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Distinct roles of HDAC1 and HDAC2 in transcription and recombination at the immunoglobulin loci in the chicken B cell line DT40

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Kohei Kurosawa, Waka Lin and Kunihiro Ohta*

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan

*Kunihiro Ohta, Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan. Tel: +81 3 5465 8834, Fax: +81 3 5465 8834, email: kohta@bio.c.u-tokyo.ac.jp

The class I histone deacetylases HDAC1 and HDAC2 are highly conserved except for their C-terminal domain, but are presumed to have distinct functions in various tissues. We investigated the division of roles between HDAC1 and HDAC2 for the control of transcription and recombination at the immunoglobulin (Ig) gene in DT40. HDAC1$^{-/-}$ knock-out cells showed an increased incidence of gene conversion and of deletion/insertion events at the Ig light chain locus (IgL), but not at the heavy chain locus (IgH). Irrespective of recombinational activity, the transcription levels at IgL and IgH were decreased in HDAC1$^{-/-}$ cells, while other genes actively transcribed in B cells were slightly up-regulated compared to the levels in wild-type cells. These observations were strikingly different from the previously reported effects in HDAC2$^{-/-}$ cells, which showed a significant enhancement of transcriptional and recombinational activities at both IgL and IgH. Swapping experiments of the C-terminal unconserved domain of HDAC2 with its HDAC1 counterpart by gene knock-in demonstrated that this domain was not responsible for the phenotypic differences of HDAC1$^{-/-}$ and HDAC2$^{-/-}$. This suggests that other features such as modifications in the *N*-terminal catalytic domain could be important to determine the functional differences of these enzymes despite their structural similarities.

Keywords: DT40 cell/gene conversion/ histone deacetylase/histone deacetylase inhibitor/ immunoglobulin.

Abbreviations: HATs, histone acetyltransferases; HDAC, histone deacetylase; Ig, immunoglobulin; IgL, immunoglobulin light chain; IgH, immunoglobulin heavy chain; NLS, nuclear localization signal; TSA, trichostatin A.

Histone acetylation has been shown to be involved in the local regulation of transcription and recombination in eukaryotic cells (*1*, *2*). The levels of histone acetylation are reversibly controlled by the balanced counteraction of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs generally function as 'corepressors' that are recruited to the target sites by DNA sequence-specific transcription repressors. In vertebrates, HDACs are classified into four subfamilies (Class I–IV). Class I, localized exclusively in the nucleus and closely related to the *Saccharomyces cerevisiae* RPD3, includes HDAC1, −2, −3 and −8. Class II HDACs (HDAC4, −5, −6, −7, −9 and −10), which can shuttle in and out of the nucleus, are similar to another yeast deacetylase, HDA1. The class III is composed of members of the SIR2/Sirtuin family which require NAD^+ for their catalytic activity. HDAC11, which is structurally and functionally distinct from the HDACs of the other three classes, belongs to Class IV (*3*, *4*). Many types of HDAC inhibitors have been developed and are evaluated in clinical trials as anti-cancer drugs. Some of them are effective against leukemia, colon and breast cancers, although HDAC inhibitors in general act unselectively against various HDACs present in different cells and tissues. Therefore, an important issue to consider is the functional differences between each of these HDACs in distinct organs.

HDAC1 and HDAC2 are very similar proteins. They share a highly conserved N-terminal region (~91% sequence identity) including the deacetylase catalytic domain. The remaining C-terminal region comprising ~40 amino acids is relatively unconserved (~34% sequence identity). Despite the overall structural similarity, some recent observations in mice and human have revealed that HDAC1 and HDAC2 may have distinct functions. For instance, mouse HDAC2 is reported to negatively regulate memory functions and the plasticity of neurons (*5*), whereas HDAC1 is not. In human cartilage cells, the unconserved C-terminal domain of HDAC1 and HDAC2 has been reported to determine their functional differences in regulating some specific genes (*6*). Since the knock-out of HDAC1 in mice leads to embryonic lethal phenotypes (*7*), precise analysis by reverse genetics reveals to be difficult. Therefore, the division of roles between HDAC1 and HDAC2 in other types of cells is mostly left uncovered.

