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Review

A comprehensive review on the chromatin conformation change code (4C) theory: A theory on ways of higher eukaryotes to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations

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ABSTRACT

In this article we first reviewed our studies on the exclusion of a large amount of IgM H- and L-chains excessively accumulated in histone deacetylase2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) during continuous cultivation as a concrete case. Based on these results, we proposed a universal hypothetic concept on the ability of higher eukaryotes for gaining un-programmed and new cell function(s), originally based on the pluri-potency, elasticity and flexibility of the chromatin structure, in order to cope with and/or overcome new environment change. We named the remarkable bio-system the chromatin conformation change code (4C) theory. Outline of the 4C theory is concretely as follows. Somatic cells of higher eukaryotes gradually acquire un-programmed and new cell functions to cope with and/or overcome environment change, by means of creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through numerous generations. Creation of chromatin structure plasticity in individual somatic cells of the same type is triggered by spontaneous unbalanced response to the environment change, followed irreversibly and diversely by its successive convergence, and accomplished in descendent cells but not in the cell which initially meets with the environment change. The environment change is recognized by putative environment change recognition receptor/site (ECRR/ECRS), and chromatin structure plasticity is irreversibly and directly created by putative chromatin conformation change complex (4C) machinery. The chromatin structure of the proximal 5'-upstream region(s) of the specific gene(s) receives the signal on the environment change, and directs the switch (on or off) for its gene expression ability through alterations between loose and tight forms. The number of codes in the 4C theory, which determines diverse cell functions and types, may be determined by the multiplication of the number of candidate genes and that (probably two) of codes for each of candidate genes.

KEYWORDS: gene targeting techniques, histone deacetylase2-deficient DT40 mutants, numerous generations during continuous cultivation,

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decreases in immunoglobulin mRNA/protein levels, changes in mRNA levels of transcription factors, epigenetic modification, chromatin structure plasticity, gain of un-programmed and new cell functions, chromatin conformation change code theory.

INTRODUCTION

In eukaryotes, both the genome information and the nuclear function are mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Both the communication and the signal transduction between the nucleus and cytoplasm, which are essential for expressions of normal cell functions, are preferentially carried out by the to and fro passage of large and/or small molecules *via* the nuclear pore that acts as a guard station at the barrier. By contrast, both the signal transduction concerning an abnormal, unexpected and/or disadvantageous change in the intra- and/or extra-cellular environment and the transport of useless molecules to the nucleus are usually prevented by this barrier system. Then, how do the higher eukaryotic cells cope with and/or overcome an abnormal, unexpected and/or disadvantageous environment change, when they firstly encounter it in their lives? Generally, this is possible by adhering to four typical countermeasures. First, in the case of a very severe change, cells die because it is far ahead of their adaptation ability. Second, in the case of a considerably severe change, cells cope with or overcome it by means of alterations in genome information, such as point mutation, insertion, deletion, duplication, multiplication, etc. in the DNA molecule through numerous generations. This mode is a basis for the evolution of species. Needless to say, the mode also occurs in all of organisms, including virus, prokaryotes, lower and higher eukaryotes and others. Third, in the case of a moderate change, cells cope with or overcome it by means of irreversible creation of chromatin structure plasticity caused by successive chromatin conformation (structure) changes with epigenetic modifications through numerous generations (cell divisions). This mode mostly occurs in higher eukaryotes and is a basis for both the development and the differentiation of cells. Fourth, in the case of a minor change, cells respond to it only through already acquired regulation mechanisms.

Since chemical modifications of histones with acetyl and methyl groups were first proposed to regulate RNA synthesis [1], the modulation of the chromatin topology has been thought to be one of the most fundamental and important events for the expression of normal cell functions in eukaryotes. The ways to modulate the chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, etc. have been intensively studied in a variety of life science fields. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones (H2A, H2B, H3 and H4) cooperatively controlled by chromatin-modifying enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), are undoubtedly the major ones [2-27]. For the past several decades, countless numbers of research reports on the acetylation and deacetylation (and also other epigenetic modifications) are accumulating without interruption in more diverse life science fields, e.g., transcription/gene expression, replication, repair plus recombination of DNA, development, differentiation, memory, pluri-potency, clinical medicine, etc. [28-39]. Naturally, as well known, many research groups have reported that alterations in the chromatin structure are remarkably involved in regulations of the lymphocyte development and differentiation [40-46]. In addition, various transcription factors, including Ikaros, PU.1, E2A, GATA-3, EBF, Pax5 and others, are involved in regulations of the development and differentiation of lymphocytes [47-56]. And then, the regulation of the IgM H-chain gene expression requires USF, TFEB, Ig/EBP, NF-IL6, OCA-b, etc. as promoter binding proteins, and Ig/EBP, NF-IL6, YY-1, E2A, PU.1, etc. as intron enhancer binding proteins.

To assess *in vivo* roles of individual members of HDACs and HATs in the above-mentioned biological events, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a specific member of HDACs and HATs [57-73], by gene targeting techniques using two different targeting vectors [74-84]. Our early findings in initially generated HDAC2-deficient

DT40 mutant cells HDAC2(-/-) revealed that HDAC2 controls the amount of IgM H-chain at the two steps of its gene expression and alternative pre-mRNA processing [57] and down-regulates the IgM L-chain gene promoter activity [60]. Moreover, the HDAC2-deficiency has varied severe and moderate effects on several cellular characteristics. That is, the deficiency represses gene expressions of HDAC7, Pax5, Aiolos, Ikaros and EBF1, elevates gene expressions of HDAC4, HDAC9, PCAF and E2A, and changes bulk acetylation levels of several specific Lys residues (K) of core histones H2A, H2B, H3 and H4 [64].

To know individual roles of these changed chromatin-modifying enzymes and transcription factors in the regulation of gene expressions of IgM H- and L-chains, we systematically generated homozygous DT40 mutant cell lines: EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), devoid of EBF1, Aiolos, E2A and Helios genes, respectively [64, 85-89], and Pax5-deficient DT40 mutant cell line Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome, which is monosomy in chickens [64, 90-93]. In addition, we generated Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene existing on chromosome 2, which is trisomy (our unpublished data). Analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of the two immunoglobulin proteins [64]. These results, together with others [94], indicated that in wild-type DT40 cells HDAC2 as a supervisor indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1, Ikaros and E2A (Figure 1-W) [64, 67].

Recently, we accidentally noticed following remarkable and important characteristics of HDAC2(-/-) DT40 mutants, which were cultivated for different long-term periods [92, 95-103].

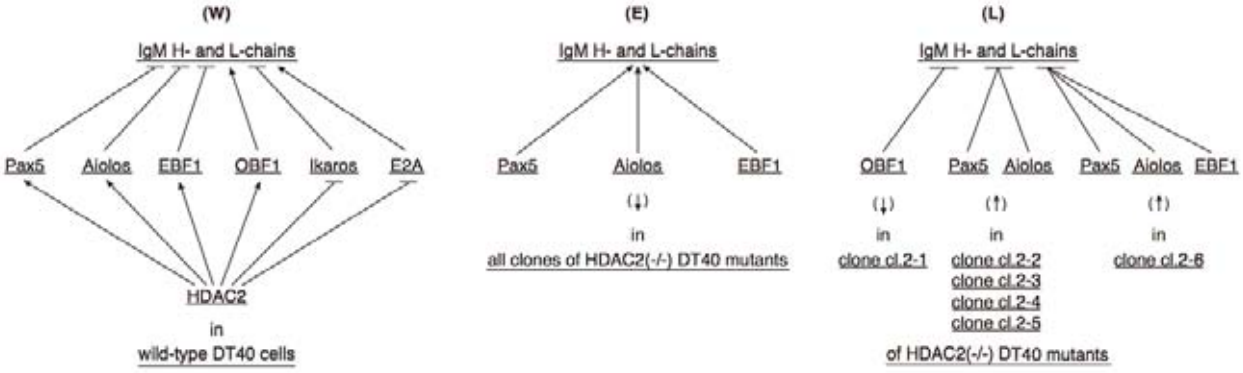

Figure 1. Ways to regulate gene expressions of IgM H- and L-chains through regulation of gene expressions of Pax5, Aiolos, EBF1, OBF1 and others in wild-type DT40 cells and all or individual clones of HDAC2(-/-) DT40 mutants at early and later stages of continuous cultivation.

In DT40 cells (W), HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulation of gene expressions of Pax5, Aiolos and EBF1, and Ikaros and E2A. At the early (E) cultivation stage, in all individual clones of HDAC2(-/-) mutants, IgM H- and L-chains are excessively accumulated based on their dramatically increased gene expressions caused by drastically decreased gene expressions of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of the two immunoglobulin proteins. At the later (L) cultivation stage, in all individual clones of HDAC2(-/-) mutants, the accumulated IgM H- and L-chains are dramatically decreased attributed to their drastically decreased gene expressions in almost the same changing pattern, caused by dramatically increased or decreased gene expressions of Pax5, Aiolos and EBF1 or OBF1 in distinct ways. Clone cl.2-1 seems to be the OBF-1-dependent type, clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be the Pax5- and Aiolos-dependent type, and clone cl.2-6 seems to be the Pax5-, Aiolos- and EBF1-dependent type. The figure is identical with Figure 1 of Refs. 102, 103 plus 126, Suppl. Figure S1 of Refs. 98 plus125, and Figure 6 of Ref. 99, and also is a set of Figures 8, 9 plus 10 of Ref. 96. These figures were first presented in Ref. 92.

IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants at the early stage of cultivation are dramatically reduced in almost similar pattern but in distinct ways in individual mutant clones through numerous generations during continuous cultivation. These distinct ways are fundamentally based on irreversible creation of varied chromatin structure plasticity of the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in individual clones of HDAC2(-/-) mutants through numerous generations during continuous cultivation [92, 97, 100]. Based on these results [92-97, 99, 100] as a concrete case, we first presented a hypothetic concept (way) to diminish IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants through numerous generations during continuous cultivation. Successively, we expanded the above-mentioned hypothetic way to a universal hypothetic concept on the ability of higher eukaryotes for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity through numerous generations, in order to adapt to an unexpected, abnormal and/or uncomfortable environment change [98, 101-103]. We named this remarkable and important bio-system chromatin conformation change (structure) code (4C) theory. In this article, we reviewed our studies on the 4C theory comprehensively. By the way, we discussed briefly the difference between the 4C theory and somewhat similar and important life phenomena, which are being rapidly studied by other groups in various organisms recently.

Protein and mRNA levels of IgM H- and L-chains are excessively elevated in both HDAC2(-/-) and Pax5(-) DT40 mutant cells at the early stage of cultivation and thereafter dramatically reduced through numerous generations during continuous cultivation

In the process of qualitative analyses of initially generated HDAC2(-/-) mutant cells [57], which were cultivated for different long-term periods, surprisingly, we accidentally noticed interesting and amazing phenomena as follows [92, 95]. In our studies, the mutant cells were cultivated on agar plate and continuously in liquid medium for at least 15-16 days before they were stocked at -80 °C. In addition, the early, middle and later stages meant relatively short-term, medium-term and long-term cultivations, respectively. At the outset, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showed that amounts of IgM H-chain and L-chain (detected as two spots) are dramatically increased at the early (E; ~10 to 20 days) stage of cultivation and thereafter gradually decreased through the middle (M; ~30 to 40 days) stage, and at the later (L; ~60 days) stage reach comparable levels in DT40 cells (Figure 2). By contrast, insignificant changes are observed for most of the other major cellular proteins during cultivation. Western blotting, which was carried out at shorter interval periods, using antibody for chicken IgM H-chain and antibody for L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron microscopy using the first antibody showed that IgM H-chain is obviously accumulated at the early stage and thereafter at the later stage reaches almost the same level as in DT40 cells (Figure 3). These results, together, indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage (Figure 1-E), but also that these accumulated immunoglobulin proteins are gradually reduced during cultivation and finally at the later stage reach comparable levels as in DT40 cells (details will be shown later) (Figure 1-L). Reverse transcription-polymerase chain reaction (RT-PCR) using primers IgM Hc plus IgM Hs showed that whole and secreted forms of IgM H-chain mRNA are dramatically increased at the early stage, and thereafter gradually reduced during cultivation and at the later stage reach very close levels as in DT40 cells (Figure 4). Remarkably, RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors, showed that gene expressions of HDAC7, HDAC9 and PCAF are gradually elevated and those of EBF1, Pax5 and Aiolos certainly change in distinct patterns during cultivation (details will be shown later). Immuno-blotting, using antibodies specific for various acetylated Lys residues (K) of core histones H2A, H2B, H3 and H4, showed that in spite of the HDAC2-deficiency, very surprisingly,

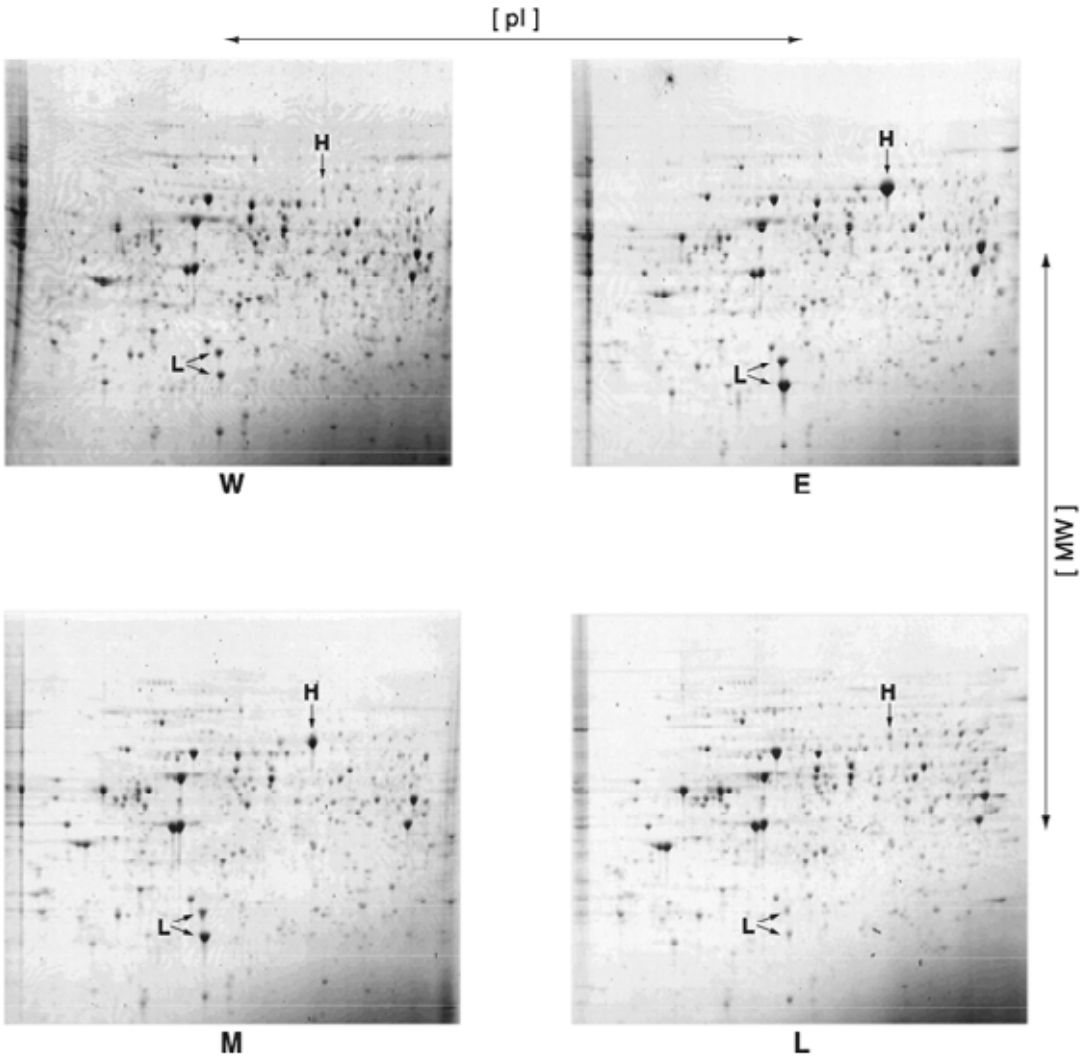

Figure 2. Alterations in the amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutants during continuous cultivation.

2D-PAGE was performed on total cellular proteins prepared from HDAC2(-/-) mutant cells at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W). H and L indicate IgM H- and L-chains, respectively. The figure is identical with Figure 2 of Refs. 102, 103 plus 126, Figure 2-1 of Ref. 92, Figure 1 of Ref. 95, and Figure 1 of Ref. 101. These figures were first presented in Ref. 92.

bulk acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) are gradually increased during cultivation, though insignificant changes are observed in most of the remaining Lys residues of core histones.

Because the gene expression of Pax5 is controlled by HDAC2 and also among the above-mentioned changed transcription factors Pax5 mainly controls gene expressions of IgM H- and L-chains [64], we studied the molecular mechanism of the gene expression of Pax5 [92, 93, 95]. Until we started this study, the Pax5 gene was not reported to exist on Z sex chromosome that is monosomy in chickens, and nucleotide sequences of its 5'-upstream region were not yet deposited in any database. Therefore, first, we directly cloned the proximal ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques [92, 95], including Southern blotting, colony hybridization and

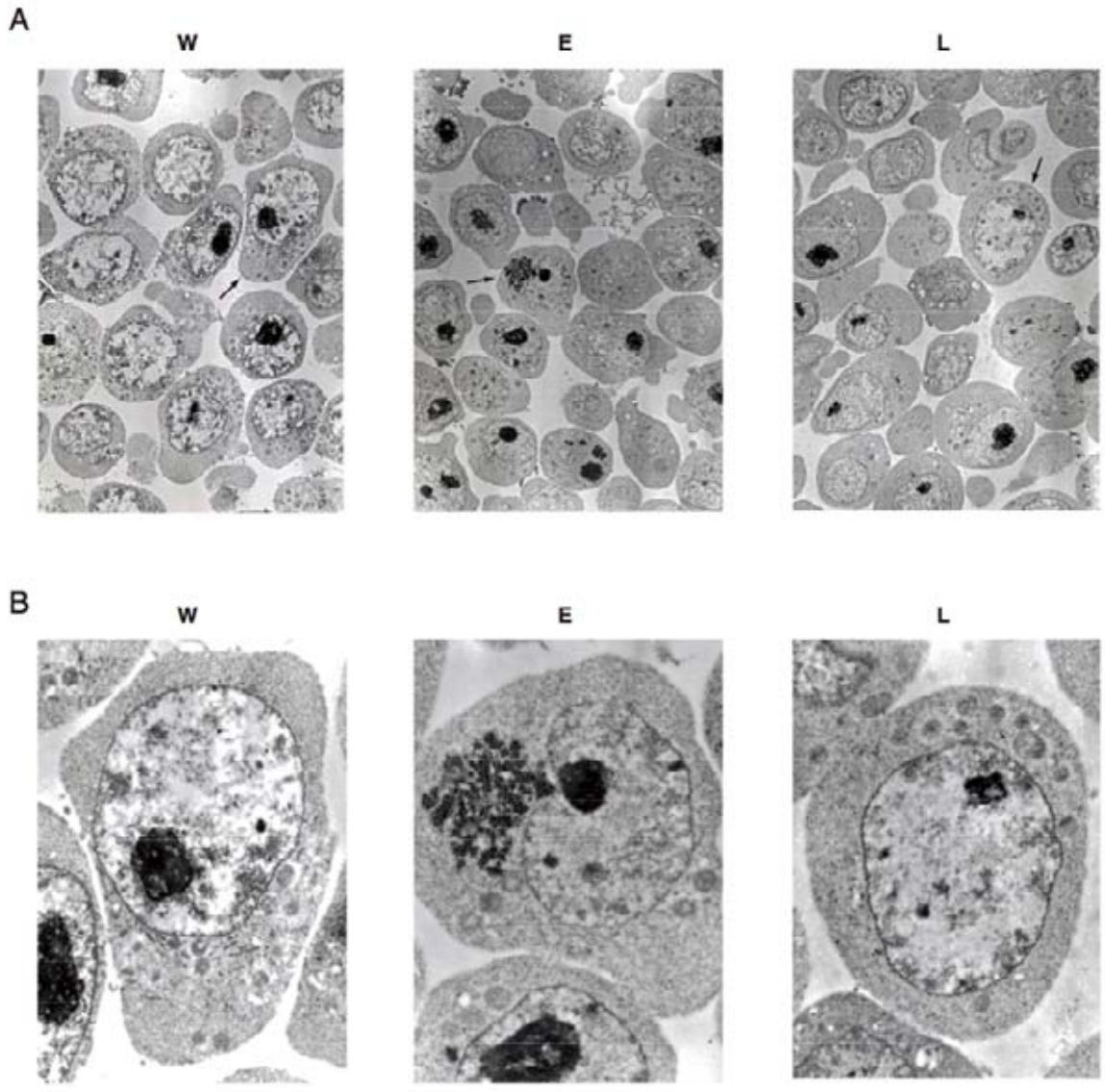

Figure 3. Alterations in the amounts of IgM H-chain proteins in HDAC2(-/-) DT40 mutants during continuous cultivation.

