

Manifestation of Functional Defects of Nervous System in *Upf3* Mutants *Drosophila melanogaster* at Larval and Adult Stages

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Abstract: Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that cleans the system from possible harmful proteins and also regulates up to 10% of normal RNAs. The essential player proteins in the NMD (core NMD factors) are Upf1, Upf2, and Upf3. Mutation of any of these NMD factors cause ranges of effects in the development of various organisms. In humans, mutation of *Upf3* was associated with neurodegenerative disorders, which include: attention deficit, schizophrenia, autism, and intellectual disability. Using functional genetics approach and behavioral analysis methods we examined the loss of function effects of *Upf3*, in the nervous system function of a *Drosophila melanogaster*. We observed certain nervous system functional defects in homozygous *Upf3* mutants. The embryos exhibited reduced and delayed hatching, the larvae manifested defects in motor function and the adults showed reduced climbing ability, defective short term memory, and learning, and notably, the adult life span was also reduced. This work has further revealed the prospect of *Upf3* as a player gene for consideration in the management of neurodegenerative diseases. We explored this using *Drosophila melanogaster* as a model organism to mimic and study the neurodegenerative traits observed in the patients suffering from *Upf3* mutation. Likewise, it suggests a further investigation into the mechanistic insight for the roles of *Upf3* in both early and late CNS development.

Keywords: Nonsense-mediated mRNA Decay, NMD Factors, Organismal Development, Neurodegenerative Behaviors, *Drosophila melanogaster*

1. Introduction

Nonsense mediated mRNA decay (NMD) is a posttranscriptional regulatory pathway that controls the quality of mRNAs and also shapes the normal transcriptome [1]. NMD is triggered by conditions such as the presence of premature termination codon (PTC) on RNA, long 3'-untranslated region (3'UTR) and upstream open reading frame (uORF) [2]. NMD pathway operates via the coordination of some functional proteins termed NMD

factors. Their structural designs allow them to identify and bind to a target mRNA for regulation. The core factors of the NMD machinery are the Up-frame shift suppressor (Upf) proteins (Upf1, Upf2, and Upf3) that are conserved in all eukaryotes studied [3]. Also crucial for the process are the Suppressor with morphological effect on genitalia (SMG) proteins; SMG1 and SMG5-9 [4].

Several lines of evidence suggest that NMD factors play various roles in the development of nervous system. For example in embryos of zebrafish, there was impaired eye

development and the diminishing of midbrain-hindbrain boundary as a result of depletion of any of the Upf1, Upf2, SMG5, and SMG6 [5]. On the contrary depletion of SMG7 presented elongated hindbrain while the midbrain-hindbrain boundary was established [5]. NMD has demonstrated significance in the development and maintenance of the architectural arrangement of the CNS in flies, as evident in the maintenance of synapse structure [6, 7] and Neural progenitors/stem cells differentiation [8, 9].

Currently, there exist over 6,000 rare genetic disorders identified, 30% of which are caused by nonsense mutations. About a quarter of these diseases contain PTC that triggers NMD [10, 11]. In humans, mutations in *Upf3B*, a homolog of (*Upf3* in *S. cerevisiae*) was associated with mental disorders, attention deficit, schizophrenia, intellectual disability and autism [12, 13]. However, due to intricacies in the eukaryotic nervous system and the presence of two copies of *Upf3* (A and B) in mammals, finding an *in vivo* model of *Upf3* for CNS remains a challenge. Nevertheless, *Drosophila* CNS has provided a simple model of studying these complex processes giving the high evolutionary conservation of the genes and even the developmental processes and pathways [14]. Moreover, unlike mammals, the *D. melanogaster* genome has only one copy of the *Upf3* gene [15], which made it a suitable model for the *in vivo* gene functional study.

In this research, we measured the impact of *Upf3* mutation on hatching ability and rate, larval locomotor activity, adult negative geotaxis, memory and learning in the *D. melanogaster* model, in an attempt to correlate the role of *Upf3*-NMD to the proper function of the nervous system.

2. Materials and Methods

2.1. Fly Stocks

The flies used in this work were either donated or generated via genetic crossings: *yw¹¹¹⁸* from Isabel Palacios Cambridge University UK, *w; Upf3¹/SM6a* from Mark Metzstein University of Utah, USA, *Upf3[Dff]/SM6*, from Bloomington (9424), and *Upf3¹/Upf3[Dff]*.

