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Methylation of the Sterol Nucleus by STRM-1 Regulates Dauer Larva Formation in *Caenorhabditis elegans*

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SUMMARY

In response to pheromone(s), *Caenorhabditis elegans* interrupts its reproductive life cycle and enters diapause as a stress-resistant dauer larva. This decision is governed by a complex system of neuronal and hormonal regulation. All the signals converge onto the nuclear hormone receptor DAF-12. A sterol-derived hormone, dafachronic acid (DA), supports reproductive development by binding to DAF-12 and inhibiting its dauer-promoting activity. Here, we identify a methyltransferase, STRM-1, that modulates DA levels and thus dauer formation. By modifying the substrates that are used for the synthesis of DA, STRM-1 can reduce the amount of hormone produced. Loss of STRM-1 function leads to elevated levels of DA and inefficient dauer formation. Sterol methylation was not previously recognized as a mechanism for regulating hormone activity. Moreover, the C-4 sterol nucleus methylation catalyzed by STRM-1 is unique to nematodes and thus could be a target for therapeutic strategies against parasitic nematode infections.

INTRODUCTION

When exposed to unfavorable conditions, such as overcrowding or scarcity of food, *C. elegans* interrupts its reproductive life cycle and enters diapause by forming a specific dauer (enduring) larva (Riddle and Albert, 1997). Dauers have increased stress resistance due to reprogramming of gene expression, and similar changes have been found in long-living mutants (Golden and Melov, 2007). Dauer or dauer-like larval stages are also widely spread among parasitic nematodes and play an important role during infection (Viney, 2009). Thus, understanding the mechanisms of dauer regulation can shed light on general processes, like aging, fertility, or endocrine circuitry, and could be used to develop novel antihelminthic treatments.

Biochemical studies and identification of genes involved in this process (*daf* genes, from dauer formation) revealed that it is initiated by specific pheromones (daumones/short chain ascarosides; Butcher et al., 2007; Jeong et al., 2005) and is controlled by TGF-β, insulin, and cyclic GMP signaling (Riddle and Albert, 1997). All these pathways converge on the activity of a nuclear hormone receptor, DAF-12 (Antebi et al., 1998, 2000), which can bind sterol-derived, bile acid-like substances: dafachronic acids (DA) (Motola et al., 2006; Figure 1A). Four isomers of DA have been described: two regioisomers, Δ^4- and Δ^7-DAs, each of which exist as 25*R*- and 25*S*-diastereomers (collectively referred to as DA). Some of the reactions for the production of DA have been clarified (Motola et al., 2006; Patel et al., 2008; Rottiers et al., 2006). The major step is performed by a cytochrome P450, DAF-9, which hydroxylates cholesterol twice in the C-26 position, producing a carboxyl group. In the presence of DA, the dauer-promoting activity of DAF-12 is suppressed and worms remain in the reproductive cycle (Motola et al., 2006). In the absence of the hormone, i.e., in *daf-9* mutants, DAF-12 together with its corepressor DIN-1 activates the diapause program (Gerisch et al., 2001; Jia et al., 2002; Ludewig et al., 2004). Regulation of reproductive growth via DA and DAF-12 is a conserved process in nematodes, as it is found in parasitic nematodes as well. In *Pristionchus pacificus* and *Strongyloides papillosus*, Δ^7-DA can inhibit the L3 larval arrest required for production of infective larvae (Ogawa et al., 2009).

As mentioned above, the major regulators of dauer formation are TGF-β, insulin, and cyclic GMP signaling pathways (Riddle and Albert, 1997). Downregulation of TGF-β (DAF-7), or the insulin receptor (DAF-2), using thermo-sensitive loss of function alleles induces dauer formation at the restrictive temperature. As activation of the dauer program via DAF-12 requires the absence of the dauer-repressing hormone, downregulation of DAF-7 or DAF-2 presumably reduces levels of DA. This could be achieved by inhibition of its synthesis, by its degradation, or by its sequestration into an inactive form. Maintaining relatively low DA levels would allow a fast response to dauer-inducing pheromones. At present, the mechanism for hormone downregulation is poorly understood.

Nematodes are auxotrophic for sterols, i.e., they cannot synthesize sterols de novo and depend on their supply from food (Entchev and Kurzchalia, 2005; Kurzchalia and Ward, 2003). More than 20 years ago it was found that in contrast to other organisms (i.e., budding yeast, plants, and mammals), *C. elegans* methylates the nucleus of sterols at the C-4 position (Chitwood et al., 1983). In organisms with de novo synthesis of

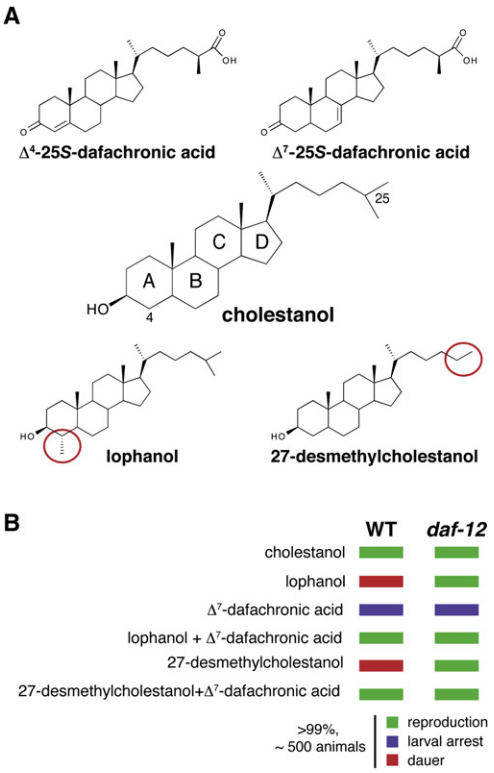

Figure 1. Effect of Different Sterols on Dauer Formation

(A) Cholesterol in the food of wild-type and *daf-12(rh61rh411)* was substituted by the depicted sterols. Red circles indicate the absence or presence of a methyl group.

(B) Worms were grown for two generations, and reproductive adults, dauer, or arrested larvae were scored.

sterols, the reverse reaction, demethylation of C-4 methylated precursors of cholesterol or ergosterol, is an essential step in sterol biosynthesis (Gaylor, 2002). The importance of C-4 sterol methylation in nematodes remained enigmatic. A possible connection to dauer formation was first indicated when cholesterol in the food of worms was substituted with 4α-methylated-sterols (4-MS), lophenol or lophanol (Figure 1A; Matyash et al., 2004), leading to dauer formation in the second generation. Also, the methyl group per se was not necessary to induce dauers, because 4α-fluoro-5α-cholestan-3β-ol had the same effect. Therefore, to produce a dauer-repressing hormone, the C-4 position of sterols must be unmodified.

Here, we present the identification of an enzyme that methylates the C-4-position of cholesterol. We named the protein STRM-1 (*st*erol A-*r*ing *m*ethylase-1). This enzyme uses the biosynthetic precursors of DAs as substrates. We also found that in the absence of DAF-9, 4-MS levels were elevated. This led us to the hypothesis that STRM-1 regulates dauer formation by reducing the amount of DA by modifying the substrate(s) of DAF-9 to make them unsuitable for DA generation. Consistent with this idea, dauer constitutive (*daf-c*) mutants, like the TGF-β mutant *daf-7(e1372)*, were more sensitive to exogenous DA in a *strm-1(tm1781)* mutant background, and *strm-1(tm1781)* animals had elevated levels of DA. As a result, several *daf-c* mutants showed a decreased frequency of dauer formation and related phenotypes when *strm-1* was deleted. Moreover, depletion of STRM-1 results in a dramatic effect under more physiological conditions: animals become almost insensitive to dauer-inducing pheromones. Thus, methylation of the sterol nucleus by STRM-1 maintains low levels of DA to facilitate regulation of dauer larva formation.

