# 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 numerous generations (3rd Edition) [**]

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**Abstract**

We report the ability of higher eukaryotes to gain un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity through numerous generations (cell divisions). Following the exclusion of excessively accumulated IgM H- and L-chains in histone deacetylase-2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) as a concrete case, we propose a universal hypothetic concept on the ability of higher eukaryotic cells to adapt to abnormal and uncomfortable environment changes. When higher eukaryotic cells firstly encounter moderate intra- and/or extra-cellular environment change, they newly acquire ability to cope with and/or overcome it by means of irreversible creation of the varied chromatin structure plasticity surrounding proximal 5'-upstream regions of the specific transcription factor and/or chromatin-modifying enzyme genes with epigenetic modifications through numerous generations. Putative environment change recognition receptor/site (ECRR/ECRS) localized nearby the nuclear membrane as a nucleus-cytoplasm barrier recognizes the new environment change. Putative chromatin conformation change complex (4C) machinery irreversibly and directly creates the varied chromatin structure plasticity of proximal 5'-upstream regions of the above-mentioned specific factor and/or enzyme genes in distinct ways in individual cells of the same type. We advance a chromatin conformation change code (4C) theory for a bio-system to gain un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity through numerous generations, in order to adapt to the new environment change.

Outline of the 4C theory, which is one of the most fundamental and important ways for life conservation and cell type determination of higher eukaryotes, is concretely as follows. 1) Somatic cells of higher eukaryotes possess the pluri-potency, elasticity and flexibility, which are basically originated from those of the chromatin structure, for gaining un-programmed and new cell functions, in order to cope with and/or overcome new environment change, when they firstly encounter with it in their lives. 2) Somatic cells gradually acquire the ability to gain un-programmed and new cell functions, in order to adapt to and/or eliminate the environment change by means of irreversible creation of chromatin structure plasticity surrounding the proximal 5'-upstream regions of specific transcription factor and/or chromatin-modifying enzyme genes through numerous generations. 3) Chromatin structure plasticity (from the tight form to loose form or vice versa) is continuously and irreversibly created based on the chromatin conformation change with epigenetic modifications through numerous generations. 4) Diversity of chromatin structure plasticity in individual cells of the same type is triggered by spontaneous unbalanced response to the environment change and accomplished by its successive convergence through numerous generations. 5) Irreversible creation of chromatin structure plasticity depends on both antecedents of somatic cells and successive response to the environment change, and occurs in descendent cells but not in the cell which initially meets with the environment change. 6) Irreversible creation of chromatin structure plasticity probably occurs inevitably but not incidentally and/or neutrally.

7) The chromatin structure of the proximal 5'-upstream region(s) of the specific gene(s), as dynamic and changeable three-dimensional conformation, receives the signal on the environment change. 8) The chromatin structure (the loose or tight form) of the proximal 5'-upstream region of the specific gene directs the switch (on or off) for its latent gene expression ability. 9) The supposed number of codes in the 4C theory, which determines both complicated cell functions and diverse cell types of higher eukaryotes, may be estimated based on combination of the number of candidate genes and that (probably two) of codes for each of these specific candidate genes. 10) The 4C theory is suitable as a powerful explanation for the ways to control development and differentiation of higher eukaryotes.

**Keywords**

Chromatin conformation change code (4C) theory, HDAC2-deficient DT40 mutants, gain of un-programmed and new cell functions, irreversible creation of chromatin structure plasticity, epigenetic modifications, pluri-potency, elasticity and flexibility of somatic cells, numerous generations, immuno-electron microscopy study, environment change recognition receptor/site (ECRR/ECRS), chromatin conformation change complex (4C) machinery, localization of IgM H- and L-chains in peri-nuclear space, decreases in IgM H- and L-chain protein and mRNA levels, changes in mRNA levels of Pax5, Aiolos, EBF1 and OBF1, changes in conformations of proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes, alterations in acetylation and deacetylation levels of K9/H3, K14/H3, K19/H3, K23/H3 and K27/H3

**Introduction**

In eukaryotes, genome information and nuclear function should be mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Additionally, both the communication and the signal transduction between the nucleus and cytoplasm, which are essential for expressions of normal cell functions, are preferentially carried out by the to and fro passage of large and/or small molecules via the nuclear pore that acts as a guard station at the barrier. By contrast, both the signal transduction concerning an abnormal, unexpected and/or disadvantageous change in the intra- and/or extra-cellular environment and the transport of useless molecules to the nucleus are usually prevented by this barrier system. Then, how do the higher eukaryotic cells cope with and/or overcome an abnormal, unexpected and/or disadvantageous environment change, when they firstly encounter it? Generally, this is possible by adhering to four typical countermeasures as follows. 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 the genome information change (mutation), such as point mutation, insertion, deletion, duplication and multiplication in the DNA molecule through numerous generations. This mode is a basis for the evolution of species. Needless to say, the mode

occurs in all of organisms including prokaryotes and eukaryotes and also virus. Third, in the case of a moderate change, cells cope with or overcome it by means of irreversible creation of the 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 simply respond to it by means of the already acquired regulation mechanisms.

Since chemical modifications of histones with acetyl and methyl groups were first proposed to regulate RNA synthesis [1], the modulation of chromatin topology has been thought to be one of the most fundamental and important events for the expression of normal cell functions in eukaryotes. The ways to modulate the chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation 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) catalyzed by chromatin-modifying enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), are undoubtedly major ones. For the past several decades, countless numbers of researches 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, development, differentiation, memory, pluri-potency, clinical medicine, etc. [2-28].

Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [29, 30], in order to know in vivo roles of specific members of HDACs, HATs, transcription factors, histones and histone chaperones in the above-mentioned biological events, we have systematically generated a number of homozygous DT40 mutants: HDAC2(-/-), EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), respectively, devoid of HDAC2, EBF1, Aiolos, E2A and Helios genes, and Pax5-deficient DT40 mutant Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens, and many other mutants [31-45]. Systematic analyses of resultant DT40 mutants HDAC2(-/-), Aiolos(-/-), Pax5(-), EBF1(-/-), Helios(-/-) and E2A(-/-) have revealed their interesting participations in gene expressions of IgM H- and L-chains as follows. In wild-type DT40 cells, HDAC2 as a supervisor indirectly regulates gene expressions of IgM H- and L-chains through opposite control of those of Pax5, Aiolos plus EBF1, and E2A plus Ikaros [31, 35]. Furthermore, in HDAC2(-/-), IgM H- and L-chains are excessively accumulated based on their dramatically increased gene expressions caused by drastic decreases in gene expressions of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of the two immunoglobulin proteins [31, 33, 35]. On the other hand, OBF1 is strongly suggested to up-regulate gene expressions of IgM H- and L-chains [46].

