Chapter 7

Review and history on the chromatin conformation change code (4C) theory: A bio-system for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations

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SUMMARY

In the chicken DT40 cell line, HDAC2 indirectly regulates transcriptions of IgM H- and L-chain genes through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1, and Ikaros plus E2A. The HDAC2-deficiency in chicken DT40 cells induces dramatic accumulations of mRNAs and proteins of IgM H- and L-chains, and thereafter these accumulated mRNAs and proteins are gradually reduced in almost similar changing pattern during cultivation in all individual clones of HDAC2(-/-) DT40 mutants. By contrast, gene expressions of Pax5, Aiolos, EBF1 and OBF1 showed remarkably distinct changing patterns during cultivation in individual HDAC2(-/-) mutant clones. At the late stage of cultivation, there exist at least three distinct manners for gene expressions of IgM H- and L-chains, i.e., OBF1-dependent, Pax5- plus Aiolos-dependent, and Pax5-, Aiolos- plus EBF1-dependent types. These distinct alterations in transcriptions of Pax5, Aiolos, EBF1 and OBF1 genes in individual clones of HDAC2(-/-) mutants should be originated from irreversible chromatin conformation (structure) changes based on varied changes in acetylation levels of particular Lys residues of histone H3 within chromatin surrounding their proximal 5'-upstream regions during cultivation. Based on these results, universally, we named bio-system for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications a chromatin conformation change code (4C) theory.

Outline of the 4C theory is concretely as follows. 1) Somatic cells of higher eukaryotes are pluri-potent, elastic and flexible for gain of new cell function to cope with and/or overcome environment change, when they firstly encounter with it, and these pluri-potency, elasticity and flexibility should be originated from those of chromatin structure. 2) New cell function to adapt for and/or eliminate environment change is gained through irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region(s) of particular gene(s). 3) Plasticity of chromatin structure (from tight to loose forms or vice versa) is continuously/irreversibly created based on chromatin conformation change with epigenetic modifications via a lot of generations (cell divisions). 4) Diversity of chromatin structure plasticity in individuals of the same cell-type should be triggered by spontaneous unbalanced response to environment change and accomplished by its successive convergence via lots of generations. 5) Irreversible creation of chromatin structure plasticity probably depends on antecedents of somatic cells and successive response to environment change, and occurs in descendent cells but not in the cell, which initially meets with it. 6) Irreversible creation of chromatin structure plasticity should occur inevitably but not incidentally and/or neutrally. 7) Environment change should be recognized by putative environment change recognition receptor/site (ECRR/ECRS) and chromatin structure plasticity should be irreversibly created by putative chromatin conformation change complex (4C) machinery. 8) Chromatin structure of proximal 5'-upstream region(s) of particular gene(s), as dynamic and changeable three-dimensional conformation, should receive signal on environment change. 9) Chromatin structure (loose or tight form) of proximal 5'-upstream region of particular gene directs switch (on or off) for its

latent transcription ability. 10) Proximal 5'-upstream chromatin region of a certain gene is regarded as "notch of chromatin" from a structural side-view and "director for transcription" from a functional side-view. 11) The number of codes in the 4C theory, which should determine complicated/varied cell functions and 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 genes.

In eukaryotes, alterations in chromatin structure are remarkably involved in regulations of gene expressions, and replication, repair plus recombination of DNA and so on [1-5, Chap. 1], and also lymphocyte development and differentiation [6-12]. On the other hand, numerous transcription factors, including Ikaros, PU.1, E2A, GATA-3, EBF, Pax5 and so on, are involved in regulations of the development and differentiation of lymphocytes [13-22]. In addition, transcriptional regulation of IgM H-chain requires USF, TFEB, Ig/EBP, NF-IL6, OCA-b and others as promoter binding proteins, and Ig/EBP, NF-IL6, YY-1, E2A, PU.1 and others as intron enhancer binding proteins. Meanwhile, of various chromatin-modifying enzymes participating in alterations in chromatin structure, histone acetyltransferase(s) (HATs) and deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of particular Lys residues of core histones (H2A, H2B, H3 and H4) [23-46]. To assess roles of individual members of HDACs and HATs, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a particular member of HDACs and HATs [47-63], by gene targeting techniques using two different targeting vectors [64-74]. Our previous findings in initially generated HDAC2-deficient mutant cells, HDAC2(-/-), revealed not only that HDAC2 controls the amount of IgM H-chain at the steps of transcription of its gene plus alternative processing of its pre-mRNA [47], but also that it down-regulates the IgM L-chain gene promoter activity [50]. Moreover, the HDAC2-deficiency has varied severe and/or moderate effects on several cellular characteristics. That is, the deficiency represses transcriptions of HDAC7, Pax5, Aiolos, Ikaros and EBF1, elevates transcriptions of HDAC4, HDAC9, PCAF and E2A, and changes bulk acetylation levels of several Lys residues of core histones [54].

To know individual roles of these altered chromatin-modifying enzymes and transcription factors on regulation of gene expressions of IgM H- and L-chains, we generated homozygous DT40 mutant cell lines: EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), respectively, devoid of EBF1, Aiolos, E2A and Helios genes [54, 75-79], and Pax5-deficient DT40 mutant cell line Pax5(-), devoid of the Pax5 gene located on Z chromosome which is monosomy in chickens (USCS Genome Browser data base) [Chap. 3]. In addition, we generated Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene located on chromosome 2 which is trisomy in chickens (our unpublished data). Analyses of these resultant mutants revealed that Pax5, EBF1, Aiolos plus Ikaros down-regulate transcriptions of IgM

H- and L-chain genes, and E2A up-regulates transcriptions of these two immunoglobulin genes [54]. These results, together with others [80], indicated that in wild-type DT40 cells HDAC2 indirectly regulates transcriptions of IgM H- and L-chain genes through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1, Ikaros and E2A (Fig. 7-1(W)) [54, 57].

Throughout the process of further qualitative analyses of characteristics of initially generated HDAC2(-/-) mutant cells [47], which were cultivated for different periods, surprisingly, we casually noticed interesting and amazing phenomena as follows [Chap. 2]. 2D-PAGE showed that amounts of IgM H-chain and L-chain (detected as two spots) were dramatically and considerably elevated at the early (~10 to 20 days) stage of cultivation, and thereafter gradually decreased via the middle (~30 to 40 days) stage and at the late (~60 days) cultivation stage reached to comparable levels in DT40 cells (Fig. 7-2). In all of our studies any cultivation stages (and days) were practically counted from the first stock day, although until then approximately 10 to 12 days had already passed since two HDAC2 alleles (or one Pax5 allele) were disrupted [47, Chaps. 2, 3, 4 and 5]. By contrast, insignificant changes were observed for most of other major cellular proteins during cultivation. Western blotting, which was carried out at shorter interval periods, using antibodies for chicken IgM H-chain and also L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that IgM H-chain was obviously accumulated at the early stage and thereafter at the late stage reduced to almost the same level in DT40 cells (Fig. 7-3). These results, together, indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage (Fig. 7-1(E)), but also that these accumulated immunoglobulin proteins are gradually reduced during cultivation and finally at the late stage reached to comparable levels in DT40 cells (details will be shown later) (Fig. 7-1(L)). RT-PCR using primers IgM Hc plus IgM Hs showed that whole and secreted forms of IgM H-chain mRNA were dramatically increased at the early stage, and thereafter gradually reduced during cultivation and at the late stage reached to very close levels in DT40 cells (Fig. 7-4). Remarkably, RT-PCR, using appropriate primers specific for various genes of chromatin-modifying enzymes and transcription factors, showed that gene expressions of HDAC7, HDAC9 and PCAF were gradually elevated, and those of EBF1, Pax5 and Aiolos were certainly altered in distinct patterns during cultivation (details will be shown later). Immuno-blotting, using available antibodies specific for various acetylated Lys (K) residues of core histones H2A, H2B, H3 and H4, showed that in spite of the HDAC2-deficiency, bulk acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) were gradually increased during cultivation, though insignificant changes were observed in those of most of remaining Lys residues of core histones.

