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ORIGINAL ARTICLE

# Changes in the position and volume of inactive X chromosomes during the G0/G1 transition

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**Abstract** In female mammals, each cell silences one X chromosome by converting it into transcriptionally inert heterochromatin. The inactivation is concomitant with epigenetic changes including methylation of specific histone residues and incorporation of macroH2A. Such epigenetic changes may exert influence on the positioning of the inactive X chromosome (Xi) within the nucleus beyond the level of chromatin structure. However, the dynamic positioning of the inactive X chromosome during cell cycle remains unclear. Here, we show that H3K27me3 is a cell-cycle-independent marker for the inactivated X chromosomes in WI38 cells. By utilizing this marker, three types of Xi locations in the nuclei are classified, which are envelope position (associated with envelope), mid-position (between the envelope and nucleolus), and nucleolus position (associated with the nucleolus). Moreover, serial-section analysis revealed that the inactive X chromosomes in the mid-position appear to be sparser and less condensed than those associated with the nuclear envelope or nucleolus. During the transition from G0 to G1 phase, the inactive X chromosomes tend to move from the envelope position to the nucleolus position in WI38 cells. Our results imply a role of chromosome positioning in maintaining the organization of the inactive X chromosomes in different cell phases.

**Keywords** X chromosome inactivation · dimethylated histone H3 on lysine 9 (H3K9me2) · trimethylated histone H3 on lysine 27 (H3K27me3) · Xi movement · G0/G1 transition

Guoliang Lyu and Tan Tan contributed equally to this work.

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## Introduction

In female mammals, one of the two X chromosomes is converted from active euchromatin into transcriptionally inert heterochromatin, so-called inactive X chromosome (Xi) or Barr body (Boumil and Lee 2001; Lyon 1972, 2002). In the context of cell differentiation, the X chromosome to be inactivated undergoes heterochromatinization with the recruitment of epigenetic enzymes, resulting in

deacetylation and methylation of specific histone residues, the accumulation of histone variant macroH2A, as well as DNA methylation (Csankovszki et al. 2001; Li et al. 2007). During this process, two important modifications, H3K9me2 and H3K27me3, are established on the Xi in a manner dependent on the transient recruitment of Eed-Enx1 polycomb group (PcG) complexes (Silva et al. 2003), which are thought to be crucial early markers of the inactive X chromosome (Heard et al. 2001; Mermoud et al. 2002; Plath et al. 2003). The X chromosome inactivation also accompanies with gene silencing and some gene reactivations may be associated with disease (Pinheiro and Heard 2017; Robert Finestra and Gribnau 2017).

The location of X chromosomes affects the process of X chromosome inactivation, during which X-X pairing and the transient co-localization of XICs (X-inactivation centers) are the prerequisite step that is influenced by X chromosome positioning (Bacher et al. 2006; Heride et al. 2010; Xu et al. 2006). Moreover, nuclear compartmentalization of the inactive X chromosome also contributes to its inactivation maintenance as a distinct entity from the activated X chromosome (Xa) (Cremer et al. 2006; Smeets et al. 2014), and the surrounding factors and the surface area are significantly different between Xi and Xa territories (Eils et al. 1996). Light and electron microscopic analyses demonstrate extensive contact between Xis and the nuclear envelope or nucleolus (Rego et al. 2008). Specifically, during the mid-to-late S phase of cell cycle, 80–90% of Xis (note: the plural of 'Xi') are associated with the nucleolus and reside within a Snf2h-enriched ring (Zhang et al. 2007). However, the behavior of the Xi during the G0/G1 transition remains unclear.

In this study, we explored the behavior of the Xi during the cell phase transition using immunofluorescence of H3K27me3 as the marker, and found that the inactive X chromosomes tend to move from the envelope to the nucleolus in WI-38 cells during the G0/G1 transition.

## Materials and methods

### Cell culture, reagents, and antibodies

WI38 cells were cultured in minimum essential medium (MEM; Invitrogen, USA) with 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 10% (*v*/*v*) fetal bovine serum (FBS; Invitrogen, USA) at 37 °C in the presence of 5% $CO_2$. Confluent WI38 cells in a cumulative population that doubled 20 to 40 times were used 10 days after plating. Reagents used in this study included anti-H3K9me2 antibodies (Upstate Biotechnology, USA, Cat. No. 05-768R), anti-H3K27me3 antibodies (Upstate Biotechnology, USA, Cat. No. 05-1951), anti-macroH2A antibodies (Santa Cruz Biotechnology, USA, Cat. No. sc-161812), BrdU antibody (Sigma, USA. Cat. No. B8434), goat anti-rabbit and goat anti-mouse IgG conjugates with FITC/rhodamine (both from Vector Laboratories, USA), and diamidino-2-phenylindole (DAPI) (Sigma, USA).

### Cell transfection assay

Recombinant vector pDsRed2-N1-fibrillarin was transiently transfected into WI38 cells using the high-efficiency transfection reagent Fugene6 according to the manufacturer's instructions (Roche, USA). At 40 hrs after transfection, the cells were plated in delta-T dishes (Biotechs, USA) or coverslips and cultured for 10 days, after which they were fixed for immunostaining or observation of living cells.

