Reduced Body Size and Decreased Intestinal Tumor Rates in HDAC2-Mutant Mice

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Abstract

Histone deacetylases (HDAC) reverse the acetylation of histone and nonhistone proteins and thereby modulate chromatin structure and function of nonhistone proteins. Many tumor cell lines and experimental tumors respond to HDAC inhibition. To assess the role of an individual HDAC isoenzyme in physiology and tumor development, HDAC2-mutant mice were generated from a gene trap embryonic stem cell clone. These mice express a catalytically inactive fusion protein of the NH_2-terminal part of HDAC2 and β-galactosidase, which fails to integrate into corepressor complexes with mSin3B. They are the first class 1 HDAC mutant mice that are viable although they are ~25% smaller than their littermates. Cell number and thickness of intestinal mucosa are reduced. Mutant embryonic fibroblasts fail to respond to insulin-like growth factor I (IGF) by the IGF-I–induced increase in cell number observed in wild-type cells. These data suggest a novel link between HDACs and IGF-I–dependent responses. Crossing of HDAC2-mutant with tumor-prone APC^{min} mice revealed tumor rates that are lower in HDAC2-deficient mice by 10% to 100% depending on segment of the gut and sex of the mice. These mice provide evidence that the key functions of HDAC2, although not essential for survival of the organism, play a rate-limiting role for tumor development *in vivo*. [Cancer Res 2007;67(19):9047–54]

Introduction

Reversible acetylation of histone proteins is considered to affect local structure of chromatin. Complex patterns of posttranslational modifications of chromatin including acetylation of histones establish combinatorial signals that are apparently interpreted by chromatin-binding proteins and the gene expression machinery to control transcriptional activity of individual genes. Histone acetylation is achieved by histone acetyltranferase whose activity is counteracted by histone deacetylases (HDAC; refs. 1, 2). Both histone acetyltranferases and HDACs are recruited to target genes through specific transcription factors and corepressor complexes (3, 4). Nuclear chromatin does not seem to be the only target of HDACs because nonhistone proteins, such as the p53 tumor suppressor, hypoxia-inducible factor 1, signal transducers and activators of transcription (STAT)-1 and STAT3, or the Ku70 protein, can be acetylated on lysine residues and have been identified as HDAC substrate proteins (5, 6). Acetylation of nonhistone proteins modulates protein-protein interactions, cellular localization, or protein stability by competition for lysine ubiquitination (7–10).

HDACs comprise four families of enzymes among which the Sir2-like enzymes in class 3 are structurally not related to the other classes. Mammalian class 1 enzymes HDAC1, HDAC2, HDAC3, and HDAC8 are related to yeast RPD3. Class 2 enzymes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 are homologous to yeast HDA1. HDAC11 constitutes its own class (i.e., class 4) with similarities to both class 1 and class 2 enzymes (4, 5, 11, 12).

Whereas the principles of action of HDACs in control of gene expression and protein function are well documented, little is known about those physiologic processes that would depend on proper function of individual HDAC isoenzymes. There are several compounds available for research in animals or clinical trials that inhibit HDAC activities (5, 13). They have a diverse spectrum of effects in the normal body (5). However, it is not clear whether the observed effects are related to the HDAC inhibitory activities or to other nonrelated activities of the compounds. For example, the HDAC inhibitor valproic acid is used over decades as an antiepileptic drug with little side effects except for teratogenicity (i.e., neural tube closure defects) in the developing embryo. Structure-activity relationships indicate that the effects on the central nervous system are not related to the HDAC inhibitory effect whereas teratogenicity is the only obvious adverse effect that is due to inhibition of HDACs (14, 15). Because those inhibitors available at present only poorly discriminate between individual isoenzymes and apparently do not achieve complete inhibition at the used doses, most information on the role of individual HDACs could only be revealed from knockout studies. The lack of individual HDAC isoenzymes affects embryonic proliferation, heart physiology, endothele function, and bone formation, indicating that all of the HDAC isoenzymes analyzed thus far serve at least some key physiologic functions that cannot be substituted for by other isoenzymes (16–20).

The concept that aberrant function of the HDAC-dependent transcriptional repression machinery plays a role in cancer was established in acute myeloid leukemia by showing that fusion proteins of the retinoic acid receptor α encoded from chromosomal translocations aberrantly repress transcription by recruitment of corepressors and HDACs (21). Based on such findings, HDAC inhibitors were used in successful treatment of specific forms of hematopoietic malignancies (5, 22–25). Much less is known about aberrant transcriptional repression and HDACs in carcinomas although HDAC inhibition in many carcinoma cell

