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ORIGINAL INVESTIGATION

The X-inactivation trans-activator *Rnf12* is negatively regulated by pluripotency factors in embryonic stem cells

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Abstract X-inactivation, the molecular mechanism enabling dosage compensation in mammals, is tightly controlled during mouse early embryogenesis. In the morula, X-inactivation is imprinted with exclusive silencing of the paternally inherited X-chromosome. In contrast, in the post-implantation epiblast, X-inactivation affects randomly either the paternal or the maternal X-chromosome. The transition from imprinted to random X-inactivation takes place in the inner cell mass (ICM) of the blastocyst from which embryonic stem (ES) cells are derived. The trigger of X-inactivation, *Xist*, is specifically downregulated in the pluripotent cells of the ICM, thereby ensuring the reactivation of the inactive paternal X-chromosome and the transient presence of two active X-chromosomes. Moreover, *Tsix*, a critical *cis*-repressor of *Xist*, is upregulated in the ICM and in ES cells where it imposes a particular chromatin state at the *Xist* promoter that ensures the establishment of random X-inactivation upon differentiation. Recently, we have shown that key transcription factors supporting pluripotency directly repress *Xist* and activate *Tsix* and thus couple *Xist*/*Tsix* control to pluripotency. In this manuscript, we report that *Rnf12*, a third X-linked gene critical for the regulation of X-inactivation, is under the control of *Nanog*, *Oct4* and *Sox2*, the three factors lying at the heart of the pluripotency network. We conclude that in mouse ES cells the pluripotency-associated machinery exerts an exhaustive control of X-inactivation by taking over the regulation of all three major regulators of X-inactivation: *Xist*, *Tsix*, and *Rnf12*.

Introduction

In her seminal manuscripts proposing random X-inactivation as the mechanism underlying dosage compensation in mammals (Lyon 1961; Lyon 1962), Mary Lyon suggested this process must occur early during female embryonic development, around the formation of the late blastocyst. This first approximation was confirmed by means of single cell blastocyst injections (Gardner and Lyon 1971), cytogenetical observation of the heterochromatic X-chromosome (Plotnick et al. 1971; Takagi and Sasaki 1975), and biochemical analysis of X-linked genes encoding metabolic enzymes at different developmental stages (Monk and Kathuria 1977): these studies established that cells of the inner cell mass (ICM) of the blastocyst carry two active X-chromosomes, whilst in the trophectoderm the paternally inherited X-chromosome is inactivated. The idea that emerged from this was that X-inactivation is initially established upon differentiation, either imprinted in extra-embryonic tissues or randomly in the embryo proper. This enduring concept was well supported by analysis of embryonic stem (ES) cells: random X-inactivation is only established upon differentiation (Rastan and Robertson

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1985). The delivery of important molecular insights of the X-inactivation process, and their study in the pre-implantation embryo challenged this initial dogma (reviewed in Navarro and Avner 2009). In particular, the discovery of the X-linked non-coding *Xist* gene (Brown et al. 1991; Borsani et al. 1991; Brockdorff et al. 1991) and of the mechanisms by which it triggers X-inactivation in *cis*, were critical milestones.

Xist produces a 17 kb-long non-coding RNA exclusively expressed from the inactive X-chromosome (Xi) of female cells that mediates X-wide silencing. *Xist* RNA structures a nuclear compartment from which the transcriptional machinery is excluded and members of the Polycomb group recruited, leading to the silencing of X-linked genes and the initiation of a cascade of chromatin events that end up with the heterochromatinisation of the X-chromosome (reviewed in Ng et al. 2007). In both male and female ES cells, *Xist* is consistently repressed and this might be sufficient to keep all X-chromosomes active (Xa) (reviewed in Navarro and Avner 2009). Indeed, forced expression of *Xist* in ES cells leads to X-inactivation even before differentiation (Wutz and Jaenisch 2000), indicating that *Xist* repression is the most critical event required to abolish X-inactivation in undifferentiated cells. Notably, upon differentiation, *Xist* is monoallelically upregulated exclusively in female cells, thereby triggering random X-inactivation (reviewed in Navarro and Avner 2009). The analysis of *Xist* RNA and associated heterochromatin marks in early mouse embryos radically changed our conception of the developmental dynamics of X-inactivation (Mak et al. 2004; Okamoto et al. 2004). Although all cells of the ICM appeared to lack an Xi, the cells of the cleavage-stage morula were found to carry an Xi. Further, it was shown that in the morula, the Xi is always the paternal X-chromosome. Therefore, X-inactivation is established much earlier than previously thought, at the two to four cells transition, under the form of imprinted X-inactivation. The acquisition of pluripotency, a characteristic restricted to the cells of the ICM, and more particularly the expression of *Nanog* (Chambers et al. 2003), a critical transcription factor specifying pluripotency, leads to the reactivation of the paternal Xi exclusively in the ICM (Silva et al. 2009). Strikingly, *Xist* silencing and Xi reactivation also accompany the acquisition of pluripotency in vitro, either after somatic-ES cells fusions (Tada et al. 2001), nuclear cloning (Eggan et al. 2000), or the generation of induced pluripotent stem cells (Stadtfeld et al. 2008). Understanding how X-inactivation is abolished in pluripotent cells is, therefore, important not only for X-inactivation, but also to the wider field of epigenetics and cellular reprogramming.

