FOXM1 promoter by CTCF *in vivo*. Importantly, depletion of CTCF by shRNA significantly inhibited tumour progression and metastasis in HCC mouse models. Our work uncovered a novel functional role of CTCF in HCC pathogenesis, which suggests that targeting CTCF could be further explored as a potential therapeutic strategy for HCC.

© 2017 The Authors. *The Journal of Pathology* published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland.

Keywords: CTCF; HCC; FOXM1; TERT; chromatin immunoprecipitation; metastasis

Received 6 March 2017; Revised 16 August 2017; Accepted 24 August 2017

No conflicts of interest were declared.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer death worldwide [1]. The prognosis for HCC is poor, because the tumour is usually diagnosed at an advanced inoperable stage when it can no longer be eradicated by surgical resection. Non-surgical treatments such as transarterial chemoembolization, systemic therapy and targeted therapy [2] for patients

with advanced-stage HCC can only modestly extend their lifespan. Therefore, a thorough understanding of the underlying mechanisms regarding tumour growth and metastasis is critical for the development of effective therapeutic targets against HCC.

CCCTC-binding factor (CTCF) is a ubiquitously expressed 11 zinc-finger protein involved in a plethora of functions, including genome isolation, enhancer blocking, transcriptional regulation, imprinting, and long-range transcriptional control [3]. There are ~14

000–30 000 CTCF-binding sites diversely located in the intergenic, promoter, exon and intron regions in the genome [4]. Through the combinatorial use of different zinc fingers, CTCF can bind to a wide range of variant sequences and differentially interact with coregulatory proteins to perform a versatile role in genome regulation [5]. Among other functions, CTCF is best known as a chromatin organizer [3], maintaining DNA topology, and as an insulator to block enhancer–promoter interactions [6]. The role of CTCF in tumourigenesis remains elusive [7–10]. Analysis of CTCF-binding sites in a panel of reference cell lines designated by the Encyclopedia of DNA Elements (ENCODE) consortium has identified ubiquitous and cell type-specific CTCF-binding sites [11], and has revealed different CTCF-binding landscapes in normal and tumour cells [12]. These findings suggest divergent and complex functional roles of CTCF in different cell types and cell states. At present, the functional significance of differential interactions between CTCF and its binding sites in different cell types remains largely unexplored. More importantly, the functional role of CTCF in HCC pathogenesis remains unknown.

In this study, we found that CTCF expression is upregulated in a subset of HCCs, and that CTCF overexpression is associated with a poorer prognosis of HCC patients. We characterized the functional role of CTCF in HCC by using cell and mouse models, and showed that it contributes to HCC tumourigenesis and metastasis.

Materials and methods

Plasmids, reagents, and antibodies

Lentivirus plasmids, i.e. pLKO.1-puro vectors containing non-targeting short hairpin RNA (shRNA) and shRNAs targeting *CTCF* (TRCN0000014549 and TRCN0000014551), were from Thermo Fisher Scientific (Waltham, MA, USA). CTCF SMARTpool small interfering RNA (siRNA) (L-20165-00-0020) was from Dharmacon (Lafayette, CO, USA). Anti-CTCF antibody for chromatin immunoprecipitation (ChIP) was from Millipore (Billerica, MA, USA); anti-TERT (1:1000, clone no. H-231), anti-telomerase repeat-binding factor 1 (TRF1) (1:1000, clone no. H-242), anti-Ki67 (1:500, clone no. MIB-1) and anti-p21 (1:1000, clone no. N-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-CTCF (1:2000, clone no. D31H2), anti-poly(1:1000, ADP-ribose) polymerase (PARP), anti-cleaved PARP (1:1000, clone no. D64E10), anti-phospho-ATM (Ser1981) (clone no. D25E5), anti-phospho-checkpoint kinase 2 (CHK2) (Thr68) (1:1000, clone no. C13C1) and anti-phospho-H2A.X (Ser139) antibodies were from Cell Signaling Technology (Beverly, MA, USA); anti-p27 (1:1000, clone no. SX53G8) antibody was from Dako (Glostrup, Denmark); anti-TRF1 (1:200, clone no. TRF-78) and anti-telomerase repeat-binding

factor 2 (TRF2) (1:200, clone no. 4A794) antibodies for immunofluorescence were from Abcam (Cambridge, MA, USA); and anti- β -actin antibody (1:5000, clone no. AC-15) was from Sigma-Aldrich (St Louis, MO, USA). The pcDNA3-HA-FOXM1C plasmid was a kind gift from K. M. Yau of The University of Hong Kong.

Cell culture

SK-HEP-1, HepG2, PLC5 and Hep3B cells from the American Type Culture Collection. Huh7 cells were from the Health Science Research Resources Bank (Osaka, Japan). BEL-7402 cells were from the Shanghai Institutes for Biological Sciences, Chinese Academy of Science. MHCC97L and HepG2.2.15 cells were from Z. Y. Tang of Fudan University and A. L. Huang of Chongqing Medical University, PR China, respectively. Cells were cultured in growth medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), and maintained in a humidified incubator at 37 °C with 5% CO₂. All cells were authenticated by short tandem repeat profiling analysis.

Human HCC specimens

Tumorous liver tissues and the corresponding adjacent non-tumorous liver tissues were collected from 47 patients who had undergone curative surgery for HCC at the Prince of Wales Hospital, Hong Kong. Frozen normal liver tissues were obtained from patients who had undergone liver resection for hepatic metastasis of colorectal cancer. Baseline clinical and laboratory parameters were retrieved from the hospital database and reviewed. The histological diagnosis of HCC was reviewed and confirmed by two pathologists (A.W.H.C. and K.F.T.). Informed consent was obtained from each recruited patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong (CREC#2013.688). Clinicopathological parameters of these patients are summarized in supplementary material, Table S1.

After liver resection, all patients with HCC were followed up with measurement of serum α -fetoprotein every 6 months, and with ultrasound or contrast computed tomography every 6–12 months. Overall survival was defined as the time from surgery to HCC-related death or the last follow-up if death had not occurred. Disease-free survival was defined as the time from surgery to radiological evidence of tumour relapse.

Human gene expression microarray analysis

Quality-controlled RNA samples were labelled with cyanine-3 dCTP (Agilent Technologies, Santa Clara, CA, USA), and hybridized on Agilent Whole Human Genome 4 × 44 K Microarrays according to the manufacturer's protocol. The arrays were washed and scanned with an Agilent scanner, and the image was digitized with Agilent Feature Extraction 11.0. Data

were analysed with GeneSpringGX 12.0 (Agilent Technologies) for '75th percentile shift' and 'baseline to median of all samples' normalization. Normalized gene expression signals were used for fold-change analysis. Gene expression array data have been submitted to the GEO (accession number GSE100533).

Lentivirus production and transduction

Lentiviruses expressing shRNAs against *CTCF*, i.e. shCTCF-1 (GCAAGGCAAGAAATGCCGTTA) and shCTCF-2 (GCGGAAAGTGAACCCATGATA), and non-targeting shRNA, i.e. shCont (CAACAA-GATGAAGAGCACCAA), were produced with the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA, USA). Cell transduction was carried out as described previously [13].

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

cDNA was synthesized from RNA by the use of PrimeScript RT Master Mix (TaKaRa), and subjected to quantitative polymerase chain reaction (qPCR) analysis with SYBR Premix Ex Taq II kits (TaKaRa Shuzo Co., Ltd., Kyoto, Japan) and the ABI PRISM 7900 Fast Real-Time PCR System. The primers used in RT-qPCR experiments are shown in supplementary material, Table S2.

Cell growth, senescence and motility assays

Senescence-associated β -galactosidase (SA- β -gal) assays, colony formation assays and soft agar assays were performed as described previously [14]. Cell migratory and invasive abilities were determined by the use of Transwell (Corning Life Sciences, Bedford, MA, USA). Matrigel (BD Biosciences, San Jose, CA, USA) invasion assays were performed as described previously [13].

