CDK8
A NEW MODULATOR FOR X-CHROMOSOME INACTIVATION

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SUMMARY

The evolution of sex chromosome dimorphism led to a gene expression imbalance of X-linked genes in mammals. To compensate for this dosage difference, all but one X-chromosomes in female mammals are silenced by a process called X-chromosome inactivation. This process is triggered by the expression of the IncRNA Xist and leads to transcriptional gene silencing of X-linked genes followed by heterochromatinisation of the inactive X-chromosome.

In order to find new factors involved in this process our group conducted a genetic screen. One of the identified candidates is Cdk8. Cdk8 is a kinase that has been associated with the Mediator complex and has been implicated in transcriptional regulation. In addition, a role of Cdk8 in several cancers has been identified.

This thesis investigates the role of Cdk8 in X-Chromosome inactivation. I can show that loss of Cdk8 reduces X-linked gene silencing. Loss of Cdk8 leads to reduced recruitment of PRC2 to the inactive X, which results in reduced deposition of heterochromatic H3K27me3 marks, associated with stable gene silencing. These results suggest a contribution of Cdk8 in regulating the chromatin modifier complex PRC2, which has been documented to be involved in X-chromosome inactivation.

In order to study the function of Cdk8, I characterised preimplantation and postimplantation Cdk8 knockout embryos. Loss of Cdk8 does show severe polymorphic phenotypes but does not lead to female specific lethality. This observation shows that Cdk8 is not crucial for X-chromosome inactivation in development. Potential modulating functions of Cdk8 in X-chromosome inactivation might be masked by redundant pathways. The severe developmental effect of Cdk8 depletion might be based on deregulated Notch signaling, which I could substantiate by in vitro analysis of Notch target gene expression.

My results show that Cdk8 is involved in X-chromosome inactivation as its loss of function reduces X-linked gene silencing in ES cells. Loss of Cdk8 kinase function leads to reduced Ezh2 recruitment and H3K27me3 deposition by Xist. In the embryo Cdk8 is crucial for development in both sexes.
ZUSAMMENFASSUNG


Zusammenfassend konnte ich die Regulation der X-Chromosomen Inaktivierung durch Cdk8 zeigen, da dessen Verlust in Zellsystemen zu reduzierter Genrepression
INTRODUCTION
INTRODUCTION

X-CHROMOSOME INACTIVATION

Sexual reproduction in eukaryotes enhances their evolutionary fitness by increasing genetic variation. Fertilisation of two haploid germ cells from sexually dimorphic parents lead to a new genetic combination in the offspring, thereby promoting genetic diversity. This sexual dimorphism resulted in the appearance of sex determining genes in a great many species. In a subset of these species, the sex determining genes were the origin of the evolution of sex chromosomes.

The sex determining locus and its surrounding genes, which gradually acquired sex determining functions by beneficial mutations, are excluded from meiotic recombination in germ cells in order to keep this sex specific region together. As a result of this suppressed recombination the sex chromosome eroded, leading to heterogamety \(^1,2\). This heterogamety is only present in one sex – harbouring the sex determining gene – whereas the other sex shows homozgyous gene levels. In eutherian mammals male heterogamety evolved, displayed by an XY pair of sex-chromosomes. The Y-chromosome harbours the Sry gene (Sex-determining region Y) as the sex determining gene \(^3\). Females have an XX genotype, thereby showing a doubled gene dose for X-chromosomal genes compared to males. This imbalance in gene expression needs to be counteracted – referred to as dosage compensation – to ensure faithful development, as unbalanced X-chromosomal gene dosage leads to failure of extra-embryonic development \(^4,5\), plausibly explained by the enrichment of placental genes on the X-chromosome.

The mechanism of dosage compensation in eutherians is based on inheritable transcriptional silencing and heterochromatinisation of all but one X-chromosomes and is therefore called X-chromosome inactivation (XCI) \(^6\). The term XCI needs to be put into perspective as some genes are escaping this process, called escapees. Recent studies estimate around 12-20% escapees in human and around 3-7% escapees in mouse. The list of these escapees naturally includes genes necessary for XCI and genes located in the pseudo autosomal region (PAR). This region is necessary for male meiotic recombination and chromosome segregation in germ cell development.
Furthermore, some of these genes escape XCI in a tissue dependent manner. It is not yet fully understood how the process of escaping is coordinated. There is however a tendency that escapees are enriched in regions that have been added to the X-chromosome more recently\(^7\) pointing to an evolutionary acquisition of XCI. XCI can be divided in two different epigenetic mechanisms: imprinted XCI (iXCI) and random XCI (rXCI). The former process leads to exclusive silencing of the paternally inherited X-chromosome (\(X^P\))\(^8\), whereas in rXCI the future inactive X-chromosome (Xi) is randomly chosen in each individual cell, leading to XCI mosaicism \(^6,9\). Most mammals use exclusively either iXCI or rXCI for dosage compensation. Marsupials are an example for iXCI only \(^10\). In rabbit \(^11\) and horse \(^12\) only rXCI can be observed. Also humans display only rXCI \(^13,14\), whereas recent studies identified an additional XCI mechanism during the first week of development. During this early phase of human development both X-chromosomes are active but their expression levels are reduced at similar rates, this mechanism is termed X-chromosome dampening \(^14\). In contrast to the just mentioned species the mouse is using iXCI \(^8,15\) and rXCI \(^6,9\) in a sequential order during development (Figure 1).
To operate such a tremendous chromosome reorganisation, a dedicated region has evolved on the X-chromosome: the X-inactivation centre (XIC). Within this centre, the driver of XCI is located – the long non-coding RNA (lncRNA) X-inactive specific transcript (Xist)\textsuperscript{16-19}. Several other genes located in the XIC promote Xist expression, for example the protein coding gene Rnf12/Rlim that controls Xist expression during iXCI\textsuperscript{20}, or the lncRNAs Jpx and Ftx, whose correct expression correlates with faithful XIC. Another set of genes in the XIC repress Xist. The most prominent negative regulator is the lncRNA Tsix\textsuperscript{21,22}. Tsix is transcribed antisense to Xist and its binding to the Xist promoter region impairs Xist expression. Regulation of Tsix expression depends on a microsatellite repeat, DXPas34, which serves as a binding platform for transcription factors\textsuperscript{23}. The expression and coating of Xist\textit{ in cis} is necessary and sufficient to silence the Xi both in iXCI\textsuperscript{8} and rXCI\textsuperscript{24}. The requirement for Xist\textit{ in rXCI} has been impressively shown by ectopic expression of Xist cDNA from autosomes\textsuperscript{25,26} and inducible expression of endogenous Xist from the male X-chromosome\textsuperscript{27}, both of which leads to silencing of the chromosome \textit{in cis}.

Upon zygotic genome activation both X-chromosomes are transcriptionally active but as soon as at the 4-cell stage one X-chromosome gets silenced. This first wave of inactivation is iXCI and leads to exclusive silencing of the X\textsuperscript{P}\textsuperscript{8}. A recent study showed that the Xist gene on the X\textsuperscript{M} is decorated with repressive H3K27me3 marks, which are established during oogenesis, thereby prohibiting Xist expression from the X\textsuperscript{M}\textsuperscript{28}. To additionally ensure inactivation of the X\textsuperscript{P} during iXCI a maternal protein – Rlim – promotes upregulation of the paternal Xist\textsuperscript{20}. IXCI is maintained in primitive endoderm and trophectoderm lineages but gets revoked in cells of the epiblast at the blastocyst stage\textsuperscript{29}.
X-chromosome reactivation is linked with the expression of pluripotency transcription factors in the epiblast. It was shown that Prdm14 promotes Rnf12 repression, thereby revoking Xist expression. Xist repression is further maintained by its antisense regulator Tsix, whose expression is upregulated by Rex1.

Reactivation is succeeded by a second wave of XCI – rXCI – which leads to random silencing of either the X\(^P\) or the X\(^M\). The exact molecular function of choosing either the X\(^M\) or the X\(^P\) for inactivation is still under investigation. Xist from the future Xi gets re-expressed by Rnf12 mediated removal of Rex1, which results in downregulation of the Xist antisense IncRNA Tsix.

Xist expression starts a cascade of events to silence the X-chromosome (Figure 2). Upon expression of Xist the ribonucleoprotein HnnpU tethers it to the future Xi. The spreading of Xist over the Xi is dependent on the 3D shape of the chromosome, whereat regions which are near the XIC in 3D space get coated and silenced first, followed by a topological rearrangement, bringing other regions in 3D vicinity of the XIC. Like other transcriptionally inactive regions in the genome the Xi localises to the nuclear periphery, which is mediated by the lamin-B receptor (Lbr) protein. A signal for these proteins to bind Xist might be a recently discovered RNA modification, namely N\(^6\)-methyladenosine (m6A). This modification is mediated by the RNA binding proteins Rbm15 and Rbm15B, which – via Wtap – recruit the m6A methyltransferase Mettl3. m6A is recognised by the m6A reader Ythdc1, which has been shown to interact with the above mentioned proteins HnnpU and Lbr and furthermore with Spen and members of the Polycomb repressive complex 1 and 2. Spen has recently been identified as a factor involved in XCI. Binding of Spen to the Xi, either via m6A or through yet unknown mechanisms, leads to the recruitment of the histone-deacetylase Hdac3. As acetylation marks on histones are correlated with transcriptionally active chromatin their removal is a crucial step in silencing the chromosome. Upon hypoacetylation of the Xi RNA Polymerase II (PolII) gets excluded from the Xi, finally abrogating transcription of the X-linked genes. Silenced genes are concentrated in the so called repressive nuclear compartment to maintain their silenced state – by additional XCI mechanisms – whereas not yet silenced genes are located at the outer borders of this compartment, which will associate with this compartment after being silenced. To maintain the repressed state chromatin is...
epigenetically modified by Polycomb repressive complex (PRC) 1 and 2. These complexes deposit repressive histone 2A lysine 119 ubiquitination (H2AK119ub) and histone 3 lysine 27 trimethylation (H3K27me3) marks, respectively, leading to the formation of facultative heterochromatin.

The interaction of these complexes with each other and Xist is subject of several recent studies. In a first step, HnrnpK recruits the non-canonical PRC1 proteins Pcgf3/5 to a specific 600bp long region of the Xist RNA. The ubiquitin ligase of this non-canonical complex, Ring1, deposits H2AK119ub marks at the corresponding X-chromosome, which serves to recruit additional non-canonical PRC1 complexes, increasing the H2AK119ub deposition through positive feedback. In parallel, these marks are recognised by the PRC2 complex, at least partially by direct binding of the PRC2 cofactor Jarid2. Pre-existing H3K27me3 marks are normally a requirement for PRC2 function as this mark is recognised by Eed, which leads to activation of the methyltransferase Ezh2, creating a positive feedback. A recent cryo-EM study proposes an alternative mechanism in which Ezh2 methylates Jarid2, resulting in an H3K27me3 mimic and thereby activating the canonical PRC2 complex.

The established heterochromatin needs to be maintained throughout development, which is achieved by further modifications of the Xi and continuous expression of Xist. One event taking place to maintain the repressed state is the exchange of histone 2A with histone macro2A1. MacroH2A1 is recognised and ubiquitinylated by PRC1 similar to H2A. The difference to the canonical histone 2A is its unresponsiveness to de-ubiquitination and therefore it can only be removed by replication associated dilution. This feature provides an epigenetic memory to maintain the repressive ubiquitin mark. A second step to ensure stable repression is DNA methylation at the level of CpG islands. Those islands are associated with promoter regions of genes and are generally un-methylated, whereas methylation of CpG islands corresponds with gene silencing. CpG methylation is mediated by the Smchd1 protein, which has been recently shown to be recruited to the Xi via the H2AK119ub modification. Binding of Smchd1 either recruits the Dnmt3b methylase directly or modifies the chromatin in a way that Dnmt3b can access the DNA. As shown for macroH2A1, depletion of Smchd1 does not lead to reactivation of X-linked genes once inactivation is established. However, it has been
reported that Smchd1 has a second, more critical role in establishing epigenetic modifications necessary for later XCI maintenance. Deletion of Smchd1 results in loss of trophoblast giant cells in female embryos contributing to female specific lethality. One hypothesis for this observation is based on deficient iXCI maintenance due to reduced deposition of repressive H3K27me3 marks. A more lineage specific mode of XCI maintenance has been attributed to the nuclear matrix Ciz1 protein. Loss of this protein leads to diffuse Xist localisation in embryonic fibroblasts and lymphoid lineages, but does not result in significant X-linked gene reactivation.

