

Memoirs of a life scientist: Interpretations and opinions on life phenomena, and experiences and contributions in life science field ~ The chromatin conformation change code (4C) theory and previous researches ~**

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Footnote

**The article is the revised version of my previous memoir article published in 2019 [198], which is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10174284>

All figures used in the article are identical with corresponding those in the previous memoir article and also are originally selected from those in the original and revised editions of my retirement commemorative monograph published and/or uploaded in 2015 and 2018 [138], respectively, which are available from following URLs: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995> and <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365>

**The article is fundamentally the re-modified version of Chapter 8 of the revised retirement commemorative monograph as follows: Nakayama, T.: Personal closing remarks on the occasion of retirement: The real course and history of our research prior to the 4C theory, personal interpretation on life phenomena, and experiences and opinions as a life scientist. 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. 1-303, 2018. The revised monograph (the original edition 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>

**The article also contains a part of the re-revised paper (which was the modified version of Chapter 6 of the above-mentioned revised monograph) as follows: 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 paper is the modified version of the original paper (which was published in 2017 and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145263>) and is available from following URL: <http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171004>

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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.
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Summary

We advanced the chromatin conformation (structure) change code (4C) theory for the bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations. The 4C theory had been studied under the worst research environments, i.e., without any research grants, in our spare time from my main research projects and that by only a womanpower. The 4C theory is the typical fruit of my serendipity and the outcome of some 10 years of my small research group. In this article, I described the detail of the 4C theory and also the outline on real course of my researches prior to the theory, my personal interpretations of the concept on life phenomena, and experiences and opinions as a life scientist.

In eukaryotes, both genome information and nuclear function are mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Additionally, 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 signal transduction concerning an abnormal, unexpected, uncomfortable 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. 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 and multiplication on the DNA molecule through numerous generations. This mode is a basis for evolution of species. Needless to say, the mode also occurs in all of organisms, including virus, prokaryotes, lower eukaryotes and others. Third, in the case of a moderate change, cells cope with or overcome it by means of irreversible creation of chromatin structure plasticity caused by successive chromatin conformational (structural) changes with epigenetic modifications through numerous generations (or cell divisions). This mode is a basis for the development and differentiation of cells. Fourth, in the case of a minor change, cells respond to it only by means of already acquired regulation mechanisms.

As will be detailed later, we accidentally discovered that IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutant cells (and also Pax5(-) DT40 mutant cells) at the early cultivation stage are dramatically decreased through numerous generations during continuous cultivation. This surprising and remarkable biological event, which should be one of the most

fundamental and important ways for the adaptation to the environment change and life conservation of higher eukaryotes, is really an example of the third case of the above-mentioned countermeasures, which are naturally classified in my own way. In addition, we advanced the chromatin conformation (structure) change code (4C) theory for the bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations (cell divisions).

Since chemical modifications of histones with acetyl and methyl groups were first proposed to regulate RNA synthesis [1], the modulation of chromatin topology has been thought to be one of the most fundamental and important events for expression of normal cell functions in eukaryotes [2-4]. The ways to modulate the chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation and others 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 [5-32]. For the past several decades, countless numbers of research papers 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, DNA replication, repair plus recombination, development, differentiation, memory, pluri-potency, clinical medicine, etc. [33-44].

Naturally, alterations in the chromatin structure are remarkably involved in regulations of the lymphocyte development and differentiation [45-51], which are closely related to one of my latter research projects as will be mentioned later. 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 [52-61]. And then, the regulation of 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.

However, my research group had mainly studied on structures (nucleotide sequences, transcriptional elements and genome organizations) of core histones and linker histone H1 of chickens until ~25 years ago (~1995) [62-72]. Prior to these researches, during more past ~25 years (started from the master course of graduate school), I had mainly studied on determination of amino acid sequences of protein (cytochrome c of bonito), in vitro and in vivo acetylation and synthesis of protein (ovalbumin) in hen's oviduct, RNA synthesis by RNA polymerase of *Bacillus subtilis* during sporulation, 2D-PAGE analysis of proteins, and structure and function of DNA binding proteins and so on [73-90]. Figuratively speaking, if I compare my whole researches to one game, those must be the pre-run of the hop, step and jump.

In the midst of the above-mentioned studies concerning structures of histone genes, the DT40 cell line, which was derived from chicken pre-B lymphocyte cells [91], was reported to possess an ability of homologous recombination with a very high frequency and to be a powerful system for gene targeting techniques [92, 93]. I thought that an approach using the gene targeting techniques in DT40 cells is very advantageous for clarifying fundamental and/or common phenomena in higher eukaryotic cells, besides specific and/or limited functions in the B lymphocyte cell lineage. I was confident that even my small group could continue any of researches using the ALV-transformed chicken DT40 cell line, although both the start and continuance of the researches using model animals (mice, rats, *C. elegans*, *Drosophila*, *Xenopus*, etc.) should be very hard for us because of insufficient grants and manpower. In addition, I was fully convinced that the peculiar knowledge on basic bio-systems obtained in DT40 cells could be universally extended to understanding fundamental and complex bio-systems in higher eukaryotes. Because almost all concepts of the most essential life phenomena, including “central dogma”, which had been elucidated in phage, prokaryote (especially *E. coli*) and/or yeast systems, could be thoroughly applicable to those of higher eukaryotes.

Based on the above-mentioned mature consideration, in parallel with studies on *in vitro* functions of histone chaperones (CAF-1 and HIRA) [94-102], I had immediately changed my main research projects to the elucidation of *in vivo* functions (and structures) of several chromatin-modifying enzymes (HATs and HDACs) and transcription factors, as well as histones and histone chaperones (CAF-1, HIRA, ASF1 and others), using the gene targeting techniques in the DT40 cell line. Of course, I had no hesitation in using the DT40 cell line as an experimental system for my research projects at all, because I had become accustomed to the chicken system through studies on both bio-synthesis and post-translational (i.e., epigenetic) modification such as acetylation and glycosylation of ovalbumin as mentioned above. First, to assess individual roles of members of histones and histone chaperones, we had comprehensively generated numerous homozygous DT40 mutant cell lines, all of which lacked their corresponding specific genes, respectively. The results obtained from resultant DT40 mutants, combined, showed that this approach was really useful and powerful to know individual functions of these specific genes *in vivo* [103-113].

In parallel and/or continuously, based on these positive results and my intrinsic interest on universal and global roles of histone acetylation and/or deacetylation, in order to assess individual roles of members of HATs and HDACs, we had systematically generated a number of homozygous DT40 mutant cell lines, each of which was devoid of a specific member of HATs and HDACs, such as GCN5, PCAF, HAT1, HDAC1, HDAC2, HDAC3 and others [114-131]. Our qualitative analyses of the HDAC2-deficient mutant cell line, HDAC2(-/-), which was initially generated by us [114], revealed not only that HDAC2 regulates the amount of IgM H-chain at the two steps of its gene expression and alternative pre-mRNA

processing [114], but also that it down-regulates IgM L-chain gene promoter activity [117]. The lacking of HDAC2 also 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 of core histones [121]. Further, we generated Pax5(-), Aiolos(-/-), Ikaros(-/-/+), EBF1(-/-), E2A(-/-), Helios(-/-) and other mutants [121, 132-134], and thereby clarified specific functions of these transcription factors in B lymphocytes [121, 129, 132-138]. In consequence, these results, together with others [139], revealed that Pax5, EBF1, Aiolos and Ikaros down-regulate gene expressions of IgM H- and L-chains, and inversely E2A and probably OBF1 up-regulate these two immunoglobulin gene expressions. Therefore, we concluded that HDAC2 as a supervisor indirectly regulates gene expressions of IgM H- and L-chains through opposite regulation of gene expressions of Pax5, EBF1, Aiolos, Ikaros, E2A and also OBF1 [121, 124, 138].

