Title: Conditional depletion reveals temporal requirements for the oscillating transcription factor NHR-23/NR1F1 in *C. elegans* larval progression

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18 **Summary Statement**

19 This work provides insight into how a recently discovered large-scale gene expression
20 oscillator promotes rhythmic skin regeneration in *C. elegans*.

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Abstract

Nematode molting is a remarkable process where the animals must essentially build a new epidermis underneath the old skin and then rapidly shed the old skin. The study of molting provides a gateway into the developmental program of many core cellular and physiological processes, such as oscillatory gene expression, coordinated intracellular trafficking, steroid hormone signaling, developmental timing, and extracellular remodeling. The nuclear hormone receptor NHR-23/NR1F1 is an important regulator of molting. Imaging and western blot time-courses revealed oscillatory NHR-23::GFP expression in the epithelium that closely followed the reported mRNA expression. Timed depletion experiments using the auxin-inducible degron system revealed that NHR-23/NR1F1 depletion early in a given larval stage caused animals to arrest with only weak molting defects, whereas later depletion resulted in highly penetrant severe molting and morphological defects. This larval arrest was independent of insulin signaling. Despite the weakly penetrant molting defects following early NHR-23/NR1F1 depletion, the epidermal barrier was defective suggesting that NHR-23/NR1F1 is necessary for establishing or maintaining this barrier. NHR-23/NR1F1 coordinates the expression of factors involved in molting, lipid transport/metabolism, and remodeling of the apical extracellular matrix. We propose that NHR-23/NR1F1 is a regulator in a recently discovered large-scale gene oscillatory network coordinating rhythmic skin regeneration.

Introduction

Molting is a critical developmental process required for the growth of all ecdysozoans, a clade comprising an estimated total of 4.5 million living species (Telford et al., 2008). Although specific to invertebrates, molting involves conserved processes such as apical extracellular matrix (aECM) remodeling, intracellular trafficking, and oscillatory gene expression (Lažetić and Fay, 2017). Molting is also a process of interest for developing new drugs against parasitic nematodes (Ghedini et al., 2007). This group of pathogens infect over one billion humans and devastate crops and livestock, yet few drugs exist to counter them and resistance is emerging (Ward, 2015b). While many genes involved in

nematode molting have been identified in *C. elegans* (Frand et al., 2005), little is known about how their gene products are coordinated to promote aECM remodeling and the generation and release of a new cuticle.

Nematodes progress through four periodic larval stages (L1-L4), prior to becoming a reproductive adult (Brenner, 1974). The end of each larval stage is punctuated by a molt that involves trafficking and secretion of aECM components, assembly of a new aECM underneath the old cuticle, followed by separation of the cuticle from the underlying aECM (apolysis) and shedding of the old cuticle (ecdysis)(Lažetić and Fay, 2017). Apolysis coincides with a sleep-like behavior called lethargus (Singh and Sulston, 1978). The cuticle is a collagenous exoskeleton secreted by hypodermal and seam epithelial cells (Page and Johnstone, 2007). The outermost layer (glycocalyx) is rich in carbohydrates and mucins and thought to be secreted by the excretory gland cell (Nelson et al., 1983; Singh and Sulston, 1978). Beneath the glycocalyx is the glycolipid- and lipid-rich epicuticle which is postulated to function as a hydrophobic surface barrier (Blaxter, 1993; Blaxter et al., 1992). The composition and biogenesis of the glycocalyx and epicuticle are poorly understood in comparison to the underlying layers composed of collagens and cuticlins.

Nuclear hormone receptor (NHR) transcription factors are key regulators of molting in insects and nematodes (King-Jones and Thummel, 2005; Taubert et al., 2011). NHRs are characterized by a ligand-binding domain (LBD) which has the potential to bind small molecules such as ligands and dietary-derived metabolites (Taubert et al., 2011). Not all NHRs have known ligands, though the ancestral proteins from which all NHRs descend bound ligands (Bridgham et al., 2010; Eick and Thornton, 2011). NHRs have a canonical zinc finger DNA-binding domain (DBD) with an unstructured hinge region between the DBD and LBD that is subject to post-translational regulation (Antebi, 2015; Campbell et al., 2008; Ward et al., 2013). NHRs can function as homodimers, heterodimers, or monomers (Sever and Glass, 2013).

There has been a large expansion of NHRs within nematode genomes, making direct comparisons to other organisms challenging (Antebi, 2015; Taubert et al., 2011). However, a single, conserved nuclear hormone receptor, NHR-23/NR1F1 (hereafter referred to as NHR-23), an ortholog of DHR3 in insects and ROR α in mammals, is a key regulator of *C. elegans* molting. *nhr-23* mutation or inactivation by RNAi leads to embryonic lethality, larval arrest, ecdysis defects, and morphology defects (Frandsen et al., 2005; Gissendanner et al., 2004; Kostrouchova et al., 1998; Kostrouchova et al., 2001). *nhr-23* mRNA expression oscillates over the course of each larval stage, peaking mid-larval stage and hitting a trough at the molt (Gissendanner et al., 2004; Kostrouchova et al., 2001). Experiments with *nhr-23* mutants and RNAi have demonstrated a role for NHR-23 in all four larval molts (Kostrouchova et al., 1998; Kostrouchova et al., 2001), but these approaches didn't determine when during a given larval stage NHR-23 was necessary. Molting must be coordinated with developmental timing, though the underlying mechanisms linking these two are poorly understood. Uncoupling of the molting and heterochronic pathway through exposure to a nicotinic agonist causes death as animals attempt to molt without completing the requisite cell divisions and differentiation (Ruaud and Bessereau, 2006). Bacterial diet can alter developmental rate in *C. elegans*, likely through modification of NHR-23 activity (Macneil et al., 2013). The NHR-23 insect ortholog (DHR3) is part of the molting gene regulatory network (Lam et al., 1997; Ruaud et al., 2010, 3) and the mammalian ortholog (ROR α) regulates circadian rhythms, lipid metabolism, and immunity (Jetten, 2009).

We found that NHR-23 protein oscillates and three distinct isoforms are expressed. Using the auxin-inducible degron system for rapid inactivation of NHR-23 during development, we discovered distinct phenotypes when NHR-23 was depleted early or late in a given larval stage. Early depletion leads to a larval arrest with mild molting defects, while later depletion resulted in penetrant molting and morphological defects. Analysis of NHR-23 target genes suggests a role in coordinating aECM assembly, remodeling, and construction of specific cuticular structures. Our work provides an entry point into understanding how NHR-23 coordinates the complex cell biology underpinning molting.

