Journal of Pathology
J Pathol 2014; **232:** 566–577
Published online 8 February 2014 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/path.4327

ORIGINAL PAPER

Identification of *ZDHHC14* as a novel human tumour suppressor gene

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Abstract

Genomic changes affecting tumour suppressor genes are fundamental to cancer. We applied SNP array analysis to a panel of testicular germ cell tumours to search for novel tumour suppressor genes and identified a frequent small deletion on 6q25.3 affecting just one gene, *ZDHHC14*. The expression of ZDHHC14, a putative protein palmitoyltransferase with unknown cellular function, was decreased at both RNA and protein levels in testicular germ cell tumours. ZDHHC14 expression was also significantly decreased in a panel of prostate cancer samples and cell lines. In addition to our findings of genetic and protein expression changes in clinical samples, inducible overexpression of ZDHHC14 led to reduced cell viability and increased apoptosis through the classic caspase-dependent apoptotic pathway and heterozygous knockout of *ZDHHC14* decreased cell colony formation ability. Finally, we confirmed our *in vitro* findings of the tumour suppressor role of ZDHHC14 in a mouse xenograft model, showing that overexpression of ZDHHC14 inhibits tumourigenesis. Thus, we have identified a novel tumour suppressor gene that is commonly down-regulated in testicular germ cell tumours and prostate cancer, as well as given insight into the cellular functional role of ZDHHC14, a potential protein palmitoyltransferase that may play a key protective role in cancer.

Keywords: ZDHHC14; tumour suppressor gene; testicular germ cell tumours; prostate cancer; apoptosis

Received 9 August 2013; Revised 20 December 2013; Accepted 3 January 2014

No conflicts of interest were declared.

Introduction

Tumourigenesis is a multistep process in which, elicited by the activation of oncogenes and the inactivation of tumour suppressor genes (TSGs), cells acquire increasingly malignant characteristics. TSGs prevent tumour initiation and progression by acting in signalling networks that regulate cell proliferation, differentiation, apoptosis, and other cellular and systemic processes. TSGs can be inactivated by deletions, point mutations [1] or by epigenetic mechanisms such as DNA methylation and histone modifications [2]. Among them, deletion is one of the most frequent genetic alterations detected in cancer cells and recurrent deletions detected in certain regions have been widely interrogated to identify TSGs. The development of comparative genomic hybridization and DNA microarray technologies has made it easy to identify genomic copy number alterations and has accelerated the discovery of TSGs. Single nucleotide polymorphism (SNP) arrays allow the determination of both genomic copy number changes and loss of heterozygosity (LOH), including copy number neutral LOH [3,4]. Many novel TSGs have been discovered in human malignancies using SNP array analysis, including *PAX5* as a key target of genetic inactivation in B-cell acute lymphocytic leukaemia [5], *PTPRD* as a tumour suppressor in lung cancer and glioma [6,7], and *IKFZF1* in acute lymphoblastic leukaemia [8].

Testicular germ cell tumours (TGCTs) are the most common malignancy in young men. Many genetic studies have been carried out in TGCTs and the

characteristic genetic change, gain of 12p [9], has been identified. Previous low-resolution genetic studies of TGCTs identified more genomic copy number gains rather than losses [9]. Interestingly, deregulation of important tumour suppressor genes such as *RB1*, *TP53*, *APC*, *WT1*, *MCC*, and *BRCA1* is rare in this type of cancer [10–12]. However, few array-based genome-wide studies have been performed for TGCTs. Using the Affymetrix 10 K SNP array, we analysed a series of TGCTs to identify genome-wide copy number changes, in particular frequently occurring small genomic region gains and losses. We found frequent deletion of a small genomic region within 6q25.3 affecting only one known gene, *ZDHHC14*. Further genetic studies revealed additional genomic alterations and reduced expression of ZDHHC14 not only in TGCTs, but also in prostate cancer. Using cell-based assays and xenografts, we performed functional studies that identified ZDHHC14 as a novel tumour suppressor.

Materials and methods

Cell lines

Three human TGCT cell lines, 833 K, Susa, and GCT27 (obtained from Jennifer Parrington, John Masters, and Lloyd Kelland, respectively), five human prostate cancer cell lines, PC-3, DU-145, LNCaP, VCaP, and 22RV1 (ATCC, Manassas, VA, USA), and one immortalized human prostate epithelial cell line, PNT2-C2 (obtained from Norman Maitland), and the human embryonic kidney HEK293 (ATCC) and T-REx™ 293 (Invitrogen, Carlsbad, CA, USA) cell lines were used in this study. All cell lines were verified by STR profiling using the ABI AmpF/STR Identifiler kit (Applied Biosystems, Foster City, CA, USA).

Clinical samples

Fresh frozen TGCT and prostate cancer samples were collected and stored in ethically approved tissue banks, and used in this study under local ethics committee approval. Archived paraffin blocks of TGCT and prostate cancer samples were collected for tissue microarray (TMA) studies following approvals obtained from local ethics committees. Written informed consent was received from participants prior to inclusion in the study.

SNP array analysis

A standard phenol/chloroform method was used to extract DNA from cell lines and fresh frozen tissues with the application of micro- or macro-dissection for clinical prostate cancer samples. Affymetrix 10 K SNP arrays (Affymetrix, Santa Clara, CA, USA) were used according to the manufacturer's instructions, with standard quality control steps in the process. Standard Affymetrix criteria for final call rate and signal intensity were applied to define a successful hybridization. Signal intensity data from SNP arrays were analysed using the commercial Partek Genomics Suite (Partek Incorporated, St Louis, MO, USA) and our in-house GOLF (V2.2.10) software [13–15]. SNP array data have been deposited in the Gene Expression Omnibus database (accession number GSE35216).

Quantitative reverse transcription PCR (Q-RT-PCR)

Total RNA was extracted using Trizol (Invitrogen) from cell lines and fresh frozen tissues and for clinical prostate cancer samples micro- or macro-dissection was used to isolate high-purity tumour cells. Standard Q-RT-PCR was performed using pre-designed Taqman® gene expression assays targeting *ZDHHC14* (Hs00257233_m1) and the endogenously expressed *GAPDH* gene (Hs99999905_m1) (Applied Biosystems).

Generation of ZDHHC14 antibody and immunohistochemistry

A polyclonal rabbit antibody targeted to two peptides (H_2N-CGLASQDSLHEDSVRG-COOH and H_2N-CPRATPDEAADLERQID-COOH) of human ZDHHC14 was generated through Eurogentec (Liège, Belgium) using the First Class program. Immunohistochemistry was performed using the Vector ABC kit (Vector Laboratories Inc, Burlingame, CA, USA) and ZDHHC14 antibody, and protein expression scored as previously described [16].

