Angelman syndrome is a neurological disorder whose symptoms include severe mental retardation, loss of motor coordination, and sleep disturbances. The disease is caused by a loss of function of UBE3A, which encodes a HECT-domain ubiquitin ligase. Evidence suggests that we can generate a *Drosophila* model for the disease. The results of several experiments show that the functions of human UBE3A and its fly counterpart, dube3a, are similar. First, expression of Dube3a is enriched in the *Drosophila* nervous system, including mushroom bodies, the seat of learning and memory. Second, we have generated dube3a null mutants, and they appear normal externally, but display abnormal locomotive behavior and circadian rhythms, and defective long-term memory. Third, flies that overexpress Dube3a in the nervous system also display locomotion defects, dependent on the ubiquitin ligase activity. Finally, missense mutations in UBE3A alleles of Angelman syndrome patients alter amino acid residues conserved in the fly protein, and when introduced into dube3a, behave as loss-of-function mutations. The simplest model for Angelman syndrome is that in the absence of UBE3A, particular substrates fail to be ubiquitinated and proteasomally degraded, accumulate in the brain, and interfere with brain function. We have generated flies useful for genetic screens to identify Dube3a substrates. These flies overexpress Dube3a in the eye or wing and display morphological abnormalities, dependent on the critical catalytic cysteine. We conclude that dube3a mutants are a valid model for Angelman syndrome, with great potential for identifying the elusive UBE3A substrates relevant to the disease.

### Results and Discussion

**Drosophila UBE3A Homolog dube3a Likely Encodes One Protein Expressed Throughout Development.** The *Drosophila* gene *CG6190*, located at polytene position 68B1 on chromosome 3L (23), has been identified as dube3a (20–22). There are 14 HECT domain E3 proteins in *Drosophila* (23), and the putative Dube3a protein is clearly the most similar to UBE3A. The fly and human proteins are similar throughout with the most similarity residing in their C-terminal HECT domains ([**Supporting information (SI) Fig. S1**](https://www.pnas.org/)). Using RT-PCR and DNA sequence determination, we found the predicted dube3a mRNA (23) in embryos, larvae, and adults (data not shown).

**Generation and Molecular Characterization of dube3a Loss-of-Function Alleles.** One mutant dube3a allele, with a P transposable element inserted in the 5′ UTR (23), was available, but dube3a expression was not obviously disrupted by the P insertion (data not shown). By mobilizing the P element, we generated three imprecise excision (deletion) alleles: dube3a606, dube3a607, and dube3a158B (Fig. 1A). In addition, we isolated a precise excision (WT) allele called dube3a60E that is isogenic with dube3a158B for the two major autosomes. Homozygotes for each of the deletion alleles, or trans-heterozygotes of each allele with *Df(3L)win5*, a

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**Author contributions:** Y.W., F.V.B., Y.F., and J.A.F. designed research; Y.W., F.V.B., Y.F., and J.A.F. wrote the paper. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0805291105/DCSupplemental.

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Mushroom bodies (MBs) are marked by nuclear GFP expressed by OK201Y methionine residue downstream of the 12400/20841 kDa produced in bacteria, that begin at either M353, the first immediate progeny. (and mainly cytoplasmic in third instar larval eye discs. (Dube3a is expressed ubiquitously in the cytoplasm, with elevated levels in the neuroblasts (which are too young to express Elav) and their immediate progeny. (6mDube3a expression in the developing brain, particularly in the hippocampal and Purkinje neurons, which are the seats of memory and motor coordination, respectively. In mice, UBE3A is broadly expressed early in embryogenesis and later concentrates in neural tissue (11). Thus, we wanted to determine whether Dube3a is expressed in the fly central nervous system during development. Using immunofluorescence, we examined expression in whole-mount embryos, larval brain and ventral nerve cord, larval eye discs, and adult brain. Because the anti-Dube3a signals were robust in embryos only, we generated flies containing genomic DNA transgenes that express N-terminal 6xmyc- or GFP-tagged Dube3a proteins from the dube3a promoter (Fig. 1A).

A Dube3a protein

B

Fig. 1. Characterization of WT and mutant dube3a alleles. (A) A diagram of the dube3a genomic region is shown at Upper (not to scale). There is ~400 bp between CG7600 and dube3a, and ~200 bp between CG6199 and dube3a. Boxes are exons, black indicates coding region, and gray indicates noncoding regions. The arrows beneath the dube3a exons indicate PCR primers used for transcript detection. Extent of the deletions in dube3a alleles is indicated: deletion 6J is ~1.7 kb, 8O is ~2.4 kb, and 15B is ~1.2 kb. Black bars beneath indicate the ~12.9 kb genomic DNA fragments in each transgene. (B) Shown is a blot of eye disc protein extracts from third instar larvae. The blots were probed with anti-Dube3a and anti-Tubulin.

