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Nuclear Hormone Receptor Regulation of microRNAs Controls Developmental Progression

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Abstract

In response to small molecule signals such as retinoids or steroids, nuclear receptors activate gene expression to regulate development in different tissues. microRNAs turn off target gene expression within cells by binding complementary regions in mRNA transcripts, and they have been broadly implicated in development and disease. Here we show that the *C. elegans* nuclear receptor DAF-12 and its steroidal ligand directly activate promoters of *let-7* microRNA family members to downregulate the microRNA target *hbl-1* and drive progression of epidermal stem cells from second to third larval stage patterns of cell division. Conversely, the unliganded receptor represses microRNA expression during developmental arrest. These findings identify microRNAs as components of a hormone-coupled molecular switch that shuts off earlier developmental programs to allow for later ones.

Lipophilic hormones coordinate organism-wide developmental progression in metazoans by binding to nuclear hormone receptors (NHR), converting the presence or absence of ligand into changes in gene expression patterns(1). This regulation is conserved in the nematode *C. elegans*, where the nuclear hormone receptor (NHR) DAF-12, a homolog of vertebrate liver-X and vitamin D receptors, regulates developmental progression or arrest in response to the environment(2,3). In favorable environments, activation of TGF- β and insulin/IGF signaling cascades result in production of the DAF-12 steroidal ligands, the dafachronic acids (e.g. Δ^4 -DA), which promote rapid progression through four larval stages (L1–L4) to reproductive adults(4). In unfavorable environments, endocrine systems are suppressed and the unliganded DAF-12 causes arrest at a stress-resistant, long-lived alternative third larval stage, called the dauer diapause (L3d) (5).

A more cell-intrinsic level of developmental control is exerted by microRNAs (miRs). miRs are ~20–22nt long RNA molecules that bind to the 3'UTR of target mRNAs and decrease their expression (6–8). Null mutants for several miR genes show tissue-selective failure of progression from one stage-specific program to the next, generally described as heterochronic phenotypes. These phenotypes are most visible in the hypodermis, where hypodermal seam cells undergo invariant asymmetric stem cell division patterns, in which one daughter cell fuses to the hypodermal syncytium whereas the other retains stem cell character and its capacity to divide (9). Only during L2, do seam cells undergo one proliferative division prior to stem cell division, and repetition or loss of this program leads to changes in overall seam cell number

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in later stages. Finally, seam cell division ceases altogether by adulthood. Seam cells in animals with mutation of the microRNA *lin-4* repeat L1 programs of asymmetric cell division during L2 stage, a triple deletion of the *let-7* microRNA homologs *mir-48*, *-84*, *-241* (referred to as *let-7s*) repeat L2 programs of cell proliferation during L3 stage, and *let-7* null mutants repeat larval stage divisions and molting behavior in adults (10–12). *daf-12(rh61rh411)* null mutants exhibit a heterochronic phenotype similar to triple deletion of *let-7* family members *mir-48, mir-241(nDf51); mir-84(n4037)*, resulting in extra seam cells at the L3 stage (Table S1) (2,11). This observation suggested that *daf-12* might directly activate the *let-7s* miRs. To test this hypothesis, we fused the miR promoters (*mir-241p* and *mir-84p*) to the luciferase gene and co-transfected the reporters with DAF-12 into human cells. DAF-12 and $\Delta 4$ -DA strongly activated *mir-241p* and *mir-84p*, whereas other promoters gave little or no signal (Fig. 1A). Deletion analysis of the *mir-241* promoter revealed that whereas several deletion mutations retained transcriptional activity in the presence of DAF-12 and $\Delta 4$ -DA, fragment #4 produced the highest fold induction and its removal substantially reduced activity (Fig. 1B). This fragment contained two pairs of DAF-12-response-elements (REs) as described by Shostak (13). In the full-length promoter context, mutation of one RE-pair in *mir-241p* and two REs in *mir-84p* led to a ~7 and ~3 fold decrease in activation, respectively. Gel mobility shift assays confirmed *in vitro* binding of DAF-12 to these REs whereas mutated versions abolished the interaction (Fig. 1C).

