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ORIGINAL RESEARCH ARTICLE

The *Drosophila foraging* gene plays a vital role at the start of metamorphosis for subsequent adult emergence

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ABSTRACT

The *foraging* (*for*) gene has been extensively studied in many species for its functions in development, physiology, and behavior. It is common for genes that influence behavior and development to be essential genes, and *for* has been found to be an essential gene in both fruit flies and mammals, with *for* mutants dying before reaching the adult stage. However, the biological process underlying the lethality associated with this gene is not known. Here, we show that in *Drosophila melanogaster*, some but not all gene products of *for* are essential for survival. Specifically, we show that promoter 3 of *for*, but not promoters 1, 2, and 4 are required for survival past pupal stage. We use full and partial genetic deletions of *for*, and temperature-restricted knock-down of the gene to further investigate the stage of lethality. While deletion analysis shows that flies lacking *for* die at the end of pupal development, as pharate adults, temperature-restricted knock-down shows that *for* is only required at the start of pupal development, for normal adult emergence (AE) and viability. We further show that the inability of these mutants to emerge from their pupal cases is linked to deficiencies in emergence behaviors, caused by a possible energy deficiency, and finally, that the lethality of *for* mutants seems to be linked to protein isoform P3, transcribed from *for* promoter 3.

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Complementation analysis; ecdysis; fat body; FOR; lethality; pleiotropy

Introduction

The cGMP-dependent protein kinase (PKG) encoded by the *foraging* (*for*) gene in *Drosophila melanogaster* has important functions in physiology and behavior (Anreiter & Sokolowski, 2019; Osborne *et al.*, 1997). In this insect, *for* underlies naturally occurring behavioral differences in feeding behavior, social behavior, learning and memory, and stress response (Anreiter & Sokolowski, 2019). Additionally, *for* regulates physiological phenotypes, such as fat storage (Allen, Anreiter, Neville, & Sokolowski, 2017) and synaptic transmission at the neuromuscular junction (Dason, Allen, Vasquez, & Sokolowski, 2019; Dason & Sokolowski, 2021). Similar functions for *for* have been reported in other animals (Anreiter & Sokolowski, 2019). In humans for instance, genetic variation in the *for* homolog, *prkg1*, is associated with behavioral self-regulation (Hawn *et al.*, 2018; Polimanti *et al.*, 2018; Struk *et al.*, 2019).

It has previously been proposed that pleiotropic genes affecting metabolism and behavior are likely to be vital (Hall, 1994). This is indeed the case for the *for* gene, as fruit fly mutants lacking the gene do not survive to adulthood, dying at the late pupal stage (Allen *et al.*, 2017). It is not known why *for* null mutants are pupal lethal. The pleiotropic effects of *for* are thought to be associated with its complex gene structure and diversity of gene products. Indeed, *for* has twenty-one reported transcripts that originate from four promoters (pr1-4) and different splice combinations (Allen *et al.*, 2017). The four *for* promoters drive gene expression in different tissues, and at different developmental stages (Allen, Anreiter, Vesterberg, Douglas, & Sokolowski, 2018; Allen & Sokolowski, 2020). It is not known if each of these transcripts produces a functional polypeptide; however, western blot analysis has revealed a large variety of FOR protein isoforms (Allen *et al.*, 2017). These FOR isoforms can each be attributed to specific promoters, with current nomenclature defining the FOR isoform(s) that originate from pr1 and pr2 as P1, the FOR isoform(s) that originate from pr3 as P3, and the FOR isoform(s) that originate from pr4 as P2 and P4. P1 can originate from either pr1 or pr2, as these transcripts share the same coding sequence, differing only in 5′UTRs. All FOR isoforms (P1–P4) encode PKGs, with a shared C-terminal kinase domain. Although FOR isoforms are predicted to differ in their dimerization, substrate binding, and cGMP

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Supplemental data for this article can be accessed here.

activation domains, specific target proteins for each of the FOR isoforms are currently not known.

More is known about how *for*-associated phenotypes map to specific promoters (Anreiter & Sokolowski, 2018, 2019). Larval nociception and *for* pathlength, are regulated by pr1 (Allen *et al.*, 2018; Dason *et al.*, 2020); adult habituation is regulated by pr1/pr3 (Eddison, Belay, Sokolowski, & Heberlein, 2012); larval fat stores are regulated by pr3 (Allen *et al.*, 2018); and adult feeding behavior and larval food intake are regulated by pr4 (Allen *et al.*, 2018; Anreiter, Kramer, & Sokolowski, 2017). It has previously been noted that loss of the *for* gene leads to late pupal lethality (pharate adult stage) (Allen *et al.*, 2017), however, this lethal phenotype has not been mapped to a specific function, tissue, or *for* isoform.

In this article, we investigate the lethal phenotype associated with the complete lack of *for* function. We find that mutants deficient in *for* consistently die as pharate adults with no escapees. We investigate larval and pupal ecdysis and find that, from a timing perspective, *for* null mutants proceed through the hallmark stages of development, including molting, but then show fatal defects in emergence behaviors. We then use a timeline of temperature-dependent knock-down of *for* to show that expression of the gene is critical in early pupal development for the later expression of normal emergence behaviors. We also performed a Gal4 screen (Brand & Perrimon, 1993) to find candidate tissues underlying the *for* lethal phenotype. We crossed 74 tissue-specific Gal4 lines to a *for* RNAi line which targets the common-coding region of the gene (Dason *et al.*, 2020). We found that driving *for* RNAi with Gal4s of broad expression (ubiquitous) induces pharate adult lethality but driving *for* RNAi with Gal4 lines of more narrow expression (tissue-specific) does not. Finally, we show that expression of P1/P3 FOR isoforms is sufficient to rescue lethality and that this appears to be linked to expression of *for* in the fat body.

Materials and methods

Drosophila *stocks and genetics*

All flies, unless otherwise specified, were reared at 25 °C, 60% humidity, and a 12 h L:D light cycle with lights on at 0800 h. For lethality scoring, 20 first instar larvae (0–4 h post-hatch) were seeded into standard rearing vials and the number of pupae and eclosing flies was scored 10–11 d after seeding. The following lines were generated in our lab and have been previously described: $Df(2L)for^{0}$ and for^{BAC} (Allen *et al.*, 2017); $for^{RNAi\text{-}exon7:8}$ (Dason *et al.*, 2020). The following lines were obtained from the Bloomington *Drosophila* Stock Center: $Df(2L)Exel^{7018}$ (stock #7789), $Df(2L)\ for^{ED243}$ (stock #24122), $Df(2L)ed^{1}$ (stock #5330), $Df(2L)drm^{P2}$ (stock #6507), $tub\text{-}Gal80^{ts}$ (stock #7108), and *tub-Gal4* (stock #5138).

Generating partial for *deletion mutants*

The partial 5′-end deletions of the *for* gene $Df(2L)for^{f0e0}$, $Df(2L)for^{d0f0}$, and $Df(2L)for^{e0f0}$ were generated using the Flp/FRT system (as described in Parks *et al.*, 2004; Thibault *et al.*, 2004). Flies carrying transposable elements with FRT sequences were obtained from the Exelixis Collection at Harvard Medical School. The insertion lines used to generate deletions were: f04293 and e02991 for $Df(2L)for^{f0e0}$; d07690 and f00049 for $Df(2L)for^{d0f0}$ and e00955 and f00049 for $Df(2L)for^{e0f0}$. The partial 3′-end deletions of the *for* gene, $Df(2L)for^{08112\text{-}3}$ and $Df(2L)for^{32122\text{-}1}$, were generated using the *hobo* transposase deletion generator system (Huet *et al.*, 2002) and the $P\{wHy\}for^{DG2311}$ element that is inserted 40 bp 5 to *for* pr 4. All deletion breakpoints were verified using PCR and Sanger sequencing.

Cloning of the P1–P3 isoform-specific RNAi line

A *for* isoform-specific RNAi line targeting exons 4 and 5 ($for^{RNAi\text{-}exon4:5}$) was generated using the pWIZ RNAi cloning vector (Lee & Carthew, 2003). A 573 bp sequence was amplified with the forward primer (GCCTGGTGGATCCGAA TTTCA) targeting the 3′-end of exon 4 and reverse primer (CCATGACATAAACGATGCTTC) targeting the 3′-end of exon 5, and then cloned as previously described (Anreiter *et al.*, 2017). P element injections into w^{1118} were performed by BestGene Inc.

