



## Regular Article

# Chromosomal distribution patterns of global 5mC and 5hmC on the ZZ/ZW and XX/XY chromosomes in the Japanese wrinkled frog, *Rana rugosa*, induced by Tet methylcytosine dioxygenase enzymes

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## Abstract

Tet methylcytosine dioxygenase enzymes catalyze the conversion of DNA methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), thereby initiating DNA demethylation. In mice, Tets also regulate gene activity by binding the regulatory elements of target genes. 5hmC is associated with euchromatic regions and epigenetic reprogramming events. In this study, we examined the levels of 5mC and 5hmC in two major groups of Japanese wrinkled frogs (*Rana rugosa*) with morphologically distinct sex chromosome constitutions, ZZ/ZW and XX/XY. Patterns of 5mC and 5hmC, as determined by immunostaining, were indistinguishable between homologous sex chromosomes in ZZ cells and XX cells. Genome-wide conversion of 5mC to 5hmC, followed by cell cycle-mediated dilution, was induced in PHA-stimulated peripheral blood cells in adult frogs. Tet enzymes may share an evolutionarily conserved function associated with a global epigenetic reprogramming event during dedifferentiation in mammals and frogs.

**Keywords:** DNA cytosine methylation, 5-hydroxymethylation, frog, sex chromosomes, peripheral blood cells

## Introduction

DNA methylcytosine (5mC) is an indispensable epigenetic mark of cell differentiation and normal embryonic development in mammals (Bird, 2002). However, 5mC is dispensable for self-renewal and proliferation potencies of mouse embryonic stem cells (ESC). This was demonstrated in null mutant ESCs for all three DNA methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b (Dnmt TKO ESC) (Tsumura *et al.*, 2006).

The Tet dioxygenase enzymes Tet1, Tet2, and Tet3 convert 5mC to 5-hydroxymethylcytosine (5hmC), which is exclusively generated by oxidation of 5mC as a substrate. A lack of all three Tets does not affect the self-renewal potency of mouse ESCs (Tet TKO ESC), but it reduces cell differentiation and proliferation potencies in Tet TKO ESCs (Dawlaty *et al.*, 2014). 5hmC is an intermediate form involved in active DNA demethylation (Tahiliani *et al.*, 2009). Genome-wide demethylation occurs in preimplantation embryos, and recent studies show that Tet3 selectively converts 5mC into 5hmC only in paternal pronuclei (patPN), leading to patPN-specific active demethylation, whereas 5mC on maternal pronuclei is erased in a cell cycle-dependent manner (Gu *et al.*, 2011; Inoue and Zhang, 2011). Differential conversion of 5mC at pronuclei is regulated by accessibility of the genome to Tets, and Tet binding is negatively regulated by an inactive chromatin mark, histone H3 lysine 9 methylation (H3K9me) (Matoba *et al.*, 2014; Nakamura *et al.*, 2012). However, patPN-specific oxidation of 5mC to 5hmC is not necessary in mouse embryogenesis (Inoue *et al.*, 2015), as both paternal 5hmC and maternal 5mC can be passively erased through cell divisions during preimplantation development. However, Tet3 is required as a maternal factor for normal embryonic development (Inoue *et al.*, 2015). Thus, for proper regulation of gene activation, site-specific binding of Tets to target genes or precise regional oxidation of 5mC is more important than initiation of genome-wide DNA demethylation. Although Tets may play different roles in embryogenesis, organogenesis, and tumorigenesis, these functions remain largely unknown. Moreover, Tets are conserved in the course of evolution to date (Almeida *et al.*, 2012). Thus, Tets may also play important roles in embryonic development, at least in animals derived from an ancestral tetrapod vertebrate.

We and other researchers reported that mouse ESCs and human induced pluripotent stem cells (iPSCs) are rich in

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5hmC; in particular, 5hmC accumulates at euchromatic regions in mouse ESCs, but is excluded from constitutive heterochromatic regions including pericentromeric regions and the Y chromosome (Ficz *et al.*, 2011; Kubiura *et al.*, 2012; Pastor *et al.*, 2011). 5hmC is also excluded from facultative heterochromatin, leading to the differential distribution of 5hmC between inactive and active X chromosomes in female somatic cells differentiated from human iPSCs (Kubiura *et al.*, 2012). Thus, 5hmC likely reflects not only previous methylation of cytosines, but also euchromatic regions and the cell's stemness.

