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Genetic characterisation of *hda1*$^+$, a putative fission yeast histone deacetylase gene

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ABSTRACT

***hda1*$^+$ (histone deacetylase 1) is a fission yeast gene which is highly similar in sequence to known histone deacetylase genes in humans and budding yeast. We have investigated if this putative histone deacetylase contributes to transcriptional silencing in the fission yeast *Schizosaccharomyces pombe*. A precise deletion allele of the *hda1*$^+$ open reading frame was created. Cells lacking the *hda1*$^+$ gene are viable. However, genetic analysis reveals that cells without *hda1*$^+$ display enhanced gene repression/silencing of marker genes, residing adjacent to telomeres, close to the silent mating-type loci and within centromere *I*. This phenotype is very similar to that recently reported for *rpd3* mutants both in *Drosophila* and budding yeast. No defects in chromosome segregation or changes in telomere length were detected. Cells lacking the *hda1*$^+$ gene display reduced sporulation. Growth of *hda1* cells is partially inhibited by low concentrations of Trichostatin A (TSA), a known inhibitor of histone deacetylase enzymes. TSA treatment is also able to overcome the enhanced silencing found in heterochromatic regions of *hda1* cells. These results indicate a genetic redundancy with respect to deacetylase genes and partially overlapping functions of these in fission yeast. The significance of these results is discussed in the light of recent discoveries from other eukaryotes.**

INTRODUCTION

It has been known for some time that histones are sometimes heavily acetylated and that this affects the stability of the resulting nucleosome (1). In addition, it was also apparent that hyperacetylation is associated with chromosome regions of high transcriptional activity, whereas transcriptionally silent regions are hypoacetylated. Since a positively charged lysine is neutralised when an acetyl group is added, this has led to the suggestion that altered charge could result in decreased nucleosomal stability and increased access for transcriptional machinery (2).

Histones can be post-translationally modified at specific lysine residues in the N termini by acetylation and deacetylation. Antibodies against acetylated forms of histones have been used to demonstrate that histones in heterochromatic regions are generally underacetylated. For example, in humans, histones H3 and H4 are hypoacetylated in both constitutive heterochromatin in centromeric regions and facultative heterochromatin of the inactive X chromosome in females (3,4). In contrast, the histones in gene-rich regions such as CpG islands, concentrated in G-bands, are acetylated (5).

For several years, the genes responsible for acetylation and deacetylation of histones were largely unknown, but recently several histone acetyl transferases (HATs) as well as histone deacetylases (HDAs) have been identified (6). The human protein HDAC1 (or HD1) was isolated by affinity purification on the histone deacetylase inhibitor trapoxin (7). This protein co-immunoprecipitates with deacetylase activity. HDAC1 is highly similar to budding yeast Rpd3, a previously known transcriptional regulator (8). It appears that deacetylase enzymes are present in complexes which contain known transcriptional repressor proteins, such as retinoblastoma (Rb), nuclear coreceptor (N-CoR), YY1, Mad/Max and Ume6 and thereby contribute to transcriptional regulation (9). Histone deacetylases are also implicated in the regulation of heterochromatin. Both *hda1* and *rpd3* deacetylase gene knockout strains of budding yeast display enhanced repression of marker genes in heterochromatin near a telomere and in *Drosophila*, the *rpd3* insertional mutant shows increased silencing of the *white* gene next to a centromere (10–12).

In the nucleus of the fission yeast, *Schizosaccharomyces pombe*, there are three heterochromatic regions, namely centromeres (*cen1*,*2*,*3*), mating-type donor loci (*mat2*,*3*) and telomeres (13). It is becoming clear that heterochromatic properties i.e. gene silencing (at all three regions) and lack of recombination (at centromeres and mating-type loci) are intimately associated with the cellular functions exerted by each region: chromosome segregation, mating-type switching processes and telomere length regulation, respectively (14–17). Inhibition of deacetylation by treating fission yeast cells with Trichostatin A (TSA) a known histone deacetylase inhibitor, causes expression of marker genes in *cen1*, *2* and *3* by increasing acetylation of histones H3 and H4 in centromeric chromatin (18). Therefore, it is plausible that in untreated cells a histone deacetylase acts to maintain centromeric heterochromatin in an underacetylated state.

