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Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression

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Histone deacetylases (HDACs) modulate chromatin structure and transcription, but little is known about their function in mammalian development. HDAC1 was implicated previously in the repression of genes required for cell proliferation and differentiation. Here we show that targeted disruption of both HDAC1 alleles results in embryonic lethality before E10.5 due to severe proliferation defects and retardation in development. HDAC1-deficient embryonic stem cells show reduced proliferation rates, which correlate with decreased cyclin-associated kinase activities and elevated levels of the cyclin-dependent kinase inhibitors p21$^{WAF1/CIP1}$ and p27^{KIP1}. Similarly, expression of p21 and p27 is up-regulated in HDAC1-null embryos. In addition, loss of HDAC1 leads to significantly reduced overall deacetylase activity, hyperacetylation of a subset of histones H3 and H4 and concomitant changes in other histone modifications. The expression of HDAC2 and HDAC3 is induced in HDAC1-deficient cells, but cannot compensate for loss of the enzyme, suggesting a unique function for HDAC1. Our study provides the first evidence that a histone deacetylase is essential for unrestricted cell proliferation by repressing the expression of selective cell cycle inhibitors.

Keywords: CDK inhibitors/chromatin/development/histone acetylation/proliferation

Introduction

Controlled gene expression and cell proliferation are essential for all organisms to ensure their integrity and survival. During development from the fertilized egg to a multicellular organism, cell fate decisions are taken and cell lineage or tissue-specific gene expression patterns have to be established and maintained. For a long time, studies aimed at elucidating the mechanisms of transcriptional activation and repression sought to determine how DNA-binding proteins could influence the initiation and elongation of transcription by RNA polymerases. Recently, it has become clear that alteration of gene expression has to occur in the context of chromatin.

The nucleosome as the basic unit of chromatin consists of ~146 bp of DNA wrapped around a histone octamer made up of two copies each of four core histones H2A, H2B, H3 and H4 (van Holde, 1988). While the structure of the core nucleosome is well defined, the basic N-terminal histone tails protrude from the core nucleosome and show no defined structure (Luger *et al*., 1997). These tail domains are subject to post-translational modifications such as acetylation, phosphorylation, methylation and ADP-ribosylation (van Holde, 1988). Recent observations indicate that these modifications occur interdependently and create a pattern that might modulate the affinity of histone-binding proteins. These findings are the basis of the histone code hypothesis (Strahl and Allis, 2000; Turner, 2000). The best studied modification of core histones is the reversible acetylation of conserved lysine residues within the N-terminal tails. Two types of enzymes, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs), control the acetylation of histones and other substrates. More than a dozen deacetylases have been identified during the last few years. According to their homology to yeast proteins, the HDACs are grouped into three classes (Gray and Ekström, 2001; Khochbin *et al*., 2001). The highly homologous class I enzymes HDAC1 and HDAC2 can heterodimerize and frequently are found in the same complexes. These deacetylases seem to be involved in more general cellular processes. In contrast, class II enzymes might have tissue-specific functions during the execution of developmental programs. According to its sequence, HDAC3 also belongs to the class I family. However, the protein can interact with both class I and class II enzymes, and might be a functional link between the two enzyme families (Grozinger *et al*., 1999; Fischle *et al*., 2001). The third mammalian HDAC class is formed of enzymes with homology to the NAD-dependent deacetylase Sir2. Human Sir2 was shown recently to be a p53 deacetylase involved in the control of cell survival (Luo *et al*., 2001; Vaziri *et al*., 2001).

HDAC1, a homolog of the yeast protein Rpd3p, was the first protein shown to have histone deacetylase activity (Vidal and Gaber, 1991; Taunton *et al*., 1996). Subsequently, HDAC1 and its closest homolog HDAC2 (Yang *et al*., 1996) were found associated with several accessory proteins such as RbAp46/48, Sin3A/Sin3B, SAP18 and SAP30 (reviewed in Ahringer, 2000). These factors seem to be required as structural components of HDACI–HDACII complexes and for the interaction with

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core histones. A variety of transcription factors including Rb, Mad, unliganded nuclear hormone receptors and p53 have been shown to regulate target genes by association with HDAC1 (reviewed in Cress and Seto, 2000; Ng and Bird, 2000). Recruitment of HDAC1 by Rb and other pocket proteins is thought to be important for the growth-inhibitory effect of these proteins (Zhang and Dean, 2001). In addition, HDAC1 interacts with the methyl-binding proteins and DNA methyltransferases, strongly suggesting cooperativity between DNA methylation and histone deacetylation in gene silencing (Dobosy and Selker, 2001; Fuks *et al*., 2001). Despite the growing knowledge on the mechanisms of HDAC-dependent gene repression, surprisingly little is known about the biological function of individual mammalian deacetylases. Most data concerning the physiological role of HDACs were obtained by using deacetylase inhibitors, which block the majority of class I and class II enzymes.

Here, we have disrupted the *Hdac1* gene in mice to examine its role during development and proliferation. We show that HDAC1 is a major deacetylase in mouse embryonic stem (ES) cells and its loss results in a substantial reduction of cellular HDAC activity and specific changes in histone modifications. The related enzymes HDAC2 and HDAC3 are up-regulated in HDAC1-null cells, but cannot compensate for the loss of HDAC1 function. Targeting of both HDAC1 alleles leads to embryonic lethality before E10.5. HDAC1-deficient embryos and HDAC1-null ES cells have proliferation defects and display increased levels of a subset of cyclin-dependent kinase (CDK) inhibitors. Our data demonstrate that HDAC1 is essential for mouse embryonic development and that the enzyme ensures cell proliferation by repressing the expression of specific growth-inhibitory genes.

Results

Expression of HDAC1 during mouse embryogenesis

Mouse HDAC1 was identified originally as a growth factor-inducible protein (Bartl *et al*., 1997). The enzyme is ubiquitously expressed in tissues of adult mice, with elevated levels in thymus, testis, ovary and placenta (Bartl *et al*., 1997). As a first step to investigate the function of HDAC1 for mammalian development, we analyzed the expression of the enzyme during different stages of mouse embryogenesis. As detected by indirect immunofluorescence microscopy, fertilized oocytes and two-cell embryos show a rather strong cytosolic HDAC1 signal that might be due to maternal storage (Figure 1A). Concomitant with zygotic genome activation in the two-cell stage, a nuclear HDAC1 staining pattern became visible in mouse pre-implantation embryos. The expression of HDAC1 was constantly high during the following cleavages to the blastocyst stage (Figure 1A). Significant HDAC1 expression levels were also detected during post-implantation development, as shown by immunostaining of E7.5 and E8.5 embryos (Figure 1B). HDAC1 was highly abundant in most embryonic and extra-embryonic tissues, with specifically strong expression in trophoblast giant cells. These large embryonic cells have an important function for the nutrition of the embryo and contain an amplified

