Misregulation of 2 μ m Circle Copy Number in a SUMO Pathway Mutant

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Attachment of the ubiquitin-like protein SUMO to other proteins is an essential process in *Saccharomyces cerevisiae*. However, yeast mutants lacking the SUMO ligases Siz1 and Siz2/Nfi1 are viable, even though they show dramatically reduced levels of SUMO conjugation. This *siz1* Δ *siz2* Δ double mutant is cold sensitive and has an unusual phenotype in that it forms irregularly shaped colonies that contain sectors of wild-type-appearing cells as well as sectors of enlarged cells that are arrested in G₂/M. We have found that these phenotypes result from misregulation of the copy number of the endogenous yeast plasmid, the 2 μ m circle. *siz1* Δ *siz2* Δ mutants have up to 40-fold-higher levels of 2 μ m than do wild-type strains. Furthermore, 2 μ m is responsible for the *siz1* Δ *siz2* Δ mutant's obvious growth defects, as *siz1* Δ *siz2* Δ [cir⁰] strains, which lack 2 μ m, are no longer heterogeneous and show growth characteristics similar to those of the wild type. Possible mechanisms for SUMO's effect on 2 μ m are suggested by the finding that both Flp1 recombinase and Rep2, two of the four proteins encoded by 2 μ m, are covalently modified by SUMO. Our data suggest that SUMO attachment negatively regulates Flp1 levels, which may partially account for the increased 2 μ m copy number in the *siz1* Δ *siz2* Δ strain.

The ubiquitin (Ub)-related protein SUMO (small ubiquitin-related modifier) functions by being covalently attached to other proteins as a posttranslational modification (12, 20, 28). Many proteins with diverse cellular functions are modified by SUMO, including at least 300 proteins in *Saccharomyces cerevisiae* (6, 13, 30, 41, 46). SUMO conjugation is essential for viability in *S. cerevisiae*, and sumoylation-defective conditional mutants arrest in the cell cycle at G₂/M. However, it is not known which substrates are involved in the essential function(s) of SUMO. SUMO conjugation is also essential in most other eukaryotic cells.

SUMOs are a family of ~93- to 98-amino-acid proteins that are ~18% identical to Ub, a 76-residue modifier protein with several functions including targeting proteins for proteasome-dependent proteolysis (15, 31). Like Ub, SUMO is attached to lysine residues in substrate proteins through an amide bond linking the C terminus of SUMO to the ϵ -amino group of the lysine residue. SUMO is often attached to the lysine in the sequence motif Ψ KXE, where Ψ is a hydrophobic residue. However, on some substrates, SUMO is attached to lysine residues in sequences that do not match this motif (20). SUMO conjugation can have a variety of different effects, including modulating protein-protein interactions, altering enzymatic activity, or blocking ubiquitylation of the substrate by competing for its ubiquitylation site lysine (12, 20, 28). SUMO does not directly target proteins for proteasome-dependent proteolysis.

SUMO is conjugated via a three-step enzyme pathway that first activates the SUMO C terminus and then modifies specific target proteins (12, 20, 28). This pathway consists of a heterodimeric SUMO-activating enzyme (E1) comprising Uba2

and Aos1, a SUMO-conjugating enzyme (E2) called Ubc9, and several SUMO ligases (E3s), which include the yeast proteins Siz1 and Siz2/Nfi1. Siz1 and Siz2 participate in sumoylation of partially distinct sets of substrates: some proteins can be sumoylated only by Siz1, others only by Siz2, and others can be sumoylated by either Siz protein (16, 22, 39; G. Bylebyl, A. Reindle, and E. S. Johnson, unpublished data). There is also some *SIZ*-independent sumoylation in yeast. Sumoylation is a reversible modification, and the SUMO-substrate bond can be cleaved by a family of SUMO-specific proteases, which includes the yeast proteins Ulp1 and Ulp2/Smt4. Ulp1 also processes the precursor form of SUMO to generate mature SUMO and therefore is required for both conjugation and deconjugation of SUMO. *UBA2*, *AOS1*, *UBC9*, and *ULP1* are all essential genes, while *ULP2*, *SIZ1*, and *SIZ2* are not. *siz1* Δ and *siz2* Δ single mutants grow quite well and do not have obvious phenotypes (22, 38, 39). However, the *siz1* Δ *siz2* Δ double mutant grows poorly at 30°C, forms irregularly shaped colonies, is cold sensitive, and contains many cells that are delayed in the cell cycle in the early stages of mitosis (22, 39). This result indicates that *SIZ1* and *SIZ2* have at least one overlapping function that contributes to these phenotypes.

We investigated the growth defects of the *siz1* Δ *siz2* Δ mutant and found that they involve the 2 μ m circle, an endogenous 6,318-bp plasmid that is found in virtually all laboratory and industrial strains of *Saccharomyces* (2). 2 μ m is a selfish DNA element whose only known activity is self propagation. 2 μ m encodes four proteins that promote efficient maintenance of the plasmid via two distinct mechanisms (2). One mechanism, which involves the 2 μ m-encoded proteins Rep1 and Rep2 as well as a *cis* element called *STB* or *REP3*, promotes accurate partitioning of 2 μ m between the mother and daughter cells at cytokinesis. In the absence of these factors, 2 μ m remains primarily in the mother cell. Rep1 and Rep2 bind *STB* and inter-

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TABLE 1. *S. cerevisiae* strains