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Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Source or reference
TD1	*h*$^{+}$/ *h*^{90} *leu1*-32/*leu1*-32 *ade6*-M210/*ade6*-M216 *ura4*-D18/*ura4*-D18	this work
TD2	*h*$^{+}$/ *h*^{90} *hda1::LEU2*/*hda1*$^{+}$ *leu1-32*/*leu1–32* *ade6*-M210/*ade6*-M216 *ura4*-D18/*ura4*-D18	this work
TO43	*h*^{90} *leu1*-32 *ade6*-M210 *ura4*-D18	this work
TO44	*h*$^{+}$ *hda1::LEU2 leu1*-32 *ade6*-M210 *ura4*-D18	this work
TO45	*h*$^{+}$ *leu1*-32 *ade6*-M216 *ura4*-D18	this work
TO46	*h*^{90} *hda1::LEU2 leu1*-32 *ade6*-M216 *ura4*-D18	this work
972	*h*$^{-}$ (wild-type)	
FY367	*h*$^{+}$ *leu1*-32 *ade6*-M210 *ura4*-D18	(18)
FY521	*h*$^{-}$ *leu1*-32 *ade6*-M210 *ura4*-DS/E [Ch16 *ade6*-M216 *m23::ura4*$^{+}$]	(15)
FY2816	*h? hda1::LEU2 leu1*-32 *ade6*-M210 *ura4*-DS/E [Ch16 *ade6*-M216 *m23::ura4*$^{+}$]	this work
FY2817	*h? hda1::LEU2 leu1*-32 *ade6*-M210 *ura4*-DS/E [Ch16 *ade6*-M216 *m23::ura4*$^{+}$]	this work
FY597	*h*^{90} *leu1-32 ade6*-M210 *ura4*-DS/E *mat3-M::ura4*$^{+}$	(15)
FY2606	*h*^{90} *hda1::LEU2 leu1-32 ade6*-M210 *ura4*-DS/E *mat3-M::ura4*$^{+}$	this work
FY2600	*h? hda1::LEU2 leu1-32 ade6*-M210 *ura4* [Ch16 *ura4*$^{+}$ *m23::ade6::tel*]	this work
FY2601	*h? leu1-32 ade6*-M210 *ura4* [Ch16 *ura4*$^{+}$ *m23::ade6::tel*]	this work
FY2603	*h*$^{-}$ *hda1::LEU2 ura4*-DS/E *leu1*-32 *ade6*-M210 [Ch16 *ade6*-M216 *m23::ura4::tel*]	this work
FY564	*h? leu1*-32 *ade6*-M210 *ura4*-DS/E [Ch16 *ade6*-M216 *m23::ura4::tel*]	(35)
FY1191	*h*$^{+}$ *leu1-32 ade6*-M210 *ura4*-DS/E *imr1L-Nco1::ura4*$^{+}$ *otr1R-Sph1::ade6*$^{+}$	(18)
FY2608	*h? hda1::LEU2 leu1-32 ade6*-M210 *ura4*-DS/E *imr1L-NcoI::ura4*$^{+}$*otr1R-Sph1::ade6*$^{+}$	this work

To investigate this possibility and to gain further insight into the function of histone deacetylase enzymes in heterochromatin regulation in fission yeast, we have identified a histone deacetylase gene, *hda1*$^{+}$. We describe the initial genetic analysis of the *hda1* null allele with respect to all three heterochromatic regions and their functions in fission yeast.

MATERIALS AND METHODS

Strains and culture conditions

The strains used in this work are listed in Table 1. Standard genetic techniques were used (19). Media were YES (0.5% yeast extract, 3% glucose), EMM with ammonium or glutamate (in PMG plates) 3.74 g/l as nitrogen source, or ME (3% malt extract, Oxoid, pH adjusted to 6.2 with NaOH), each supplemented with 100 mg/l of adenine, uracil, leucine and histidine. Half sectoring assay for chromosome loss was as described (15). For scoring of *ade6* phenotype, adenine was added to 15 mg/l. Selection for *ura4*$^{-}$ cells was done on YES plates with 5-fluoroorotic acid (5-FOA) added to 1 g/l.

Genomic disruption using a short flanking homology strategy

A linear double-stranded DNA with 80 bp of sequence homologous to the *S.pombe hda1*$^{+}$ locus flanking the *Saccharomyces cerevisiae LEU2* gene was synthesised in a two-step PCR procedure. In the first PCR, the primers were 5′-CGACCTTTTGCTACGTATTCTG-CGCTCGACGTAAAAATAATGTCCTGTACTTCCTTGTTC-3′ (oligo 1) and 5′-AGATCTTCAAGAGCTTTCGGTGTATTTTTA-TTTTCTACTTTTCCTTATCACGTTGAGCC-3′ (oligo 2); the first 40 nt of these 60 nt primers are homologous to *hda1*$^{+}$, whereas the following 20 nt are derived from *LEU2*. The product from this first reaction was diluted 1500-fold and was the substrate for the second reaction, which used the primers 5′-TTGAAGTTGT-ACATTTTATCTAATAAGGTATCGGGTTTAGCGACCTTTT-GCTACGTATTC-3′ (oligo 3) and 5′-TCCTCCTAAGTAACGT-AATTGCTCCAAGGCCCGTATTCGAAGATCTTCAAGGCT-TTCGG-3′ (oligo 4). This second pair of 60 nt primers has 20 nt of overlapping sequence with the first pair. Approximately 1 µg of the PCR product from reaction 2 was transformed into *S.pombe* strain TD1 using a lithium acetate protocol (20). Substrate DNA for diagnostic PCR was prepared from liquid microcultures using a method modified from Hoffman and Winston (21). In short, individual Leu^{+} transformants were grown in 100 µl of liquid YES. Aliquots (10 µl) of cultures were pooled in groups of 12 in

an Eppendorf tube, centrifuged for 10 s at 12 000 r.p.m. in an Eppendorf benchtop centrifuge, and resuspended in 100 µl of 2% Triton X-100; 1% sodium dodecyl sulfate; 0.1 M NaCl; 10 mM Tris–HCl, pH 8.0; 1 mM EDTA. Cells were disrupted by adding ~100 mg of glass spheres and 100 µl of phenol:chloroform (1:1, v/v) and vortexing at high speed for 4 min. dH_2O (100 µl) was added and samples were centrifuged at maximum speed for 15 min. The resulting supernatant was ethanol precipitated and dissolved in dH_2O. The pools were then screened for correct integration into the *hda1*$^+$ locus using analytical PCR with the diagnostic primers 5′-TTGATGTTTGTACGGATATC-3′ (oligo 5), 5′-TTATAAGAATGAACCAGCAC-3′ (oligo 6) and 5′-CTGGCAAAACGACGATCTTC-3′ (oligo 7) (Fig. 2B).

