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Dosage Sensing, Threshold Responses, and Epigenetic Memory: A Systems Biology Perspective on Random X-Chromosome Inactivation

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X-chromosome inactivation ensures dosage compensation between the sexes in mammals by randomly choosing one out of the two X chromosomes in females for inactivation. This process imposes a plethora of questions: How do cells count their X chromosome number and ensure that exactly one stays active? How do they randomly choose one of two identical X chromosomes for inactivation? And how do they stably maintain this state of monoallelic expression? Here, different regulatory concepts and their plausibility are evaluated in the context of theoretical studies that have investigated threshold behavior, ultrasensitivity, and bistability through mathematical modeling. It is discussed how a twofold difference between a single and a double dose of X-linked genes might be converted to an all-or-nothing response and how mutually exclusive expression can be initiated and maintained. Finally, candidate factors that might mediate the proposed regulatory principles are reviewed.

1. Introduction

The mammalian X chromosome carries about 1000 genes that mostly fulfill similar functions in males and females. They are however present as two copies in females and only as a single copy in males, resulting in dosage imbalance between the sexes. Mammals have evolved the process of X-chromosome inactivation (XCI), where one X chromosome is nearly completely silenced in females to ensure dosage compensation of X-linked genes. While XCI is imprinted in marsupials such that always the paternal X is inactivated, in placental mammals, each female cell randomly selects one X chromosome that will be silenced during early embryonic development.[1–3] Random XCI makes the female sex more robust to pathogenic X-linked mutations and might contribute to phenotypic diversity.[4]

Initiation of XCI is controlled by the X-inactivation center (*Xic*), a genomic region that encodes the long non-coding RNA (lncRNA) Xist, the master regulator of X inactivation, and a series of other genes that regulate *Xist* (**Figure 1**) (reviewed in ref. [5]). Xist is expressed exclusively from the inactive X chromosome (Xi) and coats the entire chromosome in *cis*. Acting as a scaffold for a multitude of RNA binding proteins and potentially forming a phase-separated compartment, Xist recruits members of different gene-silencing pathways, orchestrating chromosome-wide gene repression.[6–10] A small subset of genes however can resist silencing and thus escape X inactivation.[11,12]

All placental mammals studied so far express Xist from one out of two X chromosomes in female somatic cells, indicating that the outcome of XCI is similar across species. Increasing evidence suggest that at least a subset of cells will initially upregulate Xist from both chromosomes (biallelic), as observed in mice, rabbits, and humans.[15–18] Such biallelic upregulation appears to be less frequent in mice than in rabbits, but is quickly resolved to monoallelic expression in both species.[15–18] In human embryos by contrast, Xist is upregulated from both chromosomes in all cells, but initially fails to induce complete silencing and thus persists for several days.[15,19] At a later developmental time point which has not yet been observed experimentally, Xist must then be downregulated from one allele. Mice have also evolved an imprinted form of XCI, which does not occur in most other mammals, preceding random XCI in the preimplantation embryo and resulting in inactivation of the paternal X chromosome in all cells.[20,21] In the inner cell mass of the blastocyst, which will give rise to the embryo, the imprint is erased and Xist is repressed by pluripotency factors, such as Nanog and Rex1/Zfp42.[22–25] Subsequent downregulation of pluripotency factors derepresses Xist, thereby initiating random X inactivation, where either the maternal or paternal X are inactivated with equal probability. Once random XCI has been established, the inactive state is maintained in all somatic cells throughout all further cell divisions.

Whether XCI is initiated depends only on the number of X chromosomes present in the cell, but not on the Y chromosome. XO females with Turner syndrome do not undergo XCI, while XXY males with Klinefelter syndrome inactivate one of their X

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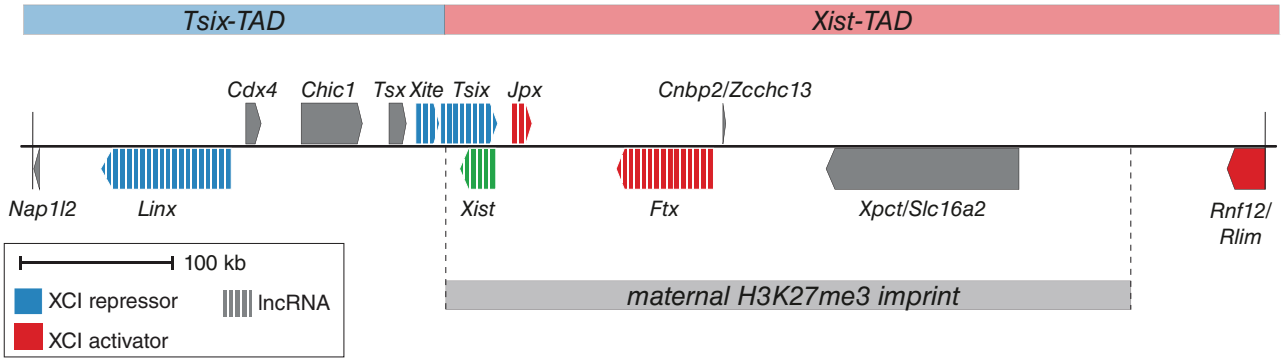


Figure 1. The mouse X-inactivation center (*Xic*). Schematic representation of the region chrX:103184059-103981285 (mm10) in the mouse genome. Genes colored in red and blue indicate Xist activators and repressors, respectively, a striped pattern marks lncRNA genes. Gene annotation and genomic coordinates from UCSC RefSeq mm10, except for Xite (depicted from the minor to the major Tsix promoter) and Linx.

chromosomes.[26] Females with X trisomy (XXX) will even inactivate two X chromosomes, implying that exactly one X chromosome remains active in diploid cells no matter how many X's are present.[27] Studies in polyploid embryos and stem cells have shown that also autosomal ploidy modulates XCI in a way that one X remains active per diploid autosome set.[28–31]

2. X-Chromosome Inactivation Concepts

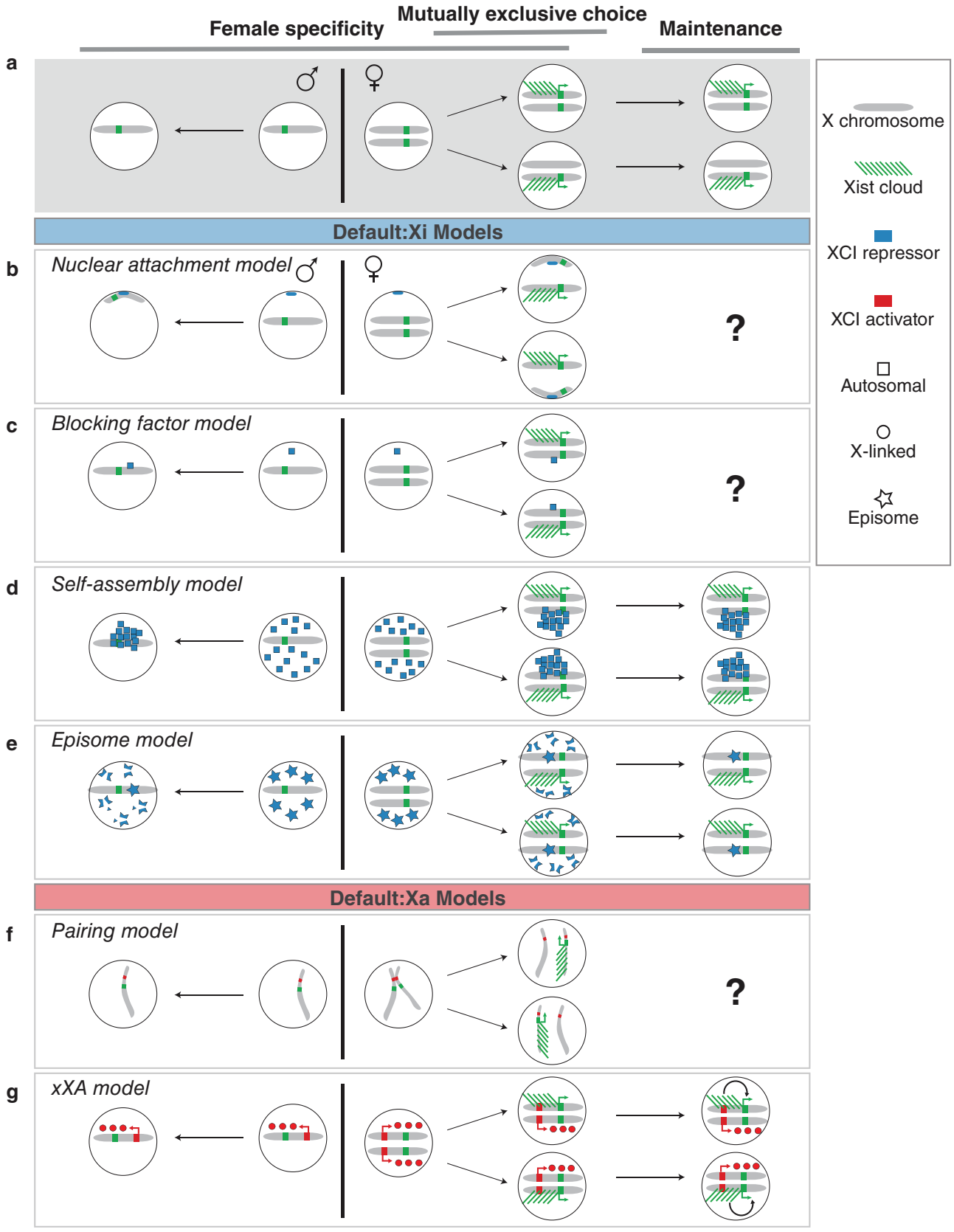
One of the most fascinating aspects of XCI is that two functionally equivalent X chromosomes within the same nucleus assume completely different fates. To this end, a cell must first assess how many X chromosomes it possesses and only initiate XCI if it has more than one (per diploid set of chromosomes). Each cell must then choose one (or more) X to inactivate (Xi) and one X to stay active (Xa). Once this decision has been made, it must be stably maintained (**Figure 2**a). The terms counting and choice have been introduced to describe these processes. Counting is the process that determines how many X chromosomes need to be inactivated while choice refers to the decision of which X to inactivate. Mary Lyon proposed the process of XCI in 1961.[1] In the almost 60 years since she postulated her hypothesis numerous concepts have been developed to explain counting and choice.