In this study, to resolve the function of Tet enzymatic activity in other vertebrates, we first analyzed the relative amounts of 5mC and 5hmC by slot blotting of genomic DNA isolated from Japanese wrinkled frogs (*Rana rugosa*, Ranidae), and then analyzed the chromosomal distribution of 5mC and 5hmC by immunostaining. *R. rugosa*, which is widely distributed in Japan, exhibits local variation in sex chromosome constitution (Miura and Ogata, 2013): the population in northwestern Japan has a ZZ/ZW sex chromosome system, whereas the population in central Japan has an XX/XY sex chromosome system (Fig. 1A). All of the sex chromosomes are derived from an ancestral chromosome 7 (Miura and Ogata, 2013) (Fig. 1B). It is possible that 5mC and/or 5hmC might be differentially distributed between the homologous ZZ sex chromosomes in male frogs and between the homologous XX sex chromosomes in female frogs, and that this difference might be involved in the dosage compensation mechanism of these amphibian sex chromosomes. Moreover, as observed between the X and Y chromosomes in male mice, it is possible that global epigenetic differences may exist

between the X and Y chromosomes and/or the Z and W chromosomes in female frogs. However, the chromosomal distribution patterns of 5mC and 5hmC have not yet been analyzed in Japanese wrinkled frogs.

In this study, we analyzed mitotic frog chromosomes from peripheral blood samples, which were prepared by stimulation with phytohemagglutinin (PHA), which promotes agglutination of erythrocytes and acts as a mitogen toward lymphocytes (Goossens *et al.*, 1994) (Fig. 1C). A major difference between the blood of frogs and mice is that frogs possess nucleated red blood cells (NRBC), whereas mice possess only a few NRBCs as fetal-type erythroblasts. In confocal microscopic analyses, we were unable to detect any inter-chromosomal differences in immunostaining of 5mC and 5hmC between the Z and W or the X and Y, but we did observe a new phenomenon pertaining to Tet regulation: PHA treatment in *R. rugosa* induced extensive oxidation of 5mC in peripheral blood cells (PBCs) rich in NRBCs, and the oxidation product 5hmC was diluted in a cell cycle-dependent manner. 5hmC was enriched in embryonic cells in both mice and frogs. These results suggest that Tet activities are generally associated with global epigenetic reprogramming during dedifferentiation, showing that 5hmC can be used as a stemness-related marker in frogs.

## Materials and methods

### Preparation of mitotic chromosome spreads

Blood samples (80–100  $\mu$ l) were collected from ~10-year-old frogs of *R. rugosa*. Samples were mixed with 2 ml of RPMI 1640 medium, supplemented with 15% fetal

