

A comprehensive review on the chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations in higher eukaryotes**

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FOOTNOTE:

****The comprehensive review is the final revision of the original and revised papers, respectively, which were published in 2017 and 2018, and are available from following URLs:**

**<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10146006> and
<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171009>**

****The comprehensive review is also the modified version of Chapter 7 of the revised monograph (the original monograph was published in 2015 and available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: All-inclusive review and history on the chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 203-260, 2018. The revised monograph [92] is available from following URL:**

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

****The comprehensive review is also the all-inclusive review on our following recent studies in [93, 95-102].**

[93] Nakayama, M. and Nakayama, T.: Generation of Pax5-deficient DT40 mutant cells, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in Pax5(-) DT40 mutants are rapidly and dramatically reduced through various generations during continuous cultivation. pp. 1-28, 2018. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145176>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170947>

[95] Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivation. pp. 1-34, 2018. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145170>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170628>

- [96] Nakayama, M. and Nakayama, T.: IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation. pp. 1-38, 2018. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145178>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170948>
- [97] Nakayama, M. and Nakayama, T.: Fundamental and distinct ways for irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gain of new cell function to exclude accumulated IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. pp. 1-70, 2018. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145181>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170993>
- [98] Nakayama, T. and Nakayama, M.: Chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. pp. 1-43, 2017. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145263>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171004>
- [99] Nakayama, M. and Nakayama, T.: IgM H- and L-chains accumulated excessively in HDAC2(-/-) DT40 mutants are dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation. Current Topics in Biochemical Research. 18, 11-25, 2017. The article is available from following website and URL: http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3 and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168788>
- [100] Nakayama, M. and Nakayama, T.: Irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications to exclude IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. Current Topics in Biochemical Research. 18, 33-56, 2017. The article is available from following website and URL: http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3 and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168794>
- [101] Nakayama, T. and Nakayama, M.: Chromatin conformation change code (4C) theory: A

bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. *Current Topics in Biochemical Research*. 18, 65-86, 2017.

The article is available from following website and URL:

http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3

and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168800>

[102] An all-inclusive review: Chromatin conformation change code (4C) theory on a bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations in higher eukaryotes. pp. 1-75, 2018. The article (the original paper was uploaded in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10146006>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171009>

Summary

In this article we review our recent studies on the ability of higher eukaryotes for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity through various generations. Following the ways to diminish IgM H- and L-chains excessively accumulated in histone deacetylase-2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) as a concrete case, we proposed a universal hypothetic concept on the ability of higher eukaryotic cells to adapt to abnormal and/or uncomfortable environment changes. In chicken 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, and Ikaros plus E2A. The HDAC2-deficiency rapidly induces dramatic accumulations of mRNAs and proteins of IgM H- and L-chains, and thereafter these accumulated mRNAs and proteins are dramatically reduced in almost similar changing pattern in all individual clones of HDAC2(-/-) DT40 mutants during simple continuous cultivation under the same conditions. By contrast, gene expressions of Pax5, Aiolos, EBF1 and OBF1 remarkably show distinct changing patterns in individual clones of HDAC2(-/-) mutants during the same simple continuous cultivation. At the later stage of cultivation, there exist at least three distinct ways for gene expressions of IgM H- and L-chains, i.e., OBF1-dependent, Pax5- plus Aiolos-dependent, and Pax5-, Aiolos- plus EBF1-dependent types. The complicated alterations in gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) mutants are based on varied irreversible chromatin conformation (structure) changes attributed to diverse changes in acetylation and/or deacetylation levels of specific Lys residues of histone H3 within their proximal 5'-upstream chromatin regions during continuous cultivation. Based on these results, we proposed a universal concept, which we named the chromatin conformation change code (4C) theory, for a bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations.

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 are pluri-potent, elastic and flexible to gain un-programmed and new cell functions to cope with and/or overcome new environment change, when they firstly encounter with it in their lives. 2) The pluri-potency, elasticity and flexibility are basically originated from those of the chromatin structure. 3) Somatic cells gradually acquire the ability to gain un-programmed and new cell functions to adapt to and/or eliminate the environment change by means of irreversible creation of chromatin structure plasticity surrounding the proximal 5'-upstream region(s) of specific transcription factor and chromatin-modifying enzyme genes through various generations (cell divisions). 4) 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 various generations. 5) Diversity of chromatin structure plasticity in individual cells of the same type is triggered by spontaneous unbalanced response to the environment change and accomplished by its successive convergence through various generations. 6) Irreversible creation of chromatin structure plasticity depends on antecedents of somatic cells and successive response to the environment change. 7) Irreversible creation of chromatin structure plasticity occurs in descendent cells but not in the cell which initially meets with the environment change. 8) Irreversible creation of chromatin structure plasticity probably occurs inevitably but not incidentally and/or neutrally. 9) 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. 10) The chromatin structure of the proximal 5'-upstream region(s) of the specific gene(s), as dynamic and changeable three-dimensional conformation, receives signal on the environment change. 11) 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. 12) The proximal 5'-upstream chromatin region of the specific gene is regarded as “notch of chromatin” from a structural point of view and “director for gene expression” from a functional point of view. 13) The supposed number of codes in the 4C theory, which determines complicated cell functions and diverse cell types in higher eukaryotes, may be estimated based on combination of the number of candidate genes and that (probably two) of codes for each of these specific candidate genes.