Because the gene expression of Pax5 is controlled by HDAC2, and among various transcription factors, mainly Pax5 controls gene expressions of IgM H- and L-chains [54], we studied molecular

mechanism of transcription of the Pax5 gene [Chap. 2]. Until we started this study, it was not reported that the Pax5 gene is located on Z chromosome that is monosomy in chickens, and nucleotide sequence data of its 5'-upstream region were not yet deposited in any database, although those of its cDNA and several homologous genes could be cited from databank. Therefore, we first cloned the ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques [54], including Southern blotting, colony hybridization and sub-cloning [81, 82]. Dual-luciferase assay carried out on this Pax5 5'-upstream region using various 5'- and 3'-deletion plasmid constructs suggested that two distinct proximal 5'-upstream regions were presumably necessary to control the gene expression negatively, whereas clearly defined promoter region(s) or element(s) has yet to be elucidated (our unpublished data). Moreover, using site-specific antibody for acetylated K9 residue of histone H3 (K9/H3) and several appropriate primers, we carried out chromatin immuno-precipitation (ChIP) assay on chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in initially generated HDAC2(-/-) mutant cells collected at the early and late cultivation stages and DT40 cells [Chap. 2]. These cultivation stages were vague and temporary, since the mutant cells tested had been already cultivated several times for other experiments. However, surprisingly, this preliminary ChIP assay suggested that in HDAC2(-/-) acetylation levels of K9/H3 within some limited segments of the proximal 5'-upstream chromatin region of the Pax5 gene were decreased at the early stage and thereafter at the late stage increased to almost the same levels in DT40 cells (details will be shown later). These results roughly agreed with the findings on changing patterns in the gene expression of Pax5 mentioned above.

As mentioned above, gene expressions of IgM H- and L-chains are mainly and indirectly regulated by HDAC2 through control of gene expressions of various transcription factors, especially Pax5 [54, Chaps. 2 and 3]. We performed time-course studies on some characteristics of Pax5(-) mutant cells at the early (~8 days), middle (~13 days) and late (~20 days) cultivation stages [Chap. 3] of relatively shorter intervals than those adopted for HDAC2(-/-) mutant cells [Chap. 2]. 2D-PAGE revealed that protein levels of IgM H- and L-chains were drastically and considerably increased at the early stage, and thereafter gradually decreased during cultivation and at the late stage reached to almost the same levels in DT40 cells (our unpublished data). Western blotting using antibody for chicken IgM L-chain (which cross-reacts with IgM H-chain) showed that IgM H-chain and L-chain (detected as two bands) were dramatically and considerably elevated at the early stage, and thereafter gradually decreased via the middle to late stages to almost the same levels in DT40 cells (Fig. 7-5). Furthermore, electron microscopy revealed that dense cytoplasmic fractions probably due to artificially accumulated IgM H- and L-chains were detected at the early stage in Pax5(-) mutant cells but not at the late stage as in DT40 cells. Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that the immunoglobulin proteins were surely accumulated at the early stage, and thereafter most of them

disappeared at the late stage as in DT40 cells. Together, these results indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage in Pax5(-), but also that these accumulated immunoglobulin proteins are rapidly reduced during cultivation and finally at the late stage reached to comparable levels in DT40 cells. Microscopy showed that in any different ranges of vision and their magnified visions, Pax5(-) was observed to be dispersive form at both the early and late stages, similar to that of DT40 cells (Fig. 7-6). Such morphological property of Pax5(-) and its changing pattern were clearly different from those of HDAC2(-/-) [see Chap. 4] as will be discussed later. RT-PCR using primers IgM Hc and IgM Hs revealed that whole and secreted forms of IgM H-chain mRNA were dramatically elevated at the first (~4 days) stage (prior to the early stage) in Pax5(-), and thereafter rapidly reduced via the early and middle stages and finally at the late stage reached to almost the same levels in DT40 cells (Fig. 7-7). On the other hand, RT-PCR using primers IgM Hm and IgM L showed that membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage, and thereafter slowly decreased via the early and middle stages and at the late stage reached to almost the same levels in DT40 cells. RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors, showed not only that PCAF and HDAC9 mRNA levels were gradually elevated from the first via early and middle stages and at the late stage reached to almost plateau levels, but also that the mRNA level of HDAC7 changed moderately during cultivation. In addition, Aiolos and OBF1 mRNA levels were gradually reduced from the first via early to middle stages and became undetectable at the late stage. Ikaros and E2A mRNA levels were drastically elevated at the first stage, and thereafter gradually and certainly decreased via the early to middle stages and at the late stage reached to almost the same levels in DT40 cells. The EBF1 mRNA level completely reduced at the first stage and remained unchanged as undetectable level throughout cultivation. The PU.1 mRNA level was obviously reduced at the first stage, and thereafter gradually elevated during cultivation and at the late stage reached to almost the same level in DT40 cells.

Based on these findings obtained by qualitative analyses of initially generated HDAC2- and Pax5-deficient DT40 mutants, HDAC2(-/-) and Pax5(-) [47, 54, Chaps. 2 and 3], we revealed that in these two mutant cells artificially accumulated immunoglobulin proteins (IgM H- and L-chains) at the early (or first) cultivation stage were diminished based on their decreased gene expressions, associated with alterations in gene expressions of various transcription factors and/or chromatin-modifying enzymes, via a lot of generations during cultivation. However, several cellular characteristics were obviously different between HDAC2(-/-) and Pax5(-). Those of HDAC2(-/-) will be more minutely shown again below.

To expand the above-mentioned results and to eliminate effects of drug-resistant genes within targeting vectors, we newly generated HDAC2-deficient mutants HDAC2(-/-) (Fig. 7-8) [Chap. 4], using

two targeting vectors different from those used previously [47]. By systematical analyses of these new HDAC2(-/-) mutant cells, we obtained again following certain and noticeable results. As shown in Figures 7-9 and 7-10, in HDAC2(-/-) mutant cells, surely, proteins and mRNAs of IgM H- and L-chains are dramatically and considerably accumulated at the early (~3 to 7 days) cultivation stage, which was earlier than that adopted for initial HDAC2(-/-) mutant cells [Chap. 2], i.e., soon after the generation (birth). It is because HDAC2 as a supervisor mainly regulates gene expressions of these two immunoglobulin proteins through opposite controls of Pax5, Aiolos, EBF1, Ikaros, OBF1 and E2A gene expressions in wild-type DT40 cells (Fig. 7-1(W)) [54]. These results obtained in HDAC2(-/-) DT40 mutants at the early stage of cultivation are schematically shown in Figure 7-1(E). The majority of artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutant cells exist as a native soluble form capable of building a high molecular weight complex with each other within endoplasmic reticulum (Fig. 7-11), since the HDAC2 mediated regulatory mechanisms should not function any longer and lacking of the mechanisms could be far superior to the capacity to secrete accumulated immunoglobulin proteins. In addition, HDAC2(-/-) mutant cells at the early stage exist as a morphologically aggregative/distorted form (Fig. 7-12), the reason for this is still unknown. Anyhow, both of the accumulation of these two immunoglobulin proteins and the aggregative form should be uncomfortable (painful) for HDAC2(-/-) mutant cells themselves. Surprisingly but somewhat expectedly, in all of individual clones of HDAC2(-/-) mutants, the artificially accumulated proteins and mRNAs of IgM H- and L-chains at the early stage are gradually reduced via the middle (~30 days) stage and at the late (~60 days) stage reached to almost the same levels in DT40 cells (Figs. 7-9, 7-10 and 7-11). Agreed with these changes, morphology of HDAC2(-/-) is also altered; i.e., the aggregative form at the early stage is altered during cultivation until the late stage to the dispersive form, which should be comfortable (peaceful) for the mutants, as for DT40 cells (Fig. 7-12). In addition to the above-mentioned findings, very recently, we first noticed following interesting and important facts on results which had been already obtained by immuno-electron microscopy of initial HDAC2(-/-) mutant cells [Chap. 6]. Artificially expressed excessive IgM H- and L-chains caused by the HDAC2-deficiency are first accumulated within endoplasmic reticulum of HDAC2(-/-) mutant cells as described above. In parallel and/or subsequently, most of these accumulated immunoglobulin proteins are gradually secreted to outside of mutant cells, however, some of them are transported to nuclear envelope but not inside of nucleus and kept in peri-nuclear space not only at the early but also at the late stages of cultivation. These findings will be discussed in detail later.

