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A steroid receptor-microRNA switch that regulates lifespan in response to signals from the gonad

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Abstract

While the gonad primarily functions in procreation, it also affects animal lifespan. Here we show that removal of the *C. elegans* germline triggers a switch in the regulatory state of the organism to promote longevity, co-opting components involved in larval developmental timing circuits. These include the DAF-12 steroid receptor, which upregulates members of the *let-7* microRNA family, involved in L2/L3 transitions. The microRNAs target an early larval nuclear factor, *lin-14*, and *akt-1*/kinase, thereby stimulating DAF-16/FOXO signaling to extend life. These studies suggest that metazoan lifespan is coupled to the gonad through elements of a developmental timer.

When germline stem cells (GSCs) are eliminated from the *C. elegans* gonad, animals live up to 60% longer (1, 2). This longevity represents more than a simple tradeoff with fertility as it depends on the presence of the somatic gonad, suggesting a model whereby GSCs antagonize life-lengthening signals from the somatic gonad. Importantly, work from *D. melanogaster* suggests that gonadal longevity is evolutionarily conserved, since flies lacking GSCs live longer than controls (3). Although the life-shortening signals from the germline remain elusive, life-lengthening signals from the somatic gonad include bile acid-like steroids, called dafachronic acids (DAs), which activate the steroid hormone receptor DAF-12, a homolog of vertebrate liver-X, farnesoid-X, and vitamin-D-receptors (4, 5). How the DAs themselves are regulated and activate downstream targets remains unclear. Evidence indicates that DA/DAF-12 signaling regulates genes important for longevity, and also converges on the DAF-16/FOXO transcription factor, by potentiating nuclear localization and augmenting transcriptional activity on longevity promoting genes (6, 7), yet the mechanisms coupling these pathways are unknown. DAF-16/FOXO is also stimulated independently by decreased insulin/IGF receptor (IR) signaling, since the longevity of *daf-2*/IR and germlineless *glp-1* mutants is additive (1).

To illuminate how germline loss stimulates longevity, we first asked whether it affects regulation of DA signaling. When we examined mRNA levels of DA signaling components by qPCR, no differences were observed between germlineless *glp-1* mutants and gonad-intact wild-type animals (WT) at the third larval (L3) stage. However, by L4 and day 1 of adulthood (D1), the hormone biosynthetic gene *daf-36*/Rieske-oxygenase was significantly upregulated in *glp-1* (Fig. 1A, Fig. S1D) but downregulated in WT (Fig. 1A). Other DA-

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biosynthetic genes including *daf-9/CYP27A1* and *dhs-16/HSD* were less affected (8) (Fig. S1A). Expressed mainly in the intestine, *daf-36* catalyzes the first step in Δ^7 -DA biosynthesis, converting cholesterol to 7-dehydrocholesterol (9, 10). Accordingly, 7-dehydrocholesterol and Δ^7 -DA, were increased 4-5-fold in *glp-1* animals as measured by GC-MS-MS (Fig. 1B–C). In D1 adults, *daf-36* upregulation was largely independent of *daf-12* and *daf-16* (Fig. S1B–C). These data suggest that a regulatory switch governs DA signaling in response to signals from the reproductive system, and reveal that germline loss stimulates the DA signaling pathway.

To see if germline loss stimulates DAF-12 transcriptional activity, we focused on *let-7*-related microRNAs, *mir-84* and *mir-241*, which are direct DAF-12 targets in larval developmental timing pathways during L2/L3 transitions (11, 12). Indeed, microRNA expression increased in *glp-1* mutants by the L4 stage, and peaked at 3-4-fold by D1 (Fig. 1D–E). MicroRNA upregulation was DA and *daf-12* dependent, whereas *daf-16* or *nhr-80*, an HNF4-like nuclear receptor regulating gonadal longevity (13), had little effect (Fig. 1F–G, Fig. S2F). Consistently, *mir-84p::gfp* and *mir-241p::gfp* promoter constructs also exhibited transcriptional upregulation in *glp-1* mutants, particularly in epidermal seam cells (*mir-84*), and intestine (*mir-241*), revealing complex tissue-specific regulation (Fig. 1E, Fig. S2E). Other members of this microRNA family, *let-7* and *mir-48*, were also upregulated, but showed no clear DA/DAF-12 dependence. By contrast, an unrelated microRNA, *mir-228*, decreased during the same time frame (Fig. S2). We conclude that germline absence upregulates DA signaling, accompanied by transcriptional activation of *let-7* family members including the DAF-12 target genes, *mir-84* and *mir-241*.

