博士論文

Involvement of the asymmetric localization of histone H3 variants between parental genomes in mouse preimplantation development

（マウス 1 細胞期胚におけるヒストン H3 変異体の局在および機能解析）

平成 28 年 12 月博士（生命科学）申請

東京大学大学院新領域創成科学研究科
先端生命科学専攻

河村　真愛

博士論文

Involvement of the asymmetric localization of histone H3 variants between parental genomes in mouse preimplantation development

（マウス 1 細胞期胚におけるヒストン H3 変異体の局在および機能解析）

平成 28 年 12 月博士（生命科学）申請

東京大学大学院新領域創成科学研究科
先端生命科学専攻

河村　真愛

指導教員：青木不学　教授

Involvement of the asymmetric localization of histone H3 variants between parental genomes in mouse preimplantation development

Machika Kawamura

2016

Department of Integrated Biosciences

Graduate School of Frontier Sciences
The University of Tokyo

Supervisor: Prof. Fugaku Aoki

CONTENTS

ACKNOWLEDGMENTS

First and foremost, I wish to express my sincere gratitude to my supervisor, Professor Fugaku Aoki of the University of Tokyo for his guidance and encouragement throughout the work. He gave me inspiring ideas and beneficial advice, continuous support, and widened my research from different perspectives. I am also grateful to Associate Professor Masataka G. Suzuki of The University of Tokyo for his precious advice, his patience, and motivation throughout this work.

I would like to thank Professor Frederic Berger of Temasek Life Sciences Laboratory, Professor Heng-Yu Fan of Zhejiang University, and Dr. Helena Fulkova of RIKEN BioResource Center. They gave me insightful ideas and motivated me throughout this work.

I would also like to my sincere appreciation to all of the former and present members of the Laboratory of Bioresource Regulation for constant support and encouragement.

Last but not the least, I would like to thank my parents and family for their continuous encouragement. They gave me precious advice and encouragement during my studies.

ABBREVIATIONS

CAF-1: Chromatin assembly factor-1

DAPI: 4', 6'-diamidino-2-phenylindole

eGFP: enhanced green fluorescent protein

H3K9me2: histone H3 dimethylated at lysine 9

H3K9me3: histone H3 trimethylated at lysine 9

H3K27me3: histone H3 trimethylated at lysine 27

HP1β: Heterochromatin protein 1 β isoform

K: lysine

MII oocyte: Metaphase II oocyte

PRC2: Polycomb Repressive Complex 2

5mC: 5-methylcytosine

SUMMARY

The 1-cell embryo, formed by the fusion of a sperm and an oocyte, is the beginning of life. The maternal and paternal genomes are first enclosed in two separate compartments, referred to as maternal pronucleus and paternal pronucleus, respectively. Although they exist in the same cytoplasm, each acquire its unique chromatin structure and characteristics. First, parental asymmetry is observed in transcriptional activity, where the paternal pronucleus has a higher transcriptional activity compared to that of maternal pronucleus. In addition, DNA replication is reported to occur asymmetrically in terms of the speed of completion. These heterogeneities can be explained by the differences in chromatin structure of maternal and paternal pronuclei. The chromatin structure of the paternal pronucleus is loosened and decondensed compared to that of maternal pronucleus. However, what determines this difference in chromatin structure and the difference in characteristics seen between the pronuclei, is not yet elucidated.

Histone variants is one of the key players in determining chromatin structure. Histone H3 has three non-centromeric variants, H3.1, H3.2, and H3.3. Although they are highly similar in amino acid sequence, each of the H3.1, H3.2, H3.3 variants are known to be associated with different histone modifications and deposition pathways. For example, H3.1 and H3.2 are deposited in both heterochromatin and euchromatin; however, H3.3 are mainly deposited in euchromatin. In addition, H3.1 and H3.2 are likely to acquire histone modifications that are associated with transcriptional repression whereas H3.3 are likely to obtain those that are associated with transcriptional activation. Taken together, H3 variants are essential in determining chromatin structure.

In this study, I hypothesize that H3 variants is one of the determinants in the

difference in characteristics of maternal and paternal pronuclei at the 1-cell stage. In chapter 1, I investigated the nuclear localization of H3 variants during the preimplantation development. As a result, asymmetric nuclear localization of H3.1/H3.2 were detected in 1-cell embryos: they are localized in the perinucleolar region in the maternal pronucleus but not in the paternal one. In addition, the nuclear localization level of H3.1/H3.2 was low compared to that of later preimplantation stages and the low nuclear localization was regulated by the low mRNA expression level and low incorporation efficiency. In chapter 2, I examined the biological significance of the low nuclear localization level of H3.1/ H3.2 in 1-cell embryos, by inducing incorporation of each of the H3 variants into chromatin of 1-cell embryos and analyzed the effect of ectopic localization of each variant in preimplantation development. The results showed that when H3.1 and H3.2 were induced into chromatin in 1-cell embryos, the developmental rate of these embryos were remarkably low compared to that of H3.3-induced and control embryos. Interestingly, the H3.1/H3.2 nuclear localization of the paternal pronucleus was altered upon induced incorporation of H3.1 and H3.2, in which the H3.1/ H3.2 was detected at the perinucleolar region, similar to that of maternal pronucleus, thus leading to a lack of asymmetry. In addition, the developmental failure in H3.1 and H3.2 overexpressed embryos were caused by the delayed DNA replication at the perinucleolar region in the paternal pronucleus whereas H3.1 and H3.2 incorporation had no effect in DNA replication in the maternal one.

This study introduced a novel insight in the role of H3.1 and H3.2, which are asymmetrically localized between the maternal and paternal pronuclei in 1-cell embryos. This difference in nuclear localization regulates DNA replication, in which the DNA replication at the perinucleolar region of the paternal pronuclei is completed earlier

compared to that of maternal pronuclei. In addition, the results suggested that it is essential that H3.1 and H3.2 is not incorporated into chromatin of 1-cell embryos, as incorporation of these variants cause defects in DNA replication of the paternal pronucleus. The nuclear localization of H3.1 and H3.2 are regulated to be low at the paternal pronuclei by lowering the mRNA expression level and incorporation efficiency into chromatin at the 1-cell stage.

GENERAL INTRODUCTION

Mammalian development begins with fertilization, where cytoplasmic fusion of sperm and the oocyte takes place, leading to a formation of a 1-cell embryo that contains each of maternal and paternal genome. The maternal chromosomes in the unfertilized oocytes are arrested at metaphase of the second meiosis. Upon fertilization, the cell cycle of the maternal chromosome resumes to complete meiosis while the sperm nuclear envelope is degenerated and paternal chromosome in the ooplasm undergoes decondensation (Adenot et al., 1991; van der Heijden et al., 2006). The two genomes are then independently enclosed into a nuclear membrane to form two nuclei, which are referred to as maternal and paternal pronuclei. The two genomes are mingled with each other during the first mitosis and then the embryos cleave into 2-cell stage embryos and then undergo subsequent cleavage to develop to term (Fig. GI-1A, B).

The parental genomes were thought to have equal roles in development until three decades ago when studies have found that each of the maternal and paternal genomes contribute differently (McGrath & Solter, 1984; Surani et al., 1984; Surani et al., 1986; Surani et al., 1987). These studies have removed either the maternal or paternal pronucleus of the 1-cell embryo and transplanted with a pronucleus of the same gender and assessed the development of these diploid parthenogenetic (lack paternal genome) or androgenetic (lack maternal genome) embryos. Although the parthenogenetic embryos were able to implant and develop until the tenth day of embryonic development, they formed poorly developed extraembryonic tissues and died. The androgenetic embryos also did not developed to term: they were poorly developed with only a few somites whereas the extraembryonic tissues was well-developed. Taken together, the maternal and

paternal genomes were found to contribute to development differently, and the presence of both pronuclei is essential to develop to term (Surani et al., 1984; Surani et al., 1986).

Although the parental pronuclei reside in the same cytoplasm, they have different characteristics in essential cellular processes such as transcription and DNA replication (Fig. GI-1C). Regarding transcription, it is known that paternal pronucleus has a greater transcriptional activity compared to that of maternal pronucleus (Bouniol et al., 1995; Aoki et al., 1997). The analysis on the BrUTP incorporation indicated that the transcriptional activity of the paternal pronucleus is greater than that of the maternal pronucleus (Aoki et al., 1997). Ram and Schultz (1993) injected SV40 early promoter-driven luciferase gene into the parental pronuclei of the 1-cell embryos. The luciferase activity was detected in the paternal pronucleus whereas not detected in the maternal pronucleus. DNA replication is also asymmetric between the parental pronuclei, regarding the time of completion and the order of spatio-temporal occurrence of DNA replication (Aoki et al., 1999). Although DNA replication is initiated synchronously between the parental pronuclei, it is completed in the paternal pronucleus earlier than the maternal one. In addition, the order of occurrence of DNA replication in nuclear regions differ between the parental pronuclei. First, DNA replication in both pronuclei occur at the nucleoplasmic region, proceeded by the DNA replication at the perinuclear and perinucleolar region. However, the site and order of DNA replication differs at the end of the process. In the paternal pronucleus, the DNA replication at the perinucleolar region is completed earlier than that of perinuclear region, resulting to the completion of DNA replication at the perinuclear region at the end of S phase. In contrast, the DNA replication at the perinuclear region of the maternal pronucleus completes earlier than that of perinucleolar region. DNA replication at the perinucleolar region of the maternal

pronucleus proceeds even after the DNA replication of the paternal pronucleus is completed. Taken together, these results suggest that the paternal pronucleus is more permissive to undergo transcription and faster DNA replication. However, the mechanism that underlies the difference in cellular processes between paternal and maternal pronuclei is not yet elucidated.

The differences of transcription and DNA replication pattern observed between parental pronuclei is thought to be regulated by chromatin structure. Chromatin consists of nucleosomes that are each wrapped around by 146 bp of DNA (Luger et al., 1997). Regions where chromatin is densely packed is referred to as heterochromatin and regions that have open and decondensed chromatin is referred to as euchromatin. The association between chromatin structure and cellular processes, such as DNA replication and transcription has been investigated in somatic cells. Generally, the regions of euchromatin are transcriptionally active whereas those of heterochromatin are transcriptionally inactive (Maison and Almouzni, 2004; Hake and Allis, 2006; Allis and Jenuwein, 2016). Furthermore, active transcription occurs in the euchromatin regions with increased nucleosome accessibility (Hake and Allis, 2006; Allis and Jenuwein, 2016). Regarding DNA replication, the analysis for BrdU incorporation have shown that at S phase, euchromatin is replicated first and then heterochromatin last (O'Keefe et al., 1992). In the 1-cell stage embryos, it has been suggested that chromatin structure is different between the parental pronuclei. In reporter gene analysis, an enhancer was required for active transcription from the injected luciferase gene in the female pronucleus but not in the male one (Wiekowski et al., 1993), suggesting that the chromatin structure is repressive for transcription in female pronucleus but permissive in the male one. Recent analysis for the chromatin structure have indicated that the looseness was different between the

parental pronuclei (Ooga et al., 2016). Fluorescence recovery after photobleaching (FRAP) analysis revealed that the exchange of histones occur more frequently in the paternal pronucleus than in the maternal one, suggesting that the paternal chromatin is more loosened compared to the maternal chromatin. Therefore, these results suggest that the paternal pronucleus has a higher transcriptional activity and faster DNA replication compared to that of maternal pronucleus, due to a loosened chromatin structure in the paternal pronucleus. Consequently, the differences in the cellular processes between the parental pronuclei seems to be regulated by factors that regulate the chromatin structure.

One of the asymmetries between the parental pronuclei extensively studied are the epigenetic modifications. Over a decade ago, it was found that chromatin structure is altered depending on the posttranslational modifications added to the histones and DNA methylation (Kouzarides, 2002; Kouzarides, 2007). These modifications are suggested to be crucial for regulation of cellular processes such as DNA replication, transcription, and cell division. Recently, there has been increasing evidence that epigenetic modifications and chromatin remodelers that regulate epigenetic modifications are asymmetric between the parental pronuclei. For example, the DNA of the maternal pronucleus is hypermethylated compared to that of paternal pronucleus, where active demethylation takes place during the 1-cell stage (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002). H3K9me2/3, histone modification associated with constitutive heterochromatin, is localized in the maternal pronuclei whereas a marginal level of localization was observed in the paternal pronucleus (Liu et al., 2004; Lepikhov and Walter, 2004; Santos et al., 2005; Puschendorf et al., 2008). H4 acetylation associated with euchromatin, becomes significantly detectable in the paternal pronucleus compared to that of maternal pronucleus right after fertilization (Adenot et al., 1997). In addition, the chromatin-bound

proteins that associate with epigenetic modifications differ between the perinucleolar region in the parental pronuclei (Tardat et al., 2015). The heterochromatin protein HP1β that is associated with constitutive heterochromatin is bound with perinucleolar region of the maternal pronucleus. In contrast, the polycomb repressive complex 1 (PRC1) and PRC2, which are associated with facultative heterochromatin (H3K27me3-rich), are recruited to the perinucleolar region of the paternal pronucleus. Taken together, these studies suggest that the chromatin structure of the paternal pronucleus is loose and decondensed compared to that of maternal one. Although recently these epigenetic asymmetries have been brought into the limelight, the biological significance of them and whether these modifications contribute to asymmetry in cellular processes observed in the parental pronuclei is not yet clarified.

Besides epigenetic modifications, histone variants were found to be one of the key determining factors of chromatin structure. The existence of histone variants were found back in 1969 (Pusarla and Bhargava, 2005) and these variants have different amino acid sequence and are coded by different genes and acquire distinct regulation in transcription and translation and chromatin incorporation (Talbert and Henikoff, 2016). Recently, it was shown to be essential regulators of cellular processes such as transcription, DNA replication, and chromosome segregation (Pusarla and Bhargava, 2005; Kamakaka and Biggins, 2005; Talbert and Henikoff, 2016). Each core histone consists of histone variants (although mammals lack H4 variants) and the exchange of histone variants to another alters the chromatin structure, thus leading to a change in cellular processes (Talbert and Henikoff, 2010; Moosmann et al., 2011; Talbert and Henikoff, 2016).

Among the core histones, histone H3 have been brought into attention due to

their high similarity of amino acid sequence yet have different characteristics. In mammals, there are three non-centromeric histone variants: H3.1, H3.2, and H3.3 (Hake and Allis, 2006). Moreover, in comparison to other core histone variants, the amino acid sequences of H3 variants are almost identical, except for few amino acid differences (Fig. GI-2A): four amino acids between H3.2 and H3.3 and five amino acids between H3.1 and H3.3, and one amino acids between H3.1 and H3.2 (Hake and Allis, 2006). Interestingly, H3.1 and H3.2 have evolved from H3.3 (Postberg et al., 2010), and the higher the class order of the organism, the greater the number of variants (Hake and Allis, 2006). For example, *S. cerevisiae* contain solely H3.3 as a non-centromeric histone variant. However, non-mammalian organisms such as *D. melanogaster* and *A. thaliana* contain H3.2 and H3.3. Interestingly, mammals contain another non-centromeric histone variant, H3.1, which is suggested to be evolved later than H3.2 and H3.3 (Postberg et al., 2010). These H3 variants differ in the regulation of expression, mechanism for chromatin incorporation, genomic region of incorporation, epigenetic modifications, and the effect on chromatin structure (Fig. GI-2B), as is described in detail below.

H3.1 and H3.2 are histone variants that are expressed and incorporated into chromatin in a DNA replication-dependent manner (Tagami et al., 2004). From ChIP-seq analyses using Flag tagged histone variants expressing embryonic stem cells, H3.1 and H3.2 are generally located in both euchromatin and heterochromatin (Yukawa et al., 2014). H3.1 is also enriched in repeat elements. In mice, there are four genes that code for H3.1 and eight genes that code for H3.2 (Wang et al., 1996a; Wang et al., 1996b). The genes that code for H3.1 and H3.2 reside in histone clusters on chromosomes 3 and 13 (Graves et al., 1985; Wang et al., 1996a; Wang et al., 1996b). H3.1 and H3.2 lack introns and H3.1 and H3.2 mRNA contain a stem loop in their 3' untranslated regions but not a polyA tail

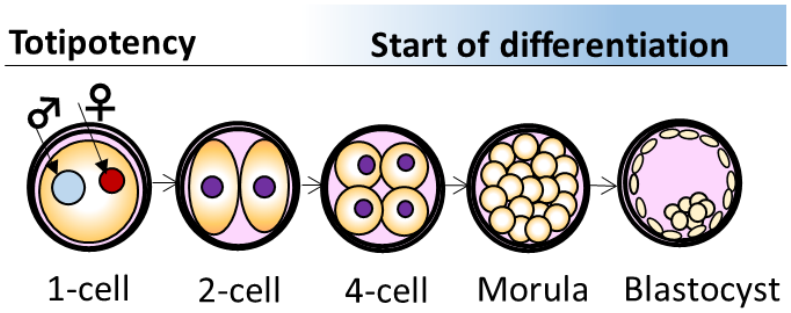
in their 3'-terminuses and is associated with stem-loop binding protein for mRNA processing (Marzluff, 2005). These histone variants are incorporated by the histone chaperone called CAF-1 (Smith and Stillman, 1989; Tagami et al., 2004; Akiyama et al., 2011) which is composed of p150, p60, and p48 (Verrault et al., 1996). H3.1 and H3.2 differ by solely one amino acid, where H3.1 possesses cysteine as 96th amino acid from the N-terminus tail instead of alanine in H3.2 and H3.3 (Hake and Allis, 2006). H3.1 is more likely to acquire H3K9me2 (Hake et al., 2006), which is a histone modification enriched in constitutive heterochromatin. In contrast, H3.2 is likely to acquire with repressive histone modifications such as H3K27 di- and tri-methylation, which is associated in facultative heterochromatin. In addition, H3.1 is only present in mammals (Hake and Allis, 2006), suggesting that H3.1 is also involved in mammalian-specific processes.

H3.3 is a histone variant, which its expression and incorporation into chromatin is DNA replication-independent (Hake and Allis, 2006). H3.3 is coded by two genes, *H3f3a* and *H3f3b*, which reside on Chromosomes 1 and 11, respectively (Maehara et al., 2015) and unlike H3.1 and H3.2, they contain introns and their transcripts have a polyA tail (Marzluff, 2005; Talbert and Henikoff, 2016). H3.3 mostly acquires modifications associated with active transcription such as H3K9ac and H3K79me2 (Hake et al., 2006; Martin and Zhang, 2005). This histone variant is generally incorporated in euchromatin via the histone chaperone, histone cell regulator (HIRA) (Ray-Gallet et al., 2002; Tagami et al., 2004). However, recently, it has been found to be incorporated in telomere repeats by another histone chaperone ATRX/DAXX (Goldberg et al., 2010). In addition, H3.3 is deposited to pericentromeric repeats (Rapkin et al., 2015), suggesting that H3.3 can be incorporated into heterochromatin. Consequently, H3.1, H3.2, and H3.3 have distinct

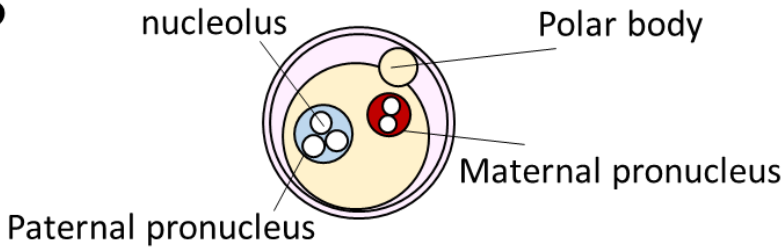
characteristics and is suggested that the replacement of these variants can alter the chromatin structure (Jin et al., 2005; Talbert and Henikoff, 2010).

My hypothesis is that H3 variants regulate the difference in characteristics of cellular processes, *i.e.* DNA replication and transcription, between the parental pronuclei. Because H3.1 and H3.2 are associated with heterochromatin, these variants may be localized in the maternal pronucleus greater to that of paternal pronucleus and H3.3 would be opposite, thus leading to an asymmetry in cellular process between the parental pronuclei. To test my hypothesis, I have examined the nuclear localization of H3 variants during the preimplantation development. Then I elucidated the regulatory mechanism of incorporation of H3.1/H3.2 into chromatin of 1-cell embryos. Finally, I investigated the biological significance of the nuclear dynamics of H3.1/H3.2 in the 1-cell stage embryos.

