X chromosome inactivation in the absence of Dicer

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Dicer is central to the RNA interference (RNAi) pathway, because it is required for processing of double-stranded RNA (dsRNA) precursors into small RNA effector molecules. In principle, any long dsRNA could serve as a substrate for Dicer. The X inactive specific transcript (*Xist*) is an untranslated RNA that is required for dosage compensation in mammals. It coats and silences 1 of the 2 X chromosomes in female cells and initiates a chromosomewide change in chromatin structure that includes the recruitment of Polycomb proteins, but it is largely unknown how *Xist* RNA mediates these processes. To investigate a potential link between the RNAi pathway and X inactivation, we generated and analyzed Dicer-deficient embryonic stem (ES) cells. In the absence of Dicer, coating by *Xist* RNA, initiation of silencing, and recruitment of Polycomb proteins occur normally. Dicer ablation had modest effects on the steady-state levels of spliced *Xist* RNA. Together our data indicate that the RNAi machinery is not essential for the initiation of X inactivation.

embryonic stem cells | gene silencing | RNA interference | Xist

Dicer is an RNase III-like enzyme involved in the generation of small double-stranded RNAs (dsRNA) from long double-stranded precursors (1, 2). Dicer cleavage products, which are classified as microRNAs (miRNAs) and endogenous small interfering RNAs (esiRNAs), are then bound by Argonautes (Ago) within multiprotein complexes and can regulate gene expression via several distinct mechanisms (3, 4). miRNAs have been extensively characterized (5), and the majority of miRNAs mediate posttranscriptional gene silencing (PTGS) by inhibiting translation of cognate mRNAs and/or promoting mRNA decay (6, 7). esiRNAS can be generated by transcription of sense and antisense transcripts, which can then form dsRNA and get processed by Dicer. The characterization of esiRNAs is currently less advanced (8). They have been cloned from the germ line of mammalian organisms (9–11), and their existence in somatic cells is anticipated (12) because any long double-stranded RNA in a cell could, in principle, be processed by Dicer.

One process that involves long, untranslated RNAs is the silencing of the X chromosome in female mammalian cells. The X inactive specific transcript (*Xist*), is a nuclear RNA transcribed from the inactive X chromosome (Xi). In female mammals, *Xist* RNA is absolutely required for dosage compensation, which is initiated during early embryogenesis (13–15). Upon counting of the X chromosomes, 1 X is designated to remain active (Xa), and *Xist* is transcriptionally up-regulated on the other X, which is destined to be inactivated. The *Xist* RNA accumulates on this chromosome in *cis* and mediates transcriptional gene silencing through unknown mechanisms. Polycomb group (PcG) proteins are recruited to the *Xist* RNA-associated chromosome and establish chromosomewide histone modifications that include histone H3 lysine 27 trimethylation (16, 17). Later in the process, additional chromatin marks, ranging from H3K9 and H4K20 methylation to DNA methylation and histone variant recruitment, become enriched on the Xi and are thought to act in concert to stably maintain the silent state of the Xi in somatic cells (18, 19). Ectopic expression of *Xist* is sufficient to initiate X inactivation because it can induce gene silencing and PcG protein recruitment (16, 20–23). Although the mechanism of PcG protein recruitment to the Xi by *Xist* is not well understood, the generation of esiRNAs formed as a result of intramolecular folding of *Xist* RNA, remains an attractive possibility (10, 11). This hypothesis is reinforced by the observation that PcG protein localization in *Drosophila* depends on an RNAi mechanism (24, 25).

Given its central role in dosage compensation, *Xist* is tightly regulated to ensure inactivation of only 1 of the 2 X chromosomes upon differentiation of embryonic cells. *Xist* action is restricted in *cis* through the action of *Tsix*, an antisense untranslated RNA that is transcribed across the entire *Xist* locus (26–30). *Tsix* is transcribed only during early embryonic development, and is shut off on the Xi and Xa with slightly different kinetics shortly after initiation of X inactivation. Recent data demonstrated that *Tsix* transcription particularly through the *Xist* promoter region is essential for *Xist* silencing and results in the modification of chromatin structure (31–33). In addition, it has also been proposed that *Xist* and *Tsix* can anneal to form a long dsRNA hybrid that can be processed by Dicer, and is important for *Xist* repression (34).

To further address a potential role of the RNAi pathway in the regulation of X inactivation, we studied different aspects of this process in Dicer-deficient embryonic stem (ES) cells. (*i*) To analyze the ability of *Xist* to coat, induce silencing, and recruit PcG proteins, we uncoupled *Xist* expression from *Tsix* control, by expressing the RNA from a tetracycline (tet)-inducible promoter in male ES cells lacking Dicer. (*ii*) To analyze the initiation of X inactivation, we derived female Dicer-deficient ES cell lines, which, upon differentiation, can recapitulate the early steps of random X inactivation that lead to silencing of only 1 X chromosome.

