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DIPLOMARBEIT

Titel der Diplomarbeit

„Characterization of heterochromatin formation in an *Xist* RNA inducible transgenic mouse line"

Verfasserin

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1 Introduction

The genome of every cell in an organism contains the information which is necessary to give rise to all specialized cell types with their different cellular functions. There are several hundred distinct cell types in a human body, which all contribute equally to the complexity of an organism. Therefore, for the successful development of a multicellular organism, cell identity is not simply defined by the genetic information provided by the DNA double helix, but also from transcriptional control of expression. Epigenetic regulation, involving chromatin remodelling, histone modifications and DNA methylation, was identified to play an important role in cell differentiation and therefore in the identity of the cell. Chromatin composition changes during development as shown by changing patterns of epigenetic modifications and the dynamics of chromatin proteins (Meshorer, et al., 2006) (Buszczak and Spradling, 2006). How chromatin modifications are regulated during development and epigenetic patterns are established is presently a focus of research. Understanding of the underlying mechanism of heterochromatin formation and stable gene silencing will help to achieve insights in the mechanism of induced pluripotency which is a current interest in regenerative medicine.

1.1 Dosage compensation

One important example of epigenetic regulation is dosage compensation, which happens in the early embryogenesis. In mammals, the unequal genetic imbalance of XY males and XX females has to be compensated. The mechanism of dosage compensation accomplishes that there is only one active X chromosome (Xa) in every cell of an organism (Lyon, 1961). In female somatic cells this is achieved by silencing all but one X chromosomes by the establishment of a heterochromatic structure and the inactive X chromosome (Xi) was first described as barr body (Barr and Bertram, 1949).

1.2 Dynamic activity state of the inactive X chromosome

The inactive X chromosome undergoes several rounds of in- and reactivation during development (Fig. 1).

During the first cleavages in early female mouse embryonic development, the paternally inherited X chromosome becomes inactive (Okamoto, et al., 2005) (Okamoto, et al., 2004) and remains inactive in extraembryonic tissues like the primitive endoderm and trophectoderm (Heard and Disteche, 2006). At the blastocyst stage at embryonic day 3.5 (E3.5), the X chromosome of cells of the inner cell mass (ICM) is reactivated. These cells give rise to the embryo proper later in development (Mak, et al., 2004). Before gastrulation at E5.5 random X inactivation is accomplished which leads to a mosaic pattern of female somatic cells either with the maternal or the paternal X chromosome inactivated (Heard and Disteche, 2006).

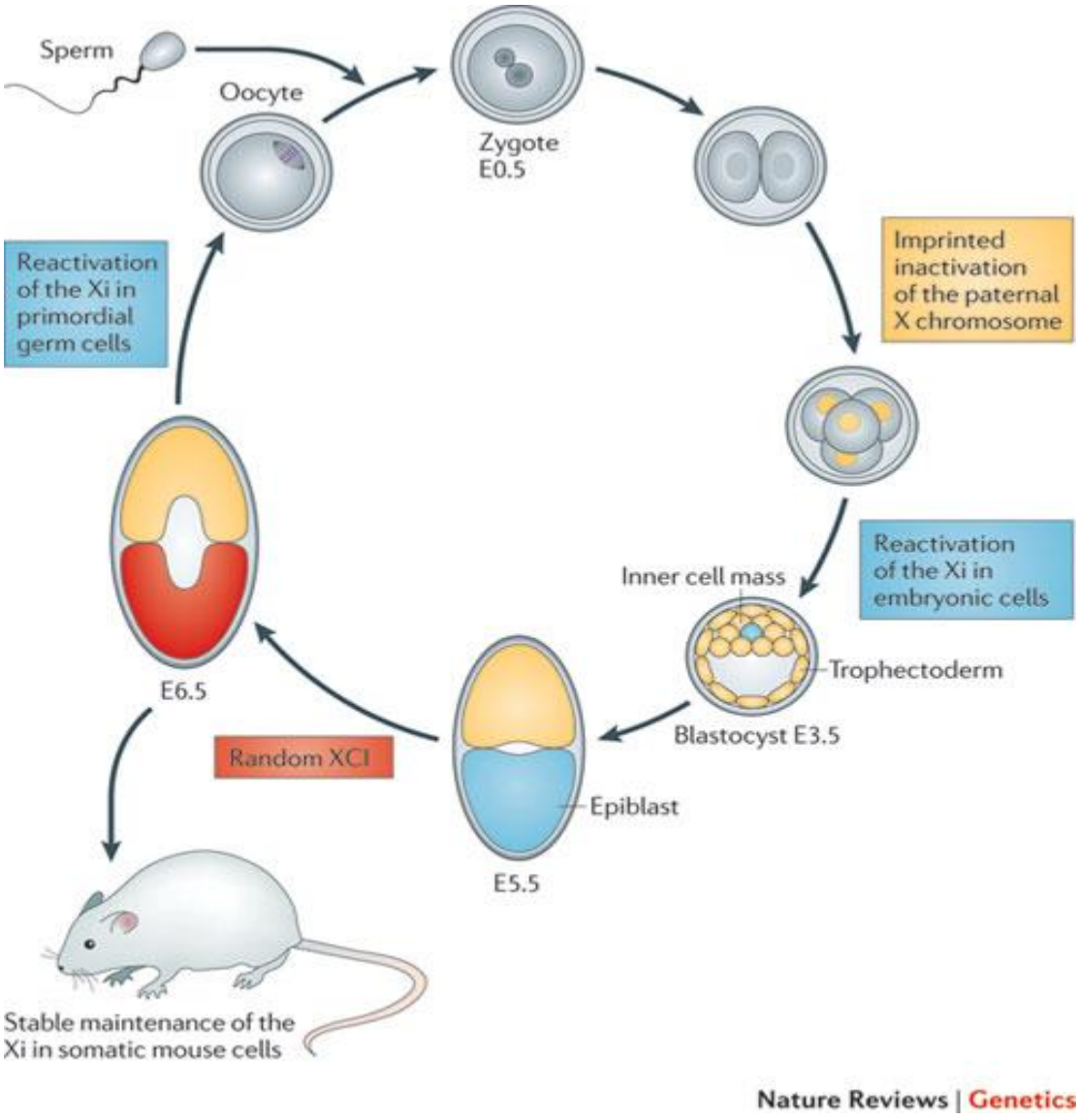


Figure 1 **X chromosome inactivation in mouse embryonic development**
adopted from review Wutz, A. (Wutz, 2011)

Fertilization of the oocyte by a spermatocyte leads to a diploid zygote consisting of a maternal and a paternal chromosome set. After the first cleavages blastocyst stage is achieved at E3.5 consisting of the trophectoderm and the ICM. The paternal X chromosome is inactivated during the first cleavages and is maintained in cells of the extraembryonic tissue. The inactive X chromosome is reactivated in cells of the ICM. In the female developing epiblast two active X chromosomes are present between E3.5 and E5.5 and before gastrulation either the maternal or the paternal X chromosome gets inactive by random X inactivation which is stable inherited in female somatic cells. Another round of reactivation takes place during migration of female primordial germ cells.

The inactive X chromosome is stable in somatic cells (Heard and Disteche, 2006) but gets reactivated in primordial germ cells and during oogenesis in female cells (Chuva de Sousa Lopes, et al., 2008). Pluripotent mouse embryonic stem cells (ESCs), which are derived from female blastocysts (Penny, et al., 1996) have two active X chromosomes. During differentiation X inactivation is established and therefore can be used as an *in vitro* model to study the mechanism of chromosome-wide silencing.

1.3 The X inactivation centre

Silencing of one of the two X chromosomes is regulated by the expression of the non-coding X inactivation specific transcript (*Xist*) from the X inactivation centre (Xic) (Brockdorff, et al., 1991) (Fig. 2). The Xic is an essential gene locus for X inactivation and plays an important role in the counting mechanism that ensures that all but one X chromosomes are silenced per cell (Monkhorst, et al., 2008). The minimal gene region for Xic in mice has been defined and lies between the T16H and the HD3 breakpoint spanning 10-20 Mb (Eicher, et al., 1972) (Rastan, 1983). *Xist* RNA expression is controlled by various regulators located at the Xic.

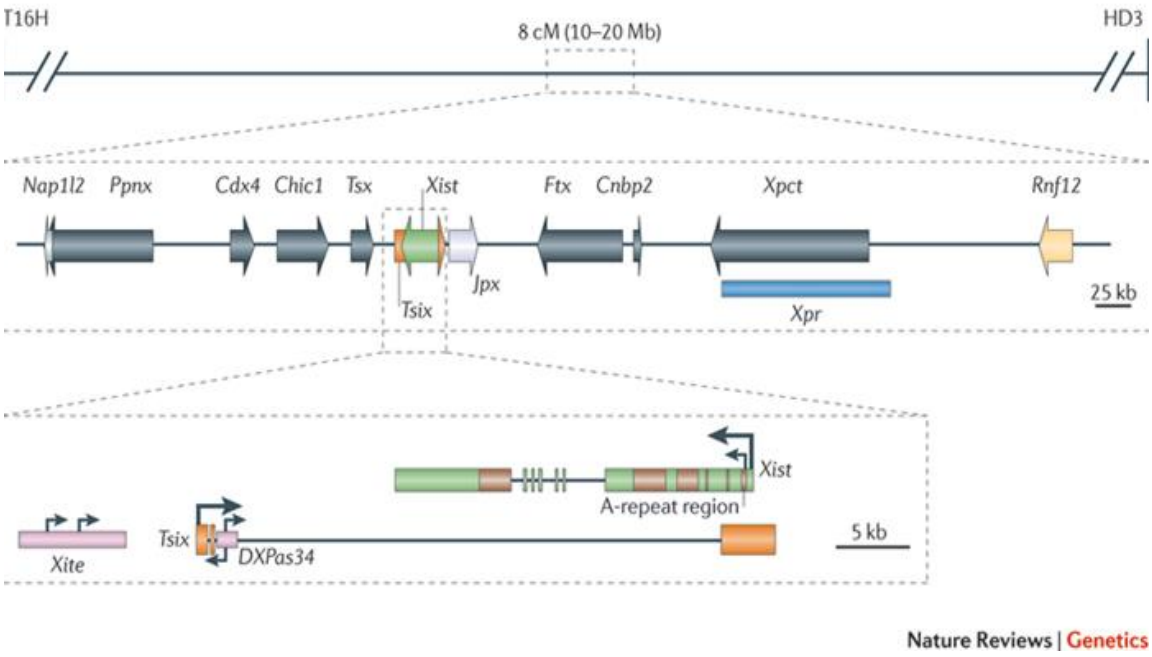


Figure 2 The X inactivation centre (Xic) adopted from (Augui, et al., 2011)

The 10-20 Mb long locus defined as X inactivation centre and surrounding elements are shown: *Nap1l2* (nucleosome assemby protein 1-like 2), *Cdx4* (caudal X linked gene), *Chic1* (cysteine rich hydrophobic1), *Tsx* (testis specific X linked), *Jpx* (expressed neighbour of *Xist*), *Ftx* (five prime to *Xist*), *Cnbp2* (cellular nucleic acid binding protein 2), *Xpct* (X-linked PEST-containing transporter) and *Rnf12* (Ring finger protein 12). *Xist* RNA locus, its antisense gene *Tsix* and the regulators *Xite* (X inactivation intergenic transcription element) and *DXPas34* are indicated in the enlarged image.

How these regulatory elements act in X inactivation still remains to be determined. Previous investigations propose that the *Jpx* locus, which produces a non-coding RNA (ncRNA) triggers female specific *Xist* activation (Tian, et al., 2010). Another ncRNA transcribed from the *Ftx* locus is believed to control the expression of *Xist*, *Tsix* and *Jpx* in male cells (Chureau, et al., 2011). Chromosomal pairing around the Xic regions, transgenic protein factors such as RNF12 and long and short transcripts have been implicated in regulating X inactivation (Augui, et al., 2007) (Bacher, et al., 2006). In addition YY1 and CTCF might structure chromatin at the Xic (Jeon and Lee, 2011). It is currently thought that all these regulatory elements convene at controlling the activation of *Xist*. Once *Xist* is upregulated it initiates inactivation of the X chromosome.

1.4 Chromosome-wide silencing triggered by *Xist* RNA

Several regulators of *Xist* RNA have been identified which include transcription factors such as Nanog and Oct4 (Guo, et al., 2009) (Navarro, et al., 2010) or the non-coding *Tsix* RNA which is transcribed antisense to *Xist* (Lee, et al., 1999). The 17-19 kb long *Xist* RNA is transcribed specifically from the inactive X chromosome and accumulates within the Xi chromosome territory in the interphase nucleus (Clemson, et al., 1996). This is accompanied by the exclusion of parts of the transcription machinery like RNA polymerase II (Pol II) and splicing factors (Okamoto, et al., 2004) (Chaumeil, et al., 2006) known as "Pol II hole". The initiation and following events of chromosome-wide silencing have been studied well in mouse ESCs with transgenes on the X chromosome as well as on autosomes (Wutz and Jaenisch, 2000). While *Xist* RNA expression is essential in the initiation process of chromosome-wide silencing, other mechanisms are responsible for the maintainance and inheritance of the inactive chromosome and *Xist* RNA can not initiate silencing in differentiated cells (Wutz and Jaenisch, 2000). The stable gene silencing on the Xi in somatic cells is maintained by epigenetic mechanisms including DNA methylation (Sado, et al., 2004) and histone H4 hypoacetylation, which is a hallmark of the Xi (Keohane, et al., 1996). After accumulation of *Xist* RNA over the chromosome, Polycomb group (PcG) complexes are recruited which play an important role as histone modifying proteins (Schwartz and Pirrotta, 2008). PcG proteins are known to contribute in development, dosage compensation and *Hox* gene regulation and two PcG complexes accomplish the establishment of chromatin modifications for gene regulation in mammals (Pietersen and van Lohuizen, 2008). The Polycomb-repressive complex 2 (PRC2) causes trimethylation of histone H3 lysine 27 (H3K27m3) (Czermin, et al., 2002) and PRC1 mediates mono-ubiquitination of histone H2A lysine 119 (H2AK119ub1) (de Napoles, et al., 2004). PRC2 consists of Eed, Suz12 and Ezh2 proteins and a mutation in Eed causes the loss of H3K27m3 (Boyer, et al., 2006). PRC1 contains the proteins Ring1A and Ring1B. The loss of Ring1B causes derepression of developmental control genes but its function in X inactivation seems to be compensated by other complexes (Leeb and Wutz, 2007).

1.5 *Xist* function during development

The ability of expressed *Xist* RNA to induce chromosome-wide silencing depends on a cellular context and timepoint in development. Up to embryonic day 9.5, *Xist* RNA remains functional in the initiation of gene silencing, whereas *Xist* in differentiated cells as mouse embryonic fibroblasts (MEFs) loses silencing function but still localizes to the chromosome (Wutz and Jaenisch, 2000). Furthermore, studies showed that expression of *Xist* RNA after embryonic day 12.5 is compatible with development but the haematopoietic system is affected leading to perinatal lethality (Savarese, et al., 2006). These investigations showed that precursor cells in the haematopoietic system possess the cellular context for *Xist* RNA initiation of gene silencing (Agrelo, et al., 2009).

1.6 *Xist* RNA composition

Established ESC systems led the way to investigate X inactivation in an *in vitro* model and introduction of transgenes carrying deletions allowed to investigate *Xist* RNA domains important for localization, modifications and gene repression (Fig. 3). A deletion study showed that the 17-19 kb long mouse *Xist* RNA contains several sequence domains that mediate localization but none of them is essential and can be compensated (Wutz, et al., 2002). The nuclear protein Saf-A/hnRNPU co-localizes with *Xist* RNA in the nucleus and is thought to contribute to chromosome attachment (Kukalev, et al., 2009). Previous investigations identified the protein YY1 contributing to *Xist* RNA loading onto the inactive X chromosome. YY1 is a bivalent protein which binds the AAnATGGCG motif on DNA and *Xist* RNA via Repeat C and is essential for *Xist* RNA localisation (Jeon and Lee, 2011).

Chromatin modifying complexes as PRC1 and PRC2 are recruited and H3K27m3, H2AK119ub1 and H4K20m1 are established at the *Xist* RNA localization site. Furthermore, the histone variant macroH2A and the chromosomal protein Ash2L are recruited to contribute to the formation of a repressive compartment (Beletskii, et al., 2001) (Pullirsch, et al., 2010). Whereas macroH2A and Ash2L require *Xist* for their recruitment to the Xi, H4 hypoacetylation is stable when *Xist* expression is lost due to a

somatic deletion (Csankovszki, et al., 1999). H4 hypoacetylation on the Xi is established at the transition to maintenance of XCI (Pullirsch, et al., 2010). How H4 hypoacetylation is maintained on the Xi independent of *Xist* is unclear.

