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Expression of human PQBP-1 in *Drosophila* impairs long-term memory and induces abnormal courtship

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Abstract **Frame shift mutations of the polyglutamine binding protein-1 (*PQBP1*) gene lead to total or partial truncation of the C-terminal domain (CTD) and cause mental retardation in human patients. Interestingly, normal *Drosophila* homologue of PQBP-1 lacks CTD. As a model to analyze the molecular network of PQBP-1 affecting intelligence, we generated transgenic flies expressing human PQBP-1 with CTD. Pavlovian olfactory conditioning revealed that the transgenic flies showed disturbance of long-term memory. In addition, they showed abnormal courtship that male flies follow male flies. Abnormal functions of PQBP-1 or its binding partner might be linked to these symptoms.**

Keywords: Mental retardation; Polyglutamine; Memory; Transcription; PQBP-1/PQBP1

1. Introduction

PQBP-1 was originally isolated as a binding protein to the polyglutamine (polyQ) sequence of a transcription factor Brn2 [1]. PQBP-1 co-localizes and interacts with mutant ataxin-1 (AT1) and huntingtin (Htt) [2,3]. PQBP-1 is highly conserved beyond species and the homologues are found in *Caenorhabditis elegans*, *Arabidopsis* and mouse. PQBP-1 possesses two conserved domains [4]. The first one is WW domain (WWD), a protein–protein interaction motif relevant to SH3 domain, which prefers proline-rich sequences [4,5]. The second one is C-terminal domain (CTD) highly conserved among PQBP-1 orthologues [4], which has no homology to any domain of other molecules. Several groups independently confirmed interaction between CTD of PQBP-1 and U5-15kD [6,7], a component of a splicing factor complex U5. A target molecule of the PQBP-1 WW domain was shown to be RNA polymerase II [2], while additional molecules may interact with PQBP-1 because WW domain is a protein-binding motif with a wide spectrum of interaction partners [4,5].

Recently, Kalscheuer et al. reported that insertions or deletions in the *PQBP1* gene at the junction region between WWD and CTD cause human mental retardation (MR) with a high frequency in Europe [8]. Another group reported MR patients carrying mutations within CTD that lead to the partial deletion of this domain [9,10]. X-linked MR disorders that had been described as Renpenning syndrome, Sutherland-Haan syndrome, Golabi-Ito-Hall syndrome and so on, turned out to be the PQBP-1 disease [11], suggesting that the frequency of the *PQBP1*-linked MR patients is relatively high among familial mental retardations [9–11]. Importantly, all the mutations reported so far lead to total or partial truncation of CTD, suggesting us significance of keeping both domains in a PQBP-1 molecule.

From this viewpoint, it is highly interesting that the *Drosophila* homologue of PQBP-1 (dPQBP-1) possesses only WWD but lacks CTD. This notion prompted us to study whether induction of human PQBP-1 (hPQBP-1) containing both WWD and CTD affects intelligence of *Drosophila*. Therefore, as the first step to investigate molecular mechanism of MR linked to the *PQBP1* mutations, we generated transgenic flies expressing human PQBP-1 and examined their memory function. As the result, we found that human PQBP-1 impairs long-term memory in the transgenic flies, suggesting that dPQBP-1 utilizes a distinct system from that of human PQBP-1 although both PQBP-1 molecules might be involved in memory formation.

2. Materials and methods

2.1. Fly stocks and generation of transgenic lines

The 0.8 kb *Eco*RI–*Xho*I fragment containing human PQBP-1 cDNA [1] was subcloned into the pUAST vector. The constructs were injected into cantonized w(cs10) preblastoderm embryos together with helper plasmid p π 25.7, containing P element with defective inverted repeats and acting as a transposase source [12]. The resulting flies were crossed with cantonized w(cs10) flies, and transgenic progeny were identified by their eye color. Multiple fly lines were generated, and chromosome localization of transgene insertions was determined by crossing the transformants with the cantonized w(cs10) balancer stock carrying dominant CyO marker for chromosome 2 and dominant TM3 or Ser marker for chromosome 3. The homozygous transgenic lines were crossed with homozygous ELAV-GAL4 driver to express human PQBP-1 protein throughout nerve system, and the progeny, UAS-PQBP-1/ELAV-GAL4, were used for further analyses. Hemizygous flies, UAS-PQBP-1/+ and ELAV-GAL4/+, served as controls. Fly

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culture and all crosses were carried out at 25 °C and 60 ± 10% humidity on standard medium according to standard protocols as described previously [13].

