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. Essentially, we 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 locomotor 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.

E6-AP | UBE3A | ubiquitin ligase | mental retardation

Several mouse models of AS have been generated including two simple knockouts of the mouse UBE3A homolog on chromosome 7 (10–13). The knockout mutants recapitulate several aspects of the disease: cerebellar and hippocampal morphology are normal, but the mice display motor dysfunction, seizures, and memory deficits. Ubiquitination has a variety of effects on protein function, depending on whether a protein is monoubiquitinated or tagged with a ubiquitin chain depending on how the ubiquitin chain is linked (14). UBE3A attaches to its substrates ubiquitin chains that are linked through the K48 residue of the first ubiquitin and the terminal G76 of the incoming ubiquitin (15). So-called K48-linked chains usually target substrates for proteasomal degradation. Thus, the simplest model to explain why loss of UBE3A activity leads to AS is that in the absence of the ubiquitin ligase, one or more UBE3A substrates accumulate in the brain and interfere with brain function. A few UBE3A substrates have been identified biochemically, but none of them has been shown to be relevant to AS (16–19).

The Drosophila genome has a UBE3A homolog called dube3a (20–22), and thus Drosophila genetics could provide a powerful means to identify Dube3a (and UBE3A) substrates relevant to AS. Here, we provide compelling evidence that Drosophila dube3a mutants are indeed a useful AS model.

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]. 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, dube3a605, and dube3a15B (Fig. 1). In addition, we isolated a precise excision (WT) allele called dube3a60E that is isogenic with dube3a15B 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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chromosome with a deletion that includes dube3a and several other genes (23), are viable, fertile, and their external morphology appears normal.

Two alleles, dube3ad6 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 dube3a80 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). 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-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
Dube3a protein is mainly cytoplasmic, expressed broadly, and in the eye disc and is cytoplasmic (Fig. 2). Developing eye disc. We find that Dube3a is expressed ubiquitously in mushroom body (Fig. 2). 6mDube3a is expressed at particularly high levels in the adult S3. As in larval brains, 6mDube3a is cytoplasmic and expressed in human UBE3A (UBE3AC941A) destroys the function of the mutant flies of similar ages were compared. Because motor coordination can be affected by age, WT and mutants is solely because of loss of function. Results on expression of the nearby gene. In summary, similar to the expression of vertebrate UBE3A, Dube3a is mainly cytoplasmic, expressed broadly, and enriched in adult mushroom bodies.

dube3a Null Mutants Display Abnormal Climbing Behavior. AS patients display loss of motor coordination, including ataxia and abnormal gait. Although the dube3a mutant flies can walk, climb, and fly, we noticed that their locomotion behaviors are abnormal. For example, the mutant flies tend to get stuck in the food, and when knocked to the bottom of a food vial, they take much longer than the WT flies to begin climbing up. We quantified the behavior defects by using an established climbing assay (24). Because motor coordination can be affected by age, WT and mutant flies of similar ages were compared.

We find that both young and older dube3a mutant flies perform markedly more poorly than WT in the climbing assay (Fig. 3A). We asked whether the climbing defect in dube3a mutants is solely because of loss of dube3a function or whether effects on expression of the nearby gene. CG7600 (Fig. 1A) might also contribute to the mutant phenotype. We generated transfectants with either of two transgenes, one with WT genomic DNA containing the dube3a gene (mdube3a) and the other with an identical genomic DNA fragment, except for a missense mutation in the codon for the critical catalytic cysteine residue in Dube3a (mdube3aC941A) (Fig. 1A). The analogous mutation in human UBE3A (UBE3A) destroys the function of the protein (25), and we show below that the C941A mutation is unlikely to destabilize Dube3a significantly. Transformant genetic backgrounds were homogenized by rounds of backcrossing with dube3a. 6mDube3a we find that a gube3a null transgene restores WT climbing ability to the dube3a null flies, whereas a gube3aC941A transgene has no rescuing activity (Fig. 3B). We conclude that the dube3a null flies climb poorly because of the dube3a mutation, and that normal locomotion depends on the ubiquitin ligase activity of Dube3a. We also tested the ability of the dube3a mutants to initiate flight (26), and we observed no obvious defect (data not shown). Because Dube3a is expressed widely, determination of whether the dube3a locomotion behaviors are because of loss of function in the nervous system requires further experiments.

Excessive Dube3a Activity in the Nervous System Also Results in Locomotion Defects. We have shown that loss of Dube3a activity results in loss of climbing ability, presumably because of accumulation of one or more Dube3a substrates. We wondered whether the motor coordination of flies is also sensitive to excessive Dube3a ligase activity, and presumably, too little substrate. To test this possibility, using the Gal4/UBA system and the pan-neural driver elav-gal4, we generated flies that overexpress dube3a or dube3aC941A in the developing and 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 find that overexpression of WT Dube3a, but not Dube3aC941A, in the eye or wing substrates. To test this possibility, using the Gal4/UBA system and the pan-neural driver elav-gal4, we generated flies that overexpress dube3a or dube3aC941A in the developing and 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.

