

further elucidate the relative contributions of intra- and interspecific diversity on community- and ecosystem-level processes.

Our work indicates two mechanisms underlying the relationships among intraspecific genotypic diversity, the diversity of associated consumers, and ecosystem processes. We explicitly showed that the effect of genotypic diversity on arthropods does not occur simply because of increased ANPP in diverse plots. It also arises because of an increase in the diversity of resources available to herbivores. These effects are nonadditive and cascade across trophic levels to structure associated communities. Our results demonstrate the need to incorporate intraspecific variation into current ecological theory that has emphasized the importance of interspecific variation (3, 4, 7, 15, 17, 18) or theory that ignores differences among species (22). Given the focus of conservation efforts on how the loss of species from communities affects ecosystem processes, our work suggests that the loss of genotypes from populations can no longer be overlooked (14, 23–25).

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19. Abundances were positively related to genotypic diversity (total: $r^2 = 0.27$, $P < 0.001$; herbivores: $r^2 = 0.29$, $P < 0.001$; predators: $r^2 = 0.07$, $P = 0.03$). There was a positive relationship between ANPP and arthropod richness (total: $r^2 = 0.24$, $P < 0.001$; herbivores: $r^2 = 0.17$, $P < 0.001$; predators: $r^2 = 0.15$, $P = 0.001$) and total abundance ($r^2 = 0.19$, $P < 0.001$) and herbivore abundance ($r^2 = 0.23$, $P < 0.001$). Arthropod richness

and abundance were correlated ($r = 0.74$, $P < 0.001$; herbivores: $r = 0.70$, $P < 0.001$; predators: $r = 0.29$, $P = 0.02$).

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Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S8

Table S1

References

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p53-Mediated Inhibition of Angiogenesis Through Up-Regulation of a Collagen Prolyl Hydroxylase

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Recent evidence suggests that antiangiogenic therapy is sensitive to p53 status in tumors, implicating a role for p53 in the regulation of angiogenesis. Here we show that p53 transcriptionally activates the α (II) collagen prolyl-4-hydroxylase [α (II)PH] gene, resulting in the extracellular release of antiangiogenic fragments of collagen type 4 and 18. Conditioned media from cells ectopically expressing either p53 or α (II)PH selectively inhibited growth of primary human endothelial cells. When expressed intracellularly or exogenously delivered, α (II)PH significantly inhibited tumor growth in mice. Our results reveal a genetic and biochemical linkage between the p53 tumor suppressor pathway and the synthesis of antiangiogenic collagen fragments.

The tumor suppressor activity of p53 results from its ability to transcriptionally activate a wide variety of target genes that in turn regulate cell cycle arrest, apoptosis, and suppression of angiogenesis (1). Although a number of p53 target genes involved in growth arrest and apoptosis have been identified, the role of p53 in the regulation of angiogenesis is

less well understood. Using a polymerase chain reaction (PCR)-based subtractive hybridization strategy (2), we identified α (II) collagen prolyl-4-hydroxylase [α (II)PH] as a p53-stimulated gene. In this screen, ecdysone-inducible p53 expression was established in the p53^{-/-} human cell line Saos-2 (Saos-2/Ec-p53 cells). Figure 1A (top) demonstrates that induction of p53 expression in these cells (bottom) stimulated transcription of α (II)PH as well as p21, a known p53 target gene (3). Up-regulation of α (II)PH was also observed when endogenous p53 expression was induced in wild-type HCT116 cells by the DNA damage-inducing agent camptothecin,

but not in a matched p53^{-/-} cell line (fig. S1). α (II)PH expression was also up-regulated upon expression of p53 from an adenovirus vector in p53^{-/-} H1299 human cancer cells (Fig. 1B). By contrast, expression of p53 had no effect on transcription of another collagen prolyl-4-hydroxylase isoform, α (I)PH, or three other human prolyl hydroxylases, PHD1, 2, and 3.

The α (II)PH promoter region contains three partially overlapping putative p53-binding half sites (see below). We derived reporter constructs by cloning sequences upstream of the α (II)PH transcription start-site or, as a control, the p21 promoter, upstream of the chloramphenicol acetyltransferase (CAT) gene. Ectopic p53 expression increased activity of both p21-CAT and α (II)PH-CAT (Fig. 1C). The chromatin immunoprecipitation (ChIP) experiment of Fig. 1D shows that p53 was recruited to the α (II)PH promoter in vivo. Collectively, the results of Fig. 1 show that α (II)PH is a direct p53 target gene.

