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The Fission Yeast Hda1p Functions on the Regulation of Proper Cell Division

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We cloned *hda1*$^+$ (*histone deacetylase 1*) of fission yeast *Schizosaccharomyces pombe*. The *hda1*$^+$ of *S. pombe* was previously reported to encode for an active histone deacetylase (Rundlett *et al.*, 1996; Olsson *et al.*, 1998). The *hda1*$^+$ is phylogenetically related to the new open reading frame *HOS2* of *Saccharomyces cerevisiae* and only shows a partial homology to the well-known histone deacetylase subclasses, *RPD3* and *HDA1*. A single *hda1* mRNA of 1.8 kb was detected at the same level in actively growing and nitrogen-starved cells. When highly over-expressed in *S. pombe* from an inducible promoter, *hda1*$^+$ inhibited cell proliferation and caused defects in morphology and cell division. The increased histone deacetylase activity was detected in *hda1*$^+$ over-expressing cells. These results suggest that the Hda1p should function on the regulation of cell division possibly by (Allfrey, 1966) direct deacetylation of cytoskeletal (Wade *et al.*, 1997) and cell division regulatory proteins, (Wolffe, 1997) or by controlling their gene expressions.

Keywords: Cell division, Histone deacetylase activity, *S. pombe*, *hda1*$^+$.

Introduction

The reversible acetylation of several proteins occurs on the ε-amino group of their lysine residues. The best understood post-translational acetylation is that of core histones that are found in all eukaryotes (Allfrey, 1966). Histone acetyltransferases (HATs) introduce an acetyl group from acetyl-CoA into the ε-amino group of lysine and histone deacetylases (HDACs) that catalyze the removal of acetate. The specific acetylation states of histones in chromatin have been correlated with transcriptional activation, chromatin assembly, and the repression of heterochromatin. Histone deacetylases (HDACs) can be grouped into two classes. The first class includes the budding yeast Rpd3-like proteins. The second class contains the budding yeast Hda1p-like proteins. Their sequences, as well as the presence of Rpd3p and Hda1p in biochemically distinct complexes, imply that these two subclasses may function in different cellular processes (Rundlett *et al.*, 1996; Leipe and Landsman, 1997; Grozinger *et al.*, 1999).

The *hda1*$^+$ of fission yeast *Schizosaccharomyces pombe* was cloned and reported to encode for an active histone deacetylase *in vitro* (Kim *et al.*, 1998; Olsson *et al.*, 1998). The deletion of *hda1*$^+$ showed no obvious defects in the mitotic cell cycle or in chromosome segregation except in reduced sporulation (Kim *et al.*, 1998; Olsson *et al.*, 1998). However, the exposure of fission yeast cells to Trichostatin A (TSA), a specific inhibitor of histone deacetylase enzymes, caused chromosome loss and structural changes of the centromeres (Ekwell *et al.*, 1997). These results suggest the redundancy of histone deacetylase activities in the fission yeast. In this study, we demonstrate that *hda1*$^+$ prevents cell proliferation and induces defects in morphology and cytokinesis when over-expressed in *S. pombe*. These results suggest the putative functions of Hda1p in the regulation of cell division.

Materials and Methods

***S. pombe* strain and media** The *S. pombe* KGY246 (*h*$^-$ *ade6-210 ura4-D18 leu1-32*) was used in this study. Culture, manipulation, and transformation of *S. pombe* were carried out essentially as described by Moreno *et al.* (Moreno *et al.*, 1991). For nitrogen starvation, MM-N was used where ammonia was omitted.

PCR cloning of *hda1*$^+$ and plasmid constructions A full-length *hda1*$^+$ gene was amplified from the *S. pombe* genomic library through a polymerase chain reaction (PCR) using two oligonucleotides: 5' oligo 5'-CGATGGATCCTATCATGGATACT CCTG-3' and 3' oligo 5'-TCCACCCGGGTCAGCCTCGAACG CGAACATC-3'. Amplified *hda1*$^+$ was subcloned into pRep1, pRM41, and pRep81 to generate pRep1/hda1, pRM41/hda1, and pRep81/hda1. The pRM41 is a derivative of pRep41 and *hda1* is

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tagged in its N-terminus with 6X histidine in pRM41/hda1. PCR was carried out with Ex-Taq polymerase (Takara Co.) and two independent PCR products were subcloned to exclude possible errors from PCR.

