A role for Spt6 in centromeric silencing in *S. pombe*

Specific Aims

The *spt6*$^+$ gene is highly conserved in eukaryotes and also has homologues in prokaryotes that share approximately 25% homology with one of its domains, the TEX domain (Pontig 2002; Anantharaman *et al.* 2002). It has been reported to be essential in *S. cerevisiae* (Clark-Adams and Winston 1987; Neigeborn *et al.* 1987), *C. elegans* (Schierenberg *et al.* 1980; Nishiwaki *et al.* 1993), *D. melanogaster* (Bourbon *et al.* 2002) and *D. rerio* (Keegan *et al.* 2002; Kok *et al.* 2007). Studies conducted in several organisms have demonstrated that Spt6 plays diverse roles in regulation of transcription, chromatin structure, and mRNA processing. We are studying Spt6 in the fission yeast, *Schizosaccharomyces pombe*, to further elucidate its role in these processes. *S. pombe* has the benefit of being genetically tractable, but it is also highly diverged from *S. cerevisiae*. As a result, it has evolved a number of characteristics that are interesting for the study of Spt6 and are potentially applicable to larger eukaryotes. First, centromeric silencing in *S. pombe* is a result of RNAi dependent heterochromatin formation. This relates to a second important difference, which is that *S. pombe* heterochromatin is modified by H3K9me and bound by Swi6, an HP1 homolog, as it is in larger eukaryotes. Together, these characteristics indicate that studying Spt6 in the context of RNAi dependent heterochromatin formation in *S. pombe* could provide interesting new insights about its function and be applicable to larger eukaryotes. Finally, nearly half of all genes are spliced in *pombe*, as compared to about 5% in *S. cerevisiae* (reviewed in Kuhn and Kaufer 2003), indicating that studying the role of Spt6 in mRNA splicing and processing in *S. pombe* could reveal new and interesting differences. We plan to take advantage of these characteristics to investigate potential roles for Spt6 in some or all of these processes.

1. Isolate and characterize *spt6* mutants to identify possible roles in transcription and chromatin structure.

2. Characterize the role of Spt6 in centromeric silencing.

Studies and results

Specific Aim 1. To characterize the role of Spt6 in *S. pombe*, we proposed to take several approaches to create mutant alleles at the endogenous *spt6*$^+$ locus and then to study their phenotypes. First, we proposed to target point mutations and deletions to alter or delete the various putative domains of Spt6 (Figure 1a), in order to better understand their roles. The two domains that were focused on initially were the helix-hairpin-helix (HhH) motif, believed to bind DNA nonspecifically (Doherty *et al.* 1990), and the Src homology 2 (SH2) domain, believed to bind RNA Polymerase II (Pol II) (Yoh *et al.* 2007). Second, we proposed to put the *spt6*$^+$ gene under the control of the thiamine repressible *nmt1*/*nmt41*/*nmt81* promoter series to conditionally reduce the levels of *spt6*$^+$ expression. Third, we proposed to subject *spt6*$^+$ to random mutagenesis to identify temperature-sensitive alleles. Finally, we proposed to delete *spt6*$^+$ in order to verify that it is essential for growth in *S. pombe*. Table 1 summarizes the mutations that were created. Except for the random mutants, which were created by transforming haploid

strains, all of these mutations were created by transforming diploids with cassettes encoding the 3' end of *spt6*$^+$ with the mutation or deletion incorporated, as well as a C'-TAP tag and a downstream KanMX cassette (Toulmay and Schneiter 2006). Transformants were selected on media containing G418 and proper integration was verified by colony PCR. These heterozygous diploids were then dissected to verify 2:2 segregation of the cassette and to create haploid strains. Deletion of the HhH domain caused a growth defect (Figure 1b). Deletion of the SH2 domain produced widely different growth phenotypes in two different transformants (Figure 1c) and the gene needs to be sequenced in order to determine whether one is carrying a second mutation in *spt6*$^+$. Repression of *spt6*$^+$ using the *nmt41* and *nmt81* promoters, but not the *nmt1* promoter, also leads to a growth defect (Figure 1d). Surprisingly, *spt6*$^+$ is not essential in *S. pombe*, although the *spt6Δ* strain is extremely sick and difficult to work with (Figure 1e).

In addition to these targeted approaches, we hoped to use random mutagenesis to identify conditional *spt6*$^+$ alleles with single mutations in each of the different domains. We isolated 21 conditional mutants but upon sequencing a subset, found that all of them contained several point mutations spanning multiple domains (Table 1). These mutants have proven somewhat useful in initial characterization studies, but are not ideal for characterizing the function of individual domains.

All mutants were initially tested for a set of possible mutant phenotypes by assessing growth under specific conditions. All the mutants had several similar sensitivities, although the severity of the phenotypes varied. In general, s*pt6* mutants grew poorly on minimal media and were temperature (37˚C) and cold (14˚C) sensitive. In addition, they were sensitive to a number of compounds, including caffeine, cycloheximide, and formamide (Figure 2a). Notably, all of the conditional mutants were sensitive to thiabendazole (Figure 2b), a phenotype that indicates a defect in chromosome segregation, often due to a defect in the RNAi-dependent formation of centromeric heterochromatin.

Specific Aim 2. The thiabendazole sensitivity of the *spt6* mutants led us to test whether Spt6 plays a role in centromeric silencing. Using *ura4*$^+$ reporters inserted at the inner and outer centromeric repeats, we demonstrated that transcriptional repression of *spt6*$^+$ with the *nmt41* and *nmt81* promoters caused a modest silencing defect (Figure 3). We also used Northern blots (Figure 4b, 5a) and real-time PCR (Figure 4c, 5b) to look at expression of the endogenous centromeric *dg* and *dh* repeats in a number of different *spt6* mutants. All *spt6* mutant alleles showed some derepression of these repeats by both of these methods, although it was fairly modest in some cases. However, the *spt6-HhHΔ* allele and the two transcriptional shut-off strains (*nmt41-spt6*$^+$ and *nmt81-spt6*$^+$) demonstrated strong derepression of these repeats. We selected the *spt6-HhHΔ* allele and the *nmt41-spt6*$^+$ strain for further study.

