thought to play a role in aging across all span, and intracellular oxidants, which are important functional relation between forkhead proteins. FKHRL1 regulates hydrogen peroxide scavenging and oxidative stress resistance. (Fig. 4. 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 FHKLR1 mutant (ΔC) isoform (B). (B) Total hydrogen peroxide scavenging capacity in control cells (Neo), cell stably expressing the activation deficient FHKLR1 mutant (ΔC) or cells expressing full-length FKHRL1 (WT). (C) Cell survival in PC12 cell lines 24 hours after exposure to hydrogen peroxide (200 μM).

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, which 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.

References and Notes
8. Supplemental materials are available on Science Online at www.sciencemag.org/cgi/content/full/1069004/DC1.
11. S. Nemoto, unpublished observations.
26. We wish to thank M. Greenberg, Y. Luo, S. G. Rhee, L. Lanfrancone, and P. G. Pelicci for reagents; S. Gutkind for helpful discussions; and I. Rivara for help with the manuscript and figures. We are particularly grateful for the help of N. J. Holbrook and X. Wang.

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 triphosphatase–activating protein) and its cofactor RanBP1 (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
cytoplasmic Ran is rapidly converted to its guanosine diphosphate (GDP)-bound form (1). During interphase, the Ran cycle regulates the binding and release of transport cargoes from their receptors in the nucleus or cytoplasm, giving directionality to nucleocytoplasmic transport (2, 3). A Ran-GTP gradient is also proposed to regulate spindle formation during mitosis (4, 5), because Ran-GTP modulates microtubule dynamics and organization (4–8). In addition, Ran appears to regulate nuclear envelope reassembly upon exit from mitosis (9, 10). Recently, the nuclear import receptor importin β has been identified as a key factor regulated by Ran-GTP during spindle morphogenesis (11–13). Importin β acts as an inhibitor of spindle assembly and is thought to function by sequestering cargoes required for microtubule polymerization and organization. Ran-GTP generated in the vicinity of chromosomes binds importin β and discharges these cargoes. However, the existence of a Ran-GTP gradient during interphase or mitosis has remained hypothetical, and although several lines of evidence support this model, it has never been demonstrated experimentally.

Based on Ran’s binding partners and regulation of nuclear transport factor–cargo interactions, we designed two chimeric biosensor probes that exhibit intramolecular fluorescence resonance energy transfer (FRET) between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), modulated by the Ran nucleotide state (Fig. 1A) (14). In the absence of binding partners, we predicted that probe flexibility should allow FRET caused by the transfer of energy from a COOH-terminal CFP to an NH2-terminal YFP, leading to a decrease of CFP (donor) emission and an increase of YFP (acceptor) emission (15). The first sensor, designed to monitor the Ran nucleotide state directly, contained the Ran-binding domain (RBD) of the yeast Ran-GAP accessory factor Yrb1 (16). The RBD has a low affinity for Ran-GDP but binds to Ran-GTP in an extended conformation with its NH2- and COOH-termini ~5.6 nm apart (17). Thus, the YFP-RBD-CFP chimera (YRC) should not undergo FRET in the presence of Ran-GTP (Fig. 1A).

The second sensor was designed to monitor the release of importin β cargoes, which is regulated by the local concentration of Ran-GTP. We used the importin β–binding domain (IBB) of importin α as a model cargo. Importin α acts as an adapter to bind a subset of nuclear cargoes and is dissociated from importin β by Ran-GTP (18–20). The free IBB domain is highly flexible (21), and the YFP-IBB-CFP (YIC) chimera should undergo FRET. However, when bound to importin β, the IBB forms an α-helical rod ~5 nm long (22), and FRET of the YIC sensor should be inhibited (Fig. 1A). Thus, this probe should directly monitor free importin β cargoes and provide an indirect measure of Ran-GTP concentration.

To characterize the behavior of the YRC and YIC chimeras, their emission profiles were examined by spectrofluorimetry with 435-nm excitation in the presence of either Ran-GDP or Ran-GTP (Fig. 1B) (23). Under opposite Ran nucleotide states, the two probes displayed an emission increase at 525 nm (YFP, acceptor) and a decrease at 474 nm (CFP, donor) indicative of FRET. YRC FRET occurred in the presence of Ran-GDP, whereas Ran-GTP induced maximal FRET of the YIC probe in the presence of importin β.

