Evolution of Distinct Responses to Low NAD+ Stress by Rewiring the Sir2 Deacetylase Network in Yeasts

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ABSTRACT Evolutionary adaptation increases the fitness of a species in its environment. It can occur through rewiring of gene regulatory networks, such that an organism responds appropriately to environmental changes. We investigated whether sirtuin deacetylases, which repress transcription and require NAD+ for activity, serve as transcriptional rewiring points that facilitate the evolution of potentially adaptive traits. If so, bringing genes under the control of sirtuins could enable organisms to mount appropriate responses to stresses that decrease NAD+ levels. To explore how the genomic targets of sirtuins shift over evolutionary time, we compared two yeast species, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, that display differences in cellular metabolism and life cycle timing in response to nutrient availability. We identified sirtuin-regulated genes through a combination of chromatin immunoprecipitation and RNA expression. In both species, regulated genes were associated with NAD+ homeostasis, mating, and sporulation, but the specific genes differed. In addition, regulated genes in *K. lactis* were associated with other processes, including utilization of nonglucose carbon sources, detoxification of arsenic, and production of the siderophore pulcherrimin. Consistent with the species-restricted regulation of these genes, sirtuin deletion affected relevant phenotypes in *K. lactis* but not *S. cerevisiae*. Finally, sirtuin-regulated gene sets were depleted for broadly conserved genes, consistent with sirtuins regulating processes restricted to a few species. Taken together, these results are consistent with the notion that sirtuins serve as rewiring points that allow species to evolve distinct responses to low NAD+ stress.

KEYWORDS Sirtuin; Sum1; nicotinamide adenine dinucleotide; sporulation

EVOLUTIONARY adaptation is the process by which species acquire traits that make them better suited to a particular environment. At the molecular level, adaptation can involve the acquisition of new protein functions or new gene expression patterns (Ohno 1970; King and Wilson 1975). In the case of new gene expression patterns, particular transcriptional regulators might be frequent rewiring points for such changes. For example, a regulator that responds to a particular stress could be redirected to new genes to elicit a distinct biological response to that stress. In this study, we explored the possibility that sirtuin deacetylases are such regulators.

Sirtuins are NAD+-dependent deacetylases that have been identified in all kingdoms of life (Greiss and Gartner 2009). These enzymes have two characteristics that are consistent with being rewiring points. First, sirtuins require NAD+ for activity (Imai *et al.* 2000) and are therefore proposed to respond to stresses that affect intracellular NAD+ levels. In particular, because NAD+ is a key redox carrier in central metabolism, changes in metabolic flux due to nutrient perturbations could affect NAD+ availability and hence the

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deacetylase activity of sirtuins. At this time, the conditions that cause low intracellular NAD^+ in yeast are not well understood. However, there is evidence that intracellular NAD^+ levels affect Sir2 activity. For example, genetic perturbations of the enzymes and transporters that maintain intracellular NAD^+ alter Sir2 activity (Smith *et al.* 2000; Anderson *et al.* 2002; Belenky *et al.* 2007), as does the presence or absence of NAD^+ precursors in the growth medium (Belenky *et al.* 2007). Moreover, intracellular NAD^+ is increased in the absence of inositol (Lee *et al.* 2013) and in aging cells (Ashrafi *et al.* 2000), and these increases correspond with enhanced Sir2 activity.

A second characteristic of sirtuins consistent with being rewiring points is that they have diverged to deacetylate a wide range of substrates. Some sirtuins, including those examined here, deacetylate histones and are targeted to specific genomic locations where they repress transcription. Thus, these sirtuins are proposed to connect gene expression to the metabolic state of the cell by sensing NAD^+ levels. Therefore, species could evolve distinct biological responses to stresses that perturb NAD^+ levels by bringing new genes under the control of sirtuins. Consistent with this idea, we have observed that in the *Candida* (CTG) clade of yeast the genomic targets of sirtuins vary from one species to another (Froyd *et al.* 2013; Kapoor *et al.* 2015; Rupert *et al.* 2016).

To explore how the genes targeted by sirtuins have shifted over evolutionary time, we compared two genetically tractable yeast species, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *S. cerevisiae* Sir2 (ScSir2) deacetylates histone tails (Braunstein *et al.* 1993; Imai *et al.* 2000) and, as part of a complex with Sir3 and Sir4, forms transcriptionally repressive chromatin at telomeres and the cryptic mating-type loci (Rine and Herskowitz 1987; Gottschling *et al.* 1990). ScSir2 also acts at the tandem ribosomal DNA (rDNA) repeats, where it reduces recombination (Gottlieb and Esposito 1989). A paralog of Sir2, Hst1 (Homolog of Sir Two), arose in a whole-genome duplication (Byrne and Wolfe 2005). *S. cerevisiae* Hst1 (ScHst1) acts with the DNA-binding protein Sum1 to repress midsporulation and NAD^+ biosynthetic genes (Xie *et al.* 1999; Bedalov *et al.* 2003). We compared the targets of ScSir2 and ScHst1 with those of Sir2 in *K. lactis* (KlSir2). *K. lactis* diverged from *S. cerevisiae* over 100 MYA (Shen *et al.* 2018) but is still more closely related to *S. cerevisiae* than the other well-known yeasts *Candida albicans* and *Schizosaccharomyces pombe*. *K. lactis* did not undergo the duplication that led to Sir2 and Hst1. Like ScSir2, KlSir2 acts at the telomeres, cryptic mating-type loci, and rDNA; and like ScHst1, KlSir2 acts at midsporulation genes (Astrom *et al.* 2000; Hickman and Rusche 2009).

An unresolved question is whether the sets of genes repressed by Sir2 and Hst1 in *S. cerevisiae* and *K. lactis* differ functionally, enabling each species to respond in a distinct way to low intracellular NAD^+. There are several differences between these species that are connected to cellular metabolism and nutrient availability. For example, in the presence of oxygen, *S. cerevisiae* processes sugars through fermentation whereas *K. lactis* favors respiration (Kiers *et al.* 1998). These distinct metabolic strategies might require different responses to low NAD^+ levels. A second difference is the coordination of the sexual cycle with nutrient availability. Newly germinated *S. cerevisiae* spores, which are haploid, mate readily in rich nutrients. The resulting diploid cells propagate mitotically until nutrients become scarce, at which point they undergo meiosis and sporulation. In contrast, newly germinated *K. lactis* spores do not mate readily but instead propagate in the haploid state until nutrients become scarce (Herman and Roman 1966; Booth *et al.* 2010). They then mate and proceed directly to meiosis and sporulation.

To determine whether Sir2 and Hst1 regulate distinct genes that would be advantageous for the different metabolic and life cycle strategies of *S. cerevisiae* and *K. lactis*, we defined the gene sets regulated by Sir2 and Hst1 in both species. To do so, we used a combination of chromatin immunoprecipitation (ChIP) and RNA expression analyses. We found that regulated genes in both species are involved in NAD^+ homeostasis, mating, and sporulation. However, the specific genes that are regulated differ. In addition, in *K. lactis*, regulated genes are associated with processes not regulated in *S. cerevisiae*, including utilization of nonglucose carbon sources, arsenic detoxification, and production of the siderophore pulcherrimin. Consistent with the species-restricted regulation of these genes, sirtuin deletion affected relevant phenotypes in *K. lactis* but not *S. cerevisiae*. We also found that the gene sets regulated by Sir2 and Hst1 are depleted for widely conserved genes. Taken together, these results are consistent with the notion that sirtuins can serve as rewiring points that allow species to evolve distinct responses to low NAD^+ stress.

Materials and Methods

Plasmids and yeast strains

Plasmids used in this study are listed in Supplemental Material, Table S1. The details of construction are provided in the Supplemental Materials and Methods. Yeast used in this study are listed in Table S2. Most *K. lactis* strains were derived from Os334 and Os335 (Heinisch *et al.* 2010), which are congenic with the reference strain CBS2359. *S. cerevisiae* strains were derived from the laboratory strain W303-1b. The details of strain construction are provided in the Supplemental Materials and Methods.

Yeast growth and transformation

Yeast were grown at 30° in YPD (1% yeast extract, 2% peptone, 2% glucose) unless otherwise stated. *S. cerevisiae* cells were transformed using the polyethylene glycol lithium acetate method (Schiestl and Gietz 1989), and *K. lactis* cells were transformed by electroporation (Hickman and Rusche 2009).

To follow growth in various carbon sources, yeast were grown in yeast minimal medium (YM) (0.67% yeast nitrogen base without amino acids) with the desired carbon source

(2% glucose or 3% glycerol). For *S. cerevisiae*, YM was supplemented with histidine, leucine, lysine, and tryptophan. Overnight (glucose) or 3-day (glycerol) cultures were diluted 50% in the same medium and grown an additional 3 hr at 30°. Cells were then diluted to OD 0.05 and placed in a 96-well clear-bottomed plate containing YM supplemented with the desired carbon source. The OD_{600} of each culture was recorded at consistent time intervals at 30° on a SpectraMax i3x microplate reader using the SoftMaxPro 6.5.1 software. To follow growth in sodium arsenate, cells were grown overnight in YPD, diluted to OD = 0.3, and allowed to grow 4 hr at 30°. Cells were then diluted to OD = 0.05 in different concentrations of sodium arsenate, and the growth was recorded as described above. To assess pulcherrimin production, cells were grown overnight in YPD, diluted to OD_{600} = 0.3 in YPD, and grown 4 hr. Cells were collected, washed, and resuspended in PBS at 1 OD/ml. Next, 10 μl of cells were spotted onto YPD plates supplemented with 3.7 mM $FeCl_3 \cdot 6H_2O$ or YM plates supplemented with various concentrations (50, 100, 200, and 400 μg/liter) of nicotinic acid. The plates were incubated for 2 days before imaging.