A



B

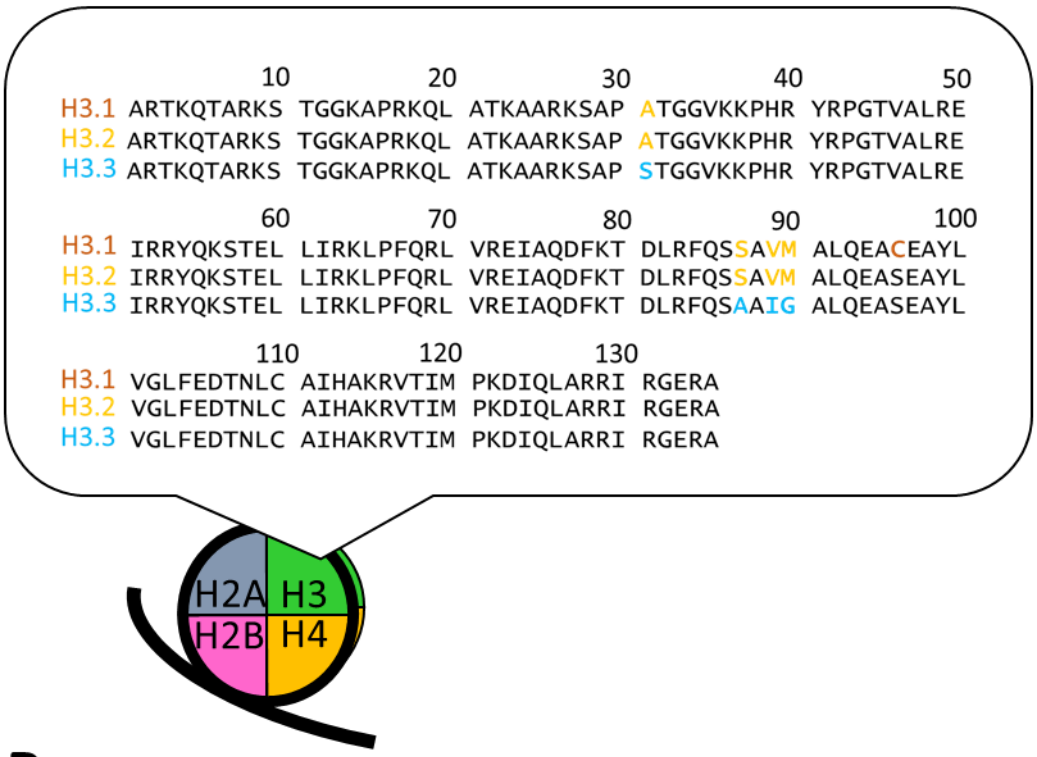


C

	Paternal ♂	Maternal ♀
Transcriptional activity	High	Low
DNA replication	Fast	Slow
Chromatin structure	Loose	Tight
H3K9 methylation	Low	High
Heterochromatin components at the perinucleolar region	PRC1 PRC2	HP1β

Figure GI-1. Illustration of preimplantation embryos and the characteristics that are asymmetrical between the parental pronuclei. **(**A) Illustration of preimplantation development. After fertilization, the parental genomes are enclosed in independent pronuclei and then mingled with each other at M phase of 1-cell stage and subsequent development occur. (B) The detailed illustration of 1-cell embryos. The maternal pronucleus is usually located proximal to the second polar body. Nucleoli reside inside the nucleus, and their number can differ among the embryos. (C) Asymmetric characteristics of the paternal and maternal pronuclei.

A



B

	H3.1	H3.2	H3.3
Method of incorporation into chromatin	Replication-dependent	Replication-dependent	Replication-independent
Region associated	Heterochromatin, Euchromatin	Heterochromatin, Euchromatin	Euchromatin

Figure GI-2. Histone H3 variants and their characteristics.

(A) Among the core histones, H3 contains three non-centromeric histone variants, H3.1, H3.2, and H3.3. The amino acid sequences are highly similar with only several amino acid difference. (B) General characteristics of histone H3 variants.

CHAPTER 1

The nuclear localization of histone H3 variants during the preimplantation development

INTRODUCTION

Histone variants are key regulators of chromatin structure. Recently, histone variants were found to be associated in development, such as gametogenesis, embryonic development, and organ development (Filipescu et al., 2013; Maze et al., 2014; Filipescu et al., 2014). Among the histone variants, there are several studies reported regarding the involvement of H3 variants in embryonic development.

The nuclear localization of H3.3 and its role in preimplantation development is analyzed in various model organisms. In *Mus musculus*, H3.3 is incorporated in both maternal and paternal pronuclei of the 1-cell embryos and is present in the nuclei throughout preimplantation development (Torres-Padilla et al., 2006; Akiyama et al., 2011). H3.3 becomes incorporated into the paternal pronucleus independent of the protamine removal (Inoue et al., 2014). H3.3 is incorporated when the paternal pronucleus is decondensed, suggesting that H3.3 incorporation facilitates decondensation of the paternal pronucleus (van der Heijden et al., 2005). In contrast, in the maternal pronucleus, H3.3 nuclear localization was temporally diminished and detected again at the G2 phase of the 1-cell embryos (Akiyama et al., 2011). Interestingly, H3.3 is incorporated in the perinucleolar region of paternal pronucleus but not the maternal pronucleus, and the mutation analysis of H3.3 that cannot be modified with trimethylation at lysine 27 have shown that this histone modification on H3.3 is essential for preimplantation development (Santenard et al., 2010).

Similar dynamics of H3.3 during preimplanation development in mammals is also reported in other non-mammalian organisms. In *Drosophila melanogaster*, the histone chaperone that incorporates H3.3 into chromatin, HIRA, and Yemanuclein (YEM)

incorporates H3.3 into the paternal pronucleus before the onset of DNA replication (Loppin et al., 2005; Orsi et al., 2013) and is involved in the decondensation of the male pronucleus (Loppin et al., 2005; Bonnefoy et al., 2007). In *Caenorhabditis elegans*, H3.3 of maternal origin is localized in both maternal and paternal pronuclei of 1-cell embryos, but the correlation between paternal pronucleus decondensation and H3.3 incorporation was not clarified (Ooi et al., 2006). Because the onset of zygotic transcription occurs at four-cell stage in *C. elegans*, the incorporation of H3.3 into chromatin in embryos is suggested to be transcription-independent. In addition, in *Arabidopsis thaliana*, the nuclear localization of H3.3 is dynamic during fertilization, in which the paternally-derived H3.3 is diminished upon double fertilization (fertilization of flowering plants) suggesting that H3.3 is involved in chromatin remodeling upon fertilization (Ingouff et al., 2007). Taken together, these studies suggest that H3.3 is involved in chromatin remodeling during preimplantation development.

In contrast to H3.3, the nuclear localization of H3.1 and H3.2 during preimplantation development remains to be elucidated. Several studies have analyzed the nuclear localization of H3.1 and/or H3.2 during preimplantation development. However, the results are contradicting. For example, Akiyama et al. (2011) microinjected cRNA that encodes for each of the H3 variants with Flag tags attached to the N terminus end, and found that H3.2 is deposited into both maternal and paternal pronuclei of the 1-cell embryos and is localized in the nuclei from this stage. In contrast to H3.2, the nuclear localization of H3.1 is observed only from 4-cell stage. Moreover, in my previous study using Flag-tagged transgenic mice that ubiquitously express H3.1, nuclear localization of H3.1 was detected from the morula stage (Kawamura et al., 2012). Even more surprising is that when antibodies specific to H3.1 and H3.2 were generated and the nuclear

localization of these histone variants were analyzed, H3.1/H3.2 was detected in the maternal pronucleus but not paternal pronucleus, suggesting asymmetry in H3.1/H3.2 nuclear localization, however with no detailed analysis of nuclear distribution (van der Heijden et al., 2005). In addition, the microinjection of cRNA that codes for H3.1 that have GFP attached to the C-terminal end into 1-cell embryos showed that H3.1 was incorporated at equal level between the parental pronuclei (Santenard et al., 2010). Thus, conflicting results have been shown whether H3.1 and H3.2 is localized in two parental pronuclei of the 1-cell stage embryos.

To investigate whether H3 variants are involved in regulating the asymmetry in cellular processes between the parental pronuclei, I attempted to elucidate the nuclear localization of H3 variants during the preimplantation development and determined whether there is an asymmetry of the nuclear localization of H3 variants seen in 1-cell embryos. Next, I clarified the mechanism underlying the nuclear localization in 1-cell embryos and why there were contradictory results regarding the analysis of nuclear localization of H3.1/H3.2.

RESULTS

The nuclear localization of histone H3 variants in the preimplantation embryos

I investigated the nuclear localization of H3 variants by immunocytochemistry using antibody that recognizes H3.1 and H3.2 (abbreviated as H3.1/H3.2) and antibody that recognizes H3.3 (H3.3). The former antibody does not discriminate between H3.1 and H3.2. The specificity of the antibody was verified by peptide adsorption (Fig. 1-1). The nuclear localization level of H3.1/H3.2 was unaltered when H3.1/H3.2 antibody was incubated with H3.3 peptide as an antigen whereas the nuclear localization decreased when H3.1/H3.2 peptide was reacted with the antibody. In contrast, the nuclear localization of H3.3 was unchanged when H3.1/H3.2 peptide was incubated with anti-H3.3 antibody whereas the nuclear localization level decreased when the antibody was reacted with H3.3 peptide. The results of immunocytochemistry showed that H3.3 was localized in the nuclei throughout the preimplantation development (Fig. 1-2A), which was consistent to previous results (Torres-Padilla et al., 2006; Akiyama et al., 2011). However, there was a low level of localization of H3.1/H3.2 in the pronuclei of 1-cell embryos compared to that of embryos from the 2-cell stage. Interestingly, when the signal intensity was increased, H3.1/H3.2 was clearly detected in the pronuclei of 1-cell embryos in which the localization was different between the parental pronuclei (Fig. 1-2B). Although the nuclear localization of H3.1/H3.2 in the nucleoplasm was the same between the two pronuclei, the localization at the perinucleolar region of the maternal pronucleus was much greater compared to that of the paternal pronuclei (Fig. 1-2C), suggesting that there is difference in heterochromatin structure of the perinucleolar region between the maternal and paternal pronuclei. It is known that constitutive heterochromatin forms

chromocenters from the 2-cell stage and appears as dots with high DNA density (Probst and Almouzni, 2011). There was a colocalization of H3.1/H3.2 and chromocenters (Fig. 1-2D) and the nuclear localization of H3.1/H3.2 increased during preimplantation development.

The involvement of DNA replication in H3.1/H3.2 deposition into chromatin of preimplantation embryos.

H3.1 and H3.2 are known to be incorporated into chromatin at S phase in somatic cells (Hake and Allis, 2006). To investigate whether H3.1/H3.2 is incorporated into chromatin in DNA replication-dependent manner, 1-cell and 2-cell embryos were treated with or without aphidicolin, an inhibitor of DNA polymerase. Immunofluorescence staining of H3.1/H3.2 revealed that in both 1-cell and 2-cell stages, H3.1/H3.2 was hardly detected in embryos treated with aphidicolin (Fig.1-3A, B), which indicated that H3.1/H3.2 is deposited into chromatin in a DNA replication-dependent manner similarly to somatic cells.

The mechanism that underlies the low nuclear localization level of H3.1/H3.2 in the 1-cell embryos

There are two possible mechanisms that cause the limited nuclear localization of H3.1/H3.2 in 1-cell embryos. First, H3.1/H3.2 mRNA ratio may be low compared to that of H3.3 at the 1-cell stage. Second, the incorporation efficiency of H3.1/H3.2 into chromatin may be low compared to that of H3.3. To examine whether the H3.1/H3.2 mRNA ratio is low compared to that of H3.3 at the 1-cell stage, the ratio of mRNA expression of H3.1, H3.2, H3.3 was analyzed using RNA-seq data (Abe et al., 2015).

Histone H3 variants are coded by multiple genes. In mice, H3.1, H3.2 and H3.3 are coded by 4, 8 and 2 genes, respectively (Wang et al., 1996a; Wang et al., 1996b; Tang et al., 2015). The RPKM values of each gene were summed up to calculate the total RPKM value for each H3 variant. The RPKM value of H3.3 at the 1-cell stage was set to 1 and the relative RPKM was calculated (Fig. 1-4). As a result, at the stages where H3.1/H3.2 nuclear localization was observed, *i.e.* 2-cell stage and onwards, the mRNA expression ratio of H3.1 and H3.2 was equivalent or above that of H3.3. However, at 1-cell stage, where the level of H3.1/H3.2 nuclear localization was low, the mRNA expression ratio of H3.1/H3.2 was low compared to that of H3.3. Therefore, low mRNA expression ratio of H3.1/H3.2 compared to that of H3.3 contributes to the low nuclear localization level of H3.1/H3.2 at the 1-cell stage.

To determine whether the incorporation efficiency of H3.1 and H3.2 is low compared to that of H3.3, cRNA that codes for H3.1, H3.2, or H3.3 with Flag-tag attached on the C-terminus were microinjected in MII stage oocytes with different concentrations (3 ng/µl, 10 ng/µl, 30 ng/µl, and 100 ng/µl) and were inseminated. The free histones in the nucleoplasm were washed away and the incorporation of Flag-tagged histones into chromatin were detected by immunofluorescence staining using anti-Flag antibody (Fig. 1-5A). The quantification of signal intensity revealed that the incorporation efficiency of H3.3 was significantly greater compared to that of H3.1 and H3.2 with cRNA concentrations below and including 30 ng/µl (Fig. 1-5B). Notably, the incorporation efficiency of the three H3 variants were similar when cRNA was microinjected with the concentration of 100 ng/µl. However, considering the microinjection of 100 ng/µl of cRNA results in more than 400 times higher concentration than endogenous mRNA in an embryo (Pikó and Clegg, 1982), the incorporation efficiency would be lower in H3.1 and

H3.2 than H3.3 at the endogenous level of mRNA. Taken together, the low level of nuclear localization of H3.1 and H3.2 in 1-cell embryos is due to the low mRNA expression and low incorporation efficiency into chromatin compared to that of H3.3.

DISCUSSION

In this chapter, I have clarified the nuclear localization of H3 variants during the preimplantation development. The results suggested that H3.1/H3.2 was localized in the pronuclei of 1-cell embryos at a low level compared to those in the nuclei of later stage of embryos (Fig.1-2A, B). I further investigated why the nuclear localization of H3.1/H3.2 is limited and examined whether the nuclear localization is regulated in both the mRNA level and the incorporation efficiency. I have shown that the mRNA expression ratio of H3.1 and H3.2 compared to that of H3.3 is low at the 1-cell stage where low nuclear localization is observed. In contrast, H3.1 and H3.2 mRNA expression ratio is greater than that of H3.3 from 2-cell stage and onwards, where nuclear localization becomes more evident. The incorporation efficiency of H3.1 and H3.2 into chromatin is significantly lower than that of H3.3 in 1-cell embryos. Taken together, the nuclear localization of H3.1 and H3.2 is low in the 1-cell stage and is regulated by both mRNA level and chromatin incorporation efficiency.

When I started this study, my hypothesis was that H3 variants are involved in the asymmetry of transcription activity and DNA replication between the parental pronuclei in 1-cell embryos. However, asymmetric localization of H3 variants was observed only for H3.1/H3.2 in perinuclear regions but not for any of H3 variants in the nucleoplasmic region. Because transcription mostly occurs in the euchromatin which is localized in the nucleoplasmic region, H3 variants do not seem to be involved in the asymmetric transcriptional regulation betwen the parental pronuclei. In contrast, the localization pattern of H3.1/H3.2 is consistent with the DNA replication pattern. A previous study showed that the maternal pronucleus has a prolonged DNA replication at the perinucleolar region whereas the DNA replication at the perinucleolar region of the paternal pronucleus

is completed at an earlier stage (Aoki et al., 1999). Since H3.1/H3.2 is associated with heterochromatin (Hake and Allis, 2006), and heterochromatic region replicates late in S phase (O'Keefe et al., 1992), the asymmetry of H3.1/H3.2 localization at the perinucleolar region is likely to cause the asymmetry in the speed and timing of DNA replication in the perinucleolar regions between the parental pronuclei.

There are several studies that have analyzed the nuclear localization of H3.1/ H3.2. The immunocytochemistry analysis by van der Heijden et al. (2005) has reported that H3.1/H3.2 was detected in both parental pronuclei at S phase. These results were consistent with my observations that the nuclear localization level of H3.1/H3.2 in the nucleoplasmic region at 10 h, when DNA replication was completed (*see* Fig. 2-5), was equal between the parental pronuclei (Fig. 1-2C). Here, I have found that there is asymmetry in the H3.1/H3.2 nuclear localization at the perinucleolar region between the parental pronuclei, although it was not mentioned in van der Heijden et al. (2005). However, the results on nuclear incorporation of H3.1/H3.2 by microinjection analysis (Santenard et al., 2010; Akiyama et al., 2011) was inconsistent to the results of immunocytochemistry (Fig. 1-2A, B, C; van der Heijden et al., 2005). When tagged H3.2 was microinjected, the nuclear localization of H3.2 was evident from the 1-cell stage (Santenard et al., 2010; Akiyama et al., 2011): in the report of Santenard et al., (2010), they describe that they cloned cDNA coding H3.1 from H3e gene, but it does not seem to be H3.1 but H3.2 because H3e encodes H3.2. The discrepancy between these results may have been caused by the concentration of injected cRNA. At a low cRNA concentration of H3.2, the chromatin incorporation efficiency is low compared to H3.3, but a high concentrations, H3.1 and H3.2 are incorporated into chromatin at almost the same efficiency as H3.3 (Fig. 1-5B). In the experiments of Santenard et al. (2010) and Akiyama

et al. (2011), 120 and 100 ng/μl cRNA was microinjected, respectively. In my results, when 100 ng/μl of cRNA was microinjected, the incorporation of H3.2 was almost same between H3.2 and H3.3. In addition, there is a possibility that the presence of a tag (GFP or Flag) or the position of the tag (C or N terminus) may also affect the protein structure and thus the interaction with the histone chaperone, leading to a different incorporation result.

My results have shown that H3.1/H3.2 nuclear localization was evident from the 2-cell stage, gradually increasing the level of nuclear localization as development progresses (Fig. 1-2A, B). This is consistent with the observation that the chromatin conformation becomes tighten and condensed during preimplantation development (Ahmed et al., 2010; Ooga et al., 2016). H3.1/H3.2 is associated with heterochromatin (Hake and Allis, 2006). Both parental chromatin organize chromocenters that are consisted of constitutive heterochromatin and become detectable from the 2-cell stage (Probst and Almouzni, 2011). This is consistent with my results where there is a colocalization of H3.1/H3.2 and chromocenter from the 2-cell stage (Fig. 1-2D). Therefore, it suggests that deposition of H3.1/H3.2 are associated with heterochromatin formation during the preimplantation development.

The paternal perinucleolar region has either low levels or absence of heterochromatin-associated features. For example, there is low level of H3.1/H3.2 (Fig. 1-2B, C), suggesting that H3.3 is the major component of the chromatin in pericentromeric regions of the paternal pronucleus. The paternal perinucleolar region lacks HP1β and H3K9me2/3, which are features associated with heterochromatin and is found in maternal pronucleus (Puschendorf et al., 2008; Tardat et al., 2015). This lack of heterochromatin components suggests that the paternal perinucleolar region is relatively

decondensed compared to that of somatic cells and female pronuclei. In fact, major satellites are expressed more in the paternal pronucleus (Probst et al., 2010). The characteristics is unique in that H3.3 is localized at the paternal perinucleolar region in that it may acquire a unique structure that is relatively loosened chromatin. Why is H3.3 but not H3.1/H3.2 enriched at the perinucleolar region of the paternal pronucleus as much as that of the maternal pronucleus? One possibility is DAXX that is a H3.3-specific histone chaperone and together with ATRX, deposits H3.3 to telomeres and pericentromeric heterochromatin (Lewis et al., 2010; Liu et al., 2012). It is suggested that although ATRX is localized in both parental nuclei, DAXX is mainly located to the paternal pericentric heterochromatin region/ perinucleolar region (De La Fuente et al., 2015). Given that DAXX has a specificity to H3.3 and does not bind with H3.1 (Lewis et al., 2010), the asymmetric recruitment of DAXX to paternal pericentromeric heterochromatin (De La Fuente et al., 2015) may facilitate the incorporation of H3.3, which in turn prevents H3.1/H3.2 incorporation, at this region.

Finally, we have revealed that the expression ratio of H3.1 and H3.2 compared to that of H3.3 in the stage where H3.1/ H3.2 nuclear localization is low in 1-cell embryos (Fig. 1-4). H3.1 and H3.2 may have low mRNA expression ratio because there are marginal levels of maternally derived H3.1 and H3.2 mRNAs. Until DNA replication occurs in the 1-cell embryo, it is suggested that there are limited mRNA that code for replication-dependent histones. H3.1 and H3.2 are encoded by genes that reside in the histone gene cluster and these genes are transcribed at S phase and the mRNA is rapidly degraded at the end of S phase (Wang, 1996a; Wang, 1996b; Koseoglu et al., 2010). Because postnatal oocytes undergo a long time span where DNA synthesis is absent until fertilization (Nashun et al., 2015), there may be marginal levels of H3.1/H3.2 before DNA

replication begins at 1-cell stage. Therefore, mRNA expression ratio of H3.1 and H3.2 may be low compared to H3.3 in 1-cell embryos.

There may be a low incorporation of H3.1/H3.2 in 1-cell stage, because histone chaperones that interact with H3.1/ H3.2 are not functional at this stage. The RNA-seq data showed that the transcripts for all three components of CAF-1 were at the similar levels between 1- and 2-cell stages (Abe et al., 2015). ASF1a, b is reported to be the upstream histone chaperone that transports H3.1 (not reported but presumably H3.2) down to CAF-1 (Tang et al., 2012; Campos et al., 2015). The mRNA expression level of these histone chaperones were also similar between 1- and 2-cell stages, thus suggesting that these histone chaperones are not responsible for the limited incorporation of H3.1/ H3.2 (Abe et al., 2015). In addition, by mass-spectrometry analyses using HeLa cells, H3.1 is reported to interact with eleven histone chaperones (Campos et al., 2015). However, the mRNA expression of these histone chaperones did not show an increase in mRNA expression level from 1-cell to 2-cell stage (Abe et al., 2015). Therefore, there may be another mechanism that suppresses CAF-1 from incorporating H3.1/H3.2.

In this chapter, I have shown that asymmetric nuclear deposition of H3.1/H3.2 reflects the differences of DNA replication between the parental pronuclei. In the next chapter, I experimentally examine whether this asymmetry of H3.1/H3.2 deposition is indeed associated with the difference of DNA replication and investigate the biological significance of the asymmetrical deposition in the preimplantation development.