This complex interplay and chronology of processes provide an archetype for epigenetic regulation and chromatin modification. Studying this process has its limitations, as it happens early in development. A tool to bypass this drawback is using an in vitro system which recapitulates the cells of the early embryo: mouse embryonic stem cells.

**Figure 2 Schematic representation of rXCI initiation and early maintenance.** (I) *Xist* recruitment and gene silencing: *Xist* is recruited to the Xi by HnmpU. The Xi is relocated to the nuclear lamina via Lbr. Rbm15 mediates Mettl3 dependent 6mA modification of *Xist*. 6mA might recruit Spen, which in turn recruits Hdac3 to deacetylate the histones finally resulting in depletion of Pol II. (II) Heterochromatinisation: HnmpK recruits non canonical PrC1 via Pcgf3/5 to *Xist* leading to H2AK119ub modification. H2AK119ub is recognised by non-canonical PrC1 leading to further H2AK119ub deposition. Jarid2 recognises H2AK119ub, which leads to PrC2 recruitment and H3K27me3 deposition, also resulting in a positive feedback of further H3K27me3 deposition. (III) Maintenance: H2A is exchanged for demethylase and deubiquitinase resistant macroH2A1, CpG islands become methylated by Dnmt3b via interaction with Smchd1.
EMBRYONIC STEM CELLS

Mouse embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of the blastocyst \(^{60,61}\). These cells self-renew indefinitely, and are able to differentiate into all three germ layers and germ cells upon stimulation. Chemically defined culture conditions \(^{62}\) allow propagation of these cells \textit{in vitro} in their naïve pluripotent \(^{63}\) state. This state is characterised by uniform expression of pluripotency transcription factors (TFs), such as Oct4, Nanog and Sox2 \(^{64}\), global hypomethylation of their genome \(^{65}\), their capability to contribute to high-level chimeras \(^{66}\) and the presence of two active X-chromosomes (X\(^A\)X\(^A\)) in female ESC \(^{67}\). This X\(^A\)X\(^A\) state has been shown to delay female development and needs to be resolved by rXCI before lineage commitment can take place \(^{68}\). This study displays the strong interplay between dosage compensation and faithful female early development.

The presence of two active X-chromosomes and the inactivation of one of them upon differentiation provide a perfect model for studying rXCI, also explaining the majority of XCI studies focusing on rXCI rather than iXCI. Not only can rXCI be investigated during \textit{in vitro} differentiation of female ESC but rather it is possible to mimic rXCI using inducible \textit{Xist} systems without disturbing the cell identity \(^{25,26}\). This system has been widely used to identify factors involved in rXCI \(^{36-38,40,41}\) as described before, showing that the main components for silencing the X-chromosome are expressed and functional in ESC.

We performed a viral gene trap mutagenesis screen in haploid ESC \(^{69}\) to find new factors involved in XCI. As haploid cells only carry one copy of each chromosome recessive mutations, which could mask a phenotype, cannot occur. These cells were derived from \textit{Xist}\(^{\text{TX/TX R26\text{nlsrTa/nlsrTa}}\) mice, which allows induction of \textit{Xist} expression by a tetracycline regulated transactivator. If \textit{Xist} is expressed in these haploid cells the only copy of the X-chromosome gets silenced leading to cell death, which provided us with a quantitative readout. Even if these cells diploidise, which can be observed after a few passages, all copies of the X-chromosome get silenced \(^{40}\) (Figure 3). The genes identified in this screen are a mixture of already published XCI factors, proving the functionality of the approach, and several new candidates. These candidates are mostly genes studied in other biological contexts, which is not surprising as also the
factors that already have been experimentally linked to XCI, like PRC1/2 or Smchd1 do not have an exclusive role in XCI. One of the highest ranked genes and most intriguing because of its already known functions in other biological processes is Cdk8.

Figure 3 Illustration of Hatx3 embryonic stem cells. Haploid ES cells were derived, carrying one copy of each chromosome. Over time, these cells diploidise due to yet unknown mechanisms resulting in an isogenic set of chromosomes. Hatx3 were derived from XistTX/TX R26nlsrtTA/nlsrtTA mice. These cells carry a transactivator in the ROSA26 locus and the Tet operator cloned upstream of the endogenous Xist promoter, which leads to Xist expression upon doxycycline treatment. Due to their isogenic chromosomes all copies of the X-chromosome harbour the Tet operator resulting in Xist mediated X-chromosome inactivation of all X-linked genes, which ultimately leads to cell death. SA: splice acceptor, nlsrtTA: nuclear localisation system - reverse tetracycline-controlled transactivator; tetO: Tet Operator; Dox: Doxycycline; Xist: X-inactive specific transcript; ESC: embryonic stem cell; XCI: X-chromosome inactivation
Cdk8 belongs to the family of cyclin dependent kinases (Cdk). These serine/threonine kinases are dependent on interaction with cyclins to fulfil their function. Cdns regulate various cellular processes like metabolism, haematopoiesis and neuronal differentiation through cell cycle or transcriptional regulation.

Cdk8 was first identified as a negative regulator of transcription. It does so by forming a repressive submodule of the Mediator complex together with Med12, Med13 and CyclinC (CycC) called kinase module. More recently also transcription activating functions of Cdk8 have been discovered. Cdk8 directly regulates transcription by phosphorylating Ser2 and Ser5 of the carboxy terminal domain (CTD) of RNA Polymerase II (PolII). These modifications lead, amongst other things, to the recruitment of the super elongation complex (SEC), which has been reported in several studies. In HCT116 cells it has been shown that Cdk8 enhances transcriptional elongation of immediate early genes upon serum stimulation. Another study in HCT116 cells showed that hypoxia inducible genes need the Cdk8-Mediator complex to release PolII pausing. In B-cell lymphoma cells it has been demonstrated that a subset of genes, most of them with metabolic functions, show enhanced transcriptional activation upon TFII-I and Ctcf mediated Cdk8 recruitment. Indication for a transcriptional repressive function of Cdk8 comes from a biochemical study in colon carcinoma cells, where it was shown that TFII-H mediated transcriptional activation is impaired upon Cdk8 dependent phosphorylation of the TFII-H subunit CyclinH.

Additionally to directly regulating the core transcriptional machinery, Cdk8 is implicated in transcriptional regulation by modifying TFs. In the case of BMP/TGF-β signalling CDK8 fulfils a dual role. First, phosphorylation of the linker domain of receptor activated SMAD1 leads to binding of the transcriptional coactivator YAP resulting in transcription activation. The same phospho-site is subsequently recognised by E3 ubiquitin ligases, leading to degradation of SMAD1 and terminating the BMP/TGF-β signal. Ifn-γ-inducible antiviral response also depends on Cdk8, as it was shown that Stat1 dependent Ifn-γ gene expression is activated upon Cdk8 mediated Stat1 phosphorylation. The same study additionally showed Stat3 S727 phosphorylation by Cdk8. Another pathway regulated by Cdk8 is the Notch signaling
pathway. Upon activation, Notch gets cleaved resulting in the Notch intracellular domain (Nicd). Nicd, with associated cofactors, binds to its target gene promoters and removes repressive TFs. This process is strictly controlled by E3 ubiquitin ligase mediated degradation of the Nicd. For the E3 ligases to be active, a preceding phosphorylation is necessary. A Nicd cofactor, mastermind, was shown to recruit Cdk8 in order to deposit this phosphorylation.

Cdk8 has been implicated in ESC maintenance and tumour growth by promoting Myc dependent transcription. An involvement of Cdk8 in embryogenesis has been proposed from an in vivo study, which reports embryonic lethality of Cdk8 null mice at embryonic day 2.5, pointing to an even earlier implication of Cdk8 in development.

An in vitro study proposes an interaction of Cdk8 and Cdk19 with Ezh2 in order to regulate retinoic acid (RA) targeted developmental genes. The study shows that Cdk8 phosphorylates Ezh2 in vitro at Thr492 and to a lesser extent at Thr350. The predominant Thr492 phosphorylation is associated with a disassembly of the PRC2 complex, which results in transcriptional active chromatin, whereas the Thr350 phosphorylation serves to recruit PRC2 to the chromatin. However, these phosphorylations were not altered upon siRNA mediated gene repression of Cdk8 or Cdk19, suggesting redundant regulatory pathways. In line with this study it has been observed that Cdk8 mediates ncRNA-dependent transcription of Polycomb target genes, which are repressed by PRC1 in the pluripotent state.

The implications of Cdk8 in transcription regulation, signalling pathways and in early development explain the findings of dysregulated Cdk8 in various cancers. This is exemplified by elevated Cdk8 levels in 60% of colorectal carcinomas (CRC). The majority of genetic alterations detected in CRC lead to aberrant Wnt signalling. Studies link exactly this aberrations to Cdk8 function. More specifically, deregulation of CDK8 promotes β-CATENIN target gene expression, a prerequisite for CRC growth. The TF E2F1 inhibits β-CATENIN/TCF dependent transcription resulting in apoptosis. This mechanism is antagonized by elevated CDK8 levels, resulting in enhanced tumour formation. CDK8 acts even more directly on β-CATENIN driven transcription as it has been shown that CDK8 associates with β-CATENIN target gene promoters, resulting in elevated expression levels. The impact of CDK8 on WNT signalling also
increases the metastatic potential of CRC cells. Another example of CDK8 acting as an oncogene has been demonstrated in acute myeloid leukaemia (AML), where it represses transcription of super-enhancer associated genes involved in cancer suppression.

The multifaceted roles of Cdk8 can also be seen in the case of CRC, as it has been reported that in the ApcMin murine tumour model Cdk8 acts as a tumour suppressor. In this model, loss of Cdk8 leads to reduced PRC2 recruitment and subsequent silencing of oncogenic signalling genes. Another study substantiates the idea of Cdk8 being a tumour suppressor by linking p53 dependent p21 transcription and subsequent apoptosis to binding of Cdk8 to the p21 promoter and thereby enhancing its transcription.

It is of note that in vertebrates paralogues of Cdk8, Med12 and Med13 evolved, namely Cdk19, Med12L and Med13L. Both complexes assemble mutually exclusive, except for their shared dependence on CycC. Cdk8 and Ckd19 share 97% sequence homology in their catalytic domain and only differ at the C-terminus (Figure 4). The critical residues of Cdk8 have been well described and consist of ATP binding at Lys52, the proton acceptor at Asp151 and Asp173, an amino acid critical for forming the activation loop, whose mutations all lead to a kinase dead protein. The same amino acids have been shown to be crucial for Cdk19 kinase function. Therefore, it is not surprising that Cdk19 shares several functions with Cdk8, such as phosphorylating Nicd, Stat1 and PolII and the oncogenic function in AML. In contrast to Cdk8, Cdk19 is dispensable for early development and compared to the ubiquitous expression of Cdk8, Cdk19 shows tissue specific expression patterns. Loss of CycC leads to profound embryo retardation and an underdeveloped placental labyrinth layer, resulting in embryonic lethality at embryonic day 10.5 suggesting additional roles of CycC in development independent of its interaction with Cdk8 and Cdk19.
**Figure 4 Cdk8 schematic representation:** ATP binding shown in green (K52), CyclinC binding site shown in red, proton acceptor shown in blue (D151), amino acid necessary for activation loop shown in green square (D173), brown squares indicate region unique to Cdk8 and not present in Cdk19. Binding of Med12/12L and Med13/13L approximately.
RESEARCH QUESTION

The aim of this thesis was to determine the impact of Cdk8 on X-chromosome inactivation and to identify the molecular basis of the function of Cdk8 in this epigenetic process.

To achieve this, I created Cdk8 loss of function ESCs harbouring a transgenic system to induce X-chromosome inactivation. These cells die upon Xist induction, which I used as a readout to define the impact of loss of Cdk8 on Xist induced X-linked gene repression. Furthermore I created Cdk8 mutant cell lines to demonstrate the requirement of the kinase function for proper X-chromosome inactivation. By analysing transcriptomic data I could show that loss of Cdk8 leads to reduced X-linked gene silencing, confirming the function of Cdk8 in X-chromosome inactivation. I found experimental indications that loss of Cdk8 might reduce heterochromatinisation by modulating PRC2, which is substantiated by a study demonstrating phosphorylation of Ezh2 by Cdk8 and reduced H3K27me3 deposition upon Cdk8 depletion.

