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Bioinformatics Analysis in Predicting Transcription Factors of *Robo3* Gene in *Drosophila melanogaster*

Muna A. Abdal Rhida[1,2]*

[1]Cell and Molecular Biology, University of Arkansas at Fayetteville, Fayetteville, AR, USA.
[2]Department of Biology, Wasit University, Kut, Iraq.
*Corresponding Author E-mail: mu.raheemn@gmail.com

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In bilaterian animals, axon guidance decisions are regulated by many transmembrane receptor proteins called Roundabout (Robo) family members. During the developmental stages of fruit flies (Drosophila melanogaster), three Robo family members play unique roles in the central nervous system. Robo3 is revolutionarily conserved among taxa and studies show that Robo3 regulates mediolateral axonal navigation. Recent studies suggest that Robo3 guides longitudinal axons in a manner independent of its ligand (slit). The expression patterns of Robo3 are controlled by transcription factors (TFs) that play a significant role in gene regulation, and it is not a fully understood mechanism. Knowing the transcription factor binding sites (TFBS) of Robo3 would help to predict TFs that regulate Robo3. In this study, bioinformatics tools MEME Suite, TOMTOM, and MAST were utilized to analyze the Robo3 DNA sequence to identify putative TFs that assist as docking regions for TFs involved in the regulation of Robo3 gene expression. We found seven putative TFs: Btd, Opa, Mad, Odd, Twi, CF2, and h. Mapping these TF motifs against the Robo3 sequence showed that these motifs are located in many regions of the Robo3 gene. Understanding the roles of these TFs in Robo3 gene regulation would help to implement novel strategies to control and overcome disorders related to the Robo3 gene. This study aims to identify the unknown TFs that may play a critical role in Robo3 gene expression.

Keywords: Bioinformatics; JASPAR; MEME Suite; Robo3; Transcription factor.

In bilaterian animals, axon guidance decisions are regulated by many tans-membrane receptor proteins called Roundabout (Robo) family [1,2]. Robo receptors control axon crossing in the central nervous system (CNS) by signaling repulsion in response to Slit ligands that are usually expressed in the midline[3–7]. Apart from midline repulsion, Robo family members in certain animals such as vertebrates and insects have taken on additional axon guidance functions in CNS [8–10]. For instance, Robo1, Robo2, and Robo3 receptors in *Drosophila* have specific functions in embryonic CNS development [11]. While Robo1 and Robo2 cooperate on midline repulsion [12–14], Robo2, and Robo3 direct axons towards a medial-lateral position of the longitudinal pathway in the ventral nerve cord[3].

Robo3 has been conserved over evolution and throughout embryonic ventral nerve cord development [15]. Its guidance of the longitudinal axon pathways at certain points along the medial-lateral axis occurs in a manner opposed to Slit-dependent midline repulsion and this role is determined by its expression pattern and structure[3].

In addition to its role in neural circuit formation, Robo3 ‘s expression has been linked to the guidance of motor axons and sensory neurons, highlighting its significance in *Drosophila’*s developing nervous system. Robo3 expression is controlled by interacting with specific transcription factors (TFs) to regulate its expression patterns that are essential for neural pathway [16]

TFs are proteins that control gene expression by binding to certain DNA sequences in the genome known as transcription factor binding sites (TFBS) [17,18]. Identifying and analyzing of TFBS of *Drosophila Robo3* will provide valuable insight into the molecular mechanisms of neural development and gene expression of Robo3. When TF binds to these sites they can either activate or repress the *Robo3* transcription. Thus, promoting different cellular processes elaborates on neural development.

Multiple TFs are incorporated in the process of DNA transcription [19]. These TFs bind to specific DNA sequences in a specific manner. TFBS placed either in the protein-coding sequences or non-coding DNA in the regulatory regions of genes called enhancers, which are orchestrated into functional units called Cis-Regulatory Modules (CRMs) could be in regions far from genes of interest and bind to specific TFs in certain developmental stages [20,21]

Enhancers, the regulatory sequences, orchestrate gene expression control in spatial and temporal patterns by governing their transcriptional processes [22]. Regulatory enhancers possess multiple (TFBS) that bind to multiple TFs. Unraveling elaborate biological networks requires a comprehensive understanding of the interaction between enhancers, TFBS, and TFs.

