Sli15-Ipl1 mediates the Cdc14 function in targeting Slk19 to the spindle midzone. In contrast, Ase1 associated with the spindle midzone of cdc14-2 and sli15-3 cells (Fig. 4C). This spindle midzone association of the stabilizer Ase1 in sli15-3 and cdc14-2 mutants partly explains why anaphase spindles of these cells do not break, although they collapse in cells that attempt anaphase in the absence of active separase (3). However, whereas anaphase spindles break in Δsli19 cells (20), they are stable in cdc14-2 cells. In both cell types, Ase1 is correctly positioned at the spindle midzone (3) (Fig. 4C). A likely explanation for this is that the spindle-associated but misplaced Slk19 of cdc14-2 cells has some residual stabilizing function.

With anaphase onset the mitotic spindle shows dramatic rearrangements, which are essential for spindle elongation, spindle stability, timely spindle disassembly at the end of anaphase, and regulation of cytokinesis (21). Here, we provide a molecular understanding of how the activation of separase at the metaphase-anaphase transition targets the chromosomal passenger proteins INCENP-Aurora to spindles. At the metaphase-anaphase transition, activated separase partially releases the phosphatase Cdc14 from the nucleolus (4, 17). Cdc14 removes inhibiting phosphates within the microtubule-binding domain of Sli15, a step that is essential and sufficient to target the Sli15-Ipl1 kinase complex to anaphase spindles. This timely regulation of Sli15-Ipl1 by Cdc14 is important to prevent aneuploidy, one of the hallmarks of cancer cells. In addition, Sli15-Ipl1 stabilizes anaphase spindles, in part by directing the Slk19 protein to the spindle midzone. The additional participation of Slk19 in the FEAR pathway indicates a complex pattern of regulation (4). A second spindle-stabilizing pathway controls Ase1. This second pathway is dependent on separase (3) but not on Cdc14 and Sli15-Ipl1. Given that the Caenorhabditis elegans ceCdc14-1 also has a function in central spindle formation (22), it is likely that the spindle-stabilizing role of Cdc14 phosphatases is conserved.

**References and Notes**

14. Materials and methods are available as supporting material on Science Online.
15. G. Pereira, E. Schiebel, unpublished observations.
24. We thank J. Hegemann, K. Labib, K. Nasmyth, and F. Uhlmann for yeast strains and plasmids. G. Walsh and members of the Paterson Institute core facility are acknowledged for excellent technical support and C. Janke for helping with the initial two-hybrid screen. We are grateful to S. Bagley for help with microscopy and K. Labib, I. Hagan, N. Jones, J. Petersen, and members of the Schiebel laboratory for helpful discussions. The work of E.S. is supported by a program grant of Cancer Research UK.

**Yeast Life-Span Extension by Calorie Restriction Is Independent of NAD Fluctuation**

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Calorie restriction (CR) slows aging in numerous species. In the yeast Saccharomyces cerevisiae, this effect requires Sir2, a conserved NAD+-dependent deacetylase. We report that CR reduces nuclear NAD− levels in vivo. Moreover, the activity of Sir2 and its human homologue SIRT1 are not affected by physiological alterations in the NAD−:NADH ratio. These data implicate alternate mechanisms of Sir2 regulation by CR.

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English metabolism. The NAD-dependent histone deacetylases Sir2 (1, 2) and SIRT1 (3, 4) are conserved from yeast to humans, and the Sir2 family is essential for yeast life-span, including heat, osmotic stress, and nitrogen starvation (5–7).

