Influence of Steroid Hormone Signaling on Life Span Control by Caenorhabditis elegans Insulin-Like Signaling

Kathleen J. Dumas,*† Chunfang Guo,‡ Hung-Jen Shih,§ and Patrick J. Hu†,*†
*Graduate Program in Cellular and Molecular Biology and †Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109, and ‡Departments of Internal Medicine and Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, Michigan 48109

ABSTRACT Steroid-sensing nuclear receptors and insulin-like growth factor signaling play evolutionarily conserved roles in the control of aging. In the nematode Caenorhabditis elegans, bile acid-like steroid hormones known as dafachronic acids (DAs) influence longevity by binding to and regulating the activity of the conserved nuclear receptor DAF-12, and the insulin receptor (InsR) ortholog DAF-2 controls life span by inhibiting the FoxO transcription factor DAF-16. How the DA/DAF-12 pathway interacts with DAF-2/InsR signaling to control life span is poorly understood. Here we specifically investigated the roles of liganded and unliganded DAF-12 in life span control in the context of reduced DAF-2/InsR signaling. In animals with reduced daf-2/InsR activity, mutations that either reduce DA biosynthesis or fully abrogate DAF-12 activity shorten life span, suggesting that liganded DAF-12 promotes longevity. In animals with reduced DAF-2/InsR activity induced by daf-2/InsR RNAi, both liganded and unliganded DAF-12 promote longevity. However, in daf-2/InsR mutants, liganded and unliganded DAF-12 act in opposition to control life span. Thus, multiple DAF-12 activities influence life span in distinct ways in contexts of reduced DAF-2/InsR signaling. Our findings establish new roles for a conserved steroid signaling pathway in life span control and elucidate interactions among DA biosynthetic pathways, DAF-12, and DAF-2/InsR signaling in aging.

Steroid hormones have critical functions in development and maintenance of homeostasis throughout metazoan phylogeny. They exert their effects largely by binding to and regulating the activity of transcription factors of the nuclear receptor superfamily (Wollam et al. 2006). Two structurally related DAs, Δ^2^- and Δ^7^-DA, differ in potency but appear to have similar functions in regulating larval development (Sharma et al. 2009).

Genetic analyses and rescue experiments with presumed DA biosynthetic intermediates are consistent with a model whereby Δ^7^- and Δ^3^-DA are synthesized from cholesterol via distinct pathways (Figure 1A) (Wollam et al. 2012). The Rieske oxygenase family member DAF-36 catalyzes the first step of Δ^7^-DA biosynthesis by synthesizing 7-dehydrocholesterol [7-DHC, (Rottiers et al. 2006; Wollam et al. 2011; Yoshiyama-Yanagawa et al. 2011)]. 7-DHC is thought to be converted into lathosterol, the 3-OH group of which is subsequently oxidized by the 3-hydroxysteroid dehydrogenase DHS-16 to create lathosterone (Rottiers et al. 2006; Wollam et al. 2012). Lathosterone is a direct Δ^7^-DA precursor and a substrate for the cytochrome P450 family member DAF-9 (Motola et al. 2006). The enzyme that catalyzes the conversion of 7-DHC into lathosterol has not been identified.

DAF-9 catalyzes the final common step of DA biosynthesis, converting lathosterone into Δ^7^-DA and 4-cholesten-3-one into Δ^4^-DA (Motola et al. 2006). Whereas Δ^7^-DA is detectable in lipid extracts from wild-type C. elegans, it is not detectable in extracts from daf-36 or daf-9 mutants, indicating that both DAF-36 and DAF-9 are...
required for Δ⁴-DA synthesis in vivo (Motola et al. 2006; Wollam et al. 2011). Δ⁴-DA has not been unequivocally identified in C. elegans extracts.

DAs and DAF-12 have multiple functions during larval development. Under conditions of high population density, food scarcity, and high temperature, wild-type C. elegans larvae undergo developmental arrest in an alternative third larval stage known as dauer. Dauer larvae are long-lived and resistant to environmental insults (Hu 2007). daf-9 mutants, which lack endogenous DAs (Motola et al. 2006), arrest as dauer larvae constitutively, even when ambient conditions favor reproductive development (Gerisch et al. 2001; Jia et al. 2002) but short-lived when cultured at temperatures between 20°C and 25°C (Gerisch et al. 2007; Gerisch et al. 2001; Jia et al. 2002; Lee and Kenyon 2009). These temperature-dependent phenotypes are suppressed by daf-12 loss-of-function mutations (Jia et al. 2002; Lee and Kenyon 2009) and exogenous DA (Gerisch et al. 2007), suggesting that unliganded DAF-12 promotes longevity at low temperatures but shortens life span at higher temperatures (Figure 1C). daf-12 mutation suppresses the life span extension conferred by daf-9 mutation at low temperatures (Ludewig et al. 2004), indicating that at 15°C, unliganded DAF-12 and DIN-1S act together to extend life span (Figure 1C).