2.1. How Can Cells Count Their X Chromosomes to Ensure Female Specificity of XCI?

Which regulatory principles ensure that XCI is initiated only in cells with two or more X chromosomes, but never in males with a single X? In principle, the default fate of the X chromosome could either be to get inactivated (default:Xi, Figures 2b–e and **3**a,b) or to stay active (default:Xa, Figures 2f,g and 3c,d). In the first case, one X in each cell, whether male or female, would have to be protected from XCI, while in the latter, cells would have to sense the presence of two X chromosomes and induce XCI only in females.[32]

Several mechanisms have been proposed to protect the active X in the default:Xi scenario. In early models, often a protective molecule or complex that is present only once in each cell was invoked. This could be a single nuclear attachment site that protects the attached chromosome from XCI (Figure 2b)[33] or a "blocking factor" that is present as a single molecule and can thus bind only one X chromosome (Figures 2c and 3a).[32,34–36] We know today, however, that the precision of gene expression is limited by the laws of thermodynamics that govern all biochemical reactions.[37–39] This makes it impossible to reliably produce a single molecule of an RNA or protein in each cell. To address this limitation, the protecting factor has been suggested to self-assemble into one large cluster of molecules (self-assembly model, Figure 2d).[40,41] Biophysical simulations have shown that this would be possible within biologically relevant timescales, if the X chromosomes are in close spatial proximity (Xic pairing, see below). Spatial colocalization would strongly reduce the search time in 3D space for individual molecules to find a cluster. One of the earliest XCI models suggested the insertion of an episomal DNA element in one X leading to activation of that chromosome (Figure 2e). Among the activated genes would be one encoding a factor that would rapidly degrade all remaining episomes in a negative feedback loop.[42] Despite large-scale sequencing efforts, however, no Xa-specific DNA insertions have been identified. Therefore, we can probably reject this model today.

Also for the alternative defaut:Xa scenario different mechanisms have been suggested (Figure 2f,g). Homologous pairing of two X chromosomes had, for example, been thought to allow sensing of the presence of more than one X (Figure 2f).[43–45] This hypothesis has however been falsified recently, because reduction of pairing through tethering one or both *Xic*'s to the nuclear lamina does not affect XCI.[46] Moreover, work in heterokaryons showed that diffusible factors are sufficient to induce XCI in an XY nucleus.[47] Already in 1971, Mary Lyon proposed a diffusible X-encoded factor to ensure female-specific XCI.[32] We will call this factor X-linked XCI activator (xXA). Because it is encoded on the X chromosome, this activator gene would be present in two copies in each female cell and its gene product (RNA or protein) would thus be present at twice the levels in females compared to males (Figures 2g and 3c). A central prediction of the xXA model is that additional X chromosomes will increase the rate with which XCI is initiated, a concept that has been verified experimentally.[31] An essential ingredient of any XCI model that relies on an xXA is that xXA initiates XCI in a switch-like manner once it exceeds a certain threshold that lies between the xXA level in male and in female cells (**Figure 4**a).[31] Accordingly,



all supernumerary X chromosomes in X-aneuploidies (e.g., XXX, XXY) are silenced until only a single active X (single xXA dose) remains.

2.2. Molecular Implementation of the XCI Initiation Threshold Required for X Dosage Sensing

The XCI initiation threshold is required to convert a twofold difference in xXA levels between males and females into a binary decision, where XCI is initiated in females only. Such a threshold response requires ultrasensitivity, where a small change in input signal (xXA dose) results in a large effect on the response (Figure 4a). Ultrasensitivity can, for example, arise from molecular titration, cooperative binding or positive feedback regulation,[48,49] and either arise globally or locally at the allele-level (Figure 4b,c).

A global threshold response could be mediated by sequestration of xXA through an (autosomal) XCI repressor or through autosomal binding sites on the DNA.[50,51] Only if xXA is

Figure 2. XCI models. a) Only female cells with two X chromosomes (right), but not males with a single X (left) initiate XCI (female specificity) through Xist upregulation (green) from one out of two X chromosomes (gray) in a monoallelic fashion (mutually exclusive choice) and stably maintain this decision throughout cell division (maintenance). b,c,d,e) Models assuming inactivation as the default fate for the X chromosome (Default:Xi). In Default:Xi scenarios female specificity is ensured through a single entity (blue) that will protect exactly one X chromosome from inactivation. This entity can be b) a nuclear attachment site, c) a single molecule blocking factor, d) a self-assembling protective cluster, or e) an episome, which is inserted into one of the two X chromosomes, followed by rapid degradation of all remaining episomes. f,g) Models assuming activity as the default fate for each X chromosome (Default:Xa). In default:Xa scenarios, presence of two X chromosomes is necessary to initiate XCI and is sensed through f) *Xic* pairing, or g) a diffusible *trans*-acting X-encoded XCI activator (xXA) that is present in a double dose in female cells and activates XCI in a dose-dependent manner.

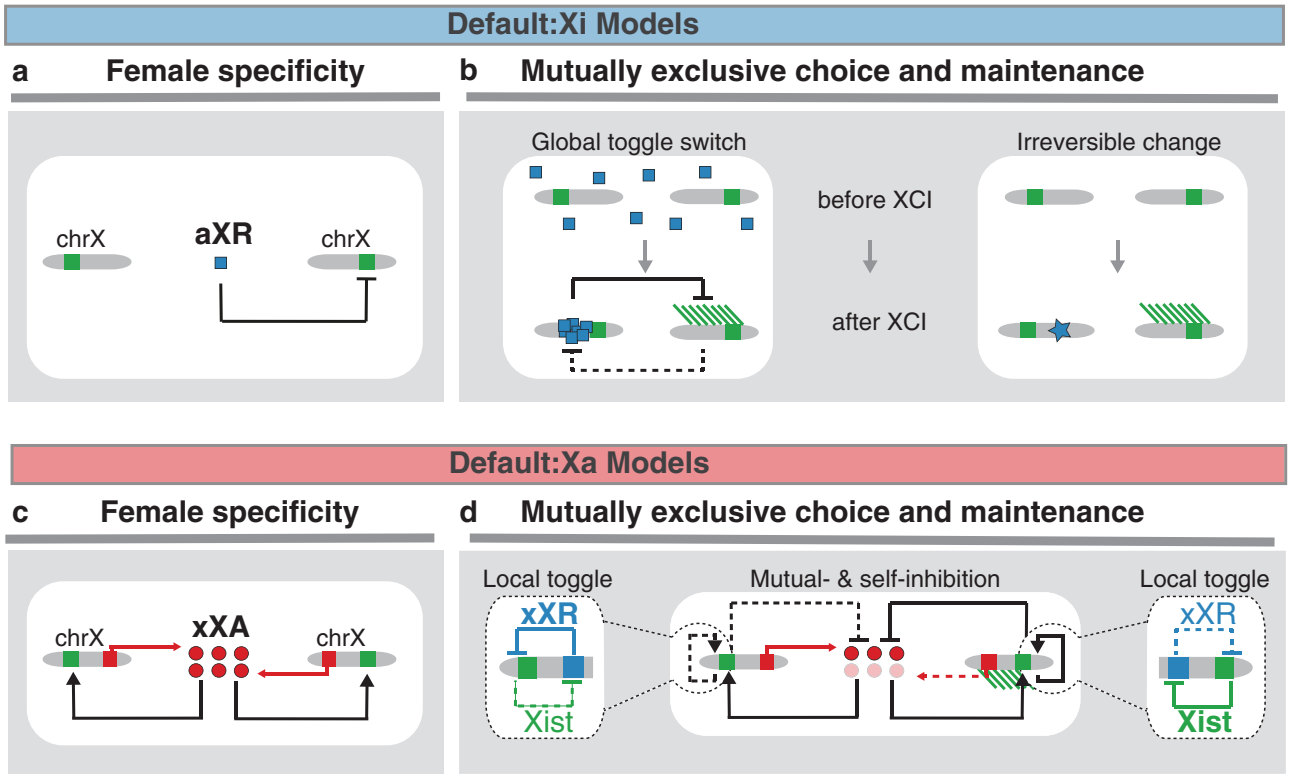


Figure 3. Network motifs implicated in the onset of XCI. a) In the Default:Xi scenarios a single entity autosomal XCI repressor (aXR, blue square) ensures female specificity by inhibiting XCI on exactly one X chromosome (gray) per nucleus. b) Stable protection of one X from inactivation could be ensured by mutual repression of the two X chromosomes (global toggle switch), for example, through a single self-assembling cluster of protective factor (blue) that associates with one X chromosome, resulting in depletion from the other X by acting as a sink for the protective factor (global toggle switch). Alternatively, an irreversible event, for example, stable DNA insertion (blue star) into one X (irreversible change) could stably protect that chromosome from inactivation. c) In the Default:Xa models female specificity is ensured through sensing the presence of more than one active X chromosome, for example, through an X-linked XCI activator (xXA, red circle). d) Upon XCI Xist will silence the *trans*-acting xXA thereby inhibiting Xist upregulation from the other Xist allele but also itself (mutual- & self-inhibition), because xXA activates both Xist alleles. To nevertheless maintain Xist expression at the Xi, an additional positive feedback is required that acts in *cis* to locally stabilize Xist expression upon xXA silencing (local toggle switch). The self-reinforcing positive feedback can be mediated by mutual repression between Xist (green) and a *cis*-acting Xist repressor (xXR, blue), for example, Tsix, which stably maintains two alternative states, where xXR dominates on the Xa (left) and Xist dominates on the Xi (right).