To test if the microRNAs function in the gonadal longevity pathway, we removed GSCs by laser microsurgery in WT and *mir-84;mir-241* mutants. As expected, lifespan was extended in germline-ablated WT compared to mock-ablated controls. Whereas gonad-intact *mir-84;mir-241* controls resembled WT, lifespan extension was strikingly abolished in germline-ablated *mir-84;mir-241* double mutants (Fig. 2A). Similarly, microRNA loss suppressed longevity and stress resistance in *glp-1* mutants (Fig. S3A–C, Table S1). By contrast, *mir-84;mir-241* mutation had little effect on longevity caused by reduced mitochondrial function (*cco-1RNAi*) or IR signaling (*daf-2RNAi*) (Fig. 2B). *mir-84* and *mir-241* transgenes driven by endogenous promoters restored stress resistance and longevity in *mir-84;mir-241;glp-1* triple mutants, but did not significantly extend lifespan in gonad-intact animals (Fig. S3B–C, Table S1). Thus, *mir-84* and *mir-241* are specifically required but not sufficient for life extension in the gonadal pathway. *mir-48* mutants also significantly decreased *glp-1* longevity, but affected WT as well (Table S1). *let-7* mutants were not analyzed because of their severe developmental defects. Therefore we focused on *mir-84* and *mir-241* for further analysis.

DAF-16/FOXO is essential for longevity in the gonadal pathway. In germlineless animals, it accumulates in intestinal nuclei, where it regulates genes important for lifespan extension (1, 6). Both DAF-12 and DAF-36 promote DAF-16 nuclear localization (4, 6), and DAF-12 and DAF-16 share transcriptional responsibilities for longevity (6). To investigate whether the microRNAs interact with DAF-16, we analyzed lifespan upon *daf-16RNAi* (*daf-16i*). If microRNA deficiency reduces *glp-1* longevity by a mechanism independent of *daf-16*, then *mir-84;mir-241;glp-1;daf-16i* animals should live even shorter than *glp-1;daf-16i* animals. Instead, *mir-84;mir-241* deletion does not further reduce life span of *glp-1* upon *daf-16i*, (Fig. 2C), suggesting that the microRNAs and *daf-16* work in the same pathway. To test this hypothesis, we examined the effect of the microRNAs on DAF-16 localization and activity. *mir-84;mir-241* deficiency modestly diminished DAF-16::GFP nuclear localization, with little effect on overall expression levels (Fig. S4A–C). Consistent with a role in regulating DAF-16 activity via DA/DAF-12 signaling, microRNA mutation significantly reduced

expression levels of 6 genes commonly regulated by DAF-12 and DAF-16 (Fig. 2D, Fig. S5A–B) (7). *mir-84;mir-241* also impacted other DAF-16 targets, including superoxide dismutase *sod-3* and the lipase *lipl-4* (Fig. S5B–C) required for gonadal longevity (14). On *daf-16i*, *mir-84;mir-241* deletion did not further decrease *daf-16*/FOXO-regulated genes (Fig. S5D), suggesting that the microRNAs control these genes' expression through *daf-16*/FOXO but not another transcription factor. By contrast, the microRNAs had little influence on the expression of *daf-12*-regulated genes, *fard-1* and *cdr-6* (Fig. S6A–C)(7), nor on the transcriptional output of other major regulators of gonadal longevity, including PHA-4/FOXO targets, *lgg-1* and *unc-51*, involved in autophagy (15), and the NHR-80 target, *fat-6*, involved in fatty acid desaturation (13)(Fig. S6D–F). These results reveal that *mir-84* and *mir-241* specifically stimulate DAF-16 localization and transcriptional activity.