The Spt6-HhHΔ protein is expressed at lower steady state levels than wild-type Spt6, so we wanted to determine whether the centromeric silencing defect was caused by deletion of the HhH domain or simply by the lowered protein levels, as in the shut-off strains. To test this, we placed *spt6-HhHΔ* under the control of the *nmt1* promoter; in the absence of thiamine this construct overexpresses the *spt6-HhHΔ* allele, producing steady state protein levels higher than those of wild-type (Figure 6a). Looking at derepression of the centromeric repeats in this overexpression strain by real-time PCR, we see that expression of the *dg* repeats is

indistinguishable from that of a *spt6-HhHΔ* mutant under the control of the endogenous promoter (Figure 6b). This indicates that the centromeric derepression in the *spt6-HhHΔ* strains is not due to reduced protein levels and may be due to a specific function of the HhH domain.

Using the *nmt41-spt6*$^+$ shut-off strain, we characterized the kinetics of centromeric derepression upon transcriptional repression of *spt6*$^+$. Cells were grown to mid-log and shifted into thiamine containing media to repress *nmt41-spt6*$^+$. Samples were then taken for 42 hours, diluting the culture back at each time point to maintain the cells in mid-log. Northern blot analysis demonstrated that *spt6*$^+$ RNA levels were barely detectable by two hours after the shift to thiamine (Figure 7a). However, derepression of the centromeric *dg* and *dh* repeats occurred significantly later, with expression starting to increase between 18-24 hours and continuing to increase at 42 hours when the time course was stopped (Figure 7b). It is currently unclear why this lag is observed, although there are many possibilities. One possibility is that the Spt6 protein levels may remain high for some time after the RNA is depleted, and this is currently being tested. Another possibility is that in its role as a transcription factor, Spt6 is responsible for expression of an RNAi or chromatin related factor, and after the Spt6 levels decrease it takes time for the protein levels of this factor to decrease. It is also possible that the process of deconstructing silent chromatin takes time due to some inherent property; for example, perhaps it requires a number of cell cycles to occur. To test whether similar kinetics might occur for an RNAi mutant, we will put genes encoding key pieces of the RNAi machinery, such as *dcr1*$^+$ (dicer) and *ago1*$^+$ (argonaute) under the control of the *nmt41* and *nmt81* promoters and monitor expression of the centromeric repeats after their transcriptional shut-off.

Significance

Given that Spt6 has been shown to be essential in *S. cerevisiae* (Clark-Adams and Winston 1987, Neigeborn *et al.* 1987) and several other organisms, it was surprising that it is not essential in *S. pombe*. BLAST searches of the *S. pombe* genome did not reveal any homologues, suggesting that it is not a simple issue of gene duplication. It is currently unclear why an *spt6Δ* strain is viable in *S. pombe*, but it indicates that there are interesting differences to study.

In addition, my results have provided the first indication that Spt6 plays a role in centromeric silencing in *S. pombe*. This is particularly interesting because of the involvement of the RNAi machinery in heterochromatin formation and maintenance. Although many questions exist about the role of Spt6 in centromeric silencing, investigating potential mechanisms could provide novel insight into regulation of the heterochromatin structure at the centromeres as well as functions of Spt6.

Plans

The current focus of this project is to characterize the function of Spt6 in centromeric silencing. In order to narrow down the possible roles, it is first important to determine if Spt6 affects silencing directly, possibly by playing a role in transcription of the centromeric repeats or modulation of the centromeric chromatin structure, or indirectly, by controlling transcription of one or more genes that regulate silencing. Two experiments will begin to address these issues. First, chromatin immunoprecipitation (ChIP) experiments will allow us to determine whether

Spt6 is normally present at the centromeric repeats. Second, we plan to conduct a microarray using the *nmt41-spt6*$^{+}$ strain in order to identify genes that are transcriptionally regulated by Spt6. In addition to providing information about whether Spt6 regulates genes involved in silencing, these results will allow us to identify other transcriptional defects when Spt6 is depleted. With respect to the silencing phenotype, the microarray could produce a clear positive result (silencing machinery is downregulated), a clear negative result (silencing machinery is unaffected) or an ambiguous result (silencing machinery is affected, but not necessarily to a degree where it rules out other scenerios). Assuming a positive result, future studies would focus on the mechanism of regulation of any affected silencing genes by Spt6. Assuming a negative or ambiguous result, many experiments remain to characterize the effect of Spt6 on silencing.

Experiments will be done to characterize the loss of centromeric silencing in an *spt6* mutant and to compare it to previously observed defects, for example, those caused by loss of the RNAi machinery. First, as mentioned previously, we plan to compare the kinetics of centromeric transcription in *spt6* mutants with that of other known silencing mutants. Second, we plan to use ChIP to analyze the chromatin state at the centromeric repeats, looking at representatives of characterized protein complexes that have been shown to be required for silencing as well as histone modifications. Potential candidates include Swi6, Ago1, Dcr1, Clr4/Rik1, Rdp1, Pol II and H3K9me$_2$. Given the recent data indicating that cell cycle plays a role in the RNAi dependent centromeric silencing (Chen *et al.* 2008; Kloc *et al.* 2008), it may also be informative to conduct some of these experiments on synchronized cells. To assess the production of proper siRNAs in an *spt6* mutant, we will look at the levels of endogenous and *ura4*$^{+}$ reporter siRNAs by Northern blot. To test whether *spt6* mutations cause chromosome segregation defects, we will assess the frequency of lagging chromosomes and perform minichromosome loss assays to quantify the level of genomic instability. Finally, we plan to investigate whether Spt6 also plays a role in silencing at the silent mating type cassettes, using silencing reporters to make an initial assessment.

