mammalian cells in that mutations in mismatch repair genes do not result in an increased tolerance to treatment with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (*30*). It is possible that genetic redundancy and the expression levels of different mismatch repair genes influence the biological consequences of a mutation in a mismatch repair gene.

It is thought that an increase in the spontaneous accumulation of mutations that are the result of an inability to process mispairs leads to the development of cancer associated with HNPCC. However, this theory does not explain the unique spectrum of tumors associated with HNPCC (predominantly colon) or the differences in the tumors observed in mismatch repair–defective mice (lymphomas) (*26*) and HNPCC patients. It is possible that the inactivation of the ability to process mispairs is not the cause of tumorigenesis in HNPCC. Nucleotide excision repair recognizes a wide spectrum of DNA lesions produced by physical and chemical agents present in the environment (*31*). Furthermore, TCR has been demonstrated for substrates of nucleotide excision repair and base excision repair (*32*). Although the enhancement of excision repair by transcription may appear subtle, if mutations in human mismatch repair genes abolish many types of TCR, then a reduction in the repair of environmentally induced DNA damage could affect the development of cancer associated with defects in mismatch repair genes.

In addition, a subtle defect in the repair of DNA damage could have a more profound impact on tumorigenesis if it is less likely to be lethal to the cell. Although predisposition to cancer is not associated with Cockayne's syndrome, these patients die on average by 12 years of age (*33*). In general, tumors associated with HNPCC develop later in a patient's life. This could explain the observation made by Parsons *et al.* (*25*) that although there appeared to be widespread mutations in non-neoplastic cells from several different tissues in a subset of HNPCC patients, there was no significant increase in the frequency of tumors. On the basis of these results, they suggested that an increase in spontaneous mutations may not be sufficient for tumorigenesis and exposure to environmental mutagens may have a part in the process.

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Linkage of Replication to Start by the Cdk Inhibitor Sic1

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In *Saccharomyces cerevisiae*, three G_1 cyclins (Clns) are important for Start, the event committing cells to division. Sic1, an inhibitor of Clb-Cdc28 kinases, became phosphorylated at Start, and this phosphorylation depended on the activity of Clns. Sic1 was subsequently lost, which depended on the activity of Clns and the ubiquitin-conjugating enzyme Cdc34. Inactivation of Sic1 was the only nonredundant essential function of Clns, because a *sic1* deletion rescued the inviability of the *cln1 cln2 cln3* triple mutant. In *sic1* mutants, DNA replication became uncoupled from budding. Thus, Sic1 may be a substrate of Cln-Cdc28 complexes, and phosphorylation and proteolysis of Sic1 may regulate commitment to replication at Start.

Before yeast can replicate DNA, they must pass Start, which requires a cyclin-dependent protein kinase composed of a catalytic subunit (Cdc28) and one of three G_1 cyclins (Cln1, -2, or -3) (*1*). After Start, B-type cyclin-Cdc28 kinases such as Clb5-Cdc28 and Clb6-Cdc28 must be activated to allow replication (*2*). Although Clb5- and Clb6-Cdc28 complexes are present in G_1 phase, they are initially inactive because of inhibition by the Sic1 protein (*2*, *3*). Activation of Clb5- and Clb6-Cdc28 occurs after Sic1 is targeted for proteolysis by the ubiquitin-conjugating enzyme Cdc34 (*2*). Thus, a *cdc34* mutant arrests with a 1N DNA content because it cannot degrade Sic1, but nevertheless buds, and duplicates its spindle pole body.

It is not known how Start triggers Sic1 inactivation or how replication is tied to other Start-dependent events such as budding and duplication of the spindle pole body. Is Start a single event that affects multiple pathways, or is Start a collection of events, one of which regulates Sic1 proteolysis and replication?

We asked whether Cln-Cdc28 complexes phosphorylate Sic1, thereby targeting it for proteolysis. Sic1 coprecipitates with

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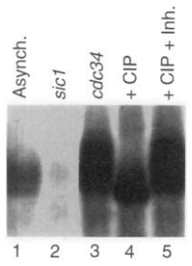

Fig. 1. Sic1 is a phosphoprotein in vivo. Extracts were made as described (*17*), and Sic1 was immunoprecipitated (*14*). The immunoprecipitates were treated or not treated with phosphatase (*18*), resolved by SDS-PAGE (*15*), blotted to nitrocellulose, and Sic1 was detected (*16*). Lane 1, asynchronous cells; lane 2, asynchronous *sic1* cells; lane 3, strain #31 (*19*) arrested at the *cdc34* block at 37°C; lane 4, as in lane 3, but treated with calf intestinal phosphatase (CIP); lane 5, as in lanes 3 and 4, but treated with CIP and the phosphatase inhibitor B-glycerolphosphate (Inh.).