Results

NHR-23 protein oscillates during development

Expression of *nhr-23* mRNA oscillates throughout each larval stage of the *C. elegans* life cycle (Gissendanner et al., 2004; Hendriks et al., 2014; Kostrouchova et al., 2001; Meeuse et al., 2020) reaching peak expression mid-intermolt and a trough prior to the completion of the next molt (Fig. 1A). However, mRNA expression profiles do not always match protein levels (de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012). For example, *C. elegans daf-12* mRNA transcripts oscillate during each larval stage, but the protein products are constitutively expressed (Antebi, 2015). To determine whether NHR-23 protein oscillates, we monitored the expression of the endogenous protein, tagged with green fluorescent protein (GFP), over the course of 28 hours. During L1, oscillating expression of NHR-23::GFP was observed in the nuclei of seam and hypodermal cells (Fig. S1). Expression of NHR-23 rises and falls prior to the completion of the 1st molt; this pattern is repeated prior to the 2nd molt as well (Fig. 1B,C), similar to the reported mRNA expression (Gissendanner et al., 2004; Kostrouchova et al., 2001).

As GFP tags can affect the expression or stability of proteins (Agbulut et al., 2006; Baens et al., 2006), we assayed the expression of NHR-23 through western blotting time-courses using an *nhr-23::AID*::3xFLAG* strain (Zhang et al., 2015). These experiments confirmed that NHR-23::AID*::3xFLAG also oscillates (Fig. 1C), similar to our NHR-23::GFP imaging experiments (Fig. 1B). We detected three distinct NHR-23 bands, consistent with the predicted sizes of *nhr-23b/f*, *nhr-23c*, and *nhr-23e* (Fig. 1C,D, Fig. S2). However, these bands could also reflect post-translational modification. All observed NHR-23 bands oscillate, though the lowest band was expressed more strongly than the other bands (Fig. 1C).

Distinct phenotypes are observed when NHR-23 is depleted early or late in L1 larvae

Given that NHR-23 isoforms oscillate, we tested when NHR-23 was necessary for molting during the L1 larval stage. We have previously shown that the auxin-inducible degron system can rapidly deplete a nuclear hormone receptor (NHR-25), with almost complete depletion within 30 minutes (Zhang et al., 2015). In a pilot experiment, we consistently found weaker depletion phenotypes using an *nhr-23::GFP::AID*::3xFLAG* strain (Ragle et al., 2020) compared to the *nhr-23::AID*::3xFLAG* strain (Zhang et al., 2015)(unpublished data). We therefore transitioned to using the *nhr-23::AID*::3xFLAG* strain for all experiments involving phenotypic analysis. We first tested NHR-23::AID*::3xFLAG depletion kinetics and found near complete depletion within 15 minutes after exposure to 4 mM auxin (Fig 2B).

We then performed timed depletion experiments in the L1 larval stage (Fig. 2A), similar to those used to determine the role of NHR-23 in spermatogenesis (Ragle et al., 2020). Experiments were conducted with an *eft-3p::TIR1::mRuby2* control strain (henceforth referred to as *TIR1*) and a strain that permits somatic depletion of NHR-23 in the presence of auxin, (*eft-3p::TIR1::mRuby2; nhr-23::AID*::3xFLAG*; henceforth referred to as *nhr-23::AID; TIR1*). Synchronized L1s were shifted onto either 4 mM auxin or control plates every two hours (Figure 2A). Animals were scored for molting defects at 20 hours post-release. 100% of *TIR1* control animals shifted onto control or auxin plates successfully molted and transitioned into the second larval stage. *nhr-23::AID; TIR1* animals shifted onto control plates also reached the L2 stage, indicating that ethanol does not affect developmental timing in this context. In contrast, *nhr-23::AID; TIR1* animals shifted onto auxin displayed multiple phenotypes including larval arrest, molting defects, vacuoles, developmental abnormalities such as disrupted tails and viable animals with a squashed morphology (“splatter”)(Fig. 2C). We then quantified the molting defects, scoring animals unable to shed their cuticles, or with morphological abnormalities (Fig. 2D). Vacuoles were observed in both the *TIR1* control and *nhr-23::AID; TIR1* strains. This could be a result of exposure to auxin or possibly an effect of the imaging process. We did not pursue the vacuole phenotype further. The percentage of *nhr-23::AID; TIR1* animals with molting defects were low (0-5%) when animals were shifted to auxin between 2-4 hours post

release (Fig. 2D). However when *nhr-23::AID; TIR1* animals were shifted 6-12 hours post release we observed a higher penetrance of molting defects (28-58%)(Fig. 2D). These

Figure 2: Distinct phenotypes are observed when NHR-23 is depleted early or late in L1 larvae. (A) Schematic of the experimental set-up. Synchronized L1 larvae were grown on control media (light grey bands) and transferred to 4mM auxin (dark grey bands) every 2 hours. Animals remained on auxin until they were imaged at 20 hours post-release. (B) Anti-FLAG immunoblot to monitor the speed of NHR-23 depletion. Synchronized *nhr-23::AID*::3xFLAG; TIR1* and *TIR1* animals were grown on MYOB plates for 12 hours, which corresponded to the peak in L1 expression from Figure 1C. Animals were then shifted onto control or auxin plates and lysates were collected at 15 and 30 minutes post-exposure. Marker size (in kDa) is provided. Stain-free analysis, which visualizes total protein on the membrane, is provided as a loading control. (C) Representative images of phenotypes observed when *nhr-23::AID*::3xFLAG; TIR1* animals were exposed to auxin in the first larval stage. (D) Percent of animals of the indicated genotypes with unshed cuticles and morphological abnormalities following exposure to auxin. The number of animals scored (n) is provided.

data could suggest that NHR-23 may not be necessary for the initiation of the molt but required for completion of the molt. Additionally, the majority of *NHR-23::AID; TIR1* animals shifted on to auxin at the 14 and 16 hour time points appeared wild type (Fig. 2D). *TIR1* and *nhr-23::AID; TIR1* animals grown on control plates developed normally with no arrest or morphological defects (N=35 for each strain). We repeated the 16 hour shift experiment and scored animals 48 hours later. Based on size, gonadal morphology, and vulval morphology, animals arrested in L2 and L3 (Figure S3). Thus depletion of NHR-23 during the final 2 hours of the L1 larval stage did not disrupt molting, suggesting that NHR-23 is not necessary during this window to complete the molt to L2.

Tissue-Specific NHR-23 depletion

We next wanted to explore the observed molting phenotypes and further identify in which tissues NHR-23 is necessary for completion of the molt. In addition to being expressed in seam and hypodermal cells (Fig. S1), single-cell RNA-seq data indicates that *nhr-23* is also expressed in rectal cells, vulval precursor cells, excretory cells (likely duct and pore cells), and glial socket cells (Cao et al., 2017). We performed auxin depletion experiments with a panel of recently described tissue-specific *TIR1* transgenes developed for hypodermal (*col-10p*), intestinal (*ges-1p*), neuronal (*rgef-1p*), seam cell (SCMmin enhancer+*pes-10delta* minimal promoter) and multi-tissue depletion (*vha-8p*: seam cell, hypodermis, gut, excretory cell, unidentified head cells)(Ashley et al., 2021). Surprisingly, no phenotypes were observed when NHR-23 was depleted using hypodermal-, intestinal-, neuronal- or seam cell-specific *TIR1* transgenes. Only depletion using the *vha-8p::TIR1* transgene was able to phenocopy the pan-somatic *TIR1* allele (*eftp::TIR1*) (Table 1). These data could reflect: i) incomplete depletion of NHR-23 in hypodermis, intestine, neurons, or seam cells; ii) NHR-23 is not necessary in those cell types; iii) NHR-23 activity is required in multiple tissues, with single-tissue depletion being insufficient to disrupt molting.