Previously, we have shown that Nanog, Oct4 and Sox2, the three main transcription factors supporting pluripotency (reviewed in Chambers and Tomlinson 2009), bind within *Xist* intron 1 in ES cells and maintain *Xist* repression until differentiation (Navarro et al. 2008). This provided a simple scenario accounting for the systematic coupling of Xi reactivation with the acquisition of pluripotency. In contrast to our expectations, however, the deletion of *Xist* intron 1 is not accompanied by increased levels of *Xist* expression in undifferentiated female ES cells (Barakat et al. 2011). This indicates that Nanog, Oct4 and Sox2 action at *Xist* is not sufficient by itself to keep *Xist* in check in undifferentiated ES cells. Hence, additional ES-specific repressive mechanisms of *Xist* must exist. In this scenario, the strong ES-specific activity of *Tsix*, a non-coding antisense chromatin repressor of *Xist* (Navarro et al. 2005, 2006; Sado et al. 2005; Sun et al. 2006, Ohhata et al. 2008, Navarro et al. 2009), and the fact that other pluripotency-associated factors, namely Klf4, c-Myc and Rex1, couple *Tsix* activity to pluripotency (Navarro et al. 2010), is compelling. However, neither *Xist* intron 1 deletion (Barakat et al. 2011) nor *Tsix* deletion (Lee and Lu 1999) can individually trigger the level of *Xist* upregulation that is compatible with the initiation of X-inactivation in undifferentiated ES cells. It remains possible that abrogating simultaneously both pluripotency-coupled repressive arms might be sufficient to do so. An alternative, not mutually exclusive, hypothesis involves a third gene recently incorporated to the short list of X-linked regulators of X-inactivation: *Rnf12*, a gene encoding an E3 ubiquitin ligase.

Rnf12, located several hundred kilobases upstream of *Xist/Tsix*, was initially identified as an inducer of *Xist* upregulation during random X-inactivation (Jonkers et al. 2009). Subsequently, the involvement of *Rnf12* during imprinted X-inactivation was demonstrated (Shin et al. 2010). Transgenic male ES cells overexpressing Rnf12 induce ectopic inactivation of the single X-chromosome upon differentiation, and in female lines a large fraction of the cells inactivates both X-chromosomes (Jonkers et al. 2009). Thus, it appears that *Xist* upregulation is triggered in differentiating female cells by virtue of the two copies of *Rnf12* that they carry, as opposed to the single copy present in male cells (Jonkers et al. 2009). In agreement, Rnf12 has been shown to act in *trans* on the minimal *Xist* promoter region without interfering with Nanog, Oct4, Sox2, and *Tsix* activity (Barakat et al. 2011). Importantly, in female ES cells overexpressing Rnf12, a significant proportion of cells already displays *Xist* RNA accumulation before differentiation (Jonkers et al. 2009). This signifies that, to ensure Xi reactivation in pluripotent cells, *Rnf12* expression must be downregulated. In this manuscript, we demonstrate the control mediated by pluripotency-factors on X-inactivation includes the downregulation of *Rnf12* by Nanog, Oct4 and Sox2.

Methods

Cell lines

All lines used in this study have been previously described. The inducible deletion of *Nanog* was performed by treating RCNβH cells with tamoxifen. These cells carry: (1) an homozygous deletion of *Nanog* endogenous alleles, (2) a Cre:ERT2 expressing transgene at the *Rosa26* locus, and (3) a loxP-flanked *Nanog* expressing transgene randomly integrated (Chambers et al. 2007). These cells were previously used to generate *Nanog*-null cells by plating tamoxifen treated cells at clonal density and expanding *Nanog*-null clones (RCNβH(t)) (Chambers et al. 2007). To restore *Nanog* expression in RCNβH(t) cells, a *Nanog* expression vector carrying a puromycin resistance gene was electroporated and cells selected for 6 days in the presence of 1 μg/ml of puromycin.

Nanog-overexpressing cells (EF4) were generated by random integration of CAG-driven *Nanog* cDNA in wild-type E14Tg2a ES cells (Chambers et al. 2003).

Oct4 inducible knock-out cells have been previously described (Niwa et al. 2000). Briefly, these cells carry two *Oct4*-null alleles and *Oct4* expression is sustained by a doxycycline suppressible transgene. Treatment of these cells with 1 μg/ml of doxycycline triggers rapid and efficient silencing of the *Oct4* expressing transgene.

Chromatin immunoprecipitation

Twenty million cells were resuspended in 3 ml of pre-warmed DMEM-FCS 10% and crosslinked with 1% formaldehyde (Sigma) for 10 min at room temperature. The reaction was quenched with 0.125 mM glycine for 5 min at room temperature. Cells were spun down for 3 min at 1,300 rpm at 4°C, and washed twice with cold PBS-1X (Invitrogen). Cell pellets were then vigorously resuspended in 300 μl of swelling buffer (5 mM Pipes pH 8, 85 mM KCl) freshly supplemented with 1X protease inhibitor cocktail (Roche) and 0.5% NP-40. The suspension was incubated for 20 min on ice with occasional shaking. Nuclei were spun down in 15 ml conical tubes for 10 min at 1,500 rpm at 4°C and resuspended in 1.5 ml of TSE150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris–HCl pH 8, 150 mM NaCl) buffer, freshly supplemented with 1X protease inhibitor cocktail. Samples were sonicated at 4°C in 15 ml conical tubes using a Bioruptor (Diagenode) for 5 cycles of 10 min divided into 30 s ON-30 s OFF subcycles at maximum power. Chromatin was then transferred into 1.5 ml tubes and centrifuged for 30 min at 14,000 rpm at 4°C. Soluble chromatin was aliquoted and stored at −80°C until use. Twenty microlitres were used for quantity and quality controls of the DNA.