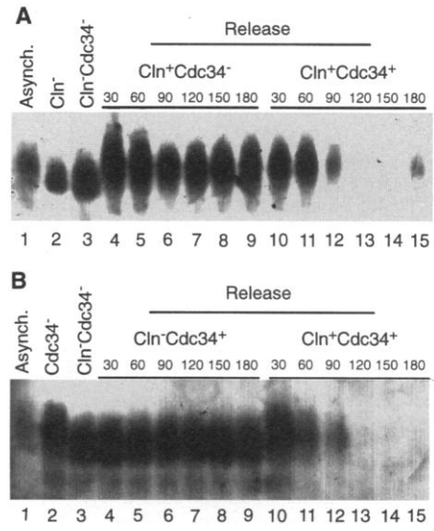

Fig. 2. Loss of Sic1 depends on *CLN*s and on *CDC34*. Abundance and phosphorylation of Sic1 were analyzed in reciprocal shift experiments (*20*). Strain #31 (*cln1 cln2 GAL-CLN3 cdc34*) (*19*) was used. (**A**) Cells were grown in galactose medium at 23°C (lane 1), shifted to glucose at 23°C for 3 hours to synchronize cells at Start (lane 2), then shifted to 37°C for another hour to inactivate Cdc34 (lane 3). Cln expression was then restored by shifting back to galactose medium, but cells were held at 37°C ($Cdc34^-$). Samples were taken every 30 min (lanes 4 to 9). As a control, Cln expression and Cdc34 function were both restored (lanes 10 to 15) to doubly blocked cells. (**B**) Cells were grown in galactose medium at 23°C (lane 1), shifted to 37°C for 3 hours to synchronize cells at the *cdc34* block (lane 2), then shifted to glucose at 37°C for 1 hour to shut off *GAL-CLN3* (lane 3). Cdc34 function was restored by a shift to 23°C, but cells were kept in glucose medium (Cln^-). Samples were taken every 30 min (lanes 4 to 9). As a control, Cdc34 function and Cln expression were both restored (lanes 10 to 15) to doubly blocked cells. FACS analysis showed that the cells in lanes 4 to 9 (A and B) failed to replicate DNA, whereas the cells in lanes 10 to 15 did replicate DNA.

Cdc28 (*4*), has one of the highest densities of potential Cdc28 phosphorylation sites of any known yeast protein (*5*), and can be phosphorylated on many sites by Cdc28 in vitro (*4*, *6*).

Sic1 is a phosphoprotein in vivo. Resolution of Sic1 by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting showed a broad, fuzzy band that may contain multiple forms of Sic1. Phosphatase treatment converted this fuzzy band (more phosphorylated form) to a band of greater mobility (less phosphorylated form) (Fig. 1).

To study the relation between the Clns, phosphorylation and proteolysis of Sic1, and DNA synthesis, we constructed a *cln1 cln2 GAL-CLN3 cdc34-2* (temperature-sensitive) strain and did reciprocal shift experiments. As expected, cells shifted from the Cln^-Cdc34^+ state to the Cln^+Cdc34^- state arrested with a $Cdc34^-$ phenotype without dividing. Sic1 accumulated in the less phosphorylated form in Cln^--arrested cells, but was phosphorylated to a greater extent when Cln was restored (*7*) (Fig. 2A, compare lanes 3 and 4). However, in the absence of Cdc34 function (Cln^+Cdc34^-), this highly phosphorylated Sic1 remained undegraded (Fig. 2A, lanes 4 to 9). In control cells arrested in the Cln^-Cdc34^+ state, then released to the Cln^+Cdc34^+ state, Sic1 became more phosphorylated when Cln was restored, and then disappeared, presumably because of proteolysis (*7*) (Fig. 2A, lanes 10 to 15). These cells then reentered a normal cell cycle. Thus, in vivo, the Cln-Cdc28 complexes are needed to generate highly phosphorylated Sic1, which is stable in the absence, but not in the presence, of Cdc34 function.