Recombineering $for^{comStop}$ *BAC*

The *galK* selection/counter-selection (as in Warming, Costantino, Court, Jenkins, & Copeland, 2005) was used to introduce a premature stop codon and transcription terminator into a bacterial artificial chromosome (BAC) containing the 35 kb *for* locus. Generation of this 35 kb construct was previously described (Allen *et al.*, 2017). The GalK sequence was PCR amplified with comStop-galK-F and comStop-galK-R primers (Table S1) and integrated into the BAC at the start of the first coding exon common to all transcripts. An *hsp70* transcription terminator was amplified with primers comStop-F and comStop-R (Table S1). A single SNP was included in the *for* specific region of the comStop-F to introduce a premature stop codon once integrated into the locus (Y573X, relative to for-PA). This PCR product was then used to replace the GalK sequence in the *for* BAC, introducing a premature stop codon and transcription terminator. The BAC was verified by PCR, restriction digest, and Sanger sequencing. The BAC was incorporated into the fly's genome using φC31 integration into the *attP2* landing site on the third chromosome (Groth, Fish, Nusse, & Calos, 2004). Transgenesis was performed by Genetic Services Inc.

Recombineering $for^{\Delta pr1}$ *and* $for^{\Delta pr2}$ *BACs*

The *galK* selection/counter-selection system (as in Warming *et al.*, 2005) was used to remove 200 bp centered around the transcription start site (TSS) of pr1 and pr2 (independently) from a BAC containing the 35 kb *for* locus. The GalK sequence was PCR amplified with deltapr1-galK-F and deltapr1-galK-R primers for the $for^{\Delta pr1}$ BAC and with deltapr2-galK-F and deltapr2-galK-R primers for the $for^{\Delta pr2}$ BAC

(Table S1). GalK sequences were independently integrated into the *for* BAC deleting each respective TSS. The GalK sequences were removed using the deltapr1-F and deltapr1-R primers for the $for^{\Delta pr1}$ and deltapr2-F and deltapr2-R primers for the $for^{\Delta pr2}$ (Table S1), generating a seamless 200 bp deletion of each TSS. The BACs were verified with PCR, restriction digest, and Sanger sequencing, and incorporated into the fly's genome using φC31 integration into the *VK00027* landing site on the third chromosome (Venken, He, Hoskins, & Bellen, 2006). Transgenesis was performed by BestGene Inc (Chino Hills, CA).

Ecdysis

Animals that had recently pupariated were examined and those containing a bubble in the midregion of the puparium (late-stage P4(i); Bainbridge & Bownes, 1981) were selected. For low-resolution video recordings of pupation, 2 rows of 10–15 animals (one of mutant animals and the other of controls) were placed on a microscope slide on double-sticky tape and the operculum was removed. The slide with pupae was then placed in a humidified Petri dish and the animals were video recorded at room temperature (ca. 22 °C) under dim transmitted light using a Leica DMLB microscope (10x magnification). For higher resolution imaging (cf. Videos 1 and 2), animals were mounted using double-stick tape on a plexiglass disc mounted on a motor that turned 5 times per hour. The operculum was then removed, and animals were covered with a large Petri dish and kept humidified using a damp piece of paper towel. The disc was then mounted under a Leica DMLB dissection microscope and images captured every time an animal entered the field of view. Matlab-based custom-made software was then used to reconstruct the time sequence of emergence behaviors for each animal. Timing and duration of the following behaviors were scored: head inflations (HIs), operculum opening (OP), and adult emergence (AE).

Temperature shift assay

Embryos of $tub\text{-}GAL80^{ts}$ *and tub-Gal4* x $for^{RNAi\text{-}exon7:8}$ were hatched on grape plates at either 30 °C or 18 °C. Fifty 0–4 h old L1 larvae were then transferred onto standard rearing vials with cornmeal-molasses fly food. For each treatment, vials were transferred to the appropriate temperature in 1-d intervals. After switching temperatures, flies were reared at that temperature until eclosion. Vials were scored for the presence of wandering third instar larvae, pupae, and eclosed individuals every day until no more individuals eclosed for 3 days.

Fat body lipid staining and analysis

Fat bodies from late third instar larvae were dissected in PBS and fixed (4% formaldehyde in PBS) for 20 min at room temperature. Tissues were then washed twice for 10 min each with PBS and incubated in 2.5 µg/mL Nile Red working solution (made from stock solution, 500 µg/mL Nile Red in acetone) for at least 5 min. Fat bodies were rinsed twice with PBS before mounting with 75% glycerol in PBS on a glass slide with a cover slip. Samples were viewed under a Leica TCS SP5 confocal laser-scanning microscope with a 20x objective and 4x zoom.

Fat body images were analyzed using ImageJ (Fiji) (Schneider et al. 2012). To measure fat content using fluorescence intensity values the images were converted to 8 bit greyscale. For whole cell area and fluorescence quantification, three cells were randomly chosen and averaged per fat body ($n = 5$ fat bodies). Cell area and mean grey value (fluorescence) were measured using the ImageJ ROI manager. To measure cell size and fluorescence, measurements were set to 'area' and 'mean grey value' under 'Analyze' -> 'Set Measurements', then cells were encircled one at a time with the 'Freehand Selections' tool and added for analysis within the ROI manager. To measure lipid droplet area and shape, 20 lipid droplets were randomly chosen per fat body and averaged per fat body ($n = 5$ fat bodies). Analysis was done as above with the ROI manager, including 'Shape Descriptors' under 'Analyze' -> 'Set Measurements'. Shape Descriptors in ImageJ include three measures of 'Roundness': Aspect Ratio (major axis/minor axis), Solidity (area/convex area) and Circularity (4 pi(area/perimeter○2), where a circularity value of 1.0 indicates a perfect circle).

Western blots

Western blots were performed as previously described (Allen *et al.*, 2017). Briefly, 20 µg of protein from whole late third instar larvae (96 ± 2 h post-hatch, 30 individuals per sample) or dissected fat bodies from late third instar larvae (96 ± 2 h post-hatch, fat bodies from five individuals were used per sample) was loaded onto a 1.5 mm 7.5% resolving SDS-PAGE gel and run at 90 V for 2 h. Proteins were transferred onto a nitrocellulose membrane at 100 V for 2 h in a 10% MeOH transfer buffer. Blots were stained with Ponceau to compare loading across lanes. Blots were blocked with blocking solution (5% non-fat milk powder, 0.1% Tween-20 in TBS) for 1 h at room temperature and incubated with 1° antibody 1:3000 anti-FOR(3) (Belay *et al.*, 2007) in blocking solution for 1 h. Membranes were washed with wash solution (0.1% Tween-20 in TBS) 3 × 5 min and incubated with 2° antibody in blocking solution for 1 h. Blots were washed with wash solution for 4 × 5 min and incubated with GE Healthcare Amersham ECL Prime Detection reagent for 5 min. Blots were visualized with X-ray film and Kodak developer and fixer.

Results

Mapping of pharate adult lethality within the for gene

To define the regions of the *for* gene that regulate the fly's terminal adult development in the pupal case, we first assessed the extent and nature of lethality of *for* mutants with available genetic deletions spanning all or parts of the *for* gene (Figure 1(a)). All *for* deletions, including a precise deletion of the whole gene ($Df(2L)for^{0}$), partial deletions of