### Cell culture and synchronization

To synchronize WI38 cells at the G0 phase, cells were seeded at a density of $2 \times 10^4/cm^2$ in flasks and cultivated without any change of medium for 10 days, after which they were replanted at a density of $1600/cm^2$ by adding low serum (0.03%) medium. After 10 days, the cells were arrested at the G0 phase. After replacing the medium with normal medium containing 10% serum, the cells entered the G1 phase. For the BrdU assay, 10 μM of BrdU was added to the media for 24 hrs incubation, and then, the media were replaced with the normal media containing 10% FBS. The cells were fixed and subjected to be immunostained at the indicated time points.

### Indirect immunofluorescence

Cells grown on coverslips were washed three times with PBS and fixed with 1.6% paraformaldehyde at room temperature (RT) for 10 min, followed by permeabilization with 0.1% Triton X-100 PBS (RT, 10 min). After washing the cells three times with PBS containing 0.1% Triton X-100 and 20 mM glycine, the coverslips were blocked with normal goat serum (containing 5%

donkey serum) in PBS (RT, 10 min), followed by incubation with primary antibodies (dilutions varied) for 6 hrs at RT. The cells were washed three times (5 min each) in PBS containing 0.1% Triton X-100 and incubated for 2 hrs at RT with secondary antibodies. Finally, the cells were washed three times in PBS containing 0.1% Triton X-100 and counterstained with 10 μg/mL DAPI.

### Microscopy and image analysis

Images of fluorescent cell nuclei (H3K27me3, MacroH2A, DAPI and BrdU staining) were collected using a Zeiss LSM700 laser scan confocal microscope equipped with a 100× Plan-Neofluar oil immersion lens (NA = 1.30). Laser power was adjusted to maximize the dynamic range of each sample. For samples with multiple stains, the adjustable fluorescence spectral window was set with a single-color control sample for each channel to avoid cross-contamination among channels. Serial optical sectioning from top to bottom along the *z*-axis (Z-stack, at least 10 slides) was applied to constitute the confocal slices (at least 10 slides) of the nuclei. We optimized the acquired fluorescent signals with the "optimal" action after setting up the "last" and "first" position. The images shown in figures were derived from the maximum projection of a series of serial sections. Image files were adjusted for brightness/contrast, superimposed, pseudocolored, and assembled using Adobe Photoshop CS 8.01 software. The volume of Barr body means the collection of H3K27me3 fluorescent territory. The Barr body was 3-D reconstructed with full projections. As for reconstruction of 3-D image, the depth was kept at the same level among the different acquisition channels. The fluorescent territory was calculated by the workstation (ZEN light edition, Carl Zeiss, Inc., Germany). The formula for calculating the volume of Barr body shows as follows:

$$V = \frac{4}{3} \times \pi \times \left(\frac{\sqrt{S}}{2}\right)^3$$

*V* means the volume of Barr body, and *S* means the fluorescent area of Barr body. The fluorescent area of Barr body was from the middle slide. For example, if the Z-stack was separated into 10 slides, we used the fifth or sixth slide to measure the fluorescent area.

### Statistical analysis

The proportion of three types of cells with different condensed statuses as indicated time point, the proportion of cells marked by H3K27me3 or MacroH2A, the volumes of Barr body at three types of positions, and the proportions of cells with different Barr body positions during G0/G1 transition were all analyzed by independent sample *t* tests. All the indicated experiments were replicated three times.

## Results

### The confluent WI38 cells with a clear Xi are at a higher proportion

Using DAPI staining, we observed three types of X chromatin with different condensation statuses in confluent human female primary fibroblasts WI38 cells, an obvious Xi in the nucleus, an less obvious Xi, and the sparsely condensed Xi chromatin scattering in the nucleus, which are designated as a clear-cut (CC) Xi, an unclear/decondensed chromatin (U/DC) Xi, and an unclear/condensed chromatin (U/CC) Xi, respectively (Fig. 1a). Moreover, we found that the percentage of CC-type Xi displayed a decreasing trend in confluent cells from 10 to 20 days, whereas the percentages of U/DC-type Xi and U/CC-type Xi showed an increasing trend (Fig. 1b). Therefore, we chose the 10-day-confluent WI38 cells for further assays.

### H3K27me3 is a cell-cycle-independent marker for Xi in WI38 cells

Although the 10-day-confluent WI38 cells stained by DAPI showed a higher percentage of cells with obvious Xis (Fig. 1), a specific marker was needed for studying the behavior of the Xi during cell cycle, since DAPI stained all the chromosomes, which is not specific for the Xi. To test the specific marker for Xi in WI-38 cells, we stained the 10-day-confluent WI38 cells with anti-trimethylated histone H3K27 (H3K27me3) or anti-macroH2A antibodies (Fig. 2a). Statistical analysis of the immunofluorescent signals indicated that nearly 100% of Xis were marked by H3K27me3 and the signal displayed as a discrete, unique, high-contrast stained body in the cells (Fig. 2a, b), while macroH2A, previously regarded as an Xi marker (Chadwick and Willard