Quantification of telomere dysfunction-induced foci (TIFs)

TIF immunofluorescence assays were performed as described previously [14]. Cells were analysed by use of a Zeiss (Jena, Germany) Axiovert 200 M fluorescence microscope, and images were captured with a CoolSNAP ES2 (Photometrics, Tucson, AZ, USA) camera. Cells with five or more phosphorylated H2AX (γ H2AX) foci colocalizing with TRF2 foci were considered to be TIF-positive.

Luciferase assays

Luciferase activity measurement was performed as described previously [15]. Measurements were conducted in cells coexpressing the indicated shRNAs and luciferase reporter, with *Renilla* luciferase reporter as a normalization control.

Chromatin immunoprecipitation

Assays were performed as described previously [16]. The primers used in ChIP-qPCR experiments are shown

in supplementary material, Table S3. Binding of CTCF was normalized to input, and expressed as a percentage of the input DNA (% input). IgG was used as a negative control. Data were obtained from at least three independent experiments.

Xenograft and orthotopic tumour models in nude mice

The protocol for the xenograft tumour model experiments was approved by the Institutional Animal Care and Use Committee of Nanjing Drum Tower Hospital, Medical School of Nanjing University. MHCC97L cells (2×10^6) transduced with lentivirus expressing shCont or shCTCF-2 were mixed with the same volume of ice-cold Matrigel, and then injected subcutaneously into the left flanks of 6-week-old female BALB/c nu/nu mice ($n = 6$ for each group). Tumour size was measured every 3 days. Mice were killed when the size of the control group tumours reached 1000 mm^3 . Tumours were fixed and then embedded in paraffin for sectioning. Tissue sections were subjected to haematoxylin and eosin (H&E) and immunohistochemical staining. The protocol for the orthotopic tumour model experiments was approved by the Committee of the Use of Live Animals in Teaching and Research at Hong Kong Polytechnic University (15-16/79-ABCT-R-GRF). Luciferase-labelled BEL-7402 cells (1×10^6), transduced with lentivirus expressing either shCont or shCTCF-2, were injected into the left lobes of the livers of BALB/c nu/nu mice as described previously [17]. Six weeks after implantation, 100 mg/kg D-luciferin was administered via peritoneal injection 5 min before bioluminescence imaging (IVIS Lumina III *in vivo* imaging system; PerkinElmer, Waltham, MA, USA). Lungs and livers were harvested postmortem for *ex vivo* imaging.

Results

Expression of CTCF in HCC cell lines and clinical specimens

Immunohistochemical analysis showed that CTCF was expressed in a number of human anatomical sites, including the colon, smooth muscle, and thyroid, but it was expressed at a very low level, if at all, in normal liver (Figure 1A). Western blotting analysis further suggested that, whereas CTCF was undetectable in normal livers, it was expressed in HCC biopsies, five HCC cell lines (PLC5, Hep3B, Huh7, HepG2, HepG2.2.15, and MHCC97L), and a cell line derived from liver adenocarcinoma (SK-HEP-1) (Figure 1B). Subsequently, the expression level of CTCF was determined with 47 pairs of primary HCCs. CTCF expression was detected in the majority of non-tumorous liver specimens, but a significant proportion of patients showed elevated CTCF levels (>2-fold) in tumour tissues (22 of 47 cases, 46.8%) relative to the adjacent non-tumorous tissues (Figure 1C, D).

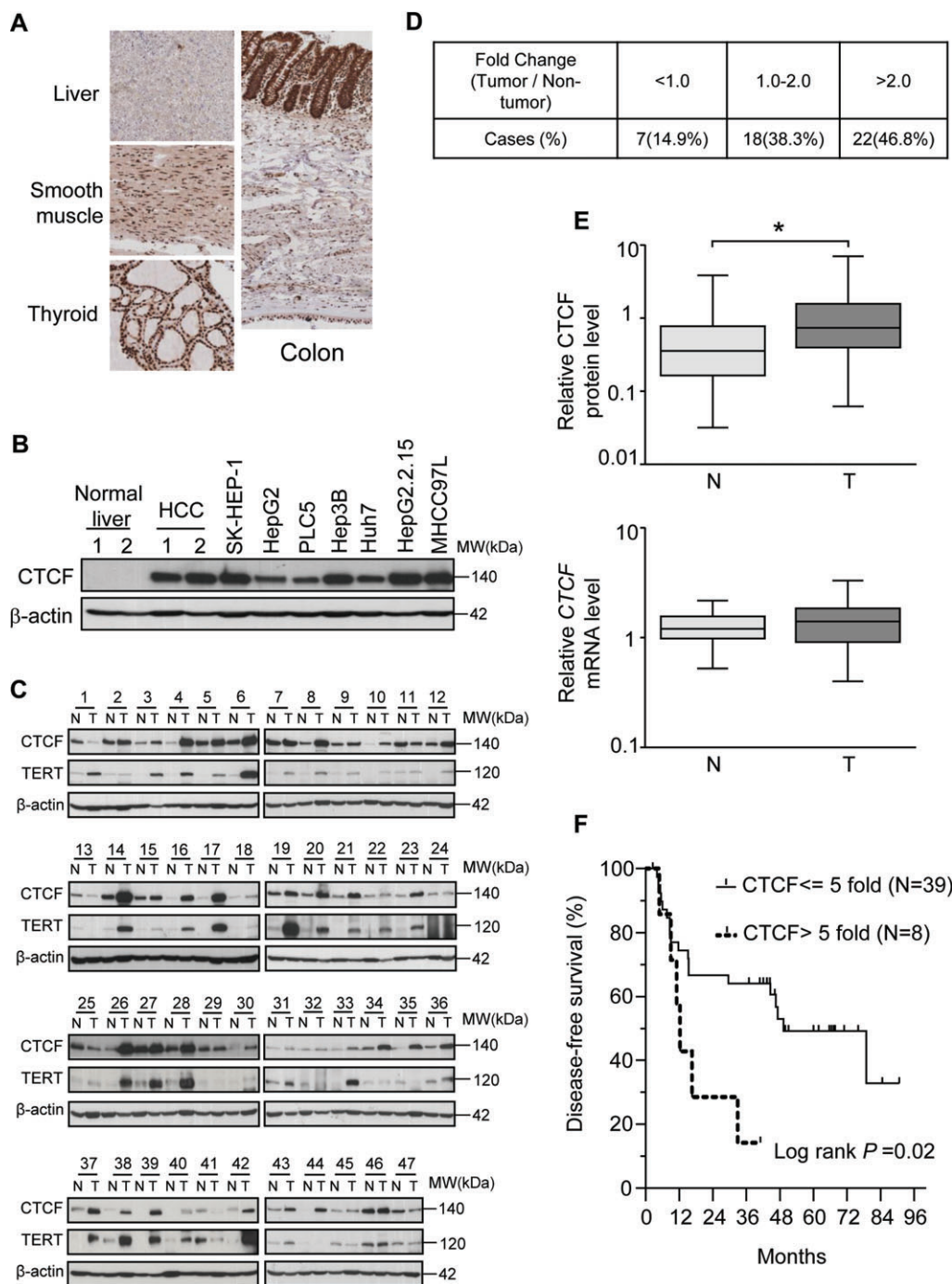


Figure 1. Overexpression of CTCF in clinical HCCs. (A) Immunohistochemical analysis of CTCF protein in normal human tissues. CTCF expression was detected in smooth muscle, colon, thyroid, and skin, but not in liver. (B) Western blotting analysis showed that CTCF was not detectable in normal liver, but was abundantly expressed in clinical HCC specimens and cell lines. (C) Western blot analysis of CTCF and TERT expression in 47 paired HCCs and adjacent non-tumoural livers. (D) Relative expression of CTCF protein in HCC specimens. Fold change was expressed as normalized CTCF expression level in HCCs relative to that in adjacent non-tumoural liver. (E) Quantification of CTCF protein (top) and *CTCF* mRNA (bottom) in HCCs and their adjacent non-tumoural liver tissues. * $P < 0.001$. (F) Kaplan–Meier analyses suggested that survival of the patients who had high CTCF expression was significantly shorter; $P = 0.02$. N, adjacent non-tumoural liver; T, HCC.