2.2. Western blot analysis

Fly heads were dissolved in sample loading buffer [62.5 mM Tris/HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 2.5% (v/v) 2-mercaptoethanol, 5% (v/v) glycerin, and 0.0025% (w/v) bromphenol blue] and heated at 100 °C for 3 min. After electrophoresis, the gels were transferred to polyvinilydene difluoride membrane (Fine Trap, Nihon Eido), which were subsequently incubated with primary antibodies, anti-PQBP-1 (C-20, Santa Cruz, 1:1000) that detects mammal CTD, for 1 h and horseradish peroxidase-conjugated second antibodies for 1 h and visualized by enhanced chemiluminescence Western Blotting Detection System (Amersham Biosciences).

2.3. Pavlovian olfactory conditioning

In this study, we use the olfactory conditioning paradigm of Tully and Quinn [12], because it produces a robust memory that allows a detailed analysis of specific memory phases. In this olfactory training protocol, about 100 flies are exposed to one odor with an electric shock reinforcement followed by another odor without punishment. We used two aversive odors, 3-octanol (OCT) and 4-methylcyclohexanol (MCH). The flies are then tested for memory in a T-maze, where they choose between the two odors used in training. Normal flies learn to avoid the shock-paired odor in a single training trial. Memory performance is calculated as the number of flies that avoid the shock-paired odor minus the number that avoid the non-shock-paired odor divided by the total number of flies. This memory score is a "half score" because normally a single performance index (PI) data point represents the average score of two experiments. In the second experiment, a

