Discovery of a Regulatory Motif for Human Satellite DNA Transcription in Response to *BATF2* Overexpression

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Abstract. *Background: One of the basic leucine zipper transcription factors, BATF2, has been found to suppress cancer growth and migration. However, little is known about the genes downstream of BATF2. Materials and Methods: HeLa cells were stably transfected with BATF2, then chromatin immunoprecipitation-sequencing was employed to identify the DNA motifs responsive to BATF2. Results: Comprehensive bioinformatics analyses indicated that the most significant motif discovered as TTCCATT[CT]GATTCCATTC[AG]AT was primarily distributed among the chromosome centromere regions and mostly within human type II satellite DNA. Such motifs were able to prime the transcription of type II satellite DNA in a directional and asymmetrical manner. Consistently, satellite II transcription was up-regulated in BATF2-overexpressing cells. Conclusion: The present study provides insight into understanding the role of BATF2 in tumours and the importance of satellite DNA in the maintenance of genomic stability.*

The basic leucine zipper transcription factor, activating transcription factor-like 2 (BATF2) belongs to a protein family that shares homologous basic leucine zipper domain at the N-terminus of the amino acid sequences. Human BATF2 [also reported as suppressor of activator protein-1(AP1), regulated by interferon (SARI)], was found to have a pronounced effect on induction of apoptosis and tumor suppression. In normal cells or transformed benign cells, such as primary human foetal astrocytes, immortal mammary epithelial cells, and immortal pancreatic mesothelial cells, *BATF2* mRNA levels were shown to be much higher than those of the malignant cells originating from the same tissue type (1, 2). Suppression of BATF2 expression initiated epithelial–mesenchymal transition in lung adenocarcinoma cell lines by modulating the Glycogen Synthase Kinase-3β-β-catenin signaling pathway (3). In clinical studies, decreased expression of BATF2 was suggested to correlate with poor prognosis in hepatocellular carcinoma (4), and down-regulation of *BATF2* mRNA levels were found in chronic myelogenous leukemia involving the suppression of breakpoint cluster region-ABL proto-oncogene 1 (BCR-ABL) activity and the downstream signaling pathways (5). BATF2 inhibited the transcriptional activity of AP1 presumably through combination with JUN proto-oncogene (c-JUN) to form a heterodimer and compete with the native FOS-JUN complex (1, 6). In our earlier studies, the overexpression of BATF2 also inhibited the transcriptional activity of nuclear factor-κB as well as AP1, suggesting more sophisticated roles for BATF2 in transcriptional regulation of gene expression than previously supposed (7). In a recent report, Cyr61-CTGF-Nov family 1 (CCN1) was suggested to be the only validated gene responsive to BATF2 overexpression by reducing AP1 binding in the promoter regions (8). Since a growing amount of evidence demonstrated the importance of BATF2 in tumor genesis and progression, intensive exploration and identification of the BATF2-regulated downstream genes at the genomic level, as well as an understanding of the role of BATF2 in normal cells, became an immediate and practical task for its mechanistic studies.

Chromatin immunoprecipitation (ChIP) assays in combination with high-throughput DNA sequencing (ChIP-sequencing) technology allow genome-wide unbiased searches for the exact binding sites of various transcription factors, especially in stably transfected cells (9). Besides classical DNA-binding motifs of transcription factors, genome-wide sequencing has also revealed an increasing set of transcribed non-coding sequences (ncRNA), including

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'pervasive transcription' controlled by heterochromatic regions (10). Such findings have greatly expanded the understanding of regulation of gene expression at the genomic level, part of which is related to transcriptional silencing and chromosomal integrity.

ChIP-sequencing technology has also been extensively used in the research of heterochromatin for the discovery of associated proteins. Heterochromatin, as the more condensed fraction of nuclear chromatin in interphase of the cell cycle, serves a variety of functions in mammalian cells, including the maintenance of genomic stability, as well as the regulation of gene expression during development and differentiation (11, 12). In the human genome, satellite DNA constitutes a significant part of heterochromatin, and satellite DNA transcripts are able to modulate gene expression and epigenetic modification of genome (13). Accumulation of satellite transcripts has been found in both human and other mammalian cell lines in response to certain stress stimuli, including DNA demethylation, heat shock, and induction of apoptosis, or gene integration and gene overexpression-induced genomic instability (14-16).

In this study, we performed ChIP-sequencing to investigate the overexpressed BATF2 regulatory circuitry by mapping the genome-wide occupancy of BATF2 binding in human HeLa cells with stable BATF2 overexpression. Our findings provided clues for understanding the role of BATF2 in tumors.

