

Functions for diverse metabolic activities in heterochromatin

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Growing evidence demonstrates that metabolism and chromatin dynamics are not separate processes but that they functionally intersect in many ways. For example, the lysine biosynthetic enzyme homocitrate synthase was recently shown to have unexpected functions in DNA damage repair, raising the question of whether other amino acid metabolic enzymes participate in chromatin regulation. Using an in silico screen combined with reporter assays, we discovered that a diverse range of metabolic enzymes function in heterochromatin regulation. Extended analysis of the glutamate dehydrogenase 1 (Gdh1) revealed that it regulates silent information regulator complex recruitment to telomeres and ribosomal DNA. Enhanced N-terminal histone H3 proteolysis is observed in *GDH1* mutants, consistent with telomeric silencing defects. A conserved catalytic Asp residue is required for Gdh1's functions in telomeric silencing and H3 clipping. Genetic modulation of α -ketoglutarate levels demonstrates a key regulatory role for this metabolite in telomeric silencing. The metabolic activity of glutamate dehydrogenase thus has important and previously unsuspected roles in regulating chromatin-related processes.

yeast chromatin | metabolism | SIR complex | epigenetic silencing | glutamate dehydrogenase

In eukaryotic nuclei, DNA is wrapped around histones to form nucleosomes, the basic subunits of chromatin (1). The physical and chemical properties of chromatin are regulated by at least two types of enzymatic activities: chromatin remodeling and posttranslational modifications of histones and chromatin-associated proteins (2, 3). These enzymatic activities directly determine the accessibility of DNA for transcription, replication, and repair.

In *Saccharomyces cerevisiae*, three genomic regions are known to contain loci repressed by chromatin-related activities. These include the *HM* silent mating-type loci (*HMR* and *HML*), regions within the ribosomal DNA (rDNA), and some telomeres. Transcriptional silencing at these loci is established and maintained by a number of factors, including the Sirtuin class III deacetylase activity of silent information protein 2 (Sir2). Notably, Sir2 uses NAD^+ as a cofactor to deacetylate histones H3 and H4 in newly deposited nucleosomes. Other than Sir2, the composition of the silencing complexes and the mechanisms of silencing are different for the three silenced regions, with the telomeres and the *HM* loci more similar to each other and the rDNA locus having distinct features (reviewed in ref. 4). Silencing at telomeres and the *HM* loci was long considered to follow a sequential model: initial deacetylation by Sir2 creates binding sites for Sir3 and Sir4, which in turn regulate the spreading of Sir2 across these regions (5). More recent high-resolution studies report a less uniform landscape for silenced chromatin (6), although the central importance of the Sir proteins remains clear. They, together with transcription factors including Rap1 and Abf1, form a multisubunit silencing complex known as the silent information regulator (SIR) complex. Silencing at the rDNA locus does not involve the SIR complex but rather requires the regulator of nucleolar silencing and telophase exit (RENT) complex, which consists of Sir2, Net1, and Cdc14 (4).

A growing field in the study of chromatin is the intersection of epigenetic and chromatin dynamics with cellular metabolic processes (7–9). Although most metabolic proteins localize to the cytoplasm, a number of metabolic proteins are found in the nucleus, where they can regulate chromatin dynamics through at least two distinct mechanisms. In one, the metabolic activities of these proteins modulate the levels of substrates or cofactors available to chromatin-modifying enzymes. For instance, the acetyl-CoA synthesizing machinery in both yeast and mammalian cells exists in multiple cellular compartments, and its metabolic activity in the nucleus directly determines the amount of acetyl-CoA available as a cofactor available for lysine acetyltransferases (10, 11).

In an alternative mechanism, metabolic proteins have evolved distinct nuclear functions. For example, the mammalian pyruvate kinase PKM2 is translocated to the nucleus when the EGF receptor is activated, where it phosphorylates histone H3 instead of its usual metabolic substrate (12). A second example is the yeast homocitrate synthase, encoded by the genes *LYS20* and *LYS21*. This protein is constitutively localized to the nucleus (13). It acts as a dosage suppressor of the DNA damage sensitivity of *esa1-414*, a hypomorphic allele of the essential lysine acetyltransferase encoded by *ESAI* (14). The nuclear function of Lys20 is independent of its lysine biosynthetic activity but requires a separate domain that facilitates the recruitment of the INO80 complex to DNA damage sites (14, 15). Lys20 is thus defined as a moonlighting protein (16, 17) because of its two distinct functions.

Significance

Eukaryotic genomes have distinct heterochromatic regions that silence gene transcription and other DNA transactions. In yeast, these regions are controlled by activities of the silent information regulator (SIR) complex, which are influenced by NAD^+ metabolism. We report results, starting from an in silico screen, that proteins functioning in a diverse range of amino acid metabolic activities influence chromatin silencing, thus expanding knowledge of connections between metabolism and epigenetic processes. In a case study, we found that glutamate dehydrogenase 1 (Gdh1) has two chromatin-based functions: controlling recruitment of the SIR complex and negatively regulating proteolysis of a conserved core histone to modulate gene expression. Mechanistically, Gdh1 contributes to silencing through α -ketoglutarate consumption, and high levels of α -ketoglutarate are shown to be detrimental to telomeric silencing.

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The findings with Lys20 prompted us to ask if other nuclear amino acid metabolic proteins function in chromatin regulation. This was an attractive possibility for two reasons. First, many amino acid metabolic reactions consume or produce metabolites, such as NAD⁺ and acetyl-CoA, that influence the activities of chromatin-modifying enzymes by modulating their cofactor pools. Second, amino acid metabolic enzymes are ancient proteins with a rich repertoire of biochemical activities, thereby making them ideal candidates to evolve multiple functions, which is indeed a common feature observed for well-established multifunctional proteins (18). By screening information curated in the *Saccharomyces* Genome Database (19), combined with results from transcriptional silencing reporter assays, we identified a diverse range of candidate proteins with potential functions in chromatin regulation. Among these, we focused on the role of glutamate dehydrogenase 1 (Gdh1) in the regulation of telomeric silencing. We report that α -ketoglutarate is an important regulatory metabolite

for telomeric silencing and that Gdh1 regulates histone H3 clipping and binding of the SIR complex to the telomeres.

Results

A Screen Revealed Diverse Amino Acid Metabolic Enzymes with Chromatin Functions. To discover additional amino acid metabolic proteins with chromatin functions, we designed an in silico screen as outlined in Fig. 1A. The screen began with a search of the yeast proteome for proteins annotated to participate in amino acid metabolism. Next, we identified proteins with reported nuclear pools (Tables S1 and S2). Transcription factors were excluded because their nuclear functions have been established. Finally, to increase the likelihood of finding candidates with chromatin regulatory functions, we selected candidates based on three additional criteria: First, they must have interactions with chromatin regulators as reported in the *Saccharomyces* Genome Database (19). Second, their metabolic function must directly involve a