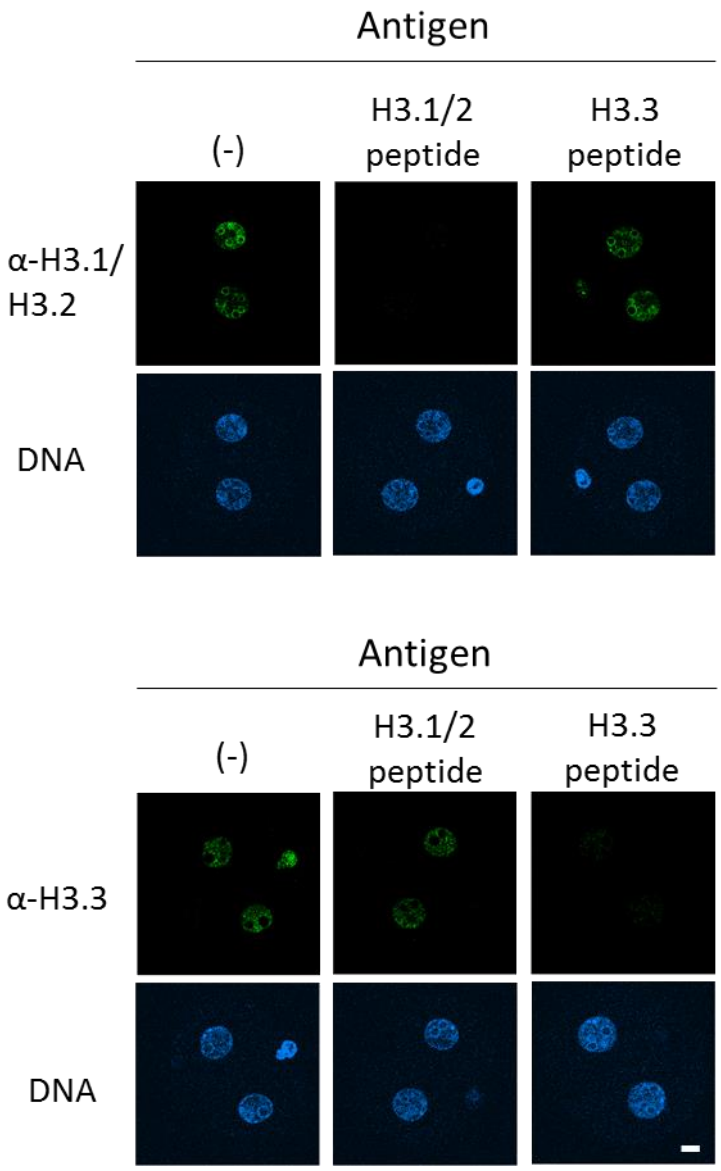


Figure 1-1. Peptide adsorption to verify specificity of anti-H3.1/ H3.2 and anti-H3.3 antibodies.

Two-cell embryos were subjected to immunocytochemistry with anti-H3.1/H3.2 and H3.3 antibodies which had been preincubated with the antigen peptides at a molecular ratio of 1:50 (antibody: peptide). A single experiment was performed, with 8-10 embryos were examined per experimental group. Scale bar = 10 μm

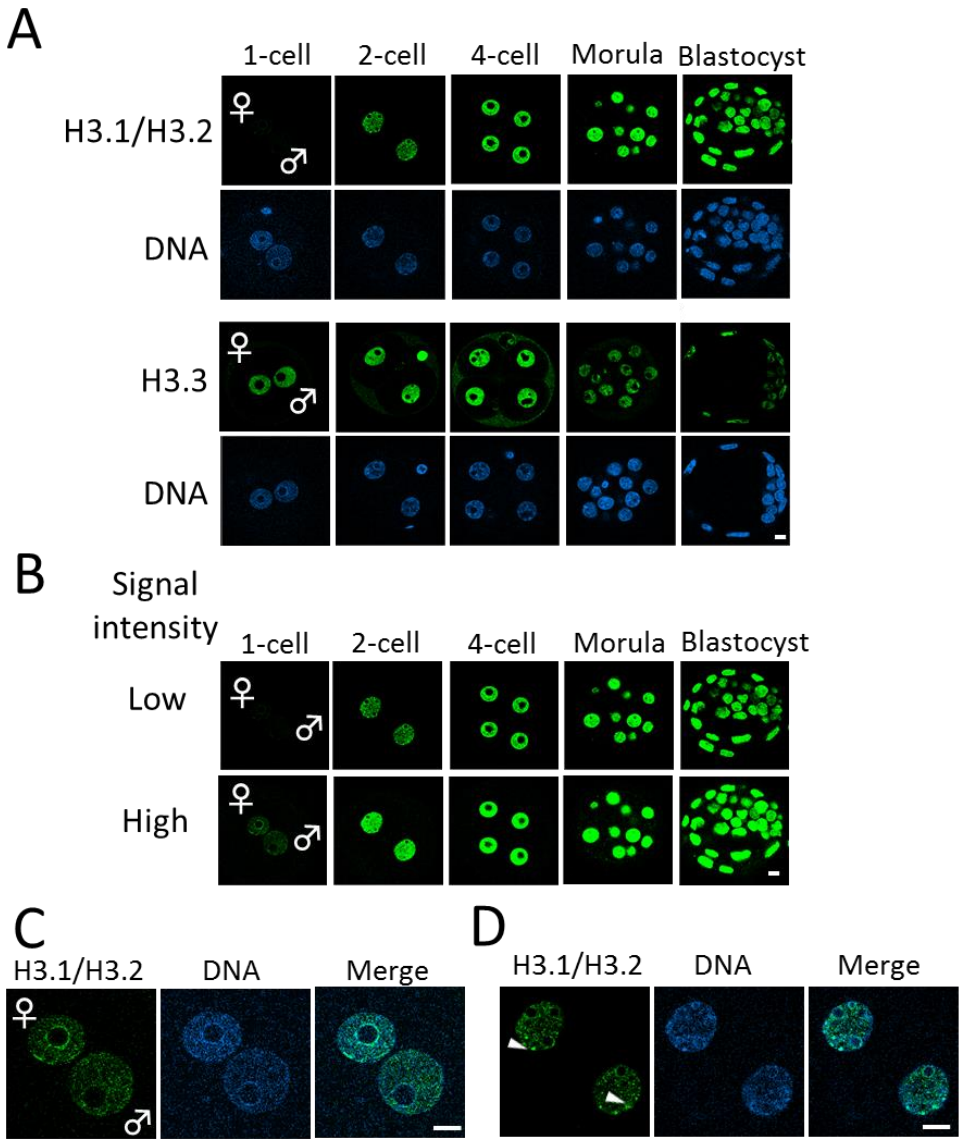


Figure 1-2. The nuclear localization of histone H3 variants in mouse preimplantation embryos.

(A). 1-cell, 2-cell, 4-cell, morula, and blastocyst embryos were immunostained using anti-H3.1/H3.2 (top half) and anti-H3.3 (bottom half) antibodies. Four or five independent experiments were performed in which eight to fifteen embryos were observed for each developmental stage in each experiment. A total of 40 to 63 embryos were analyzed. (B) The detector gain of confocal microscope of H3.1/H3.2 immunofluorescence staining in (A) was increased. (C) The enlarged image of (B) with increased detection of H3.1/H3.2 in 1-cell embryos (D) The enlarged image of 2-cell embryos from (B). For clear detection of chromocenters, the detector gain of H3.1/H3.2 immunofluorescence staining was lowered. White arrows show colocalization of H3.1/ H3.2 with DAPI-rich chromocenters. Scale bar = 10 μm.

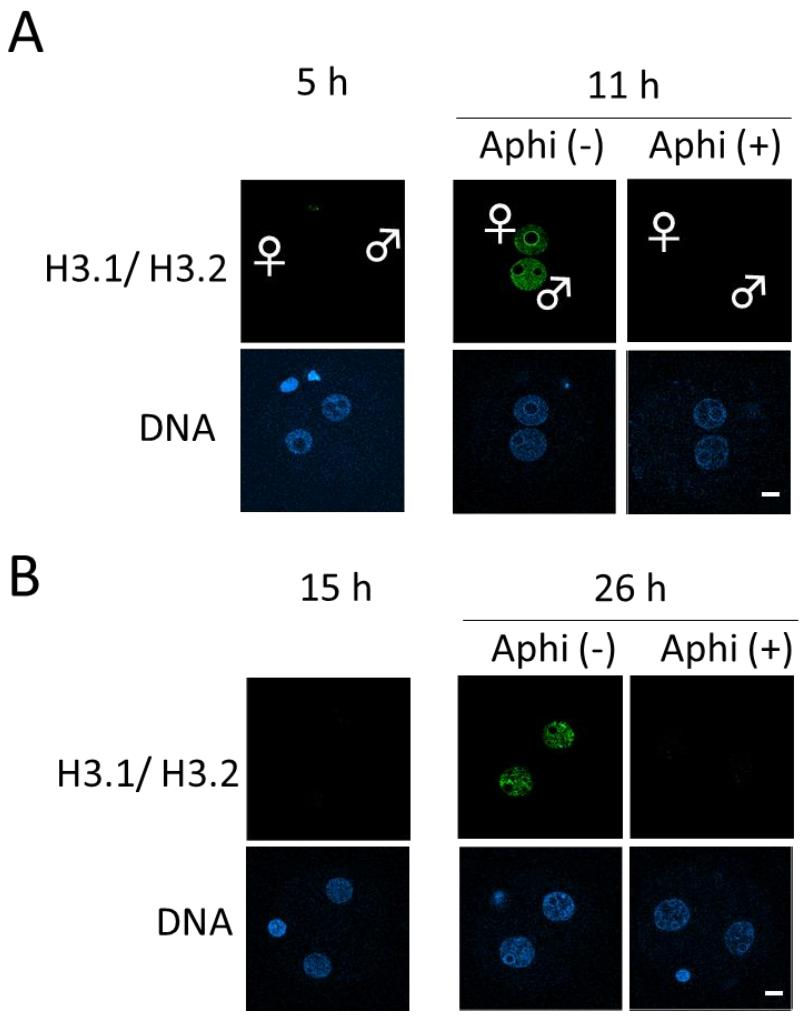


Figure 1-3. The effect of inhibition of DNA replication on H3.1/H3.2 nuclear localization in 1-cell and 2-cell embryos.

(A) The embryos were treated with aphidicolin [Aphi (+)] or DMSO [Aphi (-)] at 5 hours (h) post-insemination. The 1-cell embryos at 5 h and 11 h were collected for immunostaining to analyze the effect of DNA replication in H3.1/H3.2 nuclear localization. Three independent experiments were performed. Thirty-two to forty embryos were observed in total, with seven to sixteen embryos were observed for each sample in each experiment. (B) The embryos were treated with aphidicolin [Aphi (+)] or DMSO [Aphi (-)] at 15 h and were collected for immunostaining at 26 h to analyze the H3.1/H3.2 nuclear localization. Two independent experiments were performed, with twenty-seven embryos analyzed in total for each experimental group. Twelve to fifteen embryos were observed in each sample in each experiment. Scale bar = 10 μm.

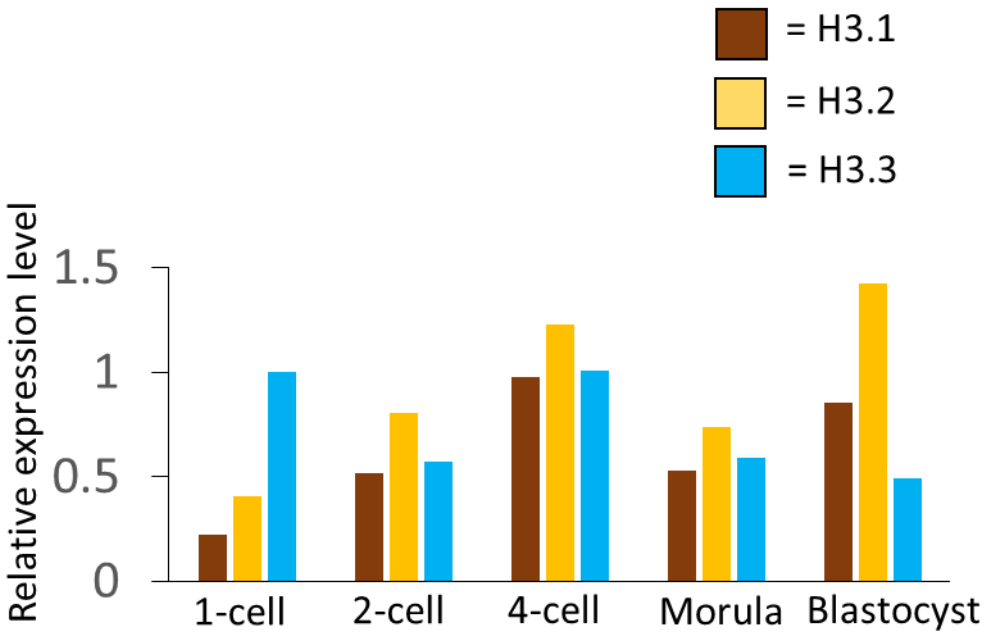


Figure 1-4. The mRNA expression ratio of histone H3 variants in each stage of preimplantation development.

RPKM values were obtained from RNA-seq. data (Abe et al., 2015). The RPKM values of each genes that code for H3.1, H3.2, and H3.3 were summed. The total RPKM of H3.3 at the 1-cell stage is expressed as 1 and the relative expression of H3.1 and H3.2 are shown for each stage.

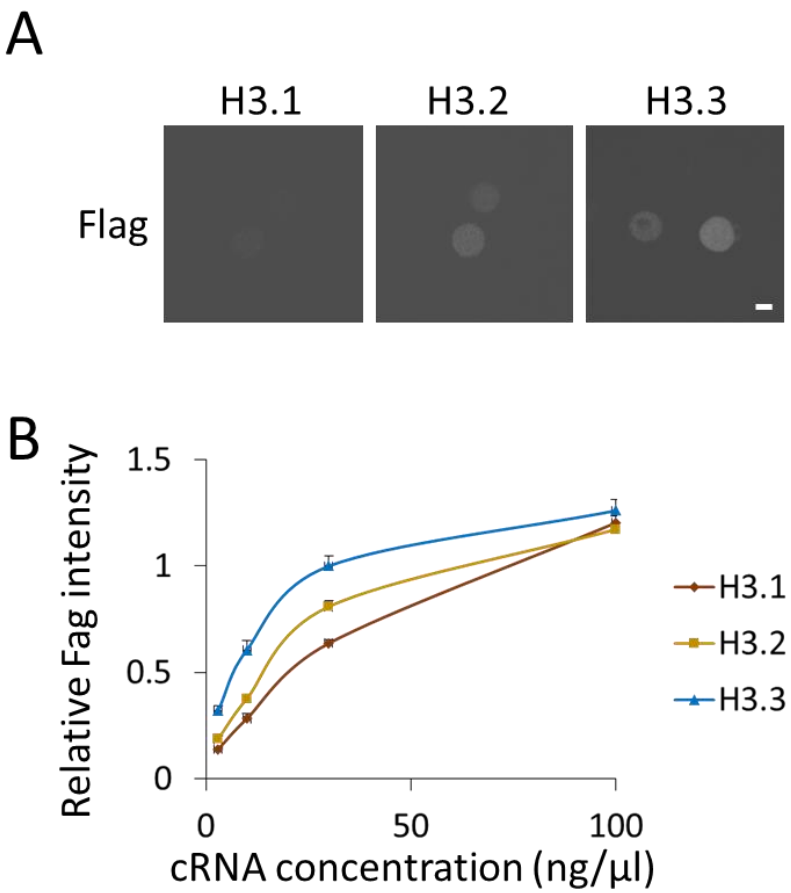


Figure 1-5. The incorporation efficiency of histone H3 variants into chromatin of 1-cell embryo.

(A) H3.1, H3.2, H3.3-Flag cRNA at the concentration of 3, 10, 30, 100 ng/μl were microinjected into mature oocytes. Embryos at 11 h were collected and immunostaining procedure was followed as described in Hajkova et al., (2010). Anti-Flag antibody was used to detect Flag-tagged histones incorporated into chromatin. Representative immunofluorescence image of 1-cell embryos when 10 ng/μl of H3.1, H3.2, H3.3-Flag was microinjected. Scale bar = 10 μm (B) The relative flag intensity when flag intensity of H3.3 microinjected at a concentration of 30 ng/μl as 1. Nine experiments were performed, using H3.3 30 ng/μl as a control in every experiment. Three or four experiments were performed for other concentrations. Ninety 1-cell embryos were analyzed for H3.3 30 ng/μl. For embryos that were microinjected with other cRNA concentrations, 26-43 embryos are analyzed in total. In each independent experiment, three to twenty 1-cell embryos were analyzed in each group. Bar indicates standard error.

CHAPTER 2

The biological significance of the nuclear dynamics of H3.1/H3.2 in 1-cell stage embryos

INTRODUCTION

During preimplantation development, the chromatin structure changes dynamically where heterochromatic regions appear as development progresses (Martin et al., 2006; Akiyama et al., 2011; Probst and Almouzni, 2011). In addition, gene expression pattern dynamically changes during the preimplantation development. There has been increasing evidence that H3 variant regulates preimplantation development, by knockdown and knockout analyses of the histone variants and the histone chaperones.

The involvement of H3.3 in the regulation of preimplantation development has been reported in several studies (Filipescu et al., 2014). Knockdown of H3.3 led to a smaller paternal pronucleus and abnormal nuclear envelope in 1-cell embryos (Inoue et al., 2014). Moreover, the depletion of H3.3 led to a lack of incorporation of other core histones, suggesting that H3.3 plays a role in establishing the nucleus and chromatin assembly. In addition, when the H3.3 that is localized at the perinucleolar chromatin of paternal pronucleus was replaced with mutated H3.3 (lysine at the 27th position from N terminus was replaced with arginine), derepression of major satellite repeats occurred at the early 2-cell stage, a stage where the expression from major satellite is silenced (Santenard et al., 2010). Moreover, introduction of mutated H3.3 in the paternal pronucleus led to a developmental arrest, suggesting that H3.3 is critical for regulating major satellite transcription and for preimplantation development. The knockdown of two genes encoding H3.3 by microinjecting morpholinos at the early 1-cell stage caused over-condensation and missegregation of chromosomes and formation of micronuclei starting from the 2-cell stage. These embryos were finally arrested at the morula stage (Lin et al., 2013). In addition, the 1-cell embryos obtained from heterozygotes knockouts of *H3f3b*,

had cleavage failure at the 1-cell stage, which suggested that H3.3 is essential for the first cleavage after fertilization (Tang et al., 2015).

It was shown that H3.1 and H3.2 are involved in the regulation of late preimplantation development. The knockout and knockdown of CAF-1 subunit p150, a chaperone of H3.1 and H3.2, caused abnormal heterochromatin formation and developmental arrest at the 8-16 cell stage (Houlard et al., 2006; Akiyama et al., 2011). This result suggested that H3.1 and H3.2 is essential for establishing heterochromatin during late preimplantation development. In addition, Hatanaka et al. (2015) have shown that depletion of CAF-1 at the morula stage leads to de-repression of class III retrotransposons and decrease in repressive histone modifications such as H3K9me3 and H4K20me3 on retrotransposons, suggesting that H3.1 and H3.2 is essential in silencing retrotransposons by their histone modifications. However, in these studies, the expression of H3.1 and H3.2 was abolished by their knockout or knockdown. Although these experiments elucidated the role of H3.1 and H3.2 in the development at the 2-cell and subsequent stages during which these proteins are present, they did not address the biological significances of low level of their nuclear deposition and their absence in the paternal perinucleolar region in 1-cell embryos.

In the previous chapter, the nuclear distribution of H3.1/H3.2 suggested that H3.1/H3.2 regulate DNA replication in 1-cell embryos. In this chapter, first, I attempted to investigate the biological significance of why H3.1 and H3.2 are localized at a low level in the pronuclei of 1-cell stage embryos. Second, I investigated the significance of why H3.1 and H3.2 show asymmetry in nuclear localization between the parental pronuclei.

RESULTS

The biological significance of the low nuclear localization of H3.1 and H3.2 in the 1-cell embryos.

In chapter 1, I have shown that the level of nuclear localization H3.1/H3.2 is low at 1-cell stage compared to the embryos at 2-cell stage and beyond. To examine the biological significance of why H3.1/H3.2 is at low level in 1-cell embryos, I induced the incorporation of H3.1/H3.2 into chromatin, and analyzed their effect on preimplantation development. The results of experiments, in which cRNA encoding Flag-tagged H3 variants were microinjected into MII stage oocytes, indicated that the incorporation efficiency of H3.1 and H3.2 into chromatin of 1-cell embryos was low compared to that of H3.3 (Fig. 1-5). However, when extremely high concentration of cRNA (100 ng/μl) was microinjected into MII stage oocytes, similar level of incorporation of the all three H3 variants were observed. Therefore, utilizing this condition, I performed overexpression of H3 variants and induced incorporation of these H3 variants into chromatin of 1-cell embryos.

I microinjected cRNA encoding for H3.1, H3.2, and H3.3, and examined the nuclear localization of H3 variants in these overexpressed embryos by immunostaining with anti-H3.1/H3.2 and anti-H3.3 antibodies (Fig. 2-1). As a result, the increased level of H3.1/H3.2 nuclear localization was observed in both maternal and paternal pronuclei of H3.1 and H3.2 overexpressed embryos, whereas H3.3 level decreased in these embryos (Fig. 2-1A). Interestingly, the pattern of H3.1/H3.2 deposition was unchanged for the maternal pronuclei whereas the pattern of H3.1/H3.2 was altered for the paternal pronuclei. Although H3.1/H3.2 was localized at the perinucleolar region of nucleolus

only in maternal pronucleus but not paternal one in no injected embryos (*see* Fig. 1-2C) and H3.3 cRNA injected ones (Fig. 2-1B), it was also deposited at the perinucleolar region of the paternal pronuclei in H3.1 and H3.2 overexpressed embryos (Fig. 2-1A). In H3.3-overexpressed embryos, the pattern of nuclear distribution of H3.1/H3.2 was unaltered (Fig. 2-1B). To verify the histones are incorporated into chromatin, I then performed immunostaining procedure in which the embryos were treated with Triton X-100 before fixation to solubilize the plasma membrane and wash away free histones present in the nucleoplasm (Fig. 2-2). Because the embryos are treated with the detergent, the nuclear structure has collapsed and the perinucleolar region is detected as a dot. Similarly to Fig. 2-1A, an increased incorporation of H3.1/H3.2 whereas a decreased incorporation level of H3.3 was detected in H3.1/H3.2 overexpressed embryos. Furthermore, an increased incorporation of H3.3 whereas a decrease in H3.1/H3.2 was observed in H3.3 overexpressed embryos. Notably, H3.1/H3.2 incorporation was detected at the perinucleolar region in the paternal pronucleus of H3.1/ H3.2 embryos similarly to Fig. 2-1A. These results suggest that the detected histones are deposited in chromatin and an alteration in chromatin distribution of H3.1/H3.2 and H3.3 occurs in H3.1 and H3.2-overexpressed embryos.

