The transcription factor FLI1 promotes cancer progression by affecting cell cycle regulation

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Introduction

Dysregulation of cell cycle control resulting in persistent cell proliferation is a hallmark of cancer.1 Altered expression and/or activity of cell cycle regulatory proteins and constitutive activation of pro-proliferative signaling pathways are among mechanisms underlying the sustained proliferative capacity that is frequently encountered in human cancers.2 There are several proteins that regulate cell cycle progression.3 This includes proteins, which interact with cyclin-dependent kinases (CDKs), cyclin-CDK complexes and kinase inhibitors. G1/S transition is a critical step in cell cycle control. In normal cells, regulatory signals are processed at this point.2 As a result of aberrant cell cycle

Binding of transcription factors to mutated DNA sequences is a likely regulator of cancer progression. Noncoding regulatory mutations such as those on the core promoter of the gene encoding human telomerase reverse transcriptase have been shown to affect gene expression in cancer. Using a protein microarray of 667 transcription factor DNA-binding domains and subsequent functional assays, we looked for transcription factors that preferentially bind the mutant hTERT promoter and characterized their downstream effects. One of them, friend leukemia integration 1 (FLI1), which belongs to the E26 transforming-specific family of transcription factors, exhibited particularly strong effects with respect to regulating hTERT expression, while the even better binding ELK3 did not. Depletion of FLI1 decreased expression of the genes for cyclin D1 (CCND1) and E2F transcription factor 2 (E2F2) resulting in a G1/S cell cycle arrest and in consequence a reduction of cell proliferation. FLI1 also affected CMTM7, another gene involved in G1/S transition, although by another process that suggests a balanced regulation of the tumor suppressor gene’s activity via opposing regulation processes. FLI1 expression was found upregulated and correlated with an increase in CCND1 expression in pancreatic cancer and brain tumors. In non-neoplastic lung cells, however, FLI1 depletion led to rapid progression through the cell cycle. This coincides with the fact that FLI1 is downregulated in lung tumors. Taken together, our data indicate a cell cycle regulatory hub involving FLI1, hTERT, CCND1 and E2F2 in a tissue- and context-dependent manner.

J.D.H. and S.L. contributed equally to this work

Additional Supporting Information may be found in the online version of this article.

Key words: hTERT, FLI1, transcription factor, cell cycle, cancer

Abbreviations: BSA: bovine serum albumin; CCND1: cyclin D1; CDKs: cyclin-dependent kinases; cDNA: complementary deoxyribonucleic acid; ChIP: chromatin immunoprecipitation; CRISPR: clustered regularly interspaced short palindromic repeats; cRNA: complementary RNA; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic; ETS: E26 transforming-specific; FLI1: friend leukemia integration; hTERT: human telomerase reverse transcriptase gene; PCR: polymerase chain reaction; PMSF: phenylmethylsulfonylfluoride; qRT-PCR: quantitative real-time polymerase chain reaction; Rb: retinoblastoma; siRNA: small interfering ribonucleic acid; TBS/T: Tris-buffered saline/Tween-20; wt: wild-type

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regulation, “stop” signals are often either attenuated or ignored in cancer, resulting in uncontrolled proliferation.\textsuperscript{4} The retinoblastoma protein (Rb) regulates G1/S transition and thus proliferation by modulating the activity of E2F transcription factors. The formation of a cyclin D1-CDK4/6 complex initiates progression to the S-phase through phosphorylation of Rb.\textsuperscript{5} The phosphorylation leads to a dissociation of the Rb-E2F complex, which allows E2F transcription factors to drive the expression of genes necessary for entry into the S-phase.\textsuperscript{6} The cyclin D1 gene (CCND1) is one of the most commonly amplified genes in human cancers and a downstream target of many oncogenic pathways. Changes in its expression have been reported to have prognostic value.\textsuperscript{7} Overexpression of CCND1 has been associated with the development of multiple cancers including breast cancer, melanoma, prostate cancer, lymphoma, colon cancer and parathyroid adenoma.\textsuperscript{8} Cellular levels of cyclin D1 are regulated by posttranscriptional and posttranslational control but also by means of protein stabilization.\textsuperscript{9}

Aberrant activation of the E26 transforming-specific (ETS) family of transcription factors has been observed throughout different stages of tumorigenesis.\textsuperscript{10} Specifically, in solid tumors, gene amplification, gene rearrangement, the formation of gain-of-function co-regulatory complexes and mutation-mediated creation of novel cis-regulatory elements are all processes by which ETS activation is mediated.\textsuperscript{10} Examples of the last kind of process are the point mutations C228T and C250T in the core promoter region of the human telomerase reverse transcriptase gene (hTERT), which is involved in the regulation of cell cycle progression. The point mutations create novel ETS binding sites.\textsuperscript{11} These mutations are encountered in 21% of medulloblastomas,\textsuperscript{12} 47% of hepatocellular carcinomas,\textsuperscript{13} 66% of bladder urothelial carcinomas,\textsuperscript{14} 71% of melanomas\textsuperscript{15} and 83% of glioblastomas.\textsuperscript{16} It is the most significant single-nucleotide mutation hotspot in noncoding regulatory regions.\textsuperscript{11} Co-existence of such activating mutations together with aberrant expression of potential trans-acting tumorigenic transcription factors may form a cancer-driving cocktail.