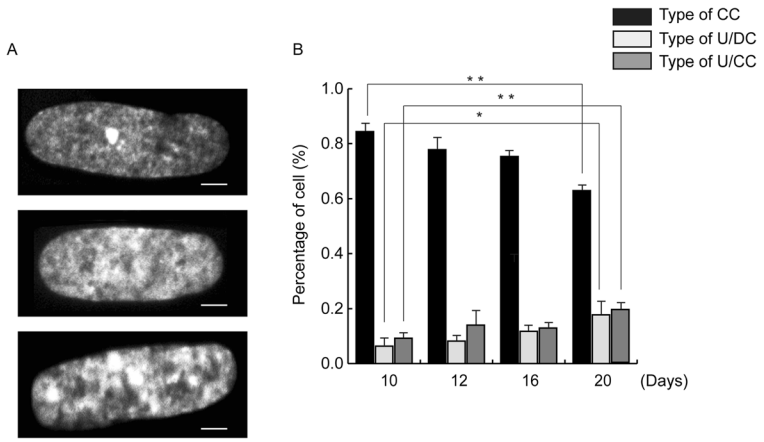



**Fig. 1** The confluent WI38 cells have clear Xi at a high proportion. **a** Three types of X chromatin with different condensation status in WI38 cells. Top: A Barr body in a nucleus (CC type). Middle: No obvious condensed chromatin and Barr body in the nucleus (U/DC type). Bottom: No obvious Barr body, but chromatin irregularly condensed in the nucleus (U/CC type). The bar represents 2 μm. **b** Calculating the proportions of three types of cells as indicated in (**a**) after 10-days plating. The proportion of cells with each type of chromatin condensation status at 20 days was significantly different from that measured at 10 days (double asterisks indicate $P < 0.01$, single asterisks indicate $P < 0.05$). The error bars indicate the s.d. More than 200 cells were used for the statistical analyses at each time point

2002; Costanzi and Pehrson 1998; Costanzi et al. 2000; Li et al. 2007; Rasmussen et al. 2000), is less specific than H3K27me3 for labelling the Xi in WI38 cells (Fig. 2a, b). When WI38 cells were synchronized at various stages of division and then stained with the anti-H3K27me3 antibodies, we found that distinct signals of the Xi not only displayed in interphase but also in the G2 phase, prophase, metaphase, and telophase (Fig. 2c), indicating that H3K27me3 is a cell-cycle-independent specific marker for Xi. In contrast, the signals stained by the H3K9me2 antibodies did not overlap well with the Xi (Fig. S1), which is consistent with our previous findings (Li et al. 2012). Therefore, we used H3K27me3 as the marker of the Xi to study its behavior in WI38 cells.

## WI38 cells have three types of Xi location

After staining with DAPI and the anti-H3K27me3 antibodies, it seems that there are three types of Xi positions, i.e., associated with the envelope (envelope position), between the envelope and the nucleolus (mid-position), and associated with the nucleolus (nucleolus position; Fig. 3a). However, one cell usually has several nucleoli, including the mini-nucleolus in mammals. To further confirm Xi positions, we transfected WI38 cells with RFP-tagged fibrillarin that marks the nucleoli, followed by immunostaining of the Xi with the anti-H3K27me3 antibodies (Fig. 3b). The results showed that WI38 cells had three types of Xi location in the nucleus, indicating a dynamic positioning of Xi.

## The Xi with different locations has different volumes

We noticed that the Xi with different locations might have different volumes. Next, we found that Xi with the envelope position occupied less area, while that with the mid-position showed a relatively larger volume, which means that the Xi was more de-condensed (Fig. 4a). To avoid the effect of fibrillarin transfection on Barr body, we further measured the volume of Xi with the three types of positions by immunofluorescent staining of only H3K27me3 (Fig. 4b). The results confirmed that the volume of the Xi in the nucleolus position was smaller than that in the mid-position and larger than that in the envelope position (Fig. 4c). We also measured the fluorescent intensity of Xis in three types of position and found that the H3K27me3 signals of the mid-position's Xi were weaker than that of the other two types of Xi (Fig. 4d). These data imply that the condensation status of the Xi in the nucleus is related to its location.

## The Xi tends to move from the envelope to the nucleolus during the G0/G1 phase transition

To explore the changes of the Xi position during G0/G1 phase transition progress, we firstly synchronized the 10-day-confluent WI38 cells in G0 phase by serum

