Induction of the human *CDC45* gene promoter activity by natural compound *trans*-resveratrol

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Abstract. GGAA motifs in the human *TP53* and *HELB* gene promoters play a part in responding to *trans*-resveratrol (Rsv) in HeLa S3 cells. This sequence is also present in the 5'-upstream region of the human *CDC45* gene, which encodes a component of CMG DNA helicase protein complex. The cells were treated with Rsv (20 μM), then transcripts and the translated protein were analyzed by quantitative RT-PCR and western blotting, respectively. The results showed that the *CDC45* gene and protein expression levels were induced after the treatment. To examine whether they were due to the activation of transcription, a 5'-upstream 556-bp of the *CDC45* gene was cloned and inserted into a multi-cloning site of the Luciferase (Luc) expression vector. In the present study, various deletion/point mutation-introduced Luc expression plasmids were constructed and they were used for the transient transfection assay. The results showed that the GGAA motif, which is included in a putative RELB protein recognizing sequence, plays a part in the promoter activity with response to Rsv in HeLa S3 cells.

Introduction

Cell division cycle 45 (CDC45) is a component of the CMG complex, which contains MCM2-7 and GINS and plays a role as an essential helicase in DNA replication in eukaryotic cells (1). The CDC45 protein, recruiting single-stranded DNA binding protein replication protein A (RPA) (2), is required for restriction of excess processing of the replication fork (3-5). In yeast, DNA damage induces Rad53, of which the homologue is CHCK2 in human cells and which causes phosphorylation of CDC45 to inhibit both initiation and elongation processes of the DNA replication (6). In addition, it has been shown that the human DNA helicase B (HDHB/HELB), which plays a part in DNA end resection (7), associates with RPA (8,9) and CDC45 (10). At the process of inter-strand crosslinks, FANCM associates with the CDC45-MCM complex while the GINS are released (11). Downregulation of GINS complex formation inhibits DNA replication, arresting the cell cycle at G_1 phase (12). On the other hand, overexpression of the CDC45 causes replication stress, including S-phase arrest, replication fork stalling and eventually apoptosis (13). These observations suggest that well balanced expression of the CDC45, functioning together with HELB, RPA and CHEK2, is essential for the regulation of DNA replication initiation, which also affects the DNA repair system in eukaryotes. However, it is not fully understood how the *CDC45* gene expression is controlled. To elucidate the gene expression regulatory mechanism, 556-bp of the 5'-upstream region of the *CDC45* gene was cloned by PCR and it was ligated into a luciferase (Luc) expression plasmid, which was used for transfection and Luc reporter assay. The results showed that the 556-bp functions as a promoter with response to *trans*-resveratrol (Rsv) in HeLa S3 cells. Analysis of the sequence using the NCBI human genomic database revealed that the 556-bp is a bidirectional promoter, containing not only the putative transcription start site (TSS) of the *CDC45* gene, but also the oppositely transcribed ubiquitin recognition factor in the ER-associated degradation 1-encoding *UFD1* gene (14). Notably, the UFD1 protein is involved in the CMG helicase disassembly process (15). The 556-bp human *CDC45* gene promoter contains duplicated GGAA and GC-box elements (16,17), which are the target of the ETS family (18) and Sp1/Sp3 (19) transcription factor (TF) proteins, respectively. Previous studies showed that they cooperatively regulate the transcription of the *HELB* and *MCM4* genes in response to Rsv in HeLa S3 cells (20,21).

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Abbreviations: C_T, threshold cycle; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Luc, luciferase; MCM, minichromosome maintenance; Rsv, *trans*-resveratrol; RT-qPCR, reverse transcription-quantitative PCR; TF, transcription factor; TSS, transcription start site

Key words: ETS, cell division cycle 45, GGAA, resveratrol, transcription

Table I. Sequences of the primers for amplifying 5' upstream regions of the human *CDC45* gene.

Primer	Sequence (5'→3')
hCDC45-9123	CCCAAGCTTAATGCAACGAAGAAACCCCGC
hCDC45-9184	TTTAAGCTTAAGCCGGTACGCCCCAGAGGCTCACC
hCDC45-9191	TTTAAGCTTACGCCCCAGAGGCTCACCGGAAGTGC
hCDC45-9191M	TTTAAGCTTACGCCCCAGAGGCTCACCCCAAGTGC
hCDC45-9234	TTTAAGCTTAGGGGGGGTGCCCGGGACAAAGCGTC
hCDC45-9234M	GCTAAGCTTAGGTGCGCTGCCCGGGACAAAGCGTC
hCDC45-9246	TTTAAGCTTCGGGACAAAGCGTCGGCTGCA
hCDC45-9285	GCCAAGCTTGGCTCTAAAACACCCTCAGTAGAAGC
hCDC45-9386	GCCAAGCTTCGTGTTGACAGTATTCCCCTCCAGAC
hCDC45-9386M1	GCCAAGCTTCGTGTTGACAGTATTGGCCTCCAGAC
hCDC45-9386M2	GCCAAGCTTCGTGTTGACAGCATTCCCCTCCAGAC
hCDC45-9477	TTCAAGCTTCAGCCATCGAGGACTCGGGCGGAACT
AhCDC45-9679	TTTAAGCTTGCGACGCTGGGCGGACATCTT
AhCDC45-9639	GATAAGCTTACTGCCTCCCACTGGGAACCCTCAGG
AhCDC45-9632	GCCAAGCTTCCCACTGGGAACCCTCAGGGAAAGTA
AhCDC45-9632MM	GCCAAGCTTCCCACTGCCAACCCTCAGCCAAAGTA
AhCDC45-9584	GGCAAGCTTCTCAGTCACATACCCAATGGGGCAGC
AhCDC45-9515	GGTAAGCTTGTAGCTTAGTTCCGCCCGAGTCCTCG
AhCDC45-9493	GGTAAGCTTCCTCGATGGCTGAAGCAGAGGCAGTC
AhCDC45-9426	CCCAAGCTTGGCCCTACTAAATTCGTCTGG

Shaded nucleotides indicate mutations that disrupt the c-ETS and GC-box consensus sequence motifs.