Next-generation sequencing

The genomic locus of *ZDHHC14* (292.42 kb) was amplified by long-range PCR using the Gene Amp XL PCR kit (Applied Biosystems) (with primers listed in Supplementary Table 1). We generated DNA libraries using the 454 GS FLX Titanium General Library kit (Roche, Basel, Switzerland) and sequenced them in a 454 Genome Sequencer FLX Instrument (Roche).

Promoter activity and DNA methylation analysis

HEK293 cells were transfected with the promoter-containing VISA plasmid and the empty VISA plasmid [17], and luciferase activity was measured using the One-Glo Luciferase Assay System (Promega, Madison, WI, USA). Genomic DNA was bisulphite-converted with the Epi-Tect Bisulfite kit (Qiagen, Venlo, The Netherlands). Biotin-labeled primers specific for three regions within the *ZDHHC14* promoter region and one upstream CpG island were used to amplify the bisulphite-converted DNA (see primer sequences in Supplementary Table 4). The amplified DNA was used for pyrosequencing as previously described [18].

Western blotting

Western blotting was performed as previously described [19] with antibodies against ZDHHC14,

cleaved caspase-7 (Asp198), PARP (Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma, St Louis, MO, USA).

Cell viability, proliferation, and apoptosis assays

Cell viability and proliferation were assessed using the CellTiter 96® AQ_{ueous} assay (Promega) following the manufacturer's instructions. 1500 cells were plated in each well of a 96-well plate. The following day, cells were treated with 1 µg/ml tetracycline to induce ZDHHC14 expression and after 72 h, cell viability and proliferation were assessed. Cell cycle distribution was established using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) as previously described [19], analysed using FlowJo 8.8.6 software. Apoptosis assays were performed using an FITC annexin V apoptosis detection kit I (Becton Dickinson) following the manufacturer's instructions.

ZDHHC14 transient overexpression and siRNA knockdown

The 22RV1 cell line was transfected with pcDNA4/TO plasmid (Invitrogen) containing *ZDHHC14* or with the empty plasmid, using the Amaxa Nucleofector Kit V (Lonza, Basel, Switzerland), and the HEK293 cell line with *ZDHHC14* WT or mutated S14 construct, using FuGENE6 (Promega), following the manufacturer's instructions. PNT2-C2 cells were transfected with *ZDHHC14* ON-TARGET plus SMARTpool siRNA (Dharmacon, Lafayette, CO, USA) at 100 nM final concentration using Oligofectamine (Invitrogen) as previously described [19].

ZDHHC14 knockout by zinc finger nucleases

PNT2-C2 cells were transfected with *ZDHHC14* CompoZr™ Knockout Zinc Finger Nucleases (CKOZFN23137-1KT, Sigma) using Lipofectamine and cultured for 3 days. Cells were split and used for the CEL-I surveyor assay (Transgenomic, Omaha, NE, USA) to confirm the action of the ZFNs, or seeded for clonal selection. One hundred clones were expanded and screened for the presence of ZFN-induced deletions by microsatellite analysis, using a 3730xl DNA analyser (Applied Biosystems). Positive clones were sequenced to confirm the deletion.

Colony formation assay

Five hundred cells were plated onto six-well plates and incubated for 1 week in 5% CO_2 at 37 °C, washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet solution. The number of visible colonies was counted.

Xenograft and bioluminescence studies

Male CD1 nude mice (Charles River Limited, UK), 6–8 weeks of age, were used in accordance with the UK Coordination Committee on Cancer Research guidelines and Home Office regulations. Mice were randomly allocated into two groups: one treated with tetracycline and the other treated with vehicle. One week after the treatment started, mice were injected subcutaneously with 2×10^6 293 T-REx™ clone Z14 3 luciferase-tagged cells into their right-side flank region and with the empty plasmid 4/TO luciferase-tagged cells into their left flank. Mice were anaesthetized and bioluminescence imaging was performed after intraperitoneal injection of D-luciferin (Caliper Life Sciences, Hopkinton, MA, USA). The IVIS Vivo-Vision 100 Imaging System (Xenogen, Alameda, CA, USA) was used for bioluminescence imaging recording and post-processing. Quantification was performed using Living Image software 3.2.

Statistics

Statistical tests for data analysis included the chi-squared test and Student's *t*-test, as described in each section. Statistical analyses were performed using the Prism 5.0b (GraphPad, La Jolla, CA, USA) statistical software package. *p* values of 0.05 or less were considered significant.

Full experimental procedures are presented in the Supplementary materials and methods.

Results

Frequent loss of 6q25.3 region affecting *ZDHHC14* in TGCTs

To search for new genomic alterations, and in particular to identify novel putative tumour suppressor genes, we performed SNP array analysis on 36 TGCT clinical samples, including five with case-matched adjacent normal tissues, and three cell lines (833 K, Susa, and GCT27). The samples without case-matched normal tissues and cell lines were normalized against the average of the five normal tissues. We confirmed previously reported frequent genomic alterations such as gain on chromosomes 7, 8, 12p, and X, and loss of 4, 5, 11, and 13 [9]. In addition, there were a number of small regions (<1 mb) with genomic copy number gains or losses that were mostly detected in one or two samples, except a loss of a small genomic region on 6q25.3 that was detected in 48.7% (19/39) of the cases. In most samples, this loss was defined by only two contiguous SNPs (Supplementary Figure 1). In three of five cases with adjacent normal tissues, this 6q25.3 deletion was observed by directly comparing the tumour against case-matched normal tissue (Figure 1), demonstrating somatic genomic alterations. The size of the deletion, based on the two deleted SNPs and the two adjacent non-deleted SNPs, can vary from 274 bp (157970668–157970942) to 275 653 bp (157769581–157985234) based on the GRCh37. This genomic region only contains one

