Developmental and Cell Cycle Quiescence Is Mediated by the Nuclear Hormone Receptor Coregulator DIN-1S in the *Caenorhabditis elegans* Dauer Larva

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ABSTRACT When faced with suboptimal growth conditions, *Caenorhabditis elegans* larvae can enter a diapause-like stage called "dauer" that is specialized for dispersal and survival. The decision to form a dauer larva is controlled by three parallel signaling pathways, whereby a compromise of TGFβ, cyclic guanosine monophosphate, or insulin/IGF-like signaling (ILS) results in dauer formation. Signals from these pathways converge on DAF-12, a nuclear hormone receptor that triggers the changes required to initiate dauer formation. DAF-12 is related to the vitamin D, liver-X, and androstane receptors, and like these human receptors, it responds to lipophilic hormone ligands. When bound to its ligand, DAF-12 acquires transcriptional activity that directs reproductive development, while unliganded DAF-12 forms a dauer-specifying complex with its interacting protein DIN-1S to regulate the transcription of genes required for dauer development. We report here that *din-1S* is required in parallel to *par-4*/LKB1 signaling within the gonad to establish cell cycle quiescence during the onset of the dauer stage. We show that *din-1S* is important for postdauer reproduction when ILS is impaired and is necessary for long-term dauer survival in response to reduced ILS. Our work uncovers several previously uncharacterized functions of DIN-1S in executing and maintaining many of the cellular and physiological processes required for appropriate dauer arrest, while also shedding light on the coordination of nuclear hormone signaling, the LKB1/AMPK signaling cascade, and ILS/TGFβ in the control of cell cycle quiescence and tissue growth: a key feature that is often misregulated in a number of hormone-dependent cancers.

KEYWORDS quiescence; dauer; nuclear hormone receptor; insulin-like signaling; DAF-12; DIN-1S; *par-4*/LKB1; *aak-2*/AMPK

LIKE many metazoans, *Caenorhabditis elegans* develops through a lengthy juvenile phase before reaching reproductive maturity; a process that includes the passage through four larval stages (L1–L4) to finally give rise to an adult hermaphrodite. However, if environmental conditions are inadequate for reproductive development, *C. elegans* possesses an effective means of modifying its life cycle allowing it to opt for an alternative mode of development referred to as the dauer stage. Dauer is a diapause-like stage that is specialized for dispersal and survival, where instead of progressing from the L2 to the L3 stage, L1 larvae will execute an alternative L2 stage (L2d) during which they alter their metabolic program to accumulate lipid reserves and subsequently enter the dauer stage (Kimura *et al.* 1997; Burnell *et al.* 2005). Dauer larvae are morphologically, metabolically, and behaviorally distinct from L3 stage larvae, while they exhibit a global state of cell cycle and developmental quiescence, presumably to conserve energy resources.

The decision to form the dauer larva is controlled by three parallel signaling pathways whereby the reduction in TGFβ, cyclic guanosine monophosphate or insulin/IGF-like signaling (ILS) will promote dauer formation. Signals from these pathways converge on DAF-12, a nuclear hormone receptor (NHR) that specifies either dauer formation or reproductive growth depending on specific environmental cues (Ren *et al.* 1996; Kimura *et al.* 1997; Antebi 2006). When environmental conditions are favorable for reproductive growth, the upstream pathways that control dauer formation activate DAF-9, a cytochrome P450 enzyme expressed in a subset of neuronal cells, which then triggers the production and release of steroid hormone ligands for DAF-12 (Gerisch and Antebi 2004;

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Gerisch *et al.* 2001, 2007; Motola *et al.* 2006). Consequently, ligand-bound DAF-12 becomes transcriptionally active, initiating the expression of numerous genes involved in directing reproductive development. These transcriptional signals will cooperate with other pathways to instruct germline development throughout the L3 and L4 stages to generate a reproductive hermaphrodite adult (Michaelson *et al.* 2010).

Conversely, unliganded DAF-12 presumably forms a dauer-specifying complex that represses the transcription of genes required for reproductive development through its association with the short isoform of the **D**AF-12 **in**teracting protein DIN-1S (Ludewig *et al.* 2004; Antebi 2006; Motola *et al.* 2006).

Considerable progress has been made in identifying the environmental elements and molecular pathways that affect dauer development; however, the downstream effectors that control the physiological changes that must take place during this stage remain uncharacterized. For example, the cyclin-dependent kinase inhibitor *cki-1* is required for the general cell cycle arrest that occurs downstream of the dauer-promoting pathways (Hong *et al.* 1998). These same effectors, or a subset thereof, also appear to upregulate the transcription of AMP-activated protein kinase (AMPK) (Narbonne and Roy 2009). How these signals impinge directly or indirectly on AMPK, *cki-1*, or additional gene products that are required for appropriate dauer quiescence, still remains unclear.

We have identified some of the factors that affect the establishment of germ cell quiescence in response to environmental conditions (Narbonne and Roy 2006). PTEN/*daf-18*, the PIP3 phosphatase that negatively regulates ILS, the catalytic alpha AMPK subunits *aak-1* and *aak-2*, and the major AMPK-activating kinase *par-4*/LKB1, are all required for germ cells to appropriately arrest their cell cycle while the animal is entering the dauer stage. These factors function either downstream of, or in parallel to Notch signaling, which, under favorable conditions, instructs nearby germ cells to divide mitotically, while concomitantly blocking entry into meiosis (Narbonne and Roy 2006). Furthermore, the timing and spatial regulation of these signals are equally very important as the germ cells progress proximally through the gonad (Killian and Hubbard 2005; McGovern *et al.* 2009).

Our analysis of the genes involved in establishing and/or maintaining germline quiescence indicated that the tumor suppressor *par-4*/LKB1, its kinase target the critical metabolic regulatory protein kinase AMPK/*aak*-2, and the tumor suppressor protein PTEN/*daf-18* were involved in this process (Narbonne and Roy 2006). But subsequent genetic analysis revealed that additional players are likely involved in the regulation of germline quiescence in the dauer larva. For example, the fact that a mutation in *par-4*/LKB1 is additive to null mutations that abolish AMPK signaling suggests that AMPK is not the sole downstream effector of *par-4*/LKB1 signaling in *C. elegans* (Narbonne and Roy 2006). To identify additional genes involved in the establishment or maintenance of germline quiescence during the dauer stage, we scaled up our initial genetic screen and isolated seven more mutant alleles displaying moderate-to-severe dauer germline hyperplasia. We report here the characterization of *din-1S*, a nuclear hormone receptor coregulator that acts in the establishment and/or maintenance of many of the key features of dauer development, including the restriction of somatic gonadal cell proliferation during this stage.

Table 1 Mutations that affect the establishment or maintenance of germline quiescence in the dauer stage

Allele	Genetic location	Gene affected
rr77	II: 0.00–1.76	Unknown
rr88	III: 7.94	*aakb-2*
rr91[a]	III: 5.70	*strd-1*
rr92[a]	III: 5.70	*strd-1*
rr93	V: 0.00–7.01	Unknown
rr94	II: 3.79	*din-1S*
rr95	IV: 0.06–4.97	Unknown

[a] Previously described allele of *strd-1* (Narbonne and Roy 2009).

Materials and Methods

Strains and culture

All *C. elegans* strains were maintained at 15° and grown according to standard procedures unless otherwise stated (Brenner 1974). N2 Bristol was used as the wild-type strain. The following alleles and transgenes were used: LGI, *rrf-1(pk1417)*; LGII, *rrf-3(pk1426)* and *din-1(rr94*, *dh127*, *dh149)*; LGIII, *daf-2(e1370)*, *daf-7(e1372)*, *glp-1(e2141)*, and *aak-1(tm1944)*; LGIV, *daf-18(ok480)*; LGV, *qIs56*[*lag-2*::GFP, *unc-119*(+)], *akt-1(ok525)*, and *par-4(it57)*; LGX, *unc-1(e719)*, *daf-12(rh61)*, *aak-2(rr48*, *ok524)*; *qIs89* [*gon-14*::GON-14::VENUS + *Saccharomyces cerevisiae* DNA]; and *syIs78* [*ajm-1*::GFP; *unc-119(+)*]. A complete list of all strains utilized in this study is available in Supplemental Material, Table S1.

Genetic screen for factors that control germ cell quiescence in response to dauer induction

A forward genetic screen was performed in order to isolate mutations in genes that regulate germ cell numbers before entering (during L2d) or during the dauer stage, as previously described (Narbonne and Roy 2006). Briefly, *daf-2(e1370)* animals are dauer constitutive at the restrictive temperature (25°). *qIs56(lag-2*::GFP) is expressed in the distal tip cells (DTCs) located at the distal ends of the gonad. *unc-1(e719)* mutants move poorly and this was useful to limit dauer loss during the screen. *daf-2(e1370)*; *qIs56*; *unc-1(e719)* L4 larvae were mutagenized with 0.03 M EMS. F_1 progeny were kept at 15° until they started laying eggs, at which point they were dispensed five per plate and upshifted to 25°. F_2 dauer larvae were screened for enlarged gonads using an increase in the displacement between the GFP signals in the DTCs as an indicator of germline hyperplasia in the dauer larva. A total of 12,400 haploid genomes were screened, and seven alleles that all exhibited increased germ cell numbers in the

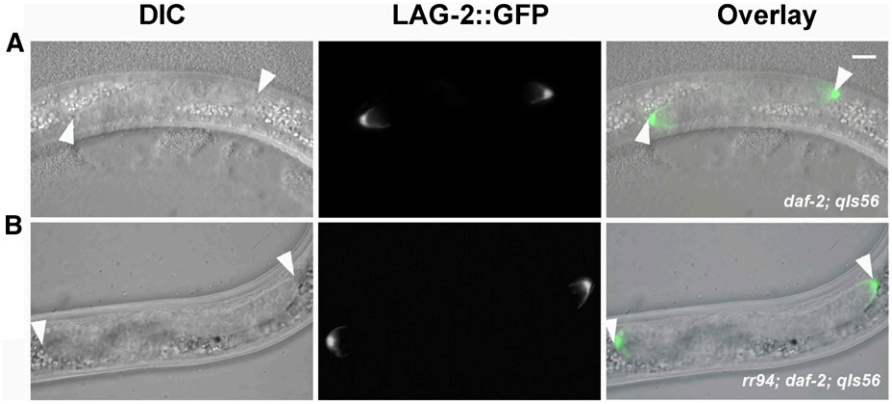

Figure 1 *rr94* causes germline hyperplasia in ILS-impaired dauer larvae. (A and B) DIC images of a control *daf-2(e1370)*; *qIs56* dauer (A) whose germline contains ~34 germ cells and (B) a *rr94*; *daf-2(e1370)*; *qIs56* mutant dauer larva whose germline contains more than twice the wild-type number of germ cells. Dark field micrographs show LAG-2::GFP expression in the gonad of the control and the *rr94* mutant dauer larvae. The overlay depicts LAG-2::GFP expression localized to the two DTCs in the control animal and the *rr94* mutant dauer larva. The DTCs are marked with arrowheads in the DIC and overlay images. Bar, 10 μm.

dauer germline were isolated (Table 1). Mutants were outcrossed four times prior to any subsequent analysis.

Mapping and cloning of rr94

rr94 was mapped to the center right of LGII by conventional linkage analysis and three factor crosses. Snip-SNP and sequencing SNP mapping (Wicks *et al.* 2001) were used to place *rr94* in a genetic interval between LGII: 3.342–4.286. Using a feeding RNA interference (RNAi) approach we targeted the genes in this interval (Kamath and Ahringer 2003) in an RNAi-sensitized *rrf-3* background (Simmer *et al.* 2002). RNAi against F07A11.6 partially phenocopied the germline hyperplasia observed in *rr94* dauer larvae. F07A11.6, or *din-1*, codes for long (*din-1L*) and short (*din-1S*) transcripts, but only *din-1S* has been shown to be involved in dauer formation (Ludewig *et al.* 2004). Independent sequencing analyses of *din-1S* identified a typical EMS-induced G/C-to-A/T transition at position 1 of predicted codon 44. This mutation alters a glutamine to an amber stop codon, resulting in a truncated protein.

Staining

For 4′,6-diamidino-2-phenylindole (DAPI) staining of whole larvae, animals were collected in 1.5-ml microfuge tubes and fixed in Carnoy's solution (60% ethanol, 30% acetic acid, 10% chloroform) while shaking for at least 4 hr at 4°. After washing twice with PBST (1× PBS + 0.1% Tween 20), animals were stained in a 0.1 mg/ml DAPI solution for 30 min. Larvae were then washed four times for at least 12 min per wash with PBST and mounted in Vectashield (Vector Laboratories, Burlingame, CA) medium.

For germline staining, gonads were extruded from animals and staining was performed as previously described using an anti-HIM-3 antibody (Zetka *et al.* 1999) or with an anti-PGL-1 antibody (Kawasaki *et al.* 1998).

