Investigation of Post-Translational Modification and Function of the Yeast Plasmid Partitioning Proteins Rep1 and Rep2

by

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ABSTRACT

The 2-micron circle of *Saccharomyces cerevisiae* is one of a small number of similar DNA plasmids found only in budding yeast. To understand how this cryptic parasite persists, despite conferring no advantage to the host, I investigated the plasmid-encoded Rep1 and Rep2 proteins. Interaction of Rep1 and Rep2 with each other and with the plasmid *STB* locus is required for equal partitioning of plasmid copies at mitosis. The Rep proteins also repress expression of Flp, the recombinase that mediates plasmid copy-number amplification. In this study, absence of Rep1 and Rep2, or over-expression of the plasmid-encoded Raf anti-repressor, increased expression of a longer, novel *FLP* transcript. Translation of this mRNA may explain elevated Flp activity at low plasmid copy number. Raf competed for Rep2 self-association and interaction with Rep1, suggesting the mechanism of Raf anti-repression. Deletion analysis identified a target site for Rep protein repression of *FLP* that is also repeated in the *STB* locus, suggesting this as the sequence required for Rep protein association with both regions of the plasmid.

Distinct roles for Rep1 and Rep2 were identified; Rep1 was found to depend on Rep2 for post-translational stability, with Rep2 dependent on Rep1 for stable association with *STB*. Lysine-to-arginine substitutions in Rep1 and Rep2 impaired their association with the host covalent-modifier protein SUMO, suggesting these were sites of sumoylation. The substitutions did not affect interaction of the Rep proteins with each other or their stability but did perturb plasmid inheritance, suggesting that Rep protein sumoylation contributes to their plasmid partitioning function. When Rep1 was mutant, both Rep proteins lost their normal localization to the nuclear foci where 2-micron plasmids cluster, and were impaired for association with *STB*, supporting this as the cause of defective plasmid inheritance. The potential sumoylation-dependent association of the Rep proteins with the 2-micron plasmid partitioning locus suggests the plasmid has acquired a strategy common to eukaryotic viral and host genomes that depend on sumoylation of their segregation proteins for faithful inheritance. Collectively, my results shed light on how the 2-micron plasmid maintains the delicate balance of persisting without harming its host.

LIST OF ABBREVIATIONS AND SYMBOLS USED

2D	two-dimensional
aa	amino acid
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
BSA	bovine serum albumin
ChIP	chromatin immunoprecipitation
CIAP	calf intestinal alkaline phosphatase
DAPI	4',6-diamidino-2-phenylindole
DIC	differential interference contrast
DIG	digoxygenin
EDTA	ethylenediaminetetraacetic acid
FRT	Flp recognition target
GFP	green fluorescent protein
GST	glutathione-S-transferase
HMW	higher-molecular-weight
IR	inverted repeat
KEOPS	kinase endopeptidase and other proteins of small size
MW	molecular weight
NP-40	Nonidet P-40
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RBE	Rep binding element
SC	synthetic complete
SD	synthetic defined
SDS	sodium dodecyl sulfate
SBP	spindle pole body
SUMO	small ubiquitin-related modifier
UTR	untranslated region
YPD	yeast extract peptone dextrose

Yeast nomenclature

ABC1	wild-type gene
abc1	mutant gene
*abc1*Δ	gene deletion
Abc1	wild-type protein
$Abc1_{XX}$	mutant protein

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CHAPTER 1. INTRODUCTION

1.1 The Budding Yeast 2μm Plasmid

The 2μm plasmid (or 2μm circle) of the baker's yeast *Saccharomyces cerevisiae* is found in virtually all laboratory and industrial strains and is one of a small number of related plasmids found only in the Saccharomycetaceae family of budding yeasts. These plasmids are effectively cryptic parasites, conferring no significant phenotype or benefit to the host, and yeast lacking the plasmid (cir^0) exhibit a slight (~1%) growth advantage over yeast containing the plasmid (cir^+) (64). Despite this, these plasmids are faithfully maintained at high copy number in the host nucleus, with their only known functions being regulation of their copy number, and equal partitioning of plasmid copies between the mother and daughter cell during host cell division (for a review, see 63). Plasmid partitioning, while not fully understood, requires the association of two plasmid-encoded proteins, Rep1 and Rep2, with the *cis*-acting plasmid stability locus, *STB* (1, 116, 126, 190, 192, 229, 230). In addition to their partitioning function, Rep1 and Rep2 also regulate plasmid gene expression, and in particular are required for controlling plasmid copy number through repression of the plasmid-encoded *FLP* gene, encoding a recombinase that mediates copy-number amplification (162, 180, 200, 228).

The overall goal of my research was to investigate how the Rep proteins function at different sites in the 2μm plasmid to maintain the remarkable persistence of the plasmid in its eukaryotic host. I have identified novel and distinct roles for Rep1 and Rep2 in assembly of the partitioning complex at the plasmid *STB* locus, and provide evidence for host-mediated post-translational modification of both Rep1 and Rep2 by a ubiquitin-like protein, SUMO, and for this modification being functionally significant. My findings also provide new insight into the mechanism of regulation of *FLP* gene expression by plasmid-encoded proteins.

1.1.1 Organization of the *S. cerevisiae* 2μm Circle Genome and Similarities to Other Yeast Plasmids

The *S. cerevisiae* 2μm plasmid (**Figure 1**) contains four protein-coding genes, *FLP*, *REP1*, *REP2*, and *RAF* (93, 210). The organization of the 2μm plasmid is similar to that of

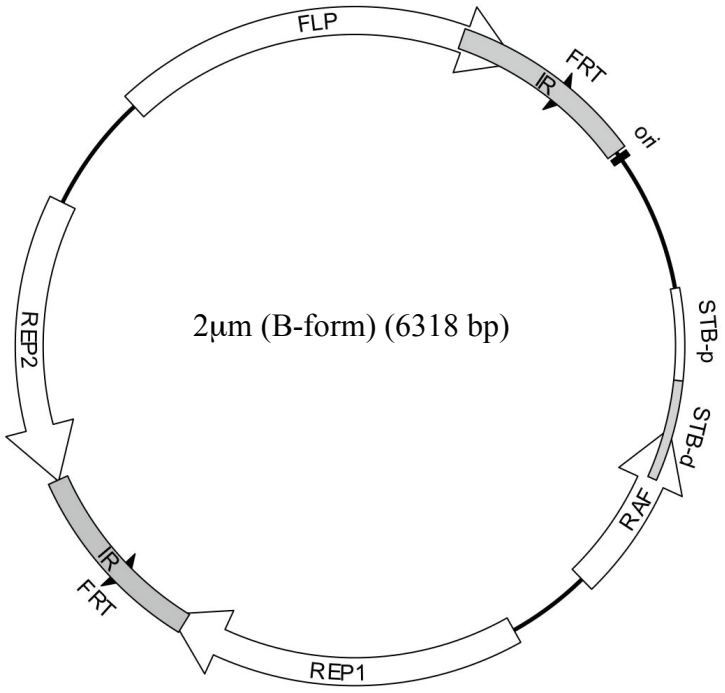

Figure 1. Map of the *S. cerevisiae* 2μm plasmid. Open reading frames (arrows), origin of replication (ori), *STB*-proximal (STB-p), *STB*-distal (STB-d), inverted repeats (IR), and Flp-recognition targets (FRT) are indicated.

2μm-like plasmids in other yeasts closely related to *S. cerevisiae*, including *Zygosaccharomyces rouxii* (219) *Zygosaccharomyces bisporus* (218) *Kluyveromyces lactis* (35, 58) *Kluyveromyces waltii* (34) *Zygosaccharomyces fermentati, Zygosaccharomyces bailii* (224) and *Torulaspora delbrueckii* (14). Plasmids harboured by these yeast species are similar in size and encode three or four predicted open reading frames (ORFs). The most striking feature that they share is the presence of a long sequence repeated in opposite orientations in two regions nearly opposite to one another in the plasmid, with one repeat adjacent to an autonomously replicating sequence (*ARS*) that functions as an origin of replication (20). In the yeast, the plasmids exist in equimolar amounts of two forms, A and B, produced by recombination between the repeats (141). This recombination is catalyzed by a site-specific recombinase called Flp, encoded by the largest ORF on all these plasmids (4, 21, 83, 149). The site recognized by Flp (*FRT*) is found in both inverted repeats (IRs) for each plasmid. The Flp protein sequence is highly conserved between yeast plasmids and is similar to the site-specific recombinase Cre encoded by bacteriophage P1 (5, 161). The other proteins encoded by the yeast plasmids lack obvious homologues.

1.1.2 Replication and Copy Number Amplification of the 2μm Circle

Studies with the *S. cerevisiae* 2μm plasmid have revealed how Flp and the conserved organization of the plasmids are involved in copy number amplification. The copy number of the 2μm plasmid is kept at a consistent level within a cell population, with strains of yeast differing in the average copy number maintained (70, 85). For some haploid yeast strains the average copy number is close to 40, while for others it is about 90 (65). Following transformation of a single 2μm plasmid into a yeast cell lacking the plasmid, the normal high plasmid copy number is restored by the time the cell forms a colony (197), indicating that the plasmid encodes an amplification system that can rapidly recover from a drop in copy number. Unlike bacterial plasmids, where DNA replication can be initiated multiple times and is unlinked to cell division (157), the 2μm plasmid origin, like all eukaryotic chromosomal origins, fires only once during S phase of the cell cycle (257). Experimental evidence supports Futcher's model of a double-rolling-circle mechanism to explain how Flp-mediated recombination between the two *FRT* sites can create multiple copies of the plasmid from a single firing of the origin (62, 231). Shortly following replication initiation, one

replication fork passes the *FRT* site located proximal to the origin. Flp-catalyzed recombination between the two *FRT* sites then orients the two replication forks in the same direction such that they chase each other around the plasmid, leading to formation of a multimeric circle containing tandem copies of 2μm. A second recombination event allows the replication forks to converge, and finally, Flp- or host-mediated recombination resolves the multimeric plasmid into monomers. Flp-mediated recombination between the two *FRT* sites leads to the two topologically different but functionally equivalent "A" and "B" forms of the plasmid.

1.1.3 The 2μm Circle Partitioning System

The single *ARS* on the 2μm plasmid that functions as the origin of replication does not differ from *ARS* elements of host chromosomes. The *ARS* elements were originally identified by their ability to confer replication competence to plasmids in yeast. Although these *ARS*-only artificial plasmids can replicate, they fail to be efficiently inherited and are retained in the mother cell during ~90% of cell divisions, with the daughter cell receiving no copies of the plasmid, a phenomenon called maternal bias of inheritance (159). A recent study has demonstrated that the maternal inheritance bias likely arises from the combination of the slow diffusion rate of plasmids, and rapid mitosis, which limits the probability that plasmids will traverse the narrow junction between the mother and daughter nucleus at anaphase before the onset of cytokinesis (68). In contrast to *ARS*-only plasmids, the 2μm circle overcomes this maternal inheritance bias to achieve efficient transmission of plasmid copies to the daughter cell at mitosis. Given that rapidly growing haploid cells produce a 2μm plasmid-free daughter only about once in every ten thousand cell divisions (64), and no detectable rate of plasmid loss has been observed in diploid cells, which contain twice the plasmid copy number of haploids (150), the efficiency of the 2μm plasmid partitioning mechanism is striking. Early studies identified the 2μm-encoded components that are both necessary and sufficient for partitioning function as the Rep1 and Rep2 proteins, and the *STB* locus (116, 126).

1.1.3.1 *STB*

STB can be divided into two functional domains, *STB*-proximal and *STB*-distal, named for their relative distances from the origin (160) (see **Figure 1**). *STB*-proximal consists of an array of tandem, imperfect AT-rich 62-63 bp repeats (93). The 2μm plasmid in standard laboratory *S. cerevisiae* strains contains 5.5 copies of the repeated sequence, while the number of repeats varies from two to nine for variants of the 2μm plasmid isolated from various laboratory and brewing strains of *S. cerevisiae* (118, 126, 244). *STB*-distal contains a transcription termination sequence that protects *STB*-proximal from transcription of the adjacent *RAF* gene (63, 66, 160, 210). As seen for centromeres (38), transcription through *STB* impairs its segregation function. *STB*-distal also contains a transcriptional silencer that represses expression of all plasmid transcripts, including non-coding transcripts, but the functional significance of this silencing activity is not known (160).

1.1.3.2 Rep1 and Rep2

Rep1 is predicted to be a 43.2-kDa 373-aa protein with a potential coiled-coil domain near its carboxy-terminus (240), but otherwise shares no similarity with any other known proteins beyond its related Rep1 homologues in 2μm-like plasmids. Rep2 is predicted to be a 33.2-kDa 296-aa, basic (pI = 9.9) protein, and has no homologues in any known species. Other 2μm-like plasmids encode a protein that is functionally analogous to Rep2, but exhibits no significant sequence similarity. Rep1 and Rep2 each contain a C-terminal nuclear-localization signal (229), and immunofluorescence experiments have demonstrated that Rep1 and Rep2 are both exclusively nucleus-localized (1, 190, 191, 229, 230).

In vivo two-hybrid protein interaction assays and *in vitro* baiting experiments have demonstrated that Rep1 and Rep2 interact with each other, and can each self-associate (1, 190, 192, 229). Interaction of Rep1 with Rep2 is required for plasmid partitioning, since point substitutions in Rep1 or Rep2 that impair their association cause plasmid instability (254, Arpita Sengupta, Dalhousie University, PhD thesis). The amino-terminal third (residues 1-129) of Rep1 is sufficient for interaction with both full-length Rep1 and Rep2 in two-hybrid and *in vitro* baiting assays (192), and Rep1 and Rep2 have been shown to compete for binding to Rep1 *in vitro* (190). It has yet to be established whether self-association of either Rep1 or Rep2 is functionally significant for plasmid partitioning.

1.1.3.3 Stoichiometry of Rep1 and Rep2

Despite each cell containing multiple copies of the 2μm plasmid and therefore of the *REP1* and *REP2* genes, Rep1 and Rep2 are not abundant proteins (our unpublished results). Rep1 is estimated to be approximately ten times more abundant than Rep2, consistent with the observation of a ~10-fold higher level of *REP1* mRNA than *REP2* mRNA (162, 190). Gene dosage experiments have demonstrated that a single copy of chromosomally-integrated *REP2*, but not *REP1* is sufficient to supply partitioning function to an *STB*-containing plasmid in *trans* (30), suggesting that the levels of Rep1 may be a limiting factor in establishing a functional Rep protein-*STB* plasmid partitioning complex.

1.1.3.4 Interaction of Rep1 and Rep2 with the Plasmid Partitioning Locus, *STB*.

In vivo one-hybrid assays and chromatin immunoprecipitation (ChIP) experiments have shown that both Rep1 and Rep2 associate with the plasmid *STB* locus (151, 229). Rep1 and Rep2 are present at *STB* throughout most of the cell cycle, losing association for a brief period of time between late G1 and early S phase (254). Plasmid partitioning is dependent on association of Rep1 with *STB*, since point substitutions in Rep1 that abolish Rep1-*STB* cause plasmid mis-segregation (254). The molecular basis for association of Rep1 and Rep2 with *STB* is not clear. Studying interactions of Rep1 and Rep2 *in vitro* is particularly challenging due to the insoluble nature of Rep1 and Rep2 when purified from yeast (87, 240). Bacterially expressed Rep1 and Rep2 were shown to be able to bind to *STB* independently of one another in surface plasmon resonance assays, but for both proteins, binding required the presence of host protein extracts (87), suggesting that the Rep proteins may be dependent on interaction with a host protein for association with *STB*. In southwestern assays, Rep2 was shown to directly bind DNA, having a higher affinity for *STB* than another sequence with lower AT-content (192). Taken together, these findings suggest that Rep1 and Rep2 can independently associate with *STB in vivo*, but at least for Rep1 the association may require the presence of host proteins. Currently, no host proteins required for association of Rep1 or Rep2 with *STB* have been identified.

1.1.4 Autoregulation of Plasmid Copy Number by Transcriptional Feedback

1.1.4.1 Response of *FLP* Expression to Changes in Plasmid Copy Number

In addition to their role in plasmid partitioning, which is described in greater detail below, the Rep proteins have also been implicated in regulating 2μm plasmid copy number by repressing expression of the plasmid genes (**Figure 2**). Tight control of *FLP* gene expression is critical, as elevated plasmid copy number is toxic (36, 51, 162, 180). Hyper-amplification of plasmid copy number, induced by *FLP* over-expression, results in a "nibbled" colony morphology with cells in some sectors blocked in cell-cycle progression (162). Cells display aberrant budding patterns and cir^0 progeny are frequently produced (162), suggesting that hyper-elevated copy number also reduces the efficiency of plasmid inheritance. Several observations have suggested that the *FLP* gene is repressed once the plasmid reaches normal copy number, with repression mediated by plasmid-encoded proteins that increase in abundance as plasmid copy number rises. Cellular Flp activity has been shown to decrease as plasmid levels increase (180). Loss of functional Rep1 or Rep2 leads to a significant increase in the level of *FLP* mRNA, indicating that Rep1 and Rep2 act together to repress *FLP* (162, 200, 228). Absence of Rep1 or Rep2 has been shown to alter nucleosome organization in the *FLP* promoter region, suggesting that Rep1 and Rep2 may bind to this region of the plasmid (227), although definitive evidence for association of Rep1 and Rep2 with the *FLP* promoter has not been demonstrated. The repressive effect of Rep1 and Rep2 on *FLP* gene expression can be alleviated by over-expression of the 2μm plasmid *RAF* gene (162, 200). *RAF* is also repressed by the combined expression of Rep1 and Rep2, although the levels of Rep proteins required to repress *RAF* are higher than those needed for maximal *FLP* repression (162). Murray *et al*. (1987) proposed that anti-repressive effects of the Raf protein might be a fine-tuning mechanism that increases sensitivity to small changes in plasmid copy number. The observations suggest a mechanism of autoregulation of plasmid copy number that could allow copy number to recover following rare events of unequal plasmid partitioning at mitosis. In cells receiving a lower-than-normal number of plasmid copies, repression of *FLP* would be alleviated because there would be less Rep proteins to repress the promoters of both the *FLP* and *RAF* genes. At normal copy number, the Rep

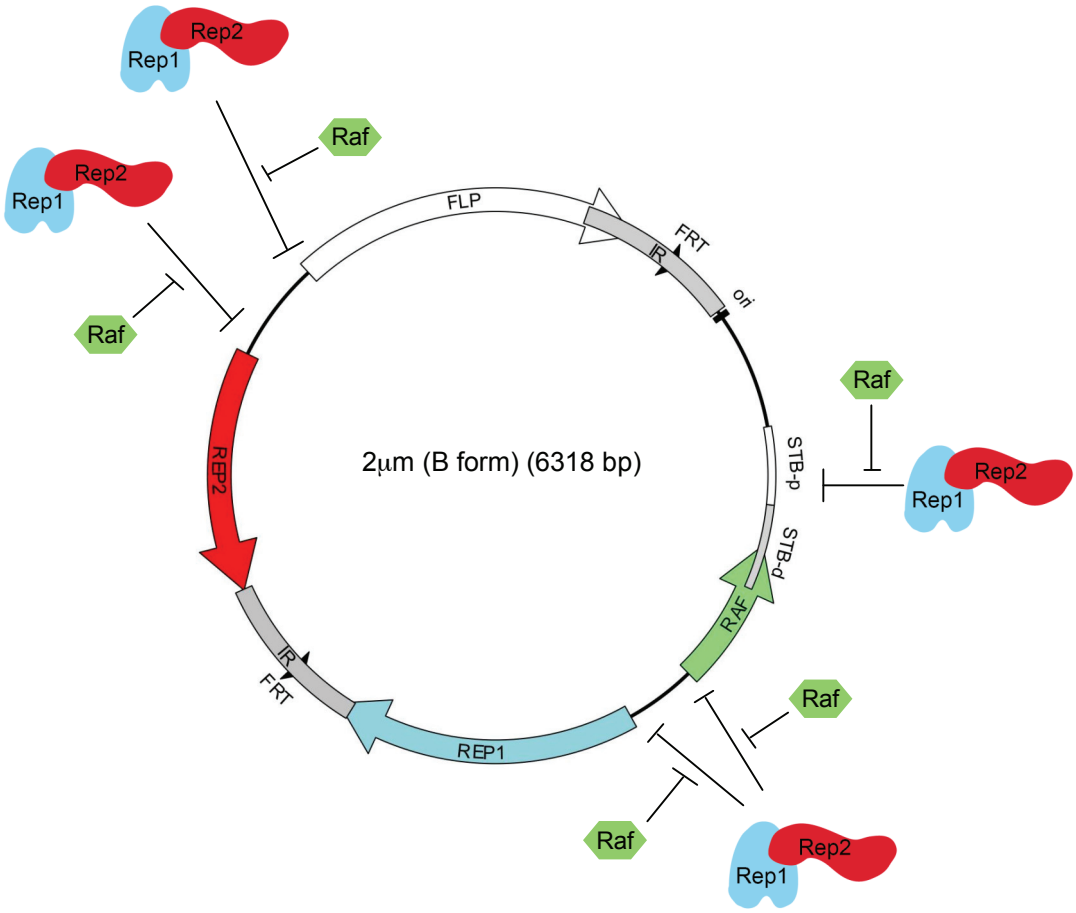

Figure 2. Model for regulation of 2μm plasmid transcription by plasmid proteins. Evidence suggests that Rep1 (blue) and Rep2 (red) together repress expression of all four protein-coding 2μm plasmid transcripts as well as non-coding transcripts originating from *STB*. Repression is antagonized by the anti-repressor Raf (green). Adapted from Velmurugan *et al*. (1998).

proteins would repress both *FLP* and *RAF* gene expression, preventing further plasmid amplification (162).

1.1.4.2 Repression of *REP1*, *REP2*, and *STB* Transcripts by Rep1 and Rep2

In addition to repressing the *FLP* and *RAF* genes, Rep1 and Rep2 also regulate expression of their own genes. *REP1* was found to be repressed by high levels of Rep1 and Rep2 (200), and in yeast expressing a temperature-sensitive *rep1* allele the levels of both *REP1* and *REP2* transcripts were increased at the non-permissive temperature (228).

Rep1 and Rep2 also regulate expression of several non-coding transcripts that are promoted by the *STB* partitioning locus (118, 228). Transcripts of ~1950 and ~600 nt initiate within *STB* and terminate in the region 5' of *REP1* and within the *RAF* ORF, respectively (118, 162, 210). The levels of these non-coding 2μm plasmid transcripts increase in the absence of functional Rep1, and also when Raf is overproduced (118, 162, 228). A function for any of these non-coding transcripts has yet to be demonstrated. The ability of the Rep proteins to repress transcription of most of the 2μm plasmid transcripts provides a clear mechanism of controlling plasmid copy number.

1.1.5 Plasmid Segregation in Bacteria

The ability of the Rep proteins to repress expression of their own genes, and their interaction with the tandem repeat sequence of the 2μm plasmid partitioning locus, is analogous to general features of partitioning of plasmids in bacteria. Homology between Flp and recombinases encoded by bacteriophage genomes suggests that yeast plasmids might be distantly related to phages. Partitioning systems of low-copy bacterial plasmids such as P1, F, and R1 (reviewed in 72, 117) is typically mediated by two plasmid-encoded "Par" proteins, which interact with each other and with a *cis*-acting "*par*" locus comprised of a repeated DNA sequence. One of the Par proteins contains an ATPase domain, and is recruited to the *par* locus through association with the other Par protein. Evidence also indicates that the Par proteins autoregulate expression of their own genes by forming a repressor-corepressor complex. This may reflect a requirement for proper stoichiometry between the two Par proteins (18). Despite the superficial similarities between the segregation systems of these bacterial plasmids with the partitioning system of the yeast 2μm plasmid, there is no

functional resemblance between the systems, reflecting the requirement to coordinate partitioning with the distinct cell division machinery of a prokaryotic versus eukaryotic host. The bacterial plasmid partitioning complex regulates localization of the plasmid, directing plasmids to the centre of the cell during replication, and closer to the two poles during cell division.

1.1.6 Co-localization of Rep1 and Rep2 with the 2μm Plasmid in Subnuclear Foci

Clues for how the 2μm plasmid is partitioned in the eukaryotic yeast host first came from immunofluorescence experiments that revealed that Rep1 and Rep2 are found in punctate foci in the nucleus (1, 190, 191, 230). Direct visualization of fluorescently tagged 2μm-based reporter plasmids in live cells suggests that 2μm plasmids are also organized in the nucleus as three to five dynamic foci that form a close-knit cluster and co-localize with Rep1 and Rep2 (191, 230). When Rep1 and Rep2 are expressed in yeast lacking an *STB*-containing plasmid, they appear to be uniformly dispersed throughout the nucleus, suggesting that their association with *STB* may be required for localization to specific subnuclear domains (191). An increase in plasmid copy number results in an increase in the size, rather than the number, of Rep protein and plasmid foci, and foci become brighter in S phase, consistent with the doubling in numbers of plasmids in these clusters (191, 230). Throughout most of the cell cycle, the plasmid foci, like those of chromosomal centromeres, are located in close proximity to spindle pole bodies (SPBs), the yeast equivalent of mammalian microtubule organizing centres (84, 119, 154, 191).

1.2 Chromosome Segregation in Budding Yeast

Although the 2μm plasmid and the Rep proteins are found in close proximity to chromosomal centromeres in the yeast nucleus, the plasmid *STB* locus shares no identity with centromeric DNA. Despite this, the plasmid is dependent on the integrity of the mitotic spindle and plasmid partitioning occurs during anaphase concomitantly to chromosome segregation, indicating that the plasmid is dependent on host for segregation (152, 230). Although the 2μm plasmid-encoded components of the partitioning system have been identified, it remains unsolved how the plasmid utilizes host cell chromosome segregation

machinery for partitioning. To fully appreciate the possible ways in which the plasmid could use the host process, the mechanism of chromosome segregation needs to be briefly reviewed. Although the mechanism is highly conserved in eukaryotes, the review focuses on what is known about the process in yeast.

1.2.1 Kinetochore-Mediated Attachment of Centromeres to the Mitotic Spindle

The centromere is the region of chromosomal DNA that specifies assembly of the kinetochore, a protein complex of more than 60 subunits (148) which mediates attachment of chromosomes to the mitotic spindle during cell division. The "inner" kinetochore proteins are tightly associated with centromeric DNA and recruit "outer" kinetochore proteins that attach chromosomes to the plus end of a spindle microtubule (103). The spindle is comprised of both nuclear and cytoplasmic microtubules, with the minus ends of both nucleated at SPBs. The plus ends of cytoplasmic microtubules are anchored in the cell cortex, and are required for correct orientation of the spindle (103). In yeast, these two classes of microtubules remain distinct during mitosis, as the nuclear membrane does not break down during cell division and the SPBs remain embedded in the nuclear membrane (24, 236).

1.2.1.1 Formation of the Inner Kinetochore at Centromeric (*CEN*) DNA

Yeast of the Saccharomycetaceae family are unusual among eukaryotes in that their centromeres are very short (~125 bp), hence are referred to as "point" centromeres, and are defined by specific sequence elements (144). In contrast, most other eukaryotes contain "regional" centromeres which are much longer, extending from a few kilobases to several megabases, and are specified epigenetically, rather than by sequence (41). Each of the 16 chromosomes in *S. cerevisiae* contains a single ~125-bp centromere consisting of three Centromere DNA Elements, CDE I-II-III, that are required for faithful segregation of chromosomes during cell division (60, 97). CDEI and CDEIII sequences are conserved in all centromeres, and while CDEII sequences are not conserved, they are all between 78-86 bp in length and have ~90% AT-content (reviewed in 33). CDEI is the binding site for Cbf1, a nonessential helix-turn-helix protein, which interacts with the CBF3 (centromere binding factor 3) complex (99), comprised of Ndc10, Cep3, Skp1, and Ctf13 (45, 132, 205). Association of the CBF3 complex with CDEIII is required for faithful chromosome segregation (67). CBF3 subunit Ndc10 mediates recruitment of Scm3, a nucleosome

assembly factor specific for forming a centromere-specific nucleosome in which the canonical histone H3 is replaced with the histone H3 variant Cse4 (28, 196). The entire centromere is wrapped around a Cse4-containing nucleosome, which protects 123-135 bp of DNA, rather than the 146 bp that is wrapped around canonical nucleosomes (44). Cse4 and its mammalian homologue, CENP-A, are found only at functional centromeres and are considered the epigenetic marker of eukaryotic centromeres (2, 153, 207). Cse4 and CBF3 are required for binding of the inner kinetochore protein Mif2 (homologue of human CENP-C) to CDEIII, at a site close to where Ndc10 binds (42). The CBF3 complex and Mif2 mediate recruitment of outer kinetochore proteins (42).

1.2.1.2 Attachment of the Outer Kinetochore to Nuclear Microtubules

Several multi-subunit protein complexes mediate attachment of the inner kinetochore to spindle microtubules. The "mid-kinetochore" proteins include the COMA and MIND complexes, while the outer kinetochore is comprised of the Dam1/DASH and NDC80 complexes (32, 48, 114, 115, 170, 234). Once kinetochores are re-assembled at the centromere following a brief loss of association during DNA replication, one of the two sister chromatids of a replicated chromosome is captured by a microtubule through attachment to the lateral surface of the microtubule mediated by the NDC80 outer kinetochore complex (147, 214). After this initial capture of the chromosome by a microtubule, the minus-end-directed motor protein Kar3 and its accessory subunit Cik1 guide the chromosome toward a SPB (213, 214). The NDC80 and Dam1 complexes mediate kinetochore attachment to the microtubule plus ends (147, 213, 233). Association of the Dam1 complex with microtubules is also dependent on phosphorylation of at least three Dam1 subunits by the Aurora B protein kinase Ipl1 (12, 31, 216). Ipl1 is a component of the multi-subunit chromosome passenger complex, involved in stabilizing microtubule cross-links at the spindle midzone (78). Bipolar attachment of the chromosome to the spindle is subsequently achieved through capturing of the other sister chromatid kinetochore by a second microtubule originating from the other SPB.

1.2.2 Regulation of Sister-Chromatid Cohesion

During the time between DNA replication and the onset of anaphase, sister chromatids are held together by a complex of proteins called cohesin. The cohesin complex

is an annular structure consisting of two rod-like proteins, Smc1 and Smc3, as well as Mcd1, Scc3, and Pds5 (165, and reviewed in 249). Cohesin is enriched at pericentomeric regions, and in yeast is found at ~11-kb intervals along the entire length of chromosomes, often in regions of convergent transcription (79). Recruitment of cohesin to pericentromeric DNA is essential to the fidelity of chromosome transmission, and is dependent on Cse4, Mif2, and Ndc10 (215). Condensin, a complex structurally similar to cohesin and comprised of Smc2, Smc4, Brn1, Ycg1, and Loc7 (131, 171, 208), contributes to cohesion at chromosome arms but not pericentromeric regions (129).

1.2.2.1 Topology of Cohesin Binding

The ring-like structure of the cohesin complex suggests it might entrap sister chromatids; however, it is still not clear if cohesion is mediated by single cohesin ring that embraces two sister chromatids, or by interactions between cohesin rings that each encircle a single chromatid (249). The topology of association of cohesin with DNA may not be universal. One hypothesis currently gaining empirical support postulates that cohesion at chromosome arms is interchromosomal, linking homologous regions of sister chromatids, while cohesion in pericentromeric regions is intrachromosomal, forming a loop of DNA that presents the kinetochore for capture by a microtubule (15, 255). Intramolecular cohesion at pericentromeric regions may strengthen centromeric DNA, which is under the greatest amount of force from the spindle, and may enable the DNA to stretch and compact in response to dynamic forces directed at the kinetochore (17).

1.2.2.2 Timing of Cohesin Association with DNA

Coordinating sister-chromatid cohesion with the cell cycle can be divided into three distinct phases: cohesion establishment, maintenance, and dissolution. Cohesin is first loaded onto chromosomes during the G1 phase of the cell cycle by Scc2-Scc4 (39), and establishment of functional sister-chromatid cohesion occurs during DNA replication, and is dependent on acetylation of cohesin subunit Smc3 by the acetyltransferase Eco1 (182, 186, 220). The cohesin subunit Pds5 is required for maintenance of cohesion from S phase to G2/M (94, 174).

1.2.3 Segregation of Chromosomes at Anaphase

The bipolar attachment of all chromosomes to the mitotic spindle signals the onset of anaphase. At anaphase, cleavage of Mcd1 by separase (Esp1) releases sister chromatids from the cohesin complex (223). Prior to chromosome segregation, separase is inhibited by securin (Pds1). Anaphase begins with activation of the anaphase promoting complex (APC), an E3 ubiquitin ligase which catalyzes ubiquitination of securin, leading to its degradation, and relieving inhibition of separase (40, 43, 252).

The plus-end-directed nuclear microtubule-motor proteins Cin8 and Kip1 cross-link nuclear microtubules and, once the linkages between the sister chromatids are released, act to push SPBs apart (108, 183), elongating the spindle and moving sister chromatids toward opposite poles of the nucleus.

1.3 The 2μm Plasmid Poaches Host-Cell Chromosome-Segregation Proteins

The 2μm plasmid lacks *cis*-acting *CEN* DNA elements required for establishing a kinetochore. However, a subset of centromere-associated proteins are recruited to *STB* in a Rep1- and Rep2-dependent manner, with the recruitment being functionally significant for plasmid partitioning (46, 88, 151).

In addition to being found in a single nucleosome at each of the 16 centromeres in budding yeast, the histone H3 variant Cse4 is incorporated into nucleosomes at *STB* (88, 110). Unlike centromeres, which depend on the CBF3 complex for assembly of a Cse4-containing nucleosome, *STB* recruits Cse4 by an alternative mechanism, since substitution of CBF3 subunit Ndc10 leads to loss of Cse4 association with centromeres but not *STB* (88) and association of the Ndc10 with *STB* has not been detected by ChIP (151). Forming a functional Cse4-containing nucleosome at *STB* requires both Rep1 and Rep2, and is dependent on the ability of Rep1 to interact with *STB*. A lack of functional Cse4 impairs Rep2-*STB* association, and causes plasmid mis-segregation (88).

Recruitment of Cse4 to *STB* is dependent on one of the two plus-end-directed motor proteins functionally redundant in chromosome segregation, Kip1, but not on its close relative Cin8 (46). Loss of Kip1 increases the frequency of mis-segregation of a 2μm plasmid (artificially restrained at one copy per cell for the assay) and association of Cse4 with *STB* is

impaired. Kip1 was found by ChIP and one-hybrid assays to interact with *STB* in a Rep1- and Rep2-dependent manner, and to co-immunoprecipitate with Rep2 (46), suggesting that the Rep proteins might directly recruit the Kip1 motor protein to the plasmid partitioning locus.

Incorporation of a Cse4-containing nucleosome at *STB* is required to recruit Rsc2, a component of the RSC (Remodel the Structure of Chromatin) chromatin-remodeling complex (88). Rsc2 contains two bromodomains, which bind acetyllysine, and an AT-hook, that binds the minor groove of AT-rich DNA (27). Rsc2 is required for establishing functional cohesion at chromosome arms, but not at pericentromeric regions, and yeast lacking Rsc2 display reduced chromosome-transmission fidelity (7, 110). Loss of Rsc2 also causes changes in chromatin architecture at *STB*, and yeast lacking Rsc2 are unable to stably maintain the native 2μm plasmid (239).

RSC-mediated chromatin remodeling of *STB* is required for loading of the cohesin complex at *STB* (88). Mehta *et al.* (2002) demonstrated using one-hybrid and ChIP assays that cohesin is associated with the 2μm plasmid *STB* locus, and that this association is required for plasmid partitioning. Unlike centromeric cohesin, loading of cohesin at *STB* is dependent on the spindle, since nocodazole-induced microtubule depolymerization disrupts association of cohesin with *STB*, but not with chromosomes (152). Cohesin loading at *STB* is dependent on both Rep1 and Rep2, but lack of functional Mcd1, a cohesin subunit, does not affect association of Rep1 or Rep2 with *STB*, indicating that the cohesin recruitment occurs downstream of Rep-*STB* association. Ghosh *et al.* have provided evidence suggesting that cohesin binds sister copies of 2μm plasmids together, in a tri-linked catenane (chain-link) topology (73). This type of linkage is consistent with the observation that 2μm plasmids are segregated in a sister-to-sister fashion at mitosis (71).

Taken together, the data suggest a hierarchical order of protein interactions at the *STB* locus, where Rep1- and Rep2-mediated recruitment of Kip1 to *STB* is required for establishing a Cse4-nucleosome, which in turn is necessary for Rsc2-mediated chromatin remodeling of *STB* and recruitment of the cohesin complex.

1.4 2μm Plasmid Maintenance is Dependent on Sumoylation

One of the primary objectives of my doctoral research was to characterize post-translational modification of the plasmid partitioning proteins Rep1 and Rep2 with the Small

Ubiquitin-like MOdifier, SUMO (encoded by the *SMT3* gene in yeast). Sumoylation is an essential conserved eukaryotic function known to regulate diverse cellular processes (120, 143), and can modulate substrate protein interactions, localization, or post-translational stability (reviewed in 120, 69 and 235).

1.4.1 The SUMO Pathway

SUMO must be activated in a series of steps catalyzed by SUMO-specific E1, E2, and E3 enzymes before being conjugated to target proteins, which occurs in a manner mechanistically similar to ubiquitination (120). The primary translation product of the *SMT3* gene must first be processed by the SUMO protease Ulp1, which cleaves the three carboxy-terminal residues to expose the diglycine motif required for conjugation (134). The carboxy terminus of this mature processed SUMO is covalently linked through a thioester bond to the Aos1/Uba2 heterodimer (E1 activating enzyme) in an ATP-dependent reaction. SUMO is then transferred to the active site cysteine residue in the SUMO E2 conjugating enzyme, Ubc9. Some target proteins may require an E3 ligase (Siz1, Siz2, Mms21, Cst9) (37, 122, 259) for recruitment to Ubc9. Ubc9 catalyzes formation of an isopeptide bond between the carboxy-terminus of SUMO and a lysine residue in the target protein.

Sumoylation is a highly reversible and dynamic modification, and SUMO itself can be sumoylated, so that lysine residues in targeted proteins can be conjugated with a chain of SUMO moieties or with SUMO monomers. In yeast, the two SUMO-specific deconjugating enzymes Ulp1 and Ulp2 cleave SUMO from distinct subsets of target proteins (134, 135). Ulp1 is essential for viability due to its role in processing SUMO to its mature, conjugatable form (134), while Ulp2 is not essential, and is primarily involved in cleavage of poly-SUMO chains (25).

1.4.2 Function of Protein Sumoylation

SUMO targets are predominately nuclear but are also found in the cytoplasm, plasma membrane, and mitochondria (69). In some cases, sumoylation of a protein regulates post-translational stability. Sumoylation can increase the half-life of a target protein in some cases by occupying a critical ubiquitination site, as has been shown for SUMO-1 modification of human IκBα (50). Sumoylation can also promote protein degradation through sumoylation-

dependent ubiquitination, which in yeast is mediated by the heterodimeric ubiquitin E3 ligase Slx5-Slx8, homologue of human RNF4. Slx5-Slx8 recognizes substrates through interaction with poly-SUMO chains covalently linked to the target protein (112, 158, 178, 209, 225, 247, 248). In other cases, sumoylation regulates the subcellular localization of a protein. For many transcription factors, sumoylation controls nucleocytoplasmic shuttling (11, 26, 138, 173, 217, 256). Sumoylation can also regulate sub-nuclear localization. For example, in mammalian cells, sumoylation targets the promyelocytic leukemia (PML) protein to PML nuclear bodies (163, 226), and is required for the nuclear trafficking regulatory protein RanGAP1 to be localized to kinetochores and the mitotic spindle during mitosis (123). In yeast, sumoylation is required for targeting the inner kinetochore protein Ndc10 to the spindle (156).

1.4.3 Consensus Motifs for Covalent and Non-Covalent Interaction with SUMO

Some proteins are dependent on sumoylation for binding to their partner proteins. Sumoylation-dependent protein interactions are often mediated through binding of one protein that is covalently conjugated to SUMO by another protein that contains a SUMO interaction motif (SIM), which recognizes SUMO non-covalently. This motif consists of a hydrophobic core (V/I-x-V/I-V/I) that is usually flanked by acidic residues, or phosphorylated serine residues (92, 201). The SIM can function in either orientation (202).

Compared to the relatively small number of SIM-containing proteins discovered to date, hundreds of sumoylated proteins have been identified, many by high-throughput studies involving mass spectrometry analysis of SUMO conjugates affinity-purified from total protein extracts (49, 92, 175, 242, 261, 262). The majority of sumoylation sites conform to the four-residue consensus sequence (V/I/L-K-x-E/D) (181), where K is the SUMO acceptor site. In some cases, conjugation to SUMO at the consensus lysine is also dependent on acidic residues (253) or phosphorylated serine residues (101) carboxy-terminal to the core consensus. Sumoylation sites that do not conform to a consensus site have also been identified. For example, the replication processivity factor PCNA contains both a consensus and a non-consensus sumoylation site (105). While the majority of sumoylation sites identified thus far conform to the consensus motif, the number of non-canonical sites may be

under-represented due to the difficulty in predicting these sites as being sumoylated based solely on examination of protein sequence.

1.4.4 Low Level of Sumoylation, Large-Scale Consequences

SUMO conjugates of proteins can be difficult to detect since they are rapidly cleaved by the SUMO isopeptidases in whole cell extracts, and often less than 1% of a protein is present as a SUMO-conjugated form at steady-state levels (120, 121). Sumoylation of a target protein can be critical for proper function despite only a small proportion being present in the SUMO-conjugated form at any given time. Sumoylation is highly dynamic, so an entire pool of a protein might pass through the modified form and back over a short period of time. Transient modification of a protein could temporarily confer a function whose effect persists following removal of SUMO. For example, in the case of transcription factors, many of which are sumoylated (76, 77), sumoylation may recruit other cofactors that remain associated with the DNA even after the transcription factor is desumoylated. Alternatively, sumoylation-dependent recruitment of chromatin-remodeling enzymes to DNA could effect a change in chromatin structure that is maintained following loss of SUMO association with chromatin (69, 96).

1.4.5 Sumoylation and 2μm Plasmid Maintenance

Initial observations linking a role for sumoylation in 2μm plasmid maintenance were reported in studies demonstrating that retention of the 2μm plasmid at the normal copy number is dependent on host cell sumoylation (22, 36, 51, 260). Combined loss of two of the SUMO E3 ligases, Siz1 and Siz2, (36), loss of the SUMO-targeted ubiquitin ligase Slx5-Slx8 (22), substitution of the SUMO protease Ulp1 (51) or loss of two proteins, Mlp1 and Mlp2, that are required for tethering Ulp1 to the nuclear pore (260) leads to pronounced increases in plasmid copy number. The inheritance of elevated plasmid copy number in these mutants is associated with a nibbled colony morphology due to G2/M arrest of cells in those sectors of the colony where the plasmid copy number has reached toxic levels (51, 106). Hyper-amplification of the 2μm plasmid in yeast containing substitutions in SUMO-pathway genes has been linked to altered sumoylation of the plasmid-encoded Flp protein. A high proportion

(~10%) of Flp is normally sumoylated at steady-state levels, and substitution of the major SUMO attachment site in Flp is sufficient to cause plasmid hyper-amplification (36, 250).

For yeast with sumoylation-deficient Flp, the increased number of plasmids is associated with the appearance of an aberrant higher-molecular-weight species containing tandem copies of the 2μm circle, as well as single-stranded DNA (250). Xiong *et al.* (2009) have proposed that formation of the higher-molecular-weight species, which is dependent on the host yeast homologous-recombination DNA-repair enzymes, results from dysfunction of sumoylation-deficient Flp, which fails to properly complete recombination between 2μm Flp target recognition sites. The product is recognized as damaged DNA and becomes a substrate for host repair enzymes which generate the aberrant multimeric form of the plasmid and resolve elevated numbers of monomeric plasmids from the multimers.

In addition to having increased copy number, yeast defective for sumoylation also frequently give rise to plasmid-free progeny (36, 51), and a fluorescently-tagged 2μm reporter plasmid was shown to mis-segregate in yeast mutant for the SUMO protease Ulp1 (51). Unequal partitioning would further exacerbate the effect of altered sumoylation on plasmid copy number independently of any effect on Flp, as some cells could acquire more than half, or even all, of an already elevated number of plasmids during a cell division. SUMO-conjugated forms of the 2μm plasmid partitioning protein Rep2 have been observed (36), and both Rep1 and Rep2 were shown to interact with SUMO in a yeast two-hybrid assay (51), suggesting that sumoylation might play a direct role in plasmid segregation by regulating Rep protein function.

1.5 Overview

In this thesis, I present the results of my studies aimed at elucidating how Rep1 and Rep2 function. I demonstrate distinct roles for Rep1 and Rep2 in plasmid partitioning, showing that Rep2 is required for Rep1 post-translational stability, while Rep1 is required for stable association of Rep2 with *STB*. I show that Rep2 is phosphorylated on multiple residues; however, no functional significance in the known roles of Rep2 in plasmid partitioning and in repression of plasmid gene expression could be attributed to Rep2 phosphorylation. I provide evidence for Rep1 and Rep2 both being targeted for sumoylation. The results suggest that sumoylation of Rep1 and Rep2 promotes their stable association with

STB, and impaired sumoylation of both Rep proteins leads to significant defects in efficiency of plasmid inheritance. From my investigations of the mechanism of regulation of *FLP* gene expression, I demonstrate that Rep1 and Rep2 are both associated with the *FLP* promoter, and specifically repress expression of one of two major *FLP* transcripts. My findings suggest that the plasmid-encoded Raf anti-repressor alleviates Rep protein-mediated repression by impairing association of Rep2 with the *FLP* promoter by competing with Rep2 for interaction Rep1, and by inhibiting Rep2 self-association. Collectively these findings significantly further understanding of the two major roles of the Rep proteins in maintenance of the 2μm plasmid.

CHAPTER 2. MATERIALS AND METHODS

2.1 Yeast Strains and Media

Standard methods were used for growth and manipulation of yeast and bacteria (184, 189). Yeast were cultured in YPAD (1% yeast extract, 2% Bacto Peptone, 0.003% adenine, 2% glucose), synthetic defined (SD) (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil, and all required amino acids), or synthetic complete (SC) medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil, 1% Difco casamino acids, 0.002% tryptophan) (184). For induction of galactose-inducible promoters, glucose was replaced with galactose (2%). Media were supplemented with 200 mg/L geneticin (G418, Sigma) and 100 mg/L nourseothricin (clonNAT, Werner BioAgents) for selection of *KanMX6*-tagged and *NatMX6*-tagged gene replacements, respectively. Yeast were transformed by the LiAC/SS-DNA/PEG method (75).

Yeast strains used in this study are listed in **Table 1**. Strains lacking the 2μm circle, designated [cir^0], were derived from strains containing the 2μm circle, [cir^+], by expression of a defective Flp recombinase from the plasmid pBIS-GALkFLP-(TRP1), kindly provided by Dr. M. Gartenberg (221). Yeast gene-deletion strains were created by targeted replacement of wild-type alleles with *KanMX6* gene-deletion alleles amplified by PCR from appropriate strains in the EUROSCARF yeast gene-deletion strain collection (237) using recommended primers and conditions. Transformed yeast were selected for G418-resistance and gene deletions confirmed by PCR.

2.2 Plasmids

Plasmids used in this study are indicated in **Table 2.**

2.2.1 2μm-Based Plasmids for Studying Partitioning Function

Plasmid pAS4, a *flp*$^-$ *ADE2*-tagged version of the 2μm circle that can be propagated in yeast and *E. coli*, has been previously described (192). The *ADE2* gene insertion disrupts the *FLP* gene, and the Flp target site between the *REP1* and *REP2* genes has been deleted and replaced with the *E.coli* vector pTZ18R (Pharmacia). pAS10 is identical to pAS4 but

Table 1. Yeast strains used in this study.

Name	Genotype	Reference
W303a/α [*cir*$^+$]	*MAT**a**/MAT*α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11/his3-11,15 trp1-1,trp1-1* [*cir*$^+$]	(185)
W303a/α [*cir*^{0}]	*MAT**a**/MAT*α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11/his3-11,15 trp1-1,trp1-1* [*cir*^{0}]	(185)
W303/1a	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1* [*cir*$^+$]	(185)
MD83/1b	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1* [*cir*^{0}]	(51)
MD83/1c	*MAT*α *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1* [*cir*^{0}]	(51)
JP91/4	*MAT*α *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 smt3Δ::KanMX6* [pRS426-*CUP1p*-HA$_3$-*SMT3(GG)*] [*cir*^{0}]	this study
HZY1017	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 HIS6FLAGSMT3::KanMX6* [*cir*^{0}]	(262)
JP65/1d	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 HIS6FLAGSMT3::KanMX6* [*cir*$^+$]	this study
JP66/1a	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 HIS6FLAGSMT3::KanMX6* [*cir*^{0}]	this study
CTY10/5d	*MAT**a** gal4 gal80 his3-200 trp1-901 ade2 ura3-52 leu2-3,112 met thr URA3::*(lexAop)$_8$-*lacZ* [*cir*$^+$]	(8)
CTMD/3a	*MAT**a** his3 trp1 leu2-3,112 ade2-1 ura3 met URA3::*(lexAop)$_8$-*lacZ* [*cir*^{0}]	M. Dobson*
MD83/29	*MAT**a** ade2-1 ura3-1 leu2-3,112 15 trp1-1 GFP-lacI::HIS3* [*cir*^{0}]	this study
EP4	*MAT**a** gal4 gal80 his3-200 trp1-901 ade2 ura3-52 leu2-3.112 met thr URA3::STB*-p-*HIS3* [*cir*$^+$]	E. Polvi*
EP4MD [*cir*$^+$]	*MAT**a**/MAT*α *gal4/GAL4 gal80/GAL80 his3-11,-15/his3-200 trp1-1/trp1-901 ade2-1/ade2 ura3-1/ura3-52 leu2-3,-112/ leu2-3,-112 MET/met THR/thr URA3:: STB-p-HIS3* [*cir*$^+$]	E. Polvi*
EP4MD [*cir*^{0}]	*MAT**a**/MAT*α *gal4/GAL4 gal80/GAL80 his3-11,-15/his3-200 trp1-1/trp1-901 ade2-1/ade2 ura3-1/ura3-52 leu2-3,-112/ leu2-3,-112 MET/met THR/thr URA3:: STB-p-HIS3* [*cir*^{0}]	E. Polvi*
EGY48 [*cir*$^+$]	*MAT*α *ura3 his3 trp1* (lexAop)$_6$*::LEU2* [*cir*$^+$]	(56)
EGY48 [*cir*^{0}]	*MAT*α *ura3 his3 trp1* (lexAop)$_6$*::LEU2* [*cir*^{0}]	(56)
JP98/2	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 rsc2Δ::NatMX6* [*cir*^{0}]	this study
JP99/2	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 kip1Δ::NatMX6* [*cir*^{0}]	this study
JP02/1a	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 bud32Δ::KanMX6* [*cir*$^+$]	this study
JP06/1b	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 cka2Δ::KanMX6* [*cir*$^+$]	this study
JP62/1b	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 kns1Δ::KanMX6* [*cir*$^+$]	this study
JP01/1b	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 bud32Δ::KanMX6* [*cir*^{0}]	this study
JP75/2c	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 slx5Δ::KanMX6* [*cir*^{0}]	this study
JP76/2d	*MAT*α *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 slx8Δ::KanMX6* [*cir*^{0}]	this study
MD144/2c	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 siz1Δ::KanMX6 siz2Δ::KanMX6* [*cir*$^+$]	this study
MD83/7b	*MAT**a** ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 ulp1ΔES::URA3* [*cir*$^+$]	(51)
BY4741	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0* [*cir*$^+$]	(19)
BY4741 *bud32*Δ	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 bud32Δ::KanMX6* [*cir*$^+$]	(237)
BY4741 *cka2*Δ	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 cka2Δ::KanMX6* [*cir*$^+$]	(237)
BY4741 *kns1*Δ	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 kns1Δ::KanMX6* [*cir*$^+$]	(237)
BY4741 *slx5*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 slx5Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *slx8*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 slx8Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *ubc2*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ubc2Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *ubc4*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ubc4Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *ubc5*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ubc5Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *ubc8*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ubc8Δ::KanMX6* [*cir*^{0}]	(74)
BY4741 *ubc13*Δ [*cir*^{0}]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 ubc13Δ::KanMX6* [*cir*^{0}]	(74)
IRS10	*MAT**a** ura3-1 leu2-3,112 his3-11,15 trp1-1* pAS10ΔORI::*ADE2* [*cir*^{0}]	this study
IRS133	*MAT**a** ura3-1 leu2-3,112 his3-11,15 trp1-1* pAS*rep1$_{3R}$*ΔORI::*ADE2* [*cir*^{0}]	this study
IRS135	*MAT**a** ura3-1 leu2-3,112 his3-11,15 trp1-1* pAS*rep2$_{13R}$*ΔORI::*ADE2* [*cir*^{0}]	this study

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Table 2. Plasmids used in this study.