Keywords

Gene targeting techniques, chicken DT40 cells, histone deacetylase-2 (HDAC2), HDAC2-deficient DT40 mutants (HDAC2(-/-)), IgM H- and L-chains, continuous cultivation, various generations, decreases in IgM H- and L-chain protein and mRNA levels, changes in mRNA levels of Pax5, Aiolos, EBF1, OBF1 genes in individual mutant clones, irreversible creation of chromatin structure plasticity, proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes, alterations in acetylation and deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3, epigenetic modifications, neighboring overlapping tailing chromatin immuno-precipitation (NotchIP) assay, environment change recognition receptor/site (ECRR/ECRS), chromatin conformation change complex (4C) machinery, chromatin conformation change code (4C) theory, gaining un-programmed and new cell function(s), pluri-potency, elasticity and flexibility of somatic cells of higher eukaryotes

Introduction

In eukaryotes, genome information and nuclear function are mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Communication and signal transduction between the nucleus and cytoplasm, both of 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 various generations. This mode is a basis for the evolution of species. 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 various generations. This mode is a basis for the development and differentiation of cells. Fourth, in the case of a minor change, cells respond to it using 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 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 mutant cells HDAC2(-/-) revealed that HDAC2 controls the amount of IgM H-chain at the 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 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 on the regulation of gene expressions of IgM H- and L-chains, we have systematically generated homozygous DT40 mutant cell lines: EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), respectively, devoid of EBF1, Aiolos, E2A and Helios genes [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 (Fig. 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, 95-102]. 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 various 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 various generations during continuous cultivation [92, 97, 100]. Based on these results [92-97, 99, 100], we proposed a hypothetic concept on the ability of higher eukaryotes for gaining un-programmed and new functions by means of irreversible creation of chromatin structure plasticity through various generations [98, 101, 102]. Following the ways to diminish IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants as a concrete case, we expanded it to a universal hypothetic concept on the ability of higher eukaryotic cells to adapt to an unexpected, abnormal and/or uncomfortable environment change.

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 various generations during continuous cultivation

In the course 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 [57, 64, 92, 93, 95-102], the cultivation stages (and days) were practically counted from the first day of cultivation from the stock at -80 °C, although until the stock at -80 °C about 15-16 days had already passed since two HDAC2 alleles (and one Pax5 allele) were disrupted. 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 (Fig. 2). By contrast, insignificant changes are observed for most of other major cellular proteins during cultivation. Western blotting, which was carried out at shorter interval periods, using two antibodies for chicken IgM H-chain and also 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 (Fig. 3). These results, together, indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage (Fig. 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) (Fig. 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 (Fig. 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, surprisingly, 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 those of most remaining Lys residues of core histones.

Because the gene expression of Pax5 is controlled by HDAC2, and then 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 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 surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in initially generated HDAC2(-/-) mutant cells collected 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 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 (~8 days), middle (~13 days) and later (~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 (Fig. 5). Furthermore, 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. 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 (Fig. 6). 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. RT-PCR using primers IgM Hc and IgM Hs revealed that the whole and secreted forms of IgM H-chain mRNA are dramatically elevated at the first (~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 (Fig. 7). 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. RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors, 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 mRNA level of HDAC7 moderately changes during cultivation. 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 cultivation. The PU.1 mRNA level is obviously reduced at the first stage, and thereafter gradually elevated during cultivation and at the later stage reach almost the same level as in DT40 cells.

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 various 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 through distinct ways of gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation

To clarify the above-mentioned results in further detail and to eliminate effects of drug-resistant genes within targeting vectors, we newly generated HDAC2-deficient DT40 mutants HDAC2(-/-) (Fig. 8) [92, 96, 99], using two targeting vectors different from those used previously [57]. By systematical analyses of these secondly generated HDAC2(-/-) mutant cells, we could obtain again following remarkable and noticeable results. As shown in Figures 9 and 10, 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) (Fig. 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 (Fig. 11) [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 (Fig. 12), 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 (Figs. 9, 10 and 11). Agreed 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 (Fig. 12). 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, the IgM H- and L-chains artificially and excessively synthesized caused 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 aggregation) may be transmitted to the chromatin (structure) within the nucleus during cultivation, though both of 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 of 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 (Fig. 10) [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.

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 (Fig. 10). 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 (Fig. 1-L). Moreover, clone cl.2-1 resembles Pax5(-) mutants in the way to decrease the accumulated immunoglobulin proteins during cultivation in appearance, 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 of 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 (Fig. 10). Thereafter, those of Pax5 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 (Fig. 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 (Fig. 10). 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 (Fig. 1-L), since the two immunoglobulin gene expressions in DT40 cells are directly and cooperatively regulated by these three transcription factors (and E2A) (Fig. 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. For instance, in DT40 cells, Pax5 regulates dramatically or moderately gene expressions of PCAF, HDAC7, HDAC9, Aiolos, OBF1, Ikaros, E2A, EBF1 and PU.1 and further Pax5 isoforms A and B regulate differentially other B cell development-related factors [92, 93]. In addition, Aiolos regulates pre-mature B cell apoptosis mediated by BCR signaling [86]. E2A regulates gene expressions of surviving, 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 C θ [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 function(s) in distinct ways through various generations during cultivation. That is, the same and new cell functions of individual HDAC2(-/-) mutant clones that we mean are both of the excluding artificially accumulated IgM H- and L-chains and the ridding themselves free from the aggregative form, since these two must be abnormal, unexpected and/or uncomfortable for the mutant cells. 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 facts that as the cell samples at the early stage of cultivation we collected the mutant cells at 15-16 days after their birth 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 were already reported to down-regulate gene expressions of IgM H- and L-chains in chicken DT40 cells by gene targeting techniques [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 various 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 various 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. It is because the chromatin structure surrounding the proximal 5'-upstream region should be directly and closely related to 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 was 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) (Figs. 13, 14, 15, 16 and 17). 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 various 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 (Fig. 13-Pax5). Consequently, 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, 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 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 (Fig. 10).

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 (Fig. 13-Aiolos). Accordingly, the chromatin structure surrounding the proximal ~2.1 kb 5'-upstream region of the gene, which may consist of ~11 nucleosomes, is 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 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 (Fig. 10).

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 (Fig. 13-EBF1). 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, 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 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 (Fig. 10).

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 (Fig. 13-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 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 (Fig. 10).

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 PCAF gene is really a very low at the early stage but thereafter gradually and dramatically increased until the later stage (Fig. 10). 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 (Fig. 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 various 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, those 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 (Fig. 14-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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, 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 (Fig. 14-Aiolos). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 (Fig. 14-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 their 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 (Fig. 14-OBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 becomes 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 (Fig. 10).

