# Supplementary Figures and Legends for Wu et al., G3 2020.

AAACCAAATATTACGTATACGAAAAATTGCGTATACGCCAGCATAATAGTTCACCCGAAATTCGCAGATCCAGTAATTCAAAGTTAGCGAATCGAAA GGAGAGCATAAGCGAAACAAAAAGCGCTACAAATGCCCCCAGAAGCGGGTCTAATCAGAGCCCGGGCGTCGGGTTCCAATCAGATCGCCAGCTGGAGA AAGTTAAATAATCATTAGTGAAAGGTCCATCTGCAGCTTCACAATCCGGCAAGCGGTCTAGTAATCAGCAGCCAGAAGGAGCAGCGCATCGCATCGT AACGCATCCTTAGTTTCGGTGGGCCAGGCCGCCAGAAAGCCGTCAAAATACGAAAGAAGGTGACCTCGAAGAAGGCCAGCGTTGTGTCGGTTGACGA ATAGACACGCCCACGCCCACGCCCGTTGACCCAGAGCAGGAGTGCCCACAGCCCAGGAGCAACCCGATCCACAGCCAGAACCAGACACCCATCGAGA TGGACGACACACAGCACTTCTGCCTGCGCTGGAACAACTACCAGAGCAGCATAACCTCGGCGTTCGAGAATCTGCGCGACGACGAGGCCTTCGTGGA TGTGACCCTCGCCTGCGAGGGACGCAGCATCAAGGCACACCGCGTCGTCCTCCCGCCTGCAGTCCCTACTTCCGCGAGCTGCTCAAGAGCACACCC TGCAAACACCCGGTCATACTGCTGCAGGATGTCAACTTCATGGACCTGCACGCCCTGGTGGAGTTCATCTACCACGGCGAGGTCAACGTGCACCAGA TCCCCGACCCTCTTCAGCCGCCAGGGAGCGGGTTCGCCACCGCCGACGGCGGTGCCATCGCTGCCCTCGCACATCAACAACCAGCTGCTGAAGCGCA TGGCCATGATGCACCGCAGCAGCAGCGCCGCCGCCGCCGAGGAGAACCTCCCCACGCCTTTAAGCGTCTGCGCGGCTCGGACAACAGCCTGCCGCTGTC  ${\tt CGGCGCCGTTGGCAGTGGCAGCAACAACAACAACAACCAGCCCCGACTTGCCGCCGCTTCATGCGCGCAGCGCCCCCAGCAGACTCCGGCCGACTTCAGT$ ACGATCAAGCACCACAACAACAACAACACGCCGCCGCCGCTGAAGGAGGAGAAGCGCAACGGAACCGCAACGGAAACTCTGGCAACGGCAATGGCA TCCAGTAGCGGCGACGGCGGCAAGGGATCCCTGAGCTCCGGCAACGACGAGGAGATCGGCGACGGACTCGCCTCCCATCATGCCGCCCCCCAGTTCA TCATGTCGCCGGCGAGAACAAGATGTTCCATGCAGCCGCCTTCAACTTTCCCAATATCGATCCCTCAGCGTTGCTAGGTCTCAGCACACAGTTGCA GCAGTCCGGTGACCTCGCCGTGTCCCCTCAAGGACCGCACAGCATCACCCGCTCCGCAGCTACCTCGCCACCAGTTCTACCTCATCGCCACCCTCG  ${\tt CCGCCCACAGCCCTGATCTCGCCGACCAGTTCGCTGAAGGGGAGTCTGGCCGCCGCCGTCTACAGCCTACACGTCACGCCCACGGCCATGTCCTTG$ GGCACGCCACCTCGCCGCCGCCGCCGCCGCGCGCGCGCGGCAGCAGTGTGGGCAGCAGCAGCCACCTCCACGGGTGTGGGGTGTCAACTCTGG