HDACs are also believed to play crucial roles in the vertebrate immune system. The acetylation of histones has been reported to regulate gene expression during immunocyte differentiation such as T and B-cell development. It also promotes the somatic recombination of immunoglobulins (Igs) and of T-cell receptor loci by enhancing V(D)J recombination (*8*–*10*), class-switch recombination (*11*, *12*) and gene conversion (*13*–*15*).

In chicken and some mammals (*16*), the diversification of the Ig variable segments primarily depends on a specific type of homologous DNA recombination called 'gene conversion'. The chicken Ig light chain (IgL) and heavy chain (IgH) contain only a single functional V-J segment in one allele which undergoes V(D)J recombination during early B-cell development (*16*–*18*). The other allele remains in the transcriptionally silent germ line configuration (*17*, *19*). Then, successive rounds of templated and unidirectional transfer of short DNA sequences are induced from the upstream pseudo-V segments clusters to the functional V region of the active allele, thereby generating sequence diversity of the Ig loci (*17*, *20*). This mechanism of Ig gene conversion, along with somatic hypermutaion and class switch recombination, has been shown to be initiated by AID, a B-cell specific activation-induced cytidine deaminase (*21*, *22*).

We previously reported that the frequency of Ig gene conversion in the chicken B cell line DT40 increases upon treatment of the cells with the HDAC inhibitor trichostatin A (*5*, *13*, *23*) or by the knock-out of the intrinsic HDAC2 gene (*14*). Moreover, the effects of TSA and of HDAC2 knock-out were shown to be distinct and additive, suggesting that other HDACs than HDAC2 might be involved in the modulation of gene conversion frequency and localization (*14*). In addition, other studies using DT40 mutants has also shown that HDAC2, but not HDAC1, is involved in the transcriptional regulation of IgH (*24*) and IgL (*25*, *26*). Thus, HDAC1 and HDAC2 are believed to have distinct roles in the regulation of transcription and recombination in DT40.

Here we report that the transcription of Ig at the active allele is hampered by the homozygous HDAC1$^{-/-}$ knock-out in DT40 cells as compared to the wild-type (WT) cells. Thus HDAC1$^{-/-}$ induced the opposite effect from the increase of Ig transcription previously shown in HDAC2$^{-/-}$ cells. Intriguingly, HDAC1$^{-/-}$ cells also showed an increased gene conversion frequency at the IgL locus, but not at IgH, whereas HDAC2$^{-/-}$ cells showed an enhanced gene conversion activity at both IgL and IgH. We also performed swapping experiments between the unconserved C-terminal domains of HDAC2 and HDAC1 and found that, contrary to the observations made in previous studies in human cartilage cells (*6*), the C-terminal domain is not responsible for the functional differences of HDAC1 and HDAC2 as regards the regulation of Ig gene expression in DT40. These results suggest that the mechanisms for the division of roles of HDACs may differ depending of the cell type and of the biological function they regulate.

Materials and Methods

Cell culture and transfection

The wild-type DT40 strain and the knock-out mutants for HDAC1 or HDAC2 were as described (*14*, *24*, *27*). Cell culture conditions were as previously described (*13*, *14*). The cell density was kept lower than 2×10^6 cells/ml. To study the effect of TSA, DT40 cells were cultured at least for 1 week in the presence of 10 nM TSA. Transfection experiments were carried out as previously described (*14*).

Extraction of genome and total RNA

Genomic DNA was prepared from 2×10^6 exponentially growing cells using the Illustra tissue and cells genomicPrep Mini Spin Kit (GE Healthcare). Total RNA was extracted using the TRIzol reagent (Invitrogen) as previously described (*14*) from 2×10^6 exponentially growing cells.

