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ORIGINAL PAPER

New mutant versions of yeast FACT subunit Spt16 affect cell integrity

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Abstract Transcription by RNA polymerase II is impeded by the nucleosomal organization of DNA; these negative effects are modulated at several stages of nucleosomal DNA transcription by FACT, a heterodimeric transcription factor. At promoters, FACT facilitates the binding of TATA-binding factor, while during transcription elongation FACT mediates the necessary destabilization of nucleosomes and subsequent restoration of nucleosome structure in the wake of the transcription elongation complex. Altered FACT activity can impair the fidelity of transcription initiation and affect transcription patterns. Using reporter genes we have identified new mutant versions of the Spt16 subunit of yeast FACT with dominant negative effects on the fidelity of transcription initiation. Two of these *spt16* mutant alleles also affect cell integrity. Cells relying on these *spt16* mutant alleles display sorbitol-remediated temperature sensitivity, altered sensitivity to detergent, and abnormal morphologies, and are further inhibited by the *ssd1-d* mutation. The overexpression of components of protein kinase C (Pkc1) signaling diminishes this *spt16 ssd1-d* temperature sensitivity, whereas gene deletions eliminating components of Pkc1 signaling further impair these *spt16* mutant cells. Thus, the FACT subunit Spt16 and Pkc1 signaling have an overlapping essential function, with an unexpected role for FACT in the maintenance of cell integrity.

Keywords Spt16 · Cryptic promoters · Spt phenotype · Ssd1 · Protein kinase C signaling · Cell wall

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Introduction

DNA in the nucleus is packaged into chromatin, mainly in the context of nucleosomes, placing the DNA in a relatively inaccessible state. However, ready access to DNA is needed for many processes, including gene transcription. This access is facilitated and/or regulated by several proteins or protein complexes (Sims et al. 2004; Workman 2006). Among these is a nuclear complex named FACT, whose subunits are encoded in all eukaryotic cells assessed so far. FACT facilitates the transcription of protein-coding genes by RNA polymerase II (RNAPII) and its accessory proteins (Belotserkovskaya and Reinberg 2004; Belotserkovskaya et al. 2004).

FACT has several activities during transcription. Most notably, FACT is involved in diminishing the nucleosomal barrier to transcription that is encountered by RNAPII, and in this way facilitates transcription elongation. This activity of FACT is seen in in vitro transcription assays (Orphanides et al. 1998, 1999). Human FACT can destabilize nucleosomes during in vitro transcription, most likely extracting one H2A–H2B heterodimer from each nucleosome (Belotserkovskaya et al. 2003). Genetic (in vivo) studies of yeast FACT are consistent with the in vitro observations (Formosa et al. 2002). These findings have led to a model in which a nucleosome is destabilized during transcription elongation by the extraction of one of the two H2A–H2B dimers (Belotserkovskaya and Reinberg 2004;

Belotserkovskaya et al. 2004). These actions may involve the $(H3–H4)_2$ tetramer of the nucleosome: FACT alters the nuclease sensitivity of DNA that contacts the H3–H4 tetramer, and both subunits of FACT bind H3–H4 (Belotserkovskaya et al. 2003; Rhoades et al. 2004; Stuwe et al. 2008). Moreover, the FACT subunit Spt16 can be mutated to suppress the deleterious effects of an H3 mutation (Duina et al. 2007). FACT localizes to transcribed regions, and may travel with RNAPII, facilitating access to nucleosomal DNA during the transcription elongation process (Belotserkovskaya et al. 2004; Kim et al. 2004; Mason and Struhl 2003; Saunders et al. 2003).

Although FACT may be a global transcription factor, recent findings show that in vivo depletion of the yeast FACT subunit Spt16, and thus of FACT activity, can have gene-specific effects on transcription (Jimeno-Gonzalez et al. 2006). In that study, these effects are attributed to differential stringency in nucleosome positioning at the 5′ ends of transcribed regions, resulting in dissimilar needs for FACT activity at different genes.

Altered FACT activity can affect transcription patterns in a different way, through promoter activation. Sequences internal to many transcribed regions have the potential to act as sites of transcription initiation, but are prevented from doing so by the inherently repressive nature of nucleosomal structure. Repression of these so-called 'cryptic' promoters during normal transcription from upstream promoters depends on the reestablishment of nucleosome structure after the passage of RNAPII. The involvement of FACT in this activity is evidenced by studies showing that, in vivo, a FACT mutation impairs histone deposition in the wake of RNAPII (Schwabish and Struhl 2004). Consequently, mutant FACT may allow transcription initiation at cryptic-promoter sites within transcribed regions (Evans et al. 1998; Kaplan et al. 2003; Lycan et al. 1994; Malone et al. 1991; Mason and Struhl 2003; Prelich and Winston 1993; Rowley et al. 1991; Xu et al. 1993). Indeed, a genome-wide survey found that impaired FACT activity permits transcription initiation at cryptic internal promoters in over 1,000 yeast genes (Cheung et al. 2008). FACT activity, through its ability to mediate the transcription-linked restoration of nucleosome structure, is thus necessary for the fidelity of transcription initiation.

FACT also has effects on transcription initiation that are more direct. In yeast, the FACT subunit Spt16 shows genetic interactions with the general transcription initiation factor TFIIA and with TATA-binding protein TBP, which binds TFIIA and promoter DNA for transcription initiation (Biswas et al. 2005). Mutating the Spt16 subunit of FACT decreases TBP binding to promoters in vivo, while in vitro assays show that FACT facilitates the cooperative binding of TBP and TFIIA to promoter sequences that are in a nucleosomal configuration (Biswas et al. 2005; Mason and Struhl 2003). These findings provide another potential mechanism for gene-specific effects of FACT mutations.

Genetic approaches can lead to significant understanding of in vivo function. Indeed, important advances in understanding Spt16 function, and thus most likely FACT function, have been gained through the study of *spt16* mutations that were identified because they confer temperature sensitivity for yeast cell proliferation (Formosa et al. 2001). However, such mutational effects need not be specific to transcription, as FACT and/or its subunits are also implicated in other activities, including DNA replication and repair, and heterochromatin function (Keller and Lu 2002; Krohn et al. 2003; Lejeune et al. 2007; Obuse et al. 2004; Okuhara et al. 1999; Schlesinger and Formosa 2000; Tackett et al. 2005; Tan et al. 2006; Tan and Lee 2004; Wittmeyer and Formosa 1997; Yarnell et al. 2001). Moreover, several of these temperature-sensitive yeast mutations destabilize the mutant Spt16 protein, making it instructive to distinguish the effects of inadequate but normal Spt16 function from mutationally impaired function (O'Donnell et al. 2004; Xu et al. 1993, 1995). We therefore sought mutant alleles of the yeast *SPT16* gene that affect the Spt16 protein and FACT in a dominant manner, reasoning that a mutant Spt16 protein would be expected to be relatively stable to produce a dominant effect. The effect we sought was impaired fidelity of transcription initiation due to defective nucleosome reassembly in the wake of transcribing RNAPII, as manifested by the activation of functional transcription from cryptic promoters in certain reporter genes (Winston 1992). Described here are the unselected characteristics of two of these mutant alleles that indicate effects on cell integrity, reflecting perturbations by these *spt16* mutations in the transcriptional responses to cell-wall stresses. These findings identify a previously unsuspected impact on yeast cell physiology of Spt16 and FACT.

