Diminishing HDACs by drugs or mutations promotes normal or abnormal sister chromatid separation by affecting APC/C and adherin

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Accepted 17 January 2008
Journal of Cell Science 121, 1107-1118 Published by The Company of Biologists 2008
doi:10.1242/jcs.024224

Summary

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) play important roles in cell regulation, including cell cycle progression, although their precise role in mitotic progression remains elusive. To address this issue, the effects of HDAC inhibition were examined upon a variety of mitotic mutants of the fission yeast *Schizosaccharomyces pombe*, which contains three HDACs that are sensitive to trichostatin A (TSA) and are similar to human HDACs. Here it is shown that HDACs are implicated in sister chromatid cohesion and separation. A mutant of the cohesin loader Mis4 (adherin) was hypersensitive to TSA and synthetically lethal with HDAC deletion mutations. TSA treatment of *mis4* mutant cells decreased chromatin-bound cohesins in the chromosome arm region. By contrast, HDAC inhibitors and *clr6* HDAC mutations rescued temperature sensitive (ts) phenotypes of the mutants of the ubiquitin ligase complex anaphase-promoting complex/cyclosome (APC/C), which display metaphase arrest. This suppression coincided with facilitated complex formation of APC/C. Moreover, our mass spectrometry analysis showed that an APC/C subunit, Cut23/APC8, is acetylated. HATs and HDACs might directly target adherin and APC/C to ensure proper chromosome segregation, and anti-tumour effects of HDAC inhibitors could be attributed to this deregulation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/7/1107/DC1

Key words: HDAC, Acetylation, Mitosis, APC, Cyclosome, Cohesion

Introduction

Two classes of enzymes control protein acetylation. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) target lysine residues in the N-terminal tails of histones. Most HATs and HDACs have been found in transcriptional factor complexes and, therefore, have been considered to regulate transcription by modulating acetylation levels of the chromatin (Kouzarides, 1999; Kuo and Allis, 1998). Acetylation may be a major protein modification in cell regulations rivalling phosphorylation (Kouzarides, 2000). Acetylation plays a role in the cell cycle, based on studies identifying the biological function of HATs and HDACs. Budding yeast Esa1, a MYST (MOZ, Ybf/Sas3, Sas2, Tip60) family HAT, is essential for cell viability, and its mutation results in G2/M phase arrest (Clarke et al., 1999). Mammalian TAF250 and its budding yeast homologue Taf145, the largest subunits of the TFIID complex having HAT activity, are required for G1 phase progression (Suzuki-Yagawa et al., 1997; Walker et al., 1997). The importance of HDACs for cell cycle progression was suggested by studies on the effects of HDAC inhibitors on cell proliferation (Johnstone and Licht, 2003; Marks et al., 2001). Low concentrations of trichostatin A (TSA), a potent HDAC inhibitor, are able to arrest mammalian cells at the G1 and G2 phases (Noh and Lee, 2003; Yoshida et al., 1990). Consistently, recent analyses by siRNA and targeted disruption demonstrated that class I HDACs are required for cell proliferation in carcinoma cells and mouse embryos (Glaser et al., 2003; Lagger et al., 2002). However, the molecular mechanisms for cell cycle arrest induced by HDAC inhibition are not well understood.

HDACs form a large protein family and are categorized into three classes according to sequence and structural similarities to budding yeast HDACs; Class I and Class II HDACs are similar to *Saccharomyces cerevisiae* Rpd3p and Hda1p, respectively, whereas newly discovered Class III HDACs are the Sir2-like NAD-dependent HDACs thought to be involved in regulating cellular NAD levels and life span regulation. Fission yeast *S. pombe* has three Class I and II HDACs, Clr6, Clr3 and Hos2/Phd1/Hda1, that are sensitive to TSA and regulate histone acetylation levels in the genome, cooperating with Class III HDACs (Bjerling et al., 2002; Kim, Y. et al., 1998; Olsson et al., 1998; Wiren et al., 2005). Mutants of *clr3* and *clr6* were originally identified as defective in silencing of the mating type locus (Ekwall and Ruusala, 1994; Grewal et al., 1998; Thon et al., 1994). Currently, it has been established that HDACs have an important role in heterochromatin formation and maintenance. Culture of *S. pombe* cells for several rounds of

doubling in the presence of TSA causes disruption of centromeric heterochromatin as well as an increased rate of chromosome loss (Ekwall et al., 1997; Olsson et al., 1999). A similar result was obtained for higher eukaryotic cells (Taddei et al., 2001). Furthermore, several lines of evidence suggest that HDACs are implicated in chromosome segregation. Clr6 is the sole essential HDAC for cell viability in fission yeast, and *clr6-1* mutants show abnormal chromosome segregation with lagging chromosomes (Grewal et al., 1998; Nakayama et al., 2003; Silverstein et al., 2003). TSA-treated mammalian cells also show chromosome segregation defects and prolonged mitotic delay (Cimini et al., 2003; Taddei et al., 2001). Centromeric heterochromatin is considered to be important for centromere formation; therefore, mitotic defects caused by HDAC inhibition may be attributable to centromere dysfunction. However, it was recently shown that a human Class I HDAC, HDAC3 modulates Aurora B kinase function in mitosis by deacetylating and converting histone H3s into preferred substrates (Li et al., 2006), implying that HDACs might have a further role in mitotic progression, independent of transcriptional regulation and heterochromatin formation.

To understand the role of HDACs in the cell cycle, especially in mitotic progression, the inhibitory effect of HDACs was studied in fission yeast *S. pombe*, a well-established model organism for cell cycle analysis. Underlying molecular mechanisms were further investigated.

Results

TSA affects cell growth in a subset of fission yeast mitotic mutants

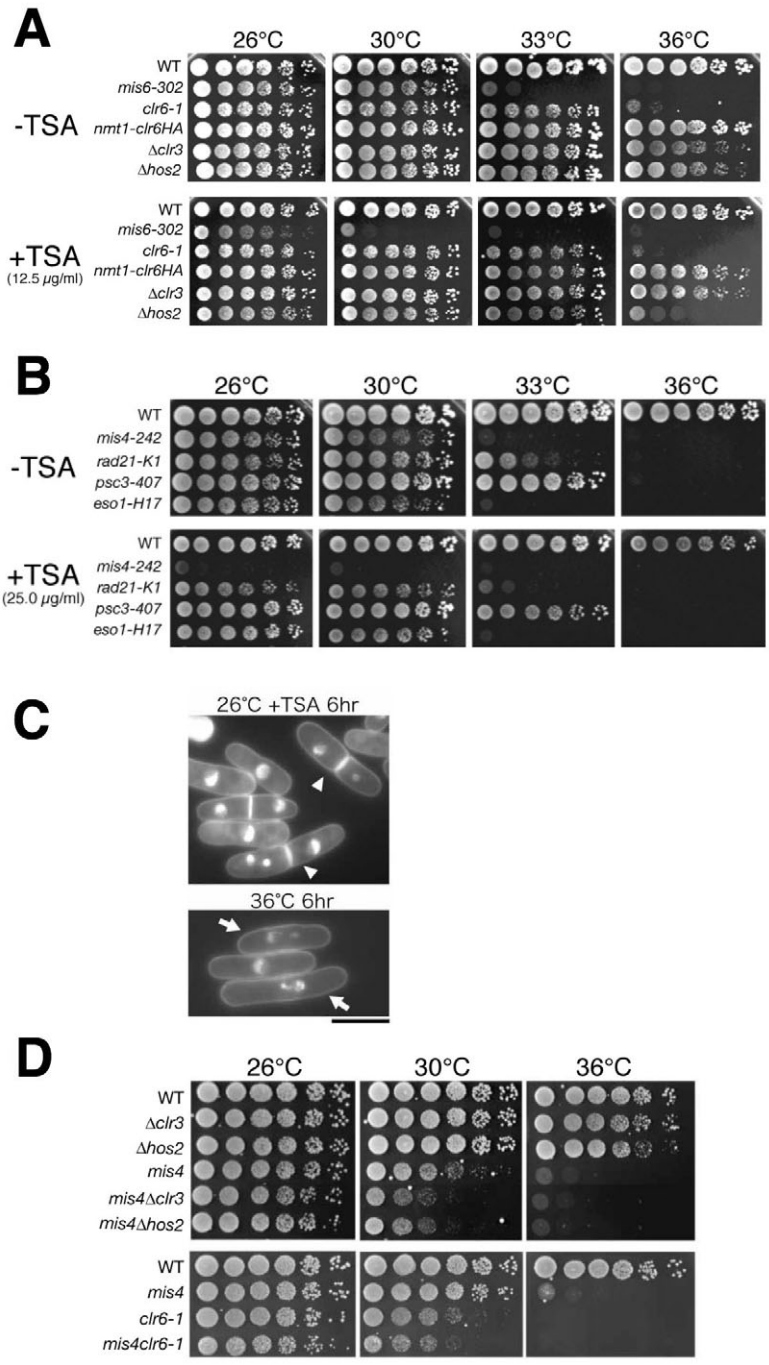
To investigate how HDACs are involved in mitotic progression, the effects of an HDAC inhibitor, TSA, were examined at the permissive temperature (26°C) on cell growth of *S. pombe* temperature-sensitive (ts) mutants defective in different aspects of mitosis (mutant strains examined are listed in Table 1). As previously shown, *S. pombe* Class I HDAC mutant *Δhos2* showed hypersensitivity to TSA (Kim, Y. et al., 1998; Olsson et al., 1998)

