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Integrative Analysis of Transcriptional Regulation of the ATG4D, an Autophagy-Related Gene, Using JASPAR and STRING databases.

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ABSTRACT: Autophagy is a catabolic cellular process that is conserved and plays an important role in sustaining cellular stability, adjusting to malnourishment, and responding to cellular stress. ATG4D is a gene that involves the formation of the autophagosome and is considered a key player in autophagy. Inclusive knowledge of how the ATG4D gene is regulated may provide insights into the gene's role in different cellular aspects. In this research, bioinformatics tools were utilized, specifically the JASPAR database, to predict the transcription factor binding sites (TFBS) and transcription factors (TFs) binding to the ATG4D promoter region. Multiple TFs were identified, and among these identified TFs, we focused on eight that were well-known for their established roles in regulating autophagy, responding to cellular stress, and promoting transcriptional regulation of ATG4D. These TFs include FOXO3, TFEB, TP53, SP1, STAT3, NF-κB, E2F1, and CREB1. To discover the functional interactions of these TFs, the STRING database was utilized to conduct protein-protein interaction analysis and functional enrichment. This analysis helped us to better comprehend the complicated regulatory network linked to autophagy and the precise role of ATG4D. Functional enrichment analysis revealed significant gene ontology terms and pathways linked with autophagy, cellular stress response, apoptosis, and transcriptional regulation. These findings indicate that the regulation of ATG4D gene expression is shaped by a complicated network of transcription factors that play significant roles in autophagy and sustaining cellular balance. Using a combination of bioinformatics tools such as JASPAR and STRING gave a thorough insight into the regulatory atmosphere of ATG4D, providing opportunities for treating diseases associated with autophagy dysregulation.

Keywords: Autophagy, ATG4D, JASPAR, STRING, TF, TFBS

1. INTRODUCTION

Autophagy is highly regulated process that occurs inside the cell in a response to specific conditions such as nutrient deficiency, hypoxia, and oxidative stress[1]. Autophagy provides essential cellular components and energy to enable cellular surviving under abnormal conditions through degradation and recycling cellular ingredients, such as damaged organelles, misfolded proteins, and other cellular debris, [1]. Autophagy means self-eating Greek language [2]. It creats a double-membrane vesicle called the autophagosome, which engulfs the targeted cellular ingredients. Many proteins are involved in this process, including ATG (autophagy-related) proteins, Beclin-1, and LC3 (microtubule-associated proteins 1A/1B light chain 3). The autophagosome fuses with lysosomes, making an autolysosome and the engulfed material is digested by lysosomal enzymes. The outcome of this process is represented by amino acids, fatty acids, and other basic molecules that return back into the cytoplasm for recycling[3]. Many diseases are connected with autophagy

such as cancer, neurodegenerative disorders, and infectious diseases[4]. It is also lined with the immune response through intracellular pathogens degradation in a process called xenophagy [5], [6]. Further, it plays an active role in supporting the immune system in order to detect and remove cancerous or infected cells by presenting antigens [6].

Several genes have been involved in autophagy process such as ATG1, ATG5, ATG7, ATG8, ATG9, BECN1, MTOR, AMPK, PIK3C3/VPS34, LAMP1 and LAMP2, Rab7, P62/SQSTM1, NBR1, HIF1A, TP53, and ATG4D [7]. However, the latter has drawn particular attention due to its exceptional and essential functions [8], [9]. ATG4D, one of the ATG4 proteases, plays a central role in the lipidation and de-lipidation of LC3 and other ATG8 family proteins, the essential steps for autophagosome development and maturation. ATG4D cleaves (pro-LC3), the precursor of LC3, to represent a glycine residue at the C-terminus. This processing step is important for the later attachment of LC3 to phosphatidylethanolamine (PE), which is crucial for the widening and sealing of autophagosome membranes. Once autophagosomes and lysosomes combine, ATG4D eradicates lipids from LC3-II, turning it back into its original cytosolic form (LC3-I). This reprocessing is important to keep a pool of LC3 prepared for future autophagy rounds [10]. Moreover, ATG4D is implicated in Mitophagy, the discriminating autophagic degradation of damaged mitochondria. Effective functioning of ATG4D is crucial for reducing faulty mitochondria, a key factor in maintaining cellular health and preventing oxidative stress [11]. ATG4D drives autophagy in neurons to ensure removing dysfunctional organelles and protein clumps, which eventually protect against neurodegenerative diseases like Parkinson's and Alzheimer's [12]. Despite other ATG4 isoforms including ATG4A, ATG4B, and ATG4C play a role in autophagy, ATG4D stands out due to its unique substrate preferences and regulation methods. This is particularly significant for specific forms of selective autophagy [13], [14].

