The *C. elegans* heterochronic gene *lin-28* coordinates the timing of hypodermal and somatic gonadal programs for hermaphrodite reproductive system morphogenesis

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Abstract

C. elegans heterochronic genes determine the timing of expression of specific cell fates in particular stages of developing larva. However, their broader roles in coordinating developmental events across diverse tissues have been less well investigated. Here, we show that loss of *lin-28*, a central heterochronic regulator of hypodermal development, causes reduced fertility associated with abnormal somatic gonadal morphology. In particular, the abnormal spermatheca-uterine valve morphology of *lin-28(lf)* hermaphrodites traps embryos in the spermatheca, which disrupts ovulation and causes embryonic lethality. The same genes that act downstream of *lin-28* in the regulation of hypodermal developmental timing also act downstream of *lin-28* in somatic gonadal morphogenesis and fertility. Importantly, we find that hypodermal expression, but not somatic gonadal expression, of *lin-28* is sufficient for restoring normal somatic gonadal morphology in *lin-28(lf)* mutants. We propose that the abnormal somatic gonadal morphogenesis of *lin-28(lf)* hermaphrodites results from temporal discoordination between the accelerated hypodermal development and normally timed somatic gonadal development. Thus, our findings exemplify how a cell-intrinsic developmental timing program can also control proper development of other interacting tissues, presumably by cell non-autonomous signal(s).

Introduction

Heterochrony refers to modes of developmental alterations of an organism in which genetic changes lead to either accelerated or delayed development of certain body parts relative to others, in the context of evolution (Keyte and Smith, 2011; Klingenberg, 1998), or in experimental organisms (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). Genetic mutations causing heterochrony in the nematode *Caenorhabditis elegans* identified a gene regulatory network, the ‘heterochronic pathway’, that governs the relative timing of developmental events during the four larval stages (L1~L4). Loss-of-function (*lf*) mutations of *lin-14* or *lin-28* result in precocious development via the skipping of hypodermal cell fates specific to one or several larval stages (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). In contrast, *lin-4(lf)* and *let-7(lf)* mutations prevent the normal progression of certain stage-specific cell fates, leading to abnormal repetition of larval stages (Ambros and Horvitz, 1984; Reinhart et al., 2000).

lin-28 encodes a conserved RNA-binding protein containing one cold shock domain and two zinc finger domains. The mammalian homolog of LIN-28 is implicated in diverse biological processes, including tumorigenesis, pluripotency, and metabolism (Piskounova et al., 2011; Yu et al., 2007; Zhang et al., 2016; Zhu et al., 2011). In *C. elegans*, expression of LIN-28 is highest in the late embryo and L1 stages and decreases from the L2 stage onwards. LIN-28 is primarily expressed in the hypodermis, neurons, and muscle. *lin-28(lf)* mutants skip L2-specific hypodermal cell fates, resulting in precocious expression of L3, L4 and adult hypodermal fates (Moss et al., 1997; Seggerson et al., 2002). Thus, in the wild type, *lin-28* functions in early larval stages to specify the proper timing of L2-to-L3 cell fate transitions. *lin-28* does this by promoting expression of the HBL-1 transcription factor, and by preventing premature expression of mature *let-7* microRNA (Abbott et al., 2005; Vadla et al., 2012; Van Wynsberghe et al., 2011).

Here, we investigated the role of *lin-28* in maintaining fertility of *C. elegans* hermaphrodites. The hermaphrodite gonad consists of germ cells, which originate from proliferation of two germline precursor cells (Z2 and Z3), and somatic gonadal tissues, which are derived from two somatic precursor cells (Z1 and Z4), located within the gonadal primordium of L1 larvae (Kimble and Hirsh, 1979). Somatic gonadal development occurs during the L1-L4 larval stages and is characterized by stage specific patterns of cell division, morphogenesis, and differentiation into tissues with specific functions, including the gonadal sheath cells, spermatheca, spermathecal-uterine valve (Sp-Ut valve), uterus, and uterine seam cells (utse). During ovulation, mature oocytes are released to the spermatheca for fertilization. Post-fertilization, the embryo exits from the spermatheca into the uterus. Efficient ovulation, fertilization, and spermathecal exit are critical for optimal reproductive capacity of *C. elegans* hermaphrodites, and mutants defective in these processes exhibit reduced fertility (Iwasaki et al., 1996; Kariya et al., 2004; Kovacevic and Cram, 2010).

Our results show that certain aspects of somatic gonadal development are abnormal in *lin-28(lf)* hermaphrodites, reflected by abnormal morphology of the uterus, uterine seam, and Sp-Ut valve. These morphological defects, particularly the abnormal Sp-Ut, dramatically limit *lin-28(lf)* fertility. Our results further indicate that the normal development of the somatic gonad relies on temporal coordination of

hypodermal developmental events with somatic gonadal events, and that *lin-28* acts in the hypodermis to specify a schedule of hypodermal events that is properly coordinated with a corresponding schedule of somatic gonadal developmental events. We demonstrate that the hypodermal function of *lin-28* is sufficient to regulate somatic gonadal development non-autonomously, consistent with a role for *lin-28*, and downstream heterochronic genes, in controlling the hypodermal components of critical developmental signaling between the gonad and hypodermis.

Results

***lin-28(lf)* mutants exhibit defects in embryo production and embryo viability**

lin-28(n719) hermaphrodites produce fewer larval progeny than wild-type hermaphrodites (Fig 1A). *lin-28(n719)* mutants are unable to lay eggs, due to precocious vulva development, which results in abnormal vulva morphogenesis (Euling and Ambros, 1996). Like other egg-laying defective mutants, *lin-28(lf)* hermaphrodites contain their entire brood of embryos trapped inside the limited space of the somatic gonad. To test whether the reduced number of progeny of *lin-28(lf)* hermaphrodites could be simply the result of their egg-laying defect, we compared the number of progeny of *lin-28(n719)* hermaphrodites with that of *lin-2(e1309)*. *lin-2(e1309)* mutants exhibit defective egg-laying due to their vulvaless phenotype, which results from cell lineage defects not related to developmental timing (Hoskins et al., 1996). *lin-28(n719)* mutants produce substantially fewer progeny than *lin-2(e1309)* (Fig 1A), suggesting the reduced brood of *lin-28(n719)* animals is not merely the result of an egg-laying defect. To quantify embryo production in *lin-28(n719)* hermaphrodites, we dissected mature, gravid hermaphrodites and counted the embryos retained inside. *lin-28(n719)* gravid adults contained fewer embryos than *lin-2(e1309)* animals (Fig 1B), indicating that *lin-28(n719)* mutants are defective in embryo production. To test embryonic viability, we harvested embryos from dissected gravid adults and counted the number that hatched and developed into larva. Approximately 70% of *lin-28(n719)* embryos failed to develop, whereas essentially all of the *lin-2(e1309)* embryos were viable (Fig 1C). These results suggest that reduced embryo production and embryonic lethality contribute to the reduced progeny number of *lin-28(n719)* mutants. *lin-28(n719)* mutants also displayed these same defects at 20°C, although at a somewhat reduced penetrance compared to 25°C (Fig. S1). We conducted all our subsequent experiments at 25°C, where those defects are most prominent.

We also performed tissue-specific RNAi experiments using *rrf-1(lf)* and *ppw-1(lf)*, and observed that somatic knockdown of *lin-28* caused greater reduction of total number of progeny than did germline knockdown of *lin-28* (Fig. S2). This suggests the fertility defects of *lin-28(lf)* mutants are mainly due to somatic absence of *lin-28*.

Defects in ovulation and spermathecal exit cause reduced embryo production in *lin-28(lf)*

To investigate the cause of reduced embryo production in *lin-28(n719)* mutants, we checked whether ovulation and spermathecal exit proceed normally. We examined the spermatheca of *lin-28(n719)* mutants using the expression of a spermatheca reporter, *fkh-6p::GFP* (Chang et al., 2004). Approximately 70% of *lin-28(n719)* adults contained embryos in their spermathecae, whereas less than 10% of wild-type spermathecae contained embryos (Fig 2A,B). Unlike in the wild type, many embryos in *lin-28(n719)* hermaphrodites had undergone multiple rounds of cell division inside the spermathecae. This suggests that *lin-28(n719)* mutants have defects in the process of spermathecal exit. We used time-lapse video microscopy to monitor the first ovulation and spermathecal exit (Movie 1, 2). In wild type hermaphrodites, ovulation, fertilization, and spermathecal exit of an individual embryo happens within 20 minutes (McCarter et al., 1999). By contrast, we observed that for ~50% of *lin-28(n719)* hermaphrodites, the first ovulated embryo remained in the spermatheca for more than 60 minutes (Fig 2E; Movie 2).

Next, we addressed whether ovulation is also defective in *lin-28(n719)* mutants. The presence of endomitotically-replicating (Emo) oocytes is a characteristic of ovulation mutants (Iwasaki et al., 1996), where oocytes undergo several rounds of DNA replication without ovulation and fertilization. Gonadal DAPI staining revealed that some *lin-28(n719)* hermaphrodites contain endomitotic oocytes (Fig 2D) in the oviduct. We speculate that the defective ovulation in *lin-28(n719)* mutants may result from impairment of the spermathecal exit process, wherein the presence of fertilized embryos trapped within the limited spermathecal space would prevent subsequent entry of mature oocytes. We conclude that the poor fertility of *lin-28(n719)* mutants is the consequence of embryos becoming trapped in the spermathecae, stalling subsequent ovulation.

Sp-Ut valve morphogenesis, uterine lumen formation, and utse migration are abnormal in *lin-28(lf)* mutants

Spermathecae in *C. elegans* hermaphrodites consist of two rows of 12 cells forming a long tube structure at the young adult stage (Gissendanner et al., 2008; Kimble and Hirsh, 1979). Long spermathecal tube structures, labeled by *fkh-6p::GFP*, are observed in the *lin-28(n719)* mutants at the 4th stage (Fig 3B), which corresponds to L4 and adult stage in wild type (Fig 6C). Wild-type spermathecae become constricted horizontally as somatic gonadal tissues continue morphogenesis before the first ovulation. *lin-28(n719)* spermathecae exhibited a similar constricted and extended morphology as in the wild type (Fig 3A,B). Overall, we did not detect appreciable differences in spermathecal morphology between wild-type animals and *lin-28(n719)* mutants.

