

Role of ZNF143 in tumor growth through transcriptional regulation of DNA replication and cell-cycle-associated genes

Hiroto Izumi,¹ Tetsuro Wakasugi,² Shohei Shimajiri,³ Akihide Tanimoto,⁴ Yasuyuki Sasaguri,³ Eiji Kashiwagi,¹ Yoshihiro Yasuniwa,¹ Masaki Akiyama,¹ Bin Han,¹ Ying Wu,¹ Takeshi Uchiumi,⁵ Tokuzo Arao,⁶ Kazuto Nishio,⁶ Ryuta Yamazaki⁷ and Kimitoshi Kohno^{1,8}

Departments of ¹Molecular Biology, ²Otorhinolaryngology and ³Pathology and Cell Biology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu; ⁴Department of Tumor Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima; ⁵Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka; ⁶Department of Genome Biology, Kinki University School of Medicine, Osaka-Sayama, Osaka; ⁷Yakult Central Institute for Microbiological Research, Tokyo, Japan

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The cell cycle is strictly regulated by numerous mechanisms to ensure cell division. The transcriptional regulation of cell-cycle-related genes is poorly understood, with the exception of the E2F family that governs the cell cycle. Here, we show that a transcription factor, zinc finger protein 143 (ZNF143), positively regulates many cell-cycle-associated genes and is highly expressed in multiple solid tumors. RNA-interference (RNAi)-mediated knockdown of ZNF143 showed that expression of 152 genes was downregulated in human prostate cancer PC3 cells. Among these ZNF143 targets, 41 genes (27%) were associated with cell cycle and DNA replication including cell division cycle 6 homolog (CDC6), polo-like kinase 1 (PLK1) and minichromosome maintenance complex component (MCM) DNA replication proteins. Furthermore, RNAi of ZNF143 induced apoptosis following G2/M cell cycle arrest. Cell growth of 10 lung cancer cell lines was significantly correlated with cellular expression of ZNF143. Our data suggest that ZNF143 might be a master regulator of the cell cycle. Our findings also indicate that ZNF143 is a member of the growing list of non-oncogenes that are promising cancer drug targets. (*Cancer Sci* 2010; 101: 2538–2545)

Transcriptional regulation of gene expression requires the orchestrated recruitment of transcription factors by sequence-specific DNA binding regulators. Staf was initially identified as the transcriptional activator of the RNA polymerase III-dependent *Xenopus* tRNA gene.⁽¹⁾ It has recently been shown that its human ortholog zinc finger protein 143 (ZNF143; formerly known as hStaf) is also involved in RNA-polymerase-II-dependent gene transcription.^(2,3) The DNA binding domain of ZNF143 is located in the central part of the protein and consists of seven zinc finger domains.^(1,4) The ZNF143 DNA binding site is thought to be at least 18 bp long because the protein has seven zinc fingers; by analogy, the well-known zinc finger transcription factor Sp family and members of the KLF family contain three zinc fingers and recognize DNA sequences approximately 6 bp long.⁽⁵⁾ Recently, it has been shown by a bioinformatics approach that ZNF143 binding sites are widely distributed in the CpG island-type promoters of the human genome.⁽⁶⁾ Functional classification of ZNF143 target genes has revealed that many of the identified genes are important for cell growth: 27% of the genes are categorized as cell cycle/DNA replication/DNA repair proteins.

We have previously reported that expression of ZNF143 is induced by DNA-damaging agents and is enhanced in cisplatin-resistant cell lines.⁽⁷⁾ ZNF143 binds preferentially to cisplatin-

modified DNA. We also found that ZNF143 binding sites are frequently found in the promoter region of DNA repair genes.

Here, we investigated the ZNF143 target genes by RNA interference (RNAi). One hundred and fifty-two genes were downregulated by ZNF143-specific small interfering RNA (siRNA) transfection. Among them, 41 genes are categorized as concerned with cell cycle and DNA replication. ZNF143 was highly expressed in cancer cells when compared with non-tumor regions. Downregulation of ZNF143 effectively induced G2/M cell cycle arrest and apoptosis, indicating that ZNF143 might be a promising molecular target for anti-cancer drugs.

Materials and Methods

Cell culture and antibodies. Human prostate cancer cells PC3,⁽⁷⁾ human cervical cancer cells HeLa⁽⁷⁾ and human bladder cancer cells T24⁽⁸⁾ were cultured in minimum essential medium (MEM). Human colon cancer cells Caco-2⁽⁹⁾ and DLD1,⁽¹⁰⁾ human glioblastoma CCF-STTG1⁽¹¹⁾ and human astrocytoma cells U343⁽¹¹⁾ were cultured in Dulbecco's modified Eagle's minimal essential medium. Human lung cancer cells A549,⁽¹²⁾ B203L,⁽¹²⁾ PC9,⁽¹²⁾ A110L,⁽¹²⁾ QG56,⁽¹²⁾ SQ1,⁽¹²⁾ B1203L,⁽¹²⁾ PC10,⁽¹²⁾ 904L⁽¹²⁾ and A529L⁽¹²⁾ were cultured in RPMI 1640 medium. These media were purchased from Nissui Seiyaku (Tokyo, Japan) and contained 10% fetal bovine serum. Cell lines were maintained in a 5% CO₂ atmosphere at 37°C. Antibodies against peroxiredoxin 4 (PRDX4) (sc-23974), minichromosome maintenance complex component 2 (MCM2) (sc-9839), MCM3 (sc-9850), MCM4 (sc-28317), MCM5 (sc-22780) and MCM6 (sc-9843) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Flag (M2) and β-actin (A5441) were purchased from Sigma Aldrich (St Louis, MO, USA). Antibodies against aurora B kinase (AURKB) (1788-1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (2073-1) were purchased from Epitomics (Burlingame, CA, USA). Antibodies against PLK1 (37-7000) and CDC6 (05-550) were purchased from Invitrogen (Carlsbad, CA, USA) and Upstate Biotech (Lake Placid, NY, USA), respectively. The polyclonal antibody against the high mobility group B2 (HMGB2) was described previously.⁽¹⁵⁾ The polyclonal antibody against ZNF143 was raised by multiple immunizations of a New Zealand white rabbit with synthetic peptides. The synthetic peptide sequences were MLLAQINRDSQGMTEFPGGGMEAQHVTLTLC and QLGEQPSLEEAIIRIASRIQQGETPGLDD for ZNF143.

