C. elegans DAF-16/FOXO interacts with TGF-β/BMP signaling to induce germline tumor formation via mTORC1 activation

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Abstract

Activation of the FOXO transcription factor DAF-16 by reduced insulin/IGF signaling (IIS) is considered to be beneficial in C. elegans due to its ability to extend lifespan and to enhance stress resistance. In the germline, cell-autonomous DAF-16 activity prevents stem cell proliferation, thus acting tumor-suppressive. In contrast, hypodermal DAF-16 causes a tumorous germline phenotype characterized by hyperproliferation of the germline stem cells and rupture of the adjacent basement membrane. Here we show that cross-talk between DAF-16 and the transforming growth factor β (TGFβ)/bone morphogenic protein (BMP) signaling pathway causes germline hyperplasia and results in disruption of the basement membrane. In addition to activating MADM/NRBP/hpo-11 gene alone, DAF-16 also directly interacts with both R-SMAD proteins SMA-2 and SMA-3 in the nucleus to regulate the expression of mTORC1 pathway. Knocking-down of BMP genes or each of the four target genes in the hypodermis was sufficient to inhibit germline proliferation, indicating a cell-non-autonomously controlled regulation of stem cell proliferation by somatic tissues. We propose the existence of two antagonistic DAF-16/FOXO functions, a cell-proliferative somatic and an anti-proliferative germline activity. Whereas germline hyperplasia under reduced IIS is inhibited by DAF-16 cell-autonomously, activation of somatic DAF-16 in the presence of active IIS promotes germline proliferation and eventually induces tumor-like germline growth. In summary, our results suggest a novel pathway crosstalk of DAF-16 and TGF-β/BMP that can modulate mTORC1 at the transcriptional level to cause stem-cell hyperproliferation. Such cell-type specific differences may help explaining why human FOXO activity is considered to be tumor-suppressive in most contexts, but may become oncogenic, e.g. in chronic and acute myeloid leukemia.
Author summary

The transcription factor FOXO is a well-known tumor suppressor whose activity is controlled by nutrients and stress signaling. In the roundworm *C. elegans*, the activity of the FOXO protein DAF-16 is best known for its beneficial role in stress response and long lifespan. However, FOXO proteins may also promote tumor cell growth and maintenance in chronic and acute myeloid leukemia, suggesting that they may have different roles in distinct contexts. Previously we have shown that selective activation of DAF-16 in the epidermis causes a tumorous growth in the stem cells of the *C. elegans* germline. Now we demonstrate that this oncogenic activity of DAF-16 is mediated by interactions with the transforming growth factor (TGFβ)/Bone Morphogenic protein (BMP) signaling pathway. In the epidermis, direct binding of DAF-16 and R-SMAD proteins of the BMP pathway helps to activate genes involved in the mTORC1 signaling pathway that is frequently activated in tumors. We propose that the transcription factor DAF-16/FOXO may be controlled in different ways in the stem cells, in which its activity normally prevents tumor formation, and in other tissues, in which defects in controlling its activity may result in overwriting the beneficial stem cell activity to eventually promote tumor cell growth.

Introduction

The *C. elegans* FOXO transcription factor DAF-16 is one of the most intensively studied transcription factors due to its ability to extend lifespan and confer stress resistance [1]. In well-fed and stress-free animals, DAF-16 is inactivated by the insulin/IGF-1 signaling (IIS) through the IIS receptor DAF-2, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and the AGC kinases AKT-1, AKT-2, and SGK-1 that promote its cytosolic localization [2–4]. Upon reduced IIS or stress stimuli DAF-16 becomes nuclearly localized and transcriptionally active. Active DAF-16 extends lifespan and increases stress resistance, indicating an overall beneficial effect of DAF-16 activation. DAF-16 activity in the germline, the source of stem cells in *C. elegans*, has a cell-autonomous inhibitory role in cell proliferation [5].

In a previous study we have shown that increasing DAF-16 activity by transgenic expression causes tumor-like germline hyperplasia that is accompanied by degradation of the extracellular matrix surrounding the gonad, and this eventually results in premature death [6]. Due to its similarity to several aspects of tumorigenesis in higher organisms, we suggest to term this phenotype a “*C. elegans* germline tumor”. The penetrance of this tumor-like phenotype is strongly enhanced in a loss-of-function mutant of *shc-1*, which encodes a DAF-2 interacting protein and homolog of the human adaptor protein Shc [6]. We had shown that DAF-16 activity exclusively in the hypodermis is sufficient to induce the tumor-like phenotype, arguing strongly in favor of a non-cell-autonomous mechanism. One of the surprising results of this study was that inactivation of *daf-2*, which should further increase DAF-16 activity according to the canonical IIS signaling pathway, rather suppresses DAF-16 mediated germline tumor phenotype. This suggests that the pro-oncogenic DAF-16 activity is positively rather than negatively regulated by IIS, providing a conundrum that could not be explained easily. Moreover, a loss-of-function mutation in *daf-18*/PTEN (Phosphatase and Tensin homolog), encoding a well-studied phosphatase with functions antagonistic to PI3K that, according to canonical insulin signaling, should enhance PI3K signaling and inactive DAF-16, further enhances this phenotype. Furthermore, the three AGC kinases AKT-1, AKT-2, and SGK-1 that in most previous studies have displayed redundant activities as negative regulators of DAF-16, have antagonistic roles in DAF-16 mediated tumor formation. AKT-1 acted as an inhibitor of germline...
tumor formation, as the *shc-1;akt-1* double mutant shows tumor-like growth similar to that observed in *shc-1(Is[daf-16::GFP])* [6]. Strikingly, down-regulation of *akt-2* or *sgk-1*, like *daf-2*, suppressed germline hyperplasia in *shc-1;akt-1* animals, indicating that the activity of DAF-2, AKT-2 or SGK-1 in this context contributed to germline tumor formation. All these observations are inconsistent with the canonical model of the linear IIS pathway, in which DAF-2 negatively regulates DAF-16. DAF-16 in turn has been shown before to inhibit germ cell proliferation cell-autonomously, and also acts to inhibit *gld-1* germline tumors [5, 7] that differ in certain aspects from the tumor-like phenotype observed here. These observations suggest a novel proliferation-promoting DAF-16/FOXO function in the presence of active IIS (a comparison of the novel versus canonical DAF-16 function and its regulation is summarized in the S1 Fig).