RNA isolation and sequencing

For sequencing, RNA was isolated, as previously described (Schmitt *et al.* 1990), from *S. cerevisiae* strains LRY3093 and 3099 and *K. lactis* strains LRY2835, 2849, and 2850 (2012 data set), or LRY2835, 2850, 3096, and 3098 (2016 data set). Cells were grown in YPD and harvested in midlog phase at OD_{600} ~1. DNA was removed from 10 μg RNA using Turbo DNase (Life Technologies), according to the manufacturer's instructions. One set of *K. lactis* RNA samples was processed and sequenced (50 bp single end) on an Illumina HiSeq2000 machine at the Duke Institute for Genome Sciences and Policy sequencing facility. A second set of *K. lactis* samples and the *S. cerevisiae* samples were processed and sequenced (50 bp single end) on an Illumina HiSeq2500 at the University at Buffalo Genomics and Bioinformatics Core facility.

ChIP and processing for microarray or sequencing

For the ChIP-sequencing (ChIP-Seq) experiment from *S. cerevisiae*, ScSir2-HA was immunoprecipitated from LRY1926, ScHst1-HA was immunoprecipitated from LRY558, and LRY1009 was used for mock immunoprecipitation (IP) and input DNA. ChIP and processing is described in the Supplemental Methods. Library preparation and sample barcoding was done at the University at Buffalo Genomics and Bioinformatics Core facility. The samples were then sequenced on an Illumina HiSeq2500 using 50 bp single-end sequencing.

For the ChIP-on-Chip experiment, KlSir2-HA was immunoprecipitated from strains LRY2021 or LRY2022. For LRY2021, the IP sample was labeled with Cy5 and the input was labeled with Cy3. For LRY2022, the dyes were swapped. Labeled DNA was hybridized to a custom ChIP-on-Chip 2 × 105K microarray (G4498A; Agilent), designed with 102,839 60-nucleotide probes tiled across the *K. lactis* genome spaced approximately every 100 bp and covering 6.17 Mbp (AMADID 018357). ChIP was conducted as previously described (Hickman and Rusche 2009) and processed as described in the Supplemental Methods. To assess the data quality, we plotted the $\log_2$ of IP/input and observed long right tails, corresponding to enrichment in the IP channel (Figure S1).

Bioinformatic analysis

ChIP-Seq reads of ScSir2 and ScHst1 were mapped to the S288c *S. cerevisiae* reference genome (GCA_000146055.2) using BWA v0.7.7-r441 (Li and Durbin 2009). Mapping rates for each sample are in Table S3. For calling enriched peak regions, MACS2 v2.0.10 (Zhang *et al.* 2008) was used with genomic input as control, and the parameters used were, "-B–nomodel–extsize 200 -q 0.01 -g 12157105." This analysis identified 159 ScSir2 peaks and 692 ScHst1 peaks. Genes associated with these peaks were defined as those genes for which either the gene body intersected with a peak or the start codon was within 1 kbp of a peak.

ChIP-on-chip signals were mapped to the *K. lactis* reference genome (https://www.ncbi.nlm.nih.gov/genome/?term=txid28985%5Borgn%5D), and KlSir2 binding sites were called using MA2C software (Song *et al.* 2007). The normalization method was set as "Robust," the false discovery rate cut-off for ChIP-enriched regions was 0.05, the BANDWIDTH was set to 500 bp, the number of MIN_PROBES was 5, and the MAX_GAP was set as 250 bp. This analysis identified 460 KlSir2 peaks. Genes associated with these peaks were defined using the same criteria as for *S. cerevisiae*.

For RNA-sequencing (RNA-Seq), the raw reads from single-end sequencing were mapped to the *K. lactis* and *S. cerevisiae* reference genomes, allowing no more than two mismatches, using tophat v2.0.10 (Kim *et al.* 2013). Mapping rates for each sample are in Table S3. Fragments per kilobase per million reads were obtained using cufflinks version 2.2.1 (Trapnell *et al.* 2013) and count data were obtained using HTSeq-count (Anders *et al.* 2015) with alignment quality cut-off set to 10. The gene annotation file for *K. lactis* was obtained from Genolevures database (https://www.ncbi.nlm.nih.gov/genome/193?genome_assembly_id=28427); the file for *S. cerevisiae* was obtained from Ensembl database (http://ftp.ensembl.org/pub/release-66/gtf/saccharomyces_cerevisiae/). edgeR (Robinson *et al.* 2010) was applied to detect genes differentially expressed between wild-type and knockout strains, with a false discovery rate cut-off of 0.05 and the absolute fold-change of ≥2.

To identify *S. cerevisiae* orthologs of *K. lactis* genes, each *K. lactis* gene served as a BLASTP query, and the top *S. cerevisiae* hits (≥70% of the maximum score for that search) were identified. Next, these *S. cerevisiae* genes were used as queries, and the top *K. lactis* hits were identified. If the second BLASTP search returned to the starting *K. lactis* gene, the genes from the two species were concluded to be orthologs. In some cases, the BLASTP search identified a multigene family or paralogs that arose through duplication in the *S. cerevisiae* lineage. These genes were all taken as orthologs.

Most of the ortholog assignments were consistent with the Yeast Gene Order Browser (Byrne and Wolfe 2005), which considers gene order as well as homology. After manual refinement, including the use of gene order to assign orthologs to some short, rapidly evolving genes, we identified *S. cerevisiae* orthologs for 109 of the 175 KlSir2-regulated genes.

Mating assays

For *S. cerevisiae* mating assays, cells of both mating types were grown separately overnight in YPD at 30° with shaking. Cultures were diluted by 50% in YPD and incubated for 3 hr, spun down, and resuspended at 10 OD/ml in YPD. A lawn was prepared using 200 μl (two OD of cells) of one mating type spread on YM plates supplemented with histidine, tryptophan, and leucine. The other stain was then used to make five 10-fold serial dilutions. Next, 200 μl of the most dilute culture (2×10^{-5} OD) was spread on YPD plates to determine the number of viable cells and over the lawn to determine the number of cells that could mate. Three mating lawns and one YPD plates were counted for each mating combination.

For *K. lactis* mating assays, *MAT***a** cells expressed mCherry, and *MAT*α cells expressed yEGFP. Cells of both mating types were cocultured on malt extract (5% malt extract and 3% agar) for 3 days at 30°. Cells were resuspended in 500 μl of sterile distilled water and then sonicated using a soniprep 150 sonicator for 5 sec at an amplitude of 5 μm. Finally, cells were examined using ImageStream cytometry (ImageStream Mark II Imaging flow cytometer; EMD Millipore) with the following settings: 488 nm laser at 100 W, 561 nm laser at 150 W, ×60 amplification. Images were collected for brightfield in channels 1 and 9, side scatter in channel 6, yEGFP in channel 2, and mCherry in channel 4. A total of 100,000 events were collected for each sample. IDEAS software (EMD Millipore) was used to identify cells that were in focus and showed both red and green fluorescence. Each of these events were manually examined for an hourglass appearance typical of zygotes.

Sporulation assay

Diploid cells were freshly grown from frozen glycerol stocks. After overnight growth on YPD plates, cells were patched onto KOAc plates (1% KOAc and 3% agar) and incubated at 30°. At 2 hr intervals, cells were resuspended in sterile distilled water, vortexed, and examined under a light microscope. All cells in three fields of vision were scored as either having sporulated (tetrad morphology) or not. The experiment was conducted twice with four biological replicates (diploid strains) each time.

Data and reagent availability statement

Strains and plasmids are available upon request. Gene expression data are available at Gene Expression Omnibus (GEO) under the accession numbers GSE92930, GSE86149, and GSE84403. ChIP-Seq data are available at GEO under the accession number GSE84552. ChIP-on-Chip data are available at GEO under the accession number GSE85574.

Table S1 lists plasmids used in this study. Table S2 lists yeast strains used in this study. Table S3 lists mapping rates for Illumina sequencing experiments. Table S4 contains RNA-Seq and ChIP-Seq data for all annotated *S. cerevisiae* genes. Table S5 contains RNA-Seq and ChIP-on-Chip data for all annotated *K. lactis* genes. Table S6 contains descriptions and comparative information for ScSir2-regulated genes. Table S7 contains descriptions and comparative information for ScHst1-regulated genes. Table S8 contains descriptions and comparative information for KlSir2-regulated genes identified based on 2016 RNA-Seq data. Table S9 contains descriptions and comparative information for KlSir2-regulated genes identified based on 2012 RNA-Seq data. Table S10 is the basis for Figure 3. It includes RNA-Seq and ChIP data for the genes known to act in each pathway represented in the figure. Table S11 lists oligos used for quantitative PCR to validate the regulation of particular genes. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11743566.