Cdc34 has been considered to act downstream of Clns and Cdc28. Surprisingly, however, cells shifted from the Cln^+Cdc34^- state to the Cln^-Cdc34^+ state did not enter S phase or divide and in all respects maintained a $Cdc34^-$ phenotype. This result suggests that the Cdc34 function cannot be completed in the absence of Cln-Cdc28 activity. Highly phosphorylated Sic1 accumulated in the Cln^+Cdc34^- cells (*7*) (Fig. 2B, lane 2); Sic1 then became less phosphorylated, but not degraded, after the shift to the Cln^-Cdc34^+ state (*7*) (Fig. 2B, lanes 4 to 9). This result suggests that the $Cdc34^-$ phenotype is maintained in the Cln^-Cdc34^+ cells because the less phosphorylated form of Sic1 cannot be degraded in the absence of Cln activity. When cells were shifted from Cln^+Cdc34^- to Cln^+Cdc34^+, the more phosphorylated form of Sic1 that had accumulated at the *cdc34* block disappeared (Fig. 2B, lanes 10 to 15), and the cells went through S phase and reentered a normal cycle. These experiments show that Sic1 loss requires Cln function as well as Cdc34 function, and that the more phosphorylated form of Sic1 is dependent on Cln activity and correlated with Sic1 loss. Because cells arrest before S phase regardless of the phosphorylation state of Sic1, both forms must inhibit Clb-Cdc28 complexes.

These results are consistent with a model wherein Cln-Cdc28 complexes phosphorylate Sic1, and this phosphorylation targets Sic1 for degradation by the Cdc34 pathway. However, the experiments are correlative, and other mechanisms are also possible. For example, Cln-Cdc28 complexes may serve to activate Cdc34 itself, and the phosphorylation of Sic1 may be a correlated but irrelevant event.

If a major function of Clns is to promote proteolysis of Sic1, then Clns should be less important in a *sic1* mutant. Indeed, a *sic1* mutation suppressed the lethality of a *cln1 cln2 cln3* triple null mutation (Fig. 3B, sectors 1, 3, and 4). Thus, the only nonredundant essential function of the Clns is to inactivate Sic1. The *cln1 cln2 cln3* triple mutation is also suppressed by a mutation

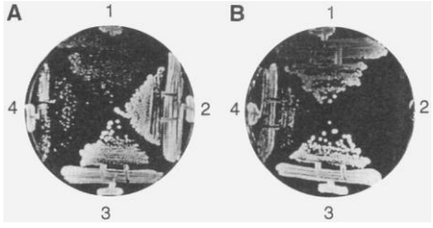

Fig. 3. A *sic1* deletion suppresses lethality of *cln1 cln2 cln3*. (**A**) YEP + 1% raffinose + 1% galactose. (**B**) YEP + 2% glucose. Plates were incubated at 30°C for 3 days. Strains were as follows: 1, BS147 (*pGAL-CLN3 Δcln*s *Δsic1*); 2, BS100 (*GAL-CLN1 Δcln*s); 3, BS178 (*GAL-CLN1 Δcln*s *Δsic1*); and 4, BS152 (*Δcln*s *Δsic1*) (*19*).

called *BYC1* (*8*), and it now appears that *BYC1* is allelic to *sic1* (*9*). This suppression by *BYC1* occurs even if *clb2*, *clb5*, or *pcl1* is also deleted (*8*). Clns1, -2, and -3 have other important functions that are compromised in the *cln1 cln2 cln3 sic1* quadruple mutant: Plating efficiency is poor, budding and cell morphology are highly abnormal, and the cells are generally sick. Presumably, budding is now mediated by combinations of other cyclins such as Pcl1, Pcl2, Clb5, and Clb6 (*10*).

If Sic1 is an important and specific inhibitor of replication, then a *sic1* mutation might uncouple DNA replication from other Start events, such as budding. To test this hypothesis, we obtained small unbudded cells from an exponential culture of *sic1* cells and examined the cells for DNA content by fluorescent-activated cell sorting (FACS). At least 20% of the unbudded cells were already 2N, whereas there were essentially no 2N cells in the equivalent fraction from a wild-type culture. After reinoculation into fresh medium, the *sic1* cells