However, until then, I had never met my own exciting research that takes me captive and makes my heart beat fast with joy, and I had not been throbbing heavily with almost all of so-called brilliant and/or fashionable achievements in life science field with a few exceptions. I chiefly owed such feelings to the fact that I have been fascinated by the word “life” from boyhood; therefore, I had a great interest in fundamental concepts of life phenomena but not so much interest in any of individual vital substances, reactions and/or functions, even though they are very important. Surely, it goes without saying that still now countless important questions remain to be resolved in the life science field. By the way, I previously reported not only that extracellular ovalbumin and egg-white proteins inhibit ovalbumin and protein syntheses in hen’s oviduct, respectively [76, 78], but also that structural changes of specific histone genes possibly result in differences in their transcriptional regulations in chicken different tissues, the reason for which is still unknown [66, 67]. In addition, by gene targeting techniques in chicken DT40 cells, we reported that all histone gene families have the inherent ability to keep constant protein levels of respective histone subtypes, all of which are very important for life conservation, when a certain gene member of the corresponding subtype is deleted [103-108, 115]. Therefore, for these above-mentioned reasons, as will be described later in detail, I had much continued to have an interest in adaptability of higher eukaryotes to environment change (one of most fundamental concepts of life phenomena), including both the structural changes of the specific genes among different cells (tissues) and the compensation ability. Because the regulation ways of ovalbumin and egg-white protein syntheses could be considered as a response ability of hen’s oviduct to extracellular environment change, the structural alterations of specific genes (and naturally their chromatin) among different tissues should be thought as a basis for determination of cell type-specificity, and also the compensation ability should be thought as a response to intracellular environment change, i.e., the decrease in protein levels of specific histone member.

In the midst of re-constitution (re-expression) experiments to study the participation of HDAC2 in the regulation of gene expressions of IgM H- and L-chains in detail, we became aware of the surprising phenomena as follows. We first introduced the tetracycline- (tet-) repressive HDAC2 cDNA into HDAC2(-/-) mutants, and isolated the transformed cell line HDAC2(-/-)tetHDAC2(+), lacking two alleles of the endogenous HDAC2 gene but possessing the artificial tet-repressive HDAC2 cDNA. Contrary to our expectation, surprisingly, protein and mRNA levels of IgM H- and L-chains were very low in HDAC2(-/-)tetHDAC2(+) even in the case of non-existence of HDAC2 due to the presence of tet, as well as both in the case of existence of HDAC2 due to the absence of the drug and in wild-type DT40 cells having the enzyme (our unpublished data).

Concerning these findings, members of my research group each had different interpretations. One of colleagues intensely asserted not only that the accumulation of IgM H- and L-chains in HDAC2(-/-) was not truly based on the HDAC2-deficiency and resulted from only unknown artificial cause of beneath our notice, but also that the accumulated immunoglobulin proteins were only reduced spontaneously during cultivation to construct HDAC2(-/-)tetHDAC2(+). Since the colleague was bigoted toward a vague biased (but ordinary in a sense) view that several properties of higher eukaryotic cells are easily changeable during cultivation. Similarly, another colleague conservatively insisted that the accumulation and diminution of IgM H- and L-chains in HDAC2(-/-) were not directly due to the HDAC2-lacking but based on only somatic mutations occurred within the genome locus of the IgM H-chain gene during cultivation to construct the transformants, since the locus was generally considered as a hot spot for the mutations [93]. Thus, they were not interested in these findings no more.

However, intuitively, I believed that the accumulation of IgM H- and L-chains in HDAC2(-/-) was really caused by the HDAC2-deficiency itself, and their diminution during cultivation to construct HDAC2(-/-)tetHDAC2(+) should be attributable to unknown important biological mechanisms. Such my own opinion was supported in part by our following findings. First, several re-experiments (2D-PAGE, Western blotting and Northern blotting) showed that protein and mRNA levels of IgM H-chain (and also L-chain) were firstly increased in several independent HDAC2(-/-) mutant clones and thereafter reproducibly decreased, whenever they were cultivated for a long period (our unpublished data). Second, by contrast, insignificant changes (increases and/or decreases) in protein and mRNA levels of IgM H- and L-chains were observed in numerous DT40 mutant cell lines (approximately 30), each of which was devoid of a member of HDACs (except HDAC2), HATs, transcription factors, histones and histone chaperones, etc. [103-109, 121, our unpublished data].

Meanwhile, in the process of the above-mentioned consideration and re-examinations, I noticed some following mistaken speculations due to qualitative results in our previous paper [114], besides those on the insufficient results in our other papers [84, 86-90]. Re-experiments showed that protein and mRNA

levels of HDAC2 in the heterozygous mutants HDAC2(-/+) were obviously decreased to ~50% of those in DT40 cells (our unpublished data), whereas these two levels had been reported to be kept constant by means of the compensation mechanism [103-109]. Re-examined RNase protection assay (and Northern blotting) showed that the amounts of IgM H-chain protein and ratio of secreted to membrane-bound forms of its mRNA were certainly increased in HDAC2(-/+), compared with those in DT40 cells (our unpublished data), though both the amounts and the ratio had been reported to remain unchanged. The growth rate of initially generated HDAC2(-/-) mutant clones re-tested (our unpublished data), as well as that of most clones of secondly generated HDAC2(-/-) mutants [121], was slightly but truly different from that of DT40 cells, though the growth rate of the initial mutants had been reported to be essentially identical with that of DT40 cells [114]. Pulse-labeling/chase experiment and Northern blotting re-examined showed that protein and mRNA of IgM L-chain really existed as large and small forms and the amounts of the two forms were surely increased associated with the HDAC2-deficiency (our unpublished data), though the small form of IgM L-chain had been considered to be a specific proteolytic product of the large form at the C-terminal region.

Based on my consideration and enthusiasm as mentioned above, I asked again some colleagues of my research group to study mechanisms of the increases and decreases in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) as a part of their own research works. However, several kinds of approaches gave only negative (unpublished) data as follows. RT-PCR performed on various transcription factors (including some promoter and intron enhancer binding proteins mentioned above) for the IgM H-chain gene expression showed only insignificant (or no) changing patterns in their mRNA levels between DT40 cells and HDAC2(-/-) mutants at any cultivation stages. MNase protection assay carried out on several regions within the chromatin surrounding the IgM H-chain gene showed insignificant changing patterns in the nucleosome ladders between DT40 cells and HDAC2(-/-) mutants at any cultivation stages. In addition, MNase protection assay performed on the chromatin of several genes encoding transcription factors (including Pax5, Aiolos, Ikaros and EBF1) also showed insignificant changing patterns in the nucleosome ladders between DT40 cells and HDAC2(-/-) mutants at any cultivation stages.

