The EMBO Journal Vol. 20 No. 8 pp. 1993–2003, 2001

Saccharomyces cerevisiae Elongator mutations confer resistance to the *Kluyveromyces lactis* zymocin

Frank Frohloff, Lars Fichtner, Daniel Jablonowski, Karin D.Breunig and Raffael Schaffrath[1]

Institut für Genetik, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 10, D-06120 Halle (Saale), Germany

[1]Corresponding author
e-mail: schaffrath@genetik.uni-halle.de

F.Frohloff and L.Fichtner contributed equally to this work

Kluyveromyces lactis **killer strains secrete a zymocin complex that inhibits proliferation of sensitive yeast genera including *Saccharomyces cerevisiae*. In search of the putative toxin target (TOT), we used mTn*3*:: tagging to isolate zymocin-resistant *tot* mutants from budding yeast. Of these we identified the *TOT1*, *TOT2* and *TOT3* genes (isoallelic with *ELP1*, *ELP2* and *ELP3*, respectively) coding for the histone acetyltransferase (HAT)-associated Elongator complex of RNA polymerase II holoenzyme. Other than the typical *elp* ts-phenotype, *tot* phenocopies hypersensitivity towards caffeine and Calcofluor White as well as slow growth and a G_1 cell cycle delay. In addition, *TOT4* and *TOT5* (isoallelic with *KTI12* and *IKI1*, respectively) code for components that associate with Elongator. Intriguingly, strains lacking non-Elongator HATs (*gcn5*Δ, *hat1*Δ, *hpa3*Δ and *sas3*Δ) or non-Elongator transcription elongation factors TFIIS (*dst1*Δ) and Spt4p (*spt4*Δ) cannot confer resistance towards the *K.lactis* zymocin, thus providing evidence that Elongator equals TOT and that Elongator plays an important role in signalling toxicity of the *K.lactis* zymocin.**

Keywords: Elongator/killer yeast/TOT/zymocin

Introduction

In competing for limited resources, microorganisms have evolved sophisticated strategies to gain selective advantages over their competitors. One of these is the secretion of toxic compounds that results in killing or growth arrest of other species or genera. Well studied cases are the dsRNA-encoded viral KT28 and K1 killer toxins of *Saccharomyces cerevisiae* (Wickner, 1996), which cause sensitive yeast cells to irreversibly block DNA synthesis and arrest in S phase (Schmitt *et al*., 1996) or to destroy cytoplasmic membrane function by *TOK1* hyperactivation and lethal ion channel formation (Ahmed *et al*., 1999). Killer dsDNA plasmid-carrying strains of the dairy yeast *Kluyveromyces lactis* also secrete a zymo-toxin (referred to as zymocin) that inhibits cellular growth of various sensitive yeast genera including *S.cerevisiae* (Stark *et al*., 1990; Schaffrath and Breunig, 2000). As judged from fluorescence-activated cell sorter (FACS) analyses, it predominantly causes sensitive budding yeast cells to arrest at the unbudded G_1 stage of the cell cycle with an unreplicated (1*n*) DNA content (Butler *et al*., 1991a). Although reminiscent of pre-START arrests induced by the pheromone cascade or displayed by *cdc28*^{ts} strains at restrictive growth temperatures (Leberer *et al*., 1997), the speculation that zymocin might act by antagonizing G_1 cyclin function does not hold true: hyperactive dominant *CLN3* alleles are not able to rescue sensitive cells from zymocin treatment (Butler *et al*., 1994). Further evidence that the zymocin might act in late G_1 before START is provided by the finding that cells that have been chemically arrested in S phase by hydroxyurea, prior to zymocin treatment, are able to commit one complete round of cell division and get arrested in the new unbudded G_1 cell cycle stage once they have been released from the chemical S block in the continued presence of zymocin (Butler *et al*., 1991a).

Despite the heterotrimeric (αβγ) structure of native holo-zymocin (Stark and Boyd, 1986), intracellular expression of its smallest subunit, the γ-toxin, alone is lethal. Thus, conditional expression of the γ gene from regulatable *GAL* promoters leads to a galactose-dependent G_1 arrest that mimics the effect of native holo-zymocin (Tokunaga *et al*., 1989; Butler *et al*., 1991b). The α and β subunits, however, are needed for holo-zymocin to act from the cell exterior. The β subunit is predicted to form four C-terminal hydrophobic domains, suggesting cell membrane association, while the α subunit exhibits an exo-chitinase activity that is essentially required for holo-zymocin function (Stark *et al*., 1990; Butler *et al*., 1991c). Sensitive yeast cells can be rescued from zymocin action by exogenously applying crude chitin preparations (D.Jablonowski and R.Schaffrath, unpublished data) and chitin-deficient *S.cerevisiae* mutants are resistant towards exo-zymocin (Takita and Castilho-Valavicius, 1993) but not against endogenous γ-toxin expression. These observations suggest a role for the α and β subunits in zymocin docking to sensitive cells, whereas cytotoxicity resides solely within the γ-toxin subunit. Based on their ability to grow in the presence of the holo-form, zymocin-resistant mutants termed *skt* (sensitivity to *K*.lactis toxin), *iki* (insensitive to killer) and *kti* (*K*.lactis toxin insensitive), respectively, have been isolated independently (Kawamoto *et al*., 1990; Butler *et al*., 1994; Kishida *et al*., 1996). Sensitivity of these mutants towards intracellular, conditional expression of the γ-toxin can distinguish zymocin binding/uptake (class I) from potential γ-toxin target site mutants (class II). Mutations in the class I genes *SKT5* and *KTI2* (isoallelic with *CHS4* and *CHS3*, respectively) affect chitin biosynthesis and only lead to exo-zymocin resistance (Kawamoto *et al*., 1993; Butler *et al*., 1994), whereas mutations in the class II genes *KTI12*, *IKI1* and *IKI3*

confer resistance against exo-zymocin and endogenously expressed γ-toxin (Butler *et al*., 1994; Yajima *et al*., 1997). Despite these recent advances, the intracellular toxin target site (TOT) still remains elusive and earlier reports that adenylate cyclase, Cdc35p, is involved in zymocin action (Sugisaki *et al*., 1983) have been ruled out (White *et al*., 1989).

To understand the zymocin mode of action we isolated several γ-toxin target site (*tot*) mutants by way of mTn*3* transposon mutagenesis and PCR-mediated gene disruption following conditional expression of the γ-toxin from within *S.cerevisiae* cells. Thus, we identified mTn*3*:: integrations and re-verified individual knockouts in several *TOT* genes. The *tot*Δ strains are resistant to both exo-zymocin and endogenous γ-toxin expression, providing evidence for true toxin target site mutants. Moreover, loss of *TOT* gene function renders yeast cells additional phenotypes: slow growth, a G_1 cell cycle delay and sensitivity towards the drugs caffeine, Calcofluor White and 6-azauracil (6-AU). Consistent with the latter, which implies a functional role in transcription elongation, we identified the *TOT* gene products as constituents of Elongator and components associated with Elongator, a multisubunit complex interacting with elongating RNA polymerase II holoenzyme.

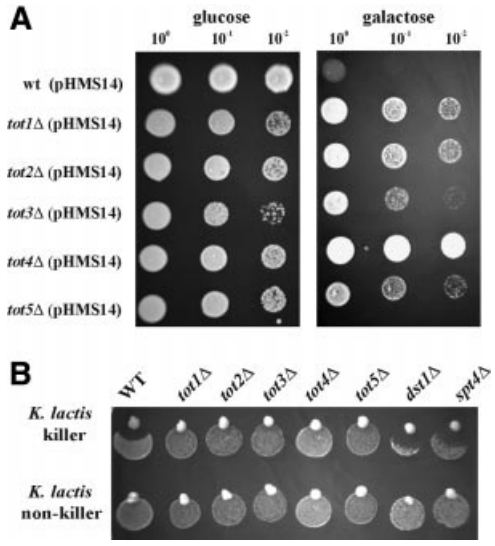

Fig. 1. The *tot*Δ mutants are resistant towards endogenously expressed γ-toxin and exogenously applied *K.lactis* zymocin. (**A**) γ-toxin assay. Serial dilutions of toxin-sensitive (wt) and -resistant (*tot1–5*Δ) cells transformed with the *GAL1*-driven γ-toxin vector pHMS14 were replica spotted on repressing (glucose) and inducing (galactose) rich medium and grown for 2 days at 30°C. Lack of growth indicates γ-toxin sensitivity. (**B**) Killer eclipse assay. Wild-type, *tot1–5*Δ strains, *dst1*Δ and *spt4*Δ cells were spotted twice on to YPD medium. A *K.lactis* strain (upper row: killer strain AWJ137; lower row: non-killer strain NK40) was set on to the edge of these spots and incubated for 2 days at 30°C. Zones of inhibition around the zymocin-secreting killer strain (upper row) indicate zymocin sensitivity; lack of growth inhibition equals zymocin resistance.