Northern blot To detect *hda1* mRNA in *S. pombe*, total RNAs were prepared from KGY246 grown in MM or MM-N [14]. 15 μg of total RNA of each was electrophoresed, transferred to the Hybond membrane (Amersham Co.), and probed with the full-length *hda1* PCR fragment.

Over-expression of Hda1p in *S. pombe* For *hda1*$^+$ over-expression, KGY246 cells were transformed with pRep1/hda1, pRM41/hda1, or pRep81/hda1, respectively. In these constructs, *hda1*$^+$ is under the control of *nmt1* promoters with different transcription efficiencies that induce *hda1*$^+$ expression in the absence of thiamine in the medium (Basi *et al.*, 1993; Maundrell, 1993). KGY246 cells, transformed respectively with pRep1, pRM41, or pRep81, were used for negative controls. Crude cell extracts were prepared as described in Song *et al.* (Song *et al.*, 1996) and over-expressed Hda1p was detected with anti-His tag (Stratagene) antisera.

Microscopic techniques Cells were fixed by adding 1/4 volume of a freshly prepared 17% (wt/vol) paraformaldehyde to an exponentially growing culture. Fixed cells were washed twice with PBS (pH 8.0) and mounted with 1 μg/ml DAPI (Sigma). Photographs were taken with Tmax 400 and the negatives were scanned for figures with a Proimager 8200 (Pixelcraft).

Assay of HDAC activity The whole cell extracts, or nuclear extracts, were prepared from the *hda1* over-expressing cells as essentially described (Moreno *et al.*, 1991; Song *et al.*, 1996) and were used as an enzyme source. The histone deacetylase (HDAC) activity was assayed by following the procedures of Carmen *et al.* (Carmen *et al.*, 1996). The whole cell extract (60 μg protein), or nuclear extract (22 μg protein), was incubated with 8 μg [^{3}H]-acetyl-labeled HeLa histones in a total volume of 200 μl assay buffer (75 mM Tris-HCl pH 7.0, 275 mM NaCl, 2.0 mM β-mercaptoethanol, 0.1 mM EDTA, and 1 mM PMSF) for 60 min at 37°. The nuclear extract (60 μg protein) of HeLa cells, where HDAC1 was induced by the Tet-off system (Clontech), was used as a positive control. The released [^{3}H]- acetate was extracted and quantified by scintillation counting. Assays were performed in duplicates and the average of the two independent assays was presented.

Results and Discussions

Identification of *hda1*$^+$ in fission yeast The *hda1*$^+$ of fission yeast *S. pombe* displayed only a partial sequence homology to *RPD3* and *HDA1* of budding yeast (Leipe and Landsman, 1997; Olsson *et al.*, 1998). We cloned the *hda1*$^+$ gene by amplifying its ORF from a *S. pombe* genomic library through a polymerase chain reaction (PCR). The 5' and 3' ends of the cloned *hda1* ORF were verified by restriction mappings and sequencing. In order to confirm the expression