⁸To whom correspondence should be addressed.
E-mail: k-kohno@med.uoeh-u.ac.jp

Plasmid construction. To prepare luciferase (luc) reporter plasmids, genomic DNA was amplified with the following primer pairs: CDC6 luc1, 5'-AGATCTCGCTCCAGCGTGTGTCGG-3' and 5'-AAGCTTACCCAGCTCCGCTGCCTCAC-3'; CDC6 luc2, 5'-AGATCTACTACGCCTCCAGCGTGTGTCGG-3' and 5'-AAGCTTACCCAGCTCCGCTGCCTCAC-3'; PLK1 luc, 5'-AGATCTCCGCATCCACGCCGGTTTGG-3' and 5'-AAGCTTGGCTGCAGACCTCGATCCGAGC-3'; AURKB luc, 5'-AGATCTCACTGGGGGAATTTGGGAAAC-3' and 5'-AAGCTTGGGGTCCAAGGCACTGCTACTC-3'; MCM2 luc, 5'-AGATCTCGAACTCTGAGCTTGATCC-3' and 5'-AAGCTTCCGCCACTACAGCAACAACC-3'; and HMGB2 luc, 5'-AGATCTGGGCGGCGCACGGGAGACCC-3' and 5'-AAGCTTCCCGCAGAGCGGCCGACCC-3'. Restriction enzyme sites are underlined. These PCR products were cloned and ligated into the *Bgl*III-*Hind*III site of the pGL3-basic vector (Promega, Madison, WI, USA). The pGL3-P2 (promoter vector) containing an SV40 promoter upstream of the luciferase gene was purchased from Promega. Preparation of 3xFlag-ZNF143 expression plasmid was described previously.⁽⁷⁾

Knockdown with siRNA. The following double-stranded RNA 25-bp oligonucleotides were commercially generated (Invitrogen): *ZNF143* siRNA, *siZNF #1*, 5'-GCTGGAAGATGGTACACAGCTTAT-3' (sense) and 5'-ATAAGCTGTGGTACCATCTTCCAGC-3' (antisense); and *siZNF #2*, 5'-GGACGACGTTGTTTCTACACAAGTA-3' (sense) and 5'-TACTTGTGTAGAAACAACGTCGTC-3' (antisense). Two hundred picomoles of siRNA were mixed with 10 μ L Lipofectamine 2000 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After 20 min, 1×10^6 cancer cells were gently mixed and incubated for a further 20 min. Transfected cells were used for oligonucleotide microarray study, cell proliferation assays, flow cytometry and western blotting.

Oligonucleotide microarray study and microarray analysis. A microarray procedure was performed as described previously.⁽¹⁴⁾ In brief, total RNA extracts were collected from PC3 cells transfected with ZNF143 #2 siRNA or control siRNA in duplicate, as described above. Eight GeneChips (Affymetrix, Santa Clara, CA, USA) were used for analysis. The microarray analysis was performed using the BRB Array Tools software ver. 3.3.0 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) developed by Dr Richard Simon and Amy Peng.

Cell proliferation assays. The cell proliferation assay was described previously.⁽¹⁵⁾ Briefly, siRNA-transfected cancer cells were seeded into 12-well plates at a density of 5×10^4 cells per well. Twenty-four hours after transfection was set as time zero. The cells were harvested by trypsinization and counted every 24 h with a Coulter-type cell size analyzer (CDA-500; Sysmex Corp., Kobe, Japan).

Flow cytometry. Flow cytometry was described previously.⁽¹⁵⁾ Briefly, siRNA-transfected PC3 cells (1×10^6) were seeded into 90-mm plates. After 72 h, the cells were analyzed using an EpicsXL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA). For assessment of apoptosis, an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit II (BD Biosciences, San Jose, CA, USA) was used.

Western blotting. Transfection of expression plasmid and preparation of nuclear proteins was described previously.⁽⁷⁾ ZNF143 expression plasmid- or siRNA-transfected PC3 cells were collected after 48–72 h and nuclear protein or whole cell lysate was subjected to western blotting. Detection was performed using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). The protein expression levels were quantitated using a Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP) assays. The cloning of PC3 cells stably expressing 3xFlag-tagged ZNF143 has been described previously.⁽⁷⁾ Purified DNA from these cells was used

for PCR analysis with the indicated primer pairs (Table S1). The PCR products were separated by electrophoresis on 2% agarose gels and were stained with ethidium bromide.

Reporter assays. One $\times 10^5$ PC3 cells per well were seeded into 12-well plates. The following day, 1 μ g luciferase reporter plasmid was transfected with 30 pmol *ZNF143* siRNA or control siRNA using 5 μ L Lipofectamine 2000 (Qiagen). After 48 h, luciferase activity was detected using a Picagene kit (Toyo Ink, Tokyo, Japan). Light intensity was measured using a luminometer (Luminescencer JNII RAB-2300; Atto, Japan). The results shown are normalized for protein concentration measured using the Bradford method, and are representative of at least three independent experiments.