Tissue specific depletion of NHR-23

TIR1 Promoter	Tissue	Larval Arrest	Molt Defects
<i>col-10</i>	<i>hypodermis</i>	no	no
<i>rgef-1</i>	<i>neurons</i>	no	no
<i>vha-8</i>	<i>neurons, gut,</i>	yes	yes
	<i>hypodermis</i>		
<i>SCMmin + pes-10</i>	<i>seam cells</i>	no	no

Depletion of NHR-23 leads to a defective cuticle barrier

We next tested the function of the epidermal barrier using an *nlp-29p::GFP* reporter. *nlp-29* is an antimicrobial peptide expressed following wounding or exposure to particular pathogens (Pujol et al., 2008). Mutations or RNAi that alter the cuticle barrier also trigger *nlp-29* expression (Dodd et al., 2018; Melo and Ruvkun, 2012; Sandhu et al., 2021; Ward et al., 2014, 25). Given the mild morphological and molting defects observed following early NHR-23 depletion in L1 larvae, we reasoned that the cuticle barrier might be intact. The *TIR1* control only had weak *nlp-29p::GFP* expression which was not noticeably different between control and auxin treated animals (Fig. 3A,B). The *nhr-23::AID*, *TIR1* animals on control media had slightly elevated levels of *nlp-29p::GFP* on control media. In contrast, both early and late exposure of *nhr-23::AID*, *TIR1* animals to auxin caused the reporter to be overexpressed, suggesting hypodermal damage in both cases (Fig. 3A,B). We further tested the cuticle integrity in a hypo-osmotic shock assay in which animals with defective cuticle barriers rapidly die following exposure to water (Kage-Nakadai et al., 2010). As this assay typically scores L4s or adults, we grew animals to early L3 (25 hours post-release), shifted animals onto control or auxin plates, and tested sensitivity to hypo-osmotic stress. *TIR1* animals grown on control media or auxin were completely viable in water (Fig. 3C). *nhr-23::AID*, *TIR1* control animals had a weakly penetrant sensitivity to hypo-osmotic shock, while the same strain grown on auxin rapidly died following hypo-osmotic shock (Fig. 3C). Similarly, *nhr-23::AID*, *TIR1* control animals also had mildly elevated *nlp-29p::GFP* expression, which may suggest the strain is a

weak hypomorph. Together, the data suggest that NHR-23 is required for maintenance of the cuticle barrier.

NHR-23 depletion causes larval arrest

nhr-23::AID; *TIR1* animals exposed to auxin at hours 2 and 4 appeared smaller than control animals, suggesting a potential developmental arrest. This arrest was not reversed by removing animals from auxin (unpublished data). Notably, *nhr-23(tm1323)* null homozygotes are reported to arrest in L1 and L2, however the parameters used to

define these arrest points were not defined (Kouns et al., 2011). To test whether NHR-23 depletion triggers a developmental arrest, we used an *hlh-8p::GFP* promoter reporter to monitor the M lineage (Harfe et al., 1998). Newly hatched L1 animals have one M cell which undergoes a stereotypical series of divisions to produce 16 M lineage cells by the L1 molt (Fig. 4A)(Sulston and Horvitz, 1977). To determine whether animals were arresting in the L1 stage, we shifted synchronized *nhr-23::AID; TIR1* animals carrying an *hlh-8p::GFP* reporter onto auxin plates at 3 hours and 9 hours post-release. We imaged animals 24 hours post-release and M cell number was scored. *nhr-23::AID; TIR1* animals exposed to auxin 3 hours post-release had an average count of 13.45 M cells (95% confidence interval=0.6) while the late exposure resulted in an average of 15.55 M cells (95% confidence interval=0.3)(Fig. 4B). As auxin concentrations of 1 mM and above can slow *C. elegans* development (Zhang et al., 2015), we shifted *TIR1, hlh-8p::GFP* animals onto auxin at 3 hours and confirmed that they had a similar number of M cells to animals grown on control plates (Fig. 4B). This difference was statistically significant (two-tailed t-test $p=8 \times 10^{-8}$). The *nhr-23::AID; TIR1* animals exposed to auxin 9 hours post release did not have significantly different M cell number than any of the controls ($p>0.3$)(Fig. 4B). We also observed that the *nhr-23::AID; TIR1, hlh-8p::GFP* animals exposed to auxin at three hours displayed more penetrant molting defects compared to the strain lacking the *hlh-8p::GFP* reporter (unpublished data). This result could indicate that the *hlh-8p::GFP* or the *dpy-20(+)* marker in the integrated transgene may be suppressing the larval arrest phenotype caused by early depletion of NHR-23 in L1 larvae. Regardless, the M cell reporter data indicates that depletion of NHR-23 3 hours post-release causes an L1 arrest.

To test whether this arrest due to early NHR-23 depletion was unique to the L1 stage, we performed similar depletion experiments shifting early L3 animals onto auxin and monitoring development by scoring vulva morphology 24 hours later (Mok et al., 2015). While NHR-23 is expressed in vulval precursor cells, we never saw aberrations in vulval morphology in *nhr-23::AID, TIR1* animals grown on auxin. The majority of *TIR1* animals developed to late L4, with auxin causing a slight developmental delay evidenced by more animals in mid-L4. *nhr-23::AID; TIR1* animals grown on control plates also reached late

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Figure 4: NHR-23 depletion causes larval arrest. (A) Depiction of the M cell mesoblast cell divisions in L1 larvae created with BioRender.com. Newly hatched animals are born with a single M cell, which undergoes the indicated set of divisions to produce 16 descendants at the L1/L2 molt. An *hih-8p::GFP* reporter can be used to monitor M cell divisions in living animals. (B and C) Animals of the indicated genotype were synchronized and shifted to either auxin or control plates at either 3 (early shift) or 9 hours (late shift) post-release and imaged at 24 hours post-release. (B) Representative images of animals of the indicated genotypes on auxin plates. (C) M cell quantification for animals of the indicated genotype shifted to control or auxin plates. Mean and standard deviation are labeled. The number of animals assayed for each genotype and condition is indicated at the top of the graph.