Name	Relevant genotype	Reference or source
DF5	<i>MATa/MATα trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 [cir⁺]</i>	10
YWO1	<i>MATα [cir⁺]</i>	35
YWO102	<i>MATα ubc9Δ::TRP1 leu2::ubc9pro-ser::LEU2 [cir⁰]</i>	35
MHY1488	<i>MATa ulp1Δ::HIS3 LEU2::ulp1-333 [cir⁰]</i>	24
JD52	<i>MATa trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 [cir⁺]</i>	J. Dohmen
<i>uba2-ts10</i>	<i>MATa uba2Δ::HIS3 pIS2-ts10 [cir⁰]</i>	23, 33
IS18-M1	<i>MATa ulp2-1 [cir⁺]</i>	33
IS30	<i>MATa ulp2::URA3 [cir⁺]</i>	33
EJY325	<i>MATa siz1Δ::LEU2 [cir⁺]</i>	22
EJY324	<i>MATa siz2Δ::TRP1 [cir⁺]</i>	22
EJY326	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺]</i>	22
EJY339	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 rad9Δ::HIS3 [cir⁰]</i>	This study
EJY340	<i>MATa/MATα siz1Δ::LEU2/SIZ1 siz2Δ::TRP1/SIZ2 [cir⁰]</i>	This study
EJY341	<i>MATa [cir⁰]</i>	This study
EJY342	<i>MATa siz1Δ::LEU2 [cir⁰]</i>	This study
EJY343	<i>MATa siz2Δ::TRP1 [cir⁰]</i>	This study
EJY344	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁰]</i>	This study
EJY345	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺ FLP1-HA::HIS3]</i>	This study
EJY346	<i>MATa [cir⁺ FLP1-HA-His_g::HIS3]</i>	This study
EJY347	<i>MATa siz1Δ::LEU2 [cir⁺ FLP1-HA-His_g::HIS3]</i>	This study
EJY348	<i>MATa siz2Δ::TRP1 [cir⁺ FLP1-HA-His_g::HIS3]</i>	This study
EJY349	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺ FLP1-HA-His_g::HIS3]</i>	This study
EJY350	<i>MATa [cir⁺ REP2-HA-His_g::HIS3]</i>	This study
EJY351	<i>MATa siz1Δ::LEU2 [cir⁺ REP2-HA-His_g::HIS3]</i>	This study
EJY352	<i>MATa siz2Δ::TRP1 [cir⁺ REP2-HA-His_g::HIS3]</i>	This study
EJY353	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺ REP2-HA-His_g::HIS3]</i>	This study
EJY354	<i>MATa [cir⁺ REP1-HA-His_g::HIS3]</i>	This study
EJY355	<i>MATa [cir⁺ RAF1-HA-His_g::HIS3]</i>	This study
EJY356	<i>MATa [cir⁺ FLP1(K375R)-HA-His_g::HIS3]</i>	This study
EJY357	<i>MATa siz1Δ::LEU2 [cir⁺ FLP1(K375R)-HA-His_g::HIS3]</i>	This study
EJY358	<i>MATa siz2Δ::TRP1 [cir⁺ FLP1(K375R)-HA-His_g::HIS3]</i>	This study
EJY359	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺ FLP1(K375R)-HA-His_g::HIS3]</i>	This study
EJY360	<i>MATa [cir⁺ flp1(Y343F)-HA-His_g::URA3]</i>	This study
EJY361	<i>MATa siz1Δ::LEU2 siz2Δ::TRP1 [cir⁺ flp1(Y343F)-HA-His_g::URA3]</i>	This study

act with the cellular cohesin complex, and these interactions are essential for accurate plasmid segregation (27, 44). The other mechanism enables 2μm to be amplified if its copy number drops. This involves a third 2μm-encoded protein, Flp1 recombinase, and two inverted repeat sequences, called FRT, that are present on opposite sides of the 2μm plasmid (2). Flp1 catalyzes intramolecular recombination between the two FRT sites, thereby inverting one side of the plasmid with respect to the other. 2μm has a single origin of replication, which fires once per S phase, but it is thought that Flp1 activity allows a single 2μm template to be copied more than once per S phase: if the Flp1-mediated intramolecular recombination takes place during S phase between the converging replication forks, it produces a replication intermediate in which both replication forks go in the same direction, chasing each other around the circle, producing multiple copies of 2μm DNA until the reaction is reversed and the structure resolved (2, 11). Expression of *FLP1* is controlled by a mechanism involving the other three 2μm-encoded proteins (Rep1, Rep2, and Raf1), such that *FLP1* expression is increased when 2μm levels are low and repressed when 2μm levels are high (2, 29, 32). This provides a feedback mechanism that promotes amplification of 2μm specifically when copy numbers drop.

2μm is generally considered to be an innocuous passenger in yeast, with only a 1.5 to 3% decrease in growth rate in a strain that contains the plasmid relative to an isogenic strain that does not (26). However, more than 20 years ago an allele called *nibbled* (*nib*) was described in which elevated levels of 2μm

resulted in irregularly shaped colonies, cold sensitivity, and cell cycle delay (18, 19). The gene responsible for this phenotype was not identified. This result suggests that yeast has an “antiviral” mechanism for preventing 2μm from accumulating to such high copy numbers that it interferes with growth of the host cell.

We have found that the *siz1Δ siz2Δ* mutant, like the *nib* strain, has elevated levels of 2μm and that 2μm is involved in the major growth defects of this strain. We investigated possible molecular mechanisms for this misregulation of 2μm and found that both Rep2 and Flp1 are modified by SUMO, suggesting that SUMO may participate in 2μm copy number control by directly targeting plasmid-encoded proteins.

MATERIALS AND METHODS

Media and genetic techniques. Standard techniques were used (1). Rich yeast medium containing 2% glucose (YPD) and synthetic yeast medium were prepared as previously described (36). α-Factor arrests were done with 10 μM α-factor (Sigma) for 3 h.

Plasmids and yeast strain constructions. *S. cerevisiae* strains used are listed in Table 1. All strains are derivatives of JD51 (8) except YWO1 and the *ubc9* and *ulp1* temperature-sensitive (*ts*) mutants, which are derived from DF5 (10). Deletions and epitope tags were made in the chromosomal copies of genes using the products of assembly PCRs as previously described (21). Construction details and oligonucleotide sequences are available on request. [cir⁰] strains were constructed by taking advantage of the fact that *rsc2Δ* mutants lose 2μm at a high rate (42). An *rsc2Δ::kan^R* mutant was constructed in the JD51 background, cultured through three 1,000-fold dilutions, plated, and screened for [cir⁰] isolates by colony PCR (42). This *rsc2Δ* [cir⁰] strain was crossed to a *siz1Δ siz2Δ rad9Δ* [cir⁰] strain (EJY339), which we had obtained unintentionally when we

attempted to construct this triple mutant in a [cir⁺] strain. A segregant containing none of the markers was selected, and this wild-type (wt) [cir⁰] strain was then mated to a *siz1 Δ siz2 Δ* [cir⁰] strain that had been made by crossing EJY339 to a *siz1 Δ siz2 Δ CDC3-HA* strain that we had also shown to be [cir⁰]. The resulting *siz1 Δ /SIZ1 siz2 Δ /SIZ2* [cir⁰] diploid strain (EJY340) sporulated well, and all spores germinated and grew to produce colonies that were homogenous and grew well at all temperatures. Strains EJY341 to EJY344 were all derived from this diploid strain. Strains containing variants of 2 μ m were made from these [cir⁰] strains by transformation as described below.