Microinjection

In order to assess cell/lineage autonomy of *din-1S(rr94)*, we microinjected constructs that included a germline-expressing or a constitutively active somatic promoter using a method that was described previously (Kelly *et al.* 1997). The DNA injection mix included 1 ng/μl PCR fragment of either *din-1S*::DIN-1S, *pgl-1*::DIN-1S, or *sur-5*::DIN-1S; 60 ng/μl *Pvu*II-digested N2 genomic DNA; 1 ng/μl *Eco*RI-digested pRF4 (*rol-6D*), and 1 ng/μl *Pst*I-digested pMR910 (*myo-2*::GFP). Injected animals were maintained at 15°. Adult F_2 Rol animals were transferred to 25° to allow F_3 descendants to form dauer larvae that were subsequently collected and stained with DAPI for germline analysis. *myo-2*::GFP was used to identify transgenic animals for cell counts.

Counting proximal somatic gonadal cells

The preparation of dauer larvae to count the numbers of proximal somatic gonad cells was performed using the GON-14::VENUS (GFP) marker to distinguish somatic cells (Z1 and Z4 descendants) from nearby germ cells. Only the nuclei located proximally were counted; those somatic gonadal cell nuclei that began migrating distally were excluded from the analysis. Once we identified the cells within the cluster, we counted nuclei based on nuclear morphology and position as described for germ cell nuclei (Narbonne and Roy 2006). We refer to these proximal somatic gonadal cells as the somatic gonadal cell cluster throughout the study.

Postdauer reproductive fitness

L1 larvae were synchronized and grown at 25° to induce dauer formation. Dauer larvae were subsequently singled and 1-, 4-, or 7-day-old dauer larvae were placed one per plate at 15° to permit their recovery and monitored daily for egg laying or bagging. Sterile worms were followed until death to ensure that reproduction was not simply delayed.

Other techniques

Microscopy and double-strand RNA (dsRNA) injections were performed as described previously (Kostic *et al.* 2003). Feeding RNAi (Kamath and Ahringer 2003), antibody staining, DAPI staining, germ cell nuclei counts, and dauer survival assays were all carried out as previously described (Narbonne and Roy 2006).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Table 2 *din-1S(rr94)* is required for normal dauer survival and the timely establishment of cell cycle quiescence in the dauer gonad

Genotype	No. of germ cells in dauer[a]	No. of proximal somatic gonadal cells in dauer[a]	Dauer survival in days[a] (*n*)
daf-2(e1370)[b]	33.6 ± 3.9	6 ± 0	33.5 ± 8.3 (207)
rr94; *daf-2(e1370)*[b]	82.0 ± 16.3	25.4 ± 19.2	14.4 ± 8.0 (191)
din-1S(dh127); *daf-2(e1370)*	64.2 ± 14.6	14.9 ± 16.3	ND
din-1S(dh149); *daf-2(e1370)*	71.9 ± 15.6	12.9 ± 15.9	ND
din-1S(rr94/dh127); *daf-2(e1370)*[c]	77.8 ± 15.2	22.1 ± 15.9	ND
din-1S(rr94/dh149); *daf-2(e1370)*[c]	83.7 ± 12.0	19.6 ± 19.5	ND
daf-2(e1370); *E.V. (RNAi)*[d]	32.9 ± 2.0	6 ± 0	ND
din-1S(RNAi); *daf-2(e1370)*	72.3 ± 21.8	20.9 ± 22.0	ND
daf-7(e1372)[b]	31.0 ± 3.2	6 ± 0	26.3 ± 8.7 (61)
rrf-3(pk1426); *daf-7(e1372)*	34.5 ± 7.0	6 ± 0	ND
rrf-1(pk1417); *daf-7(e1372)*	31.8 ± 4.4	6 ± 0	ND
rr94; *daf-7(e1372)*[b]	103.3 ± 9.0	87.6 ± 10.1	27.2 ± 7.2 (105)
daf-7 (e1372); *E.V. (RNAi)*[d]	31.2 ± 2.4	6 ± 0	ND
din-1S(RNAi); *daf-7(e1372)*	60.7 ± 13.8	27.0 ± 11.7*	ND
rrf-3(pk1426); *daf-7(e1372)*; *E.V. (RNAi)*[d]	32.4 ± 2.7	6 ± 0	ND
rrf-3(pk1426); *din-1S(RNAi)*; *daf-7(e1372)*	63.2 ± 9.9	40.6 ± 19.8*	ND
rrf-1(pk1417); *daf-7(e1372)*; *E.V. (RNAi)*[d]	33.7 ± 2.8	6 ± 0	ND
rrf-1(pk1417); *din-1S(RNAi)*; *daf-7(e1372)*	65.9 ± 24.4	14.0 ± 12.9*	ND

* $P < 0.05$ (statistical significance) between all three strains according to a two-tailed Mann–Whitney *U*-test.
[a] Mean ± SD; *n*, sample size, $n \geq 20$.
[b] Full genotype includes *qIs56*.
[c] Full genotype includes *qIs56*/+. The presence of the transgene was used as a marker for successful mating and for identification of true heterozygous progeny.
[d] E.V., empty vector was used as an RNAi control.

Results

Mutants that affect germline stem cell quiescence

In order to characterize the molecular genetic pathways governing germline quiescence in the dauer larva, we performed a forward genetic screen for mutants that exhibit dauer-specific germline hyperplasia in a *daf-2* background. From this screen, we isolated seven mutants that comprised six complementation groups (Table 1). Among these, *rr94* exhibited strong expressivity and penetrance and was therefore selected for further characterization. *rr94* is a recessive allele that causes an enlarged gonad specifically in dauer larvae (Figure 1). We did not observe supernumerary germ cell divisions in *rr94* animals in either a *daf-2* or *daf-7* background when maintained at permissive temperature (Figure S1). Other than the observed hyperplasia, *daf-2*; *rr94*; *qIs56* dauer larvae are visibly healthy and display characteristics typical of dauer larvae: morphologically they possess a radially constricted body and pharynx, they do not pump, and they express a LAG-2::GFP reporter in the IL2 head neurons (Ouellet *et al.* 2008) (Figure S2). Upon closer examination we noted that the *daf-2*; *rr94* animals do not form wild-type alae and are more sensitive to SDS than *daf-2* dauer larvae, although the seam cell numbers appear unaffected (Figure S3). This suggests that the integrity of the dauer cuticle may be affected in *rr94* animals. Curiously, both *rr94*; *daf-2* and *rr94*; *daf-7* double mutants undergo premature seam cell fusion without affecting overall cell numbers along the lateral seams (Figure S3).

Unlike *daf-2* dauer larvae that possess ~34 germ cells, *rr94*; *daf-2* dauer larvae possess threefold more germ cells in their gonad. Like the supernumerary germ cells in AMPK mutants, these cells arise due to ongoing cell divisions during the L2d period when germ cells normally begin to slow down their division rate and arrest (Narbonne and Roy 2006). In the *rr94* larvae, the germ cells continue dividing at a higher rate throughout the L2d, which is also modestly extended compared to *daf-2* animals (our unpublished results), but eventually they do arrest and remain quiescent throughout the duration of the dauer stage (Figure S4). Therefore, *rr94* is required to regulate the duration of germ cell proliferation and the rate of cell divisions preceding the onset of the dauer stage.

In addition to the increase in germ cell counts, we observed that *rr94* dauer larvae have extra cells located in the region normally occupied by a cluster of somatic gonadal cells. These additional cells arise during L2d, much like the extra germ cells that arise in these mutants (our unpublished results). In wild-type dauer larvae, the somatic gonad is composed of 12 cells that are essentially identical to those present at the end of the L2 stage, owing to the normal cell cycle arrest that occurs during this diapause-like stage. This includes two DTCs and 10 somatic cells that give rise to all the other somatic tissues of the gonad (Hubbard and Greenstein 2005). Six of these somatic cells are located proximally to the two extending gonad arms in dauer and for simplicity we refer to these cells as the somatic gonadal cell cluster. The remaining four cells migrate away from this area before dauer formation is complete. We found that *rr94*; *daf-2* dauers contain about 25 cells within the region where the somatic gonadal cell cluster would normally reside, in contrast to the six cells typical of the wild-type cluster (Table 2).

To further demonstrate that these cells are part of the developing somatic gonad, we examined the expression of

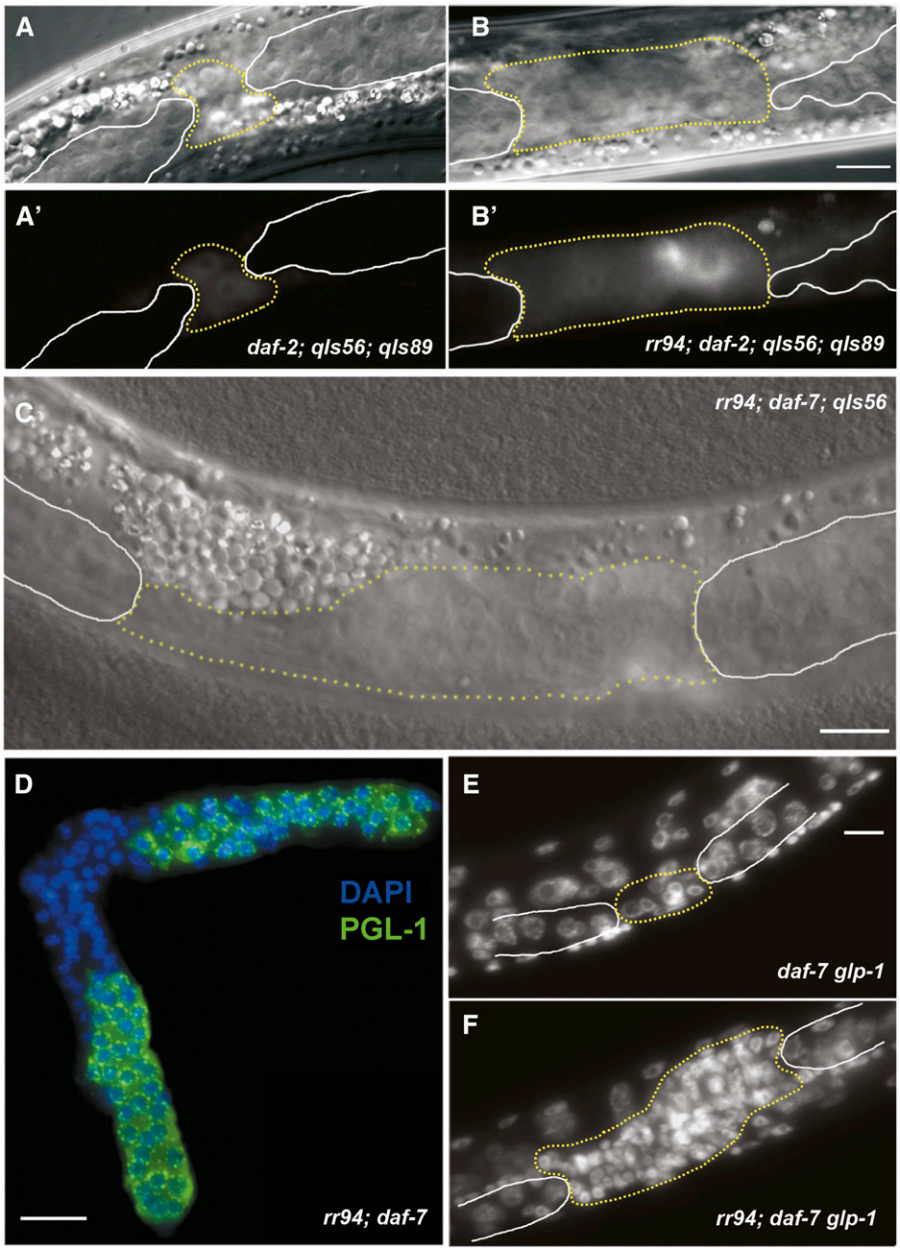

Figure 2 Supernumerary cells are present in both the germline and the somatic gonad in *rr94* dauer larvae. (A and B) DIC image of a *daf-2(e1370)*; *qIs56*; *qIs89* control dauer gonad (A) and a *rr94*; *daf-2(e1370)*; *qIs56*; *qIs89* dauer gonad (B). (A′ and B′) Image showing *qIs89* [*gon-14*::GON-14::VENUS] expression in the proximal somatic gonadal cells that form a cluster between the proximal gonad arms in both control (A′) and *rr94* (B′) dauer animals. (C) DIC image of a *rr94*; *daf-7(e1372)*; *qIs56* dauer larva depicting the enlarged proximal somatic gonadal cell cluster outlined with a dotted yellow line. (D) Extruded *rr94*; *daf-7(e1372)*; *qIs56* dauer gonad stained with DAPI (blue) and the P-granule-specific PGL-1 antibody (green). (E and F) Image of a *daf-7(e1372) glp-1(e2141)* dauer gonad (E) and a *rr94*; *daf-7(e1372) glp-1(e2141)* dauer gonad (F) stained with DAPI. The gonad arms containing a small number of germ cells are outlined by solid white lines, while dotted yellow lines indicate the proximal somatic gonadal cell cluster in A–C and E and F. Bars, 10 μm, except in D, 20 μm. The bar shown in B′ also applies to A′, and the bar in F also applies to E.