Two alleles, dube3a6J and dube3a15B, retain the transcription start (Fig. 1A), and transcripts containing exons downstream of the deletions were detected by RT-PCR in these mutant flies (data not shown). In contrast, the transcription start site is within the deletion in dube3a80, and no dube3a80 transcripts were detected (data not shown). We used a bacterially produced full-length Dube3a protein to generate polyclonal anti-Dube3a antibodies in rats and guinea pigs. Each antibody recognizes a protein of the expected size (Mr, ~107 kDa) in WT third instar larval eye discs and embryos (data not shown), and neither full-length nor truncated proteins are detected in any of the deletion mutants (Fig. 1B; data not shown). The antibody detects N-terminally truncated Dube3a proteins of Mr, ~80 kDa or ~40 kDa produced in bacteria, that begin at either M353, the first methionine residue downstream of the dube3a15B deletion breakpoint, or M639 (see Fig. S1; data not shown), respectively. Thus, proteins potentially generated by initiation at downstream start codons in dube3a6J or dube3a15B mRNAs are unlikely to have escaped detection. We conclude that the antibodies are specific for Dube3a, and that the three deletion mutants likely produce no protein. All of the behavioral studies were performed with dube3a15B (null mutant) and its isogenic counterpart dube3a6PE (WT).

Dube3a Expression Is Broad and Mainly Cytoplasmic. As is associated with loss of UBE3A expression in the developing brain, particularly in the hippocampal and Purkinje neurons, which are the seats of memory and motor coordination, respectively. In mice, UBE3A is broadly expressed early in embryogenesis and later concentrates in neural tissue (11). We wanted to determine whether Dube3a is expressed in the fly central nervous system during development. Using immunofluorescence, we examined expression in whole-mount embryos, larval brain and ventral nerve cord, larval eye discs, and adult brain. Because the anti-Dube3a signals were robust in embryos only, we generated flies containing genomic DNA transgenes that express N-terminally 6xmyc- or GFP-tagged Dube3a proteins from the dube3a promoter (Fig. 1A).

Embryos were labeled simultaneously with antibodies to Dube3a and the pan-neural nuclear protein, Elav. We find that expression of Dube3a is ubiquitous and cytoplasmic, starting early in embryogenesis and expressed in the developing nervous system (Fig. 2A and B’). The Dube3a antibody is specific in this chromosome with a deletion that includes dube3a and several other genes (23), are viable, fertile, and their external morphology appears normal.

Fig. 2. Dube3a protein in Drosophila tissues. Confocal images are shown. (A) A stage 5 embryo. Dube3a accumulates mainly in the cytoplasm and cortically. (A’) Enlargement of part of A. (B and B’) A stage 15 embryo. Dube3a is expressed broadly, including in Elav+ neural cells. (C) GFP-Dube3a expression is ubiquitous and mainly cytoplasmic in third instar larval eye discs. (D) Enlargement of an eye disc expressing 6mDube3a. (E and E’) Ventral nerve cord and brain (G and G’) of a third instar larva. Dube3a is expressed ubiquitously in the cytoplasm, with elevated levels in the neuroblasts (which are too young to express Elav) and their immediate progeny. (F, F’, H, and H’) Enlargements of regions in (E and E’) and (G and G’), respectively. NB, neuroblasts. (I–J’) show expression in adult brains. Mushroom bodies (MBs) are marked by nuclear GFP expressed by OK201Y > UAS-nucGFP. (I and J) show different planes.
Dube3a protein is mainly cytoplasmic, expressed broadly, and in the eye disc and is cytoplasmic (Fig. 2). Levels in differentiated neurons, glia, and mushroom bodies (Fig. 6) of Dube3a are particularly high in the adult brain (Fig. S4). In contrast with larval brains, Dube3a is cytoplasmic and expressed in the adult nervous system. We noticed by casual observation that the flies that overexpress dube3a, but not dube3aC941A, have locomotion difficulties similar to the dube3a loss-of-function mutant flies, and we quantified the effects in two different sets of experiments (see SI Text and Fig. S6). Because neural overexpression of Dube3a, but not Dube3aC941A, impairs climbing, we conclude that excess Dube3a ligase activity in the nervous system impairs locomotion.

