学位論文

Involvement of Histone H3 lysine 79 methylation and Dot1L in the mechanism regulating totipotency in mouse preimplantation embryos

（マウス着床前初期発生における分化全能性調節への Histone H3 lysine 79 メチル化および Dot1L の関与）

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Contents

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Abbreviations

BSA: bovine serum albumin

Dot1: Disruptor of telomeric silencing 1

Dot1L: Disruptor of telomeric silencing 1 like

GCN5: General Control Nonderepressible protein 5

GFP: green fluorescence protein

GO: growing oocytes

GV: oocytes at the germinal vesicle stage

HEK: Human Embryonic Kidney

Hpi: Hours post insemination

H3K79me2: Histone H3 lysine 79 dimethylation

K: lysine

MII: metaphase of meiosis II

NED: nuclear export domain

NES: nuclear export signal

NLS: nuclear localization signal

ubH2B: ubiquitinated histone H2B

Summary

After fertilization terminally differentiated oocytes acquire totipotency. This property is maintained during early preimplantation development, but is lost around the late-preimplantation stage. The mechanism underlying acquisition and maintenance of the totipotent state remains largely unknown. Oocytes express the genes in their specific pattern during oogenesis. This pattern is drastically altered into zygote-specific one after fertilization and thereafter it is dynamically changed during preimplantation development. In general, gene expression is epigenetically regulated. When cells are divided, some epigenetic modifications are inherited from parental cells into daughter ones to maintain the gene expression pattern. This mechanism is called as “cell memory” and the epigenetic modifications inherited into the daughter cells are referred to as cell memory markers. As gene expression pattern is dynamically changed after fertilization, cell memory seems to be detrimental mechanisms for early preimplantation embryos to acquire and maintenance of totipotency. Therefore, I hypothesized that cell memory mechanisms might not function after fertilization.

In this thesis, to understand the mechanism regulating the acquisition and maintenance of totipotency in the early preimplantation embryos, I examined the dynamic changes of Histone H3 Lysine 79

dimethylation (H3K79me2), a cell memory marker of the active genes, during oogenesis and preimplantation development, and then investigated the mechanisms that regulate H3K79me2 during 1- and 2-cell stages.

In Chapter 1, I show that H3K79me2 was present during oocytes growth and maturation. However, it was eliminated just after fertilization and the H3K79 hypomethylation state was sustained until 4-cell stage. At the blastocyst stage H3K79me2 increased. Therefore, it was possible that elimination of H3K79me2 plays a role in acquisition of totipotency. To investigate the involvement of H3K79me2 elimination in acquisition of totipotency, I examined H3K79me2 in the somatic nuclei transplanted into enucleated unfertilized oocytes in which totipotency is acquired. H3K79me2 was eliminated in the transplanted somatic nuclei soon after parthenogenetic activation. In contrast, the elimination of H3K79me2 did not occur in the nuclei transplanted into the parthenogenetically activated embryos in which totipotency is not acquired. These results suggest that the erasing of H3K79me2 is involved in the acquisition of totipotency after fertilization.

In chapter 2, I examined the dynamics of Dot1L (Disruptor of telomeric silencing 1 like) which is thought to be sole H3K79 methyltransferase to understand the mechanisms for maintenance of H3K79 hypomethylation state during 1- and 2-cell stages. At 1-cell stage, Dot1L was localized in the pronuclei. However ubiquitinated histone H2B (ubH2B),

which is required for Dot1L-mediated H3K79 methylation, was at a low level. On the other hand, at 2-cell stage, although ubH2B was detected at a high level, Dot1L was exported from the nuclei by the mechanism involving its nuclear export domain 1 (NED1) and 2 (NED2). Thus, the absence of H3K79me2 is maintained by different mechanisms during 1- and 2-cell stages. A Dot1L deletion mutant that lacks NED1 and NED2 (referred to as N393-NLS1) is localized in nuclei at 2-cell stage. Expression of N393-NLS1 caused methylation of H3K79 and developmental arrest at 2-cell stage. These results suggest that 2-cell stage-specific nuclear export of Dot1L is important for the maintenance of H3K79 hypomethylation and the progression of preimplantation development.

In conclusion, H3K79me2 was eliminated after fertilization and sustained at a low level during 1- and 2-cell stage. These results suggest that totipotency is acquired by elimination of H3K79me2 and maintained by the absence of H3K79me2.

General introduction

After fertilization, terminally differentiated oocytes acquire totipotency (Figure GI-1). This property is sustained until 2 or 4-cell stage, and then lost during late preimplantation development. Totipotency is the ability of a single cell to produce all types of the cells. Indeed, a single blastomere of 2-cell stage embryos can develop into normal and fertile animal (Tarkowski, 1959). Although blastomeres of 4-cell stage embryos can develop into blastocyst-stage embryos but not into term due to their small size and the deficiency of the number of inner cell mass (ICM) at blastocyst-stage (Rossant, 1976), Kelly (1977) provided the evidence that at least some of blastomeres of 4-cell stage embryos is totipotent. The author showed that when a blastomere of 4-cell stage embryos was aggregated with the blastomeres derived from a different embryo to complement the deficiency of the size and cell number of ICM at blastocyst stage, the aggregated blastomeres could develop into normal and fertile mice. Furthermore, when the blastomeres of 4-cell stage embryos were aggregated with even tetraploid carrier embryos, they could produce normal mice (Tarkowski et al., 2001). Since tetraploid embryos cannot develop into term, these normal adult mice should be derived from 4-cell stage blastomeres. Thus, it was suggested that blastomeres of 4-cell stage embryos are

totipotent. At the blastocyst-stage, two distinct cell lineages, trophectoderm and inner cell mass, emerge. There is an ample knowledge about the mechanism, which regulates the differentiation into trophectoderm or inner cell mass at blastocyst stage (Niwa et al., 2005; Ralston and Rossant, 2008; Strumpf et al., 2005). However, the mechanism which regulates the totipotency during early preimplantation development remains largely unknown.

When the differentiation state is thus changed after fertilization and during preimplantation development, the gene expression pattern is also drastically altered during this period (Hamatani et al., 2004; Latham and Schultz, 2001; Wang et al., 2004). Although the gene expression pattern is maintained during oogenesis as oocyte-specific pattern expressing *c-mos*, *Zp3* etc., it is drastically altered into embryo specific one after fertilization (Figure GI-1). Moreover, the gene expression pattern of embryos is dynamically changed as embryos develop. However, the mechanism underlying regulation of gene expression pattern during preimplantation development also remains to be elucidated.

In differentiated somatic cells, the gene expression pattern is maintained from parental cells to the daughter cells so that the differentiation state is maintained after cell division. This mechanism is called as “cell memory”. It has been suggested that some epigenetic modifications play a role in this mechanism as “cell memory markers.” These

modifications are sustained in the actively transcribed gene after cell division. As a result, the information of active genes is conveyed to the daughter cells. Thus, epigenetic modifications have a role in cell memory of differentiated cells to maintain their specific gene expression patterns.

Histone modifications regulate gene expression by altering chromatin structure and functioning as the binding sites to transcription factors and chromatin modifiers. An octamer of core histone proteins (H2A, H2B, H3, and H4) which is wrapped with DNA to form nucleosome is a fundamental unit of chromatin. Recently, a line of evidence that histone proteins have roles in the regulation of gene expression has been accumulated. Histone proteins have various reversible covalent modifications, *e.g.* acetylation, methylation, ubiquitination, sumoylation and phosphorylation, which alter chromatin structures. In particular, acetylation and methylation are well known to be involved in regulation of gene expression. There are many highly conserved lysine residues in N-terminal tail of histone H3 and H4. Acetylation of lysine residues within histone tail leads to loss of positive charge of histone proteins. This positive charge contributes to strengthen the interaction with DNA which is negatively charged. Therefore, histone acetylation loosens the structure of nucleosome to be accessed easily by the transcription factors. Thus, histone acetylation has been considered to have a role in gene activation. Histone methylation is also found to occur in the histone tail. The most extensively studied histone

methylation are Histone H3 lysine 4 (H3K4) and lysine 9 (H3K9) methylation. It has been considered that the histone tail protrudes from the surface of nucleosome so that it could interact with various transcriptional factors and chromatin modifiers. Additionally, there is another lysine residue methylated, histone H3 lysine 79 (H3K79), which does not exist in the histone tail but in the globular domain (Ng et al., 2002a). H3K79 is highly conserved in most eukaryotic organisms from yeast to human (Ng et al., 2002a). Although the globular domain occurs at the center of the Histone H3 polypeptide chain, it localizes on the surface of the nucleosomal structure, so that it can be accessed by transcriptional factors (Feng et al., 2002; van Leeuwen et al., 2002).

H3K79 dimethylation (H3K79me2) has a role as a cell memory marker. In yeast, genome wide analysis of H3K79me2 revealed that 90% of the genome, which was actively transcribed, has H3K79 methylation, but around 10% of genome, which is silenced by binding of Sir proteins, does not (Laurenson and Rine, 1992; van Leeuwen et al., 2002). H3K79me2 does not occur in particular genome regions, *e.g.*, telomere and mating-type loci, in which Sir2 association with chromatin leads to heterochromatin formation (Ng et al., 2003; Sims et al., 2003; van Leeuwen et al., 2002). In Drosophila, chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) revealed that H3K79me2 is mostly present in active transcribed genes and there was correlation between the levels of H3K79me2 and

transcription (Schubeler et al., 2004). In mammalian cells, H3K79me2 occurs around transcription starting site (TSS), immediately down stream regions of TSS and coding regions of active transcribed genes (Steger et al., 2008; Wang et al., 2008). Although most of transcription factors are dissociated from the chromosome during mitosis where no transcription occurs (Hershkovitz and Riggs, 1995; Martinez-Balbas et al., 1995), H3K79me2 is retained on the genes that had been actively transcribed before mitosis (Kouskouti and Talianidis, 2005). Since H3K79me2 is thus sustained after mitotic transcriptional inactivation, it provides the cells with the information of the genes which should be transcribed after the cells exit mitosis. Therefore, H3K79me2 is thought to contribute to the faithful maintenance of gene expression pattern after cell division as a cell memory marker (Kouskouti and Talianidis, 2005; Nguyen and Zhang, 2011).

H3K79 methylation is catalyzed by Dot1 (Disrupter-of-telomere-silencing 1, also called Kmt4) family proteins. Dot1 and its orthologs are known to be sole H3K79 methyltransferase, because deletion of Dot1 and its orthologs in yeasts, flies, and mice resulted in complete disappearance of H3K79 methylation (Jones et al., 2008; Nguyen and Zhang, 2011; Shanower et al., 2005; van Leeuwen et al., 2002). At first, Dot1 was identified as a gene whose overexpression disrupts telomeric silencing in *S. cerevisiae* (Singer et al., 1998). Subsequently, Dot1 and its human orthologs DOT1L (Disrupter of telomeric silencing 1 like) were found

to have methyltransferase activity toward H3K79 in *S. cerevisiae* (Ng et al., 2002a) and human (Feng et al., 2002), respectively. Interestingly, Dot1 family proteins do not contain SET domain which is highly conserved in all other histone methyltransferases (Feng et al., 2002; Ng et al., 2002a). Dot1 family proteins belong to the Class I SAM (S-adenosyl-L-methionine)-dependent methyltransferase. Moreover, despite lacking of arginine methyltransferase activity, Dot1L family proteins have structural similarity to arginine methyltransferase (Nguyen and Zhang, 2011). Thus, Dot1 and its orthologs have many unique properties compared to other histone lysine methyltransferase. Therefore, Dot1L might be regulated by an unique pathway different from that of other histone methyltransferase. It is reported that mammalian Dot1L proteins form complexes with various transcriptional factors, which involve in transcriptional elongation. Mouse Dot1L (mDot1L) was reported to interact with positive transcription elongation factor b (p-TEFb) which phosphorylates Ser2 in the RNA polymerase II (RNA pol II) C-terminal domain (Bitoun et al., 2007). This Ser2 phosphorylation is needed for the transition from initiation to elongation during transcription by RNA pol II, suggesting that mDot1L has a role in transcriptional elongation. Thus, it is likely that Dot1L methylates H3K79 to maintain transcriptional active states of the genes (Nguyen and Zhang, 2011).

As described above, gene expression pattern is dynamically changed

during early preimplantation development. Since cell memory is important in the differentiated cells to maintain their gene expression pattern, it seems that cell memory is a mechanism detrimental for the establishment and maintenance of totipotent state in the early preimplantation embryos, which led me to the hypothesis that cell memory might not function during early preimplantation development. To address this hypothesis, I examined the changes in H3K79me2 as a cell memory marker during preimplantation development, and analyzed the dynamics of Dot1L to understand the regulatory mechanism of H3K79me2.

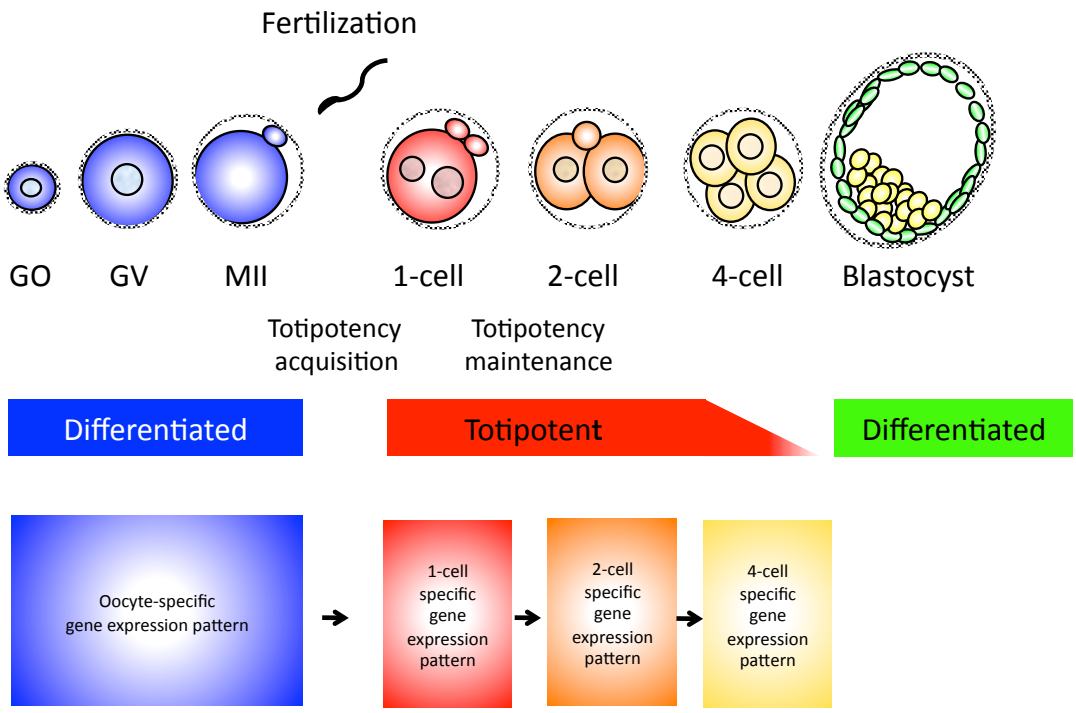

Figure GI-1. Acquisition and maintenance of totipotency during early preimplantation development.