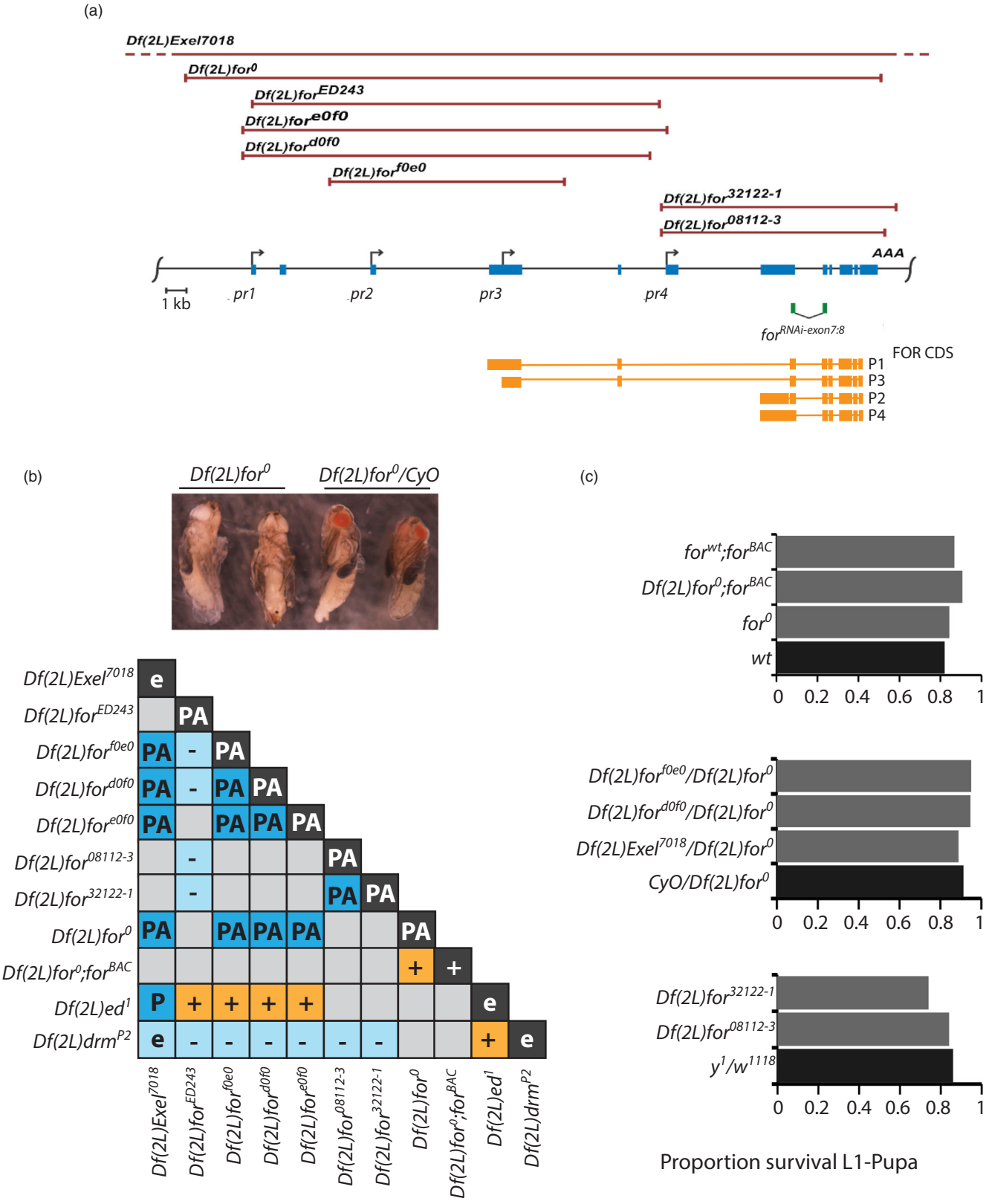

Figure 1. Animals with null deletions of the *for* gene die as pharate adults. a) Schematic of the *for* gene with exons shown as blue boxes and promoters 1–4 shown as black arrows. Genetic breakpoints of deletions used in this study are shown above the gene (red lines indicate deleted regions). The target region for the $for^{RNAi\text{-}exon7:8}$, targeting all *for* transcripts, is shown below the gene in green. Coding sequences (CDS) of FOR P1–P4 isoforms are depicted in yellow. b) Top: $Df(2L)for^{0}$ mutant (white eyes) and control (red eyes) pharate adults dissected out of their pupal cases. No observable morphological differences are evident between mutant and control animals, with eye color differing only due to the presence mini-*white* marker in control animals. Bottom: complementation analysis of *for* genetic deletion mutants. '+' viable, '−' lethal (stage not assessed), 'e' embryonic lethal, 'P' pupal lethal, 'PA' pharate adult lethal, dark grey denotes self-cross, light grey denotes not tested, dark blue denotes pharate adult lethality, light blue denotes embryonic or stage unknown lethality, yellow denotes viable, $n = 212–275$ per genotype. c) Survival from L1 larval stage to pupal stage of animals with null deletions of the *for* gene, $n = 50–120$ per genotype.

pr 1–3 *Df(2L)for*^{ED243}, *Df(2L)for*^{e0f0}, *Df(2L)fo*^{d0f0}, *Df(2L)for*^{f0e0}), and partial deletions of the 3′ end (*Df(2L)for*$^{08112\text{-}3}$ and *Df(2L)for*$^{32122\text{-}1}$), result in pharate pupal lethality, and none of these deletions complement each other for this lethality (Figure 1(b)). Mutants lacking *for* function fail to eclose but survive to the pharate adult stage. When dissected from their pupal cases at the end of metamorphosis, *for* deletion mutants look fully developed with no obvious morphological defects. When freed from their pupal cases these mutants twitch, but never stand or walk, and die soon after removal from the puparium (Figure 1(b)). As we have previously shown (Allen *et al.*, 2017), introduction of a BAC carrying the *for* gene (*for*^{BAC}) fully rescues pharate adult lethality of the precise deletion *Df(2L)for*^{0}, producing normal adults (Figure 1(b)). We additionally tested three larger deletions which have been reported to remove *for*. The first of these, *Df(2L)Exel7018,* belongs to the Exelixis deletion collection (Parks *et al.*, 2004) and spans a 127 kb genomic fragment encompassing the *for* gene, as well as 34 other genes and lcRNAs and miRNAs. This deletion results in embryonic lethality when homozygous but causes pharate pupal lethality when heterozygous with deletions limited to the *for* gene. Thus, in addition to *for, Df(2L)Exel7018* includes at least one gene that is essential for embryonic development. We also tested *Df(2L)ed*^{1} (Reuter & Szidonya, 1983) and *Df(2L)drm*^{P2} (Green, Hatini, Johansen, Liu, & Lengyel, 2002), which have been reported to delete *for* (De Belle, Sokolowski, & Hilliker, 1993; Green *et al.*, 2002) and extend out upstream (*Df(2L)ed*^{1}) and downstream (*Df(2L)drm*^{P2}) of *for*, respectively, overlapping at their proximal and distal ends (Green *et al.*, 2002). We found that while *Df(2L)ed*^{1} complemented *for* deletions within the gene, *Df(2L)drm*^{P2} did not (Figure 1(b)). The fact that *Df(2L)ed*^{1} complemented other *for* deletions suggests that this deletion does not remove all of the *for* gene as originally described (De Belle *et al.*, 1993; Green *et al.*, 2002). However, the breakpoints reported for *Df(2L)ed*^{1} might not be exact as this deletion was not mapped by sequencing but rather by polytene chromosomal analysis (Reuter & Szidonya, 1983). Like *Df(2L)Exel7018*, *Df(2L)ed*^{1} and *Df(2L)drm*^{P2} are embryonic lethal when homozygous, revealing the presence of embryonic lethal genes in the vicinity of *for*. One potential gene causing the embryonic lethality of *Df(2L)Exel7018* and *Df(2L)drm*^{P2} is *odd skipped* (Coulter *et al.*, 1990) which lies 150 Kbp downstream of *for*.

In mice, mutants of the *for* homolog *prkg1* die at different times throughout juvenile life, with fewer than 50% of animals surviving to adulthood (Feil, Lohmann, de Jonge, Walter, & Hofmann, 2003). We tested whether there is also significant lethality in *for* deletion mutants throughout larval development (Figure 1(c)). We found no significant lethality prior to pupation when comparing *Df(2L)for*^{0} mutants to *for*^{BAC} rescue or *for*^{BAC} *for* overexpression animals ($\chi^2 = 0.0047$, df = 3, $p = 0.9999$). Similarly, we found no significant pre-pupal lethality associated with *Df(2L)for*^{0} when heterozygous with the *Df(2L)Exel7018*, *Df(2L)for*^{f0e0} and *Df(2L)for*^{d0f0} deletion lines ($\chi^2 = 0.0035$, df = 3, $p = 0.9999$), or the *Df(2L)for*$^{08112\text{-}3}$ and *Df(2L)for*$^{32122\text{-}1}$ deletion lines ($\chi^2 = 1.0164$, df = 2, $p = 0.6016$), compared to their respective controls. These results indicate that lethality of *for* mutants is restricted to metamorphosis, rather than causing a gradual deterioration of health, as seen in mammals (Feil *et al.*, 2003). To further verify that a functional coding sequence of *for* is necessary for survival, we generated a BAC with an introduced premature stop codon and transcription terminator in the first coding exon common to all transcripts. This BAC was integrated into chromosome 3 and crossed into the *Df(2L)for*^{0} background. The *for*$^{BAC:comStop}$ allele (Figure S1) failed to rescue the pupal lethality of *Df(2L)for*^{0}, thereby confirming that FOR protein is required for survival.

