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The E3 ligase *ube3a* is required for learning in *Drosophila melanogaster*

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ABSTRACT

Angelman syndrome and autism are neurodevelopmental disorders linked to mutations and duplications of an E3 ligase called *ube3a* respectively. Since cognitive deficits and learning disabilities are hallmark symptoms of both these disorders, we investigated a role for *dube3a* in the learning ability of flies using the aversive phototaxis suppression assay. We show that down and up-regulation of *dube3a* are both detrimental to learning in larvae and adults. Using conditional gene expression we found that *dube3a* is required for normal brain development and during adulthood. Furthermore, we suggest that *dube3a* could be interacting with other learning and memory genes such as *derailed*. Along with firmly establishing *dube3a* as a gene that is required for learning, our work also opens avenues for further understanding the role played by this gene in brain development and behavior.

1. Introduction

Angelman syndrome (AS) is a neurodevelopmental disorder that is characterized by severe intellectual disability, developmental delay, jerky limbs, frequent seizures and uncontrollable laughter [1]. Autism, another neurodevelopmental disorder, constitutes a broad spectrum of disorders which are commonly associated with diminished social interactions, impaired communication and increased repetitive behaviors [2]. Interestingly, both these disorders are strongly associated with genetic abnormalities at the q11-13 locus of human chromosome 15 [3–5]. In particular, a gene called *ube3a* is present at this site which, when mutated, seems to be sufficient to manifest AS, and when duplicated is linked to autism. *ube3a* is known to be imprinted in neurons with the maternal allele expressed and the paternal one silenced [6]. Deletion or mutations of maternal *ube3a* result in its complete loss in neurons and cause AS [7,8]. In contrast, duplication of the maternal allele results in excess *ube3a* in the brain and may be linked to autism spectrum disorders [9]. The ube3a protein is an E3 ligase which attaches ubiquitin to its target substrates and tags them for degradation. It has also been reported that *ube3a* acts as a transcriptional co-activator of steroid hormone receptors [10].

Transgenic mice with maternally deficient *ube3a* are defective in context-dependent learning and long term potentiation with numerous signaling pathways implicated [11–14]. Fly models of AS in which the *Drosophila* homolog of *ube3a* (*dube3a*) is deleted recapitulate disease symptoms such as defects in locomotion, circadian rhythms and memory [15]. Mutant flies also have impaired dendritic branching, a phenotype that was later observed in pyramidal neurons of mice [16,17].

Since cognitive deficits and learning disabilities are common to patients of both AS and autism, we performed a detailed investigation into the effect of altered levels of *dube3a* on the learning ability of flies and demonstrate its spatial and temporal requirement for this behavior. Furthermore, we demonstrate a potential genetic interaction between *dube3a* and *derailed*, a receptor tyrosine kinase linked to learning and memory.

2. Materials and methods

2.1. Fly stocks and culture

Fly stocks were maintained at 25 ± 1 °C in BOD incubators (LD12:12) on standard sugar yeast food. Stocks – $dube3a^{PE}$,

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dube3a$^{+/-}$, myc-tagged *dube3a*$^{dup+/+}$, *UASdube3a* and *UASdube3aC941A* from Fischer laboratory [15]; *UAShube3a* generated in this study; RNAi lines from VDRC, Vienna; *dube3a* – 45876, 45875; *derailed* – 3047, 27053. All remaining stocks – Bloomington Stock Centre, Indiana University, USA. 2-3 day-old females were used for experiments.

2.2. Generating UAShube3a flies

Isoform 1 of human *ube3a* (2558bp) cDNA was cut from pGEX4T (gift from Prof. Z. Nawaz) vector using *Bam*HI and *Not*I restriction enzymes (Roche) and ligated into pUAST-attB vector (FlyC31). The clone was sent to Fly Facility, C-CAMP, Bangalore for site-specific injection.