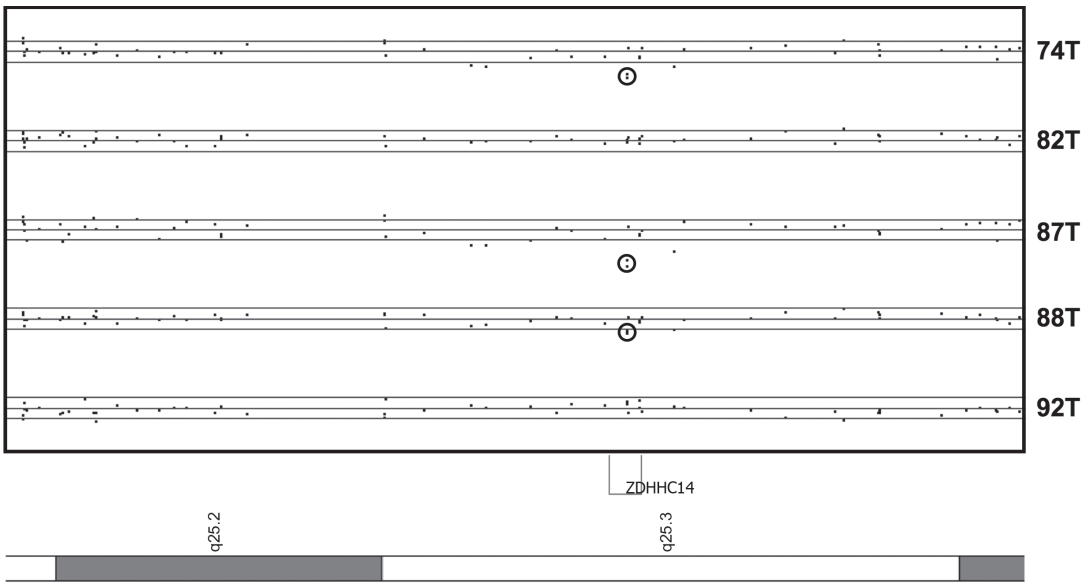


Figure 1. Frequent loss of the 6q25.3 region affecting *ZDHHC14* in TGCTs. Affymetrix 10 K SNP array results of five TGCT samples compared with case-matched normal tissues. The sub-microscopic deletion of 6q25.3 (indicated by circles) was found in three of these cases. In cases 74 T and 88T, as in most of the non-matched cases, the deletion was defined by only two contiguous SNPs. In case 87T, the SNP on the left of the two circled may also be deleted. Each dot represents an SNP and the *ZDHHC14* locus is shown within the box. In each case, the middle line represents a $\log_2$ ratio of 0 for SNP signal intensity; the top line a ratio of 1; and the bottom line a ratio of −1.

known gene, *ZDHHC14*, which encodes a protein with an unknown function (Supplementary Figure 1).

ZDHHC14 expression is commonly down-regulated in TGCTs

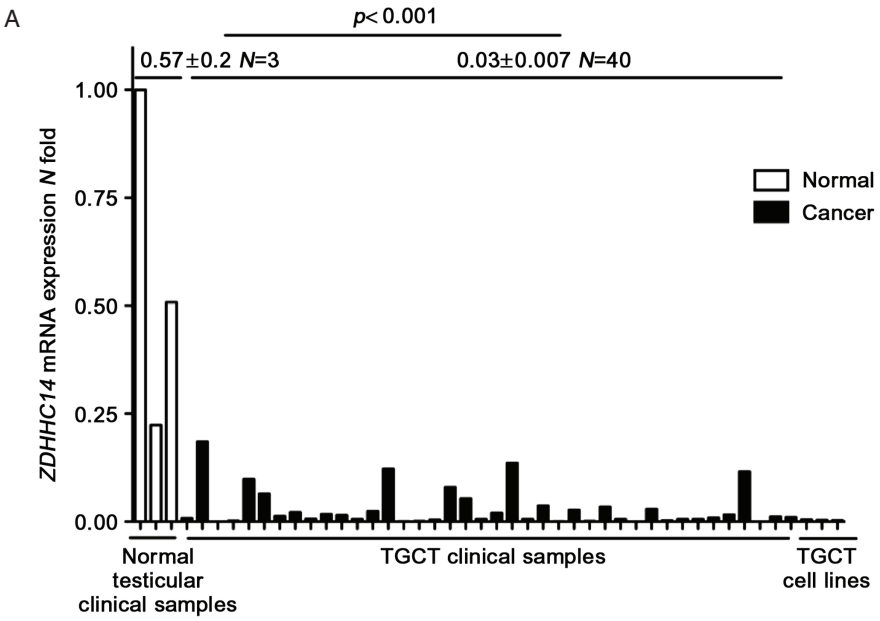
After identifying the *ZDHHC14* deletion, we investigated its consequence on expression of this gene. Firstly, we measured *ZDHHC14* mRNA expression in 40 TGCT clinical samples and three cell lines and found dramatic underexpression when compared with three normal testis tissue samples ($p < 0.0001$) (Figure 2A). We also studied ZDHHC14 protein expression by immunohistochemistry. As no commercial antibody was available, we generated an antibody against human ZDHHC14 and confirmed its specificity by western blot and immunocytochemistry (Supplementary Figures 2A–2C), and demonstrated its efficiency in staining ZDHHC14 in normal germ cells (spermatocytes) (Supplementary Figure 2D). We analysed TMAs comprising 318 cases of TGCTs for ZDHHC14 expression and found that ZDHHC14 was significantly underexpressed compared with morphologically normal seminiferous tubules adjacent to the cancers ($p = 0.0013$). ZDHHC14 levels were divided into three categories: negative, weakly positive, and strongly positive. All normal testicular tissue samples were positive, whereas 21.4% of TGCT samples were negative (Figures 2B and 2C).

ZDHHC14 expression is down-regulated in prostate cancer

Since TSGs often are involved in the genesis of more than one type of malignancy, and the Oncomine database revealed that *ZDHHC14* mRNA was also down-regulated in lymphoma, liposarcoma, brain, kidney, lung, and colorectal cancers (data from the Oncomine database http://www.oncomine.org/resource/login.html), we investigated *ZDHHC14* expression in prostate cancer. We detected a clear down-regulation of *ZDHHC14* mRNA in 38 cancer samples compared with 21 benign prostatic hyperplasia (BPH) samples ($p < 0.0001$). *ZDHHC14* mRNA was also underexpressed in the prostate cancer cell lines PC-3, DU-145, 22RV1, LNCaP, and VCaP (Figure 3A). We next studied ZDHHC14 protein expression by immunohistochemistry, analysing 483 prostate cancer clinical samples, 73 with adjacent BPH or normal prostate glands, made into 11 TMAs and 50 BPH samples from non-cancer cases constructed in one TMA. Within the prostate gland, basal cells were strongly stained. The luminal epithelial cells in all normal and BPH samples were also positively stained. However, only 53% (256/483) of the cancer samples were positively stained ($p < 0.001$; chi-squared test) (Figures 3B and 3C). This result taken with those from the Oncomine database suggests that ZDHHC14 down-regulation is a feature of many human cancers. We re-examined our published SNP array data [15,16] for genomic loss of the *ZDHHC14* region, which was found in five cases from 71 clinical samples and six cell lines.

