

Uncoupling of X-linked gene silencing from *XIST* binding by *DICER1* and chromatin modulation on human inactive X chromosome

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Abstract In mammals, X-inactivation process is achieved by the *cis*-spreading of long noncoding *Xist* RNA over one of the female X chromosomes. The *Xist* binding accumulates histones H3 methylation and H4 hypoacetylation required for X inactivation that leads to proper dosage compensation of the X-linked genes. Co-transcription of *Tsix*, an antisense copy of *Xist*, blocks the *Xist* coating on the Xi. In mice ES cells, an RNase III enzyme *Dicer1* disrupts *Xist* binding and methylated H3K27me3 accumulation on the Xi. Later, multiple reports opposed these findings raising a question regarding the possible role of *Dicer1* in murine X silencing. Here, we show that reduction of *DICER1* in human female cells increases *XIST* transcripts without compromising the binding of the *XIST* and histone tail modifications on the Xi. Moreover, *DICER1*-depleted cells show differential upregulation of many human X-linked genes by binding different amounts of acetylated histone predominantly on their active promoter sites. Therefore, X-linked gene silencing, which is thought to be coupled with the accumulation of *XIST* and heterochromatin markers on Xi can be disrupted in *DICER1* depleted human cells. These results suggest that *DICER1* has no apparent effect on the recruitment of heterochromatic markers on the Xi but is required for inactivation of differentially regulated genes for the maintenance of proper dosage compensation in differentiated cells.

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Introduction

The equalization of X-linked gene expression between two sexes despite their differences in copy number is mediated by the transcriptional silencing of one of the X chromosomes in female cells (Gribnau and Grootegoed 2012). X inactivation is a multistep process, where choice of the active X chromosome (Xa), establishment of X inactivation in early development, and its maintenance in subsequent cell divisions occur sequentially (Chan et al. 2011). The inactive state of X chromosome is associated with painting of noncoding *Xist* RNA synthesized from the same chromosome (Hall and Lawrence 2003). The *cis*-spreading of *Xist* RNA on the X establishes facultative heterochromatin formation of the inactive X chromosome (Xi), which is orchestrated by accumulation of several modified histones, including H3K27me3, H3K9me2, H4K20me, as well as accumulation of histone variant macroH2A (Payer and Lee 2008). Conversely, Xi is depleted for positive histone marks such as H3K4me2 and H3 and H4 acetylated histones inferring that Xi heterochromatin is not a homogenous entity. In human somatic cells, at least two distinct classes of heterochromatin marks were found, either the presence of *XIST* RNA, macroH2A, H3K27me3, H4K20me, or the occurrence of constitutive heterochromatin signatures, including late replication timing, H3K9me3, H4K20me, etc. (Payer and Lee 2008). These different heterochromatin domains appear to remain spatially distinct during the metaphase and the interphase (Chadwick and Willard 2004). In an instance, they might act together for bringing mitotically stable higher-order chromatin packaging through several rounds of cell divisions. However, the mechanism of

recruitment of epigenetic marks on Xi and stable maintenance of those marks in subsequent cell divisions by *XIST* RNA still remains unknown.

The regulation of *Xist* expression in random X inactivation is complex in mouse. *Tsix*, the antisense copy of murine *Xist*, produces a long (>30 kb) noncoding RNA (ncRNA) reading through same template of *Xist* coding sequence in spatial and temporal manner, which covers the entire *Xist* locus (Stavropoulos et al. 2001; Zhao et al. 2008). In mouse, *Tsix* blocks *Xist* sense RNA coating on the Xa chromosome despite two major unanswered questions, as follows: how *Tsix* stably silences *Xist* RNA on the Xa and how *Xist* induces X inactivation on the Xi. Recent studies demonstrated that *Tsix* transcripts particularly read through *Xist* promoter is essential for *Xist* silencing, which leads to the modification of chromatin organization (Ohhata et al. 2011). This routine job of *TSIX* in human dosage compensation still remained inconclusive due to the truncation of the *TSIX* transcripts during primate evolution (Migeon et al. 2002). Basically, human *TSIX* differs in its expression pattern and complementarity from its murine counterpart (Migeon et al. 2001). Human *TSIX* transcripts truncate abruptly before the four exons and the promoter of the *XIST* locus (Migeon et al. 2001; Sado et al. 2002; Tian et al. 2010; Deuve and Avner 2011) (Fig. 1a). These findings reasonably established that *TSIX* may not counteract *XIST* binding on the human Xa through conventional mechanism described in mice. Such species variations in *XIST/TSIX* structures provide different operational mechanism and accumulation of distinct components of Xi for silencing pathway in humans (Migeon 2003). The repeat A sequence in exon 1 of the *Xist* locus plays a crucial role in silencing functions of the mouse (Zhao et al. 2008; Royce-Tolland et al. 2010) and for the localization of the *XIST* RNA in humans (Chow et al. 2007). In mouse RNAi pathway, it was shown to regulate the *Xist* localization on the X chromosome probably through *Xist/Tsix*-derived small RNA (Ogawa et al. 2008). The balance of sense and antisense RNA is important in determining the probability that a given *Xist* allele will be expressed (Nesterova et al. 2008). The formation of these Xi RNAs needed *Tsix* transcription and in *Tsix*-deficient cells, their levels dropped significantly (Lee and Lu 1999; Ogawa and Lee 2003). *Dicer1* knockout female ES cells eliminated Xi RNA production and disrupted *Xist* binding and H3K27 methylation on the Xi (Kanellopoulou et al. 2005). In rare case, 5 % depletion of *Dicer1* from the normal endogenous level reduced the XiRNA level that leads to a 5–10-fold increase in *Xist* RNA in the undifferentiated ES cells (Kota 2009). However, further investigations oppose such findings by demonstrating no role of *Dicer1* in either *Xist* RNA coating or H3K27 methylation in mouse cells, which raise questions against their specific role in murine X silencing (Kanellopoulou et al. 2009; Wutz et al. 2002). Moreover, both sense and antisense transcripts

modulate *Xist* promoter DNA methylation in undifferentiated ES cells but do not have any role on *Xist* binding (Nesterova et al. 2008). In addition, a procedural change in dosage compensation in humans raised further possibility whether RNAi process has any operational role on human X silencing because repeat-associated small interfering RNA (siRNA) plays an instructive role in transcriptional silencing and heterochromatin formation in *Drosophila* (Yin and Lin 2007). In undifferentiated embryonic mouse cells, the absence of the *Tsix* or other chromatin-modulating factors often leads to a nominal increase in the *Xist* expression (Chang and Brown 2010). The deletion in *Tsix* or DNA methylation up regulates *Xist* RNA inappropriately during the onset of cell differentiation (Lee and Lu 1999). It suggests an existence of alternate possibly redundant mechanism to separate the regulation of *Xist* expression from the binding of the *Xist* and heterochromatin markers on Xi.

In mammalian RNAi, siRNA is produced by the cleavage of long dsRNA. *Dicer1* cleaves long RNA duplex into small noncoding RNA, which are later bound to *Argonaute*-associated RNAi silencing complex (Bernstein et al. 2001; Okamura et al. 2004). RNAi has also been implicated in the chromatin modifications such as DNA and histone methylations particularly histone H3 lysine methylation in plants (Stroud et al. 2012) and animals (Sinkkonen et al. 2008; Benett et al. 2008). The preferential localization of lysine 9 methylated histone H3 and associated proteins, HP1 that are required for assembly and maintenance of centromeric heterochromatin, were disrupted by the defective RNAi components in *Schizosaccharomyces pombe*, *Drosophila*, and in vertebrate cells (Peng and Karpen 2007; Kagansky et al. 2009). Loss of *Dicer1* in chicken–human hybrid cells accumulated mitotic cells due to malfunctioning of pericentric heterochromatin in which HP1 was strongly delocalized (Ohfuchi et al. 2006). Therefore, RNAi machinery might be involved in female X silencing. It offers a novel model for determining the role of RNAi in human X silencing in a manner typical to its role in centromeric heterochromatin.

To address an operational role of RNAi on the *Xist* RNA and heterochromatin proteins binding on the Xi and in human X-linked gene silencing, we have examined two above aspects in the DICER1-depleted human cells. The ability of *XIST* RNA to bind with heterochromatin factors on the Xi was also monitored. Here, we also uncoupled silencing of certain X-linked genes from the *XIST* binding on the Xi chromosome during the maintenance of human dosage compensation by the DICER1 enzyme. Our analyses also demonstrated how DICER1 upregulates X chromosomal genes expression without influencing *XIST* coating and heterochromatin maintenance of the human Xi chromosome.