DAs and DAF-12 have a profound influence on life span in animals lacking a germline. Ablation of the germline extends adult life span at 20°C by ~60%, and this life span extension requires DAF-9, DAF-36, DAF-12, and the FoxO transcription factor DAF-16 (Gerisch et al. 2007; Gerisch et al. 2001; Hsin and Kenyon 1999). Exogenous DA restores life span extension in germline-ablated animals harboring daf-9 or daf-36 mutations (Gerisch et al. 2007), indicating that liganded DAF-12 promotes longevity in this context (Figure 1C).

Similar to germline ablation, loss-of-function mutations in daf-2, which encodes the sole C. elegans insulin/insulin-like growth factor receptor family member (InsR) (Kimura et al. 1997), extend C. elegans life span in a DAF-16/FoxO-dependent manner (Kenyon et al. 1993). Both DAF-2/InsR and the germline inhibit DAF-16/FoxO activity by promoting its translocation from the nucleus to the cytoplasm (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001). In mutants that either lack a germline or have reduced DAF-2/InsR signaling, DAF-16/FoxO enters the nucleus, activating a gene regulatory program that promotes longevity (McCormick et al. 2012; Murphy et al. 2003).

How DAs and DAF-12 influence life span in the context of reduced DAF-2/InsR signaling is poorly understood. The daf-9 missense allele rh50 has distinct effects on life span in the context of specific daf-2 mutant alleles. At 15°C, daf-9(rh50) shortens the life span of both daf-2(e1368) (harboring a missense mutation in the DAF-2 ligand binding domain) and daf-2(e1370) (harboring a missense mutation in the tyrosine kinase domain) mutant animals. However, at 22.5°C, daf-9(rh50) shortens daf-2(e1368) life span but lengthens daf-2(e1370) life span (Gerisch et al. 2001). Accordingly, exogenous Δ⁴-DA prolongs the life span of daf-2(e1368) animals but does not significantly influence the life span of daf-2(e1370) animals (Gerisch et al. 2007). Furthermore, daf-12 mutant alleles influence the life span of daf-2/InsR mutants in an allele-specific manner. For example, the non-null allele daf-12(m20) (see Supporting Information, Figure S1) (Antebi et al. 2000; Snow and Larsen 2000) suppresses the extended life span phenotype of daf-2(e1368) harboring a mutation in the ligand binding domain (Patel et al. 2008) at all temperatures tested (Gems et al. 1998), whereas it enhances daf-2(e1370) life span extension at high temperatures (Gems et al. 1998; Larsen et al. 1995). In aggregate, these data underscore the need for further investigation into how steroid hormone signaling and DAF-2/InsR signaling interact in life span control. Specifically, the relative contributions of liganded and

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**Figure 1** Models of dafachronic acid (DA) biosynthetic pathways and DAF-12 complexes in the control of dauer arrest and life span. (A) Hypothetical model of DA biosynthesis adapted from Wollam et al. (2012). (B) Liganded DAF-12 promotes reproductive development, whereas unliganded DAF-12 acts with DIN-1S to promote dauer arrest. (C) Liganded DAF-12 promotes longevity in animals lacking a germline. Unliganded DAF-12 acts with DIN-1S to promote longevity at low temperatures (15°C) but shortens life span at higher temperatures (20°C–25°C). The role of DIN-1S in life span control at higher temperatures is not known.
unliganded DAF-12 to life span control have not been defined. Prior studies on the interactions of daf-12 and daf-2/InsR mutants in life span control were performed with non-null alleles of daf-12 (Gems et al. 1998; Larsen et al. 1995), complicating the interpretation of these experiments.

Here we used null alleles of daf-36 and daf-12 to explore the relationship between DA pathways and DAF-2/InsR signaling in life span regulation. Our results are consistent with a model whereby both liganded and unliganded DAF-12 influence life span. Liganded DAF-12 promotes longevity in animals with reduced DAF-2/InsR signaling. Unliganded DAF-12 also extends life span in animals subjected to daf-2/InsR RNA interference (RNAi) but shortens life span in daf-2/InsR mutants and in animals lacking a germline. These findings establish that distinct DAF-12 activities interact with DAF-2/InsR signaling to control life span.

MATERIALS AND METHODS

C. elegans strains

The wild-type N2 Bristol strain was used. Mutant alleles used are described in Table S1. Compound mutants were constructed using standard techniques.

Dauer arrest assays

Dauer arrest assays were performed at the indicated temperatures in I-36NL model incubators (Percival Scientific, Inc., Perry, IA) as described previously (Hu et al. 2006). P values were calculated using the Student t-test. Statistical analysis of all data is presented in Table S2.