RESULTS

Effects of Sterol A-Ring and Side-Chain Methyl Groups on Dauer Larva Formation

In *C. elegans*, the substitution of dietary cholesterol with 4-MS leads to dauer formation in the second generation (Matyash et al., 2004). 4-MS can neither bind to DAF-12 to inhibit dauer formation nor can they function as precursors for the reproduction hormone DA. However, 4-MS are sufficient for molting, another important function of sterols in nematodes (Entchev and Kurzchalia, 2005; Kurzchalia and Ward, 2003), as a null mutant of *daf-12* can grow and molt normally when supplied only with 4-MS (Matyash et al., 2004). We partially purified a substance that rescues the effect of 4-MS and called it gamravali (Matyash et al., 2004). Recently, Motola and colleagues identified Δ^4- and Δ^7-DAs as products of DAF-9 and ligands of the nuclear hormone receptor DAF-12 (Motola et al., 2006; Figure 1A). We synthesized DAs (Martin et al., 2008) and tested whether they abolish the 4-MS effect. We found that worms grown on a mixture of 4-MS lophanol and DA enter the reproductive cycle (Figure 1B; only Δ^7-DA is indicated). Like gamravali, DA alone cannot substitute for cholesterol, and worms are arrested as L2 larvae (Figure 1B; Matyash et al., 2004). Thus, DA fulfils the activity definition of gamravali and could potentially be identical to it. The relationship between gamravali and DA, however, can only be determined definitely when the chemical identity of gamravali is known.

The 25*S*-DAs are 3-keto derivatives of the 25*S*-cholestenoic acid, and DAF-9 is the enzyme proposed to hydroxylate the C-26 position. We tested the role of the branch point of the side chain (C-25 position) in dauer formation. We synthesized 27-desmethylcholestanol (Figure 1A; Martin et al., 2009) and fed it to worms as the sole source of sterols. 27-desmethylcholestanol led to dauer formation in the second generation (Figure 1B). Upon addition of DA, however, worms entered the reproductive cycle. Therefore, to produce a functional DA, an intact branch in the side chain is required. This suggests that either DAF-9 needs the branch for its activity or DAF-12 can bind only an acid that has this branch. Interestingly, the branch is not needed for the structural organization of the membrane, since *daf-12(rh61rh411)* mutants that cannot produce dauers grow and reproduce on 27-desmethylcholestanol (Figure 1B).

Thus, methyl groups at the C-4 and C-25 positions have opposite effects on dauer production: the presence of the former induces dauers, whereas the presence of the latter is needed for reproduction. In contrast to the C-25 methyl group missing in the artificial 27-desmethyl compound, worms can actively incorporate a missing methyl group into the C-4 position. Therefore, we wondered whether this modification could be involved in the regulation of dauer formation and the corresponding methylase could be part of the dauer-producing pathway.

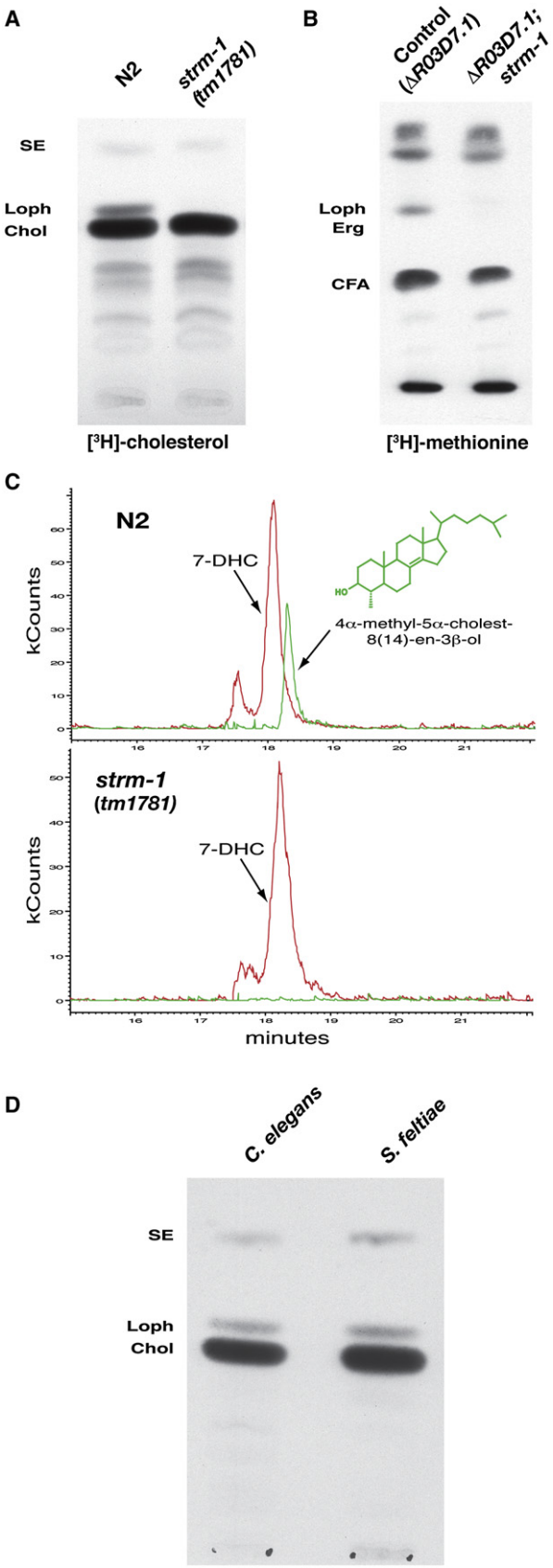

Figure 2. STRM-1 Is Responsible for C-4 Methylation in the Sterol Nucleus

(A) TLC of sterols from worms fed with [^{3}H]-cholesterol showing that *strm-1(tm1781)* does not contain 4-MS.

(B) TLC of labeled lipids from control RB755 (ΔR03D7.1) and ΔR03D7.1; *strm-1(tm1781)* fed with [^{3}H]-methionine showing that STRM-1 uses S-adenosyl methionine as a cosubstrate.

(C) GC-MS analysis of extracts from wild-type and *tm1781*(*strm-1*). Single ion modes for 7-DHC (7-dehydrocholesterol) and 4α-methyl-5α-cholest-8(14)-en-3β-ol (formula given) are shown.

(D) TLC of sterols from *Caenorhabditis elegans* and *Steinernema feltiae* fed with [^{3}H]-cholesterol showing 4-MS production in a parasitic nematode. Markers: SE, sterol esters; Loph, lophenol; Chol, cholesterol; Erg, ergosterol; CFA, 9-cyclopropylstearic acid.

