Identification of Mob2, a Novel Regulator of Larval Neuromuscular Junction Morphology, in Natural Populations of *Drosophila melanogaster*

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ABSTRACT Although evolutionary changes must take place in neural connectivity and synaptic architecture as nervous systems become more complex, we lack understanding of the general principles and specific mechanisms by which these changes occur. Previously, we found that morphology of the larval neuromuscular junction (NMJ) varies extensively among different species of *Drosophila* but is relatively conserved within a species. To identify specific genes as candidates that might underlie phenotypic differences in NMJ morphology among *Drosophila* species, we performed a genetic analysis on one of two phenotypic variants we found among 20 natural isolates of *Drosophila melanogaster*. We discovered genetic polymorphisms for both positive and negative regulators of NMJ growth segregating within the variant line. Focusing on one subline, that displayed NMJ overgrowth, we mapped the phenotype to *Mob2* [Monopolar spindle (Mps) one binding protein 2], a gene encoding a Nuclear Dbf2 (Dumbbell formation 2)-Related (NDR) kinase activator. We confirmed this identification by transformation rescue experiments and showed that presynaptic expression of *Mob2* is necessary and sufficient to regulate NMJ growth. *Mob2* interacts in a dominant, dose-dependent manner with *tricorned* but not with *warts*, to cause NMJ overgrowth, suggesting that *Mob2* specifically functions in combination with the former NDR kinase to regulate NMJ development. These results demonstrate the feasibility and utility of identifying genetic variants affecting NMJ morphology in natural populations of *Drosophila*. These variants can lead to discovery of new genes and molecular mechanisms that regulate NMJ development while also providing new information that can advance our understanding of mechanisms that underlie nervous system evolution.

THE behavior of higher organisms depends on the wiring of individual neurons into precise circuits within which information in the form of electrical signals is received, processed, and transmitted. Synapses, the specialized points of contact between neighboring cells, are the basic functional units in the nervous system where much of the information processing and integration occurs. Thus, proper neural function and behavioral output not only require establishment of synapses between the correct partners but also that these synapses be of the right size, complexity, and architecture (Houser *et al.* 2012; Ebert and Greenberg 2013). The mechanisms that regulate synaptic growth and development are

under genetic control (e.g., Collins and Diantonio 2007; West and Greenberg 2011; Krueger *et al.* 2012). Mutations that disrupt these mechanisms can have profound consequences, which in humans include conditions such as mental retardation, autism, and epilepsy (Houser *et al.* 2012; Levenga and Willemsen 2012; Peça and Feng 2012; Ebert and Greenberg 2013).

Nonetheless, synapses cannot be completely invariant on an evolutionary scale. As new species evolve, adaptive changes in behavior occur. Moreover, in more advanced organisms, nervous systems increase in complexity with addition of more neurons, creation of new neural circuits, and the rewiring of existing circuits (Ryan and Grant 2009). Although these morphological changes must also be under genetic control and are of fundamental importance to the evolution of neural systems and behavior, we lack detailed understanding of the genetic mechanisms and regulatory pathways through which these changes occur.

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We have focused on the *Drosophila* larval neuromuscular junction (NMJ) as a powerful model for studying the genetic and molecular mechanisms of synaptic growth and development. The muscles are large, arranged in an invariant, segmentally repeating pattern, and each muscle is innervated by the same identified motor neurons that form NMJs with stereotypic morphology in each animal. The NMJs are easily accessible for microscopic and electrophysiological analyses, and their morphological features, such as the number of synaptic boutons and branch points, can be readily observed and quantified. Extensive studies by many investigators have identified a number of signal transduction pathways that regulate growth and development of the larval NMJ (Collins and Diantonio 2007).

In a recent study (Campbell and Ganetzky 2012), we took advantage of the precise conservation of the overall larval body plan, musculature, and motor innervation pattern among all species of *Drosophila*, despite enormous differences in their natural history and behavior. We examined synaptic morphology of the same identified NMJ in >20 different species spanning a phylogeny of at least 40 Myr of evolutionary history (Russo *et al.* 1995). Unexpectedly, we discovered extensive variation in NMJ morphology and architecture among different species of *Drosophila*, even between closely related sibling species within the *melanogaster* clade (Campbell and Ganetzky 2012). These results raise important evolutionary questions about the functional significance of this variation in NMJ morphology and its genetic basis. To answer these questions fully, it will ultimately be necessary to determine what genetic differences underlie the variation in NMJ morphology among species, whether these differences originated before or after speciation occurred, and whether they are of any adaptive value or primarily influenced by genetic drift. Concerted effort from many investigators will likely be necessary to obtain this information.

At least much of the difficulty in trying to understand the genetic underpinnings of the variation in NMJ morphology between species is due to the fact that we have almost no knowledge about the genetics of NMJ morphology in natural populations within a species: How much genetic variation for NMJ morphology exists within a species? Which particular genes are responsible? Are these genes the same or different from those identified via mutational analysis in the laboratory as regulators of NMJ morphology? Is it even possible to identify an individual gene in nature that has strong effects on NMJ morphology, or does the phenotype depend on so many genes of small effect that it is impossible to sort them out? To begin to address these questions, we describe here a screen for variants in NMJ morphology in natural populations of *Drosophila melanogaster*. In contrast with the extensive phenotypic diversity we observed between different species of *Drosophila*, NMJ morphology in different isolates of *D. melanogaster* is mostly conserved and similar to that of laboratory wild-type strains. Nonetheless, among 20 isofemale lines examined, we did observe NMJ

overgrowth phenotypes in 2 of them. Through further genetic analysis of 1 of these variant isofemale lines, we found that it harbored at least seven different loci affecting both positive and negative regulation of NMJ growth. On the basis of its NMJ phenotype, we precisely mapped one of these loci and demonstrated that it was a hypomorphic mutation in *Mob2* (*monopolar spindle one binding protein 2*).