The *C. elegans* germline is the only tissue harboring stem cells and mitotic events throughout adulthood. The somatic distal tip cell (DTC) serves as the niche and maintains the germline stem cell fate by producing ligands to active Notch receptor on neighboring germ cells [8]. In addition, IIS promotes robust larval germline proliferation via inactivation of DAF-16 [5]. Moreover, the mammalian target of rapamycin complex one (mTORC1) signaling in the germline promotes cell proliferation and prevents differentiation [9]. Furthermore, the canonical TGF-ß/dauer signaling pathway has been shown to promote mitosis versus meiosis fate in parallel to Notch signaling [10]. Mutations causing hyperactive Notch signaling can lead to germline tumor due to excessive cell proliferation [11, 12]. In contrast, despite positive roles of IIS and mTORC1 signaling in regulating germline proliferation, active IIS contributing to germline tumor formation to our knowledge has only been reported once [6].

BMP signaling (also called the TGF-ß Sma/Mab pathway in *C. elegans*) is also highly conserved from invertebrates to humans. The BMP signaling cascade involves extracellular ligands like 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) [13–15]. BMP signaling in *C. elegans* positively regulates body size, male tail development and innate immunity [16–18]. In addition, reduction of BMP signaling and IIS delays reproductive aging by maintaining oocyte and germline quality through non-autonomous mechanisms [19].

Here, we uncover a novel interaction of BMP signaling and DAF-16 in promoting germline tumor formation. Our genetic and biochemical results suggest that the R-Smad proteins SMA-2 and SMA-3 bind directly to DAF-16 in the nucleus to regulate transcription of several common target genes of the mTORC1 pathway. R-Smad regulation of DAF-16 activity during germline tumorigenesis seems to be critical only in the hypodermis. Furthermore, we demonstrate that cell-autonomous and non-cell-autonomous activities of DAF-16 affect germline proliferation in opposite ways, suggesting that a functional balance of DAF-16 in distinct tissues may be important for rapid adaption to the diverse environmental change to ensure survival and reproduction.

## Results

### Both mTORC1 and BMP signaling pathways contribute to the DAF-16 dependent tumorous germline phenotype

We had shown previously that both *shc-1* and *daf-16* which link JNK and IIS signaling pathways contribute to tumorous germline formation [20]. In order to dissect the contribution of each pathway, we performed an RNA interference screen to identify kinases involved in germline hyperplasia. For this purpose, we screened the contribution of all kinases in the Ahringer lab [21] and the ORFeome library [22]. As a readout, we looked for suppression of the 49% penetrant sterility of *shc-1;Is[daf-16::GFP]* animals, a phenotypic aspect that is the consequence
of the tumorous germline (S2 Fig) [6]. We found that RNAi clones of three kinase genes, *sma-6, hpo-11*, and *rsks-1*, not only strongly suppressed sterility (S1 Table), but also germline hyperproliferation and rupture of the gonadal basement membrane (Fig 1A–1E, S3 Fig and S2 Table). This suggests that the activity of all three genes contributes to germline tumor formation in *shc-1;Is[daf-16::GFP]* mutant animals.

Among these identified three genes, *rsks-1* encodes the *C. elegans* S6 kinase homolog, a downstream target of mammalian target of rapamycin complex 1 (mTORC1) [23] which is
activated by the GTPase RHEB-1/Rheb and the adapter protein DAF-15/Raptor [24–26].
This suggested that mTORC1 signaling might contribute to DAF-16 dependent germline 
tumor formation. We next asked whether inactivating other mTORC1 components in C. 
elegans, let-363/TOR, rheb-1/Rheb, and daf-15/Raptor, could also suppress the shc-1;Is[daf-16: 
GFP] tumor. RNAi knock-down of let-363, daf-15 or rheb-1, respectively, resulted in constitutive L3 larval arrest in the F1 generation of shc-1;Is[daf-16::GFP] animals, substantiating 
previous findings that mTORC1 is necessary for normal development of C. elegans [27].

However, this prevented examination of the tumor phenotype that we examined at mid-late 
L3 larval stage [6]. Therefore, we initiated RNAi knock-down at larval stage L1 since such 
short-term RNAi knock-down of mTORC1 did not cause L3 larval arrest. RNAi knock-
down of daf-15, let-363 and rheb-1, respectively, strongly suppressed both germline hyper-
proliferation and disruption of the basement membrane of shc-1;Is[daf-16::GFP] mid-late L3 
animals (Fig 1B and 1E). We conclude that mTORC1 in this genetic context also has onco-
genetic activity in C. elegans.
sma-6 encodes the TGF-ß/BMP type I receptor [28], suggesting a role of BMP signaling in 
germline tumor formation. We asked whether the other genes in the BMP pathway are also 
involved in the germline phenotype. Crossing shc-1;Is[daf-16::GFP] with daf-4(m63) strongly 
enhanced the low penetrance of the constitutive dauer phenotype of daf-4, and prevented 
examination of this strain in stages later than L3. Inactivating the BMP receptor ligand gene 
dbl-1, the Co-Smad gene sma-4, the Smad genes sma-2, sma-3, or the Smad cofactor gene 
sma-9 by either mutation or RNAi fully suppressed the sterile phenotype of shc-1;Is[daf-16:: 
GFP] and strongly decreased the percentage of animals with disrupted gonadal basement 
membrane in both L3 and day one adult animals (Fig 1F and 1G, S1 and S2 Tables). Therefore, 
in addition to mTORC1, also BMP signaling contributes to DAF-16 dependent germline 
tumor formation.

In C. elegans, a second TGF-ß/dauer signaling pathway regulates dauer formation [29, 
30]. It had been suggested that neuronal TGF-ß dauer signaling promotes germline prolifera-
tion [10, 31]. We observed that RNAi knock-down of daf-1 encoding the type I TGF-ß 
receptor (specifically involved in dauer signaling) only slightly suppressed sterility, but 
failed to decrease the severity of the gonadal defect in shc-1;Is[daf-16::GFP] animals (S2 
Table). Therefore, daf-1 RNAi behaved differently from sma-6 RNAi, suggesting that 
TGF-ß/dauer signaling probably affects the DAF-16 mediated germline phenotype only 
indirectly.

The interaction of DAF-16 and BMP signaling is specific to germline signaling

Since we discovered, to our knowledge, a novel functional cross-talk between BMP signaling 
and DAF-16, we asked whether they also interact to promote additional functions of each 
other. Active DAF-16 under reduced IIS leads to constitutive dauer formation at larval stage 
and lifespan extension during adulthood [32]. Therefore, we next tested whether BMP signal-
ing is also required for DAF-16 to regulate lifespan extension or dauer formation. sma-6 RNAi 
knock-down did not shorten, but rather slightly extend the lifespan of daf-2(e1370) animals 
(Fig 2A and S3 Table). sma-6(wk7) also did not suppress constitutive dauer formation of daf-2 
(e1370) at 25˚C (S4 Table). These data suggest that SMA-6/BMP function does not seem to be 
involved in other roles of DAF-16.