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 is 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 a very low level at the early stage, as in DT40 cells, but gradually and dramatically increased until the later stage (Fig. 10). 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 to wild-type DT40 cells in the way for gene expressions of IgM H- and L-chains in appearance; and to be the major type (Fig. 1-L), like clone cl.2-4, 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 various 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 almost no binding ability based on their hyper-acetylation levels at the later stage (Fig. 15-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 (Fig. 15-Aiolos). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4 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 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 (Fig. 10).

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 (Fig. 15-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 possess 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 (Fig. 15-OBF1). Accordingly, the chromatin

structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 is the loose form in both DT40 cells and clone cl.2-4. However, as in DT40 cells, the gene expression of PCAF is really 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 (Fig. 10). 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.

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 and Aiolos, and somewhat similar to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance (Fig. 1-L) and also to be the major type.

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 various 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, those 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 no binding ability based on their hypo-acetylation levels at the later stage (Fig. 16-Pax5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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, K9/H3 and K27/H3 in particular change the binding capacity of histone H3 to DNA to a full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage (Fig. 16-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 is 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 (Fig. 10).

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 (Fig. 16-EBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 (Fig. 16-OBF1). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is 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 (Fig. 10).

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 gain 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 is the loose form in both DT40 cells and clone cl.2-6 at the middle stage but the 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 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 (Fig. 10). 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 (Fig. 1-L) and mostly similar to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance.

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 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 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 17.

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 early and later stages of continuous cultivation

Large amounts of IgM H- and L-chains caused by their excessively increased gene expressions artificially induced by the HDAC2-deficiency (Fig. 9) are first accumulated within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figs. 18 and 19) [92, 95, 96, 98, 99, 101, 102]. 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 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, and kept at the peri-nuclear space at the early and later cultivation stages (Figs. 18, 19 and 20) [92, 98, 101, 102].

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 various generations during continuous cultivation

How do the HDAC2(-/-) mutants exclude excessively accumulated IgM H- and L-chains, in order to cope with and/or overcome such as 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 decreasing ability of gene expressions of IgM H- and L-chains, and thereby decrease large amounts of these two immunoglobulin proteins. 2) They acquire a high degrading (decomposing) ability of proteins (and/or mRNAs) of IgM H- and L-chains, and thereby decrease large amounts of these

two immunoglobulin proteins. 3) They acquire a high secreting ability of IgM H- and L-chains, and thereby decrease large amounts of these 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, probably because the 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 caused by HDAC2-deficiency is kept at the peri-nuclear space at the early and later stages (Figs. 18, 19 and 20), together with the above-mentioned results (Figs. 1 to 17) 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 various generations during continuous cultivation [92, 98, 101, 102].

First, we supposed that environment change recognition repreceptor/site (ECRR/ECRS) participates in the recognition of 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) (Fig. 20). 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 specific transcription factor genes (and also to act in part in the signal transduction) (Figs. 20 and 21). Using the ECRR/ECRS, the 4C machinery and other components, the chain reaction of response to the abnormal and/or unfavorable environment change occurs as follows.

Large amounts of IgM H- and L-chains artificially caused by the HDAC2-deficiency (Fig. 9) are first accumulated within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figs. 18 and 19). 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 outside of cells), whereas, a part of them is transported to the nuclear envelope but not inside of the nucleus, and kept at the peri-nuclear space at the early and later cultivation stages (Figs. 18, 19 and 20). 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) (Fig. 20). 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 not only on the proximal 5'-upstream regions of the above-mentioned Pax5, Aiolos, EBF1, OBF1 and other genes but also in individual clones of HDAC2(-/-) mutants (Fig. 22).

In wild-type DT40 cells, in which various members of HATs and HDACs are largely expressed [64, 92], the 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes probably consists of HDAC2 as a deacetylation activity, a specific member of HATs as an acetylation activity and other factors (Fig. 21) [92, 98, 101]. On the other hand, 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 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 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 (Figs. 13, 14, 15, 16, 17 and 22). 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 (Figs. 17, 21 and 23). 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 (Figs. 21 and 23).

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 various generations during simple continuous cultivation under the same conditions (Fig. 10) [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, 100-102]. Remarkably, 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 three different cell types exhibiting distinct functions. Because they show three dissimilar ways for gene expressions of transcription factors and chromatin-modifying enzymes (such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC9, etc.), all of which are easily considered to participate in transcriptional regulations of various different genes as well as those of IgM H- and L-chains at the later cultivation stage (Figs. 1 and 10). 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. 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 way that HDAC2(-/-) DT40 mutants gain 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 many generations during continuous cultivation is obviously different from the so-called endoplasmic reticulum (ER) stress response/unfolded protein response and others.

Chromatin conformation (structure) change code (4C) theory: A universal way 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 various generations in higher eukaryotes

We expanded the above-mentioned hypothetical way concerning the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetical concept on ways for gaining 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 various generations (cell divisions) in higher eukaryotes.

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 abnormal and/or uncomfortable), they gradually gain un-programmed and new cell functions through various 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 (Figs. 20 and 21). 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) (Fig. 20). Naturally, 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. Next, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin structure within the nucleus through various generations (cell divisions). 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 acetylation and/or deacetylation events among such epigenetic modifications (Fig. 21). Positions of specific Lys residues and/or kinds of core histones are diverse. For instance, in the above-mentioned case [92, 97, 98, 100-102], 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 various 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 levels induces no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure (Fig. 21). 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 those 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 (Fig. 21). 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 (Fig. 23). 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 various 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 cells through various generations, in spite of the same environment change. In consequence, individual somatic cells of the same type are able to newly gain the same 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 an intra- and/or extra-cellular environment change, individual somatic cells of higher eukaryotes possess ability to newly gain the same 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 (Fig. 23). 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 various generations. The different ways to create varied chromatin structure plasticity in somatic cells are certainly 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 cell divisions. 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 (Fig. 22). 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 (rope-like) chromatin structure possessing self-reformation ability as three-dimensional conformation, which is dynamic and changeable between the loose and tight forms, but not mere the linear (rod-like) chromosomes and the planar (thread-like) chromatin as one- and two-dimensional conformation, which are static and unchangeable. 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. 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) 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. Moreover, in higher eukaryotes diverse kinds of somatic cells share and express cooperatively vital functions with each other.