Materials and methods

Strains and plasmids

Yeast strains used in this study are listed in Table 1; all have the S288C genetic background. Status of the *SSD1* locus was determined by sequencing. New plasmid-borne *spt16* alleles with a dominant Spt phenotype were identified in cells of strains AW11-9a and AFO1-I2. Plasmid-borne *spt16* alleles were also assessed in meiotic segregants from a diploid strain in which one *SPT16* allele is replaced by a *spt16Δ::kanMX4* deletion/replacement allele, and/or by plasmid shuffling in *spt16Δ::kanMX4* segregants, testing several independent isolates. Chromosomally integrated

Table 1 Yeast strains

Strain	Genotype	Source
BM403	*MAT***a** *his4-912δ lys2-128δ ura3-52 suc2ΔUAS spt16-G132D* (also known as *spt16-197*)	F. Winston; Xu et al. (1993)
AW11-9a	*MATα his4-912δ lys2-128δ leu2-3,112 trp1-Δ1 ura3-52 suc2ΔUAS SPT16*	Brewster et al. (2001)
AFO1-I2	*MAT***a** *his4-912δ lys2-128δ leu2-3,112 trp1-Δ1 ura3-52 suc2ΔUAS spt16-G132D*	BM403 × AW11-9A segregant
BM64	*MAT***a**/*MATα his4-912δ lys2-128δ leu2-3,112 trp1-Δ1 ura3-52 ssd1-d SPT16/spt16-101::LEU2* (homozygous unless indicated)	F. Winston; Xu et al. (1993)
KanBd	*MAT***a**/*MATα his4-912δ lys2-128δ leu2-3,112 trp1-Δ1 ura3-52 ssd1-d SPT16/spt16Δ::kanMX4* (homozygous unless indicated)	BM64 derivative; O'Donnell et al. (2004)
KanB-[plasmid]	*MATα* (or *MAT***a**) *his4-912δ lys2-128δ leu2-3,112 trp1-Δ1 ura3-52 ssd1-d spt16Δ::kanMX4* [*spt16* or *SPT16 CEN* plasmid]	KanBd segregants
FY2393	*MAT***a** *lys2-128δ his3Δ200 leu2Δ1 trp1Δ63 ura3-52 prGAL1-FLO8-HIS3::kanMX*	F. Winston; Prather et al. (2005)
Y2454	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1*	C. Boone; Tong et al. (2001)
Y2454-WT68	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1 SPT16:natMX4*	Y2454 derivative
Y2454-312-8	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1 spt16-312:natMX4*	Y2454 derivative
Y2454-319-5	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1 spt16-319:natMX4*	Y2454 derivative
Y2454-E763G	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1 spt16-E763G:natMX4*	Y2454 derivative
Y2454-E857 K	*MATα mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 ura3Δ0 MET15 lys2Δ0 SSD1 spt16-E857 K:natMX4*	Y2454 derivative
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SSD1*	C. Boone; Brachmann et al. (1998)
orfΔ	BY4741 *orfΔ::kanMX4*	C. Boone; Winzeler et al. (1999)

versions of *spt16-312* and *spt16-319* tagged with *natMX4* at a downstream *Bsr*GI site were used in analyses involving the yeast gene-deletion collection (Winzeler et al. 1999).

Plasmid-borne versions of *SSD1* and *SRP101* were generated in the *LEU2* vectors pRS315 and pRS425 by PCR amplification from the S288C-derived genomic library plasmid identified as a suppressor of *spt16-312* temperature sensitivity, using primers with *Pst*I and *Xho*I restriction sites to amplify regions spanning ~300 bp upstream and downstream of each ORF. The subcloned *SSD1* gene contained a single nucleotide difference from the reported *SSD1* sequence (Wilson et al. 1991), a C-to-T substitution at ORF nucleotide 1,442 recoding codon 481 from serine to phenylalanine, which was found to have no discernable phenotypic effect. Plasmids bearing previously characterized temperature-sensitive *spt16* mutations (Formosa et al. 2001) were gifts from T. Formosa. High-copy plasmids bearing *WSC1* (pWSC1), *MPT5* (YEpMPT5), and *PKC1* (YEpPKC1) (Hata et al. 1998; Verna and Ballester 1999) were gifts from L. Guarente, and high-copy *SLT2* (pBA958), *SWI6* (pBA1036), and *SWI4* (pBA509) plasmids were gifts from B. Andrews. *LacZ* reporter plasmids pBA251 (*SCB-lac*Z), pBA487 (*MCB-lac*Z), and pBA537 (*prPCL1-lac*Z) (Andrews and Herskowitz 1989; Ogas et al. 1991; Verma et al. 1992) were also gifts from B. Andrews, while pLGΔ-312 (*prCYC1-lac*Z), p1434 ([+2xRlm1 site]-*prCYC1-lac*Z), p1435 ([ΔRlm1 site]-*prPRM5-lac*Z) and p1366 (*prPRM5-lac*Z) (Guarente and Mason 1983; Jung et al. 2002) were gifts from D. E. Levin.

PCR mutagenesis

Mutations in *SPT16* were created in a pRS316-based plasmid using PCR followed by in vivo recombination (Muhlrad et al. 1992) to mutagenize codons 32–388, 264–813, and 712–1,035 (the C-terminal codon) of the *SPT16* ORF; some PCR reactions used decreased dATP levels to increase error frequency. Transformed *his4-912δ lys2-128δ* cells were then tested by replica plating for growth on histidine- and/or lysine-deficient medium. Our *SPT16* (strain AW11-9a; selection at 23° and 30°) and *spt16-G132D* (strain AFO1-I2; selection at 23°) recipient cells gave similar mutant yields. Plasmid dependence of an Spt

phenotype was verified by plasmid re-transformation into cells of both starting strains and/or by plasmid-loss experiments.

Gene-dosage suppression

Transformants of *spt16Δ::kanMX4* [*spt16-312* or *spt16-319*] cells harboring high-copy *LEU2* yeast genomic library plasmids were assessed for 39° growth by replica plating, yielding 64 and 72 temperature-resistant derivatives, respectively, for which 39° growth was dependent on a library plasmid. Library plasmids carrying the *SPT16* gene were identified by restriction analysis, PCR verification, and plasmid-loss experiments; the *SSD1* library plasmid insert was identified by vector-insert junction sequencing.

β-galactosidase assays

Permeabilized-cell assays were performed as described (Barnes et al. 1995), using cells grown at 30° to densities of 2–4 × 10^6 cells/ml, as determined by Coulter counter and confirmed by OD_{600} measurements. For each reporter plasmid and yeast strain, at least two sets of β-galactosidase assays were carried out in triplicate.

Northern analysis

Total RNA from cells used for *prPCL1-lacZ* and *prPRM5-lacZ* reporter-gene assays was analyzed by northern blots as described (Barnes et al. 1993). Probes were an internal *Xho*I–*Hin*dIII *ACT1* fragment, a 1-kbp PCR-generated fragment of *PCL1* encompassing the ORF, and a 960-bp PCR-generated fragment of *PRM5* encompassing the ORF. Signals were quantified by densitometry (Heikkinen et al. 2003).

Results

Dominant mutant alleles of *SPT16* can alter transcription patterns

To learn more about the Spt16 component of yeast FACT, a selection scheme to identify new mutant versions of Spt16 was undertaken based on altered expression of reporter genes. The reporter genes, *his4-912δ* and *lys2-128δ*, each have an inserted δ element that alters the transcription pattern, preventing the production of functional mRNA (Winston 1992). Mutational alterations in certain components of the transcription machinery, or in chromatin and some of its regulators, have been found to alter transcription of these reporter genes, leading to the production of functional His4 and Lys2 enzymes in what has been termed the Spt phenotype (Malone et al. 1991; Winston 1992; Winston et al. 1984).

Mutations affecting almost the entire Spt16 protein were created using error-prone PCR (Muhlrad et al. 1992) to amplify three overlapping segments of the *SPT16* ORF. The resulting PCR products, along with an appropriately digested ('gapped') plasmid-borne *SPT16* gene, were co-transformed into *his4-912δ lys2-128δ* cells, allowing full-length plasmid-borne coding sequences to be generated in vivo by homologous recombination. Transformant colonies were then tested for growth on histidine- and/or lysine-deficient medium, to identify mutagenized *SPT16* sequences with dominant effects that allow functional transcription of one or both of these reporter genes in cells despite normal Spt16 activity from the chromosomal *SPT16* locus. Although plasmids with this dominant effect were not obtained by PCR mutagenesis of the dispensable N-terminal domain (O'Donnell et al. 2004), many such

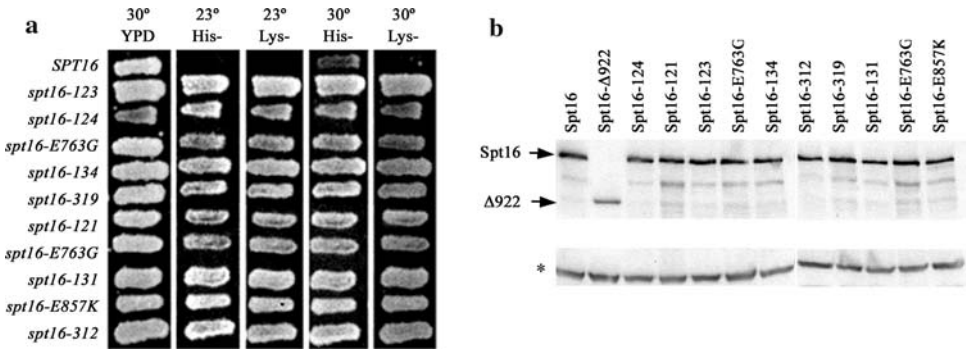

Fig. 1 New dominant negative *spt16* alleles. **a** The Spt phenotype. *SPT16 his4-912δ lys2-128δ* cells (strain AW11-9a) harboring the initially identified plasmid-borne *spt16* alleles were grown on YPD medium and then replica-plated to the conditions indicated. **b** Western analysis indicating normal abundance of Spt16 mutant proteins in *spt16Δ* cells (KanB derivatives; Table 1) relying on a low-copy *spt16* plasmid expressing the indicated mutant protein. The N-terminally truncated Spt16-Δ922 protein (Evans et al. 1998) is included to verify band identification. India ink staining of total proteins after transfer (not shown) and a 40-kDa non-specific band (*asterisk*) verified equivalent loading and transfer

