EPIGENETICS
2016, VOL. 11, NO. 9, 637–642
http://dx.doi.org/10.1080/15592294.2016.1214783

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BRIEF REPORT

OPEN ACCESS

The *Drosophila melanogaster* homolog of *UBE3A* is not imprinted in neurons

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ABSTRACT

In mammals, expression of *UBE3A* is epigenetically regulated in neurons and expression is restricted to the maternal copy of *UBE3A*. A recent report claimed that *Drosophila melanogaster UBE3A* homolog (*Dube3a*) is preferentially expressed from the maternal allele in fly brain, inferring an imprinting mechanism. However, complex epigenetic regulatory features of the mammalian imprinting center are not present in Drosophila, and allele specific expression of *Dube3a* has not been documented. We used behavioral and electrophysiological analysis of the *Dube3a* loss-of-function allele ($Dube3a^{15b}$) to investigate *Dube3a* imprinting in fly neurons. We found that motor impairment (climbing ability) and a newly-characterized defect in synaptic transmission are independent of parental inheritance of the $Dube3a^{15b}$ allele. Furthermore, expression analysis of coding single nucleotide polymorphisms (SNPs) in *Dube3a* did not reveal allele specific expression differences among reciprocal crosses. These data indicate that *Dube3a* is neither imprinted nor preferentially expressed from the maternal allele in fly neurons.

Abbreviations: AS, Angelman syndrome; *UBE3A*, *ubiquitin protein ligase E3A*; *Dube3a*, *Drosophila melanogaster* homolog of *UBE3A*; *UBE3A-AS*, *UBE3A antisense transcript*; DGRP, Drosophila genetic reference panel; SNP, single nucleotide polymorphism

ARTICLE HISTORY
Received 3 June 2016
Revised 8 July 2016
Accepted 14 July 2016

KEYWORDS
Climbing assay; DGRP; *Drosophila melanogaster*; ERG; imprinting; synaptic transmission; SNPs; *UBE3A*

Introduction

Angelman syndrome (AS) results from neuronal loss of maternally expressed *ubiquitin protein ligase E3A* (*UBE3A*) in neurons.[1] Maternal deletions of chromosome 15q11.2-q13.1 encompassing *UBE3A* are the most common cause of AS. However, maternally-inherited loss-of-function (LOF) point mutations in *UBE3A*, imprinting defects, or paternal uniparental disomy also cause AS.[2] The first evidence for imprinted expression of *UBE3A* in neurons arose from the observation that maternally derived deletions of 15q11.2-q13.1 cause AS[3] and that *UBE3A* displays maternal allele-specific expression in human brain samples.[4] In mice, *Ube3a* is expressed from the maternal allele in hippocampal, cerebellar, and olfactory bulb mitral neurons.[5] *Ube3a* is biallelically expressed in neurons born from stem cells in the adult mammalian brain and immature neurons early in development.[6] *Ube3a* expression shifts from biallelic to maternal allele specific as neurons mature.[7] *UBE3A* is biallellically expressed in human induced pluripotent stem cells and neuronal differentiation results in paternal silencing of *UBE3A*.[8] In contrast to neurons, glial cells in the mammalian brain biallelically express *UBE3A*.[6,7,9]

Imprinted expression of UBE3A in neurons is a complex mechanism mediated by the expression of a *UBE3A-antisense transcript* (*UBE3A-AS*) that interferes with expression of the sense UBE3A full-length transcript. The Prader-Willi syndrome imprinting center (PWS-IC) is maternally methylated and silenced due to an upstream Angelman syndrome imprinting-center (AS-IC) that epigenetically alters the maternal PWS-IC in the female germline via a transcriptional mechanism, leaving maternal PWS-IC nonfunctional post-fertilization.[10] Downstream from the PWS-IC is the *SNURF/SNRPN* locus, and the *SNRPN* transcript is expressed exclusively from the paternal allele due to the unmethylated PWS-IC promoter. In mature neurons, the *SNRPN* transcript progresses through a cluster of snoRNAs and spliced host genes, terminating as *UBE3A-AS*. The expression of *UBE3A-AS* on the opposite strand of *UBE3A* interferes with *UBE3A* expression on the paternal allele and is the mechanism for paternal *UBE3A* silencing.[2]