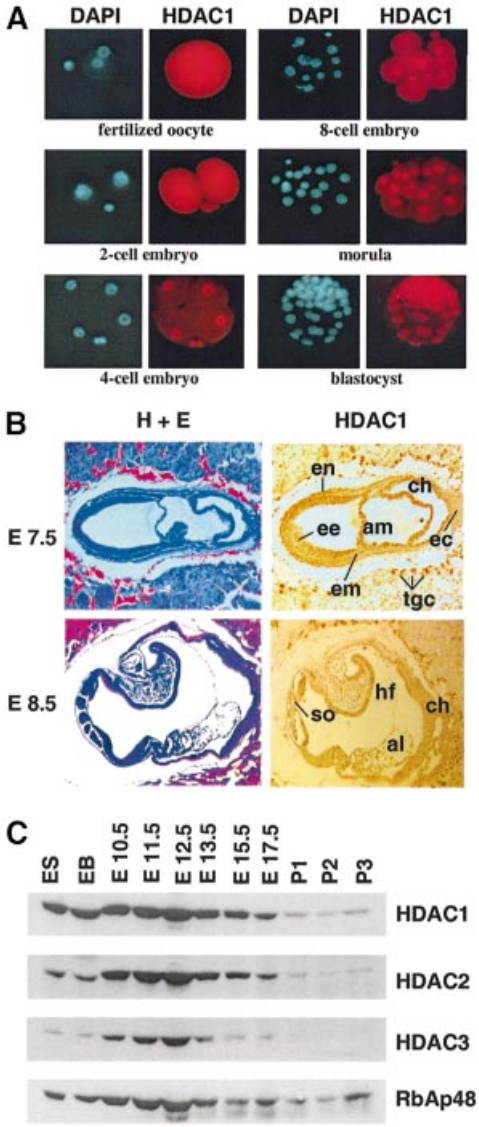

Fig. 1. HDAC1 expression during mouse embryonic development. (**A**) HDAC1 expression in pre-implantation embryos from the fertilized oocyte to the blastocyst stage was monitored in indirect immunofluorescence experiments. HDAC1 was visualized using a polyclonal HDAC1 antiserum and a secondary Texas red-conjugated antibody. Genomic DNA was stained with DAPI. (**B**) Immunohistochemical analysis of HDAC1 expression in paraffin-embedded sections of mouse embryos. Adjacent sections were stained with hematoxylin/eosin (H+E) or immunostained for HDAC1 at E7.5 (upper panels) and E8.5 (lower panels). High HDAC1 expression is found in all embryonic and extra-embryonic tissues, in particular in trophoblast giant cells. am, amnion; ch, chorion; ec, ectoplacental cone; ee, embryonic ectoderm; em, embryonic mesoderm; en, endoderm; tgc, trophoblast giant cells; al, allantois; hf, headfold; so, somites. (**C**) Expression patterns of HDAC1, HDAC2, HDAC3 and RbAp48 during mouse embryogenesis. Western blot analysis of whole-cell extracts from ES cells, embryoid bodies (EB) and 10.5- to 17.5-day-old embryos (E10.5–E17.5). P1-3 indicates 1–3 days after birth. Exposure times for ECL detection were adjusted approximately to the individual sensitivities of the HDAC antibodies.

genome. The high content of DNA in these cells correlates with elevated HDAC1 levels, but HDAC1 does not seem to be required for the endoreduplication of the trophoblast DNA (see below). Next, we compared the expression patterns of the homologous class I histone deacetylases HDAC1, HDAC2 and HDAC3 during mouse development by western blot analysis. All three enzymes were highly

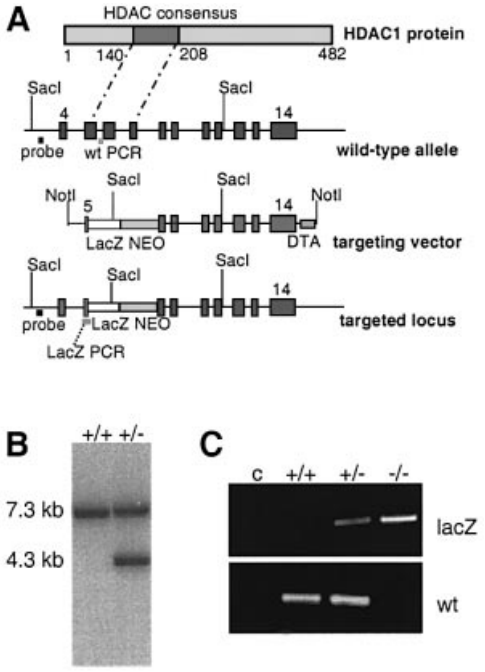

Fig. 2. Disruption of the murine HDAC1 locus. (**A**) Structure of the mouse *Hdac1* gene locus. In the targeting vector, part of exon 5 and exons 6 and 7 encoding the deacetylase consensus motif were replaced by the *lacZ/neo* cassette for G418 selection of positive clones. The diphtheria toxin gene (DTA) was inserted 3′ of the long arm to select against random integration of the targeting construct. (**B**) Southern blot analysis of tail DNA isolated from offspring of heterozygous intercrosses. Genomic DNA was digested with *Sac*I and hybridized with an intron-specific probe, which recognizes a 7.3 kb fragment in the wild-type allele and a 4.3 kb S*ac*I fragment in the targeted locus. (**C**) Yolk sac PCR analysis of E9.5 embryos.

expressed from mid to late gestation in the mouse (Figure 1C). In contrast to the other HDACs analyzed, only HDAC1 is highly abundant in ES cells and embryoid bodies (Figure 1C, ES and EB). The RbAp48 protein was shown previously to associate with HDAC1, and this interaction might be required for its full enzymatic activity (Taunton *et al*., 1996). As shown in Figure 1C, RbAp48 has an expression pattern similar to HDAC1. Together, these data suggest a specific function for HDAC1 during early embryonic development.

Targeted disruption of the Hdac1 gene

To evaluate the function of HDAC1 in mouse development, the *Hdac1* locus was inactivated by a conventional targeting approach (Figure 2A). Part of exon 5 and exons 6–7 of the murine *Hdac1* gene (Khier *et al*., 1999) were replaced by a β-galactosidase/neomycin phosphotransferase (*lacZ/neo*) cassette in E14.1 ES cells. The replaced sequence encodes the highly conserved deacetylase consensus motif (Hassig *et al*., 1998) (Figure 2A). Two independently targeted ES clones were injected into C57BL/6 mouse blastocysts and chimeras derived from both cell lines were used to generate heterozygous mice. The genotype of offspring derived from HDAC1 heterozygous intercrosses was determined by Southern blot analysis or PCR (Figure 2B, and data not shown). No HDAC1-null animals were observed in either a C57BL/6 × 129/Sv background or a pure 129/Sv background (Table I). Heterozygous animals were obtained with a

Table I. Absence of HDAC1-null animals in offspring from HDAC1 heterozygous intercrosses in a mixed C57BL/6 × 129/Sv background and a pure 129/Sv background

	+/+	+/–	–/–	ND	Total
BL/6 × 129/Sv	466	718	0	17	1201
	39%	60%	0%	1%	100%
129/Sv	114	137	0	0	251
	45%	55%	0%	0%	100%

The genotype was determined by Southern blot analysis or PCR.
ND, not determined.

frequency below the expected Mendelian ratio, but were viable and fertile and appeared to have a normal phenotype.

To determine when the HDAC mutation produces a lethal phenotype, timed matings were set up and embryos were obtained from heterozygous intercrosses at E7.5–13.5 and genotyped by PCR analysis of DNA extracted from yolk sac or total embryos (Figure 2C). Examination of embryos isolated between E7.5 and E9.5 revealed all genotypes with a Mendelian ratio. All HDAC1 mutants appeared extremely growth retarded (see below). No homozygous null embryos could be detected after E9.5, indicating that lack of HDAC1 leads to embryonic lethality before day 10.5 of gestation.

HDAC1 is indispensable for mouse embryonic development

To reveal potential developmental defects, embryos from heterozygous intercrosses were analyzed by whole-mount *in situ* hybridization with an HDAC1 riboprobe. HDAC1 wild-type or heterozygous embryos showed HDAC1 expression throughout the embryo except the developing heart (Figure 3A–C). At E9.5, HDAC1-null embryos displayed numerous abnormalities including severely disturbed head and allantois formation (Figure 3D). HDAC1–/– embryos showed normal expression of the mesoderm marker brachyury (T) (Wilkinson *et al*., 1992), excluding the possibility of a gastrulation failure (Figure 3E). We also ruled out the possibility that the HDAC1 phenotype arises due to a defect in the specification of the placenta. Trophoblast giant cells showed high HDAC1 expression levels (Figure 1B). However, analysis of placental lactogen-1 (PL-1), a marker for trophoblast giant cells (Colosi *et al*., 1987), by whole-mount *in situ* hybridization revealed a staining pattern in the null embryo similar to wild-type (Figure 3F). To exclude, that increased apoptosis accounts for the reduced size of HDAC1–/– embryos, we analyzed wild-type and null embryos by TUNEL assay. At the onset of the HDAC1-null phenotype at E7.5, only a few apoptotic cells were observed in both null and wild-type embryos (Figure 3G and H). In addition, neither wild-type nor HDAC1-null embryos show signs of necrosis at E7.5 (Figure 3I and J).