Results

Identification of genes regulated by ScSir2, ScHst1, and KlSir2

To identify the genes regulated by Sir2 and Hst1 in *S. cerevisiae* and *K. lactis*, we combined data from two experiments for each species. First, we mapped genomic loci associated with Sir2 or Hst1 using ChIP. For *S. cerevisiae*, ChIP DNA was sequenced using high-throughput Illumina technology (ChIP-Seq). For *K. lactis*, ChIP DNA was hybridized to a tiled microarray (ChIP-on-Chip). Next, we identified the genes whose transcription is influenced by Sir2 or Hst1 using Illumina sequencing of RNA (RNA-Seq). For this experiment, the cryptic mating-type loci (*HML*α and *HMR***a**) were deleted so that loss of Sir2 would not lead to simultaneous expression of **a** and α transcription factors, a condition that specifies the diploid state and causes substantial changes in gene expression. In *S. cerevisiae*, both *SIR2* and *HST1* were deleted in the same strain because these paralogs are known to substitute for one another (Xie *et al.* 1999; Hickman and Rusche 2007). Genes directly regulated by Sir2 or Hst1 were defined as those that were associated with the deacetylase in the ChIP assay and increased at least twofold in the deletion strain compared to wild type.

In *S. cerevisiae*, 171 genes increased significantly in the *sir2*Δ *hst1*Δ strain. ScHst1 associated with 974 genes, of which 115 were significantly upregulated in the *sir2*Δ *hst1*Δ strain (Figure 1A). Therefore, these 115 genes were identified as being directly regulated by ScHst1. Similarly, ScSir2 associated with a total of 176 genes, of which 10 also increased in expression and were thus identified as ScSir2-regulated (Figure 1B). In *K. lactis*, 1159 genes were associated with KlSir2, and 255 genes increased in expression in the *sir2*Δ strain. 175 of these genes had both properties and were thus determined to be directly regulated by KlSir2 (Figure 1C). Most (153) of these 175 KlSir2-regulated genes

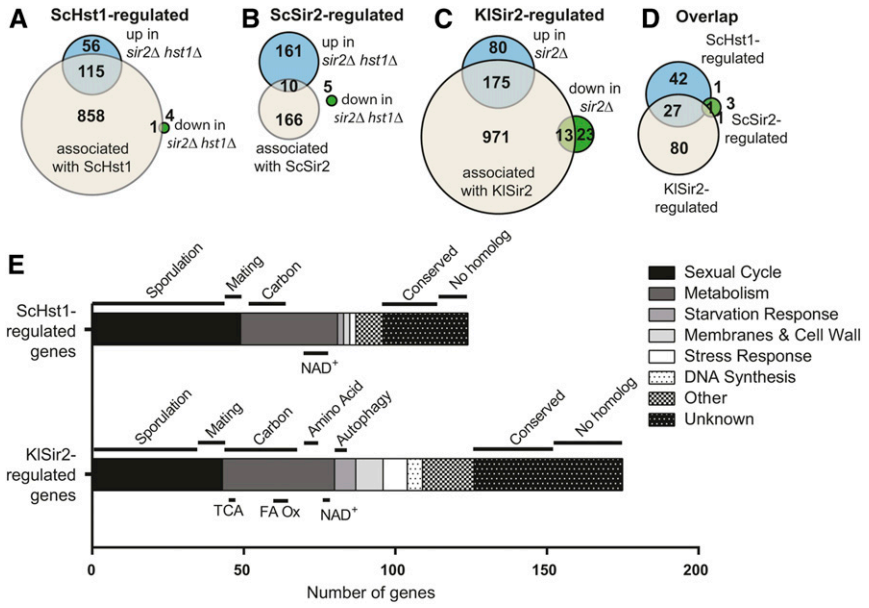

Figure 1 Identification of genes regulated by ScSir2, ScHst1, and KlSir2. (A) The overlap was determined for genes associated with ScHst1 (974), upregulated in *sir2*Δ *hst1*Δ cells (171), and downregulated in *sir2*Δ *hst1*Δ cells (five, including *SIR2* and *HST1*). (B) The overlap was determined for genes associated with ScSir2 (176), upregulated in *sir2*Δ *hst1*Δ cells (171), and downregulated in *sir2*Δ *hst1*Δ cells (five). (C) The overlap was determined for genes associated with KlSir2 (1159), upregulated in *sir2*Δ cells (255), and downregulated in *sir2*Δ cells (36, including *SIR2*). (D) The overlap was determined for genes regulated by ScSir2, ScHst1, and KlSir2. Of the 71 ScHst1-regulated genes with orthologs in *K. lactis*, 28 were also regulated by KlSir2. Of the six ScSir2-regulated genes with orthologs in *K. lactis*, two were also regulated by KlSir2. Of the 109 KlSir2-regulated genes with orthologs in *S. cerevisiae*, 28 were also regulated by ScHst1 and two were also regulated by ScSir2. (E) Genes regulated by ScHst1 and KlSir2 were manually grouped into functional categories based on Gene Ontology terms and functional information (Tables S7 and S8). The bar graph represents the number of genes in each category.

were also upregulated in a separate *K. lactis* RNA-Seq data set that we collected several years earlier (Figure S2). Unless otherwise noted, we focused on the 175 KlSir2-regulated genes (Figure 1C) that were identified based on the RNA-Seq conducted at the same time as the *S. cerevisiae* RNA-Seq. The ChIP and expression data for all annotated genes in both species are provided in Tables S4 and S5.

To confirm the genome-wide results, we examined individual genes (Figure S3). The nicotinic acid transporter *TNA1* and the spore wall synthesis gene *CDA2* were identified as regulated genes in both species. Indeed, in the absence of Sir2 or Hst1, expression of these genes was induced (Figure S3, A and B) and histone acetylation at the promoter increased (Figure S3, E and F). In addition, ScHst1 and KlSir2 were associated with the promoters of *TNA1* and *CDA2* (Figure S3, C and D). We also confirmed the regulation of three additional genes in *K. lactis*, *POX1*, *ARR3*, and *PUL2* (Figure S3). The functions of these genes and the phenotypic consequences of their regulation are presented below. *POX1* and *ARR3* were not regulated in *S. cerevisiae* (Figure S3), and *PUL2* is not found in *S. cerevisiae*.

ScSir2, ScHst1, and KlSir2 are all expected to repress transcription based on their deacetylase activity and previously described functions (Rine and Herskowitz 1987; Xie *et al.* 1999; Hickman and Rusche 2009). Consistent with this expectation, genes associated with these deacetylases were more likely to be upregulated than downregulated in the absence of the deacetylase (Figure 1, A–C). Indeed, in *S. cerevisiae* only one downregulated gene was associated with ScHst1, and none were associated with ScSir2. However, in *K. lactis* 13 downregulated genes were associated with KlSir2 (Figure 1C). To test statistically whether these genes are simply near KlSir2 peaks by chance or could be transcriptionally activated by KlSir2, we performed a Fisher's exact test. We found a strong correlation between upregulated genes and KlSir2 peaks ($P < 2.2 \times 10^{-16}$). In contrast, the correlation was less significant for downregulated genes ($P = 0.049$). Therefore, KlSir2 functions primarily as a transcriptional repressor.

Genes regulated by ScSir2 were adjacent to cryptic mating-type loci or telomeres

ScSir2 is thought to act primarily, if not exclusively, at the cryptic mating-type loci, telomeres, and rDNA (Marchfelder *et al.* 2003; Ellahi *et al.* 2015). Consistent with this expectation, all but one of the 10 genes we identified as ScSir2-regulated was near these loci (Table S6). Six genes were within 10 kb of a telomere, and three genes were adjacent to the cryptic mating-type locus *HML*. No genes near *HMR* could be identified as ScSir2-repressed because *HMR* was deleted in the strains used for RNA-Seq. However, ScSir2 was associated with *HMR* in our ChIP-Seq study. The one ScSir2-regulated gene that was not near telomeres or mating-type loci was *YAT1*. This gene was also associated with ScHst1, and thus it is not clear which of these deacetylases is the primary regulator. We note that seven of the 10 ScSir2-regulated genes were previously identified as being repressed by the Sir proteins (Ellahi *et al.* 2015). We surveyed the functions of the 10 ScSir2-regulated genes, but observed no common functional categories. We also compared the genome-wide distribution of ScSir2 observed in this study with data collected by two other labs. In agreement with (Thurtle and Rine 2014), we found that ScSir2 is focused at telomeres and mating-type loci. In contrast, Sir2 was not associated with highly expressed genes (Figure S4) as reported (Li *et al.* 2013). We note that the ChIP-Seq data that led to this conclusion had a low signal-to-noise ratio and many of the genes

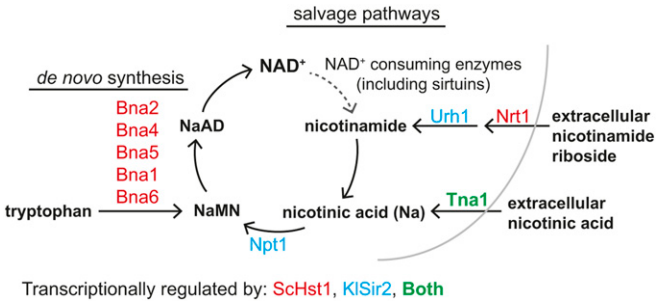

Figure 2 ScHst1 and KlSir2 regulate NAD+ homeostasis through different genes. The NAD+ *de novo* biosynthesis and salvage pathways are shown. Genes regulated by ScHst1 are colored red, genes regulated by KlSir2 are colored blue, and genes regulated by both ScHst1 and KlSir2 are green and bold. *URH1* was only upregulated in the older RNA-Seq data set. NaAD, nicotinic acid adenine dinucleotide; NAD+, nicotinamide adenine dinucleotide; NaMN, nicotinic acid mononucleotide.

identified may represent a known "hyper-ChIP" artifact (Teytelman *et al.* 2013). Therefore, our data support the previous understanding that ScSir2 performs a structural role at chromosome ends and maintains repression at the cryptic mating-type loci but does not regulate the expression of many genes. Consequently, if ScSir2 activity were affected in low NAD+, dramatic changes in gene expression would not be expected.