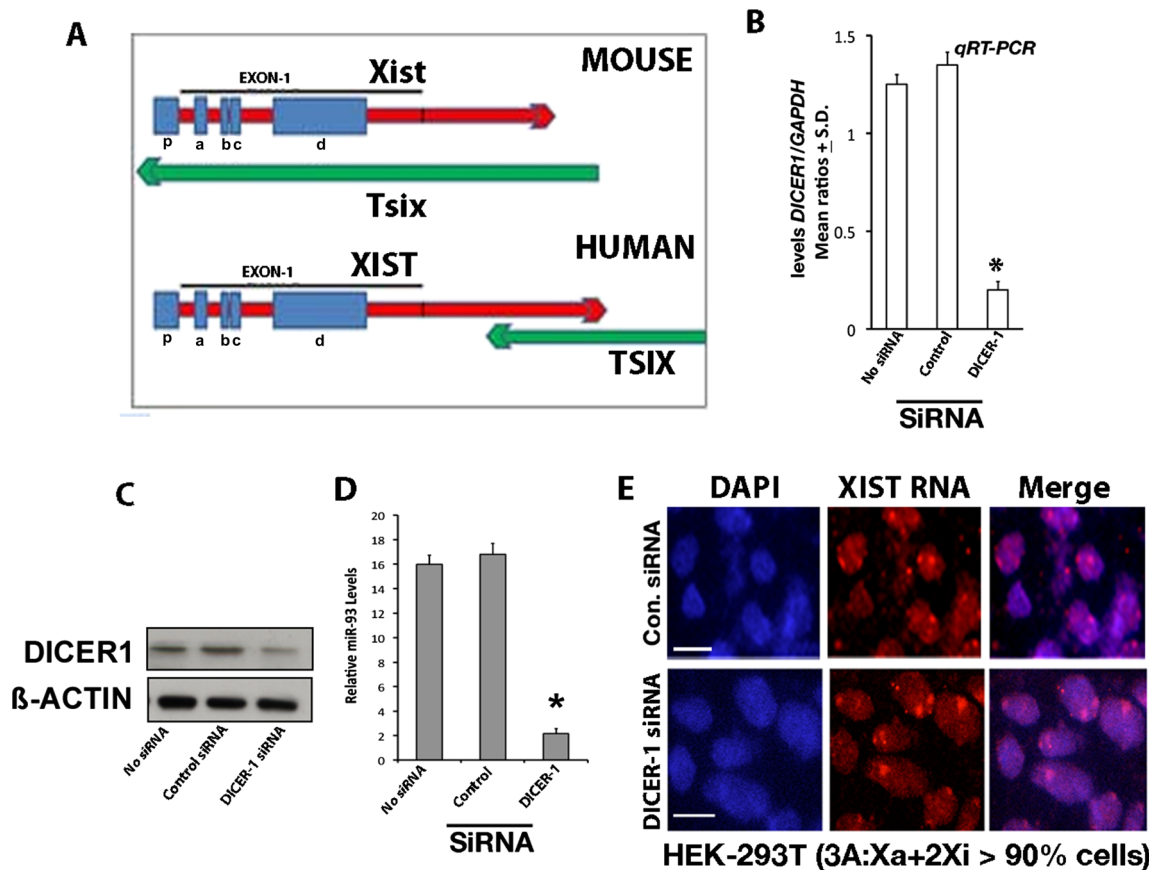


Fig. 1 Effect of *DICER1* deficiency in cells. **A** Schematic diagram showing exon 1 region of *Xist* locus. *XIST* (red) and *TSIX* (green) transcripts (in no scale) differ between human and mouse. In mouse *Tsix* overlaps *Xist* exon 1 and promoter regions while in humans *TSIX* transcript truncates before exons 1–4 and promoter regions (p: promoter region, a, b, c, d: conserved repeat elements present within the Exon-1). **B** The amount of *DICER1* transcript was estimated relative to *GAPDH* mRNA by semiquantitative RT-PCR. The mean values of the triplicate ratios (*DICER1*/*GAPDH*) were presented as a bar diagram. * $P < 0.05$, values significantly different from the no-siRNA and control siRNA transfected control. **C** Western blot hybridization was performed in no

siRNA, control siRNA, and *DICER1* siRNA-treated cells, and protein levels were estimated in comparison to the expression of β -ACTIN. **D** *DICER1*-dependent microRNA; *miR-93* levels were analyzed in no siRNA, control siRNA, and *DICER1*-transfected HEK-293T cells, and Northern blot analysis was performed. The mean values, as presented by the bar diagram, is determined by triplicate measurements. * $P < 0.05$, the average values that differ from the values of no siRNA-transfected cells at the 95 % confidence level. **E** The control siRNA and *DICER1* siRNA-transfected human HEK-293T (1Xa, 2Xi) cells were hybridized with fluorescence XIST RNA probe (Wutz and Jaenisch 2000). DNA was also counterstained with DAPI. Scale (white bar), 10 μ m

Materials and methods

SiRNA-mediated depletion

For downregulation of human *DICER1* genes by transfecting siRNA, three sets of siRNAs (as previously used, Murchison et al. 2005) were designed and synthesized (Dharmacon, USA). Three siRNAs were efficiently transfected in human culture cells HEK-293T (3A; Xa+2Xi>90 % of cells), HeLa (female Xa), and Jurkat (male Xa) using HiPerFect (Qiagen, Germany) reagent according to manufacturer's protocol (Qiagen, Germany). Transfection of the construct that reduces *DICER1* transcripts maximally was selected for further study. The sequences of *DICER1* siRNA for most effective *DICER1* depletion and control siRNA were noted below.

DICER1 siRNA (Murchison et al. 2005) GCUUGAAG CAGCUCUGGAdTdT

Control siRNA (without *DICER1*) AGGUAGUGUAAU CGCCUUGdTdT

Cell culture and transfection

The human cell lines HeLa and HEK-293T cells were grown in Dulbecco's modified Eagle's media (DMEM) with 10 % FBS and maintained at 37 °C with 5 % CO₂. Jurkat cells were grown in RPMI media supplemented with 10 % FBS under same conditions. Transfections in HeLa, Jurkat, and HEK-293T cells were carried out with HiPerFect Transfection Reagent (Qiagen, Germany). All culture conditions, including the serum batch and seeding density, were kept constant for the entire experiment.

Human cells were plated at 60 % confluency and transfected twice on alternate days, and RNA was isolated using TRIZOL reagent (Invitrogen) after 5 days. Cells were trypsinized and replated in chamber slides after second transfection.

Fluorescent in situ hybridization and immunofluorescence

Human cells were fixed with 3.7 % paraformaldehyde in PHEM buffer (60 mM PIPES, 21 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 685 mM NaCl) for 10 min and permeabilized with 1 % Triton X-100. Three types of cells were immediately used for staining or stored in 70 % alcohol at 4 °C until further use. *XIST* RNA FISH was carried out using Cy3-labeled random-primed probes as described (Wutz and Jaenisch 2000) that were synthesized using the DECAprime™ II Kit (Ambion) at 37 °C overnight. Cells were washed at 39 °C using 2× sodium citrate/sodium chloride (SSC) and deionized formamide and later with 2× SSC. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vectashield) and visualized under ×20 and ×100 objectives in confocal microscope (FV1000, Olympus). The specificity of *XIST* RNA probes (Wutz and Jaenisch 2000) was confirmed by destruction of *XIST* signals, when samples were pretreated with RNase.

For immunofluorescence, fixed cells were immunostained using 1:100 or 1:200 dilution of the following primary antibodies: rabbit or mouse H3K27me3 (Upstate, Abcam), rabbit H3K9 acetyl (Upstate) mouse H3K4 di- and trimethyl (Abcam), rabbit H3K9me3 (Upstate), rabbit BRCA1 (Upstate), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz). The 1:100 or 1:200 dilutions of secondary anti-rabbit or anti-mouse antibodies conjugated to FITC, Cy3, or Cy5 (Jackson Pvt Ltd) were used and finally counterstained with DAPI or propidium iodide (PI). The cells were observed under confocal or fluorescent microscopes.