2.2. Hatching Analysis

Forty-five adult female flies (1day old) were selected from each genotype of *yw¹¹¹⁸* (control), *Upf3¹/SM6* (*Upf3* heterozygous) and *Upf3¹* (*Upf3* homozygous). The female flies from each genotype were grouped into three, 15 flies per group. Each of these groups of female was placed in a food vial containing dry yeast with ten male flies of the same genotype. The flies were left for five days to ensure fertilization before being transferred to an apple juice agar with dry yeast for egg collection. The eggs were collected by evacuating the flies to new plates every 24 hrs. This was done five times for each group and the egg-laying and hatching capacities were assessed by counting the number of eggs laid and the number that hatched for five days. Leica MZ 9.5 was used for this experiment. During the experiment, all the flies used were kept at 25°C for

maximum yield.

2.3. Larval motor activity (Locomotors Assay)

The motor activity was assessed through analyzing the larval locomotors. Two parameters were considered, distance covered and the number of body contractions (peristaltic waves) per minute.

2.4. Larvae Collection

Three large vials of *yw¹¹¹⁸*, *Upf3¹/SM6* and *Upf3¹* containing 25 female and 10 male flies each were set. Adult flies were transferred after 16 hrs of egg-laying to new vials and the bottles were incubated at 25°C for 5 days. To collect the larvae, 100 ml of 20% sucrose was added to the bottles and allowed to sit for 20 min. The floating larvae were collected using a 25 ml serological pipette and placed in a mesh. The larvae were washed twice with deionized water.

2.5. Locomotors Assay

The clean individual larvae were transferred with a brush into a 15cm Petri dish containing 2% agarose that was previously poured and allowed to harden over a graph paper with 0.2 cm³. The larval movement was assessed by counting the number of lines crossed by a moving larva in one minute. Also, the number of peristaltic waves per minute was recorded.

2.6. Adult (Negative Geotaxis Assay)

Negative geotaxis analysis was done as described by Barone and Bohmann [16]. Age synchronized flies (7 days old) from *yw¹¹¹⁸*, *Upf3/SM6* and *Upf3¹* were selected and separated by gender. Flies from each genotype were randomly grouped into 20 – 25 per vial. Before the negative geotaxis test, the flies were placed in pre-labeled clear plastic tubes made with two empty food vials (19 cm X 2.85 cm each) joined at their openings with clear tape, without anesthetizing the flies. The flies were allowed to settle for 30 min. Different heights were marked on a piece of blotting paper and the paper was taped on a vertical surface, perpendicular to the bench, and the tubes were placed in alignment with the paper. A digital camera was set in a movie recording format and placed on a stand in front of the tube at a distance of 25 cm from the paper. The tubes were tapped for 2s and all the flies were collected at the bottom the recording and the timer were both started the flies movement was recorded for 10s. This process was repeated five times at intervals of 1min and each time the movement of the flies was recorded. The number of flies above the 2 cm mark was recorded by visual inspection of the recorded movie.

2.7. Memory and Learning

Memory and learning analysis was done as described by Malik and Hodge [17]. In this experiment, the associative olfactory learning of *Upf3* homozygous mutant *D. melanogaster* was assessed by associating an odor with

mechanical shock. 4-methylcyclohexanol and 3-octanol were diluted in mineral oil in the ratio 1:67 and 1:100 respectively as the sources of odor. The ability of the flies to associate an odor with mechanical shock was tested. Approximately 50 mixed sex adult flies were trained for conditioning 24 hrs before the experiment. Three known conditioning protocols, (the single conditioning cycle, spaced and massed protocols) [18] were used to condition the flies. A conditioning cycle was designed such that in each circle, flies had 30 s exposure to an odor under a mechanical vibration of a 2,000-rpm pulse of 1s at 5s interval on a test tube shaker. The flies were then allowed A 60 s of resting period before subjected to another circle. The flies were then exposed to the second odor for 30 s without shock. 60 s of rest marked the end of the conditioning cycles. Short term memory retention was tested after 24hrs, by transferring the flies to a central position of a T-maze, and then they were exposed to the two odors simultaneously coming from the two opposite chambers of the T-maze. They were allowed an equal opportunity to choose the odor side to follow for 60 s. The flies from each arm of the maze were counted and recorded. Those that remained at the center of the T-maze chamber were excluded from the calculation. Memory was measured in terms of percentage performance index (%PI).

