# Chapter 2

## *Rnf12 is an X-encoded dose dependent activator of X chromosome inactivation*

This chapter has been published in

Iris Jonkers*, Tahsin Stefan Barakat*, Eskeatnaf Mulugeta Achame, Kim Monkhorst, Annegien Kenter, Eveline Rentmeester, Frank Grosveld, J. Anton Grootegoed and Joost Gribnau (2009)
"RNF12 is an X-Encoded dose-dependent activator of X chromosome inactivation"
*Cell* 139:999-1011

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# RNF12 is an X-encoded dose-dependent activator of X chromosome inactivation

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## Abstract

In somatic cells of female placental mammals, one X chromosome is inactivated to minimize sex-related dosage differences of X-encoded genes. Random X chromosome inactivation (XCI) in the embryo is a stochastic process, in which each X has an independent probability to initiate XCI, triggered by the nuclear concentration of one or more X-encoded XCI-activators. Here, we identify the E3 ubiquitin ligase RNF12 as an important XCI-activator. Additional copies of mouse *Rnf12* or human *RNF12* result in initiation of XCI in male mouse ES cells and on both X chromosomes in a substantial percentage of female mouse ES cells. This activity is dependent on an intact open reading frame of *Rnf12* and correlates with the transgenic expression level of RNF12. Initiation of XCI is markedly reduced in differentiating female heterozygous *Rnf12*+/- ES cells. These findings provide evidence for a dose-dependent role of RNF12 in the XCI counting and initiation process.

## Introduction

In the mouse embryo proper, XCI is random with respect to the parental origin of the inactivated X chromosome, and is initiated around 5 days *post coitum*, or upon ES cell differentiation *in vitro* [892]. Initiation of XCI is marked by transcriptional up-regulation of the X-encoded *Xist* gene on the future inactive X chromosome (Xi). *Xist* is a non-coding, spliced and poly-adenylated RNA, which spreads over the Xi while attracting protein complexes required for the silencing process [86-87, 99]. *Tsix* and *Xite* gene sequences overlap with the *Xist* gene, but are transcribed in anti-sense direction and play an important role in suppression of *Xist* transcription during the XCI process [119, 136].

XCI starts with counting of the number of X chromosomes and selection of the future active X (Xa) and Xi. This process is stochastic, and every X chromosome has an independent probability to initiate XCI [179]. The probability for any X chromosome to be inactivated increases with an increased X to autosome ratio, suggesting involvement of an X-encoded activator in the XCI counting process [179, 189]. Studies with cell lines and mice carrying *Xist* and *Tsix* over-expression and knockout alleles have indicated that these genes play a crucial role in determining the probability to initiate XCI [104, 119, 123, 177]. *Tsix* takes part in setting up a threshold that has to be overcome by *Xist* in order to initiate XCI. In counteracting *Tsix*, X-encoded XCI-activators are responsible for dose-dependent activation of *Xist* expression, and autosomally-encoded XCI-inhibitors act as dose-dependent suppressors of *Xist*. XCI-activators might act either through activation of *Xist* directly, or by suppression of *Tsix*, thereby lowering the threshold for initiation of XCI. Similarly, XCI-inhibitors can be involved in direct suppression of *Xist* activation, or exert their activity through an indirect mechanism such as activation of *Tsix* expression.

During early embryonic development, or upon ES cell differentiation, the concentration of the XCI-activators will be sufficient, in female cells but not in male cells, to initiate XCI with a specific probability per time frame. In this working hypothesis, inactivation of both X chromosomes in female cells is prevented by *cis* inactivation of the genes encoding the XCI-activators and the stochastic nature of XCI initiation. Nonetheless,

the second X chromosome will keep a probability to initiate XCI until the XCI-activator concentration has dropped below the threshold required to initiate XCI, after silencing of the XCI-activator gene(s) in *cis*.

Recent findings indicate that CTCF, YY1, NANOG, SOX2, and OCT4 exert activities as XCI-inhibitors, by taking part in suppression of *Xist* expression [186, 191, 193], and appears to involve *Tsix*-dependent and -independent pathways. So far, X-linked genes encoding XCI-activators have not been identified. Candidate regions on the X chromosome involved in activation of XCI are delineated by different transgenes, several of them containing *Xist* and flanking sequences which have been shown to induce ectopic XCI in male cells [166-167, 169]. However, this effect was only obtained with multi-copy transgenes [175]. In addition, our finding that XCI is initiated in heterozygous ΔXTX female cells [179] indicates that *Xist*, *Tsix* and *Xite* play redundant or marginal roles in the XCI counting and initiation process, and suggest that important sequences regulating X chromosome counting and initiation of XCI are located outside the deleted region. To identify genes encoding XCI-activators we have performed a screen with male and female BAC transgenic ES cell lines. From this, we have obtained evidence that the X-encoded E3 ubiquitin ligase RNF12 is a dose-dependent factor involved in counting the number of active X chromosomes present per mammalian cell nucleus, which triggers initiation of random X chromosome inactivation.

# Results

## A screen to identify XCI-activator genes

Previous studies have indicated that genes encoding the putative XCI-activators are most likely located within a region of 10 megabases (Mb) surrounding the *Xist* locus [72-73]. To identify the XCI-activator genes, we generated transgenic male and female ES cell lines with stably integrated BAC transgenes covering part of this 10 Mb region. Our working hypothesis predicts that initiation of XCI upon differentiation in transgenic male ES cells on the single X, or initiation of XCI on both X chromosomes in a substantial percentage of transgenic female ES cells, signifies the presence of additional copies of a gene encoding an XCI-activator on the respective BAC.

Studies with mouse or human *Xist/XIST* transgenes in mouse ES cells indicated that autosomal integration of these transgenes results in activation of the endogenous *Xist* gene [167, 169, 171]. This suggests that these transgenes include sequences encoding an XCI-activator. We therefore started our screen with a BAC covering mouse *Xist*, excluding the transcription start sites of *Tsix* and *Xite*. BAC RP24-180B23 was stably transfected into male ES cells. Clones were expanded under neomycin selection and differentiated for 3 days before analysis. Besides wild type ES cells, we also made use of a male ES cell line 1.3 which contains 16 copies of an ms2 repeat integrated in exon 7 of *Xist* (**Figure 1A**). This ms2 tag does not interfere with XCI, and allows discrimination between endogenous *Xist*-ms2 and transgenic *Xist* [92]. BAC integration and copy number were determined by DNA-FISH and/or qPCR. The percentage of cells with accumulated *Xist* covering the Xi (*Xist* cloud) was determined by RNA-FISH using an *Xist* cDNA probe, which detects *Xist* and *Tsix* (in all FISH panels DNA is stained with DAPI). The pinpoint signal detected with this *Xist* cDNA probe is similar to the pinpoint signal obtained with a *Tsix* specific probe, and represents basal *Tsix/Xist* transcription which is clearly distinguishable from an *Xist*-covered Xi (**Figure 1B**). In most cell lines with an autosomal integration of the transgene, we found male cells with single *Xist* clouds (**Figure 1C**, **Supplemental Table 2A**). RNA-FISH using *Xist* (FITC) and ms2 (rhodamine red) probes performed on five different BAC transgenic 1.3 ES cell lines which were differentiated for 3 days revealed no ms2 positive clouds (**Figure 1D**), indicating that the endogenous *Xist* gene was never up-regulated (which would lead to conversion of the pinpoint signal to a cloud). This result was confirmed using a RP23-338B22 BAC sequence covering all of *Xist, Tsix,* and *Xite* (**Supplemental Table 2A**). Autosomal *Xist* spreading in differentiating male ES cells can be attributed to the absence of autosomal *Tsix* sequences (RP24-180B23), or loss of autosomal *Tsix* expression in *cis* due to truncation of one of the transgene copies or position effects (RP23-338B22, **Supplemental Figure 1A**). As a consequence of the absence of *Tsix* expression in *cis*, a relatively low XCI-activator concentration will be sufficient to induce autosomal *Xist* expression and spreading in male cells, similar to findings with male *Tsix* knockout ES cells [123, 127, 178]. Male and female mouse ES cell lines transgenic for BAC sequences CTD-2200N19 and CTD-2183M22 covering both human *XIST* and *TSIX*, or *XIST* alone (**Figure 1F**) also did not show significant induction of endogenous *Xist* in male cells (**Figure 1E**, and **Supplemental Table 2A**) or endogenous *Xist* accumulation on both X chromosomes in female cells (**Figure 1G**, and **Supplemental Figure1B** and **C**). These results indicate that the tested regions do not induce ectopic XCI in transgenic male and female cells under the conditions that we used. Our findings contrast previous claims

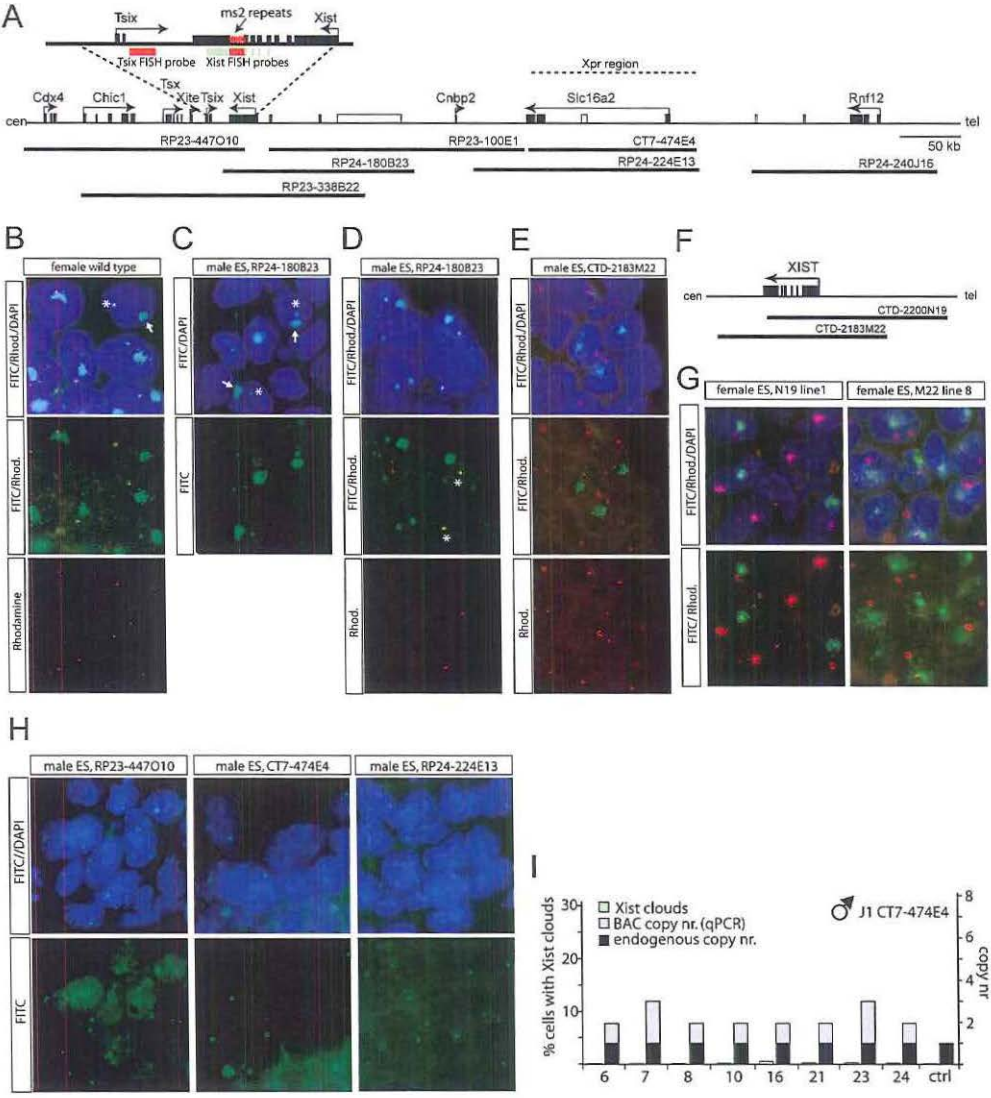



**Figure 1: BAC contig covering the X inactivation center**

**A)** Map showing part of the mouse X chromosome, the location of the BAC sequences used in this study, 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 with *Xist* (FITC) and *Tsix* probes (rhodamine red) on day 3 differentiated wild type ES cells, showing cells with *Xist* clouds only detected with the *Xist* probe (one marked with an arrow), and pinpoint signals detected with both *Xist* and *Tsix* probes (one marked with a star). **C)** RNA-FISH with an *Xist* probe (FITC) on day 3 differentiated male ES cells with an integration of BAC RP24-180B23, showing cells with *Xist* clouds (some marked with arrows), and pinpoint signals (some marked with a star). **D)** RNA-FISH with *Xist* (FITC) and ms2 (rhodamine red) probes on day 3 differentiated 1.3 male ES cells with an integration of BAC RP24-180B23, showing that *Xist* clouds are associated with autosomes, at a different location then the ms2 positive *Tsix/Xist*

that the *Xist* and *Tsix* regions are involved in the XCI counting process [166-167, 169, 171], but may be explained by a difference in transgene copy number or the number of clones that we analyzed.

We continued our search for an XCI-activator gene by generating transgenic male ES lines with BACs covering *Tsix* including a region 100 kb centromeric to *Tsix* (RP23-447O10), and BACs covering a region 300 kb telomeric to *Xist* (CT7-474E4, RP24-224E13, and RP23-100E1), including two BACs (CT7-474E4 and RP23-224E13) covering the *Xpr* region, which was recently implicated in pairing and activation of *Xist* [187]. RNA-FISH analysis only revealed pinpoint *Tsix/Xist* signals at day 3 of differentiation, indicating the absence of XCI initiation on the wild type X chromosome (**Figure 1G**, and **Supplemental Table 2B**). We also found no significant induction of XCI on both X chromosomes in female BAC RP23-100E1, CT7-474E4 and RP23-224E13 transgenic ES cells analyzed at day 3 of differentiation (**Supplemental Table 2C**). These results show that, despite involvement of the *Xpr* region in X chromosome pairing [187], additional transgenic copies of the *Xpr* region do not interfere with the XCI counting and initiation process.

### *A region on the X chromosome involved in activation of XCI*

We continued our search for an XCI-activator gene and analyzed transgenic cells with an integration of BAC RP24-240J16, which covers an area from 410 kb to 570 kb telomeric to *Xist* (**Figure 1A**). Transgenic male ES cell lines were established using three independent ES cell lines (F1 2-3, J1, and 1.3), and the BAC integration was confirmed by qPCR and/or DNA-FISH (**Figure 2B**, **2C**, and **Supplemental Figure 2A**, **2B**). Interestingly, RNA-FISH analysis of day 3 differentiated BAC transgenic ES cell lines showed several lines with a significant number of cells with *Xist* clouds, which we never observed in control male cell lines (95% confidence interval, **Figure 2A**, **2B**, **Supplemental Table 1** and **Supplemental Figure 2A**). DNA/RNA-FISH analysis detecting both the X chromosome and *Xist* RNA confirmed that these male ES cells initiated XCI on the single X chromosome (**Figure 2D**). In transgenic female cell lines we also obtained an increased percentage of cells with two *Xist* clouds (**Figure 2A** and **2C**). RNA-FISH analysis on two independent day-3-differentiated RP24-240J16 transgenic female lines, heterozygous for the ms2 tag, showed that female cells with two *Xist* clouds only had one ms2 positive cloud (**Figure 2E**). Because aneuploidy would have resulted in a significant proportion of cells with either two ms2 positive or two negative clouds, this finding confirms that the transgenic female cells contained two X chromosomes. Our results therefore suggest that BAC RP24-240J16 harbors a gene encoding an XCI-activator.

**Figure 1: continued**
pinpoint signals (star). **E)** RNA-FISH detecting mouse *Xist* (rhodamine red) and human *XIST* (FITC) on day 3 differentiated male cells transgenic for human CTD-2183M22 (**Figure 1F**). **F)** Map of human *XIST* and the location of the BAC sequences used to generate transgenic ES cell lines. **G)** RNA-FISH detecting murine *Xist* (rhodamine red) and human *XIST* (FITC) on day 3 differentiated transgenic female ES cells. **H)** Similar to C), but with BACs RP23-447O10, CT7-474E4, and RP24-224E13. **I)** Percentage of CT7-474E4 male transgenic cell lines (6-24) with *Xist* clouds (green, BAC copies in grey, endogenous copies in black, n>100 per cell line).