Finally, I determined a sex independent requirement for Cdk8 in pre- and postimplantation development, suggesting a modulating function of Cdk8 in X-chromosome inactivation in development. Embryo studies revealed a polymorphic Cdk8<sup>lox</sup> phenotype that might be based on deregulated Notch signaling, as I could show deregulation of Notch target genes and Nicd turnover in vitro.

Together these data demonstrate the implication of Cdk8 in X-chromosome inactivation in vitro presumably by modulating PRC2 function and a crucial role for Cdk8 in development, apparently by modulating additional developmental processes, exemplified by deregulated Notch signaling in vitro.

Studying the modulation of PRC2 function in a discrete and controllable system like X-chromosome inactivation will advance our understanding in the tightly controlled and regulated processes that lead to context specific epigenetic gene repression.

The appearance of polymorphic phenotypes, presumably due to deregulated Notch signaling, will allow us to further study the precise regulation of signaling necessary for proper development.
RESULTS
RESULTS

GENERATION AND CHARACTERISATION OF CDK8 LOSS OF FUNCTION MOUSE EMBRYONIC STEM CELLS

CDK8 LOSS OF FUNCTION EMBRYONIC STEM CELLS

To validate the finding of the screen that Cdk8 contributes to XCI, I generated Cdk8 loss of function mutations in ESCs (ΔCdk8). I used the cells in which the screen was conducted. The haploid ESCs were derived from Xist\textsuperscript{TX/TX} \textit{R26nlsrtTA/nlsrtTA} mice, specifically the line Hatx3\textsuperscript{40}. As I do not require a haploid genome for my analysis I used diploidised Hatx3 cells (in this study hereafter called WT). To generate loss of function mutations I deployed a two guide RNA Crispr/Cas9 strategy deleting 136bp around the start codon of Cdk8. This should abrogate transcription and introduce a frameshift in case of any alternative downstream transcripts. I obtained several independent homozygous loss of function cell lines and continued to work with three of these clones, which are ΔCdk8 #8, ΔCdk8 #11 and ΔCdk8 #15 (hereafter called #8, #11 and #15). #8 and #15 show the expected deletion (Figure 5A), whereas #11 shows an 83bp deletion (Figure 5A) upstream of the start codon and an insertion of a single thymine in the coding sequence (Figure 5B). On transcript level #8 and #15 show 85% and 90% reduction of Cdk8 mRNA, respectively, whereas #11 shows expression levels similar to WT (Figure 5C). Nonetheless, all three clones show complete absence of Cdk8 protein as confirmed by western blot (Figure 5D), demonstrating that also the particular deletion pattern of #11 leads to protein depletion.

Figure 5 Generation of Cdk8 loss of function ESC: (A) Genotypic confirmation of Crispr/Cas9 mediated DNA cleavage. Cdk8: amplicon surrounding Crispr/Cas9 deletion region; Il2: Interleukin2, loading control. WT band 1372 bp, ΔCdk8 #8 and ΔCdk8 #15 1236bp, ΔCdk8 #11 1290bp. H\textsubscript{2}O negative control. (B) Sanger sequencing data of ΔCdk8 #8, ΔCdk8 #11 and ΔCdk8 #15. Numbers above indicate genomic location; green characters indicate guideRNA binding sites; red characters indicate start codon; capital character indicates insertion. (C) Expression level analysis of ΔCdk8 cells. Fwd primer spans exon1/exon2, rev primer spans exon2/exon3; Expression normalised to Gapdh and relative to WT level. Error bars represent SD. Experiment done in biological triplicates (D) Western blot confirmation of Cdk8 depletion. β-Actin: loading control
VALIDATION OF THE SCREEN

To confirm that Cdk8 is involved in XCI I recapitulated the readout used in the screen. In Hatx3 cells doxycycline (Dox) inducible Xist expression at all copies of the X-chromosome results in repression of all X-linked transcripts and leads to cell death. To increase the stringency and the comparability of the readout I decided to use a single cell assay (Figure 6A). I quantified the ratio of colonies emerging in the induced over the uninduced condition (Figure 6B). Around 5% of WT cells grew up to colonies. In comparison, for #8 around 75%, for #11 around 55% and for #15 around 70% of the cells gave rise to colonies, respectively. To confirm that the surviving colonies still responded to Dox with Xist expression induced XCI, I replated three of the #8 colonies and withdraw Dox for 24 or 48 hours, respectively. Rapidly decreasing Xist transcript and rapidly increasing expression levels of the X-linked gene Pgk1 confirmed that the inducible system was intact in ΔCdk8 cells (Figure 6C). This result verified the initial screen and shows that the absence of Cdk8 enhances survival upon induction of Xist in this cell model.
The kinase function of Cdk8 is responsible for the observed phenotype

To ensure that the observed phenotype is based on the deletion of Cdk8 and its function as a kinase, I reintroduced Cdk8 cDNA into ΔCdk8 cells using the PiggyBac transposon system. Expression level analysis (Figure 7A) and western blot analysis (Figure 7B) shows restored Cdk8 expression in a clone derived from #8, ΔCdk8 #8TG15 (#8TG15), and in two clones derived from #11, ΔCdk8 #11TG2 (#11TG2) and ΔCdk8 #11TG14 (#11TG14). Additionally, Cdk8 cDNA was introduced in WT cells as a control (WTTG). The product of the transgenic Cdk8 is bigger than the wildtype, as the transgene has an HA and Strep-tag® II tag fused to the N-terminus. This is the reason for the double band observed in the WTTG. Overexpression of Cdk8 in the WT does not influence the survival upon Dox induction, whereas restoration of Cdk8 expression

Figure 6 Validation of Cdk8 as a candidate silencing factor. (A) Schematic representation of the single cell survival assay. Single cells are sorted in a 96well plate, in medium alternating containing Dox (+) or not (-). After two weeks emerging colonies are quantified and the ratio between colonies emerging in the XCI induced medium over colonies emerging in the wells without XCI induction is calculated (B) Ratio of Survival over no Dox control is depicted. Error bars represent SD. Experiment done in biological triplicates. (C) Validation of responsiveness of surviving colonies to Dox induced XCI. mRNA levels of Xist, lower panel, and Pgk1, upper panel, upon 24 and 48 hours of Dox withdrawal, respectively. Error bars represent SD, Experiment done in biological duplicates.
in ΔCdk8 cells decreased survival to around 15% consistent with a restoration of \textit{Xist} function (Figure 7C). The still elevated survival compared to WT indicates a significant but partial restoration of \textit{Xist} function by reintroduction of Cdk8. A full restoration of \textit{Xist} function is difficult using a transgenic system, which consists only of the coding region but lacks regulatory sequences. Restoration of \textit{Cdk8} expression in ΔCdk8 cells significantly reduces survival of these cells upon \textit{Xist} induction demonstrating the requirement of Cdk8 for proper X-chromosome inactivation.

To investigate if \textit{Xist} function depends on the kinase function of Cdk8 I introduced mutated forms of Cdk8 cDNA into the ΔCdk8 cells. These mutations are reported to abolish Cdk8 kinase function. In one construct I exchanged one amino acid, which is acting as the proton acceptor (D151) from Asp to Ala (D151A) and in a second construct I deleted an amino acid crucial for ATP binding (K52) (ΔATP). I introduced these constructs in the ΔCdk8 #8 cell line as well as in WT cells and confirmed expression of the transgenes on protein level for two clones each: ΔCdk8 #8\textsuperscript{TG} D151A#4 and ΔCdk8 #8\textsuperscript{TG} D151A#6 for the D151A mutation (Figure 8A) and ΔCdk8 #8\textsuperscript{TG} ΔATP#14 and ΔCdk8 #8\textsuperscript{TG} ΔATP#35 for the ΔATP mutation (Figure 8B). In the survival assay the mutant constructs do not significantly reduce survival as compared to the wildtype Cdk8 cDNA (Figure 8C, D). The missing rescue effect of the kinase dead mutant \textit{Cdk8}
constructs provide evidence that Xist function is dependent on the kinase function of Cdk8.

Cdk19 is a close parologue of Cdk8. It has been shown that they have overlapping functions in various contexts. To assess if Cdk19 has a function in Xist induced X-linked gene repression I created loss of function mutations for Cdk19 in WT and ΔCdk8 #8 cells. I used two guide RNAs to remove the start codon. Three independent clones from WT and ΔCdk8 background show significantly reduced mRNA levels (Figure 9A). Loss of Cdk19 does not significantly change Cdk8 expression and neither is Cdk19

**Figure 8 Cdk8 kinase dead constructs:** (A-B) Western blot confirmation of Cdk8<sup>TG</sup> restoration. HA: detection of HA-Tag fused to Cdk8; β-Actin: loading control (A) D151A kinase dead constructs (B) ΔATP kinase dead constructs (C-D) Ratio of Survival over no Dox control is depicted. Error bars represent SD, Experiment done in biological triplicates. (C) D151A kinase dead constructs (D) ΔATP kinase dead constructs.

**CDK19 DOES NOT COMPENSATE FOR CDK8 FUNCTION IN XIST MEDIATED SILENCING**

Cdk19 is a close parologue of Cdk8. It has been shown that they have overlapping functions in various contexts. To assess if Cdk19 has a function in Xist induced X-linked gene repression I created loss of function mutations for Cdk19 in WT and ΔCdk8 #8 cells. I used two guide RNAs to remove the start codon. Three independent clones from WT and ΔCdk8 background show significantly reduced mRNA levels (Figure 9A). Loss of Cdk19 does not significantly change Cdk8 expression and neither is Cdk19
upregulated in ΔCdk8 cells (Figure 9A). Applying the single cell survival assay pinpoints the survival phenotype specifically to Cdk8 loss of function, as deletion of Cdk19 does not further enhance survival upon Dox stimulation, neither in the WT background nor in the ΔCdk8 cells (Figure 9B). This experiment demonstrates that specifically Cdk8 but not Cdk19 is required for Xist function. Furthermore, loss of neither Cdk8 nor Cdk19 is compensated by transcriptional upregulation of the paralogous kinase (Figure 9A).

Figure 9 ΔCdk19 ESC: (A) Expression level analysis of ΔCdk19 cells and ΔCdk8ΔCdk19 cells. Black bar represents Cdk19 expression level, grey bar represents Cdk8 expression levels. Expression normalised to Gapdh and relative to WT level. Error bars represent SD. Experiment done in biological duplicates (B) Ratio of Survival over no Dox control is depicted. Error bars represent SD. Experiment done in biological triplicates.
THE ROLE OF CDK8 IN INITIATION OF RANDOM X-CHROMOSOME INACTIVATION

THE EFFECT OF CDK8 DEPLETION ON SILENCING OF X-LINKED GENES

Hatx3 cells die upon Dox stimulation due to repressing transcription of all copies of X-chromosomal genes. As ΔCdk8 cells survive upon induction of XCI to a much higher degree than WT cells, I performed a transcriptomic analysis to define the degree of deregulation of silencing upon Cdk8 depletion. I compared the protein coding transcriptome of WT cells and the three ΔCdk8 cell lines in untreated conditions and after 48 hours of Dox induction, a time point at which X-linked genes are mostly silenced but the cells are still viable. As expected, the WT samples uniformly and nearly completely shut down all X-linked genes subjected to XCI whereas the transcription of autosomal genes did not change (Figure 10A I and Table 1). On the other hand, ΔCdk8 cells show quite a different distribution of gene silencing. The average expression level of X-linked genes was decreased to 50% compared to nearly 0% observed in WT samples and the degree of silencing showed a higher variation, ranging from genes nearly completely silenced to genes nearly unaffected by XCI. As seen for the WT, the overall transcription did not change significantly between induced and uninduced conditions (Figure 10A II). The difference in X-linked gene expression is not based on a significant difference in transcription between the cell lines as the ratio of ΔCdk8 over WT for autosomal and X-linked gene expression in the uninduced situation demonstrates (Figure 10A III). The variation of reduced silencing does not apply for a specific region on the X-chromosome as shown by plotting the TPM values according to the chromosomal location of the genes (Figure 10B). Statistical comparison of the differentially repressed X-linked genes upon Xist expression revealed 261 significantly repressed X-linked genes in the WT compared to six significantly repressed genes in the ΔCdk8 cells (min. absolute fold change 2; max. FDR p-value 0.01). I took several of the differentially silenced genes and validated their expression levels by qRT-PCR at the same time points chosen for the RNAseq. I compared the relative gene expression decrease between uninduced and induced conditions. On average, the pyruvate dehydrogenase kinase 3 (Pdk3), a mitochondrial enzyme involved in glucose metabolism, shows 3.6 times higher relative expression and the transcription elongation factor Bex4 shows 4.9 times higher relative expression compared to the
relative expression changes in the WT, respectively. 3.1 times higher expression compared to WT was observed for the actin-binding protein Pls3 and even 5 times more transcript was detected for the glycolytic enzyme Pgk1. MeCP2 (Methyl-CpG binding protein 2), which is responsible for Rett syndrome, shows 2 times higher expression compared to WT.

