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RIF1 and KAP1 differentially regulate the choice of the inactive versus the active X chromosomes.

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ABSTRACT

The onset of random X inactivation in mouse requires the switch from a symmetric to an asymmetric state, where the identities of the future inactive and active X chromosomes are assigned. This process is known as the choice. Here we show that RIF1 and KAP1 are two fundamental factors for the definition of this transcriptional asymmetry. We found that at the onset of mESC differentiation, biallelic upregulation of the long non-coding RNA Tsix weakens the symmetric association of RIF1 with the *Xist* promoter. The *Xist* allele maintaining the association with RIF1 goes on to upregulate *Xist*, in a RIF1-dependent manner. Conversely, the promoter that loses RIF1 gains binding of KAP1, and that KAP1 is required for the boost of Tsix levels preceding the choice. We propose that the mutual exclusion of Tsix and RIF1, and of RIF1 and KAP1 at the Xist promoters establish a self-sustaining loop that transforms an initially stochastic event into a stably inherited asymmetric X chromosome state.

INTRODUCTION

X chromosome inactivation (XCI) is the process leading to the stable transcriptional silencing of one of the two X chromosomes in female placental mammals, with the aim of equalising the expression of X-linked genes between males and females (Lyon, 1961). This process represents one of the best-studied examples of how different nuclear processes, such as epigenetic control, 3D organisation of chromatin contacts, sub-nuclear positioning and, potentially, replication-timing regulation, are integrated to achieve transcriptional control. Random XCI (rXCI) is initiated when Xist, an X-encoded long non-coding RNA (lncRNA) is up-regulated from one of the two X chromosomes, the future inactive X chromosome (Xi) (Brockdorff et al., 1991, Brown et al., 1991, Marahrens et al., 1997, Penny et al., 1996). *In vivo,* this happens around the time of embryo implantation (Monk & Harper, 1978, Rastan, 1982), while in cultured female mouse embryonic stem cells (mESCs), XCI takes place during a narrow time-window at the onset of differentiation (Wutz & Jaenisch, 2000). Monoallelic up-regulation of *Xist* is coupled to loss of pluripotency and several activating and repressing factors of this process have been described (Chureau et al., 2011, Furlan et al., 2018, Gontan et al., 2012, Jonkers et al., 2009, Lee & Lu, 1999, Makhlouf et al., 2014, Navarro et al., 2008, Tian et al., 2010). Guided by the three-dimensional (3D) conformation of the X chromosome (Engreitz et al., 2013, Simon et al., 2013), Xist spreads *in cis* and recruits SPEN to enhancers and promoters of the X-linked genes to trigger their silencing (Chu et al., 2015, Dossin et al., 2020, McHugh et al., 2015, Moindrot et al., 2015, Monfort et al., 2015), and the exclusion of RNA polymerase II (Chaumeil et al., 2006, Kucera et al., 2011). This in turn promotes the recruitment of the Polycomb Repressive Complexes (PRC1/2) and the accumulation of tri-methylated H3K27 (H3K27me3) (Sun et al., 2006, Zhao et al., 2008) and

monoubiquitinated H2AK119 (H2AK119ub) (de Napoles et al., 2004) on the future inactive X chromosome (Xi). Contextually, Lamin B receptor (LBR) tethers the future Xi to the nuclear periphery to facilitate Xist spreading into active gene regions and the maintenance of the silent state (Chen et al., 2016). Finally, the entire Xi switches the replication timing to mid-late S-phase (Takagi et al., 1982). The combination of all these events facilitates the attainment of an exceptionally stable transcriptionally silent status, so robustly controlled that it is maintained throughout the entire life of the organism. One of the least understood of all these steps is the mechanism that, in the initiating phase of XCI, directs the random choice of which one of the two *Xist* alleles to up-regulate, marking the future Xi, and which to silence (marking the future active X chromosome, Xa). We will refer to this process as the "choice" (Avner & Heard, 2001). This is a key stage, as failure to establish monoallelic *Xist* expression can result in either both X chromosomes being silenced or both remaining active, consequently leading to embryonic lethality (Borensztein et al., 2017, Marahrens et al., 1997, Takagi & Abe, 1990). Tsix is a lncRNA encoded by a gene that overlaps, in the antisense orientation with *Xist*, and plays a well-established role as an *in cis* repressor of *Xist* (Lee & Lu, 1999). In female mESCs, *Tsix* is bi-allelically expressed, to become down-regulated on one of the two X chromosomes, the future Xi, at the onset of differentiation, hence allowing for *in cis Xist* up-regulation (Lee et al., 1999, Stavropoulos et al., 2001). The switch to mono-allelic expression of *Tsix* is important in determining the destinies of the future Xi (Tsix silenced) and Xa (Tsix transiently maintained). In fact, interfering with the expression of one of the two *Tsix* alleles in female mESCs results in a non-random choice, with the *Tsix*-defective chromosome predetermined as the future Xi (reviewed in (Starmer & Magnuson, 2009)). Although *Tsix* downregulation is essential for *in cis* up-regulation of Xist, the molecular

mechanism of Tsix-driven silencing is still unclear. The *Tsix* terminator region overlaps with the *Xist* promoter, and *Tsix* transcription through this region and/or Tsix RNA have been proposed to be essential for *Xist* repression (Shibata & Lee, 2004) by promoting a transient silenced chromatin state (Navarro et al., 2006, Navarro et al., 2005, Ohhata et al., 2008, Sado et al., 2005). The establishment of the opposite *Xist/Tsix* expression patterns on the two genetically identical X chromosomes, must rely on the coordinated asymmetric distribution of activators and/or repressors of transcription.

Mathematical modelling can recapitulate the experimental features of XCI by postulating the existence of an *in cis* negative regulator of *Xist* (cXR) and an *in trans*, X-linked, *Xist* activator (tXA) (Mutzel et al., 2019). While a cXR is sufficient to explain the maintenance of mono-allelic *Xist* expression, a tXA is needed to explain 1. the establishment of the *Xist* mono-allelic expression; 2. the female specificity of XCI; 3. the resolution of bi-allelic *Xist* expression detected, to various extents, in different organisms (Mutzel et al., 2019). In mouse, Tsix is the most likely cXR, while RNF12, an X-linked ubiquitin ligase that functions as a dose-dependent initiator of XCI (Gontan et al., 2012, Jonkers et al., 2009), has been proposed as one of the potential tXA. However, while over-expression of *Rlim* (*Rnf12*) in male cells can induce XCI (Jonkers et al., 2009), its deletion in females is not sufficient to prevent *Xist* up-regulation (Shin et al., 2014, Wang et al., 2017). Thus, RNF12 could account for the X-linked aspects of the tXA function, such as female specificity and resolution of bi-allelic expression, but one or multiple other transactivators must be contributing to the asymmetric control of *Xist* expression. Moreover, conceptually, the expression level of a single, X-linked gene, does not constitute a switch robust or sensitive enough to be the only element to control a clear-cut bi-stable state for *Xist* (active on one and silent on the other allele) (Mutzel & Schulz, 2020). The establishment of *in cis*, self-reinforcing and

mutually exclusive circuits on the two *Xist* alleles could create the ultrasensitivity required to generate a binary switch-type of control (Mutzel & Schulz, 2020). Key to this model, is the idea that the initial stochastic events will trigger a chain of local, mutually exclusive and self-sustaining events to bookmark both Xi and Xa.