Finally, we named our theory on such a bio-system to gain un-programed and new cell function(s) by means of irreversible creation of chromatin structure plasticity with epigenetic modifications, which is one of the most fundamental and important ways for life conservation and cell type determination of higher eukaryotes, as the chromatin conformation (structure) change code (4C) theory. 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 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. Therefore, the 4C theory can innovate the general notion on nature of somatic cells and open the door for gaining un-programmed and new cell function(s) of higher eukaryotes. On the other hand, programmed, complicated and diverse cell functions are orderly and systematically expressed throughout their development and differentiation. However, 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) throughout these two fundamental life phenomena.

There are several crucial questions 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, 95-102]. 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'-upstream 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. 10) 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 various generations and also in model animals (such as *C. elegans*, *Drosophila*, *Xenopus*, mice, rats and others) during development and differentiation is very helpful and significant, because these influences under the varied conditions can be easily studied by various research groups.

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Conflict of Interest Statement

There are no conflicts of interest.

Postscript

This comprehensive review is the revised version of our original and second review articles, which were uploaded in 2017 and 2018, and are available from following URLs: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10146006> and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171009>

And the comprehensive review is also the over-view on our previous and recent studies in [57, 64, 92-102]. The studies in Refs. 96, 97 and 98 were reviewed in Refs. 99, 100 and 101, respectively.

References

- [1] Allfrey, V., Faulker, R. M. and Mirsky, A. E.: Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. USA*, 51, 786-794, 1964.
- [2] Brownell, J. E., Zhou, J., Rannali, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. and Allis, C. D.: Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5 linking histone acetylation to gene activation. *Cell* 84, 843-851, 1996.
- [3] Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y.: The transcriptional coactivators p300 and CPB are histone acetyltransferases. *Cell* 87, 953-959, 1996.
- [4] Taunton, J., Hassig, C. A. and Schreiber, S. L.: A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* 272, 408-411, 1996.
- [5] Wolffe, A. P.: Histone deacetylase: a regulator of transcription. *Science* 272, 371-372, 1996.
- [6] Hassig, C. A. and Schreiber, S. L.: Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. *Curr. Opin. Chem. Biol.* 1, 300-308, 1997.
- [7] Davie, J. R.: Covalent modifications of histones: expression from chromatin templates. *Curr. Opin. Genet. Dev.* 8, 173-178, 1998.
- [8] Luger, K. and Richmond, T. J.: The histone tails of the nucleosome. *Curr. Opin. Genet. Dev.* 8, 140-146, 1998.

- [9] Johnson, C. A. and Turner, B. M.: Histone deacetylases: complex transducers of nuclear signals. *Semin. Cell Dev. Biol.* 10, 179-188, 1999.
- [10] Brown, C. E., Lechner, T., Howe, L. and Workman, J. L.: The many HATs of transcription coactivators. *Trends Biochem. Sci.* 25, 15-19, 2000.
- [11] Cheung, W. L., Briggs, S. D. and Allis, C. D.: Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* 12, 326-333, 2000.
- [12] Strahl, B. D. and Allis, C. D.: The language of covalent histone modifications. *Nature* 403, 41-45, 2000.
- [13] Turner, B. M.: Histone acetylation and an epigenetic code. *Bioessays* 22, 836-845, 2000.
- [14] Jenuwein, T. and Allis, C. D.: Translating the histone code. *Science* 293, 1074-1080, 2001.
- [15] Khochbin, S., Verdel, A., Lemerrier, C. and Seigneurin-Berny, D.: Functional significance of histone deacetylase diversity. *Curr. Opin. Genet. Dev.* 11, 162-166, 2001.
- [16] Roth, S. Y., Denu, J. M. and Allis, C. D.: Histone acetyltransferases. *Annu. Rev. Biochem.* 70, 81-120, 2001.
- [17] Carrozza, M. J., Utley, R. T., Workman, J. L. and Cote, J.: The diverse functions of histone acetyltransferase complexes. *Trend. Genet.* 19, 321-329, 2003.
- [18] Yang, X. J. and Seto, E.: Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Curr. Opin. Genet. Dev.* 13, 143-153, 2003.
- [19] Wang, S., Yan-Neale, Y., Zeremski, M. and Cohen, D.: Transcription regulation by histone deacetylases. *Novartis Found Symp.* 259, 238-245, 2004.
- [20] Biel, M., Wascholowski, V. and Giannis, A.: Epigenetics - an epicenter of gene regulation: histones and histone-modifying enzymes. *Angew. Chem. Int. Ed. Engl.* 44, 3186-3216, 2005.
- [21] Margueron, R., Trojer, P. and Reinberg, D.: The key to development: interpreting the histone code? *Curr. Opin. Genet. Dev.* 15, 163-176, 2005.
- [22] Kouzarides, T.: Chromatin modifications and their function. *Cell* 128, 693-705, 2007.
- [23] Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwein, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhatar, R., Shilatifard, A., Workman, J. and Zhang, Y.: New nomenclature for chromatin-modifying enzymes. *Cell* 131, 633-636, 2007.
- [24] Lee, K. K. and Workman, J. L.: Histone acetyltransferase complexes: one size doesn't fit all. *Nat. Rev. Mol. Cell Biol.* 8, 284-295, 2007.
- [25] Goldberg, A. D., Allis, C. D. and Bernstein, B. E.: Epigenetics: a landscape takes shape. *Cell* 128, 635-638, 2007.
- [26] Shahbazian, M. D. and Grunstein, M.: Functions of site-specific histone acetylation and