Changing in ATG4D expression or function might develop cancer. For instance, ATG4D upregulation can promote autophagic degradation of damaged organelles and proteins, potentially suppressing tumor growth [15]. ATG4D has also been connected to metabolic disorders [16], cardiovascular diseases, and infectious diseases[17] . Autophagy regulation is sophisticated, comprising transcriptional regulation, post-translational modification, and signaling pathways. Understanding the transcriptional regulation of ATG4D autophagy gene provides insight into how cells adjust to several physiological and pathological conditions.

Transcription factors (TFs) are proteins binding to DNA sequences, thus regulating the transcription of a gene. They regulate gene expression in response to numerous pathological and physiological stimuli [18]. Identifying the TFs that bind to the promoter region of autophagy-related genes such as ATG4D can explain the regulatory networks involved in autophagy.

JASPAR database is a popular used tool for predicting TF binding sites containing high-quality, curated assemblies of transcription factor binding profiles obtained from experimental data [19]. Analyzing the promoter region of the ATG4D gene using JASPAR, we were able to predict several TFs that might regulate the expression of ATG4D gene by examining its promoter region.

In order to understand the efficiency of these TFs and their potential connections, we utilized the STRING database. STRING is an online resource tool keen to the complete mapping and analysis of protein-protein interactions. It integrates known and predicted interactions from different sources to explore the interaction networks and functional enrichment of designated TFs [20] .

This study aims to investigate the transcriptional regulation of the ATG4D gene related with autophagy by predicting the TFs that attach to the ATG4D promoter region by using JASPAR database. Additionally, evaluate the protein interaction network and functional enrichment of these transcription factors utilizing the STRING database will provide an insight into the interaction networks and functional enrichment of designated TFs.

We focused on a group of transcription factors that play a role in autophagy and responses to cellular stress: FOXO3, TFEB, TP53, SP1, STAT3, NF-κB, E2F1, and CREB1. These transcription factors were predicted by JASPAR and have been known to play a vital role in multiple regulatory functions such as cell survival, apoptosis, and immune responses within the autophagic pathway.

2. METHODOLOGY

2.1. ATG4D Gene and Sequence Information:

The DNA sequence of the human ATG4D gene was retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (Gene ID: 84971). The promoter region, typically lies upstream of the transcription start site (TSS), was identified by Ensembl databases Genome Browser to locate the TSS of ATG4D and extract the promoter sequences (2000 bp upstream of TSS).

2.2. Predicting Transcription Factor Binding Sites (TFBS) and transcription factors (TFs) using JASPAR Databases:

JASPAR (http://jaspar.genereg.net/) was used to identify TFBS motifs. The promoter sequence of ATG4D gene retrieved from ensemble as FASTA format was scanned against the JASPAR CORE database by setting the relative profile score threshold on 80%.

2.3. Exploring protein-protein interactions and functional enrichment by STRING database

Predicted (TFs) found by JASPAR were examined to bind to the ATG4D gene by using STRING database (version 12) to explore protein-protein interactions and functional enrichment (accessed via the official website (https://string-db.org/).

3. RESULTS AND DISCUSSION

The current study aimed to predict transcription factors binding sites (TFBS) and (TFs) binding to the ATG4D gene associated with autophagy exploitation the JASPAR database. JASPAR revealed several potential TFBS and TFs within the promoter region of the ATG4D gene. Among these TFs, we chose those that were well-documented in literature for their roles in autophagy regulation and cellular stress responses, and transcriptional regulation activity proposing their noteworthy regulatory impact on ATG4D expression. These TFs are: FOXO3, TFEB, P53, NF-κB, CREB1, STAT3, SP1, E2F1(Table.1). These TFs were examined using the STRING database (https://string-db.org/) to discover their interaction network and accomplish functional enrichment analysis, focusing on autophagy-related processes. The TFs were inputted into STRING (version 12), resulting in an interaction network that visualized identified and predicted protein-protein interactions among the TFs (Figure. 1). The network was filtered to include high-confidence interactions with a minimum interaction score of 0.4. The PPI network was (1.99e-05), which demonstrated significant enrichment ($p < 0.01$), indicating that the detected interactions were biologically related and not accidental. The interaction network created by STRING discovered a highly interrelated TFs, revealing wide-ranging interaction and potential co-regulation among these TFs.

Gene Ontology analysis showed significant enrichment in processes related to regulation of autophagy, cellular response to stress, apoptotic process, and transcriptional regulation activity for all TFs. KEGG pathway analysis on the other hand highlighted pathways such as the mitophagy pathway, AMPK signaling pathway, and FoxO signaling pathway and all showed significant enrichment. While Reactome Pathway showed significant enrichment in processes related transcriptional regulation by TP53 (Table.2)

These results prove a significant connotation between the selected transcription factors and the regulation of the ATG4D gene, highlighting their potential roles in autophagy. The high-confidence interactions detected in the STRING network propose a multifaceted regulatory network that these TFs may work synergistically to control autophagy.