One of the best studied functions of BMP signaling in C. elegans is in body size regulation 
[28]. IIS also regulates body size, since daf-2 mutant animals are longer than wild type as a con-
sequence of DAF-16 activation [34], results that we confirmed here (Fig 2B and S5 Table).
However, *sma-6(wk7)* reduced body sizes of both *daf-2* and *daf-16;daf-2* animals, suggesting that BMP signaling positively regulates body size probably in parallel to DAF-16. Taken together, signaling to the germline is the only cooperative role of BMP and DAF-16 interaction that we found.
BMP signaling promotes the stem-cell proliferative activity of nuclear DAF-16

DAF-16 activity is regulated at least at two steps: via control of its nuclear translocation and, subsequently, via modifying its strength as a transcriptional activator through protein interactions and possibly protein modifications. The DAF-16(4A) variant shows constitutive nuclear localization and our previous study has indicated that both DAF-16(4A)::GFP and wild type DAF-16::GFP expressed in shc-1(ok198) background induce germline tumorigenesis [6, 35]. Nuclear localization of DAF-16(4A)::GFP was not altered by a sma-6 mutation (Fig 2C). However, sma-6(wk7) strongly reduced the penetrance of the germline phenotype in shc-1;Ex[daf-16(4A)::GFP] animals (Fig 2D and S2 Table). This suggests that SMA-6 may modulate the transcriptional activity of nuclear DAF-16.

BMP signaling and DAF-16 promote the germline hyperproliferation in a non-cell-autonomous way

Reporter gene studies have suggested that sma-6 is expressed in the hypodermis, intestine, and pharynx [28]. Using our own reporter genes, we also observed weak sma-3 expression in the somatic gonad (S4 Fig). It is not known whether sma-6 is expressed in the germline due to germline silencing of the transgenic reporter genes used in such expression analysis. To knock-down sma-6 exclusively in the germline, we performed sma-6 RNAi in the background of the rrf-1(ok589) loss-of-function mutant that limits the sensitivity against RNA interference to the germline [36]. sma-6 RNAi suppressed the gonadal integrity defects in shc-1;Is[daf-16::GFP], but not in shc-1;rrf-1;Is[daf-16::GFP] animals (Fig 3A, S1 and S2 Tables), indicating that somatic, but not germline activity of SMA-6 may contribute to DAF-16 dependent tumors formation. Since a recent report suggested that rrf-1(ok589) to some extent also might allow RNAi to work in the intestine and seam cells [37], we cautiously argue that neither seam cell nor intestinal SMA-6 activity seem to contribute to the DAF-16 dependent germline tumor phenotype.

In order to pinpoint the critical tissue for BMP activity, we expressed sma-6 under the control of tissue specific promoters in shc-1;sma-6;Is[daf-16::GFP] animals to rescue tumor suppression of sma-6(wk7) (rescue should result in reoccurrence of the tumor phenotype). Neither expression of sma-6 in the intestine, pharynx nor in the somatic gonad resulted in the reappearance of germline tumors in shc-1;sma-6;Is[daf-16::GFP] animals (Fig 3B). We tried in vain to receive stable transgenic lines of Pdpy-7:sma-6 in shc-1;sma-6;Is[daf-16::GFP] background, in which sma-6 should be expressed exclusively in the hypodermis. Although we obtained more than 200 F1 transgenic animals, we never observed sma-6 expression in the F2 generation, indicating either toxicity or silencing of this transgene. Therefore, we analyzed F1 transgenic animals with hypodermal-only sma-6 expression, but these did not show rescue. However, coexpression of sma-6 in both hypodermis and somatic gonad was sufficient to rescue sma-6 tumor suppression. In contrast, expressing sma-6 in all sma-6 positive tissues except the hypodermis (somatic gonad, intestine and pharynx), did not cause tumor reoccurrence. We conclude that the hypodermis and somatic gonad are the critical tissues for BMP signaling that promote DAF-16 tumor induction.

SMA-2 and SMA-3 bind directly to DAF-16

Since it has been shown that forkhead transcription factors can bind to Smad proteins directly [38, 39], a simple hypothesis was that DAF-16 might physically interact with R-Smad proteins
SMA-2 and/or SMA-3 in the hypodermis. We tested R-Smad and DAF-16 interactions with three different assays.

First, we performed in vivo co-immunoprecipitation experiments from C. elegans cell extracts. Extracts from animals carrying functional GFP::sma-3 were precipitated with anti-GFP antibody and the resulting precipitates were subjected to Western blotting by using anti-DAF-16 antibody to detect endogenous DAF-16 (Fig 3C). We detected DAF-16 in GFP::SMA-3 but not in GFP precipitates, suggesting a specific interaction between DAF-16 and SMA-3.
We were unable to express a functional version of tagged SMA-2 in *C. elegans*, since all fusion tags (GFP, Flag and V5 tags) we had tested resulted in loss of SMA-2 function, indicated by a failure of the respective transgene to rescue the small body size phenotype of *sma-2(e502)* animals.

Second, we performed IP with recombinant DAF-16::V5 and SMA-2::GFP/GFP::SMA-3 co-expressed in the human HEK293T cell line to verify the physical interaction. DAF-16::V5 protein was coimmunoprecipitated with both SMA-2::GFP and GFP::SMA-3 (Fig 3D), further supporting a direct interaction between the two R-Smad proteins and DAF-16.

Third, we tested whether DAF-16/R-Smad interactions occur in a yeast model. As positive control we included FTT-2, which is a *C. elegans* 14-3-3 homologue and has been shown to interact with DAF-16 [40]. We found that in a yeast-two-hybrid system, FTT-2, SMA-2, and SMA-3 all interact with DAF-16 (Fig 3E), corroborating our co-immunoprecipitation results. Even though it has been suggested previously that SMA-2 and SMA-3 form a heterodimer [41], we were unable to detect a direct interaction between SMA-2 and SMA-3 in this assay (S5 Fig).