Plasmid	Notes			Source
pBM272	*CEN*	*URA3*	*GAL1/10* promoter	(254)
pBM272-REP1	*CEN*	*URA3*	*GAL1p-REP1*	(254)
pBM272-REP2	*CEN*	*URA3*	*GAL10p-REP2*	(254)
pBM272-REP1+REP2	*CEN*	*URA3*	*GAL1p-REP1 GAL10p-REP2*	(254)
pRSFLAG-TRP-REP1	*CEN*	*TRP1*	*GAL10p-FLAGREP1*	J. Chew*
pRSFLAG-TRP-REP2	*CEN*	*TRP1*	*GAL10p-FLAGREP2*	J. Chew*
pRSFLAG-TRP	*CEN*	*TRP1*	*GAL1/10* promoter	J. Chew*
pRSFLAG-LEU	*CEN*	*LEU2*	*GAL1/10* promoter	J. Chew*
pGAL-LEU-REP1	*CEN*	*LEU2*	*GAL1p-REP1* (low level expression)	this study
pGAL-LEU-REP1(ES)	*CEN*	*LEU2*	*GAL1p-REP1*	this study
pGAL-LEU-rep1(133)	*CEN*	*LEU2*	*GAL1p-rep1*$_{3R}$	this study
pGAL-LEU-REP2	*CEN*	*LEU2*	*GAL1p-REP2* (low level expression)	this study
pGAL-LEU-REP2(ES)	*CEN*	*LEU2*	*GAL1p-REP2*	this study
pGAL-LEU-rep2(134)	*CEN*	*LEU2*	*GAL1p-rep2*$_{SA}$	this study
pGAL-LEU-rep2(135)	*CEN*	*LEU2*	*GAL1p-rep2*$_{13R}$	this study
pGAL-LEU-RAF	*CEN*	*LEU2*	*GAL1p-RAF*	this study
pGAL-TRP-REP1	*CEN*	*TRP1*	*GAL1p-REP1* (low level expression)	this study
pGAL-TRP-REP1(ES)	*CEN*	*TRP1*	*GAL1p-REP1*	this study
pGAL-TRP-rep1(133)	*CEN*	*TRP1*	*GAL1p-rep1*$_{3R}$	this study
pGAL-TRP-REP2	*CEN*	*TRP1*	*GAL1p-REP2* (low level expression)	this study
pGAL-TRP-REP2(ES)	*CEN*	*TRP1*	*GAL1p-REP2*	this study
pGAL-TRP-rep2(134)	*CEN*	*TRP1*	*GAL1p-rep2*$_{SA}$	this study
pGAL-TRP-rep2(135)	*CEN*	*TRP1*	*GAL1p-rep2*$_{13R}$	this study
pGAL-TRP-RAF	*CEN*	*TRP1*	*GAL1p-RAF*	this study
pMM2	2µm	*TRP1*	*GALp* B42$_{AD}$-HA	M. McQuaid*
pMM2-REP1	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*REP1*	M. McQuaid*
pMM2-rep1(113)	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*rep1*$_{K305R}$	this study
pMM2-rep1(133)	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*rep1*$_{3R}$	this study
pMM2-REP2	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*REP2*	M. McQuaid*
pMM2-rep2(134)	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*rep2*$_{SA}$	this study
pMM2-rep2(135)	2µm	*TRP1*	*GALp* B42$_{AD}$-HA-*rep2*$_{13R}$	this study
pMM3	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA	this study
pMM3-REP1	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*REP1*	M. McQuaid*
pMM3-rep1(113)	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*rep1*$_{K305R}$	this study
pMM3-rep1(133)	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*rep1*$_{3R}$	this study
pMM3-REP2	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*REP2*	M. McQuaid*
pMM3-rep2(134)	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*rep2*$_{SA}$	this study
pMM3-rep2(135)	*CEN*	*TRP1*	*GALp* B42$_{AD}$-HA-*rep2*$_{13R}$	this study
pGAD424	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$	Clontech
pGADREP1	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-REP1*	(192)
pGADREP2	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-REP2*	(192)
pGADSMT3	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-SMT3GG*	(51)
pSE1111	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-SNF4*	(54)
pGADBUD32	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-BUD32*	this study
pGADCKA2	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-CKA2*	this study
pGADKNS1	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-KNS1*	this study
pGADPCC1	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-PCC1*	R. Sternglanz†
pGADKAE1	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-KAE1*	this study
pGADySUMOΔGG	2µm	*LEU2*	*ADH1p GAL4*$_{AD}$*-smt3ΔGG*	M. McQuaid*
pGBDSMT3ΔGG	2µm	*URA3*	*ADH1p GAL4*$_{BD}$*-smt3ΔGG*	(92)

Plasmid	Notes			Source
pGBDUBC9	2µm	*URA3*	*ADH1p GAL4$_{BD}$-UBC9*	(92)
pSH2-1	2µm	*HIS3*	*ADH1p LexA*	(8)
pSHREP1	2µm	*HIS3*	*ADH1p LexA-REP1*	(192)
pSHrep1(113)	2µm	*HIS3*	*ADH1p LexA-rep1$_{K305R}$*	this study
pSHrep1(133)	2µm	*HIS3*	*ADH1p LexA-rep1$_{3R}$*	this study
pSHrep2(134)	2µm	*HIS3*	*ADH1p LexA-rep2$_{SA}$*	this study
pSHrep2(135)	2µm	*HIS3*	*ADH1p LexA-rep1$_{13R}$*	this study
pSHBUD32	2µm	*HIS3*	*ADH1p LexA-BUD32*	this study
pSHCKA2	2µm	*HIS3*	*ADH1p LexA-CKA2*	this study
pSHKNS1	2µm	*HIS3*	*ADH1p LexA-KNS1*	this study
pSHREP2	2µm	*HIS3*	*ADH1p LexA-REP2*	(192)
pSHNIS1$_{357-408}$	2µm	*HIS3*	*ADH1p LexA-NIS1(357-408)*	K. Williams*
pAS4	2µm	*ADE2*	*flp$^-$ REP1 REP2*	A. Sengupta*
pAS10	2µm	*ADE2*	*flp$^-$ REP1 REP2*	A. Sengupta*
pAS113	2µm	*ADE2*	*flp$^-$ rep1$_{K305R}$ REP2*	this study
pAS133	2µm	*ADE2*	*flp$^-$ rep1$_{3R}$ REP2*	this study
pAS135	2µm	*ADE2*	*flp$^-$ REP1 rep2$_{13R}$*	this study
pAS136	2µm	*ADE2*	*flp$^-$ rep1$_{3R}$ rep2$_{13R}$*	this study
pAS134	2µm	*ADE2*	*flp$^-$ REP1 rep2$_{SA}$*	this study
pKan4	2µm	*KanMX6*	*flp$^-$ REP1 REP2*	J. Wentzell*
pKan10	2µm	*KanMX6*	*flp$^-$ REP1 REP2*	R. Mackay[‡]
pKan113	2µm	*KanMX6*	*flp$^-$ rep1$_{K305R}$ REP2*	this study
pKan133	2µm	*KanMX6*	*flp$^-$ rep1$_{3R}$ REP2*	this study
pKan135	2µm	*KanMX6*	*flp$^-$ REP1 rep2$_{13R}$*	this study
pKan136	2µm	*KanMX6*	*flp$^-$ rep1$_{3R}$ rep2$_{13R}$*	this study
pKan134	2µm	*KanMX6*	*flp$^-$ REP1 rep2$_{SA}$*	this study
pKanΔrep1	2µm	*KanMX6*	*flp$^-$ rep1Δ REP2*	this study
pKanΔS	2µm	*KanMX6*	*flp$^-$ REP1 rep2Δ*	this study
pASUR1H6R2	2µm	*ADE2*	*flp$^-$ UBC9-REP1 HIS6-REP2*	this study
pASUR1H6R2(135)	2µm	*ADE2*	*flp$^-$ UBC9-REP1 HIS6-rep1$_{13R}$*	this study
pASUR2H6R1	2µm	*ADE2*	*flp$^-$ UBC9-REP2 HIS6-REP1*	this study
pASUR2H6R1(133)	2µm	*ADE2*	*flp$^-$ UBC9-REP2 HIS6-rep1$_{3R}$*	this study
pAS50Δrep1	2µm	*ADE2*	*flp$^-$ rep1Δ REP2*	this study
pAS4ΔS	2µm	*ADE2*	*flp$^-$ REP1 rep2Δ*	A. Sengupta*
pAS4ΔX	2µm	*ADE2*	*flp$^-$ REP1 REP2 stbΔ*	A. Sengupta*
pRS426-CUP1p3HASMT3GG	2µm	*URA3*	*CUP1p-3HAsmt3ΔGG*	(242)
pRS316-CUP1p3HASMT3GG	*CEN*	*URA3*	*CUP1p-3HAsmt3ΔGG*	this study
pBIS-GALkFLP-(TRP1)	*CEN*	*TRP1*	for curing strains of 2µm	(221)
pHR3	2µm	*URA3*	*FLP REP1 REP2 raf*	H. Rasmussen*
pHR5	2µm	*TRP1*	*FLP REP1 REP2 raf*	H. Rasmussen*
pHR5ΔTGCA1	2µm	*TRP1*	*rbe1*Δ	this study
pHR5ΔTGCA2	2µm	*TRP1*	*rbe2*Δ	this study
pHR5ΔTGCA1+2	2µm	*TRP1*	*rbe1*Δ *rbe2*Δ	this study
pHR5TACA1	2µm	*TRP1*	*rbe1$_{ga}$*	this study
pHR5TACA2	2µm	*TRP1*	*rbe2$_{ga}$*	this study
pHR5ΔTACA1+2	2µm	*TRP1*	*rbe1$_{ga}$ rbe2$_{ga}$*	this study
pHR5ΔR1R2	2µm	*TRP1*	*rep1*Δ *rep2*Δ (PinAI)	this study
pHR3ΔR1R2	2µm	*URA3*	*rep1*Δ *rep2*Δ	this study
pSV5	2µm	*TRP1*	*STB* and 256 *lacO* array	(151)

Plasmid	Notes	Source
pET32M		Novagen
pETBUD32	His_6Bud32	this study
pETCKA2	His_6Cka2	this study
pETKNS1	His_6Kns1	this study
pGEXREP1	GST-Rep1	(192)
pGEXrep1(1-129)	GST-$Rep1_{1-129}$	this study
pGEXrep1(130-373)	GST-$Rep1_{130-373}$	this study
pGEXrep1(62-373)	GST-$Rep1_{62-373}$	this study
pGEXrep1(77-373)	GST-$Rep1_{77-373}$	this study
pGEXrep1(62-99)	GST-$Rep1_{62-99}$	this study
pGEX4T	GST	GE Life Sciences
pGEXREP2	GST-Rep2	(192)
pGEXrep2(1-58)	GST-$Rep1_{1-58}$	this study
pGEXrep2(1-144)	GST-$Rep1_{1-144}$	this study
pGEXrep2(58-296)	GST-$Rep1_{58-296}$	this study

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with pTZ18R inserted in the opposite orientation. Site-directed mutagenesis of *REP1* and *REP2* was carried out by gap repair of plasmids pAS10 and pAS4, respectively. PCR amplicons containing either the *REP1* ORF flanked by ~650 bp upstream and ~450 bp downstream or the *REP2* ORF flanked by ~900 bp upstream and ~600 bp downstream and containing the designated point substitution(s) were created by overlap extension PCR (102, 104) and co-transformed into yeast with NruI/SalI-digested pAS10 or SphI-digested pAS4, respectively. Oligonucleotide sequences for site-directed mutagenesis of *REP1* (**Table 3**) and *REP2* (**Table 4**) are listed. Plasmids were isolated in *E. coli*, substitutions confirmed by sequencing, and were re-transformed into yeast for subsequent experiments. In order to combine various *REP1* and *REP2* alleles into a single *ADE2 flp*$^-$ 2μm plasmid, a PCR product encoding from ~900 bp upstream of the *REP2* ORF to ~980 bp into the *REP1* ORF in a pAS4-based plasmid was used for gap repair of BamHI/SphI-cut pAS10. To create *ADE2*-tagged 2μm-based plasmids lacking the *REP* genes, pAS4 was digested with SphI and the plasmid self-ligated to make pAS4Δ*REP2* (Arpita Sengupta, PhD thesis), and an XhoI site was introduced upstream of the *REP1* coding region in pAS10 by PCR and the resulting plasmid digested with XhoI and SalI and self-ligated to create pAS10Δ*REP1*. To create pKan4 in which *ADE2* is replaced with *KanMX6* in pAS4, yeast harboring pAS4 were transformed to G418-resistance with a PCR product encoding the *ade2*Δ*::KanMX6* allele. pKan4 was isolated in *E. coli* and re-transformed into yeast for all subsequent experiments. An identical strategy was used to create all other *KanMX6*-tagged 2μm plasmids derived from pAS4/pAS10-based plasmids. To create yeast strains containing the *REP1* and *REP2* genes with their flanking 2μm sequences, including *STB*, chromosomally integrated at the *ADE2* locus, pAS4 was digested with BclI and SnaBI to remove the origin of replication, and overhangs were filled in and self-ligated to create pAS10ΔORI. pAS10ΔORI was linearized with NdeI and used to transform *cir*^{0} strains to adenine prototrophy.

2.2.2 2μm-Based Plasmids Encoding Tagged Rep1 and Rep2 Proteins

ADE2-tagged 2μm plasmids directing expression of green fluorescent protein (GFP)-, Ubc9-, SUMO- or His6-tagged Rep1 and Rep2 proteins expressed from their own respective promoters were created by overlap extension PCR (107) and gap repair in a fashion similar

Table 3. Primers for site-directed mutagenesis of Rep1.

Substitution	Forward Primer (5'-3')	Reverse Primer (5'-3')
K11R	GTATTAGACAATGTATTATGCAGCAC	CATAATACATTGTCTAATACAAGCAAGCAGTC
K44,45,47R	TTATAGAAGATACAGAACGTTAGCATTTGCATTT	CGTTCTGTATCTTCTATAATTGTCAGGAACTG
K68R	AATTGAAAGAGAACTGGATTGGCCT	TCCAGTTCTCTTTCAATTACCGGTGTG
K105R	GTCAGTTAAGAGCCACCATCGGAGAG	GTGGCTCTTAACTGACTAATAAATGCA
K117R	TAATGTAAGAGGCACGCTAAACCGC	GCGTGCCTCTTACATTAATATCTAAACCC
K125R	GGGGAAGAGGTATCAGAAGGCCTA	CTGATACCTCTTCCCCTGCGGTTTAG
K131R	AAGGCCTAGAGGCGTATTTTTTAGATAC	TACGCCTCTAGGCCTTCTGATACC
K146R	AATACAAGAGTCACTGCATTCTTCTC	TGCAGTGACTCTTGTATTGACAAATGGAG
K159R	AGATTATAATAGAATTGCCTCAGAATATCA	AGGCAATTCTATTATAATCTCGAAGATAA
K169R	ATAATACTAGATTCATTCTCACGTTTTC	GAGAATGAATCTAGTATTATTGTGATATTC
K190R	CGCCTTGAGAAATGTTATTAGGTGCTCC	ATAACATTTCTCAAGGCGGAGAAGTT
K204R	TACATTTCTAGATTTGTGGAAAGAGAACAG	CCACAAATCTAGAAATGTATTCATGAATT
K212R	ACAGGATAGAGGTCATATAGGAGATC	ATATGACCTCTATCCTGTTCTCTTTCC
K261R	TGTGAAAGATGGCAGTCTGAAGCG	GACTGCCATCTTTCACATAATGAATCTATT
K290,295,297R	GAATTCGTCGTACAAGATTCAGAAGTGTCTTGTATCATATA	CTGAATCTTGTACGACGAATTCTGAGGTTCGCCATCC
K305R	ATATACTAAGAGAACTAATTCAATCTCAG	GAATTAGTTCTCTTAGTATATGATACAAGAC
K315R	AACCGTAAGAGTTTATCGCGGTAGTAG	CGATAAACTCTTACGGTTCCCTGAGA
K328R	CACACGATTCGATAAGAATAAGCTTACATTATG	TCTTATCGAATCGTGTGAAA
K348R	TTGACAGTAAGATTTGAAGAGCATTGGAA	TCTTCAAATCTTACTGTCAAGTAGACC
K354R	GCATTGGAGACCTGTTGATGTAGAGG	CAACAGGTCTCCAATGCTCTTCAAAT
K365,367,370R	GATTCAGAGAGCGAAGAGTGGATGGGTAGGTT	TCTTCGCTCTCTGAATCTGCATCTAAACTCGAC
T81A	TGCAATTGTCGATCGAATC	TCGATCGACAATTGCATTGTACACTAGTGCAG
L77,V78,Y79A	GCTGCGGCCAATACAATTGTCGATC	GTATTGGCCGCAGCTGCAGGATCAGGCCA
S330A	GCCTTACATTATGAAGAGCA	CTTCATAATGTAAGGCTATCTTTATCGAATCGTG
S330E	GAGTTACATTATGAAGAGCA	CTTCATAATGTAACTCTATCCTTATCGAATCGTG

Table 4. Primers for site-directed mutagenesis of Rep2.

Substitution	Forward Primer (5'-3')	Reverse Primer (5'-3')
K8,13R	AGAAATCTGACGGTAAGAGCACGTACAGCTTATAG	TCTTACCGTCAGATTTCTGGCTGTTTCAATGTCG
K42,44R	GAGTAGACGTAGATCTGATGAGCTACTCTT	CAGATCTACGTCTACTCTCTATATCAATATC
K91R	GCGAGAATGGTTAAGGAAAGGTT	CCTTAACCATTCTCGCAGCAGGCAAAATT
K95R	ATGGTTAGAGAAAGGTTTGATTCGATT	CAAACCTTTCTCTAACCATCTTCGCAGC
K95R*	ATGGTTAGAGAAAGGTTTGATTCGATT	CAAACCTTTCTCTAACCATTCTCGCAGC
K124R	TAACAGACTGTTAGACAATAGAAAG	TTGTCTAACAGTCTGTTATTCTGTAGCATCA
K130R	TAGAAGACAACTATACAAATCTATTG	TTTGTATAGTTGTCTTCTATTGTCTAACAGCT
K134R	CTATACAGA TCTATTGCTATAATAATAG	AGCAATAGATCTGTATAGTTGCTTTCTATT
K130,134R**	AAGACAACTATACAGATCTATTGCTATAATAATAG	GATCTGTATAGTTGTCTTCTATTGTCTAACAGTC
K146,148,149R	GAGACAGAAGGAGAGCTACCGAAATGC	GCTCTCCTTCTGTCTCTCTCGGGCAATCTTCC
K158R	AGAAGAATGGATTGTACACAGTT	TGTACAATCCATTCTTCTCATGAGCATTTCG
K177R	ATGAGACTCGTAAGCGTCGTTA	CGCTTACGAGTCTCATAACATCTTCTTCC
K208R	CTCTAAGAGATATATTCAATAGTTTCAA	TTGAATATATCTCTTAGAGATTCCGGGATG
K226,227R	CAAAGAAGAAGTGAGTTGGAAGGAAG	CAACTCACTTCTTCTTTGCTGTAAACGATTCT
S71,72A	GCTGCTGCAGAAGATTCCAGC	ATCTTCTGCAGCAGCATCAAGACCATACGG
S76,77A	GCGGCCGTATCTTCTGACTC	AGAAGATACGGCCGCATCTTCTGCGCTAGAAT
S76,77A***	GCTGCTGCAGAAGATGCGGC	ATCTTCTGCAGCAGCATCAAGACCATACGG
S79,80,82,83A	GCTGCTGACGCGGCAGCTGAGGTAATTTTGCC	CCGCGTCAGCAGCTACGCTGGAATCTTCTG
S107,108A	GCTGCACAAGAAGCAAGTCA	TGCTTCTTGTGCAGCGAGCATACCATTTCC
S79,80,82,83E	GAAGAGGACGAAGAGGCTGAGGTAATTTTGCC	CCTCTTCGTCCTCTTCTACGCTGGAATCTTCTG
I138,139,140A	GCAGCCGCAGGAAGATTGCCCGAG	TTCCTGCGGCTGCAGCAATAGATTTGTATAGT

*For template *rep2* DNA encoding K92R

**For template *rep2* DNA encoding K124R

***For template *rep2* DNA encoding S71,72A

to that used for plasmids encoding point-mutant *REP1* and *REP2* alleles. Rep proteins that were N-terminally tagged with GFP or Ubc9 contained a GGGGG peptide linker sequence and for the N-terminal SUMO-tag the linker sequence was SSGGG. Oligonucleotide sequences used for these constructs are shown in **Table 5**.

2.2.3 Plasmids for Expression of Fusion Proteins for One- and Two-Hybrid Assays

pGAD424- and pSH2-1-derived plasmids expressing Rep1, Rep2, and mature yeast SUMO as fusions with the Gal4 transcriptional activation domain, $Gal4_{AD}$, or with the bacterial LexA repressor protein, respectively, have been previously described (51). The genomic DNA encoding the Nis1 SUMO interaction motif-containing domain (92) inserted in plasmid pSH-$NIS1_{357-408}$ was isolated in a library screen (Jeremy Benjamin, Dobson lab, unpublished results). *TRP1*-based plasmids that direct galactose-inducible expression of the Rep proteins as HA-epitope-tagged B42 transcriptional activation domain ($B42_{AD}$-HA)-fusions in yeast and that were either 2μm-based (pMM2) or single-copy *CEN/ARS* (pMM3) were created by digestion of pJG4-5 (86) with BamHI and NotI. The overhangs were filled in and the plasmid was self-ligated to create pMM1. A 6.1-kb EcoRI/SphI fragment from pMM1 was ligated with EcoRI/SphI fragments from pGAD424, pGAD-REP1 and pGAD-REP2 (192) to yield pMM2, pMM2-REP1 and pMM2-REP2, respectively. The KpnI/EagI fragment containing the 2μm backbone in the pMM2-based plasmids was replaced with KpnI/EagI-digested fragment of pRS314 (198) to generate the single copy *CEN/ARS* pMM3, pMM3-REP1, and pMM3-REP2, respectively. For one-hybrid and two-hybrid analyses, the ORFs for alleles of *REP1* or *REP2* were amplified by PCR with flanking EcoRI and BamHI cleavage sites and subcloned in pMM3, pGAD424 and pSH2-1 vectors that would enable the proteins to be expressed as $B42_{AD}$-HA, $Gal4_{AD}$ and LexA fusions, respectively. All PCR-amplified genes were checked after cloning by sequencing. Plasmid pGADySUMOΔGG was created by subcloning the conjugation-defective SUMO-encoding SmaI/PstI fragment from pGBD-*smt3*Δ*GG* (92) into pGAD424. For testing two-hybrid interaction of protein kinases with Rep1 and Rep2, ORFs for *BUD32*, *KNS1*, and *CKA2* were amplified by PCR with flanking BamHI and SalI cleavage sites and subcloned into pGAD424 and pSH2-1 for

Table 5. Primers for introducing tags at the N- or C-terminus of Rep1 or Rep2.

Tag	N/C	Primer name	Sequence (5'-3')
GFP	N	REP1-GFP1	GGTGGAGGTGGCGGAATGAATGGCGAGAGAC
GFP	N	REP-GFP2	TCCGCCACCTCCACCTTTGTATAGTTCATCCATG
GFP	N	REP-GFP3	ATGAGTAAAGGAGAAGAA
GFP	N	REP1-GFP4	GTTCTTCTCCTTTACTCATATTTCAGTTATTTTCCATTA
GFP	N	REP2-GFP1	GGTGGAGGTGGCGGAATGGACGACATTGAAAC
GFP	N	REP2-GFP4	GTTCTTCTCCTTTACTCATTTTGGTTTTCTTTTACCAG
His_6	N	R1NHIS6F	ATGCATCATCACCATCACCATATGAATGGCGAGAGAC
His_6	N	R1NHIS6R	ATGGTGATGGTGATGATGCATATTTCAGTTATTTTCCATTA
His_6	N	R2NHIS6F	ATGCATCATCACCATCACCATATGGACGACATTGAAAC
His_6	N	R2NHIS6R	ATGGTGATGGTGATGATGCATTTTGGTTTTCTTTTACCAG
His_6	C	R1CHIS6F	CATCATCACCATCACCATTAGGTTATATAGGGATATA
His_6	C	R1CHIS6R	ATGGTGATGGTGATGATGCCCATCCACCTTTCG
His_6	C	R2CHIS6F	CATCATCACCATCACCATTGATCCAATATCAAAGGA
His_6	C	R2CHIS6R	ATGGTGATGGTGATGATGTACCCTAGAAGTATTACG
Ubc9	N	REP-UBC9-r	TCCGCCACCTCCACCTTTAGAGTACTGTTTAGC
Ubc9	N	REP-UBC9-f	ATGAGTAGTTTGTGTCTA
Ubc9	N	R1-UBC9-1	TAGACACAAACTACTCATATTTCAGTTATTTTCCATTA
Ubc9	N	R2-UBC9-1	TAGACACAAACTACTCATTTTGGTTTTCTTTTACCAG
SUMO	N	REP1SUMO1	TCTTCAGGTGGAGGTATGAATGGCGAGAGAC
SUMO	N	REPSUMO2	ACCTCCACCTGAAGAAATCTGTTCTCTGTGAG
SUMO	N	REPSUMO3	ATGTCGGACTCAGAAG
SUMO	N	REP1SUMO4	CTTCTGAGTCCGACATATTTCAGTTATTTTCCATTA
SUMO	N	REP2SUMO1	TCTTCAGGTGGAGGTATGGACGACATTGAAAC
SUMO	N	REP2SUMO4	CTTCTGAGTCCGACATTTTGGTTTTCTTTTACCAG

expression as $Gal4_{AD}$ and LexA fusion proteins, respectively.

2.2.4 Plasmids for Galactose-Inducible Over-Expression of 2μm-Encoded Genes

To create *TRP1*- or *LEU2*-based *CEN/ARS* plasmids for expression of 2μm plasmid genes from the *GAL1* promoter, the *REP1*, *REP2*, and *RAF* ORFs were amplified by PCR with flanking EcoRI and SalI restriction sites and cloned using a TOPO-TA cloning kit (Invitrogen). EcoRI(end-filled)/SalI-digested fragments containing the respective ORFs were then ligated into BamHI(end-filled)/SalI-digested pRSFLAG-TRP and pRSFLAG-LEU, which were created by replacement of the PvuII fragment encoding the multiple cloning site of pRS314 and pRS315 (198), respectively, with the 1.4-kb PvuII fragment from pESC-URA (Stratagene) containing the *GAL1* promoter.

Co-expression of *REP1* and *REP2* from these plasmids severely inhibited growth of co-transformants when grown in medium containing galactose. To create plasmids that directed a lower level of galactose-inducible expression of *REP1* and *REP2*, EcoRI(end-filled)/SalI fragments from pGAD-REP1 and pGAD-REP2 containing the *REP1* and *REP2* ORFs, respectively, flanked by BamHI cleavage sites, were subcloned into pRSFLAG-TRP and pRSFLAG-LEU as detailed above. Levels of Rep1 and Rep2 proteins expressed from these vectors were much lower than those expressed when the ORFs lacked flanking BamHI sites. These low-level Rep1 and Rep2 expression vectors were implemented in experiments that would be complicated by toxicity due to high levels of Rep1 and Rep2.

The *URA3*-tagged *CEN*/*ARS* pBM272-derived plasmids encoding *REP1*, *REP2*, or *REP1* and *REP2* ORFs under control of the bidirectional *GAL1*/*GAL10* promoter were kind gifts from M. Jayaram (University of Texas).

2.2.5 Plasmids for Expression of Yeast Proteins in Bacteria

For expression of Bud32, Cka2, or Kns1 protein kinases in *E. coli*, ORFs encoding the respective kinases were cloned into *E. coli* expression vector pET32 (Novagen). Plasmids directing expression of N-terminal glutathione-S-transferase (GST)-tagged Rep1 and Rep2 in *E. coli* have previously been described (192). Plasmids for expression of truncated Rep1 and Rep2 proteins fused to GST were derived from the pGAD424-based plasmids by cloning the

EcoRI/SalI fragments encoding the truncated *REP1* or *REP2* ORFs into *E. coli* expression vector pGEX4T (GE Life Sciences).

2.2.6 Amplification-Competent 2μm-Based Plasmids

URA3- and *TRP1*-tagged plasmids pHR3 and pHR5, respectively, are 2μm-based plasmids encoding a functional *FLP* gene, both intact Flp target sites, as well as *REP1*, *REP2*, and *STB* but lacking a functional *RAF* gene (constructed by Holly Rasmussen, Dobson lab). Substitutions in the *FLP* promoter region were introduced by gap repair of EagI/SphI-digested pHR5. To create derivatives of pHR3 and pHR5 that contained partial deletions in both the *REP1* and *REP2* genes, pHR3 and pHR5 were digested with PinA1 and self-ligated to create pHR3ΔR1R2 and pHR5ΔR1R2.

2.2.7 Other Plasmids

Plasmids pRS426-*CUP1p*-3HA-*SMT3(GG)* and pGBD-*smt3*Δ*GG* were generous gifts from Dr. M. Hochstrasser (Yale University) and are yeast vectors that express HA-epitope-tagged mature SUMO, and Gal4 transcription factor DNA-binding domain ($Gal4_{BD}$)-tagged conjugation-defective SUMO, respectively (92).

2.3 One-Hybrid and Two-Hybrid Assays

To test for interaction of the Rep proteins with *STB*, cir^+ and cir^0 derivatives of one-hybrid reporter strain EP4MD were transformed to tryptophan prototrophy with pMM2- or pMM3-based plasmids encoding various Rep1 and Rep2 alleles. Transformants were grown overnight in selective liquid medium containing glucose, and were serially diluted, spotted on solid medium containing galactose, and imaged after four to seven days of incubation at 28˚C. For two-hybrid assays, co-transformants in the two-hybrid reporter strain CTY10/5d (cir^+) or CTMD/3a (cir^0) were assayed for β-galactosidase expression by a filter assay. Co-transformants in the two-hybrid reporter strain EGY48 were assayed for *LEU2* expression by spotting serial dilutions of yeast on solid medium lacking leucine. Specificity of interactions was confirmed by co-expressing LexA-fusion proteins with the $Gal4_{AD}$, $Gal4_{AD}$-Snf4, or $B42_{AD}$, and $Gal4_{AD}$-fusion proteins with LexA (59).

2.4 Plasmid Loss Assays

To determine the rate of loss of *KanMX6*-tagged or *ADE2*-tagged 2μm plasmids, yeast transformants were initially grown on the appropriate solid medium (YPAD with G418, and SC lacking adenine, respectively) to maintain selection for plasmid-containing cells. The proportion of plasmid-containing cells was then determined both before and after ~15 generations of growth in non-selective medium (YPAD) by comparing plating efficiency on solid YPAD medium containing or lacking G418 (for *KanMX6*-tagged plasmids), or for *ADE2*-tagged plasmids, by comparing the number of completely red colonies (indicating absence of the plasmid) to total colonies on solid YPD medium. The rate of increase in the percentage of plasmid-free cells (rate of plasmid loss) was calculated as previously described (52) and statistical significance was determined using an unpaired t test.

2.5 Quantitative PCR

For quantitative real-time PCR (qPCR), yeast DNA was isolated using a standard glass bead disruption protocol (184). Plasmid copy number was determined by amplifying part of the plasmid *REP1* gene using primers 5'-GAATGGCGAGAGACTGCTTG and 5'-CCGATGGTGGCCTTTAACTG, and part of the chromosomal *TRP1* gene as a reference using primers 5'-CTGCATGGAGATGAGTCGTG and 5'-CCATTTGTCTCCACACCTCC. These two amplicons had virtually identical amplification efficiencies and gave the same relative values over a 10 000-fold dilution of template DNA. qPCR was carried out using a BioRad CFX96 Real-Time System and BioRad C1000 Thermal Cycler using SYBR Green SuperMix according to the manufacturer's protocol (BioRad). PCR conditions were as follows: 1 cycle at 95°C for 3 min; and 40 cycles, each consisting of 95°C for 10 s, 64°C for 10 s, and 72°C for 30 s, followed by plate reading. The cycle number for the PCR product to reach preset threshold (C_T value) was determined for three replicates for each DNA sample. The fold change in plasmid copy numbers was compared to that of the native 2μm plasmid in an isogenic yeast strain, and was calculated using the comparative C_T method (ΔΔCT) (140).

2.6 Protein Analysis

For most applications, protein was extracted from yeast by chemical lysis as described (36, 251). Briefly, ~1 x 10^8 cells were pelleted, resuspended in 200 μL of lysis

solution (1.85 M NaOH, 7.4% β-mercaptoethanol) and incubated for 10 min on ice. Protein was precipitated for 10 min by addition of 200 µL cold 50% trichloroacetic acid, pelleted by centrifugation, washed twice with 1 mL cold acetone, and thoroughly dried.

2.6.1 Western Blotting Analysis

For western blot analysis, protein was resuspended in equal volumes of urea extraction buffer (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, 50 mM Tris) and 2× protein gel loading buffer (125 mM Tris pH 6.8, 4.0% SDS, 20% glycerol, 4.0% β-mercaptoethanol, 1 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol). Protein suspensions were briefly vortexed, centrifuged at 16 000 × *g* for 1 min and supernatants analyzed by western blotting as described (192) to BioRad ImmunBlot PVDF (for chemiluminescent detection) or Amersham Hybond-LFP (for fluorescent detection). Antibodies were rabbit-derived polyclonal anti-Rep1 or anti-Rep2 (192), mouse anti-Pgk1 (Molecular Probes), mouse anti-HA, mouse anti-FLAG, rabbit anti-FLAG (Sigma), or mouse anti-LexA (Santa Cruz). Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG (KPL), anti-mouse Dylight 488, anti-mouse Dylight 549, anti-rabbit Dylight 649 (Rockland). Chemiluminescence was generated using an ImmunStar Western C kit (BioRad) and captured either by X-ray film or digitally using a charge-coupled-device camera in a VersaDoc 4000 MP imaging system (BioRad). Secondary antibodies coupled to different fluorophores allowed simultaneous detection of multiple antigens on a single blot. For detection of blots having both HRP- and fluorophore-conjugated secondary antibodies, blots were kept moist for chemiluminescent detection, then dipped briefly in methanol, thoroughly dried, and fluorescence captured.

2.6.2 Two-dimensional Gel Electrophoresis

For two-dimensional (2D) gel electrophoresis, pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1.45% DTT, 1% IPG buffer), spun at 16 000 × *g* for 5 min, and proteins were separated by isoelectric focusing using an IPGphor apparatus (Amersham Biosciences). In-gel rehydration was done on 13-cm IPG strips pH 3-10 L (Amersham Biosciences). Isoelectric focusing was performed for a total of 11 000 volt-hours. For the second dimension the IPG strips were equilibrated for 30 min in 6 M urea,

30% glycerol, 1% SDS, 2% DTT and 30 min in 6 M urea, 30% glycerol, 1% SDS, 3% iodoacetamide. Strips were loaded onto 10% SDS-PAGE gels and separated at 100 V for 5 h, and protein was transferred to PVDF membrane using a Hoefer SE 600 apparatus.

2.6.3 Enrichment of His_6-Tagged Rep1 and Rep2 Proteins in Yeast Extracts

For metal-ion affinity chromatography, dried protein pellets were resuspended in 1 mL of binding buffer (6 M guanidine HCl, 300 mM NaCl, 50 mM sodium phosphate, pH 8.0) supplemented with 10 mM N-ethylmaleimide, vortexed briefly, and clarified by centrifugation at 16 000 × *g* for 15 min. Supernatants were transferred to a new microfuge tube containing ~20 µL TALON resin (Clontech) and rocked at room temperature for 2 h. Resin was briefly washed three times with wash buffer (8 M urea, 300 mM NaCl, 50 mM sodium phosphate, 5 mM imidazole, pH 7.5), and then 4 µL of 1.5 M imidazole was added, followed by 25 µL of 2× protein gel loading buffer. Resin suspensions were boiled for 5 min, clarified by centrifugation and ~5 µL analyzed by western blotting.

2.7 Expression and Affinity Purification of Yeast Proteins in Bacteria

For expression and affinity purification of protein kinases in bacteria, *E. coli* strain BL21 transformed with pET32 plasmid encoding *BUD32*, *CKA2*, or *KNS1* was grown to saturation overnight in LB medium containing ampicillin and diluted 1:200 into 100 mL fresh medium, grown at 37˚C to an OD_{600} 0.3, shifted to 23˚C and grown to OD_{600} 0.8. Isopropyl-β-D-thiogalactopyranoside was added (0.4 mM) and the expression of the fusion protein was induced at 14˚C for 18-21 h. Cells were harvested at 4˚C, and resuspended in 3 mL purification buffer (20 mM Tris-Cl pH 7.6, 0.3 M NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and kept on ice. Lysozyme (60 µL of 10 mg/mL) was added and suspensions were incubated on ice for 20 min. Suspensions were briefly sonicated, and spun at 16 000 × *g* for 15 min at 4˚C. Supernatants were stored at -80˚C in 1 mL aliquots until ready for affinity purification. Purification was performed by addition of an equal volume of purification buffer to the aliquot, followed by a 10-min incubation at room temperature with of 30 µL TALON resin. Beads were washed with purification buffer twice and eluted in 50 µL purification buffer containing 100 mM imidazole.

For expression of GST-tagged Rep1 and Rep2 in bacteria, *E. coli* strain BL21 was transformed with pGEX-plasmid encoding full-length or truncated *REP1* and *REP2* ORFs grown overnight in LB medium containing ampicillin, diluted 1:10 into fresh medium, grown at 37˚C for 1 h; 0.3 mM IPTG was then added and the culture was incubated at 26˚C for 5 h. The following manipulations were carried out at 4˚C. Cells were harvested by centrifugation at 4000 × *g* for 10 min. Cell pellets were resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) containing 0.2% Nonidet P40 (NP-40) and a protease inhibitor cocktail (0.5 μg/mL leupeptin, 1 μg/mL aprotinin, 0.8 μg/mL pepstatin A and 1 mM PMSF, 1 mM dithiothreitol), washed twice in buffer A (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 0.1% NP-40, 2 mM methionine) and resuspended in 10 mL buffer A. Lysozyme (100 μg/mL) was added and the mixture was sonicated and centrifuged at 20 000 × *g* for 10 min. Supernatants were supplemented with 15% glycerol and stored at -80˚C.

2.8 *In Vitro* Kinase Assays

In vitro kinase assays were carried out essentially as described (57) with slight modifications. GST-tagged Rep1 and Rep2 proteins from 0.1-1 mL soluble *E. coli* extract were first immobilized on glutathione-agarose beads (GE Healthcare) by incubation for 30 min at room temperature, followed by two washes in PBS and two washes in kinase buffer (50 mM Tris pH 7.6, 10 mM $MnCl_2$, 10 mM $MgCl_2$). Beads were resuspended in 20 μL of kinase buffer, and 1-4 μL of affinity-purified protein kinase (see above) and 25 μM [γ-^{32}P] ATP (Amersham Pharmacia Biotech; specific radioactivity, 2000–3000 cpm/pmol) was added, and incubated at 37˚C for 1 h. Beads were washed twice with 400 μL kinase buffer, then 15 μL 2× protein gel loading buffer was added. Beads were heated 85˚C for 5 min, briefly centrifuged and ~5 μL supernatant resolved in 8% SDS-PAGE gels, which were dried and analyzed by autoradiography.

2.9 Fluorescence Microscopy

All images were digitally captured using a Nikon80i fluorescent microscope with a Nikon DS-Qi1Mc digital camera and processed using NIS-Elements Basic Research software. Immunofluorescence analysis of yeast was carried out essentially as described (23). Logarithmically growing yeast were fixed in 3.7% formaldehyde for 1 h, digested with

zymolyase 20T (ICN), and spotted to Superfrost Plus slides (Fisher). After 30 min the fixed spheroplasts were dried in cold methanol for 6 min and cold acetone for 30 s, and rehydrated in blocking buffer (PBS with 2% BSA). Slides were incubated at 4˚C overnight with affinity-purified anti-Rep1 or anti-Rep2 antibodies diluted in blocking buffer. Slides were washed twice in PBS, incubated at room temperature for 1 h with 1:500 AlexaFluor594-conjugated goat anti-rabbit secondary antibody (Invitrogen) in blocking buffer, and washed twice in PBS. Mounting medium containing 100 ng/mL 4',6-diamidino-2-phenylindole (DAPI) was added prior to imaging. To visualize the localization of a 2μm reporter plasmid, the *TRP1*-marked plasmid pSV5 containing the an origin of replication, the *STB* sequence, and 256 lac operator sequences (151) was visualized by expressing a GFP-LacI repressor fusion protein as described (230).

2.10 Chromatin Immunoprecipitation (ChIP)

ChIP analysis of Rep protein association with the plasmid *STB* locus was performed essentially as described (88, 124) with the following modifications. Yeast cultures (50 mL) were grown to OD_{600} ~1-2 and fixed with 1% formaldehyde for 15 min at room temperature. Crosslinking was quenched by addition of 125 mM glycine and after 5 min cells were harvested, washed with water three times and resuspended in 400 μL cold lysis buffer D (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1× complete protease inhibitor cocktail [Roche]). Yeast were kept chilled for the following steps. Cells were lysed using glass beads, and chromatin was sheared to 0.1 – 1 kbp fragments by 8 rounds of sonication for 12 s each using a Branson 250 sonifier set to an output level of 3 and 50% duty cycle. Sonicated lysates were pelleted at 16 000 × *g* for 15 min, the supernatant transferred to a new tube, centrifuged again at 16 000 × *g* for 10 min, and 20-100 μL of supernatant (whole cell extract) brought up to 400 μL with lysis buffer D, and incubated for 4 – 16 h with polyclonal anti-Rep1, anti-Rep2, or monoclonal anti-FLAG (Sigma) antibodies at 4 ºC. Protein A Sepharose CL-4B beads (GE Healthcare) were equilibrated in lysis buffer D and incubated with 20 μg of sonicated salmon sperm DNA and 100 μg bovine serum albumin for ≥ 1 h, and 20 μL of beads were added to the immunoprecipitation mixtures and incubated for 1 h. Beads were washed six times at room temperature as follows: twice with 1 mL ChIP wash buffer I (50 mM Hepes-KOH, pH 7.5,

150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, and 1% Triton X-100) for 5 min each, twice with 1 ml of ChIP wash buffer II (50 mM Hepes-KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, and 1% Triton X-100) for 5 min, and once with 1 ml of ChIP wash buffer III (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate, and 0.5% NP-40) for 5 min, and once with 1 mL of TE for 5 min. Chromatin was eluted in SDS, decrosslinked, digested with proteinase K and DNA extracted with phenol and chloroform (23). DNA was resuspended in 100 μL of TE, and serial dilutions of template DNA were amplified by 30 cycles of PCR with primers flanking *STB*, which were 5'-ATTATAGAGCGCACAAAGGAGA and 5'-TGCACTTCAATAGCATATCTTTG, or primers amplifying the *FLP* promoter region 5'-GACGGATCCAAATTGTGGCATGCTTAG and 5'-GACGGATCCTGTGCAGATCACATGTC. PCR products were resolved by agarose gel electrophoresis, strained with ethidium bromide, imaged using a VersaDoc MP 4000 imaging system (BioRad) and quantified by densitometry using QuantityOne software (BioRad). Specificity of co-immunoprecipitation was assessed by comparing the amount of *STB* DNA immunoprecipitated by anti-Rep1 and anti-Rep2 antibodies to that pulled down by the anti-FLAG antibody, and by assessing the degree of non-specific immunoprecipitation of a chromosomal target (*CEN3*) amplified using primers 5'-CATAAACATGGCATGGCGATCAG and 5'-CACCAGTAAACGTTTCATATATCC.

2.11 RNA Analysis

For RNA extraction, 50 mL of yeast were grown to OD_{600} ~1.0 and were harvested, washed with LET buffer (100 mM Tris pH 7.4, 100 mM LiCl, 0.1 mM EDTA), and frozen at ≤ -80˚C. Cell pellets were resuspended in 300 μL of LETS buffer (LET with 0.1% SDS) and the remainder of the procedure was performed on ice. Cells were lysed by vortexing with phenol/chloroform and glass beads, and the aqueous layer was extracted twice with phenol/chloroform and once with chloroform. RNA was precipitated with ethanol, resuspended in 15 mM sodium acetate pH 5.2, 0.5% SDS, precipitated with ethanol, and resuspended in 40 μL water. RNA quality and concentration were determined by analyzing 0.1 μL of RNA by agarose gel electrophoresis and densitometry, and ~20 μg of RNA was loaded onto 1.2% denaturing agarose MOPS/formaldehyde gels. Equal loading was

confirmed by in-gel ethidium straining of rRNA. RNA was capillary transferred to a nylon membrane (Biodyne B, Pall), UV-crosslinked, and hybridized to double-stranded digoxygenin (DIG)-labelled DNA probes that were synthesized by PCR according to the manufacturer's instructions (Roche). The PCR product used for detection of *FLP* mRNA corresponded to residues 449-727 of the *FLP* ORF. Hybridization was performed for 10-16 h at 42˚C as described, and chemiluminescent detection of the DIG-labelled probes was carried out using alkaline phosphatase-conjugated anti-DIG Fab fragments and CDP-Star chemiluminescent detection reagent (Roche) according to the manufacturer's instructions. Chemiluminescence was captured by X-ray film or digitally as detailed above for western blot analysis.

CHAPTER 3. RESULTS

3.1 Post-translational Stability and Modification of Rep1 and Rep2

Faithful inheritance of the 2μm plasmid is dependent on adequate levels of the two 2μm plasmid partitioning proteins, Rep1 and Rep2 (30, 52). As a starting point for investigating Rep protein function, Rep protein post-translational stability and modifications were examined.

3.1.1 Higher-Molecular Weight Forms of Rep1 and Rep2 Detected by Western Blotting

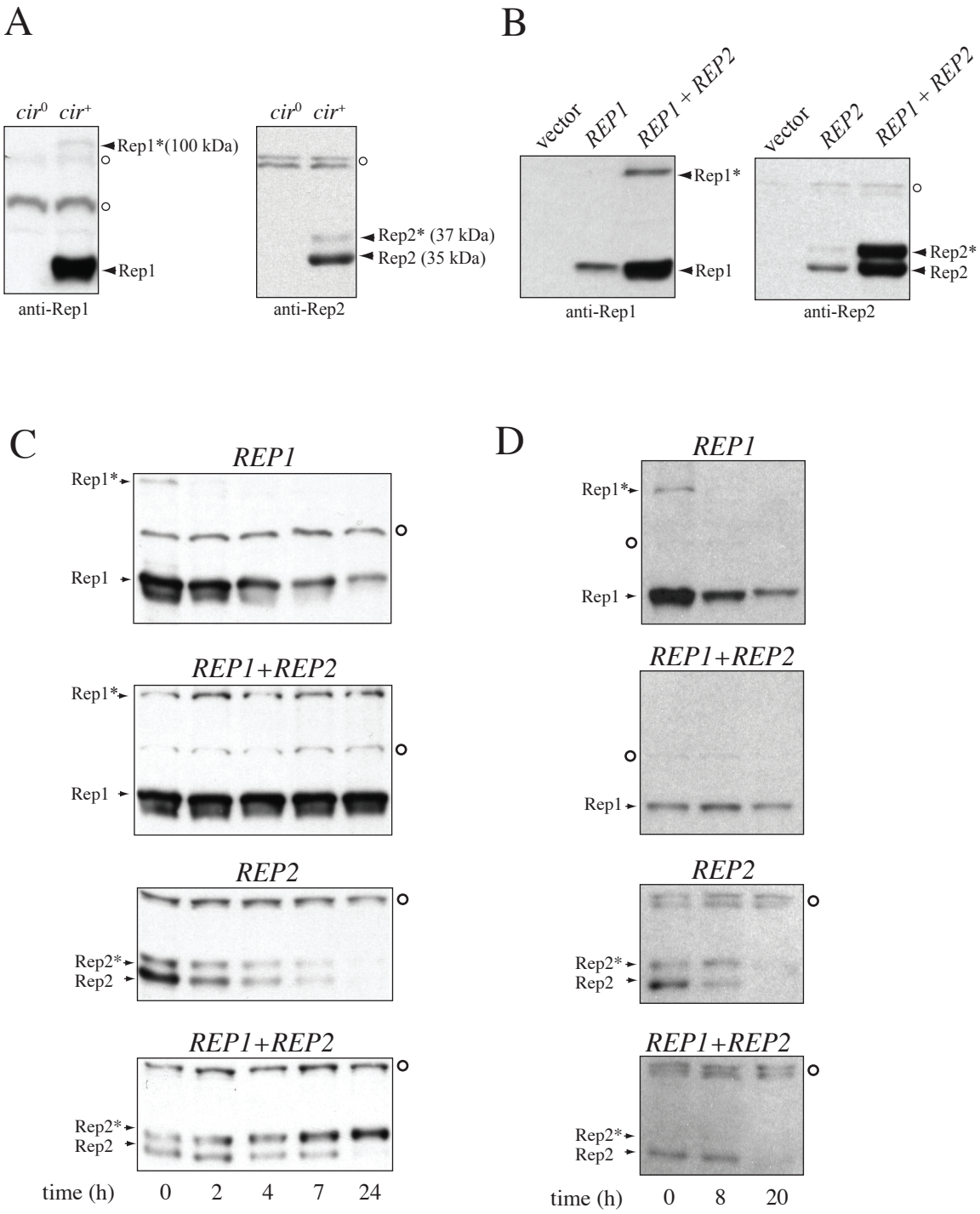
To identify potential post-translationally modified forms of Rep1 and Rep2, protein was extracted from yeast containing (*cir*$^+$) or lacking (*cir*^{0}) the native 2μm plasmid and analyzed by western blotting. Our polyclonal anti-Rep1 and anti-Rep2 antibodies did detect some proteins in both *cir*$^+$ and *cir*^{0} protein extracts (**Figure 3A**). These off-target non-Rep protein species were found to be reliable indicators of protein loading, comparable to reprobing blots with an antibody directed against 3-phosphoglycerate kinase (Pgk), and in this thesis these cross-reacting proteins were sometimes used instead of Pgk to demonstrate protein loading. In addition to these off-target species, the anti-Rep antibodies detected species present only in protein extracted from *cir*$^+$ yeast. Major species of the sizes expected for native Rep1 (43.2 kDa predicted, 50 kDa observed) and Rep2 (33.2 kDa predicted, 35 kDa observed) were detected with their respective antibodies. Interestingly, additional higher-molecular-weight (HMW) species were detected with both anti-Rep1 (~100 kDa) and anti-Rep2 (~37 kDa) antibodies.

The absence of these HMW species in protein from *cir*^{0} yeast suggested these were post-translationally modified forms of the Rep proteins.

3.1.2 Rep2 Protects Rep1 from Degradation *In Vivo*

Expression of the Rep1 and Rep2 proteins had previously been shown to be regulated at the level of transcription, with Rep1 and Rep2 acting together to repress their own genes

Figure 3. Examining stability and post-translationally modified forms of Rep1 and Rep2. (A) Total protein was extracted from isogenic *cir*$^{+}$ and *cir*^{0} yeast strains and analyzed by western blotting with antibodies against Rep1 (left) and Rep2 (right). (B) *CEN/ARS* plasmids encoding either the *REP1*, *REP2*, or both *REP1* and *REP2* ORFs under control of a galactose-inducible promoter, or with no ORF inserted (vector), were transformed into *cir*^{0} yeast and Rep protein expression was induced for 4 h by growth in medium containing galactose. Total protein was extracted and Rep1 and Rep2 levels were analyzed by western blotting. (C) Yeast transformants in (B) were grown for 12 h in medium containing galactose, cycloheximide was added, and total protein was extracted from yeast at the indicated time points and analyzed by western blotting. (D) An *ADE2*-tagged 2μm plasmid encoding both *REP1* and *REP2*, or that was deleted for either *REP1* or *REP2*, was transformed into *cir*^{0} *ade2* yeast. Transformants were grown to logarithmic phase in medium lacking adenine, and protein was analyzed by western blotting at the indicated time points following addition of cycloheximide. Apparent molecular weights (kDa) of Rep protein species detected by the antibodies are shown. Higher-molecular weight forms of the Rep proteins (asterisks), and proteins that were recognized non-specifically by the antibodies (open circles) are indicated.


A
cir0 cir+
Rep1*(100 kDa)
Rep1
anti-Rep1
cir0 cir+
Rep2* (37 kDa)
Rep2 (35 kDa)
anti-Rep2
B
vector
REP1
REP1 + REP2
Rep1*
Rep1
anti-Rep1
vector
REP2
REP1 + REP2
Rep2*
Rep2
anti-Rep2
C
REP1
Rep1*
Rep1
REP1+REP2
Rep1*
Rep1
REP2
Rep2*
Rep2
REP1+REP2
Rep2*
Rep2
time (h) 0 2 4 7 24
D
REP1
Rep1*
Rep1
REP1+REP2
Rep1
REP2
Rep2*
Rep2
REP1+REP2
Rep2*
Rep2
time (h) 0 8 20

(200, 228). Previous data from our lab suggested that Rep1 protein levels might also be regulated post-translationally by interaction with Rep2. Point substitutions in Rep1 that abolish interaction with Rep2 were consistently associated with reduced steady-state levels of the mutant Rep1 protein, suggesting that interaction of Rep1 with Rep2 increases Rep1 stability (Arpita Sengupta, PhD thesis). To further investigate this hypothesis, the effect of the absence of Rep2 on the levels of Rep1, and vice-versa, was determined. A *cir*^{0} yeast strain was transformed with a single-copy (*CEN*/*ARS*) plasmid encoding *REP1*, *REP2*, or both *REP1* and *REP2* ORFs under control of a galactose-inducible (*GAL*) promoter. Expression from non-2μm-based plasmids using a heterologous promoter ensured that Rep1 and Rep2 protein levels would not be affected by differences in plasmid copy number caused by mis-segregation or altered Rep protein-dependent regulation of *REP* gene expression. The transformed yeast were transferred to medium containing galactose to activate *GAL*-driven expression of the *REP* genes. After a brief period sufficient for Rep protein expression, protein was extracted and examined by western blotting analysis with antibodies directed against either Rep1 or Rep2 (**Figure 3B**).

When Rep1 and Rep2 were co-expressed from a galactose-inducible promoter in *cir*^{0} yeast, the single species of Rep1 and Rep2, respectively, corresponding to each unmodified Rep protein, and the single HMW form of each Rep protein were also detected. All Rep protein species appeared to be more abundant than in protein extracted from *cir*$^+$ yeast, as the signals for each species were more intense relative to those of off-target proteins detected by the antibodies, consistent with the Rep proteins being expressed at a higher level from the strong galactose-inducible promoter than when expressed from their native promoters of 2μm-plasmid-encoded *REP* genes in *cir*$^+$ yeast. When the Rep proteins were expressed individually from a galactose-inducible promoter, the species consistent with unmodified Rep1 and Rep2, as well as the 100-kDa species of Rep1 (on a longer exposure of the blot in **Figure 3B**, not shown), and the 37-kDa species of Rep2 (**Figure 3B**) were also detected, although the levels of all species of the Rep proteins were significantly lower than when the Rep proteins were co-expressed, suggesting that Rep1 and Rep2 may stabilize each other post-translationally.