RIF1 is a multifaceted protein, required for the regulation of several of the nuclear processes that take place during XCI. RIF1 is the only known genome-wide regulator of replication timing (Cornacchia et al., 2012, Foti et al., 2016, Hayano et al., 2012, Hiraga et al., 2014, Peace et al., 2014, Seller & O'Farrell, 2018, Yamazaki et al., 2012). It confines long-range chromatin contacts within the respective boundaries of the nuclear A/B compartments (Gnan et al., 2021) and plays an as yet unclear function in the control of gene expression (Daxinger et al., 2013, Foti et al., 2016, Li et al., 2017, Tanaka et al., 2016, Toteva et al., 2017, Zofall et al., 2016). RIF1 is an adaptor for Protein Phosphatase 1 (PP1), one of the main Ser/Thr phosphatases in eukaryotic cells (Alver et al., 2017, Dave et al., 2014, Hiraga et al., 2014, Hiraga et al., 2017, Mattarocci et al., 2014, Sreesankar et al., 2015, Trinkle-Mulcahy et al., 2006). In *Drosophila melanogaster,* the RIF1-PP1 interaction was shown to be essential during embryonic development (Seller & O'Farrell, 2018). In addition, the RIF1-PP1 interaction is essential for RIF1-dependent organisation of chromatin contacts (Gnan et al., 2021). Others (Chapman et al., 2013, Daxinger et al., 2013) and we (this work) have observed that mouse RIF1 deficiency is associated with a sex-linked differential lethality, with the female embryos dying around the time of implantation. These data have suggested that RIF1 could be important during XCI. Here we find that RIF1, present biallelically on the *Xist* P2-promoter in female mESCs, becomes asymmetrically enriched at P2 on the future Xi, concomitant with the choice, at the

time when *Tsix* expression switches from bi- to mono-allelic. RIF1 then plays an essential role in *Xist* up-regulation, thus determining the future Xi. The removal of RIF1 from the future Xa arises from the KAP1-dependent increase of *Tsix* bi-allelic expression that leads to the choice. Our data identify the KAP1-dependent regulation of Tsix levels and the consequent transition of RIF1 association with *Xist* promoter from symmetric to asymmetric, as key steps in the molecular cascade that leads to the identification of the future Xi and Xa.

RESULTS

RIF1 is required for X inactivation during embryonic development and for *Xist* up-regulation

The analysis of the progeny derived from inter-crosses of mice heterozygous for a *Rif1* null allele (*Rif1*^{+/-}, Appendix Fig. S1A and B) in a pure C57BL/6J genetic background has revealed that *Rif1* is essential for embryonic development (Fig. 1A). In contrast, in a mixed genetic C57BL/6J-129/SvJ background, *Rif1* deletion results in a differential lethality between the sexes (Fig. 1B). Indeed, in this case, only a small proportion of the expected *Rif1*^{-/-} mice, exclusively males, are recovered at weaning. In order to pinpoint more precisely the time of the onset of lethality, we have analysed the frequency of recovery of *Rif1*^{-/-} embryos at different stages of development in a C57BL/6J pure background. We found that, up to the blastocyst stage (E3.5), there are no obvious differences in the number of male and female *Rif1* null and wild type embryos recovered (our unpublished observation). However, by E7.5, although still recoverable, *Rif1* null female embryos are already dead/abnormal (Fig. 1C and D). In contrast, males appear to die only around mid-gestation (Fig. 1C). This early-onset

lethality observed specifically in females suggests that the lack of RIF1 could interfere with the process of XCI, as the timing coincides with the onset of random XCI.

Given the diversity of its roles, RIF1 could act at one or several of the multiple steps during XCI. To dissect at what stage(s) of the process RIF1 is required, we generated female mESCs carrying homozygous conditional *Rif1* allele (*Rif1^Flox/Flox^*) and a tamoxifen-inducible CRE recombinase (*Rosa26^Cre-ERT/+^*,(Buonomo et al., 2009)). To trigger XCI in the absence of *Rif1*, we set up a protocol in which we combined differentiation by embryoid bodies (EB) formation (Doetschman et al., 1985) and tamoxifen treatment (Fig. 2A and Materials and Methods). By RT-qPCR as well as by RNA sequencing, we found that *Rif1* deletion (Fig. 2B) severely impairs *Xist* up-regulation (Fig. 2C, Fig. EV1A, EV1B and EV2A) and, consequently, the enrichment of H3K27me3 on the future Xi (Fig. 2D). Failure of *Xist* up-regulation in the absence of *Rif1* is not due to a general defect in exit from pluripotency (Fig. EV1C and EV2B, D and E) or to failed commitment to differentiation (Fig. EV1C and EV2C, D and E). Moreover, during the early stages of differentiation the levels of the main negative regulator of Xist, Tsix, appear to be reduced faster in *Rif1* knockout cells compared to the control (Appendix Fig. S2A). Finally, the overall dynamics of RNF12 appear comparable between control and *Rif1* knock out cells (Fig. EV1B and Appendix Fig. S2B). Overall, these results indicate that failure of *Xist* up-regulation is the likely cause of defective XCI in *Rif1* null female embryos and that RIF1 could directly and positively regulate *Xist* expression.

RIF1 is a positive regulator of *Xist* and its binding specifically bookmarks the future Xi

Xist is controlled from two promoters, P1 and P2 (Johnston et al., 1998), separated by a repetitive region essential for the silencing properties of Xist (Wutz et al., 2002). While the epigenetic control of the upstream P1 promoter was shown to be important for *Xist* regulation (Navarro et al., 2005), P2 appears to serve as an internal regulatory unit, possibly modulating the expression from P1 (Makhlouf et al., 2014). We found that RIF1 is enriched specifically at *Xist* P2 promoter, both in mESCs (Fig. 3A, Appendix Fig. S2C and S2D) and in early EBs (Fig. 3B), supporting the hypothesis that RIF1 could be a direct regulator of *Xist* expression. In agreement with this, we found that P2 harbours two potential RIF1-binding sites, defined by the presence of a consensus sequence derived from the analysis of RIF1 genome-wide distribution by ChIP-seq in female mESCs (Foti et al., 2016) (Appendix Fig. S3A). To confirm that RIF1 association with *Xist* promoter has a positive effect on *Xist* expression, we used a reporter assay system, where *Xist* promoter has been cloned upstream of a firefly Luciferase gene (Gontan et al., 2012). We found that, upon differentiation, in the absence of RIF1, the induction of Luciferase from the *Xist* promoter is significantly reduced (Fig. 3C), supporting the hypothesis that RIF1 association with P2 exerts a positive, direct effect on *Xist* transcription.