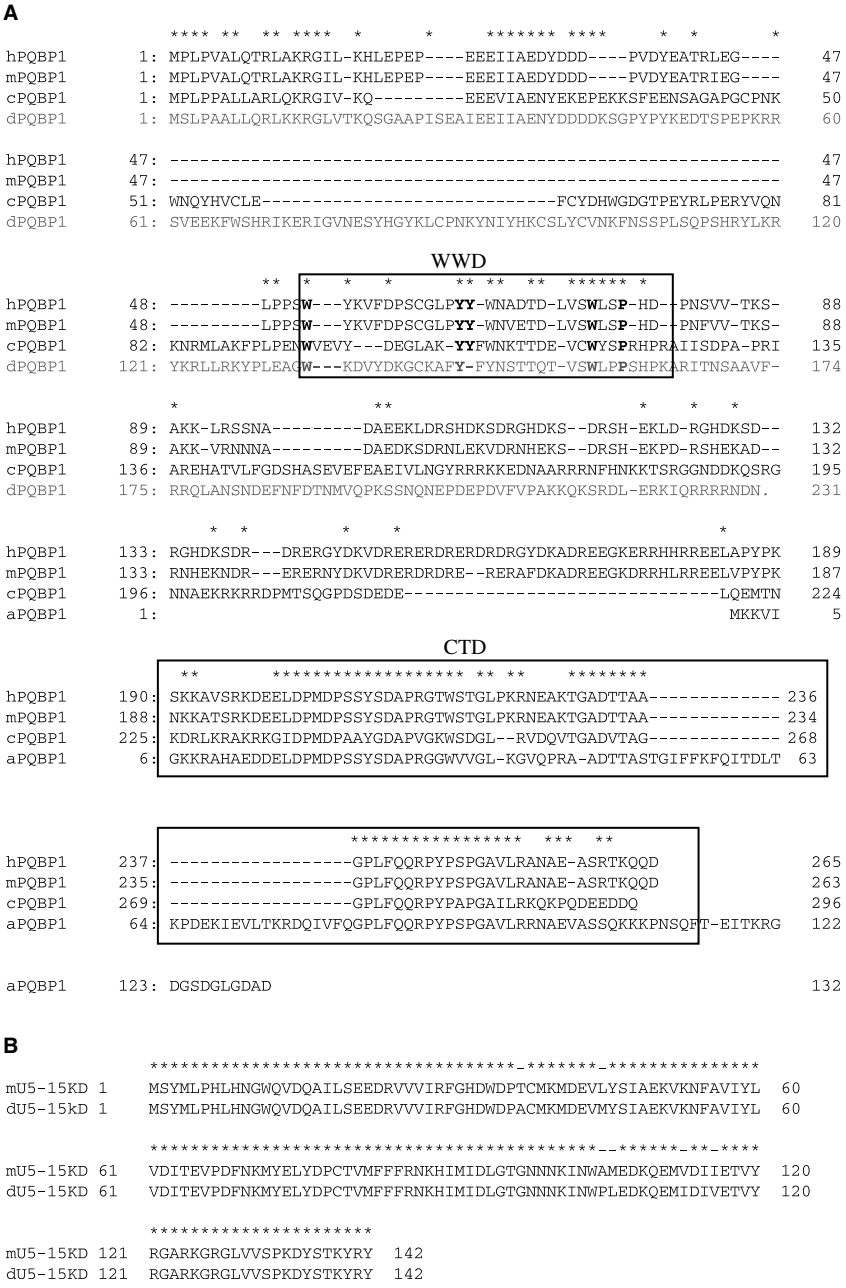

Fig. 1. (A) Alignment of PQBP1 orthologues and *Drosophila* homologue (dPQBP-1) shows that dPQBP-1 lacks C-terminal domain (CTD). hPQBP-1, mPQBP-1, cPQBP-1, dPQBP-1, aPQBP-1: human, mouse, nematoda, *Drosophila*, *Arabidopsis* PQBP-1, respectively. Asterisks indicate amino acids conserved in three species or more. (B) U5-15kD, a binding partner of PQBP-1-CTD is strictly conserved in *Drosophila*. Mouse and *Drosophila* U5-15kD are aligned. Asterisks indicate amino acids conserved.

new population of 100 flies is taught to associate the other odor with shock. 1 and 3 h memory test was performed 1 and 3 h after training respectively. For 24 h memory test, flies were trained 5 times with 1 h interval (spaced-training), and memory test was performed 24 h after the fifth training.

2.4. Evaluation of abnormal courtship

Nearly 20 male flies at 3 days were put in a culture tube and their abnormal courtship was observed for 5 min. By a stopwatch, we counted the duration when a fly in the tube followed male flies with opening its wing. The percentage duration was calculated in four independent experiments with line #2, #3 and wild-type flies: w(cs10).

3. Results

PQBP-1 is highly conserved beyond species, whereas dPQBP-1 shows a unique structure. Homology scanning with NCBI blast (http://www.ncbi.nih.gov/BLAST/) and with FlyBase (http://flybase.bio.indiana.edu/) revealed that only one homologue exists in *Drosophila* (Fig. 1A). Although the binding partner of CTD, U5-15kD is strictly conserved in *Drosophila* (Fig. 1B), dPQBP-1 lacks CTD just like mutant PQBP-1 in human MR. With the search result, we wondered whether human PQBP-1 improves intelligence of *Drosophila*. It was also possible that human PQBP-1 disturbs functions of dPQBP-1 if *Drosophila* possesses a unique molecular network with dPQBP-1.

Expression of a gene is driven by a yeast transcription factor GAL4 from the pUAST vector possessing five GAL4-binding sites. Therefore, in double transgenic flies carrying the pUAST system, it is possible to regulate expression of a gene by GAL4 with an appropriate promoter. After construction of pUAST-hPQBP1 for transgenic flies, we tested by Western blot whether PQBP-1 protein expression is induced by co-transfection of pwa-GAL4 in *Drosophila* Schneider cells. The result showing a remarkable induction of PQBP-1 protein (Fig. 2A) allowed us to proceed to generation of PQBP-1 transgenic flies.

From injection to about 600 eggs, we obtained two transgenic flies with red eyes. By crossing them with flies carrying balancer genes, localization of the transgene was determined. In both flies, pUAST-hPQBP1 is integrated into chromosome 2. Finally, we generated three lines of homozygous transgenic flies, two from the first egg and one from the second egg. The three lines (line 1–3) were crossed with the homozygous elav-GAL4 transgenic flies, in which GAL4 is expressed in all neurons, to generate double transgenic flies. Induction of the PQBP-1 protein in the three double transgenic fly groups (#1–3, originating from line 1–3, respectively) was examined by Western blot. PQBP-1 was efficiently induced in flies #2 and #3 (Fig. 2B), thus we used these lines for intelligence analyses.

