Functional Interaction Between β-Catenin and FOXO in Oxidative Stress Signaling

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β-Catenin is a multifunctional protein that mediates Wnt signaling by binding to members of the T cell factor (TCF) family of transcription factors. Here, we report an evolutionarily conserved interaction of β-catenin with FOXO transcription factors, which are regulated by insulin and oxidative stress signaling. β-Catenin binds directly to FOXO and enhances FOXO transcriptional activity in mammalian cells. In *Caenorhabditis elegans*, loss of the β-catenin BAR-1 reduces the activity of the FOXO ortholog DAF-16 in dauer formation and life span. Association of β-catenin with FOXO was enhanced in cells exposed to oxidative stress. Furthermore, BAR-1 was required for the oxidative stress–induced expression of the DAF-16 target gene *sod-3* and for resistance to oxidative damage. These results demonstrate a role for β-catenin in regulating FOXO function that is particularly important under conditions of oxidative stress.

Forkhead box O (FOXO) transcription factors are negatively regulated by the insulin signaling pathway through phosphoinositide 3-kinase (PI 3-kinase) and protein kinase B (PKB, also called c-Akt). Phosphorylation by PKB leads to nuclear exclusion of FOXO, and as a result, its function as a transcriptional activator is inhibited (*1*). In *C. elegans*, an insulin-like signaling pathway controls entry into the dauer diapause, an alternative larval stage that is induced by starvation, a dauer pheromone, or high temperature. Under dauer-inducing conditions, the FOXO homolog DAF-16 is activated, and dauer development is initiated (*2*). We observed that animals containing a null mutation in the β-catenin gene *bar-1* (*3*) are defective in starvation-induced dauer development. To investigate whether BAR-1 is required for DAF-16–dependent dauer formation, we activated DAF-16 with a temperature-sensitive loss-of-function allele of the insulin receptor–like gene *daf-2* (*4*–*6*), then, we compared dauer induction in the presence and absence of *bar-1*. At the restrictive temperature (25°C), both *daf-2* and *daf-2*; *bar-1* double mutants showed 100% dauer induction (n = >200), demonstrating that BAR-1 is not required for the execution of dauer development. At intermediate temperatures, when DAF-16 signaling was not fully activated, there was a significantly lower induction of dauer larvae in the absence of *bar-1* (Fig. 1A). This shows that BAR-1 is required for DAF-16 function when DAF-16 activity is limiting. Overexpression of BAR-1 enhanced the *daf-2*–induced formation of dauer larvae (Fig. 1A). Reduced expression of *daf-16* by RNA interference (RNAi) inhibited the dauer-inducing effect of overexpressed BAR-1 (Fig. 1B), which indicates that BAR-1 may increase dauer formation by enhancing DAF-16 activity. In addition to controlling dauer development, DAF-16 also influences longevity (*6*). Because *daf-16* null mutants show a shorter average life span, we tested whether BAR-1 was also required for normal longevity. *bar-1* mutants had a shorter life span, one that was similar to that of *daf-16* mutants (Fig. 1C). Taken together, these results show that BAR-1 is required for DAF-16 signaling in dauer induction and life-span regulation.

To investigate whether β-catenin is required for FOXO function in mammalian cells, we analyzed FOXO activity in cells that have no detectable β-catenin expression (IIAI.6 cells) (*7*, *8*). In these cells, expression of FOXO4 induced transcription of several different FOXO reporters (Fig. 2A) (*9*), and transcriptional activity was enhanced when β-catenin was also expressed. Cytoplasmic abundance of β-catenin is controlled by glycogen synthase kinase–3β (GSK-3β), a component of the β-catenin destruction complex. Stabilization of endogenous β-catenin in A14 cells (*10*) by treatment with the GSK-3β inhibitor LiCl enhanced FOXO4 activity (Fig. 2B). This effect of LiCl appeared to be