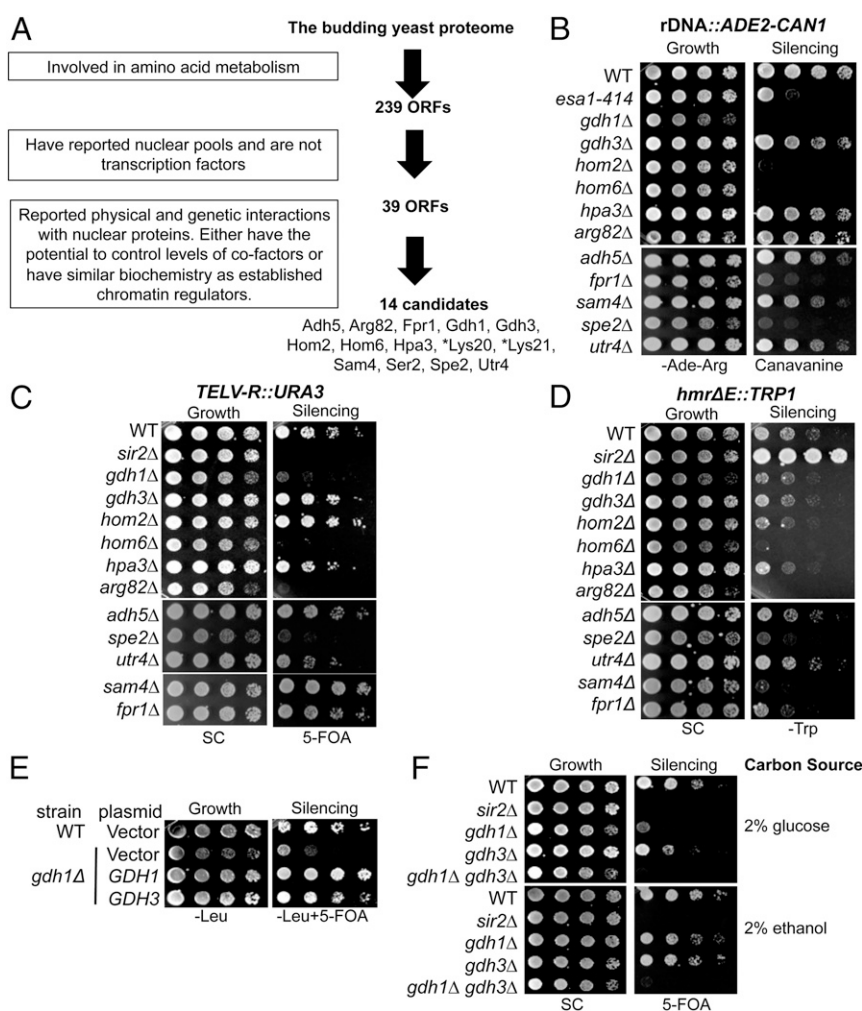


Fig. 1. A number of amino acid metabolic proteins regulate chromatin silencing. (A) The in silico screen identified 14 proteins that potentially function in chromatin regulation. The search was performed using the Advanced Search Engine in the *Saccharomyces* Genome Database: yeastmine.yeastgenome.org/yeastmine/begin.do. Search criteria for each step were boxed at Left. (B–D) Strains carrying triple silencing reporters (Table S3) were plated on the indicated media to assess chromatin silencing. The *esa1-414* and *sir2Δ* strains served as controls with established silencing defects. (B) Deletion of *FPR1*, *GDH1*, *HOM2*, *HOM6*, and *SPE2* caused defects in rDNA reporter silencing. Decreased growth on canavanine indicates defective rDNA silencing. (C) Deletion of *ARG82*, *GDH1*, *HOM6*, and *SPE2* caused defective telomeric silencing, whereas deletion of *SAM4* improved silencing. Reduced growth on 5-FOA indicates defective telomeric silencing. (D) Deletion of *ARG82*, *HOM6*, *SAM4*, and *SPE2* enhanced *HMR* silencing. Reduced growth on SC-Trp indicates enhanced *HMR* silencing. (E) Increased dosage of *GDH3* suppressed *gdh1Δ*'s silencing defect. WT and *gdh1Δ* strains were transformed with 2- μ plasmids: vector (pRS425), *GDH1* (pLP2764), or *GDH3* (pLP2662). (F) *GDH1* and *GDH3* single and double mutants showed different telomeric silencing phenotypes on glucose and ethanol-containing media. WT (LPY4916), *sir2Δ* (LPY4979), *gdh1Δ* (LPY16033), *gdh3Δ* (LPY16785), and *gdh1Δ gdh3Δ* (LPY17916) strains were plated on 5-FOA with the indicated carbon sources. **Lys20* and *Lys21* were identified in the screen but are not analyzed further here because of their previous characterization (14).

metabolite needed for chromatin-modifying enzymes. The metabolites considered included acetyl-CoA, S-adenosylmethionine, α -ketoglutarate, and NAD(P)⁺, which are cofactors for histone acetyltransferases, histone methyltransferases, JMJC-domain-containing histone demethylases, and class III histone deacetylase, respectively (20). Alternatively, metabolic enzymes with similar catalytic activities as those established for chromatin-modifying enzymes were selected, because they might act on chromatin substrates through catalytic promiscuity (21).

The screen identified 14 candidates with potential functions in chromatin regulation (Fig. 1A). Notably, the screen recovered not only Lys20 and its homolog Lys21 but also Arg82 (also known as Ipk2), another established moonlighting protein (22, 23). Therefore, the screen served as a promising approach for discovering candidate metabolic proteins with potential chromatin regulatory functions. For the new candidate proteins, the metabolic function, the rationale of the selection, and examples of nuclear proteins that they interact with are briefly summarized in Table S1.

To assess the candidates' roles in chromatin function, we took advantage of a yeast strain in which reporter genes were integrated at three silenced regions (24). Of note, we grew the null strains in synthetic complete (SC) medium throughout this study, because SC is made with defined nitrogen sources (*Materials and Methods*) and is likely to introduce fewer experimental variables than rich medium for analyzing strains deleted for amino acid metabolic genes. We did not include Lys20 and Lys21 in this analysis because their roles in silencing have been examined previously (25), and we were unable to assess the silencing function of Ser2 because of the poor growth of the null strain in our SC medium.

We first assessed rDNA silencing in strains deleted for each of the remaining 11 candidate genes. The rDNA reporter is an *ADE2-CAN1* cassette inserted at the 25S transcription unit. *CAN1* encodes a plasma membrane permease that imports arginine. Natural silencing within the rDNA locus represses *CAN1* expression, thus blocking the import of the toxic arginine analog canavanine (Fig. 1B). Mutants with rDNA silencing defects, for example *esa1-414* (26), are sensitive to canavanine, because elevated import and incorporation of canavanine compromise protein functions. Strains deleted for *FPR1*, *GDH1*, *HOM2*, *HOM6*, and *SPE2* strains were sensitive to canavanine, suggesting that these genes may normally promote rDNA silencing (Fig. 1B).

The reporter strain also has the *URA3* gene inserted on the right arm of telomere V (*TELVR*). Cells expressing *URA3* are sensitive to 5-fluoroorotic acid (5-FOA). Natural silencing at *TELVR* represses *URA3* transcription, allowing cells to grow on 5-FOA. Defective silencing, such as that caused by *SIR2* deletion, results in cell death on 5-FOA (27). Deletion of *ARG82*, *GDH1*, *HOM6*, and *SPE2* resulted in varying degrees of 5-FOA sensitivity (Fig. 1C), suggesting that their gene products normally promote telomeric silencing. In contrast, deletion of *SAM4* improved growth on 5-FOA, suggesting an inhibitory role of Sam4 in telomeric silencing. Notably, unlike the *SIR2* deletion mutant that showed complete death on 5-FOA, deleting *GDH1* and *SPE2* led to small colony size and delayed growth on 5-FOA (Fig. 1C). This phenotype is similar to that observed upon deletion of *CAC1*, which encodes a component of the CAF1 chromatin assembly complex (28–30).

