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Mutations in the Circadian Gene *CLOCK* in Colorectal Cancer

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Abstract

The circadian clock regulates daily variations in physiologic processes. *CLOCK* acts as a regulator in the circadian apparatus controlling the expression of other clock genes, including *PER1*. Clock genes have been implicated in cancer-related functions; in this work, we investigated *CLOCK* as a possible target of somatic mutations in microsatellite unstable colorectal cancers. Combining microarray gene expression data and public gene sequence information, we identified *CLOCK* as 1 of 790 putative novel microsatellite instability (MSI) target genes. A total of 101 MSI colorectal carcinomas (CRC) were sequenced for a coding microsatellite in *CLOCK*. The effect of restoring CLOCK expression was studied in LS180 cells lacking wild-type CLOCK by stably expressing GST-CLOCK or glutathione *S*-transferase empty vector and testing the effects of UV-induced apoptosis and radiation by DNA content analysis using flow cytometry. Putative novel CLOCK target genes were searched by using ChIP-seq. *CLOCK* mutations occurred in 53% of MSI CRCs. Restoring *CLOCK* expression in cells with biallelic *CLOCK* inactivation resulted in protection against UV-induced apoptosis and decreased G_2-M arrest in response to ionizing radiation. Using ChIP-Seq, novel CLOCK-binding elements were identified near DNA damage genes *p21, NBR1, BRCA1*, and *RAD50. CLOCK* is shown to be mutated in cancer, and altered response to DNA damage provides one plausible mechanism of tumorigenesis. *Mol Cancer Res; 8(7); 952–60. ©2010 AACR.*

Introduction

Circadian rhythms are daily oscillations in physiologic processes. They regulate various functions in the human body, including sleep, body temperature, hormone production, digestive secretion, and immune activity. The rhythms are controlled by an endogenous clock. At the molecular level, the circadian clock is composed of the products of at least eight core genes (*Clock, Ck1ε, Cry1, Cry2, Per1, Per2, Per3*, and *Bmal1*), which are organized as a transcriptional-translational regulatory network (1, 2). The basic helix-loop-helix and Per/Arnt/Sim (bHLH-PAS) domain transcription factor CLOCK has a pivotal role as a positive component in this autoregulatory feedback loop. CLOCK acts as a heterodimer with another transcription factor, BMAL1, to activate transcription of a large number of target genes (1). Recent data suggest that Npas2 (neuronal PAS domain protein 2) may play a redundant role in the circadian clock (3). Several of the oscillating circadian clock target genes, such as *c-MYC, MDM2, p53*, caspases, and cyclins, are involved in the regulation of the cell cycle, cellular homeostasis, and metabolism (2). Some of the circadian genes also directly modulate cell proliferation. Members of the casein kinase 1 (CK1) family, which are key kinases in establishing the circadian oscillations of the core clock (4), also phosphorylate and destabilize β-catenin (3, 5), a component in the WNT signaling pathway that plays a key role in colorectal cell proliferation and tumorigenesis (2). PER1 has been found to interact with ATM, a kinase involved in DNA double-strand break–induced events, thus playing a role in DNA damage control (6). In a recent study, BMAL1 was identified as a putative regulator of the p53 pathway (7). Cells with suppressed BMAL1 expression were unable to activate the p53 target gene $p21^{CIP1}$ and were proposed to affect the ability of p53 to induce cell cycle arrest upon cellular stress, such as DNA damage.

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Recent studies have indicated a role for the circadian clock in cancer development. The tumor-suppressor function of the circadian clock has been proposed, and the core clock genes have been hypothesized, but not shown, to be targets of somatic mutations. Studies using human cell lines and mouse genetic models have shown that disruption of the *Per1* and *Per2* circadian clock genes leads to dysfunction of cell cycle checkpoints, leading to susceptibility to DNA damage–induced cancers. Overexpression of *PER1* in human cancer cell lines results in reduced colony formation and clonogenic expansion, sensitization to radiation-induced apoptosis, and altered expression of transcriptional target genes such as *MYC* and *p21* (6). Genetic ablation of components of the circadian clock, *Per1* and *Per2*, in mice results in complete loss of circadian rhythms and premature aging (8-10). *Per2* null mice show an increase in hyperplasia and neoplasia in response to γ-radiation (9). Moreover, Per2 mutation has been shown to accelerate intestinal polyp formation in $Apc^{Min/+}$ mice (11). Shiftwork that involves circadian disruption is probably carcinogenic to humans (2, 12). Altogether, there is strong epidemiologic and increasing molecular evidence suggesting involvement of circadian clock in cancer development.

Defects in the DNA mismatch repair system lead to genetic instability referred to as microsatellite instability (MSI), which occurs in ~15% of colorectal cancers (CRC; ref. 13). MSI tumors are near diploid, and the mechanism of carcinogenesis under mismatch repair deficiency is the generation of a large number of substitution, as well as insertion and deletion mutations, which typically target microsatellite sequences. Mutations providing growth advantage to the cell are clonally selected (14). MSI-associated small insertions and deletions in coding microsatellites lead to shifts in the reading frame, resulting to truncation or other alterations of the protein product. Expression of mRNAs with such frameshift mutations is typically somewhat decreased due to the presence of premature stop codons, which result in degradation of some of the mutant mRNA through the nonsense-mediated decay pathway.