The Sp-Ut valve connects the spermatheca to the uterus in wild type hermaphrodites, and serves as a portal through which fertilized embryos exit the spermatheca into the uterus (McCarter et al., 1999). The mature Sp-Ut valve consists of the toroidal syncytium and the core cell syncytium. We characterized Sp-Ut valve structure in *lin-28(n719)* mutants using a *cog-1::GFP* reporter, which is expressed in the Sp-Ut valve core cell syncytium from the late L3 or early L4 stage (Palmer et al., 2002). In wild type animals, the

core cell syncytium stretches during the L4 stage, and in young adults, the Sp-Ut valve core forms a dumbbell-like structure with one end in the spermatheca and the other end in the uterus (Fig 3C). Although we observed apparently normal expression of *cog-1::GFP* in the Sp-Ut valve region of *lin-28(n719)* mutants in late 3rd larval stage, the core cell stretching did not occur and the Sp-Ut valve core remained as a single lobe structure in 4th stage animals (Fig 3D). This observation indicates that Sp-Ut valve morphology is abnormal in *lin-28(n719)* mutants, suggesting the connection between the spermatheca and the uterus is also disrupted. We conclude that the aberrant connection between the uterus and the spermatheca associated with the abnormal morphology of the Sp-Ut valve causes the spermathecal exit defect in *lin-28(lf)* mutants. To assess whether the abnormal Sp-Ut valve morphology of *lin-28(n719)* could reflect somatic gonadal cell lineage defects analogous to the precocious hypodermal cell lineages exhibited by *lin-28(n719)* (Ambros and Horvitz, 1984), we investigated whether the Sp-Ut valve syncytium in the mutant contains a normal number of nuclei as in the wild-type. The Sp-Ut valve is derived from daughter cells of the dorsal uterine lineage, and the valve core syncytium is comprised of a fusion of two cells (Kimble and Hirsh, 1979). We used confocal microscopy to count the number of nuclei in the Sp-Ut valve core region based on labeling by *cog-1::GFP*. Two nuclei were present in Sp-Ut core cell of both wild-type and *lin-28(n719)* mutants, suggesting the morphological defect is not caused by abnormal cell division in the lineage generating the Sp-Ut valve core (Fig. S3A,B).

In addition to the abnormal Sp-Ut valve morphology, other somatic gonadal defects were evident in *lin-28(n719)* mutants. In wild-type animals, the uterine lumen forms between the dorsal and ventral uterus during the L4 stage when uterine toroidal cells fuse to generate a syncytium (Newman et al., 1996). In *lin-28(n719)* mutants at the 4th stage, we observed abnormally small, incompletely-connected, and/or less elongated uterine luminal structures, compared to the wild type (Fig. S4B,C).

The uterine seam (utse) is a component of the hermaphrodite somatic gonad that mediates structural attachment between the hypodermis and uterus (Newman and Sternberg, 1996). The utse syncytium forms in the early L4 stage and extends laterally during progression to mid L4. The utse connects the uterus to the seam cells laterally and also to uv1 and the vulva. *egl-13::GFP* is expressed in the nuclei of π cell lineage, whose products includes the utse, from late L3 to L4 stages (Ghosh and Sternberg, 2014). Utse labeled by *egl-13p::GFP* migrated laterally in wild-type hermaphrodites (Fig. S5A,B). However, the utse nuclei did not migrate during the 4th stage in *lin-28(n719)* mutants, although *egl-13p::GFP(+)* cells were detected in the utse region (Fig. S5C,D).

lin-28(n719);lin-2(e1309) double mutants, which lack vulva formation, have the same somatic gonadal defects as *lin-28(n719)* mutants, implying these defects are not indirect consequences of the abnormal vulval morphology in *lin-28(n719)* mutants (Fig. S6).

The defects in uterine lumen formation and utse migration in *lin-28(n719)* mutants, together with their Sp-Ut valve morphological defects, suggest that *lin-28* activity is required for multiple aspects of proper hermaphrodite somatic gonadal development.

Eggshell integrity is compromised in *lin-28(lf)* mutant embryos

To investigate the embryonic lethality in *lin-28(n719)* mutants, we dissected embryos from adult hermaphrodites and imaged the isolated embryos. The *lin-28(n719)* embryos displayed abnormal, irregular shapes (Fig 4A), reminiscent of the misshapen phenotypes exhibited by egg-shell mutants (Johnston et al., 2006; Maruyama et al., 2007; Zhang et al., 2005). The *C. elegans* eggshell is formed rapidly after fertilization in the spermatheca and consists of chitin, lipid, and structural proteins, and functions as a protective barrier around the embryo. Eggshell abnormalities often result in embryo lethality (Johnston et al., 2010).

Chitin-binding domain-protein 1 (CBD-1) is a component of the eggshell cortex, and *cbd-1::mcherry* expression marks the periphery of wild-type embryos (Allen et al., 2014; Johnston et al., 2010). *cbd-1::mcherry* expression was evident surrounding embryos from *lin-28(n719)* hermaphrodites, indicating that an eggshell does form (Fig 4B). The wild type eggshell is impermeable to the lipophilic dye FM4-64 (Johnston et al., 2006), but in our study, about 50% of *lin-28(n719)* embryos were permeable to FM4-64, while around 10% of *lin-2(e1309)* embryos were permeable (Fig 4C).

This finding indicates that eggshell integrity is compromised in embryos produced by *lin-28(n719)* mutants. In support of this conclusion, *lin-28(n719)* embryos also exhibited osmotic stress sensitivity. Embryos from wild type hermaphrodites maintained their oval shape upon exposure to 0, 150, and 300 mM KCl, whereas embryos from *lin-28(n719)* animals swelled up in 0 or 150 mM KCl (Fig 4D).

Interestingly, embryos of *fln-1(tm545)* mutants, which have defective spermathecal exit (Kovacevic and Cram, 2010), also exhibited permeability to FM4-64 (Fig 4C), suggesting that abnormally prolonged residence in the spermatheca may result in compromised eggshell integrity (see Discussion).

In summary, *lin-28* is required for normal somatic gonadal development, including uterine lumen formation, uterine morphogenesis, and proper Sp-Ut valve formation. We suggest that the abnormal Sp-Ut valve structure in *lin-28(n719)* mutants causes defects in spermathecal exit and ovulation, resulting in reduced embryo production. In addition, the embryos stalled in the spermatheca seem to suffer eggshell damage, resulting in embryonic lethality (Fig 4E).

Embryonic lethality of *lin-28(n719)* is rescued by maternal expression of wild type *lin-28*

We speculated that the lethality of embryos produced by *lin-28(n719)* hermaphrodites results from defects in maternal somatic gonadal morphology (which results in trapping of embryos in the spermatheca), rather than from an absence *lin-28* function in the embryos per se. If so, then maternal expression of *lin-28* should rescue the embryonic lethality of *lin-28(n719)* homozygous embryos. To test this, we crossed *lin-28(n719)* hermaphrodites with wild-type males to obtain heterozygous mutants (*lin-28(n719)/+*), and then assessed the viability of *lin-28(n719)* homozygous self-progeny from these *lin-28(n719)/+* hermaphrodites. Viable *lin-28(n719)* homozygotes were identified by their characteristic egg-laying defective phenotype as adults. Amongst the self-progeny of heterozygous (*lin-28(n719)/+*) hermaphrodites, we observed a ratio of wild type progeny (+/+ or *lin-28(n719)/+*) to egg-laying defective progeny (*lin-28(n719)/lin-28(n719)*) of

2.89(±0.2):1 on average, which is very close to the expected 3:1 ratio for complete maternal rescue of embryonic lethality (Table 1). This finding suggests that *lin-28(n719)* embryonic lethality reflects a requirement for *lin-28* activity in the mother to enable proper development of her somatic gonad, which is required for the production of viable embryos.

Genes downstream of *lin-28* in developmental timing regulation also function downstream of *lin-28* in somatic gonadal morphogenesis and fertility

Functional interactions of *lin-28* with other heterochronic genes for the control of *C. elegans* hypodermal cell lineage developmental timing have been described previously (Ambros, 2011; Resnick et al., 2010) (see Fig 5E). *lin-28* functions upstream of *lin-46* and *hbl-1* to control the timing of L2 to L3 fate transitions in hypodermal cell lineages, and *lin-28* also acts via a pathway consisting of *let-7*, *hbl-1*, *lin-41*, and *lin-29* to regulate the transition from L4 fates to adult fates in the hypodermis.

To determine whether these same heterochronic genes that act downstream of *lin-28* for hypodermal cell fate timing also function in fertility and embryonic lethality, we examined those phenotypes in double mutants of the heterochronic genes (Fig 5A-C). The number of live progeny per animal produced by the *lin-28(n719);let-7(mn112)* and *lin-28(n719);lin-29(n836)* double mutants was significantly higher than that produced by the *lin-28(n719)* mutants (Fig 5A). Also, the number of live progeny of *lin-28(n719);lin-46(ma164)* was even greater than that of *lin-2(e1309)*, because the loss of *lin-46* rescued the vulva and egg-laying defects of *lin-28(n719)* mutants. *lin-28(n719);let-7(mn112)* and *lin-28(n719);lin-29(n836)* mutants produced more embryos per animal than *lin-28(n719)* mutants (Fig 5B) and the embryonic viability was suppressed in these double mutants, compared to *lin-28(n719)* (Fig 5C). The embryonic viability of *lin-28(n719);lin-46(ma164)* was similar to wild type animals (Fig 5C).

Consistent with the genetic epistasis observed above for fertility and embryonic viability, *lin-46(lf)*, *let-7(lf)* and *lin-29(lf)* were also epistatic to *lin-28(lf)* for somatic gonadal morphogenesis. Our RNAi knockdown experiments showed that genetic absence of *let-7*, *lin-46* or *lin-29* suppress the Sp-Ut valve defects caused by *lin-28(RNAi)*. *hbl-1(RNAi)* phenocopied the fertility phenotypes of *lin-28(n719)* mutants, including abnormal Sp-Ut valve core morphology (Fig. S7A,B). Loss of *lin-46* or *let-7* function rarely affected the Sp-Ut valve morphological defect in *hbl-1(RNAi)* animals, indicating *hbl-1* is epistatic to these genes. Finally, approximately 70% of *lin-29(lf);cog-1::GFP* animals showed the wild-type Sp-Ut valve core cell morphology when *hbl-1* function was compromised (Fig. 5A). In addition, a normal uterine lumen was observed in ~50% of *lin-28(n719);let-7(mn112)* mutants (Fig. S4D). Most *lin-28(n719);lin-29(n836)* and *lin-28(n719);lin-46(ma164)* mutants showed complete uterine lumen formation (Fig. S4E,F). Utse migration defects of *lin-28(lf)* mutants were also partially suppressed by loss of either *let-7* or *lin-46* (Fig. S5G-J).

Overall, our findings indicate that *let-7*, *lin-46*, *hbl-1*, and *lin-29* act downstream of *lin-28* for somatic gonadal development in a network configuration essentially identical to that previously-described for the control of hypodermal cell fate timing by these same genes (Fig 5E).