Results

Molecular identification of TOT genes and analysis of the tot phenotypes

Among a pool of 100 000 yeast clones carrying insertions of the mini-transposon mTn*3*::*lacZ*::*LEU2* (mTn*3*) γ-toxin-resistant clones were screened for by switching on the expression of the γ-toxin from the UAS_{GAL1} promoter on 2% galactose plates. Three hundred and twelve clones able to grow on galactose were selected over a period of 7 days. Of these, roughly one-sixth (58 clones) showed β-galactosidase expression in a filter assay, indicating in-frame *lacZ* fusions. After pHMS14 plasmid rescue, restriction pattern analysis and retransformation into non-mutagenized reporter strain LS20, 41 candidates remained to be subjected to a second viability screen using inducible γ-toxin expression from another regulatable promoter (UAS_{MET25}) to eliminate promoter-specific false positives related to UAS_{GAL1}. Fifteen clones were able to grow in the absence of added methionine. These were finally subjected to a killer eclipse assay testing exo-zymocin resistance. Only three candidates passed this test. The *TOT1–3* genes disrupted by mTn*3* in these three candidates were identified by vectorette PCR and sequencing as YLR384c (*ELP1*/*IKI1*/*TOT1*), YGR200c (*ELP2*/*TOT2*) and YPL086c (*ELP3*/*TOT3*).

To verify that zymocin resistance caused by the mTn*3* minitransposon insertion was due to inactivation of the *TOT* genes, individual knockouts of *TOT1–3* were constructed by PCR-mediated gene disruption. In addition, two more genes reported previously as zymocin resistance determinants (Butler *et al*., 1994; Yajima *et al*., 1997) were individually disrupted and shown to display a *tot* phenotype: *KTI12* (*TOT4*) and *IKI1* (*TOT5*). All these *tot*Δ cells showed resistance towards endogenously expressed γ-toxin (Figure 1A) as well as against extracellular holozymocin (Figure 1B), indicating that the *tot1–5*Δ strains are true toxin target site mutants. To analyse whether the *tot1–5*Δ mutations were dominant or recessive, the null mutants were crossed with LF20, the wild-type zymocin-sensitive strain of opposite mating type. *TOT*/*tot*Δ heterozygous diploids obtained in this way were all sensitive towards exo-zymocin and intracellular γ-toxin (data not shown), showing that each of the *tot1–5*Δ mutations behaves recessively. Since *TOT1*, *TOT2* and *TOT3* were recently identified as *ELP1*, *ELP2* and *ELP3*, structural genes encoding components of RNA polymerase II Elongator (Otero *et al*., 1999; Wittschieben *et al*., 1999; Fellows *et al*., 2000), mutants lacking transcription factors TFIIS (*dst1*Δ) (Archambault *et al*., 1992) and Spt4p (*spt4*Δ) (Hartzog *et al*., 1998; Wada *et al*., 1998), both of which affect elongation but are not components of the Elongator complex, were assayed against exo-zymocin. *dst1*Δ and *spt4*Δ cells were found to be as sensitive towards zymocin as the wild-type strain LS20 (Figure 1B), suggesting that zymocin resistance is not generally associated with elongation mutants but involves specifically *ELP*/*TOT* gene function. As shown previously by Butler *et al*. (1994), *KTI12* (*TOT4*) overexpression leads to zymocin resistance, while *IKI1* (*TOT5*) overexpression does not (Yajima *et al*., 1997). To test the overexpression phenotype of *TOT1–TOT4*, the genes were overexpressed from their native promoter on multicopy yeast episomal vectors in the wild-type background of strain LS20. *TOT4* was the only gene causing γ-toxin resistance in multicopy (data not shown).

We next analysed the *tot*Δ cells under various growth conditions. Deletion of each *TOT* gene renders cells a slow growth phenotype (Slg^+) at 30°C (Figure 2A), which leads

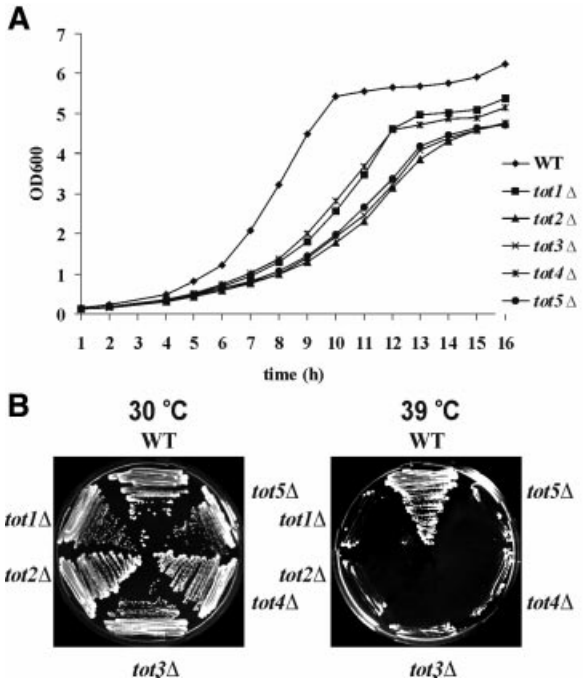

Fig. 2. Deletion of *TOT1–5* genes confers slow growth and thermosensitive phenotypes. (**A**) Growth curve of *tot1–5*Δ strains. *tot1–5*Δ cells show a slow growth phenotype and do not reach the biomass of the wild-type reference in stationary phase. Strains were grown in YPD medium (2% glucose) and growth was measured at OD_{600} over a period of 16 h. (**B**) Ts phenotype of *tot1–5*Δ strains. Strains were streaked on YPD and incubated for 3 days at 30 and 39°C.

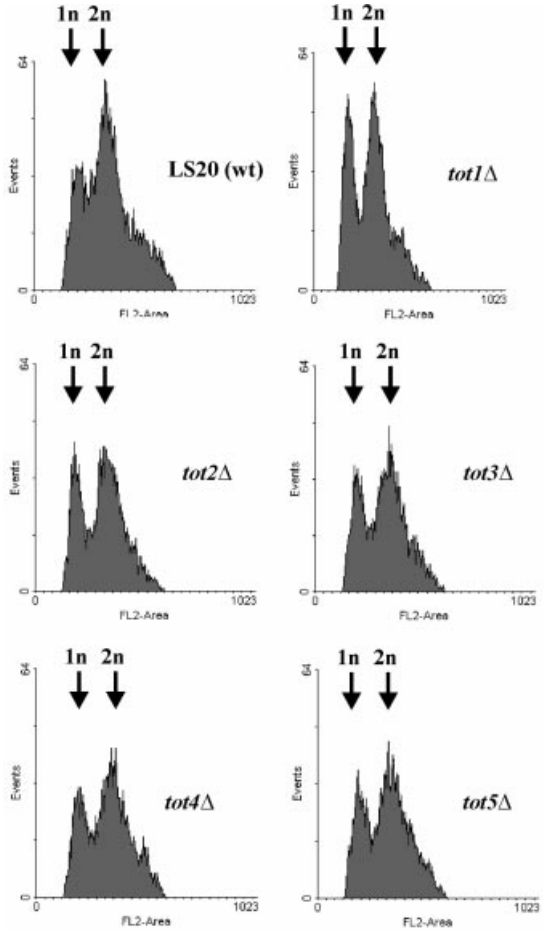

Fig. 3. The slow growth phenotype of *tot1–5*Δ strains correlates with a significant delay in the G_1 phase of the cell cycle. FACS analyses of exponential growing *tot1–5*Δ cells show an extended 1*n* peak in comparison with the wild-type strain, indicating a delay in the G_1 to S transition.

