Function and tissue focus of *daf-18*/PTEN in maintaining blast cell multipotency and quiescence in *Caenorhabditis elegans* dauer larvae

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Abstract

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Cellular quiescence, a reversible state of cell-cycle exit, and developmental potential, the ability to differentiate into appropriate cell types, are properties essential for normal development and stem cell function (reviewed in (Cheung and Rando, 2013; Fiore et al., 2018; Mihaylova et al., 2014)). Understanding the mechanisms by which cells maintain quiescence has important implications for developmental biology, as this reversible state of cell-cycle exit is a key attribute of stem cells, as well as for cancer biology, as quiescence plays a key role in tumor dormancy and metastasis. Environmental conditions are key in regulating whether stem cells maintain quiescence or exit to resume divisions and developmentally progress. I aim to investigate how the properties of quiescence and developmental potential are retained over long periods of time and how they are appropriately regulated by external environmental inputs. The nematode Caenorhabditis elegans is an excellent model for investigating both of these questions because it is capable of entering and maintaining a developmentally arrested state for an unusually long time compared to the normal lifetime of the worm, and because the decision to enter this arrest is regulated entirely by external environmental inputs (Cassada and Russell, 1975).

Upon encountering conditions unfavorable for growth, C. elegans enters an alternative, developmentally arrested state called dauer diapause in which precursor cells remain quiescent for months – a period many times the lifespan of a worm grown under favorable conditions (Cassada and Russell, 1975). Maintaining precursors in this arrested state is important in order for the worms to develop normally once conditions improve and requires components of the
conserved Insulin/Insulin-like (IIS) signaling pathway (Karp and Greenwald 2013 and this work); of note, the IIS pathway also regulates mammalian quiescence (Eijkelenboom and Burgering, 2013). Canonical regulation of dauer diapause includes IIS, TGFβ, and dafachronic acid (DA)/nuclear hormone receptor (NHR) signaling (reviewed in (Murphy and Hu, 2013a)).

Here, I investigate how DAF-18, the sole *C. elegans* ortholog of the tumor suppressor PTEN (Phosphatase and tensin homolog) (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999), maintains quiescence in dauer through regulation of these conserved signaling pathways using the *C. elegans* gonad as a model. The gonad is composed of somatic cells and the germline. Both the somatic gonad and germline develop post-embryonically from precursor cells present when dauer arrest occurs, and these precursor cells remain quiescent for the duration of dauer diapause (Cassada and Russell, 1975; Hong et al., 1998; Narbonne and Roy, 2006). After exit from dauer, division and differentiation resume.

DAF-18/PTEN is required for germline quiescence during dauer diapause (Narbonne and Roy, 2006), and my results implicate DAF-18/PTEN in the control of quiescence of the somatic tissues as well, including the somatic gonad. In this role, DAF-18/PTEN activity in the somatic gonad non-autonomously coordinates both germline stem cell (GSC) and somatic gonad blast (SGB) quiescence. I have demonstrated this somatic gonad focus through mosaic analysis, tissue-specific rescue, and tissue-specific excision mosaics. We propose that DAF-18/PTEN mediates production of a signal promoting quiescence from the somatic gonad to the SGBs and GSCs and that this signal does not absolutely require or solely target the IIS, TGFβ, or DA/NHR signaling pathways normally implicated in regulation of dauer diapause.
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Chapter 1. Introduction

1. Introduction to Caenorhabditis elegans dauer larvae:

A fundamental question in developmental biology with strong implications for human health and disease is how cells remain quiescent and multipotent for extended lengths of time. The *C. elegans* dauer larva, described further below, is an outstanding model for studying conserved mechanisms of cellular quiescence and the maintenance of multipotency: in both dauer larvae and mammals, the conserved IIS pathway components DAF-18/PTEN and DAF-16/FoxO regulate these properties and directly regulate genes that control cell cycle progression (Baugh and Sternberg, 2006; Dijkers et al., 2000; Escoté and Fajas, 2015; Mellough et al., 2015; Rafalski and Brunet, 2011). Genetic studies of dauer larvae have also contributed fundamentally to our understanding of the insulin signaling pathway. Of particular relevance to this thesis, studies in *C. elegans* clarified the role of the protein and lipid phosphatase DAF-18/PTEN (Phosphatase and tensin homolog) as the major negative regulator of insulin signaling (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999; Worby and Dixon, 2014), and first showed that the transcription factor DAF-16/FoxO (Forkhead box class O) is a major target negatively regulated by insulin signaling (Ogg et al., 1997). Both DAF-18/PTEN and DAF-16/FoxO have critical roles in human disease: PTEN is an important tumor suppressor, while FoxO can promote or suppress tumorigenesis depending on cell context (Eijkelenboom and Burgering, 2013; Milella et al., 2015; Worby and Dixon, 2014). Both PTEN and FoxO have roles in other diseases as well (Burgering and Medema, 2003; Cantley and Neel, 1999; Ogg et al., 1997). Dysregulation of PTEN or FoxO contributes to disease in part because both regulate cellular quiescence: for example, PTEN is required to maintain quiescence in primordial follicles, the source of mammalian ova (Adhikari et al., 2010; Eijkelenboom and Burgering,
2013; Milella et al., 2015). These roles in quiescence are shared by their C. elegans orthologs during larval development (Fukuyama et al., 2006, 2012; Karp and Greenwald, 2013; Narbonne and Roy, 2006; Wolf et al., 2014; Zheng et al., 2018a). We therefore expect that our studies of how DAF-18/PTEN maintains quiescence in dauer will reveal new conserved mechanisms directly relevant to the maintenance of stem cell quiescence in mammalian systems.

*C. elegans* is also an outstanding and proven model for identifying interactions between different signaling pathways in development. Investigating cellular quiescence and relevant signaling pathways in *C. elegans* dauer larvae has the potential to impact not only therapy for human cancers but also for infectious disease: the dauer larva is analogous to the infective stage of parasitic nematodes as it is long-lived, non-feeding, and capable of forming a reproductive adult (Ashton et al., 1999; O’Halloran and Burnell, 2003). In *C. elegans* dauer larvae, failure to maintain quiescence and block signaling pathways such as LIN-12/Notch prevents dauers from forming normal, reproductive adults upon recovery (Colella et al., 2016; Karp and Greenwald, 2013, this work); therefore, studies of quiescence and signaling in dauer may identify novel therapeutic targets for parasitic infections. Thus, as a proven model system amenable to rapid genetic screens, the *C. elegans* dauer is likely to elucidate therapeutically relevant mechanisms in quiescence that can then be verified in and applied to mammalian contexts.

This thesis will be concerned with the maintenance of quiescence during dauer arrest by DAF-18/PTEN in the somatic gonad and germline in dauer.

2. Development of the somatic gonad and germline in *C. elegans*

2.1 Gonadogenesis in *C. elegans* continuous development:
Under favorable conditions, the nematode *Caenorhabditis elegans* progresses through four larval stages (L1-L4) before molting into an adult (Figure 1). *C. elegans* hatch with 4 gonad precursors: Z1 and Z4 form the entire somatic gonad, and Z2 and Z3 the germline (Hubbard and Greenstein, 2005; Kimble and Hirsh, 1979). The somatic gonad then develops in two distinct phases (Figure 2A). In the first stage, Z1 and Z4 divide in L1 to form the somatic gonad primordium, which consists of twelve cells that do not divide during L2 (Kimble and Hirsh, 1979; Kimble and White, 1981). By the end of L2, three of the twelve cells are terminally differentiated, the anchor cell (AC) and two distal tip cells (DTCs), such that there are nine somatic gonad blast (SGB) cells and three specified cells (Hubbard and Greenstein, 2005; Kimble and Hirsh, 1979; Kimble and White, 1981). The SGBs include three ventral uterine (VU), four spermathecal/sheath (SS), and two dorsal uterine (DU) precursor cells (Newman et al., 1996). The SGBs and specified cells (twelve cells total) together form the somatic gonad primordium.

In the second stage of somatic gonad development, starting in L3, the SGBs divide and their descendants undergo fate specification to form the structures of the adult somatic gonad (Figure 2A).

The AC and DTCs do not divide but are essential for development of the adult reproductive system. The AC induces vulval development and development of the uterine-vulval connection, including the uterine-seam syncytium cell (the utse), which is formed from the fusion of the AC with a syncytium of VU descendants (Kimble and Hirsh, 1979; Kimble and White, 1981; Newman et al., 1996). The DTCs are required for promoting mitotic divisions in the germline stem cells (GSCs) and extension and flexion of the germline arms.
In the presence of food, the germline precursors Z2 and Z3 start dividing in L1 and the GSCs continue to divide mitotically thereafter, maintaining a pool of GSCs in the adult (Hubbard and Greenstein, 2005; Korta and Hubbard, 2010). Meiotic divisions do not begin until the L3 stage. The descendants of the first ~70 GSCs that differentiate by meiotic divisions become sperm (four per GSC), and thereafter the meiotic progeny either form oocytes or undergo apoptosis. The GSCs therefore proliferate continuously in all stages, in contrast to somatic gonad development.

The initial GSC divisions are GLP-1/Notch-independent (Austin and Kimble, 1987), and continued GSC proliferation requires activation of GLP-1/Notch in the germline precursors by the ligand LAG-2, which is produced by the DTC of each arm (reviewed in (Hubbard and Greenstein, 2005)). Notch signaling is also essential for somatic gonad development: LIN-12/Notch signaling in the somatic gonad is required for normal fate specification in somatic gonad precursors and their descendants in both stages of somatic gonad development (both described in more detail below).

Under starvation conditions, dauer entry occurs between the two phases of somatic gonad development; dauer entry additionally interrupts the continuous proliferation of germline precursors (Cassada and Russell, 1975; Hong et al., 1998), Figure 2B, described below).

2.2 Gonadogenesis in *C. elegans* with dauer life history:

In replete conditions, *C. elegans* develops rapidly and continuously through larval stages before molting into a reproductive adult with a lifespan of approximately two weeks. In contrast, under unfavorable conditions, the nematode deviates from continuous development and instead enters a larval stage of interrupted development and prolonged quiescence, dauer diapause.
(Figure 1) (Cassada and Russell, 1975). After the L1 stage, the larvae enter the L2d stage, an extended alternative to L2; if conditions do not improve, they undergo an extended L2-dauer molt to form dauers (Fielenbach and Antebi, 2008). Dauers can survive for many months (in contrast to the normal 2-3-week lifespan) without food and display increased resistance to harsh environmental conditions such as desiccation or detergents (Cassada and Russell, 1975). If conditions improve, dauers recover and resume development as post-dauer L3 (pdL3) larvae and become reproductive adults overtly indistinguishable from those without dauer life history (Cassada and Russell, 1975). However, there are some anatomical differences, including supernumerary muscle arms and changes in IL2 neuron morphology (Dixon et al., 2008; Schroeder et al., 2013), as well as differences in gene expression profiles (Hall et al., 2010).

Dauer arrest interrupts both somatic gonad and germline development: somatic gonad development is interrupted after the first stage, and the mitotic divisions of the germline that are normally continuous throughout the larval stages are halted (Figure 2B (Cassada and Russell, 1975; Hong et al., 1998)). The L2d somatic gonad and germline are similar to that of L2: the somatic gonad primordium still consists of twelve cells, nine precursors and the differentiating AC and DTCs (Colella et al., 2016; Narbonne and Roy, 2006). Germline development ceases upon dauer formation with approximately 40 GSCs in the wild type reference strain N2 (Narbonne and Roy, 2006). For the duration of dauer diapause, the nine somatic gonad precursor cells and all GSCs from L2/L2d remain quiescent until dauer recovery.

2.3 The role of LIN-12/Notch signaling in somatic gonad development:

Notch signaling is an intercellular transduction pathway that mediates cell-cell interactions and cell fate specification (Greenwald and Kovall, 2013). Studies in C. elegans first
identified many components of the Notch pathway that are conserved in other systems, including mammals, and provided key insights that helped elucidate the mechanism of Notch signal transduction (Greenwald, 2012). *C. elegans* has two Notch orthologs, *lin-12* and *glp-1*, and both are required for proper somatic gonad and germline development: *lin-12* is required for development of the somatic gonad, vulva, and gonad-vulval connection, while *glp-1* is required for GSC mitotic proliferation (Austin and Kimble, 1987; Ferguson and Horvitz, 1985; Ferguson et al., 1987; Greenwald et al., 1983; Newman et al., 1995).

LIN-12/Notch signaling is required for several distinct events during development of the normal somatic gonad:

i. AC-VU decision through lateral specification: early in gonadal development, four cells in the somatic gonad primordium have the potential to become the AC, termed the α cells and β cells. The two β cells lose this potential early and eventually become VUs. The two α cells retain AC potential longer and, through lateral specification by LIN-12/Notch signaling, small stochastic differences in initial levels of LIN-12/Notch are amplified through a feedback mechanism. Finally, the α cell with relatively lower LIN-12/Notch activity is specified as the AC, and the other α with higher LIN-12/Notch becomes the VU (Greenwald et al., 1983; Kimble and Hirsh, 1979; Kimble and White, 1981; Seydoux and Greenwald, 1989; Wilkinson et al., 1994). In LIN-12/Notch mutants, AC and VU fate specification does not occur normally: loss of LIN-12/Notch function results in specification of multiple ACs over the VU fate, while in activated dominant mutants all cells with AC potential are instead specified as VUs (Greenwald et al., 1983). The fluorescent reporter and integrated transgene *arIs107[mir-61::yfp]* is expressed in the α and β cells, and this expression becomes restricted to the VUs after AC-VU fate
specification and therefore can be used as a marker of VU cells after this decision (Yoo and Greenwald, 2005).

ii. Pi cell (defined here) fate induction and utse formation: The VUs begin dividing in L3 and their descendants adopt a variety of cell fates required for development of the adult somatic gonad. Six of the VU granddaughters adopt what is termed a \( \pi \) cell fate, and the others adopt the \( \varphi \) cell fate (Newman et al., 1995). The AC induces \( \pi \) cell fate specification in these VU descendants by activating LIN-12/Notch signaling in these cells through expression of the LIN-12/Notch ligand, LAG-2.

\( \pi \) cell daughters later fuse to form a syncytium, and after inducing vulval development (see below), the AC fuses with this syncytium (Ghosh and Sternberg, 2014; Newman and Sternberg, 1996; Newman et al., 1996). Once fusion with the AC has occurred, nuclei migrate outwards from the proximal gonad to form a mature utse. Formation of the utse requires the \( \pi \) cells and therefore also requires LIN-12/Notch signaling for specifying the \( \pi \) cell fate.

The adoption of the \( \pi \) cell fate requires cell-cell interactions and indicates cell fate determination, as \( \pi \) cell fate is determined once the VU granddaughters are born (Newman et al., 1995): ablation of the AC when the VUs have not divided or have only divided once results in failure to specify \( \pi \) cell fates in a majority of larvae, and only the \( \varphi \) cell fate is specified. However, \( \pi \) cell fates are specified normally with ablation of the AC after the VU granddaughters are born, six of which will become the \( \pi \) cells (Newman et al., 1995). Subsequent utse formation by \( \pi \) cell daughters represents terminal differentiation of these cells.

Both \( \pi \) cell fate specification and utse formation can be visualized with multicopy integrated transgenes. \texttt{syIs80[lin-11::gfp]} and \texttt{kuIs29[egl-13::gfp]} are direct transcriptional targets of LIN-12/Notch and markers of \( \pi \) cell and \( \pi \) cell daughters and are expressed once they
are specified in the late L3 (Cinar et al., 2003; Gupta et al., 2003; Hanna-Rose and Han, 1999; Oommen and Newman, 2007). \textit{arIs51[cadh-3::gfp]} is expressed in the differentiated anchor cell by the end of the L2 larval stage and its expression later expands to the entire utse syncytium when the AC fuses with the immature syncytium of \( \pi \) cell daughters in the early-to-mid L4 larval stage (Karp and Greenwald, 2003; Pettitt et al., 1996) also see (Ghosh and Sternberg, 2014).

iii. Vulval induction: in the four \( \pi \) cell daughters that do not contribute to utse formation, an alternative uv1 cell fate is specified through EGF signaling from the vulval precursor cell descendant vulF cells (Chang et al., 1999). The uv1 cells are required for proper establishment of the vulval-uterine connection and extend processes connecting to the vulval vulF cells (Newman et al., 1996). Therefore, signaling from vulval cells is required for normal somatic gonad development, and proper vulval fate specification requires LIN-12/Notch signaling: the six vulval precursor cells (VPCs) remain quiescent during the L1 and L2 stages and are induced to divide and specify a precise pattern of cell fates by the AC during the L3 (reviewed in (Greenwald and Kovall, 2013) and (Sternberg, 2005). This patterning is primarily mediated by crosstalk between the LIN-12/Notch and EGFR signaling pathways and requires the AC. Vulval development and regulation of VPC in dauer are described in further detail below.

3. Vulval development in continuous development and dauer life history

Though I have focused on the somatic gonad, one well-characterized example of the maintenance of quiescence and multipotential in somatic blast cells during dauer diapause is the vulval precursor VPCs. The VPCs remain quiescent and retain VPC potential throughout dauer diapause, and the IIS signaling target \textit{daf-16}/FoxO is required for both (Euling and Ambros, 1996; Karp and Greenwald, 2013). Additionally, in this context, blocks to both the LIN-
12/Notch and EGFR signaling pathways are required for normal post-dauer development. Here I briefly review VPC development in continuous development and in dauer.

In the L1, there are six VPCs, numbered P3.p-P8.p. Each VPC is multipotent and has the potential to adopt the 1°, 2°, or 3° fate. In the early L3 stage, an EGF-like inductive signal from the AC of the gonad activates EGFR signaling in P6.p specifying 1° fate, and P6.p in turn produces a lateral signal that activates LIN-12/Notch in its neighbors P5.p and P7.p, causing them to adopt a 2° fate (Figure 3, adapted from (Karp and Greenwald, 2013)) (Reviewed in (Sternberg, 2005)). P3.p, P4.p, and P8.p, which do not receive either of these patterning signals, adopt a 3° fate and each divides once to produce two daughters that fuse with the hypodermis. Fluorescent reporters for transcriptional targets of the EGFR or LIN-12/Notch pathways mark these fates: for example, as a result of P6.p adopting 1° fate, the LIN-12 activating ligand lag-2 is expressed and can be visualized with a fluorescent marker driven by the lag-2 promoter (Chen and Greenwald, 2004; Zhang and Greenwald, 2011). Likewise, 2° fate normally adopted by P5.p and P7.p in continuous development can be marked using a reporter for the lateral signaling target of LIN-12, lst-5 (Choi, 2009; Karp and Greenwald, 2013; Yoo and Greenwald, 2005).

The potential of VPCs to adopt 1°, 2°, or 3° fates – their multipotency – appears to involve mechanisms for blocking the activity of the EGFR and LIN-12/Notch pathways (Karp and Greenwald, 2013). Expression of LIN-12(intraΔP), a constitutively active form of LIN-12 which is stable and nuclear in dauer VPCs, induces a 2° fate marker in up to 75% of all VPCs in L2d, but this expression is completely blocked by the L2d-dauer molt such that none of these markers are expressed in dauer.

The IIS target daf-16/FoxO is required for the blocks to signal transduction of activated EGFR and LIN-12/Notch during dauer diapause, maintaining VPC quiescence and multipotency
(Karp and Greenwald, 2013). *daf-16* encodes the ortholog of mammalian FoxO, a transcription factor important for longevity, stress resistance, and dauer formation (Larsen et al., 1995; Lin et al., 1997; Vowels and Thomas, 1992) In *daf-16(0)* dauer larvae, VPCs adopt 1° and 2° fates and often divide as well, indicating that DAF-16 activity is necessary for maintaining not just VPC multipotency but also quiescence during dauer diapause (Karp and Greenwald, 2013). The role of DAF-16 in maintaining VPC multipotency and quiescence is likely autonomous to the VPCs: VPC-specific RNAi against *daf-16* in dauers results in 1° fate adoption in P6.p.