With aging, several subcellular components change in the NAD-dependent metabolism. For example, NAD is depleted in the yeast Schizosaccharomyces pombe (8) and in several human cancers (9). Moreover, in several species, calorie restriction (CR) reduces the NAD level in vivo, along with a concomitant increase in NADH (10, 11). In addition, CR increases the NAD−:NADH ratio and extends life-span (12, 13). Although it is possible to affect Sir2 activity by genetically manipulating NAD− metabolic pathways, it is not known whether NAD− is a bona fide regulator of Sir2 in normal cells. A major obstacle has been determining the effective concentrations of these metabolites in living cells, as various levels of free NAD− and NADH have been reported (11, 12). To compare the levels of freely available NAD− in the nuclei of wild-type and long-lived cells in which Sir2 is activated, a reporter assay was developed based on the Salmonella typhi murium NADR protein, a transcriptional repressor whose affinity for its recognition sequence, the NAD box, is specifically NAD−-dependent (13). NADR was converted to a yeast transcriptional activator by fusing its C terminus to a nuclear localization signal and the activation domain (AD) of the yeast Gal4 transcription factor (fig. S1) (14). Expression of the fusion protein was driven from a high-level constitutive promoter. A series of NAD boxes identical to their arrangement in the native S. typhi murium NAD regulon (fig. S2) were cloned upstream of the yeast Ase1-associated but misplaced Slk19 of cdc14-2 cells has some residual stabilizing function.
Histidine-3-AT assay medium was slower in detecting variations in nuclear NAD\(^+\). Also shown are assays of negative control strains: empty vector (no NadR-AD), mutant Nad boxes (mut) (TGTTGTA and its inverted repeat). Spots were incubated for \(-48\) hours at 30°C. (B) The Nad\(^+\) reporter system detects changes in availability of nuclear NAD\(^+\). Nad\(^+\) may be generated from nicotinic acid via the NAD\(^+\) pathway or from tryptophan via the de novo pathway, which is catalyzed by Bna1-6 (17, 18). Deletion of BN\(\text{A}6\) decreases NAD\(^+\) levels, whereas exogenously supplied acetaldehyde increases NAD\(^+\) by oxidizing the NADH pool (24). BN\(\text{A}6\) was deleted in the Nad\(^+\) reporter by replacing the entire coding region with a KanMX cassette and spotted on reporter assay media. The wild-type reporter strain was pregrown in liquid SC medium with or without histidine + 3-amino triazole (3-AT). The extent of growth on the test media is indicative of the availability of NAD\(^+\). With or without histidine, BNA6\(+/\text{H}11001\) was deleted in the NAD\(^+\) oxidizing the NADH pool (17, 18). BN\(\text{A}6\) was deleted in the NAD\(^+\) reporter by replacing the entire coding region with a KanMX cassette and spotted on reporter assay media. The wild-type reporter strain was pregrown in liquid SC medium with or without histidine (10 mM) for 2 hours and then spotted on reporter assay media containing acetaldehyde (10 mM). (C) Life-span extending manipulations do not correlate with increased nuclear NAD\(^+\). To assay calorie-restricted cells, the Nad\(^+\) reporter strain was grown for 2 hours in defined SC medium with either 2.0% glucose (standard concentration) or 0.5% glucose (CR). Cultures were washed and spotted in serial 10-fold dilutions on assay media with the same glucose concentrations. The retarded growth of the strain on restricted medium was rescued by acetaldehyde (10 mM). (D) The Nad\(^+\) reporter strain was spotted on assay media and grown under two low-intensity stresses known to extend replicative life-span: high osmolarity (4% glucose) (2) and heat stress (37°C) (8, 9).

Only the strain carrying both the NadR-AD activator and the Nadp-HIS3 reporter grew on plates lacking histidine with 3-AT (10 mM) (Fig. 1A). No growth was observed for control strains lacking the NadR-AD activator or with mutant NAD boxes (Fig. 1A), or for strains carrying other AD fusions (Fig. S3). Loss-of-function mutations in the ribonucleotide kinase or nicotinamide mononucleotide adenylyltransferase domains of NadR-AD also did not alter HIS3 expression (15). The Nad\(^+\) reporter system detected variations in nuclear NAD\(^+\) levels in vivo as a bna6\(+/\text{H}11001\) strain with \(-30\%\) lower steady-state NAD\(^+\) levels displayed retarded growth. In contrast, acetaldehyde, which regenerates NAD\(^+\) when reduced to ethanol, stimulated growth (Fig. 1B). Overexpression of HAP4, which increases respiration, also increased growth, whereas a mitochondrial mutant incapable of respiring grew very slowly (Fig. S4).

