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TGF-β and Insulin Signaling Regulate Reproductive Aging via Oocyte and Germline Quality Maintenance

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SUMMARY

Reproductive cessation is perhaps the earliest aging phenotype that humans experience. Similarly, reproduction of *Caenorhabditis elegans* ceases in mid-adulthood. Although somatic aging has been studied in both worms and humans, mechanisms regulating reproductive aging are not yet understood. Here, we show that TGF-β Sma/Mab and Insulin/IGF-1 signaling regulate *C. elegans* reproductive aging by modulating multiple aspects of the reproductive process, including embryo integrity, oocyte fertilizability, chromosome segregation fidelity, DNA damage resistance, and oocyte and germline morphology. TGF-β activity regulates reproductive span and germline/oocyte quality noncell-autonomously and is temporally and transcriptionally separable from its regulation of growth. Chromosome segregation, cell cycle, and DNA damage response genes are upregulated in TGF-β mutant oocytes, decline in aged mammalian oocytes, and are critical for oocyte quality maintenance. Our data suggest that *C. elegans* and humans share many aspects of reproductive aging, including the correlation between reproductive aging and declining oocyte quality and mechanisms determining oocyte quality.

INTRODUCTION

Many biological functions associated with quality of life decline with age, but female reproductive aging is one of the earliest declines humans experience. Although progressive loss of ovarian follicles leads to menopause between the ages of 45 and 55, the risk of infertility, birth defects, and miscarriage increase a decade earlier, likely because of age-related declines in oocyte quality (te Velde and Pearson, 2002). Although aged mammalian oocytes exhibit increased errors in fertilization, chromosome segregation, and cleavage divisions (Goud et al., 1999; te Velde and Pearson, 2002), little is known about mechanisms that regulate oocyte quality maintenance with age.

Caenorhabditis elegans is a useful model for aging studies because of its short life span and the conservation of longevity pathways from *C. elegans* to humans (Kenyon, 2005; Suh et al., 2008). Recently, *C. elegans* has also been developed as a model of reproductive aging (Andux and Ellis, 2008; Hughes et al., 2007; Luo et al., 2009). These studies established that (1) *C. elegans* reproductive aging is independent of sperm contribution; (2) simply reducing ovulation rate or progeny production do not extend reproductive span; and (3) reproductive aging is usage independent (i.e., independent of the magnitude and timing of oocyte use). That is, in worms as in humans, simply delaying the reproductive schedule does not delay reproductive aging.

One argument against using *C. elegans* as a model of human reproductive aging is that oocytes are continually produced in worms, whereas humans' total oocyte supply is produced at birth. However, both human and *C. elegans* females reproduce for about one-third to one-half of their lives, and thus undergo significant reproductive aging on proportional time scales, implying that genetic mechanisms may link reproduction to longevity in both organisms (Cant and Johnstone, 2008; Luo et al., 2009). Furthermore, both human and *C. elegans* oocytes are cell-cycle arrested at meiotic prophase I, release from arrest is coordinated with oocyte maturation in both, and the mechanisms underlying oocyte maturation are highly conserved between the two organisms (Greenstein, 2005; Mehlmann, 2005). Most importantly, human reproductive aging occurs a decade prior to the exhaustion of the oocyte supply, suggesting that oocyte quality, rather than quantity, is the limiting factor for successful reproduction with age. Thus, the critical question that we address in this study is whether worms' reproduction is similarly limited by oocyte quality, and if so, by what mechanisms.

Several long-lived *C. elegans* mutants, including the Insulin/IGF-1 receptor mutant *daf-2*, delay reproductive aging (Huang et al., 2004; Hughes et al., 2007; Luo et al., 2009). *daf-2* mutants extend life span, delay distal germline integrity decline, and extend reproductive span through the activity of the FOXO transcription factor DAF-16 (Garigan et al., 2002; Hughes et al., 2007; Kenyon et al., 1993; Luo et al., 2009), but the role of *daf-2* in oocyte quality maintenance and the mechanisms by which *daf-2* mutants extend reproductive span are unknown.

We recently found that mutants of the TGF-β Sma/Mab pathway also significantly extend reproductive span (Luo et al.,

2009), whereas mutants in the TGF-β Dauer pathway extend life span (Shaw et al., 2007) without greatly extending reproductive span (Luo et al., 2009). The TGF-β Sma/Mab pathway, which is highly conserved from worms to humans, consists of extracellular ligands (DBL-1), type I (SMA-6) and type II (DAF-4) receptors, R-Smads (SMA-2 and SMA-3), a co-Smad (SMA-4), and a transcription co-factor (SMA-9) (Massagué, 2000; Savage-Dunn, 2005). Notably, Sma/Mab regulation of reproductive span is genetically independent of Insulin/IGF-1 signaling (IIS) and Dietary Restriction (Luo et al., 2009).

Here we show that *C. elegans* oocytes, like human oocytes, degrade functionally and morphologically with age and that reduction of TGF-β Sma/Mab signaling and IIS delays reproductive aging by maintaining oocyte and germline quality. Although the TGF-β Sma/Mab pathway acts autonomously in the hypodermis to regulate body size (Wang et al., 2002), surprisingly, we find that both insulin and TGF-β Sma/Mab signaling regulate oocyte and distal germline quality maintenance nonautonomously. TGF-β regulates reproductive aging separately from the developmental regulation of growth, both temporally and transcriptionally. We find that TGF-β oocyte transcriptional targets that are required for *C. elegans* embryonic and germline integrity maintenance also change with age in mammalian oocytes. The conserved nature of these signaling pathways suggests that the mechanisms underlying the maintenance of *C. elegans* reproductive capacity with age may also influence reproductive capacity decline in higher organisms.

RESULTS

TGF-β Sma/Mab and Insulin/IGF-1 Signaling Regulate Embryo Viability and Oocyte Quality Maintenance

Wild-type *C. elegans* reproduction declines with age, but reduced Insulin/IGF-1 signaling (IIS) delays reproductive cessation (Hughes et al., 2007). We recently found that reduced TGF-β Sma/Mab signaling also significantly extends reproductive span in a manner that is independent of insulin signaling, caloric restriction, sperm contribution, and ovulation rate (Luo et al., 2009). To identify the molecular mechanisms underlying normal reproductive aging and its delay in insulin and TGF-β signaling mutants, we systematically investigated each component of the reproductive system, from fertilized embryos through the distal germline (Figure 1A).

To examine fertilized embryo quality, we determined embryonic lethality rates. Compared to age-matched *daf-2* and *sma-2* animals, older wild-type animals produced significantly more embryos that fail to hatch, though they all produced more unhatched embryos with age (Figures 1B and 1E and Figure S1A available online) and that are susceptible to damage by bleaching, a test of eggshell integrity (Figures S1C and S1D). Thus, the reproductive span extension exhibited by *daf-2* and *sma-2* mutants is at least partly a manifestation of increased embryo integrity late in reproduction.

Chromosomal abnormalities, in particular aneuploidies, are a major cause of mammalian embryonic developmental defects (Magli et al., 2007; Rubio et al., 2003), and nondisjunction rates also increase with age in *Drosophila* (Tokunaga, 1970). Increased chromosomal abnormalities, particularly autosome loss, could contribute to *C. elegans* embryonic lethality. Meiotic X chromosome nondisjunction produces males (Hodgkin et al., 1979), which in combination with embryonic lethality, provides a simple measure of chromosomal loss (Saito et al., 2009). Strikingly, the fraction of male progeny produced by wild-type mothers increased 16-fold from day 1 to day 5 (Figure 1C). By contrast, the rate of male production by *daf-2* and *sma-2* mutants was significantly lower (Figure 1C and Figure S1B). To directly test disjunction fidelity, we counted DAPI-stained bodies (Saito et al., 2009) in oocytes of spermless (*fem-1*) animals. We found that the number of oocytes with the normal number of stained bodies (six bivalents) decreased significantly with age in *fem-1*, but insignificantly in *sma-2;fem-1* and *daf-2;fem-1* animals (Figure 1D and Figure S1E), suggesting an increased frequency of chromosomal segregation errors in wild-type oocytes with age. Therefore, worms with reduced Insulin/IGF-1 and TGF-β Sma/Mab signaling better maintain oocyte chromosome segregation fidelity with age.

Oocyte quality decline is also a cause of human age-related infertility (Goud et al., 1999). To test fertilizability, we mated hermaphrodites with young adult (Day 1 or 2) wild-type males and counted the number of fertilized embryos and unfertilized oocytes produced each day (Figure 1E and Figure S1F), excluding mothers that stopped producing cross-progeny before reproductive cessation. (While fertilized embryos are ovoid with a distinct eggshell, unfertilized oocytes are fuzzy and round, as shown in Figure 1E). Aging wild-type animals produced a significant number of unfertilized oocytes with age, whereas *daf-2* and *sma-2* mutants produced almost exclusively successfully fertilized embryos (Figure 1E and Figure S1F). Although *daf-2* and *sma-2* mutants produce fewer total progeny, such usage-dependent mechanisms as total progeny number, early progeny production, and ovulation rate have been previously eliminated as contributing factors in reproductive aging (Andux and Ellis, 2008; Hughes et al., 2007; Luo et al., 2009). To ensure that sperm is not limiting in our mated assays, we examined oocytes of mated worms for ribonucleoprotein (RNP) foci, which form in sperm-depleted oocytes (Jud et al., 2007); the Day 8 nonreproductive mated worms do not form RNP foci (Figure S1G). This suggests that sufficient sperm are available throughout the reproductive period in our mating experiments, and the unfertilized oocytes that the wild-type worms produce in old age are likely due to lower oocyte quality. Mutations in both the TGF-β Sma/Mab and IIS pathways delay such decline, rendering the oocytes fertilizable longer.