Mob was originally identified in yeast as a regulator of Nuclear Dbf2 (dumbbell formation)-related (NDR) kinases involved in mitosis and cytokinesis (Luca and Winey 1998). Subsequently, these proteins were found to be conserved from yeast to man and to function in signaling pathways affecting apoptosis, mitosis, cell proliferation, and cellular morphogenesis (Hergovich 2011). In *Drosophila*, Mob2 has previously been shown to regulate photoreceptor and wing hair morphogenesis (He *et al.* 2005; Liu *et al.* 2009) but has not been implicated in regulating NMJ growth. In flies and mammals, Mob proteins and NDR kinases have been found to play a role in regulating growth and development of neuronal dendrites (Zallen *et al.* 2000; Baillat *et al.* 2001; Emoto *et al.* 2004, 2006; Lin *et al.* 2011). We show that Mob2 functions presynaptically to negatively regulate NMJ growth in concert with the NDR kinase, Tricornered (Trc), but not with the closely related NDR kinase, Warts (Wts).

Our results demonstrate that individual genes with strong phenotypic effects on NMJ morphology can be identified in natural populations of *D. melanogaster* and suggest that natural populations may be a rich source for additional such variants. Not only can these variants uncover important new pathways regulating NMJ growth and development but they also provide candidate genes whose potential contribution to the phenotypic differences in NMJ morphology between species can be further examined.

Materials and Methods

Fly stocks

Natural isolates of *D. melanogaster* from Madison, Wisconsin were collected by Helen Hartman and Josh Gnerer. African isolates were obtained from Sean Carroll (University of Wisconsin, Madison, WI) and Chung-I Wu (University of Chicago). *UAS-Mob2* was provided by Paul Adler (University of Virginia, Charlottesville, VA). *UAS-Mob2-RNAi* was obtained from the Vienna *Drosophila* RNAi Center. All other stocks were obtained from the Bloomington Stock Center.

Imaging

All larvae were obtained from low-density crosses incubated at 25°. Wandering third-instar larvae were dissected in ice-cold Ca²⁺-free saline and fixed for 15 min in 4% formaldehyde in PBS. Larvae were incubated in FITC-conjugated anti-HRP (Jackson ImmunoResearch) at 1:100 overnight at 4°. NMJs were imaged using a Zeiss 510 confocal microscope. Brightness and contrast were adjusted using Photoshop (Adobe).

Quantification of boutons and branch points was performed at NMJ4 in segments A2–A4 as described in Coyle *et al.* (2004). Branch points were determined for each bouton with branch points defined as the number of projections from that bouton minus one (excluding terminal boutons). Branch points for each bouton were then added to determine the total number of branch points per NMJ. At least 25 NMJs were quantified for each genotype.

Quantitative real-time PCR

Approximately 15 larval brains were dissected from wandering third-instar larvae of each genotype. RNA was isolated using the RNeasy Plus Mini kit (Qiagen). cDNA was generated with an iScript cDNA kit (Bio-Rad). Real-time qPCR was carried out as described by Katzenberger *et al.* (2006). Three independent biological isolations were each analyzed three times. All primer sets produced a single product of expected size. Primer sets (oriented 5' to 3') for qPCR were as follows: total *Mob2*, GTGGATCTTCCAGCTGGTTT and GTCTTCTTGCCCTTCTCGTC; RpL17, CCAATCTACGTGTG CACTTCA and ACTCCTTCTGGTCGATGACG.

Electrophysiology

Electrophysiology was performed on muscle 4 (segments 3 and 4) of wandering third-instar larvae. Standard HL-3 saline with either 1.8 mM or 0.4 mM external calcium was used for recording. Preparations were visualized on an inverted Nikon DIAPHOT200 using Hoffman optics. Microelectrodes with access resistances of ~25 M Ω were filled with 3 M KCl and nerve stimulation electrodes were filled with bath saline. Average muscle input resistances were 14.4 M Ω . Evoked excitatory junction potentials (EJPs) were recorded in current clamp using the Axoclamp 2B amplifier (Molecular Devices). Mean EJP amplitudes were determined from 100 consecutive evoked EJPs at 2-Hz stimulation. Traces were analyzed using AxonClamp software. mEJP amplitude and frequency were analyzed using Mini Analysis software 5.6.4 (Synaptosoft, Deatur, GA).

Statistical analyses

Statistical analyses were performed via Student's *t*-tests for pairwise comparisons. Significance levels of <0.05, <0.01, and <0.001 are indicated by one, two, and three asterisks, respectively. Error bars denote standard error of the mean for bouton quantifications and electrophysiological measurements and standard deviation for the quantitative PCR.

Results

NMJ morphology is conserved in natural populations of *D. melanogaster*

What is the “wild-type” phenotype for larval NMJ morphology? Some laboratory stocks have been maintained for >100 years, during which time they have been selected for those animals that thrive under laboratory conditions

(non-escapers, etc.). These laboratory strains live under optimal conditions where food is provided, temperature and humidity are controlled, mates are available, and predators are absent. In contrast, natural populations of *D. melanogaster* inhabit a wide variety of climates and habitats (e.g., temperate climates, deserts, tropical rainforests) that present numerous ecological and behavioral challenges absent in the laboratory. Successful adaptation to meet these challenges and survive in nature likely entails genetic changes to synaptic structure and function that modify behavior. Therefore, it is reasonable to ask whether the standard NMJ morphology observed in laboratory wild-type stocks is an accurate depiction of the phenotype of wild-type flies in nature. We have previously discovered that there is extensive variation in NMJ morphology between different species of *Drosophila* (Campbell and Ganetzky 2012). Here we address the question of whether variation in the NMJ morphology occurs within natural populations of one species—in particular, *D. melanogaster*.

We examined larval NMJ morphology in three laboratory wild-type stocks (Canton-S, Oregon-R, and Urbana) as well as isolates from 20 natural populations (12 inbred lines from diverse locations and eight isofemale lines from Madison, WI). To describe the phenotypes quantitatively, we counted the number of synaptic boutons (an estimate of NMJ size) and branch points (an estimate of NMJ complexity), focusing on the NMJ on muscle 4 (NMJ4) because of its relative simplicity. Despite the differences in environments from which these lines were obtained, NMJ morphology is relatively uniform among these lines (Figure 1, A–F). The three laboratory wild-type stocks show no significant differences in NMJ morphology either for bouton number or branch points. Furthermore, we found no significant variation among the inbred natural isolates. The average number of boutons per NMJ4 across these lines was 20.5 with a standard error of 0.7 between groups, which is comparable to error observed within a single population. The average number of branch points per NMJ4 across populations was 2.6 ± 0.5 . This uniformity in phenotype was largely true for the eight isofemale lines from Madison, WI, with two exceptions. These two lines, *H4* and *H5* (Figure 1, G and H), exhibit a mild overgrowth phenotype with increased branching. *H4* has an average of 25.9 ± 1.0 boutons and 6.9 ± 0.7 branch points per NMJ4. *H5* has an average of 23.8 ± 0.8 boutons and 5.7 ± 0.4 branch points per NMJ4. Although we focus here on NMJ4, we observed similar differences in phenotype for other NMJs such as NMJ6/7 and NMJ12/13 (not shown).