RESULTS

Identification and disruption of a putative fission yeast histone deacetylase gene

Histone deacetylase genes from such diverged organisms as humans, flies (*Drosophila*) and budding yeast are 60–70% identical at the amino acid sequence level. By scanning the Sanger Centre DNA *S.pombe* sequence database (http://www.sanger.ac.uk/) using the TBLASTX function for potential coding regions with similarities to the *S.cerevisiae* Rpd3 histone deacetylase protein sequence, we identified an open reading frame of 1302 bp with the potential to encode a protein of 434 amino acid residues (cosmid SPAC3G9, accession no. AL021046). The predicted Hda1p amino acid sequence is highly similar to the Rpd3-related proteins *S.cerevisiae* Rpd3 (10), *S.cerevisiae* Hos2 (12), *Homo sapiens* HD1 (or HDAC1) (7) and a *Caenorhabditis elegans* putative histone deacetylase (accession no. q09440). Within this group, it is marginally closer to the *S.cerevisiae* histone deacetylase Hos2 protein than to the other members. The region of similarity extends to at least 330 amino acid residues (Fig. 1), which corresponds to over 75% of the length of the coding regions. The remaining three *S.cerevisiae* genes encoding related proteins, *HDA1*, *HOS1* and *HOS3* (12,22), share only parts of the domains conserved in the Rpd3 group, largely the same subdomains as those previously defined as common to the superfamily comprising histone deacetylases, acetoin utilisation proteins and acetylpolyamine amidohydrolases (23,24). It has been proposed that the large 2/3 N-terminal ‘prokaryotic homology’ domain confers enzymatic activity and the more diverged C-terminal domain is involved in functional specificity (25).

The strategy for disruption of *hda1*$^+$ is shown in Figure 2A and B. Among 200 diploid Leu$^+$ transformants, one was identified which displayed the diagnostic 2.4 and 0.75 kb PCR products from the disrupted allele, as well as the 1.8 kb band originating from the wild-type allele (Fig. 2C, top panel). This diploid (TD2) was sporulated and tetrads were dissected. A 2:2 segregation of the Leu$^+$ phenotype was observed, consistent with a single integration event (Fig. 2C, bottom right panel). Haploids from the tetrads were analysed and in all cases the 2.4 and 0.75 kb diagnostic bands segregated together with the Leu$^+$ phenotype, as expected. The knockout fission yeast strain was also analysed by southern blotting. Genomic DNA was extracted from wild-type and knockout cells, digested using *Bam*H1, *Hin*dIII and *Eco*R1 restriction enzymes, and hybridisation of the resulting filter to an *hda1*$^+$ probe revealed a single band present in wild-type cells. This band was absent in *hda1::LEU2* cells (data not shown). We conclude that *hda1*$^+$ is a non-essential gene.