Real-time quantitative RT-PCR

RT-PCR was performed as previously described (*14*). Prime Script RT reagent Kit (Takara) was used for reverse transcription. Real-time RT-PCR was performed with the SYBR Premix ExTaq (Takara) using an ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems). Primer sequences are provided in Supplementary Data 1.

DNA sequence analysis of Ig V regions

The sequence patterns of the V regions in IgL and IgH were analysed as previously described (*13*, *14*). The Ig V regions were amplified by PCR using the Expand High Fidelity Plus System (Roche) from the genomic DNA of ~5000 cells as template. The amplified products were cloned into the pCR2.1TOPO vector (Invitrogen) and sequenced. The nucleic acid sequences were aligned in reference to the V pseudogene sequences (*17*, *20*) and analysed using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Western blot analysis

The protocol was followed as described (*14*). Proteins were extracted from whole cell lysates (equivalent to 1×10^5 cells), resolved in a 10% SDS–PAGE gel, and transferred onto nitrocellulose membranes. The membranes were incubated with 1 : 5,000 anti-DYKDDDDK primary antibody (Wako, cat#018-22381) then with 1 : 10,000 HRP conjugated anti-mouse IgG secondary antibody. Antigen detection was performed using ECL Western Blotting Detection Reagents (GE Healthcare).

Construction of the HDAC2::1C plasmid for knock-in experiments

To make the knock-in insert, the coding sequence of the HDAC1 C-terminal region downstream of the NLS was amplified by PCR from DT40 total cDNA. The insert was flanked by a 5′ and a 3′ region homologous to HDAC2 for targeted integration: the 5′ region was obtained by PCR amplification of a 3 kb genomic region containing exon 10, 11 and part of exon 12 (including the NLS) of HDAC2; the 3′ region was obtained by PCR amplification of a 3 kb region downstream of the HDAC2 stop codon. Using the In-Fusion PCR Cloning System (Clontech), the PCR fragments of the C-terminal HDAC1 region and of the 5′ region homologous to HDAC2 were fused and cloned between the *SpeI* and *BamHI* sites of pIELKb [a plasmid derived from pIRESneo3 (Clontech) with modified restriction sites]. Then the 3′ region homologous to HDAC2 was cloned between the *AgeI* and *PacI* sites. Finally, a BGH terminator and the blasticidin or puromycin resistance gene under control of the SV40 promoter were inserted between the *BamHI* and *SmaI* sites to make the final HDAC2::1C plasmid, named pH1Cb-3. Plasmid maps and sequences are available upon request.

Construction of plasmids for over-expression experiments

All vectors were derived from pIEn-CT, a modified pIRESneo3 plasmid (Clontech) in which the CMV promoter was replaced by the CAGGS promoter and the coding sequences of the 6xHis and the 3xFLAG (DYKDDDDK) epitope tags were added upstream of the terminator codon in the multi cloning site. The coding sequences of HDAC2, HDAC2ΔCTD and HDAC2::1C were

amplified by PCR from DT40 total cDNA and inserted between the *ClaI* and *NheI* sites of pIEn-CT. The fusion of DNA fragments was performed using the In-Fusion PCR Cloning System (Clontech).

Results and Discussion

HDAC1 and HDAC2 have distinct roles in transcription and recombination at the Ig loci in DT40