TGF-β Sma/Mab and IIS Regulate Oocyte Morphology Maintenance

To determine whether IIS and TGF-β Sma/Mab signaling regulate oocyte morphology maintenance, we examined wild-type and mutant oocytes with age. On Day 1 of adulthood, wild-type oocytes are large and closely packed with their neighboring oocytes (Figure 1F). *sma-2* mutants have fewer oocytes aligned in the gonad because of their short length, but the morphology of the oocytes in both the *daf-2* and *sma-2* mutants is similar to wild-type in early adulthood.

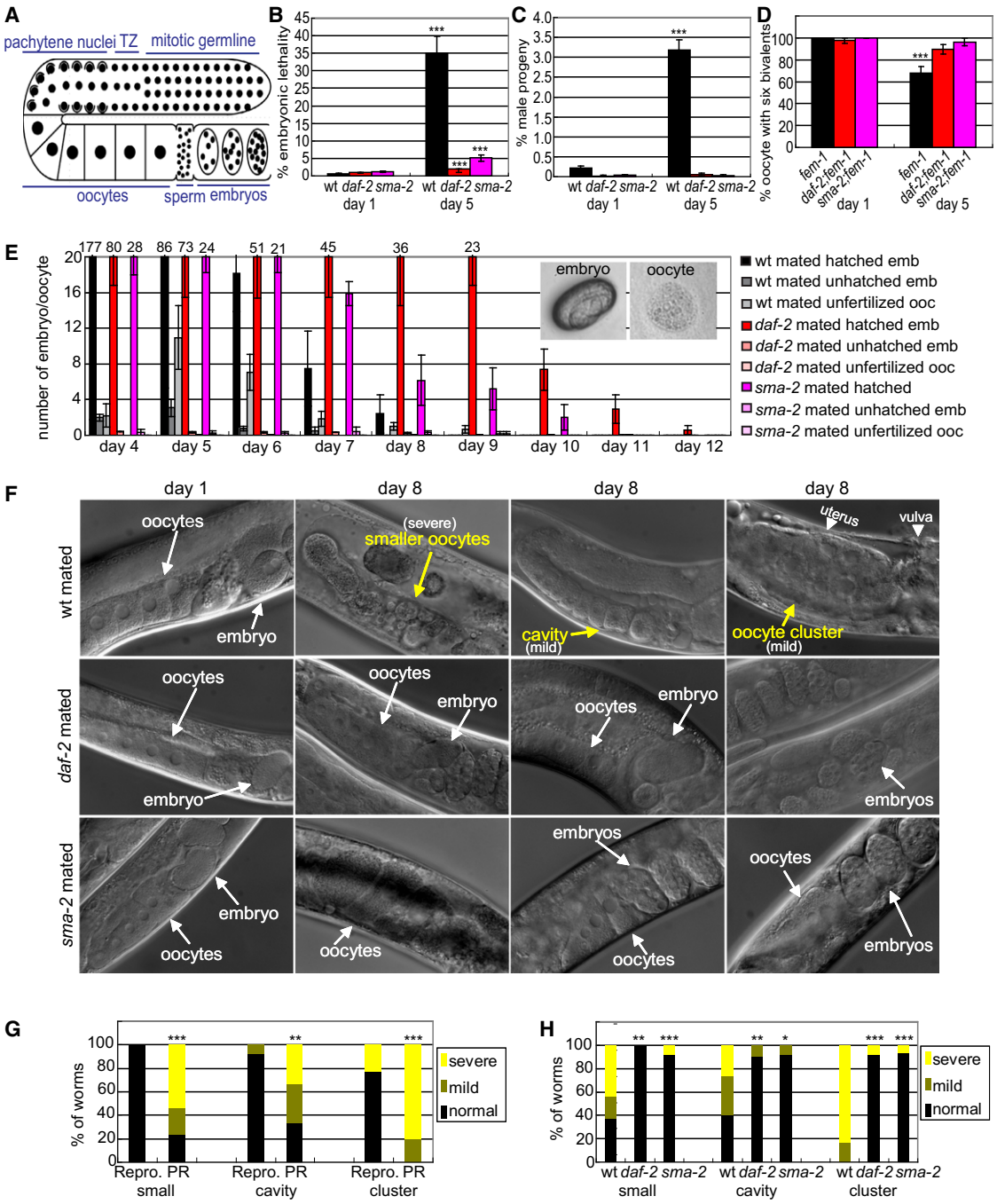


Figure 1. TGF-β Sma/Mab and Insulin/IGF-1 Signaling Regulate Embryo Viability, Oocyte Fertilizability, and Oocyte Morphology

(A) Schematic of the *C. elegans* gonad.

(B) Percentage of embryos that fail to hatch (±SEP).

(C) Percentage of male progeny (±SEP).

(D) Percentage of oocytes with 6 DAPI-stained bodies (±SEP).

(E) Number of hatched embryos (inset, left), unhatched embryos, and unfertilized oocytes (inset, right) produced each day after mating with young wild-type (wt) males (mean ± SEM); percentages shown in Figure S1F.

(F) Oocyte morphology, with defects in yellow.

(G) Oocyte morphology markers scored in mated wt animals that are either reproductive (Repro) or post-reproductive (PR).

(H) Oocyte morphology markers scored in day 8 mated worms. p-values for wild-type versus *daf-2* or *sma-2* indicated.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

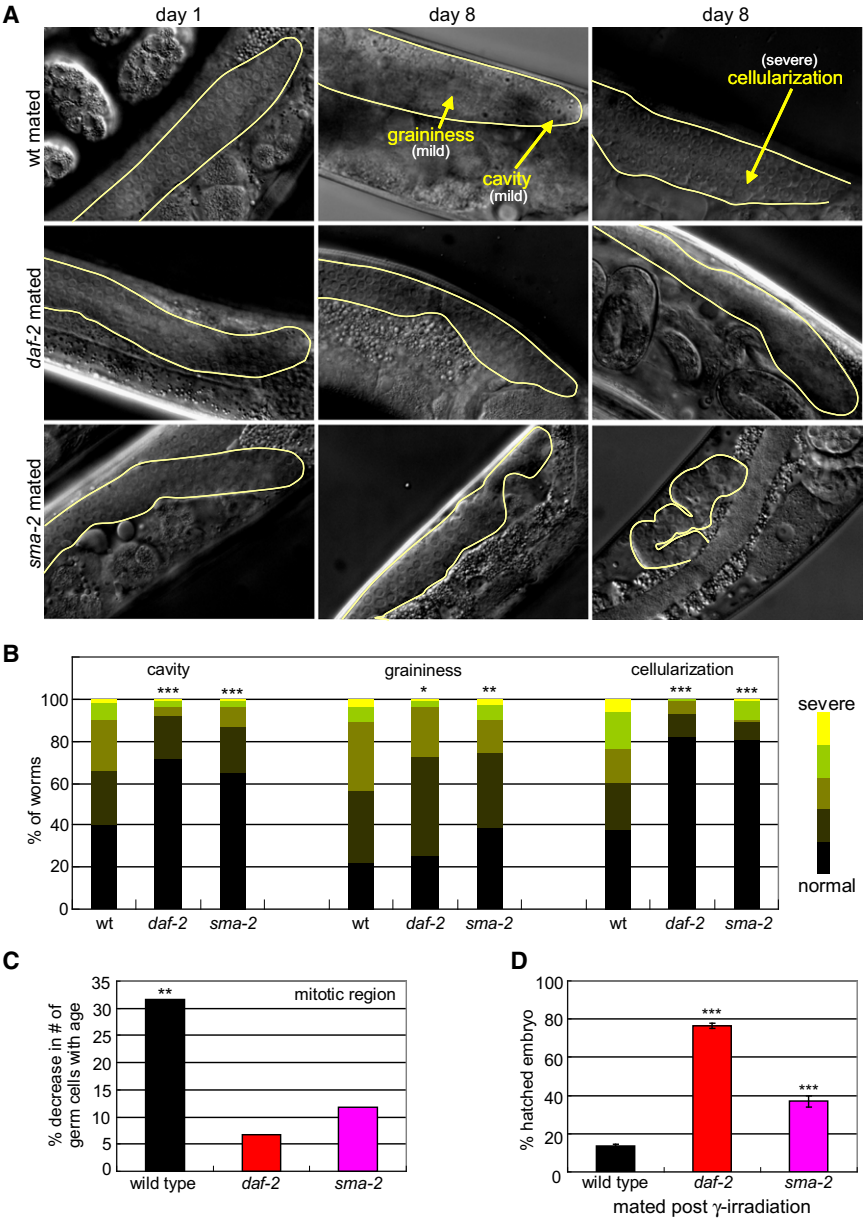


Figure 2. TGF-β Sma/Mab and Insulin/IGF-1 Signaling Regulate DNA Damage Response and Distal Germline Integrity

(A) Distal germline morphology, with defects in yellow.