The direct engagement of these transcription factors in autophagic processes is verified by enrichment in the Autophagy (Mitphagy – animal) KEGG pathway. The known roles of TFEB in regulating lysosomal function and FOXO3's effect on the expression of autophagy-related genes support the enrichment findings [21], [22]. Moreover, there was a significant enrichment observed in the "p53 signaling pathway" which highlights the double function of these pathways in regulating autophagy and apoptosis [23]. TP53 that plays a key role in the cell's response to stress shows a complicated effect on autophagy by either enhancing or hindering the process depending on the conditions[24], [25] . NF-κB, was documented for its contribution in inflammation and immune system response, by interacting with autophagic processes, it provides additional support for the functional enrichment results [26], [27], [28].

The cross-talk among these transcription factors and how they together affect ATG4D specifies a robust regulatory network controlling autophagy. To illustrate, STAT3, which is naturally linked with cytokine signaling [29] , is recognized for its involvement in controlling autophagy during stressful circumstances [30], [31], [32]. E2F1, which is mainly known for directing the cell cycle [33], shows involvement in autophagy by activating the transcription of autophagy-related genes [34], [35], [36]. Additionally, CREB1, which is responsible for responding to distinct physiological stimuli [37], showed a role in autophagy by regulating genes related to sustaining energy balance and handling stress [38], [39], [40].

These findings offer significant insights into the regulatory landscape of ATG4D, emphasizing the intricate network of interactions and the enriched biological processes and pathways. Experimental validation is need in the future work, such as Chromatin Immunoprecipitation followed by sequencing (ChIP-seq), to confirm TF binding to the ATG4D promoter in vivo. Future research should also discover the dynamic regulation of these TFs under different physiological and pathological circumstances to comprehend their roles in autophagy process.