Developmental timing of hypodermal tissues and somatic gonadal tissues are discoordinated in *lin-28(lf)* mutants

Based on the above observations indicating parallels between the control of hypodermal developmental timing and somatic gonadal morphogenesis by *lin-28* mutants, we investigated whether the stage-specificity of somatic gonadal developmental events might be altered in *lin-28(n719)* hermaphrodites, in analogy to their precocious hypodermal development. As a marker to monitor the expression of stage-specific programs in the somatic gonad and hypodermis, we employed *cog-1::GFP* (Palmer et al., 2002), which is expressed in the wild type dorsal uterus and Sp-Ut valve core, beginning from late-L3/early-L4 stage (Fig 6A). *cog-1::GFP* is also expressed in the ventral hypodermis (vulval cell lineages) beginning in the mid L4 stage, which is after the onset of *cog-1::GFP* somatic gonadal expression (Fig 6B).

We examined whether the normal relative order of *cog-1::GFP* expression in the somatic gonad and vulva is altered in *lin-28(n719)* mutants. In *lin-28(n719)* hermaphrodites, *cog-1::GFP* expression in the vulva was observed precociously in the mid-3rd larval stage (Fig 6D), consistent with the previously-described precocious vulval cell divisions of *lin-28(lf)* mutants (Euling and Ambros, 1996). However, the onset of somatic gonadal expression of *cog-1::GFP* was normal, beginning from the late 3rd larval stage in both *lin-28(n719)* and in the wild type (Fig 6E). Thus, the expression of *cog-1::GFP* in the vulva precedes the expression in somatic gonads in *lin-28(n719)* mutants. These data suggest that the somatic gonadal development, at least as reflected by *cog-1::GFP* expression, is not precocious in *lin-28(n719)*, despite precocious development of the hypodermis. Therefore, developmental events in hypodermal tissues and somatic gonadal tissues are temporally discoordinated in *lin-28(n719)* mutants (Fig 6F). We hypothesize that this discord, between the precociously-developing hypodermis, and normally-timed somatic gonad, causes abnormal somatic gonadal morphogenesis.

To investigate the hypothesis that the somatic gonadal defects of *lin-28(n719)* mutants are caused by precocious development of the hypodermis, we examined whether post-dauer development, which has been shown to suppress the precocious hypodermal development of *lin-28(n719)* (Euling and Ambros VR, 1995; Liu and Ambros, 1991), can also rescue *lin-28(n719)* somatic gonadal defects. We observed that after post-dauer development, *lin-28(n719)* adults exhibited restored fertility and embryonic viability, normal morphology of the Sp-Ut valve core, uterine lumen, and normal utse migration (Fig 5B, 5D, S7E, S4G, S5E, and S5F). This suppression of somatic gonadal defects in *lin-28(n719)* animals by post-dauer development supports the supposition that *lin-28* acts indirectly, via downstream-genes and events, to mediate normal somatic gonadal morphogenesis (Fig 5E).

Hypodermal expression, but not somatic gonadal expression, of *lin-28* rescues somatic gonadal defects, embryonic lethality, and reduced number of total progeny in *lin-28(lf)* hermaphrodites

If the somatic gonadal morphological defects of *lin-28(n719)* mutants originate from discoordination between the timing of hypodermal and somatic gonadal development, suppressing the precocious hypodermal development in *lin-28(n719)* mutants should also rescue the somatic gonadal phenotypes. To

test whether *lin-28* expression specifically in the hypodermis could rescue the Sp-Ut morphological defects of *lin-28(n719)*, we employed Mos1-mediated single copy insertion (MosSCI) transformation to generate transgenic worms expressing *lin-28::GFP::lin-28 3' UTR* driven by tissue-specific promoters; these included *lin-28* endogenous promoter sequences, a *dpy-7* hypodermal promoter (Gilleard et al., 1997), and an *ehn-3A* early somatic gonadal promoter (Large and Mathies, 2010). The endogenous *lin-28* promoter drives *lin-28::GFP* expression in neurons and hypodermis, where *lin-28* is known to be expressed (Moss et al., 1997). Using spinning disk microscopy we also detected *lin-28p::lin-28::GFP* expression in Z1 and Z4 cells, which are precursors of somatic gonadal tissues, (Fig. S8A,B). *dpy-7p::lin-28::GFP* was expressed in the hypodermis during the embryonic and L1 stages, and *ehn-3Ap::lin-28::GFP* expression was strongest in Z1 and Z4 from the late embryo to L1 stages (Fig. S8C,D). *lin-28::GFP* levels decreased from the L2 stage in all three strains, presumably due to repression mediated by the *lin-28 3' UTR*.

We crossed those MosSCI transgenic strains with *lin-28(n719);cog-1::GFP* hermaphrodites to assess the relative timing of somatic gonadal and hypodermal *cog-1::GFP* expression. *cog-1::GFP* was expressed in somatic gonadal tissues prior to the vulva in wild-type animals, whereas *cog-1::GFP* expression in the vulva was precocious in the *lin-28(n719)* mutants (Fig 6, 7A,7B). *lin-28* expression via its endogenous promoter (*lin-28p::lin-28::GFP::lin-28 3'UTR*) restored the normal relative timing of somatic gonadal and hypodermal *cog-1::GFP* expression in *lin-28(n719)* mutants (Fig 7C). Similarly, in *dpy-7p::lin-28::GFP;lin-28(n719);cog-1::GFP* animals, *cog-1::GFP* expression in the vulva occurred at the normal time, following somatic gonadal expression. Thus, hypodermal expression of *lin-28* efficiently rescues precocious hypodermal development of *lin-28(n719)* (Fig 7D). By contrast, somatic gonadal expression of *lin-28* via the *ehn-3Ap::lin-28::GFP* transgene did not rescue the precocious expression of hypodermal *cog-1::GFP* in *lin-28(n719)* mutants (Fig 7E). These results are consistent with cell-intrinsic activity of *lin-28* in the hypodermis to control hypodermal developmental timing.

Does hypodermal expression of *lin-28* also rescue the somatic gonadal morphogenesis defects of *lin-28(n719)* hermaphrodites? Indeed, *dpy-7p::lin-28::GFP* did rescue wild-type morphology of the Sp-Ut valve core in *lin-28(n719)*, (Fig 7F, H and I), normal uterine lumen formation (Fig. S4I), and normal utse migration (Fig. S5M,N). By contrast, somatic gonadal *lin-28* expression did not rescue *lin-28(n719)* somatic gonadal defects (Fig 7G,7J, S4J,S4K, S5O and S5P).

We also performed mosaic analysis to investigate if hypodermal expression of *lin-28* is necessary and sufficient for somatic gonadal morphogenesis (Yochem and Herman, 2003). We constructed *lin-28(n719);cog-1::GFP* strains with an extrachromosomal transgenic array containing a *lin-28* expression vector and a *sur-5::GFP* plasmid as a reporter for the array. 12 mosaic animals which completely lost the extra-chromosomal array in the Z1 and Z4 lineages, but not in hypodermal lineage, all showed wild type Sp-Ut valve core morphology at the young adult stage (Fig 8A, S9B). For 7 of 8 mosaic animals where *sur-5::GFP* was undetectable in the hypodermis, but clearly expressed in the Z1 and Z4 lineages, wild type Sp-Ut valve core morphology was not observed (Fig 8B, S9C). This suggests that loss of *lin-28* expression in

hypodermal tissues, but not in somatic gonadal tissues, is correlated with abnormal Sp-Ut valve morphogenesis (Fig 8C, Fig. S9).

Because the hypodermal expression of *lin-28* suppresses the morphological defects of the somatic gonad in *lin-28(n719)* mutants (Fig 7), we investigated whether embryonic lethality and fertility defects were accordingly rescued, as would be expected. Expression of either *lin-28p::lin-28::GFP* or *dpy-7p::lin-28::GFP* restored the embryo viability of *lin-28(n719)* progeny, whereas *ehn-3Ap::lin-28::GFP* expression did not restore viability of *lin-28(n719)* progeny (Fig 9A).

Interestingly, although the total number of live progeny was greater for the *dpy-7p::lin-28::GFP;lin-28(n719)* strain than for the *lin-28(n719)* mutants, for only ~15% of animals were the number of progeny comparable to the wild-type, and the other ~85% of animals produced fewer than 20 progeny at 25°C. In addition, the *dpy-7p::lin-28::GFP* transgene in the wild-type background led to a similarly reduced number of progeny, indicating that the expression of *dpy-7p::lin-28::GFP* alone is sufficient to reduce fertility, apparently by impairing egg-laying (Fig 9C). In contrast, somatic gonadal promoter-driven *lin-28::GFP* expression did not affect the number of progeny produced by *lin-28(n719)* hermaphrodites (Fig 9B).

Discussion

C. elegans lin-28(lf) hermaphrodites exhibit a dramatic reduction in fertility, in excess of what would be expected as a simple consequence of their vulval morphogenesis defects. In principle, it was possible that *lin-28* could promote fertility entirely via a germline-specific activity, analogous to the demonstrated role of mammalian Lin28 in promoting pluripotency and stem cell proliferation (Yu et al., 2007; Zhang et al., 2016). Indeed, *lin-28* has been reported to regulate the germ cell pool size in mice and in *C. elegans* hermaphrodites (Shinoda et al., 2013; Wang et al., 2017). However, the reported effects on germ cell pool size in *C. elegans*, after germline knockdown of *lin-28*, did not include substantially reduced fertility (Wang et al., 2017). Our results suggest that the reduced fertility of *lin-28(lf)* hermaphrodites is caused by fertility-promoting activities of *lin-28* outside of the germline, that is, within somatic cell lineages of the gonad and/or other tissues. Other examples in which *lin-28* controls the development of reproductive tissues other than germ cells have been reported in flies and mice. The *Drosophila* egg chamber is fused abnormally early in *lin-28* null mutants, and the development of mice vaginal openings is delayed in *lin-28a* transgenic mice (Stratoulas et al., 2014; Zhu et al., 2010).

Here we investigated the somatic function of *lin-28* in promoting *C. elegans* hermaphrodite fertility. Our results show that *lin-28* is required for the completion of certain somatic gonadal morphogenetic events, specifically, the enlargement of the uterine lumen, the migration of utse nuclei, and extension of the Sp-Ut valve core, and that these somatic gonadal morphogenesis defects likely underlie the reduced fertility of *lin-28(lf)* hermaphrodites. In particular, the abnormal Sp-Ut valve of *lin-28(lf)* animals has a potent impact on fertility by preventing fertilized embryos from entering the uterus, thereby inhibiting ovulation and resulting in reduced embryo production.

We also found compromised eggshell integrity in *lin-28(lf)* mutants, which negatively affects embryonic viability, similarly to other eggshell defective mutants (Johnston et al., 2006; Johnston et al., 2010; Maruyama et al., 2007). Moreover, we found that another spermathecal exit mutant *fln-1(lf)* has eggshell-defective phenotypes similar to *lin-28(lf)* (Fig 4C), suggesting a causal link between spermathecal retention and loss of eggshell integrity. We speculate that physical damage to the eggshells may occur when the embryos are trapped in the spermatheca. Interestingly, deficiency of *cbd-1*, an essential component of the *C. elegans* eggshell, leads to pinched-off embryos reflecting incomplete spermathecal exit (Johnston et al., 2010). This suggests that damage to the eggshell of embryos lingering too long in the spermatheca could further aggravate an underlying spermathecal exit defect. Further research is needed to clarify the relationship between spermathecal exit and eggshell integrity.