Upon differentiation, *Xist* is mono-allelically transcribed, up-regulated only from the future Xi. If RIF1 acts as a positive regulator of *Xist*, we would expect it to be associated mono-allelically, specifically with P2 on the future Xi. In order to test this hypothesis, we have taken advantage of the Fa2L cell line, in which 1. the two X chromosomes can be discriminated, as one originates from *Mus castaneus* (*cast*) and the other from *Mus musculus 129/SvJ* (*129*) mouse strains; 2. Xa (*cast*) and Xi (*129*)

are predetermined, as the *129 Tsix* allele carries a transcriptional stop signal, approximately 4kb downstream from the *Tsix* major promoter (Fig. 3D, scheme and (Luikenhuis et al., 2001)). *Xist* is therefore preferentially up-regulated from the *129*-derived X chromosome. We have analysed the association of RIF1 with *Xist* P2 promoter of the future Xa and Xi by allele-specific ChIP-qPCR (Appendix Fig. S3B) and found that RIF1 is preferentially associated with the *Xist* P2 promoter of the *129 Xist* allele (future Xi) in both mESCs (Fig. 3D) and upon differentiation (Fig. 3E). Importantly, in control wild type mESCs (bi-allelically expressed *Tsix*), also carrying one *cast* and one *129* X chromosome, RIF1 is equally distributed on both P2 promoters (Fig. 3D). This suggests that the asymmetric association of RIF1 with the future Xi is concomitant with / follows the switch from bi- to mono-allelic *Tsix* expression that accompanies the choice and allows *Xist* monoallelic up-regulation. As in the case of RIF1 conditional cells, depletion of RIF1 in Fa2L cells (Appendix Fig. S3C) also compromises *Xist* up-regulation (Appendix Fig. S3D). These data show that RIF1's asymmetric association with the future Xi parallels the choice and that it is essential for *Xist* up-regulation.

RIF1 asymmetric localisation on the future Xi is driven by *Tsix* expression

How is the transition from bi- to mono-allelic RIF1 association with *Xist* promoter regulated? While this would generally be triggered by differentiation, in undifferentiated Fa2L cells it is pre-determined and RIF1 is preferentially associated with the X chromosome that does not express full-length Tsix transcript (Fig. 3D and E). This suggests that Tsix RNA and/or transcription could destabilise RIF1 association with *Xist* promoter. In agreement with this hypothesis, we found that blocking *Tsix* expression by treating mESCs with the CDK9-inhibitor flavopiridol, which

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inhibits transcriptional elongation (Chao & Price, 2001) (Fig. EV3A) or, briefly, with triptolide, an inhibitor of transcription initiation (Fig. EV3B), is sufficient to revert RIF1 preferential association with the future Xi in the Fa2L cells to a symmetric mode of binding (Fig. 3F and EV3C). In addition, flavopiridol treatment of wild type mESCs also leads to an increased P2-association of RIF1 (Fig. 3G), indicating that this is not an effect specific to the Fa2L cells. Finally, while this work was under review, RIF1 has been found associated with Tsix RNA in mESCs (Aeby et al., 2020), supporting the hypothesis that Tsix RNA can compete for RIF1 association with *Xist* P2 in the genome.

KAP1 is important for the Xa/Xi choice

With the aim of understanding the molecular mechanism by which RIF1 regulates *Xist* expression, we have investigated whether some of the known transcriptional regulators associated with RIF1 (Sukackaite et al., 2017) are also required for XCI. We focused in particular on KAP1, as KAP1 and RIF1 have already been shown to regulate overlapping targets, such as Dux and MERVLs (Li et al., 2017, Maksakova et al., 2013, Percharde et al., 2018). We found that knock down of Kap1 (Appendix Fig. S4A and B) impairs *Xist* up-regulation (Fig. 4A and Fig. EV4A), similarly to the knockout of *Rif1*. This is not due to compromised exit from pluripotency (Fig. EV4B), impaired activation of the differentiation transcriptional program (Fig. EV4C) or reduced RIF1 levels (Fig. EV4D), suggesting that diminished *Xist* activation is not a consequence of an overall impaired cell differentiation. In addition, the dynamics of expression of RNF12 appear comparable between control and Kap1 knock down cells (Fig. EV4E). However, in contrast to the depletion of Rif1, depletion of Kap1 in Fa2L cells (Appendix Fig. S4C), where the choice is pre-determined, has no consequences

for *Xist* upregulation (Fig. 4B). These data suggest that KAP1 is required prior to or at the time of the choice, while it is dispensable once *Tsix* mono-allelic expression has been established. In agreement with a role during the choice, we found that Kap1 knock down affects Tsix dynamic regulation at the onset of differentiation. In wild type cells, during the early stages of differentiation, Tsix levels rise transiently (at 1, or 1 and 2 days of embryoid body differentiation respectively, depending on the culture conditions, Fig. 4C and Appendix Fig. S2A). The boost corresponds to an increased detection of Tsix RNA from both alleles (Fig. 4D), suggesting that this step precedes the switch to *Tsix* mono-allelic expression and the consequent choice of Xa/Xi. Upon Kap1 down-regulation, we found not only a failure in the temporary boost of Tsix levels (Fig. 4C), but also a failure to evolve towards Tsix mono-allelic expression, as Tsix becomes undetectable (Fig. 4D). In a situation of pre-determined choice (Fa2L cells), Tsix levels remain low, even upon differentiation, and Kap1 knock down has no further effect (Appendix Fig. S4D).

In summary, the failure to up-regulate *Xist* caused by *Rif1* deletion and by Kap1 knock down have very different causes. While RIF1 is directly required to promote *Xist* up-regulation, KAP1's function is to drive the transient increase of Tsix levels that precedes the choice. The consequent failure to up-regulate *Xist* when Kap1 is knocked down could be caused, in this case, by a failure to execute the choice. The low, bi-allelic Tsix levels typical of mESCs instead evolve directly towards an absence of Tsix.

RIF1 negatively regulates KAP1 association with the *Xist* promoter/*Tsix* terminator in mESCs

KAP1 is a multifunctional protein, and a key global regulator of transcription, involved in several aspects of gene expression modulation. Through its interaction with the

H3K9 histone methyltransferase SetDB1, KAP1 can promote transcriptional silencing. Alternatively, it can modulate transcriptional or transcript levels, either regulating the release of RNA polymerase II proximal pausing from the promoter (especially at genes encoding for lncRNAs (Bunch et al., 2016)), or as part of the 7SK complex (McNamara et al., 2016). This is a ribonucleoprotein complex with roles both at the promoter and in the transcriptional termination of several genes, including several lncRNAs (Castelo-Branco et al., 2013).

To gain an insight into the mechanism by which KAP1 regulates Tsix levels, we have analysed KAP1 distribution along *Tsix* regulatory regions. Consistent with a function during the choice, we could not detect KAP1 on any of the regions examined in mESCs. Instead, we found that KAP1 was specifically recruited to *Xist* P2 promoter at the onset of differentiation, around the time when Tsix levels are boosted (Fig. 5A). Taking advantage of the Fa2L cells, we could also determine that KAP1 associates preferentially with *Xist* P2 of the future Xa (*castaneus* allele, Fig. 5B and EV4F). KAP1 and RIF1 occupy, therefore, the same region, but with complementary spatial (Xa versus Xi) and temporal dynamics (KAP1 appears on *Xist* P2 on the Xa when RIF1 leaves it). In order to understand if these events are coordinated, we have investigated whether RIF1 regulates KAP1 association with *Xist* P2. We found that *Rif1* deletion leads to KAP1 binding to *Xist* promoter, even in undifferentiated cells (Fig. 5C). This is not due to a general increase of *Kap1* expression (Fig. EV1A), KAP1 protein levels (Fig. EV5A) or its overall binding to chromatin (Fig. EV5B). Moreover, KAP1 enrichment is specific for *Xist* promoter, as other regions known to be associated with KAP1 that we have tested, like *Zfp629* (Fig. 5C and our unpublished observation) (Ding et al., 2018), did not show an increased KAP1 association upon *Rif1* deletion. Importantly, the effect of RIF1 deficiency is unlikely to be due to an indirect, general