Life span assays

Life span assays were performed in I-36NL incubators (Percival) at the indicated temperatures. After alkaline hypochlorite treatment and two generations of growth, young adult animals were placed onto nematode growth media (NGM) plates containing 25 μg/ml (100 μM) 5-fluoro-2’-deoxyuridine (FUDR; Sigma) and 10 μg/ml nystatin (Sigma) that had been seeded with 20× concentrated Escherichia coli OP50. For life span assays of strains carrying glp-1(e1341), animals were raised at 25°, and sterile young adult animals were placed onto NGM plates containing nystatin but lacking FUDR as described above. Assays were conducted at 20° unless otherwise noted. Viability was assessed visually or with gentle prodding. Prism software (GraphPad Software, La Jolla, CA) was used for data representation and statistical analysis. P values were calculated using the log-rank test. Statistical analysis of all data is presented in Table S2.

RNAi

Feeding RNAi was performed using variations of standard procedures (Boulton et al. 2002). For dauer assays, NGM plates containing 5 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG) and 25 μg/ml carbencillin were seeded with 500 μl of overnight culture of E. coli HT115 harboring either control L4440 vector or daf-2 RNAi plasmid. Gravid animals cultured on control or daf-2 RNAI plates were picked to assay plates for 6-hr egg lays. Dauer larvae were scored after progeny had been incubated at 25° for 48–60 hr. For life span plates, NGM plates containing 5 mM IPTG, 25 μg/ml carbencillin, 25 μg/ml FUDR, and 10 μg/ml nystatin were seeded with 500 μl of 5× concentrated overnight culture of E. coli HT115 harboring either control L4440 vector or daf-2 RNAI plasmid. Young adult animals cultured on standard NGM plates seeded with E. coli OP50 were picked to RNAI plates and scored for viability as described above.

RESULTS

Modulation of DAF-2/InsR signaling by DAF-12

Two classes of daf-2 mutants have distinct interactions with the non-null daf-2(m20) allele (Gems et al. 1998). The dauer-constitutive phenotype of Class 1 daf-2 alleles (e.g., the ligand binding domain mutant e1368), which are also long-lived and thermotolerant (Gems et al. 1998), is suppressed by daf-12(m20). In contrast, Class 2 daf-2 alleles (e.g., the tyrosine kinase domain mutant e1370), which have pleiotropic characteristics in addition to the aforementioned Class 1 phenotypes, have a synthetic non-dauer larval arrest phenotype in combination with daf-12(m20) (Gems et al. 1998; Larsen et al. 1995; Vowels and Thomas 1992).

Notably, daf-12(m20) is a nonsense mutation that specifically affects DAF-12A isoforms; it is predicted to truncate DAF-12A upstream of the C-terminal ligand binding domain, potentially resulting in a DAF-12A polypeptide that contains an intact zinc finger in the N-terminal DNA binding domain. The DAF-12B isoform, which contains the ligand binding domain but lacks the DNA binding domain, is not affected by m20 (Figure S1) (Antebi et al. 2000; Snow and Larsen 2000). The influence of a daf-12 null allele on the dauer-constitutive phenotype of daf-2 mutants has not been explored.

To clarify the epistatic relationship between daf-2 and daf-12, we constructed daf-2daf-12 double mutants using the daf-12(n611hr1411) null allele (Figure S1 and Table S1) hereafter referred to as “daf-12 null” (Antebi et al. 2000; Snow and Larsen 2000) and performed dauer arrest assays at 25°. As expected, both the Class 1 daf-2(e1368) ligand binding domain mutant and the Class 2 daf-2(e1370) tyrosine kinase domain mutant had strong dauer-constitutive phenotypes (Figure S2). The dauer-constitutive phenotype of daf-2(e1368) was completely suppressed by daf-12(null), consistent with the effect of daf-12(m20) on other Class 1 daf-2 alleles (Gems et al. 1998). At 15°, daf-2(e1370); daf-12(null) double mutants developed reproductively into adults (data not shown). At 25°, they arrested as larvae that were longer and wider than dauer larvae and that lacked both dauer alae and pharyngeal remodeling (Figure S2 and data not shown). This phenotype is comparable to that previously described for daf-2(e1370); daf-12(m20) double mutants (Gems et al. 1998; Larsen et al. 1995; Vowels and Thomas 1992) and suggests that the DAF-12A isoforms that are affected by the m20 mutation are the isoforms that prevent non-dauer larval arrest in daf-2(e1370) mutants at 25°.