4. Regulation of dauer diapause

The cellular basis for the regulation of dauer entry is complex. Three major signaling pathways regulate dauer entry: Insulin/Insulin-like growth factor signaling (IIS), TGFβ, and DA-DAF-12/steroid hormone signaling (Antebi, 2015; Gumienny and Savage-Dunn, 2013; Hu, 2007; Murphy and Hu, 2013a) Environmental conditions detected by sensory neurons regulate the production of insulin-like growth factors, DAF-7/ TGFβ, and steroid hormone ligands (Dafachronic Acids, DA); these inputs are integrated to control the decision to proceed to reproductive adulthood or enter dauer diapause (Gerisch et al., 2004; Mak and Ruvkun, 2004). Active IIS and TGFβ signaling converge on the nuclear hormone receptor (NHR) homolog DAF-12, which promotes continuous development when bound by DA ligand (Figure 4, from (Karp and Greenwald, 2013)). When IIS and TGFβ signaling are inactive, DAF-12 ligand is absent and DAF-12 instead binds to the SHARP corepressor homolog and DAF-12 interacting protein DIN-1 to promote dauer formation (Ludewig et al., 2004). cGMP signaling also regulates dauer formation, generally upstream of the IIS and TGFβ pathways (discussed further below and reviewed in (Fielenbach and Antebi, 2008)).
The roles of some components of these pathways in regulating germline and/or somatic gonad quiescence during dauer development (L2d and the L2d-dauer molt) have been identified, though less is known about the maintenance of their quiescence during the dauer larval stage itself; these are described below.

4.1 Insulin/insulin-like signaling (IIS):

Apart from regulating the dauer entry decision and dauer formation, the IIS signaling pathway regulates many processes and aspects of development in *C. elegans*, including aging, fat metabolism, stress resistance, behavior and learning, germline proliferation, and non-dauer developmental arrest (reviewed in (Murphy and Hu, 2013a)). Active IIS promotes continuous development, while reduced IIS permits dauer entry. Loss of function of the sole *C. elegans* insulin receptor ortholog *daf-2* (Kimura et al., 1997; Ruvkun and Hobert, 1998) forces constitutive dauer formation (Gottlieb and Ruvkun, 1994). Loss of function in other conserved components of the IIS pathway, including AGE-1, PDK-1, AKT-1, and AKT-2, also results in constitutive dauer formation, and dauer formation in these mutants requires the activity of the FoxO ortholog DAF-16 (Figure 5, reviewed in (Murphy and Hu, 2013a)). As in other systems, *daf-2/IR* is regulated by insulin-like peptides; however, in *C. elegans* it is predicted that there are approximately 40 insulin-like (INS) molecules and many of these can regulate dauer formation. Additionally, some of the INS peptides are agonistic to *daf-2* while others antagonize *daf-2* activity (Cornils et al., 2011; Murphy et al., 2007; Pierce et al., 2001). The *ins* genes are mainly expressed and function in neurons, though some expression and function has been identified in the intestine as well (Murphy et al., 2007; Pierce et al., 2001).
The lipid and protein phosphatase *daf-18/PTEN* is required for germline quiescence in dauer (Narbonne and Roy, 2006) and is a conserved inhibitor of IIS signaling, therefore promoting activity of the canonical IIS target and transcription factor DAF-16/FoxO (Figure 5, adapted from (Murphy and Hu, 2013a)). *daf-18/PTEN* is described in further detail below.

Activation of the worm ortholog of the insulin receptor, DAF-2, inhibits DAF-16 and promotes continuous development, so loss of *daf-2* results in active DAF-16 and a *daf-c* phenotype (Larsen et al., 1995; Lin et al., 1997; Vowels and Thomas, 1992). Conversely, in *C. elegans*, *daf-16* is required for dauer formation and *daf-16* null mutants are *daf-d. daf-16(0)* mutants fully suppress dauer formation in *daf-c* mutants of the IIS pathway, indicating that DAF-16/FoxO is the main target of IIS in regulating dauer formation (Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Vowels and Thomas, 1992). In dauer forms using the *daf-c* mutant *daf-7(e1370)* (described below), DAF-16 regulates both quiescence and multipotency in the VPCs, in part by repression of LIN-12/Notch targets (Karp and Greenwald, 2013). *daf-16* is important for longevity, stress resistance, and dauer formation. In the presence of food, IIS promotes phosphorylation of DAF-16, leading to its cytoplasmic sequestration so it cannot activate its nuclear targets (Larsen et al., 1995; Lee et al., 2001; Lin et al., 1997, 2001; Vowels and Thomas, 1992) *daf-16* is not required for maintaining quiescence in the dauer germline (Narbonne and Roy, 2006), but it is required in the VPCs in dauer for maintenance of both quiescence and multipotency, at least in part through a DAF-16-mediated block to LIN-12/Notch signaling (described above, (Karp and Greenwald, 2013)).

Aside from *daf-18/PTEN*, the PP2A holoenzyme *pptr-1* is also an IIS inhibitor that regulates dauer entry through regulation of phosphorylation and activity of AKT-1 (Padmanabhan et al., 2009). Loss of *pptr-1* suppresses the *daf-c* phenotype in loss of function
*daf*-2 mutants, while knockdown of other PP2A subunits does not. This suppression of dauer formation is not seen in an alternative pathway *daf*-c mutant, *daf*-7/TGFβ.

### 4.2 TGFβ:

When active, the TGFβ signaling pathway, promotes continuous development (Ren et al., 1996; Schackwitz et al., 1996). Of the five TGFβ-related genes, a strong role in dauer formation has only been identified for *daf*-7/TGFβ (Gumienny and Savage-Dunn, 2013). DAF-7 signals through a canonical receptor pathway which, when active, inhibits activity of the Co-Smad DAF-3 and its partner, the Sno/Ski homolog DAF-5. *daf*-7 is expressed specifically in the ASI sensory neurons, while other components of the pathway are expressed in other tissues (Ren et al., 1996; Schackwitz et al., 1996).

In addition to regulating dauer formation, *daf*-7/TGFβ signaling regulates germline proliferation, but may have both pro- and anti-proliferative roles. *daf*-7 signaling independent of DAF-3 and DAF-5 appears to negatively regulate GSC proliferation through regulation of DTC expression of the GLP-1/Notch ligand *lag*-2 (Park et al., 2010). However, DAF-7 activity in the ASI neurons has also been identified as positively regulating GSC proliferation under favorable conditions through its receptor DAF-1, which is expressed in the DTCs (Dalfó et al., 2012). In this role, *daf*-7/TGFβ signaling likely regulates DAF-3 and DAF-5 activity as in the canonical pathway; additionally, in this role, *daf*-7 appears to promote proliferation independently of GLP-1/Notch. Finally, this positive regulatory role is also independent of factors that promote dauer formation in other pathways including DAF-18/PTEN and DAF-16/FoxO (IIS) and DAF-12 (NHR).
In dauer, however, *daf*-7/TGFβ is not required for germline progression in *daf-18(0) dauer* and the GSCs arrest appropriately in *daf-7(ts) dauer* (Narbonne and Roy, 2006). However, it is possible that other TGFβ ligands may promote gonad proliferation in *daf-18(0) dauer*. For example, the TGFβ ligand *dbl-1* has some role in repressing dauer formation: *dbl-1* null single mutants do not affect dauer formation but do enhance the *daf-c* phenotype of *daf-7(e1372)* at 20°C (Morita et al., 1999). Additionally, the biological function of the TGFβ homologs *tig-2* and *tig-3* are unknown and could have unidentified roles in dauer regulation (Suzuki et al., 1999). Analysis of other TGFβ-related genes may reveal unidentified roles in dauer regulation or functional redundancy with *daf-7*.

4.3 DA/NHR:

Nuclear hormone receptor (NHR) signaling acts downstream of both IIS and TGFβ signaling in regulating the dauer entry decision (Antebi, 2015; Gerisch et al., 2004; Mak and Ruvkun, 2004). Steroids called Dafachronic Acids (DAs) and essential ligands for the NHR DAF-12 promote continuous development. When unliganded, DAF-12/NHR promotes dauer entry by interacting with corepressors such as DIN-1S/SHARP (Ludewig et al., 2004), reviewed in (Fielenbach and Antebi, 2008). *daf-12* ligand binding domain (LBD) mutants which abrogate ligand-bound DAF-12 activity have up to twice the GSCs observed in wild-type dauers, but no somatic tissue progression (Antebi et al., 1998). In addition to diminishing ligand binding, *daf-12* LBD mutants may also affect corepressor binding and therefore unliganded DAF-12, thus forming partially defective dauers which are generally SDS-sensitive (Antebi et al., 1998; Ludewig et al., 2004). Likewise, the DAF-12 corepressor *din-1s/SHARP* is required for the proper establishment of SGB and GSC quiescence during L2d and L2d-dauer molt, and this
function may require DAF-12 activity (see below (Colella et al., 2016; Ludewig et al., 2004). 

din-1/SHARP is not required for maintenance of SGB or GSC quiescence in dauer (Colella et al., 2016). Whether DAF-12 activity regulates maintenance of quiescence in dauer in addition to its establishment is unknown; however, while daf-12/NHR is essential for dauer formation, din-1/SHARP is not, suggesting other transcriptional complexes are likely able to substitute as a partner for pro-dauer DAF-12 activity (Ludewig et al., 2004).

Production of the DAs that are essential for liganded DAF-12/NHR activity and which act as ligands for DAF-12 in promoting continuous development requires daf-9, a steroidogenic hydroxylase (Gerisch et al., 2004; Jia et al., 2002; Ludewig et al., 2004). daf-9(0) mutants are daf-c and fully arrest in dauer unconditionally; this arrest is daf-12-dependent (Albert and Riddle, 1988; Antebi et al., 1998; Gerisch, B., Antebi, 2004; Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006). DAF-9 acts non-autonomously to regulate other tissues in the neuroendocrine XXX cells of the head and the hypodermis, and this activity involves positive feedback between the XXX cells and the hypodermis; daf-9 is also expressed in the spermatheca (Gerisch, B., Antebi, 2004; Mak and Ruvkun, 2004; Schaedel et al., 2012). This positive feedback loop ensures robust and unified regulation of the decision to bypass or enter dauer in the entire organism.

4.4 Guanylyl cyclase signaling:

A guanylyl cyclase signaling pathway that regulates dauer formation has been reported to act upstream of the IIS and TGF-β pathways (reviewed in (Fielenbach and Antebi, 2008) and (Murphy and Hu, 2013a). The daf-c mutant daf-11 encodes a transmembrane guanylyl cyclase and is expressed in chemosensory neurons. Candidate downstream effectors of this pathway as
well as many components of a dauer-regulating guanylyl cyclase signaling pathway are expressed in sensory neurons as well (Coburn and Bargmann, 1996; Coburn et al., 1998; Komatsu et al., 1996).

Guanylyl cycling may also at least partially mediate pheromone-mediated signals for dauer entry. Dauer pheromone refers to the small molecule ascarosides that mediate population density signaling in C. elegans. Ascarosides have been demonstrated to induce dauer formation, though some discrepancy exists in the identity of the particular ascarosides that have this activity (Butcher et al., 2007; Jeong et al., 2005). Subsequently, GTP-binding protein (G protein)–coupled receptors (GPCRs) have been identified that mediate dauer formation in response to pheromone signaling, and biosynthetic components of the pheromone-producing pathways have been identified as well ((Butcher et al., 2009; Kim et al., 2009).

5. daf-18/PTEN in dauer

daf-18/PTEN is the sole lipid and protein phosphatase in C. elegans. The lipid phosphatase activity of daf-18/PTEN inhibits IIS by antagonizing the activity of the PI3K AGE-1, and thereby promoting activity of the IIS target DAF-16/FoxO through nuclear localization (Figure 5, adapted from (Murphy and Hu, 2013a); green, dauer-promoting activity; red, dauer-inhibiting activity, (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999; Solari et al., 2005). daf-18 null mutants fully suppress the dauer-constitutive phenotypes of mutations in the components of the IIS pathway. Based on differential suppression by a loss-of-function allele daf-18(e1375), as well as analysis of gain-of-function IIS mutants, daf-18 appears to suppress both age-1-dependent and -independent outputs of daf-2/IR activity (reviewed in (Murphy and Hu, 2013a)).
**daf-18** is required for germline quiescence in dauer, but appears to act independently of **daf-16** in this role (Masse et al., 2005; Narbonne and Roy, 2006). **daf-18** mutant dauers are dauer defective, but dauers can be obtained in a **daf-7(e1372ts)/TGFβ** or **daf-2(e1370ts)/IR** background; in the presence of the hypomorphic allele **daf-18(e1375)**, dauers in these backgrounds arrest with over twice the number of GSCs compared to control dauers (Narbonne and Roy, 2006). Additionally, **daf-18(ok480)** null mutant dauers can be obtained in **daf-7** mutants, and these dauers arrest with over four times the number of GSCs than controls. The requirement for **daf-18** in maintaining arrest of somatic tissues and the question of whether **daf-18** is required during L2d/L2d-dauer molting stages to establish quiescence, or during dauer arrest to maintain quiescence, have not been tested.

Many aspects of dauer formation and maintenance are regulated non-cell autonomously from the nervous system, intestine, and hypodermis (as in (Gerisch et al., 2004; Libina et al., 2003; Masse et al., 2005). Analysis of tissue-specific expression of **daf-18** in the null **daf-18(mg198); daf-2(e1372)** mutants has shown that **daf-18** expression in each of multiple tissues, including the intestine, nervous system, seam cells, and body wall muscle, can autonomously and non-autonomously rescue the various dauer defects observed in **daf-18(0)** mutants. Such **daf-18** expression, however, cannot rescue the “gonadal developmental arrest”; gonadal developmental arrest was only rescued by **daf-18** expression from its own widely-expressed promoter (Masse et al., 2005). Thus, the tissue focus for regulation of gonadal arrest by **daf-18** has not been determined.

Also notably, like **din-1s** (described above), the two AMP-activated kinase catalytic subunits **aak-1** and **aak-2** are required additively for establishing germline arrest in dauer, and so is the upstream AMPK activator **par-4/LKB1**, but none of these genes affect SGB quiescence in
dauer (Colella et al., 2016; Narbonne and Roy, 2006). Like *din-1s, aak-2* was explicitly found to have a role in establishing GSC quiescence during L2d/L2d-dauer molt stages but not maintaining it in dauer.

The only component reported to be required for maintaining quiescence in the somatic gonad and germline during dauer arrest, rather than establishing quiescence during L2d/L2d-dauer molt, is *cki-1*, which encodes an ortholog of the cyclin dependent kinase inhibitor p27/Kip (Hong et al., 1998). Both “ongoing divisions” were observed in the somatic gonad and germline in *daf-7(e1372); cki-1(RNAi)* dauers.

In summary, little is known about maintenance of somatic gonad and germline quiescence during dauer arrest, or which tissue (or tissues) regulates this quiescence. Only *cki-1* is definitively reported to have a role in maintenance of quiescence in these tissues (Hong et al., 1998), though explicit timing has not been tested for most genes with identified roles in establishing or maintaining somatic gonad and germline quiescence; precise time courses have been reported for *aak-2* and *din-1s* only (Colella et al., 2016; Narbonne and Roy, 2006). Whether *daf-18/PTEN* is required for establishing quiescence, maintaining it, or both in the germline is not known. *daf-18/PTEN* is required to maintain GSC quiescence in dauer, but whether it has a role in somatic gonad quiescence as well has not been tested. Additionally, its tissue focus in this role has not been investigated.

6. *daf-18/PTEN* in L1 arrest

My thesis is a study of dauer, but there is another well-studied arrested state in *C. elegans* for which *daf-18/PTEN* is required, L1 arrest. L1 arrest occurs if eggs hatch into a nutritionally
deficient environment; this arrest occurs because postembryonic development is not initiated upon the hatching of *C. elegans* eggs, but occurs only when nutrition is supplied (Lewis and Fleming, 1995). During L1 arrest, both somatic and gonad precursors remain quiescent until replete conditions are detected at which point developmental progression (Fukuyama et al., 2006; Hong et al., 1998). The genes required for quiescence of germline precursors in L1 arrest have been relatively well-characterized, and our understanding of the mechanisms for maintaining quiescence in somatic precursors has improved significantly in recent years. As described here, it is clear that *daf-18/PTEN* is required for maintenance of many precursors in L1 arrest, including the germ and somatic precursors; while other roles for *daf-18/PTEN* have been described, including for survival of L1 arrested larvae (Fukuyama et al., 2012), here I focus on its requirement to regulate tissue quiescence and the tissue focus for *daf-18/PTEN* in this role.

### 6.1 Germline precursor quiescence in L1 arrest

In L1-arrested larvae cultured without food, the germline precursors Z2 and Z3 arrest in a G2 4N stage and do not divide until L1 arrest is exited (Fukuyama et al., 2003, 2006). In *daf-18(0)* L1-arrested larvae, however, Z2 and Z3 divide, indicating that DAF-18/PTEN is required for maintenance of quiescence in the germline precursors (Fukuyama et al., 2006). The AMP-activated protein kinases (AMPKs) *aak-1* and *aak-2* are also required for maintenance of germline quiescence in L1 arrest, though redundantly. Both *daf-18* and *aak* appear to act by inhibiting TORC1 (TOR Complex 1), as loss of activity of multiple TORC1 components suppresses germ cell divisions in L1 arrest (Fukuyama et al., 2003, 2006, 2012).

Notably, the divisions of Z2 and Z3 observed in *daf-18(0)* larvae are suppressed by mutations in the *age-1* and *akt-1* components that mediate IIS signaling, but not by a loss-of-
function daf-16 mutant (Fukuyama et al., 2006). These findings suggest that DAF-18/PTEN maintains Z2/Z3 quiescence through inhibition of IIS, and that downstream effector genes other than daf-16/FoxO are involved in this role. Additionally, arrest does not require several other daf-d genes known to be required for dauer arrest including daf-3 or daf-5 (TGFβ), daf-22 (dauer pheromone production), daf-12 (NHR signaling), or daf-6 (normal amphid and phasmid morphology and function).

Cell-autonomy of daf-18 in this context has not been demonstrated. However, an extrachromosomal array carrying daf-18(+) rescues the germ precursor defect (Fukuyama et al., 2006), suggesting that this function may not be cell-autonomous as extrachromosomal arrays are often silenced in the germline (William G. Kelly, SiQun Xu, 1997). The tissue focus of aak in maintaining germline quiescence in L1 arrest has also not been determined. Both daf-18 and aak are required for L1 survival, and their cellular focus in this role has been identified: both function in the intestine, and daf-18, additionally, functions in the hypodermis, while aak functions in the neurons (Fukuyama et al., 2012). Therefore, both daf-18 and aak appear to have distinct foci for their roles in L1 arrest survival vs. maintenance of germline precursor quiescence.

6.2 Somatic precursor quiescence in L1 arrest

It has recently been demonstrated that daf-18/PTEN is required to maintain quiescence of somatic precursors during L1 arrest. Loss of developmental arrest in the form of divisions or migration is seen in at least three types of post-embryonic somatic precursors, the M mesoblast, Q neuroblast, and P neuroblast (Fukuyama et al., 2015; Zheng et al., 2018a). The developmental progression observed is characterized by divisions in the M and Q precursors and by ventral migration of the P neuroblast. Like the germline precursors, reactivation of M and P cells is
mediated by TOR signaling; however, unlike Z2 and Z3, *daf-16* also has a role, albeit lesser, in maintaining arrest of somatic precursors (Baugh and Sternberg, 2006; Fukuyama et al., 2015). In contrast, Q cell divisions in *daf-18(0) L1* arrest are induced by *mpk-1* signaling, which is activated through INS gene expression induced by the TGFβ ortholog *dbl-1/BMP* (but not *daf-7/TGFβ*) (Zheng et al., 2018a). Intriguingly, these divisions are suppressed with loss of PP2A activity in *pptr-2* and *sur-6* mutants, indicating that these PP2As promote Q cell division downstream of *akt-1*; however, the PP2A encoded by *pptr-1*, which negatively regulates the IIS pathway through AKT-1 (Padmanabhan et al., 2009), does not suppress Q cell division. Therefore, some PP2As may promote AKT-1 activity while others inhibit it; alternatively, *pptr-1* is redundant with another PP2A in this context.