### DAF-16 and BMP signaling share common target genes

Since both DAF-16 and its BMP interactors SMA-2 and SMA-3 encode transcription factors, we next sought to determine downstream genes influenced by both sets of proteins. Several reports have already documented candidate genes directly regulated by DAF-16 or BMP signaling, but genes co-regulated by DAF-16 and SMA-2/3 have not been reported so far. We therefore performed microarray assays to identify genes differentially regulated in *shc-1(ok198);Is[daf-16::GFP], shc-1(ok198) daf-16(mu86) and shc-1(ok198);sma-6(wk7);Is[daf-16::GFP] strains. Hierarchical clustering of genes differentially regulated between any of the three strains suggested a substantial effect of BMP signaling on the transcriptional output of DAF-16 (S6 Fig). To analyze the consequences of this interaction in more detail, we used a linear model analysis to select genes with a statistically significant (FDR-corrected p-value < 0.01) and robust (at least two-fold expression change) response to DAF-16 or SMA-6 activity changes. We identified 2,006 genes as regulated by DAF-16 and 1,957 genes as regulated by SMA-6. Intriguingly, 77% of these genes respond to both DAF-16 and SMA-6, which corresponds to 39% and 40% of all genes regulated by DAF-16 and by SMA-6, respectively (Fig 4A). A more detailed investigation of these genes revealed that 79% (612/777) of the genes that at least partially depend on SMA-6 are genes that are activated by DAF-16, and another 108 genes are downregulated by DAF-16 in a SMA-6-dependent manner (Fig 4B). Together this indicates that 94% of the genes responsive to both factors are controlled by them in a cooperative manner.

Next we compared the genes identified in our analysis with previously reported consensus sets of genes that are activated (class I genes) and repressed (class II genes) by DAF-16 and that were derived from the combined analysis of different genome-wide expression studies. Typically, these compared *daf-2(-)* against *daf-16(-); daf-2(-)* backgrounds [42]. We found that 33% (162 of 498) of the genes we identify as upregulated by DAF-16 in a SMA-6 independent manner are known class I target genes. Similarly, 30% (218 of 731) of the genes that we see downregulated by DAF-16 independent of SMA-6 are known class II target genes. In contrast, only 8.6% (62 of 720) of the genes either up- or downregulated cooperatively by DAF-16 and SMA-6 are known class I or class II target genes, respectively (Fig 4C). This suggests that the common targets of DAF-16 and BMP signaling are typically distinct from the DAF-16 targets that were identified by comparing *daf-2(-) and daf-16(-); daf-2(-) gene expression. To confirm this hypothesis, we also checked whether modulation of BMP signaling could alter expression of
the frequently used DAF-16 target gene sod-3. In daf-2(e1370) mutant, sod-3 expression is increased in a DAF-16 dependent manner and this can be visualized in vivo by using an integrated transcriptional GFP fusion reporter [43]. We verified this observation and showed that daf-2 animals showed increased GFP expression, and this was fully dependent on DAF-16 (Fig 5A). Knock-down of sma-6 did not significantly affect the expression level of the sod-3::GFP reporter in daf-2 background, further supporting our microarray assay results.

**TORC1 and hpo-11 are common target genes of DAF-16 and BMP signaling to promote germline tumor**

Next, we searched for common targets of DAF-16 and BMP signaling to cause germline tumor formation. Inspecting the modENCODE database, we found reports of DAF-16 occupancy on the promoter regions of hpo-11, daf-15, rks-1 and rheb-1 (S7 Fig). In addition, we identified consensus DAF-16 binding sites in each of their 5’ promoter sequences, making them candidates for being direct DAF-16 target genes. Next, we tested by mRNA quantification whether
expression of these genes is affected by DAF-16 and BMP activities. Quantitative-PCR (q-PCR) results showed that the mRNA levels of *rsks-1*, *rheb-1*, *daf-15*, and *hpo-11* in *shc-1;ls*[daf-16::GFP] animals were strongly increased when compared to wild-type, and the increase fully depended on DAF-16 (Fig 5B). Inactivation of *sma-6* significantly reduced mRNA levels of *rsks-1*, *rheb-1*, *daf-15*, but not of *hpo-11*. In summary, combinatorial activation by DAF-16 and R-Smad transcription factors in the hypodermis may activate some common transcriptional target genes to cause tumor formation in the germline.

To show direct binding of DAF-16 and SMA-3 to the promoters of the candidate target genes, we performed ChIP-qPCR experiments using the promoter regions predicted by the modENCODE project (position of primer sets for ChIP-qPCR indicated in the S7 Fig). Our data revealed binding of both DAF-16 and SMA-3 in the *rheb-1* promoter, and binding of DAF-16 in the *hpo-11* promoter (Fig 5C). Quantification of DAF-16 and SMA-3 binding on
both daf-15 and rsk-1 promoters failed, since we were unable to find primer sets suitable for q-PCR quantification (list of tested primer sets is provided in S9 Table).

**IIS controls transcriptional specificity of DAF-16 towards its target genes**

One of the surprising results in Qi et al. (2012) was that DAF-16 dependent germline tumor formation was suppressed, rather than enhanced by additional inactivation of daf-2. This indicated an obvious discrepancy to the canonical model of the IIS pathway, which would predict that down-regulation of daf-2 should enhance, rather than suppress DAF-16 activities. We asked whether inactivation of daf-2 suppresses DAF-16 dependent tumor phenotype via regulating the identified downstream genes. Whereas all four candidate genes (rks-1, rheb-1, daf-15, and hpo-11) are strongly activated in the tumor strain shc-1;Is[daf-16::GFP] that carries a daf-2(+) allele, they were differentially regulated in daf-2(-) animals (Fig 5D). In daf-2 mutant animals only hpo-11 mRNA level was up-regulated by DAF-16. In contrast, both rks-1 and daf-15 transcripts were strongly reduced and only down-regulation of daf-15 mRNA level partially depended on DAF-16. This suggests the existence of both DAF-16 dependent and independent mechanisms to inactivate mTORC1 in daf-2(-) mutant background. In addition, our results indicate that the target specificity of DAF-16 can be differentially affected by upstream IIS.