2.3. Adult learning – aversive phototaxis suppression (APS) assay

The APS assay was used as described earlier [18]. The 16 trials given were combined into 4 blocks of 4 each and data for the final block is shown. Performance index is defined as the percent of trials in which the fly chose the dark chamber. Number of flies tested is indicated in figures.

2.4. Larval learning

A taste-aversion protocol was developed in which a Petri-dish was layered with 1% agarose. Half was maintained in darkness (containing 5 mM quinine hydrochloride) and the other half in light. Ten feeding third-instar larvae were placed onto the center and allowed to roam for five minutes. The larvae were subjected to 16 consecutive trials with a 5-min rest period between each trial. The trials were combined into 4 blocks of 4 each and the last block is represented in the data. Performance index is defined as the percent of larvae that chose the light chamber. Number of larvae tested is indicated in figures.

2.5. Climbing assay and quinine sensitivity index

Climbing assay and quinine sensitivity index are previously described [18,19].

2.6. RNA and protein quantification

Semi-quantitative and quantitative PCR was performed as described [19]. Primers are listed in Supplementary Table S1. Results were normalized to *rp49*. Protein was extracted from fly heads using standard procedures. Primary antibodies – rabbit anti-E6AP (Santa Cruz), mouse anti-actin (Abcam) and rabbit anti-derailed (gift from Prof. JM Dura). Band intensities were quantified using Image J and normalized to β-actin.

2.7. Mushroom body defects

Adult brains were processed for immunostaining using standard protocols. Primary antibody – mouse anti-fasciclinII (DSHB). Brains were mounted in DAPI-mounting medium (Vectashield). Z-series images were taken on LSM 510Meta (Carl Zeiss) microscope using 40X oil objective and stacked using ImageJ. Brains were designated as defective if they showed either folded or missing α-lobes or fused β-lobes. If no obvious anatomical defects were observed, they were designated as normal.

2.8. Statistical analysis

All data was analyzed and graphs plotted using SigmaPlot 12.0. $p < 0.05$ was considered significant. Normal data is represented as histograms representing mean and error bars are s.e.m. and non-normal data is represented as box plots with the median as the line, 10th, 25th, 75th and 95th percentile as boxes and error bars respectively and dots are outliers. Two groups were compared using two-tailed Student T-test, data for more than two groups was subjected to one-way analysis of variance (ANOVA) and compared using Holm-sidak (normal data) or Dunn's (non-normal data) pairwise *post hoc* comparison. *dube3a*^{PE} served as a control for *dube3a*$^{+/-}$ mutants and OregonR + for duplication mutants. GAL4-UAS perturbations were considered significant if $p < 0.05$ upon comparison to both transgene (GAL4/UAS) controls. Rescue manipulations were considered significant if they were different from mutants but not from transgene controls. All figures were arranged in Adobe Photoshop.

3. Results and discussion

3.1. dube3a is preferentially expressed from maternal allele

The level of *dube3a* mRNA (Fig. 1A and B) and protein (Fig. 1C and D) in flies with a paternally inherited null or duplication mutation (*dube3a*$^{m+p-}$ or *dube3a*$^{dupm-/p+}$) was compared to those with a maternally inherited allele (*dube3a*$^{m-p+}$or *dube3a*$^{dupm+/p-}$) and found to be markedly different between the reciprocal heterozygotes. *dube3a* was expressed significantly less from the paternal allele than the maternal one. These results are similar to the pattern of *ube3a* expression in humans and mice, where this gene is maternally expressed and paternally silenced in neurons [6]. While the possible reasons for this difference in gene expression from the parental alleles in flies are not understood, in this study, the mutant (*dube3a*$^{+/-}$) and duplication (*dube3a*$^{dup+/-}$) alleles were inherited maternally.