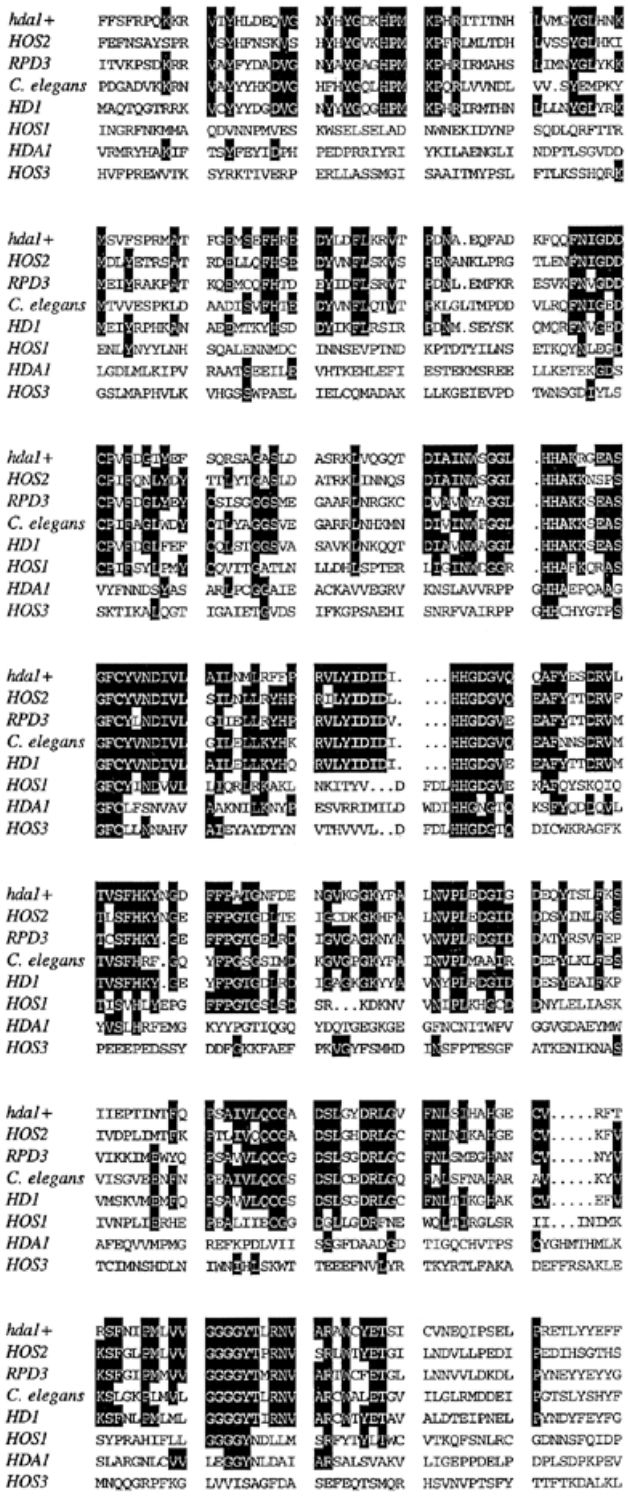

Figure 1. Sequence similarity of selected putative histone deacetylases, acetoin utilisation proteins and acetylpolyamine amidohydrolases from different species; alignment showing identical amino acid residues (black boxes). Only the region of sequence conservation is shown. Displayed sequences are: *hda1*$^+$ (*S.pombe*), accession no. AL021046; *HOS2* (*S.cerevisiae*), accession no. Z72716; *RPD3* (*S.cerevisiae*), accession no. 854536; *C.elegans* putative histone deacetylase, accession no. Q09440; *HD1* (*H.sapiens*), accession no. Q13547; *HOS1* (*S.cerevisiae*), accession no. Z49219; *HDA1* (*S.cerevisiae*), accession no. Z71297; *HOS3* (*S.cerevisiae*), accession no. U43503.

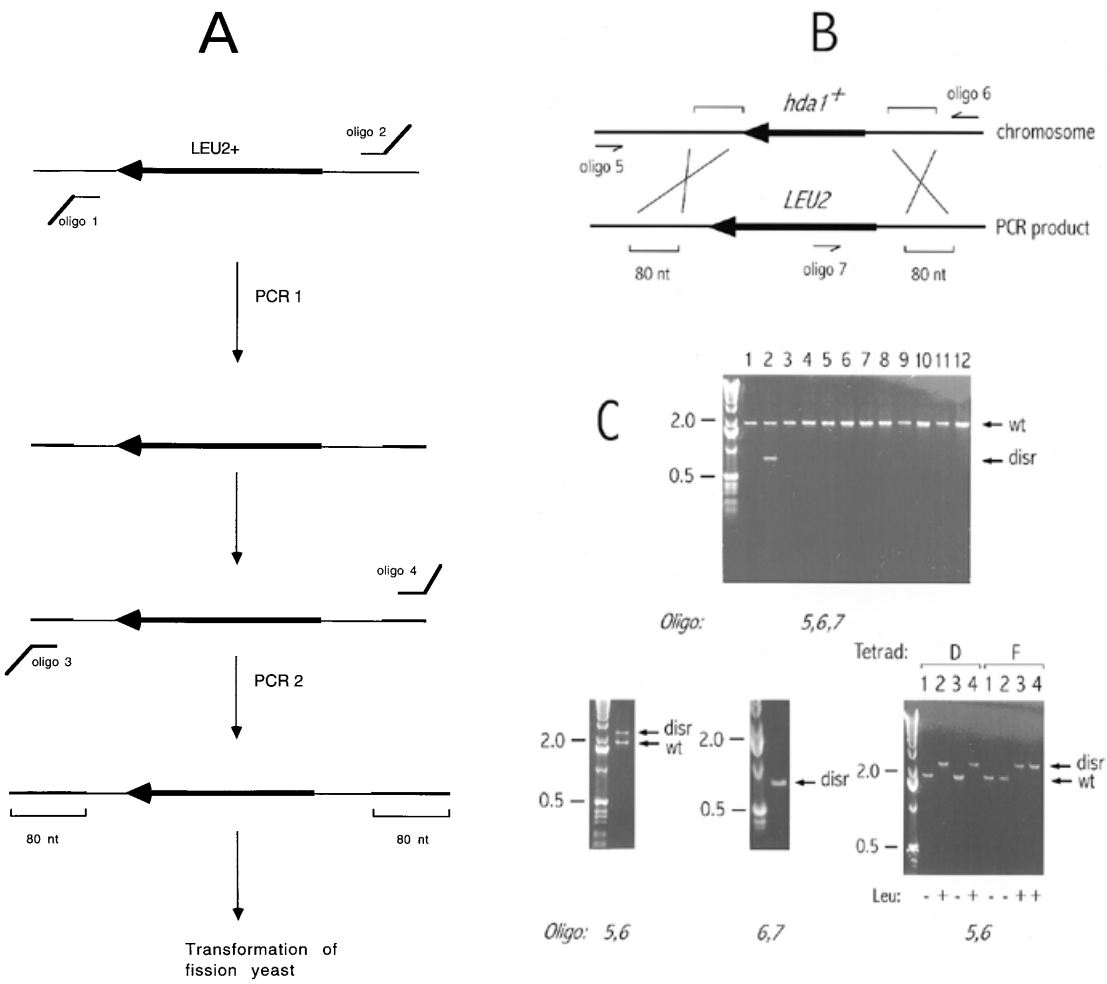

Figure 2. PCR-based disruption of the genomic *hda1*$^+$ locus. (**A**) Strategy for PCR knockout. Schematic view of PCR steps involved in engineering the *hda1::LEU2* deleted allele (disruption cassette). The location of oligos 1–4 are indicated (see Materials and Methods). (**B**) Principle of verification of correct integration by PCR. The regions of homology of oligos 5, 6 and 7 are indicated. (**C**) PCR analysis of disruptant. Top panel, PCR analysis (using oligos 5, 6 and 7 of individual diploid transformants. Lane 2 contains the 0.75 kb band indicative of the disruption (shown by arrow, 'disr'). Bottom left, analysis of transformant no. 2 using oligos 5 and 6 only. Bottom centre, analysis of transformant no. 2 using oligos 6 and 7. Only the expected 0.75 kb band is seen. Bottom right, PCR analysis, using oligos 5 and 6, of haploid segregants from two dissected tetrads from diploid transformant no. 2.