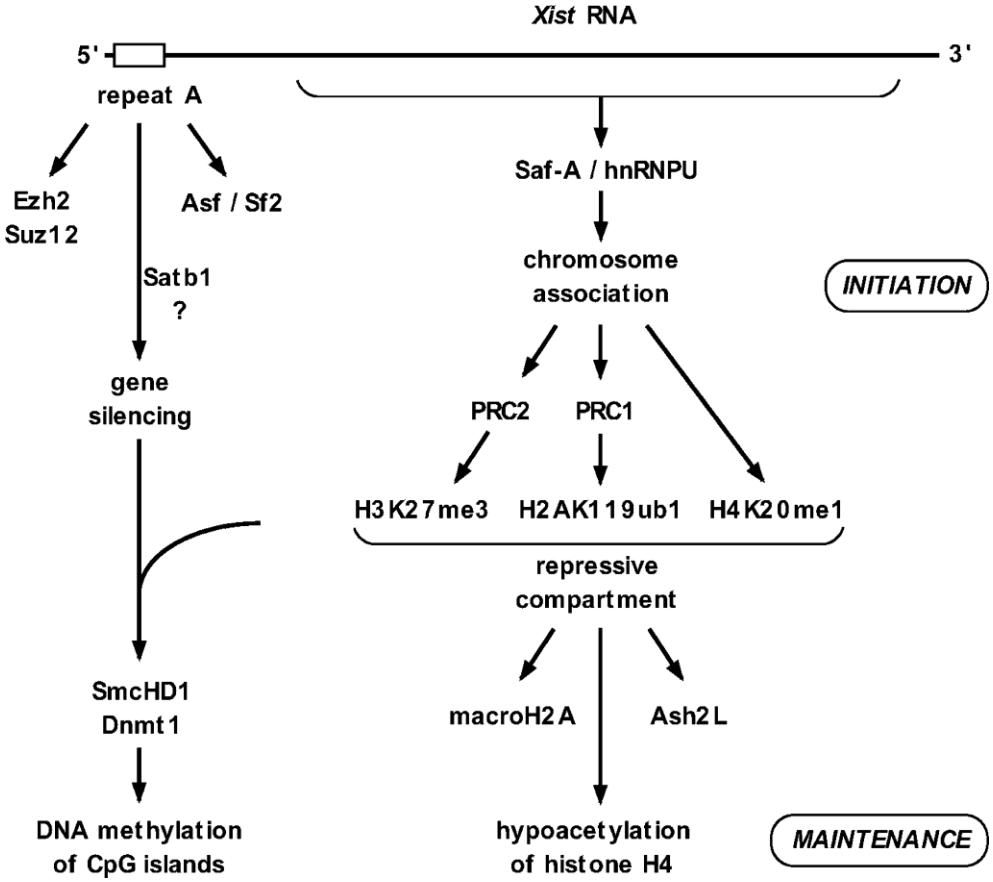


Figure 3 ***Xist* RNA sequence function** adopted from (Arthold, et al., 2011)

17-19 kb long *Xist* RNA with proteins and chromatin modifications necessary for X inactivation are indicated. The 500 nucleotide long repeat A sequence at the 5´region is essential for gene repression. Proteins such as Ezh2, Suz12, Asf/Sf2 and Satb1 are associated with this domain. More yet unknown proteins with function in the cellular context of gene silencing are indicated with a question mark. In somatic cells the maintenance of gene silencing is accomplished by DNA methylation of CpG islands by the proteins SmcHD1 and Dnmt1. A nuclear protein associated with chromosomal localization is Saf-A (also known as hnRNPU), chromatin modifying complexes as PRC1 and PRC2 and chromosomal proteins as macroH2A and Ash2L are recruited to the Xi. Histone H4 hypoacetylation is established independent of *Xist* RNA accumulation.

1.7 Repeat A and stable gene silencing

Deletion studies revealed a repeated sequence at the 5´end of *Xist* RNA which is essential for the initiation of gene repression and is called repeat A sequence (Wutz, et al., 2002). *Xist* RNA carrying this 500 bp deletion still localizes to the chromosome and all chromatin modifications are established, but no gene silencing can be observed (Chaumeil, et al., 2006). Further investigations showed that male ESCs carrying the *Xist* RNA lacking repeat A on their X chromosome are able to differentiate in vitro with just little effect on gene expression and the term of Xiag chromosome (inactive X chromosome with active genes) was established (Pullirsch, et al., 2010). Proteins interacting with repeat A sequence include Ezh2 and Suz12, which are part of the PRC2 complex (Cao, et al., 2002) and the splicing factor Asf/Sf2 (Royce-Tolland, et al.). The homeobox domain protein Satb1 plays an important role in the cellular context of gene silencing in T cell differentiation (Agrelo, et al., 2009) and proteins which might act similar in function still have to be investigated. To accomplish stable gene silencing in differentiated somatic cells, DNA methylation of CpG rich promoters are established by SmcHD1 and Dnmt1 (Blewitt, et al., 2008; Sado, et al., 2000; Vasques, et al., 2005). Studies of the effect of Dnmt1 null mutations in mice on X inactivation showed that imprinted genes in the extraembryonic tissue are uneffected whilst random X inactivation in cells of the embryo proper was unstable and imprinted genes got reactivated due to hypomethylation (Sado, et al., 2000). These investigations suggest that methylation is required to maintain stable X inactivation in cells of the embryonic lineages but imprinting in cells of the extraembryonic lineages is compatible with demethylation. Furthermore, studies on somatic cells with a conditional mutant *Xist* allele show that *Xist* RNA expression, DNA methylation and histone H4 hypoacetylation act synergistically to maintain stable gene silencing in somatic cells (Csankovszki, et al., 2001).

1.8 Model of a repressive compartment

To explain the discovery of a chromosome possessing all heterochromatin marks known from the inactive X chromosome without significant gene silencing, a model of a repressive compartment has been suggested, whereof genes and non-genic regions might be regulated separately (Chaumeil, et al., 2006) (Fig. 4). This model aims to explain how genes escape repression by coexistent formation of heterochromatin in cells expressing *Xist* RNA without repeat A. It is believed that actively transcribed genes locate on the periphery of the chromosome territory where they are accessible to components of the transcription machinery. The repressive compartment is established on the Xi chromosomal territory where *Xist* RNA accumulates. Chromatin modifying complexes as PRC1 and PRC2 are recruited to this area, components of the transcription machinery are excluded and late replication is established. It is believed that in the case of *Xist* RNA without repeat A genes might not associate with the repressive environment and escape transcriptional silencing.

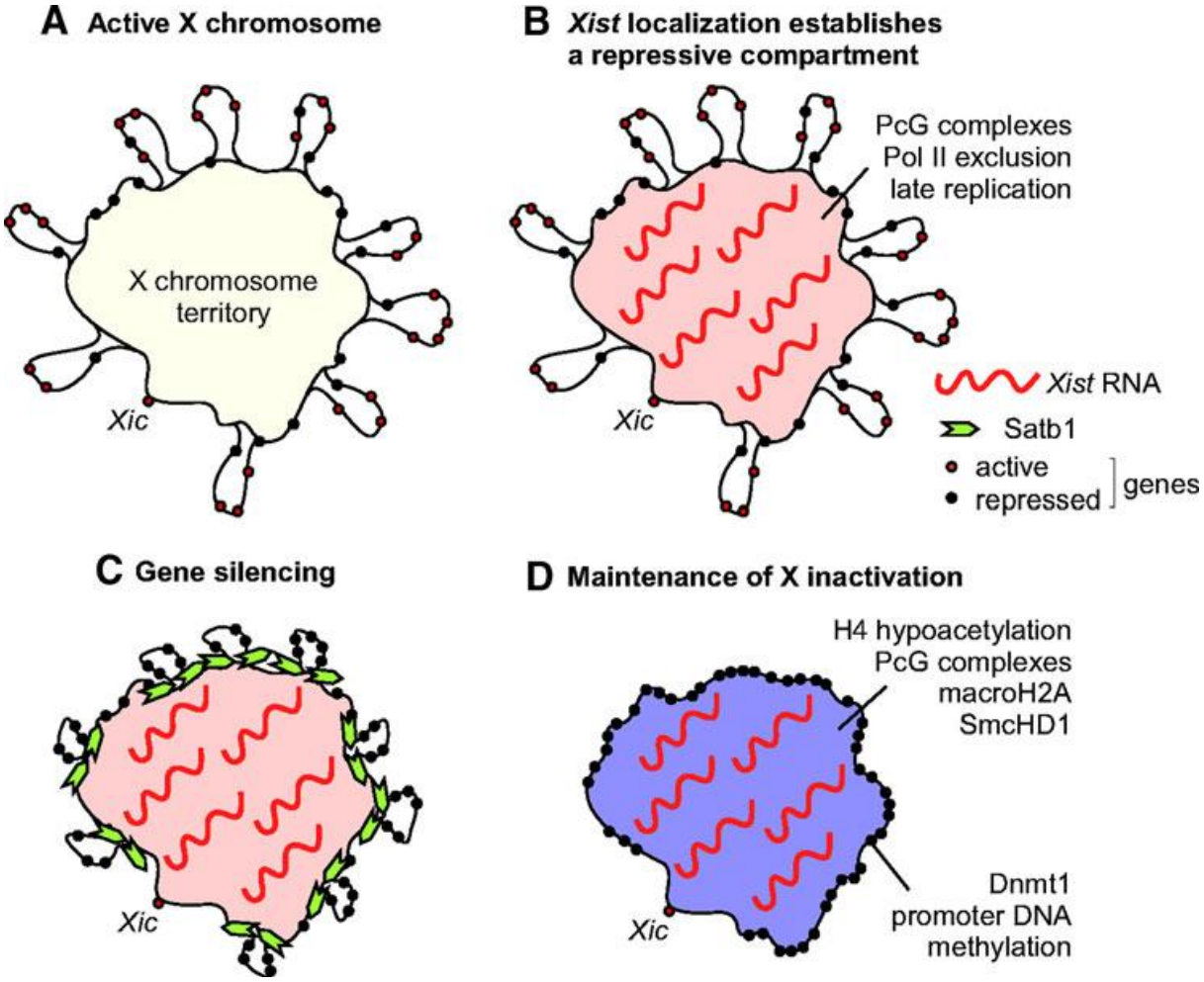


Figure 4 **Repressive compartment** adopted from (Arthold, et al., 2011)

Before inactivation, the X chromosome (A) shows active transcribed genes on the periphery of the chromosomal compartment. After *Xist* RNA expression it localizes over the territory of the inactive X chromosome (B, indicated in red). The repressive compartment is established by recruitment of chromatin modification complexes, exclusion of factors of the transcription machinery and late replication. For gene silencing a developmental context and repeat A sequence of *Xist* RNA are required. It is thought that proteins as Satb1 might be necessary to link chromatin loops to the repressive compartment and induce repression (C). The maintenance of silenced genes is accomplished by histone H4 hypoacetylation, chromatin modifications established by PcG complexes and recruitment of macroH2A, SmcHD1 and Dnmt1 (D).

1.9 Inducible *Xist* RNA expression system

For studying *Xist* RNA expression, its role in initiation of chromosome-wide silencing and the establishment of chromatin modifications, an inducible heterologous expression system in mouse ESC is used (Fig. 5). The transactivator protein nls-rtTA (indicated in blue) is expressed from the ubiquitously active Rosa26 (R26) locus. By the addition of doxycycline (dox, indicated in green) the transactivator can bind to the promoter sequence in trans on chromosome 11 and induces *Xist* RNA, without repeat A (ColXist, indicated in red) expression.

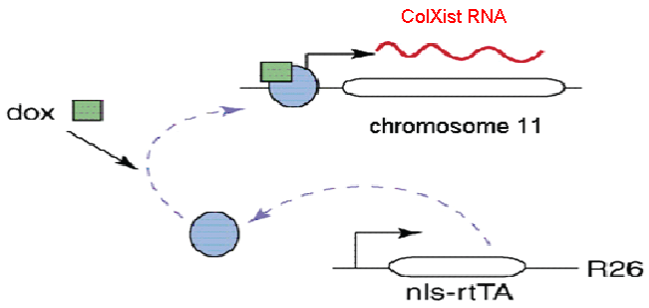


Figure 5 **Inducible *Xist* RNA expression system** adopted from (Wutz, 2007)

The used inducible *Xist* RNA expression system is shown schematically. The in trans transcribed transactivator nls-rtTA (blue) is expressed from the R26 locus and can bind to the promoter on chromosome 11 with addition of doxycycline (green). Transgene *ColXist* RNA (red) expression is induced.

This system provides for investigations on *Xist* RNA function in different timepoints in development and transgene expression on X chromosome as well as on various autosomes. Previous studies to obtain insight in the complex mechanism of X inactivation and chromosome-wide silencing were performed using this established inducible system, e.g. on investigating the developmental window in which *Xist* RNA can induce silencing (Wutz and Jaenisch, 2000), deletion studies to investigate sequence functions of Xist (Wutz, et al., 2002), studies on chromosomal protein recruitment (Pullirsch, et al.) or the role of Polycomb group in X inactivation (Leeb and Wutz, 2007) (Leeb, et al.).

1.10 Aims of this study

1.10.1 Consequences of *ColXist* RNA expression in early mouse development

Previous investigations showed that the expression of an *Xist* RNA lacking repeat A from the X chromosome in male embryonic stem cells is compatible with differentiation *in vitro* (Pullirsch, et al., 2010), which suggests no significant silencing function of the transgenic *Xist* RNA. This study aims to investigate if the expression of *Xist* RNA without repeat A, expressed from chromosome 11, is also compatible with mouse embryonic development. The phenotypic consequences of homozygous and heterozygous *ColXist* RNA expression from embryonic day 4.5 will be investigated and the consequences of chromatin modifications and gene silencing will be studied.

1.10.2 Effect of *ColXist* RNA expression in tissue stem cell niches

The second aim of this study is to investigate the effect of C*olXist* RNA expression in developing mice from embryonic day 12.5 to obtain insight on the effect in the haematopoietic system. Previous studies showed that *Xist* RNA induction after E12.5 is compatible with normal development until birth, but leads to death shortly after birth, due to severe problems in haematopoiesis (Savarese, et al., 2006). To investigate the effect of transgenic *ColXist* RNA expression from chromosome 11, heterozygous induction will be accomplished over a time period of four weeks and phenotypic consequences will be studied.

1.10.3 Genome-wide gene expression profiling

The third aim of this study is to identify to which extent homozygous expression of *ColXist* RNA lacking repeat A leads to misregulation of genes. Therefore, an established *in vitro* ESC system is used to compare expression differences of genes in cells with or without *ColXist* RNA induction in differentiation using Affymetrix analysis. Furthermore, it will be investigated if identified misregulated genes are

expressed in ES cells and induced upon lineage formation in differentiation and if these genes are Polycomb target genes or targets of other epigenetic systems.

These investigations will provide insights into the function of epigenetic modifications in development. The observation of a chromosome which carries all chromatin modifications known from the Xi (Xiag) with only little effect on gene expression was surprising (Pullirsch, et al., 2010) and with this study we hope to get insights into the underlying molecular mechanisms. Furthermore, we address the question how the non-coding *Xist* RNA lacking repeat A prevents gene upregulation in an autosomal context that is independent of its gene repression function.

2 Materials and Methods

2.1 Cultivation of eukaryotic cells

Cell culture work was performed under sterile, class II safety conditions. Used solutions were steril-filtered (0,2% gelatine was autoclaved) and all consumables desinfected with 70% ethanol before use. Cells were cultured in a CO_2 incubator (Sanyo, 37°C, 5% CO_2).

2.1.1 Consumable materials

Aspirator pipettes	FALCON™
Cell culture plates	6-well plate, 48-well plate, 96-well plate, IWAKI microplate, polystyrene
Cell culture flasks	T25, T75, IWAKI, polystyrene
Cell culture dishes	FALCON™, polystyrene
Pipettes	5 ml, 10 ml, 25 ml, COSTAR® Stripette
Tubes	15 ml, 50 ml, FALCON™

2.1.2 Solutions

Accutase	provided by tissue culture core facility
Bovine serum, heat inactivated	GIBCO, Ref# 26170-035
BSA fraction V	GIBCO, Ref# 15260-037
β-Mercaptoethanol	Aldrich, Cat# M6250
Concanavalin A	Calbiochem, Cat# 234567
DMEM	PAA, high glucose 4,5g/l, without L-glutamine, Cat# E15-009
DMEM/Ham´s F-12	PAA, without L-glutamine, Cat# E15-012
DMSO,Dimethyl Sulfoxide	Sigma, D8418
Doxycycline	1mg/ml (1000x) stock

Foetal Bovine Serum	PAA, A15-080
Fibronectin	Millipore, hu plasma fibronectin, FC010
Gelatine	0,2% in H_2O, autoclaved
HEPES buffer 1M	PAA, Cat# S11-001
L-glutamine	GIBCO, 200mM, Ref# 25030-024
M2 medium	SIGMA-ALDRICH, M7167
M16 medium	SIGMA-ALDRICH, M7292
N2B27	Ndiff®, neural differentiation medium, Stem Cells Inc., Cat# SCS-SF-NB-02
Non essential amino acids (NEAA)	GIBCO, 100x, Ref# 11140-035
PBS with Ca+Mg	PAA, Dulbecco´s 1x phosphate buffered saline, Cat# H15-001
PBS without Ca+Mg	PAA, Dulbecco´s 1x phosphate buffered saline, Cat# H15-002
PenStrep	GIBCO, 5000 units penicillin, 5000 µg streptomycin, Cat# 15070-063
RPMI-1640	GIBCO, Cat# 31870
Retinoic acid	10mg/ml stock
Sodium pyruvate	GIBCO, 100mM, Ref# 11360-039
Trypsin/0,02% EDTA	*used 0,25% in PBS,* GIBCO

2.1.3 Established cell lines

X^3 cells, mouse fibroblasts, female

X^3 cells are derived from a mammary cell line with a stable karyotype and two inactive X chromosomes (Wutz and Jaenisch, 2000).