remodelling of the *Xist* promoter chromatin, as the association of another P2-specific transcription factor and *Xist* activator, Yin-Yang-1 (YY1) (Makhlouf et al., 2014), is unchanged in *Rif1* knockout cells (Fig. EV5C). We also found that knocking down Rif1 in undifferentiated Fa2L cells (Fig. EV5D) facilitates KAP1 association to *Xist* P2 (Fig. 5D) comparably to what happens in *Rif1* conditional cells upon induction of *Rif1* deletion (Fig. 5C). Specifically, KAP1 gains access to the future Xi (*129* allele, carrying the truncated *Tsix* allele), where normally RIF1 is preferentially localised (Fig. 3D and E). Overall, these data indicate that, in mESCs, RIF1 is symmetrically associated with *Xist* P2 on both X chromosomes, protecting P2 from the binding of KAP1. Upon triggering differentiation, the bi-allelic increase of Tsix levels weakens RIF1 association with DNA, facilitating the transition of RIF1 to an asymmetric association with one of the two *Xist* promoters, the future Xi, and the consequent association of KAP1 with the other *Xist* promoter, on the future Xa. This event, in turn, sustains the KAP1-dependent increase of Tsix levels that precedes the switch to *Tsix* mono-allelic expression and the choice, further reinforcing RIF1 exclusion from P2 on the future Xa.

KAP1 recruits the 7SK complex to Tsix terminator
The timing of recruitment, the RIF1-dependent regulation and the preferential enrichment on the future Xa support the idea that KAP1 functions by promoting the choice, possibly *in cis*. The association of KAP1 with *Xist* P2 promoter on the future Xa suggests that KAP1 could repress *Xist*. However, Kap1 knock down does not induce precocious upregulation of *Xist* (Fig. 4A and Fig. EV4A), nor does KAP1 early-association with *Xist* P2 promoter in *Rif1* null mESCs and EBs lead to increased tri-methylation of histone H3K9 (Fig. 6A and B). These observations do not support the hypothesis of KAP1 regulating the choice through *Xist* repression. An alternative

hypothesis is that KAP1 could instead regulate *Tsix* either by controlling its transcriptional termination and, consequently, RNA stability (reviewed in (Peck et al., 2019)), or by promoting the formation of a terminator-promoter positive feedback loop (Tan-Wong et al., 2008), to boost *Tsix* transcription. *Xist* P2 promoter, in fact, overlaps with *Tsix* transcriptional terminator. In support of either of these hypotheses, we have found that, as in the case of KAP1, the 7SK complex component HEXIM1 is also enriched on *Xist* promoter/*Tsix* terminator in *Rif1* knockout mESCs (Fig. 6C), and it is associated with the future Xa in Fa2L cells, in a KAP1-dependent manner (Fig. 6D and E). Overall, these data suggest that KAP1 could promote the choice of the future Xa by sustaining *in cis* the increase of Tsix levels that would stabilise the asymmetric RIF1 distribution.

DISCUSSION

While marsupials have adopted an imprinted X inactivation strategy, eutherians have evolved a mechanism based on the random choice of the X chromosome to be inactivated. The latter can contribute to a higher degree of resistance of females to pathogenic X-linked mutations and increase phenotypic diversity. Despite its importance, the mechanisms guiding the random choice are still unclear, partially because of the randomness and consequent heterogeneity in the cell population, partially because of the inaccessibility of the early embryos, where the process takes place naturally and, finally, because of the inherent difficulty of identifying asymmetry involving two identical chromosomes.

Several lines of evidence suggest that *Tsix* is involved in the choice-making process. For example, introduction of a stop codon that blocks Tsix transcript before its overlap with *Xist* (Luikenhuis et al., 2001), or deletions of its major promoter

(Vigneau et al., 2006), or of the GC-rich repeat region that immediately follows it (Dxpas34) (Lee & Lu, 1999), or insertion of a gene trap in the same region, that abolishes the production of Tsix RNA (Sado et al., 2001), result in a non-random choice, with the *Tsix*-defective chromosome as the future Xi. Moreover, monoallelic downregulation of Tsix levels by deleting *Xite*, a *cis*-acting element that positively regulates *Tsix*, also skews the choice (Ogawa & Lee, 2003). Interestingly, Xist itself can influence the choice, in a yet-to-be-understood feedback control loop. *Xist* ectopic up-regulation can in fact skew the choice in favour of the *Xist*-overexpressing chromosome (Nesterova et al., 2003, Newall et al., 2001).

Our experiments show that RIF1 association with the *Xist* P2 promoter is negatively regulated by *Tsix* expression or RNA levels. Tsix could therefore be the determinant of the asymmetric association of RIF1 with the future Xi at the choice. We would like to propose a model (Fig. 7) whereby, at the onset of differentiation, the transient, bi-allelic increase of Tsix levels will promote a weaker or more dynamic association of RIF1 with *Xist* P2, thus creating a window of opportunity for KAP1 stochastic association with either allele. The KAP1-bound allele will go on to sustain higher Tsix steady state levels *in cis*, thus skewing RIF1 association with the opposite allele, and initiating a self-reinforcing loop on the future Xa. On the future Xi, RIF1 will promote *Xist* upregulation, thus establishing the inactivation. The negative effect of RIF1 on KAP1 association with *Xist* promoter in ESCs is at the heart of the mutual exclusion, reinforced by KAP1's positive effect on the levels of Tsix, that is, in turn, a negative regulator of RIF1 association with *Xist* promoter. How RIF1 excludes KAP1 is currently unclear, but we can envisage at least two potential mechanisms, based either on RIF1/KAP1 competition for binding to a shared site, RNA or protein partner, or through KAP1 de-phosphorylation by RIF1-associated PP1. Phosphorylation of KAP1 has

indeed been shown to regulate KAP1 association with heterochromatin protein 1 (HP1) (Chang et al., 2008).

In support of our model, we have shown that, the association of KAP1 with the P2 region upon differentiation coincides with the detection of higher levels of Tsix RNA (Fig. 5A), and this increase is dependent upon KAP1 (Fig. 4C and D). The molecular mechanism by which KAP1 modulates Tsix levels is currently unknown. The data presented here suggest that KAP1 could modulate *in cis Tsix* transcriptional up-regulation, termination and/or RNA stability through the 7SK complex. Finally, we cannot exclude a model where KAP1 promotes Tsix increase *in trans*, through a yet unknown differentiation-induced factor. In this case, the association of KAP1 with *Xist* P2 could contribute *in cis* to the identification of Xa, by establishing a stable repression of *Xist* promoter, with RIF1 shielding the future Xi by excluding KAP1. Although our data do not support the hypothesis of KAP1-dependent silencing of *Xist* (Fig. 4A and Fig. EV4A) through H3K9me3 (Fig. 6A and B), KAP1 could promote repression through a different mechanism, for example DNA methylation (Coluccio et al., 2018).

Our data show that the increase of Tsix that precedes and, possibly, leads to a proficient choice, requires KAP1. It has been previously shown that failure to set up the choice as a consequence of homozygous deletion of *Tsix,* leads to a mixture of cells showing either no *Xist* up-regulation or bi-allelic up-regulation during differentiation (Lee, 2002, Lee, 2005). This is different from what we observe in Kap1 knock down cells, where we detect defective *Xist* up-regulation, but not bi-allelic expression. Nonetheless, a situation where, from the start of the process in ESCs, Tsix is always absent, as in the case of *Tsix*^{-/-}*,* is clearly different from the system where Tsix levels remain physiological until differentiation is triggered, as in the case of Kap1 knock down (Fig. 4D).