AGTCCTGGCAGCCAGGCGACGGCAGCAGGAGGAGCACCGTGACCCAAGCGGGCACGCCTCCGCTACCGCCTCCGCATGCCACCGCCCACCAGCGGCGGCGCA TAAACGAGCCGCAGGAGTGCCCCTACTGCCGGANGACCTTCTCGTGCTACTACTCGCTGAAGCGGCACTTCCAGGACAAGCACGAGCAGTCNGACAC GCTGTATGTGTGCGAGTTTTGCCACCGGCGGTATCGCACCAAGAACTCGCTGACCACGACAAGAGCCTGCAGCATCGCGGCTCTAGCGGCATGCTG AAGCGGCTGCTTAAGACGACCGCCATCAAGCACGGACTGGTGGGCCACGGACATGGGCACGGACATGTCCATCACCCGCATGCGCACCACGACCACGCCC TGTCCCATCCGCGCACTAGTCTGTATGACTTCACCAGCGAGCTGGGACAGCCGCCACCGGGCATCCAATAGGAGCTAAGTTGGAACGCCATCTTTTA CCATTGTTTTCGCACTGGACCGACGATCCCCCATCGCTCATCGCTCAACGCCCATCCGCCAGGAGGGGGGGATCTAGATCTCCAGCGTGAACTAGGTC  ${\tt TTAGCTAATTAATGATTAAGCATATTAGGCATATTTTGGCGTCAACTGTTTTATTACTCTTATCCCTATGTTAGGTAATTATTACGATTATTTTTTT$ GTGTATCCTTAACTCTATTCGTACTATTAATTATTTGTGCTGCTCTCACCTACTTAGTAGTTGAAGCACCTTCGCTGCTTCATGTGCAATACCTCAA TAAAACACACAAATACAAACAGCGTGAAGTTGCGTTTAAAGAATACTTATTAGATTTAGACTTATGTTACCATTAATGACAACAAAAATGGGAAGAG TGACACGAGGGCGGGAGCGGTTGGAGCAATAACGGTACATATCATGCAACTGCAATTGCAAATGAATCTGAATCTGCAGCCAAAACTTAATTTAAAA  ${\tt CTAAAGAGAAACTAAAGCTGCATCCGAAATAGCAACCGAATCTTAAGTAGAATNTAAGCCACCTGTCGGGCGCTACCGGTACTCAGCCGCCGCCCCCC$ GTACACTCAACGGTTTTTTTTTTTTTTAATAGTTTTTATGCGCATTAAACTTATTTTATTTGCCAACTGCTGCGCCTAACGTGCGTTCTCTAAAGAGTCGG TAAATGTAGCTCATATATATAAGACTAGAGTAAGCTTAATTCAAATCTAA**TGTTTAAAGTCGGCGGGGCACGGAGAAGACAAACG**TGCAGAAGCGAA ACACAAGAGACAAAACAAAATCAACCAAACAAAAGAAAAACGTTATCAGGGATAAAATATCAAGTATGCAGTAGGAAAGTAATTTTTTTCTACTTA TGCGTATGCGCAAGCTAAGCGAAACAACTAAACTAATGTCTATGTATACATGTATATACTTTACACCATTTACCATATACAATTTATAAGCCGATAT GCCGATGATATACACATATACAATGTTAGTTTGGTGTATAGAAGACAGATGAGCTGTAAACGTTTTTGCAAATCAAGAAAATAACGTATTTAACAAA TCGAAGTAGCTAGCGACGGGAGTATTCAGAGCAGAATCATAAAGGGAATAGGGCCGCAGTTAGCACTGCCCCAAAATAGTGTCTGATCATGTTGCAT ATCTGTGTTAAGAAAATGTTTAAGTCAAAAGTACGAAGAATGTGTATCAATTCAATGTCTCAGGAATTTCTTAAAAATTTAACCAATTTACACCTTAT 