Therefore, the severely growth retarded appearance of HDAC1-deficient embryos is suggestive of a cellular proliferation defect. To test this assumption, we performed an immunohistochemical analysis of wild-type and HDAC1-deficient embryos at E7.5 for the expression of the proliferation marker Ki67 antigen (Schluter *et al*., 1993). The observed difference in size between HDAC1 wild-type and null embryos correlated well with a

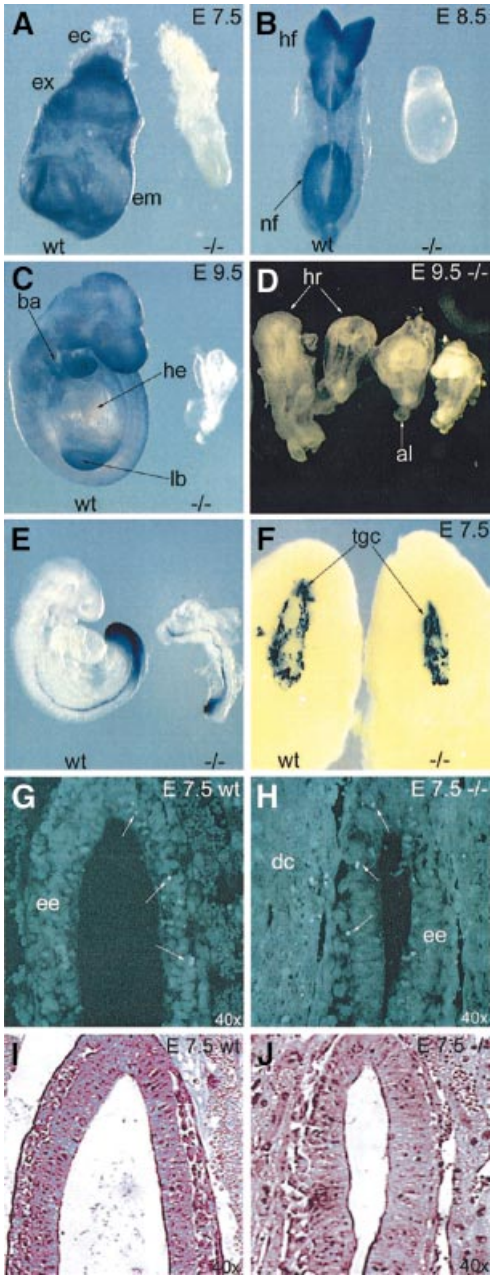

Fig. 3. Phenotypic analysis of HDAC1 mutant embryos. (A–D) Whole-mount *in situ* hybridization of wild-type and HDAC1 mutant embryos with an HDAC1 riboprobe. (**A**) HDAC1 is highly expressed in the ecto-placental cone (ec), the extra-embryonic (ex) and embryonic (em) ectoderm at E7.5. (**B**) Elevated HDAC1 levels are detected in the head fold (hf) and the neural fold (nf) at E8.5. (**C**) HDAC1 is highly expressed throughout the E9.5 embryo except in the developing heart (he), with pronounced expression in the bronchial arches (ba) and the limb bud (lb). (**D**) Spectrum of phenotypes observed for HDAC1 mutant embryos at E9.5 with misformed allantois (al) and defects in head formation; hr, head region. (**E**) Brachyury expression at E9.5 is comparable in HDAC1 wild-type and HDAC1 mutant embryos. (**F**) E7.5 wild-type and mutant embryos within the maternal decidua are shown. Placental lactogen-1 expression in trophoblast giant cells (tgc) is similar in HDAC1 wild-type and HDAC1 null embryos. (G and H) TUNEL assay on paraffin-embedded sections of E7.5 wild-type (**G**) and mutant (**H**) embryos shows similar numbers of apoptotic cells in the embryonic ectoderm (ee) of wild-type and mutant embryos. Arrows indicate apoptotic cells. (**I** and **J**) Hematoxylin/eosin staining of sections adjacent to the sections shown in (G) and (H).

significantly reduced number of proliferating cells in HDAC1-null embryos (51% Ki67 positive) compared with wild-type embryos (65% Ki67 positive) (Figure 4). Taken together, these results strongly suggest that the reduced size of HDAC1-deficient embryos at E7.5 is due to a proliferation defect rather than to increased apoptosis.

Changed histone modifications in HDAC1-null cells

To examine the effects of HDAC1 deficiency at the cellular level, we generated HDAC1-null ES cells by blastocyst outgrowth experiments (Hogan *et al*., 1994). Five independent HDAC1-deficient ES cell lines and corresponding wild-type and heterozygous control cell lines were analyzed and gave essentially the same results. Comparative analyses were performed with early passages of wild-type, heterozygous and null ES cells obtained from littermates. HDAC1 protein levels were decreased in heterozygous ES cells and undetectable in HDAC1-null cells (Figure 5A). Expression of the HDAC1-associated factor RbAp48 was unchanged in heterozygous and HDAC1-deficient cell lines. Levels for the HDAC1-related enzymes HDAC2 and HDAC3, in contrast, were found to be increased complementary to HDAC1 expression (Figure 5A). Similarly, HDAC2 protein was expressed at a higher level in various tissues of heterozygous mice (data not shown). Despite the up-regulation of HDAC2 and HDAC3, the total histone deacetylase activity was significantly decreased in HDAC1-null cells (Figure 5B). HDAC2 previously was found to be associated with HDAC1 (Hassig *et al*., 1998) and this associa-

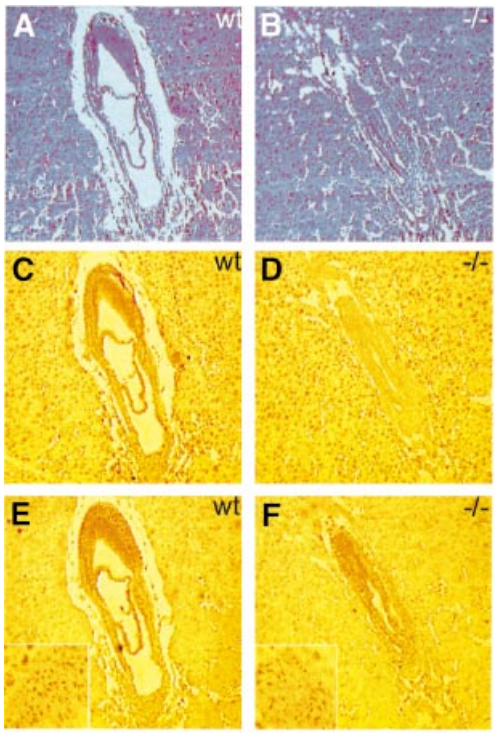

Fig. 4. Reduced proliferation of HDAC1-null embryos. Immunohistochemical analysis of HDAC1 and Ki67 antigen expression in mouse embryos at E7.5. Adjacent paraffin-embedded sagittal sections of mouse embryos were stained with hematoxylin/eosin (**A** and **B**) or immunostained for HDAC1 (**C** and **D**) or for the proliferation marker Ki67 nuclear antigen (**E** and **F**). The boxed areas show higher magnifications of the Ki67-stained embryo sections.

tion might be necessary for enzymatic activity of HDAC1–HDAC2-containing complexes. To examine whether HDAC2 activity is impaired in HDAC1-deficient ES cells, we analyzed HDAC1–HDAC2-containing complexes in fractionation experiments and HDAC activity assays. In wild-type cells, HDAC1 and HDAC2 were found in the same fractions of a 5–15% sucrose gradient (Figure 5C). In HDAC1-null cells, the corresponding fractions showed significantly reduced total histone deacetylase activity and HDAC2 displayed a slightly broader distribution profile, which might be due at least in part to the increased expression of HDAC2. HDAC2 co-sedimented in both cell lines with components of the NuRD and Sin3 complexes such as Sin3A, MTA1 and RbAp48, consistent with the presence of intact HDAC2 complexes (Figure 5C).