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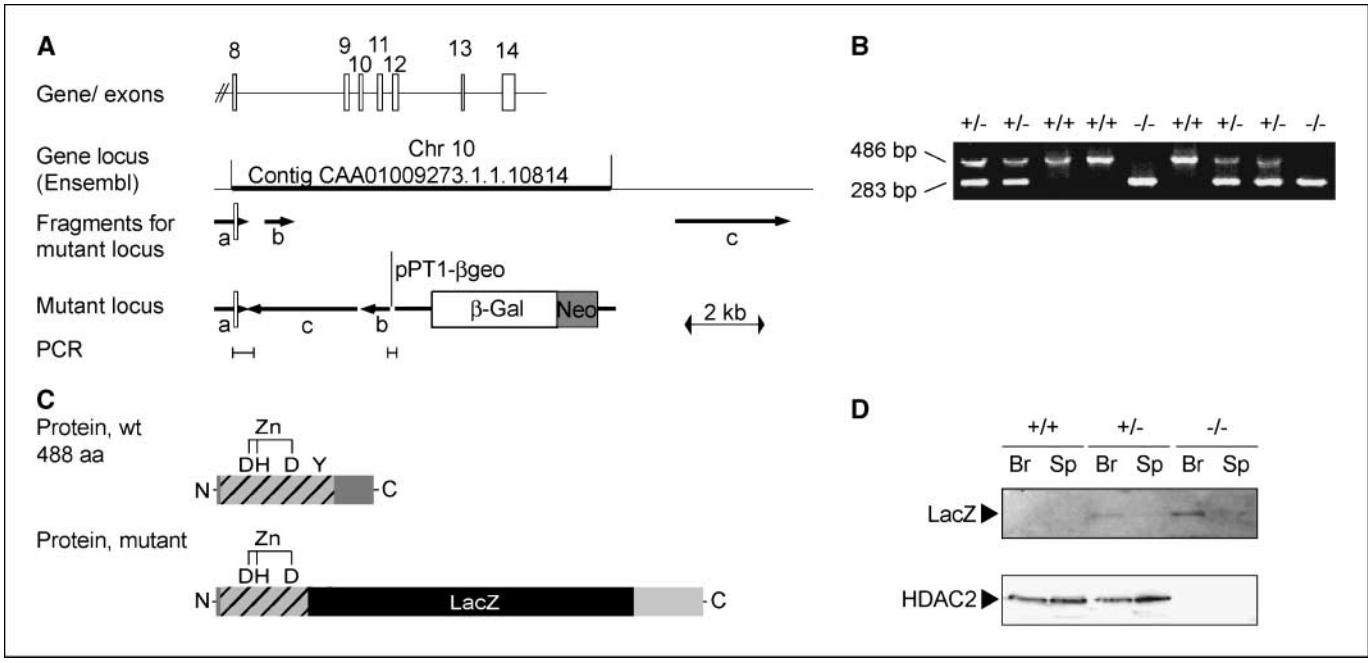

Figure 1. Insertion of the pPT1-βgeo targeting vector into the *HDAC2* gene results in expression of a HDAC2-LacZ fusion protein. *A,* wild-type and mutant *HDAC2* gene loci were amplified by PCR using primers in exon 8, exon 9, and the LacZ cassette and sequenced. Homologous sequences in wild-type and mutant gene loci are marked by letters *a* to *c*, with arrowheads indicating orientation. *B,* a representative genotype analysis from tail DNA by PCR is shown. *C,* structures of wild-type and mutant proteins. Letters indicate amino acids considered essential for HDAC activity. *D,* expression of the wild-type and LacZ fusion proteins was confirmed by Western blot analysis from brain and spleen whole-tissue extracts.

lines induces cell cycle arrest or cell death (25). EZH2 might serve as a HDAC-dependent transcriptional (co-)repressor in prostate cancer (26), and increased expression of HDAC2 on mutation of adenomatous polyposis coli (APC) might promote colonic cancer (27). The up-regulation of HDAC2 in colonic cancer (27) and the elevated levels of HDAC2 in many cell lines derived from epithelial cancers compared with normal primary cells from the same tissues (28) are prominent examples that an individual HDAC (i.e., HDAC2) might serve a key role in the development of some forms of cancer.

The aim of this study was to test whether HDAC2 serves a rate-limiting role in intestinal tumor formation. It was equally important to assess whether there are HDAC2 functions that cannot be substituted for by other HDACs during development and physiologic function of the whole organism. Because, at present, an isoenzyme-specific inhibitor is not available, the study relied on genetic inactivation of HDAC2 in mice and crossing with tumor-prone APC^{min} mice.

We now show for the first time that inactivation of a class 1 HDAC is compatible with survival of mutant embryos and mice. Nevertheless, mutant mice are smaller than their wild-type littermates, and fibroblasts derived from mutant embryos respond poorly to insulin-like growth factor I (IGF). Development of intestinal tumors due to mutation of APC is reduced in HDAC2-mutant mice, indicating that a single HDAC isoform plays a key role during intestinal tumor development.

Materials and Methods

Animal handling and analysis. Breeding of mice followed standard protocols with the addition of providing wet food to newborns and late weaning. Skeletons (29) were stained as published. APC^{min} and *HDAC2*-mutant mice were crossed by *HDAC2* heterozygous breeding with male APC^{min} mice. For tumor counting, sections of intestines fitting individual microscopic slides were rinsed in cold PBS, longitudinally cut open, and then fixed in 4% buffered formaldehyde for 4 h, stained with methylene blue, and examined by microscope (2.5× object lens). For histologic evaluation, rolls of intestinal segments were prepared with the proximal ends of the segment in the center.

Cell culture. Mouse embryonic fibroblasts (MEF) were harvested from E13.5 mouse embryos following a standard protocol.[6] For quantitative analysis of apoptosis induction, $\sim 5 \times 10^3$ cells were seeded per well in 96-well plates. IGF-I or IGF-II (Sigma) was added 5 h after seeding (20 ng/mL). Medium was collected after culture and analyzed for fragmented DNA indicating apoptosis using the Cell Death Detection $ELISA^{PLUS}$ Kit (Roche). Cells were counted in a Neubauer chamber.

Biochemical analyses. Extracts were prepared in NET-N buffer [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Igepal, 10% glycerol] with protease inhibitors (1:200, Sigma) at 4°C by Dounce homogenization (brain) or sonication (cells). Immunoprecipitates were prepared from 700 μg of extract by incubation with 1 μg of antibody overnight at 4°C. Antibodies against HDAC1 (for Western blot and immunohistochemistry), HDAC2, HDAC3, HDAC8, mSin3B, and actin were from Santa Cruz Biotechnology; anti-HDAC2 was from Upstate Biotechnologies; and anti–β-galactosidase was from Roche.