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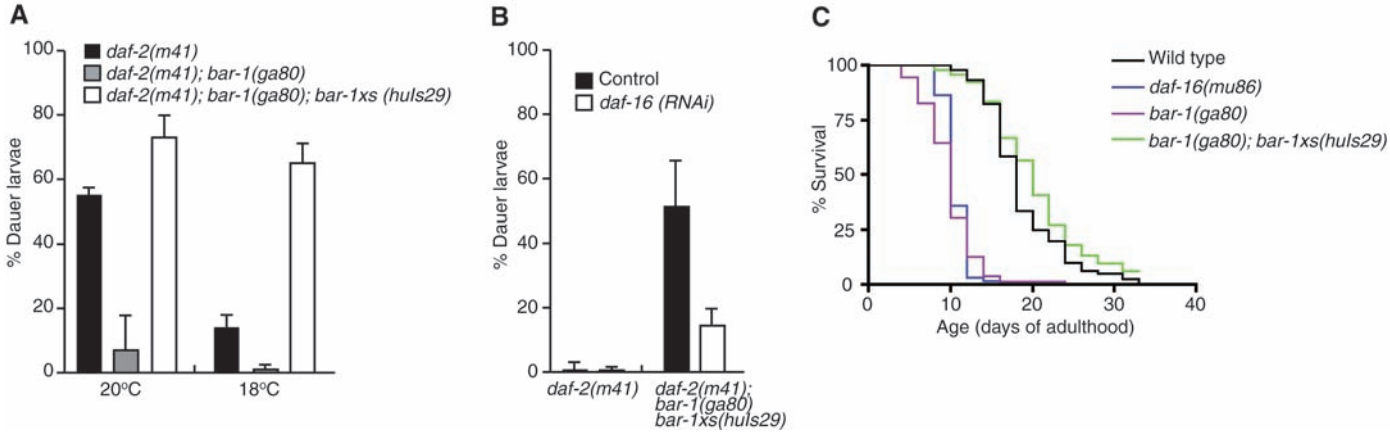

Fig. 1. Genetic interaction between *bar-1* and *daf-16* in dauer induction and life-span regulation. (**A**) Dauer larvae formation induced by *daf-2* was reduced in the absence of *bar-1* ($P < 0.01$, one-way ANOVA with Bonferroni correction), but was enhanced when BAR-1 was overexpressed (*bar-1xs*) ($P < 0.01$ at 18°C). Data are presented as means ± SD and represent the average of four independent experiments. Note that overexpression of BAR-1 did not induce dauer formation in wild-type animals (n = >1000). (**B**) The dauer-inducing effect of BAR-1 overexpression was reduced when DAF-16 function was inhibited by RNAi ($P < 0.01$). Data are presented as means ± SD and represent the average of five independent experiments. (**C**) Loss of *bar-1* reduces life span. Survival curves show that *bar-1* and *daf-16* mutants have a shorter mean life span (10 days, n = 100) than wild-type animals (18 days, n = 100) ($P < 0.01$, Kaplan-Meier survival analysis).

specific to β-catenin stabilization, because LiCl treatment in IIA6.I cells, which lack β-catenin, did not affect FOXO reporter activity (Fig. 2B). Ectopic expression of FOXO results in increased expression of the cell cycle inhibitor p27Kip1 and a consequent arrest in G_1 (*11*). Consistent with the reporter assays, β-catenin overexpression enhanced the expression of endogenous p27Kip1 (Fig. 2C) and the FOXO-induced arrest in G_1 (Fig. 2D). To further investigate the requirement for endogenous β-catenin in FOXO signaling, we used LS174 colon carcinoma cells, which express a β-catenin–specific small interfering RNA (siRNA) under the control of a tetracycline-inducible promoter (*12*). LS174 cells express a mutant form of β-catenin that is insensitive to degradation by the β-catenin destruction complex. As a consequence, T cell factor (TCF)–dependent transcription is increased. Expression of β-catenin–specific siRNA inhibited TCF reporter activity (Fig. 2E). Note that reduction of β-catenin expression also inhibited FOXO-dependent signaling. Transcription induced by FOXO4 overexpression or by activation of endogenous FOXO in cells treated with the PI 3-kinase inhibitor LY294002 was reduced after expression of β-catenin–specific siRNA (Fig. 2E). These experiments show that β-catenin is required for FOXO activity.