Genes regulated by ScHst1 function in NAD+ homeostasis, the sexual cycle, and nutrient scavenging

In contrast to ScSir2-regulated genes, ScHst1-regulated genes were distributed throughout the genome. ScHst1 is known to repress genes involved in NAD+ biosynthesis and sporulation (Xie *et al.* 1999; Bedalov *et al.* 2003), and indeed, these genes were well-represented among our 115 ScHst1-regulated genes (Figure 1E and Table S7). In particular, our list included five genes required for *de novo* synthesis of NAD+ and two that encode transporters of NAD+ precursors. It also included 43 genes involved in sporulation, most of which contribute to formation of the prospore membrane or spore wall. Moreover, 76 of the 115 genes (66%) are induced during sporulation (Friedlander *et al.* 2006; Borde *et al.* 2009). Our data are consistent with previous studies, as 84 of our ScHst1-regulated genes (73%) were previously reported to be regulated by Hst1 or its DNA-binding partner Sum1 (Bedalov *et al.* 2003; McCord *et al.* 2003).

We also observed additional groups of functionally related genes that are regulated by ScHst1. For example, six genes are involved in cell fusion during mating and are associated with the shmoo tip, four genes encode hexose transporters, two genes are involved in allantoin degradation, two genes are involved in thiamine biosynthesis (Li *et al.* 2010), and two genes are involved in pyridoxine biosynthesis. Thus, if low NAD+ triggers the induction of ScHst1-regulated genes, *S. cerevisiae* would be expected to respond by increasing the synthesis of NAD+ and other cofactors, scavenging for nutrients, and inducing genes required for mating and sporulation.

Genes regulated by KlSir2 have additional functions not observed in S. cerevisiae

The functions of most KlSir2-regulated genes had to be inferred from their *S. cerevisiae* orthologs because few of these genes have been studied experimentally in *K. lactis*. We found that some KlSir2-regulated genes have similar functions to ScHst1-regulated genes. In particular, two genes are involved in NAD+ homeostasis, 36 genes are involved in sporulation, nine genes are involved in mating, two genes are involved in allantoin metabolism, one gene is involved in thiamine biosynthesis, and one gene is involved in pyridoxine biosynthesis (Table S8). Thus, Sir2 has continued to regulate these biological processes over evolutionary time. However, we also found that a number of KlSir2-regulated genes were functionally distinct from ScHst1-regulated genes (Figure 1E). For example, KlSir2 regulates 10 genes involved in metabolizing non-glucose carbon sources, four genes involved in DNA synthesis, and seven stress-response genes that mitigate the effects of arsenate, oxidative stress, and DNA damage. Thus, if low NAD+ triggers the induction of KlSir2-regulated genes, *K. lactis* would be expected to respond not only by increasing the level of NAD+ and by facilitating mating and sporulation, but also by mounting responses that buffer against stresses.

In *K. lactis*, the nonduplicated KlSir2 displays properties similar to both of its *S. cerevisiae* paralogs ScSir2 and ScHst1 (Hickman and Rusche 2009). Therefore, we determined whether KlSir2-regulated genes are repressed in a manner similar to ScSir2, which acts with Sir4, or ScHst1, which acts with Sum1. Of the 175 KlSir2-regulated genes, 148 (84%) are repressed through a ScHst1-like mechanism, based on their increase in expression in the absence of KlSum1 (Table S8). However, only four are repressed by a ScSir2-like mechanism based on their increase in expression in the absence of KlSir4. Moreover, expression of KlSir2-regulated genes was correlated in *sir2*Δ and *sum1*Δ cells, but not in *sir2*Δ and *sir4*Δ cells (Figure S5). Therefore, most KlSir2-regulated genes are regulated by the SUM1 complex, indicating that it is appropriate to compare these genes with those regulated by ScHst1.

The gene sets regulated by ScHst1 and KlSir2 had modest overlap

Given the functional overlap between the gene sets regulated by ScHst1 and KlSir2, it might be expected that the same genes in these functional categories would be regulated by these deacetylases in both species. However, only 29 genes were regulated in both species (Figure 1D), representing 16.5% of KlSir2-regulated genes and 24% of ScHst1-regulated genes. These genes included 17 genes involved in sporulation, one gene involved in mating, and one gene involved in NAD+ homeostasis. Thus, many of these common genes do participate in the biological processes that are regulated in both species. Nevertheless, many other genes associated with these processes are only regulated by Sir2 or Hst1 in one of the two

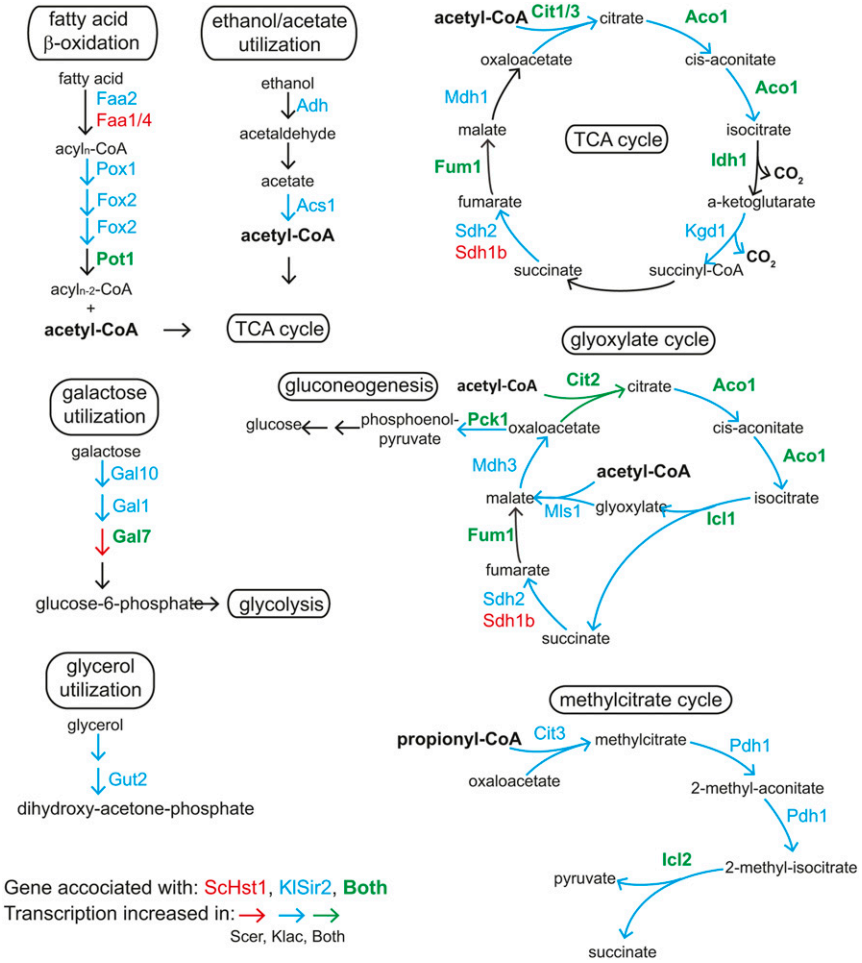

Figure 3 Metabolic pathways regulated by ScHst1 and KlSir2. Regulation by ScHst1 and KlSir2 was evaluated for each gene in metabolic pathways of interest. Gene names are colored red for association with ScHst1, blue for association with KlSir2, and green for association with both sirtuins. Arrows are colored red if the gene increased in *hst1*Δ *sir2*Δ compared to wild-type *S. cerevisiae*, blue if the gene increased in *sir2*Δ compared to wild-type *K. lactis*, and green if the gene increased in both species. For *K. lactis*, induction was evaluated using both RNA-Seq data sets. The genes evaluated are listed in Table S10. Galactose is converted to glucose-6-phosphate and then enters glycolysis. Glycerol is converted to dihydroxy-acetone-phosphate, an intermediate in glycolysis and gluconeogenesis. Non-fermentable carbon sources, including fatty acids, ethanol, and acetate, are metabolized to acetyl-CoA via the fatty acid β-oxidation and ethanol/acetate utilization pathways. The acetyl-CoA then feeds into the TCA cycle to produce energy or the glyoxylate cycle and gluconeogenesis to produce glucose. Fatty acids with odd numbers of carbons also generate propionyl-CoA, which is metabolized via the methylcitrate cycle.

species. This finding is consistent with a model in which the targets of the SUM1 complex shift over evolutionary time.