The early embryonic lethality of *Rif1*^{-/-} females described here contrasts with the milder effect of *Xist* conditional inactivation in the epiblast described previously (Yang et al., 2016). However, beside the technical differences between a conditional system, where the efficiency of the deletion can be lower than 100%, and a knockout, RIF1 has at least two other key roles, in the regulation of the replication-timing program (Cornacchia et al., 2012, Foti et al., 2016, Hayano et al., 2012, Yamazaki et al., 2012) and replication fork protection (Buonomo et al., 2009, Garzon et al., 2019). In fact, depending on the genetic background, most or some of the male embryos also die, although later during development (this work). We cannot therefore exclude that the early female lethality could derive from a synthetic effect of multiple problems, added on top of the failure of X inactivation.

In summary, we propose that, during the stochastic phase of the choice of the future Xi, Tsix-dependent destabilisation of the symmetric association of RIF1 with *Xist* P2 promoter sets in motion the establishment of two, mutually exclusive circuits that will identify Xi and Xa. RIF1's presence on P2, inhibiting KAP1 and promoting *Xist* expression will identify the future Xi. On the other allele, KAP1's presence on P2, sustaining Tsix levels and, thus, helping to exclude RIF1, will identify the Xa. The initial stochastic binding of KAP1 will thus become a binary switch, where a bi-stable, self-sustaining circuitry on the two X chromosomes is propagated.

MATERIALS AND METHODS

mESC differentiation

Wild type ESCs were plated onto non-coated petri dishes at a concentration of 1×10^6 cells/ 10 cm^2, in a volume of 10 ml medium lacking 2i and LIF. At day 4 of differentiation the aggregated embryoid bodies (EBs) were gently transferred to gelatinised tissue culture dishes. Medium was gently changed every 48 hours with minimal disruption of the EBs. EBs were grown for up to 4 or 7 days in total. In experiments where cell

differentiation was combined with *Rif1* deletion, the differentiation was preceded by 48 hours of 4-hydroxytamoxifen (OHT, #H7904, Sigma-Aldrich) treatment, at a concentration of 200 nM in ES medium containing LIF and 2i. Differentiation was then started with $2x10^6$ cells/ $10cm^2$ dish for *Rif1*$^{+/+}$ and $2.5x10^6$ cells/ $10cm^2$ for *Rif1*$^{F/F}$ cells in medium lacking 2i and LIF but containing 200 nM OHT. At 1 day of differentiation, medium was replaced with medium without OHT. At day 4 of differentiation, the EBs were transferred to gelatinised tissue culture dishes as above.

KAP1, RIF1 and HEXIM1 ChIP

Chromatin immunoprecipitation was performed according to(Bulut-Karslioglu et al., 2012). Briefly, for RIF1, KAP1 and HEXIM1 ChIP, collected cells were first crosslinked using 2mM disuccinimidyl glutarate (DSG, # BC366 Synchem UG & Co. KG) in PBS for 45 min. at RT while rotating, washed twice in PBS, followed by 10 min. of additional crosslinking in 1% formaldehyde (#252549, Sigma-Aldrich) in cross-linking buffer (50 mM HEPES pH 7.8, 150 mM NaCl, 1 mM EDTA and 500uM EGTA) at RT. Crosslinking was followed by 5 min. quenching in 0.125 M glycine at RT, washed twice in cold PBS and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, supplemented with protease inhibitor cocktail, #11873580 001, Roche). Chromatin fragmentation was performed using Soniprep 150 to produce a distribution of fragments enriched between 300 and 400 bp. The lysate was precleared by centrifugation at low speed 400 g for 20 min at 4 °C. Chromatin was quantified using Qubit dsDNA High Sensitivity assay kit (#Q32854, Life Technologies). Immunoprecipitation was performed by incubating 100 µg of chromatin diluted in 10 volumes of Dilution buffer (1% Triton X-100, 2 mM EDTA, 167 mM NaCl, 20 mM Tris-HCl pH 8.1, including Protease Inhibitor) overnight rotating at 4 °C together with either α-KAP1, α-RIF1 or α-HEXIM1 antibodies (see Appendix Table S2) or IgG only control (#sc-2026, Santa Cruz), 10% of chromatin was isolated as input control. The following day, 50 µl of Dynabeads protein G slurry (#10004D, Thermo Fisher) per ChIP sample was added and incubated rotating for another 2 hours at 4 °C. The beads were magnet-separated and washed twice with Low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH8.1), one time each with High salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl pH8.1), LiCl buffer (0.25M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM

EDTA,10 mM Tris-HCl pH 8.1) and finally TE. Each wash was performed for 5 min. on a rotating wheel at 4 °C and all buffers were supplemented with protease inhibitor cocktail (#11873580 001, Roche). Prior to elution, samples were rinsed once in TE without protease inhibitor. ChIP-DNA was eluted from the beads by rotating at RT for 1 hour in Elution buffer (1% SDS, 100 mM NaHCO$_3$). Beads were separated and the supernatants as well as input samples were subjected to RNAse A (#R5250, Sigma-Aldrich) treatment (37.5 μg/sample) for 1 hour at 37 °C followed by decrosslinking using Proteinase K (#P6556, Sigma-Aldrich) treatment (45μg/sample) overnight at 60 °C. The following day, ChIP-DNA and input samples were purified using ChIP DNA Clean and Concentrator kit (#D5205, Zymo Research) and retrieved DNA as well as input DNA was quantified using Qubit dsDNA High Sensitivity assay kit (#Q32854, Life Technologies). The concentration of ChIP-DNA and input samples were adjusted to maintain a similar ratio of ChIP-DNA:INPUT between different ChIP experiments. qPCRs were performed using the SYBR Green reaction mix (#04887352001, Roche) on a LightCycler 96 Instrument (Roche), following standard protocols. Enrichments over input control were calculated for each respective primer set. Primer sequences are presented in Appendix Table S3.

RNA extraction, reverse transcription and RT-qPCR

Frozen cell pellets were lysed and homogenised using QIAshredder column (#79656, QIAGEN) followed by RNA extraction using the RNeasy kit (#74106, QIAGEN) according to the manufacturer's instructions. On column-DNAse treatment was performed at 25 °C-30 °C for 20 min. using RQ1 RNase-Free DNase (#M6101, Promega). After elution, a second round of DNAse treatment was performed using 8U of DNase/sample, incubated at 37 °C for 20 min. The reaction was terminated by adding 1μl of RQ1 DNase Stop Solution and incubated at 65 °C for 10 min. RNA was quantified using Nanodrop, and cDNA synthesis was performed using RevertAid H Minus First Strand cDNA kit (#K1632, Thermo Scientific) using random hexamer priming. qPCRs were performed using the SYBR Green reaction mix (#04887352001, Roche) on a LightCycler 96 Instrument, following standard protocols. Gene expression data was normalised against a geometric mean generated by RT-qPCR of either: *Gapdh*, *Ubiquitin* and *β-Actin* or *Rplp0*, *Ubiquitin* and *Sdha*. For flavopiridol or triptolide

treated cells, gene expression levels were normalised against *18S* ribosomal RNA. Primer sequences are presented in Appendix Table S4.