Table 1. The effect of TSA on various *S. pombe* mutants

Function	Homologues (human/*S. cerevisiae*)	Mutant allele	TSA sensitivity 6.25 μg/ml	TSA sensitivity 12.5 μg/ml	TSA sensitivity 25.0 μg/ml	References
APC/C subunit	Apc1	*cut4-533*	++	++	++	(Yamashita et al., 1996)
	Apc3/Cdc27	*nuc2-663*	+	+	+	(Hirano et al., 1988)
	Apc4	*cut20-100*	+	+	+	(Yamashita et al., 1999)
	Apc6/Cdc16	*cut9-665*	+	+	+	(Samejima and Yanagida, 1994)
	Apc8/Cdc23	*cut23-194*	+	+	+	(Yamashita et al., 1999)
	Apc10/Doc1	*apc10-153*	++	++	++	(Matsumura et al., 2003)
		apc10-666	++	++	++	This study
APC/C E2	UbcH10/Ubc11	*ubcP4-140*	++	++	++	(Osaka et al., 1997)
APC/C activator	Cdc20	*slp1-362*	–	–	–	(Matsumoto, 1997)
	Cdh1	*Δste9*	N	N	N	(Kitamura et al., 1998)
Spindle checkpoint	Mad2	*Δmad2*	N	–	– –	(Toyoda et al., 2002)
Proteasome subunit	S4/Yhs4	*mts2-1*	N	N	N	(Gordon et al., 1993)
	S14/Min1	*mts3-1*	N	N	N	(Gordon et al., 1996)
Separase	hSeparase/Esp1	*cut1-206*	+	+	+	(Hirano et al., 1986)
		cut1-T693	++	++	++	(Funabiki et al., 1996)
		cut1-21	+	+	+	(Funabiki et al., 1996)
Securin	hPTTG/Pds1	*cut2-364*	+	+	+	(Hirano et al., 1986)
Cohesion	NIPBL/Scc2	*mis4-242*	– –	– –	– –	(Furuya et al., 1998)
	EFO1, 2/Eco1	*eso1-H17*	N	N	N	(Tanaka et al., 2000)
Cohesin subunit	Scc1	*rad21-K1*	N	–	–	(Tatebayashi et al., 1998)
	Scc3	*psc3-407*	N	N	N	(Yuasa et al., 2004)
	Smc1	*psm1-ST273*	N	N	N	This study
	Smc3	*psm3-ST426*	N	N	N	This study
Condensin subunit	HCAP-H/Brn1	*cnd2-1*	N	N	N	(Aono et al., 2002)
	HCAP-E/Smc2	*cut14-208*	N	N	N	(Saka et al., 1994)
	HCAP-C/Smc4	*cut3-447*	N	N	N	(Saka et al., 1994)
Kinetochore component	CENP-I/Ctf3	*mis6-302*	– –	– –	– –	(Saitoh et al., 1997)
	hMis12/Mtw1	*mis12-537*	N	N	N	(Goshima et al., 1999)
	Cenp-A/Cse4	*cnp1-1*	N	N	N	(Takahashi et al., 2000)
HDACs	HDAC1, 2/Rpd3	*clr6-1*	N	N	N/–	(Grewal et al., 1998)
	HDAC1, 2/Rpd3	*nmt1-clr6HA*	N	N	N	This study
	HDAC6,10?/Hda1	*Δclr3*	N	N	N	This study
	HDAC3?/Hos2	*Δhos2*	– –	– –	– –	This study

++, the growth is strongly improved; +, the growth is slightly improved; N, the growth is not affected by TSA; – –, the growth is strongly inhibited; –, the growth is slightly inhibited. The indicated temperature-sensitive mutants and null mutants grown exponentially at 26°C were diluted and spotted with wild-type cells on YPD plates containing 6.25-25.0 μg/ml TSA. Those plates were incubated at 26, 30, 33 and 36°C. Effects of TSA on the growth of those mutants were determined by comparing with the growth of the mutants in the absence of TSA, and with wild type. Names of their homologous proteins in human and *S. cerevisiae* and the references for each strain are also shown.

(Table 1 indicated by −−, and Fig. 1A shown in the presence of TSA at 36°C). A centromere/kinetochore mutant *mis6-302* was also highly sensitive to TSA at 30°C (Fig. 1A). It is known that HDACs are involved in heterochromatin formation important for centromere function; therefore, the hypersensitivity of the *mis6-302* mutant to TSA might be due to the synergistic effects of heterochromatin disruption and kinetochore defects. Here it was not further analysed. Unexpectedly, some of the mutants defective in the process of sister chromatid separation, e.g. sister chromatid cohesion and anaphase onset, showed marked responses to TSA, as shown in Table 1. So far there has been no established role for HDACs in sister chromatid separation. Hereinafter, the mechanisms underlying the effects of HDAC inhibition on these mutants are investigated in detail.



The cohesion loader mutant *mis4-242* is hypersensitive to HDAC inhibition

Two mutants defective in sister chromatid cohesion, a cohesin subunit mutant *rad21-K1* (Tatebayashi et al., 1998) and the adherin mutant *mis4-242* (Furuya et al., 1998), were sensitive to TSA, as shown in Table 1 and Fig. 1B. Whereas *rad21-K1* was only slightly sensitive to TSA, *mis4-242* was extremely hypersensitive even at the permissive temperature, 26°C. Conversely, mutants of other cohesin subunits, the cohesin establishment factor Eso1 and condensin subunits all failed to show any hypersensitivity to TSA (Table 1 and Fig. 1B). Mis4 is similar to budding yeast Scc2 and human NIPBL, which is the causal gene of Cornelia de Lange syndrome, and fly NippedB, which is implicated in gene expression for development (Dorsett, 2007). In the presence of TSA at the permissive temperature (26°C), *mis4-242* frequently revealed missegregation with unequal daughter nuclei (Fig. 1C, upper panel; indicated by arrowheads). The phenotype was different from that of *mis4-242* at the restrictive temperature (36°C) in the absence of TSA, which showed condensed chromosomes indicative of a mitotic arrest due to the spindle checkpoint activation (Fig. 1C, lower panel; indicated by arrows) (Toyoda et al., 2002).

Next, it was examined whether inhibition of any specific HDAC among the three TSA-sensitive HDACs in *S. pombe* causes the hypersensitivity of the *mis4-242* mutant to TSA. Three double mutants were constructed and examined for TSA sensitivities; *mis4-242 clr6-1*, *mis4-242 Δclr3* and *mis4-242 Δhos2*. *mis4-242* showed a certain additive effect on temperature sensitivity with *Δclr3* or *Δhos2* at 30°C, whereas single mutants *Δclr3* and *Δhos2* did not show any temperature sensitivity at all (Fig. 1D, upper panels). The *clr6-1* single mutant was temperature-sensitive, and the *mis4-242 clr6-1* double mutant showed a less additive effect, if any, on temperature sensitivity for colony formation growth than the other double mutants (Fig. 1D, lower panels).

TSA treatment decreases Mis4 protein levels and the amount of Rad21 associated with the chromosome arm region

To understand why *mis4-242* is strongly affected by TSA, the behaviour of Mis4 protein was analysed for up to 12 hours after TSA treatment at 26°C. The cell extracts of

Fig. 1. *mis4* mutants are sensitive to HDAC inhibition and show synthetic lethality with HDAC mutants. (A) Spot tests of effects of TSA on the growth of a kinetochore mutant *mis6-302* and HDAC mutants *clr6-1*, *nmt1-clr6HA*, *Δclr3*, *Δhos2* and wild type (WT). These strains were grown at 26°C and spotted at serial cell concentrations on YPD plates lacking or containing TSA (12.5 μg/ml), followed by incubation at the indicated temperatures. (B) Wild type, *mis4-242*, *rad21-K1*, *psc3-407* and *eso1-H17* mutants were spotted on YPD plates lacking or containing TSA (25.0 μg/ml). (C) *mis4-242* mutants were cultured in YPD medium containing 12.5 μg/ml TSA at 26°C (upper panel) or in YPD medium at 36°C (lower panel) for 6 hours, then fixed with glutaraldehyde and stained with DAPI. The cells showing unequal chromosome missegregation phenotypes are indicated by arrowheads, and the cells in which chromosomes were condensed because of mitotic arrest due to spindle checkpoint activation by arrows. Scale bar: 10 μm. (D) The indicated strains were grown exponentially and then spotted on YPD plates.

wild type and *mis4-242* were immunoblotted with antibodies against Rad21, Mis4, Cut9 and Cdc2. Expectedly, the levels of acetylated histones (AcH3 and AcH4) in the wild-type cells increased within 1 hour after TSA addition (Fig. 2A). In conjunction with the hypersensitivity of *mis4-242* to TSA, Mis4 protein levels were reduced in the wild-type cells and the mutant cells after TSA treatment (Fig. 2A). The amount of Mis4 protein in the mutant cells was already much lower than in wild-type cells before treatment, and it further decreased at 4 hours and became undetectable after 8 hours. In *mis4-242* mutant cells, the level of Rad21 was similar to that in wild-type cells but less phosphorylated (more abundant lower bands) (Tomonaga et al., 2000), probably due to reduced chromatin loading. Curiously, the Rad21 level increased and peaked at around 4 hours after the addition of TSA in *mis4-242* mutants. The levels of Cut9, an anaphase-promoting complex/cyclosome (APC/C) subunit, however, were not significantly affected in the presence of TSA, whereas the levels of Cdc2 (detected by PSTAIR antibody) slightly increased after the addition of TSA. mRNA levels of Mis4 were not decreased by TSA treatment, either in wild-type or *mis4-242* mutant cells (supplementary material Fig. S1). These results imply that HDAC inhibition might cause destabilization of Mis4 protein, which might account for the hypersensitivity of *mis4-242*.