Materials and Methods

Molecular cloning and reporter construction. The coding sequence of human BATF2 (NM_138456) was polymerase chain reaction (PCR) amplified and cloned into the *Eco*R I and *Xba* I (TaKaRa, Dalian, China) restriction sites of p3×Flag-CMV plasmid vector (Sigma, St. Louis, MO, USA). The primer pairs used were 5'-GGAATTC GATGCA CCTCTG TGGGGG CAATG-3' and 5'-GC TCTAGA TTAGAA GTGGAC TTGAGC AGAGGA-3'.

The pGL3-Basic vector was used to create a set of luciferase reporters to validate the regulatory functions of identified BATF2 targeted motifs. The PCR primers used for vector construction and qPCR verification are shown in Table I.

Cell culture and transfection. HeLa cells (American Type Culture Collection, Manassas, VA, USA) as well as the derived stable line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). A total of 3×10^5 cells were seeded in each well of 6-well plates for 24 h prior to the transfection of 2 μg p3×Flag-CMV-BATF2 plasmids using the X-tremeGENE HP DNA Transfection Reagent (Roche, Palo Alto, CA, USA). The transfection procedures were performed following the manufacturer's instructions.

Establishment of HeLa-B2 cells stably overexpressing BATF2. Native HeLa cells were seeded in 12-well plates at 1×10^5 per well for 24 h. Transfection of p3×Flag-CMV-BATF2 was performed. Selection with 200 μg/ml G418 (Sigma,St. Louis, MO, USA) was started after 48 h post-transfection and continued for 7 days. The obtained viable cells were then subcloned by limited dilution, and the finally diluted cells were maintained in DMEM with 10% FBS with the presence of G418 (200 μg/ml) for an additional 3 or 4 weeks. When the subcloned cells reached 70 - 80% confluence, they were transfered to 6-well plates. The subcloned cells were subjected to western blot analyses for detection of FLAG-tagged BATF2 proteins. A cell clone with moderated overexpression of the transgene and normal morphology, which was also able to expand at a normal rate in 150 μg/ml G418-containing medium, was selected (designated HeLa-B2) and subjected to further analyses.

Cell proliferation assay. The proliferation of native HeLa cells and derived HeLa-B2 cells were evaluated using the CellTiter 96^R A_{Queous} One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The readout from triplicate samples was averaged, normalized to the values at time 0, and are represented as the mean±S.E.

Flow cytometry. Cells maintained in 100 mm dishes were diluted to a density of approximately 1×10^6 viable cells/ml. Ice-cold 70% ethanol was used for fixation of cells by incubation at −20˚C for 2 h. The fixed cells were resuspended in 0.5 ml PI/RNase Staining Buffer (BD Biosciences, San Jose, CA, USA), and then immediately loaded to a BD FACSria flow cytometer for detecting the cell cycle. The data analysis was conducted using the FACSDiva 4.1 software (BD Biosciences) with appropriate parameters.

Western blot. HeLa cells were harvested and lysed with RIPA solution (Thermo, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Thermo, Waltham, MA, USA). The exact Supernatant proteins (40 μg) were loaded as each sample for SDS-polyacrylamide gel electrophoresis. Primary antibodies were as follows: anti-Flag (1:500; Cell Signaling Technology, Beverly, MA, USA), anti-BATF2 (1:500; Santa Cruz) and anti-β-actin (1:1,000; Santa Cruz, CA, USA). Goat anti-rabbit 680/790 and goat anti-mouse 680/790 (1:10,000); LI-COR Biosciences, Cambridge, UK) were used as secondary antibodies. The membranes were scanned with an Odyssey™ Infrared Imager (LI-COR).

Immunofluorescence microscopy. HeLa and HeLa-B2 cells were fixed with 4% paraformaldehyde, and permeabilized with 1% Triton X-100 in 12-well plates. The cells were then sequentially incubated with anti-Flag (1:200; Cell Signaling Technology, Beverly, MA, USA) and anti-non-histone heterochromatin protein 1 (HP1) (1:200; Cell Signaling Technology, Beverly, MA, USA) primary antibodies, and with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:1000; Santa Cruz, CA, USA). 4',6-diamidino-2-phenylindole (DAPI) was used for the staining of nuclei. Images were acquired from a Leica DM5000 B fluorescent microscope or Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) by Leica Application Suite software.

Luciferase reporter assay. HeLa cells seeded in 24 well-plates were transfected with 1 μg plasmid DNA with X-tremeGENE HP DNA Transfection Reagent. Cells were treated with 2,000 U/ml IFN-β for 12 h at 24 h post-transfection. The luciferase activities were assayed using a Luciferase Kit (Promega, Madison) following the manufacturer's instructions. The data recorded from a Lumat LB9507 luminometer (Berthold, EG&G, Wildbad, Germany) based on three independent experiments were normalized and averaged for statistics.