Western blots

The control and DICER1 siRNA-transfected and nontransfected HEK-293T and HeLa cells were washed with ice-cold phosphate-buffered saline (PBS), lysed with 1× Laemmli buffer. The protein concentration was estimated by Lowry's method. The 50 µg protein/per lane was fractionated in 12 % SDS-PAGE gel. Separated proteins were transferred to a PVDF membrane. The membranes were probed with anti-rabbit H3K9me2, H3K27me3, H3K4me3, and H3K9Ac (1:1000; Upstates), anti-rabbit histone H3 antibodies (1:1000), anti-mouse histone 3 (1:1000 dilution), and/or β-ACTIN antibody (1:500 dilution; Progen, USA). The blots were treated with goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibody (Roche, Germany) in 1:2000 dilutions and developed for color detection using NBT and BCIP.

Total RNA from cells and real-time PCR

A population of siRNA-transfected and nontransfected HEK-293T, HeLa and Jurkat cells was trypsinized and resuspended in DMEM. Cells were collected and washed with PBS and used for RNA isolation. Total cellular RNA was isolated by TRIZOL reagent (Invitrogen) and treated with RNase-free DNase I (Ambion) for 1 h at 37 °C. Two micrograms of DNase-free RNA were added as template for complementary DNA (cDNA) synthesis using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega) and random hexamers. Further amplification of cDNA was performed by PCR using gene-specific primers. The following primers were used for amplification of each gene.

XIST:

Forward primer: 5'-AGCTCCTCGGACAGCTGTAA-3',
Reverse primer: 5'-CTCCAGATAGCTGGCAACC-3';

GAPDH:

Forward primer: 5'-TGAAGGTCGGAGTCAACGGA
TTTG-3',

Reverse primer: 5'-TGATGGCATGGACTGTGGTCAT
GA-3';

Col A45:

Forward primer: 5'-ACAGCTTTTGGCTGGCAACT-3',
Reverse primer: 5'-CGGCTAATTCGTGTCCTCAAG-3';

MAGE H1:

Forward primer: 5'-CCTTTGTAGCTGAGAACGGC-3',
Reverse primer: 5'-CCTCTTCAGGAAGCAACAGC-3';

Hemophilia AF8:

Forward primer: 5'-GTGGCAGACTTATCGAGGAAA-3',
Reverse primer: 5'-TCGAGCAATAATTGGAGGGT-3';

SMARCA 1:

Forward primer: 5'-ACGAAATGCTCCCCAGTTTA-3',
Reverse primer: 5'-TCTCTCTCTTTCTCAATTTCCA-3';

KIAA1166:

Forward primer: 5'-CCCTTGGCTGGTGTATTTGT-3',
Reverse primer: 5'-CTCCAICTGCAGGGTCTTGT-3';

MOSPD2:

Forward Primer: 5'-GATCATGGCAGAGAATCACG-3',
Reverse primer: 5'-GATCATGGCAGAGAATCACG-3'.

Quantitative real-time PCR was carried out using the Roche Lightcycler® 480 II system using Sybr Fast Universal Ready Mix (KAPA Biosystems) according to manufacturer's protocol. The software in Lightcycler 480 II was used to analyze and quantify the PCR products.

Northern transfer and hybridization

Total cellular RNA from no siRNA, control siRNA, and DICER1 siRNA-transfected cells were prepared, electrophoresed, and hybridized with antisense *miRNA 93*-synthesized

probe. Blots were prepared in triplicate for each probe. As a loading control, the blots were reprobbed with β 1-tubulin RNA. Quantification was performed using a Fuji 2000 Bas Phosphorimager.

Chromatin immunoprecipitation

RNA-ChIP was performed as reported earlier (Cavalli et al. 1999; Sun et al. 2006). For a detailed method, please see [Electronic supplementary material](#).

Results

Production of DICER1-depleted human cells

Since RNase III enzyme DICER1 has a central role in RNA interference pathway, we were interested to see whether it has any influence on human dosage compensation and maintenance of X inactivation. For this, we had generated three cell types, HEK-293T (a female embryo lineage, 3A; Xa+2Xi), Jurkat (male Xa), and HeLa (female Xa) deficient for DICER1 gene. Initially, DICER1-depleted human cell lines were generated by incubating cells in DICER1 gene-specific siRNA-containing media as described earlier (Murchison et al. 2005). To ensure whether the siRNA dramatically reduced the DICER1 transcripts, quantitative real-time PCR analysis was conducted. Data showed a multifold (6-fold) reduction of DICER1 messenger RNA (mRNA) within 5 days by incorporating siRNA at a regular interval (alternate days) relative to the same mRNA from untreated (no siRNA) and control siRNA-transfected HEK-293T (3A; Xa+2Xi) cells (Fig. 1b). The reduction of DICER1 expression was also quantitated in the protein (Fig. 1c) levels (Murchison et al. 2005) using Western blot hybridization. In both cases, treatment of DICER1 inhibitory siRNA reduced transcripts and DICER1 proteins dramatically as verified by quantitative RT-PCR and Western blot analysis respectively. To verify whether this reduction of DICER1 protein is sufficient to negate its function, an ideal DICER1-dependent miRNA, *miR-93* levels were analyzed in DICER1 siRNA-treated cells by northern blot analysis. Considerable decrease of *miR-93* in DICER1-deficient HEK-293T cells (Fig. 1d) indicated a successful functional knockdown of DICER1. Cells at this stage were immediately fixed and used for further study. To test a potential link between RNAi, X chromosomal identity of noncoding RNA and X chromosomal dosage compensation in humans, three human cell lines, HEK-293T(3A; Xa+2Xi), HeLa (with no Xi-stained chromosome), Jurkat (Only male Xa) were verified by fluorescence in situ hybridization (FISH) using *XIST* RNA probe (Supplementary Fig. 1; Wutz and Jaenisch 2000). The current dogma is that noncoding *XIST* RNA is transcribed from inactive center of the

X (XIC) and is only coated on silent X chromosome in females; however, its binding on active male X was not detected (Gribnau and Grootegoed 2012; Chan et al. 2011). Generally, two inactive X chromosomes coated with *XIST* RNA were only present in HEK-293T cells (3A; Xa+2Xi) while *XIST* RNA fails to hybridize with existing X chromosome in Jurkat and HeLa cells (Supplementary Fig. 1).

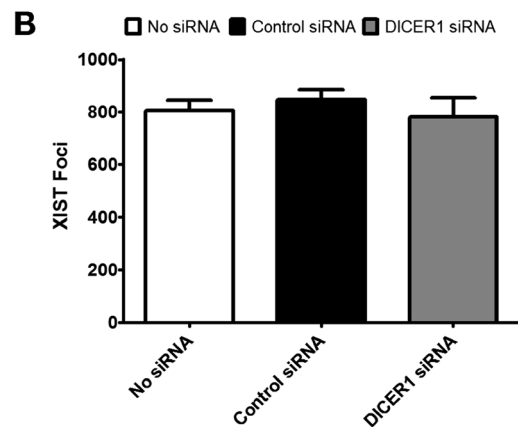
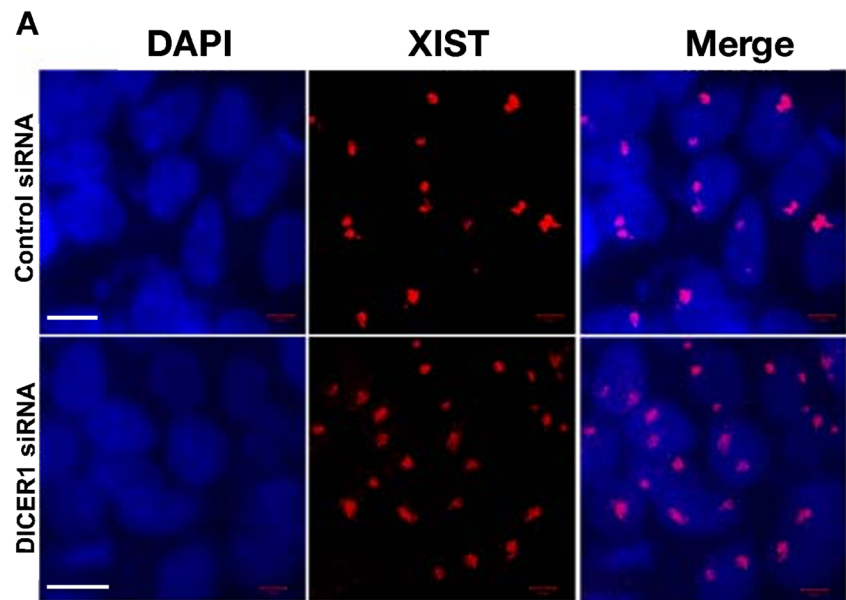
Binding of *XIST* RNA