For monitoring silent mating-type control, the reporter strain carries the *TRP1* gene in the *HMR* silent mating-type locus. Natural silencing of *HMR* represses *TRP1* transcription, causing poor growth on medium lacking tryptophan. Loss of silencing in the *SIR2* deletion strain improved growth on SC-Trp (27). In contrast, deletion of *ARG82*, *HOM6*, *SAM4*, and *SPE2* worsened growth on SC-Trp (Fig. 1D), suggesting that *HMR* silencing is enhanced in these mutants. The phenotype for the *ARG82* mutant was unexpected because it was reported to have a mating defect (31), whereas enhanced *HM* silencing usually results in a higher mating efficiency. Nonetheless, it is known that deletion

of *PLC1*, which acts upstream of *ARG82*, similarly enhances *HMR* silencing when deleted (32). Therefore, Arg82 is likely to have a complex role in the regulation of mating-type control.

The divergent results for silencing for *GDH1* and *GDH3* upon first consideration might be surprising, as the two are paralogs (33), with the two proteins more than 90% similar in amino acid sequence. However, the genes are differentially regulated by carbon sources: Gdh1 is expressed and active in medium containing either glucose or ethanol, whereas Gdh3 is only detectable and active with ethanol as a carbon source (34). We speculate that no defect in its telomeric silencing was observed upon deletion of *GDH3* because of its low level of expression in glucose. In fact, increased gene dosage of *GDH3* suppressed the telomeric silencing defect of the *gdh1* Δ mutant (Fig. 1E). Also, deletion of either *GDH* gene only caused a mild loss of silencing when grown in ethanol (Fig. 1F), suggesting that the two paralogs may have overlapping functions in telomeric silencing when they are both expressed. Moreover, the *gdh1* Δ *gdh3* Δ double mutant showed defective telomeric silencing in both glucose- and ethanol-containing media (Fig. 1F). Therefore, both proteins contribute to telomeric silencing, with Gdh1 as the primary player when glucose is the carbon source.

Overall, our screen revealed that the deletion of *ARG82*, *FPR1*, *GDH1*, *GDH3*, *HOM2*, *HOM6*, *SAM4*, and *SPE2* altered silencing of integrated reporter genes, whereas the deletion of *ADH5*, *HPA3*, and *UTR4* had no apparent effect. We focused on *GDH1* because it encodes the broadly conserved glutamate dehydrogenase enzyme that lies at the nexus between the citric acid cycle and nitrogen metabolism. Dysregulation of this enzyme has been directly associated with congenital hyperinsulinism (35) and is indirectly implicated in cancer through the glutamine production pathway (36). Therefore, it is of great importance to determine whether GDH has a role in chromatin regulation in addition to or independent of its role in metabolism.

Gdh1 Regulates Recruitment of SIR Proteins to Silent Chromatin and Is Bound to Telomeric Chromatin. Because the silencing reporters assayed in Fig. 1 are metabolic in nature and Gdh1 is a metabolic protein, we further evaluated Gdh1's role in silencing through independent assays. Recent studies showed that 5-FOA-based telomeric silencing assays could give false-positive results for mutants that elevate ribonucleotide reductase (RNR) activities (37, 38). One way to eliminate the confounding effect is to inhibit RNR activity with 10 mM hydroxyurea (HU) (37). Adding HU did not rescue the 5-FOA sensitivity of *gdh1* Δ *TELVR::URA3*, suggesting that the 5-FOA readout is likely a true reflection of *gdh1* Δ 's silencing defect (Fig. 2A).

Next, we assessed Gdh1's impact on the recruitment of the SIR proteins at natural telomeres. Deletion of *GDH1* did not change Sir2 protein levels (Fig. 2B), however it did reduce Sir2 binding at all natural telomeric loci studied (Fig. 2C). In contrast, deletion of *GDH1* did not significantly change Sir2 binding at the *HMR* locus (Fig. 2C), consistent with the result of the *hmr* Δ *E::TRP1* reporter assay. Sir3 binding was also reduced at telomeres (Fig. S1A).

To directly assess the effect of the loss of the SIR complex in the absence of Gdh1, we measured the transcript levels of three genes close to their native telomeres (Fig. 2D). We found increased gene expression in the *GDH1* deletion mutant (Fig. 2D). Although less severe than the effect of deleting *SIR2* (Fig. 2D), these results parallel those of the silencing reporter assay in which the remaining silencing activity is likely to be mediated by the residual presence of the SIR complex at the telomeres. These independent assays collectively support a role for Gdh1 in telomeric silencing through modulating the recruitment of the SIR complex.

In contrast to the silencing effects for telomeres, we found that the rDNA reporter assay was confounded by a silencing-independent effect of Gdh1 on *CAN1*. A *GDH1* deletion mutant with *CAN1* at its endogenous locus was hypersensitive to canavanine (Fig. S2A),