The tissue focus of *daf-18/PTEN* in maintaining somatic precursor quiescence is distinct for different somatic blast cells: *daf-18* is required non-autonomously in the hypodermis to prevent M cell and P cell progression and also displays some rescue in neurons and intestine, but not muscle. However, *daf-18* is required autonomously to maintain Q neuroblast quiescence (Fukuyama et al., 2015; Zheng et al., 2018a).
Chapter 1 Figures
Figure 1. Continuous development and dauer formation life cycle in *Caenorhabditis elegans*. In favorable conditions (green) *C. elegans* develops through four larval stages, L1-L4, before molting into a reproductive adult. In unfavorable conditions (orange), TGFβ and IIS signaling are low and L1s molt into an alternative to the L2 called the L2d (Cassada and Russell, 1975). If conditions improve, continuous development can be resumed, but otherwise L2ds undergo an extended molt to form dauer larvae. Dauers have an extended lifespan and increased stress resistance and, if conditions improve, recover to continuously develop through post-dauer L3 and L4 stages to become reproductive adults.
Figure 2. Somatic gonad and germline development. (A) At the start of the L1 stage, the gonad primordium consists of four cells: the somatic gonad precursors Z1 and Z4, and the germline precursors Z2 and Z3 (Kimble and Hirsh, 1979; Kimble and White, 1981). In the first phase, Z1 and Z4 generate twelve cells. Three cells are terminally differentiated: two distal tip cells (DTCs), which promote mitosis in the germline and guide later gonad outgrowth, and the anchor cell (AC), which later organizes uterine and vulval patterning (Kimble and Hirsh, 1979; Kimble and White, 1981; Newman et al., 1996). The other cells are precursors to later structures, including 3 ventral uterine precursor cells (VUs), which generate descendants that will form the ventral uterus in the second phase of somatic gonadal development. The second stage of somatic gonadal development begins in mid-L3, when the precursor cells divide. At that time, the VUs produce 12 granddaughters; in 6 of these granddaughters, LIN-12/Notch is activated by a LAG-2 signal from the AC via cell-cell interactions, causing them to adopt a fate called “pi” (Newman et al., 1995). The pi cells divide once and eight of their daughters fuse with the AC to form the Uterine Seam (utse) syncytium. (B) In dauer, somatic gonad development is interrupted after the first phase (Hong et al., 1998; Narbonne and Roy, 2006).
Figure 3. VPC fate patterning in continuous development. From (Karp and Greenwald, 2013). “(A) VPC specification in wild-type hermaphrodites. During L3, an EGF-like signal (red) from the anchor cell (AC) activates Ras signaling in P6.p, causing it to adopt 1° fate and produce ligands, including LAG-2 and APX-1, which activate LIN-12/Notch in P5.p and P7.p.” Sourced in Karp and Greenwald 2013 from (Sternberg, 2005).
Figure 4. Integration of signaling pathways regulating the decision to form dauers. From (Karp and Greenwald, 2013). The insulin/insulin-like signaling (IIS) and TGFβ signaling pathways converge on signaling mediated by the nuclear hormone receptor (NHR) DAF-12 (Gerisch, B., Antebi, 2004; Mak and Ruvkun, 2004). In favorable environments, IIS and TGFβ signaling are both active and inhibit activity of downstream targets DAF-16/FoxO and DAF-3/Smad, DAF-5/Sno/Ski, allowing production of dafachronic acids (DAs), which require activity of the steroid hydroxylase DAF-9. These DAs act as ligands for DAF-12/NHR which then promotes continuous development. In unfavorable environments, both IIS and TGFβ signaling are inactive, and DAF-12/NHR unliganded activity promotes dauer formation.
Figure 5. Insulin/insulin-like signaling pathway. Adapted from (Murphy and Hu, 2013a). Green, dauer-promoting activity (loss of function: dauer defective). Red, dauer-inhibiting activity (loss of function: dauer constitutive). Insulin/insulin-like peptides (ILPs) can have agonistic or antagonistic activity. When agonistic ILPs activate the insulin receptor DAF-2, signaling through IIS-promoting components (colored red) promote phosphorylate the canonical IIS target DAF-16/FoxO such that it is sequestered in the cytoplasm. When IIS is inactive, DAF-16/FoxO is localized in the nucleus and can activate its targets. DAF-18/PTEN promotes DAF-16/FoxO nuclear localization by antagonizing AGE-1/PI3K activity. Not shown are other IIS targets which include HSF-1 and SKN-1.
Figure 6. The daf-7/TGF-β signaling pathway. From (Gumienny and Savage-Dunn, 2013). daf-7 expressed in the ASIs promotes signaling in target cells that ultimately inhibits the functions of DAF-3/Smad and DAF-5/Sno/Ski. DAF-7/TGFβ signaling is active in favorable conditions and promotes continuous development (opposing dauer development). DAF-7/TGFβ activates receptor composed of two DAF-1 type I receptor and DAF-4 type II receptor subunits which promote signal transduction through the Smads DAF-8 and DAF-14.
Figure 7. DAF-9-mediated DA signaling through DAF-12/NHR. From (Antebi, 2006) with slight adaptations. In favorable environments, the IIS and TGFβ signaling pathways promote dafachronic acid (DA, blue circles) signaling and therefore ligand-bound DAF-12 activity, while in unfavorable conditions, ligand is absent and unliganded DAF-12 interacts with corepressors like DIN-1/SHARP to promote dauer formation. Production of DAF-12-binding DA ligands requires the steroid hydroxylase DAF-9.
Chapter 2

daf-18/PTEN acts in the somatic gonad to coordinate somatic gonad and germline development in C. elegans dauer larvae

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Summary

*C. elegans* larvae integrate environmental information with decisions about developmental progression (Antebi, 2013; Hu, 2007; Rougvie & Moss, 2013). In favorable conditions, worms develop rapidly and continuously through four larval stages into reproductive adulthood. In contrast, if conditions are unfavorable through the second larval stage, worms enter dauer diapause, a state of global and reversible developmental arrest in which precursor cells remain quiescent and preserve their developmental potential, anticipating developmental progression if conditions improve. Signaling from neurons, hypodermis, and intestine regulate the distinctive appearance and behavior of dauer larvae, and many aspects of developmental arrest of the non-gonadal soma (Antebi, 2013; Murphy & Hu, 2013; Schaedel, Gerisch, Antebi, & Sternberg, 2012). Here, our analysis of *daf-18/PTEN* has revealed that the decision of somatic gonad blast cells (SGBs) and germline stem cells (GSCs) to be quiescent or to progress developmentally is regulated differently from the nongonadal soma. We used tissue-specific rescue, Cre recombinase-mediated tissue-specific knockout, and traditional genetic mosaic analysis to show that *daf-18/PTEN* acts non-autonomously within the somatic gonad primordium to maintain developmental quiescence of both SGBs and GSCs. Our analysis suggests that *daf-18* acts in somatic gonad cells to produce a “pro-quiescence” signal or signals that acts *inter se* and between the somatic gonad and the germline. The inferred signal is not mediated by DAF-2/Insulin Receptor and does not maintain quiescence of the nearby Sex Myoblasts, and developmental progression in *daf-18(0)* does not require Dafachronic Acid ligands. Finally, we show that abrogating quiescence in dauer has deleterious consequences for post-dauer fertility. In sum, our results demonstrate a novel role for the somatic gonad as an endocrine organ to synchronize somatic gonad and germline development during dauer diapause and recovery.
Key words: *C. elegans*, dauer, diapause, DAF-18, PTEN, gonad, germline, stem cell, quiescence

Results

daf-18 activity prevents developmental progression of the somatic gonad in dauer larvae

daf-18, the sole *C. elegans* PTEN gene, is required for dauer formation through modulation of the insulin signaling pathway (Ogg & Ruvkun, 1998). *daf-18(0)* (null) mutants are therefore defective in dauer formation, but *daf-18(0)* dauers can be generated using *daf-7(e1372)/TGFβ*, a parallel input that constitutively induces dauer formation, and identified using SDS-selection for dauer larvae (Larsen, Albert, & Riddle, 1995; Vowels & Thomas, 1992). In this study, unless otherwise specified, “*daf-18(0)* dauers” refers to *daf-7(e1372)*; *daf-18(ok480null)* (Brisbin et al., 2009), and “control dauers” are *daf-7(e1372)*; *daf-18(+)*. The arrested gonad in *daf-7* control dauers resembles that of genotypically wild-type dauers, and as described below, key findings regarding *daf-18(0)* dauers were replicated using mutations in *daf-2/Insulin receptor* and *daf-9/steroidogenic hydroxlyase* to induce dauer formation.

Gonadogenesis occurs in two phases (Figure 1A). The first phase is completed during the L2 stage with the formation of the somatic gonad primordium, in which the germline stem cells (GSCs) are segregated into two arms, each capped by a distal tip cell (DTC), and a proximal region containing nine somatic gonad blast cells (SGBs) and the Anchor Cell (AC) (Kimble & Hirsh, 1979). In the second phase, beginning in the L3 stage, the SGBs divide and generate the structural cells of the gonad; the AC organizes development of the uterus and vulva; and the DTCs nurture GSC proliferation and lead outward extension of the gonad arms (Kimble, 1981). In dauer larvae, development is suspended between the first and second phases, and both GSCs and SGBs remain quiescent (Hu, 2007).
Loss of daf-18 abrogates GSC quiescence in dauer (Narbonne & Roy, 2006), and, when we marked all somatic gonad cells of daf-18(0) dauers with mCherry-histone (Figure 1 and STAR Methods), we observed a dramatic increase in the number of mCherry-labeled cells, suggesting loss of SGB quiescence as well (Figure 1B). Expression of cell fate markers for Ventral Uterine precursor cell (VU) and Sheath-Spermatheca precursor cell (SS) descendants characteristic of the second phase of gonadogenesis, including expanded arls51[cdh-3::gfp] expression indicative of utse (uterine seam syncytium) formation from terminal descendants of VUs, indicates that SGBs undergo normal lineages in daf-18(0) dauers (Figure 1C,D).

Furthermore, developmental progression of SGBs is not observed prior to daf-18(0) dauer entry, and the penetrance of dauers displaying developmental progression increases with time in dauer, consistent with a role for daf-18 in maintaining SGB quiescence in dauer (Figure S1). The role of daf-18 therefore differs from genes that act in the L2d stage: components of the AMPK pathway, which is required to establish GSC (but not SGB) quiescence (Narbonne, Hyenne, Li, Labbe, & Roy, 2010; Narbonne & Roy, 2006), and din-1/SHARP, a co-repressor for several transcription factors (Ludewig et al., 2004; Oswald et al., 2002; Shi et al., 2001), which appears to act autonomously to establish SGB and GSC quiescence in preparation for dauer entry (Colella, Li, & Roy, 2016).

In opposing dauer formation, daf-18 acts via modulation of Insulin/Insulin-like Growth Factor signaling (IIS), leading to increased cytoplasmic sequestration of DAF-16/FoxO (Murphy & Hu, 2013). daf-16 is not required to maintain quiescence of GSCs in dauer (Narbonne & Roy, 2006), but is important for maintaining quiescence of some somatic non-gonadal blast cells in dauer (Karp & Greenwald, 2013). When we tested the requirement of daf-16 for SGB progression, we observed that, whereas 88% of daf-18(0) dauers have expanded arls51[cdh-3::gfp] utse formation, only 7% of daf-16(mgDf50null); daf-7 do (Figure 1D). This observation suggests that regulation of SGB quiescence in dauer is largely independent of daf-16, and is supported by additional evidence below.
*daf-18* functions in the somatic gonad primordium to prevent SGB and GSC developmental progression in dauer

The cellular basis for the regulation of dauer entry involves multiple non-gonadal tissues. Environmental conditions detected by sensory neurons regulate the production of insulin-like growth factors, DAF-7/TGFβ, and steroid hormone ligands; these inputs are integrated to control the decision between proceeding to reproductive adulthood or entering dauer diapause (Antebi, 2013; Hu, 2007; Rougvie & Moss, 2013; Schaedel et al., 2012). Tissue-specific expression of *daf-18* activity in neurons, intestine, and the seam cells of the hypodermis, but not muscle, restores the ability to form dauers in *daf-18*(0) mutants (Masse, Molin, Billaud, & Solari, 2005).

To ascertain the cellular focus of *daf-18* activity for preventing SGB and GSC progression in dauer larvae, we first performed genetic mosaic analysis (Herman, 1984): random mitotic loss of arEx2399[*daf-18*(+)], an extrachromosomal array carrying *daf-18*(+), in a *daf-7; daf-18*(0) background, created “*daf-18*(0) mosaic dauers” lacking *daf-18*(+) activity in defined embryonic founder cells and their descendants (Figure 2 and STAR Methods). SGB progression was assessed using *arIs51[cdh-3::gfp]*, which expands from the AC to the utse near the end of the second phase of gonadogenesis (see Figure 1), and GSC progression was assessed by anatomy (STAR Methods). This analysis (Figure 2A) revealed that progression of both somatic gonad and germline development was blocked in *daf-18*(0) mosaic dauers that retained the array in the MS lineage, which gives rise to the somatic gonad, even when lineages that generate the germline, intestine, hypodermis, and most of the nervous system lacked the array [and therefore lacked *daf-18* activity]. Conversely, *daf-18*(0) mosaic dauers lacking the array in the MS lineage displayed progression of somatic gonad and germline development even when the lineages forming the germline, intestine, nervous system, and hypodermis retained *daf-18*(+). These results suggested that *daf-18* acts in the somatic gonad to maintain both SGB and GSC quiescence.
We tested this inference by restoring DAF-18 to the somatic gonad primordium using the ckb-3 promoter (Kroetz & Zarkower, 2015). In dauer larvae, ckb-3p::mCherry-histone is seen in all cells of the somatic gonad primordium and in only three other cells, tentatively identified as MS-derived pharyngeal muscle segment pm6 of the pharynx (STAR Methods). Three independent extrachromosomal arrays and a single-copy insertion transgene of ckb-3p::daf-18(+) restored SGB and GSC quiescence to daf-18(0) dauers (Figure 2B; Figure S2A), consistent with daf-18 function in the somatic gonad to prevent both somatic gonad and germline developmental progression. Additional tissue-specific rescue experiments supported the conclusion that the presence of daf-18 activity in the intestine or nervous system is not sufficient to maintain SGB and GSC quiescence (Figure S2B).

daf-18 has a diffuse cellular focus within the somatic gonad

The above analysis indicated that daf-18 acts in the somatic gonad to maintain GSC quiescence non-autonomously. Here, we ask if there is a specific cellular focus within the somatic gonad for maintaining GSC and SGB quiescence.

In each arm, the terminally-differentiated DTC forms a niche for the GSCs, and in the L4 stage of continuous development, responds to nutritional signals to regulate the number of germ cells (Pekar et al., 2017). Therefore, it seemed plausible that daf-18 might act in dauer DTCs to regulate GSC quiescence non-autonomously, but act autonomously within the SGBs to regulate their quiescence. Remarkably, expression of DAF-18 specifically in DTCs of daf-18(0) dauers restores both GSC and SGB quiescence (Figure 2B,C). Thus, daf-18 activity in the DTCs is sufficient to maintain quiescence non-autonomously of both SGBs and GSCs. However, when we created a "floxed" allele, daf-18 [loxP>daf-18(+)>loxP], and generated "excision mosaics" by expressing Cre recombinase specifically in the DTCs (Hoier, Mohler, Kim, & Hajnal, 2000; Kage-Nakada et al., 2014; Ruijtenberg & van den Heuvel, 2015) (Figure 3A), we found that
both SGBs and GSCs remained quiescent, suggesting that the DTCs are not the sole cellular focus in the somatic gonad for maintaining blast cell quiescence (Figure 3B).

We supported this conclusion by genetically ablating the DTCs (along with the AC) using *hlh-2(RNAi)* (Karp & Greenwald, 2003, 2004) in *daf-18(+) and daf-18(0)* backgrounds. The absence of DTCs did not abrogate quiescence of the SGBs in *daf-18(+) dauers* (Figure S3A) and did not restore quiescence to the SGBs *daf-18(0) dauers* (Figure S3B). GSC quiescence or progression could not be evaluated, as DTCs are required for continued GSC divisions (Kimble, 1981). These observations also suggest that the AC, the only other terminally differentiated cell present in the somatic gonad primordium, is not the sole redundant cellular focus along with the DTCs.

Since *daf-18* expression in the somatic gonad or in the DTCs is sufficient to rescue *daf-18(0)*, but *daf-18* activity in the DTCs is not necessary, we conclude that there is a diffuse cellular focus within the somatic gonad. Consistent with this view, excision mosaics lacking *daf-18* activity in all somatic gonad cells causes both SGBs and GSCs to lose quiescence (Figure 3). In addition, the excision mosaics express GFP-H2B under the control of the *daf-18* promoter, indicating that *daf-18* normally is expressed in all somatic gonad cells in dauer. Finally, as the transgene fully rescues *daf-18(0)*, and the somatic gonad excision mosaics that display progression retain the intact *loxP>daf-18(+)>loxP* transgene in the germ line (STAR Methods), our observations support a somatic gonad focus for *daf-18* rather than in the germ line in maintaining GSC quiescence in dauer.

**Loss of *daf-18* permits SGB and GSC progression in other dauer-constitutive mutant backgrounds**

To test if the effects described above depend on the *daf-7* dauer-constitutive mutant background, we also tested the effect of *daf-18(0)* in dauer-constitutive mutant backgrounds that compromise one of the other major signaling pathways that regulate dauer entry, Insulin/Insulin-
like Growth Factor signaling (IIS) or steroid hormone receptor signaling (Antebi, 2013; Murphy & Hu, 2013). Our analysis indicates that the requirement for *daf-18* to maintain SGB and GSC quiescence in dauer is not a special property of the *daf-7* background, and each also has additional implications.

In the presence of food, agonistic insulin-like peptides activate the sole Insulin Receptor (InsR) ortholog DAF-2 to promote continuous development (Hu, 2007; Murphy & Hu, 2013). In *C. elegans*, there are about 40 genes encoding insulin-like peptides, some agonistic and others antagonistic (Fernandes de Abreu et al., 2014; Pierce et al., 2001; Zheng et al., 2018). Thus, a potential mechanism by which *daf-18* may promote quiescence would be to promote the expression or activity of an antagonistic insulin in the somatic gonad primordium. This role, or any other role for IIS in this process, could be revealed if loss of *daf-2* prevented developmental progression in *daf-18*(0). We used the reference allele *daf-2(e1370ts)* to drive dauer formation at the restrictive temperature, and as *daf-18*(0) suppresses dauer formation of *daf-2(e1370ts)*, we created genetic mosaics for *daf-18*(0) by loss of *arEx2399[daf-18(+)]*. In mosaics retaining *arEx2399[daf-18(+)]* in the MS lineage, both SGBs and GSCs remained quiescent; in mosaics lacking *arEx2399[daf-18(+)]* in the MS lineage, both SGBs and GSCs developmentally progressed (Figure 4A; Figure S4). This result indicates that *daf-18* activity in the somatic gonad maintains SGB and GSC quiescence, i.e. the cellular focus for this activity in the somatic gonad are not unique to *daf-7; daf-18*(0) dauers. These results also indicate that the inferred pro-quiescence somatic gonad endocrine signal regulated by *daf-18* is not likely to be an inhibitory insulin-like peptide.