**Hypodermal DAF-16 and BMP signaling promote germline proliferation**

The results so far suggest that DAF-16 mis-regulation as a consequence of distinct genetic backgrounds requires BMP signaling to become tumorigenic. The obvious question therefore was, whether BMP signaling also contributes to normal developmental functions of DAF-16 in wild-type strains, such as controlling germline proliferation. This could be examined by counting germline nuclei in the proliferative zone of the germline which is located between the DTC and the transition zone in which germ cell differentiation is initiated. daf-16 and sma-6 animals at day one of adulthood contained approximately 20% and 63% less mitotic germline nuclei, respectively, than wild-type animals (Fig 6A), suggesting that both DAF-16 and SMA-6 positively regulate distal germline proliferation. To address tissue specificity of DAF-16 and SMA-6, we tried rescuing daf-16 and sma-6 in a tissue-specific way. Transgenic expression of both wild-type DAF-16::GFP and constitutive nuclear DAF-16(4A)::GFP in the hypodermis completely rescued the decreased number of mitotic germline nuclei in daf-16 mutant animals. DAF-16 expression in the intestine also partially rescued the germline proliferation phenotype of daf-16 mutants whereas expression in the neuron or muscle had no effect. We noticed that, whereas in the majority of animals hypodermal DAF-16 expression of either wild-type daf-16::GFP or daf-16(4A)::GFP rescued the daf-16 mutant phenotype, in approximately 10% of these animals the numbers of mitotic germline nuclei even exceeded 300, something never observed in wild type animals. This sometimes led to enlargement of distal gonads (S8 Fig). Expression sma-6 in the somatic gonad robustly rescued germline proliferation defect while hypodermis expression sma-6 showed a minor, but significant, increase of proliferative germ cell number (Fig 6B). sma-6 expression in neither intestine nor pharynx had any effect on sma-6(-) animals. Taken together, these results suggest both hypodermal DAF-16 and BMP signaling are required for wild-type germline proliferation. And they also act in additional distinct tissues.

Do DAF-16 and BMP functions in the hypodermis depend on each other? Hypodermal expression of sma-6 failed to increase germ nuclei number in the proliferative zone of daf-16 animals (Fig 6A), and hypodermal daf-16 expression also did not increase the number of proliferative germ cells in a sma-6 mutant (Fig 6B). These results suggest that the DAF-16 and BMP signaling activities in the hypodermis to regulate germline proliferation depend on each
other, consistent with our proposed model that DAF-16 and R-Smad protein interaction cooperate to control common target genes.

Next we asked whether selective down-regulation of the target genes of DAF-16/SMA-2/3 interaction in the hypodermis, namely *rsks-1*, *rheb-1*, *daf-15* and *hpo-11* affects germ cell proliferations. For this purpose, we performed hypodermis-specific RNAi knock-downs of these four genes in an *rde-1;Is[Plin-26::rde-1]* strain. This strain harbors the argonaute/PIWI gene *rde-1* required for the function of RNA interference exclusively in the hypodermis. *rheb-*
1, daf-15, or hpo-11 RNAi resulted in an approximately 20% reduction of germline nuclei in the proliferative zone and rsks-1 RNAi led to an approximately 25% reduction (Fig 6C), indicating that RHEB-1, RSKS-1, DAF-15, and HPO-11 in the hypodermis positively regulate germline proliferation. We asked whether hypodermal specific RNAi knock-downs of these four genes could further reduce number of proliferative germ nuclei in either daf-16 or sma-6 background. rheb-1, daf-15, or hpo-11 RNAi failed to further reduce number of proliferative germ nuclei in either daf-16;rde-1;Is[Plin-26::rde-1] or sma-6;rde-1;Is[Plin-26::rde-1] animals (Fig 6D and 6E). rsks-1 RNAi further reduced number of proliferative germ nuclei in these two strains but to less extent compared with rde-1;Is[Plin-26::rde-1] (15% vs. 25% reduction). Taken together, these experiments indicate that rsks-1, rheb-1, daf-15 and hpo-11 are epistatic to both DAF-16 and BMP signaling in the hypodermis to promote germline proliferation. In addition, rsks-1 might also mediate additional pathway to regulate cell proliferation.

Discussion

We identified protein interactions of DAF-1/FOXO and the Smad transcription factors of TGF-ß/BMP signaling that regulate target genes components of the mTORC1 pathway in C. elegans. This cross-talk promotes cell proliferation of germline stem cells non-cell-autonomously, and its hyper-activation results in a tumor-like germline phenotype. Another identified downstream target of IIS/DAF-16 axis encodes HPO-11, a pseudo-kinase with unknown function in C. elegans and ortholog of NRBP/MADM (nuclear receptor-binding protein, myeloid-leukemia factor-adaptor molecule), a regulator of germline stem cells and tumorigenesis in humans and Drosophila. Based on these functional similarities, we suggest that aspects of FOXO/R-Smad signaling to the germline stem cells may be conserved in evolution.

BMP signaling contributes to DAF-16 dependent germline tumor formation

DAF-16/FOXO hyperactivity in the hypodermis results in a tumor-like germline phenotype that we showed to involve germ cell hyper-proliferation and disruption of the basement membrane [6]. Now we identified genes whose down-regulation suppressed this phenotype, among them sma-2, sma-3, sma-4, sma-6 and sma-9, encoding components of the TGF-ß/BMP pathway. A combination of genetic and biochemical experiments furthermore revealed that the BMP effectors SMA-2 and SMA-3 can physically interact with DAF-16 to promote germline proliferation at wild type conditions, but may cooperate with hyper-active DAF-16 to cause tumors. This interaction is selectively required for only a subset of DAF-16 functions, based on the following arguments: (I) We show that the well-known beneficial roles of DAF-16 in extending lifespan and in promoting dauer formation in daf-2(-) animals do not involve BMP signaling (Fig 2, S3 and S4 Tables). (II) The DAF-16 dependent transcriptional activation of sod-3 in daf-2 mutant animals is also independent of BMP (Fig 5A). (III) Moreover, both BMP and IIS/DAF-16 contribute to the regulation of body size, but do so independently of one another (Fig 2C and S5 Table). Taking together, these results suggest that the functional interaction between BMP signaling and DAF-16 has consequences that seem to be specific for the germline, even though this interaction takes place in the hypodermis, a tissue adjacent to the gonad. Although both DAF-16 and R-Smad proteins are capable of shuttling between nucleus and cytoplasm, we suggest that the relevant interaction described here occurs in the nucleus, since a sma-6 mutation could still suppress the phenotype caused by constitutively nuclear DAF-16(4A);::GFP. We therefore propose that BMP signaling promotes the activity of nuclear DAF-16, rather than its nuclear entry (Fig 2C and 2D), by modulating DAF-16 binding to the promoters of downstream genes. This claim was further justified by identifying
DAF-16 and SMA-3 binding sites in the promoter of *rheb-1* gene of the mTORC1 pathway (see below).

