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Functional divergence of dafachronic acid pathways in the control of *C. elegans* development and lifespan

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ABSTRACT

Steroid hormone and insulin/insulin-like growth factor signaling (IIS) pathways control development and lifespan in the nematode *Caenorhabditis elegans* by regulating the activity of the nuclear receptor DAF-12 and the FoxO transcription factor DAF-16, respectively. The DAF-12 ligands Δ^4- and Δ^7-dafachronic acid (DA) promote bypass of the dauer diapause and proper gonadal migration during larval development; in adults, DAs influence lifespan. Whether Δ^4- and Δ^7-DA have unique biological functions is not known. We identified the 3-β-hydroxysteroid dehydrogenase (3βHSD) family member HSD-1, which participates in Δ^4-DA biosynthesis, as an inhibitor of DAF-16/FoxO activity. Whereas IIS promotes the cytoplasmic sequestration of DAF-16/FoxO, HSD-1 inhibits nuclear DAF-16/FoxO activity without affecting DAF-16/FoxO subcellular localization. Thus, HSD-1 and IIS inhibit DAF-16/FoxO activity *via* distinct and complementary mechanisms. In adults, HSD-1 was required for full lifespan extension in IIS mutants, indicating that HSD-1 interactions with IIS are context-dependent. In contrast to the Δ^7-DA biosynthetic enzyme DAF-36, HSD-1 is dispensable for proper gonadal migration and lifespan extension induced by germline ablation. These findings provide insights into the molecular interface between DA and IIS pathways and suggest that Δ^4- and Δ^7-DA pathways have unique as well as overlapping biological functions in the control of development and lifespan.

Introduction

In replete environments, *Caenorhabditis elegans* larvae undergo four molts during reproductive development. When faced with unfavorable conditions, they enter diapause, arresting in an alternative third larval stage known as dauer. Dauers are adapted for survival and dispersal and resume development to adulthood once ambient conditions improve (Riddle, 1988).

The elucidation of signal transduction pathways that regulate dauer arrest has provided a glimpse into how organisms integrate environmental cues with developmental programs. Genetic analysis has defined an intricate signaling network that controls the transition to diapause (Fielenbach and Antebi, 2008; Hu, 2007). A pathway regulated by the DAF-11 guanylyl cyclase (Birnby et al., 2000) acts in sensory neurons to promote reproductive development by potentiating the expression of insulin-like (Li et al., 2003; Pierce et al., 2001) and transforming growth factor-beta (TGFβ)-like (Murakami et al., 2001; Ren et al., 1996; Schackwitz et al., 1996) ligands. These ligands regulate gene expression by activating conserved insulin-like and TGFβ-like signal transduction pathways in target tissues.

A hormone biosynthetic pathway also regulates dauer arrest by synthesizing dafachronic acids (DAs), which are steroid ligands for the nuclear receptor DAF-12 (Motola et al., 2006). The cytochrome P450 DAF-9 (Gerisch et al., 2001; Jia et al., 2002) acts on distinct precursors to generate two DAs, Δ^4- and Δ^7-DA (Motola et al., 2006). Precursors for Δ^4- and Δ^7-DA are thought to be synthesized by the 3-β-hydroxysteroid dehydrogenase (3βHSD) family member HSD-1 (Patel et al., 2008) and the Rieske oxygenase DAF-36 (Rottiers et al., 2006), respectively (Fig. 1). DA binding to DAF-12 promotes reproductive development, whereas unliganded DAF-12 promotes dauer arrest (Motola et al., 2006). Although synthetic Δ^7-DA is more potent than synthetic Δ^4-DA in dauer rescue bioassays (Sharma et al., 2009), exogenous Δ^4-DA rescues *daf-36* mutant phenotypes (Gerisch et al., 2007; Rottiers et al., 2006), indicating that Δ^4-DA can compensate for a reduction in Δ^7-DA *in vivo*. Whether Δ^4-DA and Δ^7-DA have distinct biological functions is not known.

daf-12 mutations suppress dauer arrest in *daf-11* and *daf-7/TGFβ* pathway mutants (Thomas et al., 1993), indicating that DAF-12 is required for dauer arrest in these mutants and suggesting that DAF-12 acts downstream of DAF-11 and DAF-7/TGFβ pathways. In contrast,

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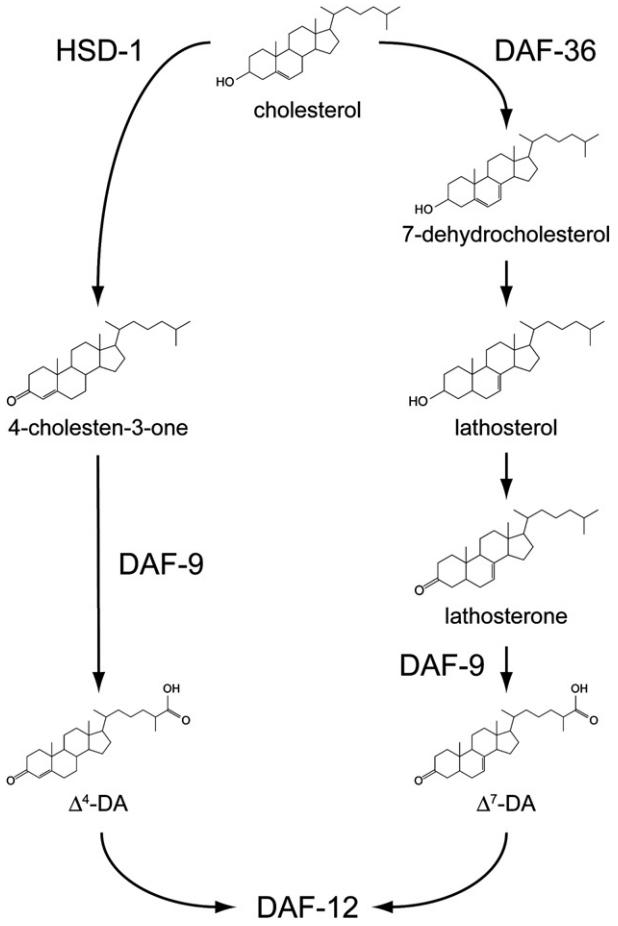