Steroids called Dafachronic Acids (DAs) are essential ligands for the nuclear hormone receptor DAF-12, which promotes continuous development in the presence of DA and dauer development in the absence of DA (Antebi, 2013). The production of DAs requires the steroidogenic hydroxylase DAF-9, which acts downstream of both IIS and DAF-7/TGFβ signaling in regulating the dauer entry decision (Figure 4A) (Gerisch & Antebi, 2004; Jia, Albert,
& Riddle, 2002; Ludewig et al., 2004). *daf-9(0)* mutants constitutively become dauer larvae, and *daf-18(0); daf-9(0)* dauers showed significantly increased SGB and GSC progression compared to *daf-9(0)* dauers (Figure 4A), indicating that developmental progression in the absence of DAF-18 does not absolutely depend on DAs. Curiously, we observed that a significant number of *daf-18(0); daf-9(0)* dauers displayed GSC progression without concomitant SGB progression (Figure 4A), an uncoupling of these processes we have not observed in other *daf-18(0)* dauer larvae.

The somatic gonad does not regulate sex myoblast quiescence in dauer larvae

Like the SGBs of the somatic gonad, the sex myoblasts (SMs) are mesodermal in origin. The SMs are born in the L1 stage and remain quiescent in the L2 stage, during which time they migrate so as to align with the center of the proximal gonad, and re-enter the cell cycle in the L3 stage (Sulston & Horvitz, 1977). This migration is guided by an FGF-like signal from the somatic gonad (Burdine, Branda, & Stern, 1998). We marked SMs using *arT133[hlh-8p::gfp]* (STAR Methods) and observed that in *daf-7* control dauers, the SMs remain quiescent in their normal positions adjacent to the proximal gonad; in contrast, in *daf-18(0)* dauers, the SMs generate multiple descendants, indicating that they have lost quiescence (Figure 4B and STAR Methods). To test if the *daf-18*-regulated endocrine signal produced by somatic gonadal cells also regulates SM quiescence, we restored quiescence to the SGBs and GSCs by expressing *daf-18(+) in the somatic gonad cells in a *daf-18(0)* mutant background. Nevertheless, we observed numerous marked SM descendants, indicating that SM quiescence was not restored (Figure 4B), and suggesting the possibility that the pro-quiescence endocrine signal produced in the somatic gonad primordium may not be freely secreted or is unable to cross the basement membrane barrier that surrounds the entire gonad.
Post-dauer defects in \textit{daf-18} somatic gonad excision mosaics reveal the importance of maintaining developmental quiescence in dauer

To determine the impact of inappropriate developmental progression due to the absence of \textit{daf-18} activity in the somatic gonad on post-dauer reproductive system development, we examined individual excision mosaic dauers in which a Cre driver induced excision of $\textit{loxP}\textit{daf-18(+)\textit{loxP}}$ in somatic gonad cells and control dauers (no Cre driver) after recovery at 15°C (STAR Methods). In recovered control dauers, 16/16 post-dauer adults were fertile and 15/16 laid eggs normally. In contrast, 12/14 excision mosaics lacking \textit{daf-18} in the somatic gonad were overtly abnormal: 9/14 were sterile (**$p < 0.001$ vs control), and 3/14 were fertile but egg-laying defective. Thus, the inability to maintain developmental quiescence in dauer has adverse consequences for fertility post-dauer, underscoring the importance of the as-yet unknown endocrine signal in dauer life history.

Discussion

Our finding that \textit{daf-18}/PTEN acts non-autonomously within the somatic gonad primordium to regulate the decision of both SGBs and GSCs to be quiescent or to resume development implicates the somatic gonad as an endocrine organ in dauer larvae, with somatic gonadal cells signaling both inter se and to the germline to regulate developmental progression. In principle, \textit{daf-18} activity could normally be required for somatic gonad cells to produce a “pro-quiescence” signal, or loss of \textit{daf-18} could lead to ectopic production of a “pro-progression” signal. Because expression of \textit{daf-18(+) in DTCs} is sufficient to restore quiescence to the SGBs and GSCs even while the remainder of the somatic gonad and other tissues are \textit{daf-18(0)}, we favor the interpretation that \textit{daf-18} activity in dauer normally promotes the production or activity of a single pro-quiescence signal, or discrete ones to the SGBs vs. GSCs, from somatic gonad cells (Figure 4C).
In many situations, PTEN acts autonomously, e.g. to regulate cell size in the Drosophila ommatidium (Huang et al., 1999) and mammalian stem cell quiescence [e.g. (Huang et al., 1999; Kwon et al., 2001; G. Li et al., 2002; Porter et al., 2016; Yilmaz et al., 2006)]. Curiously, in mammals, one PTEN isoform has a poly-arginine stretch that mediates its secretion (Hopkins et al., 2013) and PTEN has been observed in extracellular vesicles (Putz et al., 2012); although DAF-18 does not have any poly-arginine regions, extracellular vesicles have been observed in C. elegans [reviewed in (Beer & Wehman, 2017)] raising the possibility that DAF-18 movement per se could explain the apparent non-autonomy we observed. However, even if there is any such movement of DAF-18, it must be highly constrained or specifically directed, because retention of daf-18(+) in the germline while the somatic gonad is daf-18(0) does not rescue either GSC or SGB quiescence, and expression of daf-18(+) in the somatic gonad restores quiescence to SGBs and GSCs but not the nearby SMs.

Indeed, our analysis indicates that any signal regulated by daf-18 is specific to gonadal cells and/or has a limited range, and in the case of the communication between somatic gonad and GSCs, directionality. Regardless of mechanism, it is apparent that coordinated development of soma and germline mediated by daf-18 is important for post-dauer fecundity. We envisage that the somatic gonad cells receive the inputs from non-gonadal soma that regulate the global dauer entry/exit decision, such that the gonadal endocrine activity/activities diagrammed in Figure 4C not only ensure that development of somatic and germline are coordinated with each other, but also with global developmental progression.

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**Author Contributions**

C.C.T. conducted the experiments; C.C.T. and I.G. designed the experiments, interpreted results, and wrote the paper.

**Declaration of Interests**

The authors declare no competing interests.
Figure Legends

Figure 1. Developmental progression of Somatic Gonad Blast cells (SGBs) and Germline Stem Cells (GSCs) in *daf-18(0)* dauers. Both control and *daf-18(0)* dauers contain *daf-7(e1372)* and markers.

(A) Gonadogenesis. In the first phase, Z1 and Z4 give rise to the twelve cells that form the somatic gonad primordium in the L2 stage: the terminally-differentiated Anchor Cell (AC) and Distal Tip Cells (DTCs), and nine SGBs, including three Ventral Uterine precursor cells (VU, blue outline) and two Sheath-Spermathecal precursors (orange outline). The germline precursors Z2 and Z3 generate GSCs that are segregated into gonad arms when the somatic primordium forms. In the second phase, beginning in the L3 stage of continuous development, the SGBs divide; after two rounds of SGB division, the AC induces certain VU descendants to adopt the π cell fate. In the L4 stage, some π cell daughters fuse with the AC to form the multinucleate Uterine Seam cell (utse, represented as green structure) and there are 10 pairs of sheath cells (represented by orange ovals). In control dauer larvae, gonadogenesis is suspended at the end of the first phase, and progression in *daf-18(0)* dauer larvae is evident because markers characteristic of π cells, the utse, and sheath cells are expressed (see D).

(B) Developmental progression of the SGBs and GSCs in *daf-18(0)* dauers: increased cell number. Top, a control dauer showing developmental arrest of the somatic primordium and germ line, with the entire gonad visible in the photomicrograph; bottom, a *daf-18(0)* dauer at the same magnification, with only the posterior arm and proximal gonad shown at the same magnification. All somatic gonadal cells are marked by *arTi112[ckb-3p::mCherry-H2B]*, which is more highly expressed in Z1 and Z4 and becomes progressively weaker as the lineages progress; nevertheless, an increase from 12 cells of the somatic primordium in control dauers (n = 10/10) to ≥20 cells in *daf-18(0)* dauers is evident (n = 10/11). The proliferation of GSCs, which are not marked, has been quantified previously (Narbonne & Roy, 2006) and is evident in
the overt expansion in the width (double-headed arrow, maximum width of representative control arm) and/or extension of the gonad arms and in an increase in the number of germline nuclei evident by Nomarski Differential Interference Contrast microscopy (DIC). Representative pictures are orthogonal projections of Z-stacks of mCherry fluorescence collected on a spinning disk confocal (STAR Methods).

(C) Utse formation in daf-18(0) dauers. Top, arls51[cdh-3::gfp] is expressed only in the AC of a control dauer; bottom, expansion of cdh-3::gfp indicative of utse formation in a daf-18(0) dauer. Germline expansion in the daf-18(0) dauer is again evident in the greater width of the gonad arm compared to the control dauer [double-headed arrow, see above]. Strain is GS8024 daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]. Images were collected on a compound light microscope (STAR Methods).

(D) Quantification of markers indicating SGB progression. See 1B for arTi112[ckb-3p::mCherry-H2B] and 1C for arls51[cdh-3::gfp]. syIs80[lin-11::gfp] and kuIs29[egl-13::gfp] mark π cells and their daughters, descendants of the VUs. tnIs6[lim-7::gfp] marks the descendants of the sheath-spermathecal precursor (SS). Transgene details and scoring criteria are given in STAR Methods. n=20-57. (***) P < 0.001, (**) P < 0.01, (*) P < 0.05 by two-tailed Fisher's exact test.

Figure 2. daf-18 acts in the somatic gonad primordium to maintain SGB and GSC quiescence in dauer. Both control dauers and daf-18(0) dauers contain daf-7(e1372) and markers.

(A) Genetic mosaic analysis. Genetic mosaic dauers lacking daf-18(+) activity in defined lineages were isolated from strain GS8095 daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]; arEx2399[daf-18(+)] (see STAR Methods for details). arEx2399 includes fluorescent markers that are visible in E (intestinal) derivatives and body wall muscles. Each circle represents an individual with a simple loss of the array in one lineage; a square represents a compound loss.
Red, SGB and GSC progression was observed when the array was lost in lineages leading to the somatic gonad; white, losses in which both SGBs and GSCs remained quiescent, including losses in lineages giving rise to the germ line. Note that all eight losses in the MS lineage retained the array in the E lineage, all simple losses in the MS lineage retained the array in all other lineages, and tissue-specific rescue experiments in Figure S2B provide additional evidence against a cellular focus in the intestine or neurons.

SGB progression was scored as expanded cdh-3::gfp expression indicative of utse formation (see Fig. 1C); GSC progression was scored by DIC. In 23/24 mosaics, the SGBs and GSCs either progressed simultaneously or maintained quiescence.

**(B) Tissue-specific expression of daf-18(+) in the somatic gonad or DTCs rescues SGB and GSC quiescence in dauer.** SGB progression was scored by expanded arls51[cdh-3::gfp] expression indicative of utse formation, and GSC progression was scored by DIC (see Fig. 1C and STAR Methods). A representative transgene expressing in all somatic gonad cells (arTi195, ckb-3p::daf-18(+):T2A::tagBFP2; purple) or DTCs only (arTi118, hlh-12p::daf-18(+):T2A::tagBFP2; red) is shown; data for additional, independent transgenes with these constructs are shown in Figure S2A. (***) P < 0.001 by two-tailed Fisher’s exact test.

**(C) Photomicrograph showing DTC-specific expression of daf-18(+) rescues SGB and GSC progression in daf-18(0) dauers.** This individual is of genotype daf-18(0); daf-7; arEx2416[hlh-12p:S::daf-18::T2A::tagBFP2::unc-54 3’UTR]; arls51[cdh-3p::gfp], with tagBFP2 (produced from a bicistronic message due to the T2A linker (Ahier & Jarriault, 2014)) marking the DTCs (see STAR Methods). Gonad development has not progressed, as is evident from the presence of an AC expressing arls51[cdh-3::gfp] in the proximal gonad rather than expanded expression associated with a utse, and the small size of the gonad arms (compare with Figure 1).
Figure 3. *daf-18* has a diffuse cellular focus within the somatic gonad. All dauers contain *daf-7(e1372); daf-18(ok480)*, markers, excisable *daf-18(+)*, and Cre drivers.

(A) Strategy to generate excision mosaics. The conditional allele arTi179, *daf-18 [loxP>daf-18>loxP]*, restores normal dauer development in a *daf-18(0); daf-7* background. Tissue-specific expression of Cre recombinase using the single-copy insertion drivers shown (box) generates excision mosaics in which *daf-18(+) is removed from the DTCs [hlh-12p], all somatic gonad cells [ckb-3p], or variably in all/most or no somatic gonad cells [hlh-1p] because of transient expression in MS granddaughters (Krause, Harrison, Xu, Chen, & Fire, 1994; Murray et al., 2008). None of the Cre drivers used caused excision in the germline. For more details of excision patterns observed with these drivers, see STAR Methods. *daf-18(0) excision mosaics were obtained using a cross strategy (box) that (i) eliminated potential maternal contribution of *daf-18(+) by providing the DAF-18-expressing *loxP>daf-18(+)>loxP* floxed locus through the male and (ii) required a single excision event involving the *daf-18 [loxP>daf-18>loxP]* locus present in F1 heterozygous progeny, so that all GFP-positive DTCs lacked *daf-18(+) activity. For further details about generation of transgenes and additional observations about the full effects of the drivers used see STAR Methods.

(B) SGB and GSC progression in excision mosaics. The excision pattern induced by each driver shown was confirmed by both GFP expression from the excision event involving the single *daf-18 [loxP>daf-18>loxP]* locus (see above) in the mosaics scored and analysis of *hels105*, an established recombination reporter (Ruijtenberg & van den Heuvel, 2015) (STAR Methods). SGB, somatic gonad blast cell; DTC, distal tip cell; GSC, germline stem cell. SGB division progression was inferred by substantially increased number of cells expressing *ckb-3p::mCherry::H2B* and concomitant expansion by DIC, and germline divisions were scored by DIC (see Fig. 1B,C). (***) *P* ≤ 0.001 by two-tailed Fisher’s exact test.

Figure 4. Evidence for a novel signal regulated by *daf-18/PTEN* and model
(A) Effect of \textit{daf-18(0)} in dauers formed by \textit{daf-c} mutations affecting IIS or NHR signaling.

SGB progression was inferred in all dauer larvae based on expanded \textit{arls51[cdh-3::gfp]} expression indicative of utse formation and overall anatomy. GSC progression was inferred based on overtly increased size and GSC number in the gonad arms (STAR Methods).

To evaluate IIS, we tested whether SGBs and GSCs progress in \textit{daf-18(0)} dauers with the canonical loss of function mutation in \textit{daf-2\textbackslash IR}, \textit{daf-2(e1370)}. Since \textit{daf-18(0)} suppresses constitutive dauer formation caused by \textit{daf-2(e1370)}, we created \textit{daf-18(0)} mosaic dauers lacking \textit{daf-18(+)} in the MS lineage. SGB and concomitant GSC progression was observed in \textit{daf-18(0)} dauers lacking DAF-18(+) in the MS lineage, and was not observed in dauers retaining DAF-18(+) in the MS lineage. \textit{daf-7(e1372)} and \textit{daf-2(e1370)} are temperature-sensitive mutants that form dauers constitutively at 25°C.

The null allele \textit{daf-9(dh6)} was used to prevent the production of DA ligands for DAF-12/NHR, keeping it in the unliganded state the promotes dauer development. Because \textit{daf-9(dh6)} forms dauers constitutively, strains were maintained using a rescuing extrachromosomal array, which is lost meiotically to create homozygous \textit{daf-9(0)} non-mosaic dauers (Gerisch, Weitzel, Kober-Eisermann, Rottiers, & Antebi, 2001) (STAR Methods). We note also that, in \textit{daf-18(0); daf-9(0)} dauers, significantly more GSC progression was observed than SGB progression (P < 0.01).

\(***\) P ≤ 0.001, (**) P ≤ 0.01 by two-tailed Fisher’s exact test.

(B) Tissue-specific rescue of somatic gonad quiescence does not restore SM quiescence to \textit{daf-18(0)} dauers. Top, \textit{daf-7(e1372)} “control dauer” with a quiescent gonad, showing \textit{cdh-3::gfp} expression in the AC and the two SMs, labeled with \textit{hlh-8p::mCherry}. Bottom, “Rescued gonad” in which \textit{ckb-3p::daf-18(+)} restores quiescence to the somatic gonad and GSCs (see also Figure 2B) without restoring quiescence to SMs, as evidenced by multiple SM descendants labeled with \textit{hlh-8p::mCherry}. For detailed genotypes see Methods. (*** P ≤ 0.001 by two-tailed Fisher’s exact test compared with \textit{daf-7(e1372)}.}
Model for daf-18 function in regulating quiescence of GSCs and SGBs in dauer. Our analysis indicates that loss of daf-18 in the somatic cells of the gonad leads to concomitant loss of both SGB quiescence and GSC quiescence. We therefore infer that daf-18(+) activity in somatic gonad cells regulates the activity or production of a signal or signals to the germline and inter se to promote quiescence. We do not know if there is a single pro-quiescence signal that acts on both tissues or if there are distinct signals. Since restoration of daf-18(+) specifically to the somatic gonad rescues both somatic gonad and germline quiescence, a simple model is that there is a single signal that coordinates developmental progression of both tissues.

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Iva Greenwald (isg4@columbia.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans strains and transgenes

See Key Resources Table for the full list of strains. Strains carrying temperature-sensitive dauer-constitutive mutations were maintained at 15-20°C and shifted to 25°C as described in the Method Details. The dauer-defective mutations daf-16(mgDf50) I [daf-16(0)] and daf-18(ok480) IV [daf-18(0)] and the dauer-constitutive mutations daf-7(e1372) III, daf-2(e1370) III, daf-9(dh6) X [daf-9(0)] are described in WormBase.

The following transgenes were used to mark cells of the somatic gonad primordium and to ascertain their developmental arrest or progression:

arTi112[ckb-3p::mCherry-H2B] is a single-copy insertion transgene generated with the
assistance of Justin Shaffer that marks all cells of the somatic gonad primordium. As in all other constructs with the ckb-3 promoter, a synthetic intron “S” was included to increase expression efficiency [see below; (Fire, Harrison, & Dixon, 1990; Kozak, 1986)] The ckb-3p (Kroetz & Zarkower, 2015) drives highest expression in Z1 and Z4 and becomes progressively weaker as the lineages progress.

arls51[cdh-3p::gfp] (Karp & Greenwald, 2003) is expressed in the AC in the L2 and L3 stages of continuous development and in wild-type starved, daf-7(e1372), daf-2(e1370) and daf-9(dh6) dauers. GFP expands to the utse in the early L4 stage of continuous development (Ghosh & Sternberg, 2014) and in daf-18(0) dauers as indicated.

syls80[lin-11::gfp] (Gupta & Sternberg, 2002) and kuls29[egl-13::gfp] (Hanna-Rose & Han, 1999) mark π cells and their daughters.

tns6[lim-7::gfp] marks the descendants of the sheath-spermathecal precursor (SS) (Voutev, Keating, Hubbard, & Vallier, 2009).

arTi133[hlh-8p::ERK-KTRmClover::T2A::mCherry::H2B], designated arTi133[hlh-8p::mCherry::H2B] in the text, is expressed in the SMs and their descendants (de la Cova & Greenwald, 2012), and is expressed in the two SMs in dauer.

Additional transgenes generated during the course of this study for mosaic analysis and tissue-specific rescue experiments are described in the Method Details below.

METHOD DETAILS

Identifying and scoring dauer larvae

Unless otherwise specified, dauer larvae carrying dauer-constitutive (daf-c) mutations [daf-7, daf-2, or daf-9] were obtained by treating gravid hermaphrodites with bleach to release eggs using a standard protocol (Stiernagle, 2006). The eggs were incubated at 25°C on standard NGM plates containing E. coli (Brenner, 1974). When scored approximately 72 hours later,
dauers have been arrested for at least 24 hours; these are designated “1-day dauers.” When scored approximately 96 hours after eggs are shifted to 25°C, dauers with daf-c alleles have been in dauer for at least 48 hours; these are designated 2-day dauers. When scoring double mutant dauers carrying daf-c and dauer-defective [daf-d] mutations [daf-18(0) or daf-16(0)], the daf-c controls were set up and scored in parallel to daf-c; daf-d strains.