2.8. Life Span

The life span was analyzed as previously described by Langelotti *et al* [19]. One hundred and twenty synchronized adult flies were collected from each genotype of *yw¹¹¹⁸* (control), *Upf3¹/SM6* (*Upf3* heterozygous) and *Upf3¹* (*Upf3* homozygous). The flies were grouped into 6 of 20 flies each in a 1:1 male to female ratio. The flies were then transferred to fresh vials of food every two days without anesthesia. The number of flies that died was counted and removed during every flipping. Percentage survival in days was calculated from percentage death.

3. Results

The effect of *Upf3* mutation on egg hatching is shown in figure 1. It was observed that there was a significant reduction of hatching in the *Upf3¹* flies (*Upf3* homozygous mutant) when compared to the *yw¹¹¹⁸* (control) and *Upf3¹/SM6* (*Upf3* heterozygous mutant) (Figure 1A). Additionally, there was a delay of hatching in the *Upf3* homozygous mutant embryos in contrast to *Upf3* heterozygous and the control (Figure 1B). Although 47.74% and 40.75% embryos of the control and *Upf3* heterozygous respectively hatched within the first 24 hrs, only 11.92% of *Upf3* homozygous embryos hatched within this time. Approximately 73% of the *Upf3* homozygous hatched between 24 and 48 hrs while 50.04% and 54.49% of the control and the *Upf3* heterozygous hatched in the same period. The hatching of *Upf3* homozygous embryos extended up to 72 and 96 hrs by 6.28% and 7.71% respectively, which is significantly different compared to the control which is

1.71 and 0.51%, $p < 0.05$ (Figure 1B).

Larval locomotor activity was analyzed by assessing the rhythmic behavior during crawling in the *Upf3* homozygous larva in comparison with the control and the *Upf3* heterozygous. Distance covered per time and number of peristaltic waves per time unit was studied. The results showed a significant reduction ($P < 0.001$) in the distance covered by the *Upf3* homozygous when compared with the control and *Upf3* heterozygous mutant flies (Figure 2A) but there was no noticed difference in the larval peristaltic wave (Figure 2B).

The negative geotaxis of the flies was analyzed using the climbing assay as a measure of the neuromuscular function in adults. The results showed a significantly reduced climbing ability in the *Upf3* homozygous flies compared to the control and the *Upf3* heterozygous (Figure 3).

Short term memory and learning of the homozygous mutant *Upf3* flies were reduced in comparison to control and *Upf3* heterozygous mutant flies (Figure 4). Lastly, the lifespan showed a significant reduction of the mean percentage survival and the median survival rate (a day at which 50% of flies are alive; Figure 5). Interestingly, there was no significant difference between the control and the heterozygous *Upf3* mutant flies.