MicroRNAs downregulate gene expression by binding to the 3' UTR of target mRNAs and reducing stability and translation. To explore targets of *mir-84;mir-241* and how they stimulate DAF-16/FOXO activity, we searched for DAF-16/FOXO inhibitors with potential *mir-84;mir-241* binding sites in their 3' UTRs, using the bioinformatic algorithm mirWIP (16). Among predicted targets were components of IR signaling, including the PDK/AKT kinase cascade which inhibits DAF-16/FOXO (17).

Consistent with microRNA-mediated inhibition, *mir-84* and *mir-241* significantly repressed luciferase reporters containing the 3' UTRs of *akt-1* and *pdk-1* in cell culture (Fig. S7A, G–H). In *C. elegans*, *akt-1-3'UTR* dual reporter (DR) constructs were upregulated in *mir-84;mir-241* double mutants relative to WT, while an *unc-1-3'UTR-DR* control reporter remained unchanged (Fig. 3A–B, Fig. S7C), indicating an inhibition of the *akt-1* 3'UTR by *mir-84* and *mir-241* *in vivo*. Moreover, the *akt-1-3'UTR-DR* construct was downregulated 40–50% in *glp-1* animals compared to WT (Fig. 3C–D). In contrast, a *pdk-1-3'UTR-DR* construct was not downregulated in *glp-1* animals (Fig. 7I). These results indicate that *mir-84;mir-241* downregulate *akt-1* through its 3'UTR, in response to gonadal signals.

When we examined functional interactions between *akt-1* and the microRNAs, we observed that, *akt-1i* extended the lifespan of WT and *glp-1* mutants by 27% and 23% respectively (Fig. 3E, Table S1), consistent with previous results (18). Strikingly, *akt-1i* enhanced longevity in *mir-84;mir-241;glp-1* triple mutants to the same absolute extent as in *glp-1* (Fig. 3E), suggesting *akt-1*-knockdown bypasses the requirement for the microRNAs. Correlatively, *akt-1i* restored expression of *daf-16* target genes, *sod-3* and *lipl-4*, in *mir-84;mir-241;glp-1* animals (Fig. 4F, Fig. S7F). These results argue that *akt-1* acts in the gonadal pathway, where the microRNAs normally antagonize *akt-1* to promote longevity, as well as independently through canonical insulin/IGF signaling (18). Because *daf-2*/IR and gonadal longevity are additive, *akt-1* could be regulated by inputs other than *daf-2* upon germline ablation and serve as a general regulator of *daf-16*/FOXO.

The *let-7* family of microRNAs target several genes in the heterochronic pathway, a circuit that controls larval developmental timing (19). These include the zinc-finger protein *hbl-1*/hunchback and the ring-finger protein *lin-41*/trim71. By mirWIP, other heterochronic genes, including nuclear protein *lin-14* and the *let-7* binding protein *lin-28* are predicted targets. If these genes are microRNA targets in the gonadal pathway, then their downregulation should restore longevity to *mir-84;mir-241;glp-1* triple mutants. RNAi treatment from L4 onwards revealed that only *lin-14i* restored lifespan extension to the triple mutants (Fig. 4A, Table S1).

lin-14 is an intriguing candidate since Slack had previously shown that *lin-14* loss-of function extends lifespan in a *daf-16*/FOXO dependent manner, and *lin-14* gain-of-function mutations shorten lifespan (20). During development *lin-14* governs L1/L2 transitions, but

its context in aging is unclear. We found that *lin-14i* extended lifespan in WT as reported, but did not further extend that of *glp-1* mutants (Fig. 4A). To examine its relationship with *daf-16*/FOXO, we tested whether *lin-14* knockdown influenced *daf-16* target gene expression. Similar to aging experiments where longevity was restored, *lin-14i* also significantly restored *daf-16* expression of *sod-3* and *lip1-4* to *mir-84;mir-241;glp-1* (Fig. 4F, Fig. S7F). Altogether these observations suggest that the microRNAs downregulate *lin-14* and promote longevity via *daf-16*.