hTERT is normally silenced in somatic cells and hence confers a finite lifespan to the cells. However, reactivation of hTERT expression may help cells to achieve limitless proliferative capacities, a major attribute of cancer cells. In glioblastoma, the ETS transcription factors ETV4, ELF1, ETS1 and GABPA have been shown to bind mutant hTERT promoter and reactivate gene expression, which may account for disease aggressiveness.\textsuperscript{17} There is compelling evidence of aberrant expression of at least some members of the ETS family in some cancers and a correlation with outcome has been reported.\textsuperscript{18} The identification of ETS transcription factors that bind and drive hTERT gene expression further substantiates their impact on tumor biology. However, the underlying mechanism is not well understood in many cases. The binding potential of ETS transcription factors to the mutant hTERT promoter and its consequences need to be elucidated further.

We have previously shown that protein microarrays can be made for diverse applications, such as interaction studies, by means of cell-free, in vitro protein expression of frequently functional proteins.\textsuperscript{19–21} Here, we report on the use of protein microarrays for the assessment of preferential binding of 667 transcription factors to the mutant hTERT core promoter. Certain members of the ETS family exhibited a particularly strong preference. We show that Friend leukemia integration 1 (FLI1), a rarely expressed ETS transcription factor,\textsuperscript{22} not only bound to the mutated hTERT promoter preferentially but also activated its expression, while another preferentially binding transcription factor did not affect hTERT expression. In turn, hTERT expression had an effect on genes involved in the control of the cell cycle, such as CCND1, the E2F transcription factor 2 gene (E2F2) and the gene encoding the tumor-suppressive CKLIF-like MARVEL transmembrane domain-containing protein 7 (CMTM7). As a result of this, G1/S transition is influenced, leading to a promotion of cell growth and proliferation.

Materials and Methods

Microarray production

Protein microarrays were produced as previously described\textsuperscript{19–21} with slight modifications. Briefly, cDNA libraries containing either full-length transcription factors or their DNA-binding domains were used as templates for PCR-based generation of protein expression templates. Following spotting of all PCR-products onto nickel-coated epoxy-slides, 2.4 nl of the S0 T7 high-yield cell-free protein expression system (Promega, Mannheim, Germany) were placed on top of each construct and incubated for protein expression in a ventilated incubator.

Microarray screening

Slides were washed for 5 min at room temperature in assay buffer (25 mM Hepes, pH 7.9, 50 mM glutamate potassium salt monohydrate, 8 mM magnesium acetate tetrahydrate;
sterile filtered through a 0.45 μM filter) and then blocked in the same buffer supplemented with 2% bovine serum albumin and 4 μM salmon sperm DNA (blocking buffer). The arrays were again washed and incubated with an equimolar amount of commercially synthesized fluorescently labeled double-stranded mutant and wildtype DNA (Supporting Information Table S1) in a blocking buffer supplemented with 1 mM DTT, 10% glycerol and 1 mM PMSF on the microarrays at 4°C overnight. The microarrays were then washed twice and rinsed with assay buffer and double-distilled water. They were air-dried in a ventilated oven at 30°C for 30 min. Image acquisition was on a Tecan scanner (Tecan, Männedorf, Switzerland); feature extraction was done with GenePix 6.0 Pro (Molecular Devices, Sunnyvale, CA).

**Cell culture**

Different cancer cell lines were used in our study: nonsmall cell lung cancer (NCI-H1299, Research Resource Identifier RRID: CVCL_0060, hTERTwt), breast cancer (MDA-MB-231, RRID: CVCL_0062, hTERT C228T), Merkel-cell carcinoma (MCC26, RRID: CVCL_2585, hTERT C250T), pancreatic ductal adenocarcinoma (MiaPaCa-2, RRID: CVCL_0428, hTERTwt), glioblastoma (U-87MG ATCC, RRID: CVCL_0022, hTERT C228T), HEK293T cells (RRID:CVCL_0063) and a noncancer cell line (MRC5, RRID:CVCL_0440, hTERTwt). MiaPaCa-2 was purchased from ATCC, Manassas, VA. The other cell lines were obtained from the following sources: U87-MG ATTC from Karenvally Somasundaram of the Indian Institute of Science in Bangalore; MDA-MB-231 from Kristen J. Price, MD Anderson Cancer Center in Houston, TX; MCC26 from Weng-Ann Lui, Karolinska Institutet in Solna, Sweden; NCI-H1299 and MRC5 from Holger Sültmann, DKFZ in Heidelberg, Germany; HEK293-T from Andreas Trump, DKFZ in Heidelberg, Germany. All cell lines have been authenticated using SNP or STR profiling within the last 3 years and all experiments were performed with mycoplasma-free cells. The genotype of the hTERT promoter was assessed in all cell lines using the GC RICH PCR system (Sigma-Aldrich, Taufkirchen, Germany) as previously described. The hTERT promoter sequences of each cell line are presented in Supporting Information Figure S1.