To test whether DICER1 affects *XIST* painting on Xi, DICER1-depleted cells were hybridized with fluorescent *XIST* RNA probe. In mouse ES cells, loss of *Dicer1* failed to paint *Xist* RNA on Xi in the interphase nuclei (Ogawa et al. 2008). The region of the X heterochromatin in transfected nuclei showed an intense DAPI staining as a signature for strong *XIST* RNA localization (Supplementary Fig. 2). Because high chromatin compaction in heterochromatin always shows intense DAPI staining, a similar level of condensation was also noticed in DICER1-depleted HEK-293T cells (Fig. 1e). Thus, DICER1 has no apparent effect on the Xi condensation in female human cells. For better understanding of the *XIST* RNA localization on the entire inactive X, an in situ hybridization analysis was conducted using fluorescent *XIST* RNA probe in human cultured cells. The silent Xs of HEK-293T cells (3A; Xa+2Xi) were coated with two *XIST* RNA (Fig. 2a) but HeLa (female Xa) and Jurkat (male Xa) cells do not carry any *XIST*-coated X chromosome (Supplementary Fig. 1). Once the cells are transfected with DICER1 siRNA, *XIST* still binds on the inactive Xs (Fig. 2a). Enumeration of *XIST* foci in no siRNA, control siRNA, and DICER1 siRNA-transfected HEK-293T (3A; Xa+2Xi) cells showed nearly the same numbers. There is no significant difference in *XIST* foci number in three transfected cell types (Fig. 2b). Therefore, depletion of DICER1 does not compromise intense *XIST* RNA coating on the Xi chromosomes.

Recruitment of chromatin modifications

Heterochromatinization of human Xi chromosome is characterized by accumulation of more repressive histone codes associated with *XIST* RNA binding. *XIST* RNA coating is the first event in a cascade of changes on the Xi chromosome. Several early histone modifications including enrichment of macro-H2A deposition (Li 2002), histone H3K27me3 (Chan et al. 2011), and H3K9me2 (Rougeulle et al. 2004) impart profound effects on Xi heterochromatin formation coupled with X-linked gene silencing. Using immunofluorescence technique, an enrichment of modified histone isoforms including H3K27me3 on the Xi chromosome was noticed as a Xi marker (Fig. 3a) in control and DICER1 siRNA-transfected HEK-293T cells (3A; Xa+2Xi). The

Fig. 2 Localization of *XIST* RNA by degradation of human *DICER1* mRNA on multiple inactive Xs in the human HEK-293T cells. **a** Fluorescence in situ hybridization of *XIST* RNA on control siRNA or *DICER1* siRNA-transfected HEK-293T cells. Scale (white bar), 10 μ M. **b** Bar diagram showing enumeration of *XIST* foci in no siRNA, control siRNA, or *DICER1* siRNA-transfected cells. The mean values \pm S.D. from the transfected cells ($n=460$ cells) were presented by the bar diagram ($P<0.01$). The types of siRNA are noted in the diagram



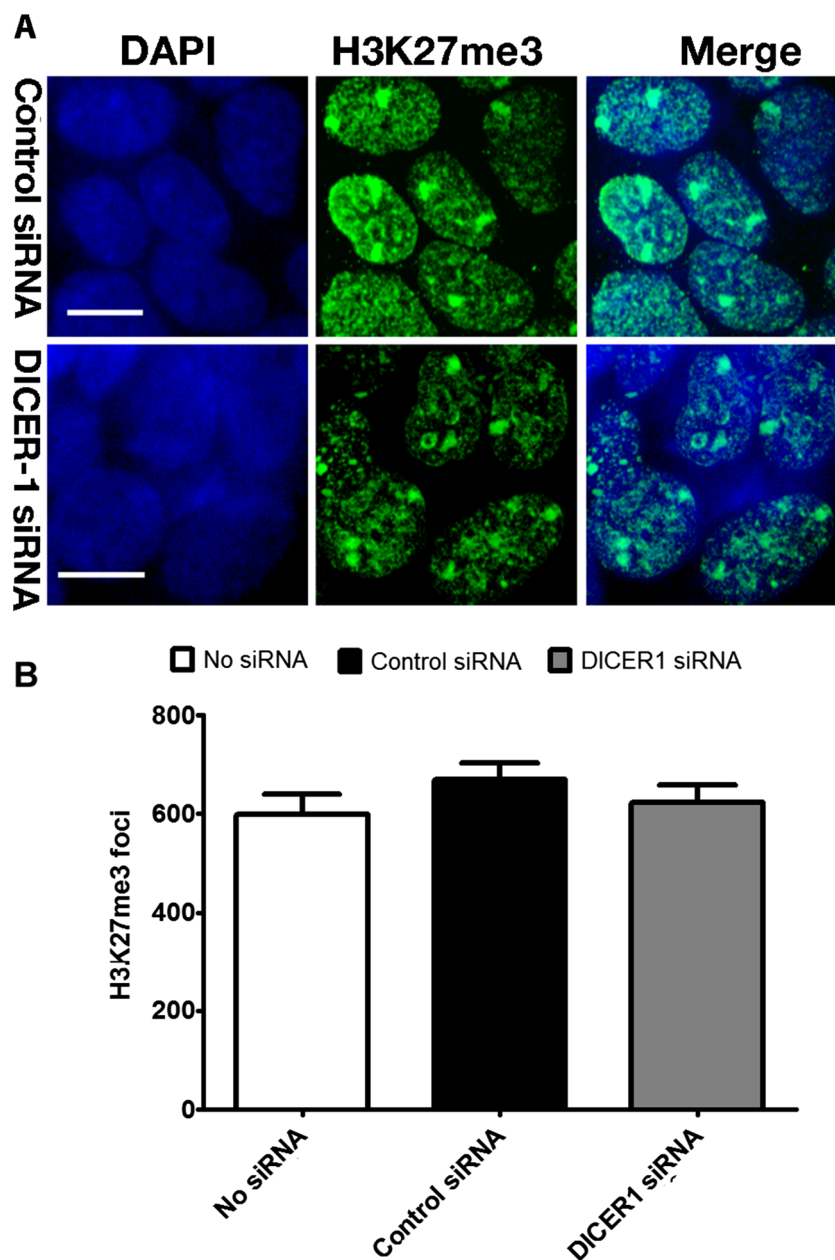
statistical analysis on H3K27me3-bound Xi revealed that nearly equal number of Xi-bound H3K27me3 foci existed in variant cells (Fig. 3b).

Apart from early chromatin modifications, H3K9 methylation and *XIST* RNA overlaps on the inactive X chromosomes suggesting that enrichment of histone H3 lysine 9 methylation might also be involved in the maintenance of stable transcriptional silencing. In most cases, major heterochromatin marks in mammalian nuclei, H3K9me2, and DNA methylation ensure complete nucleolar organization, which is essential for RNAi machinery (Peng and Karpen 2007; Kondo et al. 2008; Kagansky et al. 2009). The level of H3K9me2 and other major histone marks were estimated by Western blots from HEK-293T cells treated with no siRNA, control siRNA, and/or *DICER1* siRNA (Fig. 4a). We also measured the amount of histone H3 by the Western blot hybridization as a loading control. To avoid saturation of the gel blots, we also rehybridized the same blots with β -ACTIN probe as a gel control. The level of methylated histone H3 proteins (H3K9me2 and H3K27me3) in *DICER1* siRNA-transfected

cells was changed at a marginal level relative to no siRNA and control siRNA transfected cells. However, the total amount of extractable histone H3 and β -ACTIN proteins in both cases appear to be similar, which suggests that H3 and β -ACTIN modifications were unaffected in the *DICER1*, control, and no siRNA-incorporated cells. Thus, *DICER1* is not important for targeting *XIST* RNA and histone methylation although H3K9me2 level remained unaltered on the Xi chromosomes, which readily accounts for higher order chromosome packaging needed for Xi maintenance.