At this step, as manners to eliminate uncomfortable intra-cellular (and/or extra-cellular if existence) environment change for HDAC2(-/-), we built up the following brief working hypothesis [Chap. 4]. Putative signal(s) concerning the accumulation of IgM H- and L-chain proteins (and also probably cell aggregation) should be transmitted to chromatin (structure) within nucleus during cultivation, though the

mechanism and the machinery still remain quite unknown. Transcriptions of numerous genes encoding chromatin-modifying enzymes and/or transcription factors should change, associated with alterations in their chromatin structure. The putative signal(s) on and response(s) for the environment change could be repeatedly converged into gene expressions of PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Oct2, Blimp1, XBP-1 and others during cultivation. Interestingly, as will be described below in detail, mRNA levels (i.e., gene expressions) of these altered transcription factors and chromatin-modifying enzymes each showed distinct changing patterns during cultivation in six examined individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants (Fig. 7-10) [Chap. 4], regardless of almost similar changing pattern in mRNA and protein levels of IgM H- and L-chains and cell morphology.

In clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which are high levels in DT40 cells and down-regulate transcriptions of IgM H- and L-chain genes [54], are almost completely suppressed at the early stage and thereafter remain unchanged until the late stage (Fig. 7-10). By contrast, the mRNA level of OBF1, which is also high level in DT40 cells and probably up-regulates transcriptions of these two immunoglobulin genes [54, 80], is gradually and dramatically reduced from the early to late stages. Therefore, the manner for gene expressions of IgM H- and L-chains at the late stage in clone cl.2-1 seems to be dependent on OBF1 and considerably differ from that in wild-type DT40 cells in appearance (Fig. 7-1(L)). In clones cl.2-2 and cl.2-4, mRNA levels of Pax5, Aiolos and EBF1 are almost completely suppressed at the early stage (Fig. 7-10). Thereafter, those of Pax5 and Aiolos are gradually increased via the middle to late stages but that of EBF1 remains unchanged as undetectable level until the late stage. On the other hand, the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the late stage. Therefore, the manner for gene expressions of IgM H- and L-chains at the late stage in clones cl.2-2 and cl.2-4 (and cl.2-3 and cl.2-5) seems to be dependent on Pax5 and Aiolos and slightly similar to that in DT40 cells in appearance (Fig. 7-1(L)). Moreover, these four clones should be the major type, since four initially generated HDAC2(-/-) mutant clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) resembled these four clones in several cellular properties [Chap. 2]. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are almost completely decreased at the early stage and thereafter gradually increased via the middle to late stages (Fig. 7-10). On the other hand, the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the late stage. Therefore, the manner for gene expressions of IgM H- and L-chains at the late stage in clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 and most similar to that in DT40 cells in appearance (Fig. 7-1(L)), since these two immunoglobulin gene expressions in DT40 cells are directly/cooperatively regulated by these three transcription factors (and E2A) (see Fig. 7-1(W)). These three models for roles of Pax5, Aiolos, EBF1 and OBF1 in control of gene expressions of IgM H- and L-chains at the late stage

of cultivation in individual clones of HDAC2(-/-) DT40 mutants are schematically shown in Figure 7-1(L). However, the manners to suppress gene expressions of IgM H- and L-chains at the late cultivation stage in all individual HDAC2(-/-) mutant clones, including clone cl.2-6, should be really distinct from the ordinary and reversible transcription regulations of these two immunoglobulin genes in DT40 cells. Since all of these mutant clones are lacking HDAC2, which controls transcriptions of Pax5, Aiolos, EBF1, OBF1 and also E2A genes. If additional independent clones of HDAC2(-/-) mutants are analyzed, besides the above-mentioned three manners, other distinct manners for gene expressions of IgM H- and L-chains will be probably added. Moreover, the above-mentioned results on varied alterations in gene expressions of various transcription factors and chromatin-modifying enzymes suggested that some other unknown cellular characteristics must certainly change in individual HDAC2(-/-) mutant clones during cultivation.

Based on these findings [Chap. 4], we concluded that each individual clones of HDAC2(-/-) mutants should possess their own ability to gain the same and new cell functions in distinct manners via a lot of generations during cultivation. Namely, the same and new cell functions of individual HDAC2(-/-) mutant clones mean not only excluding artificially accumulated IgM H- and L-chains but also ridding themselves free from aggregative form, since these two should be uncomfortable for the mutant cells, themselves. Such distinct manners should bring about distinct changing patterns in transcription levels of Pax5, Aiolos, EBF1, OBF1 (and also E2A and others) in individual mutant clones, though gene expressions of IgM H- and L-chains change in almost similar pattern in all of them. In addition, we would like to emphasize that changes of any characteristics of HDAC2(-/-) (and also Pax5(-)) mutant cells should be more drastic just soon after their birth. This inference is based on the facts that we collected the mutant cells at ~10 to 12 days after their birth by gene targeting techniques [Chaps. 2, 3 and 4] and their doubling times were ~12 hrs [47, 54, 64]; therefore, they should be populations of ~20 to 25 generations even at the early stage. Of various transcription factors whose transcriptions were altered in HDAC2(-/-) mutants during cultivation, particularly, Pax5, Aiolos, EBF1 and OBF1 should be influential candidates participating in diminutions of artificially elevated gene expressions of IgM H- and L-chains. Because these four factors were already reported to regulate these two immunoglobulin gene expressions [54] and changing patterns of their gene expressions were anti-parallel or parallel with changing patterns of IgM H- and L-chain gene expressions in one or more of individual HDAC2(-/-) mutant clones [Chap. 4].