**Chromatin immunoprecipitation**

The cell lines NCI-H1299, MCC26, U-87MG ATCC and MRC5 were cultured in 15 cm plates to about 80% confluence. Cells were fixed for 10 min with freshly prepared 1% paraformaldehyde and quenched with freshly prepared 125 mM glycine solution for 5 min. The cells were then washed, scraped and pelleted in a benchtop centrifuge at 4°C for 5 min at 2,000g.

ChIP was performed using the SimpleChIP kit (Cell Signaling Technology, Frankfurt, Germany) following the manufacturer’s protocol using the respective antibody (Supporting Information Table S2); as a control for unspecific binding, a complex mixture of rabbit IgGs was applied. Enrichment of the hTERT promoter was determined by qPCR with the SsoAdvanced Universal SYBR Green Supermix (Biorad, Munich, Germany). The primer sets TERT+47 and TERT-5329 (Supporting Information Table S1) were used as previously described. The percent input method was used for normalization.

**siRNA target gene knockdown**

The siRNA molecules used are listed in Supporting Information Table S3. siRNA gene knockdown was performed with Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) following the manufacturer’s instructions, except that cells were incubated for 7 hr with DNA complexes before being replaced with normal culture medium. After 24 hr growth, the cells were harvested on ice for RNA isolation or protein extraction. Transcript levels were quantified by quantitative PCR using the Fast SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA) with HPTRT as housekeeping gene. All qPCR primers were purchased from Qiagen (Quantitect primer assay; Hilden, Germany).

**Transcriptome profiling**

Transcriptome profiling was performed on the Human Sentrix-12 BeadChip microarrays (Illumina, Munich, Germany) using 500 ng of total RNA as input. The gene expression data were normalized using quantile normalization with R using the function normalize.quantiles of the Bioconductor package “preprocessCore” (Bioconductor Release 3.8).

**Reactome and hallmark enrichment analysis**

To identify gene sets enriched upon knockdown of candidate transcription factors, gene set enrichment analyses were performed using the Broad Institute algorithm as described in the user instructions. The normalized expression signals from the transcriptional profiling formed the basis of the analysis. Gene set permutations were used that exhibited a false discovery rate significance value of 5% or less.

**CRISPR/Cas9-mediated knockout of FLI1**

Two sgRNAs targeting FLI1—cloned into the lentiviral vector pLentiCRISPR.V2—were purchased from GenScript (Piscataway, NJ; Supporting Information Table S3). Plasmids were transfected into MCC26 cells at 70–80% confluence using Lipofectamine LTX and Lipofectamine Plus reagents (Thermo Fisher Scientific). Transfected cells were selected by growth with 1 μg/ml of puromycin for 14 days. Surviving colonies were pooled and used for single-cell sub-cloning. Single-cell clones were expanded and the knockout checked by Sanger sequencing; FLI1 expression was assessed by Western blot.

**Telomerase activity assay**

Telomerase activity was determined with the TRAPEze RT kit (Merck, Darmstadt Germany) following the manufacturer’s protocol. Briefly, proteins were isolated from all cells and quantified. After dilution to a protein concentration of 0.5 μg/μl, 2 μl were mixed with 5 μl of 5xTRAPeze RT reaction mix,
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Immunohistochemistry

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Immunohistochemistry

Tissue slices were dried, fixed in –20°C-cold acetone, washed in 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% BSA (TSB) for 10 min, and then blocked with 0.3% methanol. Slides were again washed and blocked with Powerblock (DCS Innovative, Hamburg, Germany) for 20 min. They were incubated overnight with primary antibody and rinsed with 0.5 M PBS, then washed twice with 0.5 M PBS for 5 min. After another wash with TSB supplemented with 0.05% Tween20, there was incubation with the secondary antibody. The slides were briefly washed with TSB, stained with Hematoxylin (Merck), dehydrated and mounted. Images were acquired with an Axio scanner (Zeiss, Oberkochen, Germany).

Western blotting

Protein lysates were prepared and quantified using the BCA protein assay kit (Pierce, Darmstadt, Germany). The proteins were then blotted onto 0.2 μM nitrocellulose membranes and blocked with 3% milk. Membranes were incubated overnight at 4°C with the target antibodies diluted as shown in Supporting Information Table S2. Immune complexes were detected with the corresponding species-specific, HRP-conjugated secondary antibody (Supporting Information Table S2). Blots were developed by means of ECL western blot substrate (EMD Millipore, Darmstadt, Germany).

Statistical analysis

Unless indicated otherwise, all experiments were done at least three times. The statistical analysis was performed using GraphPad 6 Prism (GraphPad Software, San Diego, CA) and SPSS 24.0 (SPSS, Chicago, IL). All results are presented as the mean ± standard error. Differences were analyzed by two-tailed Student’s t-test. Values of p ≤ 0.05 were considered statistically significant. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

A more detailed experimental workflow is available in the supplementary methods within the supplementary information file.

Data availability

The raw and normalized transcriptional data are accessible at the public database ArrayExpress (ID: Reviewer_E-MTAB-7452; password: wadXxPy5). Other data will be made available upon reasonable request.