3.2. Altering endogenous levels of dube3a disrupts learning

The aversive phototaxis suppression (APS) assay explores the ability of flies to associate an aversive taste stimulus with light and thereby learn to suppress their innate phototaxis [20]. Recently, APS assay has been increasingly used to study learning in individual flies which has been particularly useful in demonstrating the effect of sleep deprivation on learning ability [18,21–23]. This assay depends on flies having proper locomotion, phototaxis and quinine sensitivity. The locomotion and quinine sensitivity of mutant and duplication lines described above were found to be the same as control (Supplementary Figure S1 and S2 respectively). In addition, the phototaxis of each individual fly was tested prior to performing the APS assay and only positively phototactic flies were used.

These flies were then subjected to the APS assay to score their learning ability. *dube3a*$^{+/-}$ were found to be significantly impaired (Fig. 2A) and this defect was rescued by re-expressing genomic *dube3a* in these flies. *dube3a*$^{dup+/+}$ also displayed defective learning (Fig. 2B). Hence both decreasing and increasing levels of dube3a appears to be detrimental to learning, thereby recapitulating learning deficits in patients of AS and autism. Mice lacking maternally inherited *ube3a* are extensively used to model AS and display many symptoms of the disease [24]. On the contrary, only recently *ube3a* gene dosage has been increased in mice and shown to lead to features characteristic of autism [25]. This report is the first to show that elevated levels of dube3a are able to affect learning ability of flies. This indicates that apart from *dube3a* deletion mutants being used to model AS, flies over-expressing *dube3a* may also serve as a

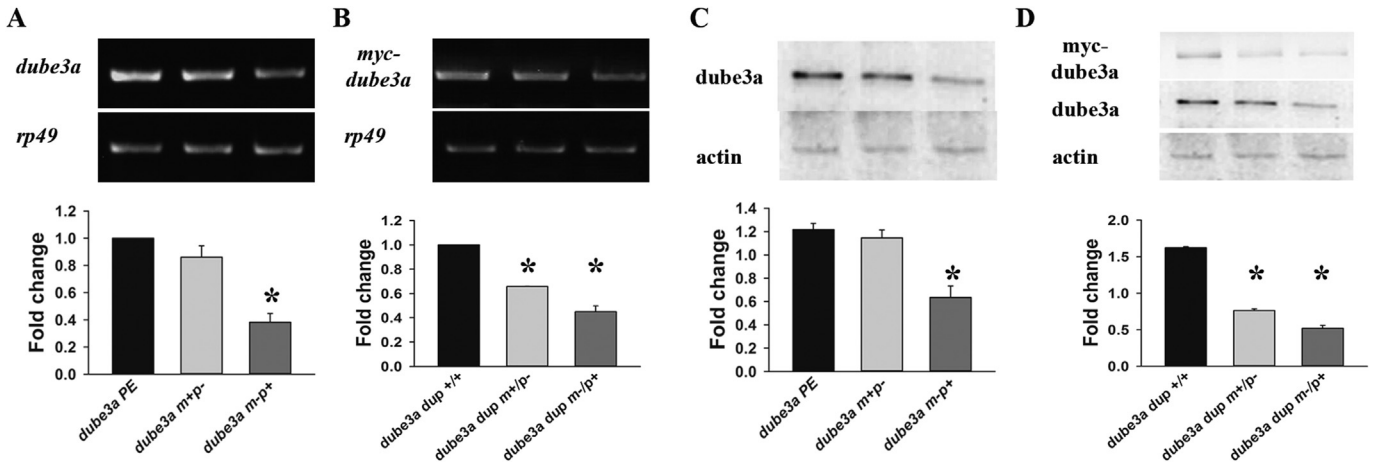

Fig. 1. *dube3a* is preferentially expressed from the maternal allele. RT-PCR of *dube3a*, *myc-tagged dube3a* and *rp49* transcript and fold change as computed by qRT-PCR in maternally and paternally inherited null (A) or duplication (B) alleles. Immunoblot for dube3a, myc-tagged dube3a and actin and fold change in maternally and paternally inherited null (C) or duplication (D) alleles. Data are mean, error bars are s.e.m.; *p < 0.05 in one-way ANOVA (Holm-sidak *post hoc* test).

model for autism [15]. Assays to measure social interactions and repetitive movements in flies have been applied to models of FragileX Syndrome [26,27]. Since these behaviors also constitute hallmark symptoms of autism, their assessment in *dube3a* over-expressing flies would strengthen the causal relationship between *ube3a* and autism.