to thermosensitivity above 38°C (Figure 2B). Doubling times are lengthened by a factor of 1.5–2.0, resulting in growth rate reductions from μ = 0.5 (wild type) to μ = 0.25–0.3 (*tot1–5*Δ). To check whether the Slg^+ phenotype was correlated with a delay in a specific phase of the cell cycle, FACS analysis of exponentially growing *tot*Δ strains was performed together with wild-type strain LS20 as a control (Figure 3). This analysis showed that during exponential growth all *tot*Δ mutant strains had a higher percentage of cells with a 1*n* DNA content than did wild-type cells (Figure 3). Thus, deletion of any *TOT* gene leads to a significant delay in the G_1 phase of the cell cycle, indicating that the *TOT* gene products are important for normal cell cycle progression. The thermosensitive phenotype of the *tot*Δ cells could be partially rescued by addition of 1 M sorbitol to the growth medium; however, the effect was not as striking as with a *chs3*Δ cell wall chitin mutant included as a positive reference strain (data not shown).

To further check whether the *tot*Δ cells might be affected in cell wall integrity, we tested growth behaviour in the presence of the purine analogue caffeine and the fluorochrome Calcofluor White, a cell wall poison with high affinity for yeast cell wall chitin (Hampsey, 1997). As compared with wild-type strain LS20, all *tot*Δ cells were hypersensitive towards Calcofluor White, indicative of a cell wall defect (Figure 4A). As a Calcofluor White-resistant positive control we included the *chs3*Δ cell wall chitin mutant (Figure 4A). Sensitivity to caffeine was also shown by all *tot*Δ cells; however, to varying degrees (Figure 4B). Thus, the *tot5*Δ strain was more sensitive than *tot1*Δ, *tot3*Δ and *tot4*Δ cells, while the *tot2*Δ mutant was less sensitive to the drug. This phenotype suggests that cell wall integrity may be affected in all the *tot*Δ cells, presumably by involvement of the Pkc1p MAP kinase signalling pathway, one of the known cellular caffeine targets (Hampsey, 1997).

Inspired by parallel identification of *TOT1–3* as transcriptional Elongator genes *ELP1–3* (Otero *et al*., 1999; Wittschieben *et al*., 1999; Fellows *et al*., 2000), we checked the effect of *TOT* gene deletion on transcriptional processes using 6-AU as indicator drug. 6-AU inhibits the nucleotide biosynthesis pathway in yeast, leading to a depletion of UTP and GTP, which in turn affects the efficacy of RNA polymerase II holoenzyme during transcriptional elongation (Exinger and Lacroute, 1992). As shown in Figure 4C, all *tot*Δ strains showed different degrees of sensitivity to 6-AU. A *dst1*Δ strain lacking the elongin TFIIS was used as positive control (Archambault *et al*., 1992). The strains *tot1*Δ, *tot3*Δ and *tot4*Δ exhibited nearly the same sensitivity as the *dst1*Δ control. Less sensitivity was shown by *tot5*Δ and *tot2*Δ. All *tot*Δ strains as well as *dst1*Δ could grow again on 6-AU when uracil was added to the media. In conclusion, this drug-induced phenotype suggests a role for the putative γ-toxin, TOT, in

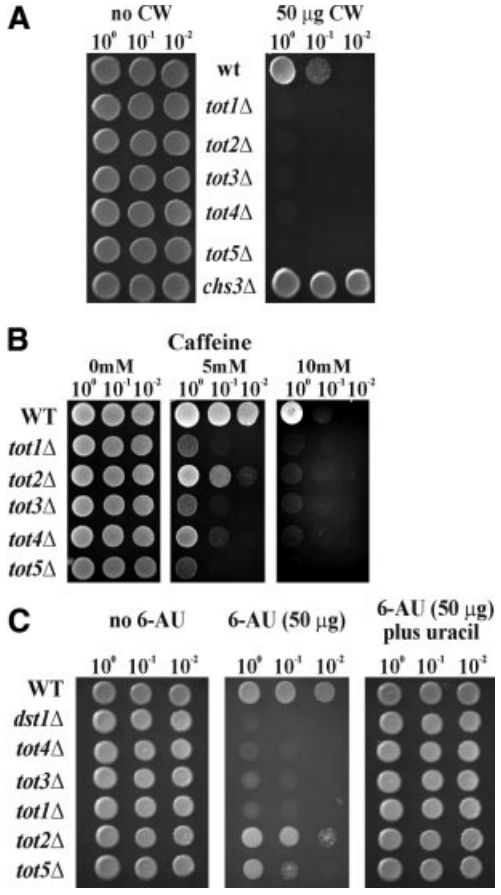

Fig. 4. More *tot* phenotypes. (**A**) Calcofluor White sensitivity. Serial dilutions of strains were replica spotted on YPD plates containing no or 50 μg of Calcofluor White (CW). All *tot*Δ strains show hypersensitivity against Calcofluor White, whereas the positive reference, *chs3*Δ, displays resistance towards the drug. (**B**) Caffeine sensitivity. Strains were spotted on YPD plates containing up to 10 mM caffeine and grown for 3 days at 30°C. All *tot*Δ strains show more or less hypersensitivity towards caffeine. (**C**) 6-AU phenotype. Strains were spotted on SD plates containing no 6-AU, 50 μg/ml 6-AU or 50 μg/ml 6-AU plus uracil. Except for the *tot2*Δ mutant, which is mildly affected by the drug, all other *tot*Δ strains show hypersensitivity towards 6-AU. The *dst1*Δ mutant served as a positive 6-AU-hypersensitive control strain.

transcript elongation *in vivo*. Moreover, loss of *TOT* gene function has a pleiotropic effect on a yeast cell's performance, leading to a complex *tot* phenotype that includes zymocin and γ-toxin resistance, slow growth, G_1 cell cycle delay and thermosensitivity as well as sensitivity towards the drugs Calcofluor White, caffeine and 6-AU. None of these *tot* phenotypes was severely amplified on combining double *tot1*Δ*tot2*Δ, *tot3*Δ*tot2*Δ, *tot4*Δ*tot2*Δ or *tot5*Δ*tot2*Δ mutations in one genetic background (data not shown), indicating that the *TOT1–5* genes are functionally related. Summing up from these phenotypic analyses, TOT can be considered to have an important role for cellular growth.

The HAT activity of Tot3p is essential for zymocin action

The *S.cerevisiae* genome encodes other histone acetyltransferase (HAT) activities than the one associated with Elp3p (Tot3p) (Wittschieben *et al*., 1999). To check

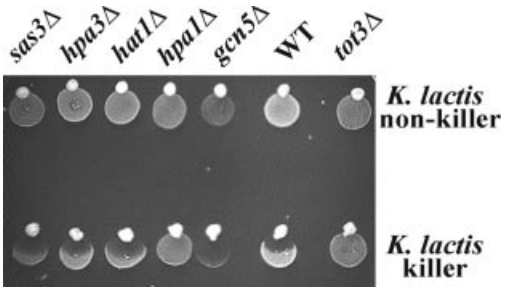

Fig. 5. Effect of HAT gene deletions on zymocin sensitivity. Strains deleted in the HAT-encoding genes *SAS3*, *HPA3*, *HAT1*, *HPA1*/*ELP3*, *GCN5* and *TOT3*/*ELP3* were subjected to a killer eclipse assay essentially as described in Figure 1. Deletion of *TOT3* confers zymocin resistance, whereas the other HAT gene deletions tested are zymocin sensitive.

whether histone acetylation is needed for zymocin action generally, we tested other HAT gene knockouts in *GCN5*, *SAS3*, *HPA1*, *HPA3* and *HAT1* (Kleff *et al*., 1995; Brownell *et al*., 1996; Reifsnyder *et al*., 1996; Brown *et al*., 2000) using zymocin eclipse assays. As illustrated in Figure 5, all HAT knockouts except for *HPA1*, which is isoallelic to *TOT3*/*ELP3*, remained zymocin sensitive. Thus, the Elp3p/Tot3p-associated HAT activity appears to be required for zymocin action, whereas other non-Elongator HATs are dispensable for zymocicity.