Next, we studied how individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 separately gain distinct manners for transcriptions of Pax5, Aiolos, EBF1, OBF1 and also PCAF genes via a lot of generations during continuous cultivation [Chap. 5]. We newly developed a chromatin immuno-precipitation (ChIP) assay on the proximal ~2.0 kb 5'-upstream chromatin region (named as

notch of chromatin) of each of the above-mentioned particular genes, using site-specific antibodies for several acetylated Lys residues of histone H3 and various appropriate primers. All segments, amplified by PCR using corresponding primers from the proximal ~2.0 kb 5'-upstream region of each of these five genes, are laid overlapping to neighboring ones each other. Actually, we performed the ChIP assay on five proximal ~2.0 kb 5'-upstream chromatin regions, each of which consist of approximately 10 to 11 nucleosomes, together with the several distal 5'-upstream and open reading frame chromatin regions of the corresponding genes. Hereafter, we designated the new ChIP assay as neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; this abbreviation means also IP on notch of chromatin) assay. Surprisingly, acetylation levels of five particular Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within the proximal ~2.0 kb 5'-upstream chromatin regions of these five genes in four mutant clones separately changed during cultivation (Figs. 7-13 to 7-16). We supposed that hyper- (high), considerable hyper-, somewhat hyper- or hypo- (low or no) acetylation levels of one or more of these five particular Lys residues (especially K9/H3 and K27/H3) within the proximal ~2.0 kb 5'-upstream chromatin regions of the five genes qualitatively induce no, weak, less or full binding ability (capacity) of histone H3 to DNA, resulting in loose, considerable loose, somewhat loose or tight form of chromatin structure (Fig. 7-17). In consequence, for example, loose or tight form of chromatin structure of a certain proximal ~2.0 kb 5'-upstream region should lead to high or low (or no) transcription level of the corresponding gene. Results obtained by the NotchIP assay [Chap. 5] and those obtained by RT-PCR [Chap. 4] on the five particular genes in the four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of HDAC2(-/-) mutants are as follows.

In DT40 cells, the five Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal 5'-upstream region from positions -1923 to +30 of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibited full binding ability of histone H3 to DNA at any cultivation stages based on their hypo-acetylation levels (Fig. 7-13). Consequently, the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells, but change to tight form at the early stage in clone cl.2-1 and remain unchanged until the late stage. These facts agreed with the findings that the gene expression of Pax5, which is high level in DT40 cells, is dramatically suppressed to low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal 5'-upstream region from positions -2250 to +145 of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibited full or less binding ability of histone H3 to DNA based on their hypo- or

somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-13). Accordingly, the structure of chromatin surrounding the proximal ~2.1 kb 5'-upstream region of the gene, which may consist of ~11 nucleosomes, should be loose form in DT40 cells, but change to tight (or somewhat loose) form at the early stage in clone cl.2-1 and remain unchanged until the late stage. These facts agreed with the findings that the gene expression of Aiolos, which is high level in DT40 cells, is drastically suppressed to low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal 5'-upstream region from positions -2031 to +200 of the EBF1 gene based on their hyper-acetylation levels. However, in clone cl.2-1, especially K9/H3, K18/H3 and K27/H3 possessed full (or less) binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-13). Accordingly, the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells, but change to tight (or somewhat loose) form at the early stage in clone cl.2-1 and remain unchanged until the late stage. These facts agreed with the findings that the gene expression of EBF1, which is high level in DT40 cells, is almost completely suppressed to low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal 5'-upstream region from positions -2138 to +164 of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-1, in particular, K9/H3 and K27/H3 (and probably K23/H3) certainly possessed weak binding ability of histone H3 to DNA based on their considerable hyper-acetylation levels at the early stage. Further, the weak binding ability was dramatically increased at the middle stage and remained unchanged at the late stage based on their hypo-acetylation levels (Fig. 7-13). Accordingly, the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells. On the other hand, in clone cl.2-1, the chromatin structure should change to considerable loose form at the early stage and thereafter change to tight form at the middle and late stages. These facts agreed with the findings that the gene expression of OBF1, which is high level in DT40 cells, is slightly decreased at the early stage in clone cl.2-1 and thereafter dramatically suppressed to very low (or no) level at the middle and late stages (see Fig. 7-10).

In DT40 cells and clone cl.2-1 at any cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no (or low) binding ability of histone H3 to DNA within chromatin surrounding the proximal 5'-upstream region from positions -2005 to +231 (and two distal 5'-upstream regions) of the PCAF gene based on their hyper-acetylation levels with insignificant changes

(in case of clone cl.2-1) (Chap. 5). Accordingly, the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, should be loose form in both DT40 cells and clone cl.2-1. However, as in DT40 cells, so in clone cl.2-1 the gene expression of the PCAF gene is really very low at the early stage but thereafter gradually and dramatically increased until the late stage (see Fig. 7-10). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, should be expected to participate in transcription of the gene, and the examined proximal and distal 5'-upstream regions are assumed to poorly correlate with its transcription, regardless of their loose form in both DT40 cells and clone cl.2-1.

These results, together with the previous inference speculated from changing patterns in gene expressions of these transcription factors [Chap. 4], surely indicated that at the late cultivation stage clone cl.2-1 seems to be dependent on OBF1 and considerably distinct from wild-type DT40 cells in the manner of gene expressions of IgM H- and L-chains in appearance (Fig. 7-1(L)).

As described above, in DT40 cells, the four Lys residues (K9/H3, K14/H3, K18/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-2, those exhibited full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage. Thereafter, very surprisingly, these four Lys residues gradually lost the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels during cultivation until the late stage (Fig. 7-14). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-2. Thereafter, the chromatin structure should change to loose form until the late stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to low (or no) level at the early stage in clone cl.2-2 and thereafter gradually and certainly elevated to high level until the late stage (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-2, those, mainly K9/H3 and K27/H3 possessed less binding ability of histone H3 to DNA based on their somewhat hyper-acetylation levels at the early and middle stages, but mainly the binding capacity of K9/H3 was certainly weakened to no (or weak) binding ability based on the considerable hyper-acetylation levels at the late stage (Fig. 7-14). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-2 and thereafter change to loose (or considerable loose) form at the late stage. These facts agreed with the findings that the gene expression of Aiolos is

dramatically suppressed to low (or no) level at the early stage in clone cl.2-2 and thereafter increased to high level at the late stage (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, especially K9/H3, K14/H3 and K27/H3 (and probably K18/H3) exhibited full binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-14). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-2 and remain unchanged via the middle to late stages. These facts agreed with the findings that the gene expression of EBF1 is almost completely suppressed to low (or no) level at the early stage in clone cl.2-2 and thereafter remains unchanged (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, mainly K9/H3 and K27/H3 exhibited full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. The less binding ability of K27/H3 was slightly increased and that of K9/H3 remained unchanged as full binding ability based on their hypo-acetylation levels at the middle stage. Thereafter, the full binding ability of K9/H3 and K27/H3 was obviously reduced to no binding ability based on their hyper- or considerable hyper-acetylation levels at the late stage (Fig. 7-14). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to considerable tight form at the early stage in clone cl.2-2 and thereafter change to tight form at the middle stage. Subsequently, the tightened chromatin structure should become loose form at the late stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-2 and thereafter obviously elevated to high level at the late stage (see Fig. 7-10).

In both DT40 cells and clone cl.2-2 at any cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene based on their hyper-acetylation levels with insignificant changes (in case of clone cl.2-2) (see Chap. 5). Therefore, the structure of chromatin surrounding the proximal 5'-upstream region of the gene should be loose form in both DT40 cells and clone cl.2-2 at any cultivation stages. However, in clone cl.2-2 the gene expression of PCAF is really very low at the early stage, as in DT40 cells, but gradually and dramatically increased until the late stage (see Fig. 7-10). Accordingly, other unknown mechanisms, including further distal 5'-upstream regions, should be assumed to participate in transcription of the PCAF gene, and the examined distal and

proximal 5'-upstream regions did not correlate directly/closely with its transcription, regardless of the loose form in both DT40 cells and clone cl.2-2.

These results, together with the previous inference speculated from changing patterns in gene expressions of these transcription factors [Chap. 4], surely indicated that at the late cultivation stage clone cl.2-2 seems to be dependent on Pax5 and Aiolos and somewhat similar to wild-type DT40 cells in the manner for gene expressions of IgM H- and L-chains in appearance, and to be the major type (Fig. 7-1(L)), like clone cl.2-4, the reason for this will be mentioned later.