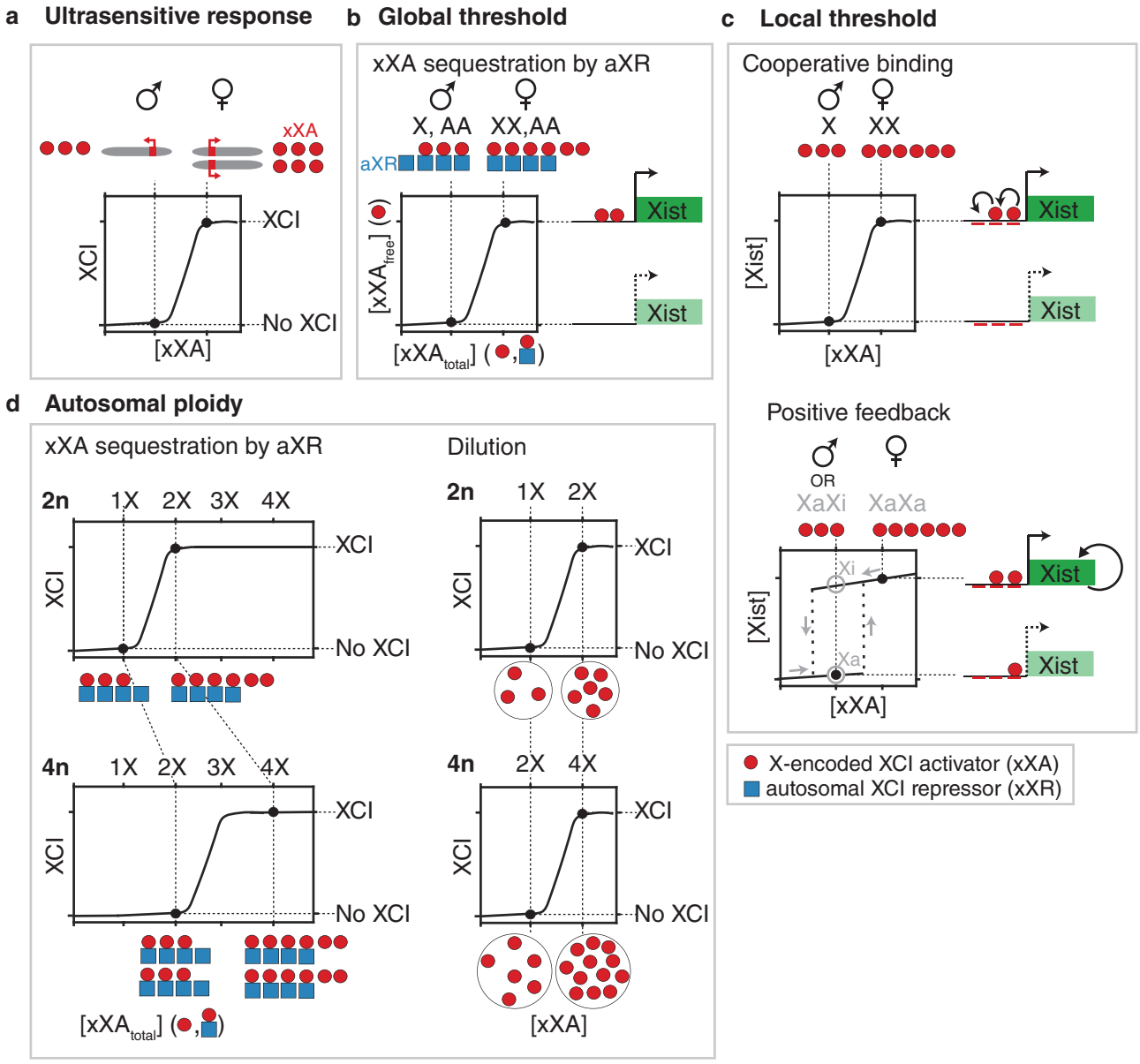


Figure 4. XCI threshold. a) An ultrasensitive stimulus-response curve is required in all xXA models. A twofold change in xXA concentration (red dots) must result in a sharp switch-like XCI response to convert a quantitative signal (1x or 2x xXA dose) into a binary all-or-nothing response (No XCI or XCI). b,c) Possible molecular implementations of the ultrasensitive threshold response. b) A global threshold at the cell level can be implemented, for example, by sequestration of xXA (red) through an autosomal XCI repressor (aXR, blue), where free xXA (xXA_{free}) becomes only available to bind and activate *Xist*, when the xXA concentration (xXA_{total}) exceeds that of aXR. c) A local threshold at the allele level can be implemented by positive cooperative binding (top) of multiple xXA molecules to a binding site cluster (red bars) at the *Xist* locus, such that initial xXA binding increases the binding affinity of the remaining unbound xXA sites. Alternatively, a local threshold can be generated by a *cis*-acting positive feedback (bottom), where Xist reinforces its own expression in *cis*. The positive feedback also has the potential to produce bistability, resulting in two alternative stable states (gray circles) in the presence of a single xXA concentration, corresponding to the Xa and Xi. Dashed lines indicate the transitions, where the low or high steady states become unstable. Gray arrows indicate hysteresis, where different steady states are reached depending on the previous state of the locus. d) Autosomal ploidy can be integrated through an aXR that counteracts xXA (see b) such that xXA is completely sequestered in both male diploid (2n1X) and tetraploid (4n2X) cells (left), or through dilution of xXA due to increased nuclear volume, resulting in the same xXA concentration in 2n1X and 4n2X cells (right).

produced from two X chromosomes some of it would remain unsequestered and could bind to the *Xist* locus to activate transcription (Figure 4b). Global threshold behavior could in principle also arise if xXA would control production of another, potentially autosomally encoded downstream activator in an ultrasensitive manner.[32,50] If the threshold was established locally at the level of Xist regulation, it could arise from cooperative binding of multiple xXA factors to the *Xist* locus, or from a *cis*-acting positive feedback loop, a concept that we have recently proposed (Figure 4c).[16]

2.3. How Does Autosomal Ploidy Modulate XCI?

Can these concepts explain how autosomal ploidy ensures that female diploid cells (XX, AA) initiate XCI while tetraploid male cells (XXYY, AAAA) do not, although they both have two X chromosomes? The default:Xi models are in general limited to diploid cells, because generation of exactly two stable protective clusters is even more difficult to envision than generation of a single one. The xXA models by contrast can explain XCI in tetraploid cells by invoking an autosomal XCI repressing factor that would scale with the number of autosomes and counteract xXA.[30,31,36,51,52] Such a repressor could, for example, sequester xXA and would thereby essentially shift the XCI initiation threshold (Figure 4d, left) or modulate a local threshold by competing with xXA for binding to the *Xic* (Figure 4c).[31,36,51] Recently, we have proposed an explanation for how autosomal ploidy might affect XCI that is independent of such an autosomal repressor. Twofold dilution of xXA factors due to an about twofold increase in nuclear volume in tetraploid cells would result in similar xXA concentrations in XY and XXYY cells,[16,53] allowing both to remain below the XCI threshold (Figure 4d, right).

2.4. How Can Mutually Exclusive Initiation of XCI from Exactly One X Chromosome Per Cell Be Ensured?

At the onset of XCI, two functionally equivalent chromosomes must assume opposing transcriptional states. The problem resembles a much better studied situation, where two similar progenitor cells assume alternative cell fates. This is often ensured through mutual inhibition of two lineage-determining transcription factors.[54,55] Such a network motif can generate a toggle switch, which can assume two alternative states, where one factor is active and represses the other one and vice versa.[56] In addition, expression of each factor is often stabilized by positive feedback regulation, giving rise to the so-called "extended toggle switch".[55] In this analogy, the two X chromosomes or factors encoded by them would resemble the two lineage-determining transcription factors, with the important difference that the two chromosomes are identical, making pure mutual inhibition more challenging. The self-assembly model would in principle generate such purely reciprocal inhibition of XCI at the two X chromosomes, because one assembled cluster would act as a sink and thus prevent binding of the factor to the other chromosome (Figures 2d and 3b, left). As discussed above, however, the self-assembly model cannot explain observations in tetraploid cells. In principle, also the Xi as a whole could act as a sink by sequestering heterochromatin components, as suggested for the Y chromosome in drosophila, and thereby prevent silencing of the second X chromosome.[57,58] The inhibition would however not be X-specific and would reduce heterochromatin formation also on autosomes.

For the models invoking an X-linked activator, Mary Lyon had already proposed in 1971 that silencing of xXA upon XCI could prevent inactivation of the other X chromosome by reducing the xXA dose to the level found in male cells, thus constituting a negative feedback loop (Figures 2g and 3d).[32] The feedback would however not be purely reciprocal: Since xXA acts on both chromosomes, silencing would also inhibit XCI on the inactive X, thus requiring additional self-reinforcing mechanisms to maintain the inactive state in the presence of reduced xXA levels, as discussed in the next section (Figure 3d). Interestingly, a similar negative feedback has already been proposed as early as 1963 in the episome model,[42] where episome integration triggers degradation of all remaining unincorporated episomes (Figure 2e).

An important requirement of any global negative feedback model is a separation of timescales. If the initial event of XCI, such as Xist upregulation, is slow and stochastic, it will generally occur at one chromosome at a time and thereby allow symmetry breaking. The subsequent negative feedback, that is, xXA silencing, must, by contrast, be fast to prevent reoccurrence of the initial event on the second X chromosome.[51] The initial probabilistic event underlying symmetry breaking could also occur upstream of Xist upregulation, for instance by switching to a permissive chromatin or conformational state (pre-emptive choice).[59,60] It is however difficult to explain how such a purely *cis*-acting mechanism could result in mutual exclusivity.

