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The Yeast Elongator Histone Acetylase Requires Sit4-dependent Dephosphorylation for Toxin-Target Capacity

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***Kluyveromyces lactis* zymocin, a heterotrimeric toxin complex, imposes a G1 cell cycle block on *Saccharomyces cerevisiae* that requires the toxin-target (TOT) function of holo-Elongator, a six-subunit histone acetylase. Here, we demonstrate that Elongator is a phospho-complex. Phosphorylation of its largest subunit Tot1 (Elp1) is supported by Kti11, an Elongator-interactor essential for zymocin action. Tot1 dephosphorylation depends on the Sit4 phosphatase and its associators Sap185 and Sap190. Zymocin-resistant cells lacking or overproducing Elongator-associator Tot4 (Kti12), respectively, abolish or intensify Tot1 phosphorylation. Excess Sit4•Sap190 antagonizes the latter scenario to reinstate zymocin sensitivity in multicopy *TOT4* cells, suggesting physical competition between Sit4 and Tot4. Consistently, Sit4 and Tot4 mutually oppose Tot1 de-/phosphorylation, which is dispensable for integrity of holo-Elongator but crucial for the TOT-dependent G1 block by zymocin. Moreover, Sit4, Tot4, and Tot1 cofractionate, Sit4 is nucleocytoplasmically localized, and *sit4*Δ-nuclei retain Tot4. Together with the findings that *sit4*Δ and *tot*Δ cells phenocopy protection against zymocin and the ceramide-induced G1 block, Sit4 is functionally linked to Elongator in cell cycle events targetable by antizymotics.**

INTRODUCTION

Reversible protein phosphorylation is an important means of regulating cellular processes such as signal transduction, gene expression, or cell cycle progression (Stark, 1998; Kobor and Greenblatt, 2002). In *Saccharomyces cerevisiae*, execution of the latter requires cyclin-dependent kinase and Sit4, a type 2A protein phosphatase (PP2A) (Cross, 1990; Sutton *et al*., 1991). Sit4 acts positively for G1 cyclin (*CLN1/2*) function, which activates cyclin-dependent kinase and G1 exit (Nasmyth and Dirick, 1991; Fernandez-Sarabia *et al*., 1992). Consistently, *sit4*^{ts} cells arrest in G1 (Sutton *et al*., 1991) and *SIT4* relates to other cell cycle-relevant genes (*CAK1*, *CLN3*, *SWI4*, and *BCK2*) (Sutton and Freiman, 1997; Munoz *et al*., 2003). Once *CLN2* is provided from a *SIT4*-independent promoter, *sit4*^{ts} cells enter S phase but remain unbudded (Fernandez-Sarabia *et al*., 1992). So, bud emergence also requires Sit4 and further studies connect Sit4 to protein kinase C, target of rapamycin (TOR), ubiquitination, and the ceramide-induced G1 block, implying that Sit4 is a multifunctional enzyme catalyzing distinctive dephosphorylation events (Di Como and Arndt, 1996; Nickels and Broach, 1996; de la Torre-Ruiz *et al*., 2002; Singer *et al*., 2003). The phenotype of *sit4* mutants depends on *SSD1*, a polymorphic gene. Whereas in *ssd1-d* strains *sit4*Δ is lethal, *SSD1-v* alleles allow *SIT4* to be deleted, although *SSD1-v sit4*Δ cells are delayed in G1 (Sutton *et al*., 1991).

In response to *Kluyveromyces lactis* zymocin or rapamycin, *S. cerevisiae* arrests in G1 (Schaffrath and Breunig, 2000; Crespo and Hall, 2002). Rapamycin inhibition of TOR leads to several read-outs that require dissociation of Sit4 from its interactor Tap42 to trigger dephosphorylation of TOR effectors (Di Como and Arndt, 1996; Crespo and Hall, 2002). Unlike rapamycin, zymocin, a three-subunit ($\alpha\beta\gamma$) toxin complex, imposes a G1 block that allows transient macromolecular synthesis (Stark and Boyd, 1986; Butler *et al*., 1991b). Zymocin docks onto cell wall chitin followed by uptake of its γ-toxin subunit (Butler *et al*., 1991a; Jablonowski *et al*., 2001b). The γ-toxin target (TOT) involves Elongator, an RNA polymerase II (pol II) associated histone acetylase (HAT) that facilitates pol II transcription, and several other factors, including PP2A Sit4 (Otero *et al*., 1999; Frohloff *et al*., 2001, 2003; Jablonowski *et al*., 2001a,c; Winkler *et al*., 2001, 2002; Kim *et al*., 2002; Fichtner *et al*., 2002a,b, 2003; Fichtner and Schaffrath, 2002; Mehlgarten and Schaffrath, 2003). As judged from the findings that modulation of pol II carboxy-terminal domain (CTD) phosphorylation alters a cell's response to zymocin, that phospho-CTD stabilizes the Elongator•pol II association, and that removal of an Elongator NLS protects against zymocin, TOT function requires nuclear Elongator•pol II contact (Otero *et al*., 1999; Jablonowski *et al*., 2001c; Fichtner *et al*., 2003).

*sit4*Δ cells phenocopy Elongator mutants, suggesting a link between Sit4 and TOT (Jablonowski *et al.* 2001a). Although both TOT function and the TOR pathway require *SIT4*, the finding that *tap42*^{ts} cells are zymocin sensitive but

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rapamycin resistant implies there is little or no TOT-TOR cross talk (Jablonowski *et al*., 2001a). Apart from Tap42, Sap155, Sap185, and Sap190 associate with Sit4 as interdependent activators (Luke *et al*., 1996). Multicopy *SAP155* confers zymocin resistance that is abrogated by excess Sap185/190, suggesting high Sap155 titrates binding of Sap185/190 to Sit4 (Jablonowski *et al*., 2001a). Consistently, *sap185*Δ*sap190*Δ cells are zymocin resistant, implying that Sit4•Sap185/190 is crucial for zymocin action. Dephosphorylation of Elongator subunit Tot1 (Elp1) is shown here to be suppressed in zymocin-resistant *sit4*Δ cells. Similarly, zymocin resistance of *sap185*Δ*sap190*Δ cells is accompanied by high phospho-Tot1 levels and strongly suggests that Tot1 dephosphorylation is Sit4•Sap185/190 dependent and required for TOT proficiency. Zymocin resistance due to removal of Elongator-associator Tot4 (Kti12) abolishes Tot1 phosphorylation, whereas excess Tot4 (and zymocin protection) is antagonized by elevated Sit4•Sap190. Our study reveals an opposing link between Tot4 and Sit4 on Elongator phosphomodification that controls the TOT-dependent G1 cell cycle block by zymocin.

MATERIALS AND METHODS

Yeast Strains, Media, K. lactis Zymocin Methods, and DNA Constructs

Yeast strains used and constructed throughout this study are listed in Table 1. Yeast cells were grown in routine yeast extract, peptone, and dextrose (YPD) or galactose (YPG) rich media (Sherman, 1991). Synthetic complete (SC) medium was prepared as described by Sherman (1991) with either glucose or galactose as carbon source. Testing the effect of C_2 ceramide (Calbiochem, San Diego, CA) involved addition (100–200 μM) to YPD plates. Zymocin sensitivity tests of *S. cerevisiae* used the colony interaction killer eclipse assay essentially as described by Kishida *et al.* (1996) together with *K. lactis* killer strains (AWJ137) and nonkiller strains (NK40) (Table 1). Analysis of gene dosage effects on zymocin sensitivity used yeast transformation (Gietz *et al*., 1992) with 2 μ yeast shuttle vectors YEplac181 (*LEU2*) carrying *TOT4/KTI12* (pJHW27) and YEp24 (*URA3*) harboring *SAP4* (CB2925), *SAP155* (CB2643), *SAP185* (CB2819), and *SAP190* (CB2606) (Butler *et al*., 1994; Luke *et al*., 1996; Jablonowski *et al*., 2001a). If required for marker convenience, the *SAP185* and *SAP190* genes were moved into YEplac112 (2 μ *TRP1*) (Gietz and Sugino, 1988) or into pRS423 (2 μ *HIS3*) (Sikorski and Hieter, 1989) by using cloning strategies described by Luke *et al.* (1996). Testing multicopy *SIT4* involved cloning a *SIT4* PCR product essentially as described previously by Posas *et al.* (1991) into YEplac181 (2 μ *LEU2*) (Gietz and Sugino, 1988) to yield YEp*SIT4*. To combine cells maintaining *SIT4* and *TOT4* in multicopy, the *TOT4* gene was removed from pJHW27 and cloned into YEplac112 (2 μ *TRP1*) by directional *Eco*RI/*Hin*dIII cloning (Butler *et al*., 1994).

Targeted Gene Disruptions and Epitope Tagging In Vivo

For PCR-mediated construction of defined *kti11*Δ, *tot1*Δ and *tot2*Δ null-alleles, the YDp-KlL plasmid carrying the *K. lactis LEU2* marker and the YDpW deleter plasmid carrying *S. cerevisae TRP1* was used (Berben *et al*., 1991; Frohloff *et al*., 2001) together with the following knockout primer pairs: FW-ko-*KTI11* (5′-ACA TAC CAC GAC TGT AAG CAC ATC ATT TGT ACA ATA CAT TAC CAG CTG AAC GAC GGC CAG TGA ATT CCC GG-3′), RV-ko-*KTI11* (5′-CTT TAT TTC TAT TTG TAT TCT CGA TCT AGC CTC TCA TCT TTA GGC AGC AGA GCT TGG CTG CAG GTC GAC GG-3′), FW-ko-*TOT1* (5′-AGA AAC AGT ACA AAT GCC TAA TGG CTT ATG GTT GAA CAT GAC AAG AGT GGC GAC GGC CAG TGA ATT CCC GG-3′), RV-ko-*TOT1* (5′-CAA TAT GAC TCT TAG GGA AAT CAT GAA TCT CTG GAA CAG GTA TTT CTG GGA GCT TGG CTG CAG GTC GAC GG-3′), FW-ko-*TOT2* (5′-ATG GTG GAA TGT ATC ACT CCC GAA GCC ATT TTT ATA GGT GCT AAC AAG CAC GAC GGC CAG TGA ATT CCC GG-3′), and RV-ko-*TOT2* (5′-CCT CAA TCT TGT AAT TTT GTC TGC TGG TGT TAT ATC CTC GTT TAG CTG CGA GCT TGG CTG CAG GTC GAC GG-3′). Leu$^+$ or Trp$^+$ transformants obtained on SC media were verified by polymerase chain reaction (PCR) by using the knockout primer pairs to amplify the foreign marker and *ORF*-specific primer pairs to check for proper integration.

