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Indicted: Worms Caught using Steroids

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Three recent papers provide new insights into endocrinology in the worm *Caenorhabditis elegans*. These studies identify natural steroid ligands for the DAF-12 nuclear receptor, define a new enzyme in the hormone biosynthetic pathway, and clarify the role of endocrine signaling in adult longevity.

Many animals rely on some form of diapause to cope with difficult environmental circumstances, allowing them to survive until conditions improve and they can return to normal reproductive life. Diapause may seem to be a developmental anomaly in a life cycle; however, detailed genetic studies in the nematode worm *Caenorhabditis elegans* have shown that this is not the case. Rather, the regulation of *C. elegans* diapause has provided key insights into the progression of normal postembryonic development—growth, sexual maturation, and senescence—critical steps in the life cycle of higher organisms.

Increased temperature, crowding, or nutritional depletion during the early stages of *C. elegans* development leads to the formation of an alternate third larval stage, the dauer larva, which is specially adapted for long-term survival. Upon a return to favorable conditions, the dauer larva emerges from diapause, resumes feeding, and continues to develop into an adult with a normal life span. Detailed studies have defined a genetic circuit that relays cues from chemosensory neurons to signal-transduction pathways that direct the choice between normal reproductive development and the dauer diapause. These studies arose from screens for mutants defective in their ability to form dauer larvae (Daf-d) or mutants that form constitutive dauers under favorable conditions (Daf-c; Riddle and Albert, 1997). Epistasis tests placed these genes in a pathway, with parallel input from TGF-β and insulin/IGF (insulin-like growth factor) signaling. This regulation makes sense as it is critical for the animal to properly assess its nutritional status prior to entering the prolonged period of diapause. During normal development, the DAF-2 insulin receptor acts through an evolutionarily conserved PI 3-kinase/AKT pathway to phosphorylate the transcription factor DAF-16, the worm ortholog of FOXO. This prevents DAF-16 translocation to the nucleus and allows normal growth to proceed (Figure 1A). In unfavorable environments, insulin/IGF signaling becomes inactive, and dephosphorylated DAF-16 is translocated to the nucleus where it blocks growth and directs dauer formation (Figure 1B). Similarly, in unfavorable conditions, the TGF-β ligand DAF-7 acts through the type II TGF-β receptor DAF-4 to regulate the downstream effectors DAF-3 (SMAD) and DAF-5 (SKI/SNO), promoting dauer formation. These parallel insulin and TGF-β signaling inputs act in a cell nonautonomous manner to control reproductive growth and converge on two genes at the end of the pathway, *daf-9* and *daf-12*, with *daf-9* acting upstream from *daf-12*.

An Endocrine Model for Reproductive Growth

The identification of *daf-9* as encoding a CYP2 cytochrome P450 enzyme and *daf-12* as encoding a nuclear hormone receptor, provided a mechanism for the cell nonautonomous relay of insulin and TGF-β inputs (Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002; Mak and Ruvkun, 2004). Based on the ability of mammalian CYP2 enzymes to metabolize steroid hormones and the identification of steroid-hormone ligands for the vertebrate orthologs of DAF-12, an endocrine signaling model was proposed in which a hormone from DAF-9 is received by DAF-12 to direct reproductive growth (Figure 1A). In unfavorable conditions, DAF-9 was proposed to be inactive, leading to a presumed repressive function for the unliganded DAF-12, directing the dauer fate (Figure 1B).

Several lines of evidence support this model. First, *daf-9* acts nonautonomously and is expressed in endocrine cells or tissues that include the hypodermis, spermatheca, and a pair of cells located in a head ganglion (Gerisch and Antebi, 2004; Jia et al., 2002; Mak and Ruvkun, 2004), whereas DAF-12 is widely expressed, consistent with its role in executing multiple developmental programs (Antebi et al., 2000). Second, the phenotypes of *daf-9* mutants resemble *daf-12* mutants that map to critical DAF-12 amino acids that are predicted to act as hormone contact sites (Gerisch et al., 2001; Jia et al., 2002). Third, cholesterol deprivation mimics *daf-9* and *daf-12* mutations and leads to a few animals that form dauer-like larvae (Gerisch et al., 2001; Jia et al., 2002; Matyash et al., 2004). Fourth, mutations in *ncr-1* and *ncr-2*, homologs of the human Niemann-Pick type C gene that are required for proper cholesterol trafficking in *C. elegans*, form transient dauer larvae that resemble cholesterol-deprived animals (Li et al., 2004). Finally, crude lipid extracts can rescue *daf-9* mutant phenotypes as well as the dauers that form in the absence of dietary cholesterol (Gill et al., 2004; Matyash et al., 2004). Taken together, these lines of evidence provide strong support for DAF-9 and DAF-12 acting in an endocrine signaling pathway that regulates postembryonic development.

