Genomic Insights into ATF3 Regulation of Cell Proliferation of HepG2 Cells

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Abstract

Previous research has indicated that increased expression of the Activating Transcription Factor 3 (ATF3) protein contributes to the inhibition of cell proliferation, migration, and growth in HepG2 hepatocarcinoma cells. In our investigation, we conducted ChIP-seq analysis to uncover the genomic loci to which ATF3 binds, aiming to elucidate the genes involved in the tumor-suppressive cascade. Our results revealed a specific ATF3 binding motif, TGACATCA, within the promoter regions of the genome. Furthermore, our study identified novel genes, including MAP2K3, which is involved in the cardiovascular fibrosis pathway response.

Introduction

Hepatocellular carcinoma (HCC) stands as a significant subclass of cancer, constituting a substantial portion of the health-cost burden, estimated at 55% (Sung et al., 2020). However, the formidable challenge lies in the drug resistance exhibited by hepatocarcinoma cells, hindering the attainment of cancer-free states post-treatment. One of the strategies proposed to regulate tumor cell suppression is Activating Transcription Factor (ATF3). ATF3 plays a crucial role in cellular stress response, significantly influencing metabolism, immunity, and tumor progression. Recognized as a master modulator in maintaining metabolic homeostasis, ATF3 functions as a central hub in coordinating cellular adaptive responses (Gilchirst et al., 2010). Research conducted by Li et al (2019),. revealed that overexpression of activating transcription factor 3 (ATF3) resulted in decreased cell proliferation, elevated cell apoptosis rates, and inhibition of cell cycle progression. In this study, we employed ChIP-seq (Chromatin Immunoprecipitation followed by Next-Generation Sequencing) to comprehensively analyze the genome-wide binding activity of ATF3 in HepG2 cells. ChIP-seq enabled high-resolution mapping of ATF3 binding sites, providing a detailed understanding of its genomic targets. By characterizing the regulatory landscape of ATF3 in HepG2 cells, our genomic data analysis uncovered novel genes interacting with ATF3, and elucidated novel pathways, thereby contributing to the broader understanding of the HCC.

Methods

Data Retrieval

The study utilizes ChIP-seq data gathered from two sets of HepG2 cells subjected to ATF3 antibody pulldown, sourced from ENCODE under the accession numbers ENCFF522PUA and ENCFF094LXX. In parallel, two sets of control datasets lacking a specific target (ENCFF247MLF and ENCFF086CF) were collected to establish a baseline. Sequencing was conducted on the Illumina HiSeq 2000 platform, generating 50nt single-end short-reads.

ChIP-seq Analysis

The computation workflow for the ChIP-seq experiment is highlighted in Figure 1 below and described in detail.

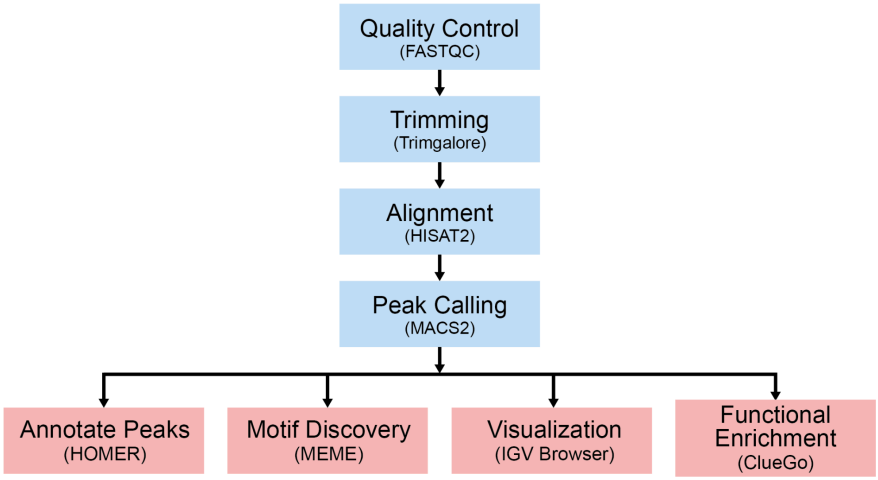

Figure 1. Computational workflow for ChIP-seq analysis.