Although it is well-established that *UBE3A* is imprinted in the mature neurons of human and mouse brain, it is unclear if *UBE3A* is imprinted in the brains of non-mammalian species including the model invertebrate organism *Drosophila melanogaster*. Flies have a single *UBE3A* homolog[11], *Dube3a*, and mutations in this gene give rise to various phenotypes independent of the parental origin of the mutation.[12] Recently, it was reported that *Dube3a* is preferentially expressed from the maternal allele in flies.[13] However, there is a lack of synteny between fly and human genomes at the *UBE3A*/*Dube3a* loci and there is no prior evidence for allele specific expression in flies. Given that flies do not have a documented imprinting center, differentially methylated region or *UBE3A-AS*, we set out to definitively determine if *Dube3a* is, in fact, preferentially expressed from the maternal allele in fly brain. Using behavioral analyses,

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electroretinograms (ERGs) and allele-specific coding single nucleotide polymorphism (SNP) gene expression studies, we found no evidence for *Dube3a* imprinting in flies. Both maternal and paternal *Dube3a* alleles are co-expressed in fly brain.

Results

Genomic regions surrounding fly and human UBE3A *are not syntenic*

The UCSC genome browser was used to examine genomic features surrounding fly *Dube3a* and human *UBE3A* (Fig. 1). In flies, the 2 genes flanking *Dube3a* are *Plod* and *CG7600*, which have human homologs located at 7q22.1 and 8q24.13, respectively. No antisense transcript has been reported to interfere with *Dube3a* expression at this locus (Fig. 1A). In contrast, the human *SNHG14* (*UBE3A-AS*) transcript clearly overlaps with the *UBE3A* transcript (Fig. 1B). Based on these data, it appears unlikely that fly *Dube3a* is preferentially expressed from the maternal allele as the surrounding genomic region is not syntenic to the human *UBE3A* region. There are no antisense transcripts detected across the *Dube3a* locus.

Climbing behavior is not dependent upon maternal Dube3a *expression*

Previous work demonstrated climbing deficiencies in homozygous $Dube3a^{15b}$ (*Dube3a* null) flies, as significantly fewer $Dube3a^{15b}$ flies climbed 3 cm within 3 s.[12] Our experiments showed a significant effect of the $Dube3a^{15b}$ allele on climbing ability ($P = 0.0021$). Tukey's multiple comparisons test indicated that $Dube3a^{15b}$ homozygous flies took significantly longer to climb to a height of 4 cm compared to control w^{1118} flies, confirming previous reports of climbing defects in homozygous $Dube3a^{15b}$ flies. Flies that inherited this $Dube3a^{15b}$ allele through either the paternal or maternal germline displayed no differences in climbing behavior from controls (Fig. 2). These data indicate that a single copy of functional *Dube3a* of either paternal or maternal origin is sufficient for normal climbing behavior, and provide evidence that *Dube3a* is neither imprinted in flies nor preferentially expressed from the maternal allele.

Synaptic transmission defects in $Dube3a^{15b}$ *mutants are present independent of parent of origin*

We previously demonstrated impaired synaptic transmission in $Dube3a^{15b}$ flies at the neuromuscular junction.[14] Here we investigated neuronal activity and synaptic transmission in photoreceptor neurons of the eye using ERGs. In the ERG signal, the "on" and "off" transients are indirect measures of synaptic transmission, while the photoreceptor potential is a measure of photoreceptor neuron depolarization.[15] Control w^{1118} flies displayed robust on/off transients and large photoreceptor potentials (Fig. 3, top trace). The $Dube3a^{15b}$ mutation significantly affected both on transients ($P \leq 0.0001$) and off transients ($P \leq 0.0001$). Tukey's post hoc multiple comparisons testing indicated that the