Acknowledgments

Author Contributions

EE has created the cellular system, performed the majority of the experiments and co-written the manuscript. RF initiated the project and performed some of the early experiments, like the staining of E3.5 embryos. LP has performed some of the ChIP experiments, the triptolide treatment and the Luciferase assay. LB, AC and NBR have performed KAP1 KD, RNA FISH and the analysis. GK has analysed the RNA seq data, supervised by MV. NBR was supervised by AC, who also critically read the manuscript. FC and AP have isolated and stained the E5.5 embryos. SBCB has conceived the project, performed some of the experiments and written the manuscript.

Conflict of interest

The authors declare no competing interests.

Data availability section

The RNA-seq are available at
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165704

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FIGURE LEGENDS

Figure 1. *Rif1* deficiency leads to female embryonic lethality at peri-implantation.

(A, B). Tables summarising the number and the sex of the pups recovered at weaning from *Rif1*$^{+/-}$ x *Rif1*$^{+/-}$ mice inter-crosses, either in a C57BL/6J **(A)** or in a mixed C57BL/6J-129/SvJ genetic background **(B)**. The observed number of mice is compared to the expected number, based on the Mendelian ratio. *p* calculated by χ^2.

(C). The table summarises the number and the sex of the embryos of the indicated genotypes, recovered from timed matings of *Rif1*$^{+/-}$ x *Rif1*$^{+/-}$ mice, in a C57BL/6J genetic background. The day of gestation (E) is indicated. **(D).** Representative images of *Rif1*$^{-/-}$ E7.5 embryos, female top and male bottom.

Figure 2. *Rif1* null female mESCs fail to upregulate *Xist* upon differentiation.

(A). Overview of the experimental design. *Rif1*$^{+/+}$ and *Rif1*$^{F/F}$ mESCs were grown for two days in medium supplemented with 4-Hydroxytamoxifen (OHT) to induce the translocation into the nucleus of the Cre-recombinase, leading to *Rif1* deletion in the Rif1$^{F/F}$ cells (*Rif1*$^{-/-}$). The embryoid body (EB) differentiation protocol was then started to trigger XCI. OHT was kept in the medium during the first 24 hours of EB differentiation. Cells were differentiated up to 4 (RNA analysis) or 7 days (H3K27me3 IF).

(B). Representative western blot to monitor RIF1 levels after Cre-mediated *Rif1* deletion and EB differentiation. SMC1: loading control.

(C). Time course analysis of Xist RNA expression by RT-qPCR during EB differentiation of *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT) and *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT) cells at the indicated timepoints. *Rif1*$^{+/+}$ (solid line) and *Rif1*$^{-/-}$ (dashed line), female (black) and male (grey). Data are presented as mean ± standard deviation from three (female lines) or two

(male lines) independent experiments. Statistical significance was determined using 2-way ANOVA comparing female *Rif1*^{+/+} to female *Rif1*^{-/-} cell lines (****p ≤ 0.0001). Xist RT-primers Xist ex7 F and R were used. Values are normalised to a geometric mean consisting of the expression of *Gapdh*, *Ubiquitin* and *β-Actin*. **(D).** Bar plot summarising the number of cells showing H3K27me3-marked Xi as a percentage of total cells counted, in *Rif1*^{+/+} (*Rif1*^{+/+} +OHT) and *Rif1*^{-/-} (*Rif1*^{F/F} +OHT) female mESCs at the indicated days of EB differentiation. Averages ±standard deviation from three (day 4 and 7) and two (day 2) independent experiments (n>200).

Figure 3. RIF1 associates with *Xist* promoter on the future Xi.

(A, B). RIF1 association with the *Xist* promoter assessed by ChIP-qPCR in two independent *Rif1*^{+/+} (*Rif1*^{+/+} +OHT, black) and two *Rif1*^{-/-} (*Rif1*^{F/F} +OHT, grey) female cell lines, in ESCs **(A)** and at 2 days of EB differentiation **(B).** P1 and P2 indicate the two *Xist* promoters, 5' indicates a region 2 kb upstream of *Xist* TSS. Inter1 and 2 are two intergenic regions that serve as negative controls. Peak and cRAD represent two previously identified regions of RIF1 association (positive control). See Appendix Fig. S2C for primer positions within Xist. Mean ±standard deviation from 3 independent experiments (A) and 2 independent experiments (B). *p* calculated by Student's two-tailed, paired *t* test comparing RIF1 association in *Rif1*^{+/+} cells on Xist P2 and P1 versus 5'. *p ≤ 0.05, ****p ≤ 0.0001 and ns=not significant.

(C). *Rif1* deletion decreases the efficiency of upregulation of a *Luciferase* reporter under the control of *Xist* promoter (Xist-2p-luc), at 2 days of EB differentiation. As a control (Luc), empty luciferase reporter vector, was transfected in parallel. The average of three independent experiments is shown. Error bars indicate the standard deviation. *p* calculated by Student's two-tailed, unpaired *t* test, for comparison of fold

activation of Xist-2p-Luc normalised to empty vector (Luc) in $Rif1^{+/+}$ versus $Rif1^{-/-}$ cells. **$p \leq 0.01$. See Appendix Material and Methods for details about the normalisation.

(D). Association of RIF1 with *Xist* P2 in the Fa2L cells (black) and a wild type female mESC line (grey), also harbouring one *castaneus* and one *129* X chromosome. Allele-specific ChIP-qPCR primers were used, *cast* indicates association with the *castaneus Xist* P2 and *129* indicates association with the *129 Xist* P2. Enrichments are presented relative to input DNA. Mean ±standard deviation from 3 independent experiments. *p* calculated by Student's two-tailed, paired *t* test comparing RIF1 association with the *castaneus* and with the *129* X chromosome Xist P2, ***$p \leq 0.001$. Below, schematic of the *Xist/Tsix* alleles in the Fa2L undifferentiated cells.

(E). Association of RIF1 with *Xist* P2 in the Fa2L cells (black) upon differentiation. The analysis was performed as in (D). Mean ±standard deviation from 3 independent experiments. *p* calculated by Student's two-tailed, paired *t* test comparing RIF1 association with the *castaneus* and with the *129* X chromosome Xist P2. *$p \leq 0.05$. Below, schematic of the *Xist/Tsix* alleles in the Fa2L differentiated cells.

(F). Quantification by ChIP-qPCR of RIF1 association with the indicated regions in the Fa2L cell line, following treatment with DMSO only (black) or flavopiridol (grey). Primers as in (E).

(G). Same as in **(F)** but for a wild type female mESC line. All enrichments are presented relative to input DNA. Mean ±standard deviation from three **(F)** and two **(G)** independent experiments are presented. Statistical significance was determined using Student's two-tailed, paired *t* test (*$p \leq 0.05$, **$p \leq 0.01$ and ns=not significant).

Figure 4. KAP1 regulates the Xa/Xi choice through Tsix

(A). Time course analysis of *Xist* expression by RT-qPCR during EB differentiation of female mESCs following knock down of Luciferase (Control, black) and Kap1 (Kap1 KD, grey), at the indicated timepoints. Data are presented as mean ±standard deviation from three independent experiments. Statistical significance was determined using 2-way ANOVA. *Xist* primers Xist ex3 F and Xist ex4 R were used. Normalisation was performed using a geometric mean consisting of the expression of *Rplp0, Ubiquitin* and *Sdha*. (*$p \leq 0.05$).

(B). RT-qPCR analysis of *Xist* expression levels during differentiation of the Fa2L cells, following expression of shRNA against Luciferase (Control, black) and Kap1 (Kap1 KD, grey), at the indicated timepoints. Mean ±standard deviation from a minimum of three independent experiments is presented. 2-way ANOVA was used to determine statistical significance. ns=not significant.