We investigated the roles of HDAC1 and HDAC2 in cell growth, transcription and recombination at the IgL and IgH loci in DT40, using the two knock-out mutants HDAC1$^{-/-}$ (*24*) and HDAC2$^{-/-}$ (*14*) cells. The growth speed of HDAC2$^{-/-}$ cells was very similar to that of the wild-type DT40 as previously reported, whereas HDAC1$^{-/-}$ exhibited a slower growth rate (Fig. 1A). Staining with trypan blue revealed no increase of dead cells, suggesting that HDAC1 plays a role in cell growth. Real-time RT-PCR analyses showed that HDAC2$^{-/-}$ and DT40 treated with 10nM TSA exhibited 1.5 to 3-fold higher levels of transcription at both IgL and IgH (Fig. 1B) as described previously (*14*, *24*). Thus, HDAC2 probably plays a repressive role for transcription at the Ig loci in DT40. In contrast, as compared to the wild-type cells, HDAC1$^{-/-}$ cells showed a substantial reduction of the transcript levels at IgL and IgH (−25 and −50%, respectively), whereas they showed normal or higher transcriptional activities at other genes involved in Ig gene conversion and somatic hypermutation, such as AID (*21*), E2A (*15*) and CTNNBL-1 (*28*), comparably to what were observed in HDAC2$^{-/-}$ and TSA-treated cells (Fig. 1C). These results suggest that HDAC1 is partly involved in the transcriptional activation of the Ig loci, which is the opposite of the HDAC2 function.

We further studied the role of HDAC1 in Ig gene conversion by analysing the DNA sequences of the Ig V segments in HDAC1$^{-/-}$ mutant cells after clonal expansion for 1 month. As previously demonstrated, the DT40 cells treated with TSA and the HDAC2$^{-/-}$ mutant cells exhibited a diversification of Ig V segments at both IgL and IgH loci (Fig. 2), largely due to an enhancement of gene conversion activity (*13*, *14*). As previously observed (*14*), the conversion tracts were scattered widely along the entire V segments of active IgL and IgH alleles in HDAC2$^{-/-}$, whereas the cells treated with TSA tended to display sequence alterations preferentially around the complementarity determining region (CDR)-1. Despite the reduction of transcriptional activity at IgL, HDAC1$^{-/-}$ also exhibited an increase of sequence alterations along the entire V segment of IgL (Fig. 2). The overall proportion of HDAC1$^{-/-}$ cells showing sequence alterations after 1 month of culture (82%) was similar to the proportion in HDAC2$^{-/-}$ cultures (80%). However, we noted that the frequency of alterations at the IgH locus in HDAC1$^{-/-}$ cells stayed at a low level, which was comparable to the untreated wild-type DT40 cells.

To further analyse the IgL-specific diversification in HDAC1$^{-/-}$, we classified the sequence alterations into four types of event: 'gene conversion',