2.1.4 Primary cell lines

MEFs derived from transgene bearing COLtetOP Xist ΔSX/+R26TA [H] mice, E9.5 to E14.5

For the isolation of MEFs, pregnant mice were sacrificed at E9.5 to E14.5, uteri dissected and embryos kept in PBS. The embryos were transferred to a sterile tissue culture hood, tissue was disintegrated with a syringe and cells cultured in MEFs medium either with or without doxycycline at 37°C/5% CO_2.

2.1.4.1 Thymus and spleen cells

Splenocyte culture medium

250ml RPMI
+ 10% heat inactivated serum
+ 2,5ml HEPES buffer
+ 2,5ml PenStrep
+ 0,7µl β-Mercaptoethanol
add 40µg/ml concanavalin A shortly before use

For the isolation of thymus and spleen cells, 19 and 20 days old mice, heterozygous for inducible *ColXist* RNA, with doxycycline induction for approximately 4 weeks, were sacrificed, thymus and spleen were dissected and kept in PBS. In a sterile cell culture hood, thymus and spleen tissues were disintegrated with a cell strainer (BD FALCON, 70µm nylon) and cultured in splenocyte medium, with or without doxycycline, at 37°C/5% CO_2. The mitogen concanavalin A was used for stimulation of proliferation *in vitro* and IF analysis was performed after two days in culture.

2.1.4.2 ESC derivation

ESC medium (serum and LIF)

200ml DMEM

+15% foetal bovine serum
+ 2ml PenStrep
+ 2ml pyruvate
+ 2ml L-glutamine
+ 2ml NEAA
+ 200µl murine LIF

stock of inhibitors

10µl PDO3
30µl Chiron
in 1ml M16 medium

For the derivation and establishment of ESC lines homozygous for *ColXist*, E3.5 pregnant mice were sacrificed and uteri were dissected. Under a brightfield microscope the uteri were flushed with M2 medium and blastocysts were collected. The blastocysts were transferred to an in vitro fertilization dish with M16 medium, 1ml M16 containing 10µl of inhibitor-stock for expansion of the inner cell mass, and incubated over night at 37°C/5% CO_2. The next day the zona pellucida was removed by incubating the blastocysts quickly in acid tyrodes. After washing in M2 medium, blastocysts were transferred to a 96-well plate in $2i^+$ medium and were incubated for 2 days. For the first passage a 48-well plate was coated for one hour with gelatine, to accomplish proper attachment. Cells were trypsinized with 25µl trypsin/EDTA and trypsinization stopped after 7 minutes by adding ESC medium (with serum and LIF). By pipetting up and down for approximately 10 times a single cell suspension was prepared and cells transferred to a 48-well plate in $2i^+$ medium. Backfreezes were made with passage 4.

2.1.5 Freezing cells

MEFs, X³ and ESCs were mixed 1:1 with 20% DMSO/80% foetal bovine serum in freezing vials (10% DMSO final concentration) and kept at -80°C.

2.1.6 Thawing cells

Cells were thawed in a 37°C waterbath, culture medium was added and cells spun down. The cell pellet was resuspended in culture medium, transferred to a tissue culture flask and further cultured according to cell type.

2.1.7 Cultivation of Mouse Embryonic Fibroblasts (MEFs) and X^3 cells

MEF medium

500ml DMEM
+ 10% foetal bovin serum
+ 5ml PenStrep
+ 5ml L-glutamine
+ 5ml Sodium pyruvate
+ 5ml Non essential amino acids (NEAA)
+ 4µl β-Mercaptoethanol

MEFs and X³ cells were split after they reached about 70% confluence. Therefore, medium was discarded and attached cells washed thoroughly two times with 1xPBS. A few drops of trypsin/EDTA were added and cells incubated at 37°C for 5 minutes. Detached cells were resuspended in 3ml medium and passaged in the correct ratio (1:3 or 1:6) to a new tissue culture flask or plate.

2.1.8 Cultivation of embryonic stem cells in 2i medium

Preparation of $2i^+$ culture medium

200ml N2B27 (either the commercial or the self-made N2B27)

+ 10ml BSA fraction V
+ 400µl LIF
+ 60µl Chiron
+ 20µl PD03

final concentrations of inhibitors are 3µM Chiron and 1µM PD03

N2B27 medium

500mL Neurobasal medium	GIBCO, Ref# 21103-049
500mL DMEM/Ham´s F-12	PAA, without L-glutamine, Cat# E15-012
10mL B-27 supplement (50x)	GIBCO, Ref# 17504-044
10mL Glutamine	
5mL N2 supplement	Batch 0410-015
8µL β-meEtOH	

washing buffer

500ml DMEM Ham´s F-12
+ 3ml BSA fraction V

Mouse embryonic stem cells were cultured and maintained under 2i conditions on gelatine-coated tissue culture plates. Therefore, all used flasks, plates and slides were coated with 0,2% gelatine for at least 30 minutes. For passing, culture medium was discarded and cells were washed 2 times with PBS. For detaching, a few drops of accutase were added and cells incubated for 5min at 37°C. To accomplish a single cell suspension, tissue culture flasks and plates were hit against the hand several times and cells were resuspended thoroughly in 3ml of washing buffer. After centrifugation for 5 minutes at 800rpm the washing buffer was discarded, the pellet resuspended in 1ml of 2i medium and cells were split according to their growth density (1:6, 1:10). ESCs were passaged every other day and cultivated at 37°C/5% CO_2.

2.1.9 ESC differentiation with retinoic acid

Differentiation medium

500ml DMEM
+ 10% foetal bovin serum
+ 5ml PenStrep
+ 5ml L-glutamine
+ 5ml Sodium pyruvate
+ 5ml Non essential amino acids (NEAA)
+ 4µl β-Mercaptoethanol
+ 2µl retinoic acid

For the differentiation of ESCs with retinoic acid, ESCs were detached and pellets resuspended as described above. ESCs were split in ratios depending on their confluence (between 1:20 to 1:40) on gelatine-coated plates in differentiation medium and cultured for 4 to 6 days at 37°C/5% CO_2. Medium was changed after 2 days of culture. Depending on the experimental approach, differentiated cells were either grown on ROBOZ slides for IF stainings or FISH for another night or cells were count and RNA isolated for further gene expression analysis.

2.2 Cytological methods

2.2.1 RNA FISH

Solutions and buffer

1xPBS

Cytosceletal buffer

100 mM NaCl
300 mM sucrose
3 mM $MgCl_2$
10 mM PIPES pH 6,8
(store at 4°C)

Cytosceletal buffer + detergent

100 mM NaCl
300 mM sucrose
3 mM $MgCl_2$
10 mM PIPES ph 6,8
0,5% Triton X-100
(store at 4°C)
(detergent and concentration vary with cell type)

4% paraformaldehyde/1xPBS

Ethanol (abs, 95%, 80%, 70%)

20x SSC

175,3 g NaCl

88,2 g sodium citrate in 800ml of H_2O

adjust pH to 7.0 with a few (!) drops of HCl (32%)

adjust volume to 1l

Formamide

Tween-20

Triton X-100

DAPI	4´,6´-diamidino-2-phenyl-indole, dihydrochloride, invitrogen
0,5M EDTA pH 8	adjust with NaOH pellets ~20-30g
3M NaOAc pH 5,2	adjust with acetic acid (glacial)
1M PIPES pH 6,8	adjust with NaOH pellets, ~2g per 50ml

1x TE buffer

10mM Tris Cl pH 7,5

1 mM EDTA pH 8

Vectashield® Mounting Medium, Vector Laboratories Cat# H-1000

Xist probe preparation

- label 1µl of tetOP-Xist plasmid by random priming using the random primer labeling kit (Agilent Technologies Prime-lt (R) II)
- in an eppendorf tube with a screw cap add
    - 22µl MilliQ (autoclaved)
    - 1µl plasmid
    - 10µl random primers
- incubate 5min at 95°C in heatblock
- put 5min on ice
- add
    - 10µl 5x dCTP
    - 0,5µl Cy3-dCTP
    - 1µl Klenow pol.

- Mix thouroughly, wrap in aluminium foil
- incubate at 37°C in incubator o/n

- the next day remove unlabelled nucleotides by using a spun column kit (clontech laboratories L-Art-Nr: 636069, Chroma spin TE, 50 col)
- shake column to loose column bed and take care not to have any buffer on the lid
- remove closure of the column, put column into an eppendorf tube with screw cap and put it into a 15ml tube
- spin to remove TE buffer, 800rpm, 5min
  use 2ml H_2O in 15ml tube as balance
- remove column, put it into a new eppendorf tube and 15ml tube

- add 1µl of 0,5M EDTA pH8 to probe (amplified the day before)
- pipett on column
- add 20µl TE buffer
- spin 1000rpm, 10min
- discard column
- labelled probe in TE buffer in the eppendorf tube in 15ml tube
- pipett out into a new eppendorf tube
- add:
    - 20µl yeast tRNA [20ng/ml]
    - 20µl salmon sperm DNA [10mg/ml] invitrogen
    - 20µl mouse Cot-1 DNA [1µ/µl]
    - 1/10 of volume of NaOAc pH 5,2 (~20µl)
    - 21/2 of volume of EtOH abs (~500µl)
- vortex!
- Store at -20°C

- spin 30 min at 13000rpm, 4°C (balance with ~700µl)
- discard supernatant
- add 1ml of 70% EtOH
- briefly vortex
- spin 5 min at 13000rpm, 4°C

- add 1ml of abs EtOH
- spin 1 min at 13000rpm, 4°C
- take off SN
- spin briefly and remove the remaining SN
- air dry pellet by opening the tube, wrap it in alumiunium foil and put it in a drawer ~30 min
- resuspend pellet by adding 80µl of hybridisation buffer (HYBRISOL VII, MP) add ~20 µl onto the pellet and break it into smaller pieces, pipet up and down with the 80µl until the whole pellet is dissolved! (it takes a few minutes)
- shake it 10 min on a shaker (wrapped in aluminium foil!)
- add 240µl of hybridisation buffer
- incubate 10 min at 74°C
- incubate 30 min at 37°C
- store at -20°C

Fixing cells

- Grow cells over night on ROBOZ slides (HTC(R), 10 wells 7mm) at 37°C / 5% CO_2
- Wash 2x mit PBS in coplin jar
- Incubate 2 min with cytoskeletal buffer + detergent
- Wash with PBS
- Fix 10 min with 4% paraformaldehyde (PFA)
- Wash with 70% EtOH
- At this step there is the possibility to store the fixed cells in 70% EtOH at 4°C
- Or continue with dehydration

Dehydration

- remove from 70% EtOH
- place in 80% EtOH for 2 min
- place in 95% EtOH for 2 min
- place in 100% EtOH for 2 min
- air dry, possibility to store slides in 70% EtOH

- add 3 µl of Xist probe/well and cover with coverslip
- place in a humid 37°C chamber o/n (use a large petry dish, layer some Whatman paper soaked in 50% formamide/2x SSC in the bottom)

Washing

- prewarm all solutions to 39°C in water bath
- remove the cover slip very carefully by dipping the slide into the washing solution before the washing steps
- wash slides in 2x SSC/50% formamide (special waste!) 3x 5min in coplin jar (~50ml)
  at 39°C water bath with agitation
- wash in 2x SSC 3x 5 min, 39°C with agitation
- wash in 1x SSC/1µl DAPI 10 min, 39°C with agitation
- add a drop of mounting media to a large cover slip, place slide on it and fix cover slip with nail polish
- image acquisition with Leica CTR6000 fluorescence microscope and LAS AF software
- store slides at 4°C in the dark

2.2.2 Immunofluorescence analysis

Immunofluorescence stainings were performed either on interphase nuclei or condensed methapase chromosome spreads. Three different staining protocols were established and used, depending if single stainings or co-stainings were performed. For immunofluorescence analysis, combined with a subsequent Xist RNA FISH, a seperate protocol was used, aiming RNase free conditions. Primary and secondary antibodies were used in different combinations and image acquistion was performed with Leica CTR6000 fluorescence microscope and LAS AF software.

solutions and buffer

KCM buffer

10mM	TrisHCl pH 8
120mM	KCl
20mM	NaCl
0,5mM	EDTA

RBS solution

10mM	TrisHCl pH 7,5
10mM	NaCl
5mM	$MgCl_2$

10% Triton X-100 stock
10% Tween-20 stock

Preparation for interphase staining

For immunofluorescence analysis on interphase nuclei, cells were grown on gelatin-coated ROBOZ slides over night at 37°C / 5% CO_2.

Preparation of metaphase chromosome spreads

For immunofluorescence analysis on condensed methaphase chromosome spreads, cells were prepared after the following protocol:

metaphase arrest

- add 1:100 demecolcine to ~70% confluent cells in flask (in case of 9ml in T25 flask add 90µl of demecolcine)
- mix well and incubate for 3-4 hours at 37°C / 5% CO_2

collecting cells

- briefly tap flask to loose the slightly adherent cells and collect the medium in a 15ml tube
- wash the attached cells 2x with 5ml PBS and trypsinize with 4 drops of trypsin for 5min at 37°C / 5% CO_2
- resuspend the cells with the medium collected earlier and spin cells down, 5min, 800rpm
- discard the medium and resuspend cells in 2ml RBS
- incubate 15min at 37°C

cytospin cells

- clean poly-lysine coated glass slides (SIGMA-ALDRICH, poly-prep™ slides, Ref# P0425-72EA) with 70% EtOH and dry them
- fix slides in special cytospin aparature as followed:
    - first add the slide (make sure to put the right side up!)
    - wet special filters (glass slide size, with holes) with PBS and put on slides
    - finally add the plastic applicator
- add 100-200µl of cell suspension per hole in the applicator
- make sure to close the rotor properly
- centrifuge in Cytospin 4 centrifuge (Shandon) for 10 min with 1200 rpm

2.2.3 Histone H3 trimethyl-lysine 27 (H3K27m3) IF-staining

This protocol was used for single H3K27m3 stainings.

- extract cells on prepared slides in KCM/0,1% Tween-20/0,1% Triton X-100 for 10min at RT in coplin jar
- fix in 4% PFA/PBS for 10min at RT
- wash in KCM/0,1% Tween-20
- block in KCM/0,1% Tween-20/5% BSA for 30min at RT

incubation with primary antibody

- dilute primary antibody
  1:500 in KCM/0,1% Tween-20/5% BSA
- add 100µl antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate either for 2 hours at RT or over night at 4°C in a humid chamber (with PBS)

washing

- remove coverslips/parafilm carefully by dipping them into washing buffer
- wash 3x 5min with KCM/0,1% Tween-20 at RT in coplin jar

incubation with secondary antibody

- dilute secondary antibody 1:500 in KCM/0,1% Tween-20/5% BSA
- add 100µl of antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate 40min at RT in the dark

washing, mounting and imaging

- wash 3x 5min in KCM/0,1% Tween-20
- counterstain in 50ml PBS/5µl DAPI (1:10000), 10min at RT
- wash with PBS
- add mouning media (Vectashield®), cover with a big cover slip, seal with nailpolish and store at 4°C in the dark

2.2.4 H4 acetylation IF-staining

This protocol was used for single H4 acetylation stainings and differs from the other protocols by the late fixation step.

- block prepared slides in KCM/0,1% Triton X-100/5% BSA for 30min at RT in coplin jar

incubation with primary antibody

- dilute primary antibody 1:500 in KCM/0,1% Triton X-100/5% BSA
- add 100µl antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate either for 2 hours at RT or over night at 4°C in a humid chamber (with PBS)

washing

- remove coverslips/parafilm carefully by dipping them into washing buffer
- wash 3x 5min with KCM/0,1% Triton X-100 at RT in coplin jar

incubation with secondary antibody

- dilute secondary antibody 1:500 in KCM/0,1% Triton X-100/5% BSA
- add 100µl of antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate 40min at RT in the dark

washing, fixing and imaging

- wash 3x 5min in KCM/0,1% Triton X-100
- fix slides in 4% PFA, 10min at RT in coplin jar
- counterstain in 50ml PBS/5µl DAPI (1:10000), 10min at RT
- wash with PBS
- add mouning media and store at 4°C in the dark

2.2.5 General Immunofluorescence protocol for co-stainings

This protocol was used for all co-stainings. For the staining of ubiquitinylated histone H2A an additional extraction step before fixation is necessary.