a GFP reporter that marks the somatic gonadal cells during this stage (*gon-14*::GON-14::VENUS) (Chesney *et al.* 2006) and that efficiently distinguishes the somatic gonadal cells in *rr94*; *daf-2* and *rr94*; *daf-7* dauer larvae. GFP is expressed in the somatic gonadal cells in *daf-2* dauer larvae where it localizes to the nuclear periphery and is diffuse in the cytoplasm of the somatic gonadal cells within the cluster, unlike in L3 stage larvae where it localizes to nuclear speckles (Chesney *et al.* 2006). On the other hand, it is undetectable in the germ cells (Figure 2A′). We observed the same diffuse cytoplasmic localization for the somatic gonadal cell marker in *rr94*; *daf-2* dauer larvae where it is expressed in the additional cells present within the proximal region of the somatic gonadal cell cluster (Figure 2B′). Our observations indicate that *rr94* controls the onset of cell cycle quiescence both in the somatic gonad and the germline stem cells. When compromised, both cell types undergo unscheduled cell divisions that give rise to pronounced hyperplasia in the lineages that constitute the dauer gonad and these abnormal cell divisions occur exclusively in animals that undergo dauer development.

rr94 is an allele of the nuclear hormone receptor coregulator din-1S

We mapped *rr94* to the center of chromosome II (see *Materials and Methods*), and feeding RNAi was performed against potential candidates in the defined interval to assess their ability to phenocopy the *rr94* defects. Of all the genes tested, only the bacterial clone that corresponded to *din-1* caused reproducible germline hyperplasia in the dauer stage.

Table 3 Loss of *daf-12* function recapitulates the dauer-associated hyperplasia in the germ line and somatic gonad typical of *din-1S(rr94)* mutants

Genotype	No. of germ cells in dauer[a]	No. of proximal somatic gonadal cells in dauer[a]
(A) *daf-2(e1370)*[b]	34.5 ± 3.5*	6 ± 0
(B) *din-1S(rr94)*; *daf-2(e1370)*[b]	49.7 ± 10.0*	9.5 ± 4.6*
(C) *daf-2(e1370)*; *daf-12(rh61)*[b]	40.4 ± 3.7*	6 ± 0
(D) *din-1S(rr94)*; *daf-2(e1370)*; *daf-12(rh61)*[b]	45.0 ± 9.9*	6 ± 0
(E) *rrf-1(pk1417)*; *daf-7(e1372)*	31.8 ± 4.4	6 ± 0
(F) *rrf-1(pk1417)*; *daf-7(e1372);E.V. (RNAi)*[c]	30.6 ± 2.9**	6 ± 0
(G) *rrf-1(pk1417)*; *daf-7(e1372)*; *daf-12(RNAi)*	40.1 ± 6.7**	8.0 ± 6.9
(H) *rrf-1(pk1417)*; *daf-2(e1370)*[d]	32.5 ± 3.8	6 ± 0
(I) *rrf-1(pk1417)*; *daf-2(e1370)*; *E.V. (RNAi)*[c,d]	31.4 ± 2.8***	6 ± 0
(J) *rrf-1(pk1417)*; *daf-2(e1370)*; *daf-12(RNAi)*[d]	37.9 ± 4.0***	6.4 ± 1.8

* $P < 0.0005$ (statistical significance) between rows B, C, and D and the control value shown in row A according to a one-tailed *t*-test with unequal variance. ** $P < 0.0005$ (statistical significance) between row G and the control value shown in row F according to a one-tailed *t*-test with unequal variance. *** $P < 0.0005$ (statistical significance) between row J and the control value shown in row I according to a one-tailed *t*-test with unequal variance.

[a] Mean ± SD; $n \geq 20$.

[b] Animals were synchronized as L1 larvae and shifted to the restrictive temperature (25°) for 48 hr before the cell counts to avoid confounding problems with *daf-12* dauer formation. At this point, although all *daf-2* animals had formed dauer larvae, the *daf-12* were in a transient dauer stage and *din-1S* animals were still in the L2d stage.

[c] E.V., empty vector was used as an RNAi control.

[d] Full genotype includes *qIs56*.

DIN-1 is a DAF-12-interacting protein with two primary isoforms: DIN-1 long (DIN-1L) and short (DIN-1S), where the *din-1S* transcript contains a large exon (exon 19) that is spliced out from the *din-1L* transcript (Ludewig *et al.* 2004). Since previous work suggested that *din-1S* is the *din-1* variant expressed during dauer, and *rr94* displays phenotypes that appear to be dauer-specific, we reasoned that *rr94* could be an allele of *din-1S* (Ludewig *et al.* 2004). In addition, two different *din-1S* alleles, *dh127* and *dh149*, phenocopied *rr94*, while these two *din-1S* alleles also failed to complement the dauer germline hyperplasia phenotype as well as the increased numbers of somatic gonadal cells in the cluster in *rr94* mutants (Table 2). Sequencing of the large *din-1S* exon identified a typical EMS-induced G/C-to-A/T transition that converted Q44 to an amber stop. We therefore conclude that *rr94* is a hypomorphic allele of the nuclear hormone corepressor/activator *din-1S*.

Dauer-specific gonadal hyperplasia is more severe in daf-7 mutant animals

To determine whether the *din-1S(rr94)* phenotypes were specific to conditions of impaired ILS, we examined the gonads of *din-1S(rr94)*; *daf-7*; *qIs56* dauer larvae and found that although the germline hyperplasia visible in the dauer larvae was comparable between the two genetic backgrounds, the number of somatic gonadal cells in *din-1S(rr94)*; *daf-7* dauer larvae was substantially higher (Table 2). This may indicate that the hyperplasia observed in the somatic gonadal cell cluster of *din-1S(rr94)* mutants is either sensitive to functional ILS and its growth promoting effects or that *daf-7*/TGFβ has a very efficient antiproliferative role that is revealed in the *din-1S(rr94)* mutant background in this developmental context.

To ensure that the extra somatic gonadal cells we observed in the gonads of *din-1S(rr94)*; *daf-7* dauer larvae were not pockets of abnormally dividing germ cells, we stained *din-1S (rr94)*; *daf-7* dauer gonads with an antibody against the germline-specific marker PGL-1. All the germ cells in *rr94* mutant dauer gonads exhibited the perinuclear enrichment typical of anti-PGL-1 staining (Figure 2D) (Kawasaki *et al.* 1998). However, the regions of presumptive somatic gonadal cell hyperplasia showed no anti-PGL signal, indicating that this hyperplasia is not due to misregulated germ cell divisions.

To further confirm the somatic nature of the observed hyperplasia in the proximal cluster, we monitored the number of somatic gonadal cells within the cluster in *din-1S(rr94)*; *daf-7 glp-1* dauer larvae (Figure 2, E and F), in which germ cell divisions are drastically reduced due to a disruption in Notch signaling (Austin and Kimble 1987). In this genetic background the degree of hyperplasia in the region of the presumptive somatic gonadal cell cluster is still very striking, strongly supporting a nongerm lineage origin of these somatic cells that overproliferate to cause the observed somatic hyperplasia. Therefore, loss of *din-1S(rr94)* function results in hyperplasia that is apparent within the gonad in dauer larvae formed due to disruption of ILS or the TGFβ pathway.

din-1S is required autonomously to appropriately establish germ cell quiescence during the dauer stage

Both the TGFβ and the ILS pathways act through the nervous system to nonautonomously regulate dauer formation and lifespan by affecting many, if not all, tissues (Inoue and Thomas 2000; Wolkow *et al.* 2000). Therefore, to determine whether *din-1S* acts cell nonautonomously in the neurons like TGFβ and ILS, or autonomously in the germ cells to establish germ cell quiescence, we performed RNAi experiments to compromise *din-1S* exclusively in the soma and/or in the germ line. Because neurons are less sensitive to dsRNA,

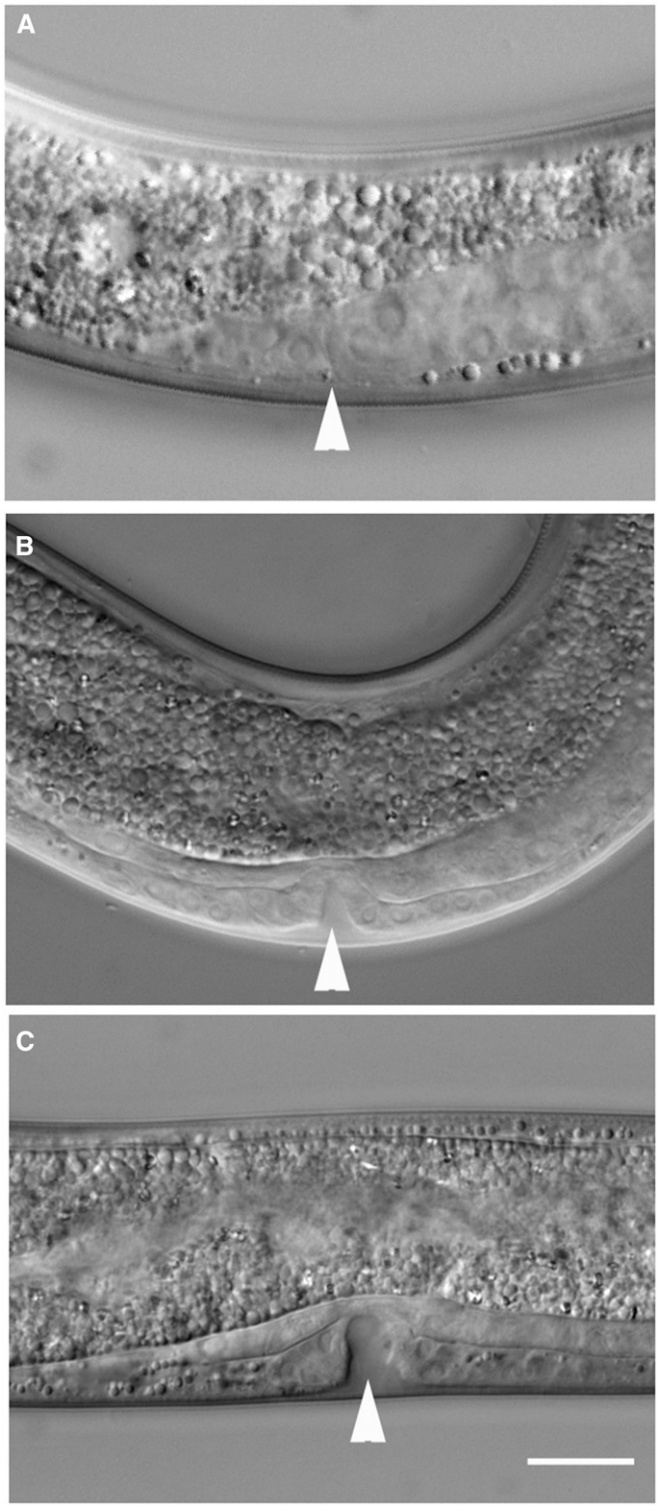

Figure 3 Vulval development proceeds inappropriately in dauer larvae with reduced nuclear hormone signaling. VPC divisions and subsequent steps in vulval morphogenesis are arrested during the dauer stage (A). Vulval divisions occur in *din-1S(rr94)*; *daf-2(e1370)* mutant dauer larvae, while cells continue to develop into a rudimentary ventral opening (B). (C) *daf-2(e1370)*; *daf-12(rh61)* dauer larva with apparently normal L4 stage vulval morphology. Bar, 10 μm.

we used an Eri mutant (*rrf-3*) that renders the neurons more responsive to RNAi (Simmer *et al.* 2002). In contrast, we performed RNAi experiments in *rrf-1* mutants that are largely defective for RNAi in the soma, and particularly in the somatic gonad, without affecting the RNAi response in the germline (Sijen *et al.* 2001; Kumsta and Hansen 2012). Neither of these mutations has been demonstrated to adversely affect the RNAi pathway in the germ line. Since the phenotype in the somatic gonad is more penetrant in *daf-7* dauer larvae than in *daf-2* animals, we performed our experiments in a *daf-7* background to facilitate quantification. We found that the number of germ cells was unchanged in somatic RNAi-deficient *daf-7* dauer larvae that were maintained on bacteria that express dsRNA against *din-1S* [compare 60.7 ± 13.8 germ cell nuclei present in *din-1S(RNAi)*; *daf-7(e1372)* *vs.* 65.9 ± 24.4 germ cell nuclei in the somatic RNAi-impaired *rrf-1(pk1417)*; *din-1S(RNAi)*; *daf-7(e1372)* in Table 2], suggesting that *din-1S* is required autonomously in germ cells to establish the timely onset of germline quiescence in preparation for dauer entry, in a manner similar to *aak-2* (Narbonne and Roy 2006).