Our results point to a close coupling between the hypodermal and somatic gonadal phenotypes in *lin-28(lf)* mutants. First, we found that a very similar configuration of the known heterochronic genes mediates the effects of *lin-28(lf)* on hypodermal developmental timing and on somatic gonadal morphogenesis (Fig 5E). The exception is *lin-41*; we could not find evidence that *lin-41*, a downstream target of *let-7* for hypodermal developmental timing (Slack et al., 2000), is involved in Sp-Ut valve core morphogenesis. The *lin-41*(RNAi) animals displayed a superficially normal dumbbell-shaped Sp-Ut valve, albeit somewhat smaller than the wild-type valve (Fig. S7C). This result may reflect either an insufficient knockdown of *lin-41* by RNAi in these experiments, or that *lin-41* does not participate in somatic gonadal development. In the latter event, *hbl-1* would appear to function as a main downstream target of *let-7* in somatic gonadal morphogenesis.

Our second finding indicating a linkage between *lin-28(lf)* hypodermal and somatic gonadal phenotypes is the observation that post-dauer development suppresses both the hypodermal and the somatic gonadal developmental defects of *lin-28(lf)* hermaphrodites. This result in particular accentuates that the somatic gonadal defects of *lin-28(lf)* do not reflect a direct role of *lin-28* in somatic gonadal development, *per se*; rather, that somatic gonadal morphogenesis fails in *lin-28* mutants as an indirect consequence of precocious development. Consistent with this idea, another precocious mutant, *lin-14(lf)* (Ambros and Horvitz, 1984), showed similar Sp-Ut valve defects to *lin-28(lf)* mutants (Fig. S7D).

Importantly, we did not find evidence for precocious development of somatic gonadal events in *lin-28(lf)*. In particular, we observed that the timing of the onset of expression of certain fluorescent markers of L3 and L4 somatic gonadal developmental events was not altered in *lin-28(lf)*, even though subsequent morphogenesis failed. Assuming that the somatic gonad and the hypodermis each has its own developmental clock, it would appear that the hypodermal developmental clock of *lin-28(lf)* mutants is accelerated, while the somatic gonadal developmental clock runs normally, resulting in discoordination of developmental timing between the two tissues during the L3 and L4 stages (Fig 6F). We propose that it is this temporal discord between the accelerated hypodermis, and the normally-scheduled somatic gonad, that results in failure of somatic gonadal morphogenesis.

The apparent absence of precocious development of the somatic gonad of *lin-28(lf)* animals suggests that *lin-28* may affect somatic gonadal morphogenesis cell non-autonomously, by controlling one or more signals from the hypodermis to the somatic gonad. In strong support for this model, we found that expression of *lin-28* specifically in the hypodermis could rescue the somatic gonadal developmental defects of *lin-28(lf)* mutants. Conversely, expression of *lin-28* specifically in the somatic gonadal precursor lineage did not rescue any *lin-28(lf)* phenotypes. Based on these observations, we propose that the principal function of *lin-28* with regards to somatic gonadal development is to act within the hypodermis to specify a schedule of hypodermal events that is properly coordinated with a corresponding schedule of somatic gonadal developmental events. Apparently, normal somatic gonadal morphogenesis requires a coordination between the hypodermis and the somatic gonad during the L3 and/or L4 stages.

Then, by what mechanisms could hypodermal activity of *lin-28* regulate the development of a different tissue? There are precedents in *C. elegans* for cell non-autonomous developmental signals originating from the hypodermis. A recent study showed that heterochronic genes acting in the hypodermis can modulate mTORC2 signaling in the intestine (Downen et al., 2016). In another example, it has been reported that migration of the hermaphrodite-specific neurons and arborization of sensory neurons are regulated by hypodermal expression of the microRNA *mir-79* and MNR-1/menonin, respectively (Pedersen et al., 2013; Salzberg et al., 2013). Hypodermal glycosylated cell surface molecules or signaling cell adhesion molecules are key downstream factors for neuronal morphogenesis in each case, implying this nonautonomous signaling may require physical contact with other tissues.

Indeed, seam cells in the hypodermis become physically connected to the utse (uterine seam cell) of *C. elegans* hermaphrodites. The connection between these two tissues is thought to be formed during the mid to late L4 stages in wild-type animals (Newman et al., 1996). The precocious hypodermal maturation in *lin-28(lf)* animals might cause this connection to be formed aberrantly, or not at all. Alternatively, the reduced number of seam cells in *lin-28(lf)* mutants may alter positioning of seam cells in the hypodermis, disrupting the normal connection between seam cells and utse. However, there is evidence that utse defects may not necessarily be associated with Sp-Ut abnormality. *lin-29* expression in the anchor cell induces signals for utse precursor cells to adopt utse fates, and *lin-29(lf)* mutants do not form a proper utse (Newman et al., 2000). However, loss of *lin-29* does not cause any detectable abnormality in Sp-Ut valve core morphology (Fig 5A). Nevertheless, it will be interesting to examine whether the physical connection between the hypodermal seam and gonadal utse is formed properly in *lin-28(lf)* hermaphrodites.

It is striking that *lin-28(lf)* mutants exhibit defects in the final stages of morphogenesis in at least three distinct somatic gonadal structures: extension of the Sp-Ut valve core, positioning of the uterine seam cell nuclei, and expansion of the lumen of the uterus. Since all three of these defects are highly penetrant in *lin-28(lf)*, and are coordinately rescued by appropriate *lin-28*-expressing transgenes, we were not able to determine if these defects are expressed independently, or whether for example, one of them is the primary defect that is linked to hypodermal developmental timing, and the other defects are secondarily precipitated

by the first. Further research is needed to identify any cause-effect relationships between utse migration, Sp-Ut valve morphogenesis, and uterine lumen formation.

Overall, our studies of *lin-28* in the context of *C. elegans* reproductive system development provides an informative model for exploring fundamental principles of multicellular development, including how the generation of organized cellular complexity requires precise temporal coordination of events across interacting tissues. Our findings exemplify how a cell-intrinsic developmental timing program can be required not only cell-autonomously to specify temporal cell fates, but also to control cell-nonautonomous signaling that is critical for proper development of interacting tissues. Our identification of cell-nonautonomous hypodermis-to-gonad developmental signaling controlled by *lin-28* and the heterochronic pathway should set the stage for future studies addressing the identity and potential evolutionary conservation of the molecular components of the downstream signal(s).

Material and Methods

Culture of *C. elegans* strains

C. elegans wild type (Strain N2) and mutant strains (listed in Supplementary Table 1) were grown and maintained (at 25°C unless otherwise noted) on nematode growth media (NGM) agar plates seeded with *E. coli* (strain HB101). A list of genotyping primers for allele confirmation can be found in Supplementary Table 2. Synchronized populations of larvae at defined developmental stages were obtained as previously described (Stiernagle, 2006). Briefly, embryos were collected using sodium hypochlorite and 5N NaOH, washed with M9 buffer, and incubated in M9 buffer overnight at 20°C, placed on NGM plates seeded with HB101, incubated for defined lengths of time at 20 °C or 25 °C, and developmental stage was assessed by DIC microscopy of a sample of worms from the population (Byerly et al., 1976).

Microscopy

For DIC and fluorescence microscopy, worms were anesthetized with 0.2 mM levamisole and mounted on 2% agarose pads. All images except the following were obtained with a ZEISS Axiocam 503 mono: Supplementary Figure 2 (Leica DM 5500Q confocal microscopy), Supplementary Figure 7 (3i (Intelligent imaging Innovations) Everest spinning disk confocal microscopy). The supplementary movies were taken with a Zeiss Axiocam 503 mono. For gonad DAPI staining, hermaphrodites were cut with a syringe needle and the extruded gonads were fixed with 95% ethanol. After washing twice with M9, the dissected gonads were incubated with 4'6'-diamidino-2-phenylindole solution (100 ng/ml) for 10 min in a humidified chamber and washed again with M9 (Modified from (Shaham, 2005)).

RNAi

RNAi by feeding worms with *E. coli* expressing double-stranded RNA was conducted as previously described (Conte et al., 2015). HT115 bacterial RNAi strains (*lin-28*, *hbl-1*, *lin-29*, *lin-46*) and an empty vector strain (L4440) from the Ahringer library were used (Kamath and Ahringer, 2003).

Analysis of fertility and embryonic viability

Individual young adult hermaphrodites were placed, one per plate, on a NGM plates seeded with HB101, and the number of live progeny from each hermaphrodite was counted 3~4 days later. To determine embryo viability, gravid adult hermaphrodites were dissected with a syringe, the released embryos were collected, and transferred to NGM plates seeded with HB101. The total number of embryos was counted immediately, and after 36 hours incubation at 25 °C, the number of live animals was counted. Viability was calculated as (Number of live animals / Total number of embryos seeded) X 100%.

Eggshell integrity

Eggshell permeability was assessed using FM 4-64 dye (Sigma, T13320), as described (Johnston et al., 2006). Briefly, embryos were dissected from gravid hermaphrodites in 150 mM KCl with 30 μM of FM4-64, and the proportion of embryos infiltrated by FM4-64 was measured using fluorescence microscopy.

Estimating the duration of spermathecal transit

Wild type and *lin-28(lf)* hermaphrodites containing a spermathecal GFP reporter (strains DZ325 and VT2930, respectively) were examined in the fluorescence dissecting microscope to identify worms that had not yet undergone their first ovulation event, and these animals were placed individually on seeded NGM plates. Each animal was then observed every 20 minutes to check for the occurrence of ovulation (evidenced by an embryo surrounded by expanded fluorescent spermathecal tissue), and/or subsequent spermathecal exit (evidenced by the embryo located in the region of the uterus and adjacent to compacted fluorescent spermathecal tissue). When an animal was first observed to contain a spermathecal-resident embryo, spermathecal entry was scored as having occurred within the previous 20 minutes. Likewise, if a

previously spermathecal-resident embryo was observed to have exited the spermatheca, the time of spermathecal exit was scored as occurring within the previous 20 minutes. The maximum duration of spermathecal transit was estimated for each embryo in terms of multiples of 20 minutes, based on the number of 20-minute intervals that encompassed spermathecal entry and spermathecal exit. For animals in which ovulation and spermathecal exit had both occurred within the previous 20 minutes, we estimated the maximum duration of spermathecal transit for that embryo as 20 minutes.

