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Research Article

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Ectopic expression of structurally similar major royal jelly proteins reveals their distinct functions in *Drosophila*

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Abstract: Royal jelly (RJ), secreted by the hypopharyngeal and mandibular glands of young worker bees, is rich in proteins, 80%-90% of which are major royal jelly proteins (MRJPs). While MRJPs from RJ have been shown to exhibit specific biological functions and are also expressed in neuronal cells, their roles *in vivo* remain poorly understood. The aim of this study was to elucidate the functional roles of individual MRJPs (MRJP1-9) *in vivo* by ectopically expressing them in *Drosophila* neurons in a binary expression system using fluorescent proteins as controls. Transcriptome sequencing revealed that although MRJP1-9 share similar tertiary structures, their overexpression affects distinct gene sets. MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 induced more differentially expressed genes, while MRJP4, MRJP6, MRJP8, and MRJP9 induced fewer such genes. Weighted gene co-expression network analysis (WGCNA) and gene set enrichment analysis (GSEA) revealed that MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 regulate overlapping gene sets, including an estradiol-responsive set, and activate cell proliferation pathways. MRJP6 lacked any significant gene set enrichment, while MRJP8 and MRJP9 modulated similar sets. Notably, the neuronal-specific overexpression of MRJP1, MRJP2, MRJP3, and MRJP5 in *Drosophila* showed activated cell proliferation-related pathways and increased body size, highlighting their functional diversity and context-dependent effects. These findings expand our understanding of the functional roles of MRJPs and provide a foundation for further exploring their biological significance in honeybees and beyond.

Key words: *Drosophila melanogaster*; Major royal jelly proteins; Ectopic expression

1 Introduction

Royal jelly (RJ), as a nutrient-rich mixture secreted by the hypopharyngeal and mandibular glands of nurse bees, plays a crucial role in the growth and development of honeybees. RJ has a relatively complex

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composition, with approximately 50% of its dry matter consisting of proteins, among which the major royal jelly proteins (MRJPs) account for over 80% of the total protein content (Collazo et al., 2021). To date, ten members have been identified in the MRJP family, namely MRJP1-10, with MRJP10 being a pseudogene, while the others (MRJP1-9) are all expressed in honeybees (Uversky et al., 2021). MRJP1 is the most abundant type (comprising 31–66% of total RJ proteins), followed by MRJP3, MRJP2 and MRJP5 (Schmitzova et al., 1998; Bilikova and Simuth, 2010). The function of MRJP1 has been shown to depend on its oligomeric state. In monomer form, MRJP1 is a 55 kDa protein, also known as royalactin, whereas its oligomeric form, apisin, is a complex of MRJP1 monomers bound to apisimin polypeptides and 24-methylenecholesterol. This oligomeric form of MRJP1 creates a fibrous network that provides RJ with the necessary viscosity (Fan et al., 2016; Tian et al., 2018). MRJP3 has been reported to have strong RNA-binding activity, which acts as an extracellular, non-sequence-specific RNA aggregation factor, protecting RNA from degradation and enhancing its bioavailability (Maori et al., 2019). Various bioactive MRJPs and their derivative peptides may benefit humans, as evidenced by studies in cell cultures, animal models and computer simulations. These bioactivities include antioxidant, antibacterial, antitumor, antihypertensive, lipid-lowering, cell growth promotion, wound healing, anti-aging, neuroprotective, anti-inflammatory, and immunomodulatory effects (Matsui et al., 2002; Nagai and Inoue, 2004; Majtan et al., 2006; Guo et al., 2009; Xin et al., 2016; Vezeteu et al., 2017; Abu-Serie and Habashy, 2019; Kim and Jin, 2019; Lin et al., 2019; Zhang et al., 2019; Park et al., 2020; Wen et al., 2024). However, beyond these examples, the functions of MRJPs within honeybees remain largely unknown.

Although RJ is primarily secreted by the head glands of worker bees, various studies have revealed that various MRJPs are also expressed in the brain cells of honeybees. Research has identified three polypeptides in the bee brain, including MRJP1-related p57, MRJP3-related p70, and a potential MRJP1 dimer, p128. In addition, proteins recognized by MRJP1 antibodies have been detected in neural fibers of the mushroom body (MB), optical lobe (OL), and antennal lobe (AL) (Peixoto et al., 2009). MRJP1 has been observed to be associated with filamentous proteins in the brain, with elevated levels of MRJP2 and MRJP7 expression noted in the brains of nurse bees compared to other workers, although their specific functions remain to be clarified (Garcia et al., 2009). Further studies have shown that MRJP1 expression in the MB varies with bee age and is reduced under conditions of social isolation, which impairs learning development (Hojo et al., 2010). It has also been confirmed that MRJP1-9 are all expressed in the bee brain, with expression levels positively correlating with those in the hypopharyngeal gland, albeit with lower levels (Dobritzsch et al., 2019). Dietary influences, such as increased 24-methylenecholesterol concentration, have been found to raise the relative abundance of MRJP1, MRJP4, MRJP5 and MRJP7 in the bee head (Chakrabarti and Sagili, 2020). Moreover, bees with gut microbial symbionts exhibited upregulated expression of odor-binding protein Obp14, olfactory receptor Or115, and MRJP1, MRJP2, and MRJP7, suggesting that gut bacteria may regulate brain gene expression to enhance learning and memory in bees (Zhang et al., 2022). Experimental interventions, such as injecting MRJP1 and MRJP3 into the bee brain, have been shown to alter worker bee behavior, increasing task reversal from foraging to nursing and reducing progression from nursing to foraging. Conversely, downregulating MRJP1 and MRJP3 via RNAi injections into the brain increased the transition of worker bees from nursing to foraging, confirming the causal role of these proteins in regulating worker bee behavior and life history (Fang et al., 2023). The above findings indicate that MRJPs may have diverse functional roles in the bee nervous system; however, studying MRJPs directly in the brain cells of bees remains challenging, and the functional differences among MRJPs *in vivo* are still poorly understood. To investigate these differences, this study conducted an experiment to ectopically express MRJP1-9 in *Drosophila* neuronal cells and performed RNA-seq analysis to elucidate the potential functional variations of MRJPs in the brain.

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2 Materials and methods

2.1 *Drosophila* culture

Flies were kept on a standard diet at 25℃ under a 12-hour light/12-hour dark cycle (12L:12D), which consisted of 218.75 g cornmeal, 112.5 g molasses, 75 g yeast, 62.5 g glucose, and 62.5 g sucrose in 3 L of water. *Act5C*-Gal4 (BS#4414), *elav*-Gal4 (BS#458), and *ppl*-Gal4 (BS#58768) flies were obtained from the Bloomington *Drosophila* Stock Center. To standardize the genetic background of flies, all were outcrossed with the *iso*^{31} (BS#5905) wild-type line for five generations.

2.2 Amplification of *mrjps* genes and construction of transgenic *Drosophila*

Total RNA was first extracted from the heads of local *Apis mellifera* using the TRIzol reagent (Vazyme, R401), then 1 μg was used for reverse transcription (Vazyme, R312). The *mrjp*1-9 genes were amplified using specific primers (Table S1) and cloned into the pUAST-attB vector after double digestion with *Xho*I and *Xba*I. The constructed plasmids, pUAST-attB-MRJPs, were microinjected into *Drosophila* line P{CaryP}attP2 (BS#25710) at 68A4 site on chrosome III. Positive transgenic offspring were identified and confirmed through red eye marker screening. To standardize the genetic background, all flies were outcrossed with the *iso*^{31} wild-type line for five generations.