When mean ratios of H3K27me3/H3K9me2 were calculated in comparison to β -ACTIN control in no, control, and *DICER1* siRNA-treated cells, almost a similar pattern was observed for those heterochromatin markers (Fig. 4b). On the other hand, both the euchromatin-activating factors, such as H3K4me3 and H3K9Ac showed nearly 1.5–2-fold enhancement in *DICER1*-depleted cells (Fig. 4b), which suggested that expression and localization of histone H3K9 and H3K27 methylations were not affected only by the depletion of *DICER1* human mRNA. The histone H3K9me2 was

Fig. 3 Localization of X silencing histone markers H3K27me3 in DICER1 knockdown cells. **a** Immunostaining was performed on control siRNA and DICER1 siRNA-treated HEK-293T cells using antibodies against H3K27me3 (*middle panel, green*) or DAPI (*left panel, blue*). Merged image is shown in the *right*. Scale (*white bar*), 10 μ M. *Inset*, the histogram of H3K27me3⁺ foci in DICER1-transfected cells, compared with cells having control siRNA and cell with no siRNA. The mean values are derived from at least three measurements of the foci. The average value with S.D. are presented by a histogram. The total ($N=375$ cells) were measured in each case



compared between cells containing intact and reduced DICER1 mRNA (Fig. 4c). The antibodies were accumulated mainly at the centromeric regions of the chromosomes in HEK-293T (Fig. 4c) as isolated spots. The strong H3K9me2 accumulation in control siRNA-transfected HEK-293T cells does not reduce markedly with DICER1 siRNAs (Fig. 4c). The level of histone H3 lysine 9 methylation associated with RNAi machinery has no apparent role on stabilization of the *XIST* RNA coating and/or maintenance of the X inactivation. These findings do not reflect the loss of higher order of chromosomal condensation in inactive X chromosome. These results ruled out the possibility that accumulation of lysine 9-methylated histone H3 on the Xi and its negligible

delocalization by the loss of DICER1 activity might be due to higher nuclear density of the silent X chromatin.

Conversely, the Xi is depleted for marks commonly linked to transcriptional activators, for example, H3K4me3 methylation (Fig. 5). These results revealed that Xi silencing is not a homogeneous entity. In human somatic cells, a consistent reduction of H3K4me2, H3K4me3 binding on the Xi was detected as colorless foci in the immunostained nuclei. Double staining with H3K4me3 and H3K27me3 (Fig. 5) antibodies showed that reduction of DICER1 did not increase the level of H3K4me3 accumulation on the Xi chromosome, which was detected by the excess H3K27me3 deposition in female cells.

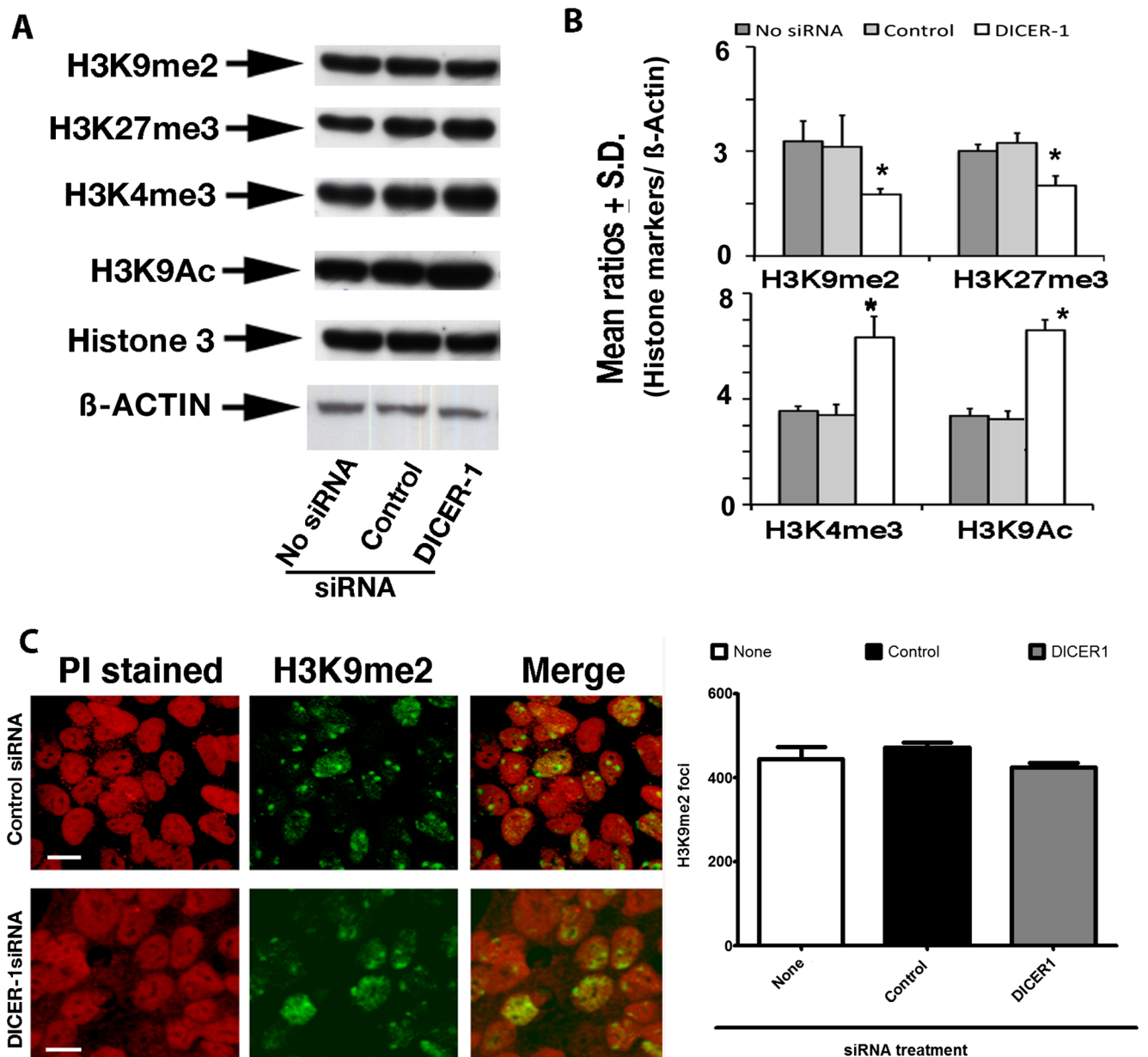


Fig. 4 Analysis of localization of heterochromatic markers with no siRNA, with control siRNA and DICER1 knockdown cells. **a** Western blot analysis of H3K9me2, H3K27me3, H3K4me3, and H3K9Ac in no siRNA, control siRNA, and DICER1 siRNA-treated HEK-293T cells. Histone 3 and β -ACTIN (nonsaturated) were also used as positive control. **b** Bar diagram showing the ratio of expression of H3K9me2, H3K4me3, H3K27me3, and H3K9Ac with respect to the expression of β -ACTIN and in no siRNA, control siRNA, and DICER1 siRNA-

transfected cells. * $P < 0.01$, the average values (triplicate mean values \pm S.D.) that differ from the no siRNA-transfected cells at the 95 % confidence level. **c** FISH analysis in no siRNA, control siRNA and DICER1-transfected cells stained with propidium iodide (PI, left panel, red) or α -H3me2K9 (middle panel, green). Right, merged pictures. Scale (white bar), 10 μ M. Inset, comparison of H3K9me2 in no siRNA, control siRNA, and DICER1 siRNA-transfected HEK-293T cells

Colocalization of BRCA1 and *Xist*

A *XIST*-interacting partner, BRCA1 functions in DNA damage pathways as a ubiquitin ligase (Stucki et al. 2005; Wu et al. 2010). *BRCA1* mutations predispose carriers of hereditary breast or ovarian cancer. Many breast cancers related to *BRCA1* deletions no longer show a Barr body, suggesting a loss or reactivation of the inactive X chromosome (Stone et al.