Mis4 (adherin) regulates the loading of the cohesin complex onto chromosomes. To investigate whether HDAC inhibition affects this cohesin loading function of Mis4, chromatin immunoprecipitation (ChIP) of Rad21 was performed before (–) and after (+) TSA treatment, using strains containing chromosomally integrated HA-tagged *rad21*$^{+}$ gene. An APC/C subunit mutant *cut9-665* was also used to arrest cells in mitosis. TSA (12.5 μg/ml) or buffer alone was added to cultures of these strains growing exponentially at 26°C, before the temperature was shifted to 36°C. After a 4 hour incubation, the cells were fixed with 1% formaldehyde. The cell extracts were then subjected to immunoprecipitation with anti-HA antibody after genomic DNA was sheared. In the cells treated with TSA, considerable reduction in the level of chromatin-bound Rad21HA was observed in the chromosome regions *lys1* and *c83* derived from non-centromeric regions of chromosome I and II, respectively (Fig. 2B). However, Rad21HA associated with chromatin in the *cnt1* and *dg* regions located in the centromere did not significantly change after TSA treatment (Fig. 2B). A similar change in chromatin association of Rad21HA as a result of TSA treatment was also observed in *cut9-665* mutants. Importantly, the total protein level of Rad21HA in the cell extracts remained unchanged (Fig. 2B, bottom panels).

The manner of the decrease was studied more precisely. No tag wild-type and rad21HA cells were grown at 26°C, followed by the addition of 12.5 μg/ml TSA to the cultures. Samples were taken 1, 2 and 4 hours after the addition of TSA, and PCR amplification was carried out with the seven primer sets indicated in Fig. 2C. The

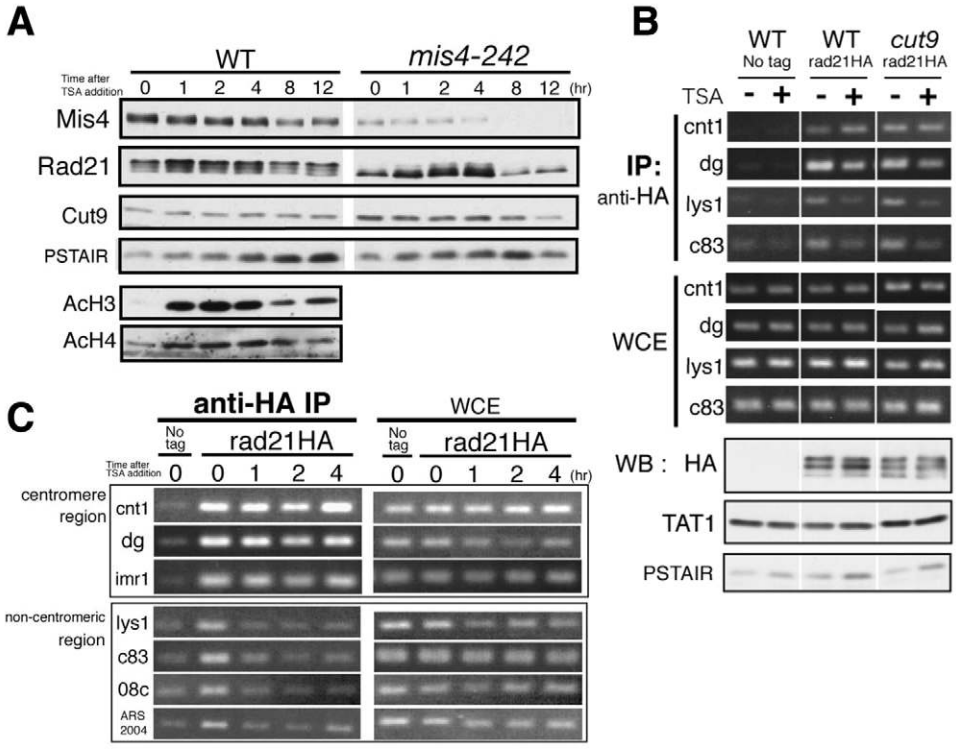


Fig. 2. TSA treatment decreases the Mis4 protein level and the chromatin-bound portion of Rad21 in the chromosome arm region. (A) Wild-type and *mis4-242* cells were cultured in YPD medium at 26°C and were harvested at the indicated time points after TSA (12.5 μg/ml) was added. Immunoblotting for the cell extracts was carried out for Mis4, Rad21, Cut9, Cdc2 (PSTAIR) and acetylated histone H3 and H4 (AcH3 and AcH4, respectively). (B) Non-tagged wild type (WT No tag), *rad21HA* integrant strains (WT rad21HA) and *cut9-665rad21HA* strains were cultured at 26°C, and then incubated at 36°C for 4 hours with or without TSA (12.5 μg/ml). ChIP for Rad21HA was performed using anti-HA antibody followed by PCR with four sets of primers (*cnt1* and *dg* for the central and outer centromere, respectively, and *lys1* and *c83* for the chromosome arm region). Immunoblotting for the whole cell extracts of the unfixed cells was also carried out using the indicated antibodies (WB). (C) Non-tagged wild-type (No tag) and *rad21HA* strains were grown exponentially at 26°C and cells were fixed at the indicated time points after TSA (12.5 μg/ml) was added to the cultures. ChIP was carried out as B using seven sets of primers (*imr1* for the central centromere on chromosome I, *08c* and *ARS2004* for the arm).

levels of Rad21 bound to the arm regions (*lys1*, *c83*, *08c* and *ARS2004*) all decreased to control levels within 1 hour after TSA treatment (Fig. 2C, lower panels). By contrast, Rad21 bound to the centromeric region (*cnt1*, *dg* and *imr1*) slightly oscillated upon TSA treatment but returned to normal levels after 4 hours (Fig. 2C, upper panels). Thus, HDAC inhibition might affect the Mis4 function of cohesin loading specifically in the chromosome arm region.

Rescue of APC/C mutants by HDAC inhibitors

In contrast to the known anti-proliferation effects on mammalian cells and the potent growth inhibition to *mis4-242*, TSA treatment greatly enhanced cell growth of the ts mutants of APC/C at the restrictive temperature (Table 1 and Fig. 3A). Every APC/C subunit mutant examined (*cut4-533*, *cut9-665*, *nuc2-663*, *cut23-194*, *cut20-100*, *apc10-153* and *apc10-666*) was rescued by TSA treatment. The suppressive effects on *cut4* and *apc10* mutants were particularly strong, as these mutants formed colonies nearly indistinguishable from wild type in the presence of TSA (12.5 μg/ml) at the restrictive temperature, 36°C (Fig. 3A). Cut4/Apc1 is the largest subunit of APC/C and is proposed to bridge two subcomplexes within APC/C, whereas Doc1/Apc10 was suggested to have a role in recognizing APC/C substrates or increasing processivity of polyubiquitination by limiting substrate dissociation (Passmore et al., 2003; Thornton et al., 2006).

Furthermore, in liquid cultures at the restrictive temperature, TSA nearly fully rescued the cellular phenotypes of *cut4-533*, and the proliferation rates were almost identical between wild type and the mutant at 36°C in the presence of TSA (Fig. 3B). In the absence of TSA, the cell proliferation of the mutant was halted at 36°C after 4 hours, with the cells showing metaphase arrest phenotypes (Fig. 3B, right top panel). Aberrant mitotic arrest phenotypes of *cut4-533* at 36°C were hardly observed in the presence of TSA (Fig. 3B, right bottom panel). Thus, TSA appears to suppress the metaphase arrest phenotypes specific to APC/C mutants.