I investigated whether the ectopic deposition of H3.1 and H3.2 at the 1-cell stage have an effect in development by observing the developmental rate until the blastocyst stage (Fig. 2-3). Drastic effect in development was observed for H3.1 and H3.2 overexpressed embryos, where only approximately 50% of embryos proceeded to 2-cell stage. This detrimental effect of H3.1 and H3.2 overexpression was more evident at the blastocyst stage. These results suggested that the low level of nuclear localization of H3.1/H3.2 at the 1-cell stage is essential for preimplantation development.

Effect of the ectopic deposition of H3.1 and H3.2 in the pronuclei on DNA replication at the 1-cell stage

To determine the mechanism underlying the developmental failure of H3.1 and H3.2-overexpressed embryos, the developmental rate of H3.1, H3.2, H3.3, GFP-overexpressed embryos and no-injected embryos were observed every four to six hours (Fig. 2-4A). Over 70% of the no-injected, GFP-, and H3.3-overexpressed embryos had cleaved into 2-cell embryos at 16 h post-insemination (h), and cleaved to 4-cell embryos at 40 h. However, more than 80% of H3.1 and H3.2 overexpressed embryos had not yet cleaved into 2-cell embryos at 16 h and most of them still remained at the 1-cell stage at 34 h. They began cleavage after 34 h and more than 70% of them developed to 2-cell and later stages at 46 h. After that, some of them developed to blastocysts, but others eventually caused fragmentation or degeneration by chronologically blastocyst stage (Fig. 2-4B). This results suggest that the cleavage from 1-cell to 2-cell is delayed in H3.1 and H3.2 overexpressed embryos, and eventually leads to developmental arrest.

The delay in cleavage from 1- to 2-cell stage in H3.1 and H3.2 overexpressed embryos may be caused by the delay in DNA replication (S phase). Therefore, to investigate whether there is a delay in DNA replication at the 1-cell stage in H3.1 and H3.2 overexpressed embryos, I examined the incorporation of BrdU into the pronuclei after the transient culture of the embryos with BrdU from 4 to 10 h (Fig. 2-5). As a result, in maternal pronuclei of no-injected embryos, GFP-, and H3.3-overexpressed embryos, DNA replication was initiated in over half of the analyzed embryos at 4 h. At 10 h, DNA replication was completed in most maternal pronuclei of no-injected, GFP-, and H3.3-overexpressed embryos. Similar to this result, the DNA replication of the maternal

pronuclei of H3.1 and H3.2 overexpressed embryos was initiated at 4 h and completed at 10 h. However, in the paternal pronuclei, the DNA replication timing of H3.1 and H3.2-overexpressed embryos was delayed when compared to that of no-injected, GFP, and H3.3-overexpressed embryos. Only less than 15% of embryos had started and only 30% of embryos completed the DNA replication at 4 and 10 h, respectively. These results suggested that the ectopic deposition of H3.1 and H3.2 into chromatin of 1-cell embryos leads to a delay in DNA replication initiation and completion in the paternal pronucleus but not the maternal one.

H3.1 and H3.2 overexpression leads to a delay in completion of DNA replication at the perinucleolar region in the paternal pronuclei

In somatic cells, DNA replication is known to occur earlier in euchromatin and later in heterchromatin regions (O'Keefe et al., 1992). Interestingly, it has been previously reported that DNA replication occurs asynchronously between maternal and paternal pronuclei (Fig. 2-6A; Aoki et al., 1999). In both paternal and maternal pronuclei, DNA replication first occurs at the nucleoplasmic region and then at the perinuclear and perinucleolar region. In the paternal pronuclei, the DNA replication at the perinucleolar region is completed earlier than the perinuclear region. In contrast, in the maternal pronuclei, DNA replication at the perinuclear region is completed earlier than that of perinucleolar region, and takes over a longer time span than the paternal pronuclei.

As shown in Fig. 2-1, the nuclear distribution of H3.1/H3.2 in the paternal pronucleus of H3.1 and H3.2 overexpressed embryos were altered to a similar distribution to that of maternal pronucleus. Considering these results, I hypothesized that the delay of DNA replication observed in paternal pronuclei of H3.1 and H3.2-overexpression

embryos (Fig. 2-5) was due to prolonged DNA replication at the perinucleolar region. To examine this hypothesis, I observed the DNA replication pattern of both maternal and paternal pronuclei at 4, 6, 8, and 10 h (Fig. 2-6B). There was no significant difference in the pattern of DNA replication in the maternal pronuclei of no-injected, GFP, H3.1, H3.2, and H3.3-overexpressed embryos in which the DNA replication occurred in the nucleoplasmic region at 4 and 6 h, at the perinucleolar region at 8 h, and completed at 10 h. However, DNA replication pattern differed in the paternal pronuclei of H3.1 and H3.2 overexpressed embryos compared that of no-injected, GFP-, and H3.3-overexpressed embryos. That is, in contrast to control and H3.3-overexpressed embryos where perinuclear region were replicated last, the DNA replication at the perinucleolar region was pursued even at 10 h (Fig. 2-6B, Fig. 2-7). The DNA replication of the nucleoplasmic region was also delayed in the paternal pronuclei of H3.1 and H3.2 overexpressed embryos (Fig. 2-5, Fig. 2-6B). However, the initiation of DNA replication in the nucleoplasmic region was delayed for less than 2 h and completed before 10 h, whereas that at the perinucleolar region was delayed for more than 4 h and still pursued at 10 h, suggesting that the delay of DNA replication at the perinucleolar region of the paternal pronucleus is the rate-limiting step and thus a cause of delayed cleavage in H3.1 and H3.2 overexpressed embryos. These results suggested that the delay in DNA replication in the paternal pronuclei of H3.1 and H3.2 overexpressed embryos is due to the ectopic deposition of H3.1 and H3.2 at the perinucleolar region, thus leading to a delay in cleavage.

H3.1 and H3.2-overexpressed parthenotes have no effect in preimplantation development.

Previous results of this current study suggested that a low level of nuclear deposition of H3.1 and H3.2 at the 1-cell stage is essential for the progression of DNA replication at the paternal pronuclei and preimplantation development. On the contrary, because DNA replication in maternal pronucleus of H3.1 and H3.2 overexpressed embryos was unaffected by the induced incorporation of H3.1 and H3.2, a low level of nuclear deposition of H3.1 and H3.2 in the maternal pronucleus is not necessary for development. To confirm it, I generated parthenotes, which are devoid of paternal genome, and examined whether or not they were capable to develop nevertheless H3.1 and H3.2 is induced into their chromatin at the 1-cell stage. Expectedly, there was no significant difference in developmental rate between H3.1 and H3.2 overexpressed parthenotes and H3.3-overexpressed, control parthenotes (Fig. 2-8A). The H3.1/H3.2 incorporation was verified in H3.2 overexpressed parthenotes and results indicated that the nuclear localization of H3.1/H3.2 was similar to that of H3.2 overexpressed embryos which had been fertilized embryos (Fig. 2-8B). This results reinforced that H3.1 and H3.2 overexpressed embryos had a delayed cleavage due to H3.1 and H3.2 deposition in the paternal pronuclei. Taken together, it is essential that H3.1and H3.2 is not incorporated at a high level in the 1-cell embryos, as a higher level of H3.1 and H3.2 deposition at the perinucleolar region of the paternal pronucleus causes the delay in DNA replication, leading to the developmental catastrophe.

The effect of H3.1 and H3.2 overexpression in epigenetic modifications.

To determine the molecular mechanism underlying the developmental failure of H3.1 and H3.2-overexpressed embryos, I investigated whether there is alteration in histone modification level in these embryos. H3K9me2/3 and H3K27me3 are histone

modifications that are associated with heterochromatin (Hake et al., 2006). These modifications are also reported to be frequently seen on H3.1 and H3.2, respectively. In addition, these modifications levels are higher in the maternal pronucleus compared to that of paternal pronucleus (Liu et al., 2004; Lepikhov and Walter, 2004; Santos et al., 2005; Puschendorf et al., 2008). Importantly, H3K9me3 is detected in the perinucleolar region of the maternal pronucleus. Because the nuclear distribution of H3.1/H3.2 in the paternal pronuclei of H3.1 and H3.2 overexpressed embryos became maternal pronucleus-like, I investigated whether there is an increase in H3K9me2 and H3K9me3 in the paternal pronucleus. However, these modifications were unaffected by H3.1/H3.2 overexpression (Fig. 2-9). Yet, this may be expected because there is no H3K9 methyltransferase activity in 1-cell embryos and only methylated histones carried over from the oocyte reside in the maternal pronucleus (Liu et al., 2004). Next, I examined whether there is change in H3K27me3 levels. Polycomb Repression Complex 2 (PRC2) is known to methylate lysine 27 in the paternal pronucleus (Tardat et al., 2015). Because there is a decrease in H3.3 levels in the paternal pronucleus in H3.1 and H3.2 overexpressed embryos (Fig. 2-1A, Fig. 2-2A, B), I hypothesized that there is a change in H3K27me3 in their paternal pronucleus (Fig. 2-10). However, H3K27me3 was unaltered in the paternal pronucleus. In addition, DNA methylation (5-methylcytosine), also associated with heterochromatin, was unchanged in the paternal pronucleus (Fig. 2-11). Therefore, these results suggest that the developmental failure of H3.1 and H3.2-overexpressed embryos is not due to the changes in global level of methylations at H3K9, H3K27 or DNA which are associated with heterochromatin formation.

DISCUSSION

In this chapter, I investigated the biological significance of why the nuclear deposition of H3.1 and H3.2 are low in 1-cell embryos compared to later preimplantation embryos, and examined whether or not the asymmetrical localization of H3.1 and H3.2 between the parental pronuclei are involved in cellular process, *i.e.* DNA replication, by overexpressing H3 variants in 1-cell embryos. The results have shown that when H3.1 and H3.2 are ectopically incorporated into chromatin, the level of nuclear deposition of these proteins increased both in male and female pronuclei (Fig. 2-1A, 2-2A, B). However, the pattern of their nuclear distribution was changed only in the male pronucleus. They became localized in the perinucleolar region by their overexpression similarly to the pattern in female pronucleus (Fig. 2-1A, Fig. 2-2A). Overexpression of H3.1 and H3.2 had a detrimental effect in development while H3.3 overexpression did not. The developmental failure was caused by the delay of DNA replication in the paternal perinucleolar region, at which H3.1/H3.2 was accumulated. To my knowledge, this is the first study that showed that H3.1 and H3.2 regulate cellular processes, *i.e.* DNA replication, in 1-cell embryos and that low nuclear localization of H3.1/H3.2 in the paternal pronucleus is essential for preimplantation development.

Here, I have shown that the DNA replication at the perinucleolar region of the paternal pronucleus was delayed in H3.1 and H3.2-overexpressed embryos (Fig. 2-6B, 2-7). The time span of the DNA replication at the perinucleolar region of the paternal pronucleus of H3.1 and H3.2-overexpressed embryos were longer than that of no-injected and control embryos. In general, the pericentromeric heterochromatin is known to be replicated late in S phase (Shermoen et al., 2010), but interestingly the pericentromeric

heterochromatin in the paternal pronucleus replicates earlier than that of its perinuclear region (Aoki et al., 1999). However, the DNA replication of paternal perinucleolar region in H3.1 and H3.2 overexpressed embryos was delayed (Fig. 2-6B, 2-7). In early embryonic development in *Drosophila*, the embryos undergo a series of S and M phases in the single cytoplasm referred to as syncytium (Su, 2010). It is known that during syncytium, the S phase gradually lengthens. One study have shown that by tracking satellite sequences that reside in pericentromeric heterochromatin by fluorescent in situ hybridization (FISH), the S phase was prolonged due to the late onset of replication of satellite repeats (Shermoen et al., 2010; Su et al., 2010). Therefore, it suggests that the replication of pericentromeric region is rate-limiting step for the completion of S phase and that prolonged replication of the pericentromeric heterochromatin in the paternal pronucleus can lead to delayed cleavage in H3.1 and H3.2 overexpressed embryos. The reason why the increase of H3.1 and H3.2 deposition in the maternal pericentromeric heterochromatin did not cause the delay in the completion of DNA replication is discussed below.

It can be debated whether ectopic deposition of H3.1 and H3.2 led to developmental failure or whether loss of H3.3 leads to developmental failure in the H3.1and H3.2 over expressed embryos. However, the analysis for the DNA replication support that H3.1 and H3.2 ectopic deposition led to developmental failure. Because, there was a delay in DNA replication in the perinucleolar region of the paternal pronucleus (Fig. 2-6B, Fig. 2-7), which was suggestive of ectopic deposition of H3.1/H3.2 in this region (Fig. 2-1A).

The ectopic deposition of H3.1 and H3.2 in 1-cell embryos led to a delayed cleavage not a complete arrest during the 46 h of culture after insemination (Fig. 2-4A).

This is supported by the fact that unlike somatic cells, the G2/M checkpoint which monitors the completion of DNA synthesis before entering the M phase are deficient in 1-cell embryos (Yukawa et al., 2007). Therefore, H3.1 and H3.2-overexpressed embryos cleaved into the 2-cell stage, nevertheless DNA replication was ongoing at a minimal level.

H3.1 and H3.2 have the same effect to preimplantation development. H3.1 and H3.2 overexpression both led to developmental failure with no significance differences in the developmental rate (Fig. 2-3). In addition, ectopic expression of H3.1 and H3.2 led to the same characteristics, regarding the delay in cleavage (Fig. 2-4A, B) and the delay in DNA replication at the perinucleolar region of the paternal pronucleus (Fig. 2-6B, 2-7). In addition, the overexpression of H3.1 and H3.2 in parthenogenetic embryos both had no significant effect in preimplantation development (Fig. 2-8), suggesting that both of these two variants have similar roles in preimplantation development and both are necessary to be incorporated at low level at the paternal perinucleolar region for preimplantation development. Although H3.1 is a mammalian-specific variant and differ by one amino acid from H3.2 (Hake and Allis, 2006), my results presented here suggest that H3.1 and H3.2 have the same roles in early preimplantation development.

Although ectopic deposition of H3.3 did not show effects on the early stages of preimplantation development, I need to mention that the developmental rate of H3.3 overexpressed embryos was significantly low compared to no-injected and GFP overexpressed embryos at morula and blastocyst stages. This may have been expected because the knockdown or knockout of CAF-1, the histone chaperone for H3.1/H3.2, led to developmental arrest at 8-16 cells (Houlard et al., 2006; Akiyama et al., 2011). Because ectopic incorporation of H3.3 led to a decrease in H3.1/H3.2 nuclear localization and

incorporation (Figs. 2-1A, 2-2A, B), this may have led to a similar phenotype observed in CAF-1 knockout or knockdown embryos (Houlard et al, 2006; Akiyama et al., 2011). Therefore, excess deposition of H3.3 may cause detrimental effects in later preimplantation embryos.

Since the DNA replication occurs later in heterochromatin than euchromatin (O'Keefe et al., 1992), I expected that histone modifications associated with heterochromatin formation, *i.e.* H3K9me2/3 and H3K27, would increase by the increased deposition of H3.1 and H3.2 in perinucleolar regions, leading to the delay in DNA replication. However, there was no change in those modifications in the paternal pronucleus of H3.1 and H3.2 overexpressed embryos (Figs 2-9, 10). Therefore, I hypothesize that the alteration of the combination of histone H3 variants and the heterochromatin associated modifications affected DNA replication timing, thus leading to a developmental failure (Fig. 2-12). The results of immunocytochemistry showed that there was no difference in H3K9me2 or me3 levels between no-injected and injected embryos (Fig.2-9). This is supported by the report that H3K9 methyltransferase is not functional in 1-cell embryos whereas there is methylation activity in the oocytes (Liu et al., 2004). Because the newly incorporated H3.1 and H3.2 are not methylated in the 1-cell stage, the H3K9me2/3 levels are unaltered in the maternal pronuclei in which only H3K9me2/3 that had been carried from oocytes remains there (Fig. 2-9). Therefore, there is no effect in the DNA replication timing in the maternal pronucleus (Fig. 2-12). Similarly, H3K27me3 level was not altered in the H3.1 and H3.2 overexpressed embryos (Fig. 2-10), because PRC2, a complex of H3K27 methyltransferase activity, is not functional at the perinucleolar region of the maternal pronucleus. It has been reported that HP1β circumvents PRC2 from binding to the maternal perinucleolar region (Tardat et al., 2015).

However, PRC2 is functional in the paternal pronucleus. In H3.1 and H3.2-overexpressed embryos, PRC2 methylates newly incorporated H3.1 and H3.2 that replaced H3.3 at the paternal perinucleolar region. Because H3K27me3 is associated with facultative heterochromatin but H3.3 is associated with euchromatin (Hake and Allis, 2006; Hake et al, 2006), I hypothesize that H3.3-H3K27me3 forms a relatively weaker heterochromatin when compared to H3.1/H3.2-H3K27me3 (Fig. 2-12). The perinucleolar region of the paternal pronucleus of H3.1- and H3.2-overexpressed embryos may be tighter and more condensed compared to that of no-injected and control embryos in which perinucleolar region is occupied with H3.3 only, leading to a delay in the DNA replication at that region. In addition, the DNA methylation was also unaltered in H3.1- and H3.2-overexpressed embryos (Fig. 2-11). This was expected because the level of H3K9me2, which is tightly associated with DNA methylation (Nakamura et al., 2012), was unaltered by the overexpression of H3.1 and H3.2 (Fig. 2-9). Finally, there may be other histone modifications that have caused developmental failure. For example, H3K64me3 is localized in the maternal pronucleus and is involved in perinucleolar heterochromatin (Lange et al., 2013). However, because this modification is H3K9me3-dependent (Lange et al., 2013) and because an increase in H3K9me3 was not detected in the paternal pronuclei of H3.1- and H3.2-overexpressed embryos (Fig. 2-9), I hypothesize this modification is not altered in paternal perinucleolar region of the overexpressed embryos. A recent study indicated that microinjection of SUV(4-20)H2, a methyltransferase of H4K20, in 1-cell embryos and overexpression at the 2-cell stage, led to developmental failure and delayed DNA replication at the 2-cell stage (Eid et al., 2016). H4K20me3 is a histone modification associated with constitutive heterochromatin and present only in the maternal pronucleus (Kourmouli et al., 2004). H3.1 and H3.2 overexpression might

promote H4K20 to be methylated, but in embryos unlike somatic cells, this modification is also associated with H3K64me3 (Eid et al., 2016) which is not supposed to alter by H3.1 and H3.2 over expression as described above. Therefore, a possible explanation of the developmental failure of H3.1 and H3.2-overexpressed embryos may be due to the change in combination of H3 variants and H3K27me3, *i.e.* from H3.3-H3K27me3 to H3.1/H3.2-H3K27me3, which led to a change in chromatin structure at the perinucleolar region of the paternal pronucleus.

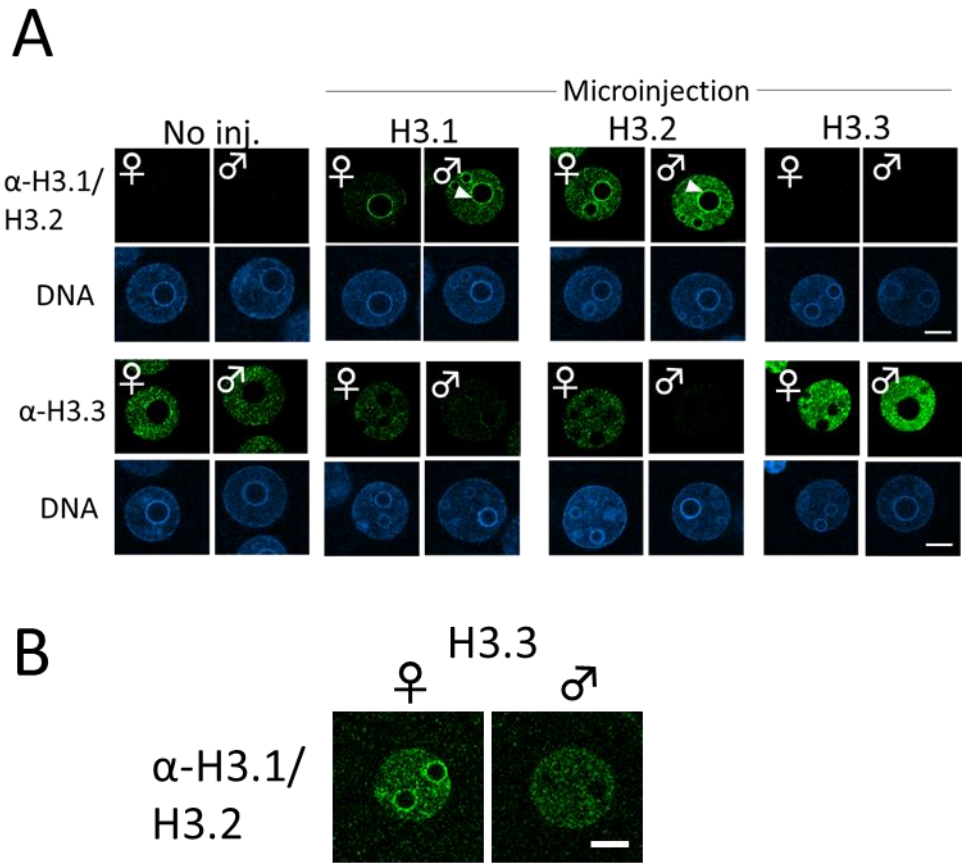


Figure 2-1. The effect of H3.1/H3.2 and H3.3 nuclear localization upon H3.1, H3.2, and H3.3 overexpression in 1-cell embryos.