Results

***Xist* RNA Can Coat the X Chromosome and Recruit Polycomb Proteins in the Absence of Dicer.** Induction of *Xist* RNA transcription is sufficient to induce chromosomewide transcriptional repression and changes in chromatin structure in *cis*. Normally, these steps only occur when female ES cells undergo differentiation, but can also be initiated when *Xist* RNA is ectopically expressed from a tetracycline (tet)-inducible promoter in undifferentiated ES cells (23, 35, 36). To directly study the ability of *Xist* RNA to coat, silence, and induce chromatin modifications in the absence of the RNAi machinery, we replaced the endogenous *Xist* promoter with a tet-inducible promoter. Specifically, tet-inducible *Xist* ES cells were engineered by sequential targeting of a reverse tetracycline transactivator (M2rtTA) into the *rosa26* locus and replacement of the endogenous *Xist* promoter with a minimal CMV promoter that carries several tet-response elements [supporting information (SI) Fig. S1]. These gene targetings were originally performed in $dcr^{fl/+}$ and $dcr^{\Delta/\Delta}$ cells

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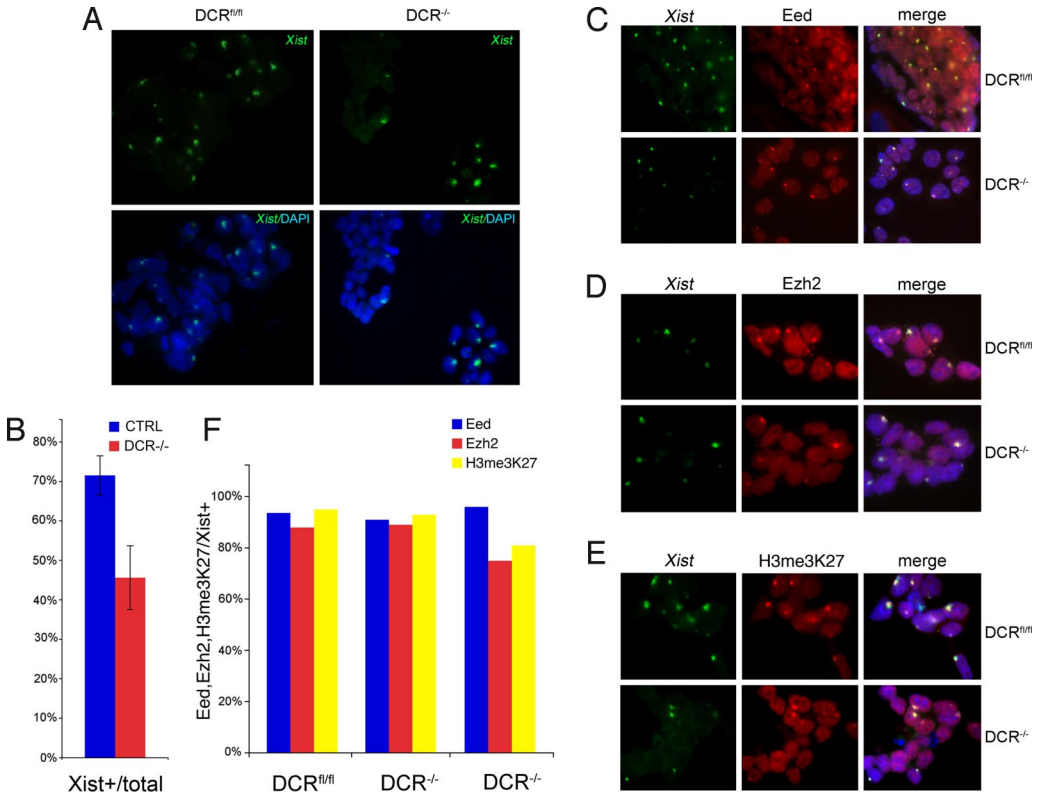

Fig. 1. *Xist* RNA coating and Polycomb protein recruitment in male Dicer KO ES cells forced to express Xist upon dox addition. (*A*) *Upper* images depict representative RNA FISH stainings for *Xist* (green) by using a strand-specific RNA probe in male $dcr^{fl/fl}$ and $dcr^{\Delta/\Delta}$ ES cells carrying the tet-inducible Xist allele, upon dox-induction of *Xist*. *Lower* images represent the merge with Hoechst staining of nuclei (blue). (*B*) Quantitation of the fraction of cells displaying robust *Xist* RNA coating. (*C*) IF/RNA FISH colocalization of *Xist* with Eed (*D*) Ezh2, and (*E*) trimethyl-H3K27, respectively, in dox-induced ES cells. *Left* images depict *Xist* RNA detected by FISH with a strand-specific RNA probe (green). *Middle* images depict immunostaining of indicated antigens (red). *Right* images depict merged images of RNA FISH, immunofluorescence and Hoechst staining. (*F*) Graphs depict quantitation of the percentage of cells with *Xist* RNA coating of the X chromosome that recruit the indicated PcG proteins and H3-m_3K27 to the *Xist* RNA associated area.

and later repeated in $dcr^{f1/f1}$ cells from which new $dcr^{\Delta/\Delta}$ cells were derived by deletion of the *loxP* flanked exons with adenovirally encoded Cre recombinase (Adeno-Cre) as described in ref. 37. The *rosa26-M2rtTA*, tetO-*Xist* promoter targetings and dcr^{f1} deletion were confirmed by Southern blot and PCR analyses (Fig. S1 and data not shown). Addition of doxycycline (dox), a tetracycline analogue, resulted in induction of *Xist* RNA and coating of the X chromosome in both $dcr^{\Delta/\Delta}$ and control cells (Fig. 1*A*), although the percentage of cells that displayed robust *Xist* RNA coating was reduced in Dicer-deficient cells (43% vs. 71%, Fig. 1*B*). No *Xist* coating was detected in the absence of dox. The results were identical with stable and de novo Cre-deleted $dcr^{\Delta/\Delta}$ cells. Furthermore, it is unlikely that there is any residual Dicer protein in these cells because colonies were expanded and harvested at least 2 weeks after Adeno-Cre deletion.