Growth of the Nad\(^+\) reporter strain on histidine-3-AT assay medium was slower in CR cells compared with the control strain (0.5% versus 2.0% glucose) (Fig. 1C) but improved with exogenous acetaldehyde, confirming that low NAD\(^+\) was responsible for slow growth. Moreover, mild osmotic stress (4% glucose medium) (2) and heat stress (37°C) (8, 9) did not increase reporter expression (Fig. 1D). Thus NAD\(^+\) levels did not correlate with yeast life-span, indicating that Sir2 is not regulated by the availability of NAD\(^+\) under these conditions.

To quantify the levels of total free NAD\(^+\) in CR cells, we used in vivo 13C NMR (carbon 13 nuclear magnetic resonance) spectroscopy, a highly accurate, noninvasive methodology. Only free, non-protein-bound NAD\(^+\) and/or free NAD\(^+\)/NADH (16) pools are available for interactions with Sir2 and could potentially play a regulatory role. Therefore, a major advantage of the NMR technique over previous studies (17, 18) is that it detects only unbound metabolites; those bound to macromolecules are unobservable as a result of severe line broadening (11, 19). Rapid small molecule equilibration across the nuclear envelope (20) suggests that measurements of free cellular NAD are likely indicative of the nuclear and cytoplasmic concentrations. Although NMR cannot distinguish NAD\(^+\) from NAD\(^-\) in living cells, the pool of NAD\(^+\) is negligible and below the threshold of detection (21) (fig. S5).

In media with 2.0% or 0.5% glucose, intracellular NAD\(^+\) concentrations were stable and NADH was below the threshold of detection (<200 \(\mu\)M) (figs. S6 and S7). Consistent with the Nad\(^+\) reporter, Nad\(^+\) concentrations were lower in CR cells than in controls (3.97 \pm 0.02 versus 4.28 \pm 0.14 mM, respectively) (Fig. 2). Addition of glucose did not affect NAD\(^-\) levels, indicating that cells were not glucose-starved. This confirms that increased NAD\(^+\) is not the explanation for increased Sir2 activity in CR cells.

We estimate the Nad\(^+\)/NADH ratio in live aerobic yeast cells to be at least 20, which agrees with classical estimates of the ratio (22, 23). Using a fluorescence-based deacetylation assay and two NAD\(^+\) concentrations (4.0 mM, as estimated by in vivo NMR, and 0.05 mM, a value in the range of previous Sir2 studies), we observed no significant effect on deacetylase activity of Sir2 or its human homologue SIRT1 within the physiological range of NAD\(^+\)/NADH (Fig. 3, A to C). Furthermore, depletion of intracellular NADH with high concentrations of acetaldehyde did not apparently stimulate Sir2 in vivo, as determined by the extent to which two rDNA reporter genes, ADE2 and MET15, were silenced (Fig. 3D). These data indicate that physiological variations in NADH are unlikely to affect the activity of Sir2 or SIRT1.