In the present study, various deletion/point mutations were made on the 556-bp region to show which elements are essential for the promoter activity with the positive response to natural compound Rsv. Additionally, reverse transcription-quantitative (RT-q) PCR and western blotting were performed to examine whether the transcripts and the translates of the *CDC45* gene accumulated following Rsv treatment of HeLa S3 cells. Collectively, the present study aimed to reveal molecular mechanisms that drive the *CDC45* gene promoter in accordance with induction of the *HELB* and *MCM4* gene expression in Rsv treated HeLa S3 cells.

Materials and methods

Materials. trans-Resveratrol (Rsv; cat. no. CAS501-36-0) was purchased from Cayman Chemical Company (20-22).

Cells and cell culture. Human cervical carcinoma (HeLa S3) cells (Institute of Medical Science, Tokyo University) (23) were grown in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.) (20-22) and supplemented with 10% fetal bovine serum (FBS; Biosera) and penicillin-streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Construction of Luc reporter plasmids. The Luc reporter plasmids, carrying 556-bp, which contains a TSS of the human *CDC45* gene, was constructed by the slight modification of a previously described procedure (20-22,24). Briefly, PCR was performed with the hCDC45-9123/AhCDC45-9679 primer pair (Table I) and genomic DNAs that were extracted from HeLa S3 cells. The amplified DNA fragment was treated with *Hin*dIII and then ligated into the multi-cloning site of pGL4.10[*luc*2] (Promega Corporation). The resultant plasmids, containing the 556-bp fragment in a correct orientation, was named pGL4-CDC45-556. Similarly, other Luc reporter plasmids were constructed by ligating a PCR-amplified DNA fragment into the *Hin*dIII site of pGL4.10[*luc*2]. The sense and anti-sense primers used for the amplification of the DNA fragments are shown in Table II. Nucleotide sequences were confirmed by a DNA sequencing service (FASMAC, Greiner Japan Inc.) with Rv or GL primers (20-22,24). The Luc reporter plasmids, pGL4-HDHB and pGL4-MCM4, were used as Rsv-inducible positive control vectors (20,21).

Transcription factor binding sequence analysis. The nucleotide sequence of the cloned 556-bp DNA fragment was subjected to analysis of human transcription factor binding elements by JASPAR 2020 (http://jaspar2020.genereg.net/).

Transient transfection and Luc assay. Luc reporter plasmids were transfected into HeLa S3 cells by a DEAE-dextran method in 96-well plates as previously described (25) and after 24 h of transfection, the culture medium was changed to Rsv (20 μM) containing DMEM with 10% FBS. After a further 24 h of incubation, cells were collected and lysed with 100 μl of 1X cell culture lysis reagent, containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid, 10% glycerol and 1% Triton X-100 and then mixed and centrifuged at 12,000 x g for 5 sec. The appropriate concentration and duration of treatment

Table II. Primer pairs used for amplifying 5' upstream regions of the human *CDC45* gene.

Plasmid	Sense primer	Anti-sense primer
pGL4-CDC45-556	hCDC45-9123	AhCDC45-9679
pGL4-CDC45-D1	hCDC45-9184	AhCDC45-9679
pGL4-CDC45-D2	hCDC45-9234	AhCDC45-9679
pGL4-CDC45-D3	hCDC45-9246	AhCDC45-9679
pGL4-CDC45-D4	hCDC45-9285	AhCDC45-9679
pGL4-CDC45-D5	hCDC45-9386	AhCDC45-9679
pGL4-CDC45-D6	hCDC45-9477	AhCDC45-9679
pGL4-CDC45-D1	hCDC45-9124	AhCDC45-9639
pGL4-CDC45-D2	hCDC45-9125	AhCDC45-9584
pGL4-CDC45-D3	hCDC45-9126	AhCDC45-9515
pGL4-CDC45-D4	hCDC45-9127	AhCDC45-9493
pGL4-CDC45-D5	hCDC45-9128	AhCDC45-9426
pGL4-CDC45-D11	hCDC45-9184	AhCDC45-9639
pGL4-CDC45-D21	hCDC45-9234	AhCDC45-9639
pGL4-CDC45-D31	hCDC45-9246	AhCDC45-9639
pGL4-CDC45-D12	hCDC45-9184	AhCDC45-9584
pGL4-CDC45-D22	hCDC45-9234	AhCDC45-9584
pGL4-CDC45-D32	hCDC45-9246	AhCDC45-9584
pGL4-CDC45-D33	hCDC45-9246	AhCDC45-9515
pGL4-CDC45-D34	hCDC45-9246	AhCDC45-9493
pGL4-CDC45-D42	hCDC45-9285	AhCDC45-9584
pGL4-CDC45-D43	hCDC45-9285	AhCDC45-9515
pGL4-CDC45-D44	hCDC45-9285	AhCDC45-9493
pGL4-CDC45-D52	hCDC45-9386	AhCDC45-9584
pGL4-CDC45-D53	hCDC45-9386	AhCDC45-9515
pGL4-CDC45-D54	hCDC45-9386	AhCDC45-9493
pGL4-CDC45-442	hCDC45-9191	AhCDC45-9632
pGL4-CDC45-442_M1	hCDC45-9191M	AhCDC45-9632
pGL4-CDC45-442_1M	hCDC45-9191	AhCDC45-9632MM
pGL4-CDC45-442_M1M	hCDC45-9191M	AhCDC45-9632MM
pGL4-CDC45-399	hCDC45-9234	AhCDC45-9632
pGL4-CDC45-399_M2	hCDC45-9234M	AhCDC45-9632
pGL4-CDC45-399_2M	hCDC45-9234	AhCDC45-9632MM
pGL4-CDC45-399_M2M	hCDC45-9234M	AhCDC45-9632MM
pGL4-CDC45-D54_M3	hCDC45-9386M1	AhCDC45-9493
pGL4-CDC45-D54_M4	hCDC45-9386M2	AhCDC45-9493