The GAL4-UAS system was used to further explore the role of dube3a in learning. Two independent RNAi lines driven by

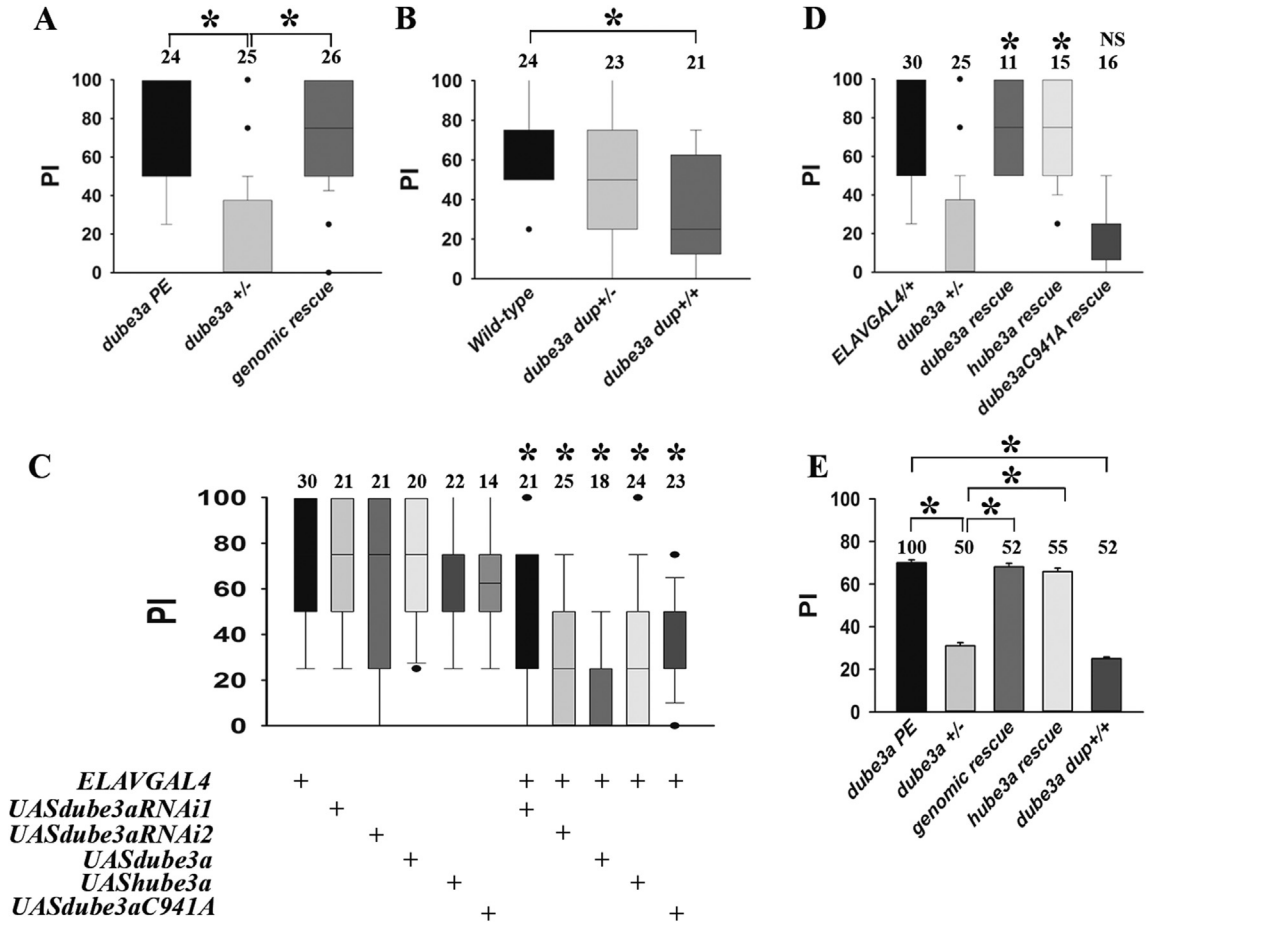

Fig. 2. Altering *dube3a* levels disrupts learning. Performance index of flies in learning assay in adults (A–D) and larvae (E). (A) $dube3a^{+/-}$ compared to controls and genomic rescue. (B) $dube3a^{dup+/-}$ and $dube3a^{dup+/+}$ compared to control. (C) *ELAVGAL4*-driven *dube3a*RNAi and over-expression of *UASdube3a*, *UAShube3a* and *UASdube3aC941A*. (D) Rescuing $dube3a^{+/-}$ mutant learning defect by *ELAVGAL4*-driven *UASdube3a*, *UAShube3a* and *UASdube3aC941A*. (E) Larval learning in $dube3a^{+/-}$and $dube3a^{dup+/+}$compared to controls and rescue of $dube3a^{+/-}$defect by expressing the genomic locus and *ELAVGAL4*-driven *UAShube3a*. A-D – Data are box plots with median as the line, 10th, 25th, 75th and 95th percentile as boxes and error bars respectively and outliers are dots. *p < 0.05 in one-way ANOVA (Dunn's *post hoc* test). NS = not significant; E – Data are mean, error bars are s.e.m.; *p < 0.05 in one-way ANOVA (Holm-sidak *post hoc* test). Numbers of flies tested are indicated above each bar/box.