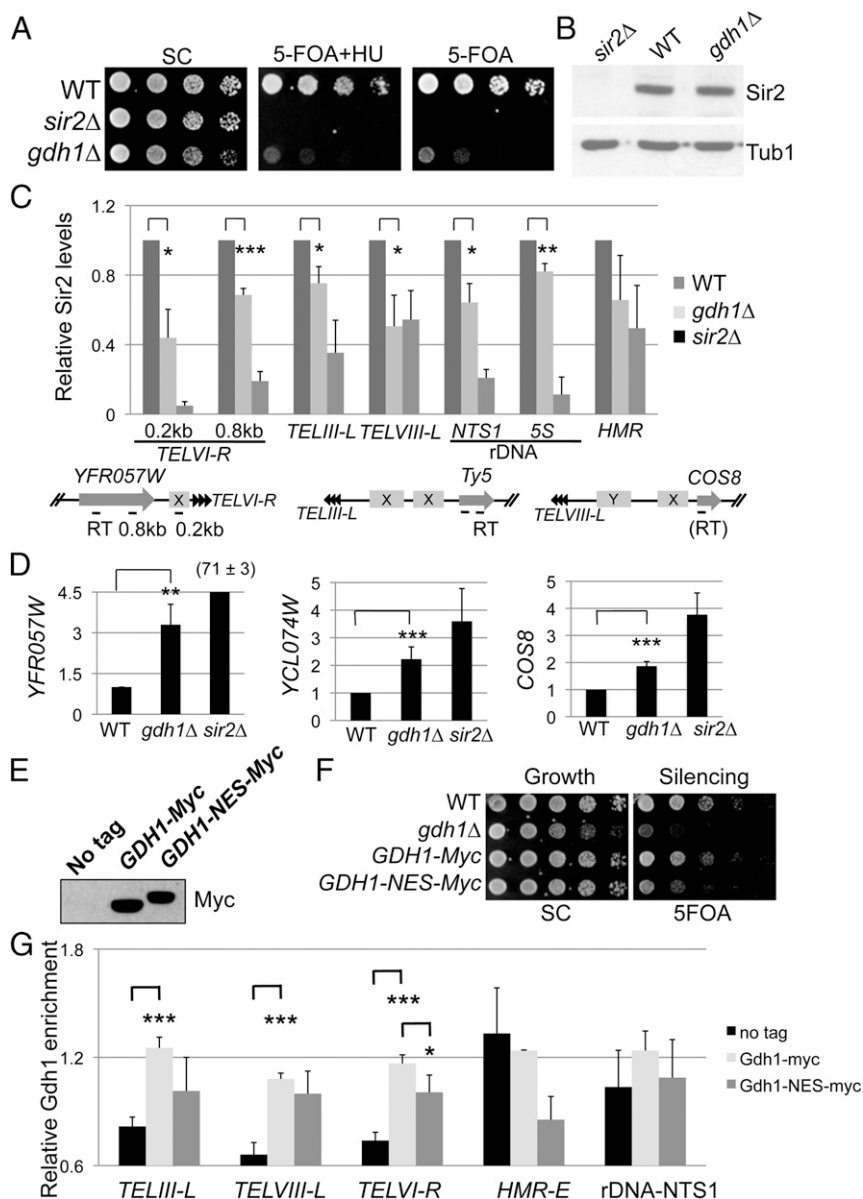


Fig. 2. Independent assays support *GDH1*'s function in telomeric silencing. (A) The *gdh1Δ* silencing phenotype on 5-FOA was not a result of elevated RNR activity. WT (LPY4916), *sir2Δ* (LPY4979), and *gdh1Δ* (LPY16033) strains were assayed on 5-FOA with 10 mM HU, a RNR inhibitor. (B) Sir2 protein levels were unaffected in the *gdh1Δ* mutant. Whole-cell extracts of WT (LPY5), *gdh1Δ* (LPY16026), and *sir2Δ* (LPY11) were immunoblotted with antiserum for Tub1 (loading control) or Sir2. (C) Sir2 binding was significantly reduced in the *gdh1Δ* mutant. Sheared chromatin was prepared from strains in *B*. Approximate positions of the primers used for ChIP analysis are indicated. Subtelomeric structures are indicated, including designations of the X and Y elements (boxed) and the TG_{1-3n} repeats (arrowheads). Sir2 enrichment at each locus was normalized to an established ChrV control locus (76) with WT set to 1 for each experiment. For all experiments of this study, error bars represent SEs and *P* values represent results of one-tailed Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.005. (D) Expression of telomere-proximal transcripts was increased in the *gdh1Δ* mutant. The mRNA from strains in *B* was analyzed by quantitative RT-PCR, using primers indicated as "RT" in *C*. Expression levels were normalized to *ACT1* with the WT value set to 1. (E) Gdh1-Myc and Gdh1-NES-Myc were stably expressed. Whole-cell extracts from no tag (LPY5), *GDH1-Myc* (LPY16784), and *GDH1-NES-Myc* (LPY17738) strains were immunoblotted for Myc. (F) Ectopic integration of an NES at the C terminus of Gdh1 caused a moderate telomeric silencing defect. WT, *gdh1Δ*, *GDH1-Myc* (LPY16782), and *GDH1-NES-Myc* (LPY17736) strains were assayed on 5-FOA for telomeric silencing. (G) Gdh1 is enriched at the telomeres, but the artificial addition of NES did not efficiently or uniformly deplete Gdh1 from the binding sites. No tag (LPY5), *GDH1-Myc* (LPY16784), and *GDH1-NES-myc* (LPY17738) strains were analyzed by ChIP. The signal at each locus was normalized to that at the ChrV control locus.

suggesting that Gdh1 influences canavanine sensitivity outside the context of rDNA. We did observe decreased Sir2 binding at the rDNA *NTS1* spacer and 5S loci (Fig. 2C). However, we did not observe loss of rDNA silencing using alternative silencing assays: The *gdh1Δ* mutant did not show changes in silencing a *URA3* reporter integrated at the *NTS1* spacer region (39) (Fig. S2B), neither did it increase the rDNA recombination rate significantly (40) (Fig. S2C).

We also assessed the effect of deletion of *GDH1* on the *HML* silent mating-type locus. The *gdh1Δ* strain silenced *HML* as efficiently as wild-type cells (Fig. S3A). Quantitative mating analysis showed that deletion of *GDH1* moderately improved mating in the *MATα* strain (Fig. S3B), suggesting that Gdh1 contributes to the regulation of silent mating type.

Because deletion of *GDH1* resulted in phenotypes consistent with contributions to chromatin-based functions and because the

protein has both nuclear and cytoplasmic pools (41, 42), it was important to establish if nuclear localization was relevant for its silencing functions. In ChIP experiments, we found that Myc-tagged Gdh1 (Fig. 2E) is enriched at all three native telomeres tested (Fig. 2G). Because Gdh1 does not have a canonical Nuclear Localization Signal, we added a well-established heterologous Nuclear Export Sequence (NES) (43) to investigate the effect of depleting the nuclear pool of Gdh1 on silencing. This construct is stably expressed (Fig. 2E) and, when provided as the only source of Gdh1 in the telomeric reporter strain, caused reduced telomeric silencing (Fig. 2F). The silencing defect of the Gdh1–NES mutant was not as strong as that of the *gdh1*Δ mutant. This may be because the endogenous localization mechanism is intact, so the nuclear pool is likely to be diminished but not eliminated. This is consistent with the observation that the artificial addition of NES did not effectively or uniformly deplete Gdh1 from the telomeric sites (Fig. 2G).

Gdh1 Is a Negative Regulator of H3 N-Terminal Clipping in Vivo. Transcriptional silencing and activation are influenced by both the structure and modification of the nucleosomal histones. For example, it is known that the N-terminal tail of histone H3 itself, along with its modifications, can be critical for gene regulation. In classic experiments, deletion of the H3 N terminus was found to aberrantly activate transcription of a number of genes (44). This interesting biological result correlates with the long-standing observation that H3 exists in two pools in vivo, one full-length and the other as a proteolytically cleaved population (45). Histone H3 “clipping” is the term used to denote the process in which the H3 N-terminal tail is

cleaved. Over the years, a number of studies have been performed to characterize clipping activity and to identify the enzyme(s) that may be responsible. Clipping was originally attributed to an unidentified serine protease, and the cleavage site was proposed to be at residue H3-S22 in budding yeast (46).

In more recent studies, glutamate dehydrogenase purified from vertebrate microsomes appeared to catalyze H3 clipping in vitro (47), suggesting a potential moonlighting role for GDH. By contrast, yeast studies showed that Gdh1 was present in a fraction with clipping activity, but whole-cell extracts from *GDH1* mutants did not affect clipping in vitro (48). Because we showed that Gdh1 had a role in regulating chromatin silencing, we asked whether it also functioned in H3 clipping in vivo through both biochemical and genetic approaches.

Nuclear extracts were prepared from log-phase cells followed by immunoblotting to evaluate both intact and clipped H3. The *gdh1*Δ mutant showed increased global H3 clipping (Fig. 3A), suggesting an inhibitory role of Gdh1 on H3 clipping under the conditions tested. The protease inhibited is likely to be a serine protease, because the H3-S22A mutation reduced the amount of clipped H3 in both the WT and the *gdh1*Δ cells (Fig. 3A). Moreover, a ChIP experiment measuring H3 N- and C-terminal signals at each locus showed that Gdh1 also inhibited H3 clipping locally at specific genomic loci, such as regions close to the telomeres but not the rDNA or the *HMR* loci (Fig. 3B).