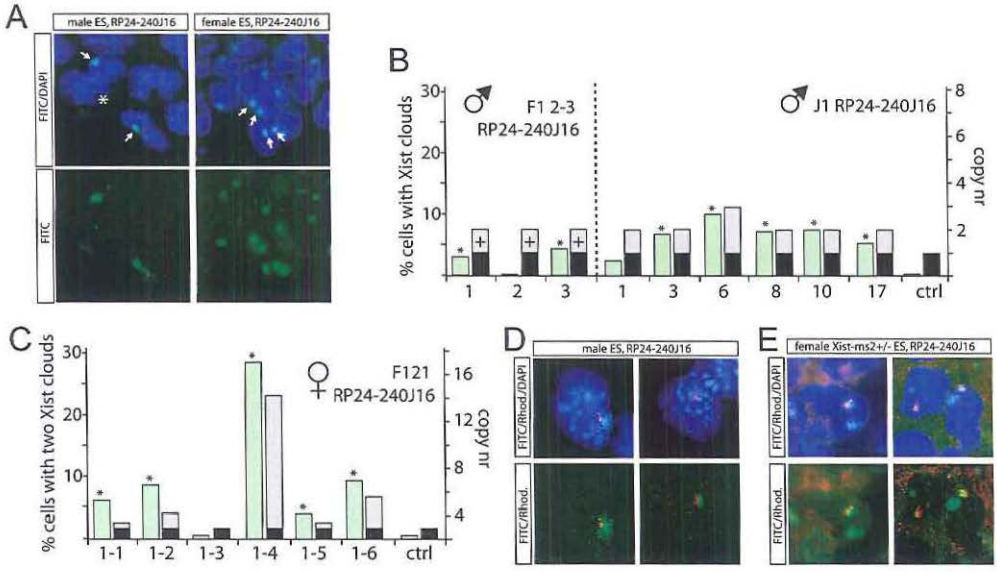



**Figure 2: Ectopic XCI in transgenic male and female ES cells**
**A)** RNA-FISH with an *Xist* probe (FITC) on day 3 differentiated ES cells with an integration of BAC RP24-240J16, showing male cells with *Xist* clouds (arrows) or pinpoint signals (star), and female cells with two *Xist* clouds (arrows). **B,C)** Percentage of male cells with single *Xist* clouds B) and female cells with two *Xist* clouds at day 3 of differentiation C), and BAC copy nr determined by qPCR (BAC copies in grey, endogenous copies of *Rnf12* in black, + indicates integration confirmed by DNA-FISH only, n>100 per cell line). Female line 1-3 has no BAC integration. ES cell lines that show a significant percentage of cells with *Xist* clouds compared to wild type control lines are indicated with an asterisk (non-overlapping 95% confidence interval, $p<0.05$). **D)** RNA/DNA-FISH detecting *Xist* (FITC) and an X chromosome specific probe (rhodamine red), on day 3 differentiated 1.3 male ES cells. **E)** RNA-FISH detecting *Xist* (FITC) and ms2 (rhodamine red) on day 3 differentiated female ES cells heterozygous for the *Xist*-ms2 tag, showing cells with two *Xist* clouds, one marked by ms2. Cells with two *Xist* clouds only showed one *Xist* and one *Xist*-ms2 cloud (Line 8 n=46, line 9 n=55).

## *Rnf12 is an XCI-activator*

BAC RP24-240J16 encompasses *Rnf12* which is ubiquitously expressed in early mouse development, and two predicted genes for which no expression data is available (**Figure 1A**). To identify the sequences required for induction of XCI in male cells, we fine-mapped the area using mouse BAC sequences covering part of BAC RP24-240J16, thereby reducing the minimal region required for ectopic XCI to 10 kb (**Figure 3A**, and **Supplemental Table 3**). Expression analysis using total RNA of day 3 differentiated female ES cells hybridized to a tiling array covering BAC RP24-240J16 provided unequivocal data that *Rnf12*, which encodes an E3 ubiquitin ligase, is the only transcribed sequence within this region, and that the 10 kb region overlaps with the promoter and exon 1 and 2 of *Rnf12* (**Figure 3B**).

To establish a direct role of RNF12 in XCI, we disrupted the open reading frame of *Rnf12* by insertion of a neomycin/kanamycin resistance cassette in two orientations into exon 5 of *Rnf12*. This mutation disrupts most of the open reading frame, but leaves the 10

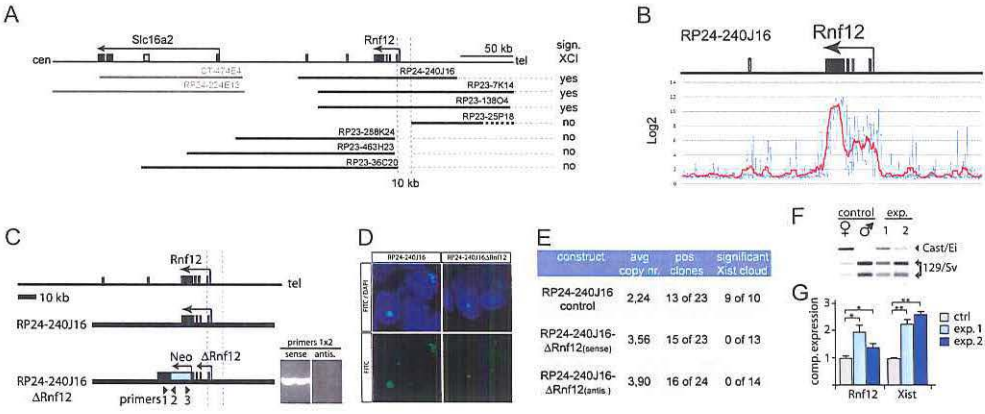



**Figure 3: *Rnf12* encodes an XCI-activator**
**A)** Position of BACs used for the fine-mapping, and their potential to stimulate XCI. BAC RP23-25P18, covering part of BAC RP24-240J16, was truncated (the part shown as a solid line was found to be present using PCR analysis; not shown). The minimal region required for ectopic XCI is marked in blue. **B)** Tiling array expression analysis with total RNA of day 3 differentiated female ES cells (top panel, moving average in red, raw data in blue, y-axis expression in log2). **C)** Map of part of the X chromosome covered by BAC RP24-240J16, and the modified BAC RP24-240J16ΔRnf12 with a neomycin resistance cassette inserted in two orientations (only the sense orientation is shown). Right panel shows confirmation of homologous recombination by PCR using the primer combination as indicated. **D)** RNA-FISH detecting *Xist* (FITC) on day 3 differentiated BAC RP24-240J16 (left panels) and RP24-240J16ΔRnf12 (right panels). **E)** Summary of the results obtained with all RP24-240J16 and RP24-240J16ΔRnf12 cell lines. Avg copy nr. is average of copynumber determined by qPCR of all clones with extra BAC integrations. Pos. clones indicates the number of clones with extra BAC integrations versus all picked clones. A 95% non-overlapping confidence interval was used for scoring ES cell lines with a significant percentage of *Xist* clouds ($p<0.05$), as determined by RNA-FISH of *Xist*. Not all positive clones with BAC integrations could be analyzed. **F)** RT-PCR analysis with RNA isolated from day 3 differentiated male (129/Sv) and female (Cast/Ei 129/Sv) control cells and male cells transiently transfected with an *Rnf12* expression construct (exp.). Analysis of NheI digested PCR products after gel electrophoresis indicates expression of transgenic *Rnf12* (Cast/Ei). **G)** RT-qPCR analysis of *Rnf12* and *Xist* expression in day 3 differentiated male cells transiently transfected with an *Rnf12* expression construct (exp. 1 and 2). Expression was compared to the expression level set at 1 in control cells (ctrl, T-test: * $p<0.05$, ** $p<0.01$).

kb minimal region required for ectopic XCI intact. This allowed us to test whether the induction of XCI is evoked by either *Rnf12*-encoded protein or by a DNA element within the 10 kb region (**Figure 3C**). Analysis of day-3-differentiated male ES cell lines with randomly integrated BAC RP24-240J16ΔRnf12 (sense and anti-sense) transgenes containing the mutated *Rnf12* gene, revealed no significant induction of XCI, in contrast to control transgenic male ES lines which showed significant induction of XCI in most cell lines (**Figure 3D**, **3E**, and **Supplemental Table 4A**). This result was confirmed with transgenic 30Δ1 female ES cells, where only random integration of the unmodified BAC RP24-240J16 resulted in an increased percentage of cells with two Xi's (**Supplemental Table 4B**),

Transient expression of the *Rnf12* cDNA might be sufficient to induce XCI in male cells. To investigate this, we transiently transfected male 1.3 ES cells with a *Rnf12* expression vector at day one of differentiation, and *Rnf12* and *Xist* expression was

determined after two more days of differentiation. *Xist* RNA-FISH experiments to test whether this transient expression of *Rnf12* resulted in activation of XCI were inconclusive, most likely due to a low transfection efficiency (<10%; measured by co-transfection of a GFP expression vector). Using RT-PCR amplification of *Rnf12* mRNA, followed by digestion with NheI, which digests an RFLP present in the endogenous *Rnf12* (129/Sv origin) PCR product but not in the transgenic *Rnf12* (Cast/Ei origin) PCR product, we could show that the transgenic *Rnf12* gene is transcribed (**Figure 3F**). In agreement with this, RT-qPCR analysis indicated an increase in transgenic *Rnf12* expression, and this correlated with increased *Xist* expression, which was up-regulated more than two-fold in *Rnf12* transfected samples (**Figure 3G**). These results support the view that *Rnf12* is involved in *Xist* up-regulation. The observed up-regulation of *Xist* was less pronounced than what would be expected from the studies with BACs containing *Rnf12*, but this is most likely due to the low transfection efficiency and cell death of male 1.3 ES cells after initiation of XCI. In addition, in the cells that have been transfected, the increased concentration of RNF12 may be lethal due to other functions of RNF12 which may be compromised when there is an overdose of the protein. Interestingly, *Rnf12* BAC transgenic ES cells did not survive freeze thawing. We think that extensive cell death may be initiated through activation of RNF12 by the thawing procedure, possibly resulting in ectopic XCI or mis-regulation of other processes.

In summary, fine mapping and expression studies revealed a 10 kb region on BAC RP24-240J16 to be required for ectopic XCI. This region overlaps with exon 1 and 2 of *Rnf12*, which is the only transcriptionally active gene covered by BAC RP24-240J16. In addition, transgenes with a disrupted *Rnf12* open reading frame do not induce ectopic XCI, whereas over-expression of *Rnf12* results in induction of *Xist* expression. These results provide strong evidence that *Rnf12* encodes an XCI-activator.

## Conservation of RNF12 and dose-dependent activation of XCI by RNF12

*Xist*-mediated XCI is present in all eutherians, and previous studies have indicated that *Rnf12* is highly conserved among mammals and other species [893]. We wanted to test whether human RNF12 can induce XCI in transgenic male and female mouse ES cells. To this end, transgenic mouse ES cells were generated containing human BAC CTD-2530H13 (**Figure 4A**), which covers an area that is homologous to the region covered by mouse RP24-240J16 (**Figure 1A**) and shows high sequence homology in the promoter and coding regions of *Rnf12/RNF12* (**Figure 4B**). Upon differentiation of several of these CTD-2530H13 transgenic cells, XCI is induced in a significant proportion of the male cells, and on both X chromosomes in an increased percentage of the female cells, suggesting that the function of RNF12 in XCI in mouse and human is conserved (**Figure 4C**, **4D** and **4E**).

To further test whether RNF12 acts as a dose-dependent activator of XCI, we EB-differentiated transgenic BAC CTD-2530H13 ES cell lines for 3 days, and determined the expression of mouse endogenous *Rnf12* and *Xist*, and human transgenic *RNF12* by RT-qPCR. Comparison of *Xist* RNA-FISH and RT-qPCR expression analysis showed a correlation between transgenic *RNF12* expression, *Xist* up-regulation, and activation of XCI on the single X in male cells (Pearson 0.77, $p<0.001$ and Pearson 0.67, $p<0.01$, respectively, **Figure 5A** and **5B**). The effect of transgenic *RNF12* on *Xist* expression,

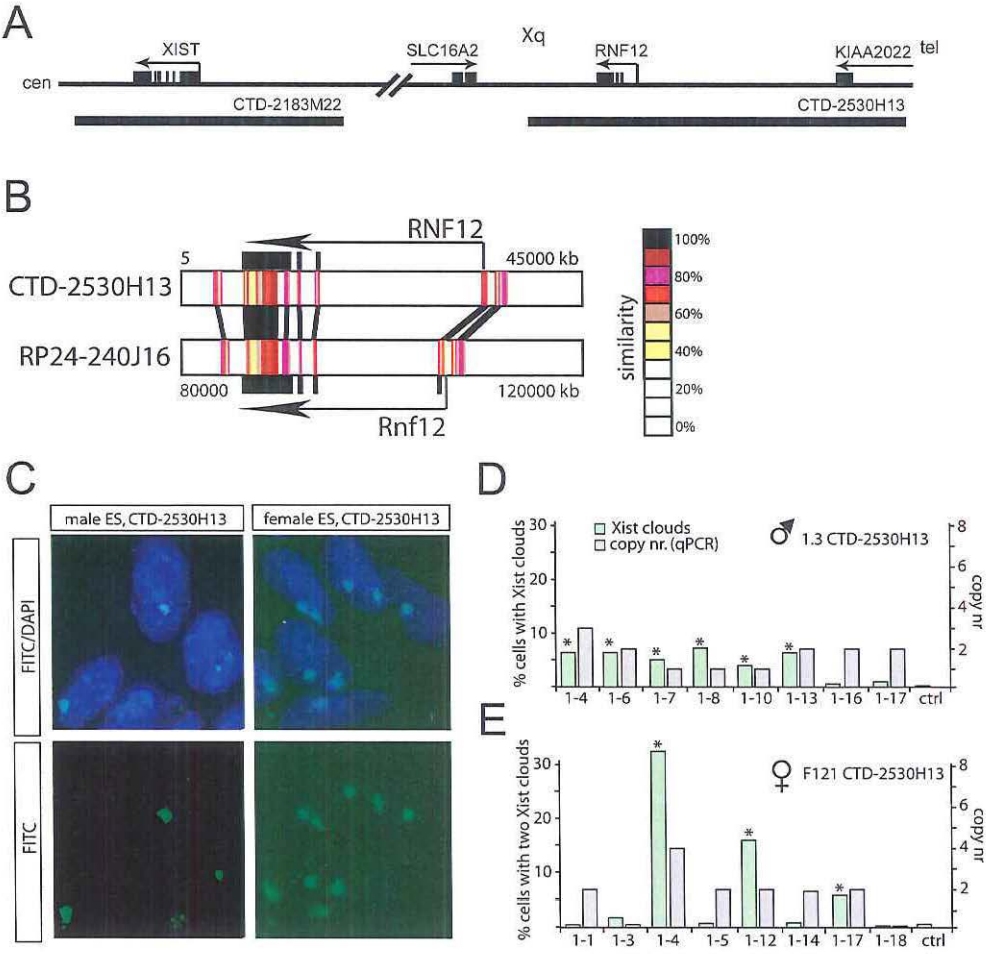
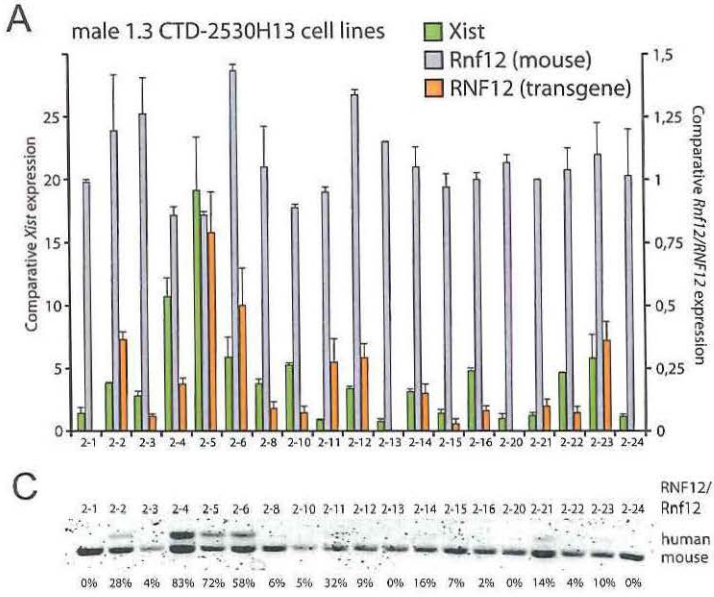


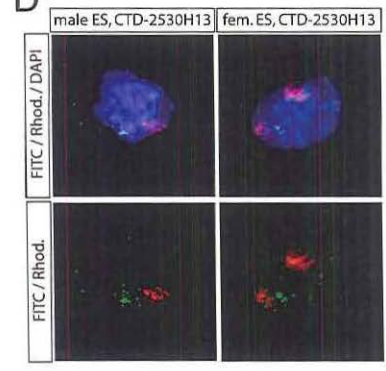

**Figure 4: The action of human RNF12 is conserved in mouse**
**A)** Schematic map of part of the human X chromosome and location of the BACs used for the analysis. **B)** Alignment of mouse (RP24-240J16) and human (CTD-2530H13) BAC sequences (bottom panel). Shown is the region containing the *Rnf12 / RNF12* gene. **C)** RNA-FISH detecting *Xist* (FITC) on day 3 differentiated CTD-2530H13 transgenic male ES cells (left panels) and female ES cells (right panels). **D, E)** Quantification of RNA-FISH presented in C), for BAC transgenic male D) and female E) cell lines, and BAC copy number determined by qPCR (grey, n>100 per cell line). Day 3 differentiated ES cell lines with a significant percentage of cells with *Xist* clouds compared to wild type control lines are indicated with an asterisk (non-overlapping 95% confidence interval, $p<0.05$).

determined by RT-qPCR, is more pronounced in male than female cell lines, because *Xist* is normalized to the level present in male and female control cell lines, respectively (**Supplemental Figure 4A** and **4B**). Some lines did not show ectopic XCI despite expression of transgenic *RNF12* (clone nrs: ♂ 2-6, 2-21 (**Figure 5B**) and ♀ 2-22 (**Supplemental Figure 4B**)), which may be attributed to truncated transgenes giving rise to