Table I. *The oligos used for vector construction and qPCR quantifications.*

Cloning primers		Sequences
Upstream	Forward	GGTACC ATCGAATGGAATCGAATGGAA ATGGAAGATGCCAAAAACAT
	Reverse	GGTACC AAGGTAAGCTAAGGTAAGCTA ATGGAAGATGCCAAAAACAT
	Scramble	GGTACC AATCGAAAAGGTTGAGGTACA ATGGAAGATGCCAAAAACAT
	Downstream (common)	GTCGAC GGATCCTTATCGATTTTACC
Detection primers		**Sequences**
SAT II		F: CGAGTCCATTCGATGATAA; R: CGGAATTGAATCGAATCGTCA
chr 2		F: TACAGAGAGATTTGAAGAGTG; R: TTCTTTATAGTCCTTCTTGGT
chr 7		F: CGTTTAAAGTATTGCTGTGTG; R: GTAGATCTGGCTTCAAGTATA
chr 16		F: ACCTACCTATAGGGGTACTCC; R: GCATCTCATGGAGTTAAACCT
chr 16'		F: GTCGCATGCTGGACACAGCAG; R: CCTCACCTATGTGCAGG

Quantitative real-time polymerase chain reaction (qPCR). The PCR was performed in triplicate using SYBR Premix Ex Taq PCR kit (Takara, Dalian, China) on an MX3000P PCR thermo-cycler (Agilent, Santa Clara, CA, USA). The parameters were set as the following: 95˚C for 5 min; followed by 95˚C for 30 s, 52˚C for 30 s and 72˚C for 15 s, for 35 cycles in total; then 72˚C for 5 min and holding at 4˚C for 15 min. The primers used are listed in Table I.

Chromatin immunoprecipitation (ChIP). HeLa and HeLa-B2 cells from 100 mm dishes at 1×10^7 were collected and treated with 1% formaldehyde for cross-linking reactions. Cells were then incubated with 125 mM glycine for 5 min to quench excess cross-linking reactants. The samples were then exposed to cold lysis buffer supplemented with protease inhibitors to allow the release of nuclei by incubation for 30 min at 4˚C. The nuclei were resuspended in nuclear lysis buffer with protease inhibitors and then sonicated to shear the chromatin to 150-500 bp. Mouse anti-Flag antibodies and nonspecific mouse IgG (Sigma, St. Louis, MO, USA) were added to the supernatant for overnight incubation at 4˚C. Additional protein G-agarose pre-blocked and suspended in ChIP dilution buffer was introduced for 3 h agitation at 4˚C. The eluates were incubated at 65˚C overnight to reverse crosslinks. Purified DNA was then subjected to sequencing analysis.

ChIP sequencing. The immunoprecipitated DNA was subjected to library preparation and sequenced on an Illumina platform at Novogene Life Sciences Company, China. Massively parallel sequencing of the ChIP products was performed using an Illumina Hiseq 2000 platform. The obtained sequences were aligned to the *Homo sapiens* reference genome version GRCh37 using bowtie (version 0.12.7) software (http://bowtie-bio.sf.net.). Regions with read enrichment were detected using Model-based Analysis of ChIP-Seq (MACS, version 1.4.1) (https://github.com/taoliu/MACS/downloads) method with the inclusion of only mapped unique reads (17). MEME (Multiple Em for Motif Elicitation) was used to identify statistically over-represented consensus motifs within the inferred binding sites using the default parameter set (18, 19). The identified motifs of most significance were matched against the human or mouse genomes (ftp://ftp.ncbi.nlm.nih.gov/ genomes/).

Results

Preparation and conduction of ChIP-sequencing assays. As current commercial antibodies for detecting BATF2 are not recommended for ChIP assays, we constructed a FLAG–BATF2 fusion construct and established stable cell lines which overexpressed *BATF2*. The expression of *BATF2* in the obtained HeLa-B2 cells was significantly increased at both mRNA (Figure 1A) and protein (Figure 1B) levels. The overexpressed BATF2 appeared to be mainly localized to the nuclei and partially among the cytoplasm (Figure 1C). The HeLa-B2 cells exhibited similar morphology to normal HeLa cells, with a slight reduction in the growth rate (Figure 1D) and cell-cycle progression (Figure 1E). Flow cytometric assay (FACS) showed that after sorting, more HeLa-B2 cells were arrested in G_2/M phase (16.93%) compared to the native HeLa cells (10.21%).