To evaluate the functional significance of H3 clipping in *GDH1*-mediated silencing, we first evaluated the N-terminal truncation (44), with a histone-shuffle experiment, in which plasmid-borne mutant or wild-type H3 is the only source of that protein in the cell

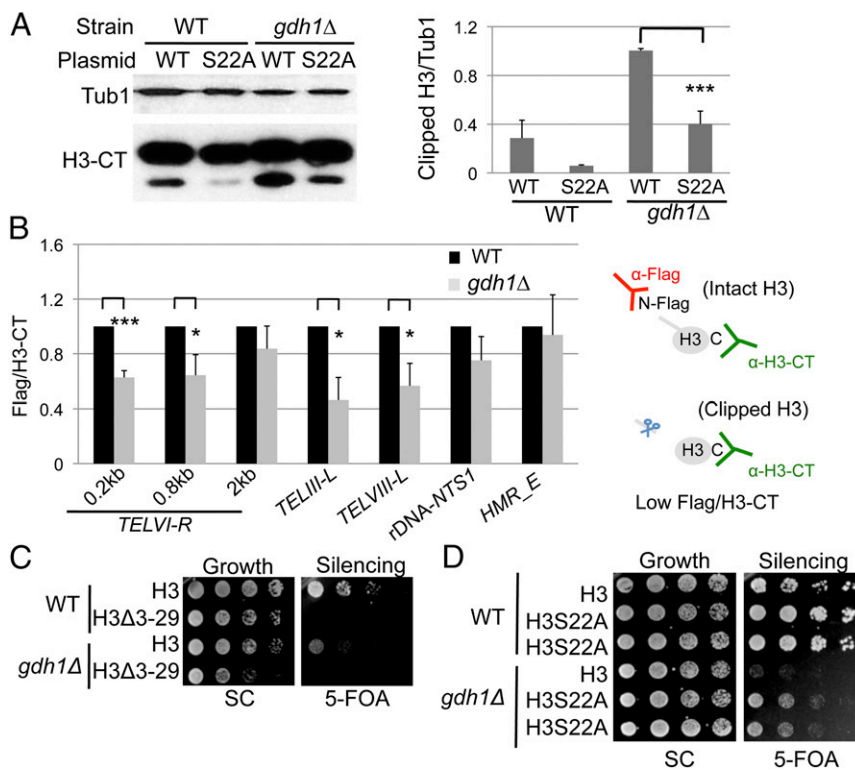


Fig. 3. Gdh1 is a negative regulator of histone H3 N-terminal clipping in budding yeast in vivo. (A) The H3S22A mutation interferes with H3 clipping. Nuclear extracts of strains were prepared from WT and *gdh1*Δ histone shuffle strains (LPY20470, -16155, -20623, and -16162) and blotted anti-H3-CT or anti-Tub1 (loading control). Blots from two sets of independent extracts were quantified. (B) H3 N-terminal clipping was increased at telomeres in the *gdh1*Δ mutant. WT (LPY19789) and *gdh1*Δ (LPY19794) histone shuffle strains carried H3 with an N-terminal Flag tag (pLP2129). Anti-Flag antibody precipitated unclipped H3, whereas anti-H3-CT antibody precipitated both clipped and unclipped H3. Flag and H3-CT signals at each locus were normalized to the respective signals for the control locus *ACT1*. The Flag/H3-CT ratio for the WT strain was set to 1 for each experiment. (C) The H3Δ3–29 mutation was epistatic to the *gdh1*Δ mutant with respect to telomeric silencing. WT and *gdh1*Δ histone shuffle strains (LPY20665, -20695, -20021, and -20023) were assayed on 5-FOA. (D) The H3S22A mutation improved silencing in both the WT and the *gdh1*Δ background. The strains used in A were assayed on 5-FOA.

(49). We found that the genetically clipped version of H3 led to loss of telomeric silencing in both WT and the *GDH1* deletion mutant and also had a modest inhibition of growth in the *GDH1* mutant (Fig. 3C). In an independent histone shuffle, in which the H3-S22A mutant that interfered with clipping was the only source of H3, we observed improved silencing in both WT and the *GDH1* mutant cells (Fig. 3D). These results support the idea that clipped H3 contributes to loss of silencing in the promotion of gene activation, consistent with the defective silencing observed in the *GDH1* deletion mutant.

Gdh1's Chromatin Functions Require Its Metabolic Activity. To determine whether Gdh1 had a distinct moonlighting activity or whether the silencing defects observed for the GDH mutants were intrinsic to the established catalytic activity, we asked if the chromatin function of Gdh1 was dependent on its metabolic activity. The well-defined functions of Gdh1 and Gdh3 in *S. cerevisiae* are in nitrogen assimilation: The enzymes catalyze the synthesis of glutamate from α -ketoglutarate and ammonium. Of

note, budding yeast has an alternative pathway to synthesize glutamate, using the glutamate synthase encoded by *GLT1* (Fig. 4A).

Studies on GDH from *Clostridium symbiosum* reported that the Asp165 residue is required for catalysis because a D165S mutant lost its catalytic activity in vitro without affecting substrate binding (50). Sequence alignment revealed that this residue is conserved in *S. cerevisiae* (Fig. 4A).

We constructed a plasmid-borne *gdh1-D150S* mutant, Myc-tagged and expressed from its endogenous promoter. The mutant protein had stable expression compared with wild type (Fig. 4B), demonstrating that the mutation did not perturb protein stability. The catalytic activity of the *gdh1-D150S* mutant was assessed in vivo by a growth assay. The assay is based on the principle that the *gdh1 Δ gdh3 Δ glt1 Δ* triple mutant strain cannot catalyze the anabolic reactions to synthesize glutamate from ammonium sulfate (Fig. 4A) and thus grows poorly in ammonium sulfate-based minimal medium. Transformation of the triple mutant with wild-type *GDH1* rescued the growth defect, whereas the *D150S* mutant was unable to do so within the same time course (Fig. 4C). Therefore, the *D150S* mutant had lost much of its catalytic activity

