from the VIGS assay described above and is a direct demonstration that rgs-CaM is a cellular suppressor of PTGS.

Gene silencing is an important genetic mechanism with both theoretical and practical implications. Mutant studies have recently led to the identification of several eukaryotic proteins that are required for PTGS and are likely components of the silencing machinery (*4*, *24*, *25*). Viral suppressors of PTGS provide a powerful tool for finding additional cellular proteins involved in PTGS, including ones that might not be easily found by using mutants. Because calmodulin and related proteins normally act by binding calcium and subsequent activation of target proteins (*26*, *27*), this finding points to a role for calcium in regulating the activity of the PTGS pathway and predicts that the downstream target protein of rgs-CaM will also be a suppressor of PTGS. Given that rgs-CaM was found by its interaction with HC-Pro and that its expression can be induced by HC-Pro, it is tempting to speculate that the suppression of PTGS by HC-Pro is mediated, at least in part, by the activation of rgs-CaM and subsequent amplification of an endogenous pathway that negatively regulates gene-silencing activity.

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19. A cDNA library prepared from a mixture of RNAs isolated from a tobacco suspension culture at various time points (0, 2, and 5 hours) after treatment with parasiticein, an elicitor of the hypersensitive-response secreted by the fungus *Phytophthora*, was cloned into the HybriZAP activation domain vector (Stratagene, La Jolla, CA). Two million colonies from this library were screened by using the pGBT9 binding domain vector with the TEV HC-Pro cloned in at the Bam HI and Pst I sites.
20. The cDNA encoding rgs-CaM was subcloned from pBluescript (Stratagene) into the binary vector pGA482, transformed into *A. tumifaciens* strain GV3101R, and used for leaf-disc transformation of *N. benthamiana* (*28*). pGA482 does not carry a promoter, thus rgs-CaM in these transgenic lines is expressed from a natural plant promoter.
21. An *N. benthamiana* transgenic line hemizygous for rgs-CaM was crossed with transgenic line 16C, generating offspring hemizygous for the GFP transgene, half of which were also hemizygous for the rgs-CaM transgene. Plants were infected with PVX-GFP and assayed for GFP expression by using long-wave UV light (*13*). RNA for Northern analysis was isolated 12 days after inoculation.
22. Seedlings of *N. benthamiana* line 16C, homozygous for the GFP transgene, were infiltrated with *A. tumifaciens* (strain C58C1) carrying a binary plasmid with GFP under control of the 35S-cauliflower mosaic virus promoter. PTGS of the GFP transgene spread systemically, and plants were silenced by 3 weeks after infiltration and then inoculated with PVX vectors as described in the text. The PVX-rgsCaM construct was made by PCR amplification of rgs-CaM cDNA and cloning into an infectious PVX-cDNA just upstream of the gene for coat protein.
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Regulation of STAT3 by Direct Binding to the Rac1 GTPase

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The signal transducers and activators of transcription (STAT) transcription factors become phosphorylated on tyrosine and translocate to the nucleus after stimulation of cells with growth factors or cytokines. We show that the Rac1 guanosine triphosphatase can bind to and regulate STAT3 activity. Dominant negative Rac1 inhibited STAT3 activation by growth factors, whereas activated Rac1 stimulated STAT3 phosphorylation on both tyrosine and serine residues. Moreover, activated Rac1 formed a complex with STAT3 in mammalian cells. Yeast two-hybrid analysis indicated that STAT3 binds directly to active but not inactive Rac1 and that the interaction occurs via the effector domain. Rac1 may serve as an alternate mechanism for targeting STAT3 to tyrosine kinase signaling complexes.