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2.3 RNA extraction and sequencing library preparation

Neuron-specific driven *ealv*-Gal4 female flies overexpressing MRJPs or the mScarlet-i fluorescent protein as a control were raised at 25℃ for 7 days, after which the heads were collected, with 30 flies per group. Total RNA was extracted from the heads using the TRIzol reagent (Vazyme, R401) following the manufacturer's instructions. RNA quality was assessed using the 5300 Bioanalyzer (Agilent Technologies), and quantification was performed with the NanoDrop™ 2000 spectrophotometer (Thermo Scientific). RNA purification, reverse transcription, library construction, and sequencing were conducted at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai). For transcriptome library preparation, 1 μg of total RNA was processed using Illumina's TruSeq™ RNA Sample Preparation Kit (San Diego, CA). Briefly, mRNA was isolated via polyA selection with oligo(dT) beads, followed by fragmentation using a fragmentation buffer. Double-stranded cDNA was synthesized with SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, CA) using random hexamer primers (Illumina). The synthesized cDNA then underwent end repair, phosphorylation, and the addition of an 'A' base while following Illumina's library construction protocol. Library size selection was performed on 2% Low-Range Ultra Agarose to obtain 300-bp cDNA target fragments, followed by 15 cycles of PCR amplification with Phusion DNA Polymerase (NEB). Quantified by TBS380, the RNA-seq libraries were loaded onto on the Illumina NovaSeq 6000 platform for paired-end sequencing.

2.4 Differential expression and functional enrichment analysis

To identify differentially expressed genes (DEGs) between two groups, gene expression levels were calculated based on transcripts per million reads (TPM). Gene abundance was quantified using RNA-Seq by Expectation-Maximization (RSEM) (http://deweylab.biostat.wisc.edu/rsem/), and differential expression analysis was performed with DESeq2 (Love et al., 2014). Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) was conducted using R version 4.10, with all DEGs between the groups serving as input. The $\log_2$FoldChange value represented the degree of expression change between the groups and was used to create a gene list sorted by gene names and values in a descending order. The *Drosophila* gene sets were retrieved from the Molecular Signatures Database (MSigDB) using the 'msigdbr' package. Enrichment analysis was then performed using the clusterProfiler package (Wu et al., 2021), a versatile tool for interpreting omics data. Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder and Horvath, 2008) was conducted using an online analysis tool provided by Majorbio Bio-Pharm Technology Co.,

Ltd. (Shanghai).

2.5 Biological validation experiments

To verify the effect of MRJPs expression in different tissues on individual size variation, transgenic UAS-MRJPs flies were crossed with different Gal4 driver lines: *Act5C*-Gal4 (all cells), *elav*-Gal4 (neuronal cells), and *ppl*-Gal4 (fat body cells). Serving as controls, each Gal4 driver line was separately crossed with *iso*^{31} flies, and different UAS-MRJP lines were separately crossed with wild-type *iso*^{31} flies as another control. After eclosion, female offspring were reared on standard food for seven days before being used for body size measurement. Flies were anesthetized on a CO_2 pad and placed on their sides to position the head antennae, balancing organ, and tail tip within the field of view. Photos were taken with a microscopic imaging system set to a uniform focal length. A smooth curve was drawn to connect the antennae, balancing organ and tail tip, allowing for pixel measurement along the curve to determine its length.

2.6 Statistical analysis

Statistical analysis was performed using Graphpad Prism 9 software for Windows (GraphPad Software, San Diego, CA, USA). The data was displayed as the mean ± standard deviation (SD), and the figure was shown as a scatter dot plot. Normality was assumed using the Shapiro-Wilk test. Differences between groups were analyzed using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test or Kruskal-Wallis test. A p-value of less than 0.05 was considered statistically significant.

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3 Results

3.1 Highly similar tertiary structures of different MRJPs

MRJPs have evolved from an ancient protein family, YELLOW, with which they share a high degree of homology, thus they are often grouped together as the MRJP/YELLOW family. The honeybee genome contains 10 *mrjp* genes (including one pseudogene) and 10 *yellow* genes (Drapeau et al., 2006). The *mrjp* genes are primarily found in *Apis* species and other Hymenoptera and are organized in a single gene cluster within the honeybee genome (Fig. 1a). The *mrjp*1-9 genes have highly similar structures (Fig. S1), with the gene family likely having had emerged from multiple duplication events from a common ancestor (Drapeau, et al., 2006). The coding sequences of *mrjp*1-9 from *Apis mellifera* were amplified and sequenced using samples collected from a local apiary. Although some polymorphic sites were identified compared to the sequences published in genome databases, the similarity between different MRJPs remains high (Fig. 1b and Table S2). Using our amplified MRJP protein sequences and the YELLOW-e3 and YELLOW-h protein sequences from databases, an unrooted phylogenetic tree was constructed using the neighbor-joining method. Phylogenetic analysis revealed a clear clustering of MRJPs within the tree branches, specifically forming distinct groups of MRJP2 + 7, MRJP5 + 6, MRJP3, MRJP1, MRJP4, and MRJP8 + 9 (Fig. 1c), each encoded by tandemly arranged, adjacent *mrjp* genes (Fig. 1a), consistent with previous findings (Drapeau, et al., 2006).

To further investigate the potential structural differences among MRJPs, their tertiary structures were examined. Prior studies have identified a 16-molecule oligomeric structure of native MRJP1, comprising four MRJP1 proteins, four apisimin molecules, and eight 24-methylenecholesterol molecules (Tian, et al., 2018). Nonetheless, the structures of other MRJPs remain unknown. The structures of the MRJP sequences that we amplified and sequenced were predicted using Alphafold3 (Abramson et al., 2024). The predicted structure of MRJP1 closely matched the experimentally determined structure of MRJP1 (Fig. S2), indicating the accuracy of the prediction. Furthermore, it was found that Alphafold3 predicted highly similar structures for MRJP1-9 (Fig. 1d), although the confidences (pLDDT scores) at the N- and C-termini were lower. This raised an intriguing question: Do proteins with such similar structures actually perform similar biological functions *in vivo*?

3.2 Efficient ectopic expression of different MRJPs in *Drosophila*

Due to the challenges associated with directly studying the functions of different MRJP proteins in honeybees, the model organism *Drosophila* was selected, as it offers a rich toolkit for genetic manipulation. *Drosophila* expresses *yellow* genes, and the YELLOW proteins in flies are ancestral members of the MRJP/YELLOW protein family, exhibiting various functions such as controlling the sex determination pathway in *Drosophila* (Radovic et al., 2002; Drapeau et al., 2003), regulating the mating success rate in males (Massey et al., 2019) and influencing development and cellular functions (Hinaux et al., 2018). Since honeybee MRJPs and *Drosophila* YELLOW proteins belong to the same MRJP/YELLOW family, it seemed feasible to study the functions of honeybee MRJPs through their ectopic expression in *Drosophila*. The Gal4/UAS binary expression system in *Drosophila* is a widely used tool for gene expression regulation in research (Fig. 2a). In this system, a tissue-specific promoter or enhancer drives the expression of the Gal4 transcription factor. By crossing flies carrying Gal4 with those carrying UAS, the resulting offspring can activate the target gene downstream of UAS in the tissues or cells expressing Gal4 (Fig. 2a). The pUAST-attB vector was selected for this purpose, and different *mrjp* genes along with the control fluorescent protein gene mScarlet-i were inserted between the XhoI and XbaI cloning sites (Fig. 2b). Importantly, different UAS-MRJPs were chosen for site-specifically integration at the attP2 site on the third chromosome of *Drosophila* (Fig. 2c) to eliminate any unintended variations that may arise from inserting at different loci. By crossing *elav*-Gal4 that drives neuronal-specific expression, with UAS-mScarlet-i, fluorescent protein expression in the *Drosophila* brain was clearly observed (Fig. 2d). In addition, via crossing *elav*-Gal4 with different UAS-MRJPs, the expression of *mrjp* genes was confirmed (Fig. 2d).