Materials and Methods

Gene expression profiling

Combining microarray gene expression data (15) and public gene sequence information, we identified *CLOCK* as 1 of 790 genes with reduced expression in MSI CRCs compared with normal colon samples, and as having a mononucleotide microsatellite in the coding region. Thus, *CLOCK* emerged as a putative novel tumor-suppressor gene in MSI CRC, along with the other candidates. We have subsequently screened all these 790 genes for mononucleotide repeat mutations (16),[11] and CLOCK was among the genes with the highest mutation frequency.

The microarray data have been described previously (15). Briefly, 33 fresh-frozen MSI colorectal tumors and 15 normal mucosa samples were analyzed using standard Affymetrix protocols. Gene expression was assessed using HG-U133A GeneChip oligonucleotide microarrays (Affymetrix; ref. 15). Mononucleotide repeats within the coding region of the genes that were underexpressed in MSI CRCs were searched by using public human genome databases (Ensembl and National Center for Biotechnology Information).

Patient samples

Colorectal adenocarcinoma and corresponding normal tissue samples were available from a Finnish sample series collected since 1994 (13, 17). Before DNA extraction, the samples were evaluated by a pathologist to confirm the presence of malignant tissue. MSI status had been determined in earlier studies (13, 17). Patient information and samples were obtained after informed consent and ethical review board approval.

Mutation detection

Mutation detection was done using genomic sequencing. All primer sequences are available on request. Fragments with T9 repeat were amplified using the proofreading enzyme Phusion (Finnzymes). Applied Biosystems 3730 BD3.1 sequencing chemistry and AB5.1 sequencing analysis software were used. Normal tissue samples were always used to confirm the somatic origin of mutations.

Statistical regression analysis

The dependency of the mutation rate on the repeat length was modeled using a general four-parameter sigmoid nonlinear regression model as suggested previously (18).

Cell culture

Cell culture was carried out using standard protocols. Cell lines were purchased from the American Type Culture Collection and the European Collection of Cell Cultures or provided by Dr. Ian Tomlinson (Laboratory of Molecular and Population Genetics, Cancer Research UK, London, UK). The following MSI CRC cell lines were used in the study: LoVo, HCT15, CCL231, HCT116, LS174T, RKO, HCT8, GP5D, HUTU80, LS180, SNUC2B, VACO5, and HCA7. Embryonic kidney cell line HEK293 was also used.

Protein expression

Proteins extracted from HEK293 and the MSI CRC cell lines HUTU80, RKO, GP5D, LoVo, LS180, LS174T, and VACO5 were analyzed. Cell pellets were lysed in radioimmunoprecipitation assay buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Diagnostics). Sample concentrations were measured by Bicinchoninic Acid Protein Assay Reagent Kit (Pierce). Protein aliquots were loaded onto a 10% polyacrylamide gel (Bio-Rad Laboratories), and fractionated proteins were transferred onto a polyvinylidene fluoride membrane (Millipore). Immunostaining with anti-CLOCK antibody (Santa Cruz Biotechnology; H-276, sc-25361, corresponding to amino acids 571–846 of human CLOCK) was carried out, after which protein expression was detected using Western

[11] Manuscript in preparation.

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Breeze Chemiluminescent Immunodetection System (Invitrogen Life Technologies). Ponceau-S staining was used as a loading control.

Luciferase assays

Luciferase assays were done in 293T cells using 30 ng reporter gene mix (25 ng 3xCLOCK reporter + 5 ng Renilla luciferase control reporter) cotransfected with 100 ng CLOCK, CLOCK-T8 mutant of green fluorescent protein (GFP) fused with 3xV5 tag or VP16 transactivation domain, and 20 ng BMAL cDNA or control plasmid. DNA was mixed with 0.225 μL Fugene HD, added to cells, and incubated for 24 hours. Luciferase activities were measured with the DualGlo kit (Promega).

Functional analyses

Full-length human CLOCK in pDEST27 and empty vector control were transfected in CLOCK-negative LS180 cells with FugeneHD and selected in the presence of 0.5 mg/mL G418. Two independent colonies expressing CLOCK as determined by Western blotting were used for functional analysis.

Stable LS180 cells were plated in 1% fetal bovine serum and cultured for 1 day to arrest cells in G_1. Cells were then mock treated or irradiated with 10 Gy using a ^{137}Cs γ-ray source (BioBeam 8000, STS) or with 30 J/m^2 UV-C (Stratalinker, Stratagene). Medium with 10% serum was added, and cells were cultured for 2 additional days before DNA content analysis with flow cytometry as described previously (19). In some experiments, caffeine was added to a 2 mmol/L final concentration 15 minutes before irradiation and kept until DNA content analysis to inhibit DNA damage checkpoint activation.

Chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation (ChIP) was done in 293T cells by transiently transfecting wild-type (WT) CLOCK-3xV5 or GFP-3xV5. Cells were incubated 24 hours after transfection followed by DNA cross-linking and immunoprecipitation with monoclonal anti-V5 antibodies (Invitrogen). Immunoprecipitated DNA was amplified and sequenced with Illumina Genome Analyzer II system using standard protocols.

Results

Somatic *CLOCK* mutations were identified in most MSI CRCs and the mutations resulted in reduced expression of CLOCK

To examine whether mutations in *CLOCK* were involved in colorectal tumorigenesis, a total of 101 MSI CRCs were analyzed for a coding microsatellite of nine thymines (T9) in *CLOCK* exon 8. *CLOCK* T9 was somatically mutated in 53 of 101 (53%) MSI CRCs. The mutations identified included a deletion of one thymine (c.368delT, T8; 47 of 53 samples), an insertion of one thymine (c.368_369insT, T10; 2 of 53), and a deletion of two thymines (c.367_368delTT, T7; 4 of 53) in the coding region of *CLOCK*. One primary cancer displayed a homozygous T8 mutation. The mutations in T9 are predicted to result in premature termination codons and truncation of the protein product (Figs. 1A–D and 2A). All mutations detected result in a truncated CLOCK protein that contains the DNA-binding bHLH domain and up to 14 amino acids of the PAS-A domain, which is required for the dimerization specificity with the other bHLH-PAS transcription factors (20, 21). The T8

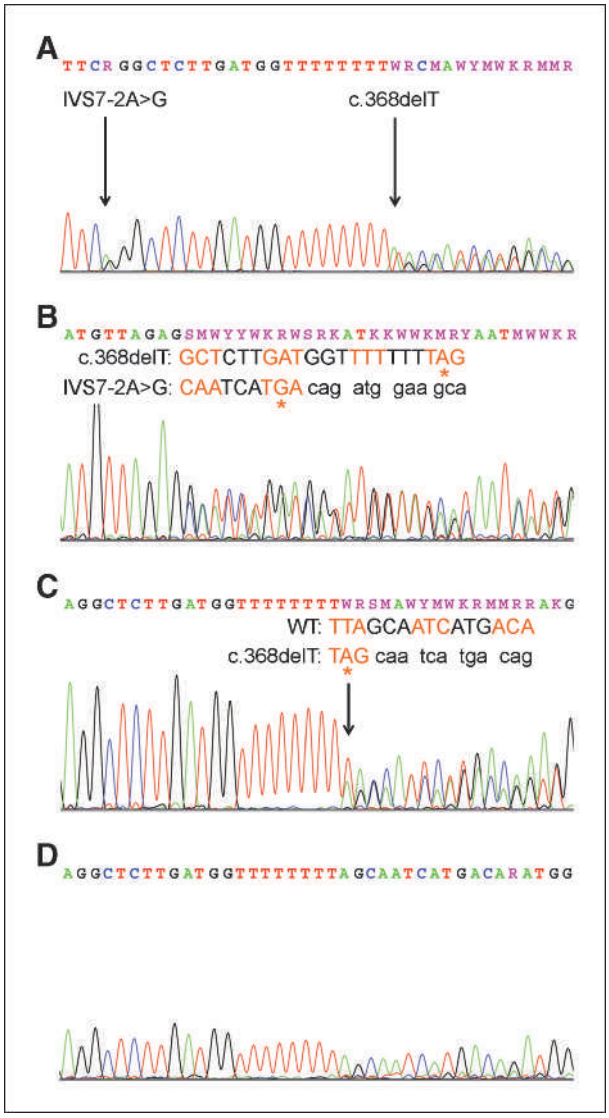

FIGURE 1. Examples of mutations observed in the *CLOCK* gene. A, genomic sequence from the RKO cell line showing the IVS7-2A>G splice site and c.368delT (A8) mutations. B, respective cDNA sequence from the RKO cell line. Transcripts of both mutant alleles are depicted above the sequence. No WT sequence is present. C, cDNA sequence from c.369delT (A8) heterozygote HCA7 cell line. Transcripts of both WT and mutant alleles are written above the sequence. The mutant allele is reduced probably due to nonsense-mediated mRNA decay, but is present in significant amounts. D, cDNA sequence graph of homozygous c.368delT deletion (A8) in MSI colorectal tumor sample. Very low WT signal originating from normal tissue contamination is also visible. *, stop codon.