For each experiment, the required amount of chromatin was defrosted and pre-cleared for 1h30 with rotation at 4°C in 1 ml of TSE150 with 50 μl of pA/pG Sepharose beads (Sigma) 50% slurry, previously blocked with 500 μg/ml of molecular grade BSA (Roche) and 1 μg/ml of yeast tRNA (Invitrogen). Pre-cleared chromatin was transferred into fresh tubes after 1 min centrifugation at 3,000 rpm and aliquoted accordingly (20 μg of DNA per ChIP). Twenty micrograms of diluted chromatin were aliquoted for input DNA extraction and precipitation. Immunoprecipitation with specific antibodies [1 μg home-made rabbit anti-Nanog (Mullin et al. 2008), 1 μg goat anti-Oct4 (Santa Cruz, sc-8628), 1 μg goat anti-Sox2 (Santa Cruz, sc-17320)] was performed overnight with rotation at 4°C, in a final volume of 500 μl. Immuno-complexes were recovered with 50 μl of blocked pA/pG Sepharose beads 50% slurry for 1h30 with rotation at 4°C. Beads were recovered by 1 min centrifugation at 3,000 rpm and washed at room temperature in 1 ml of TSE150, TSE500 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris–HCl pH8, 500 mM NaCl), washing buffer (10 mM Tris–HCl pH8, 0.25 M LiCl, 0.5% NP40, 0.5% Na-Deoxycholate, 1 mM EDTA), and twice in TE (10 mM Tris–HCl pH8, 1 mM EDTA). Each wash was performed for 5 min with rotation at room temperature. After the last wash, elution was performed in 100 μl of elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH8) for 15 min at 65°C after vigorous vortexing. Eluates were collected after 1 min centrifugation at 14,000 rpm, and the beads rinsed in 150 μl of TE-SDS 1%. After 1 min centrifugation at 14,000 rpm, the supernatant was pooled with the corresponding first eluate. Crosslinking of ChIP and input fractions was reversed overnight at 65°C, followed by proteinase K treatment (invitrogen), phenol/chloroform extraction and ethanol precipitation. DNA pellets corresponding to the input fractions were resuspended in 300 μl of water, whilst those corresponding to the ChIP fraction were resuspended in 150 μl.

RNA extraction and reverse transcription

One to five million cells were lysed in TRIZOL (Invitrogen) and RNA was then chloroform extracted and isopropanol precipitated. After DNase treatment (Quiagen), RNA was phenol/chloroform re-extracted, ethanol precipitated, resuspended in water and quantified.

One to 4 μg of RNA were used per RT reaction. RNA was denatured in the presence of 1 μg of random hexamers (Roche) for 5 min at 90°C, and reverse transcribed in a final volume of 20 μl with 100 U of SuperScriptII (Invitrogen) at 42°C for 60 min followed by heat inactivation at 70°C for 15 min. Synthesised cDNAs were diluted in 280 μl of water and stored at −20°C until used.

Real-time PCR

Q-PCR was performed in 384-well plates with a 480 LightCycler (Roche) using LightCycler 480 SYBR Green I Master (Roche). All reactions were performed in duplicate. Five microliters of DNA were used per reaction.

Standard curves of all primers were performed to check for efficient amplification (above 90%). Melting curves were also generated to verify production of single DNA species with each primer pair. All primer sequences are available upon request.

Relative levels of expression in each RT-(Q)PCR assay were obtained through the ΔΔCt method, using *Tbp* mRNA levels as a reporter in all experiments.

Enrichment levels in ChIP assays are expressed as enrichment levels as compared to the *Actin* promoter. Essentially, for each analysed position, the ΔCt method was used to calculate a ChIP over input ratio that was corrected by the appropriate dilution factor of each analysed fraction. To calculate enrichment levels, values obtained for the *Rnf12* upstream region were divided by those obtained for the *Actin* promoter.

Primer pairs to detect binding of Nanog, Oct4 and Sox2 upstream of *Rnf12* were designed across positive ChIP-Seq hits publicly available (Chen et al. 2008). The *Actin* promoter was chosen as a negative control on the basis of the absence of binding observed in the same data set. The sequences are: Rnf12-4 kb F: CAGCCTCTGGCTCTACC AGT and R: GTGACCTGCTGGGGAGAATA; Rnf12-5 kb F: GCCTGTCAAACGTCCTGTTTA and R: GGAGGTTG TGGGAGAAACAA; Actin F: CCGTTCCGAAAGTTG CCTT and R: CGCCGCCGGGTTTTATA.

Results

Rnf12 is a target of Nanog in undifferentiated mouse ES cells

Rnf12 protein levels have been shown to increase moderately during ES cells differentiation (Barakat et al. 2011). To assess whether this increase is mediated by an increase of mRNA levels, we first conducted RT-(Q)PCR in undifferentiated and differentiating ES cells. Efficient differentiation was evaluated by measuring *Nanog* mRNA levels, and as expected we found consistent downregulation of *Nanog* after 3 days of RA-mediated differentiation (Fig. 1a). Under these conditions, we found a two to threefold increase of *Rnf12* expression in RA-differentiated cells, in both males (E14Tg2a) and females (LF2) though the absolute levels of *Rnf12* mRNA showed the expected 2 to 1 ratio between XX and XY cells (Fig. 1b). This suggests that similar principles of regulation of *Rnf12* apply