The observations indicate that the levels of both major species of Rep1 and Rep2 and their respective HMW forms are higher when the Rep proteins are co-expressed compared to

when expressed in the absence of their partner Rep protein. To further investigate this, the rate at which Rep1 and Rep2 were degraded when expressed individually or together was examined. Following induction of *GAL*-driven *REP* gene expression, cycloheximide was added to block synthesis of new proteins. Cells were then harvested at various time points and Rep1 and Rep2 protein levels were monitored by western blotting (**Figure 3C**). Compared to when the Rep proteins were co-expressed, both Rep1 and Rep2 had much shorter half-lives when expressed in the absence of their partner Rep protein, suggesting that Rep1 and Rep2 protect each other from degradation. The 100-kDa species of Rep1 and 37-kDa species of Rep2 were also stabilized by expression of the partner Rep protein. Interestingly, when Rep1 and Rep2 were co-expressed, the intensity of the signal for the 37-kDa species of Rep2 increased following addition of cycloheximide, while that of the 35-kDa species decreased, suggesting that as time progressed, the 35-kDa species was being converted to the 37-kDa form.

The "co-chaperoning" activity of Rep1 and Rep2 was not dependent on association with their known binding site in the 2μm plasmid, the *STB* locus, since the yeast strains in which the proteins were expressed lacked the 2μm plasmid and therefore did not contain *STB*. Although Rep1 and Rep2 seemed to contribute significantly to the post-translational stability of their partner Rep protein, the levels of the Rep proteins obtained by *GAL*-driven expression of the *REP* genes were significantly higher than those expressed from the native 2μm plasmid. To test whether co-stabilization of Rep1 and Rep2 was also observed when the Rep proteins were expressed under the control of their native promoters, 2μm-based plasmids tagged with an *ADE2* adenine biosynthetic gene, and encoding both *REP1* and *REP2*, or lacking either *REP1* or *REP2* were created. The 2μm plasmid confers no phenotype to yeast, and insertion of the *ADE2* marker gene in the plasmid ensured that the plasmids could be retained when introduced into *ade2* mutant *cir*^{0} yeast based on their ability when present to enable the host to grow in medium lacking adenine. Maintaining selective conditions for plasmid-containing cells was particularly important for yeast transformants containing 2μm plasmids that lacked either a *REP1* or *REP2* gene, since these were expected to be inefficiently transmitted to daughter cells during cell division. The *cir*^{0} yeast transformed with the *ADE2*-tagged 2μm plasmids were grown to logarithmic phase in medium lacking adenine, and Rep protein levels were monitored following addition of cycloheximide (**Figure**

3D). As was observed when the Rep proteins were over-expressed, Rep1 expressed from the native context had a shorter half-life when Rep2 was absent compared to the almost undetectable decline in Rep1 levels 20 hours after cycloheximide addition. In contrast, for Rep2, the rate of loss no longer appeared to be influenced by co-expression with Rep1. In the absence of Rep1, a greater proportion of the Rep2 species signal was observed as the 37-kDa species compared to in the presence of Rep1, where the 37-kDa species was barely detected, but for both, all Rep2 signal was similarly lost between 8 and 20 hours after addition of cycloheximide. Taken together, these results demonstrate that Rep1 is stabilized by Rep2, and suggest that Rep2 may also be stabilized by Rep1 but only when both proteins are over-expressed, and/or are present in stoichiometric quantities that differ from their normal relative levels.

3.1.3 Ubiquitination-Dependent Regulation of Rep1 and Rep2 Stability

3.1.3.1 A Higher-Molecular-Weight Species of Rep2 Accumulates in *ubc2*Δ and *ubc4*Δ Yeast

The decreased stability of Rep1 and Rep2 when over-expressed individually suggests that Rep1 and Rep2 molecules that are not bound to each other in a heterodimeric complex might be vulnerable to proteolysis by a host-mediated mechanism. Given that the post-translational stability of many proteins is regulated by ubiquitination (for a review, see 125), I first investigated the possibility that the Rep proteins protect each other from degradation by ubiquitin-mediated proteolysis. Covalent attachment of ubiquitin to target proteins requires a ubiquitin-specific E1 activating enzyme, E2 conjugating enzyme, and E3 ligase. Yeast encode a single, essential ubiquitin activating enzyme (Uba1), eleven ubiquitin E2 conjugating enzymes (Ubc1-8, Ubc10-11, and the heterodimer Ubc13-Mms2) and many E3 ligases (125). Of the eleven E2s, two are encoded by essential genes, and of the nine encoded by non-essential genes, six (Ubc2, Ubc4, Ubc5, Ubc8, Ubc11, and Ubc13) are localized to the nucleus. To assess whether any of these six might contribute to post-translational stability of Rep1 and Rep2, *ubc*-gene deletion strains were retrieved from the EUROSCARF yeast gene-deletion collection (74) and cured of the native 2μm plasmid (221). These cir^0 derivatives were transformed with a *CEN/ARS* plasmid directing expression of either Rep1 or Rep2 from a *GAL* promoter. Yeast transformants were grown in medium containing

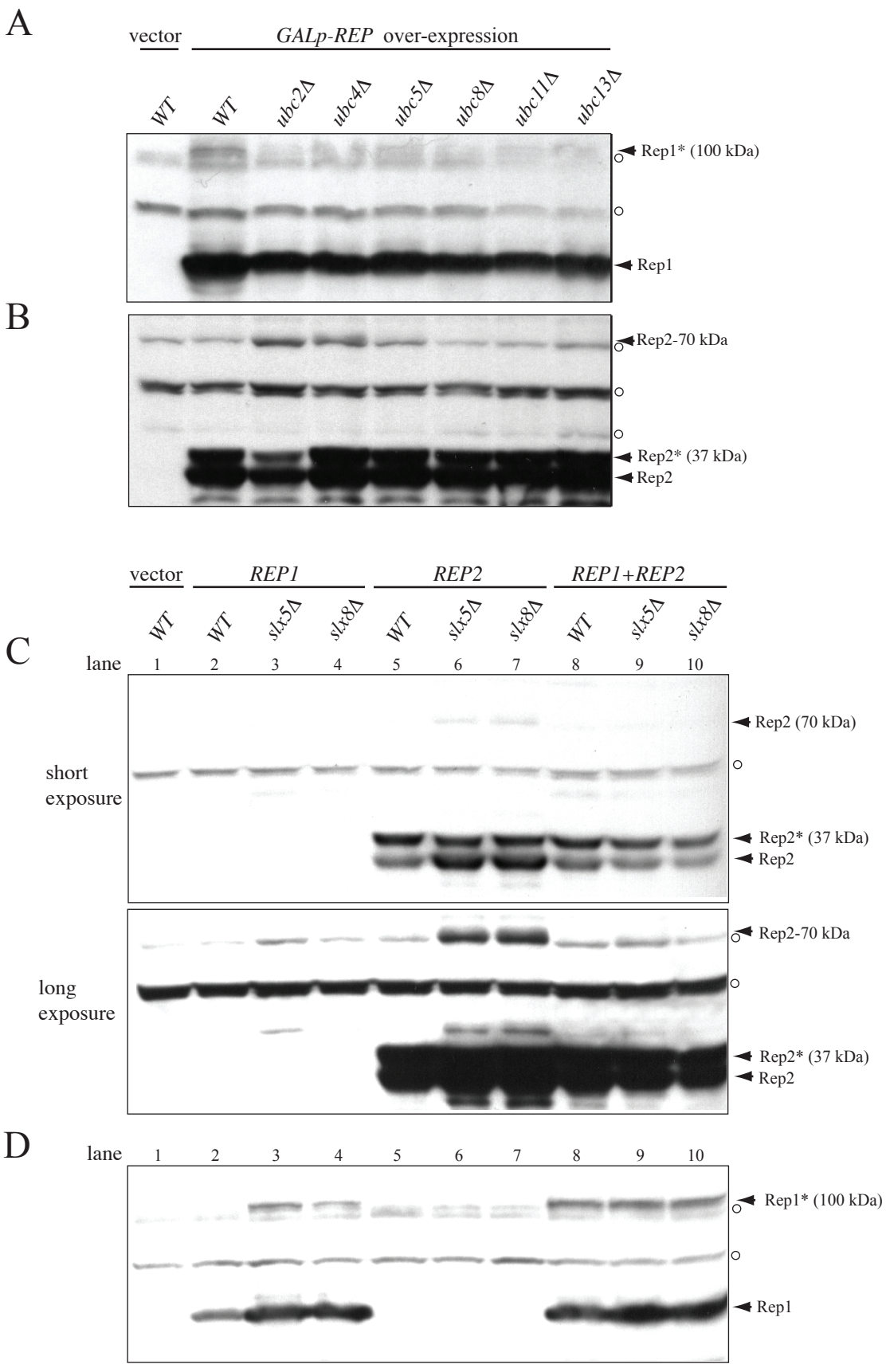
galactose to induce expression of *REP1* or *REP2*, protein was extracted and Rep1 (**Figure 4A**) and Rep2 (**Figure 4B**) levels were examined by western blotting. In this experiment, loss of a ubiquitin-conjugating enzyme normally involved in degradation of Rep1 or Rep2 would be expected to lead to an increase in Rep1 or Rep2 levels. However, the levels of Rep1 and Rep2 were not significantly increased in any of the *ubc* deletion strains, suggesting that non-dimerized Rep1 and Rep2 were not stabilized by loss of any of the ubiquitin-conjugating enzymes tested. Surprisingly, the levels of Rep2 were reduced in yeast lacking Ubc2. A reduction in Rep2 levels in the *ubc2*Δ mutant could have resulted from decreased expression from the *GAL* promoter, but this is not likely since the levels of Rep1 expressed from the *GAL* promoter were not reduced in the *ubc2*Δ strain relative to the other strains that encoded wild-type *UBC2*. An alternative explanation for the decrease in Rep2 protein levels in the *ubc2*Δ strain is that Ubc2 is required for Rep2 post-translational stability, at least under these artificial conditions in which Rep2 was over-expressed in the absence of Rep1.

Although neither Rep1 nor Rep2 accumulated in any of the *ubc* deletion strains tested, a HMW species with an apparent molecular weight (MW) of 70 kDa, ~40 kDa greater than unmodified Rep2, was detected by the anti-Rep2 antibodies in protein extracted from *ubc2*Δ, *ubc4*Δ, and *ubc5*Δ yeast. This species unfortunately also co-migrated with a non-Rep2 protein detected by the antibodies. The only reported post-translational modification to Rep2 consistent with a mobility shift of this magnitude is sumoylation (36), and 70 kDa would be consistent with the size predicted for di-sumoylated Rep2.

3.1.3.2 The 70-kDa Species of Rep2 Accumulates When Yeast are Defective for SUMO-Targeted Ubiquitination

If the 70-kDa form of Rep2 detected in protein extracts from *ubc2*Δ, *ubc4*Δ and *ubc5*Δ strains did represent a di-sumoylated form, the increased level could be due to defective SUMO-targeted ubiquitination, a pathway that specifically targets proteins that are modified by poly-SUMO chains for ubiquitination and proteasomal degradation. In yeast, SUMO-targeted ubiquitination is mediated by the heterodimeric SUMO-targeted ubiquitin E3 ligase Slx5-Slx8, and the Ubc4 and Ubc5 ubiquitin E2 conjugating enzymes (112, 158, 178, 209, 225, 247). Yeast lacking Slx5, Slx8, or both Ubc4 and Ubc5 accumulate poly-sumoylated proteins (225).

Figure 4. Analysis of Rep protein levels in ubiquitination-pathway mutants. *CEN/ARS* plasmids encoding the (A) *REP1* (B) *REP2* or (C,D) the indicated *REP* gene ORFs under control of a galactose-inducible promoter, or with no ORF inserted (vector), were transformed into *cir*^{0} yeast of the indicated genotype and Rep protein expression was induced for 8 h by growth in galactose. Total protein was extracted and Rep1 and Rep2 levels were analyzed by western blotting with anti-Rep1 (A and D) or anti-Rep2 (B and C) antibodies. HMW species of Rep1 migrating at 100 kDa and of Rep2 migrating at 37 and 70 kDa are indicated. Open circles denote species recognized non-specifically by the antibodies.


A
vector
GALp-REP over-expression
WT
WT
ubc2Δ
ubc4Δ
ubc5Δ
ubc8Δ
ubc11Δ
ubc13Δ
Rep1* (100 kDa)
Rep1
B
Rep2-70 kDa
Rep2* (37 kDa)
Rep2
vector
REP1
REP2
REP1+REP2
WT
WT
slx5Δ
slx8Δ
WT
slx5Δ
slx8Δ
WT
slx5Δ
slx8Δ
C
lane 1 2 3 4 5 6 7 8 9 10
short exposure
Rep2 (70 kDa)
Rep2* (37 kDa)
Rep2
long exposure
Rep2-70 kDa
Rep2* (37 kDa)
Rep2
D
lane 1 2 3 4 5 6 7 8 9 10
Rep1* (100 kDa)
Rep1

To test whether the loss of SUMO-targeted ubiquitination affected the level of the 70-kDa form of Rep2, or altered the overall steady-state levels of either Rep protein, Rep1 and Rep2 were over-expressed from a *GAL* promoter either individually or together in a cir^0 yeast strain that was either wild-type for *SLX5* and *SLX8*, or deleted for either gene, and the levels of Rep2 (**Figure 4C**) and Rep1 (**Figure 4D**) were analyzed by western blotting. When Rep2 was over-expressed in an *slx5*Δ or *slx8*Δ strain, a HMW species Rep2 was detected that was about the same size (~70 kDa) as that observed in extracts from the *ubc2*Δ, *ubc4*Δ and *ubc5*Δ strains (**Figure 4C, lanes 5-7**), suggesting that loss of SUMO-dependent ubiquitination causes accumulation of a 70-kDa species of Rep2. When Rep1 was over-expressed with Rep2, the 70-kDa species of Rep2 was not detected in protein extracted from either wild-type or *slx* mutant yeast (**Figure 4C, lanes 8-10**). If this is a di-sumoylated species of Rep2, the results suggest that interaction of Rep1 with Rep2 could block Rep2 sumoylation, and consequently SUMO-targeted ubiquitination by Slx5-Slx8. However, since in the absence of Rep1 expression, Rep2 levels (both the 35- and 37-kDa species) in the *slx5*Δ and *slx8*Δ strains were only modestly elevated compared to those in yeast wild-type for *SLX5* and *SLX8*, Rep1-mediated protection of Rep2 SUMO-mediated degradation may not be a major mechanism of Rep2 stabilization. Interestingly, the increase in the level of the 70-kDa species of Rep2 in the *slx* mutant yeast was accompanied by a decrease in the ratio of the level of the 37-kDa species of Rep2 relative to that of the 35-kDa species, suggesting potential interplay between different post-translational modifications of Rep2.

3.1.3.3 Rep2 May Protect Rep1 Against Ubiquitination by Slx5-Slx8

When Rep1 was expressed in the absence of Rep2, the level of Rep1 was higher in the *slx5*Δ and *slx8*Δ mutant strains compared to that in wild-type yeast (**Figure 4D, lanes 2-4**). This increase in stability was similar to that previously observed (see **Figure 3**) when Rep2 was over-expressed with Rep1 (**Figure 4D, lanes 2 and 8**) and was not further increased when Rep1 and Rep2 were co-expressed in *slx5*Δ or *slx8*Δ yeast (**Figure 4D, lanes 8-10**). These findings suggest that Rep2 may protect Rep1 from Slx5-Slx8 mediated ubiquitination, and proteasomal degradation.

3.2 Post-Translationally Modified Forms of Rep1 and Rep2

Western blotting analysis identified a 37-kDa HMW form of the 2μm plasmid partitioning protein Rep2 that suggested that Rep2 might be post-translationally modified (**Figure 3**). Preliminary experiments suggested that the 37-kDa form of Rep2 is a phosphorylated species (Jeremy Koenig, Dalhousie University, Honours thesis). To examine the potential functional significance of this 37-kDa form of Rep2, definitive support for this being phosphorylation was sought.

3.2.1 The 37-kDa Species of Rep2 is Sensitive to Phosphatase

To verify that the 37-kDa Rep2 species is a phosphorylated form, sensitivity to phosphatase was determined. Yeast were transformed with a plasmid directing expression of FLAG epitope-tagged Rep2 under control of a *GAL* promoter, and transformants were grown in medium containing galactose to induce expression of FLAG-Rep2. Spheroplasts were prepared, soluble protein was isolated and FLAG-Rep2 was immunoprecipitated and treated with calf-intestinal alkaline phosphatase in the absence or presence of sodium phosphate as a competitive inhibitor. Rep2 levels were then examined by western blotting (**Figure 5**). Two species having apparent MWs of 36 and 38 kDa were detected, consistent with the FLAG epitope adding ~1 kDa to the previously observed 35- and 37-kDa forms of untagged Rep2, respectively. Following treatment with phosphatase, the 38-kDa form of FLAG-Rep2 disappeared and the signal for the 36-kDa species was intensified. The 38-kDa form was protected when sodium phosphate was included in the reaction. These results confirm that the species of Rep2 migrating at 37-kDa represents a phosphorylated form.

3.2.2 Analysis of Post-Translationally Modified Forms of Rep1 and Rep2 by Immunoblotting of Protein Resolved by 2D Gel Electrophoresis

3.2.2.1 Rep2 is Phosphorylated at Multiple Sites

To investigate a possible role for phosphorylation of Rep2 in plasmid partitioning, my overall strategy was to identify the phosphorylation site(s) in Rep2, and then analyze Rep2 function when these target residues were mutated.

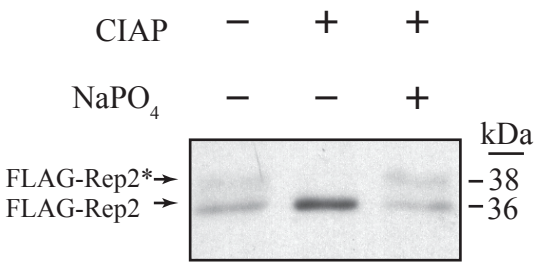


Figure 5. The 37-kDa species of Rep2 is a phosphorylated form. FLAG-tagged Rep2 was expressed in yeast from a *GAL* promoter, immunoprecipitated from cell lysates with an anti-FLAG antibody and treated with calf intestinal alkaline phosphatase (CIAP) in the presence or absence of sodium phosphate. Phosphatase reactions were then examined by immunoblotting with anti-Rep2 antibodies. A 38-kDa phosphatase-sensitive species (FLAG-Rep2*) is indicated.

As a starting point to determine the number of residues phosphorylated in the 37-kDa species of Rep2, yeast protein extracts were separated by 2D gel electrophoresis. In 2D gel electrophoresis, proteins are first separated on the basis of isoelectric point (pI) using isoelectric focusing, and subsequently resolved in the second dimension by molecular weight using standard SDS-PAGE. Separation of proteins by their charge using isoelectric focusing can resolve species that differ by even a single phosphorylated residue. To maximize the signal for Rep2 obtained from immunoblotting of the 2D gels, Rep2 was over-expressed with an N-terminal FLAG epitope tag from a *GAL* promoter in a cir^0 yeast strain. Following prolonged induction of FLAG-tagged Rep2, total protein was extracted, resolved by 2D gel electrophoresis, and analyzed by western blotting with anti-Rep2 antibodies (**Figure 6A**). In isoelectric focusing, the migration position of a protein can be predicted based on its amino acid sequence (13). The predicted MW and pI values for unmodified, as well as sumoylated and phosphorylated forms of FLAG-Rep2 (**Table 6**) were calculated using the Scansite Molecular Weight and Isoelectric Point utility (http://scansite.mit.edu).

In analysis of FLAG-Rep2 species, 13 discrete spots were detected that had apparent MW of between 36-38 kDa. None of these spots was detected by the anti-Rep2 antibodies in a parallel analysis of protein from yeast in which no Rep2 was expressed, confirming that each of the 13 spots represented a form of Rep2 (**Figure 6B**). A prominent signal with the mobility expected for unmodified FLAG-Rep2 (MW 34.4 kDa, pI 8.4) was observed (**Figure 6A**, **spot 1**). In addition, four major spots of significantly more acidic pI were observed, which also had a slightly higher apparent MW than unmodified FLAG-Rep2 (**Figure 6A**, **spots 10-13**). The higher apparent MW of the these four species makes it likely that the 38-kDa form of FLAG-Rep2 shown by western blotting to be sensitive to phosphatase (**Figure 5**) represents co-migration of these four phosphorylated forms of Rep2. These species will hereafter be collectively referred to as the "hyper-phosphorylated" species of Rep2. Approximately eight faint species (**Figure 6A, spots 2-9**) were also detected that had pI values and apparent MWs that were intermediate between the unmodified and hyper-phosphorylated forms of Rep2, consistent with these spots representing "hypo-phosphorylated" forms of Rep2.

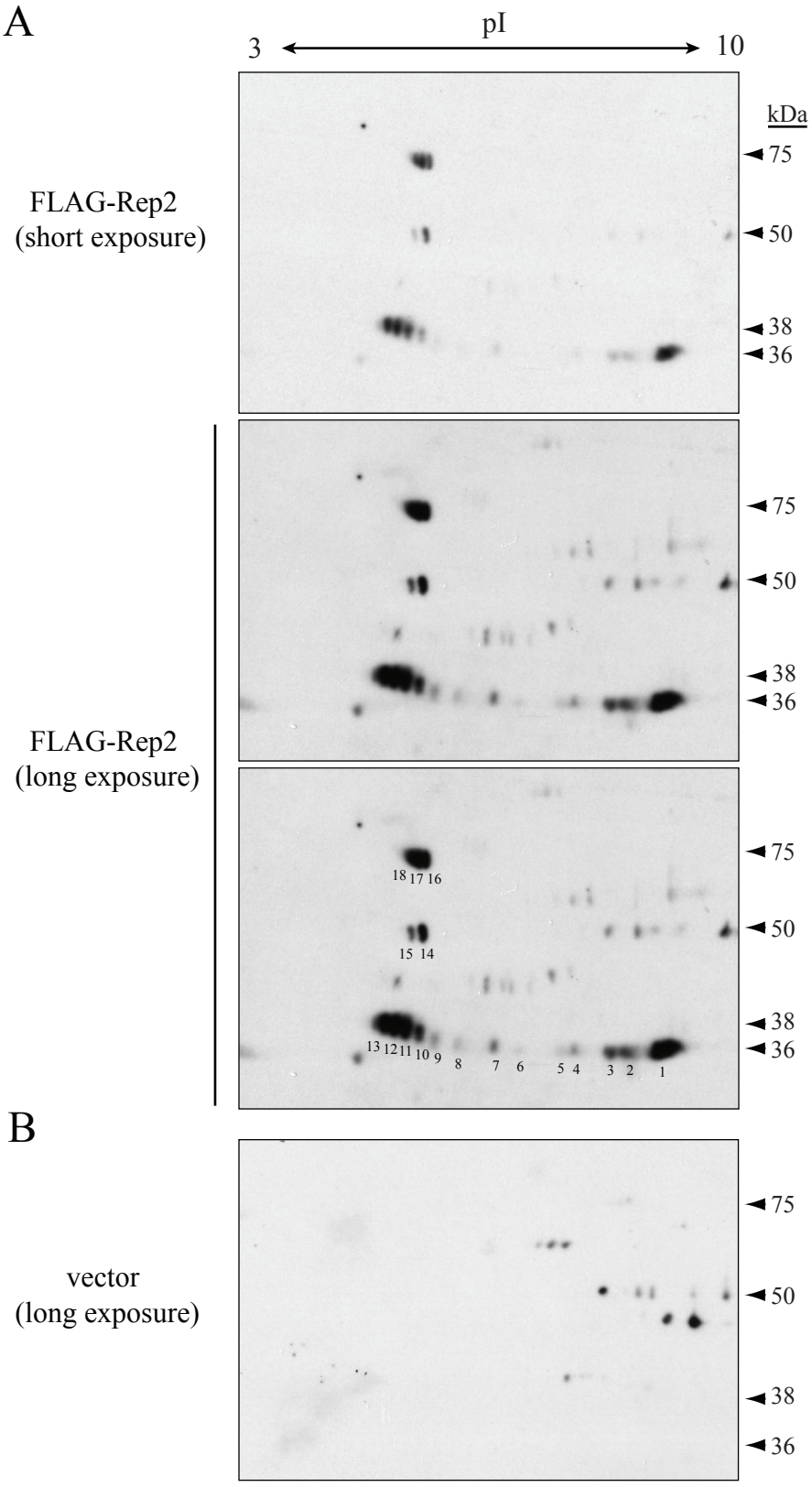
The twelve spots of increasing acidity relative to unmodified Rep2 could represent Rep2 containing between one and twelve phosphorylated residues, respectively.

Table 6. Predicted isoelectric point (pI) and molecular weight (MW, in kDa) of sumoylated and phosphorylated species of FLAG-tagged Rep2.

# phosphates	FLAG-Rep2		FLAG-Rep2-SUMO		FLAG-Rep2-2xSUMO	
	pI	MW	pI	MW*	pI	MW*
0	8.4	34.6	5.8	55	5.4	75
1	7.2	34.6	5.6	55	5.4	75
2	6.5	34.7	5.5	55	5.3	75
3	6.0	34.8	5.4	55	5.3	75
4	5.8	34.9	5.3	55	5.2	75
5	5.6	35.0	5.3	55	5.2	75
6	5.4	35.0	5.2	55	5.1	75
7	5.3	35.1	5.1	55	5.1	75
8	5.2	35.2	5.1	55	5.0	75
9	5.1	35.3	5.0	55	5.0	75
10	5.0	35.3	5.0	55	5.0	75
11	5.0	35.4	4.9	55	4.9	75
12	4.9	35.5	4.9	55	4.9	75

*predicted apparent MW

Figure 6. Multiple post-translationally modified forms of Rep2 resolved by 2D gel electrophoresis. A *cir*^{0} yeast strain was transformed with (A) pRSFLAG-REP2, which directs expression FLAG-tagged Rep2 from a galactose-inducible promoter or (B) pRSFLAG vector and transformants were grown in medium containing galactose for 16 h. Total protein was extracted, resolved by 2D gel electrophoresis, and analyzed by immunoblotting with anti-Rep2 antibodies. Signals consistently detected specifically in yeast expressing FLAG-Rep2 are numbered for reference.


A
pI
3
10
kDa
FLAG-Rep2
(short exposure)
75
50
38
36
FLAG-Rep2
(long exposure)
75
50
38
36
18 17 16
15 14
13 12 11 10 9 8 7 6 5 4 3 2 1
75
50
38
36
B
vector
(long exposure)
75
50
38
36

Alternatively, some of the Rep2 species might have slight differences in pI despite being modified by an equal number of phosphate groups, if different residues are modified. It is difficult to determine how many residues are phosphorylated in each detected species of Rep2 without accurate measurements of the pI values relative to those of known standard marker proteins, but from the analysis it is clear that Rep2 is phosphorylated on many residues.

In addition to the species of unmodified and phosphorylated Rep2 that were observed in western blot analysis of 2D gels, several species were detected that had mobilities of 50-kDa and 75-kDa (**Figure 6A, spots 14-18**), which are close to the apparent MW values predicted for mono- and di-sumoylated FLAG-Rep2, respectively. Although yeast SUMO is predicted to be 11.3 kDa, mono-sumoylation has been reported to increase the apparent MW of a targeted protein by approximately 20 kDa, while di-sumoylation would be expected to add 40 kDa (120). The 50-kDa species of Rep2 migrated as two distinct spots with slightly differing pI (**Figure 6A, spots 14-15**), while the 75-kDa species of Rep2 migrated as three spots of a similar pI (**Figure 6A, spots 16-18**). The multiple spots observed for both the 50- and 75-kDa forms of Rep2 could represent mono- and di-sumoylated species, respectively, that differ in the lysine residue(s) attached to SUMO, or could represent different phosphorylated forms of Rep2 SUMO-conjugates.

3.2.2.2 Two Species of Rep1 Detected by 2D Immunoblotting.

The observation that multiple hyper-phosphorylated species of Rep2 co-migrated as a single 37-kDa band in 1D gels raised the possibility that single band detected by anti-Rep1 antibodies in similar western blotting analysis of Rep1 also might represent more than one co-migrating species. To test this idea, *cir*^{0} yeast were transformed with a plasmid encoding FLAG-tagged Rep1, and following galactose induction of FLAG-Rep1 expression, protein was extracted, resolved by 2D gel electrophoresis, and analyzed by immunoblotting with anti-Rep1 antibodies (**Figure 7A**). Two prominent spots (**Figure 7A, spots 1-2**) were observed that had the mobilities similar to that predicted for FLAG-Rep1 (MW 44.6 kDa, pI 5.8) (**Table 7**). These spots were not detected by the anti-Rep1 antibodies in a parallel analysis of protein from yeast in which no Rep1 was expressed, indicating that the two spots represented forms of Rep1 (**Figure 7B**). The pI values for these spots are consistent with the

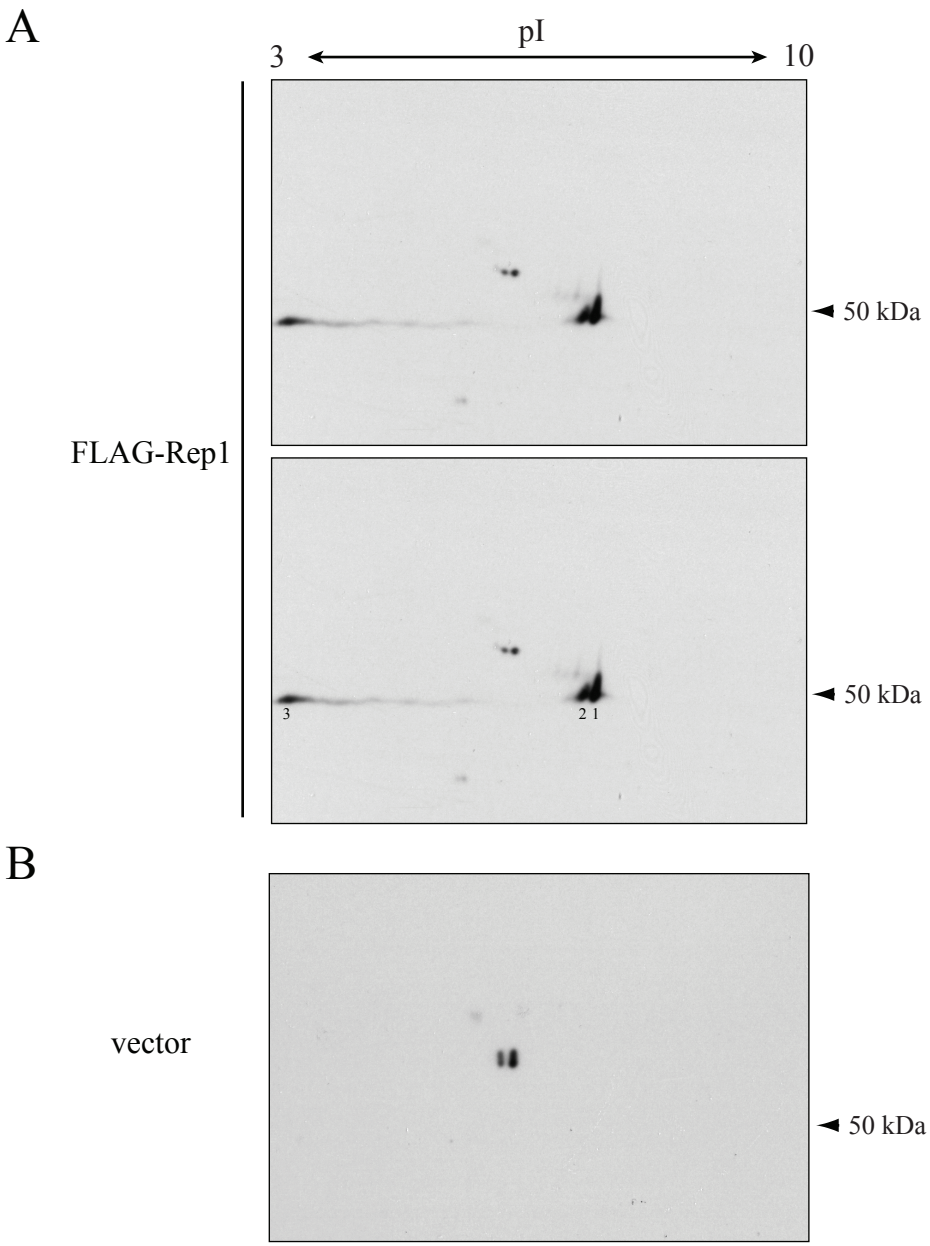


Figure 7. Analysis of Rep1 species by 2D gel electrophoresis. A *cir*^{0} yeast strain was transformed with (A) pRSFLAG-REP1, which directs expression of FLAG-tagged Rep1 from a galactose-inducible promoter, or (B) pRSFLAG vector, and transformants were grown in medium containing galactose for 16 h. Total protein was extracted, resolved by 2D gel electrophoresis, and analyzed by immunoblotting with anti-Rep1 antibodies. Signals detected specifically in yeast expressing FLAG-Rep1 are numbered for reference.

Table 7. Predicted isoelectric point (pI) and molecular weight (MW, in kDa) of sumoylated and phosphorylated species of FLAG-tagged Rep1.

# phosphates	FLAG-Rep1		FLAG-Rep1-SUMO	
	pI	MW	pI	MW*
0	5.8	45	5.5	65
1	5.7	45	5.4	65
2	5.6	46	5.4	66

*predicted apparent MW

most prominent spot being unmodified Rep1 and the more acidic species potentially representing a phosphorylated form of Rep1. However, the more acidic spot could also represent another post-translational modification that would lower the pI of Rep1, such as acetylation or methylation. Sensitivity of these species to phosphatase was not examined.

In addition, a smeared signal of extreme acidity with an apparent MW similar to that of FLAG-Rep2 was also detected (**Figure 7A, spot 3**). This could represent a highly acidic post-translationally modified form of Rep1, or might merely be an artifact of incomplete resolution of proteins during the initial isoelectric focusing step. No spots having a mobility consistent with sumoylated forms of Rep1, or with the 100-kDa species of Rep1 observed in western blotting analysis of 1D gels (**Figure 3**), were detected, even in long exposures (data not shown).

3.2.3 Phosphorylated Rep2 Accumulates in SUMO Pathway Mutants

The change in the relative levels of the hyper-phosphorylated form of Rep2 in yeast lacking the SUMO-targeted ubiquitin ligase Slx5-Slx8 (**Figure 4**), and the reduction in the number of phosphorylated forms of species consistent with sumoylated Rep2 relative to unsumoylated Rep2 species detected in immunoblotting analysis of protein separated by 2D gel electrophoresis (**Figure 6**), suggested a possible link between sumoylation and phosphorylation of Rep2. The hyper-phosphorylated form of Rep2 was shown to accumulate in yeast cells mutant for the SUMO-specific protease Ulp1 (Melanie Dobson, unpublished results). Due to the elevated plasmid copy number in *ulp1* mutant yeast (51), levels of both the 35-kDa and 37-kDa forms of Rep2 were elevated; however, preliminary data suggested the 37-kDa hyper-phosphorylated form of Rep2 was disproportionately more abundant relative to the 35-kDa form of Rep2 (Melanie Dobson, unpublished results).

To determine whether the proportion of Rep2 present in the hyper-phosphorylated form is increased in other SUMO pathway mutants, Rep2 levels were examined by western blotting of protein extracted from cir^+ yeast that were wild-type, mutant for the Ulp1 SUMO protease, or that lacked two SUMO E3 ligases, Siz1 and Siz2 (**Figure 8, lanes 2-4**). Like *ulp1* mutant yeast, the *siz1*Δ *siz2*Δ double mutant strain also had a higher level of Rep2 than wild-type yeast, consistent with previously reported observations that 2μm plasmid copy number is elevated in this strain (36). The significantly more intense signal for the Rep2 protein species

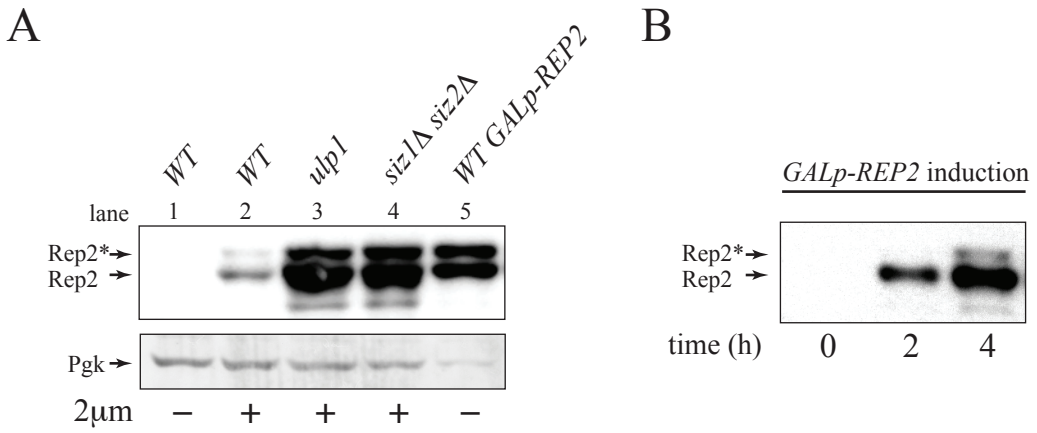


Figure 8. Hyper-phosphorylated Rep2 accumulates when Rep2 is over-expressed. (A) Protein was extracted from untransformed yeast of the indicated genotype (lanes 1-4) or from wild-type (*WT*) yeast transformed with a plasmid encoding galactose-inducible *REP2* that had been grown in medium containing galactose for 16 h (lane 5). Extracted protein was analyzed by western blotting with anti-Rep2 or anti-Pgk antibodies. (B) Yeast transformed with a plasmid encoding *GAL*-promoter-driven *REP2* were grown to saturation in medium containing raffinose as a carbon source, diluted into fresh medium containing galactose and harvested at the indicated time points following shift to galactose. Protein was extracted and analyzed as in (A). Hyper-phosphorylated Rep2 (Rep2*) is indicated.

made it difficult to determine whether the proportion of phosphorylated Rep2 relative to unmodified Rep2 was different between wild-type yeast and the SUMO pathway mutants.

3.2.4 Phosphorylated Rep2 Accumulates when Rep2 is Over-Expressed.

A possible explanation for the increased ratio of hyper-phosphorylated Rep2 relative to the 35-kDa species of Rep2 when expression of Rep1 and Rep2 was initially induced from galactose-inducible *REP* genes (**Figure 3B**), but not after prolonged galactose induction (**Figure 4C**, **lanes 5 and 8**), is that the hyper-phosphorylated form preferentially accumulates relative to the 35-kDa species of Rep2 in any condition where Rep2 levels are abnormally high. To test this possibility, the relative levels of the different forms of Rep2 were analyzed in yeast expressing *REP2* at a high level. A cir^0 yeast strain was transformed with a plasmid encoding galactose-inducible *REP2*. Transformants were grown in medium containing galactose for a prolonged period of time to induce a high level of expression of Rep2, and total protein was extracted and analyzed by western blotting (**Figure 8A, lane 5**). When *REP2* was expressed from the *GAL* promoter, levels of both the 35- and 37-kDa Rep2 species were significantly higher than those in an isogenic cir^+ yeast strain, in which Rep2 was expressed at normal levels. Notably, when Rep2 was over-expressed, the two major Rep2 species detected were approximately equally abundant, in contrast to their relative proportions in cir^+ yeast, in which there was much less of the 37-kDa hyper-phosphorylated form. This observation suggests that the hyper-phosphorylated form of Rep2 may accumulate whenever Rep2 is over-expressed. However, it is also possible that phosphorylated Rep2 accumulates in the absence of other native 2μm plasmid proteins, which were not present in the cir^0 strain in which Rep2 was over-

expressed. If this were the case, hyper-phosphorylated Rep2 would also be expected to accumulate when Rep2 is expressed at a low level in a cir^0 strain. To test this, Rep2 levels were examined at early time points following induction of *GAL* promoter-driven *REP2* (**Figure 8B**). The level of Rep2 detected early in the induction phase was markedly lower than that detected at later time points, and the amount of hyper-phosphorylated Rep2 detected was much less than the level of the 35-kDa form of Rep2. This trend suggests that the relative proportion of hyper-phosphorylated Rep2 increases with the total amount of Rep2 expressed. The data suggest that the increase in Rep2 phosphorylation observed in SUMO-

pathway mutants may simply be a consequence of higher plasmid copy number producing higher overall levels of Rep2 in these strains, and may not be related to sumoylation of Rep2.

3.2.5 Rep2 Phosphorylation is Impaired in Yeast Mutant for Protein Kinases Bud32, CK2, or Kns1

The observation that Rep2 is phosphorylated on multiple residues suggested that Rep2 may be a target of more than one protein kinase, or that Rep2 is phosphorylated on multiple residues by a single kinase. The consensus sites for many kinases have been identified to date (3, 155, 264). Identifying the kinases that phosphorylate Rep2 could aid in pinpointing which of the 49 possible phosphorylation sites (S/T/Y) in Rep2 are targeted for phosphorylation.

As a starting point to identify candidate kinases that phosphorylate Rep2, the levels of hyper-phosphorylated Rep2 were monitored in yeast strains that each lacked a single protein kinase. Based on conserved sequence motifs common to all yeast protein kinases (89, 90) yeast are predicted to contain 124 protein kinase-encoding genes (111, 264) (**Table 8**); of these, 108 are not essential for viability. To investigate whether any of these nonessential protein kinases phosphorylate Rep2, protein was extracted from 106 of the 108 viable kinase-gene deletion yeast strains (two strains were absent from our collection), and Rep2 levels were analyzed by western blotting. Complete or partial loss of Rep2 phosphorylation was anticipated to be observed as a decrease in abundance, or an increase in electrophoretic mobility of the hyper-phosphorylated species. If phosphorylation of Rep2 is important for Rep-mediated 2μm plasmid partitioning, yeast strains lacking a kinase needed for phosphorylation might also be expected to have lost the 2μm plasmid, and therefore would not express Rep2.

In protein extracts from 103 of the 106 kinase-gene deletion strains analyzed, the migration pattern of Rep2 species was indistinguishable from that of extracts from wild-type yeast (data not shown). These observations established that these 103 kinases are not essential for maintenance of the 2μm plasmid. In yeast lacking the *CKA2* or *KNS1* gene, hyper-phosphorylated forms of Rep2 had slightly increased mobility, suggesting loss of

Table 8. Predicted yeast protein-kinase genes.

Non-essential							Essential
AKL1	*ELM1*	*KDX1*	*NNK1*	*PTK2*	*SSK2*	*YAK1*	*CAK1*
ARK1	*FMP48*	*KIN1*	*NPR1*	*RCK1*	*SSK22*	*YCK1*	*CBK1*
ATG1	*FPK1*	*KIN2*	*NRK1**	*RCK2*	*SSN3*	*YCK2*	*CDC15*
BCK1	*FRK1*	*KIN3*	*PBS2*	*RIM15*	*STE11*	*YCK3*	*CDC28*
BUB1	*FUS3*	*KIN4*	*PHO85*	*RTK1*	*STE20*	*YGK3*	*CDC5*
BUD32	*GCN2*	*KIN82*	*PKH1*	*SAK1*	*STE7*	*YLR253W*	*CDC7*
CHK1	*GIN4*	*KKQ8*	*PKH2*	*SAT4*	*SWE1*	*YPK1*	*HRR25*
CKA1	*HAL5*	*KNS1*	*PKH3*	*SCH9**	*TDA1*	*YPK2*	*IPL1*
CKA2	*HOG1*	*KSP1*	*PKP1*	*SCY1*	*TEL1*	*YPK3*	*KIN28*
CLA4	*HRK1*	*KSS1*	*PKP2*	*SHA3*	*TOS3*	*YPL109C*	*MPS1*
CMK1	*HSL1*	*MCK1*	*PRK1*	*SKM1*	*TPK1*	*YPL150W*	*PKC1*
CMK2	*IKS1*	*MDS1*	*PRR1*	*SKY1*	*TPK2*	*YPL236C*	*RIO1*
CTK1	*IME2*	*MEK1*	*PRR2*	*SLT2*	*TPK3*		*RIO2*
DBF2	*IRE1*	*MKK1*	*PSK1*	*SMK1*	*TWF1*		*SGV1*
DBF20	*ISR1*	*MKK2*	*PSK2*	*SNF1*	*VHS1*		*SLN1*
DUN1	*KCC4*	*MRK1*	*PTK1*	*SPS1*	*VPS15*		*SPK1*

* absent from our deletion-strain collection

some, but not all phosphorylation of Rep2 (**Figure 9A**). Yeast lacking the *BUD32* gene expressed no Rep2 (**Figure 9A**), and were confirmed to be *cir*^{0} by PCR using 2μm-specific primers (data not shown).

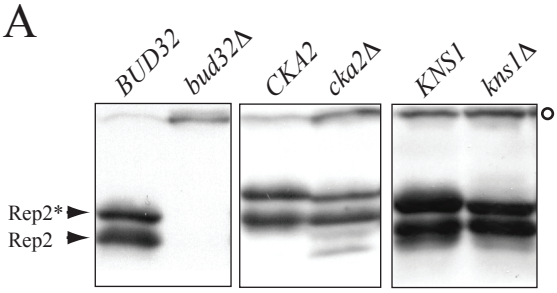
Cka2 is one of the two catalytic isoforms (the other being Cka1) of protein kinase CK2, a heterotetramer consisting of two catalytic subunits, and two regulatory subunits, (Ckb1 and Ckb2) (177). While CK2 kinases with either two Cka1 or two Cka2 subunits phosphorylate serine and threonine residues within an identical consensus sequence, depending on sequence context some substrates are specifically targeted by just one of the isoforms, and distinct functions for the two subunits have been identified (10, 29). The level of hyper-phosphorylated Rep2 could not be easily analyzed in yeast lacking both CK2 catalytic isoforms since a *cka1*Δ *cka2*Δ strain is inviable, but was not affected by deletion of *CKA1* (data not shown) suggesting that Cka2 is able to phosphorylate residues in Rep2 that are not recognized by Cka1.

Kns1 has 34% identity to Cdc28, the cyclin-dependent kinase that controls cell-cycle progression in yeast, and is a dual-specificity protein kinase, phosphorylating serine and threonine as well as tyrosine residues in substrates (133, 172). To date, the biological function of Kns1 has not been studied in depth, and consensus sequences targeted by Kns1 have not been determined.

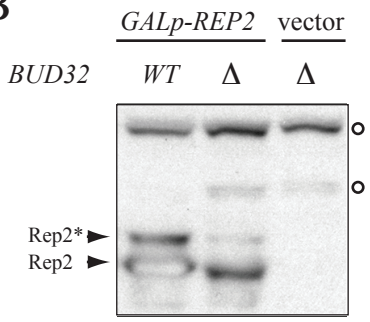
Bud32 is a component of the highly conserved KEOPS complex, which in yeast has roles in telomere maintenance, transcription, and translation (47, 53, 127, 203). Phosphorylation of Rep2 could not be assessed in the *bud32*Δ mutant since this strain lacked the 2μm plasmid. To examine phosphorylation of Rep2 in yeast lacking Bud32, *cir*^{0} yeast strains that were either wild-type or deleted for *BUD32* were transformed with a *CEN*/*ARS* plasmid directing expression of *REP2* from a *GAL* promoter. The transformed yeast were grown in galactose to induce *REP2* expression, and Rep2 levels were examined by western blotting (**Figure 9B**). The abundance of both the hyper-phosphorylated (37-kDa) and the 35-kDa species of Rep2 were reduced in the *bud32*Δ strain compared to when yeast were wild-type for *BUD32*, and the ratio of the level of 37-kDa species to that of the 35-kDa species was lower in the *bud32*Δ yeast strain. Taken together, the results from the screen of nonessential kinase gene deletion strains suggest that protein kinases CK2, Bud32, and Kns1

Figure 9. Identification of Cka2, Kns1, and Bud32 as putative Rep2 kinases. Total protein was extracted from (A) the indicated yeast deletion strains obtained from the EUROSCARF deletion-strain collection or their wild-type parental strain, grown to saturation or (B) *cir*^{0} yeast either wild-type (*WT*) or deleted (Δ) for *BUD32*, containing a plasmid encoding galactose-inducible *REP2* or with no ORF inserted (vector), and that had been grown for 8 h in medium containing galactose. Rep2 levels were analyzed by western blotting with anti-Rep2 antibodies. Hyper-phosphorylated Rep2 (Rep2*) and proteins detected by the antibodies non-specifically (open circles) are indicated. (C) A *cir*$^+$ yeast two-hybrid reporter strain was co-transformed with plasmids expressing the indicated Gal4$_{AD}$- and LexA-fusions. Co-transformants were grown for 24 h on a nitrocellulose membrane and interaction of the fusion proteins was assessed by monitoring expression of the *lacZ* reporter gene using a β-galactosidase filter assay with the substrate X-gal, which produces a dark blue precipitate upon cleavage. Vector with no insert is indicated (-). The far right column indicates observation of very strong blue (+++), strong blue (++), or pale blue (+) colour after 2 h, or lack of any detectable blue colour after 6 h (-). (D) Protein was extracted from the two-hybrid co-transformants in (C) and Rep2 levels from those expressing the indicated kinase two-hybrid fusion protein were analyzed by western blotting as in (A) and (B).

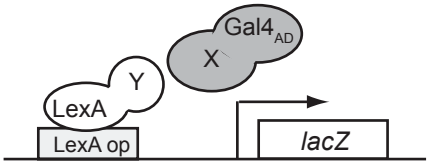
A



B

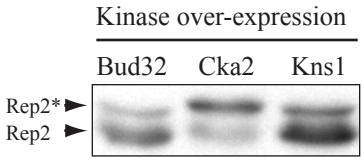


C



Gal4 $_{AD}$	LexA	*lacZ* expression
-	Bud32	-
-	Cka2	-
-	Kns1	++
Rep1	Bud32	-
Rep1	Cka2	-
Rep1	Kns1	++
Rep2	Bud32	-
Rep2	Cka2	-
Rep2	Kns1	++
SUMO	Bud32	+++
SUMO	Cka2	+++
SUMO	Kns1	+++
Bud32	Rep1	-
Bud32	Rep2	-
Bud32	-	-
Cka2	Rep1	-
Cka2	Rep 2	-
Cka2	-	-
Kns1	Rep1	+
Kns1	Rep2	+
Kns1	-	-

D



may all phosphorylate Rep2 *in vivo*, and that the Bud32 kinase might be required for maintenance of the 2μm plasmid.

3.2.6 Kns1 Interacts with Both Rep1 and Rep2 *In Vivo*

If Bud32, Cka2, and Kns1 phosphorylate Rep2, these kinases would be expected to associate with Rep2 *in vivo*. To assess this, interaction of each kinase with Rep1 and Rep2 was tested using a two-hybrid assay, with the kinases expressed as $Gal4_{AD}$ fusions, and the Rep proteins expressed as LexA fusions, and vice-versa, in a cir^+ reporter strain (**Figure 9C**). Interaction of the kinases with SUMO was also examined. While neither $Gal4_{AD}$-Bud32 nor $Gal4_{AD}$-Cka2 displayed a two-hybrid interaction with Rep1 or Rep2, $Gal4_{AD}$-Kns1 displayed robust interaction with both LexA-Rep1 and LexA-Rep2. This suggests that Kns1 might recognize both Rep proteins; however, these associations could be indirect, mediated by other nuclear proteins including endogenous Rep proteins. Neither of the Rep proteins, when expressed as $Gal4_{AD}$ fusion proteins, interacted in a two-hybrid assay with any of the three kinases, expressed as LexA fusions, although expression of LexA-Kns1 resulted in strong auto-activation of the reporter gene, which could have obscured detection of an interaction.

Interestingly, all three kinases interacted with SUMO in this assay, suggesting that they might either be sumoylated, or interact with a sumoylated protein. Two-hybrid association of Kns1 with SUMO has previously been reported (92).

3.2.7 Over-Expression of Cka2 Increases the Abundance of Hyper-Phosphorylated Rep2

Lack of two-hybrid interaction of Bud32 and Cka2 with either Rep protein does not exclude the possibility that these kinases phosphorylate Rep1 or Rep2, since interaction of protein kinases with their substrates may too transient to be detected using two-hybrid or co-immunoprecipitation assays (Paola Marignani, Dalhousie University, personal communication). As an alternative approach to test whether Bud32, Cka2, and Kns1 phosphorylate Rep2 *in vivo*, the effect of over-expressing each kinase on the level of hyper-phosphorylated Rep2 was examined. A cir^+ yeast strain was co-transformed with the plasmids used in **Figure 9C** that direct constitutive expression of Bud32, Cka2, or Kns1 as two-hybrid fusion proteins, and Rep2 levels were analyzed by western blotting (**Figure 9D**).

The proportion of Rep2 present as the 37-kDa hyper-phosphorylated form was not noticeably affected by expression of the Bud32 or Kns1 fusion proteins, but was significantly increased in yeast over-expressing the Cka2 fusions. This result provides further support for protein kinase CK2 being able to phosphorylate Rep2 *in vivo*.

3.2.8 Assessing Direct Phosphorylation of the Rep Proteins by Bud32, Cka2, and Kns1 Using *In Vitro* Kinase Assays

3.2.8.1 Bud32 and Cka2 phosphorylate Rep1 and Rep2 *in vitro*

The reduction in mobility and/or abundance of the 37-kDa Rep2 species in yeast lacking Bud32, Cka2, or Kns1 suggests that these kinases might directly phosphorylate Rep2 *in vivo*. Alternatively, the reduction in phosphorylation of Rep2 when these kinases are absent could be indirect, if these kinases stimulate activity of another kinase that phosphorylates Rep2. To see if Bud32, Cka2, and Kns1 are able to directly phosphorylate Rep1 and Rep2, *in vitro* kinase assays were performed using bacterially expressed and purified kinases and Rep proteins. Either Bud32, Cka2, or Kns1 was incubated with glutathione-S-transferase (GST)-tagged and affinity-purified Rep1 and Rep2 proteins in the presence of Mg^{2+}, Mn^{2+} and [γ^{32}P]-ATP, and reactions were resolved by SDS-PAGE and examined by Coomassie staining and autoradiography (**Figure 10**). Autophosphorylated forms of all three kinases were observed, indicating that all three kinases were catalytically active. Notably, GST-Rep1 and GST-Rep2 were both phosphorylated by Bud32 and Cka2. Bud32 and Cka2 did not phosphorylate GST, indicating that both Rep1 and Rep2 can be targeted by these two kinases *in vitro*. Kns1 phosphorylated GST alone, making it difficult to determine from this experiment whether the Rep proteins themselves could be targeted by Kns1.