On the other hand, interestingly, Northern blotting using the specific probe for the IgM H-chain gene sometimes showed a mere change in amounts of small positive-like RNA molecules having a possibility of alternative splicing product of IgM H-chain mRNA between DT40 cells and HDAC2(-/-) mutants at the early and later cultivation stages (our unpublished data). Since small RNA molecules (such as small nuclear RNA, micro RNA, small interference RNA, anti-sense RNA, etc.) were watched as important players in the regulation of cell functions at that time, we temporarily focused our attention on these molecules. However, unfortunately, these findings were not reproducible; therefore, I decided that in our case the small RNA molecules were not worth studying ever since.

For all that, I believed that these negative results should be caused only by the following facts: 1) IgM H- and L-chain genes are indirect but not direct targets of HDAC2 [121]. 2) MNase protection assay was regrettably carried out only on coding regions (open reading frame (ORF) regions) but not on proximal 5'-upstream regions of these transcription factor genes. 3) HDAC2(-/-) mutants regarded to be collected at the early stage must be actually collected at vague early cultivation stage, i.e., at fairly later and varied cultivation stages, because they had been already cultivated for a long time to use for other experiments until then. Therefore, I resolved to analyze again properties of the initially generated HDAC2(-/-) mutants collected at different cultivation periods as correctly as possible. Further, in particular, I should like to emphasize that we must newly generate HDAC2(-/-) mutants, collect them at different cultivation periods from virtual early (as early as possible) until later stages, and systematically and closely analyze their various cellular properties to solve the above-mentioned problems.

Then, I thought that a full-time researcher, but not a part-time one, must be absorbed in the project to clarify mechanisms for both of the accumulation and diminution of proteins and mRNAs of IgM H- and L-chains in HDAC2(-/-) mutants through numerous generations during continuous cultivation. I asked Dr. Masami Nakayama, my wife, to work with me as a colleague (postdoctoral researcher). She had worked on this project and others full-time, in spite of a part-time contract (few hours/day for several years and several hours/day for only one year). Further, another PhD joined my group as an assistant professor for collaboration on the project. This plan, however, came to a deadlock in a short time, because contrary to my expectation the new colleague did not contribute towards the achievement of this project at all. Based on the deliberations and results obtained throughout the above-mentioned processes, we had started and continued the research, which made my heart beat first with joy, even under the following worst research environments. Without any research grants, in our spare time from main research projects (supported by some small grants) and that by only a womanpower (of M. N.), we had undertaken the dreamiest study on the chromatin conformation change code (4C) theory for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations (cell divisions) [138]. At the start of this research, I had firmly resolved that my small research group (T. N. and M. N.) made some contribution (even if a sole and small concept) toward recent rapid development of the life science field, which had already become a big science, as well as the space science, experimental elementary particle physics, geophysics, gravity physics/gravitational wave astronomy and others. On the other hand, it was also true that we had to principally continue our ordinary projects (most of which were supported by some small grants) [98-102, 109-113, 115-130, 132-143] and collaborations (with other groups) [131, 144-163], similarly to previous our own plus collaborative researches [62-90, 94, 103-108, 114, 164-187] with ~20 unlisted reviews and others (in English and in Japanese), since minimum research conditions were

essential to execute the research on the 4C theory. Throughout whole research periods, of course, we had referred to numerous papers. Only about sixty selected randomly from them were listed as references [1-61, 91-93] in this article, although many of these referred papers were cited in our previous papers [62-90, 94-130, 132-143]. If I had not come across this biological phenomenon, besides my failure in formation of excellent research group, my life as a scientist must be ended in imperfect combustion. Anyhow, we had started the exciting research on the 4C theory with sickening anxiety, quiet fighting spirit and a wish that it will be a forerunner in future biology (life science) beyond Biochemistry, Molecular Biology and Cell Biology, all of which have mostly targeted bio-systems already acquired during evolution since the birth of living things, under following mottos:

Exploring only one, pursuing only it, by only one, and Creator of concept, rather than only one, rather than number one

The 4C theory, which is the fruit of my small serendipity and also the outcome of some 10 years of intellectual investigation spirit, intuition plus insight (of T. N.) and enthusiasm, outstanding technique plus diligent work (of M. N.), is a creative concept on ability to gain un-programmed and new cell functions and also life conservation of higher eukaryotes. In 2017, we published five original articles and one review article on the 4C theory [188-193], respectively, which were the revised versions of Chapters-2 - 7 of the retirement commemorative monograph published at my own expense in 2015 [138]. In 2017, in addition, we published three review articles [194-196], all of which reviewed the studies in the previous three original articles, respectively [190-192]. In 2018, we revised the above-mentioned retirement commemorative monograph published in 2015 [138] and also the above-mentioned five original and one review articles on the 4C theory published in 2017 [188-193]. In 2019, we revised and uploaded the second version [197] of the review article published in 2018 [193], and I first wrote and uploaded the memoir [198], which was a modified version of Chapter-8 of the retirement commemorative monograph published in 2018 [138]. Furthermore, in parallel with this article, we are now writing third version [202] of the review article (whose original and second versions were uploaded in 2018 and 2019 [193, 197], which will be uploaded in 2020 [202]. All of Figures 1 ~ 23 used in this article are identical with corresponding those in the original one [198], all of which were selected from the retirement commemorative monograph [138] and the above-mentioned five original articles [188-192].

In any case, because the 4C theory is the only visible and biggest evidence for me as a scientist, here, I would like to re-describe closely the 4C theory [193] by referring our recent review articles [194-196]. Based on our new results [138, 188-197] and previous results [114, 117, 119, 121, 124], we proposed an all-inclusive hypothetic way 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(-/-) DT40 mutants through numerous generations during continuous cultivation [138, 192, 193, 196, 197].

First, we supposed that environment change recognition receptor/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, i.e., the accumulation of IgM H- and L-chains occurs as follows.

First, 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. 22) [92, 98, 101]. However, the functions of both the ECRR/ECRS and the 4C machinery may be basically not necessary for DT40 cells, because the protein and mRNA levels of IgM H- and L-chains are very low as mentioned above.

The other hand, the case of HDAC2(-/-) mutants is as follows. Repeatedly, large amounts of IgM H- and L-chains artificially synthesized by their excessively increased gene expressions induced by the HDAC2-deficiency (Fig. 9) are first accumulated probably 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 [121]) are gradually secreted into the cultivation media (i.e. the outside of cells), whereas a part of them is transported to the nuclear envelope but not the inside of nucleus, and kept at the peri-nuclear space at the early and later cultivation stages (Figs. 18, 19 and 20). The excessively accumulated immunoglobulin proteins 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 existing 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 to the proximal 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and other genes in individual clones of HDAC2(-/-) mutants (Fig. 22).