The average level of CTCF was also significantly higher in the tumour (median 0.7383, quartiles 0.3972–1.568) than in the adjacent non-tumorous liver (median 0.3580, quartiles 0.1649–0.7731) ($P < 0.001$) (Figure 1E, upper). Nevertheless, *CTCF* mRNA levels in tumour and non-tumorous liver samples were not significantly different (Figure 1E, lower). CTCF overexpression

(>5-fold) was associated with poorer disease-free survival (1-year survival of 50% versus 76%; 5-year survival of 17% versus 51%; $P = 0.02$) (Figure 1F), but it was not associated with overall survival or any other clinicopathological parameter. Together, these data suggest that overexpression of CTCF may affect HCC progression.

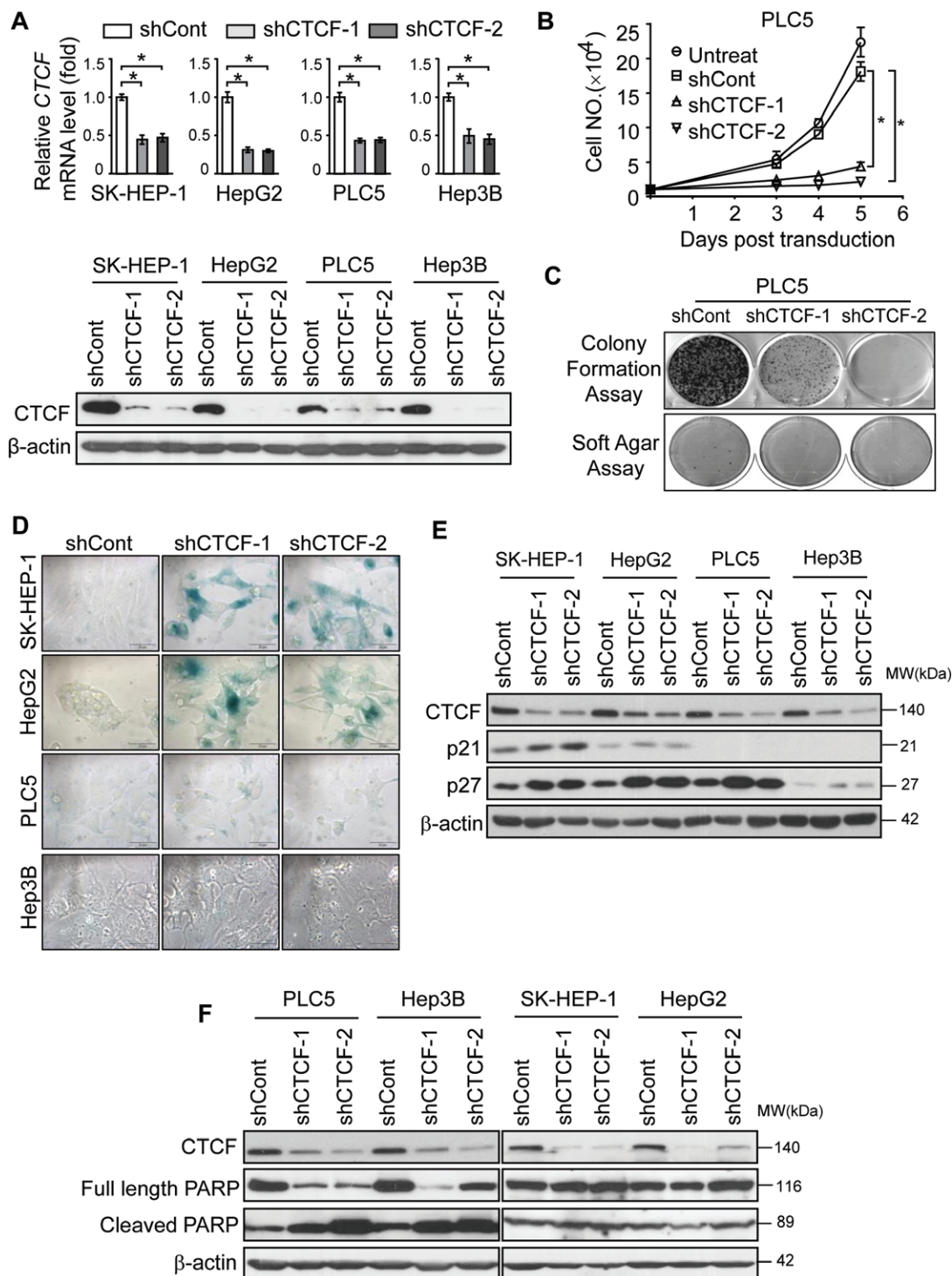


Figure 2. Cellular phenotypes induced by shRNA-mediated depletion of CTCF in HCC cells. (A) Cellular *CTCF* mRNA (upper) and CTCF protein levels (lower) were significantly reduced by lentiviruses expressing *CTCF*-targeting (shCTCF-1 and shCTCF-2) versus control (shCont) shRNA. * $P < 0.001$. (B) Proliferation of PLC5 cells was suppressed by lentiviral-mediated depletion of CTCF. * $P < 0.001$. (C) Depletion of CTCF in PLC-5 cells reduced colony formation (upper panel) and focus formation in soft agar (lower panel). (D) Depletion of CTCF led to cellular senescence in p53-positive liver cancer cells. SA- β -gal staining was conducted 6 days after cells had been transduced with lentiviruses expressing the indicated shRNA. (E) Western blot analysis of p21 and p27 in HCC cells transduced with lentiviruses expressing the indicated shRNA. (F) Western blot analysis of PARP cleavage in cells transduced with lentiviruses expressing the indicated shRNA.

Effect of CTCF silencing on HCC cell growth

Lentiviruses expressing shRNAs (shCTCF-1 and shCTCF-2) (Figure 2A) or SMARTpool siRNAs (siCTCF) (supplementary material, Figure S1A, B) were effective in depleting *CTCF* mRNA and CTCF protein

expression in a panel of liver cancer cell lines examined. Two independent CTCF shRNAs markedly reduced the proliferation of PLC5 (p53-inactivated; Figure 2B), HepG2 (p53-wild-type), SK-HEP-1 (p53-wild-type), and Hep3B (p53-deleted) cells (supplementary Figure

S1C) respectively, as compared with the untreated or scrambled shRNA (shCont)-transduced cells. Depletion of CTCF reduced the number and size of PLC5 colonies as determined by colony formation assays (Figure 2C; supplementary material, Figure S1D) and soft agar assays (Figure 2C), respectively. Furthermore, cellular senescence phenotypes, including cellular staining of SA- β -gal (Figure 2D), and induction of p21 and p27 (Figure 2E), were observed in CTCF-depleted SK-HEP-1 and HepG2 (p53-wild-type) cells, but not in Hep3B (p53-deficient) or PLC5 (p53-mutated) cells (Figure 2D, E). On the other hand, PARP cleavage was detected in CTCF-depleted PLC5 and Hep3B cells but not in SK-HEP-1 or HepG2 cells (Figure 2F). Considered together, these data are in support of depletion of CTCF preferentially inducing cellular senescence and apoptosis in p53-wild-type and p53-defective liver cancer cells, respectively.

Functional role of CTCF in telomere maintenance

Telomeric dysfunction caused by critically shortened telomeres [18] or depletion of telomere repeat binding proteins [19,20] may lead to cellular senescence characterized by a DNA damage response and the formation of TIFs. To determine whether cellular senescence induced by CTCF depletion is associated with telomeric dysfunction, we examined the expression of telomerase reverse transcriptase (*TERT*), members of the shelterin complex (*TRF1*, *TRF2*, *POT1*, *PTOP*, and *RAP1*) [21] and telomere-associated proteins (*PINX1* and *KU-70*) in CTCF-depleted cells. CTCF knockdown resulted in significant reductions in *TERT*, *TRF1* and *POT1* mRNA levels in HepG2 cells (Figure 3A), and reductions in *TERT* and *TRF1* mRNA levels in SK-HEP-1 cells (supplementary material, Figure S2A). Concordantly, significant reductions in *TERT* and *TRF1* protein expression levels were observed upon knockdown of CTCF in HepG2, PLC5, Hep3B and SK-HEP-1 cells (Figure 3B). Therefore, CTCF is essential for *TERT* and *TRF1* expression independently of p53 status. On the other hand, a positive association between tumour induction of CTCF and of *TERT* expression in HCC ($P = 0.002$) (Figures 1C and 3C) was also observed, suggesting that such a CTCF–*TERT* regulatory axis may exist *in vivo*. Consistent with the known effect of telomere dysfunction, CTCF silencing induced a DNA damage response characterized by the phosphorylation of ATM and CHK2 (Figure 3D), and the formation of TIFs characterized by colocalization of γ H2AX and TRF2 (Figure 3E).