Cells lacking the *hda1*$^+$ gene are supersensitive to TSA

TSA is a specific inhibitor of histone deacetylase enzyme (26) and TSA treatment of fission yeast cells causes expression of several genes, both in heterochromatin and euchromatin (18). We were interested to determine whether Hda1p is a target for TSA. Therefore, we investigated the growth properties of *hda1::LEU2* cells on plates in the presence of increasing concentration of TSA. It was obvious after 3 days of incubation at 32°C that growth of *hda1::LEU2* cells was partially inhibited by a lower concentration of TSA compared with wild-type cells (Fig. 3). Thus, the activity encoded by *hda1*$^+$ confers resistance to TSA. Therefore, we conclude that Hda1p is one possible target for binding of TSA.

Enhanced silencing mainly at telomeres

Since TSA derepresses silencing at centromeres and causes centromeres to become hyperacetylated (18), it is expected that a deacetylase enzyme is responsible for keeping the centromeres underacetylated. Because Hda1p may be a target for TSA, we decided to investigate whether *hda1* knockout cells display reduced silencing of centromeres. In addition, gene silencing at the mating-type region and in the vicinity of telomeres was investigated. Crosses were performed to combine the *hda1::LEU2* allele with a *ura4*$^+$ gene insertion in mating-type region, at two different sites within the centromere and close to telomeres. All these *ura4*$^+$ insertions are subject to transcriptional silencing (15). Growth of wild-type cells and *hda1::LEU2* cells harbouring these *ura4*$^+$ insertions was compared by growth on selective plates lacking uracil (–URA) and non-selective (+URA) plates (Fig. 4). It was clear that growth of *hda1::LEU2* cells was only slightly impaired as compared with growth of wild-type cells on non-selective plates, but dramatically reduced on selective plates. This indicates that silencing of the *ura4*$^+$ gene is more efficient in cells lacking *hda1*$^+$. The plating efficiencies of *hda1*$^+$ *tel3::ura4*, *hda1*$^+$ *imr1L::ura4*$^+$, and of *hda1*$^+$ *mat3::ura4*$^+$ cells were >25-fold higher than those of the corresponding *hda1::LEU2* cells. These data suggest that Hda1p is not the target

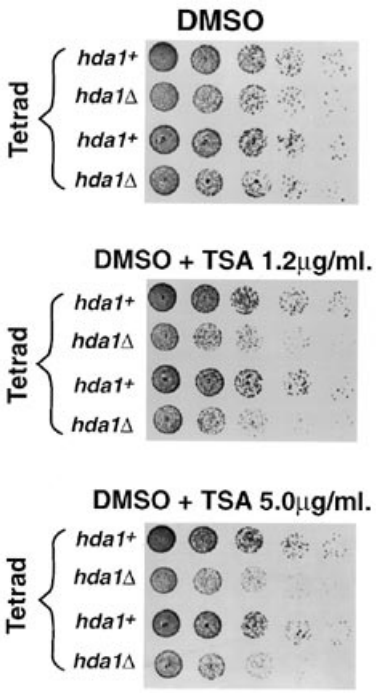

Figure 3. TSA sensitivity. A tetrad from the original TD2 *hda1::LEU2* disruptant was spotted in serial 1:3 dilutions on YES medium containing the indicated concentrations of TSA dissolved in 0.1% DMSO, or 0.1% DMSO only. Cells were grown for 3 days at 32°C.

of TSA which, when inhibited by TSA treatment, causes expression of marker genes within centromeres (18). Instead, removal of the *hda1*$^+$ gene leads to increased repression of the *ura4*$^+$ gene inserted within centromeres, at the silent mating-type locus and especially close to telomeres.

Further evidence that other histone deacetylases apart from Hda1p are the targets reponsible for derepression of markers in the centromeres, mating-type and telomere regions upon TSA pre-treatment is seen in the lower panels of Figure 4. On selective plates, survival of *hda1*$^+$ *imr1*L*::ura4*$^+$ cells increased ~25-fold in agreement with previous results (18). For *hda1::LEU2 imr1*L*::ura4*$^+$ cells, the fraction of Ura$^+$ cells was increased to about the same absolute level as for *hda1*$^+$ *imr1*L*::ura4*$^+$ cells, corresponding to a relative increase of at least 125-fold compared with untreated cells (Fig. 4 compare upper right and lower right panels). Similar effects were seen in the other heterochromatic loci. Thus, addition of TSA clearly reversed the increased silencing of *ura4*$^+$ marker genes inserted at all these loci in *hda1*Δ cells.

Maintenance of the repressed state

In a metastable gene-expression system, each locus adopts one of two possible states: repressed or expressed. The greater repression observed in *hda1::LEU2* cells could, in principle, result either from increased stability of the repressed state (maintenance of the silent state) or increased switch rate from the expressed to the repressed state (establishment of the silent state). To distinguish between these two hypotheses, the *hda1::LEU2* allele was combined with the Ch16 mini-chromosome carrying the *ade6*$^+$*::tel* marker. This marker gene, if repressed, results in formation of dark pink or red colonies, and if expressed, causes a lighter pink or white colony colour. Therefore, the rate of establishment of repressed state colonies can be estimated by replating light pink (expressed) wild-type and *hda1* null colonies and scoring frequencies of dark pink/red colonies. Similarly, the efficiency of maintenance of repressed state can be assayed by replating dark pink or red colonies and scoring occurrence of light pink/white colonies. Replating of three independent white *hda1::LEU2* and wild-type *ade6*$^+$*::tel* colonies (strains FY2600 and FY2601, respectively) produced similar frequencies (1–3%) of red colonies. In contrast, replating of three independent dark pink/red colonies from *hda1::LEU2* strain yielded a higher frequency of red colonies (64–69%) as compared with the wild-type control strain (21–31%) (Fig. 5). Thus, the maintenance of the repressed state seems to be improved in the cells lacking Hda1p (see Discussion).