Thus, overall it appears that in contrast with the extensive variation in NMJ morphology that exists among different species of *Drosophila* (Campbell and Ganetzky 2012), there is little variation in NMJ morphology among different populations of *D. melanogaster*. Nonetheless, the recovery of two isofemale lines from the Madison population with distinct NMJ morphologies indicates that at least some genetic variation for this phenotype is present in natural

Figure 1 Larval NMJ morphology is conserved in natural populations of *D. melanogaster*. Confocal images of NMJ4 labeled with FITC-anti-HRP. (A and B) Laboratory wild-type strains and inbred lines derived from natural populations in Africa (C and D) all share similar phenotypes. Six of eight isofemale lines derived from wild populations in Madison, Wisconsin also exhibit the standard NMJ phenotype as represented by the two examples (E and F). However, two of these isofemale lines exhibit variant NMJ morphology (G and H). Inset numbers represent average bouton number $\pm$ SEM. Bar, 20 μ m.

populations of *D. melanogaster*. The existence of these variants raises questions about which genes and pathways are responsible and whether they could identify novel mechanisms that regulate NMJ growth and morphology.

Both positive and negative regulators of NMJ morphology are segregating in the *H4* isofemale line

We focused on *H4*, a single isofemale variant line collected from Madison, WI, (Figure 1G) to identify individual genes responsible for the variation in NMJ morphology. Because this line, derived from a single mated female, was not isogenic, we observed phenotypic variation among her descendants over the next several generations during which the line was maintained by mass transfer. This observation suggested that multiple different genes affecting NMJ morphology were segregating within this line. To reduce this complexity to identify individual genes, we isolated single chromosomes from the *H4* line by crossing them with laboratory balancer stocks and bred them to homozygosity (Supporting Information, Figure S1). From these crosses, we generated a number of individual sublines homozygous for a single *H4* chromosome in an otherwise laboratory wild-type background. Most of the 45 semi-isogenic sublines exhibited normal NMJ morphology. However, 7 of these lines had distinctive NMJ phenotypes (Figure 2). Six of

the sublines (*H4-1*–*H4-6*) displayed an overgrowth phenotype characterized by an increase in total bouton number as well as an increase in morphological complexity as measured by the number of branch points (Figure 2, A–F). These sublines were generally even more robust than the original *H4* line (Figure 2, A–F). The remaining subline (*H4-7*) had a strong NMJ undergrowth phenotype with fewer total boutons compared with controls (Figure 2G). Complementation analysis indicated that different genes caused the similar phenotypes of the 6 sublines with NMJ overgrowth. These results, together with the isolation of a subline exhibiting NMJ undergrowth, indicate that multiple variants in both positive and negative regulators of NMJ growth were originally present in the *H4* female isolated from nature.

Identification of *Mob2* as a negative regulator of NMJ growth

We focused on subline *H4-5* (Figure 2E), homozygous for a third chromosome from nature, for subsequent analysis, to identify a specific gene(s) responsible for the observed NMJ phenotype. The overgrowth phenotype in this line was of interest because it differs in several ways from other NMJ mutants that have been described. The main axonal stalk of the NMJ is broader than normal and discrete individual boutons along that axis are not as apparent as in lab wild-type strains where they appear as beads on a string. Boutons along the main trunk in *H4-5* are larger than normal and appear almost to merge with neighboring boutons. In addition, numerous small satellite boutons branch off the main axis and hyperbudded boutons appear along the length of the NMJ and hyperbudding of terminal boutons is especially prominent. These features suggested that *H4-5* harbored a genetic variant that differed from any previously described NMJ mutant.

To determine whether the *H4-5* phenotype was caused by mutation of a single gene and, if so, to map the location of this gene, we carried out deficiency mapping of the third chromosome using the *Drosophila* deficiency collection. Although *H4-5/+* heterozygotes have a mild semidominant phenotype with increased branch points (Figure 3), this phenotype was readily distinguishable from *H4-5* homozygotes and *H4-5/Df* hemizygotes so failure of complementation could be scored (Figure 3). After testing deficiencies spanning the third chromosome, we were able to map the *H4-5* phenotype to a single small region on 3L, at cytological location 68C13. Any deletion that removed this chromosome segment uncovered the mutant phenotype when heterozygous with *H4-5* but produced NMJs of normal appearance in *Df/+* larvae. The phenotype of *H4-5/Df* is even stronger than that of *H4-5* homozygotes, suggesting that *H4-5* is a hypomorphic allele (Figure 3, A–G).

Of the 13 candidate genes within the chromosome region that uncovers *H4-5*, *Mob2* (*Monopolar spindle one binding protein*), which encodes a kinase adaptor protein, is the largest (Figure 4A). An insertion allele of *Mob2* (*Mob2^{DG30103}*), containing a *P*-element in the first intron (Figure 4C), conferred

Figure 2 Segregation of positive and negative regulators of NMJ growth in sublines derived from Madison *H4* isofemale line. Sublines from the *H4* isofemale line were derived by outcrossing single *H4* flies to laboratory balancer strains to recover individual chromosomes that were bred to homozygosity in each subline. Confocal images of NMJ4 labeled with FITC-anti-HRP from these sublines (A–G). Six of these sublines (A–F) display an NMJ overgrowth phenotype indicating loss of a negative regulator, whereas one line has an NMJ undergrowth phenotype (G), indicating loss of a positive regulator. Inset numbers are the average number of boutons per NMJ $\pm$ SEM. Bar, 20 μ m.

a mild overgrowth phenotype with increased boutons and branching when homozygous and failed to complement *H4-5* (Figure 3, E–F), suggesting that *H4-5* might contain a mutation of *Mob2*. To test this possibility further, we generated precise and imprecise excisions of the *P*-element insertion in *Mob2^{DG30103}* (Figure 4, G–L). As expected, the imprecise excision, *Mob2^{Δ18}*, still exhibits an NMJ overgrowth phenotype and fails to complement *H4-5* (Figure 3, I–J). However, the precise excision, *Mob2^{Δ17}*, is reverted to a standard NMJ morphology and it fully complements *H4-5* (Figure 3, G–H). These results support the conclusion that *H4-5* is an allele of *Mob2*. This conclusion is fully confirmed by results presented in the following sections and we will subsequently refer to the *H4-5* mutation as *Mob2^{H4-5}*.

