A *Drosophila* model for Angelman syndrome

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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. Here, 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 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.

E6-AP | UBE3A | ubiquitin ligase | mental retardation

Angelman syndrome (AS) is an inherited neurological disorder that occurs in ≈1/15,000 births and is characterized by mental retardation, minimal speech, difficulties in motor coordination, seizures, sleep disorders, and unusual behaviors such as excessive laughter, excitability, and short attention span (1, 2). AS is caused by a loss of function of a single gene on chromosome 15 called *UBE3A*, which encodes a HECT domain ubiquitin ligase or E3 protein (3, 4). Through covalent interactions with the catalytic cysteine residue, HECT domain ligases accept ubiquitin from E2 proteins and transfer the ubiquitin to specific substrates (5). UBE3A was originally called E6-AP because it was initially identified through its interactions with the viral E6 protein; binding to E6 subverts the normal substrate specificity of E6-AP to p53, and p53 degradation leads to cervical cancer (6).

There are four genetic mechanisms known for inheritance of AS (1). *UBE3A* is within a region of chromosome 15 that is paternally imprinted in the hippocampus and in the Purkinje cells of the brain (7–9). Thus, mutation of the maternally inherited gene copy underlies all four mechanisms. Most (68%) cases of AS are because of a deletion in the maternal chromosome 15 that includes *UBE3A*. Mutations in the maternal *UBE3A* gene account for another 13% of AS cases, whereas another 6% are because of imprinting defects and repression of the maternal *UBE3A* gene copy. The smallest number (3%) of AS incidences is caused by paternal uniparental disomy for chromosome 15. Finally, 10% of people with clear AS symptoms do not fall into any of the above classes, and the cause of their syndrome is unknown.

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 defects. 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: $dube3a^{80}$, $dube3a^{6J}$, and $dube3a^{15B}$ (Fig. 1*A*). In addition, we isolated a precise excision (WT) allele called $dube3a^{6PE}$ that is isogenic with $dube3a^{15B}$ for the two major autosomes. Homozygotes for each of the deletion alleles, or *trans*-heterozygotes of each allele with *Df(3L)vin5*, a

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The authors declare no conflict of interest.

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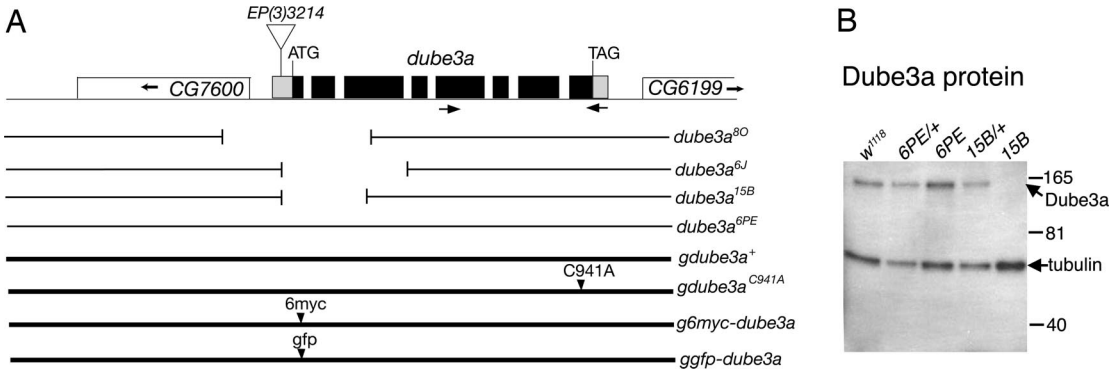

Fig. 1. Characterization of WT and mutant *dube3a* alleles. (*A*) A diagram of the *dube3a* genomic region is shown at *Upper* (not to scale). There is ≈400 bp between *CG7600* and *dube3a*, and ≈200 bp between *CG6199* and *dube3a*. Boxes are exons, black indicates coding region, and gray indicates 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.

chromosome with a deletion that includes *dube3a* and several other genes (23), are viable, fertile, and their external morphology appears normal.

Two alleles, $dube3a^{6J}$ and $dube3a^{15B}$, retain the transcription start (Fig. 1*A*), 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 $dube3a^{80}$, and no $dube3a^{80}$ 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 (M_r, ≈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. 1*B*; data not shown). The antibody detects N-terminally truncated Dube3a proteins of M_r ≈80 kDa or ≈40 kDa produced in bacteria, that begin at either M353, the first methionine residue downstream of the $dube3a^{15B}$ deletion breakpoint, or M639 (see Fig. S1; data not shown), respectively. Thus, proteins potentially generated by initiation at downstream start codons in $dube3a^{6J}$ or $dube3a^{15B}$ 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 $dube3a^{15B}$ (null mutant) and its isogenic counterpart $dube3a^{6PE}$ (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. 1*A*).