Because *Xist* can coat the X chromosome in Dicer-deficient cells, we analyzed its ability to recruit PcG proteins. These proteins accumulate on the X chromosome as soon as *Xist* RNA coats the chromosome (16, 17). Combined immunofluorescence/RNA fluorescence in situ hybridization (IF/RNA FISH) was performed 24–36 h after dox addition. In both control and Dicer-deficient ES cells, recruitment of the PcG proteins Eed, Ezh2, and Suz12 was observed to a similar extent (Fig. 1 *C* and *D* and data not shown). Approximately 80–90% of cells with *Xist* RNA foci displayed recruitment of these proteins (Fig. 1*F*).

Eed, Ezh2, and Suz12 form a complex that trimethylates histone H3K27 (16, 17) on the Xi. Because the recruitment of these proteins was unaffected in $dcr^{\Delta/\Delta}$ cells, no defect in H3K27 trimethylation (H3me3K27) was anticipated in the absence of Dicer. Indeed, comparable H3me3K27 accumulation was observed upon *Xist* induction in both $dcr^{\Delta/\Delta}$ and control cells (Fig. 1 *E* and *F*). Together, our data indicate that *Xist* RNA can coat the X chromosome in the absence of Dicer and that recruitment of PcG proteins and enrichment of the associated H3K27 methylation does not require the RNAi machinery.

Tet-Inducible *Xist* Can Silence X-Linked Genes in Dicer-Deficient Cells. It has been shown that *Xist* RNA coating is sufficient to initiate transcriptional silencing of X-linked genes and exclude the active form of RNA polymerase II (Pol II) from the *Xist*-coated territory (23, 38). To learn whether gene silencing is normal in Dicer-deficient male ES cells in which *Xist* RNA is induced by dox, we performed immunofluorescence (IF) with an antibody against the elongating form of Pol II (phosphorylated on Ser-2) followed by *Xist* RNA FISH. In all cells visualized, there was exclusion of Pol II phospho-Ser-2 from the *Xist* RNA territory (Fig. 2*A*), suggesting that the RNAi machinery is not required for *Xist* RNA-mediated initiation of silencing. Transcriptional silencing of X-linked genes was confirmed by RNA FISH for the nascent X-linked transcript *Pgk1*. Before dox addition, *Pgk1* nascent RNA foci could be detected in 95% of the control and ≈75% of the $dcr^{\Delta/\Delta}$ cells. After dox addition, *Pgk1* RNA foci could only be detected in 42% and ≈37% of the same cells (Fig. 2 *B* and *C*), demonstrating that *Pgk1* silencing can be induced equally well by *Xist* coating with or without Dicer.

In addition, silencing of *Pgk1* and another X-linked gene, *Hprt*, and *Xist* RNA induction were monitored by real-time (RT)-PCR (Fig. 2 *D* and *E*). After dox addition, *Xist* RNA was up-regulated and *Pgk1* and *Hprt* became repressed. The down-regulation of *Pgk1* or *Hprt* was not complete, but correlated well with the number of cells that displayed *Xist* RNA coating. For example, in control cells we observed *Xist* coating in ≈70% of the cells and *Hprt*/*Pgk1* levels were reduced to ≈40%, whereas in $dcr^{\Delta/\Delta}$ cells *Xist* coating was observed in ≈40% of the cells and X-linked gene expression was reduced to ≈60% (compare Fig. 2 *C* to *D*). *Xist* RNA steady-state levels were consistently higher in the $dcr^{\Delta/\Delta}$ cells but that did not result in an increase in the number of cells with *Xist* RNA coating (Figs. 1*B* and 2 *C* and *E*). Interestingly, transcription from the *Xist* locus was comparable in $dcr^{f1/f1}$ and $dcr^{\Delta/\Delta}$ cells because the levels of unspliced *Xist* mRNA were similar in both cell types (intron 3 RT-PCR in Fig. 2*E*), suggesting that a step downstream of *Xist*