Identification of a Methylase Responsible for the Methylation of the Sterol Nucleus at the C-4 Position

So far, nuclear methylation of sterols in the A-ring has been described only in nematodes (Chitwood, 1999; Chitwood et al., 1983). It is known, however, that in budding yeast Erg6p methylates sterols in the C-24 position, utilizing a Δ^{24} precursor, zymosterol (Gaber et al., 1989). Similar enzymes, called SMTs (sterol *m*ethyl *t*ransferases) have also been described in plants (Grebenok et al., 1997), *Trypanosoma* (Zhou et al., 2006), and *Leishmania* (Magaraci et al., 2003). The putative 4-methylase of nematodes could belong to the Erg6p/SMT family and contain the common methyltransferase domain (pfam08241, methyltransferase 11) as well as the specific SMT C-terminal domain (pfam08498). A phylogenetic tree of the methyltransferase 11 domain, using the SMT family and its closest homologous families, is shown in Figure S1A. The *C. elegans* genome encodes only for one gene product that clusters with Erg6p: H14E04.1 (Figure S2). A multiple sequence alignment with other members of the Erg6p/SMT family is given in Figure S1B. The S-adenosyl methionine (SAM)-binding motifs (Kagan and Clarke, 1994) are highly conserved in H14E04.1. The two SMT motifs (Grebenok et al., 1997) that are important for sterol binding in yeast Erg6p (Nes et al., 2004) are less conserved in H14E04.1, indicating a different architecture of the substrate pocket, which would allow for methylation at a different location on the sterol. Interestingly, the new diverged subfamily of SMT enzymes includes members in both free living (*C. elegans* and *C. briggsae*) and parasitic nematodes (*B. malayi*; Figure S1A).

To test whether H14E04.1 is responsible for the production of 4-MS, we used a deletion mutant affecting this locus. In the allele *tm1781*, two exons encoding conserved protein motifs are missing (Figure S2), and potential splicing would lead to a frame shift that generates an early stop codon. Thus, the allele *tm1781* should be a genetic null. The mutant was fed with radioactive cholesterol and its sterols were analyzed by TLC. As seen in Figure 2A, the deletion mutant of H14E04.1 displays no band with an R_f identical to that of 4-MS.

H14E04.1 has a readily discernible SAM-binding domain and thus should use SAM as a cosubstrate in the methylation reaction. We tested whether radioactive methionine could label 4-MS (Figure 2B). To increase the label incorporation, we exploited the deletion mutant *ok521* of methionine synthase R03D7.1, with a deficiency in methionine biosynthesis (J.T.H. and

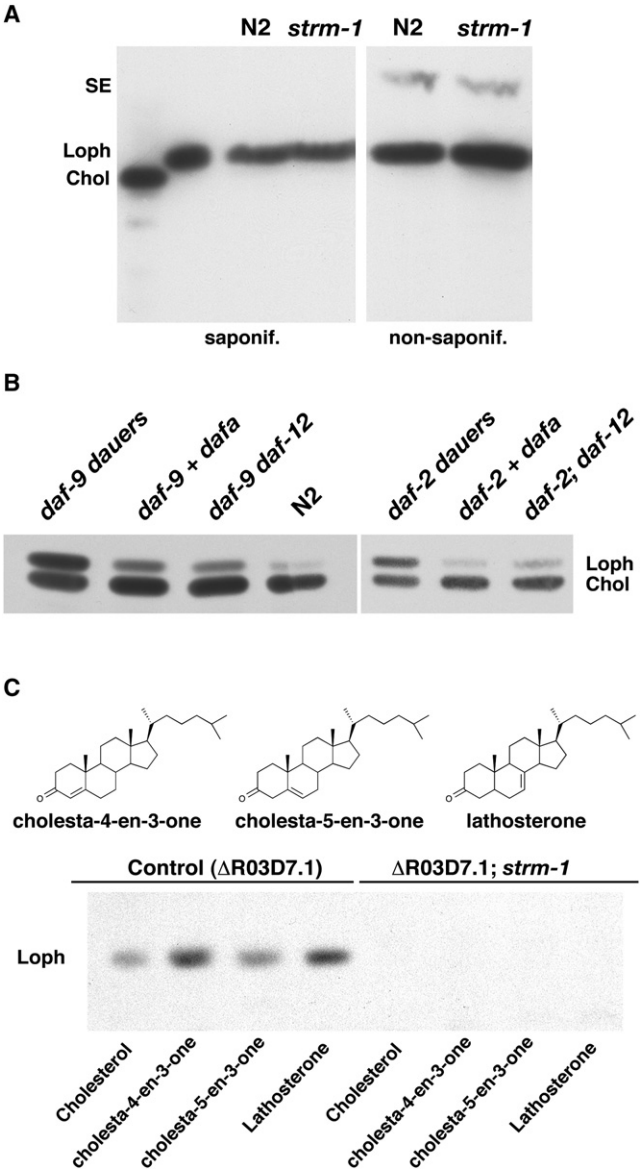

Figure 3. Irreversibility, Regulation, and Substrates of Cholesterol Nuclear Methylation

(A) TLC analysis of sterols after feeding with radioactive 4-MS with or without saponification.

(B) TLC of ^{3}H-cholesterol labeling in *daf-9(dh6)* and in *daf-2(e1370)* after feeding with DA or in a *daf-12(rh61rh411)* background.

(C) TLC of labeled 4-MS after feeding with [^{3}H]-methionine and cold cholesterol or different ketones depicted. Markers: SE, sterol esters; Loph, lophenol; Chol, cholesterol.

T.V.K., unpublished data). Due to the deletion in R03D7.1, the RB755 worm strain relies on exogenous methionine, supplied by its bacterial food source. The major band of labeled lipids corresponds to cyclopropyl fatty acids (CFA). A band with R_f identical to 4-MS present in wild-type animals is entirely absent when H14E04.1 is deleted. Note that *C. elegans* appears not to produce 24-methyl sterols (ergosterols), as a corresponding band was not detected.

The absence of 4-MS in *tm1781* was confirmed by GC-MS analysis (Figure 2C). We could also distinguish different nuclear methylated sterols (lophenol or 4α-methyl-5α-cholest-8(14)-en-3β-ol; Chitwood et al., 1983). In sterols from a mixed population of worms, we detected two peaks with a mass-to-charge ratio (m/z) of 400.3 (Figure S3A). The minor peak 2 has the same retention time and fragmentation spectrum as the lophenol standard, while the major peak elutes earlier and matches to the library spectrum of 4α-methyl-5α-cholest-8(14)-en-3β-ol (Figure S3B). After saponification of total lipid extracts from young adults of wild-type and *tm1781*, GC-MS spectra show very little over-all differences in the total ion counts (Figure S3C). The deletion mutant of H14E04.1 has comparable to wild-type amounts of desmethylsterols (shown for 7-dehydrocholesterol in Figure 2C). In *tm1781*, the peaks of 4-MS are not detected (Figure 2C), indicating that H14E04.1 is needed for their production.

To address whether H14E04.1 is specific for sterol methylation at the C-4 position, we tested whether H14E04.1 can complement *erg6*Δ in *S. cerevisiae*. Erg6p is a sterol methylase in budding yeast that methylates the side chain of zymosterol at C-24, producing fecosterol further metabolized to ergosterol (Gaber et al., 1989). H14E04.1 was cloned and expressed in yeast *erg6*Δ. We found that H14E04.1 cannot produce ergosterol to restore normal growth of the *erg6*Δ (data not shown). Therefore, H14E04.1 appears to be specific for sterol C-4 methylation.