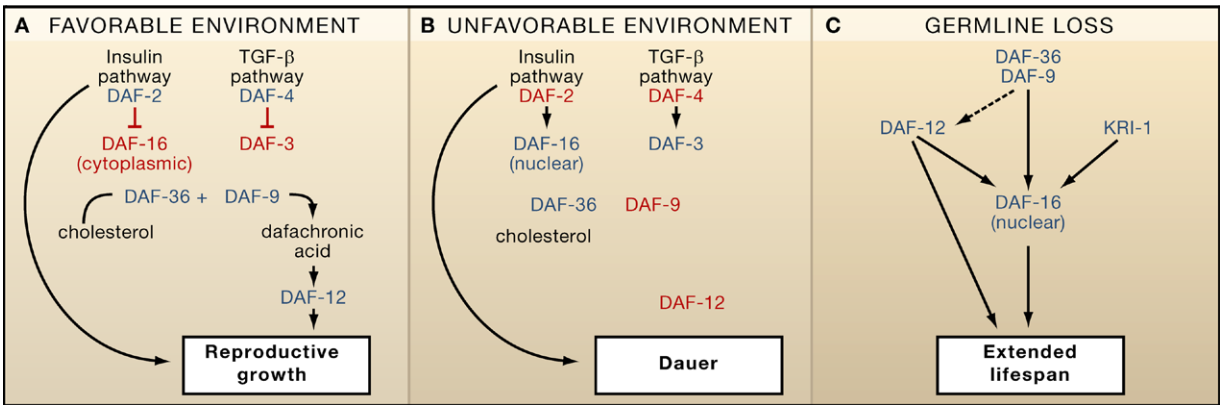

Figure 1. Regulation of Reproductive Growth, Dauer Formation, and Increased Longevity Due to Germline Ablation in the Worm
(A) Blue indicates that the factor is active; red indicates inactive. In favorable conditions, insulin signaling through the DAF-2 receptor restricts the DAF-16 FOXO transcription factor to the cytoplasm, promoting reproductive growth of the worm *C. elegans*. Active TGF-β signaling through the DAF-4 receptor inhibits the DAF-3 SMAD. In response to these parallel inputs, the DAF-36 Rieske-like oxygenase and DAF-9 cytochrome P450 enzyme produce dafachronic acid ligands that activate the DAF-12 nuclear receptor, directing reproductive growth. (B) In unfavorable conditions, DAF-16 translocates to the nucleus where it regulates genes that block growth. DAF-9 is inactive under these conditions, with the unliganded DAF-12 acting as a repressor. Only the parallel and upstream functions of DAF-2 are depicted in (A) and (B) for simplicity. (C) DAF-9, DAF-12, and KRI-1 act through DAF-16 to extend the life span of germline-deficient animals. Although it is likely that DAF-36 acts upstream of DAF-9 in this pathway, no studies have been done to place DAF-36 in this pathway.