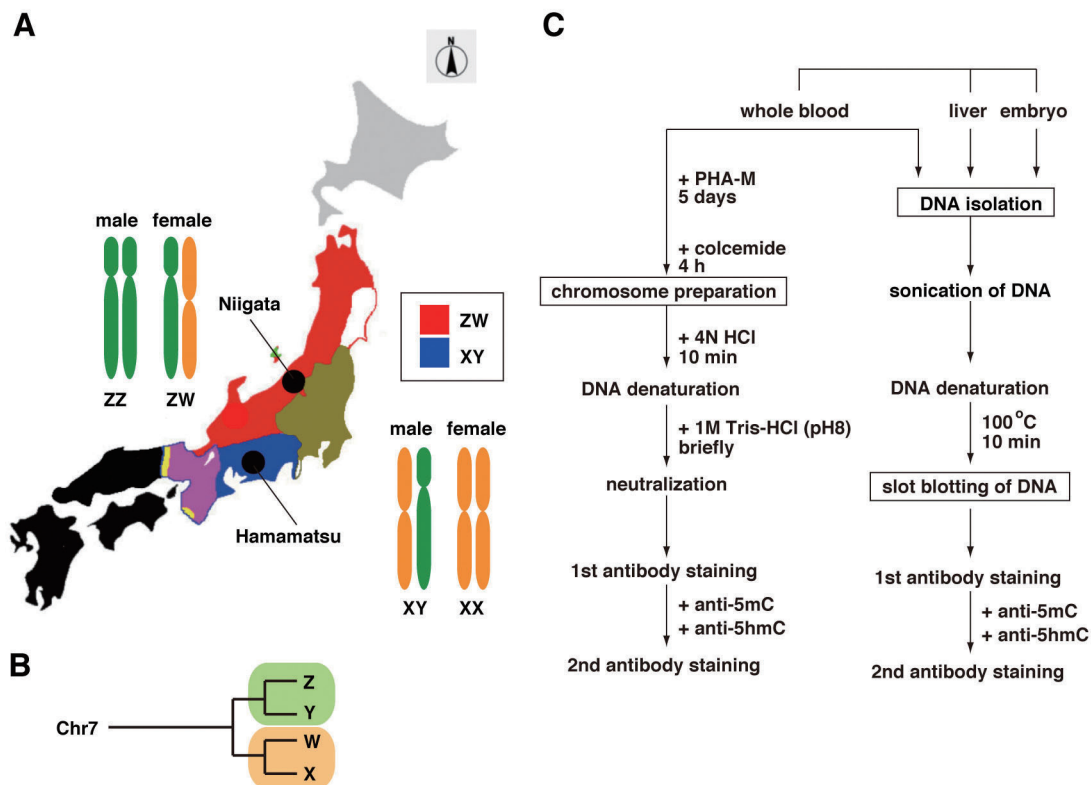


Figure 1. Intraspecific variation of sex chromosome constitution in the Japanese wrinkled frog *Rana rugosa*. **A**. Geographic distribution of two major groups: one located in the northwest of Japan (around Niigata), with a sex determination system of ZZ male and ZW female; and the other group located in central Japan (around Hamamatsu), with a sex determination system of XX female and XY male. **B**. ZW and XY sex determination systems evolved independently from an ancestral chromosome 7. **C**. Experimental schema of this study.

bovine serum, 1% penicillin/streptomycin, and 20 µg/ml of PHA-M, and then cultured for 5 days at 26°C. Most of the reagents were obtained from Gibco®/Life Technologies. Mitotic metaphases were enriched by 6 h treatment with 0.17 µg/ml of colcemide (demecolcine, Sigma-Aldrich) before harvesting as shown in Figure 1C. Cells were treated with 0.075 M KCl for 20 min at 20°C. Then, cells were fixed with 3:1 methanol:acetic acid. Chromosome spreads on glass slides were prepared by air-drying.

### **Immunostaining of chromosome spreads for 5mC and 5hmC**

Chromosomal DNA was denatured with 4 N HCl at room temperature for exactly 10 min, and then briefly neutralized in 1 M Tris-HCl (pH 8.0). Denatured chromosomes were treated with 0.1% Triton-X in PBS for 10 min at room temperature. After washing with PBS, they were treated for 30 min at room temperature with blocking solution consisting of 3% skim milk (Difco™, BD) in PBS. Primary antibodies were mouse monoclonal anti-5mC antibody (1:100, Active Motif) and rabbit polyclonal anti-5hmC antibody (1:500, Active Motif), followed by secondary antibodies, Alexa488-mouse IgG (1:500, Molecular probes®, Life Technologies) and Alexa546-rabbit IgG (1:500, Molecular Probes®, Life Technologies), respectively. All antibodies were diluted in blocking solution and treated for 1 h at room temperature. Samples were then washed three times in 0.05% Tween-20 in PBS (0.05% PBST). Finally, chromosomes were stained with ProLong® Gold Antifade Mount with DAPI (Molecular Probes®, Life Technologies). Immunostained chromosomes were observed on an LSM780 confocal microscope (Carl Zeiss).

### **Slot blotting of genomic DNA**

Genomic DNA samples were prepared from freshly isolated whole blood samples and liver tissues from adult frogs or embryonic tissues from 10-day-old feeding tadpoles according to a widely used procedure (Sambrook et al., 1989). Genomic DNA of the wild-type mouse ESC line J1 (WT ESC), derived from male blastocysts or adult mouse liver, was used as a positive control. Genomic DNA of Dnmt TKO ESCs was used as a negative control. Mouse ESCs were cultured as previously described (Kubiura et al., 2012). Fifteen micrograms of DNA from each sample was fragmented to around 500 bp using Bioruptor® (Cosmo bio Co., Ltd), and then 500 ng of fragmented DNA was incubated at 100°C for 10 min for each sample. After immediate cooling on ice, denatured DNA samples were transferred onto Hybond™-N<sup>+</sup> nylon membranes (GE Healthcare) using Bio-Dot® Microfiltration Apparatus (Bio-Rad Laboratories, Inc.). This apparatus allows spotting of many DNA samples onto a membrane at the same time without any loss of DNA, thereby enabling determination of the relative amounts of DNA samples loaded onto the same membrane. The pretreated membrane, presoaked with 6× SSC for at least 10 min, was laid over the gasket support. Next, the sample template, which enables easy application of multiple samples, was laid on top of the membrane. Five hundred microliters of Tris-EDTA (TE) buffer was applied to all of the sample slots and allowed to flow through completely. Next, 500 ng of denatured DNA samples was dissolved in 200 µl TE buffer and loaded into each slot of the sample template. A vacuum was then applied to the apparatus. After the sample filtered through,

500 µl of 2× SSC was applied to each slot and removed by vacuum. The blotted membrane was then rinsed in 2× SSC and baked at 80°C for 2 h.

