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RNF12 Activates *Xist* and Is Essential for X Chromosome Inactivation

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Abstract

In somatic cells of female placental mammals, one of the two X chromosomes is transcriptionally silenced to accomplish an equal dose of X-encoded gene products in males and females. Initiation of random X chromosome inactivation (XCI) is thought to be regulated by X-encoded activators and autosomally encoded suppressors controlling *Xist*. Spreading of *Xist* RNA leads to silencing of the X chromosome in *cis*. Here, we demonstrate that the dose dependent X-encoded XCI activator RNF12/RLIM acts in *trans* and activates *Xist*. We did not find evidence for RNF12-mediated regulation of XCI through *Tsix* or the *Xist* intron 1 region, which are both known to be involved in inhibition of *Xist*. In addition, we found that *Xist* intron 1, which contains a pluripotency factor binding site, is not required for suppression of *Xist* in undifferentiated ES cells. Analysis of female *Rnf12*$^{-/-}$ knockout ES cells showed that RNF12 is essential for initiation of XCI and is mainly involved in the regulation of *Xist*. We conclude that RNF12 is an indispensable factor in up-regulation of *Xist* transcription, thereby leading to initiation of random XCI.

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Introduction

X chromosome inactivation (XCI) in placental mammals is random with respect to the parental origin of the X chromosome that undergoes inactivation, during early embryonic development [1]. In contrast, in marsupials and mouse extra-embryonic tissues XCI is imprinted. Imprinted XCI always targets the paternally inherited X chromosome (Xp), and is initiated during the early cleavage divisions [2,3,4]. In the inner cell mass (ICM) of the mouse blastocyst, the inactive X chromosome is reactivated, after which random XCI is initiated around 5.5 days of embryonic development.

In mouse, two non-coding X-linked genes, *Xist* and *Tsix*, play a central role in the random XCI mechanism. Upon initiation of XCI, *Xist* is up-regulated on the future inactive X chromosome (Xi), and the transcribed RNA spreads along the X in *cis*, directly and indirectly recruiting chromatin modifying enzymes acting to establish the Xi [5,6,7]. *Tsix* is a negative regulator of *Xist*; the *Tsix* gene overlaps with *Xist* but is transcribed in the anti-sense direction [8,9].

Random XCI is a stochastic process in which each X chromosome has an independent probability to become inactivated [10,11]. Initiation of XCI is thought to be regulated by X-encoded activators and autosomally encoded inhibitors [11,12]. With two active X chromosomes, female cells will have a concentration of XCI activators two-fold higher than male cells, sufficiently different to drive XCI in female cells only. Rapid down-regulation of XCI activator genes in *cis*, after initiation of XCI on either one of the X chromosomes, prevents initiation of XCI on the second X chromosome.

XCI inhibitors are involved in maintaining a threshold for XCI to occur. So far, several XCI inhibitors have been identified, acting through different mechanisms, in mouse. YY1 and CTCF act as positive regulators of *Tsix*, by binding the DXpas34 *Tsix* regulatory element [13]. The pluripotency factors OCT4, SOX2 and NANOG were proposed to regulate XCI by binding to intron 1 of *Xist* and suppressing *Xist* expression directly [14]. OCT4 and SOX2 have also been implicated in the positive regulation of *Tsix* and *Xite*, the latter being an enhancer of *Tsix* [15]. These findings indicate that several proteins and pathways act in concert to suppress *Xist* transcription and to block *Xist* RNA spreading in *cis*.

XCI activators could act by activation of *Xist*, but also by suppression of negative regulators of *Xist* such as *Tsix* and the *Xist* intron 1 region. Recently, we identified RNF12 (RLIM) as the first X-linked activator of XCI [16]. This E3 ubiquitin ligase is involved in regulation of LIM-homeodomain transcription factors and telomere length homeostasis, through degradation of LDB1 and TRF1, respectively [17,18]. Previously, we found that additional transgenic copies of the *Rnf12* gene encoding this protein resulted in induction of XCI on the single X in transgenic male cells, and on both X chromosomes in a high percentage of female cells. XCI was also affected in *Rnf12*$^{+/-}$ ES cells supporting a dose-dependent role for RNF12 in activation of XCI. In the present study, we aimed to dissect the role of RNF12 in XCI, and we obtained evidence that RNF12 regulates XCI *in trans*, by

Author Summary

In all placental mammals, the males have only one X chromosome per diploid genome, as compared to the females who have two copies of this relatively large chromosome, carrying more than 1,000 genes. Hence, the evolution of the heterologous XY sex chromosome pair has resulted in an inevitable need for gene dosage compensation between males and females. This is achieved at the whole-chromosome level, by transcriptional silencing of one of the two X chromosomes in female somatic cells. Initiation of X chromosome inactivation (XCI) is regulated by X-encoded activators and autosomally encoded suppressors controlling *Xist* gene transcription. Spreading of *Xist* RNA in *cis* leads to silencing of one of the X chromosomes. Previously, we obtained evidence that the X-encoded E3 ubiquitin ligase RNF12 (RLIM) is a dose-dependent XCI activator. Here, we demonstrate that RNF12 exerts its action in *trans* and find that RNF12 regulates XCI through activation of transcription from the *Xist* promoter. Furthermore, analysis of female *Rnf12*$^{-/-}$ knockout ES cells shows that RNF12 is essential for initiation of XCI and that loss of RNF12 resulted in pronounced and exclusive down-regulation of *Xist*. It is concluded that RNF12 is an indispensable factor in *Xist* transcription and activation of XCI.

activation of the *Xist* promoter. In addition, the generation and analysis of *Rnf12*$^{-/-}$ ES cells indicated that RNF12 is required for the XCI process and appears to be involved in XCI mainly by activation of *Xist*. The results reinforce that RNF12 is a key player in regulation of the XCI process.

Results

RNF12 acts in *trans* to activate XCI

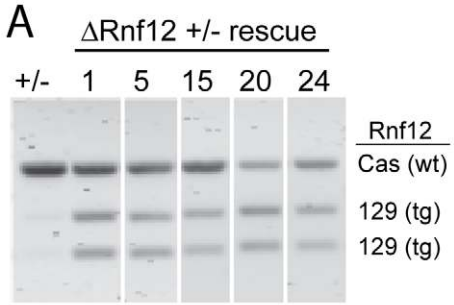
XCI is regulated by several *cis* elements, and *Rnf12* is located in close proximity to *Xist* (~500 kb). Therefore, we aimed to test whether all the activity of RNF12 is mediated in *trans*. Our previous studies showed that *Rnf12*$^{+/-}$ female ES cells induce XCI in a reduced number of ES cells. Here, we rescued 129/Sv/Cast/Ei (129/Cas) polymorphic *Rnf12*$^{+/-}$ female ES cells by introducing a 129 BAC (RP24-240J16) construct covering *Rnf12*. RT-PCR analysis followed by RFLP detection confirmed expression of the transgenic copies of *Rnf12* (Figure 1A). *Xist* RNA-FISH analysis, to detect the *Xist* coated inactive X chromosome (Xi) in day 3 differentiated transgenic ES cell lines with one additional copy of *Rnf12*, shows that XCI was restored to wild type level (Figure 1B). In line 20, with 5 transgenic copies of *Rnf12* the percentage of cells with one or two Xi's is even more pronounced, supporting a dose dependent role of RNF12 in XCI (Figure 1B, 1C). XCI is skewed in wild type 129/Cas female ES cells towards inactivation of the 129 X. This is due to the presence of different X-linked *cis* elements (Xce) that affect random choice [19]. RT-PCR detecting a length polymorphism was used to distinguish *Xist* emanating from either the 129 or the Cas alleles. We observed that skewed XCI is more pronounced in the *Rnf12*$^{+/-}$ cells, as compared to XCI in wild type cells at day 3 of differentiation (Figure 1D). This could be caused by selection against cells inactivating the wild type X chromosome, which would result in complete loss of RNF12 from these cells. However, RNF12 possibly is not essential for cell survival, also of differentiated cells, so that selection against cells inactivating the wild type X chromosome might point to a role for RNF12 in maintaining *Xist* expression. In the rescued cell lines, *Xist* was up-regulated from both alleles at day 3 of differentiation (Figure 1D). This result demonstrates that RNF12 activates XCI in *trans*.