daf-12(m20) also has disparate effects on the longevity of Class 1 and Class 2 daf-2 mutants (Gems et al. 1998; Larsen et al. 1995). To gain insight into how DAF-12 influences life span in animals with reduced DAF-2/InsR signaling, we measured life spans of daf-12(null) animals in three contexts of reduced DAF-2/InsR activity. First, we performed daf-2 RNAi in wild-type and daf-12(null) animals. daf-2 RNAi does not induce dauer arrest in wild-type animals at 25° but enhances dauer arrest at 27° (Dillin et al. 2002), suggesting that the extent to which RNAi reduces DAF-2 activity is less than that caused by Class 1 and Class 2 daf-2 mutant alleles, which all have strong dauer-constitutive phenotypes at 25° (Gems et al. 1998). As previously observed (Dillin et al. 2002), daf-2 RNAi extended life span to a degree comparable to daf-2 mutation (Figure 2, A–C). Life span extension induced by daf-2 RNAi was significantly attenuated in daf-12(null) animals (Figure 2, A and D; Table S2); daf-12(null) animals subjected to daf-2 RNAi exhibited a 34.5% decrease in median survival compared to wild-type animals on daf-2 RNAi (P < 0.0001, log-rank test), suggesting that DAF-12 is required for life span extension in animals with reduced DAF-2/InsR activity. At 25°, daf-12(null) mutants live approximately as long as wild-type animals do (Figure 2A, P = 0.0018;
Figure 2B, \( P = 0.1787 \); Figure 2C, \( P = 0.0678 \); Table S2), indicating that the effect of \( \text{daf-12(null)} \) on the life span of animals subjected to \( \text{daf-2} \) RNAi is unlikely to be due to general frailty. Furthermore, RNAi of three unrelated genes in wild-type and \( \text{daf-12(null)} \) animals revealed that \( \text{daf-12(null)} \) animals do not have an Rde (RNAi-defective) phenotype (Figure S5). This indicates that the relative reduction in life span extension caused by \( \text{daf-2} \) RNAi in \( \text{daf-12(null)} \) animals is unlikely to be due to reduced inactivation of \( \text{daf-2} \).

We also assayed the life spans of \( \text{daf-2;daf-12(null)} \) double mutants. Interestingly, although \( \text{daf-2(e1368);daf-12(null)} \) animals had a shorter life span than Class 1 \( \text{daf-2(e1368)} \) single mutants, the effect of \( \text{daf-12(null)} \) on life span extension in the context of the Class 1 \( \text{daf-2(e1368)} \) mutation was significantly smaller than its effect in the context of \( \text{daf-2} \) RNAi (Figure S3). This indicates that the relative reduction in life span extension caused by \( \text{daf-2} \) RNAi in \( \text{daf-12(null)} \) animals is unlikely to be due to reduced inactivation of \( \text{daf-2} \).

Taken together, these results suggest that \( \text{DAF-12} \) is required for life span extension in the context of RNAi knock-down of \( \text{daf-2} \), but is largely dispensable for longevity in the context of Class I \( \text{daf-2(e1368)} \) and Class II \( \text{daf-2(e1370)} \) mutants (see comparison of replicate experiments in Figure 2D).

A possible explanation for the differences in the influence of \( \text{DAF-12} \) on life span between the contexts of \( \text{daf-2} \) RNAi and mutational reduction of \( \text{DAF-2/InsR} \) activity is the distinct food sources employed in each experimental condition. RNAi by feeding, as used to reduce \( \text{daf-2/InsR} \) activity, involves the use of an \( \text{E. coli} \) strain, HT115, which is distinct from the standard lab food source, \( \text{E. coli} \) OP50. It has been shown that using HT115 in place of OP50 as a food source is sufficient to impact \( \text{C. elegans} \) longevity (Maier et al. 2010).
To test whether *E. coli* strain differences influence the effect of *daf-12* (null) on life span in the context of reduced DAF-2/InsR activity, we performed life span assays with *daf-2(e1368)* and *daf-2(e1370)* mutant animals grown on *E. coli* HT115 (expressing empty vector control RNAi) as the food source. Under these conditions, *daf-2(e1368);daf-12* double mutants were not shorter lived than *daf-2(e1368)* single mutants [Figures 2, D and E, and Table S2: *daf-2(e1368);daf-12* animals exhibited a 0% change in median life span compared to *daf-2 (e1368), P = 0.1989; *daf-2(e1370);daf-12* double mutants grown on *E. coli* HT115 were shorter lived than *daf-2(e1370)* single mutants, but the difference in median life span was not statistically significant [Figures 2, D and F, and Table S2: 12.1% decrease in median life span compared to *daf-2(e1370), P = 0.1439]. These results suggest that the food source does not account for the differential effects of *daf-12(null)* on longevity in the three contexts of reduced *daf-2/InsR* activity that we examined.