- wash prepared slides in 1xPBS
- only for ubiquitinylated histone H2A: extract 2 min with PBS/0,5% Triton X-100
-fix in 4% PFA/PBS for 10 min
- wash 2x5 min with 1xPBS
- permeabilize 3 min in PBS/0,5% Triton X-100
-wash slides in 1xPBS
-wash slides 2x5 min in PBS/0.1% Triton X-100
-block for 30 min in PBS/0,1% Triton X-100/5% BSA

- dilute primary antibody 1:500 in PBS/0,1% Triton X-100/5% BSA
- add 100µl antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate either for 2 hours at RT or over night at 4°C in a humid chamber (with PBS)

washing

- remove coverslips/parafilm carefully by dipping them into washing buffer
- wash 3x 5min with PBS/0,1% Triton X-100 at RT in coplin jar

incubation with secondary antibody

- dilute secondary antibody 1:500 in PBS/0,1% Triton X-100/5% BSA
- add 100µl of antibody dilution per slide and cover with small coverslip or with a small piece of parafilm
- incubate 40min at RT in the dark

washing, fixing and imaging

- wash 3x 5min in PBS/0,1% Triton X-100
- counterstain in 50ml PBS/5µl DAPI (1:10000), 10min at RT
- wash with PBS
- add mouning media and store at 4°C in the dark

2.2.6 Indirect immunofluorescence staining with subsequent *Xist* RNA FISH

For IF analysis with subsequent FISH, immunostaining was performed as described above. To reduce RNA degradation, Rnase Inhibitor (Rnasin®, Promega N251B, 40u/µl) was added to the blocking solution and to the primary and secondary antibody dilutions (20u/100µl) and general Rnase free work was aimed. After incubation with the secondary antibody, slides were washed 2 times with PBS/0,1% Tween-20, fixed in 4% PFA/PBS for 10 min and extracted for 5 min in cytoskeletal buffer/0,1% Triton-X100. For subsequent Xist RNA FISH analysis the FISH protocol was followed.

2.2.7 Antibodies

2.2.7.1 Primary Antibodies

Anti-hyperacetylated Histone H4 (Penta)	host: rabbit Millipore, polyclonal Antibody Cat# 06-946 Use 1:500 for IF
Anti-Histone H3 trimethyl-lysine 27	host: rabbit Active motif, polyclonal Antibody Cat# 39155 Use 1:500 for IF
	host: mouse Active motif, monoclonal Antibody Cat# 39536 Use 1:500 for IF

Anti-Histone H4 monomethyl-lysine 20	host: rabbit Active motif, polyclonal Antibody Cat# 39175 Use 1:500 for IF host: mouse Active motif, monoclonal Antibody Cat# 39727 Use 1:500 for IF
Anti-Histone macro H2A1	host: rabbit Active motif, polyclonal Antibody Cat# 39593 Use 1:500 for IF
Anti-ubiquityl-Histone H2A, clone E6C5	host: mouse Millipore, monoclonal Antibody Cat# 05-678 Use 1:500 for IF
Anti-Ring1B	host: mouse Use 1:500 for IF
Anti-ASH2	host: rabbit Bethyl Laboratories Inc., Cat# A300-107A Use 1:500 for IF
Anti-Oct-3/4	host: goat Santa Cruz Biotechnology Inc., (N-19): sc-8628 Use 1:300 for IF host: mouse Santa Cruz Biotechnology Inc. Use 1:200 for IF

Anti-Nanog	host: rabbit Use 1:200 for IF
Anti-Klf4	host: goat Use 1:20 for IF

2.2.7.2 Secondary Antibodies

Goat-anti-rabbit	Invitrogen, Alexa Fluor 555®, red, A21429
Goat-anti-mouse	Invitrogen, Alexa Fluor 488®, green, A11029
Donkey-anti-rabbit	Invitrogen, Alexa Fluor 488®, green, A21206
Donkey-anti-mouse	Invitrogen, Alexa Fluor 546®, red, A10036
Donkey-anti-goat	Invitrogen, Alexa Fluor 647®, far red, A21447

All secondary antibodies were used 1:500 for IF.

2.3 Molecular methods

2.3.1 Isolation of genomic DNA from ESC

Solutions and buffer

Ear buffer
1% SDS
20mM EDTA
50mM NaCl
50mM Tris pH7.5

Proteinase K 20mg/ml stock solution

5M NaCl

Isopropanol

70% EtOH

- cells from a confluent 6-well plate were accutazed, pellets resuspended in 500µl ear buffer/400µg/ml proteinase K and over night incubated at 55°C

DNA precipitation

- add 200µl of 5M NaCl
- vortex well
- spin 15 min at 13000 rpm, 4°C
- transfer supernatant to a new eppendorf tube and add 700µl Isopropanol (1:1)
- vortex well
- spin 30 min at 13000 rpm, 4°C
- discard supernatant
- add 500µl 70% Ethanol
- vortex
- spin 10 min at 13000 rpm, 4°C
- remove Ethanol
- airdry and resuspend DNA in 50µl TE buffer by shaking for 1 hour at 65°C
- keep at -20°C

2.3.2 Sex determination by PCR

Solutions and buffer

PCR Master Mix, 2X from Promega (Cat# M7502)

50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$

Primer

Zfy, Tsix

6x loading dye

100bp DNA ladder (BioLabs)

Approach

template	1µl
2xMM	12,5µl
forward primer	0,4µl
reverse primer	0,4µl
H2O	10,7µl
	25µl

PCR program

1	94°C	1 min
2	94°C	30 sec
3	56°C	30 sec
4	72°C	1 min
5	4°C	∞

steps 2-4 35 cycles

Preparation of 1% agarose gel

1,5g Agarose were dissolved in 150ml 1xTAE buffer and 3µl Ethidiumbromid were added.

RNA isolation for Affymetrix genome-wide expression profiling

For RNA isolation the RNeasy Kit from Qiagen (Cat# 74106) was used. Three independently derived ESC cell lines were differentiated with or without doxycycline, respectively, in 10 cm cell culture dishes for 5 days. Cells were trypsinized, 350µl of RLT buffer was added and cells disrupted and homogenized by vortexing for 1 minute. 1 volume of 70% ethanol was added, the whole sample transferred to an RNeasy spin column and centrifuged at 10000rpm for 15s. The flow-through was discarded, 350µl of RW1 added and centrifuged at 10000rpm for 15s. For additional DNase digestion, 78µl of RNase-free DNase mix was added directly on the spin column membrane and incubated for 15min at room temperature. The flow-through was discarded, 350µl of RW1 added and centrifuged at 10000rpm for 15s. 500µl of RPE buffer were added, centrifuged at 10000rpm for 2min and columns were transferred to new collection tubes. Finally the RNA was eluted by adding 30µl of RNase-free water and centrifugation at 10000rpm for 1min. RNA concentration and purity was measured with Nanodrop and RNA samples kept at -80°C. 1µg of each RNA sample was load on a 0,7% agarose gel under RNase-free conditions to check the grade of RNA degradation.

3 Results

3.1 Autosomal *Xist* mediated silencing

For investigating the effect of *Xist* RNA without repeat A expression from chromosome 11 in an *in vivo* model, mice carrying the inducible transgene were generated. First, the transgene homing cassette was integrated into the 3´end of *Col1a1* locus on chromosome 11 by homologous recombination in ES cells. This cassette carries a doxycycline inducible operator (tetOP) (Fig. 6). The *Xist* cDNA construct PGK-Xist-PA was integrated into the targeting vector Col1a1-tetOP[EV]pSF1-PGKHy by Cre recombination, thereby restoring a PGK-neo-pA gene which confirs G418 resistance (Fig. 7).

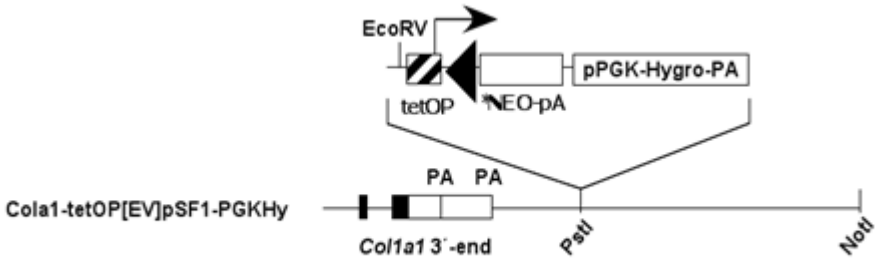


Figure 6 A transgene homing site in the *Col1a1* locus on chromosome 11

The targeting vector Col1a1-tetOP[EV]pSF1-PGKHy on the Col1a1 locus on chromosome 11 is shown. It contains the dox inducible promoter (tetOP), a loxP site (black triangle) and an antibiotic resistance marker to accomplish efficient selection of cells with integrated transgene.

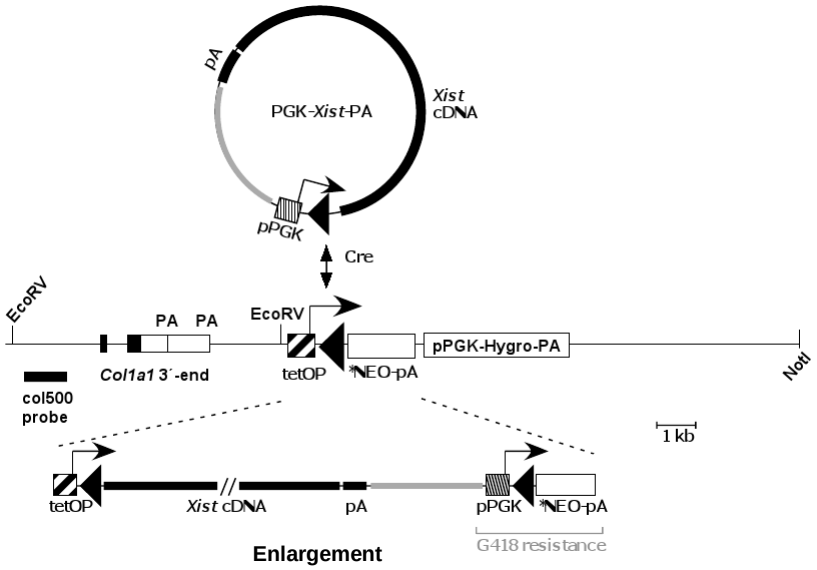


Figure 7 Cre mediated insertion on an *Xist* cDNA transgene into the homing site

Single copy integration of the PGK-Xist-PA via Cre recombination into the homing site of Col1a1 locus on chromosome 11 is indicated. LoxP sites are indicated with black triangles. Enlargement shows the restoration of G418 resistance necessary for identification of transgenic cells. The dox incucible promoter (tetOP) is indicated as a hatched box which ensures incucible expression of the transgene Xist RNA.

With the transgenic ESCs carrying inducible *Xist* RNA lacking repeat A on chromosome 11 (*ColXist* RNA) efficient germline transmission could be obtained and by breeding to mice carrying the transactivator, R26/N-nlsrtTA [BL/6,H], an inducible *in vivo* system was established.

3.2 *In vivo* expression of *ColXist* RNA from chromosome 11 in early development

Previous investigations showed that the expression of a mutant *Xist* RNA lacking repeat A can trigger the recruitment of heterochromatin marks known from the inactive X chromosome independent of gene silencing in an *in vitro* ESC system (Pullirsch, et al., 2010) (Wutz, et al., 2002). Male cells carrying the the mutant *Xist* RNA transgene on their X chromosome are capable to differentiate in culture devoid of significant cell death (Pullirsch, et al., 2010).

We aimed to investigate if the establishment of facultative heterochromatin but not gene silencing is compatible with early embryonic mouse development. Therefore crosses with different genotypes were generated (table 1). The effect of *Xist* induction on embryogenesis and survival was studied. Cytological characterization was performed to characterize *Xist* expression and chromatin modifications.

Cross	Nr. of mice	Induced ColXist expression	Dissection	Embryonic lethality	Nr. of dead embryos dead/all
Homozygous ColXist/ColXist	1	E4.5	E13.5	+	6/6
	1	E5.5	E12.5	+	8/8
Maternal transmission ColXist/+	2	E6.5	E13.5	+	14/14
	1	E2.5	E9.5	+	6/6
	2	E4.5	E11.5	+	12/12
Paternal transmission +/ColXist	1	E6.5	E13.5	+	8/8
No transgene induction Control ColXist/+	2	-	E14.5	-	0/12
No transgene Control +/+	2	E6.5	E13.5	-	0/10

Table 1 Overview of crosses

Summary of the different crosses, their genotypes and parental origin of the transgene. The number of pregnant mice is given and embryonic days (ED) of *Xist* induction and timepoint of dissection is indicated. Number of normal developed embryos is cited and embryonic lethality indicated with +/-.

3.2.1 Homozygous cross results in embryonic lethality

To investigate the effect of *ColXist* expression from chromosome 11 *in vivo* in early mouse development, two timed matings of transgenic mice (ColtetOPXistΔ) with mice carrying the transactivator (R26TA) were set up. We first aimed for mice, homozygous for the ColXist transgene, ColXist/ColXist. Expression of the mutant *Xist* RNA was induced from embryonic day E4.5 and E5.5, respectively, by adding a doxycycline/sucrose mix to the drinking water of pregnant females. At E13.5 and E12.5, pregnant mice were sacrificed and uteri were dissected. Surprisingly, six out of six and eight out of eight embryos were resorbed and showed no signs of proper development, although the placenta seemed normally developed. This severe effect on early mouse development was completely unexpected as no significant gene silencing was expected by the expression of the mutant *Xist* RNA lacking repeat A. It was not possible to isolate any MEFs for further experiments from these embryos.

3.2.2 Heterozygous maternal transgene transmission with genotype ColXist/+

To investigate if heterozygous *ColXist* expression has a severe effect on early development as well, five timed matings for ColXist/+ genotype, maternal transgene transmission, were set up and *ColXist* expression was induced with doxycycline from E6.5, E2.5 and E4.5, respectively. Mice were sacrificed and uteri dissected at E13.5, E9.5 and E11.5. Again, all the embryos (14/14, 6/6 and 12/12) showed the unexpected strong phenotype and embryos were not developed properly. However, MEFs were isolated from four resorbed embryos (induced from E2.5 and dissected at E9.5), from which two cultures could be maintained over a short period *in vitro*.

3.2.3 Transgenic *ColXist* RNA expression and accumulation in E9.5 MEFs, shown by *Xist* RNA FISH analysis

To verify inducible transgene *ColXist* RNA expression from chromosome 11, *Xist* RNA FISH analysis was performed in interphase nuclei of MEFs after short term culture with doxycycline. The hybridisation with an *Xist* RNA probe revealed one signal per nucleus in ~80% of cells in the first cell line, which showed induced *ColXist* RNA expression from chromosome 11 of male MEFs (Fig. 8 A,B). Short term cultivation of the MEF cell line without doxycycline did not reveal any *Xist* RNA signal in the nuclei, which suggests accurate heterozygous *ColXist* RNA expression from the autosome. Hybridization in the second cell line, cultured without doxycycline *in vitro* revealed one signal per interphase nucleus, which shows the endogenous expressed *Xist* RNA of the inactive X chromosome in female cells (Fig. 8 G,H). Short term cultivation with doxycycline revealed two *Xist* RNA signals per nucleus, which verified efficient *ColXist* RNA expression and accumulation on chromosome 11 (Fig. 8 I, J).

3.2.4 Establishment of facultative heterochromatin modifications in E9.5 MEFs

To investigate to what extent chromatin modifications are established on the autosome expressing the mutant *ColXist* RNA in comparison to the inactive X chromosome, immunofluorescence stainings for detecting histone modifications were accomplished. Therefore, histone H4 hypoacetylation and enriched histone H3 lysine 27 tri-methylation, known as inactive X chromosome markers, were chosen. Previous investigations indicate that H3K27m3 is an early mark of X inactivation and an enriched signal can be detected in about 70% of cells after 48 hours of *Xist* induction, independent of silencing (Kohlmaier, et al., 2004) (Plath, et al., 2003). The chromosomal analysis was mainly performed on condensed metaphase chromosome spreads in order to assess the distribution of histone modifications accurately. Male E9.5 MEFs cells reveal one chromosome with enriched H3K27m3 signal after short term cultivation *in vitro* with doxycycline (Fig. 8E). A co-staining with H4 acetylation antibody was performed to assess the characteristics of H3K27m3 enrichment and H4 hypoacetylation in the same metaphase spread. The chromosome with enhanced

H3K27m3 signal still shows H4 acetylation, although reduced near the centromeric region (Fig. 8D). The observation of a partial hypoacetylation of chromosome 11 is different to the chromosome-wide hypoacetylation of H4 reported on the inactive X chromosome (Keohane, et al., 1996). Female E9.5 MEFs reveal two enriched H3K27m3 signals after short term cultivation *in vitro* with doxycyline (Fig. 8M). The combined histone H4 acetylation staining indicate the inactive X chromosome with chromosome-wide H4 hypoacetylation and one partially hypoacetylated chromosome 11 (Fig. 8L).