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-4, these five Lys residues possessed full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage and thereafter gradually lost the binding capacity to no binding ability based on their hyper-acetylation levels until the late stage (Fig. 7-15). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-4. Thereafter, the tightened chromatin structure should change to loose form until the late stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to low (or no) level at the early stage in clone cl.2-4 and thereafter gradually and certainly elevated to high level until the late stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-4, mainly K9/H3 (and probably K18/H3 and K27/H3) possessed less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. Thereafter, mainly the binding capacity of K9/H3 was certainly weakened to no binding ability based on the considerable hyper-acetylation levels at the late stage (Fig. 7-15). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-4 and thereafter change to loose (or considerable loose) form at the late stage. These facts agreed with the findings that the gene expression of Aiolos is certainly decreased to low (or no) level at the early stage in clone cl.2-4 and thereafter increased to high level until the late stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibited full (or less) binding ability

based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-15). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-4 and remain unchanged until the late stage. These facts agreed with the findings that the gene expression of EBF1 is dramatically suppressed to very low (or no) level at the early stage in clone cl.2-4 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, mainly K9/H3 (and possibly K27/H3) possessed less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. The less binding ability remained unchanged at the middle stage but thereafter obviously decreased to no binding ability based on the hyper- (or considerable hyper-) acetylation levels at the late stage (Fig. 7-15). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to somewhat loose form at the early and middle stages in clone cl.2-4 and thereafter changed to loose form at the late stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-4 and thereafter certainly elevated to high level at the late stage (see Fig. 7-10).

In both DT40 cells and clone cl.2-4 at any cultivation stages, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels with insignificant changes (in case of clone cl.2-4) (see Chap. 5). Accordingly, the structure of chromatin surrounding the proximal (and also distal) 5'-upstream region of the gene should be loose form in both DT40 cells and clone cl.2-4. However, as in DT40 cells, the gene expression of PCAF is really very low at the early stage in clone cl.2-4 but gradually/dramatically increased from the early via middle to late stages (see Fig. 7-10). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, should be assumed to participate in transcription of the gene, and the examined distal and proximal 5'-upstream regions are assumed to poorly correlate with its transcription, regardless of their loose form in both DT40 cells and clone cl.2-4.

These results and those obtained for clone cl.2-2, together with the previous inference speculated from changing patterns in gene expressions of these transcription factors in initial and new HDAC2(-/-) mutants [Chaps. 2, 4 and 5], surely indicated that at the late cultivation stage clone cl.2-4, like clone cl.2-2 (and clones cl.2-3 and cl.2-5), seems to be dependent on Pax5 and Aiolos and somewhat similar to wild type DT40 cells in the manner of gene expressions of IgM H- and L-chains in appearance (Fig. 7-1(L)) and also to be the major type.

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-6, those exhibited full binding ability based on their hypo-acetylation levels at the early stage and thereafter gradually lost the binding capacity to no binding ability based on their hypo-acetylation levels until the late stage (Fig. 7-16). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-6. Thereafter, the tightened chromatin structure should change to loose form via the middle to late stages. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to low (or no) level at the early stage in clone cl.2-6 and thereafter gradually/dramatically elevated to high level until the late stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-6, mainly K9/H3 and K27/H3 certainly strengthened the binding capacity of histone H3 to DNA to full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage (Fig. 7-16). Thereafter, the full or less binding ability of these five Lys residues was gradually weakened to no binding ability based on their hyper-acetylation levels via the middle to late stages. Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight (or somewhat loose) form at the early stage in clone cl.2-6 and thereafter change to loose form at the late stage. These facts agreed with the findings that the gene expression of Aiolos is drastically suppressed to low (or no) level at the early stage in clone cl.2-6 and thereafter dramatically increased to high level at the late stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possessed no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-6, mainly K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibited full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage and lost the binding capacity to no binding ability based on their hyper-acetylation levels via the middle until late stages (Fig. 7-16). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-6 and thereafter change to loose form via the middle until late stages. These facts agreed with the findings that the gene expression of EBF1 is drastically suppressed to very low (or no) level at the early stage in clone cl.2-6 and thereafter gradually elevated until the late stage to almost the same levels in DT40 cells (see Fig.

7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-6, especially K9/H3 and K27/H3 (and probably K23/H3) obviously exhibited full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. Thereafter, the full or less binding ability was slightly reduced at the middle stage and further decreased to no binding ability based on their hyper-acetylation levels at the late stage (Fig. 7-16). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene, which is loose form in DT40 cells, should change to tight form at the early stage in clone cl.2-6 and thereafter change to loose form via the middle to late stages. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early stage in clone cl.2-6 and thereafter certainly elevated to high level via the middle to late stages (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA within chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels. Interestingly, in clone cl.2-6, mainly K9/H3 and K18/H3 possessed less binding ability based on their somewhat hyper-acetylation levels at the early stage, but thereafter lost the binding capacity to no binding ability based on their hyper-acetylation levels at the middle stage and again gained less binding ability based on their somewhat hyper-acetylation levels at the late stage (see Chap. 5). Accordingly, the structure of chromatin surrounding the proximal 5'-upstream region of the gene should be loose form in both DT40 cells and clone cl.2-6 at the middle stage but somewhat loose form at the early and late stages in clone cl.2-6. However, in clone cl.2-6, the gene expression of the PCAF gene is also very low at the early stage, and thereafter gradually/obviously increased until near middle stages but again dramatically decreased to very low level via the middle to late stages (see Fig. 7-10). Based on these results, other unknown mechanisms, including further distal 5'-upstream regions, should be assumed to participate in transcription of the gene, and reversely the examined distal and proximal 5'-upstream regions are not directly correlated with its transcription so much, regardless of loose form of chromatin structure in both DT40 cells and clone cl.2-6.

These results, together with the previous inference speculated from changing patterns in gene expressions of these transcription factors [Chap. 4], surely indicated that at the late cultivation stage clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 (Fig. 7-1(L)) and most similar to wild-type DT40 cells in the manner of gene expressions of IgM H- and L-chains in appearance.

CONCLUSION

As finishing touches, based on the above-mentioned results (Figs. 7-1 to 7-17) [Chaps. 2, 3, 4 and 5] and newly noticed morphological findings (Figs. 7-18 to 7-20) [Chap. 6], together with other findings [41, 50, 54, 57, 77, 80], we revised the previous brief hypothesis [Chaps. 4 and 5] and newly proposed an all-inclusive hypothesis on manners for gain of new cell function to exclude artificially accumulated IgM H- and L-chains through irreversible creation of distinct chromatin structure plasticity with epigenetic modifications in individual clones of HDAC2(-/-) mutants during continuous cultivation [Chap. 6]. We presumed that putative environment change recognition receptor/site (ECRR/ECRS) recognizes accumulation of these two immunoglobulin proteins as unfavorable environment change (and partly acts in signal transduction on the accumulation to chromatin structure). In addition, we assumed that putative chromatin conformation (structure) change complex (4C) machinery, which consists of particular member of HATs, particular member of HDACs and other components, directly/irreversibly creates chromatin structure plasticity surrounding proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of particular genes through successive conformation changes with epigenetic modifications (and partly acts in the signal transduction).