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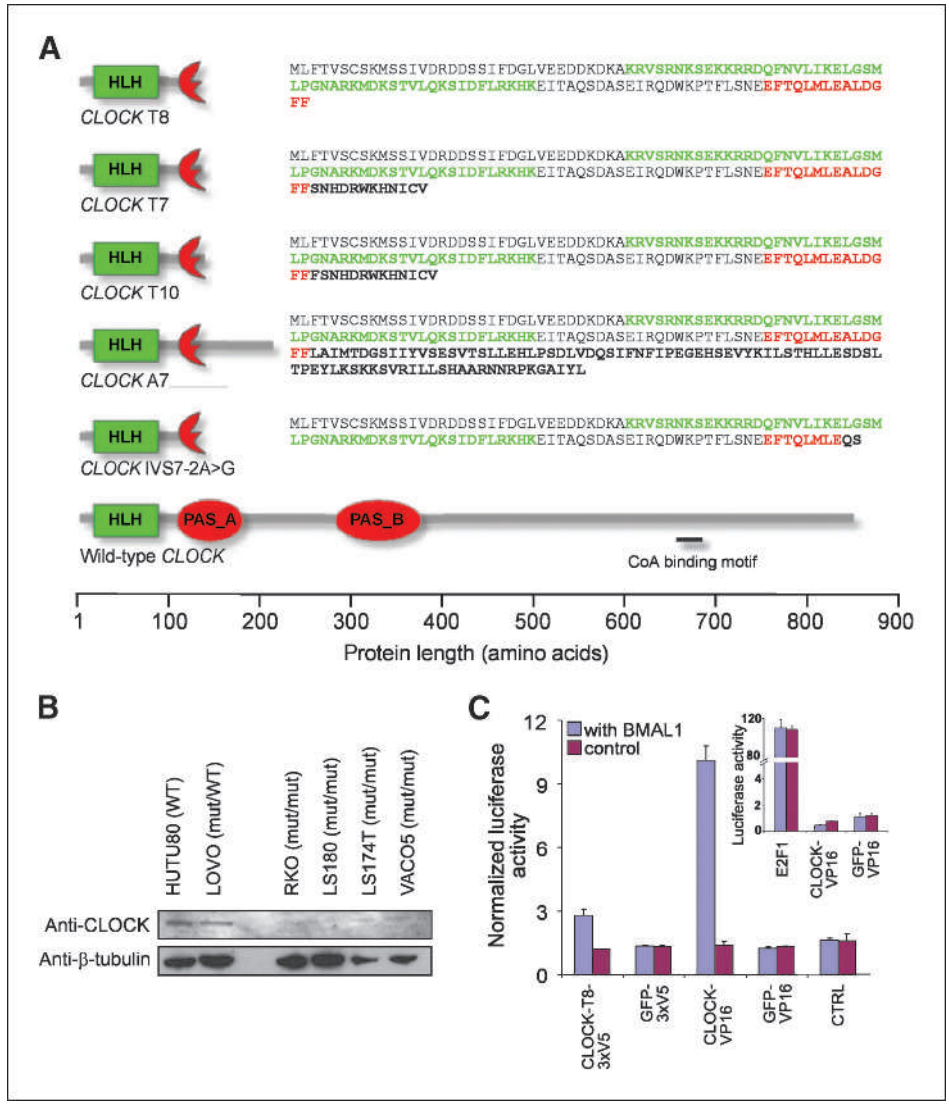

FIGURE 2. A, predicted sequences and domain structure of WT and mutated CLOCK proteins identified in MSI colorectal cancer samples. Unique sequences after the frameshift mutations that are not present in the full-length protein are in bold black typeface. The helix-loop-helix DNA binding domain (HLH) and PAS_A/B domains are in green and red, respectively. Acetyl CoA binding motif required for histone acetyl transferase activity is also indicated. B, expression of endogenous full-length human CLOCK analyzed by Western blotting in CRC cell lines with WT or heterozygous (mut/WT) and homozygous (mut/mut) CLOCK cell lines. β-Tubulin was used as a loading control. C, CLOCK-T8 protein specifically activates CLOCK reporter gene expression in the presence of BMAL1 in 293T cells. The activation is much stronger when the sequence present in CLOCK-T8 is fused to the VP16 viral transactivation domain (CLOCK-VP16). Reporter activation is specific as CLOCK-VP16 fails to induce E2F control reporter (inset).

mutation results in a truncated protein with no additional amino acid residues, whereas the other identified mutations contain 2 to 88 additional amino acid residues not present in WT CLOCK (Fig. 2A).

All 53 MSI CRCs with somatic mutations in the T9 tract were subsequently sequenced for the entire coding region of *CLOCK*. One tumor harbored an insertion in an A6 microsatellite in *CLOCK* exon 10 (c.566_567insA), resulting in premature termination codon (Fig. 2A).

Nonlinear regression analysis was done to compare mutation frequencies in the coding *CLOCK* microsatellite and in intronic presumably neutral control repeats to examine whether the mutation rate in *CLOCK* exceeded the background mutation frequency. In this analysis, *CLOCK* was significantly more often mutated than the T9 control repeats (Supplementary Fig. S1).

We next examined the possible occurrence of mutations in *CLOCK* T9 in 13 MSI CRC cell lines. LS180, LS174T (having the same origin as LS180), and VACO5 harbored a biallelic T8 mutation; GP5D harbored a T8 mutation with an insertion (T10) in the other allele; SNUC2B, RKO, HCA7, and LoVo were heterozygotes for T8; and HCT15, HCT8, CCL231, HUTU80, and HCT116 were WT for T9. A total of 30 microsatellite stable colorectal cancers were sequenced for the entire coding region of *CLOCK* with negative results.