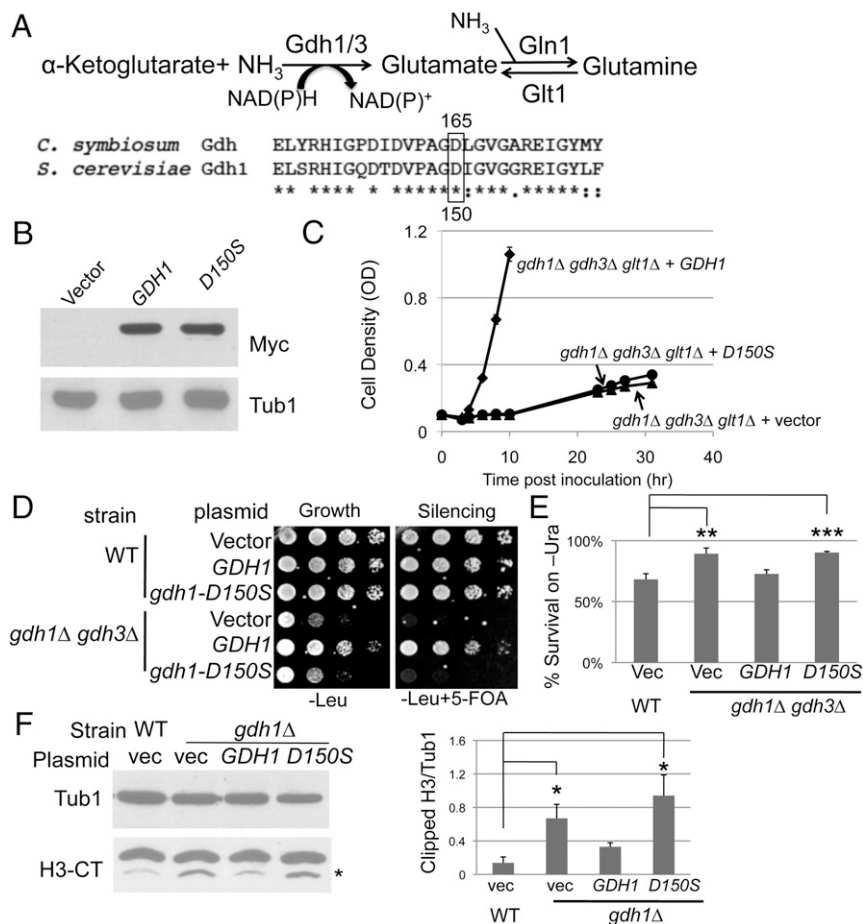


Fig. 4. The metabolic activity of the GDH homologs is important for its chromatin functions. (A) The catalytic Asp residue is conserved in *S. cerevisiae* Gdh1. (Top) The enzymatic reaction catalyzed by the GDH enzymes. (Bottom) Alignment of the *C. symbiosum* GDH with *S. cerevisiae* Gdh1. Boxed is the conserved catalytic Asp residue at position 165 in the *Clostridium* protein. (B) *gdh1-D150S*-13Myc was stably expressed. *gdh1 Δ* (LPY16026) cells were transformed with vector (pRS316), *GDH1*-13Myc (pLP2833), or *gdh1-D150S*-13Myc (pLP2834). Whole-cell extracts were immunoblotted for Myc or Tub1 (loading control). (C) The *gdh1-D150S* mutant had diminished catalytic activity required to assimilate ammonium. The *gdh1 Δ gdh3 Δ glt1 Δ* strain (LPY17131) was transformed with vector (pRS315), *GDH1* (pLP2631), or *gdh1-D150S* (pLP2638) and assayed for growth. (D) The conserved Asp residue contributes to Gdh1's function in telomeric silencing. WT and *gdh1 Δ gdh3 Δ* cells were transformed with vector (pRS425), *GDH1* (pLP2764), or *gdh1-D150S* (pLP2698), and telomeric silencing was assessed. (E) Colony counting assay on SC-Ura confirmed the lack of silencing activity of the *gdh1-D150S* mutant. The assay was described in *Materials and Methods*. (F) Gdh1's inhibitory function in H3 clipping is dependent on the conserved Asp residue. Nuclear extracts were prepared from WT (LPY4916) and *gdh1 Δ* (LPY16033) strains transformed with vector (pRS314), *GDH1* (pLP3082), or *gdh1-D150S* (pLP3083) and immunoblotted for H3-CT and Tub1 (loading control). N-terminally clipped H3 was highlighted by the asterisk. Blots from two sets of independent extracts were quantified.

in vivo. Because Gdh1 and Gdh3 were reported to form heterohexamers (34), we assessed the silencing function of the *gdh1-D150S* mutant in the *gdh1Δ gdh3Δ* background to avoid potential dominant effects. The *gdh1-D150S* mutant failed to suppress the telomeric silencing defect of the *gdh1Δ gdh3Δ* strain, as demonstrated by dilution assay (Fig. 4D) and independently counting colonies grown on medium lacking uracil (Fig. 4E). Some silencing activity was observed upon prolonged incubation, likely due to low or residual metabolic activity of the D150S mutant. Also, the conserved Asp residue is required for Gdh1's inhibitory effect on H3 clipping (Fig. 4F). Hence, we conclude that the metabolic activity of Gdh1 is required for its chromatin functions.

Elevated α -Ketoglutarate Levels Result in Telomeric Silencing Defects. Gdh1 was selected by our in silico screen because its metabolic activity involves NAD(P)⁺ and α -ketoglutarate, which serve as cofactors for class III HDACs and JMJC-domain-containing demethylases, respectively. Because we observed defective telomeric silencing in the *gdh1Δ* mutant and Sir2's HDAC activity depends on NAD⁺ (51), we asked if Gdh1's metabolic activity influences the nuclear pool of NAD⁺.

In budding yeast, nuclear NAD⁺ levels can be measured in strains with a *cis*-acting NadR binding-site box at the promoter of the *HIS3* gene that is transformed with a plasmid-borne NadR-AD transcriptional activator (Fig. 5A). The levels of free NAD⁺ in the nucleus influence the binding affinity of NadR-AD for the NAD box, thus modulating the ability of the strain to grow on medium lacking histidine (52). In this assay, the *gdh1Δ* mutant showed similar growth as the wild-type strain (Fig. 5A); thus, it is unlikely that *GDH1* influences telomeric silencing through nuclear NAD⁺ levels.

We next considered the possibility that Gdh1 regulates chromatin functions through modulating α -ketoglutarate levels. This metabolite serves as a cofactor for many enzymes, including the JMJC-domain-containing histone demethylases (53). The *gdh1Δ* mutant has reduced ability to use α -ketoglutarate as a substrate to assimilate ammonium (Fig. 4A) and caused an ~33% increase in α -ketoglutarate levels when grown in ammonium sulfate-based SC medium (34). It is worth noting that no experimental tool has yet been developed to evaluate nuclear levels of α -ketoglutarate, however our finding that an NES-tagged Gdh1 construct caused a moderate loss of telomeric silencing (Fig. 2F) suggested that the nuclear pool of α -ketoglutarate may be altered when Gdh1 is