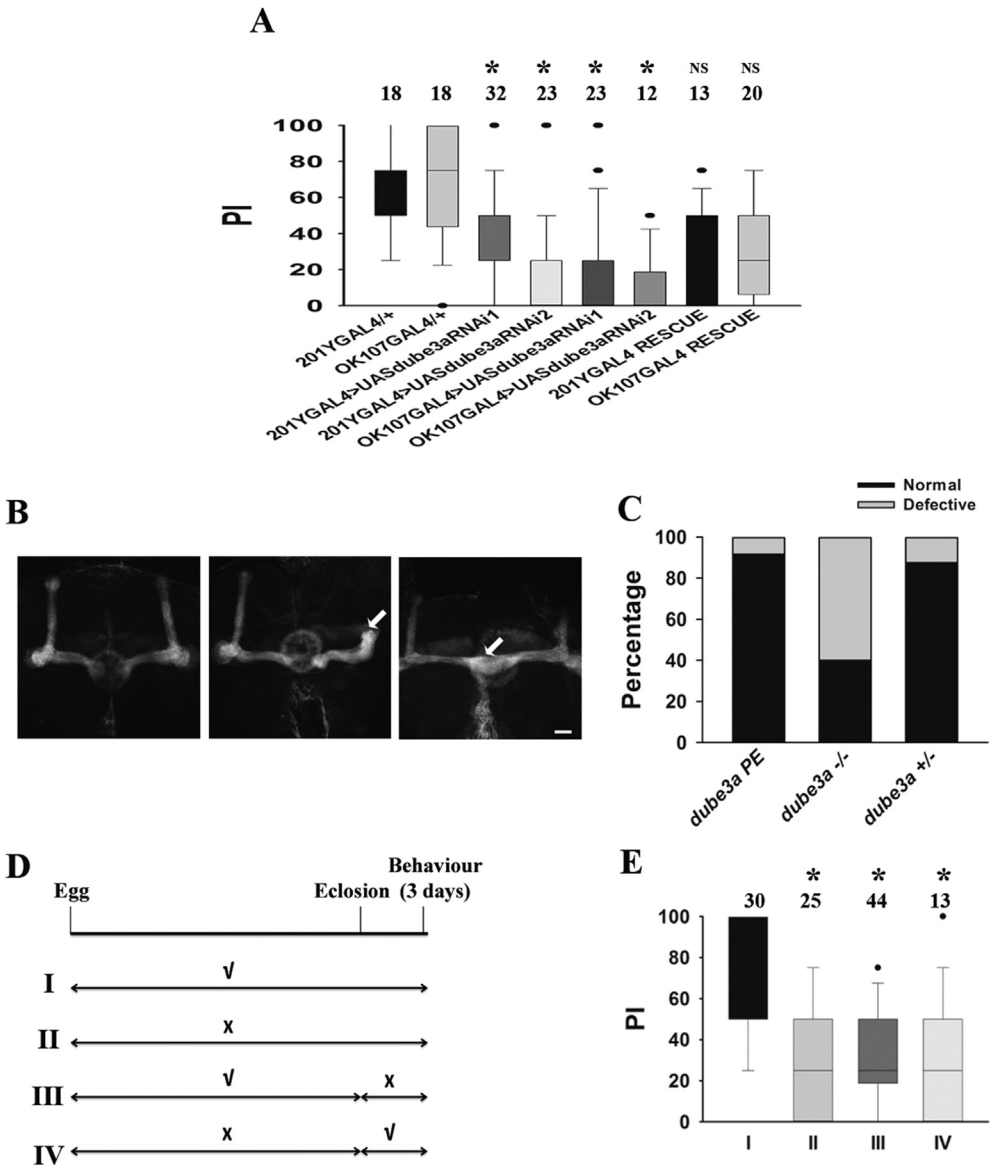

Fig. 3. *dube3a* in mushroom bodies. (A) *201YGAL4*/*OK107GAL4*-driven *dube3a*RNAi and rescue using *UAShube3a* in *dube3a*$^{+/-}$ mutant background. (B) Representative images of developmental defects in MB anatomy showing missing α-lobes and fused β-lobes as visualized by immunostaining (arrows). Scale bar: 20 μm. (C) Quantification of MB defects in controls, homozygous and heterozygous *dube3a*mutants. Data represented as percentage of brains analyzed. (D) Schematic representation of conditional *dube3a*expression. Normal (I) or reduced (II) *dube3a* during entire experiment compared to *dube3a* expression conditionally silenced either in adulthood (III) or during development (IV). (E) Performance index of flies described in (D). A, E – Data are box plots with median as the line, 10th, 25th, 75th and 95th percentile as boxes and error bars respectively and outliers are dots. *p < 0.05 in one-way ANOVA (Dunn's *post hoc* test). Numbers of flies tested are indicated above each box.

ELAVGAL4 were found to be significantly defective compared to controls. Over-expression of *dube3a* (*UASdube3a*) in neurons also adversely affected learning, confirming the defects observed in *dube3a* duplication mutants. Interestingly, flies over-expressing human *ube3a* (*UAShube3a*) also showed defects in learning while *hube3a* expression by *ELAVGAL4* in a mutant background was able to rescue mutant learning defects (Fig. 2D). There have been conflicting reports regarding the ability of flies to express functional hube3a [15,28]. In this study, the *UAShube3a* flies appear not only to be able to express functional protein but its role with respect to learning may also be conserved across species. This supports ongoing efforts using *Drosophila* to over-express *hube3a* and reveal its potential specific target substrates which are being confirmed in mice models [28–31].

To examine whether the behavioral defect observed upon elevating the protein is solely due its ligase activity, a ligase-mutant form of dube3a (*UASdube3aC941A*) was expressed which significantly reduced the learning performance (Fig. 2C). However, while expressing *UASdube3a* in a mutant background was able to rescue learning defects, *UASdube3aC941A* was not (Fig. 2D). Taken together, these results indicate that both ligase and co-activator functions of dube3a are essential for this behavior. The current view of the molecular correlates of AS pathogenesis focuses on the possibility that disruption of both these functions of ube3a could contribute to disease pathology. Previously our group has demonstrated that in AS mice, glucocorticoid-receptor mediated signaling pathway is dysregulated transcriptionally and post-translationally resulting in stress and anxiety which are characteristic features of AS patients [32]. The results described above suggest that a similar combination of defects in *dueb3a* mutants may be contributing to the learning phenotype observed in them.