Studying TFBS of *Robo3* revealed the existence of DNA conserved motifs that are recognized by *Robo3*. Researchers have been able to characterize and identify *Robo3* binding sites in different taxa through various techniques, such as electrophoretic mobility shift assay (EMSA)/gel shift assay [23], ELISA based assay[24], and chromatin immunoprecipitation followed by sequencing (CHIP-Seq)[25] . However, a few TFs have been identified to be involved in regulating *Robo3* [26]. The current study addresses this challenge by utilizing a computational process to predict TFBS within the Robo3 gene of *Drosophila melanogaster*. In silico tools such as MEME Suite, TOMTOM/JASPAR, and MAST are used to find TFBS in *Drosophila Robo3* to provide a comprehensive understanding of the regulatory network of *Robo3* and shedding light on the intricate processes that govern its activity during development (Figure.1).

Multiple motifs of *Robo3* DNA sequences were identified, characterized, mapped, and compared with an established database. We expect that these mapped sequences can serve as groundwork to predict de novo enhancers throughout the Robo3 gene of *Drosophila* using bioinformatic techniques. This study aims to identify the unknown TFs that might be critical for Robo3 gene regulation.

MATERIAL AND METHODS

Robo3 enhancer sequence identification:

The Robo3 gene sequence of *Drosophila melanogaster* is retrieved from FlyBase database ID:FBgn0041097, including the 52 and 32 UTR, introns, and exons to be tested for putative motifs that can bind TFs [27]

FlyBase is an online bioinformatics database that covers genetics and molecular information about *Drosophila* and these data are presented in various formats, such as FASTA, GFF, CSV/TSV, and JSON. FlyBase collects data from different sources such as genome projects and research literature including mutant phenotypes, gene models, molecular characterization, cytological maps, wild type expression patterns, transgenic constructs and insertions, anatomical images, sequence level gene models, and classification of gene function. Users have access to navigate the database through the sequence of DNA or proteins, genes mutant names, or ontology terms. Links between FlyBase and other databases like Beef Data and Genomics Program (BDGP) or model organism Encyclopedia of DNA Elements (modENCODE) offer more exploration choices into other organism model databases and other references of molecular information. FlyBase project involves *Drosophila* researchers and computer scientists from Harvard University, Indiana University, and University of Cambridge [27]

MEME for Motif Discovery

Multiple Em for motif elicitation (MEME)

is a tool that utilizes statistical confidence level to determine the optimal width, occurrence frequency, and description for each discovered motif [28]. In this study, MEME Suites was used to discover the motifs for *Robo3* enhancer sequences. A group of DNA or protein sequences were taken as an input and the motifs found within each enhancer sequence were taken as the output. Motifs are denoted as position-dependent letter probability matrices in MEME which indicate the likelihood of each possible letter at each position in the pattern.[21,29]

Motif comparison using TOMTOM for known motifs

TOMTOM is a tool that compares one or multiple motifs against a database of known motifs such as JASPAR, ranking and aligning significant matches. In the current work, TOMTOM was run on all motifs discovered in respective enhancer sequence found in MEME against a database of known motifs to identify those that are involved in the transcriptional regulation of *robo3*. This comparison was necessary to identify the functional motifs in the enhancer sequences so that the non-functional motifs were excluded.[30]

Using MAST for mapping predictive TFBS on the dataset

MAST is a tool for motif search and alignment. It searches sequences for matches to a set of predefined motifs[31] . In this study, MAST is utilized to map the TFBS motifs that were discovered by motif matching and functionally related to the organism mechanisms to the enhancer sequence dataset.

RESULTS

The *robo3* genomic sequence was retrieved by using the FlyBase database (ID: FBgn0041097). It is 40180 bp in length with 13 exons. This gene is located on the second chromosome 21F3-21F4; 2-2 cM.