To verify that TSA-induced suppression is due to the inhibition of the HDAC activity rather than an unknown effect of TSA, other

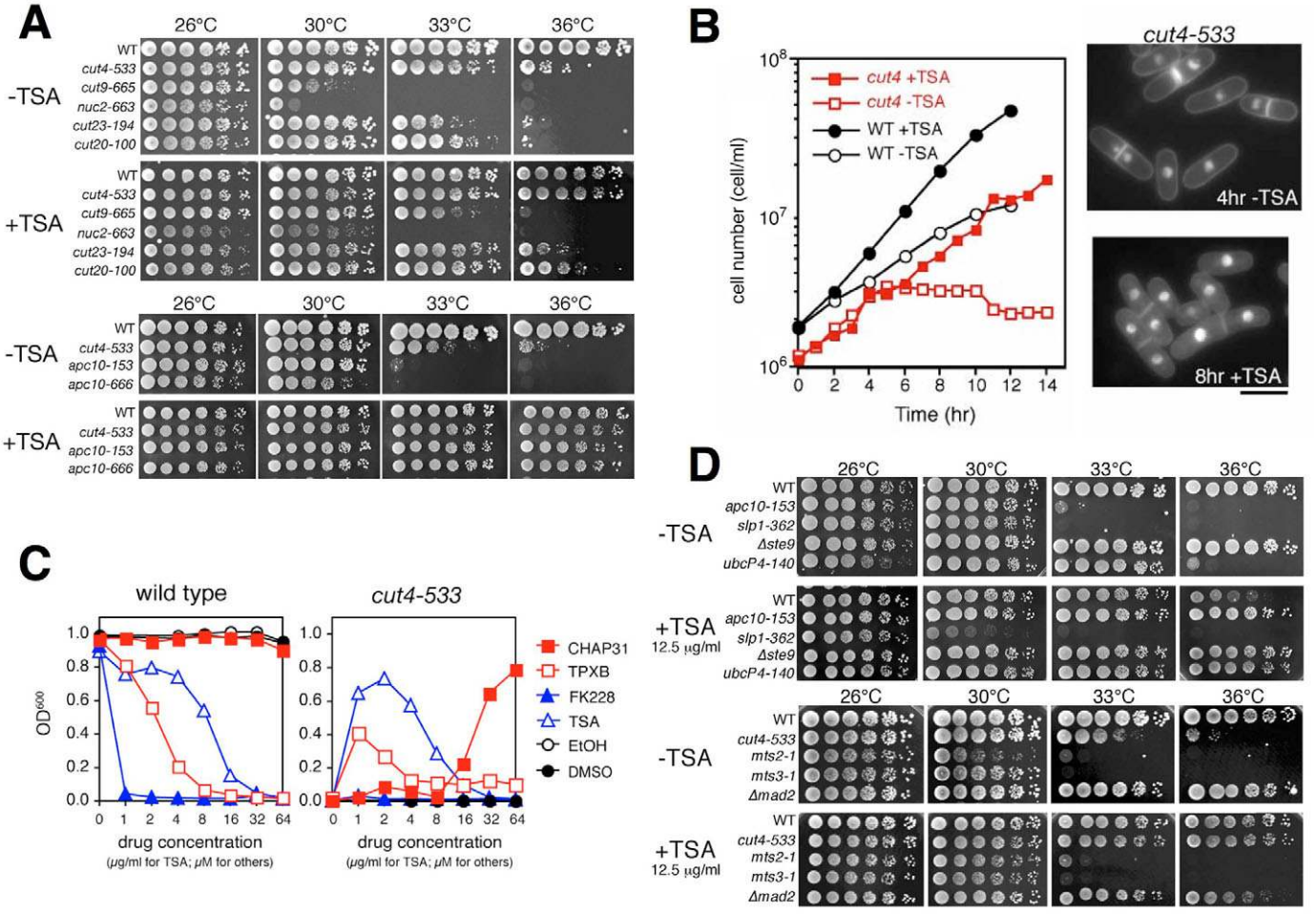


Fig. 3. Histone deacetylase inhibitors suppress temperature sensitivities of *S. pombe* APC/C mutants. (A) Wild type and APC/C subunit mutants (*cut4-533*, *cut9-665*, *nuc2-663*, *cut23-194*, *cut20-100*, *apc10-153*, *apc10-666*) were cultured at 26°C and then spotted on YPD plates lacking or containing TSA (12.5 μg/ml). (B) Wild type and *cut4-533* mutants were grown at 26°C and then shifted to 36°C (Time=0); at the same time TSA (12.5 μg/ml) was added to the cultures. Cell numbers were counted (left panel) and cytological phenotypes of *cut4-533* mutants were observed by staining with DAPI (right panel) at the indicated time points. (C) Effects of various HDAC inhibitors (TSA, CHAP31, FK228 and TPXB) on the growth of wild type and *cut4-533* mutants. Cells were cultured exponentially at 26°C, and then the temperature was shifted to 36°C after the cell numbers were adjusted to OD_{600}=0.01. HDAC inhibitors or the same volumes of the buffers (EtOH or DMSO) were added at the indicated concentrations, and cell numbers (OD_{600}) were counted after 24 hour incubation at 36°C. (D) Effects of TSA on ts or null mutants of proteins involved in the APC/C-dependent ubiquitination and proteolysis (*ubcP4-140*, *slp1-362*, Δ*ste9*, *mts2-1*, *mts3-1* and Δ*mad2*). The indicated strains were spotted on YPD plates lacking or containing TSA (12.5 μg/ml). Scale bar: 10 μm.

drugs also known to inhibit HDACs were used. A hydroxamic acid-containing peptide CHAP31, and cyclic peptides Trapoxin B (TPXB) and FK228, inhibit HDAC activity in vitro and possess anti-tumour activity (Furumai et al., 2001; Furumai et al., 2002; Kijima et al., 1993; Komatsu et al., 2001). Wild type and the *cut4-533* mutant were grown at 26°C, then shifted to 36°C in the presence of serial dilutions of these drugs. In wild type, the effects of TPXB and FK228 were stronger than that of TSA in regard to the inhibition of cell growth, whereas CHAP31 was not inhibitory at all in the range of the drug concentrations used (Fig. 3C, left panel). In *cut4-533* mutants, CHAP31, TPXB and TSA all suppressed the growth defects, with CHAP31 suppressing to the same level as TSA, confirming that the suppression effects were due to inhibition of HDACs by these drugs rather than an unknown effect of TSA (Fig. 3C, right panel). The suppression effect of FK228 was not observed at the concentrations examined, probably due to its strong toxicity.

Mutants of proteins involved in APC/C-dependent ubiquitination and proteolysis were also examined for TSA sensitivity. In agreement with the above results, the growth defect of *ubcP4-140* (Osaka et al., 1997), a mutant in the fission yeast orthologue of human UbcH10, the E2 enzyme required for the ubiquitination activity of APC/C, was also suppressed by TSA (Fig. 3D, upper panels). No significant suppression, however, was observed for *mts2-1* and *mts3-1*, ts mutants of 26S proteasome subunits, suggesting that HDAC inhibition upregulates APC/C activity rather than affects proteasome functions (Fig. 3D, lower panels). A deletion mutant of Mad2, a spindle checkpoint component known to bind the APC/C activator Slp1/Cdc20 and thus inhibit APC/C (Hwang et al., 1998; Kim, S. et al., 1998), showed hypersensitivity to TSA, which might be due to premature anaphase onset with APC/C activated by TSA treatment. A mutant of Ste9, the G1 phase-specific APC/C activator required for G1 arrest upon nitrogen starvation (Kitamura et al., 1998; Matsumoto, 1997; Yamaguchi et al., 1997), was not affected by TSA (Fig. 3D, upper panels). However, *slp1-362*, a mutant of the mitotic APC/C activator was slightly sensitive to TSA. Slp1 was suggested to have a role in mitotic entry after DNA damage checkpoint in addition to APC/C activation (Matsumoto, 1997). Thus, it is possible that TSA might affect this function of Slp1 before cells enter mitosis.