Very recently, we reported following remarkable and important phenomena by analyzing initially generated HDAC2-deficient mutants, Pax5-deficient mutants and secondly generated HDAC2-deficient mutants, all of which were continuously cultivated for varyingly long periods [47-53]. At the outset, surprisingly, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of the initially generated HDAC2(-/-) mutants revealed not only that the IgM H- and L-chains are dramatically increased at the early stage of cultivation, but also that the two accumulated immunoglobulin proteins are gradually decreased through the middle stage and at the later cultivation stage reach comparable levels as in DT40 cells [47, 48]. Successively, by making full use of reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, immunoblotting and immuno-electron microscopy, we confirmed the above-mentioned results and further revealed the important, momentous and amazing phenomena as follows [47, 50-53]. In individual clones of secondly generated HDAC2(-/-) mutants, the accumulated IgM H- and L-chains at the early cultivation stage are dramatically decreased, attributed to their drastically decreased gene expressions in almost the same changing pattern, which was caused by dramatic increases or decreases in gene expressions of Pax5, Aiolos and EBF1 or OBF1 in distinct ways during cultivation [47, 50]. These results obtained from wild-type DT40 cells and HDAC2(-/-) mutants at the early and later cultivation stages are schematically shown in Supplementary Figure S1. Such diminutions of the accumulated IgM H- and L-chains in all individual clones of HDAC2(-/-) mutants during cultivation are really examples of the third case of the above-mentioned countermeasures. Presumably, the way for diminutions of IgM H- and L-chains accumulated in Pax5-deficient mutant cells Pax5(-) during cultivation moderately resembles the case in HDAC2(-/-) mutant cells as a whole, although detailed molecular mechanisms to decrease gene expressions of the two immunoglobulin proteins should be distinct between these two mutant cell lines [47-50]. In addition, acetylation levels of specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within the chromatin surrounding proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes change in distinct ways in all individual clones of HDAC2(-/-) mutants during continuous cultivation [47, 51]. Moreover, we first noticed remarkable and important facts that a part of excessively accumulated IgM H- and L-chains is transported to nuclear envelope and kept at peri-nuclear space of initially generated HDAC2(-/-) DT40 mutant cells during continuous cultivation by immuno-electron microscopy study (details will be discussed later) [52].

In this study, based on these previously and newly obtained results from HDAC2(-/-) mutant cells, as a concrete case, we first presented a hypothetic concept (way) to diminish IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants through numerous generations during continuous cultivation. Successively, we expanded the above-mentioned hypothetic way to a universal hypothetic concept on the ability of higher eukaryotes for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity through numerous generations, in order to adapt

to an unexpected, abnormal and/or uncomfortable environment change. We named the hypothetic concept as the chromatin conformation (structure) change code (4C) theory [47, 52, 53].

## Methods

### Electron microscopy and immuno-electron microscopy

Electron microscopy and immuno-electron microscopy (using anti-chicken IgM H-chain antiserum as primary antibody) were carried out on wild-type DT40 cells and initially generated HDAC2(-/-) mutants stocked at -80 °C [31, 35] in some different methods essentially as described [47-54]. For instance, immuno-electron microscopy using pre-embedding staining procedure was done as follows. Exponentially growing HDAC2(-/-) mutant cells at the early (E; ~10 days) and later (L; ~60 days) cultivation stages and DT40 cells (W) were suspended in 1% bovine serum albumin (BSA) and collected by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 ˚C for 60 min. After washing with PB, the cell pellets were treated with or without 0.2% saponin in phosphate buffered saline (PBS) for 30 min to be permeable or non-permeable. After washing with PBS. the cell pellets were incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1000 with 1% saponin in PBS) at 4 ˚C for overnight. After washing with PBS, the cell pellets were incubated with horseradish peroxidase (HRP)-labeled affinity-purified F(ab')2 fragment Donkey anti-goat IgG(H+L) (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA; diluted 1:1000 in PBS) at 4 ˚C for overnight. After washing with PBS, the cell pellets were fixed with 1% glutaraldehyde in PBS at 4 ˚C for 5 min. After washing with 50 mM Tris-HCl buffer (pH 7.4) (TB), the cell pellets were incubated in 0.05% 3', 3'-diaminobenzidine (DAB) in TB for 20 min and in complete DAB solution containing 0.005% $H_2O_2$ for 10 min. The cell pellets were post-fixed with 1% osmium tetroxide in TB containing 1% potassium ferrocyanide for 30 min, dehydrated with a series of ethanol and embedded in epoxy resin mixture. Ultrathin sections were contrasted with lead citrate for 20 sec and examined with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan).

Immunocytochemistry using immuno-gold labeling was carried out as described [54]. Exponentially growing DT40 (W) and HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages were collected by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PB for 30 min. After washing with PB, the cells were post-fixed with 1% osmium tetroxide in PB for 60 min, washed with PB, dehydrated in ethanol and embedded in Epon. Ultrathin sections were picked up on 200-mesh gold grids coated with Formvar film and treated with 5% sodium meta-periodate in distilled water for 30 min. After rinsing in distilled water, the sections were treated with 5% normal horse serum (NHS) and 1% BSA in PBS for 10 min to block nonspecific binding and incubated with goat anti-chicken IgM H-chain antibody A30-102A (diluted 1:1500 with 5% NHS, 1%

BSA in PBS) at room temperature for 60 min. After rinsing in PBS, the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min. After washing with PBS, the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min. After washing with distilled water and drying, the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80 kV (JEOL, Tokyo, Japan). Primary antibody was omitted or replaced by normal goat serum as a control.

**Results**

**IgM H- and L-chains excessively accumulated in HDAC2(-/-) DT40 mutants are dramatically decreased in distinct ways in individual mutant clones through numerous generations during continuous cultivation**

Our new findings and previous results [31, 35, 47-51] are as follows. The HDAC2-deficiency in DT40 cells rapidly and dramatically increases mRNA (i.e., transcription/gene expression) levels of IgM H- and L-chains, followed by accumulation of the two immunoglobulin proteins probably within endoplasmic reticulum (Figs. 1, 2 and 3, and Suppl. Figs. S2 and S3). Surprisingly, excessively increased mRNAs and proteins of IgM H- and L-chains at the early stage of cultivation are gradually decreased until the later cultivation stage in almost similar pattern in all examined individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of secondly generated HDAC2(-/-) mutants (Suppl. Figs. S4 and S5). By contrast, remarkably, gene expressions of various specific transcription factors and chromatin-modifying enzymes change in distinct patterns among these individual HDAC2(-/-) mutant clones (Suppl. Fig. S5). To reduce the increased protein levels of IgM H- and L-chains resulted from their increased gene expressions, apparently, three distinct ways based on alterations in gene expressions of specific transcription factors exist at the later cultivation stage in the six individual mutant clones as follows (Suppl. Figs. S1(L) and S5) [50]. The way in clone cl.2-1 seems to be dependent on OBF1 and different from that in DT40 cells. The ways in clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be dependent on Pax5 plus Aiolos and slightly similar to that in DT40 cells in appearance. The way in clone cl.2-6 seems to be dependent on Pax5, Aiolos plus EBF1 and similar to that in DT40 cells in appearance.