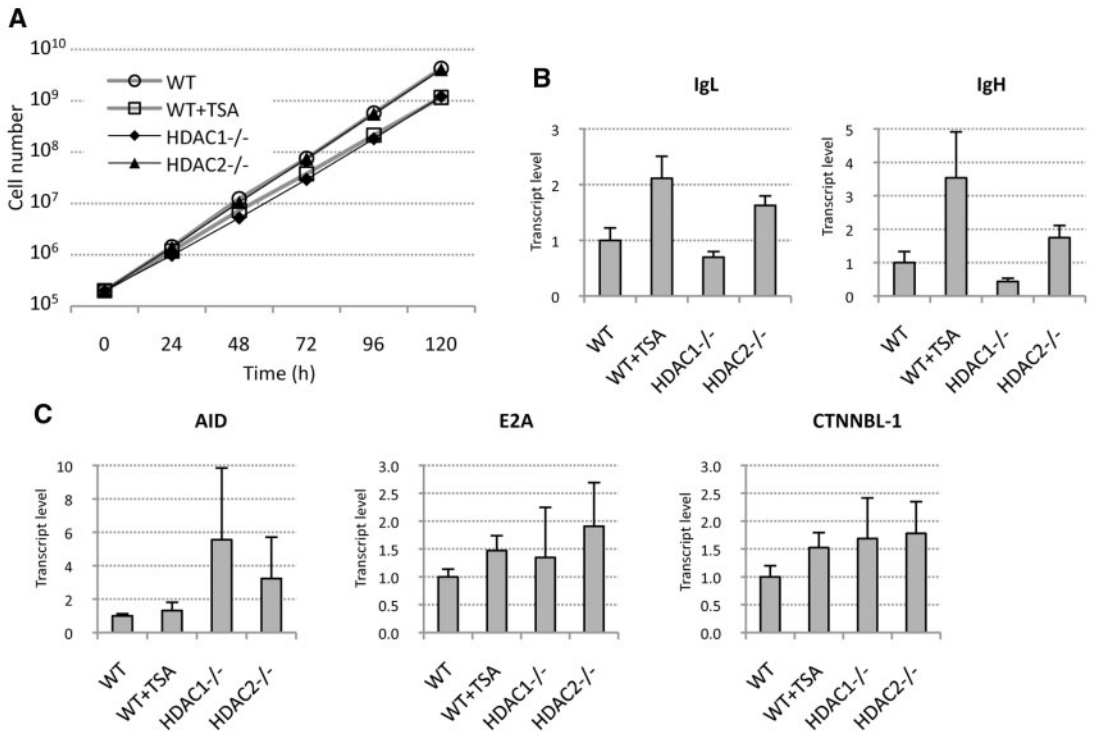

Fig. 1 Effects of HDAC1 and HDAC2 on growth speed and gene expression. (A) Growth curves of WT, WT + TSA (10 nM), HDAC1$^{-/-}$ and HDAC2$^{-/-}$ cells. Cultures were started at time 0 with 2×10^5 cells in 14 ml medium for each condition. Culture medium (2 ml) were passaged into 12 ml of fresh medium every day to maintain stable conditions. The cell density taking into account the passaged volume was determined at the indicated times and plotted on a log scale. (B) and (C) Total RNA was extracted from cells harvested at 7 and 14 days of culture and used as template for real-time RT-PCR with indicated gene specific primers. Transcript levels were normalized to β-actin levels and plotted relative to the transcript levels in WT cells. Experiments were repeated at least six times using two independent subclones for each cell type.

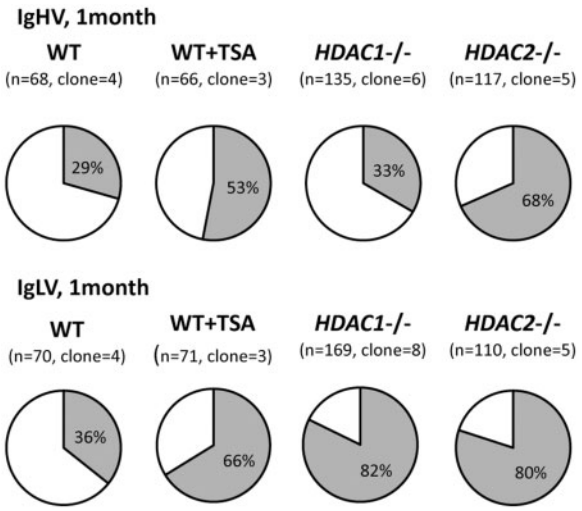

Fig. 2 Frequency of sequence alterations induced in IgLV and IgHV. The immunoglobulin variable regions were amplified from genomic DNA for each clone after 30 days of culture. After cloning of the DNA fragments and sequence analysis, we calculated the proportion of cells which showed sequence alterations (*i.e.* mutations, gene conversion events, etc.) in the variable regions compared to the original sequence before culture expansion. Gray and white sectors indicate, respectively, the percentage of cells containing sequence alterations or not. The number of independent clones ('clones') and the total number of sequences ('*n*') analysed for each culture condition are indicated in parentheses.