3.2.8.2 Bud32 and Cka2 Phosphorylate Different Regions in Rep1 and Rep2 *In Vitro*

To narrow down which residues in Rep1 and Rep2 are phosphorylated by Bud32 and Cka2 *in vitro*, the regions in the Rep proteins sufficient for being phosphorylated by the two kinases were determined. Truncated versions of Rep1 and Rep2, expressed as GST-fusions in *E. coli* and affinity-purified, were tested in kinase assays.

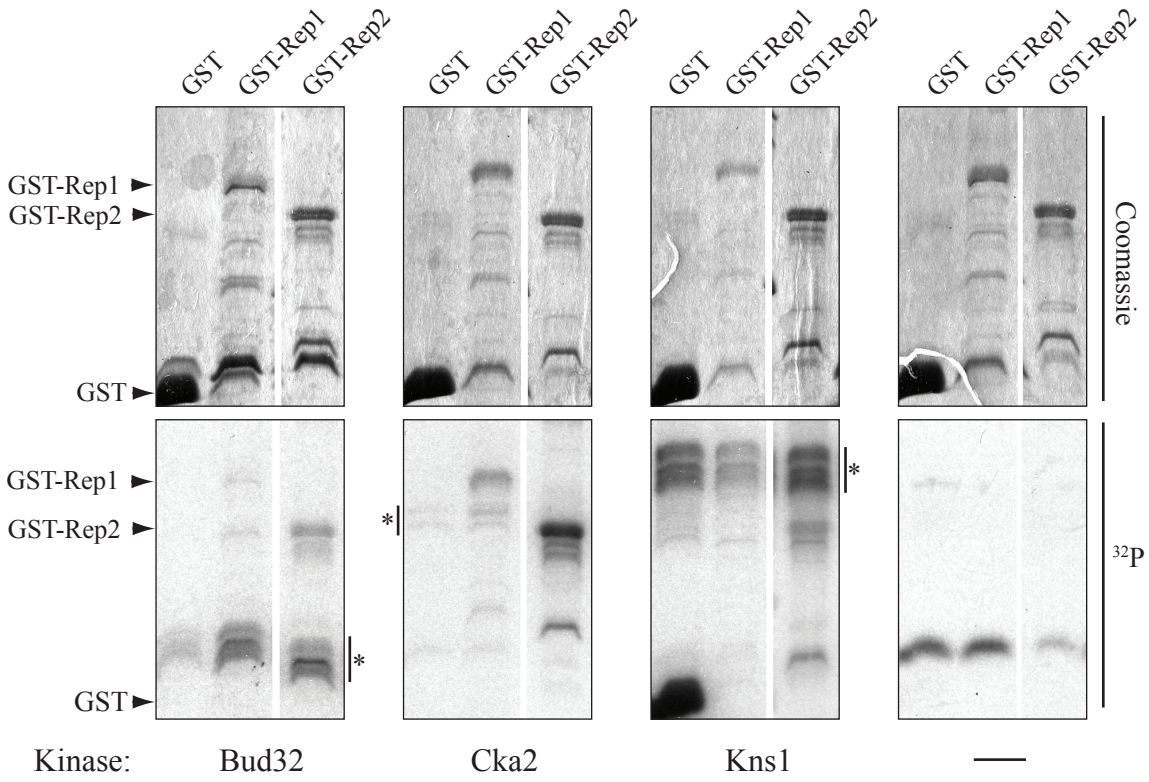


Figure 10. Examining phosphorylation of GST-Rep1 and GST-Rep2 by Bud32, Cka2, and Kns1 using *in vitro* kinase assays. *E. coli*-expressed GST-Rep1, GST-Rep2, or GST were affinity-purified and incubated with [γ-^{32}P] ATP, Mn^{2+}, Mg^{2+} and bacterially expressed and purified Bud32, Cka2, or Kns1. Reactions were resolved by SDS-PAGE, gels stained with Coomassie Blue (top) and phosphorylation detected by exposure to X-ray film (bottom). Asterisks indicate species with mobilities consistent with autophosphorylated forms of the kinases.

Both Bud32 and Cka2 phosphorylated three different N-terminally truncated Rep1 proteins, $Rep1_{62-373}$, $Rep1_{77-373}$, and $Rep1_{130-373}$ (**Figure 11**). C-terminally truncated Rep1 ($Rep1_{1-129}$) was phosphorylated by Bud32, but not Cka2; however, a shorter region of Rep1 contained within this region, $Rep1_{62-99}$, was phosphorylated by both kinases, albeit more efficiently by Bud32. These findings suggest that Bud32 phosphorylated Rep1 in both amino-terminal (aa 62-99) and carboxy-terminal (aa 130-373) regions in *vitro*, while the residues targeted by Cka2 were found within aa 130-373.

In the *in vitro* kinase assays with truncated versions of Rep2, both Bud32 and Cka2 phosphorylated $Rep2_{58-296}$, and $Rep2_{1-144}$ (**Figure 12**). Cka2 did not phosphorylate $Rep2_{1-58}$, and it was difficult to tell whether Bud32 phosphorylated this species since the signal was obscured by those of autophosphorylated forms of Bud32. Three smaller proteins also co-purified with GST-$Rep2_{1-144}$ on the glutathione-agarose resin, and therefore are likely to be C-terminally truncated forms of GST-Rep2. Cka2 phosphorylated only the largest of these three forms, suggesting that the amino terminus of Rep2 is not targeted by Cka2. These observations suggest that the residues in Rep2 targeted for phosphorylation by both Bud32 and Cka2 *in vitro* might be found within the region 59-144, which was common to the $Rep2_{58-296}$ and $Rep2_{1-144}$ truncated forms, which were both phosphorylated by each kinase, although the results do not exclude the possibility that some residues within aa 144-296 are also targeted.

3.2.9 Substitution of CK2 Consensus Sites in Rep2 Blocks Hyper-Phosphorylation

The evidence obtained thus far provides strong support for protein kinase CK2 directly phosphorylating Rep2 *in vivo*: phosphorylation of Rep2 was reduced in *cka2*Δ yeast, was increased when Cka2 was over-expressed, and Rep2 was phosphorylated by Cka2 *in vitro*. If CK2 targets Rep2 *in vivo*, substitution of CK2 consensus sites in Rep2 would be expected to reduce or abolish phosphorylation.

Kinase assays indicated that sites in Rep2 targeted for Cka2 phosphorylation *in vitro* are likely to reside between residues 59-144. Within this region, Rep2 contains four residues (S71, S72, S82, S107) that conform to the CK2 consensus motif, (S/T)-X-X-(D/E/pS), where X is any residue and pS is phosphoserine (177) (**Figure 13A**). If these sites were

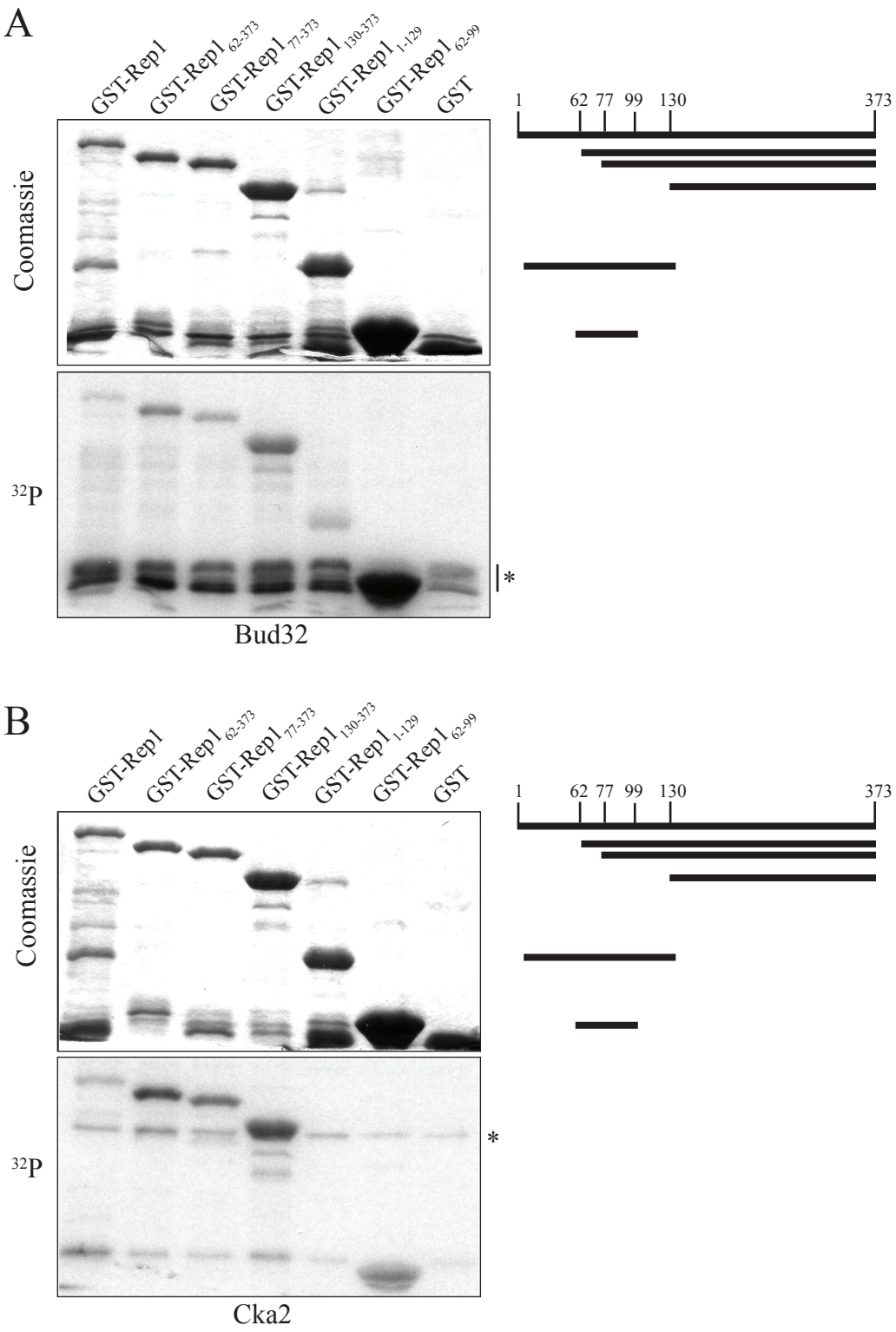


Figure 11. *In vitro* phosphorylation of truncated Rep1 proteins by Bud32 and Cka2. Phosphorylation of GST-fusions of full-length or truncated forms of Rep1 (depicted on the right) by Bud32 (A) and Cka2 (B) was assessed using *in vitro* kinase assays as described in the legend to Figure 10.

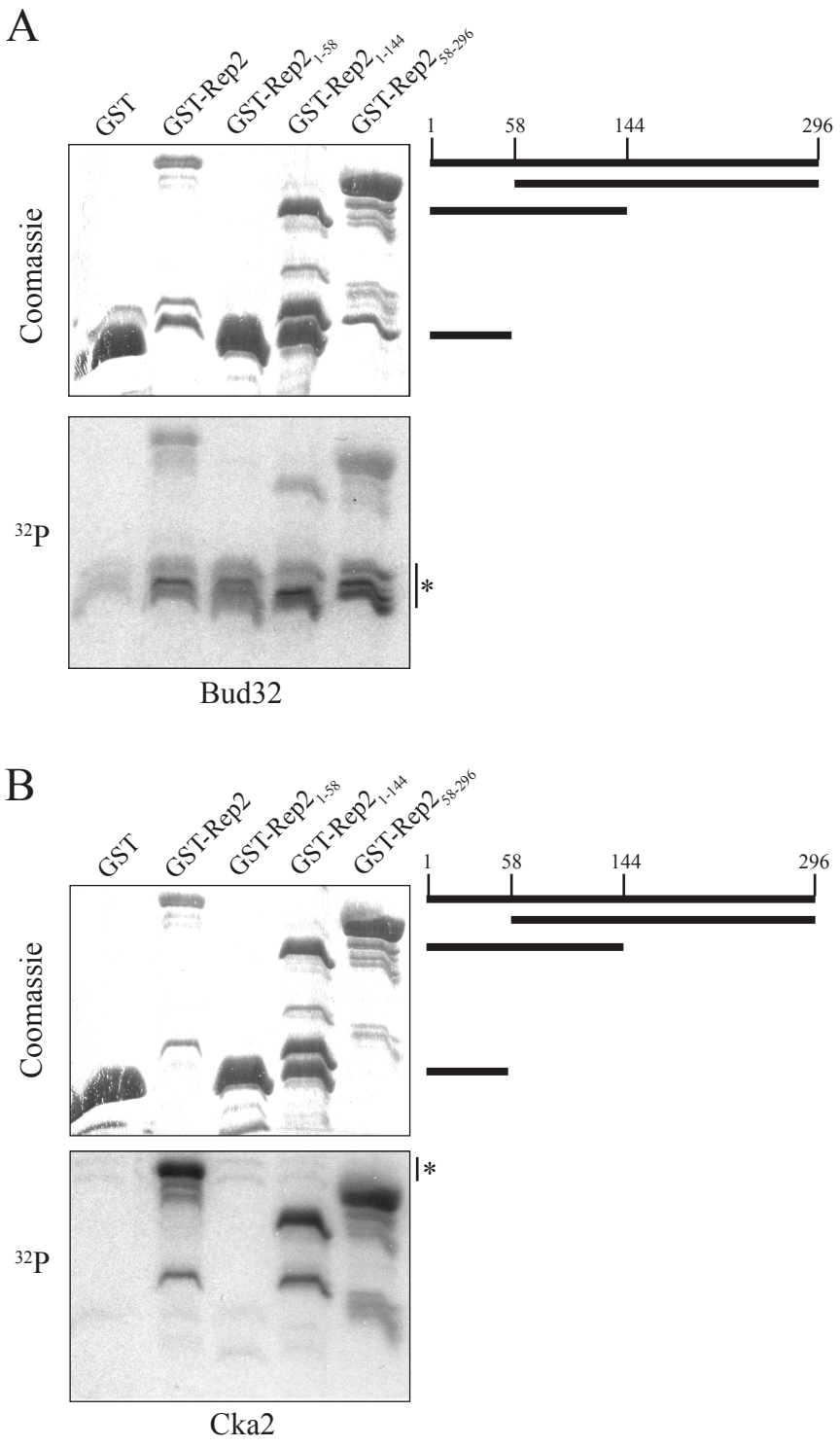


Figure 12. *In vitro* phosphorylation of truncated Rep2 proteins by Bud32 and Cka2. Phosphorylation of GST-fusions of full-length or truncated forms of Rep2 (depicted on the right) by Bud32 (A) and Cka2 (B) was assessed using *in vitro* kinase assays as described in the legend to Figure 10.

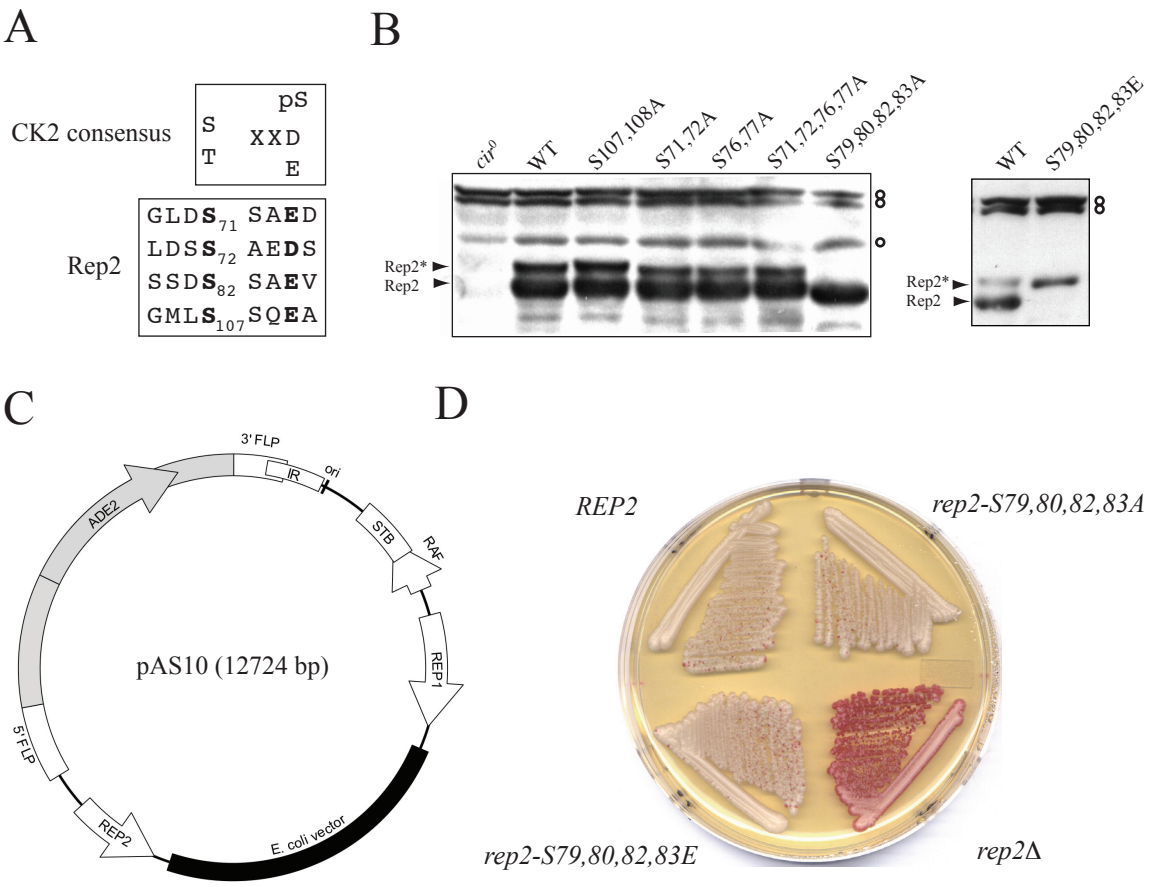


Figure 13. Mutational analysis of CK2 phosphorylation sites in Rep2. (A) Serine residues within aa 59-144 in Rep2 (bottom) with flanking sequences that conform to the protein kinase CK2 consensus (top, X can be any residue, pS is phosphoserine). (B) *ADE2*-tagged 2μm plasmids encoding Rep2 that was wild-type (WT) or contained the indicated amino acid substitutions were transformed into *cir*^{0} *ade2* yeast. Protein was extracted from the yeast transformants and also from untransformed yeast (*cir*^{0}) and analyzed by western blotting with anti-Rep2 antibodies. Hyper-phosphorylated Rep2 (Rep2*) and species detected non-specifically by the antibodies (open circles) are indicated. (C) Map of the *flp*$^-$ *ADE2*-tagged 2μm plasmid pAS10 showing disruption of the *FLP* ORF by genomic DNA encoding *ADE2* (grey) and loss of one Flp recognition site by replacement with *E. coli* vector sequences (thick black line). (D) *ADE2*-tagged 2μm plasmids encoding the indicated *REP2* alleles were transformed into *cir*^{0} *ade2* yeast, and transformants were streaked on solid YPD medium and imaged after five days of incubation at 28˚C. The efficiency of plasmid inheritance was qualitatively assessed by visualizing the red pigment that accumulates in yeast lacking a functional *ADE2* gene.

phosphorylated by CK2 *in vivo*, substitution of these residues would be expected to reduce, or possibly abolish, Rep2 phosphorylation. To test whether these sites were phosphorylated, combinations of serine residues were substituted with alanine, to block phosphorylation, or with glutamate, to mimic phosphorylation. DNA encoding the mutant *rep2* alleles was used to replace the wild-type *REP2* gene in the *ADE2*-tagged 2μm plasmid pAS4. A *cir*^{0} yeast strain was transformed with each of the plasmids, cultured in medium lacking adenine, and the effects of the substitutions on the levels of the different forms of Rep2 were analyzed by western blotting (**Figure 13B**). There was no obvious change in the abundance or mobility of the 35-kDa or hyper-phosphorylated form of the Rep2$_{S71,72A}$, Rep2$_{S76,77A}$, or Rep2$_{S71,S72,S76,77A}$ mutants. Both the 35-kDa and hyper-phosphorylated Rep2$_{S107,S108A}$ species displayed slightly retarded mobility compared to the corresponding species of wild-type Rep2, which could be due to increased Rep2 phosphorylation at other sites, but more likely can be attributed to aberrant migration of Rep2$_{S107,S108A}$ mutant that is unrelated to phosphorylation status. Notably, in the Rep2$_{S79,80,82,83A}$ mutant, hereafter referred to as Rep2$_{SA}$, the hyper-phosphorylated Rep2 species was absent, and the 35-kDa species of Rep2 also exhibited greater mobility which was more consistent with the predicted MW for unmodified Rep2 (33 kDa), suggesting that the substitutions might have abolished some of the hypo-phosphorylated forms of Rep2 as well as the hyper-phosphorylated forms. 2D gel analysis would be needed to determine whether all phosphorylation is blocked in the Rep2$_{SA}$ mutant. When the four serine residues mutated in Rep2$_{SA}$ were substituted for glutamate (Rep2$_{SE}$), only one species was observed, and had a mobility comparable to that of hyper-phosphorylated Rep2.

3.2.10 Phospho-Deficient and Phospho-Mimetic Substitutions in Rep2 Do Not Affect Plasmid Partitioning

The ability of both Rep2$_{SA}$ and Rep2$_{SE}$ to mediate 2μm plasmid inheritance was examined using a colony colour assay. A *cir*^{0} yeast strain lacking a genomic copy of the *ADE2* gene (*ade2*Δ) was transformed with derivatives of the *ADE2*-tagged 2μm plasmid pAS10 (for a map, see **Figure 13C**) encoding wild-type Rep1, and either wild-type Rep2, Rep2$_{SA}$, Rep2$_{SE}$, or that lacked a *REP2* gene altogether. Transformants were streaked to YPD medium containing limiting amounts of adenine (**Figure 13D**). Under these conditions, cells

lacking the *ADE2*-tagged plasmid can grow but form red colonies due to accumulation of a coloured intermediate in the adenine biosynthetic pathway that is the substrate for the *ADE2* gene product. As expected, when the yeast contained an *ADE2*-tagged 2μm plasmid encoding wild-type Rep2, the majority of the colonies were white, indicating that most colonies contained the plasmid, while yeast that contained a plasmid lacking a *REP2* gene exclusively formed red colonies, indicating that the plasmid was not faithfully inherited during cell division. Colonies produced by transformants containing the *ADE2*-tagged plasmids expressing $Rep2_{SA}$ or $Rep2_{SE}$ were comparable in colour to those expressing wild-type Rep2, suggesting that neither phospho-deficient nor phospho-mimetic substitutions in the Rep2 phosphorylation sites had any significant effect on Rep protein-mediated inheritance of the 2μm plasmid. Quantitative plasmid loss assays were also undertaken and confirmed that the point substitutions had no significant effect on plasmid inheritance (data not shown). Consistent with a lack of any defect in function of the phosphorylation-deficient Rep2, the $Rep2_{SA}$ mutant was not impaired for interactions known to be important for plasmid inheritance, i.e. two-hybrid interaction with Rep1 and SUMO, and association with *STB* in a one-hybrid assay (data not shown). The lack of any defect in plasmid inheritance mediated by the $Rep2_{SE}$ mutant is consistent with the lack of any significant difference in the overall levels of Rep2 expressed from the native 2μm plasmid when the levels of hyper-phosphorylated Rep2 were increased by over-expression of Cka2 (**Figure 9D**).

3.2.11 Levels of 2μm Plasmid Proteins are Reduced in *bud32*Δ Yeast

Although phosphorylation of Rep2 by CK2 did not appear to contribute significantly to Rep2-mediated 2μm plasmid inheritance, the absence of the 2μm plasmid in yeast lacking the protein kinase Bud32 (**Figure 9A**) suggested that Bud32 might have a role in plasmid maintenance. Alternatively, the *bud32*Δ strain obtained from the EUROSCARF gene-deletion strain collection might have spontaneously lost the 2μm plasmid due to a rare mis-segregation event unrelated to Bud32 function. Strains in the gene-deletion collection have been passaged for many generations since they were created. Many have acquired secondary substitutions in addition to the original gene deletions. To determine whether yeast lacking Bud32 are impaired for 2μm plasmid maintenance, new *bud32*Δ strains containing the 2μm plasmid had to be generated. To do this, one copy of the *BUD32* gene was deleted in a cir^+

diploid yeast strain, and the resulting heterozygous *BUD32*/*bud32*Δ diploid was induced to undergo sporulation to yield haploid *cir*$^+$ *BUD32* and *bud32*Δ segregants. Haploid *cir*$^+$ *cka2*Δ and *kns1*Δ yeast strains were similarly derived from *CKA2*/*cka2*Δ and *KNS1*/*kns1*Δ heterozygous *cir*$^+$ diploid strains, respectively. The haploid segregants were grown in liquid medium for the minimum number of cell divisions required to acquire enough cells for western blotting analysis (~30 generations), and Rep2 levels (as an indicator for 2μm plasmid levels) in these strains were examined by western blotting (**Figure 14A**). The level of Rep2 in protein extracted from *cka2*Δ and *kns1*Δ haploids did not significantly differ from that of wild-type yeast, but the *bud32*Δ strain had less Rep2. Lower levels of Rep2 are consistent with fewer copies of the yeast 2μm plasmid in the cell population, but could also reflect a reduction in expression of the *REP2* gene or in post-translational stability of the Rep2 protein. Rep1 levels were also reduced in the *bud32*Δ strain (data not shown) making it more likely that the drop in Rep2 levels was due to a reduction in average plasmid copy number.

A lower average plasmid copy number in the population could result from reduced copy number in each cell, but would also been seen if a significant fraction of the cells were *cir*^{0}. To assess which of these situations was more likely, inheritance of the endogenous 2μm plasmid was examined in *cir*$^+$ *bud32*Δ yeast at successive numbers of generations after loss of Bud32. A *cir*$^+$ *BUD32*/*bud32*Δ diploid yeast strain was induced to undergo sporulation and eight independent *bud32*Δ segregants were serially passaged three times on solid medium, and Rep2 levels (as an indicator for the levels of the 2μm plasmid) were examined by western blotting of protein extracted in a culture grown from a single colony from each passaged lineage (**Figure 14B**). Rep2 was expressed in all eight *bud32*Δ lineages, indicating that they all contained the 2μm plasmid. While some lineages had lower Rep2 levels than observed for yeast wild-type for *BUD32*, the lack of any *cir*^{0} colonies suggests that complete nondisjunction events, in which the daughter cell fails to receive any copies of the plasmid, are not frequent. However, the low levels of Rep2, and of the 2μm plasmid as confirmed by PCR (data not shown), in some *bud32*Δ lineages suggests that some cells might have lower than average copy number, indicating that Bud32 is required for maintenance of the plasmid at normal copy number.

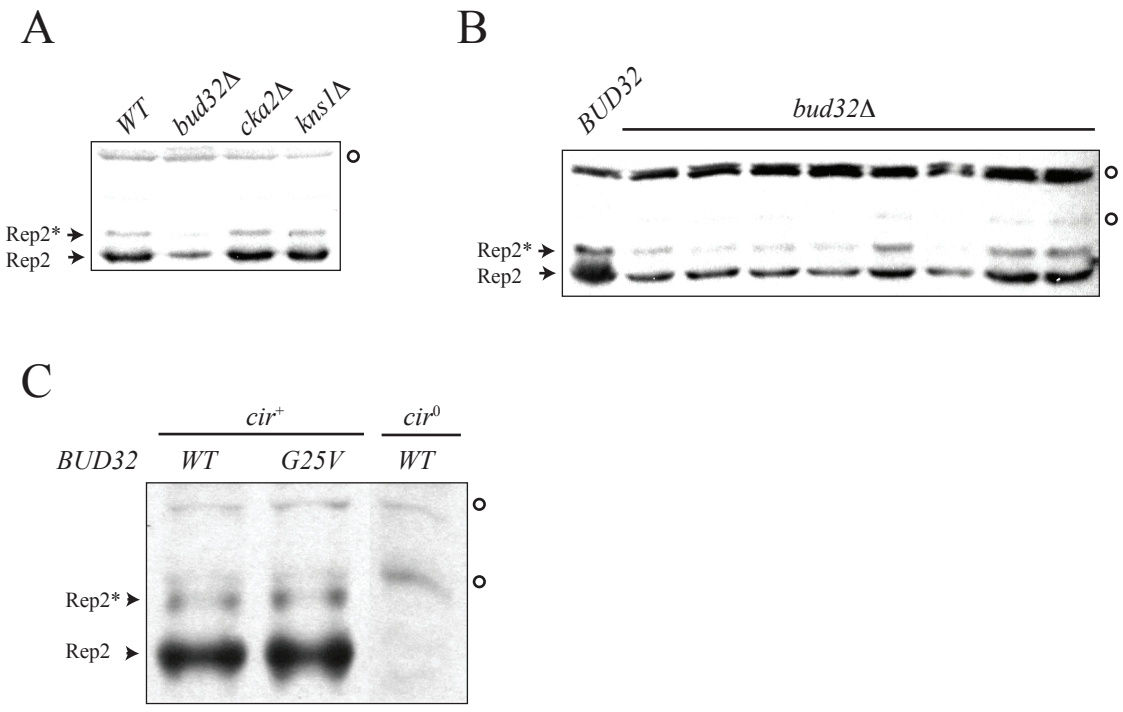


Figure 14. Bud32, but not its kinase activity, is required for plasmid maintenance. Rep2 levels were analyzed by western blotting of total protein extracted from (A) haploid yeast with the indicated genotypes ~30 generations after isolation from a *cir*$^{+}$ diploid heterozygous for the deletion, (B) haploid *BUD32* (one segregant) and *bud32*Δ (eight independent segregants) strains that were derived from a single *cir*$^{+}$ *BUD32/bud32*Δ diploid yeast strain and subsequently serially passaged three times on solid medium, (C) yeast encoding wild-type or kinase-deficient (G25V) Bud32. Hyper-phosphorylated species of Rep2 (Rep2*) and proteins detected non-specifically by the antibodies (open circles) are indicated.

3.2.12 Effects of Loss of Bud32 on 2μm Plasmid Maintenance are Not Due to Loss of Bud32 Catalytic Activity

The observation that Bud32 phosphorylates both Rep1 and Rep2 *in vitro* and that plasmid maintenance is impaired when Bud32 is absent suggests that phosphorylation of Rep1 and/or Rep2 by Bud32 *in vivo* might be important for plasmid partitioning. If this were the case, yeast encoding a catalytically inactive version of Bud32 would be expected to have a similar effect on 2μm plasmid maintenance as yeast lacking Bud32 altogether. To test this conjecture, Rep2 levels were analyzed in yeast encoding a kinase-deficient allele of Bud32 that contains a substitution in the ATP-binding pocket ($Bud32_{G25V}$) that *in vitro* was shown to reduce the catalytic activity to less than 1% of that of wild-type Bud32 (57) (**Figure 14C**). In contrast to when Bud32 was absent, when Bud32 was present but lacked catalytic activity, no reduction in either the 35-kDa or 37-kDa species of Rep2 was observed. The lack of change in the abundance or mobility of the hyper-phosphorylated species of Rep2 in yeast encoding catalytically inactive Bud32 demonstrates that this species is not a result of Bud32-mediated phosphorylation of Rep2, and the unaltered levels of Rep2 suggest that the role of Bud32 in plasmid maintenance is not dependent on its kinase activity,

3.2.13 Rep2 Interacts with KEOPS Complex Subunit Pcc1 in a Two-Hybrid Assay

Absence of Bud32 could disrupt the integrity of the KEOPS complex in which it resides (145). Although association of Bud32 with Rep1 or Rep2 was not detected in a two-hybrid assay (**Figure 9C**), if the KEOPS complex has a direct role in plasmid maintenance, other subunits of the complex might interact with Rep1 and/or Rep2. The two-hybrid interactions of Rep1 and Rep2 with two other KEOPS subunits, Pcc1 and Kae1, were examined. No interaction between Pcc1 and Rep1 was detected in this assay, but Pcc1 did interact with Rep2 (**Figure 15**). This interaction was observed using a cir^+ reporter strain but was not detected when the assay was performed using a cir^0 reporter strain. Lack of interaction of Rep2 with Pcc1 in the cir^0 strain could suggest that native plasmid proteins are required for the interaction, or could be due to a difference in the steady-state levels of the

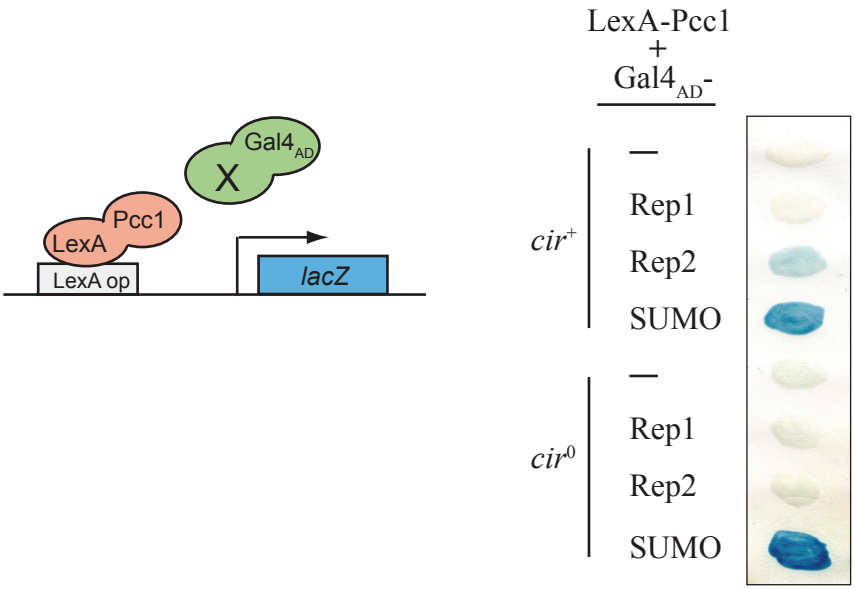


Figure 15. KEOPS subunit Pcc1 interacts with Rep2 and SUMO in a two-hybrid assay. Yeast two-hybrid reporter strains, *cir*$^{+}$ or *cir*^{0}, were co-transformed with two plasmids, one expressing LexA-Pcc1 and the other the indicated Gal4$_{AD}$-fusion protein, or unfused Gal4$_{AD}$ (-). Co-transformants were grown for 24 h on a nitrocellulose membrane and interaction of the fusion proteins was assessed by monitoring expression of the *lacZ* reporter gene using a β-galactosidase filter assay with the substrate X-gal, which produces a dark blue precipitate upon cleavage.

LexA-Rep2 fusion, which are much lower in the *cir*^{0} strain compared to the *cir*$^+$ strain (data not shown).

No two-hybrid interaction between Kae1 and Rep1 or Rep2 was detected (data not shown). Interestingly, Bud32 (**Figure 9C**), Pcc1 (**Figure 15**) and Kae1 (data not shown) all strongly interacted with SUMO in a two-hybrid assay, suggesting that one or more of the KEOPS subunits might be targeted for sumoylation.

3.3 Investigating the Function of Sumoylation of Rep1 and Rep2

A major focus of my doctoral research has been the investigation of the role of post-translational modification of the 2μm plasmid partitioning proteins, Rep1 and Rep2, by the small ubiquitin-related modifier protein, SUMO. When I initiated my research, our lab had already reported that Rep1 and Rep2 could both interact with SUMO independently of one another in a two-hybrid assay (51). To investigate the potential role of Rep1 and Rep2 sumoylation, my overall strategy was to identify sites targeted for SUMO attachment, mutate those sites to create Rep proteins that were deficient for sumoylation, and then assess the mutant proteins for their ability to direct plasmid partitioning.

3.3.1 Identification of SUMO Conjugates of Rep1 and Rep2 and Creation of Sumoylation-Deficient Substitution Mutants

As a first step to studying sumoylation of Rep1 and Rep2, I first needed a method for detecting their sumoylation. SUMO conjugates are notoriously difficult to detect because they are rapidly cleaved by the SUMO-specific proteases when cells are lysed during protein extraction procedures, and usually only a small proportion (~1%) of the total number of molecules of a targeted protein are conjugated to SUMO at any given time (120, 121). Sumoylated forms of Rep1 and Rep2 are particularly difficult to isolate due to the low abundance of the Rep proteins (our unpublished observations), and detection of Rep protein SUMO-conjugates in western blots has previously required affinity purification steps and the use of highly specific anti-SUMO antibodies which are not commercially available (36). Several strategies are commonly implemented to overcome the difficulty in detecting SUMO conjugates, including the use of yeast strains mutant for the SUMO proteases, and inhibition of SUMO protease activity during protein extractions through the use of harsh, denaturing

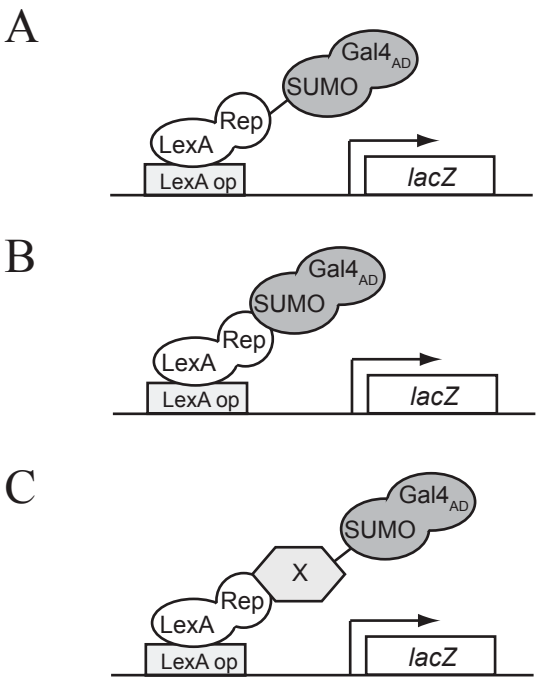
conditions, or by including the cysteine protease inhibitor N-ethylmaleimide in extraction buffers (188). Despite the use of all these approaches I was not able to detect SUMO-conjugates of Rep1 even by 2D gel electrophoresis (although another HMW form of 100 kDa was detected). For Rep2, species consistent with the sizes expected for SUMO-conjugates required over-expression from a heterologous promoter and were readily observed only by 2D gel electrophoresis and western blotting. I was also unable to detect SUMO-conjugates of Rep1 or Rep2 by western blotting using an anti-HA antibody when SUMO was expressed in yeast with an HA epitope tag.

For substitutional analysis, I needed a technique for detecting sumoylation that would reliably indicate if lysine-to-arginine substitutions introduced into the Rep proteins resulted in altered sumoylation status. Yeast two-hybrid protein interaction assays, although typically employed for examining non-covalent protein-protein interactions, have been used to covalent modification of a protein by SUMO (92, 128).

3.3.1.1 Conjugatable SUMO is Required for Two-Hybrid Interaction with Rep1 and Rep2

Although our lab had observed interaction of Rep1 and Rep2 with SUMO in a two-hybrid assay, this could reflect non-covalent binding of the Rep proteins to SUMO through a SUMO-interaction motif (SIM) in Rep1 or Rep2, or could be due to interaction of Rep1 or Rep2 with another sumoylated host protein rather than covalent attachment of SUMO to Rep1 and Rep2 (see **Figure 16**). Some proteins are both sumoylated and bind to SUMO non-covalently: for example, the human proteins Daxx and thymine DNA glycosylase (137, 211).

To test whether the two-hybrid interaction between Rep1 or Rep2 and SUMO is due to non-covalent binding to SUMO, the interaction of Rep1 or Rep2 with a non-conjugatable version of SUMO lacking the C-terminal diglycine motif (SUMOΔGG) normally required for covalent attachment (92) was assessed (**Figure 17**). Neither Rep1 nor Rep2 interacted with SUMOΔGG in a two-hybrid assay, while a fusion protein containing the previously characterized SIM domain from the yeast Nis1 protein (92) did interact with this truncated form of SUMO. The lack of interaction of the Rep proteins with SUMOΔGG indicates that neither protein contains a SIM, consistent with the results of *in vitro* protein interaction



adapted from Hannich *et al.* (2005)

Figure 16. Models for interaction of a Rep protein with SUMO in a two-hybrid assay. Rep1 or Rep2 would display a two-hybrid interaction with SUMO if they were (A) directly conjugated to SUMO through an isopeptide bond, (B) recognized SUMO non-covalently through a SUMO-interaction motif (SIM), or (C) interacted with another protein that was directly conjugated to SUMO.

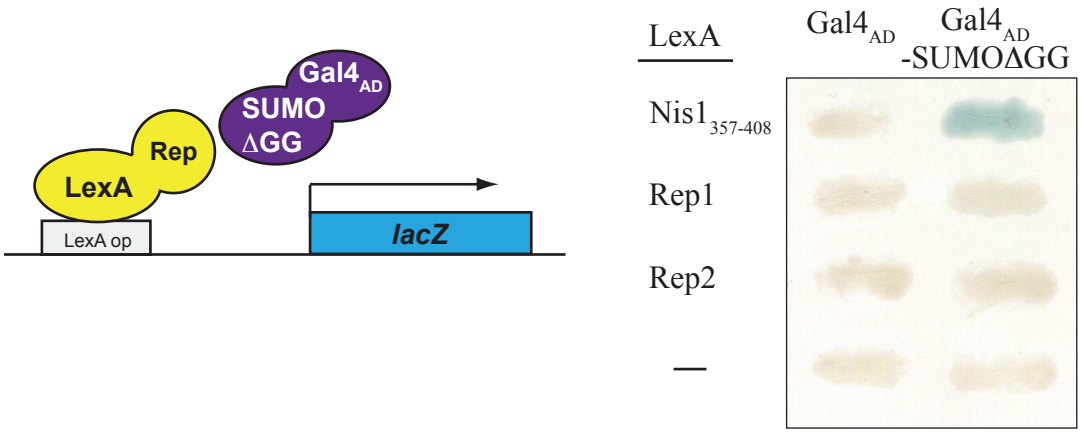


Figure 17. Rep proteins do not interact with non-conjugatable SUMO in a two-hybrid assay. A *cir*$^{+}$ yeast two-hybrid reporter strain was co-transformed with plasmids expressing the indicated $Gal4_{AD}$ and LexA fusion proteins, and interaction of the fusion proteins was assessed as described for Figure 15.

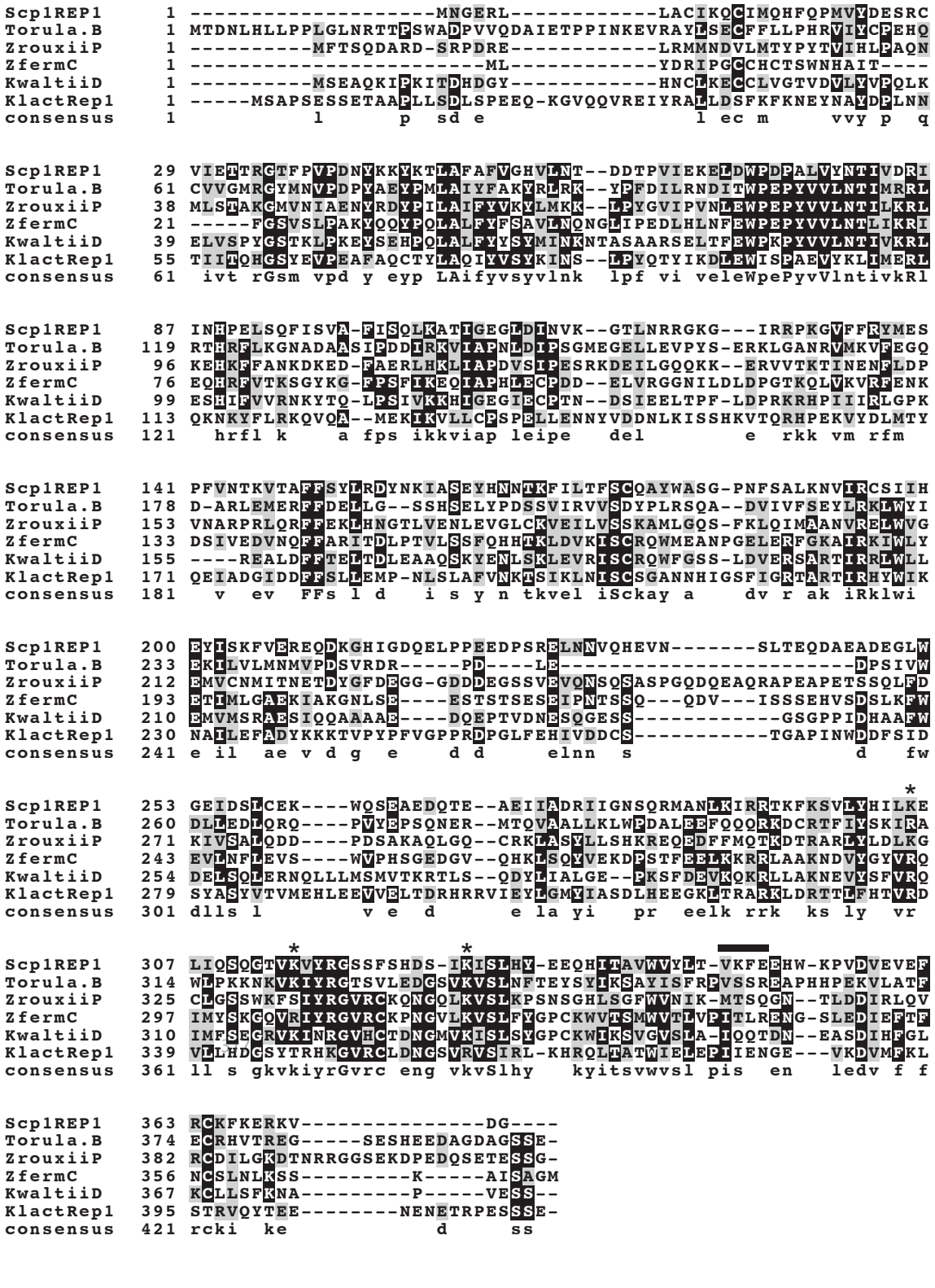
assays using purified, bacterially expressed proteins, in which Rep1 and Rep2 did not bind to SUMO (Nisa Renault, Dalhousie University, unpublished results).

Although it is possible that the two-hybrid association of the Rep proteins with SUMO could be due to interaction of Rep1 and Rep2 with a host protein that is conjugated to SUMO (**Figure 16**), previously reported observations of SUMO-conjugated forms of both Rep1 and Rep2 (36) and my own 2D gel immunoblotting analysis (**Figure 6**) suggest that two-hybrid interactions of Rep1 and Rep2 with SUMO would be expected from covalent modification. A substitutional approach was therefore undertaken based on the premise that substitution of lysine residues in Rep1 and Rep2 normally targeted for sumoylation should lead to loss of the two-hybrid interaction with SUMO if the interaction solely reflected covalent SUMO modification.

3.3.1.2 Substitution of K305, K315, and K328 in Rep1 Virtually Abolishes Interaction with SUMO in a Two-Hybrid Assay

As a first step in identifying potential sumoylation sites in Rep1, I examined its predicted amino acid sequence. SUMO is often attached to target proteins at lysine residues that conform to a four-residue consensus motif, (V/I/L)-K-x-(E/D) (181). The major sumoylation site in the 2μm plasmid Flp protein fits this motif (36). The 373-aa Rep1 protein contains 27 lysine residues, of which one, K348 (V<u>K</u>FE), matches the SUMO consensus motif. To determine whether this site was likely to be significant, I compared the sequences of Rep1 proteins encoded by several 2μm-like plasmids (**Figure 18**). However, K348 was not a conserved residue among Rep1-like proteins, and no other lysine residues were conserved in all Rep1-like proteins. Another candidate residue for a Rep1 sumoylation site was K328, which has a sequence context (I<u>K</u>IS) that is a close match to the preferred sumoylation consensus. The four-residue IKIS sequence was conserved among Rep1-like proteins encoded by various 2μm-like plasmids with the exception of Rep1 from the *Kluyveromyces lactis* 2μm-like plasmid pKD1, which had an arginine rather than a lysine at the synonymous site. To test whether either K328 or K348 serve as SUMO acceptor sites, the interaction of Rep1 containing arginine substitutions at these residues was examined in a two-hybrid assay. Arginine was chosen because the positively charged side chain most closely resembles the biochemical characteristics of the lysine side chain, and lysine-to-

Figure 18. Sequence alignment of Rep1 homologues. Clustal alignment of predicted amino acid sequences of Rep1-like proteins encoded by *S. cerevisiae* 2μm (ScpREP1), *Torulaspora delbrueckii* pTD1 (Torula.B), *Zygosaccharomyces rouxii* pSR1 (ZrouxiiP), *Z. bailii* pSB2 (ZfermC), *Kluyveromyces waltii* pKW1 (KwaltiiD), and *K. lactis* pKD1 (KlactRep1). Identical residues are in black boxes, and chemically similar residues are in gray boxes. Asterisks indicate lysine residues substituted with arginine in $Rep1_{3R}$. A thick horizontal bar indicates a sumoylation consensus motif in *S. cerevisiae* Rep1.


Scp1REP1 1 ----------MNGERL----------LACIKQCIMQHFQPMVYDESRC
Torula.B 1 MTDNLHLLPPLGLNRTTPSWADPVVQDAIETPPINKEVRAYLSECFFLLPHRVIYCPEHQ
ZrouxiiP 1 ----------MFTSQDARD-SRPDRE----------LRMMNDVLMTYPYTVIHLPAQN
ZfermC 1 ----------ML----------YDRIPGCCHCTSWNHAIT----
KwaltiiD 1 ----------MSEAQKIPKITDHDGY----------HNCLKECCLVGTVDVLYVPQLK
KlactRep1 1 -----MSAPSESSETAAPLLSDLSPEEQ-KGVQQVREIYRALLDSFKFKNEYNAYDPLNN
consensus 1 l p sd e l ec m vvy p q
Scp1REP1 29 VIETTRGTFPVPDNYKKYKTLAFAFVGHVLNT--DDTPVIEKELDWPDPALVYNTIVDRI
Torula.B 61 CVVGMRGYMNVPDPYAEYPMLAIYFAKYRLRK--YPFDILRNDITWPEPYVVLNTIMRRL
ZrouxiiP 38 MLSTAKGMVNIAENYRDYPILAIFYVKYLMKK--LPYGVIPVNLEWPEPYVVLNTILKRL
ZfermC 21 -----FGSVSLPAKYQQYPQLALFYFSAVLNQNGLIPEDLHLNFEWPEPYVVLNTLIKRI
KwaltiiD 39 ELVSPYGSTKLPKEYSEHPQLALFYYSYMINKNTASAARSELTFEWPKPYVVLNTIVKRL
KlactRep1 55 TIITQHGSYEVPEAFAQCTYLAQIYVSYKINS--LPYQTYIKDLEWISPAEVYKLIMERL
consensus 61 ivt rGsm vpd y eyp LAifyvsyvlnk lpf vi veleWpePyvVlntivkRl
Scp1REP1 87 INHPELSQFISVA-FISQLKATIGEGLDTNVK--GTLNRRGKG---IRRPKGVFFRYMES
Torula.B 119 RTHRFLKGNADAASIPDDIRKVIAPNLDIPSGMEGELLEVPYS-ERKLGANRVMKVFEGQ
ZrouxiiP 96 KEHKFFANKDKED-FAERLHKLIAPDVSIPESRKDEILGQQKK--ERVVTKTINENFLDP
ZfermC 76 EQHRFVTKSGYKG-FPSFIKEQIAPHLECPDD--ELVRGGNILDLDPGTKQLVKVRFENK
KwaltiiD 99 ESHIFVVRNKYTQ-LPSIVKKHIGEGIECPTN--DSIEELTPF-LDPRKRHPIIIRLGPK
KlactRep1 113 QKNKYFLRKQVQA--MEKIKVLLCPSPELLENNYVDDNLKISSHKVTQRHPEKVYDLMTY
consensus 121 hrfl k a fps ikkviap leipe del e rkk vm rfm
Scp1REP1 141 PFVNTKVTAFFSYLRDYNKIASEYHNNTKFILTFSCQAYWASG-PNFSALKNVIRCSIIH
Torula.B 178 D-ARLEMERFFDELLG--SSHSELYPDSSVIRVVSDYPLRSQA--DVIVFSEYLRKLWYI
ZrouxiiP 153 VNARPRLQRFFEKLHNGTLVENLEVGLCKVEILVSSKAMLGQS-FKLQIMAANVRELWVG
ZfermC 133 DSIVEDVNQFFARITDLPTVLSSFQHHTKLDVKISCRQWMEANPGELERFGKAIRKIWLY
KwaltiiD 155 ----REALDFFTELTDLEAAQSKYENLSKLEVRISCRQWFGSS-LDVERSARTIRRLWLL
KlactRep1 171 QEIADGIDDFFSLLEMP-NLSLAFVNKTSIKLNISCSGANNHIGSFIGRTARTIRHYWIK
consensus 181 v ev FFs l d i s y n tkvel iSckay a dv r ak iRklwi
Scp1REP1 200 EYISKFVEREQDKGHIGDQELPPEEDPSRELNNVQHEVN-------SLTEQDAEADEGLW
Torula.B 233 EKILVLMNMVPDSVRDR-----PD----LE----------DPSIVW
ZrouxiiP 212 EMVCNMITNETDYGFDEGG-GDDDEGSSVEVQNSQSASPGQDQEAQRAPEAPETSSQLFD
ZfermC 193 ETIMLGAEKIAKGNLSE----ESTSTSESEIPNTSSQ---QDV---ISSSEHVSDSLKFW
KwaltiiD 210 EMVMSRAESIQQAAAAE----DQEPTVDNESQGESS----------GSGPPIDHAAFW
KlactRep1 230 NAILEFADYKKKTVPYPFVGPPRDPGLFEHIVDDCS----------TGAPINWDDFSID
consensus 241 e il ae v d g e d d elnn s d fw
Scp1REP1 253 GEIDSLCEK----WQSEAEDQTE--AEIIADRIIGNSQRMANLKIRRTKFKSVLYHILKE
Torula.B 260 DLLEDLQRQ----PVYEPSQNER--MTQVAALLKLWPDALEEFQQQRKDCRTFIYSKIRA
ZrouxiiP 271 KIVSALQDD----PDSAKAQLGQ--CRKLASYLLSHKREQEDFFMQTKDTRARLYLDLKG
ZfermC 243 EVLNFLEVS----WVPHSGEDGV--QHKLSQYVEKDPSTFEELKKRRLAAKNDVYGYVRQ
KwaltiiD 254 DELSQLERNQLLLMSMVTKRTLS--QDYLIALGE--PKSFDEVKQKRLLAKNEVYSFVRQ
KlactRep1 279 SYASYVTVMEHLEEVVELTDRHRRVIEYLGMYIASDLHEEGKLTRARKLDRTTLFHTVRD
consensus 301 dlls l v e d e la yi pr eelk rrk ks ly vr
Scp1REP1 307 LIQSQGTVKVYRGSSFSHDS-IKISLHY-EEQHITAVWVYLT-VKFEEHW-KPVDVEVEF
Torula.B 314 WLPKKNKVKIYRGTSVLEDGSVKVSLNFTEYSYIKSAYISFRPVSSREAPHHPEKVLATF
ZrouxiiP 325 CLGSSWKFSIYRGVRCKQNGQLKVSLKPSNSGHLSGFWVNIK-MTSQGN--TLDDIRLQV
ZfermC 297 IMYSKGQVRIYRGVRCKPNGVLKVSLFYGPCKWVTSMWVTLVPITLRENG-SLEDIEFTF
KwaltiiD 310 IMFSEGRVKINRGVHCTDNGMVKISLSYGPCKWIKSVGVSLA-IQQTDN--EASDIHFGL
KlactRep1 339 VLLHDGSYTRHKGVRCLDNGSVRVSIRL-KHRQLTATWIELEPIIENGE---VKDVMFKL
consensus 361 ll s gkvkiyrGvrc eng vkvSlhy kyitsvwvsl pis en ledv f f
Scp1REP1 363 RCKFKERKV----------DG----
Torula.B 374 ECRHVTREG-----SESHEEDAGDAGSSE-
ZrouxiiP 382 RCDILGKDTNRRGGSEKDPEDQSETESSG-
ZfermC 356 NCSLNLKSS----------K-----AISAGM
KwaltiiD 367 KCLLSFKNA----------P-----VESS--
KlactRep1 395 STRVQYTEE---------NENETRPESSSE-
consensus 421 rcki ke d ss

arginine substitutions are used in virtually all studies of protein sumoylation (120). However, substitution of neither K348 nor K328 in Rep1 affected two-hybrid interaction of Rep1 with SUMO (**Table 9**). These observations suggest that if Rep1 were sumoylated, the targeted lysines must be at non-canonical, non-conserved sites.

