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

Muna A. Abdal Rhida[1]*

Department of Biology, College of Science, Wasit University, IRAQ

*Corresponding Author: Muna A. Abdal Rhida

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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. Autophagy-related gene 4D (ATG4D) functions in the formation of an autophagosome, which is a main player in authenticating autophagy. A comprehensive understanding of how the ATG4D gene is regulated might offer information on what parts of cellular function it involves. To predict the transcription factor (TF) binding sites and TFs governing ATG4D promoter region, bioinformatics tools such as the JASPAR database were utilized in this study. Several TFs were found, and from these regulatory agents, we concentrated on eight that are classic regulators of autophagy/transcripted to ATG4D in response to cell/autophagic stress, and promoting transcriptional regulation of ATG4D. The identified transcription factors (TFs) are FOXO3, TFEB, TP53, SP1, STAT3,YB-1,NF-kb,E2Fl and CREBl. To recognize functional interactions of the TFs, we performed protein-protein interaction analysis using STRING database crossed with Functional Enrichment. This analysis provided insights into the complex regulatory network of autophagy, and its exact function in ATG4D, which identified a variety of gene ontology terms corresponding to autophagy, the cellular stress response and apoptosis as well as transcriptional regulation at high levels. Together, these results suggest a complex transcriptional network to control ATG4D gene expression and direct the cell back into its regular homeostatic balance of autophagy. The combinatorial usage of JASPAR and STRING as bioinformatics tools provided a thorough insight into the regulatory atmosphere of ATG4D, opening new avenues for treating diseases associated with autophagy dysregulation.

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

1. INTRODUCTION

Autophagy is a quite regulated process that takes place inside the cell and in response to certain stimuli, nutrients, and incidence of deficiency, hypoxia in this atmosphere with oxidative stress [1]. Autophagy recycles building blocks and metabolic precursors to produce energy; this catabolic process is recognized as vital for the maintenance of cell metabolism. In order to ensure its cellular survival under abnormal conditions, a cell will break down and recycle ingredients like damaged protein aggregates, other organelles and cellular debris, and turnover of unfolded or misfolded proteins. Literally, autophagy is a Greek word referring to self-eating [2]. It creates an autophagosome (a double-membrane vesicle) that surrounds the cell materials to be targeted. Many proteins are engaged in this process, including ATGs (autophagy-related), Beclin-1, and LC3 (light chain 3). The autophagosome will fuse with lysosomes to form an autolysosome and the internalized cellular components in the lysosome are broken down by 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). The ability to reprocess LC3 is significant in order to sustain a pool of functional LC3 for later rounds of autophagy [10]. Additionally, ATG4D is associated with mitophagy, the selective autophagic destruction of damaged mitochondria. That is because the efficient operation of ATG4D contributes to the removal of damaged mitochondria, thereby supporting cellular health and preventing oxidative stress [11]. Neurons use autophagy to clear out old organelles and protein clumps, which maintains safety measures against declination afflictions akin to Parkinson's or Alzheimer's; this applies a specific function of autophagy ATG4D [12]. Although the other ATG4 isoforms (ATG4A, B, and C) are involved in autophagy too, distinct properties of substrate specificity and regulation distinguish ATG4D from the others. This is particularly relevant for specific types of selective autophagy [13], [14].

The alteration in expression or function of ATG4D might lead to cancer development. For example, upregulation of ATG4D may encourage the autophagic degradation of injured organelles and proteins thereby potentially inhibiting tumorigenesis [15]. Further, ATG4D is associated with metabolic and cardiovascular diseases as well as a cocktail of infectious diseases [16] [17]. The regulation of the autophagy pathway is complex, including transcriptional modulation, post-translational modifications and signaling pathways. Elucidation of the transcriptional regulation of the ATG4D autophagy gene gives an idea of how cells can cope with different physiological and pathological conditions.

Transcription factors are proteins that bind to specific DNA sequences and thereby regulate the transcription and expression of a gene in response to a wide array of pathological and physiological stimuli [18]. Identifying the TFs binding to the ATG4D prompter region can enlighten the regulatory networks connected with autophagy. JASPAR database is an eminent tool for predicting TF binding sites and consists of open access, high-quality and curated collection of transcription factor specificities derived from experimental data [19]. Exploitation of JASPAR to predict TFs on the promoter region of ATG4D gene based on its promoter, we predicted several TFs that could control this gene expression. To deduce the efficiency of these TFs or their potential interactions, we used the STRING database. STRING is an online resource aiming to provide the global mapping and analysis of protein-protein interactions. It employs information from both known and predicted interactions derived from a series of databases to investigate the interactome repeatedly involved by the planned transcription factor genes as well as their functional enrichment [20] .

The current study investigates the mechanism underlying transcriptional regulation of the autophagy-associated ATG4D gene through predictions of TFs binding to its promoter region using the JASPAR database. Moreover, a protein interaction network analysis and functional enrichment of these TFs may help better understand the function relationships among selected transcription factors so that using the STRING database for this purpose adds further information.