(A) No-injected, H3.1, H3.2, and H3.3- overexpressed 1-cell embryos at 11 h were fixed and examined for the change in nuclear localization of H3.1/H3.2 and H3.3, using anti-H3.1/H3.2 and anti-H3.3 antibodies, respectively. Eight independent experiments were performed. Two to eight embryos were analyzed in each sample per experiment. Total of 39-46 embryos were examined. White arrows indicate the presence of H3.1/H3.2 at the perinucleolar region of the paternal pronuclei. (B) The H3.1/H3.2 nuclear distribution in H3.3 overexpressed embryos, with increased intensity. Scale bar = 10 μm

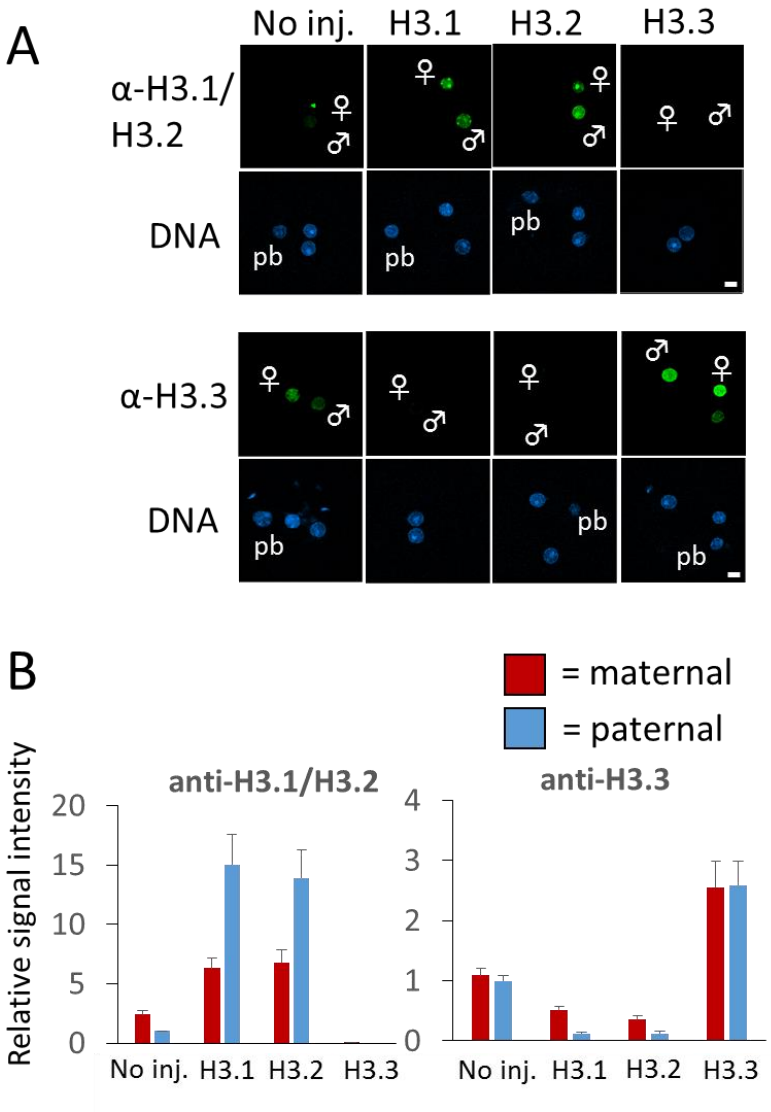


Figure 2-2. The detection of incorporated H3.1/H3.2 and H3.3 in chromatin of H3.1, H3.2, and H3.3 overexpressed 1-cell embryos.

(A) No-injected, H3.1, H3.2, and H3.3 overexpressed embryos at 11 h were collected to analyze the level of H3.1/H3.2 and H3.3 incorporated into chromatin (as described in Hajkova et al., 2010), using anti-H3.1/H3.2 and anti-H3.3 antibodies, respectively. Four (anti-H3.1/H3.2) or five independent experiments (anti-H3.3) were performed. (B) These embryos were then quantified for the incorporation level of H3.1/2 and H3.3 and corrected by DAPI signal intensity. The relative signal intensity of H3.1/H3.2 and H3.3 were shown with the intensity of paternal pronucleus of no-injected as 1. Total of 26-36 embryos (anti-H3.1/H3.2) or 29-40 embryos (anti-H3.3) were analyzed, with 3-13 embryos in each pool. pb = polar body; Scale bar = 10 μm. Bar indicates standard error.

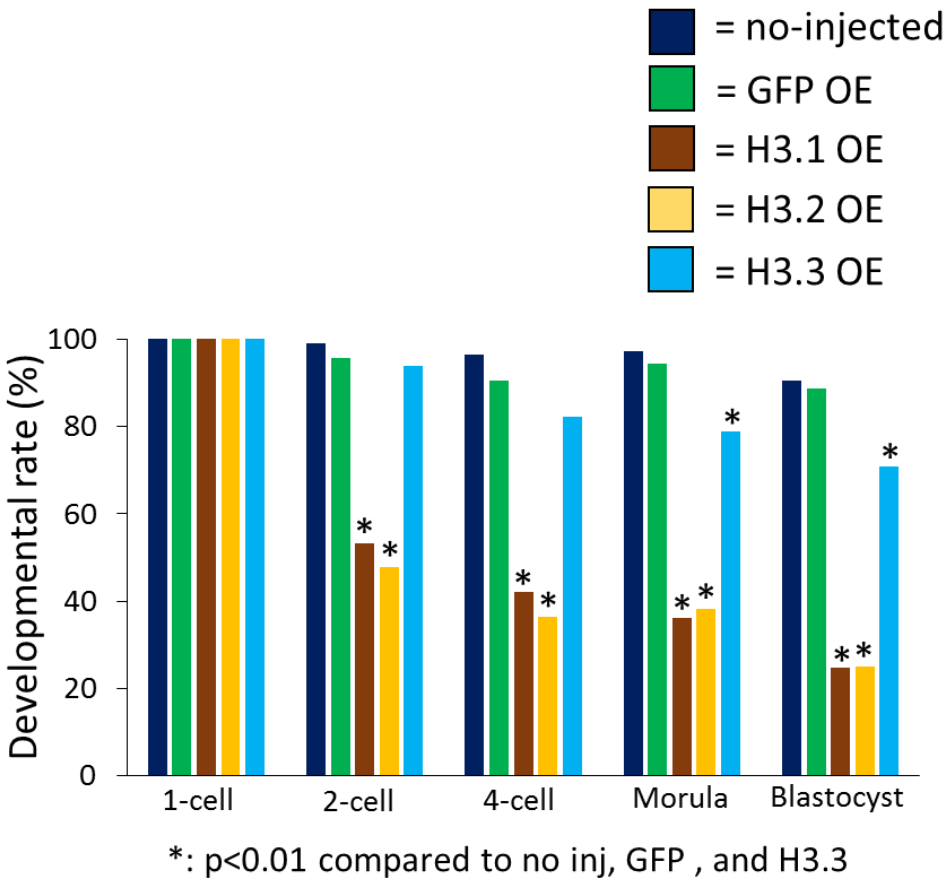


Figure 2-3. The developmental rate of no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed embryos until the blastocyst stage.

The no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed embryos that possess two pronuclei were incubated and analyzed at the following times: 2-cell (28 h), 4-cell (45-6 h), morula (72 h), and blastocyst (96 h). Eleven independent experiments were performed and seven to forty embryos are analyzed in each group per experiment. For each group, 197-228 embryos were observed in total. For H3.1, H3.2 overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to all of no-injected, GFP, and H3.3-overexpressed embryos. For H3.3-overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to both no-injected and GFP overexpressed embryos.

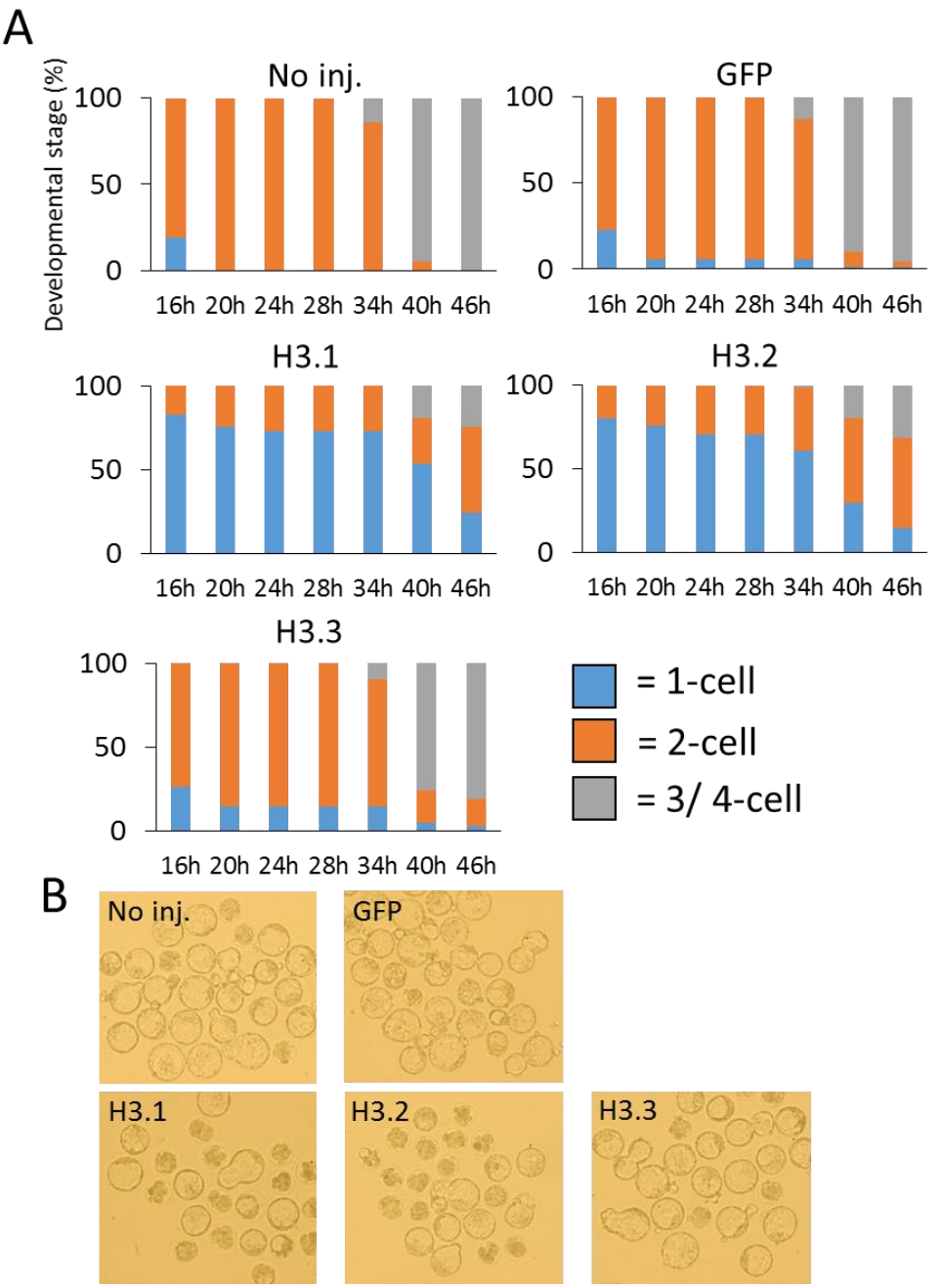


Figure 2-4. The analysis of developmental stage of no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed embryos from 16 h to 46 h.

(A) The developmental rate of no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed embryos were observed every four to six hours. Three independent experiments were performed. 41 to 71 embryos were analyzed in total, with 8-27 embryos observed in each experiment. (B)The developmental progression of no-injected and injected embryos at chronologically blastocyst stage.

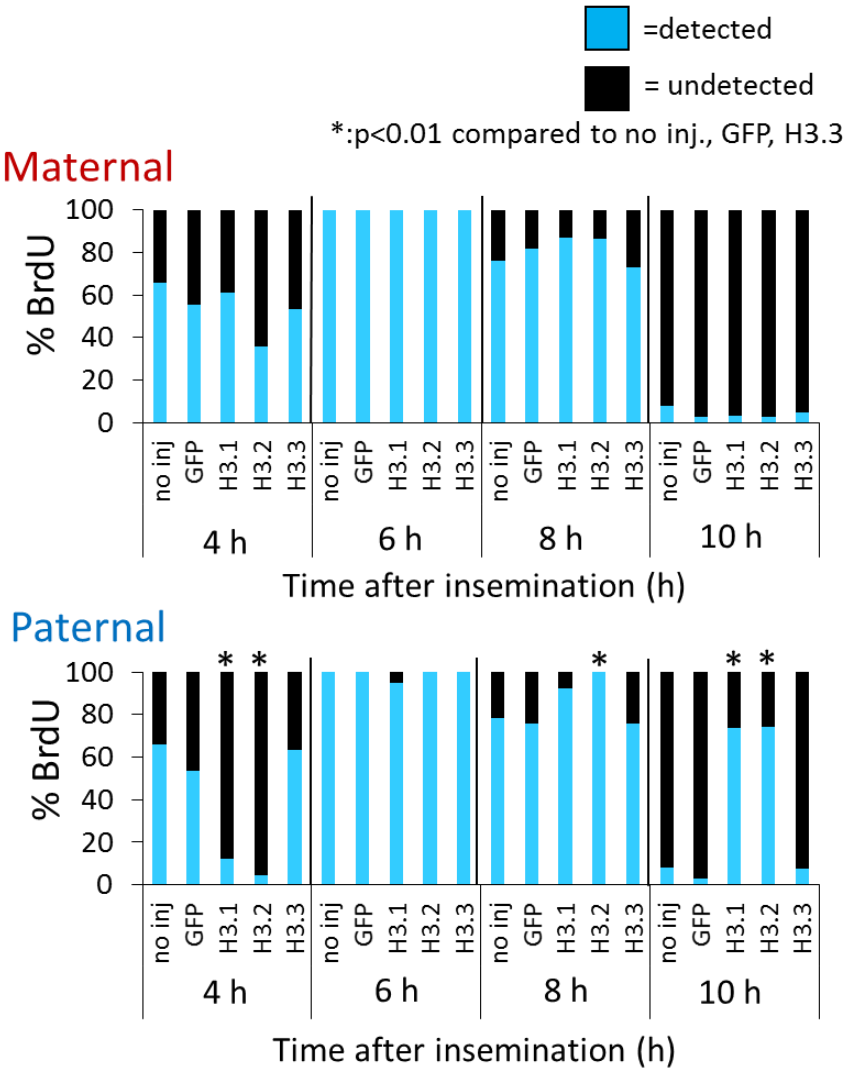


Figure 2-5. The presence of DNA replication in no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed embryos.

The presence of BrdU incorporation was analyzed at 4, 6, 8, and 10 h. Three to five independent experiments were performed. For each injected or no-injected sample, 30 to 51 embryos were analyzed in total. Three to twenty-four embryos were analyzed in each group per experiment. For H3.1 and H3.2 overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to all of no -injected, GFP, and H3.3-overexpressed embryos. For H3.3-overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to both no-injected and GFP overexpressed embryos.

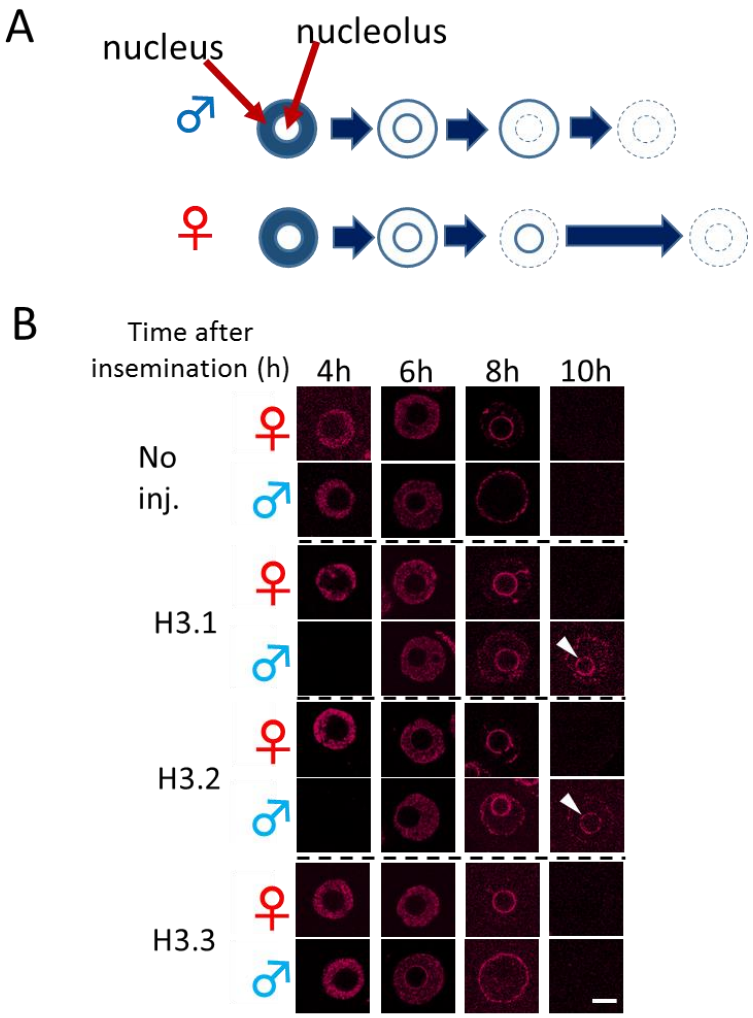


Fig. 2-6. The DNA replication pattern detected by BrdU incorporation in no-injected, H3.1, H3.2, and H3.3 overexpressed embryos.

(A) Illustration of asymmetric DNA replication in paternal and maternal pronuclei in 1-cell embryos. DNA replication start in the nucleoplasmic region in both of parental pronuclei. In the paternal pronucleus, DNA replication at the perinuclear region is completed last. However, in the maternal pronucleus, DNA replication at the perinucleolar region proceeds after the DNA replication at the paternal pronucleus completes its replication. (B) Analysis of DNA replication pattern in maternal and paternal pronuclei in no-injected, H3.1, H3.2, and H3.3 overexpressed embryos at 4 to 10 hpi. For each injected or no-injected sample, 30 to 51 embryos were analyzed in total. Three to five independent experiments were performed. Arrows indicate the presence of DNA replication at perinucleolar region in the paternal pronuclei. Scale bar = 10μm

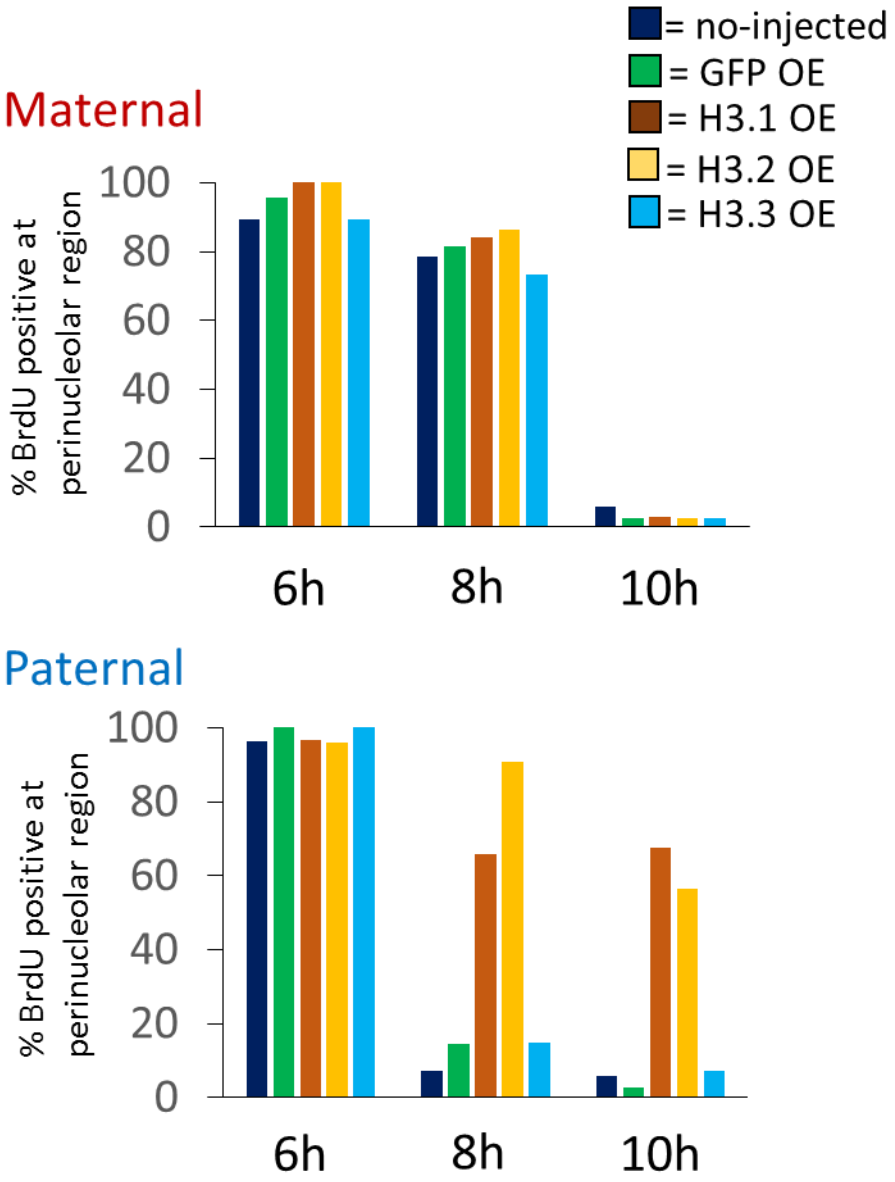


Figure 2-7. The proportion of the presence of DNA replication at the perinucleolar region in H3.1, H3.2, H3.3, GFP-overexpressed, and no-injected embryos.