To check whether the HAT-domain activity of Elp3p (Tot3p) itself is required for γ-toxin action, point mutations in *ELP3* that change two conserved tyrosine residues to alanine in the B motif of the putative catalytic HAT domain (Y540A and Y541A having <25 and ~35% of wild-type Elp3p-HAT activity, respectively) (Wittschieben *et al*., 2000) were tested. These mutant alleles were expressed from their native promoters on CEN plasmids (pBOP60-14: Y540A; pBOP60-15: Y541A; and pBOP60-13: wild-type *ELP3* allele) in the *tot3*Δ mutant and the LS20 wild-type strains co-maintaining the *GAL1*-driven γ-toxin vector pHMS14. The resulting strains were checked for complementation of the *tot3*Δ-associated γ-toxin resistance phenotype as well as suppression of γ-toxin sensitivity in wild-type LS20 (Figure 6A) using glucose to galactose shift assays.

Both the Y540A and Y541A mutant *ELP3* alleles failed to complement the resistance towards γ-toxin of *tot3*Δ cells while the wild-type *ELP3* allele was able to do so (Figure 6A). Furthermore, the Y540A allele (<25% HAT activity) slightly suppressed the sensitivity of wild-type LS20 against γ-toxin, whereas the Y541A allele (~35% HAT activity) did not (Figure 6A). When overexpressed from multicopy yeast episomal vectors, both alleles (pLF31: Y540A and pLF30: Y541A) suppressed the sensitivity of the wild-type strain LS20 towards γ-toxin, resulting in a resistance phenotype, while the wild-type *ELP3* allele did not (Figure 6B). Thus, it appears that a significant reduction of activation of the HAT encoded by *ELP3* phenocopies the effect of *TOT3*/*ELP3* gene inactivation on γ-toxin sensitivity, indicating that the HAT catalytic activity of Elp3/Tot3p is required for zymocin action. Moreover, the mutant alleles are semi-dominant over wild type, indicating that the gene products are incorporated into the Elongator complex, leading to a reduction of Elongator activity.

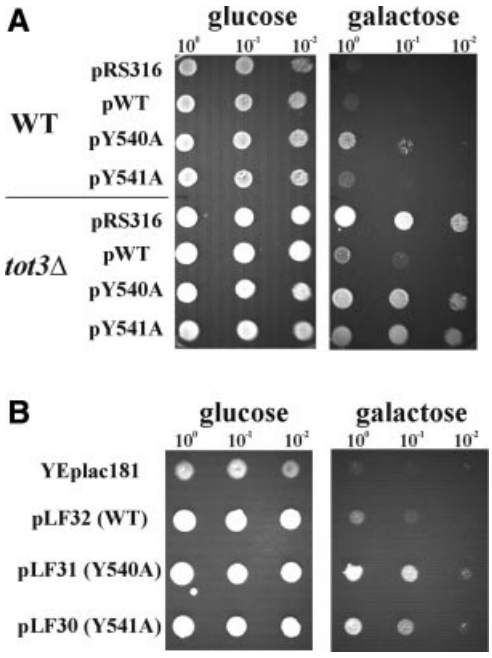

Fig. 6. The HAT activity of Elp3p/Tot3p is essential for γ-toxin action. (**A**) Wild-type and *tot3*Δ strains containing the *GAL1*-driven γ-toxin vector pHMS14 were transformed with CEN plasmids carrying no insert (pRS316), the wild-type *ELP3* allele (pWT) or mutated *elp3* alleles (pY540A and pY541A). Serial dilutions of the resulting transformants were replica spotted on glucose-repressing and galactose-inducing medium. The mutant *elp3* alleles fail to complement the *tot3*Δ-associated γ-toxin resistance phenotype. In the wild-type background, the Y540A allele slightly suppresses γ-toxin sensitivity. (**B**) When overexpressed from yeast multicopy plasmids, both the mutant *elp3* alleles (pLF31: Y540A and pLF30: Y541A) suppress the γ-toxin sensitivity of the wild-type strain co-maintaining pHMS14, whereas vector without insert (YEplac181) and the wild-type *ELP3* allele (pLF32) are not able to do so.

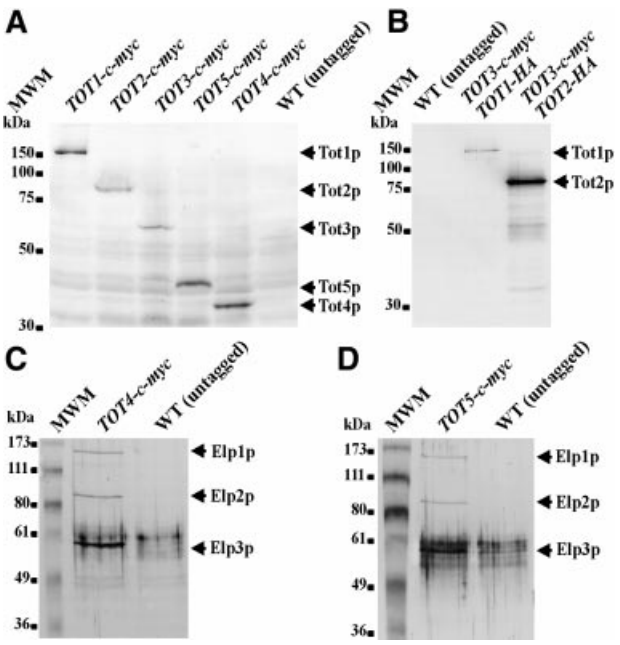

Fig. 7. Tot protein detection and co-immunoprecipitation. (**A**) Identification of *TOT1–5* as protein-encoding structural genes. Western analysis: Tot1–5p were tagged individually by one-step *in vivo* tagging with a triple c-Myc tag. Total protein extracts (50 μg) were separated using 10% SDS–PAGE, electroblotted and immunoprobed with the anti-c-Myc antibody 9E10. The position of each tagged Tot protein is indicated by an arrow. (**B**) Tot3p interacts with Tot1p and Tot2p. Protein extracts from strains expressing c-Myc-tagged Tot3p together with HA-tagged Tot1p or HA-tagged Tot2p were immunoprecipitated using the anti-c-Myc antibody 9E10. Precipitates were then separated using 10% SDS–PAGE, electroblotted and immunoprobed with the anti-HA antibody 12CA5. The positions of HA-tagged Tot1p and Tot2p are indicated by arrows. (**C** and **D**) Tot4p (C) and Tot5p (D) interact with Elp1p (Tot1p), Elp2p (Tot2p) and Elp3p (Tot3p). Protein extracts from strains expressing c-Myc-tagged Tot4p (C) or Tot5p (D) were immunoprecipitated using the anti-c-Myc antibody 9E10, separated on a 10% SDS–PAGE gel, electroblotted and immunoprobed by western analysis using polyclonal anti-Elp1p, anti-Elp2p and anti-Elp3p antibodies. The positions of co-immunoprecipitated proteins, Elp1p, Elp2p and Elp3p, are indicated by arrows.

Co-immunoprecipitation of Tot proteins

Using PCR-mediated one-step tagging *in vivo* (Knop *et al*., 1999), all five Tot proteins were C-terminally marked with the c-Myc (Figure 7A) and HA (not shown) epitope tags to analyse gene expression at the translational level and to assess protein–protein interaction using co-immunoprecipitation. As judged from zymocin eclipse assays, *TOT1–5* gene tagging had no effect on the biological activity of the individual Tot proteins, i.e. all tagged strains remained as zymocin sensitive as the wild-type reference strain (data not shown). Epitope-tagging identified *TOT1–5* as protein-encoding structural genes. Thus, total protein extracts from exponentially grown *TOT1–5-(c-myc)$_3$* cells expressed single Tot1–5 polypeptides of estimated molecular weights consistent with the predicted ones that cross-reacted with the anti-c-Myc monoclonal antibody 9E10 (Figure 7A).