2003; Pageau et al. 2007). It is established that BRCA1 protein is required for *XIST* RNA localization on the Xi (Ganesan et al. 2002). Colocalization of BRCA1 and *XIST* RNA on the Xi indicated that *BRCA1* is a transfactor agent involved in *XIST* localization. Later, a similar study failed to reproduce the overlapping binding of the *XIST* RNA and *BRCA1* in the same nuclei (Pageau et al. 2007). In another study, X-linked gene expression and XCI were not disrupted

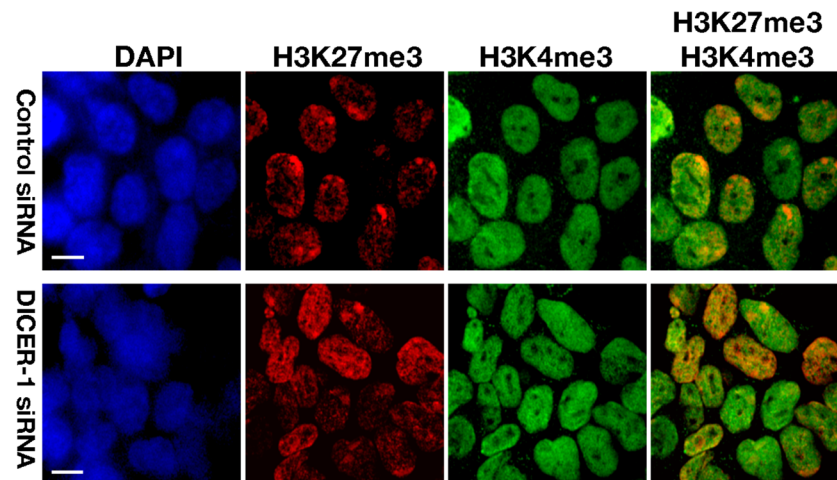


Fig. 5 Immunofluorescence of H3K4me3 in control and DICER1 knockdown human cells. Control siRNA and DICER1 siRNA-treated cells were stained with DAPI (*left, blue*), H3K27me3 (*2nd from left, red*), and H3K4me3 (*3rd from left, green*) antibodies. *Right*, H3K27me3 and

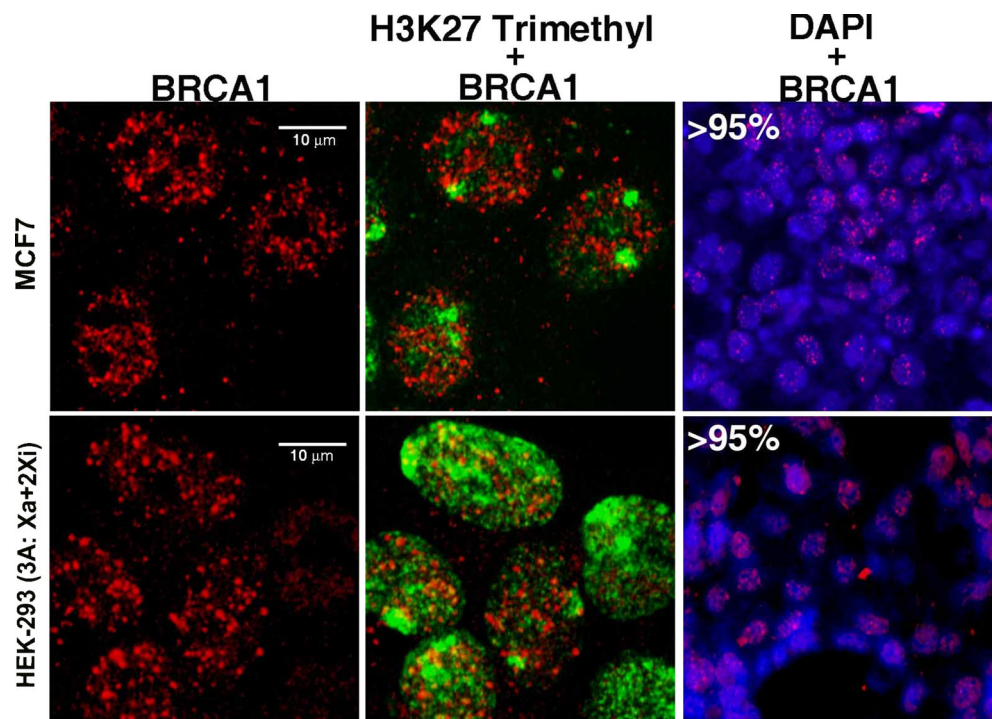
H3K4me3 colocalized image. Here, H3K27me3 was used as inactive X chromosome marker. The localization of two methylations (H3K27me3 and H3K4me3) in the cells does not overlap. *Scale (white bar)*, 10 μ M

in *BRCA1*-deficient mice (Sirchia et al. 2005). These findings are clearly hard to reconcile with each other.

To reinvestigate the overlapping binding *BRCA1* and *XIST* RNA on Xi, tumor MCF7 cells as control and nonneoplastic HEK-293T (3A; Xa+2Xi) cells were immunostained with Xi marker H3K27me3 and *BRCA1* antibodies (Fig. 6). The *BRCA1* antibody is associated with different subnuclear regions in more than 95 % of the nuclei in control MCF 7 cells and HEK-293T cells. But these bindings fail to overlap with H3K27me3-enriched region or Xi chromosome, which are

marked by the enrichment of H3K27me3 antibody in both tumor and normal cells (Fig. 6). The absence of overlapping *BRCA1* and H3K27me3 binding on the Xi suggest that *BRCA1* has no definitive role in the X-inactivation process. *DICER1*-depleted cells increased *BRCA1* binding in comparison to the control siRNA or no siRNA-transfected cells; the amount of *DICER1* depletion which enhanced the *BRCA1* binding throughout the nuclei suggested (Supplementary Fig. 3) that *DICER1* intervenes *BRCA1* binding of the human nuclei.

Fig. 6 Analysis of binding *BRCA1* and *XIST* RNA on Xi. Tumor MCF7 cells (prominent for *BRCA1* marker used as control) and nonneoplastic HEK-293T (3A; Xa+2Xi) cells were immunostained with Xi marker H3K27me3 and *BRCA1* antibodies. The *BRCA1* antibody is associated with different subnuclear regions in more than 95 % of the nuclei. *Scale (white bar)*, 10 μ M



X-linked gene expression

X silencing compensates the dosage of X-linked gene expression between male and females in mammals. But some genes escape silencing and are thus expressed for both alleles in females. In mouse, nearly 3–4 % mouse genes are expressed from the inactive X chromosome (Yang et al. 2010), whereas about 15 % of X-linked genes in human escape inactivation to some degree (Carrel and Willard 2005). Such genes potentially contribute to the sexual dimorphic traits to phenotypic variation.

To test whether the X-linked gene repression is maintained in the cells with reduced expression of DICER1, we

performed real-time PCR analysis of select transcripts that are located in many loci along the X chromosome. Consistent with the role of *Dicer1* in regulating the mouse *Xist* levels, an increase in human *XIST* levels in DICER1 knockdown cells was detected relative to no siRNA and control siRNA-transfected cells in both HEK-293T (3A; Xa+2Xi) and Jurkat (Only male Xa) (Fig. 7a). Since the increase is nearly 2–2.5-fold in case of HEK-293T cells, it suggested that XIST loci in inactive X chromosome in HEK-293T cells was expressed. This involves upregulation of XIST transcripts in DICER1-depleted HEK-293T cells. DICER1 is involved in siRNA and miRNA biogenesis in humans. The reduction of DICER1 in these two cell lines delimits the influence of siRNA and

