

Redox Regulation of Forkhead Proteins Through a *p66shc*-Dependent Signaling Pathway

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Genetic determinants of longevity include the forkhead-related transcription factor DAF-16 in the worm *Caenorhabditis elegans* and the *p66shc* locus in mice. We demonstrate that *p66shc* regulates intracellular oxidant levels in mammalian cells and that hydrogen peroxide can negatively regulate forkhead activity. In *p66shc*^{-/-} cells, the activity of the mammalian forkhead homolog FKHRL1 is increased and redox-dependent forkhead inactivation is reduced. In addition, expression of FKHRL1 results in an increase in both hydrogen peroxide scavenging and oxidative stress resistance. These results demonstrate an important functional relation between three distinct elements linked to aging: forkhead proteins, *p66shc*, and intracellular oxidants.

Intracellular reactive oxygen species (ROS) are thought to contribute to aging in a wide spectrum of organisms, although the precise mechanism of action remains elusive (1). Previous studies in the worm *C. elegans* have demonstrated that life-extending mutations in the DAF-16 pathway may require the presence of *ctl-1*, a gene encoding for a cytosolic catalase (2). Because catalase degrades hydrogen peroxide, these genetic studies suggest a possible link between forkhead activity and cellular oxidants. However, because *ctl-1* is not expressed in higher organisms, the relevance of these observations to human aging is presently unknown. Previous studies have demonstrated that for each of the three DAF-16 vertebrate homologs, FKHRL1, FKHR, or AFX, phosphorylation results in the retention of the protein in the cytosol and, hence, a reduction in forkhead-dependent transcriptional activity (3–7). To pursue observations raised by genetic studies in *C. elegans* (2), we asked whether forkhead family members respond to intracellular ROS in mammalian cells. A concentration-dependent increase in phosphorylated FKHRL1 was observed in cultured mammalian cells after treatment with hydrogen peroxide (Fig. 1A) (8). In agreement with previous observations suggesting a role for Akt in forkhead protein phosphorylation (3–7), exogenous peroxide also resulted in a concentration-dependent activation of Akt (Fig. 1B). Consistent with the known role of phosphorylation in regulating forkhead subcellular localization, FKHRL1 was predominantly in the nucleus in quiescent cells (Fig. 1C), whereas brief

hydrogen peroxide treatment resulted in the redistribution of the protein to the cytosol (Fig. 1, D and E). These results differ in some aspects from a recent report in *C. elegans* demonstrating that, under certain oxidant stress conditions, DAF-16 relocates to the nucleus (9).

To determine whether endogenous oxidants regulate forkhead activity, the effects of two different cell permeant antioxidants were assessed. FKHRL1 phosphorylation decreased in a concentration-dependent fashion when PC12 cells were treated with either the glutathione precursor *N*-acetyl-

cysteine (NAC) or the glutathione peroxidase mimetic ebselen (Fig. 1F). In agreement with the FKHRL1 phosphorylation results, levels of endogenous forkhead activity increased in cells treated with either of the cell permeant hydrogen peroxide scavengers (Fig. 1, G and H).

To date, one of the few genetic alterations demonstrated to increase life span in mammals is the homozygous deletion of *p66shc* locus (10). Based in part on the observations presented here, we reasoned that *p66shc* might regulate intracellular ROS levels and, in turn, modulate forkhead activity (8). Under basal conditions, the level of intracellular hydrogen peroxide, as assessed by the redox-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCFDA), was quite low in immortalized mouse fibroblasts derived from wild-type (*p66shc*^{+/+}) or *p66shc*^{-/-} animals (Fig. 2A). However, when these cells were subjected to serum starvation, a stimulus that increases oxidative stress, intracellular hydrogen peroxide was lower in *p66shc*^{-/-} cells compared with wild-type cells (Fig. 2, B, C, and D).