We subsequently sequenced the entire *Mob2* coding region in *Mob2^{H4-5}* but did not find any lesions, suggesting that the relevant polymorphism resides within a noncoding region and affects expression of the gene. *Mob2* is a large and complex gene that contains >30 kb of intronic sequence and encodes four distinct mRNAs, each with its own transcriptional start site, (Figure 4) making the identification of a noncoding lesion extremely difficult. To help narrow the search, we first identified regions of conserved sequence

within introns by sequence alignment with other *Drosophila* species (*D. simulans*, *D. sechellia*, *D. yakuba*, and *D. erecta*). We sequenced the regions with at least 40% identity across species in *H4-5*. Although we detected a number of single base pair polymorphisms within these domains, we found that one of them contains a more extensive sequence polymorphism. An intron segment, just upstream of the start site for the A isoform mRNA, contains a 49-bp deletion in *Mob2^{H4-5}* that is absent in laboratory wild-type strains, and in another third chromosome line, derived from *H4* with normal NMJ morphology (Figure 4C). Several additional lines of evidence make this 49-bp deletion a promising candidate responsible for mutant phenotype. First, a transposon insertion within this region is associated with a mild NMJ overgrowth phenotype and fails to complement *Mob2^{H4-5}* (Figure 3, E–H). Curiously, this region also appears to be a hotspot for transposon insertions with >30 of the 60 annotated insertions in the gene tightly clustered in a region of several hundred nucleotides. Second, a small imprecise excision of a *P*-element in this region (*Mob2^{Δ18}*) overlaps with the *Mob2^{H4-5}*-specific deletion and shares a similar NMJ overgrowth phenotype (Figure 3I). Third, one of the deletions that fails to complement *Mob2^{H4-5}* (*Df BSC577*) has an endpoint within this intron a short distance beyond the *Mob2^{H4-5}* lesion and extends to the right, leaving most of the *Mob2* gene intact (Figure 4, A and C). Fourth, as described below, we found that transcript levels are reduced in *Mob2^{H4-5}* (Figure 4D), consistent with the idea that the 49-bp deletion in *Mob2^{H4-5}* alters expression of the gene.

To test whether expression of *Mob2* mRNA is impaired in *Mob2^{H4-5}*, we performed quantitative real-time PCR on cDNA generated from larval brains. Primers were designed for the conserved portion of the gene to target all isoforms of *Mob2*. Compared with either laboratory wild-type (CS) or a different third chromosome isolate from *H4* (*H4-3*), *Mob2* mRNA levels are significantly decreased in larval brains in *Mob2^{H4-5}* homozygotes compared with the expression level of ribosomal protein L17 as an internal control (Figure 4D). Taken together with the sequence data and complementation analysis, these results support the proposal that the NMJ overgrowth phenotype in *H4-5* is due to a mutation in the *Mob2* gene that deletes 49 bp within the first intron and causes a reduction in expression of this gene. Nonetheless, this conclusion awaits complete confirmation because we cannot rule out the possibility that some other lesion elsewhere in the gene that we have not detected is the causative mutation.

***Mob2* functions presynaptically to regulate NMJ growth**

To obtain definitive evidence that *H4-5* is an allele of *Mob2*, we carried out transgenic rescue experiments using the full-length A isoform cDNA of *Mob2*. Presynaptic expression of *UAS-Mob2* using the panneuronal driver *C155-Gal4* fully rescues the *Mob2^{H4-5}* mutant phenotype both for the number of boutons and the number of branch points (Figure 5,

Figure 3 Genetic variant affecting NMJ growth in *H4-5* subline is an allele of *Mob2*. (A–F) Confocal images of NMJ4 labeled with FITC–anti-HRP. (A and D) *H4-5* homozygotes, *H4-5/Df(3L)BSC577*, and *H4-5/Mob2^{DG}* larvae exhibit similar NMJ overgrowth phenotypes compared with CS (*Canton-S*) (A) or *H4-5/+* (C) controls. (F) *P*-element insertion allele (*Mob2^{DG}*) in *Mob2* confers a mild overgrowth phenotype as a homozygote and fails to complement *Mob2^{H4-5}* (E). (G) Precise excision (*Mob2^{Δ17}*) of *Mob2^{DG}* reverts back to a standard NMJ phenotype that complements *H4-5* (*Mob2^{Δ17}/H4-5*) (H). Imprecise excision of the same element (*Mob2^{Δ18}*) (I) still exhibits an overgrowth phenotype similar to that of *H4-5* and fails to complement (J). (G and H and K and L) Quantification of the number of boutons (G and K) and branch points (H and L) at NMJ4 in larvae of the genotypes shown. Error bars denote SEM. $^{*}P < 0.01$; $^{***}P < 0.001$. Bars, 20 μ m.

C–E). In addition, neural-specific expression of RNAi directed against *Mob2* using *C155-Gal4* results in NMJ overgrowth similar to that of *Mob2^{H4-5}*. In contrast, we observe no NMJ phenotype with muscle-specific expression of *Mob2* RNAi using *24B-Gal4* (Figure 5, A and B). These results provide conclusive confirmation that *H4-5* is an allele of *Mob2* and further indicate that *Mob2* functions presynaptically to regulate NMJ development.