C-terminally tagged versions of 2 μ m-derived proteins were expressed from modified versions of the endogenous 2 μ m circle that did not contain an *Escherichia coli* origin or drug resistance marker. These were constructed by a PCR-based approach similar to the one used for adding epitope tags to chromosomal genes (21). Briefly, a PCR product containing ~500 bp of the 3' end of the desired open reading frame, followed by the tag, a STOP codon, a *HIS3* or *URA3* marker, and then the 3'-flanking region of the gene, was made by assembly PCR. This results in an insertion at the 3' end of the gene being tagged and should not affect the function of neighboring genes. The sequence of the hemagglutinin (HA) tag was GYPYDVPDYAFL and that of the HA-His₈ tag was GYPYDVPDYAFLHHHHHHHH. The K375R and Y343F versions of *FLP1* were made by similar approaches. Oligonucleotide sequences are available on request. These products were transformed into wild-type [cir⁺] yeast, and His⁺ transformants were selected. These were screened by PCR and immunoblotting for the presence of the tag and marker in the correct location. Genomic DNA (17) from these strains was then transformed into [cir⁰] versions of strains of the desired genotypes, followed by selection for His⁺ colonies. To make sure that the resulting transformants contained only the modified version of 2 μ m, colonies were screened by PCR using primers that flanked the marker in the tagged, marked plasmid (42). Colonies that contained the band diagnostic for the modified 2 μ m and lacking the band for unmodified 2 μ m were retained for further use. Primers used were 5'-GCCGTGGCCAGGACAACG-3' and 5'-GTAGCTCGTTACAGTCCG-3' for *FLP1*, 5'-GCCAGAGGATGGCGAACG-3' and 5'-GCTTCGCGTTGCATTTTCG-3' for *REP1*, 5'-CTGGCGGCAGAGAATCGT-3' and 5'-CACTGTTACACGTCGACC-3' for *REP2*, and 5'-GCACCTTCTACAATGGCTG-3' and 5'-GCTTTCGCGTTGCATTTC-3' for *RAF1*. The wild-type and K375R versions of *FLP1* were both amplified by PCR and sequenced. For the other genes, multiple independent clones were isolated and shown to have similar characteristics.

Antibodies and immunoblot analyses. Yeast whole-cell lysates were prepared by lysis in NaOH (43) and subjected to immunoblotting, followed by chemiluminescent detection (21). Antibodies were an affinity-purified rabbit polyclonal antibody (Ab) against Smt3 (SUMO) (21), the 16B12 monoclonal Ab against the HA epitope (Covance Research Products), and a goat polyclonal Ab against Rad9 (yC-20) (Santa Cruz Biotechnology). HA and His₈-tagged proteins were purified from yeast by Ni-nitrilotriacetic acid (NTA) affinity chromatography (21, 41). Strains containing tagged 2 μ m genes marked with *HIS3* were grown overnight in synthetic yeast-His medium and then diluted into 50 ml of YPD and grown to an optical density at 600 nm of ~1.0. Cells were collected by centrifugation, frozen in liquid N₂, and lysed by the addition of 500 μ l of 1.85 N NaOH and 7.4% β -mercaptoethanol on ice for 10 min. Five hundred microliters of 50% trichloroacetic acid was added, and incubation on ice was continued for 10 min. The pellet was collected by microcentrifugation and washed twice by resuspension in ice-cold acetone. After removal of the acetone, the pellet was resuspended in 1 ml of 6 M guanidine-HCl, 50 mM NaPO₄, 10 mM Tris (pH 8.0). All subsequent steps were done at room temperature. The pH was readjusted to ~8.0 by the addition of ~10 μ l of 2 M Tris base, and 10 μ l of 2 M *N*-ethylmaleimide in isopropanol was added. After the suspension was incubated for 20 min with rotation, the debris was removed by microcentrifugation for 10 min. Supernatants containing equal amounts of protein (assayed using Coomassie Plus; Pierce) were diluted to the same volume (1 ml) and supplemented to 30 mM imidazole. Ni-NTA agarose (~15 μ l; QIAGEN) was then added, followed by incubation with rotation for 2 to 16 h. The supernatant was removed, and the Ni-NTA agarose was washed four times with 8 M urea, 50 mM NaPO₄, 10 mM Tris, and 20 mM imidazole (pH 8.0). After the last wash, the beads were resuspended in 40 μ l of Laemmli loading buffer and boiled, and 5 to 10 μ l was loaded per well. Photographs of immunoblots were made using chemiluminescent detection (Supersignal; Pierce), but signals of bands were quantified with secondary antibodies coupled to fluorescent dyes IRDye 800 (Rockland Immunochemicals) and Alexa Fluor 680 (Molecular Probes) with an Odyssey infrared imaging system (LiCor Bioscience). Signals were approximately linear in the range where these bands were measured, but for some of the sumoylated species and for unmodified Flp1 in wild-type cells, the signal was not dramatically higher

than background (sometimes only two- to threefold), so variations in the background could have substantial effects on the measurements.

Analysis of 2 μ m levels. The presence of 2 μ m was assessed by colony PCR as previously described (42) using the above primers from the 3' end of *REP1*. All of the primers listed above are effective in determining whether 2 μ m is present by colony PCR.