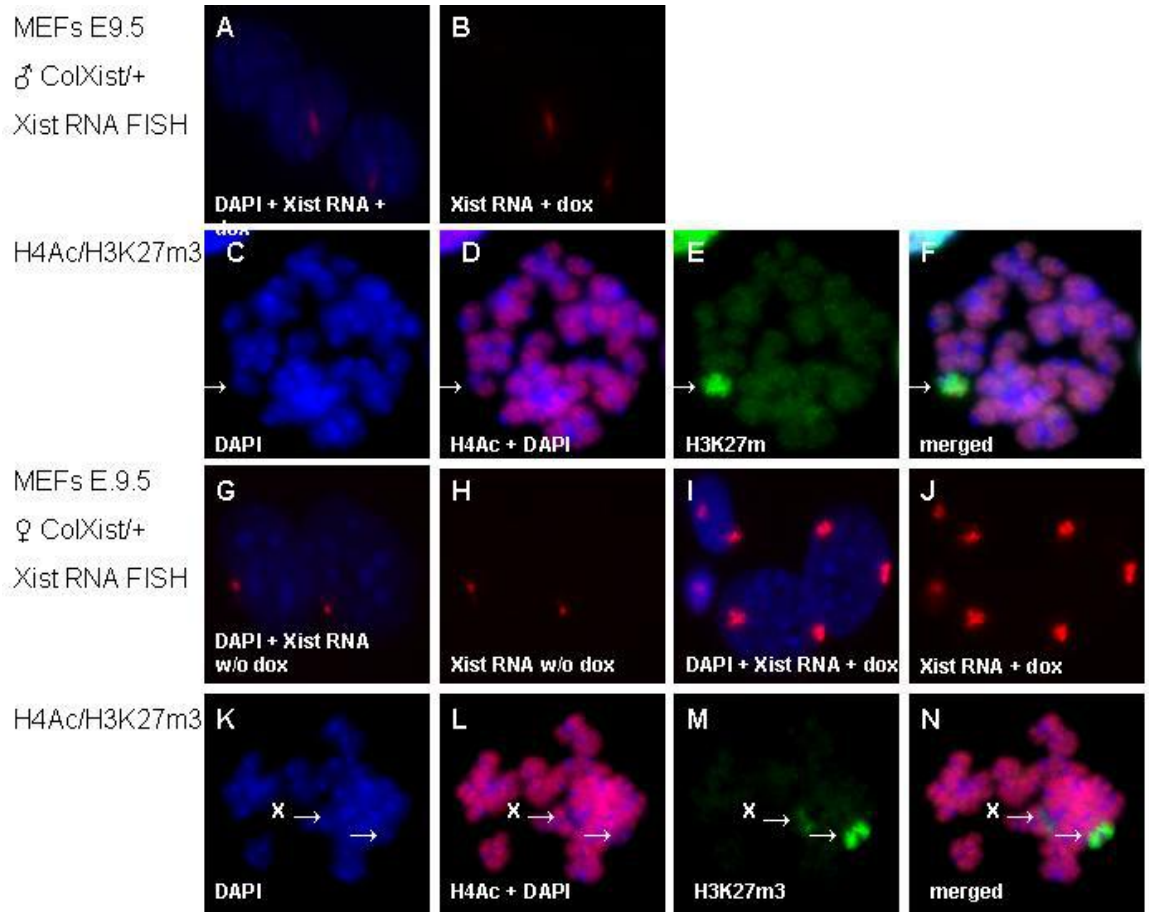


Figure 8 ***Xist* RNA FISH and IF co-stainings in heterozygous male and female E9.5 MEFs**

Xist RNA FISH in interphase nuclei of derived E9.5 male MEFs show one signal in around 80% of cells, shown in red is cy3 labelled *Xist* RNA probe (A, B). DNA is stained with DAPI, shown in blue. Derived E9.5 female MEFs reveal the endogenous *Xist* RNA accumulation without culture with doxycycline (G, H) and two signals per interphase nuclei after induced transgene expression (I, J). IF co-stainings with antibodies against H4 hyperacetylation and H3K27m3 were performed on condensed metaphase chromosome spreads. After transgene induction one chromosome shows enrichment of H3K27m3 shown in green (E) and no significant H4 hypoacetylation can be observed (D) in male cells. Female cells show two enriched H3K27m3 marks (M) after transgene induction and one hypoacetylated chromosome (L) which shows the inactive X chromosome.

3.2.5 Transgenic ColXist RNA expression in E11.5 MEFs, genotype ColXist/+

Two MEF cell lines were isolated from E11.5 resorbed embryos with transgenic *ColXist* RNA induction from E4.5, maternal transmission. Xist RNA FISH was performed with cells after short term *in vitro* cultivation with doxycycline to assess accurate transgene expression and revealed one *Xist* RNA signal in about 80% of male cells (Fig. 9, A,B,G,H). Short term cultivation of these cells without doxycycline revealed no *Xist* RNA signal in interphase nuclei, which indicates two male MEF cell lines with functional transgene expression from chromosome 11.

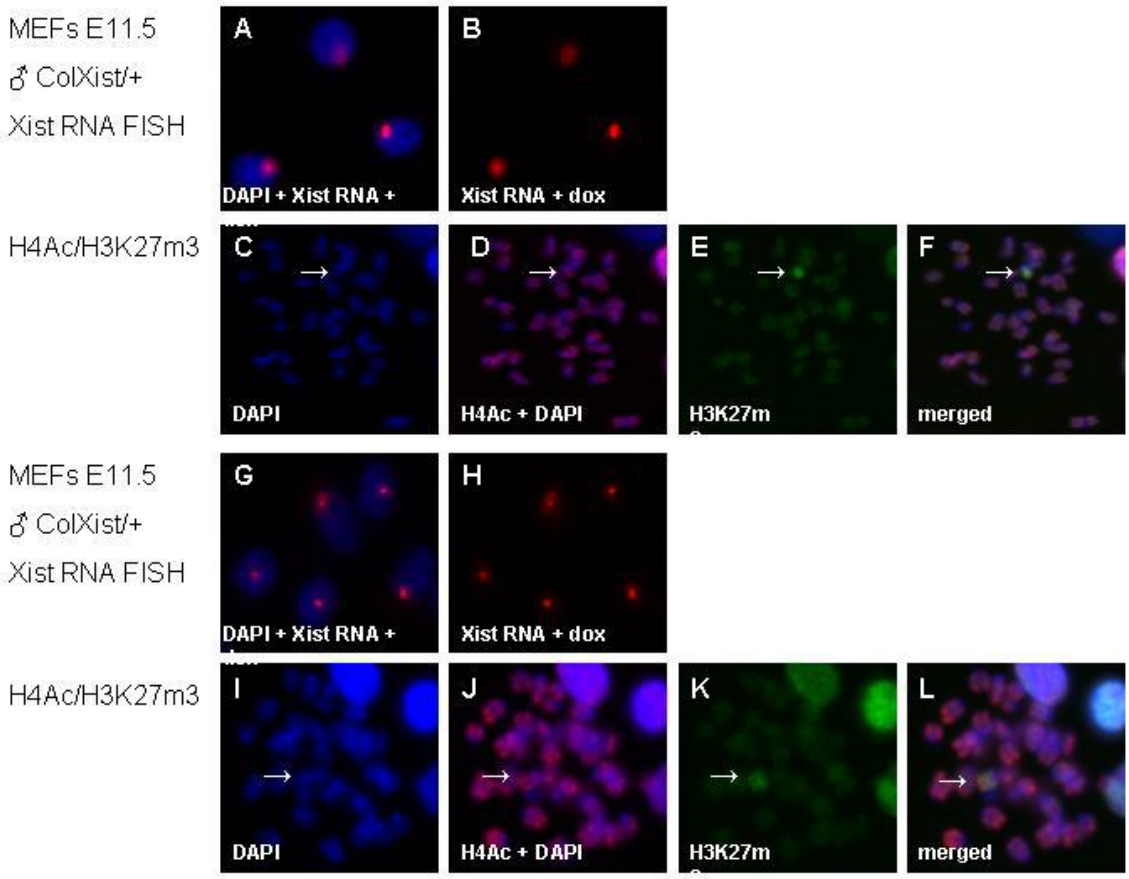


Figure 9 ***Xist* RNA FISH and IF co-stainings in heterozygous male E11.5 MEFs**

Xist RNA FISH indicates one induced *ColXist* RNA on chromosome 11 in male MEFs, shown in red (A, B, G, H). Antibodies against H3K27m3 and H4 hyperacetylation show one condensed metaphase chromosome with enriched H3K27m3, indicated in green (E, K). H4 acetylation staining in the same metaphase spread shows no significant H4 hypoacetylation on chromosome 11 (D, J).

3.2.6 Establishment of facultative heterochromatin modifications in E11.5 MEFs

For investigating the establishment of heterochromatin modifications, histone H4 hyperacetylation and H3K27m3 co-stainings were performed on condensed metaphase chromosomes. Like in E9.5 MEF cells reported above, an enrichment of H3K27m3 and partial hypoacetylation could be observed on chromosome 11 after short term *in vitro* cultivation with doxycycline (Fig. 9 C-F, I-L).

In conclusion, maternal transmission of the transgenic *ColXist* RNA, expressed heterozygously from chromosome 11 early in development, shows a surprisingly severe phenotype and no proper embryonic development can be accomplished. Cytological investigations on isolated MEFs show accurate *ColXist* expression from the autosome and establishment of facultative heterochromatin modifications. Enhanced H3K27m3 signals can be observed in around 80% of cells in both, male and female MEFs after short term cultivation with doxycycline, whereas histone H4 hypoacetylation does not appear chromosome-wide on the autosome.

3.2.7 Heterozygous paternal transgene transmission with genotype +/ColXist

To rule out any maternal effect of the transgene, one timed mating for +/ColXist genotype, paternal transmission, was set up and ColXist expression was induced with doxycycline from E6.5. Mice were sacrificed and uteri dissected at E13.5. Eight out of eight embryos showed the same severe phenotype and therefore parental transmission of the transgene does not influence the phenotype of X inactivation. No MEFs were isolated from these embryos for further cytological experiments.

3.2.8 Effect of the integrated transgene without *in vivo* induction, genotype ColXist/+

For investigating a possible effect of the integrated transgene, two timed matings for ColXist/+ genotype were set up without *ColXist* induction in the *in vivo* model and uteri were dissected at E14.5. Twelve out of twelve proper devoloped embryos could be obtained suggesting no effect of the integrated transgene in early mouse development. Twelve MEF cell lines were taken in culture to investigate accurate *ColXist* RNA expression and establishment of chromatin modifications after late induction *in vitro*. MEFs were either cultivated with or without doxycycline and sex determination by *Xist* RNA FISH analysis revealed seven female and five male cell lines whereas female cells show two signals (endogenous and transgenic *Xist*) and male cells just the transgenic *ColXist* RNA signal after doxycycline induction (Fig. 10 C,D and K,L).

For analyzing the colocalization of *Xist* and *ColXist* RNA, respectively, with the enriched heterochromatin mark H3K27m3, immunofluorescence stainings with subsequent RNA FISH analysis were performed. Focal signals of H3K27m3 in interphase nuclei can be shown which colocalize with either endogenous *Xist* RNA or transgenic *ColXist* RNA signal upon induction with doxycycline (Fig. 10 C-F). One H3K27m3 enrichment shows a weak signal which is explained by the subsequent FISH analysis treatment followed by general weaker immunofluorescence signals. Further cytological investigations showed that H3K27m3 still can be established on the autosome in around 80% of MEFs when *ColXist* expression is induced *in vitro.* The inactive X chromosome in female cells shows chromosome-wide H4 hypoacetylation in comparison to the partial deacetylation of chromosome 11 (Fig. 10 G-J, M-P), which is consistant with results from *ColXist* induction *in vivo* early in development.

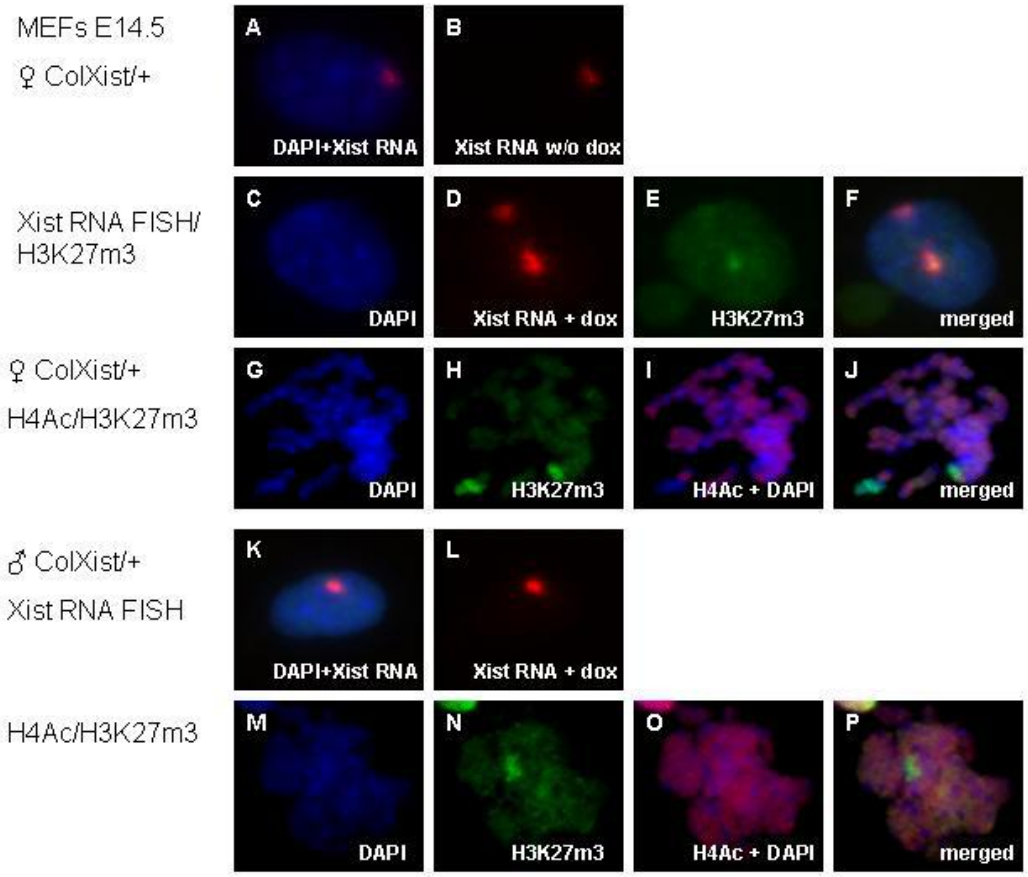


Figure 10 ***Xist*** **RNA FISH and IF co-stainings in heterozygous male and female E14.5 MEFs**

Xist RNA FISH analysis shows the endogenous *Xist* RNA in each interphase nucleus in female E14.5 MEFs without transgene induction (A, B). The co-localisation of endogenous *Xist* RNA and transgenic *ColXist* RNA, respectively, with H3K27m3 enrichment is shown (C-F). Male MEFs reveal one *Xist* RNA signal per interphase nucleus after induction (K, L). Immunofluorescence analysis on metaphase spreads show two chromosomes with enriched H3K27m3 signal in female MEFs (H), chromosome-wide hypoacetylation of the Xi and no significant decrease in acetylation on the autosome (I). In induced male MEFs chromosome 11 shows enrichement of H3K27m3 (N) and no obvious hypoacetylation (O).

3.2.9 Effect of doxycycline/sucrose mix to the drinking water, genotype +/+

To investigate a possible toxic effect of the inducer molecule doxycycline on mouse development, two timed matings of mice without the transgene with mice carrying the transactivator were set up. Doxycycline was added to the drinking water of pregnant mice starting at E6.5 and uteri were dissected at E13.5. Ten out of ten proper developed embryos could be obtained which suggests no toxic effect of doxycycline treatment.

3.2.10 ColXist expression from chromosome 11 mediates H3K27m3 but no noticeable decrease in histone H4 acetylation

Our investigations show that expression of the mutant *Xist* RNA from chromosome 11 mediates enrichment of H3K27m3 mark on chromosome 11 in around 80% of cells after induction in early development as well as in late induction in *in vitro* cultured MEFs (Fig. 11C). The enlarged images of the inactive X chromosome (Fig. 11A) and chromosome 11 (Fig. 11B) show the comparison of the chromosome-wide H4 hypoacetylation on the inactive X chromosome and partial deacetylation of chromosome 11, especially close the centromeric region.