Recombinant expression of mutant HDAC2 proteins. Expression vectors for full-length HDAC2, HDAC2 truncated after amino acid 288, and the HDAC2 truncated-*lacz* fusion encoded by the mutant *HDAC2* gene locus, together with HDAC1 for comparison, were constructed in pME18S including FLAG tags at the NH_2 terminus. The vectors were transfected into Phoenix cells with the calcium phosphate method.

Immunoprecipitation with anti-FLAG antibodies and analysis of HDAC activity were done as previously described (30).

[6] http://www.molgen.mpg.de/~rodent/MEF_protocol.pdf

Histochemical analysis. Standard procedures of paraformaldehyde fixation and paraffin embedding were used. Immunohistochemistry was done with the antibodies used for Western blotting following antigen retrieval by boiling in citrate buffer and using a horseradish peroxidase/avidin-biotin complex/3,3′-diaminobenzidine system for detection.

Results

Embryonic stem cells carrying a mutant HDAC2 locus were obtained from a library of embryonic stem cell clones that had been generated by random insertional mutagenesis with the pPT1-βgeo gene trap vector containing intronic sequences and a splice acceptor site from the *engrailed-2* gene, the *LacZ* coding sequence, and a neomycin resistance cassette for selection.[7] In the clone W035F03,[7] the 5′-rapid amplification of cDNA ends (RACE) product extending from the LacZ exon matched the HDAC2 cDNA (GenBank accession no. NM_008229, nucleotides 657–841 of coding sequence; ref. 31). The RACE product corresponds to parts of exon 7 and complete exon 8 of the *HDAC2* gene (32). The mutant locus was isolated into a PCR product of ~5 kb with primers in exon 8 and the gene trap vector, and its sequence was read (Fig. 1*A*). Comparison with the wild-type gene locus (Ensembl murine genome browser) revealed rearrangements in addition to insertion of the vector. A fragment of ~3 kb from a region downstream of the last exon had been inverted and inserted after exon 8 followed by a smaller inverted fragment of the original intron between exons 8 and 9. Thereafter, the vector was integrated with ~1 kb intronic sequence still present in the mutant *HDAC2* gene locus. Fidelity of the sequence was confirmed by generating PCR products spanning critical fusion sites between different segments from genomic DNA of embryonic stem cells and later of mutant mice (Fig. 1*A*). Mutant mice were generated from this embryonic stem cell clone by injection into blastocysts from C57Bl6 mice and backcrossing for three to seven generations on the C57Bl6 background.

The mutant gene codes for an inactive in-frame fusion product between HDAC2 and LacZ. The catalytic core is expected to be disrupted because a tyrosine residue considered to be critically important for catalysis is lost in the mutant protein (Fig. 1*C*; ref. 33). Expression of wild-type protein is lost in *HDAC2*-mutant mice whereas the mutant protein is detectable with an antibody directed against LacZ (Fig. 1*D*). The fusion protein is expressed during embryogenesis as shown by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of a mutant E13.5 embryo (data not shown). The staining of many structures is consistent with the broad expression pattern of HDAC2 found in adults (28).

Function of the HDAC2-LacZ fusion protein was characterized with respect to catalytic activity and integration into a corepressor complex with mSin3B. FLAG-tagged wild-type and mutant HDAC2 were expressed and their activities were determined in immunoprecipitates (Fig. 2*A*). The same amounts of immunoprecipitated mutant proteins did not show any activity above vector control (Fig. 2*A*). An antibody against HDAC2 coprecipitated mSin3B from wild-type tissue or MEF extracts (Fig. 2*B*). Precipitation with the HDAC2 antibody is specific because no coprecipitation of mSin3B was found in extracts from HDAC2-mutant mice or MEFs. The mutant HDAC2-LacZ fusion protein cannot be recognized by the available antibodies directed against the COOH terminus of HDAC2. The HDAC2-LacZ fusion protein was therefore precipitated with an antibody directed against LacZ, and mSin3B did not coprecipitate (Fig. 2*B*). Efficiency of precipitation is estimated to be approximately 30% to 50% by comparing amounts of HDAC2 in precipitates and supernatants or depletion of LacZ activity from immunoprecipitation supernatants (data not shown). Coprecipitation of HDAC2 wild-type protein, but not of the mutant fusion protein, with LacZ was also found in the reverse experiment (i.e., precipitations with an antibody against mSin3B; Fig. 2*C*). Furthermore, in HDAC2-mutant extracts, increased amounts of HDAC1 were coprecipitated with mSin3B (Fig. 2*C*) whereas detectable HDAC8 levels were low in either extract. In conclusion, the HDAC2-LacZ fusion protein has lost essential functions of