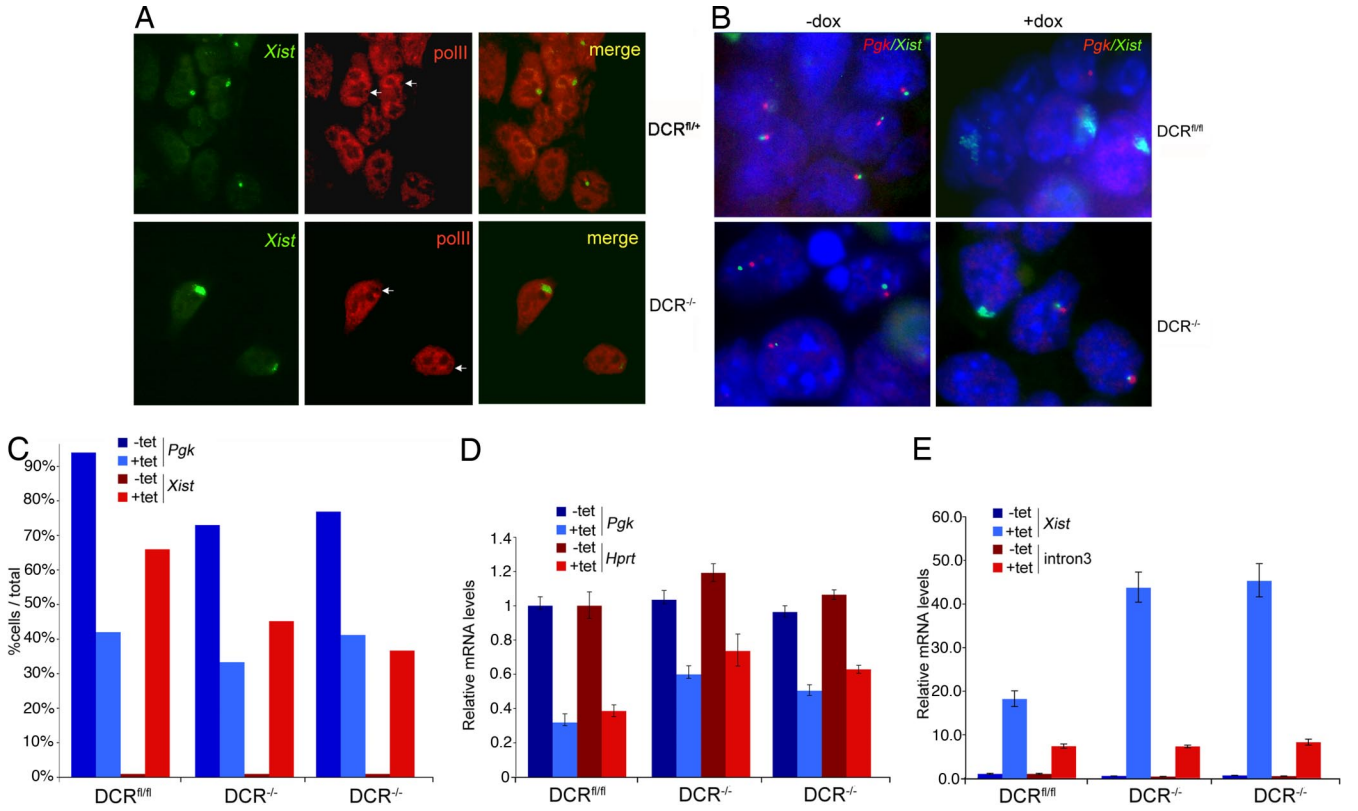

Fig. 2. Tet-induced *Xist* mediates gene silencing in male Dicer-deficient ES cells. (*A*) FISH staining for *Xist* RNA using a single-stranded RNA probe (green) and coimmunostaining of the elongating form of RNA-polymeraseII (pol II phospho-Ser-2) (red). Exclusion from the *Xist* RNA-coated territory is indicated by arrows. (*B*) FISH for *Pgk1* nascent primary transcripts (red) and *Xist* (green) in uninduced (−dox, *Left* images) and induced ES cells (+dox, *Right* images) using double stranded DNA probes. The *Xist* pinpoint signal seen is the result of *Tsix* RNA because we did not observe this signal when strand specific *Xist* RNA probes were used. Nuclei were counterstained with Hoechst 33342 (blue). (*C*) Graphs depict quantitation of the percentage of total cells which display a focus of *Pgk1* nascent transcripts (blue bars) or *Xist* RNA coating (red bars) before (−tet) and after (+tet) dox addition. (*D*) qRT-PCR analysis of *Pgk1* (blue bars) and *Hprt* (red bars) mRNA levels before (−tet) and after (+tet) dox addition (*E*) qRT-PCR analysis of spliced (blue bars) or unspliced *Xist* transcripts (intron3; red bars) before and after dox addition. *Middle* and *Right* images in *C* through (*E*) represent 2 different Dicer-deficient clones.

splicing or processing is differentially affected by Dicer deletion. However, our data show that establishment of a silent *Xist* RNA-coated chromosomal territory is not dependent on Dicer.

Inbred Female Dicer-Deficient Cells Lack 2 X Chromosomes and Cannot Be Used as a Model System for X Inactivation. The tet-inducible *Xist* expression system in male ES cells allowed us to monitor X chromosome silencing in the absence of Dicer downstream of transcriptional up-regulation of *Xist*. However, in female cells, multiple regulatory processes ensure inactivation of only 1 and not both X chromosomes (15). Female ES cells have been extensively used for the study of X inactivation. Upon differentiation, *Xist* is up-regulated on 1 X chromosome leading to inactivation of this chromosome, whereas *Xist* on the other chromosome is turned off. To address a potential role for the RNAi pathway in the context of X inactivation in female ES cells, we derived female $dcr^{fl/fl}$ ES cells from blastocysts of $dcr^{fl/fl}$ mice of the C57BL/6 background. Female ES cells that are $dcr^{\Delta/\Delta}$ were subsequently generated by Cre-mediated deletion. Cells were screened for the deletion of the *loxP*-flanked *dcr* sequences by PCR (data not shown). The presence of 2 X chromosomes was determined by real-time PCR of genomic DNA, which allowed us to calculate the X to autosome ratio (X:A). Only clones with an X:A ratio of approximately 1, which have 2 X chromosomes, were used in further experiments (Fig. S2).