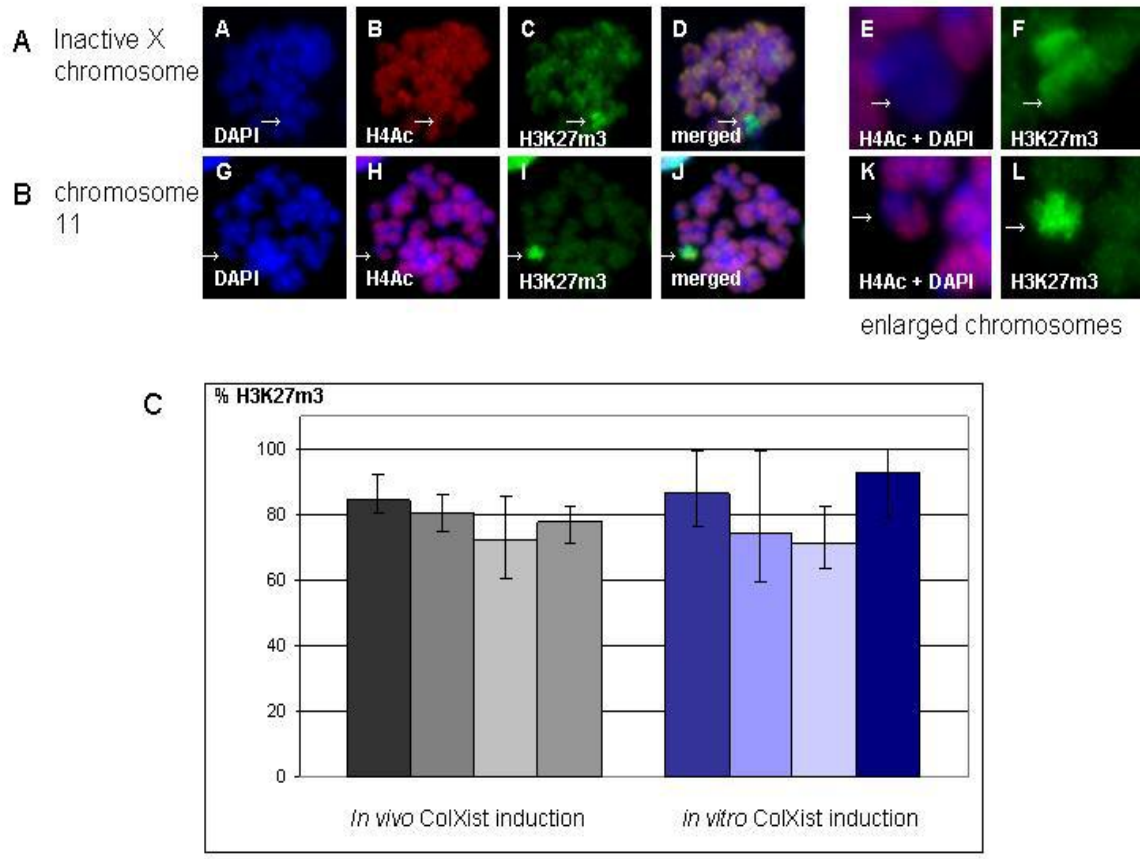


Figure 11 Immunofluorescence co-stainings and percentage of H3K27m3 after early *in vivo* and late *in vitro* induction of *ColXist* RNA expression

IF staining for H3K27m3 (green) and H4 hyperacetylation (red) reveal the differences of chromosome-wide H4 hypoacetylation of the inactive X chromosome in female control cells (E, F) and the hardly noticeable deacetylation of chromosome 11 after *ColXist* expression (K,L). The percentage of H3K27m3 on chromosome 11 after induction of *ColXist* expression *in vivo* in early development (grey bars) and *in vitro* induction with doxycycline in culture (blue bars) amounts to around 80%. Grey bars represent four indipendently derived MEF cell lines with ColXist/+ genotype and *ColXist* induction at E4.5 and 6.5, respectively and three different metaphase spreads per cell line are included. Blue bars represent four independently derived MEF cell lines with ColXist/+ genotype and *ColXist* induction in culture with three different metaphase spreads per cell line included.

3.3 *In vivo* expression of *ColXist* from chromosome 11 in late development

To investigate if expression of *ColXist* RNA at a late timepoint in development has an effect on cell differentiation in stem cell niches, we induced *ColXist* expression heterozygously from chromosome 11 from E11.5 over a time period of four weeks. A possible effect of the transgenic *Xist* RNA expression in the haematopoietic system could be investigated after all different cell types got renewed in this time period and would most likely originate in a severe phenotype. The examination of twenty days old mice showed no obvious phenotype in appearance or behaviour, suggesting that *ColXist* RNA expression has no severe effect in late embryogenesis and the turnover in the haematopoietic system. For characterising the establishment of chromatin modifications, spleen and thymus were dissected and cells taken in culture. Cell proliferation was stimulated for 48 hours by adding the T-cell mitogen concanavalin A in a concentration of 40µg/ml to the culture medium. To assess the distribution of chromatin modifications accurately, immunofluorescence stainings were performed on condensed metaphase chromosome spreads. Analysis show enrichment of H3K27m3 mark on one chromosome 11 in around 80-90% of male cells (Fig. 12B) , which suggests establishment of facultative heterochromatin marks independent of silencing function by repeat A. The co-immunofluorescence staining with H4 acetylation antibody reveals one not significantly deacetylated chromosome 11, which confirms results from *ColXist* expression in early development (Fig. 12A).

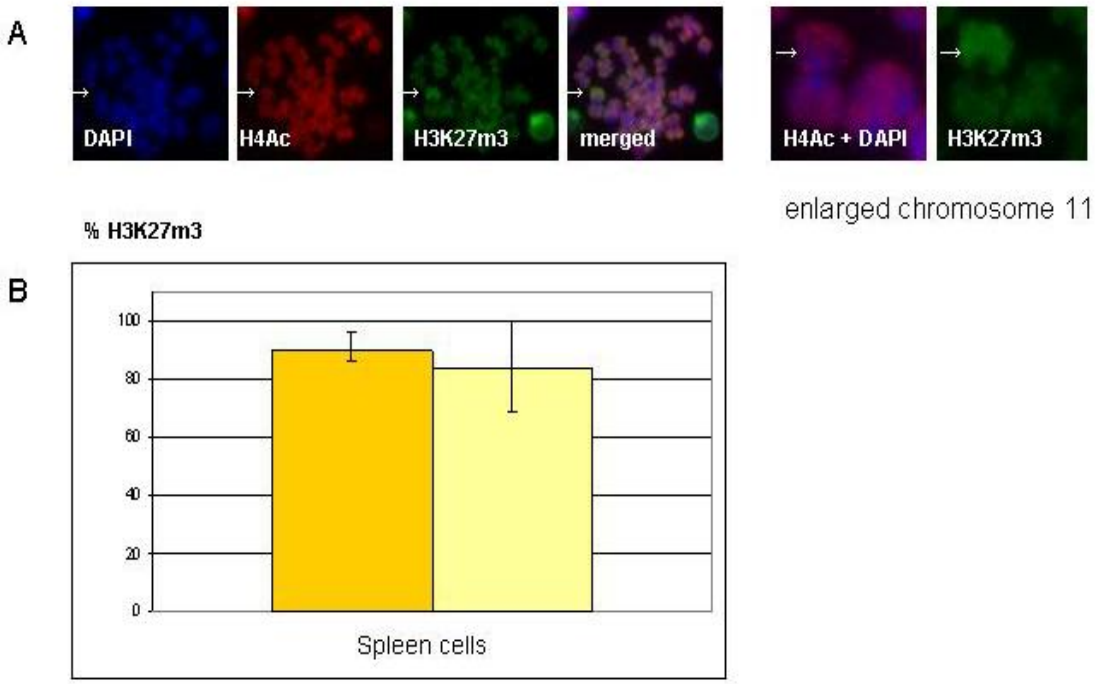


Figure 12 Immunofluorescence co-staining of isolated spleen cells and percentage of H3K27m3 enrichment

IF co-staining with H3K27m3 antibody (A, green) and H4 hyperacetylation antibody (A, red) reveals enrichment of H3K27m3 on one chromosome 11. The enlarged image shows no significant hypoacetylation of the autosome. The percentage of H3K27m3, measured in 2 different isolated male spleen cell lines, amounts to around 80%.

3.4 *ColXist* expression shows no obvious phenotype in mouse brain development

To investigate whether *ColXist* expression from a late timepoint in development has any effect on the developing mouse brain, mice were treated with doxycycline from E11.5 over a period of four weeks. Eight weeks after birth brains were dissected, weighed and compared to brains of control mice which carried no transgene but had undergone the same treatment with doxycycline. Results show no significant differences in weight between the controls and the transgene induced brains. Female brains with induced *ColXist* expression show slightly higher weight then the controls but not in a significant range and could be caused by biological differences or dissection method (Fig. 13A). For investigating if the transgene expression has any effect on the development of the

cortical layers, sagittal cryosections were prepared and histologically compared to the controls without transgene expression. Comparison between the control and the experimental sections was carried out by diverse images of sectional views and therefore inaccurate. However, no severe developmental failures of important structures as the cerebellum, the hippocampus or the cortical layers could be observed (Fig.13B).

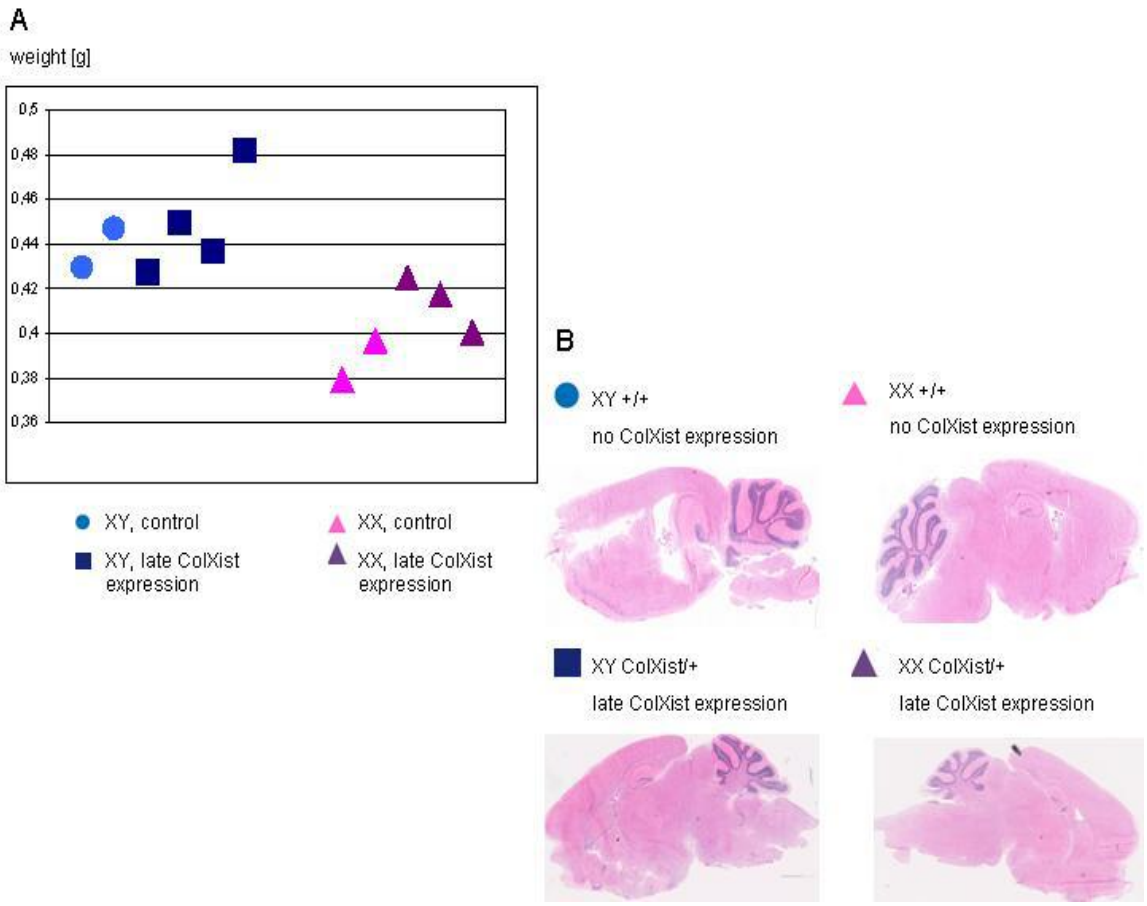


Figure 13 Influence of *ColXist* expression from E11.5 on mouse brain development

Comparison of mouse brain development without *ColXist* induction (A, male indicated with light blue dots and female with light pink triangles) and with *ColXist* induction from E11.5 over 4 weeks (A, male indicated with dark blue dots and female with dark pink triangles) shows no significant difference in weight. Sagittal cryosections of control and *ColXist* induced brains, shown in H&E stain, reveal no severe structural discrepancy (B).

3.5 Establishment of an *in vitro* system

3.5.1 Derivation of homozygous inducible ESC lines

To further investigate the molecular reason for the developmental failure, we established an *in vitro* system with ESCs homozygous for the inducible *ColXist* RNA transgene on both chromosomes 11. Thirty E3.5 blastocysts were isolated and cultured under 2i conditions to obtain ES cell lines. In addition, eight E3.5 blastocysts homozygous inducible for full length *Xist* RNA from chromosome 11 were isolated and taken in culture. Unfortunately, no ESC line with inducible full length *Xist* RNA and only seven out of thirty ESC lines inducible for *ColXist* RNA could be maintained in culture after few passages. Seven ESC lines homozygous inducible for *ColXist* RNA could be expanded in culture which maintained a typical ESC morphology (Fig. 14A).

3.5.2 Sex determination of derived ESC lines

To determine the sex of the derived ESC lines, cells were differentiated with retinoic acid for five days without the addition of doxycycline to the culture medium. Differentiated cells were grown on ROBOZ slides and *Xist* RNA FISH was performed to detect the inactive X chromosome in female cells. All of the seven cell lines showed an *Xist* RNA signal in interphase nuclei in around 40% of the cells, which refers to the inactive X chromosome in differentiation *in vitro* in female cells (Wutz and Jaenisch, 2000). To confirm inducible homozygous transgene expression from both chromosomes 11, ESCs were differentiated with retinoic acid and addition of doxycycline to the culture medium for five days. *Xist* RNA FISH analysis revealed three signals in around 40% of the cells and two signals from the transgene expression in around 80% of the cells (Fig. 14B).

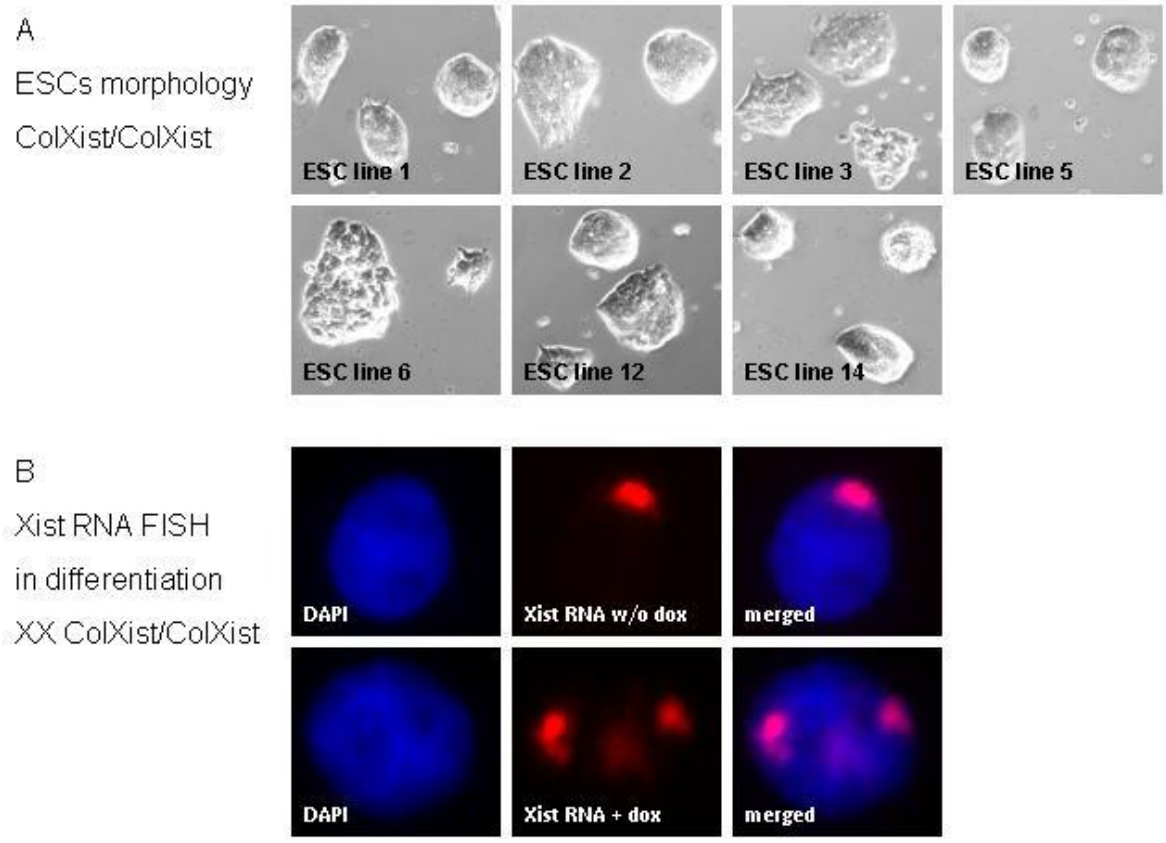


Figure 14 Morphology and *Xist* RNA FISH of seven independently derived ES cell lines homozygous for *ColXist* expression from both chromosomes 11