3.3 Distinct signaling perturbations from ectopic MRJPs expression in *Drosophila* neurons

Next, the *elav*-Gal4 driver was further utilized to induce ectopic expression of different MRJPs in neurons, followed by extracting total RNA from *Drosophila* heads for transcriptome sequencing analysis. Previous studies have reported the expression of *Drosophila* YELLOW protein in certain subpopulations of neurons in both larval and adult brains (Hinaux, et al., 2018). Following the pan-neuronal expression of MRJP1-9 in *Drosophila* heads, principal component analysis (PCA) revealed that each MRJP overexpression group distinctly separated in overall gene expression from the control group (Fig. S3), indicating that the overexpression of different MRJPs indeed causes changes in endogenous genes in *Drosophila*. Although structure predictions suggested that MRJP1-9 share relatively similar tertiary structures, the genes disrupted by their overexpression varied. Notably, MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression resulted in a higher number of differentially expressed genes, while MRJP4, MRJP6, MRJP8, and MRJP9 showed fewer differentially expressed genes (Fig. 3).

Subsequently, to identify gene association patterns between different MRJP overexpression groups and the control group, a comprehensive WGCNA was conducted on the transcriptome datasets. WGCNA is a commonly used algorithm for constructing gene co-expression networks, grouping genes with shared expression characteristics across samples into the same network. Usually, genes within the same co-expression network exhibit similar expression patterns, while these patterns for genes in different networks are distinct. Genes with similar expression profiles are clustered into groups called modules. By identifying these co-expression gene modules, we can connect them to phenotypic information of interest to explore the relationships between gene networks and phenotypes (Langfelder and Horvath, 2008). Our transcriptome data revealed four primary modules (Fig. 4a). Cluster analysis of gene-phenotype associations showed that the MRJP1, MRJP2, MRJP3, and MRJP7 overexpression groups displayed distinct patterns compared to other groups, with consistent module patterns within these groups (Fig. 4a).

Interestingly, comparative gene enrichment analysis using the screened DEGs revealed that MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression regulate similar gene sets, while MRJP6 showed no significant gene set enrichment. MRJP4 overexpression regulates a distinct gene set, whereas MRJP8 and MRJP9 regulate comparable gene sets (Fig. 4b). This finding aligns well with the phylogenetic tree clustering

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analysis (Fig. 1c). Notably, the gene sets regulated by MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression were observed to include an estradiol-responsive gene set (Fig. 4b).

Furthermore, GSEA was performed to comprehensively detect gene sets with biological significance. By ranking genes based on their differential expression between two sample types, GSEA tests whether specific gene sets are enriched at the top or bottom of the ranking list, enabling the identification of biologically relevant genes without specifying a threshold of differential expression (Mootha, et al., 2003; Subramanian, et al., 2005). The Hallmark gene sets were obtained by aggregating multiple MSigDB gene sets, designed to represent specific biological states or processes and display consistent expression (Liberzon et al., 2015). These gene sets are generated through computational methods that identify overlaps between gene sets in other MSigDB collections, retaining genes that exhibit coordinated expression. Hallmark gene sets help reduce noise and redundancy, providing a clearer biological space for GSEA. These gene sets were used to perform GSEA analysis on the differentially expressed genes from various MRJP overexpression groups relative to the control group. The results indicated that only the MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression groups significantly activated certain signature gene sets, and the Hallmark gene sets were consistently associated with cell proliferation signals, such as MYC targets, E2F targets, G2M checkpoint, and mitosis (Fig. 5).

3.4 Ectopic expression of certain MRJPs in neurons but not in fat bodies induces increased body size in *Drosophila*

MRJP overexpression activates cell proliferation-related signals. Accordingly, earlier studies have also indicated that pan-expression or fat body-specific overexpression of MRJP1 in *Drosophila* leads to increased body size (Kamakura, 2011). This study aimed to verify this observation. Initially, the pan-cell expression driver *Act5C*-Gal4 was utilized to overexpress different MRJPs, selecting approximately one-week-old Gal4-MRJPs female flies for size observation. Unfortunately, no increase in body size was observed under the pan-expression of different MRJPs (Fig. S4). In addition, the fat body-specific driver *ppl*-Gal4 was employed to overexpress various MRJPs, but again, no increased body size phenotype was observed (Fig. S5). However, when *elav*-Gal4 was used to drive the overexpression of different MRJPs in neurons, a significant increase in body size was noticed in the MRJP1, MRJP2, MRJP3, MRJP5, and MRJP8 overexpression groups, as well as a small increase in the MRJP4 overexpression group (Fig. 6).

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4 Discussion

Several unresolved questions remain regarding MRJPs, particularly their expression in tissues outside the hypopharyngeal gland, such as in brain cells. The molecular and biochemical characteristics of MRJPs in bumblebees, also members of the order Hymenoptera, suggest a non-nutritional role (Kupke et al., 2012). Consistent with findings in honeybees, MRJPs are also expressed in the bumblebee brain, particularly in the Kenyon cells of the mushroom bodies (involved in sensory integration) and in the optic lobes (Albert et al., 2014). In honeybees, *mrjp*1-5 and *mrjp*7 exhibit age-dependent expression patterns in both the worker hypopharyngeal glands and the brain, with lower abundance in the brain than in the glands. Expression levels increase from the hatchling to nurse stages but decrease in older worker bees. Unlike the other *mrjp* genes, *mrjp*6 expression shows no significant variation in the brain. The *mrjp*8 and *mrjp*9 exhibit low expression in both tissues, suggesting that they may function in other tissues, whereas *mrjp*1-7 may modulate worker phenotype plasticity and age polymorphism (Dobritzsch, et al., 2019). The expression of *mrjp*1 through *mrjp*7 is characteristic of worker bee heads, with *mrjp*1-4 and *mrjp*7 expressed at higher levels in nurse bees compared to foragers, while *mrjp*5 and *mrjp*6 being more highly expressed in foragers than in nurses, indicating additional functions beyond brood food proteins (Buttstedt et al., 2013). Our systems biology analysis from *in vivo* data further suggests that MRJP4, MRJP6, MRJP8, and MRJP9 have distinct functional

roles compared to other MRJP proteins.