Totipoitency is acquired after fertilization and maintained until 4-cell stage. Differentiation occurs at the blastocyst stage. When the differentiation state is thus changed after fertilization and during preimplantation development, the gene expression pattern is also drastically changed during this period.

Chapter 1

Changes in H3K79 methylation during preimplantation development in mice

Introduction

Once cells are terminally differentiated, their differentiation states are hardly changed. However, oocytes are unique cells which have the capability to change their differentiation state: they are converted into totipotent zygotes after fertilization. During this process, gene expression patterns are dramatically changed (Hamatani et al., 2004; Latham and Schultz, 2001; Wang et al., 2004). It seems likely that this alteration of gene expression pattern entails the elimination of cell memory markers to change the oocyte-specific gene expression pattern into the embryo specific one (Rideout et al., 2001). Since zygotic gene expression starts at mid- or late-1-cell stage (Aoki et al., 1997), the deletion of cell memory marker should occur soon after fertilization. However, the molecular mechanisms of the deletion of cell memory markers after fertilization remain to be elucidated.

It has already passed more than ten years since the report of birth of cloned sheep, Dolly (Wilmut et al., 1997). Until now, there are many reports which showed the accomplishment of the cloning of various animal species (Meissner and Jaenisch, 2006). Cloned animals were produced by nuclear transfer method in which a nucleus of somatic cell is transplanted into an enucleated unfertilized oocyte followed by parthenogenetic activation (Rideout et al., 2001; Wakayama et al., 1998; Wilmut et al., 1997). It is

therefore thought that the successful birth of cloned animals is due to the acquisition of totipotency by the transplanted nuclei in the oocytes after activation. In this process, cell memory markers would be deleted in the transplanted somatic nuclei so that somatic gene expression pattern is changed into embryonic one (Rideout et al., 2001). Thus, the nuclear transfer of somatic nuclei into oocytes is a useful model to understand the mechanisms for acquisition of totipotency.

Since H3K79me2 has been suggested to play a role as an cell memory marker as described in General introduction, there is a possibility that the removal of H3K79me2 is required for the transition from the oocytes-specific gene pattern into embryos-specific one, which leads to the establishment of totipotent state. To address this hypothesis, I examined the changes of H3K79me2 in the embryos after fertilization and in nuclear transferred embryos.

Results

Dynamics of H3K79 dimethylation during oogenesis and preimplantation development

The dynamics of H3K79me2 was analyzed by immunocytochemistry during oogenesis and preimplantation development. H3K79me2 was detected in the nuclei of growing oocytes, oocytes at the germinal vesicle stage and chromosomes of MII oocytes (Figure 1-1A). However, after fertilization, no signal of H3K79me2 was detected in the male pronucleus of the 1-cell embryos, but a low level of signal was detected in the female pronucleus. At 2-cell stage, the signal was completely lost. A weak signal appeared but still remained low at the 4-cell stage, and then the signal increased prominently at the blastocyst stage. Thus, H3K79me2 was almost lost after fertilization and maintained at the low level until 4-cell stage.

In somatic cells, H3K79me2 is regulated in a cell cycle-dependent manner in which H3K79me2 level decreases during S phase, reach the lowest point at G2 phase, increases during M phase, and is maintained at a high level during G1 phase (Feng et al., 2002). To examine whether the decrease in H3K79me2 also depends on DNA synthesis following fertilization, H3K79me2 was examined 4, 8, and 12 h after fertilization, corresponding to G1, S, and G2 phases, respectively. H3K79me2 decreased to a marginal level as early as 4 h after fertilization, at which time DNA synthesis had not yet

occurred (Figure1-1B). This result suggests that the decrease of H3K79me2 occurs soon after fertilization independently of DNA synthesis.

The specificity of the antibody used in these immunocytochemistry experiments was examined by the adsorption test with a specific antigen peptide (Figure 1-2). When anti-H3K79me2 antibody was pre-incubated with its antigen peptide, the signals were no longer detected in MII-stage oocytes (Figure 1-2), indicating that the antibody binds to H3K79me2 specifically.

These results suggest that cell memory marker H3K79me2 was eliminated after fertilization when totipotency is acquired and that the H3K79 hypomethylation state is sustained during early preimplantation development when totipotency is maintained.

Elimination of H3K79me2 from somatic nuclei transplanted into enucleated oocytes

The observation that rapid decrease of H3K79me2 occurs following fertilization supported the hypothesis that the decrease of H3K79me2 is involved in the acquisition of totipotency. To further address this hypothesis, H3K79me2 was examined in somatic nuclei transplanted into enucleated MII stage oocytes. A number of recent reports have demonstrated that somatic nuclei acquire totipotency after the transplantation into MII stage oocytes (Meissner and Jaenisch, 2006). The nucleus of NIH3T3 cell was transplanted into an enucleated MII stage oocyte and then H3K79me2 was

examined by immunocytochemistry. The results showed that the signal of H3K79me2 disappeared from the transplanted nucleus 5 h after parthenogenetic activation (Figure 1-3B and Table 1). However, it was clearly detected in the control nucleus which was embedded between the plasma membrane and the zona pellucida of the oocyte where the nucleus was not exposed to the cytoplasm of the oocytes. These results suggest that the cytoplasm of the early 1-cell embryo possesses the ability to eliminate H3K79me2 and that the decrease of H3K79me2 is involved in the acquisition of totipotency in the somatic nucleus transplanted into the oocyte.

Because it has been reported that the ability of the oocytes to confer totipotency to the transplanted somatic nuclei is lost as early as 5 to 6 h after fertilization (Wakayama et al., 2000), we examined whether the ability to eliminate H3K79me2 is also lost in the embryos around this time. The nucleus from a NIH3T3 cell was transplanted into an enucleated oocyte at 7 h after activation and then examined for H3K79me2 5 h later. We found that H3K79me2 in the transplanted nucleus remained at a high level comparable to the embedded nucleus after incubation for 5 h (Figure1-3C and Table 1). This indicates that the ability to eliminate H3K79me2 has been lost until the mid-1-cell stage.

Next, I examined whether the ability to eliminate H3K79me2 remains lost after cleaved into 2-cell stage. The nucleus of a NIH3T3 cell was transplanted into an enucleated embryo 10 h after parthenogenetic

activation and was examined for the change in H3K79me2 level after the cleavage into the 2-cell stage. Twelve hours after transplantation at which time the embryos had been cleaved into 2-cell stage, a strong signal of H3K79me2 was still detected at the level comparable to that at 2 h after transplantation (Figure1-4B). These results suggest that H3K79me2 is maintained during the late 1-cell and 2-cell stages because of no activity to eliminate H3K79 methylation at these stages. However, it is formally possible that the methyl group of H3K79 is once removed and then added during these stages. Therefore, I transplanted a pronucleus with no H3K79me2 into the parthenogenetically activated oocyte at the late 1-cell stage and allowed it to cleave into 2-cell stage. In this reconstructed oocyte, no H3K79me2 was detected (Figure1-4C), indicating that 2-cell embryos do not have the activity to methylate H3K79. Therefore, H3K79me2 in the transplanted nuclei of NIH3T3 cells seemed to be maintained in the 2-cell embryos. These results suggested that 2-cell stage embryos do not have the ability to eliminate H3K79me2. Taken together, this ability was only observed during the early 1-cell stage when the ability to confer totipotency to a transplanted somatic nucleus had been detected (Wakayama et al., 1998; Wilmut et al., 1997).

Discussion

In this chapter, I have shown that H3K79me2 which is thought to act as a cell memory marker disappeared from the genome in the 1-cell stage embryos after fertilization (Fig. 1-1). It also disappeared from the somatic nuclei transplanted into the enucleated oocytes after activation (Fig. 1-3). The gene expression patterns of differentiated oocytes and somatic nuclei transplanted into enucleated oocytes are changed into that of totipotent embryos after fertilization and activation, respectively. During these processes, cell memory markers that had previously sustained the gene expression patterns of the differentiated oocytes and somatic cells would be erased to start the new gene expression pattern. Therefore, I suggest that the disappearance of H3K79me2 is involved in deletion of the gene expression pattern of oocytes and somatic cells, which leads to acquisition of totipotency.

It was reported that some histone H2A and H3 variants, i.e. H2AZ, and H3.3, also play roles as cell memory markers (Brickner et al., 2007; Chow et al., 2005). The nucleosomes containing both of H3.3 and H2AZ are observed at transcriptional start site of actively transcribed genes (Jin et al., 2009). These variants also disappeared from the genome in the 1-cell embryos soon after fertilization (Akiyama et al., 2011; Nashun et al., 2010). Although it is not known whether H3K79me2 and these variants function for

the cell memory cooperatively or independently, H3.3 is enriched in K79 methylation when compared to other H3 variants (Hake et al., 2006). Therefore, it is possible that the chromatins of active genes are composed of H3.3 and H2A.Z at first and then Dot1L methylates K79 on their H3.3 to establish the cell memory.

The deletion of H3K79me2 occurred soon after fertilization (Fig. 1-1B). This result is consistent with the reports that transplanted somatic nuclei acquired totipotency when they were transplanted into enucleated bovine oocytes soon after the oocytes were activated (Schurmann et al., 2006; Sung et al., 2007). The ability to confer totipotency to a transplanted somatic nucleus decreases with time after the activation of enucleated oocytes; the percentage of transplanted oocytes that developed into the blastocyst stage gradually decreased by the time at which the somatic nucleus was transplanted, and no embryos that had been transplanted with the somatic nuclei 5 to 6 h after fertilization developed into the blastocyst stage (Wakayama et al., 2000). Consistent with this report, my results showed that H3K79me2 was not eliminated when the somatic nuclei had been transplanted into the enucleated embryos 7 h after activation (Figure 1-3C). Thus, I suggest that the elimination of H3K79me2 is involved in acquisition of totipotency, and the ability to erase H3K79 methylation seems to disappear during the early 1-cell stage.

The mechanism by which H3K79 methylation is deleted is unknown.

Although it is possible that an enzyme catalyzing the demethylation of H3K79 is activated soon after fertilization, such an enzyme has not yet been identified in any organisms (Nguyen and Zhang, 2011; Shukla et al., 2009). Alternatively, the histones methylated on K79 may be replaced by new ones that have not been methylated. There are three types of H3 variants, H3.1, H3.2 and H3.3, and K79 methylation is enriched on H3.3 as mentioned before (Hake et al., 2006). A previous work reported that histone H3.3 was once removed at G1 phase and then deposited at S phase after fertilization (Akiyama et al., 2011). Because of Dot1L cannot methylate free histone H3, newly deposited H3.3 is not methylated on K79 (Feng et al., 2002). The replacement of H3.3 might be thus involved in the deletion of H3K79me2.

H3K79me2 was hardly detected during early preimplantation development (Figure 1-1A). This result suggests that cell memory does not function in these stages. This is consistent with that gene expression pattern is dynamically changed during preimplantation development, because the absence of cell memory markers would facilitate the change in gene expression pattern. As described in General introduction, the embryos sustain totipotent state until 4-cell stage. Cell memory is the mechanism to maintain the differentiated state in the somatic cells. Therefore, the absence of cell memory might lead to the un-differentiated state of the early preimplantation embryos. It should be noted that H3K79me2 was slightly increased at 4-cell stage (Figure 1-1A). It was suggested that totipotency is

not an all-or-none property (Edwards and Beard, 1997) and decreases as embryos develop (Mitalipov and Wolf, 2009); 4-cell stage embryos are less totipotent than 1- and 2-cell stage embryos. Accordingly, slight increase of H3K79me2 might reflect this state of totipotency of 4-cell stage embryos.

In conclusion, H3K79me2 are removed soon after fertilization and remained at a very low level during early preimplantation development. These results suggested that the totipotency is acquired by erasing cell memory markers in the oocytes after fertilization and maintained by the absence of cell memory markers.

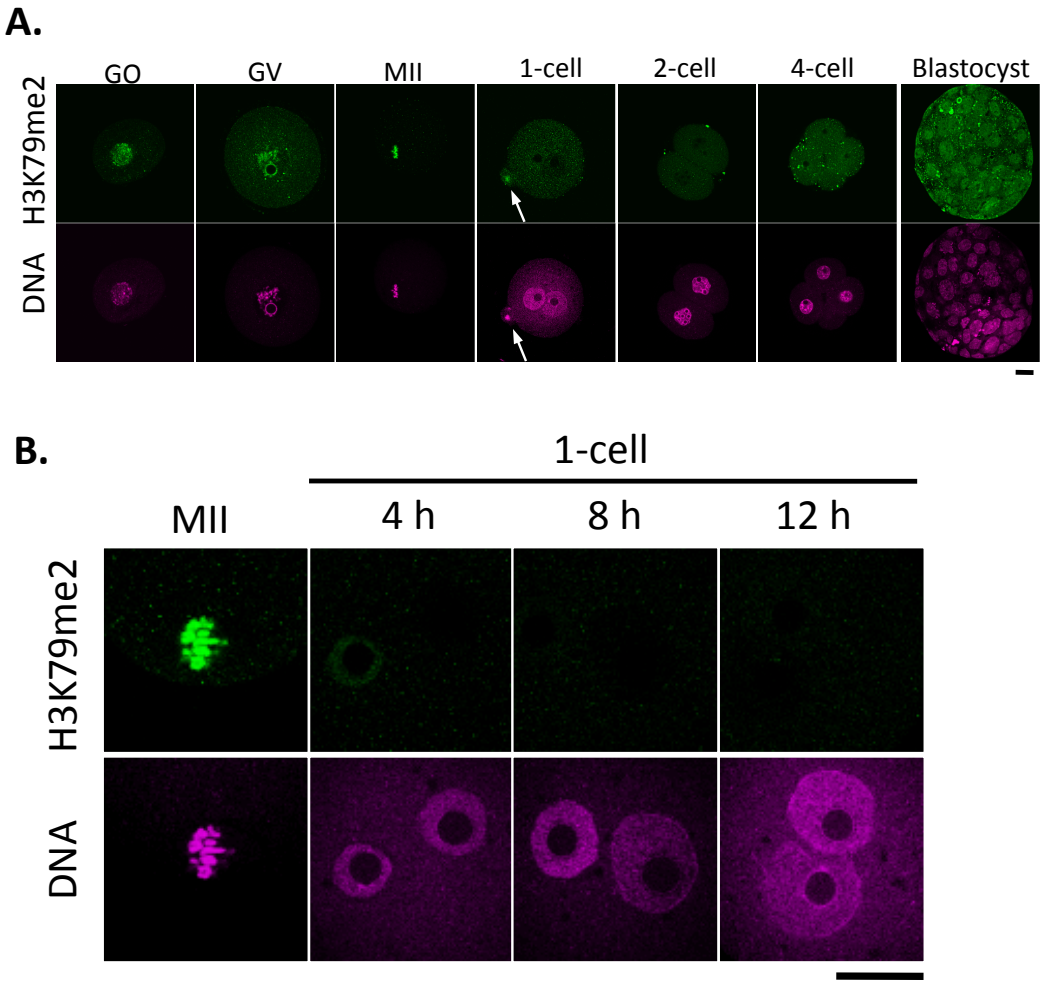

Figure 1-1 Changes in H3K79me2 during mouse oocyte growth and preimplantation development.