Conversely, we observed that the number of proximal somatic gonad precursor cells was different between Eri mutants (*rrf-3*) and the germline-specific RNAi animals (*rrf-1*) following feeding of *din-1S* dsRNA-expressing bacteria. The number of proximal somatic gonadal cells increases from an average of 27 to nearly 41 cells in the Eri mutants, while it decreases by about half when the RNAi pathway is blocked in the soma (*rrf-1*) (Table 2). It is noteworthy that although *rrf-1* mutants are defective in the RNAi amplification step, they do retain the ability to produce some small interfering RNA (siRNA) molecules corresponding to the initial RNAi-activating dsRNA (Sijen *et al.* 2001), and this could account for the small number of supernumerary proximal somatic gonadal cells in *rrf-1* dauers following *din-1S* RNAi in the germ line.

Consistent with these data, using complex extrachromosomal array-based transgenes we found that *din-1S* affected somatic gonadal cell numbers within the cluster and in the germ line in a cell-autonomous manner when expressed under the control of a somatic- *(sur-5)* or germline-specific *(pgl-1)* promoter, respectively (Figure S5). From these observations we conclude that *din-1S* acts cell autonomously and is required both in the germ line and in the soma to restrict cell division within the dauer gonad.

din-1S cooperates with daf-12 to restrict germ cell proliferation in the dauer larva

DIN-1S forms a dauer-specific complex with the nuclear hormone receptor DAF-12 that represses the transcription of genes required for reproductive development, to favor dauer formation (Ludewig *et al.* 2004; Motola *et al.* 2006). In addition, DIN-1S has been shown to interact with the ligand-binding domain (LBD) of DAF-12 similar to coregulators for other NHRs (Horlein *et al.* 1995; Hu and Lazar 1999; Oswald *et al.* 2002; Ludewig *et al.* 2004). It is therefore likely that in

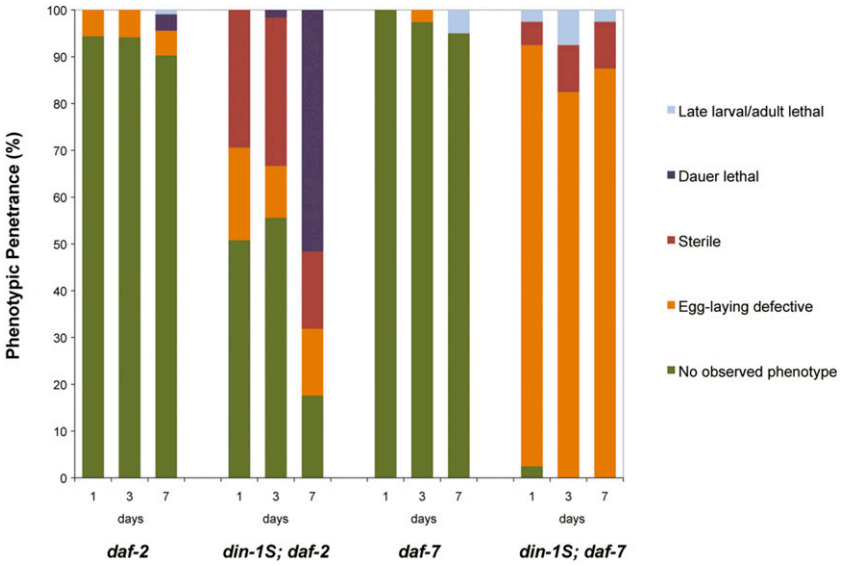

Figure 4 Postdauer-recovered *din-1S* mutants exhibit severe reproductive defects when ILS is compromised. One-, 3-, and 7-day-old control and *din-1S(rr94)* dauer larvae were placed at 15° to allow them to recover. The frequency of reproductive defects in the adults, or death either before or after recovery, was then quantified as a percentage of the total number of animals originally switched to the permissive temperature. Only very severe egg laying defects that resulted in the formation of Bags of worms were quantified. In order to ensure that the dauer larvae selected for the experiments contained two properly localized distal tip cells, and thus two intact gonad arms, all genotypes included *qIs56*. n = 91 for experiments with *daf-2*-containing strains and n = 39 for experiments with *daf-7*-containing strains.

the absence of hormone binding, the DAF-12 LBD is free to interact with DIN-1S, and the DAF-12/DIN-1S complex regulates the transcription of genes associated with dauer formation, including those required to restrict the proliferation of both the germ cells and the somatic gonadal precursors.

To determine whether this is indeed the case, we constructed *daf-2*; *daf-12* animals using a *daf-12* allele that encodes a protein that is compromised for its ability to bind DIN-1S (Ludewig *et al.* 2004). This is not a classic loss of function mutation, but rather a special allele of *daf-12* that acts as an unregulated variant. While *daf-2*; *daf-12(rh61)* animals do not form morphologically typical dauers when upshifted to the restrictive temperature, they do arrest their development and appropriately express the LAG-2::GFP marker in the IL2 neurons, indicating that the decision to form dauer has indeed been made. By examining animals after 48 hr post-temperature shift, we were able to compare germ cell numbers between the three genotypes without the confounding problem associated with the execution of the dauer stage, as *daf-12* larvae can only enter dauer transiently if at all. We found that the germ cell counts of *daf-2*; *daf-12* animals are significantly greater than those in *daf-2* controls, while we obtained comparable results when *rrf-1*; *daf-7* or *rrf-1*; *daf-2* animals were maintained on bacteria expressing *daf-12* dsRNA (Table 3). These findings are consistent with DIN-1S working in a complex with DAF-12 to restrict germline proliferation downstream of the decision to form a dauer larva.

Despite their dauer-like appearance, *din-1S*; *daf-2* and *daf-2*; *daf-12* dauer larvae are not fully developmentally quiescent. Unlike *daf-2* dauer larvae, the vulval precursor cells (VPC) of *din-1S*; *daf-2* and *daf-2*; *daf-12* dauer larvae execute multiple divisions and eventually undergo morphogenesis to generate an invaginated L4-like vulva during the dauer stage in both genetic backgrounds (Figure 3, B and C). This morphogenetic process requires exquisite coordination of cell division and cell fate specification. Therefore the invaginations that do form in these dauer larvae may or may not produce functional vulvae.

These observations taken together with the enhanced frequency of supernumerary divisions that occur in the germline and the somatic gonadal cells, and the premature fusion of the seam cells during the dauer stage, suggest that the loss of *din-1S/daf-12* allows certain cell types to remain competent to developmental signals, potentially due to their compromised ability to establish a state of quiescence. This is however not a global effect, as other cell types that would normally divide postembryonically do not undergo supernumerary cell divisions (*i.e.*,the seam cells) during the dauer stage in *din-1S* mutants (Figure 3 and Figure S3). Therefore the reduction of *din-1S* function allows some sensitive cells to continue to respond to instructive cues that would otherwise specify later larval stage events, giving rise to continued cell divisions in the gonad and untimely vulval morphogenesis.

din-1S exhibits dauer-dependent reproductive defects

C. elegans is capable of spending an extensive period in the dauer stage without adverse effects on its postrecovery reproductive fitness. In fact, studies have demonstrated that lifespan and brood size are both marginally, yet significantly, enhanced following passage through dauer (Hall *et al.* 2010, 2013).

Over 90% of *daf-2* control animals recover from the dauer stage to become healthy, egg-laying adults regardless of whether they have previously spent 1, 3, or 7 days as dauer larvae (Figure 4). Although *din-1S*; *daf-2* animals typically grow to become healthy adults, they display severe reproductive defects during the adult stage if they transit through dauer. For example, ~30% of *din-1S*; *daf-2* animals become sterile postrecovery after a 1-day or 3-day duration in dauer. In addition, 10% of the adults that do produce progeny have severe egg laying defects (Egl) and form Bags of worms (Egl/Bag), reducing their brood size significantly (Figure 4). Interestingly, over half (51.6%) of the 7-day-old *din-1S*; *daf-2*

Table 4 The reproductive defects of *din-1S* mutants correlate with the frequency of hyperplasia in the somatic gonad and postdauer germline cell division abormalities

Genotype[a]	No. of proximal somatic gonadal cells in dauer[b]	Dauer-dependent reproductive defects[c] (%)		
		Bag of worms (*n*)	Sterility (*n*)	Gonad arms containing polyploid germ cell nuclei[d] (*n*)
daf-2(e1370)	6 ± 0	4.2 (165)	0 (165)	0 (100)
din-1S(rr94); daf-2(e1370)	25.4 ± 19.2	18.2 (165)	33.9 (165)	23.9 (92)
daf-7(e1372)	6 ± 0	0 (86)	0 (86)	0 (56)
din-1S(rr94); daf-7(e1372)	87.6 ± 10.1	90 (80)	7.5 (80)	5.9 (102)

n, sample size.
[a] Full genotype includes *qIs56*.
[b] Mean ± SD; $n \geq 20$.
[c] Results for animals that spent 24 hr in dauer at 25°.
[d] Gonad arms containing polyploid germ cell nuclei were scored in animals ~24 hr after the L4/adult molt.

dauer larvae die during this stage without ever recovering (Figure 4). It was not immediately apparent why the dauer larvae expire prematurely, but they are highly vacuolated, clear, and sickly compared to control *daf-2* dauer larvae. Moreover, 34% of the 7-day-old dauer larvae that did recover became sterile adults (15 of 44 animals). Temperature had no direct effect *per se* as *din-1S*; *daf-2* dauers induced by starvation at 15° also displayed reproductive defects with comparable phenotypic penetrance following dauer recovery. Thus, *din-1S(rr94)* causes reproductive defects that are conditional upon the execution of dauer, the severity of which correlates with the duration spent in the diapause.

To determine whether the dauer-dependent reproductive defects in *din-1S*; *daf-2* animals were common to dauer larvae induced by all pathways, as is the case for the germline hyperplasia, or whether they were specific to the ILS pathway, we repeated our analyses in a *daf-7*/TGFβ background. Surprisingly we found that while at least 95% of *daf-7* control animals show no phenotype after dauer recovery, the majority (82.5% or greater) of *din-1S*; *daf-7* animals are severely Egl/Bag after recovery, regardless of the duration in dauer (see Figure 4). Animals that did not form Egl/Bags were either sterile or died as L4 larvae or young adults before reproducing.

Contrary to *din-1S*; *daf-2*, we did not observe lethality in *din-1S*; *daf-7* dauer larvae, even after 7 days, the duration associated with ~50% survival in the *din-1S*; *daf-2* animals. The penetrance of the Egl/Bag phenotype in animals that recovered from dauer correlated with the number of supernumerary proximal somatic gonadal cells present within the cluster in dauer gonads. *din-1S*; *daf-7* dauer animals consistently display at least 10 times the wild-type number of proximal somatic gonadal cells in the cluster, with an average of 87.6 cells, and accordingly, 90% of 1-day-old *din-1S*; *daf-7* dauer larvae recover to finally become Egl/Bag as adults (Table 4).

Curiously, the *din-1S*; *daf-2* dauer larvae do not always possess supernumerary somatic gonadal cells, and when they do, their numbers are limited, with an average of ~25 cells. Moreover, <19% of *din-1S*; *daf-2* dauer larvae recover to become Egl/Bag as adults.

The Egl/Bag phenotype can occur for numerous reasons, including abnormal vulval development, or from a failure to lay eggs due to defective neuromuscular function (MacLeod *et al.* 1977; Ferguson and Horvitz 1985). *din-1S* postdauer adults do not show any obvious morphological abnormalities of the vulva, nor do they exhibit any discernible motor defects. However, vulval development does occur inappropriately early during the dauer stage in both *din-1S*; *daf-2* and *daf-2*; *daf-12* dauer larvae, which may contribute to the observed egg laying defect (Figure 3). Alternatively, the extra proximal somatic gonadal cells present within the cluster in *din-1S* dauer larvae may physically interfere with the later development of the somatic gonad and/or the appropriate cell–cell interactions that are required for correct uterine–vulval communication following dauer recovery. Since we never observe abnormal vulval development in the *din-1S*; *daf-7* background, the Egl/Bag phenotype, which is most penetrant in the *din-1S*; *daf-7* dauer larvae with supernumerary somatic gonadal cells, is probably due to the latter.