Counteracting roles for RNF12 and NANOG

One possible mechanism for regulation of XCI by RNF12, might be a direct interaction with *Xist* RNA to target chromatin components. However, examination of day 3 differentiated female cells by immunocytochemistry detecting RNF12, together with the Polycomb protein SUZ12 which accumulates on the Xi [20,21], excludes this possibility (Figure 2A). Interestingly, we noticed that the RNF12 staining intensity was much more dynamic in female compared to male cells (Figure 2B, Figure S1). Also, in female cells, a SUZ12 coated Xi appeared mainly in cells with low RNF12 staining (Figure 2A, Figure S2, and data not shown). Immunostaining of differentiating female ES cells indicated a negative correlation between expression of RNF12 and NANOG, although expression was not completely mutually exclusive (Figure 2C). To analyze this in more detail, we targeted an *Rnf12* promoter-mCherry construct into ES cells, also harboring a knock-in GFP transgene in the *Nanog* and *Oct4* loci. We analyzed expanded individual clones and pooled clones and obtained similar results. FACS analysis, prior to differentiation and at different time points after differentiation of these double transgenic ES cell lines, showed a negative correlation between RNF12-mCherry and NANOG-GFP expression, but not for RNF12-mCherry and OCT4-GFP (Figure 2D, 2E, Figure S3). Our findings therefore suggest specific counteracting regulatory roles for RNF12 and NANOG in XCI, which might include an inhibitory effect of NANOG on *Rnf12* transcription. Interestingly, NANOG has been implicated in the regulation XCI by direct suppression of *Xist* in ES cells, and *Xist* suppression in the ICM of the developing blastocyst corresponds with up-regulation of NANOG expression [22]. Therefore, mutual exclusive expression of RNF12 and NANOG may be required for initiation of XCI.

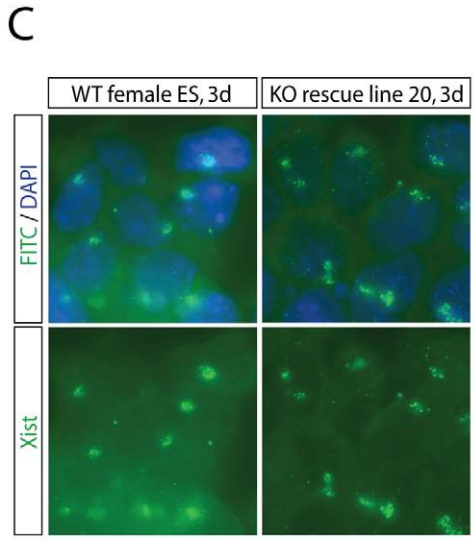
RNF12 does not regulate XCI through *Xist* intron 1

Recently, the first intron of *Xist* has been identified as a region involved in recruitment of three pluripotency factors, OCT4, NANOG and SOX2 [14]. It was shown that down-regulation of *Nanog* and *Oct4*, through gene ablation, resulted in an increase in *Xist* expression, and initiation of XCI in male cells. Interestingly, the intron 1-mediated suppression of XCI was suggested to directly act on *Xist*, without involvement of *Tsix*. To study if RNF12 might regulate XCI by interfering with binding of pluripotency factors to the intron 1 region of mouse *Xist*, we removed 1.2 kb of *Xist* intron 1 including all reported NANOG, OCT4 and SOX2 binding sites by homologous recombination with a BAC targeting construct, without disturbing the integrity of the *Xist* transcript. Targeted clones were screened by PCR amplification of a targeted RFLP (BsrgI) in female F1 2-1, 129/Cas polymorphic ES cells, which was confirmed by Southern blotting, followed by Cre mediated loop-out of the kanamycin/neomycin resistance cassette (Figure 3A, Figure S4). *Xist* RNA FISH at different time points of differentiation of several *Xist*$^{\text{intron1}+/-}$ ES cell lines indicated that XCI is initiated with the same kinetics as in wild type cells, and showed that the intron 1 region is not required for repression of *Xist* in undifferentiated ES cells or early during initiation of XCI (Figure 3B, 3C, and Figure S4G). Nevertheless, *Xist* specific RT-PCR, detecting a length polymorphism distinguishing 129 and Cas *Xist*, showed enhanced skewing at day 3 of differentiation towards 129 *Xist* expression, suggesting a role for the intron 1 region in suppressing *Xist* at later stages of differentiation, when NANOG, OCT4 and SOX2 are expressed at a lower level (Figure 3D). To test an involvement of the intron 1 region in RNF12-mediated activation of XCI, we



B

line	qPCR copy nr	RNA FISH day 3 1xXist	RNA FISH day 3 2xXist
ΔRnf12+/-	1	33%	0%
wild type	2	51%	1%
1	2	42%	0%
5	2	65%	5%
15	2	58%	2%
20	6	78%	17%
24	2	64%	4%



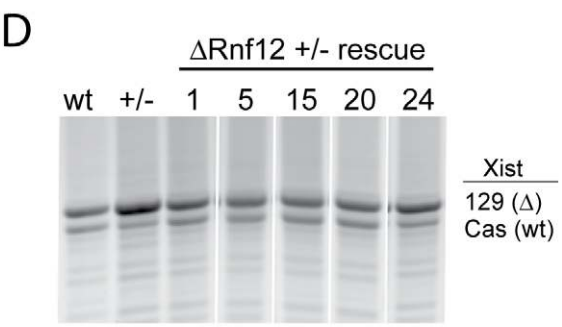


Figure 1. RNF12 activates X chromosome inactivation in *trans*. A) Allele specific RT-PCR analysis of *Rnf12* expression with RNA isolated from day 3 differentiated female *Rnf12* $^{+/-}$ ES cells (Cas/129, 129 *Rnf12* targeted), and rescued cell lines obtained after stable integration of an 129 *Rnf12* transgene. NheI digested 129 products were separated from undigested Cas products. B) Overview of RNA-FISH experiments detecting *Xist* expression in female wild type, *Rnf12* $^{+/-}$ and *Rnf12*$^{+/-}$ rescued cell lines. qPCR copynumber analysis was performed on genomic DNA. RNA-FISH analysis was performed on day 3 differentiated ES cells, and the percentage of cells harbouring one *Xist* coated X chromosome (*Xist* cloud (=Xi), 1x *Xist*) or two *Xist* coated X chromosomes (2x *Xist*) was determined. C) Representative pictures of RNA-FISH analysis, detecting *Xist* (FITC) in day 3 differentiated female wild type and *Rnf12*$^{+/-}$ rescued ES cells (line 20, *Rnf12* overexpression). DNA is counterstained with DAPI in all RNA-FISH slides. D) Allele specific RT-PCR analysis of day 3 differentiated wild type, Rnf12$^{+/-}$ and *Rnf12*$^{+/-}$ rescued cell lines, detecting an *Xist* length polymorphism that discriminates 129 and Cas *Xist*.
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introduced an *Rnf12* BAC transgene into the *Xist*$^{\text{intron1+/-}}$ ES cell lines. Additional copies of *Rnf12* resulted in induction of *Xist*, even in undifferentiated ES cells (Figure 3E, 3F, 3I), confirming our previous findings [16]. However, allele specific RT-PCR did not point to an increased preference for expression of the mutated or wild type allele, in undifferentiated ES cells (Figure 3G, 3H), indicating that RNF12-mediated action on XCI does not require the *Xist* intron 1 region (Figure 3J). At day 3 of differentiation, in several cell lines, we found higher expression of Cas *Xist* in *Rnf12* transgenic *Xist*$^{\text{intron1+/-}}$ cells compared to *Xist*$^{\text{intron1+/-}}$ only cells. We attribute this finding to an increase in the percentage of cells with two *Xist* clouds. We conclude that the *Xist* intron 1 region is not essential for suppression of XCI in undifferentiated ES cells, but may play a role later during differentiation. Furthermore, RNF12-mediated activation of XCI is independent from the *Xist* intron 1 region.