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, Dube3aR626C and Dube3aI925K, behave identically to the catalytically inactive Dube3aC941A protein in this assay; the mutant proteins accumulate to levels that are similar to WT protein but do not cause a mutant phenotype. Dube3aC555 also behaves like Dube3aC941A in that it fails to cause a mutant phenotype, but its level of accumulation is lower than that of Dube3aC555 and WT Dube3a. Finally, Dube3aT447P showed variable results. Four of six lines, at least one of which expresses similar levels of protein to WT Dube3a and Dube3aC941A, result in no phenotypes. However, one line results in lethality, and one has rough eyes. Dube3aT447P 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).

**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 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. 5A). The LTM defect after spaced training cannot be attributed to an inability of dube3a flies to react to shock or an olfaction deficit because mutant and WT performed similarly in direct tests of shock reactivity and olfaction (Fig. 5B). Moreover, the memory deficit is not because of failure to learn because immediately after a single training session, null mutants and WT performed similarly (Fig. 5A). The specificity of the defect to spaced training suggests that, similar to UBE3A mutant mice, dube3a mutant flies learn as well but cannot form LTBMs 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 with dube3a Mutations Are Defective in Long-Term Memory Formation. In flies with either the eye-specific Gal4 driver (GMR-gal4) or the wing-specific driver (MS1096-gal4). In flies with either the UAS or the Gal4 driver alone, eyes and wings are WT. (C and D) Eyes are irregular and wing tissue is curly. (E and F) Eyes and wings are WT. Five different UAS-dube3a lines and two different UAS-dube3aC941A lines gave the same results as those shown. Ubiquitous overexpression of Dube3a by using a tubulin-gal4 driver and any of the five UAS-dube3a lines was lethal, whereas ubiquitous overexpression of Dube3aC941A with either of two UAS-dube3aC941A lines had no effect. (Scale bar, 200 μm in A–F). (G and H) Tangential sections through WT eyes or eyes overexpressing Dube3a WT retina contains organized hexagonal facets, each with eight photoreceptors arranged in a trapezoid. Retinal morphology is severely disrupted by Dube3a overexpression (Scale bar, 20 μm in G and H).

**Flies with dube3a Mutations Are Defective in Long-Term Memory Formation.** In flies with either the UAS or the Gal4 driver alone, eyes and wings are WT. (A and B) or that overexpress the UAS transgene indicated with the eye-specific Gal4 driver (GMR-gal4) or the wing-specific driver (MS1096-gal4). In flies with either the UAS or the Gal4 driver alone, eyes and wings are WT. (C and D) Eyes are irregular and wing tissue is curly. (E and F) Eyes and wings are WT. Five different UAS-dube3a lines and two different UAS-dube3aC941A lines gave the same results as those shown. Ubiquitous overexpression of Dube3a by using a tubulin-gal4 driver and any of the five UAS-dube3a lines was lethal, whereas ubiquitous overexpression of Dube3aC941A with either of two UAS-dube3aC941A lines had no effect. (Scale bar, 200 μm in A–F) (G and H) Tangential sections through WT eyes or eyes overexpressing Dube3a. WT retina contains organized hexagonal facets, each with eight photoreceptors arranged in a trapezoid. Retinal morphology is severely disrupted by Dube3a overexpression (Scale bar, 20 μm in G and H).

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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 gdube3a+ transgene. Relative rhythmicity was evaluated by Fast Fourier Transformation analysis. Error bars are standard error; a bar is absent from 18–21-d males because only one fly was weakly rhythmic. Statistical significance was determined by two-tailed Student’s t test with unequal variance. *P < 0.01; **P < 0.0001.

**Materials and Methods**

**Drosophila Genetics.** The following strains were obtained from the Bloomington Drosophila stock center unless otherwise indicated. dube3a P[FD]3274 (Fbi10011388); elav-gal4 (Fbi1002910); cha-gal4 7.4 (Fbi10024050); repo-gal4 (Fbi10018692); OK107-gal4 (Fbi10004170); 201Y-gal4 (Fbi1002926); i(l)31t1 (Fbi1002098); M. Sokolowski, University of Toronto, Mississauga, ON, Canada); OK6-gal4 (Fbi10023258); B. Zhang, University of Oklahoma, Norman, OK); UAS-nucgf8 (Fbi10012493); gmr-gal4 (Fb10002994); MS1096-gal4 (Fbi1002374); tub-gal4 (Fbi1001268); Df[3]vvin5 (Fb1002457). 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-tokyo.ac.jp/Immunohistochemistry. Third instar larval eye disk immunostaining was as described in ref. 46. A complete list of antibodies and dilutions is 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 by 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).

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