Centromere function is normal in *hda1* null cells

Mutations which cause enhanced repression of marker genes inserted in fission yeast centromeres may also reduce or abolish centromere function (J.-P.Javerzat, R.C.Allshire, in preparation). Therefore, it could be argued that enhanced silencing at centromeres results in altered centromere chromatin structure which might reduce the fidelity of centromere function. Since *hda1::LEU2* cells display enhanced repression of the centromeric *ura4*$^+$ marker, we measured chromosome loss rates of the 530 kb linear non-essential mini-chromosome, Ch16, using the half-sectoring assay (15). There was no increase of Ch16 loss rates in the *hda1::LEU2* strain as compared with the wild-type control. For both strains, four independent colonies were assayed and displayed Ch16 loss rates of 0.5–1.0% per cell division at 32°C. To monitor chromosome loss or chromosome mis-segregation events cytologically, *hda1* cells and wild-type control cells were also subjected to immuno-fluoresence using anti-tubulin antibodies and Dapi staining of chromosomes. This technique allows cells with aberrant spindle morphology and/or defects in mitotic chromosome segregation such as lagging chromosomes to be identified (27). Such cells were not observed in *hda1::LEU2* cells (data not shown). In addition, sensitivity to the microtubule de-polymerising drugs methyl benzimidazole-2-yl carbamate (MBC) and thiabendazole (TBZ) was measured. Mutants which display spindle defects and centromere defects (lagging anaphase chromosomes) are often supersensitive to tubulin de-polymerising drugs (27). However, *hda1::LEU2* cells were not super-sensitive to MBC or TBZ (data not shown). Thus, the *hda1*$^+$ gene does not appear to contribute to spindle function and mitotic centromere function.

The *hda1* null mutation decreases sporulation efficiency

It is possible that *hda1*$^+$ could be involved in either mating-type expression, switching, or telomere regulation processes, all of which affect the sporulation pattern of homothallic *h*^{90} colonies. Since spores contain starch, changes in sporulation pattern can be detected using staining of colonies with iodine vapours. To investigate potential meiosis/mating-type switching phenotypes, *hda1* null Leu$^+$ segregants from the original *h*^{90}/*h*$^+$ diploid were stained with iodine vapours. The overall efficiency of sporulation was investigated macroscopically by iodine staining of colonies. We noted that the *h*^{90} *hda1*Δdisruptant stained lighter grey than the wild-type control strain (Fig. 6A), indicating a lower overall sporulation efficiency. Sporulation in wild-type versus *hda1::LEU2 h*^{90} cells was also investigated by phase contrast microscopy. The *hda1::LEU2* cells appeared to generate asci at