Hypodermal activity of BMP signaling to the germline has been described to regulate oocyte and germline quality maintenance, but this was suggested to occur independently of IIS [19]. Our newly discovered functional cross-talk between DAF-16 and BMP is distinct of the previously identified interactions of DAF-16 with the canonical TGF-β/dauer signaling pathway involved in lifespan regulation [44]. TGF-β/dauer signaling in the nervous system also promotes germline proliferation via preventing meiosis [10, 31]. Even though we observed that down-regulating the *daf-1* gene, encoding a receptor type I homologue of the TGF-β/dauer, but not the BMP signaling pathway, slightly decreased the severity of the DAF-16 dependent germline tumor phenotype, we consider the interaction between DAF-16 and canonical TGF-β/dauer pathway most likely as being indirect, since DAF-16 and DAF-1 act in different tissues [6, 10].

**BMP signaling and DAF-16 control common targets to cause tumorous germline phenotype**

We show that the R-Smad/DAF-16 interaction activates the expression of at least three genes (*rks-1*, *rheb-1*, and *daf-15*) which encode components of the mTORC1 pathway. Down-regulation of each of them strongly suppressed tumorigenesis. In addition, DAF-16 activates *hpo-11* possibly independent of BMP signaling, although the influence of *hpo-11* on wild type mitotic germ cell numbers (Fig 6C) is fully lost in both *daf-16(mu86)* and *sma-6(wk7)* mutant backgrounds (Fig 6D and 6E). All four down-stream genes contain DAF-16 binding elements (DBE) or DAF-16 associate elements (DAE) in their 5’ promoter regions and *hpo-11* has been reported to be a DAF-16 direct target [45]. Even though arguments were convincing that DAE may be a recognition site of PQM-1, the transcription factor acting antagonistically to DAF-16 [42], the *C. elegans* modENCODE project detected direct binding of DAF-16 onto the promoter regions of *rheb-1* that contains DAE, rather than DBE [46], and we confirmed DAF-16 binding to *rheb-1* by chromatin IP (Fig 5C). Our genetic data also do not find any evidence for a contribution of PQM-1 to DAF-16 dependent germline tumor formation, as *pqm-1* RNAi in our hands did not modulate the tumor phenotype (S1 and S2 Tables). Moreover, *pqm-1* was reported to be expressed exclusively in the intestine, whereas our data suggest that hypodermal expression of all four downstream genes promotes cell proliferation in the germline. Together, these data suggest that *rks-1*, *rheb-1*, *daf-15*, and *hpo-11* are candidates for direct transcriptional targets of R-Smad and/or DAF-16 to promote cell proliferation in the germline. Our results also indicate that DAF-16 can activate mTORC1 at the transcriptional level. This differs from the previous report that DAF-16 represses *daf-15* transcription in *daf-2(-)* mutant animals [26], suggesting a context dependent regulation of DAF-16 on mTORC1. We also showed that presence or absence of DAF-2 determines whether DAF-16 activates or represses transcription of mTORC1 components (Fig 5D). Similarly, as we will discuss below, presence or absence of DAF-2 also determines whether DAF-16 acts oncogenic or as a tumor suppressor. Such antagonistic effect of FOXO is also known from human tissues [47]. Finally, our results indicate that similar to the widely accepted tumorigenic activity of mTORC1 in other organisms, its *C. elegans* counterpart may fulfill a comparable role [48, 49].

**Antagonistic functions of DAF-16 in distinct tissues control germline proliferation under different physiological conditions**

The complexity of IIS signaling and DAF-16 activities in distinct tissues was highlighted by the observation of germline tumors in distinct genetic contexts. Mutant genetic backgrounds
resulting in germline tumors were shc-1::ts[daf-16::GFP], shc-1::akt-1, or shc-1::daf-18::akt-1 [6]. Common denominator is that DAF-16 is active in all strains, and shc-1 inactivation is a requirement for their solid phenotypic penetrance. However, both shc-1::ts[daf-16::GFP], and shc-1::akt-1 only resulted in tumors in the presence of daf-2(+), and daf-18/PTEN(-) exacerbated shc-1::akt-1 tumor penetrance, but did not cause germline proliferation defects in either shc-1 or akt-1 alone. These observations are, as we reported earlier [6], inconsistent with a simple model based on the canonical insulin signaling pathway, since daf-2(+) and daf-18(-) both are commonly known as negative regulators of DAF-16. Therefore, we hypothesize that down-regulation of daf-2 to suppress the DAF-16 induced germline phenotype may predominantly affect the germline rather than somatic functions of IIS [6]. It has been shown that daf-2 inactivation reduced mitotic rates of the germline stem cells via a cell-autonomous DAF-16 activity to arrest cell cycle [5]. We corroborated this result, since we counted a 40% reduction of the germline nuclei in the proliferative zone in daf-2(e1370). This reduction was almost completely dependent on DAF-16 (S9 Fig), suggesting a conserved function of IIS to tie nutrition to cell cycle control, and a cell-autonomous function of DAF-16 in the germline to prevent tumorigenesis. Our results, in contrast, suggest a distinct, germline proliferation-promoting and non-cell-autonomous role of DAF-16 which acts in the hypodermis and becomes dominant when the balance between somatic and germline IIS is perturbed. Since hypodermal expression of a daf-16 transgene in daf-16 mutant animals was sufficient to increase the number of germ cell nuclei in the proliferative zone, these two antagonistic activities of DAF-16 in the hypodermis versus the germline should function in parallel (Fig 7). In well-fed animals, DAF-2 is considered to be active, and DAF-16 therefore should be inactive. Consistently, DAF-16::GFP fusion proteins in somatic tissues have been shown to localize mostly to the cytosol. However, a pool of active DAF-16 must still be present, since daf-16 loss-of-function mutants can be phenotypically distinguished from daf-16(+)/wild type animals due to their reduced number of proliferative germ cells (Fig 6A). Therefore, we propose that, in the presence of food, residual DAF-16 activities in both tissues are balanced with pro-proliferating hypodermal dominating over anti-proliferating germline DAF-16 to allow robust germline proliferation. These two opposing functions of DAF-16 allow a balanced response to diverse environmental challenges to ensure survival and optimal reproduction. Upon food limitation and subsequent reduction of IIS, DAF-16 activity in the germline becomes dominant and inhibits germline mitosis. Such different responses of somatic and germline cells, however, require differences in the composition of the IIS pathway in both tissues, for example, tissue-specific utilization of pathway components. Candidates mediating such differences are the AGC family kinases AKT-1, AKT-2, and SGK-1. Indeed, our previous experiments had already shown that the individual distribution of AKT kinases and SGK-1 differ between individual tissues, which might explain why shc-1::akt-1 germline tumor are potently suppressed by RNAi down-regulation of either akt-2 or sgk-1 [6]. However, in certain genetic backgrounds, in which hypodermal DAF-16 activation dominates and this balance is lost, the consequence may be germline tumor.