Human tissue samples. Samples were obtained from the surgical pathology archive of the Department of Pathology and Cell Biology of The University of Occupational and Environmental Health in Kitakyushu, Japan. The selected tissues consisted of one surgically resected case each of esophageal carcinoma, gastric adenocarcinoma, squamous cell carcinoma of the lung, urothelial carcinoma of the urinary bladder, testicular seminoma and cerebral astrocytoma. These cases were classified according to the World Health Organization Histological Typing of each tissue. The diagnosis was re-evaluated and confirmed by at least three board-certified surgical pathologists who had examined formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin or other appropriate immunohistochemical stains.

Immunohistochemistry. The specimens were fixed in 20% formaldehyde and embedded in paraffin. Four micrometer-thick tissue sections were deparaffinized, dehydrated with graded xylene and alcohol, and incubated in 3% hydrogen peroxide for 5 min at room temperature to eliminate endogenous peroxidase activity. Antigen retrieval for the anti-ZNF143 antibody was performed with 0.1 M citrate buffer (pH 6.0) in an autoclave for 15 min. The sections were then incubated with anti-ZNF143 antibody (dilution ratio 1:200). The Envision plus system (Dako, Carpinteria, CA, USA) was used for antibody-bridge labeling, with hematoxylin counterstaining.

Statistical analysis. Pearson's correlation was used for statistical analysis, and significance was set at the 5% level.

Results

ZNF143 expression is required for cancer cell growth. We estimated specificity of both ZNF143 antibody and *ZNF143* siRNA. ZNF143-specific antibody recognized both endogenous 90 kDa protein and exogenous 3xFlag-ZNF143 protein (Fig. 1A). Endogenous ZNF143 protein was completely abolished with treatment of specific siRNA against ZNF143 (Fig. 1B). An *in silico* genome-wide screen for ZNF143 binding sites suggested that many of the target genes are involved in cell growth.⁽⁶⁾ To confirm these results, we first investigated whether *ZNF143* expression is required for cancer cell growth. At first, downregulation of *ZNF143* expression effectively reduced cell growth in PC3 cells (Fig. 2A). Analysis of cell cycle profiles was performed on human prostate cancer PC3 cells after transfection with two different *ZNF143*-specific siRNA. A marked increase in G2/M phase cells was observed when cells were transfected with *ZNF143* siRNA (Fig. 2B,C). In addition, knockdown of *ZNF143* expression induced apoptosis, as shown by an increased percentage of cells in the subG1 population (~5%), whereas only 1% of cells treated with control siRNA was in the subG1 population (Fig. 2C). To extend these results, the cells were stained with an anti-Annexin V-FITC antibody. Treatment with control siRNA did not induce apoptosis, whereas knockdown of *ZNF143* expression resulted in an increase in the percentage of annexin V positive cells (Fig. 2D).

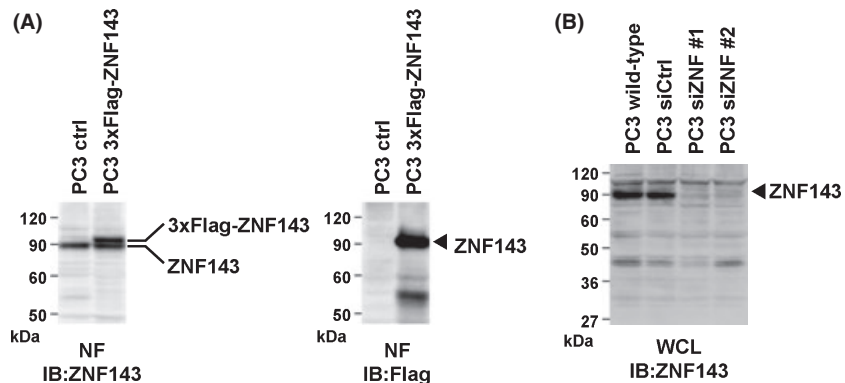


Fig. 1. Specificity of anti-zinc finger protein 143 (ZNF143) antibody and *ZNF143* small interfering RNA (siRNA). (A) PC3 cells were transfected with or without 3xFlag-ZNF143 and nuclear proteins (50 μ g) were used for western blotting with anti-ZNF143 and anti-Flag antibodies. (B) PC3 cells were transfected with or without *ZNF143* siRNA and whole cell lysate (100 μ g) were used for western blotting with anti-ZNF143 antibody. siZNF #1 and #2 indicate *ZNF143* siRNA #1 and #2, respectively.