ZDHHC14 mutations are uncommon and promoter methylation is unaffected in TGCTs and prostate cancer

The presence of mutations within the coding region would provide additional insight into the mechanism



B

	Total	Negative	Weakly positive	Strongly positive	*p* value
TGCTs in TMAs	318	68 (21.4%)	119 (37.4%)	131 (41.2%)	0.0013
Normal testicular tissue in TMAs	43	0	16 (37.2%)	27 (62.8%)	

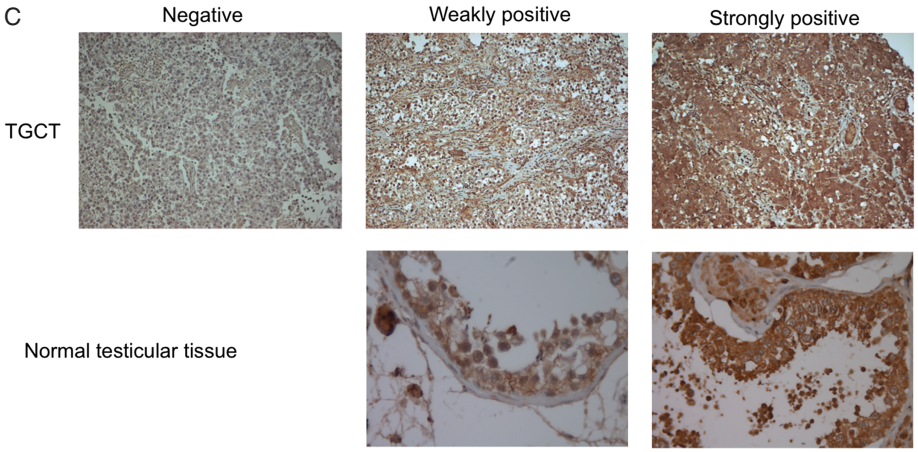


Figure 2. ZDHHC14 expression is commonly down-regulated in TGCTs. (A) Significant *ZDHHC14* mRNA underexpression in 40 primary tumours (black bars) was detected, compared with three normal testis tissues (white bars). *ZDHHC14* down-regulation was also found in three TGCT cell lines (GCT27, Susa, and 833K). Means and SEMs were calculated for both groups and a two-tailed Student's *t*-test was applied. The sample codes, in the same order as the graph, are T115, T113, T108, 9T, 10T, 18T, 29T, 33T, 58T, 66T, 75T, 86T, 90T, 93T, 94T, 105T, 108T, RH4, RH6, RH9, RH11, RH14, RH15, RH16, RH17, RH24, RH26, RH27, RH29, RH36, RH37, RH39, RH42, RH43, RH44, RH45, RH46, RH48, 1532/99, 2805/97, 3425/98, 6534/98, 7942/99, 833K, GCT27 and Susa. (B) ZDHHC14 protein expression was down-regulated in TGCTs compared with non-malignant testis tissue as detected by immunohistochemistry. ZDHHC14 expression levels were divided into three categories: negative, weakly positive, and strongly positive. This table shows the number of cases and percentages in each group and category, which were compared using a two-tailed chi-squared test. (C) Representative images of the three categories of ZDHHC14 expression from TGCTs (200× original magnification) and normal testicular tissues (400× original magnification).

of *ZDHHC14* inactivation. We therefore sequenced the nine exons of *ZDHHC14* in three TGCT clinical samples (29 T, 86 T, and 94 T) and three cell lines (833 K, GCT27, and Susa). We found one heterozygous missense point mutation in exon 4 and a 99-base pair deletion of the beginning of exon 4, corresponding to the DHHC domain, in Susa cells (Table 1). Encouraged by these results, we performed next-generation sequencing (NGS) of the entire *ZDHHC14* gene and the 5′ upstream region containing the putative promoter, in ten DNA pools containing 31 TGCT and two prostate cancer clinical samples and five prostate cancer cell lines (22RV1, DU-145, PC-3, LNCaP, and VCaP) (Supplementary Table 2).

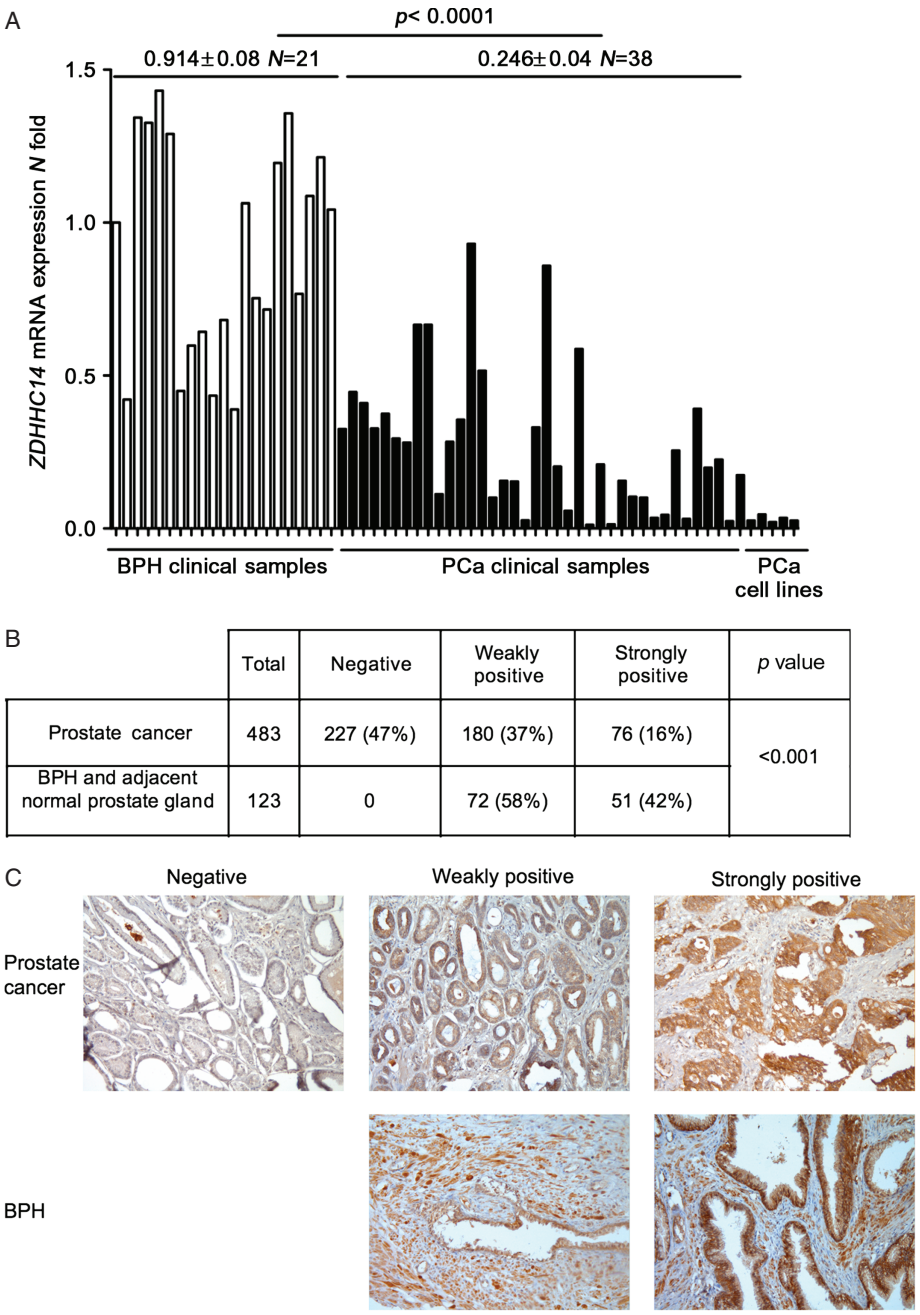


Figure 3. ZDHHC14 expression is commonly down-regulated in prostate cancer. (A) *ZDHHC14* mRNA is significantly underexpressed in 38 prostate cancer (PCa) samples (black bars) compared with 21 BPH samples (white bars). Five prostate cancer cell lines (PC-3, DU-145, 22RV1, LNCaP, and VCaP) also showed down-regulation. Means and SEMs were calculated for both groups and a two-tailed Student's *t*-test was applied. (B) Down-regulated ZDHHC14 protein expression was detected by immunohistochemical analysis in prostate cancer samples compared with BPHs and adjacent normal prostate tissues. This table shows the number of cases and percentages in each group and category, and the two-tailed chi-squared test result ($p < 0.001$). (C) Images corresponding to representative examples from each expression level category in each group (200× original magnification).