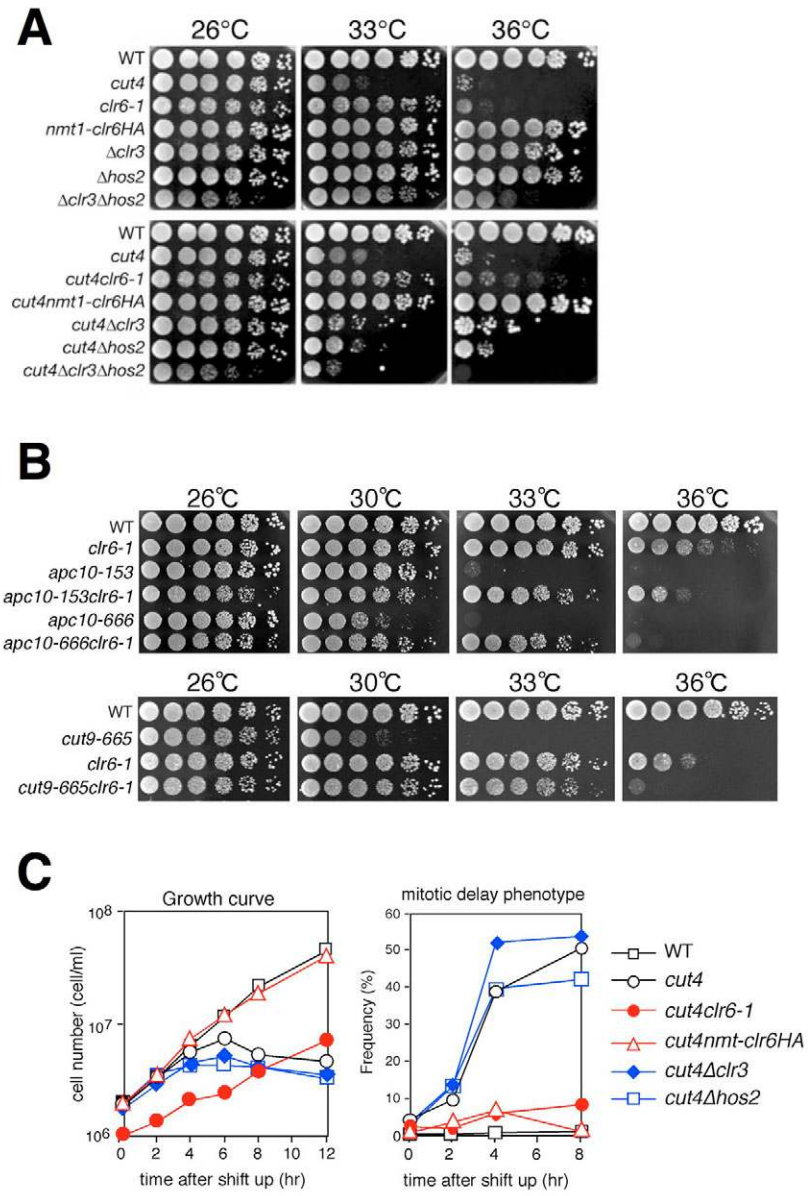


Fig. 4. Clr6 HDAC mutations suppress APC/C mutants. (A) *clr6* mutations, but not Δ*clr3* or Δ*hos2*, suppress the ts growth of *cut4-533* mutants. The indicated strains were spotted on YPD plates and incubated at the indicated temperatures. In *nmt1-clr6HA* cells, the expression of HA-tagged *clr6*$^{+}$ gene under nmt1 promoter was repressed on YPD plates. (B) The temperature sensitivities of *cut9-665*, *apc10-153* and *apc10-666* mutants were suppressed by *clr6-1* mutations. The indicated strains were spotted and incubated. (C) The growth and the frequency of mitotic arrest phenotypes of double mutants of *cut4-533* and HDAC mutants at the restrictive temperature. The indicated strains were cultured at 26°C and shifted to 36°C. The cell number (left panel) and the frequency of the mitotic delay phenotype (right panel) were measured at the indicated time points.

Suppression of APC/C mutants by Clr6 HDAC mutation

To determine which HDAC gene is implicated in the suppression of APC/C mutants, double or triple mutants with HDAC mutation or deletion were constructed. In contrast to effects seen with *mis4-242*, the double mutant *cut4-533 clr6-1* showed significantly better growth than *cut4-533* single mutants at 36°C, whereas Δ*clr3* and/or Δ*hos2* did not produce a significant suppressive effect on *cut4-533* (Fig. 4A). Another *clr6* mutant, *nmt1-clr6HA*, in which the chromosomal *clr6*$^{+}$ gene is replaced by the HA-tagged *clr6*$^{+}$ gene under the inducible *nmt1* promoter leading to repressed expression on YPD plates, almost completely rescued *cut4-533* at 36°C. The *clr6-1* mutation was also highly effective in suppressing the ts phenotypes of the two *apc10* mutants and the *cut9-665* mutant (Fig. 4B). In cytological analyses of the liquid cultures, the frequent mitotic phenotypes observed in *cut4-533* at 36°C were not observed in the double mutants *cut4-533 clr6-1* and *cut4-533 nmt1-clr6HA* (Fig. 4C). By contrast, the double mutants *cut4-533 Δclr3* and *cut4-533 Δhos2* displayed the highly frequent aberrant arrest phenotypes

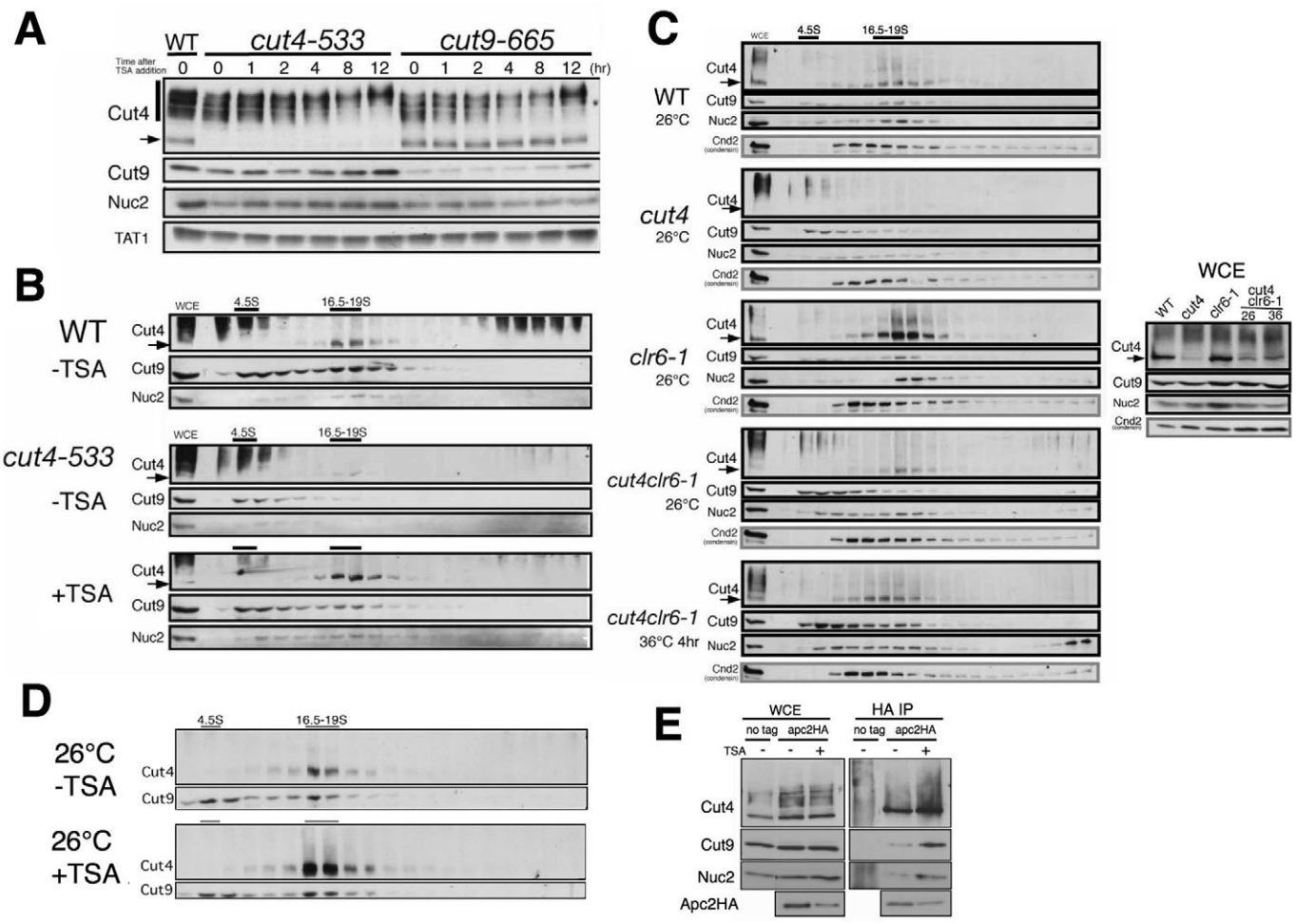


Fig. 5. Inhibition of Clr6 HDAC stimulates the formation of the 20S APC/C complex. (A) Effects of TSA treatment on cellular levels of APC/C subunit proteins. Wild type, *cut4-533* and *cut9-665* were cultured at 26°C and TSA (12.5 μg/ml) was added into the cultures. Cells were harvested at the indicated time points and the whole cell extracts were examined by imunoblotting to determine the concentrations of APC/C subunit proteins. α-tubulin was detected by TAT1 antibody as loading control. (B) Wild type and *cut4-533* mutants were cultured at 26°C and cells were harvested after 12 hour incubation in the absence or presence of 12.5 μg/ml TSA. The cell extracts were fractionated by 15-40% sucrose gradient centrifugation and APC/C subunits Cut4, Cut9 and Nuc2 were detected by immunoblotting. The fractions corresponding to 4.5S and 16.5-19S were determined using bovine serum alubmin and thyroglobulin A, respectively. (C, left panel) Sucrose gradient centrifugation was performed as in B using the cell extracts of wild-type, *cut4-533* and *cut4-533clr6-1* cells that were cultured at 26°C or 36°C for 4 hours. A condensin subunit Cnd2 was shown as a marker indicating the fraction corresponding to 13S. (C, right panel) The aliquots of the whole cell extracts of each strain were blotted. (D) Wild-type strains were cultured at 26°C and TSA (12.5 μg/ml) was added. Cells were harvested after 12 hour incubation at 26°C. The cell extract was fractionated and analysed as in B. (E) Wild type (no tag) and *apc2HA* strain were cultured at 26°C in the absence or presence of TSA (12.5 μg/ml) for 12 hours. Apc2HA protein was immunoprecipitated with anti-HA antibody. Cut4, Cut9 and Nuc2 co-immunoprecipitated with Apc2HA were detected by their specific antibodies.

seen in *cut4-533* single mutants. Therefore, it is plausible that the reduction of the Clr6 activity is responsible for the suppression of APC/C mutants by TSA treatment.