Because we and others have reported that differentiation of Dicer-deficient ES cells is compromised after embryoid body formation and outgrowth (37), we instead attempted to differentiate the cells by withdrawal of leukemia inhibitory factor (LIF) and exposure to retinoic acid (RA), a strong inducer of differentiation. In fact, both $dcr^{fl/fl}$ and $dcr^{\Delta/\Delta}$ cells responded to RA by down-regulating the ES cell markers *Oct4*, *Nanog*, and *Klf4* and up-regulating endoderm differentiation markers, such as *Hnf4* and *Gata4* (Fig. 3*A*). We also observed morphological changes consistent with cellular differentiation (Fig. 3*C*), demonstrating that Dicer-deficient ES cells efficiently respond to this differentiation protocol. In addition, *Tsix* RNA was appropriately down-regulated after addition of RA in cells of both genotypes (Fig. 3*B* and Fig. S3). However, unlike in the case of control $dcr^{fl/fl}$ ES cells, we could never detect up-regulation of *Xist* RNA by RT-PCR or *Xist* coating by RNA FISH in the female $dcr^{\Delta/\Delta}$ cells (Fig. 3 *B* and *D*).

It has been reported that Dnmt3 levels are reduced in Dicer-deficient ES cells (39, 40). Low levels of Dnmt3 are associated with global hypomethylation of genomic DNA and possibly an unstable karyotype of female ES cells, resulting in frequent loss of 1 X chromosome (41). Because the absence of Dicer could therefore lead to a pronounced XO karyotype, which cannot initiate X inactivation, we wanted to confirm that the clones used in the differentiation assays carried 2 X chromosomes. DNA FISH for the X chromosome by using X chromosome "paint" was performed 4 days after RA exposure in control and Dicer-deficient ES cells. Whereas 50% of the $dcr^{fl/fl}$ cells were XX (Fig. 3*E*), much to our surprise, the large majority of $dcr^{\Delta/\Delta}$ cells contained only 1 X chromosome. Thus, the absence of *Xist* RNA up-regulation and coating in Dicer-deficient ES cells could be attributed to the absence of a second X chromosome.

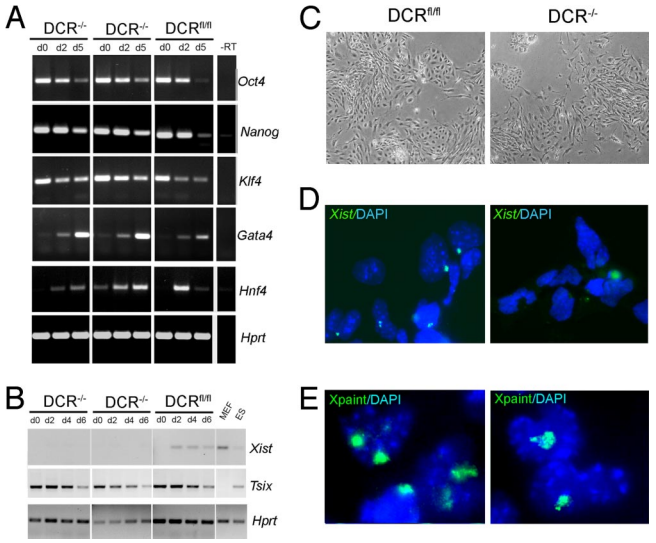

Fig. 3. Absence of X-inactivation in female C57BL/6 inbred Dicer KO ES cells. (*A*) Semiquantitative RT-PCR analysis of mRNA levels of ES cell markers (*Oct4*, *Nanog*, *Klf4*) and differentiation markers (*Hnf4*, *Gata4*) at the indicated day of RA-induced differentiation. *Hprt* served as loading control. (*B*) As in *A* except that *Xist* and *Tsix* RNA strand specific reverse transcription reactions were performed. (*C*) Phase contrast images depicting the morphological changes induced in ES cells after 4 days in RA-containing medium. (*D*) FISH for *Xist* RNA 6 days after RA addition by using a single stranded RNA probe, merged with Hoechst staining of nuclei. (*E*) Images depict FISH for the X chromosome in differentiating cultures 4 days after RA addition (green = X paint) counterstained with Hoechst.

Initiation of X Inactivation in Female C57BL/6 × CAST/Ei Dicer-Deficient ES Cells. Because loss of 1 X chromosome could account for the apparent defect in X inactivation, we attempted to derive female hybrid ES cells, which are known to be more karyotypically stable (41). $dcr^{fl/+}$ inbred mice (which are C57BL/6) were backcrossed once to outbred CAST/Ei animals, and ES cells were derived by intercrossing the progeny (F_2 cells). The resulting lines were screened using a small nucleotide polymorphism (SNP) in the X-inactivation center (XIC) to test for the presence of 1 C57BL/6 and 1 CAST/Ei allele. We succeeded in identifying 2 such female lines that were also $dcr^{fl/fl}$. We deleted the dcr^{fl} alleles in both these lines and were thus able to generate $dcr^{\Delta/\Delta}$; $X^{CAST/Ei}X^{C57BL/6}$ ES cell clones (Fig. 4*A*). At least 2 $dcr^{\Delta/\Delta}$ clones from each line were used for subsequent analyses. Dicer protein ablation was confirmed by Western blot at the time cells were used to set up differentiations (Fig. S4). In agreement with the notion that loss of Dicer affects the stability of the XX karyotype, only 50% of $dcr^{\Delta/\Delta}$ clones had 2 X chromosomes vs. 82% in the undeleted $dcr^{fl/fl}$ or $dcr^{fl/\Delta}$ cells as assessed by the XIC SNP PCR (data not shown). RA-directed differentiation of these cells was undertaken immediately after dcr^{fl} deletion to avoid progressive loss of the second X chromosome because of prolonged culturing. As expected, C57BL/6 × CAST/Ei hybrid cells differentiated properly in response to RA because *Oct4* and *Nanog* mRNA were significantly down-regulated (Fig. 4*B*).