(B) Distal germline morphology scores of day 8 mated worms; p-values compare wt versus *daf-2* or *sma-2*. *p < 0.05, **p < 0.01, and ***p < 0.001.

(C) Percentage decrease in mitotic germ cell number with age (raw values in Figure S2I).

(D) *daf-2* and *sma-2* lay significantly more hatched embryos than wild-type after γ-irradiation (% ± SEP). Animals were mated with young wt males after irradiation.

* p < 0.05, **p < 0.01, and ***p < 0.001.

On Day 8, when mated wild-type animals have nearly ceased reproduction, their oocytes have visibly degraded: some become much smaller, as previously reported (Andux and Ellis, 2008); some lose contact with their neighbors, resulting in cavities; and others fuse into large clusters packed in the uterus (Figure 1F and Figures S1H–S1K). The defects were independent of levamisole paralysis treatment used for microscopy (Figures S1L and S1M and Figures S2A and S2B). To test whether these defects are morphological predictors of reproductive capacity, we compared reproductive and post-reproductive wild-type animals; oocytes from the postreproductive animals were significantly more degraded in terms of oocyte size, cavities, and cluster formation (Figure 1G). By contrast, oocytes in aged *daf-2* and *sma-2* animals were still young-looking, with significantly fewer morphological defects than age-matched wild-type oocytes (Figure 1F and 1H and Figures S1N–S1S). Thus, reduced TGF-β Sma/Mab and IIS activity both improve oocyte morphology maintenance. Together, our data suggest that oocyte quality, as defined by chromosome segregation fidelity, fertilizability, and morphology, declines with age in *C. elegans*, and that reduced TGF-β Sma/Mab and IIS signaling delay this decline.

Distal Germline Morphology and Proliferation Is Maintained in TGF-β Sma/Mab and IIS Mutants

The distal germline undergoes significant morphological decline with age, but IIS mutations significantly slow this deterioration (Garigan et al., 2002) (Figures 2A and 2B and Figures S2C–S2F). We scored the appearance of cavities, graininess, and cellularization, the major morphological markers of germline aging (Garigan et al., 2002), and found that *sma-2* mutations also significantly slow germline deterioration (Figures 2A and 2B and Figures S2G and S2H). Although these may be independent effects of the pathways, oocyte and distal germline morphology characteristics in the same population of wild-type worms are correlated (Figure S2J).

The distal germline contains proliferating germline stem cells (GSCs) and their mitotic descendents. The number of DAPI-stained germ cell nuclei in this zone declines significantly with age in wild-type animals (Figure 2C and Figure S2I), but declines less in *daf-2* and *sma-2* animals (Figure 2C and Figure S2I), possibly because of better maintenance of proliferative ability. Together, our data suggest that both IIS and TGF-β Sma/Mab signaling may regulate the maintenance of distal germline proliferation and germline quality, as well as oocyte quality.

Physiological and DNA Damage-Induced Apoptosis Are Not Major Contributors to TGF-β and IIS Regulation of Reproductive Aging

Prior to cellularization into oocytes, germ cell nuclei undergo programmed cell death (Gumienny et al., 1999). This "physiological germ cell apoptosis" has been proposed to be an important factor in maintaining oocyte quality via resource reallocation (Andux and Ellis, 2008). We found that *sma-2* and *daf-2* mutants have higher levels of physiological apoptosis than wild-type, but wild-type levels decreased insignificantly with age (Figure S2K).

C. elegans' germline also undergoes apoptosis as a response to DNA damage from ionizing radiation (Gartner et al., 2000). We examined animals after γ-irradiation and found that DNA damage–induced apoptosis declined significantly with age in wild-type animals, but the rates in older TGF-β and IIS mutants were not significantly different from wild-type (Figure S2L). Although neither Insulin/IGF-1 nor TGF-β signaling appears to regulate this process, the significant decrease in irradiation-induced apoptosis with age likely contributes to reproductive aging in general.

DNA Damage Response Contributes to Reproductive Maintenance by TGF-β and IIS

A different aspect of the DNA damage response is improved both by reduced IIS and TGF-β signaling: the number of viable progeny produced after ionizing radiation treatment increased significantly in *daf-2* and *sma-2* mutants compared to wild-type (Figure 2D). The proportion of arrested larvae is also slightly increased in the mutants (Figure S2M), suggesting that even damaged animals are more developmentally competent than the wild-type progeny. Thus, although the rate of DNA-damage induced apoptosis is not increased, *sma-2* and *daf-2* germ cells may better repair damaged DNA or be better protected against genotoxic stress, which in turn may be partially responsible for slowed reproductive aging.

TGF-β and IIS Signaling Regulate Reproductive Aging Nonautonomously

TGF-β Sma/Mab signals cell-autonomously in the hypodermis to regulate body growth (Wang et al., 2002). To test the cell autonomy of TGF-β Sma/Mab signaling in the regulation of reproductive aging, we performed mosaic analyses. Hypodermal expression of the TGF-β Sma/Mab signal transducer SMA-3, which forms a transcriptional complex with SMA-2, is necessary and sufficient for normal body length (Wang et al., 2002). Like *sma-2* mutants, *sma-3* mutants extend reproductive span (Luo et al., 2009) and maintain oocyte and germline morphology longer with age (Figure 3C and 3D). If reproductive aging is dependent on cell-autonomous TGF-β Sma/Mab signaling in the germline, loss of the *sma-3* transgene in the germline alone should recapitulate *sma-3* reproductive span extension. Alternatively, if reproductive aging is dependent on somatic (nonautonomous) TGF-β signaling, somatic *sma-3* expression should be sufficient to suppress the long reproductive span of *sma-3*. We screened a synchronized population of *sma-3(wk30);qcEx26*[*sma-3 gDNA*;*sur-5::gfp*] transgenic animals (Wang et al., 2002), selecting worms expressing GFP in most somatic tissues, including hypodermis, but without germline fluorescence (Figure S3A–S3C) ("germline silent" animals). Because the *sma-3* transcript could still be present but undetectable, we also selected somatically fluorescent animals that produced no fluorescent progeny, indicating that they had completely lost the transgenic array in the germline ("germline lost"). As previously reported, somatic *sma-3* activity rescued body length (Figure 3A). Surprisingly, both the germline-silent and germline-lost animals had wild-type–like reproductive spans (Figure 3B), indicating that somatic *sma-3* expression is sufficient to rescue reproductive span regulation. We also found that the *sma-3* germline-silent mosaic animals reduced ovulation rate and progeny number, but have a normal reproductive span (Figure 3B and Figures S3D and S3E), underscoring our previous finding that low ovulation rates and progeny numbers do not extend reproductive span (Luo et al., 2009). Additionally, the morphology of day 8 oocytes and distal germlines of somatic *sma-3* animals were more similar to wild-type than to *sma-3* (Figures 3C and 3D). Thus, TGF-β signaling regulates reproductive aging nonautonomously, signaling from somatic tissues to the germline to maintain quality.

Expression of *sma-3* under the *vha-7* promoter, which is primarily hypodermal, rescues the small body size phenotype of *sma-3* mutants (Wang et al., 2002). To determine the tissue-specificity of nonautonomous TGF-β signaling in reproductive aging regulation, we selected large *Pvha-7::sma-3;sma-3 (wk30)* worms (Figures S3F and S3G) and found that the reproductive span extension of *sma-3* mutants was also rescued by hypodermal *sma-3* expression (Figure 3E and Figure S3H). Because we were concerned that the *vha-7* promoter might also express in somatic gonad tissues, we checked the effect of *sma-9* RNAi in a somatic-gonad-only RNAi strain (*rrf-3;rde-1;qyIs103[Pfos-1a::rde-1+Pmyo-2::yfp]*) (Hagedorn et al., 2009). Somatic gonad-specific knockdown of TGF-β signaling did not recapitulate the reproductive span extension we observed in whole-animal RNAi (Figure 3F). Together, our results suggest that TGF-β signaling in the hypodermis acts autonomously to regulate body size, but nonautonomously to regulate oocyte and distal germline quality maintenance and, subsequently, reproductive aging.

IIS acts both autonomously (Libina et al., 2003) and nonautonomously to regulate life span (Apfeld and Kenyon, 1998; Wolkow et al., 2000). We found that germline silencing of *daf-16* activity still allows *daf-2* mutant-like reproductive span extension (Figure 4A and Figure S4), suggesting that IIS also acts germline-nonautonomously to regulate reproductive aging. Tissue-specific expression analysis of the DAF-16 transcription factor showed that intestinal expression DAF-16, which increases life span (Libina et al., 2003), also significantly increased reproductive span and improved oocyte and germline morphology of *daf-16;daf-2* mutants (Figures 4B and 4D–4F). Surprisingly, muscle-expressed DAF-16, which has no effect on life span (Libina et al., 2003) also increased reproductive span and improved germline and oocyte quality significantly (Figure 4C-F), whereas neuronal DAF-16 had little effect on reproductive aging (Figures 4C–4F). Thus, IIS acts nonautonomously to regulate germline and oocyte aging, acting partially in different tissues from its nonautonomous regulation of longevity.