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. 2*A* and *B*′). The Dube3a antibody is specific in this

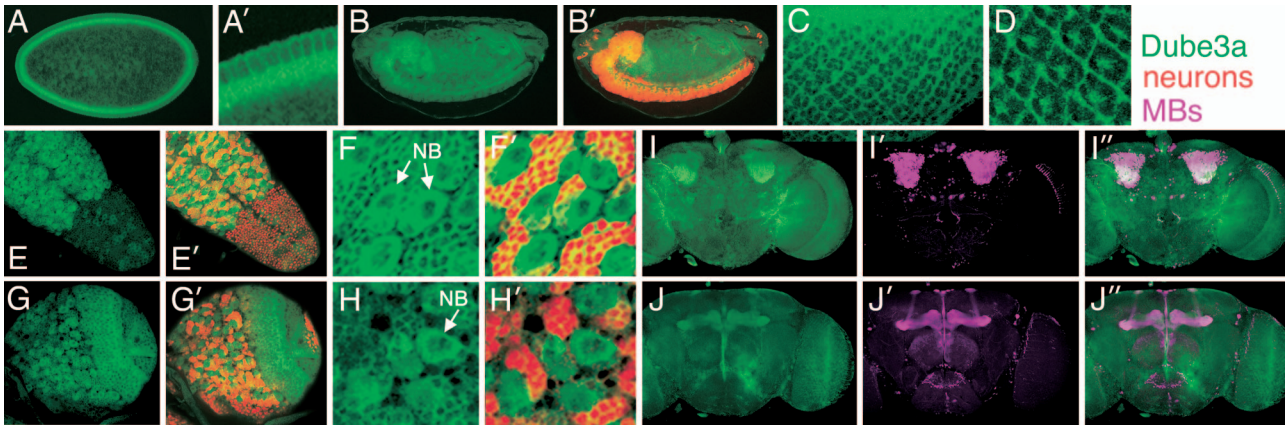

Fig. 2. Dube3a protein in *Drosophila* tissues. Confocal images are shown. (*A*) A stage 5 embryo. Dube3a accumulates mainly in the cytoplasm and cortically. (*A*′) Enlargement of part of *A*. (*B* and *B*′) A stage 15 embryo. Dube3a is expressed broadly, including in Elav$^+$ neural cells. (*C*) GFP·Dube3a 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.

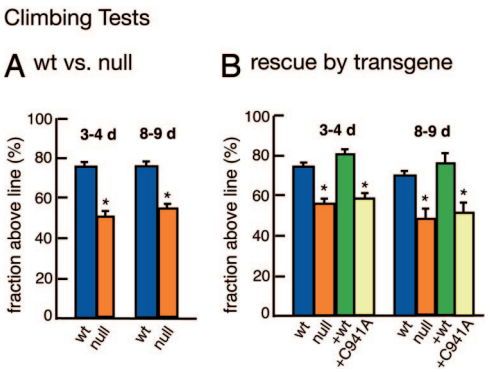

Fig. 3. Defective climbing ability because of *dube3a* loss of function. Results of climbing tests are shown as averages of 16–18 different trials, each performed on separate populations of ≈20 flies. Error bars are standard error. (*A*) At both ages tested, *dube3a* null mutants perform worse than WT flies. (*B*) At both ages tested, a *gdube3a*$^{+}$ transgene (Fig. 1*A*) rescues the *dube3a* null climbing defect to WT, whereas a *gdube3a*^{C941A} transgene (Fig. 1*A*) provides no rescue activity. ∗, $P < 0.05$

assay (Fig. S2).

Next, we examined 6xmyc-tagged Dube3a (6mDube3a) expression in the larval brain and ventral nerve cord and in adult brains. Background signal from anti-Myc is insignificant (data not shown). We colabeled with anti-Elav or coexpressed nuclear GFP by using *UAS-nGFP* and one of five different Gal4 drivers that mark glia, differentiated neurons, neuroblasts, or mushroom bodies. In the larval brain and nerve cord, 6mDube3a is concentrated in the cytoplasm of neuroblasts and their immediate progeny (Fig. 2 *E–H′* and Fig. S3) and is expressed at much lower levels in differentiated neurons, glia, and mushroom bodies (Fig. S3). As in larval brains, 6mDube3a is cytoplasmic and expressed broadly in the adult brain (Fig. S4). In contrast with larval brains, 6mDube3a is expressed at particularly high levels in the adult mushroom body (Fig. 2 *I* and *J″*).