- deacetylation. *Annu. Rev. Biochem.* 76, 75-100, 2007.
- [27] Berger, S. L.: The complex language of chromatin regulation during transcription. *Nature* 447, 407-412, 2007.
- [28] Suganuma, T. and Workman, J. L.: Crosstalk among histone modifications. *Cell* 135, 604-607, 2008.
- [29] Kohn, K. W., Aladjem, M. I., Weinstein, J. N. and Pommier, Y.: Chromatin challenges during DNA replication: A systems representation. *Mol. Biol. Cell* 19, 1-7, 2008.
- [30] Selvi, R. B. and Kundu, T. K.: Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. *Biotech. J.* 4, 375-390, 2009.
- [31] Javierre, B. M., Hemando, H. and Ballestar, E.: Environmental triggers and epigenetic deregulation in autoimmune disease. *Discov. Med.* 12, 535-545, 2011.
- [32] Bannister, A. J. and Kouzarides, T.: Regulation of chromatin by histone modifications. *Cell Res.* 21, 381-395, 2011.
- [33] Verrier, L., Vandromme, M. and Trouche, D.: Histone demethylases in chromatin cross-talks. *Biol. Cell* 103, 381-401, 2011.
- [34] Butler, J. S., Koutelou, E., Schibler, A. C. and Dent, S. Y.: Histone-modifying enzymes: regulators of developmental decisions and drivers of human disease. *Epigenomics* 4, 163-177, 2012.
- [35] Kooistra, S. M. and Helin, K.: Molecular mechanisms and potential functions of histone deacetylases. *Nat. Rev. Mol. Cell. Biol.* 13, 297-311, 2012.
- [36] Graff, J. and Tsai, L.-H.: Histone acetylation: molecular mnemonics on the chromatin. *Nat. Rev. Neurosci.* 14, 97-111, 2013.
- [37] Chen, T. and Dent, S. Y. R.: Chromatin modifiers and remodellers: regulators of cellular differentiation. *Nat. Rev. Genet.* 15, 93-106, 2014.
- [38] Tee, W.-W. and Reinberg, D.: Chromatin futures and the epigenetic regulation of pluripotency states in ESCs. *Development* 141, 2376-2390, 2014.
- [39] Morgan, M. A. and Shilatifard, A.: Chromatin signatures of cancer. *Genes and Dev.* 29, 238-249, 2015.
- [40] Staudt, L. M. and Lenardo, M. J.: Immunoglobulin gene transcription. *Annu. Rev. Immunol.* 9, 373-398, 1991.
- [41] Peterson, M. L.: RNA processing and expression of immunoglobulin genes. In *Handbook of B and T Lymphocytes* (Snow, E. C. ed.) pp321-342, 1994, Academic press, San Diego.
- [42] Calame, K. L., Lin, K. I. and Tunyaplin, C.: Regulatory mechanisms that determine the development and function of plasma cells. *Annu. Rev. Immunol.* 21, 205-230, 2003.
- [43] Busslinger, M.: Transcriptional control of early B cell development. *Annu. Rev. Immunol.* 22, 55-79,

2004.

- [44] Su, I. H. and Tarakhovsky, A.: Epigenetic control of B cell differentiation. *Semin. Immunol.* 17, 167-172, 2005.
- [45] Nutt, S. L. and Kee, B. L.: The transcriptional regulation of B cell lineage commitment. *Immunity* 26, 715-725, 2007.
- [46] Ramirez, J., Lukin, K. and Hagman, J.: From hematopoietic progenitors to B cells: mechanisms of lineage restriction and commitment. *Curr. Opin. Immunol.* 22, 177-184, 2010.
- [47] Funahashi, J., Okufuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H.: Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth Differ.* 41, 59-72, 1999.
- [48] Stevens, S., Ong, J., Kim, U., Eckhardt, L. A. and Roeder, R. G.: Role of OCA-B in 3'-IgH enhancer function. *J. Immunol.* 164, 5306-5312, 2000.
- [49] Hannon, G. J.: RNA interference. *Nature* 418, 244-251, 2002.
- [50] Shapiro-Shelef, M., Lin, K. I., McHeyzer-Williams, L. J., Liao, J., McHeyzer-Williams, M. G. and Calame, K.: Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19, 607-620, 2003.
- [51] Savitsky, D. and Calame, K.: B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. *J. Exp. Med.* 203, 2305-2314, 2006.
- [52] Nera, K.-P., Kohonen, P., Narvi, E., Peippo, A., Mustonen, L., Terho, P., Koskele, K., Buerstedde, J.-M. and Lassila, O.: Loss of Pax5 promotes plasma cell differentiation. *Immunity* 24, 283-293, 2006.
- [53] Magari, M., Aya, T., Ikeda, M., Todo, K., Kanayama, N. and Ohmori, H.: Enhancement of antibody production from a chicken B cell line DT40 by reducing Pax5 expression. *J. Biosci. Bioeng.* 107, 206-209, 2009.
- [54] Toman, I., Loree, J., Klimowicz, A. C., Bahlis, N., Lai, R., Belch, A., Pilarski, L. and Reiman, T.: Expression and prognostic significance of Oct2 and Bob1 in multiple myeloma: implications for targeted therapeutics. *Leuk. Lymphoma.* 52, 659-667, 2011.
- [55] Fujita, T. and Fujii, H.: Species-specific 5'-genomic structure and multiple transcription start sites in the chicken Pax5 gene. *Gene* 477, 24-31, 2011.
- [56] John, L. B. and Ward, A. C.: The Ikaros gene family: transcriptional regulators of hematopoiesis and immunity. *Mol. Immunol.* 48, 1272-1278, 2011.
- [57] Takami, Y., Kikuchi, H. and Nakayama, T.: Chicken histone deacetylase-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its pre-mRNA in the DT40 cell line. *J. Biol. Chem.* 274, 23977-23990, 1999.
- [58] Takami, Y. and Nakayama, T.: N-terminal region, C-terminal region, nuclear export signal and

deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. *J. Biol. Chem.* 275, 16191-16201, 2000.