Testing strain FY1679-08A for *SSD1-v* or *ssd1-d* allelism involved gene disruption of *SIT4*. To do so, we constructed a deletion cartridge in which *SIT4* of YEp*SIT4* (see above) was centrally disrupted by *LEU2*. The latter was inserted as a 1.8-kb *Bam*HI segment of YDpL (Berben *et al*., 1991) into the single *Bgl*II site of *SIT4*. The disruption (*sit4*Δ*::LEU2*) was released by *Bam*HI restriction and Leu$^+$ transformants were verified to carry the *sit4*Δ*::LEU2* disruption by using PCR and *SIT4*-A/B primers as described previously (Posas *et al*., 1991). All *sit4*Δ cells tested were found to display the characteristics of an *SSD1-v* background allowing *SIT4* to be deleted with an accompanying slow growth phenotype (Sutton *et al*., 1991). Similarly, yeast strains harboring *tot4*Δ null-alleles (DJY100, DJY101, DJY103, and DJY104; Table 1) were obtained by transforming yeast cells with the pYF6 (*kti12*Δ/*tot4*Δ*::LEU2*) deletion construct (Butler *et al*., 1994) or by PCR-mediated gene disruption by using YDp-KlL (see above) and primers FW-ko-*TOT4* (5′-AAA CTA AAC AGG CAA TTT AGT AAG AAG ATG CCA CTG GTG CTT TTT ACG GGC GAC GGC CAG TGA ATT CCC GG-3′) and RV-ko-*TOT5* (5′-ATC TCA ATT CAA GTT TTT GTT AAG ATA ATC AGC GAA AAG CGG ACC GAT CCA GCT TGG CTG CAG GTC GAC GG-3′).

Tot proteins were C-terminally tagged with hemagglutinin (HA) and c-Myc epitopes by PCR by using tagging templates and S3/S2-primer pairs as described previously (Knop *et al*., 1999; Frohloff *et al*., 2001). For Sit4, C-terminal HA tagging used S3-primers (5′-TAA GAG AAT CCA CGG CAA ACC ATA ATA ATC AAA GAG CCG GCT ATT TCT TAC GTA CGC TGC AGG TCG AC-3′) and S2-primers (5′-ATT GTG AAA ATT ATT TTT ATT CGT CGA GTT AGG GAG GGC ATG CCG TCG TGA TCG ATG AAT TCG AGC TCG-3′) and PCR with plasmid pYM2 (Knop *et al*., 1999). HA tagging Sit4 at its N terminus involved PCR reactions by using pFA6a-*TRP1*-p*GAL1*-3HA (Longtine *et al*., 1998) and the following primers: F4-*SIT4* (5′-TAT TAT TCT TCA GTC CCC TCC TCG CTC TTT TTA GAT TCG ACA TTA CAA GGG AAT TCG AGC TCG TTT AAA C-3′) and R3-*SIT4* (5′-TGG CAT TTC TTT ATT GTT TCA AGC CAT TCG TCG GGG CCT CTA GAT ACC ATG CAC TGA GCA GCG TAA TCT G-3′). YCp vector pCB243 (*LEU2 SIT4-HA*) (Sutton *et al*., 1991) was kindly provided by Dr. M. Hall (Biozentrum Basel, Switzerland) and served as an additional source for a C-terminally HA-tagged Sit4 variant expressed from its native *SIT4* promoter in strain CY3938 (*sit4*Δ; Table 1).

Immunological Techniques

Detection of tagged proteins by anti-c-Myc (9E10) and anti-HA (3F10) (Roche Diagnostics, Mannheim, Germany) antibodies was as described previously (Frohloff *et al*., 2001). Elp1/Tot1 was immunodetected using anti-Elp1/Tot1 rabbit antiserum (provided by Dr. J. Svejstrup, London Research Institute, United Kingdom) essentially as described by Otero *et al.* (1999). Protein concentrations were determined using the method of Bradford (1976), and standardized protein loadings were controlled with a rabbit antibody directed against the α and β subunits of yeast Pfk1 (provided by Dr. J. Heinisch, University of Osnabrück, Osnabrück, Germany) and diluted 1:10,000 in standard Western studies. Immunoprecipitation was performed as described previously (Zachariae *et al*., 1996; Frohloff *et al*., 2001). Analysis of Tot1 phosphomodification by Western blots with anti-phosphoserine (Q5) and anti-phosphothreonine (Q7) antibodies followed essentially the manufacturer's manual (Phospho-Protein Purification Handbook August, 2002; QIAGEN, Hilden, Germany). Cell fractionation involving sucrose-ultracentrifugation was done as described by Kölling and Hollenberg (1994). Separation of enriched yeast cytoplasmic from nuclear fractions followed the protocol of Pereira *et al.* (1998). To exclude cross-contamination between the fractions, aliquots were analyzed in parallel by anti-Nop1, anti-RFA, and anti-Cdc19 antibodies, kind gifts from Drs. J. Aris (University of Florida, Gainesville, FL), B. Stillman, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and J. Thorner, (University of California, Berkeley, Berkeley, CA), at 1:2,000 (anti-Nop1), 1:5,000 (anti-RFA), and 1:10,000 (anti-Cdc19) dilutions. Detection of Sit4 phosphatase by a Sit4-specific monoclonal antibody (kindly provided by Dr. Y. Jiang, University of Pittsburgh, Pittsburgh, PA) rather than an immunoreactive epitope-tagged version used a 1:1,000 dilution (Wang *et al*., 2003).

RNA and Reverse Transcription (RT)-PCR Methods

Total RNA was isolated from equal amounts of *SIT4* and *sit4*Δ strains cells by using the RNAeasy midi kit (QIAGEN) according to the manufacturer's recommendations. RT-PCR experiments involved equal amounts of total RNA (4 μg) with the RevertAid kit (MBI Fermentas) for 1 h at 42°C in 20-μl reaction volumes. After first-strand cDNA synthesis, 1/20 of the reaction was subjected to PCR (30 cycles) by using *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) and oligonucleotide primers (10 μM) to amplify fragments specific for Elongator subunits (*TOT1-7*) or histone (*HHT1*) and actin (*ACT1*) controls. These were as follows: *HHT1* (5′-AGC AAG AAA GTC CAC TGG TG-3′ and 5′-GAA TGG CAG CCA AGT TGG TA-3′), *ACT1* (5′-CTT CCG GTA GAA CTA CTG GT-3′ and 5′-CCT TAC GGA CAT CGA CAT CA-3′), *TOT1/ELP1* (5′-CTT GGT GTA TGA AAC TCG CG-3′ and 5′-TTC TTA CCT CTG CCA GTA CC-3′), *TOT2/ELP2* (5′-AAC CTG ATG AGA CTT CAG GC-3′ and 5′-CAA ACC TAA CAC AGG AAC GG-3′), *TOT3/ELP3* (5′-TCA GTC CTT GTA CGA AGA CG-3′ and 5′-ATA AGC TCG ACC TGA TCT GG-3′), *TOT4/KTI12* (5′-TCC GGT ATC AAC TTC ACT GC-3′ and 5′-CTT GTT CCG TTA CTT ACC CC-3′), *TOT5/ELP5* (5′-TAT TGA CGC TAC GCA GAT GG-3′ and 5′-CTC CTC TTC TTG CTT AGT GG-3′), *TOT6/ELP6* (5′-GAT GCT ACC TTC GTC AAC TC-3′ and 5′-TAC GTC CTT TGC AAA ACC GG-3′), and *TOT7/ELP4* (5′-TTT GCA AAG GAG CTA CCT GG-3′and 5′-GGA AGC AAC AGT ACA ACC C-3′). RT-PCR products were analyzed by agarose gel electrophoresis: *ACT1* (0.44 kb), *HHT1* (0.32 kb), *TOT1/ELP1* (0.49 kb), *TOT2/*