To examine miR expression *in vivo*, we generated transgenic worms containing the miR promoters fused to GFP. Wild type worms containing *mir-241p::GFP* gave a broad expression pattern as described (14). However, *daf-12* nulls showed decreased expression most noticeably in the excretory cells (exc), as well as muscles, pharynx and intestine whereas neuronal expression seemed less affected, revealing tissue selectivity of *daf-12* regulation (Fig. 2A–D). Null mutants for cytochrome P450 *daf-9(dh6)* fail to produce $\Delta 4$ -DA, and the unliganded DAF-12 interacts with its co-repressor DIN-1/SHARP to repress its transcriptional targets, causing constitutive developmental arrest and dauer larvae formation (15–17). In these hormone deficient larvae, *mir-241p::GFP* expression was tightly repressed in most tissues (Fig. 2E). Supplementation with $\Delta 4$ -DA rescued this arrest and brought *mir-241p::GFP* expression back to WT levels (Fig. 2F). Tight repression was also relieved in *daf-9(dh6); din-1(dh149)* double null mutants lacking the corepressor, with *mir-241p::GFP* expression levels similar to *daf-12* nulls (Fig. 2G,H). Unlike *daf-12* nulls, however, $\Delta 4$ -DA supplementation of *daf-9; din-1* worms restored *mir-241p::GFP* expression back to WT levels. Point mutation of all four *daf-12*-REs in *mir-241p* resulted in the same weak expression level in WT, *daf-12* null with ligand, and *daf-9* null, and *daf-9; din-1* doubles $\pm$ ligand (Fig. 2I–K, Fig. S1), revealing that these REs mediate both activation and repression. Thus, liganded DAF-12 works through its REs to mildly activate *mir-241p* in some tissues, whereas unliganded DAF-12 together with DIN-1 tightly represses expression in nearly all tissues.

As reported, *mir-84p::GFP* was expressed in pharynx, somatic gonad, seam cells, vulva cells, and occasionally in the intestine (14,18). Seam cell expression was absent in *daf-12* null and *daf-9; din-1* null worms, and expression was increased almost to WT levels in *daf-9; din-1* animals by addition of $\Delta 4$ -DA (Fig. 2L–O), explaining *daf-12* heterochronic phenotypes as a failure to activate the miRs in temporally patterned tissues. By contrast, expression in other tissues such as the pharynx, were less affected, again showing tissue-specific *daf-12* regulation (Fig. S2). The transcriptional regulation of the *let-7s* is also reflected in the abundance of total mature microRNAs as measured by Taqman Q-PCR. *daf-12* mutants and *daf-9; din-1* animals showed decreased levels compared to WT, whereas *daf-9* nulls showed tight repression of *let-7* family of miRs (Fig. 2P, Fig. S3–Fig. S5). As expected, expression in *daf-9*, *daf-9; din-1*, but not *daf-12* mutants was rescued by $\Delta 4$ -DA.

The dauer signaling pathways work upstream of *daf-12* to govern organismal developmental progression and $\Delta 4$ -DA production. We therefore sought to see if *let-7s* expression was affected by dauer constitutive mutant backgrounds of *daf-7*/TGF- β , *daf-2*/Insulin/IGF-I Receptor, and *daf-9*/CYP450. *mir-241p::GFP* expression was nearly completely repressed in these dauer larvae, whereas *mir-84p::GFP* was down regulated in some tissues such as the pharynx (Fig. 3A–D), but consistently upregulated and more penetrant in others such as the seam (Fig 3E–H). Even in reproductively growing L3 larvae, seam cell expression was more penetrant in *daf-2(e1368)* and *daf-7(e1372)* mutants than WT (Fig. 3G). $\Delta 4$ -DA supplementation largely reversed this effect in *daf-7*, but not in the *daf-2* background (Fig. 3I–M), suggesting that insulin/IGF and TGF- β signaling distinctly regulate *mir-84p* expression in a $\Delta 4$ -DA-independent and $\Delta 4$ -DA-dependent manner.