Taken together, the structural features of H14E04.1 (presence of SAM and SMT domains), the absence of 4-MS in *tm1781*, and the inability to complement *erg6*Δ indicate that H14E04.1 methylates sterols in position C-4 and is therefore the *st*erol A-*r*ing *m*ethylase (designated below as STRM-1).

We also investigated whether methylation of sterols in the ring is indeed conserved in nematodes. For this purpose, a culture of an insect parasitic nematode *Steinernema feltiae* was labeled with radioactive cholesterol. *C. elegans* and *S. feltiae* belong to different nematode suborders (Nadler et al., 2006). However, as seen in Figure 2D, the patterns of sterols in *C. elegans* and *S. feltiae* are very similar: in both species, 4-MS are major constituents.

Nuclear Methylation of Cholesterol Is Irreversible

Organisms with de novo biosynthesis of sterols have an elaborate system for demethylation of lanosterol or cycloartenol (methylated precursors of cholesterol and ergosterol or sitosterol, respectively) at the C-4 position (Benveniste, 2004; Gaylor, 2002). In contrast, nematodes actively methylate this position. Is the A-ring methylation reversible in *C. elegans*?

To investigate this question, we prepared radioactive 4-MS from worms labeled with ^{3}H-cholesterol (see Experimental Procedures). When fed to wild-type and *strm-1(tm1781)* worms, radioactive 4-MS were taken up and accumulated to significant levels (Figure 3A). These 4-MS were actively metabolized as the label was found also in sterol esters (Figure 3A, nonsaponified sample). However, even after several months of exposure, we could not detect any traces of cholesterol/desmethylsterol in either the original extracts or in saponified samples (although, a very faint hydrophilic band appeared close to the start; data not shown). The possibility that cholesterol derived from

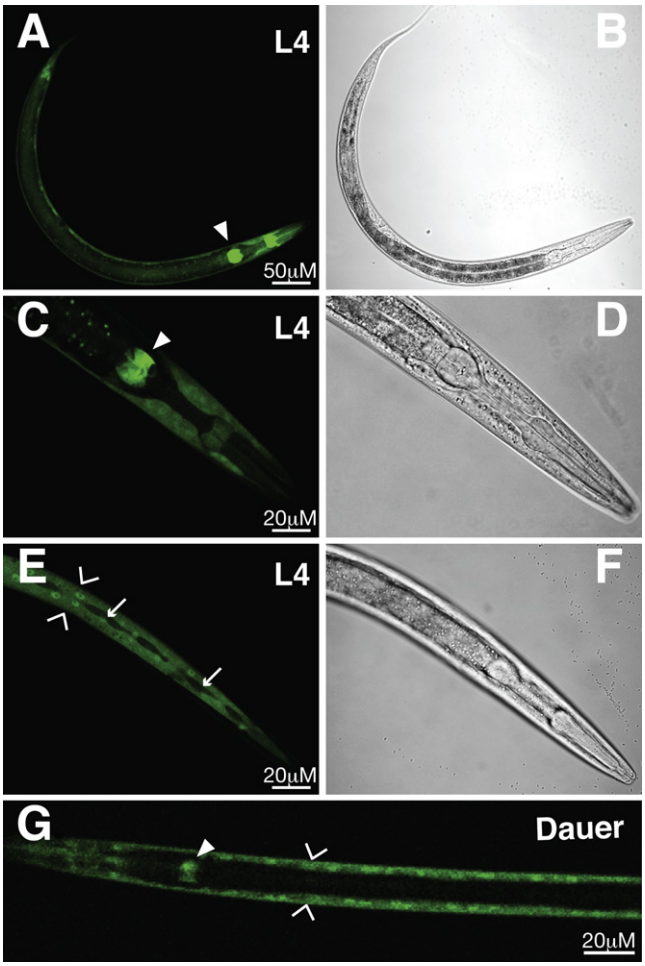

Figure 4. Expression Pattern of *strm-1*

(A, C, and E) GFP fluorescence in L4 larvae from a reporter *pstrm-1::GFP*.

(B, D, and F) Corresponding bright field images.

(G) GFP fluorescence in dauer *pstrm-1::GFP; daf-7(e1372)*. Arrowheads, pharynx bulb; arrows, seam cells; chevrons, hypodermis.

lophenol is quickly remethylated could be excluded, as *strm-1(tm1781)* also did not show any cholesterol signal (Figure 3A). Thus, methylation of the cholesterol nucleus in nematodes appears to be irreversible.

Methylated Sterols Are Strongly Elevated in Dauer Larvae, and STRM-1 Acts Downstream of DAF-9 and DAF-12

To investigate whether there is a direct connection between dauer formation and sterol methylation, we examined the levels of 4-MS in DAF-9-deficient animals (Figure 3B) by analyzing the pattern of sterols in *daf-9(dh6)* dauer-like larvae. A large homogenous population of *daf-9(dh6)* dauer-like larvae was obtained by feeding the previous generation with DA (see Experimental Procedures). In *daf-9*-deficient dauer-like larvae, 4-MS are highly increased in comparison to the reproductive stages (Figure 3B). If *daf-9(dh6)* are fed with DA, 4-MS are reduced but still elevated in comparison to the wild-type. Densitometric analysis of radiographs showed that when exogenous DA is added to *daf-9* mutants, the ratio of 4-MS/nMS is about two to three times higher than in the wild-type. This elevation can also be seen in a *daf-9(dh6) daf-12(rh61rh411)* double mutant (Figure 3B). In this strain, the receptor for DA, DAF-12, is inactive and mutants grow normally despite lacking DAF-9 and, consequently, DA. Thus, the absence of DA synthesis elevates the level of methylated sterols present.

To evaluate the significance of *daf-12* in regulating 4-MS levels in dauers, we designed an experiment in which we could obtain dauer-like larvae with inactive *daf-12*. To this end we fed radioactive cholesterol to mutants with a strong *daf-2* allele, *e1370*, which cannot completely exit the dauer stage even by feeding with DA, or in a *daf-12* null background, and in both conditions such animals remain as arrested larvae (Gems et al., 1998; Motola et al., 2006). Control *daf-2(e1370)* dauers showed a similar ratio of 4-MS/nMS to that seen in *daf-9(dh6)* dauer-like larvae (Figure 3B). Interestingly, in *daf-2(e1370)* animals fed with DA, or in a *daf-12(rh61rh411)* background, 4-MS were reduced to a ratio similar to *daf-9(dh6)* fed with DA and *daf-9(dh6) daf-12(rh61rh411)*. Taken together, these results indicate that: (1) 4-MS strongly accumulate in dauers when there is no *daf-9* activity; (2) during reproductive development, if *daf-9* is missing, there is still elevation of 4-MS; and (3) accumulation of 4-MS during dauer formation is *daf-12* dependent, as 4-MS are reduced in dauer-like *daf-2(e1370)* larvae obtained either with DA or depletion of *daf-12*. Thus, the activation of the dauer program via DAF-12 in the absence of DA leads to an increase in sterol methylation.

We hypothesized that nematodes may exploit methylation of the A-ring in sterols in order to regulate the amount of DA. One possibility is that the enzyme producing 4-MS competes with DAF-9 for a substrate(s). Δ^4- and Δ^7-DAs are produced from the corresponding cholesten-3-ones (Motola et al., 2006; formulae in Figure 3C). To test whether these cholesten-3-ones were used as substrates for the production of 4-MS, wild-type and *strm-1(tm1781)* worms were fed with cholesten-3-ones, and the methylation was followed by radioactive methionine, as described above (Figure 3C). We found that cholest-4-en-3-one and cholest-7-en-3-one (lathosterone), also substrates of DAF-9, are the most effective precursors for the production of 4-MS.