Table 1. Yeast strains used in this study

Strain	Description	Source
K. lactis		
AWJ137	α *leu2 trp1* [k1$^+$ k2$^+$] (killer)	Frohloff *et al.* (2001)
NK40	α *ade1 ade2 leu2* [k1^o k2$^+$] (nonkiller)	Frohloff *et al.* (2001)
S. cerevisiae		
JA100	*MAT*α *ura3-52 leu2-3-112 his4 trp1-1 can-1r*	Joaquin Ariño
EDN75	As JA100, but *ppz1*::*KAN*	Joaquin Ariño
JA103	As JA100, but *ppz2*::*TRP1*	Joaquin Ariño
EDN76	As JA100, but *ppz1*::*KAN ppz2*::*TRP1*	Joaquin Ariño
W303-1A	*MAT*a *ade2-1 his3-11, 15 leu2-3, -112 trp1-1 ura3-1 can1-100 ssd1-d2*	Trisha Davies
AY925	As W303-1A but *MAT*α	Kim Arndt
DEY132-1C	As AY925, but *pph21*::*HIS3*	Evans and Stark (1997)
DEY10-2B	As AY925, but *pph22*::*TRP1*	Evans and Stark (1997)
DEY132-2C	As AY925, but *pph21*::*URA3 pph22*::*TRP1*	Evans and Stark (1997)
MMY09	As W303-1A, but *cna1*::*LEU2 cna2*::*URA3*	Trisha Davies
YJN519	As W303-1A, but *cnb1*::*LEU2*	Thomas Edlind
LFY3	As W303-1A, but *tot1*Δ::*TRP1*	This work
LFY4	As W303-1A, but *tot2*Δ::*TRP1*	This work
LFY5	As W303-1A, but *tot3*Δ::*TRP1*	Jablonowski *et al.* (2001c)
LFY6	As W303-1A, but *tot4*Δ::*TRP1*	Jablonowski *et al.* (2001c)
FY1679-08A	*MAT*a *ura3-52 leu2*Δ*1 trp1*Δ*63 his3*Δ*200 GAL SSD1-v*	Euroscarf, Frankfurt
DJY1t-a	As FY1679-08A, but *TOT1-(c-myc)$_3$*::*Sphis5*$^+$	This work
FFY3t	As FY1679-08A, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$	Frohloff *et al.* (2001)
FFY4t-a	As FY1679-08A, but *TOT4-(c-myc)$_3$*::*Sphis5*$^+$	Fichtner *et al.* (2002a)
DJYS4H	As FY1679-08A, but *SIT4-(HA)$_3$*::*Sphis5*$^+$	This work
DJYHS4	As FY1679-08A, but *TRP1*::*GAL1*::*(HA)$_3$-SIT4*	This work
DJY8A-1H3	As FY1679-08A, but *TOT1-(HA)$_3$*::*Sphis5*	Fichtner *et al.* (2003)
DJYT4H	As FY1679-08A, but *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY2t1d-a	As FY1679-08A, but *TOT2-(c-myc)$_3$*::*Sphis5*$^+$ *tot1*Δ::*KlLEU2*	This work
FFY2/1dt	As FY1679-08A, but *TOT2-(c-myc)$_3$*::*Sphis5*$^+$ *TOT1-(HA)$_6$*::*KITRP1*	Fichtner *et al.* (2002a)
FFY3/1dt	As FY1679-08A, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT1-(HA)$_6$*::*KITRP1*	Fichtner *et al.* (2002b)
FFY3/2dt	As FY1679-08A, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT2-(HA)$_6$*::*KITRP1*	Fichtner *et al.* (2002a)
DJY3/2-d11	As FFY3/2dt, but *kti11*Δ::*KlLEU2*	This work
CY4029	As W303-1A, but *SSD1-v1*	Luke *et al.* (1996)
CY5224	As CY4029, but *sap185*Δ::*ADE2 sap190*Δ::*TRP1*	Luke *et al.* (1996)
CY5220	As CY4029, but *sap4*Δ::*LEU2 sap155*Δ::*HIS3*	Luke *et al.* (1996)
CY3938	As CY4029, but *sit4*Δ::*HIS3*	Luke *et al.* (1996)
DJY100	As CY4029, but *tot4*Δ::*KlLEU2*	This work
DJY101	As CY4029, but *sit4*Δ::*HIS3 tot4*Δ::*LEU2*	This work
DJY102	As CY4029, but *TOT1-(HA)$_6$*::*KITRP1*	This work
DJY103	As CY4029, but *sit4*Δ::*HIS3 TOT1-(HA)$_6$*::*KITRP1*	This work
DJY104	As CY4029, but *tot4*Δ::*LEU2 TOT1-(HA)$_6$*::*KITRP1*	This work
DJY105	As CY4029, but *sit4*Δ::*HIS3 tot4*Δ::*LEU2 TOT1-(HA)$_6$*::*KITRP1*	This work
DJY107	As CY3938, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT5-(HA)$_6$*::*KITRP1*	This work
DJY108	As CY4029, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT5-(HA)$_6$*::*KITRP1*	This work
DJY109	As CY3938, but *TOT5-(c-myc)$_3$*::*Sphis5*$^+$ *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY110	As CY4029, but *TOT5-(c-myc)$_3$*::*Sphis5*$^+$ *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY111	As CY3938, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY112	As CY4029, but *TOT3-(c-myc)$_3$*::*Sphis5*$^+$ *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY113	As CY3938, but *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY114	As CY4029, but *TOT4-(HA)$_6$*::*KITRP1*	This work
DJY115	As CY5220, but *sap4*Δ::*LEU2 sap155*Δ::*HIS3 TOT1-(HA)$_6$*::*KITRP1*	This work
DJY116	As CY4029, but *sap185*Δ::*ADE2 sap190*Δ::*LEU2 TOT1-(HA)$_6$*::*KITRP1*	This work

ELP2 (0.57 kb), *TOT3/ELP3* (0.53 kb), *TOT4/KTI12* (0.47 kb), *TOT5/ELP5* (0.5 kb), *TOT6/ELP6* (0.5 kb), and *TOT7/ELP4* (0.5 kb).

RESULTS

Nucleocytoplasmic Sit4 Mediates the Ceramide G1 Block Together with Tot4 and Elongator

Having HA-tagged Sit4 at its C terminus, we observed zymocin resistance and wild-type cell viability atypical of *sit4*Δ *SSD1-v* cells, indicating a crucial role for Sit4's C terminus in zymocicity (Figure 1A; our unpublished data). Because this region contains a conserved PP2A motif (–YFL) whose carboxymethylation mediates subunit interaction (Evans and Hemmings, 2000; Wei *et al.*, 2001), the tag may have interfered with formation of zymocin-relevant Sit4 subcomplexes. Consistent with this, an HA-tag incorporated at Sit4's C terminus upstream of this –YFL motif and expressed from single-copy vector pCB243 (Sutton *et al.*, 1991) was fully capable of complementing zymocin resistance associated with the *sit4*Δ background of strain CY3938 (Table 1 and Figure 1B, top left). An N-terminal HA-tag under *GAL1*-promoter control yielded glucose-dependent zymocin protection with *sit4*Δ *SSD1-v*-like slow growth (Figure 1A; our unpublished data). Galactose restored normal growth and zymocin inhibition, indicating that (HA)$_3$-Sit4 is functional

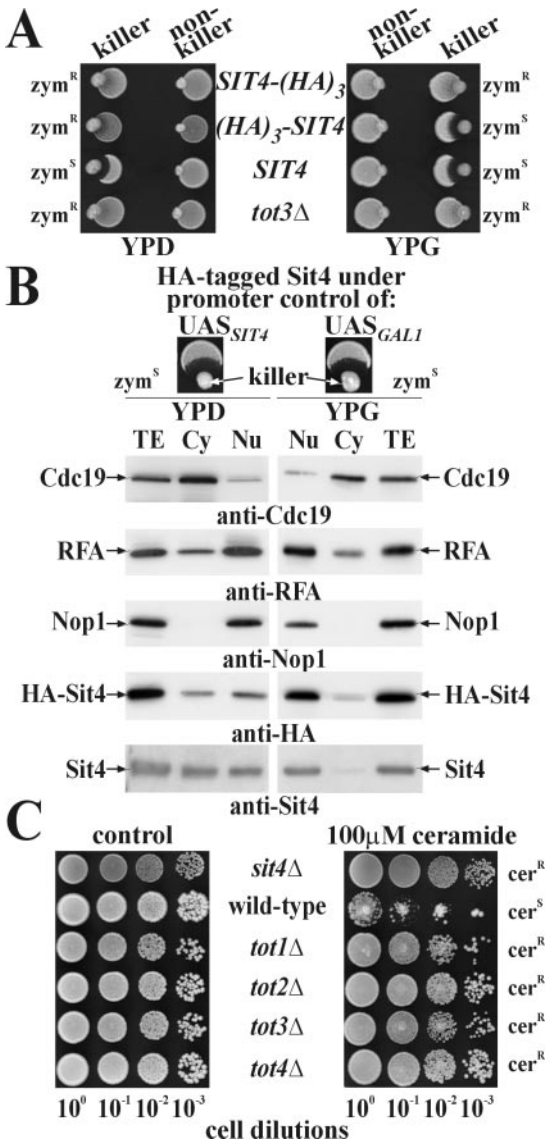

Figure 1. Sit4 can be found in the nucleus and *sit4*Δ and Tot$^-$ mutants resist ceramide. (A) The Sit4 C terminus confers zymocin sensitivity. Killer eclipse assays used *K. lactis* killer (AWJ137) and nonkiller (NK40) strains (Table 1) and *S. cerevisiae* strains expressing wild-type Sit4 (FY1679-08A: *SIT4*) or Sit4 tagged with the HA epitope at either the C terminus (DJYS4H: *SIT4*-$(HA)_3$), or at the N terminus (DJYHS4: $(HA)_3$-*SIT4*) under *GAL* promoter control. Eclipse formation around the killer strain indicates zymocin sensitivity (zym^S), whereas lack of inhibition indicates a resistant (zym^R) response. The Elongator mutant LFY5 (*tot3*Δ) served as zym^R control. Growth was on glucose (YPD) or galactose (YPG) medium. (B) Sit4 is nucleocytoplasmic. Total extracts (TE) from lysed CY3938 (*sit4*Δ) spheroplasts carrying pCB243 (UAS_{SIT4}::*SIT4-HA*) (Sutton *et al*., 1991) and DJYHS4 (UAS_{GAL1}::$(HA)_3$-*SIT4*) spheroplasts grown in YPD (left) or YPG (right) were immunoprobed together with separated cytoplasmic (Cy) and nuclear (Nu) fractions by using Sit4- and HA-specific antibodies to detect the PP2A-type Sit4. Marker distribution used anti-Nop1 (nucleus), anti-RFA (cytoplasm and nucleus), and anti-Cdc19 (predominantly cytoplasm) antibodies. To test effects of promoter-dependent *SIT4* expression on zymocin sensitivity, both promoter scenarios (UAS_{SIT4} vs. UAS_{GAL1}) were assayed by killer eclipse assays (top; also see A). (C) Ceramide assay. Tenfold dilutions of *S. cerevisiae* strains (W303-1A: wild-type, CY3938: *sit4*Δ, LFY3: *tot1*Δ, LFY4: *tot2*Δ, LFY5: *tot3*Δ and LFY6: *tot4*Δ) were spotted on YPD plates without (control) and with ceramide. Sensitivity and resistance to ceramide are indicated (cer^S and cer^R, respectively).