Table 2 *spt16* mutant alleles with dominant negative Spt phenotype

Allele	Substitutions[a]
spt16-E857K	E857K
spt16-E763G	*E763G*
spt16-121	N363S, *E763G*
spt16-122	E312D, L680P, *E763G*, R783G
spt16-123	*E656G*, *E735G*, *E763G*
spt16-124	V694A, I724T, K754R, *E763G*, F782L
spt16-131	*E763G*, *W913R*
spt16-134	*K725R*, *E763G*, K806R, I815T, V886A, E966A, Y1006C
spt16-312	*E763G*, R784G, S819P
spt16-319	*L804P*, L946S, E1004G

[a] *Italics* residues conserved in human, *D. melanogaster, C. elegans,* and *S. pombe* orthologs

plasmids were identified in the products of mid- and C-terminal-domain gap repair.

DNA sequence was obtained initially for the gap-repaired region of 11 plasmids allowing such growth (Fig. 1a), chosen to sample the products of four independent PCR amplifications of the mid and C-terminal domains. Table 2 shows that the substitution of the glutamate at position 763 with glycine (E763G) was found to be encoded in 9 of these 11 mutant alleles, with two encoding only the E763G substitution (only one included in Table 2).

A normal gene with a cryptic internal promoter is *FLO8* (Kaplan et al. 2003). This promoter is activated by mutations affecting many components of chromatin regulation, including Spt16 (Cheung et al. 2008; Kaplan et al. 2003). Testing *spt16-E763G, spt16-E57K, spt16-312,* and *spt16-319* using the *prGAL1-FLO8-HIS3* reporter gene (Cheung et al. 2008) showed that all of these *spt16* mutant alleles activate the *FLO8* cryptic promoter in dominant fashion (data not shown). Impaired cryptic-promoter repression is thus a general effect of these new *spt16* mutant alleles. The dominance suggests that these mutant Spt16 proteins interfere with the ability of normal Spt16, and FACT, to ensure the fidelity of transcription initiation. Most likely FACT containing mutant Spt16 competes with wild-type FACT for association with other transcription components, but is unable to function effectively in this context.

The new *spt16* mutant alleles, under control of the normal *SPT16* promoter and expressed from low-copy plasmids, were tested for the ability to provide essential Spt16 activity in *spt16-101::LEU2* cells or *spt16Δ::kanMX4* deletion/replacement cells. These tests indicated that the *spt16* plasmids with a dominant Spt phenotype provide essential Spt16 activities (*spt16-122* was not tested). Expression of the *his4-912δ* and *lys2-128δ* reporter genes in these cells showed that the *spt16* alleles not only supply essential function, and therefore retain several normal abilities, but also cause an Spt phenotype as the only Spt16-encoding gene (data not shown). Preliminary analysis of other features of some of these mutations has been reported (O'Donnell et al. 2004).

The dominant mutants define a new class of Spt16 functional alterations

Several *spt16* mutant alleles that have been previously identified because they confer temperature sensitivity on mutant cells also encode substitutions affecting the same segment of the Spt16 polypeptide as those described here (Formosa et al. 2001). Most of these also produce an Spt phenotype as the only Spt16-encoding gene (Formosa et al. 2001). However, direct testing of several of these plasmid-borne mutant alleles (*spt16-6, -7, -9a,* and *-11*) showed that none produced a dominant Spt phenotype in the AW11-9a tester cells (data not shown). Similarly, other *spt16* mutant alleles with an Spt phenotype, including the widely used temperature-sensitive *spt16-G132D* mutant allele, also known as *spt16-197* (Evans et al. 1998), encoding an unstable Spt16 protein (Malone et al. 1991; Rowley et al. 1991), and *spt16-Δ922*, encoding a stable but N-terminally truncated version of the Spt16 protein (Evans et al. 1998; O'Donnell et al. 2004), failed to exert dominant Spt effects (data not shown). Novel *SPT16* mutant alleles that suppress the cold sensitivity of a histone H3 mutation in dominant fashion, including one that causes a substitution (E857Q) similar to the E857K substitution reported here, also fail to exert dominant Spt effects (Duina et al. 2007).

Spt16 mutant proteins are present at normal levels

Altered levels of Spt16 protein can generate an Spt phenotype (Brewster et al. 1998; Malone et al. 1991; Xu et al. 1993). Therefore, western blot analysis was used to assess Spt16 protein levels in *spt16Δ::kanMX4* cells harboring the *spt16* mutant alleles on low-copy *CEN* plasmids. This experiment showed that the abundance of each of the mutant Spt16 proteins approximated that of wild-type Spt16 similarly expressed in these cells, showing that altered Spt16 protein abundance does not account for the Spt phenotype of the *spt16* mutant alleles (Fig. 1b).

Spt16 mutant proteins cause additional phenotypes

Some previously characterized *spt16* mutant alleles display extended phenotypes, including sensitivity to high temperature and/or hydroxyurea (Evans et al. 1998; Formosa et al. 2001; O'Donnell et al. 2004). Therefore,

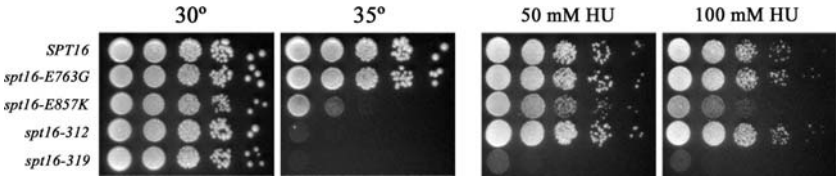

Fig. 2 Temperature and hydroxyurea (HU) sensitivities of selected *spt16* mutant alleles. *spt16Δ* cells (KanB derivatives) harboring the indicated plasmid-borne *spt16* allele were grown overnight in uracil-free liquid medium at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated for three days on YPD solid medium at the indicated temperatures or on YPD + HU at 30°

the new *spt16* mutant alleles described here, in a set of isogenic strains, were evaluated for effects on growth in *spt16Δ* cells under a variety of conditions (Hampsey 1997), including the presence of 6-azauracil, mycophenolic acid, caffeine, ethanol, formamide, and hydroxyurea, at elevated temperature and at elevated salt, glycerol, and sorbitol concentrations, at low pH, and for survival during inositol deprivation and nitrogen limitation. Under most of these conditions, cells harboring these *spt16* substitution mutations grew as robustly as did the wild-type *SPT16* control cells (data not shown). Notable exceptions were the temperature sensitivities of cells relying on *spt16-E857K*, *spt16-312* or *spt16-319*, and hydroxyurea sensitivities of cells relying on *spt16-E857K* or *spt16-319* (Fig. 2).

The temperature sensitivity of some previously reported *spt16* mutations is alleviated by increased gene dosage and consequent increased abundance of Spt16 polypeptide (O'Donnell et al. 2004; Xu et al. 1993, 1995). However, expression of the *spt16-312* and *spt16-319* mutant alleles from multicopy (2μ-based) plasmids does not alleviate the temperature sensitivities, even though these mutant proteins show normal stabilities at high temperature (O'Donnell et al. 2004). Therefore, the temperature sensitivities conferred by *spt16-312* and *spt16-319* are due to impaired function(s) of the normally abundant but mutant Spt16 proteins.

As indicated in Table 2, the single substitution that defines the *spt16-E763G* mutant allele is also present in *spt16-312*, but the latter mutant allele has extended phenotypes, including temperature sensitivity and sensitivity to cell-integrity indicators (see below), not seen for *spt16-E763G*. Molecular dissection showed that the other two *spt16-312* substitutions do not themselves cause these sensitivities (data not shown; see Fig. 4b); thus the extended phenotype of *spt16-312* results from the combination of the E763G substitution with one or both of those additional substitutions. In contrast, analogous experiments showed that the extended phenotype of the triple substitution *spt16-319* allele is caused solely by the L804P substitution, and that the other two substitutions have little or no effect (data not shown).