In a yeast two-hybrid assay, β-catenin interacted with FOXO1 and FOXO3a (table S1). The interaction required armadillo repeats 1 to 8 of β-catenin and the C-terminal half of FOXO (starting at the DNA binding domain). Binding of FOXO to the armadillo repeats of β-catenin appears to be specific, as FOXO did not bind to the armadillo repeats of APC1 or APC2 proteins. When FOXO4 tagged with

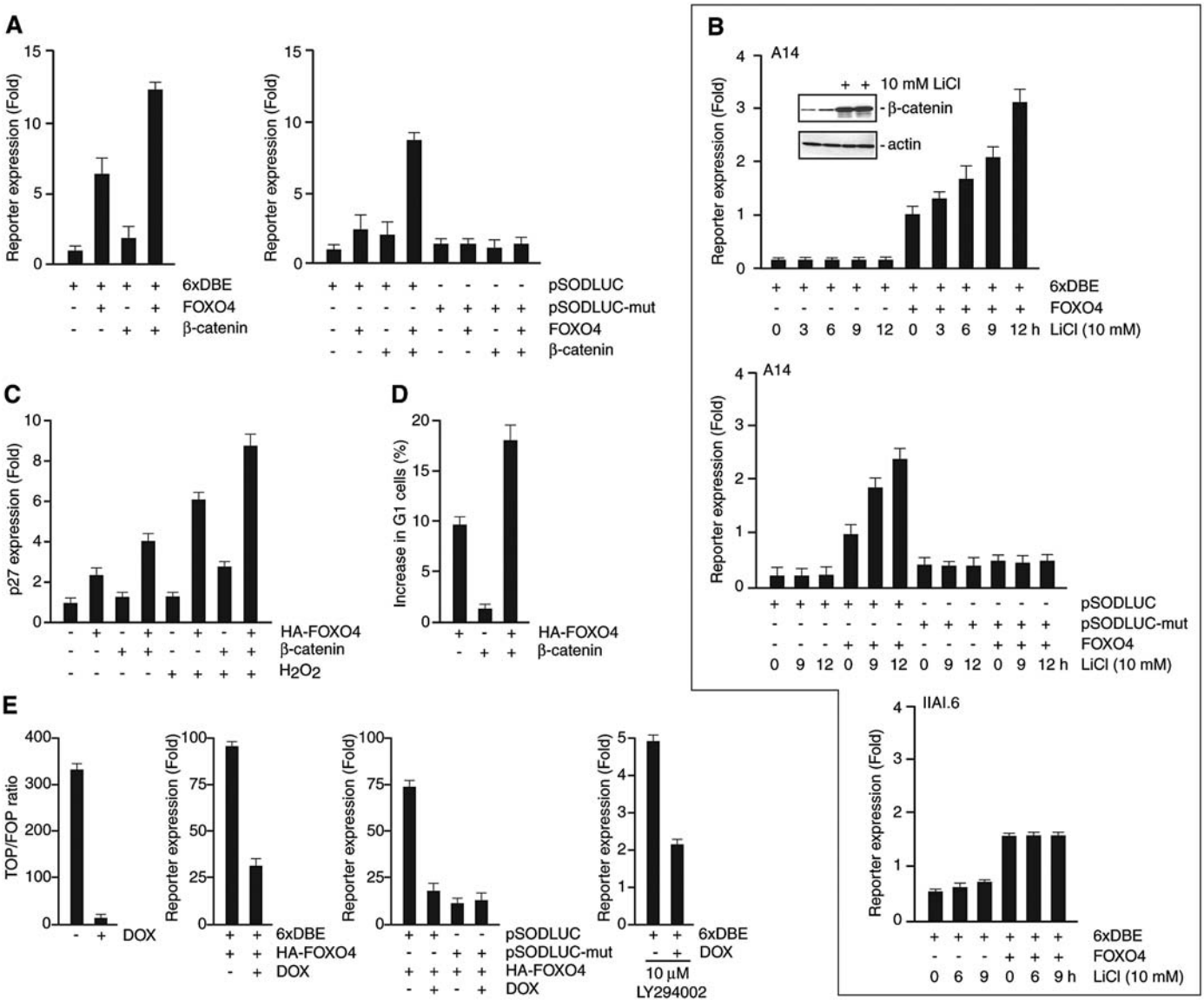

Fig. 2. Enhancement of FOXO transcriptional activity by β-catenin. FOXO activity was measured by using the reporter 6xDBE-luciferase, which contains six canonical FOXO-binding sites, or pSODLUC-3340, which contains the FOXO-binding sites of the SOD promoter. pSODLUC-3340 mutant, which has point mutations in the first and second FOXO-binding sites, was used as a control. Data are presented as means ± SD and represent three independent experiments performed in triplicate. (**A**) In IIAI.6 cells, which lack detectable β-catenin expression, coexpression of β-catenin enhanced FOXO transcriptional activity. (**B**) Stabilization of β-catenin by LiCl treatment enhanced FOXO transcriptional activity in A14 cells, but not in IIAI.6 cells. Inset shows LiCl-induced accumulation of β-catenin. (**C**) Cotransfection of β-catenin in A14 cells enhanced FOXO-induced p27Kip1 gene expression, as measured by quantitative polymerase chain reaction. Cells were treated for 16 hours with 100 μM H_2O_2 (+) or were left untreated (–). (**D**) Cotransfection of β-catenin in A14 cells enhanced the FOXO-induced arrest at G_1. (**E**) Induction of β-catenin–specific siRNA expression in LS174T cells reduced TCF and FOXO transcriptional activity. Cells were treated for 48 hours with doxycycline (DOX) to induce β-catenin siRNA expression. LY294002 treatment was for the last 16 hours. TCF reporter activity was measured by using TOP, which contains optimal TCF binding sites, and the negative control FOP.

hemagglutinin (HA) and β-catenin tagged with the Flag epitope were expressed in human embryonic kidney (HEK293T) cells, Flag–β-catenin could be coimmunoprecipitated with HA-FOXO4 and vice versa (Fig. 3A; fig. S1). In DLD-1 cells, which endogenously express β-catenin and FOXO3a, both proteins were immunoprecipitated together as well (Fig. 3B). Thus, β-catenin and FOXO physically interact in mammalian cells. This binding is likely to be