A. Oocytes and preimplantation embryos were immunostained with the anti-H3K79me2 antibody. GO indicates growing oocytes acquired from 13 days old mice. GV and MII indicate the oocytes at the germinal vesicle and MII stages, respectively. The embryos at the 1-cell stage, 1-cell; the embryos at the 2-cell stage, 2-cell; the embryos at the 4-cell stage, 4-cell; the embryos at the blastocyst stage, Blastocyst; were collected 12, 28, 45, 96 h after fertilization, respectively. Arrows indicate polar body. More than four independent experiments, in which more than 20 oocytes/embryos were observed in total, were performed in the analysis for each stage of oocytes and preimplantation embryos. Similar results were obtained in each experiment, and representative images are shown.

B. Unfertilized oocytes (MII) and 1-cell stage embryos that had been collected 4, 8, 12 h after fertilization were immunostained with the antibody against H3K79me2. More than three independent experiments, in which more than 26 embryos were analyzed in total, were conducted, except for the following analyses: 8, 10 h embryos in two experiments. Similar results were obtained in each experiment, and representative images are shown. Bar = 20 μm

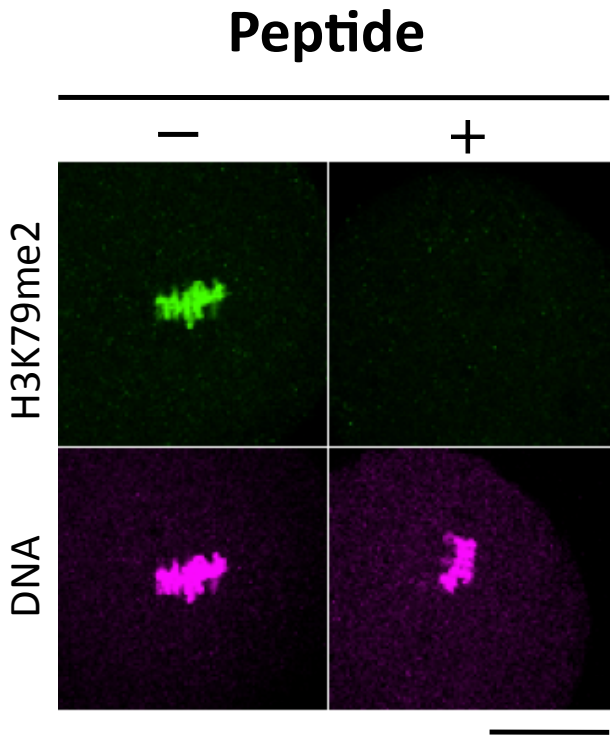

Figure 1-2. Specificity of the antibody against H3K79me2.
The antibody was incubated with (+) or without (-) 1 μg/ml antigen peptide (abcam; #ab4556) at room temperature for 1 h before immunocytochemistry of MII-stage oocytes. In two independent experiments, 13 (+) and 9 (-) oocytes were examined, respectively. Similar results were obtained in each experiment and representative images are shown above. Bar = 20 μm.

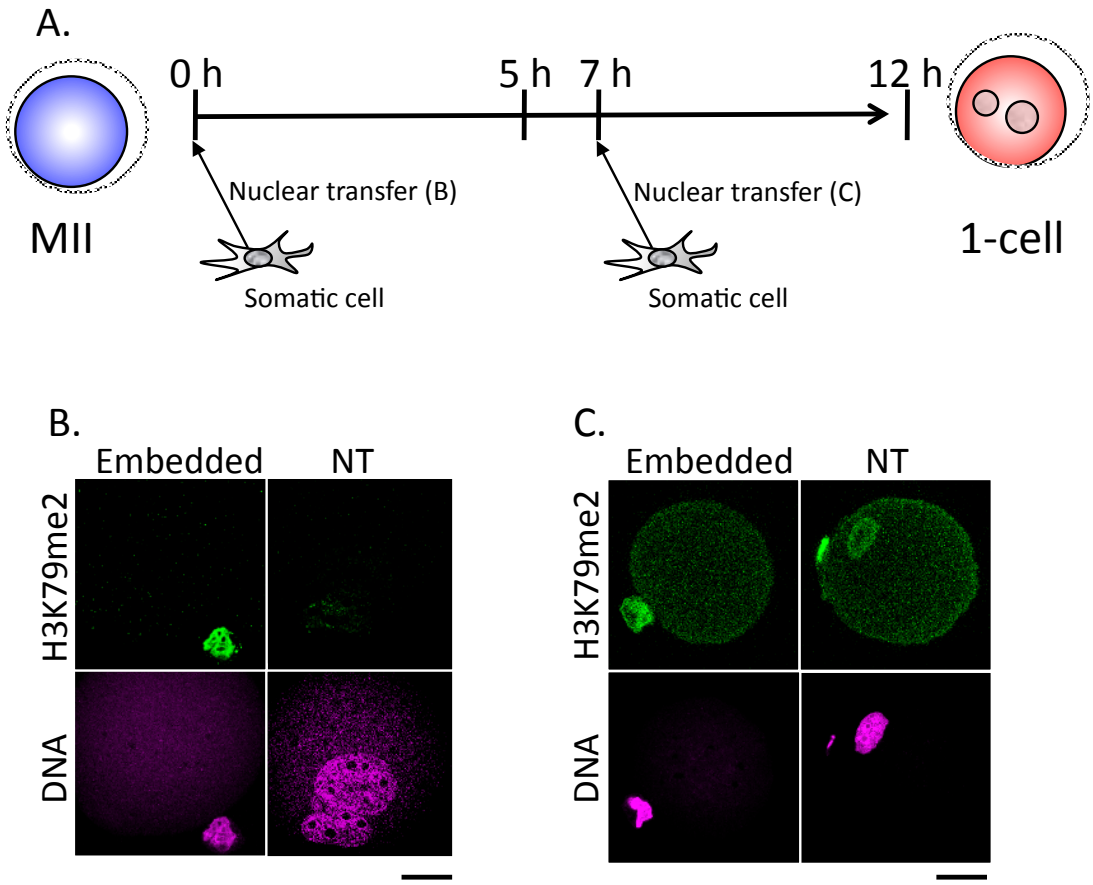

Figure 1-3. Elimination of H3K79me2 in the somatic nuclei transplanted into the enucleated oocytes.

A. A schematic of the experiments is shown. An interphase nucleus of a NIH3T3 cell was transplanted into (**B**) enucleated MII-stage oocytes and (**C**) enucleated parthenogenetically activated oocytes 7 h after activation with Sr^{2+}. After the nucleus was embedded in the perivitelline space of the oocyte, it was transplanted into the oocyte by electrofusion. Five hours later, the reconstructed oocytes (NT) were collected for immunostaining with an anti-H3K79me2 antibody. Three (B) and two (C) independent experiments were performed, in which 64 (B) and 14 (C) reconstructed oocytes were analyzed in total. Similar results were obtained in each experiment, and representative images are shown. Bars = 20 μm. Detailed results are shown in Table 1-1.

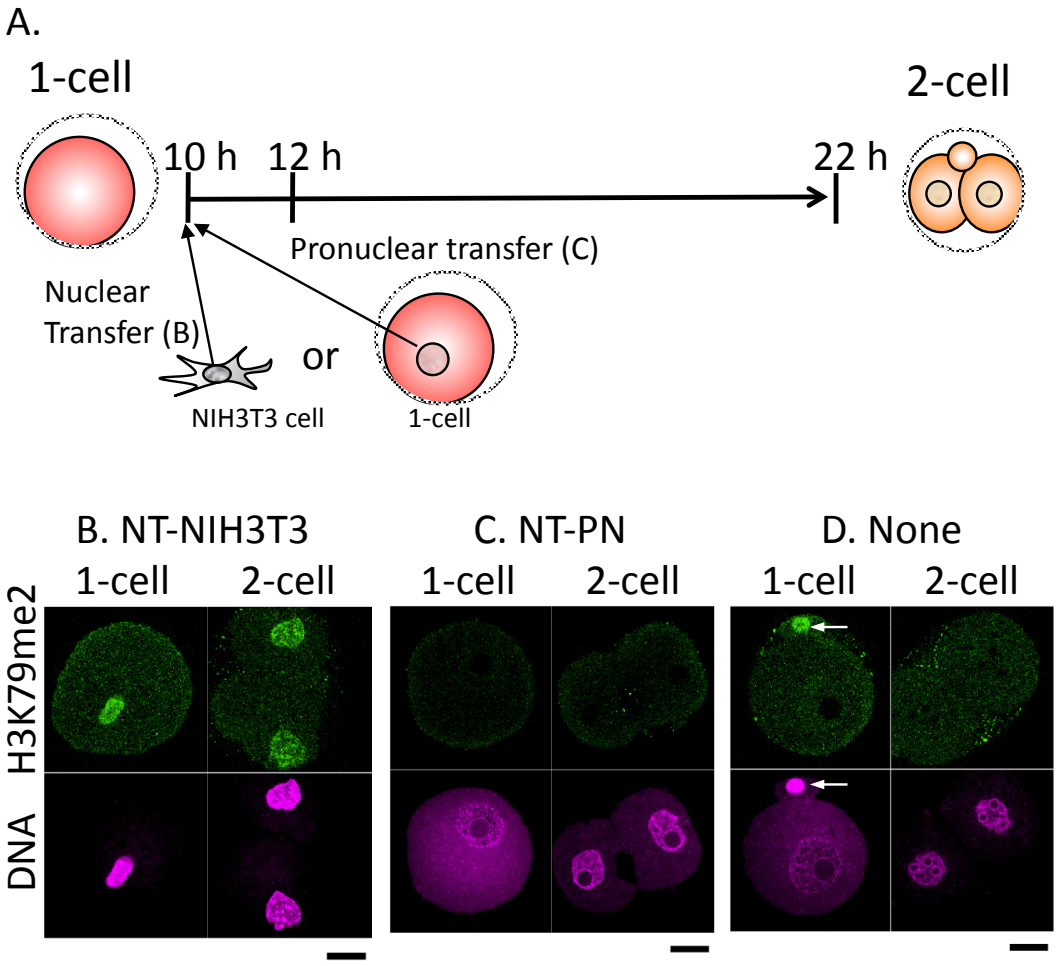

Figure 1-4. Absence of the activity to eliminate H3K79me2 in two-cell embryos.

A. A schematic of the experiments is shown. Nuclei of NIH3T3 cells (**B**: NT-NIH3T3) and pronuclei that had been collected from parthenogenetically activated oocytes 10 h after activation (**C**: NT-PN) were transplanted into enucleated parthenogenetically activated oocytes 10 h after activation and then collected 12 and 22 h after activation (2 and 12 h after transplantation, respectively) for immunostaining using the anti-H3K79me2 antibody. Parthenogenetically activated oocytes (**D**: None) were also collected 12 and 22 h after activation as a control. Three (B) and two (C, D) independent experiments were performed, and more than 10 oocytes were analyzed in total for each experimental group except for NT-PN at 12 h (seven oocytes). Similar results were obtained in each case, and representative images are shown. Arrows indicate polar body. Bars = 20 μm.

Table 1-1. Elimination of H3K79me2 in the somatic nuclei (NIH3T3) transplanted into MII stage oocytes and parthenogenetically activated embryos.

Recipient	Treatment	Total no. of cells examined	No. of nuclei showing H3K79me2 signal		
			High (%)	Low[b] (%)	None[c] (%)
Oocyte	Embeded	21	19 (90)	2 (10)	0 (0)
	NT[a]	64	0 (0)	42 (66)	22 (34)
Embryo	Embeded	8	8 (100)	0 (0)	0 (0)
	NT	14	12 (86)	1 (7)	1 (7)

[a] NT, Nuclear transfer
[b] Faint level
[c] No signal

Chapter 2

The mechanisms for maintenance of H3K79 hypomethylation during early preimplantation development

Introduction

After fertilization, embryos acquire totipotency and then they maintain the totipotent state during early preimplantation development. Since it has been thought that the acquisition of totipotency is achieved through the removal of cell memory markers (Morgan et al., 2005; Rideout et al., 2001), it is also possible that the absence of cell memory is important to maintain the totipotent state. Indeed, I have shown in chapter 1 that the hypomethylation of H3K79 is maintained during early preimplantation development (Figure 1-1A), suggesting that this state of H3K79 is involved in the maintenance of totipotency. In this chapter, to clarify the mechanism maintaining the totipotent state in the early preimplantation embryos, I seek for the mechanisms maintaining the hypomethylation of H3K79.