Expression of CLOCK protein was studied in eight MSI CRC cell lines (HUTU80, GP5D, RKO, LoVo, HCA7, LS180, LS174T, and VACO5) and in HEK293 cells. Full-length CLOCK was present in all WT cell lines (HUTU80, HEK293), but reduced in heterozygous mutant cell lines (HCA7, LoVo) and absent in homozygous mutant cell lines (LS180, LS174T, VACO5, GP5D), as well as the heterozygous mutant RKO (Fig. 2B). The lack of detectable CLOCK protein in RKO cells prompted us to sequence this cell line for the entire coding region of *CLOCK*, which

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resulted in identification of IVS7-2A>G splice site point mutation, predicted to cause protein truncation through aberrant mRNA splicing. The effect of the IVS7-2A>G mutation on CLOCK mRNA was studied by cDNA sequencing. This analysis revealed that IVS7-2A>G mutation resulted in destruction of the acceptor splice site in intron 7. As a consequence, the AG sequence in exon 8 is used in mRNA splicing, which leads to a 22-bp deletion and truncation of the predicted protein product (Figs. 1A–B and 2A). Thus, the outcome of this point mutation is very similar to the mutations in the T9 tract (Fig. 2). The splice site mutation was shown to reside in the allele WT for the T9 tract, demonstrating biallelic *CLOCK* mutation also in the RKO cell line (Fig. 1). Endogenous mutant protein was not detectable in any cell lines by Western blotting.

CLOCK T8 mutant protein heterodimerizes with BMAL1 and may act in a dominant-negative manner

As most of the primary CRCs as well as cell lines harbored monoallelic *CLOCK* mutations and as the mutations were predicted to eliminate the PAS domain, we hypothesized that the mutations might act in a dominant-negative manner, the mutated CLOCK transcript might be targeted for nonsense-mediated decay, and the consequences of the mutations might emerge through haploinsufficiency. We thus tested the ability of the CLOCK-T8 mutant protein to bind the CLOCK target element, the E-box sequence. The CLOCK-T8 mutant, but not GFP tagged with a triple-V5 tag, weakly but specifically activated a CLOCK luciferase reporter containing three E-box binding sites when coexpressed with BMAL1 cDNA. No induction of reporter activity was observed by using CLOCK-T8 or BMAL alone, indicating that CLOCK-T8 and BMAL1 cooperatively induce the CLOCK reporter activity. To further show that all features of CLOCK necessary for E-box recognition and dimerization with BMAL1 are present in the CLOCK-T8 sequence, we replaced the COOH-terminal part of WT CLOCK that contains the endogenous transactivation domain with that of VP16 viral transactivation domain. This CLOCK-VP16 construct showed robust BMAL1-dependent activation of transcription. CLOCK-T8 and BMAL1 specifically bound the E-box element as a reporter with E2F binding sites could not be induced (Fig. 2C, inset). Altogether, these data show that in the presence of BMAL1, the CLOCK-T8 fragment is sufficient for sequence-specific DNA binding, and this mutant construct is able to heterodimerize with BMAL1 despite the lack of PAS domain and could potentially act in a dominant-negative manner.

Restored CLOCK expression causes protection against UV-induced apoptosis and decreased G_2-M arrest after ionizing radiation

To analyze the functional consequences of CLOCK in colon cancer cells, we restored CLOCK expression in LS180 cells lacking WT CLOCK by stably expressing GST-CLOCK or glutathione *S*-transferase (GST) empty vector as a control. Analysis of two independent cell sublines indicated that there were no significant differences in the proliferation rate of the LS180 cells in the presence or absence of CLOCK under normal culture conditions (not shown). Significant protection against UV-induced apoptosis was seen in both sublines after restoring CLOCK expression, as detected by DNA content analysis by flow cytometry (Fig. 3A and B and not shown). CLOCK-expressing cells also showed decreased G_2-M arrest as a response to ionizing radiation (Fig. 3A and C) as observed previously with PER1 (6). This effect was specific and could be abrogated with the DNA damage checkpoint inhibitor caffeine (not shown).