We screened a group of autophagy and stress-responsive transcription factors: FOXO3, TFEB, TP53, SP1, STAT3, NF-κB, E2F1, and CREB1. Such transcription factors were inferred by JASPAR and had been reported to be involved in various regulatory functions including cell survival, apoptosis, and immune responses related to 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, and 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 highly interrelated TFs, revealing wide-ranging interaction and potential co-regulation among these TFs.

Gene Ontology analysis showed significant enrichment in processes related to the 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 the Reactome Pathway showed significant enrichment in processes related to 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 which 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 to 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 needed in 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 the autophagy process.

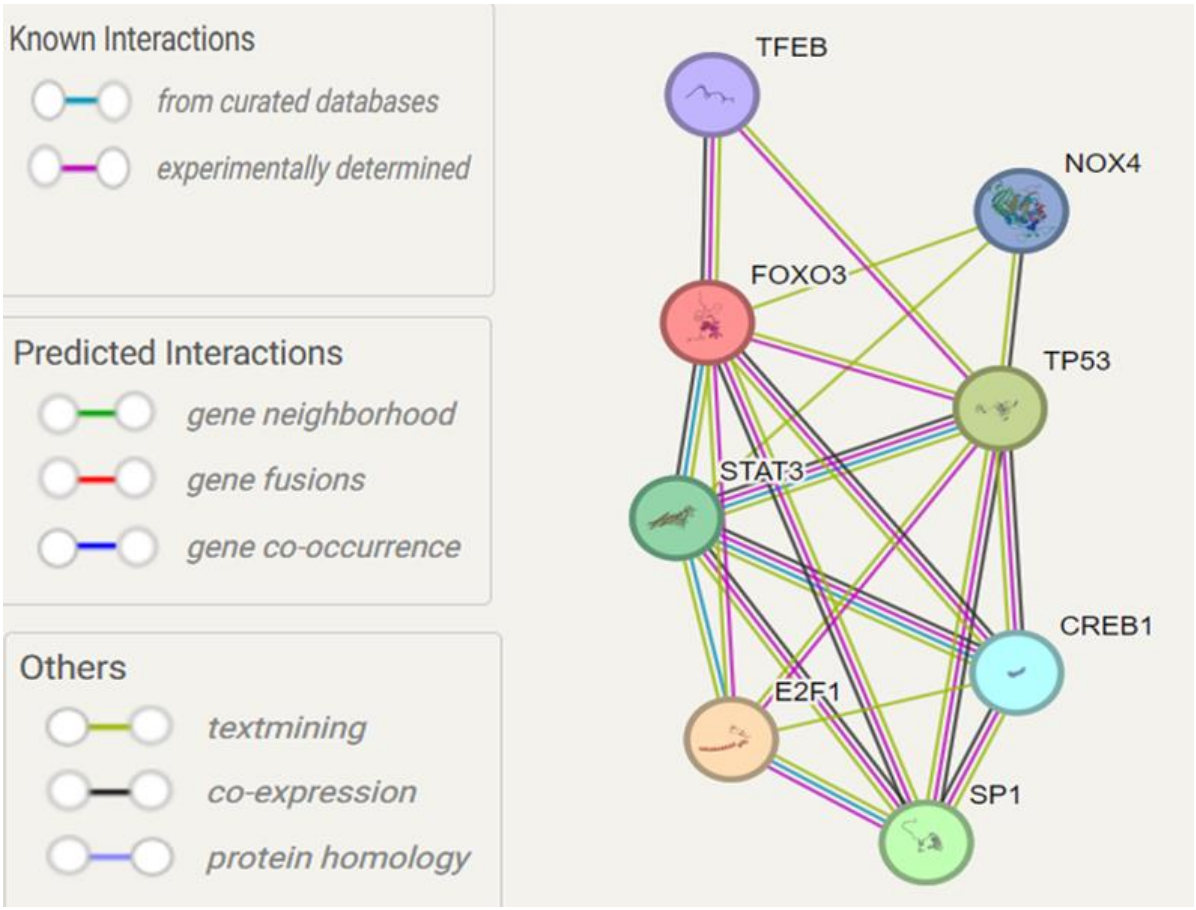

FIGURE 1. protein-protein interaction (PPI) network and construct functional enrichment in predicted transcription factors binding to ATG4D using STRNG database.

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, the STRING database was employed to construct a protein-protein interaction network and build a 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.

JASPAR and STRING-based interaction in combination with enrichment analyses of their empirically determined targets offers an outstanding platform to predict the complex regulatory coordination employed by ATG4D. The present results suggest a strong involvement of these transcription factors in the control of autophagy associated with activating cellular homeostasis and stress responses. These principles provide an important basis for subsequent investigations that seek to verify these interactions experimentally and elucidate the regulatory mechanisms acting in diverse cellular backgrounds. Comprehending the transcriptional regulation of ATG4D may discover therapeutic approaches in autophagy-related disorders, where well-controlled autophagy levels might have significant clinical benefits.

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