To test this hypothesis, we examined regulation of *lin-14* by *mir-84;mir-241*. As above, a luciferase reporter with the *lin-14*-3'UTR was downregulated by microRNAs (Fig. S7B). Similarly, a *lin-14*-3'UTR-DR construct was upregulated in *mir-84;mir-241* double mutants relative to WT, whereas the *unc-1*-3'UTR-DR controls were unchanged (Fig. 4B–C). Consistent with a role in the gonadal pathway, the *lin-14*-3'UTR-DR construct as well as full-length *lin-14::gfp* were downregulated in intestinal nuclei of germline-less animals relative to gonad-intact controls (Fig. 4D–E, Fig. S7D–E). Collectively these results reveal that *lin-14*, a core component of the developmental clock, functions in the gonadal longevity circuit, where it is downregulated by microRNAs upon germline removal.

In this work we show that components of an early-life developmental timing switch (*i.e.*, the steroid receptor DAF-12, its ligands, its target microRNAs of the *let-7* family, and LIN-14, the microRNAs' target) are used to regulate adult lifespan in response to signals from the gonad. We propose a model in which they work as part of a hormone-regulated switch between reproductive and survival modes at larval/adult stage commitments (Fig. S8). When GSC proliferation is prevented, unknown signals upregulate *daf-36* and DA production by the L4/young adult stage, subsequently activating DAF-12 and its microRNA targets, *mir-84* and *mir-241*. These microRNAs in turn downregulate *akt-1*, *lin-14*, and possibly other targets, which stimulate DAF-16/FOXO transcriptional activity, extending survival and lifespan. Because microRNA deletion does not fully abolish DAF-16 activity, other signals from either gonad or DAF-12 may also prompt gonadal longevity. Conversely, when GSC proliferation ensues, DA signaling is downregulated, microRNA expression is low, and *lin-14* and *akt-1* expression are high, resulting in normal lifespan. This switch could provide a critical link between development and longevity, serving as a checkpoint monitoring the state of the germline. For example, germline absence could mimic endogenous stress signals induced by germline quiescence or proliferative arrest in response to nutrient deprivation, infection or damage. As components of a developmental timer, the hormone-microRNA axis could ensure coordinate metabolism, maturation, and the relative timing of events between the reproductive system and the soma, with ultimate effects on lifespan. These findings extend the role of *let-7* family members beyond developmental timing and differentiation to the regulation of insulin/IGF signaling and metabolism, similar to recent studies in mammals (21). Because *let-7* family members and other components of this circuitry are evolutionarily conserved, it will be interesting to see if similar pathways impact longevity in vertebrates.

Materials and Methods

C. *elegans* strains and culture

Nematodes were grown with standard techniques at 20°C unless otherwise noted (22). All strains with *glp-1(e2141)* were maintained at 15°C and grown at 25°C to induce germlineless phenotype. NGM plates with ethanol or Δ^4 -dafachronic acid were prepared as reported (23). *daf-16(mu86);mulS109(Pdaf-16::daf-16::gfp; Podr-1::rfp)*, *pha-1(e2123);arEx1273(lin-14::gfp)*, and *nhr-80(tm1011);glp-1(e2141)* were kindly provided by Dr. Kenyon (UCSF), Dr. Greenwald (Columbia U.) and Dr. Aguilaniu (ENS de Lyon) respectively. Strains used are listed in Table S2.

Transgenes

Plasmids, *akt-1p::DR::akt-1u*, *akt-1p::DR::unc-1u*, *pdk-1p::DR::pdk-1u*, *pdk-1p::DR::unc-1u*, *lin-14p::DR::lin-15u*, and *lin-14p::DR::unc-1u* were injected into N2 to generate *dhEx765*, *dhEx768*, *dhEx772*, *dhEx776*, *dhEx779*, and *dhEx781*, respectively. For *dhEx842*, N2 was injected with *mir-241p::mir-241*, *mir-84p::mir-84*, and a co-transformation marker, *myo-2::mCherry*.

RNA interference

RNAi experiments were performed as described (24). For RNAi of *cco-1*, *daf-2* and *daf-16*, synchronized eggs were seeded on HT115 plates expressing corresponding dsRNA. For RNAi of *akt-1*, *lin-14*, and other heterochronic genes, synchronized larvae were grown on OP50 plates until late L4 and transferred to corresponding HT115 plates for RNA interference.

Lifespan assays

Ablation of germline precursor cells by laser microsurgery and adult lifespan analysis were performed as previously reported (25). Worms undergoing internal hatching, bursting vulva, or crawling off the plates were censored. Statistical analysis was performed with the Mantel-Cox Log Rank method.