with Rsv had been determined by MTS and Luc assay, respectively (24). The supernatant was stored at -80˚C. The Luc assay was performed with a Luciferase assay system (Promega Corporation) and relative Luc activities were calculated as described previously (25).

Western blotting. Cells were collected after Rsv-treatment and lysed in a RIPA buffer (20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% TritonX100, and 1% sodium deoxychlate). Protein amount was analyzed with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.) according to the manufactures protocol. After SDS-PAGE (15% acrylamide) (15-25 μg proteins/lane) and blotting onto a PVDF (Immobilon-P) membrane as previously described (20-22), Blocking was carried out in a Blocking solution, which is a TBS containing 1% Skim milk (Megmilk Snow Brand), at 4˚C for 15 h. Western blot analysis was carried out with antibodies against CDC45 (cat. no. 11881, Cell Signaling, Danvers, MA) (1:1,000), and β-actin (cat. no. A5441; MilliporeSigma) (1:1,000) at 20˚C for 1 h, followed by the incubation with horseradish peroxidase-conjugated anti-rabbit (cat. no. A0545) (1:10,000) or anti-mouse IgG (cat. no. A9917) secondary antibodies (Sigma-Aldrich; Merck KGaA) (1:10,000) at 20˚C for 1 h in a TBS containing 1% TritonX100 and 2.5% Skim milk. Signal intensities were detected with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) and quantified with a ChemiDoc image analysis system and ImageLab 6.0 software (Bio-Rad Laboratories, Inc.).