Temporal Identity and Aging: DAF-9 and DAF-12

Reproductive growth also involves the proper temporal progression of development in the third and fourth larval stages. This is reflected by the incorrect coordination of developmental programs in some *daf-12* mutants, giving phenotypes that are referred to as heterochronic because of their effects on developmental timing (Antebi et al., 2000). In addition, the DAF-9/DAF-12 pathway regulates longevity during adult stages. Germline ablation of *C. elegans* results in up to a 60% increase in adult longevity (Hsin and Kenyon, 1999). This is not due to sterility as removal of both the germline and somatic gonad does not lead to an extended adult life span. In addition, the link between the germline and adult longevity is not unique to worms because similar effects have been seen in *Drosophila* and mice (Kenyon, 2005). This coupling could have beneficial effects for survival of the species by allowing the animal to adjust its aging in response to its ability to reproduce. The effect of germline ablation on life span goes through the DAF-16 transcription factor which mediates the output of insulin/IGF and, presumably, other signaling pathways (Hsin and Kenyon, 1999; Kenyon, 2005; Tatar et al., 2003; Figure 1C). One of these pathways is associated with *daf-9* and *daf-12*, which appear to act together with *daf-16* to modulate the effects on life span seen in germline-deficient animals. The exact nature of these interactions remains unclear.

Endocrine Model Confirmed: Ligands for DAF-12

Motola et al. (2006) usher in the age of *C. elegans* molecular endocrinology by identifying the first steroid hormones in this organism—the much-awaited ligands for the DAF-12 nuclear receptor. They exploit the past studies of DAF-9 and DAF-12, using the enzyme and receptor as tools to identify small chemical compounds that link one with the other. Their initial screen showed that 3-keto-lithocholic acid, but not lithocholic acid, weakly activates DAF-12 in a tissue culture cotransfection assay, independently of coexpressed DAF-9. The identification of a C-3 ketone suggested that 3-keto-sterols may function as DAF-12 ligands. This hypothesis was confirmed by showing that two naturally occurring 3-keto sterols—4-cholesten-3-one (an oxidation product of cholesterol) and lathosterone (a *C. elegans* cholesterol metabolite)—could activate DAF-12 in the presence, but not the absence, of DAF-9. In addition, either 4-cholesten-3-one or lathosterone that had been incubated with microsomes containing DAF-9 resulted in a complete rescue of *daf-9* null mutants. This rescue was dependent upon using 3-keto-sterols as precursors and having DAF-9 in the microsomes.

Further analysis of the DAF-9 metabolites of 4-cholesten-3-one and lathosterone identified these compounds as 3-keto-4-cholestenoic acid and 3-keto-7,(5α)-cholestenoic acid, respectively. The observation that these compounds are 3-keto, C-26 oxidized derivatives of cholesterol is consistent with biochemical studies of DAF-9, which showed that it acts as a 3-keto-sterol-26-monooxygenase that modifies 3-keto-sterols through successive side chain oxidation steps to generate DAF-12 ligands. The authors named these ligands Δ^4-dafachronic acid (for 3-keto-4-cholestenoic acid) and Δ^7-dafachronic acid (for 3-keto-7,(5α)-cholestenoic acid), based on the dauer and heterochronic phenotypes of *daf-12* mutants.

Chemical synthesis of Δ^4-dafachronic acid allowed Motola et al. (2006) to show that this compound can effectively activate DAF-12 in cotransfection assays and rescue *daf-9* mutant phenotypes at nanomolar concentrations, demonstrating potent biological activity. Intermediate concentrations of Δ^4-dafachronic acid resulted

in only a partial rescue of *daf-9* phenotypes, suggesting that hormone levels are critical for DAF-12 function. In addition, Δ^4-dafachronic acid had little effect on *daf-12* mutants that carry mutations predicted to affect ligand binding, consistent with the hormone directly regulating receptor activity. Consistent with classic models for nuclear receptor regulation, Δ^4-dafachronic acid blocked the interaction between DAF-12 and its corepressor, DIN-1, and significantly enhanced the ability of DAF-12 to recruit the mammalian coactivator SRC-1. This effect of Δ^4-dafachronic acid on DAF-12 cofactor interactions also translated to DAF-12 activity, resulting in potent induction of a DAF-12-regulated luciferase reporter gene in cultured cells. Taken together, these studies suggest that Δ^4-dafachronic acid directly binds to DAF-12 to influence its interactions with cofactors and thereby regulate its transcriptional activity. Binding to Δ^4-dafachronic acid occurs with a high affinity characteristic of natural nuclear receptor ligands. Importantly, Motola et al. (2006) showed that 3-keto-cholestenoic acids could be isolated from extracts of wild-type worms, but not *daf-9* mutants, and that these molecules can activate DAF-12 and fully rescue *daf-9* mutants, defining them as natural hormones for DAF-12.