As the next step in identifying the Rep1 sumoylation sites, I created several more lysine-to-arginine point mutants of Rep1. Initially, a set of Rep1 mutants was created each having a single lysine residue, or a group of closely spaced lysine residues, substituted, that collectively comprised substitutions affecting all 27 lysine residues in Rep1. Each of the mutated Rep1 proteins was assessed for two-hybrid interaction with SUMO. When the assays were performed using the standard 2μm-containing (*cir*$^+$) reporter strain, none of these sequence alterations affected interaction of Rep1 with SUMO (data not shown). However, since the Rep proteins are known to self-associate and interact with each other, it was possible that a mutant Rep1 protein that was unable to be sumoylated (expressed as a LexA-fusion protein) could still indirectly interact with SUMO (expressed as a $Gal4_{AD}$-fusion protein) if the mutant Rep1 protein associated with endogenous Rep1 or Rep2 conjugated to $Gal4_{AD}$-SUMO (see **Figure 16C**). Therefore, assays were performed in a *cir*^{0} reporter strain to ensure that endogenous plasmid proteins would not be able to contribute to interaction with SUMO. When the Rep1 single point mutants were checked for interaction with SUMO in a *cir*^{0} two-hybrid reporter strain, $Rep1_{K305R}$ displayed reduced interaction with SUMO (**Figure 19A**). However, this interaction was not abolished, suggesting that other residues were sumoylated in the $Rep1_{K305R}$ mutant. Next, I tested interaction of SUMO with a mutant version of Rep1 in which all lysine residues except for the three most carboxy-terminal were substituted with arginine ($Rep1_{24R}$). This Rep1 mutant displayed no interaction with SUMO, suggesting loss of all SUMO acceptor sites (data not shown). To identify the residues in Rep1 targeted for sumoylation I then constructed various mutant Rep1 proteins containing subsets of the substitutions in $Rep1_{24R}$ (**Table 9, and Figure 19A**). Simultaneous substitution of three lysine residues, K305, K315, and K328 ($Rep1_{3R}$), was sufficient to reduce the two-hybrid association of Rep1 with SUMO to the level observed for $Rep1_{24R}$. These results suggest that Rep1 is sumoylated at K305, K315, and K328, with K305 serving as the major sumoylation site.

Table 9. Two-hybrid interactions of Rep1 alleles with SUMO.

Rep1 allele	SUMO interaction
WT	+++
K11,44,45,47,68,105,117,125,131,146,159,169,190,204,212,261,290,295,297,305,315,328,348R	–
K11,44,45,47,68,105,117,125R	+++
K146,159,169,190,204,212,261,290,295,297,305,315,328,348R	–
K146,159,169,190,204,212R	+++
K290,295,297,305,315,328,348R	–
K290,295,297,305,315R	+
K305,315,328,348R	–
3R = K305,315,328R	–
K305,315, 348R	+
K305,315R	+
K305R	++
K315R	+++
K328R	+++
K348R	+++

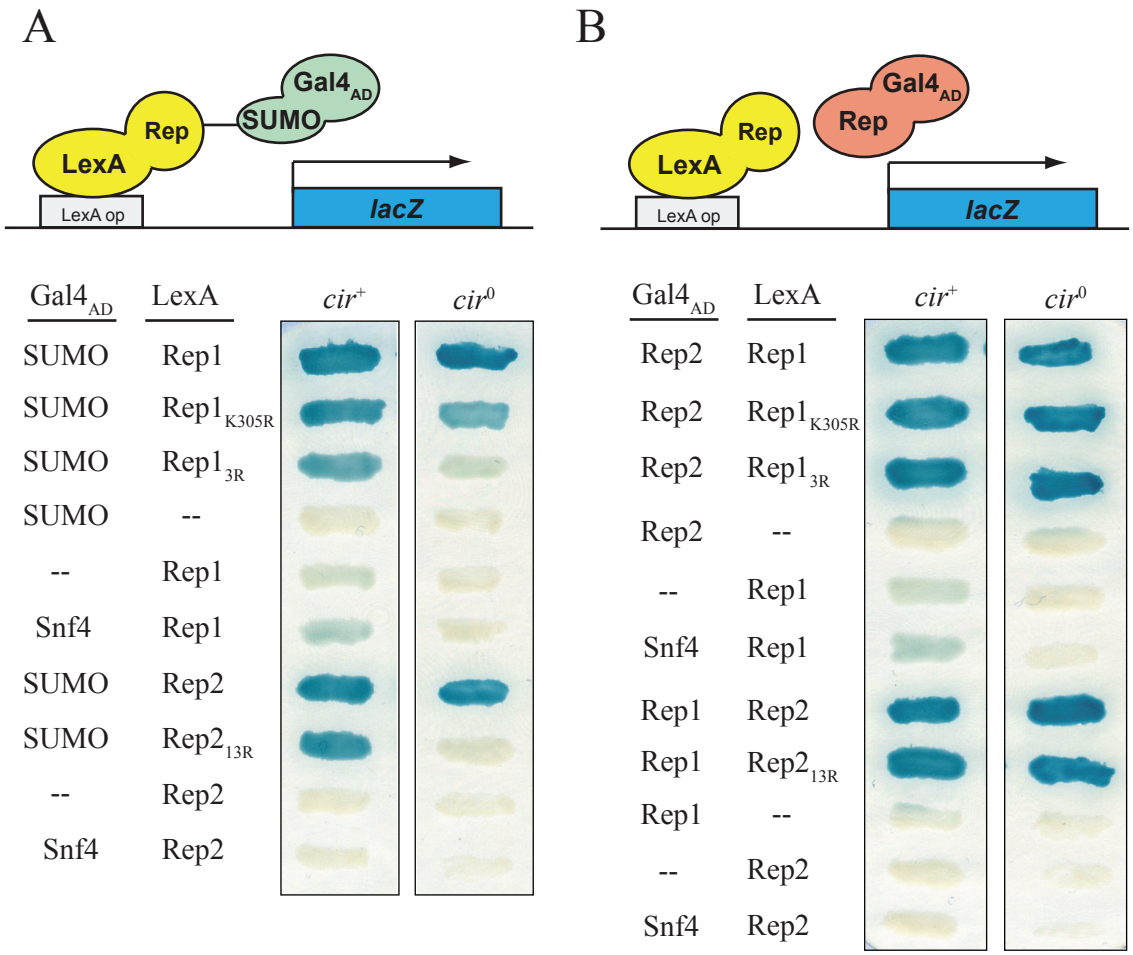


Figure 19. Lysine-to-arginine substitutions in Rep1 and Rep2 impair two-hybrid interaction with SUMO. To assess the effect of lysine-to-arginine substitutions on interaction of Rep1 and Rep2 proteins with (A) conjugatable SUMO or (B) each other, yeast two-hybrid reporter strains, *cir*$^{+}$ or *cir*^{0}, were co-transformed with plasmids expressing the indicated Gal4$_{AD}$ and LexA fusion proteins and interaction of the fusion proteins was assessed as described for Figure 15.

3.3.1.3 Multiple Lysine Substitutions in Rep2 Required to Abolish Two-Hybrid Interaction with SUMO

Identification of lysine residues in Rep2 targeted for sumoylation was undertaken by the same approach as that used for Rep1. The 296-aa Rep2 protein has 22 lysine residues, none of which match the sumoylation consensus sequence (**Figure 20**). Rep2 has no alignable homologues in other 2μm-like plasmids, so no strong candidate sumoylation sites could be identified through bioinformatic analyses. Therefore, arginine substitutions were systematically introduced at each lysine residue in Rep2, and two-hybrid interaction of these mutant versions of Rep2 with SUMO was tested (**Table 10**). In a *cir*^{0} two-hybrid reporter strain, no single lysine substitution in Rep2 abolished its two-hybrid association with SUMO; however, simultaneous substitution of thirteen lysine residues in Rep2-K42,44,92,124,130,134,146,148,149,177,208,226,227R ($Rep2_{13R}$) was found to virtually eliminate its two-hybrid interaction with SUMO (**Figure 19A**). While lysine substitutions in $Rep2_{13R}$ virtually abolished interaction with SUMO in the *cir*^{0} reporter strain, as had been observed for $Rep1_{3R}$, the substitutions in both $Rep1_{3R}$ and $Rep2_{13R}$ barely reduced the interaction in a *cir*$^+$ reporter strain, suggesting that endogenous plasmid proteins were contributing to interaction of the two-hybrid Rep fusion proteins in the *cir*$^+$ strain and highlighting the need for the assays to be performed in *cir*^{0} strains to effectively monitor covalent attachment of SUMO to Rep1 and Rep2.

3.3.1.4 Sequence alterations that Abolish Two-Hybrid Interaction of Rep1 and Rep2 with SUMO Do Not Impair Rep1-Rep2 Interaction

The amino acid substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ could have abolished the two-hybrid interaction with SUMO if they significantly reduced the steady-state levels of the Rep proteins when expressed as LexA-fusion proteins from the two-hybrid vector, rather than impairing covalent attachment to SUMO. Immunoblotting analyses established that there was no significant difference in the levels of wild-type and sumoylation-deficient Rep proteins when expressed as LexA fusions (data not shown). An alternative explanation for the loss of two-hybrid interaction was that the lysine-to-arginine substitutions, while conservative, perturbed Rep protein structure. To assess this, $Rep1_{3R}$ and $Rep2_{13R}$ were tested for their

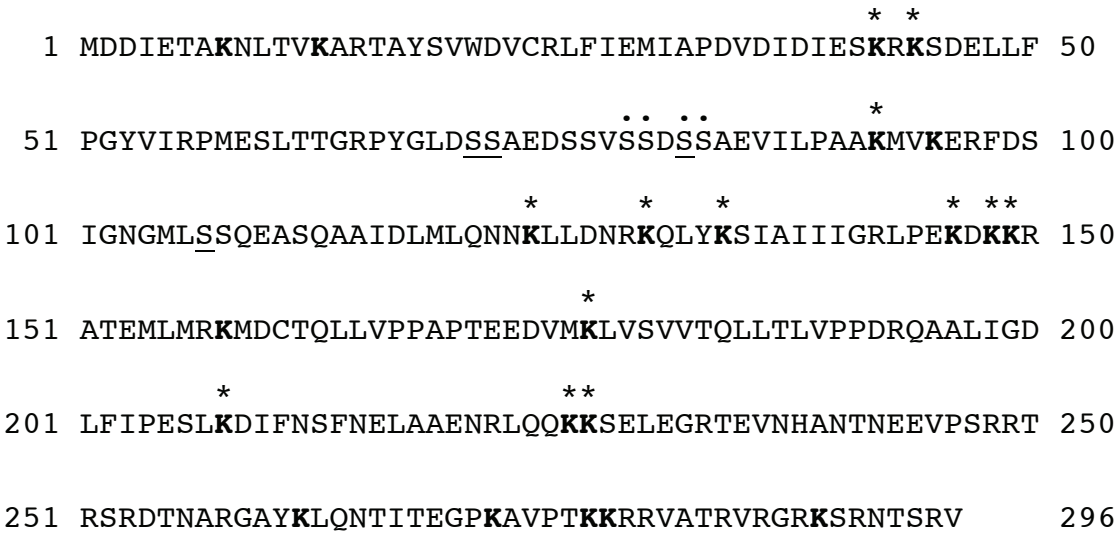


Figure 20. Amino acid sequence of Rep2. The predicted amino acid sequence of Rep2 is shown. Lysine residues substituted in $Rep2_{13R}$ (asterisks), serine residues substituted in $Rep2_{SA}$ and $Rep2_{SE}$ (dots) and serine residues conforming to CK2 consensus sites (underlined) are indicated.

Table 10. Two-hybrid interactions of Rep2 alleles with SUMO.

Rep2 allele	SUMO interaction
WT	+++
K8,13R	+++
K42,44R	+++
K92R	+++
K95R	+++
K124R	+++
K130R	+++
K134R	+++
K146,148,149R	++
K158R	+++
K177R	+++
K208R	+++
K226,227R	+++
13R = K42,44,92,124,130,134,146,148,149,177,208,226,227R	-

known two-hybrid interactions with their respective partner Rep proteins (192). The association of $Rep1_{3R}$ and $Rep2_{13R}$ with wild-type Rep2 and Rep1, respectively, was not impaired (**Figure 19B**), demonstrating that the substitutions did not affect Rep1-Rep2 interaction and suggesting they did not significantly perturb Rep protein structure.

3.3.2 Identification of SUMO Conjugates of Rep1 and Rep2 by Western Blot Analysis

The loss of two-hybrid interaction of the $Rep1_{3R}$ and $Rep2_{13R}$ proteins with SUMO caused by lysine-to-arginine substitutions strongly suggests that substitution of these residues blocks covalent attachment of SUMO. However, the loss of two-hybrid interaction with SUMO could also have been due to loss of interaction with a sumoylated host protein. Direct identification of SUMO-conjugates of Rep1 and Rep2 was necessary to establish that the substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ block covalent attachment of SUMO.

3.3.2.1 Affinity Purification of SUMO Conjugates Enables Detection of Sumoylated forms of Rep1 and Rep2

Conjugation of a single SUMO moiety to target proteins increases the apparent MW by ~20 kDa relative to the unmodified protein, while polysumoylated species are observed as multiple HMW forms increasing by ~20-kDa intervals upwards in standard western blotting analysis (120). For Rep1 and Rep2, no HMW species having mobilities consistent with sumoylation are detected in protein extracted from *cir*$^+$ yeast (**Figure 4**) even after prolonged exposures.

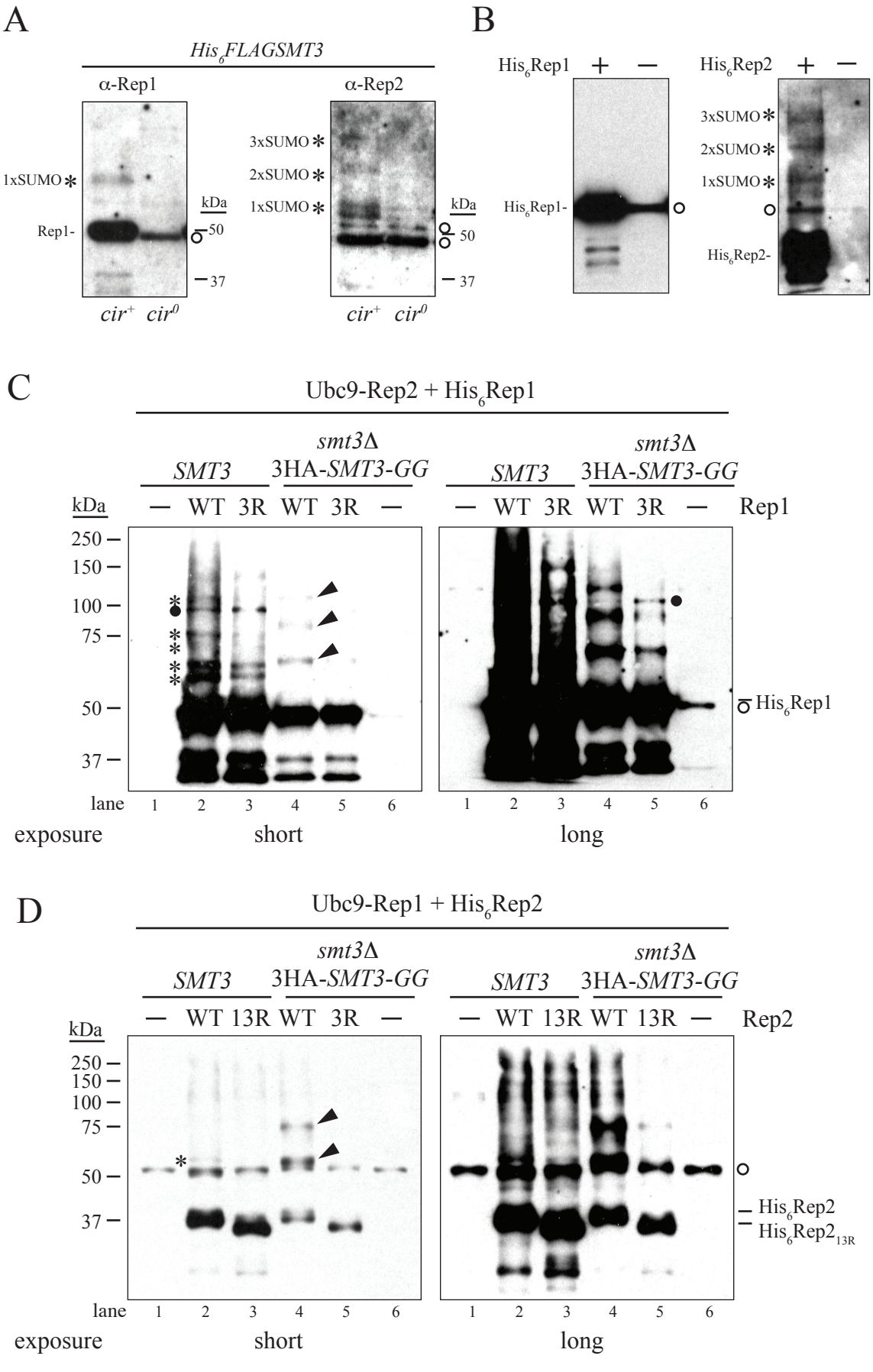
Difficulty in detecting SUMO conjugates of native Rep1 and Rep2 expressed at endogenous levels by western blotting of total yeast protein extracts may reflect the low abundance of Rep-protein SUMO conjugates, whose signal may have been obscured by those from off-target proteins detected by the antibodies. To enhance detection of sumoylated forms of Rep1 and Rep2 in western blotting, SUMO was expressed with a hexa-histidine (His_6)-tag to enable enrichment by metal-ion affinity chromatography under the denaturing conditions often required to preserve SUMO conjugates (121). To tag SUMO, DNA encoding the His_6FLAG epitope was introduced into the genome at the 5' end of the SUMO-encoding ORF (*SMT3*) locus (262). Protein was extracted from this yeast strain, and His_6FLAG-tagged SUMO conjugates were enriched by metal-ion affinity chromatography

and examined by western blotting with an anti-FLAG antibody. Multiple species were detected that were not observed when protein was extracted from yeast not expressing His_6FLAG-tagged SUMO, demonstrating that the affinity purification had been effective (data not shown). However, the pattern detected by the anti-FLAG antibody in extracts from *cir*$^+$ and *cir*^{0} strains was identical (data not shown), suggesting that if sumoylated forms of Rep1 and Rep2 were present, they were much less abundant than the SUMO conjugates of other yeast proteins, or the signal was obscured by the other SUMO-conjugated proteins. To determine whether any species of Rep1 and Rep2 had been affinity-purified from the extracts, as would be expected if the Rep proteins had been conjugated with the His_6-tagged SUMO, duplicate blots were probed with anti-Rep1 and anti-Rep2 antibodies (**Figure 21A**). A prominent species with the mobility expected for unmodified Rep1 was detected in affinity-purified extracts from *cir*$^+$ yeast, suggesting that Rep1 has affinity for the Co^{2+} resin. In yeast expressing no Rep1 or Rep2, a more faint species also having a mobility similar to that of Rep1 was also detected by both the anti-Rep1 and anti-Rep2 antibodies. This species was also observed when protein was purified from yeast expressing untagged SUMO, indicating that this likely represents a non-SUMO-conjugated protein with affinity for the Co^{2+} resin (data not shown). Notably, for protein extracted from yeast expressing Rep proteins from the native 2μm plasmid, very faint HMW species having the mobilities predicted for those of SUMO conjugates of both Rep1 and Rep2, respectively, were detected. In affinity-purified extracts from *cir*$^+$ yeast, a species with the mobility predicted for mono-sumoylated Rep1 was detected, as well as several species consistent with mono-, di-, and tri-sumoylated Rep2. These forms of Rep2 could reflect sumoylation at more than one lysine residue (multi-sumoylation) or represent a chain of SUMO proteins attached to a single lysine residue (poly-sumoylation).

3.3.2.2 Probing for Rep-Protein SUMO Conjugates Following Enrichment of His_6-Tagged Rep1 and Rep2

Although a single species of Rep1 and several species of Rep2 consistent with their respective SUMO-conjugated forms were detected in western blots of protein enriched for SUMO conjugates, the signals observed for these species were often barely detectable above background. The signal of SUMO conjugates of Rep1 and Rep2 needed to be improved to be

Figure 21. Western blot analysis of putative Rep protein SUMO-conjugates. Protein was extracted (A) from isogenic *cir*$^{+}$ and *cir*^{0} yeast strains encoding His_6FLAG-tagged SUMO (His_6FLAG*SMT3*), (B) from yeast expressing His_6Rep1, His_6Rep2, or that did not express any Rep proteins (-), and (C and D) from a *cir*^{0} yeast strain wild-type for *SMT3* that encodes endogenous SUMO, or in which *SMT3* was deleted and the yeast transformed with a plasmid expressing triple-HA-tagged mature SUMO (3HA-*SMT3-GG*) and either lacked (-) or contained an *ADE2*-tagged 2μm plasmid encoding Ubc9-Rep2 and His_6-Rep1 (C) or Ubc9-Rep1 and His_6-Rep2 (D). His_6-tagged proteins were affinity-purified with Co^{2+} resin and analyzed by western blotting with anti-Rep1 (A, B left, and C) or anti-Rep2 (A, B right, and D) antibodies. Species with mobilities consistent with those expected for the Rep proteins conjugated with endogenous or His_6FLAG-tagged SUMO (asterisks), 3HA-SUMO conjugates of the Rep proteins (arrowheads), proteins that cross-react with the antibody (open circles) and an unidentified HMW species of Rep1 (closed circle) are indicated.


A
His6FLAGSMT3
α-Rep1
α-Rep2
1xSUMO
3xSUMO
2xSUMO
Rep1-
kDa
50
37
cir+ cir0
B
His6Rep1 + −
His6Rep2 + −
His6Rep1-
His6Rep2-
C
Ubc9-Rep2 + His6Rep1
SMT3
smt3Δ 3HA-SMT3-GG
kDa
— WT 3R WT 3R —
Rep1
250
150
100
75
50
37
His6Rep1
lane 1 2 3 4 5 6
exposure short long
D
Ubc9-Rep1 + His6Rep2
— WT 13R WT 3R —
— WT 13R WT 13R —
Rep2
His6Rep2
His6Rep2 13R

able to reliably distinguish wild-type from potential sumoylation-deficient mutant Rep1 and Rep2 proteins. To improve the signal, His_6-tags were introduced at the amino termini of Rep1 and Rep2 to enable the Rep proteins to be affinity-purified from protein extracts before western blotting analysis. DNA encoding the tags was integrated into the 5' end of the *REP1* or *REP2* coding region in an *ADE2*-tagged 2μm plasmid, which could be introduced into a *cir*^{0} *ade2* yeast strain and maintained by culturing the yeast in medium lacking adenine. Protein was extracted from the transformed yeast and analyzed by western blotting with anti-Rep1 and anti-Rep2 antibodies. His_6-Rep1 and His_6-Rep2 were expressed at levels similar to those of untagged Rep1 and Rep2, respectively, expressed from an *ADE2*-tagged 2μm plasmid, and were functional for plasmid partitioning, as indicated by a colony colour assay (data not shown). To detect sumoylated forms of the Rep proteins, protein was extracted from the transformants, enriched by metal-ion affinity chromatography, and analyzed by western blotting. No HMW species of Rep1 were observed, while faint HMW species of Rep2 having the mobilities expected for the addition of one, two, and three SUMO moieties were faintly visible (**Figure 21B**). Taken together, the results indicate that detection of SUMO conjugates of Rep1 and Rep2 when expressed at normal levels from a 2μm-based plasmid is technically challenging even after affinity purification of either SUMO or the Rep proteins. Detection of SUMO conjugates at the level needed for comparing wild-type and the putative sumoylation-deficient Rep protein mutants therefore required methods of artificially increasing cellular levels of Rep-protein SUMO conjugates.

3.3.2.3 Rep Protein Sumoylation is Enhanced in *trans* by Ubc9 Fusion-Firected Sumoylation, and is Reduced in the $Rep1_{3R}$ and $Rep2_{13R}$ Mutants

To increase the levels of their SUMO conjugates, Rep1 and Rep2 were expressed as fusions with the SUMO-conjugating enzyme Ubc9. Ubc9 fusion-directed sumoylation (UFDS) is typically used to enhance auto-sumoylation of the Ubc9-chimera (113, 168, 169). HMW species of both Ubc9-Rep1 and Ubc9-Rep2 having mobilities consistent with sumoylated forms were detected (data not shown). However, because Ubc9 itself is sumoylated (92, 238, 261, 262), these sumoylated species might have represented SUMO modification of the Ubc9 moiety rather than of Rep1 or Rep2. Because Rep1 and Rep2 directly interact *in vivo*, I tested whether fusion of Ubc9 to Rep1 could enhance sumoylation

of Rep2 and vice-versa. Yeast were transformed with *ADE2*-tagged 2μm plasmids encoding one Rep protein tagged with Ubc9, and the other tagged with His_6. Total protein was extracted, and His_6-tagged Rep proteins were affinity-purified using Co^{2+} resin and analyzed by western blotting. HMW species of both Rep1 (**Figure 21C, lanes 2-3**) and Rep2 (**Figure 21D, lanes 2-3**) were detected that had the mobilities expected for sumoylated forms. The respective levels of these species were reduced but not eliminated in parallel analyses of the $Rep1_{3R}$ and $Rep2_{13R}$ mutants. The sizes of these species, and the reductions in their levels when amino acid substitutions that virtually abolished two-hybrid Rep-SUMO interaction were introduced suggest that these HMW species are sumoylated forms of Rep1 and Rep2.

To further confirm that the HMW species of His_6-Rep1 and His_6-Rep2 are sumoylated forms, similar experiments were performed in a yeast strain transformed with a plasmid directing constitutive expression of a triple-HA epitope-tagged version of mature SUMO, which, unlike endogenous SUMO, does not require proteolytic processing to be available for conjugation. The *SMT3* gene was deleted so that HA-tagged SUMO was the only version of SUMO expressed in this strain. If the HMW species of Rep1 and Rep2 observed when the yeast contained endogenous SUMO were SUMO-conjugated forms, the ~5-kDa epitope tag fused to SUMO would be expected to make these conjugates migrate more slowly due to the presence of the epitope tag in SUMO, and since 3HA-SUMO was expressed at a higher level than endogenous SUMO, the HMW forms might be increased in abundance.

When yeast expressed 3HA-SUMO, western blotting analysis of the Rep proteins revealed HMW forms of both Rep1 and Rep2 similar to those observed when yeast contained endogenous SUMO, except that the species were more abundant relative to the amount of unmodified Rep proteins, and exhibited slower electrophoretic migration (**Figure 21C-D**). Analysis of Rep1 species revealed HMW forms consistent with one, two, and three SUMO moieties conjugated to Rep1, whose abundances were reduced in the $Rep1_{3R}$ mutant (**Figure 21C, lanes 4-5**). HMW species of Rep2 with mobilities consistent with mono- and poly-sumoylated Rep2 were also readily detected, and were reduced in a similar analysis of $Rep2_{13R}$ (**Figure 21D, lanes 4-5**). These sumoylated forms were also observed when His_6-Rep1 and His_6-Rep2 were co-expressed with Ubc9 fused to $Rep2_{13R}$ and $Rep1_{3R}$, respectively (data not shown), indicating that sumoylation of the His_6-tagged Rep proteins was not

dependent on sumoylation of their respective interacting partner Rep protein fused to Ubc9. I attempted to confirm that these species were HA-tagged SUMO conjugates by probing duplicate blots with an anti-HA antibody, but species of the sizes expected for the sumoylated forms of His_6-Rep1 or His_6-Rep2 were not observed. The Rep proteins are not abundant and the level of the HA-SUMO-conjugated forms may have been below the limit of detection for the monoclonal anti-HA antibody. Although I was unable to directly confirm that the HMW species were SUMO-conjugated forms of the Rep proteins, their shifts in mobility upon introduction of an epitope tag in SUMO, and loss when lysine residues in the Rep proteins were substituted with arginine, are consistent with both Rep1 and Rep2 being directly targeted for sumoylation on these residues and with $Rep1_{3R}$ and $Rep2_{13R}$ being sumoylation-deficient.

In addition to the HMW forms of Rep1 and Rep2 that were reduced in abundance by the lysine substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ mutants, the HMW species migrating at ~100 kDa detected by the anti-Rep1 antibodies was also observed (**Figure 21C**). The species was not detected when yeast did not express Rep1, and its mobility and abundance relative to unmodified Rep1 were not altered by the lysine substitutions in $Rep1_{3R}$ or when SUMO was HA-tagged as opposed to being the endogenous form. The species was also observed even when all Rep1 lysine residues with the exception of the three most carboxy-terminal were substituted with arginine (data not shown). This species might therefore represent a post-translational modification to Rep1 other than sumoylation.

3.3.3 Analyzing the Function of Sumoylation-Deficient Rep1 and Rep2 Mutants

3.3.3.1 Plasmid Inheritance is Impaired when the Rep Proteins are Sumoylation-Deficient.

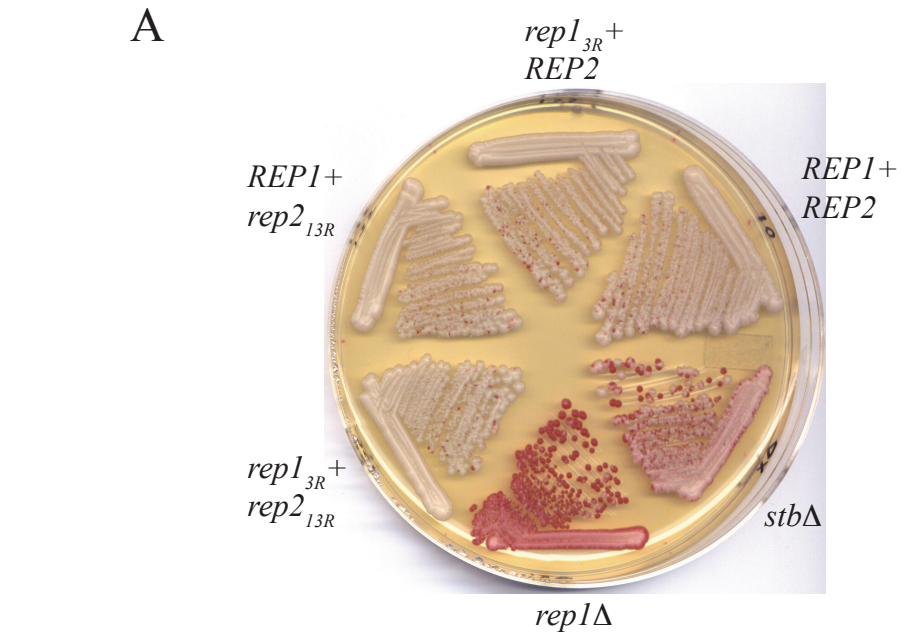
To determine whether sumoylation of Rep1 and Rep2 contributes to 2μm plasmid partitioning, I analyzed the inheritance of 2μm-based plasmids encoding either wild-type or sumoylation-deficient Rep1 and Rep2. Since the native 2μm plasmid confers no phenotype, the mutant *REP* genes encoding $Rep1_{3R}$ and $Rep2_{13R}$ were introduced into the *ADE2*-tagged 2μm plasmid, pAS10 (see **Figure 13C** for a map).

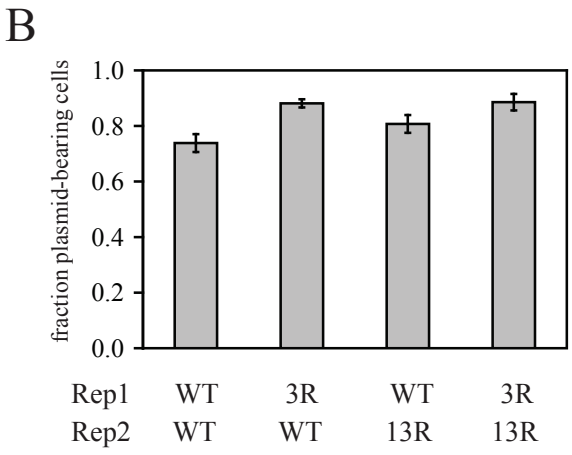
As controls for plasmids that lack a functional partitioning system, pAS10 derivatives lacking the *REP1* gene or the repeated sequences in the *STB* partitioning locus were also

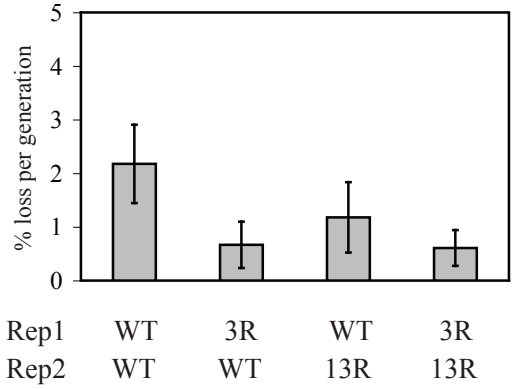
created. These plasmids were transformed into *cir*^{0} yeast and transformants were initially propagated in the absence of adenine to ensure that only plasmid-containing cells could continue to proliferate. To qualitatively analyze plasmid inheritance, transformants were streaked onto solid medium containing a limited amount of adenine, and the colour of the colonies formed was examined (**Figure 22A**). As previously observed (Arpita Sengupta, PhD thesis), yeast transformed with a plasmid encoding wild-type Rep1 and Rep2 formed mostly white colonies, indicating that the plasmid was being efficiently inherited during cell division. Yeast transformed with the plasmid lacking a *REP1* gene (*rep1*Δ) formed only red colonies, indicating a severe defect in plasmid inheritance, and consistent with previous observations that loss of Rep1 severely impairs plasmid partitioning (126). Yeast harboring the *stb*Δ plasmid formed about equal numbers of red and white colonies, indicating that partitioning was impaired, but not to the extent observed when Rep1 was absent. When Rep1, Rep2, or both Rep1 and Rep2 contained the lysine-to-arginine substitutions that abolished SUMO interaction, yeast formed mostly white colonies similar to those observed when yeast expressed wild-type Rep1 and Rep2. This finding suggests that inheritance of the *ADE2*-tagged 2μm plasmids is not affected by the amino acid substitutions that impair Rep protein sumoylation.

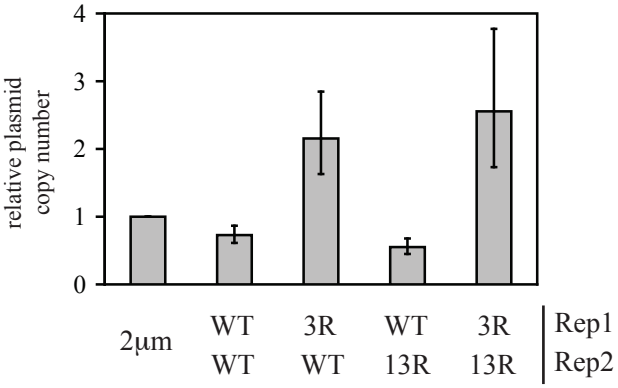
Although no differences were observed between the ability of wild-type and presumptive sumoylation-deficient Rep proteins to mediate plasmid inheritance, subtle defects in inheritance could have been obscured by the qualitative nature of the colony colour assay. To quantify plasmid inheritance, the proportion of plasmid-containing cells was determined for transformants grown in liquid medium lacking adenine (**Figure 22B**). Under these conditions, defects in plasmid partitioning can be detected as an increase in the proportion of cells auxotrophic for adenine, provided the defect is severe enough to significantly increase the proportion of daughter cells that fail to receive any copies of the plasmid during cell division. Compared to when Rep1 and Rep2 were wild-type, there was no significant difference in the fraction of plasmid-containing cells when the plasmid encoded $Rep2_{13R}$; however, when plasmids encoded $Rep1_{3R}$, the fraction of plasmid-containing cells was slightly higher, suggesting that these plasmids were inherited even more effectively than when Rep1 was wild-type. As an alternative measure of plasmid inheritance, I also determined the rate at which plasmid-free cells were generated during growth in

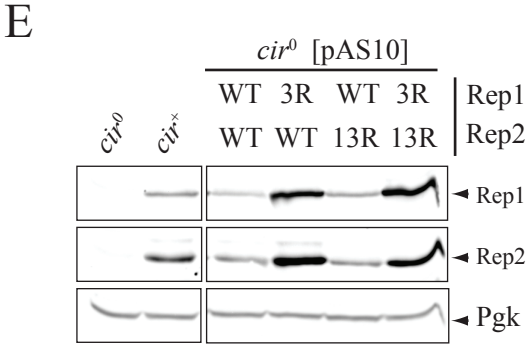
Figure 22. Inheritance and copy number of *flp*$^-$ *ADE2*-tagged 2μm plasmids encoding Rep1$_{3R}$ and Rep2$_{13R}$. (A) *ADE2*-tagged 2μm plasmids (pAS10-based) encoding the indicated *REP1* and *REP2* alleles or lacking *REP1* (*rep1*Δ) or the *STB*-proximal repeats (*stb*Δ), were transformed into *cir*^{0} *ade2* yeast. Transformants were streaked on solid YPD medium to relax selection for the *ADE2* plasmid and imaged after five days of incubation at 28˚C. The efficiency of plasmid inheritance was qualitatively assessed by visualizing the red pigment that accumulates in yeast lacking a functional *ADE2* gene. Transformants from (A) with plasmids encoding the indicated Rep1 and Rep2 alleles were grown in medium lacking adenine, and the proportion of plasmid-containing cells (B) and the rate of loss of plasmid-containing cells after transfer to medium containing adenine (C) (avg ± sd from six independent yeast transformants) were determined. (D) DNA was extracted from the indicated transformants from (A) and the copy number of the pAS10-based plasmids relative to that of the native 2μm plasmid in an isogenic *cir*$^+$ strain was determined by qPCR. Error bars indicate the variation in relative copy number values obtained between three independent PCR reactions using the same template DNA. (E) Protein was extracted from the cultures of yeast analyzed in (D) and analyzed by immunoblotting with anti-Rep1, anti-Rep2, and anti-Pgk antibodies.


A
rep1 3R+
REP2
REP1+
rep2 13R
REP1+
REP2
rep1 3R+
rep2 13R
stbΔ
rep1Δ


B
fraction plasmid-bearing cells
1.0
0.8
0.6
0.4
0.2
0.0
Rep1 WT 3R WT 3R
Rep2 WT WT 13R 13R


C
% loss per generation
5
4
3
2
1
0
Rep1 WT 3R WT 3R
Rep2 WT WT 13R 13R


D
relative plasmid copy number
4
3
2
1
0
2µm WT 3R WT 3R Rep1
WT WT 13R 13R Rep2


E
cir0 [pAS10]
WT 3R WT 3R Rep1
cir0 cir+ WT WT 13R 13R Rep2
Rep1
Rep2
Pgk

medium containing excess adenine, a condition in which plasmid-free cells are not selected against and will continue to proliferate (**Figure 22C**). Yeast transformed with plasmids encoding $Rep1_{3R}$ generated plasmid-free cells at a lower rate than when Rep1 was wild-type, consistent with the increase in the proportion of plasmid-containing cells observed when transformants were cultured in medium that maintained selection for the plasmid. Substitutions in Rep2 that impaired sumoylation had no significant effect on the rate of plasmid loss.

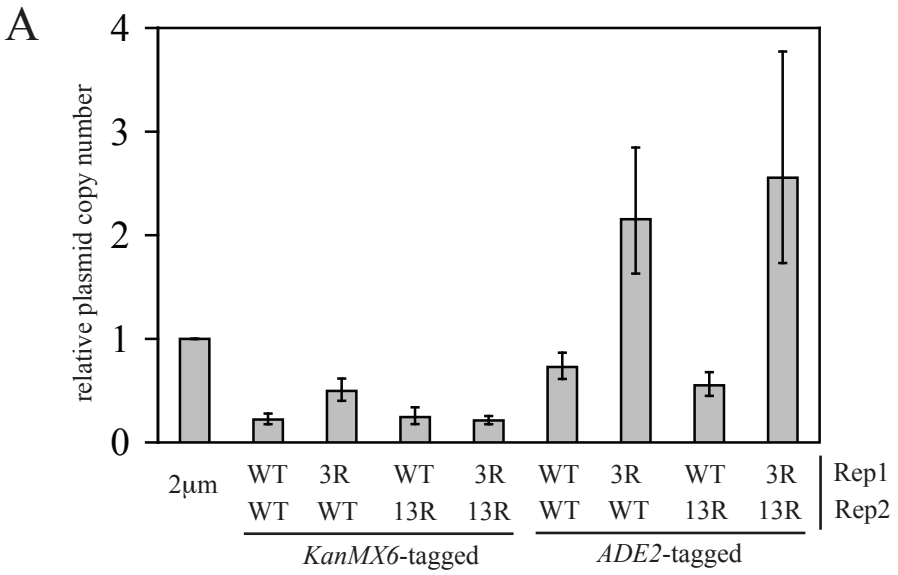
The improved inheritance of *ADE2*-tagged 2μm plasmids associated with substitutions that impaired sumoylation of Rep1 was puzzling; it was difficult to rationalize how the amino acid substitutions could improve Rep1 function. I noted that upon introduction of the *ADE2*-tagged 2μm plasmids into yeast, colonies on primary transformation plates initially grew much more slowly when the plasmids encoded $Rep1_{3R}$ compared to when Rep1 was wild-type, but after passaging onto fresh medium, all transformants grew equally well (data not shown). This observation could be explained by a partial loss of partitioning function of the $Rep1_{3R}$ mutant. Following the initial transformation event, where cells take up on average only one plasmid molecule, plasmids with a partial defect in partitioning (if this were the case for those encoding $Rep1_{3R}$) would more frequently fail to be delivered to the daughter cells during cell division. After successive rounds of plasmids being duplicated during S phase, and more frequently retained in the mother cell during mitosis, the copy number of the $Rep1_{3R}$-encoding plasmid in mother cells would eventually become exceedingly high, such that even with unequal partitioning due to partial impairment of Rep1 function, daughter cells would become more likely to inherit some copies of the plasmid. Once a stable high copy number has been established, subtle partitioning defects would be obscured, since cells having excessively high plasmid copy would rarely give rise to a plasmid-free cell, even though plasmid copies may nonetheless be unequally partitioned during mitosis. An additional reason for the improved inheritance of *ADE2*-tagged 2μm plasmid encoding $Rep1_{3R}$ would be if the *ADE2* gene at higher copy number confers a selective advantage to the yeast. Copy number of marker-tagged plasmids is driven up with increasing demand for the gene product (63, 167, 222). The *ADE2* gene confers a considerable (3%) fitness advantage to yeast, even when yeast are grown in the presence of excess adenine (64, Jessica Wentzell, Dalhousie University, Honours thesis).

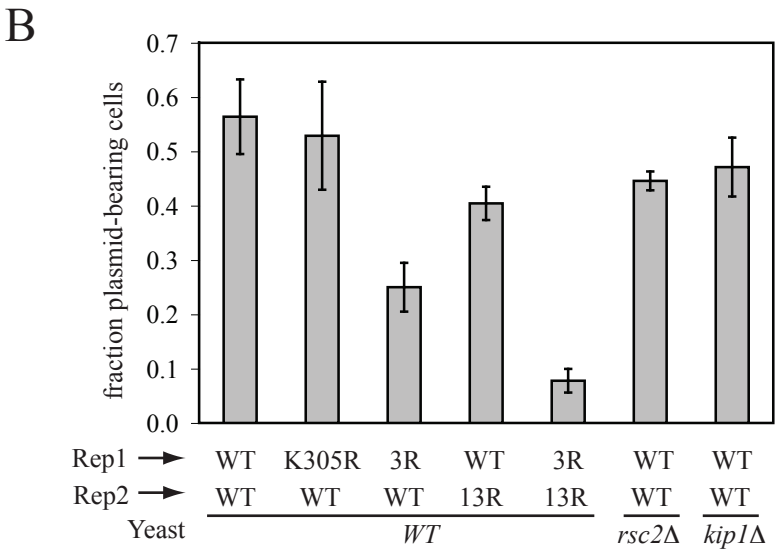
If these hypotheses are correct, and the *ADE2*-tagged 2μm plasmids encoding $Rep1_{3R}$ are poorly partitioned, their copy number would be expected to be higher than that of plasmids encoding wild-type Rep1 when yeast transformed with the plasmids are grown in medium lacking adenine. To test whether copy number of the *ADE2*-tagged 2μm plasmids was increased when they encoded the $Rep1_{3R}$ mutant, quantitative real-time PCR (qPCR) was used to measure the copy number of the pAS10-based plasmids (**Figure 21D**). The average copy number of the plasmid encoding $Rep2_{13R}$ was not significantly different than that of the plasmid encoding wild-type Rep proteins; however, the copy number of the plasmid encoding $Rep1_{3R}$ was about three-fold higher. The levels of the Rep1 and Rep2 proteins expressed from these plasmids, analyzed by western blotting, also correlated with the differences in plasmid copy number (**Figure 21E**). The elevated copy number of the *ADE2*-tagged plasmid encoding $Rep1_{3R}$ is consistent with the hypothesis that plasmid partitioning is impaired when Rep1 was sumoylation-deficient.

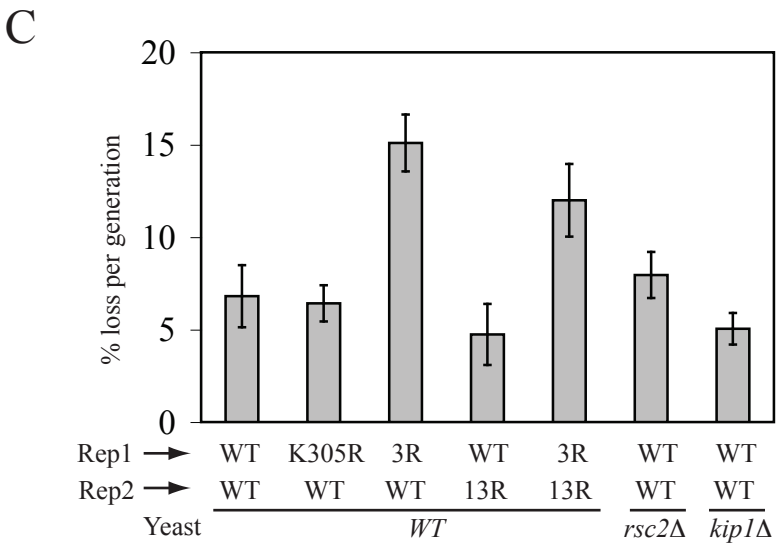
To be able to determine unequivocally whether plasmid inheritance is perturbed when Rep1 sumoylation was impaired, I tagged a 2μm plasmid with a marker gene that was less likely to confer the increased selective advantage with increased copy number that was associated with the *ADE2*-tagged plasmids. The *KanMX6* gene, which confers resistance to the aminoglycoside antibiotic G418 (232), had the desirable characteristics. The *ADE2* gene on the pAS10-based 2μm plasmids was replaced with this dominant drug-resistance marker, enabling plasmid-containing cells to be distinguished from plasmid-free cells based on their ability to grow in the presence of G418. Yeast containing the *KanMX6* gene would be expected to be equally fit compared to those lacking a *KanMX6* gene when grown in the absence of G418, since in the absence of G418 the enzyme encoded by the *KanMX6* gene has no substrate and is therefore useless to the cell.

To determine the copy number of *KanMX6*-tagged plasmids relative to otherwise identical plasmids tagged with *ADE2*, yeast were transformed with *KanMX6*- or *ADE2*-tagged plasmids encoding wild-type or presumptive sumoylation-deficient Rep proteins. The transformants were grown in medium selecting for the plasmids, DNA was extracted and the plasmid copy number was determined by qPCR (**Figure 23A**). As predicted, the average copy number of the *KanMX6*-tagged plasmids was significantly lower than their corresponding *ADE2*-tagged counterparts. There was no significant difference in the average

Figure 23. Mutations that perturb two-hybrid interaction of Rep1 and Rep2 with SUMO also impair plasmid inheritance. (A) DNA was extracted from *cir*^{0} yeast transformants harbouring *KanMX6*- or *ADE2*-tagged *flp*$^{-}$ 2μm plasmids encoding the indicated *REP1* and *REP2* alleles, and plasmid copy number relative to that of the native 2μm plasmid in an isogenic *cir*$^{+}$ strain was determined by qPCR. Error bars indicate the variation in relative copy number values obtained for three independent PCR reactions using the same template DNA. (B) *KanMX6*-tagged 2μm plasmids encoding the indicated Rep1 and Rep2 alleles were transformed into *cir*^{0} yeast of the indicated genotype, cultured in medium containing G418 and the proportion of plasmid-containing cells determined. (C) The transformants in (B) were transferred to medium lacking G418 and after ~15 generations the rate of loss of plasmid-containing cells (avg ± sd from six independent yeast transformants) was determined.


A
relative plasmid copy number
0
1
2
3
4
2µm
WT WT
3R WT
WT 13R
3R 13R
WT WT
3R WT
WT 13R
3R 13R
Rep1
Rep2
KanMX6-tagged
ADE2-tagged


B
fraction plasmid-bearing cells
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Rep1 → WT K305R 3R WT 3R WT WT
Rep2 → WT WT WT 13R 13R WT WT
Yeast
WT
rsc2Δ
kip1Δ


C
% loss per generation
0
5
10
15
20
Rep1 → WT K305R 3R WT 3R WT WT
Rep2 → WT WT WT 13R 13R WT WT
Yeast
WT
rsc2Δ
kip1Δ

copy number between *KanMX6*-tagged plasmids encoding Rep1 + Rep2, Rep1 + $Rep2_{13R}$, and $Rep1_{3R}$ + $Rep2_{13R}$, while the *KanMX6*-tagged plasmid encoding $Rep1_{3R}$ + Rep2 was approximately double that of the other three.