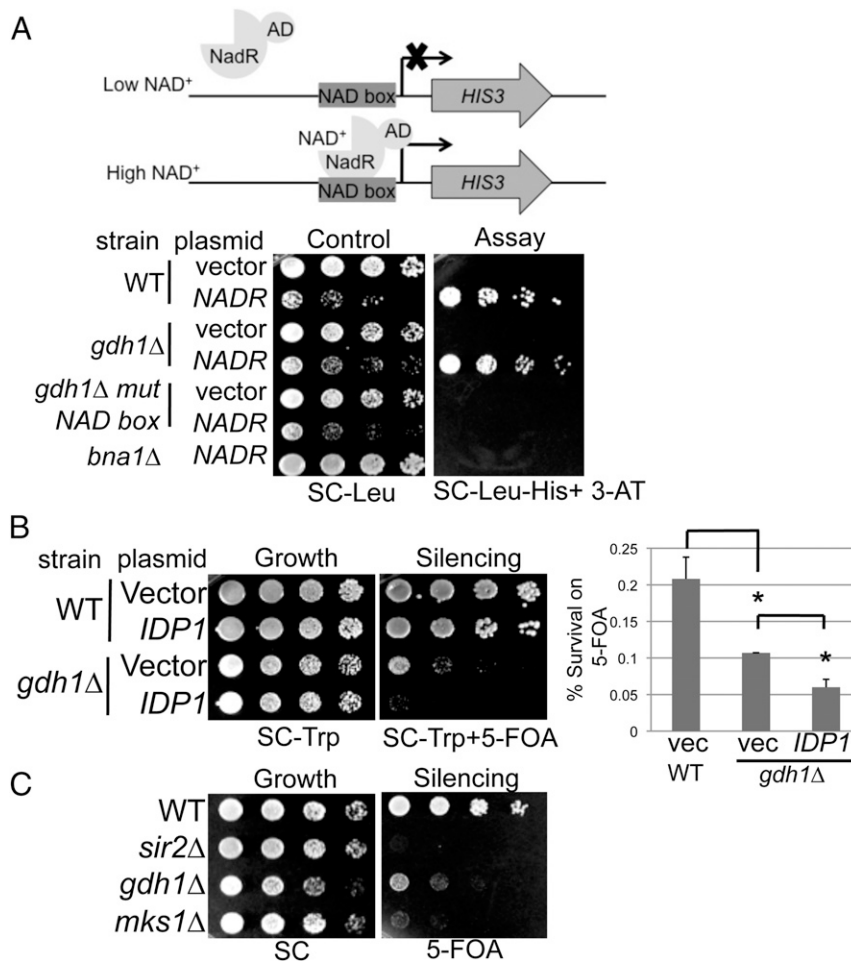


Fig. 5. Gdh1 likely regulates telomeric silencing through modulating α -ketoglutarate levels. (A) The *gdh1Δ* cells have normal levels of nuclear NAD⁺. WT (LPY20466) and *gdh1Δ* (LPY20477) reporter strains were transformed with vector (pLP3227) or NADR-AD (pLP3228). Nuclear NAD⁺ levels were measured by growth on SC-His-Trp + 10 mM 3-AT. 3-AT prevents leaky transcription of *HIS3*. The *bna1Δ* strain (LPY20468) was a positive control for reduced NAD⁺ levels, and the *gdh1Δ* strain with mutant NAD boxes (LPY20480) was a negative control to test for NadR-AD-specific effects. (B) Increased dosage of *IDP1* worsened telomeric silencing in *gdh1Δ*. WT, and *gdh1Δ* cells transformed with vector (pRS314) or *IDP1* (pLP3238) were assayed on SC-Trp + 5-FOA. Survival rate on 5-FOA was quantified as described in *Materials and Methods*. (C) Deletion of *MKS1* caused defective telomeric silencing. WT, *sir2Δ*, *gdh1Δ*, and *mks1Δ* (LPY16796) strains were assayed on 5-FOA.

depleted from the nucleus. Also, we observed physical association of Gdh1 at telomeric loci (Fig. 2G), and thus it is possible that Gdh1 influences α -ketoglutarate levels locally at specific genomic regions.

To assess whether elevated α -ketoglutarate levels could contribute to the silencing defect of the *gdh1 Δ* mutant, we took two separate approaches. First, we transformed *gdh1 Δ* with plasmid-borne *IDP1*, which encodes a mitochondrial NADP-specific isocitrate dehydrogenase (IDH) that is known to contribute to the synthesis of α -ketoglutarate (54, 55). Increased dosage of *IDP1* further worsened telomeric silencing in *gdh1 Δ* cells by \sim 50% (Fig. 5B), consistent with the concept that these two genetic manipulations had additive effects on increasing α -ketoglutarate levels. In an independent approach, we deleted the gene *MKS1*, because a previous study showed that this mutant caused a 600% increase in α -ketoglutarate levels (56). The *mks1 Δ* mutant showed an even stronger telomeric silencing defect than the *gdh1 Δ* mutant (Fig. 5C). These findings suggest that α -ketoglutarate levels are important regulators of telomeric silencing.

GDH1 and JHD2 Exhibit Complex Genetic Interactions. Because we observed that high levels of α -ketoglutarate caused a silencing defect, we sought to identify the molecular mechanism that links Gdh1 directly to telomeric silencing. The best established group of α -ketoglutarate-regulated nuclear proteins is the JMJC-domain-containing demethylases. Budding yeast has five known enzymes of this family, of which only the histone H3 Lys4 demethylase Jhd2 specifically removes methyl groups from trimethylated histone H3 Lys4 (57). Because H3 Lys4 methylation marks are known to be important for telomeric silencing (58, 59), we tested the hypothesis that the increased α -ketoglutarate levels in the *gdh1 Δ* mutant hyperactivated Jhd2 to alter telomeric silencing. To test the hypothesis, we first took a gene overexpression approach. We found that increased dosage of *JHD2* worsened telomeric silencing in the *gdh1 Δ* mutant (Fig. S4A), suggesting that the deletion of *GDH1* creates a condition under which Jhd2 has a greater impact on telomeric silencing. Furthermore, deletion of *JHD2* did not rescue the silencing defect of the *gdh1 Δ* mutant (Fig. S4B). Rather, the *jhd2 Δ gdh1 Δ* double mutant showed a silencing defect similar to each single mutant (Fig. S4B). Within the limits of these experiments, we concluded that although Gdh1 and Jhd2 may regulate telomeric silencing through a common pathway, the simple hypothesis of Jhd2 as a target of Gdh1 is unlikely to be correct. Thus, the mechanism through which Jhd2 and Gdh1 may regulate telomeric silencing is likely to be complex.

Discussion

Increasing evidence suggests that multiple pathways connect metabolism to chromatin regulation and epigenetic processes (8, 9, 20). In this study, our in silico screen revealed that multiple proteins with established roles in amino acid metabolism also have potential functions in chromatin. Our focus on glutamate dehydrogenase revealed that both Gdh1 and Gdh3 regulated telomeric silencing. Gdh1 acts as the primary regulator when glucose is the carbon source, whereby it regulated recruitment of the SIR complex to telomeres. Multiple genetic analyses demonstrated that increased α -ketoglutarate levels are detrimental to telomeric silencing and that Gdh1 has complex genetic interactions with the Jhd2 histone demethylase.

Deletion of Multiple Candidate Genes Encoding Metabolic Proteins Altered Chromatin Silencing. We found that deletion of *ARG82*, *FPRI*, *GDH1*, *GDH3*, *HOM2*, *HOM6*, *SAM4*, and *SPE2* could cause silencing defects. The gene products of these candidates represent five distinct metabolic pathways: arginine biosynthesis, proline isomerization, assimilation of ammonium, methionine and threonine biosynthesis, and SAM metabolism. Future studies will establish if the other candidate proteins contribute to chromatin-

mediated silencing through metabolism-dependent or -independent mechanisms.

It is noteworthy that our in silico search revealed that 39 amino acid metabolic proteins have reported nuclear pools, 25 of which were eliminated from immediate consideration as chromatin regulators by one or more of our search criteria (Table S2). We note, however, that some of these proteins may regulate chromatin functions in diverse ways. For example, they may use the same catalytic site for entirely different biochemical reactions (21). Further, the candidate genes whose deletion did not show silencing phenotypes may contribute to other aspects of chromatin function, such as DNA damage repair, replication, or other elements of transcriptional regulation.

Gdh1 Is a Negative Regulator of H3 N-Terminal Clipping in Vivo. The N terminus of histone H3 contributes significantly to gene regulation. In a recent report, GDH isolated from vertebrate cell microsomes was reported to clip free and chromatin-bound H3 in vitro (47). In marked contrast to this report, we found instead that deletion of *GDH1* increased H3 clipping globally and locally during log phase and that the increase was dependent on the conserved catalytic residue controlling its metabolic activity (Fig. 4F). Thus, yeast Gdh1 can inhibit rather than catalyze H3 N-terminal clipping in vivo with functional consequences for gene regulation (Fig. 3).

Current knowledge on histone H3 clipping is rather limited. Recent work suggested that a vacuolar protease encoded by *PRB1* has clipping activity in budding yeast during the early stationary phase (48). Nonetheless, it remains unclear whether, when, and how Prb1 might enter the nucleus from the vacuole to access chromatin. Also, Prb1 cleaved H3 at lysine residues in vitro, but independent studies confirmed that the in vivo clipping activity is sensitive to serine protease inhibitors (46, 48) and mutation of H3 to H3-S22A diminishes H3 clipping (Fig. 3A). Future work is required to establish the physiological conditions under which Prb1 is active, to identify any additional H3 proteases, and to determine the protease regulated by Gdh1.