### **Quantification of 5mC and 5hmC in genomic DNA**

Next, membranes were pre-treated with 3% skim milk in 0.1% PBST for 30 min at room temperature, and then incubated overnight at 4°C with primary antibody diluted in 3% skim milk in 0.1% PBST. The following primary antibodies were used: monoclonal anti-5mC antibody (1:5,000, Active Motif) or rabbit polyclonal anti-5hmC antibody (1:10,000, Active Motif), followed by secondary antibody treatment with HRP-mouse IgG (1:3,000, GE Healthcare) or HRP-rabbit IgG (1:5,000, GE Healthcare), respectively. Hybridized bands were visualized using the ECL Prime Western blotting detection kit (GE Healthcare). The relative densities of the signals were analyzed using an ImageQuant™ LAS-4000 mini imaging system (GE Healthcare). As an internal control, each value was standardized to the value obtained in mouse WT ESCs. Finally, the means ± SD of relative values for 5mC and 5hmC were compared between samples.

### **Statistical analyses**

The Mann-Whitney U-test was chosen because the sample size was limited, and it was not clear if these were samples taken from normally distributed data. As a convenient statistical method, Z values were used to determine significance. When the absolute value of the calculated Z score was greater than the absolute value of Z-critical (1.96), the difference was determined to be significant ( $P < 0.05$ ).

## **Results**

### **Amphibian RBCs are rich in 5mC but almost completely lack 5hmC**

First, because excess DNA reduces the reliability of luminometric quantitation, we determined the most appropriate volume of DNA per slot. To this end, we subjected six sets of serially diluted mouse DNA samples to slot-blot analysis for 5hmC. On the basis of this experiment, we decided that 500 ng of DNA per slot was optimal for luminometric quantitation (Fig. 2A). Caution is required when using immunostaining because anti-5mC antibody can visualize 5mC in each cell when 5mC accumulates above a threshold level on chromosomal regions. Thus, the chromosomal regions negative for 5mC signals do not always indicate unmodified regions. This is demonstrated by the fact that almost no 5mC signals were detected in WT ESCs by immunostaining (data not shown), but 5mC signals were detectable in slot-blot analyses using 500 ng of genomic DNA (Fig. 2B and D). No signal was detected in Dnmt TKO ESCs. Thus, in contrast to immunostaining methods, slot-blot analyses using 500 ng of fragmented DNA were sensitive enough to monitor global levels of 5mC. Our results clearly show that all frog DNA samples (blood samples, livers, and feeding tadpoles) contained 1.5–4-fold more 5mC than DNA from mouse WT ESCs ( $P < 0.05$ ).

The levels of 5hmC detected in frog embryos were similar to those of mouse WT ESCs. Moreover, as seen in mice, 5hmC was also detected in frog liver. 5hmC was absent in TKO ESCs, confirming the specificity of the 5hmC antibody used in this study. In slot-blot analyses,