Prolyl hydroxylation is a required, rate-limiting step in collagen biosynthesis (4), suggesting that p53-mediated stimulation of α (II)PH expression might increase collagen levels. We therefore investigated the effect of p53 expression on endogenous collagen 18 levels. Unexpectedly, the level of full-length collagen 18 was diminished in H1299 cells following expression of wild-type p53 but not a transcriptionally defective p53 mutant (Fig. 2A, left, and fig. S2) (5). One explanation for this result is that under these conditions, collagen breakdown was also stimulated. To test this hypothesis,

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lyzed conditioned media (CM) from p53-expressing H1299 cells. The results (Fig. 2A, indicate that the proteolytically pro-

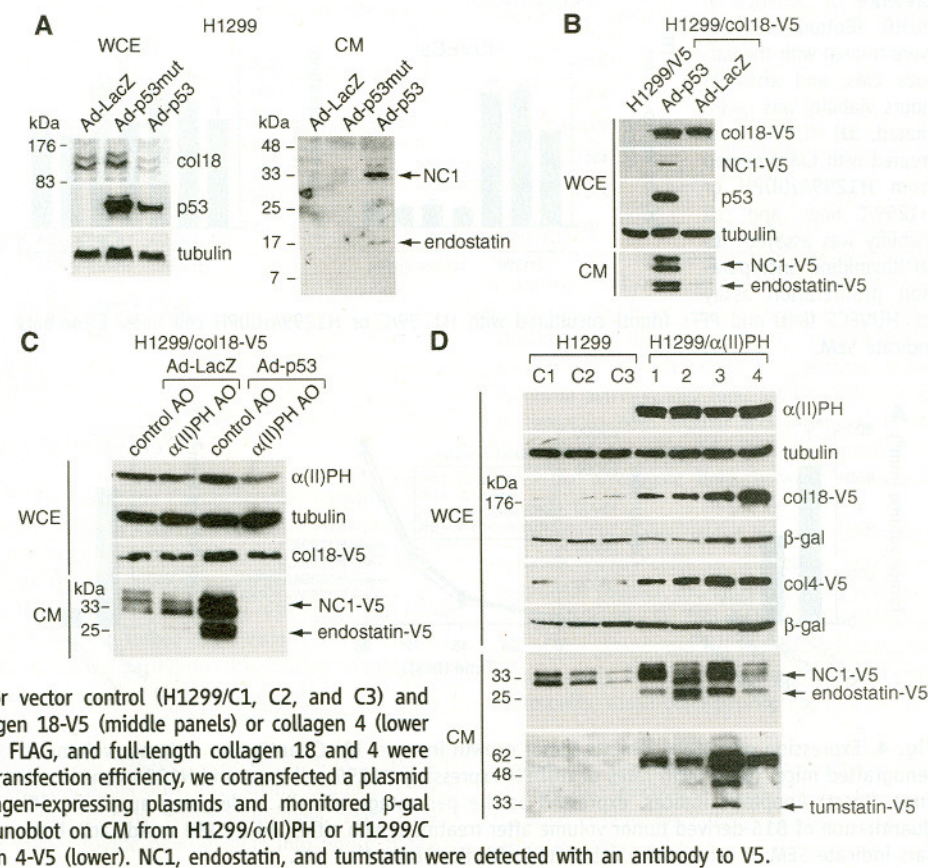
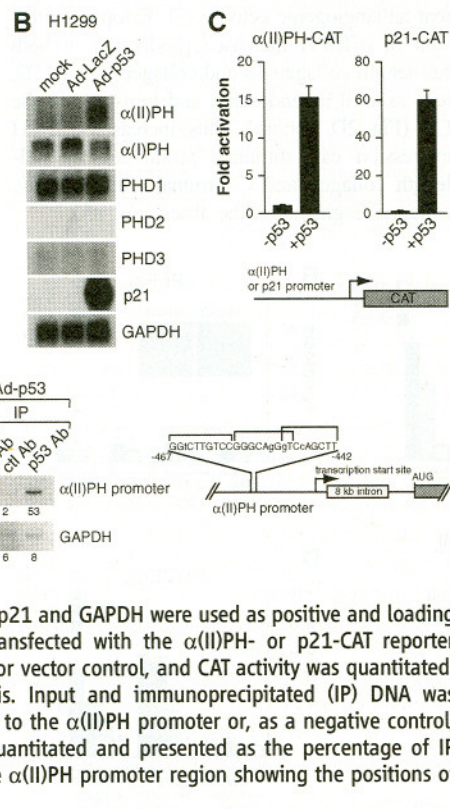
cessed ~30- and ~18-kD collagen 18 C-terminal fragments, which corresponded in size to NC1 and the well-characterized antiangiogenic

agent endostatin, respectively, were present only in CM from cells expressing wild-type p53.