[59] Nakayama, T. and Takami, Y.: Participation of histones and histone-modifying enzymes in cell functions through alterations in chromatin structure. *J. Biochem.* 129, 491-499, 2001.

[60] Takechi, S., Adachi, M. and Nakayama, T.: Chicken HDAC2 down-regulates IgM light chain gene promoter activity. *Biochem. Biophys. Res. Commun.* 299, 263-267, 2002.

[61] Kikuchi, H., Takami, Y. and Nakayama, T.: GCN5: a supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes. *Gene* 347, 83-97, 2005.

[62] Kikuchi, H., Barman, H. K., Nakayama, M., Takami, Y. and Nakayama, T.: Participation of histones, histone modifying enzymes and histone chaperones in vertebrate cell functions. *Reviews and Protocols in DT40 Research*, Springer-Verlag, Berlin, pp225-243, 2006.

[63] Barman, H. K., Takami, Y., Ono, T., Nishijima, H., Sanematsu, F., Shibahara, K. and Nakayama, T.: Histone acetyltransferase 1 is dispensable for replication-coupled chromatin assembly but contributes to recover DNA damages created following replication blockage in vertebrate cells. *Biochem. Biophys. Res. Commun.* 345, 1547-1557, 2006.

[64] Nakayama, M., Suzuki, H., Yamamoto-Nagamatsu, N., Barman, H. K., Kikuchi, H., Takami, Y., Toyonaga, K., Yamashita, K. and Nakayama, T.: HDAC2 controls IgM H and L-chain gene expressions via EBF1, Pax5, Ikaros, Aiolos and E2A gene expressions. *Genes Cells* 12, 359-373, 2007.

[65] Kikuchi, H. and Nakayama, T.: GCN5 and BCR signaling collaborate to induce pre-mature B cell apoptosis through depletion of ICAD and IAP2 and activation of caspase activities. *Gene* 419, 48-55, 2008.

[66] Barman, H. K., Takami, Y., Nishijima, H., Shibahara, K., Sanematsu, F. and Nakayama, T.: Histone acetyltransferase-1 regulates integrity of cytosolic histone H3-H4 containing complex. *Biochem. Biophys. Res. Commun.* 373, 624-630, 2008.

[67] Kikuchi, H., Barman, H. K., Nakayama, M., Takami, Y. and Nakayama, T.: Studies on epigenetic control of B cell functions using the DT40 cell line. *Advances in Genetics Research* 2, Urbano K. V. (Ed.), Nova Science Publishers, Inc. NY, pp153-166, 2010.

[68] Kikuchi, H., Kuribayashi, F., Takami, Y., Imajoh-Ohmi, S. and Nakayama, T.: GCN5 regulates the activation of PI3K/Akt survival pathway in B cells exposed to oxidative stress via controlling gene expressions of Syk and Btk. *Biochem. Biophys. Res. Commun.* 405, 657-661, 2011.

[69] Kikuchi, H., Kuribayashi, F., Kiwaki, N., Takami, Y. and Nakayama, T.: GCN5 regulates the superoxide-generating system in leukocytes via controlling gp91-phox gene expression. *J. Immunol.* 186,

3015-3022, 2011.

[70] Kikuchi, H., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, Y., Takami, Y. and Nakayama, T.: GCN5 protects vertebrate cells against UV-irradiation via controlling gene expression of DNA polymerase η . *J. Biol. Chem.* 287, 39842-39849, 2012.

[71] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: GCN5 is essential for IRF-4 gene expression followed by transcriptional activation of Blimp-1 in immature B cells. *J. Leukoc. Biol.* 95, 399-404, 2014.

[72] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: GCN5 is involved in regulation of immunoglobulin heavy chain gene expression in immature B cells. *Gene* 544, 19-24, 2014.

[73] Escaffit, F., Vaute, O., Chevillard-Briet, M., Segui, B., Takami, Y., Nakayama, T. and Trouche, D.: Cleavage and cytoplasmic relocalization of histone deacetylase 3 are important for apoptosis progression. *Mol. Cell. Biol.* 27, 554-567, 2007.

[74] Baba, T. W., Giroir, B. P. and Humphries, E. H.: Cell lines derived from avian lymphomas exhibit two distinct phenotypes. *Virology* 144, 139-151, 1985.

[75] Buerstedde, J.-M. and Takeda, S.: Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* 67, 179-188, 1991.

[76] Takeda, S., Masteller, E. L., Thompson, C. B. and Buerstedde, J.-M.: RAG-2 expression is not essential for chicken immunoglobulin gene conversion. *Proc. Natl. Acad. Sci. USA* 89, 4023-4027, 1992.

[77] Takami, Y., Takeda, S. and Nakayama, T.: Targeted disruption of an H3-IV/H3-V gene pair causes increased expression of the remaining H3 genes in the chicken DT40 cell line. *J. Mol. Biol.* 250, 420-433, 1995.

[78] Seguchi, K., Takami, Y. and Nakayama, T.: Targeted disruption of O1H1 encoding a particular H1 histone variant causes changes in protein patterns in the DT40 chicken B cell line. *J. Mol. Biol.* 254, 869-880, 1995.

[79] Takami, Y., Takeda, S. and Nakayama, T.: Targeted disruption of H2B-V encoding a particular H2B histone variant causes changes in protein patterns on two-dimensional polyacrylamide gel electrophoresis in the DT40 chicken B cell line. *J. Biol. Chem.* 270, 30664-30670, 1995.

[80] Takami, Y., Takeda, S. and Nakayama, T.: An approximately half set of histone genes is enough for cell proliferation and a lack of several histone variants causes protein pattern changes in the DT40 chicken B cell line. *J. Mol. Biol.* 265, 394-408, 1997.

[81] Takami, Y. and Nakayama, T.: One allele of the major histone gene cluster is enough for cell proliferation of the DT40 chicken B cell line. *Biochim. Biophys. Acta* 1354, 105-115, 1997.