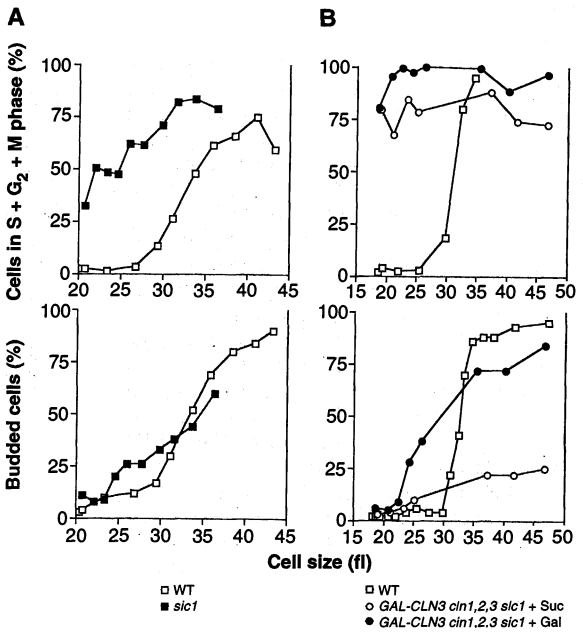

Fig. 4. A *sic1* deletion uncouples S phase from budding. (**A**) Small unbudded cells of strain W303a (*19*) (□) or its isogenic *sic1::URA3* derivative BS193 (■) were obtained by elutriation (*21*). Cells were reinoculated in fresh, warm medium, and samples were taken every 15 min and analyzed for budding, cell volume, and DNA content (FACS) (*22*). (**B**) Strain BS147 (*pGAL-CLN3 Δcln Δsic1*) (*19*) was grown in sucrose plus galactose. Cells were washed and resuspended in medium containing sucrose but no galactose to turn off *GAL-CLN3*. After 1 hour, small unbudded cells were collected by elutriation (*21*). Half the sample was reinoculated into YNB medium with 2% sucrose (*GAL-CLN3* off) (○), and the other half was reinoculated into YNB medium with 1% sucrose and 1% galactose (*GAL-CLN3* on) (●). Samples were taken every 30 min and analyzed as in (A). W303a cells (*19*) grown in YNB + 2% sucrose were elutriated and monitored after reinoculation (□).

replicated DNA much earlier than the wild-type cells, but budded at about the same time (Fig. 4A). [In other, similar experiments, the *sic1* mutation did advance budding slightly, although never as much as the advance in S phase (*2, 11*). The early activation of Clb5 that occurs in *sic1* cells may advance budding.]

In a second experiment, *cln1 cln2 GAL-CLN3 sic1* cells were grown with *GAL-CLN3* on, and then *GAL-CLN3* was turned off for 1 hour. Small unbudded cells were obtained by elutriation. Fifty to 80% of these cells had a DNA content greater than 1N, despite their lack of Cln. (The large fraction of 2N cells probably resulted from overexpression of *CLB5* induced by *GAL-CLN3*.) When the cells were released into fresh medium, efficient budding was still dependent on reexpression of Cln3, whereas S phase was not (Fig. 4B). Thus, in *sic1* mutants, replication and budding are uncoupled; they occur at different times, and budding is much more dependent on Cln than is replication.

Although phosphorylation and loss of Sic1 are dependent on both Cln and Cdc34 function, we have not shown that Sic1 is a direct substrate of the Cln-Cdc28 kinase in vivo, nor that Sic1 proteolysis is ubiquitin-mediated. However, these are both strong possibilities. Phosphorylation converts at least one other protein into a substrate for Cdc34-mediated proteolysis (*12*). Whatever the precise mechanism by which Clns and Cdc34 cause the loss of Sic1, our genetic experiments show that this loss is largely responsible for the normal dependence of DNA replication on Start.

An analogous system may be used by mammalian cells. Cyclin D–Cdk4 complexes promote S phase by inhibiting function of the retinoblastoma protein. In cells lacking retinoblastoma, the cyclin D–Cdk4 activity is no longer required (*13*).

The identification of Sic1 as a target of Clns suggests that Start consists of several component events. The Start event controlling S phase is probably phosphorylation of Sic1; phosphorylation of other substrates may control budding and duplication of the spindle pole body, and together these phosphorylations constitute Start.