The observed loss of centromeric silencing in *spt6* mutants could be the result of a global chromatin change, rather than an effect specific to the centromeres. Based on previous studies of Spt6 in *S. cerevisiae*, potential chromatin effects could include changes in nucleosome positioning and density or histone modification. Previous studies have indicated that Spt6 is responsible for replacing nucleosomes over transcribed regions, as *spt6* mutants have been shown to have local defects in nucleosome positioning over open reading frames and promoters (Kaplan *et al.* 2003; Adkins and Tyler 2006). Nucleosome positioning in *spt6* mutants could be determined by MNase digestion and analyzed locally by Southern blotting at select centromeric regions as well as other loci of interest as indicated by the *nmt41-spt6*$^{+}$ microarray, or on a genome-wide level by sequencing or microarray technology. Another study indicated that the *spt6-1004* allele (HhHΔ), but not certain other *spt6* mutants, produces global decreases in Set2 dependent H3K36me (Youdell *et al.* 2008). It could be informative to assess global levels of several histone modifications by Western blot, in order to identify any large changes in histone modifications, such as H3K36me, and other modifications that are associated with silenced (H3K9me) and activated chromatin (H3K4me). Data from these experiments could be analyzed with respect to the *nmt41-spt6*$^{+}$ microarray data to attempt to correlate changes in chromatin and transcription.

Together, these experiments will begin to identify the role that Spt6 plays in promoting centromeric silencing, but will also inform us about the global role of Spt6 in *S. pombe*. As we characterize the roles it plays in regulating transcription and chromatin, both globally and at the centromeres, it will be interesting to contrast our findings with what is known in other species, such as *S. cerevisiae*, in order to get a fuller picture of the role of *spt6*$^{+}$ in all eukaryotes.

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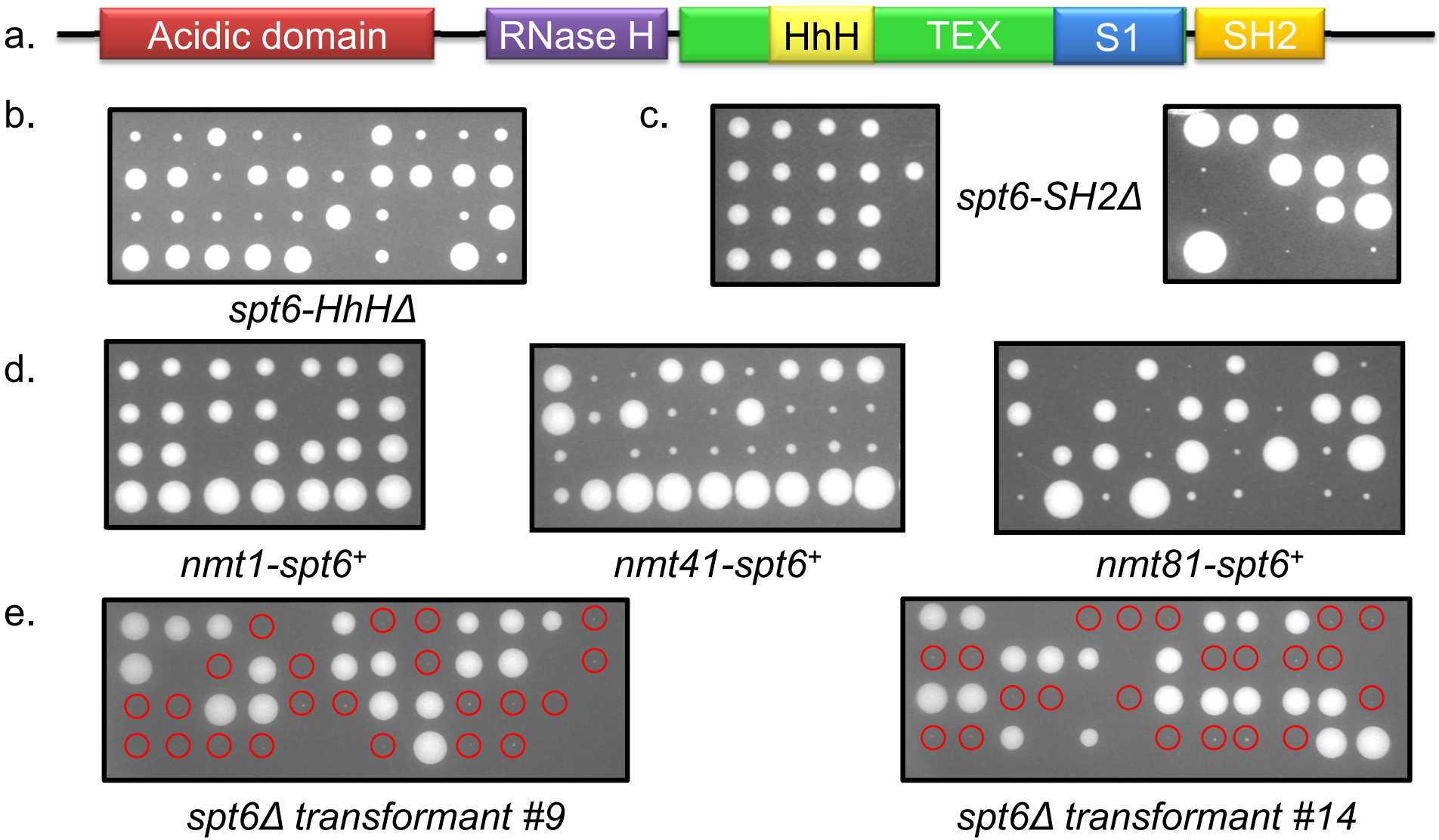