**Fundamental ways to irreversibly create the chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gaining new cell functions to diminish IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through numerous generations during continuous cultivation**

To clarify these three distinct ways in further detail, we performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP: this abbreviation also means IP on notch of chromatin) assay on the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of Pax5, Aiolos, EBF1 and OBF1 genes (Suppl. Figs. S6, S7, S8 and S9) [47, 51]. We tentatively and qualitatively deduced the binding ability (capacity) of histone H3 to DNA based on acetylation levels of one or more of the specific Lys residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3). That is, hyper- (high), considerably hyper-, somewhat hyper- and hypo- (low or no) acetylation levels of one or more of these five Lys residues should qualitatively induce no, weak, less and full binding ability of histone H3 (or corresponding Lys residue(s)) to DNA, resulting in the loose (open), considerably loose, somewhat loose and tight (closed) forms of the chromatin structure, leading to high, considerably high, somewhat high and low (or no) mRNA (i.e., transcription/gene expression) levels of corresponding gene(s). The results obtained by the NotchIP assay revealed that such distinct ways should be fundamentally originating from irreversible creation of the varied chromatin structure plasticity surrounding proximal ~2.0 kb 5'-upstream regions of corresponding transcription factor genes with epigenetic modifications through numerous generations during cultivation. To put it concretely, in wild-type DT40 cells having HDAC2 activity, the chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of four genes encoding Pax5, Aiolos and EBF1 (which down-regulate gene expressions of IgM H- and L-chains [35, 47, 50]) and OBF1 (which probably up-regulates those of the two immunoglobulin proteins [46, 50]) is in the loose (open) form due to no binding ability of histone H3 to DNA based on hyper- (high) acetylation levels of one or more of the specific Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) [51]. Therefore, these four transcription factor genes are transcribed at high levels in DT40 cells [35, 47, 48, 50]. On the other hand, in all individual clones of HDAC2(-/-) mutants having no HDAC2 activity, the chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of these four factor genes changes dramatically and severally based on varied acetylation levels of one or more of the five specific Lys residues of histone H3 during cultivation, resulting in alterations in their gene expression levels as follows [47, 48, 50, 51].

In clone cl.2-1, at the early stage of cultivation, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and EBF1 genes is in the tight (closed) form due to the binding ability of histone H3 to DNA based on hypo- (low or no) acetylation levels of one or more of the five specific Lys residues (Suppl. Fig. S6), and as a result, transcriptions of these three genes are almost completely suppressed to undetectable levels (Suppl. Fig. S5). However, that of the OBF1 gene is in the somewhat loose form due to the less binding ability of histone H3 to DNA based on slight (or considerably decreased) hyper-acetylation levels, thereby its transcription is slightly decreased. By contrast, at the later cultivation stage, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes remains in the tight form due to the binding ability of histone H3

to DNA based on hypo-acetylation levels; therefore, their transcriptions also remain unchanged at an undetectable level. On the other hand, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene changes to the tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, its transcription is suppressed to almost an undetectable or very low level. These results supported the above-mentioned inference (i.e., OBF1-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clone cl.2-1.

In clones cl.2-2 and cl.2-4, at the early stage of cultivation, as in clone cl.2-1, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose (or less tight) form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Suppl. Figs. S7 and S8). Therefore, transcriptions of the first three genes are almost completely suppressed and that of the last one gene is certainly decreased (Suppl. Fig. S5). At the later cultivation stage, contrary to this, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their transcriptions are dramatically increased. However, the chromatin structure of the EBF1 gene remains in the tight form based on hypo-acetylation levels, and its transcription remains low at almost undetectable level. These results supported the above-mentioned inference (i.e., Pax5- and Aiolos-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clones cl.2-2 and cl.2-4 (and also cl.2-3 and cl.2-5). Moreover, these four clones should be the major types, since they resemble in several cellular characteristics to four initially generated HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) [31, 35, 47, 48, 50].

In clone cl.2-6, at the early stage of cultivation, as in clones cl.2-1, cl.2-2 and cl.2-4, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Suppl. Fig. S9). Therefore, transcriptions of the first three genes are almost completely suppressed and that of the last one gene is certainly decreased (Suppl. Fig. S5). By contrast, at the later cultivation stage, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their transcriptions are dramatically increased. These results supported the above-mentioned inference (i.e., Pax5-, Aiolos- and EBF1-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clone cl.2-6.

Results on alterations in acetylation levels (hyper or hypo) of the five specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), the form (loose or tight) of the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their

mRNA (transcription/gene expression) levels (high or low) during cultivation in individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 [47, 50, 51] are roughly and schematically presented in Supplementary Figure S10. Concerning the above-mentioned results on the suppression of excessive gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation, there are some important comments as follows. Excessively accumulated IgM H- and L-chains resulting from their dramatically increased gene expressions in HDAC2(-/-) mutants just after their birth (by gene targeting techniques) [31, 35, 47, 48, 50] are probably unfavorable and useless for them; therefore, the mutant cells have come to acquire a new ability to diminish a large amount of the two immunoglobulin proteins as an abnormal and uncomfortable environment change through numerous generations during cultivation. The ways to suppress gene expressions of IgM H- and L-chains at the later cultivation stage in all of HDAC2(-/-) mutant clones, even in clone cl.2-6 [47, 50], are surely distinct from the ordinary and reversible way to regulate their gene expressions in wild-type DT40 cells. Because in DT40 cells gene expressions of IgM H- and L-chains are indirectly regulated by HDAC2 through opposite regulations of Pax5, Aiolos, EBF1, OBF1, and E2A gene expressions [35, 37]. In addition, gene expressions of various transcription factors and chromatin-modifying enzymes (i.e., Blimp1, PCAF, HDAC7, HDAC9, etc.), besides those of Pax5, Aiolos, EBF1 and OBF1, dramatically and separately change in individual HDAC2(-/-) mutant clones during cultivation (Suppl. Fig. S5) [47, 48, 50]. Therefore, remarkably, besides the alterations in gene expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and in cell morphology [47, 48, 50, 52, 53], some other unknown important cellular characteristics might be obviously changing among individual HDAC2(-/-) mutant clones during cultivation. Such presumable changeable characteristics may be complicated and diverse in individual clones of HDAC2(-/-) mutants, even though they are originally and completely the same cell type.

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

Very recently, besides the above-mentioned numerous important results, we first noticed following remarkable facts from the previously obtained findings on the initially generated HDAC2(-/-) DT40 mutant cells by means of immuno-electron microscopy study. Large amounts of IgM H- and L-chains artificially synthesized by their excessively increased gene expressions induced by the HDAC2-deficiency (Suppl. Figs. S4 and S5) are first accumulated probably within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figs. 1, 2, 3 and 4, and Suppl. Figs. S2 and S3) [35, 47, 48, 50]. Most of the accumulated IgM H- and L-chains (which probably exist as a high-molecular weight complex) [35]) are gradually secreted to outside of cells (i.e., into the cultivation media), whereas, as indicated by immuno-electron microscopy a part of them is transported to the nuclear envelope but not

inside of the nucleus, and kept at the peri-nuclear space at the early and later cultivation stages (Figs. 1, 2, 3 and 4) [47, 48, 50]. This inference should be surely supported by other results obtained from immuno-electron microscopy study [47, 48, 50].