Immuno-electron microscopy, using anti-chicken IgM H-chain antiserum, was performed on HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W) in wide range (A) and for single cell (B). A large amount of accumulated IgM H-chain proteins was detected only at the early (E) stage in HDAC2(-/-) mutant cells. The figure is identical with Figure 3 of Refs. 102, 103 plus 126, Figures 2-3 of Ref. 92, and Figure 4 of Ref. 95. These figures were first presented in Ref. 92.

sub-cloning, and determined its nucleotide sequences (GenBank accession number: LC060666). Dual-luciferase assay, using various 5'- and 3'-deletion plasmid constructs of the ~4.9 kb 5'-upstream region of the Pax5 gene, suggested that two distinct proximal 5'-upstream regions are presumably necessary to negatively control its gene expression, whereas the clearly defined promoter(s) or element(s) has yet to be elucidated (our unpublished data). Moreover, using site-specific antibody for acetylated Lys-9 residue of histone H3 (K9/H3) and several appropriate primers, we carried out chromatin immuno-precipitation (ChIP) assay on the chromatin

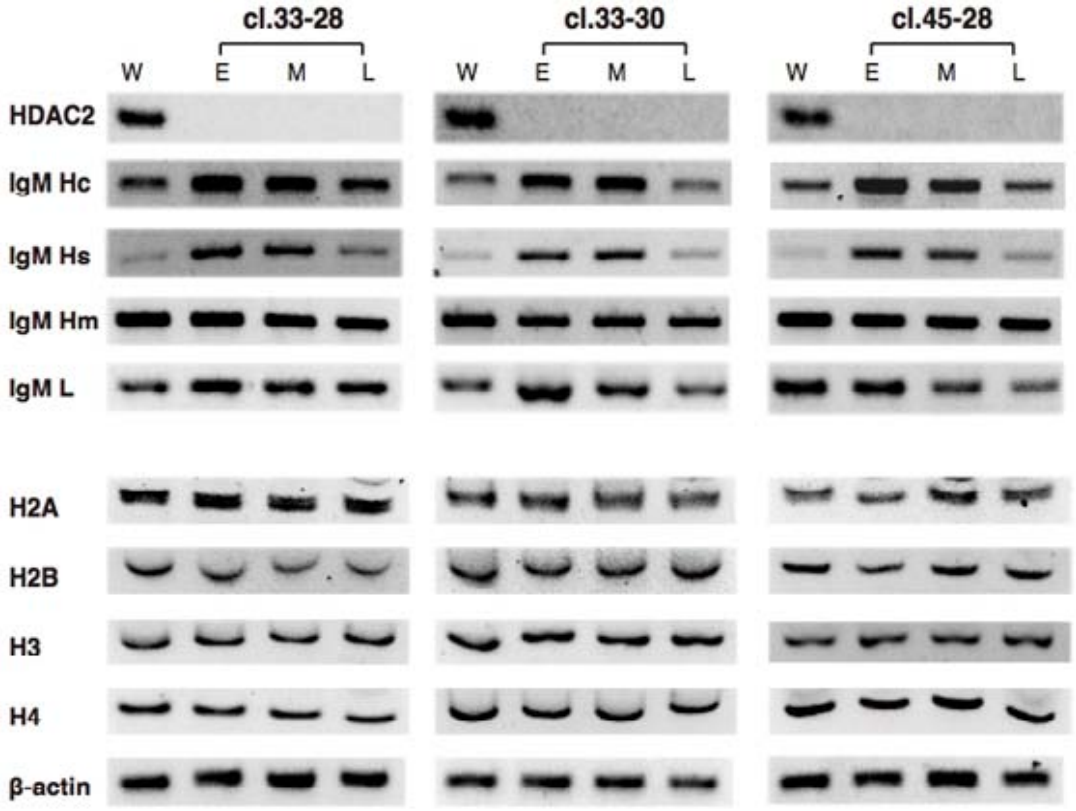

Figure 4. Alterations in gene expressions of IgM H- and L-chains and core histones in HDAC2(-/-) DT40 mutants during continuous cultivation.

Total RNAs were extracted from three individual clones of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W). RT-PCR was performed on total RNAs, using appropriate primers for HDAC2 mRNA, whole (IgM Hc), secreted (IgM Hs) plus membrane-bound (IgM Hm) forms of IgM H-chain mRNA and IgM L-chain mRNA (IgM L), and core histones H2A, H2B, H3 and H4 mRNAs. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were identical with those of Ref. 64. The figure is identical with Figure 4 of Refs. 102, 103 plus 126, Figures 2-4 of Ref. 92, and Figure 5 of Ref. 95. These figures were first presented in Ref. 92.

surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in initially generated HDAC2(-/-) mutant cells at the early and later cultivation stages and in DT40 cells [92, 95]. These cultivation stages were vague and temporary, since these tested mutant cells had been already cultivated several times for other experiments. However, surprisingly, this preliminary ChIP assay suggested that in HDAC2(-/-) mutant cells acetylation levels of K9/H3 within some limited segments of the proximal ~2.0 kb 5'-upstream chromatin region of the Pax5 gene are decreased at the early stage and thereafter at the later stage increased to almost the same levels as in DT40 cells (details will be shown later). These results roughly agreed with the findings on changing patterns in the gene expression of Pax5 mentioned above.

As mentioned above, gene expressions of IgM H- and L-chains are mainly and indirectly regulated by HDAC2 through the control of gene expressions of several transcription factors, especially Pax5 [64, 92-95]. We performed time-course studies on some characteristics of Pax5(-) mutant cells at the early (E: ~8 days), middle (M: ~13 days) and later (L: ~20 days) cultivation stages [92, 93], which were relatively shorter intervals than those adopted for HDAC2(-/-) mutant cells [92, 95]. 2D-PAGE revealed that IgM H- and L-chains are

drastically and considerably increased at the early stage, and thereafter dramatically decreased during cultivation and at the later stage reach almost the same levels as in DT40 cells (our unpublished data). Western blotting using the above-mentioned IgM L-chain antibody showed that IgM H-chain and L-chain (detected as two bands) are dramatically and considerably elevated at the early stage, and thereafter gradually decreased through the middle to later stages to almost the same levels as in DT40 cells (Figure 5). Electron microscopy revealed that dense cytoplasmic fractions probably due to artificially accumulated IgM H- and L-chains are detected in Pax5(-) mutant cells at the early stage but not at the later stage as in DT40 cells. Immuno-electron microscopy using the IgM H-chain antibody showed that the immunoglobulin proteins are surely accumulated at the early stage, and thereafter most of them disappear at the later stage as in DT40 cells. Together, these results indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage in Pax5(-) mutants, but also that these accumulated immunoglobulin proteins are rapidly reduced during cultivation and finally at the later stage reach comparable levels as in DT40 cells. Furthermore, RT-PCR using primers IgM Hc and IgM Hs revealed that the whole and secreted forms of IgM H-chain mRNA are dramatically

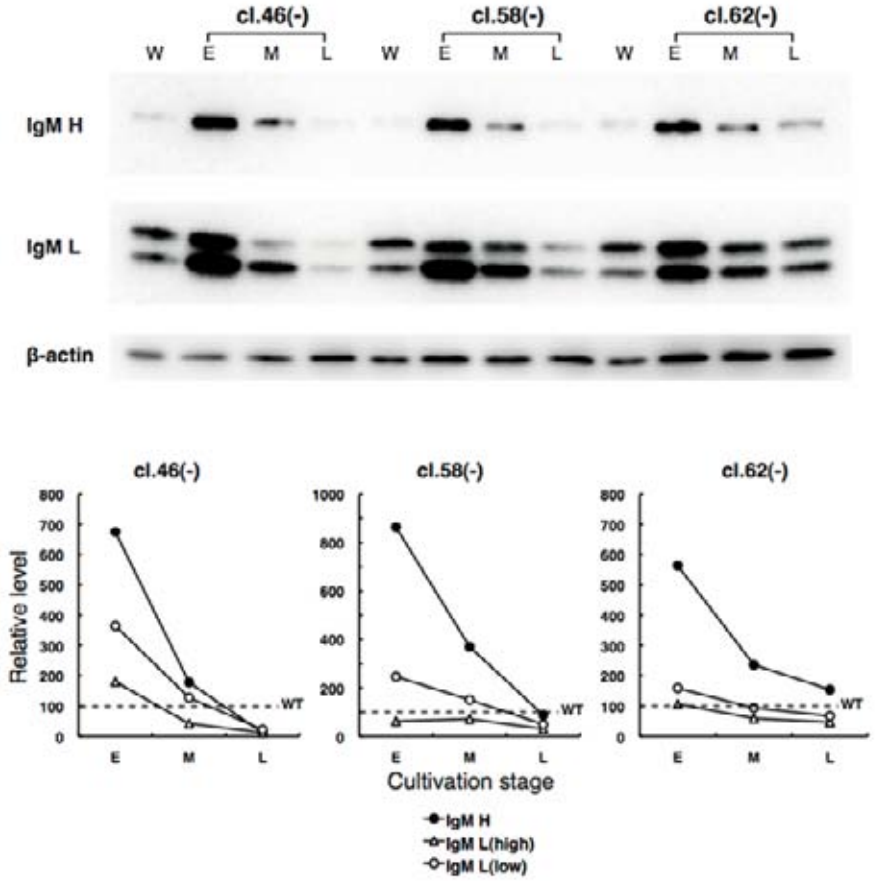

Figure 5. Alterations in amounts of IgM H- and L-chains in Pax5(-) DT40 mutants during continuous cultivation.

Western blotting was performed on total cellular proteins prepared from three Pax5(-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W), using anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain. Relative levels of IgM H-chain and large (high) plus small (low) forms of IgM L-chain are shown in the lower panel. The figure is identical with Figure 5 of Refs. 102, 103 plus 126, Figure 3-3 of Ref. 92, and Figure 3 of Ref. 93. These figures were first presented in Ref. 92.

elevated at the first (F; ~4 days) stage (prior to the early stage) in Pax5(-) mutants, and thereafter rapidly reduced through the early and middle stages and finally at the later stage reach almost the same levels as in DT40 cells (Figure 6). On the other hand, RT-PCR using primers IgM Hm and IgM L showed that the membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA are considerably increased at the first stage, and thereafter slowly decreased through the early and middle stages and at the later stage reach almost the same levels as in DT40 cells.

Interestingly, RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors in Pax5(-) mutants, showed not only that PCAF and HDAC9 mRNA levels are gradually elevated through the first, early and middle stages and at the later stage reach almost plateau levels, but also that the HDAC7 mRNA level moderately changes during cultivation (Figure 7). In addition, Aiolos and OBF1 mRNA levels are gradually reduced from the first through the early to middle stages and become undetectable at the later stage. Ikaros and E2A mRNA levels are drastically elevated at the first stage, and thereafter gradually and certainly decreased through the early to middle stages and at the later stage reach almost the same levels as in DT40 cells. The EBF1 mRNA level is completely reduced at the first stage and remains unchanged at an undetectable level during

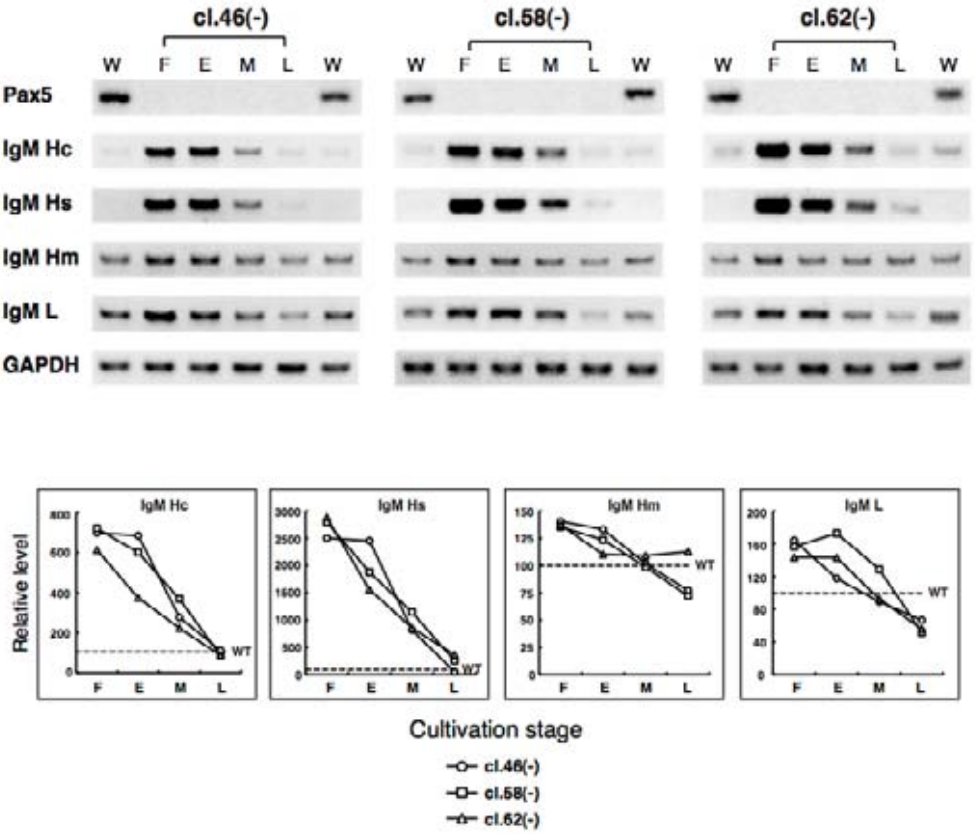

Figure 6. Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutants during continuous cultivation.

RT-PCR was performed on total RNAs prepared from three Pax5(-) mutant clones at the first (F), early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W), using appropriate primers for Pax5 mRNA, and whole (IgM Hc), secreted (IgM Hs) plus membrane-bound (IgM Hm) forms of IgM H-chain mRNA and IgM L-chain (IgM L) mRNA. Their relative mRNA levels are shown in the lower panel. The figure is identical with Figure 7 of Refs. 102, 103 plus 126, Figures 3-5 of Ref. 92, and Figure 6 of Ref. 93. These figures were first presented in Ref. 92.

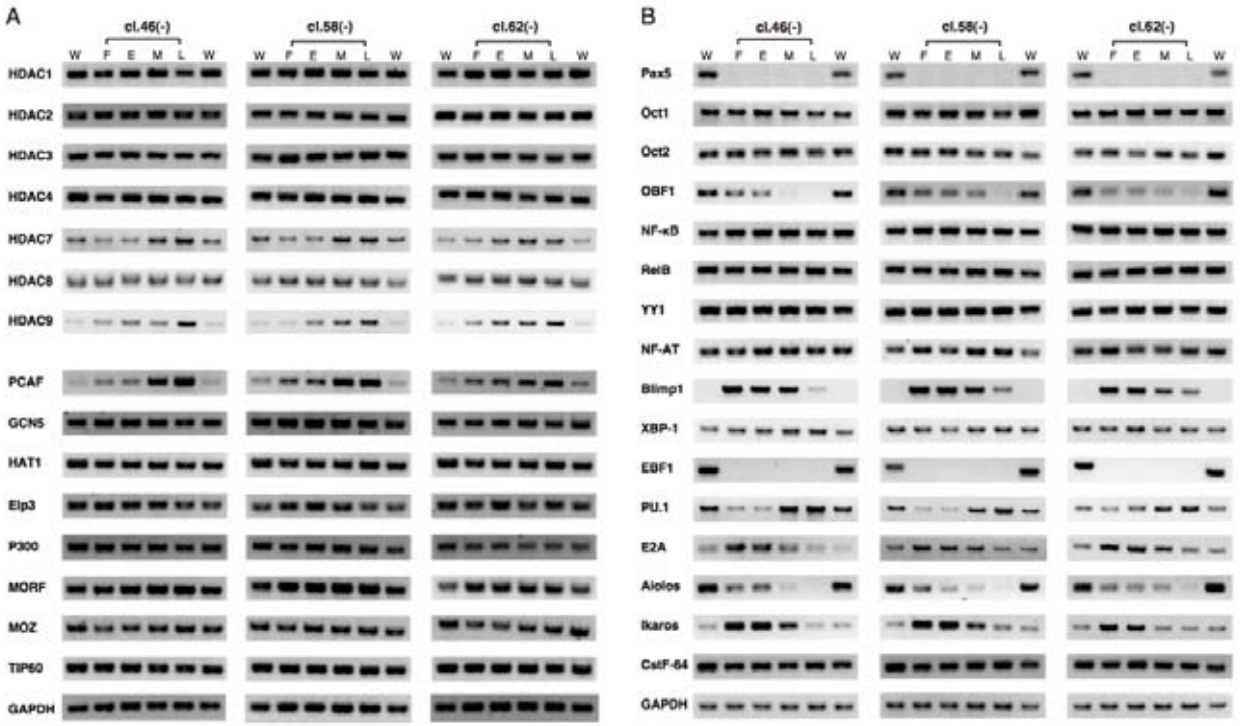

Figure 7. Alterations in gene expressions of various members of HDACs, HATs and transcription factors in Pax5(-) DT40 mutants during continuous cultivation.

RT-PCR was performed on total RNAs as described in Figure 6, using appropriate primers for various members of HDACs and HATs (A), and transcription factors (B), instead of those for IgM H- and L-chains. The figure is identical with Figure 6 of Ref. 93 and also Figures 3-6 of Ref. 92.

cultivation. The PU.1 mRNA level is obviously reduced at the first stage, and thereafter gradually elevated during cultivation and at the later stage reaches almost the same level as in DT40 cells.

By the way, microscopy showed that Pax5(-) mutants are observed to be the dispersive form at both the early and later stages, similar to that of DT40 cells (Figure 8). Such a morphological property of Pax5(-) mutants and its changing pattern are clearly different from those of HDAC2(-/-) mutants [96, 99] as will be discussed later.

Based on these findings obtained by qualitative analyses of initially generated HDAC2- and Pax5-deficient DT40 mutants, HDAC2(-/-) and Pax5(-) [57, 64, 92, 93, 95], we revealed that in these two mutant cell lines IgM H- and L-chains artificially accumulated at the early (or first) cultivation stage are diminished based on their decreased gene expressions, associated with alterations in gene expressions of various transcription factors and/or chromatin-modifying enzymes, through numerous generations during cultivation. However, several cellular characteristics are obviously different between HDAC2(-/-) and Pax5(-) mutant cells. Those of HDAC2(-/-) mutant cells will be shown in detail below.