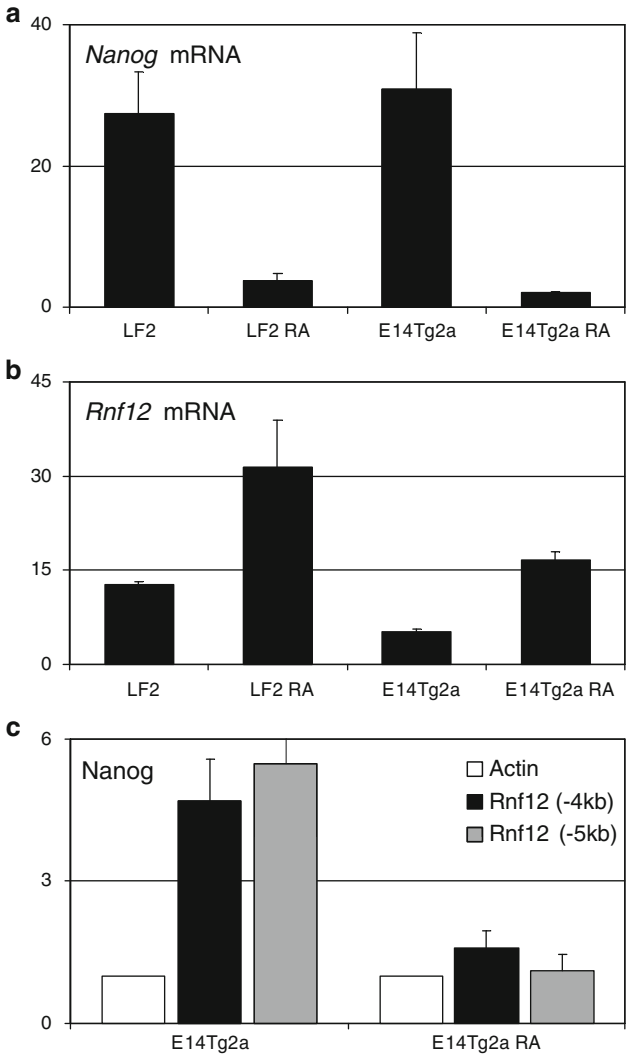

Fig. 1 *Rnf12* is a direct target of Nanog in ES cells. Male (E14Tg2a) and female (LF2) ES cells were differentiated with retinoic acid for 3 days, and *Nanog* (**a**) and *Rnf12* (**b**) mRNA levels measured by RT-(Q)PCR before and after treatment. Statistical significance of *Rnf12* expression differences was assessed with a paired *t* test [*p* value of LF2-d3/d0 <0.03 ($n = 3$) and of E14Tg2a-d3/d0 <0.01 ($n = 3$)]. **c** ChIP analysis of Nanog binding at two regions located 4 and 5 kb upstream of the transcription start site in undifferentiated and RA-differentiated male ES cells. All values represent averages of independent cell cultures ± SEM

during male and female differentiation and that the sole presence of two alleles in females underlies the female-specific action of *Rnf12*.

The correlation between *Nanog* downregulation and *Rnf12* upregulation during ES cells differentiation, and the fact that in the embryo the reactivation of the Xi closely follows Nanog protein expression in the ICM (Silva et al. 2009), suggests that Nanog might be important for *Rnf12* regulation. Interestingly, genome-wide analysis suggests *Rnf12* might be a direct target of Nanog (Chen et al. 2008). To confirm this, we determined whether two regions

located 4 and 5 kb upstream of *Rnf12* and identified by ChIP-Seq as positive Nanog hits (Chen et al. 2008) show enrichment for Nanog in ChIP-(Q)PCR assays. A five to sixfold enrichment was measured at these two regions in undifferentiated ES cells but not in RA-differentiating negative controls (Fig. 1c). These results, therefore, suggest that Nanog is a direct regulator of *Rnf12* in undifferentiated ES cells.

Nanog acts as a repressor of *Rnf12* expression in undifferentiated ES cells

The negative correlation existing between Nanog binding and *Rnf12* expression during ES cells differentiation (Fig. 1) suggests that Nanog may act as a negative regulator of *Rnf12*. To assess the modality of Nanog action at *Rnf12*, we first analysed the consequences of overexpressing Nanog. To do this, we used a cell line (EF4) carrying a randomly integrated *Nanog*-expressing transgene (Chambers et al. 2003) that confers high levels of *Nanog* mRNA expression (Fig. 2a). In EF4, binding of Nanog at both the −4 and −5 kb regions of the *Rnf12* locus is greatly increased, with enrichment levels being around two to threefold higher than in control E14Tg2a ES cells (Fig. 2b). Given the fact that in EF4 cells Nanog protein is elevated approximately fivefold relative to wild-type cells (Yates and Chambers 2005), this implies that Nanog binding at *Rnf12* in wild-type cells is not saturated, at least at the population level, though it may be saturated in EF4 cells. Since in wild-type cells Nanog is heterogeneously expressed (Chambers et al. 2007), with a significant fraction expressing low or undetectable Nanog protein, the increase of binding in EF4 likely reflects the homogeneity of Nanog expression and binding achieved by means of transgenic Nanog expression. Importantly, in EF4 ES cells, the expression of *Rnf12* is reduced by about 50% (Fig. 2c).

To consolidate this observation, we took advantage of *Nanog*-null ES cells (RCNβH(t), Chambers et al. 2007) in which neither Nanog expression (Fig. 2a) nor binding at *Rnf12* are detected (Fig. 2b). In these cells, *Rnf12* transcripts were found upregulated by around twofold, as expected for Nanog acting as a repressor (Fig. 2c). Next, we investigated whether *Rnf12* upregulation is a primary, early consequence of Nanog depletion. To do this, we used RCNβH cells, the parental line of RCNβH(t). In RCNβH ES cells, both *Nanog* alleles have been deleted and *Nanog* expression is supported by a randomly integrated transgene in which the *Nanog* open reading frame is flanked by loxP sites. Moreover, RCNβH express a Cre:ERT2 fusion protein from the constitutive *Rosa26* locus. Upon treatment of these cells with tamoxifen, the Cre:ERT2 fusion protein translocates into the nucleus and mediates the deletion of the *Nanog*-expressing transgene (Chambers et al. 2007).