RNF12 regulates *Xist*

RNF12 could regulate XCI through activation of *Xist* or suppression of *Tsix*, or both. Previously, we analyzed *Xist* transgenic male ES cell lines with a BAC RP24-180B23 integration covering *Xist* only [16], or a BAC RP23-338B22 sequence containing both *Xist* and *Tsix* (Figure 4A). These male transgenic ES cell lines also contained 16 copies of an ms2 bacteriophage repeat sequence located in exon 7 of the endogenous *Xist* gene, allowing separate detection by RNA-FISH of autosomal versus endogenous *Xist* spreading [23]. Differentiation of transgenic male ES lines containing the *Xist-Tsix* transgene resulted in expression of *Xist* from the autosomal integration site in cell lines containing multicopy integrations. Autosomal spreading of *Xist* in these cell lines is most likely due to accumulation of enough *Xist* RNA to silence at least one copy of *Tsix*, allowing spreading of *Xist* in *cis*. Integration of truncated transgenes that lack *Tsix* would facilitate this process [16]. This also explained autosomal *Xist* spreading in BAC RP-24-180B23 single copy male transgenic ES cell lines upon differentiation, because *Tsix* is not covered by this BAC [16]. We used two of these, *Xist* only, BAC RP-24-180B23 ES cell lines to introduce 129 BAC RP24-240J16 transgenes covering *Rnf12*, and found *Xist* spreading on the single endogenous X (Figure 4B and 4C), confirming previous results.

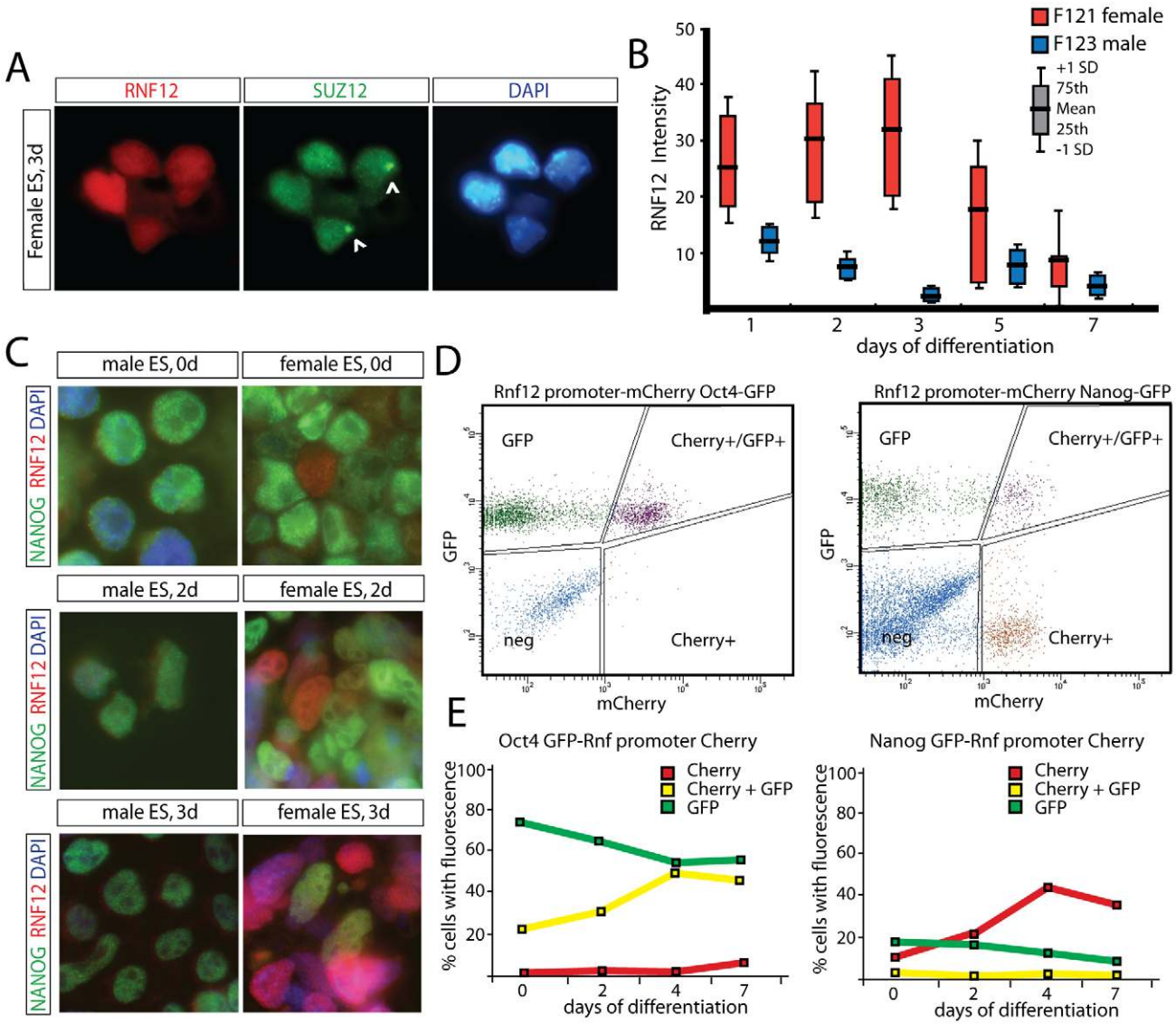

Figure 2. Counteracting roles for RNF12 and NANOG in XCI. A) Immunocytochemistry detecting RNF12 (Alexa 546) and SUZ12 (Alexa 488) in day 3 differentiated female ES cells. Cells showing accumulation of SUZ12 on the X chromosome (Xi) show low levels of nuclear RNF12, suggesting that RNF12 is downregulated upon XCI. RNF12 does not accumulate on the SUZ12 coated Xi. B) Quantification of RNF12 staining intensities in female and male ES cells at different timepoints of differentiation. Red and blue box plots show results for female and male cells, respectively. Mean, interquartile range and standard deviation are indicated. N>100 cells per timepoint. Female cells show higher staining intensities and more fluctuation of RNF12 expression compared to male cells. C) Immunocytochemistry detecting RNF12 (rhodamine) and NANOG (FITC) in undifferentiated and day 2 and 3 differentiated male and female ES cells. D) FACS analysis of NANOG-GFP (right panel) and OCT4-GFP (left panel) ES cells transgenic for an *Rnf12*-mCherry promoter construct. FACS plots show results of undifferentiated ES cells. Cells are gated for GFP+, Cherry+, GFP+Cherry+ or negative. Results of a representative experiment are shown. E) Quantification of FACS analysis of NANOG-GFP (right panel) and OCT4-GFP (left panel) ES cells transgenic for an *Rnf12* mCherry promoter construct. Cells were differentiated for up to 7 days, and the percentage of positive cells was determined (Cherry+, red line; GFP+, green line; Cherry+GFP+, yellow line).
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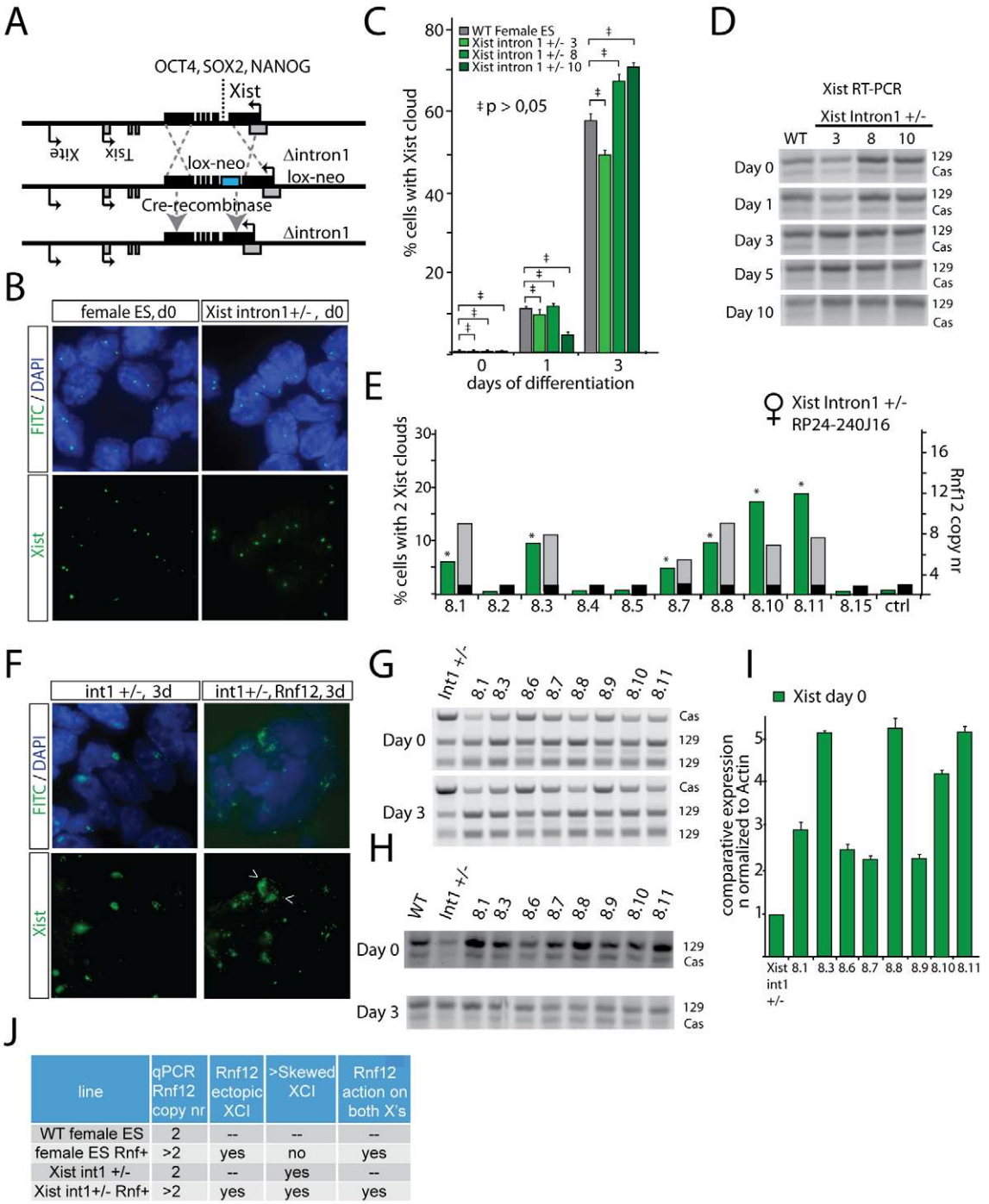
We also found a significant increase in the number of cells with autosomal *Xist* spreading, indicating that RNF12 activates XCI through *Xist*. Next, we introduced an *Rnf12* transgene (BAC RP24-240J16) in a single copy *Tsix* male transgenic ES cell line that lacks transgenic *Xist* (BAC RP23-447O10). These double transgenic ES cell lines contain a Cas X chromosome which allowed RFLP mediated discrimination of endogenous (Cas) and transgenic (129) *Tsix*. Analysis of these cell lines indicated that transgenic over-expression of RNF12 does not lead to down-regulation of *Tsix*, as measured by qPCR and by RNA-FISH examining the relative number of *Tsix* pinpoint signals (Figure 4D, 4E, 4G). Interestingly, allele specific RT-PCR indicated that endogenous *Tsix* (Cas) is even down-regulated in samples with higher *Xist* expression, indicating *Xist*-mediated silencing of *Tsix* in *cis* (Figure 4F). Taken together, these results indicate that *Xist* and not *Tsix* is the functionally most important downstream target of RNF12.

