Insights as a scientist on life phenomena: The chromatin conformation change code (4C) theory, our researches prior to the theory, and interpretations, experiences and opinions in life science field

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Footnote

**The article is fundamentally the 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

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All figures used in the article are identical with corresponding those in the following review article: Nakayama, T. and Nakayama, T.: An all-inclusive review: Chromatin conformation change code (4C) theory on a bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations in higher eukaryotes. pp. 1-75, 2018. The review article is the revised version (the original paper was published

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Summary

We advanced the chromatin conformation 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 various 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 of 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 of 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 various generations. This mode is a basis for evolution of species. Third, in the case of a moderate change, cells cope with or overcome it by means of irreversible creation of chromatin structure plasticity caused by successive chromatin conformational (structural) changes with epigenetic modifications through various 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 through 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 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 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 various 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 new 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 ~20 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 protein (ovalbumin) acetylation and synthesis 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 my whole researches are likened to a 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]. An approach using the gene targeting techniques in DT40 cells was very advantageous for clarifying not only specific and/or limited functions in the B lymphocyte cell lineage but also fundamental and/or common phenomena in higher eukaryotic cells. I was confident that even my small group could continue any of researches using the ALV-transformed chicken DT40 cell line, although both of 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 expanded for 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 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 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 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 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 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 of 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 detailed participation of HDAC2 in regulation of gene expressions of IgM H- and L-chains, 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 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 for construction of 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 for construction of 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 for construction of 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]. 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 of 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 two forms (large and small) and their amounts 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 probe specific 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 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 were 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 5’-upstream regions of these transcription factor genes. 3) HDAC2(-/-) mutants were really collected at

vague early cultivation stage. That is, HDAC2(-/-) mutants regarded to be collected at the early stage must be actually collected 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 quickly 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 during cultivation. I asked Dr. Masami Nakayama, my wife, to work with me as a colleague. She had worked on this project and others full-time, in spite of a part-time contract. 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 various 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 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 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 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 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, furthermore, we revised the above-mentioned retirement commemorative monograph published in 2015 [138] and also the above-mentioned five original articles and one review article on the 4C theory published in 2017 [188-193]. All of Figures 1 - 23 used in this article are identical with corresponding those in the review article [193], all of which were selected from the retirement commemorative monograph [138] and the above-mentioned five original articles [188-192]. This article is the revision of Chapter-8 of the retirement commemorative monograph revised in 2018 [138].

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, 188-196] 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 during continuous cultivation [138, 192, 193, 196].

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, 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 transcription factor genes (and also 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 accumulation of IgM H- and L-chains occurs as follows. Large amounts of IgM H- and L-chains artificially created 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 (outside of cells), whereas a part of them is transported to the nuclear envelope but not 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 unfavorable environment change, the signal concerning it 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 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 wild-type DT40 cells, in which various members of HATs and HDACs are largely expressed [121, 138, 188, 190, 194], the 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes probably consists of HDAC2 as a HDAC activity, a specific HAT member (e.g., GCN5) as a HAT activity and other factors (Fig. 21) [192, 196]. On the other hand, in all of individual clones of HDAC2(-/-) mutants, at the very early stage of cultivation (just soon after their birth by gene targeting techniques), bulk conformation of the 4C machinery dramatically changes to remove or drastically reduce HAT activity (of the assumed member of HATs), attributed to the HDAC2-deficiency. Throughout the above-mentioned process and following continuous cultivation, the 4C machinery newly consists of a different member of HDACs, a specific (same or different) member of HATs and other factors, and thereby becomes varied. The diversity of alterations in the chromatin structure 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, 196]. 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 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, 191-196]. 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 various 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 surviving, 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 Cθ [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 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 of the same cell type and also the established cell line [192, 193, 196].

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

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 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 various 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 abnormal, unexpected, uncomfortable 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 various generations (cell divisions). Following the initial acceptance of the putative signal(s), the 4C theory 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 several 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 structure 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 [191-196], 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 various 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 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 that immediately and rapidly cause the chromatin conformation change for expressing programmed and ordinary cell functions.