An *ssd1-d* mutation enhances *spt16* temperature sensitivity

To elucidate the mechanisms of *spt16-312* and/or *spt16-319* temperature sensitivity, genes were sought that, when present in increased gene copies, alleviate these temperature sensitivities. Growth at 39° of *spt16Δ::kanMX4* cells harboring a *spt16-312* or *spt16-319* low-copy plasmid was conferred by the presence of many high-copy library plasmids, but most of these carried the wild-type *SPT16* gene. Similar 39° growth was also made possible by *SPT16* on a low-copy *CEN* vector (data not shown). Thus, while the Spt phenotype of *spt16-312* and *spt16-319* is dominant, temperature sensitivity is a recessive trait (see also Fig. 7).

The single library plasmid allowing 39° growth that did not carry *SPT16* was found to harbor the complete coding sequences for *SSD1* and *SRP101* as well as a 3′ portion of *YDR291W* (Fig. 3a, b). Subcloning showed that *SSD1* itself diminishes the temperature sensitivity of *spt16-312* cells, while *SRP101* has no effect (Fig. 3a). A low-copy *SSD1* plasmid also alleviated the temperature sensitivities of *spt16-312* and *spt16-E857K*, suggesting that complementation of an unexpected *ssd1* mutant allele present in these cells may be involved (Fig. 3c).

The *SSD1* locus is polymorphic in yeast laboratory strains. The chromosomal *ssd1* locus present in the haploid *spt16Δ::kanMX4* cells, retrieved onto a plasmid by gap repair and sequenced, harbored a single change, the C-to-G substitution characteristic of the *ssd1-d* mutant allele that converts codon 698 of the 1,250-codon *SSD1* open reading frame into a stop codon (Sutton et al. 1991; Uesono et al. 1997). Thus, *SSD1* diminishes temperature sensitivity in these situations through complementation of a chromosomal *ssd1-d* allele. Temperature sensitivity is therefore exacerbated by the *ssd1-d* mutation.

In *ssd1-d* cells with and without a low-copy *SSD1* plasmid, the *spt16-E763G, spt16-E857K, spt16-312,* and *spt16-319* mutant alleles, and the *spt16-L804P* derivative of *spt16-319*, were all found to activate the Spt reporter genes. Similarly, *SSD1* status had no effect on the HU sensitivities of *spt16-319* and *spt16-L804P* (*spt16-E857K* was not tested). Therefore, the effects of *SSD1* status are

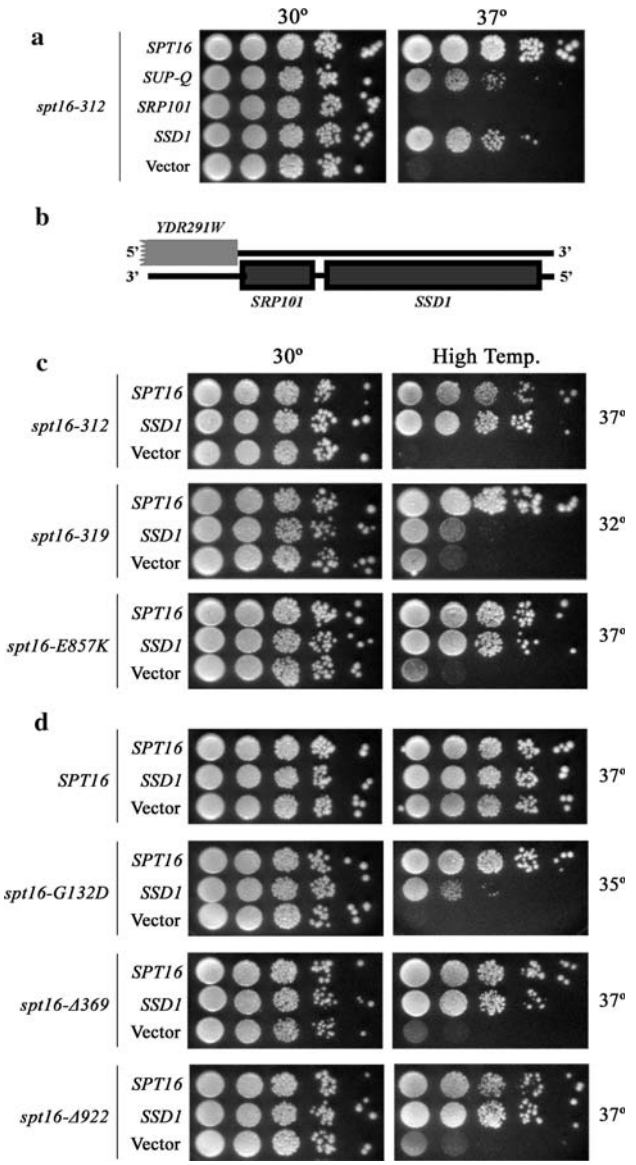

Fig. 3 Suppression of *spt16* temperature sensitivities. **a** *spt16-312* mutant cells (KanB derivative) harboring the suppressing high-copy plasmid (*SUP-Q*) or single-gene plasmids as indicated on the left were grown in leucine- and uracil-free liquid medium at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated for 3 days on YPD solid medium at the indicated temperatures. **b** Schematic of genomic DNA carried by the suppressing library plasmid. **c**, **d** Derivatives of strain KanB relying on the indicated *spt16* plasmid (far left) and harboring the indicated low-copy plasmid (near left) were incubated as in (**a**) at restrictive temperatures appropriate for each *spt16* allele (far right). *spt16-Δ922* and *spt16-Δ369* encode functional but N-terminally resected proteins (Evans et al. 1998; O'Donnell et al. 2004)

limited to certain features of these new *spt16* mutant alleles.

To determine if the temperature sensitivities conferred by other *spt16* mutant alleles are also affected by *SSD1* status, *spt16Δ::kanMX4 ssd1-d* cells harboring plasmid-borne *spt16-G132D*, *spt16-Δ922* and *spt16-Δ369* (Evans et al. 1998; O'Donnell et al. 2004), were transformed with the low-copy *SSD1* plasmid or empty vector and tested for growth at elevated temperatures. For all three mutants, the presence of the *SSD1* plasmid increased the permissive growth temperature (Fig. 3d). This interaction between *spt16* and *ssd1-d* suggests that Spt16 and Ssd1 work in parallel to mediate a common cellular process.

spt16 mutant phenotypes suggest defective cell integrity

Yeast cell growth demands continuous and controlled cell-wall remodeling, with the inherent danger of loss of cell integrity due to aberrant cell-wall remodeling. Ssd1 has been implicated in the maintenance of cell integrity. Allelic variation at the *SSD1* locus leads to altered composition of the yeast cell wall (Ibeas et al. 2001; Wheeler et al. 2003), and the *ssd1-d* allele alters sensitivities to sodium dodecylsulfate (SDS), caffeine, and Calcofluor White, which reflect perturbations of the cell wall (Kaeberlein and Guarente 2002; Martin et al. 1996). In light of the genetic interaction identified here between *ssd1* and *spt16*, phenotypes associated with perturbations of cell integrity were assessed for cells with mutant Spt16 proteins.

Sorbitol remediation of temperature sensitivity reflects the suppression, through osmotic stabilization, of cell lysis that would otherwise be brought about by impaired cell integrity (Hampsey 1997). Fig. 4a shows that the presence of 1 M sorbitol increased the range of permissive growth temperatures for *ssd1-d* mutant cells that harbor new temperature-sensitive *spt16* mutations. This suppression is consistent with an overlapping role for Spt16 and Ssd1 in the maintenance of cell integrity.

The *ssd1-d* allele in combination with mutations in other genes mediating cell integrity can cause increased sensitivity to SDS (Kaeberlein and Guarente 2002; Moriya and Isono 1999). Tryptophan auxotrophy also causes sensitivity to SDS (Liu et al. 2004); therefore, cells were made tryptophan prototrophic by transformation with a low-copy *TRP1* plasmid before testing. These experiments showed that, while *spt16-319 ssd1-d* double-mutant cells were no more sensitive to SDS than were *SPT16 ssd1-d* single-mutant cells, *spt16-312 ssd1-d* cells were SDS sensitive, and that this sensitivity was abrogated by the plasmid-borne *SSD1* gene (Fig. 4b). This detergent sensitivity suggests differences in cell-wall status due to the *spt16-312* mutation.