RNF12 is required for XCI

We previously found that the rate of initiation of XCI is reduced in differentiating female *Rnf12*$^{+/-}$ ES cells, compared to wild type ES cells [16]. The RNF12 protein level in these *Rnf12*$^{+/-}$ female



J

line	qPCR Rnf12 copy nr	Rnf12 ectopic XCI	>Skewed XCI	Rnf12 action on both X's
WT female ES	2	--	--	--
female ES Rnf+	>2	yes	no	yes
Xist int1 +/-	2	--	yes	--
Xist int1+/- Rnf+	>2	yes	yes	yes

Figure 3. RNF12 initiates XCI independent of pluripotency factor binding to *Xist* intron 1. A) Schematic representation of part of the X chromosome and the strategy to target the *Xist* intron 1 pluripotency factor binding sites. A BAC targeting construct replacing *Xist* intron 1 by a floxed neomycin resistance cassette (Neo) was used to target specifically the 129 allele in Cas/129 female ES cells. The Neo cassette was looped out after transient expression of Cre recombinase. B) RNA-FISH analysis detecting *Xist* (FITC) in undifferentiated female wild type and *Xist* $^{intron\ 1+/-}$ ES cells. In both wild type and *Xist* intron 1 deleted cells, only pinpoint signals are visible, representing basal *Xist* and *Tsix* expression. C) Bar graph showing the percentage of wild type and *Xist* $^{intron\ 1+/-}$ ES cells that initiated XCI, detected by *Xist* RNA-FISH, at different time points of EB differentiation. No statistical significant differences were noticed between the wild type control and the cell lines harbouring a deletion of *Xist* intron 1 (95% confidence interval, N>100 cells per time point ‡ p>0.05). D) Allele specific RT-PCR analysis detecting *Xist* expression in female wild type and *Xist* $^{intron\ 1+/-}$ cell lines (clone 3, 8 and 10) during differentiation. E) qPCR analysis to determine the *Rnf12* copy number in *Xist* $^{intron\ 1+/-}$ ES cells transgenic ES cell lines (transgenic, grey, and endogenous, black, copy number), and percentage of cells with two *Xist* clouds at day 3 of differentiation. F) RNA-FISH analysis detecting *Xist* (FITC) in day 3 differentiated *Xist* $^{intron\ 1+/-}$ ES cells, without (left panels) and with (right panels) an *Rnf12* transgene. The *Xist* clusters in one cell with two *Xist* clusters are indicated with arrowheads. G) RFLP RT-PCR amplifying a NheI RFLP present on the endogenous129 *Rnf12* allele, and the *Rnf12* transgene. Relative expression analysis was performed with RNA isolated from undifferentiated and day 3 differentiated ES cell lines. H) RT-PCR amplifying a length polymorphism distinguishing *Xist* emanating from the mutated 129 allele and the wild type Cas allele, with RNA isolated from undifferentiated and day 3 differentiated ES cell lines. I) *Xist* expression in undifferentiated *Rnf12* transgenic

Xist $^{\text{intron 1+/−}}$ ES cells, and an *Xist* $^{\text{intron 1+/−}}$ control cell line was quantified qPCR. J) Table summarizing the results obtained with female wild type, *Rnf12* transgenic, *Xist* $^{\text{intron 1+/−}}$ and *Xist* $^{\text{intron 1+/−}}$ *Rnf12* transgenic ES cell lines.
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cells is equal to that in male cells [16], but XCI is still occurring at a higher rate than in male cells. This indicated the presence of additional X-encoded XCI activators, but did not exclude the possibility that RNF12 is essential for XCI. To address this point, we generated *Rnf12*$^{-/-}$ female ES cells by targeting the wild type Cas *Rnf12* allele in *Rnf12*$^{+/-}$ ES cells (Figure 5A). Correct targeting was confirmed by RT-PCR, showing loss of a targeted RFLP located in exon 5 of *Rnf12* (Figure 5B). The presence of two X chromosomes in these *Rnf12*$^{-/-}$ female ES cells was ascertained by X chromosome DNA FISH analysis and amplification of an RFLP in the *Xist* gene (Figure 5C, and data not shown). Western blotting analysis confirmed the absence of RNF12 protein in the knockout cells (Figure 5D). RT-PCR and qRT-PCR of pluripotency associated genes and differentiation markers gave information that differentiation of the *Rnf12*$^{-/-}$ ES cells was not different from that of wild type ES cells (Figure 5E, 5F and Figure S5). However, *Xist* RNA FISH analysis showed that differentiating *Rnf12*$^{-/-}$ ES cells only sporadically initiate XCI (Figure 5G, 5H and 5I). QPCR analysis confirmed that *Xist* is not detectably upregulated when measured for a population of *Rnf12*−/− cells upon differentiation. Moreover, DNA-FISH detecting a whole chromosome X paint probe at day 7 and 10 of differentiation excluded X chromosome loss (Figure S5). The few *Rnf12*$^{-/-}$ cells that initiated XCI appeared in clusters, suggesting clonal expansion of a few cells that initiated XCI (Figure S5). We therefore conclude that RNF12 is an essential factor in XCI.

RNF12 activates the *Xist* promoter

Evidently, the *Rnf12*$^{-/-}$ knockout cells present the possibility to study control of gene expression by RNF12. Therefore, we next performed micro-array expression analysis comparing day 3 differentiated *Rnf12*$^{-/-}$ and wild type cells. We found that *Xist* was the only gene that was subject to differential regulation, showing pronounced down-regulation (Figure 5J). Interestingly, none of the known downstream targets of RNF12 appeared affected in our analysis. This may be due to our ES cell differentiation system resulting in a mixed population of cells at different stages of differentiation. In addition, the 3-day-time span allowed in our studies for cell differentiation may have prevented detection of effects on downstream targets which are expressed at later stages of differentiation. Nevertheless, our results indicate that the main function of RNF12 at this early stage of differentiation concerns the regulation of XCI. The observed dependency of *Xist* transcription on RNF12 might be effectuated by RNF12 acting through the *Xist* promoter. To test this, we expressed *Xist* promoter luciferase reporter constructs, both transiently and stably, in wild type female and *Rnf12*$^{-/-}$ ES cell lines and differentiated these cells for 3 days. The results revealed an unequivocal correlation between RNF12 expression and luciferase expression (Figure 5K). Our results therefore demonstrate that RNF12 activates the *Xist* promoter, although this does not exclude a role for other *cis* regulatory sequences, further away from the *Xist* promoter, in RNF12-mediated activation of XCI.