The identification of DAF-12 ligands enabled Motola et al. (2006) to determine the biological activity of dafachronic acid in the *daf-2*/insulin and *daf-4*/TGF-β pathways. Δ^4-dafachronic acid rescued the phenotypes of both *daf-7* and weak *daf-2* mutants, in agreement with epistasis studies that position these genes upstream from *daf-9* and *daf-12* in the dauer pathway. Interestingly, Δ^4-dafachronic acid was unable to fully rescue a strong *daf-2* mutant, suggesting that the insulin pathway can also act downstream of, or parallel to, *daf-12* (Figures 1A and 1B), confirming genetic studies which indicate that *daf-2* function is difficult to place relative to *daf-12* (Riddle and Albert, 1997; Vowels and Thomas, 1992). The requirement of 3-keto-sterols as DAF-9 precursors suggests that their synthesis may be an equally important regulatory step in the production of DAF-12 ligands. The identification of enzymes responsible for converting cholesterol to a 3-keto-sterol thus represents an important future goal for our understanding of how DAF-12 ligands are produced.

Initial Insights into Dafachronic Acid Biosynthesis

Rottiers et al. (2006) report a first step in this direction by showing that *daf-36* encodes a key component of the dafachronic acid biosynthetic pathway. The authors identified this gene in a screen for mutants that display heterochronic and Daf-c phenotypes similar to those of *daf-9*, reasoning that such genes might represent additional steps in the DAF-9/DAF-12 pathway. Genetic studies positioned *daf-36* downstream from TGF-β and insulin signaling and upstream from *daf-12*. Also, like *daf-9*, *daf-36* mutations block the adult longevity associated with germline ablation, clearly placing this gene in the signaling pathways that control reproductive development and adult life span.

daf-36 encodes a protein homologous to Rieske-like oxygenases found in plants, bacteria, and lower vertebrates. Interestingly, a bacterial family member that is most similar in sequence to DAF-36 can catalyze the 9-α hydroxylation of mammalian steroids, suggesting that DAF-36 may participate in the production of a DAF-12 ligand. Consistent with this, Δ^4-dafachronic acid can efficiently rescue *daf-36* mutant phenotypes at concentrations similar to those required to rescue *daf-9* mutants. Lathosterone and 4-cholesten-3-one could also rescue *daf-36* mutants—two compounds that have no effect on *daf-9* mutants—suggesting that *daf-36* acts upstream from *daf-9*. Moreover, 7-dehydrocholesterol, but not its immediate precursor, cholesterol, could rescue *daf-36* mutants, suggesting that DAF-36 acts in the first step of dafachronic acid biosynthesis, modifying cholesterol to form 7-dehydrocholesterol (Figure 1A) or in a parallel pathway. These observations provide the first evidence that Rieske-like oxygenases can function in lipophilic hormone production. This proposal is consistent with the DAF-36 expression pattern, which is most abundant in the intestine—the major tissue for lipid storage in *C. elegans*. Interestingly, DAF-36 is not detectable in the head ganglion cells that express DAF-9, suggesting that hormonal precursors from the intestine are modified by peripheral cells to produce functional dafachronic acid. DAF-36 expression in the intestine is also consistent with its role in controlling adult life span. *daf-36* mutations block the increased life span of germline-deficient animals, suggesting that it contributes to DAF-9 and DAF-12 endocrine signaling in the reproductive pathway.

Regulation of Life Span by Germline Signaling

Berman and Kenyon (2006) provide further insights into how hormone signaling from the reproductive system to the intestine can influence adult longevity. These authors identify a gene, *kri-1*, that is required for the extended life span due to germline ablation, and characterize the effects of *kri-1*, *daf-9*, and *daf-12* mutations on this pathway. The *kri-1* gene encodes a conserved protein with ankyrin repeats that is constitutively expressed in the pharynx and intestine throughout postembryonic stages. Like *daf-9* and *daf-12*, *kri-1* mutations suppress the increase in life span associated with germline loss but do not affect the life span of wild-type animals. In addition, *kri-1* mutants have no significant effect on the life span of germline-deprived *daf-2* mutants or on their Daf-c phenotype, indicating that *kri-1* acts independently of DAF-2 in the reproductive-signaling pathway.