Mob2 does not alter synaptic function

Does *Mob2^{H4-5}* affect synaptic function as well as NMJ morphology? To address this question, we performed intracellular recordings from muscle 4 to assay various parameters of synaptic function including both spontaneous and evoked transmitter release. Spontaneous fusion of single synaptic vesicles with the presynaptic terminal elicits quantal

depolarization events in the muscle called miniature EJPs (mEJPs). The amplitude of mEJPs is a measure of the amount of neurotransmitter packaged into each vesicle. The frequency of spontaneous fusion events provides an indication of the baseline properties of the synaptic release machinery. Electrical stimulation of the motor nerve generates an action potential that triggers the coordinated and simultaneous release of many synaptic vesicles that produce an EJP in the muscle, whose amplitude is a measure of the total number of synaptic vesicles released in response to an action potential. We measured each of these parameters in CS and *Mob2^{H4-5}* larvae (Figure 6). We performed the recordings in a high Ca^{2+} (1.8 mM) Ringer's solution to measure physiological excitation as well as in low Ca^{2+} (0.4 mM) Ringer's solution to avoid saturating muscle depolarization, thereby enabling better resolution of any subtle

Figure 4 *H4-5* is a polymorphism in *Mob2* that affects expression level. (A) Representation of the genomic region containing *Mob2*, adapted from FlyBase. Each gene is represented by a thick gray bar below the genomic scale. *Mob2* is the thick black bar. Below the genes are three deficiencies used to map *H4-5*. Both *Df(3L)BSC675* and *Df(3L)BSC577* (black) failed to complement the *H4-5* NMJ phenotype, while *Df(3L)ED4475* (gray) complemented *H4-5*. The dashed black lines define the genomic region containing *H4-5*. (B) The genomic segment spanned by the *Mob2* gene is shown as the solid thick bar below the genomic scale. Below this bar, the four different mRNAs encoded by *Mob2* are shown. Each of these alternatively spliced mRNAs initiates transcription from a different start site. Thick gray bars represent untranslated exon sequence; thick black bars correspond to translated regions. Thin black lines are introns. (C) Enlarged close-up view of the boxed region in B. This region contains the 49-bp deletion present in *H4-5*, the left endpoint of a deficiency coming in from the right that fails to complement *H4-5*, the site of *P*-element insertion in *Mob2^{DG}*, and the breakpoints of *Mob2^{A18}*, an imprecise excision derived from *Mob2^{DG}*. (D) Quantitative real-time qPCR was used to measure levels of *Mob2* mRNA in samples isolated from larval brains of *H4-5*, *Canton-S* (CS), and *H4-3*, another introgressed subline derived from *H4* with an NMJ overgrowth phenotype. The level of *Mob2* mRNA is significantly reduced compared with either of the controls. Error bars are standard deviation.

differences in neurotransmitter release. However, we did not observe significant differences in any of the electrophysiological parameters we measured (Figure 6) despite the overgrown morphology at *Mob2^{H4-5}* NMJs.

trc interacts with *Mob2* to regulate NMJ growth

Thus far we have demonstrated that a naturally occurring genetic variant affecting NMJ growth is defective in *Drosophila Mob2*. The overgrowth phenotype exhibited by hypomorphic alleles of *Mob2* indicates that it is a negative regulator of NMJ growth. But what is the cellular pathway by which *Mob2* exerts its effect on NMJ growth? In *Saccharomyces cerevisiae* where *Mob* proteins were originally characterized, there are two *Mob* proteins encoded by different genes. Both *Mob1* and *Mob2* activate Nuclear Dbf2 (dumb-bell formation)-related (NDR) kinases to regulate polarized cell growth, bud site selection, and cell morphology (Luca and Winey 1998). The two *Mob* proteins in yeast regulate two different NDR kinases: Dbf2 is activated by *Mob1* as part of the mitotic exit network (MEN) (Luca and Winey 1998), whereas Cbk1 is activated by *Mob2* in the RAM (regulation of Ace2 activity and cellular morphogenesis) pathway (Weiss *et al.* 2002; Nelson *et al.* 2003). It is unclear which of the two yeast *Mob* proteins is the counterpart of *Drosophila Mob2* because the *Drosophila* protein shares the same degree of identity with either yeast protein. Moreover,

it has been shown that *Drosophila Mob2* can physically interact with both *Trc* (Tricornered) and *Wts* (Warts) the two NDR kinases in *Drosophila* (He *et al.* 2005). Thus, *Mob2* could normally regulate NMJ growth via activation of *Trc*, *Wts*, or both.

To investigate these possibilities further we examined the NMJ phenotype of loss-of-function alleles of both *trc* (*trc¹*) and *wts* (*wts³⁻¹⁷*). Both of these mutations are recessive lethals, so we were not able to examine the NMJ phenotype in either mutant homozygote. However, we did observe a strong dominant genetic interaction between *trc* and *Mob2*. *trc¹/Mob2^{H4-5}* larvae exhibit an NMJ overgrowth phenotype comparable to that of *Mob2^{H4-5}* homozygotes (Figure 7, A–D). We found the same dominant interaction in heterozygotes between *Mob2^{H4-5}* and a deficiency that uncovers *trc*. In contrast, NMJs in *wts³⁻¹⁷/Mob2^{H4-5}* larvae appear phenotypically normal (Figure 7, E and F). Thus, *Mob2* appears to regulate NMJ morphology via an interaction with *Trc* and not the related kinase, *Wts*.

Discussion

The genetic variation harbored in natural populations has long been a valuable source for discovery of new mutations affecting essential biological mechanisms, particularly before the use of chemical mutagens in *Drosophila* became

Figure 5 *Mob2* acts presynaptically to regulate NMJ morphology. (A–C) Confocal images of NMJ4 labeled with FITC–anti-HRP. (A) RNAi knockdown of *Mob2* with the neuron-specific driver *C155-Gal4* causes an NMJ overgrowth phenotype similar to that of *Mob2^{H4-5}*. (B) This phenotype is not seen with RNAi knockdown of *Mob2* in muscles with the driver *24B-Gal4*. (C) Neural expression of *UAS-Mob2* by *C155-Gal4* in a *Mob2^{H4-5}* background produces significant rescue of the NMJ overgrowth phenotype. Quantification of the number of boutons (D) and branch points (E) at NMJ4 in larvae of the genotypes shown. Error bars denote SEM. ** $P < 0.01$; *** $P < 0.001$; NS, not significant ($P > 0.05$).