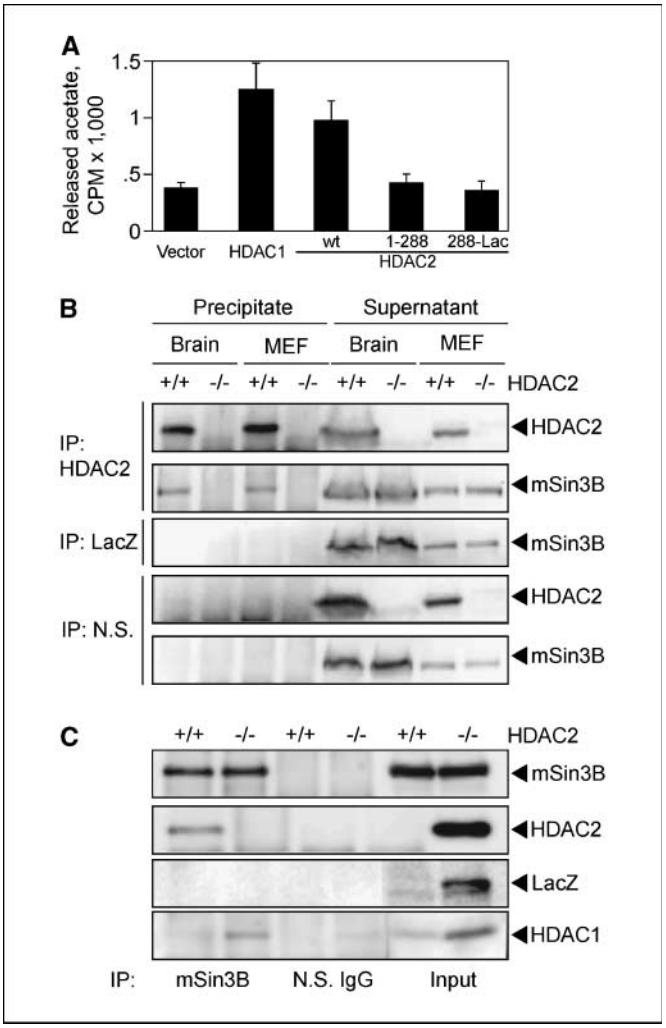

Figure 2. Lack of integration of the HDAC2-LacZ fusion protein into the mSin3B corepressor complex. *A,* equal amounts of immunoprecipitates of cells transfected with control vector or expression vectors for FLAG-tagged HDAC1, HDAC2, HDAC2 truncated after amino acid 288 (HDAC2_1-288), or the HDAC2-LacZ fusion protein encoded by the mutant *HDAC2* locus (HDAC2_288-Lac) were assayed for HDAC activity. *Columns,* average released ^{3}H-labeled acetate from three independent experiments; *bars,* SD. *B,* integration of wild-type HDAC2 into a corepressor complex was analyzed by coimmunoprecipitation of mSin3B with antibodies against HDAC2 or LacZ from brain or MEF extracts. Amounts of HDAC2 in immunoprecipitates and 30% of the supernatant after immunoprecipitation were compared with the estimated efficiency of precipitation. Immunoprecipitation with a nonspecific immune serum (*N.S.*) was done for control. *C,* coimmunopreciptitation of HDAC2, LacZ, or HDAC1 was analyzed after precipitation with an antibody against mSin3B. Representative results of at least two independent experiments.

[7] Available from: http://genetrap.de; search for vectors and clone ID.

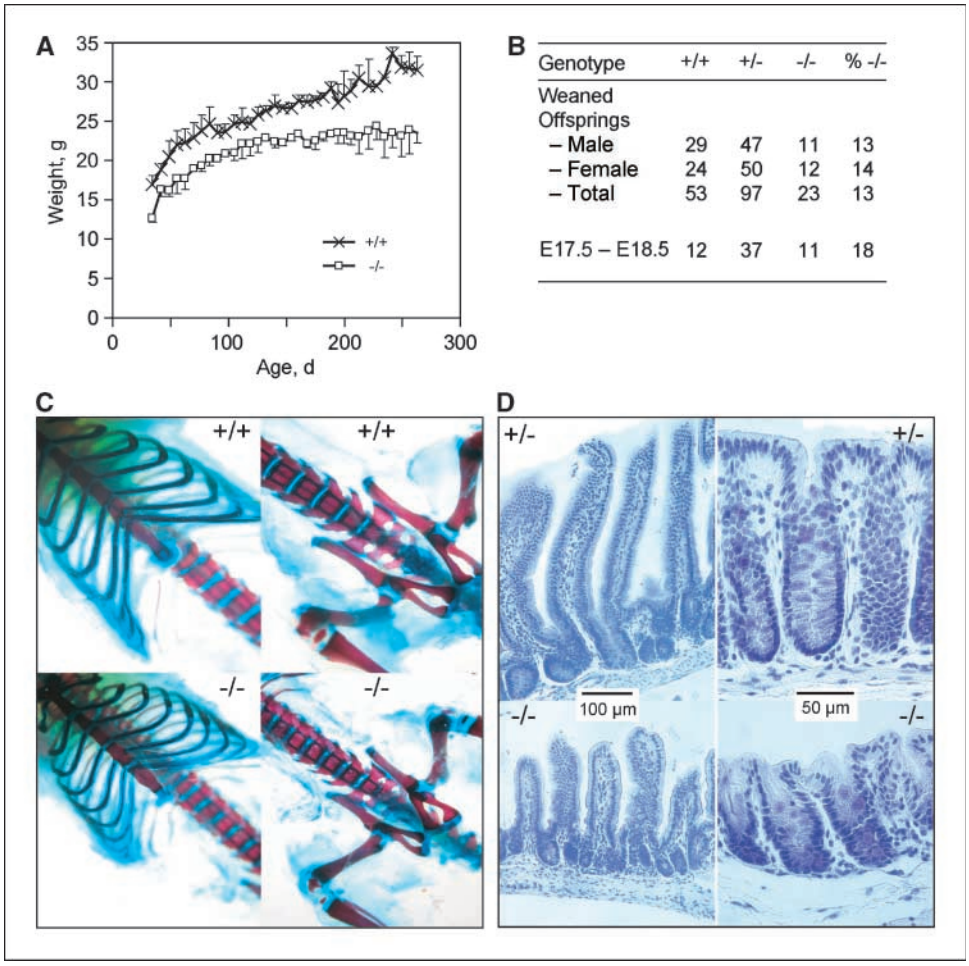

Figure 3. Reduced size and body weight of HDAC2-deficient mice. *A,* body weight was determined weekly. Results from two wild-type and two HDAC2-mutant female mice of the same date of birth. Similar results were obtained for males. *B,* genotypes from a breed of heterozygous animals on a mixed genetic background of 129Sv and C57Bl6 were collected over a 4-wk period at time of weaning; results are summarized in the table. Timed pregnancies from heterozygous parents were also evaluated for representation of embryonic genotypes. *C,* bones (Alizarin red) and cartilage (Alcian blue) were stained in 14-day-old male littermates and photographs were taken of the axial skeleton. Similar results were obtained in a second independent experiment. *D,* upper small intestine and colon of 4-week-old mice were stained with H&E. Representative frames from three pairs of littermates. Results from wild-type and heterozygous animals were identical.