'point mutation', 'ambiguous' (single nucleotide change caused by either somatic mutation or gene conversion) and 'insertion/deletion'. As shown in Table I, much more 'insertion/deletion' were found in the altered sequences of HDAC1$^{-/-}$ compared to HDAC2$^{-/-}$ or TSA-treated DT40. This result suggests that the mechanisms underlying the IgL sequence diversity in HDAC1$^{-/-}$ is different from those in HDAC2$^{-/-}$. It is possible that some machineries for non-homologous end-joining, which often leads to 'insertion/deletion' events, rather than the machineries for homologous recombination, are preferentially recruited to the IgL V region in HDAC1$^{-/-}$. Supporting this inference, DT40 cells with disrupted RAD18, which plays a role in ensuring successful homologous recombination, are known to exhibit an increase in the frequency of deletions and duplications in the V segment of IgL (*29*). We could not detect any significant difference in the transcription levels of RAD18 in HDAC1$^{-/-}$ cells (data not shown), nevertheless HDAC1 has also been reported to associate with DNA repair factors (*30*, *31*) and it is conceivable that it affects the protein activity of Rad18 or other related factors to regulate the incidence of 'insertion/deletion' events in the patterns of diversification at IgL.

Investigation of the protein domain responsible for the functional differentiation of HDAC1 and HDAC2 at the Ig loci in DT40

All the results described above indicate that HDAC1 and HDAC2 have distinct roles in controlling transcription and recombination at the Ig loci in DT40 cells. To elucidate the molecular basis for the functional differentiation of HDAC1 and HDAC2,

Table I. Variation of altered sequences.

IgLV, 1 month	WT ($n=35$)	WT + TSA ($n=36$)	HDAC1$^{-/-}$ ($n=34$)	HDAC2$^{-/-}$ ($n=34$)
Gene conversions (%)	50	52	40	52
Point mutations (%)	36	24	26	28
Ambiguous (%)	9	24	19	16
Insertions/deletions (%)	5	0	15	4

n indicates the total number of analyzed sequences.

we tried to identify the domain responsible for this disparity. As mentioned previously, the N-terminal region of HDAC1 and HDAC2 containing the deacetylase domain is highly conserved: the sequence identity in this region of ~440 amino acids is 91%. The remaining C-terminal domain (~40 amino acids) is relatively unconserved: the sequence identity is only 34%. Therefore, the C-terminal domain can be suspected to be involved in the division of functions between HDAC1 and HDAC2. In that respect, Hong *et al.* (*6*) recently conducted experiments using the transient expression of chimeric HDACs with swapped C-terminal domains in human cartilage cells. These experiments, though they cannot exclude the influence of endogenous HDACs, clearly indicated that the C-terminal unconserved domains in human HDAC1 and HDAC2 were pivotal to differentiate their functions in the regulation of some genes specific to cartilage cells.

To test whether the same is true for chicken DT40, we prepared a homozygous knock-in HDAC2 construct (HDAC2::1C/HDAC2::1C), in which each C-terminal domain of the two intrinsic HDAC2 alleles was swapped with the corresponding C-terminal domain of HDAC1, taking advantage of the gene targeting technique allowed by the high homologous recombination activity of DT40 (Fig. 3A and B). This type of reverse-genetics analysis can exclude any influence of endogenous HDACs and therefore allows a more rigorous assessment of the effect induced by the swapped chimeric HDAC2::1C construct.

We confirmed by RT-PCR analysis that HDAC2::1C is expressed in the homozygous knock-in DT40 cells at a level similar to the unmodified HDAC2 in wild-type cells (data not shown). Quantitative real-time PCR analyses of Ig transcription levels in HDAC2::1C expressing cells compared to HDAC1$^{-/-}$ and HDAC2$^{-/-}$ cells revealed that the C-terminal swapping did not affect the transcription profile: the HDAC2::1C knock-in cells in the absence of endogenous HDAC1 and HDAC2 exhibited a similar transcription profile to that of HDAC1$^{-/-}$ cells at IgL and IgH (Fig. 3C). In addition, the cell growth of HDAC2::1C knock-in cells is very similar to that of HDAC1$^{-/-}$ cells, which show a slower growth rate than wild-type and HDAC2$^{-/-}$ cells (Fig. 3B). We also analysed the diversification of Ig V segments at both IgL and IgH in the HDAC2::1C knock-in cells. The results showed that the diversification at IgL increased significantly (60% of the cells containing sequence alterations) but not at IgH, which is similar to the parental HDAC1$^{-/-}$ phenotype (data not shown).