Preventing Xist upregulation from the second X, seems not to be the only function of the proposed negative feedback loop, mediated by silencing of xXA. If cells erroneously initiated XCI on the single X in males or biallelically on both X chromosomes in females, complete xXA silencing could reverse initiation of XCI. In support of this idea, we have recently shown that biallelic XCI initiation is indeed reversible.[16] The xXA-mediated feedback loop might therefore govern the reversion of biallelic Xist expression, which is observed to varying extents in different species, to the final monoallelic expression pattern.[15–18] Moreover, it might reverse Xist upregulation in male cells, as recently observed in mouse embryos.[17]

2.5. Maintenance of Monoallelic Expression through Bistability and Epigenetic Memory

To ensure stable monoallelic XCI, the inactive state must, once established, be maintained throughout cell divisions. The biochemical reactions that govern transcriptional regulation are in general reversible except for changes to the DNA sequence. This irreversibility of DNA insertions was suggested in the episome model to allow stable maintenance (Figures 2e and 3b, right).[42] However, our recent finding that cells are able to revert biallelic to monoallelic Xist expression suggests that choice is initially reversible arguing against the occurrence of a single irreversible event.[16] It thus seems more likely that dedicated self-reinforcing mechanisms ensure stable maintenance of the active and inactive states (Figure 3d).[16,31,32] By systematic testing of different regulator types through mathematical modeling we have recently shown that such a self-reinforcing, positive feedback could be mediated by an X-linked regulator that functions as an Xist repressor in *cis*.[16] Since such a regulator would be silenced by Xist during XCI, Xist and the repressor would mutually repress each other, thus forming an additional local toggle switch in *cis* (Figure 3d). Such a switch could generate local bistability, which would allow stable maintenance of two alternative expression states at the two X chromosomes in the presence of a single activator dose (Figure 4c, bottom). Alternatively, a self-reinforcing feedback could also function upstream of Xist upregulation, for example, mediated by other

cis-acting mechanisms such as chromatin modifications and could lead to pre-emptive choice (see above).[61] As discussed above, a positive feedback loop can also give rise to ultrasensitivity to generate the threshold behavior required for female specificity and would thus ensure both, stable maintenance of alternative states and the threshold required for female-specific XCI (Figure 4c, bottom).

2.6. Emerging Concepts: Ultrasensitivity and Feedback Loops

In summary, the default fate of the X chromosome appears to be the active state and initiation of XCI is triggered by one or several X-linked XCI activators. The required threshold response could be implemented in a global manner, such that the activator is only active in female cells, for example, through sequestration by an autosomal repressor. Alternatively, the threshold could be generated through *cis*-regulatory events at the *Xist* locus for instance through cooperative binding of xXA to the *Xic*, or through local feedback regulation. Autosomal ploidy could modulate the threshold either through autosomal XCI repressors that counteract the activator or through dilution of the activator due to increased nuclear volume. Rapid silencing of the X-linked activator, once XCI has been initiated, could ensure mutually exclusive choice, if the inactive state is memorized in *cis*, for example, through a local positive feedback loop.

Similar mechanisms have been invoked in ensuring mutual exclusive expression in other biological contexts such as the differentiation of olfactory neurons, where each cell must make a stochastic but stable choice for exactly one out of about 1000 different olfactory receptors. Also here, the mutually exclusive choice of a single receptor is mediated by a global negative feedback, triggered by activation of a receptor gene, and the decision is locked in by a local positive feedback mediated by nucleosome modifications.[62–66]

3. Candidate Regulators and Mechanisms

A series of abstract concepts of how random XCI can be ensured have been proposed and partially rejected again over the years. To test the emerging concepts, we now have to identify the predicted regulators. If not stated otherwise, all observations discussed in the next sections were made in mouse embryonic stem cells (mESC), the main cell culture model of XCI.

3.1. xXA Factors: Rnf12 and Accomplices

To ensure female-specific XCI an xXA factor must be expressed at twofold higher levels in females compared to males, and it must activate XCI in a switch-like manner that allows for an all-or-nothing decision within this twofold range. To mediate the predicted negative feedback, xXA should be rapidly silenced during XCI. Moreover, its overexpression in male cells should induce ectopic XCI, while a heterozygous xXA deletion should abolish XCI in females and thus result in female-specific lethality.

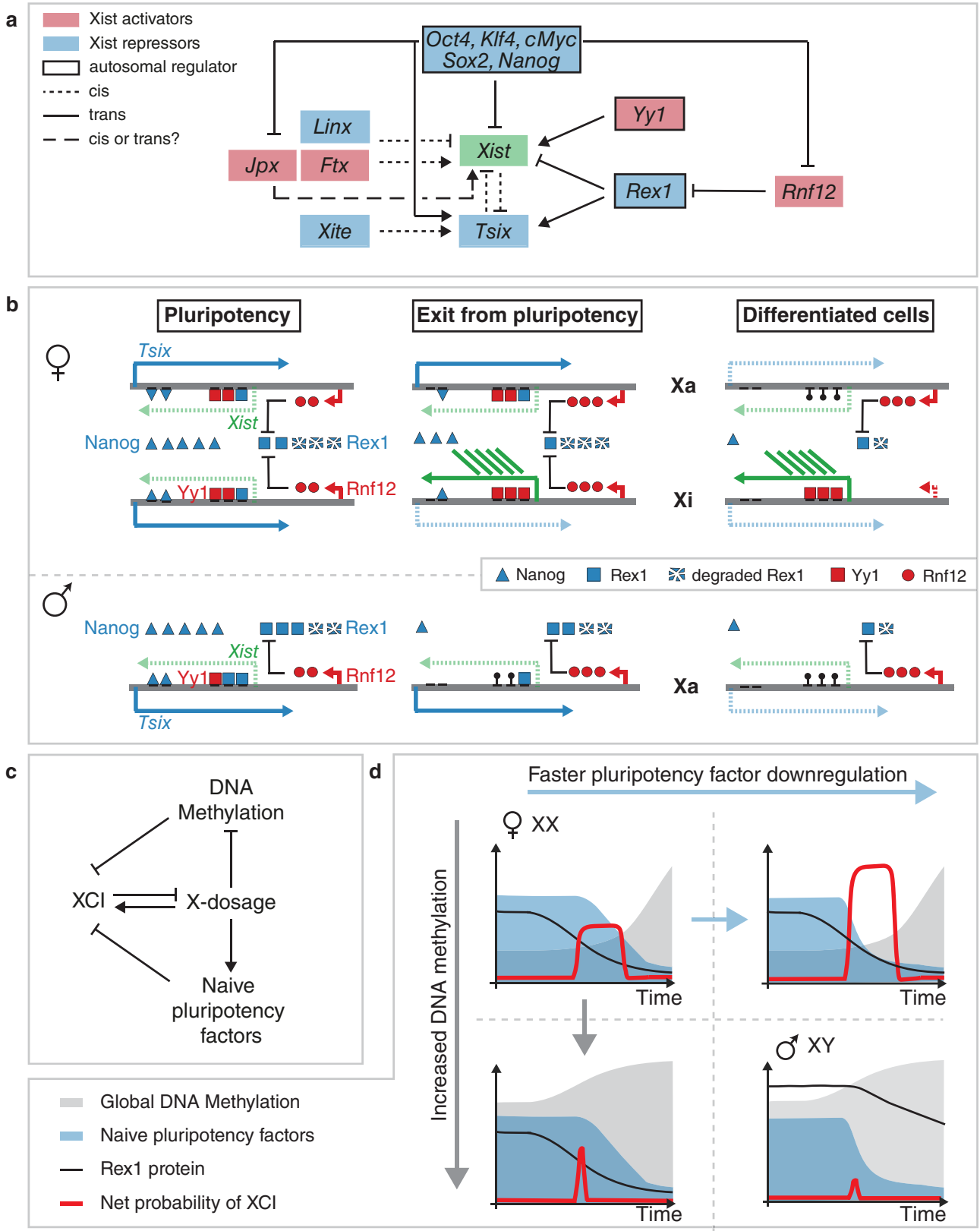
The best studied xXA factor is the *Rnf12/Rlim* gene, which encodes an E3 ubiquitin ligase and is located close to *Xist* (Figure 1).[67] Rnf12 activates Xist by targeting the autosomally encoded XCI repressor and pluripotency factor, Rex1 for degradation (**Figure 5**a).[25,68] Rnf12 clearly functions as a dose-dependent Xist activator, as its overexpression in male cells results in ectopic XCI, and is rapidly silenced by Xist.[67] Its heterozygous deletion in females, however, does not abolish, but only delay XCI.[67] Complete absence of Rnf12 by contrast prevents random XCI in differentiating mESCs and imprinted XCI in early mouse embryos, while random XCI in vivo has been reported to be unaffected.[69–71] The mESC phenotype, however, seems to depend on the precise culture conditions,[72] maybe because rapid downregulation of Rex1 under certain conditions might make XCI Rnf12-independent. Interestingly, experiments with heterozygous Rnf12 mutants where the single intact copy of Rnf12 is silenced during XCI have shown that XCI cannot be maintained without an active Rnf12 allele.[47] This supports the above-discussed idea that biallelic XCI in females might be unstable because it results in complete xXA silencing.[47] The fact that loss- and gain-of-function perturbations of Rnf12 result in asymmetric phenotypes, suggests that additional xXA factors exist. If multiple xXAs act together in a redundant or additive manner, overexpressing one xXA factor should result in ectopic XCI in males but its heterozygous deletion would not necessarily abolish XCI in females as other xXAs could compensate for the loss. Such a cooperation of multiple xXA factors might also facilitate robust X-dosage sensing, as individual genes often exhibit higher than twofold cell-to-cell expression variability.[73]

An X-linked activator that might potentially cooperate with Rnf12, is the lncRNA Jpx, which is located about 10 kb upstream of *Xist* and has been suggested to activate XCI by evicting Ctcf from the *Xist* gene (Figures 1 and 5a).[74,75] While its function as an XCI activator is well established, its mechanism of action and whether it can act in *trans* remain controversial.[47,76] Moreover, *Jpx* escapes XCI both in mice and in humans and can thus not be invoked for the predicted negative feedback regulation.[75,76] Yet another Xist-activating lncRNA, named Ftx, is located about 100 kb upstream of *Xist* (Figures 1 and 5a).[77,78] Ftx activates Xist in *cis* by the act of transcription, independent of its lncRNA and the microRNA cluster it contains, and can therefore not be one of the *trans*-acting xXA factors that have been predicted to underlie female specificity.[78] In conclusion, several positive X-encoded regulators of Xist have been identified, but additional factors necessary to mediate female-specific XCI onset remain to be found.