Discussion

In ES cells, RNF12 exerts its main function in XCI

Here, we present evidence that RNF12 is an essential activator of random XCI. RNF12 acts in *trans* on the *Xist* promoter, in differentiating mouse ES cells, to activate *Xist* transcription, leading to *Xist* RNA cloud formation and spreading of the silencing complex over the future inactive X chromosome in *cis*. Although our results show that RNF12 acts in *trans*, it is to be expected that the close proximity of the *Rnf12* gene to the *Xist* locus, taken together with the dose-dependent action of RNF12, is quite crucial for well-tuned regulation of XCI. Such proximity most likely facilitates rapid down-regulation of *Rnf12* in *cis* upon initiation of XCI, leading to a lower nuclear RNF12 content, thereby preventing inactivation of the second X chromosome.

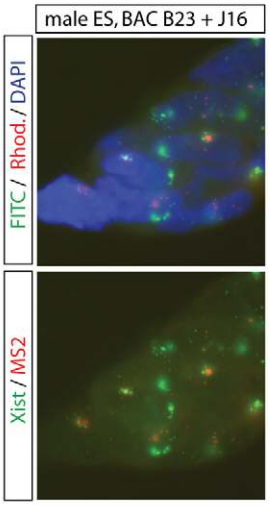
Whole genome expression analysis suggests that the major function of RNF12 in ES cells is its regulation of *Xist* RNA expression, hence XCI. This is a very surprising finding, as RNF12 has been implicated in many other biological pathways. Apparently, in the present cell differentiation system, loss of expression of RNF12 does not cause a deviation from the wild type differentiation process to such an extent that it affects gene expression other than that of *Xist*. However, also based on our studies we do not exclude a function for RNF12 at later stages of cell differentiation, or in mouse development. In addition, redundant pathways or proteins such as RNF6, a close homologue of RNF12, may prevent full phenotypic expression of loss of RNF12. However, RNF12 exerts a predominant role in targeting *Xist*, as evidenced by our observation that *Xist* is largely silenced in the RNF12 deficient cells.

While our manuscript was under review, Shin et al. (2010) published a paper suggesting that RNF12 might be required in particular for imprinted XCI in mice [24]. Remarkably, that study included the observation that RNF12 depletion did not prevent initiation of random XCI in a significant percentage of *Rnf12*−/− ES cells derived from mouse blastocysts. This discrepancy with our findings might be explained by experimental differences, such as differences concerning the design of the knockout, the genetic background of the ES cells, or the cell derivation and culture procedures. Differences in cell differentiation protocols have been shown to have a pronounced impact on the XCI process [25]. Also, ES cells derived from embryos with a different genetic background could express XCI activators and XCI inhibitors at different levels, allowing XCI in either a lower or a higher percentage of *Rnf12*−/− cells. Future studies comparing the two independently generated *Rnf12*−/− ES cell lines will yield useful information about these points.

Other XCI activators

Although our observations provide evidence that RNF12 is an essential factor for the XCI process to occur in differentiating ES cells, we anticipate that other XCI activators act in parallel, and might independently regulate *Xist* or *Tsix*, or both. Dosage compensation mechanisms in species such as *D. melanogaster* and *C. elegans* also involve multiple factors and pathways, possibly leading to increased fidelity of these mechanisms [26]. In such a mechanism involving multiple factors, RNF12 would be the dose-dependent factor that is required to exceed the cumulative threshold limit to proceed towards initiation of XCI. It is feasible that female *Rnf12*$^{-/-}$ cells sometimes do initiate XCI (Figure 6A), as a consequence of the stochasticity of the process. This would be compatible with a mechanism, in which the combined total activity of all putative XCI activators exclusive of RNF12 is just below or around the threshold to initiate XCI. Interestingly, *Xist* cloud formation is also sporadically found in male cells, but in

B



C

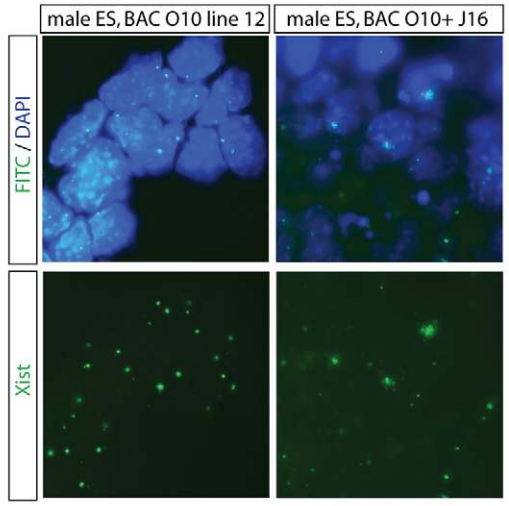
ES 1.3 B23 Line 18, RP24-240J16

line	copy nr	% Xist autosomal	% Xist endo	n
no insert	-	3%	0%	77
2	3	46%	15%	117
3	7	29%	34%	146
14	4	50%	21%	154
23	2	66%	39%	145
24	2	60%	45%	104

ES 1.3 B23 Line 21, RP24-240J16

line	copy nr	% Xist autosomal	% Xist endo	n
no insert	-	12%	0%	112
1	2	15%	8%	136
2	4	26%	35%	106
3	6	35%	53%	92
4	10	21%	67%	81
5	1	42%	26%	110

D

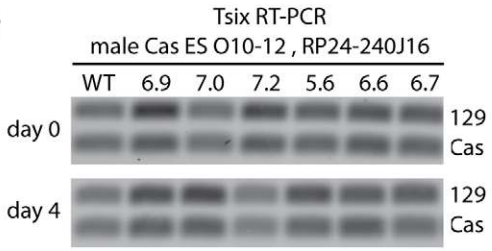


E

male Cas ES O10 Line 12, RP24-240J16

line	copy nr	% 2 Tsix pinpoints	% Xist endo	% Xist+ with Tsix pinpoint	n
no insert	-	55%	0%	-	347
6.9	6	54%	18%	69%	231
7.0	2	46%	3%	66%	119
7.2	3	69%	8%	77%	123
5.6	2	62%	4%	60%	142
6.6	2	64%	4%	50%	139
6.7	2	62%	2%	50%	132

F



G

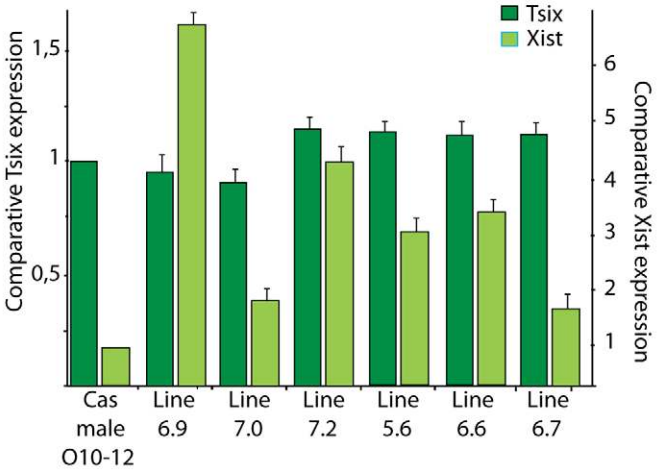


Figure 4. RNF12 activates *Xist* directly, but does not inhibit *Tsix*. A) Map showing part of the mouse X chromosome, the location of the BAC sequences used, and the position of ms2 repeats within *Xist*. RNA-FISH probes are indicated in green and red, and non-annotated genes in grey. B) RNA-FISH analysis detecting endogenous *Xist* (ms2, rhodamine and FITC positive) and exogenous *Xist* (FITC) from the autosomally integrated *Xist*-only BAC RP24-180B23 in day 3 differentiated male ES cells transgenic for *Rnf12* (BAC RP24-240J16). C) Table summarizing RNA-FISH results from B). Copy number of the *Rnf12* transgene was determined by gDNA qPCR. Shown are the percentage of autosomal and endogenous *Xist* clouds; N, number of cells analyzed. D) RNA-FISH analysis detecting endogenous and transgenic *Tsix* (FITC, pinpoint signals) and endogenous *Xist* (FITC, clouds) in day 3 differentiated *Tsix* transgenic male cells (left panels) and day 4 differentiated *Tsix* transgenic male cells with additional copies of an *Rnf12* transgene (right panels). E) Table summarizing results obtained with single copy *Tsix* transgenic male ES cell lines with a *Rnf12* transgene, 4 days after differentiation. Shown are copy number of the *Rnf12* transgene, percentage of cells with two *Tsix* signals, cells with an *Xist* cloud, and the percentage of cells with an *Xist* cloud and *Tsix* pinpoint signal (n is number of cells analyzed). F) Allele specific RT-PCR detecting transgenic (129) and endogenous (Cas) *Tsix* in undifferentiated and day 4 differentiated *Tsix*/*Rnf12* double transgenic ES cells. G) qPCR analysis to quantify *Xist* and *Tsix* expression in day 4 differentiated *Tsix*/*Rnf12* double transgenic ES cells, and a control cell line without an *Rnf12* transgene.
doi:10.1371/journal.pgen.1002001.g004