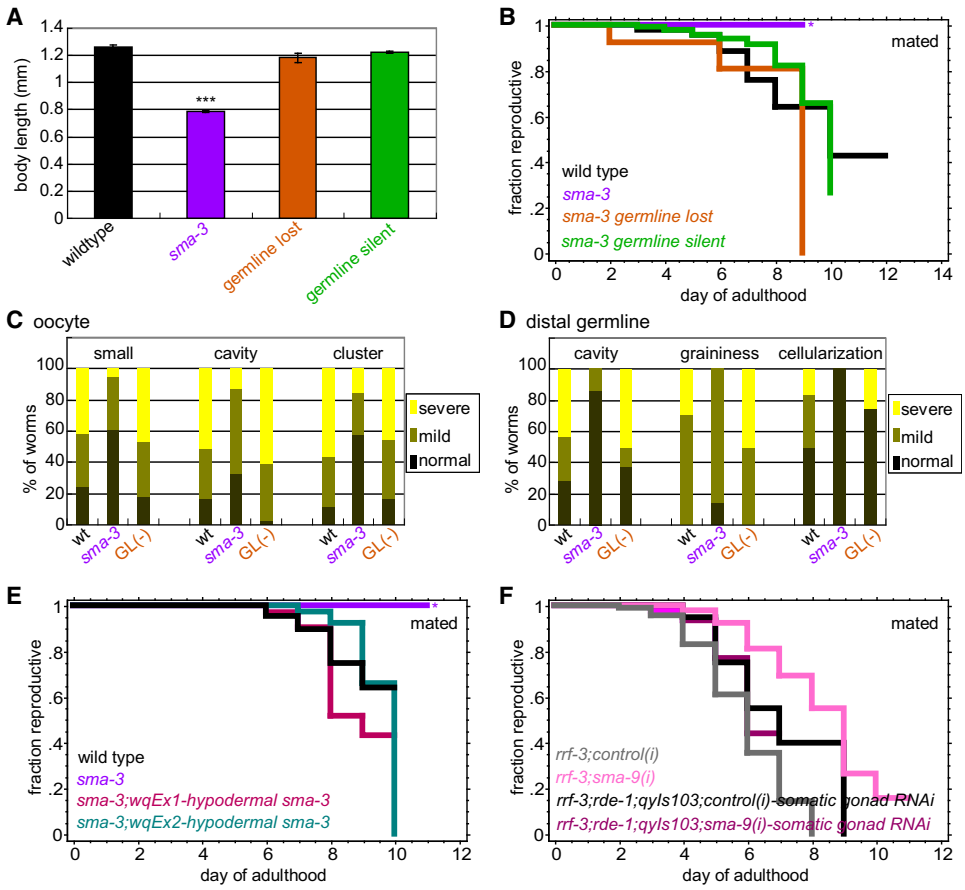


Figure 3. TGF-β Sma/Mab Signaling Regulates Reproductive Aging Nonautonomously in Hypodermis

(A) Body length of wt, *sma-3(wk30)*, *sma-3(wk30);qcEx26[sma-3 gDNA; sur-5::gfp]* animals that have lost or silenced transgenic *sma-3* expression in the germline (mean ± SEM).

(B) Mated reproductive spans of worms in (A). *High matricide rate due to internal progeny hatching. (All reproductive span statistics are shown in Table S1.)

(C and D) Scoring of oocyte (C) and distal germline (D) morphology markers in day 8 mated wt, *sma-3*, and *sma-3* germline-lost (GL) animals.

(E) Two independent transgenic lines (*sma-3(wk30);Pvha-7::gfp::sma-3*) expressing *sma-3* in the hypodermis have mated reproductive spans similar to wild-type (Table S1).

(F) *sma-9* RNAi significantly extends mated reproductive span of *rrf-3* worms, but does not extend the mated reproductive span of the somatic-gonad-only RNAi strain *rrf-3;rde-1;qyIs103[Pfos-1a::rde-1+Pmyo-2::yfp]*.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

TGF-β Sma/Mab Signaling Acts in Adulthood to Regulate Reproductive Aging

The timing of IIS' effects on reproduction and longevity largely overlap, acting primarily in adulthood with some contribution to reproduction in late larval stages (Dillin et al., 2002) (Figures S5A and S5B). By contrast, TGF-β signaling acts in earlier larval stages to regulate body size (Liang et al., 2003; Savage-Dunn et al., 2000). To determine the timing of TGF-β regulation of reproductive span, we used RNAi to knock down Sma/Mab signaling in RNAi-sensitive *rrf-3* mutants either during the animals' whole life or only during adulthood. Whole-life *sma-9 (RNAi)* treatment both reduced body size (Figure 5A and Figure S5C) and increased reproductive span (Figure 5B). *sma-9(RNAi)* treatment only in adulthood, however, did not reduce body size (Figure 5A), but increased reproductive span to the same extent as whole-life *sma-9(RNAi)* treatment ($p = 0.46$, Figure 5B). Thus, the effects of TGF-β signaling on body size are temporally separable from its effects on reproduction. Additionally, small body size is not required for extended reproductive span through TGF-β signaling. Our tissue specificity and temporal analyses suggest that the downstream effectors that control body size and reproductive aging may be distinct, despite the fact that they are both controlled by TGF-β signaling in the hypodermis.

TGF-β Oocyte Quality Targets Are Shared with Mammalian Oocyte Aging Genes

To identify the targets of TGF-β Sma/Mab signaling that regulate reproductive aging, we compared the transcription of unfertilized oocytes isolated from day 8 spermless *fem-1* and *sma-2;fem-1* worms (Figure S5D). Gene ontology (GO) analysis of significantly upregulated and downregulated TGF-β oocyte genes (Figure 5C

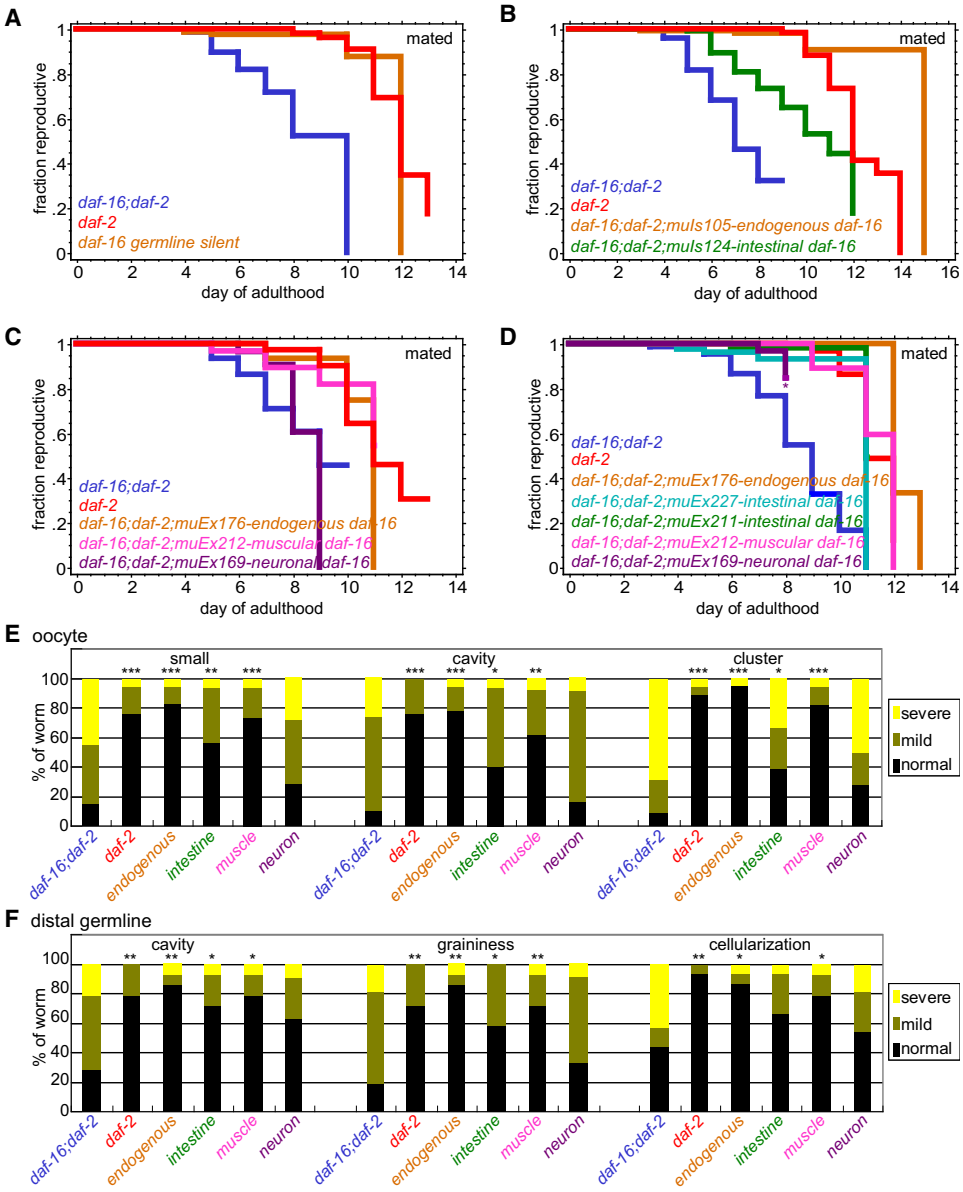


Figure 4. Insulin/IGF-1 Signaling Regulates Reproductive Aging Nonautonomously in Intestine and Muscle

(A) *daf-16 germline silent* worms (*daf-16(mu86);daf-2(e1370);muIs105 [Pdaf-16::gfp::daf-16 +rol-6(su1006)]*, Figure S4) with only somatic *daf-16* activity have a reproductive span similar to *daf-2* mutants (statistics in Table S1).