Members of the STAT family of transcription factors are unusual among transcription factors in that they have characteristics of cytoplasmic signaling molecules such as an Src-homology 2 (SH2) domain and tyrosine phosphorylation sites (*1*). The SH2 domain targets the STATs to cytokine receptors such as the interleukin-6 (IL-6) and interferon-γ receptors (*2*). Upon tyrosine phosphorylation in response to receptor activation, the STATs dimerize through interaction of the SH2 domain with phosphorylated tyrosine residues and translocate to the nucleus. There, they directly bind DNA and form complexes with other transcription factors through protein-protein interactions (*3*). Several of the STATs are also activated by growth factor receptors (*4*, *5*). Receptor kinases, Jak family kinases, and Src family kinases have been implicated in this process (*6*–*10*). Reactive oxygen species (ROS) activate the Jak2 and TYK2 kinases as well as STAT3 and participate in growth factor signaling to the STATs (*11*). Because the small guanosine triphosphatase (GTPase) Rac has been implicated in the generation of ROS in response to growth factors (*12*), we investigated whether Rac1 has a role in STAT3 activation by growth factors.

Dominant negative N17Rac was cotransfected with STAT3 into COS-1 cells and phosphorylation of STAT3 in response to epidermal growth factor (EGF) stimulation was examined with phospho-specific antibodies. EGF treatment resulted in stimulation of both tyrosine and serine phosphorylation of STAT3 (Fig. 1A). However, dominant negative Rac1 completely abolished STAT3 phosphorylation on both tyrosine and serine residues induced by EGF, but had no effect on STAT3 expression (COS-1 cells express very little endogenous STAT3). In contrast, cotransfection with wild-type Rac1 did not

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inhibit EGF activation of STAT3 (*13*). EGF effectively activated a STAT-driven luciferase gene, but this activation was inhibited by cotransfection of the cells with dominant negative Rac1 (Fig. 1B). Similar results were seen in fibroblasts treated with platelet-derived growth factor (PDGF) (*13*).

A constitutively active Rac mutant (L61Rac1) was cotransfected into COS-1 cells with a STAT3 expression vector. These cells were deprived of serum, and the extracts were analyzed for STAT3 phosphorylation by protein immunoblotting. Rac1 stimulated the phosphorylation of STAT3 on both tyrosine and serine residues (Fig. 2A). Expression of activated Rac1 also stimulated expression of a STAT-driven reporter gene (Fig. 1B). Expression of activated Rac (V12Rac1) in Rat-1 cells via an adenoviral vector resulted in tyrosine phosphorylation of endogenous STAT3 (Fig. 2B), whereas an empty vector adenovirus had no effect (*13*). Likewise, STAT DNA binding activity was induced in the Rat-1 cells infected with V12-Rac1 viruses (Fig. 2C). In contrast to PDGF, Rac1 induced primarily STAT3 homodimers (upper band, Fig. 2C) and not complexes containing STAT1 (lower bands, Fig. 2C).

Guanine nucleotide exchange factors (GEFs) activate Rac by promoting the switch of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (*14*, *15*). Tiam-1, a Rac GEF (*16*), was expressed with STAT3 in COS-1 cells and was found to induce STAT3 tyrosine and serine phosphorylation (Fig. 2D). This result indicates that activation of endogenous Rac1 can lead to the activation of STAT3.