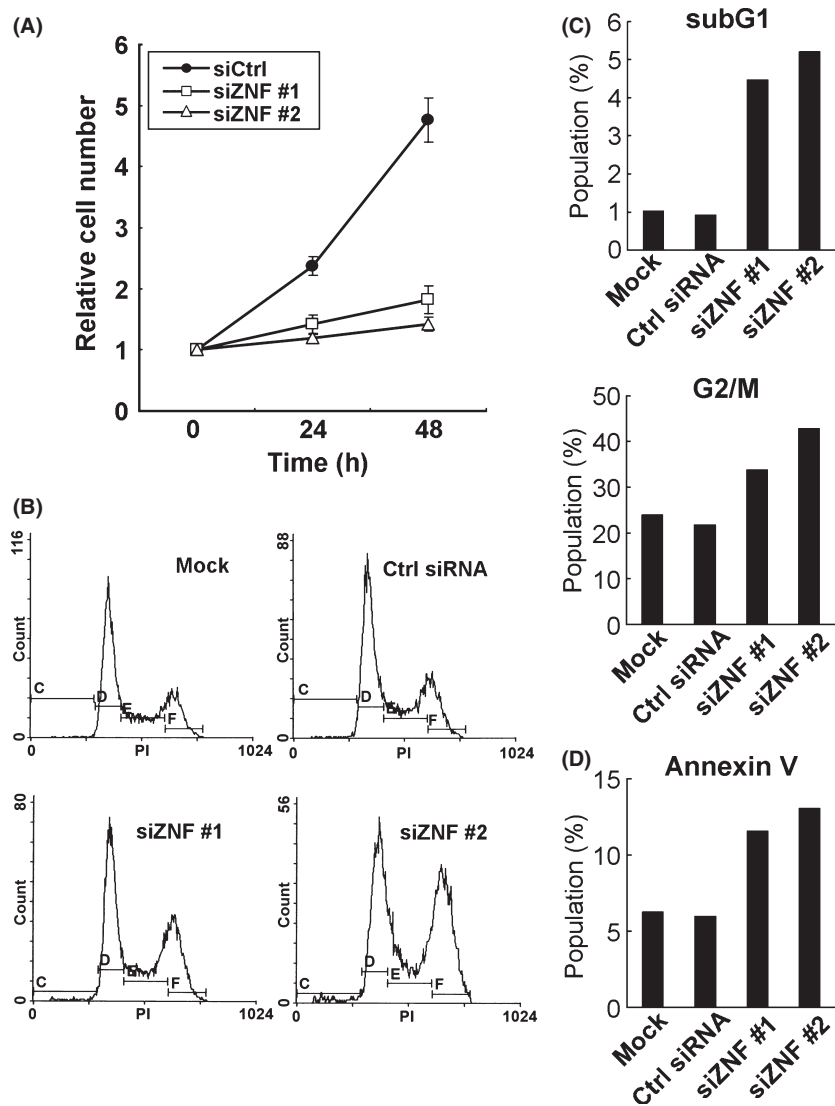


Fig. 2. Knockdown of *ZNF143* induces G2/M arrest. (A) PC3 cells were transfected with control siRNA (siCtrl), *ZNF143* siRNA #1 (siZNF #1) or *ZNF143* siRNA #2 (siZNF #2). Cells were counted after the indicated times, with time zero being 24 h after transfection. The results were normalized to cell numbers at time zero. The points represent the mean of at least three independent experiments; the bars show the SD. (B) PC3 cells were transfected with or without the indicated siRNA. Cells were harvested after 72 h and used for propidium iodide (PI) staining. DNA content in single cells was measured. siZNF #1 and #2 indicate *ZNF143* siRNAs #1 and #2, respectively. (C) SubG1 and G2/M populations were calculated from the results of part (B). (D) siRNA-transfected PC3 cells were stained with Annexin-V-fluorescein isothiocyanate and PI, and the percentage of apoptotic cells (annexin V positive and PI negative) was determined by flow cytometry. siZNF #1 and #2 indicate *ZNF143* siRNA #1 and #2, respectively.

DNA microarray analysis with *ZNF143*-specific siRNA. We then evaluated global expression profiles to determine the real *ZNF143* target genes by cDNA microarray. One hundred and fifty-two genes were downregulated <0.4 -fold after *ZNF143*-specific siRNA transfection, whereas 84 genes were upregulated more than 2.5-fold after downregulation of *ZNF143*

expression (Table S2). To categorize the 152 downregulated genes, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources Program (<http://david.abcc.ncifcrf.gov/>). Among 152 genes, 41 (27.0%) were categorized as concerned with the cell cycle/DNA replication (Table 1). Next, we searched for putative *ZNF143*

Table 1. Cell cycle/DNA replication associated genes downregulated following zinc finger protein 143 (ZNF143) knockdown

Gene ID	Symbol	Accession	Gene name	Fold change†
23397	NCAPH	NM_015341	Barren homolog 1 (Drosophila)	0.174
991	CDC20	NM_001255	CDC20 cell division cycle 20 homolog (<i>Saccharomyces cerevisiae</i>)	0.221
57405	SPC25	NM_020675	Spindle pole body component 25 homolog (<i>S. cerevisiae</i>)	0.234
4174	MCM5	NM_006739	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (<i>S. cerevisiae</i>)	0.234
9212	AURKB	NM_004217	Aurora kinase B	0.239
890	CCNA2	NM_001237	Cyclin A2	0.248
990	CDC6	NM_001254	CDC6 cell division cycle 6 homolog (<i>S. cerevisiae</i>)	0.252
4173	MCM4	NM_005914	MCM4 minichromosome maintenance deficient 4 (<i>S. cerevisiae</i>)	0.256
4176	MCM7	NM_005916	MCM7 minichromosome maintenance deficient 7 (<i>S. cerevisiae</i>)	0.258
5347	PLK1	NM_005030	Polo-like kinase 1 (Drosophila)	0.266
7465	WEE1	NM_003390	WEE1 homolog (<i>Schizosaccharomyces pombe</i>)	0.278
81620	CDT1	NM_030928	DNA replication factor	0.284
83461	CDCA3	NM_031299	Cell division cycle associated 3	0.288
5888	RAD51	NM_002875	RAD51 homolog (RECA homolog, <i>Escherichia coli</i>) (<i>S. cerevisiae</i>)	0.289
4171	MCM2	NM_004526	MCM2 minichromosome maintenance deficient 2, mitotin (<i>S. cerevisiae</i>)	0.289
56992	KIF15	NM_020242	Kinesin family member 15	0.290
3148	HMGB2	NM_002129	High-mobility group box 2	0.295
113130	CDCA5	NM_080668	Cell division cycle associated 5	0.301
54443	ANLN	NM_018685	Anillin, actin binding protein (Scraps homolog, Drosophila)	0.320
55388	MCM10	NM_018518	MCM10 minichromosome maintenance deficient 10 (<i>S. cerevisiae</i>)	0.332
4172	MCM3	NM_002388	MCM3 minichromosome maintenance deficient 3 (<i>S. cerevisiae</i>)	0.334
9918	NCAPD2	NM_014865	Chromosome condensation-related SMC-associated protein 1	0.336
11004	KIF2C	NM_006845	Kinesin family member 2C	0.348
1017	CDK2	NM_001798	Cyclin-dependent kinase 2	0.348
51512	GTSE1	NM_016426	G-2 and S-phase expressed 1	0.353
9088	PKMYT1	NM_004203	Protein kinase, membrane associated tyrosine/threonine 1	0.356
5984	RFC4	NM_002916	Replication factor C (Activator 1) 4, 37KDA	0.357
3835	KIF22	NM_007317	Kinesin family member 22	0.358
10403	NDC80	NM_006101	Kinetochore associated 2	0.359
699	BUB1	NM_004336	BUB1 budding uninhibited by benzimidazoles 1 homolog (Yeast)	0.360
9055	PRC1	NM_003981	Protein regulator of cytokinesis 1	0.363
9700	ESPL1	NM_012291	Extra spindle poles like 1 (<i>S. cerevisiae</i>)	0.365
55143	CDCA8	NM_018101	Cell division cycle associated 8	0.369
1104	RCC1	NM_001269	Regulator of chromosome condensation 1	0.376
5982	RFC2	NM_002914	Replication factor C (Activator 1) 2, 40KDA	0.376
9787	DLG7	NM_014750	Discs, large homolog 7 (Drosophila)	0.391
10051	SMC4	NM_005496	SMC4 structural maintenance of chromosomes 4-like 1 (Yeast)	0.391
9493	KIF23	NM_004856	Kinesin family member 23	0.397
4751	NEK2	NM_002497	NIMA (never in mitosis gene A)-related kinase 2	0.399
899	CCNF	NM_001761	Cyclin F	0.399
22974	TPX2	NM_012112	TPX2, microtubule-associated, homolog (<i>Xenopus laevis</i>)	0.399

