Nutrient Availability Regulates SIRT1 Through a Forkhead-Dependent Pathway

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Nutrient availability regulates life-span in a wide range of organisms. We demonstrate that in mammalian cells, acute nutrient withdrawal simultaneously augments expression of the SIRT1 deacetylase and activates the Forkhead transcription factor Foxo3a. Knockdown of Foxo3a expression inhibited the starvation-induced increase in SIRT1 expression. Stimulation of SIRT1 transcription by Foxo3a was mediated through two p53 binding sites present in the SIRT1 promoter, and a nutrient-sensitive physical interaction was observed between Foxo3a and p53. SIRT1 expression was not induced in starved p53-deficient mice. Thus, in mammalian cells, p53, Foxo3a, and SIRT1, three proteins separately implicated in aging, constitute a nutrient-sensing pathway.

In the yeast *Saccharomyces cerevisiae* and in the nematode *Caenorhabditis elegans*, life-span can be extended by increasing the expression of the deacetylase Sir2, an enzyme whose activity depends on the oxidized form of nicotinamide adenine dinucleotide (NAD) (J, 2). In these model organisms, the ability of Sir2 to extend life may be related to its role in gene silencing. In both the nematode and yeast, certain simple environmental stresses can also increase life-span. In yeast, reducing the amount of available glucose has this effect. The ability of glucose restriction to increase the life-span of yeast requires Sir2 (3). In *C. elegans*, activation of the Forkhead transcription factor DAF-16 is also associated with increased life-span (4) and its activation depends in part on nutrient availability (5). Genetic evidence further suggests that in worms, DAF-16 and Sir2 work through a common pathway (2), and recent evidence suggests that their mammalian counterparts physically interact (6, 7).

Here, we further analyzed the interrelationships of the closest mammalian orthologs

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of DAF-16 and Sir2: the Forkhead transcription factor Foxo3a and the mammalian NAD-dependent deacetylase SIRT1. Using a mammalian model of nutritional stress (8), we examined the effects of nutritional withdrawal on the activity of the SIRT1 promoter. When a mammalian cell line (PC12) was starved overnight of both serum and glucose, SIRT1 promoter activity increased by a factor of ~4 relative to cells maintained in complete medium (Fig. 1A). Levels of SIRT1 mRNA (Fig. 1B) (fig. S1) and protein expression (Fig. 1C) also increased under these conditions. Under normal nutrient conditions, a fusion protein comprising Foxo3a and green fluorescent protein (GFP) was primarily cytosolic (Fig. 1D). However, within 1 hour after nutrient withdrawal, the fusion protein was predominantly found within the nucleus (Fig. 1D) (fig. S2). Starvation also increased Foxo3a protein expression (fig. S3). The activity of a Forkhead-dependent luciferase reporter increased under starved conditions in two different mammalian cell lines (PC12 cells, Fig. 1E; HeLa cells, Fig. S4). Starvation also induced the expression of a subset of other previously identified Forkhead transcriptional targets (fig. S5). When mice were subjected to an overnight fast, SIRT1 mRNA increased in numerous tissues including skeletal muscle and liver (Fig. 1F) (fig. S6). These results suggest that in mammalian cells, nutritional stress induces both SIRT1 transcription and Foxo3a activation.

To determine whether Forkhead activity is required for the observed induction of SIRT1 expression under starved conditions, we used small interfering RNA (siRNA) to inhibit endogenous Foxo3a expression. Transient expression of a Foxo3a siRNA reduced SIRT1 promoter activity under starved conditions (Fig. 1G). In a stable cell line expressing Foxo3a siRNA constitutively, endogenous Foxo3a was greatly reduced (Fig. 1H). Similarly, whereas the control cell line exhibited a doubling of SIRT1 protein expression under starved conditions, cells with decreased Foxo3a expression had a reduced response.