DNA (X paint/XIC probe) and *Xist* RNA FISH were subsequently performed. DNA FISH showed that ≈70% of the $dcr^{fl/fl}$ and ≈50% of the $dcr^{\Delta/\Delta}$ cells contained 2 X chromosomes after 8 days of RA treatment (Fig. 4*C*), confirming the PCR results. RNA FISH analysis revealed coating by *Xist* RNA in both control and Dicer-deficient female ES cells (Fig. 4*D*). RT-PCR confirmed the presence of *Xist* transcripts in both cases (Fig. 4*E*). Surprisingly, the level of *Xist* RNA was much higher in the $dcr^{\Delta/\Delta}$ than in the $dcr^{fl/fl}$ cells even before differentiation. It is unlikely that this reflects precocious differentiation of Dicer-deficient cells, because the levels of *Oct4* and *Nanog* were comparable with $dcr^{fl/fl}$ controls. In fact, *Xist* RNA coating was detected in ≈5% of $dcr^{\Delta/\Delta}$ cells even before differentiation, whereas <1% of $Xist^{+}$ cells were observed in the $dcr^{fl/fl}$ controls. However, on differentiation (d4) ≈15% of the cells displayed *Xist* RNA coating in both cultures. In addition both $dcr^{fl/fl}$ and $dcr^{\Delta/\Delta}$ $Xist^{+}$ cells were able to recruit Ezh2 and exclude the elongating form of RNA Pol II from the Xist-coated territory (Fig. 4*F* and Fig. S5). Taken together, our data indicate that there is no major defect in the X-inactivation process in female ES cells upon Dicer ablation.

Discussion

The RNAi pathway has been implicated in RNA-mediated transcriptional gene silencing in many organisms, including mammals. The possibility that *Xist* RNA could function through a small RNA effector molecule was an attractive hypothesis. Although Dicer has no obvious role in maintaining *Xist* RNA coating and the silent state of the Xi in T cells (42), these observations did not rule out the possibility that the process of initiating X inactivation during early embryogenesis or the recruitment of PcG proteins is Dicer dependent. In this regard, it was recently reported that *Xist*/*Tsix* RNA duplexes exist and can be processed by Dicer into small RNAs, called xiRNAs, that may function in the process of X inactivation (34).

However, by using a "Tet-on" system that allows *Xist* induction from the endogenous locus, we found that neither *Xist* RNA coating nor silencing of the X chromosome required processing by Dicer. These results were identical irrespective of whether cells in which both dcr^{fl} alleles were deleted by Cre recombinase and passaged for multiple generations or de novo deleted cells were analyzed. Therefore, it is unlikely that any of the observed phenotypes could be the result of secondary mutations acquired during prolonged culture or residual Dicer protein sustaining X inactivation. In keeping with the conclusion that X inactivation in this experimental setting is not dependent on the function of a canonical RNAi system, no small RNAs from the dox-induced control ES cells could be detected by Northern blot analysis (data not shown). However, we cannot rule out limitations in the sensitivity of the assay, the probes used, or the timing of the analysis, because Ogawa *et al.* were able to show evidence of putative Dicer-processed products of *Xist*/*Tsix* RNA duplexes (34). Our results suggest a more subtle role for Dicer in the X-inactivation process, because the proportion of $dcr^{\Delta/\Delta}$ cells that displayed *Xist* RNA coating was significantly lower compared with that of the controls ($P < 0.001$), even though *Xist* RNA levels were higher in the former setting. This could indicate a modest influence of Dicer on the efficiency of *Xist* coating, which warrants further analyses. This notwithstanding, once *Xist* RNA coats the X chromosome, recruitment of the PcG protein histone modifiers (Eed, Ezh2, and Suz12) was unaffected by Dicer ablation, as was the trimethylation of H3K27, which is a hallmark of *Xist* RNA-mediated chromosome silencing. Similarly, transcriptional repression of X-linked genes and exclusion of the transcriptional machinery from the *Xist* RNA-coated territory were normal in Dicer-deficient ES cells.

In addition to the forced expression system in male ES cells, female ES cells, which recapitulate the in vivo process of X inactivation, were analyzed for their ability to initiate this process in the absence of Dicer. In C57BL/6 Dicer-deficient female ES cells, we were unable to detect *Xist* RNA induction or coating, but these cells are karyotypically unstable, and only rarely retain 2 X chromosomes. Because *Xist* RNA coating was only induced in a small percentage of cells even in the Dicer-proficient cultures, we strongly suggest that the observed lack of X inactivation is simply a product of X chromosome loss. For this reason, we derived hybrid CAST/Ei × C57BL/6 ES cells. These cells maintained 2 X chromosomes both in the Dicer-proficient and -deficient state in a large fraction of the population. Importantly, *Xist* RNA induction was normal after Dicer ablation, and *Xist* RNA coating was detected in $dcr^{\Delta/\Delta}$ subclones just like in control cells. Surprisingly, *Xist* RNA levels