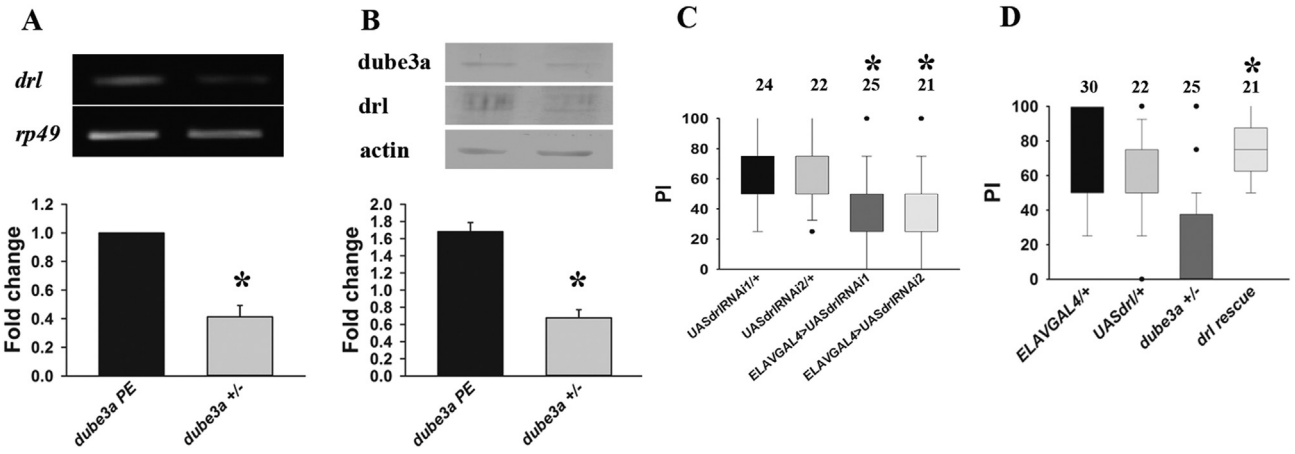

Fig. 4. *derailed* expression in *dube3a*$^{+/-}$ mutants. (A) RT-PCR of *derailed* and *rp49* transcript in *dube3a*$^{+/-}$ compared to control (*dube3a*^{PE}) and fold change as computed by qRT-PCR. (B) Immunoblot for derailed, dube3a and actin and fold change (derailed/actin). (C) *ELAVGAL4*-driven *derailed*RNAi. (D) Defective learning of *dube3a*$^{+/-}$rescued by expressing *ELAVGAL4*-driven *UASdrl*. A-B – Data are mean, error bars are s.e.m.; *$p < 0.05$ in two-tailed Student T-test. C-D – Data are box plots with median as the line, 10th, 25th, 75th and 95th percentile as boxes and error bars respectively and outliers are dots. *$p < 0.05$ in one-way ANOVA (Dunn's *post hoc* test). Numbers of flies tested are indicated above each bar/box.

3.3. Larval learning is defective in dube3a mutants

Larvae were subjected to a similar taste-aversive paradigm to assay their learning ability. When feeding third instar larvae are placed in an agar-containing Petridish and allowed to choose between light and dark chambers, they prefer darkness but when darkness is associated with the aversive taste of quinine, they learn to choose the light chamber. While wild-type larvae were able to learn this association, *dube3a*$^{+/-}$ mutants were not and this defect was rescued by introducing a *dube3a*-containing genomic transgene and by expressing *ELAVGAL4*-driven *hube3a* in a mutant background (Fig. 2E). *dube3a*$^{dup+/+}$ larvae also had impaired learning. Numerous genes important for learning and memory in adult flies also play a role in larval learning [33,34]. In this report, we demonstrate learning defects in both larvae and adults of *dube3a* mutant flies, indicating its requirement for this behavior.