Adult ecdysis (emergence) is severely affected in for deletion mutants

The inability of *for* deletion mutants to eclose from their pupal cases, despite seemingly normal development to the pharate adult stage, might indicate a deficiency in ecdysis. *Drosophila melanogaster* go through two larval ecdyses, marking the end of the first and second larval stages with the shedding of the larval cuticle, and a pupal ecdysis, which transforms the last larval instar into a pupa and marks the start of metamorphosis. The last ecdysis in the *Drosophila* life cycle happens at the end of metamorphosis with the eclosion of the adult fly. Each ecdysis is triggered by the neuropeptides Ecdysis Triggering Hormone (ETH) and Eclosion Hormone (EH) (Ewer & Reynolds, 2002; Zitnan & Adams, 2012). Interestingly, EH acts *via* cGMP (Ewer, De Vente, & Truman, 1994), which could, therefore, implicate *for* (encoding a PKG) in the transduction of EH actions. To test the hypothesis that *for* deletion mutants might be defective in ecdysis, we assessed whether these mutants show any failures at the transitions between larval stages and at pupation. We found that larval ecdyses (from larval Stage I–II and from larval Stage II–III) in *forDf(2L)for*^{0} deletion mutants proceeds normally, with stereotypical inflation of the trachea and shedding of the cuticle from the previous stage. Pupation (pupal ecdysis) also appears normal and resulting pharate adults showed standard head, wing, and leg morphology, all of which are sensitive to failures at pupal ecdysis (Lahr, Dean, & Ewer, 2012). These results were expected, as no significant lethality was observed in these mutants prior to the adult pharate stage (Figure 1(c)). We next assessed whether *for Df(2L)for*^{0} deletion mutants express normal eclosion behaviors. The typical emergence sequence includes the inflation of the trachea (which is most evident in the head) (Figure 2(a)), followed by the inflation of the ptilinum, which pushes open the operculum, culminating with the onset of anteriorly-directed (A-P) peristalsis movements that propel the fly's body out of the pupal case (Video S1–S4, 10.6084/m9.figshare.13506834). We found that emergence events (tracheal air filling) and behaviors (HI and A-P peristalses) of *Df(2L)for*^{0} deletion mutants are usually only weakly expressed or absent (Figure 2(b)). Indeed, only 15% of *Df(2L)for*^{0} animals show HI. This is significantly less than wildtype animals, where 100% of

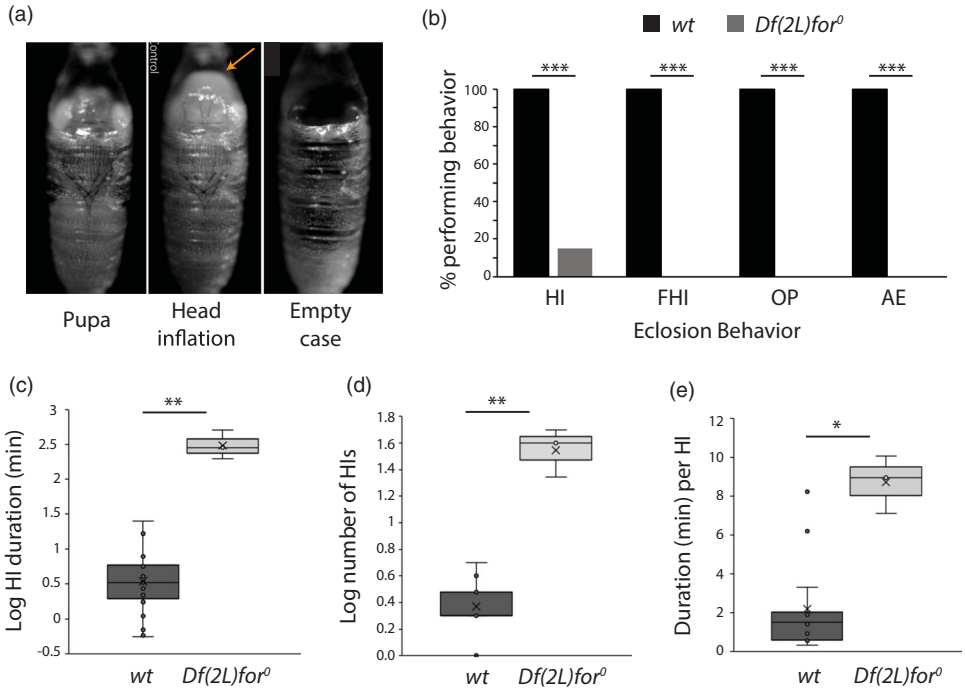

Figure 2. Animals with null deletions of the *for* gene show defects in emergence behaviors. a) Still images from video S3 depicting HI (transition from left panel to middle panel, yellow arrow) prior to emergence from the pupa case (right panel). b) Percentage of wildtype and *Df(2L)for*^{0} individuals performing HIs and subsequent emergence behaviors. HI: head inflation; FHI: final head inflation; OP: operculum opening; AE: adult emergence, $n = 19$–20 per genotype. c) Duration (min) of head inflations in wildtype and *Df(2L)for*^{0} individuals that performed the phenotype, $n = 19$ for wildtype and $n = 3$ for *Df(2L)for*^{0}. d) Number of head inflations performed by wildtype and *Df(2L)for*^{0} individuals that performed the phenotype, $n = 19$ for wildtype and $n = 3$ for *Df(2L)for*^{0}. e) Average duration of each head inflation performed by wildtype and *Df(2L)for*^{0} individuals that performed the behavior, $n = 19$ for wildtype and $n = 3$ for *Df(2L)for*^{0}. '*' denotes $p < 0.05$; '**' denotes $p < 0.01$; '***' denotes $p < 0.001$.

individuals show HI ($\chi^2 = 25.277$, df = 1, $p < 0.001$). Furthermore, 0% of *Df(2L)for*^{0} animals have robust emergence behaviors, which include the final head inflation (FHI), OP and AE. In contrast, 100% of wildtype individuals show these behaviors (FHI: $\chi^2 = 35.1$, df = 1, $p < 0.001$; OP: $\chi^2 = 36.1$, df = 1, $p < 0.001$; AE: $\chi^2 = 36.1$, df = 1, $p < 0.001$). We then analyzed the duration and number of HIs for all animals that showed this behavior. This analysis revealed that, when it occurs, HI in *Df(2L)for*^{0} animals takes significantly longer than in wildtype animals (Figure 2(c); Mann–Whitney Rank Sum Test; $U_{19,3} = 1$, $p = 0.007$). *Df(2L)for*^{0} animals also show significantly more HI bouts than wildtype animals (Figure 2(d); Mann–Whitney Rank Sum Test; $T_{19,3} = 63$, $p = 0.006$), and each HI takes longer in *Df(2L)for*^{0} animals than in wildtype flies (Figure 2(e); Mann–Whitney Rank Sum Test; $T_{19,3} = 62$, $p = 0.01$). However, these results should be interpreted with caution considering the small number of *Df(2L)for*^{0} animals that display HIs. Severe defects were also seen in the execution of the ecdysial peristalses: these typically do not occur, and, in the rare cases where they are observed, they are short-lived and do not result in successful emergence from the pupal case, with animals then stopping all movements and dying within the pupal case. In addition, it appears that the abdomen fails to contact the puparium to gain traction by pushing on the inside surface of the pupal case, making any contractions ineffective in propelling the body forward. These defects suggest a failure to sustain the expression of the ecdysial motor program and possibly also a failure in air intake, which is used to increase body volume and could aid in increasing the contact between the abdomen and the inner surface of the puparium.