Proteins and mRNAs of IgM H- and L-chains excessively accumulated at the early cultivation stage are dramatically reduced in almost similar changing pattern in distinct ways of gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

To clarify the above-mentioned results in further detail and also to eliminate effects of drug-resistant genes within targeting vectors, we newly generated HDAC2-deficient DT40 mutant cells HDAC2(-/-) (Figure 9) [92, 96, 99], using two targeting vectors different from those used

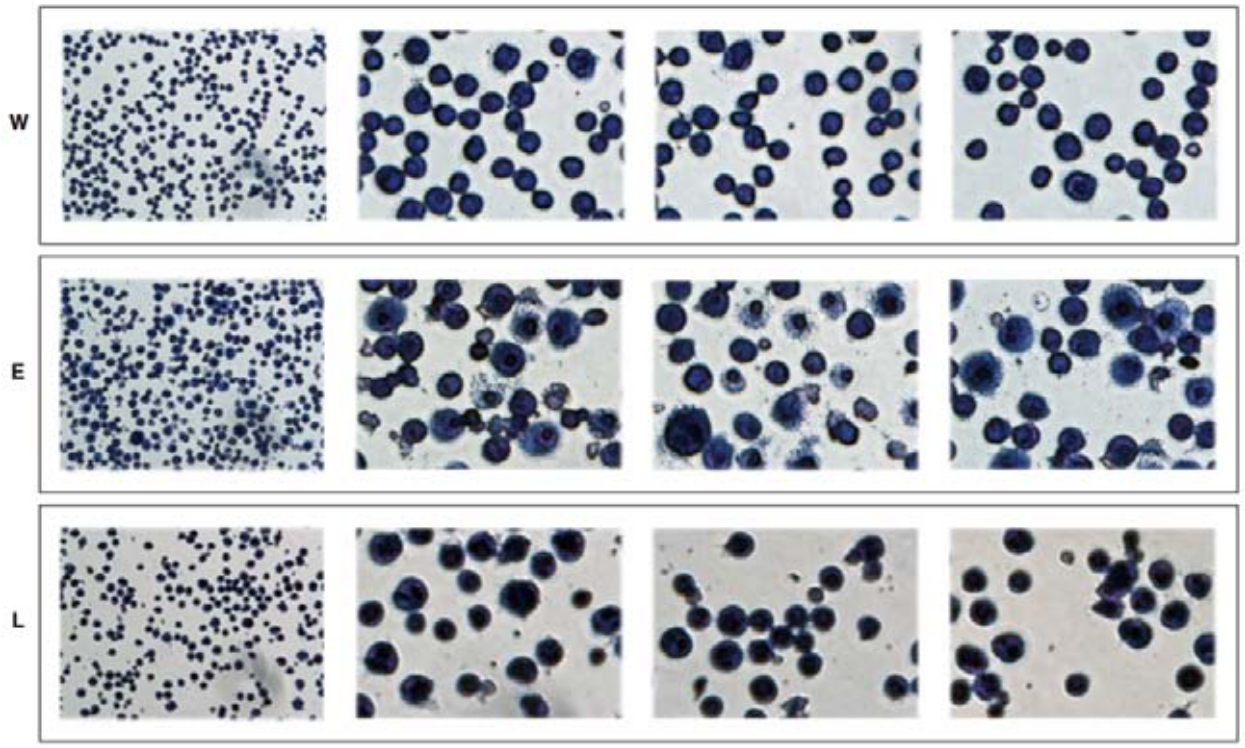

Figure 8. Morphology of Pax5(-) DT40 mutant cells during continuous cultivation.
Microscopy was performed on Pax5(-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W) at different magnifications. The figure is identical with Figure 6 of Refs. 102, 103 plus 126, Suppl. Figure 3-S1 of Ref. 92, and Figure 5 of Ref. 93. These figures were first presented in Ref. 92.

previously [57]. By systematical analyses of six individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of the 28 secondly generated HDAC2(-/-) mutant cells, we could obtain again following remarkable and noticeable results. As shown in Figures 10 and 11, in HDAC2(-/-) mutant cells, proteins and mRNAs of IgM H- and L-chains are dramatically and considerably accumulated at the early (E; ~3 to 7 days) cultivation stage, which was earlier than that adopted for initially generated HDAC2(-/-) mutant cells [92, 95], i.e., soon after the generation (birth). It is because HDAC2 as a supervisor mainly regulates gene expressions of these two immunoglobulin proteins through opposite controls of Pax5, Aiolos, EBF1, Ikaros, OBF1 and E2A gene expressions in wild-type DT40 cells (W) (Figure 1-W) [64]. These results obtained in HDAC2(-/-) mutant cells at the early stage of cultivation are schematically shown in Figure 1-E. The majority of IgM H- and L-chains artificially accumulated in HDAC2(-/-) mutant cells exist as a native soluble form capable of building a high molecular weight complex with each other within endoplasmic reticulum (Figure 12) [64], since the HDAC2 mediated regulatory mechanisms do not function any longer and lacking of the mechanisms can be far superior to the capacity to secrete accumulated immunoglobulin proteins. In addition, HDAC2(-/-) mutant cells exist as a morphologically aggregative and distorted form at the early stage (Figure 13), the reason for this is still unknown. Anyhow, both the accumulation of the two immunoglobulin proteins and the aggregative form may be abnormal and/or uncomfortable (painful) environments for HDAC2(-/-) mutant cells. Surprisingly but as expected in part, in all of individual clones of HDAC2(-/-) mutants, the proteins and mRNAs of IgM H- and L-chains artificially accumulated at the early stage are gradually reduced through the middle (M; ~30 days) stage and at the later (L; ~60 days) stage reach almost the same levels as in DT40 cells

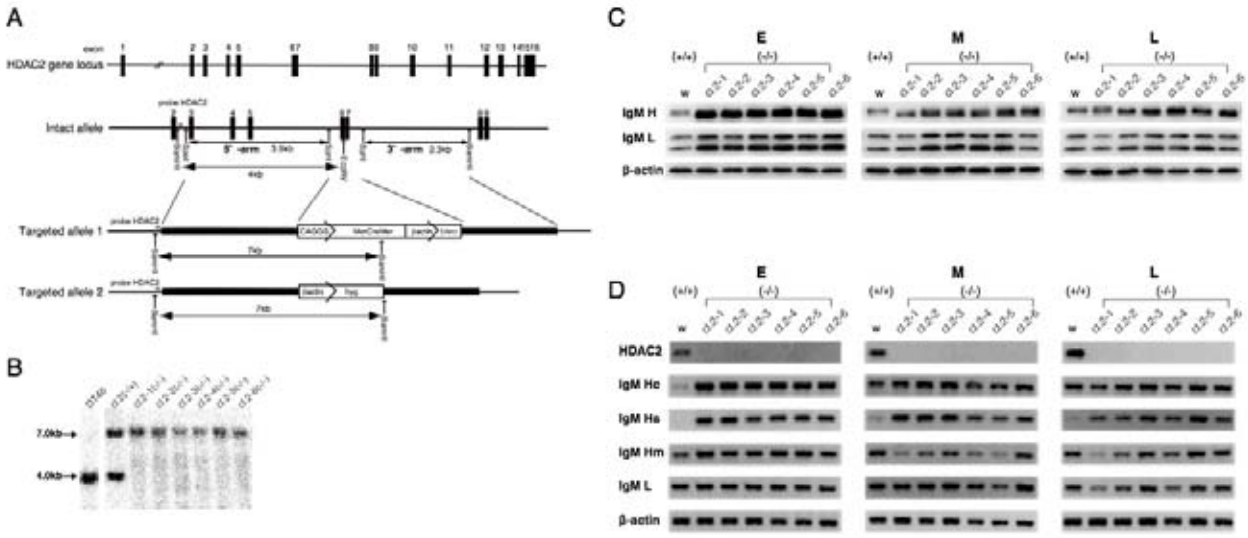

Figure 9. Genomic organization of chicken HDAC2 gene, generation of HDAC2(-/-) DT40 mutants, and alterations in protein and mRNA levels of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

A) Schematic presentation of chicken HDAC2 genomic locus (top) with enlarged drawing of its intact allele (middle) and targeted alleles (two bottoms). Locations of exons 1-16, drug resistance cassettes and probe HDAC2 are indicated by solid boxes, white boxes and gray box, respectively. Relevant fragments obtained from BamHI/EcoRV digestions are shown. **B)** Southern blotting of homologous recombination event. The BamHI/EcoRV fragments of genomic DNAs prepared from DT40, one HDAC2(-/+) mutant clone cl.2 and six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) were analyzed with probe HDAC2. **C)** Western blotting, using anti-chicken IgM L-chain and H-chain antibodies, was performed on total cellular proteins prepared from the six HDAC2(-/-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W). IgM H and L indicate IgM H- and L-chains (two bands), respectively. **D)** RT-PCR was performed on total RNAs extracted from the six HDAC2(-/-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W), using appropriate primers for whole (IgM Hc), secreted (IgM Hs) and membrane-bound (IgM Hm) forms of IgM H-chain mRNA and IgM L-chain mRNA (IgM L). The figure is identical with Figure 8 of Refs. 102, 103 plus 126, Figure 4-1 of Ref. 92, Figure 1 of Ref. 96, and Figure 1 of Ref. 99. These figures were first presented in Ref. 92.

(Figures 10, 11 and 12). Agreeing with these changes, the morphology of HDAC2(-/-) mutants also changes; i.e., the aggregative form at the early stage changes during cultivation and at the later stage becomes the dispersive form, which may be normal and/or comfortable (peaceful) for the mutants as for DT40 cells (Figure 13). In addition to the above-mentioned findings, very recently, we first noticed following interesting and important facts on results which had been already obtained by immuno-electron microscopy of initially generated HDAC2(-/-) mutant cells [64, 92, 95]. As described above, IgM H- and L-chains artificially and largely synthesized by the HDAC2-deficiency are first accumulated within endoplasmic reticulum of HDAC2(-/-) mutant cells. In parallel and/or subsequently, most of these accumulated immunoglobulin proteins are gradually secreted to outside of mutant cells (i.e., into the cultivation media); however, a part of them is transported to the nuclear envelope but not inside of the nucleus and kept in the peri-nuclear space at the early and also later stages of cultivation. These findings will be discussed in detail later.

At this step, concerning the ways to eliminate large amounts of IgM H- and L-chains as an abnormal, unexpected and uncomfortable intra- and/or extra-cellular environment change for HDAC2(-/-) mutant cells, we built up a brief working hypothesis as follows [92, 96, 99]. Putative signal(s) concerning the accumulation of IgM H- and L-chains (and also probably cell

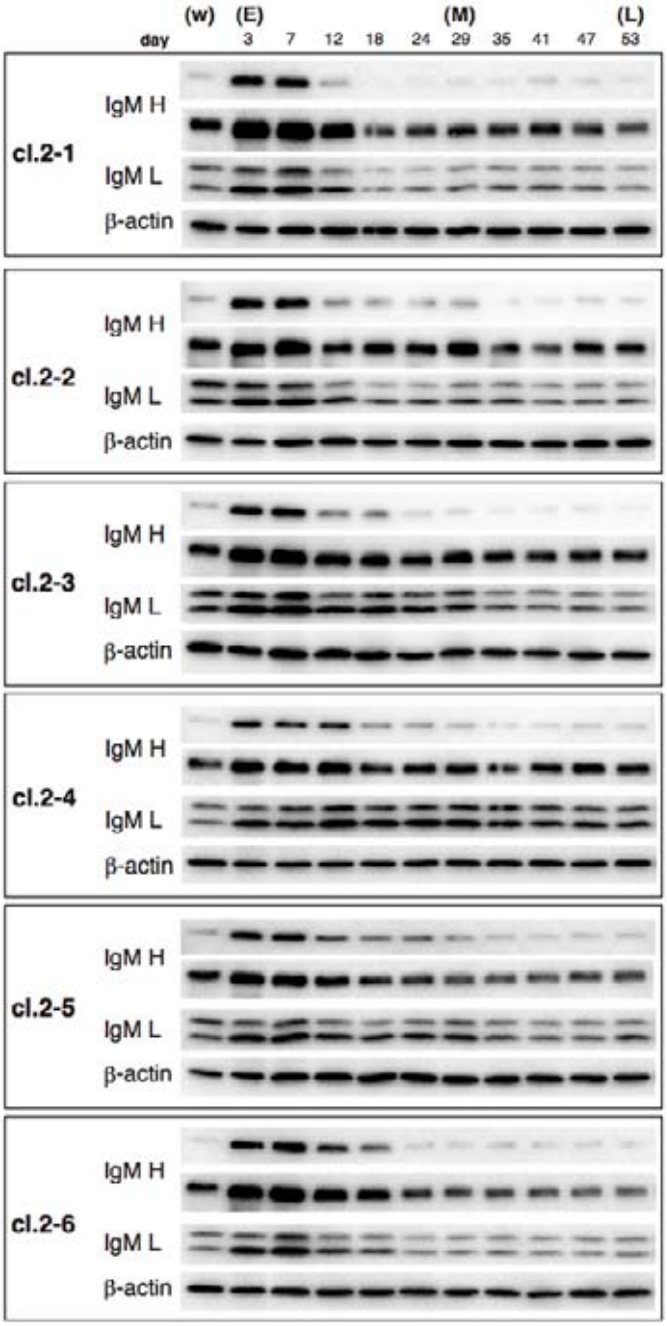

Figure 10. Alterations in amounts of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

Western blotting was performed on total cellular proteins prepared from the six HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at the indicated cultivation periods, including the early (E), middle (M) and later (L) stages and from DT40 cells (W). Proteins were sequentially detected with chicken antibodies for IgM L-chain that cross-reacts with IgM H-chain (top and third), IgM H-chain (second), and β-actin as a control. Upper and lower bands (indicated by IgM H) correspond to IgM H-chain detected by antibodies for IgM H- and L-chains, respectively. IgM L-chain (indicated by IgM L) was detected as two bands. The figure is identical with Figure 9 of Refs. 102, 103 plus 126, Figures 4-2 plus Suppl. Figure 6-S4 of Ref. 92, Figure 2 of Ref. 96, Figure 2 of Ref. 99, and Figure 2 of Ref. 101. These figures were first presented in Ref. 92.

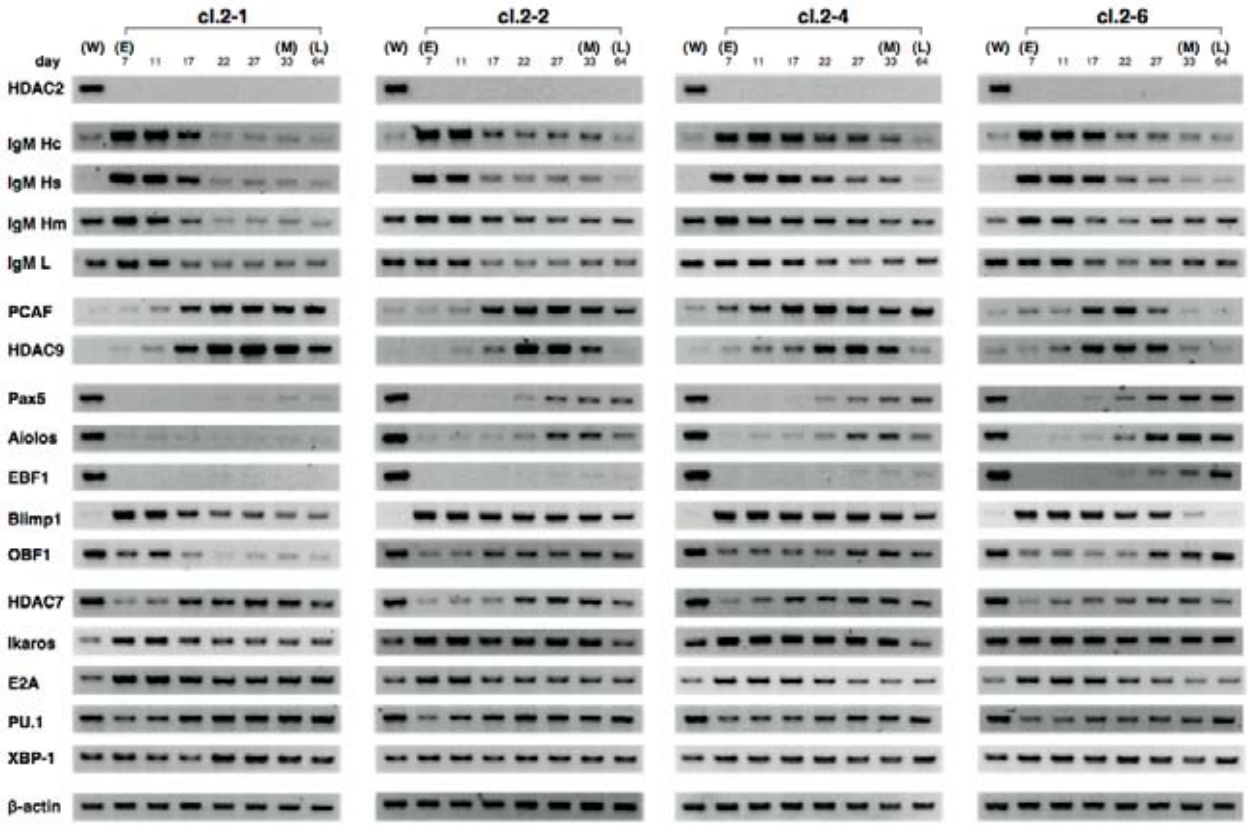

Figure 11. Alterations in gene expressions of IgM H- and L-chains, and specific members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

RT-PCR was performed on total RNAs prepared from four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the indicated periods of cultivation, including the early (E), middle (M) and later (L) stages and from DT40 cells (W), using appropriate primers for mRNAs of IgM H- and L-chains, PCAF, HDAC9, Pax5, Aiolos, EBF1, Blimp1, OBF1, HDAC7, Ikaros, E2A, PU.1 and XBP-1, the changes of which were shown in Refs. 92 and 96. The figure is identical with Figure 10 of Refs. 102, 103 plus 126, Figures 4-6 plus Suppl. Figure 6-S5 of Ref. 92, Figure 6 of Ref. 96, Figure 4 of Ref. 99, and Figure 3 of Ref. 101. These figures were first presented in Ref. 92.

aggregation) may be transmitted to the chromatin (structure) within the nucleus during cultivation, though both the mechanism and the machinery still remain quite unknown. Gene expressions of numerous chromatin-modifying enzymes and transcription factors slightly change associated with alterations in their chromatin structure. Both the putative signal(s) concerning the environment change and the response(s) to the change are repeatedly converged into the chromatin structure of the proximal 5'-upstream regions of PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Oct2, Blimp1, XBP-1 and other genes during cultivation. Interestingly, as will be described below in detail, mRNA (i.e., gene expression/transcription) levels of these changed transcription factors and chromatin-modifying enzymes show distinct changing patterns during cultivation in six examined individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants (Figure 11) [92, 96, 99], regardless of almost similar changing pattern in mRNA and protein levels of IgM H- and L-chains, and also in the cell morphology. Hereafter, the changing patterns in gene expressions of the altered specific transcription factors and other cellular characteristics of four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) will be shown in detail, because the four individual clones (cl.2-2, cl.2-3, cl.2-4 and cl.2-5) had great

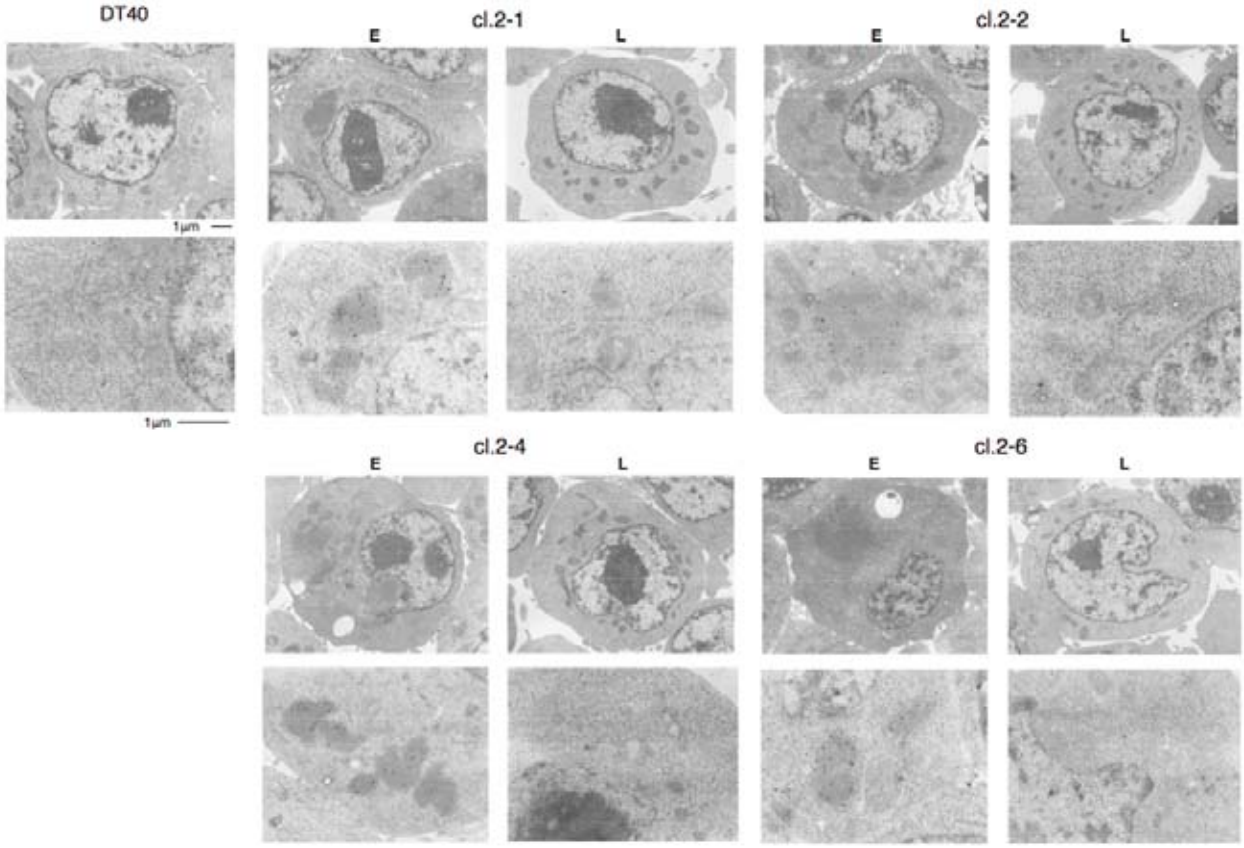

Figure 12. Alterations in amounts of IgM H-chain proteins in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

Electron microscopy (upper panels) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (lower panels) were performed on four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E) and later (L) cultivation stages and on DT40 cells (W). Dense cytoplasmic fractions due to accumulated IgM H-chains were observed only at the early (E) stage in the four mutant clones (E in upper panels). Positive signals for IgM H-chains were observed only at the early (E) stage in these mutant clones (E in lower panels). The figure is identical with Figure 11 of Refs. 102, 103 plus 126, Figure 4-4 of Ref. 92, and Figure 4 of Ref. 96. These figures were first presented in Ref. 92.

resemblance in many cellular properties with each other as mentioned above.

In clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which are high levels in DT40 cells and down-regulate gene expressions of IgM H- and L-chains [64], are almost completely decreased at the early stage and thereafter remain unchanged until the later stage (Figure 11). By contrast, the mRNA level of OBF1, which is a high level in DT40 cells and probably up-regulates gene expressions of these immunoglobulin proteins [64, 94], is dramatically reduced from the early to later stages. Therefore, the way for gene expressions of IgM H- and L-chains at the later stage in clone cl.2-1 seems to be dependent on OBF1, and considerably different from that in wild-type DT40 cells in appearance (Figure 1-L). Moreover, clone cl.2-1 resembles in appearance Pax5(-) mutants in the decreasing pattern of the accumulated IgM H- and L-chains, since the gene expression of OBF1 (associated with changes in gene expressions of Aiolos, EBF1, PCAF, HDAC7 and HDAC9) is dramatically decreased in almost similar pattern in both clone cl.2-1 [96] and Pax5(-) [93]. In clones cl.2-2 and cl.2-4, mRNA levels of Pax5, Aiolos and EBF1 are almost completely decreased at the early stage (Figure 11). Thereafter, those of Pax5

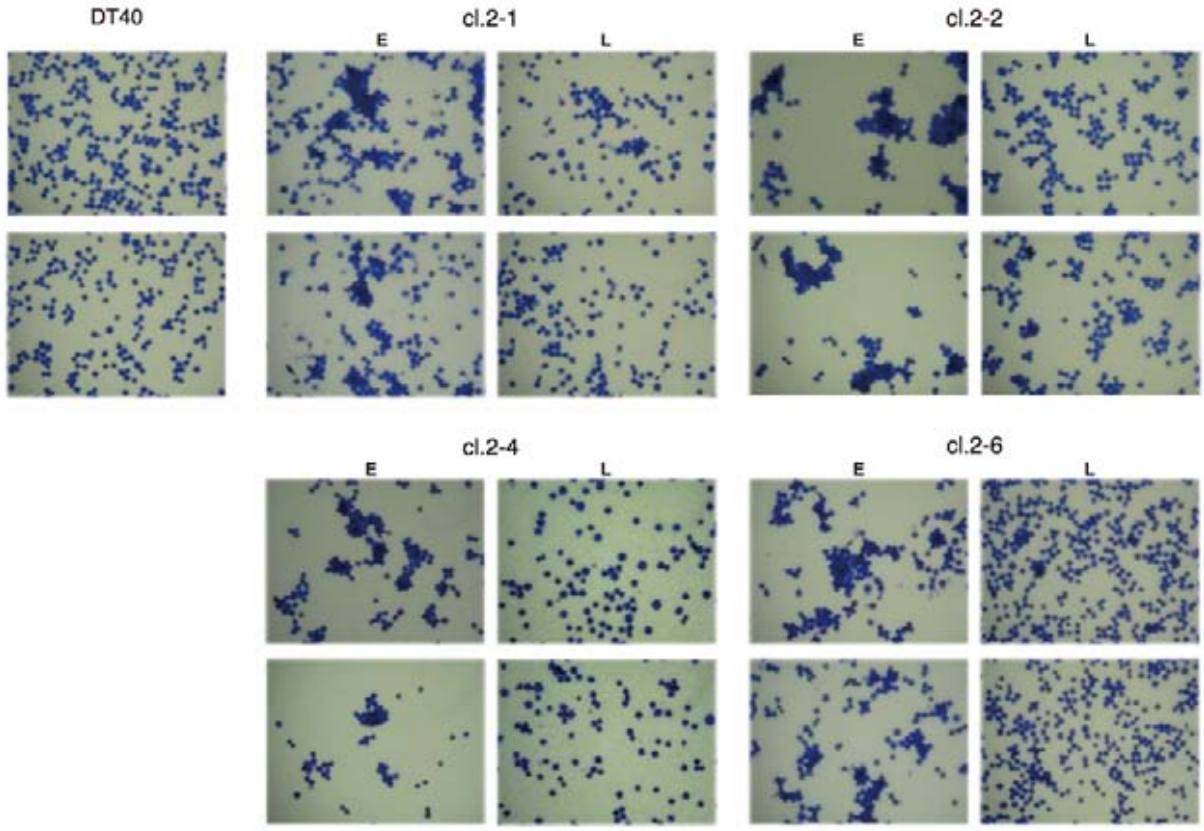

Figure 13. Alterations of morphology of individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation. Microscopy was performed on four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E) and later (L) stages of cultivation and on DT40 cells (W) in several different ranges of vision. The different ranges of vision within microscopy are presented. Aggregative form was observed only at the early (E) stage in these mutant clones. The figure is identical with Figure 12 of Refs. 102, 103 plus 126, Figures 4-5 of Ref. 92, and Figure 5 of Ref. 96. These figures were first presented in Ref. 92.

and Aiolos are gradually increased through the middle to later stages but that of EBF1 remains unchanged at an undetectable level until the later stage. On the other hand, the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the later stage. Therefore, the ways for gene expressions of IgM H- and L-chains at the later stage in clones cl.2-2 and cl.2-4 (and cl.2-3 and cl.2-5) seem to be dependent on Pax5 and Aiolos, and slightly similar to that in DT40 cells in appearance (Figure 1-L). Moreover, these four clones are the major types, since four initially generated HDAC2(-/-) mutant clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) resemble the first four clones in several cellular properties [95]. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are almost completely decreased at the early stage and thereafter dramatically increased through the middle to later stages (Figure 11). On the other hand, the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the later stage. Therefore, the way for gene expressions of IgM H- and L-chains at the later stage in clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1, and mostly similar to that in DT40 cells in appearance (Figure 1-L), since the two immunoglobulin gene expressions in DT40 cells are directly and cooperatively regulated by these three transcription factors (and E2A) (Figure 1-W). These three models for roles of Pax5, Aiolos, EBF1 and OBF1 in control of gene expressions of IgM H- and L-chains at the later stage of cultivation in individual clones of HDAC2(-/-)

DT40 mutants are schematically shown in Figure 1-L. These ways to suppress gene expressions of IgM H- and L-chains at the later cultivation stage in all individual HDAC2(-/-) mutant clones are really distinct from the ordinary and reversible transcriptional regulations of the two immunoglobulin genes in DT40 cells. It is because all of these mutant clones are lacking HDAC2, which controls gene expressions of Pax5, Aiolos, EBF1, OBF1 and E2A in DT40 cells. If additional independent clones of HDAC2(-/-) mutants are analyzed, besides the above-mentioned three ways, other distinct ways for gene expressions of IgM H- and L-chains will be probably added. Moreover, the above-mentioned results on varied alterations in gene expressions of various transcription factors and chromatin-modifying enzymes suggested that some other unknown cellular characteristics must certainly change in individual HDAC2(-/-) mutant clones during cultivation. The reasons for this inference are as follows. Pax5 clearly regulates PCAF, HDAC7, HDAC9, Aiolos, OBF1, Ikaros, E2A, PU.1 plus EBF1. Pax5 isoforms A and B differentially regulate many B cell development-related genes [64, 92, 93]. Aiolos regulates pre-mature B cell apoptosis mediated by BCR signaling [86]. E2A regulates gene expressions of survivin, IAP2 and caspase-8 [85]. Helios regulates the gene expression of protein kinase Cs [87]. EBF1 regulates dramatically gene expressions of Blimp-1 and protein kinase Cθ [88, 89].

Based on these findings [92-96, 99], we concluded that individual clones of HDAC2(-/-) mutants possess the ability to gain the same and new cell functions, i.e., to exclude large amounts of IgM H- and L-chains and to change the mutant cells from the aggregated to normal dispersed forms in distinct ways as the generation progresses during cultivation. Such distinct ways bring about different changing patterns in gene expressions of Pax5, Aiolos, EBF1, OBF1 (and E2A and others) in individual mutant clones, though gene expressions of IgM H- and L-chains change in almost similar pattern in all of them. In addition, we would like to emphasize that alterations in any characteristics of HDAC2(-/-) (and also Pax5(-)) mutant cells are more drastic just soon after their birth. This inference is based on the fact that the mutant cells 15-16 days after their birth were used as the samples at the early stage of cultivation, by gene targeting techniques [92, 93, 95, 96, 99] and their doubling times are ~12 hrs [57, 64, 77]; therefore, they are populations around 30-32 generations even at the early stage.

As described above, among transcription factors Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1 and others, whose gene expressions change in HDAC2(-/-) mutants during cultivation, Pax5, Aiolos, EBF1 and OBF1 are influential candidates participating in decreases in the elevated gene expressions of IgM H- and L-chains in individual clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6 of HDAC2(-/-) mutants [92, 96, 99]. The validity of this inference is supported by the findings that changing patterns of these four factor gene expressions are anti-parallel or parallel with those of the two immunoglobulin gene expressions in one or more of these six individual clones of HDAC2(-/-) mutants. Further, as described previously, Pax5, Aiolos and EBF1 have already been shown, by gene targeting techniques, to down-regulate gene expressions of IgM H- and L-chains in chicken DT40 cells [64, 67, 92, 93, 96, 99], and OBF1 was suggested to up-regulate these two immunoglobulin gene expressions, since it functionally activates the chicken L-chain promoter in NIH 3T3 cells [94].

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay: A new method to study fundamental ways for irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes based on varied alterations in acetylation and deacetylation levels of specific Lys residues of histone H3 in individual clones of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

We studied how individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of HDAC2(-/-) mutants differentially gain distinct ways for positive or negative gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF through numerous generations during cultivation. To execute the project, we carried out the ChIP assay on the chromatin surrounding their proximal ~2.0 kb 5'-upstream,

distal 5'-upstream and coding (open reading frame: ORF) regions. This is because the chromatin structure surrounding the proximal 5'-upstream region should be directly and closely related to the transcriptional activity of the corresponding gene, regardless of the presence or absence of transcriptional elements within the region. Moreover, as mentioned above, our previous data obtained by the dual-luciferase assay suggested that at least the proximal ~1.6 kb 5'-upstream region of the Pax5 gene is necessary for the regulation of its gene expression (data not shown) [92, 95]. We designed appropriate primers based on nucleotide sequences of the proximal 5'-upstream, distal 5'-upstream and ORF regions of these five specific genes, which were cloned and determined by us or obtained from a database. We named this ChIP assay as the neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; the abbreviation also means IP on notch of chromatin) assay. It is because all of DNA fragments amplified by PCR using appropriate primers, which were designed based on nucleotide sequences of the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of the above-mentioned genes, coincide with corresponding segments of the region and are laid overlapping to each other with neighboring ones.

We systematically carried out the NotchIP assay on the chromatin prepared from four individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E; 3 days), middle (M; 33 days) and later (L; 58 days) stages of cultivation, and from wild-type DT40 cells (W). Throughout the NotchIP assay, we used five site-specific antibodies for acetylated Lys-9 (K9/H3), Lys-14 (K14/H3), Lys-18 (K18/H3), Lys-23 (K23/H3) and Lys-27 (K27/H3) residues of histone H3 as primary antibodies, since bulk acetylation levels of these five Lys residues of histone H3 obviously changed in initially generated HDAC2(-/-) mutants during cultivation [92, 95]. However, regarding the Pax5 gene in clone cl.2-2, we used only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3 at the early and later stages of cultivation, because this case was the first attempt of the NotchIP assay. In these studies, we tentatively and qualitatively deduced the binding ability (capacity) of histone H3 to DNA based on acetylation levels of one or more of these specific Lys residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) obtained by the NotchIP assay, though it is still unclear which Lys residue(s) is really and/or mainly involved in the binding. That is, hyper- (high), considerably hyper-, somewhat hyper- and hypo- (low or no) acetylation levels of one or more of these five Lys residues should qualitatively induce no, weak, less and full binding ability of histone H3 (or corresponding Lys residue(s)) to DNA, resulting in the loose (open), considerably loose, somewhat loose and tight (closed) forms of the chromatin structure, leading to high, considerably high, somewhat high and low (or no) mRNA (i.e., transcription/gene expression) levels of corresponding gene(s) (Figures 14, 15, 16, 17 and 18). Results obtained by the NotchIP assay [92, 97, 100] and RT-PCR [92, 96, 99] on the five specific genes in the four individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants are simply shown as follows.

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes based on varied alterations in acetylation and/or deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3 for gaining new cell function to exclude IgM H- and L-chains accumulated in clone cl.2-1 of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

In DT40 cells, the five Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -1923 to +30 of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibit full binding ability of histone H3 to DNA at all cultivation stages based on their hypo-acetylation levels (Figure 14-Pax5). Consequently, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, exists as the loose form in DT40 cells, but changes to the tight

form at the early stage in clone cl.2-1 and thereafter remains unchanged until the later stage. These facts agreed with the findings that the gene expression of Pax5, which is at a high level in DT40 cells, is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (Figure 11).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2250 to +145 of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at all cultivation stages (Figure 14-Aiolos). Accordingly, the chromatin structure surrounding the proximal ~2.1 kb 5'-upstream region of the gene, which may consist of ~11 nucleosomes, exists as the loose form in DT40 cells, but changes to the tight (or somewhat loose) form at the early stage in clone cl.2-1 and thereafter remains unchanged until the later stage. These facts agreed with the findings that the gene expression of Aiolos, which is at a high level in DT40 cells, is drastically suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (Figure 11).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2031 to +200 of the EBF1 gene based on their hyper-acetylation levels. However, in clone cl.2-1, K9/H3, K18/H3 and K27/H3 in particular possess a full (or less) binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at all cultivation stages (Figure 14-EBF1). Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, exists as the loose form in DT40 cells, but changes to the tight (or somewhat loose) form at the early stage in clone cl.2-1 and thereafter remains unchanged until the later stage. These facts agreed with the findings that the gene expression of EBF1, which is at a high level in DT40 cells, is almost completely suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (Figure 11).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2138 to +164 of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-1, K9/H3 and K27/H3 (and probably K23/H3) in particular certainly possess a weak binding ability of histone H3 to DNA based on their considerably hyper-acetylation levels at the early stage. Further, the weak binding ability is dramatically increased at the middle stage and thereafter remains unchanged at the later stage based on their hypo-acetylation levels (Figure 14-OBF1). Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells. On the other hand, in clone cl.2-1, the chromatin structure changes to the considerably loose form at the early stage and thereafter changes to the tight form at the middle and later stages. These facts agreed with the findings that the gene expression of OBF1, which is at a high level in DT40 cells, is slightly decreased at the early stage in clone cl.2-1 and thereafter dramatically suppressed to a very low (or no) level at the middle and later stages (Figure 11).

In DT40 cells and clone cl.2-1 at all cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no (or a low) binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2005 to +231 (and two distal 5'-upstream regions) of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-1 [92, 97, 100]. Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in both DT40 cells and clone cl.2-1. However, as in DT40 cells, so in clone cl.2-1 the gene expression of the

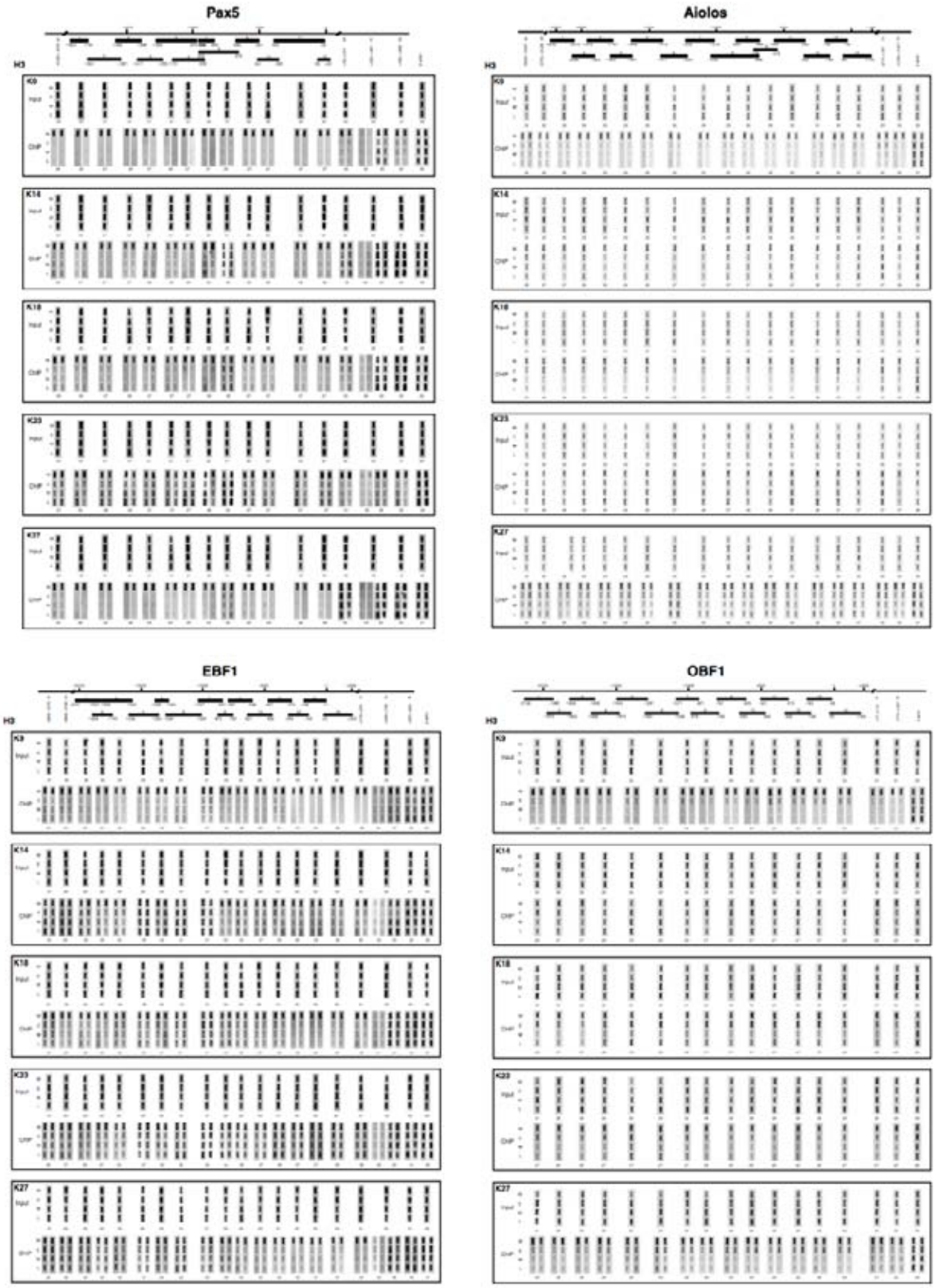

Figure 14

PCAF gene is really at a very low level at the early stage but thereafter gradually and dramatically increased until the later stage (Figure 11). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, are expected to participate in the PCAF gene expression, and the examined proximal and distal 5'-upstream regions are assumed to poorly correlate with the PCAF gene expression, regardless of their loose form in both DT40 cells and clone cl.2-1.