B

| Male 1.3 CTD-2530H13 line | qPCR copy nr | RNA-FISH 1xXist | n |
|---|---|---|---|
| 2-1 | - | 0% | 103 |
| 2-2 | ++ | 3%* | 136 |
| 2-3 | + | 4%* | 152 |
| 2-4 | ++ | 12%* | 198 |
| 2-5 | +++ | 36%* | 106 |
| 2-6 | ++ | 0% | 75 |
| 2-8 | + | no cells | - |
| 2-10 | + | 7%* | 152 |
| 2-11 | + | 5%* | 115 |
| 2-12 | + | no cells | - |
| 2-13 | - | N.D. | - |
| 2-14 | + | 5%* | 114 |
| 2-15 | + | no cells | - |
| 2-16 | + | 7%* | 114 |
| 2-20 | - | 0% | 108 |
| 2-21 | + | 0% | 133 |
| 2-22 | + | 9%* | 117 |
| 2-23 | ++ | 6%* | 108 |
| 2-24 | - | 0% | 142 |




E

| male 1.3 construct | RNF12 | 1xXist | 1xXist and RNF12 | n |
|---|---|---|---|---|
| CTD-2530H13 line 2-4 | 45% | 14% | 95% | 604 |
| CTD-2530H13 line 2-5 | 50% | 12% | 96% | 711 |

| female F1 2-1 construct | RNF12 | 1xXist | 2xXist | 2xXist and RNF12 | n |
|---|---|---|---|---|---|
| CTD-2530H13 line 2-3 | 30% | 42% | 11% | 100% | 276 |
| CTD-2530H13 line 2-5 | 21% | 46% | 7% | 90% | 268 |

**Figure 5: RNF12 is a dose-dependent activator of XCI**
**A)** Expression analysis of male 1.3 cell lines targeted with the human CTD-2530H13 BAC containing *RNF12*. RT-q-PCR was performed with primer sets for *Xist* (green), *Rnf12* (grey), and *RNF12* (orange). All samples were normalized to beta-*actin*, and expression was compared to the average of wild type cell lines 1.3 H13 1/13/20/24. Expression of human *RNF12* was compared to expression of mouse *Rnf12* of the control cell lines. **B)** Table of male 1.3 cell lines targeted with the human CTD-2530H13 BAC containing *RNF12*. The relative copy number of the BAC integration, ranging from – (no integration) to +++ (several copies). The RNA-FISH column represents percentage of cells with one *Xist* cloud (1x*Xist*, * $p<0.05$ based on a 95% confidence interval, n is number of cells counted). **C)** RT-PCR analysis detecting a length polymorphism distinguishing *Rnf12* and *RNF12*. The relative expression of *RNF12* compared to endogenous *Rnf12* is depicted below each lane. **D)** *Xist-RNF12* RNA-FISH with day 3 differentiated BAC CTD-2530H13 transgenic male and female ES cell lines, detecting *Xist* clouds (rhodamine red) and *RNF12* transcription foci (FITC). **E)** Quantification of *RNF12* transcription foci (*RNF12*), single (1x *Xist*) or double (2x *Xist*) *Xist* clouds, and the percentage of cells with a single *Xist*

a non-functional RNA, or to variegated expression through position effects resulting in only a few cells expressing the transgene at a relative high level. To compare the expression level of *RNF12* versus *Rnf12,* we amplified a length polymorphism distinguishing *RNF12* from *Rnf12* by RT-PCR (**Figure 5C**). Quantification of the relative intensity of *RNF12* and *Rnf12* confirmed a correlation between expression of transgenic *RNF12, Xist* up-regulation, and XCI (Pearson 0.67, $p<0.01$ and Pearson 0.58, $p<0.05$ respectively). Our results also indicate that transgenic expression is moderate, not reaching the endogenous level, suggesting selection against cells that express *RNF12/Rnf12* at higher levels. We further investigated whether expression of *RNF12* correlated with *Xist* cloud formation, by combined RNA-FISH detecting *Xist* and *RNF12.* Analysis of two transgenic male and female cell lines indicated that a single *Xist* cloud in male cells and two *Xist* clouds in female cells were almost exclusively present in cells which showed an *RNF12* RNA-FISH signal ($P<0.0001$, Z-test for proportion, **Figure 5D** and **5E**).

Although the function in XCI appears to be conserved, we do not know whether the specific activities of mouse and human RNF12 are equal. We therefore repeated the experiment shown in **Figure 5**, but generated 1.3 male transgenic ES cells harboring a BAC sequence covering the *Mus. musculus mollosinus Rnf12* gene, which allowed us to use a RFLP (NheI) that discriminates between endogenous and transgenic *Rnf12* cDNA. *Xist* RNA-FISH with day 3 differentiated transgenic ES cell lines containing at least one transgene copy showed induction of XCI in a significant proportion of cells for most ES cell lines (**Supplemental Figure 4D** and **4E**). RT-PCR expression analysis confirmed that *Mus. musculus mollosinus Rnf12* is expressed in the undifferentiated transgenic male cell lines that induce XCI, at 0.4- to 2.0-fold levels compared to the expression level of one endogenous *Rnf12* copy (**Supplemental Figure 4E**). In addition, Western blot analysis with anti-RNF12, and anti-ACTIN as a control, confirmed elevated expression of RNF12 in day one differentiated transgenic male ES cell lines compared to a wild type control cells (not all cell lines were analyzed, **Supplemental Figure 4F**). Expression of *Rnf12* correlated with cells that initiated XCI at day 3 of differentiation (Pearson 0.67, $p<0.01$). Similar to our findings with CTD-2530H13 transgenic ES cell lines, we only observed ES cell lines that showed moderate expression of transgenic *Rnf12*.

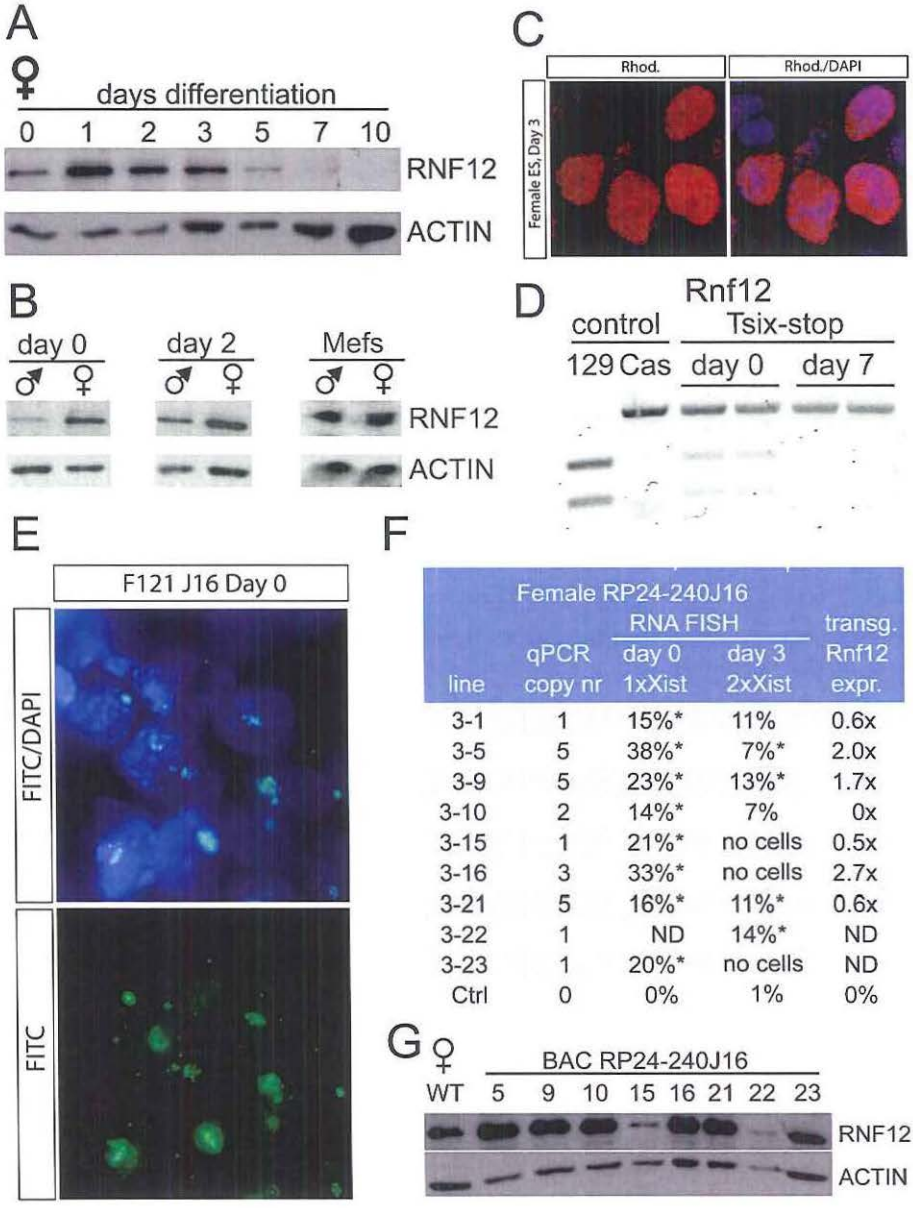
The results obtained with transgenic ES cell lines harboring human *RNF12* and *Mus. musculus mollosinus Rnf12* transgenes indicate that the function of RNF12 is most likely conserved from human to mouse. Furthermore, expression of ectopic *RNF12* positively correlates with expression of *Xist* and subsequent initiation of XCI.

**Figure 5: continued**
cloud together with an *RNF12* signal in BAC CTD-2530H13 transgenic male ES cell lines (1x*Xist* and *RNF12*), or two *Xist* clouds together with an *RNF12* signal in BAC CTD-2530H13 transgenic female ES cell lines (2x*Xist* and *RNF12*), at day 3 of differentiation (n=number of cells analyzed).

### Endogenous RNF12 expression correlates with XCI

*Rnf12* encodes an E3 ubiquitin ligase involved in the regulation of LIM-homeodomain containing factors, by targeting LIM interacting proteins LDB1 and LDB2 for degradation [893]. In addition, RNF12 also appears to be involved in gene activation, and telomere length homeostasis, indicating that this multifaceted protein is involved in many important processes besides XCI [894-895]. To play a dose-dependent role in XCI, it is to be expected that, prior to XCI, the RNF12 concentration is two-fold higher in female cells compared to male cells, and that expression of RNF12 is correlated with the developmental time period were XCI is initiated. In addition, to properly regulate XCI *Rnf12* expression should be subject to XCI to ensure down-regulation of RNF12 preventing initiation of XCI on the second X chromosome.

The endogenous RNF12 expression was estimated in nuclear extracts of male and female ES cells at different time points of differentiation, using Western blot analysis with an antibody detecting RNF12, and anti-ACTIN as a loading control. This analysis revealed that RNF12 is up-regulated in female and male cells around the time XCI is initiated in female cells, after which expression decreases in time (**Figure 6A**, and data not shown). Comparison of RNF12 expression in nuclear and cytoplasmic extracts indicated that RNF12 is almost exclusively present in the nucleus (data not shown). Immuno-cytochemistry also revealed nuclear localization of RNF12 at day 3 of differentiation (**Figure 6C**), and indicated that RNF12 did not localize to the Xi. Comparison of RNF12 expression in Cast/Ei/129/Sv male and female ES cell lines prior to XCI, and around the time XCI is initiated, confirms that RNF12 is higher expressed in female compared to male cells (**Figure 6B**). This difference in RNF12 expression was absent in male and female mouse embryonic fibroblasts (MEF), suggesting that one copy of *Rnf12* is inactivated in female MEFs. Allele specific RT-PCR analysis with RNA of undifferentiated and day-7-differentiated heterozygous *Tsix* mutant ES cells, which exclusively inactivate the mutant 129/Sv X [123], indeed confirmed that *Rnf12* expression is subject to XCI (**Figure 6D**). The above Western blot analysis indicated that RNF12 is already expressed prior to XCI, from which it can be suggested that additional copies of *Rnf12* may lead to XCI in undifferentiated ES cells. We targeted F1 2-1 female cells with BAC RP24-240J16 and picked and expanded neomycin positive clones. Undifferentiated ES cell lines with additional transgenic copies of *Rnf12*, determined by qPCR, appeared indistinguishable from wild type undifferentiated ES cells. Undifferentiated and day three differentiated ES cells were fixed and subjected to *Xist* RNA-FISH analysis. We found that all the undifferentiated cell lines with transgenic copies of *Rnf12* showed *Xist* clouds, which were not present in undifferentiated female control cells (**Figure 6E** and **6F**). Interestingly, several of the cell lines with the highest percentages of Xi's in undifferentiated ES cells, showed extensive cell loss upon differentiation, with no or a few cells left to analyze at day 3 of differentiation. The relative expression of *Rnf12* was estimated by RT-PCR amplifying an RFLP (NheI). This analysis indicated increased expression of transgenic *Rnf12* in almost all undifferentiated transgenic ES cell lines tested (**Figure 6F**), which correlated with *Xist* cloud formation (Pearson 0.86, $p<0.01$). Also the RNF12 protein levels in nuclear extracts from cells at day 3 of differentiation were increased in most transgenic ES cell lines




| Female RP24-240J16 | | RNA FISH | | transg. |
|---|---|---|---|---|
| line | qPCR copy nr | day 0 1xXist | day 3 2xXist | Rnf12 expr. |
| 3-1 | 1 | 15%* | 11% | 0.6x |
| 3-5 | 5 | 38%* | 7%* | 2.0x |
| 3-9 | 5 | 23%* | 13%* | 1.7x |
| 3-10 | 2 | 14%* | 7% | 0x |
| 3-15 | 1 | 21%* | no cells | 0.5x |
| 3-16 | 3 | 33%* | no cells | 2.7x |
| 3-21 | 5 | 16%* | 11%* | 0.6x |
| 3-22 | 1 | ND | 14%* | ND |
| 3-23 | 1 | 20%* | no cells | ND |
| Ctrl | 0 | 0% | 1% | 0% |

**Figure 6: Expression analysis of RNF12**
**A)** Western blot analysis detecting RNF12 (top panel) and ACTIN (bottom panel) in female ES cells at different time points in differentiation. **B)** Western blot analysis detecting RNF12 (top panel) and ACTIN (bottom panel), comparing RNF12 expression in male and female undifferentiated (left panel), day 2 differentiated ES cells (middle panel), and in MEFs (right panel). **C)** Immunocytochemistry on day 3 differentiated female ES cells using an antibody detecting RNF12 (rhodamine red). **D)** RT-PCR

compared to wild type female cells (**Figure 6G**), although lines 3-15 and 3-22 did not show an increase in RNF12 expression, possibly due to cell loss of cells over-expressing RNF12.

Taken together, these findings indicate that RNF12 is expressed in the time window of ES cell differentiation were XCI is initiated, with a nuclear concentration of RNF12 that is higher in female compared to male cells. Our results suggest that the RNF12 concentration is well titrated to ensure a dose-dependent role for RNF12 in activation of XCI. In agreement with this, additional copies of *Rnf12* can induce XCI in undifferentiated female ES cells, indicating that an increase in the RNF12 concentration is sufficient to trigger XCI.

**Figure 6: continued**
expression analysis of *Rnf12* with RNA isolated from undifferentiated and day 7 differentiated 129/Sv Cast/Ei *Tsix*-stop ES cells, which exhibit non-random XCI of the mutated 129/Sv X chromosome, using a NheI cleavage of the PCR product at an RFLP present in the 129/Sv allele. The left two lanes show control 129/Sv and Cast/Ei samples. **E)** RNA-FISH detecting *Xist* (FITC) on undifferentiated BAC RP24-240J16 (left panels) transgenic female ES cells. **F)** Table of female F1 2-1 cell lines targeted with BAC RP24-240J16. The copy number of the BAC integration, and the percentage of undifferentiated cells containing one *Xist* cloud (1x*Xist*) and day 3 differentiated cells with two *Xist* clouds (2x*Xist*) are depicted. The column on the right shows the level of transgene expression, determined by RFLP RT-PCR, relative to one endogenous copy of *Rnf12* in undifferentiated ES cell lines. ES cell lines that show a significant percentage of cells with *Xist* clouds compared to wild type control lines are indicated with an asterisk (confidence interval 95%, $p<0.05$). **G)** Western blot analysis detecting RNF12 (top panel) and ACTIN (bottom panel) with nuclear extracts of day 3 differentiated ES cell lines described in F).