3.2. XCI Initiation Threshold: Yy1—Rex1 Competition or Antisense Transcription?

Ultrasensitivity, which is required for the predicted XCI initiation threshold, can be generated, for example, through molecular titration, cooperative binding or positive feedback regulation as explained above.[48] Jpx has been proposed to establish an XCI threshold by titrating the autosomal XCI repressor Ctcf away from the *Xist* locus, which, when overexpressed prevents Xist upregulation.[74] It remains unclear, however, how a rather lowly expressed RNA could titrate a highly abundant protein[79] and how the effect could be specific to Xist regulation although Ctcf is found at a large number of genomic locations.

Instead, ultrasensitivity might rather arise from *cis*-regulatory events at the *Xic*. Cooperative binding of transcription factors



could in principle occur, if multiple binding sites are clustered together. Interestingly, the autosomally encoded ubiquitous transcription factor Yy1, which is essential for initiation and maintenance of Xist expression (Figure 5a), binds to a highly conserved cluster of binding sites in the 5′ region of *Xist* and also to *Tsix*.[80–83] While Yy1 is bound to all X chromosomes before XCI, its binding becomes restricted to the Xist-expressing alleles at the onset of XCI (Figure 5b, red square).[81,82] Two related transcription factors, Rex1 and Yy2, were derived from Yy1 through retrotransposition in placental mammals and have similar binding motifs.[84] The Xist repressor Rex1, which is targeted for degradation by Rnf12 (see above), indeed functions at least in part, through competing for binding with Yy1.[82] Accordingly, Yy1 knock-down dramatically impairs Xist upregulation at the onset of differentiation in female mESCs,[82] while Rex1 depletion in male cells results in ectopic Xist upregulation.[25,67] Interestingly, Yy2, which is poorly characterized, is located on the X chromosome and could thus also contribute to female specificity if functioning as an Xist activator. Taken together, the clustered Rex1/Yy1 binding sites could generate the threshold required to distinguish between a single and a double dose of Rnf12, if Yy1 bound in a cooperative manner (Figure 5b).

An alternative mechanism to generate ultrasensitivity would be positive feedback regulation, for example, mediated by mutual inhibition of Xist and an *cis*-acting Xist repressor (see above).[16] The best studied *cis* repressor is *Tsix*, a lncRNA locus antisense to *Xist*, which completely overlaps with the *Xist* gene, including its promoter (Figures 1 and 5a,b).[36,85] Tsix transcription establishes a repressed chromatin state at the *Xist* promoter and might additionally repress Xist by transcriptional interference.[16,86–89] Tsix clearly functions in *cis* only, as heterozygous Tsix deletions skew the choice of the inactive X toward the mutated allele.[36,59,90,91] Through a mathematical model of transcription at the *Xist*/*Tsix* locus, we have shown that antisense transcription can indeed maintain alternative Xist expression states on Xa and Xi.[16] Repression of Xist by Tsix also delays the kinetics of Xist upregulation and can therefore ensure the required timescale separation (slow XCI initiation, fast negative feedback). Accordingly, Tsix mutations accelerate Xist upregulation[31] such that homozygous Tsix deletions result in a weakened timescale separation between Xist upregulation and xXA silencing and a higher fraction of biallelic cells.[52]

The functional conservation of Tsix in other species is unclear. In human embryonic carcinoma cells, the overlap between XIST and TSIX is reduced, such that antisense transcription does not reach the XIST promoter.[92] Our simulations showed that, even with this reduced overlap, the required sharp threshold response can be generated within a twofold range of xXA, if we assume transcriptional interference, which is supported by experimental evidence.[16] However, TSIX transcription has not been observed in human embryos or embryonic stem cells.

Alternative *cis*-acting Xist repressors could be *Xite* and *Linx*, two other lncRNA loci that have been implicated in Xist regulation (Figures 1 and 5a).[93,14] *Xite* is a Tsix enhancer whose deletion results in downregulation of Tsix in *cis* and mildly skewed XCI.[93,94] Thus, rather than being an independent Xist repressor, Xite acts upstream of Tsix. Linx, on the other hand, represses Xist in *cis*, but independently of its transcription, thus rather functioning as a *cis*-regulatory DNA element,[95] which makes it potentially insensitive to Xist-mediated silencing and therefore incapable of mediating the double-negative feedback.

The human-specific lncRNA XACT also antagonizes XIST and its expression pattern in primed hESCs fits with a role as X-linked repressor: XACT coats the Xa while XIST coats the Xi.[96] In addition, XACT transgenes in mESCs can prevent Xist accumulation in *cis*.[97] However, XACT is initially expressed together with XIST from all X chromosomes in human embryos.[96,97] This coexpression would be compatible with an XACT-mediated positive feedback loop, if mutual repression (XACT silencing and XIST repression by XACT) only sets in once the biallelic state gets resolved to the state of a female cell with one active and one inactive X chromosome (XaXi). To test these ideas, we first need to acquire the ability to observe onset of random XCI in human embryos or in hESCs. Recent developments in human embryo culture and new culture conditions for hESCs, will hopefully soon allow us to observe this transition to the XaXi state and investigate the functional roles of XACT and potentially TSIX.[98–101]

3.3. Integration of Autosomal Ploidy: Autosomal Regulators Counteracting xXA

Autosomal ploidy modulates the XCI initiation threshold either through dilution of xXA factors or through autosomal XCI

Figure 5. XCI regulators. a) Schematic wiring diagram of Xist (green) and its regulators at the onset of XCI. b) Possible molecular implementation of female-specific monoallelic XCI onset. Shown are both X chromosomes (gray) in female (top) and the single X in male cells (bottom) before (left), during (middle) and after (right) differentiation. Transcriptional activity of Xist, Tsix, and Rnf12 is indicated by solid dark (active) or dotted light arrows (inactive). Free and chromatin-bound proteins are indicated as squares (Rex1, Yy1), circles (Rnf12), or triangles (Nanog). Yy1 competes with Rex1 for binding to *Xist*'s first exon and can therefore only bind if Rex1 levels are low and binding sites are unmethylated. In the pluripotent state repression through Tsix prevents Xist upregulation even on alleles with Yy1 binding. Upon differentiation, Rnf12 upregulation depletes Rex1 in female cells, generating the opportunity for Yy1 binding and Xist upregulation. Xist repression by other pluripotency factors such as Nanog ensures slow monoallelic on-switch of Xist. In males, binding sites remain occupied by Rex1 due to lower Rnf12 levels, and fast establishment of DNA methylation, possibly promoted by Tsix transcription, stably prevents Yy1 binding and Xist upregulation. Upon successful dosage compensation, DNA methylation levels also rise globally in female cells and prevent Yy1 binding to the Xa, so that in differentiated cells Tsix transcription is no longer necessary for Xist repression. c) Double X-dosage induces XCI mediated by xXA factors, but also halts differentiation in females by maintaining pluripotency factor expression and delaying global DNA remethylation. Faster DNA methylation might contribute to Xist repression in males, while slower downregulation of pluripotency factors might reduce the probability for Xist upregulation in females, thereby ensuring proper XCI timing and robust monoallelic XCI. d) Hypothetical roles of sex differences in DNA methylation and differentiation for correct XCI onset. Female XX cells (upper left) exhibit DNA hypomethylation (gray) and reduced protein levels of the Xist repressor Rex1 (black), but slower downregulation of other Xist repressing naive pluripotency factors (blue) compared to male XY cells (lower right). DNA hypomethylation might be required to generate an extended time window (red), where XCI can be initiated (compare left top and bottom). Slower downregulation of pluripotency factors might be important to ensure a low XCI probability (red) required to prevent biallelic Xist upregulation (compare top left and right).

repressors, as discussed above. Such an autosomal repressor is Rex1, which mediates the effect of Rnf12 on XCI (Figure 5a,b).[25] When comparing a diploid male cell (XY, AA) with a single X to a corresponding tetraploid cell (XXYY, AAAA), twofold higher Rnf12 levels in the latter could potentially be neutralized by a twofold increase in Rex1. If we assume that ubiquitinylation of Rex1 follows Michaelis-Menten kinetics, Rex1 levels would be similar in both cell types, potentially explaining why they do not initiate XCI.

Apart from Rex1, a series of additional (autosomal) pluripotency factors have been found to repress XCI either directly or via controlling Xist regulators, such as Tsix or Rnf12 (Figure 5a).[22–24,102–107] By activating Tsix or repressing Xist, they could in principle also modulate the activation threshold of the proposed Tsix-mediated positive feedback loop.[16] Given their dynamical regulation during the onset of XCI and the fact that also autosomal activators control Xist, it might however be challenging to sense autosomal ploidy via pluripotency factor levels.

3.4. Maintenance: Which *cis*-Acting Positive Feedback Mechanisms could Generate Epigentic Memory?

While Xi maintenance in somatic cells is mostly Xist independent, monoallelic silencing initially requires the continuous presence of the Xist RNA on exactly one X chromosome.[108–111] Such stable maintenance of two alternative Xist expression states on two alleles in the same nucleus requires *cis*-acting mechanisms that memorize the allelic expression states. We have recently proposed that local bistability, mediated by a *cis*-acting positive feedback loop could enable such a memory by constituting a local toggle switch.[16] This feedback might be mediated by mutual repression of Xist and Tsix and could generate two stable steady states, one in which Tsix dominates over Xist (Xa) and vice versa (Xi) (Figure 3d). If Xist-mediated silencing of an *cis*-acting Xist repressor such as Tsix would indeed be required to stabilize Xist expression, a perturbation of Xist's silencing capacity should prevent Xist upregulation, because it would block one interaction in the double-negative feedback. A failure to upregulate Xist has indeed been observed in mouse embryos carrying a deletion of the A-repeat (ΔA), which is required for Xist's silencing function,[112] but was interpreted as a disruption of an Xist enhancer element.[113] Xist upregulation was restored when the ΔA allele was put under control of a strong beta-actin promoter, which can override Tsix-mediated repression,[114] supporting a role of Xist-mediated silencing in upregulation of Xist itself. Depletion of silencing factors, such as Spen, a protein that recruits transcriptional corepressors and histone deacetylases to Xist,[7,8,115] would be predicted to have a similar effect. Interestingly, deletion of Spen or its binding site in Xist reduce Xist RNA levels even in an inducible system.[6] Also methylation of N6-methyladenosine residues (m6A) in the Xist RNA has been suggested to contribute, albeit to a lesser extent, to Xist's silencing ability.[6,116] Accordingly, Xist upregulation is indeed impaired upon knockdown of components of the m6A methylation machinery.[116] Taken together, silencing of X-linked genes seems to indeed enforce Xist expression, supporting the notion of a silencing-dependent self-reinforcing feedback. Given that Tsix expression is shut off as cells differentiate, additional mechanisms must stabilize Xist expression in somatic cells.