We considered whether some regulated genes were missed (false negatives), causing the overlap to appear smaller than it actually is. To evaluate this possibility, we postulated that false negatives would meet one, but not both, of the criteria used to call regulation (association with the sirtuin and increased expression in the absence of the sirtuin). For each regulated gene, we determined whether its ortholog in the other species met both, one, or neither of the criteria (Figure S6). This analysis revealed some genes that could be false negatives. Nevertheless, 67% of KlSir2-regulated genes and 49.5% of ScHst1-regulated genes are unlikely to be regulated in the other species, either because the ortholog met neither criterion or because there is no ortholog.

ScHst1 and KlSir2 regulate NAD⁺ homeostasis through different genes

The regulation of NAD$^+$ biosynthesis by ScHst1 has been described as a feedback loop (Bedalov *et al.* 2003). In particular, a drop in intracellular NAD$^+$ levels would reduce the activity of the NAD$^+$-dependent deacetylase ScHst1, relieving its repression of genes that boost NAD$^+$ levels. These genes include those involved in *de novo* NAD$^+$ biosynthesis (*BNA* genes) and the import of NAD$^+$ precursors (*TNA1* and *NRT1*). We also observed that these genes are regulated by ScHst1, and we found that a similar feedback loop exists for KlSir2 (Figure 2 and Tables S7 and S8). However, only one gene, the transporter *TNA1*, was regulated in both species (Figure S3). *K. lactis* lacks the genes for *de novo* biosynthesis, precluding them from regulation by KlSir2. In addition, *K. lactis* lacks a unique transporter for the NAD$^+$ precursor nicotinamide riboside. A single *K. lactis* gene, KLLA0D00550g, is related to three paralogous *S. cerevisiae* transporters, including the nicotinamide riboside transporter *NRT1* and two thiamine transporters. This arrangement may account for KlSir2 instead regulating enzymes that process NAD$^+$ precursors. In summary, both species use an NAD$^+$-dependent deacetylase as part of a feedback mechanism to maintain NAD$^+$ levels, but the particular genes involved in this circuit are different. This finding suggests that even though the specific targets of Sir2 have shifted over evolutionary time, a role for Sir2 in regulating NAD$^+$ homeostasis has been preserved. This finding also supports the notion that Sir2 and Hst1 are sensors that tune gene expression in response to fluctuations in intracellular NAD$^+$ levels.

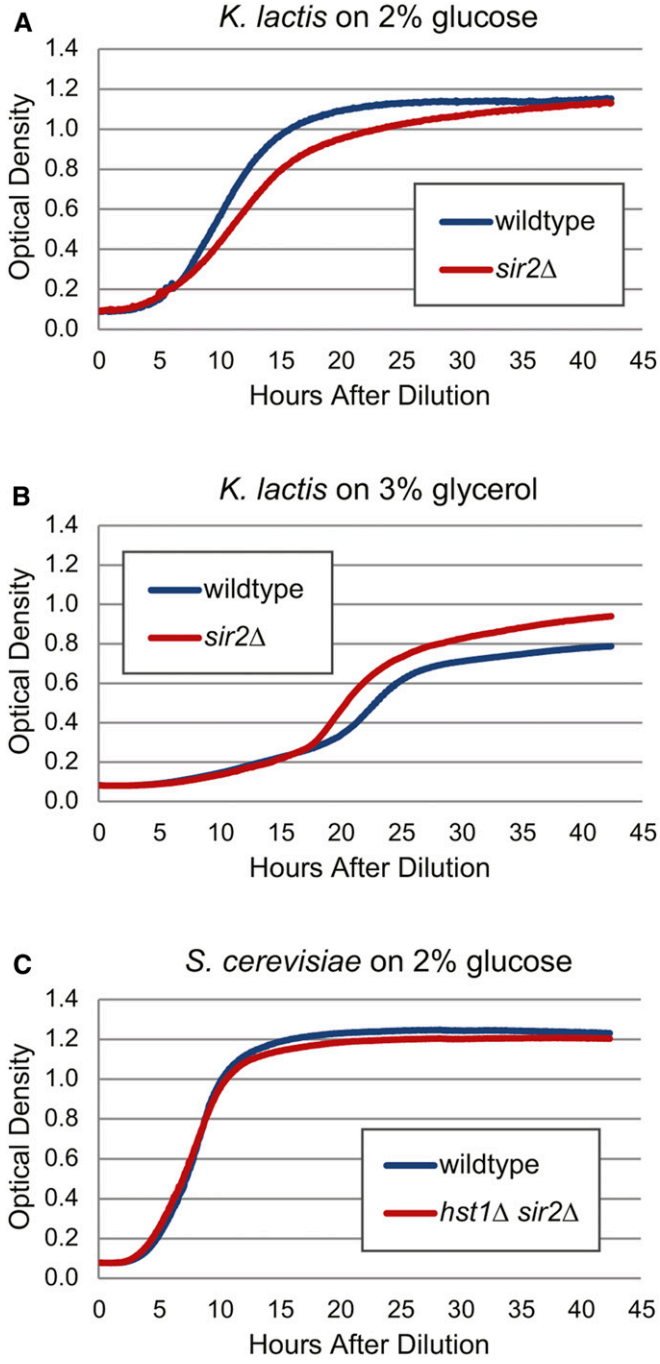

Figure 4 KlSir2 but not ScHst1 influenced growth rate in minimal medium. (A) *K. lactis* cells with or without *SIR2* were grown in minimal medium (YM) with 2% glucose, and the density of the culture was recorded over time using a microplate reader. *K. lactis* strains were *SIR2* (LRY2992) and *sir2*Δ (LRY2993). (B) The same *K. lactis* strains were grown in minimal medium with 3% glycerol. (C) *S. cerevisiae* cells with or without *SIR2* and *HST1* were grown in minimal medium with 2% glucose. The *S. cerevisiae* cells did not grow in minimal medium with 3% glycerol. *S. cerevisiae* strains were *HST1 SIR2* (LRY3093) and *hst1*Δ *sir2*Δ (LRY3099).

KlSir2 regulates genes involved in utilization of carbon sources other than glucose

We observed that genes involved in carbon metabolism were regulated by both ScHst1 and KlSir2 (Figure 3 and Tables S7–S9). Many of these genes facilitate growth in the absence of glucose. For example, some genes metabolize sugars other than glucose, such as galactose or lactose. Other genes metabolize nonsugars, such as ethanol, glycerol, fatty acids, or amino acids. We also observed regulation of genes involved in the TCA cycle and the related glyoxylate and methyl citrate cycles. The TCA cycle is required for aerobic respiration and is an alternative to fermentation, the preferred way for *S. cerevisiae* to process glucose. The methylcitrate cycle is a variation of the TCA cycle in which the three-carbon compound propionyl-CoA is metabolized in place of the two-carbon compound acetyl-CoA. This cycle enables metabolism of propionate and fatty acids with odd numbers of carbons. The glyoxylate cycle is also a variation of the TCA cycle, in which the steps that produce CO_2 are bypassed. Instead, the carbon atoms are shunted into gluconeogenesis, allowing a cell to build sugars from acetyl groups. Doing so is necessary for synthesizing nucleotides and cell wall carbohydrates when sugars are not available in the environment.

To assess the extent to which metabolic pathways are regulated by ScHst1 and KlSir2, we scored all the genes in each pathway of interest for the association of ScHst1 or KlSir2 and for induction in the absence of the deacetylase (Figure 3 and Table S10). We found some pathways, such as the TCA and glyoxylate cycles, have the potential to be regulated in both species. In contrast, other pathways are regulated by KlSir2 but not ScHst1, including the methylcitrate, fatty acid β-oxidation, glycerol utilization, and ethanol/acetate utilization pathways. We confirmed the regulation of several of these genes, including *POX1*, which is involved in fatty acid oxidation (Figure S3). Thus, in *K. lactis* a drop in intracellular NAD^+ that compromises KlSir2 activity would lead to the increased expression of genes required to utilize nonsugar carbon sources.

Given that metabolic flux in *K. lactis sir2*Δ cells might be shifted away from glucose consumption, we compared the growth of wild-type and *sir2*Δ strains in glucose and glycerol. In glucose, *K. lactis sir2*Δ cells grew more slowly than wild-type cells (Figure 4A). In contrast, in *S. cerevisiae*, loss of Sir2 and Hst1 did not affect growth in glucose (Figure 4C). Interestingly, the *K. lactis sir2*Δ cells actually grew faster than wild-type cells in glycerol (Figure 4B). For *S. cerevisiae*, growth did not occur in minimal medium with 3% glycerol. These results are consistent with the model that KlSir2 promotes the ability of *K. lactis* cells to utilize glucose efficiently by damping down pathways that favor other carbon sources. Such repression is presumably relieved in low NAD^+.