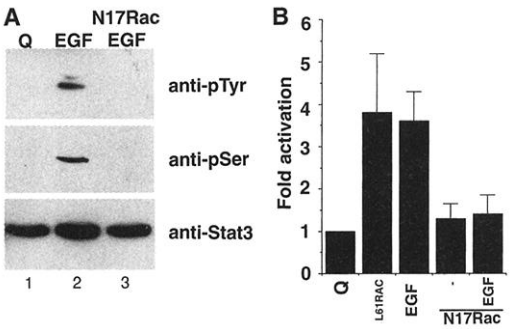

Fig. 1. Inhibition of growth factor–mediated STAT activation by N17Rac. (**A**) Inhibition of STAT3 phosphorylation by N17Rac1. COS-1 cells maintained in 10% fetal calf serum (FCS) in Dulbecco's modified Eagles' medium supplemented with L-glutamine in 60-mm plates were cotransfected with Rc/CMV-STAT3 (1 μg) and dominant negative Rac (pMT3N17Rac1) (4 μg) (*25*) with the use of Fugene-6 (Boehringer-Mannheim). The cells were placed in 0.5% FCS for 36 hours and were stimulated with EGF (100 ng/ml) (Collaborative, Bedford, Massachusetts) for 15 min before total lysates were prepared. Protein immunoblot analysis was done with antibody to pTyr705 STAT3 (anti-pTyr)(New England Biolabs, Beverly, Massachusetts) and assayed by chemiluminescence (Pierce, Bedford, Massachusetts). The resulting blot was stripped and reprobed with antibody to pSer727 STAT3 (anti-pSer) (New England Biolabs) and antibody to STAT3 (anti-Stat3) (K-15) (Santa Cruz). Q, quiescent, untreated cells. (**B**) Inhibition of growth factor–mediated stimulation of a STAT-specific promoter by N17Rac. COS-1 cells were transiently transfected with a high-affinity SIE-luciferase (hSIE) reporter plasmid (*11*) and STAT3 with the use of Fugene 6. After 2 days in 0.5% FCS, the cells were stimulated for 5 hours with 100 ng/ml EGF and were assayed for their luciferase activity as previously described (*11*). SIE reporter plasmid (200 ng), L61Rac (100 ng), or N17Rac (50 ng) and pRL-TK (renilla luciferase) normalization plasmid (20 ng) were used in each 25-mm well. Total transfected DNA was equalized with control vector DNA. Luciferase activity was normalized to a simultaneously transfected internal control plasmid (pRL-TK). The data in this graph represent the average fold increase in expression relative to quiescent unstimulated cells of four separate experiments done in duplicate with the standard deviation shown by error bars.

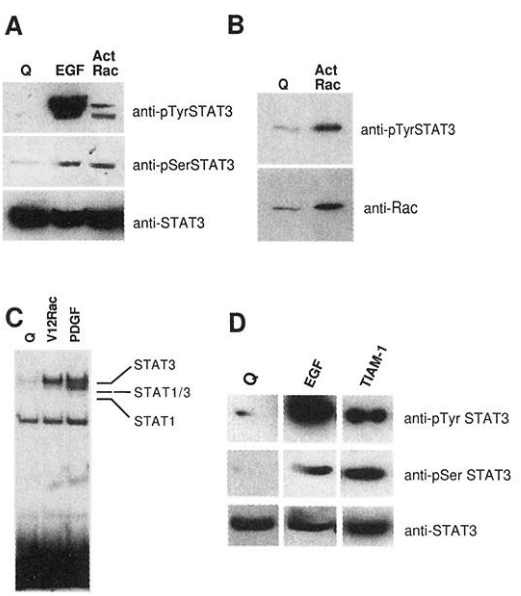

Fig. 2. STAT3 phosphorylation is induced by activated Rac. (**A**) COS-1 cells (60-mm plates) were cotransfected with STAT3 (1 μg) and activated Rac (pMT3L61Rac1) (100 ng) (Act Rac) with the use of Fugene 6. The cells were placed in 0.5% FCS for 36 hours before lysates were prepared as previously described (*11*). EGF treatment was 100 ng/ml for 15 min. Protein immunoblot analysis was done with anti-pTyr705 STAT3. The blot was stripped and reprobed with anti-pSer727 STAT3 and anti-STAT3. (**B**) Rat-1 fibroblasts were infected with adenovirus vectors containing V12Rac (AdV12Rac1) (*12*) for 6 hours with 1000 particles/cell. Cells were placed in 0.5% FCS for 24 hours before total lysates were prepared. Protein immunoblot analysis was done with anti-pTyr705 STAT3. The blot was stripped and re-probed with anti-Rac1 (Santa Cruz). Lane Q shows extracts of cells infected with an adenovirus vector without a cDNA insert. (**C**) STAT3 DNA binding activity. Adenovirus containing V12Rac was used as described above to infect Rat-1 fibroblasts. Nuclear extracts were obtained and analyzed by electrophoretic mobility shift assay with an m67-SIE probe as described previously (*11*, *26*). The dashes indicate the position of the STAT1 and STAT3 homo- and heterodimers. (**D**) STAT3 phosphorylation. COS-1 cells were cotransfected with STAT3 (1 μg) and Tiam-1 (1 μg) and were placed in 0.5% FCS for 36 hours before total lysates were obtained as described above. Protein immunoblot analysis was performed with anti-pTyr705 STAT3. The blot was stripped and reprobed with anti-pSer727 STAT3 and anti-STAT3. Comparison to EGF-stimulated cells is shown.