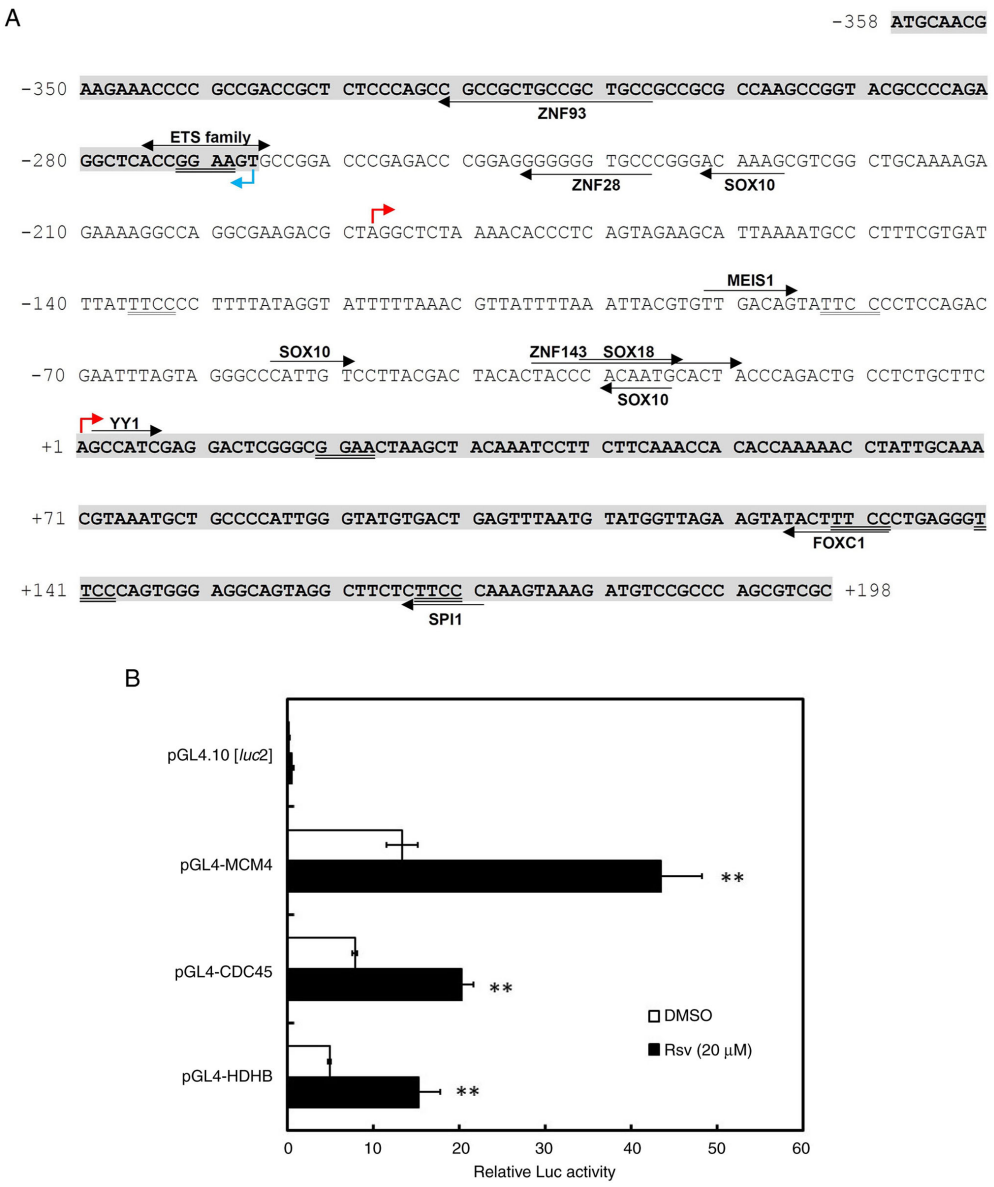

Figure 1. Characterization of the human *CDC45/UFD1* bidirectional promoter region. (A) The nucleotide sequence of the 556-bp fragment that was obtained from PCR is shown. The most upstream 5' end of the human *CDC45* (XM_011530416.1/XM_024452278.1 and XM_011530417.3/XM_011530418.3) and *UFD1* (NM_001035247.3, NM_005659.7/NM_001362910.2zcDNAs are designated by red and blue arrows, respectively. The most upstream of the *CDC45* gene transcript variants X2/X3 is designated as nucleotide number +1 and that of the X4/X5 and the *UFD1* transcript are shown by arrows on -188 and -267, respectively. Putative transcription factor-binding sites were predicted by the JASPAR2020 database program and they (relative score >98%) are indicated by arrows on or under the sequence. The 'ETS family' includes ELK1, ELK3, ELK4, ERF, ERG, ETS1, ETS2, ETV1, ETV3, ETV4, ETV5, ETV6, FEV, FLI1, GABPA and SPI1. (B) Luc reporter plasmids, pGL4.10[*luc*2], pGL4-MCM4-309, pGL4-CDC45-556 and pGL4-HDHB were transfected into HeLa S3 cells, which were treated with or without Rsv (20 μM) for 24 h. Results show relative Luc activities compared with that of pGL4-PIF1-transfected and Rsv-non-treated cells. Results are shown as means ± standard deviation from at least three independent experiments. Statistical analysis for the results between Rsv-treated and DMSO-treated control cells was performed with the Student's t-test **P<0.01 vs. DMSO. Luc, luciferase; Rsv, *trans*-resveratrol.

RT-qPCR. RNA extraction, cDNA synthesis, and qPCR were performed according to the manufacturer's protocol. First-strand cDNAs were synthesized with ReverTra Ace (Toyobo Life Science), random primers (Takara Bio, Inc.) and total RNAs extracted from HeLa S3 cells, which were cultured 1 to 5x10^6 cells/ϕ 10 cm dish. PCR analysis was carried out using a Mx3000P Real-Time qPCR System (Stratagene; Agilent Technologies, Inc.) (20-22). cDNAs were added to the Thunderbird Realtime PCR Master Mix (Toyobo Life Science), containing 0.3 μM of each primer pair. The primer pairs for amplifying the human *CDC45* and *GAPDH* transcripts were hCDC45-820: GACTGCACACGGATCTCC TT/AhCDC45-949: TCTGTCCATGCACAGACCAC and hGAPDH556/hGAPDH642 (20-22), respectively. Amplification was carried out initially for 1 min at 95˚C, followed by 40 cycles at 95˚C (15 sec) and 58˚C (30 sec). Quantitative PCR analysis for each sample was carried out in triplicate. Relative gene expression values were obtained by normalizing threshold cycle (C_T) values of target genes in comparison with C_T values of the *GAPDH* gene using the $2^{-\Delta\Delta Cq}$ method (26).

Statistical analysis. Standard deviations (S.D.) for each data were calculated and results are shown as means ± S.D. from three independent experiments. Statistical analyses were

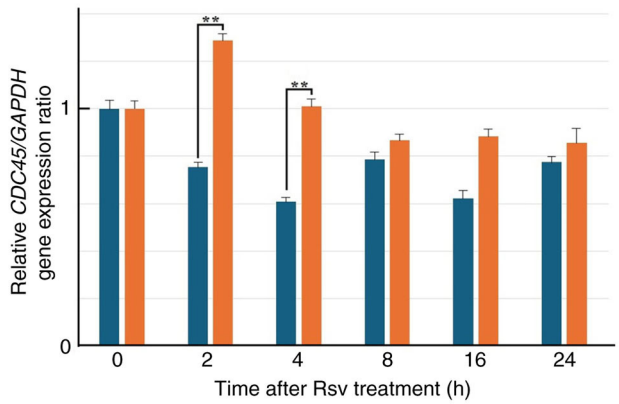

Figure 2. Effects of Rsv on *CDC45* gene expression. The culture medium of HeLa S3 cells was changed to DMEM (containing 10% FBS) with 20 μM of Rsv (orange columns). Blue columns represent control cells that were cultured without Rsv. Cells were harvested after 0, 2, 4, 8, 16 and 24 h of treatment. Total RNAs were extracted from cells and synthesized cDNAs were subjected to reverse transcription-quantitative PCR with primer pairs to amplify *CDC45* and *GAPDH* transcripts. The results show the relative *CDC45*/*GAPDH* gene expression ratio from four independent experiments. Statistical analysis for the results between Rsv-treated and non-treated cells was performed with the Student's t-test. **P<0.01. Rsv, *trans*-resveratrol; DMEM, Dulbecco's modified Eagle's medium.