Dauer larvae induced using daf-c mutations were identified by SDS selection: incubation in 1% SDS for at least 10 minutes kills all non-dauer stages except for eggs, from which dauer larvae can easily be distinguished (Cassada & Russell, 1975; Karp, 2018). Dauers induced by starvation were identified based on dauer morphology: pharyngeal constriction, radial constriction, and the presence of dauer alae.

**Imaging**

For all scoring and imaging, larvae were mounted on agarose pads and immobilized in 10 mM levamisole. To characterize arTI112[ckb-3p::mCherry::H2B] in daf-18(0) dauers (Figure 1B, 1D), mCherry-expressing cells were counted and imaged by collecting Z-stacks of mCherry fluorescence with a Zeiss spinning disk confocal dual camera system. For all other experiments, larvae were scored and/or imaged with a 40x Plan-Neo or a 63x Plan-Apo objective, either on a Zeiss Axio Imager Z1 microscope with a Hamamatsu Orca-ER camera or a Zeiss Axio Imager D1 microscope with an AxioCam MRm. An X-Cite 120Q light source from EXFO photonics solutions was used for illumination.

We note that for strains in which both tagBFP2 and GFP were assessed, for illuminating tagBFP2, we used a filter that did not admit GFP from transgene markers (excitation 379-401nm, beamsplitter 420nm, emission 435-485nm) and for illuminating GFP, we used a filter that did not admit tagBFP2 (excitation 450-490nm, beamsplitter 495nm, emission 500-550nm).
Evaluating SGB progression using fluorescent markers

*arls51[cdh-3::gfp]*: characterized in *daf-18(0)* dauers (Figure 1) and used to assess SGB progression in all mosaic analysis (Figures 2, 4 and Supplemental Figure 4), tissue-specific rescue (Figure 3, Supplemental Figure 3), alternative *daf-c* backgrounds (Figure 4 and Supplemental Figure 4), and *L2d/L2d-Dauer molt/Dauer timing experiments* (Supplemental Figure 1).

In continuous development, *arls51[cdh-3::gfp]* is expressed in the AC of the somatic primordium in the L2 stage, and remains restricted to the AC until it expands to multiple cells of the utse in the L4 stage. Dauer SGBs were considered to have progressed if they had expanded expression of GFP in multiple cells confirmed by DIC microscopy. Details on scoring *arls51[cdh-3::gfp]* in *L2d/L2d-Dauer molt/Dauer timing experiments* are described in Figure S1.

Control dauers for *daf-18* experiments are from strain GS8052 *daf-7(e1372); arls51[cdh-3::gfp]* unless otherwise specified. *daf-18(0)* dauers are from strain GS8925 *daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]* unless otherwise specified.

*arTi112[ckb-3p::mCherry::H2B]*: normally, mCherry-H2B is evident in the 12 cells of the wild-type or *daf-c* mutant dauer somatic gonad primordium; dauers with expression in >20 cells were considered to have had SGB division, indicative of developmental progression.

*syIs80[lin-11::gfp]* and *kuIs29[egl-13::gfp]*: These markers are not expressed in the somatic primordium in wild-type or *daf-c* dauers (see above); any marker expression in multiple cells of the somatic gonad indicates SGB progression.

*tnIs6[lim-7::gfp]*: This marker is expressed broadly in sheath cells and their extensions that surround the germ cell nuclei (see above), so Sheath-Spermathecal (SS) blast cell progression was inferred from any expanded expression consistent with sheath cell morphology.
Scoring GSC quiescence and progression in *daf-7(e1372)* or *daf-2(e1370)* dauers

GSC quiescence and progression had been extensively characterized in *daf-7(e1372)* and *daf-2(e1370)* control dauers, as well as *daf-18(0)* or *daf-18* partial loss of function mutant dauers (Narbonne & Roy, 2006). In these studies, precise counts of the number of GSCs revealed that the increase in numbers were accompanied by a corresponding overt increase in germline size by anatomy, indicating that anatomical enlargement of the gonad arms was a valid metric for GSC progression.

In control dauers, we observed that gonad arms arrested with a consistent width, length, extension, and overall size, containing approximately 30 GSCs total as previously reported (Figure 1B, C; (Narbonne & Roy, 2006)). In contrast, in most *daf-18(0)* dauers, one or both gonad arms appeared substantially enlarged, displaying increased width and a large number of GSCs as assessed by microscopy. GSC progression was frequently indicated by dramatic extension of the germline arms that could reach the pharynx or tail of the dauers; such extension was also accompanied by increased GSC numbers in *daf-18(0)* dauers as previously reported (Narbonne & Roy, 2006).

Gonad size was scored categorically as no/mild increase (no progression, approximately \( \leq 50 \, \mu m \) extension and \( \leq 11 \, \mu m \) width when lateral), or as having an overt increase (approximately \( \geq 80 \, \mu m \) extension or \( \geq 14 \, \mu m \) width when lateral) compared to controls. We conservatively considered dauers with a mild increase as not having GSC progression to guard against any potential distortion due to *daf-18(0)* defects in radial constriction. In dauers with moderate or extreme enlargement, evident by increased width or extension to an extent never observed in control dauers, GSCs were considered to have progressed. See Figure 1 for examples of dauers with moderate (Figure 1C) and extreme (Figure 1B) increases in germline width and length.
Scoring GSC quiescence and progression in daf-9(dh6) dauers

The *daf-9* null allele, *daf-9*(dh6), prevents DA production and results in constitutive dauer formation (Gerisch & Antebi, 2004). Strains containing *daf-9(0)* were maintained with a rescuing extrachromosomal array, and meiotic loss of the array in the parent germline led to segregation of homozygous *daf-9*(0) dauers (Gerisch et al., 2001).

*daf-18*(0); *daf-9*(0) dauers were scored categorically as no/mild increase (extension of 70-80 \( \mu \)m, no progression), or as having an overt increase (extension over 90 \( \mu \)m) compared to *daf-9*(0) control dauers. We note that *daf-9*(0) dauers arrest with more GSCs than *daf-7*(e1372) or *daf-2*(e1370) dauers; however, there is no discernable difference germline size, extension, or width between early dauer arrest (<12 hours in dauer, n=11, scored approximately 48 hours after eggs were shifted to 25\(^\circ\)) and 1-day dauers (n=20, scored 72 hours after the shift), indicating that there is no further progression of germline development in the *daf-9*(0) control dauers.

*daf-18* mosaic analysis for cellular focus

Strain GS8095 [*daf-7*(e1372); *daf-18*(ok480) arls51[cdh-3::gfp]; arEx2399[*daf-18(+)*]] was used for genetic mosaic analysis shown in Fig. 2A. Non-mosaic individuals were fully rescued for *daf-18* defects, i.e. they formed dauer larvae that displayed radial body constriction, pharyngeal constriction, and somatic gonad and germline quiescence (n=10).

**Generation of arEx2399[*daf-18(+)*] for mosaic analysis.** arEx2399 is a simple extrachromosomal array. *daf-18(+) was provided by a PCR product amplified from N2 genomic DNA, including the entirety of the 5’ UTR and 3’ UTR up to the coding regions of the genes immediately upstream and downstream of *daf-18* (IV:419,952 to 426,169, a total of 6218 base pairs; Wormbase release number WS267). The PCR product (25 ng/\( \mu \)l) was coinjected with
pDS266(glo-1p::tagRFP, 25 ng/µl, kindly provided by Dr. Daniel Shaye), pMS102 [lag-2p(FL)::2xnls::tagRFP, 25 ng/µl; (Sallee & Greenwald, 2015)], and p716(myo-3p::mCherry, 25 ng/µl) (J. Li & Greenwald, 2010) into the germline of N2 hermaphrodites. The injected hermaphrodites were placed at 15°C, and 7 days later, individual descendants were isolated from each injected parent and examined for potential array-carrying progeny based on expression of the myo-3p::mCherry and glo-1p::tagRFP coinjection markers. The lag-2p(FL)::2xnls::tagRFP could not be visualized in the expected tissues (neurons and somatic gonad), likely due to the greater relative brightness of myo-3p::mCherry. The array arEx2399 was selected to generate strain GS8095.

**Generation of daf-18(0) mosaic dauers.** To generate mosaics, we relied on random mitotic loss of arEx2399, an extrachromosomal array carrying daf-18(+) in strain GS8095. Such “daf-18(0) mosaic dauers” lack daf-18(+) activity in different embryonic founder cells and their descendants, with the losses inferred by fluorescent markers also carried on arEx2399[daf-18(+)]: myo-3p::mCherry, expressed in all body wall muscles (Okkema, Harrison, Plunger, Aryana, & Fire, 1993) (with defined muscle cells descending from the founder cells ABp, MS, C and D) and glo-1p::tagRFP, expressed in the intestine (Hermann et al., 2005), which is derived solely from E (Sulston, Schierenberg, White, & Thomson, 1983). The germline derives solely from the founder cell P₄; an array loss in P₂ would mean absence of the array in the germline precursor P₄, and a P₂ loss was inferred based on absence of the array in C- and D-derived body wall muscle cells (see Figure 2). Such P₂ losses give confidence that the lack of daf-18 activity does not abrogate GSC quiescence. Conversely, retention of the array in C and D implies likely retention in P₄ and therefore that the arEx2399[daf-18(+)] array was present in the GSCs.

Mosaic dauers were obtained by allowing 40 GS8095 daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]; arEx2399[daf-18(+)] gravid adult hermaphrodites to lay eggs at 25°C for 7-17 hours, and scored after 1-2 days in dauer (72-89 hours after egg lay). Dauer arrest was
confirmed in all mosaics scored by SDS selection (see above). Additional mosaics were identified as L1 larvae to facilitate recognition of myo-3p::mCherry expression patterns resulting from particular lineage losses. Somatic gonadal progression was assessed by arls51[cdh-3::gfp] expression and germline progression by anatomy.

**daf-18 tissue-specific rescue experiments**

We used tissue-specific promoters to drive daf-18(+) as part of our analysis of the cellular focus for daf-18 activity. The promoters ckb-3p (Kroetz & Zarkower, 2015) and hlh-12p (Tamai & Nishiwaki, 2007) have been described in continuous development; we corroborated that they have similar expression in dauer (see below).

The rescuing transgenes were generated in the form promoter::S::daf-18::T2A::tagBFP2: the viral T2A peptide triggers “ribosomal pausing” such that a single transcript produces two independent proteins (here DAF-18 and TagBFP2) (Ahier & Jarriault, 2014), the synthetic intron “S” increases expression efficiency [see below; (Fire et al., 1990; Kozak, 1986)] and the unc-54 3’UTR was used as a neutral 3’UTR (Hunt-Newbury et al., 2007; Merritt, Rasoloson, Ko, & Seydoux, 2008). Rescue was assessed in strains of genotype transgene; daf-7(e1372); daf-18(ok480) arIs51[cdh-3::gfp].

For each promoter, a single-copy insertion transgene is shown in Fig. 2A. These were generated and mapped using the standard miniMos protocol (Frokjaer-Jensen et al., 2014).

Extrachromosomal arrays were also generated with these constructs and the rescue data are shown in Supplemental Figure S2A. Complex arrays were generated by injecting Spel-digested pCT17(hlh-12p::S::daf-18::T2A::tagBFP2) or pCT19(ckb-3p::S::daf-18(+)::T2A::tagBFP2) at 2 ng/µl along with coinjection marker pCW2(ceh-22p::gfp) (expressed specifically in pharyngeal muscle) digested with Scal at 1 ng/µl and with N2 genomic DNA digested with PvuII at 50ng/µl into GS8925 daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]. F2 progeny with GFP in pharyngeal muscle were isolated to establish independent lines.
**ckb-3p-driven somatic gonad rescuing transgenes.** Single-copy insertion (Fig. 2): arTi195 [ckb-3p::daf-18::T2A::tagBFP2]. arTi195 maps to V:2.22 in an intron of the gene unc-41. Extrachromosomal arrays (Figure S2): arEx2413-arEx2415 [ckb-3p::daf-18::T2A::tagBFP2].

As it was difficult to visualize TagBFP2 from these transgenes to confirm that expression in dauer is similar to that reported in continuous development, we examined arTi112[ckb-3p::mCherry-h2b], in which H2B appears to stabilize the fluorescent protein. mCherry-H2B was observed in the somatic gonad precursors Z1 and Z4 and in all of their descendants in dauer larvae (Figure 1B) and in only three additional cells, tentatively identified as the three MS-derived pharyngeal muscle segment pm6 cells, in dauer larvae as well as in continuous development. Expression was not seen in any other cells in any other stages.

**hlh-12p-driven DTC rescuing transgenes.** hlh-12p expression is restricted to the DTCs in continuous development from L2 onwards (Tamai & Nishiwaki, 2007). We verified the specificity in dauer for arEx2416 here.

Single-copy insertion (Fig. 2): arTi118 [hlh-12p::daf-18::T2A::tagBFP2], inserted at II:-9.30 in the second exon of irld-65. tagBFP2 expression is too dim to score reliably from this transgene.

Extrachromosomal array: arEx2416 [hlh-12p::daf-18::T2A::tagBFP2] has robust tagBFP2 expression in the DTCs but was not visible in SGBs or AC in dauer (Figure 2C). tagBFP2 was visible only in the DTCs but not SGBs or the AC in 61/61 daf-18(0) dauers, and no non-gonadal expression was observed.

Expression of Cre from both the ckb-3p and hlh-12p promoters using recombination readout reporters (described below) agreed with these observations. Neither of these promoters drives germline expression in any stage, also supported by lack of Cre-mediated excision in the germline (see below).

**Extrachromosomal arrays expressing daf-18(+) in specific non-gonadal tissues.** Strains with extrachromosomal arrays expressing daf-18(+) cDNA specifically in the intestine
(veEx349[ges-1p::daf-18cDNA::myc::daf-18 3'UTR]) and neurons (veEx344[gef-1p::daf-18cDNA::myc::daf-18 3'UTR]), as well as from its native promoter (tdEx349[daf-18p::daf-18cDNA::myc::daf-18 3'UTR]), are described in (Fukuyama, Kontani, Katada, & Rougvie, 2015) and were kindly provided by Masamitsu Fukuyama and Ann Rougvie. All three arrays express functional DAF-18 based on rescue of a daf-18(0) phenotype in L1 larvae (Fukuyama et al., 2015) and, in our hands, improved non-gonadal dauer-defective phenotypes such as SDS resistance of daf-18(0) dauers. tdEx349[daf-18p] rescued all morphological defects of daf-18(0) dauer larvae (data not shown). Strains scored were of genotype Ex; daf-7(e3172); daf-18(ok480) arIs51[cdh-3::gfp]. tdEx239 may have integrated spontaneously as it segregated in our crosses in a Mendelian ratio.

**Obtaining and scoring dauer larvae for tissue-specific rescue experiments.** For arTi195 [ckb-3p::daf-18(+)] and arTi118[hlh-12p::daf-18(+)] single-copy insertion transgenes and extrachromosomal arrays veEx349, veEx344, and tdEx349, 1-day dauers were obtained and SDS-selected by the standard method described above. For other extrachromosomal arrays, 1 day dauers were obtained after an egg-lay instead of using a bleaching protocol to obtain a synchronized population, followed by SDS-selection to obtain dauers. SGB progression was assessed by arIs51[cdh-3::gfp] expression and GSC progression by microscopy as described above.

**Cre-mediated excision mosaic experiments**

**Generation of the loxP>daf-18>loxP single-copy insertion transgene arTi179.**

arTi179[daf-18p::loxP::daf-18::loxP::GFP::H2B], designated loxP>daf-18>loxP, is a single-copy insertion generated using the standard miniMos protocol (Frokjaer-Jensen et al., 2014). Insertion of arTi179 into I:4.97 in the intergenic region between F25D7.7 and mgl-2 was
identified using the standard protocol for mapping miniMos insertions (Frokjaer-Jensen et al., 2014).

arTi179 was created from plasmid pCT33. This plasmid contains the entire daf-18(+) genomic region, including introns, starting with the ATG and extending 20 base pairs into the 3’ gene eri-1, with loxP sites positioned to allow for excision of daf-18(+).

We represent the full loxP>daf-18>loxP sequence of pCT33 and arTi179 as follows: daf-18p::SbfIPacI::loxP::aa::daf-18(+)::loxP::PacI::S::GFP::his-58::unc-54 3’UTR, and contains the following elements:

- **daf-18p** is the 1021 bp 5' flanking region from the upstream gene to the ATG of daf-18.
- “aa” denotes base pairs added that, along with daf-18 native 5’ UTR, creates a Kozak sequence (Kozak, 1986).
- “**daf-18(+)**” is the genomic sequence of daf-18 including introns, starting with the ATG and extending 20 base pairs into the 3’ gene eri-1. [A one base-pair mutation within the 5th intron (a3989g) does not interfere with rescue of daf-18(0) (see below).
- “**S**” designates a synthetic intron cassette that includes common features of C. elegans introns and increases efficiency of expression (Fire et al., 1990), and the version used here also includes a Kozak sequence (Kozak, 1986).
- a **his-58** histone tag was included to improve detection of GFP fluorescence.
- the unc-54 3’UTR was used as a neutral 3’UTR (Hunt-Newbury et al., 2007; Merritt et al., 2008).

arTi179 was designed such that GFP expression is only expected with Cre-mediated recombination. GFP expression was never detected in dauer larvae except in the presence of Cre drivers, and thus tissue-specific excision of daf-18 as monitored by GFP expression was readily assessed in dauer (see below). Although effective for dauer larvae, we note that in continuous development, GFP expression cannot be used to monitor tissue-specific excision...
because there is a low-level broad GFP expression from \textit{arTi179} in all stages of continuous
development even in the absence of Cre drivers, likely due to the presence of an SL2 splice
acceptor in the \textit{daf-18} 3’ UTR as per its position as the upstream gene in an operon with \textit{eri-1}.

\textbf{Generation of single-copy insertion CRE drivers.} We used the Cre recombinase
optimized via several modifications for efficiency in \textit{C. elegans} by (Ruijtenberg & van den
Heuvel, 2015). miniMos-based single-copy insertion transgenes \textit{arTi168[hlh-12p::Cre]},
\textit{arTi236[ckb-3p::Cre]}, and \textit{arTi235[hlh-1p::Cre]} were modeled on constructs use for optimized
Cre-expressing transgenes described in (Ruijtenberg & van den Heuvel, 2015).

These driver constructs are all of the form \textit{promoter::S::Cre::tbb-2 3’UTR}, where “S”
designates a synthetic intron cassette that includes a Kozak sequence as above (Fire et al.,
1990; Kozak, 1986). Each insert was incorporated into the miniMos vector pCFJ910 (Frokjaer-
Jensen et al., 2014). \textit{arTi168[hlh-12p::CRE(opti)]} was mapped to X:24.06 in a noncoding
region. \textit{arTi179} and \textit{arTi235} were not physically mapped but insertion is inferred based
homoygosity and Mendelian behavior during strain constructions.

In pCT37, promoter = \textit{hlh-12p} and in pCT43, promoter = \textit{ckb-3p} as used for tissue-
specific rescue. In addition, pCT42, promoter = \textit{hlh-1p}, a 3130 bp region analyzed by (Krause
et al., 1994; Murray et al., 2008). Excision events caused by the Cre driver transgenes were
examined in dauer larvae via GFP expression from \textit{arTi179[loxP>daf-18>loxP]} and an
independent Cre readout reporter, \textit{hels105} (Ruijtenberg & van den Heuvel, 2015), as described
in the next section.