KlSir2 affects resistance to arsenate

Several genes regulated by KlSir2 are involved in stress responses (Table S8), including three genes responsible for removing the toxic metalloid arsenic from cells. *ARR2* encodes an arsenate reductase, which reduces arsenate to arsenite, and *ARR3* encodes an antiporter that exports arsenite out of the cell. *ARR2* and *ARR3* are regulated by KlSir2, but not by ScHst1 or ScSir2 (Tables S7 and S8). Indeed, we

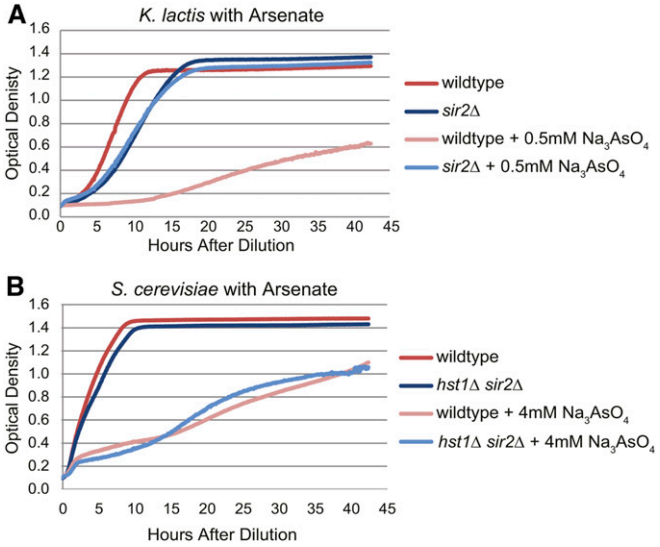

Figure 5 KlSir2 but not ScHst1 influenced growth on arsenic. (A) *K. lactis* cells with and without *SIR2* were grown in rich medium (YPD) alone or with 0.5 mM sodium arsenate, and the density of the culture was recorded over time using a microplate reader. The same strains were used as for Figure 4. (B) *S. cerevisiae* cells were grown in YPD alone or with 4 mM sodium arsenate, as described for A.

confirmed that *ARR3* is regulated by KlSir2, but not ScHst1 (Figure S3). To test whether the presence of Sir2 or Hst1 affects resistance to arsenicals, we grew wild-type and mutant cells on YPD containing sodium arsenate. We found that growth of wild-type *K. lactis* cells was severely reduced in sodium arsenate, whereas *sir2*Δ cells grew similarly in the presence or absence of sodium arsenate (Figure 5A). Note that in this medium containing glucose, the *sir2*Δ cells grew more slowly than wild-type cells, consistent with our findings in Figure 4. In contrast to *K. lactis*, wild-type and *sir2*Δ *hst1*Δ *S. cerevisiae* cells were similarly affected by sodium arsenate (Figure 5B). Thus, KlSir2 impedes the ability of *K. lactis* cells to detoxify arsenate, but ScHst1 and ScSir2 do not act similarly in *S. cerevisiae*.

Genes regulated by ScHst1 and KlSir2 are less likely than other genes to be evolutionarily conserved

In addition to the conserved genes described above that are sirtuin-regulated in *K. lactis* but not *S. cerevisiae*, we found that a disproportionately high number of regulated genes only occur in one of the two species. To identify orthologous genes, we used a two-way BLASTP procedure described in the methods. This approach allowed us to assign *S. cerevisiae* orthologs for 103 of the 175 KlSir2-regulated genes. Of the remaining genes, 56 had no significant BLASTP hit in *S. cerevisiae*. Another 16 had a hit but the two-way BLASTP search returned to a different *K. lactis* gene that had a much higher score than the starting gene, indicating that the *S. cerevisiae* hit is actually the ortholog of a different *K. lactis* gene. Thus, we identified *S. cerevisiae* orthologs for just 103 (59%) of the genes regulated by KlSir2 [Manual refinement ultimately identified orthologs for 109 (62%) genes]. In contrast, for the genome as a whole, the same analysis identified *S. cerevisiae* orthologs for 90% of all *K. lactis* genes. To estimate the statistical probability that a random set of genes would deviate so much from the genome-wide percentage, we generated 10,000 random sets of 175 *K. lactis* genes and recorded the percentage of genes in each set that had *S. cerevisiae* orthologs. As expected, the results are distributed around the genome-wide value of 90% (Figure 6A). Importantly, none of the 10,000 trials resulted in a percentage close to 59%, indicating that random chance does not explain the low percentage of KlSir2-regulated genes with *S. cerevisiae* orthologs. We extended this analysis to other species and found that KlSir2-regulated genes are also less likely to have orthologs in eight other fungal species (Figure 6B). Therefore the set of genes regulated by KlSir2 is enriched for genes that are not widely conserved. We also found the same trend for ScHst1-regulated genes, with regulated genes having a lower percentage of orthologs in other species compared to total *S. cerevisiae* genes (Figure 6C). Thus, genes regulated by Sir2 and Hst1 are more likely to be species-restricted than the average gene. This observation is consistent with the model that sirtuins regulate genes that result in species-appropriate responses to low NAD^+.

KlSir2 regulates synthesis of pulcherrimin, an iron-chelating compound not produced by most yeast

Two KlSir2-regulated genes that are not found in *S. cerevisiae* or many other yeasts are *PUL1* and *PUL2*. Together, Pul1 and Pul2 synthesize the secreted siderophore pulcherriminic acid, which chelates iron(III) to form a red-colored compound pulcherrimin (Krause *et al.* 2018). *K. lactis* scavenges iron by importing pulcherrimin via a specific transporter, Pul3. It is speculated that microbes that use pulcherrimin to sequester iron gain an advantage over neighboring microbes that lack the transporter (Sipiczki 2006; Oro *et al.* 2014; Krause *et al.* 2018). We confirmed that *PUL2* is regulated by KlSir2 (Figure S3). To determine whether KlSir2 influences the production of pulcherrimin, we spotted wild-type and *sir2*Δ cells on rich medium (YPD) supplemented with $FeCl_3$ (Figure 7A). After 2 days, we observed a red halo surrounding the *K. lactis* cells. This halo was likely due to pulcherrimin, as it did not form on plates not supplemented with $FeCl_3$. Moreover, it did not form around *S. cerevisiae* cells, which lack *PUL1* and *PUL2*. Importantly, in *sir2*Δ *K. lactis* cells, the red halo was more intense in color, consistent with KlSir2 suppressing the production of pulcherrimin.

Next, we investigated whether the availability of NAD^+ influences pulcherrimin production. When intracellular NAD^+ is low, Sir2 is predicted to be less active, permitting expression of target genes, such as *PUL1* and *PUL2*. We spotted *K. lactis* cells on minimal medium (YM) prepared with varying concentrations of nicotinic acid, a precursor of NAD^+ (Figure 2). Because *K. lactis* cannot synthesize NAD^+ *de novo*, lowering nicotinic acid should decrease the intracellular concentration of NAD^+, leading to increased expression of *PUL1* and *PUL2* and more pulcherrimin production. Indeed, wild-

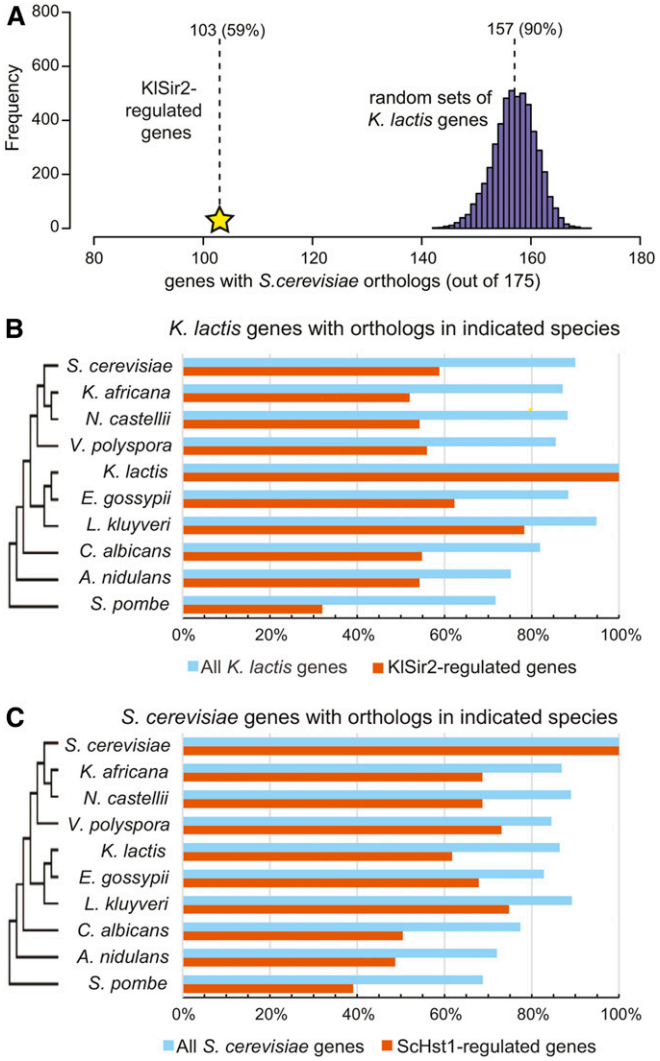

Figure 6 Genes regulated by ScHst1 and KlSir2 were less likely than other genes to be widely conserved. (A) A total of 10,000 sets of 175 randomly selected *K. lactis* genes were evaluated for the number of genes with *S. cerevisiae* orthologs. The results were distributed ~157 genes (90%). For the 175 KlSir2-regulated genes, 103 (59%) had *S. cerevisiae* orthologs. This number is well outside the distribution for the randomly selected genes. (B) For *K. lactis*, the percentage of genes with orthologs in each of nine fungal species is graphed for the 5076 total genes (blue) and the 175 KlSir2-regulated genes (red). The cladogram represents the relative relationships of the species *Kazachstania africana*, *Naumovozyma castellii*, *Vanderwaltozyma polyspora*, *Eremothecium gossypii*, *Lachancea kluyveri*, *Candida albicans*, *Aspergillus nidulans*, and *Schizosaccharomyces pombe*. (C) For *S. cerevisiae*, the percentage of genes with orthologs in each of nine species is graphed for the 5906 total genes (blue) and the 115 ScHst1-regulated genes (red).

type cells grown on lower amounts of nicotinic acid had a more intense red halo (Figure 7B). These results are consistent with decreased repression of *PUL1* and *PUL2* in low nicotinic acid.