As reported, Elp1p (Tot1p), Elp2p (Tot2p) and Elp3p (Tot3p) associate together within Elongator, a 650 kDa multisubunit complex that interacts specifically with elongating RNA polymerase II holoenzyme (Otero *et al*., 1999; Wittschieben *et al*., 1999; Fellows *et al*., 2000). Consistent with this, we could show by way of co-immunoprecipitation that Tot3p interacts with Tot1p and Tot2p (Figure 7B) and that Tot2p associates with Tot1p and Tot3p (data not shown). Moreover, all three Elongator constituents, Elp1p, Elp2p and Elp3p, could also be shown to interact with Tot4p (Figure 7C) and Tot5p (Figure 7D). These co-immunoprecipitations involved precipitation of c-Myc-tagged Tot4p and Tot5p with the anti-c-Myc antibody 9E10 covalently linked to protein A–Sepharose and detection by polyclonal anti-Elp1p, -Elp2p and -Elp3p antibodies (Otero *et al*., 1999; Wittschieben *et al*., 1999; Fellows *et al*., 2000). In conclusion, all *TOT* gene products were found to be associated in a multisubunit complex consisting of (at least) five polypeptides, three of which were recently identified as components of the Elongator complex. Thus, the *S.cerevisiae* Elongator appears to be part of or to represent the putative *K.lactis* γ-toxin target, TOT.

Gene transcription in response to the K.lactis zymocin

Since the Elp3p-associated HAT activity appears to be essential for Elongator function *in vivo* (Wittschieben *et al*., 2000), it is highly likely that TOT/Elongator plays a

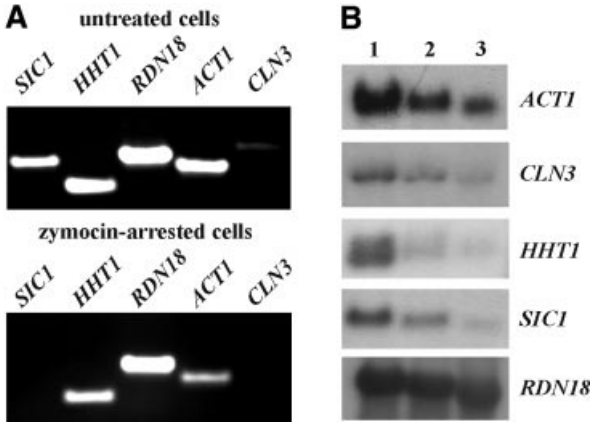

Fig. 8. Gene transcription in response to the *K.lactis* zymocin. (**A**) Identical amounts of total RNA isolated from untreated and zymocin-arrested cells were subjected to RT–PCR experiments to study the transcription of RNA polymerase I-dependent (*RDN18*) and RNA polymerase II-dependent (*SIC1*, *HHT1*, *ACT1* and *CLN3*) genes by 1% TBE–agarose gel electrophoresis. (**B**) Identical amounts of total RNA (10 μg) isolated from untreated cells (lane 1) and cells arrested by zymocin for 3 h (lane 2) and 6 h (lane 3) were subjected to northern blot analysis using probes specific for the *RDN18*, *SIC1*, *HHT1*, *ACT1* and *CLN3* genes.

role in transcript elongation or promoter remodelling by maintaining transcriptionally competent chromatin while piggybacked to the elongating form of the RNA polymerase II holoenzyme (Travers, 1999). To investigate the effect of the *K.lactis* zymocin on transcriptional processes, identical amounts of total RNA prepared from untreated and zymocin-arrested cells were subjected to RT–PCR experiments using primers specific for RNA polymerase I-dependent and RNA polymerase II-driven genes. As illustrated in Figure 8A, RNA polymerase I-dependent transcription of the 18S rRNA gene (*RDN18*) remained largely unaffected, whereas transcription of the RNA polymerase II-dependent genes (*SIC1*, *CLN3*, *HHT1* and *ACT1*) decreased significantly upon treating cells with the *K.lactis* zymocin. Similarly, this differential display was seen when using conventional northern hybridization studies (Figure 8B). Here, the effect of the *K.lactis* zymocin on RNA polymerase II-dependent transcription was particularly pronounced with regard to *SIC1*, *CLN3* and *HHT1* gene activation. Our observation that RNA polymerase I-dependent transcription remains largely unaffected by the *K.lactis* zymocin is consistent with the results of a previous study by Butler *et al*. (1991c) showing that bulk RNA synthesis, measured by incorporation of radiolabelled uracil, is not affected during zymocin treatment. In conclusion, the *K.lactis* zymocin appears to preferentially affect RNA polymerase II-dependent gene transcription.

Discussion

The *K.lactis* zymocin is toxic against a variety of sensitive yeast genera including *S.cerevisiae*. Earlier reports that the zymocin functions on *S.cerevisiae* by inhibiting the adenylate cyclase, and hence abolishing the roles of cAMP essential for mitotic growth and cell division (Sugisaki *et al*., 1983), have been disproved (White *et al*., 1989). Thus, its mode of action still remains unclear. Zymocin-resistant *S.cerevisiae skt*, *iki* and *kti* mutants have been isolated previously (Kawamoto *et al*., 1990; Butler *et al*., 1994; Kishida *et al*., 1996). Sensitivity of these towards intracellular, conditional expression of the γ-toxin from inducible promoters has distinguished zymocin binding/uptake (class I) from γ-toxin target site mutants (class II). The presence of as many as 10 distinct *kti* class II complementation groups suggests that a complex pathway transduces the zymocin's inhibitory effect (Butler *et al*., 1994). While some of them may be involved in the expression of target(s) inhibited by the γ-toxin, a number of proteins could also participate in the process that is blocked by it. These might act in a biochemical pathway or, alternatively, form a putative target complex containing several components. In favour of the latter, our genetic screens involving mTn*3* tagging and PCR-mediated gene disruption to search for potential γ-toxin target (*tot*) mutants have identified the TOT complex as a putative target. The *tot* phenotypes common to all *TOT* gene deletions provide genetic evidence that the specified Tot gene products are functionally related to one another. Thus, in addition to zymocin and γ-toxin resistance, *tot1–5*Δ cells commonly display slow growth and thermosensitive phenotypes as well as G_1 cell cycle delay and hypersensitivity towards the drugs Calcofluor White, caffeine and 6-AU. The fact that none of these phenotypes is severely amplified on combining double *tot*Δ mutations in one background can be genetically interpreted as *TOT1–5* genes functioning in the same process. Moreover, our re-identification of *ELP1/IKI3*, *ELP2* and *ELP3*, structural genes encoding Elongator complex components (Otero *et al*., 1999; Wittschieben *et al*., 1999; Fellows *et al*., 2000), as *TOT1*, *TOT2* and *TOT3*, respectively, provides substantial evidence for the 'putative target complex' hypothesis. Consistent with this, our co-immunoprecipitation experiments have shown that the putative γ-toxin target complex, TOT, consists of two more constituents encoded by *KTI12* (*TOT4*) and *IKI1* (*TOT5*) in addition to the known Elongator components. Since *tot4*Δ and *tot5*Δ cells are phenotypically indistinguishable from *tot1–3*Δ (*elp1–3*Δ) cells, it may well be envisaged that Tot4p and Tot5p constitute further as yet uncharacterized Elongator components, suggesting that the Elongator complex represents or is part of TOT.

By introducing *ELP3* HAT domain mutations into an *elp3*Δ strain, it has been shown recently that the HAT activity associated with Elp3p is essential for Elongator function *in vivo* (Wittschieben *et al*., 2000). Thus, in spite of being incorporated into Elongator complexes, the two mutant Elp3 proteins tested (Y540A and Y541A with <25 and ~35% HAT activity compared with wild-type Elp3p, respectively) failed to rescue the cells from the *elp3*Δ-associated phenotypes. We have shown here that, similarly, both mutant Y540A and Y541A Elp3 proteins failed to confer sensitivity towards the γ-toxin when re-introduced into our toxin-resistant *tot3*Δ (*elp3*Δ) reporter strain. The point mutants behaved as dominant-negative alleles with respect to γ-toxin sensitivity. Thus, γ-toxin sensitivity apparently requires the Elongator-associated HAT activity. Moreover, they conferred γ-toxin resistance when introduced on multicopy episomal vectors into a toxin-sensitive *ELP3* wild-type reporter strain. Probably, the mutant overproduced Elp3p variants compete for incorp-

oration into Elongator with wild-type Elp3p, thus reducing the concentration of active Elongator complexes. Taken together, these results indicate that not only inactivation of Elongator by way of *TOT1–5* gene deletions, which may ultimately result in non-assembly of functional Elongator complexes, but also *in vivo* assembly of Elongator complexes with significantly reduced HAT activity lead to the *tot* phenotype, including resistance towards the *K.lactis* zymocin.