Fig. 1. Hypothetical model of dafachronic acid (DA) biosynthetic pathways. Adapted from Rottiers et al. (2006) and Patel et al. (2008).

the relationship between the DA pathway and the insulin-like pathway appears to be complex. Whereas reduced insulin-like signaling results in constitutive dauer arrest, *daf-12* mutations cause a synthetic non-dauer larval arrest phenotype in the context of reduced insulin-like signaling (Larsen et al., 1995; Vowels and Thomas, 1992). Furthermore, exogenous Δ^4-DA fully rescues dauer arrest in *daf-9* and *daf-7/TGFβ* mutants but causes non-dauer larval arrest in some strains harboring mutations in the insulin receptor (InsR) ortholog DAF-2 (Motola et al., 2006). The molecular basis for these interactions between DA and insulin-like signaling remains poorly understood.

Upon engagement by insulin-like ligands, DAF-2/InsR promotes reproductive development by activating a conserved phosphoinositide 3-kinase (PI3K)/Akt pathway (Morris et al., 1996; Paradis et al., 1999; Paradis and Ruvkun, 1998). Dauer arrest caused by reductions in DAF-2/InsR signaling is fully suppressed by mutations in the FoxO transcription factor DAF-16 (Gottlieb and Ruvkun, 1994; Vowels and Thomas, 1992), indicating that DAF-16/FoxO is the major target of DAF-2/InsR pathway signaling and that DAF-2/InsR antagonizes DAF-16/FoxO. DAF-2/InsR activation of PI3K/Akt signaling results in DAF-16/FoxO phosphorylation by Akt and its subsequent cytoplasmic sequestration *via* binding to 14-3-3 proteins (Berdichevsky et al., 2006; Hertweck et al., 2004; Lee et al., 2001; Li et al., 2007; Lin et al., 2001).

Although the inhibition of FoxO transcription factors by Akt in both *C. elegans* and mammals is well established, multiple lines of evidence support the existence of a second pathway that acts in parallel to PI3K/Akt signaling to inhibit FoxO. DAF-16/FoxO that is constitutively nuclear by virtue of either mutation of its consensus Akt/PKB phosphorylation sites or inactivation of either AKT-1 or the 14-3-3 protein FTT-2 is not fully active (Berdichevsky et al., 2006; Hertweck et al., 2004; Lin et al., 2001; Zhang et al., 2008), suggesting that a pathway acting in parallel to AKT-1 inhibits the activity of nuclear DAF-16/FoxO. To identify components of this pathway, we performed a genetic screen for enhancers of the dauer arrest phenotype seen in *akt-1* null mutants (*eak* mutants) (Hu et al., 2006; Zhang et al., 2008). Here we report the cloning and characterization of *eak-2*. Surprisingly, *eak-2* is allelic to *hsd-1* (Patel et al., 2008). This finding provided us with an opportunity to examine the interface between *C. elegans* insulin-like and DA pathways in dauer regulation and lifespan control.

Materials and methods

Strains

The following strains were used: N2 Bristol (wild-type), *hsd-1 (mg345)*, *hsd-1(mg433)* (Patel et al., 2008), *daf-9(k182)* (Gerisch et al., 2001), *daf-36(k114)* (Rottiers et al., 2006), *glp-1(e2141)* (Priess et al., 1987), *daf-16(mgDf47)* (Ogg et al., 1997), *daf-12(rh61rh411)* (Antebi et al., 2000), *akt-1(mg306)* (Hu et al., 2006), *eak-3(mg344)* (Zhang et al., 2008), *daf-2(e1370)* (Kimura et al., 1997), *sqt-1(sc13) age-1(hx546)* (Morris et al., 1996), *pdk-1(sa709)* (Paradis et al., 1999), *eak-4(mg348)* (Hu et al., 2006), and *sdf-9(mg337)* (Hu et al., 2006). DAF-16::GFP localization was assayed using strain TJ356 (*zIs356)* IV, which contains an integrated C-terminal translational fusion of the DAF-16A isoform in-frame to GFP (Henderson and Johnson, 2001). Strains CF1330 and CF1371 harbor extrachromosomal arrays encoding wild-type GFP::DAF-16/FoxO and a constitutively nuclear GFP::DAF-16/FoxO mutant lacking all AKT phosphorylation sites, respectively (Lin et al., 2001).

eak-2 mutant isolation, mapping, and sequencing

Isolation, mapping, and sequencing of *eak-2* alleles were performed as described for *eak-3* (Zhang et al., 2008).

hsd-1 cDNA isolation

hsd-1 cDNA isolation was performed as described for *eak-3* (Zhang et al., 2008), except that total RNA was isolated from fluorescent XXX cells that had been FACS-purified from cultured embryonic cells containing an integrated *sdf-9*p::RFP transgene (Hu et al., 2006).

hsd-1p::GFP construction and analysis

The *hsd-1* promoter (corresponding to nucleotides 24,173–24,413 of YAC Y6B3B, as annotated by the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) was amplified from genomic DNA, a fragment containing GFP and the *unc-54* 3′ UTR was amplified using pPD95.75 as a template, and the two PCR products were fused using overlap extension PCR to create *hsd-1*p::GFP. *hsd-1*p::GFP and the transformation marker plasmid pRF4 were coinjected at concentrations of 40 ng/μl and 60 ng/μl respectively into animals containing an integrated *sdf-9*p::RFP transgene. Transgenic animals were generated, animals were visualized using an Olympus BX61 upright microscope, and fluorescence was analyzed using SlideBook 4.1 digital microscopy software (Intelligent Imaging Innovations, Inc., Denver, CO, USA) as described (Hu et al., 2006).