Figure 7

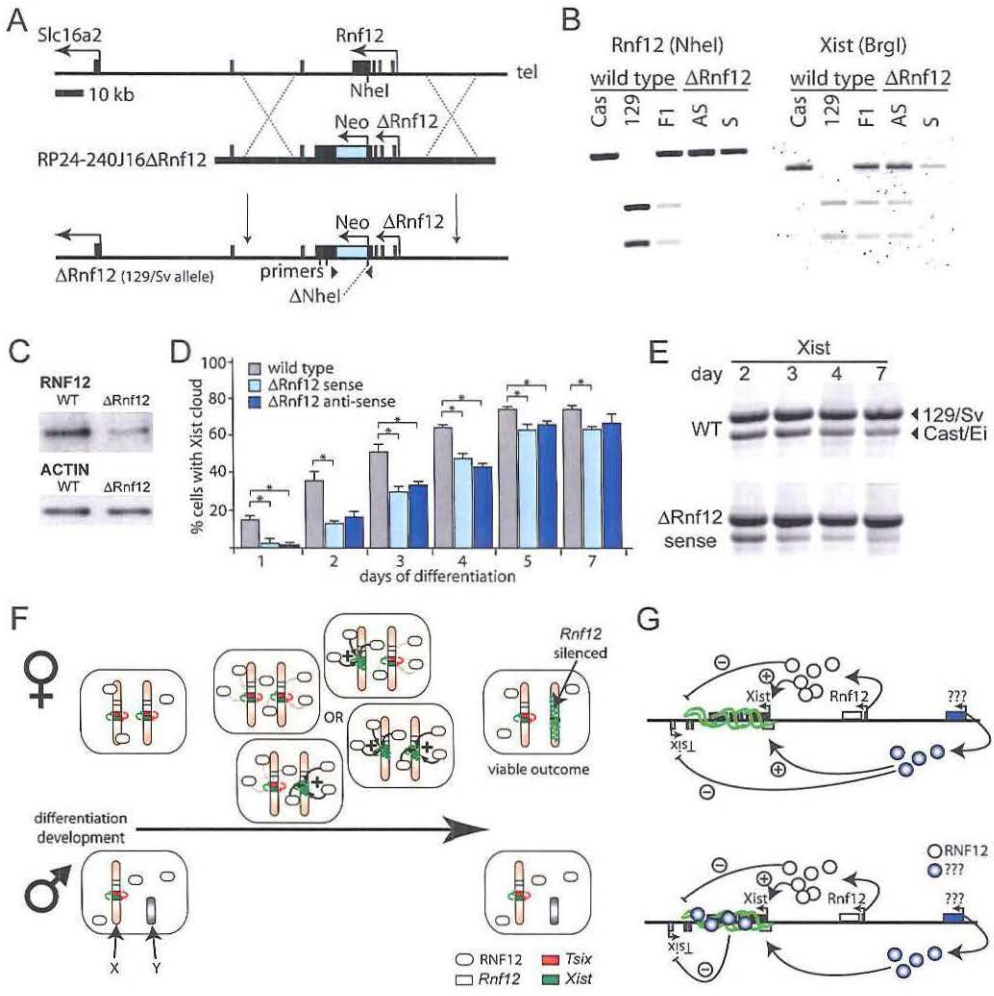



**Figure 7: Generation and analysis of *Rnf12+/-* female ES cells**
**A)** A map of part of the X chromosome and the BAC RP24-240J16ΔRnf12 targeting construct with a neomycin resistance cassette inserted in two orientations (only sense orientation is shown), and the targeted 129/Sv allele, and location of PCR primers used for genotyping. **B)** Left panel, RT-PCR with primers amplifying a RFLP (NheI) in the 129/Sv *Rnf12* allele. Right panel, RT-PCR with a primer set amplifying a RFLP (BrgI) in the 129/Sv *Xist* allele. **C)** Western blot analysis detecting RNF12 (top panel) and ACTIN (bottom panel) in nuclear extracts from a wild type female and Δ*Rnf12+/-* ES cell line. **D)** Bar graph showing the percentage of wild type and Δ*Rnf12+/-* (sense and anti sense) ES cells that initiated XCI, detected by *Xist* RNA-FISH, at different time points of EB differentiation. Significant differences are marked with an asterisk (T-test, * $p<0.05$) **E)** RT-PCR expression analysis of *Xist* originating from the 129/Sv allele (top band) and the Cast/Ei allele (bottom band) with RNA isolated from day 2, 3, 4 and

### XCI is reduced in Rnf12+/- female cells

RNF12 appears to be an important activator of X chromosome counting and initiation of XCI, but it may not act as a single factor, and the existence of one or more additional XCI-activators or cofactors with some RNF12-independent activity cannot be excluded. To study this, we deleted *Rnf12* from one X chromosome (129/Sv) by targeting BAC RP24-240J16ΔRnf12 (sense and antisense) to a polymorphic F1 2-1 female (Cast/Ei-129/SV) ES cell line (**Figure 7A**). Correct targeting was confirmed by PCR amplification of a sequence containing a RFLP (NheI) which was removed from the targeted allele (**Figure 7B**). Some 129/Sv product is still detectable due to feeder cell (129/Sv) contamination. X chromosome copy number and origin were confirmed by DNA-FISH (**Supplemental Figure 5**), qPCR (not shown) and PCR amplification of a sequence containing a RFLP (BrgI) in *Xist* (**Figure 7B**). Western blot analysis with nuclear extracts from undifferentiated wild type and Δ*Rnf12*+/- (sense) ES cells confirms that RNF12 expression is reduced in the heterozygous knockout ES cells (**Figure 7C**). Two heterozygous Δ*Rnf12*+/- (sense and anti-sense) ES cell lines were EB-differentiated, fixed at different time points, and subjected to *Xist* RNA-FISH. We found that initiation of XCI in both Δ*Rnf12*+/- ES cell lines was severely reduced, by more than 80%, at the start of differentiation (day 1-3) but recovered later in the differentiation process (**Figure 7D**). EBs generated with Δ*Rnf12*+/- ES cells appeared smaller (data not shown), indicating cell selection against cells that retain two active X chromosomes and selection against cells that inactivated the wild type endogenous *Rnf12,* which would result in a RNF12 null cell. Analysis of *Xist* expression using a length polymorphism to distinguish cDNA originating from the 129/Sv or Cast/Ei allele revealed that *Xist* is expressed from both alleles in the beginning of the XCI process (**Figure 7E**), indicating that the probability to initiate XCI is reduced for both X chromosomes in Δ*Rnf12*+/- cells and is not caused by a *cis* effect of the *Rnf12* mutation. The F1 2-1 ES cells used in our studies show skewed XCI, with a preference for *Xist* expression from, and inactivation of, the 129/Sv X chromosome, caused by the presence of two different Xce's with different allelic properties. In Δ*Rnf12*+/- cells, from day 3 of differentiation onwards, *Xist* is almost exclusively expressed from the 129/Sv allele, suggesting cell selection in favor of cells that inactivated the mutant *Rnf12* allele. The presence of female Δ*Rnf12*+/- cells that do initiate XCI indicates that one or more additional X-encoded XCI-activators are involved in initiation of XCI. Nonetheless, the severe reduction in cells that initiate XCI in the Δ*Rnf12*+/- cells, and our studies with *Rnf12/RNF12* transgenic ES cells, both emphasize an important role for RNF12 in XCI counting and initiation.

**Figure 7: continued**
7 differentiated wild type and Δ*Rnf12*+/- (sense) ES cells. **F)** Upon differentiation of the ES cells, the concentration of RNF12 and/or an unknown target or modifier of RNF12 increases, which potentiates the action of RNF12. This leads to stochastic up-regulation of *Xist* transcription (grey arrows, up-regulation low, black arrows, up-regulation high), resulting in a probability to initiate XCI, and accumulate along the X (+). Spreading of *Xist* RNA leads to silencing of *Rnf12* transcription in *cis*, resulting in a drop of the nuclear RNF12 concentration, prohibiting inactivation of the second X. In male cells, the RNF12 concentration does not reach the threshold required to start this sequence of events. **G)** *Rnf12* mediated activation of XCI, could involve activation of *Xist*, or suppression of *Tsix* through direct or indirect mechanisms (RNF12 in yellow). The action of other putative XCI-activators (blue) could involve a similar mechanism as hypothesized for RNF12 (left panel), or could involve silencing of *Tsix* by *Xist* RNA mediated recruitment (right panel).

## Discussion

Herein, we have identified RNF12 as an X-encoded activator of X chromosome inactivation (XCI) in mouse embryonic stem (ES) cells. Additional copies of mouse or human *Rnf12/RNF12* result in an increased probability to initiate XCI, resulting in XCI on the single X chromosome in male cells and on both X chromosomes in female cells. The action of RNF12 is dose-dependent, and analysis of heterozygous *ΔRnf12*+/- ES cells confirms a role for *Rnf12* in XCI. At the same time, our findings indicate that one or more additional XCI-activators are involved in counting and initiation of XCI. Still, it is evident that RNF12 plays a key role in control of X-chromosomal gene dosage between male and female cells, and is therefore the first X-linked dose-dependent activator of XCI.

### *RNF12 is a potent activator of XCI*

Previous studies have indicated that ectopic XCI is initiated in transgenic male cells containing additional copies of *Xist/XIST* and flanking sequences [166-167, 169, 171]. In the present analysis, these same regions did not evoke ectopic XCI in transgenic male and female cells. To try to explain this discrepancy, one could argue that transgene copy number was different in the various studies, and that the integration site and variegated transgene expression might be implicated. In addition, analysis of XCI at later time points of the differentiation process might reveal induction of XCI. The absence of initiation of ectopic XCI in our *Xist/Tsix* transgenic cell lines indicates that additional sequences are required for proper Xic function, which is supported by our previous finding that ΔXTX heterozygous ES cells still initiate XCI [179], and the finding that single copy *Xist/Tsix* transgenes do not induce XCI on the endogenous X chromosome [175]. The *Xpr* region located ~200 kb telomeric to *Xist* has been implicated in proposed pairing of two X chromosomes [187], and activation of *Xist*. However, our findings indicate that extra transgenic copies of the *Xpr* region are not sufficient for initiation of ectopic XCI, although this does not preclude a role for this region in the XCI process. Regarding the present studies it should be noted that almost all the male and female cell lines that we generated with a BAC transgene containing *Rnf12* resulted in XCI on the single X chromosome in male cells (32 out of 38 cell lines), and on both X chromosomes in female cells (17 out of 20 cell lines). For most cell lines activation of XCI also correlated with expression of transgenic RNF12. From this, we feel that we can safely conclude that RNF12 is a very potent activator of XCI.

The observation that the RP24-240J16 transgenic cells did not initiate XCI on all X chromosomes, can be explained by our finding that RNF12 is not the only XCI-activator. Also, cells that inactivate too many X chromosomes will be counter-selected, possibly masking the effect of *Rnf12* on XCI, and expression of the transgenes may have been variegated. More importantly, extra copies of *Rnf12/RNF12* will lead to an increased probability to initiate XCI in transgenic cells, which may never reach 100% in the time interval required for one cell division, so that always some cells can retain at least one Xa. Our expression analysis also indicates that the expression level of transgenic *Rnf12* is modest. This suggests that higher expression levels are not tolerated, because this most likely results in extensive induction of XCI already in undifferentiated ES cells, and subsequent loss of the transgenic cells. Higher RNF12 concentrations may also compromise cell viability due to mis-regulation of other pathways in which RNF12 takes part. We think

this may also explain why the effect of transient expression of RNF12 on *Xist* induction is smaller than what is obtained in low copy transgenic ES cell lines, because the effect of transient expression of RNF12 on XCI may be masked through extensive counter-selection against transfected cells.

We found that XCI can be induced in undifferentiated female transgenic ES cells expressing exogenous RNF12. This finding could suggest that RNF12 over-expression induces ES cell differentiation. However, undifferentiated wild type and transgenic ES cells appeared morphologically similar, and female ES cells with two copies of *Rnf12* have not been reported to be more differentiated than male cells with one copy of *Rnf12* [38]. Also, activation of XCI in *Rnf12* transgenic male ES cells cannot be explained by an effect of RNF12 on differentiation. It is therefore more likely that *Rnf12* transgenic ES cells remain undifferentiated, which implies that all other factors required for initiation of XCI are already present in undifferentiated ES cells, although the level of these other factors may increase upon differentiation. Our results also suggest that repression of *Xist* can be overcome by increased expression of RNF12, even in undifferentiated ES cells.

### *Stochastic initiation of XCI*

A stochastic model for XCI predicts that every X chromosome has an independent probability to be inactivated [179]. This contrasts with all other models that explain counting and initiation of XCI through mutually exclusive mechanisms [892]. Our present findings support a stochastic model for XCI and indicate that the probability to initiate XCI is directed by a chance process which is dependent on the nuclear concentration of different factors that promote or suppress XCI.

As described and referenced in the introduction, *Xist* and *Tsix* knockout and over-expression studies have indicated that the probability to initiate XCI is determined by the balance between *Xist* and *Tsix* transcription, which is regulated by X-encoded XCI-activators and autosomally encoded XCI-inhibitors. XCI-activators are involved in dose-dependent up-regulation of *Xist* expression, whereas XCI-inhibitors are involved in suppression of *Xist*. Protein expression analysis of differentiating female ES cells indicates that RNF12 is expressed and up-regulated around the time XCI is initiated. The nuclear concentration of RNF12 will reach a threshold, in female and not in male cells, that is sufficient to up-regulate *Xist* expression, either directly or indirectly, to a point where it initiates XCI (**Figure 7F**). Other XCI activators may be present at a constant concentration or reach a threshold in a similar fashion. The observed initiation of XCI in undifferentiated female ES cells upon transgenic RNF12 expression indicates that up-regulation of RNF12 may be required and sufficient to trigger XCI in female cells. However, the predicted E3 ubiquitin ligase activity of RNF12 suggests that expression or activity of additional factors, acting as substrate or modifier of RNF12 action, are likely to be involved in XCI.

RNF12 or its downstream target(s) may act through activation of *Xist* directly, or indirectly by suppression of *Tsix* (**Figure 7G**). Another possibility is that RNF12 is recruited by *Xist* RNA in order to silence *Tsix*, but RNF12 immuno-staining did not show specific accumulation of RNF12 anywhere in the nucleus suggesting that this is the least likely option. The evolutionary conservation of RNF12 and activation of XCI in mouse ES cells by human RNF12, taken together with the absence of an established role for *TSIX* in human X inactivation, makes it likely that RNF12 is involved in activation of *Xist/XIST*

transcription. This is also supported by studies with *Xist* promoter transgenes, which showed higher expression in female cells compared to male cells [89].

After initiation of XCI, the XCI-activator genes will be silenced in *cis*, providing a simple mechanism to decrease the concentration of the XCI-activators. Such a feedback mechanism will prevent inactivation of the second X chromosome in female cells, although the other X chromosome(s) still have a probability to initiate XCI, in the time window in between initiation of XCI and down-regulation of the XCI-activator concentration at the protein level. Previous studies have indicated that genes which are in close proximity to *Xist* are more likely to be inactivated at an earlier time point of development or ES cell differentiation than genes located further away. Close proximity of *Rnf12* to *Xist* may therefore facilitate a quick down-regulation of RNF12 [38]. Re-evaluation of the data used for this study indicates that *Rnf12* indeed belongs to the category of genes that is silenced early during ES cell differentiation (**Supplemental Figure 6**). Interestingly, *Rnf12* is also among the first genes to be down-regulated in imprinted XCI, which commences around the four- to eight-cell stage of embryonic development [311], and silencing of *Rnf12* is strictly dependent on *Xist* [896]. Also, the stability of RNF12 has been reported to be much lower than other nuclear proteins [897], which would facilitate a rapid feed-back mechanism to prevent inactivation of the second X chromosome.

Whether RNF12 is required for *Xist* expression after the Xi is established, remains to be determined. The observation that *Xist* remains expressed on the Xi can be explained by silencing of its negative regulator *Tsix*. On the Xi a lower XCI-activator concentration would then be sufficient for sustained expression of *Xist*. Following XCI, the reduced concentration of XCI-activators will be too low to induce XCI of the Xa, and its *Xist* gene is silenced in a *Tsix* dependent process involving *Xist* promoter specific DNA methylation and repressive histone modifications [89, 112, 898].

### *More XCI-activators involved*

*Rnf12* is ubiquitously expressed in embryos around the onset of XCI [893]. One known target of RNF12 is LDB1, which interacts with LIM-homeodomain transcription factors. RNF12 is also involved in activation of estrogen receptor α [895] and degradation of a telomere associating protein TRF1 with an effect on telomere length homeostasis [894]. Hence, RNF12 likely acts as a regulator, activator or repressor, also outside the context of XCI, but none of the known RNF12 partners from other pathways have been implicated in XCI.

Analysis of Δ*Rnf12*+/- cell lines indicates that *Rnf12* is not the only X-encoded XCI-activator regulating initiation of XCI. Similar to *Rnf12*, these activators may be involved in activation of *Xist* transcription or repression of *Tsix* through a direct mechanism or by *Xist* RNA mediated recruitment (**Figure 7G**). Studies on other organisms provide clear examples of how dosage compensation processes can be triggered by single master switch genes: *Sxl* in *Drosophila* and *Xol-1* in *C. elegans*. In both species, these master switch genes are regulated by several X-encoded numerator genes and autosomally encoded denominator genes, suggesting that more than one numerator and denominator gene is required to suppress the noise in the system [899]. For eutherians, a similar mechanism may apply, with the *Xist/Tsix* locus representing the master switch locus that is regulated by several XCI-activators and inhibitors to count the number of X chromosomes and activate

initiation of XCI. Previous studies suggest that NANOG, SOX2, OCT4, CTCF and YY1 act as XCI-inhibitors. Depletion of these factors results in increased *Xist* transcription indicating an involvement in determining the threshold that has to be overcome for initiation of XCI, either by direct repression of *Xist* or activation of *Tsix* [186, 191, 193]. However, the genes encoding the three pluripotency factors factors NANOG, SOX2, and OCT4, and also the genes encoding CTCF and YY1, are all located on autosomes, and are most likely not differentially expressed between male and female cells. Therefore, despite a clear involvement in XCI, these genes are not likely candidates to direct the XCI counting process. In contrast, the X-chromosomal *Rnf12* gene can be readily envisaged to encode a protein that acts as a numerator, to discriminate between chromosomally male and female cells in relation to XCI. RNF12 represents the first identified XCI-activator, involved in determining the probability to initiate XCI. Identification and characterization of the direct target or modifier of RNF12, and other genes encoding XCI-activators, will be the next step to further elucidate the control of counting and initiation in X chromosome inactivation.