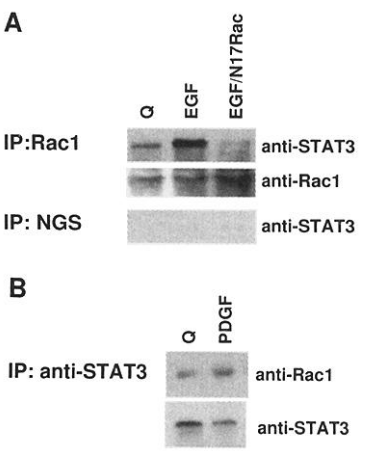

Fig. 3. (**A**) Interaction of Rac1 and STAT3 in vivo in transfected COS-1 cells. COS-1 cells (100-mm plates) were transfected with STAT3 (3 μg) and N17Rac (12 μg) as indicated with the use of Fugene 6. The cells were placed in 0.5% FCS for 36 hours and stimulated with EGF (100 ng/ml) for 15 min before total lysates were obtained as previously described. Proteins from lysates (1.5 mg) were immunoprecipitated (IP) with antibody to Rac1 (anti-Rac1) (Santa Cruz) and the immune complexes were captured with protein A–G agarose beads (50 μl) (Santa Cruz). Half of the sample was run on an 8% polyacrylamide gel and the remainder was run on a 15% polyacrylamide gel. The 8% gel was blotted with anti-STAT3 and the 15% gel was blotted with anti-Rac1 (Transduction Labs, Lexington, Kentucky). Non-immune goat serum (NGS) was used as a control. (**B**) Interaction of endogenous Rac1 and STAT3 in Rat-1 cells. Quiescent Rat-1 fibroblasts were treated with PDGF-BB (25 ng/ml) for 15 min before total lysates were obtained as described above. Protein lysates (500 μg) were immunoprecipitated with anti-STAT3 (Santa Cruz) and the immune complexes captured using 100 μl of Protein A agarose beads (Gibco, Grand Island, New York). Protein immunoblot analysis was done with anti-Rac1. The blot was stripped and reprobed with anti-STAT3.

We examined whether Rac1 exists in a complex with STAT3. Coimmunoprecipitation showed that STAT3 was associated with Rac1 and that this association was enhanced by EGF treatment of the cells (Fig. 3A). Nonimmune goat serum (NGS) did not precipitate STAT3. The tyrosine-phosphorylated form of STAT3 was also associated with Rac1 (*13*).

We examined whether dominant negative Rac1 would interfere with EGF-stimulated Rac1 association with STAT3. Dominant negative Rac was cotransfected into COS-1 cells with STAT3 and Rac1 complexes were immunoprecipitated with antibody to Rac1. Dominant negative Rac1 completely inhibited the increase of Rac1-STAT3 association induced by EGF treatment (Fig. 3A). Endogenous STAT3 associated with endogenous Rac1 in Rat-1 fibroblasts, and this association was enhanced twofold as measured by densitometry in cells treated with PDGF (Fig. 3B). Thus, the Rac1-STAT3 association is not dependent on overexpression of either Rac1 or STAT3.