Construction of plasmids

To generate transgenic strains containing tissue specific promoters driving *lin-28::GFP*, we removed the sequence between the first exon and second exon (1:8409341-1:8410415) of *lin-28a* to prevent the sequence from serving as an endogenous promoter (Moss et al., 1997). GFP sequences were adapted from XW12 (Wei et al., 2012) and were fused in frame to the carboxy terminus of *lin-28* coding sequence. The primers used for the overlapping PCRs for these procedures are listed in Supplementary Table 2. We used Gateway® Technology (Invitrogen, cat 12535-019) to construct transgenic vectors. *lin-28::GFP* was cloned into the gateway entry vector pDONR P2P3 by BP reaction. Also, the promoter regions of *enh-3A*, *lin-28*, and *dpy-7* were cloned into pDONR P4P1r by BP reaction. *lin-28* 3'UTR was cloned into pDONR P2rP3. LR reactions of these three entry vectors with pCJF150 yielded final vectors containing the following transgenes for injection: pSW40(*lin-28p*), pSW42(*ehn-3Ap*), and pSW43(*dpy-7p*). The sequences for all primers used in this procedure can be found in Supplementary Table 2 and the maps of pSW40, pSW42, and pSW43 can be found in Supplementary Figure 10.

Generation of MosSCI transgenic lines

MosSCI single-copy insertions (into the *ttTi5605* Mos1 allele, near the center of chromosome II) were obtained using the protocol previously described on the “wormbuilder” website (<http://www.wormbuilder.org/>). For each plasmid construct, MosSCI transformation was generally conducted using the direct injection approach (Frøkjær-Jensen et al., 2012), and also using the approach employing extrachromosomal array intermediates and ivermectin selection for insertion (Shirayama et al., 2012). To prepare plasmids for injection, the following plasmids were purified using a midiprep kit (Qiagen, Cat.12143): pSW40, pSW42, pSW43, pCFJ601 (*eft-3p::transposase*), pMA122 (*hspp::peel-1*), pGH8 (*rab-3p::mCherry*), pCFJ90 (*myo-2p::mCherry*), pCFJ104 (*myo-3p::mCherry*), pJL44 (*hsp-16.48p::MosTase::glh-2* 3'UTR), pCCM416 (*myo-2p::avr-15*), and pRF4 (*rol-6(su1006)*). For the direct injection method, injection mixtures consisted of pCFJ601 (50 ng/ul), pMA122(10 ng/ul), pGH8(10 ng/ul), pCFJ90(2.5 ng/ul), pCFJ104(5 ng/ul), and one of the transgene-containing plasmids (pSW40, pSW42, or pSW43(25 ng/ul); the mixture was injected into EG4322 hermaphrodites and injected animals were placed singly onto NGM plates seeded with HB101. Following 7~10 days of incubation at 25 °C, cultures were heat-shocked (35 °C, 1 hr) to kill any worms with an extrachromosomal array and surviving animals were cloned. After allowing them to produce progeny, worms are genotyped to identify single copy transgene inserted strains. For the approach using ivermectin selection, we injected a mixture of pJL44(50 ng/ul), pCCM416(50 ng/ul), and pRF4(50 ng/ul) with one of pSW40, pSW42, or pSW43(15 ng/ul) into WM186 hermaphrodites. After allowing the progeny of injected animals to grow for multiple generations, they were heat-shocked (35 °C, 1 hr) to induce heat shock promoter driven transposase expression from extra chromosomal arrays, and single single-copy transgene inserted strains were selected by ivermectin resistance (10 ng/ml) against extrachromosomal arrays. We obtained VT3702 by the direct injection method, and VT3486 and VT3392 by the extrachromosomal array intermediate method. We crossed those strains to VT2929 to obtain VT3703, VT3517 and VT3516 (Supplementary Table 1).

Mosaic analysis

A transgenic strain carrying an extrachromosomal array expressing wild type *lin-28* and *sur-5::GFP* (VT3884: *lin-28(n719)*; *syls63[cog-1::GFP]*; *maEx265[lin-28(+); sur-5::GFP]*, was generated by injection of VT2929 with a mixture of plasmids pSW40 (20ug/ml) and pTG96 (100ug/ml) (Yochem et al., 1998). Populations of VT3884 animals at the 4th stage or young adult stage were screened using fluorescence microscopy to identify animals with either of two classes of mosaic patterns of *sur-5::GFP* expression (Figure 8A, 8B). Class I: Absence of detectable *sur-5::GFP* in the hypodermis, along with *sur-5::GFP* expression retained in Z1 and Z4 (corresponding to presumed loss of the extrachromosomal array in the AB lineage, and a second loss of the array in the P2 or C blastomere). Class II: Absence of *sur-5::GFP* in Z1 and Z4, with *sur-5::GFP* expression retained in the hypodermis (consistent with loss of the extrachromosomal array in a precursor of Z1 and Z4, with retention of the array at least in the AB lineage). Sp-Ut valve core morphology was scored for each animal based on *cog-1::GFP* expression.

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Competing interests

No competing interests exist.

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Table 1. Ratio of egg laying and egg laying defective progeny from *lin-28* heterozygous mutants

<i>lin-28</i> (+) / <i>lin-28</i> (-) heterozygote	Egg laying progeny (n)	Egg laying defective progeny (n)	Ratio (Egg laying / Egg laying defective)
#1	133	43	3.09
#2	108	40	2.70
#3	89	31	2.87
#4	71	27	2.63
#5	142	46	3.09
#6	111	41	2.71
#7	102	35	2.91
#8	131	42	3.12
Average			2.89
Stdev			0.20

Figures

Figure 1. *lin-28(lf)* hermaphrodites have reduced brood size and exhibit defects both in embryo production and embryonic viability.

(A) Number of live progeny per hermaphrodite for wild type (strain N2) and for *lin-2(e1309)* and *lin-28(n719)* mutants at 25°C. *lin-2(e1309)* mutants are used as egg-laying defective controls; both *lin-2(e1309)* and *lin-28(n719)* hermaphrodites are unable to lay eggs. (B) Number of embryos per hermaphrodite for *lin-28(n719)* and *lin-2(e1309)* mutants at 25°C (dissected from gravid adults 60 hr after initiation of synchronized larval development by feeding starved L1s). (C) Viability of mutant embryos dissected from *lin-28(n719)* and *lin-2(e1309)* hermaphrodites at 25°C. % viability = 100×(viable hatched larvae/total embryos). (Number of animals ≥15 per each assay; number of independent replicate assays = 11 for *lin-28(n719)*, 4 for *lin-2(e1309)*) (A-C: Data are shown as mean ± SD, unpaired t-test, ****p<0.0001)

Figure 2. *lin-28(lf)* hermaphrodites have defects in exit of embryos from the spermatheca, and defects in ovulation.

(A, B) Spermatheca labeled by *fkh-6p::GFP* of representative wild-type and *lin-28(n719)* adult hermaphrodites. (A) In the wild type, oocytes (Ooc) pass into the spermatheca (Sp), where they are fertilized, and rapidly exit as a one-cell embryo (Emb), and so most spermathecae are not observed to contain an embryo. (B) In a *lin-28(n719)* hermaphrodite, an embryo (around ~150 cells) was trapped in the spermatheca. (C, D) DAPI staining of oocytes in wild-type (C) and *lin-28(n719)* hermaphrodites. (C) In the wild type, individual oocytes (Ooc) contained a haploid complement of condensed chromosomes (arrows). (D) In the *lin-28(n719)* mutant, endomitotic DNA was evidenced by an excessively bright DAPI signal in the oviduct before spermathecae, a characteristic of ovulation defective mutants. (Scale bar = 10 μ m in this paper, except where noted). (E) Distribution of estimated maximum duration of 1st spermathecal transit to uterus in wild type and *lin-28(lf)* mutants (See Material and Methods)

Figure 3. *lin-28(lf)* mutants show essentially normal spermathecal primordium structure, but display defects in spermathecal-uterine (Sp-Ut) valve morphology.

Spermathecal primordium visualized by *fkh-6p::GFP* expression (A, B) and Sp-Ut valve core structure visualized by *cog-1::GFP* expression (C, D) in wild-type and *lin-28(n719)* hermaphrodites at successive times in the advancement towards the first ovulation. In each panel, the upper images are of hermaphrodites at the L4 or early young adult stage, and the lower images are of hermaphrodites somewhat later in development, just before the time of first ovulation. (*lin-28(n719)* hermaphrodites skipped one larval stage; therefore, the "4th stage" corresponds to L4 and adult stage in wild-type [See Fig 6].) (A, B) In both wild type and *lin-28(n719)* mutants, similar tube-shaped spermathecal primordia were detected, which contracted horizontally to form similar sac-like structures in older adults (A, B lower panels). (C, D) Sp-Ut valve core structure (Sp-Ut, dashed oval) labeled by *cog-1::GFP* at successive stages in L4-adult developmental progression of a wild type and *lin-28(n719)* hermaphrodite. (C) In wild type, the Sp-Ut valve core stretches to form a fully developed "dumbbell" structure, with one side residing in the spermatheca and the other side in the uterus. The distance between each side shown here was ~10 μ m. (D) In *lin-28(n719)* mutants, the Sp-Ut valve core does not stretch and remained as "single lobe" structure, indicating an abnormal connection between the spermatheca and uterus in the mutants

Figure 4. *lin-28(lf)* embryos are misshapen and are defective in eggshell integrity.

(A) DIC images of wild-type, *lin-2(e1309)* and *lin-28(n719)* embryos from dissected adult animals. Wild type and *lin-2(e1309)* mutants produce ovoid embryos, but *lin-28(n719)* embryos exhibit irregular shapes. (B) *cbd-1::mCherry* expression indicates that eggshells were present in *lin-28(n719)* embryos despite their misshapen morphology (lower panels). (C) Eggshell permeability of embryos produced by *lin-2(e1309)*, *lin-28(n719)*, *egg-1(tm1071)*, and *fln-1(tm545)* hermaphrodites. Eggshell mutant *egg-1(tm1071)* served as a control. Embryos from *lin-28(n719)*, *fln-1(tm545)*, and *egg-1(tm1071)* were more permeable to the lipophilic dye FM 4-64 than *lin-2(e1309)* embryos. Like *lin-28(n719)*, *fln-1(tm545)* hermaphrodites exhibit defects in spermathecal exit. Permeability was calculated as the percentage of permeable embryos/total embryos from dissected adult animals (Number of animals ≥ 15 per each assay; number of independent replicate assays = 3 for each strain, Data are shown as mean $\pm$ SD. unpaired t-test compared to *lin-2(e1309)*, * $p < 0.05$). (D) Morphology of embryos under different osmotic conditions. *lin-28(n719)* embryos were more sensitive than wild type to low salt conditions, indicating a lack of protection from osmotic stress. (E) Model for physiological causes of fertility defects in *lin-28(lf)* mutants. *lin-28(lf)* animals have abnormal Sp-Ut valve structure, which leads to defects in spermathecal exit and ovulation, and hence a reduced embryo production. In addition, retention of embryos in the spermatheca compromises eggshell integrity, which causes embryonic lethality.