The Expression Patterns of *strm-1* and *daf-9* Partially Overlap

Competition between enzymes for a substrate is more likely if they are expressed in the same cells. *daf-9* is expressed in the XXX cells, the hypodermis, and the spermatheca (Gerisch and Antebi, 2004; Gerisch et al., 2001; Jia et al., 2002; Mak and Ruvkun, 2004). We investigated the expression pattern of *strm-1* using a GFP reporter. *strm-1* expression starts late in embryogenesis and it is high throughout larval stages and in adults (data not shown and Figures 4A and 4B). During reproductive development, the strongest expression was detected in the pharynx (Figures 4C and 4D), which is one of the major sites of sterol accumulation (Matyash et al., 2001). The hypodermal syncytium also showed high levels of fluorescence (Figures 4E and 4F) in contrast to the seam cells (white arrows). In order to follow *strm-1* expression in dauer larvae, we introduced the reporter in *daf-7(e1372)* animals, which produce dauers at

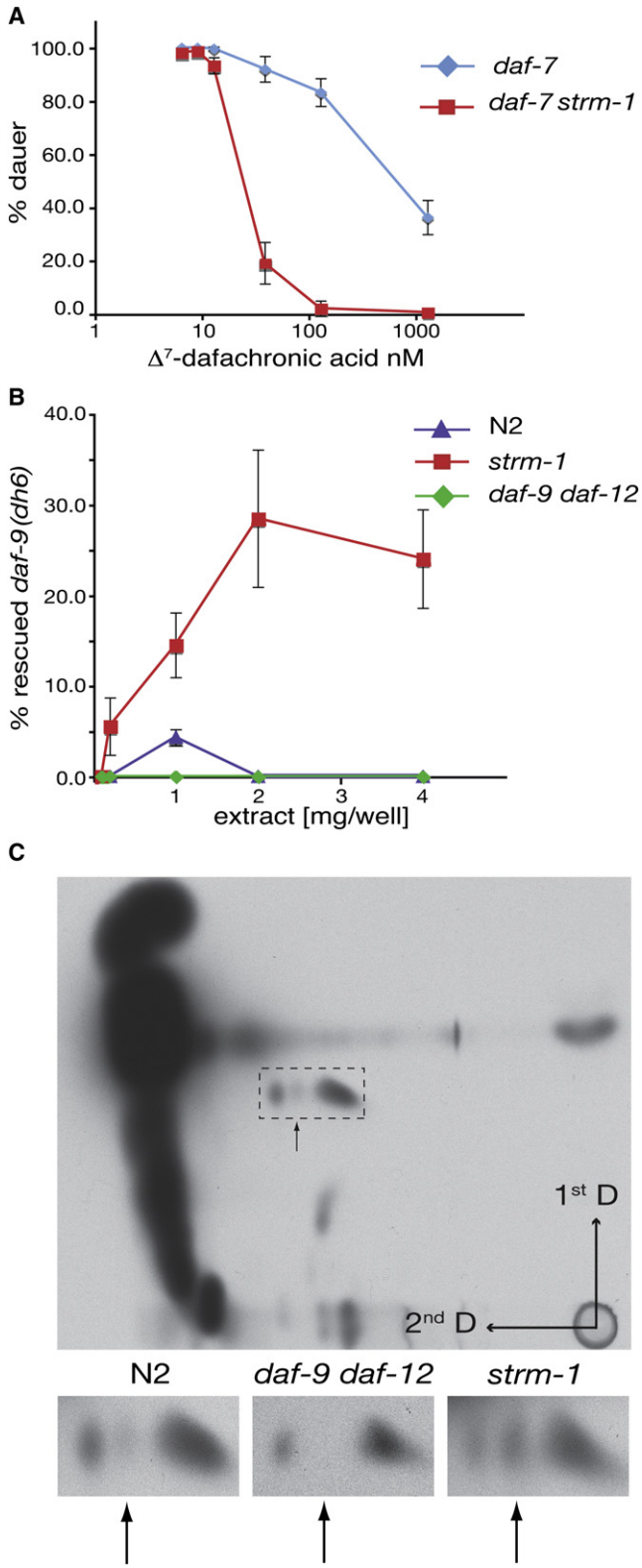

Figure 5. Deletion Mutants of *strm-1* Contain Elevated Amounts of DA

(A) Bypass of dauer formation in *daf-7(e1372)* and *daf-7(e1372) strm-1 (tm1781)* after feeding with DA (n = 3). Error bars represent ±SD.

(B) Rescue of *daf-9(dh6)* dauer-like larvae by crude hydrophobic extracts (n = 3). Error bars represent ±SD.

(C) Direct detection of elevated amounts of DAs in *strm-1(tm1781)* by 2D TLC. Upper panel: a complete TLC plate from control worms (arrows, first and second dimensions of the TLC). Lower panels: blowups of regions with DA (arrows, DA spot).

25°C (Figure 4G). While pharyngeal fluorescence in dauers decreases significantly, GFP in the hypodermis remains at high levels, indicating high expression of *strm-1* in dauers.

Previously, it was shown that *daf-9* is expressed in two neuroendocrine XXX cells that are important in preventing dauer arrest (Gerisch et al., 2001; Jia et al., 2002). *daf-9* is also strongly expressed in the hypodermis during reproductive growth, but is shut down in dauers (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). Thus, STRM-1 and DAF-9 are present in the same hypodermal cells and would be able to compete for substrates (cholesten-3-ones) during reproductive development. In addition, *strm-1* continues to be expressed in dauers, which accumulate 4-MS (Matyash et al., 2004; Figure 3B) and, presumably, do not produce DA.

Deletion Mutants of *strm-1* Contain Higher Amounts of DA

We documented above that the absence of DAF-9 can elevate 4-MS. A related question is whether deletion of *strm-1* elevates DA and thus makes the production of dauer larvae more difficult. To investigate the influence of *strm-1* on dauer formation, we produced double mutants of *strm-1(tm1781)* and *daf-7(e1372)*, which at 25°C produces dauers. *daf-7* mutants can be rescued by feeding with DA (Motola et al., 2006). If *daf-7(e1372) strm-1 (tm1781)* have elevated DA, then worms grown on different concentrations of hormone should be rescued to a greater extent than *strm-1*-positive animals. At high concentrations, DA rescues dauer formation in both *daf-7(e1372)* and in *daf-7(e1372) strm-1 (tm1781)* (Figure 5A). The differences become evident at lower concentrations: ten times less DA still can fully rescue the double mutant. Remarkably, the addition of lophenol/lophanol to the double mutants does not decrease their sensitivity to DA (data not shown). This again indicates that 4-MS do not actively induce dauer formation but instead suggests that their biosynthesis influences amounts of the hormone by competing with substrates of DAF-9 (see also the effect of lophenol later).

Next we decided to compare the level of DA in wild-type and *strm-1(tm1781)* animals based on its bio-activity, using the rescue of *daf-9(dh6)* mutants as an assay (Gerisch et al., 2007; Motola et al., 2006). DA rescues DAF-9 deficiency in a concentration-dependent manner (Motola et al., 2006). The activity of extracts from wild-type, *strm-1(tm1781)*, and *daf-9(dh6) daf-12 (rh61rh411)* worms was tested (Figure 5B). As expected, the extract from *daf-9(dh6) daf-12(rh61rh411)* was inactive (note that addition of DA to the extract rescued *daf-9* deficient animals; Figure S4A). In contrast, crude extracts from wild-type and *strm-1 (tm1781)* rescued *daf-9* deficiency, with the *strm-1(tm1781)* extract being more active. It could be diluted 5- to 10-fold and still had activity comparable to wild-type extracts.