To verify that p53 expression leads to the production of collagen 18 C-terminal fragments, we derived an H1299 cell line stably expressing full-length collagen 18 bearing a C-terminal V5 epitope tag (H1299/col18-V5 cells). Expression of p53 in these cells resulted in the production of the NC1 fragment and endostatin, which was not observed in control H1299/col18-V5 cells or in cells expressing LacZ (Fig. 2B). Analysis of CM from these cells showed that NC1 and endostatin were present only following expression of p53. Treatment with the caspase inhibitor zVADfmk had no effect on the production of NC1 and endostatin (fig. S3), indicating that processing of collagen 18 is not a nonspecific consequence of p53-mediated apoptosis.

To confirm that α (II)PH activity is necessary for the p53-dependent production of collagen-derived antiangiogenic peptides, we analyzed NC1 and endostatin levels following inhibition of α (II)PH using two strategies. First, in CM from p53-expressing H1299/col18-V5 cells prepared in the presence of the prolyl hydroxylase inhibitor ethyl-3,4-dihydroxy benzoate (EDHB), the levels of NC1 and endostatin were substantially lower (fig. S4). Second, we used an antisense oligonucleotide (AO) to specifically inhibit the production of α (II)PH in p53-expressing H1299/col18-V5 cells. An AO against α (II)PH, but not a control AO, prevented both the induction of α (II)PH observed upon p53

2. Expression of p53 stimulates the extracellular release of antiangiogenic collagen 4 and C-terminal fragments. (A) (Left) Immunoblot monitoring expression of endogenous collagen 18 in cell extracts (WCE) from H1299 cells transfected with Ad-LacZ, Ad-p53, or Ad-p53mut. Endogenous collagen 18 levels were analyzed by immunoblotting with an antibody against the C terminus; expression of p53 and tubulin were monitored as controls. Molecular size markers are indicated on the left. (Right) Endogenous NC1 and endostatin levels were analyzed by immunoblotting of conditioned media (CM) with an antibody against the C terminus. (B) Immunoblot monitoring WCE and CM from H1299/col18-V5 cells transfected with Ad-LacZ or Ad-p53. V5-tagged full-length collagen 18 and C-terminal proteolytic fragments were detected with an antibody to V5. Expression of p53 and tubulin were monitored as controls. (C) Immunoblot on WCE and CM from H1299/col18-V5 cells infected with Ad-LacZ or Ad-p53 and transfected with either an α (II)PH AO or control AO. α (II)PH levels were monitored with a polyclonal antibody. (D) (Top) Immunoblot on WCE from cell lines stably expressing FLAG- α (II)PH [H1299/ α (II)PH1, 2, 3, and 4] or vector control (H1299/C1, C2, and C3) and infected with a plasmid expressing either collagen 18-V5 (middle panels) or collagen 4 (lower panels). α (II)PH was detected with an antibody to FLAG, and full-length collagen 18 and 4 were detected with an antibody to V5. To normalize for transfection efficiency, we cotransfected a plasmid expressing β -galactosidase (β -gal) with the collagen-expressing plasmids and monitored β -gal with an antibody to β -gal. (Bottom) Immunoblot on CM from H1299/ α (II)PH or H1299/C expressing collagen 18-V5 (upper) or collagen 4-V5 (lower). NC1, endostatin, and tumstatin were detected with an antibody to V5.



expression and the appearance of NC1 and endostatin in the CM (Fig. 2C) (6). We conclude that α (II)PH expression is necessary for the p53-mediated production of antiangiogenic collagen fragments.