In *Drosophila*, the YELLOW protein is expressed in certain central neurons from the second larval stage to adulthood, including a group of neurons adjacent to clock neurons marked by the PDF neuropetide (Hinaux, et al., 2018). The *yellow* gene, positioned downstream of *fru* in the third instar larval brain, is a crucial component of the somatic sex determination pathway in *Drosophila* and plays an important role in the development of male wing extension during courtship (Drapeau, et al., 2003). Some studies have also suggested that the influence of YELLOW on male mating success is mediated by pigmentation of a male-specific leg structure called the sex comb rather than through neural circuit regulation (Massey, et al., 2019). Also, it has been reported that the *yellow* gene modulates behavioral plasticity by inhibiting courtship in butterfly species with dimorphic males (Connahs et al., 2022). While numerous studies have reported the expression of MRJPs in brain cells of honeybees, research into the biological functions of MRJPs in the brain remains limited. In one study, MRJP proteins were injected directly into the honeybee brain or, conversely, RNA interference or corresponding antibodies were used to inhibit MRJP functions in the brain, demonstrating that certain MRJP proteins play a role in regulating worker bee behavior and life history (Fang, et al., 2023). However, the precise neurobiological functions of MRJPs are yet to be understood.

The intriguing question is: Why do MRJPs, which have such similar predicted structures, exhibit distinct functions *in vivo*? This functional divergence may be related to post-translational modifications of the proteins. Previous studies have shown that proteins in royal jelly undergo glycosylation, with asparagine-linked (N-linked) glycosylation being one of the most common types of post-translational modifications in eukaryotes. All MRJPs are predicted to have 1–8 N-glycosylation sites; the glycosylation of MRJP1–4, MRJP6, MRJP7, and MRJP9 has been experimentally confirmed (Okamoto et al., 2003; Kimura et al., 2010; Zhang et al., 2014). Research indicates that MRJP1 and MRJP2 exhibit different glycosylation patterns, and this differential glycosylation indeed affects the antibacterial activity of MRJP2 (Bilikova et al., 2009). Moreover, glycosylation may influence the IgE-binding capacity of MRJP1 and MRJP2 (Hayashi et al., 2011). These findings suggest that glycosylation plays a significant role in modulating the biological activity of MRJPs.

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Several studies have reported the cell proliferation-promoting potential of MRJP proteins extracted from RJ (Mureşan et al., 2022). MRJP1, in particular, exhibits growth factor-like activity: it enhances DNA synthesis in rat hepatocytes, maintains proliferation and inhibits apoptosis (Kamakura et al., 2001; Kamakura and Sakaki, 2006). Furthermore, MRJP1 stimulates the growth of human bone marrow cells (Watanabe et al., 1998) and human lymphocytes (Moriyama et al., 2015). The 290-kDa oligomer composed of MRJP1 and apisimin enhances lymphocyte proliferation in humans (Tamura et al., 2009). A protein component primarily containing MRJP2, MRJP3 and/or MRJP7 exhibited potential wound-healing bioactivity, promoting proliferation and migration of human epidermal keratinocytes in an *in vitro* scratch model (Lin, et al., 2019). In its monomeric form, MRJP1 can activate a pluripotency gene network to support the self-renewal of mouse embryonic stem cells (mESCs) (Wan et al., 2018). Even recombinant MRJP1 from *Apis cerana* stimulated cell growth in lepidopteran cell lines, further validating these findings (Shen et al., 2010). MRJPs have also been shown to extend the lifespan of *Drosophila* by promoting signaling through the epidermal growth factor receptor (EGFR) pathway (Xin, et al., 2016), and monomeric MRJP1 similarly influences lifespan in *Caenorhabditis elegans* by promoting the EGF-EGFR pathway (Detienne et al., 2014). Enhanced EGFR signaling is a key pathway for stimulating cell division and differentiation. In this study, we systematically compared the functions of different MRJPs for the first time, and demonstrated at the molecular network regulation level that the overexpression of MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 significantly activates cell proliferation-related signaling pathways.

Previous research has proposed that royalactin (MRJP1) in royal jelly drives queen development in bees through an EGFR-mediated signaling pathway (Kamakura, 2011). In this study, the Gal4-UAS system was used to overexpress MRJP1 in *Drosophila*. Compared to the UAS-MRJP1 control, *Drosophila* with pan-cellular MRJP1 expression exhibited increased body size, cell size, fertility, and lifespan, along with a

shortened development time (Kamakura, 2011). Furthermore, similar phenotypic changes were observed when MRJP1 was specifically overexpressing in the fat bodies, suggesting that this tissue-specific expression can also replicate the effects of pan-cellular overexpression (Kamakura, 2011). However, these findings have been challenged by subsequent research, arguing that royalactin does not play a definitive role in queen development (Buttstedt et al., 2016). Analyzing the *Drosophila* genetic experiments in Kamakura's study, it was noted that only UAS-MRJP1 control was used, without a strictly designed additional Gal4 control group. In this study, we included control groups where Gal4 lines and UAS lines were crossed with wild-type lines to drive MRJP1 overexpression either pan-cellularly or in fat body cells. Contrary to Kamakura's findings, we did not reproduce the increased body size when MRJP1 was overexpressed with pan-cell or fat body-specific drivers in *Drosophila*. Interestingly, when MRJPs were overexpressed in neurons, some of them did show an increase in body size, a phenomenon warranting further investigation into its underlying mechanisms.

5 Conclusions

In summary, this study highlights the structural and functional diversity of MRJPs within the MRJP/YELLOW family (Fig. 7). It has been shown that despite their highly similar tertiary structures, MRJPs exhibit distinct biological impacts when ectopically expressed in *Drosophila* neurons. Phylogenetic and structural analyses have confirmed the evolutionary relationships among MRJPs, while transcriptome and gene network analyses revealed unique gene expression patterns and signaling perturbations associated with specific MRJP overexpression groups. Notably, MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression induced the significant activation of cell proliferation-related pathways, consistent with their phylogenetic clustering and gene enrichment profiles. Interestingly, among these five MRJPs, the neuronal-specific expression of MRJP1, MRJP2, MRJP3, and MRJP5 led to increased body size in *Drosophila*, whereas their expression in the fat bodies or across all cells did not, highlighting the context-dependent effects of MRJPs on physiological phenotypes. These findings expand our understanding of MRJP functional roles and provide a foundation for further exploring their biological significance in honeybees and beyond.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA020151) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

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Author contributions

Wenfeng CHEN and Yufeng YANG conceived and designed the experiments; Lingqi YU, Danfeng WANG, Jiayu XIE, Dongjing WEN, Yi ZHANG and Xuanhao CHEN performed the experiments; Wenfeng CHEN, Lingqi YU, Danfeng WANG, Zhenxing LIU, and Lirong SHEN drafted and revised the manuscript. All authors read and approved the final manuscript and, therefore, had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

All authors declare that they have no conflict of interest. This study used the invertebrate model organism *Drosophila melanogaster*, which is exempt from ethical approval requirements.