Excessive amounts of IgM H- and L-chains caused artificially by the HDAC2-deficiency are first accumulated (probably as their large protein complex with each other [54]) within endoplasmic reticulum of HDAC2(-/-) mutant cells. Most of these accumulated immunoglobulin proteins are gradually secreted to outside of cells, whereas, some of them are transported to nuclear envelope but not inside of nucleus and kept at peri-nuclear space not only at the early but also at the late cultivation stages (Figs. 7-18 and 7-19) [Chap. 6]. These accumulated immunoglobulin proteins at peri-nuclear space should bind to ECRR/ECRS localized on inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 7-20). After ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as unfavorable environment change, the putative signal concerning it is genome-widely transmitted to chromatin structure surrounding proximal 5'-upstream regions of numerous genes (probably located on several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes and/or related factors/enzymes (Figs. 7-20 and 7-21). Successively, the spontaneous unbalanced signal transduction and response for the environment change are consecutively and separately converged not only on the particular genes but also in individual clones of HDAC2(-/-) mutants. The 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes in wild-type DT40 cells probably contains HDAC2 as HDAC activity and a particular HAT member (e.g., GCN5) as HAT activity and many other factors (Fig. 7-21). By contrast, in all of individual HDAC2(-/-) mutant clones, at the very early stage (just soon after the birth by gene targeting techniques), bulk conformation of the 4C machinery should be dramatically changed to remove or drastically reduce the HAT activity (of the assumed HAT member) associated with the HDAC2-deficiency. Through the above-mentioned processes during cultivation, the 4C machinery

should come to newly contain another particular member of HDACs (except HDAC2), particular member of HATs and many other factors, and thereby becomes diverse.

The diversity of alterations in chromatin structure is preferentially originated from varied acetylation and/or deacetylation levels of one or more of the particular Lys residues at N-terminal tail of histone H3 [Chap. 5], which are cooperatively caused by proper member of HATs and proper member of HDACs in the protean 4C machinery. These successive epigenetic modifications with acetyl group of particular Lys residues, such as K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3), result in irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions for the above-mentioned targeted genes. By contrast, this protean 4C machinery should not be able to change chromatin structure surrounding open reading frames (coding regions) of these targeted genes so much. As a result, the proximal 5'-upstream chromatin region possessing hyper-acetylation levels of one or more of the particular Lys residues is loose (open) form based on no binding ability of histone H3 to DNA, and that possessing hypo-acetylation levels of one or more of the particular Lys residues is tight (closed) form based on binding ability of histone H3 to DNA (Figs. 7-17 and 7-21). Thus, as the need arises, transcription factor complex (TFC) machinery, which consists of RNA polymerase, proper transcription factors, particular member of HATs, particular member of HDACs and other factors (Fig. 7-21), is able to bind to promoter regions (or elements) within loose (but not tight) form of chromatin structure surrounding proximal 5'-upstream regions of the targeted (but not untargeted) genes, which have become latently active (but not inactive) state, followed by initiation of their transcriptions. Consequently, individual clones of HDAC2(-/-) mutants exhibit flexible, elastic and pluri-potent ability for gain of new cell function to diminish increased gene expressions of IgM H- and L-chains in distinct manners, i.e., increases and/or decreases in transcriptions of Pax5, Aiolos, EBF1 and OBF1 genes through irreversible creation of distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation, even though they are completely the same cell type having the same genotype and are also established cell line (Fig. 7-21) [54, Chaps. 2, 4, 5 and 6].

We expanded the above-mentioned hypothesis on the diminution of excessively accumulated IgM H- and L-chains in HDAC2(-/-) mutants to a universal hypothetic concept (principle) for gain of new cell function through irreversible creation of distinct chromatin structure plasticity with epigenetic modifications in higher eukaryotic cells via a lot of generations (cell divisions) [Chap. 6]. When higher eukaryotic somatic cells firstly in their life encounter with the change in intra- and/or extra-cellular environment, in order to adapt for or eliminate it (if uncomfortable), they have gradually come to gain new cell function via a lot of generations. Namely, the somatic cells have acquired the ability to adapt themselves to newly encountered environment change and/or to eliminate the painful environment change.

Using ECRR/ECRS, the 4C machinery and other components, the cells should cause the chain reaction of response for the environment change as follows. First of all, the environment change should be recognized by means of ECRR/ECRS, which may be localized nearby nuclear membrane as a cytoplasm-nucleus barrier (probably at inner nuclear membrane where hetero-chromatin is possibly located) (Fig. 7-20). There is a possibility that at this step putative intermediary sensor molecule(s) should act for the recognition of the environment change. As a next step, the putative signal(s) on the change should be genome-widely transmitted to chromatin structure within nucleus via numerous generations (cell divisions). Following the initial acceptance of the putative signal(s) at chromatin structure, gene expressions of various chromatin-modifying enzymes, transcription factors and/or related enzymes/factors may slightly change, coupled with a slight alteration in their chromatin conformation. Both of the signal transduction and the response for the environment change should be successively repeated and converged into restricted chromatin structure surrounding proximal 5'-upstream regions of particular targeted factor and/or enzyme genes. Finally, these successive signal transduction and response concerning the environment change should cause various epigenetic modifications of histones and/or DNA within the restricted chromatin regions with acetyl group, methyl group, phosphate group and/or others. Of these various epigenetic modifications, acetylation and/or deacetylation of particular Lys residues of core histones (H2A, H2B, H3 and H4) may be major ones [23-26, 28-37]. The 4C machinery, which comprises particular member of HATs, particular member of HDACs and also other factors, should be preferentially involved in the epigenetic modifications with acetyl group. Participating positions of Lys residues and/or kinds of core histones should be diverse. For example, in the above-mentioned case [Chap. 5], acetylation and/or deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. In consequence, epigenetic modifications of one or more of these particular Lys residues of histone H3 with acetyl group should change within proximal 5'-upstream chromatin regions of particular targeted genes via a lot of generations (cell divisions). Distinct functions of the protean 4C machineries on such acetylation levels should be mainly based on different combinations of each member of HATs and HDACs in them, because any HAT and HDAC members' own activities must be unchangeable, just as those of almost all enzymes in any biological reactions. By contrast, these protean 4C machineries may not act so much in alterations in chromatin structure surrounding open reading frames of corresponding genes.

Furthermore, the binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from acetylation levels of one or more of these particular Lys residues, though which Lys residue(s) is really and/or mainly involved in the binding is still undefined. Namely, hyper- (high) or hypo- (low or no) acetylation levels should induce no binding or binding ability of histone H3, resulting in loose (open) or tight (closed) form of chromatin structure. Thus, chromatin structure plasticity should be created irreversibly based on the successive conformation changes (Figs. 7-21 and

7-22). Whenever the need arises, the TFC machinery can bind to promoter regions (or elements) within loose form of chromatin structure surrounding proximal 5'-upstream regions of targeted genes (which are latently active state), and thereby can initiate their transcriptions (Fig. 7-21). By contrast, the TFC machinery cannot bind to promoter regions (or elements) within tight form of chromatin structure surrounding proximal 5'-upstream regions of untargeted genes (which are latently inactive state), followed by no initiation of their transcriptions. Consequently, loose or tight form of chromatin structure surrounding proximal 5'-upstream region should cause high or low (or no) transcription level of corresponding gene. Remarkably, manners for irreversible creation of chromatin structure plasticity should be distinct in individual clones of the same cell type having the same genotype, even though the environment change and signal(s) on it are the same for all of them. That is, to gain new cell function, in spite of the same environment change, individual cell clones each may possess ability not only to alter complicatedly/diversely chromatin structure surrounding proximal 5'-upstream regions of numerous genes but also to change that surrounding the proximal 5'-upstream region of the same gene into diverse forms. Consequently, gene expressions of a set of various particular enzymes and factors should be changed differently in individual cell clones via repeated cell divisions, and as a result they are able to newly/separately gain the same (or distinct) cell function(s) in distinct manners via a lot of generations.