Dot1L is thought to be sole H3K79 methyltransferase (Jones et al., 2008; Nguyen and Zhang, 2011; Shanower et al., 2005; van Leeuwen et al., 2002). Dot1L preferentially methylates nucleosomal histone but not free histone, implying possibility that Dot1L recognizes additional molecular targets on the nucleosome besides H3K79 (Nguyen and Zhang, 2011). It was reported that ubiquitinated histone H2B (ubH2B) is required for H3K79 methylation by Dot1L (Briggs et al., 2002; Mohan et al., 2010; Ng et al., 2002b; Shahbazian et al., 2005) (Figure 2-1A). There are some mechanisms by which ubH2B affects H3K79 methylation by Dot1L. First, ubH2B recruits

bridging factors which interact with Dot1L so that H3K79 methylation is facilitated (Lee et al., 2007). Second, ubH2B directly interacts with Dot1L to cause H3K79 methylation (Oh et al., 2010). Third, ubH2B affects nucleosomal structures and increases their accessibilities to Dot1L (Fierz et al., 2011). Thus, it has been suggested that ubH2B regulates H3K79 methylation catalyzed by Dot1L through various and mutually non-exclusive mechanisms (Nguyen and Zhang, 2011).

In addition to ubiquitination of H2B, it was suggested that H3K79 methylation is regulated via Dot1L sub-cellular localization (Reisenauer et al., 2009; Reisenauer et al., 2010) (Figure 2-1B). AF17, which is dominantly localized in the cytoplasm, binds to the domain of 479-659 amino acids in Dot1L. It has been shown that they were co-localized in the cytoplasm of human embryonic kidney 293T (HEK 293T) cells (Reisenauer et al., 2009). Knockdown of AF17 caused enrichment of nuclear localization of Dot1L, which led to H3K79 hypermethylation (Reisenauer et al., 2009). Thus, AF17 binding to 479-659 domain of Dot1L reduces its nuclear localization, leading to the decrease in H3K79 methylation. Reisenauer and her colleagues (2010) also found that mammalian Dot1 ortholog (Dot1L) has three nuclear localization signals (NLSs: NLS1, NLS2 and NLS3). When GFP-Dot1L was transiently expressed in HEK 293T cells, the proteins were dominantly localized in the nucleus or the cytoplasm in 60% or 40% of the cells, respectively (Reisenauer et al., 2010). However, the deletion of one of NLSs

decreased the percentage of the cells with nuclear localized Dot1L (Reisenauer et al., 2010). Thus, these three NLSs in Dot1L are important in its sub-cellular distribution.

In this chapter, I analyze the dynamics of Dot1L and ubH2B to understand the mechanism regulating hypomethylation state of H3K79 during early preimplantation development.

Results

Expression of Dot1L mRNA in the oocytes and preimplantation embryos

In order to elucidate the mechanisms to maintain hypomethylation of H3K79 during 1- and 2-cell stage, I examined the mRNA expression level of Dot1L in the oocytes and preimplantation embryos. GV stage oocytes, 1-, 2-, 4-cell and blastocyst stage embryos were subjected to semi-quantitative RT-PCR. The results showed that Dot1L mRNA was always expressed through these developmental stages (Figure 2-2).

Sub-cellular localization of Dot1L in the oocytes and preimplantation embryos

It has been suggested that Dot1L-mediated H3K79 methylation is regulated by the Dot1L sub-cellular localization in somatic cells (Reisenauer et al., 2009) (Figure 2-1B). Therefore, I examined the localization of Dot1L in the oocytes and preimplantation embryos. Immunocytochemistry using the anti-Dot1L antibody revealed that Dot1L was localized in the nuclei of GO, GV and 1-cell embryos, but not 2-cell ones (Figure 2-3). A low level of Dot1L was observed in the nuclei at 4-cell stage and then its level increased at blastocyst stage. These results suggested that the hypomethylation of H3K79me2 at 2-cell stage was caused by the absence of Dot1L from the

nucleus. However, at 1-cell stage, the nuclear localization of Dot1L was observed in spite of H3K79 hypomethylation. Therefore, hypomethylation state of H3K79 seems to be regulated by different mechanisms between 1- and 2-cell embryos.

The specificity of anti-Dot1L antibodies was confirmed by the adsorption test by using the antigen peptide (Figure 2-4).

The dynamics of ubiquinated histone H2B during preimplantation development

It has been known that Dot1L mediated H3K79 methylation requires ubiquitination of histone H2B (ubH2B) (Briggs et al., 2002; Mohan et al., 2010; Ng et al., 2002b; Shahbazian et al., 2005) (Figure 2-1A). Hence, I hypothesized that the level of H3K79 methylation is regulated by that of ubH2B at 1-cell stage. To address this hypothesis, I examined the change in ubH2B level after fertilization. In immunocytochemistry using anti-ubH2B antibody, only a weak signal was detected in the 1-cell embryos (Figure2-5), whereas much stronger signal of ubH2B was observed in growing oocytes, 2-, 4-cell and blastocyst stage embryos. These results suggested that H3K79 hypomethylation was caused by the low level of ubH2B at 1-cell stage, although Dot1L is localized in the nuclei at this stage.

Sub-cellular distribution of flag-tagged Dot1L in 1- and 2-cell

stage embryos

As described above, the hypomethylation of H3K79 seems to be caused by the absence of Dot1L from the nucleus at 2-cell stage (Fig. 2-3). To address whether this absence is due to no transportation of Dot1L into the nucleus, I examined the sub-cellular localization of flag-tagged Dot1L in the 1- and 2-cell embryos. The experimental scheme is shown in Figure 2-6A. The mRNA encoding flag-tagged Dot1L was injected into 1-cell embryos 2 h after fertilization and then the embryos were collected at 12 h (at 1-cell stage) and 28 h (at 2-cell stage) after fertilization. Although the nuclear localization of flag-Dot1L was clearly observed at 1-cell stage (Figure 2-6B), it was barely detected at 2-cell stage with the exception for a small part of embryos in which the protein was predominantly localized in the nucleus. These results suggest that there is a mechanism by which Dot1L cannot be localized in the nucleus at 2-cell stage.

Analysis for the domain of Dot1L responsible for the sub-cellular localization in HEK293T cells

It has been reported using HEK293T cells that Dot1L has three nuclear localization signals (NLSs: NLS1, NLS2, and NLS3) and a nuclear export domain (NED1) which are involved in the mechanism regulating its sub-cellular localization (Reisenauer et al., 2009; Reisenauer et al., 2010). However, the detail of this mechanism has not been known well. Therefore, I

generated the constructs of Dot1L deletion mutants fused with EGFP to analyze the roles of NLSs and NED1 in the nuclear localization of Dot1L. These constructs were transiently transfected into HEK293T cells. When full length of Dot1L was transfected, it was dominantly localized in the nucleus in 74% of cells and in the cytoplasm in 26% of cells (Figure 2-7A). The fragment of N-terminal 393 amino acid residues (hereafter referred as N393) which contains only a conserved catalytic core domain but no NLSs or NED1 was dominantly localized in the cytoplasm in 95% of the cells (Figure 2-7B). When NLS1 was added to this catalytic core domain (N393-NLS1), the mutant fusion protein became to localize in the nucleus in most of cells (89%; Figure 2-7C). However, by adding NED1 to N393-NLS1 (N393-NLS1-NED1), the resultant mutant fusion protein was localized in the cytoplasm again in almost all of the cells (98%; Figure 2-7D), indicating that NED1 functions to export the protein to the cytoplasm even in the presence of NLS1. Finally, when NLS2 and NLS3 (NLS2/3) were added to N393-NLS1-NED1 (N393-NLS1-NED1-NLS2/3), the protein was localized in the nucleus in most of the cells (93%; Figure 2-7E). It should be noted that N393-NLS1-NED1-NLS2/3 is the mutant protein in which only 660-1088 region was deleted from the full length and that it showed significantly higher tendency to localize in the nucleus than full length protein ($P < 0.01$ by χ^2 test) (93% vs 74%), suggesting that 660-1088 region functions in nuclear export of Dot1L.

Analysis for the domain responsible for the cytoplasmic localization of Dot1L in the 2-cell stage embryos

As described above, Dot1L was not localized in the nucleus in the 2-cell stage embryos. It is possible that NLSs do not function at this stage. To address this, I generated mRNAs encoding flag-tagged proteins consisted of the catalytic domain and NLSs, and examined their sub-cellular localization in the 2-cell embryos. First, I confirmed that the flag-tagged protein of only the catalytic domain (N393) was not localized in the nucleus (Figure 2-8A). However, when NLS1 or NLS2/3 was added to this catalytic domain (N393-NLS1 or N393-NLS2/3, respectively), both of them localized in the nucleus in most of blastomeres of 2-cell embryos (96% and 85%, respectively; Figure 2-8B, C). Thus, both of NLS1 and NLS2/3 functioned at 2-cell stage, suggesting that the other factor(s) than NLSs inhibit(s) the nuclear localization of Dot1L.

To examine whether NED1 and 660-1088 domain were involved in the inhibition of the nuclear localization of Dot1L at 2-cell stage, deletion mutants of these domains were generated and analyzed for their sub-cellular localization. Although full length Dot1L was dominantly localized in the nucleus only in 16% of the blastomeres of 2-cell embryos (Figure 2-9A), the deletion of NED1 (ΔNED1) or 660-1088 region (Δ660-1088) significantly increased the percentage of nuclear localization into 88% or 51%, respectively ($P < 0.01$, by χ^2 test) (Figure 2-9B, C). To identify the domain

responsible for nuclear export in the 660-1088 region, I divided this region into two parts, 660-972 and 973-1088. The deletion of 660-972 (Δ660-972) did not affect the nuclear localization so that the percentage of the blastomeres with nuclear localization was almost the same between this deletion mutant and full-length protein, *i.e.*18% vs. 16% (Figure 2-9D). However, the deletion of 973-1088 (Δ973-1088) significantly increased the nuclear localization ($P < 0.01$, by χ^2 test): the mutant protein was dominantly localized in the nucleus in 68% of the blastomeres (Figure 2-9E). These results suggested that 973-1088 domain is responsible for nuclear export of Dot1L at 2-cell stage. Accordingly, this 973-1088 domain was hereafter referred to as NED2. Taken together, my results suggest that Dot1L is exported from the nucleus by the mechanism in which NED1 and NED2 play roles at 2-cell stage.

Analysis for the expression of AF17 in the preimplantation embryos

It has been reported that, AF17 was identified as a protein interacting with Dot1L (Reisenauer et al., 2009). It binds to NED1 to export Dot1L from nucleus in HEK 293T cells. Accordingly, I hypothesized that AF17 plays a role in Dot1L nuclear export in the 2-cell stage embryos. In order to investigate this possibility, I examined the mRNA expression level of AF17 in the oocytes and preimplantation embryos. The results of semi-quantitative RT-PCR assay showed that although the expression level

of AF17 was high at 4-cell and blastocyst stages, it was relatively much lower at GV, 1- and 2-cell stages (Figure 2-10). In particular, it was at the lowest level at the 2-cell stage, which suggests that AF17 is not important in the nuclear export of Dot1L in the 2-cell embryos.

Analysis for the role of 2-cell-specific nuclear export of Dot1L in preimplantation development

I have shown that H3K79me2 is absent and Dot1L is not localized in the nucleus in the 2-cell stage embryos (Figure 1-1 and 2-3). These results prompted me to explore two questions: the first one was whether or not the absence of H3K79me2 is merely caused by the absence of Dot1L in the nucleus and the second was whether the absence of Dot1L in the nucleus at 2-cell stage is the phenomenon necessary for the preimplantation development. To address these questions, Flag-N393-NLS1 which has been shown to localize in the nucleus (Figure 2-8B) was expressed in the 2-cell embryos and examined for H3K79me2 and preimplantation development. The results showed that Flag-N393-NLS1 induced H3K79me2 in 2-cell stage embryos (Figure 2-11A). Most of these embryos were arrested at 2-cell stage and only 1% of the embryos developed to the blastocyst stage (Figure 2-11B). The control embryos which had been injected with H_2O showed no increase in H3K79me2 and 96% of them developed to the blastocyst stage (Figure 2-11-A, B). These results suggested that the absence of Dot1L at 2-cell stage

is important for the maintenance of hypomethylation of H3K79me2 and required for the early preimplantation development.

Discussion

In this chapter, to clarify the mechanisms to sustain hypomethylation state of H3K79 during early preimplantation development, I examined the dynamics of Dot1L and ubH2B. At 1-cell stage, although Dot1L was localized in the pronuclei, only a low level of ubH2B was observed (Figure 2-3 and 2-5). By contrast, at 2-cell stage, although high level of ubH2B was observed, Dot1L was exported from the nuclei (Figure 2-3 and 2-5). Thus, it seems that hypomethylation of H3K79me2 is maintained by different mechanisms between these two stages.

It is possible that Dot1L has a function other than H3K79 methylation in the pronuclei at 1-cell stage. It was reported that the methyltransferase domain of yeast Dot1 reactivated the genes which had been silenced in heterochromatin, independently of H3K79 methylation activity (Stulemeijer et al., 2011). This function involves Gcn5 (General control nonderepressible protein 5), a histone acetyltransferase, which catalyzes acetylation towards H3K9, K14 and K18 (Grant et al., 1999; Stulemeijer et al., 2011). Acetylation of histones plays a role in gene activation (Grunstein, 1997). Therefore, Dot1L may be involved in gene activation after fertilization thorough the function other than H3K79 methylation. Indeed, it was reported that the acetylation of H3K9 and K14 increased after fertilization (Hara, 2004).

ubH2B was present at a low level at 1-cell stage (Figure 2-5). This low level of ubH2B may be caused by a low transcriptional activity. H2B ubiquitination is catalyzed by E2 ubiquitin conjugating enzyme, Rad6 and E3 ubiquitin ligase, Bre1 (Nguyen and Zhang, 2011; Robzyk et al., 2000; Wood et al., 2003). These enzymes complex was associated with elongating RNA pol II (Martin and Zhang, 2005; Xiao et al., 2005), suggesting that ubiquitination of H2B depends on transcriptional activity. Indeed, when the cells were treated with α-amanitin, a potent inhibitor of RNA polymerase II, ubH2B level decreased (Zhang and Yu, 2011). After fertilization, gene expression resumes at mid- or late-1-cell stage in mice (Aoki et al., 1997). However, the transcriptional activity is still low at this stage (Aoki et al., 1997). Thus, ubiquitination of H2B seems to be sustained at a low level in 1-cell stage embryos due to the low transcriptional activity, which may lead to the hypomethylation of H3K79me2.