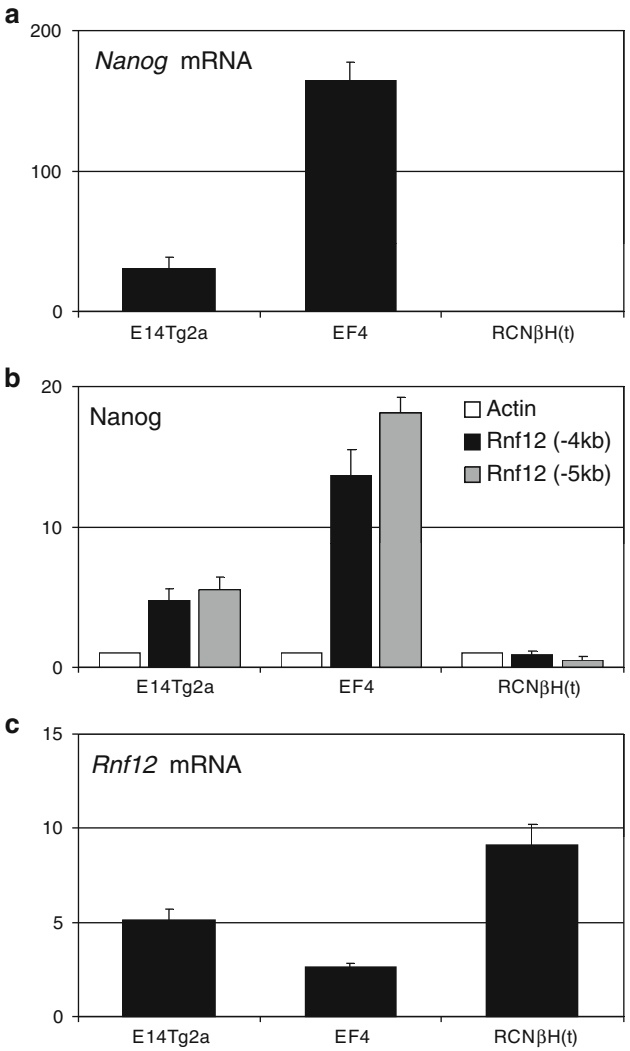

Fig. 2 *Rnf12* is downregulated by Nanog in ES cells. Analysis of *Nanog* mRNA levels (**a**) binding at the *Rnf12* locus (**b**) and *Rnf12* mRNA levels (**c**) in wild-type E14Tg2a ES cells, *Nanog*-overexpressing ES cells (EF4), and *Nanog*-null ES cells (RCNβH(t)). All values represent averages of independent cell cultures ± SEM. Statistical significance of *Rnf12* expression differences was assessed with an unpaired *t* test [*p* value of EF4/wt <0.03 ($n = 3$) and of RCNβH(t)/wt <0.03 ($n = 3$)]

RCNβH cells were treated with tamoxifen for 3 days and RNAs collected daily. *Nanog* mRNA levels decrease strongly within the first 24 h of treatment, and reach background levels after 48 h (Fig. 3a). Within the first 24 h of tamoxifen treatment, *Rnf12* mRNA increases to levels similar to that detected in established *Nanog*-null cells (Fig. 3b). Together with our ChIP analysis, this result suggests that the alteration of *Rnf12* expression observed in established *Nanog*-null or overexpressing lines is due to a direct action of Nanog on *Rnf12*.

Next, we tested the reversibility of *Rnf12* upregulation observed in *Nanog*-null cells. To do this, a Nanog-expression vector was stably integrated in RCNβH(t) ES cells and RNA

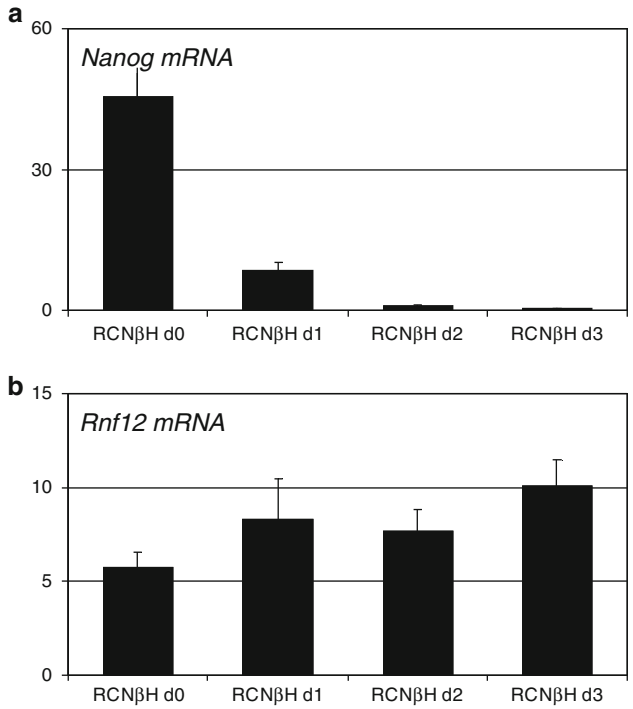

Fig. 3 *Rnf12* upregulation arises rapidly following Nanog depletion. Analysis of *Nanog* (**a**) and *Rnf12* (**b**) mRNA levels in inducible *Nanog*-null RCNβH cells treated with tamoxifen for the indicated number of days. All values represent averages of independent cell cultures ± SEM. Statistical significance of *Rnf12* expression differences was assessed with a paired *t*-test. Although there is a clear trend from day 1 onwards, clear statistical significance is only reached after 3 days of treatment [*p* value of d3/d0 <0.03 ($n = 10$)], when *Nanog* expression is reduced to about 0.1% of untreated cells