Transient expression in PC12 cells of a constitutively active mutant of Foxo3a (Foxo3a-TM) stimulated the activity of a ~2.8-kb SIRT1 promoter fragment in the presence of nutrients (Fig. 2A). Successive deletions of the SIRT1 promoter revealed that the stimulatory effect of Foxo3a-TM was lost between positions ~202 and ~91. Lack of Forkhead binding sites in this region suggested that the stimulatory effect of Foxo3a-TM could be indirect. Interestingly, this region contains two consensus binding sites for the tumor suppressor protein p53 (Fig. 2B). To determine whether Foxo3a-TM might stimulate the SIRT1 promoter through these p53-binding motifs, we compared the stimulatory effects of Foxo3a-TM on the wild-type 202–base pair (bp) fragment, or with promoter fragments in which one or both of the p53-consensus binding sites were mutated. Mutation of either p53-binding site reduced the stimulatory effects of Foxo3a-TM (Fig. 2C). In the absence of both binding sites, Foxo3a-TM–dependent stimulation was reduced more than 90%. Furthermore, a synthetic promoter containing three tandem repeats of a 25-bp SIRT1 promoter fragment containing both p53 binding sites was activated by Foxo3a-TM to a degree that equaled or exceeded the observed effects of Forkhead proteins on the full-length SIRT1 promoter (Fig. 2D).
These results raise the possibility that Foxo3a and p53 might physically and/or functionally interact. The Forkhead-associated (FHA) domain may mediate protein-protein interaction, and some FHA family members directly interact with p53 (9–12). Using purified recombinant proteins, we observed that p53 and Foxo3a appeared to directly interact in vitro (Fig. 3A). To localize the potential region of Foxo3a needed for p53 interaction, we generated a series of truncation mutants of Foxo3a in which successively more of the C terminus of the protein was deleted (Fig. 3B). Full-length and truncated mutants were expressed as GFP fusion proteins in PC12 cells to assess subcellular distribution. Under normal nutrient conditions, Foxo3a-TM was constitutively nuclear, whereas the distributions of the truncated Foxo3a mutants, Foxo3a-K4 and Foxo3a-k5, were cytosolic (similar to wild-type Foxo3a, Fig. 1D). In contrast, the location of the Foxo3a-w61 truncation mutant was constitutively nuclear, presumably because of the lack of a nuclear export signal.

When overexpressed in HeLa cells, p53 immunoprecipitated with wild-type Foxo3a and each of the truncation mutants (Fig. 3C). The amount of associated p53 correlated better with the amount of nuclear, rather than total, Foxo3a. For instance, even though the overall expression level of Foxo3a-TM was considerably lower than that of wild-type Foxo3a, the amount of coimmunoprecipitated p53 was roughly similar. The amount of p53 after Foxo3a immunoprecipitation was greatest when the constitutively nuclear Foxo3a-K61 mutant was expressed. This mutant also showed that the C terminus of Foxo3a is not required for interaction with p53. The interaction between Foxo3a and p53 was also observed when we performed reciprocal immunoprecipitation of p53 followed by Western blot analysis for Foxo3a (Fig. 3D). Previous studies have identified a conserved histidine as essential for the interaction between certain FHA proteins and phosphopeptides (9, 10). Nonetheless, mutation of the corresponding histidine residue in Foxo3a did not affect binding to p53, as assessed by coimmunoprecipitation (Fig. S7). However, the interaction between wild-type Foxo3a and p53 was strongly dependent on nutrient availability (Fig. 3E). This may simply reflect a difference in subcellular distribution of Foxo3a under normal nutrient or starved conditions (Fig. S2); however, we cannot rule out the possibility that starvation-induced posttranslational modifications to either p53 or Foxo3a are important for their increased interaction.

p53 can function as either a transcriptional repressor or activator (14). Expression of p53 repressed the transcriptional activity of a reporter construct under the control of three tandem copies of a 25-bp element derived from the SIRT1 promoter (Fig. 3F). Coexpression of Foxo3a-TM relieved this inhibition. In contrast, p53 expression modestly activated transcription of an alternative synthetic p53 response element derived from the human ribosomal gene cluster. Coexpression of Foxo3a-TM inhibited p53-dependent transcriptional activity of this synthetic reporter (Fig. 3G). Thus, in PC12 cells, the physical interaction between Foxo3a and p53 antagonizes p53 function. Functional interaction between these two proteins was also observed in HeLa cells (Fig. S8).

These in vitro results suggest a complex role for p53 in SIRT1 regulation. Under normal nutrient conditions, the predominant effect of p53 involves repression of SIRT1 (Fig. 3F). In contrast, under starved conditions, the ability of activated Foxo3a to stimulate SIRT1 expression requires p53 (Fig. 2C). These data suggest that in the absence of p53, the basal expression level of SIRT1 might rise but the starvation-induced increase would be blunted. To test this hypothesis, we analyzed SIRT1
expression in mice with a targeted deletion in p53. Basal SIRT1 expression was higher in adipose tissue of p53−/− mice than in wild-type controls (Fig. 4A). This result is particularly interesting given the recent observation that SIRT1 plays a prominent role in fat usage (15). A survey of other tissues revealed a more modest but consistent increase in basal SIRT1 expression in the p53−/− mice (fig. S9). However, SIRT1 mRNA did not appreciably change in either liver or skeletal muscle of p53−/− mice after overnight fasting (Figs. 1F and 4B). This lack of starvation-induced SIRT1 expression occurred even though, relative to wild-type animals, p53−/− mice had an even greater drop in their fasting glucose after food withdrawal (wild-type mice, 102 ± 7 mg/dl; p53−/− mice, 76 ± 8 mg/dl; n = 4 each, P < 0.01). These in vivo results further support a role for p53 in SIRT1 regulation.