Figure 1. Creation of *spt6* mutants. (a) The *spt6*$^{+}$ gene encodes conserved domains, including an N' acidic domain that may mediate histone interactions, RNaseH, TEX and S1 domains that typically bind nucleic acids, and a C' SH2 domain that may mediate interactions with RNA Pol II. The TEX domain also encodes an HhH motif that binds DNA in a non-sequence dependent manner. As described in the text, targeted mutations were created in diploids and dissected. **(b)** Deletion of the HhH domain produced a growth defect that segregated 2:2. **(c)** Two independent SH2 transformants displayed widely different growth properties. These are being sequenced, since one of them is likely to have an additional mutation in *spt6*. **(d)** Repression of *spt6*$^{+}$ expression by the *nmt41* and *nmt81* promoters , but not the *nmt1* promoter, causes a growth defect. **(e)** The *spt6Δ* colonies were extremely sick, but germinated with the same efficiency as the WT colonies, indicating that the viability is not likely due to a suppressor. Some of the colonies are poorly visible by eye, but the red circles indicate *spt6Δ* colonies that were present and grew when re-gridded.

Mutant	Approach	Mutations	Strong phenotypes
spt6-a3	Random mutagenesis	Mutations in TEX and SH2 domains and other regions (6 total)	Cold, cycloheximide, minimal media
spt6-b23	Random mutagenesis	Mutations in RNase H and S1 domains and 3' end (4 total)	Cold, formamide
spt6-b25	Random mutagenesis	Mutations in RNaseH, HhH and S1 domains (5 total)	Heat, cold, phleomycin, formamide, minimal media
spt6-b38	Random mutagenesis	Mutations in SH2 domain and 3' end (5 total)	Cold, caffeine, cycloheximide, formamide, hydroxyurea, minimal media, phleomycin
spt6-HhHΔ	Targeted mutagenesis	Deletion of the HhH motif	Heat, cold, caffeine, cycloheximide, formamide, minimal media
spt6-SH2Δ	Targeted mutagenesis	Deletion of the SH2 domain	In progress
nmt1-spt6^{+}	Transcriptional shut-off	Insertion of the thiamine-repressible *nmt1* promoter	None
nmt41-spt6^{+}	Transcriptional shut-off	Insertion of the thiamine-repressible *nmt41* promoter	Heat, formamide, thiabendazole
nmt81-spt6^{+}	Transcriptional shut-off	Insertion of the thiamine-repressible *nmt81* promoter	Heat, cold, caffeine, formamide, thiabendazole

Table 1. Summary of conditional *spt6* mutants. The conditional alleles created by random mutagenesis contained several mutations spread across multiple domains. As a result, targeted deletions and transcriptional shut-off strains were used for follow-up experiments.

