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[**]

Tatsuo Nakayama[#, $, *] and Masami Nakayama[#]

[#]Section of Biochemistry and Molecular Biology, Department of Medical Sciences, Faculty of Medicine, [$]Department of Life Science, Frontier Science Research Center, University of Miyazaki, 5200, Kihara, Kiyotake, Miyazaki, 889-1692, Japan

[*]Corresponding author: tnakayam@med.miyazaki-u.ac.jp (T. Nakayama)

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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 various generations. 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 proposed 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 chromatin-modifying enzyme genes with epigenetic modifications through various generations. Putative environment change recognition receptor/site (ECRR/ECRS) recognizes the new environment change. Putative chromatin conformation change complex (4C) machinery irreversibly and separately creates the varied chromatin structure plasticity of proximal 5'-upstream regions of the above-mentioned specific factor and 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 by the 4C machinery through various generations, in order to adapt to the new environment change recognized by the ECRR/ECRS. The 4C theory is suitable as an explanation for the ways to control development and differentiation of higher eukaryotes.

Keywords

Chromatin conformation change code (4C) theory, gain of un-programmed and new cell function, irreversible creation of chromatin structure plasticity, epigenetic modifications, various generations, immuno-electron microscopy study, pluri-potency, elasticity and flexibility of somatic cells

Introduction

In eukaryotes, genome information and nuclear function should be mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Additionally, communication and signal transduction between the nucleus and cytoplasm, both of which are essential for expressions of normal cell functions, are preferentially carried out by the to and fro passage of large and/or small molecules via the nuclear pore that acts as a guard station at the barrier. By contrast, both 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 alterations in genome information, such as point mutation, insertion, deletion, duplication and multiplication in the DNA molecule through various generations. This mode is a basis for the evolution of species. Third, in the case of a moderate change, cells cope with or overcome it by means of irreversible creation of the chromatin structure plasticity caused by successive chromatin conformational (structural) changes with epigenetic modifications through various generations. This mode is a basis for the development and differentiation of cells. Fourth, in the case of a minor change, cells respond to it only through already acquired regulation mechanisms.

Since chemical modifications of histones with acetyl and methyl groups were first proposed to regulate RNA synthesis [1], the modulation of chromatin topology has been thought to be one of the most fundamental and important events for expression of normal cell functions in eukaryotes. The ways to modulate the chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation 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], we have systematically generated various mutants, all of which were devoid of specific members of HDACs, HATs and transcription factors [31-45], besides those lacking numerous members of histones and histone chaperones. Systematic analyses of resultant DT40 mutants HDAC2(-/-), Aiolos(-/-), Pax5(-), EBF1(-/-), Ikaros(-/-/+), 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 Ikaros plus E2A [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 [47], Pax5-deficient mutants [48] and secondly generated HDAC2-deficient mutants [49], all of which were continuously cultivated for varyingly long periods. 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]. In individual clones of secondly generated HDAC2(-/-) mutants, the accumulated IgM H- and L-chains at the early cultivation stage are dramatically decreased, which are attributed to their drastically decreased gene expressions in almost the same changing pattern, caused by dramatic increases or decreases in gene expressions of Pax5, Aiolos and EBF1 or OBF1 in distinct ways during cultivation (details will be discussed later) [49]. 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 [48, 49]. 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 (details will be discussed later) [50].

In this study, based on our previously and newly obtained results from HDAC2(-/-) mutant cells, as a fundamental way for higher eukaryotes to adapt to environment change, we proposed a universal concept, which we named the chromatin conformation change code (4C) theory, for the bio-system to gain un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications through various generations in higher eukaryotes.

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 [47-51]. 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 ℃ 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 ℃ 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 ℃ for overnight. After washing with PBS, the cell pellets were fixed with 1% glutaraldehyde in PBS at 4 ℃ 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 [51]. 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 various generations during continuous cultivation

New findings and previous results [31, 35, 47-50] 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, following 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 (Suppl. Figs. S1(L) and S5) [49]. 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 various 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) [50]. 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 various 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, 49]) and

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OBF1 (which probably up-regulates those of the two immunoglobulin proteins [46, 49]) 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) [50]. Therefore, these four transcription factor genes are transcribed at high levels in DT40 cells [35, 47, 49]. 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, 49, 50].