Because phosphorylation of *p66shc* at a position-36 serine residue is required for conferring increased susceptibility to cell death induced by oxidative stress (10), mutation of this residue should confer a dominant interfering phenotype. PC12 cell lines expressing empty vector (Neo), wild-type *p66shc* (WT), or a position-36 mutant of *p66shc* (SA) were established in which levels of overexpressed proteins were comparable (Fig. 2E, inset).

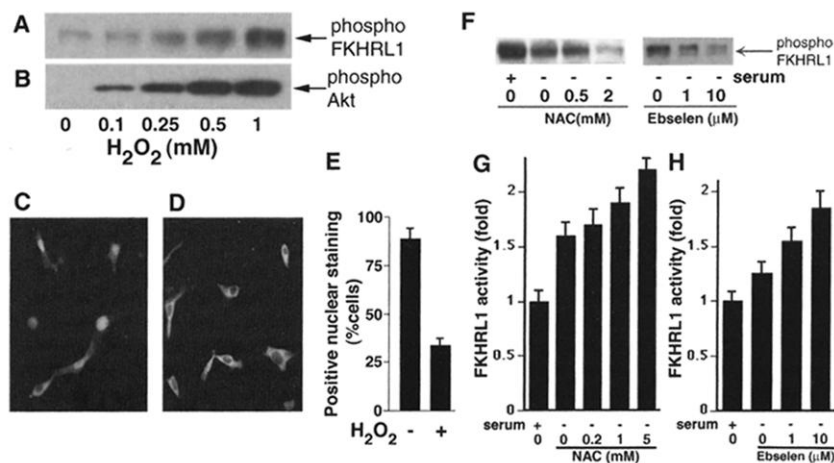


Fig. 1. Hydrogen peroxide regulates forkhead phosphorylation. (A) Endogenous FKHRL1 phosphorylation in PC12 cells after exposure to the indicated concentration of hydrogen peroxide for 20 min, was detected by Western blot analysis of cell lysates with an antibody to phospho-FKHRL1 (8). (B) Phospho-Akt levels following peroxide stimulation were determined with an antibody to phosphoserine 473 Akt (8). (C) Representative fields of indirect immunofluorescence of transfected PC12 cells expressing epitope-tagged FKHRL1 under serum free, quiescent conditions, or (D) in the presence of 0.5 mM hydrogen peroxide for 20 min (8). (E) Quantitation of cells exhibiting positive nuclear staining (combination of cell exhibiting either nuclear staining only or cells exhibiting both nuclear and cytoplasmic staining). (F) Levels of endogenous phosphorylated FKHRL1 in PC12 cells using an antibody to phospho-FKHRL1 15 hours after serum withdrawal with or without the concomitant addition of the antioxidant NAC or the glutathione peroxidase mimetic, ebselen. Two separate representative experiments of endogenous forkhead activity in cells subjected to serum withdrawal and treated with increasing concentrations of (G) NAC or (H) ebselen (8).

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Similar to what was observed in *p66shc*^{-/-} cells, levels of ROS were reduced in cells expressing the dominant interfering *p66shc* mutant (Fig. 2E).

Genetic studies from *C. elegans* longevity mutants have inferred that increased life-span correlates with a moderate increase in forkhead activity. Augmented forkhead-dependent transcriptional activity was also observed in *p66shc*^{-/-} cells (Fig. 3A). This was particularly evident after serum withdrawal, a condition accompanied by detectable oxidative stress. This effect was not a result of differences in serum responsiveness or a global increase in transcription in *p66shc*^{-/-} cells because serum response element (SRE)-dependent transcription was identical between these two cell types (11). Stable expression of the dominant interfering mutant of *p66shc* also increased FKHLR1 activity (Fig. 3B). It is important to note that in addition to increasing ROS levels, serum withdrawal activates numerous other intracellular pathways, which undoubtedly affect forkhead activity. Although serum starvation increases intracellular oxidants (Fig. 2B), our results, and others (3), suggest that the net effect of serum starvation is a small increase in forkhead activity. This is presumably a result of serum withdrawal activating redox-insensitive pathways that increase FKHLR1 activity. Nonetheless, our data suggests that in the setting of serum starvation, inhibiting the increase in ROS either by blocking *p66shc* activity (Fig. 3, A and B) or by treating with cell permeant antioxidants (Fig. 1, G and H), further augments forkhead activity.