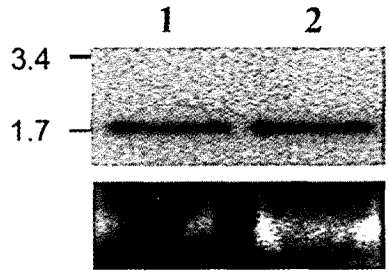
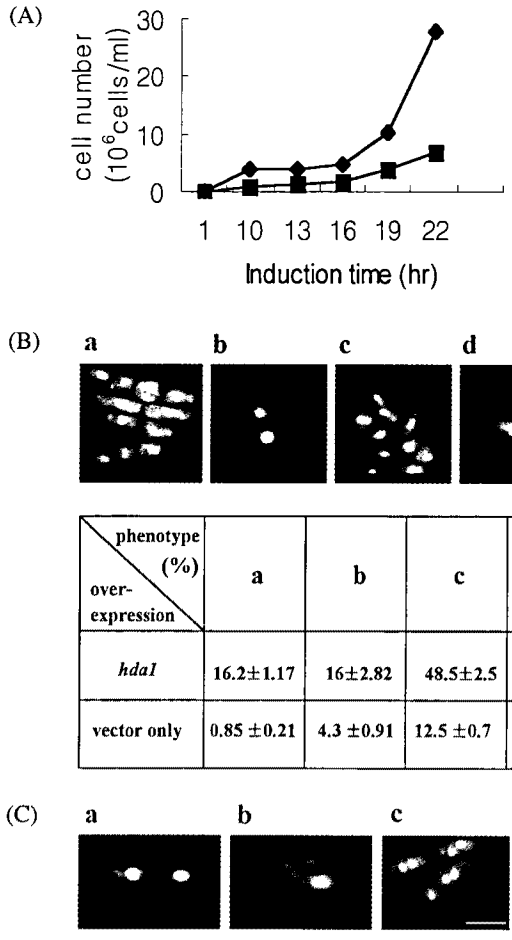


Fig. 1. Detection of *hda1*$^+$ mRNA by Northern hybridization. 15 μg of total RNAs from (1) actively growing and (2) nitrogenstarved cells were separated and probed with a fragment of the *hda1* gene prepared by PCR. Approximately equal amounts of RNAs were loaded in each lane, as illustrated by ethidium bromide staining of 23S rRNA (lower).

of *hda1*$^+$ in *S. pombe*, we detected the *hda1*$^+$ mRNA by Northern analysis. A single mRNA of approximately 1.8 kb (expected size by ORF) was present at the same level in the actively growing, as well as in the nitrogen-starved cells (Fig. 1). A previous report showed the decreased sporulation efficiency in the *hda1* null mutant, proposing its function on sporulation (Olsson *et al.*, 1998). However, this Northern analysis indicated that the *hda1*$^+$ is actively expressed in vegetative growth, as well as in the nitrogen-starved cells when sporulation was induced.

***hda1*$^+$ over-expression and its phenotypes** Previous studies showed that the deletion of *hda1* had no defects in the mitotic cell cycle and chromosome segregation (Kim *et al.*, 1998; Olsson *et al*, 1998). As an approach to cellular functions of *hda1*$^+$, we over-expressed *hda1*$^+$ and examined its phenotypes. The over-expression of *hda1*$^+$ was not lethal, but cell proliferation was severely diminished by its over-expression (Fig. 2A). The over-expression of *hda1*$^+$ also led to defects in the cell morphology and cell division (Fig. 2B). Approximately 50% of the *hda1*$^+$ over-expressed cells demonstrated bulb-like shapes instead of rod shapes of wild type cells (Fig. 2B-c). More than 30% of the cells became elongated and were frequently multi-septated (Fig. 2B-a, b). Nuclei were mislocated, or missegregated, in about 10% of these cells (Fig. 2C). As shown in Fig. 2C-a, b, these cells frequently contained no nucleus, and for those containing one, it recurrently did not lie in the middle. About 17% of the elongated cells contain multiple nuclei that are not properly separated by septum, as shown in Fig. 2C-c. These phenotypes are typically observed in the mutants of the genes for cytoskeleton and cell division control (Snell and Nurse, 1993).

We examined the *hda1*$^+$ over-expression phenotypes by changing the transcription efficiency of *hda1*$^+$ with different thiamine-repressible expression vectors. Because of the mutations in the *nmt1* promoter, the transcription efficiency of pRep1 was reported to be approximately 80 fold higher than that of pRep81 and 7 fold higher than that of pRM41, when



phenotype (%) / over-expression	a	b	c	d
hda1	16.2±1.17	16±2.82	48.5±2.5	19.3±3.3
vector only	0.85 ±0.21	4.3 ±0.91	12.5 ±0.7	84.5 ±0.7