(C). Tsix RNA levels in female mESCs infected with shRNA directed against Luciferase (Control, black) and KAP1 (Kap1 KD, grey), during differentiation. Mean ±standard deviation from four independent experiments are shown. Statistical significance was determined using 2-way ANOVA. (**$p \leq 0.01$). Values have first been normalised to a geometric mean consisting of the expression of *Rplp0, Ubiquitin* and *Sdha*.

(D). RNA FISH analysis of *Tsix* expression during differentiation of female mESCs expressing an shRNA directed against Luciferase (control) or against Kap1 (Kap1 KD). Left: Cells with no (0, red), one (1, grey) and two or more (2, black) Tsix foci were counted in two independent experiments, shown averaged. Statistical significance was determined by χ^2. A minimum of 110 cells were counted per each time point for each line (**$p \leq 0.01$, ****$p \leq 0.0001$). Right: examples of cells with one (top) or two (central and bottom) Tsix FISH signals. Scale bars: 5µm.

Figure 5. RIF1 negatively regulates KAP1 association with *Xist* P2.

(A). ChIP-qPCR analysis of KAP1 association with the indicated sites in wild type female mESCs (*Rif1⁺/⁺,* same as used in Fig. 3B but without OHT) and during early differentiation. ZFP629 is a well-characterised KAP1 associated region (positive control). Xite A and C indicate two regions within the Tsix enhancer *Xite*, Tsix region 1 indicates Tsix major promoter, Tsix region 2 indicates the Dxpas34 region, Tsix region 3 indicates a region slightly downstream of the Dxpas34 region. P1 and P2 indicate the two *Xist* promoters, 5' indicates a region 2 kb upstream of *Xist* TSS. Inter1 is an intergenic region. See Appendix Fig. S2C for the positions of the primers within *Xist* and *Tsix*. The data are presented as mean ±standard deviation from three (2d EB and 1d EB) and two (ESCs) independent experiments. Statistical significance was calculated by Student's two-tailed unpaired *t* test comparing RIF1 association to Xist P2 and P1 in 2d EB versus 1d EB (*$p \leq 0.05$ and ns=not significant). In the inset, Tsix RNA levels were quantified by RT-qPCR during the differentiation of wild type female ESCs shown in Fig. 4F. The average of two experiments is shown. Tsix values are normalised to a geometric mean consisting of the expression of *Rplp0, Ubiquitin* and *Sdha.* Error bars indicate standard deviations.

(B). Using allele-specific primers, ChIP-qPCR was used to measure the association of KAP1 with *Xist* P2 in the Fa2L cells (black) and a wild type female mESC line also harbouring one *castaneus* and one *129* X chromosome (grey). *cast* indicates association with the *castaneus Xist* P2 and *129* indicates association with the *129 Xist* P2. Enrichments are presented relative to input DNA. Mean ±standard deviation from a minimum of three independent experiments. Statistical significance was determined

by Student's two-tailed, paired *t* test. Below, schematic of the *Xist/Tsix* alleles in the Fa2L cells.

(C). KAP1 association with *Xist* promoter in two independent *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT, black) and two *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT, grey) female mESC cell lines. *Ezr* is an additional region known to be associated with KAP1 in mESCs. Enrichments are presented relative to input DNA. Mean ±standard deviation from a minimum of three independent experiments per cell line are displayed. Statistical significance was determined using Student's two-tailed, unpaired *t* test comparing the KAP1 association with Xist P2 and P1 in *Rif1*$^{+/+}$ versus *Rif1*$^{-/-}$ cells (**p ≤ 0.01).

(D). Allele-specific KAP1 association with *Xist* P2 in Fa2L cells following knock down of Luciferase (Control, black) and Rif1 (Rif1 KD, grey). *cast* indicates association with the *castaneus Xist* P2 promoter and *129* indicates association with the *129 Xist* P2 promoter. Enrichments are presented relative to input DNA. Average ±standard deviation of two independent experiments.

Figure 6. KAP1 recruits the 7SK complex to the *Xist* P2 promoter/*Tsix* terminator of the future Xa.

(A, B). H3K9me3 association with the *Xist* promoter (P1 and P2) in two independent *Rif1*$^{+/+}$ and *Rif1*$^{-/-}$ cell lines, analysed by ChIP-pPCR in mESCs **(A)** and 2d EBs **(B)**. Myf5 serves as a positive control region, β actin negative. Average ±standard deviation of two independent experiments.

(C). HEXIM1 association with the *Xist* promoter (P1 and P2) in two independent *Rif1*$^{+/+}$ and two *Rif1*$^{-/-}$ mESC lines, analysed by ChIP-qPCR. Tkbp1 and Myf5 are two control regions. As in the case of KAP1 association, deletion of *Rif1* induces accumulation of HEXIM1 on *Xist* P1 and P2. Average ±standard deviation of two independent

Figure 7. Model for RIF1 and KAP1-dependent bookmarking of Xi and Xa respectively.

The low bi-allelic expression of *Tsix* in mESCs allows the association of RIF1 with P2 on both *Xist* alleles. However, the presence of pluripotency-dependent inhibitors will not allow *Xist* up-regulation, despite the presence of RIF1. (1) Upon differentiation, the increase of Tsix levels weakens the association of RIF1 with P2. This opens the opportunity for a stochastic KAP1 binding to P2 of one of the two alleles (2). KAP1 is required for sustained high levels of Tsix, further reinforcing RIF1 exclusion from the KAP1-bound/Tsix high allele and establishing the asymmetry. It is not known whether KAP1 gains access to P2 to promote the increase of Tsix levels first, or whether the increase of Tsix levels is initially triggered by a differentiation-dependent factor. (3). The pluripotency Xist inhibitors having been silenced, RIF1 promotes *Xist* expression on the future Xi. A self-sustainable binary switch is thus created and it consolidates the choice of the future Xi and Xa.

EXPANDED VIEW FIGURE LEGENDS

Figure EV1. Transcriptome analysis in *Rif1* null ESCs and early-stage EBs.

(A). Volcano plot summarising the top differentially expressed genes in *Rif1⁻/⁻* (*Rif1^F/F*
+OHT) ESCs (left) and EBs (right), compared to *Rif1⁺/⁺ (Rif1⁺/⁺*+OHT). Two
independent cell lines were analysed per genotype. In red, genes whose differential
expression is statistically significant (FDR<0.05). *Trim28* (*Kap1*) expression is not
upregulated in *Rif1* null cells, whilst *Xist* expression levels are significantly down-
regulated in EBs.

(B). Heat-maps summarising the logarithmically transformed, normalised expression
levels of randomly-chosen X-linked genes in *Rif1⁺/⁺* and *Rif1⁻/⁻* ESCs (brown) and 2
days EBs (blue). Failure to up-regulate *Xist* in *Rif1⁻/⁻* EBs is specific, as other X-linked
genes do not show the same behaviour. *Rlim* (*Rnf12*) expression, for example, is not
affected by *Rif1* deletion (highlighted, along with Xist, in the red box).

(C). Pie charts summarising the biological processes (GO enrichment analysis,
GOrilla) most represented among the genes whose expression is significantly
deregulated upon *Rif1* deletion in ESCs (top) and EBs (bottom). Differentially
expressed genes were obtained from the DESeq2 (adjusted p-value, 0.05 and
log2FCl>0.5). Genes linked to developmental processes (GO5) represent only a small
percentage of differentially expressed genes (about 50% up-regulated and 50% down-
regulated).