Expression of for during early pupal development is necessary and sufficient for adult viability

Given that *for* null mutant animals (*Df(2L)for*^{0}) do not show defects in the timing of pupal ecdysis, lethality of these mutants at AE is likely linked to the timing of a different process during metamorphosis. To gain a better understanding of what this process might be, we performed a conditional *for* knock-down assay to determine the exact pupal stage at which *for* is needed for viability. We used a UAS-driven *for* RNAi line (*UAS-for*$^{RNAi\text{-}exon7:8}$) in combination with the ubiquitous *Tubulin* GAL driver and temperature-sensitive GAL80 (GAL80^{ts}) (McGuire, Mao, & Davis, 2004) to knock-down *for* during specific intervals of pupal development (Figure 3(a)). At low temperatures (18 °C) GAL80^{ts} represses the transcriptional activity of Gal4, preventing *for*$^{RNAi\text{-}exon7:8}$ expression. At high temperatures (30 °C) this repression is relieved, allowing Gal4 to drive *for*$^{RNAi\text{-}exon7:8}$ expression, thereby causing the knock-down of *for*. This strategy allows *for* expression to be knocked down at specific time intervals by shifting flies from the restrictive (18 °C) to the permissive (30 °C) temperature, and vice versa (Figure 3(a)). We shifted flies in one-day intervals in both directions and determined the timepoint during development at which *for* needs to be expressed for adult viability (Figure 3(b)). We found that when shifting from the restrictive (18 °C) to the permissive (30 °C) temperature, flies survive when *UAS-*

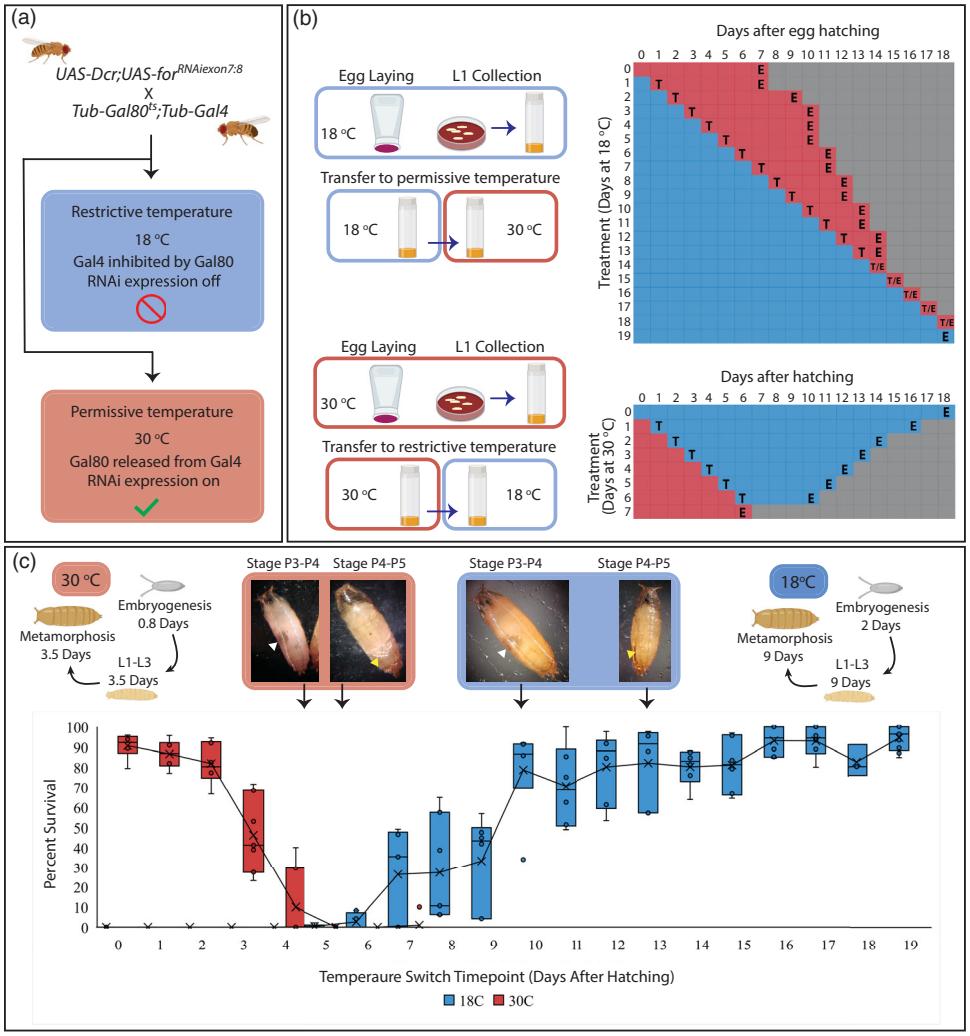

Figure 3. Expression of *for* is required around the time of the start of metamorphosis for normal adult emergence. a) Schematic of the experimental design that used temporally restricted GAL80^{ts} repression of Gal4 to limit *for*$^{RNAi\text{-}exon7:8}$ expression to specific periods during development. b) Experimental design of temperature shift experiments. Embryos are hatched at either the permissive (30 °C, red) or the restrictive (18 °C, blue) temperature and vials are transferred to the restrictive (18 °C) or permissive (30 °C) temperatures on different days after hatching (X-axis). After switching temperatures, flies are reared at that temperature until eclosion. Temperature regimes are color-coded along the Y-axis: grey squares mark days past adult emergence at each temperature regime; 'T' marks the day flies are transferred to new temperature; 'E' marks the day flies eclose. c) Survival of flies as a function of the day of post-embryonic development when they are switched from the permissive (30 °C) to the restrictive (18 °C) temperature (red boxplots), or vice versa (blue boxplots). As developmental time is sensitive to temperature, life cycles at each temperature are illustrated on the top left (30 °C) and right (18 °C). Representative images of pupae at the development point where *for* starts being required for viability (red) or is no longer necessary for viability (blue) are shown. For both treatment regimens, this turning point occurs at Bainbridge and Bownes pupal development stage P3–P4 (Bainbridge & Bownes, 1981). White arrow demarks the characteristic bubble in the abdomen visible at this stage. Pupal development stage P4–P5 is also shown for both temperature regimens, with the yellow arrow demarking the appendages that become visible at this stage. Boxplots show inclusive median and interquartile range, whiskers show minimum and maximum values; all data points are shown on plot, $n = 4$–6 vials per treatment (50 larvae per vial).

for$^{RNAi\text{-}exon7:8}$ is active after day 10, which at 18 °C corresponds approximately to pupal stage P2-P4 (bubble stage) (Bainbridge & Bownes, 1981; Tyler, 2000) (Figure 3(c)). When *UAS-for*$^{RNAi\text{-}exon7:8}$ is expressed before day 3 of development at 30 °C, corresponding to the same pupal P2–P4 stage as day 10 of development at 18 °C, adult flies also emerge normally (Figure 3(c)). Pupal stage P2–P4 occurs early in pupal development (12 h after puparium formation at 25 °C) and is marked by a clearly visible gas bubble in the pupal abdomen (Figure 3(c)). Our results show that *for* is not necessary for viability before or after pupal stage P2–P4 of development. Consistent with our deletion analysis of the *for* gene, which showed that *for* mutants die as pharate adults, time-restricted knock-down of *for* during early metamorphosis resulted in pharate adult lethality. These results show that *for* is essential in a process that occurs early during metamorphosis (between pupariation and pupation) in order for the adult fly to emerge normally. However, metamorphosis continues for animals in which *for* was knocked down early during metamorphosis, with death occurring in morphologically normal-looking pharate adults.

To further narrow down the process for which *for* might be essential, we performed a Gal4 screen (Brand & Perrimon, 1993) to identify drivers for which knock-down of *for* caused pharate adult lethality. For this, we crossed UAS-*for*$^{RNAi\text{-}exon7:8}$ to 74 different Gal4 drivers with different levels of cell specificity (Table S2). Using four ubiquitous Gal4 drivers, *tubulin-Gal4*, *daughterless-Gal4*, *actin-Gal4*,