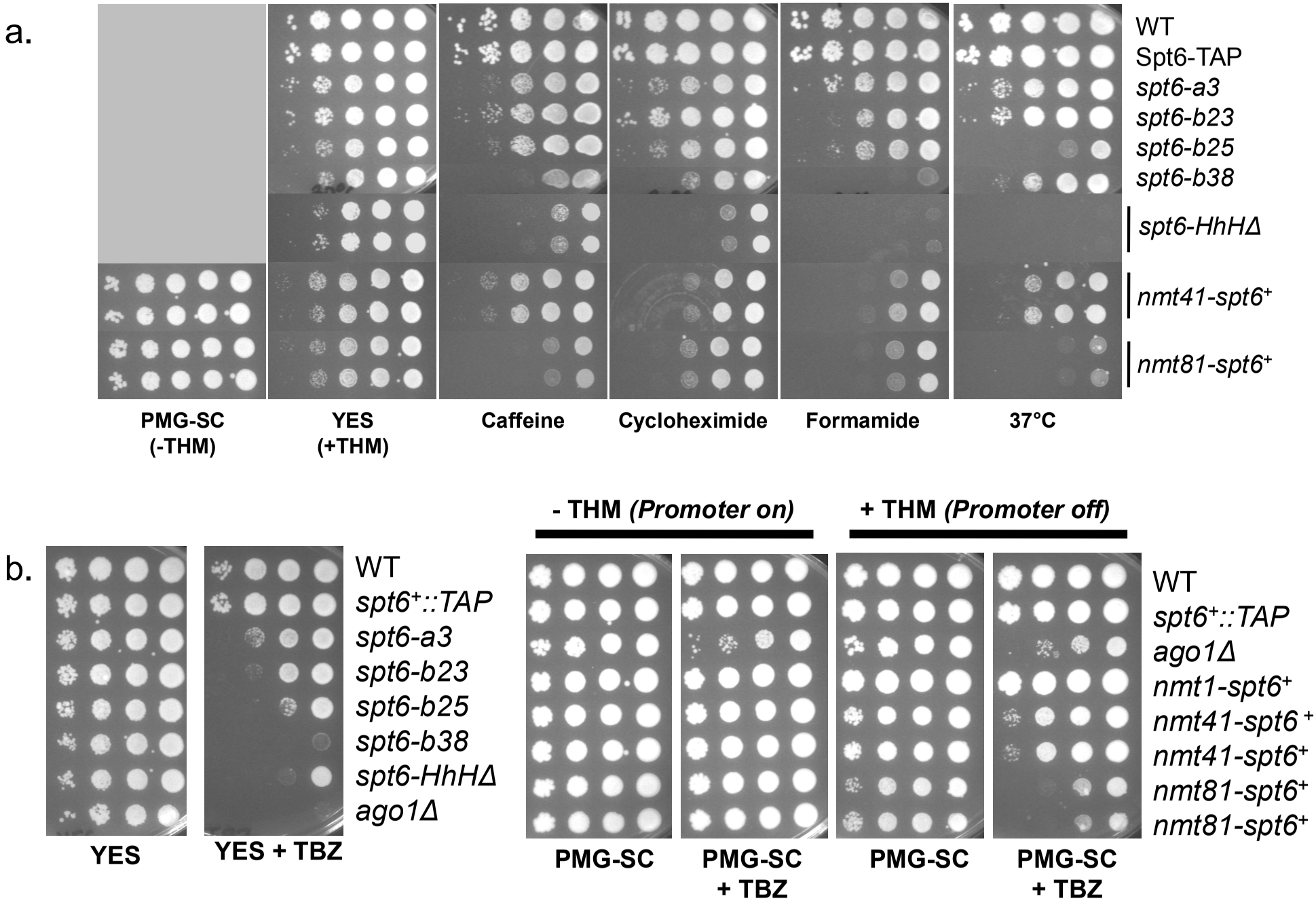

Figure 2. Phenotypic analysis of *spt6* mutants. Plate phenotypes were assessed by growing cells to saturation, making serial 10-fold dilutions and spotting them on solid media. **(a)** Mutant alleles of *spt6* demonstrated sensitivity to 10 mM caffeine, 10 µg/mL cycloheximide and 2% formamide, as well as temperature sensitivity. **(b)** The *spt6* mutants also displayed a sensitivity to 17 µg/mL thiabendazole, indicating that they may have a centromeric heterochromatin defect.

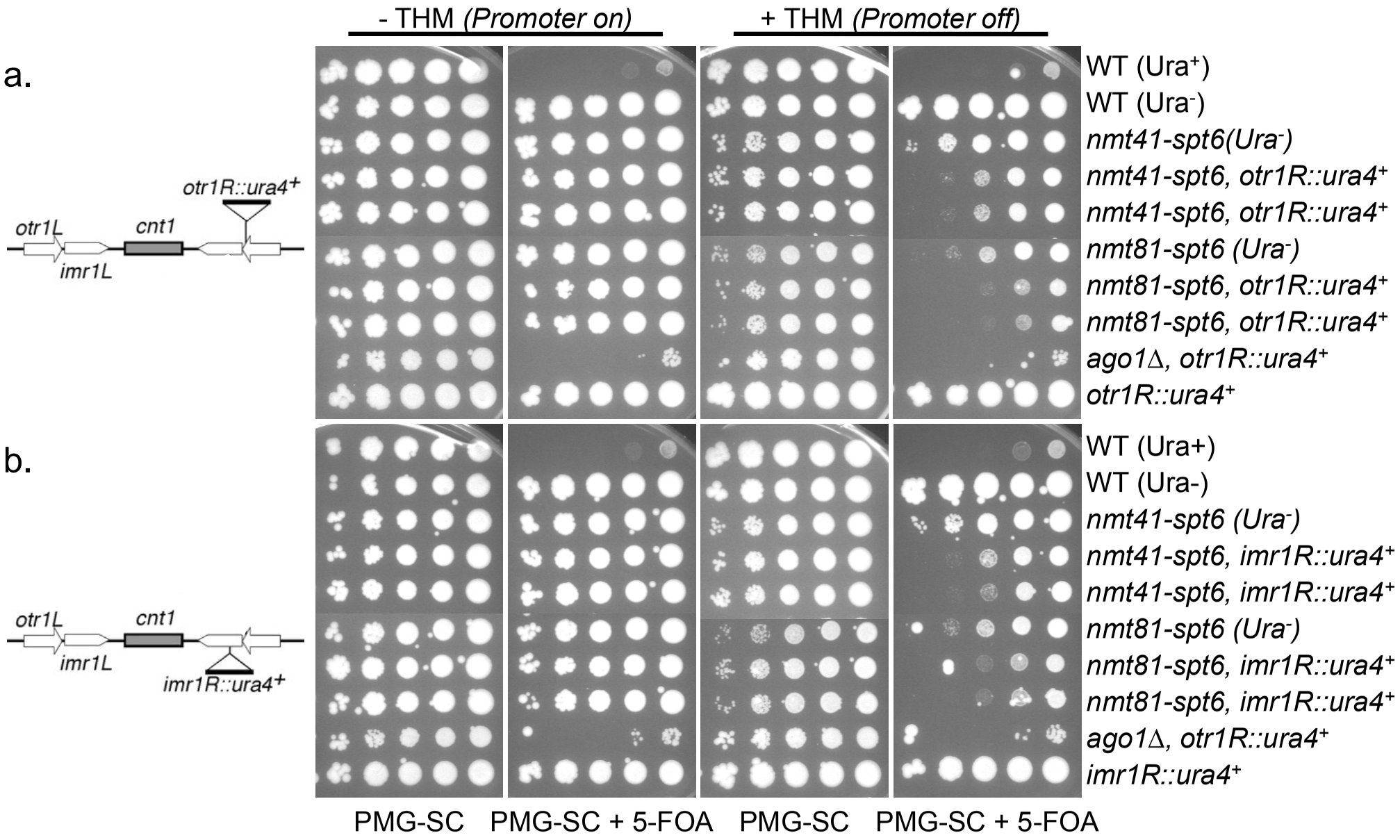

Figure 3. Transcriptional shut-off of *spt6*⁺ derepresses centromeric silencing reporters. The *nmt41/81-spt6*⁺ strains were crossed to the *otr1R::ura4*⁺ (outer repeat) and *imr1R::ura4*⁺ (inner repeat) centromeric silencing reporter strains. Cells were grown to saturation and serial 10-fold dilutions were spotted onto PMG-SC media with and without 15 μM thiamine (THM) and 0.1% 5-FOA. The plates were monitored and these photos taken on day 7. Expression of the *ura4*⁺ cassette at both the **(a)** *otr1R* and **(b)** *imr1R* reporter was increased in the presence of THM, as shown by sensitivity to 5-FOA, in comparison to the *ago1Δ* strain. This indicates that transcriptional shut-off of *spt6*⁺ leads to derepression of centromeric transcription.