Bioinformatic analyses of the post-sequencing ChIP-sequencing data. Following sequencing with the Illumina Hi-Seq system, a total of 29.3 and 30.1 million reads were obtained from FLAG and IgG pulled-down samples of HeLa-B2 DNAs, respectively. The data from the two parallel sets displayed similar quality indices, which was sufficient to allow subsequent screening of differential components. To determine if there were any preferred sequences of DNA-binding sites associated with BATF2, we used the motif-finding program MEME. The most significant motif identified was a 21-nucleotide motif for BATF2 binding, with a stringent *e-value* (Figure 2A). By searching the motif across the entire human genome using the *grep* tool, we noticed that it was distributed unevenly independently of the chromosome length and concentrated on chromosomes 1, 2, 4, 7, 10, 16, 17, 21 and 22 (Figure 2B). Interestingly, at the centromere or pericentromeric regions, the BATF2-binding sites occurred more frequently compared to other locations

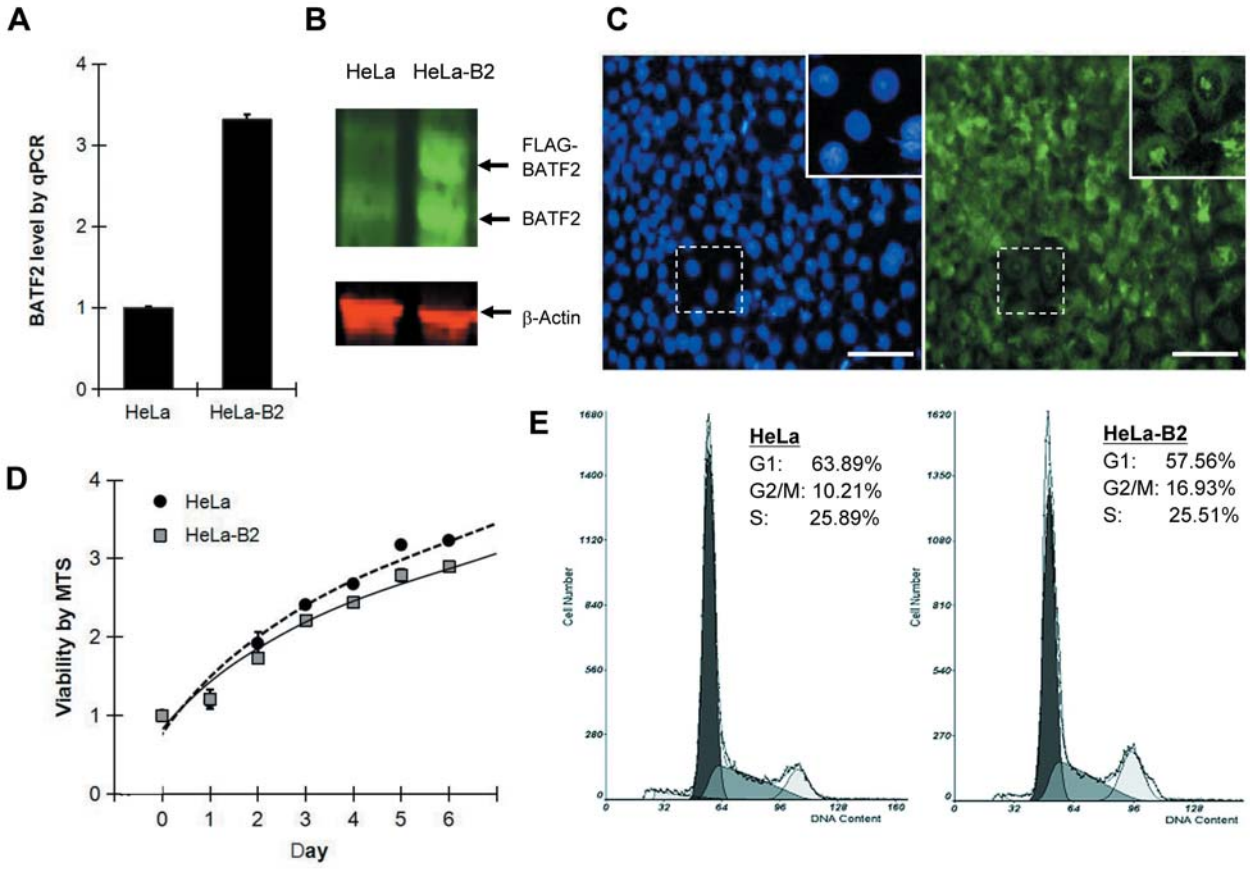

Figure 1. *Characterization of stable BATF2 overexpression HeLa cells. A: BATF2 mRNA levels in HeLa-BATF2 (HeLa-B2) cells by RT-qPCR compared to the native HeLa cells. B: Identification of BATF2 protein level in HeLa and HeLa-B2 cells by western blotting with antibodies to BATF2 and FLAG. C: Immunofluorescence of BATF2 over-expression in HeLa-B2 cells using a FLAG antibody. DAPI was used for the staining of nuclei. The bar represents 100 μm. D: The proliferation of HeLa-B2 and native HeLa cells determined by the CellTiter Proliferation Assays. E: FACS cell-cycle analyses based on propidium iodide staining of HeLa (left) and HeLa-B2 cells (right).*

(Figure 2D). By extended matching of these motifs with sub-categorization into the non-coding region, intron, exon and promoter of upstream 5,000 bp regions, consistent results were obtained, as the motif was only found in 1% in the coding regions of the human genome (Figure 2C).