Forkhead box protein M1 (FOXM1) mediates the effect of CTCF on motility and invasiveness of HCC cells

To reveal other potential CTCF-regulated genes, PLC5 cells transduced with shCont or shCTCF-1 were subjected to gene expression profiling analysis. Using a two-fold cutoff, we found that 3357 genes were

downregulated and 8243 genes were upregulated in CTCF-depleted cells (supplementary material, Table S4). We noticed that depletion of CTCF also resulted in substantial reduction in *FOXM1* expression. *FOXM1* is implicated in the tumorigenesis and metastasis of a variety of tumours, including HCCs [22–25]. Downregulation of *FOXM1* in CTCF-depleted liver cancer cells (HepG2, PLC5 and Hep3B, SK-HEP-1) was confirmed by RT-qPCR (supplementary material, Figure S2B) and western blot (Figure 4A) analyses. In the same cohort of HCC biopsies, *FOXM1* expression levels were significantly elevated in HCCs (median 3.22, quartiles 2.28–4.68) as compared with the adjacent non-tumorous liver (median 0.26, quartiles 0.17–0.34) ($P < 0.00001$) (Figure 4B). A significant positive correlation (Spearman's rank = 0.63, $P < 0.01$) (Figure 4C) between the CTCF protein and *FOXM1* mRNA levels was observed in these specimens, suggesting that CTCF may regulate *FOXM1* expression *in vivo*. In agreement with the role of *FOXM1* in metastasis [26], depletion of CTCF also markedly reduced cell migration and impaired invasion of PLC5 cells. Importantly, ectopic expression of *FOXM1* restored the cell migration and invasive activity of CTCF-depleted cells (Figure 5A, B), suggesting that *FOXM1* is one of the major downstream effectors of CTCF to promote tumour cell dissemination. To further understand the role of CTCF in metastasis, we assessed the expression of E-cadherin, α -catenin, β -catenin, vimentin, and N-cadherin, which are proteins involved in epithelial–mesenchymal transition (EMT). Unexpectedly, the expression of these proteins was not altered appreciably in CTCF-depleted cells (Figure 5C). Nevertheless, there were reductions in cortical localization of F-actin, membrane protrusions, and stress fibres (Figure 5D).

Transcriptional regulation of *TERT* and *FOXM1* by CTCF

The data above suggested that CTCF may regulate gene expression of *TERT* and *FOXM1*. We therefore conducted reporter gene assays to determine whether CTCF regulates *TERT* and *FOXM1* promoter activity. As shown in Figure 5E, depletion of CTCF significantly attenuated luciferase reporter activity driven by the *TERT* 5' regulatory sequence (–923 to +77 bp relative to the *TERT* transcriptional start site) (pGL3-*TERT*-1 kb) and *FOXM1* 5' regulatory sequence (–955 to +45 bp relative to the *FOXM1* transcriptional start site) (pGL3-*FOXM1*-1 kb), whereas it did not alter the activity of the *SV40* promoter (pGL3-*SV40*). Bioinformatics analysis using CTCFBSDB 2.0 (<http://insulatordb.uthsc.edu>) revealed putative binding sites located at –990, –884, –629 and –28 bp of *TERT*, and at –816 and –107 bp of *FOXM1*, respectively (supplementary material, Figure S3). Furthermore, analysis of the ChIP-sequencing data in ENCODE further revealed that, in HepG2 cells, the CTCF peak region was located between –120 to –269 bp of *FOXM1* (supplementary material, Figure S3). To further confirm the