The higher activity of the extract from *strm-1(tm1781)* could be based on the ability of STRM-1 to methylate DA, leading to inactivation of the hormone. In order to investigate this, we exploited

the sensitivity of *daf-9(dh6)* mutants to exogenously supplied DAs in a *strm-1(tm1781)* background. We reasoned that if STRM-1 is inactivating DA, the double mutant *strm-1(tm1781); daf-9(dh6)* would be more sensitive to exogenously supplied hormone than the single *daf-9(dh6)* mutant. This could be detected either by rescue at low concentrations that have no effect on *dh6* alone, or by increased rescue at suboptimal concentrations. As seen in Figure S5, *strm-1(tm1781)* does not lead to increased sensitivity to exogenous DAs in this *daf-9* null background, since both lines need similar amounts of DAs to enter into reproduction. Thus, direct inactivation of the hormone by methylation seems very improbable.

Obviously, the differences in amounts of DA between wild-type N2 and *strm-1(1781)* should be demonstrated directly. To detect DA(s) on TLC, we designed a 2D-system where acidic sterols were clearly separated from noncharged ones. (Separation of DA, cholestenoic acid, and cholesterol is shown in Figure S4B.) In an extract from control N2 worms, three cholesterol-derived spots with acidic properties appeared in the region where the DA standard is located (Figure 5C, rectangle). The right and left spots are present in *daf-9 daf-12* (lower panel) as well as in dauers (data not shown). The middle one, however, with identical R_f values to the DA standard, is missing in the extracts from *daf-9 daf-12* (lower panel) or dauers. We conclude that this spot is DA. Remarkably, the intensity of the spot increases significantly in the extract from *strm-1(tm1781)*. This spot is very faint even in N2, which explains the low activity of this extract (Figure 5B). All attempts to detect this difference by LC-MS and thus quantify the increase of DA in *strm-1(tm1781)* have been unsuccessful. Thus, it seems that the absence of STRM-1 leads to an elevated level of DA, and consequently this methylase may well regulate dauer formation.

Deletion Mutants of *strm-1* Are Inefficient in Dauer Larva Production

We compared the efficiency of dauer formation of *daf-7(e1372)* and *daf-7(e1372) strm-1(tm1781)* at different temperatures (Figure 6A). Although both mutant strains produce dauers at 25°C, at lower temperatures the number of reproductive animals in the double mutant was much higher (Figure 6A). The *daf-7 (e1372)* mutant produces dauers even at the permissive temperature (15°C) (Swanson and Riddle, 1981) (Figure 6A; about 50% at 15°C). In contrast to this, only about 3% dauers were observed in the *daf-7(e1372) strm-1(tm1781)* double mutant.

The influence of STRM-1 on the efficiency of dauer formation was also tested in other genetic backgrounds (*daf-2(e1368)*, *daf-36(k114)*, and *daf-9(rh50)*) (Figure S6). In all cases, a very similar tendency to that seen in *daf-7* mutants was observed. Remarkably, *strm-1(tm1781)* was very effective in rescuing the distal tip cell migration defect (Figure S6B), a characteristic of some *daf-c* hypomorphs, which develops at low levels of hormone (Gerisch et al., 2001; Jia et al., 2002; Rottiers et al., 2006). Thus, hypomorphic *daf-9(rh50)* mutants could be rescued almost entirely by *strm-1(tm1781)*. These data argue that there is a genetic interaction between *daf-9* and *strm-1*.

The most direct proof that STRM-1 regulates the process of dauer formation under natural conditions was obtained using dauer-inducing pheromones (daumone/short ascarosides). These substances induce dauers effectively in wild-type worms

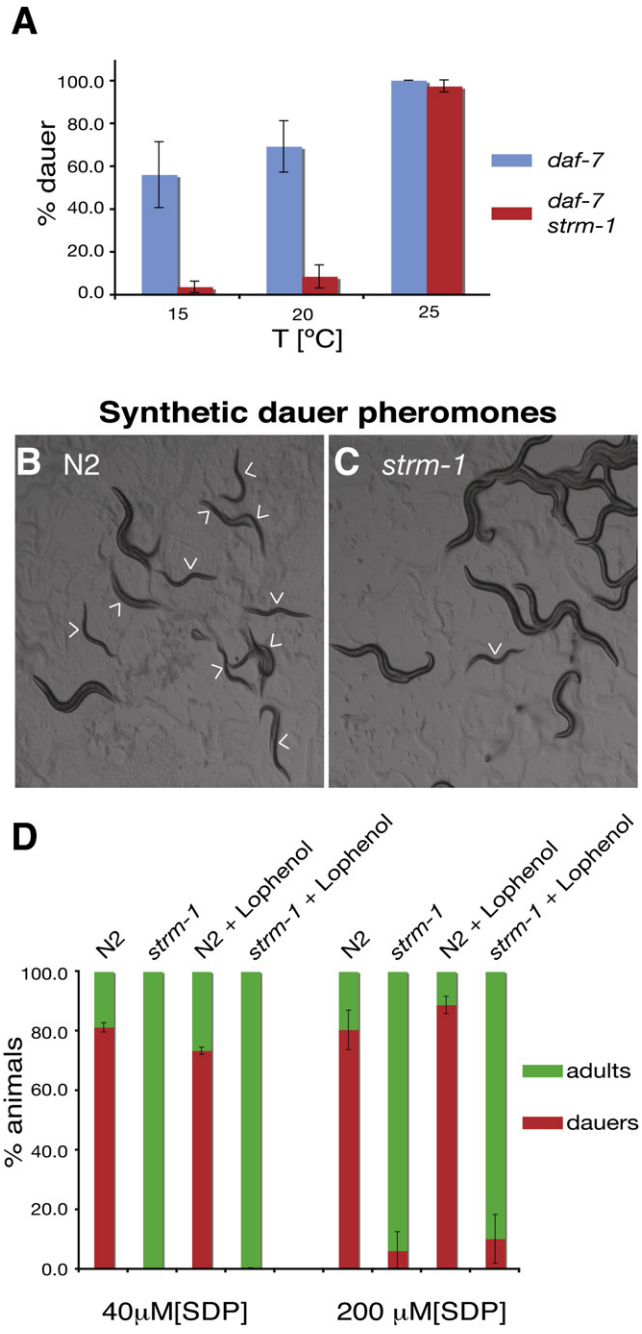

Figure 6. Deletion Mutants of *strm-1* Are Inefficient in Dauer Larva Production and Do Not Respond to Dauer-Inducing Pheromones

(A) Dauer formation in *daf-7(e1372)* and *daf-7(e1372) strm-1(tm1781)* at different temperatures (n = 12). Error bars represent ±SD.

(B–D) Diminished response to synthetic dauer pheromones in *strm-1(tm1781)*. Overview of pheromone assay plates of wild-type (B) and *strm-1(tm1781)* (C). Chevrons indicate dauers; note, there is only a single dauer in (C). (D) Quantification of pheromone assays at different pheromone concentrations and in the presence or absence of lophenol (n = 3). Error bars represent ±SD.