Modulation of DAF-2/InsR signaling by DA biosynthetic components

Since DAF-12 is regulated by DA ligands (Motola et al. 2006), we explored the influence of mutations in DA biosynthetic pathway components on dauer arrest and life span in animals with reduced DAF-2/InsR activity. Mutations in two genes encoding components of DA biosynthetic pathways, *daf-36* and *daf-9*, cause a dauer-constitutive phenotype (Gerisch et al. 2001; Jia et al. 2002; Rottiers et al. 2006). The null allele *daf-36(k114)* (Rottiers et al. 2006) (hereafter referred to as "*daf-36(null)"") and the partial loss-of-function allele *daf-9(k182)* enhanced the dauer-constitutive phenotype induced by *daf-2 RNAi* at 25°C (Figure 3A). They also enhanced the dauer-constitutive phenotype of the Class 1 *daf-2(e1368)* ligand binding domain mutant at 20°C (Figure 3B) and 15°C (Figure S4).

To elucidate interactions between DA biosynthetic pathways and DAF-2/InsR signaling in life span control, we performed life span assays in *daf-36(null)* and *daf-9(k182)* mutants in two contexts of reduced DAF-2/InsR activity: *daf-2 RNAi* and *daf-2(e1368), daf-36(null)* and *daf-9(k182)* mutations both reduced life span extension induced by *daf-2 RNAi* [Figure 3C: *daf-36(null)* exhibited a 25.8% decrease in median life span compared to wild-type animals on *daf-2 RNAi, P < 0.0001; Figure 3D: *daf-9(k182)* exhibited a 28.1% decrease in median life span compared to wild-type, P < 0.0001; Table S2]. Because neither Δ1 nor Δ2-DA is detectable in *daf-36 null* mutants (Wollam et al. 2011), these results suggest that DAs are required for maximal life span extension in animals subjected to *daf-2 RNAi*. Similar to the case for *daf-2/InsR* (Figure 2), the requirement for DA biosynthesis in life span extension induced by reduced DAF-2/InsR activity is context-dependent, as the magnitude of life span reduction caused by *daf-36* and *daf-9* mutations was smaller in animals harboring the Class 1 *daf-2(e1368)* allele than in animals subjected to *daf-2 RNAi* [Figure 3E: *daf-2(e1368);daf-36(null)* median life span was 10.3% less than that of *daf-2(e1368), P < 0.0001; Figure 3F: *daf-2(e1368);daf-9(k182)* median life span was 10.3% less than that of *daf-2(e1368), P < 0.0001; compare to Figures 3, C and D; Table S2; results summarized in Table 1]. The difference in response on *daf-2 RNAi* compared to *daf-2/InsR* genetic mutation was not due to differences in the *E. coli* strain used as a food source, as *daf-2(e1368)* mutant animals had similar life spans when assayed on *E. coli* HT115 (with vector control RNAI) and *E. coli* OP50 [Figure 3G: on HT115, median life span of *daf-2(e1368);daf-36(null)* animals was 7.4% shorter than *daf-2(e1368), P = 0.4328; Figure 3H: on HT115, median life span of *daf-2(e1368);daf-9(k182)* animals was 7.4% shorter than *daf-2(e1368), P = 0.2991; Table S2]. Collectively, these data suggest that both DAs and DAF-12 contribute to life span extension in animals with reduced DAF-2/InsR activity.

Role of unliganded DAF-12 in life span control by DAF-2/InsR signaling

To elucidate the relative contributions of liganded and unliganded DAF-12 to life span control in animals with reduced DAF-2/InsR signaling, we determined the influence of *daf-12(null)* mutation on the life spans of *daf-36(null)* animals with reduced DAF-2/InsR activity. Since *daf-36(null)* animals do not make Δ1- or Δ2-DA (Wollam et al. 2011), DAF-12 activity in the context of *daf-36(null)* is largely attributable to unliganded DAF-12.

In animals subjected to *daf-2 RNAi*, *daf-36(null)* mutation reduced life span (Figures 3C and 4A), as did the hypomorphic *daf-9(k182)* mutation (Figure 3D). *daf-36(null);daf-12(null)* animals subjected to *daf-2 RNAi* had even shorter life spans than *daf-36(null) single mutants subjected to *daf-2 RNAi* [Figure 4A and Table S2: *daf-36(null);daf-12(null)* had a 25.9% decrease in median life span compared to *daf-36(null) on *daf-2 RNAi, P < 0.0001]. From this finding, we infer that unliganded DAF-12 promotes longevity in the context of *daf-2 RNAi*.