Sensitivity to the anionic dye Congo Red is another hallmark of cell-wall defects (Ram and Klis 2006). Both *spt16-312 ssd1-d* and *spt16-319 ssd1-d* cells showed increased sensitivity to Congo Red compared to *SPT16*

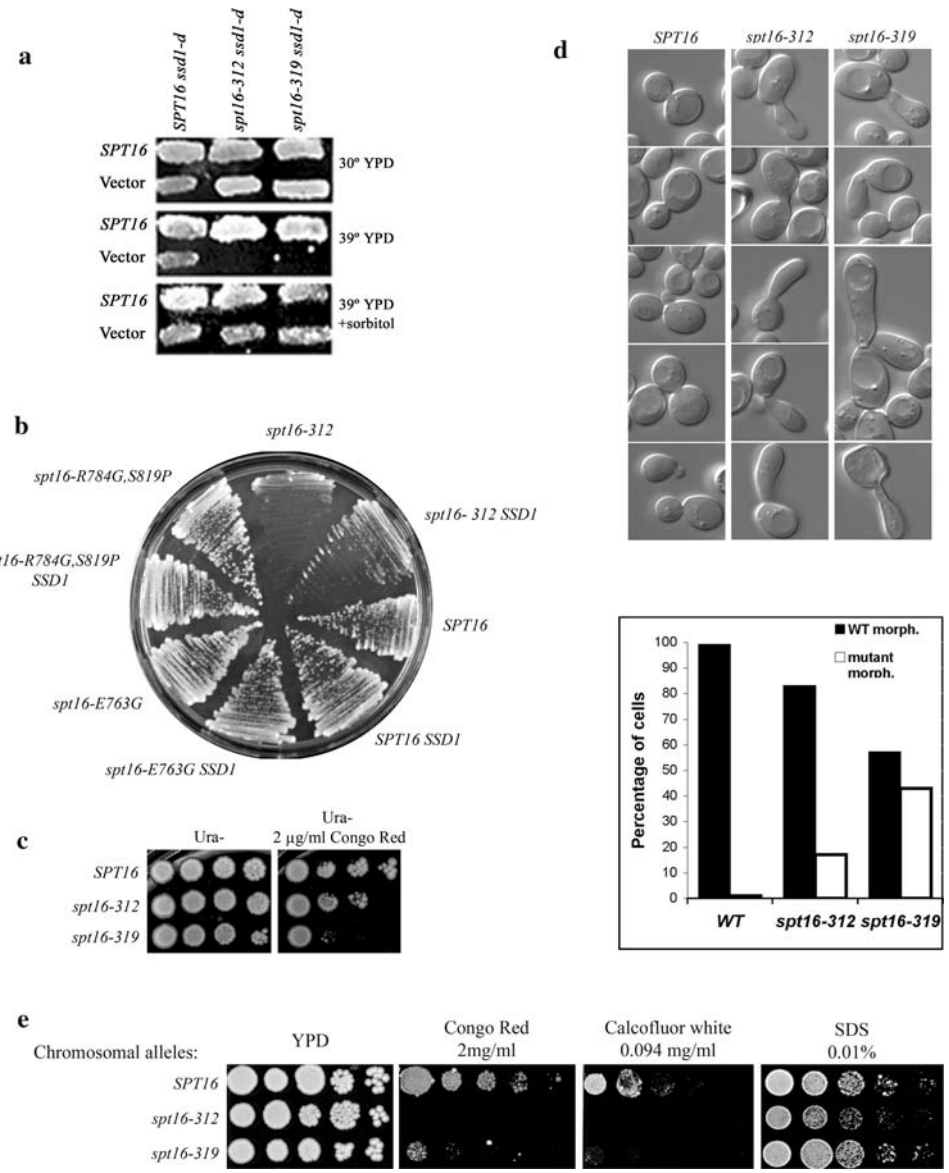

Fig. 4 *spt16* phenotypes indicative of cell-integrity defects. **a** Cells of plasmid-bearing *ssd1-d* KanB derivatives with the genotype indicated above each column, and harboring the low-copy plasmid indicated on the left, were grown on YPD and then replica-plated to YPD and YPD + 1 M sorbitol, and incubated at the temperatures indicated. **b** *spt16Δ ssd1-d* (KanB) cells harboring the indicated *spt16* or *SPT16* plasmid and either a low-copy *SSD1* plasmid or vector control were grown in selective medium to maintain both plasmids and streaked onto YPD + 0.02% SDS medium and incubated at 30°. **c** *spt16Δ ssd1-d* (KanB) cells harboring the indicated *spt16* or *SPT16* plasmid were grown in uracil-free liquid medium at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated at 30° on SC-uracil medium with or without Congo Red (2 µg/ml). **d** Representative morphological anomalies found by differential interference contrast microscopy for *spt16 ssd1-d* cells (plasmid-bearing KanB derivatives) grown to liquid rich medium at 30°. **e** Derivatives of strain Y2454 with an intact *SSD1* gene and chromosomally integrated *spt16* or *SPT16* alleles (Table 1) were grown in rich liquid medium at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated at 30° on YPD containing the indicated agents. These tests used a higher concentration of Congo Red than for panel (**c**). In all panels on this figure, comparisons were between isogenic cells

ssd1-d cells, another suggestion of a role for Spt16 in the maintenance of cell integrity (Fig. 4c).

Cell-integrity mutations in combination with *ssd1* often lead to abnormal cell morphologies, including elongated and/or misshapen buds and multi-budded cells indicative of impaired cytokinesis and/or cell septation (Du and Novick 2002; Kaeberlein and Guarente 2002; Moriya and Isono 1999). Similar morphological defects were seen for *spt16 ssd1-d* cells growing at 30°, but not for *SPT16 ssd1-d* or *spt16 SSD1* cells (Fig. 4d, and data not shown); the percentage of *spt16 ssd1-d* cells displaying these defects increased after cells were shifted to 38° for further

incubation (data not shown). Diamidino-2-phenylindole (DAPI) staining of DNA in *spt16 ssd1-d* cells growing at 30° showed that each of the cell bodies in a multiply budded cell or cell 'chain' contained a nucleus, suggesting that nuclear division is not significantly affected (data not shown). This finding is consistent with the nuclear staining reported for other situations in which mutations combined with *ssd1-d* result in the formation of 'chains' of septation-deficient cells (Costigan et al. 1994; Moriya and Isono 1999).

The above investigations indicating cell-wall defects were carried out in *spt16Δ* mutant cells harboring a low-copy *SPT16* or *spt16* plasmid. Similar determinations were made, in an *SSD1* genetic background (Jorgensen et al. 2002), using cells with the *spt16-312* and *spt16-319* alleles genetically marked and integrated at the *SPT16* chromosomal locus (Fig. 4e). These tests showed an analogous, but less marked, sensitivity to SDS caused by the *spt16-312* mutation, and sensitivities to the anionic dye Calcofluor White, another indicator of cell-wall effects (Ram and Klis 2006), caused by both *spt16-312* and *spt16-319*. As seen for cells with plasmid-borne *spt16* mutant alleles (Fig. 4c), the cells relying on chromosomal *spt16* mutant alleles were also sensitive to Congo Red (Fig. 4e). These observations reinforce the view that these *spt16* mutant alleles affect cell integrity through cell-wall effects.

Genetic interactions with the Pkc1 pathway indicate a role for Spt16 in cell integrity

Cell integrity is also maintained through Pkc1-mediated signal transduction, which occurs via a phosphorylation cascade from the Pkc1 protein kinase to the Mkk1, Mkk2 and Slt2/Mpk1 kinases (Heinisch et al. 1999), with cell-surface sensor proteins such as Wsc1/Slg1 as initiators of Pkc1 signaling (Verna et al. 1997). In light of the above phenotypic and genetic evidence suggesting that Spt16 mediates cell integrity and morphogenesis in parallel with Ssd1, genetic interactions were sought between *spt16* mutant alleles and determinants of Pkc1 cell-integrity signaling.

The first approach was to assess whether the overexpression of Pkc1-pathway components alleviates the temperature sensitivities of *spt16 ssd1-d* cells. Whereas a multicopy *SLT2* plasmid had no effect on *spt16-319* temperature sensitivity and in fact inhibited *spt16-312* cells, increased dosage of *WSC1* and *PKC1* weakly suppressed the temperature sensitivities of *spt16-312* and *spt16-319* cells, suggesting that these *spt16* mutations impair Pkc1 signaling for cell integrity (Fig. 5a).