**Proposed ways for gaining un-programmed and new cell functions 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**

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

Based on the morphological but insufficient findings, together with previous results mentioned above and others [31, 33, 35, 37, 46-51], we slightly revised our previous hypothesis [50] and proposed an all-inclusive hypothetic ways for gaining un-programmed and new cell functions to diminish artificially accumulated IgM H- and L-chains by means of irreversible creation of the varied chromatin structure plasticity of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications through numerous generations during continuous cultivation in individual clones of HDAC2(-/-) mutants [47, 52].

First, we assumed the environment change recognition receptor/site (ECRR/ECRS), which participates in the recognition of accumulation of IgM H- and L-chains as an abnormal and unfavorable environment change (and probably acts in part in the signal transduction concerning the IgM H- and L-chain accumulation to the chromatin structure) (Fig. 4). In addition, the 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 the chromatin structure plasticity surrounding proximal 5'-upstream regions of the above-mentioned specific transcription factor genes (and also to act in part in the signal transduction) (Figs. 4 and 5). 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.

First, in wild-type DT40 cells, in which various members of HATs and HDACs are largely expressed [35, 50], the 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes probably contains HDAC2 as a HDAC activity, a specific HAT member (e.g., GCN5) as a HAT activity and other factors (Fig. 5). On the other hand, in all of the 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 comes to newly consist of a different member of HDACs, a specific (same or different) member of HATs and other factors, and thereby becomes varied.

The excessively accumulated IgM H- and L-chains lying in the peri-nuclear space bind to the ECRR/ECRS localized at the inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 4). 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 environment change is consecutively and separately converged to the proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1, OBF1 and other genes in individual clones of HDAC2(-/-) mutants.

The diversity of alterations in the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned specific Pax5, Aiolos, EBF1 and OBF1 genes is preferentially attributed to varied acetylation and deacetylation levels of one or more of the five specific Lys residues at N-terminal tail of histone H3 [50] caused by the collaboration of proper members of HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications of K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3) with acetyl group lead to irreversible creation of the distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of the Pax5, Aiolos, EBF1 and OBF1 genes (Suppl. Figs. S6, S7, S8, S9 and S10, and Fig. 6). In general, 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 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 form based on its binding ability to DNA (Fig. 6). By contrast, probably, the 4C machinery cannot change the chromatin structure surrounding open reading frames (ORF: coding regions) of these target genes by much [51-53]. Thus, as the need arises, transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain member of each 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 these target (but not un-target) genes (i.e., Pax5, Aiolos, EBF1 and OBF1 genes) (which have become latently active (but not inactive) state), and thereby initiates their gene expressions (Figs. 5 and 6). As a concrete result, individual HDAC2(-/-) mutant clones 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 (Suppl. Fig. S5) [35, 47, 48, 50]. Naturally, 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 [51].

Remarkably, the six tested individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants can be clearly classified into the above-mentioned three different cell types exhibiting distinct functions, because they show three dissimilar ways for gene expressions of transcription factors and chromatin-modifying enzymes (such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC9 and others) at the later cultivation stage (Suppl. Figs. S1 and S5). Besides, in one or more of these six mutant clones, as a whole, 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, there is a great possibility that additional different cell types might exist, if other individual mutant clones obtained [50, 52, 54] are analyzed. Consequently, individual clones of HDAC2(-/-) mutants acquire flexible, elastic and pluri-potential ability not only to adapt in distinct ways to an environment change but also to branch off into diverse derivative cell types, which may exhibit varied characteristics (functions), even though they are originally the same cell type and also the established cell line.

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

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 way for gaining un-programmed and new cell function(s) by means of irreversible creation of the varied chromatin structure plasticity of proximal 5'-upatream regions of specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications though numerous generations (cell divisions) in higher eukaryotes [47, 52, 53].

When higher eukaryotic cells firstly encounter the change in an intra- and/or extra-cellular

environment in their lives, in order to adapt to or eliminate the change (if abnormal and/or uncomfortable), they gradually gain un-programmed and new cell function(s) through numerous generations. Namely, the cells acquire the ability to adapt themselves to newly encountered environment change and/or to exclude the painful environment change. Using the ECRR/ECRS, the 4C machinery and other components, the eukaryotic cells create a chain reaction of response to the environment change as follows (Figs. 4 and 5). 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. 4). There is a possibility that putative specific molecule(s) acts as an intermediary sensor at this step in the way to recognize the new environment change. Naturally, there is certainly another possibility that ECRR/ECRS should be newly created through gathering of its components based on the fluid mosaic model of membrane, if it consists of them. As a next step, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin structure within the nucleus through numerous generations (cell divisions). The initial acceptance of putative signal(s) may induce a slight alteration in the chromatin structure of numerous 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 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 major ones.

The 4C machinery, which consists of a specific member of the HATs and HDACs and other factors, preferentially participates in the acetylation and deacetylation among such epigenetic modifications (Fig. 5). Participating positions of specific Lys residues and kinds of core histones are diverse. For instance, in the above-mentioned case [51], 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 Lys residues of histone H3 with acetyl group change within the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned specific target genes through numerous generations. Distinct functions of the protean 4C machinery on such acetylation and deacetylation levels are mainly based on different 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 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. Namely, hyper- (high) or hypo- (low or no) acetylation levels induces no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure (Fig. 5). Thus, the chromatin structure plasticity is irreversibly created based on successive conformation changes with epigenetic modifications. These ways to gradually and tardily create the chromatin structure plasticity for gaining un-programmed and new cell function(s) are obviously different from those that immediately and rapidly cause the chromatin conformation change for expressing programmed and ordinary cell function(s).

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. 5). 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. 6). Notably, there is a great possibility that the ways for irreversible creation of the chromatin structure plasticity are distinct among individual cells of the same type, even though the new environment change and signal(s) on it are the same for all of them. That is, in order to gain un-programmed and new cell function(s), individual cells possess ability not only to complicatedly and diversely change the chromatin structure surrounding proximal 5'-upstream regions of various specific genes but also to separately change the chromatin structure of the same proximal 5'-upstream region of a certain gene into varied forms. Thus, gene expressions of the specific transcription factors and chromatin-modifying enzymes change diversely among individual cells through numerous generations, in spite of the same environment change. As a consequence, individual cells of the same type are able to newly gain the same and/or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment change.