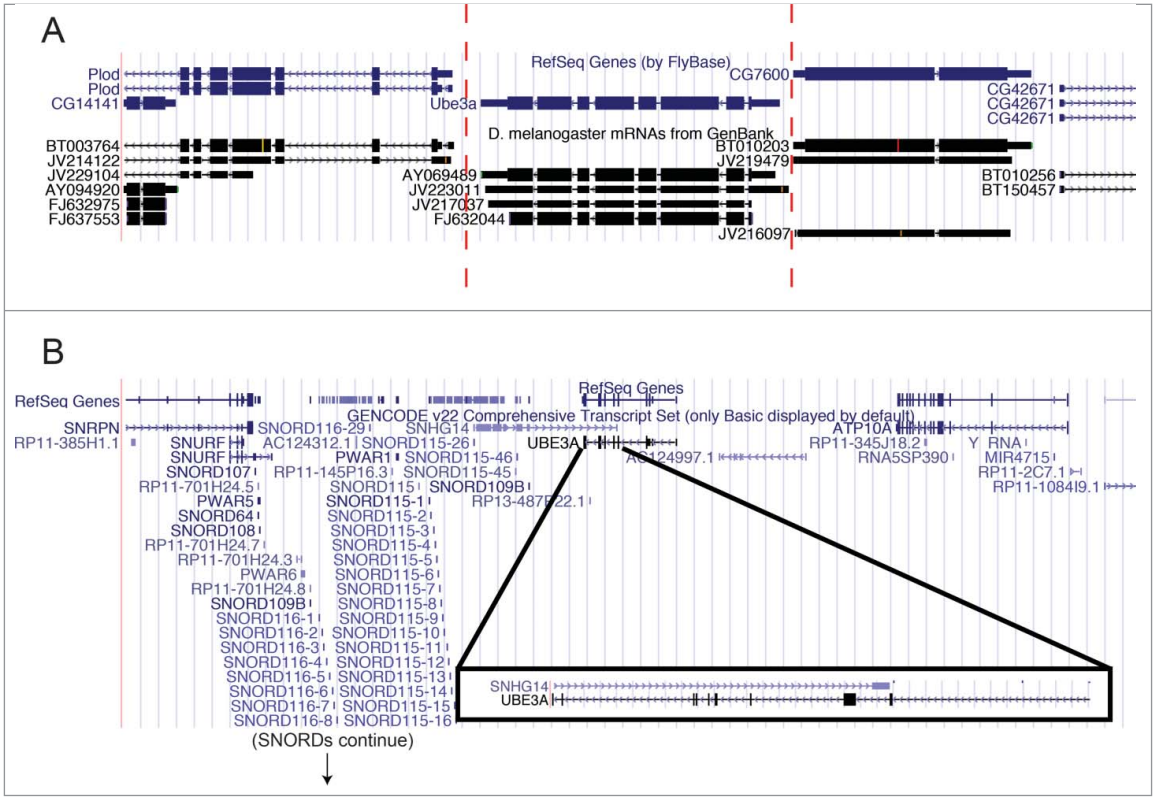

Figure 1. The genomic region surrounding *UBE3A* is not syntenic between flies and humans. Images from the UCSC genome browser (http://genome.ucsc.edu/) for the 10 kb region surrounding *Dube3a* in flies (A) and 1 Mb surrounding *UBE3A* in humans (B; note: the *SNORD* cluster has been truncated). In flies, there is no cluster of *snoRNAs* or the presence of *SNURF/SNRPN* upstream or downstream of *Dube3a*. There is also no RNA transcript that overlaps with *Dube3a* (A; red bars). In humans, note the presence of *SNHG14* (*UBE3A-AS*) that overlaps with *UBE3A* transcript (B, boxed region).