To further confirm that the redox-dependent forkhead inactivation was regulated by *p66shc*,

cells were directly challenged with exogenous oxidants. In wild-type fibroblasts, stimulation with either insulin or hydrogen peroxide led to a rapid and significant increase in FKHLR1 phosphorylation (8). However, in cells deficient in *p66shc*, no phosphorylation of FKHLR1 was observed after oxidative stress, though FKHLR1 phosphorylation was observed in response to insulin (Fig. 3C). Cells without *p66shc* also had reduced Akt activation following treatment with hydrogen peroxide while the level of FKHLR1 phosphorylation observed in *p66shc*^{-/-} cells in response to another stress stimuli, ultraviolet (UV) light, was not substantially altered (11). A similar pattern of FKHLR1 regulation was observed in the PC12 cells expressing mutant *p66shc* (SA) (Fig. 3D). Only expression of wild-type *p66shc* was capable of restoring oxidative stress-induced FKHLR1 phosphorylation in *p66shc*^{-/-} fibroblasts (Fig. 3E). Thus, oxidant-mediated forkhead inhibition requires *p66shc*.

In *C. elegans*, DAF-16 appears to regulate oxidative stress in part due to the ability of DAF-16 to directly or indirectly transactivate a number of antioxidant enzymes and stress-related gene products (2, 12–14). Previous studies have also demonstrated an increase in resistance of *p66shc*^{-/-} cells to oxidative stress (10). To address whether increased FKHLR1 transcriptional activity augments antioxidant scavenging and oxidative stress resistance in mammalian cells, we analyzed FKHLR1-mediated transactivation of the catalase promoter (8). We identified a number of putative FKHLR1 consensus binding sequences beginning at position -2339 and -1667 of the human catalase promoter (15). Wild-type FKHLR1 transactivated a reporter

construct containing an approximate 3 kb catalase promoter fragment but not a shorter 1.6 kb fragment that lacked consensus binding sites (Fig. 4A). The degree of transcriptional activity of the catalase fragment was similar in magnitude to a synthetic reporter containing three tandem FKHLR1 sites. As expected, no stimulation was observed for either the full-length catalase promoter or the synthetic forkhead promoter when an activation domain deficient FKHLR1 mutant (ΔC) was transfected. In addition, only PC12 cells overexpressing wild-type FKHLR1 showed an increase in hydrogen peroxide scavenging capacity (Fig. 4B). Although transient expression of FKHLR1 has been associated with growth arrest (16), our stable overexpressing cell lines appeared to grow at the same rate as control cells (11). Only PC12 cells expressing wild-type FKHLR1 had in-

Fig. 2. Absence of *p66shc* lowers intracellular hydrogen peroxide levels. (A) Western blot analysis of shc isoform expression in fibroblasts derived from wild-type animals (+/+) or from *p66shc*-null animals (-/-). (B) Quantification of DCFDA fluorescence under basal conditions or following serum withdrawal in wild-type cells (gray bars) and null cells (black bars). Representative DCFDA fluorescence 24 hours after serum withdrawal in (C) wild-type cells and (D) null cells (8). (E) Levels of observed DCFDA fluorescence 24 hours after serum withdrawal in cell lines transfected with vector only (Neo), with wild-type shc (WT), or with a dominant interfering position-36 mutant (SA). Inset, expression levels of epitope-tagged transfected *p66shc* in PC12 cell lines. The position-36 mutant consistently migrated with a faster mobility.