List of cell cycle/DNA-replication-associated genes with fold change marked <0.4. †Fold change indicates the average of expression data of ZNF143 siRNA/control siRNA.

binding sites in the promoter region of these genes. All 41 genes had at least one ZNF143 binding site within 1 kb of the putative transcription start site (Table 1).

Validation of the ZNF143 target genes. To validate the microarray expression data, we performed western blotting, ChIP assays and reporter assays. We obtained eight specific antibodies for proteins among the 41 cell cycle/DNA replication regulators with ZNF143 binding site(s). As shown in Figure 3, the expression of nine proteins was decreased by ZNF143 siRNA transfection. In particular, PLK1, CDC6 and HMGB2 were substantially decreased, whereas β -actin and PRDX4, which do not have a ZNF143 binding site, were not affected by ZNF143 siRNA transfection. Next, we examined whether ZNF143 directly regulates the expression of these genes using ChIP and luciferase reporter assays. For ChIP assays, we used stable transfectants that expressed three flag tags at the N-terminus of ZNF143.⁽⁷⁾ As shown in Figure 4A, ZNF143 bound to the promoters of the seven genes that con-

tained a ZNF143 binding site. The promoter region of PRDX4, which does not have a ZNF143 binding site, was used as a negative control. We also investigated the luciferase reporter activities of five genes. The promoter activities of CDC6, PLK1, AURKB, MCM2 and HMGB2 were clearly decreased by transfection with ZNF143-specific siRNA #2 (Fig. 4B). Myslinski *et al.*⁽⁶⁾ reported that 58% of genes that had a ZNF143 binding site contained the submotif ACTACN in their 5' regions, and that their promoter activities were higher than those of promoters that lacked the submotif. To confirm these results, we prepared two CDC6 promoters: CDC6 luc2 contained the ACTACN sequence and CDC6 luc1 did not. As shown in Figure 4C, our results were consistent with the findings of Myslinski *et al.*, and ZNF143 siRNA reduced both CDC6 promoter activities, with or without the ACTACN motif, in the same ratio (Fig. 4B).

Correlation between ZNF143 expression and cell growth in human lung cancer. ZNF143 regulates the gene expressions

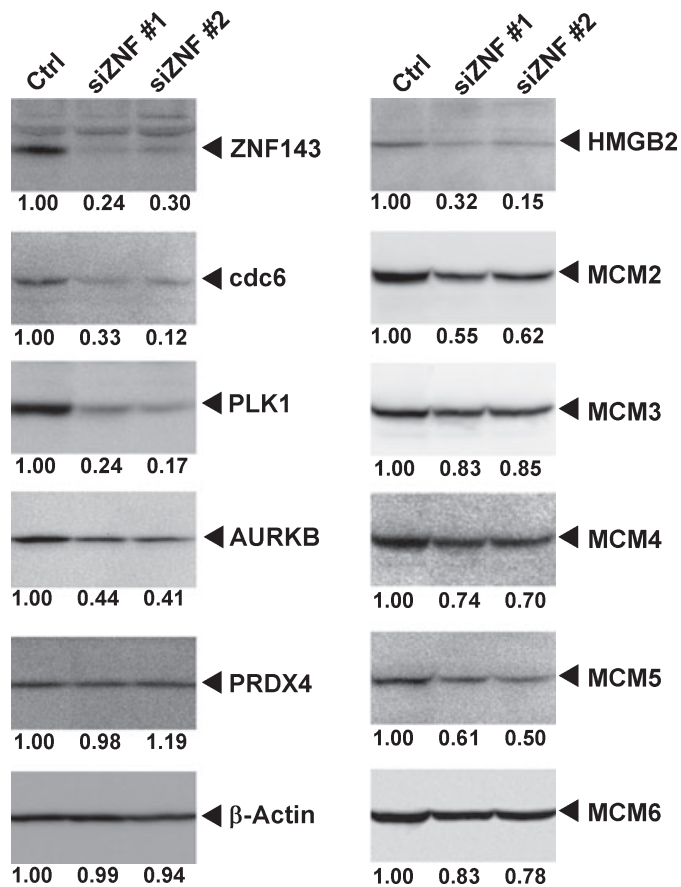


Fig. 3. *ZNF143* small interfering RNA (siRNA) reduced the expression of cell cycle/DNA replication-related genes. siRNA-transfected PC3 cells were used for western blotting. Quantitation of protein expression levels is shown under the panels. siZNF #1 and #2 indicate *ZNF143* siRNA #1 and #2, respectively.

associated with the cell cycle and DNA replication. We investigated the cellular expression level of *ZNF143* and doubling time with 10 lung cancer cell lines. *ZNF143* expression was different in each cancer cell line as compared with Nrf2 and inversely correlated with doubling time (Fig. 5A,B). This result is consistent with repression of cell growth by downregulation of *ZNF143* in lung cell cancer A549 and 904L (Fig. 5C). Repression of cell growth by downregulation of *ZNF143* was also observed in HeLa, DLD1, Caco-2, T24, CCF-STTG1 and U343 (data not shown).