## Acknowledgements

We would like to thank Nilhan Gunhanlar, Cristina Gontan Pardo, Akiko Inagaki, Bas de Hoon, and Maureen Eijpe for help with experiments. We also thank all department members for helpful discussions. This work was supported by HFSP CDA, NWO-VIDI and TOP grants to J.G., and a grant from the Dutch government (BSIK programme 03038, SCDD).

## Supplemental Data

The Supplemental Information contains BAC engineering procedures, procedures for BAC copy number determination and expression analysis, statistical procedures, six figures and four tables.

## Methods

### Cell lines

Transgenic ES cell lines were generated using wild type male J1 (129/Sv), F1 2-3 (129/Sv-Cast/Ei) ES lines or a wild type female line F1 2-1 (129/Sv-Cast/Ei). For determination of the origin of *Xist* a male line 1.3 and female line 30Δ1 were used, which both contain one *Xist* allele with 16 ms2 repeats integrated in exon 7 [92]. ES cells were grown and differentiated as described [179]. *Rnf12* transgenic ES cells did not survive freeze thawing and were made fresh prior to analysis. All wild type cell lines and modified BACs will be made available on request.

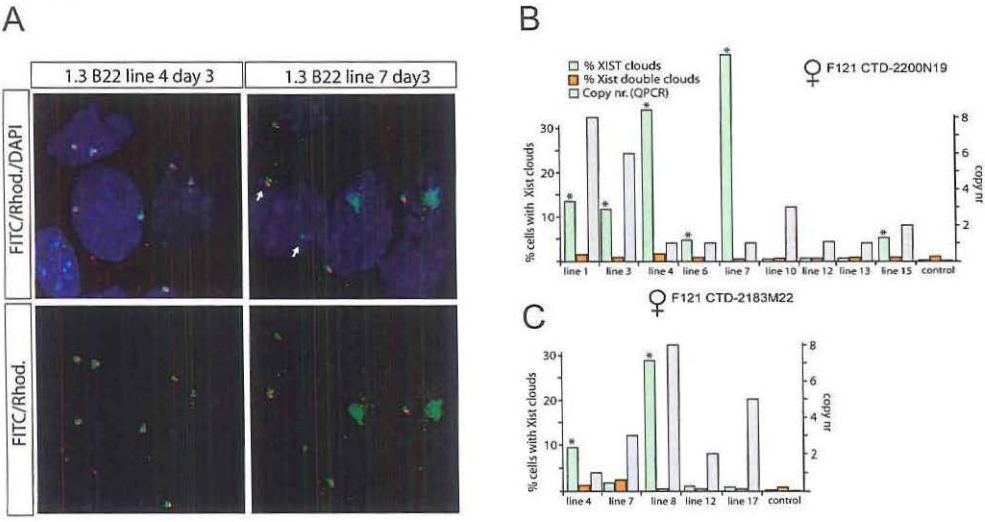
### Transient expression of Rnf12

Cast/Ei *Rnf12* cDNA was PCR amplified and cloned into pEGFP-N1 (Clontech), replacing the EGFP coding sequence. Male 1.3 ES cells were EB differentiated for one day, and then transfected with or without the *Rnf12* expression construct using lipofectamine (Invitrogen). The cells were co-transfected with a GFP expression construct for determining the transfection efficiency. The cells were harvested at day three of differentiation.

### RNA and RNA/DNA-FISH

RNA-FISH and RNA/DNA-FISH were performed as described [92, 179]. For detection of the region surrounding *Xist* (Xic probe) a cocktail of biotin labeled BAC sequences was used (CT7-474E4, CT7-45N16, CT7-155J2 and CT7-211B4). The *Tsix* RNA-FISH probe was a 5 kb SacII-SalI fragment (134071-139156 in AJ421479)

## Supplemental Figures

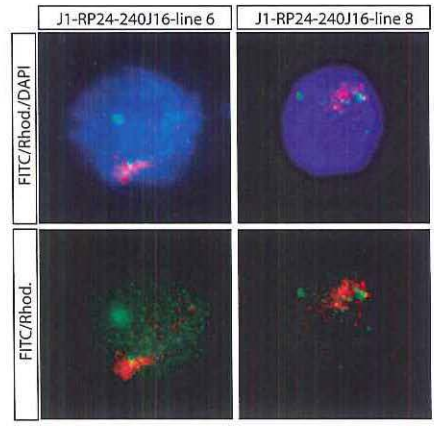



**Supplemental Figure 1: XCI in ES cell lines with a mouse and human *Xist/XIST* transgenes**
**A)** RNA-FISH with *Xist* (FITC) and *Tsix* probes (rhodamine red) on two day three differentiated ES cell lines with different integrations of a single copy BAC RP23-338B22 transgene. In line 1.3 RP23-338B22-7 that harbors one copy of the transgene and shows autosomal *Xist* accumulation, combined *Xist-Tsix* RNA-FISH revealed that a substantial proportion of cells displayed two *Xist* pinpoint signals, at day 1 of differentiation, of which only one colocalized with a *Tsix* specific pinpoint signal. We never observed that RP23-338B22-7 cells contained two *Tsix* pinpoint signals, in contrast to line 1.3 RP23-338B22-4 that did not show autosomal *Xist* accumulation. This indicates that, in the RP23-338B22-7 cells that showed autosomal spreading of *Xist*, the autosomal *Tsix* gene was already silent. **B,C)** Percentage of CTD-2200N19 (B) and CTD-2183M22 (C) female transgenic cells with two murine *Xist* clouds (orange) and a single human *XIST* cloud (green), and BAC copy number as determined by qPCR (BAC copies in grey, n>100 per cell line, * $p<0.05$, confidence interval 95%).

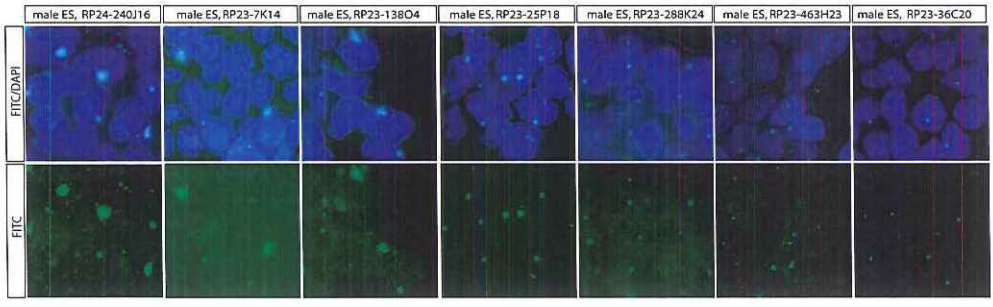
A ♂

| RP24-240J16, male ES 1.3 | | | |
|---|---|---|---|
| line | copy nr | % Xist | n |
| 15 | 3 | 4%* | 204 |
| 17 | 4 | 9%* | 156 |

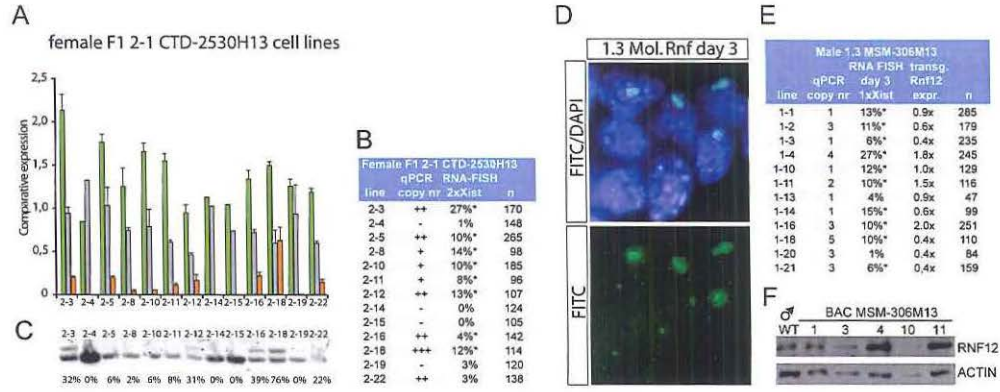
B



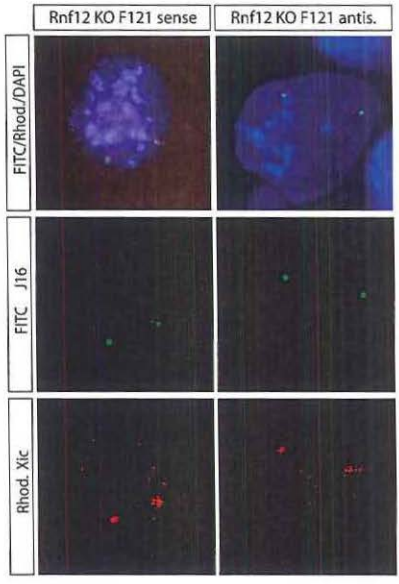

**Supplemental Figure 2: XCI in RP24-240J16 transgenic male ES cell lines**
**A)** Copy number determination and quantification of the percentage of cells that initiated XCI at day 3 of differentiation in 1.3 male ES cell lines transgenic for RP24-240J16 (* $P<0.05$, confidence interval 95%). **B)** DNA-FISH analysis with a RP24-240J16 BAC probe (FITC) and an X chromosome paint probe (Cy3), showing the autosomal integration of the BAC in J1 transgenic lines 6 and 8. Note that the intensity of the FITC signal correlates with the copy number determined by qPCR (**Figure 2B**).



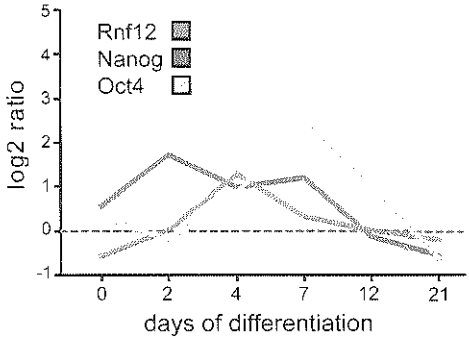

**Supplemental Figure 3: A 10 kb region from BAC contig RP24-240J16 activates XCI in male cells**
RNA-FISH detecting *Xist* (FITC) on day 3 differentiated male ES cells with different BAC transgenes (**Figure 3A**), showing that the 10 kb region depicted in **Figure 3A** is required for initiation of XCI in male cells.




**Supplemental Figure 4: Expression of transgenic RNF12 and *Rnf12* correlates with XCI**
**A)** Expression analysis of female F1 2-1 cell lines targeted with the human CTD-2530H13 BAC containing *RNF12*. RT-qPCR was performed as in **Figure 5A**. Expression was compared to the average of wild type cell lines F1 2-1 H13 4/14/15, which did not display ectopic expression of *RNF12*. **B)** Table of female F1 2-1 cell lines targeted with the human CTD-2530H13 BAC containing *RNF12* as in **Figure 5B** (* P<0.05, confidence interval 95%). **C)** RT-PCR analysis detecting a length polymorphism distinguishing *Rnf12* from *RNF12* expression. The relative expression *RNF12/Rnf12* is depicted below each lane. **D)** RNA-FISH with an *Xist* probe (FITC) on day 3 differentiated BAC MSM-306M13 transgenic 1.3 male ES cell lines. **E)** Copy number determination and quantification of the percentage of cells that initiated XCI at day 3 of differentiation in 1.3 male ES cell lines transgenic for BAC MSM-306M13. The right two columns show the level of transgene expression of *Rnf12* relative to the endogenous copy of *Rnf12*, determined by RT-PCR followed by RFLP digestion, and the number of cells analyzed for determining the percentage of *Xist* clouds (* P<0.05, confidence interval 95%). **F)** Western blot analysis detecting RNF12 (top panel) and ACTIN (bottom panel) with nuclear extracts of wild type male cells and MSM-306M13 transgenic ES cell lines at day 1 of differentiation.




**Supplemental Figure 5: Establishment of *ΔRnf12+/-* ES cell lines**
DNA-FISH on two different *Rnf12* knockout cell lines (sense and antisense) with a BAC RP24-240J16 probe (FITC) and a probe detecting the Xic (rhodamine red), confirming the correct integration of the targeting construct.




**Supplemental Figure 6: RNA expression analysis of *Rnf12*, *Nanog* and *Oct4* in differentiating female ES cells**
Expression data for *Rnf12*, *Nanog* and *Oct4* in differentiating female ES cells by Lin et al. [38], were retrieved and the log2 expression ratio was plotted in time.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| F121-9 | 0% | 0 | 235 | F121-9 | 50% | ± 5.1 | 0.27% | ±0,53 | 370 |
| F123 | 0% | -- | 773 | F123 | 0% | | -- | | 862 |
| J1 | 0% | -- | 704 | J1 | 0.14% | ±0.27 | -- | | 720 |
| 1.3 | 0% | -- | 653 | 1.3 | 0% | | -- | | 526 |

**Supplemental Table 1: XCI in control cell lines**
XCI was determined by *Xist* RNA-FISH in undifferentiated (left table) and day 3 differentiated (right table) male and female control cell lines.

A ♂

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | 8% | 90 | 4 | 1 | 0% | 1% | 142 | ± 1.6 | 1 | 2 | 0% | 0% | 125 | | 10 | 1 | 0% | 0% | 118 |
| 3 | - | 10% | 455 | 6 | 2 | 7% | 0% | 165 | | 3 | 2 | 0% | 1% | 101 | ± 1.9 | 11 | 4 | 50% | 0% | 224 |
| 18 | + | 3% | 77 | 7 | 1 | 24% | 1% | 144 | ± 1.6 | 5 | 2 | 40% | 0% | 152 | | 14 | 1 | 0% | 0% | 122 |
| 21 | + | 12% | 112 | 9 | 5 | 66% | 1% | 140 | ± 1.6 | 6 | 2 | 14% | 0% | 116 | | 15 | 1 | 49% | 0% | 212 |
| 25 | + | 16% | 272 | 10 | 1 | 1% | 1% | 110 | ± 1.9 | 7 | 3 | 0% | 2% | 124 | ± 2.5 | 16 | 2 | 0% | 0% | 140 |
| | | | | 12 | 3 | 31% | 0% | 100 | | 8 | 1 | 38% | 0% | 240 | | | | | | |
| | | | | | | | | | | 9 | 1 | 37% | 0% | 189 | | | | | | |

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| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 2 | 0% | 73 | 2 | 2 | 0% | >100 | | | 2 | 2 | 0% | >100 | | | 4 | 3 | 0% | >100 |
| 14 | 5 | 0% | 46 | 3 | 2 | 0% | >100 | | | 3 | 2 | 0% | >100 | | | 7 | 2 | 0% | >100 |
| | | | | 4 | 2 | 0% | >100 | | | 11 | 2 | 0% | 94 | | | 14 | 2 | 0% | >100 |
| | | | | 14 | 3 | 0% | 112 | | | 13 | 4 | 0% | 85 | | | 15 | 2 | 0% | >100 |
| | | | | 15 | 3 | 0% | 143 | | | | | | | | | | | | |

C ♀

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 3 | 0% | 110 | | 2 | + | 1% | 117 | ± 1.8 | 1 | + | 2% | 49 | ± 3.9 |
| 8 | 12 | 0% | 61 | | 3 | + | 2% | 70 | ± 3.3 | 2 | + | 4% | 49 | ± 5.0 |
| 10 | 2 | 1% | 177 | ± 1.5 | 4 | + | 0% | 90 | | 3 | + | 2% | 43 | ± 4.2 |
| | | | | | 5 | + | 1% | 72 | ± 2.3 | 4 | + | 0% | 40 | |
| | | | | | 6 | + | 1% | 133 | ± 1.7 | 13 | + | 0% | 38 | |
| | | | | | | | | | | 14 | + | 0% | 40 | |

**Supplemental Table 2: XCI in BAC transgenic male and female ES cell lines**
**A)** Copy number determination by qPCR and quantification of the percentage of cells that show accumulated *XIST* and/or *Xist* at day 3 of differentiation in male ES cell lines transgenic for RP24-180B23, RP23-338B22 and CTD-2183M22. (+), Integration confirmed by DNA-FISH only. For BAC RP23-338B22 and CTD-2183M22 transgenic ES cell lines the 95% confidence interval of endogenous mouse *Xist* accumulation is shown. **B)** Copy number determination by qPCR and quantification of the percentage of cells that initiated XCI at day 3 of differentiation in male ES cell lines transgenic for RP23-447O10, RP23-100E1, CT7-474E4, and RP24-224E13 as determined by presence of an *Xist* cloud. **C)** Copy number determination by qPCR and quantification of the percentage of cells that initiated XCI on two X chromosomes at day 3 of differentiation in female ES cell lines transgenic for RP23-447O10, CT7-474E4, and RP24-224E13 as determined by presence of two *Xist* clouds. (+), Integration confirmed by DNA-FISH only. The 95% confidence interval of endogenous mouse *Xist* accumulation is shown in the right column.