Figure EV2. *Rif1⁻/⁻* mESCs can exit pluripotency and commit to differentiation.

(A). Xist RNA levels monitored by RT-qPCR in two independent *Rif1⁺/⁺* (black) and
three independent *Rif1^F/F* (grey) female mESC lines, during EB differentiation, in the

absence of OHT. Xist RT-primers Xist ex7 F and R were used. Values are normalised to a geometric mean consisting of the expression of *Gapdh, Ubiquitin* and *β-Actin.* Expression levels are plotted as mean ±standard deviation of a minimum of three individual experiments with statistical significance determined using 2-way ANOVA. ns=not significant.

(B, C). Time course analysis of expression levels for the indicated genes, during EB differentiation of two individual *Rif1$^{+/+}$* (*Rif1$^{+/+}$* +OHT, black) and three individual *Rif1$^{-/-}$* (*Rif1$^{F/F}$* +OHT*,* grey) female mESC lines*.* Expression levels are first normalised to a geometric mean consisting of the expression of *Gapdh, Ubiquitin* and *β-Actin* and then plotted relative to pre-samples (day -2), as mean ±standard deviation of three or four individual experiments with statistical significance determined using 2-way ANOVA (**$p ≤ 0.01$, ***$p ≤ 0.001$, ****$p ≤ 0.0001$ and ns=not significant) comparing *Rif1$^{+/+}$* to *Rif1$^{-/-}$* cells. **(B)**. Pluripotency-associated genes and **(C)** genes expressed during early-stages of differentiation.

(D). The analysis of gene expression in *Rif1* wild type and null female ESCs and EBs shows that the data cluster first according to the cell type (ESCs versus EBs, PC1) and then according to the genotype (*Rif1$^{+/+}$* versus *Rif1$^{-/-}$*, PC2). The wider distance between the gene expression data of wild type and *Rif1* null EBs as compared to ESCs is in agreement with the mixture of cell types that is characteristic of EBs.

(E). The analysis of the expression of key lineage markers in *Rif1* wild type and null female cells, both ESCs and EBs, shows that *Rif1* null cells differentiate in all 3 lineages, with some individual genes displaying some degree of variable level of expression.

Figure EV3. Inhibiting Tsix transcription in Fa2L cells reverts RIF1 association with P2 from asymmetric to symmetric.

(A, B). Tsix RNA levels following flavopiridol **(A)** and triptolide **(B)** treatment, relative to DMSO treated Fa2L cells. RNA levels were first normalised to *18S* ribosomal RNA and plotted as mean from three individual experiments ±standard deviations. Statistical significance was determined using Student's two-tailed unpaired *t* test (****$p \leq 0.0001$).

(C). Allele-specific RIF1 association with *Xist* P2 in Fa2L cells following treatment with DMSO only (black) or triptolide (grey). *cast* indicates association with the *castaneus Xist* P2 promoter and *129* indicates association with the *129 Xist* P2 promoter. Enrichments are presented relative to input DNA. Mean ±standard deviation of three independent experiments. *p* calculated by Student's two-tailed paired *t* test. (*$p \leq 0.05$).

Figure EV4. Defective *Xist* upregulation in KAP1 knockdown cells is not due to lack of differentiation.

(A). Quantification of Xist cloud formation (1 or 2 clouds, open or filled bars, respectively) by RNA FISH in Control and Kap1 KD female mESCs, differentiated into EBs for 3 days. The average of two experiments is shown. N>400 cells. The error bars represent ± standard deviation. *p* was calculated by χ^2 (***$p \leq 0.001$). Below, an example of Xist cloud RNA FISH signal. Green=Xist, blue=DAPI.

(B, C). Time course analysis of gene expression levels quantified by RT-qPCR during EB differentiation of female mESCs infected with shRNA directed against Luciferase (control-black) and Kap1 (grey), at the indicated timepoints. Values have first been normalised to a geometric mean consisting of the expression of *Rplp0, Ubiquitin* and

Sdha and then presented relative to pre-samples (day: -4) Mean ±standard deviation from three independent experiments are shown. Statistical significance was determined using 2-way ANOVA (*$p \leq 0.05$, **$p \leq 0.01$ and ns=not significant). **(B).** pluripotency-associated genes and **(C)** genes expressed during early-stages of differentiation.

(D). Representative western blot analysis of RIF1 and KAP1 levels in Kap1 KD female mESCs. SMC1: loading control. Quantification of RIF1 and KAP1 protein levels normalised to SMC1 and relative to control cells are shown below.

(E). Representative western blot analysis of RNF12 levels in proteins extracted from female mESC infected with shRNA directed against Luciferase (control) and Kap1, at the indicated timepoints during EB differentiation. LAMIN B1 (LMNB1): loading control. Below, quantification of RNF12 protein levels relative to day 0 of control and Kap1 KD cells respectively. Values normalised to LMNB1.

(F). Using allele-specific primers, ChIP-qPCR was used to analyse the association of KAP1 with *Xist* P2 in differentiating Fa2L cells. *cast* indicates association with the *castaneus Xist* P2 and *129* indicates association with the *129 Xist* P2. Enrichments are presented relative to input DNA. Mean ±standard deviation from a minimum of three independent experiments. *p* was calculated by Student's two-tailed paired *t* test (*$p \leq 0.05$).

Figure EV5. The overall binding of KAP1 to chromatin is unaffected in *Rif1*$^{-/-}$ mESCs.

(A). RIF1 and KAP1 levels analysed by western blot in protein extracts from two *Rif1*$^{+/+}$ (*Rif1*$^{+/+}$ +OHT) and two *Rif1*$^{-/-}$ (*Rif1*$^{F/F}$ +OHT) independent female mESC lines, following 2 days of OHT treatment and at 2 days EB. SMC1: loading control. Below,

quantifications of RIF1 and KAP1 protein levels shown as relative levels compared to one of the *Rif1^+/+* cells. Values normalised to SMC1.

(B). Western blot analysis of KAP1 levels in protein extracts from the indicated cell fractions, from two *Rif1^+/+* (*Rif1^+/+* +OHT) *and* two *Rif1^-/-* (*Rif1^F/F* +OHT) independent female mESC lines, following 2 days of OHT treatment. β-TUBULIN: marker for the cytosolic fraction. LMNB1 and histone H2A: markers for the chromatin/insoluble fraction. Below, quantifications of KAP1 protein levels detected in the chromatin fractions shown as relative levels compared to one of the control cells. Values normalised to H2A.

(C). YY1 association with the *Xist* promoter in two independent *Rif1^+/+* (*Rif1^+/+* +OHT, black) and two *Rif1^-/-* (*Rif1^F/F* +OHT, grey) female mESC lines, analysed by ChIP-qPCR. Ex1 indicates a region within Xist exon 1, 2.5 kb downstream of *Xist* transcriptional start site (TSS), P2 indicates *Xist* promoter P2 spanning the YY1 consensus motif. Peg3 indicates a known YY1-associated site on the *Peg3* gene. Mean ±standard deviation from two independent experiments is presented.

(D). Western blot analysis of RIF1 levels in protein extracts from Fa2L cells after Rif1 knock down. SMC1: loading control. Below, quantification of RIF1 protein levels compared to control cells.