One such candidate mechanism is DNA methylation since knock-out of the maintenance DNA methyltransferase Dnmt1 results in derepression of Xist on the Xa in somatic cells.[117,118] DNA methylation also plays a key role in genomic imprinting, where two alleles of the same gene acquire alternative expression states predetermined by their parental origin.[119,120] Interestingly, the regulatory region within *Xist* that is bound by Yy1 at the Xist-expressing chromosome, is methylated on the other allele which prevents Yy1 binding.[81,82,121] This allelic asymmetry suggests that DNA methylation might be involved in a potential chromatin-based feedback loop to sustain epigenetic memory at the *Xist* locus (Figure 5b). However, even in the absence of de novo methylation, most cells still exhibit the correct Xist expression pattern in vivo, suggesting that DNA methylation is not essential to establish alternative Xist expression states and that additional redundant mechanisms are in place to lock in these states.[122] Since Tsix transcription has been shown to promote DNA methylation, the Tsix-mediated feedback and a potential chromatin-based feedback loop might be coupled and the latter could take over stabilization of the two states, once Tsix transcription ceases (Figure 5b).[87]

3.5. Global X-Dosage Effects Might Help to Create a Female-Specific Window of Opportunity

Interestingly, the presence of two active X chromosomes affects also autosomal gene expression.[123] Double X-dosage shifts cells toward the naive stem cell state, such that they express higher levels of naive pluripotency factors, exhibit global DNA hypomethylation, a hallmark of the naive state, and decreased activity of the differentiation-promoting MAP kinase signalling pathway (Figure 5c).[124,125] Moreover, female cells differentiate more slowly and can only leave the pluripotent state once X-dosage compensation has occurred.[124] An intriguing hypothesis that has not been explored so far is that these sex differences could also contribute to female specificity or robustness of XCI (Figure 5d). X inactivation is initiated upon the exit from the naive pluripotent state, which is associated with global DNA hypomethylation and thus coincides with global remethylation of the entire genome.[120] DNA hypomethylation and potentially slower remethylation due to delayed differentiation in cells with two X chromosomes might be important to create a "window of opportunity" for Xist upregulation (Figure 5d, left, compare top to bottom).[124,126] In addition, the slower downregulation of pluripotency factors in XX females might be required to limit the speed of Xist upregulation in order to maintain the timescale separation between upregulation and silencing (see above) (Figure 5d, top, compare left to right).

4. Conclusion and Prospects

In summary, a global negative feedback mediated by X-linked XCI activators combined with a *cis*-acting positive feedback can in principle ensure female-specific and monoallelic XCI. Experimental evidence for the existence of the negative feedback comes from the observation that cells are able to reverse biallelic Xist expression.[16] The Rnf12-Rex1 axis clearly contributes to the

feedback but additional xXA factors must exist to ensure female-specific XCI onset. To identify these factors, their ability to induce XCI when overexpressed in male cells should be assessed, as this allows to also uncover regulators that act in combination. To identify how the required threshold response is ensured, Xist dose-response curves should be measured, for example, by titrating a known xXA factor, such as Rnf12. Transient overexpression of the xXA factor might even allow us to observe the hysteresis effect experimentally. Overexpression should induce Xist and the positive feedback should maintain expression upon subsequent reduction of the activator level. Once the predicted non-linear response and the memory are directly observable, the underlying mechanisms can be identified by experimental perturbation of candidate regulators such as Tsix transcription, Yy1 binding or DNA methylation.

The reason why the mechanisms mediating the two feedback loops remain incompletely understood, might be their highly redundant implementation. Given the challenge to maintain the correct Xist expression pattern throughout all somatic cell divisions and in diverse cell types, such redundant regulation might be required to ensure robustness. To dissect the contribution of the different mechanisms, it will be essential to perturb them in combination and to assess the contribution of each one in a quantitative fashion. Mathematical models provide a useful tool for understanding the regulation of such fascinating biological processes as random XCI because they formalize conceptual ideas and make predictions that can be rigorously tested in experiments.