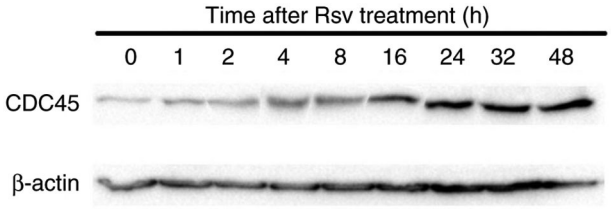

Figure 3. Effects of Rsv on CDC45 protein amount in HeLa S3 cells. HeLa S3 cells were collected after 0 to 48 h of Rsv (20 μM) treatment. The extracted proteins were separated by a 15% SDS-PAGE and western blotting was performed with primary antibodies against CDC45 and β-actin (upper and lower rows, respectively). The signal of each band was quantified and the results show the relative CDC45/β-actin expression ratio compared with that of the non-treated control cells (0 h treatment). Results are shown as means ± standard deviation from three independent experiments. Rsv, *trans*-resveratrol.

performed with the Student's t-test and asterisks indicate values of *P<0.05, **P<0.01 and ***P<0.005. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of the human CDC45 promoter region. Duplicated GGAA motifs in the *HELB* and *MCM4* promoters respond to Rsv (20,21), which can up-regulate the NAD^+/NADPH ratio in HeLa S3 cells (27). The HELB is known to interact with CDC45 that associates with MCM helicase to develop the CDC45-MCM-GINS complex (1,12). To examine whether the *CDC45* promoter responds to Rsv accompanied with the *HELB* and *MCM4* promoter, the present study amplified and isolated the 556-bp of the 5'-flanking sequence of the *CDC45* gene by PCR. Sequence analysis revealed that the pGL4-CDC45-556 contained a nucleotide identical to NCBI Sequence ID NC_000022.11 (nucleotide from 19419724-19479679) and that it covers the sequence of the most upstream 5' end of the cDNA (Sequence IDs: XM_011530416.1 and XM_024452278.1, for *CDC45* gene transcript variants X2 and X3, respectively and XM_011530417.3 and XM_011530418.3, for transcript variants X4 and X5, respectively; GENE ID, CDC45: 8318). This 556-bp region also contains a 5' upstream end of the *UFD1* mRNAs (sequence ID: NM_001035247.3, NM_005659.7 and NM_001362910.2; GENE ID, UFD1: 7253) in a reverse orientation to that of the *CDC45* gene. The TSS was tentatively set as +1 at the most upstream 5' end of the *CDC45* transcript variants X2 and X3. The JASPAR2020 database program (jaspar.genereg.net/) indicated that the consensus recognition sequences of several known transcription factors are present within the 556-bp (Fig. 1A). This DNA sequence has no obvious TATA box or CCAAT motif but contains putative binding sites of ZNF93 (-321 to -307), ETS family (-275 to -266), ZNF28 (-246 to -237), SOX10 (-232 to -227, -55 to -50, -30 to -25), ZNF143 (-35 to -20), SOX18 (-31 to -24), YY1 (+2 to +7), FOXC1 (+125 to +132) and SPI1 (+166 to +171). To examine whether the 556-bp DNA fragment functioned as a promoter, Luc reporter plasmids, pGL4-HDHB (20), pGL4-MCM4 (21) and pGL4-CDC45-556, were transiently transfected into HeLa S3 cells. All the relative Luc activities of the plasmids-transfected cells increased after the addition of Rsv to the cell culture (Fig. 1B). Based on this observation, it was decided to examine the *CDC45* gene/protein expression and promoter activity.

Effects of Rsv on CDC45 gene expression and its protein amount in HeLa S3 cells. Next, total RNAs were extracted from cells after adding Rsv to the culture medium and RT-qPCR was carried out (Fig. 2). The relative gene expression of *CDC45* compared with that of the *GAPDH* gene increased approximately two-fold at 2 to 4 h after Rsv treatment. western blotting showed that the amount of CDC45 protein accumulated from 16 to 48 h after the treatment (Fig. 3).

Determination of Rsv-response element(s) in the CDC45 promoter. To examine the Rsv-responsive sequence, deletion from the 5' and 3' ends of the 556-bp *CDC45* promoter region was introduced into the pGL4-CDC45-556 (Fig. 4A and B). The positive response to Rsv was observed in the cells that were transfected with pGL4-CDC45-D5. On the other hand, no apparent Luc activity was detected in pGL4-CDC45-D6-transfected cells (Fig. 4A), indicating that the region from -96 to -6 functions as a *CDC45* gene promoter that positively responds to Rsv. In addition, comparing the Luc activities of the cells transfected with pGL4-CDC45-D4 and -D5, it was suggested that the region from -55 to +12 is essentially required for *CDC45* promoter activity and its positive response to Rsv (Fig. 4B). Because the deletions from -358 to -299 or -248 (Fig. 4A) and from +198 to +159 (Fig. 4B) gave higher Luc activities, these regions may have negative regulatory element(s). The deletion from +158 to +104 from D11 and D12 constructs raised the basal promoter activity, suggesting that the 56 nucleotide contains negative elements) (Fig. 4C). Other deletions both from the 5'- and 3'- were examined in a similar transfection experiment (Fig. 4D). However, apparent Luc activities with positive response to Rsv were observed in cells that were transfected by