Fig. 2. Growth inhibition and phenotypes by *hda1*$^+$ over-expression.
KGY246 was transformed with pRM41/hda1 and pRM41. Transformants were induced for 16-18 hrs in a selective medium lacking thiamine. (A) *hda1*$^+$ over-expression inhibits cell proliferation. Cells were counted every 3 hr. Square (■) indicates *hda1*$^+$ over-expressing cells and diamond (◆) indicates a vector-only negative control. (B) Assorted phenotypes of the *hda1*$^+$ over-expressed cells (a-d). Each phenotype (a-d) in the top panel matches with the labeling (a-d) in the bottom table. The top panel shows the phenotype of (a) multi-septated, (b) elongated, (c) bulb shape, (d) normal rod shape. Bar, 5 μm. An average of two independent experiments was presented. 300 cells were counted in each experiment to determine the phenotypes. (C) (a) Panel shows a cell of unequal nuclear division and a cell with a nucleus not in the center. (b) Panel shows an anucleated cell. (c) Panel shows the elongated cells with multiple nuclei. Bar, 5 μm.

induced in the absence of thiamine (Basi *et al.*, 1993; Maundrell *et al.*, 1993). Compared with the *hda1*$^+$ over-expression phenotypes by pRM41 (described previously), the phenotypes by over-expression in pRep1 are consistent and more severe. The phenotypes by over-expression in pRep81, however, are less severe than that in pRM41 (data not shown). These differential phenotypes by the differential expression of

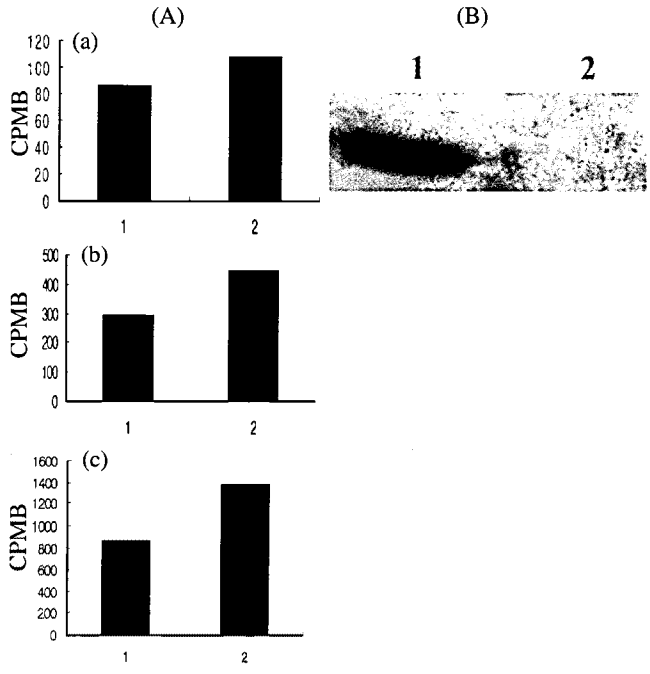


Fig. 3. Expression and histone deacetylase (HDA) activity of the Hda1p.
(A) HDA activity of the Hda1p-overexpressing cells. For (a) and (b), KGY246 cells were transformed with pRM41/hda1 and His-tagged Hda1p was induced as described. Both (a) crude extracts and (b) nuclear extracts were prepared and their HDA activities were tested. In graphs (a) and (b), (1) and (2) represent a vector-only induced negative control and His-tagged Hda1p over-expressing cells, respectively. For (c) expression of the human HDAC1 was induced by adding tetracycline (Tet-off system, Clontech) in HeLa cells transfected with HDAC1-FLAG episome. In (c), the HDA activities of the nuclear extract from (1) HDAC1 uninduced and (2) HDAC1 induced cells. (B) Detection of His-tagged Hda1p by a western blot. Lane 1 represents the cells of induced His-tagged Hda1p and lane 2 represents the uninduced.

hda1$^+$ confirm that the defects in cell proliferation and cell division are due to the over-expression of *hda1*$^+$.

The decreased sporulation efficiency has been reported in the *hda1* null mutant, proposing its function on sporulation (Olsson *et al.*, 1998). We could not investigate the effects of the *hda1*$^+$ over-expression on sporulation, since over-expression of *hda1*$^+$ led to defects in the cell morphology and cell division. Since the *S. pombe* cells should be in the G1 stage of the cell cycle for proper conjugation and sporulation, the analysis of sporulation efficiency in cells with cell cycle defects could be meaningless.