These results, together with the previous inference speculated from changing patterns in gene expressions of Pax5, Aiolos, EBF1 and OBF1 [93], indicated that at the later cultivation stage clone cl.2-1 seems to be dependent on OBF1 and considerably distinct from wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance (Figure 1-L).

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes based on varied alterations in acetylation and/or deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3 for gaining new cell function to exclude IgM H- and L-chains accumulated in clone cl.2-2 of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

As described above, in DT40 cells, the four Lys residues (K9/H3, K14/H3, K18/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-2, they exhibit a full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage. Thereafter, very surprisingly, the level of binding capacity of these four Lys residues of histone H3 to DNA gradually decreases during cultivation and finally reaches the state of no binding ability based on their hyper-acetylation levels at the later stage (Figure 15-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2. Thereafter, the chromatin structure changes to the loose form until the later stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter gradually and certainly elevated to a high level until the later stage (Figure 11).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-2, those Lys residues mainly K9/H3 and K27/H3 possess a less binding ability of histone H3 to DNA based on their somewhat hyper-acetylation levels at the early and middle stages, but mainly the binding capacity of K9/H3 is certainly decreased to the state of no (or weak) binding ability based on the considerably hyper-acetylation levels at the later stage (Figure 15-Aiolos).

Legend to Figure 14. Alterations in acetylation levels of specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation.

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and from DT40 (W), and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for the indicated segments of each of Pax5, Aiolos, EBF1 and OBF1 genes once or twice. Chicken β-actin was used as a control. A portion of cell supernatants as an input was amplified once by PCR using the same primers. PCR products were analyzed by agarose gel electrophoresis. The figure is identical with Figure 13 of Refs. 102, 103 plus 126, and is a set of Figures 5-1, 5-5, 5-9 plus 5-13 of Ref. 92, and is identical with Suppl. Figure 6-S6 of Ref. 92, and a set of Figures 1, 5, 9 plus 13 of Ref. 97, and is identical with Figure 4 of Ref. 101. These figures were first presented in Ref. 92.

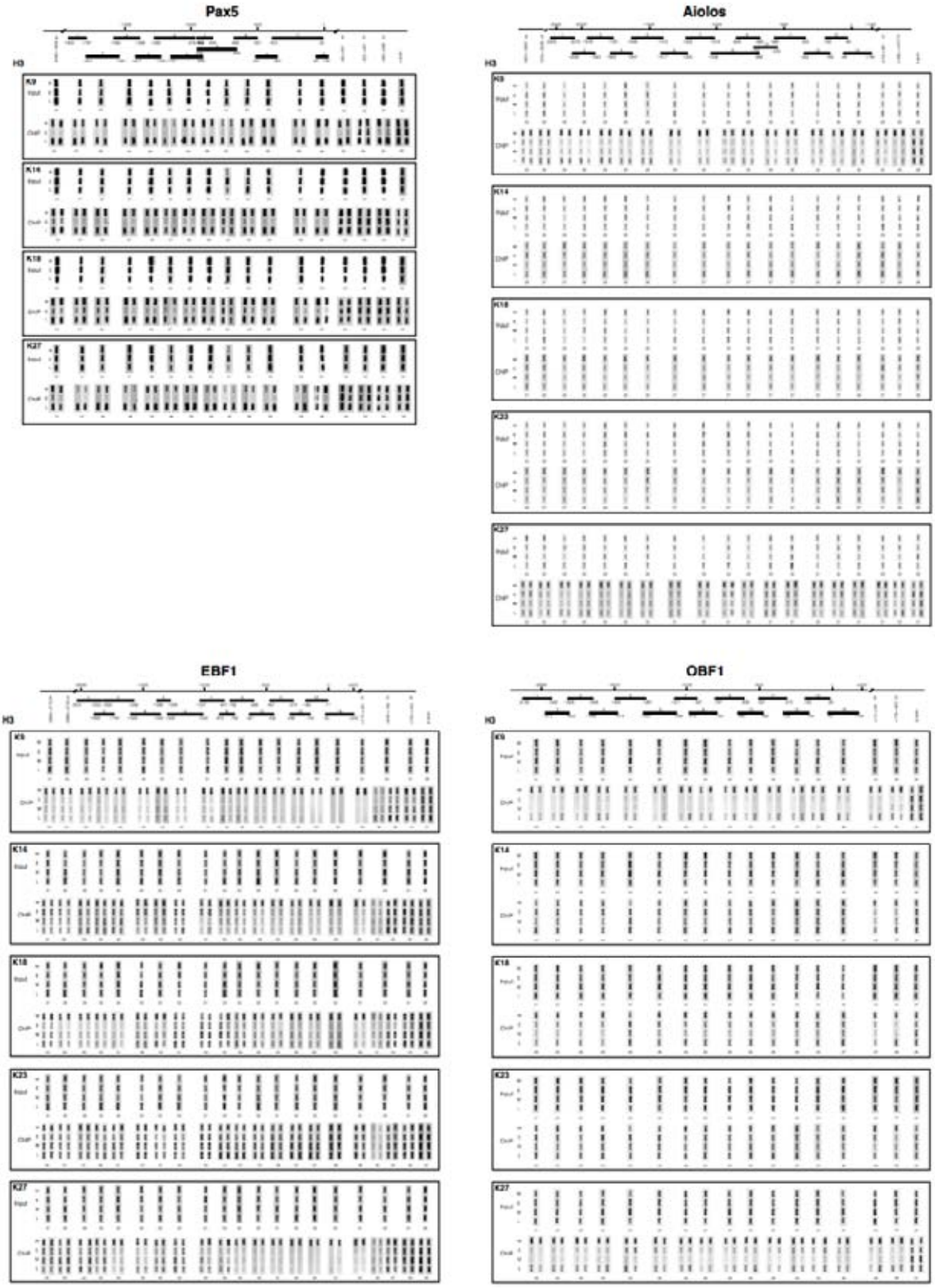

Figure 15

Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2 and thereafter changes to the loose (or considerably loose) form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter increased to a high level at the later stage (Figure 11).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, K9/H3, K14/H3 and K27/H3 (and probably K18/H3) in particular exhibit a full binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at all cultivation stages (Figure 15-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2 and remains unchanged through the middle to later stages. These facts agreed with the findings that the gene expression of EBF1 is almost completely suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter remains unchanged (Figure 11).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, K9/H3 and K27/H3 in particular exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. The less binding ability of K27/H3 is slightly increased and that of K9/H3 remains unchanged as a full binding ability based on the hypo-acetylation levels at the middle stage. Thereafter, the full binding ability of K9/H3 and K27/H3 is obviously reduced to the state of almost no binding ability based on their hyper- or considerably hyper-acetylation levels at the later stage (Figure 15-OBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the considerably tight form at the early stage in clone cl.2-2 and thereafter changes to the tight form at the middle stage. Subsequently, the tightened chromatin structure changes to the loose form at the later stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-2 and thereafter obviously elevated to a high level at the later stage (Figure 11).

In both DT40 cells and clone cl.2-2 at all cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-2 [92, 97, 101]. Therefore, the chromatin structure surrounding the proximal 5'-upstream region of the gene exists as the loose form in both DT40 cells and clone cl.2-2 at all cultivation stages. However, in clone cl.2-2 the gene expression of PCAF is really at a very low level at the early stage, as in DT40 cells,

Legend to Figure 15. Alterations in acetylation levels of specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-2 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and in DT40 (W) as described in the legend for Figure 14. The figure is identical with Figure 14 of Refs. 102, 103 plus 126, and is a set of Figures 5-2, 5-6, 5-10 plus 5-14 of Ref. 92, and is identical with Suppl. Figure 6-S7 of Ref. 92, and a set of Figures 2, 6, 10 plus 14 of Ref. 97, and is identical with Figure 5 of Ref. 101. These figures were first presented in Ref. 92.

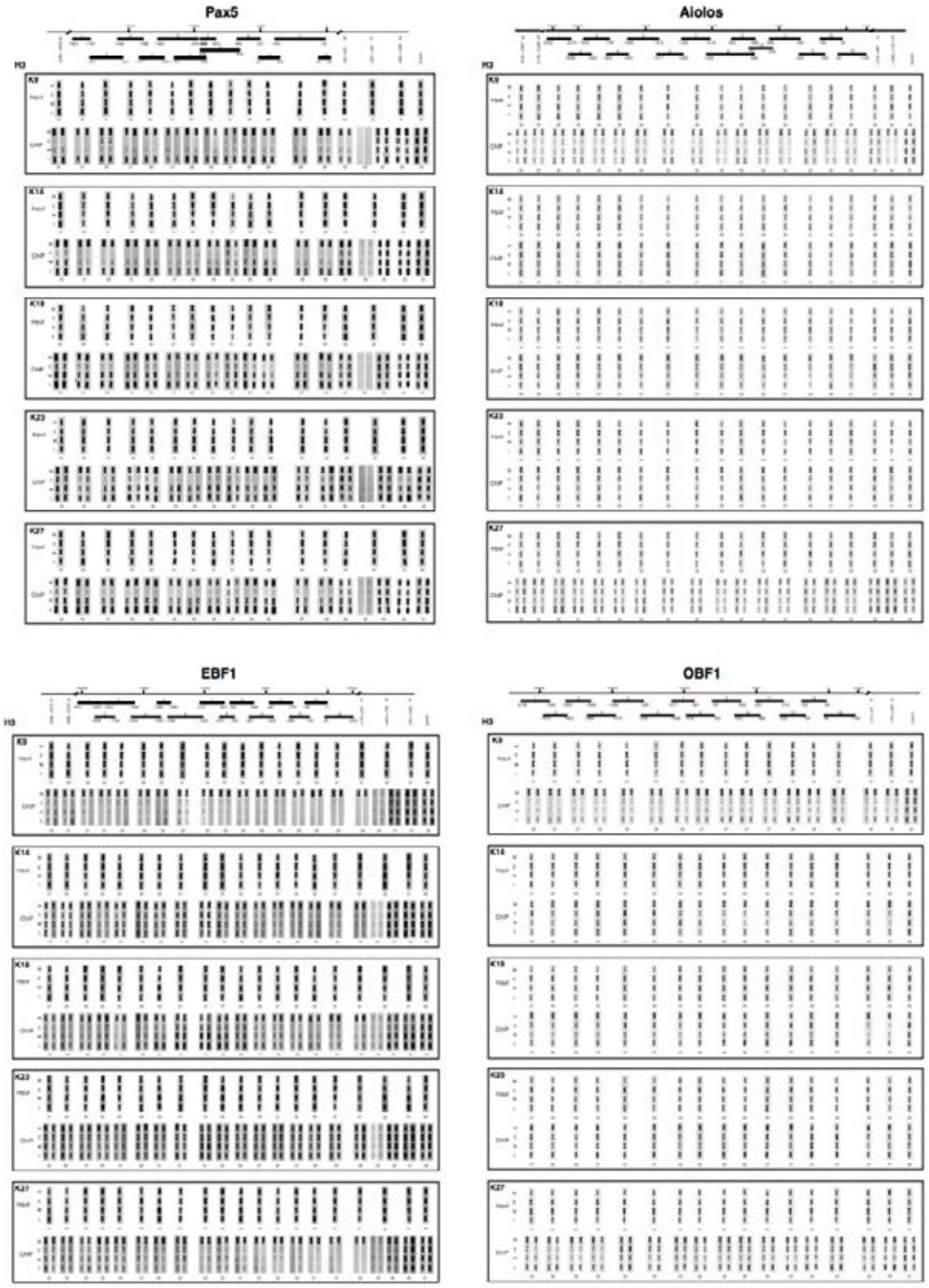

Figure 16

but gradually and dramatically increased until the later stage (Figure 11). Accordingly, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in the PCAF gene expression, and the examined distal and proximal 5'-upstream regions do not correlate directly and closely with the PCAF gene expression, regardless of the loose form in both DT40 cells and clone cl.2-2.

These results, together with the previous inference speculated from changing patterns in gene expressions of Pax5, Aiolos, EBF1 and OBF1 [92, 96, 99], indicated that at the later cultivation stage clone cl.2-2 seems to be dependent on Pax5 and Aiolos, and somewhat similar in appearance to wild-type DT40 cells in the way for gene expressions of IgM H- and L-chains. In addition, clone cl.2-2, like clone cl.2-4, should be a member of the major clone type among at least three different HDAC2(-/-) mutant clone types (Figure 1-L); the reason for this will be mentioned later.

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes based on varied alterations in acetylation and/or deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3 for gaining new cell function to exclude IgM H- and L-chains accumulated in clone cl.2-4 of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-4, the five Lys residues possess a full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage. Thereafter, the binding capacity of these five Lys residues to DNA decreases during cultivation and finally reaches the state of almost no binding ability based on their hyper-acetylation levels at the later stage (Figure 16-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4. Thereafter, the tightened chromatin structure changes to the loose form until the later stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-4 and thereafter gradually and certainly elevated to a high level until the later stage (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-4, K9/H3 (and probably K18/H3 and K27/H3) mainly possess a less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. Thereafter, mainly, the binding capacity of K9/H3 is certainly decreased during cultivation and finally reaches the state of almost no binding ability based on the considerably hyper-acetylation levels at the later stage (Figure 16-Aiolos). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4 and

Legend to Figure 16. Alterations in acetylation levels of specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-4 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and in DT40 (W) as described in the legend for Figure 14. The figure is identical with Figure 15 of Refs. 102, 103 plus 126, and is a set of Figures 5-3, 5-7, 5-11 plus 5-15 of Ref. 92, and is identical with Suppl. Figure 6-S8 of Ref. 92, and a set of Figures 3, 7, 11 plus 15 of Ref. 97, and is identical with Figure 6 of Ref. 101. These figures were first presented in Ref. 92.

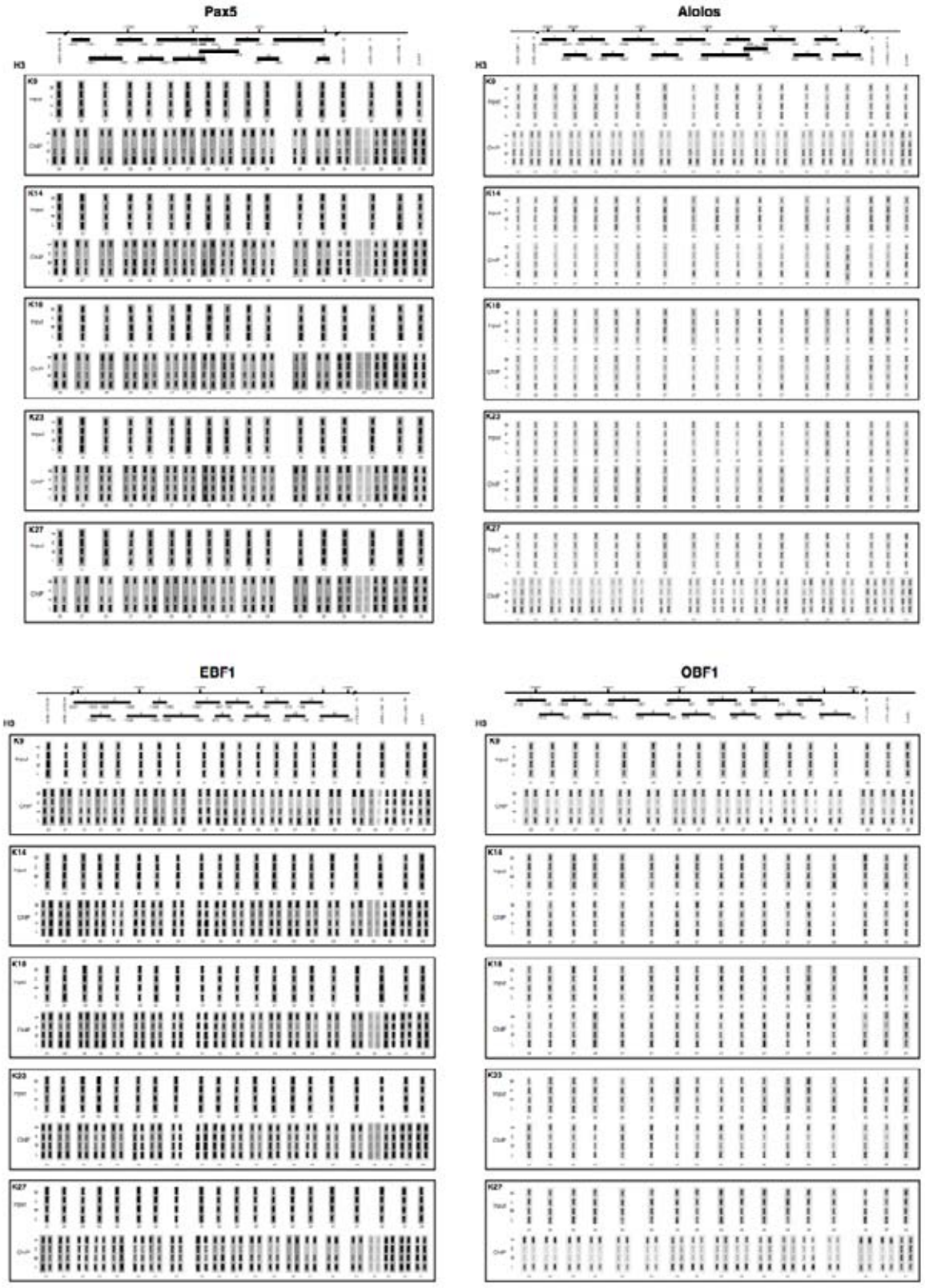

Figure 17

thereafter changes to the loose (or considerably loose) form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is certainly decreased to a low (or no) level at the early stage in clone cl.2-4 and thereafter increased to a high level until the later stage (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) in particular exhibit a full (or less) binding ability based on their hypo- or somewhat hyper-acetylation levels at all cultivation stages (Figure 16-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4 and remains unchanged until the later stage. These facts agreed with the findings that the gene expression of EBF1 is dramatically suppressed to a very low (or no) level at the early stage in clone cl.2-4 and thereafter remains unchanged during cultivation (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, K9/H3 (and possibly K27/H3) mainly possesses a less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. The less binding ability remains unchanged at the middle stage but thereafter is obviously decreased to no binding ability based on the hyper- (or considerably hyper-) acetylation levels at the later stage (Figure 16-OBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the somewhat loose form at the early and middle stages in clone cl.2-4 and thereafter changes to the loose form at the later stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-4 and thereafter certainly elevated to a high level at the later stage (Figure 11).

In both DT40 cells and clone cl.2-4 at all cultivation stages, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-4 [92, 97, 100]. Accordingly, the chromatin structure surrounding the proximal (and also distal) 5'-upstream region of the gene exists as the loose form in both DT40 cells and clone cl.2-4. However, as in DT40 cells, the gene expression of PCAF is really at a very low level at the early stage in clone cl.2-4 but gradually and dramatically increased from the early through middle to later stages (Figure 11). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in the PCAF gene expression, and the examined distal and proximal 5'-upstream regions are assumed to poorly correlate with the PCAF gene expression, regardless of the loose form in both DT40 cells and clone cl.2-4.

Legend to Figure 17. Alterations in acetylation levels of specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-6 of HDAC2(-/-) mutants during continuous cultivation.

NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and in DT40 (W) as described in the legend for Figure 14. The figure is identical with Figure 16 of Refs. 102, 103 plus 126, and is a set of Figures 5-4, 5-8, 5-12 plus 5-16 of Ref. 92, and is identical with Suppl. Figure 6-S9 of Ref. 92, and a set of Figures 4, 8, 12 plus 16 of Ref. 97, and is identical with Figure 7 of Ref. 101. These figures were first presented in Ref. 92.

These results and those obtained for clone cl.2-2, together with the previous inference speculated from changing patterns in gene expressions of Pax5, Aiolos, EBF1 and OBF1 in initially and secondly generated HDAC2(-/-) mutants [92, 95-97, 99], indicated that at the later cultivation stage clones cl.2-4 and cl.2-2 (and clones cl.2-3 and cl.2-5) seem to be dependent on Pax5 plus Aiolos, somewhat similar in appearance to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains (Figure 1-L), and members of major clone type among at least three different HDAC2(-/-) mutant clone types.