For quantitative real-time PCR (qPCR), yeast DNA was isolated using glass beads and phenol-chloroform (17). Levels of 2 μ m were measured using a sequence from the Y' subtelomeric element as the reference. Primers for measuring 2 μ m DNA were 5'-CACAAAGATAGTACCGCAAAACGA-3' and 5'-CACTTTGCTGCTTTTCCTTAAT-3', which amplify a 65-bp region between *FLP1* and *REP2*. The Y'-derived primers were 5'-ACAATGGCCTTCGACTCTGGTTC and 5'-ATCACAGCCCGAAGAAGCACT-3'. These two amplicons had virtually identical amplification efficiencies and gave the same relative values over a 100-fold dilution of the template DNA. qPCR was carried out with an Opticon System DNA engine (MJ Research) using the DyNAmo HS SYBR green qPCR kit (MJ Research). Each reaction contained 15 μ l of SYBR Green Master Mix, optimized concentrations of one of the primer sets (0.9 μ M for each 2 μ m primer and 0.6 μ M for each reference primer), 0.2 to 20 ng genomic DNA, and distilled H₂O to a 30- μ l final volume. PCR conditions were as follows: 1 cycle at 95°C for 14 min; and 40 cycles, each consisting of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s, followed by plate reading. The cycle number for the PCR product to reach preset threshold (C_T number) was determined for three to six replicates for each DNA sample. The fold change of the 2 μ m number compared to that of wild-type yeast DNA was calculated by $2^{-\Delta\Delta C_T}$ methods (25). Values were compared by the Student *t* test.

Southern blot hybridization was performed as previously described (1). Lanes were loaded with 0.5 μ g of yeast DNA, prepared as previously described (17), and digested with PstI. The probe consisted of a 2,246-bp ScaI-PstI fragment containing 2 μ m sequences from pRS426 (5). The probe was labeled using the DIG High Prime DNA labeling kit (Roche Applied Science), and the signal was detected according to the manufacturer's instructions.

Microscopy. Cells were stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) after fixation in 90% ethanol. DAPI-stained and differential interference contrast micrographs were taken using a 63 $\times$ oil objective on a Leica DM. RXA microscope with a Cool SNAP fx digital camera (Roper Scientific) and IP Lab software (Scanalytics). Photographs of yeast colonies were taken using a Nikon tetrad dissecting microscope with a 10 $\times$ objective and 15 $\times$ ocular by holding an Olympus Stylus 300 digital camera at 3 $\times$ zoom to the ocular of the microscope.

Plasmid loss assays. To measure the loss rate of *HIS3*-marked versions of 2 μ m, single colonies from plates lacking His were grown for 15 to 25 generations in YPD, and the fraction of cells lacking the plasmid before and after growth in YPD was determined by plating ~300 cells on a YPD plate, allowing colonies to grow, and replica plating onto a plate lacking His to test for the plasmid. Loss rate was calculated as previously described (7). For Flp1-Y343F-containing *URA3*-marked plasmids, single colonies from plates lacking uracil were diluted and plated directly onto YPD plates, followed by replica plating onto plates lacking uracil. The results are the percentages of cells that have lost the plasmid while growing under selective conditions, which should represent the loss rate, assuming minimal cross-feeding within the colony.

RESULTS

***siz1 Δ siz2 Δ* mutants exist in different heritable states.** A *siz1 Δ siz2 Δ* double mutant formed colonies of varied size and cell morphology when grown at 30°C (Fig. 1) (22). Some colonies were near-wild-type sized and contained mostly small, normal-looking cells, but they also had some sectors of enlarged poorly growing cells, giving the colonies an irregular shape. Other colonies consisted entirely of the enlarged, poorly growing cells. While these often grew to contain several hundred cells, they usually could not be restreaked to form viable colonies. This effect depends on temperature: at 36°C, the highest temperature where the parental strain can grow, the double mutant is virtually indistinguishable from the wild type in its growth rate; whereas at 20°C, most cells are of the poorly growing type, and the strain is virtually inviable (22). This pattern of growth suggested that these mutant cells can grow in at least two different "states," both of which are heritable: cells

FIG. 1. *siz1Δ siz2Δ* strains form irregularly shaped colonies containing enlarged cells. Wild-type (left) or *siz1Δ siz2Δ* (right) cells were streaked on YPD plates and incubated at 30°C for 1 day (bottom) or 3 days (top). Top panels show visible colonies, while images in bottom panels were taken with a tetrad dissecting microscope (see Materials and Methods).

in one state grow well and give rise to daughter cells that mostly grow well, whereas cells in the other state grow poorly and give rise to daughter cells that also grow poorly. The evidence also suggested that at certain rates, cells in either state could generate cells in the other state. For example, the healthy colonies contained sectors of poorly growing cells, suggesting that the cell that initiated the colony was of the rapidly growing type but that slowly growing cells were occasionally generated. The evidence for conversion in the other direction was that *siz1Δ siz2Δ* double mutants derived from tetrad segregation initially formed colonies consisting exclusively of enlarged, slow-growing cells, but eventually all spore-derived colonies generated the faster-growing, more normal-looking cells (data not shown).

***siz1Δ siz2Δ* mutants have high 2 μ m copy numbers.** It was pointed out to us that irregular colony shape, cold sensitivity, and G₂/M arrest are characteristics of strains containing the *nibbled* (*nib*) allele, which was originally identified in a strain of *Saccharomyces carlsbergensis* (18, 19). These phenotypes of *nib* are associated with a high copy number of the 2 μ m circle. Consequently, we asked whether 2 μ m copy number was altered in *siz* mutants. *siz1Δ* and *siz2Δ* single mutants had 2 μ m copy numbers similar to that of the wt, but *siz1Δ siz2Δ* double mutant isolates had 7- to 40-fold-higher levels, as measured by quantitative PCR (Fig. 2A and B). 2 μ m levels varied substantially from isolate to isolate, which was not surprising, because these cultures were very heterogeneous in their growth rates. *siz1Δ siz2Δ* strains seemed to adapt as they were maintained in culture so that they grew better and had lower 2 μ m copy number levels. The isolate shown in Fig. 2A, lane 6, grew notably better than the others analyzed in the experiment shown in this figure (data not shown).

We also tested whether the cold sensitivity of *siz1Δ siz2Δ*

correlated with differences in 2 μ m copy numbers at different temperatures. Four large round *siz1Δ siz2Δ* colonies growing at 36°C were each split and restreaked on YPD plates and grown for 2 days at 20°C, 30°C, and 36°C. Cells were grown on plates to prevent rapidly growing lineages from taking over the culture. 2 μ m copy numbers were then measured (Fig. 2B). Cells grown at 36°C contained 2- to 5-fold-higher levels of 2 μ m than wt cells, but cells grown at 30°C or 20°C contained 15- to 40-fold-higher levels. There was no significant difference in copy number between cells grown at 20°C and 30°C, even though the strain grew better at 30°C than at 20°C. Thus, 2 μ m levels partially correlated with growth rate, consistent with the idea that the higher the 2 μ m copy number, the slower the growth rate. There may be some other factor that also contributes to slow growth at 20°C.