Cell cycle quiescence is compromised in the proximal germline in din-1S; daf-2 mutants

While almost 20% of *din-1S*; *daf-2* animals recover from dauer to eventually form Egl/Bag adults, we were curious as to why *din-1S*; *daf-2* animals display almost 34% dauer-dependent sterility, while *din-1S*; *daf-7* animals recover from dauer to become sterile adults at a comparatively lower frequency (Table 4). To better delineate the basis of this discrepancy, we DAPI-stained dissected postdauer adult gonads to find that almost 24% of *din-1S*; *daf-2* postdauer gonads contained large masses of germ cells, while these tumor-like growths were observed in only 6% of *din-1S*; *daf-7* animals (Figure 5 and Table 4). The frequency of this phenotype correlates well with the observed sterility in the two mutant genotypes.

When we observed *din-1S*; *daf-2* animals in the earlier L4 stage, small anomalous cell populations were found proximal to pachytene nuclei in 20% of the gonads examined. The loss of ILS in *din-1S* mutants therefore causes some germ cells to undergo abnormal cell divisions and/or endomitotic cycles instead of progressing through meiotic prophase (Figure 5).

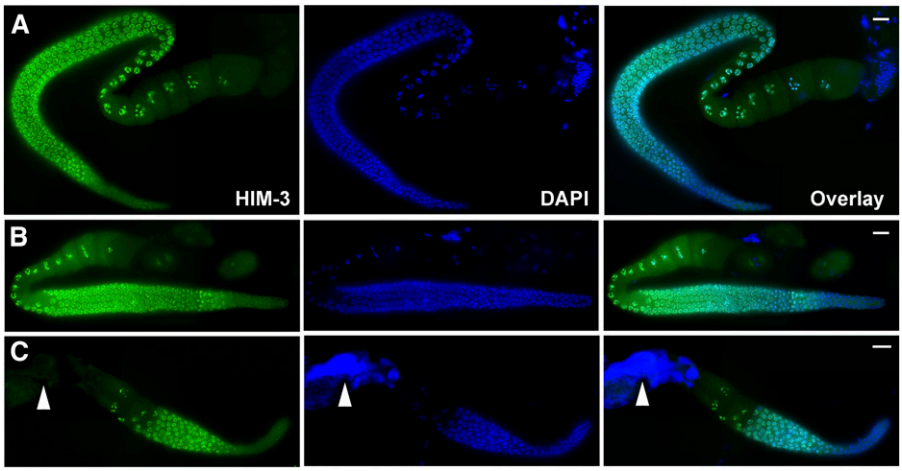

Figure 5 Impaired ILS causes cell cycle abnormalities in the germ cells of postdauer *din-1S* adults. Representative gonads extruded from postdauer-recovered adult hermaphrodites were counterstained with DAPI (blue) and stained with anti-HIM-3 (green) to highlight cells that have entered meiotic prophase. Overlay depicts the combined signals obtained from the two channels for each genotype/condition. (A) *daf-2(e1370)* adults maintained at 15°. (B) *din-1S*; *daf-2(e1370)* adult animals that were maintained at 15° that did not transit the dauer stage. (C) *din-1S*; *daf-2(e1370)* that transitted through the dauer stage and were downshifted and maintained at 15° for 48 hr prior to analysis. Morphological and cytological abnormalities are apparent in the postdauer *din-1S*; *daf-2(e1370)* gonad that contain endomitotic cells that contain excess DNA (arrowhead) and germ cells that have undergone aberrant proliferation causing anomalous tumor-like growths. Bars, 10 μm.

din-1S is required for long-term dauer survival in response to reduced insulin signaling

Examination of reproductive fitness of the *din-1S* mutants showed that about half of the 7-day-old *din-1S*; *daf-2* dauer larvae failed to recover when transferred to 15° (Figure 4). Although these dauers lived for several days at 15°, they did not survive for the several weeks or months typical of wild-type dauer larvae. Other mutations that affect germline stem cell quiescence also affect dauer survival through effects on energy resource utilization (Narbonne and Roy 2006, 2009). We found that *din-1S*; *daf-2* dauer larvae do not survive for long durations, with an average of <15–16 days (Figure 6 and Table 2). This is significantly different from the *daf-2* mean dauer survival of 33.5 days using our assay method. In comparison, the mean dauer survival of *daf-2*; *aak-2* dauers is <11 days. Therefore, similar to *aak-2*, *din-1S* is required for the long-term survival of the dauer larvae when ILS is compromised.

As described earlier, unlike *din-1S*; *daf-2* animals, *din-1S*; *daf-7* animals recovered from the dauer stage regardless of the duration, and dauer lethality was never observed in *din-1S*; *daf-7* animals. Consistent with the effects of *din-1S* on reproductive fitness in *daf-7* dauers, the survival of *din-1S*; *daf-7* animals is not reduced compared to *daf-7* control animals (Table 2). *din-1S* is therefore not required for the long-term survival of dauers induced by reduction of TGFβ signaling.

din-1S acts in parallel with the LKB1/AMPK signaling cascade to regulate germline stem cell quiescence and long-term survival in the dauer larva

Genetic evidence suggests that *aak-1* and *aak-2* are not the sole downstream effectors of *par-4*/LKB1 signaling and/or that other AMPK activators may also function to control germline quiescence (Kahn *et al.* 2005; Narbonne and Roy 2006). To determine whether *din-1S* represents one of these unknown players, we tested whether it functions together with *aak-2*/AMPK to regulate germline quiescence. The degree of hyperplasia in the germline of the *din-1S*; *daf-2*; *aak-2* mutant dauer larvae (an average of 152.6) is nearly equal to the sum of the average number of germ cells observed in the *din-1S*; *daf-2* and the *daf-2*; *aak-2* mutant dauer larvae (Table 5, rows B and E). This additive role of each of these genetic pathways was further demonstrated using animals that are completely null for AMPK signaling (Table 5, rows K and L). Furthermore, we found that the 5.8-day dauer survival of the *din-1S*; *daf-2*; *aak-2* dauer larvae was significantly shorter than that of either *din-1S*; *daf-2* or *daf-2*; *aak-2* double mutants (Figure 6). These results suggest that *din-1S* and *aak-2* function in parallel to independently regulate germline quiescence and long-term survival during the dauer stage.

Interestingly, when *din-1S*; *daf-2*; *aak-2* animals were placed at 25° in order to induce dauer formation, we observed that a proportion of the triple mutant dauer larvae recovered from the diapause. Although the premature dauer recovery of *aak-2* mutants has been previously described (Apfeld *et al.* 2004; Cunningham *et al.* 2014), in our hands only very few if any of the *daf-2*; *aak-2* or *daf-2* control dauer larvae recover after three days, while over 14% of *din-1S*; *daf-2*; *aak-2* dauer larvae had recovered (Table 5). The observation that *din-1S* and *aak-2* undergo premature dauer recovery is suggestive of their activity being required downstream of, or in parallel to, ILS to maintain the dauer state, much like that observed in mutants that lack serotonin signaling (Cunningham *et al.* 2014).

In addition, although *aak-2* has no effect alone on somatic gonadal cell quiescence during the dauer stage, it enhanced the *din-1S* supernumerary proximal somatic gonadal cell defect almost twofold (Table 5, rows E and G, and F and H). Therefore, the loss of *din-1S* sensitizes the somatic gonadal cells to a reduction in AMPK signaling, despite the fact that loss of AMPK alone has no effect on the somatic gonadal cell numbers.

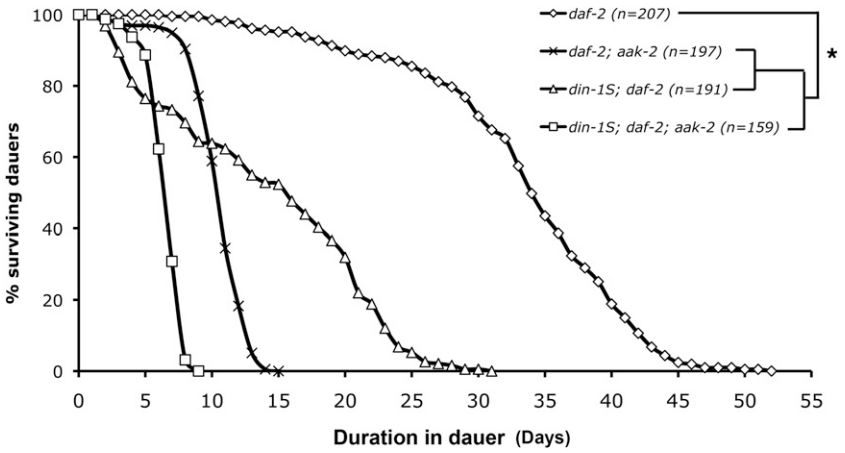

Figure 6 *din-1S* is required for typical dauer survival in ILS mutants. Animals were induced to form dauer by upshift to 25°, and after 24 hr they were subsequently singled into drops of buffer and monitored for recovery and survival every 24 hr thereafter. * $P = 0.0001$ between all four strains using a log rank test. All strains carry the *qIs56* transgene initially used for DTC visualization. The experiment was repeated at least three times.

Our observations with *din-1S*; *aak-2* double mutants motivated us to examine whether similar relationships exist between *din-1S* and other members of the *par-4*/LKB1 signaling pathway including another AMPK catalytic subunit ortholog, *aak-1*. We found that *din-1S*; *aak-1* double mutant dauer larvae contain the same number of germ cells (mean of 126.9) as *din-1S*; *aak-2* mutants (Table 5, rows D and H) while the population of proximal somatic gonadal cells in *din-1S*; *daf-2*; *aak-1* mutant dauer larvae is also significantly higher than that observed in *daf-2*; *aak-1* animals. *din-1S*; *par-4* and *din-1S*; *aak-1*; *aak-2* mutants display similar relationships in both cell types (Table 5, rows J and L). These data suggest that *din-1S* acts additively with the LKB1/AMPK signaling pathway to regulate the appropriate onset of cell cycle quiescence in both the germline and the somatic gonad in the dauer larva.

Discussion

Complex organisms have evolved diverse mechanisms for responding to environmental stresses that can influence developmental outcomes and/or fitness. In *C. elegans*, unfavorable growth conditions promote entry into the developmentally arrested dauer stage. However, while the signaling pathways that affect the dauer decision have been dissected, the downstream factors that execute these changes still remain largely uncharacterized. Our laboratory has previously reported the identification of some of the key factors responsible for establishing the timely onset of quiescence of the germline stem cells in the dauer larva (Narbonne and Roy 2006, 2009). We report here that the NHR coregulator DIN-1S is required for long-term dauer survival in ILS mutants as well as postdauer reproductive fitness when either ILS or TGFβ signaling is impaired. Furthermore, our genetic analyses suggest that *din-1S* is required cell autonomously to regulate the timely onset of quiescence in the germline stem cell population and the somatic cells that contribute to the adult gonad.

DIN-1S forms a dauer-specific complex with DAF-12 that represses the transcription of genes required for reproductive development, while favoring that of a dauer-specific gene expression repertoire (Ludewig *et al.* 2004; Motola *et al.* 2006). We have shown that *daf-2*; *daf-12* animals also arrest their development and exhibit hyperplasia within the germline similar to *din-1S* mutants, consistent with DIN-1S working with DAF-12 to restrict germline proliferation as the larva prepares to execute the dauer stage.

The *daf-2*; *din-1S* animals resemble and behave like dauer larvae by most criteria. However, *din-1S* mutants enter the dauer stage at the global, organismal level, while at the individual cellular level the commitment is both incomplete and cell type/tissue specific, not unlike certain *daf-12* mutants (Antebi *et al.* 1998).

Our data suggest that *din-1S* is required to regulate both the duration and the rate of the somatic and germ cell divisions during the L2d period that precedes dauer quiescence both in animals with compromised ILS or TGFβ signaling. These cell divisions usually begin to slow at the end of L2d, but in *rr94* mutants, the same decrease in division rate occurs, but only after a prolonged period of proliferation that continues throughout an extended L2d period. Curiously, none of the mutants we have identified to date undergo continuous cell divisions during the dauer stage, suggesting that a novel class of mutants has yet to be isolated and characterized.

It is interesting that the *din-1S(rr94)* has distinct effects in two different dauer-constitutive backgrounds. The number of somatic gonadal cells is more than three times greater in a *daf-7* background than in a *daf-2* background, suggesting that an intact ILS pathway enhances the growth and/or proliferation of these cells when *din-1S* is disrupted. Consistent with this observation, *daf-2* mutations have been shown to decrease the volume of mitotic germline tumors (Pinkston *et al.* 2006), while a role for ILS in ensuring proper expansion of the germline and the somatic gonad that occurs during the L3 stage has been proposed (Michaelson *et al.* 2010). Alternatively, TGFβ could play a critical role in establishing cell cycle quiescence in an ILS-independent manner during this stage. Based on our current data we cannot distinguish which of these two scenarios might account for the differences in germ cell numbers in each of these Daf backgrounds.