If the role of p53 in the production of antiangiogenic collagen fragments is to increase α (II)PH expression, then ectopic overexpression of α (II)PH should stimulate production of C-terminal collagen fragments even in the absence of p53. To test this prediction, we derived four independent H1299 cell lines stably ex-

pressing α (II)PH [H1299/ α (II)PH1, 2, 3, and 4] and transfected into each a plasmid expressing C-terminal V5-tagged collagen 18 or collagen 4, whose C-terminal fragment, tumstatin, has potent antiangiogenic activity (7). Ectopic expression of α (II)PH increased production of both full-length collagen 18 and collagen 4 (Fig. 2D, top) as well as endostatin and tumstatin in the CM (Fig. 2D, bottom). Thus, increased α (II)PH expression can stimulate production of full-length collagen and C-terminal antiangiogenic collagen fragments in the absence of p53.

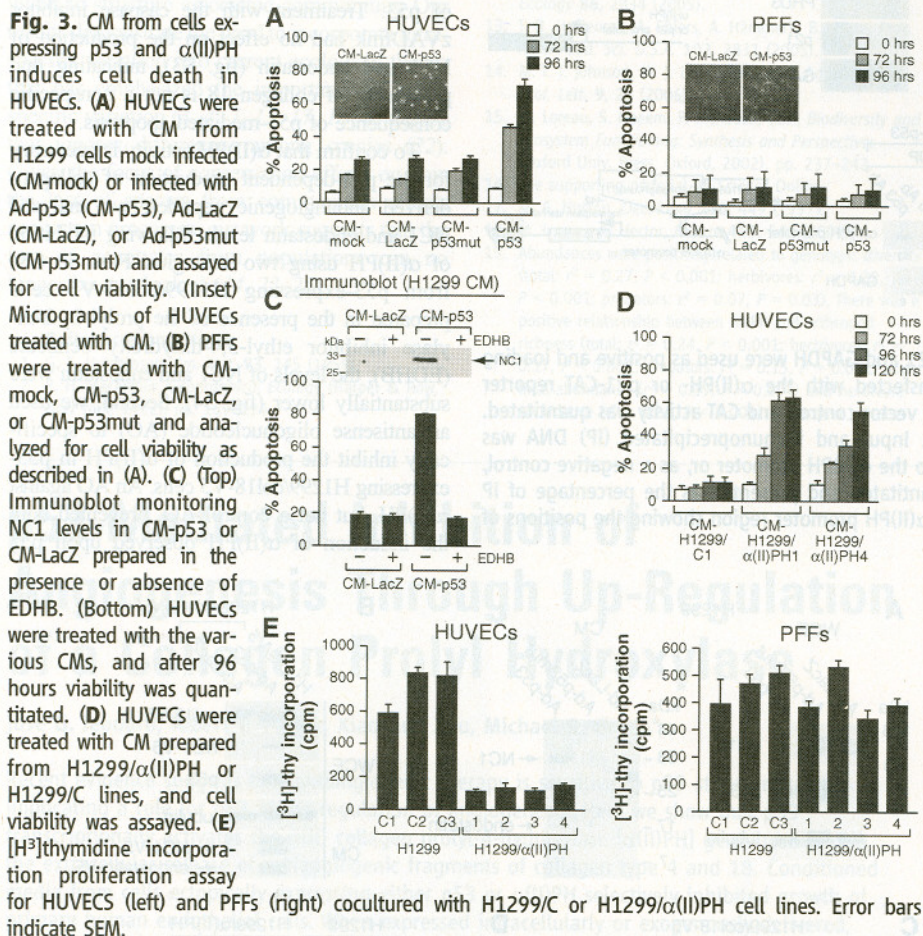


Fig. 3. CM from cells expressing p53 and α (II)PH induces cell death in HUVECs. (A) HUVECs were treated with CM from H1299 cells mock infected (CM-mock) or infected with Ad-p53 (CM-p53), Ad-LacZ (CM-LacZ), or Ad-p53mut (CM-p53mut) and assayed for cell viability. (Inset) Micrographs of HUVECs treated with CM. (B) PFFs were treated with CM-mock, CM-p53, CM-LacZ, or CM-p53mut and analyzed for cell viability as described in (A). (C) (Top) Immunoblot monitoring NC1 levels in CM-p53 or CM-LacZ prepared in the presence or absence of EDHB. (Bottom) HUVECs were treated with the various CMs, and after 96 hours viability was quantitated. (D) HUVECs were treated with CM prepared from H1299/ α (II)PH or H1299/C lines, and cell viability was assayed. (E) [3 H]thymidine incorporation proliferation assay for HUVECs (left) and PFFs (right) cocultured with H1299/C or H1299/ α (II)PH cell lines. Error bars indicate SEM.