\textbf{Testing the Cre drivers using \textit{hels105}.} In the absence of Cre driver-induced excision,
the single-copy miniMos insertion \textit{hels105[rps-27::loxP::nls::mCherry::let-858 3’
UTR::loxP::nls::GFP::let-858 3’ UTR]} expresses mCherry ubiquitously but not GFP; upon Cre-
mediated excision, it expresses GFP but not mCherry (Ruijtenberg & van den Heuvel, 2015).

We assessed the patterns of Cre-mediated excision in the presence of our drivers using
the \textit{hels105} reporter in dauers formed by starvation at 25°C and in continuously-developing L2
larvae prepared by timed egg lay at 25°C. GFP expression resulting from the *hlh-12p* and *ckb-3p* drivers using the *hels105* reporter was broader than expected. We catalogue these differences here, but importantly, they do not change our conclusions about the cellular focus of *daf-18*. We speculate that the differences reflect the low-level, transient expression of Cre at an early point of a lineage, such that excision occurring early in a lineage as a result of transient expression of Cre is inherited in all lineal descendants even if there is no expression from the promoter in those cells thereafter, while rescue experiments likely require stronger or sustained expression at the time and place of action, as would visualization of the fluorescent reporters generally used to characterize the promoters.

*arTi168[hlh-12p::Cre]* with *hels105*: in addition to the expected DTCs, we observed GFP expression three cells in the tail, some pharyngeal muscle cells, and some cells of the excretory system. Importantly, no proximal gonad excision was observed (*n* =7 dauer, *n*=10 L2). Furthermore, ectopic Cre excision mediated by this driver does not affect the interpretation that the DTCs are not required for SGB and GSC quiescence in dauer larvae, since excision of *arTi179[loxP>daf-18>loxP]* using this driver in cells did not cause SGB and GSC progression.

*arTi236[ckb-3p::Cre]* with *hels105* (*n*=10 dauer, *n*=10 L2): in addition to the expected expression of GFP in all somatic gonad cells, we also observed expression in some cells of the body wall muscle, pharynx, and tail. This excision pattern is consistent with expression of the driver earlier than expected in the MS lineage. We also observed expression in an occasional intestinal or hypodermal cell.

*arTi235[hlh-1p::Cre] and hels105* (*n*=10 dauer, *n*=10 L2): in this case, the observed pattern of excision was as expected. We always observed expression in all body wall muscle cells, where *hlh-1* is a main muscle determinant (Fukushige, Brodigan, Schriefer, Waterston, & Krause, 2006) and in many pharyngeal cells and sometimes in the somatic gonad cells as expected from the reported transient expression in MS granddaughters (Krause et al., 1994;
Murray et al., 2008). We were able to exploit this variability for somatic gonad excision to support the conclusion that \textit{daf-18} acts in the somatic gonad (Figure 3B).

We also note that we never saw evidence for germline excision caused by these drivers. For both \textit{hels105} and \textit{arTi179 [loxP>\textit{daf-18}>loxP]}, we examined progeny of hermaphrodites for ubiquitous somatic GFP expression that would have been indicative of a rearrangement in the parent germline. For \textit{arTi179 [loxP>\textit{daf-18}>loxP]}, we also directly inspected the germline of excision mosaics for GFP expression.

**Obtaining tissue-specific excision mosaic dauer larvae.** Strains of genotype \textit{daf-7(e1372); daf-18(ok480); p::Cre} were generated for each Cre driver used. As males, and some hermaphrodites, of genotype \textit{arTi179[loxP>\textit{daf-18}(+)>loxP]; daf-7(e1372); daf-18(ok480); arTi112[ckb03p::mCherry::H2B]} constitutively become dauers even at the permissive temperature, we moved dauer larvae to fresh plates at 15°C and used the recovered, post-dauer males for the cross strategy and to maintain the strain.

The strategy shown in Figure 2 was used: \textit{daf-18(0)} males homozygous for \textit{loxP>\textit{daf-18}(+)>loxP} were mated to \textit{daf-18(0); hermaphrodites carrying a Cre driver (Figure 3A)}, ensuring that \textit{daf-18(0)} F1 hermaphrodite progeny that were scored had no maternal contribution from \textit{loxP>\textit{daf-18}(+)>loxP}. This strategy also ensured that the \textit{daf-18(0)} F1 progeny were heterozygous for \textit{loxP>\textit{daf-18}(+)>loxP}, so that only one excision event would be necessary to generate a \textit{daf-18(0)} excision mosaic; since Cre-mediated recombination of \textit{loxP>\textit{daf-18}(+)>loxP} results in \textit{\textit{daf-18}(+)>loxP::\textit{loxP::GFP::H2B}}, cellular expression of GFP-H2B in larvae heterozygous for \textit{loxP>\textit{daf-18}(+)>loxP} indicates complete excision and loss of \textit{\textit{daf-18}(+)} in that cell.

Crosses were initiated with twelve L4 \textit{loxP>\textit{daf-18}(+)>loxP; daf-18(0)} and four L4 \textit{daf-18(0); p::Cre} hermaphrodites at 20° C. The parent crosses were moved to new plates periodically over 2-3 days and each egg lay was shifted to 25°C once the parents were removed.
Scoring tissue-specific excision mosaic dauer larvae. Dauers were selected by incubation in 1% SDS and scored 65-100 hours after egg lay, which corresponds to 1-2 days after dauer formation (Karp, 2018). Only hermaphrodite dauers were scored. As per the strategy above and in Fig. 3A, expression of GFP indicates an excision mosaic lacking daf-18(+) in the GFP-expressing cell. Somatic gonad cells were marked with arTi112[ckb-3p::mCherry-H2B]. SGB progression was scored by increased numbers of cells with mCherry::H2B and confirmed by anatomy, and GSC progression was scored by anatomy. Individual observations of somatic gonad excision of loxP>daf-18(+)>loxP in dauer by driver are:

arTi236[ckb-3p::Cre]: GFP was expressed in cells of the proximal somatic gonad and the DTCs. In addition to implicating the somatic gonad as the cellular focus, the observation that the daf-18 promoter in daf-18p::loxP::gfp drives expression of GFP in the somatic gonad indicates that the daf-18 gene is expressed the dauer somatic gonad.

arTi235[hlh-1p::Cre]: Two classes of excision mosaics were identified: (1) individuals that did not express GFP in the somatic gonad even when consistent GFP was seen in the entire body wall muscle (n = 18 in Fig. 3A), and (2) individuals that expressed GFP in the somatic gonad as well as the body wall muscle (n = 7 in Fig. 3A). That excision mosaics of class 1 do not display SGB and GSC progression while excision mosaics of class 2 do strongly supports the conclusion that the somatic gonad is the cellular focus of daf-18 for maintaining quiescence of these blast cells in dauer larvae.

arTi168[hlh-12p::Cre] GFP is expressed in the DTCs but not the proximal gonad. GFP expression was observed in both DTCs in 11/13 dauers and in at least one DTC in the remaining 2 dauers, where autofluorescence may have obscured the second DTC. SGB progression was scored by increased numbers of cells with mCherry::H2B and confirmed by anatomy, and GSC progression was scored by anatomy.
Generating *daf-18(0)* mosaics in a *daf-2(e1370)* background

Strain GS9117 *daf-2(e1370); daf-18(ok480) arls51[cdh-3::gfp]; arEx2399[daf-18(+)] was used to generate dauers retaining or lacking *daf-18(0)* in the MS lineage (Fig. 4) as described above. Since *daf-18(0)* fully suppresses the dauer constitutive phenotype of *daf-2*, the ability to form dauer larvae is one indication that the array rescues *daf-18(0)*. In addition, non-mosaic individuals formed dauer larvae that displayed radial body constriction, pharyngeal constriction, and somatic gonad and germline quiescence (n=7). Mosaic dauers were obtained from 40 GS9117 *daf-2(e1370); daf-18(ok480) arls51[cdh-3::gfp]; arEx2399[daf-18(+)] gravid hermaphrodites carrying the array grown at 15°C that laid eggs at 25°C for approximately 24 hours. Dauers were scored after approximately 1-2 days in dauer (65-101 hours after eggs were laid at 25°C); selection and scoring criteria were conducted as described above for mosaic analysis of GS8095 dauers.

We scored specific lineage losses of *arEx2399* in each *daf-2(e1370); daf-18(ok480) arls51[cdh-3::gfp]* dauer and grouped simple (lost in one founder cell lineage) and complex (more than one founder cell lineage) losses based on retention or loss of *arEx2399[daf-18(+)]* in the MS lineage (Figure 4). These data are represented alongside the mosaic data from GS8095 *daf-7(e1372); daf-18(ok480) arls51[cdh-3::gfp]; arEx2399[daf-18(+)]* (see above) analyzed the same way. Specific lineage losses are shown in Figure S4. Three mosaic dauers had simple losses in P2 [germline therefore inferred to be *daf-18(0)*]: 0/3 had SGB or GSC progression. In 2/2 simple MS losses, both the SGBs and GSCs progressed. Thus, mosaic analysis of *daf-18* in both the *daf-2* and *daf-7* backgrounds indicate a cellular focus for *daf-18(0)* within the MS lineage for regulating SGB and GSC progression, indicating cell non-autonomy for GSC progression.

Scoring SM progression in *daf-18(0)* and *daf-18(+) expression mosaics
To assess SM progression in *daf-7; daf-18(0)* dauers, SM descendants were fluorescently marked with *arTi133[hlh-8p::ERK-KTRmClover::T2A::mCherry::H2B]* (abbreviated in this text as *arTi133[hlh-8p::mCherry::H2B]*, de la Cova et al. 2017). In all control dauers, the two SMs are positioned lateral to the center of the gonad and do not divide (*n=24*, Figure 4B), but the SMs progressed in 7/7 *daf-18(0)* dauers (P < 0.001), evident by 8-16 mCherry::H2B-expressing cells in two clusters lateral to the central gonad.

To assess SM progression when SGB and GSC quiescence was rescued, we examined strain GS9083 *arTi133[hlh-8p::ERK-KTRmClover::T2A::mCherry::H2B]; daf-7(e1372); daf-18(ok480) arIs51[cdh-3::gfp]; arTi195[ckb-3p::daf-18(+)::T2A::tagBFP2]*. SMs were scored as progressing if more than two mCherry-expressing cells were visible in the gonad region, and in 19/20 scored, > seven SM descendants were observed and 1/20 had 3 marked cells. In these dauers, SGB quiescence was confirmed by *arIs51[cdh-3::gfp]* expression restriction to the AC and GSC with anatomy as described above.

**Genetic ablation of AC and DTCs by *hlh-2(RNAi)***

The DTCs and AC can be genetically ablated by feeding *hlh-2(RNAi)* to L1 larvae (Karp & Greenwald, 2003, 2004) (Figure S3). The *lag-2* reporter *arls131[lag-2p::2Xnls-yfp]* (ref) marks the specified DTCs and AC, so absence of YFP in both DTCs and the AC, as well as abrogation of gonad arm extension indicate successful DTC and ATC “ablation”. SGB divisions were scored using *arTi112[ckb-3p::mCherry::H2B]* and germline progression by anatomy.

**Post-dauer phenotypes of gonad-specific excision mosaics**

After tissue-specific excision of *loxP>daf-18(+)>loxP* (Figure 4), 1-2 day dauers were obtained by SDS selection, placed on individual plates containing *E. coli*, and allowed to recover at the permissive temperature, 15° C. Post-dauer adult hermaphrodites were scored for sterility and
egg-laying defects. Sterility would be a direct effect of aberrant somatic gonad and/or germline development.

Egg-laying defects, evident by the presence of live larvae inside the adult hermaphrodite from egg retention and hatching, may result from abnormal development of the somatic gonad, vulva, vulval-uterine connection, or reproductive muscles.

QUANTIFICATION AND STATISTICAL ANALYSIS

When comparing two genotypes for the frequency of two outcomes, a two-tailed 2x2 Fisher’s exact test was used. Differences were considered significant if the p-value is less than or equal to 0.05, and specific p-values for each comparison are given in the figure legends or main text.
Chapter 2 Figures
A Gonadogenesis

Phase 1

L1
Z1 Z2 Z3 Z4

GSC

Somatic gonad

L2
GSC

DTC

Phase 2

L3
GSC

L4
utse

B SGB and GSC divisions in daf-18(0) dauers

Control, 2 arms

daf-18(0), 1 arm

10µ

Control, 2 arms

daf-18(0), 1 arm

10µ

C utse formation and GSC expansion in daf-18(0) dauers

Control, 2 arms

daf-18(0), 1 arm

utse

D Quantification of SGB progression in daf-18(0) dauers

control dauers: 0% for all markers

All somatic gonad ckb-3p::mCherry::H2B

0% 50% 100%

lin-11::gfp

egl-13::gfp

lim-7::gfp

utse cdh-3::gfp

daf-16(0) utse cdh-3::gfp

Fig. 1 Tenen and Greenwald
Figure 2

A  Mosaic analysis: inferred loss of daf-18(+) array and observed effect on SGBs and GSCs

SGBs and GSCs:
○ Progress
□ Quiescent

B  Tissue-specific expression: rescue of SGB and GSC progression in daf-18(0)

SGB  GSC

%daf-18(0) background

0%  50%  100%

no transgene

ckb-3p::daf-18

hlh-12p::daf-18

C  daf-18(+) in the DTCs maintains SGB and GSC quiescence

Figure 2
Tenen and Greenwald
Figure 3

A Excision mosaic strategy

\[ \text{loxP} > \text{daf-18(+) > loxP}: \text{ before excision, expresses DAF-18} \]

\[ \text{loxP} > \text{daf-18(+) genomic> loxP} \]

\[ \text{GFP::H2B} \star \text{ 3'UTR} \]

after excision: expresses GFP-H2B

\[ \text{loxP > daf-18(+) > loxP; daf-18(0) ♀ × daf-18(0): p::Cre ♂} \]

score excision in F1 heterozygotes

B SGB and GSC progression in daf-18 excision mosaics

<table>
<thead>
<tr>
<th>somatic gonad excision</th>
<th>n</th>
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<th>50%</th>
<th>100%</th>
</tr>
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<tr>
<td>SGB progression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>GSC progression</td>
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</tr>
<tr>
<td>none</td>
<td>21</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>only DTCs</td>
<td>13</td>
<td>hlh-12p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>18</td>
<td>hlh-1p</td>
<td></td>
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</tr>
<tr>
<td>all somatic gonad cells</td>
<td>7</td>
<td>hlh-1p</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>7</td>
<td>ckb-3p</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3
Tenen and Greenwald
Figure 4

A Alternative *dauer-constitutive* mutants

B Tissue-specific rescue of somatic gonad quiescence does not restore SM quiescence

Control gonad (quiescent SMs)  SM

ventral view  SM:eGFP::mCherry  10 µ

"Rescued gonad": daf-18(0); ckb-3p::daf-18(+)

SM descendants

daf-18(0); daf-7; ckb-3p::daf-18(+)

20/20 display SM progression***

C Model: *daf-18* regulates a quiescence promoting signal or signals

From the somatic gonad to promote GSC quiescence

WT or control dauers

Within the somatic gonad to promote SGB quiescence

WT or control dauers

✓ pro-quiescence signal

Fig. 4

Tenen and Greenwald
Figure S1. Time course of SGB progression in daf-18(0) dauers. Related to Figure 1.
Dauer and pre-dauer larvae were obtained as described in STAR Methods. *p<0.05, **p<0.01, ***p<0.001 by two-tailed Fisher's exact test.

A. SGB progression in daf-18(0) dauers occurs after dauer entry. “+” = control, GS8052 daf-7; arls51[cdh-3::gfp] and “(0)” = GS8024 daf-7; daf-18(0); arls51[cdh-3::gfp]. Control dauers develop more slowly than daf-18(0) dauers, as IIS controls growth rate regardless of dauer life history (Karp, 2018; Ruaud, 2011). Therefore, staging and time in dauer were determined by a combination of morphological criteria and time.

Morphological criteria: “No progression” = GFP in AC only and somatic gonad size is wild type. Progression to “Pre-utse” stage=GFP expression has not expanded from the AC, but the somatic gonad is substantially increased in size and number of cells (see Figure 1B, 1C). “Utse initiated,” GFP expansion was evident, but could not be confirmed to encompass multiple cells by fluorescence or DIC microscopy. “Utse formed”=GFP expansion was expanded to multiple cells, confirmed by fluorescence or DIC.

Staging criteria: Described in (Karp, 2018). L2d: daf-7(e1372); daf-18(ok480) larvae scored at 26 hours after egg preparation had L2d morphological characteristics (granule accumulation, increased length compared to continuous L2) without the radial constriction that initiates during the L2d-dauer molt. L2d-dauer molt: confirmed by morphology and timing. Morphological criteria included increased length compared to L2d larvae, initiation of radial constriction, initiation of dauer, and partial pharyngeal constriction. Melting animals were further subdivided into “early” and “late” based on presence of partial dauer alae, as dauer alae begin to form late in the L2d-dauer molt.

Dauer (no SDS): Control larvae: full pharyngeal constriction, radial constriction, and dauer alae were observed. daf-18(0) dauer: full dauer alae were observed (radial and pharyngeal constriction is not normal in daf-18(0) dauers). Subdivision by time in dauer: “initial” dauer larvae (estimated as <4 hrs in dauer) were still encased in a shed molt cuticle and would still be SDS-sensitive; dauers with full alae, cuticle was shed, and scored at later time points were estimated to be between 1-15 hours in dauer based on the earliest time points at which dauer entry was observed.

B. SGB progression in daf-18(0) increases with time in dauer. “+” = as above. “(0)” = GS8925 daf-7; daf-18(0); arls51[cdh-3::gfp]. All dauers were SDS-selected at different time points after eggs were moved to 25°C. Because the timing of dauer entry differs for daf-18(+) and daf-18(0), time in dauer was adjusted based on morphological criteria (see above) and the developmental time point at which each genotype acquires SDS resistance. Hours after eggs at 25C by time in dauer are: <9hrs dauer/50hr control/40hr daf-18(0); 1-19hr dauer/66hr control/50hr daf-18(0); ≥1 day dauer/72hr control/72hr daf-18(0); ≥2 day dauer/96hr control/ND daf-18(0). “1 day” and “2 day” dauers are estimated to have been in dauer at for least 24 or 48 hours respectively. n=17-33.
**A** *daf-18*(+) expressed in DTCs or all somatic gonad cells restores SGB quiescence in *daf-18*(0) dauers

<table>
<thead>
<tr>
<th>promoter::<em>daf-18</em>(+)</th>
<th>0%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
</tr>
<tr>
<td><em>hlh-12p</em> (DTCs)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
</tr>
<tr>
<td><em>ckb-3p</em> (all somatic gonad)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
</tr>
</tbody>
</table>

**B** *daf-18*(+) expressed in intestine or neurons does not restore SGB quiescence in *daf-18*(0) dauers

<table>
<thead>
<tr>
<th>promoter::<em>daf-18</em>(+)</th>
<th>0%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No array, <em>daf-18</em>(0) background</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
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<tr>
<td><em>daf-18p</em>: native</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
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<tr>
<td><em>ges-1p</em>: intestine</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
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<tr>
<td><em>rgef-1p</em>: neurons</td>
<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
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<td>![bar graph](cdh-3::gfp+ utse in dauer.png)</td>
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</tbody>
</table>

Figure S2. Additional tissue-specific *daf-18*(+) rescue experiments. Related to Figure 2.

Extrachromosomal arrays were assayed in the background of *daf-7*(e1372); *daf-18*(ok480) *arts51*[cdh-3::gfp], and progression was assayed by expansion of GFP expression indicative of utse formation. n = 20-22.

A Extrachromosomal arrays expressing *daf-18*(+) in *daf-18*(0) dauer somatic gonad rescue VU arrest. The arrays were formed from plasmid pCT17(*hlh-12p*::S::*daf-18*::T2A::tagBFP2) or pCT19(*ckb-3p*::S::*daf-18*::T2A::tagBFP2) (see STAR Methods). (***) *P* < 0.0001 by two-tailed Fisher’s exact test compared with the control, *daf-18*(0).