The coverage of the *ZDHHC14* gene in the pools ranged from 78% to 92%, with the average depth for each sample from 11 to 145 reads. The quality (Phred score) was above 30 in all the samples (Supplementary Figure 3 and Supplementary Table 3). We detected two missense point mutations in exons 3 and 9 in the prostate cancer cell line pool, none of them annotated as SNPs in the 1000 Genomes database (http://www.1000genomes.org/home). The missense point mutations in exons 3 and 9 were confirmed in the LNCaP cell line by Sanger sequencing (Table 1). Although the missense mutations were scored as damaging using SIFT mutation prediction software (http://sift.jcvi.org/), the finding of mutations solely in cell lines indicates that *ZDHHC14* mutation may not greatly contribute to its inactivation in those two tumour types. We also assessed promoter methylation status as a possible cause leading to ZDHHC14 down-regulation. The promoter region was unmethylated in both non-malignant and malignant prostate and

Table 1. List of the mutations found in the *ZDHHC14* gene

Sample	Method	Gene location	Genomic position	Type of mutation	Size (bp)	NT change	AA change
TGCT Susa cell line	Exon sequencing	Exon 4	158,049,459	Point mutation	1	T > C	L215S
TGCT Susa cell line	Exon sequencing	Exon 4	158,049,380–479	Deletion	99	NA	NA
LNCaP PCa cell line	NGS	Exon 3	158,014,155	Point mutation	1	G > A	C181Y
LNCaP PCa cell line	NGS	Exon 9	158,093,804	Point mutation	1	G > A	A373Y

TGCT = testicular germ cell tumour; PCa = prostate cancer; NGS = next-generation sequencing; NA = not applicable.

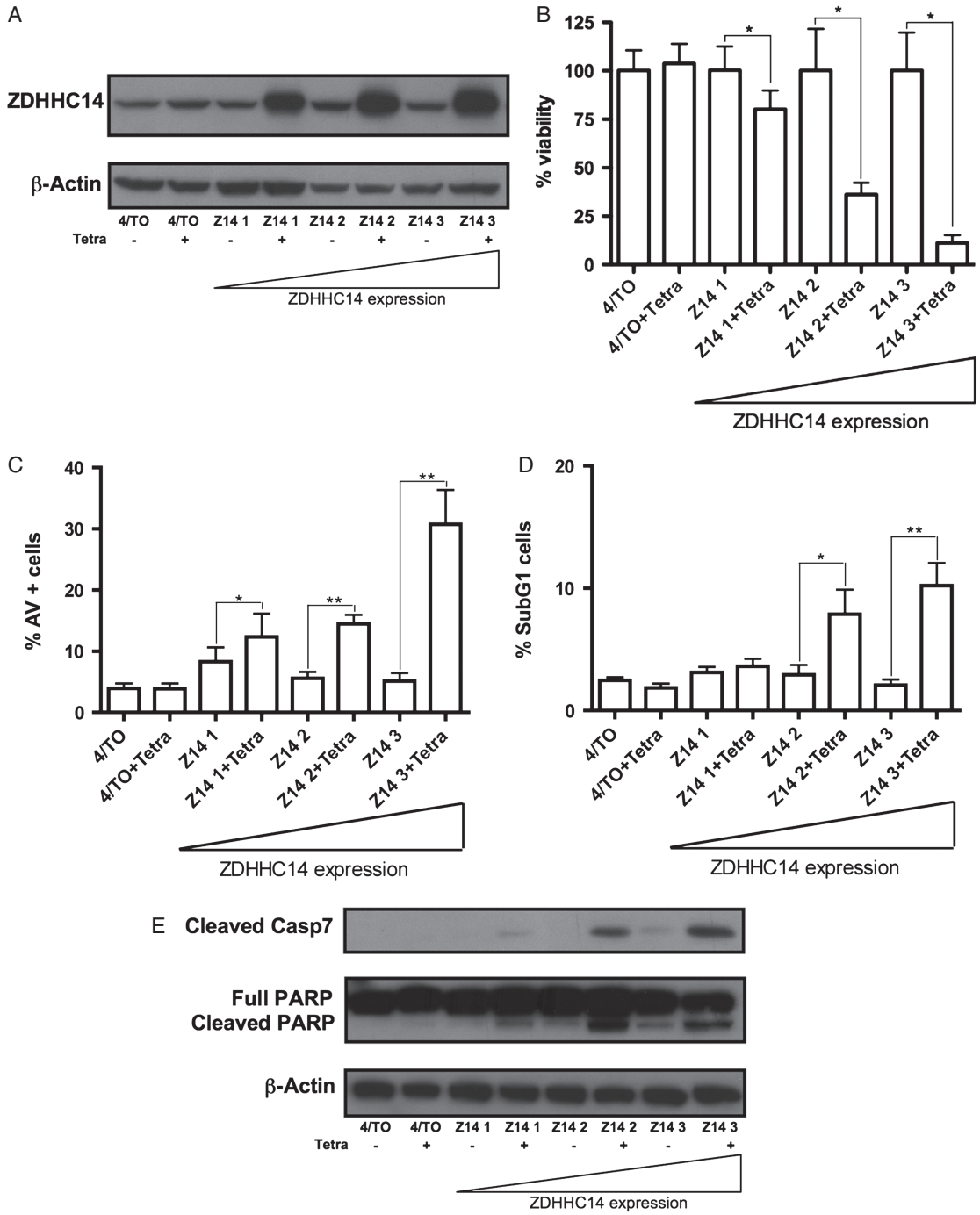


Figure 4. Decreased cell viability by activation of caspase-dependent apoptosis in 293 T-REx ZDHHC14-overexpressing clones. (A) Using 293 T-REx cells, we generated three tetracycline-inducible clones stably overexpressing ZDHHC14 (Z14 1, 2, and 3) and an empty plasmid-expressing clone (4/TO), to be used as a control. Treatment with tetracycline (Tetra) for 72 h induced different ZDHHC14 overexpression levels in the three clones. A representative example of three independent experiments is shown. (B) Decreased cell viability, directly proportional to ZDHHC14 overexpression, was detected in all three clones by MTS assay after 72 h of tetracycline treatment. (C) Apoptosis was quantified by FACS analysis of annexin V-positive cells (AV+) and (D) subG1 cells after 72 h tetracycline treatment. Means and SEMs of triplicates were calculated and a two-tailed Student's *t*-test was applied. (E) Cleavage of the classic apoptotic markers caspase-7 (top row) and PARP (middle row) in ZDHHC14-overexpressing HEK293 T-REx cells. Western blotting was performed after 72 h tetracycline treatment. A representative example of three independent experiments is shown.

testicular clinical samples (Supplementary Figure 4), ruling out promoter hypermethylation as the cause for ZDHHC14 underexpression in prostate cancer and TGCTs.