TSA treatment and *clr6* mutation enhance the assembly of 20S APC/C

These results raised a question as to how TSA could suppress the phenotype of APC/C mutants. HDACs are known to be involved in repression of gene transcription, and, accordingly, HDAC inhibition could affect protein expression. To examine the possibility that HDAC inhibition suppresses APC/C mutants via increased expression of the mutant proteins, the levels of various APC/C subunits were examined in wild type, *cut4-533* and *cut9-665*. Cell extracts were prepared from cells cultured at 26°C in the presence of TSA for 12 hours. As shown in Fig. 5A, no significant increase in the cellular concentration of Cut4 and Cut9 mutant proteins was observed in the presence of TSA. However, after TSA addition, due to unknown protein modifications the upper bands of Cut4 (Yamashita et al., 1996) migrated more slowly and the level of Cut9 protein appeared to slightly increase. Overexpression of wild-type Cut9 did not suppress the ts phenotypes in *cut4-533* (Y.K., unpublished); therefore, it is unlikely that the increase of the Cut9 protein is the chief reason for the suppression of APC/C mutants by TSA treatment.

In fission yeast, APC/C is known to sediment as a 20S particle, which was disrupted in *cut4-533*, *cut9-665* and *nuc2-663* mutant cells (Yamada et al., 1997; Yamashita et al., 1996). To examine the possibility that formation of the 20S APC/C complex might be affected by HDAC inhibition, sucrose gradient centrifugation was performed on the extracts from *cut4-533* mutants cultured in the absence or presence of TSA. In wild type, the subunits of APC/C,

Cut4, Cut9 and Nuc2, enriched in the fractions around 20S (Fig. 5B, WT –TSA). In *cut4-533* mutant cells, the levels of APC/C subunits that sedimented around 20S were much lower even at the permissive temperature 26°C in the absence of TSA; however, their levels at 20S greatly increased in the presence of TSA (Fig, 5B, *cut4-533* –TSA and +TSA). Recovery of the 20S complex occurred within 2 hours after TSA addition in *cut4-533* mutants and was also observed in *cut9-665* mutants (supplementary material Fig. S2A,B) (Y.K., unpublished). Thus, the 20S complex can re-form in APC/C mutant cells if TSA is added into cultures, suggesting that suppression of APC/C mutants by TSA treatment might be due to the restoration of APC/C complex formation.

To examine whether the *clr6-1* mutation also affects the APC/C complex formation in a manner similar to the addition of TSA, cell extracts of the *cut4-533 clr6-1* double mutant were analysed by sucrose gradient centrifugation along with three other strains, wild type, single *clr6-1* and single *cut4-533* mutant cells (Fig. 5C). Cnd2, a component of the condensin complex, was used as a sedimentation marker for the position of 13S (Sutani and Yanagida, 1997). Expectedly, the levels of Cut4, Cut9 and Nuc2 in the 20S fractions were found to greatly increase in *cut4-533clr6-1* double mutant cells cultured at both 26 and 36°C, compared with those in single *cut4-533* mutant extracts (Fig. 5C, left panels). Thus, Clr6 plays a main role in restoring the level of the 20S APC/C complex by TSA treatment. In the whole cell extracts, a slight increase in the levels of non-modified Cut4 mutant protein (indicated by the arrows) and the Cut9 protein was detected in the double mutants at both 26 and 36°C, compared to the single *cut4* mutants (Fig. 5C, right panels). APC/C subunit proteins might therefore be stabilized when the 20S complex is properly assembled.

It was also asked whether the wild-type APC/C complex is affected by TSA. Cell extracts prepared from wild-type cells cultured in the presence or absence of TSA were examined using sucrose gradient centrifugation. As shown in Fig. 5D, the 20S peak of the APC/C subunits was observed in both extracts, and the Cut4 band was more intense in the presence of TSA. To confirm that these APC/C subunits actually form the complex, immunoprecipitation was performed using the strain containing chromosomally integrated *apc2*$^{+}$ gene C-terminally tagged with 3HA (apc2HA). As shown in Fig. 5E, more Cut4, Cut9 and Nuc2 proteins were indeed co-precipitated with Apc2HA in the presence of TSA. Thus, HDAC inhibition appears to enhance APC/C assembly in wild-type cells as well as in APC/C mutant cells.

Cut23, a subunit of APC/C, is acetylated

The above results hinted that the ability of APC/C subunits to assemble into the complex might be regulated by acetylation-deacetylation cycle of APC/C subunits. To examine this possibility, 6His-tagged APC/C subunits (Cut4, Apc2, Nuc2, Cut9, Cut23, Apc10, Apc11 and Hcn1) were overexpressed in wild-type cells grown in the presence or absence of TSA, then purified by nickel beads under denaturing conditions and immunoblotted by anti-acetyl-lysine antibodies. No change in acetyl-lysine levels was observed in the presence of TSA for any of the proteins analysed (Y.K. and A.M., unpublished). The antibodies used in this experiment were raised against acetylated histones or bovine serum