Because we use the eye for the overexpression experiments below, we wanted to know whether Dube3a is expressed in the developing eye disc. We find that Dube3a is expressed ubiquitously in the eye disc and is cytoplasmic (Fig. 2 *C* and *D* and Fig. S5).

In summary, similar to the expression of vertebrate UBE3A, Dube3a protein 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. 3*A*). 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. 1*A*) might also contribute to the mutant phenotype. We generated transformants with each of two transgenes, one with WT genomic DNA containing the *dube3a* gene (*gdube3a*$^{+}$) 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 (*gdube3a*^{C941A}) (Fig. 1*A*). The analogous mutation in human UBE3A (UBE3A^{C820E}) 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*^{6PE}. We find that a *gdube3a*$^{+}$ transgene restores WT climbing ability to the *dube3a* null flies, whereas a *gdube3a*^{C941A} transgene has no rescuing activity (Fig. 3*B*). 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/UAS* system and the pan-neural driver *elav-gal4*, we generated flies that overexpress *dube3a* or *dube3a*^{C941A} in the developing and adult nervous system. We noticed by casual observation that the flies that overexpress *dube3a*, but not *dube3a*^{C941A}, 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 Dube3a^{C941A}, 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 Dube3a^{C941A}, in the eye or wing results in severe abnormalities, and that ubiquitous overexpression of Dube3a, but not Dube3a^{C941A}, is lethal (Fig. 4). We also generated several lines that overexpress N-terminally 3xmyc-tagged versions of Dube3a and Dube3a^{C941A}, and the results were identical to those with their untagged counterparts (Fig. S7*A*). 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 3mDube3a^{C941A} were similar (Fig. S7*B*). 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. S7*A*. We

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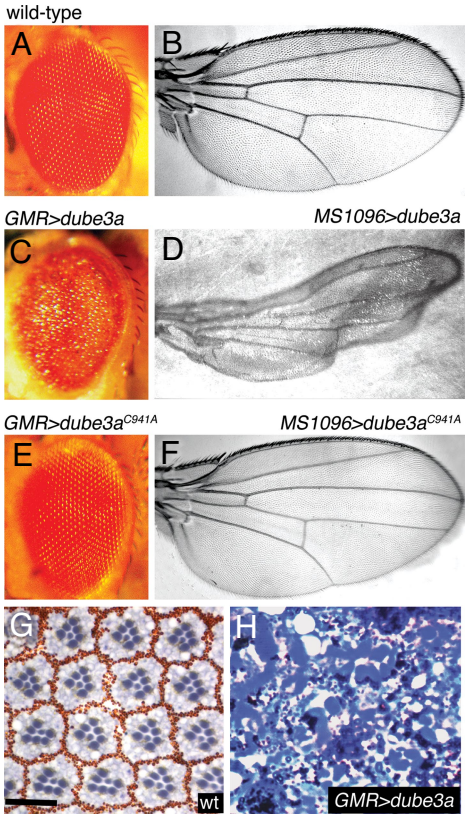

Fig. 4. Morphological defects in flies that overexpress *dube3a*$^{+}$ in the eye or wing. Shown are external eyes (*A*, *C*, and *E*) and wings (*B*, *D*, and *F*) of flies that are WT (*A* and *B*) or that overexpress the *UAS* transgene indicated with the eye-specific Gal4 driver (*GMR-gal4*) or the 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-dube3a*^{C941A} 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 Dube3a^{C941A} with either of two *UAS-dube3a*^{C941A} lines had no effect. (Scale bar, 200 μm in *A–F*) (*G* and *H*) Tangential sections through WT eyes or eyes overexpressing Dube3a. WT retinas contain 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*).

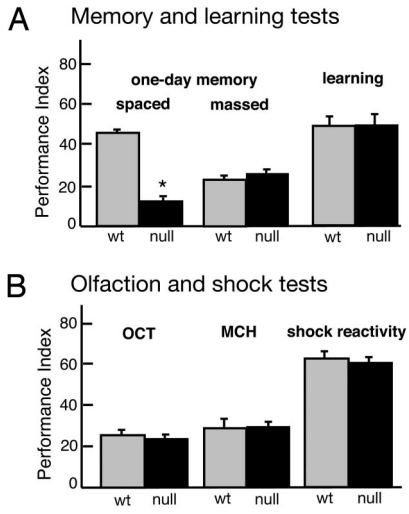

Fig. 5. Defective 1-d memory in *dube3a* mutants specifically after spaced training. (*A*) Results of 1-d memory and learning tests are shown. *dube3a* null mutants perform worse than WT flies after spaced training but not after massed training or immediately after a single training session (learning) (8 PI per genotype; *, $P < 0.0001$). (*B*) Results of behavioral experiments for task-relevant olfactory acuity and shock reactivity are shown. No significant differences were observed between *dube3a* null mutants and WT (8 PI per genotype; $P = 0.61$ for 3-octanol (OCT); $P = 0.98$ for 4-methylcyclohexanol (MCH). Aversion to OCT or MCH was similar in mutant vs. control flies ($P = 0.55$ and $P = 0.32$, respectively; data not shown). There is no significant difference between *dube3a* null mutants and WT controls (4 PI per genotype; $P = 0.78$). Error bars, standard error; PI, performance index; OCT, 3-octanol; MCH, 4-methylcyclohexanol. See *Materials and Methods* for complete description of methods and calculation of PI.