ZDHHC14 activates a classic caspase-dependent apoptotic pathway

To confirm the tumour suppressor role of ZDHHC14 and also to attempt to elucidate its cellular function, we generated a tetracycline-inducible ZDHHC14-overexpressing stable 293 T-REx cell line and isolated three clones that overexpressed ZDHHC14 at different levels (Figure 4A). Overexpression of ZDHHC14 decreased cell number in all three clones, with this decrease being directly proportional to ZDHHC14 expression levels (Figure 4B). This result was due to ZDHHC14 overexpression inducing apoptosis, as determined by quantification of annexin V-positive cells and sub-G1 apoptotic cells by FACS analysis (Figures 4C and 4D), which were again proportional to ZDHHC14 levels. We also found cleavage of caspase-7 and PARP (Figure 4E), showing that the classic caspase-dependent pathway was involved. We then performed transient overexpression of ZDHHC14 in 22RV1 prostate cancer cells, which led to an increase in sub-G1 apoptotic cells together with the cleavage of caspase-7 and PARP (Figure 5). This result confirms the involvement of ZDHHC14 in inducing apoptosis and supports its role as a tumour suppressor in prostate cancer.

In order to determine if ZDHHC14's apoptotic function was due to its palmitoyltransferase activity, we transiently transfected HEK293 cells with either wild-type ZDHHC14 or a mutated catalytic DHHC domain construct. We detected that the decrease in cell viability and increase in apoptosis were lower in the mutant construct transfected cells than in cells transfected with the wild type, although the mutated construct still had lower viability levels than the empty vector transfected cells, probably due to the inherent toxicity of overexpressing a protein (Supplementary Figure 5). These data demonstrate the involvement of the catalytic DHHC domain in inducing apoptosis.

ZDHHC14 heterozygous deletion increases colony formation

To further confirm the tumour suppressor role of ZDHHC14, we attempted its knockdown by siRNA in several cell lines, obtaining efficient mRNA knockdown, but no decrease at protein level. This was a consequence of the slow turnover rate of ZDHHC14 protein, as determined by persistent detection of ZDHHC14 expression after 6 days' treatment with the protein synthesis inhibitor cycloheximide (Supplementary Figure 6). shRNA stable knockdown was also attempted but without efficient knockdown even at the RNA level. Finally, we used zinc finger nucleases (ZFNs) to knock out *ZDHHC14* in immortalized prostate epithelial PNT2-C2 cells. After screening 100 clones derived from cells transfected with *ZDHHC14* ZFNs, we found a clone with an 11 bp deletion in one of the two alleles. This deletion generates a stop codon immediately after it (Figure 6A). This heterozygous deletion decreased ZDHHC14 protein expression compared with a mock transfected clone and with the parental cells (Figure 6B and Supplementary Figure 2). Colony formation assays showed both more and larger colonies in the *ZDHHC14* heterozygous deleted cells than in parental and mock transfected cells (Figures 6C and 6D), further confirming the tumour suppressor role of *ZDHHC14*.

Increased ZDHHC14 expression suppresses xenograft tumour initiation

To assess the role of ZDHHC14 in tumour growth *in vivo*, we employed xenograft experiments using the 293 T-REx cells described above. We chose clone 3, with the highest ZDHHC14 levels, and the empty plasmid clone as a control. We randomly allocated ten mice into two groups and initiated the treatment by administrating tetracycline in drinking water, or vehicle for the control group. After 7 days, all mice

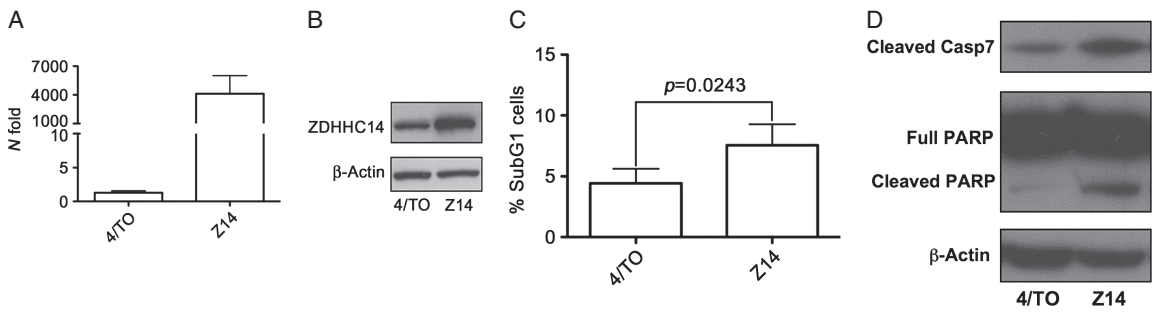


Figure 5. Transient overexpression of ZDHHC14 in the prostate cancer cell line 22RV1 induces caspase-dependent apoptosis. 22RV1 prostate cancer cells were transiently transfected with ZDHHC14 (Z14) or empty plasmid (4/TO) and checked for *ZDHHC14* mRNA expression after 24 h by Q-RT-PCR (A), and for protein expression after 48 h by western blotting (B). (C) Induced apoptosis by ZDHHC14 overexpression shown as an increase of subG1 population by FACS analysis 48 h after transfection. Means and SEMs of triplicates were calculated and a two-tailed Student's *t*-test was applied. (D) Cleavage of caspase-7 (top row) and PARP (middle row) detected 48 h after transfection by western blotting. A representative example is shown. Z14 = ZDHHC14 transfected; 4/TO = empty plasmid transfected cells.