I. *Data Preprocessing*
Single end reads of 50bp length underwent quality assessment with FastQC and were subsequently processed by trimming Illumina adaptor sequences using TrimGalore.

II. *Read Mapping and Format Conversion*
Processed reads were aligned to the GRCh38 genome assembly using HISAT2. Further, SAMtools was utilized to convert SAM files to BAM files, as well as to sort and index the resulting BAM files.

III. *Peak Calling and Consensus Analysis*
ATF3 transcription factor-DNA binding sites were identified using MACS2 with the narrow peaks' parameter. Consensus peaks between replicates were determined using bedtools intersect. The consensus peaks served as input for downstream analysis.

IV. *Genomic Annotation and Pathway Analysis*
Genomic features of consensus peaks were annotated using HOMER annotatePeaks.pl. Proportions of genomic features and gene type distributions were computed. Functional enrichment analysis was employed by the ClueGo plugin within Cytoscape to identify biological pathways the ATF3-bound genes were involved in. The BAM files from each replicate were used for visualization of enrichment sites on the IGV browser.

V. *Motif Discovery*
Before conducting motif discovery analysis, the nucleotide sequences corresponding to the consensus peak coordinates were retrieved from the GRCh38 genome using BEDtool's getfasta feature. These sequences were then utilized as input for the motif discovery analysis performed with MEME.

Results

Peak Identification and Genomic Distribution

The two treatment replicates contain 24.6 million and 36.1 million reads, and two control replicates containing 42.7 million and 46.3 million reads, respectively, post quality control test and trimming. On peak calling there were 22,151 enriched peaks for region 1 and 26,835 enriched peaks for region 2 identified. Consensus analysis yielded 15,918 highly enriched and reproducible peaks. The distribution of these peaks across genomic regions revealed that the majority of ATF3 peaks mapped to intronic

(49.47%), intergenic (39.61%), and promoter-transcription start site (6.37%) regions (Figure 2A). Further examination of the fraction of gene types exhibiting enriched peaks, in Figure 2B, showed that most were in the protein-coding areas (73.15%).

Identification of Conserved Binding Site Motifs

The exploration of DNA sequence motifs linked to the binding of the ATF3 transcription factor through motif discovery analysis revealed a 7-nucleotide motif, 'TGAGTCA' (Figure 2C), identified in 5,043 out of 15,918 peaks (31.68%). The motif was validated by Zhao et al. (2016), where the researchers noticed the top 12 motifs contained the AP-1 sequence TGAGTCA. This suggests that ATF3 may bind to DNA sequences containing the AP-1 motif, which is consistent with its known ability to form heterodimers with other proteins, including those in the AP-1 complex.

Identification and Visualization of ATF3-Bound Promoter-TSS Regions

To glean insights into the interaction between ATF3 and the genome, we visually represented promoter regions of genes exhibiting significant binding in Figure 2D This visualization highlights the robust activity of ATF3 across the promoters of STAT6 and MAP2K3 in both treatment samples. It's been reported STAT6 is highly expressed in human cancers, including HCC, and is associated with poor prognosis in HCC patients (Qing et al., 2017). MAP3K3 kinase is activated by mitogenic substances and environmental stress and is necessary for the expression of glucose transporter (Fujishiro et al., 2001). Activation of MAPK14 and confers abnormal cell division which can play a role in the proliferation of cancer

Functional Enrichment Analysis of Promoter-Bound Peaks

Given the pivotal role of ATF3 in the binding to promoter regions, our focus turned to genes associated with peaks in these regions. We identified 1,012 genes that show significant ATF3 enrichment in promoter-TSS regions. Functional enrichment analysis resulted in 183 Gene Ontology (GO) terms, in which 5 specific pathways piqued particular interest. In hepatocellular carcinoma (HCC), cancer cells have been shown to promote tumor invasion and metastasis through the stimulation of fibrinolysis, a process that breaks down fibrin, a protein integral to blood clot formation (Figure 2E). This dysregulated fibrinolysis, as demonstrated by Chen et al. (2018), disrupts clot stability within the tumor microenvironment, facilitating tumor invasion into surrounding tissues and metastasis to distant sites. ATF3, a transcription factor, emerges as a potential regulator in this pathway, exerting indirect influence through its regulatory effects on gene expression. By modulating the expression of genes involved in fibrinolysis, ATF3 may impact the extent and dynamics of fibrin degradation, thereby influencing the aggressiveness of HCC.