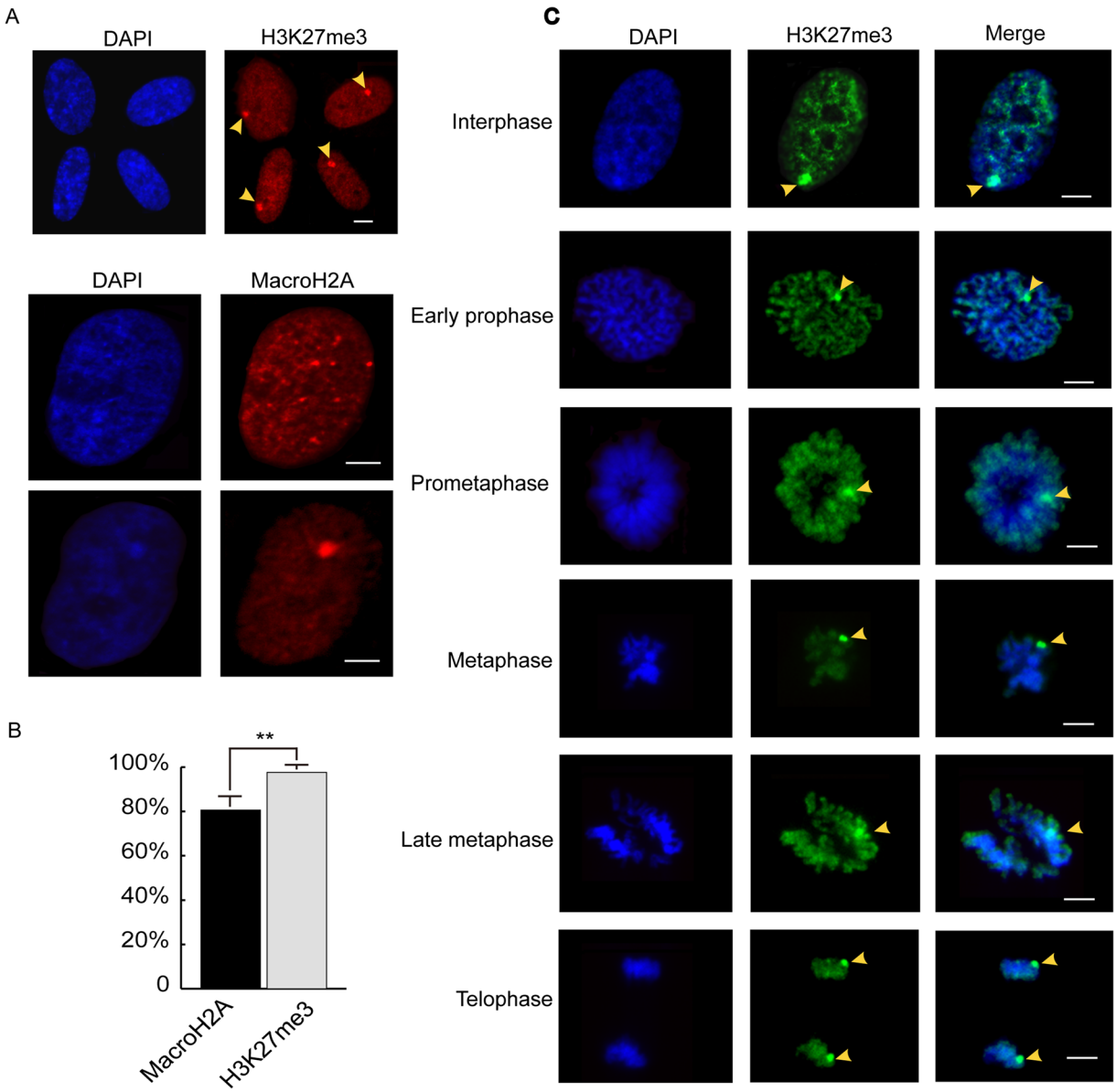



**Fig. 2** H3K27me3 is a cell-cycle-independent marker for Xi in WI38 cells. **a** Top: WI38 cells were stained by DAPI (blue) and anti-H3K27me3 antibodies (red). Bottom: WI38 cells were stained by DAPI (blue) and anti-MacroH2A antibodies (red). The bar presents 2 μm. The arrowheads show Barr bodies. **b** Calculating the proportions of WI38 cells marked by H3K27me3 or MacroH2A in random cell phase. More than 200 cells were used for the statistical analyses. The proportion of WI38 cells marked by H3K27me3 is more than MacroH2A (double asterisks indicate $P < 0.01$). The error bars indicate the s.d. **c** H3K27me3 can serve as a marker of a Barr body-like chromatin structure during mitotic phases. The Barr body-like chromatin structure was stained by anti-H3K27me3 antibodies (green) and DNA was stained by DAPI (blue). The bar represents 2 μm. The arrowheads show Barr bodies

starvation, and then re-feeding the cells with normal media to induce the G0/G1 phase cell transition. After DAPI staining, we found that the number of nuclei with de-condensed chromatin and no obvious Xis increased dramatically from the beginning of serum stimulation, peaked at 30% after stimulating for 2 hrs, and then slowly decreased until 6 hrs to become to be nearly the same as the beginning of the stimulation, whereas the percentage of nuclei containing distinct Xis showed an opposite pattern (Fig. 5a). Moreover, the proportion of