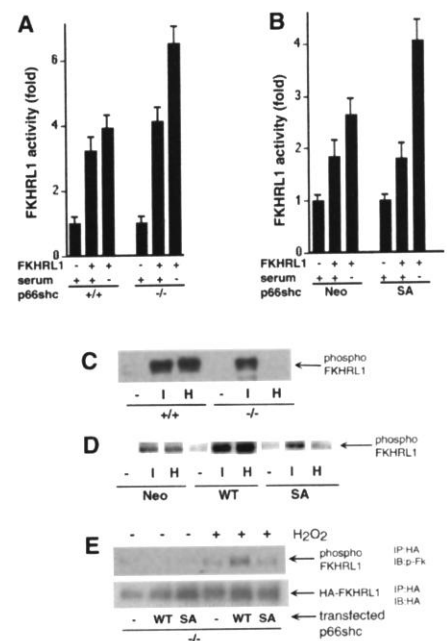
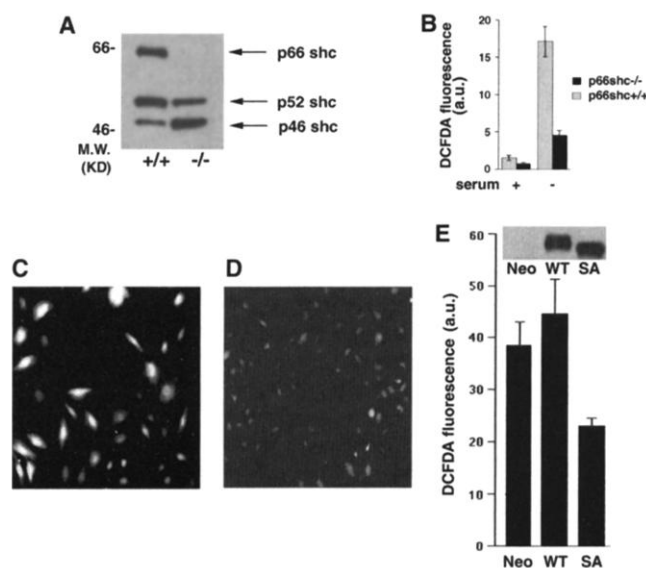


Fig. 3. Regulation of forkhead activity by *p66shc*. (A) Forkhead activity in the presence and absence of serum in wild-type (+/+) and null (-/-) mouse fibroblasts or (B) in PC12 cells stably transfected with empty vector or a dominant interfering mutant of *p66shc* (8). Levels of activity are expressed as fold change from basal forkhead activity observed in each cell line in the presence of serum and without exogenous FKHLR1 transfection. (C) Endogenous FKHLR1 phosphorylation under quiescent conditions (-), 10 min after insulin (I) stimulation or 20 min after hydrogen peroxide (H) stimulation in mouse fibroblasts derived from wild-type animals or *p66shc*-null animals. (D) FKHLR1 phosphorylation in corresponding stable PC12 cell lines stimulated with insulin or hydrogen peroxide as in (C). (E) Transient transfection of wild-type *p66shc* restores hydrogen peroxide-stimulated FKHLR1 phosphorylation in null cells. Cells were stimulated with hydrogen peroxide (1 mM) for 20 min before harvest.