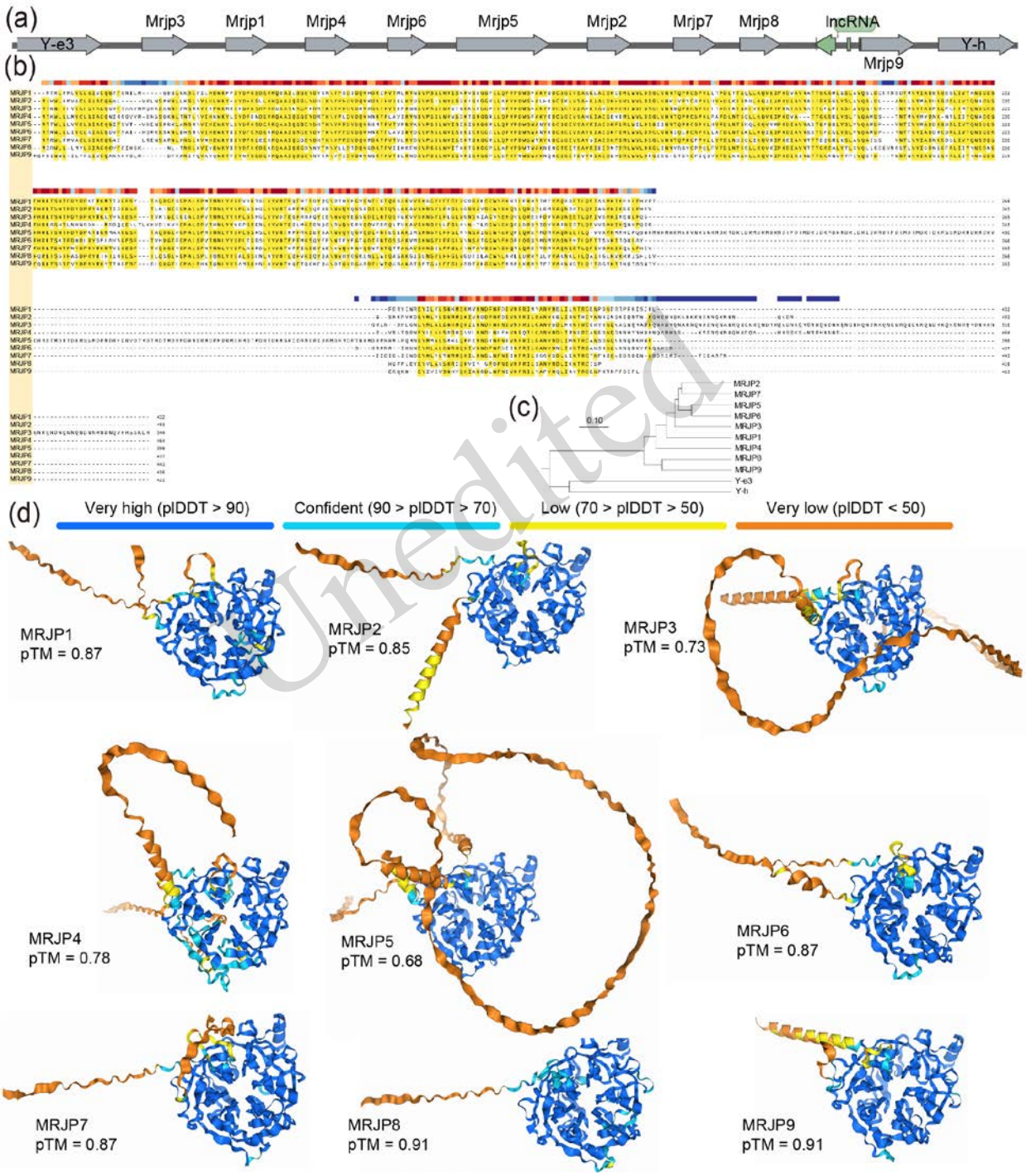

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Fig. 1 Comparative analysis of protein sequences and tertiary structures of different MRJPs.

(a) Schematic diagram of *mrjp* and partial *yellow* genes in the honeybee genome; (b) Alignment results of MRJP protein sequences obtained from our cloning and sequencing data; (c) Phylogenetic tree analysis of MRJP protein sequences based on our cloning and sequencing data. An unrooted phylogenetic tree based on the neighbor-joining method was constructed using MEGA X (Kumar et al., 2018); (d) Three-dimensional structures of different MRJPs predicted by Alphafold3. AlphaFold produces a per-residue model confidence score (pLDDT) between 0 and 100. Some regions below 50 pLDDT may be

unstructured in isolation. The predicted template modeling (pTM) score is derived from the template modeling (TM) score, a metric that evaluates the accuracy of an entire structure. A pTM score above 0.5 indicates that the overall predicted folding of the complex may closely resemble the true structure.

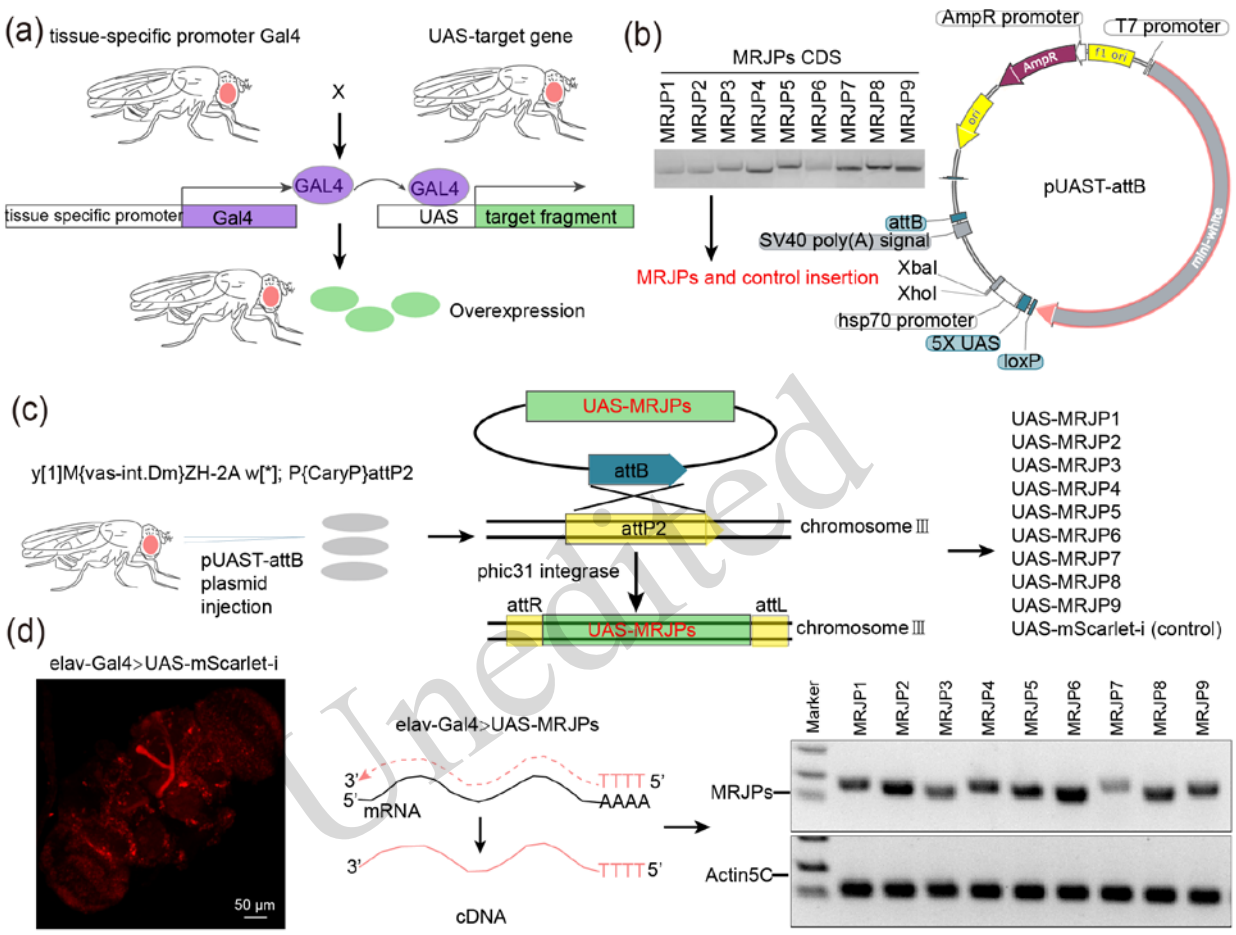

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Fig. 2 Construction and expression analysis of transgenic *Drosophila* with different UAS-MRJPs.
(a) The Gal4/UAS binary expression system in *Drosophila*; (b) The pUAST-attB plasmid used for cloning various *mrjp* genes, enabling site-specific insertion into the *Drosophila* chromosome; (c) Site-specific insertion of pUAST-MRJPs-attB into the attP2 locus on the third chromosome of *Drosophila*, mediated by integrase; (d) Validation of UAS-MRJPs and UAS-mScarlet-i expression.