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 surrounding proximal 5'-upstream regions of these transcription factor genes is preferentially attributed to varied acetylation and deacetylation levels of one or more of the five specific Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) at the N-terminal tail of histone H3 [191, 195] caused by the collaboration of proper member 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 generally, the chromatin structure surrounding 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 the chromatin structure surrounding 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 (coding regions) of these specific transcription factor genes by much [191, 192, 195-197]. As the need arises, the 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 promoter regions (or elements) within the loose (but not tight) 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 numerous generations during simple continuous cultivation under the same conditions (Fig. 10) [121, 138, 188, 190, 194]. Such distinct ways are not under the control of HDAC2 but originally based on irreversible creation of their distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation [138, 191-198, 202]. 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 more 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 gene expressions of numerous different genes, as well as those of IgM H- and L-chains at the later cultivation stage (Figs. 1 and 10). 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 [134, 138, 189]. In addition, Aiolos regulates pre-mature B cell apoptosis mediated by BCR signaling [141]. E2A regulates gene expressions of survivin, IAP2 and caspase-8 [140]. Helios regulates the gene expression of protein kinase Cs [139]. EBF1 regulates dramatically gene expressions of Blimp-1 and protein kinase C θ [129, 142]. Besides, in one or more of these six mutant clones, as a whole, respective gene expression patterns of PCAF, HDAC7, HDAC9, Ikaros and OBF1 are spontaneously and complicatedly reversed 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 [190, 194] are analyzed, there is a 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 to an abnormal, unexpected and/or unfavorable environment change in distinct ways 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 [192, 193, 196].

The above-mentioned way that HDAC2(-/-) DT40 mutants gain un-programmed and new cell function to diminish excessively accumulated IgM H- and L-chains as unnecessary materials by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations during continuous cultivation is obviously different from the so-called endoplasmic reticulum (ER) stress response/unfolded protein response and others.

We expanded the above-mentioned hypothetic way on the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetic concept on ways for gaining un-programmed and new cell functions by means of irreversible creation of varied chromatin structure plasticity surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through numerous generations (cell divisions) in higher eukaryotes.

When higher eukaryotic cells firstly encounter an intra- and/or extra-cellular environment change in their lives, they gradually acquire the ability to adapt themselves to the new environment change or

exclude it (if unnecessary, abnormal, unexpected, uncomfortable and/or painful) through various generations (cell divisions). Using the ECRR/ECRS, the 4C machinery and other components, the eukaryotic cells start 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 the intermediary sensor at this step 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 numerous generations (cell divisions). Following the initial acceptance of the putative signal(s), the 4C machinery induces a slight alteration in the chromatin structure surrounding proximal 5'-upstream regions 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 the HATs and HDACs and other factors, preferentially participates in the acetylation and deacetylation among such epigenetic modifications by rights (Fig. 21). Positions of specific Lys residues and kinds of core histones are diverse. For instance, in the above-mentioned case [191-198, 202], the acetylation and 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 target genes through numerous generations. Varied functions of the protean 4C machinery on such acetylation and 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 can not alter the chromatin structure surrounding ORF regions (coding 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 the acetylation and deacetylation levels of one or more of the 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 induce no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure (Fig. 21). Thus, chromatin structure plasticity is irreversibly created based on successive conformation changes with epigenetic modifications. These ways to gradually and tardily create chromatin structure plasticity for gaining un-programmed and new cell functions are obviously different from the ways to 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 promoter regions (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned specific 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 promoter regions (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 the ability not only to complicatedly and diversely alter the chromatin structure surrounding proximal 5'-upstream regions of various specific genes but also to separately alter 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 alter among individual cells through numerous generations, in spite of the same environment change. As a consequence, individual somatic cells of the same type are able to newly gain the same and/or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment change.

In conclusion, 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 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 the ability to newly gain the same and/or distinct un-programmed cell function(s) in different ways by means of irreversible creation of varied chromatin structure plasticity with epigenetic modifications, i.e., from the loose to tight forms or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (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. Creation of varied chromatin structure plasticity in individual somatic cells is triggered by the spontaneous unbalanced response to the new environment change when they firstly encounter with it and then irreversibly accomplished by the successive unbalanced convergence of the response through numerous generations. The different ways to create varied chromatin structure plasticity in somatic cells are 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 "director for gene expression" from a functional point of view, besides as "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 concerning the environment change may be just the steric (rope-like) chromatin structure (possessing self-reforming ability) itself 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 themselves 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. The self-reforming ability (we named here), which is based on the 4C theory to irreversibly create plasticity of three-dimensional chromatin conformation (structure) in higher eukaryotes, is obviously different from the well-known rearrangement of antibody genes and others, because these are somatic recombination (rearrangement) of DNA structures (base/nucleotide sequences) themselves. Naturally, concrete data are not enough to support these ideas. As a consequence of these complicated biological events, higher eukaryotes acquire pluri-potential, elastic and flexible ability to create diverse derivative cell types possessing varied characteristics/functions (e.g., varied component cells of diversely

differentiated organs) in distinct ways from the same type of somatic cells 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 possibly regarded as extra-cellular environment for themselves. Moreover, in higher eukaryotes diverse kinds of somatic cells share and express orderly and cooperatively vital functions with each other. As concrete examples for the maintenance of homeostasis that is one of vital functions, 1) hypothalamus synthesizes and secretes corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), luteinizing hormone-releasing hormone (LHRH), and thyrotropin-releasing hormone (TRH), 2) anterior pituitary synthesizes and secretes growth hormone (GH), luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), and 3) posterior pituitary synthesizes and secretes antidiuretic hormone (ADH), vasopressin and oxytocin (OT). In addition, 4) A (α), B (β), D (δ) and PP cells of Langerhans islet of pancreas synthesize and secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively.

Finally, as mentioned above, we named our hypothetic theory on such a bio-system that gains un-programmed and new cell function(s) by means of irreversible creation of chromatin structure plasticity with epigenetic modifications, which is one of the most fundamental and important ways for life conservation and cell type determination of higher eukaryotes, as the chromatin conformation (structure) change code (4C) theory [138, 192, 193, 196]. By the way, here, I want to briefly explain on simple concepts and molecular mechanisms of the two well-known code theories (such as the genetic code and histone code), which are closely correlated to chromatin (or DNA) functions (especially gene expression) but different from the 4C theory, because we did not mention about the two until now in this article at all. First, the genetic code is the biochemical basis of heredity consisting of codons (triplet: three consecutive base/nucleotide sequences) in DNA and mRNA that determine the specific amino acid sequences in proteins (and polypeptides) at the translation step on the ribosome. The molecular mechanism of the translation is based on that codon on mRNA summons (or is recognized and accessed by) aminoacyl-tRNA through base pair formation by hydrogen bond between the former and later ones. Needless to say, the genetic code appears to be uniform for nearly all known organisms, therefore, in life phenomena it is one of the most fundamental and important concepts, as well as the double helix structure of DNA. Second, the histone code, which is an epigenetic code as well as DNA methylation, will be explained a little more, since it is a bit like the 4C theory apparently. The histone code is a hypothesis that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications of histones within their flexible N-terminal tails protruding from the nucleosome. Because, as mentioned previously, many of the histone tail modifications participate very well in chromatin structure, and both the histone modification state and chromatin structure correlate well to gene expression levels. The critical concept of the histone code hypothesis is that the histone modifications serve to recruit other enzymes and/or factors by specific recognition of the modified histone (as a