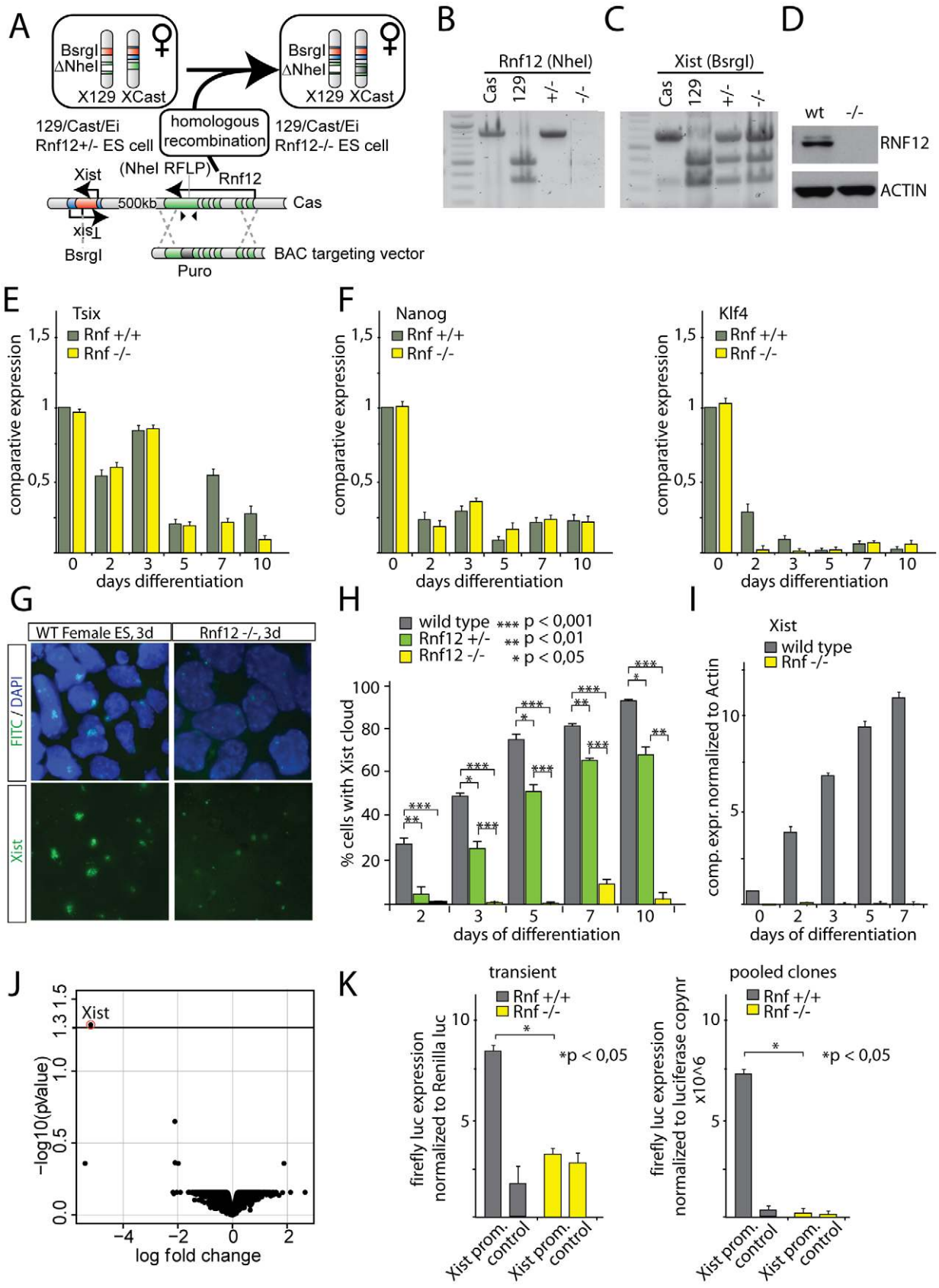


Figure 5. RNF12 is essential for XCI. A) Targeting strategy to generate *Rnf12*$^{-/-}$ ES cells. The Cas *Rnf12* allele of the previously generated heterozygous *Rnf12*$^{+/-}$ ES cells (Cas/129) was targeted with a BAC construct containing a puromycin selection cassette disrupting the open reading frame of *Rnf12*. B) PCR RFLP analysis with primers spanning a NheI RFLP descriminating the Cas (no NheI site) and the 129 (NheI site present) alleles,

which was used to insert the targeting cassette. C) PCR RFLP analysis confirming the presence of two X chromosomes in *Rnf12*$^{-/-}$ ES cells. PCR primers span a BsrgI RFLP located in *Xist*. D) Western analysis of RNF12 protein and ACTIN in wild type and *Rnf12*$^{-/-}$ ES cells. E) qRT-PCR analysis detecting *Tsix* expression in female wild type and *Rnf12*$^{-/-}$ ES cells differentiated for up to 10 days. Results were normalized to Actin. F) qRT-PCR analysis as in (H), but now detecting Nanog (left graph) and Klf4 (right graph) expression. G) RNA-FISH analysis detecting *Xist* (FITC) in day 3 differentiated female wild type and *Rnf12*$^{-/-}$ ES cells. H) Bar graph showing the percentage of female wild type, *Rnf12*$^{+/-}$ and *Rnf12*$^{-/-}$ ES cells that initiated XCI, as determined by *Xist* RNA-FISH, at different time points of differentiation. *** p<0,001; ** p<0,01; * p<0,05, Student's T-test. I) qRT-PCR detecting *Xist* in female wild type and *Rnf12*$^{-/-}$ ES cells differentiated for up to 7 days. Results were normalized to *Actin*. J) Genome wide expression analysis comparing day 3 differentiated *Rnf12*$^{-/-}$ and wild type ES cells. Shown are the Log fold expression change and the adjusted P value. K) Luciferase assay detecting expression of an *Xist*-promoter-luciferase construct in female wild type and *Rnf12*$^{-/-}$ ES cells differentiated for 3 days. For transient experiments, cells were co-transfected at day 0 with the *Xist*-promoter-luciferase or control vector (empty luciferase vector) and a Renilla plasmid. Results were normalized to Renilla expression. For stable pooled clones, the promoter constructs were transfected, clones were pooled after selection and differentiated 3 days prior to analysis.
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contrast to female *Rnf12*$^{-/-}$ cells, this represents a lethal condition and will be selected against.

Our studies indicate that RNF12 participates in *Xist* promoter activation, through an action which requires the presence of the minimal promoter. Although the direct protein target(s) of RNF12 remain elusive, its reported E3 ubiquitin ligase activity [17] would be compatible with RNF12 targeting an inhibitor of *Xist* transcription through proteasome-mediated degradation. This does not exclude that RNF12 might be involved, in addition or alternatively, in activation of a transcription factor driving *Xist* expression through positive regulation of transcription. Furthermore, RNF12 could be involved in regulation of *cis*-regulatory sequences other than the *Xist* promoter, yet to be identified and further away from the *Xist* locus.