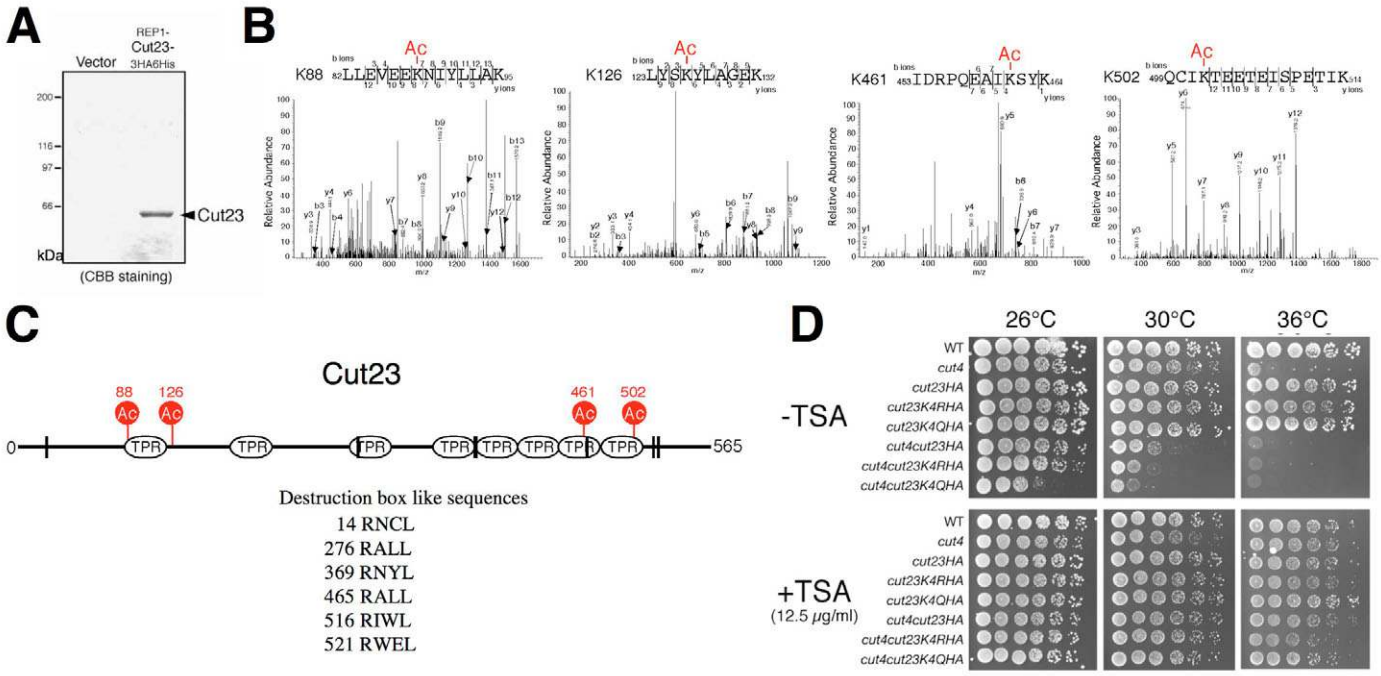


Fig. 6. An APC/C subunit Cut23 is acetylated in vivo. (A) 3HA6His-tagged Cut23 overproduced from pREP1 plasmid in wild type was purified using nickel beads under denaturing conditions and was separated by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. (B) The band of purified Cut23-3HA6His shown in (A) (~64 kDa) was cut out and subjected to survey of the acetylation site using LC-MS/MS after trypsin digestion (Ohta et al., 2002). The MS/MS spectra of the tryptic acetylated peptide amino acids 82-95, 123-132, 499-502 of Cut23 obtained by collision-induced dissociation of the $[M + 2H]^{2+}$ or $[M + 3H]^{3+}$ precursor ions, *m/z* 858.55, *m/z* 606.87, *m/z* 496.91, *m/z* 946.02. Each peptide has one possible acetylated lysine residue, K88 in 82-95, K126 in 123-132, K461 in 453-464 or K502 in 499-514. Peptides containing unmodified lysine residues were also detected for each region and these four acetylated peptides were detected in two independent experiments. (C) Localization of the four putative acetylated sites in Cut23 protein. The acetylation sites (Ac) and TPR domains are shown in the diagram. Six destruction box-like sequences (RxxL) in Cut23 are also shown as black bars. (D) Spot tests for indicated strains on YPD plates in the absence or the presence of 12.5 μg/ml TSA.

albumins so that they might not be able to detect acetylated APC/C subunits. Therefore, mass spectrometry was used to analyse individual APC/C subunits. 3HA6His-tagged APC/C subunits were overexpressed in wild-type cells grown in the presence of TSA and purified by nickel beads under denaturing conditions. Cut23/APC8 was found to be acetylated. The purified Cut23 protein was separated by SDS-PAGE and stained with Coomassie Blue (Fig. 6A), and the band corresponding to the Cut23 subunit was cut out and digested by trypsin. By liquid chromatography and tandem mass spectrometry (LC-MS/MS) analyses (Ohta et al., 2002), four putatively acetylated peptides were identified in Cut23 protein (Fig. 6B). Among the four acetylation sites in Cut23, only the K461 residue is conserved from yeast to human. Cut23 contains six putative destruction box sequences (RxxL), which are known as substrate recognition motifs for APC/C, and one of them (^{465}RALL) is close to the conserved acetylated sequence (Fig. 6C). Three of the four putative acetylation residues, including K461, were located within the tetratricopeptide repeat (TPR) domain of Cut23 involved in protein-protein interaction (Fig. 6C). To investigate the functions of these acetylation sites, strains that contain HA-tagged Cut23 with all four sites mutated to arginine or glutamine (*cut23K4RHA* and *cut23K4QHA*, respectively) were constructed, as well as a strain carrying HA-tagged wild-type Cut23 (*cut23HA*). C-terminal HA-tagging alone partially affects the function of Cut23 protein, as the ts growth defect of *cut4-533* was increased when it was combined with *cut23HA* (Fig. 6D, –TSA). Notably, the growth of *cut4-533cut23K4RHA* and *cut4-533cut23K4QHA* was even slower than *cut4-533cut23HA* at 26 and 30°C, implying that the acetylation sites have some role in Cut23 functions. However, temperature sensitivity of all double mutants was suppressed by TSA treatment or *clr6-1* mutation (Fig. 6D) (Y.K., unpublished). Therefore, the four acetylation sites in the Cut23 protein are not essential for suppression of APC/C mutants by HDAC inhibition, at least not on their own. As yet unidentified acetylation sites on Cut23 and/or other APC/C subunits might therefore be important for the formation of APC/C.

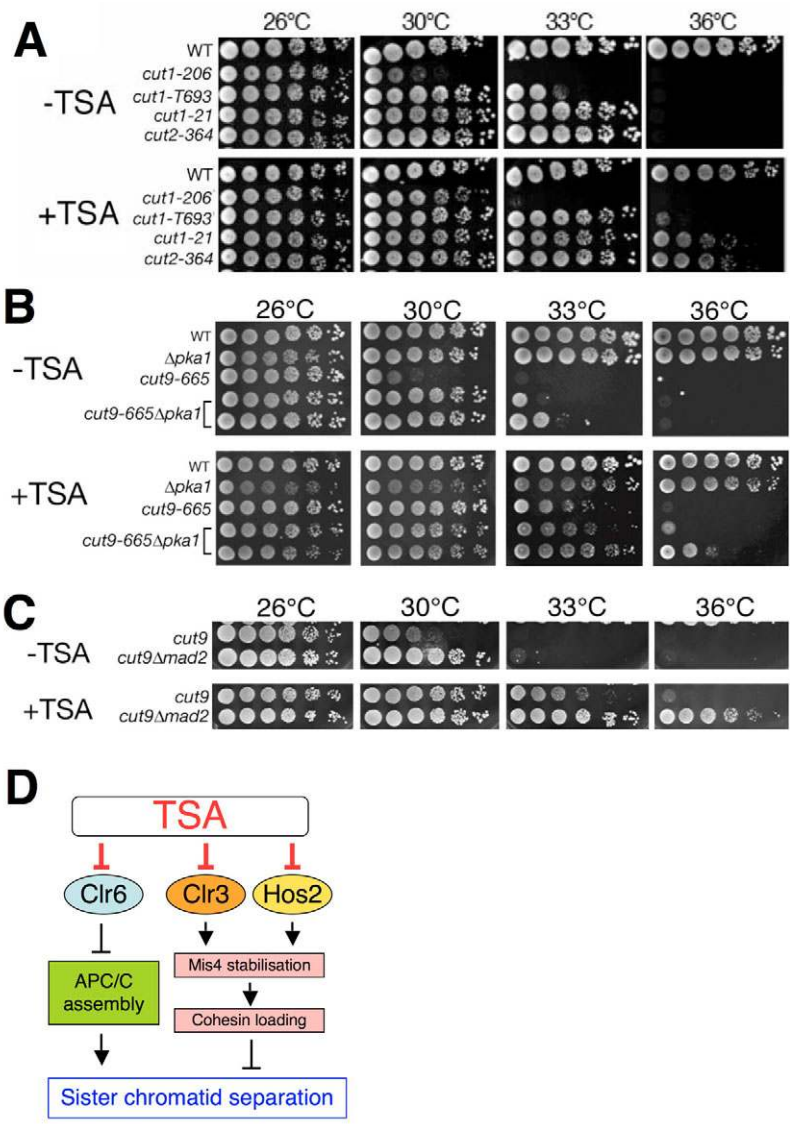


Fig. 7. HDAC inhibition facilitates sister chromatid separation in *S. pombe* mutants. (A) ts mutants of *S. pombe* separase Cut1 and securin Cut2 were suppressed by TSA. *cut1-206*, *cut1-T693*, *cut1-21* and *cut2-364* growing exponentially at 26°C were spotted on YPD plates lacking or containing TSA (12.5 μg/ml), and then incubated at 26, 30, 33 and 36°C. (B) Wild type, *cut9-665*, *Δpka1* and *cut9-665Δpka1* mutants grown exponentially were diluted and spotted on YPD plates lacking or containing TSA (12.5 μg/m). (C) *cut9-665* and *cut9-665Δmad2* stains were spotted as B. *cut9-665Δmad2* double mutants grew better than the *cut9-665* single mutants; however, the temperature sensitivities were further suppressed in the presence of TSA. (D) Results presented in the present study are summarized. Clr6, Clr3 and Hos2 are three HDACs that are sensitive to TSA. Clr6 inhibition facilitates the assembly of APC/C ubiquitin ligase complex in the mutant cells. Inhibition of Clr3 and Hos2 deteriorates chromatin loading of cohesin complex through Mis4 destablisation. In both cases, sister chromatid separation is promoted.

HDAC inhibition facilitates sister chromatid separation

At the onset of anaphase Cut2/Securin is polyubiquitinated for destruction by APC/C allowing activation of Cut1/Separase, which in turn cleaves cohesin subunits. If HDAC inhibition causes the facilitation of anaphase according to our hypothesis, it is expected that *cut1* and *cut2* mutants defective in the activation of Cut1 and sister chromatid separation (Funabiki et al., 1996; Kumada et al., 1998) would also be suppressed by TSA. Consistently, the ts phenotypes of separase mutants, *cut1-206*, *cut1-T693* and *cut1-21*, and the securin mutant, *cut2-364*, were all partially rescued in the presence of TSA (Fig. 7A). Mitotic phenotypes of *cut1-206* in the liquid culture at the restrictive temperature were significantly suppressed by the addition of 12.5 μg/ml TSA (K.N., unpublished).

The spindle checkpoint and the cAMP/PKA pathway are known to negatively regulate APC/C (Kim, S. et al., 1998; Yamashita et al., 1996). We then examined whether suppression of APC/C mutants by TSA requires the presence of cyclic AMP-dependent protein kinase Pka1 and/or spindle checkpoint protein Mad2. Suppression took place in the double mutants *cut9 Δpka1* and *cut9 Δmad2* in the presence of TSA (Fig. 7B,C), indicating that neither PKA or Mad2 pathways were essential for the suppression.