widespread (e.g., Sandler *et al.* 1968). Naturally occurring variants affecting behavioral traits are of particular interest because of their potential contribution to the behavioral differences that have emerged in the evolution of closely related species and, in some cases, may have even contributed to speciation (Yamamoto and Ishikawa 2013). For example, latitudinal variation in the length of a Thr-Gly repeat in the *Per* protein in *D. melanogaster* is inferred to reflect an important role in thermal adaptation (Sawyer *et al.* 1997). Similarly, naturally occurring variants in the *foraging* (*for*) gene, which encodes a cGMP-dependent protein kinase has been linked with differences in thermotolerance of neural function and behavior in *D. melanogaster* (Dawson-Scully *et al.* 2007). Although synapses are the basic units of neural communication and ultimately responsible for generating all behavior in higher organisms, there have been no systematic studies of genetic variation in synaptic morphology and architecture in natural populations in any organism. Here, we have used the larval NMJ of *D. melanogaster* as a model synapse to begin to investigate the genetics of synaptic morphology in natural populations. We have found that although NMJ morphology is largely uniform throughout *D. melanogaster* populations in many parts of the world, genetic variants affecting NMJ growth and architecture can be identified in these populations, especially when homozygous, isogenic lines are established. Thus, significant hidden genetic variation for NMJ morphology exists in natural populations of *D. melanogaster*. We pursued the analysis of one of the variants and identified *Mob2* as a novel regulator of NMJ morphology. These results demonstrate the feasibility and utility of identifying variants affecting NMJ morphology in natural populations of *Drosophila*. Clearly, much additional work is needed to determine which variants may confer any adaptive advantage or are of potential evolutionary significance. In particular, we have not addressed these issues for *Mob2*. As one of many variants that we found

segregating in natural populations there is no evidence to suggest that *Mob2* itself is of special evolutionary relevance. Nonetheless, further identification and analysis of naturally occurring variants can lead to discovery of new genes and molecular mechanisms that regulate NMJ development while ultimately also providing new information about the genetic mechanisms that contribute to nervous system evolution.

We previously discovered that NMJ morphology varies enormously among different species of *Drosophila*. Furthermore, even when comparing sibling species that have diverged for <0.5 million years (for example within the *melanogaster* clade) there were significant differences in NMJ morphology, indicating that this phenotype can evolve very rapidly by comparison with other morphological traits (Campbell and Ganetzky 2012). In contrast, among the various populations we have examined for a single species, *D. melanogaster*, we find a more uniform morphology. These results raised broad questions about how NMJ morphology in *Drosophila* has evolved. Although extensive further studies will be necessary to resolve these questions, our analysis here has provided some potentially relevant information. In general, we uncovered more phenotypic variation as we bred individual chromosomes to homozygosity. This observation suggests, not surprisingly, that most of the genetic variants segregating in natural populations of *melanogaster* are heterozygous and likely rare in the population as a whole. Utilizing the variation in NMJ morphology that we did find, we further scrutinized one of those lines by isolating single chromosomes to generate homozygotes from a genetically diverse and likely heterozygous, natural population. Moreover, by examining individual sublines derived from a single wild-caught female, we found that the phenotypic variants we initially identified harbored multiple different genetic polymorphisms affecting NMJ growth and morphology some of which even exerted opposite effects on the NMJ phenotype.

Figure 6 Morphological change in *Mob2^{H4-5}* does not correlate with synaptic function. Intracellular recordings of evoked excitatory junction potentials (EJP) and spontaneous miniature EJPs (mEJP) amplitude and frequency and are from muscle 4 in 0.4 mM and 1.8 mM external calcium. (A) Representative mEJP recordings, quantified in B and C. (D) Representative EJPs evoked by electrical stimulation of the appropriate segmental nerve bundle. (E) Average EJP amplitude, a measure of the number of vesicles released in response to an evoked action potential, is quantified for CS and *Mob2^{H4-5}*. Error bars indicate SEM (minimum $n = 12$).

We observed comparable results when we performed a similar genetic analysis beginning with a natural isolate exhibiting “normal” NMJ morphology (not shown). By deriving individual sublines from this isolate, each of which was homozygous for a particular autosome, we were able to find multiple genetic polymorphisms affecting NMJ growth segregating in the genome of the original isolate. The normal appearance of the NMJ in the original isolate thus was not because it lacked genetic variants that altered the phenotype, but rather because it harbored multiple variants that either were recessive and initially heterozygous or acted in concert to confer a normal phenotype. It is thus possible that stabilizing selection acts to maintain NMJ growth within optimal parameters for *D. melanogaster*. Because many of the genes that regulate NMJ morphology in *Drosophila* are known to have pleiotropic effects, it is possible that a polymorphism in such a gene could be advantageous for reasons having nothing to do with NMJ morphology but nonetheless perturb NMJ development so that it falls outside the optimal range. A secondary modifier that restores the NMJ phenotype back to the normal range without compromising the advantageous effects of the initial polymorphism would thus have some selective advantage. In this manner, multiple polymorphisms in both positive and negative regulators of NMJ growth could accumulate within a population and be segregating within the genome of an individual fly. Despite the multiple polymorphisms, the overall NMJ phenotype would appear normal until the various genetic factors were segregated into different sublines.

Further investigation is needed to determine if this model is supported by additional data.

In addition to understanding the evolutionary forces that are acting to shape NMJ morphology, another important question is which specific genes and molecular pathways underlie these evolutionary changes? Although it is of particular interest to know which genes are responsible for the extensive variation in NMJ phenotype we observe among different species of *Drosophila*, performing interspecific crosses between these species is still sufficiently challenging to make this approach difficult. Instead, to begin addressing this question, we focused on the occasional variants in NMJ morphology that could be found in natural populations of *D. melanogaster*. By identifying novel regulators of NMJ morphology in natural populations of *D. melanogaster*, we expand our knowledge of genes that could potentially play a role in determining the phenotypic differences in NMJ morphology between different species. Moreover, by focusing on *D. melanogaster*, we can take advantage of the full range of genetic and molecular tools available in this organism.

The studies reported here provide a demonstration of the effectiveness and usefulness of this approach. Beginning with a variant in NMJ morphology found among the offspring of a mated female obtained from nature, we were able to identify a single gene, *Mob2*, that plays a major role in determining this phenotype. Furthermore, because *Mob2* has not previously been implicated in regulating development of presynaptic terminals, identification of this

Figure 7 Mob2 genetically interacts with *trc* but not with *wts* to regulate NMJ growth. (A–F) Confocal images of NMJ4 labeled with FITC–anti-HRP. *trc¹/Mob2^{H4-5}* double heterozygotes exhibit significantly enhanced NMJ overgrowth compared with either single heterozygote alone, whereas NMJs of *wts³⁻¹⁷/Mob2^{H4-5}* double heterozygotes do not differ from single heterozygote controls. Quantification of the number of boutons (G) and branch points (H) at NMJ4 in larvae of the genotypes is shown. Error bars denote SEM. *** $P < 0.001$; NS, not significant ($P > 0.05$).

naturally occurring variant has also revealed an important new regulatory pathway that merits further analysis.