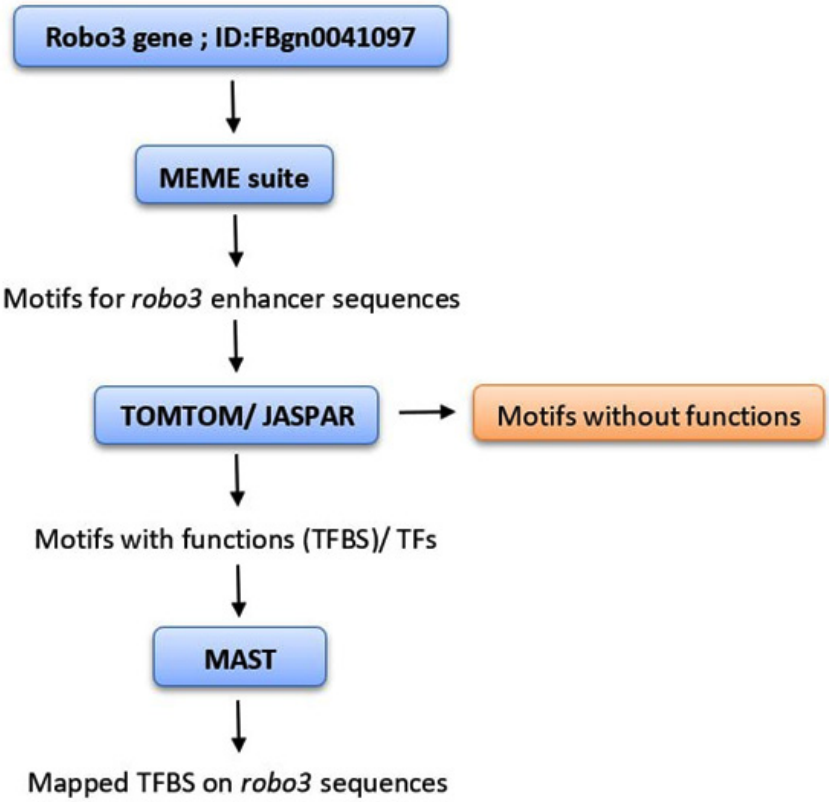


Fig. 1. A schematic of using bioinformatic tools (MEME Suit, TOMTOM/JASPAR, and MAST to predict transcription factor binding sites(TFBS) and transcription factors(TF) related to *Robo3* gene

After retrieving the whole gene sequence of *robo3* from FlyBase, the MEME Suite web server (Figure 2) was used to provide access to identify motifs, a three-dimensional structure composed of specific amino acid sequences in proteins linked with specific functions [32] . These motifs were identified according to various parameters such as motif maximum and minimum length, motif number to be identified, and the target strand. MEME Suite is considered a gateway for motif sequence identification and analysis represented by DNA binding sites and protein interaction domains. It utilizes a training set of protein sequences to produce MEME motif results, including a description of the sequences, motif occurrences organized by significance value, and visual illustration of motif distributions [33]. In this study, the maximum number of requested motifs was 10, the maximum motif width ranged from six to ten, and the maximum sites per motif ranged from two to five.

Motifs found by MEME consist of all sequence sites no matter what functional significance they have. Comparing these motifs with known ones in the TF family database aids in identifying them as potential TFBS motifs. Thus, JASPAR CORE insect, a specific database within the JASPAR database, emphases on providing high-quality, curated groups of transcription factor binding sites, that are crucial for comprehending gene regulation. It contains binding profiles for transcription factors precisely from insect species, including *Drosophila melanogaster*. These profiles are characterized as position frequency matrices (PFMs), which designate the preferred binding patterns of transcription factors to DNA sequences [34]

TFBS motifs were identified by using TOMTOM, the motif comparison tool, in the motif database by aligning target and query motifs with the best hit detected according to the E-value and p-value (Figure 3). TOMTOM measures the resemblance between two motifs offers a numerical score for the two motifs match and assesses a statistical evaluation of the score significance. Only matches whose significance is less than or equal to the threshold specified by the thresh switch were displayed. The matches q-value were used to measure the significance by default. The alignments that have the lowest E-value score were the only alignments to be evaluated and the query motif matching that score was recorded in a table including both the alignment and the TFBS identifier [35]. Some motifs shared the same TFs due to their sequence similarities. Thus, we identified only seven TFs for the 10 putative motifs as shown in Table 1. These TFs were Odd, Twi, CF2, Btd, Mad, Opa, and h. (Table1).

The potential motifs were then mapped against the enhancer sequence array by MAST (Motif Alignment and Search Tool). For each motif in the sequence, MAST found the top

Fig. 2. MEME outcome page displaying recognized motifs in our dataset, with amino acid position-specific scores presented as logos and PSSM.

match, put together the scores for these matches, and got an E-value for each sequence. MAST output contained an E-value which was below the set threshold. The block diagram (Figure 4) displays the corresponding positions of the top motif matches in high-scoring sequences and the annotated alignments. The motif match score is computed by adding the score from every column of the position-dependent scoring matrix that matches the letter at the location in the sequence (Figure 4).