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6. Two-dimensional gel electrophoresis of Sic1 phosphorylated in vitro by Cdc28 showed 13 labeled charge isoforms, suggesting 13 phosphorylation sites. There are nine Ser-Pro or Thr-Pro sites in Sic1. Even the most highly phosphorylated Sic1 showed only a modest change in mobility in the SDS-PAGE dimension, consistent with the data shown in Figs. 1 and 2.
7. Sic1 from cells arrested in various states was treated with phosphatase as in Fig. 1 to show that the mobility shift was due to a change in phosphorylation. Consistent with these results, the mobility of Sic1 is altered in *cdc28* mutants (*2*).
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14. Antibody to rabbit Sic1 (12.5 μl) [J. D. Donovan, J. H. Toyn, A. L. Johnson, L. H. Johnston, *Genes Dev.* **8**, 1640 (1994)] was added to a 3-mg cell extract. After incubation for 1 hour at 0°C, protein A beads (30 μl) were added, and the mixture was rocked at 4°C for 1 hour. Beads were washed four times with alkaline phosphatase buffer (APB) [50 mM tris-HCl (pH 8), 10 mM dithiothreitol, 0.6 mM dimethylaminopurine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), tosyl-L-phenylalanine-chloromethyl ketone (10 μg/ml), pepstatin (5 μg/ml), and soybean trypsin inhibitor (10 μg/ml)].
15. Extract (50 μg) was loaded per lane on a 16 cm by 18 cm by 0.75 mm gel and run for 20 hours at 100 V.
16. Proteins were transferred to nitrocellulose for 30 min at 10 V. Blots were blocked by using non-fat milk (5%) in tris-buffered saline [TBS; 140 mM NaCl, 2.5 mM KCl, 25 mM tris-HCl (pH 7.4)] for 1 hour. Blots were incubated overnight with a 1:100 dilution of rabbit antibody to Sic1 (*14*). Blots were washed four times in TBS, then incubated with a 1:2000 dilution of alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G (Pierce) for 1.5 hours, washed, and finally incubated at room temperature with 10 ml of NBT-BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolphosphate *p*-toluidine) (Gibco-BRL) for 5 to 10 min. β-Tubulin was used as a loading control.
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18. Beads (30 μl) carrying immunoprecipitated Sic1 (*14*) were divided into three portions (10 μl), and these were treated with APB (10 μl) (*14*), APB (8 μl) plus calf intestinal phosphatase (CIP) (2 μl, 2 U) (Boehringer), or APB (7 μl), CIP (2 μl), and B-glycerolphosphate (1 μl of 1 M).
19. Strains were W303a (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1-100 ssd1-d* [*psi*$^+$]) [B. J. Thomas and R. Rothstein, *Cell* **56**, 619 (1989)], #31 (*MATα cdc34-2 cln1::HIS3 cln2::TRP1 ura3::GAL-CLN3 leu2 ura3*), BS100 (*MATα cln1::LEU2-GAL-CLN1-HA3 cln2::TRP1 cln3::HIS3 leu2 his3 ura3 ade2 trp1*), BS147 (*MAT***a** *cln1 cln2 cln3 sic1::TRP1* [*pGAL-CLN3 CEN URA3*] *ura3 leu2 trp1 his2 ade1*), BS152 (*cln1 cln2 cln3 sic1::TRP1*) (derived from BS147 by plasmid loss), and BS178 [(*MAT***a** *cln1::LEU2-GAL-CLN1-HA3 cln2 cln3 sic1::TRP1 ura3 trp1 his*(2 or 3)]. The *sic1::TRP1* allele was from M. Tyers; the parent of BS147 was from F. Cross.
20. Cells were grown to 1 × 10^7 cells per milliliter. The galactose medium was YEP (1% yeast extract, 1% peptone) with 1% raffinose and 1% galactose; the glucose medium was YEP with 1% raffinose and 2% glucose. Before shifting from one medium to another, cells were first washed twice with the new medium that had been prewarmed to the target temperature. Sic1 was detected as described (*15, 16*). Representative samples were treated with phosphatase as shown (Fig. 1) to demonstrate that the mobility shift was due to phosphorylation.
21. Cells were grown in SD medium [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)] with required amino acids to 2 × 10^7 cells per milliliter with 2% filter-sterilized sucrose (W303 and BS193) or 1% filter-sterilized sucrose plus 1% galactose (BS147). Cells were centrifuged, sonicated, and elutriated in medium at 30°C.
22. A computer curve-fitting algorithm estimated the number of cells with DNA content of 1N, 2N, or between 1N and 2N.

23. We thank L. Johnston for antibody to Sic1; M. Mendenhall, M. Tyers (who independently found suppression of the *cln1 cln2 cln3* mutant by *sic1*), and F. Cross for strains, reagents, helpful discussions, and communication of unpublished results; and M. Cleary and M. Luke for reading the manuscript. Supported by NIH grant GM 39978 and U.S. Army Breast Cancer grant DAMD17-94-J-4050 to A.B.F.

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