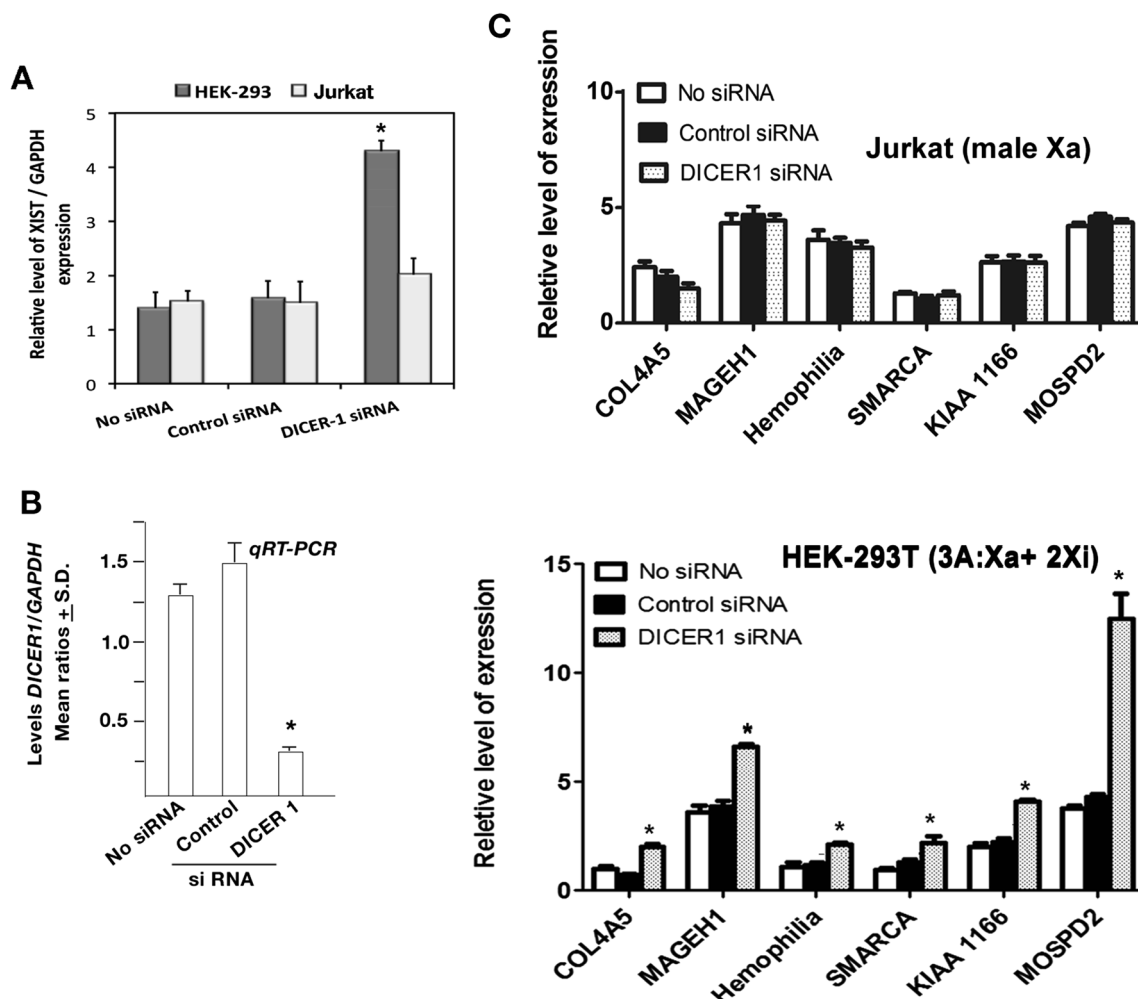


Fig. 7 Upregulation of X-linked gene expression: **a** quantitative estimation XIST mRNA (real-time PCR) from no siRNA, control siRNA, and DICER1 siRNA-transfected HEK-293T (3A; Xa+2Xi) and Jurkat (only male Xa) (*top*) cells were presented. The relative ratios ($n=3$) of gene/GAPDH of triplicate values were plotted in a *bar diagram*. The standard deviations of each mean values were present at the *top of each bar*. The name of each gene was sited at the *bottom of the histogram*. * $P<0.05$, values were significantly different from the respective control. **b** The amount of DICER1 transcript was assayed relative to *GAPDH* mRNA by semiquantitative RT-PCR in the Jurkat cells. The mean values of the

triplicate ratios (DICER1/*GAPDH*) were presented as a *bar diagram*. * $P<0.05$, values are significantly different from the no siRNA-transfected control. **c** Expression of six X-associated transcripts, *COL4A5*, *MAGEH1*, *hemophilia A F8*, *SMARCA1*, *KIAA1166*, and *MOSPD2* analyzed by RT-PCR in Jurkat (*top*) and HEK-293T(*bottom*) cell lines. The quantitative values of six different transcripts of control siRNA and DICER1 siRNA-transfected cells relative to no siRNA cells with S.D. noted in the key, is represented by the *bar diagram*. Each ratio is determined by triplicate measurements. * $P<0.05$, 95 % confidence interval

endogenous microRNA in both cases on the X-linked transcripts. We used these cells for further experiments.

To determine whether repression of RNAi factor has no impact on the inactive X chromosome restoring its activity, we have measured six different X-linked transcripts, viz., collagen alpha-5(IV) chain (*COL4A5*), melanoma-associated antigen H1 (*MAGEH1*), hemophilia A factor VIII (*hemophilia A F8*), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 1 (*SMARCA1*), hepatocellular carcinoma-associated antigen 127 (*KIAA1166*), motile sperm domain-containing 2 (*MOSPD2*), from the DICER1-transfected, no siRNA, and control siRNA-transfected cells (Fig. 7a, b). Selection of six X chromosomal genes is important, and they are not involved in sexual dimorphism or phenotypic variation in humans. Six X-associated transcripts, *COL4A5*, *MAGEH1*, *hemophilia A F8*, *SMARCA1*, *KIAA1166*, and *MOSPD2* were increased significantly in DICER1-transfected HEK-293T cells relative to the amount in no siRNA and control siRNA-transfected cells (Fig. 7b). However, the amount was almost similar in three cases of no siRNA, control siRNA, and DICER1 siRNA-transfected Jurkat cells. It indicated that 2Xi chromosome containing female cells (HEK-293T, 3A; Xa+2Xi) increases the transcription compared with Xa-containing Jurkat cells. The elevated X transcript in general revealed that the genes were transcriptionally active regardless of their influence of siRNA and miRNA in DICER1-reduced cells (Fig. 7a, b).

A consistent but different degree of upregulation of X-linked mRNA was observed in DICER1-depleted HEK-293T cells relative to DICER1-reduced Jurkat cells. A comparison with control siRNA-transfected cells demonstrating that DICER1 is involved in a post-establishment Xi process for maintaining the balance of certain X-linked gene dosage in human somatic cells revealed that DICER1 is a transcriptional target for individual gene expression; therefore, DICER1 uncoupled functionally the X-linked gene expression from the chromatin modifications including *XIST* binding on the silent X chromosome during maintenance of human dosage compensation. These outcomes are consistent with earlier findings that DICER1 depletion in fact had a global effect on gene expression, including upregulation of nearly >2000 genes in HEK-293T cells (Tang et al. 2007).

Recruitment of chromatin modifiers on X-linked gene promoters

To correlate possible chromatin changes occurring gene activation, we determined by a series of ChIP assays coupled with real-time PCR the histone methylation state at the regulatory regions of the four X-linked genes, *COL4A5*, *MAGEH1*, *KIAA1166*, and *MOSPD2* (Fig. 8a, b). We have analyzed major histone methylation, histone H3K27me3 and H3K9me2, whose excess accumulation is required for chromatin changes of Xi chromosome. The enrichment of histone