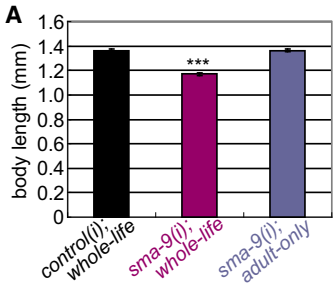
(B–D) *daf-16* activity in intestine (B and D) and muscle (C and D) significantly restores reproductive span extension, whereas neuronal *daf-16* activity (C and D) does not.

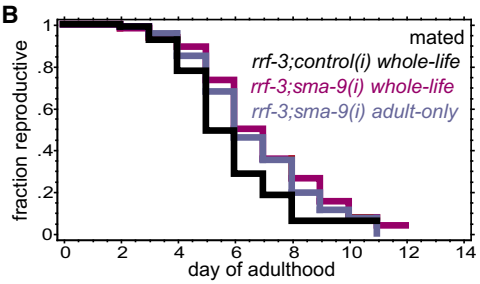
(E and F) Oocyte (E) and distal germline (F) morphology scores of day 8 mated *daf-16;daf-2*, *daf-2*, endogenous-promoter-driven and tissue-specific promoter-driven *daf-16* transgenic animals. *p < 0.05, **p < 0.01, and ***p < 0.001 for *daf-16;daf-2* versus other genotypes.

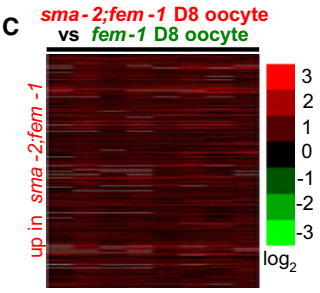
* p < 0.05, **p < 0.01, and ***p < 0.001.

and Figure S5F; Tables S2A and S2B) identified such categories as oogenesis, cell cycle, chromosome segregation and organization, DNA damage response, proteolysis, ATP binding, signaling, transcription regulation, protein transport, aging, GTP binding, and oxidoreductases (Figures 5D and 5E Figure S5G, Table S3, and Table S4). More than 70% of the *sma-2*-regulated genes are regulated in the same direction in young relative to old (day 8) *fem-1* oocytes (Figures S5E and S5F), and similar GO terms are also enriched (Figure 5D), suggesting that these genes are good markers of the "youthfulness" of oocytes.

A striking number of the genes and GO terms identified in our array analysis of *sma-2;fem-1* and *fem-1* oocytes that were associated with "youthful" oocytes are shared with genes







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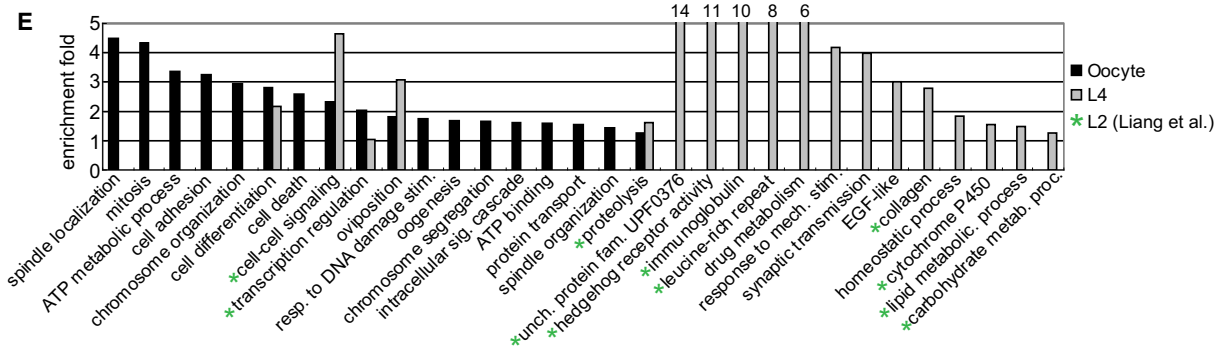
Gene Ontology category	Gene count	Genes from oocyte array studies (homologs)		
		Worm (up in *sma-2*)	Mouse *Hamatani, et al. 2004*	Human *Steuerwald, et al. 2007*
Cell cycle				
mitosis H M W	8	***cyb-1***(Ccnb2), ***cyb-3***(Ccnb3), ***cdc-25.2***(Cdc25a), *cki-1**	***Ccnb2***, ***Ccna2***, ***Cdc16***	***CCNA2***, ***CCNG1***, *CDK7*
Chromosome segregation, org'n				
chromosome segregation H M W	7	***smc-4***(Smc4l1), *klp-7*, *frm-5*	***Smc4l1***, *Nin*, *Kif3b*, *Bub1*	***Smc3l1***, *BUB1B*, *BUB3*
spindle localization M	4	*gad-1*, *mes-1*, *par-3*	*Hook1*, *Nin*, *Rnf19*	
spindle organization M	3	*mbk-2*, *sur-6*, *goa-1*	*Tuba2*, *Tubd1*, *Pcnt2*	
chromosome organization M W	12	*spr-5*, *nurf-1*, *hpl-1*, *hil-2*	*Hdac2*, *Morf4l2*, *Rbbp7*	
DNA damage response and repair				***MBD4*** *(interacts with MLH1), ATR, NBS1*
response to DNA damage stim. H M	4	***mlh-1***(MLH1), *clk-2*, *pme-5*, *uev-2**	***Msh-3***, *Exo1*, *Shprh*	
Proteolytic pathway				
proteolysis H M	19	***ubc-1***(Ube2a), ***ubc-2***(Ube2d1), *ulp-1*	***Ube2a***, *Ubc*, *Usp1*	*USP1*, *CTSC*, *GRP58*
Energy pathway, mitochondrial fn.				
ATP metabolic process M	7	***pmr-1***(Atp2c1), ***vha-13***(Atp6v1a), ***tat-5***(Atp9b)	***Atp2c1***, ***Atp6v1d***, ***Atp5b***	
ATP binding H M W	42	***pgp-7***(Abcb11), ***mrp-2***(Abcc3), ***psa-4***(SMARCA5), *pdk-1*, *akt-2*	***Abcb6***, ***Abcf3***, *Cct2*	***ABCC4***, ***SMARCA5***, *SUV3*
Cell signalling and communication				
intracellular signaling cascade H M W	11	***cdc-42***(RHO GTPase), *vhp-1*, *sel-12*	***Rhoh***, *Kras2*, *Mek1*	*ATF1*, *CREB1*, *CLK1*
cell-cell signaling M W	5	*unc-18*, *ace-1*, *cab-1*	*Gja7*, *Shroom3*, *Mmp2*	
Protein transport				
protein transport H M W	11	***arf-1.1***(Arf1), ***arl-13***(Arl13b), ***rab-6.2***(Rab6)	***Arf1***, ***Arl4***, ***Rab1***	***ARF4***, ***ARF6***, ***RAB11a***
Transcription regulation H M W	19	*hlh-1*, *efl-1*, *spt-5*	*Phtf1*, *Crsp6*, *Lhx8*	*PHTF1*, *NFE2L2*, *EIF2AK2*
Reproductive process				
oogenesis M	5	*hrp-1*, *goa-1*, *fem-3*	*Nalp5*, *Padi5*, *Nalp9a*	
oviposition W	15	*unc-84*, *cki-1*, *mtm-3*		
Other				
cell death M W	7	*ced-1*, *ced-8*, *crn-4*	*Tnfaip8*, *Mdm4*, *Bcl2l10*	
cell differentiation W	24	*par-1*, *eor-2*, *lin-28*		
cell adhesion M W	6	***epi-1***(Lama2), ***hmr-1***(Cdh11), ***cdh-3***(Cdh23)	***Lama2***, ***Cdh2***, ***Pcdhb17***	

E



downregulated in aging mouse and human oocytes (Hamatani et al., 2004; Steuerwald et al., 2007) (Figure 5D), such as mitotic cell cycle regulation, chromosome segregation, response to DNA damage, proteolysis, ATP binding, signaling, transcriptional regulation, and protein transport. The condensin SMC is upregulated in *sma-2* oocytes and declines in both mouse and human oocytes with age, suggesting that chromosome segregation is a shared key determinant of oocyte quality. Cell cycle regulators (CYB-1/3) and DNA mismatch repair proteins (e.g., MLH-1 and MBD4) are also higher in *sma-2* oocytes and decline with age in mammalian oocytes. Interestingly, several TGF-β signaling genes are upregulated with age in mouse oocytes (Hamatani et al., 2004), paralleling our observations on the extension of reproductive span in *C. elegans* TGF-β mutants.