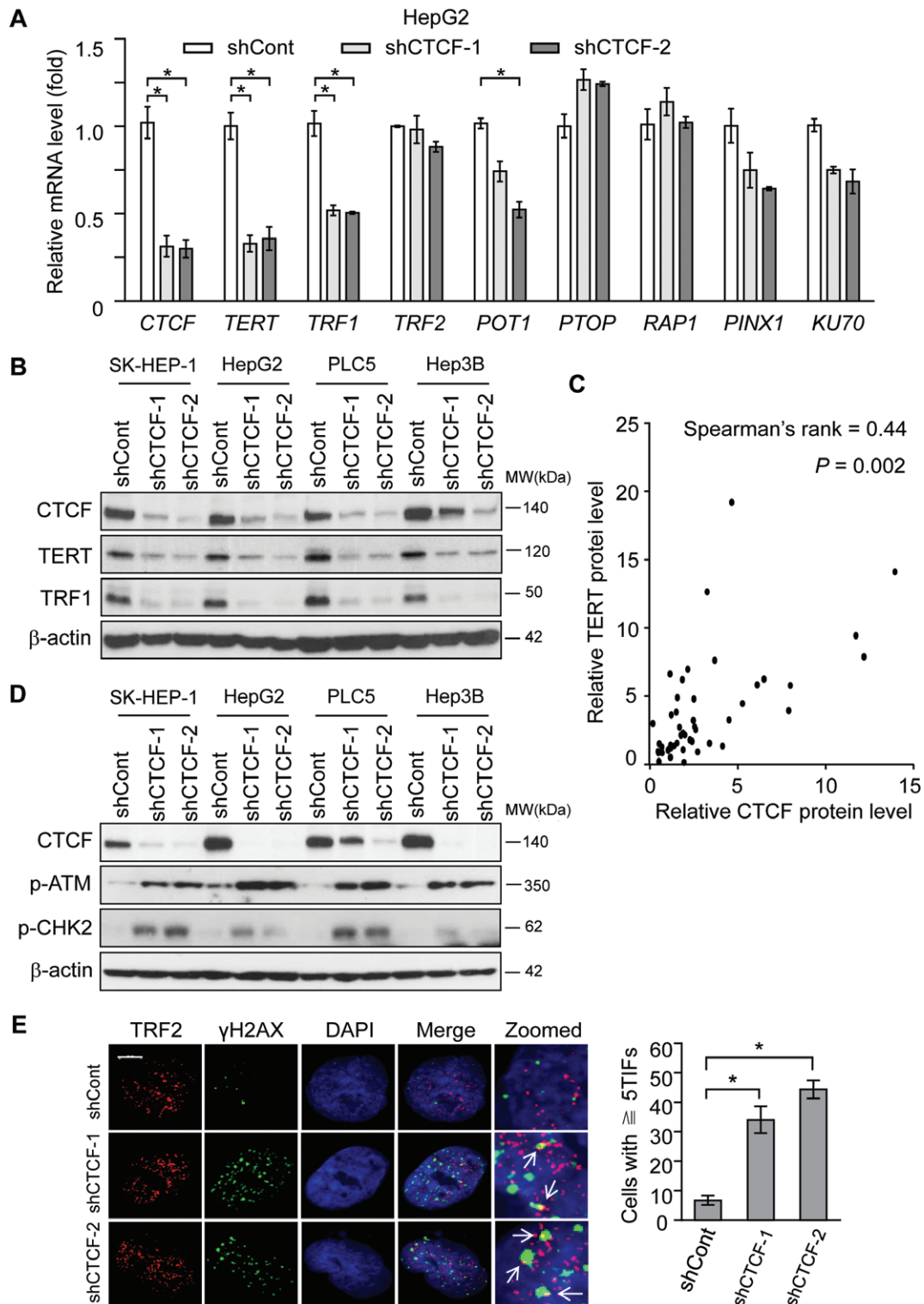


Figure 3. Depletion of CTCF repressed TERT and TRF1 expression and induced telomere abnormality. (A) RT-qPCR analysis of mRNA levels of genes related to telomere maintenance in HepG2 cells transduced with lentiviruses expressing the indicated shRNA. *ACTB* expression was used as an internal control. Values represent the mean \pm standard deviation (SD) of three independent experiments. * $P < 0.01$. (B) Western blot analysis of CTCF, TERT and TRF1 in liver cancer cells 5 days after transduction with lentiviruses expressing the indicated shRNA. (C) Upregulation of CTCF protein was positively correlated with upregulation of TERT protein in clinical HCC ($P = 0.002$). Fold induction (HCC/non-tumoural liver) of CTCF was plotted against fold induction of TERT (Figure 1C), and analysed with Spearman's σ rank test. (D) Western blot analysis of CTCF, phosphorylated ATM, and phosphorylated CHK2 in different liver cancer cells 5 days after transduction with lentiviruses expressing the indicated shRNA. (E) Immunofluorescence staining of TRF2 (red) and γ H2AX (green) in SK-HEP-1 cells. Cells were analysed 5 days after transduction with lentivirus expressing the indicated shRNA. Arrows indicate telomere-associated γ H2AX foci. Quantification of TIFs is shown on the right. In each experiment, 100 nuclei from five randomly picked fields were analysed. Scale bar: 20 μ m. Data are mean \pm SD. * $P < 0.05$. DAPI, 4',6-diamidino-2-phenylindole.

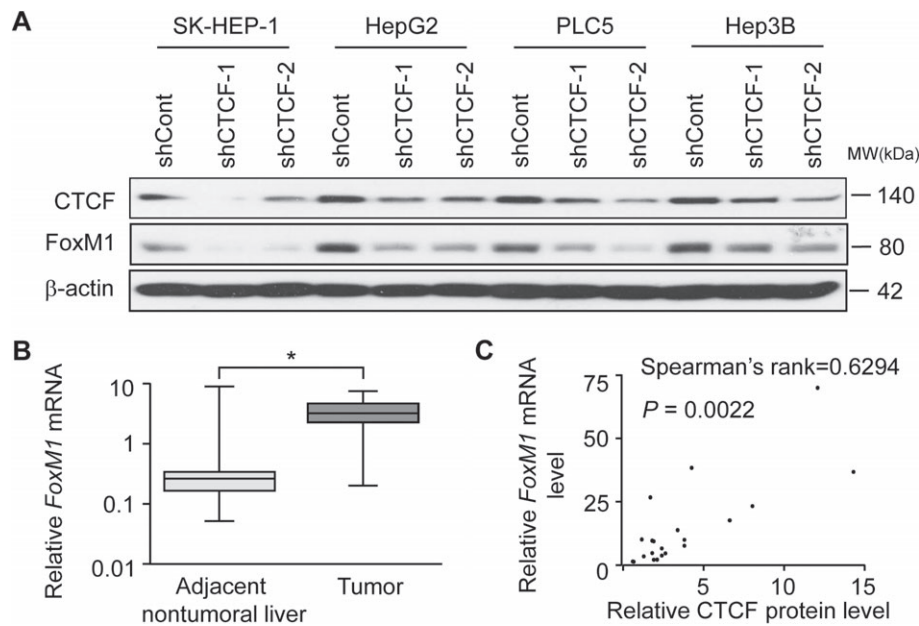


Figure 4. Correlation between CTCF and FOXM1 levels. (A) FOXM1 protein levels in HCC cells were significantly reduced by lentiviruses expressing CTCF-targeting (shCTCF-1 and shCTCF-2) versus control (shCont) shRNA. (B) *FOXM1* mRNA levels were significantly elevated in HCCs as compared with the adjacent non-tumoural livers. * $P < 0.001$. (C) Upregulation of *FOXM1* mRNA was positively correlated with upregulation of CTCF protein in clinical HCC ($P = 0.002$). Fold induction (HCC/non-tumoural liver) of *FOXM1* mRNA was plotted against fold induction of TERT, and analysed by Spearman's σ rank test.

interaction between CTCF and the *FOXM1* promoter *in vivo*, ChIP analysis was conducted with anti-CTCF antibodies, followed by qPCR amplification of amplicons that spread across the 12.6-kb region of *FOXM1* (Figure 5F). ChIP-qPCR analysis showed differential enhancement of signal at the imprinted-control region of the *IGF2/H19* locus in CTCF-expressing versus CTCF-depleted cells (Figure 5F) [27], indicating the validity of the assay. On the other hand, significant enhancement of CTCF signal was observed in the TSS (amplicon c) and the intron 1 (amplicon d) regions of *FOXM1*, respectively, and it was abolished by CTCF depletion. In contrast, no significant enhancement of signal was observed in exon 4 (amplicon f), where there is no putative CTCF-binding site around this locus (Figure 5F). Considered together, these data are in support of CTCF regulating the expression of *FOXM1* via interaction with the CTCF-binding site(s) located in the proximal promoter of the gene.

Role of CTCF in tumour progression and metastasis *in vivo*

To further ascertain the role of CTCF in HCC growth *in vivo*, a xenograft model was prepared by subcutaneous implantation of human MHCC97L cells expressing shCont or shCTCF-2. Cells expressing shCTCF-2 showed substantial repression of CTCF expression and a profound reduction in tumour growth over a period of 28 days as compared with cells expressing shCont (Figure 6A). On the 28th day, shCTCF-2-expressing tumours were also smaller in size (Figure 6B, upper) and lower in weight (Figure 6B, lower). Immunohistochemical analysis of tumour sections revealed that shCTCF-2-expressing cells showed clear reductions

in staining for CTCF, FOXM1, and the proliferation marker Ki67. However, no significant differences in terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) staining were detected (Figure 6C).

Next, an orthotopic HCC metastatic model was employed to test the *in vivo* metastatic role of CTCF. Highly metastatic BEL-7402 cells expressing shCont or shCTCF-2 (Figure 6D, upper) were injected into the left lobes of the livers of nude mice, and, 35 days later, the formation of tumour foci in the livers and lungs of the mice was evaluated with a Xenogen imaging system. Consistent with the results of the xenograft experiment above, BEL-7402 cells expressing shCont formed liver tumours with a higher tumour load than the tumours formed by cells expressing shCTCF-2 (Figure 6D, upper, and Figure 6E). On the other hand, three of five (60%) mice with cells expressing shCont showed extensive tumour metastasis to the lungs, whereas the tumour load in the lungs of mice with liver tumours expressing shCTCF-2 was minimal (Figure 6D, lower). Taken together, these data support the hypothesis that CTCF contributes to tumour growth and metastasis.

Discussion

In this study, we elucidated the functional role of CTCF in HCC oncogenesis. CTCF is an essential genome organizer and insulator, and is known for its role in establishing the three-dimensional structure of the genome [3]. Intriguingly, however, CTCF expression was not detected in the three human normal liver specimens that we analysed (two by western blotting and one by