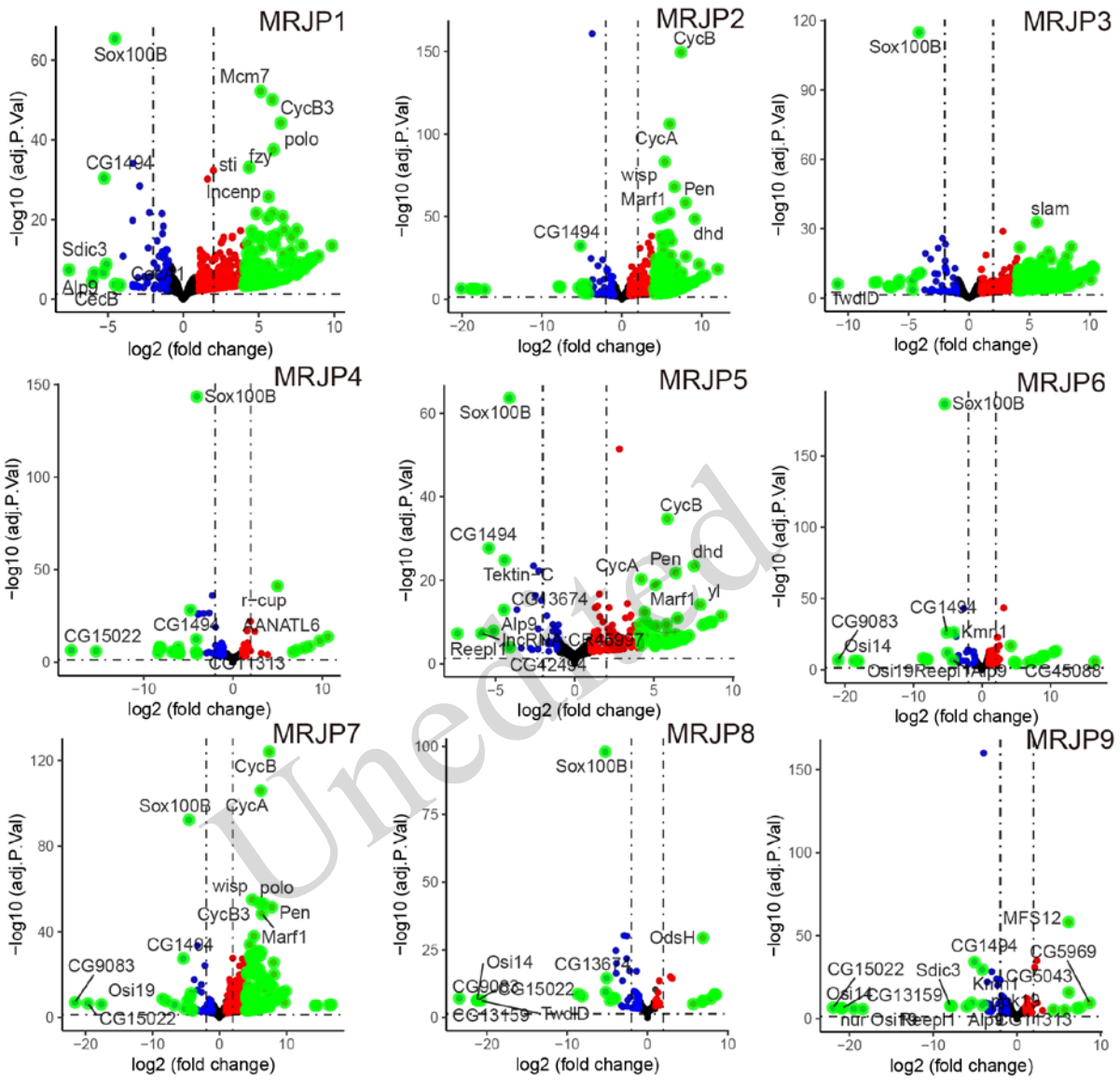

Fig. 3 Differentially expressed genes following the overexpression of different MRJPs in neurons of *Drosophila* heads. Quantitative analysis of transcriptome data was performed using DESeq2, with the x-axis representing the $\log_2$(fold change) of differentially expressed genes and the y-axis representing the adjusted P-value. Genes with P-value < 0.05 and the absolute value of $\log_2$(fold change) > 0.5 were considered differentially expressed. Red dots indicate the value of $\log_2$(fold change) > 1, blue dots indicate the value of $\log_2$(fold change) < -1, and green dots represent genes with absolute value of $\log_2$(fold change) ≥ 4.

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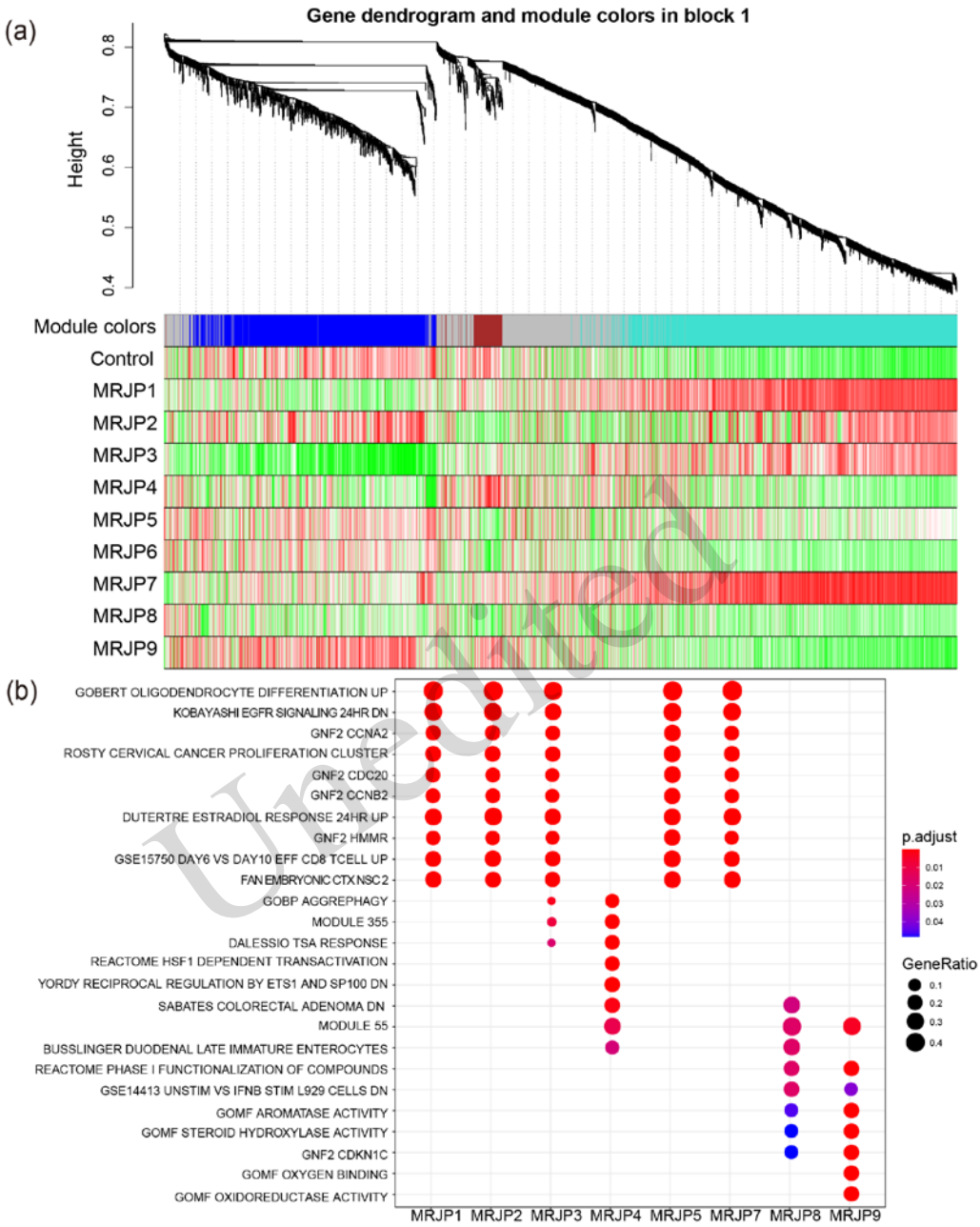