The variation in silencing between the different genes and also between the three ΔCdk8 cell lines, together with the unaffected silencing, confirmed by qRT-PCR, of the Hmgn5 gene, only 1.1 times relative expression difference between ΔCdk8 and WT, recapitulates the findings of the RNAseq analysis, which shows variation of X-linked gene expression in ΔCdk8 cells upon Xist induction. The housekeeping gene Rrm2 serves as a control for unaffected transcription of autosomal genes (Figure 10C). Comparison of the expression levels of these genes in WT and ΔCdk8 cells in uninduced conditions do not show significant expression differences, which could have been an reason for the observed differences in relative expression changes between uninduced and induced samples (Figure 10D). These data show a significant reduction of gene silencing by Xist in the absence of Cdk8 confirming the effect of Cdk8 on X-chromosome inactivation.

Figure 10 Transcriptomic analysis of X-linked genes: (A) Representation of transcriptomic changes upon Xist induction based on TPM (transcripts per kilobase million) normalised expression values (I) WT and (II) ΔCdk8 samples; blue curves: ratio of autosomal gene expression treated over untreated conditions; red curves: ratio of X-linked gene expression treated over untreated conditions, dashed line represents median over all genes in the indicated subset (III) Transcriptomic changes due to Cdk8 loss of function in uninduced conditions; blue curve: ratio of autosomal gene expression ΔCdk8 over WT; red curve: ratio of X-chromosomal gene expression ΔCdk8 over WT; dashed line represents median over all genes in the indicated subset. (B) Heatmap showing TPM normalised values for X-linked genes according to their chromosomal location, no clustering and normalisation applied; illustration of X-chromosome modified from https://www.sciencedirect.com/science/article/pii/S0168952511000059 (C) qRT-PCR expression level analysis to confirm differentially silenced X-linked genes after 48 hours of Xist induction. Expression normalised to Gapdh and relative to untreated expression. Rrm2 serves as an autosomal control gene, error bars represent SD. Experiment done in biological triplicates. (D) Expression level analysis of differentially silenced X-linked genes in untreated conditions. Expression normalised to Gapdh and relative to WT. Rrm2 serves as an autosomal control gene, error bars represent SD. Experiment done in biological triplicates.
Table 1: Top 100 repressed X-linked genes in WT upon Xist induction. Sorted column-wise for descending fold change (FC) in the WT samples. FDR p-value: false discovery rate corrected p-value; WT: data for WT samples; ΔCdk8: corresponding FC and FDR p-value for ΔCdk8 samples; Red: genes significantly downregulated in WT and ΔCdk8 samples.
**Xist Expression and Localisation**

The first step in initiating XCI is expression of Xist and its localisation at the future Xi. To check if Xist gets expressed and localised in ΔCdk8 cells I performed RNA-FISH experiments to quantify the formation of Xist clouds (Figure 11A). After 24 hours of Dox induction WT cells show well-defined Xist cluster in 57% of counted nuclei. The ΔCdk8 cell line #8 has 62%, #11 has 58% and #15 has 45% detectable Xist cluster per counted nuclei, respectively. None of the cell lines show Xist cluster before Dox induction (Figure 11B). This result indicates that the ectopic expression of Xist and its localisation vary between the clones but Xist localisation is not perturbed upon Cdk8 depletion. However, quantification of Xist RNA levels by qRT-PCR shows a fourfold reduction of Xist expression in the absence of Cdk8 compared to WT. As the reduced Xist levels cannot be rescued by transgenic expression of Cdk8 and they are also observed in the WT<sup>TG</sup> cells, it is likely a clonal or manipulation based effect rather than a Cdk8-dependent effect (Figure 11C). Taken together these data demonstrate that Cdk8 does not abrogate Xist expression nor Xist localisation which indicates that Cdk8 is acting at a later step of XCI.
Figure 11 Xist localisation and expression: (A) Xist-FISH in WT, left row, and ∆Cdk8 cells, rows 2-4. Upper column: Cluster formation in induced samples, Lower column pinpoint signal from binding to antisense Tsix, detectable by the double-stranded FISH probe, in uninduced samples; 100x magnification; red: Xist probe; blue: DAPI nuclear stain; scale bar 10µm. (B) Quantification of Xist cluster formation per 100 nuclei in uninduced and induced conditions, error bar represents SD. Induced samples quantified in biological triplicates. (C) Expression analysis of Xist in WT, ∆Cdk8 and rescue cells 48 hours after XCI induction. Expression normalised to Gapdh and relative to untreated expression, error bars represent SD. Asterisks indicate significance (P-value smaller 0.05) calculated by one-way ANOVA. Experiment done in biological triplicates.
Following *Xist* coating and gene silencing, chromatin modifications ensure stable silencing of the Xi. This is achieved, amongst others, by the PRC2 complex. In order to quantify the efficiency of PRC2 recruitment and its capacity to deposit the repressing H3K27me3 mark I performed immunofluorescence experiments combined with RNA-FISH. Introducing Cdk8 cDNA, wild type and mutant constructs, does not change the formation of *Xist* cluster (Figure 12B, C). I quantified the formation of PRC2 clusters, represented by the methyltransferase subunit Ezh2, and the formation of H3K27me3 clusters as a function of *Xist* cluster formation (Figure 12A). The WT shows 67% Ezh2 recruitment and 43% of histone modification per counted FISH cluster. The Ezh2 recruitment is reduced, on average, 3.4 times in the loss of function cells and the histone tri-methylation is reduced 1.7 fold. Reintroducing Cdk8 cDNA in the loss of function cells restores cluster formation to 71% for Ezh2 compared to WT recruitment and close to WT levels of H3K27me3. Expression of Cdk8 cDNA in the WT cells does not change H3K27me3 cluster formation but these cells show less Ezh2 recruitment compared to their corresponding WT, more comparable to the rescued ΔCdk8 cells (Figure 12D). The reduced deposition of heterochromatin marks cannot be restored by introducing neither the D151A nor the ΔATP Cdk8 cDNA variants that lack kinase activity (Figure 12E, F). Taken together these results further indicate that Cdk8 is acting downstream of *Xist* expression and localisation and that Cdk8 in its function as a kinase is required for correct recruitment or function of PRC2 at the inactive X-chromosome.

Taken together I could show that loss of Cdk8 reduces *Xist* mediated cell death, which is based on reduced X-linked gene silencing. The observed reduction of silencing is dependent on the kinase function of Cdk8, as kinase dead constructs cannot significantly rescue the survival phenotype. Cdk8 loss of function does not abolish *Xist* expression and localisation suggesting a subsequent role in XCI. This function could be modulation of PRC2 as its recruitment to the Xi and the deposition of the repressive histone mark is markedly reduced in the absence of Cdk8 and can only be restored by introducing a wild type rescue construct but not by kinase dead variants.
To further investigate the effect of Cdk8 on X-chromosome inactivation and its potential role in modulating the Polycomb repressive Complex 2 the next step was to study the role of Cdk8 in vivo. In doing so, I could avoid an impact of the transgenic Xist induction system of the Hatx3 cell system, which could distort the in vivo function of Cdk8. Furthermore, my in vitro data indicated a female specific function of Cdk8 in early development, which demands in vivo confirmation. Finally, studying the potential molecular mechanism of modulating PRC2 in vivo would increase the knowledge about Cdk8 as the function of PRC2 during early development is well documented, allowing me to characterise eventual developmental Cdk8 loss of function phenotypes based on published data.

**The Role of Cdk8 in Early Mouse Development**

**Analysis of Cdk8 Mutant Mice**

It has been reported that a Cdk8 mutation results in embryonic lethality at embryonic day 2.5\(^4\). Therefore, I generated an epiblast specific Cdk8 knockout by using a Cre recombinase under the control of the epiblast specific promoter of the Sox2 gene\(^{103}\). To avoid deleter activity due to Cre protein in the oocyte\(^{104}\), resulting in preimplantation knockout of Cdk8\(^{104}\), I crossed Sox2-Cre\(^{+/-}\) Cdk8\(^{WT/-}\) males, which have been generated by crossing B6.Cg-Tg(Sox2-cre)1Amc/J\(^{103}\) (Sox2-Cre\(^{+/-}\)) and B6.CDK8tm1(fl/fl)Eucomm animals (http://www.mousephenotype.org/about-
ikmc/eucomm) (Cdk8\textsuperscript{f/f}), with Cdk8\textsuperscript{g/g} females. Heterozygous progeny was obtained at Mendelian ratios. No homozygous mutants were born indicating a requirement of Cdk8 for embryonic development.

To investigate a potential difference between male and female embryonic development of Cdk8\textsuperscript{lox/lox} mutants I collected embryos at embryonic day 12.5 and analysed them for their phenotype (Figure 13A) and genotype (Figure 13B). I obtained 78 embryos with a ratio of 46% male to 54% female. As expected, 54% of the embryos inherited the Cre-recombinase, in a Mendelian ratio of 55% male to 45% female. The ratio of Cdk8\textsuperscript{lox/lox} was 25%, as expected.

The Cdk8 knockout embryos showed a 50% male to 50% female sex ratio questioning a female specific phenotype of Cdk8 loss of function at this stage of development (Figure 13B). However, the knockout embryos displayed a polymorphic developmental phenotype (Figure 13A III-VI). Half of the mutant embryos showed a more severe developmental retardation and the onset of embryo degradation became observable (Figure 13A III, IV). 2/3 of these strongly affected knockout embryos were female and 1/3 male. The less pronounced phenotype shows defective head formation, possibly due to neural tube closure defects, and hyper-vascularisation. Two thirds of the embryos displaying this less pronounced phenotype were male and one third were female. Therefore, a slight bias towards females for phenotypic severity is observed. However, female development can proceed without Cdk8. In none of the analysed knockout embryos residual WT alleles could be detected demonstrating a complete Cre-recombinase mediated excision of the floxed alleles at this stage of development. These observations show that heterozygous Cdk8 expression is sufficient for postimplantation development but its loss of function is incompatible with full embryonic development. Furthermore, I could show that loss of Cdk8 leads to a polymorphic phenotype, which is not exclusively female specific at the developmental stage analysed.
CDK8 IN PREIMPLANTATION DEVELOPMENT

I could show that Cdk8 has a crucial function in postimplantation development irrespective of the sex. To investigate a potential involvement of Cdk8 in preimplantation development and imprinted XCI, as it has been shown for Rnf12, I crossed Sox2-Cre+/- Cdk8WT/- females with Cdk8fl/fl males, which should lead to a full knockout. This effect is presumably based on active Sox2 promoter expression in female germ cells leading to Cre-recombinase protein transmission in the oocytes.

I obtained 39 embryos at embryonic day 12.5 in a 38% to 62% ratio of male to female.

Half of the embryos had the knockout genotype, which is expected, as all oocytes, harbouring the Cre-recombinase transgene or not, transmit the Cre recombinase. The knockout embryos displayed the same polymorphic phenotypes observed for the epiblast specific knockout using paternally transmitted Sox2-Cre (Figure 13A) with a ratio of 75% showing the less severe phenotype, displayed by defective head formation, and 25% showing the more pronounced phenotype of severe developmental retardation.