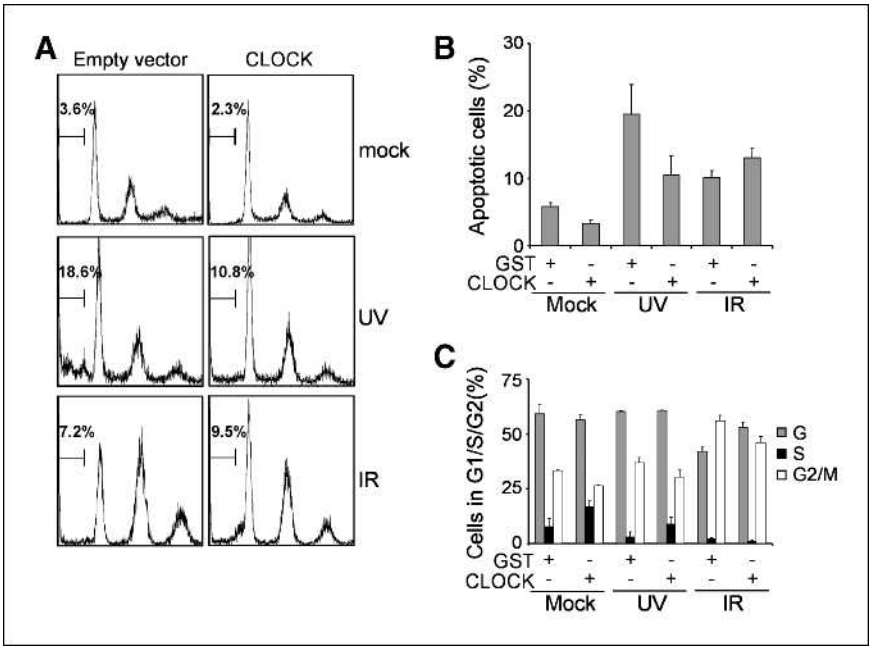

FIGURE 3. Biallelic *CLOCK* T8 mutation and cellular DNA damage response. A, cell cycle profiles of empty vector and CLOCK-expressing LS180 cells under normal culture conditions (mock) or after DNA damage caused by UV light (UV) or ionizing radiation (IR). The percentage of cells in the sub-G_1 fraction corresponding to apoptotic cells is indicated with bars. B, quantification of apoptotic effect in UV- and IR-treated LS180 cells. Note that IR treatment induces apoptosis to similar levels in both control and CLOCK-expressing cells. C, percentage of cells in different cell cycle phases after mock, UV, or IR treatment. CLOCK-negative cells are strongly arrested in G_2-M.

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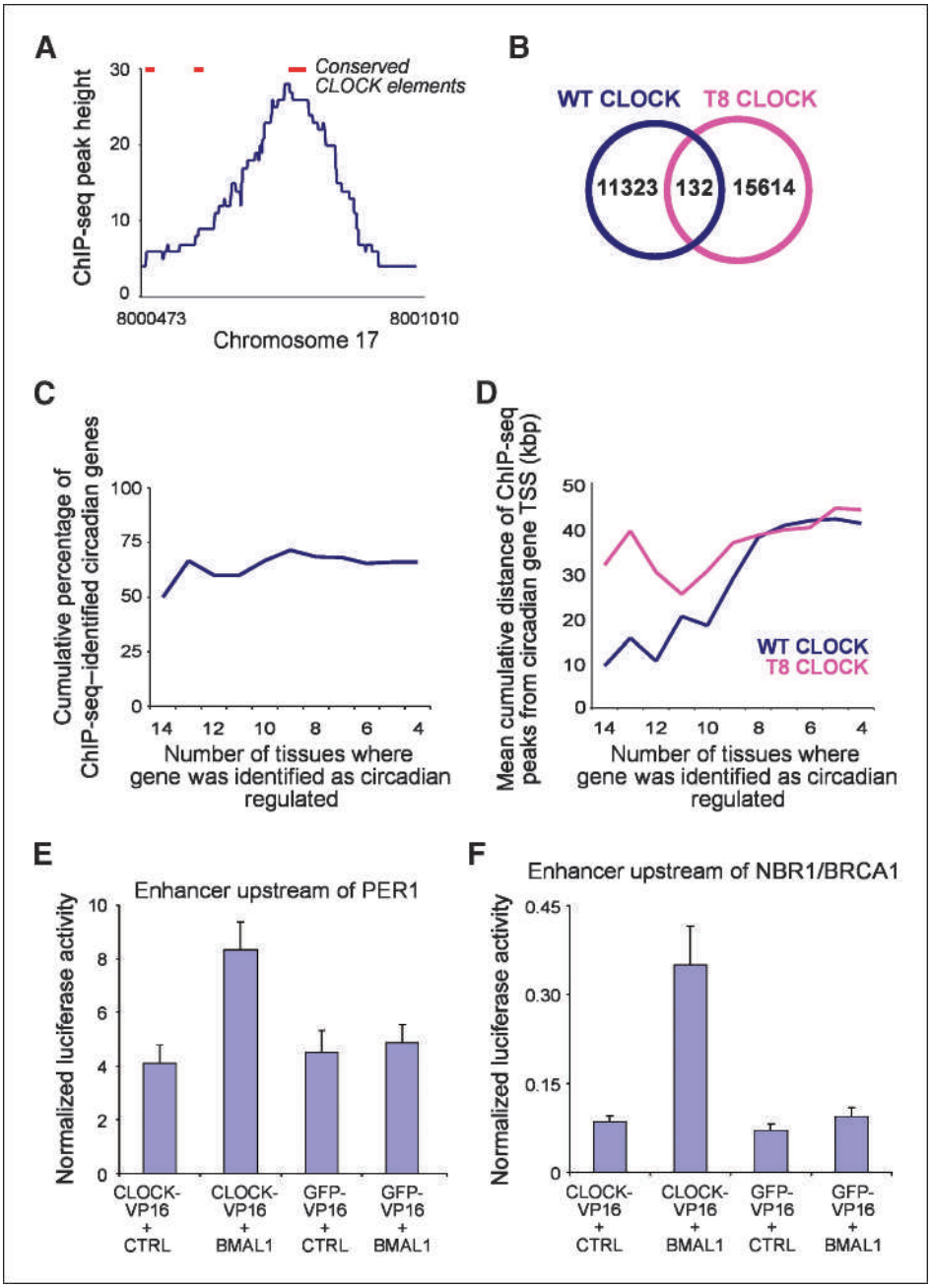