direct, as the two proteins can interact in yeast. Coimmunoprecipitation experiments with BAR-1 tagged with vesicular stomatitis virus glycoprotein (VSV) and Flag–DAF-16 expressed in HEK293T cells showed a detectable interaction between BAR-1 and DAF-16 (Fig. 3C). DAF-16 did not interact with HMP-2 or WRM-1, two β-catenin–like proteins that function in adhesion and noncanonical Wnt signaling, respectively (Fig. 3D) (*9*, *13*). Thus, the interaction between β-catenin and FOXO appears to be evolutionarily conserved.

In *C. elegans*, as well as in mammalian cells, oxidative stress activates FOXO signaling by stimulating its relocation from the cytoplasm to the nucleus (*4*, *14*, *15*). We therefore investigated whether oxidative stress influences the binding between FOXO and β-catenin. Treatment of cells with 200 μM hydrogen peroxide resulted in enhanced binding between ectopically expressed β-catenin and FOXO4 (Fig. 3A), whereas treatment of cells with insulin, which reduces FOXO activity, did not affect the binding (*9*). A small increase in binding (about twofold) in response to oxidative stress was also observed between the endogenously expressed proteins in DLD-1 cells (Fig. 3B). The interaction between exogenous BAR-1 and DAF-16 expressed in HEK293T cells was increased in response to oxidative stress as well (Fig. 3C). We tested whether BAR-1 is required for oxidative stress–induced DAF-16 activity in *C. elegans*. One FOXO target gene that undergoes increased expression after oxidative stress is manganese superoxide dismutase (MnSOD) (*16*). We examined expression of the MnSOD homolog *sod-3* (*17*) in transgenic animals using a green fluorescent protein (GFP)–based reporter (*sod-3::gfp*). There was a strong increase in *sod-3* expression when animals were grown in the presence of 0.25 mM paraquat, a herbicide that induces the formation of reactive oxygen species (*18*) (Fig. 4A). This effect appeared to be DAF-16 dependent, as there was no increase in *sod-3* expression levels in *daf-16* mutants. The paraquat-induced expression of *sod-3* was also reduced in *bar-1* mutants, a defect that was rescued by overexpression of wild-type BAR-1 (Fig. 4B). This shows that BAR-1 is required for oxidative stress–induced DAF-16 signaling. Indeed, overexpression of BAR-1 (in a wild-type *bar-1* background) led to a modest, *daf-16*–