As a result of the theoretical prediction mentioned above, in higher eukaryotes, for gain of new cell function, somatic cells (and also tumor cells) exhibit pluri-potency, elasticity and flexibility, all of which should be basically originated from those of their chromatin structure. That is, somatic cells of higher eukaryotes possess ability to gain new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications, i.e., from tight to loose forms or vice versa of chromatin structure surrounding proximal 5'-upstream region(s) of particular gene(s), in order to adapt themselves to intra- and/or extra-cellular environment change (Fig. 7-23). Such loose or tight form of the proximal 5'-upstream chromatin structure should be latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region (as mere nucleotide sequences) of the gene is probably potential but silent state for its transcription. Variety of irreversible creation of chromatin structure plasticity in individual clones of the same cell type should be basically triggered by the spontaneous unbalanced response for the environment change when they firstly encounter with it and accomplished by the successive convergence of the unbalanced response via a lot of generations (cell divisions). Moreover, chromatin structure plasticity in somatic cells should be created irreversibly in distinct manners, which are probably dependent upon their antecedents. Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, should be inherited to descendent generations associated with or without additional structural change via cell divisions. Remarkably, irreversible creation of chromatin structure plasticity should occur 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/or enzyme reactions occur in the cell itself which accepts proper signal (Fig. 7-22). With the intention of adapting to the environment change, irreversible creation of chromatin structure plasticity should occur inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region, as if it is coxswain, should direct the switch for latent transcription ability of the corresponding gene through irreversible creation of chromatin structure plasticity (Fig. 7-23). Therefore, besides "notch of chromatin" from a structural side-view as mentioned above, the proximal 5'-upstream chromatin region could be regarded as "director for transcription" from a functional side-view. Of course, the notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator and others) and their neighboring nucleotide sequences. We should like to emphasize that real recipient of signal on the environment change may be just chromatin structure itself as three-dimensional conformation which is dynamic and changeable between loose and tight forms, but not mere chromatin and chromosome themselves as one- (or two-) dimensional conformation which is static and unchangeable. Naturally, chromatin structure of proximal 5'-upstream chromatin region (notch or director), as dynamic and changeable three-dimensional conformation, undertakes 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 conformational change responsive to the signal. These ideas are based on the facts that almost all of macromolecules (such as proteins) generally exhibit characteristic steric conformations and thereby possess abilities to receive particular signal(s) and to express their own biological functions. However, concrete data are not enough to support these ideas so far.

Finally, we named such bio-system for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications, which should be one of the most important manners for life maintenance and cell-type determination of higher eukaryotes, a chromatin conformation (structure) change code (4C) theory [see Chap. 6]. The 4C theory should open the door for gain of new cell function of higher eukaryotes and innovate the general concept on nature of somatic cells. Presumably, in the 4C theory, the supposed number of codes, which should determine complicated/varied characteristics of higher eukaryotic cells, could be roughly estimated based on combination/multiplication of the number of candidate genes and that of codes for each of these genes as follows. Most influential candidates should be genes of chromatin-modifying enzymes, transcription factors and related enzymes/factors, which are necessary for gain of new cell function and cell-type determination of higher eukaryotes. The number of codes for each of these candidate genes should be two, i.e., loose and tight forms of proximal 5'-upstream chromatin region, which directs the switch (on or off) for latent transcription ability of the corresponding gene. Incidentally, the 4C theory should be suitable as an explanation for development and differentiation of higher eukaryotes, because behavior of putative signal

concerning the environment change seems to considerably resemble that of certain substances (such as hormone, cytokine and nerve-transmission substance), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

In the 4C theory on exclusion of artificially accumulated IgM H- and L-chains in HDAC2(-/-) DT40 mutants during cultivation, following crucial questions remain to be clarified yet [Chaps. 2, 4, 5 and 6]. 1) Despite deficiency of HDAC2 activity, why acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes are decreased at the early cultivation stage in HDAC2(-/-) mutant cells. 2) Why the decreased acetylation levels of one or more of K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 within proximal 5'-upstream chromatin regions of these genes are increased during cultivation. Why the case of the OBF1 gene is opposite. 3) It must be determined which Lys residue(s) of K9, K14, K18, K23 and K27 of histone H3 really and/or mainly binds to DNA within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes. 4) Steric and functional differences between loose and tight forms (which should be based on hyper- and hypo-acetylation levels of one or more of the particular Lys residues of histone H3) of chromatin structure surrounding proximal 5'-upstream regions of these genes must be more minutely clarified. 5) Why changing patterns in acetylation levels of the above-mentioned particular Lys residues of histone H3 during cultivation in individual transcription factor genes differ among each mutant clone. 6) Why changing patterns in acetylation levels of the above-mentioned particular Lys residues of histone H3 during cultivation in each transcription factor gene differ within individual mutant clones. 7) How the 4C machinery (which acts in irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions) differs from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in transcriptions of open reading frames of corresponding genes) may be almost the same as the well-known transcription machinery. 8) To demonstrate the 4C theory, both of ECRR/ECRS as a first player to recognize the environment change and the 4C machinery as a final player to create irreversibly chromatin structure plasticity must be clarified. 9) Clarification of effects of changes in temperature, atmosphere and nutrition on ability to gain new cell function in established cell lines via a lot of cell divisions and in model animals (such as mice and rats) during development/differentiation may be effective and powerful as concrete approach to generalize the 4C theory.

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FIGURE LEGENDS

Figure 7-1. Manners for control of gene expressions of IgM H- and L-chains through control of gene expressions of particular transcription factors in the presence or absence of HDAC2 in wild-type DT40 cells (W) or all and individual clones of HDAC2(-/-) mutant cells at early (E) and late (L) cultivation stages

The figure is a set of Figs. 4-8, 4-9 and 4-10 in [Chap. 4] and also identical with Suppl. Fig. 6-S1 in [Chap. 6].

Figure 7-2. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutant cells during cultivation

2D-PAGE was performed on total cellular proteins prepared from HDAC2(-/-) mutant cells at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W). H and L indicate IgM H- and L-chains. The figure is identical with Fig. 2-1 in [Chap. 2].

Figure 7-3. Alterations in amounts of IgM H-chain proteins in HDAC2(-/-) DT40 mutant cells during cultivation

Immuno-electron microscopy was performed on HDAC2(-/-) mutant cells at the early (E) and late (L) cultivation stages and DT40 cells (W), using anti-chicken IgM H-chain antiserum. A large amount of accumulated IgM H-chain proteins were detected only at the early (E) stage in mutant cells. The figure is identical with Fig. 2-3 in [Chap. 2].

Figure 7-4. Alterations in gene expressions of IgM H- and L-chains, and core histones in HDAC2(-/-) DT40 mutant cells during cultivation

Total RNAs were extracted from three HDAC2(-/-) mutant clones at the early (E; ~20 days), middle (M; ~40 days) and late (L; ~60 days) cultivation stages and DT40 cells (W). RT-PCR was performed on total RNAs, using appropriate primers for HDAC2, whole, secreted plus membrane-bound forms of IgM H-chain and IgM L-chain, and core histones H2A, H2B, H3 and H4. The figure is identical with Fig. 2-4 in [Chap. 2].

Figure 7-5. Alterations in amounts of IgM H- and L-chains in Pax5(-) DT40 mutant cells during cultivation

Western blotting was performed on total cellular proteins prepared from three Pax5(-) mutant clones at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W), using anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain. The figure is identical with Fig. 3-3 in [Chap. 3].

Figure 7-6. Morphology of Pax5(-) DT40 mutant cells during cultivation

Microscopy was performed on Pax5(-) mutant cells at the early (E) and late (L) cultivation stages and DT40 cells (W). The figure is identical with Suppl. Fig. 3-S1 in [Chap. 3].