Results

ETS transcription factors bind to the mutant hTERT promoter

We generated protein microarrays of the DNA-binding domains of 667 transcription factors as well as full-length molecules of some (Supporting Information Table S4). They have been described in detail before28 and were used for defining binding motifs. Also, it was demonstrated that the full-length transcription factor proteins and their DNA binding domains have a very similar binding behavior. The complete expression of the proteins or the respective binding domains on the microarray was assessed by immunostaining the proteins’ N-terminal 6xHis- and C-terminal V5-tags that were introduced during protein synthesis (Fig. 1a). About 90% of the transcription factors were expressed completely. In some cases, the C-terminal signal intensity was higher than the N-terminal one, although protein synthesis proceeds from the N- to the C-terminus. However, as reported in detail before,19 access of the antibodies to the terminal tags varies due to protein folding and attachment of the proteins to the solid support.

Fluorescently labeled DNA-fragments of 36 bp were synthesized that represented the wildtype promoter sequence or the C228T and C250T variant (Supporting Information Table S1). Using equimolar amounts, we incubated them on the microarrays for the identification of candidate binders (Figs. 1b–1d). While several transcription factors exhibited binding to the 17.6 μl water, 0.4 μl TITANIUM Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France) and analyzed on an Applied Biosystems ViiA7 Real-Time PCR system (Thermo Fisher Scientific).

Luciferase promoter assay

A 474 bp fragment of the hTERT promoter region was amplified from different cell lines using the GC RICH PCR SYSTEM (Sigma-Aldrich) and the primers listed in Supporting Information Table S1. Ligated into pGL3basic using the Quick ligation kit (New England Biolabs, Frankfurt, Germany), it was then transformed into One Shot chemically competent Escherichia coli cells (Invitrogen). Cells were grown to 30–50% confluence before FLI1 knockdown was performed. The cells were grown further for 24 hr and then transfected with the luciferase constructs. Cells were harvested and luciferase activity measured with the Dual-Luciferase Reporter Assay kit (Promega).

Cell viability assay and cell cycle analyses

Cell viability was analyzed using resazurin (Fisher Scientific, Schwerte, Germany) and fluorescence was measured using the FLUOstar Galaxy system (BMG, Ortenberg, Germany). For cell cycle analyses, cells were grown to 70–80% confluence and analyzed by propidium iodide in a Canto two-laser FACS analyzer (BD, Franklin Lakes, NJ). FACS data were analyzed using Modfit LT V10 (Verity Software House, Topsham, ME).

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wildtype and mutated DNA-sequences, only five of them—ELK3, FLI1, ETV5, ETV1 and ZNF358—showed a more than twofold preference compared to the wildtype sequence (Figs. 1e and 1f); all but the last transcription factor belong to the ETS family and are known to recognize the GGAA motif generated upon hTERT promoter mutation.29,30 For the

Figure 1. Identification of transcription factors that bind preferentially to the mutant hTERT promoter. (a) The protein microarray is shown that presents 667 transcription factor DNA binding domains and four transcription factors as full-length proteins. Proteins were immunostained with fluorescently labeled antibodies that target terminal tags. Incubation of a 35 bp fragment resembling (b) the mutated or (c) the wildtype version of the promoter sequence identified specific binding; in (d), a merger of the images is shown. (e) The ratio, resulting from four independent experiments, is presented of the normalized binding signals produced by the mutant and wild-type DNA-fragments. Transcription factors are highlighted in red, which exhibited at least twofold stronger binding to the mutated sequence. (f) The results of few transcription factors are presented in more detail (left panel). In addition, the relative protein level was deduced from the antibody labeling of their terminal tags (right panel). Green and orange bars indicate the signal intensities obtained at the N- and C-termini, respectively. (g) Sequence recognition motifs of the three best binding transcription factors (extracted from Motifdb and JASPR; Refs. 48,49) are depicted in comparison to the binding motif generated by the mutations (leftmost panel). (h) The enrichment of the promoter region (blue) is shown in comparison to the results obtained with an entirely unrelated DNA region located some 5 kb upstream of the promoter and used as an internal control (red). (i) The genotype of the hTERT promoter was confirmed experimentally for the cell lines used in the analyses; three relevant results are shown. Since the complementary strand was sequenced the C228T and C250T conversions are represented by sequence changes from G to A. ns = not significant; ***p ≤ 0.001.

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transcription factors that exhibited less binding to the mutated sequence, the recognition motifs are mostly not known. For ZBTB7A, two motifs are described of which one overlaps with the mutation sequences. In order to control that not largely different protein expression levels influenced the binding behavior, we determined qualitatively the actual protein amount present on the microarrays by analyzing the binding of antibodies to the terminal 6xHis- and V5-tags. Comparable amounts of protein were found to be present. Also, the N- and C-terminal signal intensities were similar (Fig. 1f) indicating mostly full-length protein expression. The ETS transcription factors with the most prominent binding to the mutated sequence have DNA-recognition motifs that are similar to the one in the mutant promoter (Fig. 1g). We performed binding experiments also on microarrays on which 95 transcription factors were present as full-length molecules (Supporting Information Table S5), including FLI1 and ELK3. They exhibited the same binding specificity to the mutated promoter sequence as their DNA binding domains (e.g., Supporting Information Fig. S2a). In addition, we confirmed the binding of DNA-fragments with only one of the two mutations being present, respectively (e.g., Supporting Information Fig. S2b).