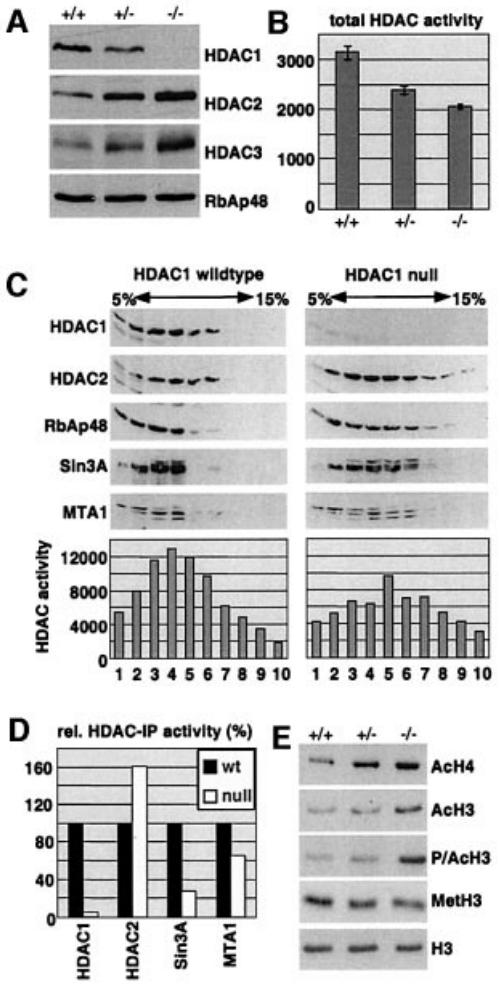

Fig. 5. HDAC1-deficient ES cells display decreased histone deacetylase activity and changes in histone modifications. (**A**) Western blot analysis of homologous HDAC proteins of total protein extracts prepared from wild-type, heterozygous and null ES cell lines. The blot was incubated sequentially with antibodies directed against HDAC1, HDAC2, HDAC3 and RbAp48, respectively. (**B**) Equal amounts of extracts described in (A) were analyzed for deacetylase activity with tritium acetate-labeled histones as substrates. Counted radioactivity corresponds to the amount of released acetyl moieties per hour and 10 μg protein and reflects the relative HDAC activity. Results are shown as mean values of three independent experiments. (**C**) Co-sedimentation analysis of HDAC1–HDAC2-containing complexes in wild-type and HDAC1-null cells. One aliquot of each fraction was analyzed on western blots for the presence of components of HDAC1–HDAC2 complexes. A second aliquot of each fraction was tested for total HDAC activity. (**D**) Comparison of deacetylase activities associated with components of the HDAC1–HDAC2 complexes. HDAC1, HDAC2, Sin3A and MTA1 were immunoprecipitated from whole-cell extracts prepared from wild-type or HDAC1-null ES cells and analyzed for associated HDAC activity as described in (B). The data shown are representative of three independent experiments. (**E**) Changes in core histone modifications in HDAC1 heterozygous and homozygous ES cells. Lack of HDAC1 resulted in H3 and H4 hyperacetylation, increased S10/K14 phosphoacetylation and reduced K9 methylation of histone H3. Histones were extracted and analyzed on western blots with antibodies recognizing acetyl-histone H4 (AcH4), acetyl-histone H3 (AcH3), histone H3-phosphoS10-acetylK14 (P/AcH3) and histone H3-methylK9 (MetH3). Equal loading was controlled by probing with an H3 antibody (H3).

To test directly a requirement for HDAC1 for HDAC2 enzymatic activity, we analyzed the deacetylase activity of immunoprecipitated HDAC1 and HDAC2. As expected, immunoprecipitates obtained with HDAC1 antibodies from null ES cell extracts showed only background activity, while HDAC2 activity was significantly increased in HDAC1-deficient cells (Figure 5D). In contrast, enzymatic activity associated with Sin3A and MTA1 immunoprecipitates was clearly reduced in HDAC1-null cells. Taken together, these findings argue against a strict requirement for HDAC1 for HDAC2 enzyme function, but indicate a potential role for HDAC1 for a subset of deacetylase complexes that contain both enzymes (Humphrey *et al*., 2001). Our data show that HDAC1 is a major histone deacetylase in mouse ES cells.

Next, we analyzed the effects of HDAC1 deficiency on modifications of histone tails. While the acetylation of bulk histones as detected on acidic Triton–urea gels was not significantly affected by the loss of HDAC1 (data not shown), western blot analysis with modification-specific antibodies revealed increased acetylation levels of a subset of histones H3 and H4 in HDAC1-null cells (Figure 5E). The difference in the results obtained by the two methods is due to the higher sensitivity of the western blot analysis and indicates that only a relatively small portion of core histones are hyperacetylated in HDAC1-deficient cells (see Discussion).

Different modifications of N-terminal histone tails such as phosphorylation, methylation and acetylation have been shown to be interdependent (Cheung *et al*., 2000b; Clayton *et al*., 2000; Lo *et al*., 2000; Rea *et al*., 2000; Nakayama *et al*., 2001). In good agreement with these findings, we observed an increase in histone H3 phosphorylation on Ser10 and phosphoacetylation on Ser10 and Lys14 in HDAC1-null ES cells (Figure 5E and data not shown). Phosphoacetylation of histone H3 was shown to play an important role for transcriptional activation (Cheung *et al*., 2000a; Clayton *et al*., 2000). In contrast, histone H3 methylation on Lys9 was implicated in transcriptional silencing (Jenuwein, 2001). As shown in Figure 5E, histone H3 methylation was slightly reduced in HDAC1–/– ES cells. Our results support the idea of cooperation between histone acetylating and phosphorylating enzymes and suggest that deacetylation by HDAC1 might be linked to efficient histone methylation.

HDAC1 is essential for unrestricted cell proliferation of mouse ES cells

ES cells, when cultured under defined conditions and in the absence of leukemia inhibitory factor (LIF), differentiate

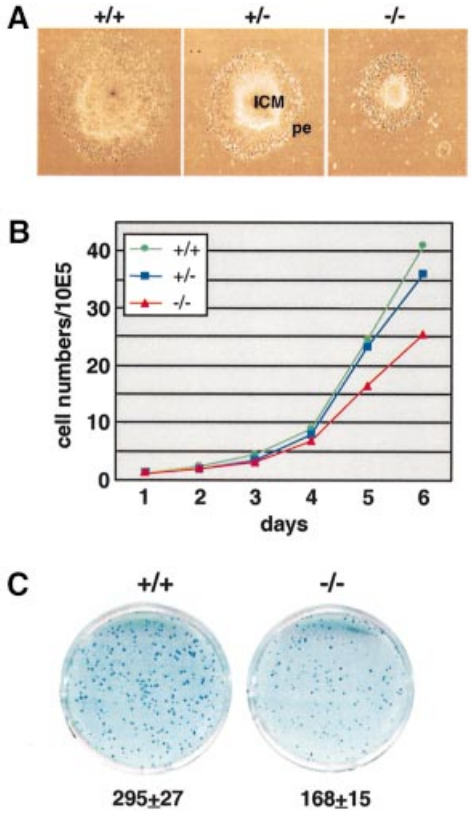

Fig. 6. Loss of HDAC1 in ES cells leads to impaired proliferation. (**A**) Three ES cell lines of different genotype were subjected to differentiation. The HDAC1-null embryoid bodies show a reduced inner cell mass (ICM), but normal differentiation. pe, parietal endoderm. (**B**) Growth curves of HDAC1 wild-type, heterozygous and null ES cell lines. Equal numbers of cells (5×10^5) were seeded in triplicate and aliquots were counted daily during a time period of 6 days. (**C**) Colony formation assay with wild-type and HDAC1 mutant ES cells. A total of 1.5×10^3 cells were seeded on SNL feeder layers and cultivated for 8 days. Cells were fixed and stained with methylene blue and colonies were counted. The average clone numbers of four plates are indicated with the respective standard deviations.

into embryoid bodies (Desbaillets *et al*., 2000). Embryoid bodies were generated from HDAC1 wild-type, heterozygous or null ES cell lines. ES cells showed an HDAC1 dose-dependent size of the inner cell mass, suggesting a prolonged generation time for HDAC1-deficient ES cells (Figure 6A). The differentiation of ES cells, however, was not affected by the absence of HDAC1 as judged by parietal endoderm formation (Figure 6A) and the spectrum of differentiated cells that appeared during embryoid body formation (data not shown). These *in vitro* differentiation results complement the observation that HDAC1 mutant embryos contain differentiated cell structures. To analyze the observed proliferation defect in more detail, we performed proliferation assays with non-differentiated ES cells. Equal numbers of cells were seeded in triplicate and aliquots were counted daily for up to 6 days. As shown in Figure 6B, HDAC1-null ES cells displayed impaired proliferation in comparison with wild-type and heterozygous cell lines. Similar data were obtained with 'split-count' experiments, where equal numbers were plated, counted after 2 days and plated again repeatedly with the original cell number (data not shown). In addition, we measured the plating efficiency of wild-type and null ES cells. Equal numbers of cells (1.5×10^3) were plated in quadruplicate on layers of mitomycin C-treated SNL feeder cells and colonies were counted after 8 days. As shown in Figure 6C, HDAC1-deficient cells showed a significantly reduced number of colonies (168 ± 15 for HDAC1-null cells versus 295 ± 27 for wild-type cells). In agreement with the results shown for the E7.5 embryos (Figure 3), TUNEL assay with wild-type and HDAC1-null cells confirmed that the reduced number of HDAC1-deficient ES cells is not caused by increased apoptosis (data not shown). Together with the observed growth defect of HDAC1-null embryos (Figures 3 and 4), these data suggest a crucial role for HDAC1 in proliferation of embryonic mouse cells.