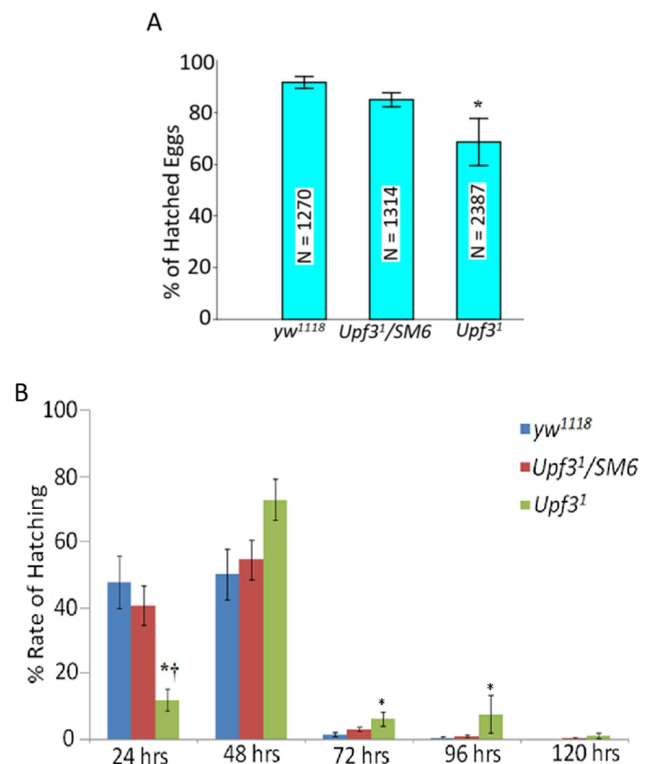


Figure 1. Egg hatching rate and timing of *Upf3* embryos. (A) Percentage of hatched eggs in control (*yw¹¹¹⁸*), heterozygous *Upf3* mutant (*Upf3¹/SM6*) and homozygous *Upf3* mutant (*Upf3¹*). * indicates a significant reduction when compared with the control ($p < 0.01$). (B) The average percentage of eggs hatched grouped per time point (24, 48, 72, 96 and 120 hrs). * indicate statistical difference when compared with control ($p < 0.05$) and † indicate statistical difference when compared with *Upf3¹/SM6* ($p < 0.005$). Data are plotted as means \pm SEM. Data were analyzed using One-way ANOVA and then Tukey's pairwise.

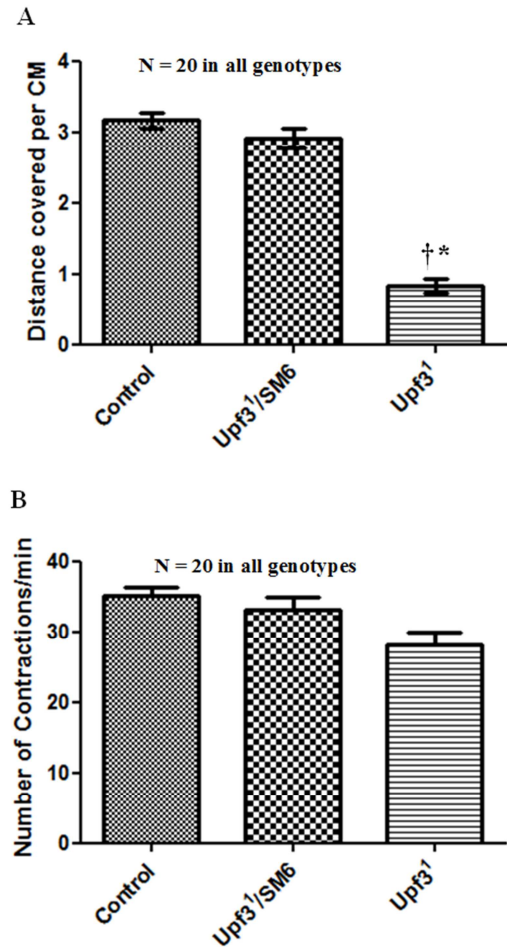


Figure 2. Larval movement of Upf3 mutants. Analysis of Larval movement in the genotypes *yw*¹¹¹⁸ (control), Upf3¹/SM6 (Upf3 heterozygous) and Upf3¹ (Upf3 homozygous) mutant flies (A) Mean distance covered by the larvae in cm per minute. *Indicate a significant difference between the Upf3 homozygous compared to Control, $p < 0.001$. † indicates significant difference compared to Upf3 heterozygous mutants, $p < 0.001$ ANOVA and Tukey's pairwise (B) The average number of body contractions per minute as the measure of the peristaltic wave of a larva.

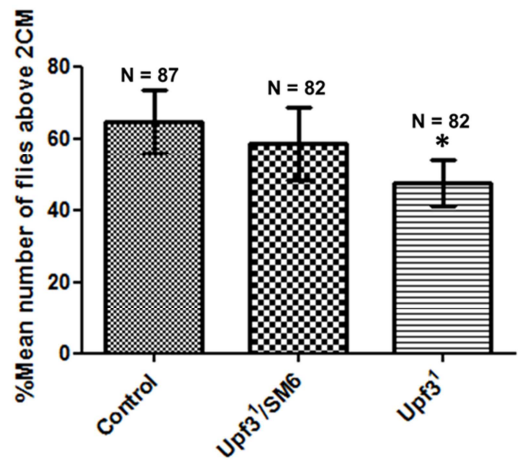


Figure 3. Negative geotaxis analysis of Upf3 mutant flies. The climbing ability was measured by the percentage number of flies able to climb above 2cm in ten seconds. *Indicate a significant difference when compared with the *yw*¹¹¹⁸ (control) and Upf3¹/SM6 (Upf3 heterozygous). ANOVA and Tukey's pairwise $p < 0.05$. Data are presented in mean \pm SEM.