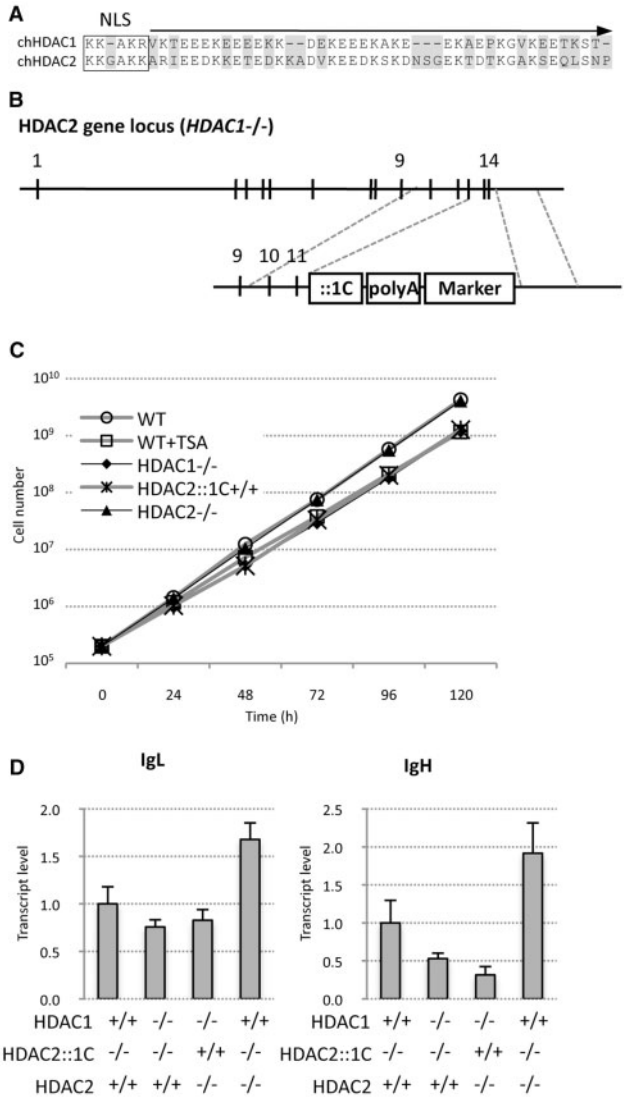

Fig. 3 Characterization of the HDAC2::1C mutant.
(A) Alignment of the C-terminal regions of chicken HDAC1 and HDAC2. Non-similar residues are indicated in gray. The NLS is indicated by the black-lined box. The horizontal arrow indicates the region selected for the swapping and deletion experiments. (B) Schematic diagram of the genomic organization of HDAC2 and of the targeting vector resulting in the HDAC2::1C construct. Numbered vertical bars indicate the locations of exons. The boxed '::1C' represents the coding sequence of the HDAC1 C-terminal region downstream of the NLS. (C) Growth curves of WT, WT + TSA (10 nM), HDAC1$^{-/-}$, HDAC2::1C$^{+/+}$ and HDAC2$^{-/-}$. Cell density was determined at the indicated time as in Fig. 1A. (D) Effects on the transcription levels of IgL and IgH. Real-time RT-PCR was performed as in Fig. 1B. Experiments were repeated two times using two subclones for each cell type, except for the HDAC2::1C$^{+/+}$ mutant for which we used four independent subclones.