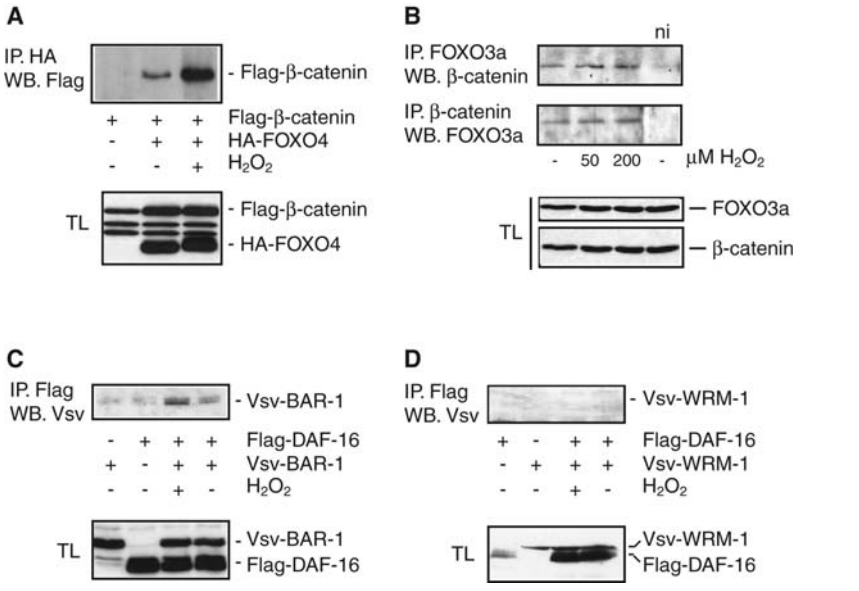

Fig. 3. Binding of β-catenin to FOXO transcription factors. (**A**) Coimmunoprecipitation between transfected Flag–β-catenin and HA-FOXO4 in HEK293T cells. The bottom panel shows expression of the transfected constructs (TL, total cell lysate). (**B**) Coimmunoprecipitation between endogenous β-catenin and FOXO3a in DLD1 human colon carcinoma cells. (**C**) Coimmunoprecipitation between transfected VSV–BAR-1 and Flag–DAF-16 in HEK293T cells. (**D**) VSV–WRM-1 and Flag–DAF-16 do not bind in HEK293T cells. In (A, C, and D), HEK293T cells were treated for 1 hour with 200 μM H_2O_2 (+) or were left untreated (–).