Appendix

Autosomes	All chromosomes that are not sex chromosomes. The number of autosome sets that a cell possesses determines its ploidy. Mice and humans are diploid (2n).
Bistability	The coexistence of two stable steady states for the same system's parameters. For instance, high Xist expression on Xi and low Xist expression on Xa in the presence of a single xXA dose.
Choice	Process by which a female cell decides which of its two X chromosomes to inactivate. In random XCI the probability for the maternal and paternal X to become inactivated is equal. However, certain *Xic* mutations or polymorphisms can result in preferential inactivation of one X chromosome (skewing).
Cis	A *cis* regulator locally affects the chromosome on which it is encoded. *cis*-regulatory events are necessary to allow stable opposing expression states on the active and Xis. Long non-coding RNAs (lncRNAs) often act as *cis* regulators (Tsix, Linx as *cis*-Xist repressors, Jpx, Ftx as *cis*-Xist activators).
Cooperativity	If two molecules bind in a cooperative manner, binding of the first molecule increases affinity of the second molecule.
Counting	Process by which a cell determines its X-to-autosome ratio to decide how many X chromosomes must be inactivated in order to achieve a ratio of one active X per diploid set of autosomes.
Female specificity	XCI only initiates in XX females but not in XY males. An exception to this are males who have more than one X chromosome (e.g., XXY, Klinefelter syndrome) and females with a single X chromosome (XO, Turner syndrome)
Hysteresis	Memory of the system's previous state, meaning that the system exhibits a different response depending on whether it was previously in its "on" or "off" state. This allows Xist to stay "off" on the Xa, while being "on" on the Xi.
Maintenance	Stable propagation of the decision for an active and Xi throughout all further cell divisions.
Monoallelic	Expression of a gene from only one of multiple existing (and genetically identical) copies of DNA.
Toggle switch	Mutual cross-inhibition between two entities resulting in bistability.[56,127] The paradigm for mutual exclusivity in biology that can explain numerous decision-making processes, such as the lysis-lysogeny decision of phage lambda, which is mediated by mutual repression between two transcription factors (for a review see refs. [128,129]).
Trans	A *trans*-acting regulator affects all copies of its target gene and is usually a diffusible molecule, such as a protein or RNA. *trans*-regulatory events are necessary to allow communication between the X chromosomes in a cell.
Ultrasensitivity	Threshold behavior. Small changes in the stimulus close to the threshold result in a large change in the response.
Xic	The X inactivation center (Figure 1) has been defined as the minimal region sufficient to trigger XCI if present in two copies.[130] Among other important XCI regulators it contains the *Xist* gene, the master regulator of XCI.
XO	Female genotype with only one X and no Y chromosome.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] M. F. Lyon, *Nature* **1961**, *190*, 372.
[2] G. B. Sharman, *Nature* **1971**, *230*, 231.
[3] R. Galupa, E. Heard, *Curr. Opin. Genet. Dev.* **2015**, *31*, 57.
[4] M. F. Lyon, *Acta Paediatrica* **2007**, *91*, 107.
[5] R. Galupa, E. Heard, *Annu. Rev. Genet.* **2018**, *52*, 535.
[6] T. B. Nesterova, G. Wei, H. Coker, G. Pintacuda, J. S. Bowness, T. Zhang, M. Almeida, B. Bloechl, B. Moindrot, E. J. Carter, I. A. Rodrigo, Q. Pan, Y. Bi, C.-X. Song, N. Brockdorff, *Nat. Commun.* **2019**, *10*, 3129.
[7] C. A. McHugh, C.-K. Chen, A. Chow, C. F. Surka, C. Tran, P. McDonel, A. Pandya-Jones, M. Blanco, C. Burghard, A. Moradian, M. J. Sweredoski, A. A. Shishkin, J. Su, E. S. Lander, S. Hess, K. Plath, M. Guttman, *Nature* **2015**, *521*, 232.
[8] C. Chu, Q. C. Zhang, S. T. da Rocha, R. A. Flynn, M. Bharadwaj, J. M. Calabrese, T. Magnuson, E. Heard, H. Y. Chang, *Cell* **2015**, *161*, 404.
[9] S. T. da Rocha, E. Heard, *Nat. Struct. Mol. Biol.* **2017**, *24*, 197.
[10] A. Cerase, A. Armaos, C. Neumayer, P. Avner, M. Guttman, G. G. Tartaglia, *Nat. Struct. Mol. Biol.* **2019**, *26*, 331.
[11] F. Yang, T. Babak, J. Shendure, C. M. Disteche, *Genome Res.* **2010**, *20*, 614.
[12] L. Carrel, C. J. Brown, *Philos. Trans. R. Soc., B* **2017**, *372*, 20160355.
[13] W. J. Kent, *Genome Res.* **2002**, *12*, 996.
[14] E. P. Nora, B. R. Lajoie, E. G. Schulz, L. Giorgetti, I. Okamoto, N. Servant, T. Piolot, N. L. van Berkum, J. Meisig, J. Sedat, J. Gribnau, E. Barillot, N. Blüthgen, J. Dekker, E. Heard, *Nature* **2012**, *485*, 381.
[15] I. Okamoto, C. Patrat, D. Thépot, N. Peynot, P. Fauque, N. Daniel, P. Diabangouaya, J.-P. Wolf, J.-P. Renard, V. Duranthon, E. Heard, *Nature* **2011**, *472*, 370.
[16] V. Mutzel, I. Okamoto, I. Dunkel, M. Saitou, L. Giorgetti, E. Heard, E. G. Schulz, *Nat. Struct. Mol. Biol.* **2019**, *26*, 350.
[17] E. J. Sousa, H. T. Stuart, L. E. Bates, M. Ghorbani, J. Nichols, S. Dietmann, J. C. R. Silva, *Cell Stem Cell* **2018**, *22*, 919.
[18] H. Shiura, K. Abe, *Sci. Rep.* **2019**, *9*, 3637.
[19] S. Petropoulos, D. Edsgärd, B. Reinius, Q. Deng, S. P. Panula, S. Codeluppi, A. P. Reyes, S. Linnarsson, R. Sandberg, F. Lanner, *Cell* **2016**, *165*, 1012.
[20] G. F. Kay, S. C. Barton, M. A. Surani, S. Rastan, *Cell* **1994**, *77*, 639.
[21] I. Okamoto, A. P. Otte, C. D. Allis, D. Reinberg, E. Heard, *Science* **2004**, *303*, 644.
[22] P. Navarro, I. Chambers, V. Karwacki-Neisius, C. Chureau, C. Morey, C. Rougeulle, P. Avner, *Science* **2008**, *321*, 1693.
[23] M. E. Donohoe, S. S. Silva, S. F. Pinter, N. Xu, J. T. Lee, *Nature* **2009**, *460*, 128.
[24] J. Silva, J. Nichols, T. W. Theunissen, G. Guo, A. L. van Oosten, O. Barrandon, J. Wray, S. Yamanaka, I. Chambers, A. Smith, *Cell* **2009**, *138*, 722.
[25] C. Gontan, E. M. Achame, J. Demmers, T. S. Barakat, E. Rentmeester, W. van IJcken, J. A. Grootegoed, J. Gribnau, *Nature* **2012**, *485*, 386.
[26] P. A. Jacobs, J. A. Strong, *Nature* **1959**, *183*, 302.
[27] M. L. Barr, D. H. Carr, *Can. Med. Assoc. J.* **1960**, *83*, 979.
[28] S. Speirs, J. M. Cross, M. H. Kaufman, *Genet. Res.* **1990**, *56*, 107.
[29] S. Endo, N. Takagi, M. Sasaki, *Dev. Genet.* **1982**, *3*, 165.
[30] K. Monkhorst, B. de Hoon, I. Jonkers, E. Mulugeta Achame, W. Monkhorst, J. Hoogerbrugge, E. Rentmeester, H. V. Westerhoff, F. Grosveld, J. A. Grootegoed, J. Gribnau, *PLoS One* **2009**, *4*, e5616.
[31] K. Monkhorst, I. Jonkers, E. Rentmeester, F. Grosveld, J. Gribnau, *Cell* **2008**, *132*, 410.
[32] M. Lyon, *Nature, New Biol.* **1971**, *232*, 229.
[33] D. E. Comings, *Am. J. Hum. Genet.* **1968**, *20*, 440.
[34] S. Ohno, *Annu. Rev. Genet.* **1969**, *3*, 495.
[35] S. Rastan, *Development* **1983**, *22*, 1.
[36] J. T. Lee, N. Lu, *Cell* **1999**, *99*, 47.
[37] A. Raj, A. van Oudenaarden, *Cell* **2008**, *135*, 216.
[38] T. B. Kepler, T. C. Elston, *Biophys. J.* **2001**, *81*, 3116.
[39] J. L. Spudich, D. E. Koshland, *Nature* **1976**, *262*, 467.
[40] M. Nicodemi, A. Prisco, *PLoS Comput. Biol.* **2007**, *3*, e210.
[41] M. Nicodemi, A. Prisco, *Phys. Rev. Lett.* **2007**, *98*, 108104.
[42] M. M. Grumbach, A. Morishima, J. H. Taylor, *Proc. Natl. Acad. Sci. USA* **1963**, *49*, 581.
[43] C. P. Bacher, M. Guggiari, B. Brors, S. Augui, P. Clerc, P. Avner, R. Eils, E. Heard, *Nat. Cell Biol.* **2006**, *8*, 293.
[44] N. Xu, C.-L. Tsai, J. T. Lee, *Science* **2006**, *311*, 1149.
[45] S. Augui, G. J. Filion, S. Huart, E. Nora, M. Guggiari, M. Maresca, A. F. Stewart, E. Heard, *Science* **2007**, *318*, 1632.
[46] T. Pollex, E. Heard, *Nat. Genet.* **2019**, *51*, 285.
[47] T. S. Barakat, F. Loos, S. van Staveren, E. Myronova, M. Ghazvini, J. A. Grootegoed, J. Gribnau, *Mol. Cell* **2014**, *53*, 965.
[48] Q. Zhang, S. Bhattacharya, M. E. Andersen, *Open Biol.* **2013**, *3*, 130031.
[49] J. E. Ferrell, S. H. Ha, *Trends Biochem. Sci.* **2014**, *39*, 556.
[50] J. Starmer, T. Magnuson, *Development* **2009**, *136*, 1.
[51] S. M. Gartler, A. D. Riggs, *Annu. Rev. Genet.* **1983**, *17*, 155.
[52] J. T. Lee, *Science* **2005**, *309*, 768.
[53] C. C. Henery, J. B. Bard, M. H. Kaufman, *Dev. Biol.* **1992**, *152*, 233.
[54] J. E. Ferrell, *Curr. Opin. Cell Biol.* **2002**, *14*, 140.
[55] J. X. Zhou, S. Huang, *Trends Genet.* **2011**, *27*, 55.
[56] T. S. Gardner, C. R. Cantor, J. J. Collins, *Nature* **2000**, *403*, 339.
[57] F. O. Francisco, B. Lemos, *J. Genom.* **2014**, *2*, 94.
[58] P. J. Wijchers, R. J. Festenstein, *Trends Genet.* **2011**, *27*, 132.
[59] N. Stavropoulos, N. Lu, J. T. Lee, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10232.
[60] S. Mlynarczyk-Evans, M. Royce-Tolland, M. K. Alexander, A. A. Andersen, S. Kalantry, J. Gribnau, B. Panning, *PLoS Biol.* **2006**, *4*, e159.
[61] I. B. Dodd, M. A. Micheelsen, K. Sneppen, G. Thon, *Cell* **2007**, *129*, 813.
[62] A. K. Alsing, K. Sneppen, *Nucleic Acids Res.* **2013**, *41*, 4755.
[63] D. B. Lyons, A. Magklara, T. Goh, S. C. Sampath, A. Schaefer, G. Schotta, S. Lomvardas, *Cell Rep.* **2014**, *9*, 884.
[64] T. Ferreira, S. R. Wilson, Y. G. Choi, D. Risso, S. Dudoit, T. P. Speed, J. Ngai, *Neuron* **2014**, *81*, 847.