The role of BATF2-binding motif as a regulatory element of gene transcription. As BATF2 is a transcription factor in nature, we performed transient expression reporter assays to test whether the BATF2-binding motif exerts transcription-regulatory activity. The results in Figure 3A indicate that the motif in reverse orientation manifested an increase of 2.7-fold in luciferase activity and that the forward motif had a similar activity compared to that of the scrambled control group, indicating that transcriptional regulation of the reverse motif was specific rather than a random effect. When two reverse motifs were used in tandem, a 4.7-fold increase in luciferase activity was detected for the forward motif sequence. However, such a dose effect of luciferase reporters was not further observed when the reverse tandem sequences were increased three and four times. Early studies demonstrated that the suppressive activity of BATF2 on *AP1* transcription could be amplified by interferon-β (IFN-β) (1). We found that the transcription activities driven by the reverse motif were indeed enhanced by IFNβ (Figure 3B).

BATF2 promotes human satellite DNA transcription. Comparison of the motif sequence with human satellite DNAs showed that the core sequence of the reverse motif TCCATT matched a signature of human satellite DNA SAT II (20-22) (Figure 3C). The quantitative PCR analyses showed drastically elevated transcription levels of human satellite DNA SAT II in HeLa-B2 cells, specifically under the control of the reverse motifs (Figure 3D). As we have previously found that the