Mob1 and Mob2 were originally identified in yeast as NDR kinase adaptor proteins that regulate aspects of mitosis and budding (Luca and Winey 1998). Yeast Mob1 is a member of the MEN, a pathway that initiates cytokinesis during the cell cycle (Luca and Winey 1998). Yeast Mob2 is a member of the RAM signaling network, which is involved in regulating cell polarity and daughter cell-specific transcription (Weiss *et al.* 2002; Nelson *et al.* 2003). Beyond yeast, the Mob family of genes has expanded, which presumably enables members of this family to subserve additional specialized functions in more complex organisms. For example, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio* each have four different Mob genes while in mammals, including humans, there are seven (Li *et al.* 2006; Mrkobrada *et al.* 2006).

Although the role of Mob proteins in mitosis is evolutionarily conserved (Lai *et al.* 2005; He *et al.* 2005; Shimizu *et al.* 2008; Trammell *et al.* 2008), Mob proteins have also been found to regulate various other developmental processes in postmitotic cells. For example, Mob2 in *Drosophila* regulates morphogenesis of photoreceptors and wing hairs (He *et al.* 2005; Liu *et al.* 2009) and Mob4 (pheocin), a divergent member of this gene family negatively regulates NMJ growth apparently through its effects on axonal transport and microtubule dynamics (Schulte *et al.* 2010). In addition, mammalian Mob proteins have been localized to neuronal dendrites where NDR kinases regulate branching and tiling (Zallen *et al.* 2000; Baillat *et al.* 2001). Our discovery of Mob2 as a regulatory protein that acts presynaptically to restrict NMJ growth further emphasizes the importance of a more complete investigation of the signaling pathways through which Mob2 and its relatives act to

regulate growth and morphogenesis of presynaptic and postsynaptic structures.

Despite the fact that Mob2 is known to function in multiple different developmental pathways, we do not observe any phenotypes in other potentially affected structures such as compound eyes or wing hairs in *Mob2^{H4-5}*, the naturally occurring variant or the *Mob2^{Δ18}* excision allele. This result is consistent with the polymorphism we identified in *Mob2^{H4-5}*, which appears to be a regulatory mutation that affects expression levels of Mob2 in larval brains, suggesting that expression might be differentially altered in different tissues minimizing pleiotropic effects. The presence of a regulatory mutation in *Mob2* is also in accord with a growing body of evidence that regulatory mutations rather than coding sequence polymorphisms contribute to most important evolutionary changes in nature (Carroll 2005; Stern and Orgogozo 2009). In this instance, however, we do not have any evidence that Mob2 is involved in any of the evolutionary changes in NMJ morphology that we have observed. Rather, *Mob2^{H4-5}* appears to be a potential genetic variant on which evolutionary mechanisms could act to modify NMJ morphology in certain ways. It will be of interest to examine Mob2 as a potential candidate gene that contributes to the variation in NMJ morphology observed in other species of *Drosophila*, particularly in species with NMJs exhibiting hyperbudded boutons.

A key functional aspect of Mob proteins is their interaction with NDR/Dbf2 kinases. Two Dbf2 kinases are found in *Drosophila*: Tricornered (Trc) and Warts (Wts). In regulating morphogenesis of the compound eye, Mob2 has been found to interact physically with both Trc and Wts and to exhibit genetic interactions with both Dbf2 kinases as well (He *et al.* 2005). In contrast, for NMJ development, we found that *Mob2* interacts only with *trc* but not with *wts*,

suggesting that Mob2 can mediate distinct developmental pathways via interactions with different Dbf2 partners. Identification of other components of the Trc-Mob2 signaling pathway involved in regulating NMJ growth will be an important future goal to unravel the mechanism by which this pathway mediates synaptic development.

In summary, through the analysis of naturally occurring variants affecting NMJ morphology in wild populations of *Drosophila melanogaster*, we have uncovered an important new pathway regulating NMJ growth. We anticipate that natural populations will be a rich source of other such variants and that further investigations similar to those described here will continue to be extremely valuable.