Table 5 *din-1S* cooperates with *ILS* and the LKB1/AMPK signaling pathway to regulate quiescence in the germline and in the somatic gonad of the dauer larva

Genotype	No. of germ cells in dauer[a]	No. of proximal somatic gonadal cells in dauer[a]	Dauer survival (in days)[a]
(A) *daf-2(e1370)*[b]	33.6 ± 3.9	6 ± 0	33.5 ± 8.3 (207)
(B) *din-1S(rr94)*; *daf-2(e1370)*[b]	82.0 ± 16.3	25.4 ± 19.2	14.4 ± 8.0 (191)
(C) *daf-2(e1370) aak-1(tm1944)*	50.3 ± 7.8	6 ± 0	ND
(D) *din-1S(rr94)*; *daf-2(e1370) aak-1(tm1944)*[b]	126.9 ± 20.4	60.3 ± 14.3	ND
(E) *daf-2(e1370)*; *aak-2(rr48)*[b]	76.1 ± 15.2	6 ± 0	10.7 ± 2.2 (197)
(F) *daf-2(e1370)*; *aak-2(ok524)*	67.5 ± 9.7	6 ± 0	ND
(G) *din-1S(rr94)*; *daf-2(e1370)*; *aak-2(rr48)*[b,c]	152.6 ± 28.7	52.5 ± 25.5	5.8 ± 1.3 (159)
(H) *din-1S(rr94)*; *daf-2(e1370)*; *aak-2(ok524)*	126.0 ± 24.8	58.9 ± 23.2	ND
(I) *daf-2(e1370)*; *par-4(it57)*	129.8 ± 19.9	6 ± 0	ND
(J) *din-1S(rr94)*; *daf-2(e1370)*; *par-4(it57)*[d]	211.8 ± 25.9	75.5 ± 13.2	ND
(K) *daf-2(e1370) aak-1(tm1944)*; *aak-2(ok524)*	168.6 ± 39.3	6 ± 0	ND
(L) *din-1S(rr94)*; *daf-2(e1370) aak-1(tm1944)*; *aak-2(ok524)*[d]	211.5 ± 39.3	71.9 ± 12.2	ND

n, sample size. ND, not determined.
[a] Mean ± SD; $n \geq 20$ unless otherwise stated.
[b] Full genotype includes *qIs56*.
[c] *din-1S*; *daf-2*; *aak-2* mutant animals showed a synthetic dauer recovery phenotype where 14.6% of animals had recovered inappropriately after 72 hr in dauer at 25°. None of the *daf-2(e1370)*, *din-1S(rr94)*; *daf-2(e1370)*, or *daf-2(e1370)*; *aak-2(rr48)* animals recover prematurely under our culture conditions. $n = 300$.
[d] $n = 15$.

During conditions of impaired ILS, *din-1S* is required for normal reproductive fitness following exit from the dauer stage. While sterile *din-1S*; *daf-2* adults were observed about a third of the time among postdauer animals, they were rarely observed with the *din-1S*; *daf-7* genotype, or in animals that had never entered the dauer stage. We subsequently observed that the proportion of postdauer gonads containing large, tumor-like cell aggregates correlates well with the observed frequency and degree of sterility in both genotypes, suggesting that these cell aggregates either prevent normal reproductive activity in the gonad or are evidence of defective gametogenesis and/or gonadogenesis. When we observed younger postdauer gonads, we found that they too contained cell aggregates between the regions of the gonad where the primary spermatocytes reside and the region that contains differentiated sperm. It is unlikely that the sterility we observe in postdauer *din-1S* animals results exclusively from defects in the sperm since wild-type male sperm could not rescue the sterility of mated postdauer *din-1S* hermaphrodites (our unpublished results). At present our data cannot distinguish whether the germ cells that give rise to the tumor-like cell aggregates are delayed in their meiotic entry and eventually undergo endomitotic cycles, or whether they actually initiate meiosis and exit aberrantly (Subramaniam and Seydoux 2003).

These aggregates only occur in *din-1S* animals that have previously formed dauer and have impaired ILS. The compromise of *din-1S* may sensitize the germ cells to the wave of insulin signaling that occurs as animals are downshifted to permissive temperature to allow for dauer recovery. Moreover, proliferative cues may arise from displaced supernumerary somatic gonadal cells that would normally enhance proliferation in the mitotic regions of the gonad (Killian and Hubbard 2005). These foci could form a latent niche to promote division rather than the meiotic quiescence typical of the more proximal L3/L4 germ cells (McGovern *et al.* 2009).

The genetic interactions presented here are consistent with a model where at least two distinct signaling pathways act in parallel to establish and/or maintain germline quiescence in the dauer larva. These pathways function in the soma, namely in the vulva and the somatic gonad, and the germline downstream of dauer-inducing signals released in the soma from the ILS and TGFβ pathways. In addition to their effects on the germ line, the compromise of either *par-4*/LKB1 or *aak-2*/AMPK also resulted in increased supernumerary divisions in the somatic gonadal precursors, well beyond that normally observed in *din-1S* single mutants. Therefore, in addition to its regulatory effects in the dauer germline, the LKB1/AMPK signaling pathway also contributes to quiescence in the soma by preventing the untimely proliferation of the cells within the undeveloped gonad as the animal transits through L2d in preparation for the quiescent dauer stage. Perturbations in this pathway alone are not sufficient to trigger unscheduled divisions in the somatic gonad, and only when mutations arise in a second parallel pathway, like in animals that lack the *din-1S* coregulator, is this function revealed.

This is not unlike the situation in various tumor contexts where disruption of a given tumor suppressor can initiate tumorigenesis in more than one cell type or tissue depending on the functional state of specific combinatorial drivers in the affected cells (Davoli *et al.* 2013). In fact, in addition to the germline mutations of LKB1 that lead to the cancer-predisposing Peutz-Jeghers syndrome, spontaneous mutations in LKB1 have also been identified in lung, cervical, and pancreatic cancers, perhaps due to cooperation, or the lack thereof, with other cell-specific factors that are important

to control tissue homeostasis (Avizienyte *et al.* 1999; Carretero *et al.* 2004; Herrmann *et al.* 2011).

The role of the nuclear receptors in hormone-sensitive cancers has been well described (Heinlein and Chang 2004; Tokunaga *et al.* 2014). Our genetic approach to identify factors involved in germline quiescence has enabled us to identify and characterize how NHR signaling can cooperate with additional pathways to implement this cell cycle arrest such that when they are perturbed, unscheduled cell proliferation occurs in tissues that would otherwise remain quiescent. Not surprisingly, many of the genes we have uncovered through our analyses are themselves orthologs of tumor suppressors or gene products that have been implicated in various cancer-predisposing disorders in humans (Di Cristofano and Pandolfi 2000; Luo *et al.* 2005; Sanchez-Cespedes 2007). Future experiments designed to follow up on phenotypes observed in *din-1S* animals should allow the identification of additional previously uncharacterized targets downstream of insulin/IGF-like signaling, while the continued characterization of *din-1S* mutants will enhance our understanding of the coactivator- and corepressor-mediated mechanism of NHR function during diverse cellular and/or developmental contexts.

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GENETICS

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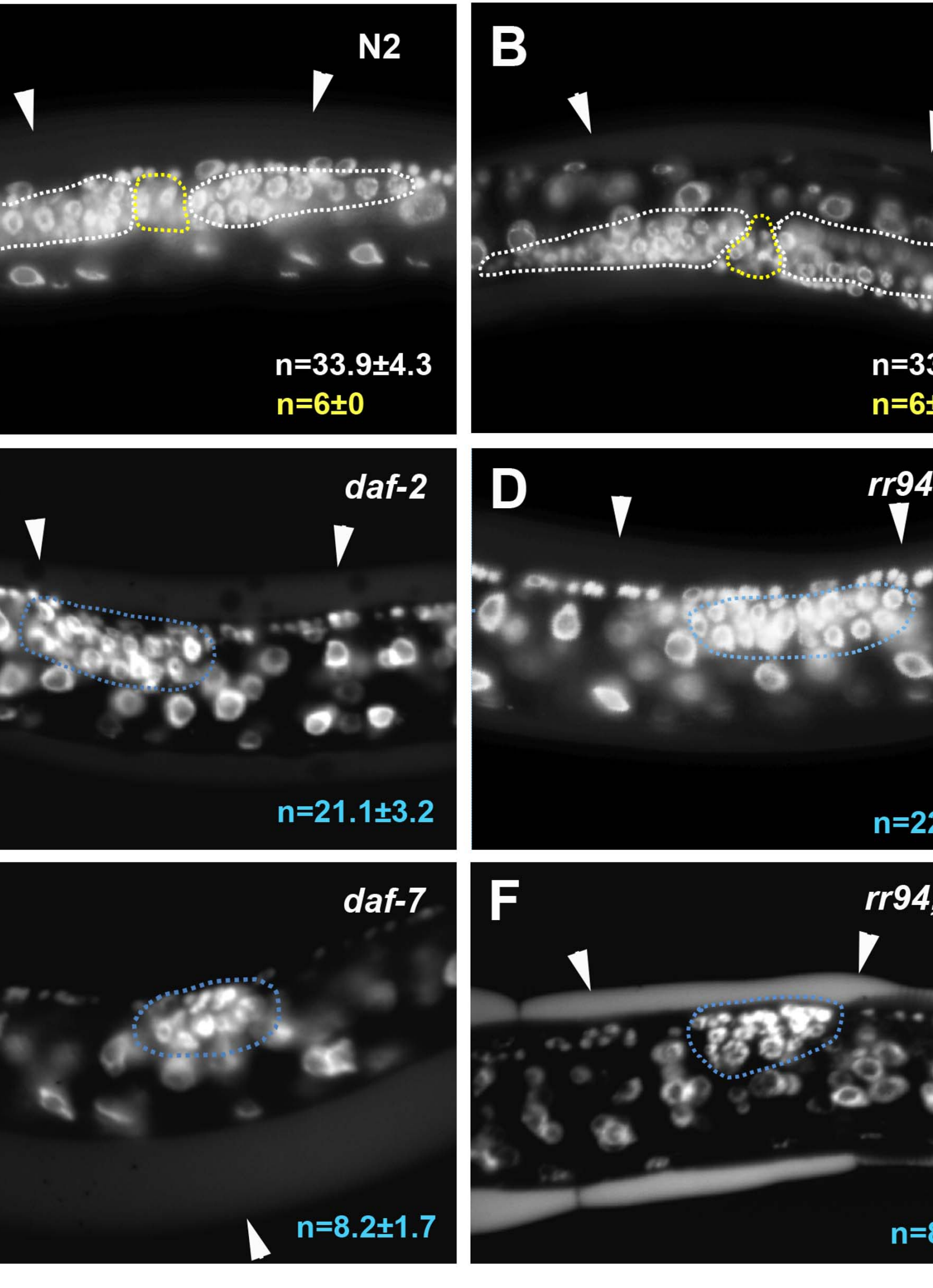
Developmental and Cell Cycle Quiescence Is Mediated by the Nuclear Hormone Receptor Coregulator DIN-1S in the *Caenorhabditis elegans* Dauer Larva