A yeast two-hybrid screen of mouse embryo and HeLa cell cDNA libraries was performed with the use of V12Rac1 as bait. Among the V12Rac1-specific interacting clones isolated were clones coding for the Rac binding domain of murine p21-activated kinase (PAK-A) and full-length human STAT3. Activated Rac (V12Rac1) but not inactive Rac (N17Rac1) interacted with GAL4-STAT3 in the yeast assay (Fig. 4A). Thus, the GTP-bound form of Rac has a higher affinity for STAT3 than the GDP bound form. The Rac1-STAT3 interaction is specific because another member of the Ras family of GTPases, V12H-Ras, did not interact with STAT3. Mutation of phenylalanine to alanine at position 37 (F37A) in the effector domain of V12Rac1 completely abolished interaction with STAT3, as did mutation of phenylalanine to leucine (F37L) (*17*). Neither of the F37 mutations affected the interaction of V12Rac1 with the Rac binding domain of murine PAK-A. Mutation of tyrosine 40 of Rac1 to cysteine (Y40C) did not effect the interaction of Rac1 and STAT3.

In COS-1 cells, the Rac1(F37A) mutation abolished association with STAT3 and stimulation of STAT3 tyrosine phosphorylation even when expressed at higher levels than wild-type activated Rac (Fig. 4B). The Y40C mutation did not affect either of these Rac activities. These results suggest that the interaction between activated Rac and STAT3 is direct, depends on the effector domain, and requires the GTP-bound form of Rac.

The best known STAT tyrosine kinases are those of the Jak family kinases. Therefore, we examined COS-1 cells for evidence of Jak kinase activation in response to L61Rac (Fig. 5). Jak2 becomes activated in COS-1 cells in response to activated Rac1, as judged by protein immunoblotting with antibody to Jak2 that recognizes the tyrosine-phosphorylated sites required for kinase activation (*18*). This phosphorylation was inhibited by the Jak2 kinase inhibitor AG490. Immunoprecipitation (IP) kinase assay confirmed that Jak2 activity was increased in response to activated Rac1 (*13*). We were unable to detect activation of other Jak family kinases in response to Rac.

Recruitment of the STATs to cytokine receptors by interaction of the STAT SH2 domain with receptor phosphotyrosines is important for STAT activation and specificity (*19*, *20*). It seems likely that the Rac1-STAT3 interaction could play an analogous role for STAT3 activation by growth factors by recruiting STAT3 to kinase signaling complexes at the membrane or cytoskeleton. Similarly, Rac2 has been shown to play an integral role in the assembly and activation of the multisubunit NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase enzyme complex (*21*).

Current evidence indicates that STAT3 activation by growth factors is not mediated solely by receptor kinases but in addition by non-receptor tyrosine kinases such as Jak and Src family members (*7*, *9*, *10*, *22*). It is possible that Rac is required for the activation of the Jak or Src family kinases, or both, in response to receptor kinase activation. Rac1 mediates generation of ROS in response to