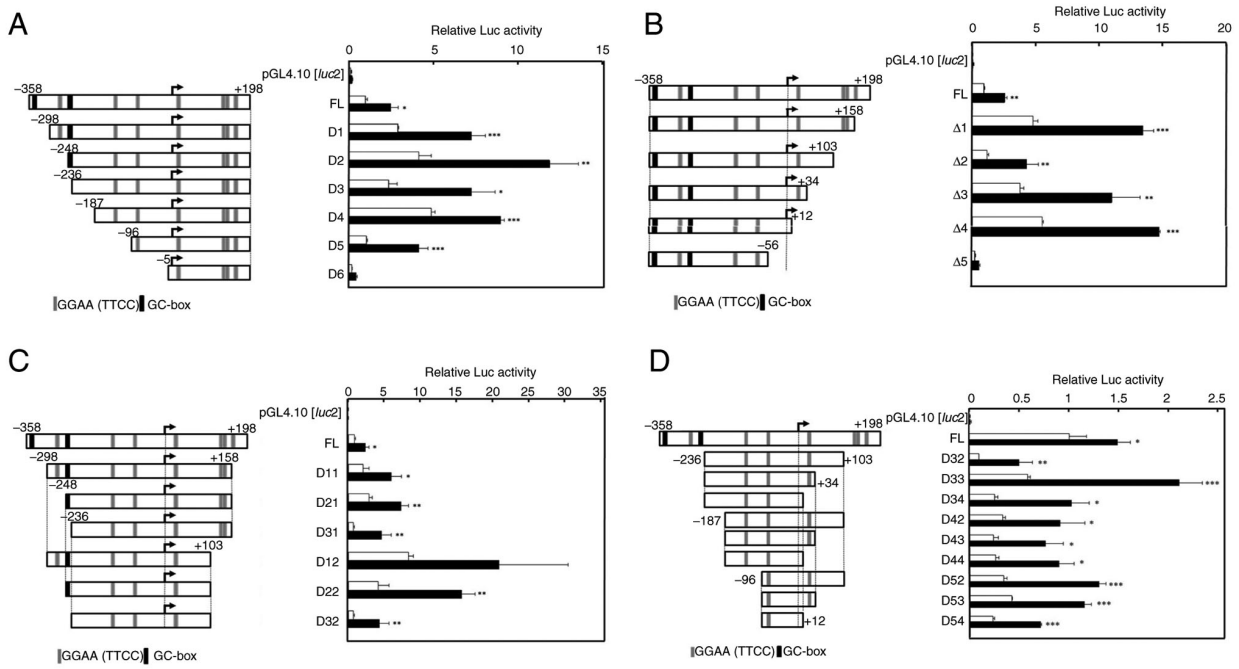

Figure 4. Effect of Rsv on human *CDC45* promoter activity. (Left panels) The 5' upstream end of the human *CDC45* gene, which has been ligated upstream of the *Luciferase* gene of the pGL4.10[*luc*2], is shown. The 5'-end of the cDNA of the X2 and X3 transcript variants is designated +1. The GGAA (TTCC) and GC-box elements are schematically shown. (Right panels) Luc reporter plasmids were transiently transfected into HeLa S3 cells and treated with (closed bars) or without (open bars) Rsv (20 μM) for 24 h. (A) Transfection was performed with (A) pGL4-CDC45-556, pGL4-CDC45-D1, -D2, -D3, -D4, -D5 and -D6, (B) pGL4-CDC45-556, pGL4-CDC45-D1, -D2, -D3, -D4, -D5 and -D6, (C) pGL4-CDC45-556, pGL4-CDC45-D11, -D21, -D31, -D12, -D11 and -D32, (D) GL4-CDC45-556, pGL4-CDC45-D32, -D33, -D34, -D42, -D43, -D44, -D52, -D53 and -D54. Luc activities were normalized to that of the pGL4-PIF1-transfected cells. Histograms show relative Luc activities of deletion-introduced plasmid-transfected cells compared with that of the pGL4-CDC45-556-transfected cells without Rsv treatment. Results are shown as means ± standard deviation from three independent experiments. Statistical analysis for the results between Rsv-treated and non-treated cells was performed with the Student's t-test. *P<0.05, **P<0.01 and ***P<0.005. Rsv, *trans*-resveratrol; Luc, luciferase.

these plasmids, including pGL4-CDC45-D54, which has the shortest 108-bp sequence. The obtained results totally showed that the region from -96-+12 functions as a minimum promoter that has Rsv response.

Introduction of point mutations on the CDC45 promoter. To indicate the Rsv-responsive sequence more precisely, point mutations were introduced to the *CDC45* promoter (Fig. 5). To examine the contribution of the GGAA (TTCC) motifs (ACC GGAAGT; -275 to -267 and ATACTTTCCCTGAGGGTTCCC AGTG; +124 to +148) and the GC-box (GGAGGGGGGTGCC; -249 to -237) to the drug response, point mutations were introduced on pGL4-CDC45-442 and -399 (Fig. 5A). Transfection and Luc assay showed that most mutation-introduced plasmids had a positive response to Rsv. However, the response was much reduced in pGL4-CDC45-399-M2 and -M2M-transfected cells (Fig. 5A), suggesting that the GC-box, which was predicted as ZNF28 recognition sequence by JASPAR2020 program, plays a part in the regulation of *CDC45* transcription.

Next, the minimum core Rsv-responding 108-bp from -96 to +12 was examined to see whether it contained a Rsv response element(s) (Fig. 5B). Previous studies on the human *HELB*, *MCM4* and *TP53* gene promoters showed that a GGAA (TTCC) motif plays a primarily important role in the response to Rsv (20-22). Therefore, it was hypothesized that the GGAA (TTCC) core motif also regulates the *CDC45* promoter activity. However, no TF-binding sites with GGAA (TTCC) were indicated by the JASPAR2020 program when the threshold was set at 98% (Fig. 1A). Therefore, the present study examined setting the threshold at 95%. This time, the putative RELB binding sequence, which is located just 3'-downstream of the MEIS1 binding motif was indicated (Fig. 5B). Compared with the wild type pGL4-CDC45-D54, mutation in the pGL4-CDC45-D54_M3 reduced the Luc activity, but a positive response to Rsv was still observed (Fig. 5B). On the other hand, the response was completely abolished in the pGL4-CDC45-D54_M4. These results suggested that the sequence between putative MEIS1 and RELB recognition sites is of primary importance for the *CDC45* promoter activity and the positive response to Rsv.