Therefore, we next investigated the molecular basis of the proliferation defects in HDAC1-deficient ES cells. Cell cycle regulators such as cyclins and CDK inhibitors have been shown to be important for the anti-proliferative effects of HDAC inhibitors (Archer *et al*., 1998; Vaziri *et al*., 1998; Wharton *et al*., 2000). Along this line, impaired cellular proliferation of HDAC1-deficient ES cells correlated with the up-regulation of a subset of CDK inhibitors. Expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ was found to be increased in HDAC1-null cells (Figure 7A), while $p16^{INK4A}$ and $p19^{ARF}$ were undetectable in any of the ES cell lines (data not shown). A function of HDAC1 in the regulation of p21 and p27 is also illustrated by the finding that the histone deacetylase inhibitor trichostatin A induced the expression of both CDK inhibitors in wild-type ES cells (data not shown). Protein levels of cyclin A, cyclin B, cyclin E, RbAp48 and p53 were unchanged in all ES lines, while Cdk2 expression was increased in HDAC1-null cells (Figure 7A and data not shown). In agreement with induced levels of p21 and p27, cyclin A- and cyclin E-associated kinase activity was significantly decreased in HDAC1-null ES cell lines (Figure 7B).

To reveal a direct link between the deregulation of the two CDK inhibitors and the proliferation defect of HDAC1 mutant embryos, we examined their expression in HDAC1 wild-type and null embryos. As shown in Figure 7C, RT–PCR analysis of E9.5 embryos revealed an up-regulation of p21 and p27 in HDAC1–/– embryos. Together, these results suggest that the reduced proliferation capacity of HDAC1-deficient cell lines and the retardation of the mutant embryos is due at least in part to increased expression of the CDK inhibitors p21 and p27.

Finally, we tested whether the increased expression of p21 and p27 in HDAC1-null cells correlates with hyperacetylation of histones associated with the CDK inhibitor genes. To this end, we performed chromatin immunoprecipitation experiments with antibodies specific for acetylated histone H3 and H4 isoforms. As shown in Figure 7D, the proximal p21 promoter was associated with hyperacetylated histone H3 in HDAC1-null ES cells, while the distal promoter region did not seem to be sensitive to loss of HDAC1. Similarly, increased expression of HDAC2 and p27 in HDAC1-null cells (Figures 5A and 7A) was paralleled by hyperacetylation of histone H3 at the corresponding promoters. We also observed an increase in acetylated histone H4 at the HDAC2 promoter in the absence of HDAC1, while histone H4 acetylation at the promoters of the p21 and the p27 gene remained

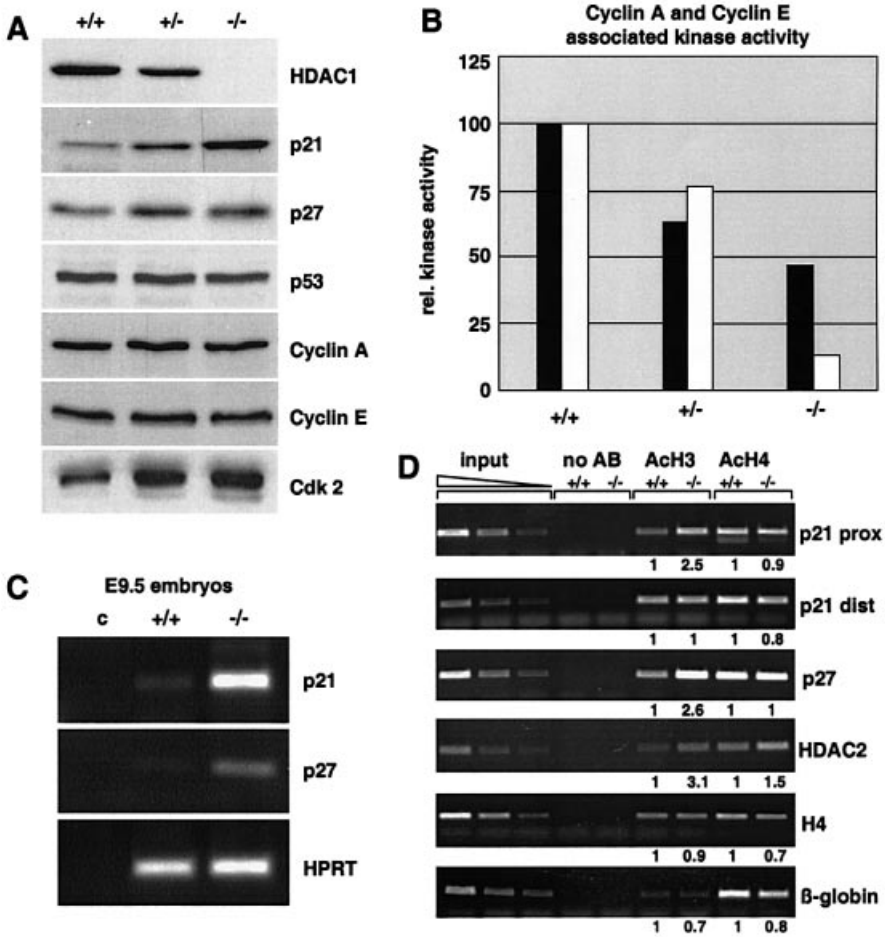

Fig. 7. The CDK inhibitors p21 and p27 are up-regulated in HDAC1-deficient ES cells. (**A**) Western blot analysis of whole-cell extracts prepared from wild-type, heterozygous and null ES cell lines. Western blots were probed sequentially with the indicated antibodies. (**B**) HDAC1-deficient ES cells show reduced cyclin A- and cyclin E-associated kinase activity. Cyclin A or cyclin E complexes were immunoprecipitated from ES cell extracts and incubated with histone H1 in the presence of [γ-^{32}P]ATP. Phospholabeled histone H1 was quantified and is shown for cyclin A (black bars) and cyclin E (white bars) relative to the kinase activity of wild-type cells arbitrarily set as 100. The results are representative of three independent experiments. (**C**) p21 and p27 expression is increased in E9.5 mutant embryos. HPRT was used as a control. The levels of p21, p27 and HPRT mRNA were determined by semiquantitative RT–PCR analysis. (**D**) Hyperacetylation of specific target promoters in HDAC1-null cells. Chromatin isolated from wild-type or HDAC1-deficient cells was precipitated in the absence of specific antibodies (no AB) or with antibodies specific for acetylated histone H3 (AcH3) or acetylated histone H4 (AcH4). Total input DNA (1×, 0.25× and 0.0625×) and DNA from the antibody-bound fractions were analyzed by quantitative PCR. Amounts of amplified DNA were quantified and are indicated relative to the signals from wild-type cells (set to 1).

unchanged. The acetylation levels of histones H3 and H4 at two control genes (histone H4 and β-globin) were comparable in wild-type and HDAC1-null ES cells. These data provide a direct link between loss of HDAC1, changes in histone acetylation and the activation of specific target genes.