***ZNF143* expression in human cancer specimens.** To investigate further whether highly proliferative cancer cells are characterized by increased *ZNF143* expression, tissue sections of surgical specimens from cancer patients were analyzed by immunohistochemistry (Fig. 6). Six solid tumors (esophageal carcinoma, gastric adenocarcinoma, squamous cell carcinoma of the lung, urothelial carcinoma of the urinary bladder, testicular seminoma and cerebral astrocytoma) were analyzed in comparison with adjacent non-tumor samples. Nuclear-localized *ZNF143* was strongly expressed in cancer cells. Expression of *ZNF143* was low or weak in non-tumor tissue.

Discussion

We have identified a number of genes that are induced by treatment with anti-cancer agents and/or overexpressed in drug-resistant cells.^(7,14,16–24) The drug sensitivity of cancer cells is

governed by the complex pathways involved in the pathogenesis of cancer,^(25,26) and identification of the molecules governing cancer cell growth is emerging as a primary theme of molecular therapies. The transcription factor *ZNF143* is stress-inducible and overexpressed in cisplatin-resistant cells.^(7,27) We found *ZNF143* binding sites in the promoter region of many DNA repair genes and propose that *ZNF143* regulates the expression of DNA repair genes. It has been shown recently that *ZNF143* binding sites are widely distributed in the human genome and are located in the promoters of approximately 1000 human genes.⁽⁶⁾ This suggests that *ZNF143* might regulate basic cellular functions at the transcriptional level.

Many studies have indicated that the cellular content of cell-cycle-related gene products is strictly regulated post-translationally by the ubiquitin–proteasome system.^(28,29) On the other hand, little is known about the transcriptional regulation of cell-cycle-related genes. The E2F family of transcription factors is well known to be a master regulator of cell proliferation and is regulated by the retinoblastoma tumor suppressor gene.⁽³⁰⁾ In this report, downregulation of *ZNF143* strongly induced G2/M arrest. On the contrary, downregulation of E2F induced G1 cell cycle arrest, indicating that the role of *ZNF143* may be quite distinct from that of E2F in the cell cycle. *ZNF143* target genes have been identified by a combination of *in silico* and experimental approaches.⁽⁶⁾ Myslinski *et al.*⁽⁶⁾ used eleven 18-bp binding sites in a genome-wide motif search and identified approximately 1000 genes as *ZNF143* target genes. Functional classification of these genes indicated that the majority of the identified genes are important for cell growth. Consistently, our results clearly showed that *ZNF143* is required for cancer cell growth (Figs 2,5). RNAi-mediated knockdown of *ZNF143* showed that the expression of 152 genes was downregulated in human prostate cancer PC3 cells (Table S1). Among these, 41 genes are categorized as concerned with cell cycle/DNA replication (Table 1). The human genome database suggests that approximately 500–600 genes are assigned for cell cycle/DNA replication. Based on our data, approximately 7.5% (41 genes/500–600 genes) of cell cycle/DNA replication genes might be directly or indirectly regulated by *ZNF143*. On the other hand, *ZNF143* regulates only approximately 0.5% (110 genes/23 000–27 000 genes) of other genes except for cell cycle/DNA replication. It is noteworthy that important cell-cycle-associated kinases such as *PLK1*, *AURKB*, *WEE1*, budding uninhibited by benzimidazoles (*BUB1*) and cyclin-dependent kinase 2 (*CDK2*) are target genes for *ZNF143*. Only nine (*SPC25*, *CDC6*, *PLK1*, *RAD51*, *MCM3*, *KIF2C*, *GTSE1*, *ESPL1*, *CDCA8*) (22%) of 41 genes were present in the *ZNF143* target gene list identified by the *in silico* genome screen.⁽⁶⁾ We also found putative *ZNF143* binding sites in many DNA repair genes;⁽⁷⁾ however, only four (*Rad51*, *BRCA1*, *FEN1*, *EXO1*) genes were downregulated by *ZNF143* siRNA. It has been shown that BUB family expression is required for cancer cell growth but not for normal cells, indicating that the BUB family is one of the genes involved in non-oncogene addiction.^(31,32) It is an interesting finding that *BUB1* and *BUB1B*⁽³³⁾ are target genes of *ZNF143*. These indicate that *ZNF143* specifically regulates genes associated with cell cycle/DNA replication and is one of the master regulators of DNA metabolism including cell cycle and cell growth.