mark/modifier) via protein domains specialized for such purposes, rather than through simply stabilizing or destabilizing the interaction between the histone and the specified DNA region. These recruited enzymes and/or factors then act to alter chromatin structure actively or to promote transcription. In other words, the molecular mechanism of the histone code is based on that histones are modified (marked) by several small molecules, and the mark summons (or is recognized and accessed by) various enzymes and/or factors, which participate in the expression of various chromatin functions (especially gene expression). Finally, I repeat the description on the 4C theory with a slight modification, the molecular mechanism of the 4C theory is based on that the plasticity of proximal 5'-upstream chromatin regions of various genes are irreversibly created by the 4C machinery through epigenetic modification with acetyl group (and others), and the loose (open) or tight (closed) form of chromatin structure summons or rejects (or is recognized and accessed by, or is reversely un-recognized and un-accessed by) various transcription factors and/or chromatin-modifying enzymes, which participate in the expression of numerous chromatin functions (including gene expression). Therefore, needless to mention again, regarding the expression of genome (chromatin) functions including the gene expression, the 4C theory obviously functions upstream both the histone code and the genetic code. Probably, the supposed number of codes in the 4C theory, which determines complicated and varied characteristics (functions) of higher eukaryotic cells, can be roughly estimated based on combination (multiplication) of the number of candidate genes and that of codes for each of these genes. The most influential candidates are various specific genes encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes, all of which are necessary for gaining varied cell functions and specificities of higher eukaryotes. The number of codes for each of these candidate genes is probably two. This is because the loose or tight form of proximal 5'-upstream chromatin region, 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 the nature of somatic cells and open the door for gaining un-programmed and new cell function(s) of higher eukaryotes. On the other hand, almost all of programmed, complicated and diverse cell functions, which were acquired through a long evolutionary process, 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.

At the time of final check of this article, I noticed some former experimental reports on changes in phenotypes of living things through enormous generations during continuous cultivation, depending on secondary sources but not original reports, which are sure to exist. Both the mutation of *E. coli* [199]

and the temperature adaptation of *Drosophila melanogaster* [200, 201] should be typical examples of these phenotypic changes. Naturally, the 4C theory is obviously different from these phenomena, which are closely related to the above-mentioned second countermeasure (as alterations in genome information) for the ways to cope with and/or overcome the environment change, although it is still unknown whether the real basis of the case of *Drosophila* is genome information change or chromatin conformation change. Moreover, the 4C theory is clearly different from the well-known phenomena on alterations in the colors of snowshoe hare's hairs and ptarmigan's feathers between summer and winter seasons and almost all of similar phenomena, because these are programmed functions responded to environment change, although the related genome information is not changed. In addition, very recently, I found the most interesting paper on the very important life phenomena that are not directly related to the ability to gain un-programed and new cell functions as follows [202]. Here, by borrowing a part of description in the new paper, with some repetitions of the above description, as added articles, I wish to describe again the remarkable life phenomena that are being rapidly researched recently. Needless to mention, differentiation of higher eukaryotes means life events that a fertilized egg differentiates into numerous cell types to make a complete multicellular organism, and is preferentially accompanied with epigenetic modification of chromatin structure, leading to both gradual loss of pluripotency and reversely acquisition of cell-type-specific functions (characteristics) [203, 204]. Epigenetic modifications act as intermediary between environment and gene expression, and thereby participate in various organismal phenomena, including homeostasis (e.g., oxidative stress). For example, among many epigenetic modifications (such as methylation, acetylation and others) of histones H2A, H2B, H3 and H4, Lys-27 of histone H3 (H3/K27) modifiers regulate systemic stress resistance (such as oxidative stress) and lifespan in *C. elegans* [205] and *D. melanogaster* [206]. In addition, the activation of a stress response in one tissue elicits sometimes a systemic stress response [207, 208], suggesting that intertissue communication is important to regulate stress resistance. Interestingly, in various species the epigenetic alteration induced by genetic manipulation and environmental trajectory can sometimes be taken over through many generations, even when the first stimulus no longer exists [209-212]. For instance, in *C. elegans* increased stress resistance induced by low-level stress exposure during development can be transmitted to the descendant generations through soma-to-germ communication [212]. Moreover, small RNAs are involved in both the responses to environmental changes and the transgenerational epigenetic inheritance [213, 214], suggesting that systemic regulation of epigenetic information by means of small RNAs is essential for transgenerational epigenetic inheritance, which induces phenotypic alteration in descendant generations. Very recently, noticeably, the intestine-to-germline communication of epigenetic states was identified to intergenerationally enhances stress resistance in *C. elegans* [202]. Concretely, the alterations in epigenetic states caused by deficiency of the histone H3K4me3 modifier ASH-2 in the intestine or germline increase organismal stress resistance abrogated by knockdown of the H3K4

demethylase RBR-2. Remarkably, the increase in stress resistance induced by the ASH-2 deficiency in the intestine is abrogated by RBR-2 knockdown in the germline, suggesting the intestine-to-germline transmission of epigenetic information. This communication from intestine to germline in the parental generation increases stress resistance in the next generation. Thus, together, these results reveal that intertissue communication of epigenetic information provides mechanisms for intergenerational regulation of systemic stress resistance, indicating that acquired characteristics (traits) of somatic cells of higher eukaryotes are intergenerationally inherited into descendant generations. Naturally, the 4C theory is obviously different from these important 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 [138, 188, 190-198, 215]. 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) In order 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 numerous generations and also in model animals (such as *C. elegans*, *Drosophila*, *Xenopus*, mice, rats and others) during the development and differentiation is very helpful and significant. Because these influences under the varied conditions can be easily studied by various research groups, judging from the fact that previous researches in *Drosophila* were already conducted, although they were not related to molecular mechanisms [198, 199].

In any case, if we have been able to study this biological subject in favorable research surroundings, some of the above-mentioned questions (which had already been presented in our monograph, original articles and review articles [138, 188-198, 215]) concerning the 4C theory on the exclusion of artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutants during cultivation must have been resolved. Hence, I earnestly crave that somebody has an interest in universal concept on the 4C theory, takes over the theory and elucidates some momentous questions on the theory including the above-mentioned ones in near future.