Validating the specificity of FLI1 binding to mutant promoter sequence
Although recognition by FLI1 of the GGAA motif generated upon the hTERT promoter mutation had been described,29,30 we performed chromatin immunoprecipitation (ChIP) for confirming the result of the microarray assay. Since the hTERT promoter mutations were observed in different cancers, but could play diverse roles depending on the biological context, we investigated the relevance of FLI1 in cell lines originating from different cancer entities: NCI-H1299 (nonsmall cell lung cancer), MCC26 (Merkel-cell carcinoma), U-87MG ATCC (glioblastoma) and the noncancer lung fibroblast cell line MRC5. The hTERT promoter region of all cells was sequenced, confirming their sequence status (Supporting Information Fig. S1). In the ChIP analysis, quantitative PCR was employed for detecting sequence enrichment (Fig. 1h). As a control, an arbitrarily selected, unrelated DNA segment located some

Figure 2. FLI1- and ELK3-based gene regulation. (a) Quantitative real-time PCR measurements of the hTERT transcript levels were performed in the respective cell line without gene inhibition (red bars) or upon knockdown with siRNAs targeting ELK3 (orange bars) or FLI1 (green bars). (b) For confirmation of the siRNAs’ activity, their effects on the transcript levels of FLI1 or ELK3, respectively, are shown. (c) Heatmap showing the genes that were consistently downregulated upon FLI1 knockdown in the cancer cell lines. Each column represents the results obtained in NCI-H1299, MCC26 and MiaPaCa-2 (left to right). Next to the results with knocking down FLI1, the results with ELK3 knockdown are shown. In addition, variation caused by transfection with an siRNA of scrambled sequence are shown compared to the transcript levels of untreated cells (control). (d) Reactome gene set enrichment analysis based on the microarray data predicted pathways and activities that were significantly enriched in control cells compared to FLI1 knockdown cells. (e) The plot shows predicted pathways and activities that resulted from a similar enrichment analysis on the hallmark gene set. NES, normalized enrichment score; ns, not significant; **p ≤ 0.01; ***p ≤ 0.001.
5,000 bp upstream of the hTERT promoter was used. MRC5, which lacks the relevant mutations, produced a background level signal. It was comparable to that of a negative control, in which a complex mixture of rabbit IgGs isolated from pooled rabbit sera was used in the immunoprecipitation rather than a FLI1-specific antibody. Cell line NCI-H1299 was meant to act as another control, since none of the two hTERT mutations is present in its promoter. However, there was an enrichment of the promoter region well above that of the control sequence. This signal is likely to be a result of FLI1 binding to a native binding motif that has been described to be present in the region. The site is also present in U-87MG ATCC and MCC26. The additional C228T mutation in U-87MG ATCC did not lead to the further enrichment of the core promoter sequence. In contrast, the value nearly tripled with MCC26, which carries the C250T mutation, confirming binding of FLI1 to this sequence motif.

**FLI1 but not ELK3 regulates the expression of hTERT**

We focused further analyses on ELK3 and FLI1 since they exhibited the strongest binding to the mutant promoter sequence. In order to determine if their binding affects the expression of hTERT and in consequence downstream molecules, siRNA-mediated RNA interference was performed on

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**Figure 3. Expression regulation.** (a) The relative luciferase activity is shown after cloning wild-type or mutated promoter sequences from the named cell lines into a construct encoding for the luciferase gene. The luminescence of the expressed protein was measured as an indicator of promoter activity (red bars). Apart from differences between promoters, there was a clear reduction of activity upon FLI1 knockdown (green bars). (b) Telomerase activity measurement in three cancer cell lines (red bars) and cells, in which FLI1 (green bars) or hTERT (blue bars) had been knocked down. (c) Quantitative real-time PCR measurement of the CCND1 transcript levels (upper panel) in control cells (red bars) and cells, in which FLI1 (green bars) or hTERT (blue bars) had been knocked down. The same is shown for E2F2 transcript levels in the lower panel. (d) Quantitative real-time PCR measurement of the CMTM7 transcript levels under conditions as described above. siRNA control stands for using an siRNA of scrambled sequence. ns, not significant; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

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four cell lines—NCI-H1299 (wildtype; wt) and MCC26 (C250T) as well as the pancreatic and breast cancer cell lines MiaPaCa-2 (wt) and MDA-MB-231 (C228T). Subsequently, the transcript levels of hTERT were measured by quantitative RT-PCR. In all four cell lines, FLI1 knockdown resulted in a significant reduction of hTERT transcript levels (Fig. 2a). The effect was weaker in the cell lines with mutations (MCC26 and MDA-MB-231) that exhibit two FLI1 binding sites than in the wildtype cell lines with one binding site (NCI-H1299 and MiaPaCa-2). In contrast, the knockdown of ELK3 did not yield a comparable result. On the contrary, hTERT transcript levels even increased slightly in MCC26 and MiaPaCa-2. This effect was not due to a lacking activity of the siRNA targeting ELK3. Quantitative PCR confirmed that both the siRNA targeting FLI1 and the one acting on ELK3 had a strong knockdown effect (Fig. 2b).