Figure 5. Genetic epistasis analysis of *lin-28* and other heterochronic genes for effects on fertility, embryonic lethality, and Sp-Ut valve morphogenesis.

(A) Total number of viable larva progeny of *lin-2(e1309)*, *lin-28(n719)*, *lin-28(n719);let-7(mn112)*, *lin-28(n719);lin-29(n836)*, *lin-46(ma164);lin-28(n719)* mutants, post-dauer *lin-28(n719)*, and wild type. (B) Embryo production of *lin-2(e1309)*, *lin-28(n719)*, *lin-28(n719);let-7(mn112)*, *lin-28(n719);lin-29(n836)* mutants. (C) Embryonic viability of *lin-2(e1309)*, *lin-28(n719)*, *lin-28(n719);let-7(mn112)*, *lin-28(n719);lin-29(n836)*, *lin-46(ma164);lin-28(n719)* mutants, post-dauer *lin-28(n719)*, and wild type. (Number of animals ≥ 15 per each assay; each dot represents independent replicate assay) The reduction in the number of progeny of *lin-28(n719)* mutants is increased by loss of *let-7*, *lin-29* or *lin-46*. (A). Both embryo production (B) and embryo viability (C) are improved by loss of *let-7*, *lin-29* or *lin-46*. Total number of progeny and embryonic viability of *lin-28(lf)* mutants are also improved by post-dauer development (A,C). (A-C: Data are shown as mean $\pm$ SD, unpaired t-test compared to *lin-28(lf)*, NS; not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.)

(D) The percentage of animals with normal Sp-Ut valve core morphology, visualized by *cog-1::GFP* expression in wild type, *let-7(mn112)*, *lin-46(ma164)* and *lin-29(n836)* mutants, treated with control RNAi

(EV, empty vector strain L4440), *lin-28*(RNAi) or *hbl-1*(RNAi). Wild type, *lin-46*(*ma164*), and *lin-29*(*n836*) mutants treated with L4440 empty vector RNAi rarely showed Sp-Ut valve defects. *let-7*(*mn112*) mutants with control RNAi showed less than 10% of Sp-Ut valve defects. *lin-28*(RNAi) treatment of wild type led to ~95% Sp-Ut valve defects, an effect that was partially or fully suppressed by *let-7*(*mn112*), *lin-46*(*ma164*) and *lin-29*(*n836*). *hbl-1*(RNAi) treatment of wild type also led to Sp-Ut valve defects that were rarely suppressed by *let-7*(*mn112*) and *lin-46*(*ma164*) and moderately suppressed (~70%) by *lin-29*(*n836*). (Number of animals ≥ 12 per each assay; each dot represents independent replicate assay, Data are shown as mean $\pm$ SD, unpaired t-test, p-values denoted as asterisks or “NS” were calculated by each mutant compared to wild type in each RNAi set. NS; not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) (E) The genetic regulatory pathway model for somatic gonadal morphogenesis, derived from the results of epistasis experiments presented in Fig 5A–D, is highly similar to the model for temporal regulation of hypodermal cell fates derived from previous studies (Ambros, 2011; Resnik & Rougvié 2010, See Discussion section regarding possible involvement of *lin-41* in somatic gonadal morphogenesis). We suggest that the specification of proper hypodermal developmental timing by these genes is essential for somatic gonadal morphogenesis.

Figure 6. Heterochronic development of the hypodermis, relative to the somatic gonad, in *lin-28(lf)* hermaphrodites.

(A, B, D, E) *cog-1::GFP* expression patterns in wild type and *lin-28(n719)* hermaphrodites at indicated developmental stages. The temporal order of the onset of *cog-1::GFP* expression in the somatic gonad and vulva is reversed in *lin-28(lf)* compared to the wild type. *cog-1::GFP* is expressed in both somatic gonadal tissues (uterus and Sp-Ut valve) and hypodermal tissue (vulva). The timing of expression in wild type and *lin-28(n719)* mutants is summarized in (C). In wild type, *cog-1::GFP* is expressed in somatic gonadal tissues at the late 3rd larval stage (A) and in the vulva in the middle of the 4th larval stage (B). In *lin-28(n719)* mutants, vulval expression of *cog-1::GFP* occurs precociously in the middle of the L3 stages (D), while somatic gonadal expression of *cog-1::GFP* starts at the normal time, in the late 3rd larval stage (E). Both vulval and somatic gonadal expression are detected at 3rd lethargus (E). (F) Model for the importance of *lin-28* activity for somatic gonadal development: In wild type animals, timing of hypodermis development and somatic gonadal development are synchronized. In *lin-28(lf)* mutants, hypodermal development happens precociously while somatic gonadal development does not, resulting in heterochronic development between two tissues. We suggest this heterochrony causes somatic gonadal morphogenesis defects.

Figure 7. Hypodermal expression of *lin-28* rescues developmental timing defects and Sp-Ut valve morphogenesis in *lin-28(lf)* mutants.

cog-1::GFP expression in the somatic gonad and vulva in late 3rd or early 4th larval stage hermaphrodites (A-E), or in the Sp-Ut valve in adult or 4th stage hermaphrodites (F-J), of the wild type (A, F), *lin-28(lf)* (B, G), and *lin-28(lf)* with *lin-28* expression driven by various promoters (C-E, H-J). *dpy-7p* (D, I) is a hypodermal-specific promoter (Gilleard et al., 1997) and *ehnl-3Ap* (E, J) is an early somatic gonadal-specific promoter (Large and Mathies, 2010). Note: In these experiments, the promoter-driven *lin-28* transgene (C-E, H-J) is also tagged with GFP, but *lin-28::GFP* expression is not detectable at these stages, so all the GFP signal shown here corresponds to *cog-1::GFP*. *lin-28p::lin-28::GFP;lin-28(n719)* (C) and *dpy-7p::lin-28::GFP;lin-28(n719)* (D) expresses *cog-1::GFP* in the somatic gonad earlier than in the vulva, as in wild type (A). In contrast, both *lin-28(n719)* mutants (B) and *ehnl-3Ap::lin-28::GFP;lin-28(n719)* (E) expresses *cog-1::GFP* precociously in the vulva. 83% of *lin-28p::lin-28::GFP;lin-28(n719)* (H) and 95% of *dpy-7p::lin-28::GFP;lin-28(n719)* (I) animals restore the Sp-Ut core dumbbell structure observed in wild type (F). However, the Sp-Ut valve core structure remains as a single lobe shape in both *ehnl-3Ap::lin-28::GFP;lin-28(n719)* (J) and *lin-28(n719)* animals (94% and 98% respectively) (G). These data suggest that hypodermal expression of *lin-28* is sufficient to rescue heterochronic development (A-E) and abnormal Sp-Ut valve core morphogenesis (F-J) in *lin-28(n719)* mutants, but somatic gonadal expression of *lin-28* cannot rescue either defect.

Figure 8. *lin-28(lf)* mosaic analysis for Sp-Ut valve morphogenesis

(A) (B) Summary of mosaic expression patterns of *sur-5::GFP* in VT3884 animals where the extrachromosomal array *maEx265[sur-5::GFP; lin-28p::lin-28::GFP]* was lost either (A) in a precursor of Z1 and Z4, or (B) in hypodermal lineages (the AB lineage, and probably also in P2 or C). (See Fig. S9 for more detailed analysis of individual mosaic animals and non-mosaic controls). The observed *sur-5::GFP* expression pattern for these two classes of mosaic animals are color coded: Green, confirmed *sur-5::GFP* expression; Black, Confirmed absence of *sur-5::GFP* expression; Light green, *sur-5::GFP* expression not determined. Dotted lines represent multiple cell divisions not shown in these abbreviated cell lineage diagrams. (Note: Expression in the E lineage was determined in some mosaic animals, but was not relevant to Sp-Ut valve core morphogenesis; see Fig. S9B and S9C). (C) Summary of the phenotypes exhibited by Class I and Class II mosaic animals and non-mosaic controls. Presence of wild type Sp-Ut valve core morphology was determined in mosaic animals (A, B) as well as in the control animals which contained or completely lost extrachromosomal arrays in both hypodermal and somatic gonadal lineages (Fig. S9A and S9D). (Chi-square test, NS; not significant, **** $p < 0.0001$)

Figure 9. Hypodermal expression of *lin-28* enhances embryonic viability and number of progeny of *lin-28(lf)* mutants.

(A) Embryonic viability of *lin-28p::lin-28::GFP;lin-28(n719)* and *dpy-7p::lin-28::GFP;lin-28(n719)* are restored to a level similar to that in wild type or *lin-2(e1309)* mutants. However, expression of *ehnl-3Ap::lin-28::GFP* does not enhance the viability of *lin-28(n719)* embryos. (Number of animals ≥ 15 per each assay; number of independent replicate assays = 4 for *lin-28(n719)*, 3 for all other strains.) (B) The number of live larva progeny is increased in *lin-28p::lin-28::GFP;lin-28(n719)* ($n=36$) and slightly enhanced in *dpy-7p::lin-28::GFP;lin-28(n719)* ($n=53$) compared to *lin-28(n719)* mutants ($n=25$). Progeny numbers for *dpy-7p::lin-28::GFP* insertional lines without *lin-28(n719)* ($n=11$) are similar to those of *dpy-7p::lin-28::GFP;lin-28(n719)* (p value=0.80), suggesting that expression of *dpy-7p::lin-28::GFP* induces fertility defects regardless of *lin-28(n719)*. Progeny numbers for *ehnl-3Ap::lin-28::GFP;lin-28(n719)* ($n=40$) are similar to those of *lin-28(n719)* mutants. (A,B: Unpaired t-test compared to *lin-28(lf)*, NS; not significant, * $p<0.05$, **** $p<0.0001$) (C) Embryos of *dpy-7p::lin-28::GFP* (left panel) and *dpy-7p::lin-28::GFP;lin-28(n719)* (right panel) are trapped inside adult hermaphrodites, indicating that defective egg laying is a cause of the reduced fertility in these animals. (Scale bar = 100 μ m).

Supplementary Information

Supplementary Figure 9 is moved to supplementary figure 2, and other supplementary figures are renumbered accordingly

The *C. elegans* heterochronic gene *lin-28* coordinates the timing of hypodermal and somatic gonadal programs for hermaphrodite reproductive system morphogenesis

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Figure S1. Fertility phenotypes of *lin-28(lf)* mutants at 20°C and 25°C.