Figure 13 Cdk8 knockout: (A) Representative pictures of embryonic day 12.5 embryos from Sox2-Cre+/- Cdk8WT/- ♂ X Cdk8fl/fl ♀ crosses. I: Cdk8WT/fl embryo, II: Cdk8WT/- embryo, III-VI: Cdk8/- embryos displaying phenotypic variation. (B) Genotypic analysis of the representative embryos shown in (A); Deletion: double band shows WT and floxed Cdk8 allele, lower band shows floxed out allele; loxP: presence of loxP sequences, upper band loxP integrated in DNA, lower band: WT band; Cre: presence of Cre recombinase transgene. Sex: Sex determination, upper band: Sry, lower band: Xist; Hatx3: Control for ESC line without floxed alleles. H2O: Negative control (C) Genotypic analysis of one litter of preimplantation Cdk8 knockout; Deletion: Upper band WT allele, lower band floxed out allele, double band in 2nd -/- might be product of incorrect recombination; Cre: Cre-recombinase DNA; Sry: male sex; Xist: female sex; Ctrl: E12.5 male epiblast specific WT/fl embryo DNA (D-E) Cdk8flox ESC. (D) Genotypic analysis; Deletion: double band shows WT and floxed Cdk8 allele, lower band shows floxed out allele. Cre: presence of Cre recombinase transgene. Sry and Xist: Sex determination. II2: Interleukin2, loading control. Hatx3: Control for ESC line without floxed alleles. H2O: Negative control (E) Western blot validation of Cdk8 deletion. β-Actin: loading control.
Genotypic analysis showed that 65% of the knockouts were female and 35% male. The sex ratio for the more severe phenotype was equally distributed between male and female and of the head development phenotype knockout embryos two thirds were female and one third male. No residual WT allele could be detected in the knockout embryos (Figure 13C).

These results show that, with the strategy used in his study, preimplantation loss of Cdk8 is compatible with development in contrast to a published report 84. My data suggest that Cdk8 is not crucial for imprinted XCI as female knockout embryos could be collected at embryonic day 12.5. The difference in the distribution of the retarded head development phenotype and the severe developmental retardation phenotype between postimplantation (50:50) and preimplantation (25:75) knockout strategy might reflect a lower number of embryos studied for the preimplantation knockout.

The results of the embryo analysis dispute a crucial role of Cdk8 in X-chromosome inactivation in vivo as female embryos were obtained beyond published embryonic lethality caused by non-functional XCI 5,105,106. Furthermore, the in vitro postulated PRC2 modulation by Cdk8 might be rather subtle, as the observed embryo phenotypes do not recapitulate PRC2 loss of function phenotypes 107-110.

To further study the effect of Cdk8 on early development I generated Cdk8 knockout (Cdk8\(^{1\text{lox}}\)) ESC newly derived from Sox2-Cre\(^{+/-}\) Cdk8\(^{WT/-}\) male x Cdk8\(^{fl/fl}\) female breedings. Genotyping and western blot analysis shows the successful derivation of three female and two male Cdk8\(^{1\text{lox}}\) ESC lines (Figure 13D, E).

**EFFECT OF CDK8 LOSS OF FUNCTION ON MOUSE EMBRYONIC STEM CELLS**

To elucidate the phenotypes observed in Cdk8\(^{1\text{lox}}\) embryos I compared the RNAseq data of WT cells and ΔCdk8 cells in untreated conditions obtained from the transcriptomic analysis performed for silencing of X-linked genes. 210 genes show upregulated transcription and 105 genes show downregulated transcription upon Cdk8 depletion (min. fold change 2; max. FDR p-value 0.01) (Figure 14A and Table 2).

Within the downregulated genes was the β-catenin target *Lef1*, which serves as a positive control of gene deregulation upon Cdk8 depletion as it has been published
that Cdk8 has a synergistic effect on β-catenin target gene transcription. Indeed, qRT-PCR analysis of *Lef1* in ΔCdk8 cells showed an average downregulation of 60% in ΔCdk8 cells compared to WT (Figure 14B). This result is confirmed by qRT-PCR analysis of *Lef1* in *Cdk8*^{flox} cells, which show on average 60% reduction of mRNA for the female *Cdk8*^{flox} cells and 75% reduction for the male *Cdk8*^{flox} cells (Figure 14C).

Across the set of deregulated genes no clustering for a specific pathway was apparent, neither any accumulation of PRC2 target genes, further indicating a subtle role of Cdk8 mediated PRC2 modulation. There were however differentially expressed genes known to be regulated by Notch signaling.

It is published that Cdk8 regulates Notch1 intracellular domain (Nicd) stability and in the transcriptomic analysis the Notch target genes Hes1, and members of the Hey gene family where deregulated upon Cdk8 depletion. I investigated this function in more detail as deregulated Notch signaling is implicated in defective mouse embryonic development. qRT-PCR shows that the Notch target gene *Hes1* shows on average a 2.6 times higher expression in the ΔCdk8 cells compared to WT (Figure 14D). Expression analysis of the *Cdk8*^{1lox} cells show a non-significant difference of about 20% overexpression of *Hes1* in female cells and insignificant changes in male cells (Figure 14E). Preliminary data suggest that the elevated gene level correlates with elevated Nicd protein levels in ΔCdk8 cells detected by western blot (Figure 14F). Another preliminary observation is the detection of Nicd protein in Dox induced ΔCdk8 samples but not in WT cells, which could be a relic of the elevated protein level seen in the untreated samples.

Cdk8 has been implicated in increasing Stat1 and Stat3 activity by phosphorylating Ser727. Using ΔCdk8 cells I could not observe differences in phosphorylation compared to WT, neither for Stat1 (Figure 14G) nor for Stat3 (Figure 14H), neither in the untreated nor in the treated samples.
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Table 2: Top 50 differentially expressed genes in ΔCdk8 compared to WT. Left column: Activated genes. Right column: Repressed genes. Sorted for descending fold change (FC). FDR p-value: false discovery rate corrected p-value.
In summary I could confirm several deregulated genes upon Cdk8 depletion that were initially found in the RNAseq. However, none of these genes provides a further link to X-chromosome inactivation. Furthermore, these data suggest the observed modulation of PRC2 by Cdk8 might be a XCI specific function as transcriptomic analysis did not provide any transcriptionally significantly deregulated PRC2 target genes upon Cdk8 depletion. Besides I could show that neither Stat1 nor Stat3 phosphorylation is deregulated in Cdk8 mutant embryonic stem cells.

In contrast I could confirm transcriptional deregulation of the Notch target gene Hes1, which points to reduced Nicd turnover, as observed with a western blot in Cdk8 mutant cells. Deregulation of Notch signaling due to a lack of Cdk8 could likely contribute to the observed phenotypes in embryonic development. Confirmation will need further investigations in the future.

**Figure 14 Cdk8 targets:** (A) Volcano plot displaying differential gene expression between ΔCdk8 and WT cells. Red dots: significant differentially regulated genes (min. fold change 2; max FDR p-value: 0.01) (B-E) Validation of differentially regulated genes. Gene expression normalised to Gapdh relative to indicated WT. error bars represent SD, experiments done in biological triplicates. Asterisks indicate significance (p-value smaller 0.05) calculated by one-way ANOVA (B) Lef1 gene expression in ΔCdk8 cells (C) Lef1 gene expression in Cdk8$^{1lox}$ ESC (D) Hes1 gene expression in ΔCdk8 cells (E) Hes1 expression in Cdk8$^{1lox}$ ESC (F-H) Western blot analysis of published Cdk8 target proteins in ΔCdk8 cells in the absence (-) and presence (+) of XCI induction, Antibodies: α-Cdk8: validation of Cdk8 depletion. α-β-Actin: loading control (F) Nicd protein levels, * indicates transmembrane domain, # double band displays Next and Nicd domain of cleaved Notch1 protein (G) α-Stat1 phospho S727 protein levels, α-Stat1: total Stat1 protein levels, double band reflects phosphorylated Stat1 protein (H) α-Stat3 phospho S727 protein levels, α-Stat3: total Stat3 protein levels, double band displays Stat3 isoforms.
DISCUSSION
DISCUSSION

CDK8 IS INVOLVED IN X-CHROMOSOME INACTIVATION

CDK8 LOSS OF FUNCTION INCREASES SURVIVAL OF ESC BY REDUCING X-LINKED GENE REPRESSION BY XIST

The screen for X-inactivation factors performed in our group provided a list of candidates, which to date have not been implicated in dosage compensation. One of the highest ranked candidates is the cyclin dependent kinase 8 (Cdk8). In this thesis I could show that Cdk8 is indeed a protein involved in XCI.

Deploying the Crispr/Cas9 system allowed me to generate several independent Cdk8 loss of function ES cells to study the effect on XCI. In our model system induction of XCI by Dox induced Xist expression results in cell death due to the repression of all copies of X-linked genes. I used the survival rate of these cells as readout for the effect of Cdk8 on the ability of Xist to induce gene repression. In the absence of Cdk8 the survival of cells upon Xist expression was approximately ten fold increased over WT cells. This result recapitulates the finding of the screen that loss of Cdk8 increases cell survival upon Xist expression.

Comparison of mRNA sequencing data obtained from WT cells and ΔCdk8 cells in non-treated conditions and after 48 hours of Xist expression confirmed that the enhanced survival is correlated with a reduction of Xist induced X-linked gene repression. WT cells show a near complete shutdown of X-linked genes compared to non-treated conditions. In contrast, ΔCdk8 cells show approximately 50% residual X-linked gene expression after Xist induction. The effect on gene expression in ΔCdk8 cells is gene specific as residual X-linked gene expression upon Xist induction varies across the X-chromosome. This variation might be caused by a more specialised function of Cdk8 on a set of genes rather than a chromosome wide function in silencing or by differences in chromatin structure, which arise upon Cdk8 depletion.
THE CATALYTIC FUNCTION OF CDK8 IS CRUCIAL FOR GENE REPRESSOR BY XIST

I could show that the function of Cdk8 is dependent on its catalytic function as a kinase. Reintroducing Cdk8 cDNA in ΔCdk8 cells reduced survival from 50% to 15%. This significant but not complete rescue can originate from the use of the transgenic PiggyBac system for restoring Cdk8 expression. This approach only provides the coding region of Cdk8 without regulatory sequences and the plasmid randomly integrates in the genome. These differences can affect the function of the transgene compared to the endogenous gene.

Survival was only reduced when I introduced the WT cDNA. Mutated constructs that abrogate the kinase function do not rescue gene repression by Xist. These results demonstrate that Cdk8 is regulating XCI via phosphorylation.

CDK19 CANNOT COMPENSATE FOR CDK8 LOSS OF FUNCTION

I could show that loss of Cdk19, albeit having significant sequence and functional overlap with Cdk8, is not able to increase survival upon Xist induction neither in WT cells nor in ΔCdk8 cells. Additionally, reduction of neither of the kinase transcripts leads to a transcriptional deregulation of their paralogue kinase transcript. A specificity for Cdk8 could be in accordance with the necessity of Cdk8 but not Cdk19 in early development. Mutation of Cdk8 leads to early embryonic lethality 84, whereas a mutation of Cdk19 can lead to homozygous null offspring, though not at expected ratios 100.

As Cdk8 and Cdk19 show significant sequence homology and conservation of crucial catalytic amino acids, the molecular difference in the XCI phenotype might be found in the C-terminus of these proteins. The C-terminus of Cdk8 consists of 37 amino acids that are not found in Cdk19. These extra amino acids might mediate the XCI specific function of Cdk8 in embryonic stem cells. Either by assembling with the Med12/13 Mediator subunits, opposed to Med12L/13L for Cdk19, or by providing a not yet characterised specificity of Cdk8 for target proteins. To address this question one could assess the rescue potential of mutant constructs, containing either deletions of...
these unique amino acids in Cdk8 cDNA or integrations of these residues in Cdk19 cDNA.

**THE EFFECT OF Cdk8 ON Xist EXPRESSION AND LOCALISATION**

Loss of Cdk8 does not impair the expression and localisation of Xist to the inactive X-chromosome as judged from RNA-FISH experiments. However, expression analysis by qRT-PCR showed a fourfold decrease in Xist RNA levels in ΔCdk8 cells compared to WT. The reduction was not restored by introduction of either cDNA construct, wild type or kinase dead mutant. Reintroducing WT Cdk8 cDNA in ΔCdk8 cells significantly reduces the increased survival observed for ΔCdk8 cells compared to WT cells. It is therefore plausible that the observed differences in Xist levels, measured by qRT-PCR, are likely based on a clonal effect of the cells rather than a consequence of Cdk8 depletion. When I compared the Xist cluster formation between ΔCdk8 and WT cells I could not detect differences in the size of the cluster. Since immunofluorescence comparisons are not quantitative, computationally calculated averages of Xist cluster sizes in the different cells would be needed to identify differences in Xist cluster sizes, indirectly indicative for difference in Xist abundance.