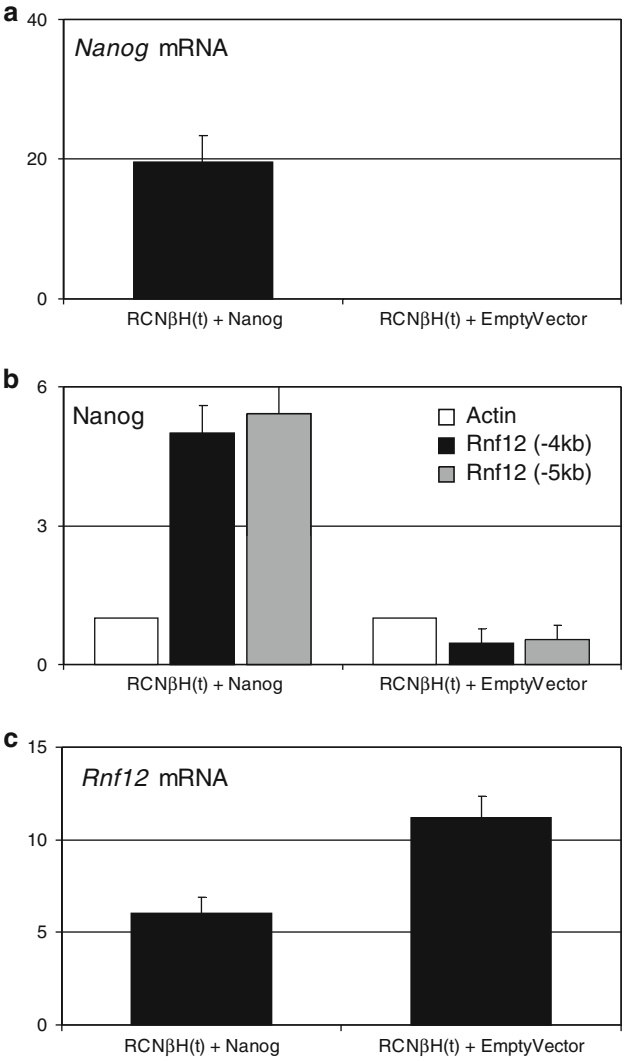

Fig. 4 *Rnf12* upregulation in *Nanog*-null ES cells is reversible. Analysis of *Nanog* mRNA levels (**a**) binding at the *Rnf12* locus (**b**) and *Rnf12* mRNA levels (**c**) in RCNβH(t) *Nanog*-null cells transfected with either a *Nanog*-expression vector or an empty vector. All values represent averages of independent cell cultures ± SEM. Statistical significance of *Rnf12* expression differences was assessed with a paired *t* test (*p* value of Nanog/empty <0.03 ($n = 3$)]

and chromatin prepared. Evaluation of *Nanog* mRNA levels (Fig. 4a) indicates restoration of *Nanog* expression to levels similar to that of wild-type ES cells (Fig. 1a), indicating that the analysis is performed in physiological conditions regarding the level of *Nanog* expression. Compared to empty vector transfectants, the re-expression of *Nanog* in *Nanog*-null cells leads to efficient binding of Nanog at the −4 and −5 kb sites of the *Rnf12* upstream region (Fig. 4b), with identical levels of enrichment to those observed in wild-type cells (Fig. 1c). Under these conditions, *Rnf12* mRNA levels (Fig. 4c) decline back to wild-type levels (Fig. 1b), whilst the cells transfected with the empty vector express similar levels of *Rnf12* than untransfected RCNβH(t) ES cells (Fig. 2c). Taking all our observations together, we conclude that Nanog acts as a reversible repressor of *Rnf12* expression in ES cells.

Nanog-independent binding of Oct4 and Sox2 at the *Rnf12* upstream region

In the absence of Nanog binding to the *Rnf12* upstream region, *Rnf12* is twofold upregulated in male ES cells. However, the level reached in differentiating male ES cells is about threefold as compared to that observed in wild-type undifferentiated cells. This indicates that Nanog depletion in ES cells does not recapitulate entirely the mechanisms leading to *Rnf12* upregulation during differentiation. Additional repressors of *Rnf12* might exist in ES cells.

Genome-wide localisation studies of transcription factor binding in ES cells have shown that Nanog tends to bind at chromatin sites overlapping or just adjacent to those bound by Oct4 and Sox2 (Chen et al. 2008). More importantly, at *Xist* intron 1 Nanog binds with Oct4 and Sox2 to repress *Xist* transcription (Navarro et al. 2008). Therefore, we

hypothesised that Oct4 and Sox2 bind with Nanog at the *Rnf12* upstream region. We performed ChIP assays with Oct4 (Fig. 5a) and Sox2 (Fig. 5b) antibodies in ES cells. Both factors were found to bind at the −4 and −5 kb sites of the *Rnf12* upstream region, with levels of enrichment about four to fivefold higher than those measured at the beta-actin negative control. Importantly, in RA-treated differentiating ES cells, binding of both Oct4 and Sox2 is totally abolished. This result suggests that Oct4 and Sox2 cooperate with Nanog to bring about efficient downregulation of *Rnf12*.

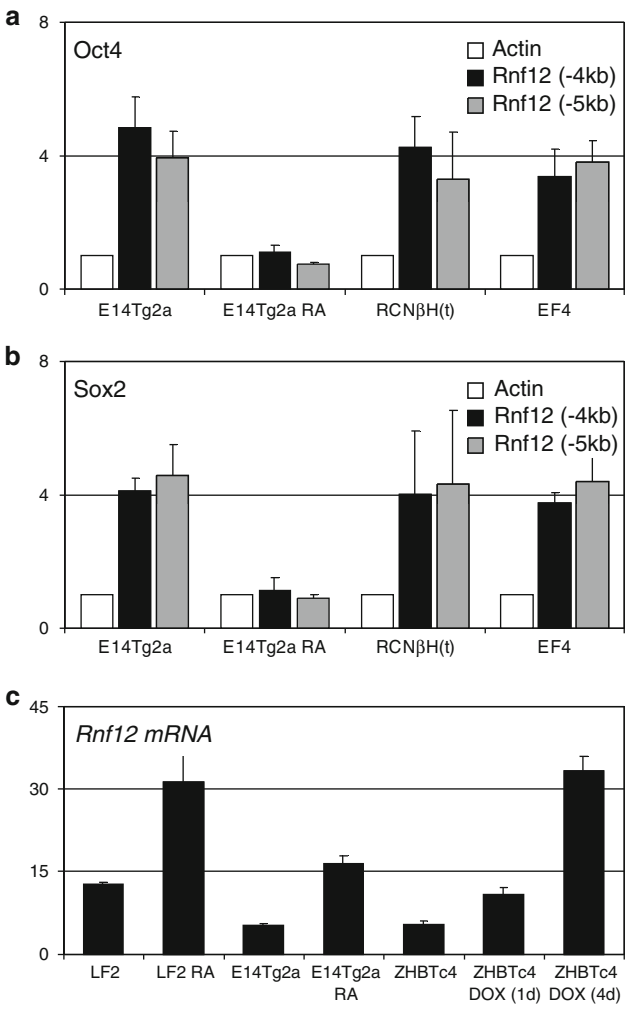

Fig. 5 Oct4 and Sox2 also act as *Rnf12* repressors in ES cells. Analysis of Oct4 (**a**) and Sox2 (**b**) binding at the *Rnf12* upstream region in the indicated cell lines. (**c**) Comparison of *Rnf12* expression in different cell lines and conditions. ZHBTc4 ES cells are doxycycline inducible *Oct4*-null ES cells. All values represent averages of independent cell cultures ± SEM. Statistical significance of *Rnf12* expression differences in doxycycline-treated ZHBTc4 cells was assessed with a paired *t* test [*p* value of d1/d0 and d4/d0 <0.01 ($n = 6$ and 4, respectively)]. An unpaired *t* test further confirmed that the level of *Rnf12* mRNA reached in doxycycline-treated ZHBTc4 is significantly higher than that measured in RA-treated E14Tg2a cells (*p* value <0.01)