Gonadal migration assays

Egg lays were performed on standard NGM plates at 20 °C, and L4 larvae were scored for gonadal migration defects (Mig phenotype) using a Nikon SMZ800 stereomicroscope. Mig phenotypes were confirmed by visualization on an Olympus BX61 upright microscope at 200× magnification.

Lifespan assays

Lifespan assays were performed as described (Hu et al., 2006), except that the 5-fluorodeoxyuridine (FUDR) concentration used in lifespan assays was 0.025 mg/ml. For lifespan assays on *glp-1(e2141)* strains, animals were cultured at 25 °C after egg lay, and L4 animals were picked to NGM plates containing nystatin but lacking FUDR and incubated at 20 °C.

Dauer assays

Dauer assays were performed as described (Hu et al., 2006). To test rescue of dauer arrest by various sterols, 10 µl of 100 µM sterol stock solution was mixed with 90 µl of concentrated *E. coli* OP50 suspension, and the mix was aliquoted onto 6-cm NGM plates and allowed to dry overnight at room temperature. These plates were then used for standard dauer assays on *hsd-1;akt-1* double mutant animals at 25 °C. Final concentration of exogenous sterols was 111 nM. For assays of strains harboring extrachromosomal DAF-16/FoxO transgenes, gravid transgenic animals were picked for egg lays based on the Rol phenotype. Progeny were incubated at 25 °C and assayed for dauer arrest 48–60 h after egg lay. Animals that bypassed dauer arrest were scored as Rol or non-Rol. Only Rol bypassors were included in calculations of dauer arrest penetrance.

Quantitative real-time reverse transcriptase PCR (qPCR)

After a four-hour egg lay, animals were grown at 25 °C for 24 h prior to harvesting. Total RNA isolation, cDNA synthesis, and quantitative real-time reverse transcriptase PCR were performed using gene-specific primers as described (Zhang et al., 2008).

DAF-16::GFP localization

Animals harboring an integrated DAF-16::GFP transgene were grown at 25 °C and analyzed as described (Zhang et al., 2008). To obtain a distribution of DAF-16::GFP localization patterns in a population of animals, single animals were categorized as exhibiting nuclear, nuclear and cytoplasmic, or cytoplasmic fluorescence, and percentages of animals exhibiting each pattern were calculated for each strain.

Results

Animals harboring the *akt-1(mg306)* null mutation arrest as dauers at 27 °C but develop reproductively at 25 °C (Hu et al., 2006). To identify mutations that enhance the *akt-1(mg306)* dauer arrest phenotype, we mutagenized *akt-1(mg306)* animals and screened for rare F_2 dauers at 25 °C. After excluding mutants whose dauer arrest phenotype was either not suppressed by *daf-16/FoxO* RNAi or did not require the presence of the *akt-1(mg306)* mutation, we identified 21 independent mutants that define seven *eak* genes. Four *eak* genes have been cloned: *eak-3*, *eak-4*, *sdf-9* (which is allelic to *eak-5*), and *eak-6*. They encode plasma membrane-associated proteins that function in a single pathway to promote reproductive development by acting in parallel to AKT-1 to inhibit DAF-16/FoxO activity nonautonomously (Hu et al., 2006; Zhang et al., 2008).

eak-2 is defined by two independent alleles, *mg334* and *mg345*. We cloned *eak-2* and found that it is allelic to the 3βHSD family member *hsd-1* (see Supplementary data for details), which was originally identified in a screen for enhancers of the *ncr-1* dauer arrest phenotype (Patel et al., 2008). HSD-1 is thought to participate in the biosynthesis of DAs. DAF-9, which is expressed in the XXX cells, the hypodermis, and the hermaphrodite spermatheca (Gerisch et al., 2001; Jia et al., 2002), catalyzes the final steps in the biosynthesis of two distinct DAs, Δ^4-DA and Δ^7-DA, from the precursors 4-cholesten-3-one and lathosterone (Motola et al., 2006) (Fig. 1). The predicted biochemical activity of HSD-1 and the expression pattern of *hsd-1*p::GFP (Fig. S3; (Patel et al., 2008) suggest that HSD-1 and DAF-9 participate in Δ^4-DA biosynthesis in the XXX cells, whereas DAF-36 and hypodermal DAF-9 synthesize Δ^7-DA (Fig. 1).

hsd-1 mutants undergo dauer arrest in a DAF-16/FoxO- and DAF-12-dependent manner

hsd-1 single mutants do not have a strong dauer arrest phenotype at 25 °C (Figs. 2B and C). However, they do undergo dauer arrest at 27 °C when cultured on NGM plates lacking supplemental cholesterol (Fig. 2A). Dauer arrest in these conditions required both DAF-16/FoxO and DAF-12, indicating that HSD-1 promotes reproductive development by antagonizing DAF-16/FoxO and DAF-12. *hsd-1* mutation also enhanced the dauer arrest phenotype of other DA biosynthetic mutants (Fig. 2B). As previously shown (Patel et al., 2008), precursors of both Δ^4- and Δ^7-DA rescued the *hsd-1* dauer arrest phenotype (Fig. S4).