The next enriched pathway is the response to unfolded protein which is common in HCC due to factors such as viral infections, hypoxia, and metabolic dysregulation (Figure 2E). ATF3 is involved in the unfolded protein response (UPR), a cellular pathway activated in response to endoplasmic reticulum (ER) stress, suggesting its potential role in HCC pathogenesis (Wei & Fang, 2021).

Lastly, the response to organic substances, cellular response to chemical stimuli, and acute inflammatory response all significantly contribute to the development and progression of hepatocellular carcinoma (HCC), with ATF3 playing a crucial role in each process (Figure 2E). The exposure to various organic substances, such as toxins and carcinogens, activates ATF3 as a stress-responsive transcription factor, influencing gene expression patterns pivotal in cell proliferation, apoptosis, and DNA repair, thereby impacting HCC initiation (Liu et al., 2024). Additionally, ATF3 is implicated in the

cellular response to chemical stimuli, regulating genes involved in cell survival and inflammation, affecting HCC progression and therapeutic responses. In the context of HCC-related inflammation, ATF3's dual role as a pro-inflammatory and anti-inflammatory mediator modulates the tumor microenvironment, immune cell dynamics, and cytokine signaling, influencing tumor growth, invasion, and metastasis (Hai et al., 2010).

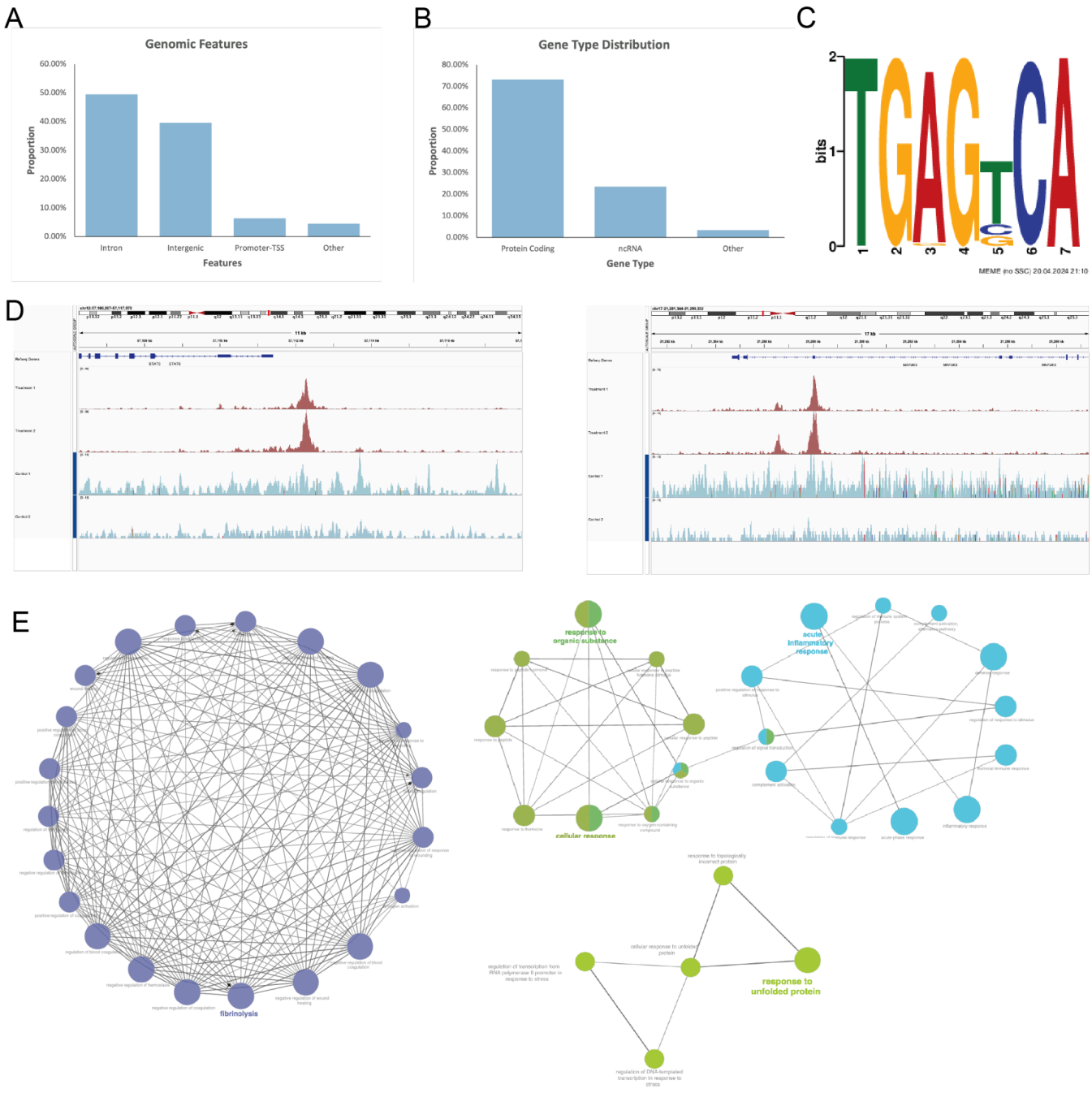

Figure 2 ChIPseq Analysis Results. A. B. C. The identified binding motif, TGAGTCA. D. Genomic tracks showing binding activity at the upstream promoter region of the STAT6 (left) and MAP2K3 (right) genes. E. The networks are displaying fibrinolysis, response to unfolded protein, response to organic substances, cellular response, and acute inflammatory response pathways.

Discussion

The ATF3 protein is widely recognized as a pivotal regulator in cellular stress responses. Previous research has highlighted a correlation between elevated ATF3 expression and suppressed tumor cell proliferation in hepatocarcinoma cell lines, notably HepG2. In our investigation, we pinpointed novel gene regions where ATF3 binds to for transcription, including Mitogen-Activated Protein