[65] S. Serizawa, T. Ishii, H. Nakatani, A. Tsuboi, F. Nagawa, M. Asano, K. Sudo, J. Sakagami, H. Sakano, T. Ijiri, Y. Matsuda, M. Suzuki, T. Yamamori, Y. Iwakura, H. Sakano, *Nat. Neurosci.* **2000**, *3*, 687.
[66] B. M. Shykind, S. C. Rohani, S. O'Donnell, A. Nemes, M. Mendelsohn, Y. Sun, R. Axel, G. Barnea, *Cell* **2004**, *117*, 801.
[67] I. Jonkers, T. S. Barakat, E. M. Achame, K. Monkhorst, A. Kenter, E. Rentmeester, F. Grosveld, J. A. Grootegoed, J. Gribnau, *Cell* **2009**, *139*, 999.
[68] C. Gontan, H. Mira-Bontenbal, A. Magaraki, C. Dupont, T. S. Barakat, E. Rentmeester, J. Demmers, J. Gribnau, *Nat. Commun.* **2018**, *9*, 4752.
[69] T. S. Barakat, N. Gunhanlar, C. G. Pardo, E. M. Achame, M. Ghazvini, R. Boers, A. Kenter, E. Rentmeester, J. A. Grootegoed, J. Gribnau, *PLoS Genet.* **2011**, *7*, e1002001.
[70] J. Shin, M. Bossenz, Y. Chung, H. Ma, M. Byron, N. Taniguchi-Ishigaki, X. Zhu, B. Jiao, L. L. Hall, M. R. Green, S. N. Jones, I. Hermans-Borgmeyer, J. B. Lawrence, I. Bach, *Nature* **2010**, *467*, 977.
[71] J. Shin, M. C. Wallingford, J. Gallant, C. Marcho, B. Jiao, M. Byron, M. Bossenz, J. B. Lawrence, S. N. Jones, J. Mager, I. Bach, *Nature* **2014**, *511*, 86.
[72] F. Wang, K. N. McCannell, A. Bošković, X. Zhu, J. Shin, J. Yu, J. Gallant, M. Byron, J. B. Lawrence, L. J. Zhu, S. N. Jones, O. J. Rando, T. G. Fazzio, I. Bach, *Cell Rep.* **2017**, *21*, 3691.
[73] A. Sigal, R. Milo, A. Cohen, N. Geva-Zatorsky, Y. Klein, Y. Liron, N. Rosenfeld, T. Danon, N. Perzov, U. Alon, *Nature* **2006**, *444*, 643.
[74] S. Sun, B. C. Del Rosario, A. Szanto, Y. Ogawa, Y. Jeon, J. T. Lee, *Cell* **2013**, *153*, 1537.
[75] D. Tian, S. Sun, J. T. Lee, *Cell* **2010**, *143*, 390.
[76] O. Rosspopoff, C. Huret, A. J. Collier, M. Casanova, P. J. Rugg-Gunn, J.-F. Ouimette, C. Rougeulle, *BioRxiv* **2019**. https://doi.org/10.1101/689430
[77] C. Chureau, S. Chantalat, A. Romito, A. Galvani, L. Duret, P. Avner, C. Rougeulle, *Hum. Mol. Genet.* **2011**, *20*, 705.
[78] G. Furlan, N. G. Hernandez, C. Huret, R. Galupa, J. G. van Bemmel, A. Romito, E. Heard, C. Morey, C. Rougeulle, *Mol. Cell* **2018**, *70*, 462.
[79] J. Holzmann, A. Z. Politi, K. Nagasaka, M. Hantsche-Grininger, N. Walther, B. Koch, J. Fuchs, G. Dürnberger, W. Tang, R. Ladurner, R. R. Stocsits, G. A. Busslinger, B. Novák, K. Mechtler, I. F. Davidson, J. Ellenberg, J.-M. Peters, *eLife* **2019**, *8*, e46269.
[80] C. M. Syrett, V. Sindhava, S. Hodawadekar, A. Myles, G. Liang, Y. Zhang, S. Nandi, M. Cancro, M. Atchison, M. C. Anguera, *PLoS Genet.* **2017**, *13*, e1007050.
[81] A. G. Chapman, A. M. Cotton, A. D. Kelsey, C. J. Brown, *BMC Genet.* **2014**, *15*, 89.
[82] M. Makhlouf, J.-F. Ouimette, A. Oldfield, P. Navarro, D. Neuillet, C. Rougeulle, *Nat. Commun.* **2014**, *5*, 4878.
[83] M. E. Donohoe, L.-F. Zhang, N. Xu, Y. Shi, J. T. Lee, *Mol. Cell* **2007**, *25*, 43.
[84] J. D. Kim, C. Faulk, J. Kim, *Nucleic Acids Res.* **2007**, *35*, 3442.
[85] J. T. Lee, L. S. Davidow, D. Warshawsky, *Nat. Genet.* **1999**, *21*, 400.
[86] T. Sado, Y. Hoki, H. Sasaki, *Dev. Cell* **2005**, *9*, 159.
[87] P. Navarro, D. R. Page, P. Avner, C. Rougeulle, *Genes Dev.* **2006**, *20*, 2787.
[88] P. Navarro, S. Pichard, C. Ciaudo, P. Avner, C. Rougeulle, *Genes Dev.* **2005**, *19*, 1474.
[89] T. Ohhata, Y. Hoki, H. Sasaki, T. Sado, *Development* **2008**, *135*, 227.
[90] S. Luikenhuis, A. Wutz, R. Jaenisch, *Mol. Cell. Biol.* **2001**, *21*, 8512.
[91] T. B. Nesterova, C. M. Johnston, R. Appanah, A. E. T. Newall, J. Godwin, M. Alexiou, N. Brockdorff, *Genes Dev.* **2003**, *17*, 2177.
[92] B. R. Migeon, A. K. Chowdhury, J. A. Dunston, I. McIntosh, *Am. J. Hum. Genet.* **2001**, *69*, 951.
[93] Y. Ogawa, J. T. Lee, *Mol. Cell* **2003**, *11*, 731.
[94] N. Stavropoulos, R. K. Rowntree, J. T. Lee, *Mol. Cell. Biol.* **2005**, *25*, 2757.
[95] R. Galupa, E. P. Nora, R. Worsley-Hunt, C. Picard, C. Gard, J. G. van Bemmel, N. Servant, Y. Zhan, F. El Marjou, C. Johanneau, P. Diabangouaya, A. Le Saux, S. Lameiras, J. P. da Fonseca, F. Loos, J. Gribnau, S. Baulande, U. Ohler, L. Giorgetti, E. Heard, *Mol. Cell* **2020**, *77*, 352.
[96] C. Vallot, C. Huret, Y. Lesecque, A. Resch, N. Oudrhiri, A. Bennaceur-Griscelli, L. Duret, C. Rougeulle, *Nat. Genet.* **2013**, *45*, 239.
[97] C. Vallot, C. Patrat, A. J. Collier, C. Huret, M. Casanova, T. M. Liyakat Ali, M. Tosolini, N. Frydman, E. Heard, P. J. Rugg-Gunn, C. Rougeulle, *Cell Stem Cell* **2017**, *20*, 102.
[98] G. Guo, F. von Meyenn, M. Rostovskaya, J. Clarke, S. Dietmann, D. Baker, A. Sahakyan, S. Myers, P. Bertone, W. Reik, K. Plath, A. Smith, *Development* **2018**, *145*, dev166397.
[99] A. Sahakyan, R. Kim, C. Chronis, S. Sabri, G. Bonora, T. W. Theunissen, E. Kuoy, J. Langerman, A. T. Clark, R. Jaenisch, K. Plath, *Cell Stem Cell* **2017**, *20*, 87.
[100] T. W. Theunissen, M. Friedli, Y. He, E. Planet, R. C. O'Neil, S. Markoulaki, J. Pontis, H. Wang, A. Iouranova, M. Imbeault, J. Duc, M. A. Cohen, K. J. Wert, R. Castanon, Z. Zhang, Y. Huang, J. R. Nery, J. Drotar, T. Lungjangwa, D. Trono, R. Jaenisch, *Cell Stem Cell* **2016**, *19*, 502.
[101] S. Kilens, D. Meistermann, D. Moreno, C. Chariau, A. Gaignerie, A. Reignier, Y. Lelièvre, M. Casanova, C. Vallot, S. Nedellec, L. Flippe, J. Firmin, J. Song, E. Charpentier, J. Lammers, A. Donnart, N. Marec, W. Deb, A. Bihouée, C. Le Caignec, Milieu Intérieur Consortium, *Nat. Commun.* **2018**, *9*, 360.
[102] P. Navarro, M. Moffat, N. P. Mullin, I. Chambers, *Hum. Genet.* **2011**, *130*, 255.
[103] Z. Ma, T. Swigut, A. Valouev, A. Rada-Iglesias, J. Wysocka, *Nat. Struct. Mol. Biol.* **2011**, *18*, 120.
[104] P. Navarro, A. Oldfield, J. Legoupi, N. Festuccia, A. Dubois, M. Attia, J. Schoorlemmer, C. Rougeulle, I. Chambers, P. Avner, *Nature* **2010**, *468*, 457.
[105] F. Loos, C. Maduro, A. Loda, J. Lehmann, G.-J. Kremers, D. Ten Berge, J. A. Grootegoed, J. Gribnau, *Mol. Cell. Biol.* **2016**, *36*, 2656.
[106] X. Chen, H. Xu, P. Yuan, F. Fang, M. Huss, V. B. Vega, E. Wong, Y. L. Orlov, W. Zhang, J. Jiang, Y.-H. Loh, H. C. Yeo, Z. X. Yeo, V. Narang, K. R. Govindarajan, B. Leong, A. Shahab, Y. Ruan, G. Bourque, W.-K. Sung, H.-H. Ng, *Cell* **2008**, *133*, 1106.
[107] A. Marson, S. S. Levine, M. F. Cole, G. M. Frampton, T. Brambrink, S. Johnstone, M. G. Guenther, W. K. Johnston, M. Wernig, J. Newman, J. M. Calabrese, L. M. Dennis, T. L. Volkert, S. Gupta, J. Love, N. Hannett, P. A. Sharp, D. P. Bartel, R. Jaenisch, R. A. Young, *Cell* **2008**, *134*, 521.
[108] G. D. Penny, G. F. Kay, S. A. Sheardown, S. Rastan, N. Brockdorff, *Nature* **1996**, *379*, 131.
[109] C. J. Brown, H. F. Willard, *Nature* **1994**, *368*, 154.
[110] G. Csankovszki, B. Panning, B. Bates, J. R. Pehrson, R. Jaenisch, *Nat. Genet.* **1999**, *22*, 323.
[111] A. Wutz, R. Jaenisch, *Mol. Cell* **2000**, *5*, 695.
[112] A. Wutz, T. P. Rasmussen, R. Jaenisch, *Nat. Genet.* **2002**, *30*, 167.
[113] Y. Hoki, N. Kimura, M. Kanbayashi, Y. Amakawa, T. Ohhata, H. Sasaki, T. Sado, *Development* **2009**, *136*, 139.
[114] Y. Sakata, K. Nagao, Y. Hoki, H. Sasaki, C. Obuse, T. Sado, *Development* **2017**, *144*, 2784.
[115] A. Minajigi, J. E. Froberg, C. Wei, H. Sunwoo, B. Kesner, D. Colognori, D. Lessing, B. Payer, M. Boukhali, W. Haas, J. T. Lee, *Science* **2015**, *349*, 282.
[116] D. P. Patil, C.-K. Chen, B. F. Pickering, A. Chow, C. Jackson, M. Guttman, S. R. Jaffrey, *Nature* **2016**, *537*, 369.
[117] C. Beard, E. Li, R. Jaenisch, *Genes Dev.* **1995**, *9*, 2325.
[118] B. Panning, R. Jaenisch, *Genes Dev.* **1996**, *10*, 1991.
[119] R. Duffié, D. Bourc'his, *Curr. Top. Dev. Biol.* **2013**, *104*, 293.

[120] M. V. C. Greenberg, D. Bourc'his, *Nat. Rev. Mol. Cell Biol.* **2019**, *20*, 590.
[121] D. P. Norris, D. Patel, G. F. Kay, G. D. Penny, N. Brockdorff, S. A. Sheardown, S. Rastan, *Cell* **1994**, *77*, 41.
[122] T. Sado, M. Okano, E. Li, H. Sasaki, *Development* **2004**, *131*, 975.
[123] E. G. Schulz, *Philos. Trans. R. Soc., B* **2017**, *372*, 20160366.
[124] E. G. Schulz, J. Meisig, T. Nakamura, I. Okamoto, A. Sieber, C. Picard, M. Borensztein, M. Saitou, N. Blüthgen, E. Heard, *Cell Stem Cell* **2014**, *14*, 203.
[125] J. Song, A. Janiszewski, N. De Geest, L. Vanheer, I. Talon, M. El Bakkali, T. Oh, V. Pasque, *Stem Cell Rep.* **2019**, *12*, 333.
[126] I. Zvetkova, A. Apedaile, B. Ramsahoye, J. E. Mermoud, L. A. Crompton, R. John, R. Feil, N. Brockdorff, *Nat. Genet.* **2005**, *37*, 1274.
[127] M. Delbrück, *Colloq. Int. C. N. R. S.* **1949**, *2*, 33.
[128] A. B. Oppenheim, O. Kobiler, J. Stavans, D. L. Court, S. Adhya, *Annu. Rev. Genet.* **2005**, *39*, 409.
[129] K. Sneppen, *Rep. Prog. Phys.* **2017**, *80*, 042601.
[130] S. Rastan, E. Robertson, *J. Embryol. Exp. Morphol.* **1985**, *388*, 379.