No morphological anomaly was observed in flies #2 and #3 in which human PQBP-1 was expressed by elav-GAL4 (Fig. 3A) and their body weights were not significantly different from control flies w(cs10) (data not shown). No locomotive impairment was observed in double transgenic flies #2 and #3. Because learning disturbance in MR might be relevant to impairment of memory, we applied the odor-conditioned memory test to evaluate intelligence of human PQBP-1 transgenic flies. As described in Section 2, flies were electrically shocked with one of the two odors, and their escaping behav-

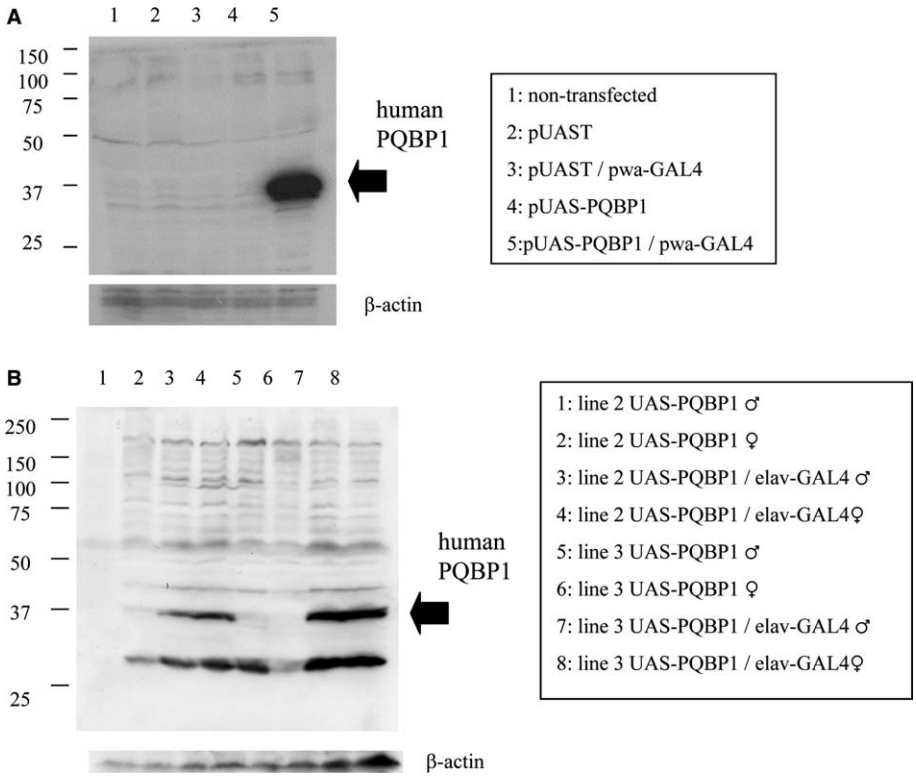

Fig. 2. (A) Expression of human PQBP-1 with UAS-GAL4 system was checked in *Drosophila* Schneider cells by Western blot. Lane 5 shows remarkable induction of human PQBP-1 from UAS promoter by pwa-GAL4. (B) Expression of human PQBP-1 in transgenic flies. Human PQBP-1 is induced male and female double-transgenic flies carrying UAS-PQBP1 and elav-GAL4. Elav is a promoter that drives expression in all neurons.