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Fig. 4 Co-expression network analysis and gene enrichment comparison analysis of head transcriptomes in different MRJP overexpression and control groups.

(a) Gene co-expression network analysis. The upper part of the image displays the hierarchical clustering tree of genes, the middle section shows the modules to which the genes belong, and the lower part illustrates the heatmap of gene-phenotype correlations within the modules, where each row represents a phenotype and each column corresponds to a gene within the module. The color indicates the strength of the correlation, with red denoting a positive correlation and green denoting a negative correlation. Module means a highly interconnected set of genes. In an undirected network, genes within a module are highly correlated; (b) Gene enrichment comparison analysis: We performed enrichment analysis of DEGs from various MRJP overexpression groups compared to the fluorescent protein mScarlet-i overexpression control group using the gene set resources annotated for *Drosophila* provided by the MSigDB for GSEA software. The analysis utilized the compareCluster function from the clusterProfiler package, including only genes with an adjusted p-value (adj.P.Val) < 0.05 and an absolute log fold change ($|\log_2(\text{fold change})|$) > 1.

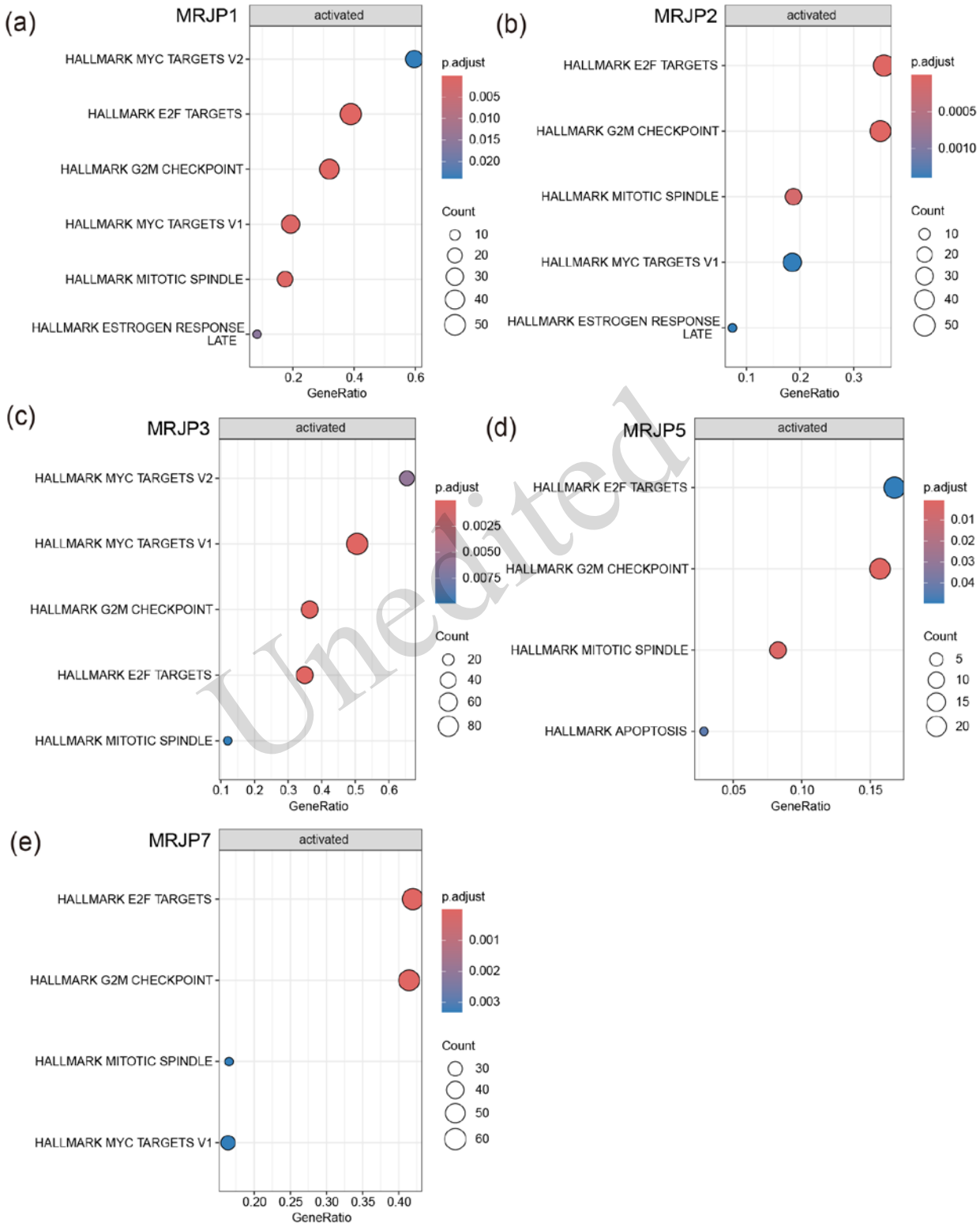

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Fig. 5 Gene set enrichment analysis of Hallmark gene sets in different MRJP overexpression groups.
(a) GSEA of MRJP1 overexpression group. (b) GSEA of MRJP2 overexpression group. (c) GSEA of MRJP3 overexpression group. (d) GSEA of MRJP5 overexpression group. (e) GSEA of MRJP7 overexpression group.