Since all the *TOT* genes are non-essential, the zymocin cannot be considered to cause a G_1 arrest by inhibition/inactivation, direct or otherwise, of Elongator. Instead, sensitive *S.cerevisiae* cells require a functional Elongator in order to be able to respond to the *K.lactis* zymocin. This requirement for Elongator is genetically sustained by the observation that cells lacking either non-Elongator HATs (*gcn5*Δ, *hpa3*Δ, *hat1*Δ and *sas2*Δ) (Kleff *et al*., 1995; Brownell *et al*., 1996; Reifsnyder *et al*., 1996; Brown *et al*., 2000) or non-Elongator transcription elongation factors (*dst1*Δ and *spt4*Δ) (Archambault *et al*., 1992; Hartzog *et al*., 1998; Wada *et al*., 1998) are not able to alter γ-toxin sensitivity in a way the *tot1–5*Δ strains can. This implies that the *K.lactis* zymocin can activate a pathway that is inhibitory to the cell cycle and of which TOT/Elongator is a part.

As for the molecular mode of action of the *K.lactis* zymocin, our results provide at least two possible models. First, one might envisage direct interaction of the γ-toxin with fully assembled TOT/Elongator complexes in order to promote the observed G_1 cell cycle arrest. Thus, γ-toxin-bound TOT/Elongator and free Elongator would compete for a limited supply of a downstream effector molecule that is a key positive regulator of the cell cycle needed for events in late G_1. Given the fact that Elongator has recently been demonstrated to associate with the CTD-hyperphosphorylated form of RNA polymerase II and proposed to be exchanged for Mediator upon CTD dephosphorylation after transcription termination (Otero *et al*., 1999; Wittschieben *et al*., 1999), it is conceivable to speculate that RNA polymerase II itself may be a prime candidate for this critical effector protein. In the absence of the γ-toxin, RNA polymerase II would associate with TOT/Elongator complexes to acquire full transcriptional activity and to promote a normal G_1 to S transition by activation of START-specific genes. In the presence of the γ-toxin, RNA polymerase II would be sequestered and inactivated by the γ-toxin-bound TOT/Elongator complex, leading to a G_1 cell cycle arrest, e.g. γ-toxin-bound TOT/Elongator complexes associated with RNA polymerase II may prevent exchange for Mediator during or after transcription termination, thus lowering the concentration of RNA polymerase II able to initiate transcription of START-specific genes needed for a proper G_1 to S cell cycle progression. Consistent with this model, we found that transcription of RNA polymerase II-dependent genes (*SIC1*, *CLN3*, *HHT1* and *ACT1*) was specifically down-regulated in zymocin-arrested cells, whereas RNA polymerase I-dependent transcription of the 18S rRNA gene (*RDN18*) remained largely unaffected. Therefore, we favour the hypothesis that the *K.lactis* zymocin and its cytotoxic γ subunit have the TOT/Elongator-associated RNA polymerase II holoenzyme as a putative direct target. Alternatively, one might envisage that the γ-toxin target could be a gene product whose transcript is down-regulated in strains lacking TOT/Elongator function. To address this question in more detail we are currently designing genome-wide micro-array approaches to identify genes whose transcription is particularly affected in the *tot* mutants.

Materials and methods

Strains, media, DNA constructs and general methods

Routine bacterial transformations used for constructing recombinant DNA, yeast plasmid shuttles and rescues as well as amplification of the mTn*3*-tagged yeast DNA library involved electroporation of *Escherichia coli* strains DH5α, XL1-Blue and TOP10F′. These were grown in Luria–Bertani medium supplemented with one or several of the following additions: ampicillin (100 μg/ml), kanamycin (80 μg/ml), X-Gal (80 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μg/ml). All yeast strains used or generated in this study are described in Table I. Standard rich and minimal growth media, YEPD and SC, respectively, were prepared essentially as described by Sherman (1991). For phenotypic analysis, these media were supplemented with 6-AU (50 μg/ml), caffeine (1–20 mM) or Calcofluor White (50–1000 μg/ml). For 6-AU assays, Ura^+ transformants (YCplac33) (Gietz and Sugino, 1988) of the *tot*Δ strains were used. Yeast transformations involved electroporation (Becker and Guarente, 1991) as well as the lithium acetate method (Gietz *et al*., 1992). Mating type switching of LL20 (*MAT*α, see Table I) to generate *MAT***a** cells (LF20, see Table I) was accomplished using *HO* gene expression from plasmid pHAL24 (YEplac181 carrying the *HO* gene on a 2.5 kb genomic *Hin*dIII fragment) followed by plasmid segregation. To create Ura^- derivatives of strain LL20, yeast cells were transformed with a *ura3*Δ deleter plasmid followed by selection against *URA3* on SC minimal medium containing uracil and 5-fluoroorotic acid (5-FOA). The deleter plasmid was constructed by autoligation of the YDp-U backbone fragment (Berben *et al*., 1991) obtained by double digestion with *Eco*RV and *Sma*I to delete an internal *URA3*-borne segment and reconstitute a non-functional *ura3*Δ allele. Among Ura^- transformants (termed LS20, see Table I), *ura3*-linked auxotrophy was distinguished from *ura5* mutants by single copy *URA3* gene complementation (YCplac33). To construct the methionine-repressible γ-toxin expression vector pHAL9, the γ-toxin gene was fused as an *Xho*I–*Sal*I restriction fragment from plasmid pARB1 (Butler *et al*., 1994) to the UAS_{MET25} promoter of *Sal*I-cut vector p413*MET25* (Mumberg *et al*., 1994). The latter was obtained by deleting the *GFP* gene from pGFP-NFUS (Niedenthal *et al*., 1996) using *Bam*HI and autoligation of the vector backbone. Galactose-inducible expression of the γ-toxin was achieved using pHMS14, a p413-based vector (Mumberg *et al*., 1995), carrying the UAS_{GAL1}–γ-toxin fusion on a 1.4 kb *Sst*I–*Eco*RI fragment from pARB1 (Butler *et al*., 1994; Schaffrath *et al*., 1997). As a UAS_{GAL1} vector control, plasmid pHMS22 (p413 carrying the UAS_{GAL1} promoter only) was used. Plasmids for overexpression of Tot1p (pFF14), Tot2p (pFF10) and Tot3p (pFF9) were constructed by cloning the coding sequence with UAS and terminator into YEplac195 using *Sac*I–*Sal*I (pFF14), *Pst*I–*Bam*HI (pFF10) and *Hin*dIII–*Sal*I (pFF9). Mutated alleles of *ELP3/TOT3* (Y540A, Y541A) were cloned for overexpression in YEplac181 by using *Bam*HI–*Kpn*I restriction fragments of pBOP60-13, pBOP60-14 and pBOP60-15 (Wittschieben *et al*., 2000), generating pLF30 (Y541A), pLF31 (Y540A) and pLF32 (wild type).