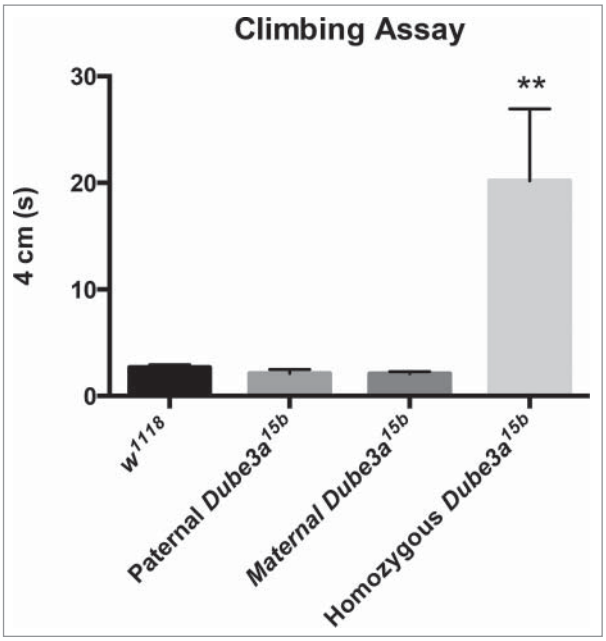

Figure 2. Only *Dube3a*^{15b} homozygous LOF flies display climbing deficits. At 5 d of age, flies were tested for motor function. Homozygous *Dube3a*^{15b} flies had significantly impaired climbing ability compared to control *w*^{1118} flies. Paternally or maternally inherited *Dube3a*^{15b} heterozygotes did not differ from controls in climbing ability ($P = 0.0021$). Data are presented as mean with standard error, n = 6 for all groups.

presence of the *Dube3a*^{15b} allele (either homozygous or heterozygous) reduced both on and off transients regardless of the parent of origin of the mutant allele as compared to control flies. No significant differences were observed in on/off transients among *Dube3a*^{15b} allele carrying flies (heterozygous or homozygous). We observed no effect of this mutation on photoreceptor potentials (Fig. 3; $P = 0.057$). Thus, the *Dube3a*^{15b} allele appears to effect synaptic transmission components of the ERG signal without interfering with photoreceptor neuron depolarization. Haploinsufficiency for Dube3a is also sufficient to decrease synaptic transmission regardless of the parental origin of the mutation, thus indicating that *Dube3a* is not preferentially expressed from either allele in the brain.

Allele-specific expression of Dube3a

To investigate *Dube3a* allele specific expression at the molecular level, we performed reciprocal crosses with flies from the DGRP containing coding SNPs in *Dube3a*. Using the UCSC genome browser of the DGRP2 data[16] (Located at: dgrp2.gnets.ncsu.edu), we selected coding SNPs located in *Dube3a*. Three separate DGRP lines were used; RAL-239 (G117T), RAL-313 (G78T), and RAL-517 (C171A), which each contain coding SNPs in *Dube3a* as compared to the reference line RAL-21. Female RAL-239 crossed to male RAL-21 and female RAL-21 crossed to male RAL-239 showed 2 similarly sized (~50%) peaks in the chromatogram at position 117 in the *Dube3a* cDNA sequence (Fig. 4A). Similar results were observed between reciprocal crosses of RAL-313 crossed to RAL-21 at position 78 (Fig. 4B), and RAL-517 and RAL-21 at position 171 (Fig. 4C). These data align with our previous experiments and support the conclusion that *Dube3a* is biallelically expressed in the fly brain, and neither imprinted nor preferentially expressed from the maternal allele.

Discussion

Our behavioral and molecular analyses indicate that neural expression of *Dube3a* alleles is independent of parental origin. Using the same *Dube3a*^{15b} allele as Wu et al., we confirmed climbing deficiency in *Dube3a* homozygous LOF flies.[12] Here we demonstrated that paternally- or maternally-deficient *Dube3a*^{15b} flies do not display climbing difficulties, indicating that one functional copy of *Dube3a* is sufficient for normal motor function independent of parent of origin. Our lab previously demonstrated synaptic transmission deficits at the neuromuscular junction in *Dube3a*^{15b} homozygous flies[14], and we confirmed synaptic transmission deficits here using ERGs. In our previous work at the 3rd instar neuromuscular junction, we reported that excitatory junction potentials decreased in amplitude faster in *Dube3a* LOF flies in comparison with controls,[14] possibly due to a depletion of synaptic vesicles in the readily releasable pool.[17] Here we show reductions in on/off transients in the ERG signal, which may also be due to a depletion of synaptic vesicles. Regardless, on/off transients were reduced in *Dube3a* homozygous and reciprocal heterozygous *Dube3a*^{15b} flies, supporting the conclusion that *Dube3a* is neither imprinted nor preferentially expressed and that *Dube3a* haploinsufficiency is sufficient to cause synaptic transmission defects. The strongest piece of evidence indicating that *Dube3a* is not imprinted in flies comes from sequencing several different wild-type alleles of *Dube3a*. Reciprocal crosses for 3 separate wild-type *Dube3a* alleles indicated similar, if not identical, expression of both maternally- and paternally-inherited alleles.