Excessive Dube3a Activity During Development Results in Abnormal Morphology or Lethality. We wondered whether dube3a overexpression with strong eye- or wing-specific drivers might generate morphological defects. We found that overexpression of WT Dube3a, but not Dube3aC941A, in the eye or wing results in severe abnormalities, and that ubiquitous overexpression of Dube3a, but not Dube3aC941A, is lethal (Fig. 4). We also generated several lines that overexpress N-terminally 3xmyc-tagged versions of Dube3a and Dube3aC941A, and the results were identical to those with their untagged counterparts (Fig. S7A). The different effects of the two proteins are not because of differences in protein levels, because we found lines where the levels of 3mDube3a and 3mDube3aC941A were similar (Fig. S7B). We conclude that whereas the absence of Dube3a causes no noticeable eye or wing defects, nor kills the flies, excessive Dube3a ligase activity in developing eyes or wings results in aberrant morphology, and ubiquitous ligase overactivity kills the animals.

To test whether the human gene would affect fly morphology similarly to dube3a, we attempted to overexpress human UBE3A in Drosophila by using the same methods, but we were unable to do so. This could be because of problems with codon usage or gene product stability (see SI Text).

Fly dube3a Genes with AS Missense Mutations Act as Loss-of-Function Mutants. Eight different missense mutant alleles of human UBE3A that result in loss of function have been identified in Angelman syndrome patients (27–32). In support of the idea that the fly and human genes function similarly, all but one of these mutations are in amino acids conserved between the two species (Fig. S1). If Drosophila Dube3a functions as human UBE3A does, we expect that these point mutations would also result in loss of function of the fly gene. To test this idea, we separately introduced four of these point mutations into fly 3mdube3a genes and, by using eye-specific and ubiquitous Gal4 drivers, tested each mutant gene for function in the overexpression assay described above. The results are summarized in Fig. S7A. We
also assayed Dube3a protein accumulation in several lines, and representative results are shown in Fig. S7B. We find that two of the mutant proteins, Dube3a<sup>R626C</sup> and Dube3a<sup>E325K</sup>, behave identically to the catalytically inactive Dube3a<sup>C941A</sup> protein in this assay; the mutant proteins accumulate to levels that are similar to WT protein but do not cause a mutant phenotype. Dube3a<sup>C55Y</sup> also behaves like Dube3a<sup>C941A</sup> in that it fails to cause a mutant phenotype, but its level of accumulation is lower than that of Dube3a<sup>C941A</sup> and WT Dube3a. Finally, Dube3a<sup>1447P</sup> showed variable results. Four of six lines, at least one of which expresses similar levels of protein to WT Dube3a and Dube3a<sup>C941A</sup>, result in no phenotypes. However, one line results in lethal phenotypes and one has rough eye phenotype. Dube3a<sup>T447P</sup> may be a partially functional protein that can cause a mutant phenotype only when expressed at higher levels than Dube3a. We conclude that the fly counterparts of all four of the disease missense alleles behave as loss-of-function mutations in the fly, probably through a variety of different mechanisms (33) (see SI Text).

**Fig. S7.** A representation of a diagram for the localization of two strains of flies. The diagram shows the localization of the WT and mutant strains in the context of the neural tissue.

**Flies with dube3a Mutations Are Defective in Long-Term Memory Formation.** AS patients suffer severe cognitive impairment. UBE3A mutant mice may be similarly impaired; they can learn to associate a tone with a shock (fear conditioning) but have impaired context dependent long-term memory (LTM) (10). We tested dube3a mutant flies for defects in olfactory learning and memory; the flies were trained to associate an odor with a shock and were then tested for avoidance of the odor (34, 35). As in mice, LTM in *Drosophila* requires repeated training (34–36). “Spaced training,” in which 10 training sessions (12 trials in a session) are separated by 15-min rest intervals, produces LTM that requires protein synthesis and lasts more than one week (34–36). In contrast, the same 10 training sessions without the rest intervals, known as “massed training,” induces a different kind of memory that is protein synthesis independent and decays within 4 d (34–36). One-day memory performance is usually higher after spaced training than massed training (34–36). To determine whether *dube3a* mutant flies have memory defects, we tested null mutants for 1-d memory after spaced or massed training, and we found that after spaced training, their memories were significantly defective when compared with WT, but similar to WT after massed training (Fig. S4). The LTM defect after spaced training cannot be attributed to an inability of *dube3a* flies to react to shock or an olfactory deficit because mutant and WT performed similarly (Fig. S5). The memory deficit is not because of failure to learn because immediately after a single training session, null mutants and WT performed similarly (Fig. S4). The specificity of the defect to spaced training suggests that, similar to *UBE3A* mutant mice, *dube3a* mutant flies learn as well but cannot form LMTs as well as WT flies. Because LTM requires particular transcription factors and protein synthesis (37–39), Dube3a activity may be involved in regulation of gene expression.