(A) Total number of live larval progeny per animal for wild-type N2 animals ($n=5$), *lin-2(e1309)* mutants ($n=17$ for 25°C, $n=16$ for 20°C) and *lin-28(n719)* ($n=19$) mutants at 20°C and 25°C. (B) Embryonic lethality for *lin-2(e1309)* and *lin-28(n719)* mutants at 20°C and 25°C. (Number of animals ≥ 15 per each assay; number of independent replicate assays = 3) (C) The number of embryos produced at varying time points after feeding of synchronized L1 larvae, for *lin-2(e1309)* and *lin-28(n719)* mutants at 20°C and 25°C. ($n=10,8,8$ (*lin-2(e1309)*), $12,15,13$ (*lin-28(n719)*) for 61hr, 69hr, and 77hr respectively at 20°C. $n=12,10,10,0$ (*lin-2(e1309)*), $15,13,12,3$ (*lin-28(n719)*) for 48hr, 53hr, 60hr and 69hr respectively at 25°C.) (A-C: Data are shown as mean $\pm$ SD. Unpaired t-test compared to *lin-2(lf)*, **** $p<0.0001$)

Figure S2. Reduction of *lin-28* function in the soma decreases fertility of hermaphrodites more than does reduction of *lin-28* function in the germline.

Wild type, *rrf-1(pk1417)* mutants, and *ppw-1(pk2505)* mutants were fed with *lin-28* RNAi and empty vector RNAi, and the number of progeny produced by each animal was determined. *ppw-1(pk2505) lin-28(RNAi)* animals all exhibited reduced fertility similar to *lin-28(RNAi)* animals. However, *rrf-1(pk1417) lin-28(RNAi)* animals were, overall, less affected by *lin-28(RNAi)*, with only a minority of animals exhibiting reduced fertility. (Error bar shows standard deviation. unpaired t-test, each strain with *lin-28(RNAi)* was compared to corresponding strain with empty vector(RNAi). NS; not significant, *****p*<0.0001.)

Figure S3. Both wild type and *lin-28(lf)* mutants have two nuclei that comprise Sp-Ut valve core syncytium.

Confocal microscopic images of Sp-Ut valve core syncytium labeled by *cog-1::GFP*. Both wild type animals (A) and *lin-28(lf)* mutants (B) exhibit two nuclei in their Sp-Ut valve core region (dashed oval), suggesting cell division in the Sp-Ut valve core occurs normally in *lin-28(lf)* mutants. (All Scale bars = 10 μm hereafter)

Figure S4. Defective formation of the uterine lumen in *lin-28(lf)* mutants is restored by the loss of function of *lin-28* downstream genes, or post-dauer development, or hypodermal *lin-28* expression.

(A-C) Uterine lumen formation in wild type and *lin-28(n719)* mutants. (A) Wild-type animals form a long uterine lumen between the dorsal uterus and ventral uterus in the mid L4 stage (red dashed oval), whereas, (B) the majority of *lin-28(n719)* mutants at an analogous point in 4th stage development exhibit an immature, partially-formed lumen. (C) Some 4th stage *lin-28(n719)* mutants show a connected lumen, which is shorter and rounder than that in wild-type animals. (D-F) Uterine lumen formation in *lin-28(n719);let-7(mn112)*, *lin-28(n719);lin-29(n836)*, and *lin-28(n719);lin-46(ma164)* double mutants. (D) Lumen formation is restored in ~50% of *lin-28(n719);let-7(mn112)* mutants. (E) The majority of *lin-28(n719);lin-29(n836)* animals, and (F) the majority of *lin-28(n719);lin-46(ma164)* mutants show uterine lumen formation similar to the wild type. (G) *lin-28(n719)* mutants form an elongated uterine lumen similar to wild type after post-dauer development. (H-K) Rescue of *lin-28(lf)* uterine lumen phenotype by transgenes expressing LIN-28 driven by specific promoters. *lin-28p::lin-28::GFP;lin-28(n719)* (H) and *dpy-7p::lin-28::GFP;lin-28(n719)* (I) show normal uterine lumen formation as wild-type animals. *ehn-3Ap::lin-28::GFP;lin-28(n719)* show only partial uterine lumen (J) or shorter and rounder uterine lumen (K) similar to the defects exhibited by *lin-28(n719)* alone (B and C, respectively).

egl-13p::GFP

lin-28(lf);egl-13p::GFP

Post-dauer lin-28(lf);egl-13p::GFP

lin-28(lf);let-7(lf);egl-13p::GFP

lin-28(lf);egl-13p::GFP;lin-46(RNAi)

lin-28p::lin-28::GFP; lin-28(lf);egl-13p::GFP

dpy-7p::lin-28::GFP;lin-28(lf);egl-13p::GFP

ehn-3Ap::lin-28::GFP;lin-28(lf);egl-13p::GFP

Figure S5. Defective migration of uterine seam cell (utse) nuclei in *lin-28(lf)* mutants is rescued by loss of function of *lin-28* downstream genes, or by post-dauer development, or by hypodermal *lin-28* expression. (A-D) utse nuclei, labeled by *egl-13p::GFP*, in wild type and *lin-28(n719)* mutants. *egl-13p::GFP* expression in the utse region (dashed oval) is shown in early L4 (A) and mid-L4 (B) of the wild-type, and at analogous steps (C, D) of 4th stage *lin-28(n719)* animals. utse nuclei expressing *egl-13p::GFP* migrate laterally during the early to mid L4 stage in wild type (A, B), but no such migration occurs in *lin-28(n719)* mutants (C, D). (E,F) utse migration defects of *lin-28(n719)* mutants are suppressed when the mutants develop via post-dauer stages. (G-J) Loss of function of *let-7* or *lin-46* can partially suppress the utse migration defects in *lin-28(lf)* mutants. (G,H) *lin-28(n719);let-7(mn112)* mutants show a more normal migration of utse nuclei compared to *lin-28(n719)* mutants (C, D). (I,J) utse nuclei of *lin-28(n719)* mutants migrated laterally when *lin-46* function was knocked down by RNAi. (K-P) Rescue of *lin-28(lf)* utse migration phenotype by transgenes expressing LIN-28 driven by specific promoters. *lin-28* expression with *lin-28* endogenous promoter (*lin-28p::lin-28::GFP;lin-28(n719);egl-13p::GFP*) restores utse migration as wild type (K, L). Hypodermal expression of *lin-28* (*dpy-7p::lin-28::GFP;lin-28(n719);egl-13p::GFP*) also rescues utse migration defects in *lin-28(n719)* mutants (M, N), whereas *lin-28* expression driven by early somatic gonadal promoter (*ehn-3Ap::lin-28::GFP;lin-28(n719);egl-13p::GFP*) does not rescue the phenotype (O, P). (Note: In these experiments (K-P), the promoter-driven *lin-28* transgene is also tagged with GFP, but *lin-28::GFP* expression is not detectable at these stages, so all the GFP signal here corresponds to *egl-13p::GFP*.)

Figure S6. Defective Sp-Ut valve morphogenesis, uterine lumen formation, and utse migration are not results of the abnormal vulval morphogenesis of *lin-28(lf)* animals.

(A, B) Sp-Ut valve core structure (dashed oval) visualized by *cog-1::GFP* in *lin-2(e1309)* and *lin-2(e1309);lin-28(n719)* animals. (A) The "dumbbell" structure of Sp-Ut valve core cell is shown in *lin-2(e1309)* mutants like wild type animals (Fig 3C). (B) *lin-2(e1309);lin-28(n719)* mutants exhibit the "single-lobe" structure of Sp-Ut valve core, similar to *lin-28(n719)* mutants (Fig 3D). (C, D) Uterine lumen formation (dashed oval) visualized by DIC in *lin-2(e1309)* and *lin-2(e1309);lin-28(n719)* animals. (C) Fully extended and connected uterine lumen (red-dashed oval) is formed in *lin-2(e1309)* mutants like wild type animals (Fig S3A). (D) By contrast, *lin-2(e1309);lin-28(n719)* mutants form only partial uterine lumen, similar to *lin-28(n719)* (Fig S3B). (E, F) utse nuclei (surrounded by a dashed line) are labeled by *egl-13p::GFP* in *lin-2(e1309)* and *lin-2(e1309);lin-28(n719)* animals. (E) utse migration appears normal in *lin-2(e1309)* (Fig S4B), whereas (F) utse nuclei remain tightly clustered in *lin-2(e1309);lin-28(n719)* animals, similar to *lin-28(n719)* (Fig S4D).

Figure S7. Sp-Ut valve core cell morphology *hbl-1(lf)*, *lin-41(lf)* and *lin-14(lf)* mutants, and post-dauer suppression of *lin-28(lf)* morphological defects.

(A-E) Sp-Ut valve core morphology is visualized by *cog-1::GFP*, shown alone (right panel), and overlaid with DIC (left panel). (A) Animals treated with empty vector RNAi have normal Sp-Ut morphology. (B) *hbl-1(RNAi)* animals. (C) The Sp-Ut valve of *lin-41(RNAi)* animals appeared essentially normal in morphology, except for a somewhat reduced size. (D) *lin-14(n179)* exhibit abnormal Sp-Ut morphology. (E) Post-dauer development of *lin-28(n719)* mutants restored an Sp-Ut valve morphology similar to wild type animals.

Figure S8. Both *lin-28* endogenous promoter and *ehn-3A* promoter drives early somatic gonadal expression of *lin-28::GFP*.

(A,B) Two *lin-28p::lin-28::GFP* L1 larvae where *GFP* expression was detected in Z1 and Z4 cell by spinning disk microscopy. (C,D) Z1 and Z4 expression of *lin-28::GFP* driven by early somatic gonadal promoter (*ehn-3Ap::lin-28::GFP*). *GFP* expression in Z1 and Z4 cells were detected by fluorescence microscopy beginning in late embryogenesis (C) also L1 larvae (D).

Figure S9. *lin-28(lf)* mosaic analysis for Sp-Ut valve morphogenesis

(A-D) Expression patterns of *sur-5::GFP* in VT3884 animals where the extrachromosomal array *maEx265[sur-5::GFP; lin-28p::lin-28::GFP]* was (A) retained in all hypodermal lineages, somatic gonad (Z1,Z4), and intestinal lineages, (B) lost in a precursor to Z1 and Z4, (C) lost in the hypodermal cell lineages, or (D) lost in all cell lineages. The observed *sur-5::GFP* expression patterns are color coded as in Figure 8: Green, confirmed *sur-5::GFP* expression; Black, Confirmed absence of *sur-5::GFP* expression; Light green, *sur-5::GFP* expression not determined. Dotted lines represent multiple cell divisions not shown in these abbreviated cell lineage diagrams. (E-T) Representative DIC or GFP images of animals in each of the indicated classes of animals (A-D). Note that *sur-5::GFP* is expressed only in nucleus and is hence distinctive from *cog-1::GFP* which is expressed also in the cytoplasm. (A, E-H) This category of animals expressed *sur-5::GFP* in (E, F) spermathecal and uterine cells (*), and also in (G, H) hypodermal cells (arrows). Of 44 animals which belong to this category, 43 animals showed dumbbell-shaped Sp-Ut valve core as wild type (F, dotted outline). (B, I-L) These mosaic animals expressed *sur-5::GFP* in (K, L) hypodermal cell lineages (arrows), but not in (I, J) somatic gonadal lineages. We found 12 mosaic animals showing this pattern and all showed wild type dumbbell-shaped Sp-Ut valve core seen by *cog-1::GFP* (J, dotted outline). 5 animals did and 6 animals did not retain the array in intestinal cell lines. We didn't determine intestinal expression of the array in 1 animal. (C, M-R) In these mosaic animals *sur-5::GFP* was expressed in (M, N) the Z1 and Z4 lineages, but was not detected in (Q, R) the hypodermal lineages. (Q) DIC image of the animal in (R); arrows indicate hypodermal cell nuclei. (R) Merged image of DIC and GFP of the same animal. GFP signals do not match with nucleus of hypodermal cells (dotted outlines) suggesting that GFP signals were not from hypodermal cells. We found 8 mosaic animals in this category, and 7 animals did not have wild type dumbbell-shaped Sp-Ut valve core (P, dotted outline). (Of those 7 animals, 6 animals expressed *sur-5::GFP* in the intestinal (E) cell lineage, and 1 did not express *sur-5::GFP* in the intestine.) 1 animal showed wild type dumbbell-shaped Sp-Ut valve core in anterior somatic gonadal region, but not in posterior region. This animal expressed *sur-5::GFP* in the intestinal region. (D, S, T) Animals that did not express *sur-5::GFP* in any cells: 28 out of 30 such animals showed the single-lobe shaped abnormal Sp-Ut valve core morphology characteristic of *lin-28(lf)* mutants(S,T).