FIGURE 4. ChIP sequencing of CLOCK identifies true circadian-regulated genes. A, the most significant peak observed in 293T cells is located 4 kb upstream of PER1 and contains four conserved previously identified CLOCK-binding elements (red lines; ref. 20). B, Venn diagram showing the overlap of T8 CLOCK mutant and WT CLOCK ChIP-seq peaks. C, ChIP-seq with WT CLOCK identified most known circadian-regulated genes. Cumulative percentage of identified circadian genes is plotted as the function of being circadian regulated as determined by detection of circadian expression in different tissues starting from the highest confidence circadian genes. Expression in a higher number of tissues is considered as higher confidence for circadian regulation. D, mean distance of significant ChIP-seq peaks from the transcription start sites (TSS) of the circadian-regulated gene is shown for T8-CLOCK mutant (pink) and WT CLOCK (blue). E, activation of luciferase reporter containing the CLOCK-binding PER1 −4 kb region in 293T cells with the coexpression of CLOCK-VP16 and BMAL1 but not with GPF-VP16 or CLOCK-VP16 alone. F, activation of luciferase reporter containing a CLOCK-binding element from the vicinity of *NBR1* and *BRCA1* genes in 293T cells. Error bars, 1 SD ($n = 3$).

Identification of novel CLOCK target genes using ChIP-seq

To distinguish between direct and p53-dependent indirect regulation of p21, and to search for other putative CLOCK-regulated genes that affect DNA damage and colon cancer progression, we performed ChIP followed by massive parallel sequencing (ChIP-seq) in 293T cells transfected with V5 epitope–tagged WT CLOCK, T8-CLOCK mutant, or GFP as a negative control. To date, no ChIP-seq experiments have been described for the identification of circadian-controlled genes in any organism. Altogether, 14,086 peaks were identified in the CLOCK ChIP-seq sample; 11,315 of these peaks showed statistically significant enrichment ($P < 0.05$) compared with the GFP-transfected sample.

The strongest peak in CLOCK ChIP-seq sample was located 4 kb from the PER1 transcription start site, a known target gene of CLOCK. In a previous bioinformatics-based identification of putative CLOCK-binding elements (22), this peak contained four CLOCK binding sites that are conserved between mouse and human (Fig. 4A; Supplementary Fig. S2). A smaller peak was additionally found

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14 kb upstream of the PER1 transcription start site, thus further validating our ChIP-seq approach. In contrast, the T8-CLOCK mutant showed a completely different pattern of ChIP-seq peaks. Only 132 overlapping peaks between these samples were identified; the overlapping peaks did not include PER1 or any of the other highest scoring peaks in the WT CLOCK sample (Fig. 4B). This suggests that the consequence of T8 mutation would be a complete loss of normal CLOCK target gene regulation.

To further confirm the validity of our ChIP-seq analysis, we compared the genes identified in the vicinity of the ChIP-seq peaks with genes having cycling expression pattern in a previous meta-analysis of circadian genes in mammalian tissues (23). These genes were ranked based on the number of tissues (out of 14) in which they show cycling behavior. Using a ±150 kbp maximum distance between transcription start site and the ChIP-seq peaks, we could identify CLOCK binding sites in the vicinity of 65% of all cyclically expressed circadian genes (Fig. 4C). In the WT clock sample, but not in the T8-CLOCK mutant sample, the mean distance of ChIP-seq peaks from the transcription start sites of the putative circadian genes increased with decreasing circadian confidence (Fig. 4D), indicating that the CLOCK binding sites in the highest confidence circadian genes are in close proximity to the transcription start site. The putative circadian genes and their distance to the nearest CLOCK ChIP-seq peaks are shown in Supplementary Table S1. Circadian genes near which we found no enrichment of CLOCK are listed in Supplementary Table S2 and are possibly driven by other transcription factors.

The peak –4 kbp from the PER1 transcription start site also drove luciferase expression when cotransfected with both CLOCK-VP16 and BMAL1 (Fig. 4E). Another tested CLOCK binding element identified by ChIP-seq, located near *NBR1* and *BRCA1* genes, also strongly induced luciferase reporter expression with CLOCK-VP16 and BMAL1 cotransfection (Fig. 4F), indicating that the ChIP-seq approach was able to identify CLOCK-regulated enhancer elements.

We also detected significant enrichment of ChIP-seq peaks 20 kbp upstream and 54 kbp downstream from the transcription start site of *CDKN1A*, the gene encoding the cell cycle inhibitor p21. Both of these elements contained a single match to the E-box element when analyzed with the HMMER software (Supplementary Fig. S3), although these scored lower than the matches in the PER1 element. No ChIP-seq peaks were found near the *p53* gene, suggesting that the regulation of p21 by CLOCK is direct and p53 independent. Furthermore, *CDKN1A* is one of the highest confidence circadian-regulated genes with cyclic expression detected in 10 out of 14 mammalian tissues analyzed (Supplementary Table S1; ref. 23).