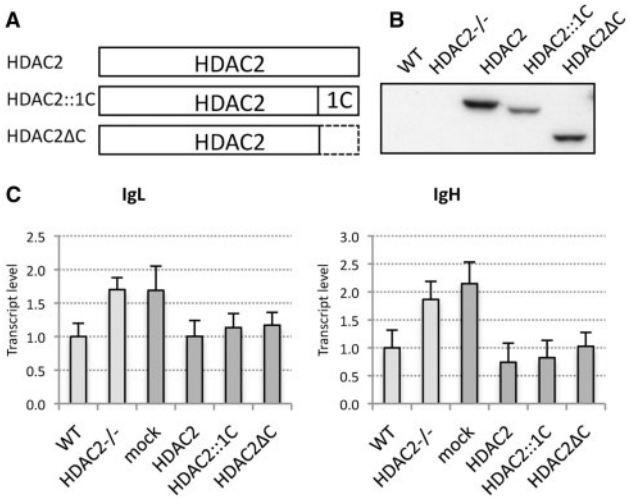

Fig. 4 Over-expression experiments in HDAC2$^{-/-}$ cells.
(A) Schematic diagram of the HDAC2 variants used for over-expression experiments. 'HDAC2' is the native chicken wild-type HDAC2. 'HDAC2::1C' is the chimera mutant in which the C-terminal region downstream of NLS was swapped with the C-terminus of HDAC1. HDAC2ΔC is lacking the C-terminal region. Each expression vector were transfected into HDAC2$^{-/-}$ cells to construct stable cell lines. (B) Immunoblot of the whole cell extracts from the transfected cells using a FLAG-specific antibody. (C) Effects on the transcription levels of IgL and IgH. Real-time RT-PCR was performed as in Fig. 1B. Experiments were repeated at least three times using three subclones for each cell type.

These results clearly indicate that HDAC2::1C acts as a fully functional HDAC2 protein, instead of complementing the absence of HDAC1.

To further verify this result, we conducted complementation experiments in HDAC2$^{-/-}$ cells with the ectopic expression of the full-length HDAC2 protein, a C-terminus truncated version of HDAC2 (HDAC2-ΔC, deleted for the 44 amino acids downstream of the nuclear localization signal (NLS), and the chimeric HDAC2::1C (Fig. 4A and B). Quantitative real-time PCR analyses showed that the full-length HDAC2, HDAC2::1C and HDAC2-ΔC can all fully compensate for the HDAC2$^{-/-}$ deficiency regarding transcription at the Ig loci (Fig. 4C).

Taken together, these results lead to the conclusion that the C-terminal region of HDAC1 and HDAC2 is dispensable for the division of their functions, at least in the DT40 cell line. This raises the possibility that subtle structural differences in the N-terminal region containing the HDAC domain may define the enzyme selectivity for the targets of deacetylation. There seems to be no significant difference in the amino acid residues forming the histone deacetylase catalytic core nor in its secondary structure (*32*). However, we noted the existence of differences in the secondary structure of the remaining regions, which may be significant for the differentiation of the roles of HDAC1 and HDAC2 and be worth investigating in the future. Another possibility is that HDAC1 and HDAC2 themselves acquire different post-translational modifications such as acetylation and phosphorylation, which could be essential for the modulation of their activity. Notably, other groups reported that several lysine residues of HDAC1 are acetylated and that the activity of acetylated HDAC1 is reduced compared to non-acetylated HDAC1(*33*, *34*). One of these lysine residues (Lys-432) is not conserved in HDAC2, and they showed that when a region containing this residue in HDAC1 was swapped by its corresponding region of HDAC2, the acetylation level of the enzyme was reduced and its activity restored. Although we focused on the extreme C-terminal domain for our current

study, further analysis of such post-translational modifications in other regions might help to understand the basis of the functional differences between HDAC1 and HDAC2.

Our data suggest that the mechanisms for the control of various biological processes by HDACs may differ from one cell type to the other or depending on the targeted locus. Presumably, the differential activities of distinct HDAC-interacting proteins allow a precise control case by case depending on the cell context. Further investigation of the molecular basis of HDAC targeting in different types of cells shall provide important knowledge about the effects of HDAC inhibitors on various human diseases.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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