(Figure 1A). On separating the cytoplasmic and nuclear fractions from galactose-grown $(HA)_3$-*SIT4* cells, Sit4 was found to be predominantly nuclear localized with a minor cytoplasmic pool (Figure 1B, right). Similarly, in *sit4*Δ cells, HA-tagged Sit4 expressed from its natural promotor on pCB243 (Sutton *et al*., 1991) showed up in both cytoplasmic and nuclear fractions (Figure 1B, left). Therefore, we conclude that contrary to data inferred from indirect immunofluorescence microscopy by using multicopy *SIT4* cells (Sutton *et al*., 1991), Sit4 cannot be considered to be exclusively cytoplasmic, a notion supported by several independently observed nuclear Sit4 interactions (Arndt *et al*., 1989; Ho *et al*., 2002, Singer *et al*., 2003). As for its zymocin relevant function, it is noteworthy that comparably with balanced nucleocytoplasmic localization in the *SIT4* wild-type promoter context (Figure 1B, top left), *GAL1*-promoter induced nuclear accumulation of Sit4 did not alter a yeast cell's vulnerability to the toxin complex (Figure 1B, top right). This supports a nuclear-associated role of Sit4 for zymocin's lethal action and is in line with fractionation data that recently reported Elongator to be preferentially found in the nucleus (Fichtner *et al*., 2003). Comparing functional linkage between Sit4, Elongator and Tot4, an Elongator partner protein (Fichtner *et al*., 2002a,b), we checked how *tot1-4*Δ and *sit4*Δ cells responded to C2-ceramide, another *SIT4*-dependent G1 cell cycle blocker (Nickels and Broach, 1996). Unlike wild-type cells and consistent with phenocopying zymocin protection, cells lacking Elongator, Tot4, or Sit4 efficiently resisted ceramide (Figure 1C). So, Elongator, Tot4, and Sit4 function together in cell cycle-related processes susceptible to antizymotics.

Sit4•Sap155 Is Dispensable for Elongator Expression and Elongator Complex Integrity

Because *sit4* mutants display pol II transcription defects (Arndt *et al*., 1989), we checked whether *sit4*Δ cells affect Elongator gene expression, a condition predicted to induce zymocin resistance (Frohloff *et al*., 2001). We examined transcription of *TOT1-7* by RT-PCR and found essentially identical mRNA levels for these Elongator subunits in *SIT4* and *sit4*Δ cells (Figure 2A; our unpublished data). Comparison of Tot1-5 levels in multicopy *SAP155* cells, which phenocopy loss of *SIT4* function with regards to zymocin resistance (Jablonowski *et al*., 2001a), also failed to show an effect of Sit4•Sap155 on Elongator expression at the translational level. Thus, c-Myc–tagged Tot protein levels were hardly affected by excess Sap155 (Figure 2B; our unpublished data). Intriguingly, c-Myc-tagged Tot1 (Elp1), separated as two distinct forms (Figure 2B), confirming recent data that Tot1 may undergo proteolysis (Fichtner *et al*., 2003). Our failure to detect alterations in Elongator subunit expression prompted us to study whether Sit4 affects Elongator assembly, disruption of which protects from zymocin (Fichtner *et al*., 2002b; Frohloff *et al*., 2003). Coimmune precipitation between Elongator subunits in the presence or absence of Sit4 revealed that all the individual subunit interactions tested remained unaffected in *sit4*Δ cells; contacts between Tot3-Tot5, Tot4-Tot3, and Tot4-Tot5 (Figure 2C) were unaltered. In conclusion, zymocin-resistance of *sit4*Δ or multicopy *SAP155* cells is not based on deregulation of Elongator subunit expression or interference with Elongator complex assembly or integrity.

Elongator Is a Phospho-Complex

To test whether Sit4 rather acts posttranslationally, we asked whether Elongator is phosphomodified. Protein extracts obtained from *TOT2-(c-myc)*$_3$ cells coexpressing *TOT1-(HA)*$_6$,

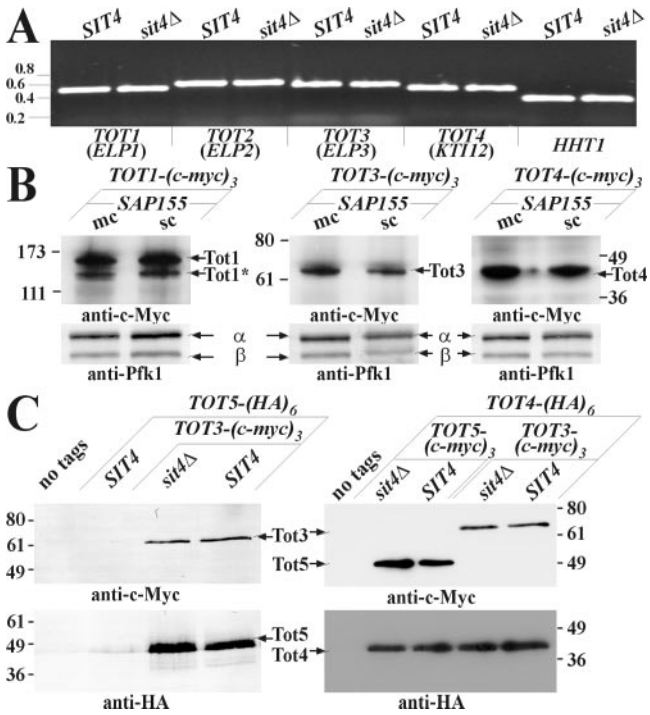

Figure 2. Elongator expression and complex assembly in correlation to *SIT4* and *SAP155*. (A) Elongator gene transcription (*TOT1-4*) is unaffected by *sit4*Δ. RT-PCR was used to compare expression of *TOT1-4* in CY4029 (*SIT4*) and CY3938 (*sit4*Δ) strains. Histone H3 (*HHT1*) served as control. Numbers refer to kilobases (Gene Ruler; MBI Fermentas). (B) Elongator expression is unaffected by multicopy *SAP155*. Identical amounts of protein extracts from c-Myc–tagged Elongator strains DJY1t-a (*TOT1-*$(c\text{-}myc)_3$), FFY3t (*TOT3-*$(c\text{-}myc)_3$) and FFY4t-a (*TOT4-*$(c\text{-}myc)_3$) carrying multi- (mc) or single copy (sc) *SAP155* were immunoprobed by 9E10 (anti-c-Myc). Loading was followed using anti-Pfk1 antibody (recognizing phosphofructokinase α and β subunits as indicated). Positions of c-Myc-tagged Tot proteins are marked by arrows. Tot1 separates (at least) in two forms, with the faster (*) being N-terminally truncated (Fichtner *et al*., 2003). (C). Loss of *SIT4* does not affect Elongator assembly. Equal amounts of protein extracts obtained from wild-type *S1T4* DJY108, DJY110, and DJY112 and *sit4*Δ DJY107, DJY109, and DJY111 strains expressing the indicated tagged Tot proteins were subjected to 9E10 (anti-c-Myc) immunoprecipitations. Detection of Tot3 and Tot5 used 9E10 (anti-c-Myc), monitoring HA-tagged Tot5 or Tot4 used 3F10 (anti-HA). Numbering (B and C) refers to kilodaltons of molecular markers (Invitrogen, Carlsbad, CA).

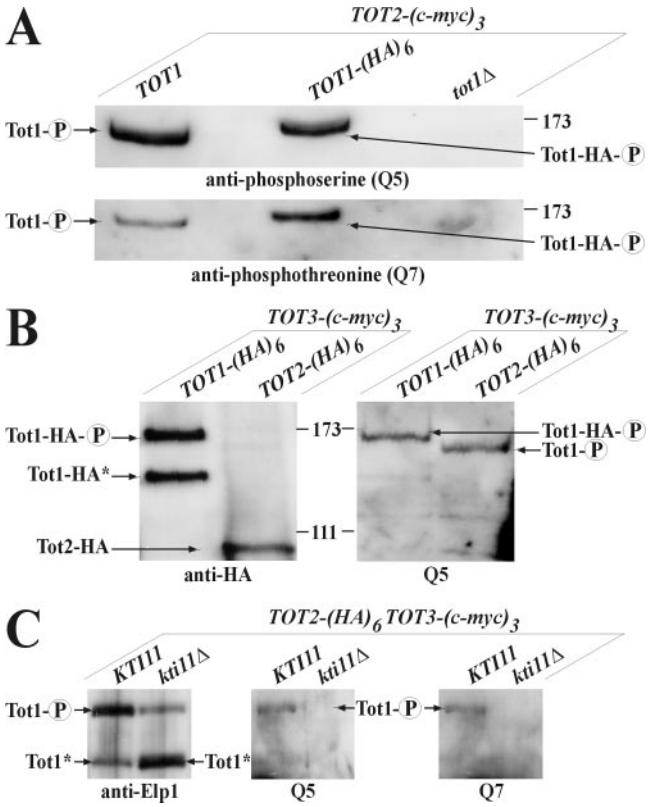

Figure 3. Elongator is a phospho-complex. (A) 9E10 (anti-c-Myc) immunoprecipitates of strains FFY2t-a (*TOT2-*$(c\text{-}myc)_3$), FFY2/1dt (*TOT2-*$(c\text{-}myc)_3$ *TOT1-*$(HA)_6$), and DJY2t1d-a (*TOT2-*$(c\text{-}myc)_3$ *tot1*Δ) were subjected to 6% SDS-PAGE analysis and probed with Q5 and Q7 antibodies, respectively. Phosphoforms (Ⓟ) of Tot1 and Tot1-HA are shown by arrows. (B) Phosphorylation of Tot1 requires full-length protein. 9E10 (anti-c-Myc) immunoprecipitates from strains FFY3/1dt (*TOT3-*$(c\text{-}myc)_3$ *TOT1-*$(HA)_6$) and FFY3/2dt (*TOT3-*$(c\text{-}myc)_3$ *TOT2-*$(HA)_6$) were probed with 3F10 (anti-HA) and Q5 to detect Tot1-HA, Tot2-HA, and phosphoforms (Ⓟ) of Tot1 and Tot1-HA. The truncated (~20 kDa) Tot1-HA form is indicated (*) (also see Figure 2B). (C) Phosphorylation of Tot1 is supported by Elongator partner protein Kti11. 9E10 (anti-c-Myc) immunoprecipitates from the strains FFY3/2dt (*KTI11*) and DJY3/2-d11 (*kti11*Δ) coexpressing epitope-tagged Tot2 and Tot3 were probed with anti-Elp1/Tot1, Q5 and Q7 antibodies. Truncated Tot1 is indicated (*). Numbers (A and B) refer to protein marker sizes in kilodaltons (Invitrogen).