Fig 4 legend, continued

(D) Animals of the indicated genotype were synchronized and shifted onto auxin at 25 hours post-release and imaged 23 hours later (48 hours post-release). Animals were staged based on vulval morphology (Mok et al., 2015). L4 larval stages were grouped as Early L4 (4.0-4.2), Mid L4 (4.3-4.5), and Late L4 (4.6-4.9). The number of animals assayed for each genotype and condition is indicated at the top of the graph. (E) In a repeated experiment animals were synchronized and shifted onto auxin at 25 hours post-release and imaged 48 hours post shift. Representative images of vulva stages observed in animals for each indicated genotype and condition.

mid-L4. In contrast, when *nhr-23::AID*; *TIR1* animals were shifted to auxin, the vast majority of animals were arrested in early L4 with a fraction of the population remaining in L3. Repeating the experiment and scoring developmental progression 48 hours post shift to control or auxin plates confirmed that *nhr-23::AID*; *TIR1* animals grown on auxin were arrested predominantly in L4 (representative image in Fig 4E). *TIR1* animals on either control or auxin plates and *nhr-23::AID*; *TIR1* animals on control plates all reached adulthood (unpublished data. These data indicate that NHR-23 activity is required in multiple larval stages for developmental progression.

Larval arrest following NHR-23 depletion is independent of the insulin signaling pathway

There are well-characterized developmental checkpoints that promote larval arrest in the absence of food (Fielenbach and Antebi, 2008; Kaplan and Baugh, 2016). *daf-16* is a transcription factor which is part of the insulin signaling pathway and is necessary for starvation-induced L1, L3, and L4 arrest (Baugh and Sternberg, 2006; Schindler et al., 2014). Starved *daf-16* mutants fail to arrest at these checkpoints and continue development (Baugh and Sternberg, 2006; Schindler et al., 2014). We crossed a *daf-16(mu86)* null allele into our *TIR-1*; *hlh-8p::GFP* and *nhr-23::AID*; *TIR1*; *hlh-8p::GFP* strains and grew synchronized L1 larvae on control or auxin plates. *daf-16(mu86)*, *TIR1* animals were able to reach adulthood on both control and auxin plates. *nhr-23::AID*, *TIR1*, *daf-16(mu86)* animals reached adulthood on control plates, but were arrested as larvae when grown on auxin. To determine whether this arrest was in L1, we repeated this experiment and monitored the myoblast lineage. *nhr-23::AID*, *TIR1*, *daf-16(mu86)* animals shifted onto control plates had similar M cell counts to *TIR1*, *daf-16(mu86)* animals grown on either control or auxin plates (Fig. 5A). These M cell counts were consistent with animals having made it to the L1 molt. In contrast, *nhr-23::AID*, *TIR1*, *daf-*

16(*mu86*) animals shifted onto auxin averaged 12.85 M cells (95% confidence interval=2), which was significantly different from the controls (two-tailed t-test $p=4 \times 10^{-7}$) and indicative of a larval arrest (Fig. 5A).

Overexpression of the cytochrome P450 gene *daf-9* (*daf-9::GFP*) was shown to act in the *daf-16* pathway and produce an even more robust bypass of the L3 and L4 nutritional checkpoint (Schindler et al., 2014). This strain overexpresses *daf-9::GFP* in the two endocrine XXX cells, though under starvation conditions *daf-9::GFP* will be overexpressed in the hypodermis (Gerisch et al., 2001). NHR-23 depletion did not trigger *daf-9::GFP* hypodermal expression, suggesting that animals were not starving (Fig. S4). The majority of *TIR1; daf-9::GFP* animals shifted to control or auxin plates in early L3 reached the late L4 stage (Fig. 5C). Similarly, *nhr-23::AID, TIR; daf-9::GFP* animals shifted onto control plates reached the late L4 stage (Fig. 5C). When *nhr-23::AID, TIR; daf-9::GFP* animals were shifted onto auxin the majority of animals were in mid-L4, with a smaller fraction of the population in L3, early L4, or late L4 (Fig. 5C). When we repeated the experiment and scored animals 48 hours after the auxin shift, *nhr-23::AID, TIR; daf-9::GFP* animals were still in early to mid L4 suggesting that we were observing an arrest rather than developmental delay (representative image in Fig. 5C). Repeating the experiment and scoring animals 48 hours post-shift onto control or auxin plates confirmed that *nhr-23::AID, TIR; daf-9::GFP* animals were arresting in L4 on auxin plates. While *daf-9::GFP* overexpression allowed animals to progress later in L4 compared to NHR-23 depletion alone, these animals never molted into young adults and we classified this phenotype as an L4 arrest. These results suggest that the larval arrest caused by NHR-23 depletion is independent of the insulin signaling pathway.

NHR-23 regulates oscillating genes involved in aECM biogenesis, lipid transport, and signal transduction

To gain insight into how depletion of NHR-23 at different points in larval development affects molting, we analyzed the expression of NHR-23-regulated genes by mining existing microarray (Kouns et al., 2011) and oscillatory gene expression data (Hendriks et al., 2014)(Table S1). Of the 265 NHR-23 regulated genes, 236 (89%) were oscillatory (Table S1); a similar trend was observed in a recent analysis of these datasets (Tsiairis and Großhans, 2021). In contrast, 10-20% of *C. elegans* genes oscillate in expression (Hendriks et al., 2014; Kim et al., 2013; Meeuse et al., 2020)(Two-tailed Chi-squared test $p < 0.0001$). To test whether these *nhr-23*-regulated oscillating genes were enriched for

particular biological functions, we performed gene ontology analysis which indicated an enrichment in genes implicated in: i) molting and/or cuticle structure; ii) lipid metabolism and transport; iii) protease inhibition; iv) cell adhesion; and v) protein autoprocesing (genes related to hedgehog signaling)(Fig. S5). Interestingly, while the *C. elegans* genome encodes 181 collagen genes (Teuscher et al., 2019), *nhr-23* only regulated 13 collagens but these include all of the furrow collagens implicated in epidermal damage sensing (Table S1; *dpy-2*, *dpy-3*, *dpy-7*, *dpy-10*)(Dodd et al., 2018). Other regulated collagens have well-described roles in body morphology (*sqt-1*, *sqt-2*, *rol-6*)(Kramer and Johnson, 1993). There are also several sets of genes involved in apical extracellular matrix biogenesis and remodeling, such as lipocalins, proteases, protease inhibitors, fibrillin, PAN and ZP domain-containing proteins, and leucine-rich repeat proteins. Although not enriched as a gene ontology class, NHR-23 also regulates several transcription factors implicated in molting or energy metabolism (*peb-1*, *nhr-91*, *dpy-20*)(Clark et al., 1995, 20; Fernandez et al., 2004; Kasuga et al., 2013). Most of the genes in these ontology classes have a peak phase following that of *nhr-23* (Fig. 6C). (modENCODE Consortium et al., 2010). As expected, NHR-23 was enriched in the promoter region of genes (Fig. S6). There was also notable enrichment downstream of the transcriptional end site (Fig. S6). We then examined whether NHR-23 was enriched near *nhr-23*-regulated oscillating genes. We converted the period to developmental time assuming a 9 hour larval stage which makes each hour equivalent to 40°. We separated the genes into three groups based on their peak phase. There were low levels of NHR-23 binding in genes that peaked in expression between 0 and 3 hours (Fig. 6B). For reference, *nhr-23* peaks at 178.11°/4.45 hours (Meeuse et al., 2020). In contrast, the genes that peaked in expression between 3 and 6 hours had much higher NHR-23 binding (Fig. 6B). The genes peaking in expression later (6-9 hours) had reduced NHR-23 binding, but still higher levels than the genes peaking between 0 and 3 hours (Fig. 6B). Thus genes which peak in expression close to when *nhr-23* mRNA levels peak tend to have higher NHR-23 levels upstream, downstream, and within their gene bodies (Fig. 6B).