DISCUSSION

The essential functions of Robo3 in central nervous system development, mediolateral axonal guidance, and slit-independent guidance of longitudinal axons are highlighted by the developmental processes in *Drosophila*. These functions are significantly mediated by TFs. Our research employs bioinformatics tools to investigate the regulatory mechanisms of Robo3, specifically by identifying (TFBS) within its DNA sequence.

The comprehensive investigation of the robo3 genomic sequence retrieved from the FlyBase database (ID: FBgn0041097) using MEME Suite, JASPAR, TOMTOM, and MAST has revealed several potential TFBS motifs and TFs. They are more likely to be related to Robo3 gene regulation since many studies have shown that they have transcription factor properties. For instance, it has been shown that CF2 plays a significant role in determining the fate of follicular cells during the oogenesis of *Drosophila* and is involved in the developing muscles of the embryo during myoblast fusion at stage twelve.[35] Another study indicated that CF2 suppressed the expression of the Actin 88F gene and kept the balance of filament in *Drosophila* during the development of indirect flight muscles. [36]

Previous research has shown that Opa and MAD, found in this study, were involved in gene expression. Opa is a common timing (late–acting) pioneer factor that triggers a subsequent burst of zygotic gene expression [37]. It is involved in changing the pair-rule gene regulatory network to control frequency doubling in *Drosophila* segmentation . While Mad family transcription factors act as repressors throughout the process of differentiation and development [38]

In addition, Btd was related with an important role in the DNA-binding region and interacts with TATA box-binding protein-associated factors. It has a critical role in the development of the antennal, intercalary, and mandibular segments of the head [39]

On the other hand, studies showed that Twist TF (Twi) in *Drosophila* is a basic helix-loop-helix transcription factor that is considered a candidate regulator of mesodermal differentiation and myogenesis [40], and elevated its levels were necessary for somatic myogenesis and had inhibitory actin to mesodermal derivatives [41]

Fig. 3. TOMTOM result page. MEME motifs were compared to known motifs from JASPAR CORE insects

These findings are a critical milestone in discovering multiple unknown TFBS and TFs that drive *Robo3* transcription which involves in neural development. Experimentally future work is needed to confirm these results such as the Chromatin Immunoprecipitation (CHIP) assay or luciferase assay to determine if these TFs can bind and regulate the expression of the Robo3 gene.