In addition to the genes that are shared between *sma-2* mutants and age-regulated in mouse and human oocytes, our analysis has uncovered new genes that are potential regulators of reproductive aging. *lin-28*, which is important in reproductive development regulation (Hartge, 2009) and the reprogramming of differentiated cells into induced pluripotent stem cells (Nimmo and Slack, 2009), and *clk-2*, a telomere length regulator that is involved in DNA damage response and cell cycle checkpoint, are also significantly upregulated in *sma-2* oocytes (Table S2A). Several Class 2 longevity genes, including *dod-23* and *dod-24* (Murphy et al., 2003), as well as many oxidoreductases and protein metabolism genes, are significantly downregulated in *sma-2* oocytes (Table S2B), suggesting additional novel mechanisms that may contribute to the regulation of oocyte aging. Finally, expression of the insulin-like peptide genes *ins-22* and *ins-23* is significantly upregulated in *sma-2* oocytes, whereas *ins-7* (Murphy et al., 2003) is downregulated, possibly indicating insulin signaling from the oocytes themselves.

TGF-β Somatic and Oocyte Transcriptional Targets Are Distinct

Sma/Mab L2 transcriptional targets regulate body size and male tail patterning (Liang et al., 2007). We compared the expression profiles of Sma/Mab mutant and wild-type early L4 whole animals, prior to oocyte development; these targets are similar to the L2 targets of Liang et al. (2007) (Figure 5E). In contrast to *sma-2* oocyte gene expression, the genes upregulated in Sma/Mab larvae (Table S2C) include the GO terms of hedgehog signaling, immunoglobulin domain proteins, leucine-rich repeat proteins, cuticle collagens, and lipid and carbohydrate metabolism genes (Figure 5E). Thus, at both the gene and GO term level, the targets of Sma/Mab signaling in body size and oocyte quality regulation are largely nonoverlapping (Figure 5E).

TGF-β Oocyte Targets Are Required for Reproductive Quality Maintenance

To test candidate genes for their roles in reproduction, we used RNAi knockdown to screen the top-ranking oocyte target genes for their effects on *sma-2* late embryo hatching, reasoning that loss of important *sma-2*-upregulated genes might reduce reproductive success. Of 60 genes tested, 27 reduced *sma-2* embryo-hatching rates (Figure 6A and Figure S6A). We then tested the genes with the strongest hatching effects for their contributions to reproductive span determination and embryo/oocyte quality (Figures 6B–6J and Figure S6). Three genes, *smc-4* (*condensin*, structural maintenance of chromosomes), *cyb-3* (*cyclin B*, sister chromatid segregation), and E03H4.8 (unknown, predicted vesicle coat complex), shortened *sma-2* reproductive span substantially, from *sma-2*'s mean of 9 days to < 3 days (Figure 6B). We found that these "early effect" genes also had severe effects on *sma-2* embryonic viability, producing almost exclusively unhatched embryos (Figures 6C–6F and Figure S6B). These genes are critical for oocyte quality, because knocking them down in wild-type also resulted in severe effects on embryonic viability (Figures S6D–S6F). Knockdown of these genes also severely affected germline and oocyte morphology; oocytes were largely unidentifiable, distal germline cells were not well defined, and the gonads themselves were misshapen (Figures 6G–6J and Figure S6C). The loss of other *sma-2*-oocyte regulated genes also increased the rate of unhatched embryos and/or unfertilized oocytes with age in both *sma-2* and wild-type, but later or more mildly (Figures 7B–7D and Figure S7); these include *math-33* (putative apoptosis gene), F47G4.4 (putative chromosome segregation gene), F52D10.2 and C06E7.4 (both unknown), and F21F3.3 (methyltransferase).

Because we had observed that progeny survival after DNA damage was increased in *sma-2* mutants (Figure 2D), and a number of the DNA damage response genes upregulated in *sma-2* oocytes and were required for embryo viability (Figure 5D and Table S3), we investigated these genes' effects on *sma-2*'s oocyte quality and post-γ-irradiation embryonic lethality. We found that loss of *mlh-1*, a DNA mismatch repair homolog of human MLH1, increased the rate of unhatched embryos and unfertilized oocytes late in *sma-2* reproduction (days 7–10; Figure 7A, Figure 6A, and Figure S6A). Loss of *uev-2* (stress/DNA damage response) and *pme-5* (PARP/tankyrase) had milder effects on hatching (Figures 7E and 7F, Figure 6A, and Figure S6A). However, loss of *uev-2* had a significant effect on post-γ-irradiation *sma-2* embryonic lethality (Figure 7G).

Figure 5. TGF-β Sma/Mab Signaling Regulates Oocyte Quality and Body Size through Distinct Sets of Downstream Targets

(A) *sma-9* RNAi adult-only treatment reduces body size significantly ($p < 0.001$, 14% decrease), whereas adult-only treatment does not (mean ± SEM).

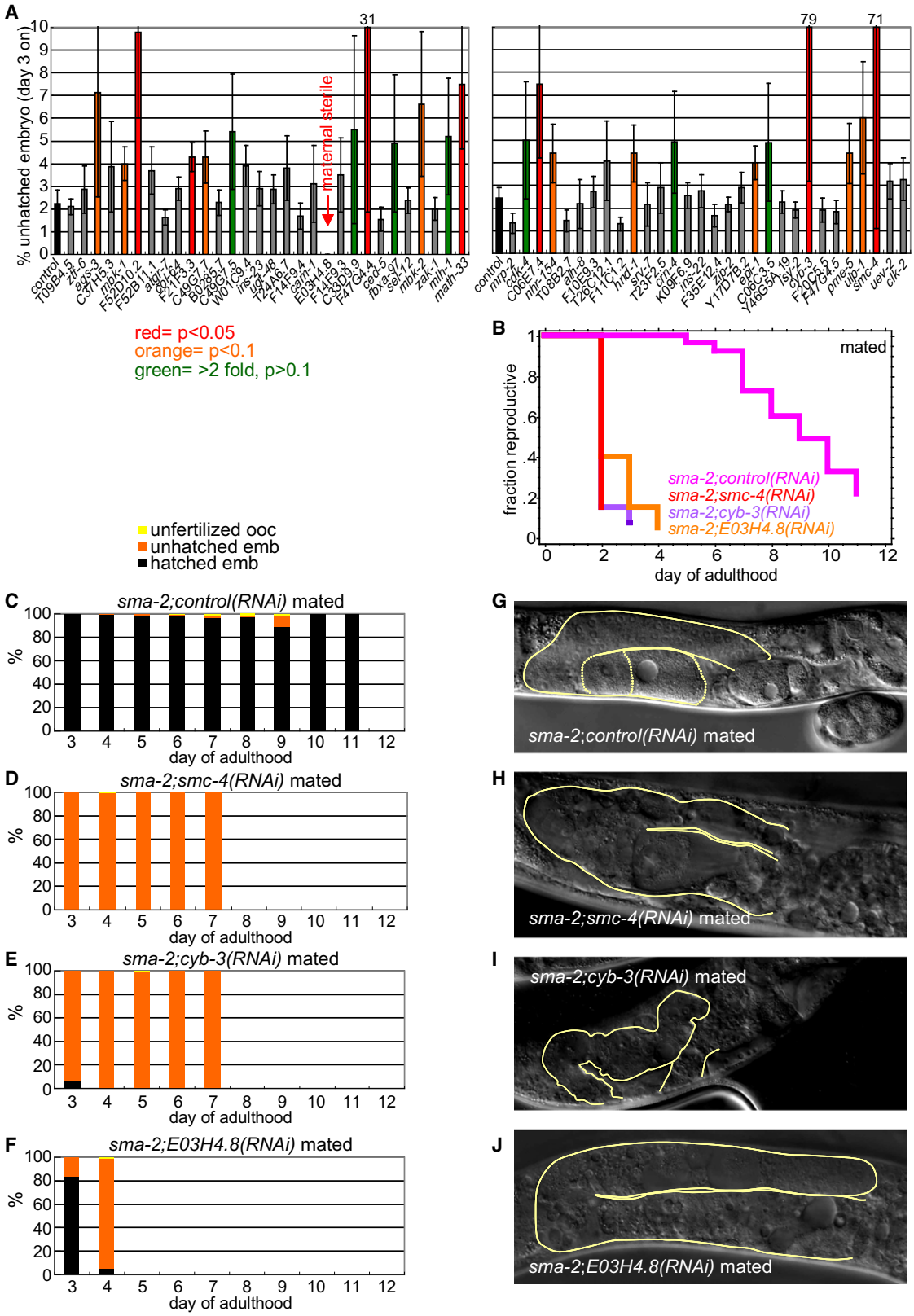
(B) Mated reproductive spans of *rrf-3* animals treated with control RNAi whole-life, with *sma-9* RNAi whole-life, or with *sma-9* RNAi in adulthood only (Table S1).