To confirm the nucleotide dependence of the binding reactions biochemically, pull-down experiments were performed with purified proteins (Fig. 1C) (24). YRC bound Ran-GTP but not Ran-GDP. The interaction was fully reversible, because YRC that had bound to Ran-GTP could be released by the addition of Ran-GAP to induce Ran-GTP hydrolysis. Like full-length importin α, YIC bound to importin β only in the absence of Ran-GTP.

Fig. 1. (A) Design of FRET probes. The YRC probe, consisting of a RBD fused to YFP and CFP, should undergo FRET in the presence of Ran-GDP but not when bound to Ran-GTP. The YIC probe consisting of the importin β–binding domain of importin α (IBB) fused to YFP and CFP should undergo FRET when released from importin β by Ran-GTP. [B] through [D] Characterization of FRET probes with purified recombinant proteins. (B) Emission profiles showing the decrease in the CFP (donor) fluorescence peak at 474 nm and increase in the YFP (acceptor) fluorescence peak at 525 nm when FRET occurred. Reactions contained 0.2 μM RCC1, 2 μM Ran, and either 1 mM GDP (blue line) or 1 mM GTP (red line) with 1 μM YRC (upper panel) or 1 μM YIC plus 1.5 μM importin β (lower panel). Samples were excited at 435 nm, and emission was measured from 460 to 550 nm at 0.5-nm increments. (C) Ran nucleotide state–dependent binding of probes can be detected biochemically. Ran-GTP but not Ran-GDP pulled down YRC, and this binding was abolished upon GTP hydrolysis induced by addition of Ran-GAP (upper panel). Importin β pulled down importin α or YIC only in the absence of Ran-GTP (lower panel). (D) Reversibility of probe interactions. [Upper panel] FRET of YRC was diminished fractionally to a baseline level by addition of increasing amounts of Ran-GTP (arrowheads) but restored by subsequent addition of Ran-GAP causing GTP hydrolysis (arrow). [Lower panel] FRET of YIC was diminished by addition of importin β (arrow) but recovered upon addition of Ran-GTP (arrowheads). Experiments were performed with continuous stirring in a volume of 500 μl. Samples were excited at 435 nm every 2 s, and emission at 474 nm (IcFP), 525 nm (IyFP), and the 525/474 (IyFP/IcFP) ratio were obtained simultaneously.
The fluorescent tags did not alter the affinity of the probe for importin β as compared to IBB or importin α (25, 26). The rapid reversibility of FRET sensors was demonstrated by monitoring of emission intensity over time while the Ran nucleotide state was manipulated (Fig. 1D). The YRC FRET ratio calculated as the FRET emission at 535 nm upon excitation at 435 nm, divided by CFP emission at 474 nm (I \text{FRET} / I \text{CFP}) decreased rapidly when its binding partner Ran-GTP was added and was restored upon addition of Ran-GAP. In contrast, the I \text{FRET} / I \text{CFP} Ratio of YIC decreased quickly after the addition of importin β but was recovered upon Ran-GTP–induced release of the sensor.

Next we examined whether the FRET probes could also be used to monitor differences in the Ran nucleotide state in mitotic cytoplasmic extracts prepared from unfertilized Xenopus eggs (Fig. 2, A through C) (27, 28). Analogous to results with purified proteins (Fig. 1B), addition of a nonhydrolyzable Ran mutant loaded with GTP (Ran-Q69L-GTP) to extracts containing YRC caused a decrease in YFP acceptor emission and an increase in CFP donor emission, indicating a loss of YRC FRET (Fig. 2A), whereas YIC FRET in extract increased in the presence of Ran-Q69L-GTP (Fig. 2B). Similar results were obtained with the addition of RCC1, whereas addition of a large excess of Ran-GAP did not significantly alter FRET ratios of YRC (Fig. 2C). Thus, at equilibrium, free Ran was predominantly in the GDP-bound form in cytoplasmic egg extracts.