Kinase 3 (MAP2K3), crucial for glucose transporter expression. MAP2K3, in turn, phosphorylates and activates MAPK14/p38-MAPK, leading to the accumulation of its active form and continuous activation of MAPK14, fostering oncogenic transformation in primary cells. Notably, multiple transcript variants of MAP2K3 have been identified through alternative splicing. This prompts further inquiry into the reasons behind ATF3 binding to this kinase genomic region, especially considering ATF3's role as a tumor suppressor juxtaposed with MAP2K3's association with oncogenic transformation, despite both being activated by cellular stress. Furthermore, on analysis of functionally enriched pathways we discovered ATF3 is associated with response to unfolded protein which are common in HCC due to factors such as viral infections, hypoxia, and metabolic dysregulation. ATF3 is involved in the unfolded protein response (UPR), a cellular pathway activated in response to ER stress, suggesting its potential role in HCC pathogenesis. In light of our findings, future investigations could employ RT-qPCR to validate the expression changes of genes following ATF3 modulation, providing deeper insights into the regulatory mechanisms at play. Additionally, ChIP-qPCR assays could be conducted to validate the direct binding between ATF3 and the genes of interest, thereby confirming the specificity of ATF3's genomic interactions. These findings underscore the potential for further exploration into ATF3's impact on gene regulation, offering promising prospects for elucidating the molecular mechanisms underlying hepatocellular carcinoma and identifying potential therapeutic targets.

Contributions

Chandrima identified the transcription factor of interest, downloaded the data and performed data preprocessing and alignment. Chandrima and Sahiti developed the research objective. Sahiti laid out the steps of the analysis and performed peak calling and peak visualization. Rameesha performed peak annotation, functional enrichment, and motif discovery. Throughout the project, all team members collaborated extensively, each making equal contributions across all project facets.

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