The zinc finger protein *hbl-1*/hunchback is responsible for L2 proliferative programs of seam cells (11). *hbl-1* loss leads to a loss of proliferative programs, and hence a decreased seam cell number (Table S1). *let-7s* is proposed to inhibit *hbl-1*, through microRNA-mediated repression of its 3'UTR because *let-7s* mutants result in both increased seam number and *hbl-1::GFP* expression at L3 (11,19). Given that DAF-12 activates *let-7s*, we sought to examine interactions with *hbl-1*. Consistent with NHR signaling promoting *hbl-1* inhibition, *daf-12* mutants and *daf-9*;*din-1* double mutants have extra seam cells, the latter reversed by $\Delta 4$ -DA (Table S1). Moreover, the penetrant extra seam phenotype of *daf-12(rh61)* ligand binding domain mutant was dependent on functional *hbl-1*(+), placing *daf-12* upstream of *hbl-1* by genetic epistasis. Accordingly, we observed consistent upregulated hypodermal expression of *hbl-1p::GFP::hbl-1-3'UTR* during L3 in *daf-12(rh61)*, but not in WT (Fig. 4AB). This upregulation was likely due to loss of UTR-mediated repression, as exchange of the *hbl-1-3'UTR* for a non-repressed *unc-54-3'UTR* led to equal hypodermal expression levels in both WT and *daf-12(rh61)* background (Fig. 4CD).

In this work, we show that the nuclear hormone receptor DAF-12 directly regulates the *let-7* relatives, *mir-84* and *mir-241*, connecting organism-wide commitments to cell intrinsic programs (Fig. 4E). These studies suggest a model whereby in unfavorable environments, downregulated insulin/IGF-1 and TGF- β pathways suppress dafachronic acid production and unliganded DAF-12 together with corepressor DIN-1 repress miR expression in most tissues and specify developmental arrest. Conversely, in favorable environments, stimulation of insulin/IGF-1 and TGF- β growth signaling pathways results in dafachronic acid production. Liganded DAF-12 activates *let-7* homologs, which in turn downregulate their target, *hbl-1*, allowing L2/L3 transitions in the hypodermis. The use of a NHR-miR coupled molecular switch to turn off earlier programs to allow for later ones is a function likely to be conserved, and may be a paradigm for understanding hormone- dependent developmental progression, stem cell differentiation, maturation or tumor formation in metazoans. In particular, activation of new programs, and inhibition of earlier ones is critical for the fidelity of distinct developmental states, which may be less apparent in more complex animals whose cell lineages are unknown. In fact, DAF-12 itself is downregulated by *let-7* at later stages, suggesting that both feedforward and feedback loops drive transitions (20). In *D. melanogaster*, the steroid hormone ecdysone and its cognate receptor regulate developmental progression in part via *mir-14*, but it is unknown whether regulation is direct or indirect (21). Our studies also reveal the intricacy of NHR signaling in an organismal context. They give visible evidence that liganded receptors can activate their targets, while unliganded receptors can repress them, with vastly different outcomes for progression or arrest. This hormone dependent modulation of target gene expression around basal transcription mirrors that seen with the vertebrate homolog LXR (22). Finally, DAF-12/NHR regulation of the miRs is highly tissue- and stage-specific, implicating other transcription factors, coregulators, and chromatin factors in the control of miR expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DAF-12 and dafachronic acid (Δ 4-DA) activate microRNA promoters *in vitro*

A. Activation of miR promoters in HEK293T cells. Promoters of *let-7* homologs, *mir-84* and *mir-241*, are strongly activated in the presence of DAF-12 and 400nM Δ 4-DA, whereas other microRNAs are relatively unaffected. Luciferase assays were measured in triplicate, (with SD). EtOH, ethanol vehicle control; *ptk-luc*, empty luciferase vector.

B. Mutation analysis of *mir-241* and *mir-84* promoters reveals DAF-12/ Δ 4-DA activating elements. Deletion analysis of the *mir-241p* shows that the highest relative induction occurs with fragment #4, which contains four DAF-12 REs, 241abcd. Deletion or point mutation of 241ab elements (in red) abolishes activation (blue bars). Similarly, point mutation of DAF-12 REs in the *mir-84* promoter, *mir-84a* and *mir-84b* reduce expression (red bars).