and *armadillo-Gal4* we found that three drivers (*tubulin-Gal4*, *daughterless-Gal4*, and *actin-Gal4*) induce pharate adult lethality, as expected. One driver (*armadillo-Gal4*) fails to cause lethality but instead results in adult flies with wrinkled wings. The expression of *for* RNAi in wing discs has previously been reported to result in wing defects (Swarup, Pradhan-Sundd, & Verheyen, 2015), and *armadillo* acts as an essential molecule in the *wingless* signaling pathway, which is essential for wing development (Sanson, White, & Vincent, 1996). As *for* is a highly pleiotropic gene, this wing phenotype effect could be independent from *for*'s essential function leading to lethality. More likely, and consistent with the defects we see in AE, the wrinkly wing phenotype could be a result of wings not being expanded properly at emergence or not being formed properly at pupation when the wing disc is everted. Either way, these results indicate that a strong knock-down of *for* is sufficient to induce lethality (Figure S2), with *armadillo-Gal4* having perhaps a weaker ubiquitous knock-down, resulting in viable adult flies. We next tested seven *for-Gal4* lines. Two of these are gene traps inserted downstream of *for* pr4 ($for^{CR00867\text{-}TG4.2}$ and $for^{MI01791\text{-}TG4.1}$). The $for^{CR00867\text{-}TG4.2}$ line is itself pupal lethal when homozygous, as it 'traps' (prevents) expression from all four *for* promoters. Driving $for^{RNAi\text{-}exon7:8}$ with this line results in pharate adult lethality with <10% escapees. Driving *UAS-*$for^{RNAi\text{-}exon7:8}$ with $for^{MI01791\text{-}TG4.1}$ on the other hand does not cause lethality. The difference between the results obtained using these 2 drivers could be because $for^{CR00867\text{-}TG4.2}$ drives stronger gene expression (Allen *et al.*, 2018). We further tested four promoter-Gal4 lines driven by different fragments of upstream *for* DNA sequence, which included each of the four *for* TSSs (for^{pr1}-*Gal4*, for^{pr2}-*Gal4*, for^{pr3}-*Gal4*, and for^{pr4}-*Gal4*) (Allen *et al.*, 2018). None of these lines induces lethality, suggesting that RNAi expression driven by these Gal4 lines may be too weak to drive a strong knock-down of *for,* or that they do not recapitulate *for* expression in the tissue(s) responsible for the lethal phenotype. Lastly, we tested for^{NP7361}, an enhancer trap line in which Gal4 is inserted downstream of *for* pr1 (Hayashi *et al.*, 2002). This line also does not induce lethality, supporting our results below that pr1 is not responsible for the lethality phenotype. However, we cannot rule out that for^{NP7361} drives expression too weakly to cause a visible phenotype. We then tested a total of 64 tissue-specific Gal4 lines (Table S2), including 18 neuronal drivers, seven glial drivers, six muscle drivers, four gut drivers, three trachea drivers, four fat body drivers, three imaginal disc drivers, and a maternal loading driver (driving expression in cells that load maternal RNAs into the egg). None of these drivers resulted in lethality. Together these drivers target all tissues in which a function of *for* has been described. The expression of most drivers is well described in embryonic, larval, and adult stages. However, it is unclear how strongly and where these drivers are active throughout metamorphosis and specifically during the pupal bubble stage, when *for* plays a role that is essential for subsequent AE. Furthermore, it is possible that *for* is needed in a combination of tissues, rather than a single one, during early pupal development.

Lethality is rescued by FOR P1/P3 expression in the fat body

As we could not determine the tissue where *for* is essential for viability with our Gal4/RNAi knockdown screen, we aimed to test whether the lethal phenotype of *for* could be rescued driving a *UAS-*for^{cDNA} construct with different Gal4 drivers in a *for Df(2L)*for^{0} background. We used a P1 *UAS-*for^{cDNA} construct (Belay *et al.*, 2007), which encompasses the largest *for* open reading frame, corresponding to the P1 FOR protein isoform (Figures 4(a) and S3). Curiously, crossing of the *UAS-*for^{cDNA} into the *Df(2L)*for^{0} *for* deletion line rescues lethality in the absence of a Gal4 driver. An independent insert of this transgene similarly rescues the lethality of the *Df(2L)*for^{0} mutant. This result indicates that the *UAS-* for^{cDNA} construct has leaky expression, and that this leaky expression is sufficient to rescue the *for* lethal phenotype. This expression could result either from enhancers close to the *UAS-*for^{cDNA} insertion site driving expression of P1, or from *for* pr3, which resides within an exon of the P1 isoform and is thus included in the *UAS-*for^{cDNA} construct (Figure 4(a)). We assessed the leaky expression of *UAS-*for^{cDNA} in *Df(2L)*for^{0};*UAS-*for^{cDNA} animals by western blot, and found strong (leaky) expression in whole late third instar larvae of FOR P3 isoform (driven by *for* pr3, Figure 4(a), ~72–83 kDa bands). In third instar larvae *for* pr3 expresses in the larval fat body (Allen *et al.*, 2018), a tissue that dissociates (e.g. fat body cells separate and are absorbed) during the initial pupal stages and provides energy for metamorphosis. Mapping of the regulatory elements of *for* pr3 found that the fat body *cis*-regulatory element resides close to TTS of the promoter and is included in the *UAS-*for^{cDNA} construct (Figure 4(a); Allen & Sokolowski, 2021). We thus dissected fat bodies of *Df(2L)*for^{0};*UAS-*for^{cDNA} animals and probed for FOR expression by western blot. In the absence of a Gal4 driver, we detected high FOR expression (from *UAS-*for^{cDNA}) in the larval fat body, and no expression in larvae without fat bodies (Figure 4(a)). Although we cannot exclude leaky expression of *UAS-*for^{cDNA} in sparse cells outside the fat body or in other tissues in early pupae, these results indicate that the leaky expression of *UAS-*for^{cDNA} originates mostly from the fat body. Curiously, dissected fat bodies also show expression of higher molecular weight FOR protein isoforms (Figure 4(a), >100 kDa bands). These bands could correspond to FOR P1 isoform expression driven by nearby enhancers, or they could be post-translationally modified *for* proteins produced by transcripts that arise from pr3. This suggests that *for*'s lethal phenotype is linked to the fat body. However, knock--down of *for* with the larval fat body drivers *Lsp2-Gal4*, *ppl-Gal4*, *r4-Gal4*, and *Cg-Gal4*, did not result in lethality (Table S2), but the expression of these drivers after pupariation might differ from expression in larval stages.

To determine if homozygous *Df(2L)*for^{0} deletion mutants have fat body defects, we stained fat bodies of third instar