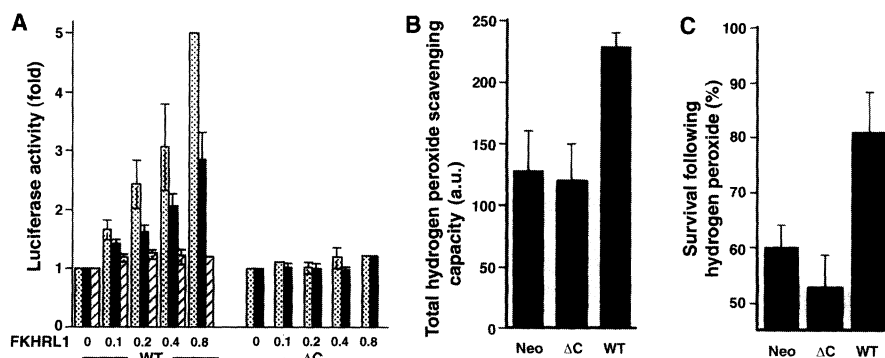


Fig. 4. FKHRL1 regulates hydrogen peroxide scavenging and oxidative stress resistance. (A) FKHRL1 stimulated transcriptional activity using a catalase promoter beginning at -3179 (black bar) or at -1645 (striped bar) or with a synthetic promoter containing three tandem forkhead binding sites (stippled bar). Transcriptional activity was determined after transfection with increasing amounts of either full-length FKHRL1 (WT) or the activation domain deficient FKHRL1 mutant (ΔC) isoform (8). (B) Total hydrogen peroxide scavenging capacity in control cells (Neo), cell stably expressing the activation deficient FKHRL1 mutant (ΔC) or cells expressing full-length FKHRL1 (WT). (C) Cell survival in PC12 cell lines 24 hours after exposure to hydrogen peroxide ($200 \mu M$).

creased survival after direct hydrogen peroxide challenge (Fig. 4C).

Our results, therefore, demonstrate an important functional relation between forkhead proteins that regulate longevity in *C. elegans*, *p66shc* that is implicated in mammalian life span, and intracellular oxidants, which are thought to play a role in aging across all species (1). We have also demonstrated a role for FKHRL1 expression in the regulation of cellular oxidative stress resistance. This is consistent with previous studies demonstrating that DAF-16 can act as a potential transcriptional activator of several antioxidant scavengers and stress resistance genes in *C. elegans* including superoxide dismutase (SOD), catalase, and OLD-1 (2, 12–14). In contrast, most evidence in mammalian cells to date suggests that, after trophic withdrawal, transient activation of forkhead proteins is associated with increased cell death (3, 17, 18). Similarly, a number of studies have indicated a potentially protective effect of Akt activation after exposure to hydrogen peroxide (19–22). These observed differences in the protective versus harmful effects of Akt and forkhead proteins may relate to the levels of activation seen in these various studies, the nature of stimulus used, the differences between transient and stable FKHRL1 overexpression, or differences in the cell lines used. Whereas small increases in DAF-16 activity are associated with longevity in *C. elegans*, more robust activation results in a dauer-like state characterized by growth arrest (9, 23). In mammalian cells, although increased forkhead activity can be associated with cell death, inhibiting forkhead activity can also result in apoptosis (24).

Lastly, since the initial description over 40 years ago of the free radical theory of aging (25) the implication has been that ROS act solely as random, destructive agents. The

observation that forkhead activity is regulated by intracellular ROS in a *p66shc*-dependent fashion suggests that intracellular hydrogen peroxide might also provide a specific signaling function. Given that oxidative stress has been implicated in a host of human diseases as well as human aging, this expanded role for ROS as signaling agents may have important therapeutic implications.

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Visualization of a Ran-GTP Gradient in Interphase and Mitotic *Xenopus* Egg Extracts

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The small guanosine triphosphatase Ran is loaded with guanosine triphosphate (GTP) by the chromatin-bound guanine nucleotide exchange factor RCC1 and releases import cargoes in the nucleus during interphase. In mitosis, Ran-GTP promotes spindle assembly around chromosomes by locally discharging cargoes that regulate microtubule dynamics and organization. We used fluorescence resonance energy transfer-based biosensors to visualize gradients of Ran-GTP and liberated cargoes around chromosomes in mitotic *Xenopus* egg extracts. Both gradients were required to assemble and maintain spindle structure. During interphase, Ran-GTP was highly enriched in the nucleoplasm, and a steep concentration difference between nuclear and cytoplasmic Ran-GTP was established, providing evidence for a Ran-GTP gradient surrounding chromosomes throughout the cell cycle.

Because of the chromosomal localization of the Ran-guanine nucleotide exchange factor (GEF) RCC1 (regulator of chromosome condensation 1) and the cytoplasmic localization of Ran-GAP (Ran-guanosine triphospha-

tase-activating protein) and its cofactor Ran-BP1 (Ran-binding protein 1), Ran-GTP is predicted to exist exclusively in the interphase nucleus or in the immediate proximity of mitotic chromosomes, whereas the bulk of