It is well established that transcription factors do not make good targets for drug design, because transcription factors form multiple complexes with other cofactors. Our immunohistochemical studies have shown that *ZNF143* is highly expressed in cancer cells (Fig. 6). RNAi-mediated knockdown of *ZNF143* induced apoptosis following G2/M cell cycle arrest (Fig. 2). Because cell-cycle-associated kinases have a critical role in cell cycle progression, these kinases comprise a promising set of targets

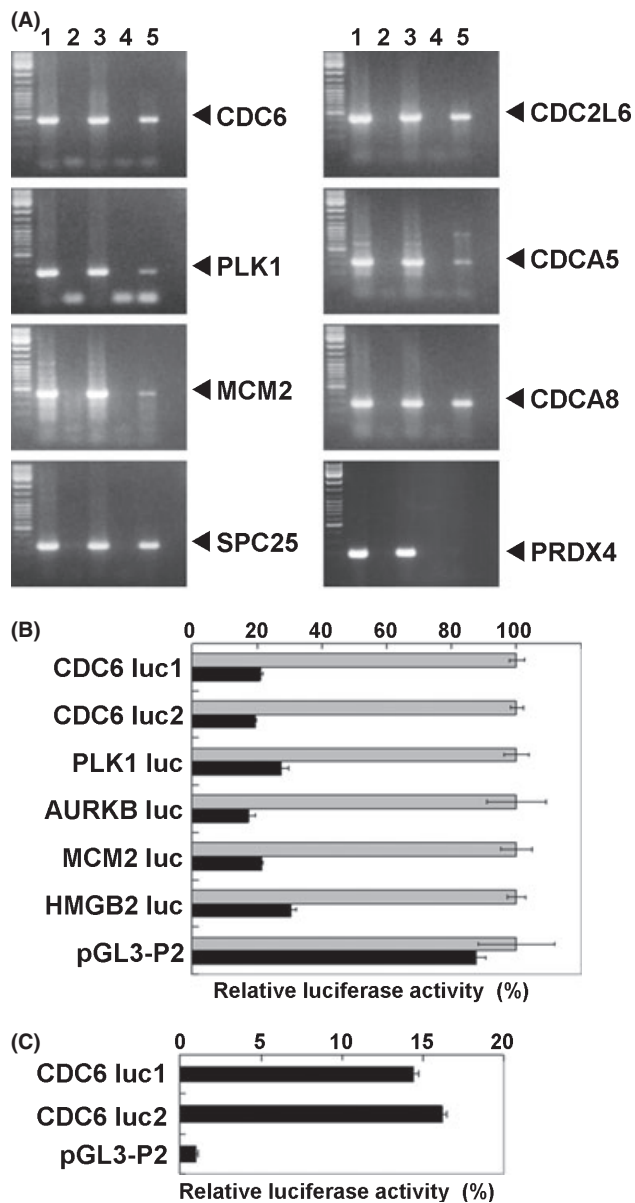


Fig. 4. Luciferase reporter and chromatin immunoprecipitation assays. (A) Soluble chromatin was prepared from flag tagged zinc finger protein 143 (ZNF143) stable transfectants (lanes 3–5) or mock transfectants (lanes 1 and 2), and immunoprecipitated with anti-mouse IgG (lane 4) or anti-Flag (M2) antibodies (lanes 2 and 5). Extracted immunoprecipitated DNA (lanes 2, 4 and 5) and soluble chromatin (lanes 1 and 3) were amplified using specific primer pairs for the indicated promoter regions. The PCR products for *CDC6*, *PLK1*, *MCM2*, *SPC25*, *CDC2L6*, *CDCA5*, *CDCA8* and *PRDX4* were 463, 296, 423, 258, 411, 342, 260 and 153 bp, respectively. (B) PC3 cells were transfected with reporter plasmid and *ZNF143* small interfering RNA (siRNA). Luciferase activities were normalized to each reporter activity with control siRNA. The gray and black bars indicate control siRNA and *ZNF143* siRNA #2, respectively. Bars represent \pm SD. (C) PC3 cells were transfected with reporter plasmid and *ZNF143* small interfering RNA (siRNA). Luciferase activities were normalized to the empty vector pGL3-P2. Bars represent \pm SD.

for anti-cancer drugs.^(34,35) Among the ZNF143 target cell-cycle-associated kinases, both *PLK1* and *AURKB* are overexpressed in many types of cancer.^(36–39) Our data showed that