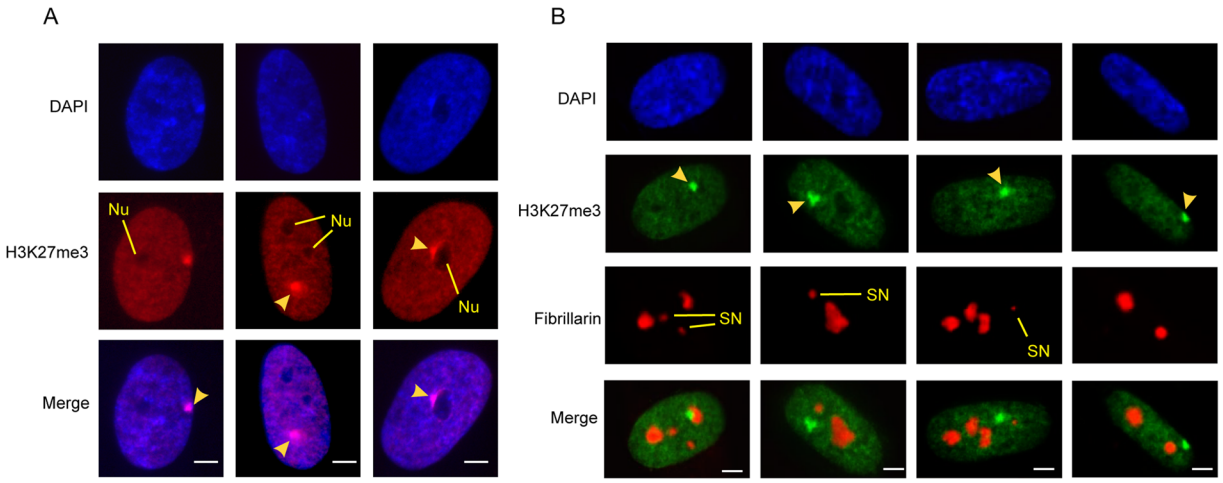


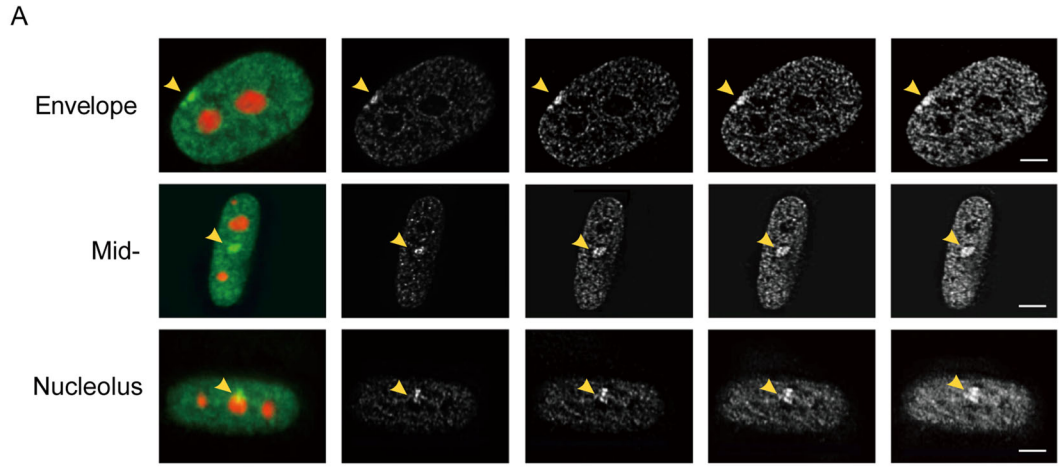

**Fig. 3** WI38 cells have three types of Xi location. **a** Confirmation of Barr body position in the nucleus. WI38 cells were stained by anti-H3K27me3 antibodies (red) and DAPI (blue). First lane: The Barr body is associated with the envelope (envelope position); Second lane: The Barr body is between the envelope and the nucleolus (mid-position); Third lane: The Barr body is associated with the nucleolus (nucleolus position). The bar represents 2 μm. The arrowheads show Barr bodies. "Nu" stands for nucleolus. **b** RFP-fibrillarin-transfected WI38 cells were stained by anti-H3K27me3 antibodies (green), and DAPI (blue). The bar represents 2 μm. The arrowheads show Barr bodies. "SN" stands for small nucleolus

chromatin de-condensed cells with distinct Xis remained stable after 6 hrs. These results implied that the condensation status of Xis in WI38 cells possibly change during G0/G1 phase transition and this process lasts about 6 hrs.

Next, we analyzed the changes of the Xi position after stimulating the G0 phase cells entering into G1 phase by labelling the Xis with anti-H3K27me3 antibodies. Interestingly, the proportion of the mid-position Xis increased after serum stimulation for 2 hrs, reached approximately 30%, and then gradually declined. Simultaneously, the proportion of Xis at the envelope position decreased after 4 hrs serum stimulation, while the proportion of Xis associated with the nucleolus increased (Fig. 5b). These results indicate that the Xi tends to move from the envelope to the nucleolus during the G0/G1 phase transition.