For understanding the genome-wide action spectrum resulting from knocking down FLI1 and ELK3, we performed transcriptome profiling in NCI-H1299, MCC26 and MiaPaCa-2. Transcript levels in cells transfected with a relevant siRNA were compared to those of cells transfected with an unspecified siRNA or scrambled sequence. We looked for significant variations that happened consistently in all three cell lines (Fig. 2c). In accordance with the observation for hTERT, genes whose transcript levels were consistently reduced upon FLI1 knockdown did not show such a result for ELK3 knockdown.

FLI1 knockdown triggered lower transcript levels of relatively few genes, suggesting a rather specific regulative process. A gene set enrichment analysis was performed based on the variations in transcript patterns between control and FLI1-depleted cells. There was a strong enrichment of E2F targets as well as gene sets that are associated with cell cycle control, G1/S transition and S-phase (Figs. 2d and 2e). Therefore, we focused our analysis on FLI1 and postulated that its effect on hTERT expression may regulate cell cycle progression via cell cycle checkpoints.

**Mutation of the hTERT promoter enhances FLI1-driven gene expression**

To analyze the impact of the ETS sites created by the C250T and C228T mutations in the hTERT promoter with regard to FLI1 binding and hTERT expression, we cloned the core promoter region of the tumor cells MiaPaCa-2 (wt), MCC26 (C250T) and MDA-MB-231 (C228T) as well as the noncancer cell line MRC5 into a luciferase reporter vector. The luciferase activity of the promoters with either of the novel ETS sites was significantly higher than the activity in MRC5 and MiaPaCa-2 cells, both of which harbor a wildtype promoter.
This is in line with the stronger affinity of FLI1 to the mutant compared to the wildtype variant. It also shows that FLI1 does not only bind to the mutant hTERT promoter but also to the wildtype promoter (Supporting Information Fig. S1). In all cell lines, there was a marked decrease in luciferase activity upon depletion of endogenous FLI1 (Fig. 3a) confirming the regulative activity of FLI1.

To demonstrate that the influence of FLI1 on hTERT is not only detectable in an artificial system, we studied the enzymatic telomerase activity in NCI-H1299, MCC26 and MDA-MB-231. In all of them, knockdown of FLI1 reduced the activity significantly (Fig. 3b). As expected, however, the effect was less pronounced as a direct knockdown of hTERT.

FLI1 regulates the expression of CCND1

To follow-up our hypothesis of preferential FLI1 binding to the mutated hTERT promoter affecting cell cycle checkpoints, we validated the transcriptional variations of relevant genes identified in the genome-wide analysis using quantitative RT-PCR. The expression levels of both cyclin D1 (CCND1) and transcription factor E2F2 (E2F2) were confirmed to be strongly reduced upon depletion of FLI1. A knockdown of hTERT had very similar effects (Fig. 3c). As expected in the genome-wide profiling, depletion of ELK3 did not change the expression of these genes in the same manner (Supporting Information Fig. S3a). We also checked the effect of a FLI1 and ELK3 knockdown on the expression of the E2F1 gene, which is another member of the E2F transcription factor family to which E2F2 belongs. E2F1 had not been affected in the genome-wide transcriptional profiling. This was confirmed by quantitative RT-PCR; a much less pronounced or even no reduction was observed in response to treatment with siRNA-FLI1 or siRNA-ELK3 (Supporting Information Fig. S3b).

CMTM7, which, like CCND1 and E2F2, is known to influence the G1/S transition and acts as a tumor suppressor, produced different results. As expected on the basis of the transcriptome analysis, knockdown with siRNA-FLI1 resulted in a strong reduction of CMTM7 expression (Fig. 3d), while siRNA-ELK3 had no such effect (Supporting Information Fig. S3c). However, as opposed to the response seen for CCND1 and E2F2, a knockdown of hTERT did not have the same effect. In fact, not only was there no significant change in CMTM7 expression in cell lines NCI-H1299 and MiaPaCa-2, CMTM7 expression increased strongly in MCC26 and MDA-MB-231 upon treatment with siRNA-hTERT. This indicates a regulatory process that is different to the one that CCND1 and E2F2 are involved in.

For confirmation of the results obtained at the RNA level, we also looked at the protein expression of relevant molecules. As predicted from the transcriptional studies, reduced expression of cyclin D1 could be seen after knockdown of FLI1 or...
hTERT (Supporting Information Fig. S4). Knockdown of hTERT led to a slight increase in the expression of FLI1, implying a possible feedback loop between these molecules.

**FLI1 promotes G1/S transition in cancer cells**

Next, we studied if depletion of FLI1 influences cell growth. siRNA-FLI1 reduced cell growth in U-87MG ATCC (C228T) as well as MCC26 (C250T; Fig. 4a). To see if this effect was stable over time, we created a CRISPR/Cas9-mediated knockout of FLI1 in cell line MCC26. Although a rather good knockdown of FLI1 was achieved with siRNA, the inhibitory effect of siRNA could get diluted over longer periods of cell growth; no such dilution is possible with a knockout variant. Several single-cell FLI1-knockout clones were picked and expanded. The correct knockout assembly was confirmed by DNA sequencing and FLI1 expression was assessed on Western blots.