HDAC2 such as catalytic activity and integration into mSin3B-containing corepressor complexes.

The prominent phenotype of HDAC2-mutant mice is a reduction in body size. Size of mutant offsprings at the time of weaning is reduced (Fig. 3*A*). Differences in body weight persist under our conditions of animal housing over lifetime. The difference at the age of 95 weeks was confirmed in an additional experiment that showed no difference between wild-type (n = 8) and heterozygous (n = 9) female mice whereas the weight of HDAC2-mutant mice (n = 6) was reduced by 40%. Genotype determination at the age of weaning indicated the loss of about half of the mutant mice (Fig. 3*B*). Recovery could be improved by proper care to support survival of smaller and apparently weaker mutant offsprings. Recovery of mutant embryos without apparent defects just before birth at a frequency only slightly lower (18%) than expected from Mendelian ratios (25%) supports the conclusion that most of the mutant mice are lost after birth due to cannibalism of smaller and weaker pups (Fig. 3*B*). On the basis of 50 embryos, the percentage of mutants seems to be already slightly reduced from E10.5 to E13.5 onward and some mutant embryos seem to be smaller than wild-type embryos at that age (data not shown). Otherwise, no obvious alterations were found by macroscopic and histologic evaluation at E7, E13.5, E15.5, or E18.5 so that the reason for the slightly reduced recovery of mutant embryos remains unclear. HDAC2-mutant mice have normal life spans because death rates are similar in wild-type and mutant mice up to 95 weeks.

Reduction in weight of adult mice differentially affects the different compartments of the body. For example, heart weights of wild-type and mutant mice are almost identical, whereas weights of brains and testes were decreased in mutant mice by ~25% (data not shown). Histologic examination of liver, heart, brain, and thymus did not reveal obvious differences between 9-month-old wild-type and mutant animals. Alterations in hematopoiesis might have been expected based on the role of HDACs in leukemia and the effects of HDAC inhibitors on hematopoietic stem cells (34). However, cell counts from peripheral blood at 9 months of age did not show alterations in mutant mice with respect to erythrocytes, granulocytes, platelets, and lymphocytes including B-cell, T-helper, and CTL subpopulations (data not shown). Homozygously *HDAC2*-mutant females are subfertile due to unknown reasons.

The axial skeleton of young mice revealed reduced size including smaller vertebrae and hip bones (Fig. 3*C*). Other malformations such as vertebral defects were not found. In addition, there was no indication for enhanced ossification of the rib cartilages as has been described for *HDAC4*-mutant mice. An additional consequence of HDAC2 deficiency was observed in the intestines of 1- to 4-month-old mice. Macroscopically, thickness and length of the gut were reduced in mutant mice (data not shown). Mucosa thickness was reduced by about a third both in small intestine and in the colon. Representative frames from the upper jejunum (Fig. 3*D, left*) and colon (Fig. 3*D, right*) are shown. In colon, cell numbers of crypts are reduced proportionally to crypt depth by 32%

($P < 0.0003$) and 29% ($P < 0.003$), respectively. Thus, numbers rather than cell sizes are affected. Representation of different cell types in the intestine was comparable between all genotypes. In summary, reduced body size and slightly thinner intestinal mucosa were the prominent observed phenotypes of HDAC2-mutant mice.

Primary fibroblasts were prepared from E13.5 embryos to test the hypothesis that relevant aspects that control growth *in vivo* might be conserved in cultured cells. Growth indicated by cell numbers was identical in cultures established from wild-type or HDAC2-mutant embryos during a 3T3 protocol over 35 passages (Fig. 4*A*). In addition, sizes of cells as assessed by the forward scatter signal in flow cytometry and cell morphology did not differ (data not shown). Abundance of key regulators of cell cycle progression is similar in wild-type and HDAC2-deficient cells with only a slightly increased expression of $p21^{Cip}$ and cyclin E as well as a slightly decreased expression of $p27^{Kip1}$ (data not shown).