Our results show that in mammalian cells, a simple model of acute nutritional stress results in a Foxo3a-dependent increase in SIRT1 levels. Interestingly, chronic caloric restriction also increases SIRT1 expression (16). Foxo3a regulation of SIRT1 expression occurs through an interaction with p53. In this regard, it is interesting to note that Forkhead proteins and p53 share a number of similarities (14, 17). In the worm, Forkhead proteins respond to nutrient availability and a homolog of p53 regulates starvation response (18). In mammals, although p53 is often linked to cancer and Forkhead proteins are commonly associated with aging, recent evidence has suggested a role for Forkhead proteins in tumorigenesis (19, 20) and a role for p53 in life-span (21, 22). Finally, both Foxo3a and p53 directly and independently bind to SIRT1 (6, 7, 23, 24). Taken together, these results suggest a complicated but undoubtedly important homoeostatic regulatory network involving p53, Foxo3a, and SIRT1. Further analysis of this network may help us to understand how adaptation to certain cellular stresses, including nutrient availability, may modulate mammalian life-span.

References and Notes
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Cofolding Organizes Alfalfa Mosaic Virus RNA and Coat Protein for Replication
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Alfalfa mosaic virus genomic RNAs are infectious only when the viral coat protein binds to the RNA 3′ termini. The crystal structure of an alfalfa mosaic virus RNA-peptide complex reveals that conserved AUGC repeats and Pro-Thr-x-Arg-Ser-x-x-Tyr coat protein amino acids cofold upon interacting. Alternating AUGC residues have opposite orientation, and they base pair in different adjacent duplexes. Localized RNA backbone reorientations stabilized by arginine-guanine interactions place the adenosines and guanines in reverse order in the duplex. The results suggest that a uniform, organized 3′ conformation, similar to that found on viral RNAs with transfer RNA-like ends, may be essential for replication.

A general problem in positive-strand RNA virology is understanding how viral RNA replication is initiated by the RNA-dependent RNA polymerase (replicase) on the correct template and nucleotide in an infected cell.

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Alfalfa mosaic virus (AMV) and ilarviruses are unusual positive-sense viruses, the genomic RNAs of which are replicated only in the presence of the viral coat protein (CP) (1, 2). These viruses are distinguished from many other members of the virus family Bromoviridae because they lack canonical features of the tRNA-like structure (TLS) common at the 3′ termini of the viral RNA genomes. The TLS is a necessary and sufficient feature for recruitment of the bromoviral replicase (3, 4). CP-induced structural organization of the AMV RNA 3′ terminus may create a functional homolog of the tRNA tail and thereby permit recognition by the RNA-dependent RNA polymerase.

CP binds specifically to the 3′ untranslated regions (3′UTRs) found on all four RNAs of the segmented AMV genome (5). The 180-nucleotide 3′ UTR secondary structure likely consists of six hairpins, most of which are separated by single-stranded tetranucleotide AUGC repeats (5–8). These repeats are characteristic of AMV and ilarvirus RNA sequences and are important for CP binding (8–11). We previously identified a 39-nucleotide minimal high affinity AMV CP-binding site, consisting of the two terminal hairpins and their flanking AUGC nucleotides (nucleotides 843 to 881 in RNA4; i.e., AMV843–881) (8, 12, 13) (fig. S1A). This fragment is competent to bind either full-length CP or a 26- amino acid peptide (CP26, fig. S1B) (13) representing the N-terminal RNA binding domain (14). The CP N terminus contains a Pro-Thr-x-Arg-Ser-x-x-Tyr (PTxRXxXY) RNA binding domain conserved among AMV and ilarvirus CPs (14). The arginine at position 17 is critical for both RNA binding and virus replication (14–16). Circular dichroism experiments suggest that the CP N terminus is unstructured in solution (17). Previous virus crystallization attempts required proteolytic cleavage of the AMV CP N terminus (18, 19).

Crystals of the AMV N-terminal CP peptide CP26 in complex with 5-bromouridine–labeled AMV843–881 RNA were grown in hanging drops by vapor diffusion. The structure was...