Discussion

HDAC1 and embryonic development

Here we show that disruption of *Hdac1* causes embryonic lethality prior to E10.5. This is the first report demonstrating that a chromatin-associated factor with histone deacetylase activity is crucial for mouse embryonic development. Previously, HDACs have been shown to be required for embryonic development of invertebrates (reviewed in Ahringer, 2000). In addition, loss of specific components of the Sin3 and the NuRD complexes such as RbAp46/48 (lin-53, rba-1), Sin3 (dSin3A), Mi-2 (dMi-2, chd-3, chd-4) and MTA1/MTA2 (egl-27, egr1) affect embryonic viability and development of *Drosophila melanogaster* and *Caenorhabditis elegans*. In this study, we observed that HDAC1–HDAC2-containing complexes such as the Sin3 and the NuRD complexes show significantly reduced deacetylase activity in HDAC1-deficient mouse cells. HDAC1 is also part of the EED–EZH complex that is involved in epigenetic control of gene expression. The mouse polycomb proteins Eed (Faust *et al*., 1998) and Ezh2 (O'Carroll *et al*., 2001) are required for embryonic development, suggesting that the maintenance of transcriptional repression is crucial for mouse embryogenesis. Several lines of evidence support the link between homeotic gene silencing, histone deacetylation and embryonic development. The transcription factor YY1 was shown to exert its repressive function by recruiting HDAC1, HDAC2 and HDAC3 (Yang *et al*., 1996, 1997). Disruption of the *YY1* gene, the mammalian homolog of the *Drosophila* polycomb gene *pleiohomeotic*, results in

embryonic lethality (Brown *et al*., 1998; Donohoe *et al*., 1999). *Drosophila* HDAC1 was found to be essential for polycomb-mediated gene silencing in fly embryos (Chang *et al*., 2001). Taken together, these findings indicate that function and integrity of HDAC1-containing complexes are crucial for the execution of developmental programs.

HDAC1 and histone modifications

Our data show a significant loss of total deacetylase activity in HDAC1-deficient ES cells. The actual contribution of HDAC1 to the overall activity might in fact be underestimated, because the homologous enzymes HDAC2 and HDAC3 are up-regulated in the absence of HDAC1. The specific increase in histone acetylation on the HDAC2 promoter suggests that the rise in HDAC2 expression in HDAC1-null cells is a direct consequence of loss of HDAC1. A feedback regulation of class I HDAC levels in response to changes in intracellular deacetylase activity was also observed in HDAC1-overexpressing Swiss 3T3 cells, since protein expression of endogenous HDAC1, HDAC2 and HDAC3 was significantly reduced in these cells (G.Lagger, K.Kroboth and C.Seiser; unpublished observations). In addition, HDAC1-overexpressing mouse fibroblasts showed reduced proliferation rates (Bartl *et al*., 1997). Taken together, these findings indicate that mammalian cells have to maintain specific levels of deacetylase activities in order to ensure unperturbed cell cycle progression. Furthermore, we show here that acetylation of a subpopulation of histones H3 and H4 is increased in HDAC1-deficient cells, suggesting that these core histones are *in vivo* substrates for HDAC1. While most mammalian deacetylases efficiently recognize acetylated histones or acetylated histone-derived peptides as substrates in *in vitro* assays, data concerning *in vivo* substrates were obtained mainly by the use of inhibitors which affect numerous deacetylating enzymes. Thus, our data provide for the first time direct evidence that a mammalian HDAC deacetylates core histones *in vivo*. In addition to increased histone acetylation, we observed concomitant changes in histone H3 phosphorylation and methylation in HDAC1-null cells. These results suggest a general function for HDAC1 in the modulation of chromatin structures, thereby lending further support to the histone code hypothesis (Strahl and Allis, 2000; Turner, 2000; Jenuwein and Allis, 2001).

HDAC1 and gene expression

The subpopulation of core histones found to be hyperacetylated in HDAC1-null cells might be part of particular chromatin regions whose reversible acetylation is controlled by the simultaneous presence of HDAC1 and its counterparts, the acetyltransferases. Loss of HDAC1 thus results in increased acetylation, while the part of the chromatin which is devoid of histone acetyltransferases and HDAC1 remains unaffected. In agreement with this idea, only a subset of genes including HDAC2, p21 and p27 displayed altered expression levels in HDAC1-null cells, indicating a specific function for HDAC1 in the regulation of gene expression. Furthermore, we observed a specific increase in the acetylation level of core histones associated with these target genes. However, it is important to note that our findings do not exclude the possibility that increased acetylation of transcription factors in HDAC1-null cells might also affect the expression of specific genes. In the future, the comparison of gene expression patterns in wild-type and HDAC1-null cells will allow the systematic identification of HDAC1 target genes and the analysis of the regulatory function of HDAC1 in more detail.

HDAC1 and proliferation

HDAC1-deficient embryos (Figure 4) and HDAC1-null cells (Figure 6) show proliferation defects. Together with previous data showing high expression levels of HDAC1 in proliferating cells (Bartl *et al*., 1997), these results are suggestive of a proliferation-linked function for the enzyme. Paradoxically, the recruitment of class I HDACs by Rb seems to be important for the repression of proliferation-associated genes, and HDAC1 should therefore act rather as a growth inhibitor (discussed in Cress and Seto, 2000; Zhang and Dean, 2001). However, the data shown in this report demonstrate that one of the key functions of HDAC1 is to prevent the expression of antiproliferative genes in cycling cells. These findings indicate that deacetylases other than HDAC1 as well as deacetylase-independent mechanisms ensure the proper regulation of Rb target genes in HDAC1-null cells.

Here, we present evidence that HDAC1 controls the expression of a specific subset of CDK inhibitors. The induction of p21 and p27 in HDAC1-null cells correlated with the hyperacetylation of the corresponding promoters. The specificity of this response is underlined by the fact that only the proximal but not the distal portion of the p21 promoter was found to be associated with hyperacetylated histone H3. The proximal p21 promoter contains Sp1-binding sites that are required for the induction of the p21 gene by deacetylase inhibitors (Sowa *et al*., 1997). Activation of tumor suppressors was shown to be a crucial function of HDAC inhibitors as anti-cancer drugs in human cells (reviewed in Cress and Seto, 2000; Kramer *et al*., 2001). Our results strongly support the idea that HDAC1 might be a relevant target in tumor treatment.

Materials and methods

Gene targeting and generation of mice

The HDAC1 targeting vector was designed to replace part of exon 5, exons 6 and 7 with the *lacZ*/*neo* cassette (O'Carroll *et al*., 2001). The cassette was flanked by a 1.3 kb *Sac*II–*Eco*RI upstream fragment covering most of intron 4 and the first 46 bp of exon 5, and a 5 kb *Kpn*I–*Eco*RI fragment reaching from intron 7 to sequences downstream of the last exon (Khier *et al*., 1999) in the pGNA-T-derived targeting vector (O'Carroll *et al*., 2001). The encoded protein consists of the 132 N-terminal residues of the HDAC1 protein fused to LacZ and completely lacks the deacetylase consensus motif and most of the catalytic domain. Targeting of the HDAC1 gene was performed in the ES cell line E14.1 as described (O'Carroll *et al*., 2001). Targeted clones were identified by nested PCR analysis and Southern blotting (see below). Targeting had no effect on the expression levels of the neighboring MRP gene (Khier *et al*., 1999). Two independent clones were injected into C57BL/6 blastocysts as described (O'Carroll *et al*., 2001). Chimeric mice were mated to C57BL/6 and 129/Sv animals, and agouti offspring were genotyped. Germline transmission of the disrupted HDAC1 allele was detected by either Southern blot or PCR analysis of tail DNA.

Isolation and culture of embryonic stem cells

ES cells were isolated from blastocysts as described (Hogan *et al*., 1994). All experiments were performed with ES cell lines obtained from littermates. For *in vitro* differentiation experiments, ES cells were aggregated in hanging drop cultures as described before (Bader *et al*.,

2000). All experiments described herein were performed with early passages of ES cell lines.

Southern blot and PCR analysis

Genomic DNA of tail biopsies, embryos or ES cells was isolated as described (O'Carroll *et al*., 2001). Southern blot analysis was performed as described previously (Khier *et al*., 1999). To determine the genotype, total genomic DNA was digested with *Sac*I and analyzed on Southern blots with a probe encompassing a portion of intron 4 which was not part of the targeting vector. For PCR analysis, an external intron 4-specific primer KoFo (5′-CCTCCCATTGAGTTGGCAGGGCAAG-3′) and a primer specific for the *lacZ* gene, LacOut (5′-AACCCGTCGGATTCT-CCGTGGGAAC-3′) were used to amplify a 490 bp fragment of the targeted allele. To detect the wild-type allele, primers WtFo (5′-GGC-CTTGTGTCTTGGAAGAGCACC-3′) and WtRev (5′-GCTGAAGGA-AGGTGGAAGAGTGGC-3′), both specific for intron 5, were used to generate a 316 bp product.