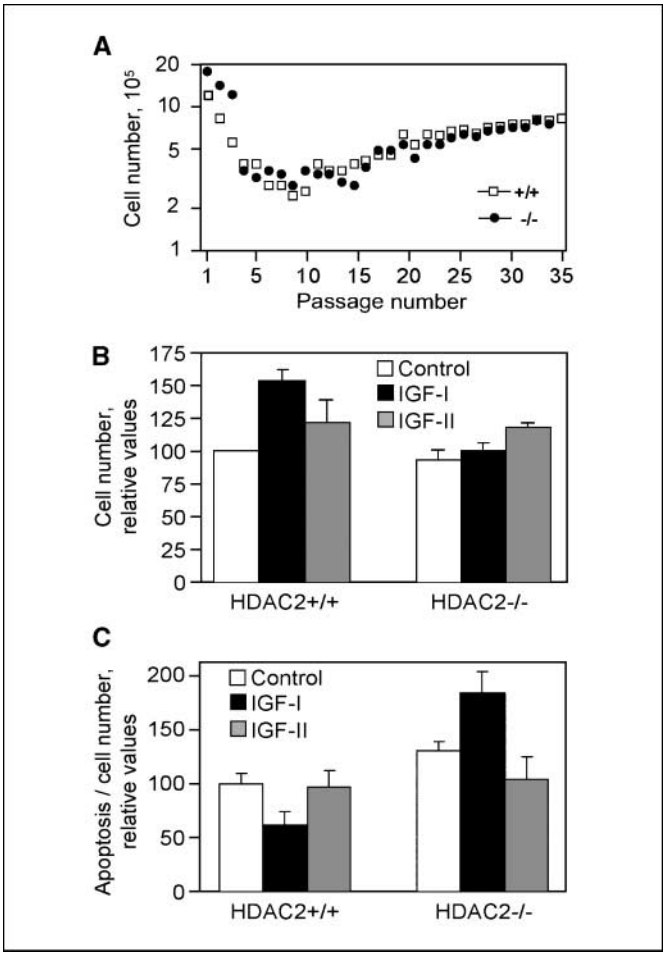

Figure 4. Reduced response to IGF-I of HDAC2-deficient embryonic fibroblasts. *A,* MEFs were established according to a 3T3 protocol from wild-type and homozygously HDAC2-deficient E13.5 embryos. At each passage, 150,000 cells were seeded and the number of cells after 3 d of culture is shown. *Points,* mean of triplicate determinations, each with two independent preparations of MEFs per genotype. *B,* identical numbers of MEFs were seeded per culture and stimulated by IGF-I or IGF-II (20 ng/mL each). Cell numbers were counted after 3 d when cultures were almost confluent, and the number in untreated wild-type cultures was set to 100%. *Columns,* average from seven (IGF-I) or four (IGF-II) experiments with two independent pools of MEFs per genotype; *bars,* SD. *C,* rates of apoptosis were determined after the 3rd day of culture by measuring total released fragmented DNA and dividing that value by the cell number. *Columns,* average from three independent experiments; *bars,* SD.

Because IGFs are key regulators of growth, we tested whether the responsiveness to IGFs is impaired in HDAC2-mutant MEFs. IGF-I and, to a lesser extent, IGF-II stimulated growth of wild-type MEFs as assessed by accumulation of cells in the nearly confluent state at 72 h of culture (Fig. 4*B*). This number of cells reflects the equilibrium between proliferation, sensitivity to contact inhibition, and cell loss by apoptosis. The IGF-I–induced increase in cell number was absent in HDAC2-mutant cultures whereas the response to IGF-II was not impaired. The differential response is plausible because the concentration of IGF-II was apparently not sufficient to fully stimulate the IGF-I receptor pathway and IGF-II signals to additional pathways (35). The difference between wild-type and mutant cells in the response to IGF-I could not be explained by differences in the rate of cell proliferation. Both wild-type and HDAC2-mutant cultures showed comparable basal and slightly IGF-inducible rates of proliferation as assessed by DNA synthesis rates per total culture in the confluent (72 h) state (data not shown). IGF-I treatment reduced the rate of apoptosis per number of cells by 40% in wild-type, but not in HDAC2-mutant, cells (Fig. 4*C*). The response of apoptosis rates to IGF-I could fully account for the increased number of cells in the confluent state of wild-type cells and the lack of response in HDAC2-mutant cultures. In HDAC2-mutant cells, rates of apoptosis were even increased by IGF-I, possibly to compensate the IGF-I–induced rate of proliferation in confluent cultures (Fig. 4*C*). These data suggest that IGF-I responsiveness and, particularly, the antiapoptotic activity of IGF-I are impaired in HDAC2-mutant MEFs.

Impaired responsiveness to IGFs might account at least for some aspects of the phenotype in HDAC2-deficient mice, but nevertheless, the questions remain: Why are only certain tissues affected? Why is the lack of HDAC2 not compensated by other class 1 HDACs? In this study, the hypothesis was tested that the structures affected most are those in which the expression of other class 1 HDACs is low compared with nonaffected structures. In colon mucosa, HDAC8 expression substantially decreases toward the apical parts of the crypts (Fig. 5), whereas HDAC3 is expressed throughout all levels of the mucosa at comparable levels (data not shown). HDAC1 expression is substantially lower in colon than in small intestine (Fig. 5). In small intestine, HDAC8 is barely detectable (data not shown) and HDAC3 levels decrease substantially from the bottoms of the crypts toward the tips of the villi (Fig. 5). Expression patterns and levels are similar in wild-type and HDAC2-mutant mice (data not shown). In summary, cells in different segments of the gut and parts of the mucosa express different levels and combinations of class 1 HDACs. Therefore, it is plausible that particularly toward the apical parts of the mucosa, combinations occur that are not sufficient to compensate the lack of HDAC2 in mutant mice.

Elevated levels of HDAC2 protein expression had been found in human colonic carcinomas and murine intestinal tumors induced by APC mutation, and survival of colonic carcinoma cells seemed to critically depend on HDAC2 expression (27). To test the role of elevated HDAC2 expression in intestinal tumor development *in vivo*, we crossed *HDAC2*-mutant with APC^{min} mice and assessed intestinal tumor formation (Fig. 6). Tumor numbers were significantly reduced in HDAC2-deficient mice compared with HDAC2 wild-type APC^{min} mice in all segments of the gut. As expected from the APC^{min} mouse model, most of the tumors were found in the small intestine, but also few tumors were detected in duodenum or colon. Consistently, the numbers of

tumors were lower in the HDAC2-deficient mice but the extent varied between 10% and 100%, depending on the segment of the gut and, remarkably, the gender of the mice. In the group with the highest tumor incidence (i.e., the small intestine of female mice), tumor number was reduced by ~60% in HDAC2-deficient animals, and in the colon of HDAC2-deficient male APC^{min} mice, no single tumor was found at all. The median tumor number in colon both from HDAC2-deficient male and female mice was zero. These effects on tumor rates are substantially more pronounced than those on thickness of normal gut mucosa and indicate that HDAC2 is not only present at elevated levels in tumors of the gut but also plays an important role for tumor development *in vivo*.