Figure 7-7. Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutant cells during cultivation

RT-PCR was performed on total RNAs prepared from three Pax5(-) mutant clones at the first (F), early (E), middle (M) and late (L) cultivation stages and DT40 cells (W), using appropriate primers for Pax5, and whole, secreted plus membrane-bound forms of IgM H-chain and IgM L-chain. The figure is identical with Fig. 3-5 in [Chap. 3].

Figure 7-8. Genomic organization of chicken HDAC2 gene, generation of HDAC2(-/-) DT40 mutant cells and alterations in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) mutant clones during cultivation

A) Schematic representation of chicken HDAC2 genomic locus (top) with enlarged drawing of the targeted region (middle) and its targeted alleles (two bottoms). B) Southern blotting of homologous recombination for DT40, one heterozygous mutant clone (-/+) and six homozygous mutant clones (-/-). C) Western blotting for six HDAC2(-/-) mutant clones at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W). IgM H and L indicate IgM H-chain and IgM L-chain. D) RT-PCR for six HDAC2(-/-) mutant clones at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W). The figure is identical with Fig. 4-1 in [Chap. 4].

Figure 7-9. Alterations in amounts of IgM H- and L-chains in individual HDAC2(-/-) DT40 mutant clones during cultivation

Western blotting was performed on total cellular proteins prepared from six HDAC2(-/-) mutant clones at indicated cultivation periods, including the early (E), middle (M) and late (L) stages and DT40 cells (W). Proteins were sequentially detected with anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain (top and third) and anti-chicken IgM H-chain antiserum (second). The figure is identical with Fig. 4-2 in [Chap. 4] and shown with slight modifications.

Figure 7-10. Alterations in gene expressions of IgM H- and L-chains, members of HATs, HDACs and transcription factors in individual HDAC2(-/-) DT40 mutant clones during cultivation

RT-PCR was performed on total RNAs prepared from four HDAC2(-/-) mutant clones at indicated periods of cultivation, including the early (E), middle (M) and late (L) stages and DT40 cells (W), using appropriate primers for IgM H- and L-chains, PCAF, HDAC9, Pax5, Aiolos, EBF1, Blimp1, OBF1,

HDAC7, Ikaros, E2A, PU.1 and XBP-1. The figure is identical with Fig. 4-6 in [Chap. 4] and shown with slight modifications.

Figure 7-11. Alterations in amounts of IgM H-chain in HDAC2(-/-) DT40 mutant clones during cultivation

Electron microscopy (upper panels) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (lower panels) were carried out on four HDAC2(-/-) mutant clones collected at the early (E; ~5 days) and late (L; ~60 days) cultivation stages and DT40 cells (W). Dense cytoplasmic fractions due to accumulated IgM H-chain were observed only at the early (E) stage in four mutant clones (E in upper panels). Positive signals for IgM H-chains were observed only at the early (E) stage in four mutant clones (E in lower panels). The figure is identical with Fig. 4-4 in [Chap. 4].

Figure 7-12. Alterations of morphology of individual HDAC2(-/-) DT40 mutant clones during cultivation

Microscopy was performed on four HDAC2(-/-) mutant clones at the early (E) and late (L) cultivation stages and DT40 cells (W). Aggregative form was observed only at the early (E) stage in all of mutant clones. The figure is identical with Fig. 4-5 in [Chap. 4].

Figure 7-13. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-) mutant clone cl.2-1 during cultivation

The figure is a set of Figs. 5-1, 5-5, 5-9 and 5-13 in [Chap. 5].

Figure 7-14. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-) mutant clone cl.2-2 during cultivation

The figure is a set of Figs. 5-2, 5-6, 5-10 and 5-14 in [Chap. 5].

Figure 7-15. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-) mutant clone cl.2-4 during cultivation

The figure is a set of Figs. 5-3, 5-7, 5-11 and 5-15 in [Chap. 5].

Figure 7-16. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-)

mutant clone cl.2-6 during cultivation

The figure is a set of Figs. 5-4, 5-8, 5-12 and 5-16 in [Chap. 5].

Figure 7-17. Summary on alterations in mRNA levels (high or low), acetylation levels (hyper or hypo) of particular Lys residues of histone H3 and chromatin structure (loose or tight) within proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in four HDAC2(-/-) mutant clones at early (E), middle (M) and late (L) cultivation stages and DT40 cells (W)

The figure is identical with Suppl. Fig. 6-S10 in [Chap. 6] and shown with some modifications.

Figure 7-18. Localization of IgM H-chain proteins at peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was performed on HDAC2(-/-) mutant cells at the early cultivation stage after treatment without (A) or with saponin (B ~ E). Arrows P, E and S indicate IgM H-chain proteins localized at peri-nuclear space, endoplasmic reticulum and surface. The figure is identical with Fig. 6-1 in [Chap. 6].

Figure 7-19. Localization of IgM H-chain proteins in peri-nuclear space of HDAC2(-/-) DT40 mutant cells at early and late stages of cultivation

Immuno-electron microscopy was carried out on HDAC2(-/-) mutant cells collected at the early (E) and late (L) cultivation stages, using anti-chicken IgM H-chain antiserum. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early 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 HDAC2(-/-) mutant cells at the late stage. E), F), H), I) and K) Enlarged versions of parts indicated by arrows e, f, h, i and k in D), G) and J). The mutant cell in G) was the same one in [Chap. 2]. Accumulated IgM H-chain proteins were observed in peri-nuclear space of all HDAC2(-/-) mutant cells at the early and late stages. The figure is identical with Fig. 6-3 in [Chap. 6].

Figure 7-20. A model for signal transduction on IgM H- and L-chains accumulated in peri-nuclear space to chromatin structure in HDAC2(-/-) DT40 mutant cells

Left panel: Immuno-electron microscopy revealed that artificially accumulated IgM H-chain proteins were localized in peri-nuclear space of HDAC2(-/-) mutant cells. Right panel: A model for signal transduction on accumulation of IgM H- and L-chains. Signal on excessively accumulated IgM H- and L-chains in peri-nuclear space of HDAC2(-/-) mutant cells was repeatedly transmitted to chromatin structure, followed by unbalanced correspondence and convergence for the signal to particular genes (Pax5, Aiolos, EBF1, OBF1, etc.) in individual mutant clones. ECRR/ECRS: environment change

recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery. The figure is identical with Fig. 6-4 in [Chap. 6].

Figure 7-21. Summary on alterations in mRNA levels of IgM H- and L-chains, Pax5, Aiolos, EBF1 and OBF1 genes, and acetylation levels of particular Lys residues of histone H3 and chromatin structure of their proximal 5'-upstream regions in individual HDAC2(-/-) DT40 mutant clones during cultivation

Alterations in mRNA levels of IgM H- and L-chains, Pax5, Aiolos, EBF1 and OBF1 genes, and acetylation levels (Ac) of particular Lys residues of histone H3 and chromatin structure (loose or tight) of the proximal 5'-upstream regions of the latter four genes in four HDAC2(-/-) mutant clones at the early (E) and late (L) cultivation stages and DT40 cells (W) are schematically represented. The 4C machinery should contain particular member of each of HATs and HDACs and others. The TFC machinery should contain RNA polymerase, particular transcription factors and others. The figure is identical with Fig. 6-5 in [Chap. 6].

Figure 7-22. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region in descendent cells and reversible regulations of gene expression and enzyme reaction in cells which initially accept proper signal

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region of particular gene occurs in descendent cells but not in initial cells that accept signal on environment change (upper panel). Regulations of ordinary gene expressions and enzyme reactions occur reversibly in the cells that accept proper signal (middle and lower panels). The figure is identical with Fig. 6-7 in [Chap. 6].

Figure 7-23. Schematic representation of chromatin conformation change code (4C) theory for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations

The figure is identical with Fig. 6-6 in [Chap. 6].