A pathway mediating cell integrity in parallel with Pkc1 signal transduction and Ssd1 involves the Mpt5 protein (Hata et al. 1998; Kaeberlein and Guarente 2002). However, increased *MPT5* gene dosage was unable to relieve the temperature sensitivities of *spt16-312* and *spt16-319* cells (Fig. 5a), and in fact decreased the maximal growth temperature of *spt16-312* cells. Thus, Mpt5-mediated activity promoting cell integrity may not be functionally linked to the activities affected by these *spt16* mutations.

As another approach to characterizing the effects of *spt16* mutations, deleterious genetic interactions were assessed for *spt16* cells deleted for other genes. Synthetic genetic array (SGA) analysis (Tong et al. 2001) was carried out at 30°, in an *SSD1* genetic background, using cells with chromosomal *spt16-312* and *spt16-319* alleles. This analysis (data not shown) indicated that *spt16-312* has a deleterious genetic interaction with *smi1Δ/knr4Δ*, which affects cell-integrity signaling (Martin-Yken et al. 2003), and that *spt16-319* interacts genetically with *swi4Δ*, which eliminates a transcription activator that is an effector of Pkc1 signaling (Baetz et al. 2001; Madden et al. 1997). Further evidence for genetic interactions with deletions eliminating these and other Pkc1-signaling components was therefore sought.

Tetrad analysis was carried out, in an *SSD1* genetic background, using parental cells with integrated and genetically marked *spt16* mutations and others with gene deletion/replacement mutations (Winzeler et al. 1999). These experiments showed good double-mutant viability at 30° for *spt16-319* and *spt16-312* in combination with *smi1Δ*, but the *spt16-312 smi1Δ* double mutants were more temperature sensitive for growth (data not shown). Similarly, there was good double-mutant viability involving deletions of the *MKK1*, *SLT2*, and *BCK2* genes, which encode components of Pkc1 signaling (Fig. 5b). However, double mutants combining *spt16-312* and *spt16-319* with *slt2Δ* and *bck2Δ* were significantly impaired at 37° in comparison to single-mutant cells (Fig. 5c). These observations indicate that Spt16 and components of each branch of the Pkc1 signaling pathway, represented by Slt2 and Bck2, share an overlapping essential function.

The two branches of Pkc1-mediated signaling are thought to act in parallel to promote cell integrity, with the Swi4-containing transcription activator SBF as a common downstream target (Heinisch et al. 1999; Madden et al. 1997; Wijnen and Futcher 1999). Although the overexpression from multicopy plasmids of Swi4 or the other SBF component, Swi6, did not affect the temperature sensitivity of *spt16-312* or *spt16-319* mutant cells (*ssd1-d* genetic background; Fig. 5a), tetrad analysis (*SSD1* genetic background) verified that deletion of the *SWI4* gene causes synthetic impairment in combination with *spt16-319* (Fig. 5b, c). Some *spt16-319 swi4Δ* double-mutant segregants did germinate to produce cells that grew reasonably well at 30°, as evident from the large colonies on

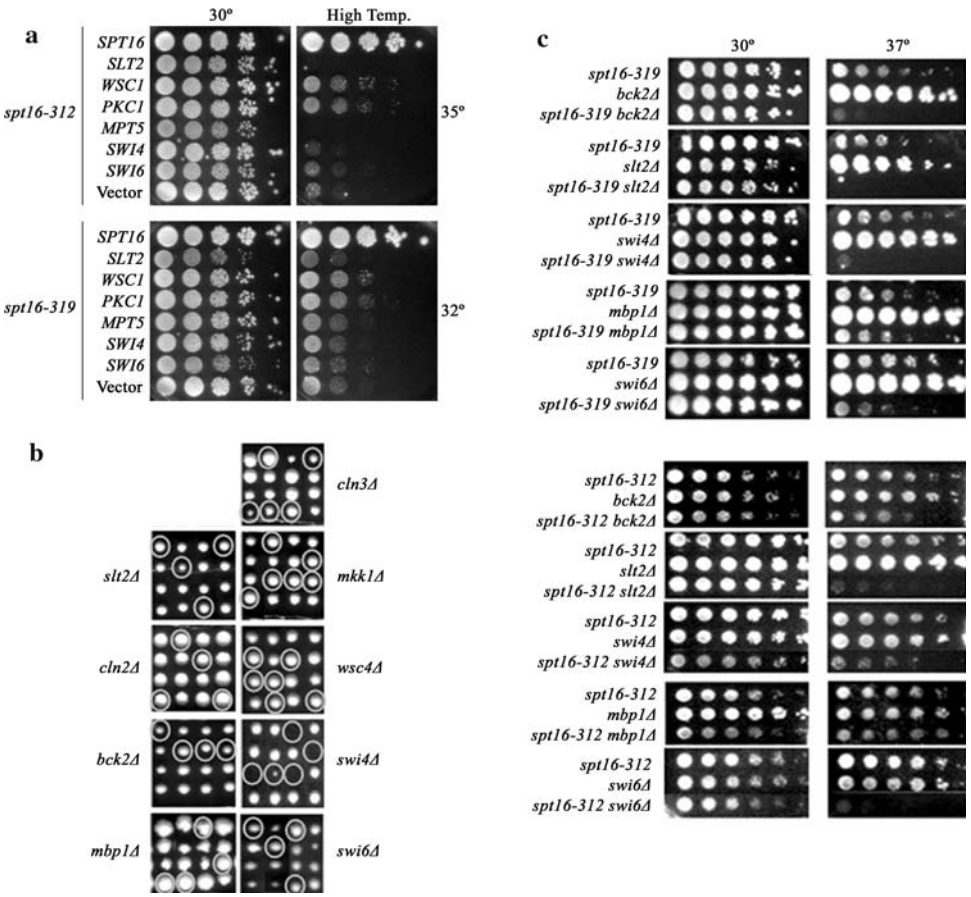

Fig. 5 Genetic interactions between *spt16* and genes encoding Pkc1-signaling components. **a** Increased dosage of *PKC1*and *WSC1* suppresses the temperature sensitivities of *spt16-312* and, to a lesser extent, *spt16-319* cells. *spt16Δ ssd1-d* cells (KanB derivatives) relying on the indicated *spt16 URA3* plasmid (far left) and harboring the indicated multi-copy *LEU2* plasmid (near left) were grown in leucine- and uracil-free liquid medium at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated for 3 days on YPD solid medium at the indicated temperatures. **b** Tetrad analysis of *SSD1* homozygous diploids (A2454 derivative crossed with *orfΔ* BY4741 derivative) heterozygous for *spt16-319:natMX4* and the gene deletion indicated beside each panel. At least eight complete tetrads were analyzed for each cross; representative tetrads are shown. Double-mutant segregants, including those inferred by marker segregation, are *circled*. **c** Temperature sensitivities. *SSD1* cells containing the indicated *spt16:natMX4* allele (A2454 derivative), the indicated gene deletion (*orfΔ* BY4741 derivative), and a representative double-mutant segregant harboring both mutations were grown to stationary phase in YEPD liquid medium, diluted to 1×10^7 cells/ml, and spotted in fivefold serial dilutions onto YPD for further incubation

random-spore double-mutant selection medium (data not shown), but these double mutants still showed synthetic growth defects at elevated temperatures (Fig. 5c). These findings suggest that spore germination may be a problem for *spt16-319 swi4Δ* meiotic progeny. Direct testing showed similar findings for *spt16-312 swi4Δ* double-mutant cells (Fig. 5c).

The combination of each of the *spt16* mutant alleles with *swi6Δ* also gave viable double mutants that were temperature sensitive for growth at 37° (Fig. 5c). Swi6 interacts not only with Swi4, but also with Mbp1 to form the transcription factor MBF (Breeden 1996). However, neither overexpression nor deletion of the *MBP1* gene affected the growth of *spt16-312* or *spt16-319* mutant cells (Fig. 5c**,** and data not shown). The genetic interactions for these *spt16* mutations are therefore limited to SBF, and perhaps Swi4 itself, which activates certain genes independently of Swi6 in response to the Pck1-pathway signal transducer Slt2 (Baetz et al. 2001).