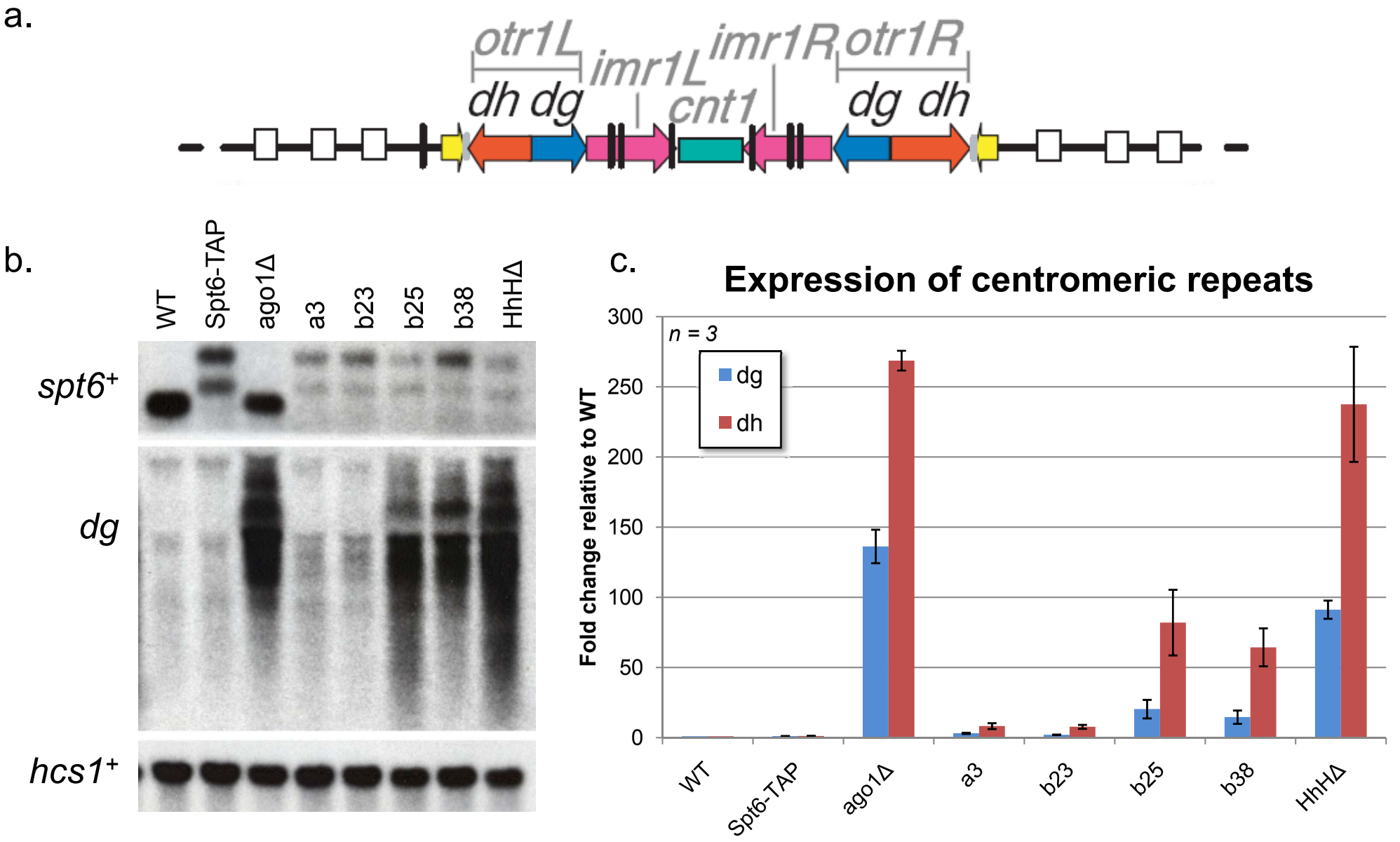

Figure 4. Strains with mutations in *spt6* have increased expression of endogenous centromeric transcripts. **(a)** In order to assess centromeric desilencing, we measured steady state expression levels of the endogenous *dg* and *dh* repeats found in the outermost centromeric repeats, as shown on chromosome 1 (adapted from Cam *et al.* 2005). **(b)** Northern blot analysis of the dg repeats showed significant derepression of the *dg* repeats in a number of these mutants. **(c)** Real-time qPCR analysis of the dg and dh repeats confirmed these results (n=3, error bars represent SEM). The Spt6-HhHΔ strain showed the most significant derepression, compared to the positive control (ago1Δ).