**Interaction between FOXO and BMP signaling**

FOXO transcription factors play an essential role in maintaining hematopoietic stem cells [51]. An important function of FOXO3a was also proposed in the maintenance of cancer stem cells that are responsible for the reoccurrence of chronic myeloid leukemia [52]. In addition, FOXO 3/4 has been shown to promote acute myeloid leukemia (AML) via inhibiting myeloid maturation and apoptosis [47]. Due to a critical role of the PI3K/Akt/mTOR signaling pathway in survival and growth of malignant cells, mTOR inhibitors like rapamycin are used in treating AML. We showed here that mTORC1 can be activated by DAF-16 and BMP signaling to
promote germline tumor and it remains to be tested whether this phenotype is sensitive to rapamycin treatment. Even more interesting is the identification of hpo-11 as a novel DAF-16 target gene, because its mammalian ortholog MADM (Mlf-1 adaptor molecule) physically interacts with myeloid leukemia factor 1 (Mlf1), which is involved in AML [53]. Interestingly, a recent publication had suggested that Drosophila MADM regulates the competition between germline stem cells and somatic cyst stem cells, for niche occupancy and also to control tumor-igenesis [54]. This may suggest that the molecular mechanisms contributing to stem cell tumors that we identified here could be evolutionarily conserved. To our knowledge there is only one study suggesting that FOXO1/3 regulates follicle growth or death by interacting with the BMP pathways in granulosa cells [55]. It will be of great interest to test whether functional
interaction between DAF-16 and BMP signaling is also conserved in *Drosophila* and in mammalian tumorigenesis, especially in AML.

**Materials and methods**

Information about strains and constructs used is described in the S1 Materials and methods.

**Scoring disruption of the gonadal basement membrane**

Scoring of disruption of the gonadal basement membrane at L3 larval stage was performed with MitoTracker staining which has been described to label basement membrane [56]. Basement membranes were stained by placing animals in a solution of 20 μM MitoTracker Red CMXRos (Molecular Probes) in M9 buffer at 23˚C for 1 hr. The animals were allowed to recover for 30 min on NGM agar plate. The mitochondria staining with MitoTracker was photobleached using a 30 s exposure to fluorescence, leaving the basement membrane staining which is resistant to photobleaching.

For scoring disruption of the gonadal basement membrane in day one adults, animals were raised at 20˚C and analyzed via DIC microscopy 24 hr after L4 stage. Germ cell outside of the gonad was indicator of gonad disruption. Per test at least thirty animals were examined and each test was performed at least three times.

**Quantification of the germ cell nuclei in L3 animals**

For counting total numbers of the germ cell nuclei, the numbers of anti-PGL-1 positive cells were quantified at the mid-late L3 stage. Antibody staining was performed as described before [6]. For the whole worm staining, 1:200 anti-PGL-1 antibody was used (a gift from Dr. Susan Strome).

**Lifespan assay**

Lifespan assays were initiated at the L4 larval stage. Synchronized animals were raised at 15˚C prior to lifespan analysis. In order to inactivate *fer-15*, L4 animals were transferred to 25˚C for the assays and examined every day. Animals that showed no response to touch were scored as dead. All of the lifespan assays were performed three times.

**Quantification of germ cell nuclei in the proliferative zone via DAPI staining**

The ‘number of nuclei in the proliferative zone’ included all the germ nuclei between the distal tip and the transition zone of day one adult animals (24 hr after mid-L4 larval stage). For visualizing and counting germ cell nuclei in the proliferating zone of the gonad, dissected gonads were fixed in methanol and resuspended in PBST containing 0.1% Tween 20 and 2 μg/ml 4’6-diamidino-2-phenylindole (DAPI) before microscopy. Z-stack images of animals were collected and the numbers of the germ cells were counted by using a ImageJ Cell Counter plug-in originally written by Kurt De Vos at the University of Sheffield, Academic Neurology.

**Yeast two-hybrid assay**

The cDNAs of interaction partners were cloned into pGBKT7 and pGADT7 yeast vectors. Competent cells of the yeast strain AH109 were cultured at 30˚C until OD₆₀₀nm value 0.8–1.2. After centrifugation, the pellets were resuspended in 1 ml LiAc-TE solution (100 mM LiAc, 10 mM Tris, pH 7.4, 1 mM EDTA). The transformation was conducted by adding 10 μg boiled salmon sperm DNA (2 mg/ml), 500 μl PEG (50% PEG MG3350) and 1 μg of each plasmid.
DNA to 100 μl yeast aliquot. After incubation at 30˚C for 15 min, 50 μl DMSO were added. After heat shocking at 42˚C for 15 min, the cells were spread onto plates without Trp or Leu. The interaction was double determined by spreading the transformed yeast onto the two selective plates (-Leu/-Trp/-His with 3 mM 3-AT and -Leu/-Trp/-Ade).

Microarray analyses
Total RNA was prepared from L3 animals raised at 20˚C by using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). RNA integrity was analyzed by capillary electrophoresis using a Fragment Analyser (Advanced Analytical Technologies, Inc. Ames, IA). RNA samples had RNA quality numbers (RQN) between X and Y and were further processed with the Affymetrix WT Plus kit and hybridized to GeneChip C. elegans 1.0 ST arrays as described by the manufacturers. Partek Genomics Suite software was used for analysis (Partek Inc., St.Louis, MI). CEL files were imported including control and interrogating probes, pre-background adjustment was set to adjust for GC content and probe sequence, and RNA background correction was performed. Arrays were normalized using Quantile normalization and probe set summarization was done using Median Polish. Probe values were log2 transformed. For the statistical analysis of differentially expressed genes log-scale gene expression values were imported into R and analyzed using the limma package [57]. For GO term analysis we used g:Profiler (http://biit.cs.ut.ee/gprofiler).

Quantification of mRNA level via Quantitative RT-PCR (q-PCR)
Total RNA was prepared from L3 animals raised at 20˚C by using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). act-4 was used as internal control. q-PCR primer sequences daf-15 for: GACAAACCGGAAGAATTATGA, daf-15 rev: CGCGAGAAGTTGAGGAGA, hpo-11 for: CACACGTTTTATGTGTCAGC, hpo-11 rev: TTTCCGAAACACCTTCGCAAT, rsks-1 for: TCCACCAATGTTCGTGTG, rsks-1 rev: GTGCTTTTTTCGCACATTTGGA, rheb-1 for GAAATCGCGTGTGATCTT, rheb-1 rev: GGAAACAAGTCTCGGAAAAC. act-4 for: CCACCAGTACCCAGGAATC, act-4 rev: GTGCGGGGATGATCTTGA.