To determine if Nanog is required or at least influences the binding of Oct4 and Sox2 at the *Rnf12* locus, we first analysed Oct4 and Sox2 binding at the −4 and −5 kb regions in cells displaying increased binding of Nanog and found that neither Oct4 nor Sox2 exhibit increased binding at *Rnf12* (EF4, Fig. 5a, b). Moreover, in *Nanog*-null ES cells both Oct4 and Sox2 remain associated to the *Rnf12* upstream region at levels of enrichment indistinguishable from those observed in wild-type E14Tg2a ES cells (RCNβH(t), Fig. 5a, b). We conclude that Oct4 and Sox2 bind at the *Rnf12* locus in a Nanog-independent fashion.

Inappropriate high levels of *Rnf12* expression in inducible *Oct4*-null male ES cells

Should Oct4 and Sox2 act as repressors of *Rnf12*, then their permanent binding in the absence of Nanog might explain the mild upregulation of *Rnf12* observed in *Nanog*-null ES cells. This situation would be strikingly similar to that previously observed for *Xist*. In the absence of Nanog, *Xist* is moderately upregulated in undifferentiated male ES cells. However, the acute silencing of *Oct4* experimentally triggered in inducible *Oct4*-null male ES cells leads to differentiation and ectopic accumulation of *Xist* RNA at levels compatible with X-inactivation (Navarro et al. 2008). We sought to test whether in these particular conditions *Rnf12* is expressed at female-like mRNA levels. To do this, we used ZHBTc4 male ES cells (Niwa et al. 2000). In these cells, both *Oct4* alleles are deleted and a doxycycline-repressible *Oct4* transgene supports *Oct4* expression. Twenty-four hours after addition of doxycycline, *Oct4* mRNA levels are completely abolished, whilst *Nanog* and *Sox2* remain expressed (Online Resource 1). This is associated with a 2 to threefold increase of *Rnf12* expression (Fig. 5c), indicating that Oct4 acts as a repressor of *Rnf12*. Strikingly, after 4 days of treatment, when *Nanog* and *Sox2* are silenced (Online Resource) and *Xist* RNA levels were previously shown to be similar to those measured in differentiating female ES cells, *Rnf12* expression further increases to levels compatible with X-inactivation as evaluated in differentiating female ES cells (Fig. 5c). Therefore, we conclude that *Rnf12* regulation by Nanog, Oct4 and Sox2 follows virtually identical principles than *Xist* regulation: Nanog, Oct4 and Sox2 synergistically downregulate *Rnf12* in undifferentiated ES cells.

Discussion

Previously, we and others have shown that in undifferentiated ES cells *Xist* is silenced by two independent

mechanisms: (1) a direct transcriptional repression mediated by Nanog, Oct4 and Sox2 binding at *Xist* intron 1 (Navarro et al. 2008), and (2) a chromatin repression mediated by *Tsix* (reviewed in Navarro and Avner 2010), which is upregulated by the direct action of c-Myc, Klf4 and Rex1 binding at the primary *Tsix* enhancer *DXPas34* (Navarro et al. 2010) and by Oct4, Sox2 and Klf4 binding at the secondary *Tsix* enhancer *Xite* (Donohoe et al. 2009; Navarro et al. 2010). Neither *Xist* intron 1 deletion (Barakat et al. 2011), nor *Tsix* mutation (Lee and Lu 1999) is individually able to trigger X-inactivation in undifferentiated ES cells, suggesting that either both systems cooperate to silence *Xist*, and/or that other systems of repression act in ES cells. It must be mentioned, however, that further analyses of the effects of *Xist* intron 1 deletion are required before rigorously concluding that it does not strongly affect *Xist* regulation. In undifferentiated ES cells, the *Xist* promoter is unable to efficiently recruit the transcriptional machinery, regardless of *Tsix* activity (Navarro et al. 2005). However, in the absence of *Tsix, Xist* RNA levels increase considerably in undifferentiated ES cells (Morey et al. 2001; Vigneau et al. 2006), suggesting *Tsix* might have post-transcriptional effects on *Xist* expression. Therefore, it is possible that in the absence of *Xist* intron 1, the transcriptional machinery is indeed efficiently recruited at the *Xist* promoter, *Xist* transcription readily enhanced, but *Xist* RNA post-transcriptionally repressed by *Tsix* RNA. Thus, a ChIP analysis of *Xist* promoter state in *Xist* intron 1-deleted ES cells is absolutely required. Also, the analysis of double *Xist* intron 1/*Tsix*-mutants is required, and future work should specifically address this critical point.