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes based on varied alterations in acetylation and/or deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3 for gaining new cell function to exclude IgM H- and L-chains accumulated in clone cl.2-6 of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-6, they exhibit a full binding ability to DNA based on their hypo-acetylation levels at the early stage. Thereafter, the binding capacity of these Lys residues to DNA is decreased during cultivation and reaches the state of no binding ability based on their hypo-acetylation levels at the later stage (Figure 17-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6. Thereafter, the tightened chromatin structure changes to the loose form through the middle to later stages. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-6 and thereafter gradually and dramatically elevated to a high level until the later stage (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-6, the binding capacity K9/H3 and K27/H3 of histone H3 to DNA, in particular, changes to a full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage (Figure 17-Aiolos). Thereafter, the full or less binding ability of these five Lys residues is gradually decreased to no binding ability based on their hyper-acetylation levels through the middle to later stages. Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight (or somewhat loose) form at the early stage in clone cl.2-6 and thereafter changes to the loose form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is drastically suppressed to a low (or no) level at the early stage in clone cl.2-6 and thereafter dramatically increased to a high level at the later stage (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-6, K9/H3 and K27/H3 (and probably K14/H3 and K18/H3), in particular, exhibit a full or less binding ability to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. Thereafter, these Lys residues reach the state of almost no binding ability based on their hyper-acetylation levels through the middle to later stages (Figure 17-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6 and thereafter changes to the loose form through the middle to later stages. These facts agreed with the findings that the gene expression of EBF1 is drastically suppressed to a very low (or no) level at the early stage in clone cl.2-6 and thereafter gradually elevated until the later stage to almost the same levels as in DT40 cells (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-6, K9/H3 and K27/H3 (and probably K23/H3) in particular exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. Thereafter, the full or less binding ability is slightly reduced at the middle stage and further decreased to no binding ability based on their hyper-acetylation levels at the later stage (Figure 17-OBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which exists as the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6 and thereafter changes to the loose form through the middle to later stages. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early stage in clone cl.2-6 and thereafter certainly elevated to a high level through the middle to later stages (Figure 11).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels. Interestingly, in clone cl.2-6, K9/H3 and K18/H3 in particular possess a less binding ability to DNA based on their somewhat hyper-acetylation levels at the early stage, but thereafter the binding capacity of these Lys residues to DNA is decreased to no binding ability based on their hyper-acetylation levels at the middle stage and again increased to a less binding ability based on their somewhat hyper-acetylation levels at the later stage [92, 97, 101]. Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene exists as the loose form in both DT40 cells and clone cl.2-6 at the middle stage but as somewhat loose form at the early and later stages in clone cl.2-6. However, in clone cl.2-6, the gene expression of the PCAF gene is also at a very low level at the early stage, and thereafter gradually and obviously increases until near middle stages but again dramatically decreases to a very low level through the middle to later stages (Figure 11). Based on these results, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in the PCAF gene expression, and the examined distal and proximal 5'-upstream regions are not directly correlated with the PCAF gene expression by much, regardless of the loose form of the chromatin structure in both DT40 cells and clone cl.2-6.

These results, together with the previous inference speculated from changing patterns in gene expressions of Pax5, Aiolos, EBF1 and OBF1 [92, 96, 99], indicated that at the later cultivation stage clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 (Figure 1-L), and mostly similar in appearance to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains.

Summary of alterations in acetylation levels of specific Lys residues of histone H3, the chromatin structure of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their gene expression levels in individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) DT40 mutants at the early, middle and later stages of continuous cultivation

In summary, alterations in acetylation levels (hyper or hypo) of specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), the chromatin structure (loose or tight form) of the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (i.e., gene expression/transcription) levels (high or low) in DT40 cells (W) and clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) DT40 mutants at the early (E), middle (M) and later (L) stages of cultivation are schematically shown in Figure 18.

A part of IgM H- and L-chains excessively accumulated in HDAC2(-/-) DT40 mutants is transported to nuclear envelope but not inside of nucleus, and kept at peri-nuclear space at the early and later stages of continuous cultivation

Large amounts of IgM H- and L-chains caused by their excessively increased gene expressions

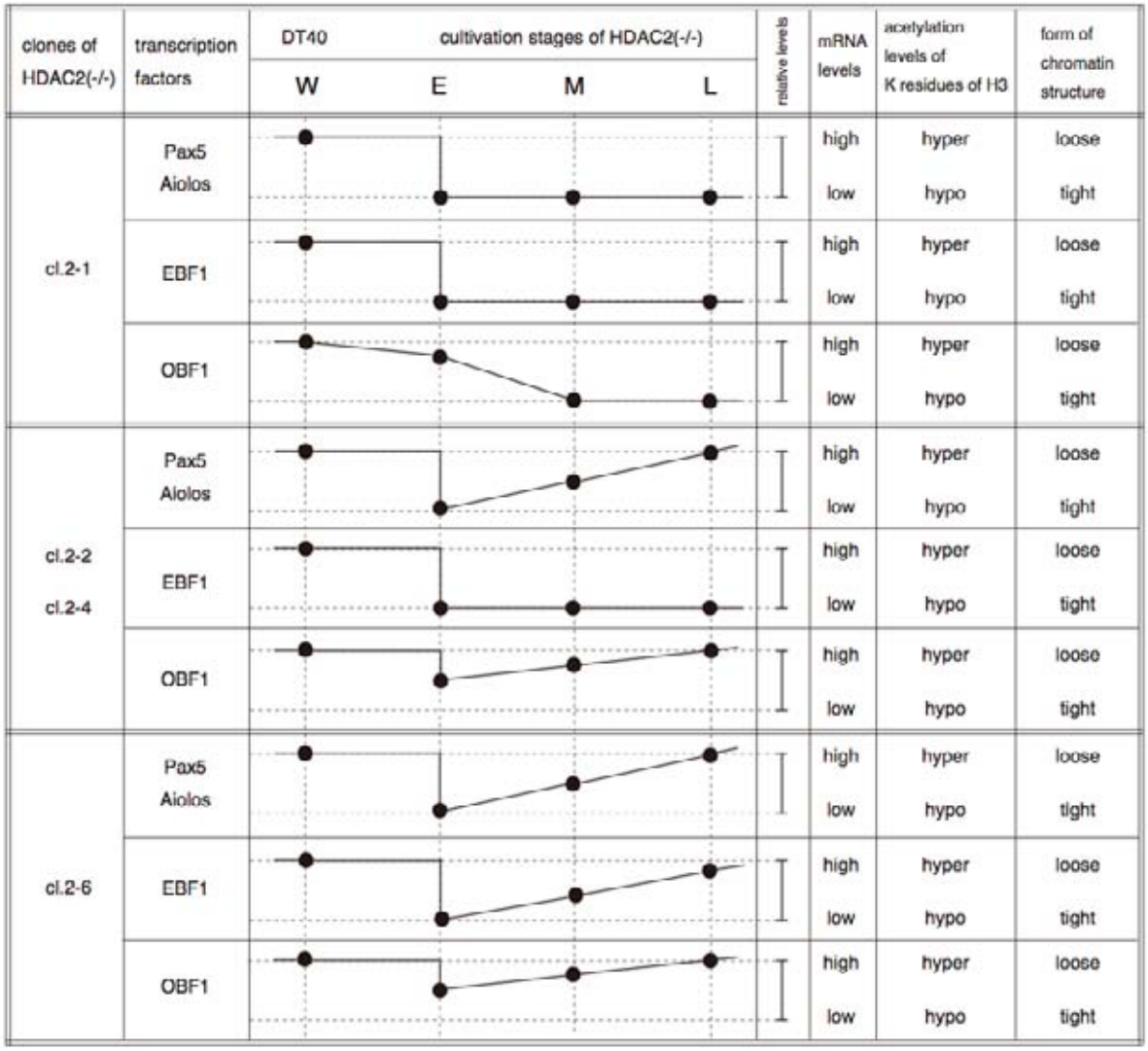

Figure 18. Summary of alterations in acetylation levels of specific Lys residues (K) of histone H3, chromatin structure of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their gene expression levels in DT40 cells (W) and in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) DT40 mutants at early (E), middle (M) and later (L) stages of continuous cultivation.

Alterations in acetylation levels (hyper or hypo) of specific Lys residues (K) of histone H3, the chromatin structure (the loose or tight form) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (gene expression/transcription) levels (high or low) in DT40 cells (W) and in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) stages of cultivation are schematically shown. The figure is identical with Figure 17 of Refs. 102, 103 plus 126, Figure 5-21 plus Suppl. Figure 6-S10 of Ref. 92, Figure 21 of Ref. 97, Suppl. Figure S9 of Refs. 98 plus 125, and Figure 5 of Ref. 100. These figures were first presented in Ref. 92.

artificially induced by the HDAC2-deficiency (Figure 10) are first accumulated within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figures 19 and 20) [92, 95, 96, 98, 99, 101-103]. Most of the accumulated IgM H- and L-chains (which probably exist as a high molecular weight complex with each other) [64] are gradually secreted to the outside of cells (i.e., into cultivation media), whereas, a part of them is transported to the nuclear envelope but not inside of the nucleus,

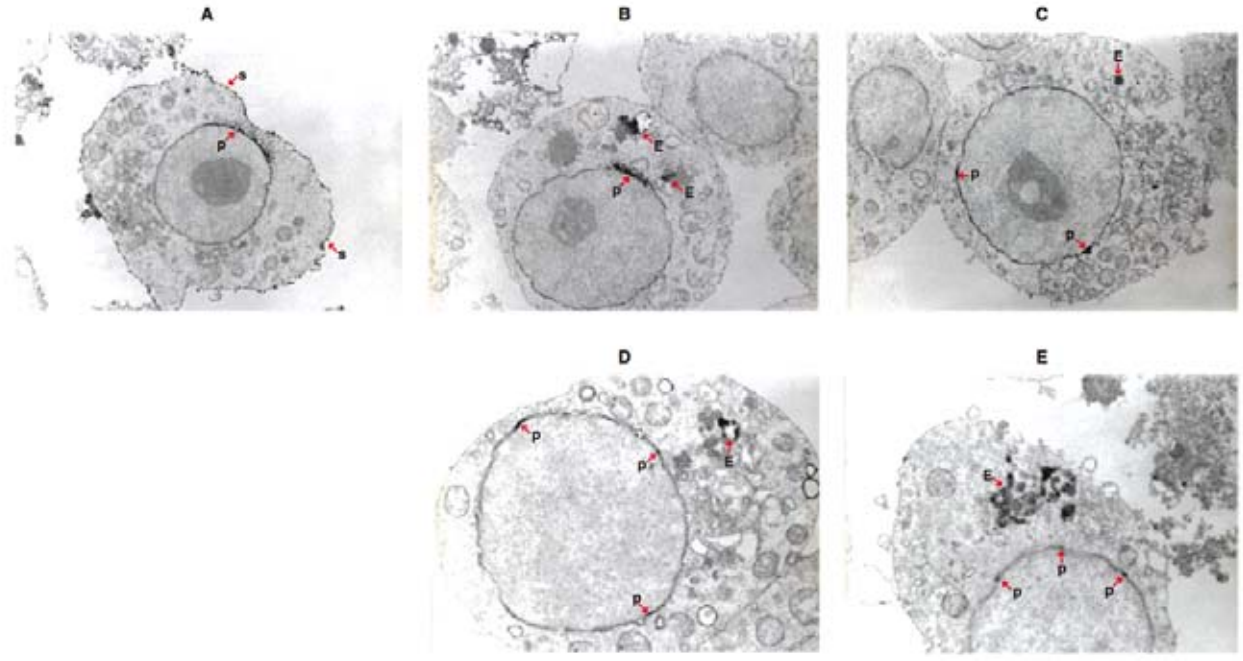

Figure 19. Localization of IgM H-chain proteins at peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells.

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out on HDAC2(-/-) mutant cells at the early (E) stage of cultivation. A) Immuno-electron microscopy without the saponin-treatment. B) ~ E) Immuno-electron microscopy with the saponin-treatment. Arrows P, E and S indicate positive signals of IgM H-chains localized at the peri-nuclear space, endoplasmic reticulum and cell surface, respectively. A large amount of accumulated IgM H-chain proteins was observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early (E) cultivation stage. The figure is identical with Figure 18 of Refs. 102, 103 plus 126, Figure 6-1 of Ref. 92, Figure 1 of Refs. 98 plus 125, and Figure 8 of Ref. 101. These figures were first presented in Ref. 92.

and kept at the peri-nuclear space at the early and later cultivation stages (Figures 19, 20 and 21) [92, 98, 101-103].

Proposed ways for gaining un-programmed and new cell function to diminish artificially accumulated IgM H- and L-chains by means of irreversible creation of varied chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in individual clones of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation

How do the HDAC2(-/-) mutants exclude excessively accumulated IgM H- and L-chains as unnecessary materials, in order to cope with and/or overcome an abnormal, unexpected, unfavorable, uncomfortable and useless environment change? Generally, this is possible by adhering to three following typical ways. 1) They acquire a high suppression ability of gene expressions of IgM H- and L-chains, and thereby decrease large amounts of the two immunoglobulin proteins. 2) They acquire a high decomposition (degradation) ability of proteins (and/or mRNAs) of IgM H- and L-chains, and thereby decrease large amounts of the two immunoglobulin proteins. 3) They acquire a high secretory ability of IgM H- and L-chains, and thereby decrease large amounts of the two immunoglobulin proteins within cells. In fact, in order to diminish excessively accumulated IgM H- and L-chains, the HDAC2(-/-) mutants have selected the first way among the above-mentioned three ways as follows, because this way should be most effective from the physiological and energy-saving point of view.

Based on our morphological findings that a part of artificially accumulated IgM H- and L-chains

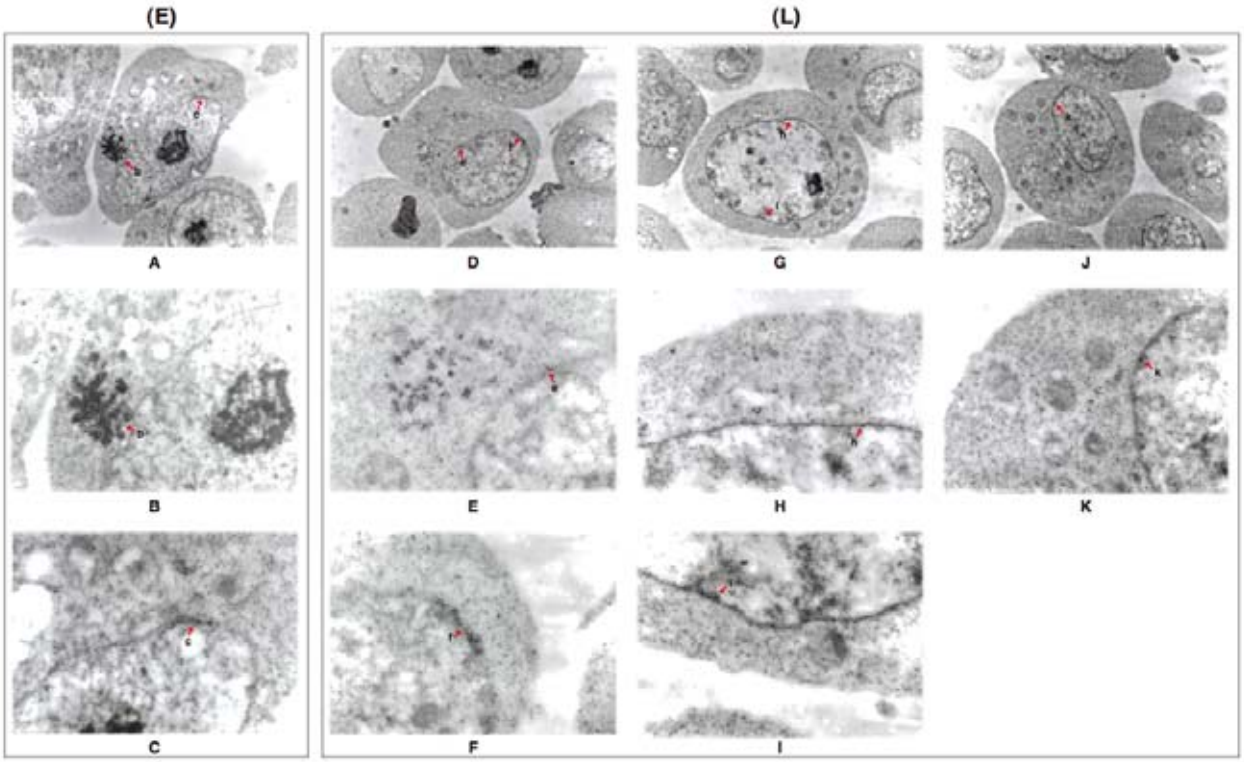

Figure 20. Localization of IgM H-chain proteins in peri-nuclear space of HDAC2(-/-) DT40 mutants at early and later stages of continuous cultivation.

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out on HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early (E) stage. B) and C) Enlarged versions of parts indicated by arrows b and c in A). D), G) and J) Immuno-electron microscopy of three individual HDAC2(-/-) mutant cells at the later (L) stage. E), F), H), I) and K) Enlarged versions of parts indicated by arrows e, f, h, i and k in D), G) and J). The mutant cell in G) was identical to the cell shown in Ref. 95. A large amount of accumulated IgM H-chain proteins was observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early (E) and later (L) stages. The figure is identical with Figure 19 of Refs. 122, 123 plus 126, Figure 6-3 of Ref. 92, and Figure 3 of Refs. 98 plus 125. These figures were first presented in Ref. 92.

caused by the HDAC2-deficiency is kept at the peri-nuclear space at the early and later stages (Figures 19, 20 and 21), together with the above-mentioned results (Figures 1 to 18) and others [57, 60, 64, 67, 92-97, 99, 100], we proposed an all-inclusive hypothetic concept on ways for gaining un-programmed and new cell function to diminish excessively accumulated IgM H- and L-chains by means of irreversible creation of varied chromatin structure plasticity of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in individual clones of HDAC2(-/-) mutants through numerous generations during continuous cultivation [92, 98, 101-103].

First of all, we supposed that environment change recognition receptor/site (ECRR/ECRS) recognizes the accumulation of IgM H- and L-chains as an abnormal, unexpected and/or unfavorable environment change (and probably acts in part in the signal transduction concerning the accumulation to the chromatin structure) (Figure 21). In addition, chromatin conformation (structure) change complex (4C) machinery, which is diverse and consists of a member of each of HATs plus HDACs and other factors, is supposed to directly and irreversibly create plasticity of the chromatin structure surrounding proximal 5'-upstream regions of the above- mentioned

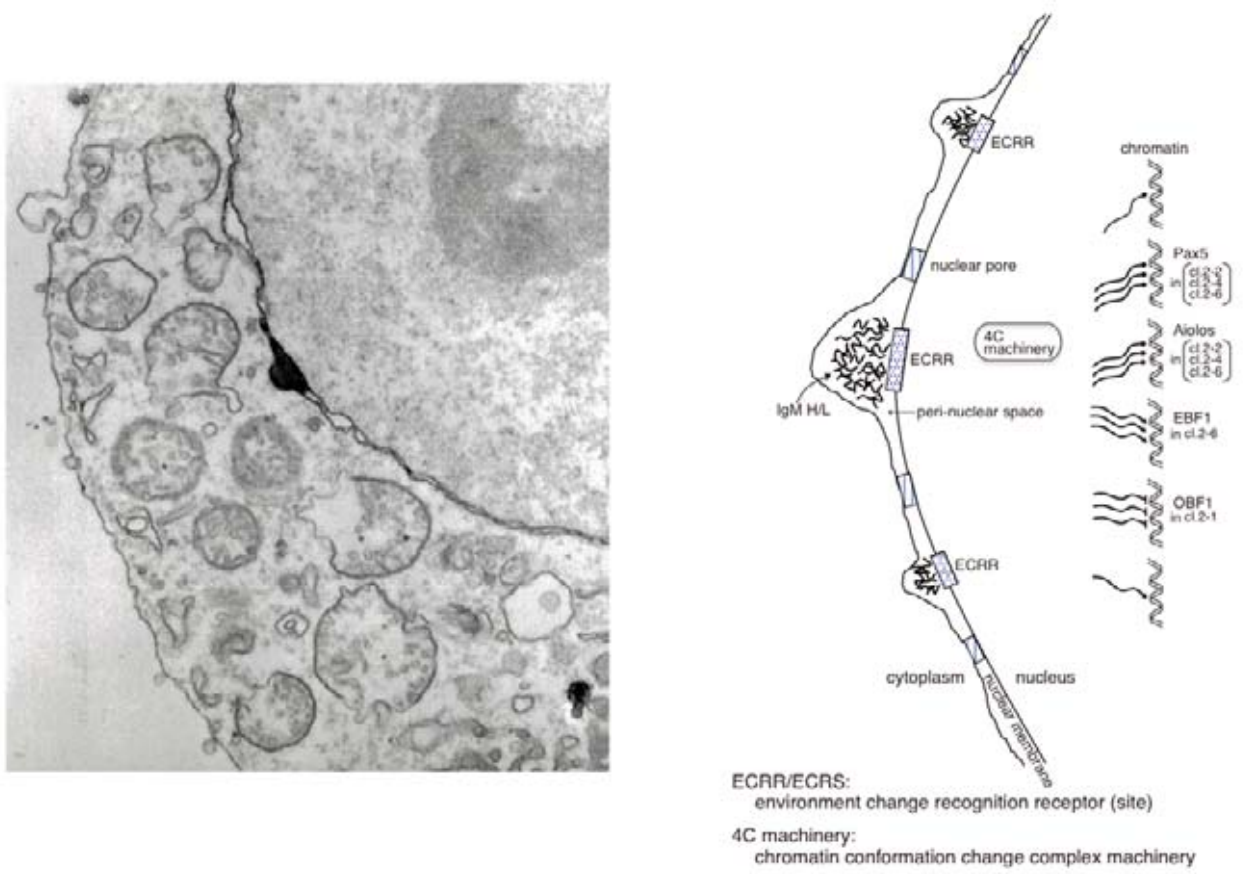

Figure 21. Localization of IgM H-chain proteins at peri-nuclear space, and a model for signal transduction concerning accumulated IgM H- and L-chains to chromatin in HDAC2(-/-) DT40 mutants during continuous cultivation.