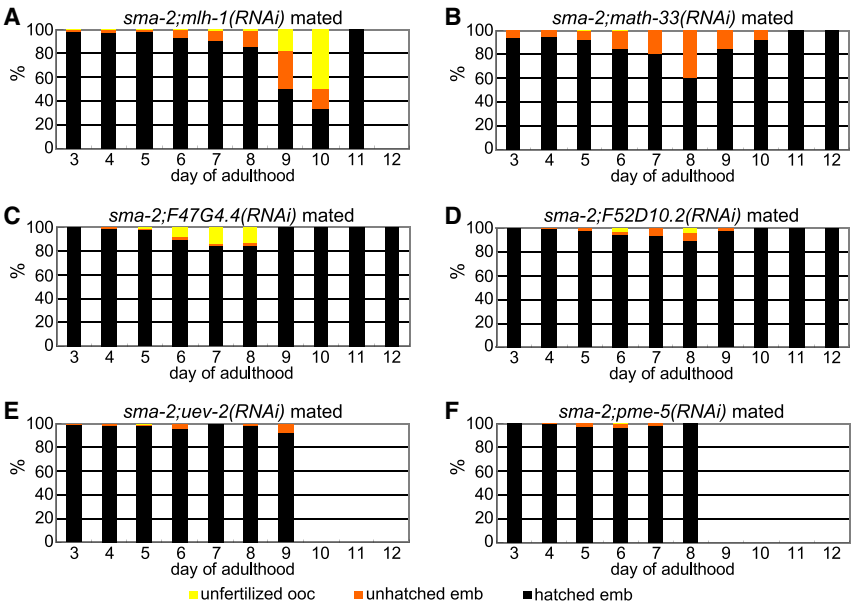
(C) Expression heat map of 386 genes significantly upregulated in *sma-2;fem-1* oocytes (FDR = 0%).

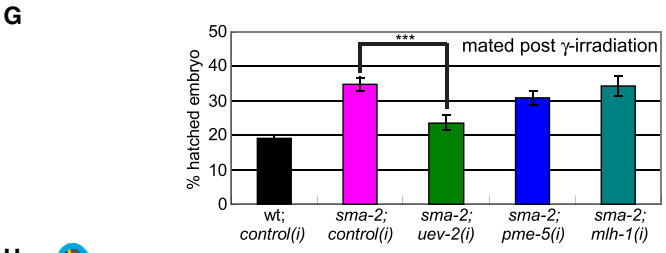
(D) Enriched GO terms for genes in (C). Example genes from this study (worm) and genes upregulated in young versus old mouse (Hamatani et al., 2004) or human (Steuerwald et al., 2007) oocytes are listed, with highly homologous or important interacting genes in bold. (Expanded gene list is provided in Table S3.) GO terms also enriched in younger human (H), mouse (W), or worm (W) oocytes are labeled with corresponding superscript letters. Asterisk indicates a gene involved in corresponding GO function but failed to be recognized by DAVID (not included in gene counts).

(E) GO terms enriched in TGF-β Sma/Mab mutant oocytes are largely distinct from those enriched in Sma/Mab L4 and L2 (Liang et al., 2007) larvae.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.


A
% unhatched embryo (day 3 on)
maternal sterile
31
79
71
red= p<0.05
orange= p<0.1
green= >2 fold, p>0.1
B
fraction reproductive
mated
sma-2;control(RNAi)
sma-2;smc-4(RNAi)
sma-2;cyb-3(RNAi)
sma-2;E03H4.8(RNAi)
day of adulthood
unfertilized ooc
unhatched emb
hatched emb
C
sma-2;control(RNAi) mated
D
sma-2;smc-4(RNAi) mated
E
sma-2;cyb-3(RNAi) mated
F
sma-2;E03H4.8(RNAi) mated
%
day of adulthood
G
sma-2;control(RNAi) mated
H
sma-2;smc-4(RNAi) mated
I
sma-2;cyb-3(RNAi) mated
J
sma-2;E03H4.8(RNAi) mated





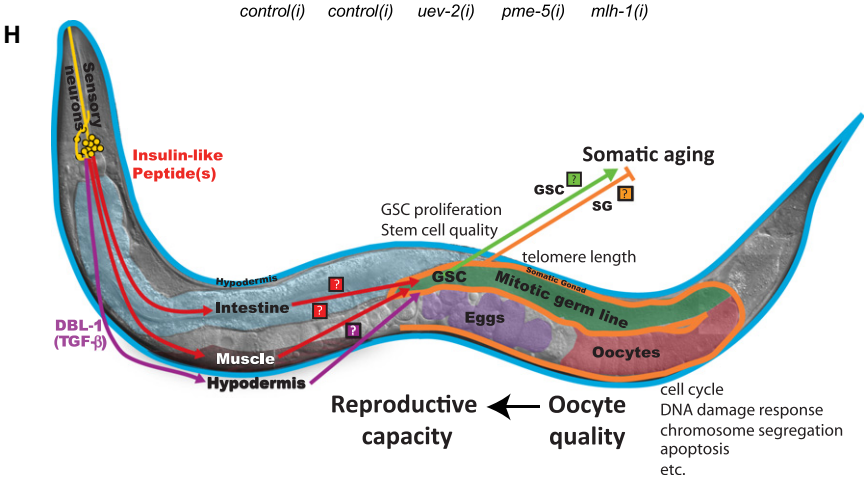


Figure 7. TGF-β Sma/Mab Signaling Regulates Genes Important for Age-Associated Oocyte Quality Maintenance

(A–F) RNAi treatments of TGF-β target genes accelerate oocyte quality decline, increasing the percentage of unhatched embryos (orange) and/or unfertilized oocytes (yellow) earlier in life (compare with Figure 6C). *mlh-1*, *math-33*, and F47G4.4 RNAis have greater effects (A-C), whereas F52D10.2, *uev-2*, and *pme-5* have milder effects (D-F). Wild-type treated with RNAis shown in Figures S7C–S7F and S7I–S7J).

(G) *uev-2* RNAi treatment significantly increases *sma-2*'s production of unhatched embryos (% ± SEP) after γ-irradiation, whereas *pme-5* and *mlh-1* do not. Animals were mated with young wt males after irradiation.

(H) Model of reproductive aging regulation by the TGF-β Sma/Mab (pink) and insulin/IGF-1 signaling (red) pathways. Ligands (Insulin-Like Peptides, TGF-β DBL-1) are secreted neuronally and mediate signaling to the soma (hypodermis, intestine, and muscle), generating as yet unidentified secondary signals to regulate reproduction. These secondary signals block distal germline and oocyte integrity maintenance with age, resulting in germline morphology decline, slowed germ cell proliferation, and a decline in oocyte quality. Downstream effectors in oocytes include chromosome segregation, cell cycle, DNA damage response/repair genes, and so forth. Declines in embryonic viability and infertility mark reproductive cessation. The germline and somatic gonad regulate somatic aging (Hsin and Kenyon, 1999), suggesting a bi-directional signaling flow in the coordination of somatic and germline aging. (Photo courtesy of Ian Chin-Sang.)

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Together, our expression results show that *sma-2* regulates a distinct set of genes in oocytes from its targets in body size determination. Furthermore, our functional analyses of *sma-2* oocyte targets suggest several mechanisms that are required for successful extended reproduction, and many of these mechanisms are shared with mammalian oocytes. Chromosome segregation and cell cycle genes are more highly expressed in *sma-2* than in wild-type worms, and their loss causes severe functional and morphological germline defects, suppressing reproduction. DNA damage response and other novel genes, by contrast, are required later in reproduction to maintain reproductive fidelity.

Figure 6. TGF-β Sma/Mab Signaling Regulates Genes Essential for Embryonic Viability in Oocytes

(A) RNAi knockdown of many *sma-2*-regulated oocyte targets increase *sma-2* mutant's embryonic lethality (mean ± SEM).

(B) RNAi knockdown of *smc-4*, *cyb-3*, and E03H4.8 have early and severe effects on reproductive span (Table S1).

(C–F) RNAi knockdown of *smc-4*, *cyb-3*, and E03H4.8 greatly increase the percentage of unhatched embryos (orange) in mated *sma-2* mutants (compare D-F with C). Wild-type treated with RNAis shown in Figures S6D–S6F.

(G-J) *smc-4*, *cyb-3*, and E03H4.8 RNAi-treated *sma-2* animals exhibit severely degraded germlines at day 8 (compare H–J with G). Contours of gonads shown in yellow, visible oocytes outlined by dotted lines in (G).

DISCUSSION

Here we have systematically examined the processes involved in reproduction, from embryonic viability through distal germline morphology, to determine which are most susceptible to aging and which are altered in mutants with extended reproductive spans. Our data establish that oocyte and distal germline quality correlate well with reproductive success and that TGF-β Sma/Mab and Insulin/IGF-1 signaling regulate reproductive aging primarily through their control of these aspects of reproduction.