The presence of DNA replication at the perinucleolar region in no-injected, H3.1, H3.2, and H3.3 overexpressed embryos at 6, 8, and 10 h. For each injected or no-injected sample, 19 to 51 embryos were analyzed in total. Three or four independent experiments were performed as described in Fig. 2-6. Three to twenty-four embryos were analyzed in each group per experiment.

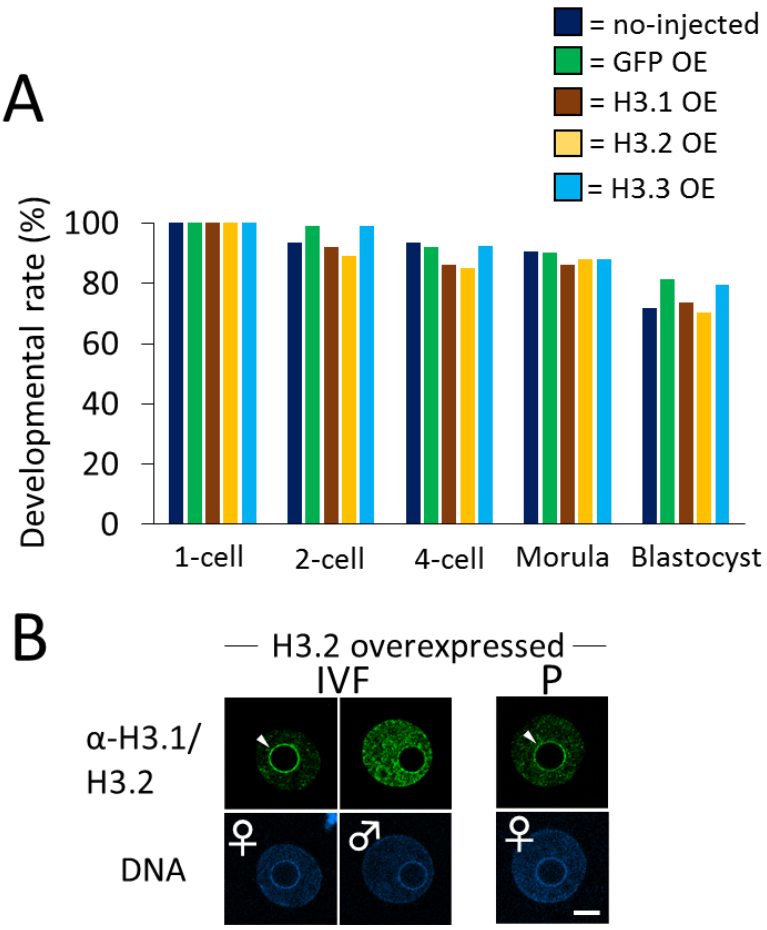


Figure 2-8. The effect of H3.1 and H3.2 overexpression on development of parthenogenetic embryos.

(A) The developmental rate of no-injected, GFP, H3.1, H3.2, and H3.3 overexpressed parthenogenetic embryos. Six independent experiments were performed. 101-117 embryos were observed, with 7-35 embryos observed in each group per experiment. For H3.1 and H3.2 overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to all of no -injected, GFP, and H3.3-overexpressed embryos. For H3.3-overexpressed embryos, χ^2 test or Fischer's exact test was performed and considered significant if $p<0.01$ to both no-injected and GFP overexpressed embryos. (B) The comparison of nuclear localization of H3.1/H3.2 between H3.2-overexpressed in vitro fertilized (IVF) embryos and parthenogenetic (P) embryos. Three independent experiments were performed, with 11 to 16 embryos observed in each group per experiment. Arrow indicates perinucleolar localization of H3.1/H3.2 in the female pronucleus.

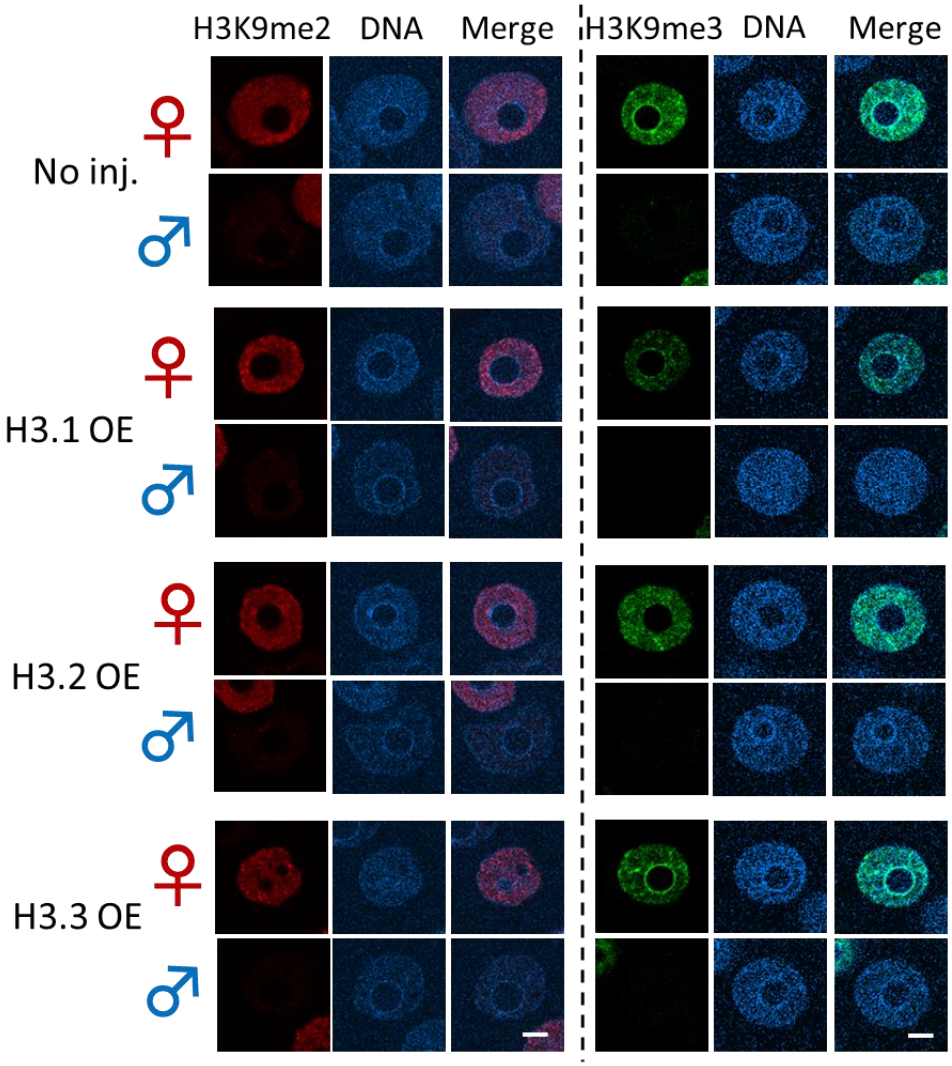


Figure 2-9. The effect of H3.1, H3.2, and H3.3 overexpression on H3K9me2/3 levels.

The H3K9me2/3 level of no-injected, H3.1, H3.2, and H3.3 overexpressed embryos were analyzed at 11 h. Three independent experiments were performed except H3.1 overexpressed embryos were analyzed in two independent experiments. A total of 13 to 34 embryos were analyzed for no-injected, injected embryos, where 4 to 14 embryos were analyzed in each experimental group. For H3K9me3 immunostaining, two independent experiments were performed, where 9 to 14 embryos were observed in total. Three to seven embryos were analyzed in each experimental group. Scale bar =10μm

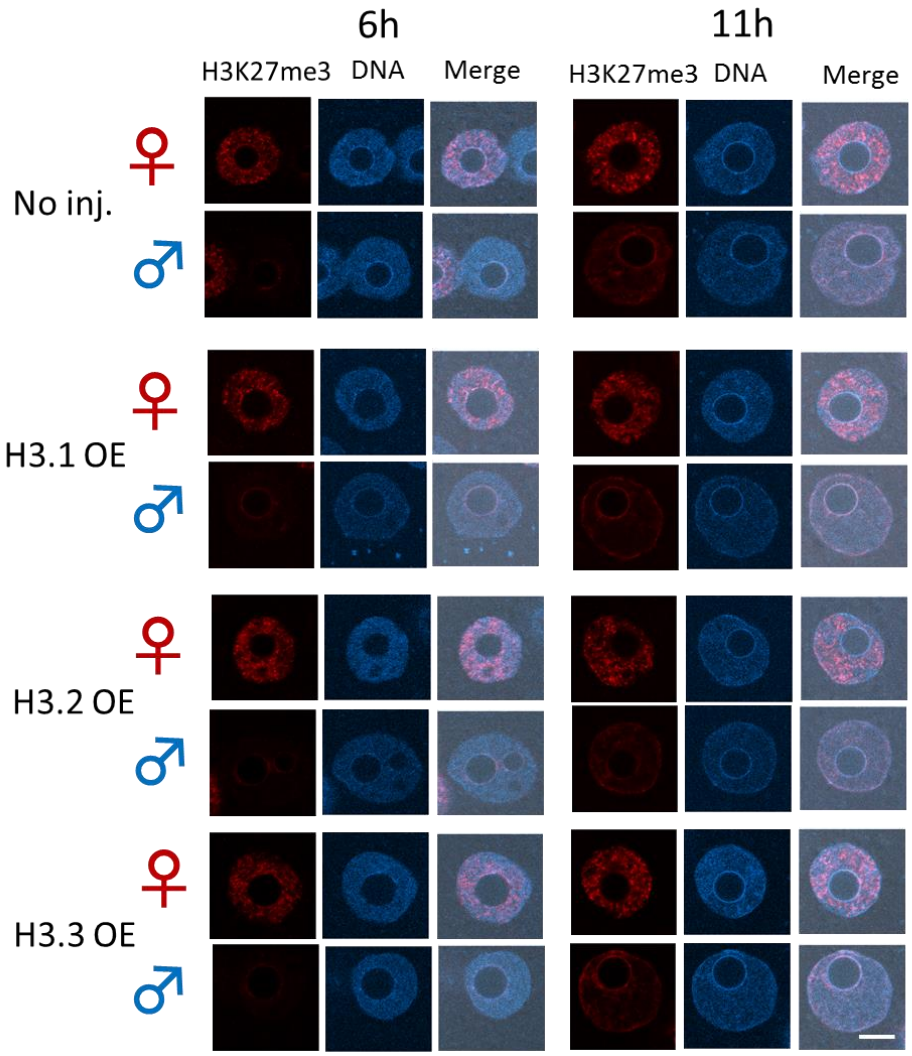


Figure 2-10. The effect of H3K27me3 levels in H3.1, H3.2, and H3.3 overexpressed embryos

The levels of H3K27me3 were analyzed in no-injected, H3.1, H3.2, and H3.3 overexpressed embryos. Two independent experiments were performed for 6 h. Four independent experiments for 11 h, except H3.1 overexpressed embryos were analyzed in three independent experiments. For 6 h, 11-17 embryos were analyzed for no-injected and injected embryos, with 3-9 embryos observed in each experimental group. For 11 h, 23-32 embryos were analyzed for no-injected and injected embryos, with 4-10 embryos observed in each experimental group.

Scale bar = 10 μm

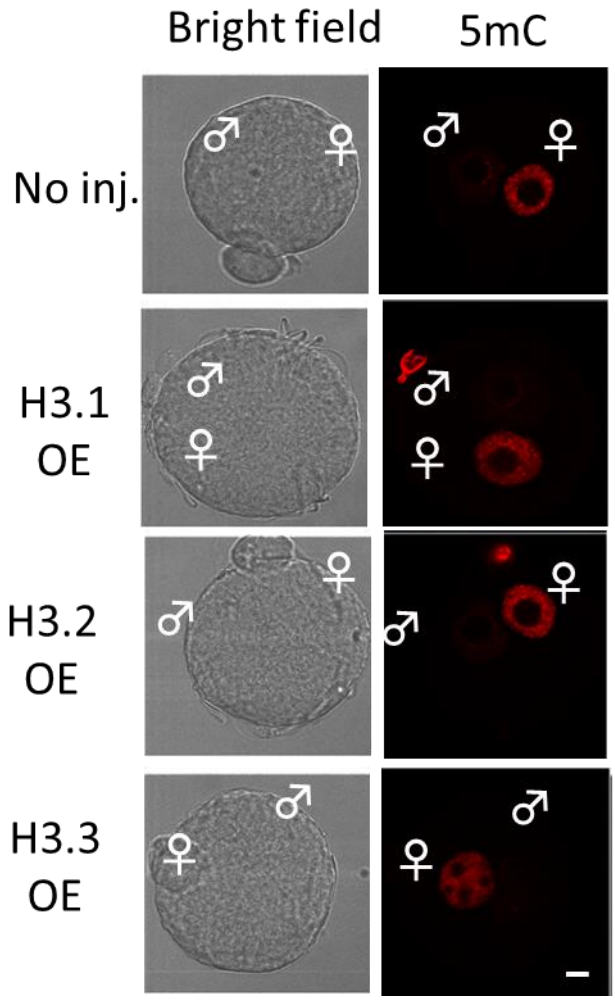


Figure 2-11. The effect on DNA methylation in H3.1, H3.2, and H3.3 overexpressed embryos.

No-injected, H3.1, H3.2, and H3.3-overexpressed embryos were collected at 11 h to examine the DNA methylation (5 methylcytosine) levels. One independent experiment was performed, in which 9 to 13 embryos were observed. Scale bar = 10 μm

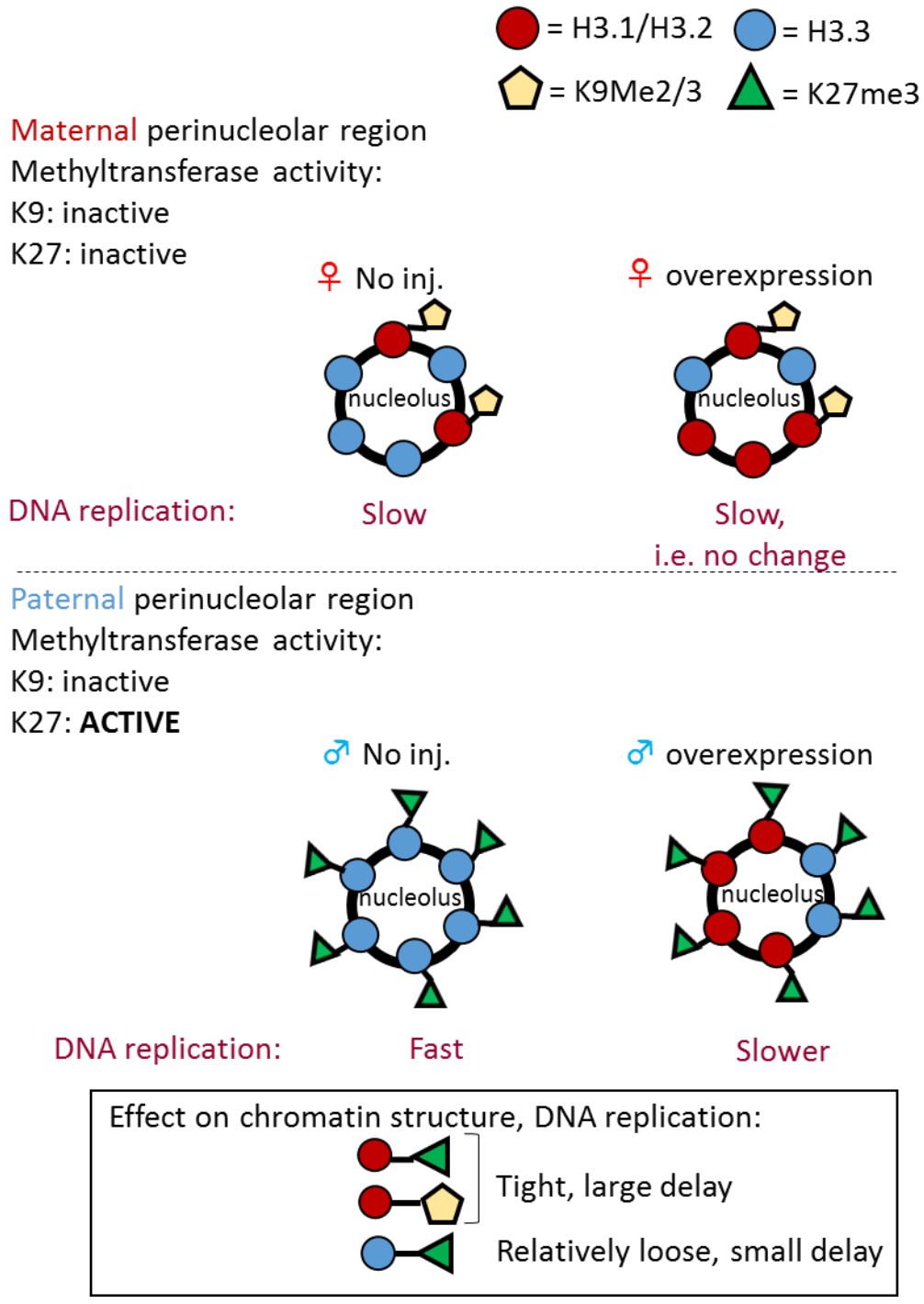


Figure 2-12. Illustration of the hypothesis to explain why the DNA replication is delayed in the paternal pronucleus but not the maternal one of the H3.1 or H3.2 overexpressed embryo. In contrast to the maternal pronucleus, there is K27 methyltransferase activity in the paternal pronucleus. The combination of H3.1/H3.2-H3K27me3 may lead to a change in chromatin tighteness, leading to delay in DNA replication.

GENERAL DISCUSSION

In chapter 1, I have shown that the nuclear localization of H3.1/H3.2 is low in 1-cell embryos compared to other preimplantation embryos. The low nuclear localization of H3.1/H3.2 was regulated by low mRNA expression and low chromatin incorporation efficiency at this stage. I have also shown asymmetrical nuclear localization of H3.1/H3.2 in 1-cell embryos. In Chapter 2, I have shown that the low level of nuclear deposition of H3.1 and H3.2 is necessary for timely completion of DNA replication in the paternal pronucleus. Taken together, this study to my knowledge, is the first one reporting that H3.1/H3.2 regulates asymmetry in cellular processes and that it is essential for the paternal pronucleus to have minimal nuclear localization of H3.1/H3.2 for proper development.

I hypothesize that the biological significance of lowering the localization level of H3.1 and H3.2 is to prevent detrimental effects of the deposition of these proteins in the paternal pronucleus and thus to prevent developmental failure. In the paternal pronucleus, the nuclear localization of H3.1/H3.2 at the perinucleolar region is equivalent or less compared to the nucleoplasmic and perinuclear regions. Therefore, the DNA replication at the perinucleolar region is completed earlier than these regions. In contrast, H3.1/H3.2 is localized at the perinucleolar region of the maternal pronucleus. Thus, the DNA replication at this region is completed last in the maternal pronucleus. The increased deposition of H3.1/H3.2 caused the delay in completion of DNA replication in the paternal pronucleus, especially at the perinucleolar region where pericentromeric heterochromatin is localized. Therefore, in 1-cell embryos, a mechanism would be required to decrease the deposition of H3.1/H3.2 in the paternal pronucleus by decreasing the overall mRNA expression and the efficiency of chromatin incorporation of H3.1 and

H3.2 (Fig. GD-1).

It is reported that major satellite repeats reside in the pericentromeric heterochromatin, which are found at the perinucleolar region of 1-cell embryos (Probst and Almouzni, 2011). It is known that there is asymmetry in major satellite transcription where it is transcribed more in the paternal pronucleus than the maternal pronucleus (Probst et al., 2010). However, whether H3.1 and H3.2 overexpression affects major satellite transcription is yet to be clarified. A study indicated that inhibition of major satellite transcription at the 1-cell stage led to developmental arrest at the 2-cell stage (Probst et al., 2010). Because H3.1 and H3.2 is mislocalized in the paternal perinucleolar region when these variants were ectopically deposited (Fig. 2-1) and since H3.1 and H3.2 are associated with heterochromatin (Hake and Allis, 2006), H3.1 and H3.2 overexpression may repress major satellite transcription in the paternal pronucleus. However, since the developmental delay occurred at the 1-cell stage but not 2-cell stage in the H3.1 and H3.2 overexpressed embryos, the repression of major satellite transcription would not be involved in the developmental failure in these embryos.