My results suggest that Dot1L is exported from nucleus at 2-cell stage by NED1- and NED2-mediated mechanism. NED1 is bound to AF17 which is dominantly localized in the cytoplasm of HEK293T cells and then Dot1L is exported to cytoplasm (Reisenauer et al., 2009). However, AF17 showed the lowest expression at 2-cell stage during preimplantation development (Figure 2-10). These results suggested that NED1 plays a role in exporting Dot1L from nucleus independently of AF17 at 2-cell stage. Since there is no report about the function of NED2, I sought for functional motifs

in NED2. First, I analyzed the amino acids sequences of NED1 and NED2 by using NETNES1.1 Server (www.cbs.dtu.dk/services/NetNES/) and found that putative functional nuclear export signal (NES) is present in NED1 but not in NED2. Next, I tried to find the amino acid sequence which is evolutionary conserved in vertebrates. Although the comparison of amino acid sequence of NED2 for various vertebrates, *i.e.* human, mouse, rat, chicken and frog, showed that their similarities are low in total, I found some highly conserved motifs consisting of several amino acids in all vertebrates or mammals (Figure 2-12). These motifs may be involved in nuclear export of Dot1L in the 2-cell stage embryos.

The expression of Dot1L-N393-NLS1, which was localized in the nuclei even at 2-cell stage, caused the methylation of H3K79 and developmental arrest at 2-cell stage (Figure 2-11A, B). These results suggested that absence of Dot1L from the nuclei is important for the maintenance of hypomethylation state of H3K79 and the progression of preimplantation development into 4-cell stage. There are large differences of gene expression pattern between early- and late-2-cell stage embryos (Wang et al., 2004). It is possible that methylation of H3K79me2 induced by Dot1L-N393-NLS1 expression inhibited the changes in gene expression pattern during 2-cell stage, which led to the developmental arrest at 2-cell stage.

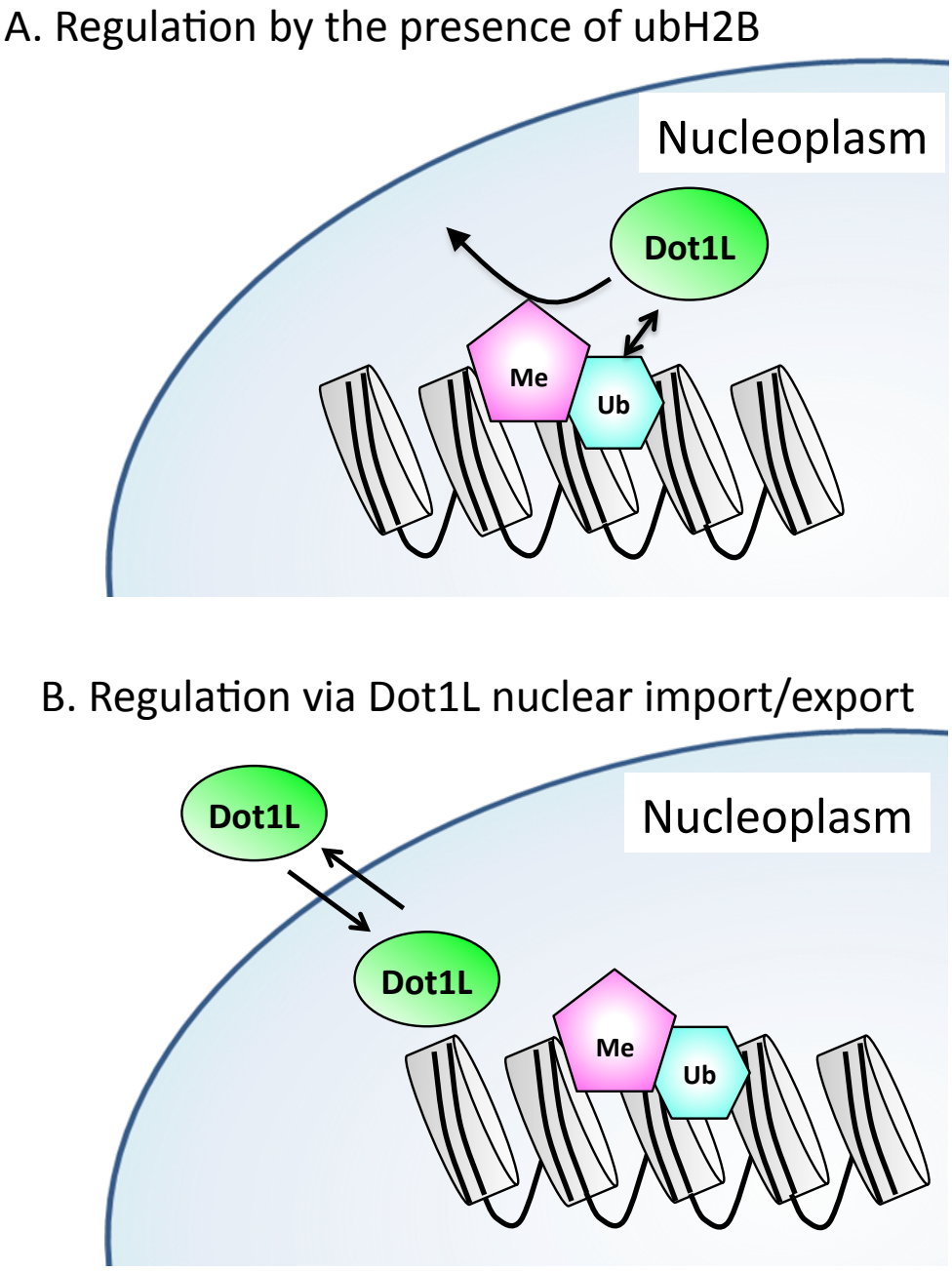

Figure2-1 Two regulatory mechanisms of Dot1L-mediated H3K79 methylation in somatic cells.

Two models of the regulatory mechanisms for H3K79 methylation are shown. **A.** Dot1L methyltransferase activity requires ubiquitinated H2B co-existing with H3K79 on the same nucleosome. **B.** H3K79me2 is also regulated via Dot1L nuclear import/export.
Me: H3K79 methylation, Ub: Histone H2B ubiquitination.

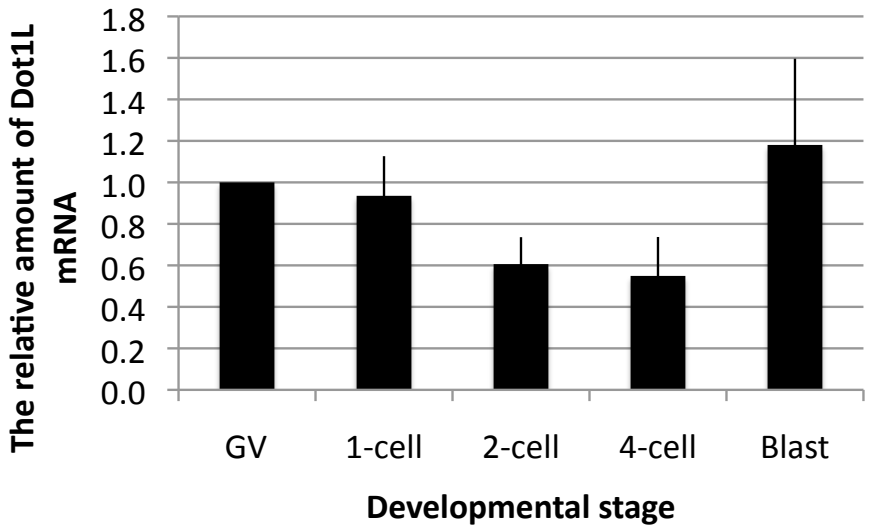

Figure 2-2. Analysis for Dot1L expression in the oocytes and preimplantation embryos by semi-quantitative RT-PCR.

The expression of Dot1L mRNA was examined by RT-PCR in GV stage oocytes (GV), 1-cell, 2-cell, 4-cell and blastocyst (Blast) stage embryos. Six independent experiments were performed. The value of GV stage oocytes was set at 1.0, and the values for other preimplantation embryos are expressed relative to this value. Bars represent SD.

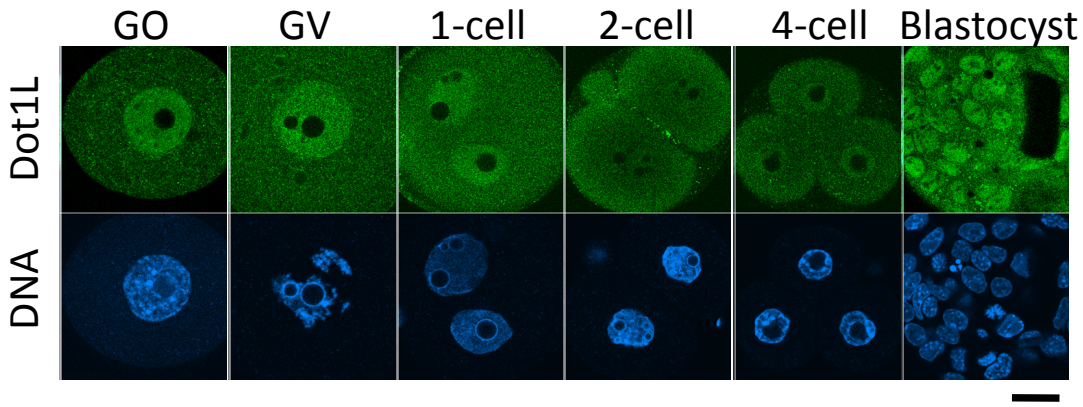

Figure 2-3. Dynamics of Dot1L during oocyte growth and preimplantation development.

Oocytes and preimplantation embryos were immunostained with an anti-Dot1L antibody. GO and GV indicate growing oocytes acquired from 13 days old mice and those at the germinal vesicle stage, respectively. The embryos at the 1-cell, 2-cell, 4-cell, and blastocyst stages were collected 12, 28, 45, 96 h after insemination, respectively. More than three independent experiments, in which more than 20 oocytes/embryos were observed in total, were performed in the analysis for each stage of oocytes and preimplantation embryos. Similar results were obtained in each experiment, and representative images are shown. Bar = 20 µm

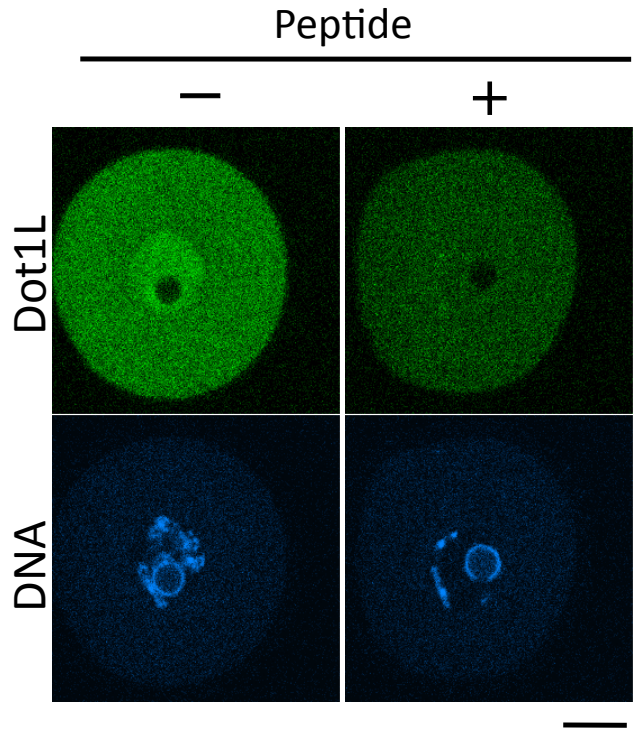

Figure 2-4. Specificity of the antibody against Dot1L.
The antibody was incubated with (+) or without (-) 0.5 μg/ml antigen peptide (abcam; #ab71337) at room temperature for 1 h before immunocytochemistry of GV-stage oocytes. In four independent experiments, 28 (+) and 33 (-) oocytes were examined, respectively. Similar results were obtained in each experiment and representative images are shown above. Bar = 20 μm.

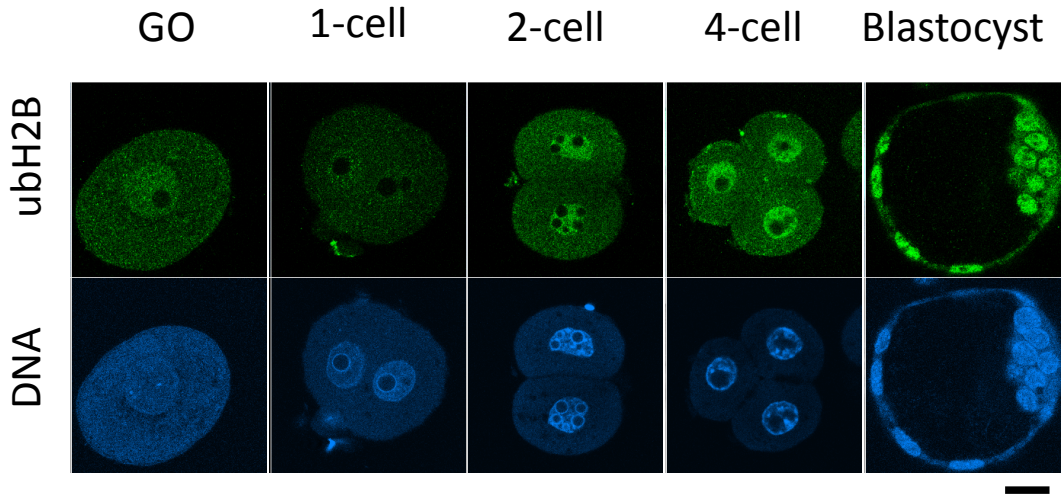

Figure 2-5. Changes in ubH2B during preimplantation development.
The cells were immunostained with anti-ubiquitinated H2B antibodies. GO indicates growing oocytes acquired from 13 days old mice. The embryos at the 1-cell, 2-cell, 4-cell, and blastocyst stages were collected 12, 28, 45, 96 h after insemination, respectively. More than three independent experiments, in which more than 21 oocytes/embryos were observed in total, were performed in the analysis for each stage of oocytes and preimplantation embryos. Similar results were obtained in each experiment, and representative images are shown. Bar = 20 μm