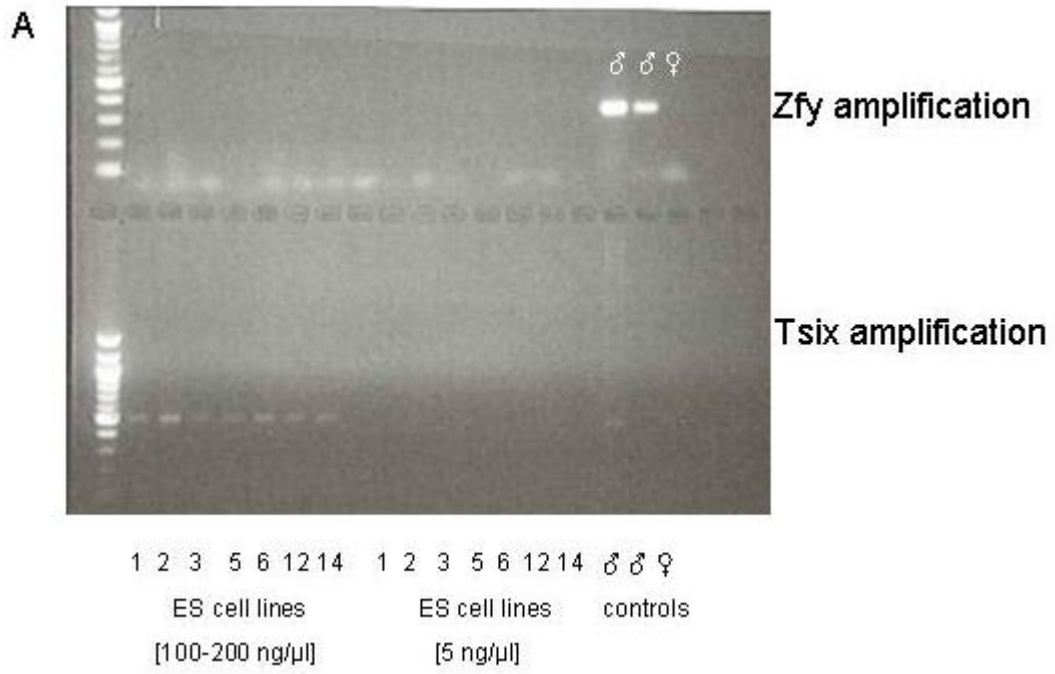
Phase contrast images show typical ESC morphology of the seven derived ESC lines under 2i culture conditions (A). *Xist* RNA FISH analysis after 5 days of differentiation without doxycycline reveals one inactive X chromosome per interphase nucleus in around 40% of cells (B). After 5 days of differentiation with doxycycline 3 *Xist* RNA signals can be detected per nucleus in around 40% of cells, whereof 2 signals show homozygous *ColXist* expression from chromosome 11 and one signal indicates the endogenous *Xist* RNA expression from the Xi.

To confirm the sex determination by *Xist* RNA FISH analysis, genomic DNA was isolated from the seven derived ESC lines and PCR was performed with primers specifically for amplification of a Y chromosome linked gene (*Zfy*) and Tsix amplification as an experimental control (Fig. 15B). The male positive control, with concentration of 200 ng/µl genomic DNA, shows the amplified *Zfy* product and *Tsix* amplification. The ESC samples with concentrations of 100-200 ng/µl genomic DNA

show no amplified *Zfy* product which suggests that all of the seven derived ESC lines are female.

3.5.3 Homozygous *ColXist* expression in differentiation *in vitro* shows no significant cell death

To investigate the effect of *ColXist* RNA expression from both chromosomes 11 *in vitro*, ESCs were differentiated with retinoic acid for five days with and without the addition of doxycycline to the culture medium, respectively. Cell counting revealed no significant cell death after homozygous induction of the transgenic *ColXist* RNA which is consistent with previous findings in male cells bearing the mutant *Xist* RNA without repeat A on their X chromosome (Pullirsch, et al., 2010) (Fig.15A). Slightly lower cell counts could be observed in five cell lines, which could be explained by little effect of doxycycline addition to the culture medium. TXY cells, which are male cells carrying an inducible *Xist* RNA transgene on their X chromosome, were used as a control. The induction of *Xist* RNA in these cells results in silencing of their X chromosome and is therefore not compatible with differentiation.



B

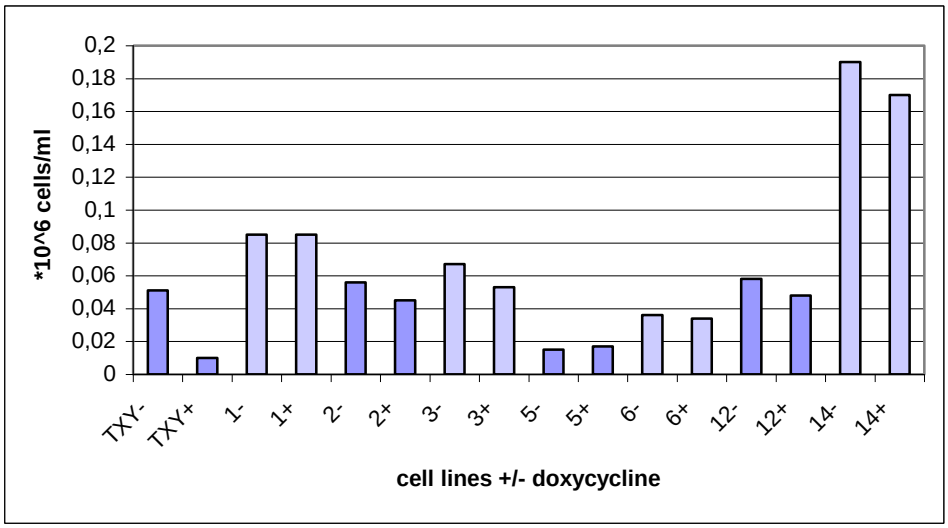


Figure 15 Sex determination of derived ES cell lines with PCR amplification and cell counting after 5 days of differentiation with retinoic acid

The amplification of a Y chromosome linked gene (Zfy) by PCR is shown for sex determination of the seven derived ES cell lines (A). Genomic DNA of the ESCs were used in two different concentrations, 5 ng/µl and 100-200 ng/µl. As controls two male samples were used, 5 ng/µl and 200 ng/µl, respectively and one female sample. The amplification of *Tsix* was used as an experimental control. The two male control samples show amplification of the male specific *Zfy* gene (318 bp) and the 200 ng/µl sample shows *Tsix* (494 bp) amplification. The 5 ng/µl concentrated sample shows no *Tsix* amplification which can be explained by the low concentration. ESC samples with 100-200 ng/µl show no *Zfy* amplification, but positive *Tsix* controls which suggests that all the seven derived ESC lines are female. Cell counting was performed with Vi-CELL viable cell counter. Comparison of counted cells after five days of differentiation with (+) or without (-) doxycycline of each derived cell line is shown (B). TXY cells, used as a control, show cell death after silencing the X chromosome by transgenic *Xist* RNA induction in differentiation (TXY+). The seven derived cell lines (1,2,3,5,6,12,14) show no significant cell death after *ColXist* induction.

3.5.4 Genome-wide gene expression profiling

To investigate if genes are misregulated by the expression of the mutant *Xist* RNA from both chromosomes 11 we have performed a genome-wide gene expression profiling using Affymetrix 3´IVT expression analysis, GeneChip Mouse Genome 430 2.0 from Atlas Biolabs company. We aimed to compare differences in the expression profiles of three independently derived ES cell lines either differentiated with or without doxycycline for five days. The genome-wide expression analysis shows no major differences in the expression profile between the transgene induced and not induced cell lines (Fig. 16, A). *Xist* RNA is clearly overexpressed after differentiation with induced *ColXist* RNA expression and the most downregulated genes are expressed from chromosome 11, which include Tom1l1 (target of myb1-like 1 (chicken)), Pctp (phosphatidylcholine transfer protein) and Mmd (monocyte to macrophage differentiation-associated). In the scatter plot diagram of chromosome 11 genes it is shown that gene expression differences between differentiation with doxycycline and without doxycycline do not vary strongly and most genes are located on the diagonal (Fig. 16, B). The illustration of the expression profiles of all genes with fold change $\geq$ 1.0 and p-value $\leq$ 0.05 shows an equal distribution of up- (967) and downregulated (933) genes (Fig. 16, E). In contrast chromosome 11 genes show a shift to downregulation with ¼ up- and ¾ downregulated genes (Fig. 16, F).

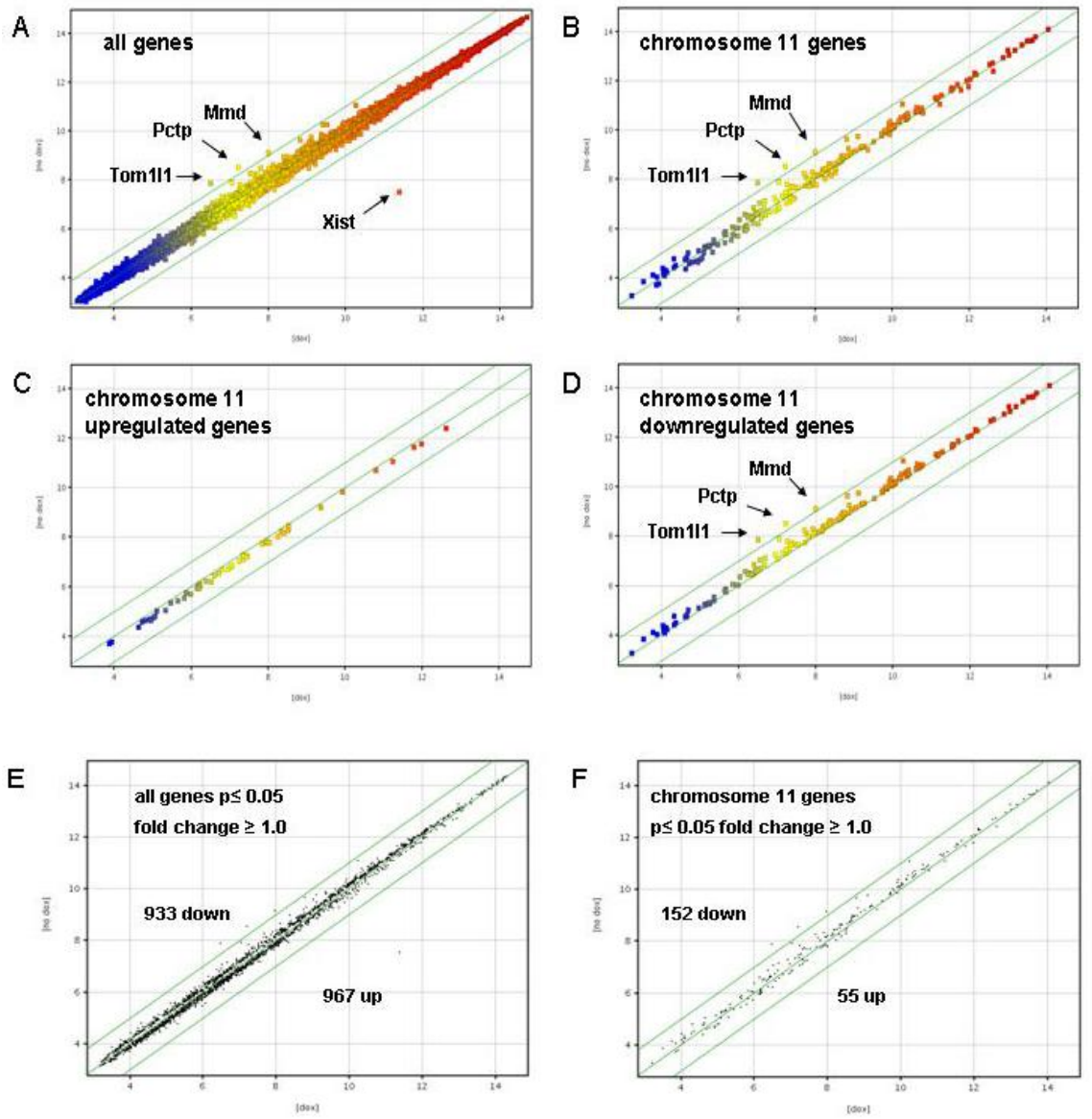


Figure 16 Scatter plot diagrams of gene expression differences

The scatter plot displays the expression difference between differentiated cells with doxycycline (X-axis) and without doxycycline (Y-axis) of the whole mouse genome. Every dot represents a gene, low expressed genes are indicated in blue and highly expressed genes in red. Genes which are expressed at a same level in differentiation with and without ColXist induction are arranged on a diagonal. The scatter plot of all 45101 genes shows no significant misregulation in the majority of genes (A). Xist RNA is clearly upregulated after transgene induction with doxycycline. The three major downregulated genes, Tom1l1, Pctp and Mmd are indicated which are all expressed from chromosome 11. All chromosome 11 genes with fold change ≥ 1.0 and p-value ≤ 0.05 are shown (B), 55 upregulated (C) and 152 downregulated (D) chromosome 11 genes are indicated in seperate diagrams.
Scatter plot of all genes with fold change ≥ 1.0 and p-value ≤ 0.05 shows 1900 genes with equal distribution of upregulated (967) and downregulated (933) genes (E). In comparison genes on chromosome 11 show a shift to downregulation with ¼ upregulated and ¾ downregulated genes (F).

Statistical analysis revealed 55 up- and 152 downregulated chromosome 11 genes with significant cut off at fold change ≥ 1.0 and p-value ≤ 0.05. The 55 upregulated genes show highest fold changes of 1,2 which shows only low significance. The six most significant downregulated genes with fold change >1,5 were compared with published data of predicted Polycomb target genes (Boyer, et al., 2006) which shows that five of these genes are not targets of PcG complexes (Table 2). No reference was found for Riken cDNA 6720460F02. Comparison to the ES cell differentiation level in 36 cells (Wutz and Jaenisch, 2000) shows that Tom1l1, Pctp and Pttg1 (pituitary tumor-transforming gene 1) are downregulated in differentiation without doxycycline, and even more with differentiation with doxycycline. For Mmd, Riken cDNA 6720460F02 and Tmem98 (transmembrane protein 98) no references could be found.

Gene	Fold change	PcG target	Regulation in differentiation –dox
Tom1l1	-2,62	-	-1,31
Pctp	-2,52	-	-1,21
Mmd	-2,24	-	?
6720460F02Rik	-1,83	?	?
Tmem98	-1,79	-	?
Pttg1	-1,56	-	-6,20

Table 2 Downregulated chromosome 11 genes with fold change >1,5

Six downregulated genes with fold change >1,5 and p-value <0,05 are shown which are not Polycomb target genes. No reference for Riken cDNA 6720460F02 was found. Tom1l1, Pctp and Pttg1 are downregulated in differentiation without doxycycline and even more in differentiation with doxycycline.

Gene	Fold change
Myo1d	-1,47
Stxbp4	-1,47
Prkca	-1,40
A530047J11Rik	-1,40
Nog	-1,37
Sox9	-1,36
Cox11	-1,35
Ccdc99	-1,32
6330403K07Rik	-1,31
Taf15	-1,30
Gabra1	-1,29
Eme1	-1,29
Wscd1	-1,29
Cox10	-1,28
Usp43	-1,28
2810001G20Rik	-1,28
Samd14	-1,28
Gsg2	-1,28
LOC100048559 /// Sfrs1	-1,27
Rnf145	-1,24
Lrrc59	-1,23
Ttc1	-1,23
Utp18	-1,22
Mrpl27	-1,22
Stat5a	1,22
Gna13	-1,21
Sfrs2	-1,20

Table 3 Up- and downregulated chromosome 11 genes with fold change >1,2

Up- and downregulated genes with fold change >1,2 and p-value <0,05 are shown. Only one of 27 genes is upregulated in differentiation with doxycycline.

4 Discussion

The role of *Xist* RNA expression in induction and maintenance of chromosome-wide gene silencing has been intensely studied over the last decades. The establishment of inducible expression systems and integration of transgenes on the X chromosome as well as on autosomes represent a useful tool to investigate *Xist* RNA function in development (Wutz and Jaenisch, 2000). In this study we investigated the consequences of expression of a mutant *Xist* RNA lacking repeat A from chromosome 11 on mouse embryonic development and tried to explain the molecular reason for the unexpected severe phenotype.

4.1 Homozygous and heterozygous *ColXist* expression in early development leads to embryonic lethality most likely before E9.5

Our investigations show that neither homozygous nor heterozygous expression of *ColXist* RNA is compatible with mouse embryonic development. This result was surprising due to previous observations that the mutant *Xist* RNA lacking repeat A has only little effect on the differentation potential and cell survival (Pullirsch, et al., 2010). However, these investigations were observed *in vitro* and with transgene expression from the X chromosome in male cells, the *in vivo* effect remains to be investigated.