Recently, epigenetic modifications have been studied extensively and the asymmetry of them between the parental pronuclei has been brought into the limelight (Hemberger et al., 2009; Burton and Torres-Padilla, 2010; Beaujean, 2014). However, the biological significance of the asymmetry in most of these modifications and their associations to cellular processes has not been yet clarified. For instance, the parental asymmetry of global DNA methylation has been well-known and intensively studied for the mechanism regulating this asymmetry, its biological significance or association to a cellular process it has not yet been elucidated, but rather there are some reports indicating that it is not involved in the regulation of development (Beaujean et al., 2004; Tsukada et

al., 2015). Here, I propose a different notion in which the nuclear distribution of histone variants, the core components of chromatin, is asymmetric between the parental pronuclei and they regulate the DNA replication in 1-cell embryos. It would be thus interesting to further investigate what other variants of core histones, together with H3.1/H3.2, are involved in the regulation of the cellular processes in the 1-cell embryos and what factors prevent the incorporation of H3.1/H3.2 in the pronucleus to maintain proper cellular processes and development.

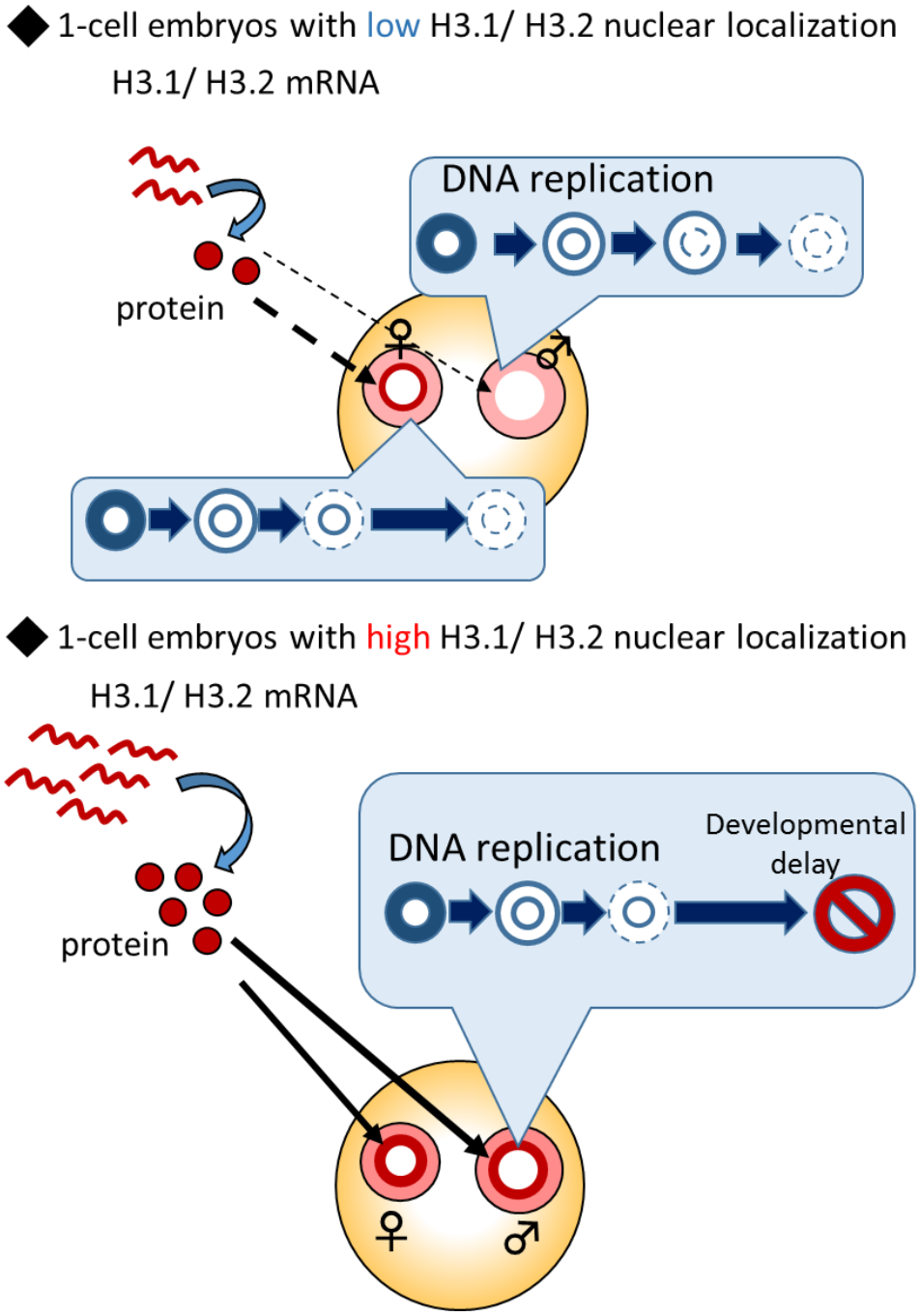


Figure GD-1. The biological significance of the low nuclear localization of H3.1/H3.2 in 1-cell embryos.

When H3.1/H3.2 is expressed at a high level, mislocalization of H3.1/H3.2 to the paternal pronucleus occurs, leading to a delayed DNA replication at the perinucleolar region and thus developmental delay. To prevent this, there is a mechanism where overall H3.1/H3.2 mRNA expression ratio and incorporation efficiency is maintained low.

MATERIALS AND METHODS

Culture condition

All oocytes and embryos were incubated in droplets of medium that were covered in mineral oil (Sigma-Aldrich, St. Louis, MO). The samples were incubated in 5% CO_2, 38ºC.

Collection of preimplantation embryos

MII stage oocytes were collected from three-week old BDF1 (DBA2 x B6Ncr Jms Slc) mice (SLC Japan, Inc., Shizuoka, Japan; CLEA Inc., Tokyo). The mice was first injected with 6 I.U. pregnant mare's serum gonadotropin (PMSG; ASKA Pharmaceutical Co, Ltd, Tokyo) to promote oocyte growth. The mice was then injected with 7.5 I.U. of human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co., Ltd) 46-50 h post-PMSG injection to induce the resumption of meiosis and ovulation. The ampulla of the oviduct were removed from mice 14-18 h post-hCG injection. The mature oocytes surrounded by cumulus cells are obtained by penetrating the ampulla of the oviduct using a 30-gauge needle (Terumo, Tokyo) and placed into 200 μl human tubal fluid (HTF) medium (Quinn and Begley, 1984).

In vitro fertilization was performed to obtain preimplantation embryos. The sperm was obtained from the caudal epididymis of 6 month old or older ICR mice and were placed in HTF medium (Quinn and Begley, 1984) supplemented with 10 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO). The sperm was incubated for 2 h before insemination for capacitation.

The oocytes were inseminated using capacitated sperm at the concentration of

200,000~ 1,000,000 sperm/ml. The embryos and/or unfertilized oocytes were placed and washed in KSOM medium (Lawitts and Biggers, 1993) 6 h post-insemination to remove cumulus cells, using glass capillaries. The presence of 2 pronuclei was examined 6 to 10 h post-insemination and 1-cell embryos with multipronuclei or one pronucleus were removed. The 1-cell embryos with 2 pronuclei were transferred into a fresh KSOM medium and were cultured until the blastocyst stage.

In vitro fertilization of denuded oocytes was conducted for microinjection analyses. Capacitated sperm were placed into 50 μl HTF medium and were incubated for 1-2 min. The oocytes were then placed into the same medium. Each sample was observed 10-15 min post-insemination to verify the presence of sperm surrounding the oocyte. Embryos and/or unfertilized oocytes were washed as the method described above. The embryos at the 1-cell, 2-cell, 4-cell embryos, morula, and blastocyst stages were collected or observed at the following times: 1-cell (10-11 h), 2-cell (28-30 h), 4-cell (45-46 h), morula (72 h), and blastocyst (96 h).

Immunofluorescence

For nuclear localization of H3.1/H3.2 and H3.3, preimplantation embryos were fixed with 3.7% paraformaldehyde (PFA) and 0.2% Triton X-100 in PBS for 20 min at room temperature. The oocytes and preimplantation embryos were washed in PBS containing 1% BSA (BSA/PBS) three times and were incubated overnight with mouse anti-H3.1/2 (1:500; CE-039B; Cosmo Bio, Tokyo) and rat anti-H3.3 (1:100; CE-040B; Cosmo Bio) antibodies in BSA/PBS containing 0.2% Tween 20. The samples were then washed in BSA/PBS three times and were incubated in secondary antibodies, Alexa Fluor 488 anti-mouse or rat IgG (1:100; Molecular Probes, Invitrogen, Carlsbad, CA), for 1 h

at room temperature. The samples were washed in BSA/PBS three times and were mounted on a glass slide with Vector Shield (Vector Laboratories, Burlingame, CA) containing 1.6 ng/µl 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto). To detect the histones that are incorporated into chromatin, the procedures described in Hajkova et al. (2010) were followed. Briefly, before fixation with 4% PFA/PBS, the embryos were treated with PBS containing 50 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100, 300 mM sucrose, and 25mM Hepes (pH=7.4) on ice for 10 min. Flag-tagged histones were detected using anti-Flag (1:1000; Sigma) and Alexa Fluor 568 anti-rabbit IgG (1:100). The endogenous histones were detected by anti-H3.1/H3.2 and anti-H3.3 antibodies with the dilutions described above. These antibodies were diluted in 1% BSA/PBS containing 0.1% Triton X-100. The second antibodies were also diluted with 1% BSA/PBS containing 0.1% Triton X-100. The embryos were washed three times in 1% PBS/PBS and in increasing concentration of Vecta Shield (Vector Laboratories) 1.6 ng/µl containing DAPI (Dojindo) before mounting on glass slides.

For the analysis of histone H3 di- or trimethylated at lysine 9 (H3K9me2 and H3K27me3), the embryos were fixed in 3.7% PFA/PBS for 1 h or 20 min, respectively, and permeabilized with 0.5% Triton X-100 for 15 min. The mouse anti-H3K9me2 (ab1220; Abcam, Cambridge) and anti-mouse H3K27me3 (05-851; Upstate/Millipore, Darmstadt) antibodies were diluted 1:100 in 1% BSA/PBS. For the analysis of H3K9me3, the embryos were fixed in 3.7% PFA/PBS containing 0.2% Triton X-100 for 20-25 min and the rabbit anti-H3K9me3 antibody (04-772; Millipore) was diluted 1:100. For secondary antibodies, Alexa Fluor 568 anti-mouse IgG or Alexa Fluor 488 anti-mouse 488 (Molecular Probes) or Fluorescein (FITC)-conjugated donkey anti-rabbit (Jackson ImmunoResearch Inc, West Grove, PA), were used and slides were prepared as described

above.

For the analysis of DNA methylation (5-methylcytosine), the following protocol was followed. The embryos were fixed in 3.7% PFA/PBS for 20 min and washed in 1% BSA/PBS and then permeabilized in 0.5% Triton/ PBS for 15 min at room temperature. The embryos were then washed in 4N HCl/ ddH2O containing 0.02% Triton X-100 three times, incubated in the last wash for 15 min. The embryos were then washed in 0.1 M Tris-HCl (pH8.5)/ ddH2O containing 0.02% Triton X-100 for three times, in which the embryos were incubated for 15 min in the last drop. The embryos were washed in 1% BSA/PBS three times and incubated for overnight at room temperature. The samples were incubated in anti-mouse 5-methylcytosine (1:2000; NA81; Calbiochem/ Millipore) in 1% BSA/PBS for 4 h at room temperature. The embryos were washed three times in 1% BSA/PBS, with an incubation of 1 h at the last wash. The embryos were then incubated with Alexa Fluor 647 anti-mouse IgG (1:250; Molecular Probes) for 1 h at room temperature. The embryos were washed three times in 1% BSA/PBS and slides were prepared as described above.

Plasmid construction

eGFP-polyA pcDNA3.1 vector (Yamagata et al., 2005) was used to generate Kozak-GFP cRNA as control for microinjection. This vector was used for the backbone for other constructed vectors. Kozak-H3.1, Kozak-H3.2, Kozak-H3.3-polyA pcDNA3.1 vectors were constructed by inverse PCR using Kozak-Flag-H3.1, Kozak-Flag-H3.2, Kozak-Flag-H3.3-polyA pcDNA3.1 vector as templates. Kozak-Flag-H3.1 and Kozak-Flag-H3.2 pcDNA3.1 vector had been constructed previously by Akiyama et al. (2011). To prepare Kozak-Flag-H3.3-polyA pcDNA3.1 vector, Kozak-Flag-H3.3 were

constructed by amplifying Kozak-Flag-H3.3 using Flag-H3.3 pCRII Topo vector which had been prepared by Akiyama et al. (2011) as a template and then inserted into pCRII vector. The insert was subcloned into pcDNA3.1 vector by digesting both ends with EcoRI (Fermentas/Thermo Fischer Scientific Inc. Waltham, MA). Kozak-H3.1-Flag, Kozak-H3.2-Flag, and Kozak-H3.2-Flag pcDNA3.1 were constructed by first amplifying Kozak-H3.1-Flag, Kozak-H3.2-Flag, and Kozak-H3.3-Flag using pCRII vector containing Flag-H3.1, Flag-H3.2 and Flag-H3.3 which had been prepared by Akiyama et al. (2011) and were inserted into pCR4 vector (Invitrogen, Life technologies). Then the inserts were subcloned into pcDNA3.1 vector by digesting both ends with EcoRI (Fermentas/Thermo Fischer Scientific).

The sequences of Kozak-H3.1, Kozak-H3.2, and Kozak-H3.3 insert is as follows:

Kozak-H3.1:

GCCACCATGGCTCGTACTAAGCAGACCGCTCGCAAGTCTACCGGCGGCAAGG
CCCCGCGCAAGCAGCTGGCCACCAAGGCCGCCCGCAAGAGCGCCCCGGCCA
CCGGCGGCGTGAAGAAGCCTCACCGCTACCGTCCCGGCACTGTGGCGCTGC
GCGAGATCCGGCGCTACCAGAAGTCGACCGAGCTGCTGATCCGCAAGCTGC
CGTTCCAGCGCCTGGTGCGCGAGATCGCGCAGGACTTCAAGACCGACCTGC
GCTTCCAGAGCTCGGCCGTCATGGCTCTGCAGGAGGCCTGTGAGGCCTACCT
CGTGGGTCTGTTTGAGGACACCAACCTGTGCGCCATCCACGCCAAGCGTGTC
ACCATCATGCCCAAGGACATCCAGCTGGCCCGTCGCATCCGCGGGGAGAGG
GCTTAA

Kozak-H3.2:

GCCACCATGGCTCGTACGAAGCAGACCGCTCGCAAGTCCACTGGCGGCAAG

GCCCCGCGCAAGCAGCTGGCCACCAAGGCCGCCCGCAAGAGCGCCCCGGCC
ACCGGCGGCGTGAAGAAACCTCACCGCTACCGTCCCGGCACCGTGGCGCTG
CGCGAGATCCGGCGCTACCAGAAGTCGACCGAGCTGCTGATCCGCAAGCTG
CCGTTCCAGCGCCTGGTGCGCGAGATCGCGCAGGACTTCAAGACCGACCTG
CGCTTCCAGAGCTCGGCCGTCATGGCTCTGCAGGAGGCGAGCGAGGCCTAC
CTTGTGGGTCTGTTTGAGGACACCAACCTGTGCGCCATCCACGCCAAGCGTG
TCACCATCATGCCCAAGGACATCCAGCTGGCCCGCCGTATCCGCGGCGAGCG
GGCTTAA

Kozak-H3.3:

GCCACCATGGCACGTACCAAGCAAACAGCCCGTAAATCGACCGGAGGCAAG
GCGCCCCGCAAGCAGCTGGCCACCAAGGCGGCCCGTAAATCGGCGCCATCC
ACCGGCGGAGTGAAGAAGCCACATCGCTACCGTCCTGGAACGGTGGCCCTG
CGTGAGATTCGTCGCTACCAGAAGTCCACGGAGCTGCTCATCCGCAAGCTGC
CGTTCCAGCGTCTGGTGCGCGAGATAGCCCAGGACTTCAAGACCGATCTGCG
CTTCCAGTCGGCGGCCATTGGAGCCCTACAGGAGGCCAGCGAGGCGTACCT
GGTCGGTCTGTTCGAGGACACCAATCTGTGCGCCATTCACGCCAAGCGCGTC
ACCATTATGCCCAAGGACATCCAGCTGGCCAGACGCATCCGTGGCGAGCGGG
CCTAA

linker sequence and Flag sequence attached to the C terminus is as follows:

GGAGGATCGGGAGGAGATTACAAGGATGACGACGATAAG

Generation of complimentary RNA (cRNA) for microinjection

The plasmids were linearized by digesting with XhoI (TaKaRa) overnight. After verifying the plasmids were linearized, they were purified by phenol-chloroform (Wako, Tokyo). The supernatant was then further purified by adding chloroform iso-amyl alcohol (Wako) to remove remaining chloroform. The precipitated sample was rinsed with 70% ethanol and dried in a desiccator. In vitro transcription was performed using T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX, U.S.A.). Lithium precipitation was performed for the RNA purification.

Microinjection

Mature oocytes were collected in α-MEM (Gibco-BRL, Grand Island, NY) containing 5% FBS (Sigma-Aldrich) and 10 ng/ml EGF (Sigma-Aldrich). To remove the cumulus cells, hyaluronidase (Sigma-Aldrich) at a final concentration of 300 μg/ml was added to the medium and incubated for 5 min at 38°C, 5% CO_2. The cumulus cells were removed by pipetting the oocytes with glass capillaries and stored in α-MEM medium containing 5% FBS (Sigma-Aldrich) and 5 ng/ml EGF. Microinjection at mature oocytes were performed in HEPES-buffered KSOM (Lawitts and Biggers, 1993), using an inverted microscope (Eclipse TE300, Nikon Corporation, Tokyo) attached with a micromanipulator and microinjector (Narishige Co., Tokyo). The borosilicate glass capillaries to hold the oocyte (Microcaps, Drummond Scientific, Broomall, PA) and narrow glass capillaries to inject cRNA (GC100 TF-10; Harvard Apparatus Ltd, Cambridge) were prepared using a Model P-97/IVF puller (Sutter Instrument, Co., California) and Microforge MF-900 (Narishige). cRNA was microinjected at a concentration and amount of 100 ng/μl and 10 pl, respectively, into the mature oocytes. After microinjection, the oocytes were washed in α-MEM (Gibco-BRL) containing 5%

FBS and 10 ng/ml EGF. The oocytes were microinjected within 1.5-5 h after oocyte collection and incubated for another 2 h in α-MEM to allow translation of injected histones before carrying out in vitro fertilization. The microinjected 1-cell embryos were washed in KSOM medium (Lawitts and Biggers, 1993) and incubated at 38°C, supplied with 5% CO_2 until the blastocyst stage. For in vitro fertilization of denuded oocytes, *see* the above section, *Collection of preimplantation embryos*.

Parthenogenesis

Parthenogenetic embryos were produced by following the procedure established by Kishigami and Wakayama (2007). Mature oocytes were microinjected within 1.5-5 h after oocyte collection and incubated for another 2 h to allow translation of histones inα-MEM containing 5% fetal bovine serum and 10 ng/ml epidermal factor. The mature oocytes were then activated for 3 h at 5% CO_2, 38°C, in KSOM (Lawitts and Biggers, 1993) containing 2 mM EGTA, 5 mM SrCl2, 5 μg/ml Cytochalasin B (Sigma-Aldrich) to generate parthenogenetic embryos with 2 pronuclei. Parthenogenetic embryos with 2 pronuclei were produced to generate embryos with the same amount of histones as the in vitro fertilized embryos. After 3 h of activation, the embryos were washed in KSOM (Lawitts and Biggers, 1993) and checked for parthenogenetic embryos with two pronuclei. After selection of parthenogenetic embryos, the embryos were cultured in KSOM medium until the blastocyst stage.

Analysis for mRNA expression ratio among H3 variants

The RPKM values of genes that code for H3.1 (*Hist1h3a*, *Hist1h3g*, *Hist1h3h*, *Hist1h3i*) and H3.2 (*Hist1h3f*, *Hist1h3b*, *Hist1h3d*, *Hist1h3e*, *Hist2h3b*, *Hist1h3c*,

Hist2h3c2, *Hist2h3c1*), and H3.3 (*H3f3a*, *H3f3b*) were obtained from Abe et al., (2015). Those values were summed in each H3 variant to compare the total expression levels among the variants. The expression level of H3.3 at 1-cell stage is set to 1 and the expression ratio is calculated.

Flag quantification

To determine the cRNA concentration of histone H3.1, H3.2, H3.3 that yields equal amount of histone incorporation, Flag-tagged H3.1, H3.2, H3.3 were used. Several concentrations of Flag-H3 variant cRNA were prepared by diluting cRNA in nuclease-free water: 3 ng/μl, 10 ng/μl, 30 ng/μl, and 100 ng/μl. Microinjected samples were collected 11 h post-insemination. The free histones in the nucleoplasm were washed away using the permeabilization solution (Hajkova et al., 2010). The immunofluorescence staining were performed using anti-Flag antibody (Sigma; 1:1000) and secondary antibody, Alexa Fluor 568 anti-rabbit IgG (Molecular Probes). The slides were prepared as described above. The signal intensity of Flag antibody and DAPI was quantified using Image J software (NIH, Bethesda, Maryland). The Flag intensity was corrected with the signal of DAPI. The maternal and paternal pronuclei were distinguished from each other by their size and the proximity to the polar body: the maternal pronucleus is smaller and proximal to the polar body.