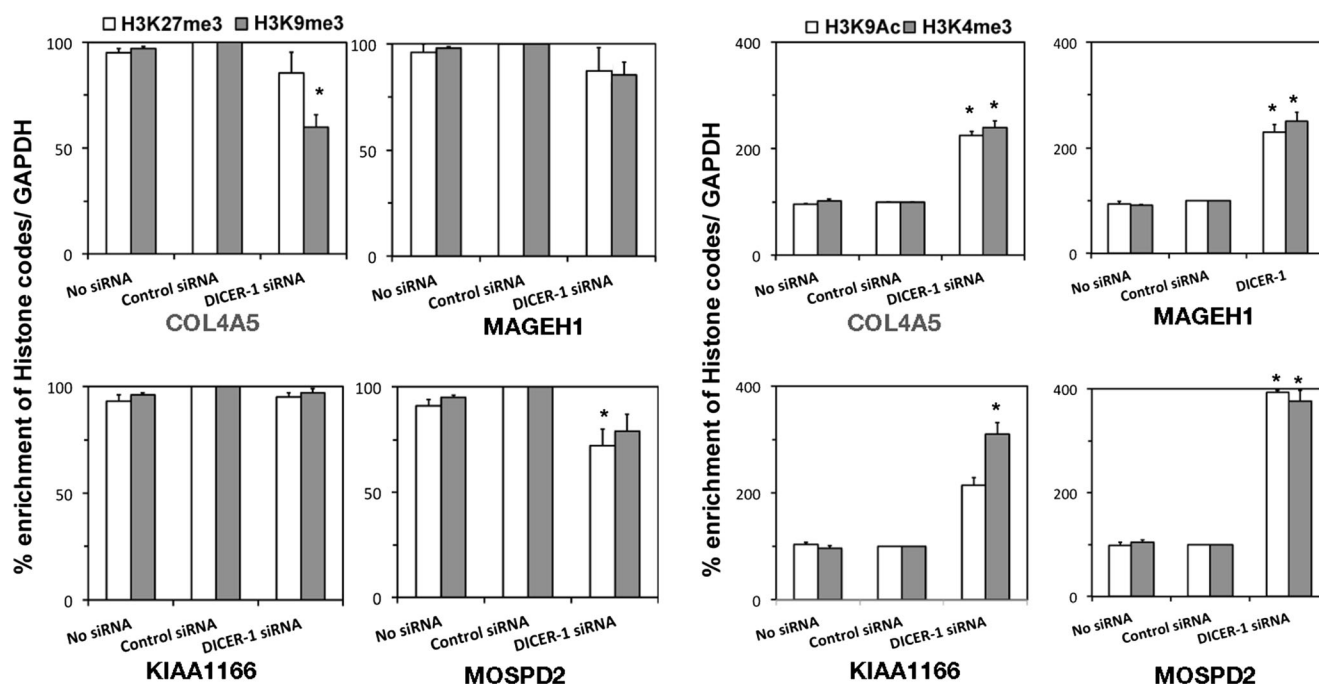


Fig. 8 Variable enrichment of four histone codes in four X-linked gene promoters. **a** Accumulation of histone H3K27me3 and H3K9me2 proteins and **b** histone H3K9Ac and H3K4me3 proteins in *COL4A5*, *MAGEH1*, and *KIAA1166* *MOSPD2* gene promoters in no siRNA, control siRNA, and DICER1 siRNA-transfected HEK-293T cells were

shown. The quantitative data with respect to the X-linked gene as compared to the accumulation of GAPDH were represented by the *bar diagrams*. Each ratio (mean values±S.D.) is determined by triplicate measurements. * $P < 0.01$, average values that differ from the no siRNA-transfected cells at the 95 % confidence level

methylation H3K27me3 and H3K9me2 is commonly associated with gene repression. Results showed that H3K9me2 expression was nominally decreased in two genes (*COL4A5* and *MOSPD2*) in the DICER1-deficient cells relative to the no siRNA and nonspecific siRNA-transfected controls in a sex-specific manner, while no significant changes were observed of their accumulation at the promoter region of *MAGEH1* and *KIAA1166* genes (Fig. 8a, Supplementary Fig. 4). Furthermore, we analyzed the levels of trimethylated H3K27, a histone modifier that has shown an enriched accumulation on the Xi. However, their enriched accumulation in Xi does not lead to their higher accumulation at the promoters of all four tested genes in DICER1-depleted cells (Fig. 8a, b). Therefore, DICER1 has no contribution on the localized chromatin modification related to the upregulated genes. The upregulation of X-linked gene activities and chromatin modulations via histone methylation are two coexisting but separate processes, which might be differentiated by the DICER1 activity in humans. However, when we tested whether the euchromatin-activating factors H3K9Ac and H3K4me3 are associated with the expression of the abovementioned four X-linked genes, we found a great enhancement of expression of all the four genes in both H3K9Ac and H3K4me3 (Fig. 8b).

Discussion

In different ways, human dosage compensation is distinct from its mouse counterpart. These differences in origin and function of X-inactivation process might underlie the subtle variations of the dosage compensation mechanism between primate and nonprimate mammals. In humans, truncation of *TSLX* during evolution and its negligible role in Xi imprinting supports this view (Migeon et al. 2001). Contrary to the earlier observations in mice ES cells (Shekar et al. 2011), our results indicate DICER1 has no role in the maintenance of *XIST* or H3K27 marks on the Xi. It is likely that general epigenetic marks including *XIST* RNA and other histone modifications once established during embryonic development allows DICER1-independent maintenance of Xi chromosomes. On the other hand, the existence of temporally regulated *Xist* and *Tsix* duplexes and probable processing of these duplexes into small XiRNA and that the absence of functional *Dicer1* in mouse ES cells leads to loss of *Xist* and H3K27 trimethylation on Xi (Shekar et al. 2011). These changes in *Dicer1*-depleted mouse ES cells may be caused by amenability of ES cell chromatin to short-term changes and recruitment of epigenetic activators of the Xi genes. Surprisingly, in agreement with our present report that DICER1 has no significant role in binding of heterochromatin marks on human Xi, several groups demonstrated that mouse Xi is faithfully propagated in *Dicer1*-deficient mature T cells (Liston et al. 2008), in mouse embryos

(Kanellopoulou et al. 2005), and in mouse male and female ES cells (Murchison et al. 2005). Even *Dicer1* hypomorphic mice also showed no differences in genomic imprinting in tested loci (Fukasawa et al. 2006). These contrasting results relating to the function of mammalian *Dicer1* in nuclear-related transcriptional processes are surprising considering the evolutionary conservation in function of these enzymes in post-transcriptional gene regulation. Knowledge regarding the specific cofactors that aid or mediate the functions of *Dicer1* in distinct cellular compartments will definitely help in dissecting the roles of small RNA in epigenetic processes.

Together, our results show that *XIST* binding and hypermethylation of histone H3K27 contributed in Xi establishment. However, DICER1 knockdown resulted in elevation of X-linked gene expression. Some genes (*XIST*, *MOSPD2*, *COL4A5*, and *MAGEH1*) are more sensitive than others, suggesting a selective and preferential effect of DICER1 in human dosage compensation (Supplementary Fig. 3). Gene regulation profiles in mouse and humans in the Xi chromosome is quite distinct. Mice have significantly fewer escape genes compared to humans. Furthermore, escape genes do not cluster in mouse, unlike the large escape domains in humans, suggesting that expression is regulated at the level of individual genes (Yang et al. 2010), whereas the proportion of genes in human escaping inactivation differs dramatically between different regions of the X chromosome, reflecting the evolutionary history of the sex chromosome (Carrel and Willard 2005). Even a whole genome expression analysis in DICER1-deficient cells also showed upregulation of more than 2000 genes (Tang et al. 2007). Therefore, it is now an open question whether DICER1 has any role in sex chromosome evolution or not.

Dicer1 has been shown to aid in establishment of proper DNA methylation by regulating repressors of DNA methyl transferases (DNMTs) through *miRNA-290* cluster-mediated inhibition in mouse ES cells and through unknown mechanism in human cancer cell lines. The functional role of *Dicer1*-dependent RNAi process is a global increase of X-linked gene expression that may be uncoupled from the binding of *Xist* and heterochromatin markers on the Xi chromosome. Therefore, the study only showed the maintenance of silencing and related chromatin modifications on the Xi chromosome in consecutive cell divisions. It is functionally different from its individual control on specific gene expression. The influence of DICER1-associated complex on gene expression might be contributed by the direct or indirect miRNA-mediated functions of *Dicer1* rather than through the modification of chromatin state. Similarly, it is also possible that upregulation of gene expression is also an effect of disruption of microRNA-mediated gene regulation. Studies with human female ES cells will be useful in determining their specific functions in DICER1 in Xi initiation and establishment and also provide a new platform for investigating the

role of nuclear RNAi in heritability and maintenance of higher-order Xi chromatin structure in primates.

Xist locus controls the process of X inactivation and recruitment of heterochromatin markers on the silent X chromosome during initiation phase of X inactivation (Wutz and Jaenisch 2000). Its role is thought to be redundant for maintenance of X inactivation. Our data shows that XIST locus in humans is indispensable for successive maintenance of X-mediated gene expression and genome stability. Moreover, it would be interesting to see the expression and the binding role of *XIST* in humans at the initiation of X-inactivation process, where *XIST* RNA along with its partner *TSIX* is known to mediate the counting and choice of Xi. Consistent with our presumption, whole genomic microarray analysis of *XIST*/*TSIX* small RNA <200 nt revealed extensive presence of small RNA including –35 and –50 nt small RNA at several gene loci (Kapranov et al. 2007), some of which are conserved between mouse and humans, hinting at plausible important regulatory roles played by these classes of RNA in genome maintenance and organization.

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Authors' contributions SKK conducted majority of cytological experiments except Fig. 1e and initial write up. LKR contributed in the concept of repeat association of Xi. DRC and VP repeated the experiments and RT-PCR data. UB designed and analyzed the experimental data, compiled the final correction, and final write up. UB and LS commented on the final write up. All authors discussed the results and approved the final version of the manuscript.

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