**Conclusions and Discussion**

In conclusion, when higher eukaryotes first encounter abnormal, unexpected, unfavorable, disadvantageous and/or even advantageous environment changes in their lives, they acquire the noteworthy ability, i.e., the bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity, in order to cope with, overcome and/or adapt to these environment changes [47-53, 55-57]. As a base of the way to gain un-programmed and new cell functions, somatic cells (and even tumor cells) of higher eukaryotes become pluri-potent, elastic and

flexible, all of which are basically originated from pluri-potency, elasticity and flexibility of the chromatin structure. That is, in order to adapt to an intra- and/or extra-cellular environment change, individual somatic cells of higher eukaryotes possess the ability to newly gain the same and/or distinct un-programmed cell function(s) in different ways by means of irreversible creation of the varied chromatin structure plasticity with epigenetic modifications, i.e., from the loose form to tight form or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (Fig. 6). Such a loose or tight form of the chromatin structure surrounding the proximal 5'-upstream region is in the latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences is in the silent state for expressions of most of the genome functions. The creation of the varied 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 numerous generations. Naturally, the different ways to create the varied chromatin structure plasticity in somatic cells are certainly dependent on their antecedents. Moreover, the 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 structural change through cell divisions. Thus, remarkably, irreversible creation of the 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. 7). Probably, irreversible creation of the 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 the chromatin structure plasticity; therefore, besides as a "notch" from a structural point of view as mentioned above, the 5'-upstream chromatin region can be regarded as a "director" from a functional point of view. Naturally, 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 the self-reformation ability itself as three-dimensional conformation, which is dynamic and changeable between the loose and tight forms, but not merely the chromatin and chromosomes themselves as one- (or 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 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, although concrete data are not enough to support these ideas so far. 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. Naturally, in some cases, even neighboring cells derived from the same cell type are probably regarded as extra-cellular environment for themselves. Moreover, in higher eukaryotes diverse kinds of somatic cells share and express cooperatively vital functions with each other.

Finally, we name our theory on such a bio-system that gains un-programmed and new cell function(s) by means of irreversible creation of the 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 [47, 50-53, 55-57]. Probably, the supposed number of codes in the 4C theory, which determines complicated and varied characteristics (functions) of higher eukaryotic cells, can be roughly estimated based on the combination (multiplication) of the number of candidate genes and that of codes for each of these genes as follows. 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. Because the loose or tight form of the 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 nature of somatic cells. On the other hand, programmed, complicated and diverse cell functions are orderly and systematically expressed throughout their development and differentiation. Nevertheless, the 4C theory is suitable as a powerful explanation for the development and differentiation 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 exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [47, 48, 50-53, 55-57]. 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 the 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 the 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 the proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes must be determined. 5) Functional and steric differences between the loose form and tight form (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding the proximal 5'-upstream regions of these specific 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 the chromatin structure plasticity of the proximal 5'-upstream region) differs from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in transcription of ORF regions of the corresponding genes) may be the same as the well-known transcription machinery. 9) To demonstrate the 4C theory, it is essential to establish both of the putative ECRR/ECRS as a first player to recognize the environment change and the putative 4C machinery as a final player to irreversibly and directly create the 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 in model animals (such as C. elegans, Drosophila, Xenopus, mice, rats and others) during development and differentiation is very effective and powerful, because these influences under the varied conditions can be easily studied by various research groups.

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**Conflict of interest statement**

There are no conflicts of interest.

**Supplementary Remarks**

The studies in Refs. 50 and 51 were reviewed in Refs. 55 and 56, respectively. The studies in this article were reviewed in Ref. 57.

All of old URLs for the first and revised editions of Monograph, Papers and Reviews, all of which are related to the chromatin conformation change code (4C) theory, were changed to new URLs as follows. Therefore, all of these first and revised editions of Monograph, Papers and Reviews, most of which were used as References in this article, are now available from new URLs but not from old URLs.

[Monograph]:

# 1st Edition (2015):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995

New URL: http://hdl.handle.net/10458/5293

# 2nd Edition (2018):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365

New URL: http://hdl.handle.net/10458/6460

[Papers]:

Paper-1 of 1st HDAC2(-/-):

# 1st Edition (2017):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10145170

New URL: http://hdl.handle.net/10458/5933

# 2nd Edition (2018):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10170628

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Paper-2 of Pax5(-):

# 1st Edition (2017):

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Paper-3 of 2nd HDAC2(-/-):

# 1st Edition (2017):

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Paper-4 of NotchIP:

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New URL: http://hdl.handle.net/10458/6505

Paper-5 of the 4C Theory:

# 1st Edition (2017):

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# 2nd Edition (2018):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10171004

New URL: http://hdl.handle.net/10458/6506

Paper-6 of Comprehensive (All-inclusive) Review:

# 1st Edition (2017):

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New URL: http://hdl.handle.net/10458/6507

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New URL: http://hdl.handle.net/10458/6613

# 4th Edition (2020):

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New URL: http://hdl.handle.net/10458/6902

Paper-7 of Memoirs (Insights):

# 1st Edition (2019):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10174284

New URL: http://hdl.handle.net/10458/6614

# 2nd Edition (2020):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10185502

New URL: http://hdl.handle.net/10458/6949

Paper-8 of Japanese Review:

# 1st Edition (2019):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10174562

New URL: http://hdl.handle.net/10458/6615

# 2nd Edition (2020):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10185510

New URL: http://hdl.handle.net/10458/6950

[Review articles in Current Topics in Biochemical Research]:

# Review-1 (Current Topics in Biochemical Research, Vol.18, pp.11-25, 2017):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168788

New URL: http://hdl.handle.net/10458/6375

# Review-2 (Current Topics in Biochemical Research, Vol.18, pp.33-56, 2017):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168794

New URL: http://hdl.handle.net/10458/6376

# Review-3 (Current Topics in Biochemical Research, Vol.18, pp.65-86, 2017):

Old URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10168800

New URL: http://hdl.handle.net/10458/6377

# Current Topics in Biochemical Research, Vol.18, pp.11-25, pp.33-56, pp.65-86, 2017:

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

Numbers of bibliographic and text references of old URLs of the Monograph, Papers and Reviews by August/2020 were as follows:

# Numbers of bibliographic and text references of old URL of the Monograph (1st Edition published in 2015) were 495 and 423, respectively. Numbers of bibliographic and text references of old URL of the Monograph (2nd Edition published in 2018) were 67 and 292, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-1 of 1st HDAC2(-/-) (1st Edition published in 2017) were 138 and 69, respectively. Numbers of bibliographic and text references of old URL of the Paper-1 of 1st HDAC2(-/-) (2nd Edition published in 2018) were 62 and 41, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-2 of Pax5(-) (1st Edition published in 2017) were 120 and 62, respectively. Numbers of bibliographic and text references of old URL of the Paper-2 of Pax5(-) (2nd Edition published in 2018) were 60 and 39, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-3 of 2nd HDAC2(-/-) (1st Edition published in 2017) were 112 and 58, respectively. Numbers of bibliographic and text references of old URL of the Paper-3 of 2nd HDAC2(-/-) (2nd Edition published in 2018) were 34 and 35, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-4 of NotchIP (1st Edition published in 2017) were 114 and 121, respectively. Numbers of bibliographic and text references of old

URL of the Paper-4 of NotchIP (2nd Edition published in 2018) were 31 and 69, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-5 of the 4C Theory (1st Edition published in 2017) were 208 and 132, respectively. Numbers of bibliographic and text references of old URL of the Paper-5 of the 4C Theory (2nd Edition published in 2018) were 62 and 72, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-6 of Comprehensive (All-inclusive) Review (1st Edition published in 2017) were 131 and 152, respectively. Numbers of bibliographic and text references of old URL of the Paper-6 of Comprehensive (All-inclusive) Review (2nd Edition published in 2018) were 43 and 70, respectively. Numbers of bibliographic and text references of old URL of the Paper-6 of Comprehensive (All-inclusive) Review (3rd Edition published in 2019) were 32 and 59, respectively. Numbers of bibliographic and text references of old URL of the Paper-6 of Comprehensive (All-inclusive) Review (4th Edition published in 2020) were overlooked, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-7 of Memoirs (Insights) (1st Edition published in 2019) were 28 and 59, respectively. Numbers of bibliographic and text references of old URL of the Paper-7 of Memoirs (Insights) (2nd Edition published in 2020) were overlooked, respectively.