As in solutions of pure proteins, FRET in the extract required both fluorophores to be on the same probe (29) and reflected specific binding reactions tightly regulated by Ran-GTP (Fig. 2D). Biotinylated sensors were retrieved from extracts by means of streptavidin beads (30). YRC did not bind endogenous Ran in untreated extracts but could capture exogenously added Ran-Q69L-GTP. Under these conditions, endogenous Ran also associated with YRC, indicating that addition of Ran-Q69L altered the Ran nucleotide state equilibrium in the extract to favor the GTP form. YIC was found associated with endogenous importin β in extracts, but this interaction was lost upon addition of Ran-Q69L-GTP. Thus, our FRET probes detected dynamic changes in the Ran nucleotide state and importin β–cargo binding in egg extracts and indicate that endogenous Ran in egg extracts is predominantly in its GDP-bound form.

To test whether our sensors could allow visualization of a Ran-GTP gradient generated by chromosome-bound RCC1 during mitosis, Xenopus sperm nuclei were added to mitotic egg extracts supplemented with FRET probes and rhodamine-labeled tubulin (Fig. 3A). Spindle assembly and FRET were both monitored by fluorescence microscopy (31). After 30 min of incubation, spindle assembly was apparent, as rhodamine-labeled tubulin incorporated into bipolar structures with chromosomes positioned at the metaphase plate. Visualization of the YRC probe revealed a gain in CFP fluorescence intensity (I \text{CFP}) and a relative decrease in FRET emission intensity (I \text{FRET}) in the immediate vicinity of the chromosomes. As a result, the I \text{FRET} / I \text{CFP} ratio of the YRC probe was the lowest in the central spindle, indicating localized loss of YRC intramolecular FRET due to its binding to Ran-GTP generated by chromosome-bound RCC1. Conversely, the YIC probe exhibited a decreased I \text{CFP} signal and an increased I \text{FRET} signal in the region surrounding the chromosomes of the spindle.

The high FRET ratio (I \text{FRET} / I \text{CFP}) levels in the central spindle thus revealed a gradient of YIC liberated from importin β, indicating localized release of cargoes by Ran-GTP. Results obtained with I \text{FRET} / I \text{CFP} ratio imaging were supported by FRET images normalized for probe concentration and channel cross-bleed (32, 33) that displayed YRC and YIC FRET gradients of similar proportions and steepness (29). When probes were substituted with a mixture of individually labeled RBD-YFP and RBD-CFP or IBB-YFP and IBB-CFP constructs or with a sensor unresponsive to the Ran nucleotide state, no FRET signal was detectable, demonstrating that the changes were specific and caused by intramolecular FRET (29).

The establishment of Ran-GTP and liberated cargo gradients preceded microtubule polymerization into polarized arrays, because YRC and YIC I \text{FRET} / I \text{CFP} ratio gradients were visible around sperm nuclei within several minutes after the initiation of spindle assembly reactions (29).

If chromatin-bound RCC1 is responsible for formation of the mitotic Ran-GTP gradient and the downstream gradient of released cargoes, then inhibition of RCC1 function should disrupt localized FRET of both the YRC and YIC probes. Furthermore, if these gradients are physiologically meaningful, their disruption should affect spindle assembly. Addition of Ran-T24N, a potent inhibitor of RCC1, eliminated FRET ratio gradients of both probes and caused a decrease in microtubule density in spindles (Fig. 3B). In contrast, addition of nonhydrolyzable Ran-Q69L-GTP led to spontaneous microtubule polymerization and formation of spindle-like structures independent of chromosomes (4–8). Under these conditions, fluorescence microscopy revealed uniform I \text{FRET} / I \text{CFP} signals without a gradient surrounding chromosomes, with overall lower YRC and elevated YIC I \text{FRET} / I \text{CFP} ratios consistent with results obtained by fluorimetry in the presence of Ran-Q69L-GTP (Fig. 2, A through C). In contrast, addition of the Ran-GTP–independ-
Addition of 30 m spindle morphology. (abolishing its FRET signal and demonstrating that proper cargo release was required to maintain the Ran-GTP gradient visualized with the YRC sensor. Thus, importin β cargo release functions downstream of the Ran-GTP gradient, and, consistent with previous data, sequestering of importin β cargoes caused defects in microtubule polymerization and organization (11).