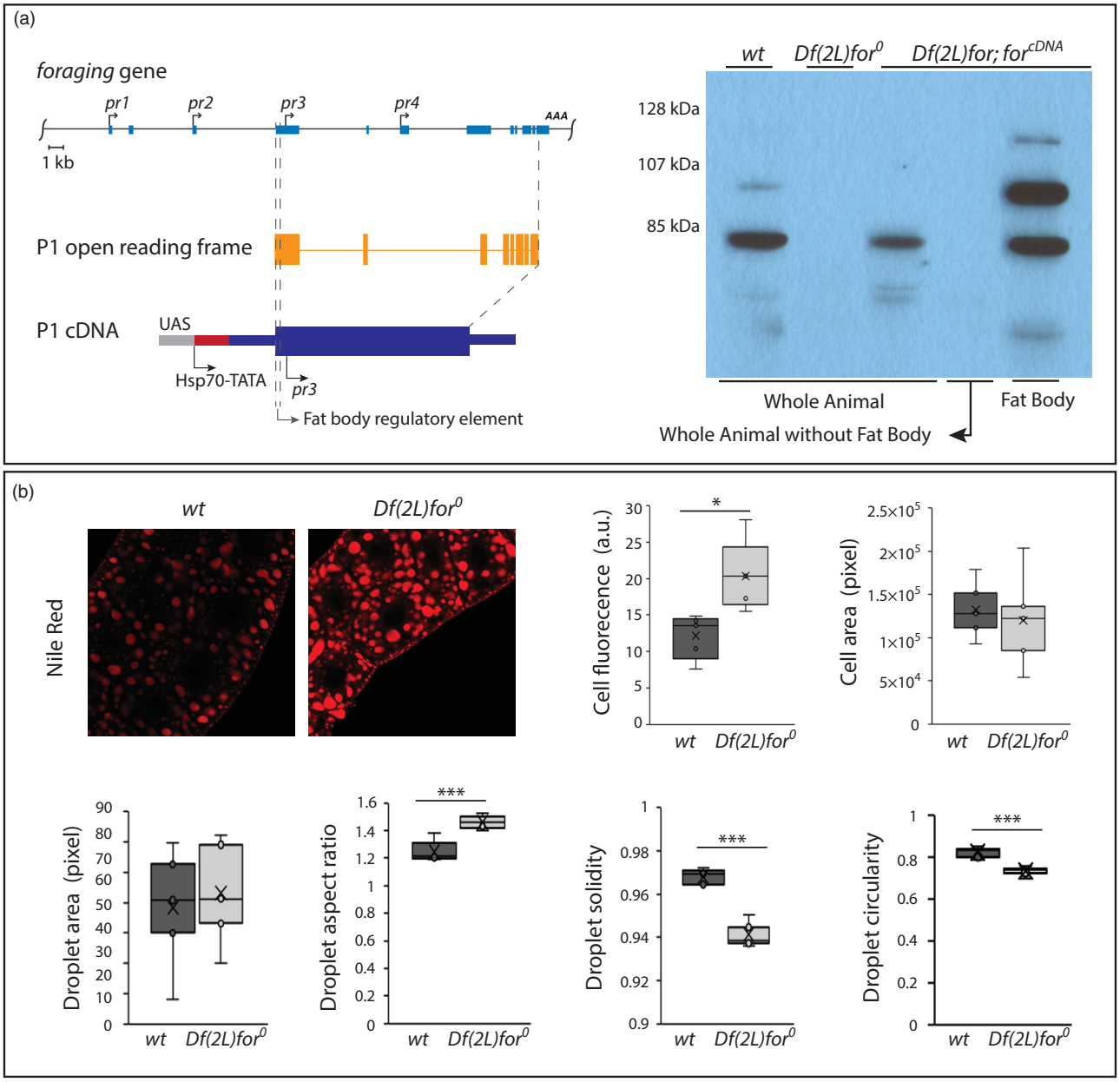

Figure 4. Fat body expression of for rescues lethality and for affects fat body morphology and fat storage. a) Left: schematic of the *UAS-for*^{cDNA}. Top: schematic of the *for* gene with exons shown as blue boxes and promoters 1–4 shown as black arrows. The *UAS-for*^{cDNA} open reading frame is shown in yellow. The *UAS-for*^{cDNA} construct is shown at the bottom. Dashed black lines indicate correspondence with genomic regions. Right: western blot showing FOR expression in whole animals, dissected fat bodies, and whole animals without fat bodies of wildtype (wt), *Df(2L)for*^{0} deletion mutants, and *UAS-for*^{cDNA} (no Gal4 diver present). For Ponceau loading control see Figure S3. b) Nile Red staining (red) of larval fat bodies. Representative staining images shown on top left. Plots show quantification of cell area (size), cell fluorescence, lipid droplet size, and lipid droplet shape descriptors (aspect ratio, solidity, and circularity) for wildtype (wt) and Df(2L)for0 deletion mutants. Boxplots show inclusive median and interquartile range, whiskers show minimum and maximum values, all data points are shown on the plot, $n = 5$ fat bodies per genotype. '*' denotes $p < 0.05$; '***' denotes $p < 0.001$.

larvae with Nile Red to assess fat body morphology and fat storage. Nile Red staining showed that *Df(2L)for*^{0} mutants have altered fat body morphology compared to wildtype animals. *Df(2L)for*^{0} mutants have significantly more fat storage, as indicated by higher fat body cell fluorescence (Figure 4(b); t-test; $t_8 = -3.238$, $p = 0.012$). Fat body cell size (cell area) is similar between wildtype and *Df(2L)for*^{0} mutants (Figure 4(b); t-test; $t_8 = 0.422$, $p = 0.684$). Lipid droplets within fat body cells differ significantly in shape: lipid droplets in *Df(2L)for*^{0} fat bodies have significantly higher droplet aspect ratio (Figure 4(b); t-test; $t_8 = -5.340$, $p < 0.001$), significantly lower solidity (Figure 4(b); t-test; $t_8 = 8.385$, $p < 0.001$), and significantly lower circularity (Figure 4(b); t-test; $t_8 = 5.716$, $p < 0.001$) than wildtype fat body cells. Size (area) of wildtype and *Df(2L)for*^{0} mutants lipid droplets is not significantly different (Figure 4(b); t-test; $t_8 = -0.409$, $p = 0.693$). The differences in droplet shape show a loss of structural integrity of lipid droplets in *Df(2L)for*^{0} mutants. Together these findings indicate that *for* plays a role in fat body storage and lipid droplet morphology.

Isoform-specific requirements of for *for viability*

The different behavioral functions of *for* have been associated with different isoforms of the gene (Anreiter & Sokolowski, 2019), however, which isoform is required for

viability has not been previously described. Rescue of the *Df(2L)for*^{0} allele's lethality with the *UAS-for*^{cDNA}, shows that either the P1 or P3 isoform is sufficient to rescue lethality. The P3 isoform is completely nested within the P1 isoform making the effects of P1 and P3 difficult to disentangle, as any mutation of P3 will also be a mutation of P1. However, analysis of the lethal effects of different transposable element insertions within *for* suggests that P3, but not P1, may underlie the lethality phenotype associated with *for*. Transgenic transposable elements, carrying phenotypic markers, often act as splice traps in the genes for which they are inserted (as in Goodwin *et al.*, 2000). We tested several transposable elements that are inserted upstream of pr3 and found that they are homozygous viable. This includes *for*^{e02991}, used to generate the *Df(2L)for*^{f0e0}, and the enhancer trap *for*^{NP7361}. However, all transposable elements inserted downstream of pr3 are pharate adult lethal. As insertions upstream of pr3 disrupt only FOR P1, but insertions downstream of pr3 disrupt P1 and P3, these findings suggest that pr3 (FOR P3), but not pr1 and pr2 (FOR P1), is necessary for viability. To further test if pr1 or pr2 are required for viability, we generated two BACs carrying deletions of *for* pr1 and *for* pr2 (Figure S1(b)). Both of these BACs are able to rescue the lethality of the *Df(2L)for*^{0} deletion, confirming that neither pr1 nor pr2 are required for *for*'s viability. However, since both pr1 and pr2 produce FOR P1 protein, there could be functional redundancy between these two promoters. Deletion of both pr1 and pr2 would confirm that P3 is the only isoforms necessary for viability. Nevertheless, the viability of flies carrying the *for*^{e02991} and *for*^{NP7361} elements inserted downstream of pr1 and pr2, strongly suggest that pr3, which drives expression in larval fat body, is necessary and sufficient for *for*'s viability.

Our data also allows us to confidently exclude a role for pr4 in the lethality phenotype, as the *Df(2L)for*^{ED245} deletion, which we find is pharate adult lethal, deletes pr1-3, but not pr4. This line has previously been reported to have active pr4 expression, but no pr1-3 expression (Belay *et al.*, 2007). The pharate adult lethality observed in the *Df(2L)for*^{f0e0} deletion, which deletes exon 3 and 4 belonging to pr1-3 isoforms, but preserves the pr4 regulatory regions and transcripts, further supports the finding that pr4 is not essential for viability. To confirm these results, we generated an isoform-specific RNAi line to target exons 4 and 5, which contain the P1 and P3 specific coding sequence (Figure S1(a)). Driving this isoform-specific RNAi, *UAS-for*$^{RNAi\text{-}exon4:5}$, with *tubulin-Gal4* or *daughterless-Gal4* severely reduces transcript and protein levels (Figure S1(b,c)), and results in pharate adult lethality. Whereas driving isoform-specific RNAi targeting exons 7, specific to pr4 (P2 and P4 protein isoforms), does not induce lethality (Anreiter *et al.*, 2017). These data allow us to effectively rule out pr4, and thus protein isoforms P2 and P4, from being essential for viability.

Discussion

The *for* gene is an essential gene with behavioral, physiological, and developmental functions. These functions are independently regulated by different promoters and transcripts of the gene (Anreiter & Sokolowski, 2019). Mutations in *for* have previously been described as pharate adult lethal (Allen *et al.*, 2017), but this phenotype had not been further investigated until now. Here, we show that although *for* null mutants die as pharate adults, *for* gene function is only required during early metamorphosis.