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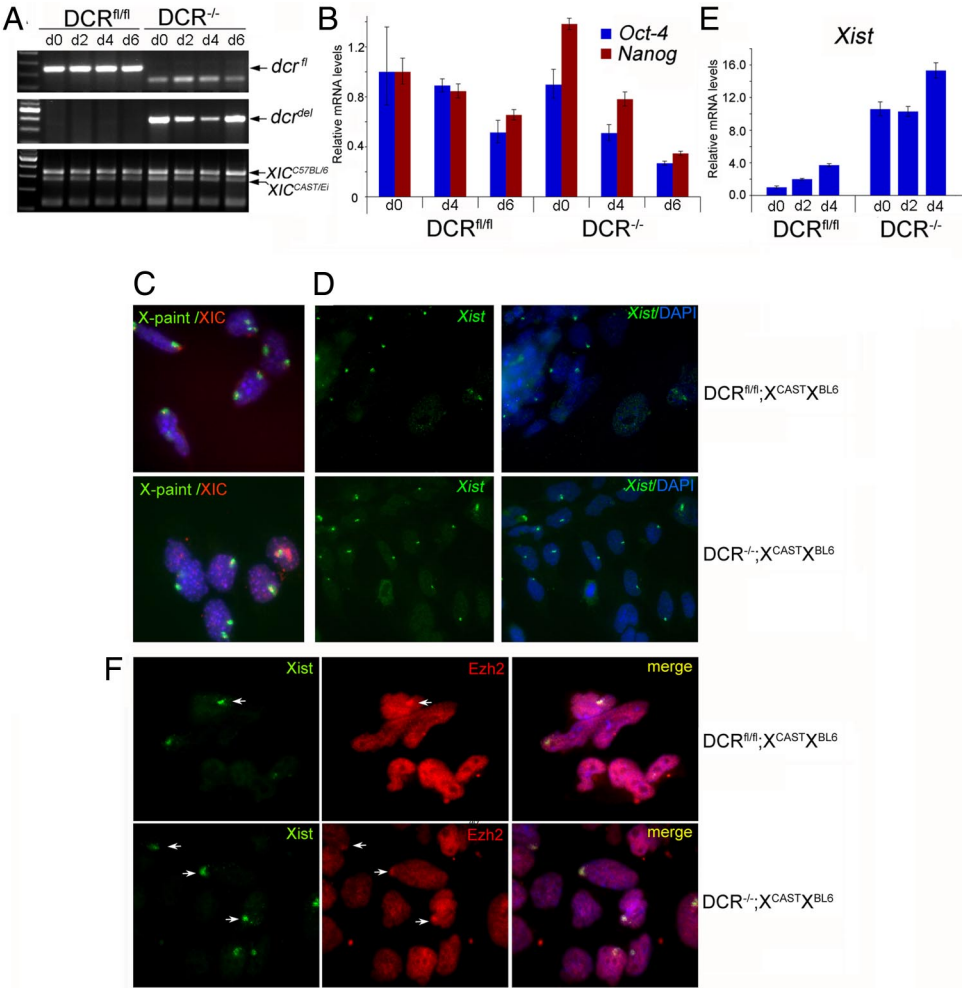

Fig. 4. X-inactivation in female hybrid CAST/Ei xC57BL/6 Dicer KO ES cells. (*A*) PCR analysis of genomic DNA for the presence of dcr^{fl}, dcr^{Δ} and CAST and C57BL/6 SNP alleles (SNP RS29080486). (*B*) qRT-PCR for *Oct4* and *Nanog* mRNA before (d0) and after RA exposure (d2 and d6). (*C*) DNA FISH indicating the presence of 2 X chromosomes in differentiating ES cells 8 days after RA exposure using X paint (green) and XIC probe (red). (*D*) *Left* images, FISH for *Xist* RNA (green) with a single-stranded RNA probe in RA-treated $dcr^{fl/fl}$ and $dcr^{\Delta/\Delta}$ cultures at day 6 of differentiation. *Right* images depict merged images of *Xist* RNA (green) and nuclear Hoechst staining (blue). (*E*) Strand-specific qRT-PCR for *Xist* RNA before (d0) and after RA exposure (d2 and d4). (*F*) Combined IF-FISH for *Xist* RNA (green, *Left* images) and Ezh2 (red, *Middle* images) in RA-treated $dcr^{fl/fl}$ and $dcr^{\Delta/\Delta}$ cultures at day 2 of differentiation. *Right* images depict merged images of *Xist* RNA (green), Ezh2 (red) and nuclear Hoechst staining (blue). Arrows indicate regions of Xist/Ezh2 co-localization.

were significantly higher in female $dcr^{\Delta/\Delta}$ cultures, even before differentiation, which appears to lead to coating of the X chromosome in the undifferentiated state (data not shown). This observation is in accordance with a recent publication by Nesterova *et al.*, which showed that Xist promoter methylation is reduced in male Dicer-deficient ES cells (43). Higher *Xist* RNA levels were also observed after dox induction. In the tet-inducible system, the levels of unspliced *Xist* RNA were not affected, suggesting that there is no difference in transcription but perhaps in the processing or stability of *Xist* RNA. The increased level of *Xist* RNA in Dicer-deficient cells might be related to a function of the antisense RNA *Tsix*. Upon deletion of *Tsix*, *Xist* RNA levels increase and coating by *Xist* RNA can sometimes be observed before differentiation (44). It is possible that only mature *Xist* transcripts are directly processed by Dicer (34). Alternatively, nuclear mRNA degradation pathways have recently been implicated in the regulation of spliced *Xist* RNA levels (45) and might have links to the RNAi system (46).