- [82] Takami, Y. and Nakayama, T.: A single copy of linker H1 genes is enough for proliferation of the DT40 chicken B cell line, and linker H1 variants participate in regulation of gene expression. *Genes Cells* 2, 711-723, 1997.
- [83] Sanematsu, F., Takami, Y., Barman, H. K., Fukagawa, T., Ono, T., Shibahara, K. and Nakayama, T.: Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. *J. Biol. Chem.* 281, 13817-13827, 2006.
- [84] Takami, Y., Ono, T., Fukagawa, T., Shibahara, K. and Nakayama, T.: Essential role of CAF-1-mediated rapid nucleosome assembly for DNA replication and cell division in vertebrate cells. *Mol. Biol. Cell* 18, 129-141, 2007.
- [85] Toyonaga, K., Kikuchi, H., Yamashita, K., Nakayama, M., Chijiwa, K. and Nakayama, T.: E2A participates in a fine control of pre-mature B cell apoptosis mediated by B cell receptor signaling via transcription regulations of survivin, IAP2 and caspase-8 genes. *FEBS J.* 276/5, 1418-1428, 2009.
- [86] Kikuchi, H., Yamashita, K., Nakayama, M., Toyonaga, K., Tsuneyoshi, I., Takasaki, M. and Nakayama, T.: Lacking of Aiolos accelerates pre-mature B cell apoptosis mediated by BCR signaling through elevation in cytochrome c release. *BBA-Molecular Cell Research* 1793, 1304-1314, 2009.
- [87] Kikuchi, H., Nakayama, M., Takami, Y., Kuribayashi, F. and Nakayama, T.: Possible involvement of Helios in controlling the immature B cell functions via transcriptional regulation of protein kinase Cs. *Results Immunol.* 1, 88-94, 2011.
- [88] Kikuchi, H., Nakayama, M., Takami, Y., Kuribayashi, F. and Nakayama, T.: EBF1 acts as a powerful repressor of Blimp-1 gene expression in immature B cells. *Biochem. Biophys. Res. Commun.* 422, 780-785, 2012.
- [89] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama, T.: Protein kinase C θ gene expression is oppositely regulated by EBF1 and GCN5 in immature B cells. *FEBS Lett.* 588, 1739-1742, 2014.
- [90] Fukagawa, T., Mikami, Y., Nishihara, A., Regnier, V., Haraguchi, T., Hiraoka, Y., Sugata, N., Todokodo, K., Brown, W. and Ikemura, T.: CENP-P, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells. *EMBO J.* 20, 4603-4617, 2001.
- [91] Chang, H. and Delany, M.-E.: Karyotype stability of the DT40 chicken B cell line: macrochromosome variation and cytogenetic mosaicism. *Chromosome Res.* 12, 299-307, 2004.
- [92] Nakayama, T. and Nakayama, M.: Chromatin Conformation Change Code (4C) theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations. pp. 1-303, 2018. The revised monograph (the original monograph was published in 2015 and is available from following URL:

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) is available from following URL:

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[93] Nakayama, M. and Nakayama, T.: Generation of Pax5-deficient DT40 mutant cells, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in Pax5(-) DT40 mutants are rapidly and dramatically reduced through various generations during continuous cultivation. pp. 1-28, 2018. The revised article (the original paper was uploaded in 2017 and is available from a following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145176>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170947>

The revised article is also the modified version of Chapter 3 of the revised monograph (the original monograph was published in 2015 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: Generation of Pax5-deficient DT40 mutants, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially accumulated in Pax5(-) are rapidly and dramatically reduced through various generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 45-71, 2018. The revised monograph is available from following URL:

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[94] Takechi, S., Adachi, M. and Nakayama, T.: Cloning and characterization of the chick Oct binding factor OBF-1. Biochim. Biophysica Acta 1577, 466-470, 2002.

[95] Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivation. pp. 1-34, 2018. The revised article (the original paper was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145170>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170628>

The revised article is also the modified version of Chapter 2 of the revised monograph (the first monograph was published in 2015 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications

through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 11-44, 2018. The revised monograph is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[96] Nakayama, M. and Nakayama, T.: IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation. pp. 1-38, 2018. The revised article (the original paper was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145178>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170948>

The revised article is also the modified version of Chapter 4 of the revised monograph (the original monograph was published in 2015 and available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: IgM H- and L-chains accumulated artificially and excessively in HDAC2(-/-) DT40 mutants are dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 72-104, 2018. The revised monograph is available from following URL:

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[97] Nakayama, M. and Nakayama, T.: Fundamental and distinct ways for irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gain of new cell function to exclude accumulated IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. pp. 1-70, 2018. The revised article (the original paper was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145181>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170993>

The revised article is also the modified version of Chapter 5 of the revised monograph (the original monograph published in 2015 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: A fundamental way for irreversible creation of chromatin structure plasticity with epigenetic modifications for gaining new cell function to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T.

and Nakayama, M. (Eds.), pp. 105-167, 2018. The revised monograph is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[98] Nakayama, T. and Nakayama, M.: Chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. pp. 1-43, 2018. The revised article (the original paper was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145263>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171004>

The revised article is also the modified version of Chapter 6 of the revised monograph (the original monograph was published in 2015 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, M. and Nakayama, T.: Chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 168-202, 2018. The revised monograph is available from following URL:

<http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

[99] Nakayama, M. and Nakayama, T.: IgM H- and L-chains accumulated excessively in HDAC2(-/-) DT40 mutants are dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation. Current Topics in Biochemical Research. 18, 11-25, 2017.

The article is available from following website and URL:

http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3

and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168788>

[100] Nakayama, M. and Nakayama, T.: Irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications to exclude IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. Current Topics in Biochemical Research. 18, 33-56, 2017.