**dube3a Null Mutants Have Abnormal Circadian Rhythms.** Because many AS patients suffer sleep disturbance, we asked whether *dube3a* mutant flies are defective in rest/activity rhythms. Flies...
were entrained to a 12 h:12 h, light:dark cycle for 3 d before placing them in locomotor activity monitors that record the frequency with which a fly crosses an infrared beam passed through the chamber (40). Activity of the flies was monitored for 12–14 d in constant darkness, which allows for the determination of free-running circadian rhythms. We tested the behavior of 

dube3a

null and WT flies, and mutants containing a copy of the

gdbe3a

transgene (Fig. 1A), and used the data to determine the periodicity and strength (consolidation) of the rhythm. Because flies undergo age associated changes in rhythm strength and period, and the pattern of activity varies with gender (41), young (4–7 d) and old (18–21 d) flies of each sex were tested separately. In all three genotypes, we observed periods within the WT range in rhythmic flies (≈23.5 h; data not shown). However, when compared with both controls, a larger fraction of 

dube3a

null mutant males were arrhythmic (Table S1), and rhythm strength was reduced in young males and older flies of both sexes (Fig. 6). Core proteins of the molecular clock are regulated by ubiquitin-mediated proteolysis, and two ubiquitin ligases, Slimb and Jetlag, mediate their degradation (42, 43). More experiments are required to determine whether Dube3a functions similarly.

**Concluding Remarks.** We have generated loss-of-function and gain-of-function 

dube3a

mutants with great potential for use in genome-wide, genetic screens to reveal substrates of Dube3a and UBE3A relevant to AS. Moreover, the fly model provides a means to test the potential relevance to AS of a 

dube3a

substrate; overexpression in the brain of relevant substrates should result in behavior defects similar to those observed in 

dube3a

loss-of-function mutations. Genes that pass this test can then be tested in the mouse model.

**Materials and Methods**

**Drosophila Genetics.** The following strains were obtained from the Bloomington Drosophila stock center unless otherwise indicated. 

\[dube3a^{P(T)}3274\] (Fbi10011388); elav-gal4 (Fbi10072910); cha-gal4 (Fbi10042505); repo-gal4 (Fbi10018692); OK107-gal4 (Fbi10081470); 201Y-gal4 (Fbi10002924); Il31t-gal4 (Fbi10002096); M. Sokolowski, University of Toronto, Mississauga, ON, Canada); OK64-gal4 (Fbi10023258); B. Zhang, University of Oklahoma, Norman, OK); UAS-nuc-gfp (Fbi10012493); GMR-gal4 (Fbi10002994); MS1096-gal4 (Fbi10002374); tub-gal4 (Fbi10012687); Df(3L)vins (Fba10002457). A complete description of the generation and characterization of 

dube3a

mutants is in the SI Text.

**Immunohistochemistry.** Embryo immunostaining was performed as described in ref. 45. Brain immunostaining was as described at: jfly.iam.u-toyko.ac.jp/immunostaining/Immunostaining.pdf. Third instar larval eye disk immunostaining was as described in ref. 46. A complete list of antibodies and dilutions are in the SI Text. Tissues were mounted in VectaShield (Vector Laboratories). Images were acquired with a Leica SP2 AOBS or a TCS-SP confocal microscope and were manipulated using Adobe Photoshop.

**Molecular Biology.** A complete description of standard methods used for 

dube3a

mRNA analysis, plasmid constructions, protein blotting, and generation of 

Dube3a antisera is in the SI Text.

**Behavioral Assays.** Climbing (24), flight (25, 26), learning and memory (34, 35), olfaction (47), shock reactivity (48), circadian rhythm (40), and stress tests (49) were as described. Complete descriptions of each assay are in the SI Text.

**Analysis of Eyes and Wings.** Adult eyes were sectioned and photographed, and wings were mounted and photographed as described (46, 50).

**Acknowledgments.** We thank B. Zhang, S. Waddell, X. Wang, M. Sokolowski, and J. Hrubgretse for flies, help and advice, Y. Ting-Chun for help with controls, J. Hrubgretse, L. Stevens, and D. Stein for plasmids, and P. Macdonald for use of his confocal microscope. This work was supported by National Institutes of Health Grants R01-HD30680 and R21-NS051307 (to J.A.F.) and R01-NS48471 (to Y.F.). F.B. received support from the Dart NeuroGenomics Alliance. A.S. is an investigator of the Howard Hughes Medical Institute.