C. Gel mobility shift assay of DAF-12 and *mir-241p*. ³²P-radiolabeled oligos containing the WT 241b element are shifted (s) by nuclear extracts expressing DAF-12::FLAG and supershifted (ss) in the presence of anti-FLAG antibody. Unlabeled WT 241b-oligo competes away the shift but addition of point-mutated oligo does not.

Figure 2. DAF-12 and $\Delta 4$ -DA regulate microRNA promoters *in vivo*

Panels A–K, *mir-241p::GFP*. Images show representative L3 animals, with indicated cell types (white arrowhead, excretory cell, exc; outlined arrowheads, neuron, neu; muscle, mus; intestine, int.; pharynx, ph). Bar graphs quantify the percentage of worms with excretory cell GFP expression as either strong (green), weak (yellow), or off (red), (two independent experiments, left and right, n=10 animals each). *mir-241p::GFP* expression level in WT (N2) grown without ligand (EtOH) (A) is decreased in *daf-12(rh61rh411)*/NHR null (C), strongly repressed in *daf-9(dh6)*/CYP450 null (E) or not activated in *daf-9(dh6)*/CYP450;*din-1(dh149)*/SHARP double null (G) but rescued nearly to WT level by growth on 250 nM $\Delta 4$ -DA (F, H). $\Delta 4$ -DA has no effect in WT (B) or *daf-12* null (D) animals. Point mutation of all four DAF-12 REs abolishes differences of tested genetic backgrounds or ligand (I–K, and Fig S1).

Panels L–O, *mir-84p::GFP*. Epidermal seam cells (arrowheads) express *mir-84p::GFP* in WT N2 (L), but not in *daf-12* nulls (M). Seam cell expression is absent in hormone deficient *daf-9*;*din-1* animals (N), but restored to nearly WT levels by $\Delta 4$ -DA supplementation (O).

Left, percentage of L3 animals showing weak or no seam cell expression (2 independent experiments, n=20 animals each).

(P) Relative quantification of microRNAs by Q-PCR. miR expression is decreased in *daf-12*, *daf-9;din-1*, and repressed in *daf-9* mutants. In *daf-9* genotypes, expression was $\Delta 4$ -DA dependent. Q-PCR was carried out using the TaqMan system (see Fig S5 and SOM for data analysis).

Figure 3. microRNA regulation by dauer signaling pathways

mir-241p::GFP shows high expression in continuously growing WT (A), but low expression in *daf-7(e1372)* dauer larvae (B). *mir-84p::GFP* shows high expression in the pharynx of continuously growing WT (C) but low expression in *daf-2(e1368)* dauers (D). *mir-84p::GFP* seam cell expression (E) is elevated in *daf-2* and *daf-9* mutants during dauer stage (F, H) and is even higher in *daf-7* mutants during reproductive growth at 20°C (G). Penetrant seam expression is reversed by 500 nM $\Delta 4$ -DA in *daf-7* but not *daf-2* during reproductive growth (I-M). Animals were assayed during L3/L3d stages, $n>20$. Red bars, ethanol vehicle, green bars, $\Delta 4$ -DA (SEM).

Figure 4. *let-7s* repression target, *hbl-1*, is regulated by DAF-12

A GFP-fusion to *hbl-1* promoter and 3'-UTR is repressed in hypodermis at mid L3 (28h) in WT (A). In the *daf-12(rh61)* mutant, reporter signal is upregulated in the hypodermis (arrows) and other tissues (B) (exposure 250ms). A GFP-fusion to the *hbl-1* promoter, containing the *unc-54* 3'-UTR lacks substantial up-regulation in the hypodermis (C, D), though body muscles show modest reporter upregulation (D) (exposure time 50ms)

Model for nuclear receptor/microRNA signaling cascades (E). In response to favorable environmental signals, activated insulin/IGF and TGF-β pathways induce Δ4-DA biosynthesis through DAF-9/CYP450. Right: Liganded DAF-12 activates L3 programs and expression of *let-7s* and thereby inhibits HBL-1 and genes of L2 programs, resulting in developmental

progression (E). Left: During unfavorable conditions, unliganded DAF-12 together with DIN-1, repress L3 programs and *let-7s*, allowing derepression of L2 programs and/or developmental arrest. Dauer signaling also has $\Delta 4$ -DA-independent outputs onto miRs.