Our results differ from those reported by others who suggest that Dicer is required for initiation of X inactivation in female ES cells (34). We present evidence that Dicer function is not required for this process in the ES cells studied here but we cannot rule out the possibility that, when forced to differentiate by a different methodology or when ES cells of a different genetic background are analyzed, Dicer would play a role in this process. In addition the observed increase in X chromosome loss in both C57BL/6 and CAST/Ei × C57BL/6 Dicer-deficient ES cells may provide another explanation for the observed X-inactivation defect by Ogawa *et al.*, because their cells were not screened for the presence of 2 X chromosomes by DNA FISH after differentiation. In support of our data, Dicer does not play an essential role in the maintenance of X inactivation, given that the Xi was readily detected in T lymphocytes from mice that were rendered Dicer deficient in cells of the T cell lineage (42), nor in Dicer-deficient embryos (43). Furthermore, Ago2-deficient mouse embryos do not show any evidence of defective X inactivation (47). This could be the result of genetic redundancy with other Ago family proteins (Ago1, −3, and −4), although Ago2 is the only member of this family with demonstrable "slicer" activity (48). Ago slicer activity is required for RNAi-mediated transcriptional gene silencing (48).

Collectively, our data indicate that Dicer does not play an essential role in the process of X inactivation. However, *Xist* RNA stability and its ability to coat the X chromosome are affected by Dicer loss. The effect of Dicer ablation on generation of miRNAs, up-regulation of miRNA targets, a link to the normal RNA degradation/processing machinery and direct cleavage of *Xist* transcripts could all account for the observed phenotype. Further analyses are necessary to elucidate a possible interplay of Dicer and *Xist* RNA processing and the physiological significance of such an interaction.

Materials and Methods

ES Cell Culture, Derivation, and Differentiation. ES cells were cultured as described in ref. 37. Briefly, the cells were maintained on a monolayer of irradiated male mouse embryonic fibroblasts (MEFs) in the presence of LIF (Chemicon). Expression from the tet-inducible promoter was induced by addition of dox (2 μg/ml) for 24–36 h. For ES cell differentiation, feeder cells were removed by preplating, and ES cells were plated on gelatin coated coverslips and differenti-

ated by withdrawal of LIF and treatment with 1 μM all-*trans*retinoic acid (Sigma) for the indicated number of days. ES cells were derived from blastocysts in the presence of the MEK-1 inhibitor (PD98059, Cell Signaling) at a final concentration of 50 μM to prevent differentiation.

RNA Preparation and RT-PCR. Total RNA was prepared using TRIzol reagent according to the manufacturer's instructions (Invitrogen). RNA was treated with DNase I (Invitrogen) for 1 h at 37°C, and cDNA was prepared using a First Strand synthesis kit (Invitrogen) using strand-specific primers or random hexamers. A list of all primers used is provided in *SI* Table S1. Relative transcript levels were estimated by qPCR using the comparative C_T ($\Delta\Delta$CT) method (49), with either SYBR Green (BioRad) or TaqMan probes (Applied Biosystems) for signal detection. The ribosomal protein gene *RPLP0* (36B4) was used for input RNA normalization unless otherwise indicated.

RNA and DNA Fluorescence in Situ Hybridization. RNA FISH was preformed as described in ref. 50 using double-stranded DNA probes for *Xist* and *Pgk1* or a strand-specific RNA probe for *Xist*. The double-stranded *Pgk1* DNA probe was generated with the Vysis Nick translation kit (Abbott Molecular) using the pCAB17 plasmid as a template; the *Xist* DNA probe was prepared using the Bioprime kit (Invitrogen) with the *Xist* cDNA as template, and the fluorescein-labeled (Roche) *Xist*-specific RNA probe by in vitro transcription. Cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100, dehydrated through a 70–80–90–100% EtOH series and air dried. Probes were added to the dry coverslips immediately after denaturation at 85°C for 10 min. DNA FISH probe (XIC probe) was prepared using an XIC containing BAC (RP23–338B22) and labeled with the Bioprime labeling kit and Cy3-dUTP (Amersham). The XIC BAC probe was mixed with X paint (Cambio) for combined X paint/XIC DNA FISHes. Samples were prepared as for RNA FISH but were denatured for 25 min at 80°C in 50% formamide, 2xSSC, 2 mM Na_2HPO_4. Hybridization was performed overnight at 37°C in a humidified chamber and washes were done according to the X paint protocol. Nuclei were counterstained for 2 min with Hoechst 33342 (Invitrogen) before mounting with Vectashield (Vector Labs).

Immunofluorescence and Antibodies. Cells were washed 2 times with PBS, fixed in 4% PFA, and permeabilized with 0.5% Triton X-100. Cells were then incubated in blocking solution (PBS-T, 50 mM glycine, BSA, gelatin) for 30 min at room temperature. Primary antibodies against Ezh2 (BD Biosciences, no. 612666), H3me3K27 (Upstate no. 05–851), RNA Pol II phospho-Ser-2 (H5, Covance no. MMS-129R), and Eed (51) were diluted in blocking buffer at appropriate concentrations. Fluorescein or rhodamine-coupled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

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