2 μ m is responsible for major growth defects of *siz1Δ siz2Δ* mutants. To test whether absence of 2 μ m can suppress the cold sensitivity, heterogeneity, and abnormal colony and cell morphology of the *siz1Δ siz2Δ* strain, we constructed a diploid

FIG. 2. *siz1Δ siz2Δ* strains contain elevated levels of the 2 μ m circle. (A) Genomic DNA was isolated from cultures of the indicated strains grown in YPD at 30°C. Equal amounts of DNA were digested with PstI and analyzed by Southern blotting. The four *siz1Δ siz2Δ* lanes represent four different isolates of the same strain (EJY326). Numbers under the lanes represent the relative amounts of 2 μ m DNA in each of these DNA samples as determined by qPCR (see Materials and Methods). Levels were normalized relative to that of the wild type. (B) Single large round colonies of wt (JD52) or *siz1Δ siz2Δ* (EJY326) strains that had been grown on YPD plates at 36°C were split and restreaked on three different YPD plates and incubated at 36, 30, or 20°C for 2 days. Genomic DNA was isolated from the resulting cells and analyzed by qPCR (see Materials and Methods). Each of the grouped sets of results represents cells from the same original colony grown at each of the three temperatures. Error bars reflect the difference between replicate PCRs using the same DNA sample.

FIG. 3. Many phenotypes of the *siz1Δ siz2Δ* strain are suppressed by elimination of 2μm. (A) Cells of the indicated genotypes were streaked on a YPD plate and incubated at 20°C for 4 days. (B) Microscopy of *wt* (top), *siz1Δ siz2Δ [cir⁺]* (middle), or *siz1Δ siz2Δ [cir⁻]* (bottom) cells grown at 30°C using differential interference contrast (DIC) Nomarski optics (left) or DAPI staining (right). Bar, 5 μm. (C) Whole-cell lysates from cultures of the indicated genotypes were analyzed by SDS-PAGE and immunoblotting with an Ab against Rad9. Bands corresponding to Rad9 are indicated.

[*cir⁰*] strain that was heterozygous for both *siz1Δ* and *siz2Δ* (see Materials and Methods). This diploid strain sporulated like the wild type; all spores derived from this strain, including *siz1Δ siz2Δ* segregants, germinated and grew to produce colonies that were homogenous, grew well at all temperatures and were virtually indistinguishable at a superficial level from those of the wild type (Fig. 3A). If we reintroduced 2μm into the haploid *siz1Δ siz2Δ [cir⁰]* strain by transforming it with genomic DNA from a yeast strain where a marker had been introduced into 2μm, the transformed colonies were cold sensitive, showed the nibbled appearance, and contained enlarged arrested cells (Fig. 3A and data not shown). These results demonstrate that absence of 2μm suppresses *siz1Δ siz2Δ* phenotypes. Cultures of the *siz1Δ siz2Δ [cir⁰]* strain also contained many fewer enlarged cells with undivided nuclei than did *siz1Δ siz2Δ [cir⁺]* cultures (Fig. 3B; Table 2). However, the *siz1Δ siz2Δ [cir⁰]* strain did contain slightly more of these arrested cells than did a *wt* culture (Table 2), suggesting that this defect is not entirely dependent on 2μm. Table 2 shows the percentages of G₂/M-phase (undivided nucleus) and M-phase (divided nucleus) cells present in cultures that had been exposed to α-factor, which causes cells to arrest in G₁. α-Factor was used to facilitate the counting of cells arrested at other points in the cell cycle. We do not believe that *siz* mutations or 2μm affected the response to α-factor itself, because most cells arrested and formed mating projections like *wt* and because we also saw arrested cells in log-phase cultures.

We also observed that *siz1Δ siz2Δ [cir⁺]* strains contained high levels of slow-migrating forms of Rad9 (Fig. 3C), which likely represent the hyperphosphorylated forms that are generated upon DNA damage (9, 40). Levels of these forms of Rad9 were dramatically reduced in the *siz1Δ siz2Δ [cir⁰]* strain and were restored by reintroducing 2μm (Fig. 3C). This result

suggests that some feature of 2μm in these cells activates the Rad9-dependent checkpoint. However, the *siz1Δ siz2Δ [cir⁰]* strain contained reproducibly higher levels of these forms than did the wild type, suggesting that there is some 2μm-independent activation of the Rad9-dependent checkpoint in this mutant.

The strong suppression of the *siz1Δ siz2Δ* strain's phenotypes by the loss of 2μm prompted us to test whether the absence of 2μm would also suppress the lethality of a strain entirely lacking SUMO, which in *S. cerevisiae* is encoded by the *SMT3* gene. We sporulated a *siz1Δ/SIZ1 siz2Δ/SIZ2 smt3Δ/SMT3 [cir⁰]* diploid and found that all *smt3Δ* segregants arrested permanently after only three to four cell divisions, indicating that the lethal phenotype of the *smt3Δ* strain does not result from a defect in 2μm copy number control. We also assayed a number of other previously isolated SUMO pathway mutants for 2μm and found that our isolates of *ts* mutants in *ubc9* (*ubc9-P69S*) (35), *uba2* (*uba2-ts10*) (23, 33) and *ulp1* (*ulp1-333*) (24) were all [*cir⁰*], even though their parental strains were [*cir⁺*] (Fig. 4). This result suggests that either

TABLE 2. Percentage of cells at various stages of the cell cycle in α-factor-arrested cultures^a

Strain (genotype)	Stage		
	G ₁	G ₂ /M	M
JD52 (<i>wt [cir⁺]</i>)	99.8 ± 0.1	0.2 ± 0.1	
EJY341 (<i>wt [cir⁰]</i>)	99.7 ± 0.2	0.3 ± 0.2	
EJY326 (<i>siz1Δsiz2Δ [cir⁺]</i>)	79 ± 2	17 ± 2	4.0 ± 0.8
EJY344 (<i>siz1Δsiz2Δ [cir⁰]</i>)	97.8 ± 0.2	1.7 ± 0.3	0.5 ± 0.2

^a Cultures were treated with α-factor for 3 h. A total of >1,500 cells of each strain were counted.