Next we used the YRC probe to analyze the distribution of the Ran nucleotide state in nuclei formed in interphase egg extracts (Fig. 3C). Although the YRC probe was enriched in the nucleus, specific loss of \( I_{\text{FRET}}/I_{\text{CFP}} \) was observed in this compartment, revealing a very high enrichment of Ran in its GTP-bound form in the nucleoplasm. An increase in YRC FRET in the nucleus was observed upon addition of either the RCC1 inhibitor Ran-T24N or the Ran-GTP binding domain of importin β (Fig. 3C) (29). Based on the Ran-GTP occupancy of the YRC sensor and its affinity for Ran-GTP, we estimated that the concentration difference between free nuclear and cytoplasmic Ran-GTP was at least 200-fold.

The FRET probes reported here enable highly sensitive monitoring of spatial and temporal changes in both the Ran nucleotide state and importin β–cargo interactions throughout the cell cycle. This analysis reveals that mitotic chromosomes are surrounded by a Ran-GTP gradient that induces a gradient of liberated cargoes and that a steep Ran-GTP concentration difference exists between the nucleus and cytoplasm. Our observations provide direct evidence that a chromatin-generated Ran-GTP gradient is maintained throughout the cell cycle and support the model that Ran-GTP acts as a positional marker of the genome.

References and Notes
14. The RBD-encoding region of Saccharomyces cerevisiae Yrb1 (RBD, corresponding to amino acids 71 through 201), the importin β-binding domain of importin β (88, corresponding to amino acids 1 through 65), and CFP and YFP (Clontech) were amplified by polymerase chain reaction and/or subcloned to generate 6XHIS tagged YRC (pKW 966), Ry (pKW 965), RC (pKW 984), YIC (pKW 970), and IC (pKW 960) expression constructs in pGEM® (Invitrogen) that were verified by sequencing. All YFP and CFP fusion proteins were expressed in bacterial strain BL21 DE3 at room temperature.
Systemic RNAi in C. elegans Requires the Putative Transmembrane Protein SID-1

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Double-stranded RNA-mediated gene interference (RNAi) in Caenorhabditis elegans systemically inhibits gene expression throughout the organism. To investigate how gene-specific silencing information is transmitted between cells, we constructed a strain that permits visualization of systemic RNAi. We used this strain to identify systemic RNA interference–deficient (sid) loci required to spread gene-silencing information between tissues but not to initiate or maintain an RNAi response. One of these loci, sid-1, encodes a conserved protein with predicted transmembrane domains. SID-1 is expressed in cells sensitive to RNAi, is localized to the cell periphery, and is required cell-autonomously for systemic RNAi.

One of the first reported and still mysterious aspects of RNAi in C. elegans is that it is systemic. Injection of gene-specific double-stranded RNA (dsRNA) into one tissue leads to the posttranscriptional silencing of that gene in other tissues and in that worm’s progeny (1). The systemic nature of RNAi also provides for initiation of RNAi by soaking animals in dsRNA (2, 3) or by cultivating worms on bacteria expressing dsRNA (4, 5). Although systemic RNAi has not been demonstrated in any other animal, posttranscriptional gene silencing (PTGS) effects in plants are well established (6). A variety of plants are resistant to RNAi (7), though the mechanism is not well understood. In C. elegans, systemic RNAi has been demonstrated in the pharynx-specific myo-2::GFP promoter (8, 9), but it is not known whether systemic RNAi also occurs in other body parts. Here, we report that a strain of C. elegans lacking systemic RNAi (sid-1 null) expresses a variant of an putative transmembrane protein, SID-1, that is required for systemic RNAi.

SID-1 is predicted to be a transmembrane protein with a large extracellular domain. SID-1 is required for systemic RNAi, as judged by the absence of dsRNA-induced silencing in sid-1 null mutants. Several lines of evidence suggest that SID-1 is involved in systemic RNAi. First, dsRNA targeting sid-1 causes a severe reduction in RNAi. Second, SID-1 is required for the efficient systemic RNAi of myo-2::GFP dsRNA. Third, SID-1 is expressed in cells that are sensitive to RNAi.

Systemic RNAi may require the efficient transport of dsRNA from its source to target cells. SID-1 may be involved in this process. Two lines of evidence support this idea. First, the sid-1 null mutation reduces the efficiency of systemic RNAi. Second, SID-1 is expressed in cells that are sensitive to RNAi.

We thank A. Dernburg and members of the Heald and Weis labs for helpful discussions and/or comments on the manuscript. M. Welch for help with fluorimetry. Y. Azuma for help with RCC1 purification, M. Ignatius for help with microscopy, and G. O. Nads for data.