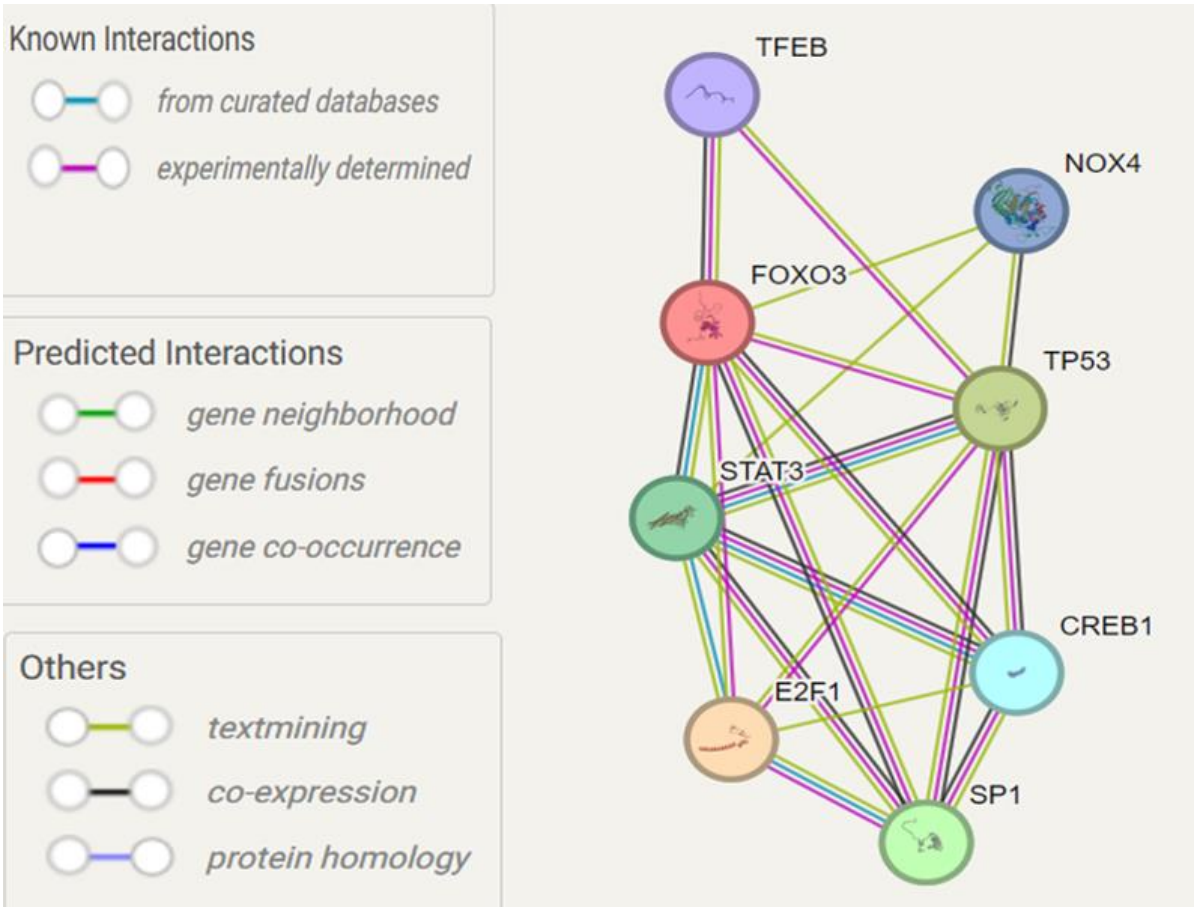

FIGURE 1. protein-protein interaction (PPI) network and construct functional enrichment in predicted transcription factors binding to ATG4D using STRNG database.

Table 1. - The putative Transcription Factor Binding Site motifs and TFs identified by JASPAR.

Name	ID	Species	Class	Family	Motif Logo
FOXO3	MA0157.2	Homo Sapiens	Forked head/winged helix factors	Fox	
TFEB	MA0692.2	Homo Sapiens	Basic helix-loop-helix factors (bHLH)	bHLH-ZIP	
TP53	MA0106.1	Homo Sapiens	P53 domain factors	P53-related factors	

NF-KB	MA0105.1	Homo Sapiens Oryctolagus cuniclus Mus musculus Rattus norvegicus	Rel homology region (RHR) factors	NF-Kappa B- related factors	
CREB1	MA0018.2	Homo Sapiens Mus musculus Rattus norvegicus	Basic leucine zipper factors (bZIP)	CREB-related factors	
STAT3	MA0144.1	Homo Sapiens	STAT domain factors	STAT factors	
SP1	MA0079.1	Homo Sapiens	C2H2 zinc finger factors	Three-zinc finger Kruppel-related	
E2F1	MA0024.1	Homo Sapiens	Forkhead/winged helix factors	E2F	

Table 2. - Summary of significantly enriched gene ontology terms (GO) and pathways for transcription factors binding to ATG4D.

Category	Term Description	Count in Network	Enrichment Strength	FDR
GO Biological Process (G0:0010506)	Regulation of autophagy	4	1.45	0.0025
GO Biological Process (G0:0006950)	Cellular response to stress	7	0.71	0.0075
GO Biological Process G0:0097190)	Apoptotic process	3	1.37	0.0255
GO Molecular Function (G0:0140297)	DNA-binding transcription factor	5	1.41	0.00016
GO Molecular Function (GO:008134)	Transcription regulatory region DNA binding	6	1.4	3.93e-05
GO Cellular Component (G0:0000785)	Chromatin	7	1.13	3.96e-05
GO Cellular Component (GO:0005667)	Transcription factor complex	6	1.46	1.86e-05
KEGG Pathway (hsa04137)	Mitophagy - animal	5	2.28	8.47e-09
KEGG Pathway (hsa04152)	AMPK signaling pathway	2	1.61	0.0097
KEGG Pathway	FoxO signaling pathway	2	1.59	0.0101

(hsa04068)				
Reactome Pathway (HSA-6804116)	Transcriptional regulation by TP53	2	2.55	0.0030

Count in Network: The number indicates how many proteins in the network are annotated with a particular term.
Enrichment Strength: Log10(observed / expected). This measure describes how large the enrichment effect is.
False Discovery Rate (FDR): Describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

4. CONCLUSION

This research provides a comprehensive analysis of the transcriptional regulation of the ATG4D gene, a key element in the autophagy pathway. exploiting the JASPAR database, we identified crucial transcription factors (FOXO3, TFEB, TP53, SP1, STAT3, NF-κB, E2F1, and CREB1) that possibly bind to the promoter region of ATG4D. These transcription factors are well-documented as regulators of autophagy and cellular stress responses, indicating their possible role in regulating ATG4D expression.

To more examine the functional inferences of these transcription factors, STRING database was employed to construct a protein-protein interaction (PPI) network and build functional enrichment analysis. The high-confidence interaction network showed significant contacts among the selected transcription factors, signifying a synchronized regulatory mechanism. The Functional enrichment analysis recognized several enriched gene ontology terms and pathways linked to autophagy, cellular stress response, apoptosis, and transcriptional regulation.

The incorporation of JASPAR with STRING-based interaction and enrichment analyses delivers a strong framework for understanding the intricate regulatory landscape of ATG4D. These findings point to the significant role that these transcription factors play a in regulating autophagy, potentially inducing cellular homeostasis and responses to stress. These perceptions offer a valued groundwork for future research intended at validating these interactions experimentally and discovering their regulatory mechanisms in distinct cellular contexts. comprehending the transcriptional regulation of ATG4D can enlighten therapeutic strategies targeting autophagy-associated diseases, where accurate modulation of autophagy might have substantial clinical profits.

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