(Figures 6B and 6D). In contrast, the ability of *strm-1(tm1781)* mutants to produce dauers was dramatically impaired, and the inducing pheromones were needed in at least an order of

magnitude higher amounts. The addition of lophenol had no effect on the production of dauers in the absence or presence of pheromone. As the amount of pheromone is directly proportional to the number of worms, animals lacking *strm-1* will produce dauers only under very overcrowded conditions. This makes the process very unlikely and again demonstrates that STRM-1 activity is a major component in the regulation of dauer formation.

DISCUSSION

The ability of nematodes to methylate the nucleus of cholesterol was discovered about 20 years ago. This reaction stands in stark contrast to the situation in organisms that produce sterols *de novo,* which possess a very effective system to demethylate the cyclization products of squalene: more than 10 enzymes and 19 steps are needed to produce cholesterol from lanosterol. Thus, the physiological relevance of the nuclear methylation in nematodes remained puzzling. Here, we have identified the sterol methylase that is responsible for the C-4 methylation of the A-ring, STRM-1. STRM-1 belongs to a diverged subfamily of SMTs containing proteins from both free-living (*C. elegans* and *C. briggsae*) as well as parasitic nematodes (*B. malayi*).

Our data show that the activity of STRM-1 is directly associated with the process of dauer larva formation and regulation of the amount of DA. Previously, we have shown that methylation per se is not important, but the unmodified C-4 position is critical for the production of the hormone that inhibits dauer formation (Matyash et al., 2004). The free C-4 position might be required for the production of the cholestenone that is later metabolized by DAF-9 to produce DA. It has been proposed that there are Δ^4- and Δ^7-DA pathways, which are controlled by two weak *daf* genes: *hsd-1* and *daf-36*, respectively (Patel et al., 2008; Rottiers et al., 2006). *hsd-1* is a 3-hydroxysteroid dehydrogenase/Δ^5/Δ^4 isomerase, and *daf-36* is a Rieske-like oxygenase. We suggest a hypothesis in which STRM-1 interferes with these two pathways by introducing a methyl group at the C-4 position. STRM-1 can therefore control the process of dauer larva formation by limiting the pool of unmethylated sterols, which are essential precursors of DA. Several lines of evidence supporting this conclusion are provided in this paper: (1) Mutants lacking DAF-9 have elevated amounts of methylated sterols. (2) Reciprocally, mutants lacking STRM-1 have elevated DA levels. (3) Both enzymes, DAF-9 and STRM-1, are strongly expressed in the same hypodermal cells. (4) The increased sensitivity of *daf-7 (e1372) strm-1(tm1781)* to DA is not abolished by the addition of external lophenol (the product of the reaction). (5) Several *daf-c* mutants (*daf-7(e1372)*, *daf-2(e1368)*, *daf-36(k114)*, and *daf-9(rh50)*) show a decreased frequency of dauer formation or the related *mig* phenotype upon deletion of *strm-1*. (6) STRM-1 does not inactivate DA directly by methylating it, since deletion of *strm-1* does not increase sensitivity to exogenous DA in the absence of *daf-9*. (7) The most convincing argument for the involvement of STRM-1 in the regulation of dauer formation is a dramatically decreased responsiveness of *strm-1*-deficient animals to pheromone.

Based on our data we suggest that A ring methylation can regulate the overall amount of DA (Figure 7). This is achieved by utilization of cholestenones as substrates for methylation,

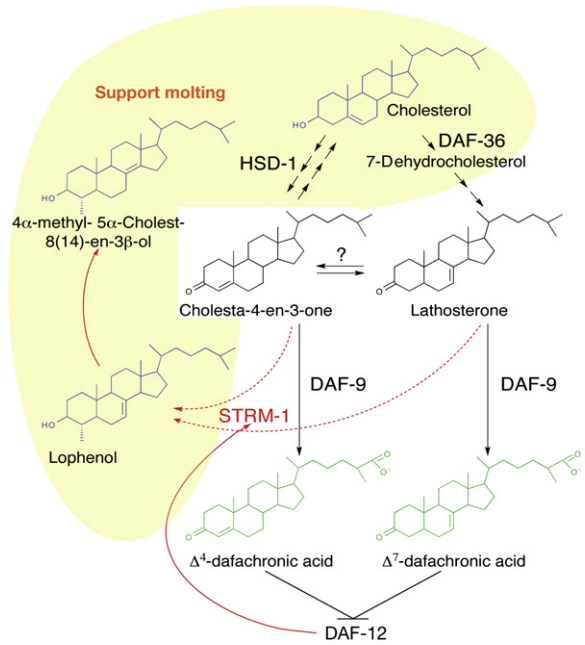

Figure 7. STRM-1 Regulates the Dauer Formation Process
Cholesterol is a precursor for ketones (cholesta-4-en-3-one and lathosterone), which are substrates for DAF-9, an enzyme that produces DAs. STRM-1 uses the same substrates to produce lophenol and, consequently, other 4-MS. Since the methylation of cholesterol is not reversible, 4-MS cannot be used to produce DA. Thus, STRM-1 can regulate levels of the latter and, consequently, the entry into the dauer state. Note that in contrast to DA, 4-MS can support the molting process.

which are also substrates of DAF-9. As the methylation is irreversible, the methylated sterols cannot be used for the production of hormone. At the same time methyl sterols, in contrast to DA, are fully active in the process of molting. Thus, methylated sterols build a pool of sterols that are used only for molting but not for hormone production. A very important element of this scheme is the existence of a positive loop for *strm-1* regulation by *daf-12*. Upon initiation of the dauer program, DA is decreased and DAF-12 promotes dauer formation. This leads, among other processes, to the increase of 4-MS via STRM-1 activity, which indirectly decreases DA even further, and the process becomes irreversible.

The study of dauer formation and its hormonal control has already proven useful in understanding other more general processes like aging, endocrine circuitry, and energy homeostasis. The identification of an important new player in nematodal hormone regulation involved in dauer formation might also lead to additional insight into other processes governed by sterol-derived signals. Those could include gonad development and fertility, growth, molting, and general lipid homeostasis. Even though C-4 methylation seems to be a nematode-specific reaction, the regulated removal of hormonal precursors could emerge as a more general mechanism in development. In addition, the uniqueness of C-4 methylation in nematodes, both free-living and parasitic, might have a medical relevance. Dauer larvae resemble, in many aspects, infective larvae from parasitic nematodes (Viney, 2009). *Brugia malayi*, a filarial nematode causing lymphatic filariasis, enters the human host as an arrested L3

infective larva following a bite of the arthropod vector (Smith, 2000). Traveling through subcutaneous tissue for several days, the larva does not complete development through L4 to adulthood until it reaches the lymphatic system. This process must be tightly regulated, most likely by hormones. Recently, it was shown that the DA/DAF-12 system is conserved in parasitic nematodes, and Δ^7-DA can suppress formation of infective larvae and turn parasitic nematodes into free-living ones (Ogawa et al., 2009). Inhibitors of C-24 methylases, like Erg6p from yeast and several other species, have been developed successfully and are being tested as a treatment against parasitic protozoans, like *Trypanosoma* or *Leishmania* (Visbal et al., 2008). It is tempting to speculate that similar inhibitors could be used to specifically disrupt developmental hormone regulation of parasitic nematodes to fight infection at a very early stage, before irreversible damage has been done to the lymphatic system.