Our genetic analysis shows that precise and partial deletions of the 3′-end of *for* result in pharate adult lethality, ruling out any effects of deleting the neighboring *CG15418* and *Ugt36A1* genes and internal *mir-4972* miRNA. Additionally, we show here that knock-down of P1 and P3 with the isoform specific *for*$^{RNAi\text{-}exon7:8}$ also results in pharate adult lethality, while we have previously shown that knock-down of P2 and P4 does not cause lethality (Anreiter *et al.*, 2017). These results indicate that the P1 and/or P3 are the FOR isoforms that are essential for viability. We have also shown that *for* BACs containing deletions of pr1 and pr2 TSSs are able to rescue *Df(2L)for*^{0} lethality. Furthermore, splice trap transposable elements downstream of pr3, but not downstream of pr1–2 are lethal. Pr1–2 produce FOR P1, while pr3 produces FOR P3. These data indicate that the vital function of *for* resides in the expression of the P3 protein isoform from pr3. Previous work has shown that for pr1 is important for larval locomotion during feeding (Allen *et al.*, 2018) and larval nociception (Dason *et al.*, 2020), and that *for* pr4 is involved in regulating both larval (Allen *et al.*, 2018) and adult food intake (Anreiter *et al.*, 2017). While *for* pr1 and pr4, the promoters associated with behavioral functions, are 'narrow' promoters (transcription initiating at a single or few base pairs), pr3, the promoter associated with viability, is a 'broad' promoter (transcription initiating over a range of base pairs) (Allen *et al.*, 2017). Interestingly, broad promoters tend to be associated with broad expression and housekeeping functions, whereas narrow promoters are associated with restricted expression and function (Bhardwaj, Semplicio, Erdogdu, Manke, & Akhtar, 2019; Hoskins *et al.*, 2011; Schor *et al.*, 2017). While it is not clear if *for* has housekeeping functions *per se*, our study finds that its most vital function, linked to pupal lethality, is associated with expression from its broadest promoter. The gene's narrower promoters on the other hand, control non-vital behavioral functions. This suggests that the association between promoter 'shape' and function would seem to hold true for the *for* gene.

Our genetic analysis also found that the *Df(2L)ed*^{1} deletion complements *for*-dependent pupal lethality, contrary to previously described (De Belle, Hilliker, & Sokolowski, 1989). The reason for this likely lies in the location of the lethal complementation groups used in De Belle *et al.* This study used gamma mutagenesis to generate a lethal tag of the *for* gene (lethal *for*-tag) to facilitate genomic mapping of the gene (the gene was named after its larval behavioral phenotype, but its exact location was not known). De Belle *et al.* simultaneously screened for recessive lethality and a change in larval *for* pathlength phenotype from rover (long paths) to sitter (short paths). The authors postulated that the lethal tag could reside in *for* or an adjacent gene, but that in either case the lethal tag would facilitate the localization of the gene influencing the rover-sitter differences in behavior

(*for*). The authors isolated three recessive lethal mutants that had a change in behavior from rover to sitter. These and other alleles isolated in a subsequent EMS screen defined a lethal *for*-tagged complementation group (previously called the *for* complementation group), which resides in the distal end of the *Df(2L)ed*^{1} deletion (previously called *ed*^{Sz}) and breaks within the 5′ end of *for* (De Belle *et al.*, 1993; Osborne, 2002). Although both the lethal tag and the behavioral larval phenotype were instrumental in mapping *for* to the *dg2* gene (now called *for*), our findings suggest that lethality seen in the original *for*-tag complementation group is genetically separable from *for*'s pupal lethal phenotype. Hence, the lethal *for*-tag generated in De Belle *et al.* (1989) identified a pupal lethal complementation group adjacent to *for/dg2*, most likely uncovering the pupal lethality of the adjacent gene *Dgrx*. *for* and *Dgrx* are transcribed in opposite directions from each other, with the lethal *for*-tag complementation group likely residing in their overlapping 5′ regulatory regions (Osborne, 2002). Both *Drgx* and *for* are pupal lethal. However, as our results show, *for*'s pupal lethality is associated with *for* pr3, which is likely not disrupted by *Df(2L)ed*^{1}. The larval pathlength behavioral phenotype however, has previously been associated with *for* pr1 (Allen *et al.*, 2018), which is likely disrupted by the *Df(2L)ed*^{1} deletion. Hence, these findings lend further support that *for* pr3 is responsible for *for*'s lethal phenotype and explain why the *Df(2L)ed*^{1} uncovered *for*'s larval pathlength behavioral phenotype in previous studies (De Belle *et al.*, 1989; 1993; Sokolowski, Pereira, & Hughes, 1997), despite complementing *for*'s lethality phenotype.

Pupal lethality could indicate a deficiency cGMP, one of the key neuropeptides involved in triggering ecdysis (Ewer & Reynolds, 2002), and the second messenger for EH. Yet our findings suggest that these actions are likely not mediated by FOR (PKG). Indeed, *Eh* null mutants express severe defects at larval ecdysis (Kruger, Mena, Lahr, Johnson, & Ewer, 2015). By contrast, we found that animals lacking *for* developed normally until AE, showing no lethality during the larval stages, and producing pharate adults with normal-looking legs and wings, suggesting normal pupal ecdysis. And although they expressed defects at AE, these appeared to be due to failures in the sustained expression of emergence behaviors rather than to an inability to express the behaviors themselves. In addition, a role of *for* in adult ecdysis would be expected to require PKG function when EH is released, shortly before emergence. However, we found that pharate lethality was caused by knocking down *for* much earlier, around the time of pupation. Coupled with the spatial requirement for *for*, which appears to be in fat body, the function of *for* is intriguing, raising many questions regarding its vital function and mechanism of action.

We found that *UAS-for*^{cDNA} produces leaky *for* expression in the fat body of third instar larvae, and that this expression, or possibly other leaky expression during pupal development, is sufficient to rescue the lethal phenotype of the *Df(2L)for*^{0} deletion mutant. It is important to note that the leaky expression of *UAS-for*^{cDNA} complicates tissue-specific rescue of *for* phenotypes, as the basal fat body expression will always be present without the need of a driver. Phenotypes not affected by the basal expression of this construct (e.g. where the *UAS-for*^{cDNA} control line does not rescue the phenotype in the absence of a GAL4 driver) might still be rescued with tissue-specific drivers, however, future studies using this tool should be aware of this problem and control for its basal expression. We further found that the morphology and lipid storage of fat bodies is affected in *Df(2L)for*^{0} mutants. *Df(2L)for*^{0} mutants have previously been shown to have increased fat storage and that this phenotype is linked to *for* pr3 (Allen *et al.*, 2018). The fat body plays an essential role in providing the required energy for metamorphosis. During the first 3 days of pupal development the larval fat body is dissociated into single cells that are gradually broken down (Butterworth, Emerson, & Rasch, 1988). Interestingly, this time period coincides with the time when *for* expression is necessary and sufficient for adult viability. However, some of the dissociated larval fat body cells persist in newly emerged adult flies, where they play an important nutritional role (Aguila, Suszko, Gibbs, & Hoshizaki, 2007). It might thus be that the lethality of *for* deletion mutants occurs due to deficiencies in fat body remodeling, resulting in insufficient energy for survival of pharate adults. PKG also plays a major role in human and murine adipose tissue, where it functions as a major receptor of cGMP, and promotes a brown fat cell adipogenic program (Bordicchia *et al.*, 2012; Haas *et al.*, 2009). Mammalian brown fat cells contain large numbers of mitochondria and are thought to play a role in body weight regulation and energy expenditure (Bordicchia *et al.*, 2012). This suggests that *for* might play a conserved role in fat tissue regulation, although the mechanism by which *for* regulates fat body morphology and storage in *Drosophila* remains a subject of future investigations. It is also possible that *for* might play a role in endocrine signaling associated with the fat body. The larval fat body secretes endocrine hormones and polypeptides in response to ecdysone signaling (Hyun, 2018). The mammalian homolog of *for*, *prkg1*, has been found to play a role in adipose tissue development and adipokine secretion (Mitschke *et al.*, 2013). Thus, lethality of *for* deletion mutants might occur due to deficiencies in hormonal signaling during early pupal development. Future studies could investigate what molecular or cellular mechanism(s) are regulated by *for* in the *Drosophila* fat body. Additionally, it will be important to further address how this complex gene is regulated to mediate its many functions.

In sum, we find that *for* is an essential gene for early pupal development, and that deletion or strong ubiquitous knock-down of this gene results in pharate adult lethality. Complete knock-out of the gene results in severe defects in emergence behaviors, despite the timing of larval and pupal ecdysis occurring normally. Furthermore, these mutants show defects in fat body morphology, indicating a possible energy deficiency underlying the lethal phenotypes, which is further supported by observations that animals are unable to perform the behaviors necessary to emerge from their pupal cases. Lastly, our findings indicate that the P3 (but not P1/P2/P4) isoform of the gene are necessary for survival, with

its removal resulting in pharate adult lethality. These findings provide new insights the isoform-specific effects and timing underlying the vital function of *for*, providing a basis for further investigating the biological mechanism underlying *for*-linked lethality phenotype.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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