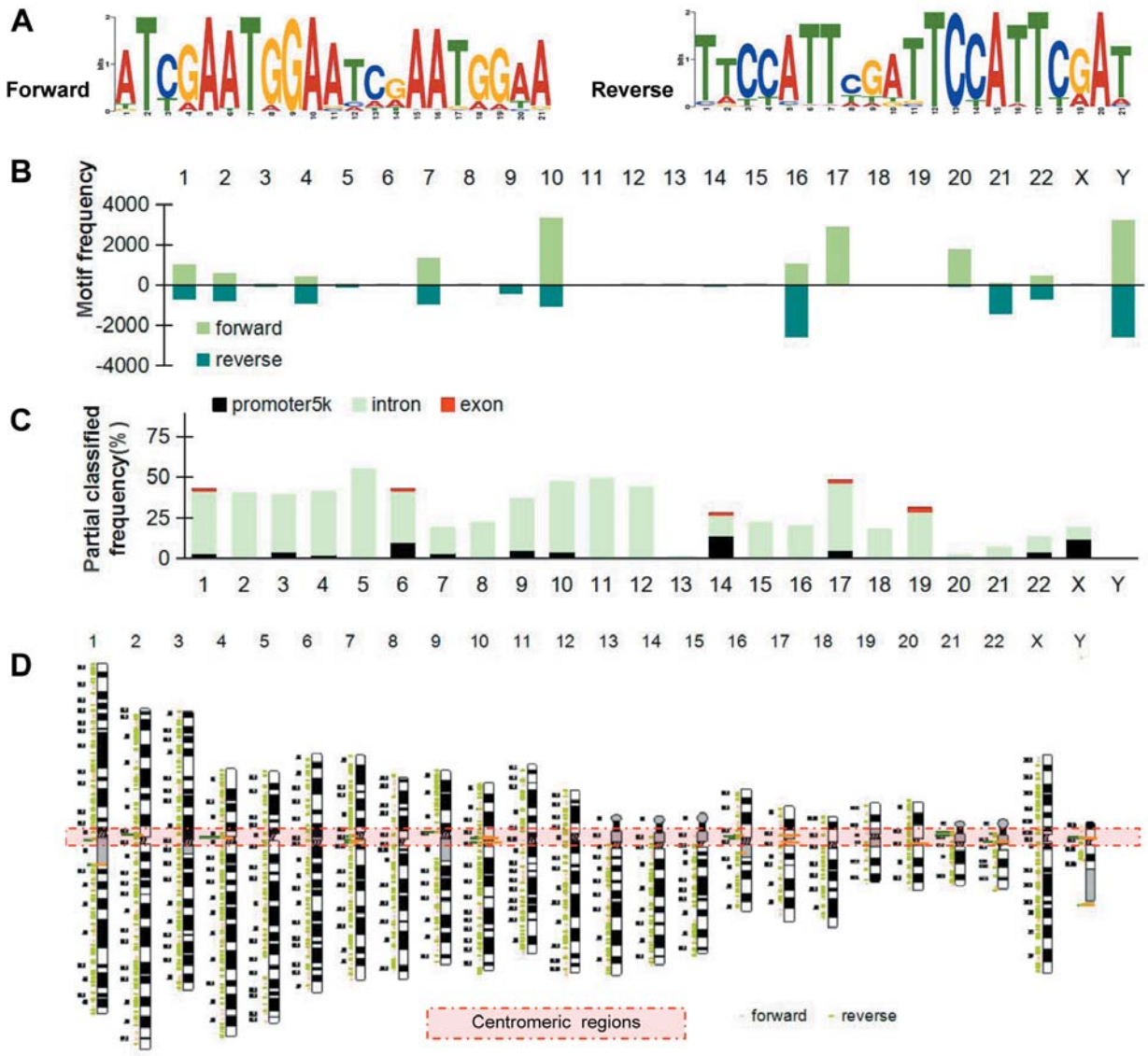

Figure 2. *Identification and pan-genomic distribution of the BATF2-interaction motif in human chromosomes. A: The motif sequence (forward and reverse complement) of the top score from the de novo motif analyses as plotted by WebLogo (http://weblogo.berkeley.edu/). B: The distribution frequency of the identified motif in reverse complement among human chromosomes. C: Sub-categorization of both the forward and reverse complement motif sequences in each human chromosome based on the gene structure annotations. D: The genomic distribution and frequencies of the forward and reverse complementary motifs.*

scattering patterns of the motif in either direction were also different among chromosomes, we further attempted to address whether there was a chromosomal selectivity of the genomic context. The data show that the SAT II transcripts from chromosome 2, 7 and 16 were significantly increased, that could be expected from the chromosomal distribution of BATF2-binding motifs (Figure 3E).

BATF2 may be involved in heterochromatin reorganization processes. In the bioinformatic analyses comparing the BATF2-binding motif with methylated H3 and HP1 targeted sites, we found a possible correlation on multiple occasions, including the coincident pattern of HP1 and BATF2 peaks at the pericentromeric chromosomal regions. We then compared the expression levels and nuclear localization of HP1 protein in both HeLa-B2 and native HeLa cells (Figure 4). In BATF2-overexpressing cells, HP1 displayed faint staining and a diffused distribution pattern in the nucleus, suggesting that BATF2 is able to participate in heterochromatin reorganization processes in coordination with HP1.