The antiangiogenic fragments of several human collagens, including collagen 4 and 18, can inhibit endothelial cell proliferation either by inducing growth arrest or apoptosis (8). This suggested that CM from cells ectopically expressing p53, which contains antiangiogenic collagen fragments, could inhibit endothelial cell proliferation. To test this possibility, we conditioned low-serum media in the presence of H1299 cells infected with an adenovirus expressing wild-type p53, mutant p53, or LacZ. CM from H1299 cells expressing p53, but not mutant p53 or LacZ, efficiently induced apoptosis in primary human umbilical vein endothelial cells (HUVECs) (Fig. 3A). By contrast, CM from p53-expressing H1299 cells had no effect on primary foreskin fibroblast (PFF) cells (Fig. 3B), indicating that the death-promoting activity was cell-type specific. To verify that prolyl hydroxylase activity is necessary for the p53-dependent promotion of HUVEC death, we prepared CM from p53-expressing H1299 cells in the presence of EDHB. CM from EDHB-treated, p53-expressing cells lacked the collagen 18 NC1 fragment (Fig. 3C, top) and failed to kill HUVECs (Fig. 3C, bottom) (9).

If increased amounts of antiangiogenic collagen fragments is the primary mechanism by which CM from p53-expressing cells inhibits HUVEC growth, then CM from cells expressing α (II)PH should have a similar activity. CM prepared from two of the H1299/ α (II)PH cell lines, described above, induced HUVEC death (Fig. 3D). Coculture with all four H1299/ α (II)PH cell lines inhibited HUVEC proliferation (Fig. 3E, left), as assessed by [3 H]-labeled thymidine incorporation, but had no effect on PFFs (Fig. 3E, right). Thus, α (II)PH expression in tumor cells is sufficient to inhibit proliferation of cocultured primary HUVECs.

Inhibition of endothelial cell growth is predicted to inhibit vascularization and limit tumor growth (10). To test this possibility, we xenografted two of the α (II)PH-expressing H1299 cell lines into the flanks of nude mice and analyzed tumor growth. Notably, ectopic expression of α (II)PH resulted in near-complete suppression of H1299 tumor growth, as compared with two independently derived control lines, which developed large tumors within a month after injection (Fig. 4A and fig. S5). Because differences in tumor growth rates could be due to clonal variability, we further confirmed these results by injecting into mice a polyclonal population (a pool of ~500 clones) of α (II)PH-expressing [H1299/ α (II)PH(p)] or vector control [H1299/C(p)] H1299 cell lines. The tumors derived from mice injected with H1299/ α (II)PH(p) cells were significantly reduced in mass (fig. S6A), contained ischemic regions (fig. S6B), and were substantially less vascularized (fig. S6C) than tumors derived from mice injected with H1299/C(p) cells. Compared to control H1299/C(p) tumors, H1299/ α (II)PH(p) tumors contained higher levels of tumstatin and

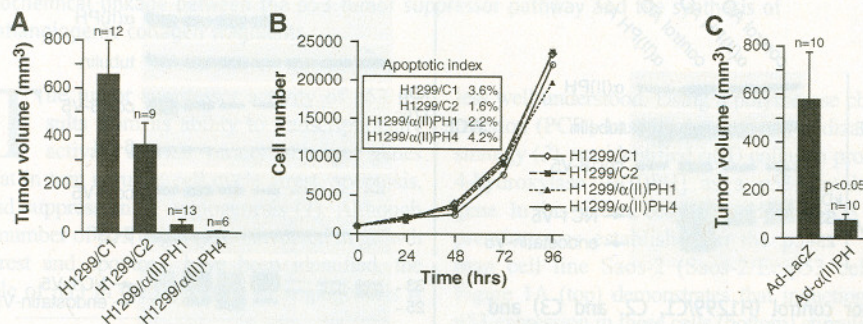


Fig. 4. Expression of α (II)PH inhibits tumor growth in mice. (A) Quantitation of tumor volume in xenografted mice. (B) Growth rates of α (II)PH-expressing H1299 cell lines and H1299 control cell lines. (Inset) Apoptotic indices, expressed as the percentage of cells undergoing apoptosis. (C) Quantitation of B16-derived tumor volume after treatment with either Ad- α (II)PH or Ad-LacZ. Error bars indicate SEM; *P* values were calculated with the Student's *t* test.

endostatin, as well as regions of intense tumstatin and endostatin accumulation (fig. S6D). These results are consistent with a model in which α (II)PH expression augments production of antiangiogenic collagen fragments. In culture, α (II)PH-expressing H1299 cells and control H1299 cells had comparable growth rates and apoptotic indices (Fig. 4B, inset). Thus, the differences in tumor size in xenografted mice were not because α (II)PH expression reduced the intrinsic growth rate of, or induced apoptosis in, H1299 cells.