Our measurements of intracellular NAD\(^+\) demonstrate that, under aerobic conditions, the steady-state levels of NAD\(^+\) do not fluctuate greatly; moreover, its fluctuations during CR negatively correlate with Sir2 activity. The redox state of NAD is also unlikely to regulate Sir2. Nicotinamide is a negative regulator of Sir2 activity in vivo, but whether this is the main mechanism of Sir2 regulation, or whether there are other regulatory mechanisms, remains to be determined.
Fig. 3. Effect of NADH on yeast Sir2 and human SIRT1 activity. Error bars represent standard error of the mean. (A) Sir2 and SIRT1 deacetylation assays with 4 mM NAD<sup>+</sup> and increasing concentrations of NADH. Activity values shown are normalized against the activities of the recombinant proteins in the absence of NADH. Activities were 23 and 100 pmol hour<sup>–1</sup> g<sup>–1</sup> for Sir2 at 0.05 mM and 4 mM NAD<sup>+</sup> respectively and 160 and 500 pmol hour<sup>–1</sup> g<sup>–1</sup> for SIRT1. (B) Sir2 and SIRT1 deacetylation assays with 0.05 mM NAD<sup>+</sup> and increasing concentrations of NADH. For clarity, data is shown on two different scales, the upper panel for 0 to 1 μM NADH and the lower panel for higher NADH concentrations. (D) Ribosomal DNA locus (RDNT) silencing assays with or without acetaldehyde (10 mM). Cells were pretreated for 2 to 3 hours and then spotted to assay plates in 10-fold dilutions or single spots for the ADE2 and MET15 assay, respectively. Silencing of the RDNT::ADE2 reporter results in growth retardation on plates lacking adenine, whereas silencing of the RDNT::MET15 reporter leads to a brown coloration on plates containing medium.

References and Notes
14. Materials and methods are available as supporting material on Science Online.
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25. We are grateful to D. Moazed, P. Frey, M. Agosto, R. Veech, J. Foster (pFW38–46), L. Guarente (pADH-HAP4), and B. Forrester (NS)(164), for advice and/or reagents. Synthesis of labeled nicotinic acid was carried out by R. Ventura and C. Maycock. This work was supported by the National Institute on Aging and the Harvard-Armenise Foundation. D.S. is an Ellison Medical Research Foundation Special Fellow. R.A. is supported by a John Talpin Postdoctoral Fellowship, O.M. by the American Federation of Aging Research.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1088697/DC1
Materials and Methods
SOM Text
Figs. S1 to S7
References and Notes
2 July 2003; accepted 23 October 2003
Published online 6 November 2003; 10.1126/science.1088697
Include this information when citing this paper.

Dual Activation of the Drosophila Toll Pathway by Two Pattern Recognition Receptors

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The Toll-dependent defense against Gram-positive bacterial infections in Drosophila is mediated through the peptidoglycan recognition protein SA (PGRP-SA). A mutation termed osiris disrupts the Gram-negative binding protein 1 (GNBP1) gene and leads to compromised survival of mutant flies after Gram-positive infections, but not after fungal or Gram-negative bacterial challenge. Our results demonstrate that GNBP1 and PGRP-SA can jointly activate the Toll pathway. The potential for a combination of distinct proteins to mediate detection of infectious nonself in the fly will refine the concept of pattern recognition in insects.

A hallmark of the host response of Drosophila to infection is the expression of antimicrobial peptide genes. Two intracellular signaling pathways regulate this expression. The Toll pathway is activated primarily by Gram-positive bacterial or fungal infections and, in particular, directs the expression of the anti-microbial peptide gene Drosomycin. In contrast, the immune deficiency (IMD) pathway responds predominantly to infections by Gram-negative bacteria and induces expression of several antibacterial peptide genes, including Dipterericin (1). Fungal and Gram-positive infections lead to the proteolytic processing of a cytokine, the growth factor-like polypeptide Spätzle, which in its cleaved form binds directly to the transmembrane receptor Toll (2–8). This binding triggers an intracellular signaling cascade that culminates in the up-regulated transcription of multiple target genes, through the nuclear factor κB-related protein Dorsal-related immune factor (DIF) (2, 9–14).

How bacterial infection leads to processing of Spätzle and subsequent activation of Toll has remained unclear. In the course of a large-scale screen designed to isolate mutants of the innate immune response of Drosophila, we identified a new mutation that affected the inducibility of Drosomycin by Gram-

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2126 19 DECEMBER 2003 VOL 302 SCIENCE www.sciencemag.org