α -Ketoglutarate Is an Important Metabolic Regulator of Telomeric Silencing. We showed that Gdh1's roles in both telomeric silencing and H3 clipping are dependent on its metabolic activity and that high α -ketoglutarate levels are detrimental to telomeric silencing. Recent studies revealed that oncogenic mutations in human IDH result in the synthesis of 2-hydroxyglutarate (2-HG) instead of the normal α -ketoglutarate product. 2-HG competitively inhibits JMJC-domain histone demethylases, resulting in increased H3K9 methylation (60, 61). Further, levels of α -ketoglutarate are key for transcriptional and epigenetic processes in stem cell maintenance (62). Our work demonstrates that elevated α -ketoglutarate levels caused telomeric silencing defects (Fig. 5B and C). These findings collectively suggest that it is crucial to maintain the homeostasis of α -ketoglutarate, as both low and high levels have detrimental effects on chromatin functions. This point will be particularly important when developing therapies against the increasingly recognized IDH mutant-bearing tumors (63, 64), because strategies to balance or compensate for decreased α -ketoglutarate may cause negative secondary effects.

In sum, we identified glutamate dehydrogenase homologs Gdh1 and Gdh3 as positive regulators of telomeric silencing. Gdh1's silencing function requires its catalytic activity, and high α -ketoglutarate levels are detrimental to silencing. Gdh1 represses H3 N-terminal clipping and regulates the recruitment of the SIR complex to the telomeres. These results, and the other candidates identified in our screen (Fig. 1), thus emphasize the emerging concept that epigenetic processes are tightly regulated by cellular metabolic status and that mutations in metabolic genes may cause diseases through changes both in the cytoplasm and in the nucleus.

Materials and Methods

Yeast Strains and Plasmids. Strains are listed in Table S3. All mutants are null alleles except *esa1-414* (26). Gene deletions were constructed by amplifying *kanMX* from the *Saccharomyces* Genome Deletion Project strains (65, 66) (oligonucleotides are listed in Table S4) and transforming it into the silencing reporter strain (24). Strains for the *GDH1* study were backcrossed before use. Double mutants were constructed through standard crosses. Histone shuffle strains were chromosomally deleted for both *HHT-HHF* loci and were originally covered with a plasmid carrying the wild-type copy of *HHT2-HHF2* (67). Histone mutant strains were made by transforming the shuffle strains with plasmids carrying histone mutants and counterselecting the wild-type plasmid.

Plasmids are listed in Table S5. Each gene was subcloned from the Yeast Genomic Tiling Library (68), including endogenous 5' and 3' sequences. The D150S mutation was introduced by site-directed mutagenesis using primers listed in Table S4. Plasmid-borne Myc-tagged *Gdh1* was constructed by replacing the natural stop codon of *GDH1* with an XmaI site using primers oLP1598 and oLP1599 and ligating the digested plasmid with an XmaI-digested DNA fragment containing the 13Myc epitope and a terminator sequence made by amplification of pLP1651 with primers oLP1629 and oLP1753.

The chromosomal *GDH1* locus was tagged by amplifying the 13Myc-*kanMX* cassette from pLP1651 (69) with oligonucleotides OLP1629 and OLP1630 and transforming it into the WT W303 strain. The NES used was a variant of the strong NES from PKI with the sequence ELAKLAGLDINLI (43). A 13Myc-Gly-Gly-NES-*kanMX* fragment was integrated to tag the C terminus of *Gdh1* by amplifying this cassette from pLP2829 with OLP1629 and OLP1630 and transforming it into the WT W303 strain.

Growth Assays and Silencing Reporter Assays. SC medium was made with a 0.67% ammonium sulfate-based yeast nitrogen base without amino acids (Difco) supplemented with amino acids (standard laboratory recipe). For all experiments in this study, freshly thawed/transformed cells were inoculated in 3 mL SC medium for 2 d before plating/diluting for log-phase growth. For dilution assays, A_{600} of 1 OD of culture ($\sim 6 \times 10^6$ cells) was pelleted, resuspended in 1 mL H_2O , plated in fivefold serial dilutions, and incubated at 30 °C. For rDNA silencing assays, strains were plated on SC-Ade-Arg (growth control) and SC-Ade-Arg + 8 μ g/mL L-canavanine (Sigma). For *HM* silencing assays, strains were plated on SC (growth control) and SC-Trp (silencing). For telomeric silencing assays, strains were plated on SC (growth control) and SC + 1 g/L 5-FOA (US Biological). For the nuclear NAD^+ assays, strains were plated on SC-Leu (growth control) and SC-Leu-His + 10 mM 3-amino-1,2,4-triazole (3-AT) (Sigma). Images were captured after 1.5–3 d. To assess the catalytic activity of *gdh1-D150S*, starter cultures of transformants were diluted to A_{600} of 0.1 in minimal medium [0.67% ammonium sulfate-based yeast nitrogen base supplemented with required amino acids and 2% (wt/vol) glucose]. A_{600} was measured at indicated times. Quantification of growth on media lacking uracil

was done by plating 200–500 log-phase cells on SC-Trp (control) or SC-Trp-Ura (silencing) media. Quantification of survival rate on 5-FOA was done by plating 500–1,000 log-phase cells on SC-Trp (control) and SC-Trp + 5-FOA (silencing). For both quantitative assays, cells were counted 48 h after plating.

mRNA Quantification. Starter cultures were diluted in 50 mL SC medium and harvested at A_{600} of 0.8–1.0 ($\sim 5 \times 10^6$ – 6.5×10^6 cells per mL). RNA was extracted using the hot acid phenol method (70), except that harvested cells were resuspended in sodium acetate buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA). RNA was reverse transcribed with random hexamers using TaqMan Reverse Transcription Reagents (Life Technologies). cDNA was diluted five- or 10-fold and analyzed by real-time PCR on DNA Engine Opticon 2 (MJ Research) with primers listed in Table S4.

ChIP. Strains were grown as for mRNA quantification and fixed in 1% formaldehyde for 15 min (25 min for anti-Myc ChIP). ChIP was performed as described (71). Briefly, sheared chromatin was incubated overnight at 4 °C with anti-Sir2 (1:1,000) (72), anti-Myc 9E10 (1:100, Sigma), anti-Flag (1:1,000, Sigma), or anti-H3-CT (1:500, Millipore). Input DNA and immunoprecipitated (IP) samples were diluted 10-fold and analyzed by real-time PCR (primers listed in Table S4). Anti-Flag specificity was verified with an untagged strain.

Preparation of Yeast Nuclei and Whole-Cell Extract. Strains were grown as for mRNA quantification. Nuclei were prepared as described (73). Whole-cell extract was prepared by resuspending each A_{600} 1.0 of cells in 25 μ L PBS with protease inhibitors and vortexing it with glass beads. Protein samples were denatured by boiling in sample loading buffer.

Protein Immunoblotting. Proteins were separated on SDS-polyacrylamide gels (18% acrylamide for the separation of histones and 8% for the other proteins) and transferred to 0.2 μ m nitrocellulose. Primary antisera used were anti-H3-CT (1:10,000, Millipore), anti-Myc (1:10,000) (74), anti-Sir2 (1:10,000) (72), and anti- β -tubulin (1:10,000) (75). Secondary antisera used were goat anti-mouse for anti-Myc or goat anti-rabbit (conjugated to horseradish peroxidase, 1:10,000, Promega). Signals were detected with Pierce ECL substrate (Thermo Scientific) on Hyblot CL films (Denville Scientific). Quantification was done using ImageJ.

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