Eileen Colella, Shaolin Li, and Richard Roy

DOI: 10.1534/genetics.116.191858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n=6±0
B
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n=6
daf-2
n=21.1±3.2
D
rr94
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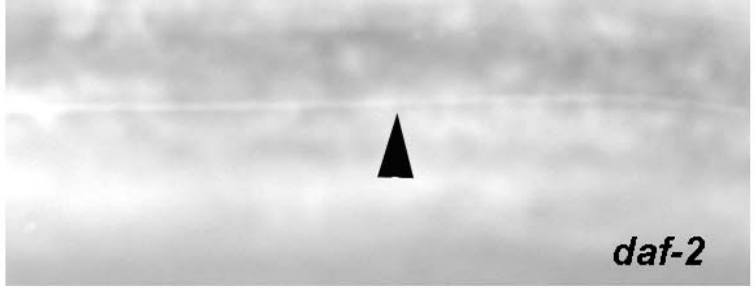
daf-7
n=8.2±1.7
F
rr94
n=8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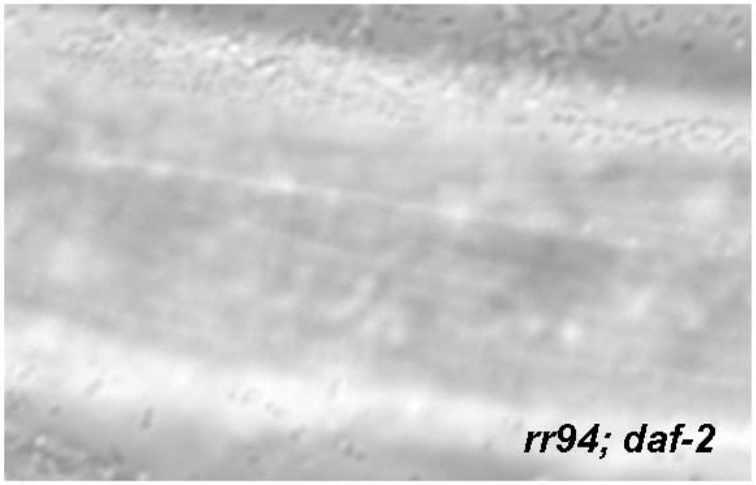
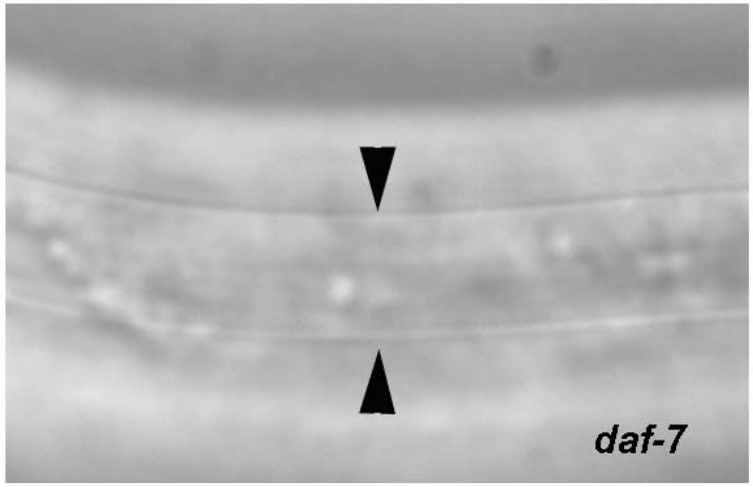

rr94; daf-2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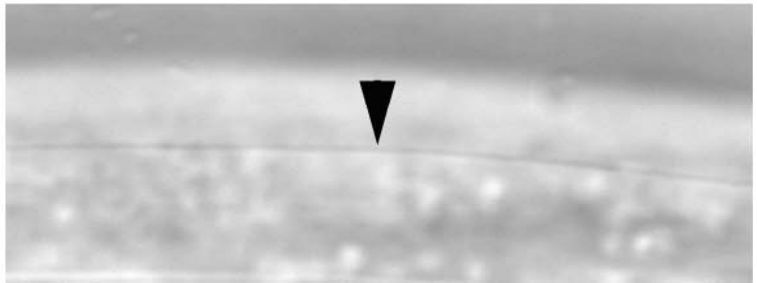

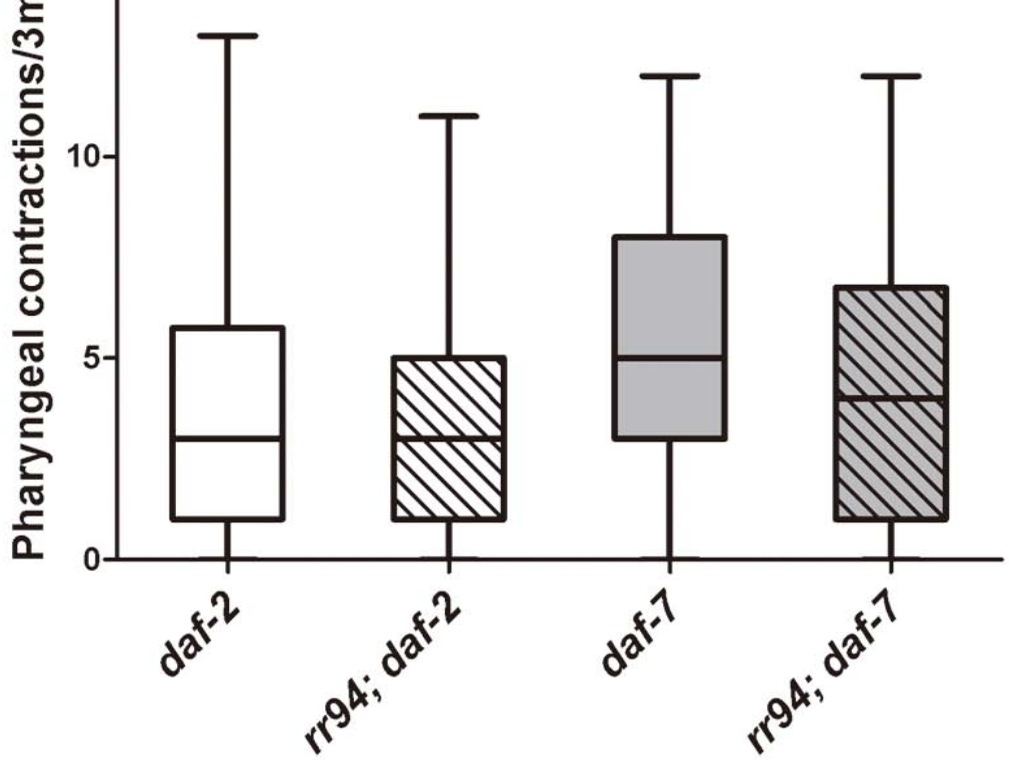
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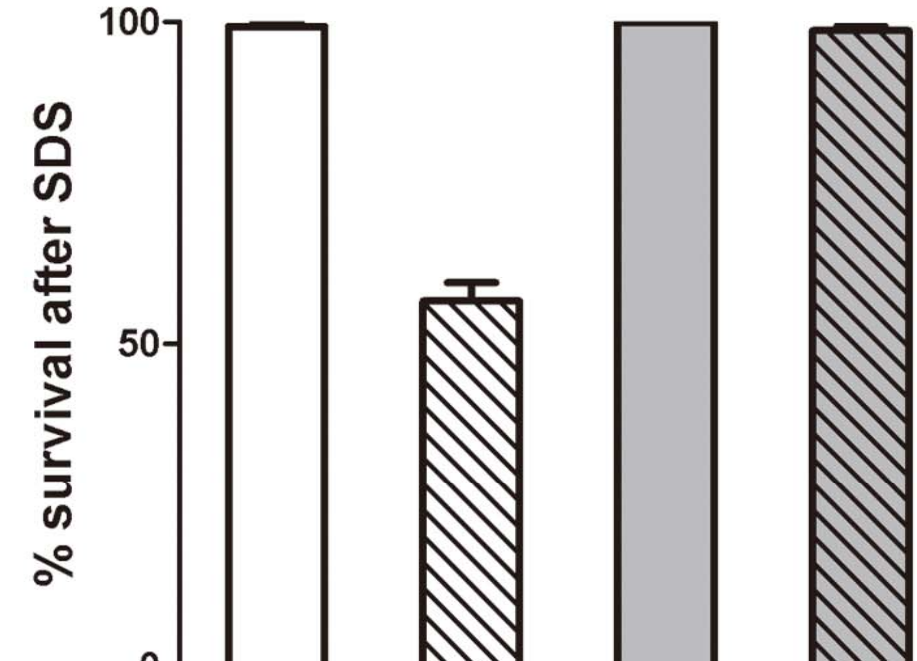
5
0
daf-2
rr94; daf-2
daf-7
rr94; daf-7

C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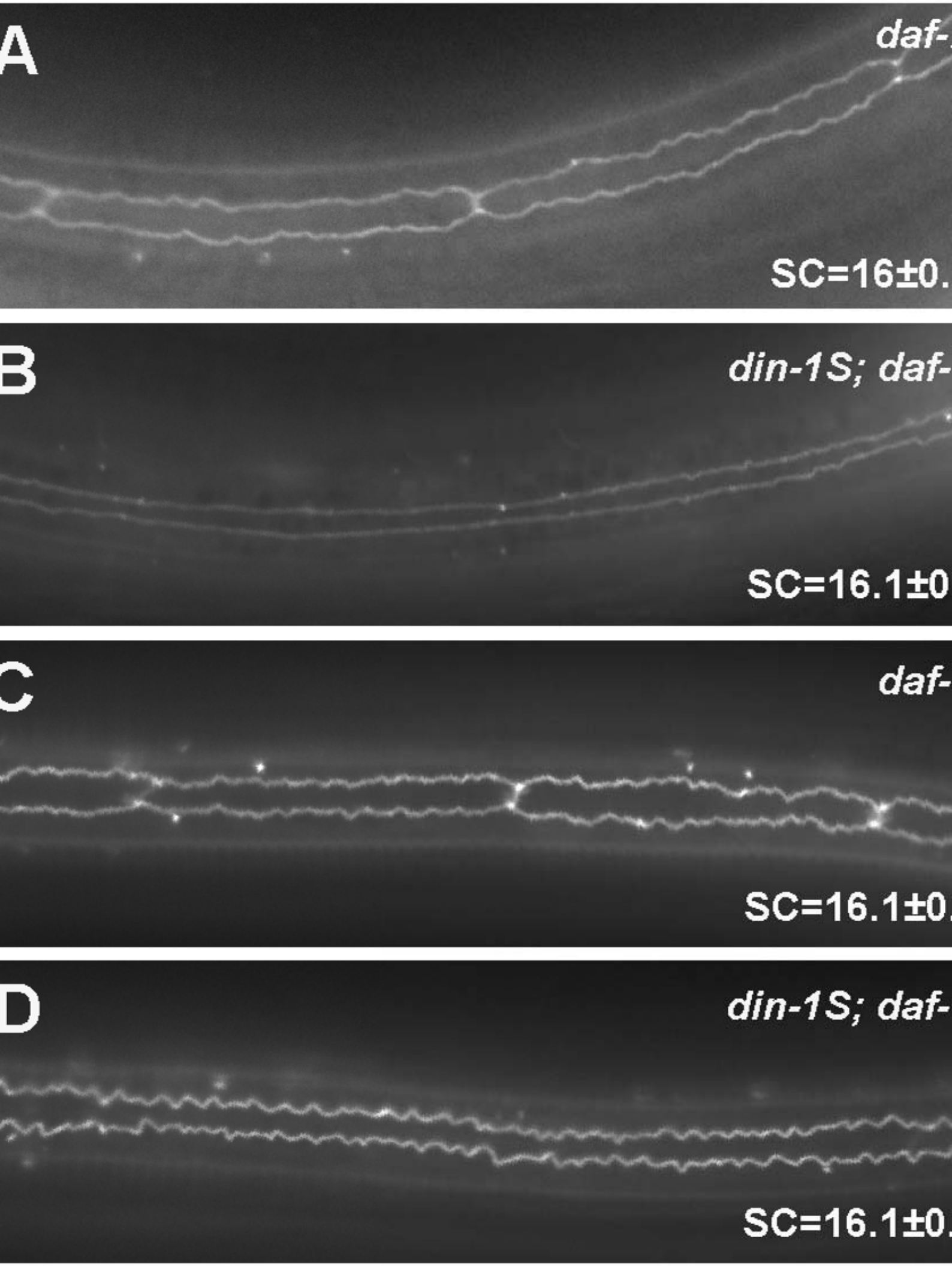
50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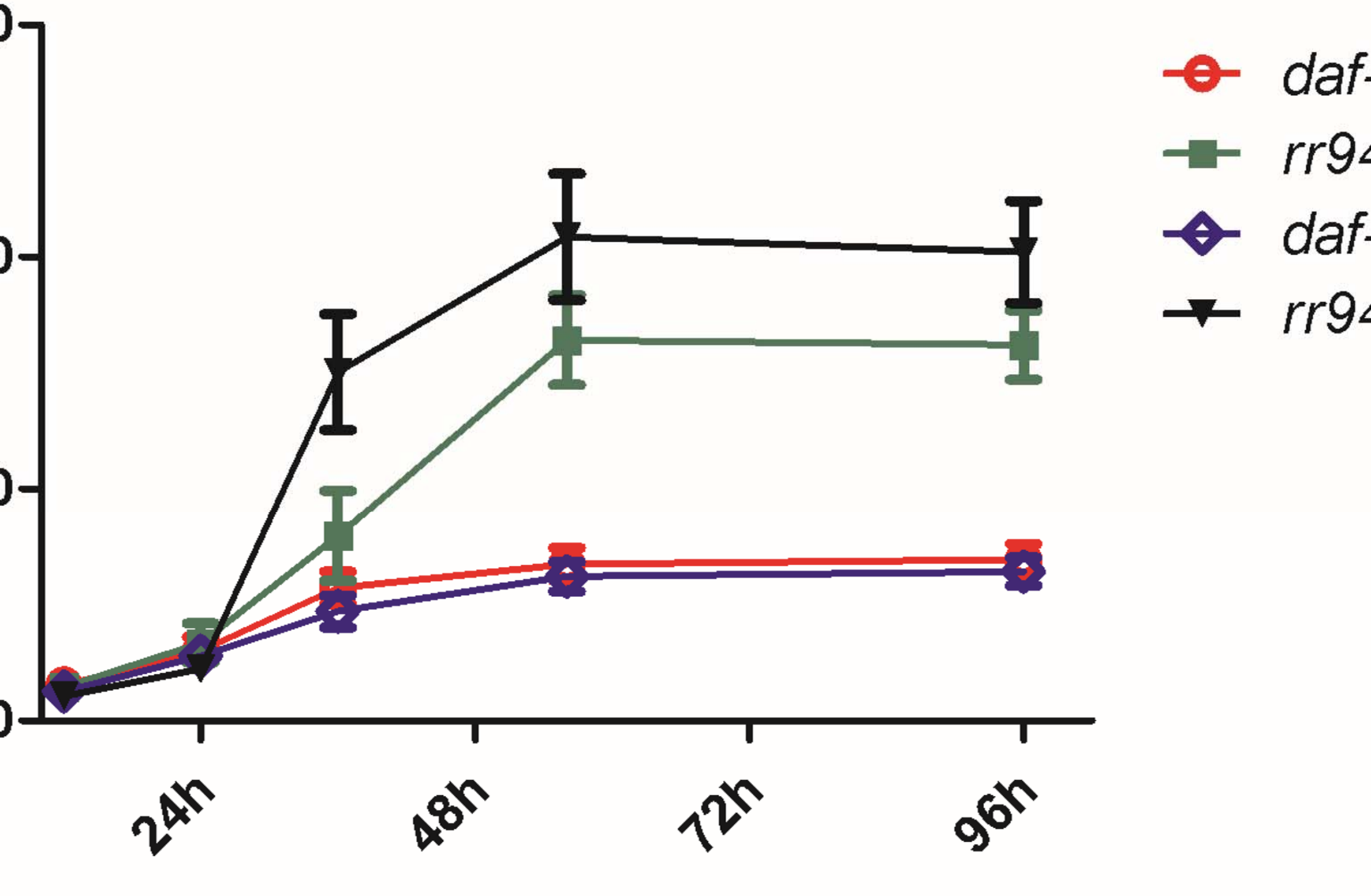
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rr94
daf-
rr94
24h
48h
72h
96h