Since *hsd-1* mutations strongly enhance the dauer arrest phenotype of *akt-1* mutants (Figs. 2B and C), we tested the interactions of *hsd-1* with other *daf-2/InsR* pathway mutants. *hsd-1* mutations also enhanced the dauer arrest phenotypes of *age-1/PI3K* and *pdk-1* mutants, consistent with HSD-1 acting in parallel to the PI3K/Akt pathway (Fig. 2C). The strong dauer arrest phenotype of *hsd-1;akt-1* double mutants also required both DAF-16/FoxO and DAF-12 function (Fig. 2C). *hsd-1* mutation also enhanced the dauer arrest phenotype of *daf-2(e1370)* mutants; whereas *daf-2(e1370)* animals develop reproductively at 15 °C, a significant fraction of *hsd-1;daf-2* double mutant animals arrested as dauers at 15 °C (Fig. 2D). *daf-2* mutants harboring the *hsd-1(mg433)* molecular null mutation had a stronger dauer arrest phenotype than *daf-2* mutants harboring the *hsd-1(mg345)* missense mutation (Fig. 2D), suggesting that *hsd-1(mg345)* is a non-null allele.

Since HSD-1 likely functions in DA biosynthesis, we tested other DA biosynthetic mutants for their ability to enhance the dauer arrest phenotype of *akt-1(mg306)*. The *daf-9(k182)* partial loss-of-function mutation (Gerisch et al., 2001) also enhanced the dauer arrest phenotype of *akt-1* mutants (Fig. 2B). The *daf-36* gene is located on the same cosmid as *akt-1*, hindering the construction of a *daf-36 akt-1* double mutant. However, we previously showed that RNAi of *akt-1* enhances dauer arrest in a *daf-36* mutant background (Zhang et al., 2008). Thus, mutations predicted to reduce the synthesis of either Δ^4- or Δ^7-DA or both DAs enhance the dauer arrest phenotype of *akt-1* mutants. These findings indicate that DAs act in parallel to AKT-1 to inhibit dauer arrest. Taken together with the observation that precursors of both Δ^4- and Δ^7-DA rescue dauer arrest in *hsd-1;akt-1* double mutants (Fig. S4), they suggest that Δ^4- and Δ^7-DA act redundantly to promote reproductive development.

hsd-1 mutation did not enhance the dauer arrest phenotype of other *eak* mutants (Figs. 2B and C), suggesting that HSD-1 and the other EAK proteins act in the same pathway and in parallel to the PI3K/Akt pathway in dauer regulation.

HSD-1 regulates DAF-16/FoxO target gene expression in a DAF-16/FoxO and DAF-12-dependent manner

The requirement of DAF-16/FoxO for dauer arrest in *hsd-1* mutants suggests that *hsd-1* mutation increases DAF-16/FoxO activity. To test this, we used quantitative real-time reverse transcriptase PCR (qPCR) to measure endogenous mRNA levels of three direct DAF-16/FoxO target genes: *sod-3* (Oh et al., 2006), *mtl-1* (Murphy et al., 2003), and *dod-3* (Murphy et al., 2003) (Fig. 3). The expression profile across six strains was qualitatively similar for all three DAF-16/FoxO target genes. *hsd-1* mutation had modest effects on transcript levels. As previously observed (Zhang et al., 2008), *akt-1* mutation increased transcript levels of all three genes. This is

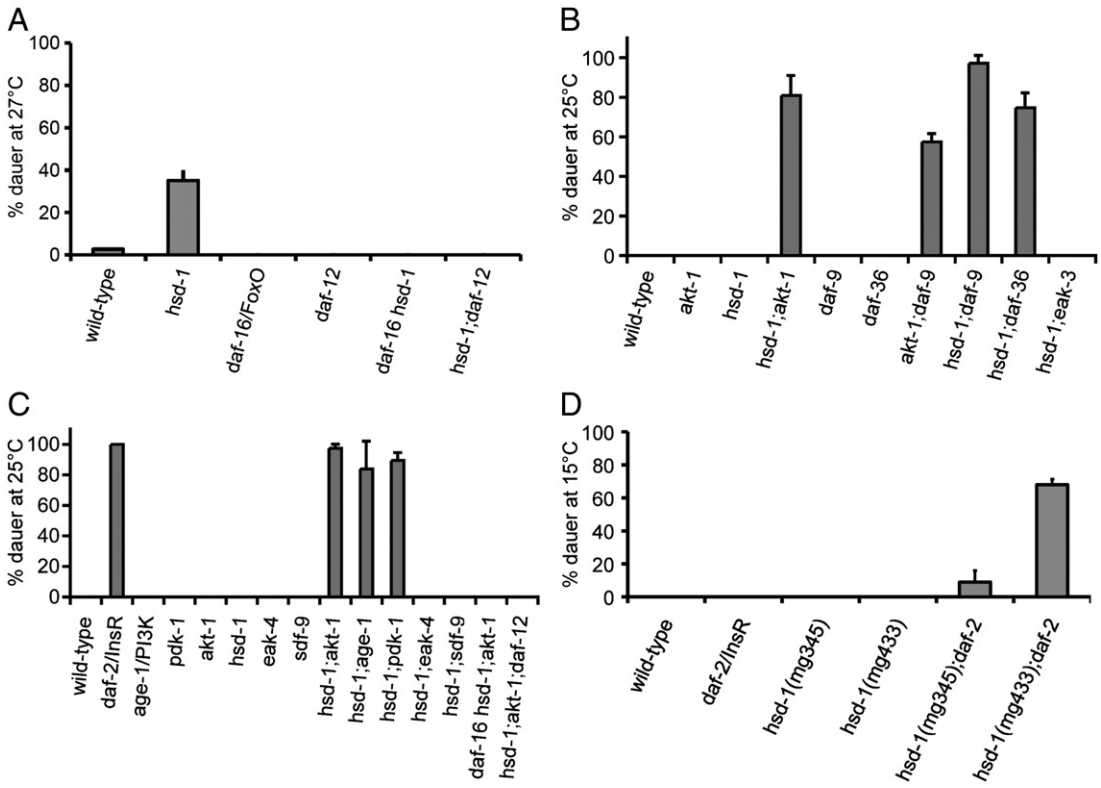

Fig. 2. HSD-1 regulation of dauer arrest. A. *hsd-1(mg433)* animals arrest as dauers at 27 °C on NGM plates lacking supplemental cholesterol in a DAF-16/FoxO- and DAF-12-dependent manner. B. Synthetic dauer arrest phenotypes at 25 °C in combinations of *hsd-1* and *akt-1* mutations with mutations in DA biosynthetic pathway components. C. The *hsd-1 (mg433)* mutation enhances dauer arrest at 25 °C in PI3K/Akt pathway mutants but not in other *eak* mutants. D. *hsd-1* mutations enhance dauer arrest at 15 °C in *daf-2(e1370)* mutants. Error bars: s.d.