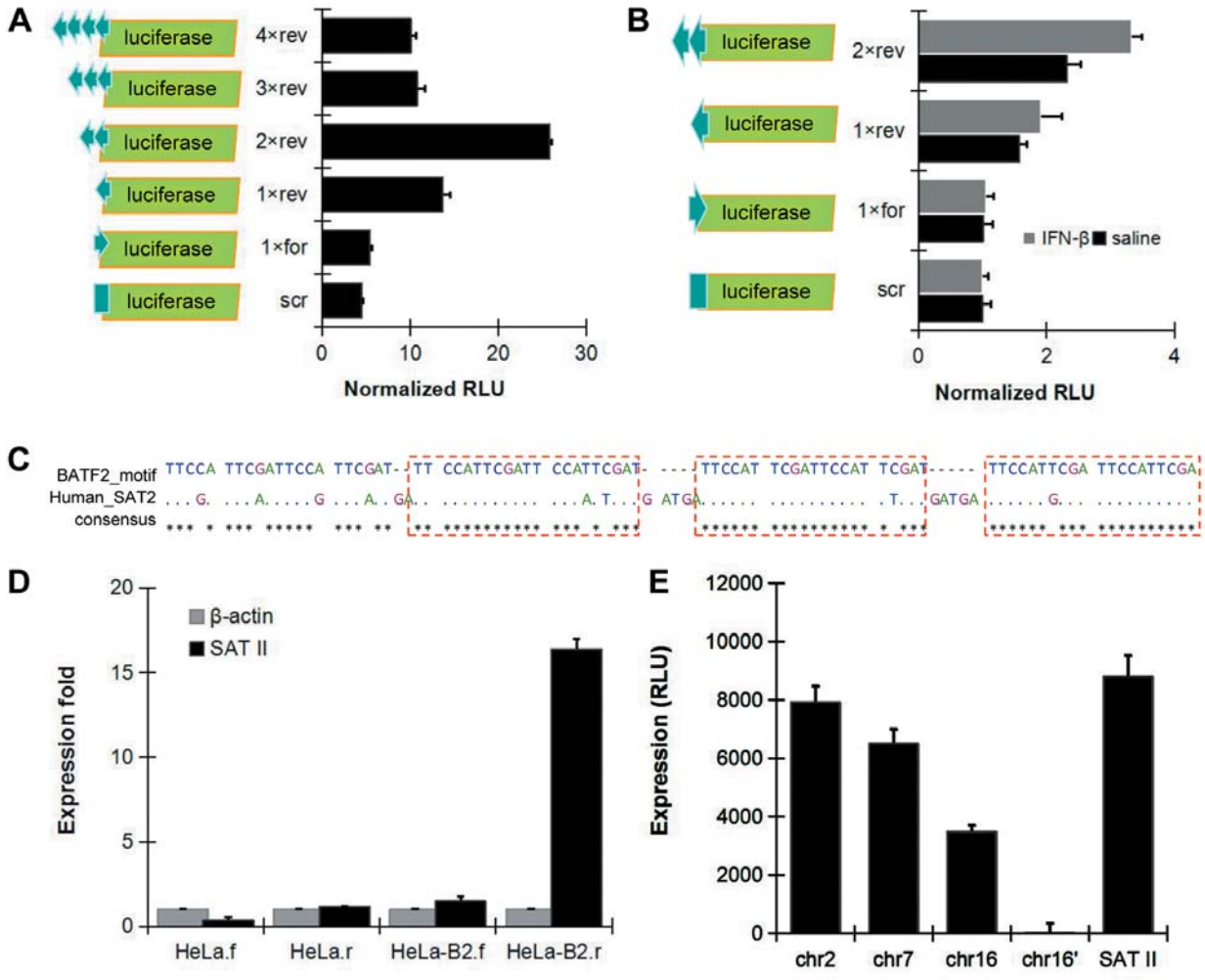

Figure 3. *The transcriptional regulatory activities of BATF2 motifs by luciferase reporter assays in HeLa cells and quantification of satellite transcripts under BATF2 overexpression in HeLa cells. A: HeLa cells were transfected with luciferase reporter constructs driven by scramble or BATF2 motifs in forward or 1×, 2×, 3× and 4× reverse orientations. B: The effects of IFN-βtreatments on the transcriptional activity of BATF2 motifs. HeLa cells were transfected with reporter vectors for 24 h, then treated with 2000 U/ml IFN-βfor 12 h before luciferase activity was assayed. C: Conserved elements in human satellite DNA (SAT II) overlapped with BATF2 motifs. D: Comparison of satellite transcript abundance downstream of the forward or reverse BATF2 motifs. The cDNAs from HeLa and HeLa-BATF2 cells were obtained using motif-based enrichment primers. The resulted fold changes were normalized to the measures of β-actin. E: RT-qPCR analyses of the transcription of SAT II in different chromosomal locations. Data from three independent experiments were normalized and presented as mean±S.E. Differences were considered significant at $p \leq 0.05$.*

Discussion

Genome-wide profiling of DNA-binding sites for proteins has emerged as a well-accepted method for identifying the particular elements for molecular interactions and allows integrated analyses of signal transduction pathways. Motif discovery is an essential process for revealing the structural bases for the underlining transcription-regulatory mechanism. In this study, we identified a 21-nucleotide DNA motif of BATF2 in a stringent *e-value* from motif discovery analyses. Within this motif, there seemed to be a repetitive consensus element of TCCATT separated by a few random nucleotides and the frequencies of such motif on each chromosome appeared to be uneven and independent of the chromosomal length. However, this distribution pattern was not seen in mouse chromosomes, indicating potential species specificity (data not shown). It was also reported that the TCCATT sequence could be a signature of human class II satellite DNA (23, 24), that might not only provide an explanation of the motif distribution pattern and species specificity (as satellite DNAs often differ between species), but also implies a possible role of BATF2 beyond being one of the common transcription factors.