To examine inheritance of the *KanMX6*-tagged 2μm plasmids encoding wild-type or mutant Rep1 and Rep2, cir^0 yeast transformed with the *KanMX6*-tagged plasmids were grown in the presence of G418 to ensure that only plasmid-containing cells could continue to proliferate. Plasmid inheritance was examined by determining the proportion of G418-sensitive cells in the population (**Figure 23B**). Substitution of only the single, potentially major sumoylation site in Rep1, K305, did not significantly reduce the proportion of plasmid-containing cells. However, when the 2μm plasmid encoded the $Rep1_{3R}$ or $Rep2_{13R}$ mutants, the fraction of plasmid-containing cells was significantly lower than when Rep1 and Rep2 were wild-type, with substitution of Rep1 having a more significant impact on plasmid inheritance than substitution of Rep2 (**Figure 23B**). When both Rep1 and Rep2 were mutant, the fraction of cells containing plasmid was reduced further still, indicating that an even higher proportion of daughter cells was consistently receiving no copies of the plasmid during cell division. To gauge the severity of this defect relative to that caused by chromosomal substitutions previously reported to impair plasmid partitioning, I transformed yeast lacking either the *RSC2* or *KIP1* gene with the *KanMX6*-tagged 2μm plasmid encoding wild-type Rep1 and Rep2. *RSC2* encodes a regulatory subunit of one form of the RSC chromatin remodeling complex (27) and *rsc2*Δ yeast are unable to maintain the 2μm plasmid (239). *KIP1* encodes a plus end-directed microtubule motor protein that is recruited to *STB* by Rep1 and Rep2 (46). Deletion of *KIP1* prevents incorporation of the centromere-specific histone H3 variant Cse4 in *STB* nucleosomes, resulting in an increased rate of plasmid mis-segregation (46). The reduction in the proportion of cells containing the *KanMX6*-tagged 2μm plasmid in yeast lacking *RSC2* was comparable to that observed when yeast wild-type for *RSC2* contained the plasmid encoding the $Rep2_{13R}$ mutant, and was not as severe as that observed when Rep1, or Rep1 and Rep2 were mutant, suggesting that Rep protein sumoylation is critical for plasmid maintenance in the cell population (**Figure 23B**). Deletion of *KIP1* did not significantly affect inheritance of the plasmid, suggesting that while use of the *KanMX6*-tagged plasmids demonstrated defects in plasmid inheritance that were not observed when *ADE2*-tagged plasmids were employed, these assays may still be relatively

insensitive to slight perturbations in 2μm plasmid partitioning compared to the artificial single-copy 2μm plasmid that was required to show the partitioning defect in *kip1*Δ yeast (46).

To further quantify the impact of impaired sumoylation of Rep1 and Rep2 on plasmid partitioning, I determined the rate at which plasmid-free cells were generated during growth in medium lacking G418, a condition in which plasmid-free cells are not selected against and will continue to proliferate. Yeast transformed with a plasmid encoding $Rep1_{3R}$ gave rise to plasmid-free cells at roughly twice the frequency compared to when Rep1 was wild-type (**Figure 23C**). A similar increase in the rate of plasmid loss was observed when plasmids encoded both $Rep1_{3R}$ and $Rep2_{13R}$. Interestingly, the rate of loss for plasmids when only Rep2 was mutant was not significantly increased compared to those encoding wild-type Rep proteins. This finding is consistent with the observation that the $Rep1_{3R}$ mutant produced a more severe effect than the $Rep2_{13R}$ mutant on the proportion of plasmid-containing cells when transformants were grown under selective conditions. Loss of Rep2 sumoylation appears to be less detrimental to plasmid maintenance.

3.3.3.2 Impaired Sumoylation of Rep1 and Rep2 Does Not Alter Their Post-Translational Stability or the Ability of Rep2 to Chaperone Rep1, but Does Not Alter Rep2 Phosphorylation Status

The defect in 2μm plasmid maintenance associated with the $Rep1_{3R}$ and $Rep2_{13R}$ mutants could be due to altered Rep protein levels. Substitution of lysine residues in the Rep proteins could alter their half-life if sumoylation of these sites contributes to Rep1 and Rep2 post-translational stability. Alternatively, the lysine residues might normally be directly targeted for ubiquitin-mediated proteolysis, or substitution of these residues could significantly perturb protein folding or other post-translational modifications of the Rep proteins. To examine Rep1 and Rep2 post-translational stability, cir^0 yeast were transformed with single-copy (*CEN*/*ARS*) plasmids encoding *REP1* or *REP2* under control of a galactose-inducible promoter, and the levels of Rep1 and Rep2 were monitored following addition of cycloheximide to the cultures as was performed in **Section 3.1.2**. The levels of a highly stable glycolytic enzyme, 3-phosphoglycerate kinase (Pgk) (9), were simultaneously examined (**Figure 24 A-B**). Neither mutant protein differed significantly in stability from

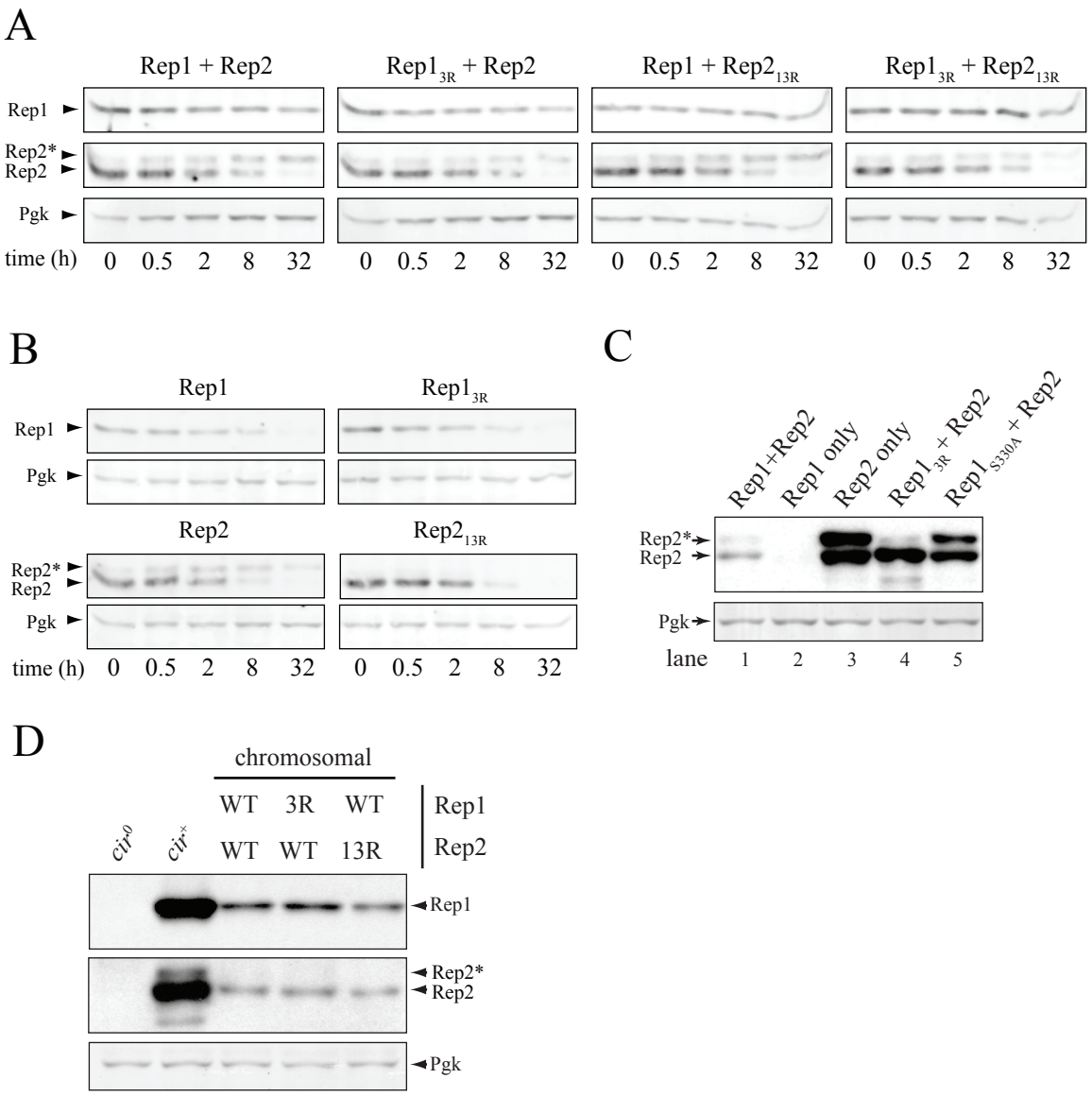


Figure 24. Levels and post-translational stabilities of wild-type and mutant Rep proteins. The indicated wild-type or mutant versions of Rep1 and Rep2 were either (A) co-expressed or (B) expressed individually in *cir*^{0} yeast under the control of a galactose-inducible promoter from single-copy *CEN/ARS* plasmids, or were expressed in *cir*^{0} yeast from *REP* genes (C) in their normal sequence context in an *ADE2*-tagged 2μm plasmid or (D) chromosomally-integrated at one copy per cell. Total protein was extracted from the yeast at the indicated time points following addition of cycloheximide (A, B) or grown logarithmically without cycloheximide (C, D) and was analyzed by western blotting with anti-Rep1, anti-Rep2, and anti-Pgk antibodies. Hyper-phosphorylated Rep2 (Rep2*) is indicated.

wild-type when expressed with a wild-type partner Rep protein or when both mutant Rep proteins were expressed together. The unaltered half-lives of the mutant Rep proteins is consistent with the lack of effect of the substitutions on the two-hybrid interaction of Rep1 with Rep2 (**Figure 19**). As previously observed for wild-type Rep1, the $Rep1_{3R}$ mutant protein had a shorter half-life when expressed in the absence of Rep2. For Rep2 expressed in the absence of Rep1, although the signal disappeared after the 2 h time point, for both wild-type and the $Rep2_{13R}$ mutant the proportion of Rep2 observed as the 37-kDa hyper-phosphorylated form differed significantly. The $Rep2_{13R}$ mutant was only detected as the 35-kDa species, suggesting that in the absence of Rep1, phosphorylation of sumoylation-deficient Rep2 is inhibited. Alternatively, $Rep2_{13R}$ is more accessible to a phosphatase. While the levels of the hyper-phosphorylated forms of Rep2 were not affected when Rep2 was co-over-expressed with $Rep1_{3R}$ from galactose-inducible promoters, when wild-type Rep2 and $Rep1_{3R}$ were expressed under control of their native promoters from *REP* genes encoded by an *ADE2*-tagged 2μm plasmid, the level of the hyper-phosphorylated form of Rep2 was reduced relative to the amount of the 35-kDa Rep2 species (**Figure 24C)**. Conversely, the levels of hyper-phosphorylated Rep2 approached those of the 35-kDa species when Rep1 was either absent or contained a substitution in a residue (S330) previously reported to be significant for interaction with *STB* (254) (**Figure 24C**). The reduction in hyper-phosphorylated Rep2 relative to the 35-kDa Rep2 species, when Rep2 was expressed with $Rep1_{3R}$ at normal stoichiometric levels from a 2μm plasmid, suggests that sumoylation of Rep1 may promote phosphorylation of Rep2.

Although substitution of the sites targeted for sumoylation did not reduce the half-life of the Rep proteins expressed from the *GAL* promoter, the high level of expression might have masked proteolytic turnover that would alter steady-state levels if the proteins were expressed from their native promoters. Comparison of the levels of wild-type versus $Rep1_{3R}$ and $Rep2_{13R}$ mutant proteins expressed from plasmids was precluded by differences in plasmid copy number resulting from defective partitioning of plasmids encoding mutant Rep proteins (**Figure 23A, and Figure 24C lanes 1 and 4**). To circumvent the plasmid copy-number differences, the *REP1* and *REP2* genes were integrated into the chromosome in a cir^0 yeast strain, and steady-state Rep protein levels were examined by western blotting (**Figure 24D**). The levels of $Rep1_{3R}$ and $Rep2_{13R}$ did not significantly differ from wild-type,

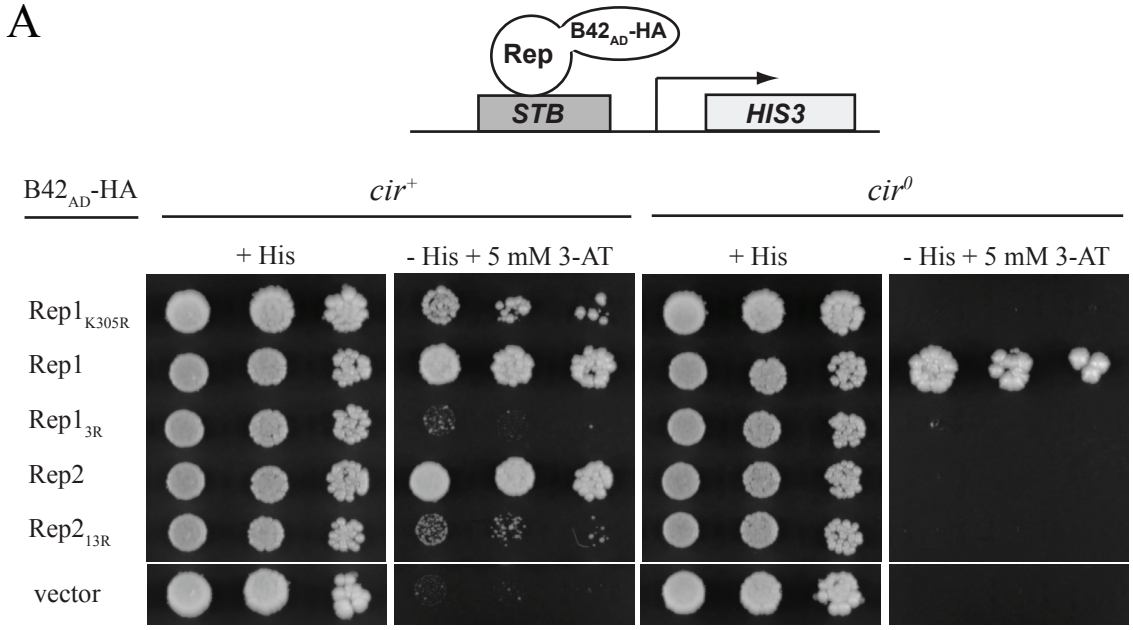
indicating that the lysine substitutions did not affect Rep1 stability and suggesting that no critical ubiquitination sites were destroyed in the process of eliminating Rep1 SUMO-attachment sites. A slight reduction in the levels of both Rep1 and Rep2 was observed when $Rep2_{13R}$ was expressed. This could indicate a slight reduction in stability of the mutant $Rep2_{13R}$ protein, which might have reduced the amount of Rep2 available to chaperone Rep1. Additionally, since the *REP* genes were expressed from their own promoters, reduced Rep protein levels could indicate a slight reduction in transcription of both *REP* genes when Rep2 is sumoylation-deficient, since expression of both *REP* genes is regulated by Rep1 and Rep2.

3.3.3.3 Sumoylation of Both Rep1 and Rep2 Promotes Their Association with the Plasmid Partitioning Locus

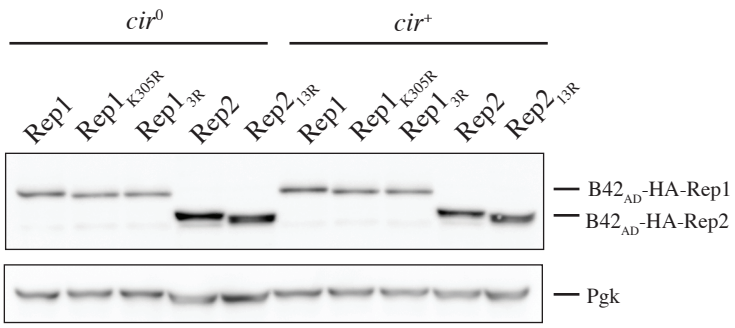
Amino acid substitutions in Rep1 that prevent its recruitment to the 2µm plasmid *STB* locus have previously been shown to result in plasmid mis-segregation (254). To examine the ability of Rep1 and Rep2 sumoylation mutants to associate with *STB in vivo*, I used a one-hybrid assay. Rep proteins were expressed as HA epitope-tagged, viral B42 activation-domain ($B42_{AD}$-HA) fusion proteins in a reporter yeast strain in which the plasmid *STB* locus was integrated in the genome upstream of a *HIS3* histidine biosynthetic reporter gene. In this assay, expression of *HIS3* is dependent on interaction of the respective Rep fusion protein with *STB* and can be assessed by monitoring growth of the yeast on medium lacking histidine and supplemented with 3-aminotriazole (3-AT), a competitive inhibitor of the *HIS3* gene product (229) (**Figure 25A**). Yeast expressing $B42_{AD}$-HA alone (vector) exhibited no growth, indicating that the viral activation domain cannot be recruited to *STB* by itself. As previously reported, in a cir^+ reporter strain, expression of wild-type Rep1 or Rep2 fused to $B42_{AD}$-HA led to robust growth, indicating that both Rep proteins are recruited to *STB* (229). The single K305R point substitution in Rep1 that only slightly diminished its interaction with SUMO in a two-hybrid assay modestly reduced one-hybrid *STB* association, while the substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ that virtually abolished two-hybrid interaction with SUMO severely impaired the abilities of these mutant proteins to associate with *STB* in the one-hybrid assay. I also examined the ability of the Rep proteins to associate with *STB* in an isogenic cir^0 strain where any contribution to the interaction from endogenous plasmid proteins would be lost (**Figure 25A**). The difference in *STB* interaction between wild-type

Figure 25. $Rep1_{3R}$ and $Rep2_{13R}$ mutants are defective for association with *STB*. (A) Yeast one-hybrid reporter strains (*cir*$^+$ or *cir*^{0}) encoding *STB* upstream of a *HIS3* reporter gene were transformed with plasmids encoding the indicated Rep1 and Rep2 proteins fused to $B42_{AD}$-HA, and five-fold serial dilutions of the transformants were spotted onto galactose-containing media to induce expression of fusion proteins. Recruitment of the Rep proteins to *STB* was monitored by growth on medium lacking histidine supplemented with 5 mM 3-aminotriazole. (B) The yeast transformants in (A) were cultured in galactose-containing medium, total protein was extracted and levels of the $B42_{AD}$-HA fusion proteins analyzed by western blotting with anti-HA antibodies. (C) ChIP assays were performed with anti-Rep1, anti-Rep2, or anti-FLAG antibodies and the precipitated DNA was amplified using primers specific for *STB*. ChIP efficiency (avg ± sd from triplicate assays) was calculated as the percent of *STB* DNA amplified from the template DNA after as compared to before immunoprecipitation (left) and ethidium-stained agarose gels of PCR products from a representative assay are shown (right). "Input" PCR reactions contained 40% of the corresponding template DNA used for immunoprecipitation.

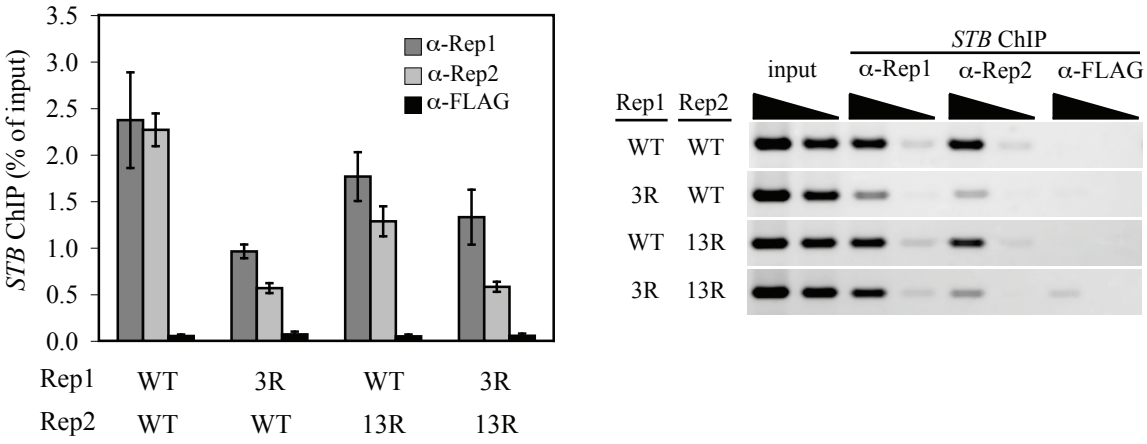
A


Rep
B42AD-HA
STB
HIS3
B42AD-HA
cir+
cir0
+ His
- His + 5 mM 3-AT
+ His
- His + 5 mM 3-AT
Rep1K305R
Rep1
Rep13R
Rep2
Rep213R
vector

B


cir0
cir+
Rep1
Rep1K305R
Rep13R
Rep2
Rep213R
Rep1
Rep1K305R
Rep13R
Rep2
Rep213R
B42AD-HA-Rep1
B42AD-HA-Rep2
Pgk

C


STB ChIP (% of input)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
α-Rep1
α-Rep2
α-FLAG
Rep1 WT 3R WT 3R
Rep2 WT WT 13R 13R
STB ChIP
input
α-Rep1
α-Rep2
α-FLAG
Rep1 Rep2
WT WT
3R WT
WT 13R
3R 13R

Rep1 and the two sumoylation-deficient Rep1 mutants was more pronounced in the *cir*^{0} reporter strain. The Rep1-K305R mutant expressed as a B42$_{AD}$-HA fusion protein did not result in growth of the reporter strain, suggesting that loss of this single sumoylation site is sufficient to significantly reduce association of Rep1 with the *STB* locus in the absence of native plasmid proteins. Expression of native Rep1 or Rep2 individually from a second plasmid in the *cir*^{0} reporter strain also expressing B42$_{AD}$-HA-Rep1$_{K305R}$ or B42$_{AD}$-HA-Rep1$_{3R}$ did not result in any growth, suggesting that the combined presence of endogenous Rep1 and Rep2 proteins was required for the limited association of the sumoylation-deficient Rep1 mutants with *STB* observed in the *cir*$^+$ reporter strain (data not shown).

Interestingly, although wild-type Rep2 expressed as a B42$_{AD}$-HA fusion protein could activate the *STB*-driven *HIS3* reporter gene in a *cir*$^+$ strain, no growth was observed for the *cir*^{0} reporter strain, consistent with observations from our lab that association of Rep2 with *STB* requires endogenous plasmid proteins (April Saunders, Dalhousie University, unpublished results).

A reduction in the steady-state levels of the mutant Rep1 and Rep2 fusion proteins could also explain the reduced one-hybrid association with *STB*. However, no significant differences in Rep fusion protein levels in the *cir*$^+$ or *cir*^{0} reporter strains were observed, (**Figure 25B**) suggesting that reduced activation of the reporter gene by the mutant Rep fusion proteins was a consequence of reduced interaction with the *STB* sequence in the promoter rather than being due to lower abundance.

To determine whether Rep1$_{3R}$ and Rep2$_{13R}$ are impaired for interaction with *STB* in a native context, the efficiency of chromatin immunoprecipitation (ChIP) of the *STB* locus with anti-Rep1 or anti-Rep2 antibodies was examined for 2μm-based plasmids encoding wild-type or mutant Rep proteins (**Figure 25C**). Consistent with results of the one-hybrid assays, *STB* DNA co-immunoprecipitated less efficiently when the plasmid encoded Rep1$_{3R}$ or Rep2$_{13R}$ rather than wild-type Rep1 and Rep2. Taken together, the results of the one-hybrid and ChIP assays indicate that defects in the inheritance of plasmids encoding Rep1$_{3R}$ and Rep2$_{13R}$ mutants may be due to impaired interaction of Rep1 and Rep2 with the plasmid partitioning locus. *STB* was also less efficiently co-immunoprecipitated with anti-Rep2 antibodies in yeast expressing Rep1$_{3R}$, suggesting that association of Rep2 with *STB* is, in part, dependent on the stable association of Rep1 with *STB*. In contrast, there was no significant difference

observed in the amount of *STB* immunoprecipitated by anti-Rep1 antibodies when the plasmid encoded wild-type Rep1 and the $Rep2_{13R}$ mutant compared to when Rep1 and Rep2 were both wild-type, suggesting Rep1 association with *STB* was not affected by the substitutions in $Rep2_{13R}$.

3.3.3.4 Rep2 Depends on Rep1 for Robust Association with *STB*

The impaired association of Rep2 with *STB* when Rep1 was absent (**Figure 25A**) or when Rep1 interaction with *STB* was reduced due the lysine substitutions in $Rep1_{3R}$ (**Figure 25C**) suggested that Rep2 may depend on interaction with Rep1 for stable association with *STB*. Although previous work has shown that Rep2, when over-expressed, is able to interact with *STB* in the absence of Rep1 (151), the degree to which Rep1 and Rep2 associate with *STB* in the absence of the partner protein has not been examined. To investigate this issue, I assessed the interaction of $B42_{AD}$-HA-Rep2 with *STB* in a cir^0 one-hybrid reporter strain in which untagged Rep1 or Rep2 were expressed individually from a *GAL* promoter (**Figure 26A**). In this assay, expression of Rep1, but not Rep2, promoted association of $B42_{AD}$-HA-Rep2 with *STB*. To examine the dependence of Rep1 and Rep2 on each other for their association with *STB* in a native context, I performed ChIP assays using yeast transformed with an *ADE2*-tagged 2μm plasmid encoding Rep1 and Rep2, or derivatives that lacked either the *REP1* or *REP2* gene (**Figure 26B**). While *STB* did co-immunoprecipitate with anti-Rep2 antibodies when Rep1 was absent, the yield was significantly lower than when Rep1 was present. In contrast, absence of Rep2 did not reduce association of Rep1 with *STB*. These results suggest that *STB*-bound Rep1 may promote more stable association of Rep2 with the plasmid partitioning locus.

3.3.3.5 Impaired Rep1 Sumoylation Alters Rep1 and Rep2 Sub-Nuclear Localization.

The multiple copies of the 2μm plasmid are organized in a small number of nuclear foci that co-localize with Rep1 and Rep2 (190, 230). The impaired interaction of $Rep1_{3R}$ and $Rep2_{13R}$ with the plasmid *STB* locus observed in the one-hybrid and ChIP assays suggested that sumoylation may contribute to Rep protein sub-nuclear localization. To assess this idea, I used indirect immunofluorescence to compare localization of wild-type Rep proteins and the $Rep1_{3R}$ and $Rep2_{13R}$ mutants expressed from *ADE2*-tagged 2μm plasmids (**Figure 27A**). As previously reported, wild-type Rep1 was present in distinct nuclear foci (190, 191, 230)

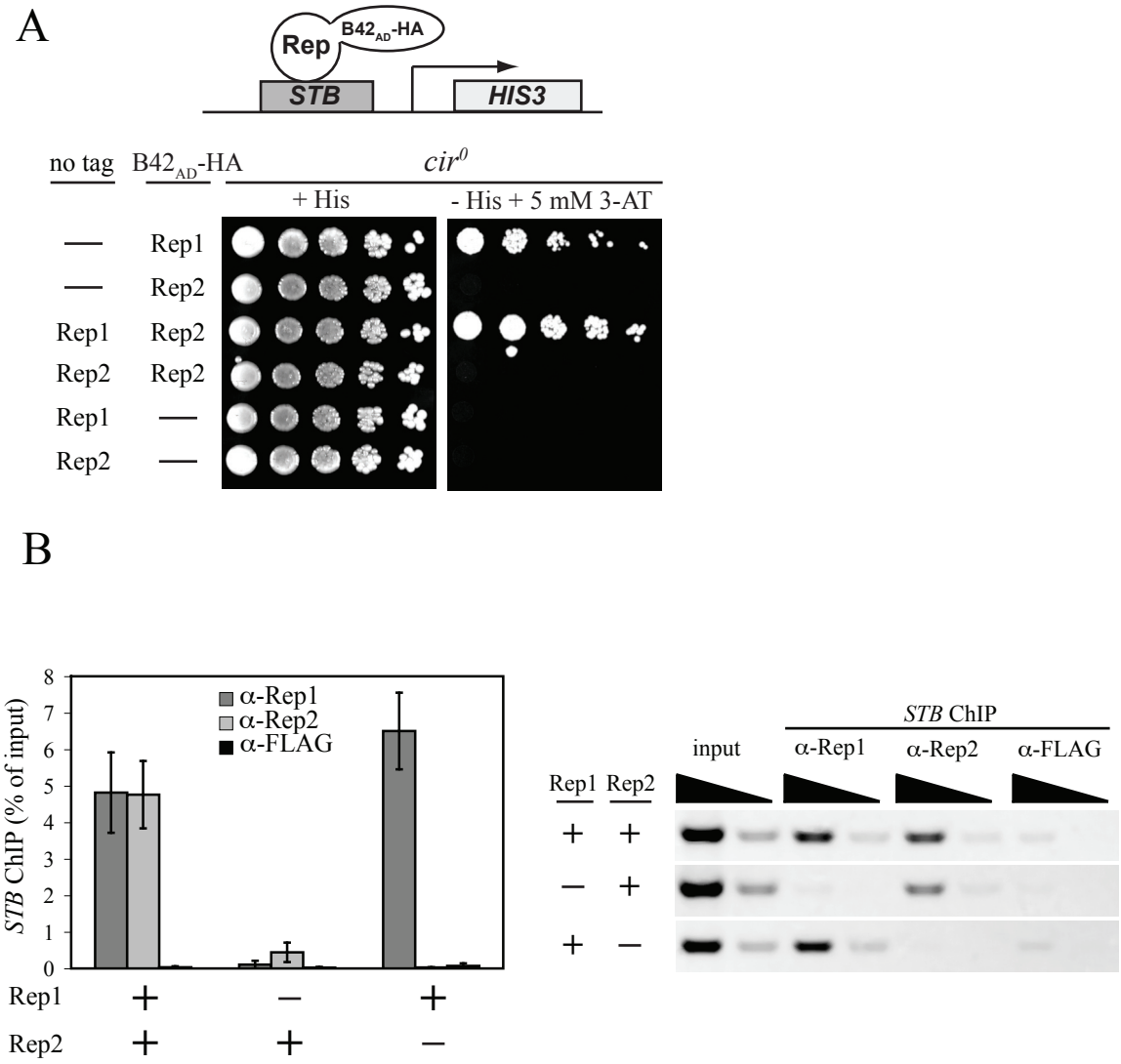
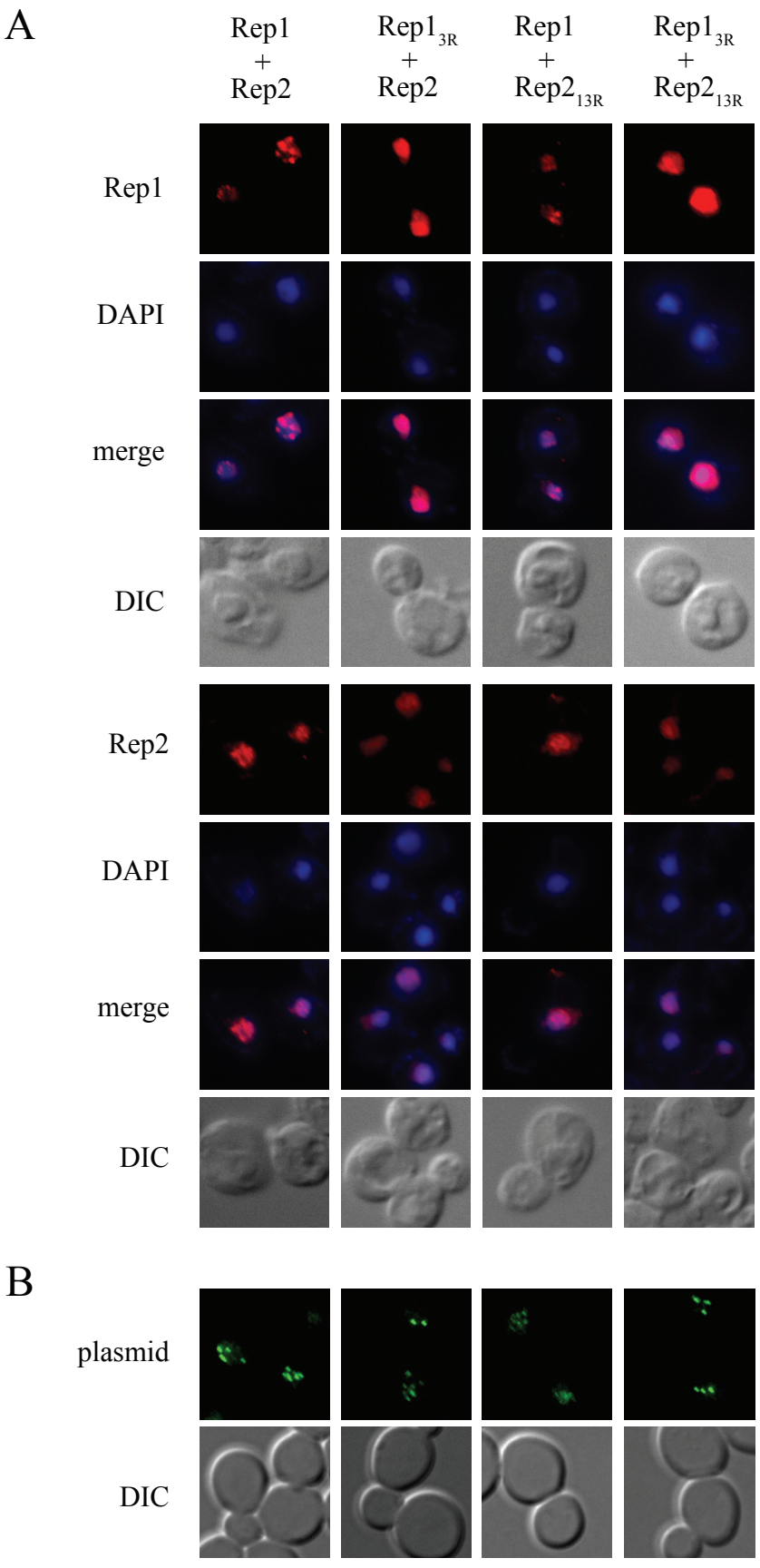


Figure 26. Stable association of Rep2 with *STB* depends on Rep1. (A) A *cir*^{0} yeast one-hybrid reporter strain was co-transformed with single-copy plasmids encoding untagged and B42$_{AD}$-HA-tagged Rep1 or Rep2, and expression of the *STB*-driven *HIS3* reporter gene monitored by growth on solid media containing galactose as described in the legend to Figure 25. (B) Chromatin was extracted from yeast expressing Rep1, Rep2, or both Rep1 and Rep2 from a 2μm-based plasmid and ChIP was performed and analyzed as detailed in the legend to Figure 25.

Figure 27. Substitutions in $Rep1_{3R}$ cause mislocalization of Rep1 and Rep2, but do not disrupt localization of plasmids in nuclear foci. (A) Spheroplasts were prepared from *cir*^{0} yeast expressing wild-type or mutant Rep1 and Rep2 from an *ADE2*-tagged 2μm plasmid and the Rep proteins were visualized by indirect immunofluorescence. Bulk chromatin was visualized by DAPI staining and cells by light microscopy (DIC). (B) Yeast were co-transformed with an *ADE2*-tagged 2μm plasmid encoding the indicated Rep1 and Rep2 alleles and a plasmid containing the *STB* locus and 256 *lacO* repeats. Plasmid localization was visualized by fluorescence microscopy following induction of GFP-LacI repressor fusion-protein expression.


A
Rep1 + Rep2
Rep1$_{3R}$ + Rep2
Rep1 + Rep2$_{13R}$
Rep1$_{3R}$ + Rep2$_{13R}$
Rep1
DAPI
merge
DIC
Rep2
DAPI
merge
DIC
B
plasmid
DIC

(**Figure 27A, column 1**); however, $Rep1_{3R}$ showed diffuse nuclear staining, suggesting that, consistent with the results of the one-hybrid and ChIP assays, sumoylation is required for Rep1 localization to the distinct sub-nuclear plasmid-containing domains (**Figure 27A, columns 2 and 4**). Wild-type Rep2, when co-expressed with wild-type Rep1, exhibited a less sharply punctate but still uneven nuclear staining pattern as has been previously reported (190, 230) (**Figure 27A, column 1**). However, Rep2 expressed with $Rep1_{3R}$ displayed a more uniform, pan-nuclear staining pattern (**Figure 27A, column 2**), consistent with results of ChIP assays that demonstrated that association of Rep2 with *STB* was impaired when Rep2 was co-expressed with $Rep1_{3R}$ (**Figure 25C**). When $Rep2_{13R}$ was expressed with wild-type Rep1 (**Figure 27A, column 3**), the Rep2 punctate staining pattern was similar to that of wild-type Rep2, suggesting Rep2 is not dependent on being sumoylated for its nuclear distribution. The respective staining patterns of Rep1 and Rep2 when both were sumoylation-deficient (**Figure 27A, column 4**) were similar to those observed when only Rep1 was mutant.

3.3.3.6 Deficient Rep1 Sumoylation Does Not Disrupt Plasmid Foci.

The mislocalization of Rep1 and Rep2 when Rep1 was sumoylation-deficient prompted us to examine plasmid localization in the presence of $Rep1_{3R}$ and $Rep2_{13R}$ mutants. To visualize 2μm plasmid foci, a 2μm reporter plasmid containing 256 *lac* operator sequences was introduced in yeast that expressed a GFP-tagged LacI repressor protein (**Figure 27B**) (230). As has been previously reported, the plasmid was localized into a small number of distinct nuclear foci in cells expressing wild-type Rep1 and Rep2 (191, 230). These foci were not disrupted even when both Rep1 and Rep2 contained the lysine-to-arginine substitutions that blocked SUMO interaction, suggesting that clustering of plasmid copies is not dependent on Rep protein sumoylation. Although the number of foci was not influenced by Rep protein sumoylation, we did observe that foci were generally brighter when Rep1 was sumoylation-deficient. This is likely a consequence of increased copy number of the lacO-*STB* plasmid due to defective partitioning when Rep1 sumoylation is impaired, and is consistent with the increase in copy number observed for marker-tagged 2μm plasmids encoding $Rep1_{3R}$ (**Figure 23A**).

3.3.3.7 Translational Fusion of SUMO to Rep1 and Rep2 Impairs Plasmid Inheritance

In some cases, the function of a sumoylation-deficient mutant protein can be restored by mimicking constitutive sumoylation by translational fusion of SUMO to the mutant protein. To test whether fusion of SUMO to $Rep1_{3R}$ and $Rep2_{13R}$ could restore their ability to mediate plasmid partitioning, DNA encoding mature SUMO was inserted in-frame upstream of the wild-type or mutant *REP1* or *REP2* ORFs in *ADE2*-tagged 2μm plasmids. However, plasmid encoding SUMO-Rep protein fusions were not inherited as effectively as those encoding untagged Rep proteins, suggesting that fusion of SUMO to either wild-type or mutant Rep protein impaired plasmid partitioning function (data not shown).

3.4 Investigating Regulation of *FLP* Expression by Plasmid Proteins

In addition to their function in 2μm plasmid partitioning, Rep1 and Rep2 also contribute to plasmid maintenance through their repression of 2μm plasmid-encoded genes (162, 180, 200, 228). Repression of *FLP* is particularly important for preventing hyper-amplification of the plasmid, which is toxic to the host and perturbs plasmid inheritance (162). The mechanism of Rep-protein-mediated repression of *FLP*, and antagonism of this repressive activity by the plasmid-encoded Raf protein, was investigated.

3.4.1 Rep protein-mediated repression of *FLP*

3.4.1.1 Rep1 and Rep2 Associate with the *FLP* Promoter and Depend on Sumoylation for Stable Association

While plasmid partitioning is dependent on association of Rep1 and Rep2 with the *STB*-proximal repeats in the 2μm plasmid, the Rep proteins may also bind at additional locations in the plasmid. Altered nuclease sensitivity of regions in the plasmid other than *STB* when the Rep proteins were absent suggested that the Rep proteins might influence expression of plasmid genes by binding to sites in their promoters (227).

FLP and *REP2* are divergently transcribed genes separated by just 372 bp of DNA. To test for association of Rep1 and Rep2 with the region of the 2μm plasmid spanning the *FLP* and *REP2* promoters, ChIP assays were performed. A cir^0 yeast strain was transformed with an *ADE2*-tagged 2μm plasmid and the amount of the *FLP/REP2* promoter DNA

immunoprecipitated by anti-Rep1 and anti-Rep2 antibodies was examined (**Figure 28**). The *FLP/REP2* promoter region specifically was immunoprecipitated with both anti-Rep1 and anti-Rep2 antibodies, indicating that both Rep proteins were likely associated somewhere within this region.

Having established that Rep1 and Rep2 containing lysine substitutions that blocked their two-hybrid interaction with SUMO (**Figure 19**) were impaired for stable association with *STB* (**Figure 25**), ChIP assays were performed to assess whether $Rep1_{3R}$ and $Rep2_{13R}$ were also impaired for association with the *FLP/REP2* promoter region (**Figure 28**). As observed for *STB*, the $Rep1_{3R}$ and $Rep2_{13R}$ mutants were defective for interaction with the *FLP/REP2* promoter. Association of wild-type Rep2 with the *FLP/REP2* promoter was also reduced when Rep1 was mutant ($Rep1_{3R}$), suggesting that, as was demonstrated for association of Rep2 with *STB* (**Figure 26**), association of the Rep2 with the *FLP/REP2* promoter is dependent on stable association of Rep1.

3.4.1.2 Deletion of a Putative Rep Protein Binding Element Upstream of *FLP* Leads to Plasmid Hyper-Amplification

The finding that Rep1 and Rep2 associated with the region of the plasmid between *FLP* and *REP2* raised the possibility that the Rep proteins might also bind to the region between the divergently transcribed *REP1* and *RAF* genes (**Figure 29**), since all four of these genes are subject to Rep protein-mediated repression (162, 180, 200, 228). The molecular basis for association of Rep1 and Rep2 with DNA *in vivo* is not known. The Rep proteins could bind to DNA directly, or could interact with host proteins that are directly bound to DNA. In either case, if the association with DNA were sequence-specific, a common sequence would be expected to be found in the *STB* repeats, as well as in the promoter regions for all four plasmid genes. A 9-bp sequence (5'-TGCATTTTT) has previously been identified as occurring in all of these regions of the 2μm plasmid (200, 228). The sequence, hereafter termed a putative Rep Binding Element (*RBE*) is found five times in *STB*-proximal, twice in the *FLP/REP2* promoter region, once in the *REP1/RAF* promoter region, and also once between the origin and *STB*-proximal. The locations of the nine *RBE* sequences in the 2μm plasmid are indicated in **Figure 29**. The possibility that the Rep proteins specifically recognize this sequence (or its complement) has not been investigated, perhaps because Rep1

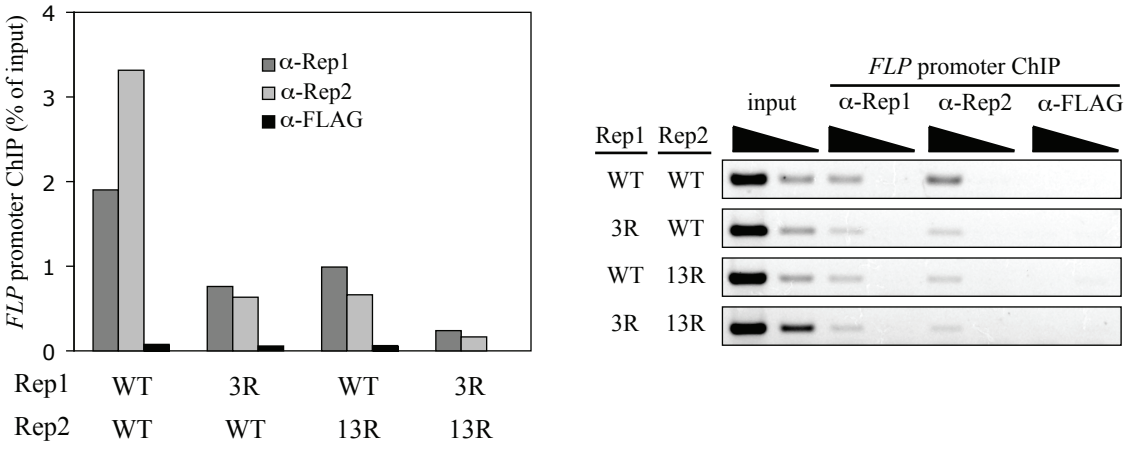


Figure 28. $Rep1_{3R}$ and $Rep2_{13R}$ mutants are impaired for association with the *FLP* promoter. A *cir*^{0} yeast strain was transformed an *ADE2*-tagged 2μm plasmid encoding the indicated Rep1 and Rep2 alleles. Chromatin was extracted from the transformants and ChIP assays were performed as described in Figure 25 using primers specific for the *FLP* promoter region.

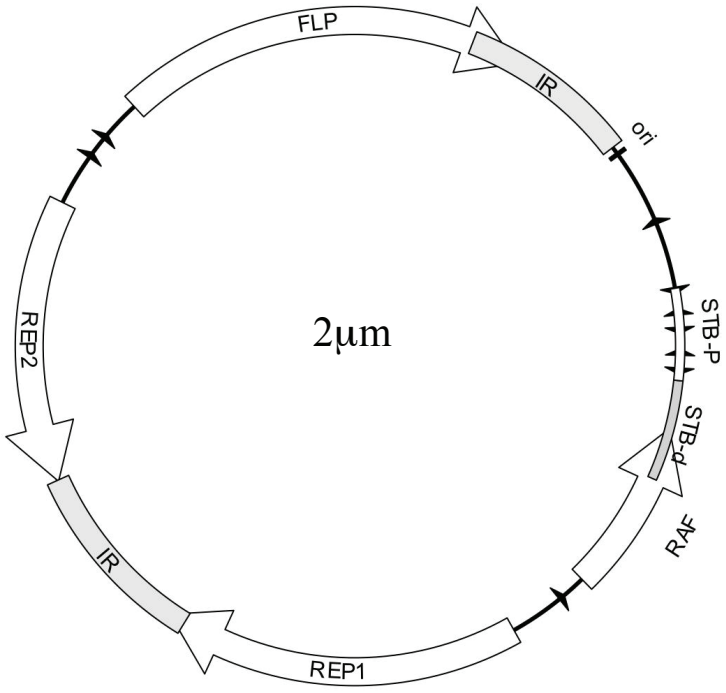


Figure 29. Locations of *RBE* elements in the 2μm plasmid. The locations and orientations of the nine *RBE* sequence elements (5'-TGCATTTTT) in the 2μm plasmid (B form) are indicated by black arrowheads.

does not display DNA-binding activity *in vitro*, and while Rep2 has been shown to bind to DNA *in vitro* using a southwestern assay, sequence specificity has not been demonstrated (192).

The *TRP1* marker-tagged 2μm plasmid pHR5 was used for assessing function of the two *RBE* elements in the *FLP/REP2* promoter region (see **Figure 30A** for a map). Unlike the *ADE2*- and *KanMX6*-tagged 2μm plasmids previously used for analyzing plasmid partitioning, pHR5 has a functional Flp amplification system, with copy number dependent on normal *FLP* gene expression. The *RAF* gene was disrupted in pHR5 so that the effects of Raf, which functions to alleviate Rep protein-mediated repression, would not mask any loss of Rep protein function.

To examine whether the *RBE* elements are required for repression of *FLP*, pHR5 derivatives were created that were deleted for the putative Rep protein binding element closest to *FLP* (*RBE1*), the element closest to *REP2* (*RBE2*), or both elements simultaneously. Loss of binding of Rep1 and Rep2 to this region would be expected to alleviate repression of *FLP*, which would be predicted to cause plasmid hyper-amplification. Hyper-amplification of the 2μm plasmid due to increased expression of *FLP* can be phenotypically observed, as yeast containing elevated 2μm plasmid copy number grow poorly and form colonies with uneven "nibbled" edges (162). A cir^0 yeast strain was transformed with pHR5 or its derivatives, and transformants were streaked to solid medium to assess colony size and morphology (**Figure 30B**).

Yeast transformed with pHR5 formed large, round colonies with smooth edges. However, yeast transformed with pHR5 containing a deletion in *RBE1* grew poorly and formed nibbled colonies, reminiscent of those observed when *FLP* was over-expressed in a cir^+ yeast strain (162). The formation of nibbled colonies suggests that the copy number of pHR5 had drastically increased when *RBE1* was deleted, consistent with Rep1 and Rep2 being unable to repress *FLP*.

In contrast to when the *RBE1* was deleted, when *RBE2* was deleted, yeast formed large colonies with smooth edges, suggesting that plasmid copy number was not significantly elevated and that *RBE2* may not be required for repression of *FLP*. Yeast transformed with derivatives of pHR5 lacking both *RBE1* and *RBE2* appeared identical to those containing plasmids deleted for just *RBE1*.

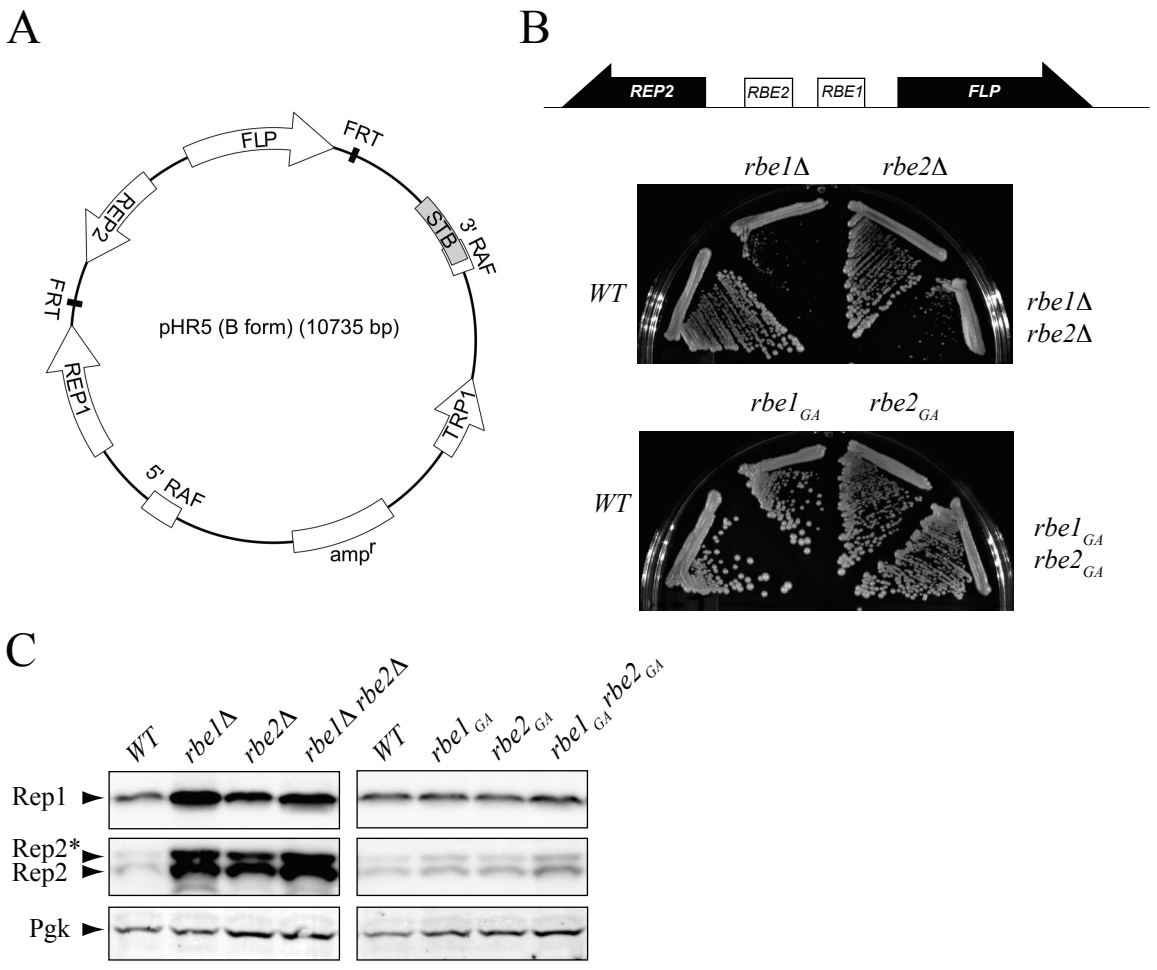


Figure 30. Effects of mutation of *RBE* elements in the *FLP/REP2* intergenic region on plasmid copy number and Rep protein levels. (A) Map of the amplification-competent *TRP1*-tagged plasmid pHR5 in which either one or both of the two *RBE* elements between the *FLP* and *REP2* ORFs was either deleted (*rbe*Δ) or contained a G-to-A substitution (rbe_{GA}). These plasmids were introduced into cir^0 *trp1* yeast and transformants were (B) assayed for their ability to form colonies on solid medium lacking tryptophan and (C) grown in liquid medium lacking tryptophan, after which protein was extracted and analyzed by western blotting with antibodies specific for Rep1, Rep2, and Pgk.

3.4.1.3 Deletion of *RBE2* in a Marker-Tagged 2μm Plasmid Leads to Modest Increases in Rep1 and Rep2 Protein Levels

To determine whether the nibbled colony morphology observed when the *RBE1* element was deleted was likely to be due to elevated plasmid copy number, the levels of both Rep2 and Rep1 were examined by western blotting analysis of protein extracted from yeast transformed with pHR5 or its *rbe1*Δ*, rbe2*Δ, or *rbe1*Δ *rbe2*Δ derivatives (**Figure 30C**). For protein extracted from yeast containing pHR5 with only *RBE1* or both *RBE1* and *RBE2* deleted, the levels of Rep1 and Rep2 were significantly higher than when only *RBE2* was deleted or when the *FLP/REP2* promoter region was wild-type. This increase is consistent with the copy number of pHR5 being higher for the *rbe1*Δ plasmids, as suggested by the poor growth and nibbled colony morphology of the yeast transformants. When *RBE2* alone was deleted, the levels of both Rep1 and Rep2 were also elevated, though not to the extent that they were when *RBE1* was deleted. This suggests that either the copy number of pHR5 was higher when *RBE2* is deleted, and/or expression of *REP2* (per plasmid copy) was elevated. An increase in Rep2 levels would be expected to lead to a subsequent increase in Rep1 levels, since Rep2 stabilizes Rep1 post-translationally (see **Section 3.1.2**). Copy number measurements will be needed to discern between these possibilities.

3.4.1.4 Disrupting the Short Palindrome in *RBE* Does Not Affect Function

The TGCA palindrome in the *RBE* sequences could form a secondary structure or be a required motif enabling the *RBE* to be bound by the Rep proteins and mediate repression. To investigate this possibility, the palindrome was disrupted by a single G-to-A substitution in *RBE1* and/or *RBE2* in pHR5. These plasmids were transformed into yeast, and colony morphology and Rep protein levels were examined as above (**Figure 30B-C**). Unlike deletion of *RBE1*, the G-to-A substitution in *RBE1* did not cause yeast transformants to form nibbled colonies, nor were Rep protein levels significantly elevated when yeast were transformed with pHR5 encoding the single nucleotide change in *RBE1* and/or *RBE2*. This suggests that the palindromic feature of the *RBE* elements is not important for *RBE* function. Further work is needed to identify the nucleotides that are critical for function of the 9-bp *RBE* sequence.