In addition, we also investigated the relationship between the volume of Xi and its position during the G0/G1 transition. Two hours after stimulation with serum, the volume of the mid-position Xis marked by H3K27me3 began to increase, and then gradually decreased until to 6 hrs (Fig. 5c). This is coincident with the change of the proportion of the mid-position Xis in the phase transition process (Fig. 5b). Besides, the volume of the Xis in the envelope position showed changes similar to the percentage change of the mid-position Xi over time (Fig. 5b, c). We also used BrdU staining to check the DNA synthesis capacity of the cells and categorized the cells into different cell cycle phases by analyzing the BrdU intensity. The results showed that the low BrdU intensity in cells represented that the cells were in G0 or G1 phase, while the high BrdU intensity indicated that cells were in S phase (Fig. 5d, e). Taken together, these results suggest that the Xis have a tendency to move from the envelope to the nucleolus during the G0-to G1-phase transition which is accompanied by the volume change.

**Fig. 4** The Xi with different locations has different volumes. **a** ▶ RFP-fibrillarin-transfected WI38 cells were stained by anti-H3K27me3 antibodies (green) and DAPI (blue). Three types of Xi location, e.g., envelope position, mid-position, and nucleolus position were visualized by confocal microscope with selected Z-stack of slides. The bar represents 2 μm. The arrowheads show Barr bodies. **b** WI38 cells were with stained by anti-H3K27me3 antibodies and further visualized by confocal microscope with overlapping the fluorescence signaling from all the cell levels. The bar represents 2 μm. **c** Statistical analysis of Barr body volume at three types of positions based on (**b**). The *Y*-axis represented the proportion of each volume of nuclei that was marked by H3K27me3. More than 200 cells were assessed in three experimental replicates. Double asterisks indicate $P < 0.01$; single asterisk indicates $P < 0.05$. The error bars indicate the s.d. **d** Statistical analysis of Barr body's fluorescent intensity at three types of positions was based on (**b**). The *Y*-axis represented the relative fluorescent intensity of Xis that was marked by H3K27me3. Two hundred cells were assessed in three experimental replicates. Double asterisks indicate $P < 0.01$. The error bars indicated the s.d.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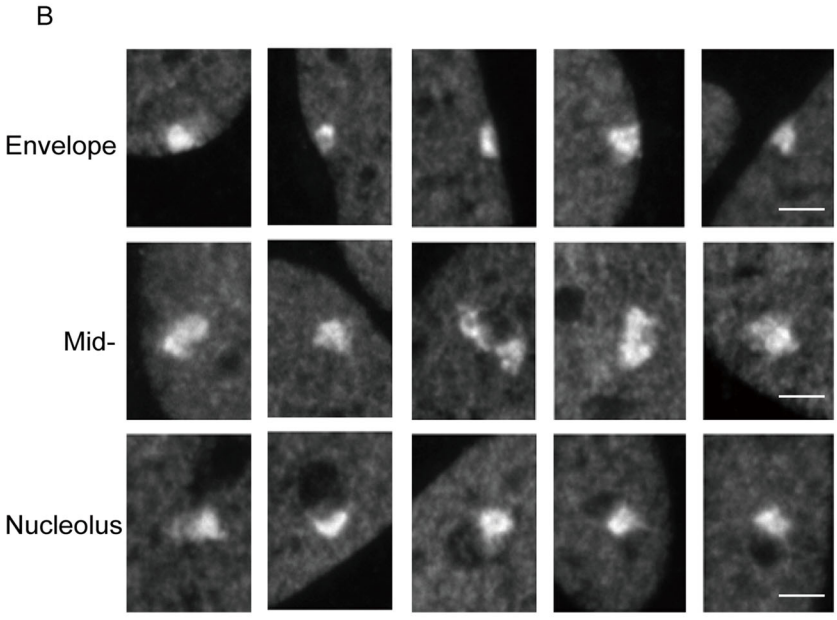
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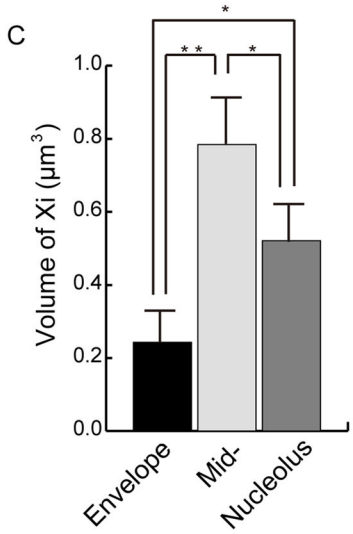
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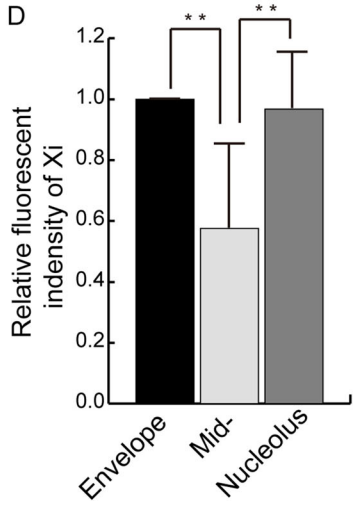
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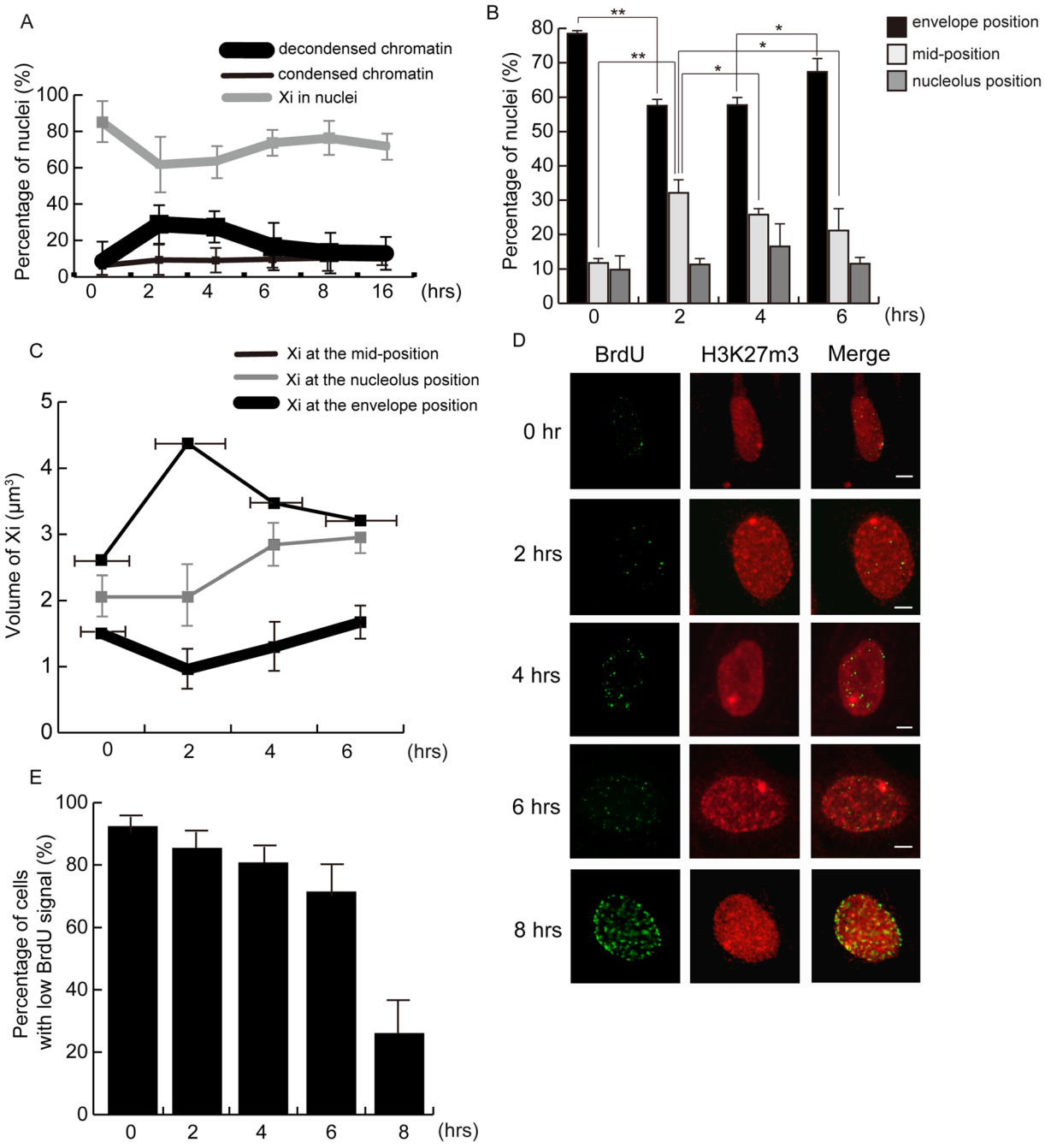