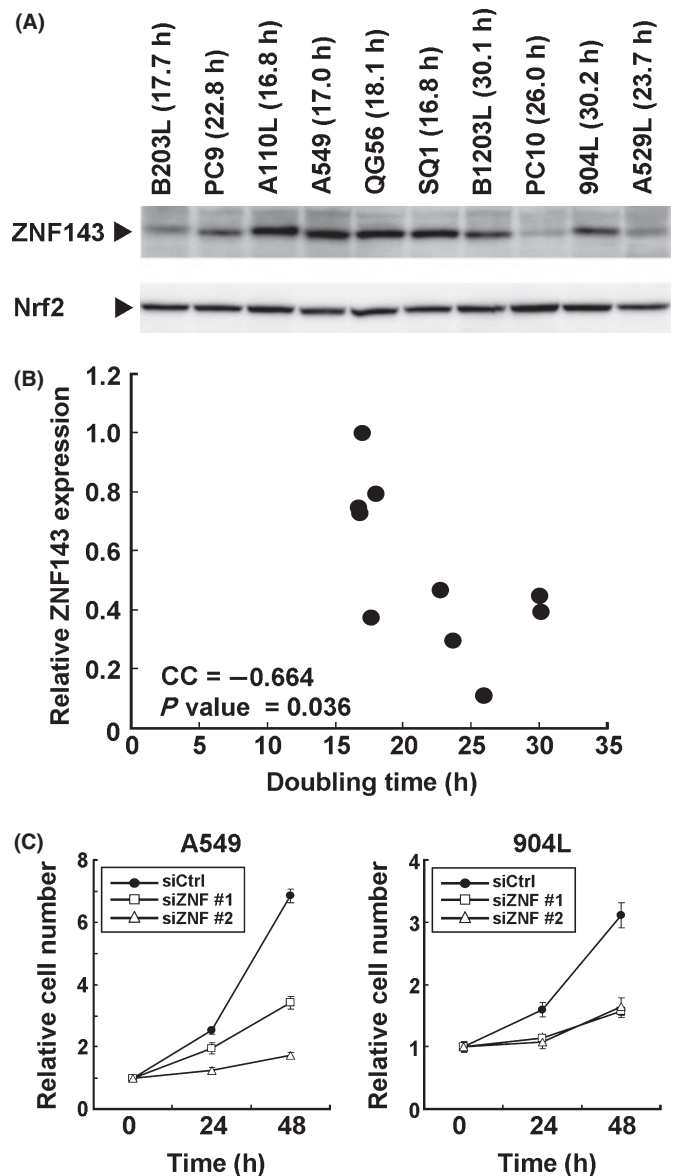


Fig. 5. Correlation between zinc finger protein 143 (ZNF143) expression and cell growth. (A) Nuclear protein (50 μ g) was used for western blotting with anti-ZNF143 and anti-Nrf2 antibodies. The doubling time for each cell lines is also shown. (B) Expression of ZNF143 and Nrf2 were quantitated using a Multi Gauge Version 3.0 using Figure 5A, and ZNF143 expression was normalized by Nrf2. The maximum expression levels of ZNF143 were set to 100. CC, coefficient of correlation. (C) A549 and 904L cells were transfected with control siRNA (siCtrl), *ZNF143* siRNA #1 (siZNF #1) or *ZNF143* siRNA #2 (siZNF #2). The cell proliferation assay is described in Figure 2A.

RNAi-mediated knockdown of *ZNF143* effectively downregulated the expression of both *PLK1* and *AURKB* (Fig. 3). The MCM proteins are required for pre-replication complex formation, DNA replication initiation and DNA synthesis. It is well known that the MCM proteins are highly expressed in human cancers. Therefore, the MCM proteins are diagnostic markers and promising targets for anti-cancer drug development.⁽⁴⁰⁾ As shown in Figure 3, several MCM proteins are targets of ZNF143. Because ZNF143 regulates the expression of both DNA replication and cell cycle regulatory members, ZNF143 may be a promising target for cancer diagnostics and therapies.

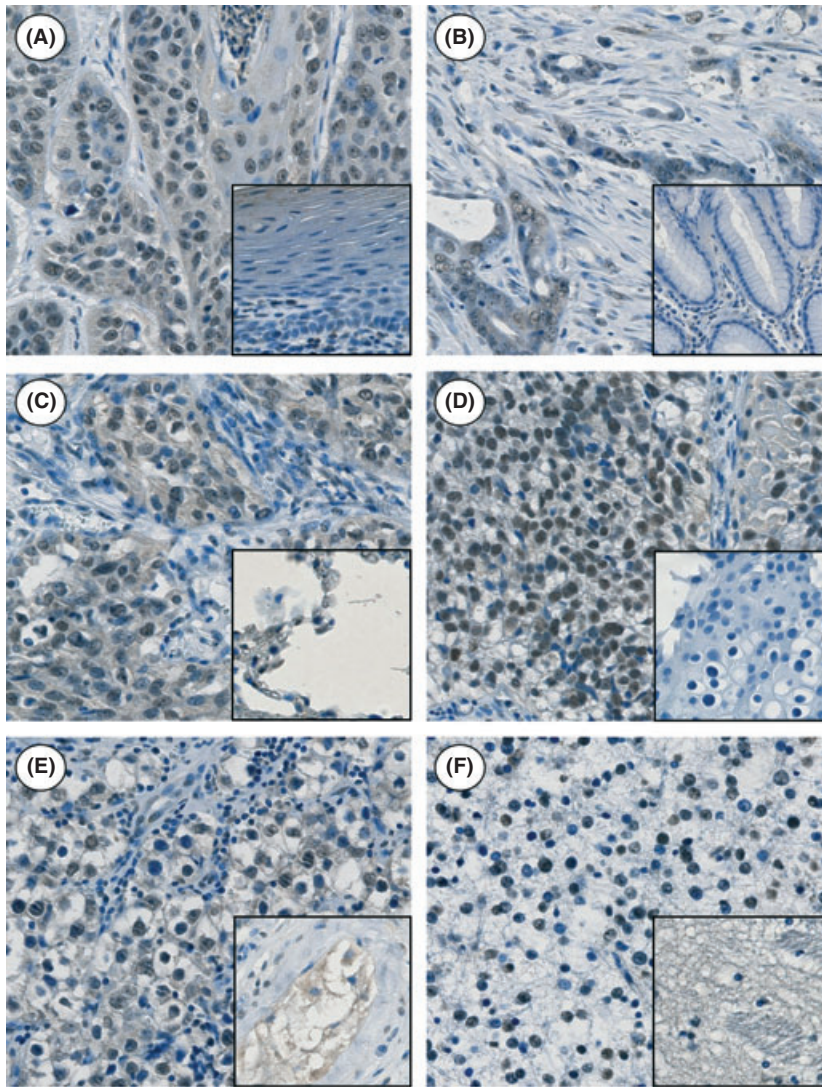


Fig. 6. Immunohistochemical analysis of zinc finger protein 143 (ZNF143) expression in various human tumor tissues. Nuclear expression of the ZNF143 protein was seen in all cancer cells, while low or weak nuclear expression was seen in normal adjacent tissues (inset). The selected tissues consisted of surgically resected cases of esophageal carcinoma (A), gastric adenocarcinoma (B), squamous cell carcinoma of the lung (C), urothelial carcinoma of the urinary bladder (D), testicular seminoma (E) and cerebral astrocytoma (F).

We found Ets binding sites in the promoter region of the ZNF143 gene. The Ets family is one of the largest families of transcription factors and is known to be involved in diverse cellular functions. The Ets family is a proto-oncogene and highly expressed in various cancers,⁽⁴¹⁾ indicating that ZNF143 expression might be regulated by the Ets family. Therefore, further studies to investigate the correlation between Ets and ZNF143 expression with clinicopathological data should help in understanding the role of ZNF143 in cancer.

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Disclosure Statement

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Supporting Information

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

Table S1. Primer pairs used for Chromatin immunoprecipitation (ChIP) assays.

Table S2. The list of selected genes with fold change marked >2.5 or <0.4.

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