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, 49].

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 [49, 50] 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, 49] 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 various 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 [49], are surely distinct from the ordinary and reversible way to regulate their gene expressions in 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, 49].   Therefore, remarkably, besides the alterations in gene

expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and in cell morphology, 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, 49]. 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, 49]. This inference should be surely supported by other results obtained from immuno-electron microscopy study [47, 49].

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 various generations during continuous cultivation

How do the HDAC2(-/-) mutants exclude excessively accumulated IgM H- and L-chains, in order to cope with and/or overcome such as an abnormal, unexpected, unfavorable, uncomfortable and useless environment change. Generally, this is possible by adhering to three following typical ways. 1) They acquire a high decreasing ability of gene expressions of IgM H- and L-chains, and thereby decrease large amounts of these two immunoglobulin proteins. 2) They acquire a high degrading (decomposing) ability of proteins (and/or mRNAs) of IgM H- and L-chains, and thereby decrease large amounts of these two immunoglobulin proteins. 3) They acquire a high secreting ability of IgM H- and L-chains, and thereby decrease large amounts of these two immunoglobulin proteins. 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, 46-50], 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 various generations during continuous cultivation in individual clones of HDAC2(-/-) mutants.

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, 49], 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 surounding 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 (coding regions) of these target genes by much [50]. 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, 49]. 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 [50].

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,

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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 [49] 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 specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications in higher eukaryotes through various 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 specific transcription factor and chromatin-modifying factor genes with epigenetic modifications though various generations (cell divisions) in higher eukaryotes.

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 various 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).   Naturally, 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.   As a next step, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin structure within the nucleus through various 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 [50], 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 various 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 programed 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.

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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 various generations, in spite of the same environment change.   As a consequence, individual cells of the same type are able to newly gain the same 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, 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. 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 to tight forms or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (Fig. 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 various 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.

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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. 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

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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. By contrast, programmed, complicated and diverse cell functions are orderly and systematically expressed throughout their development and differentiation. In spite, the 4C theory is suitable as an explanation for the development and differentiation (and so-called robustness) of higher eukaryotes, because action of putative signal(s) concerning environment changes seems to fairly resemble that of certain players (such as hormone, cytokine, nerve-transmission substance, etc.), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

There are several crucial questions to be clarified in the 4C theory on exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [47, 49, 50]. 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 and tight forms (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding 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 region of the corresponding gene) may be the same as the well-known transcription machinery. 9) To demonstrate the 4C theory, it is essential to establish both 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 various 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.

Acknowledgments

Conflict of interest statement

There are no conflicts of interest.

Postscript

The studies in Refs. 49 and 50 were reviewed in Refs. 52 and 53, respectively.   The studies in the article were reviewed in Ref. 54.

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The revised article is also the modified version of Chapter 2 of the revised monograph (the original monograph was published in 2015 and is available from following URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995) as follows: Nakayama, M. and Nakayama, T.: Protein and mRNA levels of IgM H- and L-chains artificially accumulated in HDAC2-deficient DT40 mutants are dramatically reduced through various generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of Un-programmed and New Cell Functions by Means of Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications through Various Generations, Nakayama, T. and Nakayama, M. (Eds.), pp. 11-44, 2018. The revised monograph is available from following URL: http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10169365

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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.

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.

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. 47.   Accumulated IgM H-chain proteins were observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early and later cultivation stages.

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.

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.

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 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.

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.

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 a set of Figures 8, 9 and 10 of Ref. 49.

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.

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.

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 Figure 2 of Ref. 49.

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 Figure 6 of Ref. 49 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 a set of Figures 1, 5, 9 and 13 of Ref. 50.

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 a set of Figures 2, 6, 10 and 14 of Ref. 50.

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 a set of Figures 3, 7, 11 and 15 of Ref. 50.

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 a set of Figures 4, 8, 12 and 16 of Ref. 50.

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 Figure 21 of Ref. 50 and shown with some modifications.