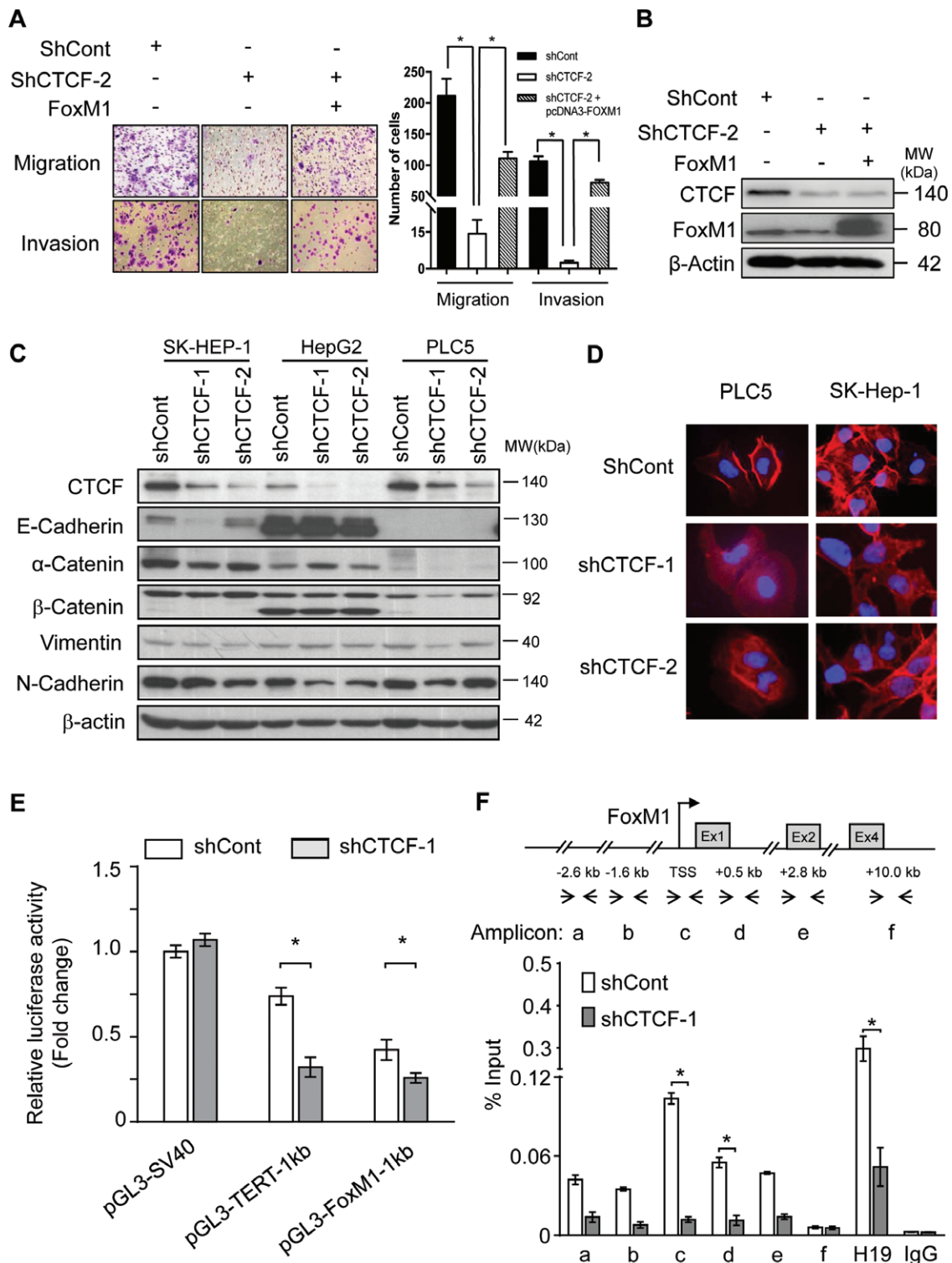


Figure 5. CTCF controls the motility and invasiveness of HCC cells through regulation of FOXM1. (A) Effect of CTCF depletion, and subsequent ectopic FOXM1 expression, on the motility and invasiveness of PLC5 cells. Left: representative images of cells that have migrated through Transwell pores (upper panel) and invasion through Matrigel (lower panel) under different conditions. Right: quantification of cell migration and invasion under different conditions. For each condition, five different fields were counted. **P* < 0.001. (B) Western blot analysis showing the knockdown of CTCF and the rescue of FOXM1 expression in PLC5 cells. (C) Western blot analysis of EMT markers in liver cancer cells depleted of CTCF. (D) Phalloidin staining for F-actin (red) in PLC5 and SK-Hep1 cells showed a reduction in the number of stress fibre filaments in cells expressing shRNA against *CTCF*. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). (E) Normalized luciferase activity of *TERT* and *FOXM1* reporter constructs. *SV40* promoter-driven luciferase reporter vector (pGL3-SV40) was used as a control. Normalized luciferase activity of SK-Hep-1 cells expressing pGL3-SV40 and shCont was designated as 1. **P* < 0.01. (F) Upper panel: a schematic diagram of *FOXM1* genomic structure and PCR amplicons for ChIP-qPCR analysis. Lower panel: ChIP-qPCR analyses of CTCF occupancy of the *FOXM1* gene of SK-Hep-1 cells. IgG, PCR signal at the TSS (amplicon c) region of *FOXM1* when IgG was used instead of CTCF antibody for ChIP. **P* < 0.05, one-way ANOVA. Results show means ± standard deviations from triplicate experiments.

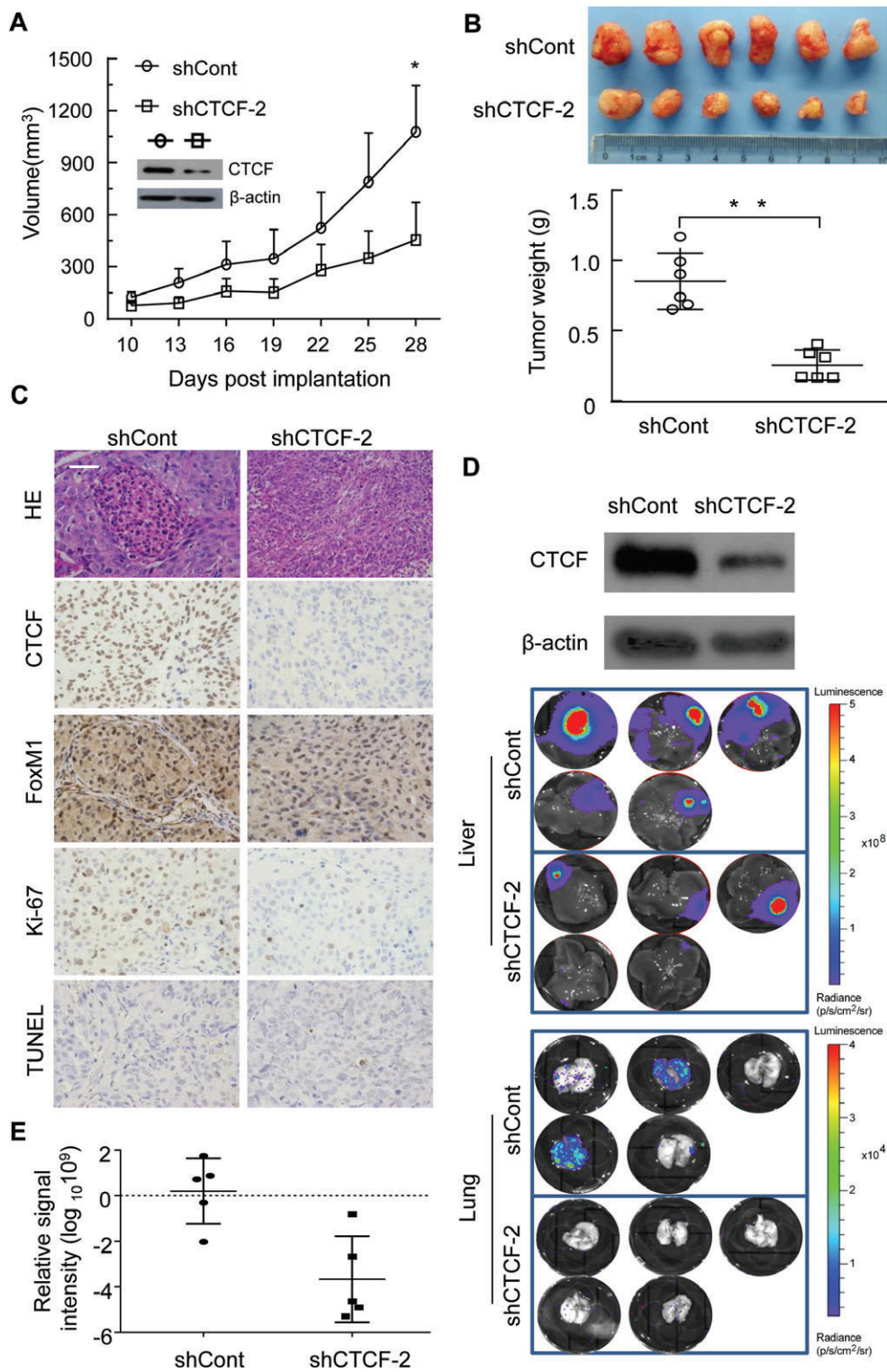


Figure 6. (A) Growth curves of xenograft tumours after subcutaneous implantation of MHCC97L cells. Insert: western blot analysis showing the expression of CTCF in shCont-expressing versus ShCTCF-2 expressing cells. **P* < 0.01, Student's *t*-test. (B) Representative images (upper) and weights (lower) of xenograft tumours formed by MHCC97L cells expressing shCont and shCTCF-2. Data represent the mean ± standard deviation. **P* < 0.05, Student's *t*-test. (C) Representative images of immunohistochemical staining of CTCF, FOXM1, and Ki67, and TUNEL staining, in tumour xenografts. Magnification: ×400. Scale bar: 50 μm. (D) Western blot showing the expression of CTCF in BEL-4402 cells expressing shCTCF-2 (upper panel). An orthotopic HCC metastatic model showed that BEL-7402 cells had undergone extensive lung metastasis *in vivo*, which was abrogated by CTCF knockdown (4/5 versus 0/5 for lung metastasis) (lower panel). (E) Volume of orthotopic liver tumours formed by BEL-7402 cells, expressed as relative luciferase signal intensity. The relative signal intensity of tumour expressing shCTCF-2 was apparently lower than that of tumour expressing shCont, but this trend did not reach statistical significance.