wild-type *TOT1* or carrying a *tot1*Δ null-allele were subjected to immunoprecipitation followed by Western blots by using anti-phosphoserine (Q5) and anti-phosphothreonine (Q7) antibodies. Remarkably, Tot1 produced strong Q5/Q7 signals, which were lost in *tot1*Δ cells as expected (Figure 3A). Precipitates from *TOT1-*$(HA)_6$ expressors produced a Q5/Q7-immunoreactive form of Tot1-HA that, compared with wild-type Tot1, showed an electrophoretic up-shift of ~10 kDa, which is in line with the expected ~9 kDa $(HA)_6$-tag extension (Figure 3A). Immunoprecipitates obtained from *TOT3-*$(c\text{-}myc)_3$ cells coexpressing *TOT1-*$(HA)_6$ or *TOT2-*$(HA)_6$ were analyzed by Western blots with 3F10 (anti-HA) and Q5 antibodies. Again, Q5 signals correlating to the slowest migrating Tot1-HA form (Figure 3B) were found. Wild-type Tot1 produced a Q5 signal down-shifted by ~10 kDa relative to Tot1-HA as expected (Figure 3B). In contrast, an electrophoretically faster Tot1-HA* variant, down-shifted by ~20 kDa, was not Q5-responsive (Figure 3B). Thus, Elongator is a phospho-complex, consisting of (at least) two Tot1 species that differ from each other by phosphorylation and by a ~20-kDa mobility shift. Because the latter is likely to involve proteolytic truncation in a manner suppressed by Kti11, an Elongator partner protein vital for zymocin and diphtheria toxin sensitivity (Fichtner and Schaffrath, 2002; Fichtner *et al*., 2003; Liu and Leppla, 2003), we tested whether *KTI11* affects Elongator phosphorylation. We immunoprecipitated Elongator from *KTI11* or *kti11*Δ cells coexpressing *TOT3-*$(c\text{-}myc)_3$ and *TOT2-*$(HA)_6$ and probed the precipitates by using anti-Elp1/Tot1 (Otero *et al*., 1999), Q5, and Q7 antibodies. In *kti11*Δ cells, the Tot1 down-shift (~20 kDa) was significantly pronounced as judged from a steep decrease of full-length Tot1 and a parallel increase of truncated Tot1 (Figure 3C). Markedly, *kti11*Δ cells no longer produced the Q5/Q7-signals corresponding to full-length Tot1 of *KTI11* cells (Figure 3C). Thus, Tot1 phosphorylation is coupled to the full-length protein. Given the low levels of

full-length Tot1 in *kti11*Δ cells, however, it remains to be seen whether Tot1 phosphorylation depends on *KTI11* gene function or whether it evaded detection due to underrepresentation. Nonetheless, Kti11 is biochemically and genetically linked to Elongator, and *kti11*Δ cells induce TOT deficiency (Fichtner and Schaffrath, 2002; Fichtner *et al*., 2003).

Dephosphorylation of Elongator Depends on Sit4•Sap185/190

To examine the relationship between Tot1 phosphomodification and Sit4 function, Elongator was immunoprecipitated from a *SIT4* strain and its isogenic *sit4*Δ knockout. Tot1 was identified using the anti-Elp1/Tot1 antibody (Figure 4A). Importantly, Tot1 isolated from *sit4*Δ cells electrophoretically migrated more slowly than Tot1 isolated from an equivalent *SIT4* strain (Figure 4A). That this reflected phosphorylation of the slower Tot1 band is supported by Western blots by using the Q5 antibody on Elongator immunoprecipitates from the same *SIT4* and *sit4*Δ strains. Again, whereas in *sit4*Δ cells, a more abundant, up-shifted phospho-Tot1 species accumulated (Figure 4A), *SIT4* cells contained an electrophoretically faster and hypophosphorylated Tot1 form (Figure 4A) that may reflect a minor Sit4-insensitive Tot1 pool or a steady-state balance with phospho-Tot1 being underrepresented in *SIT4* cells (Figure 6A). Collectively, loss of *SIT4* suppresses Tot1 dephosphorylation. Among the Saps, Sit4-specific associators that act as Sit4 activators (Luke *et al*., 1996), multicopy *SAP155* has been shown to confer zymocin resistance in a manner antagonized by excess Sap185 and Sap190 (Jablonowski *et al*., 2001a). To check whether high Sap155 out-titrates binding of Sap185/190 to Sit4, we compared Tot1-HA from *sit4*Δ cells and from *SAP4*, *SAP155*, *SAP185*, and *SAP190* overexpressors. Analysis of total protein extracts revealed identical electrophoretic up-shifts of HA-tagged Tot1 produced from *sit4*Δ and multicopy *SAP155* cells. Thus, consistent with phenocopying zymocin resistance of *sit4*Δ cells, excess Sap155 sustains phospho-Tot1 levels. In contrast, multicopy *SAP4*, *SAP185*, and *SAP190* produced Tot1 patterns similar to *SIT4* wild-type cells (Figure 4B). To test whether the effect of multicopy *SAP155* on sustaining phospho-Tot1 levels could be counteracted by excess Sap185 and Sap190, we cointroduced multicopy *SAP185*, *SAP190*, or both into *SAP155* overexpressors and investigated the electrophoretic behavior of Tot1-HA. Consistent with bypassing zymocin resistance, multicopy *SAP190* and *SAP185/190* suppressed the effect of multicopy *SAP155* on Tot1 phosphorylation (Figure 4C). Thus, as judged from suppressing the electrophoretic up-shift of Tot1 produced in multicopy *SAP155* cells, Sap185/190 specifically counteract Sap155 (Figure 4C). Contrary to *sap4*Δ*sap155*Δ cells, the *sap185*Δ*sap190*Δ double mutant up-regulated Tot1 phosphorylation (Figure 4D) and induced *sit4*Δ-like zymocin resistance (Figure 5A). Collectively, these data indicate that Tot1 dephosphorylation requires the Sit4 phosphatase in combination with Sap185 and/or Sap190.

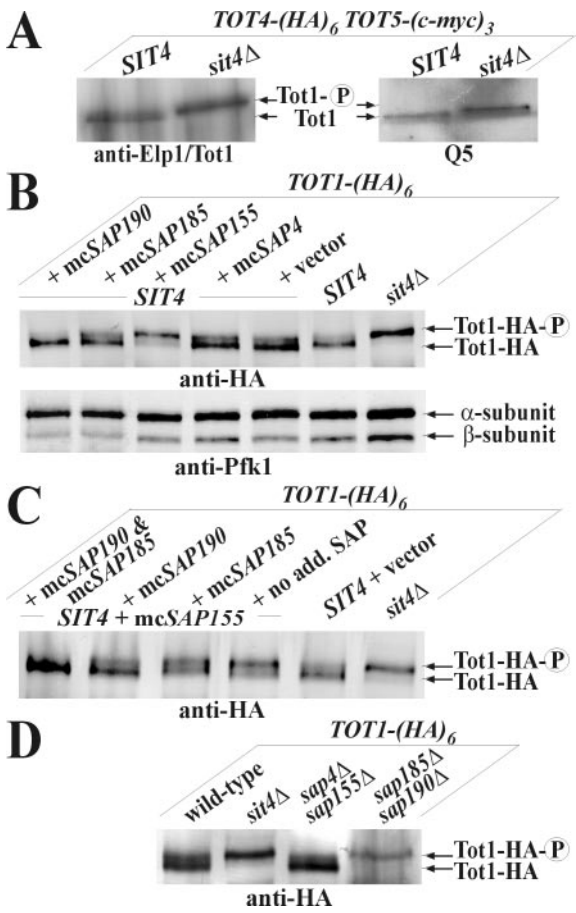

Figure 4. Sit4•Sap185/190 is specific for Tot1 dephosphorylation. (A) *sit4*Δ cells suppress Tot1 dephosphorylation. 9E10 (anti-c-Myc) Elongator immunoprecipitates from strains DJY110 (*SIT4 TOT5-(c-myc)*$_3$ *TOT4-(HA)*$_6$) and DJY109 (*sit4*Δ *TOT5-(c-myc)*$_3$ *TOT4-(HA)*$_6$) were probed with anti-Elp1/Tot1 and Q5 antibodies. The positions of hypo- (Tot1) and hyperphosphorylated Tot1 (Ⓟ) forms are shown. *sit4*Δ cells accumulate the up-shifted Tot1-Ⓟ form. (B) *sit4*Δ or multicopy *SAP155* cells suppress Tot1 dephosphorylation. Protein extracts from strains DJY103 (*sit4*Δ *TOT1-(HA)*$_6$), DJY102 (*SIT4 TOT1-(HA)*$_6$), and DJY102 carrying the indicated multicopy *SAP* constellations were standardized by anti-Pfk1 (see Figure 2B) and probed with 3F10 (anti-HA). (C) Suppressed Tot1 dephosphorylation by multicopy *SAP155* is antagonized by excess Sap185/190. Protein extracts from indicated *TOT-(HA)*$_6$ expressors were probed with 3F10 (anti-HA) to monitor Tot1-HA migration dependent on *SAP* copy number. (D) *sap185*Δ*sap190*Δ cells phenocopy high phospho-Tot1 levels of *sit4*Δ cells. Strains DJY102 (*SIT4*), DJY103 (*sit4*Δ), DJY115 (*sap4*Δ*sap155*Δ), and DJY116 (*sap185*Δ*sap190*Δ) expressing *TOT1-(HA)*$_6$ were analyzed as described above (B and C). Non- and phosphorylated forms (Ⓟ) of Tot1-HA (B–D) are shown by arrows; vector control (B and C) denotes empty YEp24 vector used to clone the *SAP* genes in multicopy (Luke *et al*., 1996).