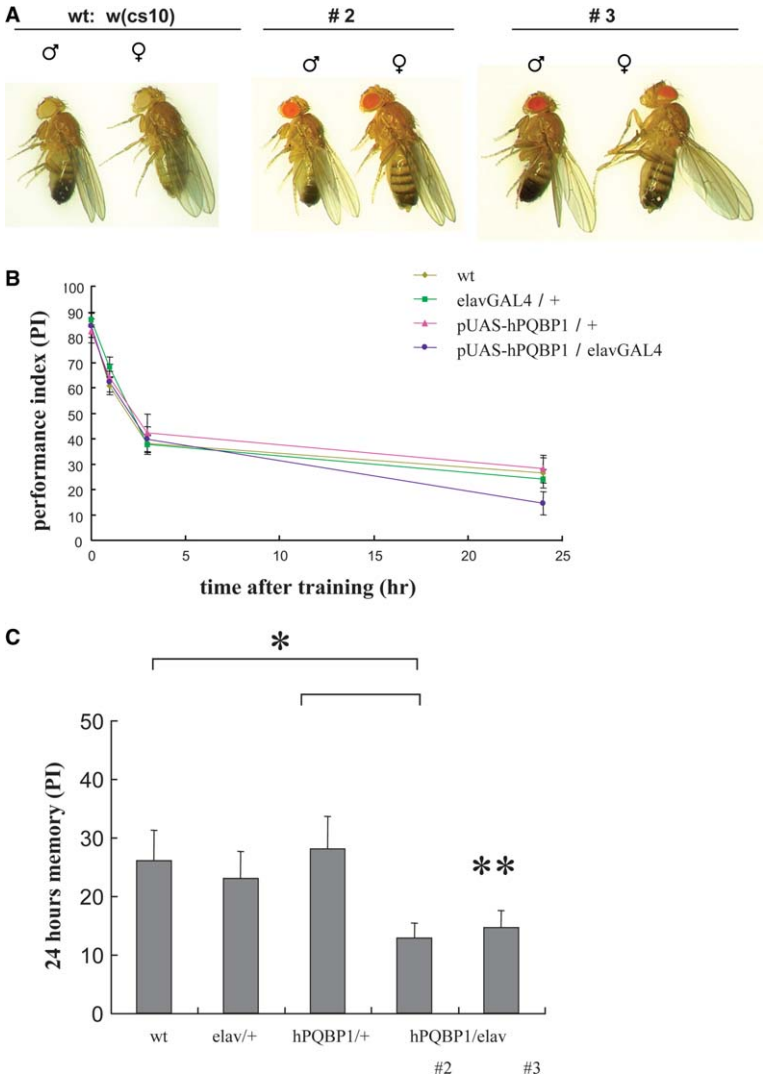

Fig. 3. (A) Macroscopic morphology of double transgenic flies #2 and #3. No anomaly was observed. (B) Odor-conditioned memory test of #2 transgenic flies expressing human PQBP-1. Retention of memory was decreased at 24 h after conditioning. (C) Quantitative analyses confirmed statistically significant declines of the long-term memory in #2 and #3 flies at 24 h. Mean + S.D. were shown. Asterisk indicates $P < 0.01$ by Student's *t*-test. Double asterisk indicates $P = 0.04$.

ior was tested in two odors after a certain period. Then, the experiment was repeated with electric stimulation in the other odor. Mean of the performance index (PI) values in two experiments was recorded as the final PI in a single trial. More than 4 trials (4–8) were performed to estimate a group of transgenic flies at one time point. *Drosophila* memory components are generally classified into four categories: short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM) and long-term memory (LTM). Corresponding with these memory components, we tested the memory of flies at 0, 1, 3, 24 h after conditioning.

We found that long-term memory is declined in #2 flies in comparison to control flies (Fig. 3B and C, $P < 0.01$). Declines to about 60% in long-term memory were also observed in double transgenic flies #3, although the difference from the control was not highly significant ($P = 0.04$) (data not shown). We could not find statistically significant decline in the other memory component from 1 to 3 h of #2 and #3 flies. It is also of note that PI value at 0 h (corresponding to learning ability) was not inferior to that of control flies.

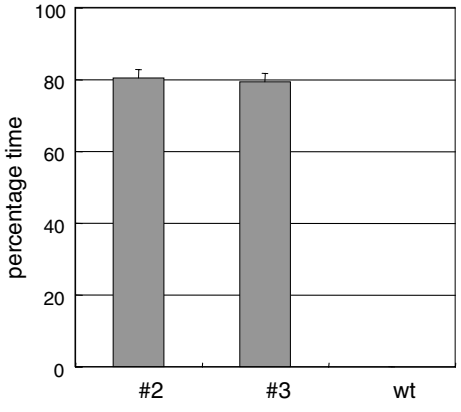

Fig. 4. Analysis of abnormal courtship. Percentage time when male transgenic flies follow male transgenic flies with opening one wing was analyzed in double transgenic flies (#2 and #3) and in the background w(cs10) flies. Abnormal courtship was not observed in the control, while double transgenic flies showed the abnormal behavior almost all the time during observation. Mean + S.D. were shown.