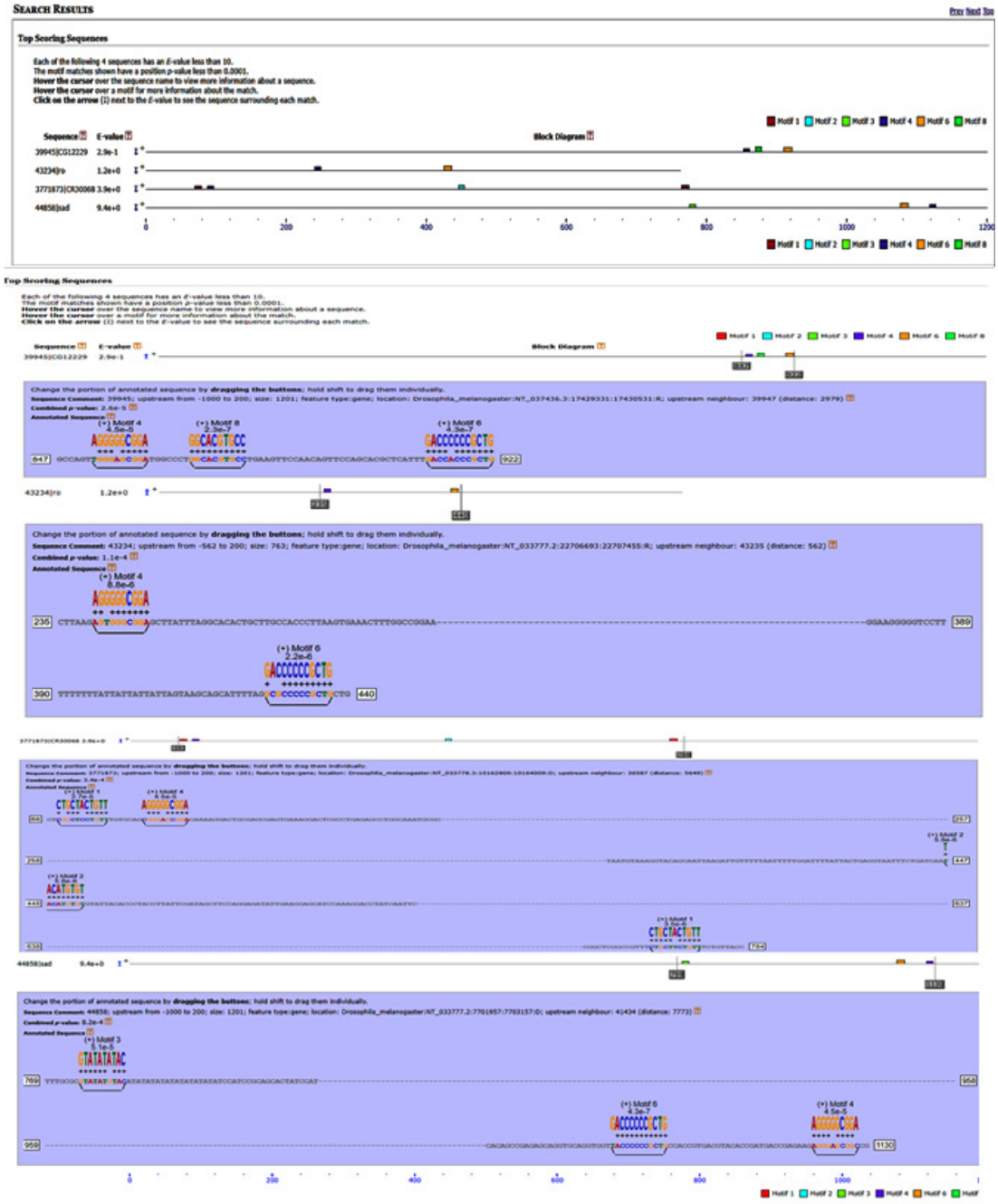
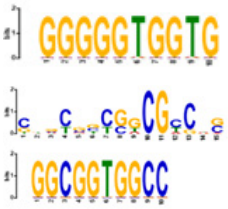
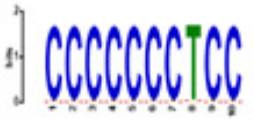
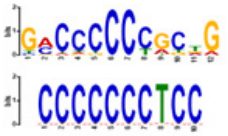
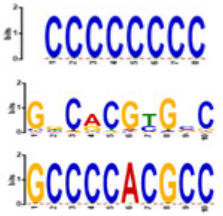
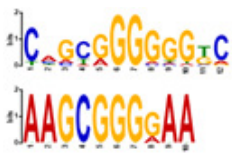
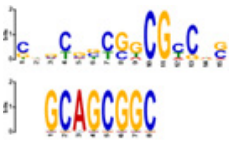


Fig. 4. The motif mapping based on MAST. Robo3 sequence with the mapped motifs detected in addition to the E-value prediction.

Table 1. The putative Transcription Factor Binding Site motifs and their alignment from an identified database.

	Motif	Sequence Logo	JASPAR Core Insect ID	Alignment	TF Binding Site
1.	GCTGCTGCTG		MA0454.1		odd
2.	TGTGTGTGTG		MA0249.2		twi
3.	ACACACACAC		MA0015.1		Cf2
4.	GGGGGTGGTG		MA0443.1		btd
5.	GGCGGTGGCC		MA0535.1		Mad
6.	CCCCCCCTCC		MA0456.1		opa
7.	CCCCCCCC		MA0443.1		btd
8.	GCCCCACGCC		MA0449.1		h
9.	AAGCGGG[GA]AA		MA0456.1		opa
10.	GCAGCGGC		MA0535.1		Mad

CONCLUSION

The developmental progressions in *Drosophila* underscore the critical roles played by *Robo3* in orchestrating central nervous system development, mediating mediolateral axonal navigation, and its involvement in slit-independent guidance of longitudinal axons. This study utilizes bioinformatics tools to explore deeper into *Robo3*'s regulatory mechanisms, precisely focusing on the identification of putative TFBS within its DNA sequence. Overall, in this investigation, we have successfully identified potential binding sites for TFs within the Robo3 gene sequence which are involved in the neural development and regulatory network. These identified sequences hold significant importance as they enable the discovery of additional genomic regions harboring similar or identical sets of binding motifs. Understanding these motifs is vital as it provides insights into which specific TFs will interact with particular DNA sequences. The interaction between these sequences and TFs ultimately regulates gene expression. Further investigation of these regulatory mechanisms promises to expand our understanding of *robo3*'s role in neural development and may eventually lead to revelations pertinent to human neurodevelopmental disorders.

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Conflict of Interest

The author declares no conflict of interest for the publishing of this paper.

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