Whenever the need arises, the TFC machinery is able to bind to promoter regions (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of 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 latently in the 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 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 are diversely altered among individual cells through various generations, in spite of the same environment change. As a consequence, individual cells of the same type are able to newly gain the same or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment 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 variety of chromatin structure plasticity in individual somatic cells is triggered by the spontaneous unbalanced response to the environment change when they firstly encounter with it and then irreversibly accomplished by the successive unbalanced convergence of the response through various generations. The different ways to create varied chromatin structure plasticity in somatic cells are certainly dependent on their antecedents. Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, is inherited to descendant generations associated with or without additional conformation change through cell divisions. Remarkably, irreversible creation of chromatin structure plasticity occurs in descendant cells but not in the cell which initially accepts the signal on the environment change, although reversible regulations of ordinary gene expressions and enzyme reactions occur in the cell itself which accepts proper signal (Fig. 22). Probably, irreversible creation of chromatin structure plasticity, with the intention of adapting to the environment change, occurs inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as the loose or tight form) directs the switch (on or off) for latent transcription ability of the corresponding gene by means of irreversible creation of chromatin structure plasticity; therefore, the 5'-upstream chromatin region can be regarded as "director" from a

functional point of view, besides as "notch" 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 is just the chromatin structure (possessing self-reformation ability) itself as three-dimensional conformation, which is dynamic and changeable (between the loose and tight forms), but not mere the chromosomes and 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 through its irreversible chromatin conformation change that is responsive to the signal. Naturally, concrete data are not enough to support these ideas. As a consequence of these complicated biological events, higher eukaryotes acquire pluri-potential, elastic and flexible ability to create diverse derivative cell types possessing varied characteristics (functions) in distinct ways from the same type of somatic cells, in order to accommodate themselves to new intra- and/or extra-cellular environment. In some cases, even neighboring cells derived from the same cell type are probably regarded as extra-cellular environment for themselves. Moreover, in higher eukaryotes diverse kinds of somatic cells share and express orderly and cooperatively vital functions with each other.

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]. 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 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 open the door for gaining un-programmed and new cell function(s) of higher eukaryotes and innovate the general notion on the nature of somatic cells. On the other hand, programmed, complicated and diverse cell functions are orderly and systematically expressed throughout their development and differentiation. However, the 4C theory is suitable as an

explanation for the development and differentiation (and also so-called robustness) of higher eukaryotes, because action of putative signal(s) concerning environment changes seems to fairly resemble that of certain players (such as hormone, cytokine, nerve-transmission substance, etc.), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

There are several crucial questions to be clarified in the 4C theory on the exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [138, 188, 190-196]. 1) Despite the HDAC2-deficiency, why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes decrease at the early stage of cultivation in HDAC2(-/-) mutants. 2) Why do the decreased acetylation levels of one or more of the specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these three genes increase during cultivation. 3) Why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding the proximal 5'-upstrean region of the OBF1 gene decrease during cultivation. 4) Which Lys residue(s) of K9, K14, K18, K23 and K27 of histone H3 is really and/or mainly involved in its binding to DNA within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes must be determined. 5) Functional and steric differences between the loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified more precisely. 6) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor gene during cultivation differ among individual mutant clones. 7) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor genes during cultivation differ within individual mutant clone. 8) How does the 4C machinery (which acts in the 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 various generations and 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.

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, 188-196]) 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 various 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 various 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 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, 188-196].

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 various 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 of 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 of 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 of 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 and opinions as follows. 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 proud of the 4C theory [138, 188-196] 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 only 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 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 been 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 stead 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, I had seriously started writing of rough drafts and manuscripts for 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, in the worst case, I decided to publish the retirement commemorative monograph at my own expense 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 huge unquestionable data

remain unpublished and then 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 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 (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 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 at least, 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), in order to adapt to, cope with and/or overcome 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 various 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 open the door for acquisition of adaptation ability of higher eukaryotes to the environment changes and thereby innovate the common concept on the nature of somatic cells. 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 this finally revised article in 2018-2019, I should like to add following postscripts. First, 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 following personal postscripts (with a part of the repeating of the above descriptions) on the process for publishing both of 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]. 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 the publication of both of the monograph and 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 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, as a first attempt 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 the description had the fault of over-minuteness.

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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 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 OBF-1-dependent type, clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be the Pax5- and Aiolos-dependent type, and clone cl.2-6 seems to be the Pax5-, Aiolos- and EBF1-dependent type. The figure is identical with Fig. 1 of Ref. 193 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. 193 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). A large amount 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. 193 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. 193 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. 193 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. 193 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. 193 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(-/-) mutants during continuous cultivation.

A) Schematic presentation of chicken HDAC2 genomic locus (top) with enlarged drawing of its intact allele (middle) and targeted alleles (two bottoms). Locations of exons 1-16, drug resistance cassettes

and probe HDAC2 are indicated by solid boxes, white boxes and 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. 193 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. 193 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. 193 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. 193 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. 193 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 immuno-precipitation (NotchIP) assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and from DT40 (W), and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for the indicated segments of each of Pax5, Aiolos, EBF1 and OBF1 genes. 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. 193 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. 193 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. 193 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. 193 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(-/-) ~~DT40~~ 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. 193 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. A large amount 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. 193 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. A large amount 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. 193 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 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. 193 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. 193 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 regions 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. 193 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. 193 and Fig. 11 of Ref. 196. These figures were first presented in Ref. 138.