Open reading frame 3 predicted let-7 binding sites 1 predicted miR-125 binding site Oligos used for RACE

## Figure S1. Sequence of broad-Z3 cDNA. Sequence of broad-Z3 cDNA identified by

RACE. Nested oligos used for RACE are indicated. The resulting sequence was

compiled with available *br-RA* sequence to show relative locations of predicted *miR-125* and *let-7* binding sites. This sequence has been deposited in GenBank (accession number MN990459).

### Α

#### Conservation of let-7 binding site sequences

D.melanogaster	1	AC-TATT-AATTATTTGT	CTGCTCTC	ACCTACTTAGTAGTTGAAGCAC-	CTTC GCTGC	TTC ATGTGCAAT.	CCTCAAGA	77
D.sechellia	1	AC-TATT-AGTTATTTGT	CTGCTCTC	-ACCTACTTAGTAGTTGAAGCAC-	CTTCGCTGC	TTC <mark>ATGTGCAAT.</mark>	CCTCAAGA	77
D.yakuba	1	AC-TATT-AGTTATTTGT	CTGCTCTC	ACCTACTTAGTAGTTGAAGCAC-	CTTCGCTGC	TTC ATGTGCAAT.	CCTCAAGA	77
D.erecta	1	AC-TATT-AGTTATTTGT	CTGCTCTC	ACCTACTTAGTAGTTGAAGCAC-	CTTCGCTGC	TTC ATGTGCAAT.	CCTCAAGA	77
D.eugracilis	1	AC-TATT-AGTTATTTGT	CTGCTCTC	ACCTACTTAGTAGTTGAAGCAT-	CTTCGCTGC	TTC <mark>ATGTGCAAT.</mark>	CCTCAAGA	77
D.ficusphila	1	AC-CATT-AGTTATTTGT	CTGCTCTC	ACCTACTTAGTAGTTGAAGCAC-	CTTCGTTGC	TTC ATGTGCAAT.	CCTCAAAA	77
D.pseudoobscura	1	AT-TAGT-AGTTCTTTGT	CTGCTCTC	AACCTACTTAGTAGTTGAAGCAAC	GACAGACATC GCTGC	TTC ATGTGCAAT.	CCTCAAGA	86
D.perimilis	1	AT-TAGT-AGTTCTT-TGT	CTGCTCTC	AAACCTACTTAGTAGTTGAAGCAAC	GACAGACATC GCTGC	TTC <mark>ATGTGCAAT.</mark>	CCTCAAGA	86
D.miranda	1	AT-TAGT-AGTTCTT-TGT	CTGCTCTC	AAACCTACTTAGTAGTTGAAGCAAC	GACAGACATC GCTGC	TTC <mark>ATGTGCAAT.</mark>	CCTCAAGA	86
D.virilis	1	AT-CATAAAATTAATTGTGT	CTGCTCTC	C-AACTACTTAGTAGTTGAAGCATC	GA-TGACATC GTTGC	TTC <mark>ATGTGCAAT.</mark>	CCTCAACA	84
D.grimshawi	1	ATCTAGA-AATTAGTTGTGT	CTGCTCTC	C-AACTACTTAGTAGTTGAAGCATC	GC-TGACATCGTTGC	TTC <mark>ATGTGCAAT.</mark>	ACTCAAGA	85
				•				
			Seed 1		Seed	12	Seed 3	

### В

Conservation of miR-125 binding site sequences

D.melanogaster	1	ACAAAAGAAAAACGTTA TCAG-GGAT	25
D.sechellia	1	ACAAAAGAAAAACGTTATCAG-GGAT	25
D.yakuba	1	ACAAAAGAAAAACGTTATCAG-GGAT	25
D.erecta	1	ACAAAAGAAAAACGTTATCAG-GGAT	25
D.eugracilis	1	ACAAAAGAAAAACGTTATCAG-GGAT	25
D.ficusphila	1	ACAAAAGTAAAACGTTATCAG-GGAT	25
D.pseudoobscura	1	ACAGAAGTAAAACGTTATCAG-GGAT	25
D.perimilis	1	ACAGAAGTAAAACGTTATCAG-GGAT	25
D.miranda	1	ACAGAAGTAAAACGTTATCAG-GGAT	25
D.virilis	1	CACCACGAATTAAAACGTTA TCAGGGGAT	29
D.mojavenis	1	AAAAC-AAAGTAAAACGTT7 TCAG-GGAT	27
		Seed	