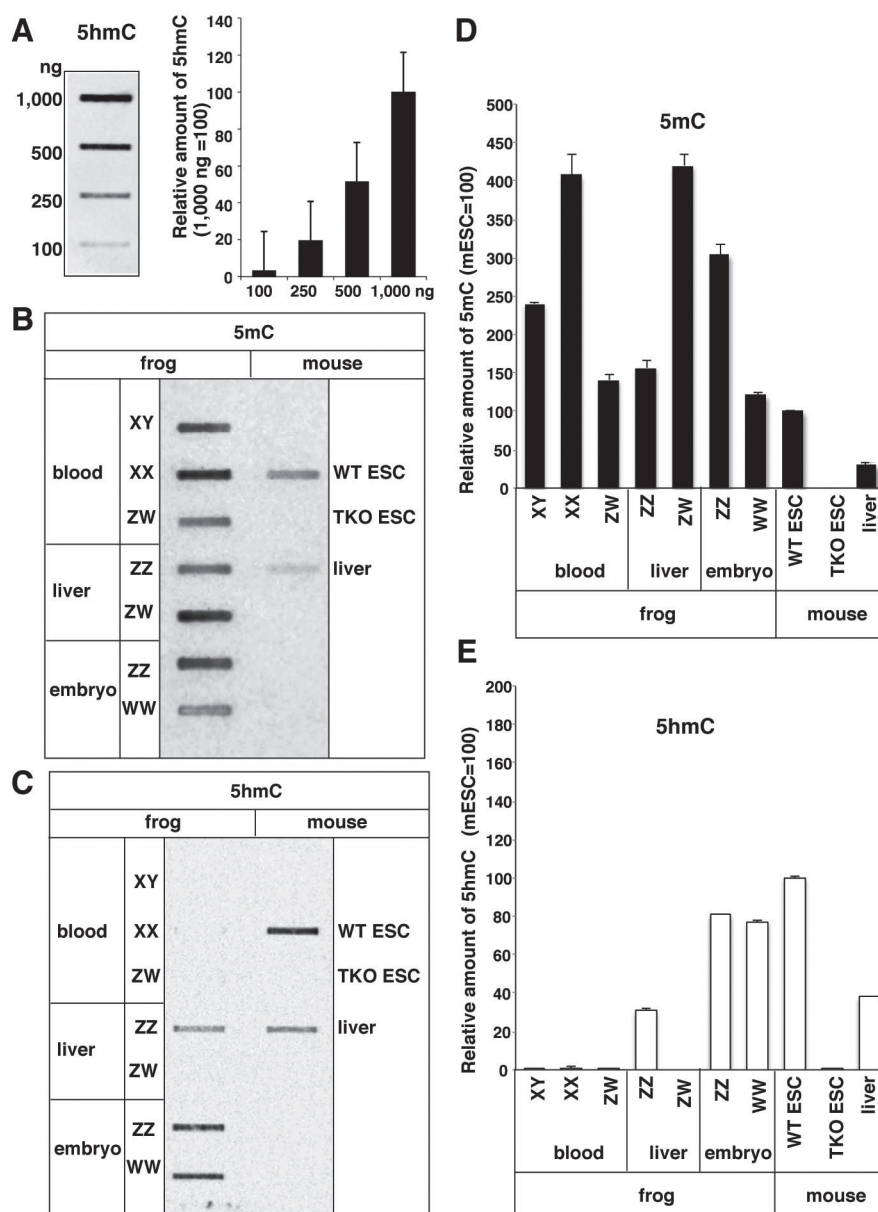


Figure 2. *Rana rugosa* genomes are rich in 5mC but almost entirely negative for 5hmC in peripheral blood. **A**. A representative slot-blot image for 5hmC, obtained using a serial dilution of mouse hepatocyte derived genomic DNA. The histogram shows the mean  $\pm$  standard deviation of the amount of DNA relative to the 1,000 ng standard (defined as 100), calculated from six sets of samples. Slot-blot analyses for 5mC (**B**) and 5hmC (**C**). Histograms for 5mC (**D**) and 5hmC (**E**) show the means  $\pm$  standard deviation, which were calculated from  $n = 3$  technical triplicates. Embryos, 10-day-old feeding tadpoles; WT ESC, wild-type mouse ESCs; TKO ESC, Dnmt triple-knockout mouse ESCs. Mann-Whitney U-test showed that every sample was significantly different from WT ESC ( $P < 0.05$ ).

we could not detect any 5hmC signals in PBC DNA from three individuals (Fig. 2C and E). These observations demonstrate that frog PBC has almost no mRNA, protein, or enzymatic activity of the Tet genes.

#### PHA-stimulated amphibian RBCs become rich in 5hmC

We stained mitotic chromosomes with anti-5mC and 5hmC antibodies in mitotic cells, which were transformed by stimulation with PHA-M for 5 days. Because frogs contain large numbers of NRBCs, it is not clear whether the mitotic cells were lymphoid cells or erythroid cells. We expected from slot-blot data that frog chromosomes derived from PBCs would be rich in 5mC, but less rich

in 5hmC. Surprisingly, although 5hmC was not detected in frog blood by slot blotting in the absence of PHA-M treatment, abundant 5hmC was detected in all frog chromosomes (Figs 3 and 4).

#### No obvious difference was detected among Z, W, X, and Y in chromosomal distribution pattern for 5mC and 5hmC

Next, we analyzed the distribution patterns of 5mC and 5hmC on sex chromosomes in ZZ male cells (Fig. 3A) and ZW female cells (Fig. 3B) in order to find inter-chromosomal differences between the two Z chromosomes and between Z and W, respectively, by immunostaining. These frogs originated in Niigata prefecture, Japan (Fig.