In connection with the 4C theory, here, I wish to briefly express my personal interpretations concerning the conception of complicated and diverse life phenomena. First, I would like to mention again the description in the beginning of this personal article with a slight modification as follows. Higher eukaryotic cells possess four possible countermeasures to cope with and/or overcome an abnormal, unexpected and disadvantageous environment change, when they firstly encounter it. 1) When cells encounter a very severe change, they die because it is far ahead of their adaptation ability. 2) When cells encounter a considerably severe change, they cope with or overcome it by means of alterations in genome information, such as point mutation, insertion, deletion, duplication and multiplication on the DNA molecule through numerous generations. 3) When cells encounter a moderate change, they cope with or overcome it by means of irreversible creation of chromatin structure plasticity caused by successive chromatin conformation changes with epigenetic modifications through numerous generations. 4) When cells encounter a minor change, they respond to it only by means of the already acquired regulation mechanism(s). As described above, IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants (and also in Pax5(-) DT40 mutants) are dramatically decreased through numerous generations during continuous cultivation. Although the above-mentioned classification is in my own way, the aforesaid remarkable biological event, which is a typical example of the most fundamental and important ways for adaptation to the environment change and life conservation of higher eukaryotes, seems to be really an example of the third case of the above-mentioned countermeasures [138, 188-198, 215].

In addition, I have a following harmless insight about the development and differentiation of higher eukaryotes, which are certainly and closely related to irreversible creation of chromatin structure

plasticity. For instance, when ovum and sperm are fertilized, all components of the chromatin (or chromosomes) (except DNA) existing in the former are generally considered to be evenly and symmetrically divided into two chromatin sets. However, in fact, an asymmetrical allotment of the chromatin components must spontaneously become in a few unbalance; i.e., two daughter cells after the first cell division of fertilized ovum have already somewhat of quantitative and qualitative differences in the constituent elements of their chromatin (chromosomes). Successively, the unbalanced distribution of the chromatin components is repeated and converged to create varied chromatin structure plasticity surrounding proximal 5'-upstream regions of different sets of numerous specific genes in individual cells through numerous cell divisions. Furthermore, such a spontaneous and asymmetrical allotment of the chromatin components at first cell division (followed by its successive convergence) universally occurs in all fertilized ova. Consequently, the complexity and diversity of chromatin structure plasticity cause those of cell specificity, i.e., distinct functions of the chromatin, nucleus and/or cell, leading to irreversible creation of variety of specific cell (or tissue) types of higher eukaryotes.

Well, genome information, which is inherited to descendant generations through cell divisions (and naturally, fertilization or pollination of generative cells), is generally preserved in nucleotide sequences of DNA and translated into amino acid sequences of protein (or polypeptide) through transcription into nucleotide sequences of RNA. In eukaryotes, as mentioned above, both genome information and nuclear function are mainly protected by the nuclear membrane that acts as a cytoplasm-nucleus barrier; naturally, there are two other typical and important barrier systems for conservation (protection) of life, e.g., a blood-brain barrier for conservation of individuals (and/or maintenance of homeostasis) in higher animals and an insemination (sperm-ovum fertilization or stamen-pistil pollination) barrier for conservation of species in higher animals or plants. Moreover, both communication and signal transduction between the nucleus and cytoplasm, which are essential for normal expressions of cell functions, are preferentially performed 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 transport of useless molecules and the signal transduction concerning an abnormal, unexpected and/or unfavorable change in the intra- and/or extra-cellular environment to the nucleus are usually shielded by this barrier system.

Hereafter, I wish to largely digress from the subject concerning researches and to mainly change it to my personal experiences, interpretations and opinions, and then to randomly mention each of these as follows. First, I organized "The 9th MBSJ Spring Symposium in Miyazaki" held on 2009 (MBSJ: The Molecular Biology Society of Japan). The theme was "New Quickening of Molecular Biology ~ Its Dawn of Daybreak from Miyazaki (in Japanese) ~". As a matter of fact, I secretly and individually included the meaning of the 4C theory, which was still immature and unpublished at that time, in the theme of the formal symposium. Anyhow, I am not a little proud of the 4C theory [138, 188-198, 215]

and our other researches concerning histones, histone chaperones, chromatin-modifying enzymes and transcription factors in the DT40 cell line by gene targeting techniques [94-130, 132-137, 139-143]. It is because all of these studies were originally started and achieved only by myself and my small group, except some technical helps, and not directly succeeded to those of any related scientists and/or groups. In particular, I have a credit for the 4C theory, a new fundamental concept on life phenomena, which was created based on a few and insufficient data in our solitary step, i.e., by a very small group (M. N. and T. N.), in spare time from our other main projects and with no grants.

Incidentally, as easily perceived by the above-mentioned facts, prior to the first paper on the iPS cells, in fact, we had started the research on the 4C theory concerning the exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation. However, while I was in active service, all of data on the 4C theory for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity could not be published as papers in Journals and presentations in meetings. Because I thought it was the minimum requirement that besides all of our results obtained by that time, more additional data should be published at the same time, in order to win recognition of scientist community. Therefore, we first published the data concerning the 4C theory as the retirement commemorative monograph at my own expense in 2015, instead of original papers in formal Journals. Further, when I retired, to my regret, nobody took over the research concerning the 4C theory. And I had to abandon all of my own old samples, finally submitted manuscripts and Experimental Notes (containing raw data, most of which had been already published), which were kept from graduate school (except some samples and almost all data of M. N. concerning the 4C theory), because I lost both the academic and private spaces to keep them. It goes without saying that my colleagues probably keep their own samples and raw data concerning our other research projects. On the occasion of my thorough retirement, for the first time, as mentioned above, I had seriously started writing of rough drafts and manuscripts for most of Chapters of the retirement commemorative monograph on the 4C theory in the form of original papers.

Meanwhile, concerning the acquisition of the pluri-potency of higher eukaryotic cells, following the iPS cells, the analogous cells triggered by simple stress of various reagent-treatments were reported in rapid succession. Nevertheless, to my great surprise, two papers on the STAP cells were splendidly and showily reported toward the end of the writing of our manuscripts of the 4C theory. However, after a little while these two papers were retracted and thereby the existence of the STAP cells came to naught. Regrettably and sorrowfully, the so-called STAP scandal was degraded itself to one of the worst injustices in the whole science world, and thereby the Japanese science world, especially the Japanese life science field, was got a violent blow. Naturally, I was afraid that the scandal must have indirect effects (such as severe criticisms of reviewers) on our research concerning the 4C theory not a little, while it has not

direct effects at all.

Anyhow, regardless of the STAP scandal, I decided to publish the retirement commemorative monograph at my own expense in the worst case as a scientist out of office at least, since I could not obtain additional data any more and thereby feared that our papers should be rejected by severe referees in the peer review process, when those are submitted. Based on these my own experiences, concerning a means for further efficient development of science, I should like to express my brief opinion as follows. First, when scientists retire thoroughly, I guess that most of them have a lot of certain but immature and unpublished data, which are taken over by nobody. In order to prevent that the enormous unquestionable data remain unpublished and thereby come to naught secretly, it is necessary for scientist community to make suitable resorts for publishing such unpublished data (of course, their reliabilities are essential) as the gemstone with a hidden potential, besides so-called formal Journals, in which mature results are published as papers and/or reports in complete forms, because open-access Journals of bad reputation rapidly go on increasing now. Although this idea may be contrary to the ordinary opinion of the community and also the general public now, especially after the historic STAP scandal and other injustices, the reason is as follows. If it is possible, in future, someone can initiate and develop new research projects using some of these authentic data just as the start line without waste of time, effort and grant, even though those are incompletely and immaturely published.