Xist expression in the used ES cells is under control of a transgenic system that consists of a doxycycline responsive transacting transcriptional activator protein. This protein binds an operator upstream of the endogenous Xist promoter and thereby induces Xist expression. It might be possible that Cdk8 is involved in proper gene expression activation driven by the transgenic activator protein. However, it is published that this transgene mediated gene activation is dependent on a mediator complex that lacks the Cdk8 module \(^{113}\). This is an argument that loss of Cdk8 is not the cause for the observed reduction in Xist levels.

If reduced Xist levels are a consequence of XCI it could be explained by changed stability of the transcript. Due to reduced X-chromosomal silencing, Xist is not provided with an optimal chromatin landscape for stably binding to the Xi, which results in reduced stability of Xist transcript. This argument however is difficult to bring in line with the transgenic regulation of Xist expression in our model system.
To further investigate this observation Xist expression and localisation needs to be analysed in differentiating Cdk8^\text{lox}\_\text{lox} female ES cells. With this setup I can study a possible effect of Cdk8 on Xist expression independent of the transgenic system. However, with this approach one has to take endogenous Xist and XCI regulations into considerations, which appear during in differentiation and might not have had an influence in our transgenic ESC system.

**PRC2 Recruitment to the Xi is Reduced in ΔCdk8 Cells upon Xist Expression**

One step in XCI involves formation of heterochromatin in order to stably repress transcription. One complex responsible for it is the PRC2 complex. I demonstrated that in ΔCdk8 cells recruitment of the catalytic subunit of PRC2, Ezh2, and the deposition of its heterochromatic mark, H3K27me3, is reduced. This effect seems to be independent of the reduced Xist expression, which drives all downstream processes of XCI. I quantified recruitment of Ezh2 and H3K27me3 relative to Xist cluster, which are similar in my mutant cells compared to WT, albeit the observed reduction of Xist expression levels in the mutant cell lines, as observed by qRT-PCR. The reduced Ezh2 and H3K27me3 cluster formation can be restored by reintroducing WT transgenic Cdk8 but not by kinase dead variants. This result further substantiates the requirement of the Cdk8 kinase function for PRC2 recruitment.

Reduced PRC2 recruitment can be overserved in other studies working on XCI factors, therefore it could be interpreted as a secondary effect triggered by inefficient XCI. Theoretically, Cdk8 could deposit repressive histone phosphorylation marks, as it has been shown for H2A serine1 phosphorylation by the Msk1 kinase in vitro. Therefore the observed reduction in recruitment of Ezh2 to the Xi in ΔCdk8 cells upon Xist expression could be a secondary effect based on deregulated gene repression due to absent repressive histone phosphorylations. However, this is an unlikely possibility as histone phosphorylation is generally associated with active transcription and so far no phosphorylation of histones by Cdk8 has been documented. In vitro it has been shown that Cdk8 but also Cdk19 are able to phosphorylate Ezh2 at Thr492 and to a lesser extent Thr350. However, this phosphorylation was not altered upon Cdk8 or Cdk19 transcriptional repression and Thr492 phosphorylation is associated with PRC2 disassembly. Apart from this in vitro study, a physical interaction between
Cdk8 and PRC2 proteins has not been documented so far\textsuperscript{115-117}. However, a study in the \textit{Apc}\textsubscript{Min} tumour model shows that loss of Cdk8 leads to reduced H3K27me3 marks at oncogenic signaling genes, resulting in their upregulation\textsuperscript{92}, which indicates that the observed reduction of PRC2 recruitment upon \textit{Xist} expression could be a consequence of Cdk8 depletion. The molecular details of how Cdk8 regulates Ezh2 remain to be elucidated.

**Finding the Connection Between Cdk8 and PRC2**

The published \textit{in vitro} study on Ezh2 phosphorylation by Cdk8\textsuperscript{85} does not provide a mechanism for the reduced PRC2 recruitment to the Xi upon Cdk8 depletion. The effect was only observed in a kinase assay and it can be catalysed by Cdk8 and Cdk19, which disputes the specificity of Cdk8 in the increased survival of transgenic ES cells upon \textit{Xist} expression. Finally, the more prominent observed Thr492 phosphorylation is associated with PRC2 dissociation, which is the opposite of the suspected function of increased PRC2 function in the presence of Cdk8.

A possible activity enhancing link between cyclin dependent kinases and EZH2 has been shown in 293 cells, where a canonical cyclin dependent kinase, CDK1, can directly phosphorylate EZH2\textsuperscript{118}. This study describes a cell cycle dependent phosphorylation of EZH2 at serine 345 by CDK1 specifically during S/G2 cell cycle phase, which according to the study increases its binding to the lncRNA HOTAIR. This lncRNA has been demonstrated to repress developmental Hox genes\textsuperscript{119}. Despite molecular differences in HOTAIR mediated gene repression, the interaction of a lncRNA and Ezh2 mediated by a cyclin dependent kinase in order to repress gene expression shows similarities to the epigenetic processes seen in XCI and the possible influence of Cdk8 on this process. Additionally to the serine 345 phosphorylation by CDK1 it has been shown in pancreatic cancer cells that EZH2 also can be phosphorylated at threonine 350 by CDK1 and CDK2\textsuperscript{120}. The studies on CDK1 and CDK2 conclude that the absence of these phosphorylation does not affect the methyltransferase activity of EZH2 but rather impairs PRC2 recruitment\textsuperscript{118,120}. These studies propose a model of how newly integrated histone 3 is decorated with its repressive K27me3 mark during DNA replication in order to inherit the repressed chromatin state during cell divisions.
It has to be mentioned that a recent study investigated phosphoproteomic changes upon chemical inhibition of Cdk8 and could not detect changes in phosphorylation of PRC complex members. However, this result needs to be put in perspective as the same study observed differences in transcriptomic changes between chemical inhibition of Cdk8 and shRNA mediated Cdk8 knockdown. This discrepancy between chemical inhibition and genetic manipulation could be observed in other reports studying Cdk8 \(^{87,91,121,122}\). A possible explanation for this frequent finding could be the accessibility of chemical compounds to Cdk8, insofar that nucleoplasmic Cdk8 might be inhibited by the chemicals, whereas protein- or chromatin-bound Cdk8, which is catalysing phosphorylations might not be accessible for these compounds.

Based on these findings, together with the reduced Ezh2 function upon Cdk8 depletion in the \(Apc^{Min}\) model, one could propose a function for Cdk8 in PRC2 recruitment similar to CDK1 and CDK2.

To investigate a possible effect of PRC2 recruitment to the Xi as a function of Cdk8 depletion it will be necessary to compare total and phosphorylated Ezh2 protein abundance in chromatin bound protein fractions of WT and Cdk8 loss of function cells. Chromatin-Immunoprecipitation experiments of Ezh2 will further help to understand if loss of Cdk8 leads to reduced recruitment of PRC2 to either X-linked gene regulatory sequences upon \(Xist\) expression or if a global reduction in recruitment can be observed. Applying chemical inhibition of Ezh2 \(^{123}\) in WT and Cdk8 loss of function cells could be an approach to disentangle reduced recruitment from reduced functionality. If loss of Cdk8 reduces functionality of Ezh2, catalytic inhibition should not show a further increase in cell survival nor a further reduction in Ezh2 localisation upon \(Xist\) induction in the Hatx3 system. On the other hand, if chemical Ezh2 inhibition shows even higher survival in \(\Delta\)Cdk8 cells this could point to a disturbed recruitment of Ezh2 upon Cdk8 depletion independent of a modulation of the Ezh2 catalytic function.

To discriminate an X-chromosome specific from a global effect of Cdk8 depletion on Ezh2 recruitment or function, it would be helpful to compare chromatin wide H3K27me3 abundance between WT and Cdk8 loss of function cells. A global difference in H3K27me3 abundance between WT and mutant cells should be apparent
without Xist induction and detectable on a western blot, whereas an X-chromosome specific reduction in H3K27me3 marks should only be observed upon Xist induction and might require proteomic analysis\textsuperscript{124} as differences between the cell types are expected to be much smaller, as only one chromosome is affected.

A genetic approach to address if the reduced X-linked gene silencing seen in ΔCdk8 cells is a cause of reduced PRC2 recruitment could be transgenic expression of WT Ezh2 and phosphomimic mutants of Ezh2 and their specific tethering to the X-chromosome using a modified Crispr/Cas9 system\textsuperscript{125} to see if these constructs are able to rescue the observed ΔCdk8 phenotype. If Cdk8 affects recruitment of PRC2 irrespective of phosphorylating Ezh2, the WT Ezh2 transgene should be able to rescue the phenotype. However, if only phosphomimic Ezh2 mutants show a rescue effect it would point to a Ezh2 phosphorylating function of Cdk8, although it would not discriminate between phosphorylation dependent recruitment and function of PRC2.

To discriminate between an effect on recruitment and an effect on functionality one could apply a kinase assay to assess if Cdk8 has the capability to phosphorylate Ezh2\textit{ in vitro}, which in a second step might be combined with a methyl-transferase assay to see if a potential\textit{ in vitro} phosphorylation of Ezh2 by Cdk8 is affecting the methyl-transferase activity of Ezh2.

If these experiments display Cdk8 dependent differences in recruitment or functionality of PRC2 it will be beneficial to perform phospho-proteomics in cellular fractions of WT cells and ΔCdk8 cells in presence and absence of Dox to see if loss of Cdk8 is resulting in differentially phosphorylated Ezh2 in the cellular environment.

The reduced recruitment of Ezh2 observed in ΔCdk8 cells upon Xist induction could also be based on a reduced H2AK119ub deposited by non-canonical PRC1, which is the first step in chromatin modifier complex recruitment during XCI according to the current working hypothesis\textsuperscript{36,49}. This possibility will need to be addressed by PRC1 specific immunofluorescence or Chromatin-Immunoprecipitation experiments.
CDK8 IN EARLY DEVELOPMENT

EMBRYONIC X-CHROMOSOME INACTIVATION IS NOT IMPAIRED BY LOSS OF CDK8

Using an epiblast specific Cre recombinase allowed me to circumvent a previously reported preimplantation lethality of Cdk8 knockout mice. To achieve this epiblast specific knockout I crossed Sox2-Cre\textsuperscript{+/−} Cdk8\textsuperscript{+/−} males with Cdk8\textsuperscript{fl/fl} females. This mating scheme is critical to avoid maternal inherited Cre activity, which would result in recombination of a Cdk8 floxed allele in preimplantation embryos. With this setup I could show that Cdk8 expression is critical for proper development, as heterozygous progeny was obtained at Mendelian ratios, whereas no Cdk8\textsuperscript{lox} pups were born.

Studying embryos at embryonic day 12.5 revealed no sex specific phenotype, as knockouts were equally distributed between the sexes.

I further crossed Sox2-Cre\textsuperscript{+/−} females with Cdk8\textsuperscript{fl/fl} males to generate full knockouts due to maternal Cre protein transmission to investigate if imprinted XCI is affected. Contrary to the published role of Cdk8 in preimplantation development I could collect embryos at embryonic day 12.5, which resembled the polymorphic phenotypes observed in the epiblast specific loss of function embryos. These experiments yielded 50% knockouts because Cre recombinase activity is inherited through the female germline, presumably through expression of the Sox2 promoter in the female germline. Therefore all embryos that inherit the maternally deleted Cdk8 allele are homozygous knockout as the paternal floxed Cdk8 allele will be excised in the zygote.

The difference to the reported early lethality might be based on the different strategies used for creating the null mutation. I use Cre recombinase mediated deletion of the floxed Cdk8 allele to create a knockout whereas Westerling et al. crossed heterozygous Cdk8\textsuperscript{+/−} mice. It is plausible that the process of recombination needs time depending on the locus that is flanked by the loxP sites to be completed and that this temporal difference to the heterozygous Cdk8\textsuperscript{+/−} crosses explain the difference in the reported observations. Another explanation could be the design of the mutation. In the Cre-lox approach the loxP sites are flanking Cdk8 exon 2, which contains critical residues for the catalytic function of Cdk8, whereas in the study by Westerling et al. a
gene trap was integrated into Cdk8 intron 4, resulting in a fusion protein consisting of the N-terminus of Cdk8 and the β-galactosidase-neomycin selection marker \(^\text{84}\).

The observations of female Cdk8 mutant development indicates that Cdk8 is not crucial for X-chromosome inactivation in the embryo as an earlier lethality would be expected upon abrogated XCI \(^5,\text{105,106}\). This observation does not rule out a contribution of Cdk8 to XCI in development, albeit it might be likely redundant or modulating.