Discussion

Each HDAC isoform that has been analyzed by genetic inactivation in mice serves functions that cannot be compensated for by other HDACs. The phenotype of mice expressing a mutant HDAC2, which lacks catalytic activity and integration into mSin3B-dependent corepressor complexes, indicates that the key functions of HDAC2, although not essential for survival, play important roles for growth, body size, thickness of intestinal mucosa, and intestinal tumor development.

Specific phenotype of HDAC2-deficient mice. HDAC2 is the first class 1 HDAC whose mutation is compatible with survival of mice. The phenotype of reduced body size, weight, and female fertility is also apparent in homozygously mutant mice after breeding on the C57Bl6 background for seven generations. Deficiencies in other HDACs show phenotypes different from that of HDAC2-mutant mice. Lack of HDAC1 is embryonic lethal presumably due to increased expression of cell cycle inhibitors (16). The knockouts of HDAC5 and HDAC9 show phenotypes in cardiac morphology and an overshooting hypertrophic response to stress (17, 18, 36). In contrast, HDAC2-mutant mice are resistant to the induction of cardiac hypertrophy (37). HDAC7 plays a critical role for endothelial cell-cell adhesion, and therefore HDAC7-mutant embryos die by E11 due to impaired vessel integrity (20). The body size of HDAC4-deficient mice is reduced apparently similarly to that of HDAC2-mutant mice. The HDAC4 phenotype has been attributed to premature ossification during enchondral bone formation (19). The phenotype of HDAC2-mutant mice, however, has other causes because we did not observe enhanced ossification in, for example, rib cartilage.

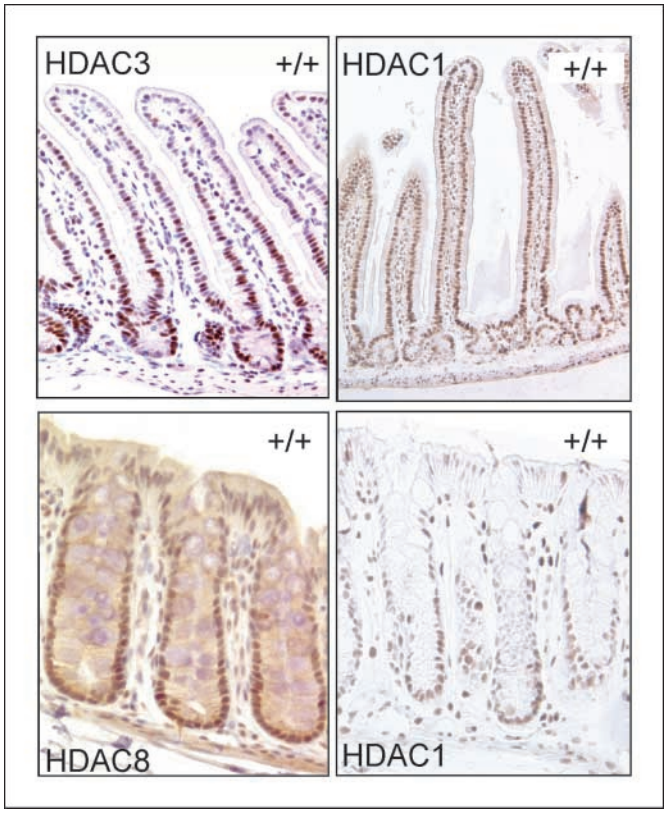

Figure 5. Expression of class 1 HDAC isoenzymes in the gut. Class 1 HDACs were detected by immunohistochemical staining of wild-type mucosae of colon and small intestine. Representative frames for differential expression in various parts of the mucosa. Frames from small intestine and colon were stained for HDAC1 under identical conditions and staining intensities were reproduced by identical procedures.

The loss of individual HDAC isoenzymes may induce different phenotypes due to several reasons such as expression patterns of different HDACs in a certain tissue, different requirements for individual HDACs for composition of specific corepressor complexes, or different specificities of individual HDACs for partner and target proteins (38). Our data suggest that HDAC1 can substitute for HDAC2 in mSin3B complexes but not fully compensate the lack of HDAC2 functions *in vivo*. It is plausible that HDAC2 cannot compensate the lack of HDAC1 in the embryo around E7.5 because HDAC2 expression is low during early embryogenesis (16). It is also plausible that HDAC2-deficient embryos can survive this period of embryogenesis and develop their phenotype later in tissues where expression of HDAC1 and possibly other HDACs is too low to compensate the lack of HDAC2 functions. In support of this proposition, we could show that HDAC2 deficiency affects structures in which the abundance of other class 1 enzymes is relatively low, such as the apical parts of intestinal mucosa. Similarly, the cartilage primordia of vertebrae and skull bones in E13.5 embryos show low expression of class 1 HDACs compared with other tissues. The latter finding might explain why the phenotype of HDAC2 deficiency at that age is restricted to a subtle and apparently transient delay in skeletal growth (data not shown).

Phenotypic similarities of IGF-I–deficient and HDAC2-deficient mice. Reduced responsiveness of HDAC2-mutant MEFs to IGF-I suggests an alternative HDAC-dependent mechanism of growth control in addition to the increased expression of cell cycle inhibitors in HDAC1-mutant mice (i.e., the requirement of HDAC2 for intact IGF-I signaling). In MEFs, however, reduced responsiveness to IGF-I does not seem to depend on increased expression of inositol polyphosphate-5-phosphatase F (data not shown) that has been associated with the failure of the heart to respond to hypertrophic stimuli (37). Body size, chondrocyte proliferation, and responsiveness of gut mucosa to growth-stimulating hormones are reduced in IGF-I–deficient mice (39–41). Growth retardation is also induced by Akt/protein kinase B mutation in this pathway (42). Thus, similar phenotypes depending on mutations in HDAC2 or the IGF-I pathway support the hypothesis that the consequences of HDAC2 deficiency are mediated, at least in part, by reduced signaling through the IGF-I signaling pathway.