## Discussion

In this study, we showed that H3K27me3 is a stable epigenetic marker of the Xi throughout the cell cycle in WI38 cells, while MacroH2A is not (Fig. 2b, c), consistent with a previous finding that the localization pattern of macroH2A in chromatin is cell-cycle dependent, and cells lose their macrochromatin body in certain cell phases (Chadwick and Willard 2002). H3K9me2 is thought to be an early marker of the Xi that is established after Xist RNA coating (Heard et al. 2001), and we found here that it is not an obvious Xi marker in

◀ **Fig. 5** The Xi tends to move from the envelope to the nucleolus during the G0/G1 phase transition. **a** Statistical analysis of three types of Barr body statuses after stimulating cells with serum. More than 200 cells were used for the statistical analyses at each time point. **b** Change of Barr body location from the G0 to G1 phase. Simultaneous analysis of three types of Barr body positions was performed after stimulating cells with serum. The proportions of cells with each of the three types of Barr body positions were significantly different between the indicated two time points. Double asterisks indicate $P < 0.01$; single asterisk indicates $P < 0.05$. The error bars indicate s.d. More than 200 cells were used for the statistical analyses at each time point. The experiments were replicated three times. **c** Simultaneous analysis of Barr body volume at each of the three types of positions over a time course. More than 200 cells were assessed in each experiment. The error bars indicate the s.d. **d** The WI38 cells were stained by BrdU (green) and anti-H3K27me3 antibodies (red) at indicated time point. The bar represents 2 μm. **e** Statistical analysis of the proportion of the cells with low BrdU intensity was based on (**d**). When the BrdU intensity was less than 10% of the total H3K27me3 intensity in the same cell, this cell was defined as a low BrdU intensity cell. More than 200 cells were used for the statistical analyses at each time point. The experiments were replicated three times. The error bars indicated the s.d.