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Figure 6: NHR-23 regulates oscillating genes involved in aECM biogenesis, lipid transport, and signal transduction. (A) A radar chart plotting amplitude over the phase of peak expression of *nhr-23*-regulated genes from Kouns *et al.* (2011). The dotted circles indicated amplitude, with the innermost circle representing an amplitude of 1 and the outermost circle representing an amplitude of 4. (B) Average signal from NHR-23 ChIP-seq data for *nhr-23*-regulated oscillating genes which peak phases between 0°-120° (Early), 120-240° (Middle), and 240°-360°. For reference, *nhr-23* mRNA peaks at 178.11°. The average NHR-23 signal for all 21,600 *C. elegans* genes (All genes) and the 20% of genes with the lowest expression (No expression) are shown for reference. The mean signal is plotted with a line and the mean's 95% confidence interval is indicated by the shaded area. (C) The data from (A) was plotted as a scatter plot and functionally annotated (see Table S1). To provide developmental context, we converted the 360° oscillation to developmental timing over a 9 hour larval stage, such as the L3 or L4 stage at 20°C. In this context, 1 hour=40° and this converted developmental time is used in B and C. *nhr-23* mRNA has a peak phase of 178.11° (4.45 hours) with an amplitude of 2.11 (Meeuse *et al.*, 2020). The green shaded area represents NHR-23 expression based on RNA-seq (Meeuse *et al.*, 2020), imaging (Figure 1B), and western blotting data (Figure 1C). Dotted lines indicate boundaries between the early, middle, and late *nhr-23*-regulated oscillating genes from B.

Discussion

C. elegans molting is a powerful model to understand how a large-scale gene oscillator is integrated into rhythmic epidermal regeneration. In this study, we identify distinct windows of NHR-23 activity required for initiation and execution of the molt program and formation of the epithelial barrier.

A model for the role of NHR-23 in molting

Depletion of NHR-23 early in a larval stage before NHR-23 levels start rising produces a larval arrest with only weakly penetrant molting defects (Fig 2). In contrast, depletion after NHR-23 levels have risen produces molting and developmental defects (Fig. 2). The first larval stage appeared to be most sensitive to NHR-23 depletion, as all animals appeared to arrest in the L1 stage. In contrast, depletion at the start of L2 produced arrests in both L2 and L3 larvae, and depletion at the start of L3 produced arrests in L3 and L4 larvae. It is unclear whether these differences are technical, such as NHR-23 depletion is more effective in L1 because of smaller animal volume or the longer duration of the L1 stage affords more time to deplete NHR-23 below a critical threshold. Alternatively, NHR-23 might play a more prominent role in L1 vs other larval stages.

Larval arrest following NHR-23 depletion was not suppressed by *daf-16* inactivation, so is not an insulin/IGF-regulated development checkpoint. There were no molting defects nor arrests observed when animals were exposed to auxin following the NHR-23 peak in expression, suggesting that these animals were able to molt and arrest in the next larval stage. NHR-23 depletion phenotypes might represent defects in molting initiation (early depletion) or molting execution (late depletion). The arrest caused by early NHR-23 depletion might reflect failure to activate the next tier of a gene regulatory program. This model would be consistent with insect molting, in which the hormone ecdysone triggers a regulatory cascade driven by nuclear hormone receptors, including the NHR-23 ortholog DHR3 (King-Jones and Thummel, 2005; Lam et al., 1997; Ruaud et al., 2010; Thummel, 1990). Consistent with this model, *nhr-23* regulates *peb-1* and *dpy-20*, transcription factors with known roles in molting (Clark et al., 1995, 20; Fernandez et al., 2004, 1). *nhr-23* also regulates *nhr-91* (Kouns et al., 2011)(Table S1), a nuclear hormone

receptor involved in blast cell progression in response to nutrients (Kasuga et al., 2013). An alternate, non-exclusive model is that physiological defects trigger an arrest. Early *nhr-23*-regulated genes include a set of apical ECM components required to preserve epithelial integrity during morphogenesis (*fbn-1*, *sym-1*, *let-4*, *noah-1*, *noah-2*) (Kelley et al., 2015, 1; Vuong-Brender et al., 2017). An equivalent structure may be required in larvae prior to apical ECM remodeling to ensure that the internal turgor pressure does not cause epithelial rupture. One intriguing possibility is that the earlier NHR-23-regulated genes may be more sensitive to NHR-23 levels. This model would align with *E. coli* amino acid biosynthesis gene regulation where enzymes earlier in the pathway have more responsive promoters with higher activity (Zaslaver et al., 2004). Accordingly, there is an enrichment for NHR-23 binding flanking and within oscillating genes that peak in expression close to the NHR-23 peak in expression (Fig. 6B). However, formal testing of this model would require an intensive study involving biochemical assessment of NHR-23 affinity for candidate binding sites and experimental manipulation *in vivo* of the candidate binding sites.

Later depletion of NHR-23 might allow the molt to initiate, but the loss or reduction of critical aECM components and remodeling factors, signaling proteins, and metabolic enzymes could cause the molt to fail resulting in the observed developmental and ecdysis defects (Fig. 6C). *nhr-23* regulates the expression of five lipocalin genes all peaking within a 15° period (equivalent to 15 minutes in a 9 hour larval stage) (Table S1). Two of these lipocalins (LPR-1 and LPR-3) are required for aECM organization, with LPR-3 playing an additional role in ecdysis (Forman-Rubinsky et al., 2017). Some lipocalins can bind matrix metalloproteases (Leng et al., 2009; Yan et al., 2001). Notably, *nhr-23* also regulates *nas-37*, a conserved matrix metalloprotease with roles in ecdysis (Davis et al., 2004) as well as two uncharacterized predicted proteases (*nas-4*, *spg-7*). Metalloprotease activity must presumably be tightly regulated to prevent premature ecdysis. *mlt-11* is a poorly characterized protease inhibitor with nine Kunitz protease inhibitor domains, and peaks in expression prior to these proteases so might function to regulate the timing of ecdysis. *nhr-23*-regulated genes predicted to function in lipid metabolism or transport could also contribute to the late NHR-23 depletion phenotype. There are a number of hedgehog-

related (*qua-1*, *wrt-1*, *wrt-4*) and Patched or Patched-related genes (*ptc-3*, *ptr-4*, *ptr-10*, *ptr-11*, *ptr-18*), a subset of which are implicated in molting or lipid transport (Chiyoda et al., 2021, 4; Cohen et al., 2021, 4; Hao et al., 2006; Zugasti et al., 2005). It has been hypothesized that a sterol ligand regulates nematode molting as cholesterol depletion in *C. elegans* phenocopies *nhr-23* inactivation and insect molting is initiated by a pulse of the steroid hormone ecdysone (Lažetić and Fay, 2017; Thummel, 1990); *nhr-23* regulates two genes predicted to be involved in steroid metabolism (*dhs-16*, *mboa-1*) (Kouns et al., 2011) (Table S1). Future analysis of these candidate *nhr-23* targets will illuminate how collagen secretion, apical ECM remodeling, apolysis, and ecdysis are coordinated with developmental progression.