Despite the exacerbating effects of the *ssd1-d* mutation on the temperature sensitivity caused by the widely used *spt16-G132D* mutation (Fig. 3d), this mutant allele does not cause the cell-integrity phenotype seen for the *spt16-312* and *spt16-319* mutant alleles: unlike *spt16-312* and *spt16-319*, *spt16-G132D* did not sensitize *ssd1-d* cells (strain KanB) to Congo Red, and as a chromosomal allele in *SSD1* cells (Y2454 derivative) did not cause enhanced temperature sensitivity in combination with *slt2Δ* (data not shown). These findings underscore the distinct nature of the *spt16* mutant alleles described here.

spt16-319 increases transcription indicative of cell-wall stress

The effects of the *spt16-312* and *spt16-319* mutations on SBF, Swi4, and MBF activation of transcription were assessed directly in *ssd1-d* cells, using *lacZ* reporter genes activated by these specific factors. As a control, the activation by MBF was also assessed. Expression of the Swi4-activated reporter was significantly increased in *spt16-312* and *spt16-319* cells, while significantly increased expression from the SBF-activated reporter and the MBF-activated reporter was evident for *spt16-319* cells (Table 3). This increased reporter-gene expression may not result from increased expression of Swi4 and/or Swi6 in *spt16* cells: northern analysis showed no significant changes in *SWI4* and *SWI6* mRNA abundance due to the *spt16-319* or *spt16-312* mutation (data not shown), despite the involvement of Spt16 in *SWI4* and *SWI6* gene activation (Lycan et al. 1994). RNA levels from the endogenous Swi4-regulated *PCL1* gene in *spt16* mutant cells reflected the *prPCL1-lacZ* reporter-gene activities in these cells (Fig. 6). Therefore, increased signaling is likely the cause of the increased activities of the SBF and Swi4 transcription activators in *spt16 ssd1-d* double-mutant cells.

Another effector of Pkc1-mediated cell-integrity signaling is the transcription activator Rlm1 (Jung et al. 2002). The *lacZ* reporter gene driven by the *YIL117C/PRM5* promoter, which is activated during cell-wall stress by a single Rlm1-binding site (Jung et al. 2002), and by the *CYC1* promoter in which the *CYC1* UASs are replaced by tandem Rlm1-binding sites, was significantly activated in *spt16-319 ssd1-d* mutant cells (Table 3). Deletion of the Rlm1-binding site in the *YIL117C* promoter abolished virtually all expression (Table 3), suggesting that these effects of the *spt16* mutation depend on the Rlm1 activator. As expected, RNA levels from the endogenous Rlm1-regulated *YIL117C/PRM5* gene were also elevated in *spt16-319 ssd1-d* mutant cells (Fig. 6). However, the combination of an *spt16* mutation with *rlm1Δ* caused neither growth impairment nor temperature sensitivity (data not shown), suggesting that the Rlm1 branch of

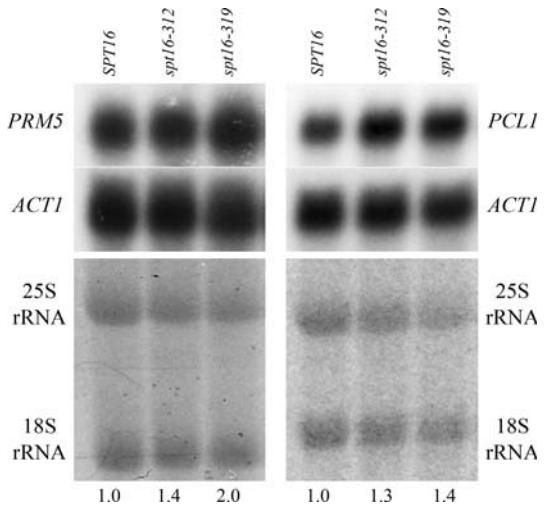

Fig. 6 *spt16* mutant alleles affect mRNA levels. *spt16Δ ssd1-d* cells (KanB derivatives) harboring an *spt16-312, spt16-319,* or *SPT16* low-copy plasmid, and the *prPRM5-lacZ* plasmid, were grown at 30° to 2–4 × 10^6 cells/ml in uracil- and leucine-free medium and harvested for RNA preparation. Total RNA was resolved electrophoretically and probed for endogenous *PCL1*, *PRM5*, and *ACT1* (as a loading control) mRNAs; equal loading was confirmed by ethidium staining of rRNA bands. Numerical values are *PCL1* and *PRM5* mRNA intensities quantified with respect to the 25S rRNA signal; virtually identical values were obtained by quantifying with respect to the *ACT1* signal. Similar results were obtained for strains containing the *PCL1-lacZ* plasmid (data not shown)

Table 3 Reporter-gene β-galactosidase activities

Activators	Reporter gene	*SPT16* allele		
		SPT16	*spt16-312*	*spt16-319*
SBF (Swi4/Swi6)	*SCB-lacZ*	38.1 ± 2.0 (1.0)	37.8 ± 1.7 (0.99)	81.6 ± 3.9 (2.1)
Swi4	*prPCL1-lacZ*	3.9 ± 0.3 (1.0)	10.6 ± 0.4 (2.7)	19.3 ± 2.3 (4.9)
MBF (Mbp1/Swi6)	*MCB-lacZ*	11.5 ± 1.3 (1.0)	8.6 ± 1.9 (0.7)	25.6 ± 2.0 (2.2)
Gcn4; Bas1/Pho2	*prHIS4-lacZ* (His + medium)	23.5 ± 1.0 (1.0)	12.5 ± 0.2 (0.5)	24.1 ± 3.2 (1.0)
Gcn4: Bas1/Pho2	*prHIS4-lacZ* (His– medium)	160.0 ± 13.3 (1.0)	140.5 ± 7.4 (0.9)	145.8 ± 12.2 (0.9)
Hap1; Hap2/3/4/5	*prCYC1-lacZ*	13.8 ± 0.4 (1.0)	7.4 ± 1.6 (0.5)	27.9 ± 4.2 (2.0)
Rlm1	*(+2 Rlm1 sites)-prCYC1-lacZ*	56.9 ± 3.2 (1.0)	99.8 ± 3.6 (1.8)	172.1 ± 8.5 (3.0)
Rlm1	*prPRM5-lacZ*	12.6 ± 0.7 (1.0)	12.2 ± 0.3 (1.0)	33.8 ± 0.6 (2.7)
–	*(Δ Rlm1 site)-prPRM5-lacZ*	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

Plasmid-borne *lacZ* reporter-gene expression was quantified in *spt16Δ ssd1-d* cells harboring *spt16-312, spt16-319,* or *SPT16* low-copy plasmids. All reporter plasmids are multicopy except for *prHIS4-lacZ*. The SBF reporter plasmid (Andrews and Herskowitz 1989) has four copies of the SCB element (Breeden 1996); Swi4 activates the *PCL1* promoter (*prPCL1*) (Baetz et al. 2001; Ogas et al. 1991); the MBF reporter plasmid (Verma et al. 1992) has four copies of the MCB element (Breeden 1996). Numbers in parentheses are fold changes with respect to the values for *SPT16* cells

Pkc1 signaling does not have a major role in these *spt16* mutant cells.

The *CYC1* gene itself is not thought to be regulated by cell-wall stress, so the activation of the *prCYC1-lacZ* reporter gene in *spt16-319* mutant cells was unexpected; however, analogous activation was not seen for another control promoter, that from the *HIS4* gene (Table 3). In any case, the increased expression of reporter genes in *spt16-319 ssd1-d* mutant cells, as well as the phenotype of these cells, suggests that increased cellular 'stress' due to the *spt16-319* mutation activates the Pkc1 cell-integrity signaling pathway.

spt16-319 and *spt16-312* cause temperature sensitivity through the same mechanism

The different effects of the mutant alleles on transcriptional activation (Table 3), coupled with the related but non-identical phenotypes described above, suggested the possibility that these mutant alleles may affect cell integrity through different mechanisms. However, diploid cells harboring chromosomal mutant alleles showed a lack of complementation between *spt16-319* and *spt16-312* for temperature sensitivity, regardless of whether the cells had Ssd1 activity (Fig. 7, compare the growth of the *SSD1/SSD1* and *ssd1/ssd1* diploid cells in panel b with the growth of *SSD1* haploid cells in panel a). These findings therefore indicate that, despite significant allele specificity in genetic interactions, these particular *spt16* mutant alleles affect cell integrity through the same mechanism.