A function for RNF12 in maintaining *Xist* expression

Selection against cells inactivating the X chromosome containing the wild type allele of *Rnf12* in the heterozygous *Rnf12*+/− ES cells could point to a continued requirement for *Rnf12* in maintaining *Xist* expression, following the early stages of differentiation. From the fact that male *Rnf12*−/*Y* knockout male mice are viable [24], it can be concluded that RNF12 deficiency is compatible with survival of differentiated cells in which XCI does not play any role. Hence, it would be difficult to explain the observed selection against cells inactivating the wild type X chromosome in the heterozygous *Rnf12*+/− ES cells by loss of any possible function of RNF12 independent of XCI. If RNF12 would be required for maintaining *Xist* expression and XCI, the cells inactivating the wild type allele and becoming deficient in RNF12 can be expected to lose *Xist* expression and to reactivate the Xi. In contrast, cells inactivating the X chromosome containing the mutated allele, keeping one functional allele of *Rnf12*, will be able to maintain *Xist* expression and XCI. In a population of cells this will lead in skewed XCI of the mutated allele. In fact, such a mechanism might also be relevant to explain the reported defect in imprinted XCI resulting from an *Rnf12* mutation [24].

Imprinted XCI involves activation of *Xist* on the Xp, and the observed phenotype concerns lack of this imprinted XCI of the Xp when the mutant *Rnf12* allele is inherited from the mother. It was observed that no female embryos were born, inheriting a mutated *Rnf12* allele from either a *Rnf12*$^{-/-}$ or a *Rnf12*$^{+/-}$ mother in crosses with wild type males. In contrast, the mutated allele was transmitted to male offspring. Maternal storage of RNF12 in the oocyte was proposed to play a crucial role in imprinted silencing of the Xp in the early embryo [24]. *Rnf12* is at a 46 cM distance of the centromere, so that it can be expected that many haploid oocytes generated by the first meiotic division (the reduction division) of *Rnf12*$^{+/-}$ oocytes, which occurs at the time of ovulation, will contain both wild type and *Rnf12* mutated alleles, as a consequence of meiotic recombination. Hence, we anticipate that there will be ongoing expression of *Rnf12* in a high percentage of oocytes transmitting the mutated *Rnf12* allele, until fertilization triggers meiotic division II. The recombined wild type and mutant alleles which are present within one haploid oocyte, will be exposed to the same maternal storage of RNF12. Taken together with the observation that *Rnf12*$^{+/-}$ oocytes did not give rise to

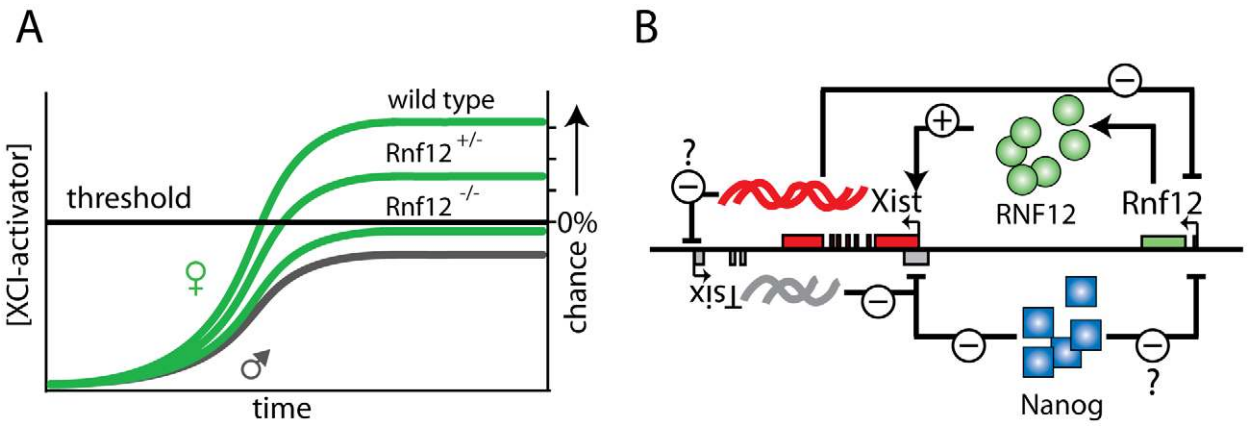

Figure 6. Rnf12 and its role in the XCI regulatory network. A) In wild type and *Rnf12*$^{+/-}$ cells the XCI activator concentration is above the threshold required to generate a probability to initiate XCI. In contrast, in most *Rnf12*$^{-/-}$ cells the XCI activator concentration is not sufficient to reach the threshold required to initiate XCI. B) The regulatory network of XCI. *Xist* is repressed in *Tsix* dependent and independent pathways (NANOG binding in intron 1). Activation of XCI is accomplished by RNF12 through activation of the *Xist* promoter, and possibly *Xist* mediated silencing of *Tsix*. Finally, *Rnf12* is repressed by *Xist* and possibly NANOG.
doi:10.1371/journal.pgen.1002001.g006

female offspring carrying the mutant allele, whereas female offspring carrying the wild type allele were obtained at the expected mendelian ratio from these oocytes [24], this argues against a predominant role for maternal storage in imprinted XCI. Rather, we favor the hypothesis that continued transcription of *Rnf12* throughout ovulation and after fertilization is required for sustained expression of RNF12, activation of *Xist* from the Xp, and maintenance of the inactive Xp. Future research will be required to address this hypothesis.

The link to pluripotency

Our results indicate a negative correlation between NANOG and RNF12 expression. NANOG and the other pluripotency factors OCT4 and SOX2 have been shown to be recruited to the *Xist* intron 1 region in undifferentiated ES cells, and were proposed to play a role in *Tsix* independent suppression of *Xist* [14]. In this regulatory mechanism, ablation of *Tsix* did not result in up-regulation of *Xist* in undifferentiated ES cells, and *Tsix* was not required for repression of *Xist* located on the inactivated paternal X chromosome in the inner cell mass. This pointed to an important role for recruitment of NANOG, OCT4 and SOX2 to *Xist* intron 1 in suppression of *Xist* in ES and ICM cells [14]. However, the present findings show that the intron 1 region is dispensable, in silencing the XCI process in undifferentiated ES cells. Deletion of *Xist* intron 1 caused an effect, but only in the form of skewing of XCI, which was notable at later stages of differentiation. Interestingly, a previous study analyzing an *Xist* mutant allele that lacks the intron 1 region but leaves the *Xist* promoter intact, also did not show up-regulation of the mutated allele in undifferentiated ES cells [27]. Although these latter results support our findings, they should be interpreted with caution because the selection cassette was still present in the cells analyzed by Marahrens et al. [27].

Like for the role of RNF12, this points to the presence of additional mechanisms, involved in suppression of XCI. *Tsix* and *Xite* are the most likely candidate genes taking part, and the combined action of these repressive mechanisms may be sufficient to suppress *Xist*. However, even with all the repressive elements in place RNF12 can induce *Xist* expression and XCI in undifferentiated ES cells [16]. This points towards another mechanism involved in *Xist* suppression, in which the nuclear concentration of the XCI activator may be too low in undifferentiated ES cells and ICM cells to allow *Xist* expression and initiation of XCI, even in the absence of repressive elements such as the intron 1 region. Future research should clarify whether these mechanisms indeed act synergistically in silencing the XCI process.

The negative correlation of RNF12 and NANOG expression that we report could reflect the differentiation state of the ES cells, and does not necessarily entail a cross-regulatory role for these proteins. Nevertheless, NANOG and other pluripotency factors are also recruited to the *Rnf12* promoter in ES cells, where it might be involved in down-regulation of *Rnf12* (Figure 6B) [28], which opens the intriguing possibility that NANOG might also be implicated in regulation of the initiation of XCI through suppression of *Rnf12*. This highlights the complexity of the overall mechanism and the interconnection of the different players involved in XCI, but also reinforces the predominant role of RNF12 in this process.

Methods

ES cell culture

ES cells were grown in standard ES medium containing DMEM, 15% foetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, non-essential amino acids, 0.1 mM β-mercaptoethanol, and 1000 U ml^{-1} LIF. To induce differentiation, ES cells were split, and pre-plated on non-gelatinised cell culture dishes for 60 minutes. ES cells were then seeded in non-gelatinised bacterial culture dishes containing differentiation medium to induce embryoid body (EB) formation. EB-medium consisted of IMDM-glutamax, 15% foetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, non-essential amino acids, 37.8 µl l^{-1} monothioglycerol and 50 µg/ml ascorbic acid. EBs were plated on coverslips 1 day prior to harvesting, and allowed to grow out.