Left panel: A portion of the peri-nuclear space (where IgM H- and L-chains were accumulated) of the HDAC2(-/-) mutant cell (indicated by an arrow P at lower position in Fig. 19C) was reversely enlarged. **Right panel:** A model for the signal transduction concerning accumulation of IgM H- and L-chains. Signal concerning IgM H- and L-chains artificially accumulated in the peri-nuclear space of HDAC2(-/-) mutants was repeatedly transmitted to the chromatin structure, followed by unbalanced response to the signal and its convergence to various specific transcription factor genes (such as Pax5, Aiolos, EBF1, OBF1, etc.) in individual mutant clones. ECRR/ECRS: environment change recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery. The figure is identical with Fig. 20 of Refs. 102, 103 plus 126, Fig. 6-4 of Ref. 92, Fig. 4 of Refs. 98 plus 125, and Fig. 9 of Ref. 101. These figures were first presented in Ref. 92.

specific transcription factor genes (and also to act in part in the signal transduction) (Figures 21 and 22). Using the ECRR/ECRS, the 4C machinery and other components, the chain reaction of response to the abnormal and/or unfavorable environment change such as the accumulation of IgM H- and L-chains occurs as follows.

In wild-type DT40 cells where various members of HATs and HDACs are largely expressed [64, 92], the 4C machinery for Pax5, Aiolos, EBF1 and OBF1 genes probably contains HDAC2 and a specific member of HATs as its components (Figure 22) [92, 98, 101]. However, the functions of both the ECRR/ECRS and the 4C machinery may be basically not necessary for DT40 cells, because the protein and mRNA levels of IgM H- and L-chains are very low as mentioned above.

On the other hand, the case of HDAC2(-/-) mutants is as follows. Repeatedly, large amounts of IgM H- and L-chains artificially created by the

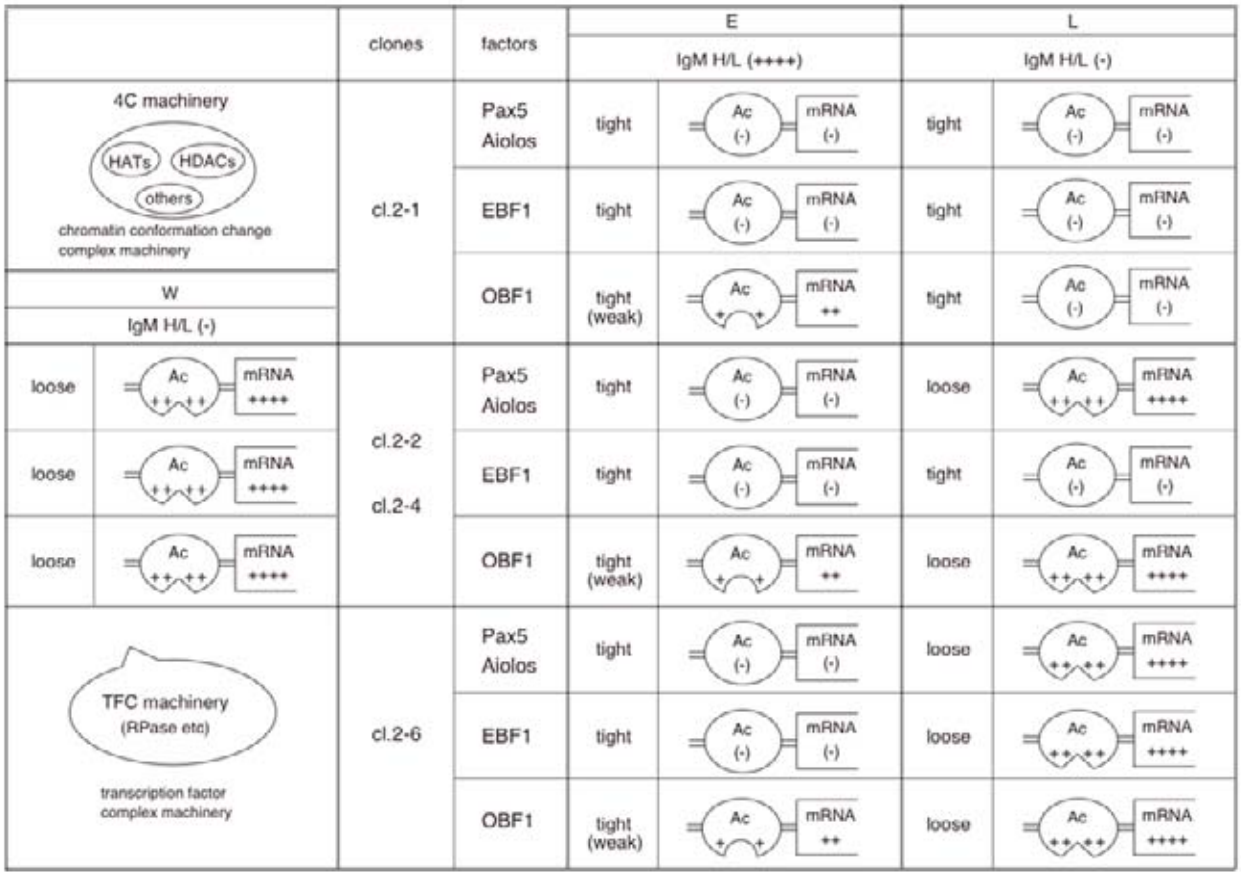

Figure 22. Summary of alterations in gene expression levels, acetylation levels of specific Lys residues (K) of histone H3, and chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.

Alterations in mRNA (gene expression) levels (-, ++ or ++++), acetylation levels (Ac; -, ++ or ++++) of specific Lys residues (K) of histone H3 and the chromatin structure (the loose or tight form) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E) and later (L) stages of cultivation and in DT40 cells (W) are schematically presented. Alterations in mRNA levels (- or ++++) of IgM H- and L-chains are also presented. Chromatin conformation change complex (4C) machinery generally contains a specific member of each of HATs plus HDACs and other factors. Transcription factor complex (TFC) machinery generally contains RNA polymerase (RPase), specific transcription factors and others. The figure is identical with Figure 21 of Refs. 102, 103 plus 126, Figure 6-5 of Ref. 92, Figure 5 of Refs. 98 plus 125, and Figure 10 of Ref. 101. These figures were first presented in Ref. 92.

HDAC2-deficiency (Figure 10) are first accumulated within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figures 19 and 20). Most of the accumulated IgM H- and L-chains (which probably exist as a high molecular weight complex with each other [64, 67]) are gradually secreted into the cultivation media (i.e., to the outside of cells), whereas, a part of them is transported to the nuclear envelope but not the inside of the nucleus, and kept at the peri-nuclear space at the early and later cultivation stages (Figures 19, 20 and 21). The accumulated immunoglobulin proteins laying at the peri-nuclear space bind to the ECRR/ECRS localized at the inner nuclear membrane (where hetero-chromatin is possibly located) (Figure 21). After the ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as an abnormal and/or unfavorable environment change, the signal concerning the environment change is genome-widely transmitted to the chromatin structure

surrounding proximal 5'-upstream regions of numerous genes (probably exist on several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes. Following the initial signal transduction, a spontaneous unbalanced response to the abnormal and/or unfavorable environment change is consecutively and separately converged on the proximal 5'-upstream regions of the above-mentioned Pax5, Aiolos, EBF1, OBF1 and other genes in individual clones of HDAC2(-/-) mutants (Figure 23).

Next, what should be emphasized is as follows. In all of individual clones of HDAC2(-/-) mutants at the very early stage of cultivation (just soon after their birth by gene targeting techniques), bulk conformation of the 4C machinery dramatically changes to remove or drastically reduce HAT activity (of the assumed member of HATs), attributed to the HDAC2-deficiency. Throughout the above-mentioned process and following continuous cultivation, the 4C machinery newly consists of a different member of HDACs, a specific (same or different) member of HATs and

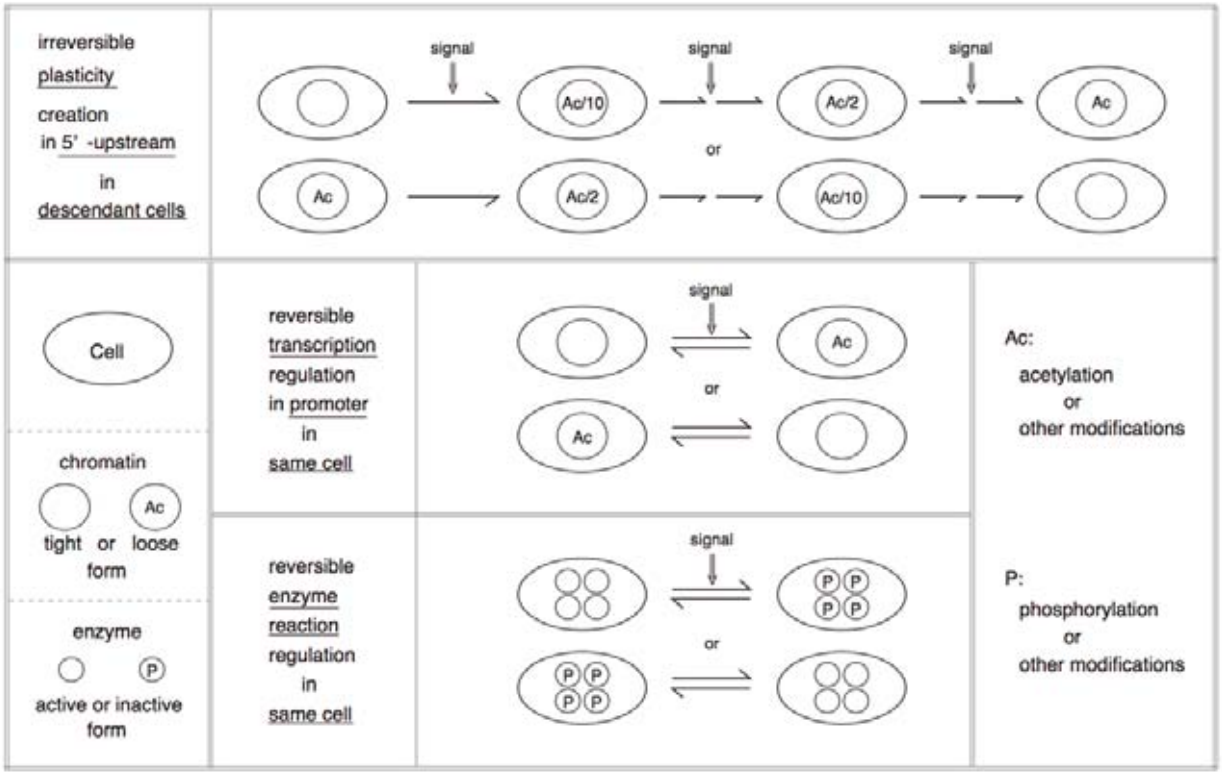

Figure 23. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region with epigenetic modifications in descendent cells, and reversible regulations of ordinary gene expression and enzyme reaction in cells that initially accept proper signal.

Upper panel: Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region(s) of specific transcription factor and chromatin-modifying enzyme gene(s) with epigenetic modifications occurs in descendant cells but not in initial cell that accepts the environment change signal. Ac, Ac/2 and Ac/10 qualitatively indicate hyper-, considerably hyper- and somewhat hyper-acetylation levels of specific Lys residues (K) of core histones (e.g., histone H3), respectively. **Middle and Lower panels:** Regulations of ordinary gene expression (on transcriptional regulatory elements) and enzyme reaction reversibly occur in the cell that accepts proper signal. Ac and P indicate acetylation, phosphorylation and/or other chemical modifications, respectively. The figure is identical with Figure 22 of Refs. 102, 103 plus 126, Figures 6-7 of Ref. 92, Figure 7 of Refs. 98 plus 125, and Figure 12 of Ref. 101. These figures were first presented in Ref. 92.

other factors, and thereby becomes varied. The diversity of alterations in the chromatin structure is preferentially attributed to varied acetylation and deacetylation levels of one or more of the five specific Lys residues at N-terminal tail of histone H3 [92, 97, 100] caused by the collaboration of proper members of HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications of K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3) with acetyl group lead to irreversible creation of distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes (Figures 14, 15, 16, 17, 18 and 23). In general, the chromatin structure of proximal 5'-upstream regions possessing hyper-acetylation levels of one or more of the specific Lys residues of histone H3 is in the loose (open) form based on its no-binding ability to DNA, but that of proximal 5'-upstream regions possessing hypo-acetylation levels of one or more of the specific Lys residues of histone H3 is in the tight (closed) form based on its binding ability to DNA (Figures 18, 22 and 24). By contrast, probably, the 4C machinery cannot change the chromatin structure surrounding ORF regions of these specific transcription factor genes by much [92, 97, 98, 100-102]. As the need arises, transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain members of HATs and HDACs and other factors) is able to bind to promoters (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes (which have become latently active state), and thereby initiates their gene expressions (Figures 22 and 24).

As a concrete result, individual clones of HDAC2(-/-) mutants gain the same, un-programmed and new cell function to reduce increased gene expressions of IgM H- and L-chains (resulting in their decreased protein levels) in almost the same changing pattern through increased or decreased gene expressions of Pax5, Aiolos plus EBF1 or OBF1 in distinct ways through numerous generations during simple continuous cultivation

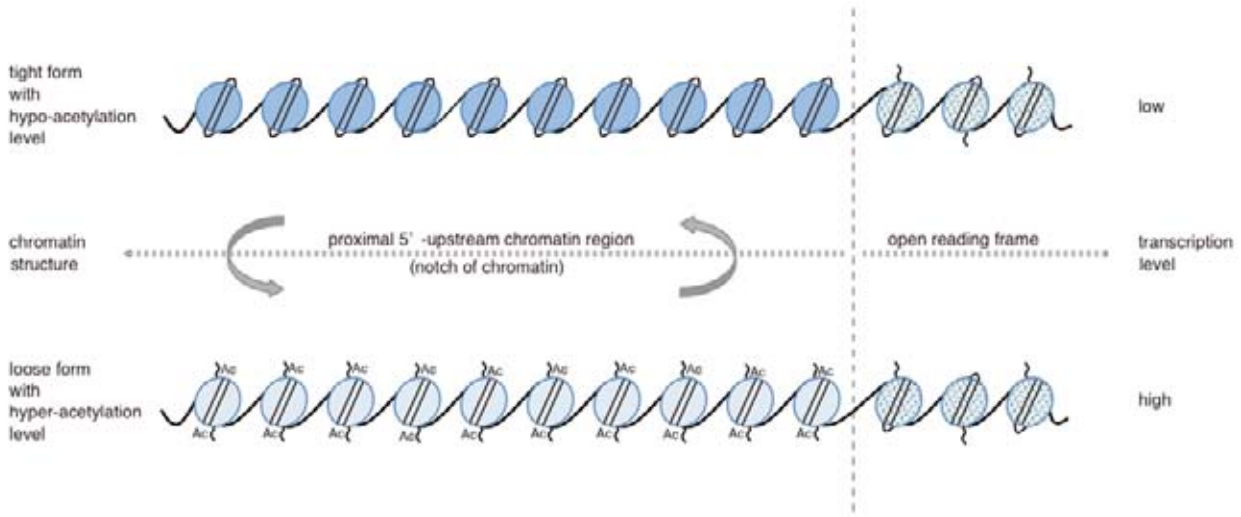

Figure 24. Chromatin conformation change code (4C) theory: A model for gaining un-programmed and new cell function(s) by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations.

Irreversible creation of chromatin structure plasticity with epigenetic modifications occurs surrounding proximal 5'-upstream region (notch of chromatin) but not ORF region of specific transcription factor and chromatin-modifying enzyme gene(s) through numerous generations (cell divisions). The tight or loose form of the chromatin structure is based on hypo- or hyper-acetylation levels of specific Lys residues (K) of histone H3, and causes low or high transcription (gene expression) levels. The figure is identical with Figure 23 of Refs. 102, 103 plus 126, Figure 6-6 of Ref. 92, Figure 6 of Refs. 98 plus 125, and Figure 11 of Ref. 101. These figures were first presented in Ref. 92.

under the same conditions (Figure 11) [64, 92, 95, 96, 99]. Naturally, such distinct ways are not under the control of HDAC2 but originally based on irreversible creation of their varied chromatin structure plasticity with epigenetic modifications during continuous cultivation [92, 97, 98, 100-103]. Remarkably, at the later cultivation stage the six individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants can be clearly classified into the above-mentioned OBF1-dependent, Pax5- and Aiolos-dependent, and Pax5-, Aiolos- and EBF1-dependent cell types having quite distinct functions. Because these three cell types show the different gene expressions of Pax5, Aiolos, EBF1, OBF1, Blimp-1, PCAF and HDAC9, all of which are probably involved in many other gene expressions in complicated and separate manners (Figures 1 and 11). Besides, in one or more of these six HDAC2(-/-) mutant clones, as a whole, gene expression patterns of PCAF, HDAC7, HDAC9, Ikaros and OBF1 are spontaneously and complicatedly reverse in the midst of simple continuous cultivation, although those of Pax5, Aiolos, EBF1, E2A, PU.1 and Blimp1 do not change while passing from the early to later cultivation stages. Furthermore, if other individual mutant clones obtained [96] are analyzed, there is a great possibility that additional distinct cell types might exist. These results indicate that there are many different ways (molecular mechanisms) for HDAC2(-/-) mutants to newly gain a same un-programmed cell function as if there exist many distinct mountain trails to climb the top of a mountain. Consequently, individual clones of HDAC2(-/-) mutants acquire flexible, elastic and pluri-potential ability not only to adapt in distinct ways to an abnormal, unexpected and/or unfavorable environment change but also to branch off into diverse derivative cell types, which may exhibit varied characteristics (functions), even though they are originally the same cell type and also the established cell line.

The above-mentioned ways that HDAC2(-/-) DT40 mutants gain un-programmed and new cell function to diminish excessively accumulated IgM H- and L-chains by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations during continuous cultivation are obviously different in molecular mechanisms from the so-called endoplasmic reticulum (ER) stress response/ unfolded protein response. Naturally, the ways to eliminate unnecessary products (large amounts of IgM H- and L-chains) are clearly different from the many well-known life phenomena, e.g., the alterations in the colors of snowshoe hare's hairs and ptarmigan's feathers between summer and winter seasons, and the mimicries of Philomachus pugnax and Papilio polytes probably based on supergenes, because these are programmed functions in response to environment change and their related genome information is unchanged.