B *daf-18*(+) expressed in intestine or neurons does not restore SGB quiescence in *daf-18*(0) dauers. Extrachromosomal arrays *tdEx349*[*daf-18*::*daf-18*cDNA::myc::*daf-18* 3’UTR], *veEx349*[*ges-1p*::*daf-18*cDNA::myc::*daf-18* 3’UTR], and *veEx344*[*rgef-1p*::*daf-18*cDNA::myc::*daf-18* 3’UTR] rescued a *daf-18*(0) phenotype in starved L1 larvae (Fukuyama et al., 2015). These arrays improved the SDS-resistance of *daf-18*(0) dauers (data not shown), but restoration of SGB quiescence was only seen when *daf-18*(+) was expressed using its native promoter and not the tissue-specific intestinal or neuronal promoters. A strain expressing DAF-18(+) specifically in the hypodermis, *veEx383* [*dpy-7p*::*daf-18* cDNA::myc::*daf-18* 3’UTR], was inviable in the *daf-7*(e1372); *daf-18*(ok480) *arts51* background and therefore could not be scored. (***) *P* < 0.0001 by two-tailed Fisher’s exact test compared with *daf-18*(+).
Figure S3. Genetic ablation of the DTCs and AC by hlh-2(RNAi). Related to Figure 3.

hlh-2 is critical to specify the DTCs and the AC, so hlh-2(RNAi), performed by feeding L1 larvae bacteria expressing double-stranded RNA, results in genetic ablation of the DTCs and the AC (Karp and Greenwald, 2003; Karp and Greenwald, 2004). Feeding bacteria expressing lacZ double-stranded RNA serves as a negative control. All dauers were SDS selected as described in STAR Methods.

A. hlh-2(RNAi) performed on strain GS8525 daf-7(e1372) arls131[lag-2p::2xnls::yfp]; arTi112[ckb-3p::mCherry::H2B]; nre-1(hd20) lin-15b(hd126) did not abrogate quiescence of the SGBs in 10/10 daf-18(+)) SDS-selected dauers, as evidenced by the overall small size of the gonad (dotted lines) and presence of the nine SGBs.

B. hlh-2(RNAi) performed on strain GS8604 daf-7(e1372) arls131[lag-2p::2xnls::yfp]; arTi112[ckb-3p::mCherry::H2B]; nre-1(hd20) lin-15b(hd126) did not restore quiescence to the SGBs in 11/11 daf-18(0) dauers, as evidenced by the numerous mCherry-labeled somatic gonadal cells and growth of the gonad.
Fig. S4. Genetic mosaics lacking daf-18 activity in defined lineages. Related to Figure 4. Strain is GS9117 daf-2(e1370); daf-18(ok480) arls51[cddh-3::gfp]; arEx2399[daf-18(+)]. See STAR Methods for experimental details.
Chapter 3. General Discussion

My goal was to investigate how cellular quiescence is maintained over long periods of time in response to the variable environmental inputs encountered in development. To address this aim, I studied maintenance of quiescence in the C. elegans somatic gonad and germline during dauer arrest. This process requires tissue arrest for periods of time much longer than occurs in continuous development under replete conditions, and which is directed in response to adverse environmental conditions. I have found that daf-18/PTEN, which has a defined role in regulating GSC quiescence in dauer (Narbonne and Roy, 2006), is also required for quiescence of SGBs, and that it acts in the somatic gonad to coordinate quiescence of both the SGBs and the GSCs. My work further suggests that daf-18 is required for maintenance of this quiescence during dauer arrest rather than establishing it during dauer entry. The mechanism by which daf-18/PTEN signals from the somatic gonad to the germline to maintain this quiescence does not appear to involve the canonical signaling pathways that regulate dauer formation.

Many questions about the role of daf-18/PTEN in maintaining quiescence of the SGBs and GSCs in dauer remain to be answered. Of these, determining the nature and identity of the signal by which daf-18 can non-autonomously coordinate SGB and GSC quiescence is likely one of the most intriguing and is discussed first. Determining what redundant cellular foci exist for DAF-18/PTEN activity in the dauer somatic gonad is also of great interest because it has implications for whether DAF-18/PTEN acts cell-autonomously as well as non-autonomously in this context. Other outstanding questions include whether DAF-18/PTEN may itself be secreted to maintain tissue quiescence non-autonomously in C. elegans, and whether mechanisms that mediate soma-germline communication in other contexts are involved here.

In this chapter I will discuss these questions.
3.1 Signal regulated by DAF-18/PTEN to regulate SGB and/or GSC quiescence

Probably the major question addressed in this work is how DAF-18/PTEN can non-autonomously regulate SGB and GSC quiescence. There must be some non-autonomous signal from the somatic gonad to the SGCs, and possibly from the somatic gonad to the SGBs as well; alternatively, though PTEN usually acts autonomously, it has also been reported to be secreted intercellularly, and the possibility that DAF-18/PTEN secretion non-autonomously regulates quiescence of the SGBs and/or GSCs in dauer is discussed separately below. For the purposes of this initial discussion, we assume the existence of a distinct signal or signals between the tissues.

In this thesis, I have demonstrated that DAF-18/PTEN is capable of non-autonomously regulating both SGB and GSC quiescence and that in this role, its activity is required in the somatic gonad but not the germline. My work also suggests that daf-18 has a redundant cellular focus in the somatic gonad and likely the SGBs, and therefore I do not know whether a non-autonomous signal mediated by daf-18 is required for SGB quiescence in wild-type dauers, or rather simply sufficient in tissue-specific rescue experiments. Here, I discuss the nature of the signal, whether there may be distinct signals for maintaining SGB vs. GSC quiescence, and what the mechanism of the signal might be.

3.1.1 Nature of the DAF-18/PTEN-mediated and somatic gonad-derived signal

My results demonstrating sufficiency of and requirement for daf-18/PTEN in the somatic gonad but not the germline to maintain SGB and GSC quiescence in dauer suggests the somatic gonad provides a signal to the germline. Furthermore, DAF-18/PTEN activity in the DTCs is sufficient but not necessary to non-autonomously rescue SGB and GSC quiescence in dauer; however, it is likely necessary in the somatic gonad to do so, suggesting daf-18 has redundant cellular foci in the somatic gonad. Whether there is one signal from the somatic gonad to the
SGBs and GSCs or distinct signals for each tissue is discussed in the next section; the discussion here assumes a singular signal, and implications for multiple signals are discussed below.

A simple model to explain the requirement of *daf-18* to coordinate SGB and GSC quiescence is that DAF-18/PTEN activity may promote a single “pro-quiescence” signal in wild-type dauers that actively maintains quiescence in both tissues; alternatively, dauer entry could establish a default state of quiescence and the progression observed in *daf-18(0)* dauers is a result of an ectopic “pro-progression” signal produced by the somatic gonad upon loss of *daf-18* in dauer. In the case of a “pro-quiescence” tissue, one would expect that any cellular focus capable of producing the signal can rescue quiescence even if other foci are *daf-18(0)*. Conversely, if the signal is an ectopic “pro-progression” signal, one would expect that all cellular foci capable of producing such a “pro-progression” signal must jointly be *daf-18(+) in order to maintain quiescence. Together the observations that *daf-18* is sufficient to rescue both SGB and GSC quiescence in dauer from the DTCs but also not necessary suggest that not all redundant cellular foci of *daf-18* are required to be *daf-18(+) for maintenance of quiescence, and therefore we favor the first model in which DAF-18/PTEN activity is required for a “pro-quiescence” signal.

Of course, more complex models are possible in which multiple signals are produced (discussed below) or in which levels of the signal must surpass a certain threshold: perhaps both the DTCs and an unidentified redundant cellular focus must jointly express a “pro-progression” signal to reach levels sufficient to cause progression. Consistent with this possibility, differences in PTEN dosage levels have been found to be important in regulating tumor progression in mice (Alimonti et al., 2010; Shen-Li et al., 2010). Additionally, feedback loops could be involved which ensure a joint decision is made between all SGBs and GSCs, consistent with mechanisms that coordinate dauer entry in the entire organism via feedback loops between pharyngeal cells and the hypodermis (Gerisch et al., 2004; Mak and Ruvkun, 2004; Schaedel et al., 2012).
It is also possible that there are both a wild-type “pro-quiescence” signal that actively maintains quiescence, requiring DAF-18 activity, and also a “pro-progression” signal produced with loss of daf-18, and that a balance between the two determines whether the tissues progress or remain quiescent. However, while the phenotype of both SGB and GSC progression is observed at high penetrance in daf-18(0) dauers (80-90%), this progression is disordered and results in abnormal gonadal and germline morphology. This abnormal morphology is evidenced both by anatomy and by fluorescent markers; for example, π cell markers are frequently expressed in up to 20 cells in daf-18(0) dauers (8/20 SDS-selected dauers), but only 12 in wild type continuous development. These observations suggest that while normal lineal descendants are observed in daf-18(0) dauers, the developmental progression observed in daf-18(0) dauers does not constitute normal continuous developmental progression. Therefore, any “pro-progression” signal is likely to be either abnormal in nature or insufficient to coordinate normal developmental progression in the somatic gonad and germline.

Some of these possibilities can be addressed with distinct experiments. For example, whether a certain threshold of DAF-18/PTEN activity is required could be assessed either by testing weaker tissue-specific promoters for rescue of somatic gonad and germline quiescence, or by testing whether rescue is still observed from the single-copy somatic gonad-specific arTi195[ckb-3p::daf-18] (described in Chapter 2) in heterozygous dauers, such that one less copy of the insertion is present and expression of daf-18 is presumably halved.

However, discerning the nature of the rescuing signal will most likely require 1) identifying the signal and 2) characterizing the redundant cellular source (or sources) for daf-18/PTEN within the somatic gonad. Experiments proposed to address these aims are discussed next.
3.1.2 Tissue focus for daf-18/PTEN: cellular redundancy within the somatic gonad

Our findings that daf-18 is sufficient and necessary in the MS lineage, and likely the somatic gonad in particular, suggest that the sole tissue focus for daf-18 in maintaining SGB and GSC is the somatic gonad. Additionally, we have found DTC-specific daf-18 expression rescues quiescence of both SGBs and GSCs in dauer but that it is not necessary; consistent with this lack of requirement for daf-18(+) in the DTCs, loss of fate specification and differentiation of the DTCs does not recapitulate or suppress the SGB progression in a daf-18(+) or daf-18(0) background, respectively. All of these findings are described in more detail in Chapter 2.

We therefore propose that there is at least one additional redundant source with the DTCs in the somatic gonad. One obvious candidate for such a source is the only other differentiated cell in the dauer somatic gonad primordium, the AC. However, two results suggest this is not the case:

1. AC genetic ablation: we tested whether the function of the AC or DTCs is required for either maintaining SGB quiescence in daf-18(+) dauers or for promoting progression in daf-18(0) dauers through genetic ablation by hlh-2(RNAi) (Karp and Greenwald, 2003, 2004). As described in Chapter 2, this ablation did not abrogate quiescence of SGBs in daf-18(+) dauers or their progression in daf-18(0). GSC quiescence is abrogated by loss of DTC function and therefore a requirement for DAF-18 activity in the AC and/or DTCs to maintain GSC quiescence could not be evaluated with this experiment. These results suggest that the AC is not a sole redundant cellular focus with the DTCs, though they do not show the AC is not one of multiple cellular foci for DAF-18/PTEN activity in this role.

2. Tissue-specific rescue experiments, however, indicate that the AC is not a redundant focus for daf-18 in maintaining SGB or GSC quiescence in dauer. We tested for tissue-specific rescue from the AC using hlh-2prox, which is a portion of the hlh-2 promoter that in continuous development is expressed in the cells that become the AC and VUs; expression in the VUs decreases significantly after the AC-VU decision until expression is only visible in
the AC (Sallee and Greenwald, 2015). This promoter is expressed visibly only in the AC in dauer (unpublished observations and personal communication). We generated two miniMos single copy insertions of $\text{hlh-2prox::daf-18::T2A::tagBFP2}$ (Frøkjær-Jensen et al., 2014) and neither rescued SGB progression in $\text{daf-18}(0)$ dauer, suggesting that not only is AC differentiation not required to maintain somatic gonad quiescence in dauer, but also that $\text{daf-18}$ expression in the ACs is not sufficient to maintain SGB or GSC quiescence in dauer (Figure 1).

If the DTCs are not the sole focus for maintaining SGB and GSC quiescence in dauer, and the only other specified cell in the dauer somatic gonad primordium is not a redundant source, then what is the redundant cellular source for $\text{daf-18}$?

One intriguing candidate is the spermathecal/sheath cell precursor (SS) cells. Four SS precursors exist in the dauer somatic gonad primordium, and some of their descendants later differentiate to form the sheath cells (Kimble and Hirsh, 1979). These sheath cells have identified roles in supporting germline proliferation: ablation of SS cells or of their most distal descendants results in a 50-60% decrease in the number of adult proliferating GSCs (Killian and Hubbard, 2005; McCarter et al., 1997). In dauer, the SS cells have extended processes that, together with the extensions of the DTCs, result in both germline arms of the somatic gonad primordium being surrounded entirely by the somatic gonad (Figure 2). This morphology can also be seen using a $\text{qIs90[ceh-22b::yfp]}$ fluorescent marker that expresses brightly in the DTCs and their sisters, two of the SS cells, in dauer (Figure 3). Therefore, the SS precursors are located such that they could, in theory, relay signals to both the germline and the proximal and distal somatic gonad in dauer. However, testing this hypothesis is problematic due to lack of sufficiently precise promoters with which to do tissue-specific rescue or excision experiments.

Two strategies that may work to test the SS precursors as a redundant focus include:
1. Tissue-specific rescue with DTC ablation: expression of \textit{daf-18(+)} from the \textit{ceh-22b} promoter may provide tissue-specific rescue to the DTCs and their sister SS cells; if this construct does rescue, subsequently testing for rescue of SGB quiescence with concurrent genetic ablation of the DTCs by \textit{hlh-2(RNAi)} could indicate whether SS-specific \textit{daf-18} is able to rescue SGB quiescence in \textit{daf-18(0)} dauers. This experiment is quite feasible with the appropriate reagents, but contingent on first confirming that ablation of the DTCs by \textit{hlh-2(RNAi)} abrogates the rescue imparted by DTC-specific expression of \textit{daf-18} from the \textit{hlh-12} promoter; otherwise rescue from \textit{ceh-22b::daf-18} would not be indicative of \textit{daf-18} in the SS cells per se.

2. Mosaic analysis: by marking an extrachromosomal array with a somatic gonad-specific fluorescent marker (for example, with the promoter \textit{ckb-3p}), one could analyze mosaic losses within the somatic gonad of an extrachromosomal array carrying \textit{daf-18(+)}. One could then analyze whether concurrent loss of the array in the DTCs and SS precursors of \textit{daf-18(0)} dauers causes progression of the SGBs and/or GSCs. This experiment may not be feasible for two reasons. Firstly, it is contingent on the requirement for \textit{daf-18} being distinct in each gonad arm, e.g. retention of \textit{daf-18} in one gonad arm does not rescue the other. We do not know whether the output of DAF-18/PTEN activity is specific to each arm, though this is possible, as signaling that regulates GSC proliferation in adults has been reported to act locally in each germline arm (Narbonne et al., 2015). Secondly, loss of the array in all four SS cells of the somatic gonad primordium would require two independent losses of the array within the somatic gonad (e.g. in Z1.a and Z1.pa with retention in Z1.pp) and therefore would occur infrequently.

Identifying the redundant cellular focus for \textit{daf-18/PTEN} in maintaining somatic gonad and germline quiescence in dauer will help determine whether DAF-18/PTEN acts both cell-autonomously and non-autonomously in this context: for example, if the SS cells are found to be the redundant cellular focus, DAF-18/PTEN activity is likely regulating SS cell quiescence.
autonomously but must rescue VU quiescence non-autonomously, and therefore may regulate quiescence by different mechanisms even within the somatic gonad. The identification of any redundant cellular source(s) will also help confirm whether DAF-18/PTEN is likely to regulate SGB quiescence non-autonomously in a wild-type context.

3.1.3 Screening for clues to the identity of the signal

Any signal and/or signaling mechanism regulated by DAF-18/PTEN activity in the somatic gonad to maintain SGB and/or GSC quiescence in dauer could be mediated by, or could regulate, myriad developmental pathways involved in dauer regulation, somatic gonad or germline development, developmental regulation from environmental conditions, or any other signaling mechanism in C. elegans. Therefore, a screen to ascertain the identity of the signal may be necessary to discover its nature. Even if such a screen does not directly yield the identity of the signal itself, it may give insights into the components required for production of the signal or into targets of the signal, both of which may help identify the signal. Given its short life cycle and the ability to visualize fluorescent reporters in vivo, C. elegans is extremely amenable to screens. While many different methods of screening exist, I propose initiating by a screen using RNAi interference, as the use of daf-7(e1372) allows for administration of RNAi by feeding and synchronized dauer formation. The RNAi sensitizer nre-1 lin-15b can be combined with daf-7 and other markers to enhance RNAi effectiveness without disrupting dauer formation or affecting somatic gonad and germline quiescence (this thesis).

The phenotype of SGB progression in daf-18(0) dauers as assessed either by arIs51[cdh-3::gfp] or tnIs6[lim-7::gfp] markers is visible on a dissecting microscope, allowing for easy and rapid screening of many RNAi clones. I would start with a more targeted screen involving yet untested components of dauer formation regulating pathways as well as pathways known to have
prominent roles in continuous larval development, particularly ones with roles in the somatic gonad.

3.1.4 Secreted PTEN

Many known roles for \textit{daf-18}/PTEN in maintaining stem cell quiescence in mammals, mice, and Drosophila are reported to be autonomous; particularly relevant is a study demonstrating that specific knockout of \textit{pten} in the primordial germ cells of mice results in impaired mitotic arrest and testicular teratoma formation, contrasting with our findings (Huang et al., 1999; Kimura et al., 2003; Kwon et al., 2001; Li et al., 2002; Porter et al., 2016; Yilmaz et al., 2006). However, non-autonomy has also been reported not in the form of a signal per se, but rather through intercellular transfer of PTEN itself, and this non-autonomous activity has been suggested to occur through two mechanisms. One mechanism by which PTEN is transported intercellularly is via exosomes, and a second is through secretion of an alternative isoform (reviewed in (Hopkins et al., 2014)).

Endogenous PTEN was found to be secreted in exosomes from both mouse embryonic fibroblasts as well as human embryonic kidney cells. These exosomes were then internalized by recipient cells and provided functional PTEN activity, as measured by Akt phosphorylation and reduced cellular proliferation (Putz et al., 2012). A different study reported that PTEN secretion in exosomes was specific to cancerous cell lines and not present in exosomes from non-cancerous cells (Gabriel et al., 2013). Also in 2013, Hopkins et al. identified PTEN-Long, a translational variant of PTEN that results from an alternative translation site and encodes 173 additional amino acids N-terminal to the canonical PTEN protein. This alternative protein is membrane-permeable and was reported to be both secreted from cells and able to enter other cells. Indeed, PTEN-Long promoted tumor regression \textit{in vitro} and \textit{in vivo} through antagonism of
the PI3K pathway. Investigations of neural stem cell proliferation, which responds to signals that reflect population density for proper development, found that nanovesicles (a category that includes exosomes) in embryonic cerebrospinal fluid carry both protein and microRNA components of the insulin-like growth factor signaling pathway. These vesicles were able to promote neural stem cell divisions, but they also identified PTEN in the nanovesicles and proposed that in different contexts, the vesicles could promote or inhibit stem cell amplification and provide a mechanism for a binary “on-or-off” regulatory signal (Feliciano et al., 2014).