In contrast to Chakraborty et al.[13], we found no evidence that *Dube3a* is imprinted or preferentially expressed from the maternal allele in *Drosophila melanogaster*. On the other hand, our data is compatible with the previously reported learning deficits in *Dube3a* maternally-deficient flies since we found that *Dube3a* haploinsufficiency results in ERG on/off transient deficiencies regardless of parental origin. As the *UBE3A* imprinting mechanism in mature mammalian neurons is complex and similar PWS/AS-ICR or antisense transcripts have not been detected in *Drosophila*, our behavioral, electrophysiological, and *Dube3a* allele-specific expression data suggest that *Dube3a* is neither imprinted nor preferentially expressed in fruit flies.

Materials and methods

Drosophila stocks

All flies were maintained at 25°C on a 12-hour light/dark cycle and raised on standard corn meal media (Bloomington Stock Center: http://tinyurl.com/zs5tpbf). *Dube3a*^{15b} is a null LOF allele that produces a truncated transcript that does not make functional Dube3a protein.[11] *w*^{1118} flies were used as controls. The following *Drosophila* Genetic Reference Panel (DGRP) lines[16] were also obtained from the Bloomington *Drosophila* Stock Center: RAL-21, RAL-239, RAL-313, and RAL-517 (stock numbers 28122, 28161, 25180, and 25197, respectively).