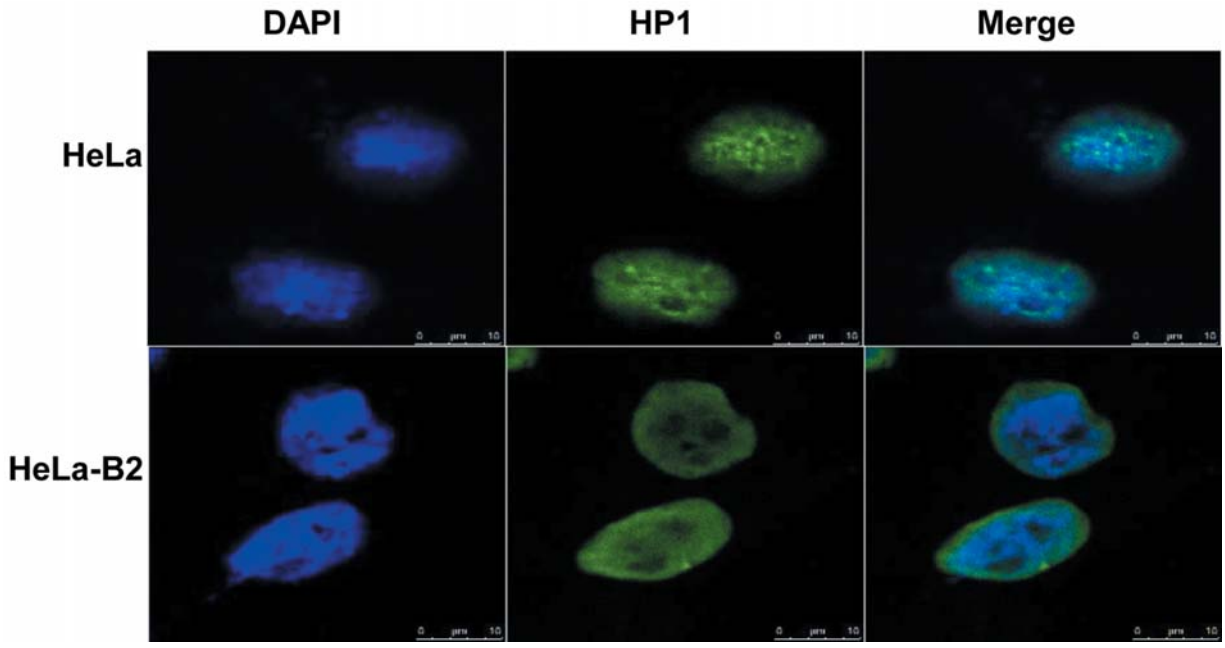

Figure 4. *Immunofluorescent analyses of HP1α in interphase patterns of HeLa cells and BATF2-overexpressing HeLa cells (HeLa-B2). The HP1α signals were displayed in green. DAPI was used for nucleic staining. Fluorescent images were acquired with a Leica SP5 confocal microscope under a x63 oil objective. The bar represents 10 μm.*

Recently, the transcripts from satellite DNA have gradually become an attractive subject in genomic and cancer-related studies. One of a few currently known candidates, CCCTC-binding factor (CTCF) was reported to up-regulate satellite transcription. *CTCF* overexpression reduced the binding of HP1α to the satellite II locus (25). The human T-cell leukemia virus type-I bZIP factor (HBZ) inhibited the DNA-binding activity of centromere protein B (CENP-B) to α-satellite DNA (26). In our study, the BATF2-binding motif increased DNA transcription in a directional and dose-dependent fashion. We also confirmed that BATF2 indeed elevated the levels of satellite transcripts with relative specificity to chromosomal locations. Our results might imply that BATF2 action on satellite transcription was likely to follow DNA polymerase II mediated principles, or involve other transcription factors that might interact with the leucine zipper domains within BATF2. These findings outline certain unusual descriptive characteristics of centrometric satellite transcription processes or regulations.

Mammalian HP1 plays a significant role in chromatin remodelling. HP1 was found to reposition in the nucleus, causing dynamic reorganization of chromatin in parallel with epigenetic modification changes under histone deacetylase inhibitors (27). The role of satellite DNA in maintenance of heterochromatin integrity was shown in a recent investigation of the tumor suppressor, breast cancer type 1 susceptibility protein (BRCA1), where the level of condensed DNA was reduced and loss of ubiquitylation of histone H2A was found at the satellite repeats within the centromere and pericentromeric regions (28, 29). Thus, the opening of centromeric chromosomal regions and priming of satellite DNA transcription can be i) an indicator of the increased potential for genetic instability, possibly through mitotic segregation errors; and ii) a survival strategy for cells to escape from genotoxic stress, including the overdose of particular transcription factors or imbalance of transcription regulatory factors, by shuffling the access of DNA-binding proteins to the repetitive non-coding DNA regions.

In summary, our present study shed light upon the possible explanation for why *BATF2* overexpression displayed a tight association with repeat satellite DNA. Although the functions of satellite transcripts are largely unknown, it is clear that satellite DNA is extremely divergent. A common feature of the satellite DNA is the irregular distribution of sequence variability along the monomer sequence. The probable reason for the diversity of repetitive tandem DNA might be evolution under selective constraints, which may protect the coding DNA from variation under changing circumstances, such as tumorigenesis induction. The suggestion here is that BATF2 may exert selective pressure on the recombination machinery *via* a repetitive motif-based mechanism for the maintenance of heterochromatin stability.

Accession Numbers

The ChIP-seq data from this study were submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/, GSE67258).

Acknowledgements

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