Protein analysis

Protein extraction, histone isolation and western blot analyses were performed as described previously (Bartl *et al*., 1997; Taplick *et al*., 1998; Khier *et al*., 1999). HDAC protein complexes were resolved in continuous 5–15% (w/v) sucrose gradients in a Beckman ultracentrifuge (SW-Ti41, 2 h, 38 000 r.p.m., 4°C). The following antibodies were used in this study: HDAC1 (polyclonal rabbit antibody and monoclonal mouse antibody), RbAp48, HDAC2, histone H3-phosphoS10, histone H3-phosphoS10-acetylK14, acetyl-histone H3, acetyl-histone H4 from Upstate Biotechnology; cyclin A, cyclin E, p21, p27, Cdk2, MTA1 and Sin3A from Santa Cruz, and Ki67 antigen from Novo Castra. The methylK9-H3 antibody was described previously (Peters *et al*., 2001) and the HDAC3 antibody was a kind gift from Claude Sardet.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were carried out as described previously (Chadee *et al*., 1999; Cheung *et al*., 2000b) with a few modifications. Chromatin was cross-linked for 10 min using formaldehyde. The sonicated chromatin solution was diluted 1:10 and precipitated with 10 µl of acetyl-specific histone antibodies (Upstate Biotechnology). The following day, chromatin–antibody complexes were isolated from the solution by incubation with 30 µl of protein A–Sepharose beads (50% slurry, 100 µg/ml salmon sperm DNA, 500 µg/ml bovine serum albumin) while rocking at 4°C for 2 h. The beads were harvested and washed as already described. Chromatin–antibody complexes were eluted from the protein A–Sepharose beads by addition of 2% SDS, 0.1 M $NaHCO_3$ and 10 mM dithiothreitol to the pellet. Cross-linking was reversed by addition of 0.05 vol. of 4 M NaCl and incubation of the eluted samples for 6 h at 65°C. The DNA was extracted with phenol–chloroform, precipitated with ethanol and dissolved in water.

PCR analysis of immunoprecipitated DNA

All PCRs were performed on a Biometra D3 thermocycler using Promega PCR Master Mix. The primer pairs 5′-CATAGATGTATGTGGCTC-TGGC-3′ and 5′-GTCTGGATATCGCTGTGGATC-3′ (p21 proximal) or 5′-AGAGACATGGAGGAAGGTGGGA-3′ and 5′-GACCACACTAAT-GGCTACCTGC-3′ (p21 distal) were used to analyze the p21 promoter region. Primers 5′-GAGCCAATGAGGGACAACAAG-3′ and 5′-GGT-CTTACGCGCACTGGTTG-3′ were used to amplify the HDAC2 promoter. The primer pair 5′-GAGCAGGTTTGTTGGCAGTCG-3′ and 5′-GTGGAAGGGAGGCTGACGAAG-3′ was used to analyze the p27 promoter. H4 and β-globin DNA sequences were amplified as control fragments, using primers 5′-GACACCGCATGCAAAGAATAGCTG-3′ and 5′-CTTTCCCAAGGCCTTTACCACC-3′ specific for the H4 gene and primers 5′-AGGCTGCTGGTTGTCTACCCTTG-3′ and 5′-AGC-TCACTGAGGCTGGCAAAGGTG-3′ specific for the β-globin locus. The linear range for each primer pair was determined empirically using different amounts of genomic DNA. PCRs with increasing amounts of genomic DNA were carried out along with the immunoprecipitated DNA. PCR products were resolved on 2% agarose–TAE gels and quantified using the ImageQuant program (Molecular Dynamics).

Enzyme assays

Histone deacetylase enzyme activity in whole-cell extracts was determined with [^{3}H]acetate-labeled chicken erythrocyte histones (kindly provided by G.Brosch, University of Innsbruck) as described by Lechner *et al*. (1996). Cyclin A- or cyclin E-associated kinase activity was determined as described previously (Schuchner and Wintersberger, 1999) with the exception that the kinase reaction contained only 2 µCi of [γ-^{32}P]ATP. Phosphorylated histone H1 was quantified using a phosphorimager (Storm 840, Molecular Dynamics) and ImageQuant Software.

Semiquantitative RT–PCR analysis

Successive isolation of total RNA and genomic DNA of E9.5 embryos was done with Trizol reagent (Gibco-BRL) as recommended by the manufacturer. After confirmation of the genotype, 2 µg of RNA were reverse transcribed with the AMV reverse transcriptase (Promega), using an oligo(dT) primer, in a total volume of 25 µl. PCR was performed with 1 µl of the reverse transcription reaction, using primers for p21, p27 and HPRT, and conditions as described (Grayson *et al*., 2001).

Immunohistochemistry and TUNEL assay

Fertilized oocytes and pre-implantation embryos were isolated from superovulating female mice. Embryos were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in blocking solution, and HDAC1 was detected with a monoclonal HDAC1 antibody (Upstate Biotechnology) followed by a secondary Texas red-coupled antibody. For immunostaining of paraffin-embedded sections, decidual swellings were isolated in ice-cold phosphate-buffered saline, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, embedded in paraffin and sectioned at 5 µm. Adjacent sections were stained with hematoxylin and eosin or processed for immunohistochemistry using an HDAC1 rabbit polyclonal antiserum (Upstate Biotechnology) or the monoclonal Ki67 antigen antibody (Novo Castra). An ultrasensitive avidin-biotinylated enzyme complex (ABC) staining kit (ABC Universal or Rabbit IgG kit, Vector) was used according to the manufacturer's instructions. Apoptotic cells on paraffin-embedded sections were detected by using an *in situ* Cell Death Detection Kit (Roche).

Whole-mount in situ hybridization

Whole-mount RNA *in situ* hybridization was performed essentially as described previously (Henrique *et al*., 1995) using BM purple (Roche) as the color substrate. The following digoxygenin-labeled antisense riboprobes were used: brachyury (T) (Wilkinson *et al*., 1992), PL-1 (Colosi *et al*., 1987) and HDAC1 (3′ fragment encompassing bp 1041–1550) (Bartl *et al*., 1997).

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References

Ahringer,J. (2000) NuRD and SIN3 histone deacetylase complexes in development. *Trends Genet.*, **16**, 351–356.

Archer,S.Y., Meng,S., Shei,A. and Hodin,R.A. (1998) p21(Waf1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc. Natl Acad. Sci. USA*, **95**, 6791–6796.

Bader,A., Al Dubai,H. and Weitzer,G. (2000) Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions. *Circ. Res.*, **86**, 787–794.

Bartl,S., Taplick,J., Lagger,G., Khier,H., Kuchler,K. and Seiser,C. (1997) Identification of mouse histone deacetylase 1 as a growth factor-inducible gene. *Mol. Cell. Biol.*, **17**, 5033–5043.

Brown,J.L., Mucci,D., Whiteley,M., Dirksen,M.L. and Kassis,J.A. (1998) The *Drosophila* Polycomb group gene *pleiohomeotic* encodes a DNA binding protein with homology to the transcription factor YY1. *Mol. Cell*, **1**, 1057–1064.

Chadee,D.N., Hendzel,M.J., Tylipski,C.P., Allis,C.D., Bazett Jones,D.P., Wright,J.A. and Davie,J.R. (1999) Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. *J. Biol. Chem.*, **274**, 24914–24920.

Chang,Y.L., Peng,Y.H., Pan,I.C., Sun,D.S., King,B. and Huang,D.H. (2001) Essential role of *Drosophila* Hdac1 in homeotic gene silencing. *Proc. Natl Acad. Sci. USA*, **98**, 9730–9735.

Cheung,P., Allis,C.D. and Sassone-Corsi,P. (2000a) Signaling to chromatin through histone modifications. *Cell*, **103**, 263–271.

Cheung,P., Tanner,K.G., Cheung,W.L., Sassone Corsi,P., Denu,J.M. and Allis,C.D. (2000b) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol. Cell*, **5**, 905–915.

Clayton,A.L., Rose,S., Barratt,M.J. and Mahadevan,L.C. (2000) Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *EMBO J.*, **19**, 3714–3726.