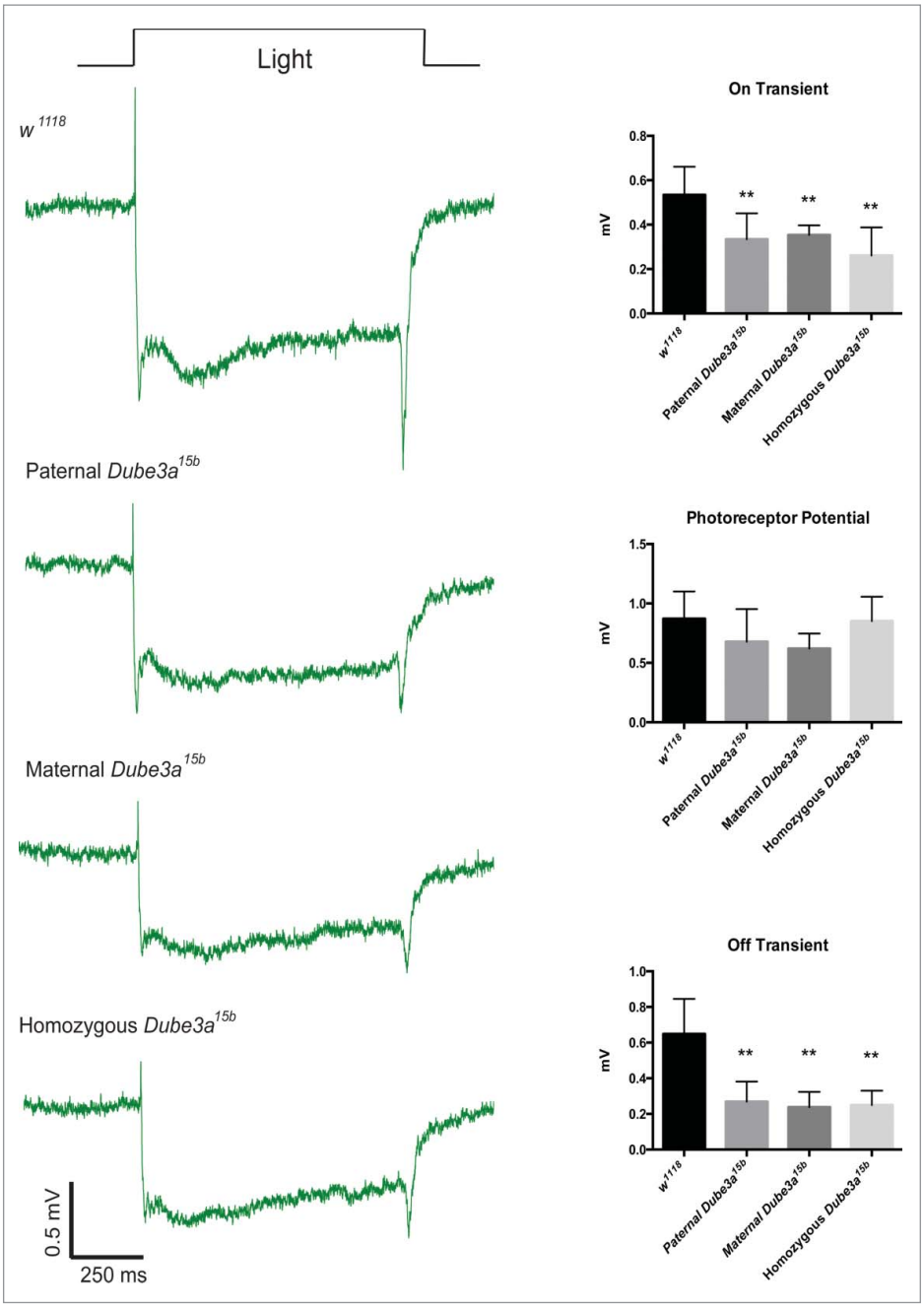

Figure 3. Heterozygous *Dube3a*^{15b} flies have reduced on/off transients in the ERG signal independent of parent of inheritance. Control w^{1118} flies (n = 12) display robust on/off transients that are significantly reduced in paternal (n = 6), maternal (n = 7), and homozygous *Dube3a*^{15b} (n = 11) flies (On Transient, $P < 0.0001$; Off transient, $P < 0.0001$). Note that the *Dube3a*^{15b} mutation does not impair photoreceptor potentials. Data are presented as means with standard error.

Genome comparisons

Genomic regions surrounding *Dube3a* in *Drosophila melanogaster* were compared to human *UBE3A* using the UCSC genome browser (http://genome.ucsc.edu/).[18] We used fly BDGP Release 6 + ISO1 MT/dm6 assembly and human GRCh38/hg38 assembly for comparisons.

Climbing assay

Climbing assays were performed as previously described.[19] In brief, 3 flies of each genotype at 5 d post eclosure were transferred to empty vials and allowed 1 min to acclimate. Flies were tapped to the bottom of the vial and the time was measured for the first fly to climb past a 4 cm vertical mark. Three trials were averaged per group of flies with 1 min intervals.

Electroretinogram (ERG)

ERGs were performed as previously described[20] with modifications. Flies were immobilized on ice for 2 min, transferred to a glass slide, and held in place with dental wax. Tungsten wire electrodes were gently inserted into the right eye (recording electrode) and abdomen (ground electrode). Data was recorded using a Model 1800 Microelectrode AC Amplifier (A-M Systems), digitized with a Micro3 1401 digitizer (Cambridge