Sit4•Sap190 Opposes Zymocin Resistance Induced by Multicopy TOT4

Because defects in several other Ser/Thr phosphatases (*pph21*Δ, *pph22*Δ, *pph21*Δ*pph22*Δ, *ppz1*Δ, *ppz2*Δ, *ppz1*Δ*ppz2*Δ, *pph3*Δ, and *ppg1*Δ), including calcineurin (*cna1*Δ*cna2*Δ and *cnb1*Δ), neither affected phospho-Tot1 levels nor zymocin sensitivity (Figure 5A; our unpublished data), dephosphorylation of Elongator is specifically linked to the PP2A-type phosphatase Sit4. To study a possible role in Elongator regulation, we compared the relationship between Sit4, Tot4, and the lethal response toward zymocin. Tot4, a potential Elongator-pol II up-loader, is able to protect cells against zymocin either when overexpressed or removed from the cell (Frohloff *et al*., 2001, 2003; Fichtner *et al*., 2002a,b). Whereas multicopy *TOT4* cells and *tot4*Δ cells show similar zymocin resistance, multicopy *SIT4* on its own has no effect (Figure 5B). However, we found that when multicopy *SIT4* was cointroduced into multicopy *TOT4* cells, zymocin protection associated with high copy *TOT4* was slightly antagonized in the presence of extra Sit4 (Figure

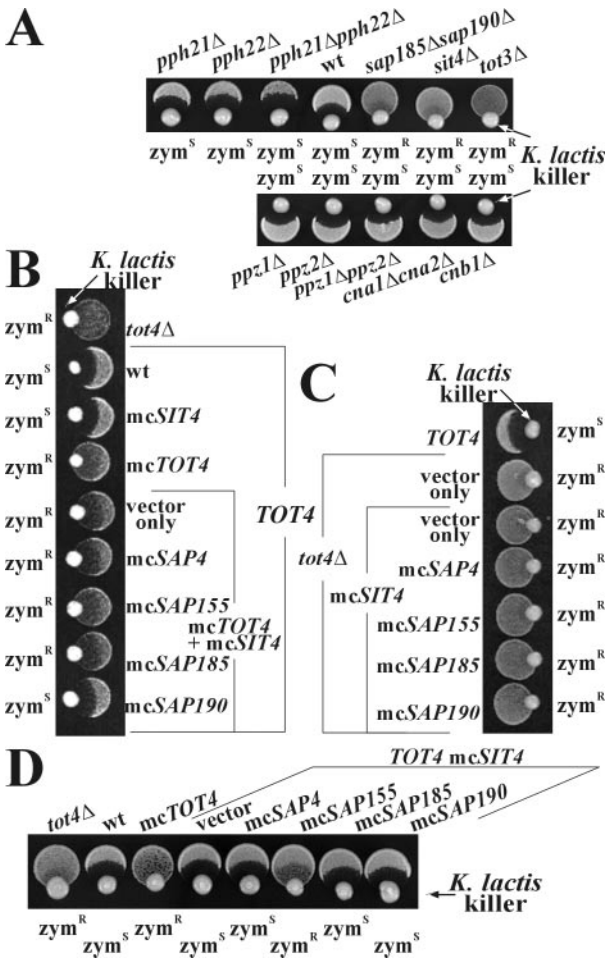

Figure 5. Zymocicity strictly depends on Sit4•Sap185/190 and high copy *TOT4* zymocin resistance is suppressed by Sit4•Sap190. (A) *K. lactis* killer eclipse assays (see Figure 1A) involved *S. cerevisiae* strains LFY5 (*tot3*Δ), AY925 (wild-type (wt)), and phosphatase mutants CY3938 (*sit4*Δ), CY5224 (*sap185*Δ*sap190*Δ), DEY132-1C (*pph21*Δ), DEY10-2B (*pph22*Δ), DEY132-2C (*pph21*Δ*pph22*Δ), EDN75 (*ppz1*Δ), JA103 (*ppz2*Δ), EDN76 (*ppz1*Δ*ppz2*Δ), MMY09 (*cna1*Δ*cna2*Δ), and YJN519 (*cnb1*Δ). (B) Eclipse assays involving resistant control DYJ100 (*tot4*Δ) and wild-type *TOT4* strain CY4029 carrying multicopy (mc) *TOT4*, *SIT4*, and/or *SAP* genes. (C) Eclipse assays involving mc*SIT4/SAP* genes maintained in strain DYJ100 (*tot4*Δ) and compared with wild-type *TOT4* CY4029 cells. (D) Eclipse assays of zymocin-resistant strain DYJ100 strain (*tot4*Δ) and wild-type CY4029 (*TOT4*) cells carrying the indicated mc*SIT4/SAP* genes. As a high copy control served mc*TOT4*. For phenotypic zymocin read-outs, see Figure 1A. Vector controls (B–D) refer to empty YEplac181, YEp24, and YEplac112 plasmids used to clone the *SIT4*, *SAP*, and *TOT4* genes, respectively (Butler *et al.*, 1994; Luke *et al.*, 1996; Jablonowski *et al.*, 2001c).

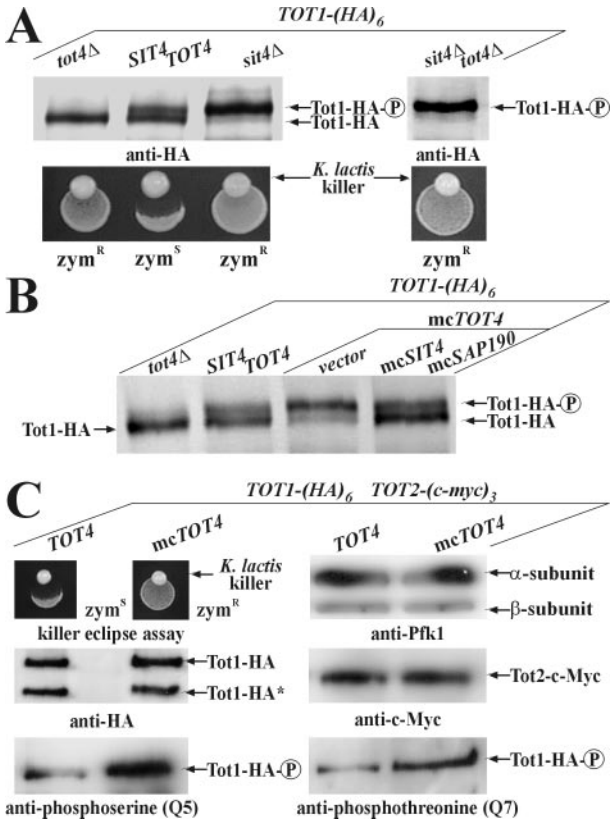

Figure 6. Balanced de-/phospho-Tot1 ratios involve opposing effects of Tot4 and Sit4. (A) *tot4*Δ cells enhance Tot1 dephosphorylation. Standardized protein extracts from indicated *TOT1-*$(HA)_6$ expressing strains DJY102 (*SIT4 TOT4*), DJY103 (*sit4*Δ), DJY104 (*tot4*Δ), and DJY105 (*sit4*Δ*tot4*Δ) were probed with 3F10 (anti-HA). The positions of non- and phosphorylated (Ⓟ) Tot1-HA forms are shown. (B) Excess Tot4 shifts electrophoretic mobility of Tot1, a situation antagonized by excess Sit4•Sap190. Standardized protein extracts from strain DJY102 (*SIT4 TOT4*) expressing *TOT1-*$(HA)_6$ and maintaining multicopy (mc) *TOT4*, *SIT4*, and *SAP190* genes as indicated were probed with 3F10 (anti-HA). As a control served DJY104 (*tot4*Δ) (see A). (C) mc*TOT4* intensifies Tot1 phosphorylation. 9E10 (anti-c-Myc) immunoprecipitates from strain FFY2/1dt (*TOT1-*$(HA)_6$ *TOT2-*$(c\text{-}myc)_3$) maintaining single copy or mc*TOT4* were probed with 3F10 (anti-HA), 9E10 (antic-Myc), Q5 (anti-phosphoserine) and Q7 (anti-phosphothreonine) antibodies to detect Tot1-HA, Tot2-c-Myc and phospho-Tot1-HA (Ⓟ). Before immunoprecipitation, extracts were standardized by anti-Pfk1 to follow content of Pfk1 α and β subunits (Figure 2B). Tot1-HA truncation is indicated (*). For zymocin read-outs (A and C), see Figure 1A.