consistent with the increased nuclear localization of DAF-16/FoxO caused by reduction of AKT-1 activity (Hertweck et al., 2004; Zhang et al., 2008). *hsd-1* mutation synergized with *akt-1* mutation to further increase DAF-16/FoxO target gene expression. This increase required both DAF-12 and DAF-16/FoxO activity, consistent with what we have previously observed with *eak-3* mutation (Zhang et al., 2008). Thus, HSD-1 inhibits DAF-16/FoxO target gene expression in a DAF-12- and DAF-16/FoxO-dependent manner, and both DAF-12 and DAF-16/FoxO are required for maximal DAF-16/FoxO target gene expression in *hsd-1;akt-1* double mutants. Since DAF-16/FoxO activates *sod-3* transcription directly (Oh et al., 2006), this result suggests that in this context, DAF-12 acts either upstream of or in parallel to DAF-16/FoxO to promote *sod-3* expression.

HSD-1 inhibits nuclear DAF-16/FoxO activity without influencing DAF-16/FoxO subcellular localization

To gain further insight into how HSD-1 regulates DAF-16/FoxO activity, we examined the localization of a functional DAF-16::GFP fusion protein (Henderson and Johnson, 2001) in wild-type, *akt-1* mutant, and *hsd-1* mutant animals (Fig. S5 and 4A). As previously reported, most wild-type animals exhibited diffuse fluorescence corresponding to cytoplasmic localization (Fig. 4A; Henderson and Johnson, 2001; Zhang et al., 2008). *akt-1* mutation promoted nuclear localization of DAF-16::GFP, as shown by the increase in percentage of animals exhibiting nuclear or nuclear and cytoplasmic fluorescence compared to wild-type (two-sided *T*-test: $p = 0.017$; Fig. 4A). In contrast, the distribution of DAF-16::GFP fluorescence patterns in *hsd-1* single mutants was indistinguishable from that of wild-type animals (two-sided *T*-test: $p = 0.562$; Fig. 4A). Thus, in contrast to AKT-1, which inhibits DAF-16/FoxO by promoting its translocation from the nucleus to the cytoplasm, HSD-1 inhibits DAF-16/FoxO activity without influencing its subcellular localization. Multiple efforts to construct an *hsd-1;akt-1* double mutant strain harboring DAF-16::GFP were unsuccessful, preventing us from determining whether *hsd-1* mutation promotes the nuclear localization of DAF-16::GFP in an *akt-1* mutant background.

To directly test the hypothesis that HSD-1 inhibits nuclear DAF-16/FoxO activity, we assayed the ability of *hsd-1* mutation to activate a constitutively nuclear GFP::DAF-16 fusion protein that lacks all Akt phosphorylation sites (DAF-16^{AM}) (Lin et al., 2001). Transgenic animals expressing DAF-16^{AM} exhibit constitutive nuclear fluorescence but do not arrest as dauers or live long (Lin et al., 2001), suggesting that in wild-type animals, nuclear DAF-16/FoxO is inhibited by a pathway that does not promote its relocalization to the cytoplasm. As previously reported, DAF-16^{AM} animals did not arrest as dauers at 25 °C in a wild-type background (Fig. 4B). *hsd-1* mutation strongly enhanced the 25 °C dauer arrest phenotype of animals harboring DAF-16^{AM} but did not promote dauer arrest in animals harboring a wild-type GFP::DAF-16 transgene (Fig. 4B). Thus, the effect of *hsd-1* mutation on dauer arrest is greatly enhanced in contexts in which DAF-16/FoxO is nuclear, strongly suggesting that HSD-1 inhibits nuclear DAF-16/FoxO activity.

HSD-1 is required for lifespan extension in DAF-2/InsR mutants

To determine whether HSD-1 plays a role in lifespan control, we assayed the lifespan of *hsd-1* mutants (Fig. 5). The lifespans of *hsd-1* single mutants were comparable to that of wild-type animals. We previously showed that *akt-1* mutants exhibit a slight increase in mean and median lifespan (Hu et al., 2006; Zhang et al., 2008); upon reduction of the FUDR concentration by 75%, we now observe significant lifespan extension in *akt-1* mutants compared to wild-type or *hsd-1* mutants (Fig. 5A; $p < 0.0001$ by the log-rank test). Two independent *hsd-1* mutant alleles modestly suppressed the lifespan extension of *akt-1* mutants, and this was of borderline statistical

significance ($p = 0.0128$ for *akt-1* vs. *hsd-1(mg345);akt-1* and 0.0548 for *akt-1* vs. *hsd-1(mg433);akt-1*). This result contrasts with the strong enhancement of dauer arrest in *akt-1* mutants by *hsd-1* mutation (Figs. 2B and C).

In light of the trend toward suppression of lifespan extension of *akt-1* mutants by *hsd-1* mutation, we determined the effect of *hsd-1* mutation on the lifespan of *daf-2/InsR* mutants. Surprisingly, *hsd-1* mutation significantly reduced the lifespan of *daf-2(e1370)* mutants (Fig. 5B; $p<0.0001$). This effect is unlikely to be a consequence of general frailty due to *hsd-1* mutation, as *hsd-1* single mutants have lifespans that are comparable to wild-type (Figs. 5A and B). Thus, although HSD-1 does not influence lifespan substantially in wild-type animals, it is required for maximal lifespan extension when insulin-like signaling is reduced. The suppression of *daf-2(e1370)* lifespan extension by *hsd-1* mutation contrasts with its enhancement of *daf-2 (e1370)* dauer arrest (Fig. 2D) and suggests that HSD-1 can either antagonize or potentiate DAF-16/FoxO activity, depending upon the biological context.