3.4. dube3a in mushroom bodies

In the adult fly brain, dube3a is highly expressed in mushroom bodies (MBs), the learning and memory center [15]. In order to demonstrate that its presence there is necessary for learning, RNAi was driven using two independent MB drivers – *201YGAL4* and *OK107GAL4* (Fig. 3A) and found to significantly impair learning. However, the learning defect of mutants could not be rescued by expressing *hube3a* using either MB driver. These results indicate that dube3a in MBs is necessary but not sufficient for this behavior and other brain areas may be contributing to it.

When MB anatomy was visualized homozygous mutants frequently had severe defects such as fused β-lobes and missing or folded α-lobes (Fig. 3B, arrows). Such MB defects have also been demonstrated the fly model of FragileX Syndrome [35]. Interestingly, heterozygous mutants used in the behavioral assays described above did not show MB defects (Fig. 3C). However, it is possible that heterozygotes have subtle structural or functional developmental defects in the brain that are not visible. A role for dube3a in dendritic arborization has already been demonstrated in fly and mice models of AS [16,17]. Additionally, there are strong indications from AS mice that ube3a plays a role in functioning of synapses by regulating various target proteins [36–38]. Thus we speculate that subtle defects in the dendrites and synapses of neurons may exist in the brains of these flies which finally manifest as impairment of behavior.

In order to delineate the role of dube3a in development and adulthood, it was conditionally silenced in these two stages using temperature-sensitive *tubulin-GAL80*. Fig. 3D shows the conditions used in this assay. Flies having normal dube3a levels (I) and those lacking dube3a (II) during the entire course of the experiment were used as controls. The efficacy of the conditions was confirmed by visually monitoring *UASGFP* expression (recombined with *ELAVGAL4*) in these flies. When dube3a was either silenced only during adulthood (III) or development (IV), learning was found to be defective. Taken together, these results indicate that dube3a expression is essential in both stages to manifest this behavior.

3.5. dube3a and other learning-related genes

The ability of dube3a to regulate transcription of learning-related genes was investigated. Ten candidate genes expressed predominantly in the MBs were chosen and their transcript levels were compared between *dube3a*$^{+/-}$and controls (Supplementary Table S1). Of these genes, two were significantly down-regulated (*derailed* and *pastrel*) while one was up-regulated (*amnesiac*) in mutants. Since this screen is far from exhaustive, we suggest that dube3a may have many other transcriptional targets relevant to learning which are yet to be revealed.

The gene *derailed/linotte* (*drl/lio*) encodes a receptor tyrosine kinase involved in WNT signaling [39]. derailed mutants are defective in learning as assayed by olfactory conditioning and APS [18,40]. In *dube3a*$^{+/-}$ *drl* mRNA and protein were both significantly reduced (Fig. 4A and B). Further, *ELAVGAL4*-driven *drl*RNAi led to impairment in learning consistent with its established role in this behavior (Fig. 4C). Interestingly, expressing *UASdrl* using *ELAVGAL4* rescued the learning defect of *dube3a*$^{+/-}$ mutants (Fig. 4D) indicating that derailed is downstream of dube3a. This observation suggests that reduced *derailed* expression in *dube3a*$^{+/-}$ could be contributing to their behavioral defects. derailed is also known to be involved in axon guidance in the fly embryo and *derailed* mutants have mushroom body defects similar to those observed in *dube3a*$^{+/-}$ [41–43]. While the nature of the interaction between ube3a and derailed begs further exploration, this is the first report implicating a component of the WNT-signaling pathway in AS pathology.

We believe that we have firmly established a role for *dube3a* in learning. This work also provides a strong foundation for using the fly to model both AS and autism and further our understanding of the contribution of *ube3a* towards normal brain development.

Conflict of interest

None.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.110.

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