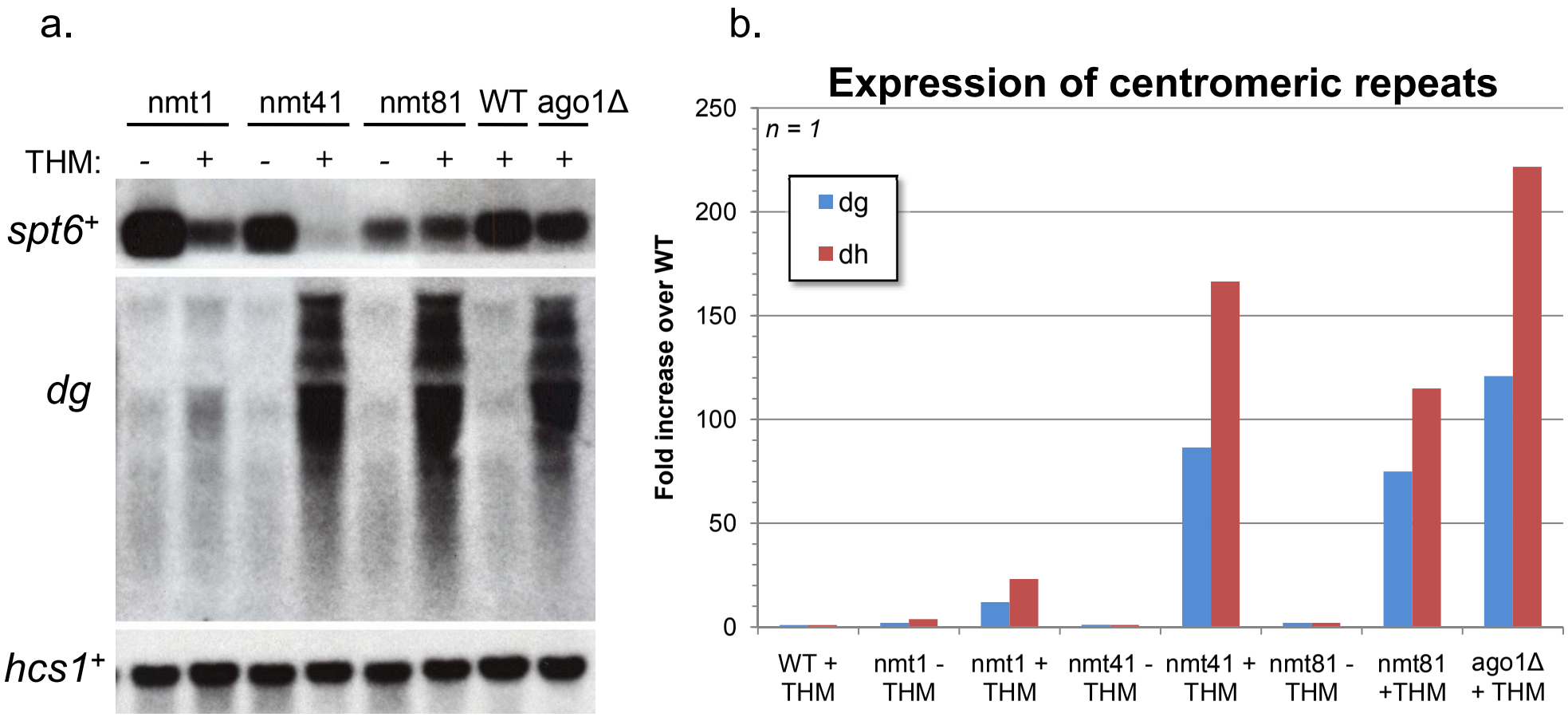

Figure 5. Transcriptional shut-off of *spt6*⁺ leads to increased expression of endogenous centromeric transcripts. (a) Northern blot analysis of the dg repeats showed significant derepression of the *dg* repeats when *spt6*⁺ levels were reduced by growing the $nmt41_{pr}$ and $nmt81_{pr}$ strains in thiamine. **(b)** Real-time qPCR analysis of the dg and dh repeats confirmed these results, but demonstrated some variability in the amount of derepression. These cultures had been inoculated into thiamine containing overnight cultures and then diluted back in thiamine containing media and grown to mid-log. In an attempt to get more consistent results, we decided to use a shift to assess derepression (see figure 7).