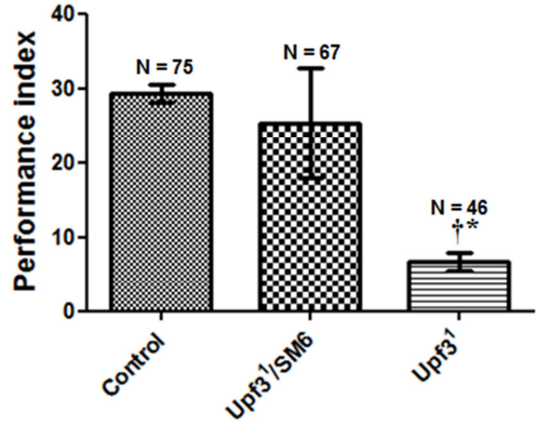


Figure 4. Memory and learning capacity of Upf3 mutant flies. The performance index (PI) of memory and learning analysis obtained among the genotype *yw*¹¹¹⁸ (control), Upf3¹/SM6 (Upf3 heterozygous) and Upf3¹ (Upf3 homozygous) mutant flies. The PI was computer using the formula, $OnS = OS/N \times 100$; OS = number of flies in the region of odor not associated with shock, OS = number of flies in the region of odor associated with shock, N = total number of flies. * indicates a significant difference compared with the control (ANOVA and Tukey's pairwise $p < 0.001$). † indicates a significant difference compared with the Upf3 homozygous (ANOVA and Tukey's pairwise $p < 0.05$). Data is presented in mean \pm SEM.

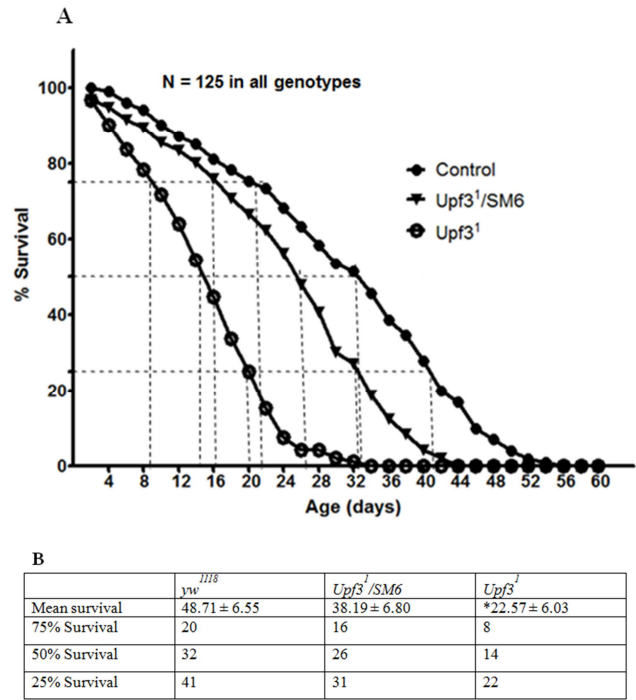


Figure 5. Percentage survival of Upf3 mutant flies. (A) Percentage survival in days calculated from percentage death ($\%death = (D + nD)/N \times 100$, where D = number of dead flies in the day of counting; nD = total number of death in the prior days before the day of counting; N = the total number of Death at the end of the observation). The dashed horizontal lines mark 25%, 50%, and 75% while dashed vertical lines lead to extrapolation of 25, 50, and 75, percentile survival. *yw*¹¹¹⁸ = control flies; Upf3¹/SM6= heterozygous Upf3 mutants and Upf3¹= homozygous Upf3 mutants in both A and B. (B) Interpolation from the graph of life span analysis, median longevity presented as (mean \pm SEM). Survival time points (days) equivalent to 75%, 50% (median survival rate) and 25% survival, extrapolated from the graph for each of *yw*¹¹¹⁸, Upf3¹/SM6 and Upf3¹. Statistical analysis was done using one-way ANOVA followed by Tukey's pairwise. * indicates significant difference ($p < 0.05$) when compared with the control.

4. Discussion

Understanding the intricacies of neuronal development is the primary goal of neuroscience and a key to solving various neurodegenerative problems that are commonly associated with old age in humans. Decades of research have documented a tremendous amount of data on the vast processes and numerous genes involved in the neurogenesis [20, 21]. The *Drosophila* CNS serves as a valuable model in studying single gene manipulations during the complex brain circuitry development due to the simplicity in the origin and the lineage groupings of the neurons, a well-defined developmental timeline, high evolutionary conservation of the genes, and the availability of genetic manipulation methods among the *Drosophila* researchers [22]. Moreover, the *Upf3* gene functional study is easier in *D. melanogaster* because its genome has only one copy of the gene unlike mammals that have 2 copies, *Upf3A* and *Upf3B* [4].