Although the catalytic mechanism of STRM-1 still needs a significant amount of characterization, it is already apparent that the enzyme catalyzes a novel sterol reaction. To our knowledge, it is the first methylase that is capable of introducing a methyl group at a specific position in the sterol nucleus and not its side chain. This feature can now be used in the chemical synthesis of new substances and may serve as a tool to investigate the role of 4-MS in a diverse range of species. For example, in fission yeast, it has been proposed that 4-MS are involved in oxygen sensing (Hughes et al., 2007). This hypothesis can now be tested directly by targeted production of C-4 methylated sterols.

EXPERIMENTAL PROCEDURES

Materials

Lophenol was purchased from Research Plus (Manasquan, NJ), [1,2,6,7-^{3}H]-cholesterol from Biotrends (Cologne, Germany), [methyl-^{3}H]-methionine from GE Healthcare Europe GmbH (Freiburg, Germany), and synthetic daumone from KDR Biotech Co., LTD (South Korea). Lophanol, 27-desmethylcholestanol, short ascaroside derivative of 8*R*-hydroxy-2*E*-nonenoic acid, (25*S*)-cholestenoic, (25*S*)-Δ^4-, (25*S*)-Δ^7-, and (25*S*)-DAs were produced in the Knölker Laboratory (Martin et al., 2008, 2009; short chain ascaroside synthesis, R.M. and H.J.K., unpublished data). All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany); Dulbecco's medium (DMEM) was from Invitrogen (Karlsruhe, Germany).

A list of *C. elegans* strains used in this study can be found in the Supplemental Data. *Steinernema feltiae* strain was purchased from E∼nema GmbH (Schwentinental, Germany) and grown on NGM agar plates seeded with *Xenorhabdus bovienii* at 25°C. Bacteria *Xenorhabdus bovienii* were grown in LB media at 30°C, to saturation.

Metabolic Labeling

Cholesterol Labeling

About 3000 bleached embryos were put on seeded plates containing 5–10 μCi of [1,2,6,7-^{3}H]-cholesterol and grown for 4 days until early adulthood. Large amounts of homogenous *daf-9(dh6)* dauer-like larvae were obtained by growing *daf-9(dh6); dhEx24* (see below) on NGM plates with 100 nM Δ^7-DA and isolating nonfluorescent adults (homozygous for *daf-9(dh6)*). The following generation was labeled as described above with or without DA in the medium. For *S. feltiae* labeling, adult worms were transferred to NGM plates seeded with *X. bovienii* mixed with 10 μCi [1,2,6,7-^{3}H] cholesterol and grown for 6 days at 25°C.

Methylation Labeling

To increase label incorporation, RB755 strains were used and putative substrate sterols were added directly to the bacteria at 130 μM. For each strain and condition, about 100,000 bleached embryos were put on three 15 cm seeded plates containing 100 μCi [methyl-^{3}H]-methionine and were grown until early adulthood.

Lophenol Labeling

About 40,000 *daf-7(e1372)* worms were labeled with 100 μCi [1,2,6,7-^{3}H]-cholesterol for 2 weeks at 25°C, and lipids were extracted from the resulting dauers and saponified (see below). Methylated sterols were scraped from a TLC plate, extracted from the silica powder according to (Folch et al., 1957), dried, resuspended in ethanol, mixed with sterol-free NA22 bacteria, seeded on sterol-free agarose plates, and fed to worms (Matyash et al., 2004). Worms were grown for 4 days until adulthood.

Lipid Extraction and TLC Analysis

Worms were lysed by freezing/thawing and lipids were extracted according to Bligh and Dyer (1957) with the exception of extracts for DA analyses, which were extracted according to Gill et al. (2004). In cases when saponification was performed, dried lipids were heated in 3M KOH:methanol (1:9,v:v) for 1 hr at 80°C. Unsaponifiable lipids were extracted three times with hexane, pooled, dried, and used for further analysis. Cholesterol labeling samples were normalized for total radioactivity before TLC analysis; samples from the methionine labeling were normalized for total radioactivity before saponification.

Thin-layer chromatography was performed on silica gel 60 or HPTLC plates (both from Merck, Darmstadt, Germany) using chloroform:methanol (24:1) as the running system. 2D TLC for the detection of DAs was done using chloroform:methanol (24:1) as the first running system and chloroform:methanol:ammonia (60:35:5) as the second. After chromatography, plates were sprayed with scintillation fluid (Lumasafe, Lumac LSC B.V., Groningen, The Netherlands) and exposed to a film (Kodak Biomax MR, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Dauer Formation Assay

Worms grown at 15°C were bleached, and approximately 100–150 embryos were transferred to NGM 12-well plates seeded with NA22 bacteria, grown at 15°C, 20°C, 22.5°C, 25°C or 27°C, and scored for percentage of dauer larva after 3–5 days depending on the temperature. In the case of *daf-36(k114)* and *daf-9(rh50)*, non-*mig* adults, *mig* adults and larvae, dead or arrested animals with extruded gonads or molting defects, and dauer/dauer-like larvae were scored. To determine DA sensitivity in *daf-7(e1372)* and *daf-7(e1372) strm-1(tm1781)*, NGM 12-well plates containing different concentrations of DA (ranging from 13 to 1300 nM) were used. To determine sensitivity of *daf-9 (dh6)* and *strm-1(tm1781); daf-9(dh6)* to exogenously supplied DA, adult *daf-9(dh6); dhEx24* or *strm-1(tm1781); daf-9(dh6); dhEx24* were placed to lay about 100 eggs in 6-well plates containing different amounts of DAs. *dhEx24* is an extra-chromosomal array carrying fully active DAF-9 and also a nuclear GFP (Gerisch et al., 2001). After 3 days, *daf-9(dh6)* were identified based on the absence of GFP fluorescence and the indicated phenotypes were scored.

Gamravali Assay and DAF-9 Activity Assay

The gamravali assay in Figure 1 was done according to Matyash et al. (2004). For the *daf-9* activity assay, extraction was done according to Gill et al. (2004), and the rescue assay was performed according to Motola et al. (2006). Approximately 500,000 bleached embryos of N2, *strm-1(tm1781)*, and *daf-9 (dh6) daf-12(rh61rh411)* were grown until L3 larval stage, lysed by freezing /thawing, diluted in 15 ml TBS, mixed with 2 ml zirconium beads, and extracted four times with 30 ml diethyl ether following extensive vortexing. Different dilutions of dried extracts in DMSO were mixed with plated bacteria and dried. The *daf-9(dh6)* rescue assay was performed as described above.

Induction of Dauer Formation with Synthetic Pheromones

The assay was done according to Butcher et al. (2007), but two synthetic dauer inducing pheromones (daumone [Jeong et al., 2005] and short ascaroside derivative of 8*R*-hydroxy-2*E*-nonenoic acid [Butcher et al., 2007]) were used in combination at relatively high concentrations to ensure a strong and robust response in wild-type (see Figure 6). Pheromones were mixed both to NGM and to heat-inactivated bacteria. In the indicated cases, lophenol (13 μM) was added to the seeded bacteria. Adult worms were placed to lay eggs (approximately 150 per well in 6-well plates). After 2 days at 25°C, dauers and L4 larvae, or young adults were counted.

Cloning of *pstrm-1::GFP*, cloning of *ADH1::STRM-1*, sequence and phylogenetic analyses, and sterol analyses by mass spectrometry are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00174-9/.

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