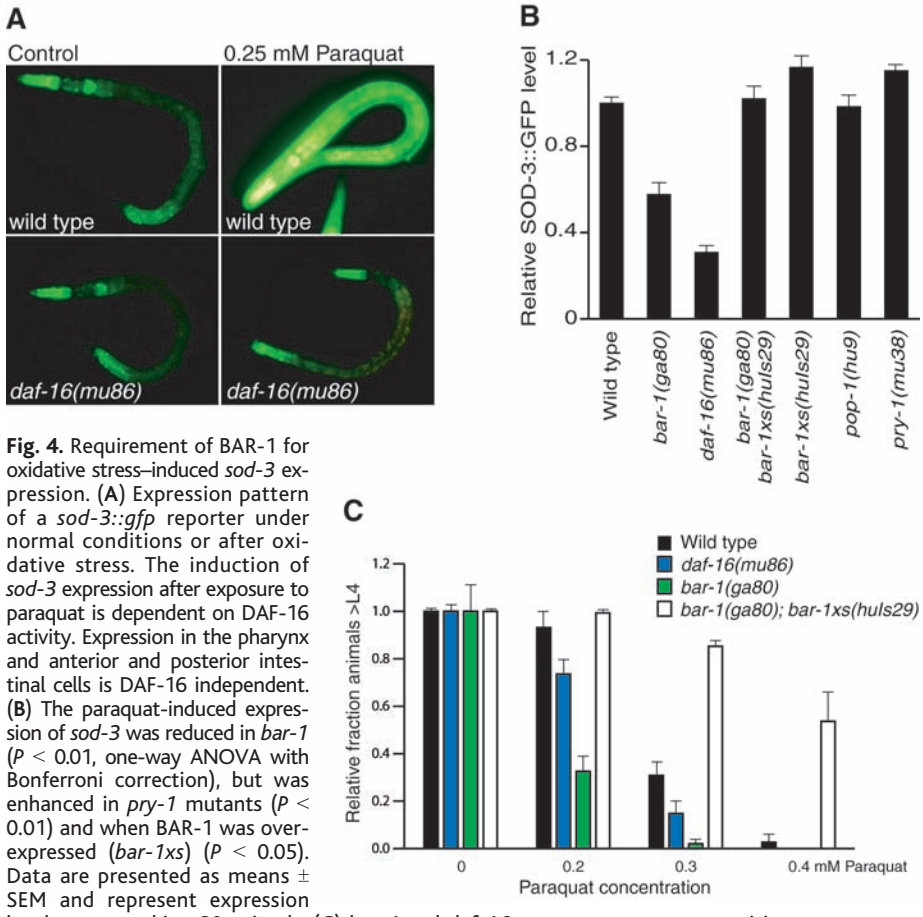

Fig. 4. Requirement of BAR-1 for oxidative stress–induced *sod-3* expression. (**A**) Expression pattern of a *sod-3::gfp* reporter under normal conditions or after oxidative stress. The induction of *sod-3* expression after exposure to paraquat is dependent on DAF-16 activity. Expression in the pharynx and anterior and posterior intestinal cells is DAF-16 independent. (**B**) The paraquat-induced expression of *sod-3* was reduced in *bar-1* ($P < 0.01$, one-way ANOVA with Bonferroni correction), but was enhanced in *pry-1* mutants ($P < 0.01$) and when BAR-1 was overexpressed (*bar-1xs*) ($P < 0.05$). Data are presented as means ± SEM and represent expression levels measured in >20 animals. (**C**) *bar-1* and *daf-16* mutants were more sensitive to paraquat-induced oxidative stress, whereas animals that overexpress BAR-1 were more resistant. The relative fraction of animals that have reached the L4 stage is indicated. Data are presented as means ± SD and represent the average of three independent experiments.

dependent increase in *sod-3* expression (Fig. 4B; fig. S2). A similar increase in *sod-3* expression was observed in the absence of PRY-1, a negative regulator of BAR-1 (*19*). To exclude the possibility that BAR-1 might affect *sod-3* expression through an interaction with the TCF homolog POP-1, we investigated *pop-1(hu9)*, which contains a mutation that specifically disrupts the function of POP-1 in canonical Wnt signaling (*19*), to find out whether it has an effect on paraquat–induced *sod-3* expression. There was no difference in *sod-3* expression between the wild type and *pop-1* mutants, demonstrating that this function of BAR-1 does not require TCF signaling. Because MnSOD is required for the detoxification of reactive oxygen species, we tested whether *bar-1* mutants were more sensitive to oxidative stress. *daf-16* and *bar-1* mutants were more sensitive to paraquat than wild-type animals (Fig. 4C). Furthermore, overexpression of BAR-1 resulted in resistance to oxidative stress. Taken together, these results show that BAR-1 is required for the DAF-16–mediated response to oxidative stress. This is consistent with our finding that the expression of the FOXO target gene, the gene for p27Kip1, is enhanced when β-catenin is overexpressed in the presence of oxidative stress (Fig. 2C).

Our results reveal an evolutionarily conserved function of β-catenin that is independent of TCF signaling. We have previously shown that FOXO transcription factors inhibit cell cycle progression (*11*, *20*), and here we show that this function is enhanced by β-catenin. In contrast, the β-catenin–TCF interaction stimulates cell cycle progression, and deregulated β-catenin–TCF signaling has a major role in the development of cancer. Thus, β-catenin appears to fulfill a critical function in balancing positive (through TCF) and negative (through FOXO) regulation of cell cycle progression. Different modes of signaling input, including insulin signaling and oxidative stress, may shift the balance between FOXO and TCF. In *C. elegans*, there is a strong correlation between DAF-16 function, the response to oxidative damage, and life span. Consistent with this, we find that loss of *bar-1* negatively affects both stress resistance and longevity. Given the known role of β-catenin in the development of cancer, our results suggest that a shift in β-catenin regulating FOXO and TCF signaling could link aging, the response to oxidative stress, and the development of certain types of cancer.

References and Notes

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Supporting Online Material

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