Figure S10. Vector maps of pSW40, pSW42, and pSW43 injected to make transgenic animals.

Table S1. *C. elegans* strains used in this study

Strain Name	Genotype
N2	
VT2932	<i>lin-28(n719)I</i>
CB1309	<i>lin-2(e1309)X</i>
PS3662	<i>syIs63[cog-1::GFP + unc-119(+)]</i>
DZ325	<i>ezIs2[ffkh-6p::GFP + unc-119(+)]</i> ; III; <i>him-8(e1489) IV</i>
VT2929	<i>lin-28(n719)I</i> ; <i>syIs63</i>
VT2930	<i>lin-28(n719)I</i> ; <i>ezIs2</i>
VT866	<i>lin-28(n719)I</i> ; <i>let-7(mn112) unc-3(e151) X</i>
MT2001	<i>lin-28(n719)I</i> ; <i>lin-29(n333) II</i>
VT937	<i>lin-28(n719)I</i> ; <i>lin-46(ma164) V</i>
AG212	<i>unc-119(ed3)</i> ; <i>avIs143 [pDNL10 (unc-119(+)) + cbd-1p::CBD-1::mCherry::cbd-1 3'UTR]</i>
VT3454	<i>lin-28(n719)</i> ; <i>avIs143</i>
UN0810	<i>fln-1(tm545)</i>
VT3660	<i>lin-14(n179)</i> ; <i>syIs63</i>
VT3730	<i>let-7(mn112)</i> ; <i>mnDP1</i> ; <i>syIs63</i>
VT3580	<i>lin-29(n836)</i> ; <i>syIs63</i>
VT3581	<i>lin-46(ma164) mals105 [col-19::GFP]</i> ; <i>syIs63</i>
MH1319	<i>kuls29 [egl-13p::GFP+unc-119(+)]</i>
VT3661	<i>lin-28(n719)</i> ; <i>kuls29</i>
VT3731	<i>lin-28(n719)</i> ; <i>let-7(mn112)</i> ; <i>kuls29</i>
VT3665	<i>lin-2(e1309)</i> ; <i>syIs63</i>
VT3664	<i>lin-2(e1309)</i> ; <i>lin-28(n719)</i> ; <i>syIs63</i>
VT3733	<i>lin-2(e1309)</i> ; <i>kuls29</i>
VT3732	<i>lin-2(e1309)</i> ; <i>lin-28(n719)</i> ; <i>kuls29</i>
EG4322	<i>ttTi5605 II</i> ; <i>unc-119(ed3) III</i>
WM186	<i>avr-14(ad1302) I</i> ; <i>ttTi5605 II</i> ; <i>unc-119(ed3) III</i> ; <i>avr-15(ad1051) glc-1(pk54) V</i>
VT3392	<i>avr-14(ad1302) I</i> ; <i>mals402[unc-119(+); ehn-3Ap::lin-28::GFP::lin-28 3'UTR]</i> ; <i>unc-119(ed3) III</i> ; <i>avr-15(ad1051) glc-1(pk54) V</i>
VT3486	<i>avr-14(ad1302) I</i> ; <i>mals403[unc-119(+); lin-28p::lin-28::GFP::lin-28 3'UTR]</i> ; <i>unc-119(ed3) III</i> ; <i>avr-15(ad1051) glc-1(pk54) V</i>
VT3702	<i>mals409[unc-119(+); dpy-7p::lin-28::GFP::lin-28 3'UTR]</i> ; <i>unc-119(ed3) III</i>
VT3517	<i>lin-28(n719)</i> ; <i>mals402</i> ; <i>syIs63</i>
VT3516	<i>lin-28(n719)</i> ; <i>mals403</i> ; <i>syIs63</i>
VT3703	<i>lin-28(n719)</i> ; <i>mals409</i> ; <i>syIs63</i>
VT3734	<i>lin-28(n719)</i> ; <i>mals402</i> ; <i>kuls29</i>
VT3735	<i>lin-28(n719)</i> ; <i>mals403</i> ; <i>kuls29</i>
VT3736	<i>lin-28(n719)</i> ; <i>mals409</i> ; <i>kuls29</i>
NL2098	<i>rrf-1(pk1417)</i>
NL2550	<i>ppw-1(pk2505)</i>
VT3884	<i>lin-28(n719)I</i> ; <i>syIs63</i> ; <i>maEx265[sur-5::GFP; lin-28p::GFP; lin-28 3'UTR]</i>

Table S2. Primer sequences used in this study

Primers	Sequences	
1 lin-28(n719) genotyping F	ttataataaaagtcggag	sequencing to
2 lin-28(n719) genotyping R	ccttcagtcctgtcctctac	confirm n719
3 let-7(mn112) genotyping F	gataccatggaggacgacgg	WT :476 bp
4 let-7(mn112) genotyping R	gtagaaaattgcatagtca	mn112: 263 bp
5 lin-29(n836) genotyping F	ggcttatcagttgatggca	WT:273bp
6 lin-29(n836) genotyping R	ccgcgcaattccggaatc	n836: 200bp
7 lin-46(ma164) genotyping F	gaattcaagattcctactgtag	sequencing to
8 lin-46(ma164) genotyping R	gaaatcacgacaattgtagacattg	confirm ma164
9 lin-14(n179) genotyping F	gaaacagctccaccactc	sequencing to
10 lin-14(n179) genotyping R	gttctgacactggtcgg	confirm n179
11 attB4+ lin-28 promoter F	ggggcaacttgtatagaaaagttgga ttctggtaaaactcttcaagc	lin-28 Promoter:PCR amplified,
12 attB1r+ lin-28 promoter R	ggggctgctttttgtacaaaacttgt cctgaaaaagatttttaaaatttt	followed by BP reaction with pDONR P4P1r
13 attB4+ dpy-7 promoter F	ggggcaacttgtatagaaaagttgga aatctcattccacgattctt	dpy-7 promoter: PCR amplified,
14 attB1r + dpy-7 promoter R	ggggctgctttttgtacaaaacttgt ttatctggaacaaaatgta	followed by BP reaction with pDONR P4P1r
15 attB4+ehn-3 promoter F	ggggcaacttgtatagaaaagttgga ctaatctagaaaaatacgcaca	enh-3A promoter:PCR amplified,
16 attB1r+ eh-3 promoter R	ggggctgctttttgtacaaaacttgt ttgttaattggaagctgg	followed by BP reaction with pDONR P4P1r
17 attB1+lin-28 gene F	ggggacaagttgtacaaaagcaggcttc gttcagcaatgcttttaatta	100bp+lin-28:GFP (P:Primer, T:template, PCR
18 lin-28 1st exon 3'(overlapping)	agggtgttgga cgaggcctctcgaaggaag	product:A~D)
19 lin-28 2nd exon 5'(overlapping)	gaggctccg tcaccaacacctcgatacttgg	1. A: (P) 20/18, (T) gDNA
20 lin-28 100bp of 5' upstream	gttcagcaatgcttttaatta	2. B: (P) 19/22, (T) gDNA
21 Primer for GFP fusionF	ggcgcgcctctagaggatc	3. C: (P)20/22 (T) A,B -> Removal of the first exon
22 Primer for GFP fusion R(Overlapping)	cggggatcctctagaggcgccgccc ttcatcagaggaattactattcttttc	4. D: (P) 21/23, (T) XW12 (Wei et al., 2012)
23 GFP R	ctattgtatagttcatccatgccca	5. E: (P) 17/24 (T) C,D ->GFP fusion
24 attB2+lin-28::GFP gene R	ggggaccacttgtacaagaagctgggtt ctattgtatagttcatccatgccca	6. BP reaction E with pDONR 221
25 attB2r+lin28_3UTR(5)	ggggcagcttctgtacaaaagtgga aatcatctagacactgagaata	lin-28 3'UTR:PCR amplified,
26 attB3 +lin-28_3UTR(3)	ggggcaacttgtataataaagttgt gccaaactgtgaggattgtaa	followed by BP reaction with pDONR P2R-P3
27 ttTi5605 genotyping F	tgacattgtcgaatgtcttc	ttTi5605: 1411bp
28 ttTi5605 genotyping R	gttatcagaagacggttacg	transgene inserted: 7kb<
29 ttTi5605 genotyping 2F	tctggctctgcttctcggtt	ttTi5605: 0bp
30 ttTi5605 genotyping 2R	caattcatcccggttctgt	transgene inserted: 1772bp

Movies

Movie 1. The first ovulation and spermathecal exit of a wild type animal

The first ovulation and spermathecal exit of a wild type animal (with *fkh-6p::GFP*, DZ325) was monitored using a time-lapse video taken for approximately 40 minutes. The first ovulation occurred around 20 minutes after the recording started, and spermathecal exit was completed approximately 15 minutes after the ovulation occurred. After the recording, a fertilized embryo was seen in the uterus.

Movie 2. The first ovulation and defective spermathecal exit in each gonad arm of a *lin-28(lf)* mutant

The first ovulation and following process of a *lin-28(lf)* mutant (with *fkh-6p::GFP*, VT2930) was monitored using a time-lapse video. Two consecutive videos of one animal were taken for approximately 40 minutes each, with ~2 minutes of interval in between. The first ovulation in the posterior arm and the anterior arm occurred around 1~2 minutes, and 9~10 minutes after the recording started, respectively. However, the spermathecal exits of both ovulated embryos to the uterus were not completed approximately 82 minutes after the recording. As a result, parts of both embryos were seen in the spermathecal region labeled by *fkh-6::GFP*.