NHR-23 is necessary for epithelial barrier establishment and/or maintenance

Early NHR-23 depletion caused sensitivity to hypo-osmotic stress and activation of an *nlp-29p::GFP* reporter (Fig 3). These data suggest that despite looking superficially wild-type that there are cuticular abnormalities. There are numerous *nhr-23*-regulated genes which are implicated in the epithelial barrier. The cuticle furrow formed by six *nhr-23*-regulated collagens (*dpy-2*, *dpy-3*, *dpy-7*, *dpy-8*, *dpy-9*, *dpy-10*) is thought to be monitored by a sensor which coordinates a number of stress responses following detection (Dodd et al., 2018). An RNAi screen of 91 collagens found that inactivation of only these six collagens caused a barrier defect (Sandhu et al., 2021). Inactivation of a subset of the furrow collagens also causes elevated *nlp-29p::GFP* reporter activity (Dodd et al., 2018), consistent with our NHR-23-depletion data (Fig. 3). *bus-8* is a predicted glycosyltransferase that plays a role in the epithelial barrier and is also *nhr-23*-regulated and its peak expression follows that of the furrow collagens (Table S1). Screening *nhr-23* regulated genes may find other genes implicated in the epithelial barrier and the peak phase could provide insight into how this barrier is constructed.

NHR-23 isoforms

We detected three bands on our western blots, the size of which are consistent with isoforms confirmed by Nanopore direct mRNA sequencing (NHR-23b/f, NHR-23c, NHR-23e) (Roach et al., 2020). NHR-23b/f is predicted to contain an N-terminal extension with

an alpha helix and an unstructured domain not found in NHR-23c (Fig. S7,S8). In many NHRs, unstructured regions in the N-termini, C-termini, or flexible region between the DBD and LBD (hinge region) contain co-regulator docking sites (Krasowski et al., 2008). It's possible that these isoforms form different transcriptional regulatory complexes. Alternatively, the sequences flanking the DBD can impact binding site preference of NHRs (Li et al., 2017; Simmons et al., 2008), meaning that these isoforms could regulate different subsets of genes.

The most robustly expressed isoform is consistent with the size of NHR-23e, which peaks prior to an abrupt decrease in all NHR-23 isoforms levels prior to the molt. NHR-23e lacks all critical residues required for forming a functional DNA-binding domain, but contains the hinge region and LBD (Fig. S7). A subset of *C. elegans* NHRs (36/278; Tables S2,3) also have isoforms that lack DNA binding domains. This group includes 10 of the 34 functionally characterized NHRs, with several involved in nutrient sensing, dietary restriction-mediated longevity, metabolism, and energy homeostasis (DAF-12, NHR-23, NHR-25, NHR-49, NHR-62, NHR-68, NHR-91, NHR-114)(Antebi, 2015; Gerisch and Antebi, 2004, 12; Gracida and Eckmann, 2013; Kasuga et al., 2013; Macneil et al., 2013; Mullaney et al., 2010; Van Gilst et al., 2005a; Van Gilst et al., 2005b). While the function of these DBDless isoforms in *C. elegans* has not been determined, they may function as dominant-negative repressors. A well-characterized transcriptional regulatory mechanism involves transcription factor dimerization where one of the monomers lacking a DBD can still heterodimerize but serves as a “poison” subunit (Amoutzias et al., 2008).

Is NHR-23 a cog in a gene expression oscillator?

Recent work has revealed that ~10-20% of the *C. elegans* transcriptome undergoes rhythmic oscillations, peaking each larval stage and likely controlling molting (Hendriks et al., 2014; Kim et al., 2013; Meeuse et al., 2020). This process of rhythmic skin regeneration has interesting parallels to postnatal mammalian hair follicle regeneration as both involve non-circadian oscillations and are regulated by BLMP-1/BLMP1 (Hauser et al., 2021; Paus and Foitzik, 2004; Stec et al., 2021). How the *C. elegans* gene expression oscillator functions is only starting to be determined. The conserved

transcription factor BLMP-1/BLIMP1 shapes oscillatory gene expression (Hauser et al., 2021) and acts as a pioneer factor to decompact chromatin (Stec et al., 2021). NHR-23 is a strong candidate to regulate a subset of molting genes in this oscillator. ChIP-seq data indicates that BLMP-1 binds upstream of the NHR-23 transcription start site (modENCODE Consortium et al., 2010). RNA-seq time courses indicate that *nhr-23* is one of the earliest expressed transcription factors implicated in molting (Hendriks et al., 2014; Meeuse et al., 2020). *nhr-23*-regulated genes are enriched for molting factors (Fig 6, Table S1)(Kouns et al., 2011), and 236 of the 265 NHR-23-regulated genes oscillate (89%). The mammalian homolog of NHR-23 (RORalpha) regulates circadian rhythms, and both NHR-23 and RORalpha proteins oscillate (Akashi and Takumi, 2005)(Figure 1).

Analysis of the *C. elegans* oscillator indicates that it operates on a Saddle Node on Invariant Cycle (SNIC) bifurcation (Meeuse et al., 2020). An important characteristic of a SNIC oscillator is arrest at specific phases, and the *C. elegans* oscillator can be arrested at points corresponding with developmental checkpoints in each larval stage. Notably, NHR-23 levels are at their lowest during these arrest points. Meeuse et al. hypothesized that signals linked to metabolism or the nutrition state of the animal control the oscillator at these checkpoints. Consistent with NHR-23 playing a role in the oscillator, it controls developmental rate in response to diet likely through modulation of NHR-23 activity, not levels (Macneil et al., 2013). Consistent with this model, the mammalian orthologs of NHR-23 bind various sterol ligands (Kallen et al., 2002; Kallen et al., 2004; Soroosh et al., 2014) and a ligand has been suggested to drive molting (Lažetić and Fay, 2017). A SNIC oscillator is also characterized by interlinked positive and negative feedback loops. In addition to the DBD-less isoform, which could function as a dominant negative regulator, *nhr-23* is negatively regulated by the *let-7* microRNA and also positively regulates this microRNA (Patel and Frand, 2018). This feedback loop represents a point where the heterochronic pathway controlling developmental timing intersects with the molting program. NHR-23 levels drop during the molt and NHR-23-depleted animals shifted off auxin do not resume development, suggesting that NHR-23 is not likely responsible for restarting the oscillation during a checkpoint but rather promoting

epidermal remodeling. Going forward, determining the factors that promote *nhr-23* expression will be critical for defining how the oscillator functions.