FIG. 4. Isolates of many SUMO pathway mutants are $[cir^0]$. Previously isolated SUMO pathway mutants were analyzed for the presence of $2\mu m$ DNA by colony PCR (see Materials and Methods), followed by agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the band that is diagnostic for the presence of $2\mu m$.

these strains have a high loss rate of $2\mu m$, impose a selection for $[cir^0]$ derivatives, or both. Two different *ulp2* mutants that we tested were both $[cir^+]$, and when we constructed *ulp2Δ* $[cir^0]$ cells, as described above for *smt3Δ*, the *ulp2Δ* $[cir^0]$ cells still had notable growth defects, indicating that the most conspicuous phenotypes of *ulp2Δ* cells are not dependent on $2\mu m$ (data not shown).

Flp1 and Rep2 are modified by SUMO. $2\mu m$ encodes four proteins, and one possible cause of the increased $2\mu m$ copy number in *siz1Δ siz2Δ* is that it results from insufficient sumoylation of one of these proteins. The other possibility is that under-sumoylation of a yeast host protein is involved. To examine sumoylation of the $2\mu m$ -encoded proteins, we constructed strains containing versions of endogenous full-length $2\mu m$ in which each of the four proteins was C-terminally tagged with HA and His₈ tags. These strains contained only the tagged version of $2\mu m$, with none of the original native plasmid (see Materials and Methods). While the plasmids expressing tagged Rep1 and Rep2 were not maintained as well as the Flp1- and Raf1-expressing plasmids ($\sim 1\%$ loss per generation for tagged Rep1 and Rep2 versus 0.3% for Flp1 and 0.08% for Raf1) (see Materials and Methods), these loss rates are an order of magnitude lower than those seen when the plasmid segregation system is inactive (34, 42, 44). This suggested that tagged Rep1 and Rep2 were at least partially active. The Flp1-HA-His₈ and the Flp1-K375R-HA-His₈ discussed below were also active, as the plasmids containing them were present in both Flp1-convertible inversion isomers, even though the original transformants presumably contained only one isomer (data not shown). Also, a C-terminal tag has been shown previously not to affect Flp1 activity in vitro (4). However, as discussed below, none of these tagged versions of $2\mu m$, which also contained a *HIS3* marker, accumulated to as high copy numbers in a *siz1Δ siz2Δ* strain as did unmodified $2\mu m$, suggesting that the marker and/or the tags interfered somewhat with $2\mu m$ biology. Also, we suspect that Rep1-HA-His₈ was present at reduced levels relative to untagged Rep1, because similar plasmids with only the HA tag produced a much stronger signal on Western blotting with anti-HA. However, the His₈ tag is essential for this experiment.

Tagged proteins were enriched by Ni^{2+} affinity chromatography, followed by immunoblotting with Abs against the HA epitope or against Smt3 (SUMO). This experiment demonstrated that both Flp1 and Rep2 were modified by SUMO, with three modified forms of Flp1 and at least three of Rep2 (Fig.

5A). (Unsumoylated Rep2 ran as two major bands, and there appeared to be two corresponding sumoylated forms with each of one, two, etc., SUMO moieties.) It is likely that these represent SUMO attachment at three different sites on Flp1 and

FIG. 5. Flp1 and Rep2 are modified by SUMO. (A) Lysates from cells containing versions of $2\mu m$ in which different $2\mu m$ -encoded proteins were tagged with the HA epitope and a His₈ tag were subjected to Ni-NTA affinity chromatography (see Materials and Methods). Eluates were analyzed by SDS-PAGE and immunoblotting with Abs against HA (left) or Smt3 (right). Tagged proteins are indicated over the lanes. Bands corresponding to unmodified Rep1, Flp1, Rep2, and Raf1 are indicated. A band that cross-reacted with the anti-HA antibody is indicated by an asterisk. (B) Strains of the indicated genotypes containing $2\mu m$ with Flp1-HA-His₈ (left panels) or Rep2-HA-His₈ (right panels) were analyzed as in panel A using antibodies against HA (bottom panels) or Smt3 (top panels). Arrowheads indicate the unmodified proteins (there were two major bands of Rep2), and lines indicate SUMO-modified forms. Asterisks indicate bands that cross-reacted with the antibodies. Numbers under the lanes indicate the relative quantities of the unmodified Flp1 or Rep2 band, the monosumoylated band, and the amount of $2\mu m$ DNA in the same culture from which protein bands were quantified (see Materials and Methods). Quantities were normalized relative to the wild type. For Rep2, the first two sumoylated bands, which likely represent monosumoylation of the two different forms of Rep2, were pooled for protein quantification.

FIG. 6. Lys³⁷⁵ of Flp1 is a major sumoylation site, and mutating it increases the amount of Flp1 protein. (A) Yeast strains of the indicated genotypes contained either native 2 μ m or 2 μ m with wild-type Flp1-HA-His₈ or Flp1-K375R-HA-His₈, as indicated. Analysis was as described in the legend to Fig. 5 with immunoblotting with anti-Smt3 (top) or anti-HA (bottom). Designations are as described in the legend to Fig. 5, except that sumoylated bands are designated with open circles placed on top of the image. Numbers under the lanes indicate the quantity of unsumoylated Flp1 relative to that in the strain with wild-type tagged Flp1. (B) Genomic DNA was isolated from cells of the indicated genotypes containing the indicated version of 2 μ m and analyzed by qPCR (see Materials and Methods). HH, HA-His₈ tag. Quantities were normalized to the value in wt cells containing unmodified 2 μ m. Error bars represent variations between DNA preparations from three separate isolates of each strain. (C) Microscopy of colonies from wild-type strains containing HA-His₈ versions of wild-type Flp1 (top) or K375R-Flp1 (bottom) that were grown for 2 days on a YPD plate at 20°C.