Transgenesis and generation of knockout ES cell lines

For the Rnf12 rescue experiments, an Ampicilin-Puromycin resistance cassette was inserted in the backbone of BAC RP24-240J16 by homologous recombination in bacteria. The modified BAC was electroporated in to female heterozygous *Rnf12*+/− cells [16], and colonies were picked after 8–10 days of Puromycin selection, expanded and differentiated. BAC copynumber was determined by qPCR, and transgene specific expression was determined by allele specific RT-PCR, as described previously [16].

To generate the female homozygous *Rnf12* −/− ES cell line, the previously generated *Rnf12*+/− ES cell line was targeted with an *Rnf12* BAC targeting construct containing an Ampicilin-Puromycin cassette disrupting the open reading frame of *Rnf12*. To generate this targeting construct, targeting arms were PCR amplified using primers GCCTTCGAACATCTCTGAGC, GAGCCGGACTAATCCAAACA, cloned into pCR-BluntII-TOPO (Invitrogen), and linearized with NheI to introduce an Ampicilin-Puromycin cassette from pBluescript. The targeting cassette was inserted in a Cast/Ei *Rnf12* BAC RP26-81P4 by homologous recombination in bacteria, and the resulting construct was used to target specifically the Cast/Ei X chromosome of the *Rnf12* +/− ES cell line. Colonies were selected under Neomycin and Puromycin selection, and the absence of *Rnf12* expression was confirmed by Western analysis.

To generate the *Xist* intron 1 deletion, a BAC targeting construct was generated by homologous recombination, replacing intron 1 by a floxed Neomycin cassette. Targetting arms were PCR amplified using primers 5′Forw:CATCAGGCTTGGCAGCAAGT, 5′R: CCTTGTTGGTCCAGACGACTATT and 3′Forw: CCAGACCAGGTCTTTGTATGCA, 3′Rev: GTGCTCCTGCCTCAAGAAGAA. Correctly targeted clones were identified by allele specific RFLP analysis using primers CAGTGGTAGCTCGAGCCTTT and CCAGAAGAGGGAGTCAGACG, followed by BsrGI digestion. The Neomycin cassette was removed by transient transfection with a CrePAC vector and selection with puromycin. The final cell lines were verified by Southern blotting.

Rnf12 and *Xist* reporter constructs

To generate the *Rnf12* promoter cherry reporter cell lines, the *Rnf12* promoter was PCR amplified using previous described primers [29], and cloned into pCR-BluntII-TOPO and sequence verified. The *Rnf12* promoter was then released from pCR-BluntII-TOPO by digestion with SacI and KpnI, and blunt cloned into an AseI-BamHI fragment from pmCherry-N1 (Clonetech), thereby replacing the pCMV promoter of pmCherry-N1 with the *Rnf12* promoter. The resulting construct was used to electroporate in Oct-GFP and Nanog-GFP ES cell lines. Both pooled cell lines and single colonies were expanded, and cherry expression was analysed by FACS analysis using a BD FACSAria apparatus.

The *Xist* promoter was amplified using primers: TCCCAAGGTATGGAGTCACC, and GGAGAGAAACCACGGAAGAA, and cloned into pGL3-basic vector. As a control, the promoter less pGL3-basic vector was transfected.

Stable pooled cell lines of wild type or *Rnf12* −/− ES cells were generated by co-transfection with a puromycin or hygromycin selection vector. Expression of Luciferase was determined using the Bright-Glo luciferase assay system (Promega) and measured using a Promega luminometer. Results were normalized to the amount of protein present in the cell lysate measured by nanodrop, and copynumber of *Xist* promoter integration determined by qPCR. qRT-PCR using primers detecting luciferase (TCTAAGGAAGTCGGGGAAGC and CCCTCGGGTGTAATCAGAAT) confirmed the results obtained. For transient luciferase experiments, cells were co-transfected using the *Xist* reporter constructs and a control Renilla construct, using Lipofectamine 2000. Luciferase activity was measured using the Dual Glo luciferase system (Promega).

Xist RNA FISH, immunofluorescence, and Western analysis

Xist RNA-FISH was performed as described [11,16]. Immunofluorescence was performed using standard procedures. RNF12 and NANOG were detected using a mouse anti- RNF12 antibody (1:250, Abnova), and a rabbit anti-NANOG antibody (1:100, SC1000, Calbiochem). ImageJ software was used to measure staining intensities; at least 100 cells were measured for each indicated time point, and background correction was performed. Western blotting was performed as previously described [16].

Expression analysis

RNA was isolated using Trizol reagent (Invitrogen) using manufacturers instructions. DNAse treatment was performed, and cDNA was prepared using SuperScriptII (Invitrogen), using random hexamers. qRT-PCR was performed using a Biorad thermocycler, using primers described in Table S1. Results were normalized to *Actin*, using the ΔCT method.

Whole genome wide expression analysis of female wild type and *Rnf12*−/− ES cells differentiated for 3 days was performed with Affymetrix Mouse Genome 430 2.0 Arrays. Differentially expressed genes were identified using Limma (Bioconductor package) in R software.

Supporting Information

Figure S1 Immunocytochemistry detecting *RNF12* (rhodamine) at different stages of differentiation of female and male ES cells.
(TIF)

Figure S2 Immunocytochemistry detecting *RNF12* (rhodamine) and *SUZ12* (FITC) in day 3 differentiated female ES cells. *SUZ12* accumulations on the Xi are indicated with arrowheads.
(TIF)

Figure S3 FACS analysis of NANOG-GFP (right panel) and OCT4-GFP (left panel) ES cells transgenic for an *Rnf12*-mCherry promoter construct. FACS plots show results of day 4 (upper panel) and day 7 (lower panel). Cells are gated for GFP+, Cherry+, GFP+Cherry+ or negative. 10.000 cells analyzed per time point.
(TIF)

Figure S4 A) RFLP-PCR amplifying a BsrgI RFLP located in *Xist* intron 1. The BsrgI site is present on the targeted 129 allele, and is disrupted by the targeting event. B,C) Analysis of RFLPs in *Rnf12* (B) and *Atrx* (C) confirmed the presence of 2 X chromosomes in *Xist* intron 1 targeted ES cells. D) Confirmation of loop out of the neomycin resistance cassette. Primers amplify a region across the neomycin cassette. E) Map and targeting strategy for the intron 1 deletion. The map shows expected allele specific fragment sizes prior to, and after targeting and Cre mediated loopout. Bottom panel shows Southern analysis of female wild type, *Xist* $^{\text{intron 1+/-}}$ neo clone (neo) and neo loop out clones 3, 8 and 10. F) DNA FISH detecting the *Xist* locus in *Xist* $^{\text{intron 1+/−}}$ cells (left panels), and X-paint DNA-FISH analysis (Rhodamine red, right panels). G) qRT-PCR analyzing *Xist* expression in female wild type and *Xist* $^{\text{intron 1+/−}}$ ES cells during a differentiation assay for up to 5 days. Results were normalized to *Actin*.
(TIF)

Figure S5 A) RNA-FISH analysis detecting *Xist* (FITC) in day 10 differentiated female wild type and *Rnf12*$^{-/-}$ ES cells. B) Example of clonal cluster of cells which have initiated XCI in *Rnf12*$^{-/-}$ ES cells differentiated for 10 days as detected by *Xist* RNA-FISH. C) RT-PCR examining expression of several differentiation markers with RNA of day 7 (left panel) and day 10 (right panel) differentiated female wild type, *Rnf12*$^{+/-}$ and *Rnf12*$^{-/-}$ ES cells. D) Allele specific RT-PCR determining *Hprt* with a 129 and Cas origin, indicating the presence of both X chromosomes in all cell lines tested. E) DNA-FISH detecting a whole chromosome X paint probe in wild type, *Rnf12*$^{+/-}$ and *Rnf12*$^{-/-}$ ES cells differentiated for 7 or 10 days. Slides were first used for RNA-FISH, and subsequently denatured and hybridized to the DNA probe. Virtually all cells in the *Rnf12*$^{-/-}$ ES cells which did not show Xist clouds stained positive for the presence of two X chromosomes.
(TIF)

Table S1 Primers used in this study.
(DOC)

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Author Contributions

Conceived and designed the experiments: JG. Performed the experiments: TSB NG CGP EMA MG RB ER JG. Analyzed the data: TSB NG CGP EMA JAG JG. Contributed reagents/materials/analysis tools: TSB NG CGP EMA MG RB AK ER JG. Wrote the paper: TSB JG.

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