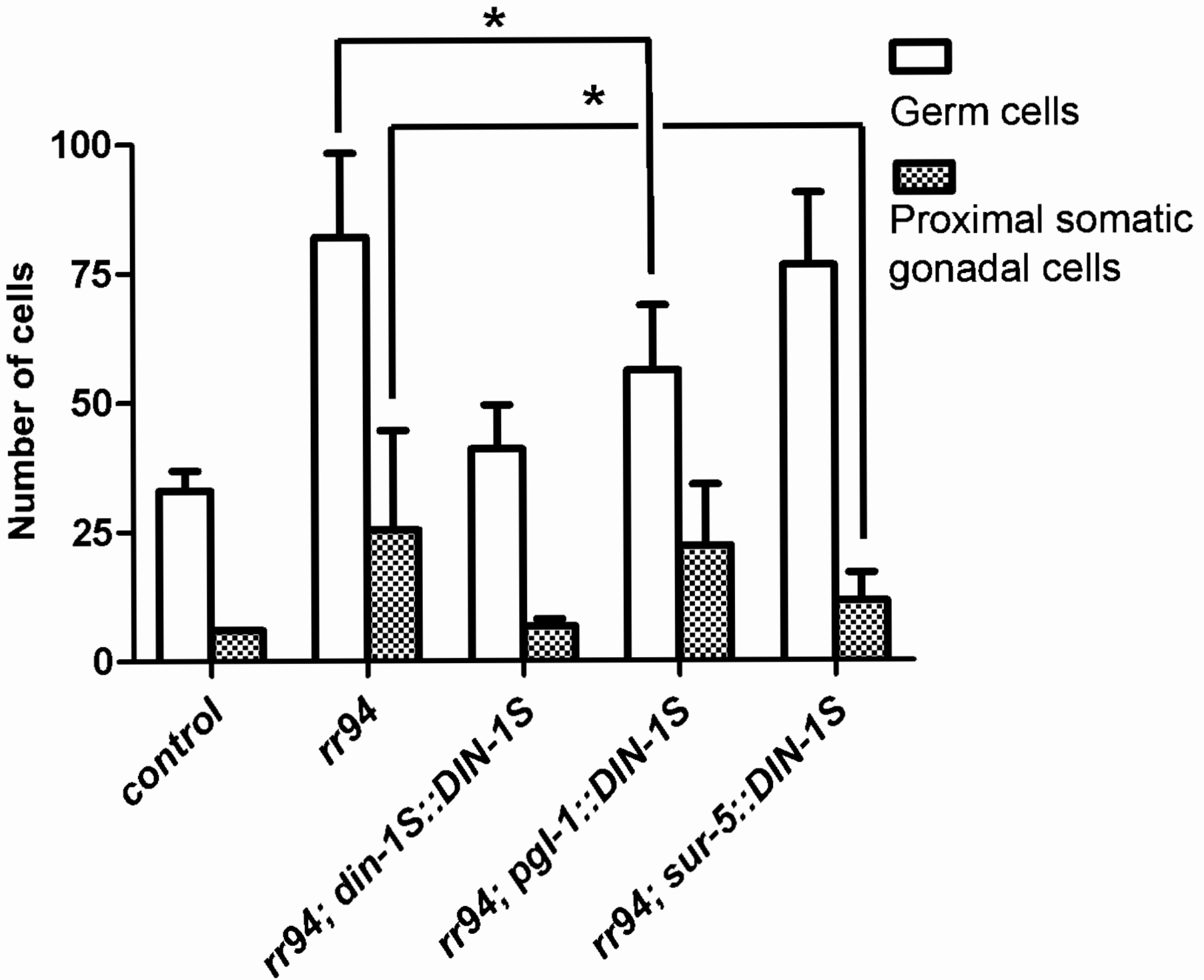
*
*
Germ cells
Proximal somatic gonadal cells
Number of cells
100
75
50
25
0
control
rr94
rr94; din-1S::DIN-1S
rr94; pgl-1::DIN-1S
rr94; sur-5::DIN-1S

Table S1. Strains used in this study

Strain name	Genotype
CB1370	*daf-2(e1370)*
MR452	*daf-2(e1370); qIs56; unc-1(e719)*
MR977	*din-1S(rr94); daf-2(e1370); qIs56*
MR1365	*din-1S(dh127); daf-2(e1370)*
MR1361	*din-1S(dh149); daf-2(e1370)*
MR1366	*daf-2(e1370); daf-12(rh61)*
MR1411	*din-1S(rr94); daf-2(e1370); daf-12(rh61)*
MR1030	*rrf-1(pk1417); daf-2(e1370); qIs56*
MR971	*daf-2(e1370) aak-1(tm1944)*
MR1356	*din-1S(rr94); daf-2(e1370) aak-1(tm1944); qIs56*
MR343	*daf-2(e1370); qIS56; aak-2(rr48)*
MR569	*daf-2(e1370); aak-2(ok524)*
MR1266	*din-1S(rr94); daf-2(e1370); qIs56; aak-2(rr48)*
MR1383	*din-1S(rr94); daf-2(e1370); aak-2(ok524)*
MR593	*daf-2(e1370); par-4(it57)*
MR1341	*din-1S(rr94); daf-2(e1370); par-4(it57)*
MR1000	*daf-2(e1370) aak-1(tm1944); aak-2(ok524)*
MR1375	*din-1S(rr94); daf-2(e1370) aak-1(tm1944); aak-2(ok524)*
MR1460	*din-1(rr94); daf-2(e1370); qIs56 ; qIs89*
MR1461	*daf-2(e1370); qIs56 ; qIs89*
CB1372	*daf-7(e1372)*
MR1201	*din-1S(rr94); daf-7(e1372); qIs56*
MR1322	*rrf-3(pk1426); daf-7(e1372)*
MR721	*rrf-1(pk1417); daf-7(e1372)*

MR170	*daf-7(e1372) glp-1(e2141)*
MR1288	*din-1(rr94); daf-7(e1372) glp-1(e2141)*
MR1973	*din-1S(rr94); daf-2(e1370); qIs56; rrEx1973[din-1S::DIN-1S; rol-6(D); myo-2::GFP]*
MR1974	*din-1S(rr94); daf-2(e1370); qIs56; rrEx1974[pgl-1::DIN-1S; rol-6(D); myo-2::GFP]*
MR1975	*din-1S(rr94); daf-2(e1370); qIs56; rrEx1975[sur-5::DIN-1S; rol-6(D); myo-2::GFP]*

LEGENDS TO SUPPLEMENTARY FIGURES

Fig. S1. *rr94* mutants display dauer-specific cell cycle defects in the germ line and the somatic gonad

Larvae maintained at 15˚C were collected at the L2 to L3 molt and stained with DAPI to label nuclei. (A,B) Germ cells (white dotted line) and somatic gonadal cells within the proximal cluster (yellow dotted line) are highlighted in the micrograph. Only larvae that were clearly in the molt (presence of the old cuticle indicated by the arrowhead) were counted. The white "n" refers to germ cell numbers, while yellow "n" refers to the somatic gonadal cells. N2 and *rr94* larvae were examined after 39h (A,B), while *daf-2*, *rr94; daf-2*, *daf-7*, and *rr94*; *daf-7* were delayed by 30min (C,D and E,F, respectively). Due to the difficulty in accurately distinguishing the somatic gonadal cell cluster from germ cells in this non-transgenic line and mostly due to the high degree of compaction among these cells, we counted the total number of cells within the gonad (Blue dotted line). The blue "n" refers to the total number of cells in the gonad (germ cells and proximal somatic gonadal cells combined) in *daf-2*, *daf-7* and double mutants with *rr94*.

Fig. S2. *rr94* dauer larvae appear morphologically normal although ILS-impaired *rr94* dauer larvae exhibit cuticular defects

(A) DIC images of dauer alae formed in *daf-2, rr94; daf-2 and daf-7* and *rr94; daf-7* dauer larvae showing the focal plane to visualize the typical alae present on the dauer larva cuticle (black arrowheads). *rr94; daf-2* do not make normal alae although *rr94; daf-7* animals form alae that appears morphologically normal. (B) *rr94* mutants do not show defects in their ability to block pharyngeal contractions

during the dauer stage. Pharyngeal contractions were quantified over a three-minute duration to compare frequencies. (C) Dauer larvae were subjected to SDS treatment and survival was quantified post-SDS soaking. *rr94* compromise does not affect SDS resistance in *daf-7* dauer larvae, but it did partially compromise post-SDS viability in *daf-2* dauer larvae.

Fig. S3. *rr94* mutants undergo premature seam cell fusion during the dauer stage.

(A-D) Seam cell fusion normally occurs at the L4-Adult molt, but in *rr94* dauer larvae this occurs prematurely during the dauer stage. In both *daf-2* and *daf-7* mutant dauer larvae the seam cell boundaries are visible using an *ajm-1*::GFP reporter for cell junctions. However, in *daf-2* or *daf-7* double mutants with *rr94,* the seams fuse prematurely during the dauer stage in both backgrounds. This premature differentiation of the seams may reflect the inabililty of certain cell types to execute dauer-specific programs, a heterochronic feature of *rr94* mutants, or both, which manifest during the dauer stage. The average numbers of the lateral cells included in the seam visualized by *ajm-1*::GFP is shown as SC and does not vary in a *rr94*-dependent manner. n≥20 animal

Fig. S4. Germ cell divisions continue in an extended L2d in *rr94* prior to establishment of quiescence during the dauer stage.

Following the switch to restrictive temperature both *daf-2* and *daf-7* animals will decrease their germ cell division rate during L2d in preparation for dauer entry. The germ cell divisions continue during an extended L2d period when *daf-2* and *daf-7* are combined with *rr94*. After this point the germ cell divisions arrest and the germ

line remains quiescent for the remainder of the dauer stage. Data represent the average germ cell numbers ±SD; n=20.

Fig. S5. *din-1S* acts autonomously to establish cell cycle quiescence in both the germ line and the somatic gonad.

din-1S was expressed by conventional transgenesis using complex arrays, either under the control of its own promoter (*din-1S*::DIN-1S); or specifically in the germ line using the *pgl-1* promoter (*pgl-1*::DIN-1S); or in the soma using the constitutively expressed somatic promoter *sur-5* (*sur-5*::DIN-1S). *din-1S*::DIN-1S can restore cell numbers to nearly wild type levels in the mutant *din-1S(rr94)* background. *pgl-1*::DIN-1S restores germ cell numbers with little effect on the SGPs, while *sur-5*::DIN-1S corrects somatic gonadal cell numbers in the proximal SGP cluster with little effect on the germ cell abundance. All strains carried *daf-2(1370)* including the dauer "Control", while the transgenic strains were *din-1S(rr94); daf-2(1370)* in addition to expressing the endogenously driven (*din-1S*::DIN-1S), the germline- (*pgl-1*::DIN-1S), or the soma-specific (*sur-5*::DIN-1S) transgenes.

* indicates significantly different cell counts ($P \leq 0.05$) between the strains compared indicated by the brackets using Student's T-test with unequal variance.