Rep2. With a very long exposure, we could also detect a band that might be a sumoylated form of Rep1 (data not shown). Next, versions of 2 μ m containing tagged Flp1 and Rep2 were introduced into *siz1Δ*, *siz2Δ*, and *siz1Δ siz2Δ* strains, and the same experiment was performed (Fig. 5B). Several results were notable in the Flp1 experiment. One was that the fraction of Flp1 that was sumoylated was increased ~2 to 3-fold in the *siz2Δ* strain but decreased by about 95% in the *siz1Δ siz2Δ* mutant. Also, there was a 5- to 10-fold more Flp1 protein in the *siz1Δ siz2Δ* mutant than in the wild type. A priori, this increase in Flp1 levels could be either a cause or an effect of the high 2 μ m levels in this mutant. However, these tagged, marked versions of 2 μ m did not accumulate to as high copy numbers in the *siz1Δ siz2Δ* strain as did unmodified 2 μ m (data not shown; Fig. 5B), and analysis of a DNA sample taken from the same culture as the protein sample showed that the cells where Flp1 levels were almost 10-fold higher contained only about 2-fold more 2 μ m DNA (Fig. 5B). This result suggests that high levels of 2 μ m cannot fully account for the increase in Flp1 levels. In contrast to Flp1, the fraction of sumoylated Rep2 was only slightly lower (20 to 50%) in the *siz1Δ siz2Δ* double mutant

than in the wild type. These results are consistent with a model where the *siz1Δ siz2Δ* mutant affects 2 μ m via an effect on Flp1.

To test this, we wanted to mutate the SUMO attachment sites in Flp1. The fact that there were three apparent bands of sumoylated Flp suggests that Flp1 is sumoylated at three separate sites. However, Flp1 contains only one sequence that matches the SUMO attachment site consensus sequence, at Lys³⁷⁵ in the sequence LKDE. (Rep2 contains no consensus sequences.) We constructed a version of 2 μ m where HA-His₈-tagged Flp1 contained a Lys-to-Arg mutation at this position and found that this dramatically reduced the levels of sumoylated Flp1 (Fig. 6A), suggesting that Lys³⁷⁵ is a major site of Flp1 sumoylation. The two remaining sumoylated forms in this mutant migrated more rapidly during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than the corresponding wild-type forms did, suggesting that they were different from those seen in the wild type, possibly because they contained linkages at different sites. Furthermore, in the *siz1Δ* mutant especially, there were two bands of the approximate size of monosumoylated Flp1, which may represent forms that are each monosumoylated a different site. These results are

consistent with a model where Lys³⁷⁵ is a major sumoylation site in Flp1 but where there are at least two other sites.

Like the *siz1Δ siz2Δ* mutant, the wild-type (*SIZ1 SIZ2*) strain containing Flp1-K375R had increased levels (~4-fold) of Flp1 (Fig. 6A), suggesting that deficient sumoylation of Flp1 may result in higher Flp1 levels. *SIZ1 SIZ2* strains containing Flp1-K375R-HA-His₈ also reproducibly had slightly higher levels of 2μm DNA than cells containing wild-type Flp1-HA-His₈ (Fig. 6B). The difference was quite small, only ~2-fold, but significant, with a *P* value of <0.05. Similar results were also obtained using different plasmids that expressed Flp1-HA and Flp1-K375R-HA. This result is consistent with a model where deficient sumoylation of Flp1 contributes to increased 2μm levels. However, levels of 2μm in the *SIZ1 SIZ2* Flp1-K375R-HA-His₈ strain were significantly lower than in *siz1Δ siz2Δ* strains containing either wild-type or mutant Flp1. This result suggests that reduced sumoylation at Lys³⁷⁵ only partially accounts for the increased 2μm levels in *siz1Δ siz2Δ* strains. The difference between the *siz1Δ siz2Δ* strains containing wild-type versus mutant Flp1 was not significant. Consistent with a partial role for sumoylation of Flp1 Lys³⁷⁵ in maintaining normal 2μm levels, the *SIZ1 SIZ2* strain containing Flp1-K375R-HA-His₈ produced slightly more abnormal colonies when grown at 20°C than did a strain with wild-type Flp1-HA-His₈ (Fig. 6C). In general, however, the Flp1-K375R-HA-His₈ strain grew at approximately wild-type rates at all temperatures. To confirm that accumulation of 2μm in *siz1 siz2* strains was indeed Flp1 dependent, we examined 2μm copy number and plasmid loss rates of a version of 2μm in which the active site Tyr-343 of Flp had been mutated to Phe. The copy number of this plasmid was the same in wt and *siz1 siz2* strains (0.5 ± 0.1 for wt cells versus 0.6 ± 0.3 for *siz1 siz2* cells, both values relative to the copy number of native 2μm in wt [JD52] cells), as was the plasmid loss rate ($6\% \pm 6\%$ for the wt versus $6\% \pm 3\%$ for *siz1 siz2*). A similar experiment with a 2μm-based plasmid completely lacking Flp1 (pAS4) (34) gave similar results (data not shown). As would be expected, *siz1 siz2* strains containing these plasmids did not contain high fractions of abnormal cells. In summary, these results show conclusively that the Flp1-K375R mutation increases Flp1 protein levels and suggest that deficient sumoylation of Lys³⁷⁵ may partially account for the effect of *siz1Δ siz2Δ* mutants on 2μm copy numbers.

DISCUSSION

We investigated the cold sensitivity, irregular colony shape, and cell cycle delay of *siz1Δ siz2Δ* mutants and found that all of these phenotypes are dependent on the endogenous 2μm circle plasmid. *siz1Δ siz2Δ* cultures contained up to 40-fold-higher levels of 2μm than the wild type, and this high copy number is likely to be responsible for the phenotypes. We also found that Flp1 and Rep2, two of the four proteins encoded by 2μm, were modified by SUMO. Flp1 sumoylation was dramatically reduced in the *siz1Δ siz2Δ* mutant, and this strain also contained significantly increased levels of Flp1 protein, suggesting that the SUMO pathway may negatively regulate Flp1. Additional support for this idea comes from the observation that mutating a major SUMO attachment site in Flp1 also increased Flp1 protein levels. Increased expression of *FLP1* has been shown previously to increase 2μm levels (29, 32), raising the possibil-

ity that the high Flp1 levels may be partially responsible for the accumulation of 2μm in these strains.