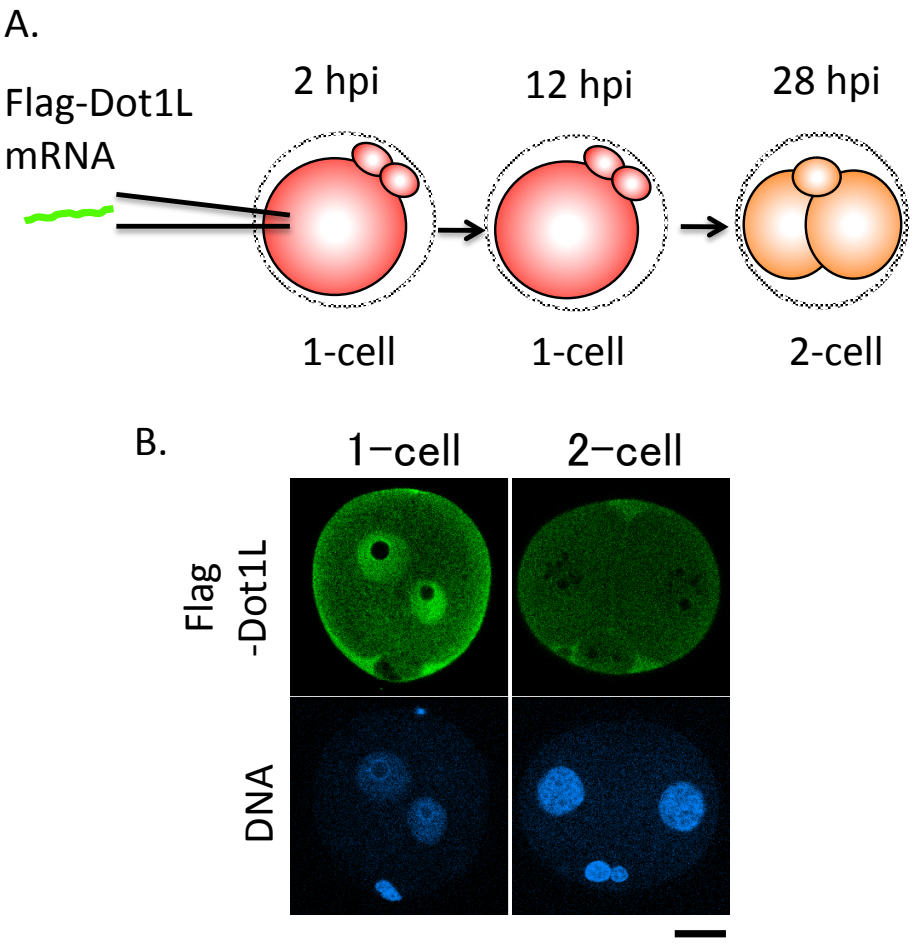

Figure 2-6. Nuclear localization of Flag-Dot1L in 1-cell and 2-cell stage embryos.
A. The scheme of the experiment is shown. Flag tagged full length Dot1L mRNA was microinjected into the 1-cell stage embryo (2 h post insemination: hpi) and collected 12 and 28 hpi, at which the embryos were at 1- and 2-cell stages, respectively.
B. The embryos were immunostained with an anti-Flag antibody. The experiments were performed at least 4 times; 39 1-cell and 37 2-cell embryos were examined in total. Bar = 20 μm

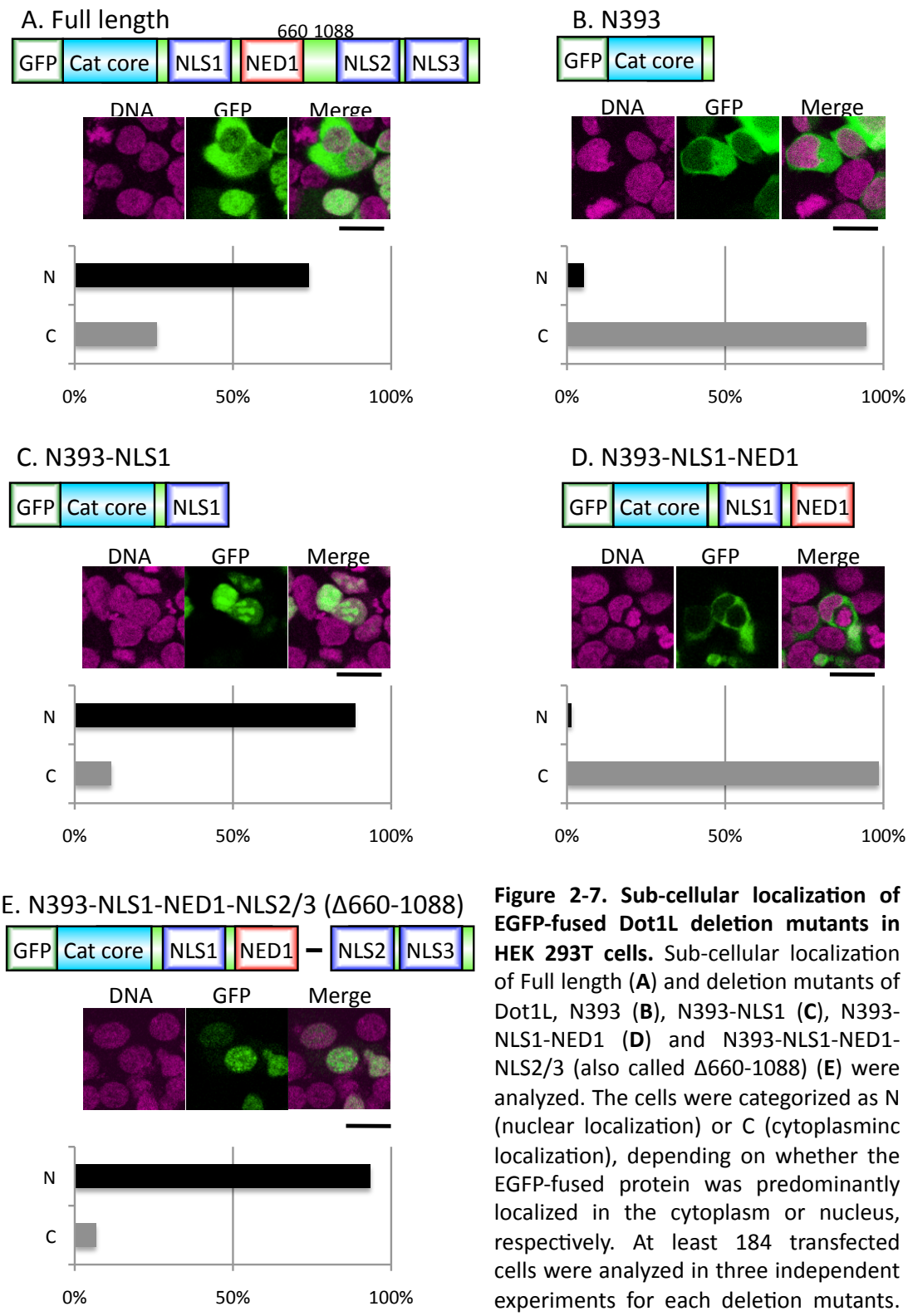

Figure 2-7. Sub-cellular localization of EGFP-fused Dot1L deletion mutants in HEK 293T cells. Sub-cellular localization of Full length (**A**) and deletion mutants of Dot1L, N393 (**B**), N393-NLS1 (**C**), N393-NLS1-NED1 (**D**) and N393-NLS1-NED1-NLS2/3 (also called Δ660-1088) (**E**) were analyzed. The cells were categorized as N (nuclear localization) or C (cytoplasminc localization), depending on whether the EGFP-fused protein was predominantly localized in the cytoplasm or nucleus, respectively. At least 184 transfected cells were analyzed in three independent experiments for each deletion mutants. Bar = 20 μm.

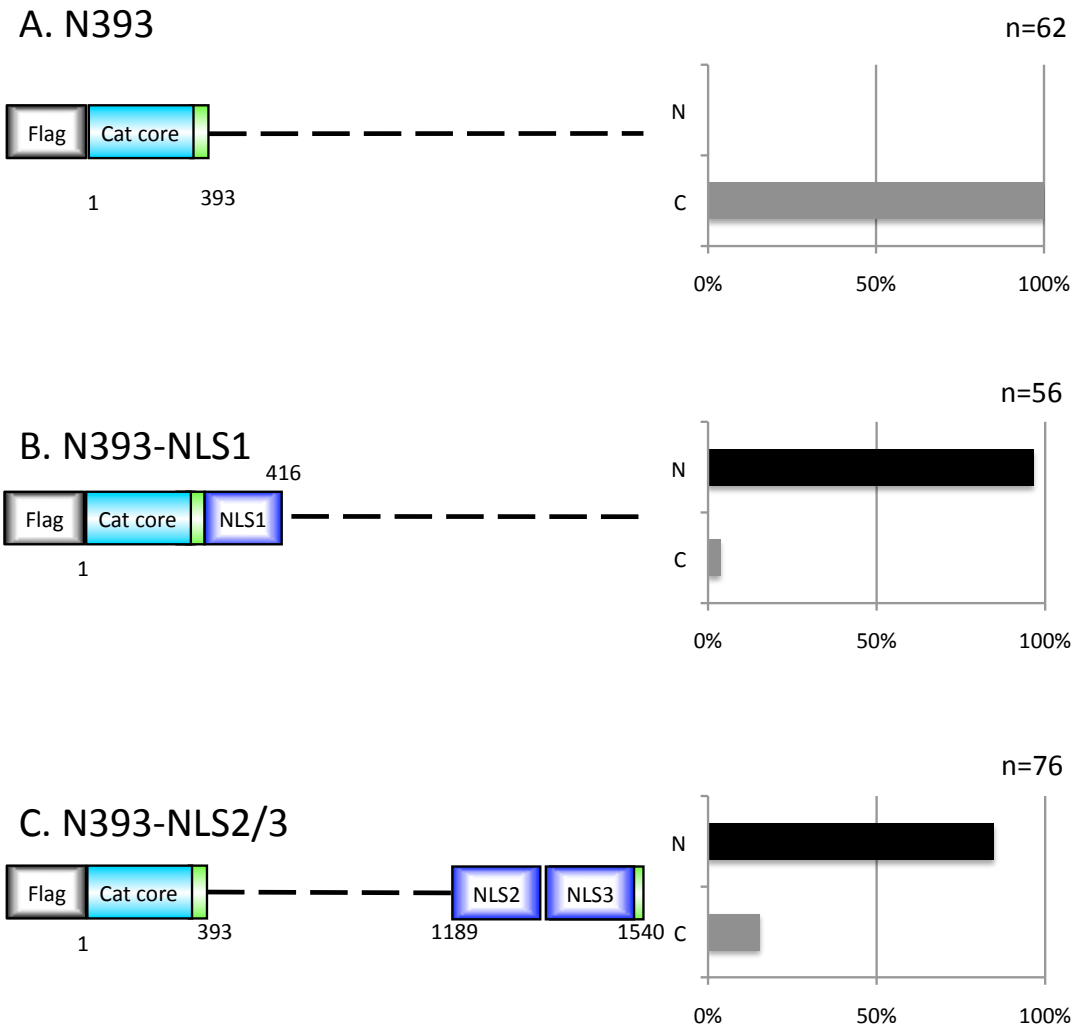

Figure 2-8. Analysis for the function of NLSs in the 2-cell stage embryos.
Two-cell stage embryos, which had been injected with mRNA encoding N393 (**A**), N393-NLS1 (**B**), or N393-NLS2/3 (**C**) at 2 h after fertilization, were collected 26 h later and immunostained with an anti-Flag antibody. The blastomeres were categorized as N (nuclear localization) or C (cytoplasminc localization), depending on whether the Flag-fused protein was predominantly localized in the cytoplasm or nucleus, respectively. "n" indicates the total number of blastomeres examined in at least 3 independent experiments.

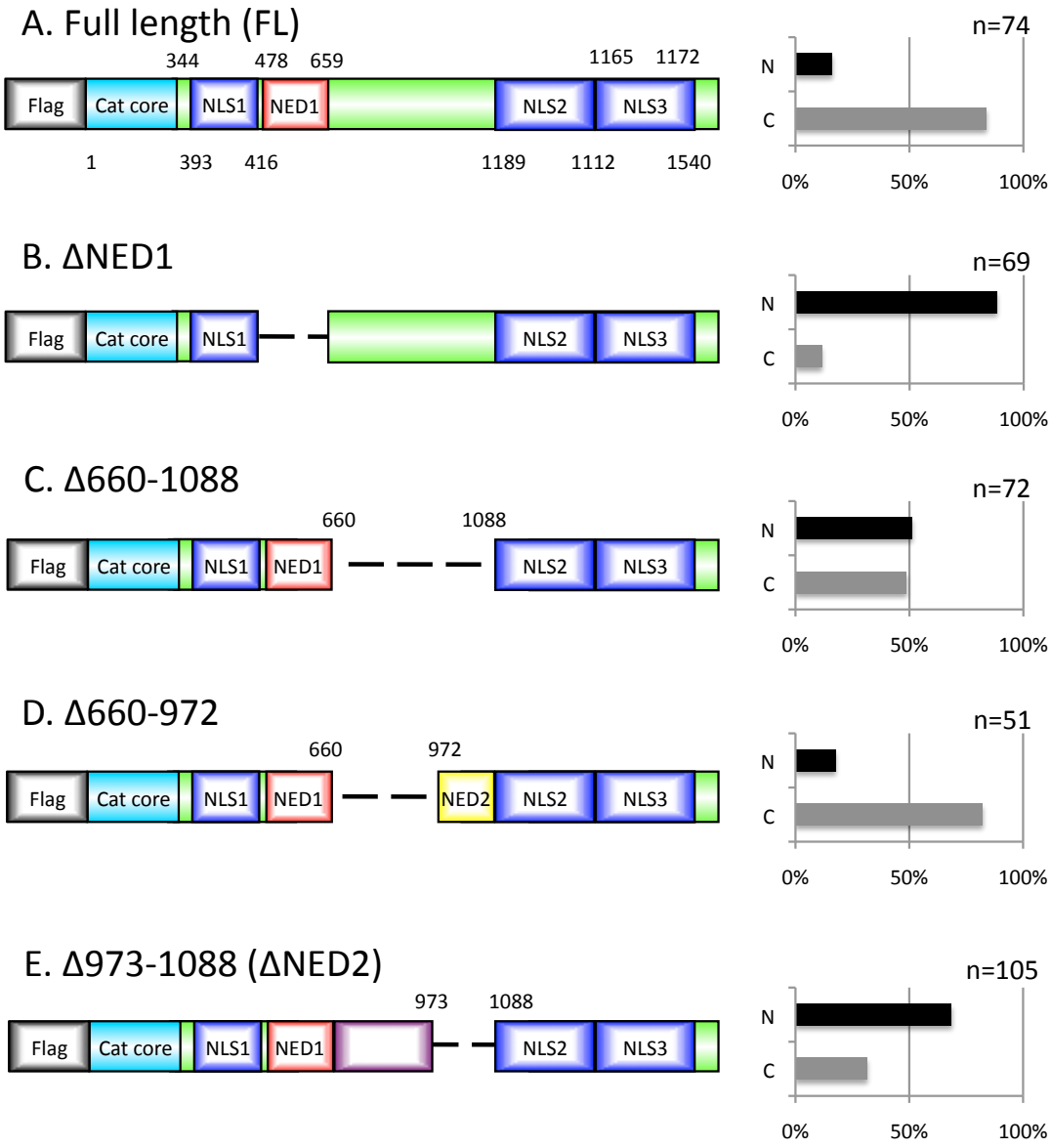

Figure 2-9. Involvement of NED1 and 973-1088 domain in the inhibition of nuclear localization of Dot1L in 2-cell stage embryos.