♂

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 2 | 9%* | 142 ± 4.7 | 6 | 5 | 5%* | 377 ± 2.2 | 1 | 2 | 0% | >100 |
| 18 | 2 | 2% | 205 ± 1.9 | 9 | 2 | 5%* | 320 ± 2.4 | 2 | 2 | 0% | >100 |
| | | | | 13 | 4 | 6%* | 228 ± 3.1 | 3 | 2 | 0% | >100 |
| | | | | | | | | 4 | 2 | 0% | >100 |
| | | | | | | | | 10 | 2 | 0% | >100 |
| | | | | | | | | 13 | 8 | 0% | >100 |
| | | | | | | | | 16 | 12 | 0% | >100 |
| | | | | | | | | 23 | 5 | 0% | >100 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 3 | 0% | 140 | 2 | 2 | 0% | 140 | 1 | 6 | 0% | 133 |
| 3 | 3 | 0% | 106 | 3 | 3 | 0% | 106 | 2 | 3 | 0% | 111 |
| 5 | 3 | 0% | 102 | 5 | 2 | 0% | 102 | 3 | 3 | 0% | 92 |
| 13 | 15 | 0% | 122 | 13 | 15 | 0% | 122 | 15 | 3 | 0% | 75 |
| 16 | 1 | 0% | 109 | 16 | 2 | 0% | 109 | | | | |

**Supplemental Table 3: XCI in BAC transgenic male ES cell lines covering part of BAC RP24-240J16**

Copy number determination by qPCR and quantification of the percentage of cells that initiated XCI at day 3 of differentiation as determined by presence of an *Xist* cloud in J1 male transgenic RP23-7K14, RP23-138O4, RP23-25P18, RP23-288K24, RP23-463H23 and RP23-36C20 ES cell lines. For BAC RP23-7K14 and RP23-138O4 transgenic ES cell lines the 95% confidence interval of endogenous mouse *Xist* accumulation is shown (* $P<0.05$).

A

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-4 | 2 | 6%* | 68 | ± 5.6 | 1 | 2 | 0% | 101 | | 1 | 5 | 0% | 119 | |
| 2-5 | 2 | 11%* | 122 | ± 5.5 | 5 | 3 | 0% | 121 | | 3 | 2 | 0% | 130 | |
| 2-9 | 3 | 10%* | 144 | ± 4.9 | 6 | 4 | 0% | 114 | | 5 | 7 | 0% | 110 | |
| 2-11 | 2 | 11%* | 68 | ± 7.4 | 9 | 6 | 0% | 136 | | 6 | 2 | 0% | 102 | |
| 2-14 | 3 | 21%* | 105 | ± 7.8 | 11 | 3 | 0% | 133 | | 9 | 3 | 0% | 112 | |
| 2-15 | 2 | 7%* | 145 | ± 4.2 | 12 | 6 | 1% | 113 | ± 1.8 | 11 | 8 | 1% | 161 | ± 1.5 |
| 2-16 | 2 | 0% | 105 | | 13 | 2 | 0% | 114 | | 14 | 3 | 0% | 134 | |
| 2-21 | 2 | 9%* | 58 | ± 7.4 | 14 | 2 | 0% | 107 | | 15 | 2 | 0% | 113 | |
| 2-23 | 3 | 9%* | 58 | ± 7.4 | 15 | 2 | 0% | 108 | | 16 | 2 | 0% | 121 | |
| 2-24 | 2 | 13%* | 174 | ± 5.0 | 16 | 5 | 0% | 108 | | 19 | 2 | 0% | 116 | |
| | | | | | 17 | 2 | 0% | 147 | | 20 | 13 | 0% | 150 | |
| | | | | | 19 | 5 | 1% | 139 | ± 1.7 | 21 | 2 | 0% | 136 | |
| | | | | | 22 | 3 | 1% | 105 | ± 1.9 | 23 | 4 | 1% | 156 | ± 1.6 |
| | | | | | | | | | | 24 | 3 | 0% | 162 | |

B

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-2 | 3 | 2%* | 232 | ± 1.8 | 1 | 3 | 0% | 63 | | 1 | 5 | 1% | 134 | ± 1.6 |
| 2-3 | 3 | 8%* | 79 | ± 5.9 | 3 | 3 | 0% | 127 | | 3 | 3 | 0% | 114 | |
| 2-5 | 3 | 4%* | 117 | ± 3.6 | 6 | 3 | 0% | 134 | | 7 | 6 | 0% | 121 | |
| 2-6 | 5 | 7%* | 105 | ± 4.9 | | | | | | | | | | |

**Supplemental Table 4: XCI in BAC transgenic RP24-240J16 and RP24-240J16ΔRnf12 male ES cell lines**

**A)** Copy number determination and quantification by qPCR of the percentage of cells that initiated XCI at day 3 of differentiation in 1.3 male transgenic RP24-240J16, RP24-240J16ΔRnf12(sense), and RP24-240J16ΔRnf12(anti-sense) ES cell lines as determined by presence of an *Xist* cloud. The 95% confidence interval of endogenous mouse *Xist* accumulation is shown in the right column (* $p<0.05$).

**B)** Copy number determination by qPCR and quantification of the percentage of cells that initiated XCI on both X chromosomes at day 3 of differentiation in 30Δ1 female transgenic RP24-240J16, RP24-240J16ΔRnf12(sense), and RP24-240J16ΔRnf12(anti-sense) ES cell lines as determined by presence of two *Xist* clouds. The 95% confidence interval of endogenous mouse *Xist* accumulation is shown in the right column (* $P<0.05$).

# Supplemental Methods

## *Modification of BACs*

BACs were acquired from BACPAC (C57/B6 libraries), Resgen (129/Sv library) or Riken (*Mus musculus mollosinus*), and a kanamycin/neomycin resistance cassette was introduced by *in vitro* lox recombination. This cassette was generated by introduction of a lox sequence and SceI site BglII-NotI into pEGFP-N1 (Clontech). RP24-240J16ΔRnf12 was generated by homologous recombination in bacteria [900]. The targeting cassette was PCR amplified using primers (GCCTTCGAACATCTCTGAGC, GAGCCGGACTAATCCAAACA), cloned into pCR-BluntII-TOPO (Invitrogen), and linearized with NheI to introduce a kanamycin/neomycin cassette AflII-EcoO109I excised from EGFP-N1. Homologous recombination in bacteria was confirmed by PCR with primers 1, GGCAGAGAGCCACTTTCATC, 2, CTGGCACTCTGTCGATACCC, 3, TTCCACAGCTGGTTCTTTCC, and gel electrophoresis. BACs were SceI linearized and electroporated into female F1 2-1 ES cells. Homologous recombination in ES cells was confirmed with primers GCCTTCGAACATCTCTGAGC, GAGCCGGACTAATCCAAACA, amplifying a NheI polymorphism present in the wild type 129/Sv allele. The presence of two polymorphic X chromosomes was confirmed by amplification of a sequence located in *Xist*, with the primers CAGTGGTAGCTCGAGCCTTT and CCAGAAGAGGGAGTCAGACG, and subsequent digestion with BrgI which digests a RFLP present in the 129SV allele.

## *Expression analysis*

For allele specific *Rnf12* RT-PCR analysis, RNA was reverse transcribed (Invitrogen Superscript III) and amplified with primers TAAAGAGGGTCCACCACCAC and GGCAGAGAGCCACTTTCATC. PCR products were purified and digested with NheI, which digests the 129/Sv but not the Cast/Ei PCR product. For RT-qPCR expression studies, *Xist* was amplified with GCCTCAAGAAGAAGGATTGC and GGGATTGTTTGTCCCTTTGG; *Rnf12* with CCCCAGGTGAAAGTACTGAGG and CTCTCCAGCTCTATTTTCATCG; *RNF12* with TGAGAGATAACAATTTGCTAGGC and GTGGGCCTTCTTTAATTTGC; and *Actin* with ACTATTGGCAACGAGCGGTTC and AGAGGTCTTTACGGATGTCAACG. Co-amplifycation of *Rnf12/RNF12* was performed with AAGAAGAGTTCGTCCTGGAGAATA and CGAAGTTTGTTGCCTTCTGT.

RNF12 protein was detected with a mouse anti RNF12 antibody (Abnova), ACTIN was detected with a mouse anti ACTIN antibody (Sigma). For expression analysis of the region covered by RP24-240J16 total RNA was isolated from two day 3 differentiated wild type female ES lines, labeled and hybridized to Niblegene tiling arrays, covering the X chromosome with 30 bp intervals, excluding repetitive and non annotated sequences.

### BAC copy number determination

BAC copy number was determined with qPCR using primers;
GTTCTTACCACCAATTGAAAACG, CAAAACAGACTCCAAATTCATCC, for RP24-180B23 and RP23-338B22,
ACCATGACCAAAGCAACTCC, CTCCTCCAGTACCATGTCTGC, for RP23-447O10
CCGCTGAAGATAGCTCTTGG, GCCACAACCAAACAGAATCC for RP24-224E13 and CT7-474E4,
ATCTCACCGTACCCATGAGC, CCTCTGGTACGACCTCTTGC, for RP23-100E1,
AGCCCCGATGAAAATAGAGG, GGCATTTCTGGATAATCTTTGG for RP24-240J16, RP23-7K14, RP23-138O4, RP23-288K24, RP23-463H23 and RP23-36C20,
AGTCATTGGCTGGTCACTCC, ATCAACCCAGACACCAAACC, for RP23-25P18,
GATAGCAGGTCAGGCAGAGG, ACGCAAAGCTCCTAACAAGC, for CTD-2183M22 and CTD-2200N19,
CTCATTTTGAGCCCTTCTGC, ACCACATTTGCCTCAGATCC, for CTD-2530H13 and GCACCCATATCCGCATCCAC, GCATTTCTTCCCGGCCTTTG, for Zfp-42 as an autosomal normalization control.

### Statistics

The 95% confidence intervals for the proportion were calculated by:

$$p-\left[1.96\times\sqrt{\frac{p(1-p)}{n}}\right] \text{ to } p+\left[1.96\times\sqrt{\frac{p(1-p)}{n}}\right]$$, with n for the number of cells analyzed, and p representing the percentage of *Xist* clouds measured. Non overlapping intervals between transgenic and non transgenic control cells were scored as significant ($p<0.05$).

The Z-test for proportion was calculated by:

$$z=\frac{|p-\pi|-\frac{1}{2n}}{\sqrt{px\frac{(1-p)}{n}}}$$, with n for the number of cells analyzed, and p and $\pi$ representing the proportion and average proportion.

The correlation coefficient, Pearsons r was determined by:

$$r=\frac{\sum(xi-\bar{x})(yi-\bar{y})}{\sqrt{\sum(xi-\bar{x})^2\sum(yi-\bar{y})^2}}$$, where xi and yi are the values of X and Y for the $i^{th}$ measurement, followed by $r\sqrt{\frac{n-2}{1-r^2}}$, to determine the p value, with n representing the degrees of freedom.

# Chapter 3

## *Precise BAC targeting of genetically polymorphic mouse ES cells*

This chapter has been published in

Tahsin Stefan Barakat, Eveline Rentmeester, Frank Sleutels, J. Anton Grootegoed and Joost Gribnau (2011)
"Precise BAC targeting of genetically polymorphic mouse ES cells"
*Nucleic Acids Res* 39:e121
&
Tahsin Stefan Barakat and Joost Gribnau
"A Restriction Fragment Length Polymorphism based Bacterial Artificial Chromosome targeting strategy for efficient and fast generation of knockout alleles in polymorphic mouse Embryonic Stem cells"
(*Manuscript in preparation*)

# Precise BAC targeting of genetically polymorphic mouse ES cells

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## Abstract

The use of bacterial artificial chromosomes (BACs) provides a consistent and high targeting efficiency of homologous recombination in ES cells, facilitated by long stretches of sequence homology. Here, we introduce a BAC targeting method which employs restriction fragment length polymorphisms (RFLP) in targeted polymorphic C57Bl/6 / Cast/Ei F1 mouse ES cell lines to identify properly targeted ES cell clones. We demonstrate that knockout alleles can be generated either by targeting of an RFLP located in the open reading frame thereby disrupting the RFLP and ablating gene function, or by introduction of a transcription stop cassette that prematurely stops transcription of an RFLP located downstream of the stop cassette. With both methods we have generated *Rnf12* heterozygous knockout ES cells, which were identified by allele specific PCR using genomic DNA or cDNA as a template. Our results indicate that this novel strategy is efficient and precise, by combining a high targeting efficiency with a convenient PCR based readout and reliable detection of correct targeting events.

## Introduction

The discovery of mouse embryonic stem (ES) cells and the possibility to manipulate the ES cell genome through homologous recombination has provided a powerful methodology to study gene function *in vitro* and *in vivo* [55-56, 901]. Initial studies indicated that key factors important for efficient gene targeting include the length of the targeting arms, which positively correlates with the targeting efficiency [902-903], and the use of isogenic DNA for the generation of targeting constructs, as the presence of SNPs in a targeting vector would reduce the targeting efficiency [902, 904]. Increased targeting efficiency was obtained by targeting of mouse ES cells with a BAC (bacterial artificial chromosomes) strategy. Several annotated BAC libraries are now available for different mouse laboratory strains, to target a variety of different ES cell lines with isogenic targeting vectors (http://www.ncbi.nlm.nih.gov/clone/). Correct targeting with BAC targeting vectors is generally verified by quantitative real time PCR (qPCR) amplifying a fragment spanning the projected deletion/insertion, together with a qPCR amplifying a fragment located in one of the arms [905]. Also DNA-FISH has been applied to determine a correct genetic modification [906]. However, because conventional Southern blotting techniques cannot be applied, these techniques are prone to detect false positive and negative clones. To avoid this problem, BAC targeting vectors are used that have both short and long targeting arms, allowing detection and/or confirmation of positive clones by Southern blotting using an external probe [907]. This requires a BAC that is properly positioned around the insertion site, or is modified by trimming one of the arms through homologous recombination in bacteria. Together, the current strategy still is associated with several problems. In view of this, we have developed a new BAC targeting strategy which makes use of RFLPs present in genetically polymorphic ES hybrid cell lines, generated by crossing C57Bl/6 female mice with Cast/Ei male mice, providing a convenient readout for proper gene targeting. In the present study, the proof of principle target gene was *Rnf12*, which encodes a nuclear

factor involved in X chromosome inactivation (XCI) [174]. For this gene, our results indicate that the new strategy can be used to efficiently introduce genetic modifications in ES cells using BAC targeting cassettes combined with a reliable readout based on allele specific PCR.

## Methods

### ES cell derivation and cell culture

Female C57Bl/6 mice were crossed to male Cast/Ei mice, and blastocysts were seeded onto irradiated mouse embryonic fibroblasts (MEFs) in DMEM, 15% v/v knockout serum replacement (Invitrogen), 100 U $ml^{-1}$ penicillin, 100 mg $ml^{-1}$ streptomycin, non-essential amino acids, 0.1mM ß-mercaptoethanol, 5000 U $ml^{-1}$ leukaemia inhibitory factor (LIF) and 50 µM MEK1 inhibitor (New England Biolabs). The dissociated inner cell mass outgrowth was seeded on new feeders and after one passage grown in standard ES medium containing DMEM, 15% v/v foetal calf serum, 100 U $ml^{-1}$ penicillin, 100 mg $ml^{-1}$ streptomycin, non-essential amino acids, 0.1mM ß-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% v/v 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. To generate chimaeras, C57Bl/6 mice where superovulated and mated, and day 3,5 blastocysts were isolated. ES cells were injected, and the embryos were transfered to pseudopregnant foster mothers. Chimeras were crossed to C57Bl/6 mice, and germ line transmission was judged by coat color. All animal experiments were in accordance with the regulations of the Erasmus MC Animal Experimental commission.

### RFLP analysis and genotyping

To confirm the parental origin of the derived C57Bl/6 / Cast/Ei hybrid mouse ES cells, RFLP analysis on genomic DNA was performed by PCR followed by restriction digestion using the following primers and enzymes: *Xist* CAGTGGTAGCTCGAGCCTTT and CCAGAAGAGGGAGTCAGACG, BsrGI; *Cdyl*: ACAGGCAGAAGGAGCTGTGT, and CCCAGCTGTAAAGGCTTCAG, ZraI. *Sry* was amplified using ATTTATGGTGTGGTCCCGTGGT and TATGTGATGGCATGTGGGTTCC.

### Karyotyping, RNA-FISH and DNA-FISH

For karyotyping, cells were blocked in metaphase using colcemid, and metaphase spreads were prepared by hypotonic treatment, followed by fixation in methanol acetic acid (3:1 v/v), according to standard procedures. *Xist* RNA-FISH and DNA-FISH were performed as described [174]. For DNA-FISH, a mouse BAC probe (RP24-240J16) containing the *Rnf12* gene was digoxigenin-labelled and used to determine the number of integration sites of the *Rnf12* targeting constructs. A cocktail containing biotin-labelled BAC sequences containing *Xist* (CT7-474E4, CT7-45N16, CT7-155J2 and CT7-211B4) was used as a probe to determine the number of X chromosomes.