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and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168794>

[101] Nakayama, T. and Nakayama, M.: Chromatin conformation change code (4C) theory: A bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure

plasticity with epigenetic modifications through various generations. *Current Topics in Biochemical Research*. 18, 65-86, 2017. The article is available from following website and URL:

http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3

and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168800>

[102] Nakayama, T. and Nakayama, M.: An all-inclusive review: Chromatin conformation change code (4C) theory on a bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations in higher eukaryotes. pp. 1-75, 2018. The revised article (the original paper was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10146006>) is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171009>

The revised article is also the modified version of Chapter 7 of the revised self-publishing monograph (the original self-publishing monograph published in 2015 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995>) as follows: Nakayama, T. and Nakayama, M.: All-inclusive review and history on the chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. In: *Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations*, Nakayama, T. and Nakayama, M. (Eds.), pp. 203-260, 2018. The revised monograph is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

Figure legends

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 OBF1-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 Fig. 1 of Ref. 102 and Suppl. Fig. S1 of Ref. 98 and Fig. 6 of Ref. 99 and also is a set of Figs. 8, 9 and 10 of Ref. 96.

Figure 2. Alterations in 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 Fig. 2 of Ref. 102 and Fig. 2-1 of Ref. 92 and Fig. 1 of Ref. 95 and with Fig. 1 of Ref. 101.

Figure 3. Alterations in 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 Fig. 3 of Ref. 102 and Fig. 2-3 of Ref. 92 and with Fig. 4 of Ref. 95.

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 Fig. 4 of Ref. 102 and Fig. 2-4 of Ref. 92 and Fig. 5 of Ref. 95.

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 Fig. 5 of Ref. 102 and Fig. 3-3 of Ref. 92 and Fig. 3 of Ref. 93.

Figure 6. 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) in distinct ranges of vision with magnified visions. The figure is identical with Fig. 6 of Ref. 102 and Suppl. Fig. 3-S1 of Ref. 92 and Fig. 5 of Ref. 93.

Figure 7. 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 Fig. 7 of Ref. 102 and Fig. 3-5 of Ref. 92 and Fig. 6 of Ref. 93.

Figure 8. 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. 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. 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 Fig. 8 of Ref. 102 and Fig. 4-1 of Ref. 92 and Fig. 1 of Ref. 96 and Fig. 1 of Ref. 99.

Figure 9. 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 Fig. 9 of Ref. 102 and Figs. 4-2 and Suppl. Fig. 6-S4 of Ref. 92 and Fig. 2 of Ref. 96 and Fig. 2 of Ref. 99 and Fig. 2 of Ref. 101.

Figure 10. 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, gene expressions of which changed in Refs. 92 and 96. The figure is identical with Fig. 10 of Ref. 102 and Fig. 4-6 and Suppl. Fig. 6-S5 of Ref. 92 and Fig. 6 of Ref. 96 and Fig. 4 of Ref. 99 and Fig. 3 of Ref. 101.

Figure 11. 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 Fig. 11 of Ref. 102 and Fig. 4-4 of Ref. 92 and Fig. 4 of Ref. 96.

Figure 12. 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 Fig. 12 of Ref. 102 and Fig. 4-5 of Ref. 92 and Fig. 5 of Ref. 96.

Figure 13. 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 Fig. 13 of Ref. 102 and is a set of Figs. 5-1, 5-5, 5-9 and 5-13 of Ref. 92 and is identical with Suppl. Fig. 6-S6 of Ref. 92 and a set of Figs. 1, 5, 9 and 13 of Ref. 97 and is identical with Fig. 4 of Ref. 101.

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.2-2 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 Fig. 13. The figure is identical with Fig. 14 of Ref. 102 and is a set of Figs. 5-2, 5-6, 5-10 and 5-14 of Ref. 92 and is identical with Suppl. Fig. 6-S7 of Ref. 92 and a set of Figs. 2, 6, 10 and 14 of Ref. 97 and is identical with Fig. 5 of Ref. 101.

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.2-4 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 Fig. 13. The figure is identical with Fig. 15 of Ref. 102 and is a set of Figs. 5-3, 5-7, 5-11 and 5-15 of Ref. 92 and is identical with Suppl. Fig. 6-S8 of Ref. 92 and a set of Figs. 3, 7, 11 and 15 of Ref. 97 and is identical with Fig. 6 of Ref. 101.

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.2-6 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 Fig. 13. The figure is identical with Fig. 16 of Ref. 102 and is a set of Figs. 5-4, 5-8, 5-12 and 5-16 of Ref. 92 and is identical with Suppl. Fig. 6-S9 of Ref. 92 and a set of Figs. 4, 8, 12 and 16 of Ref. 97 and is

identical with Fig. 7 of Ref. 101.

Figure 17. 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 Fig. 17 of Ref. 102 and Fig. 5-21 and Suppl. Fig. 6-S10 of Ref. 92 and Fig. 21 of Ref. 97 and Suppl. Fig. S9 of Ref. 98 and Fig.5 of Ref. 100.

Figure 18. 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 Fig. 18 of Ref. 102 and Fig. 6-1 of Ref. 92 and Fig. 1 of Ref. 98 and Fig. 8 of Ref. 101.

Figure 19. 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 the same one of 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 Fig. 19 of Ref. 102 and Fig. 6-3 of Ref. 92 and Fig. 3 of Ref. 98.

Figure 20. 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. 18C) 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 Ref. 102 and Fig. 6-4 of Ref. 92 and Fig. 4 of Ref. 98 and Fig. 9 of Ref. 101.

Figure 21. 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 Fig. 21 of Ref. 102 and Fig. 6-5 of Ref. 92 and Fig. 5 of Ref. 98 and Fig. 10 of Ref. 101.

Figure 22. 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 Fig. 22 of Ref. 102 and Fig. 6-7 of Ref. 92 and Fig. 7 of Ref. 98 and Fig. 12 of Ref. 101.

Figure 23. 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 various 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 various 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 Fig. 23 of Ref. 102 and Fig. 6-6 of Ref. 92 and Fig. 6 of Ref. 98 and Fig. 11 of Ref. 101.