also assayed Dube3a protein accumulation in several lines, and representative results are shown in Fig. S7*B*. We find that two of the mutant proteins, Dube3a^{R626C} and Dube3a^{I925K}, behave identically to the catalytically inactive Dube3a^{C941A} protein in this assay; the mutant proteins accumulate to levels that are similar to WT protein but do not cause a mutant phenotype. Dube3a^{C55Y} also behaves like Dube3a^{C941A} in that it fails to cause a mutant phenotype, but its level of accumulation is lower than that of Dube3a^{C941A} and WT Dube3a. Finally, Dube3a^{T447P} showed variable results. Four of six lines, at least one of which expresses similar levels of protein to WT Dube3a and Dube3a^{C941A}, result in no phenotypes. However, one line results in lethality, and one has rough eyes. Dube3a^{T447P} 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 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. 5*A*). 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. 5*B*). 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. 5*A*). The specificity of the defect to spaced training suggests that, similar to *UBE3A* mutant mice, *dube3a* mutant flies learn as well but cannot form LTMs 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

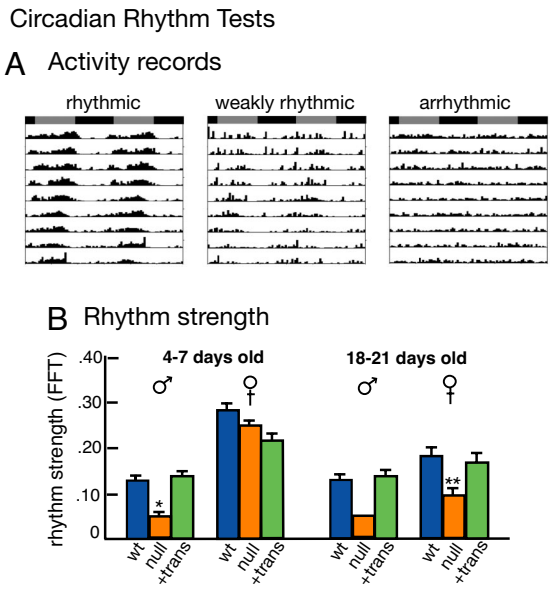

Fig. 6. Rest/activity rhythms are weaker in *dube3a* null flies. (*A*) Representative locomotor activity records of flies (see *Materials and Methods* for classification criteria). (*B*) Summary of rhythm strength: wt = $dube3a^{6PE}$, null = $dube3a^{15B}$, and +trans = $dube3a^{15B}$ containing one copy of a $gdube3a^{+}$ 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. *, $P < 0.01$; **, $P < 0.0001$.

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 (Fig. 1*A*), 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* 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.

***dube3a* Mutant Flies Are Not Susceptible to Seizures.** Because many AS patients experience seizures, we also subjected *dube3a* mutant flies to mechanical stress by vortexing them or heat shocking them briefly. Subjecting the flies to mechanical or thermal stress induces paralysis in susceptible flies, a phenomenon that is thought to be related to human seizures (44). *dube3a* mutant flies of two ages were subjected to each stress, and none of the flies showed any signs of paralysis (data not shown). The seizure phenotype is not only variable in AS patients (1, 2) but also in mouse models (10, 13).

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. $dube3a^{EP(3)3214}$ (FBti0011388); *elav-gal4* (FBti0072910); *cha-gal4.7.4* (FBti0024050); *repo-gal4* (FBti0018692); *OK107-gal4* (FBti0004170); *201Y-gal4* (FBti0002924); *l(3)31-gal4* (FBti0002096; M. Sokolowski, University of Toronto, Mississauga, ON, Canada); *OK6-gal4* (FBti0023258; B. Zhang, University of Oklahoma, Norman, OK); *UAS-nucgfp8* (FBti0012493); *GMR-gal4* (FBti0002994); *MS1096-gal4* (FBti0002374); *tub-gal4* (FBti0012687); *Df(3L)vin5* (FBab0002457). 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/html/manuals/pdf/E_Staining_ItoKei.pdf. 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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