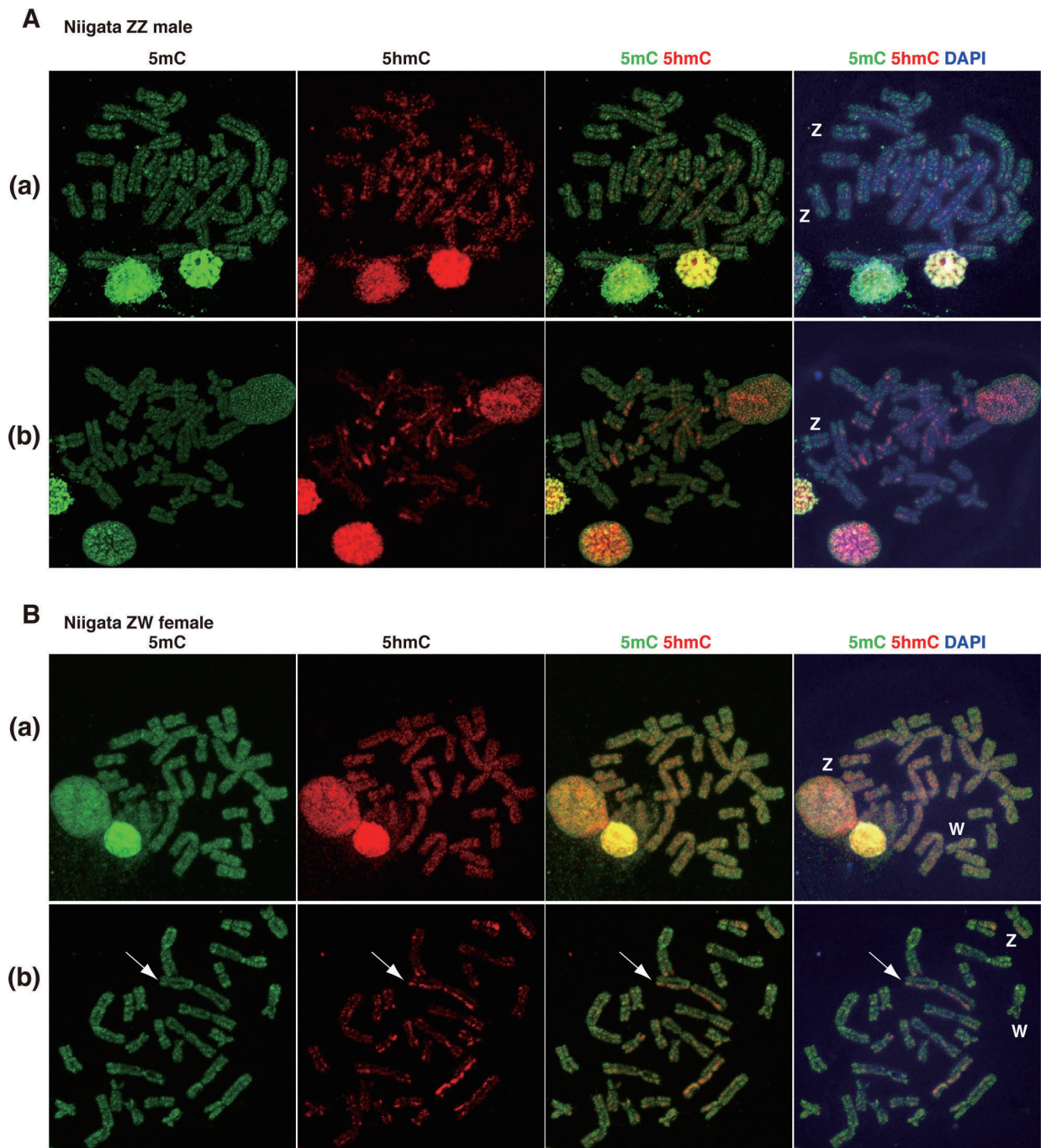


Figure 3. Chromosomal localization of 5mC and 5hmC in PHA-stimulated PBCs from ZZ and ZW frogs. (A) ZZ male cells and (B) ZW female cells from the Niigata population. Two sister chromatids are stained in a similar fashion with anti-5hmC antibody in (a), but differentially stained in (b).

1A, the northwest of Japan). However, the Z chromosome exhibited behavior similar to that of the W, as well as the autosomes, in each cell (Fig. 3A(a) and B(a)). Similarly, we could not detect any differences between the two X chromosomes in XX female cells or between the X and Y in XY male cells (Fig. 4A(a) and B(a)) from a domestic population. These frogs originated in Hamamatsu prefecture, Japan (Fig. 1A, central Japan). More importantly, the 5hmC signals localized on one side of two sister chromatids in many mitotic frog cells from ZZ, ZW, XX, and XY blood samples (Figs 3A(b), 3B(b), 4A(b), and 4B(b)).

Taken together with the slot-blotting data, these results

suggest that oxidation of 5mC is initiated synchronously when cells are treated with PHA, and that the oxidation product 5hmC is diluted in a cell cycle-dependent manner through at least two cell cycles (Fig. 5). This may be due to a transient activation of Tet enzymes and the unique property of 5hmC that prevents Dnmt1 target recognition (Valinluck and Sowers, 2007), leading to passive loss of 5hmC through cell division.