Future perspectives

This work highlights the power of timed protein depletion for dissecting the role of oscillating developmental regulators in development. We have found distinct phenotypes depending on when we deplete NHR-23 in a given larval stage. Going forward it will be important to determine whether NHR-23 acts cell-autonomously or non-autonomously, and identify the critical tissues in which NHR-23 activity is necessary. As transcription factors coordinate the expression of batteries of genes in a given biological process, future work will reveal how NHR-23 coordinates apical ECM remodeling, apolysis, and ecdysis. NHR-23 oscillates in the soma yet is constitutively expressed in the germline (Ragle et al., 2020). Exploring the differences between oscillatory and constitutive NHR-23 expression could shed light on mammalian gene regulation, as several circadian rhythm regulators oscillate in the soma but are constitutively expressed in the testes (Alvarez et al., 2008; Kang et al., 2010; Kennaway et al., 2012; Morse et al., 2003)

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Materials and Methods

C. elegans Strains and Culture

C. elegans strains were cultured as originally described (Brenner, 1974), except worms were grown on MYOB instead of NGM. MYOB was made as previously described (Church et al., 1995). Animals were cultured at 20°C for all assays, unless otherwise indicated. For general strain propagation, animals were grown at 15°C according to standard protocols.

Strains used in this study

Strain	Genotype
JDW3	<i>nhr-23(wrd1[GFP^ΔBioTag-degron-3xFLAG]) I; ; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+)] II</i> (Ragle et al., 2020)
JDW230	<i>wrdSi40 [SCMp[*]::TIR1::F2A::mTagBFP2::AID[*]::NLS::tbb-2 +SEC II:0.77]</i> (Ashley et al., 2021)
JDW258	<i>wrdSi22 [eft-3p::TIR1::F2A::mTagBFP2::AID[*]::NLS::tbb-2 3'UTR+SEC, I:-5.32] ; nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG)) I</i> (Ashley et al., 2021)
JDW290	<i>nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG))I;</i> <i>re313 [col-10p::TIR1::F2A::mTagBFP2::AID[*]::NLS::tbb-2 3'UTR] II:0.77, II</i>
JDW291	<i>nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG))I;</i> <i>reSi7 [rgef-1p::TIR1::F2A::mTagBFP2::AID[*]::NLS::tbb-2 3'UTR I:-5.32], I</i>
JDW292	<i>nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG))I; ieSi61</i> <i>[ges-1p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II.</i>
JDW294	<i>nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG))I; cpSi171</i> <i>[vha-8p::TIR1::F2A::BFP::AID[*]::NLS::tbb-2 3'UTR I:-5.32] I</i>
JDW310	<i>nhr-23(kry61(nhr-23::AID[*]-TEV-3xFLAG))I; wrdSi40</i> <i>[SCMp[*]::TIR1::F2A::mTagBFP2::AID[*]::NLS::tbb-2 +SEC II:0.77]</i>

JDW311	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I; wrdSi44 [SCMp*::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR, II:0.77 ttTi5605]</i>
JDW348	<i>ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; frls7 [nlp-29p::GFP + col-12p::DsRed] IV</i>
JDW349	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; frls7 [nlp-29p::GFP + col-12p::DsRed] IV</i>
JDW350	<i>ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; dhls64 [daf-9p::daf-9::GFP + lin-15(+)]</i>
JDW351	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; dhls64 [daf-9p::daf-9::GFP + lin-15(+)]</i>
JDW352	<i>ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>
JDW353	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+?)] II; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>
JDW354	<i>wrdSi60 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR, I:-5.32] I; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>
JDW355	<i>wrdSi61 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR, I:-5.32] ; nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG)) I; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>
JDW356	<i>wrdSi59 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR, I:-5.32], daf-16(mu86) I; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>
JDW357	<i>wrdSi59 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR, I:-5.32], nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG)), daf-16(mu86) I; ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i>

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KRY87	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I</i> (Zhang et al., 2015)
KRY88	<i>nhr-23(kry61(nhr-23::AID*-TEV-3xFLAG))I; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+)] II</i> (Zhang et al., 2015)

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Strains provided by the CGC

Strain	Genotype
AA277	<i>lin-15B&lin-15A(n765) X; dhls64 [daf-9p::daf-9::GFP + lin-15(+)]</i> (Gerisch and Antebi, 2004)
PD4666	<i>ayls6 [hlh-8::GFP fusion + dpy-20(+)] X</i> (Harfe et al., 1998)
CA1200	<i>unc-119(ed3); ieSi57 [Peft-3::TIR1::mRuby::unc-54 3'UTR, cb-unc-119(+)] II</i> (Zhang et al., 2015)
CA1209	<i>ieSi61 [ges-1p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II</i>
CF1038	<i>daf-16(mu86) I</i> (Lin et al., 1997)
DV3800	<i>reSi2 [col-10p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR II:0.77]</i> (Ashley et al., 2021)
DV3805	<i>reSi7 [rgef-1p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]</i> (Ashley et al., 2021)
JDW118	<i>wrdSi22 [eft-3p::TIR1::F2A::mTagBFP2::tbb-2 3'UTR+SEC, I:-5.32]</i> (Vo et al., 2021)
JDW224	<i>wrdSi22 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR+SEC, I:-5.32]</i> (Ashley et al., 2021)
LP869	<i>cpSi171 [vha-8p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]</i> (Ashley et al., 2021)

864

865 **Auxin treatment**

866 Control and auxin media/plates were made as described in Ragle et al. 2020. Control
867 media consisted of MYOB agar+0.25% ethanol. Auxin media was made by dissolving
868 indole 3-acetic acid (Alfa Aesar, AAA1055622) in 100% ethanol to 1.6 M and then mixing
869 it into melted MYOB agar at 55°C to a final concentration of 4 mM prior to pouring plates.
870 Temperature of the media was monitored with a Lasergrip 1080 infrared thermometer gun
871 (Etekcity). Plates were seeded with *E. coli* OP50 and incubated overnight at room
872 temperature. Plates were stored for up to one month at 4°C prior to use. For all auxin
873 treatment experiments, animals were synchronized by alkaline bleaching. The collected
874 eggs were incubated in M9 buffer supplemented with 5mg/mL cholesterol at 20°C for 24
875 hours and arrested L1 larvae were released onto the indicated type of MYOB plate.

876

877 **Microscopy**

878 Synchronized animals were collected from MYOB, control, or auxin plates at the indicated
879 time points using 1000µl of M9 + 2% gelatin. Animals were spun at 700xg for 1 min. The
880 media was then aspirated off and animals were resuspended in 500µl M9 + 2% gelatin
881 with 5 mM levamisole. 12µl of animals in M9 +gel with levamisole solution were placed
882 on slides with a 2% agarose pad and secured with a coverslip. Images were acquired
883 using a Plan-Apochromat 40x/1.3 Oil DIC lens on an AxioImager M2 microscope (Carl
884 Zeiss Microscopy, LLC) equipped with a Colibri 7 LED light source and an Axiocam 506
885 mono camera. Acquired images were processed through Fiji software (version: 2.0.0- rc-
886 69/1.52p). For direct comparisons within a figure, we set the exposure conditions to avoid
887 pixel saturation of the brightest sample and kept equivalent exposure for imaging of the
888 other samples.