Experiments with different timepoints of *ColXist* induction and dissection suggest aberrant development most likely before E9.5. The dissected embryos showed a resorbed morphology, although the placenta seemed normally developed. This could indicate a failure in lineage differentiation of the embryonic tissues, although it remains speculative if the extraembryonic development is not affected. Expression of *ColXist* RNA might implicate a malfunction in regulation of genes in development which could be explained either by the disruption of gene expression patterns by epigenetic modifications or the sequestration of epigenetic regulators. Thereby, the *ColXist* RNA-coated chromosome 11 could act as a sink for heterochromatic factors like heterochromatin protein-1 (HP1), limiting the availability of these factors for silencing of other heterochromatic loci (Chadwick and Willard, 2003)

Notably, the phenotype can be observed in mice heterozygous for the transgenic *ColXist* RNA as well and is independent of the parental origin of the transgene whereby, a maternal effect can be excluded. The targeted single copy insertion of the transgene at the 3´end of *Col1a1* locus on chromosome 11 precludes the possibility of *Col1a1* disruption which would implicate amongst others severe defects in blood vessel formation, which could cause the observed phenotype. This is confirmed by a control experiment in which embryos carrying the maternal transmitted transgene, without induction by doxycycline, develop normally until E14.5. Furthermore, a toxic effect of doxycycline can be excluded regarding to a control experiment where embryos, lacking the transgene, were treated with doxycycline and normally developed E13.5 embryos could be observed.

4.2 No obvious phenotype can be observed after heterozygous *ColXist* expression from E11.5

Studies on *Xist* function in different cellular contexts and timepoints in development revealed its silencing function up to E9.5 and showed that expression after E12.5 was compatible with normal development until birth. However, *Xist* expression has an effect on the haematopoeitic system which leads to death shortly after birth (Savarese, et al., 2006). These investigations show the susceptibility to silencing by *Xist* in stem cell niches like the haematopoeitic system, which remains over the lifespan of mice (Agrelo, et al., 2009).

We aimed to investigate the effect of heterozygous *ColXist* RNA expression from E11.5 over a time period of four weeks on mouse development and the haematopoeitic system. Our observations showed no postnatal lethality and no obvious phenotype in born mice. We conclude that the heterozygous expression of *ColXist* RNA from chromosome 11 has no severe effect on the differentiation potential of precursor cells in the haematopoeitic lineage and is compatible with normal development at least until eight weeks after birth.

Furthermore, we aimed to observe any obvious effect in late development. Therefore, *ColXist* RNA was induced from E11.5 over a period of four weeks and mice were examined for any obvious peculiarity in behaviour and appearance. Mice showed normal motoric behaviour and two out of twelve mice revealed one cloudy eye each,

which could indicate a possible transgene effect. However, this observation applied only to a small fraction of mice and has to be further investigated for significance.
The dissected brains of *ColXist* RNA induced mice and control mice without the transgene expression showed no significant difference in weight. Furthermore, no severe structural discrepancy could be observed by comparison of sagittal sections.
We conclude that *ColXist* RNA expression from a late timepoint in development (E11.5) and continued postnatal induction of the transgene has no obvious effect on mouse development.

4.3 Trimethylation of histone H3 lysine 27 (H3K27m3) is established by *ColXist* RNA expression

Immunofluorescence analysis show that the prominent heterochromatin mark H3K27m3 is established on chromosome 11 by the expression of *ColXist* RNA. The trimethylation of histone H3 lysine 27 is caused by PRC2 (Plath, et al., 2003) and indicates successful recruitment by the transgenic *Xist* RNA lacking repeat A to the autosome.
We could observe established H3K27m3 after *in vivo ColXist* induction in early development as well as after induction in isolated MEFs in culture. Previous investigations showed that *Xist* loses its silencing function in differentiation and a cellular context is necessary (Savarese, et al., 2006) (Wutz and Jaenisch, 2000). However, it has been shown that in differentiated cells *Xist* RNA still localizes to the chromosome and H3K27m3 can be established independently of silencing (Kohlmaier, et al., 2004).
Our results confirm these data and show establishment of H3K27m3 at an average of 80% both after *in vivo* induction from E4.5 and *in vitro* induction in MEFs in culture. In female cells heterozygous for transgenic *ColXist* RNA the inactive X chromosome and chromosome 11 can be observed in condensed metaphase spreads. Furthermore, the co-localization of *Xist* RNA and H3K27m3 can be observed in interphase nuclei.
The recruitment of the histone variant macroH2A has been shown to be dependent of *Xist* expression and is involved in chromosomal memory (Pullirsch, et al., 2010). Previous investigations showed that recruitment of macroH2A by a mutant *Xist* RNA lacking repeat A can be observed by a low percentage of around 30% (Pullirsch, et al., 2010). Our investigations showed weak signals of co-localization of *Xist* RNA and

macroH2A by a low percentage (data not shown) and further investigations would give insights of the recruitment of other known heterochromatin proteins.
Furthermore, our results show the establishment of H3K27m3 in isolated spleen cells by an average of 80% of cells. We conclude that PRC2 can be recruited by the mutant *ColXist* RNA without repeat A and establishes H3K27m3 on chromosome 11. This applies to induction at an early timepoint in development, induction in MEFs in culture as well as in cells of haematopoeitic system after incuction from E11.5 independently of silencing.

4.4 No noticeable hypoacetylation of histone H4 on chromosome 11 can be observed

Histone H4 hypoacetylation is a prominent mark of the Xi in differentiated cells (Keohane, et al., 1996), its maintenance is independent of *Xist* expression and contributes to stable gene silencing on the inactive X chromosome (Csankovszki, et al., 1999). Previous investigations on the requirement of repeat A and gene silencing for chromosome-wide histone H4 hypoacetylation revealed a barely deacetylated X chromosome with bands of acetylated histone H4 which suggested regions of active genes (Pullirsch, et al.). These observations were in contrast with the chromosome-wide hypoacetylation known from the Xi (Keohane, et al., 1996).
Immunofluorescence co-stainings of H3K27m3 and histone H4 acetylation reveal no noticeable H4 hypoacetylation of chromosome 11 in contrast to the chromosome-wide deacetylation of the inactive X chromosome. Enlarged images of the autosome suggest only partial deacetylation near the centromeric region. We obtained this result after in vivo *ColXist* induction in early development as well as after in vitro induction in MEFs in culture and in isolated spleen cells. We assume that the presence of active gene regions on chromosome 11 is responsible for the acetylation mark.

In summary, we observe that even heterozygous *ColXist* expression from chromosome 11 in vivo is not compatible with embryonic development and leads to embryonic lethality most likely before E9.5. In contrast, heterozygous *ColXist* expression from E11.5 has no effect on late mouse development and lineage specific differentiation of the haematopoeitic system seems to be unaffected. Trimethylation of histone H3 lysine

27 can be observed in an average of 80% of cells after both, in vivo induction in development, as well as after in vitro induction in differentiated cells. No noticable hypoacetylation of histone H4 can be observed though.

4.5 Only female ES cell lines homozygous inducible for *ColXist* expression could be derived

For investigating the molecular reason of the severe phenotype we established ES cell lines homozygous for inducible *ColXist* RNA from both chromosomes 11. Seven cell lines out of thirty isolated E3.5 blastocysts could be established and surprisingly, all seven cell lines were female. The remaining *ColXist* ES cells could not be maintained in culture under 2i conditions. Furthermore, the establishment of an ES cell line with inducible full lenght *Xist* transcript from chromosome 11 could not be obtained either.
It can be speculated that the isolation of seven female ES cell lines happened by chance and any significance has to be recapitulated by repeating this experiment. However, this would be an unlikely coincidence. Furthermore, it has to be noted that no transgene expression was induced before blastocyst isolation. Therefore, any gene silencing function or sequestration of heterochromatin proteins can be excluded for explaining this result. Another possible explanation could be an involved counting mechanism, which achieves survival of female cells only. At the moment this remains speculative as the cells only carry the mutated *Xist* RNA and no other regulatory elements of the X inactivation centre are present, which is believed to be involved in the counting mechanism (Monkhorst, et al., 2008). Future investigation of a potential role of the ColXist transgene in regulation of X inactivation during ES cell derivation could clarify this observation.

4.6 In vitro differentiation shows no significant cell death

For investigating the effect of homozygous *ColXist* RNA expression from both chromosomes 11 in differentiation in vitro, we used the established ES cell lines and differentiated them for five days with retinoic acid with and without doxycycline, respectively. Our results show no significant cell death after homozygous induction of the transgenic *Xist* RNA. This is consistent with observations from previous investigations where only three genes on chromosome 11 were significantly downregulated by Xist in homozygous ES cells at day 4 of differentiation which shows only little effect of Xist lacking repeat A on gene expression (Pullirsch, et al., 2010).
From the obtained results we claim that *ColXist* RNA expression from chromosome 11 might prevent upregulation of genes involved in lineage formation at an early timepoint in development. We suggest that an early lineage decision is affected, which results in a developmental failure but shows only little effect on differentiation *in vitro*.

4.7 Genome-wide gene expression profiling

Our results of genome-wide gene expression profiling shows only slightly changes in gene expression patterns. This result supports our expectation of no significant gene silencing function of the mutant *Xist* RNA without repeat A. The statistical analysis of the experiment with three independently derived cell lines shows highly upregulated *Xist* RNA expression, which approves the experimental approach, and slightly downregulated genes, which are mainly chromosome 11 genes. These include Tom1l1 (target of myb1-like 1 (chicken)), Pctp (phosphatidylcholine transfer protein) and Mmd (monocyte to macrophage differentiation-associated). If the low downregulation of one of these genes or interaction of several misregulated genes on chromosome 11 is responsible for the observed phenotype remains to be investigated.
Our investigations suggest that slightly misregulation of gene expression patterns has severe consequences on mouse embryonic development. These minor changes might not be investigated after *in vitro* differentiation with retinoic acid. Another approach might be the diffferentiation into embryonic bodies to exclude lineage specific differentiation promoted by retinoic acid. Furthermore, quantitative expression analysis with reverse transcriptase real time PCR would provide further information about up- or

downregulation of specific genes in differentiation compared to the ES cell expression profile.

In summary the genome-wide gene expression profiling reveals a shift to downregulation of chromosome 11 genes. Other chromosomes do not seem to be affected by transgenic *Xist* RNA expression due to equal distribution of up- and downregulated genes. It is clearly shown that ¾ of chromosome 11 genes are downregulated in differentiation with *ColXist* RNA expression. However, fold changes do not excess 2,6 which is relatively low. Furthermore, our investigations show that downregulated genes are no Polycomb target genes (Boyer, et al., 2006) and seem to be downregulated upon differentiation. This is contrary to our assumption that affected genes are prevented from upregulation in differentiation by *ColXist* RNA expression. Overall, our data suggest that the transgenic *Xist* RNA lacking repeat A remains some silencing function on chromosome 11.

5 Summary

The non-coding *Xist* RNA is the trigger of chromosome-wide gene repression during X inactivation in mammals. Gene repression depends on a repeated sequence on the 5´ of *Xist*. Studies in mouse embryonic stem cells (ESCs) have shown that this repeat A element is essential for the initiation of gene silencing by *Xist*. Expression of Xist lacking repeat A has been shown to lead to an X chromosome which possesses all chromatin modifications of the inactive X chromosome (Xi) but genes remain active.

Here, we investigate if such a chromosome configuration can be compatible with development. For this we have established mice harbouring transgene for inducible expression of repeat A deleted *Xist* RNA from the *Col1a1* locus on chromosome 11. We show that induction of *Xist* lacking repeat A in embryonic cells leads to histone H3 lysine 27 tri-methylation (H3K27m3) but no noticeable decrease in histone H4 acetylation. Our experiments reveal an unexpected strong phenotype leading to embryonic lethality most likely before embryonic day 9.5. Notably, the phenotype was observed also in mice heterozygous for the transgenic chromosome 11 and independent of the parental origin of the transgene. However, no obvious phenotype could be observed after late induction of *Xist*. This suggests that in early development gene expression patterns might be disrupted by epigenetic modifications triggered by *Xist* without repeat A.

To investigate the molecular basis of this phenotype we have established ESCs from homozygous transgene bearing blastocysts and performed genome-wide gene expression profiling using Affymetrix mouse array analysis of three independently derived ES cell lines either with induction of *Xist* or without. We compared expression differences between induced and non-induced cells after 5 days of differentiation with retinoic acid. Our results show only little misregulation of genes with low fold changes. However, we could investigate a higher percentage of downregulated genes on chromosome 11 which suggests remaining silencing function of transgenic *Xist* RNA lacking repeat A in an autosomal context. Most significantly downregulated genes are no Polycomb target genes and seem to be downregulated upon differentiation. The assumption that developmental genes are prevented to be upregulated upon differentiation by transgenic *Xist* RNA expression could not be confirmed.

Overall, our results show that expression of *Xist* RNA lacking repeat A from chromosome 11 establishes H3K27m3, but no significant H4 hypoacetylation. Genes on this modified chromosome are slightly downregulated which is obviously not compatible with mouse embryonic development. We suggest that misregulated genes on chromosome 11 are necessary for a lineage specific differentiation pathway early in development and already little downregulation causes embryonic lethality.

6 Zusammenfassung

Die nicht-kodierende *Xist* RNA ist entscheidend für die Initiation der chromosomweiten Genrepression während der X-Inaktivierung in weiblichen Säugetierzellen. Die Stilllegung X-spezifischer Gene ist abhängig von einer sich wiederholenden Sequenz am 5´Ende von *Xist*. Untersuchungen an murinen embryonalen Stammzellen zeigten, dass dieses sogenannte „repeat A"-Element essenziell für die Initiation der Genrepression durch *Xist* ist. Es wurde gezeigt, dass die Expression einer *Xist* RNA mit fehlendem repeat A zwar alle charakteristischen Chromatinmodifikationen herbeiführen kann, Gene an diesem Chromosom jedoch aktiv bleiben.

In diesem Projekt wird untersucht, ob eine solche Chromosomen-Konfiguration mit der embryonalen Entwicklung vereinbar ist. Dafür wurde ein transgenes Mausmodell etabliert, bei dem die Expression einer *Xist* RNA mit fehlendem repeat A am 3´Ende des *Col1a1* Locus am Chromosom 11 mittels Doxyzyklin induzierbar ist. Es wird gezeigt, dass die Expression der mutierten *Xist* RNA zu Histon H3 - Lysin 27-trimethylierung (H3K27m3) führt, jedoch keine erkennbare Histon H4 - Deacetylierung beobachtet werden kann.

Die Expression der transgenen *Xist* RNA ohne repeat A *in vivo* in der frühen embryonalen Entwicklung zeigt einen starken, unerwarteten Phänotyp, der zu embryonaler Letalität vor Tag 9.5 der Embryonalentwicklung führt (E9.5). Interessanterweise wurde dieser Phänotyp ebenso bei heterozygoter Expression des Transgens beobachtet und ist unabhängig von der parentalen Herkunft der *Xist* RNA. Expression in einem späteren Stadium der Entwicklung (E11.5) zeigt keinen offensichtlichen Phänotyp was darauf hinweisen könnte, dass Genexpressionsmuster in der frühen embryonalen Entwicklung durch epigenetische Modifikationen gestört werden.

Um den molekularen Mechanismus des beobachteten Phänotyps zu untersuchen, wurden embryonale Stammzelllinien aus Blastocysten etabliert, welche homozygot induzierbare *Xist* RNA ohne repeat A am Chromosom 11 tragen. Mit Hilfe dieses *in vitro* Systems wurde eine genomweite Genexpressionsanalyse mittels Affymetrix Maus Array durchgeführt. Drei unabhängig voneinander isolierte embryonale Stammzelllinien wurden mit Retinolsäure für 5 Tage entweder mit oder ohne Doxyzyklin differenziert und die Unterschiede in der Genexpression zwischen

induzierten und nicht induzierten Zelllinien verglichen. Die Ergebnisse zeigen nur minimale Unterschiede in der Genexpression. Ein höherer Prozentsatz an herunterregulierten Genen konnte am Chromosom 11 beobachtet werden was darauf hinweist, dass Expression der transgenen *Xist* RNA ohne repeat A eine verbleibende Funktion in der Gen-Stilllegung im autosomalen Kontext aufweist. Die am stärksten herunterregulierten Gene sind keine Zielgene für Polycomb-Komplexe und scheinen in der Differenzierung herunterreguliert zu werden.

Zusammenfassend zeigen die Ergebnisse, dass die Expression einer transgenen *Xist* RNA ohne repeat A vom Chromosom 11 zwar H3K27m3 etabliert, jedoch keine signifikante H4 Deacetylierung beobachtet werden kann. Dreiviertel der Gene, die am Chromosom 11 lokalisiert sind, werden in geringem Ausmaß herunterreguliert, was offensichtlich mit der embryonalen Entwicklung der Maus nicht vereinbar ist. Es ist anzunehmen, dass die betroffenen Gene notwendig sind für spezifische Differenzierungswege in der frühen embryonalen Entwicklung und bereits geringe Änderungen in der Expression zu embryonaler Letalität führen.

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