### RT-PCR

RNA was isolated from undifferentiated ES cells using Trizol reagent (Invitrogen), according to manufacturer's instructions, and cDNA was prepared using the SuperScript TM III First-Strand Synthesis System (Invitrogen). RT-PCR for pluripotency markers was performed using the following gene specific primers: *Oct4*: CCCCAATGCCGTGAAGTTG, TCAGCAGCTTGGCAAACTGTT; *Nanog*: AGGATGAAGTGCAAGCGGTG, TGCTGAGCCCTTCTGAATCAG; *Sox2*: CACAGATGCAACCGATGCA, GGTGCCCTGCTGCGAGTA; *β-Actin*: CAACGAGCGGTTCCGATG, GCCACAGGATTCCATACCCA.

### Construction of targeting constructs

The *Rnf12* targeting construct has been described [174]. To generate the SA-tpA stop constructs, a cassette containing a floxed splice acceptor and polyadenylation sequence and a Frt site flanked neomycin/kanamycin fusion gene was generated, starting with a pEGFP-NI vector (Clontech). A linker containing a Lox66 and EcoRV, BglII and BamHI sites together with a linker containing a Lox71 and Frt sites flanking a ScaI site were cloned BglII-NotI into pEGFP-N1, releasing EGFP (complete sequence: GATCTAATATAACTTCGTATAGCATACATTATACGAACGGTAGATATC AGATCTGGATCCTATTGAAGCATATTACATACGATATGCTTGCCATTTAATTCC GGAGAATCCGGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGTACTGA AGTTCCTATTCTCTAGAAAGTATAGGAACTTCTAGG). The ScaI site was used to insert a DraIII-AseI kanamycin/neomycin fragment. The SA-tpA sequence was a BamHI fragment from pSStpA [123], inserted in the BamHI site of the linker. Three unique restriction sites in introns 2, 3 and 4 of *Rnf12* were PCR amplified, with 500 bp of flanking region, and cloned into pPCR-Topo-bluntII, using the following primers and unique restriction sites: intron 2 BglII GGGCTACACAGAGAAAGAAACC, AGCCATGCATGCTTGTGTTA; intron 3 NheI GAAACAGCTTGTTTTATAATGTTTCTT, TTGAACATGTGTTGCAAAATTAC; intron 4 AvrII ACATTTTGTTTGGGGAGGTG, GAATTGTGCAACTCGGAACA. The SA-tpA kanamycin/neomycin cassette was NheI-AflII released and inserted into the unique restriction sites of the intronic targeting constructs. The final constructs were used for homologous recombination in bacteria of a C57Bl/6 BAC RP24-240J16 [900]. Positive clones were screened by PCR for the correct recombination event using primers spanning the homologous recombination arms of the targeting constructs and insert specific primers: *Rnf12* intron 2 AAAGGTTTTGGCTGGATGGT, rev TGTGCCATAATGCTTGGCTA, *Rnf12* intron 3 CCCAGGTAAGCTGCATGTAA, rev TGTAGTCTTCTGAGCAACTCTTCC, *Rnf12* intron 4 ACAGAGCCCCGATGAAAAT, rev ACACGATTAGGACACTCATGG.

### Targeting of ES cells

Targeting constructs were linearized by PI-SceI digestion. For each electroporation, approximately 40 µg of DNA and 1.0 x $10^6$ ES cells were used. Cells were seeded on drug resistant male feeder cells (MEFs), and selection with neomycin (270 µg $ml^{-1}$, active) was started 24 hours post-transfection and continued for 7-12 days. Drug resistant clones were picked and expanded.

### RFLP analysis of targeted ES clones

Genomic DNA (gDNA) of ES clones was isolated, and RFLP analysis was performed using the following primers and enzymes: *Rnf12* GCCTTCGAACATCTCTGAGC, GAGCCGGACTAATCCAAACA, NheI; *Xist* CAGTGGTAGCTCGAGCCTTT and CCAGAAGAGGGAGTCAGACG, BsrGI. For analysis of cell lines targeted with the stop constructs, RNA was isolated, cDNA prepared and expression of *Rnf12* was analyzed by RFLP analysis using primers TAAAGAGGGTCCACCACCAC and GGCAGAGAGCCACTTTCATC followed by NheI restriction digestion.

### qPCR copy number analysis

Copy number of genomic sequences was determined by real time qPCR with genomic DNA using the following primers: *Rnf12* (NheI site): GTTCGTCCTGGAGAATACCG, GGAAAAGGTACGCCTAAAACC; *Rnf12*: AGCCCCGATGAAAATAGAGG, GGCATTTCTGGATAATCTTTGG ; *Zfp42*: GCACCCATATCCGCATCCAC, GCATTTCTTCCCGGCCTTTG.

### Southern blotting

Five to 10 µg of genomic DNA was digested overnight with NheI, and separated on a 0,7% agarose gel. DNA was blotted to Hybond membranes using standard procedures, and the blot was hybridized with a PCR amplified probe (primers: GGCAGAGAGCCACTTTCATC, GCCAAAGACCTCCAACCATA)

## Results

Existing methodology for screening positive clones after homologous recombination with BAC targeting cassettes involves qPCR or DNA-FISH. These methods are prone to detect false positive and negative clones, and we therefore set out to develop a method to screen targeting events by using RFLPs in F1 hybrid cell lines. In the first approach the BAC targeting vector destroys the RFLP and inserts a kanamycin/neomycin resistance cassette in the open reading frame of the gene of interest, thereby ablating gene function (**Figure 1A-I**). Removal of the RFLP can be screened by PCR using genomic DNA as a template and subsequent digestion of the PCR product with the restriction enzyme recognizing the targeted RFLP. In another approach, we introduce a splice acceptor poly-adenylation transcription stop cassette (SA-tpA) in an intron of the gene of interest, prematurely abrogating gene transcription resulting in a non-functional protein. For this second approach, positive clones are identified by RT-PCR amplification of a cDNA sequence, which contains an RFLP that is located downstream of the transcription stop cassette (**Figure 1A-II**).

### Generation and targeting of F1 ES hybrid cell lines

To obtain genetically polymorphic ES cell lines with a high number of RFLPs that could be used for gene targeting, we generated F1 hybrid ES cell lines by crossing C57Bl/6 female mice with Cast/Ei male mice. The C57Bl/6 classical inbred *Mus musculus* mouse strain is among the most widely used and best characterized mouse strains. The C57Bl/6 mouse genome has been sequenced, and several well-annotated BAC libraries have been generated [908]. The Cast/Ei inbred strain has been derived from a wild population of the subspecies *Mus musculus castaneus*, is more difficult to breed, but offers advantages related to its variant genetic background [909]. Intercrosses between Cast/Ei and other strains have been extensively used for SNP based distinction of the maternal and paternal genome, for instance to study genomic imprinting and XCI. The C57Bl/6 and Cast/Ei mouse strains are highly polymorphic, with an estimate of one SNP per 311 base pairs, providing a sufficient number of RFLPs to allow targeting of almost every gene [909]. The Cast/Ei *Mus m.* subspecies is currently being sequenced, a BAC library is available, and a SNP database is publically accessible (www.perlegen.com).

We generated five different F1 ES cell lines, with the proper karyotype and ES cell morphology, which were successfully differentiated into embryoid bodies (EB) (**Figure 1B**). RT-PCR expression analysis confirmed expression of the pluripotency markers *Oct4*, *Sox2* and *Nanog* (**Figure 1C**). Karyotyping and PCR analysis of genomic DNA with a primer set specific for *Sry* showed that three ES cell lines were male and two female (**Figure 1D**). We confirmed the C57Bl/6 / Cast/Ei F1 genotype by PCR amplification of the autosomal gene *Cdyl* and the X-linked gene *Xist*, and digestion with restriction enzymes specific for RFLPs that discriminate between a C57Bl/6 or Cast/Ei PCR product (ZraI for *Cdyl*, and BsrGI for *Xist*). Digestion of the *Xist* PCR products from genomic DNA (gDNA) of the male ES cell lines revealed only a C57Bl/6 product, as expected, because the single X chromosome in male cells is inherited from the C57Bl/6 mother. Two male ES cell lines, E3 and E14, were injected in C57Bl/6 blastocysts and several founders (5 for E3, 2 for E14) were retrieved, all showing high coat color contribution (representative animals are shown in **Supplementary Figure 1**). Different founders were crossed with C57Bl/6 females and all tested animals showed germline transmission (**Figure 1E**). Taken together, we generated three male and two female C57Bl/6 / Cast/Ei F1 ES cell lines. Because of our interest in the female specific X chromosome inactivation process female ES line E15 was used for further targeting studies.

### Targeted disruption of the Rnf12 open reading frame

For BAC mediated targeting of an RFLP we selected the X-chromosomal *Rnf12* gene as a target (**Figure 2A**). We have recently shown that the encoded RNF12 acts as a dose-dependent activator of X chromosome inactivation (XCI) in female ES cells [174, 910-911]. RNF12 is an E3 ubiquitin ligase that regulates XCI through activation of the X-linked gene *Xist* [911]. The transcribed *Xist* RNA coats the inactive X chromosome in *cis* (**Figure 1B**), thereby attracting chromatin modifying enzymes involved in establishing inactive chromatin [892].

*Rnf12* consists of five exons, spanning 24 kb. In the SNP database we identified NheI as an RFLP located in exon 5 of the C57Bl/6 *Rnf12* allele, and confirmed the RFLP

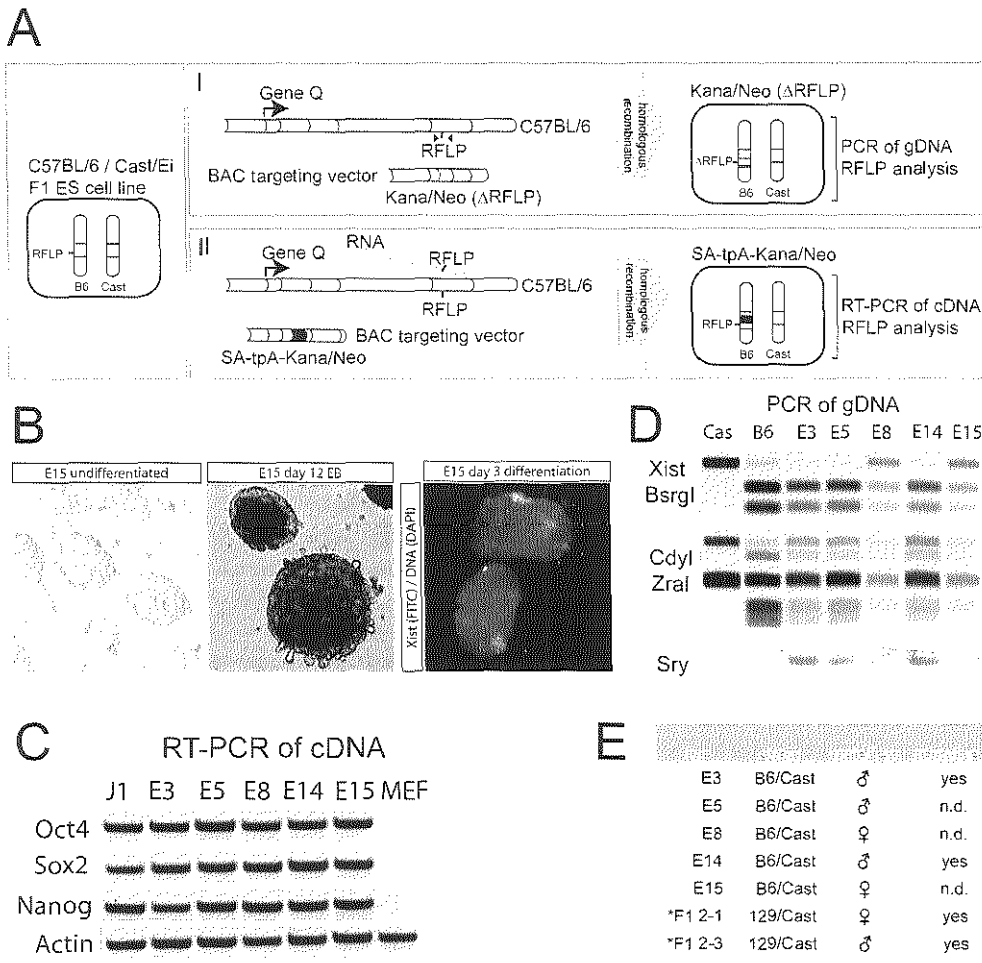



**Figure 1: Two approaches of a new strategy for manipulation of hybrid mouse ES cell lines**
**A)** Schematic overview of RFLP mediated BAC targeting of hybrid C57Bl/6 / Cast/Ei ES cells. Chromosomes from C57Bl/6 are indicated with B6 and from Cast/Ei with Cast. Exons of the gene of interest are indicated in green, the kanamycin/neomycin resistance cassette in white, and the transcription stop cassette in black. **B)** C57Bl/6 / Cast/Ei hybrid ES cells show proper ES cell morphology (left panel), are able to differentiate *in vitro* in embryoid bodies (middle panel), and female lines can initiate X chromosome inactivation upon differentiation (right panel, showing *Xist* RNA labelled in FITC, and DNA stained with DAPI). **C)** RT-PCR of pluripotency genes. The generated C57Bl/6 / Cast/Ei ES cells (E3-E15) express the pluripotency factors *Oct4*, *Sox2* and *Nanog*. J1 is a male control ES cell line, MEFs were used as negative control, and *Actin* is a control mRNA. **D)** PCR amplification of gDNA of different C57Bl/6 / Cast/Ei ES cell lines (E3-E15) and control gDNA (Cas and B6), and digestion with restriction enzymes identifying allele specific products for *Xist* (X-encoded, BsrGI, top panel), *Cdyl* (autosomal, ZraI, middle panel) and PCR amplification of *Sry* (Y-encoded, bottom panel).
**E)** Table summarizing the characteristics of the C57Bl/6 / Cast/Ei ES cell lines analyzed in this study, and two other polymorphic 129/Sv / Cast/Ei cell lines (*) that have been used in other gene targeting studies [174].

by sequencing analysis of gDNA isolated from C57Bl/6 and Cast/Ei mice. Disruption of RNF12 activity by insertion of a neomycin/kanamycin resistance cassette in this NheI RFLP would lead to a premature translation stop of RNF12, resulting in a 331 aa protein lacking the RING finger that is crucial for RNF12 activity. Based on this, we generated a targeting construct to disrupt *Rnf12*, by PCR amplification of the NheI RFLP and 500 bp of flanking sequence, and subsequent insertion of a kanamycin/neomycin resistance cassette in the NheI site. The C57Bl/6 BAC RP24-240J16, covering the *Rnf12* gene, was targeted through homologous recombination in bacteria [900], and correct targeting was confirmed by PCR amplification using primers inside the resistance cassette and outside the targeting arms (data not shown). The targeting efficiency in bacteria was >80%. The modified BAC sequence was linearized with SceI and targeted to female ES cell line E15, and subsequent to neomycin selection clones were picked and expanded for further analysis. Genomic DNA of these clones was subjected to PCR using primers spanning the targeted NheI site, and the PCR product was digested with NheI. Correctly targeted clones are expected to have an undigested Cast/Ei product only, although contamination by feeder cells (C57Bl/6) might result in the presence of some digested material (**Figure 2B**). We therefore also grew the targeted clones without feeders, which indicated that 12% of the picked clones showed a loss of the C57Bl/6 specific PCR product (**Figure 2C**, and **Figure 3C**).

Our results were confirmed by qPCR analysis with primers spanning the NheI site, which indicated a reduction in copy number from two to one, and primers amplifying a region proximal to the NheI site, indicating no change in the copy number (**Figure 2E**). The loss of one NheI site could be attributed to a correct targeting event, or loss of an X chromosome. Although the qPCR results indicated that both X chromosomes were present, we also performed a PCR amplification of a BsrGI polymorphism in the *Xist* gene. Digestion of the PCR products with BsrGI, which only digests the C57Bl/6 PCR product, indicated that both X chromosomes were present in all clones that showed a loss of the C57Bl/6 *Rnf12* PCR product (**Figure 2D**). We confirmed this finding by DNA-FISH with two different probes, one covering the BAC used for targeting the ES cells, and the other covering *Xist* and flanking sequences. We found that clones that lost the RFLP had retained the expected 40,XX karyotype, providing evidence for a correct targeting event, and precluding the presence of randomly integrated BACs (**Figure 2F**). Finally, genomic DNA of targeted clones was subjected to Southern blotting analysis, which indicated the correct targeting event; loss of the C57Bl/6 specific 5.4 kb band in knockout clones that were selected based on the RFLP PCR assay and no loss in control clones (**Figure 2G**).

Analysis of all our targeted clones indicated that targeting with a C57Bl/6 construct, was specific for the C57Bl/6 allele. However, our previous studies also indicated that the same construct can be used to target the 129/Sv allele in F1 2-1 129/Sv / Cast/Ei female ES cells [174]. The targeting efficiency for this experiment was lower, probably due to the presence of SNPs in the targeting construct (**Figure 3C**). Using a Cast/Ei targeting construct in which kanamycin/neomycin was replaced by a ampicillin/puromycin resistance cassette, we were also able to target the Cast/Ei allele, resulting in a homozygous *Rnf12*-/- ES cell line [911]. In this experiment the efficiency was lower (3%) than found for the C57Bl/6 construct targeting the C57Bl/6 allele, possibly due to selection against cells deficient for *Rnf12* (**Figure 3C**).