Loss of Hst1 and Sir2 reduced mating in both S. cerevisiae and K. lactis

We did observe some functional categories of genes that are regulated by both ScHst1 and KlSir2, including genes required for mating. In *S. cerevisiae*, these genes act relatively late in the mating pathway (Table S7). Specifically, four of the six mating genes encode proteins that are localized to the shmoo tip and are involved in cell fusion, another gene is involved in nuclear fusion, and the last damps down pheromone signaling. Similarly, three KlSir2-regulated genes also encode proteins associated with the shmoo tip and cell fusion. However, there are also four KlSir2-regulated genes involved in the earliest steps of mating (Table S8). These include a pheromone (α-factor) and two subunits of the G protein that signals pheromone binding. Given that *K. lactis* delays mating until it encounters nutrient deprivation, an appealing hypothesis is that nutrient deprivation is associated with a drop in intracellular NAD^+, which in turn triggers a reduction of KlSir2 activity and increased expression of mating genes. Thus, KlSir2 could help restrict mating to nutrient poor conditions. In contrast, ScHst1 may not play this role, as *S. cerevisiae* mates in rich nutrient conditions.

To test the hypothesis that KlSir2 hinders mating by repressing mating genes, we developed a quantitative mating assay based on imaging cytometry. This approach was necessary because *K. lactis* mates at a low frequency. For both wild-type and *sir2*Δ strains, we generated *MAT***a** cells that expressed GFP and *MAT*α cells that expressed mCherry. In addition, the cryptic mating-type loci were deleted so *sir2*Δ cells would not be sterile pseudodiploids, and the strains were made prototrophic to eliminate nutritional dependencies that might influence mating. *MAT***a** and *MAT*α haploid cells were grown together on malt extract to induce mating and were then examined using an ImageStream cytometer. Mated zygotes were identified as those cells that were both red and green and had the classic hourglass shape. Consistent with previous studies (Herman and Roman 1966), the efficiency of mating was very low. Nevertheless, zygote formation was significantly lower in the *sir2*Δ strain, compared to the wild type (Figure 8A). This result indicated that although Sir2 does influence the efficiency of mating, the direction of change was opposite to our prediction that increased expression of mating genes in the *sir2*Δ strain would enhance mating.

For comparison, we also examined whether the absence of ScHst1 and ScSir2 influenced the ability of *S. cerevisiae* cells to mate. In this case, it was not necessary to use imaging cytometry because the efficiency of mating in *S. cerevisiae* is much higher. Instead, a known number of haploid cells of one mating type was spread on a lawn of the opposite mating type. Only diploids had the necessary markers to grow on the selective plate, allowing us to determine the fraction of cells that mated. As for *K. lactis*, the silent mating-type loci were deleted so the loss of Sir2 silencing would not lead to a sterile pseudodiploid state. The *sir2*Δ *hst1*Δ strains had slightly reduced mating when the *MAT***a** stain was used as the lawn and a more pronounced decrease in mating when the *MAT*α stain was used as the lawn (Figure 8B). Thus, the loss of ScSir2 and ScHst1 reduced mating in *S. cerevisiae*, just as the loss of KlSir2 did in *K. lactis*. Therefore, although only

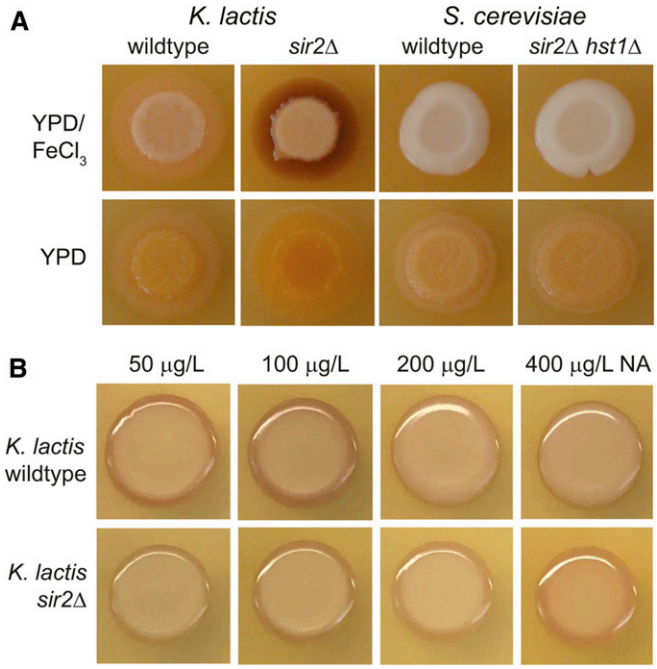

Figure 7 KlSir2 influenced production of the siderophore pulcherrimin. (A) Exponentially growing cells were spotted on rich medium (YPD) with or without 3.7 mM $FeCl_3$. The plates were incubated 2 days and then imaged. The same strains were used as for Figure 4. (B) The same *K. lactis* strains were spotted on minimal medium (YM) prepared with various concentrations of nicotinic acid (NA), a precursor of NAD^+. The standard concentration of nicotinic acid in yeast minimal medium is 400 μg/liter.

KlSir2 regulates early mating genes, ScHst1 and KlSir2 both affect the mating process similarly. We speculate that the timing of mating events may be perturbed in the absence of ScHst1 or KlSir2.

Loss of Hst1 and Sir2 shortened the time to sporulation in both S. cerevisiae and K. lactis

When diploid yeast cells are starved for nitrogen, they initiate meiosis. The four resulting haploid nuclei are each encased in a special cell wall and become spores. Both ScHst1 and KlSir2 repress sporulation genes (Figure 1E) (Xie *et al.* 1999; Hickman and Rusche 2009). However, different classes of genes are regulated in the two species. In *S. cerevisiae*, 84% (36/43) of ScHst1-regulated sporulation genes are "midsporulation" genes involved in formation of the spore membrane and wall. In contrast, in *K. lactis* only 64% (23 of 36) of KlSir2-regulated genes are involved in this phase of sporulation. Other genes are involved in earlier steps of meiosis, including four genes involved in chromosome pairing and segregation. It is particularly striking that KlSir2 regulates three meiotic transcription factors, including *IME1*, the master inducer of meiosis (Table S8). Based on this observation, we hypothesized that loss of KlSir2 would advance the timing of sporulation in *K. lactis* whereas loss of ScHst1 would not do so in *S. cerevisiae*. To test this hypothesis, diploid cells freshly grown from freezer stocks were placed on sporulation medium. These cells were examined microscopically every 2 hr, and the fraction of tetrads (products of sporulation) was scored. As predicted, we found that at each time point a greater percentage of *K. lactis sir2*Δ cells had sporulated compared to wild-type cells (Figure 8C). Surprisingly, however, we found the same trend for *S. cerevisiae* cells (Figure 8D). Therefore, both KlSir2 and ScHst1 delay sporulation. Although KlSir2 regulates more early-sporulation genes than ScHst1 does, both deacetylases affect the sporulation process similarly.

Discussion

In this study, we examined the hypothesis that Sir2 functions as a transcriptional rewiring point, potentially leading to species-appropriate adaptive responses to conditions that decrease intracellular NAD^+ levels. We compared genes regulated by Sir2 and its paralog Hst1 in two yeast species that diverged over 100 MYA (Shen *et al.* 2018), and we found that some biological processes regulated by these deacetylases are common to both species. Nevertheless, the specific genes that are regulated are distinct, indicating significant plasticity in the targets of Sir2 and Hst1 over evolutionary time. In addition, KlSir2 regulates genes in functional categories not regulated by ScHst1 or ScSir2, such as utilization of nonglucose carbon sources, resistance to arsenate, and production of the siderophore pulcherrimin. These findings indicate that Sir2 can serve as a transcriptional rewiring point.