# Numbers of bibliographic and text references of old URL of the Paper-8 of Japanese Review (1st Edition published in 2019) were 54 and 99, respectively. Numbers of bibliographic and text references of old URL of the Paper-8 of Japanese Review (2nd Edition published in 2020) were overlooked, respectively.

# Numbers of bibliographic and text references of old URL of Review-1 of Current Topics in Biochemical Research (Vol.18, pp.11-25, 2017) were 53 and 11, respectively.

# Numbers of bibliographic and text references of old URL of Review-2 of Current Topics in Biochemical Research (Vol.18, pp.33-56, 2017) were 46 and 6, respectively.

# Numbers of bibliographic and text references of old URL of Review-3 of Current Topics in Biochemical Research (Vol.18, pp.65-86, 2017) were 66 and 11, respectively.

**References**

[1] Allfrey, V., Faulker, R. M. and Mirsky, A. E.: Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. USA, 51, 786-794, 1964.

[2] Brownell, J. E., Zhou, J., Rannali, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. and Allis, C. D.: Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5 linking histone acetylation to gene activation. Cell 84, 843-851, 1996.

[3] Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y.: The transcriptional coactivators p300 and CPB are histone acetyltransferases. Cell 87, 953-959, 1996.

[4] Taunton, J., Hassig, C. A. and Schreiber, S. L.: A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272, 408-411, 1996.

[5] Brown, C. E., Lechner, T., Howe, L. and Workman, J. L.: The many HATs of transcription coactivators. Trends Biochem. Sci. 25, 15-19, 2000.

[6] Cheung, W. L., Briggs, S. D. and Allis, C. D.: Acetylation and chromosomal functions. Curr. Opin. Cell Biol. 12, 326-333, 2000.

[7] Turner, B. M.: Histone acetylation and an epigenetic code. Bioessays 22, 836-845, 2000.

[8] Roth, S. Y. Denu, J. M. and Allis, C. D.: Histone acetyltransferases. Annu. Rev. Biochem. 70, 81-120, 2001.

[9] Carrozza, M. J., Utley, R. T., Workman, J. L. and Cote, J.: The diverse functions of histone acetyltransferase complexes. Trend. Genet. 19, 321-329, 2003.

[10] Yang, X. J. and Seto, E.: Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. Curr. Opin. Genet. Dev. 13, 143-153, 2003.

[11] Margueron, R., Trojer, P. and Reinberg, D.: The key to development: interpreting the histone code? Curr. Opin. Genet. Dev. 15, 163-176, 2005.

[12] Goldberg, A. D., Allis, C. D. and Bernstein, B. E.: Epigenetics: a landscape takes shape. Cell 128, 635-638, 2007.

[13] Shahbazian, M. D. and Grunstein, M.: Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. 76, 75-100, 2007.

[14] Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhattar, R., Shilatifard, A., Workman, J. and Zhang, Y.: New nomenclature for chromatin-modifying enzymes. Cell 131, 633-636, 2007.

[15] Lee, K. K. and Workman, J. L.: Histone acetyltransferase complexes: one size doesn't fit all. Nat. Rev. Mol. Cell Biol. 8, 284-295, 2007.

[16] Berger, S. L.: The complex language of chromatin regulation during transcription. Nature 447, 407-412, 2007.

[17] Suganuma, T. and Workman, J. L.: Crosstalk among histone modifications. Cell 135, 604-607, 2008.

[18] Kohn, K. W., Aladjem, M. I., Weinstein, J. N. and Pommier, Y.: Chromatin challenges during DNA replication: A systems representation. Mol. Biol. Cell 19, 1-7, 2008.

[19] Selvi, R. B. and Kundu, T. K.: Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. Biotech. J. 4, 375-390, 2009.

[20] Javierre, B. M., Hemando, H. and Ballestar, E.: Environmental triggers and epigenetic deregulation in autoimmune disease. Discov. Med. 12, 535-545, 2011.

[21] Bannister, A. J. and Kouzarides, T.: Regulation of chromatin by histone modifications. Cell Res. 21, 381-395, 2011.

[22] Verrier, L., Vandromme, M. and Trouche, D.: Histone demethylases in chromatin cross-talks. Biol. Cell 103, 381-401, 2011.

[23] Butler, J. S., Koutelou, E., Schibler, A. C. and Dent, S. Y.: Histone-modifying enzymes: regulators of developmental decisions and drivers of human disease. Epigenomics 4, 163-177, 2012.

[24] Kooistra, S. M. and Helin, K.: Molecular mechanisms and potential functions of histone deacetylases. Nat. Rev. Mol. Cell. Biol. 13, 297-311, 2012.

[25] Graff, J. and Tsai, L.-H.: Histone acetylation: molecular mnemonics on the chromatin. Nat. Rev. Neurosci. 14, 97-111, 2013.

[26] Chen, T. and Dent, S. Y. R.: Chromatin modifiers and remodellers: regulators of cellular differentiation. Nat. Rev. Genet. 15, 93-106, 2014.

[27] T. W.-W. and Reinberg, D.: Chromatin futures and the epigenetic regulation of pluripotency states in ESCs. Development 141, 2376-2390, 2014.

[28] Morgan, M. A. and Shilatifard, A.: Chromatin signatures of cancer. Genes and Dev. 29, 238-249, 2015.

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

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

[31] Takami, Y., Kikuchi, H. and Nakayama, T.: Chicken histone deacetylase-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its pre-mRNA in the DT40 cell line. J. Biol. Chem. 274, 23977-23990, 1999.

[32] Takami, Y. and Nakayama, T.: N-terminal region, C-terminal region, nuclear export signal and deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. J. Biol. Chem. 275, 16191-16201, 2000.

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

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

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

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

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

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

[39] Kikuchi, H., Kuribayashi, F., Kiwaki, N., Takami, Y. and Nakayama, T.: GCN5 regulates the superoxide-generating system in leukocytes via controlling gp91-phox gene expression. J. Immunol. 186, 3015-3022, 2011.