Next, I wished to change the subject to life scientist's types (or styles) having no direct connection with the 4C theory. Since old times, roughly speaking, there are three typical researcher's types (or styles) based on means of taking influential and important data in life science field as follows. The first type is a category of researchers who find new biological concepts and/or phenomena based on morphological nature observation, like C. R. Darwin. Most of primate biologists and ecologists (in fieldwork) are classified into this category. The second type is a category of researchers who find new biological concepts, phenomena, functions and/or molecules based on their own experimental results, like G. J. Mendel. Most of experimental biologists (in wet laboratory) are classified into this category. The third type is a category of researchers who find new biological concepts, phenomena, functions and/or molecules based on data (or results) of other scientist(s), like J. D. Watson and F. H. C. Crick, and M. Kimura. Most of molecular evolutionists and informatics biologists (in dry/silico laboratory) are classified into this category. Of course, I belonged thoroughly to the second category; therefore, we could not add any more data to the 4C theory after my thorough retirement. To return from the digression, I should like to emphasize once again that these facts were just the reason and/or the excuse for the above-mentioned decision, i.e., I decided to publish our data concerning the 4C theory as the retirement commemorative monograph at my own expense, followed by registration as the repository of University of Miyazaki Library, as an amateur or an old fossil-like and analog-type scientist out of office.

I should like to just mention a following word in conclusion. Future Biology that I mean must preferentially focus on the bio-system (we named the 4C theory) for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity (which should inevitably occur) rather than alterations in genome information (which should incidentally and/or neutrally occur). Because the 4C theory is a new hypothetic concept on the bio-system to cope with, overcome and/or adapt to abnormal, unexpected, disadvantageous and/or even advantageous environment changes, when higher living things firstly encounter them. As concrete approaches 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 functions through numerous generations (cell divisions) in the established cell lines and/or during the development and differentiation of model animals (such as mice, rats, *C. elegans*, *Drosophila*, *Xenopus* and others) may be very significant and helpful, because these influences under the varied conditions can be easily studied by various research groups. Consequently, the 4C theory can innovate the common concept on the nature of somatic cells and open the door for acquisition of adaptation ability of higher eukaryotes to the environment changes. It is because the bio-system (the 4C theory) to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications is one of the most fundamental and important phenomena for the life conservation and cell-type determination of higher eukaryotes.

On the occasion of closing these first and revised memoirs published in 2019 [198] and in 2020, I should like to add following postscripts. First of all, I added the following common comment on the techniques rapidly developed in life science field. For instance, because the genome editing techniques make impacts of right and wrong for mankind without fail, all of natural science, social science and the humanities must cooperatively solve the troublesome problems must occur now and in near future. In addition, I added the personal postscripts on the publication process (with repeating a part of the above descriptions) of contents concerning the 4C theory as follows. I first negotiated with Springer Japan about the publication of the retirement commemorative monograph on the 4C theory at my own expense several times. However, the publisher could not consider my proposal, probably because it did not match with the guidelines. Therefore, I asked for a small printing office in Miyazaki to publish the retirement commemorative monograph on the 4C theory at my own expense. In succession, I added the following personal postscripts on the process for publishing both the revised editions of the retirement commemorative monograph (which was first registered as the institution repository in 2015 and is available from our URL [138]) and the five original and one review articles (all of which were first registered as the institution repositories in 2017 and are available from our six URLs [188-193]) in 2018.

First, I sent the first edition of the monograph with my compliments to many Japanese scientists in the

chromatin field and other life science fields and also informed many foreign scientists in the chromatin field its URL after registration as the institution repository [138]. Moreover, I informed the above-mentioned Japanese and foreign scientists the URLs of the six first articles which were the modified versions of Chapters 2-7 of the monograph. Unfortunately, contrary to my expectation, regardless of whether comments were positive or negative, there were no responses with the exception of a slight comment. However, after a little while of both the publication of the monograph and the uploading of the repository concerning the 4C theory [138], as mentioned above, some scientists suggested that we have better to submit the contents concerning the 4C theory to certain ordinary Journals. Following their suggestions, we formally prepared the six manuscripts of the five original and one review articles [188-193], all of which were the revisions of Chapters 2-7 of the retirement commemorative monograph in a relatively simple description. Then, we successively tried to submit the revision of Chapter 2 to two open-access Journals (F1000Research and bioRxiv) as the first attempt. However, the two Journals were not able to consider our submissions, because our manuscripts did not match with their guidelines (policies), i.e., in the case of F1000Research the raw data for important findings must be submitted at the time of submission and in the case of bioRxiv the content must be unpublished, respectively. The reasons why I could not answer to their guidelines were as follows. 1) Almost all figures of the retirement commemorative monograph (and also the five original and one review articles) consisted of PCR product bands cut off from more than 5000 (including ~200 of the first paper) original photographs of gel electrophoretic patterns (i.e., raw data) of ChIP and NotchIP assays and others. Unfortunately, we were not able to provide the raw data that F1000Research means, because all of the equipment used were already production stoppage and went wrong, and also their license could not be used now; therefore, we have only printed matters and useless data files of all original gel electrophoretic patterns. 2) As mentioned above, the contents concerning the 4C theory had been already published as the retirement commemorative monograph and uploaded as the institution repository available from our URL [138]; therefore, all data in the manuscripts of the six articles were regarded as the published ones by bioRxiv. These facts strongly suggested that any ordinary Journals should not accept our revised manuscripts concerning the 4C theory. Therefore, I decided to publish the revised versions of Chapters 2-7 of the retirement commemorative monograph concerning the 4C theory as the five original and one review articles with corrections in quite some detail and to register them as the institution repositories, which are available from corresponding URLs [188-193]. Fortunately, at length we had the opportunity to publish the researches on the 4C theory (especially the researches in Refs. 190-192) as three review articles in Current Topics in Biochemical Research in 2017 [194-196]. Finally, in 2018, we revised the retirement commemorative monograph and the six articles, and thereafter registered these revisions as the institution repositories, all of which are available from the above-mentioned respective URLs [138, 188-193]. And then, in the three reviews in "Current Topics in Biochemical Research" and also in the

revised monograph and six papers, I could able to describe almost all the contents that I want to write based on our data concerning the 4C theory, even though there must be a possibility of some over-descriptions.

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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 other genes in wild-type DT40 cells or in all and 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, OBF1 and EBF1, and Ikaros and E2A. In all clones of HDAC2(-/-) mutants, at the early (E) cultivation stage, IgM H- and L-chains are excessively accumulated attributed to 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. In individual clones of HDAC2(-/-) mutants, at the later (L) cultivation stage, 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. 198. The figure is also identical with Fig. 1 of Refs. 193 plus 197 and Fig. 6 of Ref. 194 and is a set of Figs. 8, 9 and 10 of Ref. 190. These figures were first presented in Ref. 138.

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. The figure is identical with Fig. 2 of Ref. 198. The figure is also identical with Fig. 2 of Refs. 193 plus 197 and Fig. 1 of Ref. 196 and Fig. 1 of Ref. 192 and Fig. 1 of Ref. 188. These figures were first presented in Ref. 138.