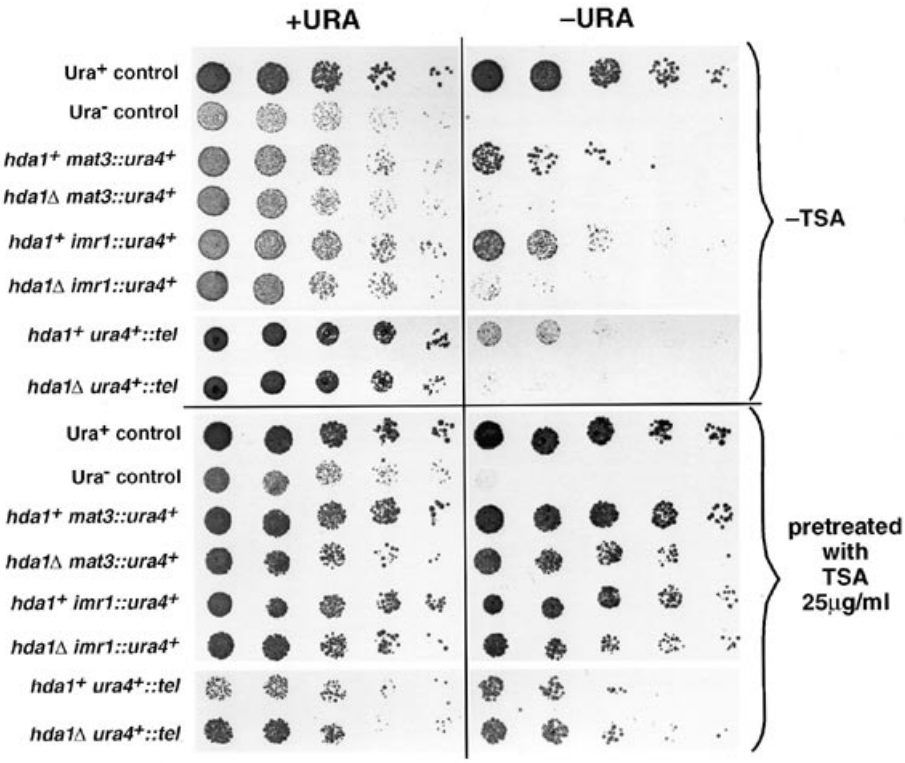

Figure 4. Effect of the *hda1*Δ mutation on the *ura4*$^+$ gene integrated at various heterochromatic loci. Upper panels, enhanced silencing in *hda1*Δ cells. Left panel, non-selective medium (+URA); right panels, selective medium lacking uracil (–URA). Cells were serially diluted 1:5 and allowed to grow for 3 days at 32°C. Strains used were: 972*h*$^-$ (Ura$^+$ control); FY367 (Ura$^-$ control); FY597 (*hda1*$^+$ *mat3::ura4*$^+$, integration adjacent to the mating-type locus); FY2606 (*hda1*Δ *mat3::ura4*$^+$); FY1193 (*hda1*$^+$ *imr1*L*::ura4*$^+$, integration in centromere); FY2608 (*hda1*Δ *imr1*L*::ura4*$^+$); FY564 (*hda1*$^+$ *ura4*$^+$*::tel*, integration adjacent to telomere of Ch16 mini-chromosome); FY2603 (*hda1*Δ *ura4*$^+$*::tel*). Lower panels, derepression by pretreatment with TSA (25 μg/ml for 6 cell doublings at 32°C). Cells were spotted on selective or non-selective media as above; same strains and culture conditions as in upper panels.

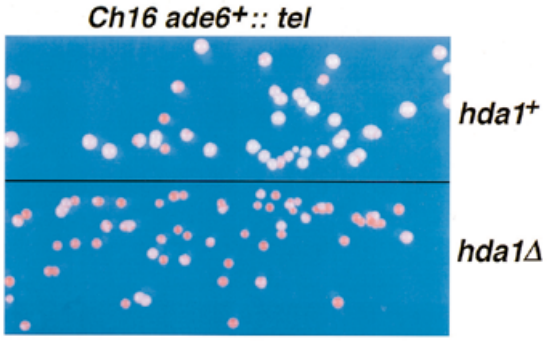

Figure 5. Increased maintenance of the repressed state of the *ade6*$^+$ gene integrated adjacent to a telomere. Comparison of maintenance of red repressed state of *Ch16-ade6*$^+$*::tel* in *hda1*$^+$ versus *hda1*Δ cells. The strains FY2600 (*hda1*$^+$) and FY2601 (*hda1*Δ) were streaked on low adenine medium (4 mg/l). A red (repressed) colony from each strain was selected, restreaked on low adenine medium and incubated at 32°C for four days.

a lower rate compared with the wild-type, since a larger fraction of zygotes and a smaller fraction of matured tetrads was observed as compared with wild-type (Fig. 6B and C, and Table 2). No spores arising from haploid cells (i.e. two-spored asci) which would indicate expression of silent mating-type loci were observed (28) and no other obvious anomalies in formation of spores or tetrads were noted.

Table 2. Kinetics of meiosis and sporulation in wild-type and *hda1*Δ mutant cells

Days	TO43 (wt)		TO46 (*hda1*Δ)	
	Zygotes (%)	Tetrads (%)	Zygotes (%)	Tetrads (%)
0	0	0	0	0
1	10.9	4.9	2.6	1.0
2	8.3	41.7	13.4	11.8
3	6.7	65.3	16.3	17.6
4	7.6	62.3	13.3	20.3

Cells were transferred from vegetative (YES) to sporulation medium (ME) on day 0, and progression of meiosis at 25°C was followed microscopically for the indicated number of days. Each figure represents counting of at least 300 cells.

Reduced sporulation of an h^{90} strain is usually associated with either expression (derepression) of the silent mating-type cassettes which leads to haploid meiosis and reduced mating efficiency (14,16) or with a reduced rate of mating-type switching causing less mating events within the colony (see also Discussion). The possibility that *hda1::LEU2* allele causes increased expression is unlikely because of absence of haploid meiosis (see above). A possible effect of *hda1::LEU2* allele on switching was tested by combining with a mating-type region harbouring opposite information at the silent mating-type loci (h^{09}) (29). The rationale of such an experiment is that if the mutation affects the

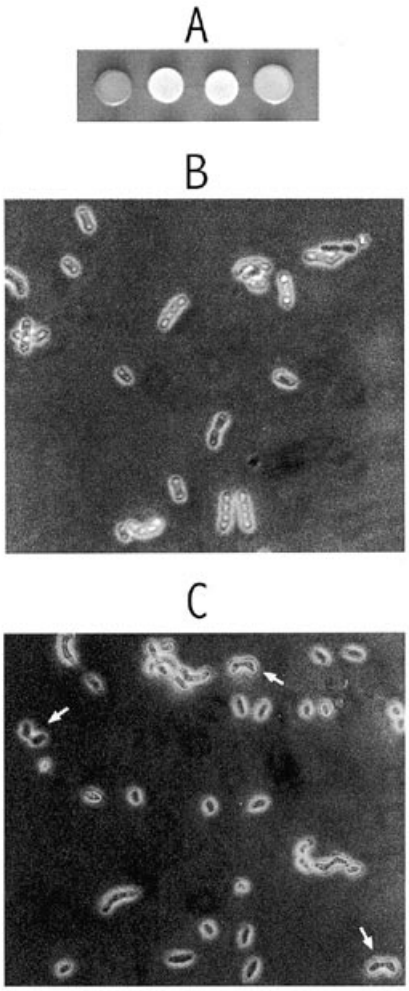

Figure 6. Effect of *hda1* disruption on sporulation. (**A**) Iodine staining of spores in segregants from the h^+/h^{90} *hda1*Δ/*hda1*$^+$ diploid disruptant TD2; same tetrad D as shown in Fig. 2 (see also Table 2). From left: colony no. 1 represents wild-type h^{90} cells; 2, h^+ *hda1*Δ; 3, wild-type h^+; 4, h^{90} *hda1*Δ cells. (**B**) micrographs of wild-type h^{90} cells after 3 days in ME sporulation medium, at 25°C. (**C**) h^{90} *hda1*Δ cells under the same conditions as in (B). Note the absence of mature (four-spored) asci and the accumulation of immature tetrads (zygotes; marked by arrows) in the mutant strain.

directionality of mating-type switching, one should be able to detect this as an enhanced sporulation in this background, something we did not observe (data not shown).