GC/MS/MS analysis

Worms were synchronized by bleaching and grown until day 1 of adulthood for analysis. Lipids were extracted from worms *via* the Bligh and Dyer method before derivitizing for GC-MS analysis (26). 7-dehydrocholesterol and dafachronic acid (DA) levels were analyzed by GC/MS/MS on a 7000A Triple Quadrupole GC/MS instrument (Agilent Technologies) equipped with an ESI source and an HP5-ms column. Whole-worm lipid extracts were spiked with cholesterol-*d*₇ and 5 β -cholanic acid as internal standards and then derivitized with either Fluka III (Sigma) or trimethylsilyldiazomethane (Sigma) for 7-dehydrocholesterol or DA analysis, respectively. Compounds were analyzed in MRM mode using the following transitions: cholesterol-*d*₇ (*m/z* 465.4→360.3), 7-dehydrocholesterol (*m/z* 350.2→195.0), 5 β -cholanic acid (*m/z* 374.3→264.0) and Δ^7 -DA (*m/z* 428.3→229). The results were from three independent experiments.

Plasmid construction

To generate pDR, pMyo-LD17, a gift from Dr. Lo, was modified by first removing the *myo-2* promoter and L-HDAg gene within, then replacing the original *unc-54* 3'UTR with *unc-1* 3'UTR. The Dual Reporter (DR) module in pDR consists of *gfp::icr::rfp* as reported (27). DR reporters were subsequently generated from pDR as follows:

akt-1p::DR::akt-1u* and *akt-1p::DR::unc-1u—The *akt-1* promoter of 3.2 kb was amplified from genomic DNA by PCR and inserted in front of the DR module. For *akt-1p::DR::akt-1u*, a 444 bp *akt-1* 3'UTR was amplified from genomic DNA and cloned after the DR module.

pdk-1p::DR::pdk-1u* and *pdk-1p::DR::unc-1u—The *pdk-1* promoter of 2.9 kb was amplified from genomic by PCR and inserted in front of the DR module. For *pdk-1p::DR::pdk-1u*, *pdk-1* 3'UTR of 492 bp was amplified from genomic DNA and cloned after the DR module.

lin-14p::DR::lin-14u* and *lin-14p::DR::unc-1u—The *lin-14* promoter (5.2 kb) was amplified from genomic DNA by PCR and cloned before the DR module. For

lin-14p::DR::lin-14u, a 1.5 kb *lin-14* 3'UTR was amplified from genomic DNA and cloned after the DR module.

To generate *mir-241p::mir-241* and *mir-84p::mir84*, a 2.0 kb *mir-241* promoter, a 2.8 kb *mir-84* promoter, and the microRNA stem-loops together with ~200 bp of flanking sequence were PCR amplified from genomic DNA. PCR fragments were subsequently cloned into L3781 (Fire vector kit 1997).

For luciferase assays, 3'UTRs of interest were amplified from genomic DNA by PCR and cloned after the luciferase gene in pMIR-REPORT Luciferase (Ambion). For plasmids expressing mature microRNAs, the stem-loop of tested microRNA and ~200 bp of flanking sequence were amplified from genomic DNA by PCR and cloned into the 3'UTR of GFP in pEGFP-C1.

Cell culture and transfection

HEK293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (Gibco). Transfections were performed with FuGENE HD (Roche) according to the manufacturer's instruction.

Luciferase assay

Plasmids expressing microRNAs were co-transfected into HEK293T cells with firefly and renilla luciferase reporters. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega) 24 h post transfection. The 3'UTR of interest was fused with firefly luciferase and renilla luciferase serves as an internal control. At least three independent experiments were performed.

qRT-PCR

Synchronized worms were grown until day 1 of adulthood unless otherwise noted. Afterwards, worms were collected in TRIzol (Invitrogen) and frozen in liquid nitrogen. For mRNA, total RNA was prepared by RNeasy Mini kit (QIAGEN) and cDNA was subsequently generated by Superscript III First Strand Synthesis System with random hexamers (Invitrogen). For microRNA, miRNeasy Mini kit (QIAGEN) and TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) were used for total RNA and cDNA preparation respectively. qRT-PCR was performed with Power SYBR Green master mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Three or four technical replicates were performed in each reaction. *ama-1* and Sno-RNA U18 were used as internal controls for mRNA and microRNA qRT-PCR respectively. The results were from at least three biological replicates. Primer sequences are listed in Table S3.