In the *daf-2(e1368)* background, *daf-36(null)* and *daf-9(k182)* mutations also reduced life span (Figures 3, E and F, and 4B). However, in contrast to our findings with *daf-2 RNAi*, *daf-12(null)* mutation did not further shorten the life spans of *daf-2(e1368);daf-36(null) double mutant animals. Whereas *daf-12(null)* mutation shortened the life span of *daf-36(null)* animals subjected to *daf-2 RNAi* (Figure 4A, 25.9% decrease in median life span, P < 0.0001), it extended the life span of *daf-2(e1368);daf-36(null) animals fed *E. coli* OP50 [Figure 4B and Table S2: 29.2% increase in median life span of *daf-2(e1368);daf-36(null);daf-12(null) compared to *daf-2(e1368);daf-36(null), P < 0.0001]. The food source does not account for this difference; in contrast to the context of *daf-2 RNAi*, *daf-12(null)* mutation did not shorten the life spans of *daf-2(e1368);daf-36(null)* mutant animals when animals were grown on *E. coli* HT115 [Figure 4C and Table S2; 0% change in median life span comparing *daf-2(e1368);daf-36(null);daf-12(null) to *daf-2(e1368);daf-36(null), P = 0.6097]. Because *daf-12 null* mutation in the context of *daf-2(e1368)* mutation and the absence of DA is either beneficial or neutral to life span (Figure 4, B and C), we conclude that unliganded DAF-12 shortens life span in *daf-2(e1368)* mutant animals. We were unsuccessful in our efforts to construct *daf-2(e1370);daf-36(null) double mutants; this precluded an assessment of the influence of *daf-36(null) mutation on life span in the *daf-2(e1370)* mutant background.

In aggregate, our results (summarized in Table 1) support roles for both liganded and unliganded DAF-12 in life span control in animals with reduced DAF-2/InsR signaling. Liganded DAF-12 promotes longevity in all contexts tested, whereas unliganded DAF-12 modulates life span in a context-dependent manner; in the context of reduced DAF-2/InsR signaling via *daf-2 RNAi*, unliganded DAF-12 promotes longevity. In contrast, in the context of DAF-2/InsR signaling reduction via *daf-2(e1368) mutation*, unliganded DAF-12 is detrimental to life span.

Role of unliganded DAF-12 in life span control by the germline

Although both DAF-2/InsR and the germline control life span by regulating DAF-16/FoxO activity, they do so through distinct molecular pathways (Berman and Kenyon 2006; Ghazi et al. 2009; Hsin and Kenyon 1999). DA biosynthetic enzymes and DAF-12 are required for life span extension induced by germline ablation (Gerisch et al. 2007;
Gerisch et al. 2001; Hsin and Kenyon 1999; Yamawaki et al. 2010), suggesting that liganded DAF-12 is important in promoting longevity in animals lacking a germline. In light of our finding that unliganded DAF-12 can shorten life span in daf-2/InsR mutants (Figure 4B), we sought to determine whether unliganded DAF-12 also shortens life span in animals lacking a germline.

Figure 3 Mutations that reduce DA biosynthesis promote dauer arrest and shorten life span in animals with reduced DAF-2/InsR signaling. (A and B) daf-36(null) and daf-9(k182) mutations enhance dauer arrest of animals subjected to daf-2 RNAi (A) [wild-type on daf-2 RNAi vs. daf-36(null) on daf-2 RNAi, \( P = 0.0009 \); wild-type on daf-2 RNAi vs. daf-9(k182) on daf-2 RNAi, \( P = 0.0689 \)], or harboring the Class I daf-2(e1368) allele]; (B) [daf-2(e1368) vs. daf-2(e1368);daf-36(null), \( P = 0.0017 \); daf-2(e1368) vs. daf-2(e1368);daf-9(k182), \( P = 0.0072 \)]. Data represent the averages of three replicate experiments with a minimum of 400 animals scored per genotype. Error bars indicate SEM. (C and D) daf-36(null) or daf-9(k182) mutations reduce life span of animals subjected to daf-2 RNAi [\( P < 0.0001 \)] or (E and F) harboring the Class I daf-2(e1368) allele [\( P < 0.0001 \)]. (G and H) Food source control experiments for (E) and (F), respectively. E. coli HT115 expressing vector control RNAi was used as the assay food source as opposed to E. coli OP50. For each life span experiment, more than 60 animals were assayed per genotype. All raw data and statistics, including data from experimental replicates, are presented in Table S2.
Table 1 Summary of effects of daf-12 and daf-36 null mutations on life span in three contexts of DAF-16/FoxO activation