HSD-1 is not required for proper gonadal migration or lifespan extension in animals lacking a germline

In addition to their dauer-constitutive phenotypes, *daf-9* and *daf-36* loss-of-function mutants and *daf-12* ligand binding domain mutants exhibit developmental abnormalities in gonadal migration (Mig phenotype) (Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002; Rottiers et al., 2006). In these mutants, the gonadal leader cells fail to turn dorsally and continue to migrate away from the primordial vulva (Fig. S6A). Since exogenous Δ^4-DA rescues the Mig phenotype of *daf-9* mutants (Motola et al., 2006) and HSD-1 is thought to participate in Δ^4-DA biosynthesis (Patel et al., 2008), we examined *hsd-1* mutant animals for the Mig phenotype. Surprisingly, in contrast to *daf-9* and *daf-36* mutants, which exhibited high penetrance Mig phenotypes (Fig. 5C), *hsd-1* null mutants did not have discernible gonadal migration abnormalities (Figs. S6B and 5C). Thus, unlike DAF-9 and DAF-36, HSD-1 is not required for proper gonadal migration during larval development.

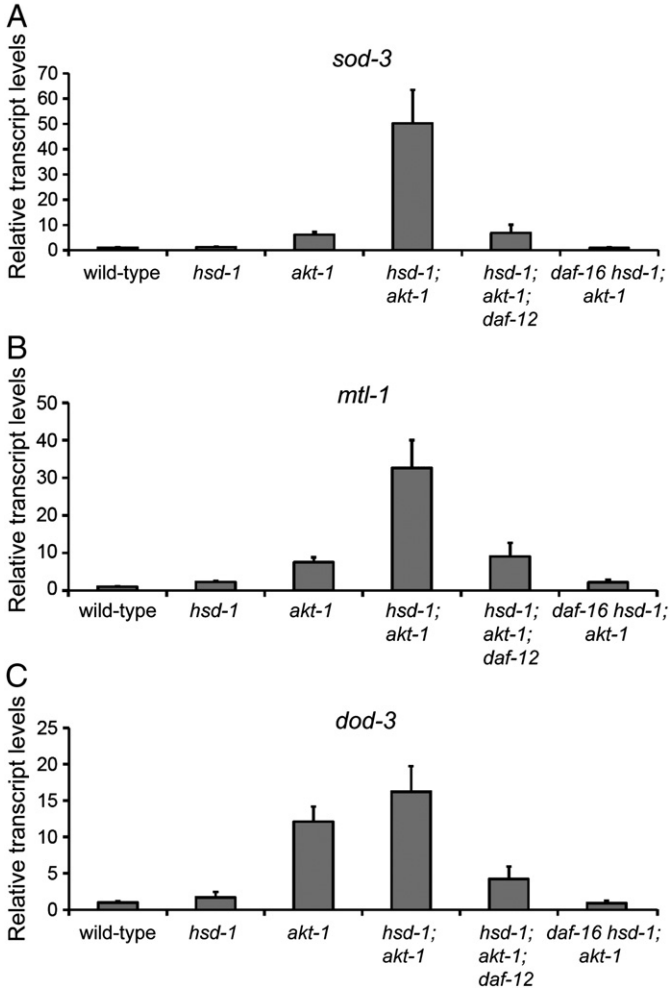

Fig. 3. HSD-1 regulation of DAF-16/FoxO target gene expression. Quantification of endogenous transcripts corresponding to the DAF-16/FoxO target genes A. *sod-3*, B. *mtl-1*, and C. *dod-3* in various mutant strains. Error bars: s.d.

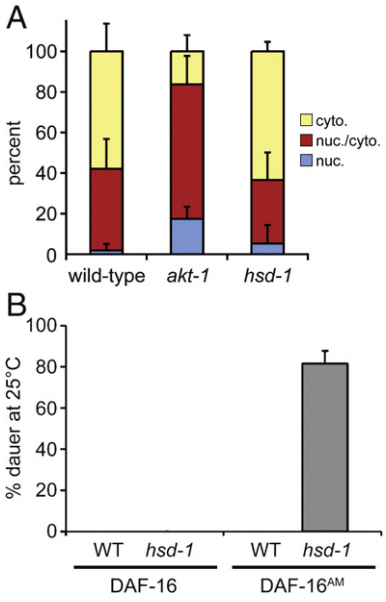

Fig. 4. HSD-1 inhibits nuclear DAF-16/FoxO activity without promoting its translocation from the nucleus to the cytoplasm. A. Percentage of animals exhibiting cytoplasmic, nuclear and cytoplasmic, or nuclear DAF-16::GFP localization. Error bars: s.d. $N = 62$ (wild-type), 77 (*akt-1(mg306)*), and 62 (*hsd-1(mg433)*). B. The *hsd-1 (mg433)* mutation enhances dauer arrest at 25 °C in animals harboring a constitutively nuclear GFP::DAF-16/FoxO mutant lacking all Akt phosphorylation sites (DAF-16^{AM}) but not in animals harboring wild-type GFP::DAF-16. Error bars: s.d.

DAF-9, DAF-36, and DAF-12 are also required for lifespan extension in animals lacking a germline (Berman and Kenyon, 2006; Rottiers et al., 2006). Since HSD-1 is required for full lifespan extension in *daf-2(e1370)* mutant animals (Fig. 5B), we tested the role of HSD-1 in lifespan extension caused by germline ablation. As previously shown (Rottiers et al., 2006), DAF-36 was required for lifespan extension in *glp-1(e2141)* mutants lacking a germline (Fig. 5D). In contrast, HSD-1 was completely dispensable for lifespan extension in *glp-1* mutants. Thus, whereas HSD-1 and DAF-36 play similar roles in dauer regulation (Fig. 2B), they have distinct functions in the control of gonadal migration and lifespan.