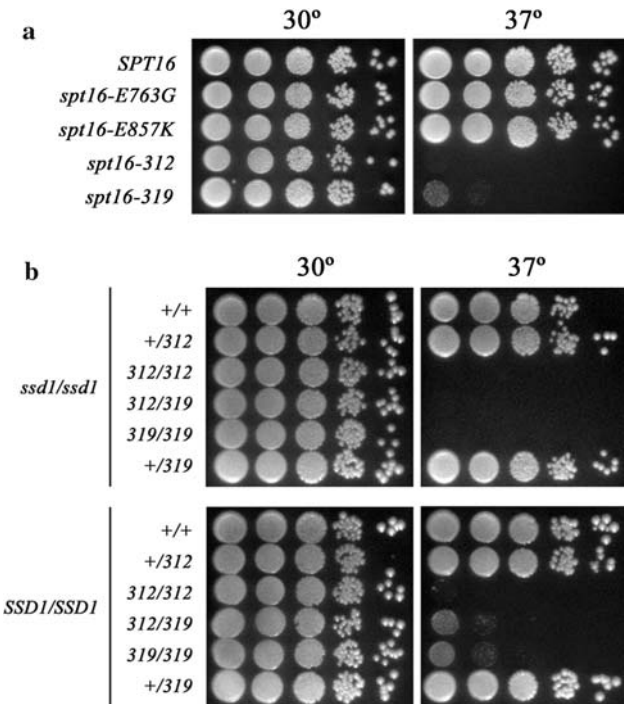

Fig. 7 Non-complementation between *spt16-312* and *spt16-319*. **a** Haploid *SSD1* cells with an *spt16:natMX4* allele integrated at the *SPT16* locus (Y2454 derivatives) were grown overnight in YEPD at 28°, diluted to 1×10^7 cells/ml, spotted in tenfold serial dilutions, and incubated for 3 days on YPD solid medium at the indicated temperatures. **b** Diploids with integrated *STP16::natMX4* and/or *spt16::natMX4* alleles, with or without the *SSD1* gene, were derived by crossing appropriate segregants from matings between the haploid cells in panel (**a**) and BY4741-derived *ssd1Δ::kanMX4* cells (Table 1). These diploids were tested for growth as in panel (**a**)

Discussion

The work presented here describes the identification and characterization of several new yeast mutations affecting the Spt16 subunit of FACT. This transcription factor is important for transcription in a nucleosomal context. One function ascribed to FACT is the restoration of proper nucleosome structure in the wake of the transcription elongation complex, most likely by nucleosome reassembly. Inadequate restoration of nucleosome structure can result in aberrant transcription originating from 'cryptic' promoters, which would otherwise be maintained in a repressed state by nucleosomal structure (Kaplan et al. 2003). New mutations affecting Spt16 were selected by the ability to bring about functional transcription from such cryptic promoters, suggesting that these mutations interfere with transcription-linked restoration of nucleosome structure.

Nucleosomes impede transcription initiation and elongation by RNAPII; effective transcription therefore entails the reorganization of nucleosomes to allow DNA access by the transcription complex. FACT has also been implicated in the nucleosome reorganization that allows transcription elongation. Finally, FACT has been found to have effects at transcription initiation. As discussed below, perturbations at any of these levels may be responsible for some of the transcriptional effects reported here that appear to be gene specific rather than global in nature.

Cells relying on two of these new *spt16* mutant alleles were found to display several phenotypes characteristic of defects in cell integrity. Furthermore, the *ssd1-d* mutant allele was shown to impair the high-temperature growth of cells relying on these *spt16* mutant alleles; analogous genetic interactions have implicated Ssd1 protein function, along with the Pkc1 signal-transduction pathway, in maintaining cell integrity and normal morphogenesis (Kaeberlein and Guarente 2002). The overexpression of genes encoding components of Pkc1 signaling mitigated the temperature sensitivity of *spt16 ssd1-d* mutant cells, whereas gene deletions eliminating members of each of the two branches of the Pkc1 signaling cascade resulted in significant growth impairments for these *spt16* mutant cells. Finally, the expression levels of reporter genes

indicate that increased signaling through the Pkc1 pathway is brought about by *spt16* effects. These findings point to an unexpected involvement of FACT in the maintenance of cell integrity and regulation of cell morphogenesis.

The influence of Spt16 and FACT in the maintenance of cell integrity probably involves the altered expression of gene(s) whose products are important for cell-wall metabolism. Transcriptional profiling (microarray analysis) identified ~16 genes with transcript levels up or down by 2-fold or more in *spt16-312* cells, and ~185 genes analogously affected in *spt16-319* cells, but failed to detect altered transcript levels for any genes with functions related to cell-wall integrity (unpublished observations). However, this analysis did not take into consideration the possibility of differential activation of cryptic internal promoters, as seen upon analysis of purpose-built microarrays (Cheung et al. 2008). In any event, these gene-expression effects are likely to be gene specific, rather than global. Two mechanisms for gene-specific effects due to altered FACT activity have been suggested. The first is a consequence of the nucleosome reorganization activity proposed or demonstrated for FACT. The depletion of normal FACT from yeast cells has been found to have at least two effects on transcription (Jimeno-Gonzalez et al. 2006). FACT depletion activates a cryptic promoter in the *FLO8* gene that is normally repressed by nucleosomal configuration (Kaplan et al. 2003). In addition, FACT depletion has gene-specific transcriptional effects, both positive and negative, that have been functionally linked to nucleosome positioning in the proximal portions of transcribed regions. Genes with a promoter-proximal nucleosome that is precisely positioned along the DNA, and thus potentially more stable, suffer greater inhibition through FACT depletion (Jimeno-Gonzalez et al. 2006). Analogous gene-specific effects upon Spt16 depletion have been seen in human cells (Li et al. 2007). Therefore, the effects of the *spt16* mutants described here may reflect altered nucleosome reorganization abilities of the mutant FACT in these cells.

Alterations in FACT activity may also have gene-specific effects through actions at promoters. Depletion of yeast FACT activity diminishes promoter occupancy by transcription initiation factors TBP and TFIIB, and *spt16* mutations have strong genetic interactions with mutations affecting TBP and TFIIA (Biswas et al. 2005; Mason and Struhl 2003), although neither of these effects is known to be gene specific. FACT also associates with certain transcription regulators. In *Drosophila*, FACT associates with GAGA factor and facilitates the transcription of several *Hox* genes through relief of transcriptional silencing (Nakayama et al. 2007; Shimojima et al. 2003), while mammalian FACT, or a FACT subunit, stimulates SRF-dependent activation by association with the transcription factors MKL1 and SRF itself (Kihara et al. 2008; Spencer et al. 1999). Like FACT, other proteins considered to be global transcription elongation factors also have gene- or promoter-specific effects; included here are Spt5, which in *Drosophila* has locus-specific effects on transcription activation and repression (Jennings et al. 2004), and Spt6, which mediates the repression of several yeast genes through nucleosome reassembly at promoters (Adkins and Tyler 2006).

FACT is not the only component of transcription elongation with effects on cell integrity; another is the Paf1 complex (Paf1C). Paf1C is a multi-subunit assembly that associates with transcriptionally engaged RNAPII enzyme and facilitates the ubiquitination of histone H2B in transcribed regions (Kim et al. 2004; Ng et al. 2003; Wood et al. 2003; Xiao et al. 2005). This modification is important for FACT activity during transcription along a nucleosomal template in vitro and in vivo (Fleming et al. 2008; Pavri et al. 2006). Mutations affecting Paf1C have gene-specific effects and can impair the transcription of cell-integrity genes, with severe effects on cell integrity; consistent with these effects, deleterious genetic interactions are seen for the Paf1C mutations *paf1Δ, cdc73Δ,* and *ctr9Δ* in combination with *swi4Δ* (Betz et al. 2002; Chang et al. 1999; Porter et al. 2002). In contrast, genetic interactions are not seen for the combinations *paf1Δ pkc1Δ* and *paf1Δ mpk1Δ*, or for *paf1Δ rlm1Δ* and *cdc73Δ rlm1Δ* (Betz et al. 2002; Chang et al. 1999). These genetic relationships for Paf1C components resemble those described here for *spt16* mutations, implying that FACT and Paf1C may share an overlapping role in maintaining cell integrity through the same mechanism. On the other hand, the *spt16-319* allele was found to have deleterious genetic interactions with deletions eliminating several Paf1C components (*cdc73Δ, leo1Δ, rtf1Δ*; unpublished observations), suggesting that FACT and Paf1C mediate an important function in different ways. This function could be related to the cell-integrity role described here, but could also reflect other transcription-related actions of FACT and Paf1C.

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