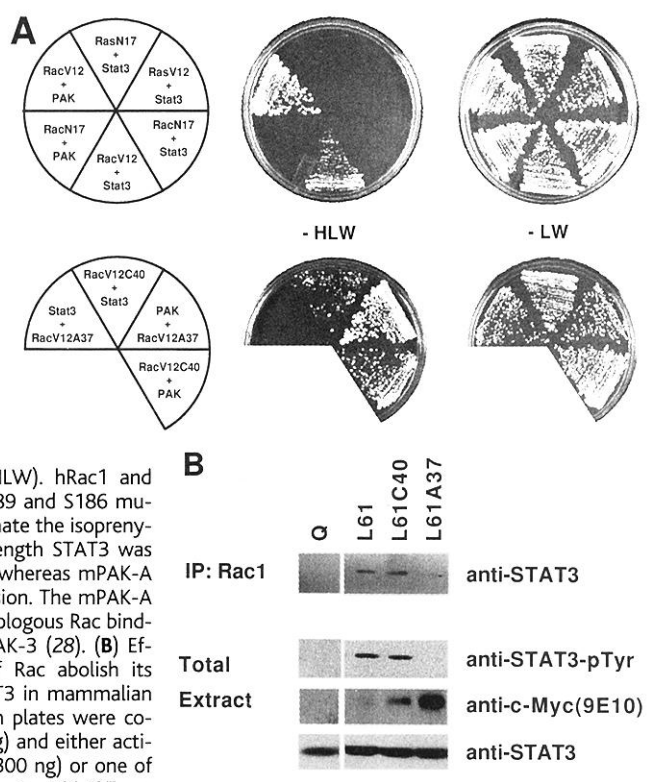

Fig. 4. (**A**) Rac1 specifically interacts with STAT3 in a yeast two-hybrid interaction assay. V12S189Rac1, which was fused to the DNA binding domain of LexA (*27*), was used as bait to screen 2×10^6 colonies of a human HeLa cell cDNA library (Stratagene, La Jolla, California) and 1.5×10^6 colonies of a mouse embryo 9.5- and 10.5-day-old library. Once the STAT3 clones were isolated, the indicated bait and target plasmids were tested for interaction on SC media lacking histidine, leucine, and tryptophan (–HLW). hRac1 and H-Ras also contained the S189 and S186 mutations, respectively, to eliminate the isoprenylation of the GTPases. Full length STAT3 was expressed as a GAL4 fusion, whereas mPAK-A was expressed as a VP-16 fusion. The mPAK-A construct contained the homologous Rac binding domain sequence of mPAK-3 (*28*). (**B**) Effector domain mutations of Rac abolish its ability to complex with STAT3 in mammalian cells. COS-1 cells in 100-mm plates were cotransfected with STAT3 (3 μg) and either activated pRK5-myc-L61hRac1 (300 ng) or one of the effector domain mutants L61A37 or L61C40 (*29*) with the use of Fugene 6. The cells were placed in 0.5% FCS for 24 hours before total lysates were obtained. (Top) Association of Rac1 and STAT3. The protein lysates (500 μg) were immunoprecipitated with anti-Rac1 and the immune complexes captured with Protein A agarose beads (100 μl). Protein immunoblot analysis was done with anti-STAT3. (Bottom) Stimulation of STAT3 tyrosine phosphorylation. Protein immunoblot analysis of the same extracts with anti-pTyr705 STAT3. The blot was stripped and reprobed with anti-STAT3 and then with anti-myc (9E10) antibody to detect the overexpression of the different Rac constructs.

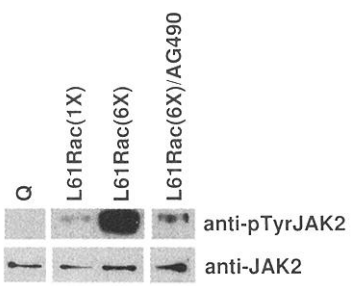

Fig. 5. Activation of Jak2 in cells expressing L61Rac. COS-1 cells (100-mm plates) were cotransfected with STAT3 (3 μg) and activated pMT3L61Rac1 [300 ng (1X) or 2 μg (6X)] with the use of Fugene 6. The cells were placed in 0.5 % FCS for 24 hours and, where indicated, were treated with 50 μM AG490 (Calbiochem, San Diego, California), a specific Jak2 inhibitor, for 60 min before total lysates were obtained. Immunoblotting was done with an anti-Jak2 phosphotyrosine (Y1007/1008) antibody (Affinity Bioreagents, Golden Colorado). The blot was stripped and reprobed with an anti-Jak2 (Upstate Biotech, Lake Placid, New York).

growth factor stimulation, and ROS is known to activate Src and Jak family kinases (*11*, *23*, *24*). Thus, Rac1 may both localize STAT3 to kinase complexes and contribute to the activation of the kinases themselves.

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Regulation of *C. elegans* Life-Span by Insulinlike Signaling in the Nervous System

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An insulinlike signaling pathway controls *Caenorhabditis elegans* aging, metabolism, and development. Mutations in the *daf-2* insulin receptor–like gene or the downstream *age-1* phosphoinositide 3-kinase gene extend adult life-span by two- to threefold. To identify tissues where this pathway regulates aging and metabolism, we restored *daf-2* pathway signaling to only neurons, muscle, or intestine. Insulinlike signaling in neurons alone was sufficient to specify wild-type life-span, but muscle or intestinal signaling was not. However, restoring *daf-2* pathway signaling to muscle rescued metabolic defects, thus decoupling regulation of life-span and metabolism. These findings point to the nervous system as a central regulator of animal longevity.