Despite these considerations, additional mechanisms coupled to pluripotency might be involved in *Xist* suppression in ES cells. In this context, the results we report here expand our previous work on the coupling between X-inactivation and pluripotency regulators. Indeed, we demonstrate *Rnf12* is downregulated by Nanog, Oct4 and Sox2, the three main actors of pluripotency. Interestingly, the sole overexpression of Rnf12 in female ES cells leads to X-inactivation in the undifferentiated state (Barakat et al. 2011). Although the mechanisms of Rnf12 action at *Xist* are unknown, good evidence suggests it may activate the *Xist* promoter (Barakat et al. 2011). Therefore, the fact that *Rnf12* is downregulated by Nanog, Oct4 and Sox2 appears to be of critical importance in maintaining *Xist* silencing in undifferentiated ES cells. It is noteworthy that in all *Nanog*- and *Oct4*-null experimental conditions in which we previously reported ectopic *Xist* upregulation in male cells (Navarro et al. 2008), *Rnf12* is also upregulated. Given the positive action exerted by Rnf12 on *Xist*, this suggests that all our previous observations on *Xist* might at least in part be due to *Rnf12* upregulation rather than to a direct action of Nanog, Oct4 and Sox2 through *Xist* intron 1 binding. If this was to be true, the binding of these factors to *Xist* intron 1 would be extremely mysterious and perhaps functionally linked to the choice of the X-chromosome to be inactivated, as recently suggested (Barakat et al. 2011). We reiterate, however, that further work on the effects of *Xist* intron 1 deletion in undifferentiated ES cells is necessary before reaching these conclusions. Similarly, unequivocal proof that the binding events we have identified here directly inhibit *Rnf12* transcription should be provided in the future by targeting specific mutations that abrogate the binding.

Immuno-fluorescence analysis of Nanog and Rnf12 in ES cells has previously shown that Rnf12 is only consistently detected in Nanog-negative ES cells (Barakat et al. 2011). Nanog-negative and -positive cells have been shown to interconvert (Chambers et al. 2007), implying that Rnf12-negative and -positive cells may interconvert as well. This suggests that Nanog action at Rnf12 is reversible and strictly dependent on its expression, as supported by the restoration of normal levels of *Rnf12* upon re-expression of *Nanog* in *Nanog*-null ES cells. To ultimately prove this single cell analysis, ideally by immunofluorescence, should be performed to follow the evolution of Nanog and Rnf12 protein levels during the growth of ES clones derived from single cells.

Initially viewed as a simple *Xist*-Nanog/Oct4/Sox2 connection, the system coupling X-inactivation with pluripotency is rapidly expanding, first by the inclusion of *Tsix* as a direct target of Klf4, Rex1 and c-Myc, and now with the repressive action of Nanog, Oct4 and Sox2 on *Rnf12* expression (Fig. 6). Strikingly, although our study has been restricted to Nanog, Oct4 and Sox2, additional transcription factors such as Klf4 and Esrrb, which have important roles in pluripotency (reviewed in Chambers and Tomlinson 2009), might also contribute to *Rnf12* control as suggested by available ChIP-Seq data sets (Online Resource 2). This leads to a remarkably complex network of functional interactions between pluripotency and X-inactivation regulators, a major emergent property of which might be the increase of the robustness of X-inactivation inhibition in pluripotent compartments of the early embryo such as the ICM and the germ line. Furthermore, we believe the number of actors and nodes of this network is likely to grow further both by discovering other pluripotency factors acting on *Xist*, *Tsix* and *Rnf12*, and by finding new X-linked regulators of X-inactivation. For instance, two additional X-linked non-coding genes have been recently shown to play a role in *Xist* regulation in ES cells: *Ftx* (Chureau et al. 2011) and *Jpx* (Tian et al. 2010). Both are located between *Rnf12* and *Xist* and have been suggested to act as *Xist* activators through yet unknown mechanisms. Although it is unknown whether *Ftx* and/or *Jpx* overexpression leads to X-inactivation in the undifferentiated state, their downregulation in ES cells may be important

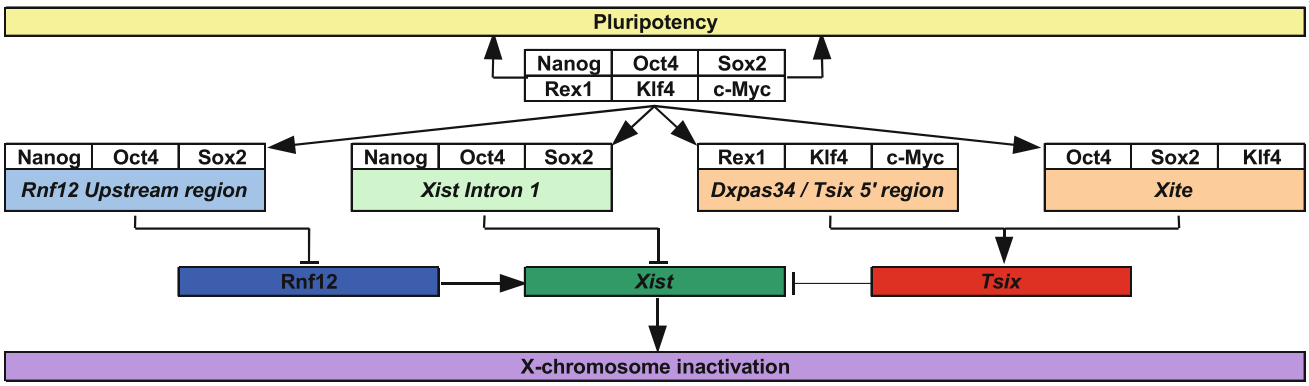

Fig. 6 Extended network coupling X-inactivation and pluripotency. The diagram depicts all known functional interactions between pluripotency and X-inactivation regulators

for keeping *Xist* silent before differentiation. Strikingly, extensive binding of Nanog but not Oct4/Sox2 is observed around *Ftx* in available ChIP-Seq data sets (Online Resource 2). In contrast, *Jpx* does not appear in ChIP-Seq studies as a target of Nanog or of any other pluripotency-associated factor. Although additional work needs to be done to elucidate whether these two non-coding genes are also controlled by pluripotency transcription factors, it is increasingly clear that the road to pluripotency and the path of X-inactivation are intrinsically connected through the direct control of many, if not all, X-inactivation regulatory genes by the transcriptional machinery dedicated to pluripotency. The future establishment of the contribution of each individual factor to the control of X-inactivation will undoubtedly shed light on our understanding of how genetic and epigenetic regulators functionally interact to bring the phenotype into being.

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