889

890 **Phenotypic analysis**

891 For the phenotypic analysis in Figure 2, synchronized CA1200 or KRY88 larvae were
892 released onto MYOB plates and then shifted onto control or auxin plates every two hours
893 up to 16 hours. Animals were collected as described in the Microscopy section and
894 imaged by DIC microscopy to score for morphology and shedding of the L1 cuticles. For

the M cell lineage experiments in Figures 4C and 5A, synchronized animals of the indicated genotypes were released onto MYOB plates and shifted onto control or auxin plates at 3 hours (early shift) or 9 hours (late shift) post-release and then imaged at 24 hours post-release, as described in the Microscopy section. M cells were counted and recorded. For the L3 shift experiments (Fig. 4D,5B) synchronized animals of the indicated genotype were grown on MYOB. At 25 hours post-release, they were shifted onto 6-well control or auxin MYOB plates seeded with OP50. 24 hours later, animals were collected as described in the Microscopy section and imaged by DIC microscopy to stage animals according to vulval morphology (Mok et al., 2015).

Hypo-osmotic stress sensitivity

Hypo-osmotic stress sensitivity assays were performed on L4 stage larvae as described (Ward et al., 2014), except we used 20µl of dH₂O. Each strain was assayed in triplicate.

Western Blots

Animals were synchronized by alkaline bleaching, as described in the Auxin Treatment section. For the western blots in Figure 1C and 2B, 6000 KRY88 animals per well were transferred onto a 6-well MYOB plate seeded with OP50. Animals were cultured at 20°C and collected at the indicated timepoints as previously described by washing animals out of the well with M9+0.05% gelatin (VWR, 97062-620), transferring to a 1.5 ml tube and washing twice more with M9+0.05% gelatin (Vo et al., 2021). Animals were pelleted, transferred in a 30 µl volume to a new 1.5 ml tube, and flash frozen in liquid nitrogen. For the western blot in Figure 2B, synchronized KRY87 animals were grown at 20°C on MYOB plates, before being washed off in M9+0.05% gelatin. A 0 minute time point was collected as described above, and then 6000 animals were transferred into the wells of a 6-well control or auxin plate seeded with OP50. Samples were collected at 15 and 30 minutes following transfer into the well, as described above. For the western blots in Figure 3B, 500 animals of the indicated genotype were transferred into wells of a 6-well control or auxin plate seeded with OP50. Animals were incubated for 24 hours at 20°C and collected as described above. To generate the lysate for western blot analysis, animals were freeze-cracked twice in liquid nitrogen, and Laemmli sample buffer was

added to 1X and samples were heated to 95°C for 10 minutes. The resulting supernatants were used for western blot analysis, as described below.

For the blots in Figures 1C and 2B 10 µl of lysate and 7.5 µl 1:1 mix of Amersham ECL Rainbow Molecular Weight Markers (95040-114) and Precision Plus Protein Unstained Standards (1610363) was resolved by SDS-PAGE using precast 4-20% MiniProtean TGX Stain Free Gels (Bio-Rad). For the blot in Figure 3B, the lysates had more variable levels of total protein, particularly the NHR-23 depleted samples that produced an early larval arrest. Therefore we quantified the signal of the most intense band by Stain Free imaging (Posch et al., 2013) using a Bio-Rad ChemiDoc imaging system, and ran a new gel with normalized loading. Proteins were transferred to a polyvinylidene difluoride membrane by semi-dry transfer with a TransBlot Turbo (Bio-Rad). Total protein, pre- and post-transfer, was monitored using the stain-free fluorophore as described (Posch et al., 2013; Ward, 2015a). Membranes were washed in TBST and blocked in TBST+5% milk (TBST-M; Nestle Carnation Instant Nonfat Dry Milk, 25.6-Ounce, Amazon) for one hour at room temperature. Blots were rocked in primary antibodies in TBST-M overnight at 4°C and then washed 4x5min with TBST. For primary antibodies conjugated with horseradish peroxidase (HRP) the blots were developed after the last TBST wash. Otherwise, blots were incubated with HRP-conjugated secondary antibodies in TBST-M for one hour at room temperature followed by 4x5min TBST washes and then developed as described below.

The primary antibody used for Figure 1C and 2B was horseradish peroxidase (HRP) conjugated anti-FLAG M2 (Sigma-Aldrich, A8592-5x1MG, Lot #SLCB9703) at a 1:2000 dilution. Precision Protein StrepTactin-HRP Conjugate (Bio-Rad, #1610381, Lot #64426657) was included with the primary antibody at a 1:10,000 dilution to visualize the protein size standard during blot imaging. For the blot in Figure 3B, we used the following 1° antibodies diluted at 1:2000: rabbit anti-GFP antibody (Sigma Millipore, C1544-100µg) and mouse anti-alpha-Tubulin 12G10 (Developmental Studies Hybridoma Bank, we used the “-c” concentrated supernatant). The secondary antibodies were Digital anti-Rabbit (Kindle Biosciences LLC, R1006) and Digital anti-mouse (Kindle Biosciences LLC,

R1005), both diluted 1:1000. Streptactin-HRP was included at 1:1000 with the 2° antibody incubations, similar to the anti-FLAG M2-HRP blots. Blots were incubated for 5 minutes with 1 ml of Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095) and the final blot were imaged using the 'chemi high-resolution' setting on a Bio-Rad ChemiDoc MP System.

Bioinformatics

Figure 6A was generated in R (R Core Team). Phase and amplitude in Table S1 were converted to x and y coordinates for each gene by calculating $x = \text{amplitude} * \cos(\text{phase})$ and $y = \text{amplitude} * \sin(\text{phase})$. For Figure 6B and S6, L3 NHR-23 ChIP-Seq data was downloaded from <http://www.modencode.org>, accession ID modEncode_3837. Wig files were converted to bigwig format and loaded into the Bioconductor package SeqPlots (cite_SeqPlots)(Huber et al., 2015; Stempor and Ahringer, 2016). A bed file with the start and end coordinates of all genes' CDS was generated and loaded into SeqPlots. SeqPlots aligned all genes by the start and end position and scaled the coding sequence of each gene to 2 kb. The average signal over all aligned genes was calculated in 25 bp windows from 1 kb upstream of the start site to 1kb downstream of the end site. For Fig. 6C the phase for the oscillating genes in Table S1 was converted to time during the molting cycle by assuming a 9 hour larval stage which make each hour=40°. The phase in hours was then plotted along the x axis and the oscillating genes' amplitude was plotted on the y axis. The gene annotation was based on the Concise Description and Automated Description downloaded from Wormbase.

Statistical Analysis

Statistical tests and numbers of animals analyzed are detailed in figure legends.

Competing interests

The authors declare no competing or financial interests.

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