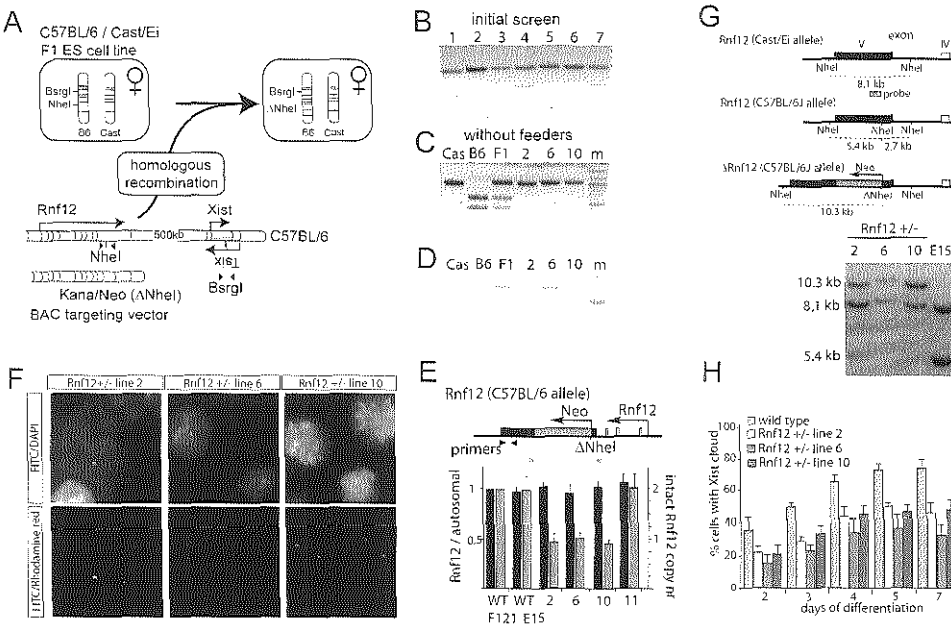
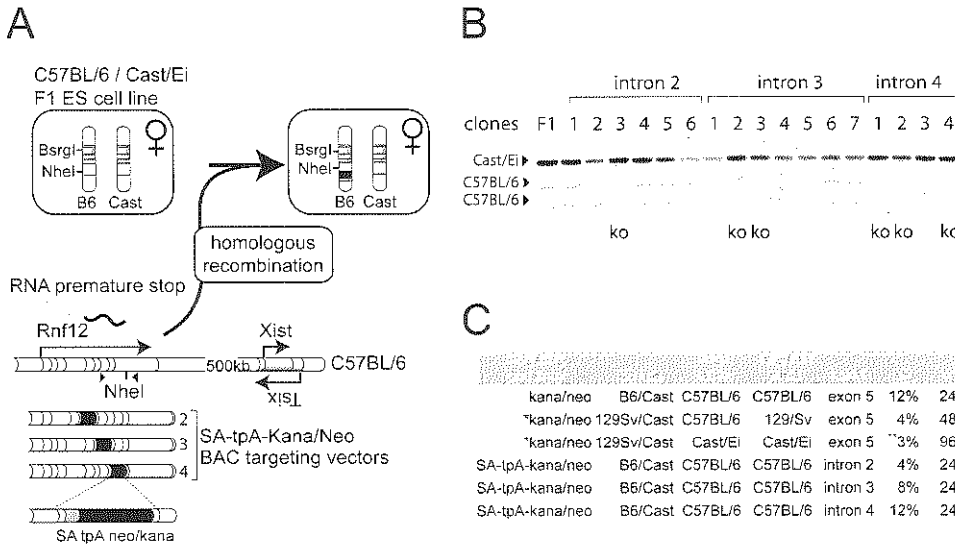



**Figure 2: Generation of *Rnf12* knockout ES cell lines by disruption of the *Rnf12* open reading frame**

**A)** Targeting of *Rnf12* by insertion of a kanamycin/neomycin resistance cassette into the exon containing a NheI RFLP destroys the RFLP (present in the C57Bl/6 allele). **B)** PCR analysis with primers indicated in **Figure 2A** amplifying the NheI RFLP located in *Rnf12* using genomic DNA of targeted clones 1-7. PCR fragments were digested with NheI. **C)** Same as in **Figure 2B**, but with gDNA isolated from clones 2, 6 and 10, grown without feeder cells. Cast/Ei (Cas), C57Bl/6 (B6) and Cast/Ei / C57Bl/6 (F1) DNA was used as a control (m=marker). **D)** Same as in Figure 2C, but PCR analysis amplifying the BsrGI RFLP located in *Xist* using gDNA of the targeted clones. PCR fragments were digested with BsrGI. **E)** qPCR analysis with primers indicated using gDNA of different targeted clones. Values were normalized to values obtained with a primer set amplifying the autosomal *Zfp42* gene. **F)** DNA-FISH analysis with a BAC probe covering the targeting cassette (FITC), and a mix of BAC probes detecting *Xist* and flanking regions (Rhodamine red) (DNA stained with DAPI). **G)** Southern blotting analysis, using a probe that distinguishes between the Cast/Ei (8.1 kb) and C57Bl/6 (5.4 kb) alleles. A neo insertion event destroying the NheI RFLP present on the C57Bl/6 allele results in a 10.3 kb fragment (clones 2, 6 and 10). **H)** Three different *Rnf12*+/- ES cell lines and a wild type female ES cell line were differentiated, and subjected to *Xist* RNA-FISH. The relative number of cells showing an *Xist* cloud, indicative for initiation of XCI, is shown at different time points of differentiation (N>100 per time point, error bars represent 95% confidence intervals).

To demonstrate the value of the strategy described herein for studying a specific cellular process, we tested the effect of the heterozygous *Rnf12* deletion on XCI. We analyzed the percentage of cells that initiated XCI, by *Xist* RNA-FISH at different time points of EB differentiation, for three *Rnf12*+/- clones. Previously, we found that a heterozygous deletion of *Rnf12* in female cells results in a significant reduction of XCI, as part of the evidence that RNF12 is an important activator of XCI [174]. In agreement with this, analysis of the present C57Bl/6 / Cast/Ei *Rnf12* +/- female ES lines also showed a reduction in the number of cells that initiated XCI (**Figure 2H**). These results indicate that




| | | | | | | |
|---|---|---|---|---|---|---|
| kana/neo | B6/Cast | C57BL/6 | C57BL/6 | exon 5 | 12% | 24 |
| *kana/neo | 129Sv/Cast | C57BL/6 | 129/Sv | exon 5 | 4% | 48 |
| *kana/neo | 129Sv/Cast | Cast/Ei | Cast/Ei | exon 5 | **3% | 96 |
| SA-tpA-kana/neo | B6/Cast | C57BL/6 | C57BL/6 | intron 2 | 4% | 24 |
| SA-tpA-kana/neo | B6/Cast | C57BL/6 | C57BL/6 | intron 3 | 8% | 24 |
| SA-tpA-kana/neo | B6/Cast | C57BL/6 | C57BL/6 | intron 4 | 12% | 24 |

**Figure 3: Generation of *Rnf12* knockout ES cell lines by insertion of a transcription stop cassette**
**A)** Generation of *Rnf12* knockout ES cell lines by integration of a splice acceptor triple poly-adenylation sequence (SA-tpA) together with a kanamycin/neomycin resistance cassette. Different targeting constructs were generated to target the stop cassette to one of the introns 2, 3 or 4. **B)** RT-PCR analysis with cDNA form clones targeted with cassettes targeting different introns, using primers amplifying the NheI RFLP located in exon 5. RT-PCR products were digested with NheI to reveal allele specific PCR products. Correctly targeted clones are indicated (ko). **C)** Table summarizing the targeting efficiency of the constructs described for targeting the C57Bl/6 allele (n=number of clones picked). (*) Targeting efficiencies of previously described experiments involving C57Bl/6, and Cast/Ei exon 5 targeting constructs used to target the 129/Sv and Cast/Ei alleles in F1 2-1 129/Sv / Cast/Ei female ES cells [174, 911]. (**) The Cast/Ei allele was targeted in *Rnf12*+/- (129/Sv / Cast/Ei) ES cells creating a *Rnf12* null ES cell, which may affect the targeting efficiency.

homologous recombination of RFLPs with BAC targeting constructs provides an efficient and precise method to generate knockout ES cell lines.

### *Premature abrogation of Rnf12 transcription*

Even in the highly polymorphic ES cells used for the present study, a relatively small percentage of all genes will not contain suitable RFLPs to allow targeting of a resistance cassette abolishing expression of a functional transcript. For such genes, a splice acceptor triple poly-adenylation (SA-tpA) cassette could be used to insert into an intronic RFLP, thereby leading to a premature stop of transcription. Alternatively, a SA-tpA cassette could be inserted into an intron of the gene of interest, using a transcribed RFLP located downstream of the insertion site to screen for proper integration. Both modifications of the present method provide many more possible targeting sequences. As proof of principle, we generated targeting vectors aimed to prematurely stop transcription in one of the introns 2, 3 or 4 of *Rnf12* (**Figure 3A**). BAC targeting cassettes were generated through homologous recombination of a SA-tpA cassette flanked by a kanamycin/neomycin resistance cassette

in different introns of *Rnf12* in C57Bl/6 BAC RP24-240J16. Proper integration of the SA-tpA cassette was verified by PCR (not shown), and the targeting vectors were electroporated into female E15 C57Bl/6 / Cast/Ei ES cells. Neomycin resistant clones were expanded without feeder cells for RNA isolation. To determine the clones with a proper integration of the SA-tpA cassette abolishing *Rnf12* expression from the targeted C57Bl/6 allele, we generated cDNA and performed RT-PCR amplifying the exon 4-5 junction of *Rnf12* including the previously described NheI RFLP. Allele specific expression was determined by digestion of the RT-PCR product with NheI, which only digests the C57Bl/6 PCR product. For each targeting vector we obtained positive clones which showed expression of the Cast/Ei allele only, indicating proper integration of the transcription stop cassette (**Figure 3B**). Loss of an X chromosome was ruled out by PCR amplification of a BsrGI RFLP located in the *Xist* gene, using gDNA and subsequent digestion with BsrGI (data not shown). The targeting efficiencies were 4%, 8%, and 12%, for targeting of introns 2, 3, and 4, respectively, which is in the range of previously reported targeting efficiencies of BAC targeting constructs [906]. These results indicate that different targeting methods, either removing an RFLP or ablating expression of an RFLP combined with an allele specific PCR to detect a correct targeting event, can be applied to introduce genetic modifications in ES cells.

## Discussion

Previous attempts to generate genetically modified ES cell lines by homologous recombination of BAC targeting vectors have been challenging, mainly because a clear readout for proper integration of the targeting cassette was missing. Here, we have generated and targeted F1 C57Bl/6 / Cast/Ei hybrid ES cell lines, which are genetically polymorphic, carrying a high density of RFLPs.

We have opted for two different approaches for BAC mediated gene targeting, both using RFLPs as a readout for properly targeted clones. In one strategy a resistance cassette is integrated into the open reading frame of a gene, ablating gene function and disrupting the RFLP. In another approach, a splice acceptor transcription stop cassette is inserted in a transcribed RFLP, or in a genomic location upstream of an RFLP, and subsequently the RFLP is used to detect loss of expression of the targeted allele. The last strategy can even be used to generate conditional knockout or rescue alleles, by using inverted asymmetric lox sites. The combination of these approaches provides an opportunity to disrupt a wide selection of candidate target genes. Nonetheless, even in the F1 C57Bl/6 / Cast/Ei ES cell lines for some genes no RFLPs will be available. Especially, single exon genes or genes covering a relative small genomic region may not entail enough SNPs for the design of a proper targeting approach. Also, the targeting strategy based on screening of expressed RLFPs requires the targeted gene to be expressed in ES cells. Fortunately, this is true for many genes, and even genes which only play a role in embryonic development and differentiation processes appear to be expressed at a base level in ES cells, sufficient to allow application of the second approach.

The targeted F1 C57Bl/6 / Cast/Ei ES cell lines can directly be used to study the effect of the mutation introduced *in vitro*, or can be used for the generation of mice, either

through the generation of chimaeric mice by injection of ES cells in a diploid blastocyst or by the tetraploid complementation technology [912]. For several studies, the analysis of mutant mice generated with these hybrid ES cell lines requires extensive back crossing. However, mice generated by tetraploid complementation could avoid this problem. Also, the effect of many mutations can be studied in hybrid mice, and for studies involving genomic imprinting and X inactivation a hybrid back ground is even the preferred back ground for studying the consequence of an introduced mutation.

Regarding efficiency of the new strategy, for targeting of the *Rnf12* locus, we find that analysis of less than a hundred clones results in a sufficient number of properly targeted cell lines (**Figure 3C**), in agreement with previous findings using homologous recombination with BAC targeting vectors [906]. Although there is neither a gain or loss in efficiency, the use of RFLPs to perform an allele specific PCR profoundly facilitates the detection of positive clones, and omits less reliable and more laborious techniques such as DNA-FISH and qPCR. Therefore, our new strategy for gene targeting combines the high efficiency of BAC targeting technology with a convenient readout to screen for positive targeting events. The highly polymorphic C57Bl/6 / Cast/Ei ES cell lines we generated, in combination with the different approaches described here, for targeting a gene of interest, provides many options for efficient and precise BAC targeting of almost every gene in the mouse genome.

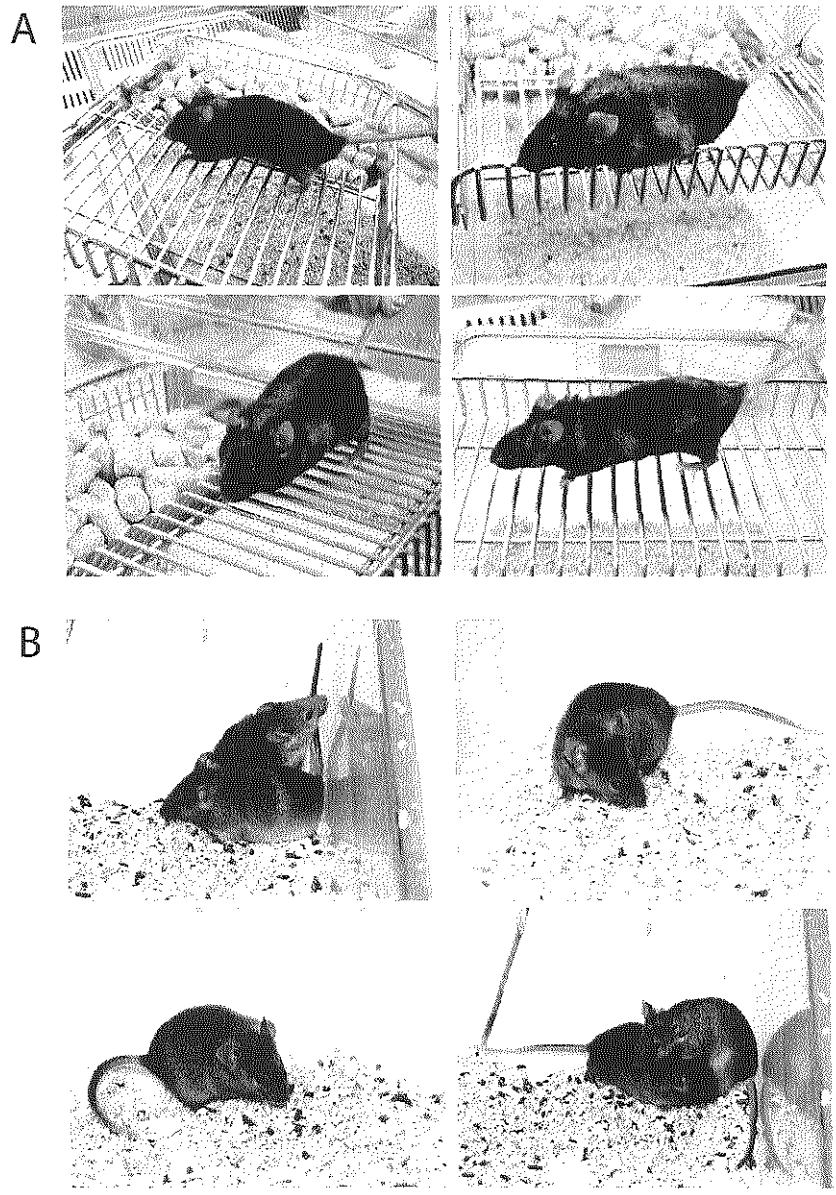
## Acknowledgements

We would like to thank all laboratory members for helpful discussions. This work was supported by grants from the Dutch Research Council (NWO-TOP, and -VICI) to J.G.

## Supplemental data

Supplemental data contains one supplementary figure.

## Supplementary Figure




**Supplementary Figure 1: Germ line transmission of C57Bl/6 / Cast/Ei ES cell lines**
**A)** Founders generated by injection of E3 (top two panels), and E14 (bottom two panels), show high coat color contribution. **B)** Offspring of founders shown in **A**, indicating germ line transmission.