Figure 3. Alterations in amounts of IgM H-chain 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 a single cell (B). Large amounts of accumulated IgM H-chain were detected only at the early (E) stage in HDAC2(-/-) mutant cells. The figure is identical with Fig. 3 of Ref. 198. The figure is also identical with Fig. 3 of Refs. 193 plus 197 and Fig. 4 of Ref. 188. These figures were first presented in Ref. 138.

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, whole (IgM Hc), secreted (IgM Hs) plus membrane-bound (IgM Hm) forms of IgM H-chain and IgM L-chain (IgM L), and core histones H2A, H2B, H3 and H4. Some data for DT40 (W) and HDAC2(-/-) at the early (E) stage were identical with those of Ref. 124. The figure is identical with Fig. 4 of Ref. 198. The figure is also identical with Fig. 4 of Refs. 193 plus 197 and Fig. 5 of Ref. 188. These figures were first presented in Ref. 138.

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. 198. The figure is also identical with Fig. 5 of Refs. 193 plus 197 and Fig. 3 of Ref. 189. These figures were first presented in Ref. 138.

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. 198. The figure is identical with Fig. 6 of Refs. 193 plus 197 and Fig. 5 of Ref. 189. These figures were first presented in Ref. 138.

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, and whole (IgM Hc), secreted (IgM Hs) plus membrane-bound (IgM Hm) forms of IgM H-chain and IgM L-chain (IgM L). Their relative mRNA levels are shown in the lower panel. The figure is identical with Fig. 7 of Ref. 198. The figure is also identical with Fig. 7 of Refs. 193 plus 197 and Fig. 6 of Ref. 189. These figures were first presented in Ref. 138.

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 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 a 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 three 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. 198. The figure is also identical with Fig. 8 of Refs. 193 plus 197 and Fig. 1 of Ref. 194 and Fig. 1 of Ref. 190. These figures were first presented in Ref. 138.

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. 198. The figure is also identical with Fig. 9 of Refs. 193 plus 197 and Fig. 2 of Ref. 196 (and Fig. 2 of Ref. 194 and Fig. 2 of Ref. 190, which were slightly modified). These figures were first presented in Ref. 138.

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 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 Ref. 96. The figure is identical with Fig. 10 of Ref. 198. The figure is also identical with Fig. 10 of Refs. 193 plus 197 and Fig. 3 of Ref. 196 (and Fig. 4 of Ref. 194 and Fig. 4 of Ref. 190, which were slightly modified). These figures were first presented in Ref. 138.

Figure 11. Alterations in amounts of IgM H-chain 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-chain 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. 198. The figure is also identical with Fig. 11 of Refs. 193 plus 197 and Fig. 4 of Ref. 190. These figures were first presented in Ref. 138.

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. The aggregative form was observed only at the early (E) stage in these four mutant clones. The figure is identical with Fig. 12 of Ref. 198. The figure is also identical with Fig. 12 of Refs. 193 plus 197 and Fig. 5 of Ref. 190. These figures were first presented in Ref. 138.

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 immunoprecipitation (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 chromatin was 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 chromatin was amplified by PCR using appropriate primers for

the indicated segments of each of Pax5, Aiolos, EBF1 and OBF1 genes. β -actin was used as a control. A portion of cell supernatants as an input was amplified once by PCR using the same primers, and PCR products were analyzed by agarose gel electrophoresis. The figure is identical with Fig. 13 of Ref. 198. The figure is also identical with Fig. 13 of Refs. 193 plus 197 and Fig. 4 of Ref. 196 and is a set of Figs. 1, 5, 9 and 13 of Ref. 191. These figures were first presented in Ref. 138.

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. 198. The figure is also identical with Fig. 14 of Refs. 193 plus 197 and Fig. 5 of Ref. 196 and is a set of Figs. 2, 6, 10 and 14 of Ref. 191. These figures were first presented in Ref. 138.

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. 198. The figure is also identical with Fig. 15 of Refs. 193 plus 197 and Fig. 6 of Ref. 196 and is a set of Figs. 3, 7, 11 and 15 of Ref. 191. These figures were first presented in Ref. 138.

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(-/-) 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.2-6 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. 198. The figure is also identical with Fig. 16 of Refs. 193 plus 197 and Fig. 7 of Ref. 196 and is a set of Figs. 4, 8, 12 and 16 of Ref. 191. These figures were first presented in Ref. 138.

Figure 17. Summary of alterations in acetylation levels of specific Lys residues (K) of histone H3, chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their gene expression levels in DT40 cells (W) and 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 (loose or tight form) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (gene expression) levels (high or low) in DT40 cells (W) and four 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. 198. The figure is also identical with Fig. 17 of Refs. 193 plus 197 and is the slightly modified version of Fig.5 of Ref. 195. These figures were first presented in Ref. 138.

Figure 18. Localization of IgM H-chain 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 stage of cultivation. A) Immuno-electron microscopy without saponin-treatment. B) ~ E) Immuno-electron microscopy with saponin-treatment. Arrows P, E and S indicate positive signals of IgM H-chain proteins localized at the peri-nuclear space, endoplasmic reticulum and cell surface, respectively. Large amounts of accumulated IgM H-chain were observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early cultivation stage. The figure is identical with Fig. 18 of Ref. 198. The figure is also identical with Fig. 18 of Refs. 193 plus 197 and Fig. 8 of Ref. 196. These figures were first presented in Ref. 138.

Figure 19. Localization of IgM H-chain 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. Large amounts of accumulated IgM H-chain were 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. 198. The figure is also identical with Fig. 19 of Refs. 193 plus 197 and Fig. 3 of Ref. 192. These figures were first

presented in Ref. 138.

Figure 20. Localization of IgM H-chain 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 on the 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 for the signal and its convergence to various specific 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. 198. The figure is also identical with Fig. 20 of Refs. 193 plus 197 and Fig. 9 of Ref. 196. These figures were first presented in Ref. 138.

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 (loose or tight form) surrounding 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. 198. The figure is also identical with Fig. 21 of Refs. 193 plus 197 and Fig. 10 of Ref. 196. These figures were first presented in Ref. 138.

Figure 22. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region(s) of specific gene(s) 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 gene(s) with epigenetic modifications occurs in descendant cells but not in initial cell that accepts environment change signal. Ac, Ac/2 and Ac/10 indicate qualitatively 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 (at transcriptional regulatory elements) and enzyme reaction reversibly occur in the cell that accepts proper signal. Ac and P indicate acetylation, phosphorylation, ADP-ribosylation and/or other chemical modifications, respectively. The figure is identical with Fig. 22 of Ref. 198. The figure is also identical with Fig. 22 of Refs. 193 plus 197 and Fig. 12 of Ref. 196. These figures were first presented in Ref. 138.

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 surrounding proximal 5'-upstream region(s) of specific gene(s) with epigenetic modifications through various generations.

Irreversible creation of chromatin structure plasticity with epigenetic modifications occurs surrounding proximal 5'-upstream region(s) (notch of chromatin) but not ORF region(s) of specific 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. 198. The figure is also identical with Fig. 23 of Refs. 193 plus 197 and Fig. 11 of Ref. 196. These figures were first presented in Ref. 138.