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Literature Cited

- Baillat, G., A. Moqrich, F. Castets, A. Baude, Y. Bailly *et al.*, 2001 Molecular cloning and characterization of phoecein, a protein found from the Golgi complex to dendritic spines. *Mol. Biol. Cell* 12: 663–673.
- Campbell, M., and B. Ganetzky, 2012 Extensive morphological divergence and rapid evolution of the larval neuromuscular junction in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 109: E645–E655.
- Carroll, S. B., 2005 Evolution at two levels: on genes and form. *PLoS Biol.* 3: e245.
- Collins, C. A., and A. DiAntonio, 2007 Synaptic development: insights from *Drosophila*. *Curr. Opin. Neurobiol.* 17: 35–42.
- Coyle, I. P., Y. H. Koh, W. C. Lee, J. Slind, T. Fergestad *et al.*, 2004 Nervous Wreck, an SH3 adaptor protein that interacts with Wsp, regulates synaptic growth in *Drosophila*. *Neuron* 41: 521–534.
- Dawson-Scully, K., G. A. Armstrong, C. Kent, R. M. Robertson, and M. B. Sokolowski, 2007 Natural variation in the thermotolerance of neural function and behavior due to a cGMP-dependent protein kinase. *PLoS ONE* 2: e773.
- Ebert, D. H., and M. E. Greenberg, 2013 Activity-dependent neuronal signaling and autism spectrum disorders. *Nature* 493: 327–337.
- Emoto, K., Y. He, B. Ye, W. B. Grueber, P. N. Adler *et al.*, 2004 Control of dendritic branching and tiling by the Tricornered-kinase/Fury signaling pathway in *Drosophila* sensory neurons. *Cell* 119: 245–256.
- Emoto, K., J. Z. Parrish, L. Y. Jan, and Y. N. Jan, 2006 The tumor suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. *Nature* 443: 210–213.
- He, Y., K. Emoto, C. Fang, N. Ren, X. Tian *et al.*, 2005 *Drosophila* Mob family proteins interact with the related Tricornered (Trc) and Warts (Wts) kinases. *Mol. Biol. Cell* 16: 4139–4152.
- Hergovich, A., 2011 MOB control: reviewing a conserved family of kinase regulators. *Cell. Signal.* 23: 1433–1440.
- Houser, C. R., N. Zhang, Z. Peng, C. S. Huang, and Y. Cetina, 2012 Neuroanatomical clues to altered neuronal activity in epilepsy: from ultrastructure to signaling pathways of dentate granule cells. *Epilepsia* 53(Suppl 1): 67–77.
- Katzenberger, R., M. A. Marengo, and D. A. Wassarman, 2006 ATM and ATR pathways signal alternative splicing of *Drosophila* TAF1 Pre-mRNA in response to DNA damage. *Mol. Cell. Biol.* 26: 9256–9267.
- Krueger, D. D., L. P. Tuffy, T. Papadopoulos, and N. Brose, 2012 The role of neurexins and neuroligins in the formation, maturation, and function of vertebrate synapses. *Curr. Opin. Neurobiol.* 22: 412–422.
- Lai, Z. C., X. Wei, T. Shimizu, E. Ramos, M. Rohrbaugh *et al.*, 2005 Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* 120: 675–685.
- Levenga, J., and R. Willemsen, 2012 Perturbation of dendritic protrusions in intellectual disability. *Prog. Brain Res.* 197: 153–168.
- Li, H., A. Coghlan, J. Ruan, L. J. Coin, J. K. Heriche *et al.*, 2006 TreeFam: a curated database of phylogenetic trees of animal gene families. *Nucleic Acid Res.* 34: D572–D580.
- Lin, C. H., M. Hsieh, and S. S. Fan, 2011 The promotion of neurite formation in Neuro2A cells by mouse Mob2 protein. *FEBS Lett.* 585: 523–530.
- Liu, L.-Y., C.-H. Lin, and S.-S. Fan, 2009 Function of *Drosophila* mob2 in photoreceptor morphogenesis. *Cell Tissue Res.* 338: 377–389.
- Luca, F. C., and M. Winey, 1998 MOB1, an essential yeast gene required for completion of mitosis and maintenance of ploidy. *Mol. Biol. Cell* 9: 29–46.
- Mrkobrada S., L. Boucher, D. F. Ceccarelli, M. Tyers, and F. Sicheri, 2006 Structural and functional analysis of *Saccharomyces cerevisiae* Mob1. *J. Mol. Biol.* 362: 430–440.
- Nelson, B., C. Kurischko, J. Horecka, M. Mody, P. Nair *et al.*, 2003 RAM: a conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. *Mol. Biol. Cell* 14: 3782–3803.
- Peça, J., and G. Feng, 2012 Cellular and synaptic network defects in autism. *Curr. Opin. Neurobiol.* 22: 866–872.
- Russo, C. A. M., N. Takezaki, and M. Nei, 1995 Molecular Phylogeny and Divergence Times of *Drosophilid* Species. *Mol. Biol. Evol.* 12: 391–404.
- Ryan, T. J., and S. G. N. Grant, 2009 The origin and evolution of synapses. *Nat. Rev. Neurosci.* 10: 701–712.
- Sandler, L., D. L. Lindsley, B. Nicoletti, and G. Trippa, 1968 Mutants affecting meiosis in natural populations of *Drosophila melanogaster*. *Genetics* 60: 525–558.
- Sawyer, L. A., J. Hennessy, A. A. Peixoto, E. Rosato, H. Parkinson *et al.*, 1997 Natural variation in a *Drosophila* clock gene and temperature compensation. *Science* 278: 2117–2120.
- Schulte, J., K. J. Sepp, R. A. Jorquera, C. Wu, Y. Song *et al.*, 2010 DMob4/Phoecein regulates synapse formation, axonal transport, and microtubule organization. *J. Neurosci.* 30: 5189–5203.
- Shimizu, T., L. L. Ho, and Z. C. Lai, 2008 The mob as tumor suppressor gene is essential for early development and regulates tissue growth in *Drosophila*. *Genetics* 178: 957–965.
- Stern, D. L., and V. Orgogozo, 2009 Is genetic evolution predictable? *Science* 323: 746–751.
- Trammell, M. A., N. M. Mahoney, D. A. Agard, and R. D. Vale, 2008 Mob4 plays a role in spindle focusing in *Drosophila* S2 cells. *J. Cell Sci.* 121: 1284–1292.

- Weiss, E. L., C. Kurischko, C. Zhang, K. Shokat, D. G. Drubin *et al.*, 2002 The *Saccharomyces cerevisiae* Mob2p-Cbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter cell-specific localization of Ace2 transcription factor. *J. Cell Biol.* 158: 885–900.
- West, A. E., and M. E. Greenberg, 2011 Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb. Perspect. Biol.* 3: 1–21.
- Yamamoto, D., and Y. Ishikawa, 2013 Genetic and neural bases for species-specific behavior in *Drosophila* species. *J. Neurogenet.* 27: 130–142.
- Zallen, J. A., E. L. Peckol, D. M. Tobin, and C. I. Bargmann, 2000 Neuronal cell shape and neurite initiation are regulated by the Ndr kinase SAX-1, a member of the Orb6/COT-1/warts serine/threonine kinase family. *Mol. Biol. Cell* 11: 3177–3190.

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Supporting Information

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Identification of Mob2, a Novel Regulator of Larval Neuromuscular Junction Morphology, in Natural Populations of *Drosophila melanogaster*

Megan Campbell and Barry Ganetzky

Creating semi-isogenic lines by isolating natural X and 3rd chromosomes:

Isogenizing line with isolated natural 3rd chromosome:

Figure S1 Crossing scheme used to isolate single chromosomes from natural populations. The original isofemale line, *H4*, was crossed to a third chromosome balancer line carrying an attached-X chromosome. F1 males were crossed back to the original balancer stock, whose selected progeny were used to generate a stock with a single natural third and first chromosome and a variable second chromosome. To specifically isolate only the natural third chromosome, attached-X females from the generated stock were crossed to a line with second and third chromosome balancers. F1 males were then crossed to a third chromosome balancer stock and progeny were crossed to generate a line with a single isolated third chromosome in a lab wild-type background.