**Figure 6.** Expression of FLI1 in healthy and tumor tissues. (a) Results are presented that were extracted from genome-wide transcriptional studies on pancreatic ductal adenocarcinoma, brain tumors (astrocytoma, glioblastoma, ependymoma and medulloblastoma) as well as nonsmall cell lung cancer in comparison to the transcript levels observed in the respective healthy tissues. ns, not significant; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. (b) Immunohistostaining was performed on healthy and diseased brain and lung tissues. Typical results are shown.
(Fig. 4b). All clones analyzed exhibited the same result with respect to sequence and FLI1 expression. Clone 1 was used for an assay of cell growth that lasted 150 hr. The difference in cell numbers with wild-type sequence and FLI1-knockout was found to be stable over time (Fig. 4c).

Since we found FLI1 to bind both the mutant and wildtype promoter variants, although with a preference for the mutated sequence, we investigated the impact of FLI1 knockdown on cell cycle progression. Irrespective of the cancer type, FLI1 depletion resulted in less cell cycle progression, with an accumulation of cells in the G0/G1 phase and a concomitant decrease in the number of cells in the S-phase. An hTERT knockdown led to an equally strong accumulation of cells in the G0/G1 phase (Fig. 5a). Interestingly, however, in the noncancerous cell line MRC5, a knockdown of FLI1 resulted in a more rapid progression through the cell cycle, with fewer cells accumulating in the G0/G1 phase (about 10%) while there were concomitantly more cells in the S phase (approximately 4%; Fig. 5b). While FLI1 knockdown in cells MCC26 resulted in changes that followed the trend seen for the other cancer cells, the observed changes were not significant. Therefore, we performed an analysis with the FLI1 knockout MCC26 cells in addition, which resulted in a clear increase in cell number in G0/G1 phase from 42.4% to 69.7% (p < 0.05) and a concomitant decrease in S phase from 40.6% to 9.6% (p > 0.05). As before, the G2/M phase was not affected (3.3–3.0%; p > 0.05).

### CCND1 and FLI1 expression in tumors
To confirm that the relationship of FLI1 and CCND1 can be observed also in human tumor tissues rather than merely in cell lines, we performed database mining, extracting transcript expression data for several human cancer entities: pancreatic, brain, lung, colorectal and ovarian tumors as well as melanoma. In pancreatic ductal adenocarcinoma and all forms of brain tumor, except for medulloblastoma, both genes were more expressed in tumor than noncancerous tissue (Fig. 6a). The upregulation of FLI1 is not common to all ETS-family transcription factors. While some are highly expressed in tumors, including molecules such as GABP and ETV1 reported earlier, others were expressed preferentially in healthy pancreas tissue (Supporting Information Fig. S5a). Also, in medulloblastoma, the expression of FLI1 and CCND1 correlated, but there was no expression difference between samples from healthy individuals and cancer patients.

All other tumor samples analyzed showed profiles different from pancreatic and brain tumors and different from each other. In addition, they lack direct co-expression between FLI1 and CCND1. Nonsmall cell lung cancer exhibited lower expression of FLI1 in tumor than noncancerous tissues, while no difference could be observed for CCND1. In melanoma, there was higher expression of FLI1 in tumor than in noncancerous tissue. Contrary to pancreatic and brain tumors, however, CCND1 expression was lower in tumor compared to healthy tissue (Supporting Information Fig. S5b). Colorectal cancer samples yielded a pattern inverse to that of melanoma, with less FLI1 but more CCND1 expression in tumor. In ovarian cancer, finally, there was no change in the expression of FLI1, but CCND1 was upregulated in tumor (Supporting Information Fig. S5b).

Complementing the transcriptional profiles, we performed immunohistochemical staining of FLI1 in tissues from patients with glioblastoma and nonsmall cell lung cancer. Consistent with the transcriptome data, we found high nuclear expression of FLI1 in glioblastoma and basal levels in healthy tissues (Fig. 6b). Also, the results on nonsmall cell lung tumors were in concordance with the transcriptome data; there was higher nuclear expression of FLI1 in normal lung than tumor tissue. In both lung tumor and adjacent healthy tissue, we observed cytoplasmic staining of FLI1, which was not detected in glioblastoma.

### Discussion
Given the size of the human genome, still relatively few cases are identified in which somatic mutations in noncoding regions create cis-acting regulatory elements. This illustrates the fact that the current understanding of the genetic background of relevant diseases is likely to be far from complete. There are tens of thousands of possible enhancers, for example, which are located either within introns or at intergenic sites located kilobases away from their target genes. Mutations in such regulative regions could have profound consequences on health matters. Here, we looked at two known hotspot mutations in the core region of the hTERT promoter that create novel transcription factor binding sites. Using a microarray exhibiting transcription factors, we screened for molecules that bind preferentially the mutant hTERT promoter. The observed binding selectivity supported earlier observations that a substantial proportion of arrayed proteins exhibit proper functionality although being produced in situ by cell-free protein expression. Several transcription factors were detected that preferred the mutated sequence. Mere binding, however, proved insufficient for triggering gene activity. The transcription factor ELK3, for instance, showed similarly specific binding to the mutated hTERT promoter as FLI1 but did not have a comparable effect on the gene’s expression. Since formation of a multi-protein complex is the next step toward activating transcription, differences between ELK3 and FLI1 at this level are probably responsible for this.