While 2μm is responsible for the more conspicuous defects of the *siz1Δ siz2Δ* strain, 2μm copy number control is not the only function of *SIZ1* and *SIZ2*. Siz1 and Siz2 are responsible for sumoylating many proteins, most of which presumably do not affect 2μm. For example, sumoylation of yeast PCNA requires *SIZ1*, and there are DNA repair defects associated with the loss of PCNA sumoylation (14, 16, 37). Furthermore, *siz1Δ siz2Δ* [cir⁰] strains contained slightly higher levels of highly phosphorylated Rad9 than did wt cells, suggesting that they contain damage in their chromosomal DNA.

Previous discussions of the *nib* phenotype have argued that *nib* could either be the cause of high 2μm levels or that the *nib* strains might merely be hypersensitive to higher than normal levels of 2μm (2, 18). Absence of *SIZ1* and *SIZ2* clearly caused the elevated 2μm copy number here. Entire cultures grown from *siz1Δ siz2Δ* [cir⁺] segregants, which were derived from heterozygous diploid strains with normal 2μm copy numbers, had dramatically higher copy numbers than wild-type segregants from the same tetrads. The cells in these colonies were uniformly sick within a few cell divisions of germination and only later developed sectors that grew better and had lower 2μm levels. The role of *SIZ1* and *SIZ2* in 2μm biology is, almost certainly, to promote SUMO conjugation; there is currently no evidence that *SIZ1* and *SIZ2* have any functions independent of SUMO conjugation, although the related mammalian PIAS proteins can have SUMO-independent effects (20). Furthermore, other mutants in the SUMO pathway also have 2μm-related defects. The original *nib* allele has been mapped to a region near *RAD1* (19), which is very close to *ULP1*, a gene encoding a SUMO cleaving enzyme. Recently, it has been shown that *nib* is indeed an allele of *ULP1* (M. Dobson, personal communication). Very recently, it has also been shown that a mutant that mislocalizes Ulp1 also accumulates 2μm (45). We also found that our isolates of *ts* mutants in *ubc9*, *uba2*, and *ulp1* are all [cir⁰], although their parental strains are [cir⁺], suggesting that growth of these strains may have selected for the loss of 2μm.

What are the SUMO substrates involved in preventing accumulation of 2μm? Our results suggest that sumoylation of Flp1 may account at least partially for this role of the SUMO pathway. Flp1 sumoylation was at near or above wild-type levels in *siz1Δ* and *siz2Δ* single mutants but was reduced by about 95% in the double mutant. This result is consistent with the genetics of 2μm accumulation, where 2μm levels were about the same as in the wt in both single mutants but were dramatically increased in the double mutant. Furthermore, total amounts of Flp1 were increased 5 to 10 fold in the *siz1Δ siz2Δ* double mutant. This suggests a possible mechanism for how 2μm levels become elevated, as it has been shown that high levels of Flp1 are sufficient to increase 2μm copy number (29, 32). On the other hand, one could argue that reduced sumoylation of some other protein might cause the increase in 2μm levels and that the increased 2μm levels would cause the increase in Flp1. This idea is contrary to the model for feedback regulation of *FLP1* expression and is also made less likely by our observation that a strain containing 10-fold-higher levels of Flp1 contained only ~2-fold-more 2μm than the wild type. Another argument that changes in Flp1 levels may be the

cause rather than the effect is that the Flp1-K375R mutant, which eliminated a major sumoylation site in Flp1, also showed elevated levels of Flp1 protein. In this case, elevated Flp1 levels cannot be a secondary effect from deficient sumoylation of a different protein. Of course, this mutation could have effects on Flp1 other than just reducing sumoylation, but the fact that this mutation and the *siz1 Δ siz2 Δ* mutant had similar effects is consistent with a model where sumoylation of Flp1 at this site leads to a reduction in Flp1 protein levels. Lys³⁷⁵ is in a C-terminal domain of Flp1 that is not present in the related Cre recombinase (3), consistent with the possibility that this is a regulatory domain unique to Flp1.

However, reduced sumoylation of Flp1 at Lys³⁷⁵ does not account fully for the high levels of 2 μ m seen in *siz1 Δ siz2 Δ* mutants. Significantly higher levels of Flp-K375R-containing 2 μ m DNA were present in the *siz1 Δ siz2 Δ* strain than in the wild-type strain, indicating that *SIZ1* and *SIZ2* have other roles in 2 μ m control. This difference could reflect a requirement for *SIZ1* and *SIZ2* in attaching SUMO to Rep2 (or possibly Rep1), to host proteins or to other sites in Flp1. There was not a dramatic difference in sumoylation levels of Rep2 in the *siz1 Δ siz2 Δ* strain compared to the wild type, which might suggest that Rep2 is less likely to play a major role in the phenomenon. Sumoylation of the other sites in Flp1 was Siz dependent. Several yeast proteins involved in 2 μ m partitioning have recently been shown to be sumoylated, including the chromatin remodeling complex subunit Rsc2 and the cohesin subunits Smc1 and Scc1/Mcd1 (27, 41, 42, 44). It is not known to what extent sumoylation of these proteins is controlled by Siz1 and Siz2.

While this discussion has focused on how alterations in Flp1 could produce high levels of 2 μ m DNA, defects in plasmid partitioning could also lead to accumulation of 2 μ m. Unequal plasmid segregation would be followed by amplification in the progeny cell (most likely the daughter cell) that receives the lower level of plasmid. This would cause an overall accumulation of 2 μ m, as long as complete loss of the plasmid was infrequent. Flp1 would be required for 2 μ m accumulation under this model as well: 2 μ m copy numbers would not be expected to rise overall without Flp1-dependent amplification in the cells receiving fewer copies. Our results using Flp1-deficient versions of 2 μ m showed that 2 μ m is indeed amplified by a Flp1-dependent mechanism in *siz1 siz2* strains. This is consistent with either the hyperactive Flp1 model or the defective partitioning model for accumulation of 2 μ m.

Further studies will be needed to resolve the many remaining questions about this system, including the identities of the other SUMO attachment sites in Flp1 and Rep2, the roles of other host proteins in controlling SUMO-dependent 2 μ m-related functions, and the mechanism by which 2 μ m amplification is triggered in some cells but not in others.

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