## Discussion

Here, we demonstrated that the frog *R. rugosa* possesses a 5mC oxidation system. The oxidized product 5hmC was

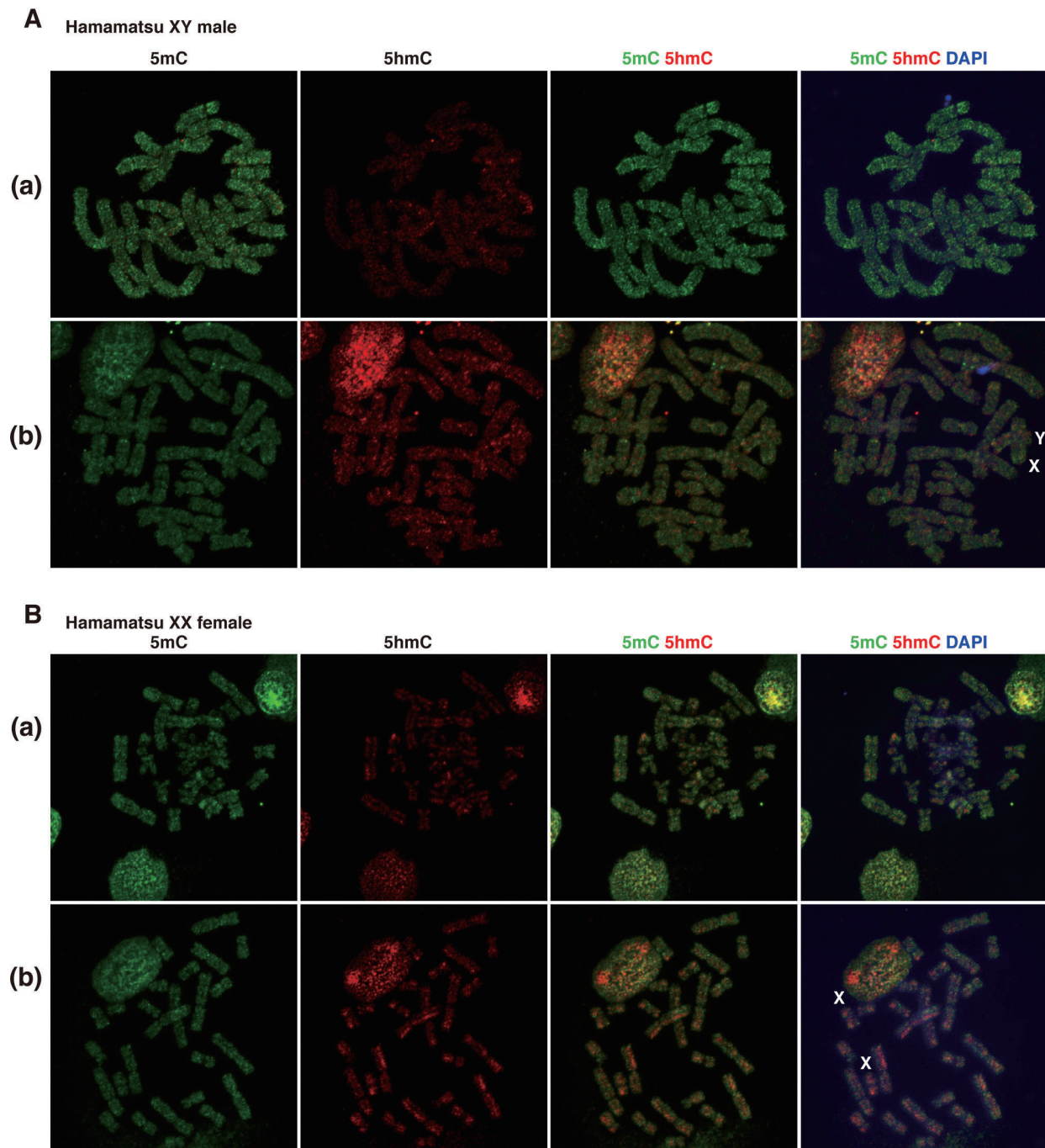


Figure 4. Chromosomal localization of 5mC and 5hmC in PHA-stimulated PBCs from XX and XY frogs. (A) XY male cells and (B) XX female cells from the Hamamatsu population. Two sister chromatids are stained in similar fashion with anti-5hmC antibody in (a), but they are differentially stained in (b).

detectable in frog embryos, but less obvious in somatic cells, as seen in mice. Although mammalian erythrocytes do not possess a nucleus, a variety of vertebrate species possesses nuclei in their red blood cells. Slot-blot analyses revealed that genomic DNA of PBCs was rich in 5mC, but almost completely lacked 5hmC. Immunostaining of frog chromosomes revealed that 5mC throughout the genome is converted to 5hmC in PHA-stimulated PBCs. Next, the oxidized product of 5mC becomes diluted in a cell cycle-dependent manner. These evidences show that PHA likely initiates dedifferentiation of a type of frog PBC through genome-wide epigenetic reprogramming.