Each species has a characteristic life-span, ranging from 10 days for the nematode *Caenorhabditis elegans* to 80 years for humans. Despite these vast differences in life-span, shared features of aging in diverse species support the existence of a common mechanism for life-span determination (*1*). Reductions in caloric intake, insulin/insulinlike growth factor–I (IGF-I) signaling, and free radical levels can lengthen the life-span of animals as divergent as nematodes, *Drosophila*, and mammals (*1–3*). Mutations that decrease *C. elegans daf-2* insulin/IGF-I–like receptor or *age-1* phosphoinositide 3-kinase signaling result in severalfold extension of adult life-span (*2*, *4*, *5*) and increased accumulation of fat (*2*, *6–8*). Null mutations in *daf-2* or *age-1* cause constitutive arrest at the dauer larval stage; dauer larvae have slowed metabolic rates, store large amounts of fat, express high levels of antioxidant enzymes such as catalase and superoxide dismutase (SOD), and live longer than reproductive adults (*9*). One reasonable hypothesis is that free radicals generated as by-products of metabolism damage cellular components (*10*). The lower level of free radicals in *daf-2* insulinlike signaling mutants is essential for life-span extension: The life-span extension in a *daf-2* mutant requires the activity of a cytosolic catalase *ctl-1* (*11*).

The cells where *daf-2* pathway signaling is required for signaling normal life-span are not known. Insulinlike signaling may regulate metabolism and free radical production directly in aging skin or muscle, or these pathways may act in key signaling centers that then coordinately control the senescence of the entire organism. In addition, it is not clear whether insulin/IGF-I regulation of life-span is simply coregulated with metabolism or whether the metabolic shifts are mechanistically connected to the life-span regulation. Several components of the *daf-2* pathway, such as *akt-1*, *pdk-1*, and *daf-16*, are widely expressed throughout development (*12–14*). Studies of *daf-2* genetic mosaic animals showed that animals lacking *daf-2* activity from the entire AB cell lineage, which generates nearly all of the hypodermis and nervous system and half of the pharynx, have extended life-spans (*15*). However, mosaic animals lacking *daf-2* activity from blastomere daughters of AB, which generate about half of the hypodermis, nervous system, and pharynx, did not show extended life-spans. These studies showed that *daf-2* can act nonautonomously to regulate life-span but did not assign *daf-2* longevity control to particular cell types.

To define the cell type(s) from which the *daf-2* insulinlike signaling pathway functions to control *C. elegans* life-span, metabolism, and development, we restored *daf-2* pathway function to restricted cell types by using distinct promoters to express *daf-2* or *age-1* cDNAs in either neurons, intestine, or muscle cells of a *daf-2* or *age-1* mutant (*16–22*). Long life-span, metabolic changes, and dauer arrest were tested in these transgenic animals (Table 1). Because regulation of longevity may require gene activity over the entire life of the animal, the expression of green fluorescent protein (GFP) fusions to these promoters was confirmed to continue in aged animals (*23*).

The long life-span of *daf-2* and *age-1* mutants was rescued by neuronal expression of *daf-2* or *age-1*, respectively, with the panneuronal *unc-14* promoter (*16*, *24*). Neuronally restricted *age-1* expression fully restored wild-type adult life-span to an *age-1(mg44)* null mutant (Fig. 1). This rescue is comparable to the positive control, ubiquitous expression of *age-1* from the *dpy-30* promoter in the *age-1* mutant (*17*, *25*). Neuronally restricted *daf-2* expression from the *unc-14* promoter also rescued the long life-span of *daf-2(e1370)* mutants, although not

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