We then searched for other putative novel target genes with a potential role in colon cancer progression. Interestingly, the second most significant ChIP-seq peak was found in chromosome 5 and was located 73 kb from the transcription start site of the DNA repair protein RAD50. RAD50 protein is part of the MRE11, RAD50, and NBS1 (MRN) complex that responds to DNA double-strand breaks. Components of the MRN complex are mutated in mismatch repair–deficient cancers leading to decreased levels of all MRN complex components (24). Cells depleted for RAD50 became hypersensitive to UV exposure and a defective intra–S phase checkpoint on exposure to ionizing radiation that leads to radioresistant DNA synthesis (25). Our results with stable CLOCK transfectants in RKO cells are also consistent with the phenotype of RAD50 RNAi-depleted cells, as we observe reduced apoptosis in UV-treated CLOCK-expressing cells and G_1 arrest in γ-irradiated cells. The full list of CLOCK ChIP-seq peaks and the annotated genes within 150 kbp of them are shown in Supplementary Table S3. ChIP-seq peaks in the vicinity of multiple DNA damage genes suggest that CLOCK mutation has a widespread effect on cellular homeostasis.

Discussion

In this study, we found *CLOCK* mutations in the majority of the 101 MSI CRCs cancers examined. These mutations result in truncation of the respective predicted protein products. Most cancers displayed only one mutation, although biallelic inactivation was also observed (Fig. 1D), suggesting that a second mutational hit might provide an additional phenotypic effect. As the mutations were predicted to disrupt the PAS domain, we hypothesized that the mutations might act in a dominant-negative manner. The mutated CLOCK transcript could also be targeted for nonsense-mediated decay, and the consequences of the mutations might emerge through haploinsufficiency. Alternatively, all these mechanisms could be at work.

Any mutant protein expressed would contain an intact DNA-binding helix-loop-helix domain and a fragment of the PAS-A domain (Fig. 2A). The bHLH domain is responsible for the DNA binding activity and dimerization of the bHLH proteins. The PAS domains are required for the dimerization specificity with the other bHLH-PAS transcription factors, such as aryl hydrocarbon receptor, hypoxia-inducible factor HIF-1α, the aryl hydrocarbon receptor nuclear translocator, and the CLOCK dimerization partner BMAL1 (20, 21). DNA binding of the bHLH domain, in the absence of the PAS domain, has been investigated using the aryl hydrocarbon receptor as a model and indicates that the PAS domain conformationally regulates the DNA-binding specificity of the bHLH domain. The bHLH domain only may also dimerize with other bHLH domain proteins (21). Our ChIP-sequencing data are in accordance with these studies and show that the CLOCK T8 construct changes the genome-wide locations of this transcription factor completely, although DNA-binding specificity is retained as observed by luciferase reporter assay (Fig. 2C). As the most COOH-terminal portion of the CLOCK protein is required for transactivation, it is evident that the mutant form of CLOCK does not induce expression of new target genes.

Our findings are consistent with those observed in an animal model. In mice, a dominant-negative *Clock* mutant with a 51-amino-acid in-frame deletion in the COOH

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terminus of Clock binds DNA and dimerizes with Bmal1 similarly to the WT protein, but does not activate transcription (26). These mice have a phenotype affecting both the periodicity and persistence of circadian rhythms. Mice with homozygous *Clock* mutation respond to low doses of ionizing radiation by developing premature aging (27). Thus, also in colon cancer, the first mutational hit in *CLOCK* may be the most efficient for tumorigenesis, and the subsequent elimination of the remaining WT activity may provide additional phenotypic effect exactly as predicted in 1997 by King et al. (28) based on mouse phenotypes.

We analyzed the effect of CLOCK expression in CLOCK-mutant cells and found no difference in the proliferation rate in the presence or absence of CLOCK under normal culture conditions. This is in accordance with previous observations showing that Clock mutant mice display normal embryogenesis (29). Interestingly, significant protection against UV-induced apoptosis was observed in CLOCK-expressing cells, consistent with a tumor-suppressing function. Moreover, CLOCK-expressing cells showed decreased G_2-M arrest as a response to ionizing radiation, similar to previous observations made in PER1-overexpressing cells (6). Cellular responses to DNA damage include a network of checkpoint pathways involved in activation of DNA repair, cell cycle arrest, and apoptosis. Following genotoxic stress, tumor-suppressor genes, such as *p53*, act primarily by inducing growth arrest, DNA repair, or apoptosis. We show that CLOCK operates on these pathways. Our findings that CLOCK mutations do not directly affect CRC cell growth but instead alter cellular responses to DNA damage suggest that CLOCK may act as a "caretaker" rather than a classic tumor-suppressor gene (30).

Although the exact mechanisms of CLOCK-related tumorigenesis remain to be clarified and likely involve regulation of multiple cellular processes, the present data show that loss of *CLOCK* confers a cancer-prone cellular phenotype. *CLOCK* is thus the first example of a circadian clock gene directly targeted in cancer by somatic mutations. The effect of the mutations on therapeutic efficiency is an intriguing target for translational studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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