Discussion

The present study showed that treatment with Rsv (20 μM) induced *CDC45* gene and protein expression in HeLa S3 cells. Deletion and mutation analyses revealed that the putative RELB binding sequence was required for the *CDC45* promoter activity to positively respond to Rsv.

The *CDC45-UFD1* bidirectional promoter has been isolated and characterized to suggest a putative TATA-box within the region (28). Although the TATA-like sequence, TTTTATAGG (from -129 to -122) is contained in the pGL4-CDC45-D5 construct, it does not have promoter activity (Figs. 4 and 5B), indicating that the sequence is not essential for *CDC45* promoter in HeLa S3 cells. The 556-bp,

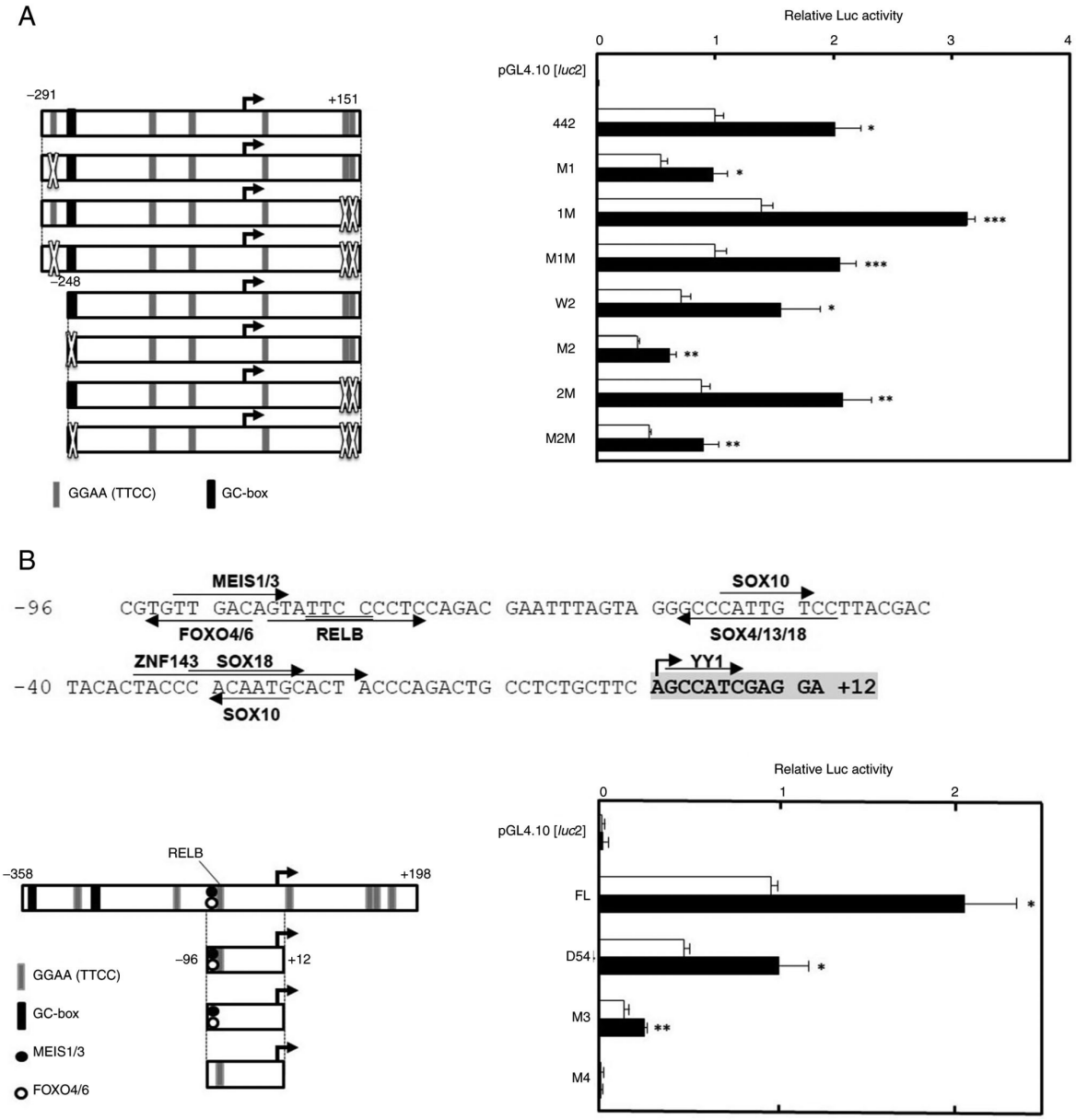

Figure 5. Effect of point mutations on the *CDC45* promoter. (A) Point mutations on the GGAA motifs or GC-box were introduced in the 449-bp and 406-bp, which are contained in the pGL4-CDC45-W1 and -W2, respectively. Similar transfection experiments were carried out as described under the legend to Fig. 4. Histograms show relative Luc activities of deletion construct-transfected cells compared with that of the pGL4-CDC45-W1-transfected cells without Rsv treatment. (B) Nucleotide sequences from -96 to +12 that are contained in the Luc reporter plasmids pGL4-CDC45-D54 are shown. The JASPAR2020 database program was applied to the 108-bp and the predicted TF-binding sites are indicated by arrows (relative score >95%). Histograms show relative Luc activities of point mutation-introduced construct-transfected cells compared with that of the pGL4-CDC45-556-transfected cells without Rsv treatment. (A and B) Results are shown as means ± standard deviation from three independent experiments. Statistical analysis for the results between Rsv-treated and non-treated cells was performed with the Student's t-test. *P<0.05, **P<0.01 and ***P<0.005 vs. pGL4-CDC45-556-transfected cells without Rsv treatment. Luc, luciferase; Rsv, *trans*-resveratrol; TF, transcription factor;