Two-cell stage embryos, which had been injected mRNA encoding full length, ΔNED1, Δ660-1088, Δ660-972 and Δ973-1088 2 h after fertilization, were collected 26 h later and immunostained with an anti-Flag antibody. The blastomeres were categorized as N (nuclear localization) or C (cytoplasminc localization), depending on whether the Flag-fused protein was predominantly localized in the cytoplasm or nucleus, respectively. “n” indicates the total number of blastomeres examined in at least 3 independent experiments.

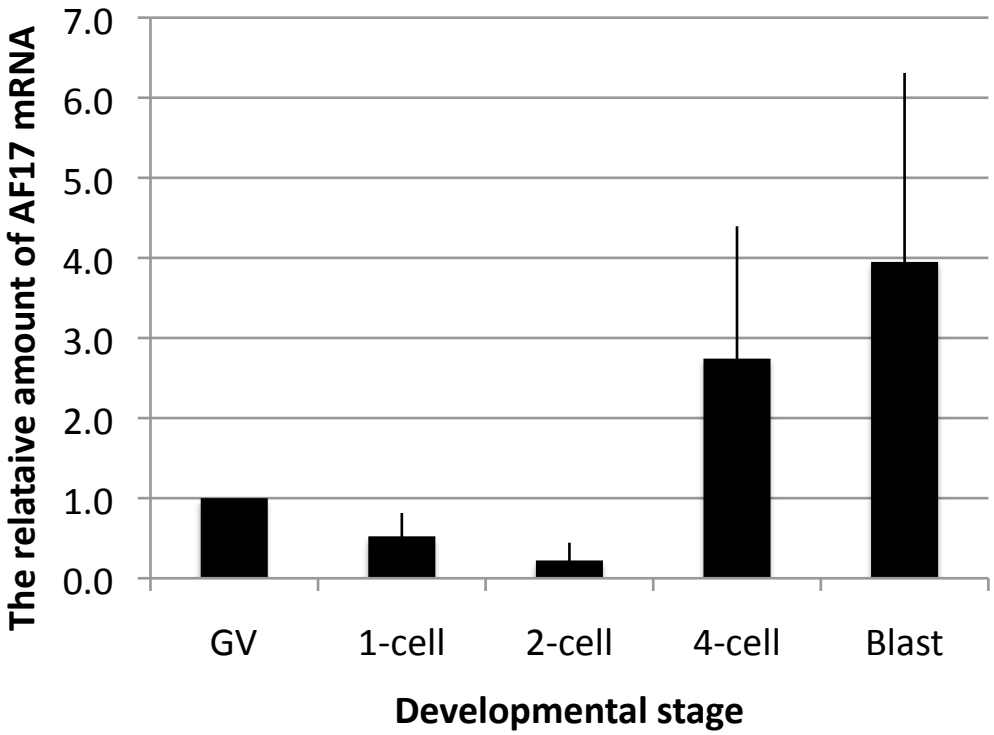

Figure 2-10. Analysis of AF17 expression in the oocytes and preimplantation embryos by semi-quantitative RT-PCR. The expression levels of AF17 mRNA was examined by RT-PCR in GV stage oocytes (GV) and the embryos at 1-cell, 2-cell, 4-cell, Blastocyst (Blast) stages. Three independent duplicate experiments were performed. The average value of GV was set at 1.0, and the values for preimplantation embryos are expressed relative to this value. Bars represent S.D.

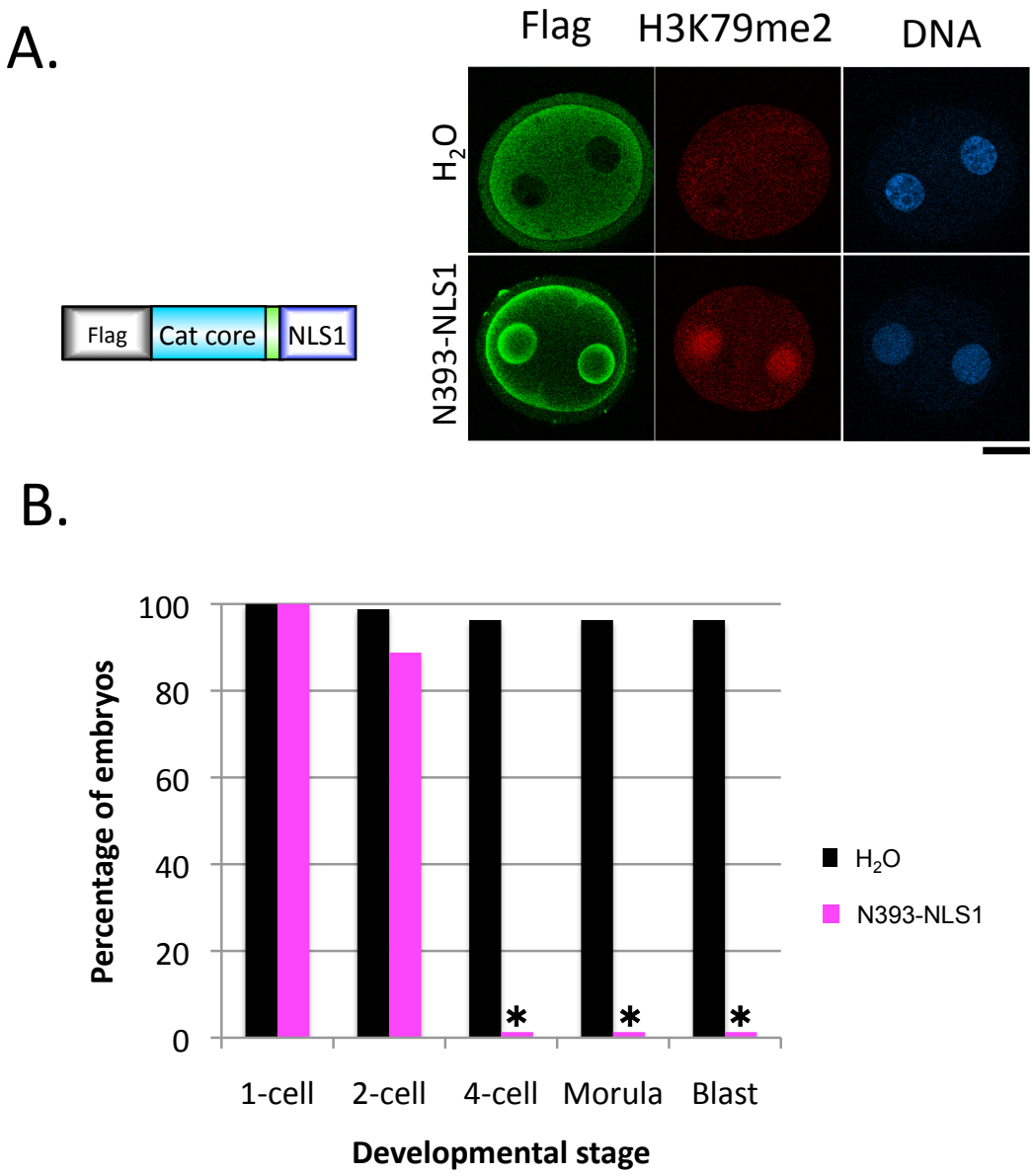

Figure 2-11. H3K79 methylation and developmental arrest in the 2 cell stage embryos expressing N393-NLS1.

Two-cell stage embryos which had been injected with mRNA encoding Dot1L deletion mutant, N393-NLS1, or sterilized water (H_2O) as a control, were examined for H3K79me2 and preimplantation development. **A.** The embryos was immunostained with the antibodies against flag and H3K79me2 to examine the nuclear localization of N393-NLS1 and changes in H3K79me2 level, respectively. Bar = 20 um. **B.** The developmental rate of the embryos expressing N393-NLS1. The values are expressed as the averaged percentages of the 4 independent experiments. In each experiment, 20 embryos were used. Asterisks indicate that the value is statistically different from control (H_2O) ($P < 0.01$; χ^2-test).

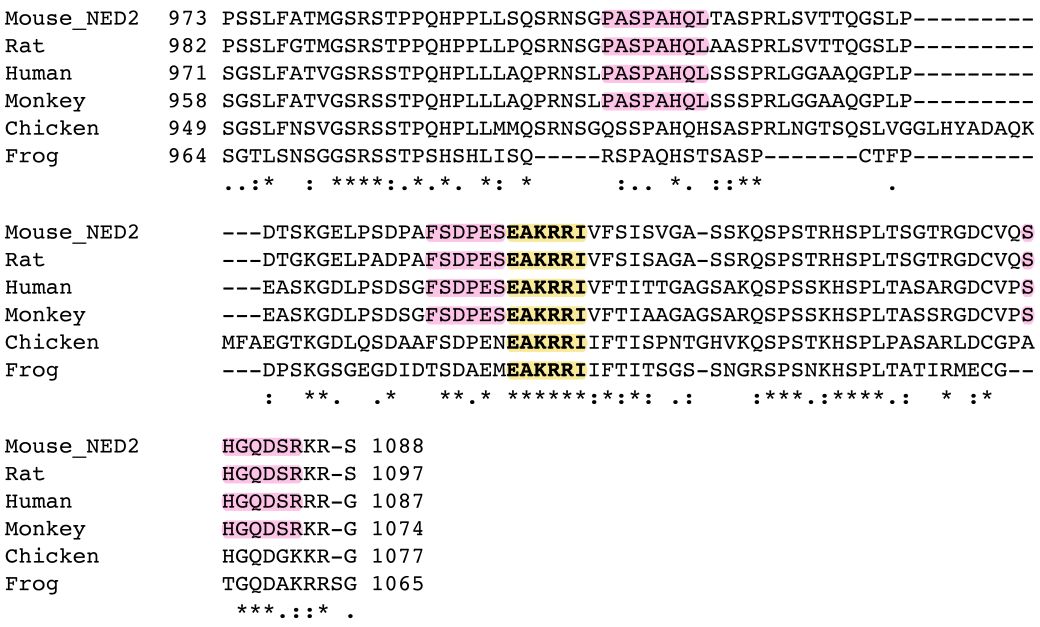

Figure 2-12. The multiple alignment of NED2 amino acid sequences of various vertebrates.

Amino acid sequences of Dot1L of various vertebrates are subjected to multiple alignment analysis to find conserved amino acid residues in NED2. The multiple alignment analysis was performed by using CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/top-j.html). Highly conserved amino acid sequences in all examined vertebrates or mammals were highlighted with yellow or pink, respectively. Asterisks indicate the positions of amino acids that are conserved in all vertebrates. Double and single dot mean the positions of amino-acids that are conserved in most but not all vertebrates and their different amino-acids have very similar and similar, respectively, properties between each other. Gaps are represented by "- - -". The numbers represent of amino acids positions on respective proteins.

Conclusion and perspective

In this thesis, I analyzed H3K79me2 in oocytes, preimplantation embryos and nuclear transferred embryos, and investigated the mechanism by which hypomethylation state of H3K79 was maintained during early preimplantation development. In chapter 1, I demonstrated that H3K79me2 was eliminated from the genome in the early 1-cell stage embryos and nuclear transferred embryos and that the hypomethylation state of H3K79 was maintained during early preimplantation development. These results suggest that totipotency is acquired by erasing of H3K79me2 and maintained by its absence from the nuclei. In chapter 2, I showed that ubH2B was at a low level in 1-cell stage embryos and Dot1L was exported from nucleus by the NED1- and NED2-mediated mechanism in 2-cell stage embryos. In addition, the expression of Dot1L mutants (N393-NLS1) that was localized in the nuclei induced the increase of H3K79me2 and developmental arrest at 2-cell stage. These results suggest that the maintenance of hypomethylation state of H3K79 is regulated by different mechanisms between 1- and 2-cell stage embryos and that the 2-cell specific mechanism for nuclear export of Dot1L is important for the progression of preimplantation development. A schematic of the mechanisms regulating H3K79me2 in the preimplantation embryos is shown in Figure C&P-1. In conclusion, I suggest that totipotency is acquired by erasing of cell memory

markers, maintained by their absence, and lost by their re-appearance (Figure C&P-2).

In the future, it is important to elucidate the mechanism(s) by which H3K79me2 is eliminated after fertilization. If a specific H3K79 demethylase or the chaperon that specifically evicts H3.3 from chromatin is identified, the biological function of H3K79me2 removal after fertilization could be clarified by knocking them out. In this thesis, NED1 and NED2 were shown to be involved in the nuclear export of Dot1L at 2-cell stage. However, the details in molecular mechanisms of nuclear export of Dot1L remain to be elucidated. Identification of the factors that interact with NED1 or NED2 at 2-cell stage might make it possible to clarify the mechanisms for 2-cell specific nuclear export of Dot1L and for the maintenance of hypomethylation state of H3K79me2. The results of these investigations will elucidate the molecular mechanisms for the acquisition and maintenance of totipotency during early preimplantation development.