BrdU incorporation assay for DNA replication

DNA replication was analyzed by examining the 5-bromo-2’-deoxyuridine (BrdU) incorporation in 1-cell embryos at 4, 6, 8, 10 h after insemination. BrdU (Roche,

Basel, Switzerland) at a final concentration of 10 µM was added to the KSOM (Lawitts and Biggers, 1993) and incubated at 38oC 30 min before sampling. After 30 min, the embryos were transferred into the KSOM medium with 10 µM BrdU and incubated 1 h at 38ºC, 5% CO_2. The embryos were then washed in 1% BSA/PBS for three times and fixed with 3.7% PFA/PBS for 1 h at room temperature. After fixation, the samples were washed with 1% BSA/PBS three times and were washed in PBS containing 0.05% Tween 20 three times. The samples were then placed under 2N HCl containing 0.1% Triton X-100 for 1 h at 37ºC. The samples were washed with 1% BSA/PBS three times and transferred into 0.1M Tris-HCl (pH8.5)/ PBS containing 0.02% Triton X for 15 min at room temperature in a humid chamber. The samples were washed three times with 1% BSA/PBS and incubated overnight with primary antibodies: mouse anti-BrdU (1:100; Roche), rabbit anti- H3K9me3 (1:1000; Millipore). Alexa Fluor 647 anti-mouse IgG (Molecular Probes) and Fluorescein (FITC)-conjugated donkey anti-rabbit (Jackson ImmunoResearch Inc.) were used as secondary antibodies and slides were prepared as described above.

REFERENCES

Abe, K., R. Yamamoto, V. Franke, M. Cao, Y. Suzuki, M. G. Suzuki, K. Vlahovicek, P. Svoboda, R. M. Schultz and F. Aoki (2015). "The first murine zygotic transcription is promiscuous and uncoupled from splicing and 3' processing." Embo j **34**(11): 1523-1537.

Adenot, P. G., Y. Mercier, J. P. Renard and E. M. Thompson (1997). "Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos." Development **124**(22): 4615-4625.

Adenot, P. G., M. S. Szollosi, M. Geze, J. P. Renard and P. Debey (1991). "Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization." Mol Reprod Dev **28**(1): 23-34.

Ahmed, K., H. Dehghani, P. Rugg-Gunn, E. Fussner, J. Rossant and D. P. Bazett-Jones (2010). "Global chromatin architecture reflects pluripotency and lineage commitment in the early mouse embryo." PLoS One **5**(5): e10531.

Akiyama, T., O. Suzuki, J. Matsuda and F. Aoki (2011). "Dynamic replacement of histone H3 variants reprograms epigenetic marks in early mouse embryos." PLoS Genet **7**(10): e1002279.

Allis, C. D. and T. Jenuwein (2016). "The molecular hallmarks of epigenetic control." Nat Rev Genet **17**(8): 487-500.

Aoki, F. and R. M. Schultz (1999). "DNA replication in the 1-cell mouse embryo: stimulatory effect of histone acetylation." Zygote **7**(2): 165-172.

Aoki, F., D. M. Worrad and R. M. Schultz (1997). "Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo." Dev Biol **181**(2): 296-307.

Beaujean, N. (2014). "Histone post-translational modifications in preimplantation mouse embryos and their role in nuclear architecture." Mol Reprod Dev **81**(2): 100-112.

Beaujean, N., G. Hartshorne, J. Cavilla, J. Taylor, J. Gardner, I. Wilmut, R. Meehan and L. Young (2004). "Non-conservation of mammalian preimplantation methylation dynamics." Curr Biol **14**(7): R266-267.

Bonnefoy, E., G. A. Orsi, P. Couble and B. Loppin (2007). "The essential role of Drosophila HIRA for de novo assembly of paternal chromatin at fertilization." PLoS Genet **3**(10): 1991-2006.

Bouniol, C., E. Nguyen and P. Debey (1995). "Endogenous transcription occurs at the 1-cell stage in the mouse embryo." Exp Cell Res **218**(1): 57-62.

Burton, A. and M. E. Torres-Padilla (2010). "Epigenetic reprogramming and development: a unique heterochromatin organization in the preimplantation mouse embryo." Brief Funct Genomics **9**(5-6): 444-454.

Campos, E. I., A. H. Smits, Y. H. Kang, S. Landry, T. M. Escobar, S. Nayak, B. M. Ueberheide, D. Durocher, M. Vermeulen, J. Hurwitz and D. Reinberg (2015). "Analysis of the Histone H3.1 Interactome: A Suitable Chaperone for the Right Event." Mol Cell **60**(4): 697-709.

De La Fuente, R., C. Baumann and M. M. Viveiros (2015). "ATRX contributes to epigenetic asymmetry and silencing of major satellite transcripts in the maternal genome of the mouse embryo." Development **142**(10): 1806-1817.

Eid, A., D. Rodriguez-Terrones, A. Burton and M. E. Torres-Padilla (2016). "SUV4-20 activity in the preimplantation mouse embryo controls timely replication." Genes Dev **30**(22): 2513-2526.

Filipescu, D., S. Müller and G. Almouzni (2014). "Histone H3 variants and their chaperones during development and disease: contributing to epiegenetic control." Annu Rev Cell Dev Biol **30**: 615-646.

Filipescu, D., E. Szenker and G. Almouzni (2013). "Developmental roles of histone H3 variants and their chaperones." Trends Genet **29**(11): 630-640.

Goldberg, A. D., L. A. Banaszynski, K. M. Noh, P. W. Lewis, S. J. Elsaesser, S. Stadler, S. Dewell, M. Law, X. Guo, X. Li, D. Wen, A. Chapgier, R. C. DeKelver, J. C. Miller, Y. L. Lee, E. A. Boydston, M. C. Holmes, P. D. Gregory, J. M. Greally, S. Rafii, C. Yang, P. J. Scambler, D. Garrick, R. J. Gibbons, D. R. Higgs, I. M. Cristea, F. D. Urnov, D. Zheng and C. D. Allis (2010). "Distinct factors control histone variant H3.3 localization at specific genomic regions." Cell **140**(5): 678-691.

Graves, R. A., S. E. Wellman, I. M. Chiu and W. F. Marzluff (1985). "Differential expression of two clusters of mouse histone genes." J Mol Biol **183**(2): 179-194.

Hajkova, P., S. J. Jeffries, C. Lee, N. Miller, S. P. Jackson and M. A. Surani (2010). "Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway." Science **329**(5897): 78-82.

Hake, S. B. and C. D. Allis (2006). "Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis"." Proc Natl Acad Sci U S A **103**(17): 6428-6435.

Hake, S. B., B. A. Garcia, E. M. Duncan, M. Kauer, G. Dellaire, J. Shabanowitz, D. P. Bazett-Jones, C. D. Allis and D. F. Hunt (2006). "Expression patterns and post-translational modifications associated with mammalian histone H3 variants." J Biol Chem **281**(1): 559-568.

Hatanaka, Y., K. Inoue, M. Oikawa, S. Kamimura, N. Ogonuki, E. N. Kodama, Y. Ohkawa, Y. Tsukada and A. Ogura (2015). "Histone chaperone CAF-1 mediates repressive histone modifications to protect preimplantation mouse embryos from endogenous retrotransposons." Proc Natl Acad Sci U S A **112**(47): 14641-14646.

Hemberger, M., W. Dean and W. Reik (2009). "Epigenetic dynamics of stem cells and cell

lineage commitment: digging Waddington's canal." Nat Rev Mol Cell Biol **10**(8): 526-537.

Houlard, M., S. Berlivet, A. V. Probst, J. P. Quivy, P. Hery, G. Almouzni and M. Gerard (2006). "CAF-1 is essential for heterochromatin organization in pluripotent embryonic cells." PLoS Genet **2**(11): e181.

Ingouff, M., Y. Hamamura, M. Gourgues, T. Higashiyama and F. Berger (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.

Inoue, A. and Y. Zhang (2014). "Nucleosome assembly is required for nuclear pore complex assembly in mouse zygotes." Nat Struct Mol Biol **21**(7): 609-616.

Jin, J., Y. Cai, B. Li, R. C. Conaway, J. L. Workwan, J. W. Conaway and T Kusch (2005). "In and out: histone variant exchange in chromatin." Trends Biochem Sci **30**(12): 680-687.

Kamakaka, R. T. and S. Biggins (2005). "Histone variants: deviants?" Genes Dev **19**(3): 295-310.

Kawamura, M., T. Akiyama, S. Tsukamoto, M. G. Suzuki and F. Aoki (2012). "The expression and nuclear deposition of histone H3.1 in murine oocytes and preimplantation embryos." J Reprod Dev **58**(5): 557-562.

Kishigami, S. and T. Wakayama (2007). "Efficient strontium-induced activation of mouse oocytes in standard culture media by chelating calcium." J Reprod Dev **53**(6): 1207-1215.

Koseoglu, M. M., J. Dong and W. F. Marzluff (2010). "Coordinate regulation of histone mRNA metabolism and DNA replication: cyclin A/cdk1 is involved in inactivation of histone mRNA metabolism and DNA replication at the end of S phase." Cell Cycle **9**(19): 3857-3863.

Kourmouli, N., P. Jeppesen, S. Mahadevhaiah, P. Burgoyne, R. Wu, D. M. Gilbert, S. Bongiorni, G. Prantera, L. Fanti, S. Pimpinelli, W. Shi, R. Fundele and P. B. Singh (2004). "Heterochromatin and tri-methylated lysine 20 of histone H4 in animals." J Cell Sci **117**(Pt 12): 2491-2501.

Kouzarides, T. (2002). "Histone methylation in transcriptional control." Curr Opin Genet Dev **12**(2): 198-209.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Lange, U. C., S. Siebert, M. Wossidlo, T. Weiss, C. Ziegler-Birling, J. Walter, M. E. Torres-Padilla, S. Daujat and R. Schneider (2013). "Dissecting the role of H3K64me3 in mouse pericentromeric heterochromatin." Nat Commun **4**: 2233.

Lawitts, J. A. and J. D. Biggers (1993). "Culture of preimplantation embryos." Methods Enzymol **225**: 153-164.

Lepikhov, K. and J. Walter (2004). "Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote." BMC Dev Biol **4**: 12.

Lewis, P. W., S. J. Elsaesser, K. M. Noh, S. C. Stadler and C. D. Allis (2010). "Daxx is an

H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres." Proc Natl Acad Sci U S A **107**(32): 14075-14080.

Lin, C. J., M. Conti and M. Ramalho-Santos (2013). "Histone variant H3.3 maintains a decondensed chromatin state essential for mouse preimplantation development." Development **140**(17): 3624-3634.

Liu, C. P., C. Xiong, M. Wang, Z. Yu, N. Yang, P. Chen, Z. Zhang, G. Li and R. M. Xu (2012). "Structure of the variant histone H3.3-H4 heterodimer in complex with its chaperone DAXX." Nat Struct Mol Biol **19**(12): 1287-1292.

Liu, H., J. M. Kim and F. Aoki (2004). "Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos." Development **131**(10): 2269-2280.

Loppin, B., E. Bonnefoy, C. Anselme, A. Laurencon, T. L. Karr and P. Couble (2005). "The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus." Nature **437**(7063): 1386-1390.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-260.

Maehara, K., A. Harada, Y. Sato, M. Matsumoto, K. I. Nakayama, H. Kimura and Y. Ohkawa (2015). "Tissue-specific expression of histone H3 variants diversified after species separation." Epigenetics Chromatin **8**: 35.

Maison, C. and G. Almouzni (2004). "HP1 and the dynamics of heterochromatin maintenance." Nat Rev Mol Cell Biol **5**(4): 296-304.

Martin, C., N. Beaujean, V. Brochard, C. Audouard, D. Zink and P. Debey (2006). "Genome restructuring in mouse embryos during reprogramming and early development." Dev Biol **292**(2): 317-332.

Martin, C. and Y. Zhang (2005). "The diverse functions of histone lysine methylation." Nat Rev Mol Cell Biol **6**(11): 838-849.

Marzluff, W. F. (2005). "Metazoan replication-dependent histone mRNAs: a distinct set of RNA polymerase II transcripts." Curr Opin Cell Biol **17**(3): 274-280.

Mayer, W., A. Niveleau, J. Walter, R. Fundele and T. Haaf (2000). "Demethylation of the zygotic paternal genome." Nature **403**(6769): 501-502.

Maze, I., K. M. Noh, A. A. Soshnev and C. D. Allis (2014). "Every amino acid matters: essential contributions of histone variants to mammalian development and disease." Nat Rev Genet **15**(4): 259-271.

McGrath, J. and D. Solter (1984). "Completion of mouse embryogenesis requires both the maternal and paternal genomes." Cell **37**(1): 179-183.

Moosmann, A., C. Campsteijn, P. W. Jansen, C. Nasrallah, M. Raasholm, H. G. Stunnenberg and E. M. Thompson (2011). "Histone variant innovation in a rapidly evolving chordate lineage."

BMC Evol Biol **11**: 208.

Nakamura, T., Y. J. Liu, H. Nakashima, H. Umehara, K. Inoue, S. Matoba, M. Tachibana, A. Ogura, Y. Shinkai and T. Nakano (2012). "PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos." Nature **486**(7403): 415-419.

Nashun, B., P. W. Hill, S. A. Smallwood, G. Dharmalingam, R. Amouroux, S. J. Clark, V. Sharma, E. Ndjetehe, P. Pelczar, R. J. Festenstein, G. Kelsey and P. Hajkova (2015). "Continuous Histone Replacement by Hira Is Essential for Normal Transcriptional Regulation and De Novo DNA Methylation during Mouse Oogenesis." Mol Cell **60**(4): 611-625.

O'Keefe, R. T., S. C. Henderson and D. L. Spector (1992). "Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences." J Cell Biol **116**(5): 1095-1110.

Ooga, M., H. Fulka, S. Hashimoto, M. G. Suzuki and F. Aoki (2016). "Analysis of chromatin structure in mouse preimplantation embryos by fluorescent recovery after photobleaching." Epigenetics **11**(1): 85-94.

Ooi, S. L., J. R. Priess and S. Henikoff (2006). "Histone H3.3 variant dynamics in the germline of Caenorhabditis elegans." PLoS Genet **2**(6): e97.

Orsi, G. A., A. Algazeery, R. E. Meyer, M. Capri, L. M. Sapey-Triomphe, B. Horard, H. Gruffat, P. Couble, O. Ait-Ahmed and B. Loppin (2013). "Drosophila Yemanuclein and HIRA cooperate for de novo assembly of H3.3-containing nucleosomes in the male pronucleus." PLoS Genet **9**(2): e1003285.

Oswald, J., S. Engemann, N. Lane, W. Mayer, A. Olek, R. Fundele, W. Dean, W. Reik and J. Walter (2000). "Active demethylation of the paternal genome in the mouse zygote." Curr Biol **10**(8): 475-478.

Pikó, L. and K. Clegg (1982). "Quantitative changes in total RNA, total Poly(A), and ribosomes in early mouse embryo." Dev Biol **89**(2): 362-378

Postberg, J., S. Forcob, W. J. Chang and H. J. Lipps (2010). "The evolutionary history of histone H3 suggests a deep eukaryotic root of chromatin modifying mechanisms." BMC Evol Biol **10**: 259.

Probst, A. V. and G. Almouzni (2011). "Heterochromatin establishment in the context of genome-wide epigenetic reprogramming." Trends Genet **27**(5): 177-185.

Probst, A. V., I. Okamoto, M. Casanova, F. El Marjou, P. Le Baccon and G. Almouzni (2010). "A strand-specific burst in transcription of pericentric satellites is required for chromocenter formation and early mouse development." Dev Cell **19**(4): 625-638.

Pusarla, R. H. and P. Bhargava (2005). "Histones in functional diversification. Core histone variants." Febs j **272**(20): 5149-5168.

Puschendorf, M., R. Terranova, E. Boutsma, X. Mao, K. Isono, U. Brykczynska, C. Kolb, A.

P. Otte, H. Koseki, S. H. Orkin, M. van Lohuizen and A. H. Peters (2008). "PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos." Nat Genet **40**(4): 411-420.

Quinn, P. and A. J. Begley (1984). "Effect of human seminal plasma and mouse accessory gland extracts on mouse fertilization in vitro." Aust J Biol Sci **37**(3): 147-152.

Ram, P. T. and R. M. Schultz (1993). "Reporter gene expression in G2 of the 1-cell mouse embryo." Dev Biol **156**(2): 552-556.

Rapkin, L. M., K. Ahmed, S. Dulev, R. Li, H. Kimura, A. M. Ishov and D. P. Bazett-Jones (2015). "The histone chaperone DAXX maintains the structural organization of heterochromatin domains." Epigenetics Chromatin **8**: 44.

Ray-Gallet, D., J. P. Quivy, C. Scamps, E. M. Martini, M. Lipinski and G. Almouzni (2002). "HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis." Mol Cell **9**(5): 1091-1100.

Santenard, A., C. Ziegler-Birling, M. Koch, L. Tora, A. J. Bannister and M. E. Torres-Padilla (2010). "Heterochromatin formation in the mouse embryo requires critical residues of the histone variant H3.3." Nat Cell Biol **12**(9): 853-862.

Santos, F., B. Hendrich, W. Reik and W. Dean (2002). "Dynamic reprogramming of DNA methylation in the early mouse embryo." Dev Biol **241**(1): 172-182.

Santos, F., A. H. Peters, A. P. Otte, W. Reik and W. Dean (2005). "Dynamic chromatin modifications characterise the first cell cycle in mouse embryos." Dev Biol **280**(1): 225-236.

Shermoen, A. W., M. L. McCleland and P. H. O'Farrell (2010). "Developmental control of late replication and S phase length." Curr Biol **20**(23): 2067-2077.

Smith, S. and B. Stillman (1989). "Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro." Cell **58**(1): 15-25.

Su, T. T. (2010). "Heterochromatin replication: better late than ever." Curr Biol **20**(23): R1018-1020.

Surani, M. A., S. C. Barton and M. L. Norris (1984). "Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis." Nature **308**(5959): 548-550.

Surani, M. A., S. C. Barton and M. L. Norris (1986). "Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome." Cell **45**(1): 127-136.

Surani, M. A., S. C. Barton and M. L. Norris (1987). "Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development." Biol Reprod **36**(1): 1-16.

Tagami, H., D. Ray-Gallet, G. Almouzni and Y. Nakatani (2004). "Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis." Cell **116**(1): 51-61.

Talbert, P. B. and S. Henikoff (2010). "Histone variants--ancient wrap artists of the epigenome." Nat Rev Mol Cell Biol **11**(4): 264-275.

Talbert, P. B. and S. Henikoff (2016). "Histone variants on the move: substrates for chromatin dynamics." Nat Rev Mol Cell Biol.

Tang, M. C., S. A. Jacobs, D. M. Mattiske, Y. M. Soh, A. N. Graham, A. Tran, S. L. Lim, D. F. Hudson, P. Kalitsis, M. K. O'Bryan, L. H Wong and J. R. Mann (2015). "Contribution of the two genes encoding histone variant h3.3 to viability and fertility in mice." PLoS Genet **11**(2):e1004964.

Tang, Y., M. V. Poustovoitov, K. Zhao, M. Garfinkel, A. Canutescu, R. Dunbrack, P. D. Adams and R. Marmorstein (2012). "Structure of a human ASF1a-HIRA complex and insights into specificity of histone chaperone complex assembly." Nat Struct Mol Biol **13**(10): 921-929.

Tardat, M., M. Albert, R. Kunzmann, Z. Liu, L. Kaustov, R. Thierry, S. Duan, U. Brykczynska, C. H. Arrowsmith and A. H. Peters (2015). "Cbx2 targets PRC1 to constitutive heterochromatin in mouse zygotes in a parent-of-origin-dependent manner." Mol Cell **58**(1): 157-171.

Torres-Padilla, M. E., A. J. Bannister, P. J. Hurd, T. Kouzarides and M. Zernicka-Goetz (2006). "Dynamic distribution of the replacement histone variant H3.3 in the mouse oocyte and preimplantation embryos." Int J Dev Biol **50**(5): 455-461.

Tsukada, Y., T. Akiyama and K. I. Nakayama (2015). "Maternal TET3 is dispensable for embryonic development but is required for neonatal growth." Sci Rep **5**: 15876.

van der Heijden, G. W., A. A. Derijck, L. Ramos, M. Giele, J. van der Vlag and P. de Boer (2006). "Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin." Dev Biol **298**(2): 458-469.

van der Heijden, G. W., J. W. Dieker, A. A. Derijck, S. Muller, J. H. Berden, D. D. Braat, J. van der Vlag and P. de Boer (2005). "Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote." Mech Dev **122**(9): 1008-1022.

Verreault, A., P. D. Kaufman, R. Kobayashi and B. Stillman (1996). "Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4." Cell **87**(1): 95-104.

Wang, Z. F., T. Krasikov, M. R. Frey, J. Wang, A. G. Matera and W. F. Marzluff (1996). "Characterization of the mouse histone gene cluster on chromosome 13: 45 histone genes in three patches spread over 1Mb." Genome Res **6**(8): 688-701.

Wang, Z. F., R. Tisovec, R. W. Debry, M. R. Frey, A. G. Matera and W. F. Marzluff (1996). "Characterization of the 55-kb mouse histone gene cluster on chromosome 3." Genome Res **6**(8): 702-714.

Wiekowski, M., M. Miranda and M. L. DePamphilis (1993). "Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic

nuclei." Dev Biol **159**(1): 366-378.

Yamagata, K., T. Yamazaki, M. Yamashita, Y. Hara, N. Ogonuki and A. Ogura (2005). "Noninvasive visualization of molecular events in the mammalian zygote." Genesis **43**(2): 71-79.

Yukawa, M., T. Akiyama, V. Franke, N. Mise, T. Isagawa, Y. Suzuki, M. G. Suzuki, K. Vlahovicek, K. Abe, H. Aburatani and F. Aoki (2014). "Genome-wide analysis of the chromatin composition of histone H2A and H3 variants in mouse embryonic stem cells." PLoS One **9**(3): e92689.

Yukawa, M., S. Oda, H. Mitani, M. Nagata and F. Aoki (2007). "Deficiency in the response to DNA double-strand breaks in mouse early preimplantation embryos." Biochem Biophys Res Commun **358**(2): 578-584.