Chromatin IP followed by q-PCR
ChIP was performed as described in Kaletsky et al. [58] by using anti-GFP antibody in wild type (untagged), shc-1(ok198);Is[daf-16::GFP], daf-2(e1370);Is[daf-16::GFP] and a somatic exclusive GFP::sma-3 strain (CS119 sma-3(wk30)III;him-5(e1490)V;qcEx24[GFP::sma-3;rol-6]). The relative fold change of qPCR was normalized to the intern negative control which was daf-15 3’UTR sequence.

Supporting information

S1 Materials and Methods. Information about strains and constructs used.

(PDF)

S1 Fig. Canonical vs. novel DAF-16 functions. Left side (grey): The canonical model of DAF-16 regulation proposes a linear pathway, in which active insulin receptor DAF-2 activates the three down-stream AGC kinases AKT-1, AKT-2 and SGK-1 [59]. AKT-1 and AKT-2 act redundantly and phosphorylate DAF-16 to prevent its nuclear localization and activation. The role of SGK-1 is debated, as different studies propose distinct SGK-1 functions either as DAF-16 inhibitor or activator [60]. DAF-18 and SHC-1 antagonize insulin signalling, thus eventually contributing to DAF-16 activation [20]. Active DAF-16 under reduced insulin signalling extends lifespan, enhances stress resistance and inhibits germline proliferation [5]. Right side (white): Qi et al. (2012) proposed a model according to which hypodermal DAF-16 activation opposes cell-autonomous DAF-16 that has been shown to act in the germline to inhibit germ cell proliferation [6]. Imbalance in DAF-16 inputs into the germline may cause germline tumor formation and reduce lifespan. shc-1;akt-1 double inactivation induce germline tumor formation. In addition, akt-2 or sgk-1 downregulation prevent, whereas daf-18(-) increases the penetrance of shc-1;akt-1 tumorous phenotype. It is currently not known whether AKT-2 and SGK-1 antagonize AKT-1 in the same or different tissues. daf-2(-) prevents tumor formation, likely because tumor-promoting DAF-16 activity is blocked, whereas cell-autonomous tumor-suppressive function of DAF-16 is activated.

(TIF)

S2 Fig. Representative pictures of shc-1(ok198);Is[daf-16::GFP] animals fed with E. coli HT115 carrying empty vector (L4440) or suppressor RNAi. (A) About 50% of the shc-1;Is[daf-16::GFP] animals are sterile due to tumorous germline, the remaining have strongly reduced brood sizes (typically <50 progeny). Therefore, embryos and larval progeny are rarely observed on plates. (B) Suppression of the germline tumor restored reproduction, as obvious from the accumulation of embryos and larval progeny on the plate. Black arrows: P0 adult animals, red arrows: F1 progeny.

(TIF)

S3 Fig. Classification of gonadal basement membrane phenotype. Shown is a representative picture of wild type (intact) and mutant (intermediate or severe defect) gonadal basement membrane stained with MitoTracker. Arrows point to disruptions of gonadal basement membrane in L3 animals of the “intermediate” group. In severe cases, the basement membrane is so substantially disturbed, that the shape and organization of the gonad is no longer recognizable. Note that MitoTracker also stains basement membranes of the other tissues.

(TIF)

S4 Fig. sma-3 is expressed in the somatic gonad. The arrows in this representative picture points to an L3 somatic gonad cell expressing the GFP::sma-3 transgene (byEx1236[Psma-3::sma-3::GFP]).

(TIF)

S5 Fig. No interaction between SMA-2 and SMA-3 in yeast-two-hybrid assay. Yeast-two-hybrid interactions testing different combinations of bait and prey plasmids on two distinct selective media (labelled on top). SMA-2AD and SMA-3AD interact with DAF-16BD, FT2BD interacts with DAF-16AD (positive control). No interaction was observed between SMA-2 and SMA-3.

(TIF)
**S6 Fig. Microarray result.** Heatmap (red/blue = high/low expression levels) of microarray results for the *shc-1;Is[daf-16::GFP]* (bottom row), the *shc-1 daf-16* (middle) and the *shc-1;sma-6;Is[daf-16::GFP]* (top) strains. Common target genes of DAF-16 and BMP signaling are found in clusters for which the coloring of the bottom row differs from that in the top row and in the middle row.

(TIF)

**S7 Fig. DAF-16 binds to promoters of hpo-11, rheb-1, daf-15 and rsks-1.** Wormbase (www.wormbase.org) gene models for *hpo-11, rheb-1, daf-15* and *rsks-1* are shown with modENCODE data from DAF-16 ChIP-seq experiments. The positions of primer sets for ChIP-qPCR and predicted DAF-16 binding sites [42, 61] are pointed in the promoter regions. Red line: DAF-16 binding element (DBE) GTAAAC/TAA or TTA/GTTTAC; black line: DAF-16 associate element (DAE); blue line: the predicted DAF-16 binding sites TGGAA/CAAT or ATTG/TTCCA.

(TIF)

**S8 Fig. DAPI staining of representative germlines of day one adult animals of the indicated genotypes.** The white lines depict the borders of the transition zones between mitotic and meiotic germ cell nuclei. The white arrows indicate the position of the distal tip cells, the stem cell niche. Scale bar: 10 μm.

(TIF)

**S9 Fig. DAF-16 inhibits germline proliferation upon reduced IIS.** The average numbers of germ cell nuclei in the proliferative zone are: wild-type: 249 ± 18 (n = 26); *daf-2(e1370)*: 155 ± 18 (n = 23); *daf-16(mu86);daf-2(e1370)*: 234 ± 24 (n = 19).

(TIF)

**S1 Table. Sterility.**

(PDF)

**S2 Table. Defects in gonadal integrity.**

(PDF)

**S3 Table. Summary of lifespan.**

(PDF)

**S4 Table. Summary of dauer formation at 25˚C.**

(PDF)

**S5 Table. Summary of body length.**

(PDF)

**S6 Table. Transgenic animals generated for this work.**

(PDF)

**S7 Table. Enriched GO terms for genes which are upregulated both by DAF-16 and BMP signaling in *shc-1;Is[daf-16::GFP]* L3 animals.**

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**S8 Table. Summary of quantification of relative mRNA level.**

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**S9 Table. daf-15 and rsks-1 primers tested for ChIP-qPCR.**

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S10 Table. Numbers of germ nuclei in the proliferation zone of day one adults.

(PDF)

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