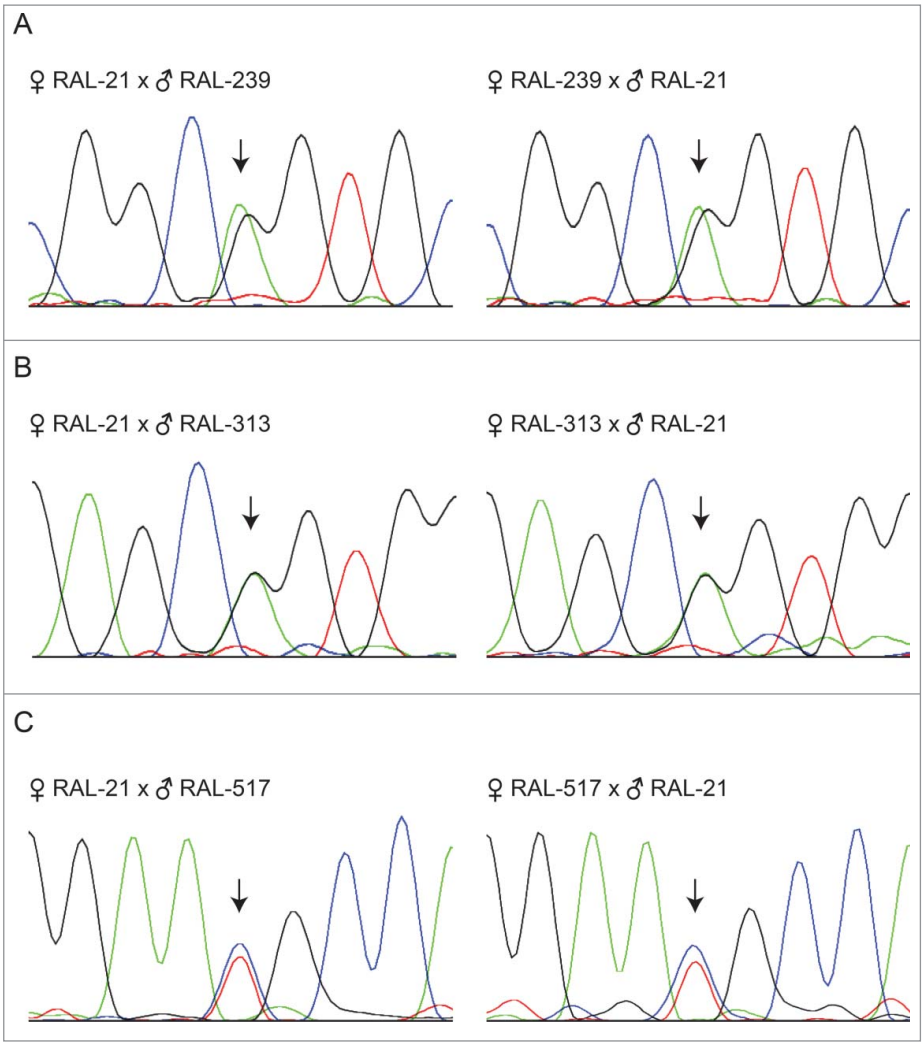

Figure 4. Sanger sequencing chromatograms of different wild-type *Dube3a* coding SNPs does not indicate imprinting or preferential expression. Total fly head RNA from reciprocal crosses with different coding SNPs present in *Dube3a* was DNase I treated and converted to cDNA. Sanger sequencing was performed on amplified cDNA using primers flanking the SNP to identify the expression of paternally or maternally inherited *Dube3a* alleles. In all alleles tested, we found similar expression levels of maternally and paternally inherited alleles as indicated by 2 peaks at each SNP of the same height (arrows).

Electronic Design), and analyzed with Spike2 software (Cambridge Electronic Design). Fly eyes were stimulated using 1-s light pulses from a 5 mm white LED (Radioshack).

Statistical analysis

Significance testing was performed by 1-way ANOVA analysis. All statistics were performed using Prism version 6.0 (GraphPad).

Allele-specific Dube3a *sequencing*

Using the DGRP and the UCSC Genome browser of DGRP2 data (dgrp2.gnets.ncsu.edu[16]), we selected 3 lines with single nucleotide polymorphisms (SNPs) in coding exons of *Dube3a*. RAL-21 was used as a reference line, and lines RAL-313, RAL-239, and RAL-517 harbored G78T, G117T, and C171A SNPs, respectively. We performed reciprocal crosses and extracted RNA from heads for cDNA synthesis. Totals of 20–30 fly heads from each cross were removed and homogenized in TRI Reagent Solution (Ambion). Total RNA was extracted using Directzol RNA MiniPrep Plus (Zymo Research Corp) according to the manufacturer's instructions, which include a DNAse step to remove genomic DNA. RNA was converted to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). A 950 bp fragment of *Dube3a* encompassing our chosen SNPs was PCR amplified with Phusion polymerase (Thermofisher Scientific) using the following forward and reverse primers, respectively: 5′-ATGAACGGTGGCGGGG-3′, and 5′-CGCTGCTTTGGGA TGAACAC-3′. PCR products were sent for Sanger sequencing (GENEWIZ) using the following sequencing primer: 5′-AAT-GAAGCTCTTGCCCAGTC-3′. Sequence data was analyzed using CodonCode Aligner (CodonCode Corporation).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported in part by a pre-doctoral fellowship to KAH from the Duplication 15q Alliance and the UTHSC Neuroscience Institute. MSL was supported by the Dorothy/Daniel Gerwin Parkinson Research Fund,

and National Institutes of Health grants R01 NS069936 and R21 GM118962. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

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