| Genetic Context | Percentage of Life Span Shortened by daf-12(null) [P value] | Percentage of Life Span Shortened by daf-36(null) [P value] | Effect of daf-12(null) Mutation on Life Span in daf-36(null) [P value] | Effect of Liganded DAF-12 on Life Span | Effect of Unliganded DAF-12 on Life Span |
|-----------------|------------------------------------------------------------|------------------------------------------------------------|------------------------------------------------|--------------------------------------|---------------------------------------|
| daf-2 RNAi      | 34.5 (<0.0001) (Figure 2A)                                 | 10.0 (<0.0001) (Figure 4A)                                 | 25.9 (<0.0001) ↓                              | ↑                                   | ↑                                    |
| daf-2(e1368)    | 10.3 (<0.0001) (Figure 2B)                                 | 29.4 (<0.0001) (Figure 4B)                                 | 29.2 (<0.0001) ↑                              | ↑                                   | ↓                                    |
| glp-1(e2141)    | 60.7 (<0.0001) (Figure 4D)                                 | 60.7 (<0.0001) (Figure 4D)                                 | 27.3 (<0.0001) ↑                              | ↑                                   | ↓                                    |

daf-2 RNAi, daf-2(e1368) mutation and germline ablation (glp-1(e2141) animals, raised at the restrictive temperature) were used to induce DAF-16/FoxO-dependent life span extension. Percentage of changes in median life span vs. the comparator (P values) are shown for each indicated experiment. See Table S2 for replicate experiments. Arrows indicate the direction of effect on life span (↑, decrease; ↓, increase). The two right columns indicate the relative effects of liganded and unliganded DAF-12 on life span.

We confirmed previously established requirements for daf-36 and daf-12 in life span extension induced by germline ablation (Figure 4C) (Gerisch et al. 2007; Hsin and Kenyon 1999; Yamawaki et al. 2010). Notably, in three of five replicate experiments, we found that glp-1; daf-12(null) animals lived longer than glp-1;daf-36(null) animals (Figure 4D and Table S2). Because daf-36(null) animals do not make δ- or Δ-DA (Wollam et al. 2011), this result suggested the possibility that unliganded DAF-12 shortens life span in animals lacking a germline. To determine whether this was the case, we examined the influence of daf-12(null) mutation on life span in germline-ablated daf-36(null) animals. glp-1;daf-36(null);daf-12(null) triple-mutation animals lived significantly longer than glp-1;daf-36(null) double-mutation animals [Figure 4D and Table S2: 27.3% increase in median life span of glp-1;daf-36(null);daf-12(null) compared to glp-1;daf-36(null), P < 0.0001], indicating that unliganded DAF-12 also shortens life span in germline-ablated animals. This result was recapitulated by control experiments performed on E. coli HT115 as the food source (Figure 4E and Table S2: 18.2% increase in median life span of daf-12;glp-1;daf-36(null) compared to glp-1;daf-36(null), P < 0.0001). Thus, in daf-36(null) animals lacking a germline, DIN-1S plays a major role in shortening life span.

**DISCUSSION**

Although the interface between *C. elegans* hormone signaling and the DAF-2/InsR pathway has been explored previously (Gems et al. 1998; Larsen et al. 1995), how these pathways interact to influence longevity remains obscure. Our work provides novel insights into the genetic interactions of liganded and unliganded DAF-12 with DAF-2/InsR signaling in life span control.

**Liganded DAF-12 promotes longevity in animals with reduced DAF-2/InsR activity**

Ambiguity about the role of DAF-12 in determining longevity is due at least in part to the use of the non-null daf-12(m20) allele in previous investigations (Gems et al. 1998; Larsen et al. 1995). We now show that the daf-12(rh61rh411) null allele and the non-null daf-12(m20) allele have distinct effects on the life spans of animals with reduced DAF-2/InsR signaling (Figure 2) (Gems et al. 1998; Larsen et al. 1995; McCulloch and Gems 2007). Our results indicate that at high temperatures, DAF-12 promotes longevity in animals with reduced DAF-2/InsR signaling (Figure 2). The magnitude of this life-span-extending effect of DAF-12 is greater in animals subjected to daf-2 RNAi than in animals harboring daf-2 mutation, indicating that the specific context of reduced DAF-2/InsR activity influences the role of DAF-12 in life span control (Table 1). The disparity between our results and those obtained with the non-null daf-12(m20) allele (Gems et al. 1998; Larsen et al. 1995) suggests that the longevity-promoting effect of daf-12(m20) and other non-null daf-12 mutations (that specifically affect DAF-12A isofoms) on the life span of daf-2(e1370) and other Class 2 daf-2 mutants (Antebi et al. 2000; Gems et al. 1998; Larsen et al. 1995; McCulloch and Gems 2007) may be attributable to a life-span-extending activity of either the DAF-12B isoform, which contains a ligand binding domain but no DNA binding domain, or truncated DAF-12A polypeptides containing most of the DNA binding domain but lacking the ligand binding domain (Antebi et al. 2000; Snow and Larsen 2000). These DAF-12 polypeptides do not play a significant role in dauer regulation by DAF-2/InsR, as daf-12(null) and daf-12(m20) have similar effects on the dauer-constitutive phenotypes of daf-2 mutants (Figure S1).