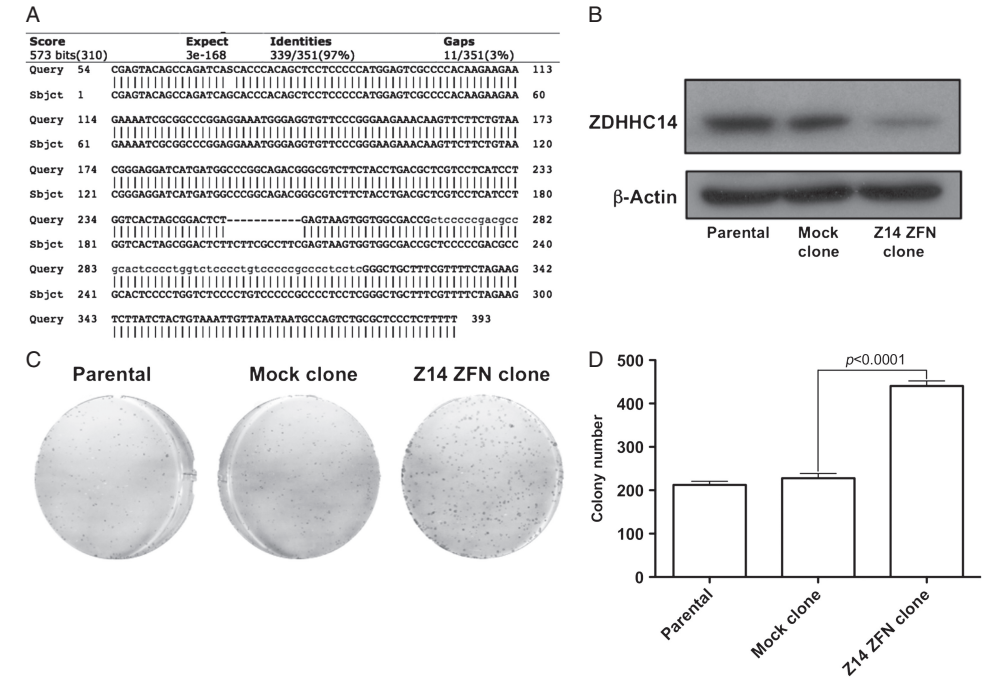


Figure 6. Heterozygous deletion of *ZDHHC14* generated by zinc finger nucleases in the immortalized prostate epithelial cell line PNT2-C2 increases colony formation ability. (A) Sequence alignment of wild-type *ZDHHC14* (Sbjct) and heterozygous deleted clone (Query) showing an 11 bp deletion in exon 1. The T before and the GA after the deletion generate a stop codon (TGA). (B) Western blot showing decreased ZDHHC14 expression in the heterozygous deleted clone (Z14 ZFN) compared with mock transfected clone and the parental cells. (C) Colony formation assay showing increased colony formation, in both numbers and sizes, in the heterozygous deleted clone compared with a mock transfected clone and parental cells. (D) Quantification of colony numbers expressed as means and SEMs of triplicates; a two-tailed paired Student's *t*-test was applied.

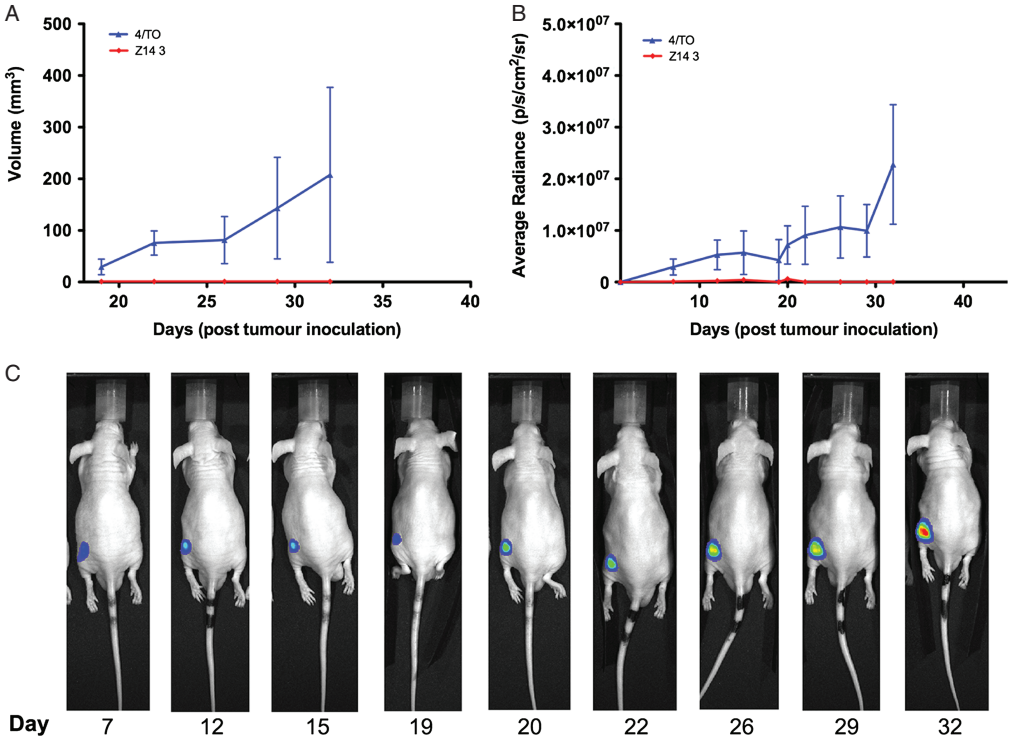


Figure 7. Increased ZDHHC14 expression suppresses xenograft tumour initiation. (A) Growth of xenograft tumours measured by volume from 293 T-REx ZDHHC14 transfected cells (Z14 3) injected into the right flank (red line) and empty plasmid transfected cells (4/TO) injected into the left flank (blue line) of five nude mice treated with tetracycline. (B) Tumour growth curves measured by quantification of the bioluminescence that emanated from the luciferase-tagged 293 T-REx ZDHHC14 or 4/TO cells. (C) Representative bioluminescence imaging of a tetracycline-treated mouse at different dates post-inoculation, showing tumour growth only in the left flank, where empty plasmid transfected cells were injected. Bioluminescence is presented as a pseudo-colour scale: red, highest photon flux; blue, lowest photon flux.

in each group were injected subcutaneously with the *ZDHHC14* plasmid-containing cells into the right flank and with the empty plasmid cells into the left flank, using each mouse as its own control. The presence of tumour cells and tumour growth was checked periodically, showing that tetracycline-dependent ZDHHC14 overexpression completely blocked tumour initiation, since none of these mice developed tumours in the right flank (Figure 7). In contrast, the same cells in the vehicle-treated mice grew to form a tumour in all cases (data not shown). As expected, cells transfected with empty plasmid developed tumours in all the mice in the tetracycline group (Figure 7) and in the vehicle group (data not shown). These results demonstrate that ZDHHC14 has a tumour suppressor role *in vivo*.