Upon germline ablation, DAF-16 in the intestine translocates from the cytoplasm to the nucleus, where its activity accounts for the entire increase in life span (Libina et al., 2003). By using a DAF-16::GFP fusion protein, Berman and Kenyon (2006) show that *kri-1* is required for DAF-16 nuclear localization in the intestinal cells of germline-deficient animals. A less dramatic but significant reduction in nuclear DAF-16::GFP was also seen in *daf-9* or *daf-12* mutants. The nuclear localization of DAF-16 seen in *daf-2* mutants, however, is not

affected by *kri-1*, *daf-9*, or *daf-12* mutations. This indicates that the roles of *kri-1* and lipophilic hormone production on DAF-16 nuclear localization are specific to the reproductive pathway and act largely independently of insulin signaling (Figure 1C).

The authors use a constitutively nuclear active DAF-16 protein to perform epistasis tests with *kri-1*, *daf-9*, and *daf-12* mutations to address the long-standing question of how DAF-9 and DAF-12 contribute to the longevity of germline-deficient animals. As expected, nuclear DAF-16 extends the life span of *daf-16* mutants that lack a germline. Similarly, nuclear DAF-16 rescues the increase in longevity seen upon germline ablation of *kri-1* mutants, demonstrating that a key function for KRI-1 is to facilitate this localization of DAF-16 in the intestine. Interestingly, a *daf-12* null mutation strongly blocked the longevity of germline-deficient animals that express nuclear DAF-16, indicating that DAF-12 can control longevity independently of DAF-16. Even more remarkable, a strong *daf-9* allele has no effect on the longevity of germline-deficient animals that express nuclear DAF-16, indicating that *daf-9* acts upstream from DAF-16 and that DAF-12 has functions in the germline-longevity pathway that are independent of lipophilic hormone signaling (Figure 1C). The identification of dafachronic-acid ligands for DAF-12 provides new directions for these studies. It will be interesting to determine how the hormone affects DAF-16 nuclear localization and adult life span in both wild-type and mutant worms.

Perspectives

It is impressive that many of the predictions drawn from in vivo studies of *daf-9* and *daf-12* mutants are confirmed by the biochemical pathways outlined by Motola et al. (2006) and Rottiers et al. (2006)—yet more evidence of the elegance of *C. elegans*. Given the central role of DAF-12 in *C. elegans* biology, it is likely that the discovery of dafachronic-acid ligands for this receptor will have a major impact on the field. As Motola et al. (2006) mention, the identification of two natural DAF-12 ligands suggests that these hormones may have distinct biological functions. A critical first step for these studies will be the chemical synthesis of Δ^7-dafachronic acid, which appears to be more efficacious and abundant in the animal than Δ^4-dafachronic acid. These ligands will facilitate more detailed functional characterization of DAF-9 and DAF-12, as well as provide many future directions for studying the critical decision between reproductive growth and dauer diapause and how the germline signals to the intestine to control adult longevity. The development of DAF-12 agonists and antagonists will provide invaluable tools for these studies, as well as a new basis for controlling parasitic nematodes, a major cause of crop damage and human disease in developing countries. In addition, further characterization of DAF-36 and the dafachronic acid biosynthetic pathway will provide critical insights into how hormone production is controlled to coordinate growth, maturation, and aging. Finally, these studies are not just about how nematodes develop but rather have wider implications. Endocrine circuits regulate progression through the life cycle and aging in all higher organisms, from *C. elegans* up to humans (Pardee et al., 2004; Tatar et al., 2003). Recent studies in *Drosophila* have shown that both insulin and TGF-β signaling impact the activity of the EcR ecdysteroid receptor, controlling organismal growth and maturation (King-Jones and Thummel, 2005; Zheng et al., 2003). Similar signaling pathways play a central role in normal mammalian development and human disease. It is likely that the breakthroughs described in these papers will have a major impact on our broader understanding of postembryonic development, providing a better basis for determining how endocrine signaling helps the animal complete its long and difficult journey through life.

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