Upf3B was associated with cognitive function in humans. Its mutation was shown to cause neurodevelopmental disorders, including, Attention deficit, Autism, Reduced memory retention and Schizophrenia [23]. This has proved the importance of *Upf3* NMD in the development and maturation of CNS. Owing to the complexity of human and mice brains, we generated homozygous *Upf3* knockdown flies using functional genetics. Our data show reduced and delayed hatching in the *Upf3* embryos. Severe defects in the negative geotaxis, reduction in short-term memory retention and severe locomotor alteration with behavioral and movement deficits were also observed. Indecisiveness and lack of coordination in the head directional movement; reverse, and zigzag movements, and sometimes peristaltic wave without covering any distance were the characteristic patterns observed in the *Upf3* mutant larvae. Collectively these features point to a defective motor and neural function in these flies which also mimicked the defects observed in the human *Upf3B* patients.

Our previous unpublished work of *Upf3* mutant larval brain analysis showed a fully developed central brain, ventral nerve cord and olfactory lobe without any major anatomical defect noticed. This is consistent with the findings in human patients and mice with *Upf3B* mutation, that showed alterations of behavior but their brains appeared normal when gross anatomy was observed [2, 9]. However, confocal microscopy analysis in *Upf3B* mutant mice revealed delayed stem cell differentiation, defect in sensory-motor gating and slightly sparse neurons and granules of the hippocampus, the memory center of the brain [2]. This may account for the cognitive defects observed in the fly models.

Interestingly, while working with the adults, their survival rate was reduced by two folds in the absence of *Upf3* when compared to the control and heterozygous *Upf3* mutants. Previous research established that *Upf3* expression is higher in the adult brain than in the early stages of the mouse [2], thus, reduced life span may be attributed to several factors which include among others; Gradual accumulation of toxic proteins as a result of reduced efficiency of *Upf3*-dependent

NMD in the absence of *Upf3* [24, 25]. Endoplasmic reticulum (ER) cellular stress augmented by the inhibition of NMD [26], which triggers cellular destructive processes that results in cellular and organismal death. Furthermore, Rho GTPase activating protein is established to be a target of *Upf3* in both humans and flies. This gene in *Drosophila* (RhoGAP68F) is involved in processes of early embryonic development, cell cycle arrest, neural development and morphogenesis [27, 28]. These functions point towards the explanation of the reduction in the survival of the *Upf3* mutant flies, even though *Upf3* is not lethal. This gene's function contribute in the completion of embryonic development during gastrulation via Rho GTPase activating protein. Additionally, this will also provide a target explanation in the neurodegenerative phenotypes noticed in the *Upf3* mutants. In humans, the homolog Rho GTPase activating protein 24 (ARHGAP24) in addition to regulation of neuronal growth [29] regulates cell polarity, inhibits cell cycle progression and induces apoptosis in renal carcinoma [30, 31]. Another factor that may partially contribute to the short life span in the *Upf3* mutant adults is the *Upf3* acquired role in telomere maintenance and DNA repair in both humans and *Drosophila* [4]. The depletion of *Upf3* to some extent may have increased the rate of telomere shortening and accumulation of DNA damages that could result in a reduced life span seen in the *Drosophila* model.

5. Conclusion

We have shown that the *Upf3* knockout model of *D. melanogaster* mimicked the neurodegenerative behaviors seen in *Upf3B* null patients and mice. The initial analysis of *Upf3* mutant flies showed that *Upf3* plays a role in embryonic development and at adult stages since *Upf3* mutant embryos exhibited reduced and delayed hatching and shortened lifespan. Using the functional genetics approach we successfully showed that *Upf3* homozygous mutants have neurophysiologic defects including reduced and aberrant motility at larval and adult stages and reduced memory and learning capacity in adults.

Although *D. melanogaster* has shown the potential of modeling the neurodegenerative disorders as a result of *Upf3* mutation, these results call for caution and thorough analysis when exploring the potential of interfering with the *Upf3* function to treat PTC-induced disorders, due to lack of total conformity between humans and flies.

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