3.4.2 Examining Alleviation of Rep Protein-Mediated Repression by Raf

The 2μm plasmid Raf protein has been proposed to function in fine-tuning of plasmid copy-number control, suggested by its ability to antagonize Rep-mediated repression of the *FLP* gene. Over-expression of *RAF* has been shown to lead to rapid derepression of the *FLP* gene, causing drastic increases in 2μm plasmid copy number (162). The mechanism of Raf-mediated derepression of *FLP* was investigated.

3.4.2.1 Over-Expression of Raf, or Combined Absence of Rep1 and Rep2, Activates Production of a Longer *FLP* Transcript

As a first step to studying the function of Raf, changes in the levels of *FLP* mRNA caused by over-expression of Rep1 and Rep2, with or without simultaneous expression of Raf, were examined. A cir^+ yeast strain was transformed with a *CEN/ARS* plasmid encoding galactose-inducible *RAF*, and transformants were grown in medium containing galactose to activate expression of *RAF*. Total RNA was extracted from yeast shortly following induction of *RAF*, and analyzed by northern blotting using a double-stranded DNA probe homologous to a ~300-bp region of the *FLP* ORF (**Figure 31A**).

The *FLP* probe detected a single major transcript of ~1300 nt that corresponds to the reported size of the major *FLP* transcript for RNA extracted from cir^+ yeast in the absence of Raf over-expression (210). This species was not detected in RNA from cir^0 yeast. Surprisingly, in addition to the 1300-nt transcript, a ~1400-nt transcript was detected when Raf, Rep1, and Rep2 were over-expressed. The 1400-nt transcript could reflect use of an alternative transcription start site upstream of that reported for the 1300-nt transcript, an alternate transcription termination site, or, because the probe was double-stranded, a novel anti-sense mRNA. However, given that over-expression of Raf did not significantly alter the level of the 1300-nt sense transcript, and that elevated Flp activity has been reported when Raf is over-expressed (162), the 1400-nt RNA is more likely to be a novel *FLP* sense transcript.

Next, I tested whether expression of the 1400-nt transcript induced by Raf was dependent on the presence of Rep1 and Rep2. To test this possibility, I analyzed *FLP* transcripts by northern blotting of total RNA extracted from cir^0 yeast transformed with pHR5ΔR1R2, an amplification-competent *TRP1*-tagged 2μm-based plasmid encoding a

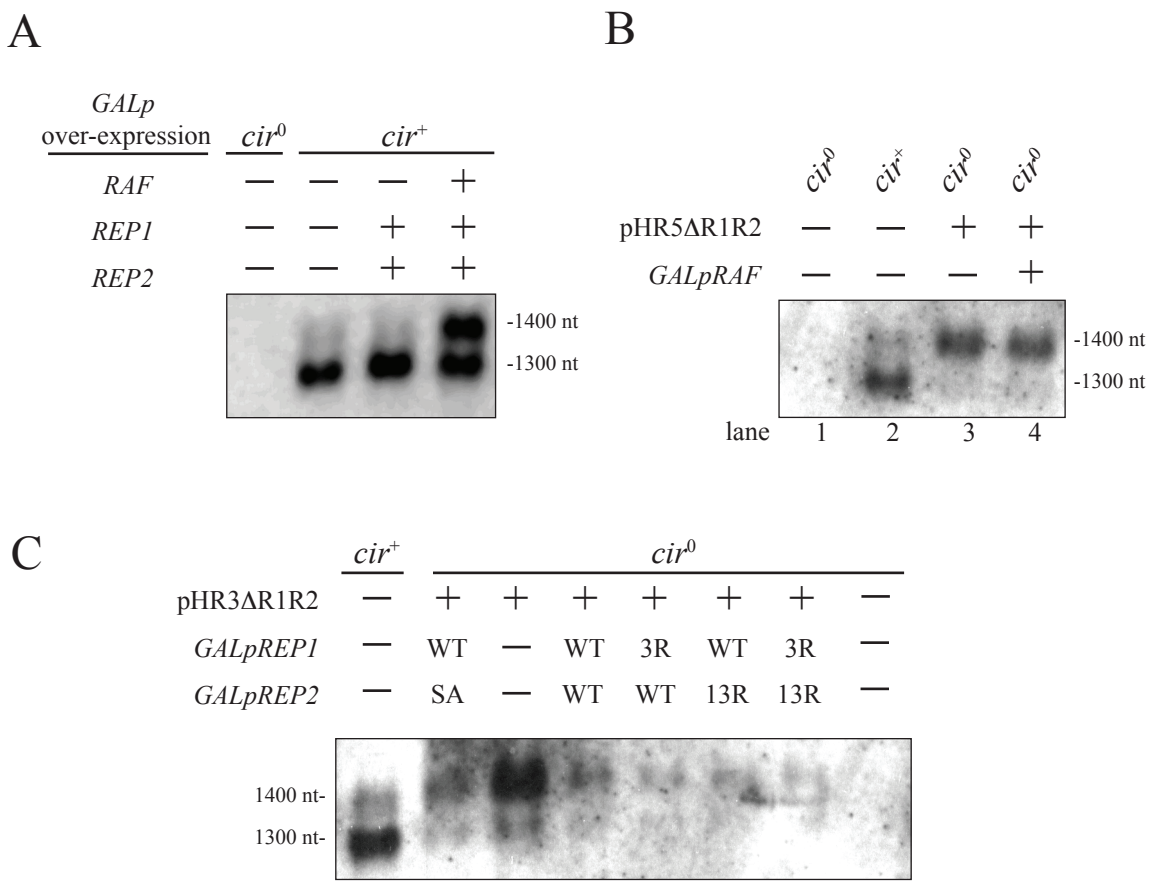


Figure 31. Effects of plasmid protein levels on *FLP* expression. (A) Single-copy plasmids encoding the indicated 2μm plasmid genes under the control of a galactose-inducible promoter (*GALp*), or without a gene inserted (-) were introduced into *cir*^{0} or *cir*$^+$ yeast, and transformants were grown in galactose for 3 h. Total RNA was extracted and levels of *FLP* mRNA were examined by northern blotting and hybridization with a DIG-labelled PCR product encoding residues 449-727 of the *FLP* ORF. The approximate sizes (in nucleotides, nt) of the transcripts detected are indicated. (B) Yeast were transformed with a *TRP1*-tagged 2μm plasmid encoding *FLP* but lacking *REP1*, *REP2*, and *RAF* genes (pHR5ΔR1R2, lanes 3-4) and a single-copy plasmid encoding the *GAL*-driven *RAF* (lane 4), or with no gene inserted (-) (lane 3). Total RNA was extracted from the transformants and from untransformed *cir*$^+$ and *cir*^{0} yeast cultured under the same conditions was examined by northern blotting following induction of *RAF* as in (A). (C) Yeast were transformed with a *URA3*-tagged 2μm plasmid encoding *FLP* but lacking *REP1*, *REP2*, and *RAF* genes (pHR3ΔR1R2), and also with single-copy plasmids encoding the indicated wild-type or mutant alleles of *REP1* and *REP2* under control of a galactose-inducible promoter. The level of *FLP* mRNA in the yeast transformants was examined by northern blotting as described in (A) following galactose-induction of *REP* gene expression.

functional *FLP* gene but lacking functional *REP1*, *REP2*, and *RAF* genes. This plasmid, which was expected to be inefficiently partitioned due to the absence of Rep1 and Rep2, was maintained by culturing yeast in medium lacking tryptophan. Total RNA was extracted from the transformants and levels of *FLP* mRNA were analyzed by northern blotting (**Figure 31B**). In these transformants, only the 1400-nt *FLP* transcript was detected, and the level of this transcript was not further increased by over-expression of Raf, consistent with the previously reported finding that Raf is an anti-repressor and not a transcriptional activator (162). These results further support the view that the 1400-nt transcript is a relevant functional *FLP* transcript, since its increase in the absence of the Rep proteins correlates with the increase in Flp activity when Rep protein levels are low (180).

3.4.2.2 Repression of *FLP* is Not Affected when Phosphorylation of Rep2 or Sumoylation of Rep1 and Rep2 is Impaired

To see whether post-translational modifications to the Rep proteins affect Rep protein repression of the 1400-nt *FLP* transcript, $Rep1_{3R}$, $Rep2_{13R}$, and $Rep2_{SA}$ mutants were over-expressed from *CEN/ARS* plasmids under the control of galactose-inducible promoters in a cir^0 yeast strain also containing an amplification-competent *URA3*-tagged 2μm plasmid that lacked functional *REP1*, *REP2*, and *RAF* genes (pHR3ΔR1R2). Expression of the *REP* genes was briefly induced and the level of *FLP* mRNA was analyzed by northern blotting, with parallel analysis of RNA extracted for cir^+ and cir^0 yeast (**Figure 31C**). For RNA from the native plasmid, as previously observed (**Figure 31A**), the 1300-nt species was the major transcript. For RNA from pHR3ΔR1R2, the 1400-nt species was the major transcript in the absence of Rep1 and Rep2 being expressed in *trans*. When Rep1 and Rep2 were over-expressed, expression of the 1400-nt transcript from the pHR3ΔR1R2 plasmid was efficiently repressed. Expression of the 1400-nt transcript was also repressed by the mutant Rep proteins, suggesting that deficient sumoylation of $Rep1_{3R}$ and $Rep2_{13R}$, and loss of phosphorylation of $Rep2_{SA}$ did not affect Rep protein-mediated repression of *FLP*.

3.4.2.3 Over-Expression of *RAF* Causes Accumulation of Rep1 and Rep2 in Yeast Containing the Native 2μm Plasmid

Northern blotting analyses indicated that Raf selectively promotes upregulation of a 1400-nt *FLP* transcript that is normally repressed by Rep1 and Rep2. A clue toward the mechanism of Raf activity was obtained by current doctoral student Mary McQuaid, who demonstrated that Raf interacts with both Rep1 and Rep2 independently in a two-hybrid assay (unpublished results). One possible mechanism for the alleviation of Rep-mediated repression is that Raf promotes degradation of Rep1 and/or Rep2. The effect of Raf over-expression on Rep1 and Rep2 protein levels was therefore examined. A cir^+ yeast strain was transformed with a *CEN/ARS* plasmid encoding galactose-inducible *RAF*, and Rep1 and Rep2 levels were analyzed by western blotting following induction of *RAF* (**Figure 32A, lanes 1-2**). Rep1 and Rep2 protein levels were both significantly elevated when Raf was over-expressed. However, since Raf over-expression increases the copy number of the 2μm plasmid by alleviating Rep protein-mediated repression of *FLP*, the increase in Rep1 and Rep2 levels might merely reflect elevated 2μm plasmid copy number, rather than altered Rep protein stability or *REP* gene expression.

3.4.2.4 Over-Expression of *RAF* Increases the Levels of Rep1, but Not Rep2, Expressed from 2μm Plasmids Lacking an Amplification System

To test whether over-expression of *RAF* affected the levels of Rep1 and Rep2 expressed from a 2μm plasmid that lacked an amplification system, a cir^0 yeast strain was transformed with a *CEN/ARS* plasmid encoding galactose-inducible *RAF*, and a flp^- *ADE2*-tagged 2μm plasmid (pAS4). Expression of *RAF* was briefly induced, and Rep protein levels were analyzed by western blotting (**Figure 32A, lanes 4-5**). Raf over-expression modestly increased the level of Rep1, but not of Rep2. This finding suggested that high levels of Raf upregulate expression of the *REP1* gene and/or increase Rep1 post-translational stability.

3.4.2.5 Raf Over-Expression Stabilizes Rep1, and Inhibits Phosphorylation of Rep2

Given that Raf expression can indirectly regulate 2μm plasmid copy number, and possibly also expression of the *REP1* and *REP2* genes, for the analysis of the effect of Raf over-expression on Rep1 and Rep2 post-translational stability the *REP* ORFs were placed

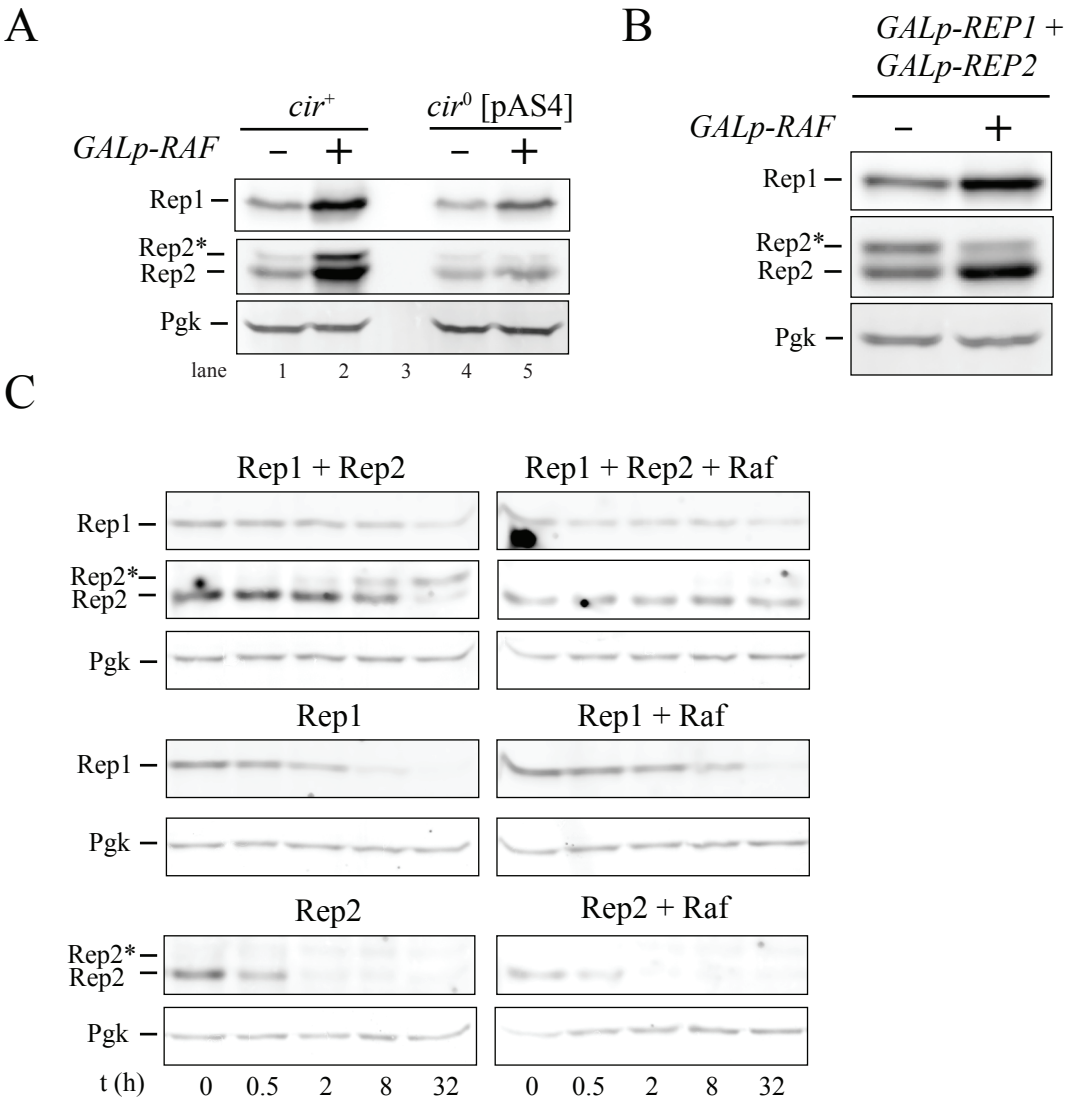


Figure 32. Effects of *RAF* over-expression on Rep1 and Rep2 protein levels and post-translational stabilities. A *CEN/ARS* plasmid directing expression of galactose-inducible *RAF* (*GALpRAF*), or with no gene inserted (-), was transformed into yeast containing (A) the native 2μm plasmid (lanes 1-2) or the *flp*$^{-}$ *ADE2*-tagged 2μm plasmid pAS4 (lanes 4-5), or (B, C) co-transformed with a second *CEN/ARS* plasmid encoding galactose-inducible *REP1* (*GALpREP1*) and/or *REP2* (*GALpREP2*). Yeast were grown in medium containing galactose and protein was extracted (A, B) during logarithmic growth or (C) at the indicated time points following addition of cycloheximide. Protein extracts were analyzed by western blotting with antibodies against Rep1, Rep2, and Pgk.

under control of heterologous galactose-inducible promoters on non-2μm-based plasmids. The plasmid was introduced into *cir*^{0} yeast along with a *CEN/ARS* plasmid encoding galactose-inducible *RAF*, or empty vector (**Figure 32B**). Rep1 levels were elevated when *RAF* was over-expressed, suggesting that interaction of Raf with Rep1 may protect Rep1 from degradation. For Rep2, when *RAF* was over-expressed there were altered proportions of the two major Rep2 species, with less observed in the hyper-phosphorylated form, making it difficult to determine whether there had been a significant change in total Rep2 levels. The reduced level of the 37-kDa form of Rep2 suggests that interaction of Raf with Rep2 might protect Rep2 from a kinase. Consistent with this hypothesis, mapping of Rep2 domains sufficient for interaction with Raf identified a region of Rep2 that contains the sites phosphorylated by protein kinase CK2 (Mary McQuaid, unpublished data).

To further examine the effect of Raf on post-translational stability of Rep1 and Rep2, Rep proteins were expressed either in the presence or absence of *RAF* over-expression, and the levels of Rep1 and Rep2 were monitored at successive time points after addition of cycloheximide (**Figure 32C**). Under these conditions, the rate of degradation for Rep1 and Rep2 expressed individually or together was not affected by *RAF* over-expression. While the observations that Raf increases steady-state levels of Rep1 but does not significantly increase the half-life of Rep1 following treatment of yeast with cycloheximide appear to conflict, they could be consistent with Raf having a very short half-life relative to that of Rep1, such that following treatment with cycloheximide Raf is degraded, leaving Rep1 more susceptible to proteolysis.

3.4.2.6 Raf Over-Expression Inhibits Association of Rep2 with the Plasmid Partitioning Locus

The lack of a decrease in Rep protein stability when Raf was over-expressed suggested that Raf does not alleviate Rep protein-mediated repression of *FLP* by promoting degradation of Rep1 and Rep2. Raf has been found to associate with *STB* in a one-hybrid assay, with association dependent on the presence of Rep1 (Mary McQuaid, unpublished observations). To test whether Raf over-expression affects recruitment of Rep1 and Rep2 to *STB*, one-hybrid assays were performed (**Figure 33A**). A *cir*$^+$ reporter strain was co-transformed with a *CEN/ARS* plasmid directing expression of Rep1 or Rep2 as B42$_{AD}$-HA

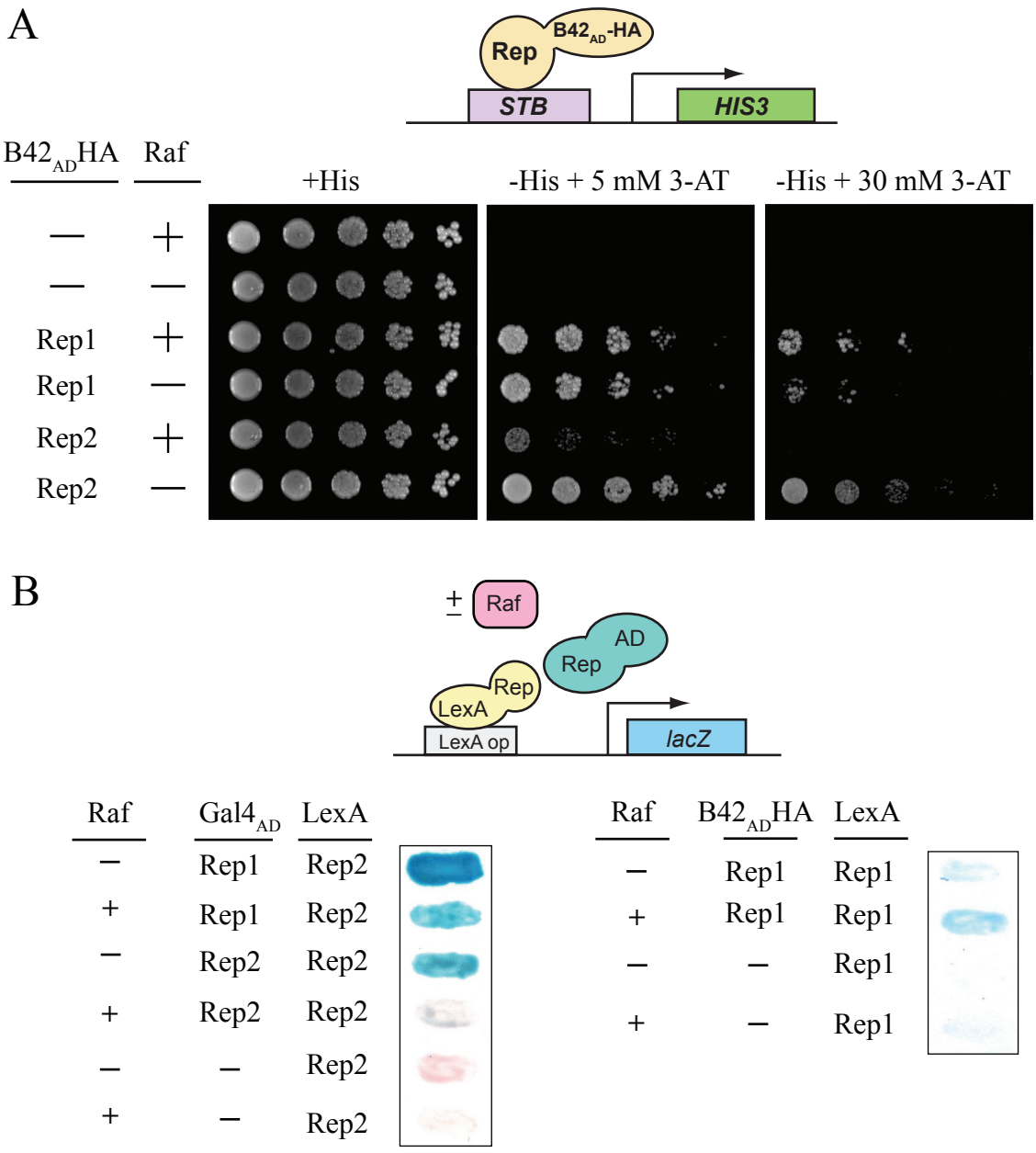


Figure 33. Over-expression of *RAF* impairs Rep2 interaction with *STB* and Rep1, and Rep2 self-association. (A) A *cir*$^{+}$ yeast one-hybrid reporter strain encoding *STB* upstream of a *HIS3* reporter gene was co-transformed with a plasmid directing expression of the indicated B42$_{AD}$-HA fusions, and a plasmid encoding galactose-inducible *RAF* or with no gene inserted (-), and five-fold serial dilutions of the co-transformants were spotted onto media containing galactose. Association of the Rep proteins with *STB* was monitored by growth on medium lacking histidine supplemented with 3-aminotriazole (3-AT). (B) A *cir*^{0} yeast two-hybrid reporter strain was co-transformed with plasmids expressing the indicated Gal4$_{AD}$, LexA, and B42$_{AD}$ fusion proteins, as well as a plasmid encoding galactose-inducible *RAF* or vector (-). Co-transformants were grown for 24 h on a nitrocellulose membrane overlaid on solid medium containing galactose, and the effect of Raf over-expression on Rep1-Rep2 and Rep2-Rep2 interactions (left) and Rep1-Rep1 interaction (right) were assessed as described in the legend to Figure 15.

fusion proteins, and a plasmid that expresses untagged Raf from a *GAL* promoter. Recruitment of the Rep fusions was monitored by growth on medium lacking histidine supplemented with 3-AT. In this assay, Raf over-expression led to a slightly higher level of association of Rep1 with *STB*, which may reflect an increased level of the $B42_{AD}$-Rep1 fusion protein due to the Rep1-stabilizing activity of Raf. In contrast, Raf over-expression led to decreased association of $B42_{AD}$-HA-Rep2 with *STB* in this assay. If Raf has the same effect on association of the Rep proteins at the *FLP* promoter as at *STB*, these results would suggest that Raf might alleviate repression of *FLP* by inhibiting Rep2 association with the *FLP* promoter.

3.4.2.7 Raf Over-Expression Inhibits Rep1-Rep2 and Rep2-Rep2 Associations in a Two-Hybrid Assay

The mechanism by which Raf prevents association of Rep2 with *STB* (and, presumably, all sites of Rep protein binding in the 2μm plasmid) is not clear. One possible mechanism of inhibiting association of Rep2 with 2μm plasmid target sites would be if Raf competes with Rep2 for binding to Rep1, since Rep2 depends on Rep1 for stable association with *STB* (**Figure 26**).

To analyze the effect of high levels of Raf on cross- and self-associations of the Rep proteins, two-hybrid assays were performed (**Figure 33B**). A cir^0 two-hybrid reporter strain was co-transformed with plasmids directing expression of Rep proteins as $Gal4_{AD}$ and LexA fusions. These co-transformants also contained either a *CEN/ARS* plasmid directing expression of *RAF* under control of a *GAL* promoter, or empty vector. Expression of Raf decreased two-hybrid interaction of Rep1 with Rep2, as well as Rep2 self-association, suggesting that Raf physically impedes these interactions. Detection of Rep1 self-association in a two-hybrid assay requires over-expression of Rep1 as a $B42_{AD}$HA-fusion protein from a galactose-inducible promoter (our unpublished results). When interaction of the $B42_{AD}$-HA-Rep1 fusion protein with LexA-Rep1 was assessed, Raf over-expression increased the two-hybrid interaction. This increase could represent improved Rep1 self-association but more likely reflected the elevation in Rep1 protein levels expected to result from Raf over-expression. The increased Rep1-Rep1 two-hybrid interaction in the presence of Raf also suggests Raf does not compete for Rep1 self-association.

3.4.2.8 Over-Expression of Raf Ablates Toxicity Due to High Levels of Rep1 and Rep2

In analyzing the effects of Raf over-expression combined with simultaneous over-expression of Rep1 and Rep2, I noticed that following galactose-induced expression of the three plasmid proteins, transformants expressing Raf in addition to Rep1 and Rep2 grew much better than those expressing Rep1 and Rep2 only. Combined over-expression of Rep1 and Rep2 has previously been reported to be toxic (180). To determine whether Raf over-expression alleviates this toxicity, transformants expressing combinations of galactose-inducible *REP1*, *REP2*, or *RAF* genes were serially diluted and spotted onto solid medium containing galactose (**Figure 34A**). As expected, combined over-expression of Rep1 and Rep2 severely inhibited growth, while over-expression of Rep1 or Rep2 individually had no significant effect on growth. When Raf was over-expressed in yeast already expressing Rep1 and Rep2, yeast grew as well as when no 2μm plasmid proteins were expressed, indicating that the toxicity due to Rep1 and Rep2 over-expression was virtually completely alleviated by Raf.

3.4.2.9 Deficient Sumoylation of Rep2 Alleviates Rep Protein Toxicity

The mechanism by which Raf over-expression alleviates toxicity of combined over-expression of Rep1 and Rep2 cannot be by decreasing Rep1 and Rep2 protein levels, since Rep1 proteins levels are increased when Raf is over-expressed, and Rep2 levels are not significantly changed (**Figure 32B**). Raf could alleviate Rep protein-induced toxicity if phosphorylation of Rep2 is required for toxicity, as Raf over-expression was shown to reduce the level of hyper-phosphorylated Rep2 (**Figure 32B**).

To test whether substitutions that affect post-translational modifications of Rep1 and Rep2 are required for the toxic effects of Rep protein over-expression, yeast were transformed with single-copy plasmids expressing wild-type Rep proteins or $Rep1_{3R}$, $Rep2_{13R}$, or $Rep2_{SA}$ from galactose-inducible promoters. Transformants were serially diluted and spotted onto medium containing galactose to examine Rep protein toxicity (**Figure 34B**). The lysine substitutions in $Rep1_{3R}$ did not alleviate Rep protein toxicity, while those in $Rep2_{13R}$ significantly reduced toxicity. In contrast, phosphorylation-deficient $Rep2_{SA}$ was equally as toxic as wild-type Rep2, when co-expressed with Rep1. The results suggest that

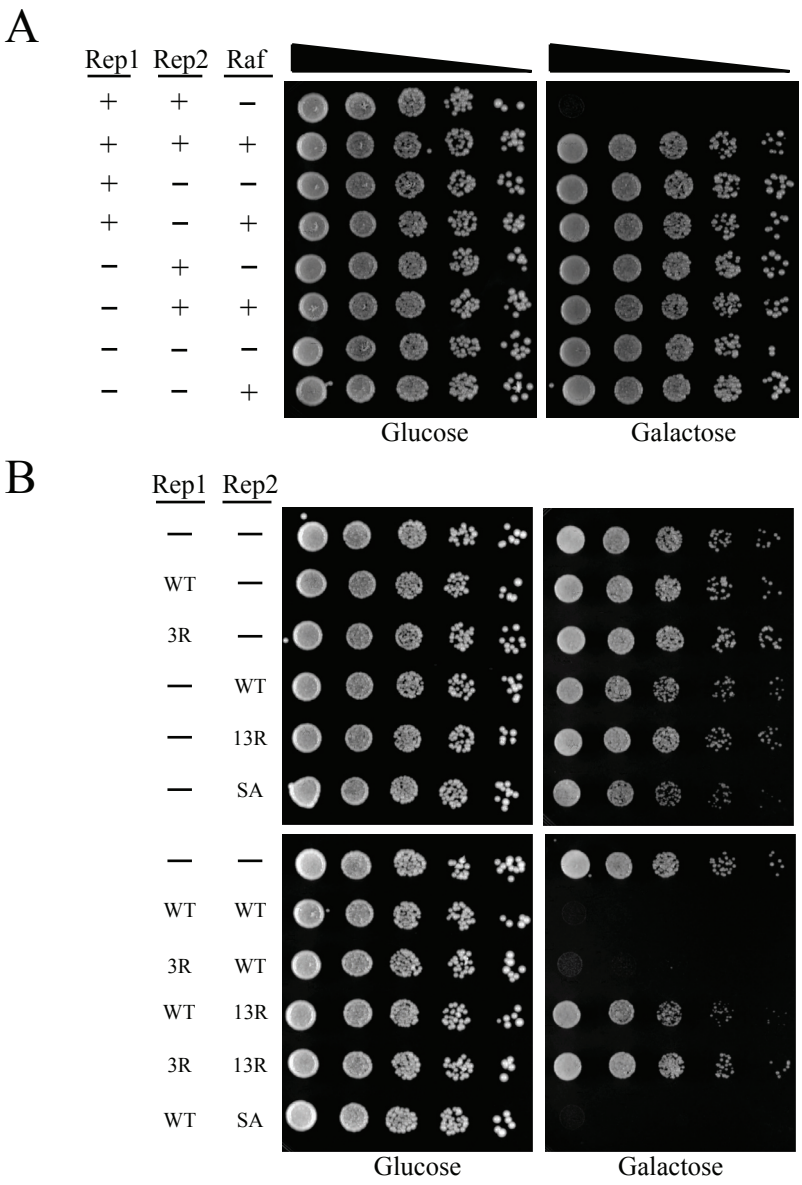


Figure 34. Effect of *RAF* over-expression and amino acid substitutions in Rep1 and Rep2 on Rep protein toxicity. *CEN/ARS* plasmids encoding the indicated plasmid proteins expressed under the control of a galactose-inducible promoter or without a plasmid gene inserted (-) were introduced into *cir*^{0} yeast. Transformants were grown overnight in medium that selected for retention of the plasmids and contained glucose to maintain repression of the *GAL* promoters, and five-fold serial dilutions were spotted on solid medium containing either glucose (left) or galactose (right) as a carbon source and imaged after five days of incubation at 28°C.

sumoylation of Rep2 might be required for Rep protein toxicity, and indicate Raf does not alleviate toxicity by reducing the levels of hyper-phosphorylated Rep2.

CHAPTER 4. DISCUSSION

4.1 General Overview

The two major functions of 2μm plasmid partitioning proteins Rep1 and Rep2 are to ensure that plasmid copies are equally partitioned between the mother and daughter cell during asymmetric cell division, and to regulate plasmid copy number by repressing expression of the plasmid-encoded Flp recombinase (63).

My studies have identified distinct roles for Rep1 and Rep2 which are novel, since prior to my research Rep1 and Rep2 seemed to have equally essential contributions to plasmid maintenance, with both being required for repression of plasmid genes (162, 180, 200, 228) and both being needed to recruit key host factors to *STB* during assembly of the partitioning complex (46, 88, 151, 239). My results suggest that *STB*-bound Rep1 is required for stable association of Rep2 with *STB,* while interaction of Rep2 with Rep1 increases Rep1 post-translational stability, possibly by protecting Rep1 against ubiquitination by the SUMO-targeted ubiquitin E3 ligase Slx5-Slx8.

I demonstrated that Rep2 is hyper-phosphorylated, and results from genetic and biochemical assays suggested that protein kinases CK2 and Kns1 target at least some of the residues that are phosphorylated in Rep2. Loss of Kns1 or either catalytic subunit of CK2 had no significant effect on inheritance of the native 2μm plasmid, and substitution of serine residues in Rep2 that abolished the hyper-phosphorylated species had no apparent consequence on the ability of Rep2 to mediate plasmid partitioning, or repress *FLP*, suggesting that phosphorylation of Rep2 by those kinases is not required for the functions of Rep2 that are currently known.

A screen of the single kinase-gene deletion strains identified yeast lacking Bud32, the kinase component of the conserved KEOPS complex, as being unable to maintain the native 2μm plasmid. While the catalytic activity of Bud32 was not required for plasmid maintenance, the inability of *bud32*Δ yeast to maintain the plasmid at normal copy number, and two-hybrid association of Rep2 with another KEOPS complex subunit, Pcc1, suggested that the KEOPS complex may play a direct role in plasmid maintenance.

My findings provide support for Rep1 being a target for the small ubiquitin-related modifier protein SUMO, and verify that Rep2 is also targeted for sumoylation as previously shown (36). Substitution-mutant versions of Rep1 and Rep2 that were deficient for interaction with conjugatable SUMO in a two-hybrid assay were each shown to be defective in mediating plasmid partitioning. These Rep protein mutants were both impaired for association with the plasmid *STB* locus, and both Rep proteins lost their normal punctate nuclear distribution when Rep1 was mutant, consistent with their loss of localization to the plasmid *STB* locus.

Finally, I investigated the mechanism of Rep1 and Rep2-mediated repression of *FLP*, and how Raf alleviates repression. While only a single transcript had previously been reported for *FLP* (162, 199, 210, 228), I identified two major *FLP* transcripts by northern blotting analysis, a constitutively expressed transcript unregulated by Rep1, Rep2 and Raf with a length consistent with that previously reported (210), and a longer transcript, whose abundance changed in response to altered levels of these plasmid proteins, indicating that plasmid proteins specifically regulate expression of this longer transcript. Possible mechanisms for alleviation of Rep protein-mediated repression by Raf were investigated by analyzing the effects of Raf over-expression on known interactions of the Rep proteins. ChIP assays confirmed the known association of the Rep proteins with *STB*, and demonstrated that both proteins associate near the *FLP* promoter region, with the lysine-to-arginine substitutions in Rep1 and Rep2 that impaired their association with *STB* also destabilizing their association with the *FLP* promoter. Over-expression of Raf inhibited association of Rep2, but not Rep1, with *STB*. My findings suggest that reduced association of Rep2 with *STB* could be a result of Raf competing with Rep2 for interaction with Rep1 and/or inhibiting Rep2 self-association. Interestingly, both overproduction of Raf, and lysine-to-arginine substitutions in Rep2 mitigated the toxicity associated with combined over-expression of Rep1 and Rep2.

A 9-bp sequence present at multiple locations in the 2μm plasmid and previously proposed to be the site of Rep protein action (200) was shown here to be absolutely critical for repression of *FLP*, consistent with a model in which association of the Rep proteins with the plasmid, either by direct contact with the DNA or through interaction with a DNA-bound host protein, is sequence-specific.

My findings enable further understanding of the function of Rep1 and Rep2 in their dual roles in maintenance of the yeast 2μm plasmid, and provide further evidence for the functional significance of post-translational modification by SUMO as a process essential to the inheritance of both parasitic and host genomes in yeast and in higher eukaryotes.

4.2 Distinct Roles for Rep1 and Rep2

Prior to my investigations, Rep1 and Rep2 were thought to have functionally equivalent roles in plasmid maintenance. Absence of either Rep protein results in a similar failure to recruit critical host proteins to *STB* (46, 88, 151, 254) and leads to an equally severe defect in plasmid partitioning (30, 116, 126). Neither Rep1 nor Rep2 alone is sufficient for mediating repression of plasmid genes (162, 180, 200, 228). My findings suggest why absence of either protein would produce a similar outcome.

Post-translational stability of Rep1 was found to be reduced when Rep1 was expressed in the absence of its partner protein, Rep2, suggesting that Rep2 protects Rep1 against degradation. This protection was not dependent on interaction of the Rep proteins with their cognate binding sites in the 2μm plasmid, since the chaperoning activity was also observed when the Rep proteins were expressed in a yeast strain lacking the 2μm plasmid. Rather, Rep2 likely stabilizes Rep1 through direct interaction, since previous data from our lab demonstrated that substitutions in Rep1 that impair interaction with Rep2 were correlated with a reduction in the steady-state levels of the mutant Rep1 proteins (Arpita Sengupta, unpublished results). My findings suggest that Rep1 molecules that are not bound to Rep2 may be vulnerable to degradation by the heterodimeric SUMO-targeted E3 ubiquitin ligase Slx5-Slx8, since in yeast lacking either subunit of Slx5-Slx8, Rep1 levels were not reduced in the absence of Rep2. Slx5-Slx8 has been shown to specifically target sumoylated proteins for degradation by recognizing SUMO chains on polysumoylated substrates through multiple SUMO interaction motifs (SIMs) in Slx5 (112, 158, 178, 209, 225, 247, 248). If Slx5-Slx8 does directly ubiquitinate Rep1, it might be expected that sumoylation of Rep1 would be a prerequisite to Slx5-Slx8-mediated degradation. However, when the lysine residues presumed to be the three major sumoylation attachment sites in Rep1 were substituted with arginine ($Rep1_{3R}$), the stability of Rep1, when expressed either with or without Rep2, was not increased. A residual level of sumoylation of the mutant $Rep1_{3R}$ protein might have been

sufficient to allow Rep1 to still be efficiently recognized by Slx5-Slx8. Alternatively, Slx5-Slx8 might recognize Rep1 independently of Rep1 sumoylation. Recently, Slx5-Slx8 was shown to effectively ubiquitinate the MATα2 repressor in a manner that did not depend on either sumoylation of MATα2, or the SIMs in Slx5, demonstrating that Slx5-Slx8 recognizes and ubiquitinates some proteins in a sumoylation-independent manner (248).

Loss of single subunits in a protein complex often reduces the stability of the other subunits, for example in the case of the yeast SAGA transcriptional coactivator complex (206). It is unclear whether degradation of Rep1 by Slx5-Slx8 is functionally relevant, since loss of Slx5-Slx8 only affected Rep1 levels when Rep2 was absent, a condition that would never occur outside a laboratory setting. However, yeast lacking either Slx5 or Slx8 are incapable of maintaining the 2μm plasmid (22) implicating this ubiquitin E3 ligase in plasmid maintenance. While some evidence points to Slx5-Slx8 being important for regulating Flp activity (250), findings presented here raise the possibility that Slx5-Slx8 might also contribute to plasmid maintenance by regulating Rep1 stability.

While Rep2 is required for stability of Rep1, Rep1 was shown to be required for stable association of Rep2 with the plasmid *STB* partitioning locus. In our one-hybrid assay system, detectable association of Rep2 with *STB* was absolutely dependent on the presence of Rep1. ChIP assays also supported this result, showing that the amount of *STB* DNA that co-immunoprecipitated with Rep2 was markedly reduced when Rep1 was absent. Taken together, these results suggest that Rep1 promotes association of Rep2 with *STB*. Conversely, results from one-hybrid and ChIP experiments indicated that Rep1 association with *STB* is not dependent on the presence of Rep2. My results, combined with those of earlier studies (87, 192, 254) suggest that Rep2 does recognize *STB* in the absence of Rep1, but this limited association is inadequate for function. A reduction in Rep1 levels when Rep2 is absent and a requirement for Rep1 to stabilize Rep2 association with *STB* would explain why absence of either Rep protein would result in a similar failure to recruit critical host proteins to *STB* (46, 88, 151, 254). Although Rep1 and Rep2 are able to bind to *STB* independently of one another, they may bind to *STB* as a dimer, with Rep2 that is bound to Rep1 having a higher affinity for target sites in *STB* than when not bound to Rep1. In diploid yeast, haploid-specific genes are repressed by cooperative binding of MAT**a**1 and MATα2, whose heterodimerization alters their sequence specificity and increases their affinity for operator

sites upstream of haploid-specific genes (80-82). Alternatively, Rep1 may first associate with *STB*, where it stabilizes association of Rep2 with *STB* through direct Rep1-Rep2 interaction. The increase in sensitivity of *STB* chromatin to micrococcal nuclease in yeast lacking Rep1 or both Rep1 and Rep2, but not if only Rep2 is absent (227), supports the hypothesis that Rep1 is either more directly or more tightly associated with *STB* than Rep2. Co-evolution of *STB* and Rep1 sequence variants in 2μm plasmids isolated from several laboratory and industrial *S. cerevisiae* strains (245, 246) also supports a model in which Rep1 recognition of *STB* is a critical prerequisite to productive association of Rep2 with *STB*. A hierarchical assembly of Rep protein sub-complexes has previously been proposed to mediate recruitment of the microtubule motor protein Kip1 to *STB*. While Kip1 was shown to co-immunoprecipitate with Rep2, but not with Rep1, recruitment of Kip1 to *STB* was dependent on both Rep1 and Rep2, and was required for efficient plasmid partitioning (46). These observations are consistent with Rep2 depending on Rep1 for association with *STB*, and suggest that Rep1 has an indirect role in recruiting Kip1.

4.3 Evidence for Sumoylation of Rep1 and Rep2 Identification of Sites Targeted for Sumoylation

One of the key objectives of my doctoral research has been an investigation of the functional consequences of sumoylation of the Rep1 and Rep2 plasmid partitioning proteins. When I initiated my studies, Rep2 was known to be sumoylated, and there was evidence to suggest that Rep1 was also sumoylated (36, 51). Obtaining definitive evidence for sumoylated forms both Rep proteins has not been straightforward. For most sumoylated proteins, only a small fraction of the targeted protein is modified at any one time (69, 120), and for the Rep proteins this was further complicated by their low abundance. I was able to provide further support for Rep protein sumoylation by demonstrating that Rep1 and Rep2 could interact with a conjugatable form of SUMO in a two-hybrid assay, while they did not recognize SUMO non-covalently. Substitution of lysine residues in Rep1 and Rep2 with arginine ($Rep1_{3R}$ and $Rep2_{13R}$ mutants) blocked two-hybrid interaction with SUMO, consistent with these lysine residues being targeted for sumoylation. Multiple sites had to be simultaneously abolished in both to eliminate two-hybrid interaction with SUMO, suggesting that multiple sites are targeted. For Rep1, K305 may be the major site targeted for

sumoylation, since substitution of this one site did reduce two-hybrid interaction with SUMO. Multiple lysine substitutions have similarly been needed to eliminate sumoylation of other proteins. For example, more than four sumoylation sites had to be substituted in yeast histone H2B to completely block sumoylation (166). As an extreme example, thirteen lysine residues had to be substituted to abolish sumoylation of mammalian heterochromatin protein 1 (HP1) (142). Although it is possible that the thirteen residues mutated in $Rep2_{13R}$ each normally serve as a SUMO acceptor site, this possibility seems unlikely. No individual lysine residue substitution reduced the two-hybrid interaction with SUMO, and after substituting several combinations of the thirteen residues I was unable to delineate a set of fewer substitutions that was sufficient for abolishing SUMO interaction (data not shown). In studies of other sumoylated proteins, abolishing major sumoylation sites was found to lead to increased SUMO conjugation at a less preferred site (55, 137), and this may also be the case for Rep2. Even though the multiple substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ abolished two-hybrid interaction with SUMO, faint HMW Rep protein species were detected in western blot analysis, suggesting that if these species are SUMO-conjugated forms of Rep1 and Rep2, sumoylation was still occurring, albeit at a low level, on remaining lysine residues. To demonstrate unequivocally that the HMW species of the sizes expected for SUMO-conjugates of the Rep proteins that are detected by our anti-Rep1 and anti-Rep2 antibodies and are diminished in abundance by lysine substitutions in Rep1 and Rep2 that abolish two-hybrid interaction with SUMO are indeed sumoylated forms of Rep1 and Rep2 lacks one final piece of evidence. I have yet to detect these HMW species using an antibody directed against the epitope tag incorporated into the only form of SUMO that was over-expressed in the cells during this experiment. Although the HMW forms can be detected with our anti-Rep antibodies and are shifted to a slightly higher MW when the HA epitope tag is added to SUMO as compared to when untagged SUMO is over-expressed (**Figure 21**), consistent with the extra ~5 kDa added by the triple-HA tag, no species were detected of these mobilities by an anti-HA antibody (data not shown). Our anti-Rep antibodies are affinity-purified to a high titre, so the monoclonal anti-HA signal for HA-SUMO-conjugated Rep proteins may be below the threshold of detection. Experiments are currently in progress to further enrich the Rep protein SUMO conjugates by an additional immunoprecipitation step so they can be detected by an epitope tagged to the SUMO moiety, and loss of SUMO conjugates for the

Rep1$_{3R}$ and Rep2$_{13R}$ mutants verified. Although the observed HMW species of the Rep proteins need to be confirmed as SUMO-conjugated forms, their reduction when yeast contained 2μm plasmids encoding Rep1$_{3R}$ or Rep2$_{13R}$ mutants, and the perturbed inheritance of these plasmids, suggest that the Rep proteins are targeted for sumoylation, and that this modification contributes to their partitioning function.

4.4 Rep1$_{3R}$ and Rep2$_{13R}$ Mutants are Impaired for Association with the Plasmid *STB* Locus and for Plasmid-Partitioning Function

Since sumoylation can have diverse effects, altering the activity, interactions, localization and/or stability of a targeted protein (120), I investigated which of these might be altered for the Rep1$_{3R}$ and Rep2$_{13R}$ mutants. Sumoylation of the 2μm plasmid Flp recombinase has been proposed to regulate its plasmid copy-number amplification activity by acting as a signal for ubiquitination by the SUMO-targeted ubiquitin ligase Slx5-Slx8 (250), and substitution of lysine residues could lead to loss of key ubiquitination sites. I did not detect any difference in post-translational stability of the Rep1$_{3R}$ and Rep2$_{13R}$ mutants, making it less likely that their loss of function was simply due to altered ubiquitination.

Substitutions in Rep1 or Rep2 that prevent their interaction with each other have previously been found to significantly impair 2μm plasmid partitioning (254, Arpita Sengupta, PhD thesis). Loss of sumoylation might affect this interaction, but I determined that two-hybrid interaction of Rep1 with Rep2 was not altered by the substitutions in Rep1$_{3R}$ and Rep2$_{13R}$, suggesting that overall Rep protein structure was not significantly perturbed by the lysine substitutions, and if these represent sumoylation sites, demonstrating that the Rep proteins are not dependent on sumoylation for their association *in vivo*. This picture is consistent with observations that bacterially expressed Rep1 and Rep2 interact in *in vitro* baiting assays (1, 190, 192), demonstrating that their interaction is not dependent on any yeast host-mediated post-translational modifications such as sumoylation.

Although interaction of the Rep proteins with each other was not perturbed, Rep1$_{3R}$ and Rep2$_{13R}$ proteins were found to be defective for association with *STB* in both one-hybrid and ChIP assays (**Figure 25**). Even substitution of just K305, which is likely to be the major sumoylation site in Rep1, was sufficient to produce a modest reduction in Rep1-*STB* one-hybrid association. Although substitution of K305 with arginine in Rep1 did not measurably

affect plasmid inheritance using our standard assays, when yeast were transformed with the *flp*$^-$ marker-tagged 2μm plasmids and cultured in selective medium, the copy number was significantly higher when the plasmid encoded Rep1$_{K305R}$ rather than wild-type Rep1, but not as high as when the plasmid encoded Rep1$_{3R}$ (data not shown). For these *flp*$^-$ plasmids, for which copy number is dictated by partitioning efficiency, this difference in plasmid copy number suggests that partitioning of the plasmid is perturbed by the single K305R substitution in Rep1, although the defect is not severe enough to be detected as a difference in efficiency of plasmid inheritance. The results suggest that substitution of a single sumoylation site in Rep1 modestly reduced its ability to associate with *STB*, and slightly impaired partitioning, while substitution of three sumoylation sites in Rep1 severely impaired association with *STB* and significantly affected inheritance of the plasmid. This correlation between the degree of loss of two-hybrid interaction with SUMO and the degree of impairment of both *STB* association and plasmid partitioning suggests that at least for the Rep1 lysine-to-arginine mutants, impaired sumoylation is most likely responsible for the observed defects, as opposed to other effects of the substitutions that are unrelated to sumoylation. Since efficient partitioning of the 2μm plasmid is dependent on association of Rep1 and Rep2 with the plasmid *STB* partitioning locus (254), the impaired ability of the Rep1 and Rep2 lysine substitution proteins to stably associate with *STB* is likely to be the primary defect leading to the observed loss of efficient partitioning.

While the Rep1$_{3R}$ and Rep2$_{13R}$ mutants were both impaired for association with *STB*, of the two, the substitutions in Rep1$_{3R}$ led to more severe defects in maintenance of a marker-tagged 2μm plasmid. ChIP assays also supported this difference in impact of Rep1$_{3R}$ compared to Rep2$_{13R}$ on partitioning function, showing that the amount of *STB* that co-immunoprecipitated with Rep2 was markedly reduced when wild-type Rep2 was co-expressed with Rep1$_{3R}$ rather than with wild-type Rep1. This observation was consistent with my discovery that Rep2 is dependent on the presence of Rep1 for stable association with *STB* (discussed in **Section 4.2**); loss of Rep1 association would be expected to lead to reduced association of Rep2. My findings suggest that the efficiency of plasmid partitioning correlates with the association of Rep2 with *STB*. Both the association of Rep2 with *STB* and the inheritance of a 2μm plasmid were more severely perturbed by the substitutions in Rep1$_{3R}$ compared to those in Rep2$_{13R}$. The much milder defect in plasmid partitioning when

only Rep2 was mutant suggests that sumoylation-deficient Rep2, recruited to *STB* through its interaction with sumoylation-competent Rep1, can confer some partitioning function. Although the severity of the partitioning defect associated with $Rep2_{13R}$ was mild compared to that of $Rep1_{3R}$, it was similar to that observed when the RSC2 chromatin remodeling complex is impaired, a defect severe enough to make yeast unable to maintain the native 2μm plasmid (239).

Consistent with defective assembly of the Rep1-Rep2 complex at *STB* for the $Rep1_{3R}$ mutant, Rep1 and Rep2 proteins were no longer found in discrete nuclear foci, which were previously shown to co-localize with the 2μm plasmid (190, 191, 230), when yeast contained $Rep1_{3R}$ instead of wild-type Rep1. For many SUMO targets, including transcription factors, sumoylation controls nucleocytoplasmic shuttling (11, 26, 138, 173, 217, 256); however, no cytoplasmic staining of the $Rep1_{3R}$ and $Rep2_{13R}$ mutants was observed, suggesting that sumoylation is not required for their nuclear targeting or retention. Sumoylation also often controls the sub-nuclear localization of proteins, a notable example being the SUMO-dependent localization of promyelocytic leukemia (PML) protein to PML nuclear bodies in mammalian cells (163, 226). In my study, 2μm plasmid foci were observed even when the plasmid encoded $Rep1_{3R}$ and $Rep2_{13R}$. The mutant Rep proteins might still be recruited to *STB* at a level sufficient to mediate plasmid association in clusters, but be insufficient or compromised for formation of the normal higher-order associations required for efficient partitioning. Further investigation is needed to assess whether localization of plasmid clusters at their normal spindle-pole-proximal nuclear address (152, 230) is altered by changes in Rep1 and Rep2 sumoylation status.

Association of Rep1 and Rep2 with *STB* is a fundamental step in mediating downstream events required for establishing the functional 2μm plasmid partitioning complex, recruiting to *STB* the motor protein Kip1, the centromere-specific histone H3 variant Cse4, the RSC2 complex, and ultimately the cohesin complex (46, 88, 151, 239, 254). While I cannot exclude the possibility that the lysine substitutions in $Rep1_{3R}$ and $Rep2_{13R}$ lead to impaired interactions with *STB* DNA or with host proteins that is independent of their effect on Rep protein sumoylation, I propose that sumoylation promotes recruitment or retention of the Rep proteins at *STB*. *STB* might be bound by a host protein that recognizes SUMO non-covalently by containing a SIM, and would be able to recruit or stabilize

association of sumoylated Rep proteins with *STB*. If this were the case, a conjugation-defective version of SUMO (SUMOΔGG) might be expected to be recruited to *STB* by interaction with the SIM of the bound host protein. However, no association of non-conjugatable SUMO with *STB* has been observed in our one-hybrid assay system (Mary McQuaid, Dalhousie University, unpublished results). Recently, localization of HP1 to chromosomal pericentromeric regions in mouse cells was shown to be dependent on SUMO-1 modification of HP1 (142). The sumoylated form of HP1 was shown to bind to a non-coding transcript originating from satellite repeats in pericentromeric regions, and this initial recruitment was proposed to "seed" further recruitment of HP1 and other proteins. Initial targeting of sumoylated Rep proteins to *STB* could subsequently recruit non-sumoylated Rep proteins through the known cross- and self-associations of Rep1 and Rep2.