Isolation and genetic analysis of tot mutants

A yeast transformant population carrying mini-transposon (mTn*3*) insertions randomly integrated into the genome was constructed as follows. First, strain LS20 was transformed to histidine prototrophy with pHMS14 carrying the UAS_{GAL1}–γ-toxin fusion. Several His^+ candidates (termed LS20′) were checked for γ-toxin sensitivity by replica plating on to galactose SC his^- medium, resulting in a Gal^- phenotype. Next, LS20′ was subjected to transposon mutagenesis using electroporation-mediated transformation with the *Not*I-digested mTn*3*::yeast insertion library (Burns *et al*., 1994) selecting for the mTn*3*-based *LEU2* marker on SC his^-, leu^- medium. His^+ Leu^+ yeast transformants were subsequently replica plated on galactose medium and incubated for up to 7 days to identify Gal^+ tox^R candidates. To distinguish genomic mTn*3*::integrations from plasmid-borne ones that might have caused inactivation of the UAS_{GAL1}–γ-toxin fusion on pHMS14, total DNA preparations obtained from selected Gal^+ isolates were used for yeast–*E.coli* plasmid rescue and

Table I. Yeast strains

Strain	Genotype	Reference
K.lactis		
NK40	*MATα, ade1, ade2, leu2 [k1^0 k2$^+$]*	Gunge *et al.* (1981)
AWJ137	*MAT***a**, *leu2*, *trp1* [k1$^+$ k2$^+$]	Kämper *et al.* (1991)
S.cerevisiae		
FY1679-08A	*MAT***a**, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *his3Δ200*, *GAL*	Euroscarf
FY1646	*MATα*, *his4-912δ*, *lys2-128δ*, *leu2Δ1*, *spt4Δ::HIS3*	F.Winston
GMY27	*MATα*, *ade2-101*, *leu2-3,-112*, *his3Δ200*, *ura3-52*, *lys2*, *gcn5Δ::hisG*	Anthony Wright
LPY2121	*MAT***a**, *ade2-101*, *his3Δ200*, *leu2Δ1*, *lys2-801*, *TELadh4::URA3*, *ura3-52*, *trp1Δ1 sas3Δ::HIS3*	Darryl Auston
W303-1a	*MAT***a**, *ura3-1*, *leu2-3*, *-112*, *his3-11*, *-15*, *trp1-1*, *ade2-1*, *can 100-1*	Anne Sutton
RS1236 (SK56)	as W303-1a, but *hat1Δ::TRP1*	Anne Sutton
RS1392 (YCW2)	as W303-1a, but *hpa1Δ::URA3*	Anne Sutton
YRP13	as W303-1a, but *hpa3Δ::HIS3*	Anne Sutton
LL20	*MATα leu2-3, -112, his3-11, -15, GAL*	NCYC 1445
LS20	as LL20, but *ura3*	this work
LF20	as LL20, but *MAT***a**	this work
LS20′	as LS20 plus pHMS14 (CEN4/*HIS3*/UAS_{GAL1}–γ-toxin)	this work
LFY12	as LS20, but *tot4Δ::LEU2 GAL*	this work
FFY5	as LS20, but *tot5Δ::KlLEU2 GAL*	this work
FFY6	as LS20, but *dst1Δ::KlLEU2 GAL*	this work
DJY3	as LS20, but *chs3Δ::KlLEU2 GAL*	this work
LFY1a	as LS20, but *TOT4-(c-myc)$_3$::SpHIS5*	this work
FFY1t	as LS20, but *TOT1-(c-myc)$_3$::SpHIS5*	this work
FFY2t	as LS20, but *TOT2-(c-myc)$_3$::SpHIS5*	this work
FFY3t	as FY1679-08A, but *TOT3-(c-myc)$_3$::SpHIS5*	this work
FFY5t	as FY1679-08A, but *TOT5-(c-myc)$_3$::SpHIS5*	this work
FFY2-1dt	as FY1679-08A, but *TOT3-(c-myc)$_3$::SpHIS5*, *TOT1-(HA)$_6$::KlTRP1*	this work
FFY2-3dt	as FY1679-08A, but *TOT3-(c-myc)$_3$::SpHIS5*, *TOT2-(HA)$_6$::KlTRP1*	this work

restriction enzyme analysis, using DNA of starting vector pHMS14 as positive control. Rescued plasmid DNAs identical to the *Sal*I pattern of pHMS14 were retransformed into fresh recipient strain LS20 and checked for tox^S by conditionally switching on γ-toxin expression on galactose SC (his$^-$) plates. Clones that passed this test were next checked for UAS_{GAL1}-specific false positives by using a second conditional γ-toxin expression approach involving the methionine-regulated promoter UAS_{MET25} on vector pHAL9. Using this second expression approach, clones that were able to grow under inducing conditions, i.e. in the absence of methionine, were obtained. In addition, the Gal$^+$ Leu$^+$ His$^+$ tox^R integrants were screened for in-frame fusions of the start-codon-less *lacZ* gene carried on the mTn*3* portion to yeast coding regions by checking β-galactosidase production on qualitative filter assays essentially as described (Ross-Macdonald *et al*., 1997). Resistance towards exo-zymocin was assayed using the killer eclipse assay (Kishida *et al*., 1996). To identify the yeast DNA immediately adjacent to the mTn*3* integration site of the mutants, the vectorette PCR approach was used (Ross-Macdonald *et al*., 1998). PCR products that were specifically amplified from mTn*3*-containing fragments were identified and directly subcloned into vector pCR2.1-TOPO using the topoisomerase cloning kit TOPO TA Version H (Invitrogen). Next, two independent subclones were sequenced for each candidate with the universal M13 reverse (5′-CAGGAAACAGCTATGAC-3′) and –20 forward primers (5′-GTAAAACGACGGCCAG-3′) and analysed using the BLAST and FASTA network services. To analyse whether the mTn*3*:: marked gene disruptions were recessive or dominant, the individual integrants (*MAT***a**, mTn*3*::*LEU2*, *ura3*, pHMS14 [*HIS3*]) were crossed to LF20 (*MAT***a**, *leu2*, *his3*, *URA3*) and diploids selected on SC medium were assayed for γ-toxin sensitivity/resistance by conditionally switching on γ expression on galactose medium. For PCR-mediated gene targeting (Wach *et al*., 1997) and construction of defined *tot*Δ null alleles, the original YDp plasmid set (Berben *et al*., 1991) was modified with non-*Saccharomyces* markers to utilize YDp-KlL (*K.lactis LEU2*: Zhang *et al*., 1992), YDp-KlU (*K.lactis URA3*: Längle-Rouault and Jacobs, 1995) and YDp-SpH (*S.pombe HIS5*: Wach *et al*., 1997). Knockout primers (Table II) usually consisted of 50 unique nucleotides homologous to the 5′- and 3′-regions of the yeast gene of interest plus a common 21 nucleotide stretch homologous to the multiple cloning site of plasmid pUC9H-STOP (Berben *et al*., 1991), the backbone of the YDp plasmid set used as yeast marker templates for PCR-mediated gene targeting. For generating the *tot4*Δ allele, a deletion construct, pYF6 (Butler *et al*., 1994), was alternatively used.

Flow cytometric determination of cellular DNA content

Cells from exponential growing cultures were fixed in 70% (v/v) ethanol and stored at –20°C. Aliquots (5×10^7 cells) of each sample were washed once with 1 ml of 50 mM sodium citrate solution and incubated in the dark for 30 min at 37°C in 1 ml of 1× PBS containing 1 mg of RNase A and 20 µg of propidium iodide. Each sample was analysed using a Becton-Dickinson FACS. The FACS contained a 15 mW argon laser with an excitation wavelength of 488 nm. Fluorescence was measured at 585 nm. Data were collected on 10 000 cells per sample. Under these conditions, fluorescence is considered to be proportional to DNA content (Hutter and Eipel, 1979).

Epitope tagging and immunological techniques

Epitopes were fused to genes by using PCR-based one-step *in vivo* epitope-tagging methods and tools as described by Knop *et al*. (1999). For primers used see Table II. For detection of epitope-tagged proteins, 9E10 mouse monoclonal antibody recognizing the c-Myc epitope and 3F10 rat antibody recognizing the HA epitope (Roche) were used as described (Schaffrath and Meacock, 1996). Polyclonal rabbit Elp1, Elp2 and Elp3 antibodies were kindly provided by Jesper Q.Svejstrup (ICRF, South Mimms, UK). Secondary alkaline phosphatase and peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch. Antibody cross-linking to protein A–Sepharose, preparation of protein extract and co-immunoprecipitation were carried out as described previously (Zachariae *et al*., 1996) using B60 buffer. Probes were then checked by western analysis. For all protein methods, proteinase inhibitors (Roche) and 0.5–1 mM phenylmethylsulfonyl fluoride were used.