**Role of the transcriptional coregulator DIN-1S in life span control by the germline**

In animals lacking DAs, DAF-1S, the short isoform of the transcriptional coregulator DIN-1, acts in a complex with unliganded DAF-12 to promote dauer arrest (Figure 1B) (Ludwig et al. 2004). Since unliganded DAF-12 shortens life span in germline-ablated animals (Figure 4D), we examined the role of DIN-1S in life span control in animals lacking a germline by determining the impact of the din-1S null mutation dh127 (Ludwig et al. 2004) (hereafter referred to as “din-1S(null)” on the life spans of glp-1;daf-36(null) double-mutation animals. din-1S(null) animals have life spans comparable to wild-type animals at 15° (Ludwig et al. 2004). Surprisingly, din-1S(null) completely suppressed the life span shortening effect of daf-36(null) on germline-ablated animals fed *E. coli* OP50 (Figure 4D and Table S2: P = 0.8829 for the comparison of din-1S(null);glp-1;daf-36(null) to glp-1 single mutant; din-1S(null);glp-1;daf-36(null) median life span was between 35.3% and 118.2% longer than that of glp-1;daf-36(null) in four replicate experiments, P < 0.0001 for each experiment). This result was replicated with *E. coli* HT115 as the food source [Figure 4E and Table S2: P = 0.0389 for the comparison of din-1S(null);glp-1;daf-36(null) to glp-1 single mutant; din-1S(null);glp-1;daf-36(null) median life span was 54.4% longer than that of glp-1;daf-36(null), P < 0.0001]. Thus, in daf-36(null) animals lacking a germline, DIN-1S is required for DAF-12-mediated life span control as well as for DAF-12-mediated dauer arrest.
The observation that mutations in either \textit{daf-12} (Figure 2) or genes encoding DA biosynthetic components (Figure 3) reduce life span in animals with reduced DAF-2/InsR signaling is consistent with a model where liganded DAF-12 promotes longevity when DAF-2/InsR signaling is reduced (Figure 5A). Similar results indicate that liganded DAF-12 also promotes longevity in germline-ablated animals (Figure 4D) (Gerisch et al. 2007; Gerisch et al. 2001; Hsin and Kenyon 1999; Yamawaki et al. 2010). The magnitude of the effect of reducing the activity of DA biosynthetic components or DAF-12 on life span is greater in animals lacking a germline than in animals with reduced DAF-2/InsR activity (Figures 2–4) (Gerisch et al. 2007; Gerisch et al. 2001; Hsin and Kenyon 1999; Yamawaki et al. 2010). The molecular basis for this observation is not known.

Unliganded DAF-12 has context-dependent influences on life span in animals with reduced DAF-2/InsR activity

Unliganded DAF-12 promotes longevity in animals cultured at low temperatures (Gerisch et al. 2001; Jia et al. 2002) but shortens life span in animals that are cultured at high temperatures (Lee and Kenyon 2009). Here we show that in the context of reduced DAF-2/InsR signaling, unliganded DAF-12 can either extend or shorten life span. In \textit{daf-36(null)} animals, which lack both D4-and D7-DA (Wollam et al. 2011), DAF-12 extends life span in the context of \textit{daf-2} RNAi (Figure 4A) but shortens life span in the context of the Class 1 \textit{daf-2(e1368)} allele (Figure 4B) and germline ablation (Figure 4D). Since DAF-16/FoxO is a major target of both DAF-2/InsR signaling and germline signaling in life span control (Hsin and Kenyon 1999; Kenyon et al. 1993), it is likely that the impact of unliganded DAF-12 on longevity is strongly influenced by relative levels of DAF-16/FoxO activity. This notion is supported by a recent report demonstrating that DAF-12 and DAF-16/FoxO mutually influence target gene expression in animals lacking a germline (McCormick et al. 2011).

Transcriptional coregulator DIN-1S shortens life span in animals lacking a germline

DIN-1S acts together with unliganded DAF-12 at 15°C to promote longevity (Ludewig et al. 2004). Here we show for the first time that the DAF-12 coregulator DIN-1S plays a major role in life span control.
Our results define new functions for DAF-12 complexes in life span control and underscores the context-dependence of these activities (Figure 5). As summarized in Table 1, liganded DAF-12 promotes longevity in animals subjected to daf-2 RNAi (B) but shortens life span in daf-2 mutant animals (C). (D) Unliganded DAF-12 acts together with DIN-1S to shorten life span in animals lacking a germline.

Context-dependent life span control by DAF-12 complexes

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