It is striking that even though *S. cerevisiae* and *K. lactis* have evolved separately for over 100 MY, many of the same biological processes are regulated by Sir2 and Hst1 in the two species. These processes include NAD^+ homeostasis, mating, and sporulation. This finding suggests that the last common ancestor of *S. cerevisiae* and *K. lactis* also employed Sir2 to regulate these processes, and that connecting these processes to NAD^+ levels has remained evolutionarily advantageous. Indeed, there is a clear benefit to regulating NAD^+ homeostasis genes through a feedback loop in which a drop in NAD^+ levels relieves repression of these genes. It may also be advantageous for mating and sporulation to be regulated by an NAD^+-dependent repressor, as these events often occur under low nutrient conditions that could coincide with decreased availability of NAD^+.

It is also striking that different genes involved in the same biological processes are regulated by ScHst1 and KlSir2. For example, only 17 out of 43 ScHst1-regulated sporulation genes (40%) are also regulated in *K. lactis*. This finding could indicate that it is not critical which specific genes within a functional category are regulated. Alternatively, it could indicate nuanced differences in how the two species mount developmental programs such as mating or sporulation. For example, it might be more advantageous for *K. lactis* than *S. cerevisiae* to employ Sir2 to integrate information about NAD^+ levels into the expression of early sporulation genes, including the master regulator *IME1*. If so, there might be particular situations in which fluctuations in NAD^+ levels

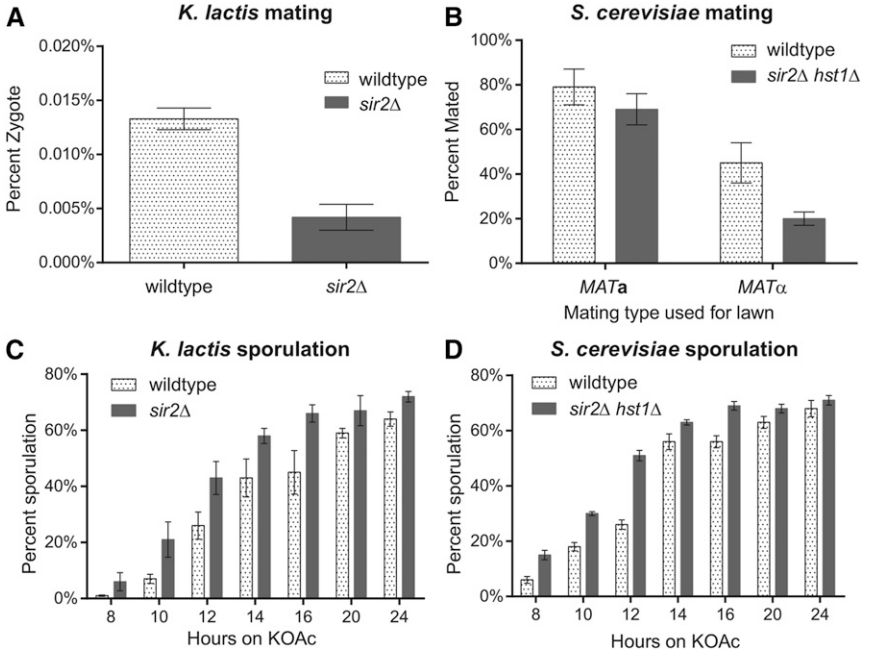

Figure 8 Loss of ScHst1 and KlSir2 decreased mating and increased sporulation. (A) Percentage of observed *K. lactis* cells that were zygotes. *MAT***a** and *MAT*α cells were cocultured on malt extract for 3 days, resuspended in water, sonicated, and then examined using ImageStream cytometry. For each sample, 100,000 events were collected. Cells that were in focus and showed both red and green fluorescence were identified using IDEAS software (EMD Millipore). These cells were then examined manually for an hourglass appearance typical of zygotes. Strains were *MAT***a** *SIR2* mCherry (LRY3037 and 3038), *MAT***a** *sir2*Δ mCherry (LRY3039 and 3040), *MAT*α *SIR2* GFP (LRY3053 and 3054), and *MAT*α *sir2*Δ GFP (LRY3055 and 3056). All strains lacked *HML* and *HMR*. (B) Percentage of *S. cerevisiae* haploids that mated to produce diploids. A lawn of one mating-type was prepared using 2 OD of cells spread on minimal plates supplemented with histidine, tryptophan, and leucine. The other mating type (2×10^{-5} OD) was spread over the lawn to determine the number of cells that could mate. Only diploid cells could grow on these selective plates. The same volume of cells was also spread on rich (YPD) plates to determine the number of viable cells. Strains were *MAT***a** *HST1 SIR2 hml*αΔ (LRY3104), *MAT***a** *hst1*Δ *sir2*Δ *hml*αΔ (LRY3101), *MAT*α *HST1 SIR2 hmr*aΔ (LRY3093), and *MAT*α *hst1*Δ *sir2*Δ *hmr*aΔ (LRY3099). (C) Percent sporulation over time for wild-type and *sir2*Δ *K. lactis* cells. Freshly grown diploid cells were patched onto KOAc sporulation plates and incubated at 30°C. At 2 hr intervals, cells were resuspended in water and examined microscopically. All cells in three fields of vision were scored as either having sporulated (tetrad morphology) or not. The experiment was conducted twice with four biological replicates (diploid strains) each time. Strains used were *SIR2* (LRY3019, 3029-31) and *sir2*Δ (LRY3020, 3032-34). (D) Percent sporulation for wild-type and *hst1*Δ *sir2*Δ *S. cerevisiae* cells. The assay was conducted as described for B. Strains used were *HST1 SIR2* (LRY3108-3111) and *hst1*Δ *sir2*Δ (LRY3112-3115).

would affect mating or sporulation in one species but not the other. Nevertheless, under the conditions we examined, the loss of the Sir2 and Hst1 affected mating and sporulation similarly in both species.

An important finding is that KlSir2 regulates additional genes not associated with the functional categories regulated by ScHst1. Induction of these genes in low NAD^+ might be adaptive for *K. lactis* but not for *S. cerevisiae*. For example, KlSir2 regulates genes that metabolize non-glucose carbon sources. The regulation of these genes in *K. lactis* might relate to its use of respiration rather than fermentation in the presence of oxygen. KlSir2 also regulates genes involved in mitigating the toxicity of arsenicals and synthesizing the siderophore pulcherrimin. Although it is not clear why induction of these genes in low NAD^+ might be advantageous for *K. lactis*, one possibility is that *K. lactis*, which lacks NAD^+ biosynthesis genes, experiences greater fluctuations in NAD^+ levels than *S. cerevisiae*. As a consequence, *K. lactis* may employ NAD^+ as a general signal of suboptimal environmental conditions. Consistent with this idea, a number of stress response genes are regulated by KlSir2, including the *ARR* and *PUL* genes. It is also possible that low NAD^+ coincides with an elevated risk of arsenical exposure. Arsenite is taken up by hexose transporters when there are no competing sugars (Liu *et al.* 2004), a situation that could coincide with low NAD^+. In the case of pulcherrimin, it may be a way to produce NAD^+ from NADH through the reduction of ferric iron, as recently suggested (Sherman *et al.* 2018). Others have found that the pathogenic yeast *C. glabrata* uses Sir2 and Hst1 to regulate genes that favor growth in a mammalian host (De Las Penas *et al.* 2003; Orta-Zavalza *et al.* 2013). These observations suggest that Sir2 does serve as a rewiring point, such that some processes are linked to NAD^+ availability only in certain species.

It is notable that a higher than expected number of Sir2- and Hst1-regulated genes lack orthologs in other fungal species. This observation is consistent with sirtuin deacetylases contributing to the acquisition of distinct responses to low NAD^+. Only 59% of KlSir2-regulated genes have *S. cerevisiae* orthologs, whereas 90% of all *K. lactis* genes do. Similarly, 62% of ScHst1-regulated genes compared to 86% of all *S. cerevisiae* genes have *K. lactis* orthologs. Thus, Sir2- and Hst1-repressed genes are more likely than the average gene to be restricted to a few species. Such genes are likely to provide unique functions, such as siderophore production, to the species in which they reside, and hence their being regulated by Sir2 or Hst1 is consistent with the hypothesis that bringing new processes under the control of sirtuins is associated with organism-specific responses to low NAD^+ stress. In the future, it will be interesting to determine the functions of these species-restricted genes.

An important technical consideration that emerged during this study is that some regulated genes were likely missed by the approach of combining ChIP and gene expression data. In particular, some Sir2- and Hst1-repressed genes might require a transcriptional activator for expression but that activator might not have been available under the standard growth conditions we used. Consequently, such a gene would

not be induced in the absence of the sirtuin repressor. This scenario could account for the larger number of genes that were associated with Sir2 or Hst1 than were induced in the absence of these sirtuins. It is therefore probable that under other growth conditions, additional Sir2- and Hst1-regulated genes could be identified.

In summary, our results are consistent with the hypothesis that sirtuins are rewiring points that allow species to evolve distinct responses to low NAD^+ stress. Because sirtuins require NAD^+ for enzymatic activity, they are hard-wired to respond to fluctuations in intracellular NAD^+ and can be thought of as dedicated rewiring points for making a cellular process sensitive to NAD^+ levels. Bringing new genes under the control of Sir2 or Hst1 enables yeast species to develop patterns of gene expression that elicit an appropriate response to low NAD^+, potentially increasing fitness.

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