Discussion

HDAC inhibitors are a promising class of anticancer agents (Bolden et al., 2006; Johnstone and Licht, 2003; Yoshida et al., 2003). However, our understanding of how HDAC inhibitors act within cells and why different cell types respond in different ways is limited. We employed the fission yeast *S. pombe* as a model, because this organism is sensitive to HDAC inhibitors and contains a set of HDAC genes similar to those of mammalian organisms. In the present study, we focused on the mitotic functions of three TSA-sensitive HDACs in fission yeast. Our results suggest that Clr6 negatively regulates APC/C independently of the PKA pathway and Mad2. By contrast, Mis4, the cohesin loader, is positively controlled by HDACs. HDAC inhibitors thus reduce the level of Mis4 and facilitate the exit from mitosis via the assembly of APC/C complex, leading to the direction of sister chromatid separation in dividing cells (Fig. 7D).

Given the known anti-proliferation effects of HDAC inhibitors on tumour cells, our result in *S. pombe* is surprising, as APC/C mutants could produce colonies by TSA treatment, *clr6-1* ts mutation or repression of Clr6 expression. Three features are prominent for this suppression of APC/C mutants by HDAC inhibition. The first is the universality of suppression: all the APC/C mutants examined were suppressible by the drugs used. In addition, *ubcP4-140*, the mutant of APC/C-specific E2, was also suppressed. The effect of TSA and *clr6-1* hence is confined to ubiquitination, as 26S proteasome subunit mutants are not suppressed. The second is that Clr6, rather than Hos2 and Clr3, appears to be the target of HDAC inhibition for the suppression. The third is that HDAC inhibition apparently affects the level of assembled APC/C rather than the expression of individual APC/C subunits. Restoration of the 20S APC/C complex seems to correspond to the recovery of APC/C activity. Similar correlation between the ts suppression and the 20S APC/C assembly was reported in the suppression of APC/C mutant *cut4-533* via the inactivation of the cAMP/PKA pathway (Yamashita et al., 1996). However, the suppression of APC/C mutants by TSA or *clr6* mutation required neither Pka1 or Mad2, thus the effect of HDAC Clr6 on APC/C seems to occur through a mechanism independent of the cAMP/PKA pathway and the spindle checkpoint. In any case, the use of HDAC inhibitors does not necessarily cause the arrest of the cell proliferation in *S. pombe*. In certain mutants described in this study, HDAC inhibitors appear to facilitate the exit from mitosis and so normal cell division.

The additive lethality of the cohesin loader mutant *mis4-242* by HDAC inhibition is interpreted as a result of the diminished levels of Mis4 under HDAC inhibition and so the stability of Mis4 may be under the positive regulation of HDACs. TSA treatment results in strong growth inhibition in *mis4-242* but not in the mutants of cohesin subunits and the establishment factor Eso1. Mis4 or its partner molecule are possibly regulated by direct acetylation for the stability of Mis4. Curiously, TSA treatment decreases the chromatin-bound cohesin subunit Rad21, especially in the non-centromeric regions. The level of centromeric Rad21 only slightly decreases 2 hours after TSA treatment and recovers to a normal level by 4 hours. Mis4 or budding yeast adherin Scc2p are required for cohesin loading on both centromere and arm regions (Ciosk et al., 2000; Tomonaga et al., 2000) and localizes to the entire chromatin region in a punctate manner (Furuya et al., 1998). However, the chromatin localization of Mis4-GFP was not affected by TSA treatment (Y.K., unpublished). We have no obvious explanation for this arm-specific cohesin unloading promoted by HDAC inhibition. Thus, HDAC inhibition seems to affect only a part of the Mis4 functions.

TSA treatment of *S. pombe* cells leads to disruption of heterochromatin, which in turn causes lagging chromosomes and increased rates of chromosome loss (Ekwall et al., 1997). However, in our experimental conditions described (6.25~25.0 μg/ml TSA, for 12 hours), no significant chromosome segregation phenotype was observed in wild-type cells, implying that the centromeric heterochromatin was not strongly affected. Consistently, the levels of Rad21 bound to the centromeric heterochromatic region (dg), recruited by Swi6/HP1 (Bernard et al., 2001; Nonaka et al., 2002), were not significantly changed after TSA treatment (Fig. 2B,C). Strong effects of HDAC inhibition are detected only in the mutant strains. The converse is seen in APC/C and *mis4* mutants, where sister chromatid separation is facilitated. For APC/C mutants, the drug promotes apparently normal sister chromatid separation and viable cell division; however, for Mis4 mutant cells, sister chromatid separation is premature and consequently becomes lethal. Thus, in *S. pombe*, HDAC inhibitors can be inhibitory or enhancing for cell proliferation under different genetic backgrounds, whereas the drug promotes sister chromatid separation via different HDACS. This variation of effects might be applicable for human cells and should be taken into consideration when the effects of HDAC inhibitors on different types of tumour are assessed.

Materials and Methods

Strains, media and TSA sensitivity test

All the mutant strains used have the genetic background of h^- *leu1-32*. The strain *apc2HA::LEU2* was constructed by transforming wild-type cells with pYC11-ΔNapc2-3HAHis6 plasmid, which contains LEU2 gene as a selection marker and the C-terminus of fission yeast *apc2*$^+$ gene (from an internal *Nhe*I restriction site) followed by the sequences coding 3HAHis6 tag at its 3′ end. The strains *nmt1-clr6HA*, Δ*clr3* and Δ*hos2* were constructed by a PCR-based method slightly modified from Bähler et al. (Bähler et al., 1998). *apc10-666* was isolated as a ts mutant showing metaphase arrest from the ts library described in Yamashita et al. (Yamashita et al., 1999). Strains *psm1-ST273* and *psm3-ST426* were isolated through screening mutants resistant to overexpression of Cut2 central fragments (Nagao and Yanagida, 2006). To examine TSA sensitivity, cells growing exponentially at 26°C were diluted with EMM2 medium and spotted at a series of concentrations: 5×10^4, 1×10^4, 2×10^3, 4×10^2, 80 and 16 cells per spot, on YPD plates containing TSA (6.25-25 μg/ml).

Antibodies

Anti-acetylated histone H3 and H4 antibodies (upstate #06-599 and #06-598, respectively), and anti-HA antibody (12CA5, Roche) were used for immunoblotting and immunoprecipitation. Polyclonal antibodies specific for Cut4, Cut9, Nuc2, Cnd2, Mis4 and Rad21 were used as previously described (Aono et al., 2002; Tomonaga et al., 2000; Yamashita et al., 1996). Anti-PSTAIR and TAT-1 monoclonal antibodies are gifts from Dr Y. Nagahama and Dr K. Gull, respectively.

Sucrose gradient centrifugation and ChIP

Sucrose gradient centrifugation was performed as previously described (Yamashita et al., 1996). ChIP of Rad21HA was performed following the previously described procedure (Tomonaga et al., 2000). The primer sets for *cnt1*, *dg* and *lys1* are the same as those previously used (Saitoh et al., 1997; Tomonaga et al., 2000). The primers used for *c83*, *08c* and *ARS2004* were newly synthesized to amplify the non-coding region on the arm of chromosome II corresponding to the cosmid SPBC83, 30244-30440, the coding region of the *SPBC28F2.08c* gene and the non-coding region in the *ARS2004* on the arm of chromosome II, respectively.

Purification of Cut23 protein and mass spectrometry analysis

To purify Cut23 protein from fission yeast cells, Cut23-3HAHis6 was overexpressed by the pREP1 vector in the presence or absence of 12.5 μg/ml TSA. The 2×10^9 cells were collected and disrupted by glass beads in 1 ml NG8 buffer (10 mM Tris-HCl at pH 8.0, containing 100 mM Na-phosphate, 50 mM NaCl, 6 M guanidine hydrochloride and 0.1% NP-40). The extracts were centrifuged at 14 000 rpm for 15 minutes, and 100 μl of Ni-NTA beads (QIAGEN) were added to the supernatant. After incubation for 2 hours at room temperature, the beads were washed twice with NG8 buffer, twice with NG7 (pH 7.0) and then twice

with buffer N8 (as NG8 but without guanidine hydrochloride). The beads were boiled in SDS sample buffer after the addition of 100 mM EDTA. Mass spectrometry analysis using in-gel digested peptides was performed as described in Ohta et al. (Ohta et al., 2002).

We thank Dr A. Klar for *clr6-1* strain, Drs Y. Nagahama and K. Gull for the antibodies and Drs H. Yamano, C. Goding, A. McAinsh, M. Trickey and S. McClelland for reading the manuscript and discussion. This study was supported by the CREST Research Project of the Japan Science and Technology Corporation (JST), the COE Research Unit of the Ministry of Education, Culture, Sports, Science and Technology, and Marie Curie Cancer Care.

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