The possibility of DAF-18 itself being secreted is unlikely, though transfer in extracellular vesicles is possible, as discussed in Chapter 2. If DAF-18 is transported intercellularly, this transport must be very tissue-specific and unidirectional, since daf-18(+) genomic fragments in the germline (multicopy array or single copy insertion) do not rescue SGB or GSC quiescence. Furthermore, tissue-specific transgenes that rescue from the somatic gonad or from the DTCs specifically do not rescue quiescence in the neighboring sex myoblasts, as reported above.

However, directional vesicle signaling between the somatic gonad and germline is possible, as endocytosis of specific membrane-associated components from the somatic gonad to oocytes has been observed (Starich et al., 2014). Additionally, communication via endosomal trafficking between the somatic gonad and germline and soma-to-soma is important for RNAi silencing, and, in this context, extracellular silencing RNA uptake into both somatic and germline cells requires rsd-3 (Imae et al., 2016). Therefore, in the absence of an identified signal, it would be interesting to test whether endosome-mediated intercellular transport of DAF-18 or possibly daf-18 mRNA is required for maintaining somatic gonad or germline quiescence; alternatively, it is possible that DAF-18 is not transferred, but other components of extracellular vesicles compose the signal.
This question could be addressed by testing whether loss of components required for endosome formation or uptake results in SGB and/or GSC progression in dauer as in *daf-18(0)* mutant dauers. I would start testing this question with mutants of components essential for endocytosis, such as the clathrin component *cht-1*, which is essential for clathrin-mediated endocytosis (Sato et al., 2009). However, many of the components required for endocytosis are also required for viability; therefore, some form of tissue-specific depletion would be necessary. Such a tissue-specific experiment could be performed using the endogenous *zif-1-ZF1* degradation system (Armenti et al., 2014). This system was originally precluded for genes required during embryogenesis, but a method compatible with such genes has been recently described (Sallee et al., 2018). Alternatively, one could feed *cht-1* dsRNA to larvae prior to dauer entry in a *daf-c* background to avoid the embryonic lethality associated with *cht-1(RNAi)*, or use a temperature-sensitive allele *cht-1(b1025ts)* whose restrictive temperature is compatible with the temperatures required to induce dauer formation in *daf-c* mutants such as *daf-7(e1372)* and *daf-2(e1370)* (Zhang et al., 2013). Tissue specificity would be preferable for confirming directionality and specifically testing whether endocytosis is required in the GSCs, SGBs, or both for maintaining quiescence in dauer.

If the SGBs and/or GSCs do indeed progress with loss of *cht-1*, I would next test whether DAF-18 itself is transferred in endosomes by tagging it with a bright fluorescent protein and expressing it specifically in the DTCs. I would then examine the proximal somatic gonad for fluorescent expression. This experiment could be done in parallel with or prior to testing endocytic components, but a negative result may simply indicate the expression is too dim or diffuse to visualize in other tissues.

### 3.1.5 Other signal mechanism candidates
Quiescence is, of course, not limited to the developmentally arrested states of dauer diapause and L1 arrest and is also required in the course of normal development. While signaling pathways required for dauer diapause or L1 arrest are obvious candidates for the mechanism by which DAF-18/PTEN activity regulates quiescence non-autonomously, our studies so far suggest they may not be involved. Other signaling pathways present intriguing candidates as well, and some are discussed here.

3.1.6 Soma-germline crosstalk: sid-1 and small RNA signaling

In *C. elegans*, environmental stressors including, but not limited to, starvation and dauer formation have been found to regulate reproductive plasticity, and this regulation depends on soma-germline crosstalk and on RNA interference (Ow et al., 2018). Changes in expression pattern in both the somatic tissues and germline were observed differentially in post-dauer adults in response to different stressors, and these changes had consequences for post-dauer reproductive fitness. We therefore aimed to test whether *daf-18* non-autonomously regulates germline progression in dauer through RNA-mediated signals. We chose to first test this question using a null allele for *sid-1*, a dsRNA transmembrane channel that was identified as a regulator of changes in gene expression and reproductive fitness resulting from dauer life history (Feinberg and Hunter, 2003; Ow et al., 2018). *sid-1* is selective for dsRNA and is required cell-autonomously for spread of systemic RNAi between tissues, including both soma and germline, and therefore also for RNAi inheritance (Feinberg and Hunter, 2003; Li et al., 2015; Shih and Hunter, 2011; Winston et al., 2002). *sid-1* is required for import of dsRNA into cells but not for its export (Jose et al., 2009), and is also capable of transporting various small molecules containing both double- and single-stranded RNA (Shih and Hunter, 2011). RNA signaling could provide an active signal in dauer to promote somatic gonad and germline arrest non-
autonomously, in which case \textit{sid-1} mutants should phenocopy \textit{daf-18}, or could result in an ectopic signal in \textit{daf-18(0)} mutants that promotes progression, in which case \textit{sid-1} loss may suppress \textit{daf-18(0)} somatic gonad and/or germline progression.

We tested whether loss of \textit{sid-1} caused progression in the somatic gonad or germline in \textit{daf-7} dauers or suppressed it in \textit{daf-7}; \textit{daf-18} dauers using \textit{cdh-3::gfp} expression to evaluate the the somatic gonad and DIC for the germline. Preliminary results (pending genotype confirmation) suggest that loss of \textit{sid-1} in \textit{daf-7(e1372); arl51[cdh-3::gfp]; sid-1(qt9)} dauers does not phenocopy \textit{daf-18(0)} since the somatic gonad and germline maintain quiescence in 20/20 dauers; this \textit{sid-1(0)} mutant also does not suppress progression observed in \textit{daf-18(0)} dauers (\textit{cdh-3::gfp} expression in the utse in 11/13 and GSC progression in 13/13 \textit{daf-7}; \textit{daf-18(0)} \textit{arl51[cdh-3::gfp]; sid-1(0)} dauers). Additionally, no defects in reproduction were observed in post-dauer \textit{daf-7; sid-1(0)} hermaphrodites (14/14 post-dauer adults were fertile), and all \textit{daf-18(0)} post-dauer adults were infertile (21/21), consistent with our analysis of post-dauer hermaphrodites mosaic for \textit{daf-18(0)} described in Chapter 2 (21/21). If confirmed, these preliminary results suggest that although RNAi interference signaling between soma and germline regulate reproductive plasticity, and this regulation is dictated by environmental inputs, this signaling is not involved in maintenance of SGB and GSC quiescence during dauer diapause or in normal reproductive development upon dauer recovery.

3.2 DAF-18/PTEN activity autonomous to quiescence-regulating cells

While the mechanism by which DAF-18/PTEN non-autonomously regulates cellular quiescence of the SGBs and GSCs is of great interest, another unanswered question is how DAF-18/PTEN acts to produce a non-autonomous signal from the cells that actually regulate
quiescence, including the DTCs. DAF-18 may regulate a variety of signaling pathways in this role, and these are explored here, starting with its canonical IIS regulatory role.

### 3.2.1 DAF-18/PTEN is not likely to inhibit Insulin/insulin-like signaling (IIS) to non-autonomously maintain cellular quiescence

*daf-18(0)* mutation fully suppresses the dauer-constitutive phenotype of mutants of the insulin/insulin-like growth factor receptor ortholog *daf-2* (Solari et al., 2005). Therefore, in *daf-18/PTEN* null mutants, the resulting ectopically activated IIS signaling downstream of *daf-2* activity is sufficient to promote continuous development and bypass dauer even in the absence of *daf-2* activity. This ectopic activation could take place in the dauer somatic gonad of *daf-18(0)* dauers and promote progression of the SGBs and GSCs.

My findings suggest that DAF-18/PTEN activity is not likely to non-autonomously regulate IIS signaling to maintain SGB or GSC quiescence in dauer: if DAF-18/PTEN inhibited IIS to maintain quiescence in the target SGB and GSC cells, one would expect loss of IIS activity in the target cells by *daf-2* mutation would suppress the SGB and GSC divisions observed in *daf-18(0)* mutant dauers. However, mosaic analysis in *daf-2(ts); daf-18(0)* mutants suggests loss of IIS in the SGBs and GSCs does not suppress progression in *daf-18(0)* mutants (Chapter 2), and therefore DAF-18/PTEN does not mediate production of a signal that inhibits IIS in these cells. Though the SGBs and GSCs may lack *daf-18(+) in these mosaics, since *daf-18* is not required autonomously in these cells to maintain quiescence in dauer, my analysis should not be affected by the absence of *daf-18* in these cells.

However, my mosaic analysis in *daf-2(ts); daf-18(0)* dauers does not test whether DAF-18/PTEN cell-autonomously inhibits IIS to produce the signal(s) that non-autonomously regulates SGB and GSC quiescence: we test for suppression of GSC and SGB progression in
daft-2(ts); daf-18(0); arEx2399[daf-18(+)] dauers with loss of daf-18(+) in the MS lineage. Therefore, if IIS is inhibited autonomously by DAF-18/PTEN activity, loss of daf-18 in the MS lineage precludes analysis of autonomous action due to the potential of ectopic daf-2 activation in those cells.

In contrast, our analysis of SGB progression in daf-16/FoxO null mutants is more informative regarding daf-18/PTEN cell-autonomous activity: nuclear localization and therefore activity of the transcription factor and ortholog of FoxO, DAF-16, is inhibited by IIS signaling, and loss of daf-16 function suppresses the constitutive dauer formation of daf-2/IR mutants completely (Larsen et al., 1995; Vowels and Thomas, 1992). DAF-16/FOXO is therefore most likely a major target of IIS in dauer formation. However, we very rarely observed the utse formation that reflects SGB progression in daf-16/FoxO null mutants (Chapter 2). Additionally, the GSC progression that occurs in daf-18/PTEN null dauers appears to be absent in daf-16/FoxO null dauers (Narbonne and Roy, 2006). Therefore, we conclude that DAF-18/PTEN is not likely to maintain SGB or GSC quiescence through its canonical role as an IIS inhibitor.

To bolster this conclusion, additional experiments would be necessary: apart from DAF-16/FoxO, other targets of IIS exist in C. elegans, including the Nrf transcription factor SKN-1 (Tullet et al., 2008), the zinc finger transcription factor PQM-1 (Tepper et al., 2013), and the heat shock transcription factor HSF-1 (Chiang et al., 2012; Morley and Morimoto, 2004). Some of these transcription factors may act redundantly to DAF-16/FoxO, and mutants could be tested for SGB and GSC progression in dauer. Additionally, one could test whether tissue-specific activation of IIS components downstream of daf-2/IR in the DTCs is sufficient to drive SGB or GSC progression; however, to my knowledge, a gain-of-function allele in C. elegans is only characterized for components downstream of age-1/PI3K (e.g. akt-1) (Paradis and Ruvkun, 1998), which may not specifically indicate a role for IIS signaling as discussed below.
In sum, we have not completely ruled out tested whether DAF-18/PTEN cell-autonomous activity requires its canonical role as an IIS inhibitor in the somatic gonad cells to maintain SGB and GSC quiescence in dauer, but our analysis so far using the IIS target DAF-16/FoxO suggests otherwise.

### 3.2.2 PI3K signaling and non-IIS pathways

DAF-18/PTEN targets both lipids and protein substrates for dephosphorylation, and the lipid phosphatase activity of DAF-18/PTEN inhibits IIS by antagonizing the activity of the PI3K AGE-1 (see Chapter 1). However, regulation of both dauer and lifespan extension by DAF-18/PTEN requires mediation of phosphatidylinositol 3, 4, 5 triphosphate (PIP3) levels, since expression of transgenes encoding mutant DAF-18/PTEN that can only recognize protein substrates does not rescue dauer formation or lifespan defects of daf-18(0) mutants (Solari et al., 2005). It is therefore likely that DAF-18/PTEN lipid phosphatase activity (not protein phosphatase activity) is required for maintaining quiescence in the SGBs and GSCs in dauer as well, and by extension that PIP3 levels are important in this process; this inference could be confirmed by expressing this DAF-18/PTEN mutant with loss of lipid phosphatase activity in the somatic gonad and/or DTCs and testing for rescue of SGB and GSC quiescence.

If DAF-18/PTEN does regulate PIP3 levels to maintain quiescence of the SGBs and GSCs in dauer, it does not necessarily regulate IIS, or it may regulate other signaling pathways via IIS signaling inhibition independently of DAF-16/FoxO: the lipid phosphatase activity of mammalian PTEN also antagonizes the mTOR pathway to regulate many cellular processes that include proliferation (reviewed in (Song et al., 2012). Consistent with regulating of mTOR signaling, daf-18/PTEN is non-autonomously required for quiescence of some somatic progenitors during L1 arrest in particular nutrient contexts, and though IIS activation reactivates
progenitor cells, this reactivation requires mTOR signaling ([Fukuyama et al., 2015], see Chapter 1). Additionally, mTOR signaling pathway mutants are defective for dauer formation, suggesting mTOR plays a role in dauer regulation (Jia et al., 2004). Altogether, it is possible that DAF-18/PTEN may autonomously control both IIS and mTOR signaling, or specifically mTOR signaling, to mediate production of a quiescence-promoting intercellular signal from the somatic gonad.

Another (speculative) possibility for DAF-18/PTEN’s mechanism of action in the somatic gonad cells is regulation of DA-DAF-12/NHR signaling. My results show a difference between somatic gonad and germline progression in daf-9(0) mutants which do not produce DAs (see Chapter 2): while germline progression occurs in approximately 60-70% of daf-18(0); daf-9(0) dauers (no significant difference compared to germline progression in daf-7(ts); daf-18(0) dauers), the somatic gonad progresses in only approximately 30% of daf-18(0); daf-9(0) dauers (p<0.01 compared to germline progression). This discrepancy suggests that while DA-DAF-12 signaling may not be required for germline progression in daf-18(0); daf-9(0) dauers, it may be partially required for somatic gonad progression. These experiments do not identify the cellular focus for DA-DAF-12 activity in this context. However, a DAF-12::GFP fusion reporter is expressed in the somatic gonad in dauer, suggestive that ectopic DA activation of DAF-12 is possible in the dauer somatic gonad (reporter expression was downregulated in all tissues in dauer but perdured in the somatic gonad and occasional neurons) (Antebi et al., 2000). If DA-DAF-12 signaling is partially required for somatic gonad progression in dauer, one possible mechanism by which DAF-18 could maintain quiescence is by inhibiting this signaling pathway in the somatic gonad. This mechanism would likely be distinct from maintenance of germline quiescence by DAF-18. Some examples of nuclear hormone receptor regulation by human PTEN in vitro (both PI3K-dependedent and independent) exist (Blind et al., 2012; Lin et al., 2004), and
it would be interesting to test whether DAF-18 may be regulating NHR signaling in the dauer somatic gonad as well. How and if this function would enable non-autonomous regulation of quiescence within the somatic gonad distinctly from the germline is unclear. One hypothesis is that DAF-18 regulates DA production and/or secretion downstream of DAF-9 activity; however, a daf-9 fusion reporter was not noted to be expressed in the dauer somatic gonad (Jia et al., 2002), making cell-autonomous regulation by DAF-18 of DA release within the somatic gonad less likely.

3.3 Concluding remarks

Many additional questions remain regarding how DAF-18/PTEN maintains quiescence in, and coordinates it between, the somatic gonad and germline in dauer. The mechanism by which the somatic gonad signals both inter se and to the germline to maintain quiescence is intriguing and unanswered, if partially characterized. Another captivating question is how somatic gonad and germline arrest is coordinated with that of non-gonadal tissues in dauer, and whether the decision to enter dauer is relayed through the somatic gonad to the germline rather than directly to both tissues from external sources. Other questions include:

Does DAF-9-mediated dafachronic acid signaling support progression differentially between the somatic gonad and germline? Does this progression require daf-12?

Do canonical dauer entry pathways regulate somatic gonad quiescence upstream of the somatic gonad-derived signal?

How does DAF-18/PTEN activity prevent cellular fate specification and differentiation in the SGB descendants? Is daf-18 also required to prevent differentiation of the GSCs in dauer?

What causes the abnormalities seen in somatic gonad and germline morphology in daf-18(0) dauers, and is the observed loss of quiescence controlled or uncontrolled proliferation?
What role, if any, does *daf-16/FoxO* play in preventing fate specification in the somatic gonad in dauer? Is LIN-12/Notch signaling blocked in the dauer VUs, and if so, does this block require *daf-16/FoxO* as in the VPCs? Is the low-penetrance loss of quiescence observed in the VUs of *daf-16(0)* dauers a result of LIN-12/Notch activation?
Chapter 3 Figures
Figure 1. Tissue-specific expression of daf-18(+) in the somatic gonad rescues somatic gonad and germline quiescence in dauer. All strains have daf-7(e1372) and markers (A) Expression pattern of the ckb-3, hlh-12, and hlh-2prox promoters are indicated in the somatic gonad primordium. ckb-3(784)p is expressed in the somatic gonad precursors Z1 and Z4 (Kroetz and Zarkower, 2015) and in all of their descendants in the somatic gonad primordium during dauer (described in Chapter 2). hlh-12(1080)p expression is restricted to the DTCs in continuous development from L2 onwards (Tamai and Nishiwaki, 2007) and was also visible only in the DTCs in daf-7(e1372); daf-18(ok480) arIs51[cdh-3::gfp]; arEx2416[hlh-12(1080)p::daf-18::T2A::tagBFP2::unc-54 3'UTR] dauers (further details in Chapter 2). hlh-2prox is a fragment of the hlh-2 promoter expressed in the four cells that become the AC and VUs (Sallee and Greenwald, 2015) and is expressed in the AC in dauer (personal communication from Catherine O’Keeffe). (B) daf-7(e1372); daf-18(ok480) arIs51[cdh-3::gfp] dauers were scored for somatic gonad development in dauer if cdh-3::gfp expression was seen in the utse of the somatic gonad. With rare exceptions, individuals in which somatic gonad developmental arrest was rescued by daf-18(+) constructs also maintained germline arrest as assessed by Nomarski differential interference contrast. Single-copy transgenes expressing daf-18cDNA::T2A::tagBFP2::unc-54 3’UTR under the hlh-12, ckb-3, or hlh-2prox promoters were generated by miniMos insertion (Frokjær-Jensen et al., 2014), with two independent insertion lines scored for hlh-2prox. A viral 2A peptide, T2A, triggers “ribosomal pausing” such that a single transcript produces two independent proteins (here DAF-18 and tagBFP2) (Ahier and Jarriault, 2014). n = 22-37. (*** P < 0.001 by two-tailed Fisher’s exact test compared with daf-18(0). NS, not significant.
Figure 2. The somatic gonad primordium envelops both germline arms in dauer. Expression of the somatic gonad marker ckb-3p::mCherry in the dauer somatic gonad. Top, Nomarksi differential interference contrast (DIC) merge with mCherry; bottom, mCherry fluorescent expression. Pictures show the entire somatic gonad and germline region of the dauer. The center of the “bowtie” pattern of fluorescence is expression in the proximal somatic gonad, with extended expression around each germline arm from DTCs and SS precursors (no expression is visible in the germline, as expected). Expression shown is from arEx2418[ckb-3p::mCherry::unc-54 3’UTR] in starved dauers selected by morphological criteria including presence of dauer alae, radial constriction, and pharyngeal constriction. Identical expression pattern was observed in 7/7 dauers examined. Strain is GS8215 and was incubated at 25°C for this experiment.
In dauers, the transgene *qls90[ceh-22b::yfp]* is brightly expressed in the DTCs (Z1.aa and Z4.pp) and the two SS cells that are DTC lineal sisters (Z1.ap and Z4.pa). This pattern is consistent with the reported expression pattern in continuous development L2 larvae (Lam et al., 2006). Dim expression is also visible in other cells of the somatic gonad primordium, consistent with published observations that this transgene is expressed in Z1 and Z4 in the L1 and becomes primarily restricted to the DTC and DTC sisters by L2 (Lam et al., 2006). This expression pattern was observed in 23/23 *qls90[ceh-22b::yfp]; daf-7(e1372)* dauers isolated by SDS selection after incubation from eggs at 25°C for 72 hours (1-day dauers).
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