Inhibition of FLI1 expression or induced reduction of hTERT expression led to much lower transcript levels of both CCND1 and E2F2, suggesting that the two latter genes are affected by hTERT expression, which in turn varies according to FLI1 binding and therefore the presence of the hotspot mutations. Inhibition of the EWSR1/FLI1 fusion in Ewing sarcoma has equally been shown to reduce CCND1 expression, pointing to the fact that the FLI1-domain could be responsible for the oncogenic activity of the EWSR1/FLI1 fusion. There has also been a report about the regulation of CCND1 by
hTERT expression variation.\textsuperscript{37} Cyclin D1 and E2F2 are part of well-defined molecular processes involved in the regulation of the cell cycle\textsuperscript{38} by influencing G1/S transition during cell cycle progression.\textsuperscript{39} Cyclin D1 forms a complex with CDK4 leading to phosphorylation of the retinoblastoma protein Rb. Rb is a negative regulator of cell cycle progression by sequestering members of the E2F transcription factor family and thereby preventing the expression of genes necessary for entry into the S-phase.\textsuperscript{40,41} Upon Rb phosphorylation, E2F2 and other transcription factors are released and their nuclear translocation leads to cell cycle progression.\textsuperscript{42} As expected, siRNA knockdown of FLI1 and hTERT led to cell cycle arrest in the G1/S phase irrespective of hTERT promoter mutation. This is in line with a recent report about the fact that depletion of a FLI1 fusion protein affects cell cycle regulators in Ewing sarcoma.\textsuperscript{36} It is also possible that FLI1 modulates the transcriptional activity of CCND1 directly by affecting its elongation.\textsuperscript{43}

There was a positive correlating co-expression between FLI1 and CCND1 expression in pancreatic and brain tumors. In other cancer entities, expression varied indicating a tissue-specific control of the process. Interestingly, pancreatic ductal adenocarcinoma and glioblastoma belong to the most aggressive forms of cancer.\textsuperscript{44} A reduction of FLI1 expression resulted in a reduction of hTERT and CCND1 and E2F2 transcript levels and less proliferation. Similar observations had already been made for CCND1 in astrocytoma.\textsuperscript{36,45,46} Strikingly, however, FLI1 knockdown in a normal lung cell line led to rapid progression through the cell cycle. Also, we found lower expression of FLI1 in lung cancer tumors compared to healthy lung tissue. This could indicate that downregulation of FLI1 in the lung may be necessary for tumor progression. This corroborates previous reports about an anti-proliferative role of FLI1 in breast cancer\textsuperscript{47} and further highlights the protein’s apparently tissue- and context-dependent function. In addition, FLI1 may not be the unique driver of CCND1 expression in all human cancers and also cyclins other than cyclin D1 may play a role in G1/S transition in some cancer entities. Next to regulating CCND1 and E2F2, FLI1 also affected CMTM7, another gene involved in G1/S transition. Of interest is the observation of direct co-expression of FLI1 and CMTM7 expression on the one hand and inverse correlation of hTERT and CMTM7 expression on the other. This suggests a balanced regulation of the activity of the CMTM7 tumor suppressor gene via opposing processes. There are eight potential FLI1 binding sites in the CMTM7 promoter region. Upon FLI1 binding, CMTM7 expression triggers suppression of proliferation. However, this could be overridden by the simultaneous FLI1 binding to hTERT. With a FLI1 binding motif in the non-mutated hTERT promoter, comparably limited hTERT expression would be responsible for only a slight downregulation of CMTM7. If the C228T or C250T mutations are present, however, the better binding of FLI1 increases hTERT expression. In turn, CMTM7 gets more strongly downregulated by hTERT, possibly offsetting the direct control of CMTM7 by FLI1.

The expression and cellular localization of FLI1 is clearly tissue-dependent. No FLI1 expression is seen in healthy brain tissue, for example, in which the ETS transcription factor GABPA is known to regulate hTERT expression. In glioblastoma, FLI1 expression is exclusively nuclear and may therefore be driving transcriptional processes. While FLI1 expression is also mostly nuclear in healthy lung tissue, it becomes cytoplasmic in lung tumors. These observations suggest that different ETS transcription factors may regulate hTERT expression in different tissues and disease states.

Conclusion

Our data clearly indicates the strong effect of transcription factor FLI1 on cell cycle control and particularly G1/S translation. Also, the human telomerase reverse transcriptase was found to play a role in the regulation, in addition to or as a consequence of its enzymatic activity, which is crucial for highly proliferating tumor cells. Apparently, there are several processes involved in all this. This finding fits the assumption that the net result of several regulative pathways is responsible for the control of proliferation, thus permitting fine-tuning of the process. Further studies are required in order to identify and elucidate the entire regulative network for more targeted interference.

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Conflict of interest

The authors disclose no potential conflicts of interest.

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