# С

Predicted sites near Aedes aegypti Br-Z3 stop codon

position 203 target 5' A CG U 3' U ACAGCUUAUU UCUUA A UGUUGGAUGA GGAGU	position 1485 target 5' G UUAU UG G 3' ACA CCU GCCUCA UGU GGA UGGAGU	position 1895 target 5' C AA G A 3' CUG ACAA ACUGC UCA GAU UGUU UGAUG AGU
miRNA 3'UGUA U 5'	miRNA 3' UGAUA U UGA 5'	miRNA 3'U A GGA G 5'
miR-125		
position 639 target 5' U U AU U A 3' UCA CAAG AG UCUUA GGGA AGU GUUC UC AGAGU CCCU	position 1527 target 5' C UAU G A 3' UUAC GGUU GUUU CAGGGA AGUG UCAA CAGA GUCCCU	
miRNA 3' AA CC 5'	miRNA 3' U UCC 5'	

# D

Predicted sites near Anopheles gambiae Br-Z3 stop codon

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let-7

position 1877 uU u 3'

cANAN UGCANU GAUGCAG

miRNA 3' U G US'

miRNA 3' U G US'

position 1116

target 5' G AA GOGGUUU AGC G

GUUU UCCCAGA UCC C'

miRNA 3' AG UC CAAGUCCC

miRNA 3' AG UC C' 5'

miRNA 3' AG UC C' 5'
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## Figure S2. Conservation of let-7 and miR-125 binding sites. (A) Alignment of

*Drosophila melanogaster broad-Z3* 3'UTR sequence that contains three *let-7* binding sites with sequence from ten other *Drosophila* species. (**B**) Alignment of *Drosophila melanogaster broad-Z3* 3'UTR sequence that contains one *miR-125* binding site with sequence from ten other *Drosophila* species. (**C-D**) Predicted *let-7* and *miR-125* binding sites in sequence 3' to the stop codon of Aedes aegypti Br-Z3 (C) or Anopheles gambiae Br-Z3. Position is indicated relative to the first nucleotide after the stop codon.



**Figure S3.** *Verification of new rabbit polyclonal anti-Br-Z3 antibodies.* (A-F) Confocal z-stacks of an entire CNS from third instar wildtype (A,D), *npr6* (B, E), and *hs-Z3* (C, F) larvae stained with anti-Br-Z3 (green in A-C and white in D-F). These samples were also counterstained with neuropil marker anti-nc82 antibodies (magenta in A-C). The yellow box in D-F highlights the thoracic hemimeres of the ventral nerve cord, the area of the CNS containing the adult lineages investigated in this study. Anti-Z3 antibodies clearly detect adult lineage neurons in wildtype tissue (D). This staining is sharply reduced in the *npr6* mutant (E) and elevated in *hs-Z3* larvae relative to wildtype. Images shown in A-F were taken under identical settings and processed in parallel, allowing their direct comparison. (G-I) Higher resolution confocal stacks of just the dorsal thoracic hemimeres from third instar wildtype (G), *npr6* (H), and *hs-Z3* (I) larvae

stained with anti-Br-Z3 (white). Adult lineage neurons are clearly detected in G and I, but not H. While some individual cells are stained in H (yellow arrowheads), this background staining can be clearly distinguished from the signal in G given the larger size and cytoplasmic (as opposed to nuclear) staining of these cells relative to the adult lineage cells stained in G. The cause of this background staining is unclear. Images shown in G-I were taken under identical settings and processed in parallel. Scale bars: A-F, 100 μm; G-I, 50 μm