Colosi,P., Talamantes,F. and Linzer,D.I. (1987) Molecular cloning and expression of mouse placental lactogen I complementary deoxyribonucleic acid. *Mol. Endocrinol.*, **1**, 767–776.

Cress,W.D. and Seto,E. (2000) Histone deacetylases, transcriptional control and cancer. *J. Cell Physiol.*, **184**, 1–16.

Desbaillets,I., Ziegler,U., Groscurth,P. and Gassmann,M. (2000) Embryoid bodies: an *in vitro* model of mouse embryogenesis. *Exp. Physiol.*, **85**, 645–651.

Dobosy,J.R. and Selker,E.U. (2001) Emerging connections between DNA methylation and histone acetylation. *Cell Mol. Life Sci.*, **58**, 721–727.

Donohoe,M.E., Zhang,X., McGinnis,L., Biggers,J., Li,E. and Shi,Y. (1999) Targeted disruption of mouse Yin Yang 1 transcription factor results in peri-implantation lethality. *Mol. Cell. Biol.*, **19**, 7237–7244.

Faust,C., Lawson,K.A., Schork,N.J., Thiel,B. and Magnuson,T. (1998) The Polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development*, **125**, 4495–4506.

Fischle,W., Dequiedt,F., Fillion,M., Hendzel,M.J., Voelter,W. and Verdin,E. (2001) Human HDAC7 histone deacetylase activity is associated with HDAC3 *in vivo*. *J. Biol. Chem.*, **276**, 35826–35835.

Fuks,F., Burgers,W.A., Godin,N., Kasai,M. and Kouzarides,T. (2001) Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J.*, **20**, 2536–2544.

Gray,S.G. and Ekström,T.J. (2001) The human histone deacetylase family. *Exp. Cell Res.*, **262**, 75–83.

Grayson,J.M., Murali Krishna,K., Altman,J.D. and Ahmed,R. (2001) Gene expression in antigen-specific $CD8^+$ T cells during viral infection. *J. Immunol.*, **166**, 795–799.

Grozinger,C.M., Hassig,C.A. and Schreiber,S.L. (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl Acad. Sci. USA*, **96**, 4868–4873.

Hassig,C.A., Tong,J.K., Fleischer,T.C., Owa,T., Grable,P.G., Ayer,D.E. and Schreiber,S.L. (1998) A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc. Natl Acad. Sci. USA*, **95**, 3519–3524.

Henrique,D., Adam,J., Myat,A., Chitnis,A., Lewis,J. and Ish Horowicz,D. (1995) Expression of a Delta homologue in prospective neurons in the chick. *Nature*, **375**, 787–790.

Hogan,B., Beddington,R., Costantini,F. and Lacy,E. (1994) *Manipulating the Mouse Embryo. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Humphrey,G.W., Wang,Y., Russanova,V.R., Hirai,T., Qin,J., Nakatani,Y. and Howard,B.H. (2001) Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. *J. Biol. Chem.*, **276**, 6817–6824.

Jenuwein,T. (2001) Re-SET-ting heterochromatin by histone methyltransferases. *Trends Cell Biol.*, **11**, 266–273.

Jenuwein,T. and Allis,C.D. (2001) Translating the histone code. *Science*, **293**, 1074–1080.

Khier,H., Bartl,S., Schuettengruber,B. and Seiser,C. (1999) Cloning and characterization of the mouse histone deacetylase 1 gene: integration of a retrovirus in 129SV mice. *Biochim. Biophys. Acta*, **1489**, 365–373.

Khochbin,S., Verdel,A., Lemercier,C. and Seigneurin Berny,D. (2001) Functional significance of histone deacetylase diversity. *Curr. Opin. Genet. Dev.*, **11**, 162–166.

Kramer,O.H., Gottlicher,M. and Heinzel,T. (2001) Histone deacetylase as a therapeutic target. *Trends Endocrinol. Metab.*, **12**, 294–300.

Lechner,T., Lusser,A., Brosch,G., Eberharter,A., Goralik Schramel,M. and Loidl,P. (1996) A comparative study of histone deacetylases of plant, fungal and vertebrate cells. *Biochim. Biophys. Acta*, **1296**, 181–188.

Lo,W.S., Trievel,R.C., Rojas,J.R., Duggan,L., Hsu,J.Y., Allis,C.D., Marmorstein,R. and Berger,S.L. (2000) Phosphorylation of serine 10 in histone H3 is functionally linked *in vitro* and *in vivo* to Gcn5-mediated acetylation at lysine 14. *Mol. Cell*, **5**, 917–926.

Luger,K., Mader,A.W., Richmond,R.K., Sargent,D.F. and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, **389**, 251–260.

Luo,J., Nikolaev,A.Y., Imai,S.-I., Shiloh,A., Guarente,L. and Gu,W. (2001) Negative control of p53 by Sir2 promotes cell survival under stress. *Cell*, **107**, 137–148.

Nakayama,J., Rice,J.C., Strahl,B.D., Allis,C.D. and Grewal,S.I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science*, **292**, 110–113.

Ng,H.H. and Bird,A. (2000) Histone deacetylases: silencers for hire. *Trends Biochem. Sci.*, **25**, 121–126.

O'Carroll,D., Erhardt,S., Pagani,M., Barton,S.C., Surani,M.A. and Jenuwein,T. (2001) The polycomb-group gene *ezh2* is required for early mouse development. *Mol. Cell. Biol.*, **21**, 4330–4336.

Peters,A.H.F.M. *et al*. (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell*, **107**, 323–337.

Rea,S. *et al*. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature*, **406**, 593–599.

Schluter,C., Duchrow,M., Wohlenberg,C., Becker,M.H., Key,G., Flad,H.D. and Gerdes,J. (1993) The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *J. Cell Biol.*, **123**, 513–522.

Schuchner,S. and Wintersberger,E. (1999) Binding of polyomavirus small T antigen to protein phosphatase 2A is required for elimination of p27 and support of S-phase induction in concert with large T antigen. *J. Virol.*, **73**, 9266–9273.

Sowa,Y., Orita,T., Minamikawa,S., Nakano,K., Mizuno,T., Nomura,H. and Sakai,T. (1997) Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. *Biochem. Biophys. Res. Commun.*, **241**, 142–150.

Strahl,B.D. and Allis,C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41–45.

Taplick,J., Kurtev,V., Lagger,G. and Seiser,C. (1998) Histone H4 acetylation during interleukin-2 stimulation of mouse T cells. *FEBS Lett.*, **436**, 349–352.

Taunton,J., Hassig,C.A. and Schreiber,S.L. (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science*, **272**, 408–411.

Turner,B.M. (2000) Histone acetylation and an epigenetic code. *Bioessays*, **22**, 836–845.

van Holde,K.E. (1988) *Chromatin*. Springer, New York, NY.

Vaziri,C., Stice,L. and Faller,D.V. (1998) Butyrate-induced G_1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. *Cell Growth Differ.*, **9**, 465–474.

Vaziri,H., Dessain,S.K., Ng Eaton,E., Shin-Ichiro Imai,S.-I., Frye,R.A., Pandita,T.K., Guarente,L. and Weinberg,R.A. (2001) *hSIR2 SIRT1* functions as an NAD-dependent p53 deacetylase. *Cell*, **107**, 149–159.

Vidal,M. and Gaber,R.F. (1991) RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 6317–6327.

Wharton,W., Savell,J., Cress,W.D., Seto,E. and Pledger,W.J. (2000) Inhibition of mitogenesis in Balb/c-3T3 cells by trichostatin A. Multiple alterations in the induction and activation of cyclin–cyclin-dependent kinase complexes. *J. Biol. Chem.*, **275**, 33981–33987.

Wilkinson,D.G., Bhatt,S. and Herrmann,B.G. (1992) Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature*, **343**, 657–659.

Yang,W.-M., Inouye,C., Zeng,Y., Bearss,D. and Seto,E. (1996) Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc. Natl Acad. Sci. USA*, **93**, 12845–12850.

Yang,W.M., Yao,Y.L., Sun,J.M., Davie,J.R. and Seto,E. (1997) Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. *J. Biol. Chem.*, **272**, 28001–28007.

Zhang,H.S. and Dean,D.C. (2001) Rb-mediated chromatin structure regulation and transcriptional repression. *Oncogene*, **20**, 3134–3138.

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