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

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

[42] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: Protein kinase Cθ is oppositely regulated by GCN5 and RBF1 in immature B cells. FEBS Lett. 588, 1739-1742, 2014.

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

[44] Kikuchi, H., Nakayama, M., Kawai, C., Kuribayashi, F., Mimuro, H., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: The histone acetyltransferase p300/CBP-associated factor acts as an effective suppressor of secretory immunoglobulin synthesis in immature cells. Microbiol. Immunol. 59, 243-247, 2015.

[45] Kikuchi, H., Kuribayashi, F., Mimuro, H., Imajoh-Ohmi, S., Nakayama, M., Takami, Y., Nishitoh, H. and Nakayama, T.: Lack of GCN5 remarkably enhances the resistance against prolonged endoplasmic reticulum stress-induced apoptosis through up-regulation of Bcl-2 gene expression. Biochem. Biophys. Res. Commun. 463, 870-875, 2015.

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

[47] Nakayama, T. and Nakayama, M.: Chromatin Conformation Change Code (4C) theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations. pp. 1-303, 2018. The 2nd Edition of the retirement commemorative monograph (Monograph) (the 1st Edition of the self-publishing

Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) is available from following new URL: http://hdl.handle.net/10458/6460

[48] Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivation. pp. 1-34, 2018. The 2nd Edition of the Paper-1 (the 1st Edition of the Paper-1 published in 2017 is available from following new URL: http://hdl.handle.net/10458/5933) is available from following new URL: http://hdl.handle.net/10458/6500

The 2nd Edition of the Paper-1 is also the modified version of Chapter-2 of the 2nd Edition of the Monograph (the 1st Edition of the Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 11-44, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460

[49] Nakayama, M. and Nakayama, T.: Generation of Pax5-deficient DT40 mutant cells, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in Pax5(-) DT40 mutants are rapidly and dramatically reduced through various generations during continuous cultivation. pp. 1-28, 2018. The 2nd Edition of the Paper-2 (the 1st Edition of the Paper-2 published in 2017 is available from following new URL:http://hdl.handle.net/10458/5934) is available from following new URL: http://hdl.handle.net/10458/6503

The 2nd Edition of the Paper-2 is also the modified version of Chapter-3 of the 2nd Edition of the Monograph (the 1st Edition of the Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, M. and Nakayama, T.: Generation of Pax5-deficient DT40 mutants, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially accumulated in Pax5(-) are rapidly and dramatically reduced through various generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 45-71, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460

[50] Nakayama, M. and Nakayama, T.: IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivation. pp. 1-38, 2018. The 2nd Edition of

the Paaper-3 (the 1st Edition of the Paper-3 published in 2017 is available from following new URL: http://hdl.handle.net/10458/5935) is available from following new URL: http://hdl.handle.net/10458/6504
The 2nd Edition of the Paper-3 is also the modified version of Chapter-4 of the 2nd Edition of the Monograph (the 1st Edition of the Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, M. and Nakayama, T.: IgM H- and L-chains accumulated artificially and excessively in HDAC2(-/-) DT40 mutants are dramatically reduced in distinct ways in individual mutant clones through various generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 72-104, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460
[51] Nakayama, M. and Nakayama, T.: Fundamental and distinct ways for irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gain of new cell function to exclude accumulated IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. pp. 1-70, 2018. The 2nd Edition of the Paper-4 (the 1st Edition of the Paper-4 published in 2017 is available from following new URL: http://hdl.handle.net/10458/5936) is available from following new URL: http://hdl.handle.net/10458/6505
The 2nd Edition of the Paper-4 is also the modified version of Chapter-5 of the 2nd Edition of the Monograph (the 1st Edition of the Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, M. and Nakayama, T.: A fundamental way for irreversible creation of chromatin structure plasticity with epigenetic modifications for gaining new cell function to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 105-167, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460
[52] 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 2nd Edition of the Paper-5 (the 1st Edition of the Paper-5 published in 2017 is available from following new URL: http://hdl.handle.net/10458/5937) is available from following new URL: http://hdl.handle.net/10458/6506
The 2nd Edition of the Paper-5 is also the modified version of Chapter-6 of the 2nd Edition of the

Monograph (the 1st Edition of the Monograph was published in 2015 and is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, M. and Nakayama, T.: Chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 168-202, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460

[53] Nakayama, T. and Nakayama, M.: A comprehensive and detailed review on the chromatin conformation change code (4C) theory: A theory on ways to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through numerous generations in higher eukaryotes. pp.1-80, 2020. The 4th Edition of the Paper-6 (the 1st, 2nd and 3rd Editions published in 2017, 2018 and 2019 are available from following new URLs: http://hdl.handle.net/10458/5941, http://hdl.handle.net/10458/6507 and http://hdl.handle.net/10458/6613) is available from following new URL: http://hdl.handle.net/10458/6902

The 4th Edition of the Paper-6 is also the modified version of Chapter-7 of the 2nd Edition of the Monograph (the 1st Monograph published in 2015 is available from following new URL: http://hdl.handle.net/10458/5293) as follows: Nakayama, T. and Nakayama, M.: All-inclusive review and history on the chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 203-260, 2018. The 2nd Edition of the Monograph is available from following new URL: http://hdl.handle.net/10458/6460

[54] Bendayan, M. and Zollinger, M.: Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. 31, 101-109, 1983.

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

The article is available from following Website and new URL.

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

and http://hdl.handle.net/10458/6375

[56] Nakayama, M. and Nakayama, T.: Irreversible creation of chromatin structure plasticity of proximal

5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications to exclude IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation. Current Topics in Biochemical Research. 18, 33-56, 2017.
The article is available from following Website and new URL.
http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3
and http://hdl.handle.net/10458/6376
[57] Nakayama, T. and Nakayama, M.: Chromatin conformation change code (4C) theory: A bio-system to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. Current Topics in Biochemical Research. 18, 65-86, 2017.
The article is available from following Website and new URL.
http://www.researchtrends.net/tia/title_issue.asp?id=40&in=0&vn=18&type=3
and http://hdl.handle.net/10458/6377

**Figure legends**

**Figure 1. Localization of IgM H-chain at peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutants.**

HDAC2(-/-) mutant cells were collected at the early stage of cultivation. Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out. A) Immuno-electron microscopy after treatment without saponin. B) ~ E) Immuno-electron microscopy after treatment with saponin. 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. The figure is identical with Figure 6-1 of Ref. 47.

**Figure 2. Localization of IgM H-chain at peri-nuclear space of HDAC2(-/-) DT40 mutants.**

HDAC2(-/-) mutant cells were collected at the early stage of cultivation, and immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out. A) and B) Immuno-electron microscopy of two individual mutant cells. D) and E) Enlarged versions of parts indicated by arrows in A) and B), respectively. C) and F) Enlarged versions of immuno-electron microscopy of the peri-nuclear space of one DT40 cell and another HDAC2(-/-) mutant cell, respectively. Accumulated IgM H-chain proteins were observed in the peri-nuclear space (indicated by arrows) of all HDAC2(-/-) mutant cells. The figure is identical with Figure 6-2 of Ref. 47.