WI38 cells; H3K9me2 does not overlap well with Xis, supporting the idea that H3K9me2 is widely distributed throughout the genome (Chadwick and Willard 2004; Rougeulle et al. 2004; Silva et al. 2003). Thus, H3K27me3 is a highly specific marker for the Xi chromosome in WI38 cells. The polycomb repressive complex 2 (PRC2), recruited with XIST, methylates histone H3 at lysine 27 (Conway et al. 2015) and LncRNA Firre and its binding protein, CTCF, are also required for maintaining H3K27me3 when a Barr body associates with a nucleolus (Yang et al. 2015).

Human Xi is capable of forming a heterochromatic body that exists at the periphery of the nucleus or nucleolus (Barr and Bertram 1949), and the perinuclear association is relatively constant in comparison with the perinucleolar association (Zhang et al. 2007). Here, we showed three types of Xi localization in WI38 cells, including a position between the envelope and the nucleolus, which has not been reported previously. Notably, the number of Xis at the mid-position (between the nucleolus and envelope) increased for 2 hrs after stimulating G0 phase cells with serum. The nucleolus-positioned Xis also showed a significant increase with a corresponding decrease of Xis associated with the nuclear envelope. The change in the number of Xis in different positions indicates that the Xis possibly moved from the envelope position to the nucleolus during the G0/G1 transition (Fig. 6). It is likely that the Xi prefers to contact the nucleolus during the transition, which may serve as a mediator that plays a role in maintaining gene silencing and the heterochromatin state of the Xi.

Along with this altered condensation status, the volume of the Xi also changed as the Xi moved from an envelope-associated position to a nucleolus-associated position. In comparison with Xis located close to the envelope or nucleolus, Xis located between the envelope and nucleolus were larger in volume, suggesting that the Xis have a relatively loose chromatin structure when leaving from the envelope. Given that the inactivation-escaping genes on Xi are usually organized into outer rims of Xi and the internal cores contain silenced genes (Clemson et al. 2006), the reorganization of chromatin structure

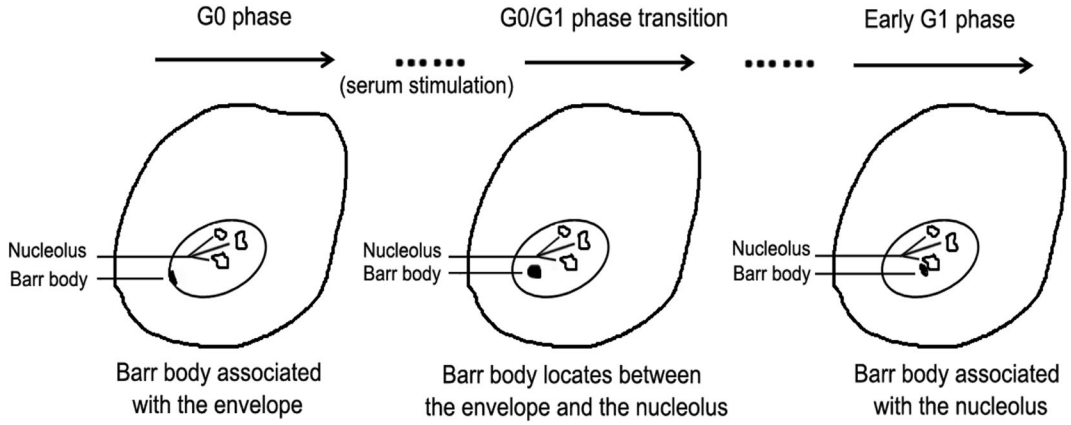


**Fig. 6** The model of Xi movement from the envelope to the nucleolus during the G0/G1 phase transition. In G0 phase, the Barr body is close to the envelope. After serum stimulation, the cell undergoes the G0/G1 phase transition with the location and volume change of Barr body

on inactive chromosomes may enhance the expression of inactivation-escaping genes.

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**Authors' contributions** W.T. and Q.L. conceived, designed and supervised the project, and modified the manuscript. T.T. and Q.L. analyzed the data and wrote the paper. G.L. Y.G. L.S. W.T. designed and performed most of the experiments and contributed to the interpretation of results.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Abbreviations** *CC*, clear-cut; *U/DC*, unclear/decondensed chromatin; *U/CC*, unclear/condensed chromatin; *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *H3K9me2*, histone H3 lysine 9 dimethylation; *H3K27me3*, histone H3 lysine 27 trimethylation; *Xi*, inactivated X chromosome; *Xa*, activated X chromosome; *Xist*, X-inactivation specific transcript; *BrdU*, 5′-bromodeoxyuridine

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