5hmC is present in all types of mouse somatic cells at

levels between 0.03% and 0.69% of dG, with the highest concentrations in neuronal cells of the central nervous system and lower concentrations in several tissues, including the liver (Golobisch, D *et al.*, 2010). Moreover, Tet1 and Tet2 are highly expressed under the control of the pluripotent cell-specific transcriptional factor Oct4, leading to higher expression in the germline (Sohni, A. *et al.*, 2015), lower Tet activity in somatic tissues, and age-dependent differences in 5hmC levels among individuals. In this study, we used wild frogs, in which we expect considerable differences in age and health conditions among specimens because their feeding and breeding environments have not been strictly regulated. Therefore, although 5hmC in liver



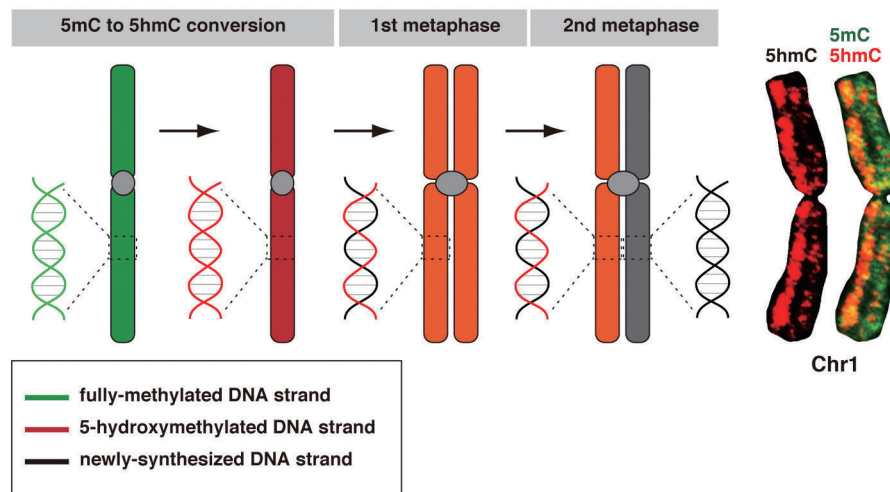


Figure 5. A possible course of cell cycle-dependent dilution of 5hmC in PHA-stimulated PBCs. Changes in a major DNA modification are speculated from mitotic chromosomes, each of which contains two sister chromatids differentially stained with anti-5hmC antibody: one is positive, but the other is negative for 5hmC. Chr1 is shown as a typical autosome seen in a ZW cell of *Rana rugosa* (arrow in Fig. 3B(b)). To obtain this staining pattern, DNA modification status changes from 5mC (green) to unmodified cytosine (gray) through 5hmC (red), followed by hemi-5hmC (orange).

samples was detected only in a ZZ frog, but not in a ZW female (Fig. 2C and E), this may reflect variation among individuals (as also observed in mice). It is possible that 5hmC signals may partly reflect proliferation of hepatic progenitors during hepatic regeneration. Orthologs of Tet2 and Tet3 were found in amphibian genomes, but Tet1 has not yet been detected: Tet3 is expressed from the gastrulation stage in various adult tissues, whereas Tet2 is enriched in neural tissues in *Xenopus tropicalis* (Almeida *et al.*, 2012). Thus, in embryonic and somatic tissues of *R. rugosa*, 5hmC may mostly be the product of Tet3-mediated oxidation. Tet3 is indispensable for normal embryonic development in African clawed frogs (*Xenopus laevis*); in particular, Tet3 regulates target gene activities for eye and neural development *via* CXXC domain-mediated binding to the genome, as well as *via* its enzymatic activity (Xu *et al.*, 2012). Tet2 negatively regulates self-renewal and long-term repopulation capacity of hematopoietic stem cells (HSCs), whereas Tet2 positively regulates the differentiation of mature blood cells for hematopoiesis in both fetal liver and adult bone marrow (Kunimoto *et al.*, 2012). Although it is still uncertain which Tet enzymes are involved, it is certain that Tet enzymes are involved in PHA-stimulated transformation of adult PBCs from a quiescent state to a mitotically active state, whether those mitotic cells are erythroid cells, lymphoid cells, fetal-type hepatic precursors, adult HSCs, or other types.

Mammalian cells contain an active X chromosome in male cells, whereas two X chromosomes are present in female cells. Dosage compensation system between male and female cells is evolutionarily established in addition to morphological divergence between sex chromosomes. In mammalian XX female somatic cells, only one X chromosome is activated. In this study, we could not detect an obvious difference between sex chromosomes (Z, W, X, or Y) and autosomes in PHA-stimulated frog PBCs. This might indicate that not much genetic divergence has yet accumulated between the Z and W and the X and Y, although these chromosomes are all derived from an ancestral chromosome 7, and none of them is genetically

identical (Miura *et al.*, 2012; Uno *et al.*, 2008). However, further precise analyses will be required to reach a conclusion.

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