One indication for a potential XCI modulating function of Cdk8 arises from the observation of polymorphic Cdk8\(^\text{1lox}\) embryos in the postimplantation knockout strategy. Half of the knockout embryos displayed a more severe phenotype which was characterised by severe developmental retardation and the onset of degradation. The other 50% of the knockout embryos displayed a defect in head formation, potentially due to a defect in neural tube closure, and hyper vascularisation. Even though the sex ratio over all knockout embryos was equally distributed, within the two observed phenotypes the sex ratio was skewed. Two thirds of the embryos with the more severe phenotype were female and only one third was male. The opposite was observed for the less severe phenotype. This accumulation of the more severe phenotype in females could point to an effect of Cdk8 on X-chromosome inactivation in development. Analysis of preimplantation Cdk8\(^\text{1lox}\) embryos displayed a 65% female to 35% male sex ratio. This could be indicative for a stronger effect of preimplantation loss of Cdk8 for female development. However, contrary to the epiblast specific Cdk8 knockout, the preimplantation loss of Cdk8 leads to the defective head formation phenotype in 75% of the analysed embryos. These results might be based on the reduced number of embryos analysed.

To substantiate this observation it will be necessary to collect embryos of earlier stages to separate possible XCI dependent from XCI independent effects of Cdk8 loss of function in development. Analysing embryos from earlier stages might also address the observations of the polymorphic phenotypes. It is plausible that the more severe phenotype arose from embryos in which the loss of the second Cdk8 allele occurred at an earlier time point than the less severe, still lethal, phenotype. This temporal discrepancy in deletion might allow development to proceed further. This assumption is based on the fact that the time point of Sox2 driven Cre recombination at embryonic
day 6.5 was determined by the efficiency of removing a stop cassette in front of a β-galactosidase reporter, which was integrated in the ROSA26 locus\textsuperscript{103,126}, a genetic region which has been found to have high recombination frequency\textsuperscript{127}. There is a chance that the kinetics at the ROSA26 locus are different to the Cdk8 locus, leading to a delayed removal of the floxed allele. This could contribute to the observed polymorphic phenotype. Because also in the preimplantation knockout embryos the polymorphic phenotypes could be observed, even though not in equal ratios, it is theoretically possible that Cre-recombinase is acting with different kinetics at the Cdk8 locus and not all cells lose the second Cdk8 allele prior to implantation because of dilution of the Cre recombinase during cell division. This hypothesis might be confirmed by performing aggregation experiments to see if there is a certain threshold of definite Cdk8 knockout cells in the blastocyst that leads to the more pronounced phenotype.

**LOSS OF A CDK BINDING PROTEIN LEADS TO $X_{ist}$ DISPERSION IN SOMATIC CELLS**

The skewed sex ratio in the more severe phenotype of Cdk8\textsuperscript{1lox} embryos might indicate a modulating or lineage specific function of Cdk8 in X-chromosome inactivation in mouse development. Lineage specific modulations have been documented in a recent study, which identified the protein Ciz1 as a factor in XCI. Loss of Ciz1 leads to viable female offspring but shows female specific $X_{ist}$ dispersion and loss of repressive H3K27me3 marks in lymphoid lineages and embryonic fibroblasts\textsuperscript{59}. This study shows that Ciz1 interacts with $X_{ist}$ and serves as an anchor to the nuclear lamina. This function is likely redundant in early embryogenesis, as neither embryo development is impaired upon Ciz1 depletion, nor is localisation of $X_{ist}$ perturbed in ΔCiz1 transgenic ES cells.

Additionally to anchoring $X_{ist}$ to the nuclear lamina, Ciz1 has been shown to interact with Cdns and thereby regulating mammalian DNA replication \textit{in vitro}. Ciz1 binds CyclinA/Cdk2 in late G1 cell cycle phase and shuttles it to the DNA, thereby replacing CyclinE/Cdk2\textsuperscript{128}.

Cdk8 loss of function does not lead to $X_{ist}$ dispersion in transgenic ES cells, therefore analysing Cdk8\textsuperscript{1lox} derived embryonic fibroblasts will show if loss of Cdk8 leads to dispersion of $X_{ist}$ in development, similarly to loss of Ciz1. If a similar
dispersion effect can be observed, it will be helpful to investigate a potential Ciz1 – CyclinC interaction, as it is already shown that Ciz1 is interacting with CyclinA and CyclinE. Furthermore, analysis of Ciz1 depletion in our transgenic ESC model system might reveal a possible common pathway that regulates Xist localisation in development.

**Modulation of PRC2 Function in Early Development**

The observed *in vitro* effect of loss of Cdk8 on Ezh2 recruitment or function cannot explain the embryo phenotypes, as *Ezh2* knockout embryos die at embryonic day 7.5, due to a failure in completing gastrulation \(^{110}\). Similarly, knockouts of other members of the PRC2 complex, *Eed* and *Suz12*, display more profound developmental defects, both resembling the gastrulation defect observed for the *Ezh2* knockout \(^{107,109}\). The difference between PRC2 knockout phenotypes and the loss of Cdk8 phenotype in embryogenesis can be explained by either potential compensatory effects *in vivo* or it can be attributed to a role of Cdk8 in fine tuning Ezh2 recruitment or function.

The identification of the Cdk1/2 binding protein Ciz1 as a factor involved in X-chromosome inactivation might highlight a function of Cdk8 in modulating PRC2 function. In pancreatic cancer cells it has been shown that Cdk2 phosphorylates Ezh2 \(^{120}\) where it is supposed to sustain repressive H3K27me3 marks during cell division. It is shown that Ciz1 is binding Cdk2 in late G1 phase but not in S phase. Assuming that Ciz1 is able to bind CyclinC one could speculate that during S phase Ciz1 binds CyclinC/Cdk8 and recruits it to the nuclear lamina in order to maintain repressive H3K27me3 marks at the X-chromosome during cell division.

**Approaching a Molecular Cause for the Cdk8 Loss of Function Phenotype**

Transcriptomic analysis of WT and ΔCdk8 ESCs showed minor transcriptional changes upon Cdk8 depletion. This analysis indicates that the observed PRC2 recruitment phenotype in ΔCdk8 cells is not a global effect, as autosomal genes silenced by PRC2 are not differentially expressed. However, some of the differentially expressed genes cluster in the Notch signaling pathway. Tightly regulated Notch
signaling is crucial for proper embryonic development and might therefore be an explanation for the observed $Cdk8^{\text{lox}}$ phenotypes.

As pluripotent stem cells are apparently unaffected by loss of Cdk8 it might be helpful to analyse the transcriptome of the knockout embryos at different developmental stages or in different parts of the embryos to further delineate aberrant transcriptional changes that lead to impaired development in the absence of Cdk8.

**CDK8 REGULATES NOTCH SIGNALING BY NICD TURNOVER**

One potential candidate network responsible for the observed developmental phenotypes is the Notch signaling pathway as I could show in $\Delta Cdk8$ ESCs and, to a lesser extent, in $Cdk8^{\text{lox}}$ cells that the Notch target gene Hes1 is differentially expressed. This observation is based on deregulated Nicd turnover, which I could observe at protein levels.

It has been shown that forced activation of Notch does not impair development of neural precursors in the embryo but inhibits their neuronal differentiation $^{112}$, which is substantiated by a study performed in ESC $^{111}$. This inhibition of neuronal differentiation leads to impaired development of the anterior-most neurectoderm, which will give rise to the midbrain $^{112}$. A molecular mechanism for this observation could be explained by upregulation of the Notch target gene DTX-1, which was shown to repress p300 mediated transcriptional activation of the neural specific transcription factor $ASCL1 / MASH1 ^{129}$. Impaired $ASCL1$ transcription results in impaired expression of neuronal differentiation genes.

The published phenotype of forced Notch activation shows severe developmental abnormalities leading to developmental arrest by mid-gestation $^{112}$. The $Cdk8^{\text{lox}}$ embryos show less severe developmental abnormalities as development can proceed further, indicating that Notch is either less active compared to the published study or the deregulation is restricted to certain cell types. This suggests a modulating role for Cdk8 in Notch signaling.

The study of forced Notch signaling also could partially explain the observed polymorphic $Cdk8^{\text{lox}}$ phenotype. It has been demonstrated that Nicd expression can
be detected starting at embryonic day 4.5 and that the first morphological abnormal embryos can be observed at embryonic day 7.5. Assuming that the deletion of the floxed Cdk8 can vary between the embryos or between cells of an embryo, it might be possible that the potentially Notch related Cdk8<sup>lox</sup> phenotype arises in embryos were Cre recombinase mediated excision of the Cdk8 allele happens at a later time point, resulting in the suspected Notch deregulation. On the other hand, earlier excision of the remaining Cdk8 allele leads to a hitherto unidentified and more profound deregulation of development and thereby masking the later occurring potential Notch phenotype.

Independent of a potential temporal discrepancy in the Cre-lox system, the Cdk8 knockout embryos could be exquisitely sensitive to Notch levels, in a way that slight variations in signaling, which occur stochastically during development, result in an unproportional developmental effect. In a wild type situation these variation would be balanced by Cdk8 driven Nicd turnover, which is absent in the Cdk8 knockout embryos. Depending on the degree of the variation in Notch levels, this could lead to the more or the less severe Cdk8<sup>lox</sup> phenotype. This non linearity between signaling strength and phenotypic severity is has been observed for Fgf8 and sonic hedgehog in facial development.

It remains to be seen to what extent Notch signaling is deregulated in Cdk8 knockout embryos as well as if this potential deregulation has an effect on the differentiation of ΔCdk8 cells in vitro. To investigate the potential implication of Cdk8 in Notch signaling one could address the differentiation potential of ΔCdk8 and Cdk8<sup>lox</sup> embryonic stem cells towards the neural lineage and further neuronal differentiation. To investigate the effect of Cdk8 on Notch signaling in the embryo, one could analyse Notch target gene expression, like the Hes gene family or Dtx1 and its target gene in neural development Ascl1 in defined regions of the developing WT and Cdk8<sup>lox</sup> embryos. Furthermore, analysis of expression levels of marker genes for published deregulated embryonic tissues, such as Sox7, could unravel a potential correlation between loss of Cdk8 and impaired embryonic development due to deregulated Notch signaling. As the material in an embryo is limiting it might be more promising to analyse Notch target gene expression and localisation by
immunocytochemistry on embryo sections and compare the expression pattern of WT and $Cdk8^{\text{lox}}$ embryos.
**CONCLUSION**

In this thesis I provide evidence that Cdk8 is a modulator of X-chromosome inactivation in embryonic stem cells. Furthermore I can show that the effect is specific to Cdk8 and it depends on its function as a kinase. Cdk8 does not have a measurable effect on imprinted X-chromosome inactivation and a possible effect on fine tuning random X-chromosome inactivation in female mouse development remains elusive. A modulatory effect might be masked *in vivo* by additional contributions of Cdk8 to early development that are sex independent.

The observed effect on X-chromosome inactivation in embryonic stem cells might be based on deregulated PRC2 function or recruitment resulting in reduced epigenetic gene repression. This function might be redundantly controlled in development, or masked by additional deregulated processes in Cdk8\textsuperscript{lox} embryos.

The observation of reduced *Xist* levels in ΔCdk8 embryonic stem cells together with the published *Xist* dispersion phenotype for Ciz1 knockout *in vivo* could point to not yet identified lineage or context specific *Xist* regulation or localisation mechanisms in random X-chromosome inactivation in the embryo. Additionally, characterisation of loss of Cdk8 in the context of an eventual interaction with Ciz1 could help to advance our understanding of maintaining the epigenetically silenced state of the X-chromosome during cell divisions.

Finally, I could show that loss of Cdk8 does not lead to preimplantation lethality and is compatible with epiblast self-renewal, which provides us newly derived *Cdk8* knockout ES cells to study the role of this kinase in early development.

The observed polymorphic *Cdk8* knockout phenotypes provide us a system to study effects of signal variations on mouse development in a sensitised environment and will advance our understanding in the tightly regulated and controlled signaling pathways in embryogenesis.
EXPERIMENTAL PROCEDURES
EXPERIMENTAL PROCEDURES

CELL CULTURE AND GENERATION OF TRANSGENIC CELL LINES

CELL CULTURE

Hatx3 cells, mutant derivatives and established ESC lines derived from mice are cultured as previously described \[^{25}\]. Briefly, cells are plated on gelatine coated dishes in medium composed of high glucose DMEM supplemented with 15% foetal bovine serum (Pan, P140402), 1% each of non-essential amino acids, Sodium Pyruvate and L-Glutamine, respectively, 8\(\mu\)l/l \(\beta\)-mercaptoethanol and 1000units/ml LIF (homemade).