Discussion

We have shown that the 3βHSD family member HSD-1 acts in parallel to AKT-1 to regulate dauer arrest via DAF-16/FoxO and DAF-12. This is supported by three major lines of evidence. First, *hsd-1* mutation strongly enhances the dauer arrest phenotype of an *akt-1* null mutant in a DAF-16/FoxO- and DAF-12-dependent manner (Fig. 2C). *hsd-1* mutation also synergizes with *akt-1* mutation to enhance expression of the DAF-16/FoxO target gene *sod-3* in a DAF-16/FoxO- and DAF-12-dependent manner (Fig. 3A). Finally, *hsd-1* mutation enhances dauer arrest in animals expressing a constitutively nuclear DAF-16/FoxO mutant that lacks all Akt phosphorylation sites (Fig. 4B). The observations that *daf-9* mutation also enhances dauer arrest in an *akt-1* null mutant (Fig. 2B) and that *akt-1* RNAi induces

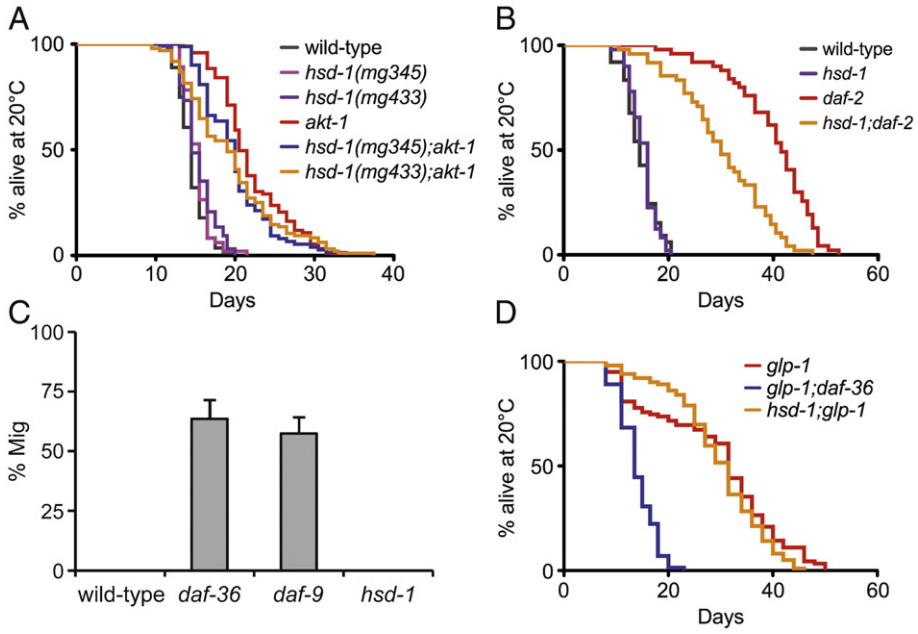

Fig. 5. Impact of *hsd-1* mutation on lifespan and gonadal migration. A. Effect of *hsd-1* mutations on lifespan at 20 °C in various genetic backgrounds. B. Effect of the *hsd-1(mg433)* mutation on lifespan extension in *daf-2(e1370)* mutants at 20 °C. C. Percentage of wild-type and mutant populations exhibiting the Mig phenotype at 20 °C. Error bars: s.d. D. Lifespan extension in *glp-1(e2141)* mutant animals does not require *hsd-1*.

dauer arrest and DAF-16/FoxO target gene expression in a *daf-36* mutant (Zhang et al., 2008) are consistent with the postulated role of HSD-1 in synthesizing the Δ^4-DA precursor 4-cholesten-3-one (Patel et al., 2008) (Fig. 1). Taken together, these data suggest that DAs act in parallel to AKT-1 to promote reproductive development *via* DAF-16/FoxO and DAF-12.

Based on their observation that DAF-16::GFP localizes to the nucleus in *hsd-1;ncr-1* double mutants, Patel et al. (2008) have proposed that steroid signaling promotes reproductive development by inhibiting DAF-16/FoxO nuclear localization. However, they did not address the question of how HSD-1 itself regulates DAF-16/FoxO activity. We find that, in contrast to *akt-1* mutation, which promotes the translocation of a functional DAF-16::GFP fusion protein from the cytoplasm to the nucleus, *hsd-1* mutation does not influence DAF-16::GFP localization (Fig. 4A). Furthermore, *hsd-1* mutation strongly promotes dauer arrest in animals expressing a constitutively nuclear DAF-16/FoxO mutant that lacks all of its Akt phosphorylation sites while having no effect on animals expressing wild-type DAF-16/FoxO (Fig. 4B). HSD-1 also acts in the same dauer regulatory pathway as other EAK proteins (Figs. 2B and C), none of which influences DAF-16/FoxO localization (Zhang et al., 2008). Based on these data, we favor a model whereby HSD-1 inhibits the activity of nuclear-localized DAF-16/FoxO without promoting its translocation from the nucleus to the cytoplasm. It is possible that *hsd-1* mutation has indirect effects on other signaling pathways that may promote DAF-16/FoxO nuclear translocation in an *ncr-1* mutant background (Patel et al., 2008).

Surprisingly, whereas *hsd-1* mutation enhanced the dauer arrest phenotype of DAF-2/InsR pathway mutants, including *daf-2(e1370)* (Figs. 2B–D), it suppressed the lifespan extension phenotype of *daf-2(e1370)* (Fig. 5B). Thus, HSD-1 can either potentiate or antagonize DAF-2/InsR signaling depending upon the biological context (Fig. 6). This could be due to context-dependent effects of the Δ^4-DA/DAF-12 interaction, the interaction of Δ^4-DA with nuclear receptors other than DAF-12 during adulthood, or a possible role for HSD-1 in the biosynthesis of distinct steroid hormones during larval development and adulthood.