Role of HDAC2 in cancer development. Lack of HDAC2 has not been described in the context of human genetically inherited disease syndromes, but overexpression has been associated with

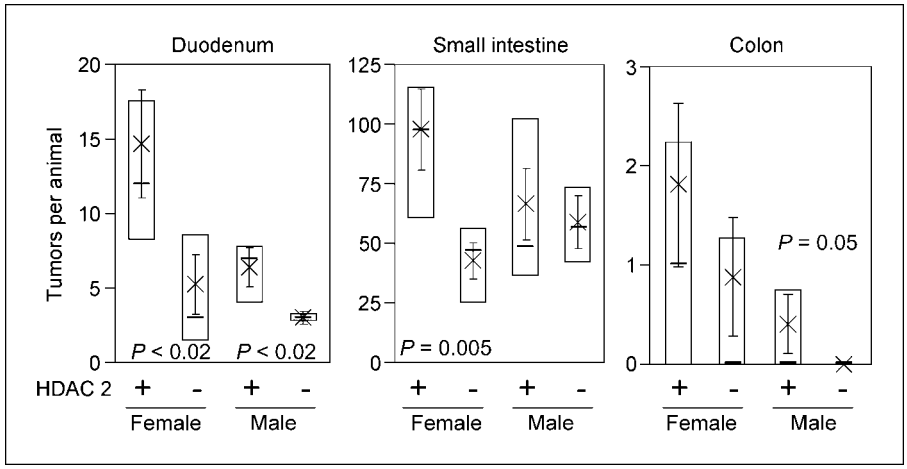

Figure 6. Intestinal tumor rates depend on HDAC2 expression in *APC*^{min} mice. HDAC2-deficient mice were crossed with *APC*^{min} mice and tumors in the different gut segments were counted at 110 d of age. *Crosses,* mean; *bars,* SE. *Columns,* 25th and 75th percentiles; *horizontal lines,* median tumor number. Numbers of animals were HDAC2 wild-type, 12 females and 10 males; HDAC2-mutant, 8 females and 4 males. Significance levels were calculated by Student's *t* test.

the phenotype of many cancer cell lines cultured *in vitro* (28) and colonic cancer *in vivo* (27, 43). The present study provides for the first time evidence that, indeed, a single HDAC (i.e., HDAC2) plays a key role during tumor development and that deficiency with respect to this single HDAC reduces tumor formation. Importantly, those regions of the gut that are affected most prominently are also the critical sites of tumor development on mutation of *APC* in humans (i.e., colon and duodenum; ref. 44). The precise mechanism(s) behind the tumor-suppressing activity of HDAC2 is not known, but the antiapoptotic activity shown previously (27) could likely be part of that mechanism. The modulation of IGF-I signaling is most likely a second mechanism because IGF signaling has been shown to promote colonic cancer development by experimental and epidemiologic approaches (45, 46).

It seems possible that HDAC2 plays a particularly important role in intestinal tumor formation because it serves functions that cannot be substituted for by other HDACs. Specificity might, however, simply rely on the expression patterns of HDAC2. Thus, it is only HDAC2, and not HDAC1 and HDAC3, that is overexpressed on loss of APC tumor suppressor function in cells and mice (27). Furthermore, abundance of individual HDACs in different segments of the gut might also provide an explanation for the differences in the susceptibility to tumor development on HDAC2 deficiency. HDAC8 was found to be only poorly expressed in the mucosa of the small intestine and more abundantly in the colon. Those HDACs that were more abundantly expressed in the small intestine were found at low levels in the colon (i.e., HDAC1 and HDAC3). Thus, the patterns of other class 1 HDACs that could compensate the lack of HDAC2 functions during tumor development are different between the segments of the gut and might differ in the efficiency to promote tumor development. There is, however, no indication for direct compensation of HDAC2 deficiency by increased expression of other class 1 HDACs in HDAC2-mutant mice (data not shown).

Genetic evidence for a specific role of HDAC2 for tumor development could lead to the development of inhibitors directed specifically against this specific isoenzyme. Development of inhibitors with selective isoenzyme specificities seems to be feasible on the level of enzyme inhibition (15, 47–49) and HDAC protein degradation (50). The genetically defined model expressing a mutant protein that is inactive and does not integrate into multiprotein complexes (e.g., corepressor) corresponds closely to drug-induced degradation of HDAC2 but not fully to inhibition of the enzyme. The lack of functional protein could, in some tissues, be compensated by recruitment of other HDACs such as HDAC1 into protein complexes. Inhibition of catalytic activity within the complex, however, would not permit this mechanism of compensation. Therefore, the effect of a specific inhibitor might be more pronounced than the lack of an individual isoenzyme. Certainly, the rate-limiting role of HDAC2 for intestinal tumor development is to be expected under either condition and could even be more pronounced on chemical inhibition compared with mutation. In addition, effects on physiologic conditions and dose-limiting toxicities could be more pronounced on application of a specific inhibitor compared with the mutation. However, one could expect that the side effects of a HDAC2-specific inhibitor are more restricted than those of broad-specificity inhibitors because the lack of functional HDAC2 in contrast to other HDACs permits almost unaffected embryonic and postnatal development.

In summary, the present mutational analysis indicates that intestinal tumor development in APC mutant mice is critically affected by the lack of HDAC2 key functions, whereas in other tissues this lack is tolerated or compensated to an extent that allows only minor impairments of embryonic development or physiology of the adult at most. In addition, human colorectal carcinogenesis is, in the majority of cases, related to increased expression of HDAC2. It has to be established to which extent and in which types of individual cases tumor progression and therapy response depend on this HDAC isoenzyme. Selective inhibition of HDAC2, when appropriate inhibitors become available, could help in solving these questions and become a novel component in therapy of colorectal cancer.

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