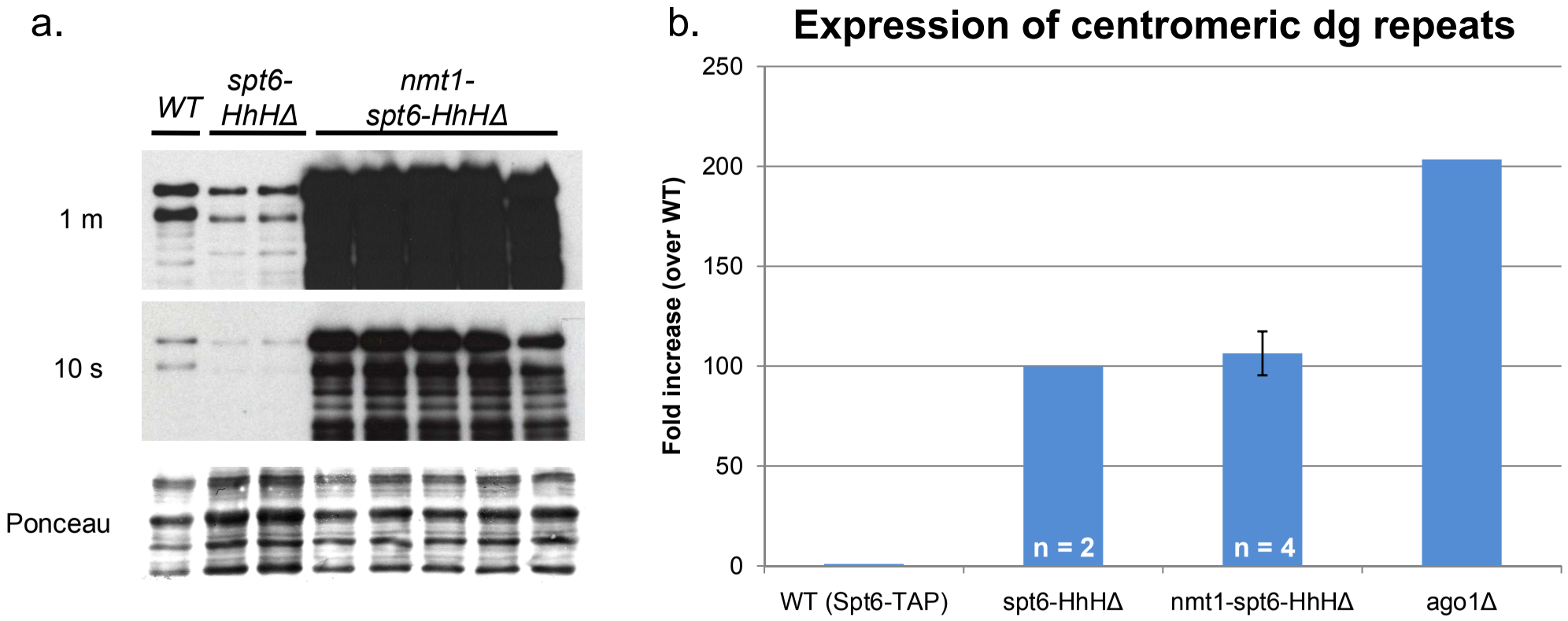

Figure 6. Derepression of centromeric repeats in HhHΔ is not due to decreased expression levels. (a) Western blot analysis of *spt6*$^{+}$ levels demonstrates that Spt6-HhHΔ is expressed at a lower steady state level than wild-type Spt6, but is overexpressed by the *nmt1* promoter in the absence of thiamine. Two exposures are shown: 1 m and 10 s, as well as a Ponceau stain loading control. **(b)** Real-time qPCR analysis of the dg repeats in these strains showed that they are derepressed comparably in *spt6-HhHΔ* strains when the protein is under- and overexpressed. This indicates that the silencing defect is not due to the reduced levels of Spt6-HhHΔ protein, and may be due to a specific role of the HhH domain.

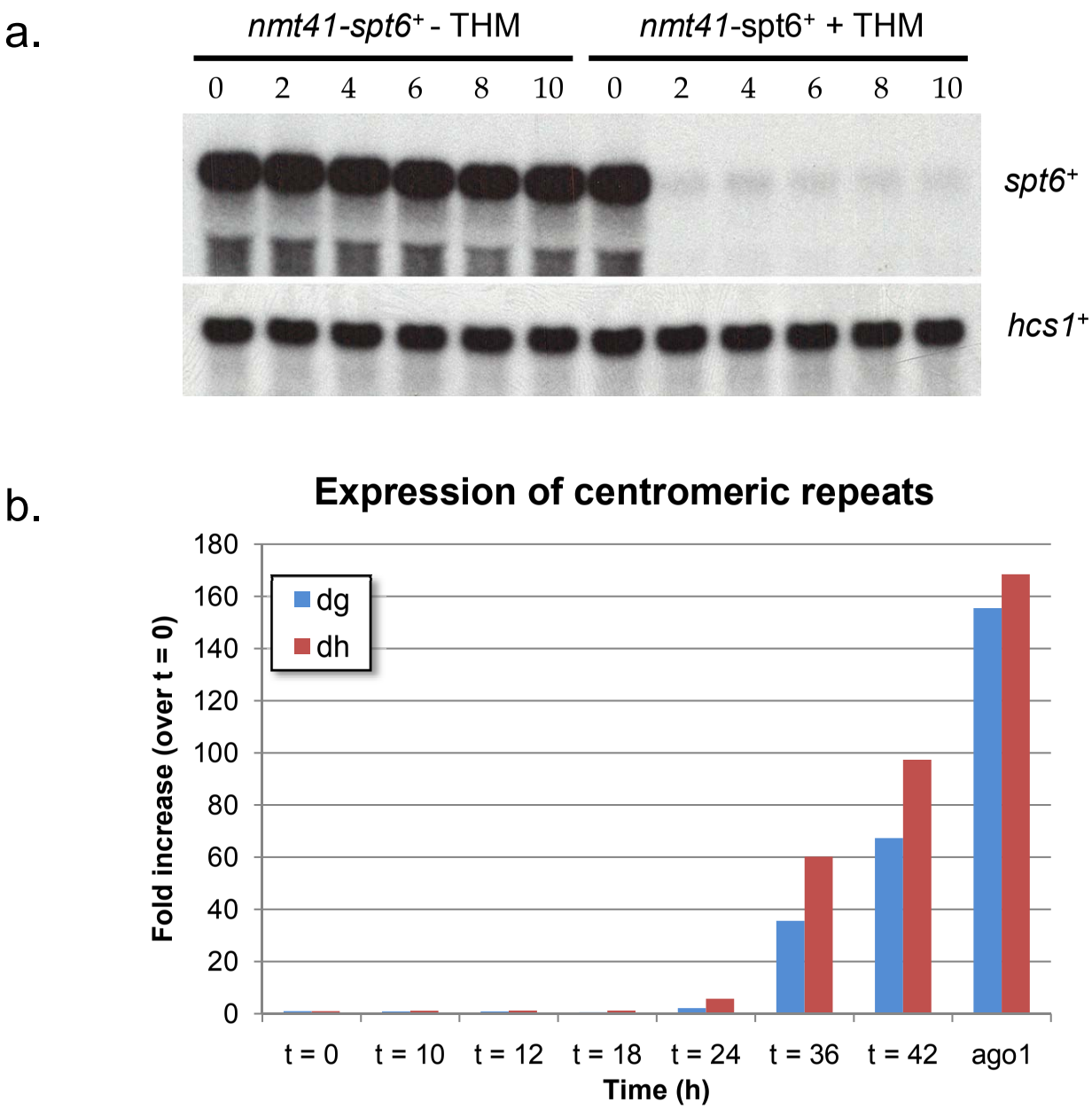

Figure 7. Derepression of centromeric repeats is delayed after depletion of *spt6*⁺ transcript. (a) Northern blot analysis of *spt6*⁺ demonstrates that the transcript is depleted by 2 hours in thiamine. Analysis of the kinetics of protein depletion is currently underway. **(b)** Real-time qPCR analysis of the dg and dh repeats showed that expression of the centromeric repeats began to increase between 18 and 24 hours and continued to increase until the end of the time course at 42 hours.