5B). This effect was significantly enhanced by cointroducing multicopy *SAP190*, which reinstated wild-type zymocin sensitivity to the multicopy *SIT4 TOT4* strain (Figure 5B). The action is specific because *SAP4*, *SAP155*, and *SAP185* remained ineffective in comparison with *SAP190* (Figure 5B). *tot4*Δ-associated resistance, however, was not opposed by multicopy *SIT4 SAP190* (Figure 5C), nor was normal zymocin sensitivity of single copy *TOT4* cells affected by multicopy *SIT4 SAP190* (Figure 5D). In a single copy *TOT4* strain, only multicopy *SIT4 SAP155* suppressed zymocin sensitivity (Figure 5D), most likely as a result of outcompeting Sap185/190 from Sit4 binding and augmenting Tot1 phosphorylation (Figure 4B). In conclusion, suppression of excess Tot4 by elevated Sit4•Sap190 implies competition between Tot4 and Sit4•Sap190 for a shared factor (TOT/Elongator) and opposing effects on Tot1 phosphorylation.

Tot4 Antagonizes Sit4-dependent Dephosphorylation of Elongator

To correlate genetic suppression between Sit4 and Tot4 on zymocin action (Figure 5B) with Tot1 dephosphorylation, we analyzed Tot1-HA expressed from *sit4*Δ, *tot4*Δ, *tot4*Δ*sit4*Δ, *TOT4 SIT4* wild-type and multicopy *TOT4* cells by using the anti-HA antibody in Western blots. Although zymocin-resistant *sit4*Δ cells exclusively reproduced high phospho-Tot1 levels (Figure 6A), sensitive *TOT4 SIT4* wild-

type cells expressed a balanced de-/phospho-Tot1 ratio with phospho-Tot1 being slightly underrepresented (Figure 6A). Conversely, in zymocin-resistant *tot4*Δ cells, phospho-Tot1 was abolished, whereas a zymocin-resistant *tot4*Δ*sit4*Δ double mutant phenocopied the phospho-Tot1 levels present in single *sit4*Δ cells (Figure 6A). Thus, removal of Sit4 or Tot4 affect Tot1 phosphomodification in opposite ways, and *tot4*Δ cells intensify Tot1 dephosphorylation. In line with *TOT4* influencing Elongator phosphorylation, zymocin resistant-multicopy *TOT4* cells up-regulated phospho-Tot1 levels compared with balanced de-/phosphorylation of zymocin-sensitive wild-type *TOT4* cells (Figure 6B). Remarkably, stimulation of Tot1 phosphorylation by excess Tot4 as detected by intensified Q5/Q7-immunoreactivity of Tot1 in Elongator immunoprecipitations (Figure 6C) did not interfere with Elongator complex assembly or subunit interaction: stoichiometric coimmunoprecipitation of Elongator subunits Tot1 and Tot2 was unaffected by multicopy *TOT4* cells (Figure 6C). Consistent with genetic suppression and functional antagonism, excess Sit4•Sap190 enhanced Tot1 dephosphorylation in multicopy *TOT4* cells, reinstated Tot1's wild-type phospho-balance (Figure 6B), and reinstalled zymocin sensitivity (Figure 5B). Thus, a fine-tuned balance of Tot1 de-/phosphomodification that is critical for Elongator's TOT function is mutually controlled by *SIT4* and *TOT4*, and our data provide evidence to suggest that locking Tot1 into either a hyper- or hypophosphorylated state is detrimental to Elongator function and protects against the G1 cell cycle block by zymocin.

*Tot1, Tot4, and Sit4 Cofractionate and sit4*Δ*-Nuclei Retain Tot4*

Consistent with Sit4-dependent Tot1 dephosphorylation being under Tot4 control, sucrose gradient cell fractionations demonstrated that HA-tagged Sit4 and Tot4 essentially comigrated (Figure 7A). A strikingly similar fractionation pattern was produced by HA-tagged Tot1, and peak fractions coincided with maximal levels of either Tot4 or Sit4 (Figure 7A, lanes 2 and 3). This implies both a physical and functional relationship among Sit4, Tot4, and Elongator. Intriguingly, although the Tot4•Elongator contact does not require *SIT4* function (our unpublished data), Sit4 seems to impact the subcellular distribution of Tot4 itself. In contrast to *SIT4* cells, which showed a balanced distribution of cytoplasmic and nuclear Tot4 pools, Tot4 was preferentially retained in *sit4*Δ-nuclei with a parallel drop in the cytoplasmic pool (Figure 7B). Provided this was due to prolonged pol II•Elongator association, Sit4 may act following the assembly of the HAT-productive holo-Elongator (Winkler *et al*., 2002) by recycling the potential up-loader Tot4. So, Sit4 likely plays a nuclear role for Elongator's TOT function and zymocin-resistant *sit4*Δ cells may have an Elongator•pol II up-loading defect.

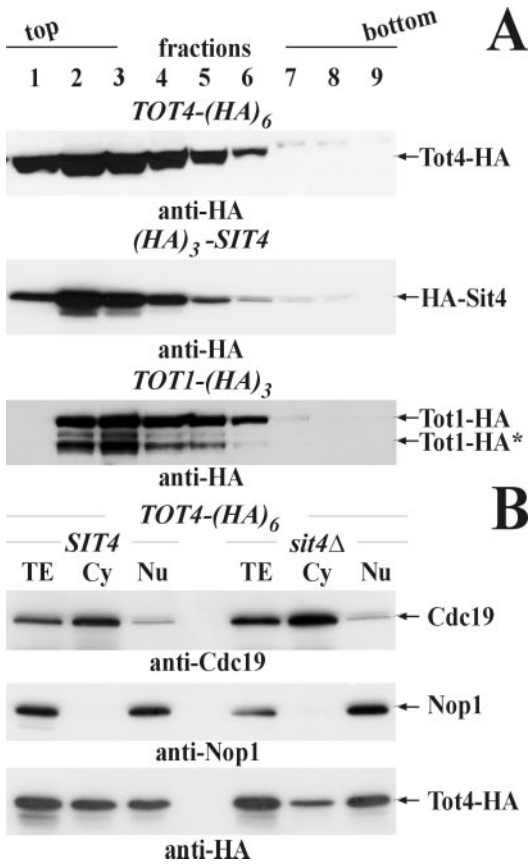

Figure 7. Sit4, Tot4, and Tot1 cofractionate and Tot4 is retained in *sit4*Δ-nuclei. (A) Cell fractionation. Tot4-HA, HA-Sit4, and Tot1-HA were detected in sucrose-gradient fractions (lanes 1–9 from a total of 14) from strains DJY114 (*TOT4-*$(HA)_6$), DJYHS4 ($(HA)_3$-*SIT4*), and DJY8A-1H3 (*TOT1-*$(HA)_3$) by using 3F10 (anti-HA). Their positions are indicated by arrows. The truncated Tot1-HA form (see Figure 3B) is indicated (*). (B) Tot4 is retained in *sit4*Δ-nuclei. Fractions of DJY114 (*SIT4*) or DJY113 (*sit4*Δ) cells expressing *TOT4-*$(HA)_6$ were probed using 3F10 (anti-HA) and compared with nuclear and cytoplasmic marker proteins Nop1 and Cdc19, respectively (see Figure 1C). Their positions are indicated by arrows.

DISCUSSION

Dephosphorylation of Elongator Subunit 1 Depends on Sit4•Sap185/190

Like Elongator mutants, cells lacking the phosphatase Sit4 or its associators Sap185 and Sap190 survive the *K. lactis* zymocin (Frohloff *et al*., 2001; Jablonowski *et al*., 2001a,c). In line with a link to pol II Elongator, *SIT4* was originally identified as a transcriptional suppressor, and *sit4* mutations combined with pol II defects are synthetically lethal (Arndt *et al*., 1989). Our findings that *sit4*Δ and *tot*Δ cells equally well resist the ceramide-induced G1 block (Nickels and Broach, 1996) (Figure 1C) reinforce functional linkage between Sit4 and pol II Elongator in processes required for progression through G1. Our finding that directly tagging Sit4's C-terminal –YFL motif elicits zymocin resistance (Figure 1A) may be interpreted that this PP2A-type domain known to mediate subunit interaction enables Sit4 to form subcomplexes with zymocin-relevant associators (Evans and Hemmings, 2000; Wei *et al*., 2001). Consistently, here we underscore that Sit4 associators Sap4, Sap155, Sap185, and Sap190 act in two functional subfamilies (Sap4/155 and Sap185/190). Only combined loss of Sap185 and Sap190 leads to zymocin resistance (Figure 5B), whereas *sap4*Δ*sap155*Δ cells and triple *sap* deletion strains still containing either Sap185 or Sap190 are zymocin sensitive (our unpublished data). As for Elongator, a phospho-complex (Figure 3), dephosphorylation of its largest subunit (Tot1/Elp1) is shown here to depend on Sit4•Sap185 and/or Sit4•Sap190 (Figure 4). Intriguingly, *SAP185* cannot effectively replace *SAP190* in suppressing multicopy *TOT4* zymocin resistance (Figure 5B). With regard to zymocin action, Sap190 thus seems to be the more potent member of the Sap185/190 subfamily. Contrary to *sap185*Δ*sap190*Δ, *sap4*Δ*sap155*Δ cells have normal phospho-

Tot1 levels (Figure 4D). However, multicopy *SAP155* induces zymocin resistance with high phospho-Tot1 levels similar to *sit4*Δ and *sap185*Δ*sap190*Δ cells (Figure 4, B and D). Because this effect is antagonized by multicopy *SAP185* or *SAP190* (Figure 4C), excess Sap155 is likely to abduct Sit4 from its TOT-relevant associators Sap185/190, a notion consistent with the observation that Sap155 is particularly important for the TOR pathway (Jacinto *et al*., 2001). This adds to our proposal that despite a shared requirement for *SIT4*, there is hardly any evidence for Sit4-mediated TOT-TOR cross talk (Jablonowski *et al*., 2001a). In line, excess Sap185/190 opposes Sap155's ability to remove Sit4 and reinstates wild-type Tot1 phospho-balance and zymocin sensitivity. Collectively, besides other dephosphorylation events related to the functioning of the TOR pathway (Crespo and Hall, 2002), Sit4•Sap185/190 promotes Elongator dephosphorylation and conditions the zymocin G1 block.