**Figure 3. Localization of IgM H-chain at peri-nuclear space of HDAC2(-/-) DT40 mutants at early and later stages of cultivation.**

HDAC2(-/-) mutant cells were collected at the early (E) and later (L) stages of cultivation, and immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early cultivation 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 independent HDAC2(-/-) mutant cells at the later cultivation stage. E), F), H), I) and K) Enlarged versions of parts indicated by arrows e, f, h, i and k in D), G) and J). The mutant cell in G) was identical with that of Ref. 48. Accumulated IgM H-chain proteins were observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early and later cultivation stages. The figure is identical with Figure 6-3 of Ref. 47.

**Figure 4. Localization of IgM H-chain at peri-nuclear space, and a model of signal transduction concerning accumulated IgM H- and L-chains to chromatin structure in HDAC2(-/-) DT40 mutants during continuous cultivation.**

**Left panel:** Localization of IgM H-chain proteins. A portion of the peri-nuclear space (where IgM

H-chain proteins were accumulated) of the HDAC2(-/-) mutant cell (indicated by an arrow P at lower position in Figure 1C) was reversely enlarged. **Right panel:** A model of signal transduction concerning the accumulation of IgM H- and L-chains to the chromatin. Signal concerning artificially accumulated IgM H- and L-chains in the peri-nuclear space of the HDAC2(-/-) mutant cell was repeatedly transmitted to the chromatin structure, followed by unbalanced response to 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 Figure 6-4 of Ref. 47.

**Figure 5. Summary of alterations in mRNA (gene expression/transcription) levels, acetylation levels of specific Lys residues of histone H3, and chromatin structure of 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/transcription) levels (-, ++ or ++++), acetylation levels (Ac; -, ++ or ++++) of specific Lys residues of histone H3, and the chromatin structure (form; loose or tight) of the 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. Chromatin conformation change complex (4C) machinery should generally contain a specific member of HATs plus HDACs and other factors. Transcription factor complex (TFC) machinery should generally contain RNA polymerase (RPase), specific transcription factors and others. The figure is identical with Figure 6-5 of Ref. 47.

**Figure 6. Chromatin conformation change code (4C) theory: A model for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations.**

Irreversible creation of the chromatin structure plasticity with epigenetic modifications occurs within the proximal 5'-upstream region (notch of chromatin) but not within ORF region of corresponding specific transcription factor gene through various generations (cell divisions). The tight form or loose form of the chromatin structure surrounding the proximal 5'-upstream region is based on hypo- or hyper-acetylation levels of specific Lys residues of histone H3, and causes low or high transcription (gene expression) levels. The figure is identical with Figure 6-6 of Ref. 47.

**Figure 7. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region with epigenetic modifications in descendent cells, and reversible regulations of ordinary gene expression and enzyme reaction in cells that initially accept proper signal.**

**Upper panel:** Irreversible creation of the chromatin structure plasticity. Irreversible creation of the chromatin structure plasticity surrounding the proximal 5'-upstream region of corresponding specific gene with epigenetic modifications occurs in descendant cells but not in initial cell that accepts the environment change signal. Ac, Ac/2 and Ac/10 qualitatively indicate hyper-, considerable 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 and enzyme reaction. Regulations of ordinary gene expression (on transcriptional regulatory elements) and enzyme reaction reversibly occur in the cell itself which accepts the proper signal. Ac and P indicate acetylation, phosphorylation and/or other chemical modifications, respectively. The figure is identical with Figure 6-7 of Ref. 47.

**Supplementary Figure S1. Ways to control gene expressions of IgM H- and L-chains through control of gene expressions of specific transcription factors in the presence or absence of HDAC2 in wild-type DT40 cells (W) or all and individual clones of HDAC2(-/-) DT40 mutants at early (E) and later (L) stages of cultivation.**

The figure is identical with Supplementary Figure 6-S1 of Ref. 47, and also a set of Figures 8, 9 and 10 of Ref. 50.

**Supplementary Figure S2. Localization of IgM H-chain in wild-type DT40 cells and HDAC2(-/-) DT40 mutants.**

**Upper panel:** Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out on wild-type DT40 cells (left) and HDAC2(-/-) mutants at the early cultivation stage (center and right). **Lower panel:** Enlarged versions of DT40 and HDAC2(-/-) mutant cells indicated by arrows in the upper panel. Positive signals of IgM H-chains were observed both at the cell surface and cytoplasm of HDAC2(-/-) mutant cells but only at the cell surface of DT40 cells. The figure is identical with Supplementary Figure 6-S2 of Ref. 47.

**Supplementary Figure S3. Localization of dense fraction due to accumulated IgM H-chain proteins in HDAC2(-/-) DT40 mutants.**

Electron microscopy was carried out on DT40 (upper panel; left and center) and HDAC2(-/-) mutant cells at the early cultivation stage (lower panel; left and center). Enlarged versions of DT40 and HDAC2(-/-) mutant cells indicated by arrows in the centers are shown in the right of upper and lower panels, respectively. Dense fractions due to accumulated IgM H-chains were observed only in HDAC2(-/-) mutant cells. The figure is identical with Supplementary Figure 6-S3 of Ref. 47.

**Supplementary Figure S4. Alterations in protein levels of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.**

The figure is identical with Supplementary Figure 6-S4 of Ref. 47, and also identical with Figure 2 of Ref. 50.

**Supplementary Figure S5. Alterations in gene expressions of IgM H- and L-chains, members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation.**

The figure is identical with Supplementary Figure 6-S5 of Ref. 47, and also identical with Figure 6 of Ref. 50 and shown with some modifications.

**Supplementary Figure S6. Alterations in acetylation levels of specific Lys residues 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.**

The figure is identical with Supplementary Figure 6-S6 of Ref. 47, and also a set of Figures 1, 5, 9 and 13 of Ref. 51.

**Supplementary Figure S7. Alterations in acetylation levels of specific Lys residues 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.**

The figure is identical with Supplementary Figure 6-S7 of Ref. 47, and also a set of Figures 2, 6, 10 and 14 of Ref. 51.

**Supplementary Figure S8. Alterations in acetylation levels of specific Lys residues 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.**

The figure is identical with Supplementary Figure 6-S8 of Ref. 47, and also a set of Figures 3, 7, 11 and 15 of Ref. 51.

**Supplementary Figure S9. Alterations in acetylation levels of specific Lys residues 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.**

The figure is identical with Supplementary Figure 6-S9 of Ref. 47, and also a set of Figures 4, 8, 12 and 16 of Ref. 51.

**Supplementary Figure S10. Summary of alterations in acetylation levels (hyper or hypo) of specific Lys residues of histone H3, the chromatin structure (form: loose or tight) of the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (gene expression/transcription) levels (high or low) in DT40 cells (W) and 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 cultivation.**

The figure is identical with Supplementary Figure 6-S10 of Ref. 47, and also identical with Figure 21 of Ref. 51 and shown with some modifications.