HDA activity of Hda1p The over-expression phenotypes of Hda1p suggest an increased activity of histone deacetylase. To confirm this, we measured the histone deacetylase activity of Hda1p over-expressing cells. We over-expressed Hda1p as a His-tagged protein (approximately 100 to 200-fold induction in transcription (Basi *et al.*, 1993)) and detected it by a

western blot with anti-His antibodies (Fig. 3B). The over-expression of the Hda1p was verified by phenotypes as well (data not shown). When compared to that of the wild type cells, the histone deacetylase activity of *hda1* over-expressing cells increased approximately 20% in crude extracts and 50% in nuclear extracts, respectively (Fig. 3A-a, b). Similarly, when the human *HDAC1* was over-expressed in HeLa cells by the Tet-off system (100 to 1000-fold induction in transcription (Yin *et al.*, 1996)) as a control, the histone deacetylase activity increased about 60% in the nuclear extracts (Fig. 3A-c).

Possible functions of Hda1p Three putative histone deacetylase genes have been reported in the *S. pombe*: *hda1*, *clr3*, and the new ORF (accession no. AL023589) (Grewal *et al.*, 1998; Kim *et al.*, 1998; Olsson *et al.*, 1998). However, the biochemical activity of the histone deacetylase was only verified in *hda1*. The *clr3* is phylogenetically homologous to the *HDA1* of *S. cerevisiae* and the new ORF is to *RPD3* of *S. cerevisiae* (65% identity in 396 amino acid overlap), respectively (Altschul *et al.*, 1997). A sequence comparison of the *S. pombe hda1*$^+$ with other putative histone deacetylases indicated that the *hda1*$^+$ phylogenetically belongs to the *HOS2* of *S. cerevisiae*, a putative histone deacetylase of unknown function. The *hda1*$^+$ only shows a partial homology to the well-known histone deacetylase subclasses, *RPD3* and *HDA1*. This suggests that it may function in cellular processes distinct from those of *RPD3* and *HDA1* (Altschul *et al.*, 1997; Leipe and Landsman, 1997).

Cells lacking *hda1*$^+$ displayed a reduced sporulation and repression of marker genes, but no obvious defects were observed in the mitotic cell cycle and chromosome segregation (Kim *et al.*, 1998; Olsson *et al.*, 1998). However, the exposure of fission yeast cells to TSA, a specific inhibitor of HDA, caused chromosome loss and structural changes of centromeres (Ekwell *et al.*, 1997). These results suggest that histone deacetylase activity is required for proper cell division, but is genetically redundant and partially overlapped in the fission yeast *S. pombe*. In this study, we showed that the over-expression of *hda1*$^+$ in the fission yeast prevented cell proliferation and caused defects in morphology, proper nuclear division, and cytokinesis (Fig. 2). Consistent with the over-expression phenotypes, the histone deacetylase activity increased in the *hda1*$^+$ over-expressing fission yeast cells as well as in the *HDAC1* over-expressing HeLa cells.

The *hda1*$^+$ over-expression phenotypes imply that *hda1*$^+$ may have a function in the control of cell division and cytoskeleton. These *hda1*$^+$ over-expression phenotypes are consistent with the former report that inhibition of histone deacetylase activities with Trichostatin A (TSA) in fission yeast caused chromosome loss (Ekwell *et al.*, 1997). Regulatory proteins for cell cycle and cytoskeleton could be direct targets of Hda1p for deacetylation or Hda1p might affect these proteins by controlling their gene expressions. The *hda1*$^+$ over-expressing cells with mislocated, or missegregated nuclei, strongly suggest defects in the functions of microtubules. The reversible acetylation of alpha-tubulin has been reported in *Chlamydomonas* and *Xenopus* embryos, but the exact function of this modification is still unclear (Maruta, 1986; MacRAE, 1997). The tubulins of yeast, including *S. pombe*, are the few that do not contain a conserved acetylation site L40. Therefore, these phenotypes might not be due to the direct deacetylation of tubulins. The identification of other target proteins that are regulated by reversible acetylation, as well as the investigation of the genes, whose transcriptions are regulated by *hda1*, will help to understand the exact function of *hda1* in the cell division of fission yeast.

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