Unlike suppression of multicopy *TOT4* zymocin resistance, Sit4•Sap190 fails to antagonize zymocin protection of *tot4*Δ cells (Figure 5C). Thus, suppression requires Tot4 to be physically present. This reinforces the TOT relevant role of Sit4 and implies that Sit4 competes with or opposes Tot4 (for a shared Elongator substrate). Consistently, phospho-Tot1 levels are entirely abolished in *tot4*Δ cells, whereas excess Tot4 intensifies Tot1 phosphorylation (Figure 6). This indicates that Sit4 may be kept in check by Tot4 inhibition. Based on its capability to occupy promoter DNA and to associate with pol II and Elongator, Tot4 has been proposed to play a regulatory role, possibly as a candidate pol II-Elongator up-loader (Fichtner *et al*., 2002a,b; Frohloff *et al*., 2003). Our findings that Sit4, Tot4, and Tot1 cofractionate (Figure 7A) and that *sit4*Δ cells increase, whereas *tot4*Δ abolish Tot1 phosphorylation (Figures 4A and 6A) suggest Elongator loading via Tot4 may require *SIT4*. Accordingly, high Sit4•Sap190 levels could correct deregulated loading due to excess Tot4 by enhancing dephosphorylation of Tot1 (or increasing Tot4 recycling). Consistently, Tot4 is retained in *sit4*Δ nuclei (Figure 7B), a scenario that may reflect a loading or recycling defect. Together with the findings that high phospho-Tot1 levels typical of *sit4*Δ cells remain unaltered in *sit4*Δ*tot4*Δ cells (Figure 6A) and that Tot4 does not relate to any known protein kinase, Tot4 is unlikely to act as a Sit4-antagonizing Elongator kinase.

Despite the elusive nature of an Elongator kinase, we report here on a supporting activity of Kti11 on Tot1 phosphorylation (Figure 3C). Kti11, recently identified as Elongator partner protein and zymocin and diphtheria toxin sensitivity factor, also interacts with components of the diphthamide synthesis pathway crucial for bacterial toxins to ADP-ribosylate elongation translation factor EF2. *kti11*Δ cells lack EF2 ADP-ribosyl acceptor activity, survive zymocin, and accumulate a truncated Tot1 form shown here to be phosphorylation-resistant (Fichtner and Schffrath, 2002; Fichtner *et al*., 2003; Liu and Leppla, 2003). If Elongator phosphoacceptor sites are identifiable by comparative mass spectrometry on *SIT4*, *sit4*Δ, and *kti11*Δ cells and whether Elongator links up to diphthamidisation via Kti11 are intriguing questions presently under investigation.

A Model for Sit4's Role in the G1 Block by K. lactis Zymocin

A model (Figure 8) that combines a yeast cell's requirement for Sit4 and Elongator to become blocked in G1 by zymocin's γ-toxin subunit can be conceived. In the absence of γ-toxin, either an alternative pathway (in *SSD1-v* strains) or TOT/Elongator dephosphorylation dependent on Sit4 can activate a key cell cycle regulator needed for G1 exit (Figure 8A). In the presence of γ-toxin, this key activator is sequestered in combination with TOT, the possible Sit4 substrate, regardless of whether the alternative *SSD1-v* pathway is normally sufficient and hence Sit4 dispensable (Figure 8B). Eventually, this leads to inactivation of the crucial activator, preventing START execution and G1 exit. If cells fail to dephosphorylate Tot1 (*sit4*Δ) (Figure 8C) or do not assemble Elongator (*tot*Δ), they express TOT deficiency that no longer permits γ-toxin to hijack Elongator (Figure 8C). As a result, *sit4*Δ and *tot*Δ cells resist γ-toxin at the expense of a G1 delay due to an Elongator defect. Consequently, reduced activation by the alternative *SSD1-v* pathway alone may cause a cell cycle delay. The findings that *tot*Δ, *sit4*Δ, and *ssd1*Δ cells are each cell cycle affected support these predictions (Sutton *et al*., 1991; Frohloff *et al*., 2001). The model's key activator ought to physically contact TOT/Elongator. As judged from *SSD1-v*–dependent pol II stimulation, copurification between Elongator and pol II and assistance of pol II transcription through chromatin by the Elongator HAT, we propose pol II as this activator (Stettler *et al*., 1993; Otero *et al*., 1999; Winkler *et al*., 2001, 2002; Kim *et al*., 2002). In line, pol II mutations can induce a G1 block per se (Sugaya *et al*., 2001), suggesting that zymocin-dependent Elongator intoxication and pol II limitation may prevent G1 exit. Indeed, zymocin affects pol II performance and CTD phosphorylation

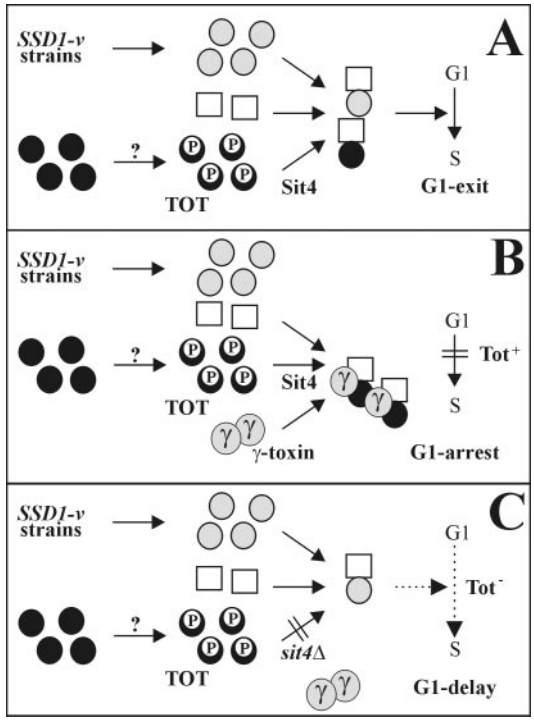

Figure 8. Model for the role of Sit4 in the cell cycle arrest imposed by *K. lactis* zymocin's γ-toxin subunit. In the absence of γ-toxin (A), the *SSD1-v* pathway (◯) and Sit4-dependent dephosphorylation of the toxin-target (TOT) substrate (Ⓟ) activate a key cell cycle regulator (□) required for processes preSTART. In the presence of γ-toxin (B), the critical protein can be sequestered and inactivated in combination with Sit4's dephosphorylated TOT substrate (●) to induce the G1 arrest (Tot^+), irrespective of whether the alternative, Sit4-dispensable *SSD1-v* pathway would be sufficient. Failure to dephosphorylate TOT (C) no longer permits inactivation of the crucial regulator and causes *sit4*Δ cells to resist γ-toxicity (Tot^-) at the expense of being G1 cell cycle delayed. The as yet elusive Elongator-specific kinase is marked by ?.

(Frohloff *et al*., 2001; Jablonowski and Schaffrath, 2002). The findings that phospho-CTD stabilizes Elongator•pol II association, that CTD phosphatase modulation alters the zymocin response and that a Tot1 NLS truncation yields resistance, suggest that zymocin requires nuclear Elongator pools to physically contact pol II (Otero *et al*., 1999; Jablonowski *et al*., 2001c; Kitamoto *et al*., 2002, Fichtner *et al*., 2003).

Because neither assembly of Elongator nor its interaction with Tot4 are Sit4 sensitive (Figure 2C), Sit4 is likely to act after assembly of the Elongator HAT complex. Previous data demonstrated that Elongator HAT productivity conditions TOT proficiency (Frohloff *et al*., 2001; Winkler *et al*., 2001). TOT deficiency due to up-regulated (*sit4*Δ, *sap185*Δ*sap190*Δ, and high copy *SAP155* or *TOT4*) (Figure 4) or down-regulated (*tot4*Δ) phospho-Tot1 levels (Figure 6, A and B) points to HAT regulation by a fine-tuned Tot1 phospho-balance (via *SIT4* and/or *TOT4*). Mutual control of Tot1 de-/phosphomodification by *SIT4* and *TOT4* suggests that phosphorylation of Tot1 does not seem to act as a functional Elongator "on/off" switch; thus, locking Tot1 into either hyper- or hypophosphorylated states impairs Elongator function as assessed by Tot$^-$ phenotypes of multicopy *SAP155* and *TOT4* cells as well as *sit4*Δ, *tot4*Δ, *sit4*Δ*tot4*Δ, and *sap185*Δ*sap190*Δ mutants. Possibly, Elongator's TOT function is supported by alternating de-/phosphorylation cycles. Whether Tot1 dephosphorylation depends directly or indirectly on Sit4 remains open. Although seemingly inconsistent with the cytoplasmic distribution deduced from immunolocalization studies on multicopy *SIT4* cells (Sutton *et al*., 1991), our data that Sit4 is nucleocytoplasmically localized (Figure 1B) suggest a significant nuclear Sit4 pool. Consistently, *sit4* mutations combined with nuclear ubiquitin or pol II defects are lethal, Sit4 physically interacts with Ctk1, a nuclear pol II CTD kinase, and with Hrr25/Kti14, a predominantly nuclear casein kinase I that copurifies with Sap185 and Sap190 (Arndt *et al*., 1989; Ho *et al*., 2002; Singer *et al*., 2003). Intriguingly, like Tot$^-$ Elongator mutants and *sit4*Δ cells, kinase-minus *kti14* mutants survive zymocin (Mehlgarten and Schaffrath, 2003). A possible role of Kti14 as a Sit4 antagonist relevant to Elongator's TOT function and phosphomodification is under investigation.

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