Alternatively, sumoylation of Rep1 and Rep2 could promote their retention at *STB*, rather than recruitment. Weak association of Rep1 and Rep2 with *STB* could be stabilized through conjugation to SUMO, perhaps mediated by a SUMO ligase that is associated with *STB*. Localization of SUMO ligases at centromeres has been implicated in upregulating sumoylation of kinetochore proteins in higher eukaryotes. Accumulation of SUMO-2/3 conjugates at centromeres in *Xenopus* was shown to be dependent on localization of the SUMO E3 ligase PIASy to centromeres through direct interaction of PIASy with kinetochore proteins (187). Another possibility for how sumoylation might enable stable association of the Rep proteins with *STB* is by causing a conformational change in Rep1 and Rep2 that increases their affinity for *STB*, locking them in a specific DNA-binding conformation that persists even after SUMO is removed by the SUMO proteases. If sumoylation of Rep1 and Rep2 is only transiently required to enable stable binding, rapid recycling to the unmodified forms could explain how low steady-state levels of Rep protein SUMO conjugates are compatible with sumoylation being critical for stable Rep protein-*STB* association. Regulation of protein conformation by SUMO modification has been demonstrated as a mechanism of controlling the enzyme activity of human thymine-DNA glycosylase (204). Perhaps sumoylation of Rep1 is required for it to specifically recognize *STB* DNA, explaining why purified, bacterially expressed Rep1 does not display DNA-binding activity *in vitro* (192).

The hypothesis that Rep protein sumoylation promotes stable association of the Rep proteins with 2μm plasmid DNA in discrete nuclear foci is reminiscent of the requirement for sumoylation in targeting proteins to centromeres in yeast and in higher eukaryotes. In human cells, proteins conjugated with SUMO isoforms 2 and 3 are enriched at centromeres (258). In yeast, topoisomerase II is robustly targeted to pericentromeric DNA when translationally fused to SUMO (212), and yeast kinetochore proteins Ndc10, Cep3, Bir1, and Ndc80 are all SUMO targets (156), with sumoylation of Ndc10 being functionally relevant. Sumoylation-deficient Ndc10 fails to localize to the mitotic spindle, resulting in defective chromosome segregation (156). The short defined point centromeres to which Ndc10 binds are unique to the Saccharomycetaceae family of budding yeast, and were recently proposed to have arisen by replacement of a typical epigenetic fungal centromere with an ancestral 2μm plasmid-derived partitioning system (144). While rapid evolution may have obscured sequence homology between the 2μm plasmid and chromosomal segregation proteins, post-translational modification of segregation proteins with SUMO might be a conserved process, essential for their common function.

In addition to the 2μm plasmid, other parasitic DNA elements exploit the host cell SUMO pathway for their maintenance (16). Many viral proteins involved in maintenance of the episomes that encode them are SUMO-modified. Members of the human papillomavirus E2 family of proteins are dependent on sumoylation for their ability to tether viral genomes to host chromosomes to ensure faithful segregation (241). Host sumoylation has therefore frequently been exploited to ensure maintenance of parasitic genomes in eukaryotic cells, and here I have provided evidence that the yeast 2μm plasmid also co-opts this essential cellular process to ensure its efficient segregation during host cell division.

4.5 Phosphorylation of Rep2 and Dependence on Rep Protein Sumoylation

When I began my research, evidence from our lab had indicated that Rep2 was phosphorylated. Post-translationally modified forms of Rep1 and Rep2 were initially examined by western blotting analyses of total yeast protein probed with Rep1- and Rep2-specific antibodies. Rep2, predicted to have a MW of 33.2 kDa, migrated as two major species having apparent MWs of 35 and 37 kDa. Phosphatase-sensitivity assays and immunoblotting analysis of protein separated by 2D gel electrophoresis revealed that the 37-

kDa species represented multiple hyper-phosphorylated forms of Rep2, while unmodified and hypo-phosphorylated forms of Rep2 comprised the signal for the 35-kDa species.

HMW species of Rep2 having apparent MWs of ~50 and ~70 kDa were also detected. The size and pI values for these proteins corresponded to those predicted for mono- and di-sumoylated Rep2, respectively, consistent with the previously reported observation that Rep2 is polysumoylated (36), and supported by evidence presented in this thesis (**Figure 21**).

Strikingly, while 13 species were detected that had apparent MWs consistent with unsumoylated Rep2, only two and three distinct species of the 50- and 70-kDa forms of Rep2, respectively, were detected. The differently charged species of the 50- and 70-kda forms of Rep2 could represent Rep2 SUMO conjugates that are also phosphorylated on residues in either Rep2, or SUMO itself. Serine-2 in yeast SUMO and in its closest homologue in higher eukaryotes, SUMO-1, is phosphorylated *in vivo* (146). Attachment of SUMO that is either phosphorylated or unphosphorylated to Rep2 would also explain why only two spots were observed for mono-sumoylated Rep2, while three were observed for di-sumoylated Rep2.

A second possibility for the multiple species of mono-sumoylated and di-sumoylated Rep2 is that they represent attachment of SUMO to two different lysine residues in Rep2. Substitutional analyses indicated that at least two lysine residues in Rep2 are able to serve as SUMO attachment sites. Two different mono-sumoylated forms of Rep2 might be detected as two different species, while di-sumoylated Rep2 could have three possible forms: Rep2 containing a single SUMO moiety attached to each site, or a chain of two SUMOs attached to one site or the other.

The 50- and 70-kDa species of Rep2 could also represent Rep2 that was sumoylated and also contained one to two phosphorylated residues, or could represent hyper-phosphorylated SUMO-conjugates of Rep2. To distinguish between these two possibilities, the change in the charge of these species following phosphatase treatment will need to be examined. If these species represent phosphorylation at only one or two sites, this low level of phosphorylation of the sumoylated forms of Rep2 might indicate that sumoylation of Rep2 might inhibit phosphorylation, or vice versa. If these species represent hyper-phosphorylated forms of Rep2 SUMO-conjugates, the results would suggest that sumoylated Rep2 is required for hyper-phosphorylation, or vice versa. In support of Rep2 sumoylation not being

dependent on hyper-phosphorylation, in analyzing interaction of Rep2 with SUMO in a two-hybrid assay, which my results suggest is indicative of the ability of Rep2 to be sumoylated, the serine-to-alanine substitutions in the phosphorylation-deficient $Rep2_{SA}$ mutant did not impair interaction with SUMO. Several lines of evidence suggest that sumoylation might be required for phosphorylation of Rep2. When yeast lacked either subunit of Slx5-Slx8, the abundance of a Rep2 species having an apparent MW of about 70 kDa, consistent with di-sumoylated Rep2, was increased, and was accompanied by a decrease in the levels of the 37-kDa unsumoylated, hyper-phosphorylated forms Rep2 relative to those of the 35-kDa species (**Figure 4**). The decrease in abundance of hyper-phosphorylated forms of unsumoylated Rep2 could be due to the hyper-phosphorylated forms accumulating in the sumoylated forms of Rep2 instead. If sumoylation of Rep2 promotes hyper-phosphorylation, impaired sumoylation of Rep2 might also inhibit phosphorylation. In support of this hypothesis, when the $Rep2_{13R}$ mutant, which does not interact with SUMO in a two-hybrid assay, was expressed in the absence of Rep1, the levels of the hyper-phosphorylated forms were reduced, suggesting that in the absence of Rep1, Rep2 requires sumoylation to be hyper-phosphorylated (**Figure 24B**). Sumoylation may localize Rep2 to a sub-nuclear domain near the kinase that phosphorylates Rep2. Surprisingly, when wild-type Rep2 was expressed along with the $Rep1_{3R}$ mutant, which is impaired for interaction with SUMO in a two-hybrid assay, the relative levels of hyper-phosphorylated forms of Rep2 were also reduced, suggesting that when Rep2 is expressed with Rep1, Rep2 is dependent on sumoylation of Rep1 for phosphorylation (**Figure 24C**). The reduced levels of the hyper-phosphorylated forms of Rep2 when expressed with $Rep1_{3R}$ were only observed when the Rep proteins were expressed from their native promoters in a 2μm-based plasmid; the abundance of the hyper-phosphorylated forms of Rep2 was not affected by the lysine substitutions in $Rep1_{3R}$ when both Rep proteins were over-expressed from galactose-inducible promoters. This discrepancy may reflect different abundance and/or stoichiometry of the Rep proteins when the *REP* ORFs were expressed from galactose-inducible promoters compared to their native promoters. The levels of *REP1* transcripts are roughly ten times those of *REP2* when the *REP* ORFs are expressed from their endogenous promoters in a cir^+ yeast strain, and the steady-state levels of Rep1 and Rep2 proteins relative to each other also reflect the difference in mRNA levels (162, 190). Therefore, when both *REP1* and *REP2* are expressed from *GAL*-

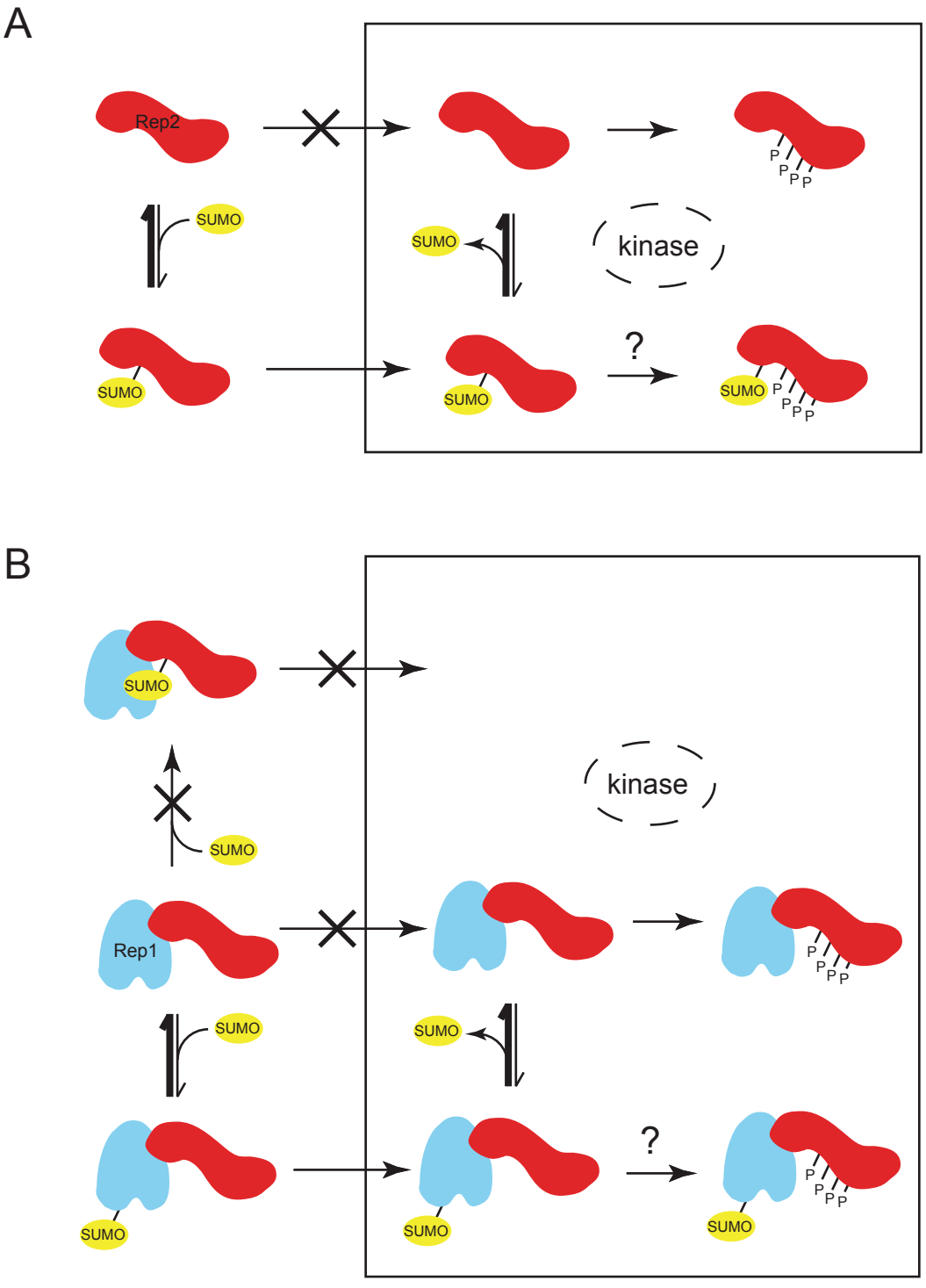
promoters in single-copy plasmids, the abundance of Rep2 relative to that of Rep1 would be expected to be higher than when both proteins are expressed at endogenous levels.

These observations could be explained by a model (**Figure 35**) in which sumoylation of the Rep proteins targets them to the kinase that phosphorylates Rep2. In this model, when Rep2 is expressed in the absence of Rep1, sumoylation of Rep2 is required for localization to the kinase. When Rep2 is expressed with Rep1, Rep2 sumoylation is inhibited and therefore is dependent on sumoylation of its partner protein Rep1 for targeting to a kinase. According to this model, binding of Rep1 to Rep2 would impair sumoylation of Rep2. This possibility is supported by two observations. Two-hybrid interaction of Rep2 with SUMO is weaker in a cir^+ yeast strain than in a cir^0 strain, suggesting that plasmid proteins may impair the Rep2-SUMO interaction (data not shown). Additionally, when Rep2 was over-expressed in the absence of Rep1 in *slx5*Δ or *slx8*Δ yeast, a 70-kDa HMW species consistent with di-sumoylated Rep2 was observed, but was not detected when Rep1 was also over-expressed (**Figure 4**).

4.6 Post-translationally Modified Forms of Rep1

In analyzing modified forms of Rep1 by immunoblotting, a faint ~100-kDa anti-Rep1 reactive species was consistently detected. When Rep1 was expressed with various epitope tags, the species was also detected by antibodies directed against the respective tag. The nature of this species is still unknown. Substitution of all Rep1 lysine residues except for the three most C-terminal with arginine did not abolish this species, indicating that the species in not likely a lysine-dependent post-translational modification. Further work is needed to determine the nature of this HMW species of Rep1. A more negatively charged post-translationally modified form of Rep1 was also detected in immunoblotting analysis of yeast proteins separated 2D gel electrophoresis. This species had the mobility predicted for Rep1 if it were phosphorylated on a limited number of sites; however, it could also represent another modification to Rep1 that would decrease the pI value, such as acetylation, or methylation of lysine residues. Some support for this being a phosphorylated species comes from the finding that Rep1 could be phosphorylated *in vitro* by the kinase Cka2, which was found to phosphorylate Rep2 *in vivo*. Since Rep1 and Rep2 co-localize, Rep1 may be in close proximity to CK2 and could be phosphorylated if the CK2 recognition sites are accessible to

Figure 35. Model for sumoylation-dependent targeting of Rep2 to a kinase. One model for sumoylation-dependent phosphorylation of Rep2 supported by findings in this study is shown. Modification of Rep1 (blue) and Rep2 (red) by SUMO (yellow) may target the Rep proteins to a sub-nuclear domain (black box) near the kinase (possibly CK2) that phosphorylates Rep2. (A) When Rep2 is expressed in the absence of Rep1, sumoylation of Rep2 is required for localization to the kinase. It remains to be established whether the SUMO-conjugated form of Rep2 is recognized by the kinase or if SUMO must be removed before Rep2 can be phosphorylated. (B) When Rep2 is co-expressed with Rep1, binding of Rep1 to Rep2 blocks sumoylation of Rep2. In this case, localization of the Rep1-Rep2 heterodimer to the kinase is dependent on sumoylation of Rep1.


A
Rep2
SUMO
SUMO
kinase
SUMO
?
SUMO
P
P
P
P
B
SUMO
SUMO
kinase
Rep1
SUMO
SUMO
SUMO
?
SUMO
P
P
P
P

CK2 *in vivo*. Treatment with phosphatase prior to 2D gel electrophoresis and immunoblot analysis of protein extracted from yeast lacking the kinase potentially responsible would be required to definitively determine whether this species of Rep1 is a phosphorylated form.

4.7 Phosphorylation of Rep2: a Non-Functional Modification?

Based on phosphatase-sensitivity and 2D-gel immunoblotting analyses, I identified multiple phosphorylated forms of Rep2. Examining for altered mobility or abundance of the hyper-phosphorylated forms in yeast lacking different kinases identified Kns1, CK2 and Bud32 as candidates for the kinases that phosphorylate Rep2 *in vivo*. Loss of Kns1 led to reduced phosphorylation of Rep2, and Kns1 interacted with both Rep2 and Rep1 in a two-hybrid assay, suggesting that Kns1 might phosphorylate both Rep proteins. However, 2μm plasmid levels were not affected by loss of Kns1, suggesting that even if Kns1 interacts with and/or phosphorylates the Rep proteins, the kinase is not required for maintenance of the 2μm plasmid.

Several lines of evidence indicated that protein kinase CK2 phosphorylates Rep2 *in vivo*. First, loss of Cka2, one of the two catalytic subunits of CK2, led to an increase in mobility of hyper-phosphorylated Rep2 in western blotting analysis. Second, Cka2 over-expression increased the abundance of hyper-phosphorylated Rep2. Third, Cka2 phosphorylated Rep2 *in vitro*, suggesting that CK2 can directly recognize Rep2. Finally, substituting alanine residues for CK2 consensus serines in Rep2 ($Rep2_{SA}$) blocked hyper-phosphorylation of Rep2 *in vivo*. Phosphorylation of Rep2 was only slightly reduced in *cka2*Δ yeast and was not significantly affected in yeast lacking the other CK2 catalytic subunit, Cka1. Cka1 and Cka2 could target overlapping sets of residues in Rep2, and completely blocking CK2 phosphorylation may require combined loss of both Cka1 and Cka2, which is lethal to yeast and therefore difficult to test.

The catalytic subunits of CK2 have both shared and independent functions (29). Cka1 is implicated in cell polarity (179) while Cka2 is involved in cell cycle progression (91). CK2 was recently shown to play an important role in chromosome segregation, phosphorylating the kinetochore proteins Mif2 and Ndc10 (176). Phosphorylation of Ndc10 by CK2 and the yeast Aurora B kinase, Ipl1, negatively regulates association of Ndc10 with centromeres (176). While phosphorylation of chromosome-segregation proteins by CK2 is required for

faithful segregation of chromosomes, my analyses suggest that CK2-mediated phosphorylation of the plasmid segregation protein Rep2 does not affect Rep2 function. Over-expression of Cka2 significantly increased the level of hyper-phosphorylated Rep2, but had no apparent effect on overall levels of Rep2, suggesting that the native 2μm plasmid was still faithfully maintained at normal copy number. Substitution of serine residues in Rep2 to alanine ($Rep2_{SA}$), to block hyper-phosphorylation, or to glutamate ($Rep2_{SE}$), to mimic constitutive phosphorylation, also did not affect efficiency of plasmid inheritance, suggesting these amino acid substitutions had little impact on Rep2 function. Phosphorylation-deficient Rep2 was also not impaired for its ability to, along with Rep1, repress the *FLP* gene (**Figure 31**), nor was the mutant protein impaired for interaction with Rep1 or SUMO in a two-hybrid assay, or for association with *STB* in a one-hybrid assay (data not shown). These observations suggest that phosphorylation of Rep2 by CK2 may not be functionally significant, at least at the phosphorylation sites abolished in the mutant $Rep2_{SA}$ protein. However, phosphorylation might contribute to a yet-undiscovered function of Rep2. Alternatively, the contribution of Rep2 phosphorylation to the efficiency of plasmid partitioning could have been too small to detect in the assays that were used to assess plasmid inheritance. The assay in which the function of $Rep2_{SA}$ was found not to be significantly impaired also failed to demonstrate a defect in plasmid inheritance for yeast lacking the Kip1 motor protein (**Figure 23**). Absence of Kip1 has been reported to cause an increased frequency of 2μm plasmid mis-segregation (46), but this defect can only be detected when a single-copy derivative of the 2μm plasmid is assessed for inheritance. Yeast lacking Kip1 do not lose the native 2μm plasmid during repeated passaging and over many generations (data not shown). Presumably, the very slight defect in partitioning efficiency is offset by the high copy number of the native plasmid and the presence of a functional amplification system that can correct the effect of unequal partitioning. To definitively establish that loss of the CK2 kinase or the CK2 phosphorylation target sites in Rep2 does not affect 2μm plasmid partitioning, the more sensitive single-copy plasmid assay that can detect more subtle perturbations in plasmid segregation would need to be employed.

The possibility that phosphorylation of Rep2 is not significant is supported by findings from one study that estimated phosphorylation at up to 65% of targeted sites may be non-consequential (130). Phosphorylation sites that are likely to be functionally significant

are more stringently conserved through evolution compared to phosphorylation sites that are less likely to contribute significantly to protein function (130). In yeast, phosphorylated S/T residues evolve more rapidly than randomly selected S/T residues. The authors propose that this phenomenon is a result of the majority of phosphorylated residues being located in disordered regions of proteins, which evolve more rapidly than structured regions (130). Rep2 is a rapidly evolving protein, bearing almost no resemblance to the partitioning proteins encoded by other 2μm-like plasmids from closely related yeast species; in contrast, the Flp and Rep1 proteins are recognizably conserved among different plasmids (161). It may seem puzzling that cells would waste ATP by phosphorylating irrelevant sites. Lienhard (136) proposed that cells have not evolved to the point where kinases only phosphorylate substrate proteins whose function is controlled by that phosphorylation event. This idea is supported by the high degree of substrate promiscuity of kinases observed *in vitro*. As long as non-functional phosphorylation events impose no significant detriment to the organism, there may be little evolutionary pressure for the off-target kinase-substrate interaction to be prevented. The relevance for the understanding of the 2μm plasmid may be that Rep2 phosphorylation at least indicates proximity or access to the Kns1 and CK2 kinases *in vivo*, which my findings suggest may be dependent on Rep protein sumoylation status (**Section 4.5** and **Figure 35**).

4.8 A Role for KEOPS Complex in 2μm Plasmid Maintenance

My initial discovery that a yeast strain lacking the Bud32 kinase had lost the 2μm plasmid identified a potential role for the evolutionarily conserved KEOPS complex in 2μm plasmid maintenance. The Bud32 kinase is a component of the KEOPS complex that in yeast has roles in telomere maintenance, transcription, and translation (47, 53, 127, 203). Although the *bud32*Δ strain from the EUROSCARF yeast deletion strain collection had lost the 2μm plasmid, when plasmid maintenance was examined in freshly derived cir^+ *bud32*Δ yeast for many generations following the loss of Bud32, no cir^0 lineages were found, even though the average plasmid copy number was generally lower than that for wild-type *BUD32* yeast. Inheritance of a marker-tagged 2μm plasmid was also not significantly affected by deletion of *BUD32* (Ron MacKay, Mount Saint Vincent University, unpublished results), further evidence that in *bud32*Δ yeast, complete mis-segregation (nondisjunction) of plasmids is

rare, while not excluding the possibility that plasmid copies might still be partitioned unequally. While plasmid partitioning did not appear to be defective in *bud32*Δ yeast, the average plasmid copy number was reduced, suggesting that Flp-mediated copy number amplification could be perturbed. Preliminary experiments have indicated that transcription of *FLP* might be affected by loss of Bud32 (data not shown), and this would be sufficient to explain the lower plasmid copy number.

The 2μm plasmid was maintained at normal copy number in yeast encoding a catalytically-inactive mutant of Bud32, suggesting that the defect associated with the loss of Bud32 might not be specific to the kinase activity of Bud32, but rather is a result of the loss of a scaffolding function. Bud32 may be needed to maintain the integrity of the KEOPS complex. Since the KEOPS complex has such diverse roles in transcription and translation, the effect of impaired KEOPS activity in 2μm plasmid maintenance could be an indirect consequence of altered expression of host genes. However, the two-hybrid interaction of Rep2 with the KEOPS subunit Pcc1 suggests a direct role for the KEOPS complex in plasmid maintenance. Further investigation is required to determine whether the Rep proteins recruit the KEOPS complex to the 2μm plasmid, or vice versa, and to identify the specific defect in plasmid maintenance that results from loss of KEOPS activity. Interestingly, KEOPS subunits Bud32, Pcc1 and Kae1 all exhibited robust association with SUMO in a two-hybrid assay, suggesting that at least one subunit of the KEOPS complex might be sumoylated. Perhaps sumoylation is required for targeting the KEOPS complex, as has been suggested here for Rep1, to specific plasmid-containing sub-nuclear domains.

4.9 Identification of a Recurring Sequence Element in the 2μm Plasmid Specifying Sites of Rep Protein Association

Besides their role in plasmid partitioning, Rep1 and Rep2 have an additional function in plasmid maintenance, the regulation of Flp-mediated copy-number amplification through repression of the *FLP* gene (162, 180, 200, 228). Repression of *FLP* is essential for long-term persistence of the plasmid, with unregulated expression resulting in over-amplification of the plasmid, which is toxic to the host (162). The presence of Rep1 and Rep2 reduces expression of all four 2μm plasmid genes, as well as non-coding transcripts driven by *STB* (162, 180, 200, 228) (**Figure 2**). These observations suggested that the Rep proteins might

bind to the promoters of these genes as well as to the *STB* repeats. Here I demonstrated using ChIP assays that Rep1 and Rep2 are both associated with the region between the divergently transcribed *FLP* and *REP2* genes, the first direct evidence that Rep1 and Rep2 associate with a region in the 2μm plasmid other than *STB*.

Som *et al*. (200) had identified a 9-bp sequence, 5'-TGCATTTTT present in each of the five *STB* repeats and at least once upstream of each of the four plasmid ORFs. The presence of this site at each region where the Rep proteins would be expected to carry out their known functions – at *STB* for plasmid partitioning, and at promoter regions for repression of plasmid genes – suggested that the 9-bp sequence, here referred to as a Rep Binding Element (*RBE*), might be the site targeted for Rep protein binding (200, 228).

Findings presented in this thesis support idea that the *RBE* element dictates the sites of Rep protein association in the plasmid. The effect of deletion of each of the two *RBE* elements located between the divergently transcribed *FLP* and *REP2* genes was investigated. Deletion of the *RBE* element closest to *FLP* (*rbe1*Δ) led to hyper-amplification of the plasmid to a toxic level, consistent with loss of Rep-mediated repression of *FLP*. When the *RBE* element closest to *REP2* was deleted (*rbe2*Δ), plasmid copy number did not accumulate to a toxic level; however, Rep1 and Rep2 levels were significantly increased. The elevated levels of both Rep1 and Rep2 could be explained by either a modest increase in plasmid copy number to a level below the threshold required to be toxic, or from increased *REP2* expression, leading to an elevated level of Rep2 that would be accompanied by an increase in Rep1 levels due to the Rep1-chaperoning activity Rep2. The latter possibility is consistent with the *RBE2* element being required for repression of the *REP2* gene.

The nucleotides most critical to the 9-bp element remain to be established. A single base change (G to A) introduced into either *RBE* element in the *FLP/REP2* promoter region did not have a significant effect on either plasmid copy number or Rep protein levels.

My findings demonstrated that deletion of each *RBE* element in the *FLP/REP2* intergenic region had effects consistent with loss of Rep protein-mediated repression. To determine whether this apparent loss of repression is due to an inability of the Rep proteins to bind to the *FLP/REP2* intergenic region, ChIP assays will need to be performed to determine whether the association of the Rep proteins within this region is affected by deletion of the *RBE* elements. The *RBE* elements in the *STB* locus may also be functionally significant for

Rep protein-mediated plasmid partitioning, since substitution of the element in the *STB* repeats was recently demonstrated to impair plasmid inheritance (Elizabeth Polvi, Dalhousie University, unpublished results). Taken together, these findings suggest that the *RBE* mediates association of the Rep proteins with the plasmid, enabling them to carry out their roles in plasmid partitioning and gene repression. This hypothesis had been proposed in early reports after it was found that the *RBE* elements correlated with regions that displayed Rep1-dependent changes in chromatin structure, supporting their identification as regions recognized by Rep1 (200, 227, 228). However, findings from subsequent bioinformatic studies suggested that the sequence might not be functionally significant, since it is not conserved in other 2μm plasmids. Xiao *et al.* (244) analyzed *STB* sequences from variants of the 2μm plasmid isolated from industrial strains of *S. cerevisiae* to identify conserved regions that were most likely to be critical for the function of *STB*. They found that one of the *STB* loci (in variant 7754-2μm) lacks the conserved TGCATTTTT but was still functional for partitioning, and therefore postulated that this element was not required for Rep protein association (244). However, the authors had neglected to point out that the 9-bp element is present elsewhere in the *STB* sequence, and therefore does not align with the elements present in the other *STB* loci. Perhaps their conclusion, along with the failure to demonstrate direct binding of Rep1 with *STB* DNA *in vitro* (87, 192) discouraged further investigation into Rep protein recognition sites at that time.

4.10 Recognition of 2μm Plasmid Target Sites by Rep1 and Rep2

The results of studies of the *FLP* promoter suggest that the *RBE* sequence defines sites of Rep protein association with the 2μm plasmid. The Rep proteins may be recruited to the plasmid by association with a host protein that recognizes this element. Supporting this possibility, one study demonstrated that Rep1 could bind to *STB in vitro*, but only in the presence of yeast protein extracts (87). Several large-scale ChIP studies have identified preferred binding sites for many yeast transcription factors, but to date none of the consensus sequences identified strongly conforms to the *RBE* sequence (6, 263).

Alternatively, the Rep proteins may bind to the *RBE* sequence directly. The co-evolution of sequences encoding Rep1 proteins and *STB* loci in variants of the 2μm plasmid harbored in various industrial strains of *S. cerevisiae* is support for *STB* being directly

recognized by Rep1 (245). Although no DNA-binding activity of Rep1 has been detectable *in vitro*, in light of the finding that the $Rep1_{3R}$ mutant, which is likely to be sumoylation-deficient, was impaired for association with both *STB* and the *FLP* promoter *in vivo*, it would be interesting to test whether *in vitro*-sumoylated Rep1 is able to bind *in vitro* to 2μm sequences containing the *RBE* element. While association of the $Rep1_{3R}$ and $Rep2_{13R}$ mutants with *STB* and with the *FLP* promoter was significantly impaired, it was not totally abolished. This limited association could be mediated by residual sumoylation of the $Rep1_{3R}$ and $Rep2_{13R}$ mutants, or could be due to a sumoylation-independent interaction of these proteins with their target sites in the plasmid. Bacterially expressed Rep2 has DNA-binding ability *in vitro* in the absence of any other yeast proteins (192), demonstrating that, at least for Rep2, recognition of DNA is not strictly dependent on sumoylation or other host-dependent post-translational modifications.

The impaired association of the $Rep1_{3R}$ and $Rep2_{13R}$ mutants with both the *FLP* promoter and *STB* and similar loss of association of wild-type Rep2 with the both of these regions of the plasmid when Rep2 was expressed with $Rep1_{3R}$ suggests that, as was demonstrated for *STB*, Rep2 may depend on Rep1 for stable association with the *FLP* promoter and that sumoylation contributes to this association. Collectively, these results suggest that the mechanism for the initial steps in assembly of the Rep protein complex may be universal for all *RBE* sequences in the plasmid. However, repression of the *FLP* promoter did not appear to be impaired even when both Rep1 and Rep2 contained the lysine-to-arginine substitutions that abolished their two-hybrid interactions with SUMO. A defect in the ability of Rep protein sumoylation mutants to repress the *FLP* promoter could have been obscured if the levels of Rep1 and Rep2, provided from *REP* genes expressed from the strong *GAL1* promoter, were much higher than the threshold level of Rep proteins required for achieving maximal repression of *FLP*. The levels of $Rep1_{3R}$ and $Rep2_{13R}$ proteins associated with the *FLP* promoter, although limited, may have been sufficient for potent repression, and a difference in their ability to repress *FLP* might only be observed when expressed at endogenous levels.

Taken together, the results suggest that sumoylation of Rep1 and Rep2 promotes their association with the *RBE* elements in the plasmid, with Rep2 being dependent on interaction with DNA-bound Rep1 for stable association. Binding of Rep1 to the *RBE* element may

stabilize the association of Rep2 with DNA adjacent to the *RBE* element. Supporting this hypothesis, Veit and Fangman (228) showed that an additional, albeit less strictly conserved, sequence is found approximately eight nucleotides 3' to most *RBE* elements. Perhaps the specificity of association of Rep2 with DNA is achieved through cooperative binding to DNA-bound Rep1 and to a preferred binding site adjacent to the *RBE* sequence.

If Rep1 and Rep2 bind to regions on the plasmid other than the *STB* partitioning locus, why are those regions unable to confer partitioning function? Interestingly, while Rep1, Rep2, and *STB* are together sufficient for plasmid partitioning (52, 126), deletion of the portion of *STB* encoding five of the repeats is not as detrimental to plasmid inheritance as deletion of either the *REP1* or *REP2* gene (**Figure 22**, and Arpita Sengupta, PhD thesis). This observation suggests that other *cis*-acting sequences in the plasmid may contribute a limited degree of Rep-mediated partitioning function when *STB* is absent. This residual partitioning function may be dependent on interaction of Rep1 and Rep2 with one or more of the four *RBE* elements in the plasmid that are not found within the repeated sequence of *STB*. Sequences flanking *STB* repeats show some similarity to the repeated sequence and might represent degenerate binding sites.

While the effects of association of Rep1 and Rep2 with their binding sites in the 2μm plasmid appear to be universal with respect to repression of transcription initiation at each of these sites, a key functional difference between association of the Rep proteins with *STB* and other regions of the plasmid is that the Rep proteins recruit host proteins such as the motor protein Kip1, the histone H3 variant Cse4 and the cohesin complex to *STB*, but not to any other sites in the plasmid (46, 88, 151). Recent findings suggested a minimum of three *STB* repeats are required for recruitment of Cse4 (109), and variants of the 2μm plasmid having just two repeats have been isolated (244), suggesting that as few as two repeats might be adequate for Cse4 recruitment, if located within an appropriate chromatin environment. It has previously been noted that the combined length of two adjacent *STB* repeats, which are between 62 and 63 bp in length, is 125 bp, virtually identical to the length of the conserved sequence that characterizes budding yeast point centromeres, the only region of DNA in the host genome that is bound by a Cse4-containing nucleosome (153).

A Cse4-containing nucleosome may be able to form at *STB* because of other features of the *STB* repeat sequence or the greater number of *RBE* elements that distinguish this

region from others, where Rep protein association does not mediate formation of the partitioning complex. *STB* repeats may contain a binding site for a host factor required for recruitment of Cse4. The Rep proteins may recruit a host protein to *STB* through direct interaction with the protein, or could indirectly be required for binding of a host protein, possibly by altering nucleosome positioning to render a binding site accessible to a host factor. Scm3, the nucleosome assembly factor specific to production of Cse4-containing nucleosomes, was recently shown to bind to the central AT-rich sequence element at centromeres to assemble a functional centromeric nucleosome (243), and Scm3 has also been shown to be required for incorporation of Cse4 in *STB* nucleosomes (109). Binding of Rep1 and Rep2 to *STB* DNA might expose an AT-rich tract of nucleotides that allows access of Scm3.

4.11 Mechanism of Rep1- and Rep2-Mediated Repression of *FLP*

An early study of transcription initiation and termination sites within the 2μm plasmid identified a 1300-nt mRNA species that initiates at about 14 bp upstream of the *FLP* start codon as the single major *FLP* transcript (210). In my investigation of the regulation of *FLP* expression, I also observed a single RNA of approximately 1300-nt as the most abundant *FLP* transcript in a cir^+ yeast strain. However, the abundance of the 1300-nt transcript was not affected by Rep1, Rep2 or Raf, while a ~1400-nt *FLP* transcript that was barely detectable in RNA from cir^+ yeast was strongly upregulated by the loss of either Rep1 and Rep2, or by overproduction of Raf. This finding suggested that the Rep proteins specifically repress transcription from an alternative start site about 100 bp upstream of that previously reported, although my analyses could not rule out the possibility that the 1400-nt transcript was either a 3'-extended *FLP* transcript, or a transcript running anti-sense through *FLP*. There are no start codons in the region ~100 nt upstream of the normal start site; the nearest ATG lies 151 bp upstream of the *FLP* ORF. Translation of the longer mRNA would therefore still initiate at the start of the *FLP* ORF to produce Flp protein. Since the levels of the 1400-nt transcript were higher when the Rep proteins were absent, a situation in which cellular Flp activity is elevated, and were lower when the Rep proteins were over-expressed in the absence of Raf, conditions in which Flp activity is known to be low (162, 180), translation of the 1400-nt transcript is the most reasonable explanation for the observed

changes in the level of functional Flp protein. Specific repression of an upstream start site by Rep1 and Rep2 was previously suggested for two non-coding transcripts initiating within *STB*, which were shown be ~100 nt longer when Rep1 or Rep2 was absent (228). If the 1400-nt transcript is a result of initiation from an alternative transcription start site ~100 bp upstream of that reported (210), the upstream start site would be located in close proximity to the *RBE* element that my analyses indicate is critical for Rep-mediated repression of *FLP*. Binding of Rep1 and Rep2 near a transcription start site may mediate repression by directly impeding access of transcription factors or RNA polymerase II to the DNA. Alternatively, association of Rep proteins at the *FLP* promoter could indirectly inhibit binding of transcription factors by remodeling the chromatin in such a way that the preferred binding site for a transcription factor is inaccessible, or the transcription machinery needed for expression of the 1400-nt transcript cannot access the start site. While expression of the 1400-nt transcript could lead to higher Flp levels simply because it increases the total abundance of Flp-encoding transcripts (1300-nt plus 1400-nt), the 1400-nt transcript might also be translated more effectively than the 1300-nt due to an extended 5'-untranslated region (UTR), as the 5'-UTR of the 1300-nt transcript is only 14 nt long and might be inefficiently recognized by ribosomes. Nonetheless, a 5'-UTR of only 14 nt may still be effectively translated, as the median length for the 5'-UTRs in yeast is ~50 nt, with a length shorter than 10 nt observed for many yeast genes (164).

The regulation of expression of the *FLP* gene by the two proteins needed for plasmid partitioning, critical for control of 2μm plasmid copy number, suggests relationships that may have existed in the ancestral version of the 2μm plasmid. Flp is the only protein that has any obvious homologues in any other non-yeast organism. Flp exhibits significant sequence homology to site-specific recombinases encoded by bacteriophages, such as the Cre recombinase of phage P1 and the Int recombinase of λ phage, suggesting that the 2μm plasmid genome may be of viral descent (5). Repression of gene expression in phages is often mediated by a repressor binding to a promoter in a manner that obstructs accessibility to RNA polymerase. For example, in λ phage the cI repressor binds to the O_{R1} operator in the P_R promoter in a region overlapping the binding site for RNA polymerase (95). Association of a repressor with promoter DNA is not always sequence-specific. For example, repression of the viral C_2 promoter in *Bacillus subtilis* phage ϕ29 occurs though "creeping" of a

nucleoprotein complex comprised of the phage protein p6, which, starting from an initial preferred binding site, multimerizes along the DNA, eventually covering the C_2 promoter and preventing RNA polymerase from binding (61, 100, 193, 194). This mechanism of repression by a higher-order protein-DNA complex is an attractive model for the mechanism of *FLP* regulation, given that Rep1 and Rep2 can each self-associate and interact with each other (1, 190, 192, 229). Rep1 induces changes in chromatin structure at the 5' *FLP* region independently of the presence of Rep2 (227), suggesting that Rep1 does bind to this region in the absence of Rep2; however, expression of Rep1 alone is insufficient for repression of *FLP* (162, 200). Rep1 may act as a seed for higher-order multimers of Rep2 to extend the coverage of the Rep protein complex, effectively blocking access of RNA polymerase II to an upstream transcription start site.

General features of the 2μm plasmid partitioning system have been noted to superficially resemble those of partitioning systems encoded by bacterial plasmids (72, 117). In yeast and bacteria, partitioning requires association of two proteins with each other, and with a *cis*-acting, repeated DNA sequence. Like Rep1 and Rep2, partitioning proteins encoded by bacterial plasmids also repress expression of their own genes. Although features of assembly of the core partitioning complex do appear to be similar for yeast and low-copy bacterial plasmids, the mechanisms of partitioning are not at all alike, perhaps reflecting different strategies for propagation during eukaryotic versus prokaryotic cell division.

4.12 Antagonism of Rep1- and Rep2-Mediated Repression of Plasmid Genes by Raf

Although Raf has been proposed to be involved in control of plasmid copy number by derepressing the *FLP* gene, Raf may alleviate repression of all 2μm plasmid transcripts that are repressed by the Rep proteins, since over-expression of Raf increases the level of the *FLP*, *REP1*, and *REP2* transcripts as well as the 1950-nt non-coding transcript initiated within by *STB* (162). My findings suggest that Raf does not alleviate repression through competition with the Rep proteins for their DNA binding site, but rather that Raf abrogates Rep-mediated repression through non-covalent binding with Rep1 and/or Rep2. Overproduction of Raf impaired association of Rep2, but not Rep1, with *STB*, indicating that repression may be alleviated by specifically obstructing association of Rep2 with DNA. This

would be consistent with my finding that, in the absence of Rep2, Rep1 still associates with its target sites *in vivo*, and also with results of earlier studies showing that expression of Rep1 in the absence of Rep2 is not sufficient for repression (162, 200), so Raf overproduction is unlikely to alleviate repression by affecting Rep1-DNA interaction. Rep1 self-association was also not impaired by over-expression of Raf. My results support association of Rep2 with 2μm target sites being dependent on interaction of Rep2 with Rep1. This interaction was inhibited by over-expression of Raf, suggesting that Raf could limit association of Rep2 with DNA by preventing interaction with Rep1. Overproduction of Raf also inhibited Rep2 self-association. While it is not known whether Rep2 self-association contributes to the ability of Rep2 to stably associate with DNA, findings from domain mapping and competition experiments suggest that Rep2 that is bound to Rep1 remains accessible for binding to additional Rep2 monomers (192). The region in Rep2 required for homo-dimerization is different from that required for interaction with Rep1 (192), and Rep1 does not compete for Rep2 self-association *in vitro* (190).

It cannot be determined from my investigations whether Raf competes with Rep1 for binding to Rep2, or vice-versa. Both of these possibilities are plausible given that Raf was shown to interact with both Rep1 and Rep2 independently in a two-hybrid assay (Mary McQuaid, unpublished results). Domain mapping experiments indicated that the amino-terminal third of Rep1 ($Rep1_{1-129}$) is sufficient for interaction with both Rep2 and Raf, suggesting that Raf might compete with Rep2 for interaction with Rep1 (192, Mary McQuaid, Dalhousie University, unpublished results). Analyzing the function of point-mutant versions of Raf that are specifically impaired for interaction with either Rep1 or Rep2 would be essential to identifying the Raf-Rep protein interactions that are critical for Raf anti-repressor function.

Combinatorial control of gene expression by competition between transcriptional repressors and anti-repressors is seen in phages, where alleviation of repression is often mediated by binding of the anti-repressor to the repressor, as opposed to competition for a common DNA binding site. The Tum anti-repressor of coliphage 186 binds to the repressor CI, preventing binding to its operator sites (195). Similarly, the Coi anti-repressor from phage P1 alleviates C1-mediated repression by binding to C1 in a 1:1 ratio (98). The satellite phage P4 E anti-repressor promotes expression of genes from its helper phage, P2, by

alleviating repression of the P2 C repressor through formation of higher-order complexes of C and E that obstruct interaction of C with its DNA operator sequence (139).

I demonstrated that in addition to functioning as an anti-repressor, Raf stabilizes Rep1. However, unlike stabilization of Rep1 by Rep2, the half-life of Rep1 was not significantly increased in the presence of high levels of Raf when measured by examining Rep1 post-translational stability following inhibition of mRNA translation with cycloheximide. This discrepancy might be explained if Raf has a very short half-life relative to Rep1, so that the stabilizing effect on Rep1 by Raf would quickly be lost following the block in protein synthesis. This observation highlights a general caveat of studying protein half-life using cycloheximide to block all protein synthesis, since effects on protein half-life may be mis-interpreted if the other proteins (e.g. chaperones, proteases) that modulate the half-life of the protein being analyzed are themselves rapidly degraded.

The observation that both Raf and Rep2 bind within the same region of Rep1 (residues 1-129) (192, Mary McQuaid, unpublished results) and that Raf and Rep2 both increase Rep1 post-translational stability suggests that Rep1 is protected from degradation by binding of this amino-terminal region to either Raf or Rep2. Stabilization of Rep1 by Raf might be important when plasmid copy number is low following unequal partitioning of plasmid copies at mitosis (190). Rep1 molecules are normally about 10 times more abundant than Rep2, and partitioning function appears to be more sensitive to low levels of Rep1 compared to low levels of Rep2 (30). At low copy number, Raf may aid in maintaining Rep1 at a level sufficiently high to ensure that partitioning function is sustained for the following cell division, while still allowing copy-number recovery by *FLP* by limiting association of Rep2 with the *FLP* promoter. While over-expression of Raf was found to impair association of Rep2 with *STB*, this reduction in association of Rep2 was not severe enough to affect inheritance of a marker-tagged 2μm plasmid (Mary McQuaid, unpublished results). When plasmid copy number is low, decreased association of the Rep proteins at the *FLP* and *RAF* promoters would enable copy-number recovery, while upregulation of *REP1* and *REP2* may enable expression of adequate levels of the Rep proteins to ensure that plasmid copies are equally partitioned during the next cell division.

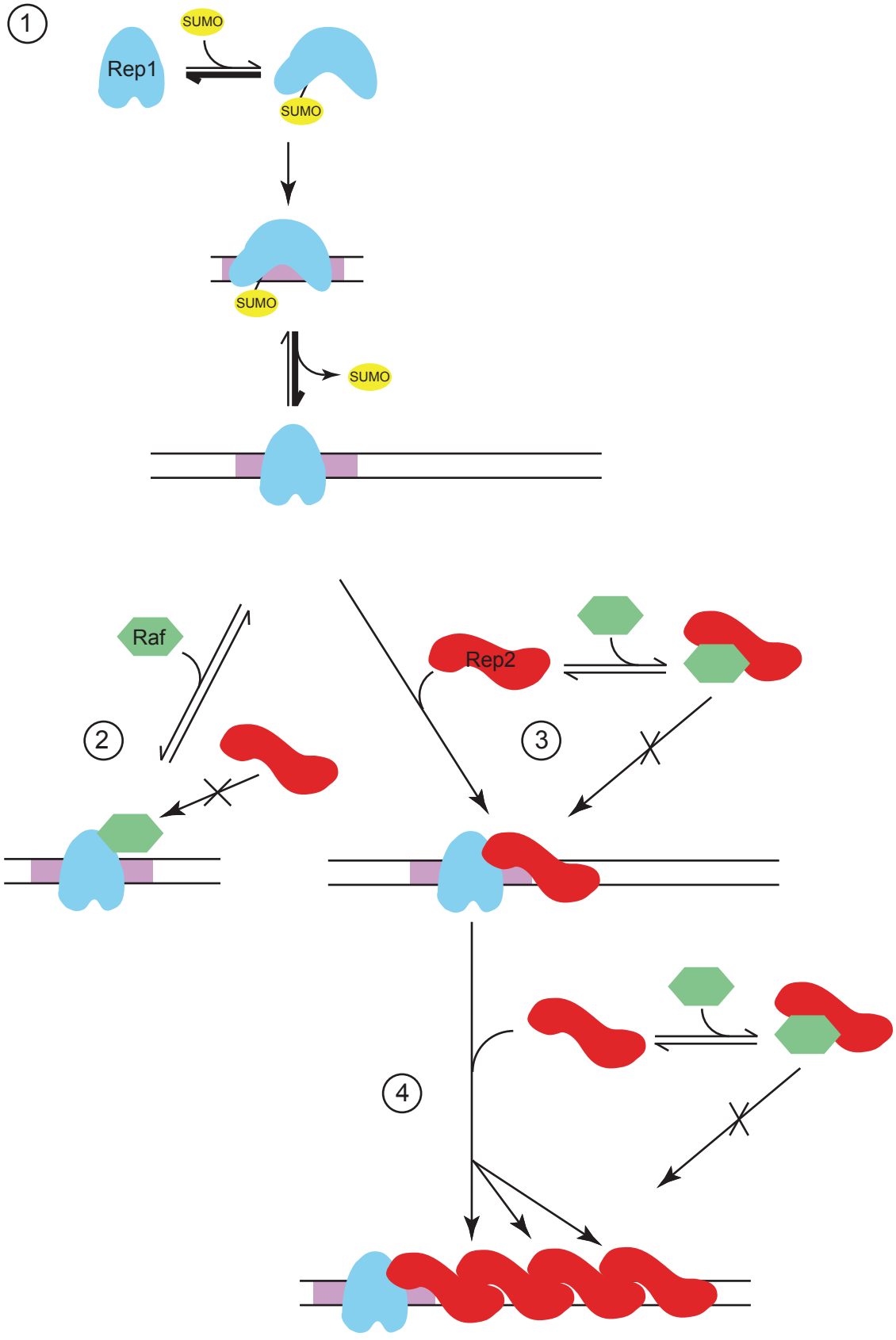
4.13 Model for Interaction of Rep1, and Rep2, and Raf with Their Target Sites in the 2μm Plasmid

One possible model for assembly of the Rep protein complex at *STB* that incorporates new findings presented in this thesis with previously reported data on the interactions of Rep1 and Rep2 with each other and with DNA is depicted in **Figure 36**. Rep1 associates with an *RBE* element in the plasmid, either directly or through interaction with a host protein, in a manner that is transiently dependent on Rep1 sumoylation, perhaps by SUMO altering the conformation of Rep1 to enable this association. Rep2 binds to DNA near regions of Rep1 binding in a manner that is promoted by sumoylation, and is highly dependent on interaction with Rep1 to maintain stable association with DNA. DNA-bound Rep2 could then self-associate with other Rep2 monomers, forming a higher-order nucleoprotein complex along the DNA. These higher-order structures might depend on any or all of the following: Rep2 self-association, sumoylation of Rep2, and the DNA-binding activity of Rep2. Based on domain mapping and competition experiments, Rep1 that is bound to Rep2 cannot bind to additional Rep1, Rep2, or Raf monomers (190, 192, Mary McQuaid, unpublished results). If Rep2 interacts with DNA-bound Rep1, these Rep2 molecules would still be accessible for association with other Rep2 molecules, since the regions of Rep2 required for self-association are different than those sufficient for interaction with Rep1. Raf might inhibit formation of this higher-order complex by competing for Rep1-Rep2 interaction and/or Rep2-Rep2 interaction, both of which would impair association of Rep2, but not Rep1 with the Rep protein target sites in the plasmid.

4.14 Concluding Remarks

Much of my research was directed at investigating how maintenance of the budding-yeast 2μm plasmid is affected by host-mediated post-translational modification of the plasmid-encoded Rep1 and Rep2 proteins. I demonstrated that Rep2 is phosphorylated on multiple residues, but have yet to uncover a function for Rep2 phosphorylation in either plasmid partitioning or repression of plasmid genes. However, I obtained evidence that supports sumoylation of both Rep1 and Rep2 as being functionally significant. I identified lysine residues in both Rep1 and Rep2 required for two-hybrid interaction with SUMO and provided evidence that substitution of these sites reduces Rep protein sumoylation. Analysis

Figure 36. Model for assembly of the Rep protein complex. One possible model for assembly of the Rep protein complex at 2μm plasmid target sites is shown. (1) Modification of Rep1 (blue) by SUMO (yellow) plays a catalytic role in promoting association with Rep protein target sites in the plasmid (purple), possibly by causing a conformational change in Rep1 that specifically increases the affinity of Rep1 for DNA, or promotes interaction with a host protein bound on the DNA. Association of Rep1 with its recognition site persists following removal of SUMO. (2) Stable association of Rep2 (red) with DNA is dependent on interaction of Rep2 with Rep1. Raf (green) may compete with Rep2 for interaction with Rep1, or (3) Raf may also compete with Rep1 for interaction with Rep2. (4) Rep2 that is bound to Rep1 is accessible to binding by other Rep2 monomers, which cooperatively bind to Rep2 and the adjacent DNA, forming a higher-order nucleoprotein complex. Formation of the higher-order complex is inhibited by Raf, which competes for Rep2 self-association.


1
SUMO
Rep1
SUMO
SUMO
SUMO
Raf
2
Rep2
3
4

of these mutant Rep proteins supports sumoylation contributing to proper localization of the Rep proteins and to robust association with their binding sites in the plasmid, and crucially, to being required for efficient transmission of plasmid copies to the daughter cell at mitosis. My findings also suggest that sumoylation of the 2μm plasmid partitioning proteins is critical for their localization in specific sub-nuclear domains, where the plasmid presumably needs to be for its efficient inheritance. The dependence of the yeast 2μm plasmid on sumoylation of its maintenance proteins for stable propagation in the host is yet another example of a survival strategy common to viral genomes in higher eukaryotes.

I identified separate functions for Rep1 and Rep2, with Rep2 being required to stabilize Rep1, and Rep1 being required for stable association of Rep2 with the *STB* partitioning locus and with the *FLP* promoter. My findings suggest that a conserved sequence element in the 2μm plasmid appears to serve as the recognition site for Rep1 and Rep2 association. Results from my investigation of the regulation of *FLP* gene expression suggested that Rep1 and Rep2 specifically repress transcription initiation from an unreported upstream start site, and that the previously reported *FLP* transcript is constitutively expressed and insensitive to changes in levels of plasmid proteins. My findings also provided insight into the mechanism of Raf-mediated anti-repression, showing that Raf specifically blocks association of Rep2 with Rep protein target sites in the 2μm plasmid, possibly by competing with Rep2 for interaction with Rep1 or by inhibiting Rep2 self-association. The mechanism of regulation of 2μm plasmid gene expression by plasmid-encoded proteins highlights common features reminiscent of regulation of phage gene expression, which might not be unexpected if the yeast plasmid originally derived from a prokaryotic phage that crossed kingdom boundaries.

It may seem perplexing that a relatively small (~6 kb) plasmid such as the 2μm circle, present at 60-100 copies per cell, would rarely be transmitted to the daughter cell through passive diffusion in the absence of an active partitioning system. However, computational models suggest that the 2μm plasmid requires a partitioning system to overcome the physical restraint imposed by the dumbbell-like shape of the yeast nucleus during cell division (68). My studies have provided further insight into how the 2μm plasmid Rep1 and Rep2 proteins contribute to ensuring stable propagation of this selfish DNA in an asymmetrically dividing eukaryotic host.

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