Loss of p53 function is a common event in tumor progression (11), and our data suggest that p53-negative tumors may lack an important mechanism for limiting tumor vascularization. In support of this idea, xenografted tumors derived from p53^{-/-} HCT116 cells were significantly more vascularized and had large areas lacking tumstatin staining compared with tumors derived from wild-type HCT116 cells (fig. S7).

We next asked whether adenovirus-mediated α (II)PH expression could reduce growth of tumors derived from B16 cells, a highly aggressive mouse melanoma cell line (12). Treatment of B16-derived melanomas with α (II)PH-expressing adenovirus significantly reduced tumor volume (Fig. 4C). Thus, α (II)PH can inhibit tumor growth when intracellularly expressed or exogenously delivered.

Although it is well established that C-terminal collagen fragments have an antiangiogenic activity (13), their physiological role and connection to cellular growth pathways have not been elucidated. Our results reveal a genetic and biochemical linkage between the p53 tumor suppressor pathway and the production of antiangiogenic collagen fragments. We propose that α (II)PH induction by p53 results in increased synthesis and secretion of full-length collagens,

which are then proteolytically processed in the extracellular matrix to produce antiangiogenic peptides. Although this extracellular proteolytic processing occurs, at least to some extent, constitutively in the absence of p53 (Fig. 2D), p53 expression greatly enhances the processing of full-length collagen 18 to endostatin. On the basis of these observations, we propose that p53 activates a transcriptional program that increases synthesis and processing of collagen-derived antiangiogenic peptides. Several alternative mechanisms by which p53 could negatively affect angiogenesis in tumors have also been suggested (14–16).

In this study, we focused on collagen 18 because its antiangiogenic properties have been extensively characterized. Consistent with our observations, a recent report has demonstrated that a modest increase (~1.6-fold) in the synthesis of endostatin can lead to marked reduction in tumor growth rates in mice (17). Our data indicate that p53-dependent up-regulation of α (II)PH also results in increased synthesis of the collagen 4 C-terminal fragment, tumstatin; by analogy, increased synthesis of other collagen 4 C-terminal fragments, such as canstatin and arretin, seems likely. The shedding of multiple collagen-derived antiangiogenic fragments at the tumor-host interface may be part of a general p53-dependent mechanism of inhibiting tumor vascularization and growth.

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Mutations That Increase the Life Span of *C. elegans* Inhibit Tumor Growth

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Mutations in *gld-1* cause lethal germline tumors in the nematode *Caenorhabditis elegans*. We find that a wide variety of mutations that extend *C. elegans*' life span confer resistance to these tumors. The long life spans of *daf-2/insulin-receptor* mutants were not shortened at all by *gld-1* mutations; we attribute this finding to decreased cell division and increased DAF-16/p53-dependent apoptosis within the tumors. Mutations that increase life span by restricting food intake or inhibiting respiration did not affect apoptosis but reduced tumor cell division. Unexpectedly, none of these longevity mutations affected mitosis in normal germlines; this finding suggests that cellular changes that lead to longevity preferentially antagonize tumor cell growth.

In nature, there is a strong correlation between physiological aging and tumor susceptibility. Mice, which have short (2-year) mean life spans, frequently acquire tumors after

~1 year, whereas dogs do so after ~10 years and humans only after many decades. Understanding how youthful animals resist tumors may provide new insights into tumor biology,

particularly if genes that regulate aging influence tumor susceptibility. Many apoptotic, signaling, and other genes that affect tumors in mammals have orthologs in *C. elegans*. Moreover, mutations affecting insulin/IGF-1 (insulin-like growth factor-1) signaling, mitochondrial activity, and food intake each extend life span in both worms and mammals (1–4). Thus, *C. elegans* may be a valuable organism in which to investigate links between aging and tumors.

In *C. elegans* *gld-1* tumor-suppressor mutants, germ cells in the early stages of oogenesis reenter the mitotic cell cycle and overproliferate (5). The cells eventually break out of the gonad and fill the body, killing the animal early in life. Like transformed vertebrate cells, these cells proliferate in a growth

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