immunohistochemistry). Our observation was consistent with data in The Human Protein Atlas [28], which showed that CTCF was expressed at a lower level in the liver than in other organs. Therefore, we hypothesized that there is a novel insulator responsible for maintaining the genome architecture of normal liver cells. Alternatively, liver cells may express a tissue-specific CTCF isoform that is not recognized by the existing CTCF antibodies. Elucidating how genome topology is organized in liver cells will provide novel insights into the cell-specific function of CTCF.

The majority of evidence has suggested that CTCF is a tumour suppressor. Through the interaction with CTCF-binding sites known as anchor sequences, CTCF partitions chromosomes into megabase-sized topologically associated domains (TADs). Within TADs, CTCF further segregates DNA into loop structures known as insulated neighbourhoods. These chromosome territories are essential for constraining enhancer–gene interactions and gene expression. Consequently, defective interactions between CTCF and anchor sequences lead to aberrant expression of genes, including oncogenes [29]. Concordantly, CTCF was shown to be sporadically inactivated or truncated in a range of tumours, such as Wilms' tumour, leukaemia, and breast, endometrial and prostate cancers [30–34]. Furthermore, *Ctcf* hemizygous knockout mice are predisposed to the development of cancer in a wide range of tissues [35]. CTCF-binding sites were also found to be some of the most frequently mutated transcription factor-binding sites in cancers [29,36,37], including HCC [38]. On the other hand, emerging evidence has suggested that CTCF overexpression or gain-of-function mutation may promote tumour growth in cell models [9,30,39]. Our study has made the pivotal discovery that CTCF is overexpressed in liver tumours, and has extended our current understanding of the pro-tumourigenic function of CTCF. We showed that CTCF promotes HCC cell growth and metastasis by directly regulating expression of *TERT* and *FOXM1*. Recent findings have suggested that a subset of CTCF-binding sites, instead of forming insulated neighbourhood loops, are located near the enhancers and serve as anchors to recruit co-activators for gene transcription [40], and this suggests a potential mechanism for CTCF function in HCC. Subsequent analysis will attempt to identify the molecules involved in the process. At present, whether enhanced CTCF protein expression in HCCs is mediated by post-translational mechanisms remains to be determined. Earlier studies showed that CTCF is subject to phosphorylation and poly(ADP-ribosylation) [41,42], but whether these modifications, or other unknown modifications, contribute to protein stability remains to be determined. On the other hand, we observed that many of the HCCs overexpressing CTCF concordantly overexpressed CTCF in the adjacent non-tumoural liver tissues, although the expression level was lower than in the tumours in general. Aetiologically, these patients were hepatitis B virus-positive or hepatitis C virus-positive, so their livers were subjected to various degrees of inflammation,

fibrosis, or cirrhosis, which are conditions that predispose to HCC development. Therefore, CTCF upregulation in non-tumoural livers may represent an important alteration that drives HCC oncogenesis.

Our study also revealed a novel CTCF–FOXM1 regulatory axis in HCC. *FOXM1* is one of the most frequently upregulated genes in human solid tumours, including HCC [25,43]. Its overexpression is also associated with poorer prognosis in HCC patients. FOXM1 is a transcriptional regulator that is implicated in a variety of biological processes, including cancer cell migration and invasion [44]. Accordingly, CTCF-depleted cells showed the reduced motility and invasiveness that are typical results of FOXM1 inhibition, but these effects were readily alleviated by ectopic expression of FOXM1, suggesting that FOXM1 is one of the important effectors of CTCF activity. Intriguingly, although an earlier study suggested that inhibition of FoxM1 leads to repression of genes involved in EMT [45], CTCF depletion did not alter the expression of these genes. Nevertheless, it led to the rearrangement of actin stress fibres of HCC cells. We postulate that, instead of modulating expression of genes that are directly involved in EMT, CTCF might regulate the motility and invasiveness of HCC cells via regulation of genes involved in the arrangement of actin stress fibres.

We speculate that CTCF overexpression may play a more widespread role during the carcinogenesis of cancer types other than HCC. However, the correlation may not be revealed readily, because differential CTCF overexpression was not detectable at the mRNA level. Given that 30–60% of the CTCF-binding sites are cell type-specific [4,11,46,47], and substantial differences in the CTCF-binding landscape were observed between normal and tumour cells [12], dysregulated CTCF expression is likely to alter distinctive genetic programmes in a cancer cell-specific manner. In HCC, CTCF depletion also led to altered expression of a vast array of genes besides *FOXM1*. A detailed functional analysis of these genes may provide a more thorough understanding of CTCF function during HCC carcinogenesis. In the future, we will address whether CTCF overexpression is a driver for HCC tumourigenesis. Furthermore, our study also raises the possibility that targeting CTCF could be further explored as a potential HCC therapeutic strategy.

Acknowledgements

This work was supported by the National Science Foundation of China (81272764), the Shenzhen Basic Research Programme (JCYJ20160531185413087), the Health and Medical Research Fund (04150406), and the Polytechnic University of Hong Kong fund (YBAZ and GUA1M) to BCBK, and the Polytechnic University Area of Strategic Importance project (1-ZE22) to LMCC.

Author contributions statement

The authors contributed in the following way: BZ, HL, YZ, KKYC: performed most of the experimental work; AWHC, PBSL, YSC: provided HCC specimens and conducted histological diagnosis; RZ, TKWL, EYTL, NPYH, XZ, VK, LMCC, ASC: contributed to animal studies; KWC, HKW: helped with microarray analysis; BCBK: responsible for the design and analysis of the experiments. All authors contributed to the writing of the manuscript.