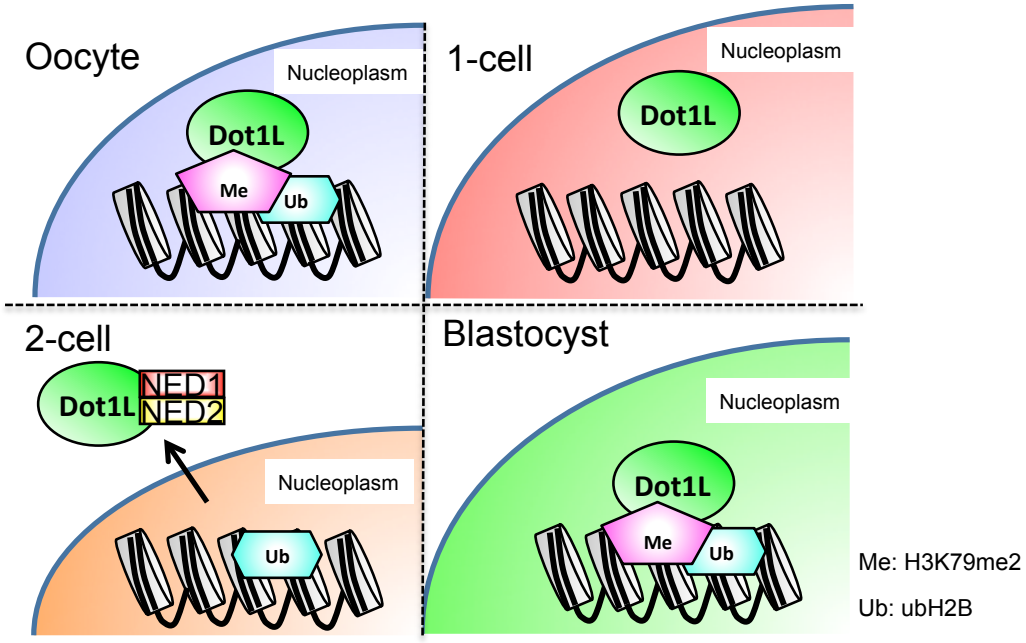

Figure C&P1. A schematic illustration of the mechanisms regulating H3K79me2 in the oocytes and preimplantation embryos.

In differentiated oocytes, H3K79me2 and Dot1L are present in the nuclei. After fertilization, H3K79me2 is removed. The hypomethylation state of H3K79me2 is maintained because of a low level of ubH2B during 1-cell stage, although Dot1L is localized in the pronuclei. At 2-cell stage, Dot1L is exported from nuclei by the mechanism involving its NED1 and NED2 domain, which results in hypomethylation of H3K79me2. Hypomethylation of H3K79me2 is thus maintained by the different mechanisms between 1- and 2-cell embryos. H3K79me2 is increased in blastocyst stage embryos with nuclear-localized Dot1L and a high level of ubH2B.

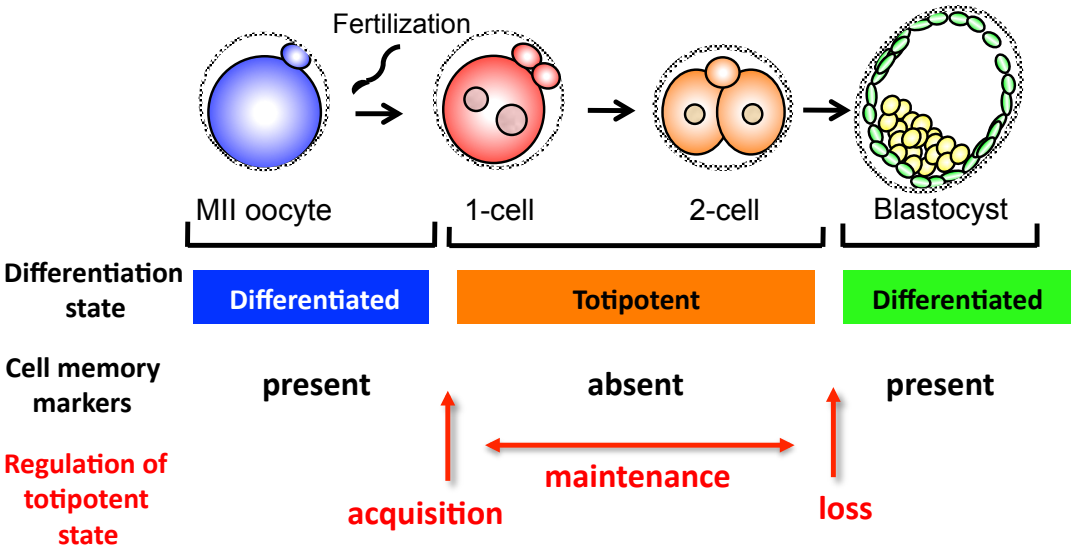

Figure C&P2. A hypothetical model of regulatory mechanisms for totipotency.
Totipotency is acquired by the elimination of cell memory markers including H3K79me2 after fertilization. It is maintained by the absence of cell memory markers during early preimplantation development, and then lost by re-appearance of cell memory markers at the late preimplantation stage.

Materials and methods

Collection and culture of oocytes and embryos

Growing oocytes were obtained from 13 day-old B6CDF1 (BDF1) mice. Oocytes at the germinal vesicle stage were collected from the ovaries of 3-weeks-old BDF1 mice (CLEA Japan Inc., Tokyo, Japan) that had been injected with 5 IU pregnant mare's serum gonadtropin (PMSG; Sankyo Co. Ltd., Tokyo, Japan). Follicles within ovaries were punctured with a sterile 30-gauge needle to release the oocytes surrounded with cumulus cells in K^{2+}-modified simplex optimized medium (KSOM; (Erbach et al., 1994)) containing 10 mM HEPES and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma Chemical Co.). The cumulus cells were removed by repeated aspiration through a glass pipette, the tip diameter of which was slightly larger than that of an oocyte. Unfertilized MII-stage oocytes were collected from the oviducts of 3-weeks-old BDF1 mice that had been superovulated by the injection of 5 IU PMSG followed by 5 IU human chorionic gonadtropin (hCG; Sankyo) 48 h later, and then placed in KSOM containing 10 mM HEPES. Spermatozoa were collected from the cauda epididymis of mature male ICR mice (SLC, Shizuoka, Japan) and were placed in human tubal fluid medium (HTF; (Quinn and Begley, 1984) supplemented with 10 mg/ml BSA. For *in vitro* fertilization, oocytes were transferred into HTF medium and were inseminated with spermatozoa that had been incubated for 2 h at 38°C.

The embryos were washed with KSOM containing 3 mg/ml BSA (KSOM/BSA) 5 h after insemination and then cultured in a humidified 5% CO_2/95% air atmosphere at 38°C. In some case, the embryos were treated with bovine testicular hyaluronidase (0.3 mg/ml) in a few minutes to be free from the surrounding cumulus cells.

Immunocytochemistry

Oocytes and embryos were fixed for 1 h in 3.7% paraformaldehyde (PFA; Wako) in PBS at room temperature, and were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 15 min. The cells were washed with 0.1% BSA/PBS and then incubated in the primary antibody against H3K79me2 (Abcam; #ab3594; 1:100) or Dot1L (Abcam; #ab71337; 1:400). To detect ubH2B, the cells were fixed in 3.7% PFA/PBS for 15 min at room temperature and permeabilized with 0.2% Triton X-100 for 30 min. The cells were pre-treated with 4 N HCl containing 0.1% Triton X-100 for 10 min at room temperature and then neutralized for 30 min with 0.1 M Tris-HCl (pH 8.5). The cells were washed in 1% BSA/PBS and were then incubated with anti-ubH2B antibody (Medimabs; #MM-0029-p; 1:200). To detect Flag tag fused proteins, the cells were fixed for 40 min in 3.7% PFA/PBS containing 0.2% Triton X-100. After washing with 1% BSA/PBS containing 0.2% Tween 20 (MP biomedicals), the cells were incubated with anti-Flag antibody (Sigma-aldrich; #F1804; 1:1000). The treatment with primary antibodies

described above was performed at 4°C overnight. Primary antibodies that were bound to the cells were probed by incubating for 45 min with the following secondary antibodies: Alexa fluor 488 conjugated anti-mouse IgG (Molecular probes, #A11001), Alexa fluor 568 conjugated anti-rabbit IgG (Molecular probes, #A10042) or FITC-conjugated anti-rabbit IgG antibodies (Jackson immunoresearch; #711-095-152). The cells were then mounted on a glass slide in Vectashield antibleaching solution (Vector Laboratories) containing 3 μg/ml 4',6-diamino-2-phenylindole (DAPI; Dojindo) or 100 μg/ml propidium iodide (PI; Sigma-Aldrich) to stain DNA. Fluorescence was detected using a Carl Zeiss 510 laser scanning confocal microscope.

Parthenogenesis

MII-stage oocytes were collected from oviducts and placed in KSOM containing 0.3 mg/ml bovine testicular hyaluronidase (Sigma Chemical). After they were cultured for 1 h in KSOM, the oocytes were treated with 10 mM Sr^{2+} for 4 h in Ca^{2+}-free KSOM. To obtain diploid parthenogenetic embryos, cytochalasin B (1% dimethylsulfoxide) was added to the KSOM/Sr^{2+} medium.

Nuclear transfer into unfertilized oocytes

Enucleation of oocytes was performed by the procedure which had been described by (Kim et al., 2002). The nuclei of NIH3T3 cells were introduced

into enucleated oocytes by electrofusion using a DC pulse of 1500 V/cm for 20 μsec in 300 mM mannitol containing 0.1 mM $MgSO_4$, 0.1 mg/ml polyvinyl alcohol, and 3 mg/ml BSA. As a control, the nucleus was embedded in the perivitelline space of the oocyte but not subjected to electrofusion.

Nuclear transfer into parthenogenetically activated embryos

Parthenogenetically activated embryos were prepared as described above. At 7 h after activation, they were enucleated and transplanted with the nuclei of NIH3T3 cells by electrofusion, using a DC pulse of 1500 V/cm for 20 μsec in 300 mM mannitol that contained 0.1 mM $MgSO_4$, 0.1 mg/ml polyvinyl alcohol, and 3 mg/ml BSA. As the control, the nucleus was embedded in the perivitelline space of the embryo but not subjected to electrofusion.

Plasmid construction

To generate Flag-tagged full-length mouse *Dot1L* (Flag-Dot1L) cDNA, Flag-Dot1L cDNA were PCR-amplified using a plasmid encoding mouse full-length *Dot1L* (Zhang et al., 2004; Zhang et al., 2006) as a template and the PCR primer added with the sequences of Kozak and Flag in its N-terminus, with KOD-plus-DNA polymerase (TOYOBO). A deoxyriboadenosine was added into the 3' terminal of PCR-amplified Flag-Dot1L fragments by Ex Taq polymerase (Takara) for TA cloning. The

PCR product was cloned into pCRII topo vector by using a TA cloning kit (Invitrogen) according to manufacturer's instructions. The resulting plasmid was named pTOPO-Flag-Dot1L.

Plasmids encoding GFP or Flag-tagged deletion mutants of Dot1L were produced by inverse PCR using PrimeSTAR MAX DNA polymerase (Takara) according to manufacturer's instructions. pEGFPC3-Dot1L (Zhang et al., 2004; Zhang et al., 2006) or pTOPO-Flag-Dot1L were used as a template to construct the deletion mutants listed above.

To increase expression of Flag-N393-NLS1, its cDNA were sub-cloned from pTOPO vector containing Flag-Dot1L-N393-NLS1 (N393-NLS1) into the *Eco*RI site of the pcDNA3.1EGFP-poly(A) (Yamagata et al., 2005), thereby deleting the EGFP cassette. The mRNA transcribed from this vector is efficiently translated in the embryos, since the vector encodes 3'-UTR region of TRF2 (TBP-related factors 2) and stretched poly(A) sequence at downstream of multi-cloning site. All constructs were verified by DNA sequencing.

Synthesis of mRNA *in vitro*

Flag-Dot1L and Flag-tagged deletion mutants of Dot1L expression vectors were digested with *Not*I or *Xho*I restriction enzymes. Using these DNA fragments as templates, capped mRNAs were synthesized by *in vitro* transcription by use of SP6 or T7 mMASSAGE mMACHINE kit (Ambion)

followed by the addition of poly(A) tails using Poly(A) Tailing Kit (Ambion), according to the manufacturer's instructions. To avoid the integration of the template DNA into the embryo genome, reaction mixtures were treated with TURBO DNase (provided with in vitro transcription kit). Synthesized mRNAs were precipitated by using lithium chloride precipitation solution and then diluted in nuclease-free water and stored at –80°C until use.

Microinjection of mRNA

Microinjection was performed on an inverted microscope (Nikon Corporation, ECLIPSE TE300) using a micromanipulator (Narishige) and microinjector (Narishige; IM300,). About 10 μl mRNA (1 mg/ml) was microinjected into cytoplasm of embryos in Hepes-buffered KSOM using narrow glass capillaries (Harvard Apparatus; GC100 TF-10).

Analysis of the sub-cellular localization of GFP-Dot1L protein in cultured cells

HEK293T cells (5×10^5 / well) were cultured on cover slips that had been coated with poly-L-lysine solution (Invitrogen; #P8920) in 1.5 ml Dulbecco's modified Eagle's medium (Sigma-aldrich) supplemented with fetal bovine serum (Invitrogen) and 0.4% Penicillin-Streptomycin (Gibco) on 6-well dishes (Nunc). After overnight of culture, the cells were then transiently transfected by adding 4 μg of the plasmids that encode

GFP-Dot1L constructs mixed with 10 ul Lipofectamine 2000 (Invitrogen; #11668-027) in 500 μl Opti-MEM I reduced serum medium (Invitrogen). Twenty-four hours after transfection, the cells were washed twice with PBS and fixed with 3.7% PFA for 15 min at room temperature and then the cells were washed twice with PBS. After washing, the cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature. The cover slips attached by the cells were then mounted on a glass slide in Vectashield antibleaching solution (Vector Laboratories) containing 3 μg/ml DAPI. Sub-cellular GFP signals were observed using a Carl Zeiss 510 laser scanning confocal microscope.

Reverse transcription (RT)-PCR analysis

Fifteen oocytes or preimplantation embryos were dissolved in ISOGEN (Nippon Gene). As an external control, 50 pg of rabbit α-globin mRNA was added before the isolation of total RNA. RNA isolation was performed according to manufacturer's instructions. Reverse transcription was performed using PrimeScript RT-PCR kit (Takara) according to manufacturer's instruction. Oligo dT primers were used in the reverse transcription reaction. For semi-quantitative PCR, cDNA corresponding to 1.5 oocytes or embryos was used as a template. Amplifications were performed using Ex Taq DNA polymerase (Takara). PCR products were separated on 2% agarose gels and stained with ethidium bromide. The gel

image was obtained using the DT-20MP UV illuminator (ATTO). The intensities of the bands of PCR products on the gels were measured by image J (NIH Image). The primers and the condition of PCR are shown as follows.

Gene	Forward	Reverse	Cycles
Dot1L	5'-caagaaaatgagtgctgcca-3'	5'-gagctgtgtgttcttctcct -3'	38
AF17	5'-ggccagttagactccacagc-3'	5'-ttcgctgtcaggctaaggat-3'	38
α-globin	5'-gtgggacaggagcttgaaat-3'	5'-gcagccacggtggcgagtat-3'	25

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