A Model for TGF-β Sma/Mab and IIS Regulation of Reproductive Aging

Our mosaic and hypodermal rescue data suggest that the TGF-β pathway regulates reproductive aging through an interaction between the soma and germline. We previously showed that TGF-β signaling regulates reproductive aging independently of such somatically controlled mechanical processes as ovulation and body growth (Luo et al., 2009), and our mosaic analysis supports this uncoupling of reproductive span and ovulation (Figures S3D and S3E). Thus, the interaction between the soma and germline to regulate reproductive aging is likely to be mediated by molecular signals. These secondary signals must originate in somatic (hypodermal) tissues downstream of TGF-β signaling, and subsequently act in the germline to control quality (Figure 7H). Similarly, IIS acts in the muscle and intestine to regulate germline and oocyte maintenance. Although the specific signals have not yet been identified, insulin-like peptides are regulated by IIS and coordinate the state of the insulin pathway between tissues (Murphy et al., 2007), and a nuclear hormone receptor is required for starvation-induced adult reproductive diapause (Angelo and Van Gilst, 2009). Together with our data, the observation that signals from the germline and somatic gonad regulate longevity (Flatt et al., 2008; Ghazi et al., 2009; Hsin and Kenyon, 1999), suggests a bidirectional flow of information between somatic and reproductive tissues normally coordinates their rates of aging.

The Distal Germline and Reproductive Aging

TGF-β Sma/Mab and IIS mutations prevent age-related decline in the integrity of the distal germline-containing germline stem cells, and the quality of the distal germline and oocytes are correlated (Figure S2J). Interestingly, germline stem cells protected by starvation have the capacity to regenerate and reestablish reproduction, even after a long period of quiescence (Angelo and Van Gilst, 2009). Although this is the first report of *C. elegans* TGF-β signaling possibly regulating germline stem cell activity in *C. elegans*, TGF-β/BMP signaling is known to affect GSC development in other organisms, including *Drosophila* germline and mammalian muscles (Carlson et al., 2009; Yamashita et al., 2005; Zhao et al., 2008). The upregulation of LIN-28, a key regulator of stem cell induction, in the TGF-β mutant reproductive system is particularly intriguing.

TGF-β Sma/Mab Signaling Regulates Reproductive Aging Distinctly from Body Growth

Although TGF-β Sma/Mab signaling regulates both body growth and reproductive aging, the downstream molecular mechanisms of these two processes are distinct. First, Sma/Mab signaling is required for body size regulation during development, before gametogenesis (Liang et al., 2003; Savage-Dunn et al., 2000), whereas Sma/Mab regulation of germ line aging is carried out in adulthood (Figure 5B). Second, body size and reproductive span are not correlated (Luo et al., 2009). Furthermore, despite the fact that Sma/Mab activity in the hypodermis directs both body growth and oocyte quality, the Sma/Mab pathway has distinct transcriptional targets in the body and oocytes. Interestingly, we find that these oocyte-specific targets can be separated into early- and late-effect genes, with chromosome segregation and cell cycle genes having early and severe effects on reproductive tissues, and DNA damage response genes primarily regulating late effects. The late effects are particularly interesting, as they are most likely to become increasingly important as oocytes age.

C. elegans as a Model of Reproductive Aging

Although worms and humans have vastly different life spans and reproductive strategies, the cellular and molecular bases of reproductive span regulation are strikingly similar. As we have shown here for *C. elegans*, oocyte quality decline is the major reason for human reproductive capacity decline, resulting in sterility and developmental birth defects. Chromosomal abnormalities, in particular aneuploidies, are the major defect in human embryos from aging mothers (te Velde and Pearson, 2002). Worms also increase chromosome nondisjunction rates with age (Rose and Baillie, 1979; Tang et al., 2010) (Figures 1B–1D), and we find that mutants with extended reproductive success significantly reduce chromosomal nondisjunction rates. Oocyte fertilizability, stress resistance, and morphology are compromised with age in humans (Blondin et al., 1997; Goud et al., 1999); we found that this is also the case for *C. elegans*, but these declines are delayed in TGF-β and IIS mutants. Finally, our oocyte transcriptional and functional analyses show that genes upregulated in TGF-β mutants are strikingly similar to mammalian oocyte genes that decline with age, suggesting that many of the molecular mechanisms underlying reproductive cessation are shared between *C. elegans* and humans. Therefore, *C. elegans* not only regulates reproductive aging through oocyte quality control, as do humans, but also, such control is mediated through the regulation of similar oocyte quality maintenance mechanisms.

The fact that both Insulin/IGF-1 and TGF-β signaling, two pathways that are evolutionarily conserved from worms to humans, have significant roles in regulating the rate of reproductive decline and utilize similar mechanisms, suggests that these pathways are also likely to be important in the regulation of human reproductive decline. Several recent genome-wide association studies of human reproductive development and menopause identified genes that regulate development and longevity in *C. elegans* (Ong et al., 2009; Stolk et al., 2009). These genes include FOXO3a, the human homolog of the DAF-16/FoxO transcription factor downstream of the Insulin/IGF-1 signaling pathway, and LIN-28, which we find is regulated by TGF-β signaling in oocytes.

TGF-β signaling has also been implicated in several aspects of mammalian reproduction and reproductive aging. TGF-β members are upregulated in mouse oocytes with age (Hamatani et al., 2004) and many TGF-β superfamily ligands regulate folliculogenesis (Knight and Glister, 2006; Trombly et al., 2009). Although humans have a more complex TGF-β pathway family

that performs many different functions, it is likely that TGF-β signaling may be involved in regulation of reproductive cessation. Therefore, the similarities in the regulation of reproductive aging in worms and humans may allow us to use worms as genetic and molecular models to study this important human problem, enabling the development of therapies to address maternal age-related birth defects and reproductive decline.

EXPERIMENTAL PROCEDURES

Extended Experimental Procedures are presented in Supplemental Information, and include *C. elegans* strains used and analyses of embryonic lethality, male progeny production, chromosome bivalents, oocyte fertilizability, RNP foci, mitotic germ cell number, physiological and irradiation-induced apoptosis, reproductive span, ovulation rate, body length, and temporal RNAi effects.

Oocyte Morphology Analysis

For each oocyte image, a score was assigned for each of the three signs of deterioration (cavities, graininess, and cellularization), according to the severity of the phenotype, with 1 equals normal, 2 equals mild, and 3 equals severe. Mann-Whitney analysis was used to determine whether there were significant differences in pairwise comparisons. An individual who was blind to the genotypes scored the images independently.

Distal Germline Morphology Analysis

For each distal germline image, a score was assigned for each of the three signs of deterioration (cavities, graininess, and cellularization), according to the severity of the phenotype, with 1 equals normal, and 5 (or 3 for Figure 3D and Figure 4F) equals most severe, by four individuals (three were blind to the genotypes) and averaged. Mann-Whitney (pairwise) analyses were used as described above.

Immunostaining

Staining with RME-2 antibody, a gift from Dr. Barth Grant, was performed as described elsewhere (Grant and Hirsh, 1999).

Mosaic Analysis

Developmentally synchronized *sma-3(wk30) III;qcEx26 X [pCS29+sur-5::gfp]* worms with somatic GFP expression were picked; green fluorescence in tissues including hypodermis, intestine, neurons, but not germline, was verified at high magnification (Figures S3A–S3C). Worms were screened for large body size before mating. Animals with no fluorescent progeny are "germline-lost" worms.

Hypodermal Rescue Strain Construction

sma-3(wk30) were injected with pCS227[P*vha-7::sma-3]* at 90 ng/μl (strains and plasmid kindly provided by Dr. Cathy Savage-Dunn) with *Pmyo-2::mCherry* (PFC590, Addgene) as a coinjection marker (5 ng/μl). Large F1s were picked to establish independent lines for follow-up analysis.

Oocyte and L4 Microarrays

Hypochlorite-synchronized wild-type and *sma-2* or *sma-4* larvae were collected at mid-L4. Oocytes were isolated (Miller, 2006) from *fem-1* (day 3 and day 8) and *sma-2;fem-1* (day 8) adults; RNA was extracted, and cRNA was linearly amplified, Cy3/Cy5 labeled, hybridized to the Agilent 44k *C. elegans* microarray, and analyzed as described elsewhere (Shaw et al., 2007). GO analysis was performed using DAVID (Dennis et al., 2003; Huang et al., 2009) on significantly differentially expressed genes (FDR = 0%, SAM; Tusher et al., 2001).

ACCESSION NUMBERS

The microarray data can be found in the Gene Expression Omnibus (GEO) of NCBI through accession number GSE23509 or in PUMAdb (http://puma.princeton.edu).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at doi:10.1016/j.cell.2010.09.013.

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S.L. and C.T.M. planned the experiments and wrote the manuscript; S.L. performed all the experiments except L4 microarrays (W.M.S.), with assistance from G.A.K. (Figure 1D and Figure S1E, generation of mosaic and tissue-specific transgenic animals for Figure 3, Figure S3, and Figure 7H), and J.M.A. (technical assistance).

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