Gene transcription analyses

Total RNA was isolated from equal amounts of zymocin-arrested and untreated *S.cerevisiae* LS20 cells using the RNAeasy midi kit (Qiagen) according to the manufacturer's recommendations. Zymocin treatment and arrest were carried out and monitored as described (D.Jablonowski, L.Fichtner, V.J.Martin, R.Klassen, F.Meinhardt, M.J.R.Stark and R.Schaffrath, in preparation). 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) using *Taq* polymerase and oligonucleotide primers (10 µM) (Table II) to amplify fragments specific for the 18S rRNA (*RDN18*; 0.52 kb), the histone H3 (*HHT1*; 0.32 kb), the actin (*ACT1*; 0.44 kb), the G_1 cyclin

Table II. Oligonucleotide primers used in this study

Name	Description	Sequence
FF1	FW ko-primer *TOT1*	5′-AGAAACAGTACAAATGCCTAATGGCTTATGGTTGAACATGACAAGAGTGGCGAC GGCCAGTGAATTCCCGG-3′
FF2	RV ko-primer *TOT1*	5′-CAATATGACTCTTAGGGAAATCATGAATCTCTGGAACAGGTATTTCTGGGAGCTT GGCTGCAGGTCGACGG-3′
FF3	FW ko-primer *TOT2*	5′-ATGGTGGAATGTATCACTCCCGAAGCCATTTTTATAGGTGCTAACAAGCACGACG GCCAGTGAATTCCCGG-3′
FF4	RV ko-primer *TOT2*	5′-CCTCAATCTTGTAATTTTGTCTGCTGGTGTTATATCCTCGTTTAGCTGCGAGCTTGG CTGCAGGTCGACGG-3′
FF5	FW ko-primer *TOT3*	5′-AGATGGCTCGTCATGGAAAAGGCCCAAAAACTAACAAAAAAAAGCTAGCACGAC GGCCAGTGAATTCCCGG-3′
FF6	RV ko-primer *TOT3*	5′-CCAGAAATAACAGAAATTTTCTCTGAACCATGCTCTTCCTTGGCGATTCTAGCTTG GCTGCAGGTCGACGG-3′
LF13	FW ko-primer *TOT4*	5′-AAACTAAACAGGCAATTTAGTAAGAAGATGCCACTGGTGCTTTTTACGGGCGACG GCCAGTGAATTCCCGG-3′
LF14	RV ko-primer *TOT4*	5′-ATCTCAATTCAAGTTTTTGTTAAGATAATCAGCGAAAAGCGGACCGATCCAGCTTG GCTGCAGGTCGACGG-3′
FF7	FW ko-primer *TOT5*	5′ CTATTGCTACAGGTAGAACAAGATATAATGGCCAGTTCGTCACATAACCCCGACGG CCAGTGAATTCCCGG-3′
FF8	RV ko-primer *TOT5*	5′-AAAAGGGATCCTCATATGGATCCTCTTCATCATAATCGTCATCCTTTTCGAGCTTGG CTGCAGGTCGACGG-3′
FF9	FW ko-primer *DST1*	5′-GTAGTCAGTCCGCATAAGAGCATTCATCATGGATAGTAAGGAAGTACTGGCGACGG CCAGTGAATTCCCGG-3′
FF10	RV ko-primer *DST1*	5′-TCTGTTACCACATGCTTCACATGTACAGAAAGTGGTCAATGGTTCATCCGAGCTTGG CTGCAGGTCGACGG-3′
DJ5	FW ko-primer *CHS3*	5′-TCCGCAGGAAAGAAATTAGAATGACCGGCTTGAATGGAGATGATCCTGATCGACGG CCAGTGAATTCCCGG-3′
DJ6	RV ko-primer *CHS3*	5′-GTCTATGCAACGAAGGAGTCACTTTCCTCCTTCCGATTGAGAATATCTTCAGCTTGG CTGCAGGTCGACGG-3′
S3-*TOT1*	one-step *in vivo* tagging *TOT1*	5′-TACCTGTTCCAGAGATTCATGATTTCCCTAAGAGTCATATTGTTGATTTTCGTACGCT GCAGGTCGAC-3′
S2-*TOT1*	one-step *in vivo* tagging *TOT1*	5′-CTTTACGAGCACTATAGACAGTAATTTATATAACTAAGAAAATGGTATGCATCGATG AATTCGAGCTCG-3′
S3-*TOT2*	one-step *in vivo* tagging *TOT2*	5′-GTGTAGGAAGTAGTGATTTGTCCACCCGTATATACTCATTAGCATATGAACGTACGC TGCAGGTCGAC-3′
S2-*TOT2*	one-step *in vivo* tagging *TOT2*	5′-ATTAACTTATTATCCTCTTCTTTTCACATGAGAAATGATATAGATATTGCATCGATGA ATTCGAGCTCG-3′
S3-*TOT3*	one-step *in vivo* tagging *TOT3*	5′-ATGGTAAACTAGGATATGAACTAGACGGTCCATACATGTCGAAAAGAATTCGTACGC TGCAGGTCGAC-3′
S2-*TOT3*	one-step *in vivo* tagging *TOT3*	5′-CTGCTTGGAAAACCGGCCATGTCGGCGGCACATAAAAGTTCTATTTACCTATCGATG AATTCGAGCTCG-3′
S3-*TOT4*	one-step *in vivo* tagging *TOT4*	5′-AGGATCGGTCCGCTTTTCGCTGATTATCTTAACAAAAACTTGAATCGTACGCTGCAG GTCGAC-3′
S2-*TOT4*	one-step *in vivo* tagging *TOT4*	5′-ATTTCGTCTTGCCATTTACCTTCTGATATTAATCACATGTATATCATCGATGAATTCG AGCTCG-3′
S3-*TOT5*	one-step *in vivo* tagging *TOT5*	5′-ACGAAAAGGATGACGATTATGATGAAGAGGATCCATATGAGGATCCCTTTCGTACG CTGCAGGTCGAC-3′
S2-*TOT5*	one-step *in vivo* tagging *TOT5*	5′-TAGTTTACATAATCTGGAAGCACTCACTATTTACCATCAGTTTCTACTTTATCGATG AATTCGAGCTCG-3′
RDN18 FW	RT–PCR	5′-CGCGCAAATTACCCAATCCT-3′
RDN18 RV	RT–PCR	5′-GGCAAATGCTTTCGCAGTAG-3′
ACT1 FW	RT–PCR	5′-CTTCCGGTAGAACTACTGGT-3′
ACT1 RV	RT–PCR	5′-CCTTACGGACATCGACATCA-3′
HHT1 FW	RT–PCR	5′-AGCAAGAAAGTCCACTGGTG-3′
HHT1 RV	RT–PCR	5′-GAATGGCAGCCAAGTTGGTA-3′
SIC1 FW	RT–PCR	5′-TTCACAGAACCTAGTCCCTG-3′
SIC1 RV	RT–PCR	5′-ACTCCTGGCGTCATTTTTCG-3′
CLN3 FW	RT–PCR	5′-CAATCTACGTCCCCGTTATC-3′
CLN3 RV	RT–PCR	5′-CGCTCTTTGGAGTAGTAGCA-3′

(*CLN3*; 0.61 kb) and the CKI (*SIC1*; 0.48 kb) genes. Northern blot analysis was carried out according to standard techniques (Sambrook *et al*., 1989).

Acknowledgements

We are indebted to Mike Stark, Fred Winston, Elmar Schiebel, Jesper Svejstrup, Mike Snyder, Rolf Sternglanz, Anne Sutton, Lorraine Pillus, Darryl Auston and Tony Wright for providing strains, plasmids, antibodies and other tools, and to T.Klapperstück for help with the FACS analysis. The project was supported by grants from the DFG (Scha 750/2) to R.S., the Martin-Luther-Universität to L.F., the Graduierten Förderung to D.J. and the AvH Foundation to R.S. (FLF-DEU/1037031).

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Received December 18, 2000; revised January 30, 2001; accepted February 28, 2001