Whereas mutations in *hsd-1* and *daf-36* have similar effects on dauer arrest (Fig. 2B; Rottiers et al., 2006), they differ in their impact

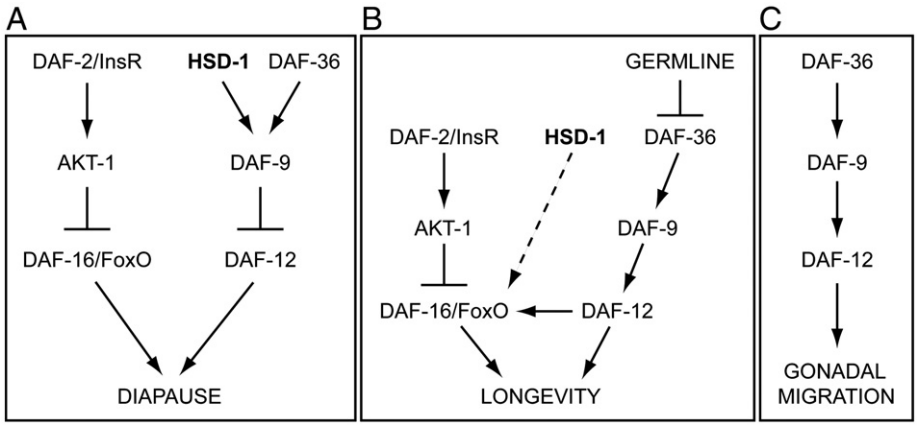

Fig. 6. Hypothetical models of insulin-like and DA pathways in the control of diapause, longevity, and gonadal migration. A. HSD-1 and DAF-36 act in canonical DA biosynthetic pathways to promote reproductive development. B. HSD-1 does not participate in lifespan control by the germline pathway but promotes lifespan extension in animals with reduced insulin-like signaling. C. Regulation of gonadal migration does not require HSD-1.

on lifespan extension and gonadal migration (Figs. 5C, D, and S6; Antebi et al., 2000; Gerisch et al., 2001; Rottiers et al., 2006). In contrast to *daf-36* mutation, *hsd-1* mutation does not cause defects in gonadal migration and does not suppress lifespan extension caused by germline ablation (Figs. 5C and D). Thus, Δ^4- and Δ^7-DA pathways may have overlapping and distinct functions in the control of development and lifespan. Since HSD-1 is the only putative DA biosynthetic enzyme that is specifically expressed in the XXX cells (Fig. S3; Gerisch et al., 2001; Patel et al., 2008; Rottiers et al., 2006), this result suggests that Δ^4-DA synthesized in the XXX cells does not influence gonadal migration or lifespan in the context of germline ablation. This could be due to the presence of DAs synthesized in other tissues that suffice to promote normal gonadal migration in *hsd-1* mutants and lifespan extension in *hsd-1;glp-1* double mutants. Indeed, the ability of exogenous Δ^4-DA to rescue both the gonadal migration phenotype as well as the suppression of *glp-1(e2141)* lifespan extension caused by *daf-9* mutation (Gerisch et al., 2007; Motola et al., 2006) indicates that Δ^4-DA can regulate these processes. However, the partial suppression of lifespan extension in *daf-2 (e1370)* mutants by *hsd-1* mutation suggests that other DAs that may compensate for loss of HSD-1 activity to promote normal gonadal migration and lifespan in germline-ablated animals are not sufficient to do so in the context of reduced insulin-like signaling.

In aggregate, our data support distinct roles for DA pathways in dauer regulation, the control of lifespan, and gonadal migration (Fig. 6). In dauer regulation, HSD-1 and DAF-36 act in parallel to AKT-1 to promote reproductive development *via* DAF-12 and DAF-16/FoxO (Fig. 6A). During adulthood, DAF-36 is required for lifespan extension in animals lacking a germline. In contrast, HSD-1 is dispensable for lifespan extension caused by germline ablation but required for lifespan extension in animals with reduced insulin-like signaling (Fig. 6B). HSD-1 has no apparent role in the regulation of gonadal migration by DAs (Fig. 6C). Since neither the biochemical activities of HSD-1 and DAF-36 nor the steroid profiles of *hsd-1* and *daf-36* mutants have been characterized, the caveat must be considered that differences in levels of and/or the anatomical site of DA synthesis could contribute to differences in *hsd-1* and *daf-36* mutant phenotypes.

Our model of HSD-1 as a regulator of nuclear DAF-16/FoxO activity suggests that human SDR42E1, the putative HSD-1 ortholog, could play an important role in the regulation of FoxO transcription factor activity in physiological or pathophysiological states in which PI3K/Akt pathway activity is low. For example, FoxO1 localizes to hepatocyte nuclei in fasted wild-type as well as diabetic mice (Aoyama et al., 2006). In light of recent reports highlighting the role of hepatic FoxO1 in glucose homeostasis (Dong et al., 2008; Matsumoto et al., 2007), SDR42E1 could be a critical regulator of nuclear FoxO1 activity and glucose metabolism in the liver. FoxO1 also localizes to the nucleus in response to dietary restriction in tumors lacking activating PI3K/Akt pathway mutations, and FoxO1 overexpression sensitizes such tumors to dietary-restriction-induced apoptosis (Kalaany and Sabatini, 2009); in this context, SDR42E1 could modulate the apoptotic response of tumors to dietary restriction. Finally, the association of FoxO3 polymorphisms with extreme human longevity (Flachsbart et al., 2009; Willcox et al., 2008) suggests the possibility that HSD-1 control of lifespan may also be phylogenetically conserved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.022.

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