DISCUSSION

Using a recently developed PCR method, we have precisely disrupted the *hda1*$^+$ gene encoding a putative histone deacetylase. This disruption method is similar to that described for budding yeast and recently reported for fission yeast (30). *hda1*$^+$ is so far the only identified member of the histone deacetylase gene family in *S.pombe*. However, genomic sequencing of *S.cerevisiae* has revealed no less than five putative histone deacetylase genes (22), of which two (*RPD3* and *HOS2*) are closely related to *hda1*$^+$. The *S.pombe* genome is at present only ~50% sequenced. We expect that additional family members will be identified as sequencing of chromosomes *II* and *III* proceeds.

The distinct but relatively moderate phenotypes we have observed in *hda1* mutants could thus be explained by partially overlapping roles of several histone deacetylases. In this context, we note that the magnitude of the effects on repression seen in *S.cerevisiae hda1* knockout strains (12) is comparable with what we find here. In strains with the *mat3*-M::*ura4*$^+$ insertion, only a weakly enhanced silencing phenotype was seen. We cannot exclude an effect of similar scale also on the mating-type cassettes, but this has not been investigated since enhanced silencing of these loci is impossible to detect.

In contrast with the general view of a correlation between decreased histone acetylation and the repressed heterochromatic state, both in *S.cerevisiae rpd3* and *hda1* mutants (10–12) as well as in the *S.pombe hda1* deletion (this paper), increased repression in heterochromatic regions is found. Indeed, it has been proposed that some forms of heterochromatin are enriched specifically in acetylation of H4 lysine 12 (10). H4 tri-acetylated at lysines 5, 8 and 12 is associated with the chromatin assembly factor (CAF) nucleosome assembly complex (31). Therefore, acetylation of lysine 12 may be required for efficient nucleosome assembly. Mutations in budding yeast components of CAF (Rlf2 and Cac1) display reduced maintenance of silent states at telomeres (32,33). Thus, it seems as if H4 acetylated isoforms which show efficient binding to CAF also favour efficient maintainence of repressed state. Therefore, removal of a histone deacetylase with activity specific for a particular amino acid residue may produce more robust heterochromatin and increase repression, rather than the opposite. Thus, the phenotypic data is consistent with this hypothesis, but nothing is yet known about the specificity of *S.pombe* Hda1p, or the state of chromatin at telomeres in wild-type and *hda1* null mutant backgrounds.

In an alternative interpretation, the observed phenotypes could be indirectly caused by *hda1*. With all deacetylases, one expects global changes in gene expression. It has already been demonstrated that TSA causes expression of normally repressed euchromatic genes such as *nmt1*$^+$ and *fbp1*$^+$ (18). Altered expression of other genes involved in chromatin regulation, caused by removal of Hda1p, may indirectly affect telomere, mating-type and centromeric loci so that the repressed state is maintained more efficiently.

It is plausible that there are at least two TSA-sensitive deacetylase complexes/enzymes in fission yeast: Hda1p described here and another one which is responsible for the TSA-induced centromere phenotypes (18). Our finding that TSA relieves repression at centromeric as well as other heterochromatic loci to virtually the same extent in *hda1* as in wild-type cells is a strong indication that this is indeed the case. It is also possible that these two complexes have different sensitivity to TSA, with the centromeric histone deacetylase being most sensitive, given the readily observed centromere defects with TSA treatment (18). There is precedence for differential sensitivity in budding yeast where the HDA complex is 10 times more sensitive to TSA than the HDB complex (34). Our finding that *hda1* cells are sensitive to TSA indicates the existence of TSA-sensitive cellular targets (histone deacetylase/s) other than Hda1p. Since *hda1*$^+$ is more similar to *RPD3* than to *HDA1* of *S.cerevisiae* (Fig. 1) and Rpd3 is a component of the relatively TSA-insensitive HDB complex (9,34), it is possible that other *S.pombe* putative histone deacetylase/s are more sensitive to TSA. This could explain the difference in phenotypes obtained after TSA treatment of *S.pombe* cells and those of *hda1* null mutant.

hda1 mutants displayed a lower sporulation efficiency than wild-type. There are several possible explanations for this; however some of these we have ruled out or made less likely. First, changes in histone acetylation patterns of heterochromatin could result in derepression of the silent mating-type loci. The absence of haploid spores in an h^{90} *hda1*Δ strain and the reduced silencing observed for *mat3::ura4*$^+$ (Fig. 3) both argue against this possibility. Second, impaired directionality of the switching could cause a lower overall efficiency of the process. However, the h^{09} *hda1*Δ strain did not display a more efficient switching and therefore this is not likely to be the explanation. Third, *hda1* could be indirectly involved in telomere length regulation. Genes involved in regulation of telomere length, e.g. *taz1*$^+$ (17), could be mis-regulated in *hda1*Δ cells or changes in telomere chromatin histone acetylation patterns could lead to defective meiosis. This explanation is made less likely because the *hda1* null mutation does not seem to affect telomere length (data not shown). A major remaining area is the meiotic pathway *per se*, where regulation of genes involved in one or several steps, including recombination, could be affected. Finally, although we have no indication that mitotic centromere function in the mutants is affected since we see no elevated mini-chromosome loss, and no MBC or TBZ supersensitivity, it is still possible that meiotic chromosome segregation is affected, and that could explain the reduced tetrad formation.

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