Discussion

Tumour suppressor genes play critical roles in preventing tumourigenesis and are frequently inactivated during tumour development and progression. Deletion of 6q has been reported frequently in human tumours and one or more TSGs located within this region have been proposed [20–22]. Using SNP microarrays on TGCTs, we detected a frequent small deletion in chromosome region 6q25.3 containing only one known gene, *ZDHHC14*. This region is also deleted in prostate cancer samples, although at a much lower frequency [15,16]. These data suggest that *ZDHHC14* may be a putative TSG. This hypothesis has been further supported by the dramatic ZDHHC14 down-regulation found in TGCT and prostate cancer. Currently, there are only three studies on ZDHHC14, which paradoxically reported a putative oncogenic role for ZDHHC14 based on its up-regulation in gastric cancer [23], diffuse large B-cell and mantle cell lymphomas [24], and acute biphenotypic leukaemia and subsets of acute myeloid leukaemia [25]. Based on the different substrate specificities of DHHC proteins [26], it is plausible that ZDHHC14 has several substrates and, depending on the cellular type and context, can regulate different pathways. Therefore, ZDHHC14 might play oncogenic roles in some cell types and tumour suppressor roles in others. However, in publicly accessible databases, there is much evidence to support the tumour suppressor role of *ZDHHC14*. *ZDHHC14* is underexpressed in many human cancers, including lymphoma, liposarcoma, and brain, kidney, lung and colorectal cancers (data from the Oncomine database http://www.oncomine.org/resource/login.html). In addition, since the cancer genome sequencing efforts started, 36 mutations affecting the *ZDHHC14* coding region have been described and *ZDHHC14* gene mutation recurrence has been found in colon adenocarcinoma (4/257 samples), lung adenocarcinoma (5/305 samples) (data from the COSMIC database http://www.sanger.ac.uk/genetics/CGP/cosmic/ and the International Cancer Genome Consortium database http://dcc.icgc.org/), lung squamous cell carcinoma (3/178 samples) [27], uterine corpus endometrioid carcinoma (4/240 samples) [28], colorectal cancer (2/72 samples) [29], and skin cutaneous melanoma (4/228 samples) (data from The Cancer Genome Atlas database http://cancergenome.nih.gov/). All these data support *ZDHHC14* as a putative TSG that is commonly affected in many types of human cancer.

The inactivation of a TSG can be achieved by its complete switching off (two-hit mechanism), by haploinsufficiency, or even by its subtle down-regulation (quasi-insufficiency) [30]. In this study, nearly all *ZDHHC14* deletions were heterozygous and in most cases, ZDHHC14 was down-regulated but not completely lost. In addition, the limited number of missense mutations found means that in most of the cases, the other allele may remain functional. These results suggest that haploinsufficiency or quasi-insufficiency is enough to inactivate the tumour suppressor role of ZDHHC14. This mechanism is also supported by our functional studies, which showed a ZDHHC14 dose response effect in suppressing cell growth and activating apoptosis, and an increased colony formation ability by heterozygous knockout.

Regarding the mechanism leading to *ZDHHC14* down-regulation, *ZDHHC14* deletion has a pivotal role in TGCTs. However, some samples without the deletion expressed lower *ZDHHC14* levels than samples with the deletion, indicating that other mechanisms may be involved. In both TGCTs and prostate cancer, our data showed that *ZDHHC14* mutation and DNA methylation may not contribute much to its inactivation. Therefore, other transcriptional or post-transcriptional mechanisms should be the main cause of *ZDHHC14* down-regulation in prostate cancer cases without genomic deletions.

Our final approach to confirm the tumour suppressor role of *ZDHHC14* was to perform functional studies. While its cellular function has not been studied, ZDHHC14 belongs to the DHHC palmitoyltransferase family. It was recently discovered that the main function of DHHC proteins is protein palmitoylation, a reversible post-translational lipid modification that regulates membrane tethering and localization/function of key proteins in cell signalling and membrane trafficking [26,31]. Deregulation of certain palmitoyltransferases has been reported in several diseases including neurological disorders and cancer [32]. Although protein palmitoylation plays an important role in cellular function, it is poorly understood [31,33] and DHHC proteins remain understudied. There are 23 DHHC proteins in humans, of which a small number, including DHHC9 [34,35], HIP14/DHHC17 [36], Aph2/DHHC16 [37,38], and DHHC2 [39–41], have reported roles in cancer. We showed *in vitro* that *ZDHHC14* heterozygous deletion increases colony formation and that overexpression suppresses cell growth by promoting apoptosis through the classic caspase-dependent pathway, which also explains why ZDHHC14 overexpression in xenograft tumours

totally blocks tumour initiation. Our functional analysis using a DHHC domain mutated construct showed that palmitoylation is partially responsible for the pro-apoptotic role of ZDHHC14, which is further supported by the deletion found in exon 4 in Susa cells affecting the DHHC domain. There are also several examples linking palmitoylation and apoptosis [37,38,42–45]. However, further studies are required to fully dissect the molecular mechanism linking ZDHHC14 and caspase-dependent apoptosis.

In summary, we have demonstrated that *ZDHHC14*, a palmitoyltransferase DHHC family gene, is commonly inactivated in human cancers and that it may exert a tumour suppressor role through the induction of apoptosis. This is the first study showing the involvement of ZDHHC14 in a specific pathway, the classic caspase-dependent apoptotic pathway. These data, together with our *in vivo* data showing the blocking of tumour xenograft initiation, demonstrate that *ZDHHC14* is a bona fide tumour suppressor gene.

Acknowledgments

We thank H Schmidt and J Andow for their technical assistance and J Yang and EE Noel for sample preparation. We also thank M-Q Hung for generously supplying the VISA plasmid. This work was supported by the Association of International Cancer Research (AICR) (grant reference 09-0512) and the Orchid.

Author contribution statement

MYV, XM, RG, ATL, RTDO, GMT, JMS, SJM, DMB, BDY, and YJL conceived and designed the experiments. MYV, XM, SCK, DL, TC, LX, MX, JMF, SSK, SYJ, AMC, NV, and DG performed the experiments. MYV, XM, RG, JM, CC, DMB, BDY, and YJL analysed the data. MYV, XM, RG, and YJL wrote the manuscript. All authors were involved in approving the final version of the manuscript prior to submission.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Supplementary materials and methods.

Table S1. List of primers used to amplify *ZDHHC14* genomic locus for next-generation sequencing.

Table S2. DNA pools used for the generation of next-generation sequencing libraries.

Table S3. Next-generation sequencing coverage, depth, and Phred-score of the DNA samples.

Table S4. List of primer sets used to amplify the bisulphite-converted DNA and perform pyrosequencing for DNA methylation analysis.

Figure S1. Affymetrix 10 K SNP array results of all unpaired TGCT samples and three cell lines.

Figure S2. ZDHHC14 antibody specificity.

Figure S3. Next-generation sequencing coverage and depth of the DNA samples.

Figure S4. ZDHHC14 down-regulation in prostate cancer is not caused by promoter hypermethylation.

Figure S5. DHHC domain is involved in apoptosis induction.

Figure S6. ZDHHC14 is a highly stable protein.