In addition to the long-term memory disturbance, we noticed abnormal sexual behavior that male flies follow male flies with spreading one of the wings (Supplementary Movie). This abnormal courtship was observed both in double transgenic flies #2 and #3 (Fig. 4). Since lines 2 and 3 originated from distinct eggs, this abnormal behavior could not be attributed to the transgene locus effect on surrounding genes but to the effect of human PQBP-1 itself.

4. Discussion

In this study, we made transgenic flies expressing human PQBP-1 to examine whether *Drosophila* PQBP-1 is involved in memory. Although normal *Drosophila* PQBP-1 is homologous to human mutant PQBP-1 lacking CTD, expression of human PQBP-1 in *Drosophila* did not improve memory function. Instead, human PQBP-1 disturbed long-term memory in *Drosophila*. Since U5-15kD, the binding partner of PQBP-1-CTD, is strictly conserved in *Drosophila*, the memory disturbance is probably due to a dominant negative effect of human PQBP-1 on *Drosophila* U5-15kD or on *Drosophila* PQBP-1. In any case, the result suggests involvement of the PQBP-1/U5-15kD system in long-term memory formation as a possible mechanism of human MR symptoms. PQBP-1 has been implicated in transcription in neurons [1,2,4] and long-term memory is dependent on de-novo protein synthesis, therefore this symptom might be relevant to transcriptional disturbance in *Drosophila*. Meanwhile, as far as we have analyzed, no morphological anomaly was detected in the nervous system including mushroom body of the double transgenic flies (data not shown). In addition, no pathological examination was reported in human MR patients linked to *PQBP1* mutations. Therefore, morphological and molecular bases of MR symptoms in the *PQBP1* gene mutations await further analyses.

Unexpectedly, we observed abnormal courtship that male flies follow male flies. As the phenomenon was observed in two independent lines, the symptom can be considered as a result of human PQBP-1 expression in *Drosophila*. Mutations of protein kinase-A, calcium channel, an unknown cytoplasmic protein, and a putative RNA-binding protein nonA have been reported to cause abnormal courtship in *Drosophila* [14–17], respectively. Although the last gene nonA might have a certain relevance to U5-15kD, an RNA-binding protein involved in splicing, it is so far unclear how abnormal courtship is induced in *Drosophila* in general. Although psychological analyses of PQBP-1-linked MR patients have not been reported, it might be necessary to examine their symptoms from this viewpoint.

Transgenic flies have been intensively used for investigation of a major X-linked human MR, fragile X syndrome. One of the reasons for the intensive use is that, although three closely related genes (*fmr1*, *fxr1*, *fxr2*) exist in mammals, *Drosophila* has only one homologue (*dfmr1*) [18,19]. A number of studies have revealed that the *dfmr1* deletion induces various symptoms such as motor disability, circadian rhythm defects, sperm defects, abnormal neuron structure, increased synapse number, abnormal synapse functions, and so on (see review [20]). Abnormal courtship was also observed in *dfmr1*-deficient flies although in this case the male flies fail to maintain courtship interest [21]. Naturally, intelligence of *dfmr1*-deficient flies is a main focus of interest. However, due to the severe disturbance of motor activities of these *dfmr1* mutant flies [19,20], it is impossible to estimate their abilities in learning and memory tests. The lack of information on MR-relevant symptoms significantly limits the development of the *Drosophila* FraX model (see review [20]). In this sense, our transgenic flies are the first successful case in which a clinical symptom of *Drosophila* relevant to MR is disturbed by manipulation of a causative gene of MR.

In summary, our results in human PQBP-1-expressing transgenic flies suggest disturbance of long-term memory formation as a possible mechanism of MR. The finding would be useful for clinical evaluation of MR patients linked the *PQBP1* gene mutations and might become a cue for understanding human MR symptoms. We are now generating conditional knockout mice and analyzing mutant flies whose expression of *dPQBP1* is very low. Results form these animal models would promote us to understand the mechanisms of the *PQBP1*-linked MR more deeply.

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Appendix A. Supplementary movie

The upper tube includes male transgenic flies (#2) expressing human PQBP-1 and the lower tube includes the control, w(cs10). Abnormal courtship was observed (upper tube). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.03.056.

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