References

- El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557–2576.
- Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020–1022.
- Phillips JE, Corces VG. CTCF: master weaver of the genome. *Cell* 2009; **137**: 1194–1211.
- Kim TH, Abdullaev ZK, Smith AD, *et al.* Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell* 2007; **128**: 1231–1245.
- Filippova GN, Fagerlie S, Klenova EM, *et al.* An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol* 1996; **16**: 2802–2813.
- Ohlsson R, Bartkuhn M, Renkawitz R. CTCF shapes chromatin by multiple mechanisms: the impact of 20 years of CTCF research on understanding the workings of chromatin. *Chromosoma* 2010; **119**: 351–360.
- Torrano V, Chernukhin I, Docquier F, *et al.* CTCF regulates growth and erythroid differentiation of human myeloid leukemia cells. *J Biol Chem* 2005; **280**: 28152–28161.
- Rasko JE, Klenova EM, León J, *et al.* Cell growth inhibition by the multifunctional multivalent zinc-finger factor CTCF. *Cancer Res* 2001; **61**: 6002–6007.
- Docquier F, Farrar D, D'Arcy V, *et al.* Heightened expression of CTCF in breast cancer cells is associated with resistance to apoptosis. *Cancer Res* 2005; **65**: 5112–5122.
- Fiorentino FP, Macaluso M, Miranda F, *et al.* CTCF and BORIS regulate Rb2/p130 gene transcription: a novel mechanism and a new paradigm for understanding the biology of lung cancer. *Mol Cancer Res* 2011; **9**: 225–233.
- Chen H, Tian Y, Shu W, *et al.* Comprehensive identification and annotation of cell type-specific and ubiquitous CTCF-binding sites in the human genome. *PLoS One* 2012; **7**: e41374.
- Wang H, Maurano MT, Qu H, *et al.* Widespread plasticity in CTCF occupancy linked to DNA methylation. *Genome Res* 2012; **22**: 1680–1688.
- Chen J, Chan AW-H, To K-F, *et al.* SIRT2 overexpression in hepatocellular carcinoma mediates epithelial to mesenchymal transition by protein kinase B/glycogen synthase kinase-3 β / β -catenin signaling. *Hepatology* 2013; **57**: 2287–2298.
- Chen J, Zhang B, Wong N, *et al.* Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth. *Cancer Res* 2011; **71**: 4138–4149.
- Ko BCB, Lam AKM, Kapus A, *et al.* Fyn and p38 signaling are both required for maximal hypertonic activation of the osmotic response element-binding protein/tonicity-responsive enhancer-binding protein (OREBP/TonEBP). *J Biol Chem* 2002; **277**: 46085–46092.
- Zhang B, Chen J, Cheng ASL, *et al.* Depletion of sirtuin 1 (SIRT1) leads to epigenetic modifications of telomerase (TERT) gene in hepatocellular carcinoma cells. *PLoS One* 2014; **9**: e84931.
- Lau EYT, Lo J, Cheng BYL, *et al.* Cancer-associated fibroblasts regulate tumor-initiating cell plasticity in hepatocellular carcinoma through c-Met/FRA1/HEY1 signaling. *Cell Rep* 2016; **15**: 1175–1189.
- Deng Y, Chan SS, Chang S. Telomere dysfunction and tumour suppression: the senescence connection. *Nat Rev Cancer* 2008; **8**: 450–458.
- Kim S-H, Davalos AR, Heo S-J, *et al.* Telomere dysfunction and cell survival: roles for distinct TIN2-containing complexes. *J Cell Biol* 2008; **181**: 447–460.
- Veldman T, Etheridge KT, Counter CM. Loss of hPot1 function leads to telomere instability and a cut-like phenotype. *Curr Biol* 2004; **14**: 2264–2270.
- de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005; **19**: 2100–2110.
- Kopanja D, Pandey A, Kiefer M, *et al.* Essential roles of FoxM1 in Ras-induced liver cancer progression and in cancer cells with stem cell features. *J Hepatol* 2015; **63**: 429–436.
- Ho DW, Kai AK, Ng IO. TCGA whole-transcriptome sequencing data reveals significantly dysregulated genes and signaling pathways in hepatocellular carcinoma. *Front Med* 2015; **9**: 322–330.
- Xia L, Huang W, Tian D, *et al.* ACP5, a direct transcriptional target of FoxM1, promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Oncogene* 2014; **33**: 1395–1406.
- Meerzaman DM, Yan C, Chen QR, *et al.* Genome-wide transcriptional sequencing identifies novel mutations in metabolic genes in human hepatocellular carcinoma. *Cancer Genomics Proteomics* 2014; **11**: 1–12.
- Raychaudhuri P, Park HJ. FoxM1: a master regulator of tumor metastasis. *Cancer Res* 2011; **71**: 4329–4333.
- Hark AT, Schoenherr CJ, Katz DJ, *et al.* CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 2000; **405**: 486–489.
- Uhlén M, Fagerberg L, Hallström BM, *et al.* Proteomics. Tissue-based map of the human proteome. *Science* 2015; **347**: 1260419.
- Hnisz D, Weintraub AS, Day DS, *et al.* Activation of proto-oncogenes by disruption of chromosome neighborhoods. *Science* 2016; **351**: 1454–1458.
- Marshall AD, Bailey CG, Champ K, *et al.* CTCF genetic alterations in endometrial carcinoma are pro-tumorigenic. *Oncogene* 2017; **36**: 4100–4110.
- Aulmann S, Bläker H, Penzel R, *et al.* CTCF gene mutations in invasive ductal breast cancer. *Breast Cancer Res Treat* 2003; **80**: 347–352.
- Filippova GN, Qi C-F, Ulmer JE, *et al.* Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter its DNA-binding specificity. *Cancer Res* 2002; **62**: 48–52.
- Yoshida K, Toki T, Okuno Y, *et al.* The landscape of somatic mutations in Down syndrome-related myeloid disorders. *Nat Genet* 2013; **45**: 1293–1299.
- Zhang J, Ding L, Holmfeldt L, Wu G, *et al.* The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature* 2012; **481**: 157–163.
- Kemp CJ, Moore JM, Moser R, *et al.* CTCF haploinsufficiency destabilizes DNA methylation and predisposes to cancer. *Cell Rep* 2014; **7**: 1020–1029.
- Flavahan WA, Drier Y, Liao BB, *et al.* Insulator dysfunction and oncogene activation in IDH mutant gliomas. *Nature* 2016; **529**: 110–114.

37. Katainen R, Dave K, Pitkäinen E, *et al.* CTCF/cohesin-binding sites are frequently mutated in cancer. *Nat Genet* 2015; **47**: 818–821.
38. Fujimoto A, Furuta M, Totoki Y, *et al.* Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet* 2016; **48**: 500–509.
39. Méndez-Catalá CF, Gretton S, Vostrov A, *et al.* A novel mechanism for CTCF in the epigenetic regulation of Bax in breast cancer cells. *Neoplasia* 2013; **15**: 898–912.
40. Ong C-T, Corces VG. CTCF: an architectural protein bridging genome topology and function. *Nat Rev Genet* 2014; **15**: 234–246.
41. Yu W, Ginjala V, Pant V, *et al.* Poly(ADP-ribosyl)ation regulates CTCF-dependent chromatin insulation. *Nat Genet* 2004; **36**: 1105–1110.
42. Klenova EM, Chernukhin IV, El-Kady A, *et al.* Functional phosphorylation sites in the C-terminal region of the multivalent multifunctional transcriptional factor CTCF. *Mol Cell Biol* 2001; **21**: 2221–2234.
43. Okabe H, Satoh S, Kato T, *et al.* Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001; **61**: 2129–2137.
44. Koo C-Y, Muir KW, Lam EW-F. FOXM1: from cancer initiation to progression and treatment. *Biochim Biophys Acta* 2012; **1819**: 28–37.
45. Meng FD, Wei JC, Qu K, *et al.* FoxM1 overexpression promotes epithelial–mesenchymal transition and metastasis of hepatocellular carcinoma. *World J Gastroenterol* 2015; **21**: 196–213.
46. Cuddapah S, Jothi R, Schones DE, *et al.* Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. *Genome Res* 2009; **19**: 24–32.
47. Schmidt D, Schwalie PC, Wilson MD, *et al.* Waves of retrotransposon expansion remodel genome organization and CTCF binding in multiple mammalian lineages. *Cell* 2012; **148**: 335–348.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. CTCF expression, cell proliferation and colony formation following transduction with siRNA and shRNAs

Figure S2. Analysis of mRNA levels relevant to telomere maintenance in cell lines following transduction with shRNA

Figure S3. Location of putative CTCF-binding sites in the *TERT* and *FOXM1* gene promoter regions

Table S1. Clinicopathological information of hepatocellular carcinoma patients

Table S2. Primer sequences for Reverse Transcription-qPCR analyses

Table S3. Primer sequences for ChIP-qPCR analysis

Table S4. Human expression array analysis of PLC5 cells 2 days after transduction with shCont or shCTCF-2. Only annotated genes altered by ≥ 2.0 -fold on depletion of CTCF are listed

50 Years ago in the *Journal of Pathology*...

Histamine-sensitising factor of *Bordetella pertussis* differentiated from immunogens by neutralisation and passive protection tests

Noel W. Preston and Pamela Garrity

To view these articles, and more, please visit:

www.thejournalofpathology.com

Click 'ALL ISSUES (1892 - 2017)', to read articles going right back to Volume 1, Issue 1.

The Journal of Pathology
Understanding Disease