Chromatin conformation (structure) change code (4C) theory: A universal way of higher eukaryotes for gaining un-programmed and new cell functions by means of irreversible creation of varied chromatin structure plasticity of proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through numerous generations

We expanded the above-mentioned hypothetic ways concerning the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetic way of higher eukaryotes to gain un-programmed and new cell functions by means of irreversible creation of varied chromatin structure plasticity of specific transcription factor, chromatin-modifying enzyme and related genes with epigenetic modifications through numerous generations (cell divisions). And then we advanced a chromatin conformation change (structure) code (4C) theory for this remarkable and important bio-system, the details of which are as follows.

When higher eukaryotic cells firstly encounter an intra- and/or extra-cellular environment change in their lives, in order to adapt to or eliminate the change (if unnecessary, abnormal and/or uncomfortable), they gradually gain un-programmed and new cell functions through numerous generations. That is, somatic cells of higher eukaryotes acquire the ability to adapt themselves to a newly encountered environment change and/or to exclude the abnormal and/or painful environment change. Using the ECRR/ECRS, the 4C machinery and other components, the somatic

cells create a chain reaction of response to the new environment change as follows (Figures 21 and 22). First of all, the new environment change is recognized by means of the ECRR/ECRS, which may be localized nearby the nuclear membrane as a nucleus-cytoplasm barrier (probably at the inner nuclear membrane where hetero-chromatin is possibly located) (Figure 21). There is a possibility that putative specific molecule(s) acts as intermediary sensor at this step in the way to recognize the new environment change. Naturally, there is certainly another possibility that ECRR/ECRS should be newly created through gathering of its components based on the fluid mosaic model of membrane. Next, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin structure within the nucleus through numerous generations. Following the initial acceptance of the putative signal(s), the 4C machinery induces a slight alteration in the chromatin structure of numerous genes encoding chromatin-modifying enzymes, transcription factors, and related enzymes and factors with epigenetic modifications. The transduction of the signal(s) and spontaneous unbalanced response to the new environment change are successively repeated and converged into the restricted chromatin structure surrounding proximal 5'-upstream regions of various specific members of the above-mentioned factor and enzyme genes. Finally, this successive signal transduction concerning the new environment change causes various epigenetic modifications of histones and/or DNA within the restricted chromatin regions of the aforesaid specific genes with acetyl, methyl, phosphate, ubiquitin and ADP-ribose groups and/or others. Of these various epigenetic modifications, acetylation and deacetylation of several specific Lys residues of core histones H2A, H2B, H3 and H4 may be the major ones.

The 4C machinery, which consists of a specific member of each of HATs and HDACs and other factors, preferentially participates in the acetylation and/or deacetylation events among such epigenetic modifications (Figure 22). Positions of specific Lys residues and/or kinds of core histones are diverse. For instance, in the above-mentioned case [92, 97, 98, 100-103], the acetylation and/or deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, epigenetic modifications of one or more of these specific Lys residues of histone H3 with acetyl group change within the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned specific target genes through numerous generations. Varied functions of the protean 4C machinery on such acetylation and/or deacetylation levels are mainly based on distinct combinations of each member of HATs and HDACs as the components, because any HAT and HDAC members' own activities are probably unchangeable. By contrast, the protean 4C machinery cannot change the chromatin structure surrounding ORF regions of corresponding genes by much. The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from acetylation and/or deacetylation levels of one or more of these specific Lys residues, though which Lys residue(s) really and/or mainly participates in the binding is still undefined. That is, hyper- (high) or hypo- (low or no) acetylation level induces no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure (Figure 22). Thus, the chromatin structure plasticity is irreversibly created based on successive conformation changes with epigenetic modifications. These ways to gradually and tardily create the chromatin structure plasticity for gaining un-programmed and new cell functions are obviously different from the ways that immediately and rapidly cause the chromatin conformation change for expressing programmed and ordinary cell functions.

Whenever the need arises, the TFC machinery is able to bind to promoters (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned target genes (which are in the latently active state), followed by initiation of their gene expressions (Figure 22). By contrast, the TFC machinery cannot bind to promoters (or elements) within the tight form of the chromatin structure surrounding proximal 5'-upstream regions of un-target genes (which are in the latently inactive state), and thereby cannot initiate their gene expressions. Consequently, the loose or tight form

of the chromatin structure surrounding proximal 5'-upstream regions causes high or low (or no) transcription levels of corresponding genes (Figure 24). Notably, there is a great possibility that the ways for irreversible creation of chromatin structure plasticity are varied among individual cells of the same type, even though the new environment change and signal(s) on the environment change are the same for all of them. That is, in order to gain un-programmed and new cell function(s), individual cells possess ability not only to complicatedly and diversely change the chromatin structure surrounding proximal 5'-upstream regions of numerous specific genes but also to separately change the chromatin structure of the same proximal 5'-upstream region of a certain gene into varied forms. Thus, gene expressions of the specific chromatin-modifying enzymes and transcription factors diversely change among individual somatic cells through various generations, in spite of the same environment change. In consequence, individual cells of the same type are able to newly gain the same and/or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment.

CONCLUSION AND DISCUSSION

In order to gain un-programmed and new cell function(s), somatic cells (and even tumor cells) of higher eukaryotes become pluri-potent, elastic and flexible, all of which are basically originated from pluri-potency, elasticity and flexibility of the chromatin structure. That is, in order to adapt to intra- and/or extra-cellular environment changes, individual somatic cells possess the ability to newly gain the same and/or distinct un-programmed cell function(s) in different ways by means of irreversible creation of varied chromatin structure plasticity with epigenetic modifications, i.e., from the loose to tight forms or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (Figure 24). Such a loose or tight form of the chromatin structure surrounding the proximal 5'-upstream region is in the latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences is in the silent state for expressions of most of the genome functions. The creation of varied chromatin structure plasticity in individual somatic cells is triggered by the spontaneous unbalanced response to the new environment change when they firstly encounter with it and then irreversibly accomplished by the successive unbalanced convergence of the response through numerous generations. The different ways to create varied chromatin structure plasticity in somatic cells are probably dependent on their antecedents. Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, is inherited to descendant generations associated with or without additional conformation change through numerous generations. Remarkably, irreversible creation of chromatin structure plasticity occurs in descendant cells but not in the cell which initially accepts the signal on the environment change, although reversible regulations of ordinary gene expressions and enzyme reactions occur in the cell itself which accepts proper signal (Figure 23). Probably, irreversible creation of chromatin structure plasticity, with the intention of adapting to the environment change, occurs inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as the loose or tight form) directs the switch (on or off) for latent transcription ability of the corresponding gene by means of irreversible creation of chromatin structure plasticity; therefore, the 5'-upstream chromatin region can be regarded as a "director for gene expression" from a functional point of view, besides as a "notch of chromatin" from a structural point of view as mentioned above. The notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator, enhancer, etc.) and also their neighboring nucleotide sequences. Remarkably, the real recipient of the signal on the environment change may be just the steric (as if loose rope-like) chromatin structure possessing self-conversion ability of conformation (we named here), which dynamically changes between the loose and tight forms, but not the static (as if rod-like) chromosome, which is in the unchanging state. That is, the chromatin structure of proximal 5'-upstream region (notch or director),

as a dynamic and changeable three-dimensional conformation, possesses two fundamental abilities, i.e., to receive the signal concerning the intra- and/or extra-cellular environment change and to direct the switch (on or off) for latent transcription ability of the corresponding gene by means of its irreversible chromatin conformation change that is responsive to the signal. The self-conversion ability of conformation, which is based on the 4C theory to irreversibly create plasticity of three-dimensional chromatin conformation (structure) in higher eukaryotes, is obviously different from the well-known rearrangement of antibody genes in immune systems and others, because these latter ones are somatic recombination (rearrangement) of DNA structures (base/nucleotide sequences) themselves. Naturally, concrete data are not enough to support these ideas. As a consequence of these complicated biological events, higher eukaryotes acquire pluri-potential, elastic and flexible ability to create diverse derivative cell types possessing varied characteristics (functions) (e.g., varied constituent cells of diversely differentiated organs) from the same somatic cell type in distinct ways, in order to accommodate themselves to new intra- and/or extra-cellular environment. In some cases, even neighboring cells derived from the same cell type are probably regarded as extra-cellular environment for themselves with each other. Naturally, in higher eukaryotes diverse kinds of somatic cells share and express cooperatively vital functions with each other. For concrete typical examples, 1) there are innumerable cell types that make up the brain and nerves. In addition, there are other examples for the maintenance of homeostasis that is also one of the vital functions as follows. 2) hypothalamus synthesizes and secretes corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), luteinizing hormone-releasing hormone (LHRH), and thyrotropin-releasing hormone (TRH), 3) anterior pituitary synthesizes and secretes growth hormone (GH), luteinizing hormone (LH) and adrenocorticotropic hormone (ACTH), and 4) posterior pituitary synthesizes and secretes antidiuretic hormone (ADH), vasopressin and oxytocin (OT). Moreover, 5) A (α), B (β), D (δ) and PP cells of Langerhans islet of pancreas synthesize and secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively.

As mentioned above, we named our theory on such a bio-system to gain un-programmed and new cell function(s) by means of irreversible creation of chromatin structure plasticity with epigenetic modifications, which is one of the most important and fundamental ways for life conservation and cell type determination of higher eukaryotes, as the chromatin conformation (structure) change code (4C) theory. By the way, here, we would like to briefly explain the simple concepts and molecular mechanisms of the two well-known, most important and fundamental code theories (i.e., the genetic code and the histone code), which are closely correlated to chromatin (or DNA) functions (especially gene expression) but different from the 4C theory, because we did not mention about these two until now in this article at all. First, the genetic code is the biochemical basis of heredity and is based on codons (triplet: three consecutive base/nucleotide sequences) in both DNA and mRNA, and the codons determine the specific amino acid sequences in proteins (and polypeptides) at the translation step on the ribosome. The translation/protein synthesis occurs through the base pair formation by the hydrogen bond between the codon in mRNA and the anticodon in aminoacyl-tRNA. Needless to say, the genetic code appears to be uniform for nearly all known organisms; therefore, in life phenomena it is one of the most fundamental and important concepts, as well as the double helix structure of DNA. Second, the histone code, which is an epigenetic code as well as DNA methylation and others, will be explained a little more, since it is a bit like the 4C theory apparently. The histone code is a hypothesis that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications of histones within their flexible N-terminal tails protruding from the nucleosome. In particular, as is well known, many histone modifications obviously participate in chromatin structure change and thereby alter expression levels of numerous genes. The critical concept of the histone code hypothesis is that the histone modifications serve to recruit other enzymes and/or factors by specific recognition of the modified histone (as a mark/modifier) *via* protein domains specialized for such purposes, rather than through simply stabilizing or destabilizing the

interaction between the histone molecule and the specified DNA region. These recruited enzymes and/or factors then act to alter chromatin structure or to promote transcription actively. In other words, the molecular mechanism of the histone code is based on the facts that histones are modified (marked) by several small molecules, and the mark summons (or is recognized and accessed by) various enzymes and/or factors, which participate in the expression of various chromatin functions (especially gene expression). Finally, we repeat the description on the 4C theory with a slight modification as follows. The molecular mechanism of the 4C theory is based on that the plasticity of proximal 5'-upstrean chromatin regions of various genes are irreversibly created by the 4C machinery through epigenetic modifications with acetyl group (and others), and the loose (open) or tight (closed) form of chromatin structure summons or rejects (or is recognized and accessed by, or is reversely un-recognized and un-accessed by) various transcription factors and/or chromatin-modifying enzymes, which participate in the expression of numerous chromatin functions (including gene expression). Therefore, needless to mention again, regarding the expression of genome (chromatin) functions including the gene expression, the 4C theory obviously functions upstream both the histone code and the genetic code. Probably, the supposed number of codes in the 4C theory, which determines complicated and varied characteristics (functions) of higher eukaryotic cells, can be roughly estimated based on the combination (multiplication) of the number of candidate genes and that of codes for each of these genes. The most influential candidates are various specific genes encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes, all of which are necessary for gaining varied cell functions and specificities of higher eukaryotes. The number of codes for each of these candidate genes is probably two. This is because the proximal 5'-upstream chromatin region (the loose or tight form), as latently active or inactive state, directs the switch (on or off) for transcription ability of the corresponding gene. Thus, in higher eukaryotes un-programmed and new cell function(s) can be newly gained by means of the 4C theory, although almost all of programmed, complicated and diverse cell functions, which had been acquired through a long evolutionary process, are orderly and systematically expressed throughout their development and differentiation. Naturally, the 4C theory is suitable as an explanation for the development and differentiation (and also so-called robustness) of higher eukaryotes, because action of putative signal(s) concerning environment changes seems to fairly resemble that of certain players (such as hormone, cytokine, nerve-transmission substance, etc.), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) through these two fundamental life phenomena.

Here, we would like to change the topic on the 4C theory to recent research results on important life phenomena. First of all, we wanted to describe briefly our impressions on genome editing techniques, which have been recently developed in life science field very rapidly and widely. Because the techniques make impacts of right and wrong for mankind without fail, all of natural science, social science and humanities must cooperatively solve the troublesome problems, which must obviously occur now and in near future. However, we did not comment further on the techniques like recombinant DNA techniques. By the way, from old to present, a lot of ways to newly acquire cell functions, which are somewhat similar to the 4C theory apparently, are being actively studied by many scientists in various organisms (prokaryotes and eukaryotes) as follows [104-108]. There are the experimental evolutions on phenotypic changes based on mutations (genome information changes) through enormous generations during long continuous cultivation and breeding periods in *Escherichia coli* (*E. coli*) and *Drosophila melanogaster* (*D. melanogaster*). For example, *E. coli* cells propagated for numerous generations obtained the ability to use citrate in media as a nutrient, although those cannot usually use citrate as a nutrient source. In addition, the temperature adaptability of two natural populations of *D. melanogaster* living in two areas with different annual temperatures was obviously different from each other after bred under the same temperature environment.

Recently, the very important life phenomena (e.g., inter-tissue communication, intestine-to-germline transmission of epigenetic information and soma-to-germ communication, etc.), which are closely related to alterations in chromatin structure, are being rapidly researched in *Caenorhabditis elegans* (*C. elegans*), *D. melanogaster* and others [109-124]. Here, by borrowing a part of description in the remarkable paper [109], we mention briefly recent achievements on these important life phenomena as follows. Epigenetic modifications of chromatin structure (such as methylation, acetylation and others of histones H2A, H2B, H3 and H4) act as intermediary between environment and gene expression, followed by participation in various organismal phenomena. The results obtained are as follows. For example, in *C. elegans* the alterations in epigenetic states caused by the deficiency of the histone H3K4me3 modifier ASH-2 in the intestine or germline increase organismal stress resistance abrogated by knockdown of the H3K4 demethylase RBR-2. The increase in stress resistance induced by the ASH-2 deficiency in the intestine is abrogated by RBR-2 knockdown in the germline, suggesting the intestine-to-germline transmission of epigenetic information. This communication from intestine to germline in the parental generation increases stress resistance in the next generation. In addition, in various species the epigenetic alterations induced by genetic manipulation and environmental trajectory can sometimes be taken over through many generations, even when the first stimulus no longer exists, e. g., the increased stress resistance induced by low-level stress exposure during development in *C. elegans* can be transmitted to the descendant generations through soma-to-germ communication. Remarkably, the systemic regulation of epigenetic information by means of small RNAs is reported to be essential for transgenerational epigenetic inheritance, which induces phenotypic alteration in descendant generations. These results, together with others, revealed that inter-tissue communication of epigenetic information provides mechanisms for intergenerational regulation of systemic stress resistance, indicating that acquired characteristics (traits) of somatic cells of higher eukaryotes are intergenerationally inherited into descendant generations.

Returning to the original topic on the 4C theory again, we listed below with partial repeat both the general concepts of the 4C theory and the unsolved crucial issues of the theory on the exclusion of excessive IgM H- and L-chains in bullet points. Outline of the 4C theory, which is one of the most fundamental and important ways for life conservation and cell type determination of higher eukaryotes, is concretely as follows. 1) Somatic cells of higher eukaryotes possess pluri-potency, elasticity and flexibility, which are basically originated from those of the chromatin structure, for gaining un-programmed and new cell functions, in order to cope with and/or overcome environment change. 2) Somatic cells gradually acquire the un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity surrounding the proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes through numerous cell divisions (generations). 3) Chromatin structure plasticity (from the tight to loose forms or vice versa) is continuously and irreversibly created based on the chromatin conformation change with epigenetic modifications through numerous cell divisions (generations). 4) Diversity of chromatin structure plasticity in individual somatic cells of the same type is triggered by spontaneous unbalanced response to the environment change and thereafter accomplished by its successive convergence through numerous cell divisions (generations). 5) Irreversible creation of chromatin structure plasticity depends on both antecedents of somatic cells and successive response to the environment change, completes in descendent cells but not in the cell which initially meets with the environment change, and probably occurs inevitably but not incidentally and/or neutrally. 6) The environment change is recognized by putative environment change recognition receptor/site (ECRR/ECRS), and chromatin structure plasticity is irreversibly and directly created by putative chromatin conformation change complex (4C) machinery. 7) The chromatin structure of the proximal 5'-upstream region(s) of specific transcription factor and/or chromatin-modifying gene(s), as dynamic and changeable three-dimensional conformation, receives the signal on the environment change. 8) The chromatin structure (the loose or tight form)

of the proximal 5'-upstream region of the specific gene directs the switch (on or off) for its latent gene expression ability. 9) The supposed number of codes in the 4C theory, which preferentially participates in both complicated cell functions and diverse cell types of higher eukaryotes, may be determined by the multiplication of the number of specific candidate genes and that (probably two) of codes for each of these candidate genes.

There are several crucial issues to be clarified in the 4C theory on the exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [92, 95-103, 125, 126]. 1) Despite the HDAC2-deficiency, why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes decrease at the early stage of cultivation in HDAC2(-/-) mutants? 2) Why do the decreased acetylation levels of one or more of the specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these three genes increase during cultivation? 3) Why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding the proximal 5'-upstrean region of the OBF1 gene decrease during cultivation? 4) Which Lys residue(s) of K9, K14, K18, K23 and K27 of histone H3 is really and/or mainly involved in its binding to DNA within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes must be determined. 5) Functional and steric differences between the loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified more precisely. 6) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor gene during cultivation differ among individual mutant clones? 7) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor genes during cultivation differ within individual mutant clone? 8) How does the 4C machinery (which acts in irreversible creation of chromatin structure plasticity of proximal 5'-upstream region) differ from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in the transcription of ORF region of the corresponding gene) may be the same as the well-known transcription machinery? 9) To demonstrate the 4C theory, it is essential to establish both the putative ECRR/ECRS as the first player to recognize the environment change and the putative 4C machinery as the final player to directly and irreversibly create chromatin structure plasticity.

Finally, we would like to emphasize the following matters. The 4C theory [92, 93, 95-103, 125, 126] is an innovative hypothetic concept on the bio-system to newly cope with, overcome and/or adapt to abnormal, unexpected, disadvantageous and/or even advantageous environment changes, when higher eukaryotic cells firstly encounter these changes. Therefore, it should be one of the most fundamental and important phenomena for the life conservation and cell-type determination of higher eukaryotes. The 4C theory occurs preferentially through the gaining of un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications (which should inevitably occur) but not through alterations in genome information (which should incidentally and/or neutrally occur). Naturally, the 4C theory is obviously different in concepts and in molecular mechanisms from the two above-mentioned remarkable life phenomena, i.e., the experimental evolution [104-108] and the transgenerational epigenetic inheritances [109-124]. As a concrete approach to generalize the 4C theory, for instance, elucidation of the influences of changes in temperature, atmosphere and/or nutrition on the ability to gain un-programmed and new cell function(s) in established cell lines through numerous generations and also in model animals (such as *C. elegans*, *D. melanogaster*, Xenopus, mice, rats and others) during development and differentiation is very powerful and significant. Because the influences under the varied conditions can be easily studied by various groups, judging from the facts that many researches in *D. melanogaster* and others were already conducted, although those were not

so much related to molecular mechanisms. When the 4C theory becomes clear in future, it can innovate significantly the general notion on the nature of somatic cells and open the door for the elucidation of the ways to gain the adaptation ability of higher eukaryotes to various intra- and/or extra-cellular environment changes.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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