containing TSSs of both genes, possesses a duplicated GGAA (TTCC) motif, which is contained in the promoter regions of many genes that encode DNA repair and genome maintenance factors (16,29). The duplicated GGAA motif in the human *TP53* promoter has been shown to have an essential role to respond to Rsv in HeLa S3 cells (22). However, the duplicated GGAA (TTCC) (from +124 to +148) in the 556-bp of the human *CDC45* promoter seems to be non-essential (Fig. 5A). The GC-box, which is a Sp1-recognitionsequence that is commonly contained in the *HELB* and *MCM4* promoter regions, serves a part in the response to Rsv (20,21). However, the Rsv-responding sequence from -96 to +12 (Fig. 5B) does not contain any GC-boxes or GC-box like motifs. In the present study, the functional transcription promoter with the response to Rsv was shown to be present in the sequence 5'-CAGTATTCCCCTCC-3' (-88 to -75), which overlaps with the MEIS1 and RELB recognition motifs (Fig. 5B). *MEIS1* gene expression is prominently suppressed when THP-1 cells are induced to differentiate into monocyte-macrophage-like cells by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (30). In murine myeloid cells, MEIS1 acts as a suppressor of granulocyte colony stimulation factor-induced differentiation (31).

The amount of RELB protein increases in granulocyte macrophage colony stimulation factor-induced macrophage cells only following stimulation by lipopolysaccharide (32). In addition, RELB is required for TNF-induced differentiation of bone marrow cells into M1 macrophages in mice (33). Our previous studies indicated that the duplicated GGAA-motif is responsible for the activation of the *E2F4* and *ZNFX1* genes during TPA-induced macrophage-like differentiation of HL-60 cells (34,35). In HeLa S3 cells, the Sp1/PU.1 ratio is notably increased following the Rsv treatment (21). It should be determined whether the RELB/MEIS1 ratio in HeLa S3 cells is increased in response to Rsv and if they are competing to bind to the Rsv response element in the *CDC45* promoter.

The natural compound Rsv has been shown to upregulate expression of the *HELB* and *MCM4* genes and its encoded proteins in HeLa S3 cells (20,21). The human *HELB* (*HDHB*) gene encodes a DNA replication-associated helicase (36), which binds to DNA double-strand breaks to inhibit DNA end resection (7). In addition, the HELB protein is known to interact with the CDC45 at initiation of DNA replication (10,37). Notably, the MCM complex, whose structure was revealed by cryo-electron microscopy (38), associates with CDC45 and GINS (1,39). The timing of CMG complex formation at the origin of replication should be limited to initiate DNA replication at a suitable time (40). The duplicated GGAA (TTCC) motifs are not only present in the 5' upstream regions of the *CDKN1A*, *RB1*, *TP53* and *MCM4* genes (21,22), but also in that of the *GINS1*, *GINS3* and *GINS4* genes (41). Expression of genes encoding proteins that regulate entering the S-phase could be accurately regulated by GGAA-recognizing TFs.

The present study showed that human *CDC45* gene promoter can be activated by Rsv in HeLa S3 cells. CDC45 is a component of CMG helicase which is essential for responses to replicative stress and stability of mammalian genomes (37,42). If, in some cancer cells, the CMG helicase play an important role as a tumor suppressor, maintaining required expression level would stop aberrant proliferation. By contrast, in other types of cancer, hyper expression of the *CDC45* gene may be harmful, functioning as an oncogenic factor (43). In this case, the CDC45 might be one of the targets, inhibition of which can lead to apoptosis or programmed cell death. Rsv can prolong the life span of organisms (44,45) and has been revealed to have beneficial effects for health (46). Further investigations are required to elucidate the mechanisms by which Rsv-induced signals regulate DNA replication/repair-associated gene expression. The present study not only examine biological effect of the Rsv, but also suggested a molecular mechanism how Rsv affects transcription of *CDC45* gene. Evaluation of the efficacies of other candidate drugs, gene expression vectors and nucleotides, including miRNAs, the multiple transfection assay would be useful to indicate which gene promoters are activated. The limitation of the present study is that it cannot be directly applied to diagnosis with tissue samples obtained by biopsy. However, it could be applied on iPS cells or patient-derived cell lines. Another concern is that the experimental system to analyze multiple gene promoter activities is only applicable with the use of/verification in only one cell line at the same time. Although it needs to be improved, this multiple promoter analysis, an easy, reproducible and cost-effective method, which does not always need transfection-efficiency estimation, can suggest which drugs or gene expression vectors should be used for the treatment.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JA and FU confirm the authenticity of all the raw data. JA and HK constructed the Luc reporter plasmid and performed experiments and analyzed the data (transfection assay, RT-qPCR and western blotting). FU interpreted the data and wrote the manuscript. ST collected and analyzed/interpreted the data. TM, YO and MA interpreted the data and edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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