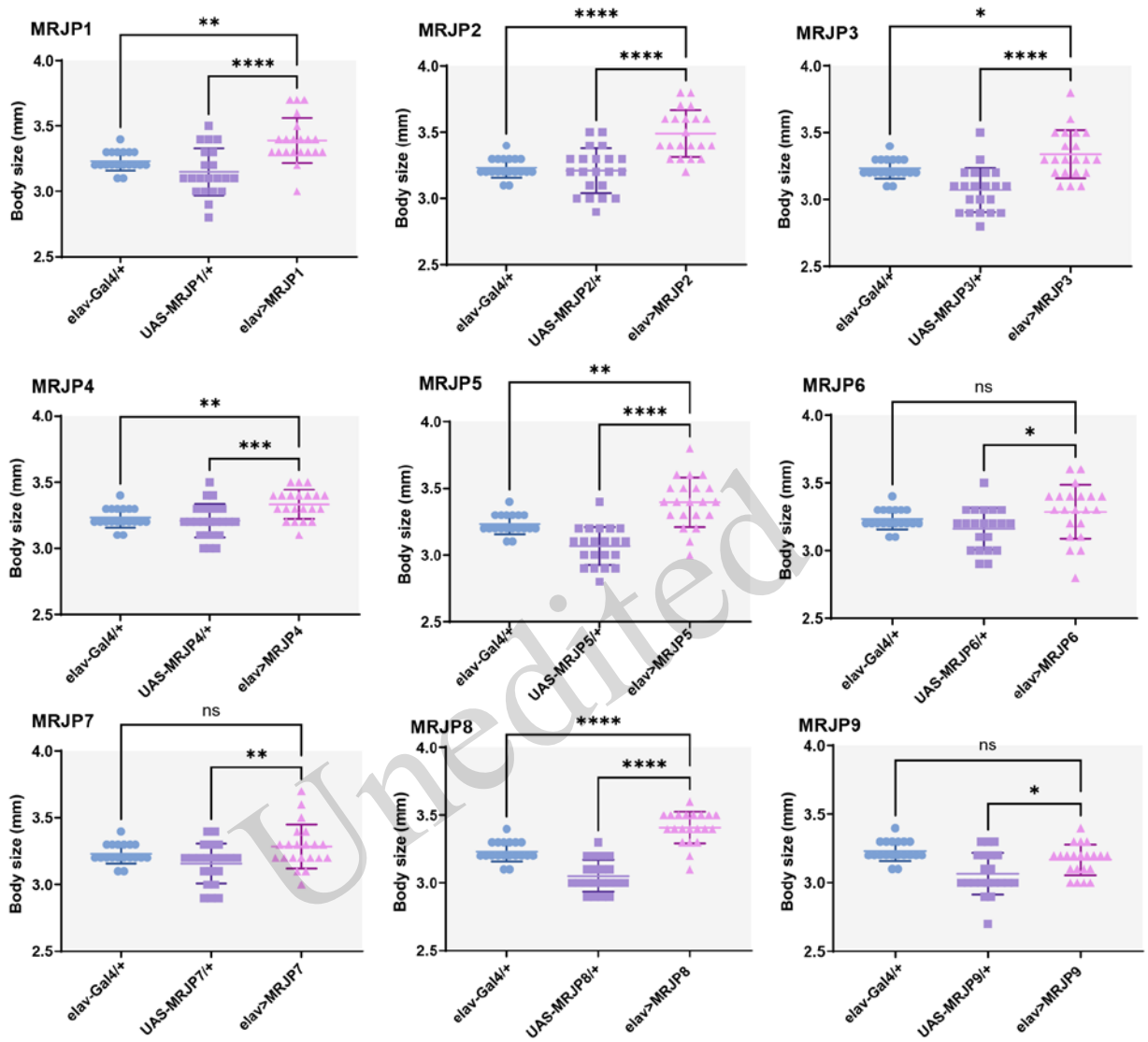

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Fig. 6 Changes in the body size of *Drosophila* following neuronal overexpression of different MRJPs.
Data are presented as mean ± SD, with values displayed in scatter dot plots (n=19-24). ns, no significance, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

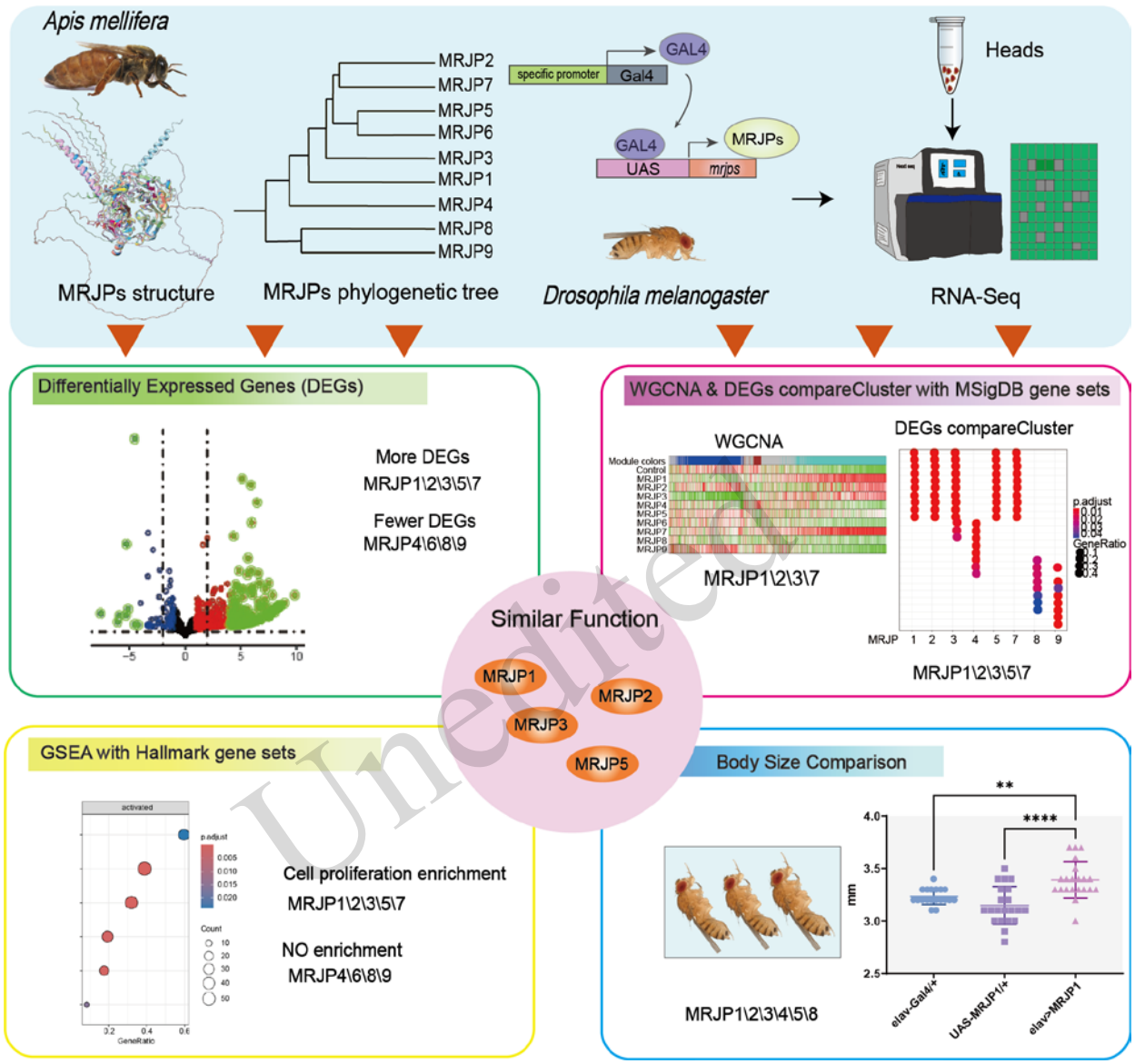

Fig. 7 Workflow summary.

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Supplementary information

Figs. S1–S5; Tables S1 and S2

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