Analysis of fluorescence intensity

Fluorescence intensity was measured by COPAS Biosort (Union Biometrica), or from microscopic pictures by Photoshop (Adobe) or ImageJ (<http://rsbweb.nih.gov/ij/>) as indicated. For microscopic pictures, background signal was subtracted as reported (28). Fluorescence microscopy was performed on a Carl Zeiss Axio Imager Z1. For analysis of *mir-84p::GFP* and *mir-241p::GFP*, images of indicated tissues were calibrated with InSpeck Green Microscopy Image Intensity Calibration Beads (Molecular Probes).

Statistical analysis

Results are presented as mean±SD unless noted otherwise. Statistical tests were performed with either One-way ANOVA with Tukey test (ANOVA) or Student's t-test (t-test) as indicated using GraphPad Prism (GraphPad software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DA	dafachronic acid
IR	Insulin/IGF receptor
GSC	germline stem cell
DR	dual reporter

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Fig. 1. Ablation of the germline upregulates DA/DAF-12 signaling

(A) Timecourse of *daf-36* mRNA level in WT and germlineless animals (*glp-1*).

Error bars: SEM.

(B–C) Δ^7 -DA (C) and 7-dehydrocholesterol (B) are elevated in germlineless animals. t-test, ** $P < 0.01$, *** $P < 0.001$.

(D) Timecourse of *mir-84* and *mir-241* levels in WT and *glp-1* animals.

(E) In *glp-1* animals, *mir-84p::gfp* and *mir-241p::gfp* expression are upregulated in seam cells (arrowheads) and intestine (arrows) respectively.

(F–G) Germline absence results in upregulation of *mir-84* and *mir-241* in a DAF-12/DA - dependent manner. Strains were grown on plates supplemented with ethanol (EtOH) or Δ^4 -dafachronic acid (DA). ANOVA, vs *glp-1* EtOH, ns: non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. DAF-12 target microRNAs are required for gonadal longevity through DAF-16/FOXO
(A) *mir-84* and *mir-241* are required for gonadal longevity. Laser microsurgery was performed to ablate the germline.
(B) *mir-84* and *mir-241* do not affect the longevity by reduced IR signaling (*daf-2RNAi*) or mitochondrial function (*cco-1RNAi*).
(C) Lifespan analysis of indicated strains on *daf-16RNAi*.
(D) qRT-PCR of DAF-16/FOXO target genes (*T08B1.1* and *btb-14*) in worms of indicated genotypes. ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. *akt-1* kinase is a microRNA target influencing gonadal longevity
(A–B) *mir-84* and *mir-241* target the 3'UTR of *akt-1* but not *unc-1* in vivo. While GFP driven by the *akt-1* promoter in the DR construct remains similar, RFP controlled by the *akt-1* 3'UTR is significantly elevated in *mir-84;mir-241*.
(C–D) *akt-1*-3'UTR-DR is downregulated in germlineless animals. 17 WT and 29 *glp-1* worms at D1 were examined from microscopic photos.
 t-test, *** $P < 0.001$.
(E) *akt-1* RNAi increases the lifespan of *mir-84;mir-241;glp-1* and *glp-1* to the same absolute extent.

Fig. 4. The *lin-14* heterochronic locus is a microRNA target regulating gonadal longevity
(A) *lin-14i* restores longevity to *mir-84;mir-241;glp-1*.
(B–C) DR construct with the 3'UTR of *lin-14* but not of *unc-1* is upregulated in *mir-84;mir-241* animals compared to WT. Arrowheads: intestinal nuclei.
(D–E) *lin-14-3'UTR-DR* is downregulated in *glp-1*. Arrowheads: intestinal nuclei. For each genotype, 60 intestinal cells from 20 worms were analyzed. t-test, *** $P < 0.001$.
(F) *lin-14i* or *akt-1i* increases *lip-4* expression in *mir-84;mir-241;glp-1*. ANOVA, * $P < 0.05$, ** $P < 0.01$.