For maintenance culture, 3\(\mu\)M Gsk3\(\beta\) inhibitor (Chir99021, Axon Medchem) and 1\(\mu\)M Mek1/2 inhibitor (PD035901, Axon Medchem) are added \[^{62}\]. To induce XCI in Hatx3 and mutant derivatives, 1\(\mu\)g/ml doxycycline is administered.

TRANSGENIC CELL LINES

Cdk8 and Cdk19 loss of function mutations were generated using the Crispr/Cas9 strategy described previously \[^{40}\]. Two guide RNAs targeting the region around the start codon were designed using the MIT algorithm (crispr.mit.edu) (Cdk8: gRNA1: 5'-ccggtccccaccgcgggccct-3', gRNA2: 5'-aagttggtcgaggcacttac-3'; Cdk19: gRNA1: 5'-ccacggtttcaagcggctgtg-3', gRNA2: 5'-caccgtaaagcgccgagcggaggt-3'). Annealed guide RNAs were inserted into the Bbs1 restriction site of the PX458 vector (Addgene #48138), which encodes for the Cas9 nuclease and harbours GFP DNA for selection. Plasmids were sequenced for correct integration using a primer targeting the U6 promoter (5'-gactatcatatgcttaccgt-3'). Corresponding vectors, Cdk8 gRNA1/gRNA2 or Cdk19 gRNA1/gRNA2, were lipofected (Lipofectamine 3000, Thermo Fisher Scientific) into Hatx3 cells and after 48 hours cells were sorted for green fluorescence (MoFlo Astrios EQ, Beckman Coulter) and plated at limiting dilution in order to obtain clonal populations. Deletions were detected using PCR on genomic DNA (Cdk8: 5'-tctctcgaggtggtccacggtc-3' and 5'-caaaacctgtatgtcaccgacacag-3'; Cdk19: 5'-ccaggtttcataaaaagggaga-3' and 5'-accctaaactccacacctca-3'; Control: Interleukin2, 5'-ttaggggaaatctgatct-3' and 5'-gtaggttgaaaattcagcatc-3') and validated by Sanger sequencing.
Rescue and kinase dead constructs were generated using the PiggyBac transposase system. Wild type Cdk8 cDNA was inserted into the EcoRI digested PB-EF1α-MCS-IRE-Neo vector (PB-EF1α-MCS-IRE-Neo cDNA cloning and expression vector, System Biosciences) using a directional seamless cloning kit (In-Fusion HD Cloning Plus, Takara Bio) (Primer: 5'-gcggccgatgactatgactttaaagtgaggctagcag-3' and 5'-ccgatttaaattcgaatttcatccgtatgtctgtgagtc-3'). Additionally, DNA coding for an HA-Strep-tagII tag (Primer: 5'-ctctagagctagcgaattatgtacccatacgtgtccttcgcgccttttcgaac-3' and 5'-gtcatagtcatgccccgccccgttttcgaac-3') was cloned upstream of the Cdk8 cDNA. Based on the created rescue vector I performed site-directed mutagenesis (In-Fusion HD Cloning Plus, Takara Bio) to either exchange an amino acid (D151A) or remove one amino acid (ΔATP). To generate these vectors, an inverse PCR of the WT Cdk8 cDNA plasmid was performed with primers overlapping at their 5'ends and harbouring the desired mutated sequence resulting in a linearized plasmid, which re-circularises because of the overlaps introduced in the primers (D151A: 5'-aggccctttgaaaaacccgctaatctttatatggg-3' and 5'-gtttcaagccctgtgcaaccc-3'; ΔATP: 5'-aaagactacgctttacaaatagaagggactgtttcaagccc-3' and 5'-agttcacatctctattgtttaagcgtaagttcatct-3'). Sequence validated plasmids were lipofected into corresponding cells together with hyperactive PiggyBac transposase and a tdTomato fluorescent reporter in a ration of 10:10:1 and sorted for tdTomato expression after 48 hours. Independent clones were derived, selected for plasmid integration by addition of 5mg/ml G418 Sulphate, PCR screened for plasmid insertion (5'-gacccctgctgttacactct-3' and 5'-tatagacacacgccacacgg-3') and validated by Sanger sequencing using the PCR screening primer.

**SINGLE CELL SURVIVAL ASSAY**

In order to quantify the effect of Cdk8 loss of function on XCI mediated cell death I developed a single cell read-out. Cells were resuspended in PBS, without Calcium and Magnesium, supplemented with 1mM EDTA and 2% foetal bovine serum and strained through a 30μm filter. Single cells were sorted into 96well tissue culture plates containing ES medium without Gsk3β inhibitor and Mek1/2 inhibitor, which – within a plate – alternately contains 1μg/ml Dox. The medium was changed after five days and emerging colonies were quantified after 12 to 14 days. The experiment is performed in technical duplicates and at least in biological triplicates.
MOLECULAR BIOLOGY TECHNIQUES

DNA EXTRACTION

DNA extraction from cells, embryos and ear clip samples is performed using tail buffer (10mM Tris pH 8.0, 100mM NaCl, 10mM EDTA and 0.5% SDS) supplemented with 1mg/ml Proteinase K. Cell pellets are resuspended in buffer and incubated at a thermo shaker for two hours at 55°C at 1400 rpm. Saturated NaCl is added to a final concentration of 33% v/v and incubated another five minutes at the shaker. Lysate is cleared by centrifugation for 15 minutes at 17000g at 4°C. Isopropanol is added to the cleared lysate to a final concentration of 70% v/v. After shaking at room temperature for five minutes, samples are centrifuged for 30 minutes at 17000g at 4°C to pellet the DNA. Pellet is washed with 70% Ethanol, air dried for ten minutes, resuspended in T/E buffer (10mM Tris pH 8.0, 1mM EDTA) and put on the shaker at 55°C to facilitate solubilisation of the DNA. DNA extraction from embryos and ear clip samples is essentially performed as described above, with the exception that digestion is performed over night at 37°C to fully disintegrate the material. DNA concentration was determined using a NanoDrop and subjected to PCR.

RNA EXTRACTION AND qRT-PCR

RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol, including an on-column DNA digest using RNase-free DNase (QIAGEN). RNA concentration was determined using a NanoDrop. Equal amounts of RNA were deployed to reverse transcription, using the SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific). Oligo(dT)$_{15}$ primer (Promega) were used to specifically reverse transcribe polyadenylated transcripts. q-PCR experiments were performed in technical duplicates and biological triplicates at a 384well format on a Roche 480 Lightcycler® instrument using the SYBR Green method (KAPA SYBR® FAST qPCR KIT, Kapa Biosystems). Fold change expression was calculated by the ΔΔct method. Gapdh expression was used for normalisation. Primer used for gene expression analysis are listed in Table 3.
## Western Blot

Protein lysates were prepared by resuspending cell pellets in TNTE buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% Triton X-100 and 1mM EDTA) supplemented with 1mM PMSF, 10mM MgCl₂, 5mM CaCl₂ and 3000U/ml DNase I, incubation on a rotating wheel at 4°C for 20 minutes and centrifugation at 17000g for 15 minutes. Protein concentration of the cleared lysate was determined using the Bio Rad DC™ Protein Assay (DC™ Protein Assay Kit II, Bio Rad). Equal concentration of protein lysate were denatured in 1x Laemmli buffer for five minutes at 95°C. Vertical PAGE was performed using the Mini-PROTEAN® system (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, Bio Rad) with self-casted gels, using 40% Acrylamide/Bis solution 37.5:1, and Towbin buffer supplemented with 1g/L SDS. Protein transfer on 0.22μm PVDF membranes was performed by the wet tank method using Towbin buffer without SDS. Membranes were blocked using 5% non-fat dry milk in TBS (50mM Tris pH 7.5 and 150mM NaCl) supplemented with 0.1% Tween20 (TBS-T) for 60 minutes at room temperature. Incubation with primary antibodies was performed in blocking reagent at

### Table 3: Oligonucleotides used for expression level analysis by q-PCR. All nucleotides purchased from Microsynth

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<td>acaagc gagcatatctctt</td>
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Table 3: Oligonucleotides used for expression level analysis by q-PCR. All nucleotides purchased from Microsynth.
indicated dilutions (Table 4) over night at 4°C. Subsequently, membranes were washed three times 15 minutes each in TBS-T and incubated with HRP-coupled secondary antibodies at a concentration of 1:20000 (Table 5) in blocking reagent for 60 minutes at room temperature. Following three washes in TBS-T for five minutes each, the blots were developed using the enhanced chemiluminescence detection method on a Bio Rad ChemiDoc™ system. Clarity™ Western ECL substrate (Bio Rad) was used for abundant proteins and SuperSignal™ West Femto Maximum Sensitivity Substrate was applied for low expressed proteins.

<table>
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<tr>
<th>ANTIBODY</th>
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<tr>
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<tr>
<td>Stat3 (124H6)</td>
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<td>Mouse</td>
<td>1:1000</td>
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*Table 4:* Primary antibodies used for western blot detection

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<td>715-035-180</td>
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<tr>
<td>Peroxidase AffiniPure Donkey α-Rabbit IgG (H+L)</td>
<td>711-035-152</td>
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</table>

*Table 5:* HRP coupled secondary antibodies. Purchased from Jackson Immuno Research, Working concentration 1:20000
**RNA-FISH**

RNA FISH was performed as previously described. Cells were seeded on Laminin coated (5μg/ml in PBS) Roboz slides (Cellpoint Scientific) in ES medium without Gsk3β and Mek1/2 inhibitor, respectively, in the absence or presence of Dox. After 24 hours, cells were rinsed in PBS, washed in CSK buffer (10mM PIPES pH 6.8, 100mM NaCl, 300mM sucrose and 3mM MgCl₂) and proteins were extracted by incubation in CSK buffer supplemented with 0.5% Triton X-100 for seven minutes. After one wash in CSK buffer, cells were fixed in 4% paraformaldehyde in PBS for ten minutes at room temperature. Slides are dehydrated in an ethanol series of 70%, 80%, 95% and 100%. FISH probe was applied to the cells and incubated overnight at 37°C in a light protected humidified chamber. Slides were washed three times each for five minutes at 39°C with 50% formamide/2xSSC (300mM NaCl, 30mM sodium citrate, pH 7.0) and 2xSSC, respectively, followed by one wash with 1xSSC. Cellular DNA is stained with DAPI and slides are mounted with mounting medium (Vectashield H-1000, Vectorlabs).

FISH probes were prepared using the random primer labelling technique (Prime-It II random primer labelling kit, Stratagene). Briefly, 1ug of template DNA plasmid (Blueprint vector containing Xist cDNA, pBP5.6) was denatured, random primer were annealed and DNA with incorporated Cy3-dCTP (GE Healthcare Amersham) was synthesised using a Klenow reaction. After overnight incubation at 37°C, the reaction was cleaned over a column (Min Elute PCR Purification Kit, QIAGEN), supplemented with Cot-1 DNA and salmon sperm DNA to compete for repetitive elements and yeast tRNA to sequester potential RNases. The probe was precipitated in 2.5 volumes Ethanol/0.1 volumes 3M sodium acetate and resuspended in hybridisation mix (Hybrisol VII, MP Biomedicals). The probe was denatured at 74°C for ten minutes and annealed at 37°C for 30 minutes.

**COMBINED IMMUNOFLUORESCENCE AND RNA-FISH**

This experiment was performed as described previously with minor modifications. Cells grown as mentioned for RNA-FISH, were rinsed with ice cold PBS and fixed for ten minutes in 4% paraformaldehyde in PBS at room temperature. After
two washes in PBS, cells were permeabilised in 0.1% sodium citrate/0.5% Triton X-100 in PBS supplemented with 20mM Ribonucleoside Vanadyl Complex (RVC) for ten minutes on ice. Slides were washed two times in ice cold PBS+0.1% Tween20 (PBS-T) and blocked for 45 minutes at room temperature in 2.5% BSA in PBS-T supplemented with 20mM RVC. Primary antibodies (mouse α-Ezh2 (AC22): dilution 1:100, Cell Signaling Technology #3147; rabbit α-H3K27me3: dilution 1:200, Active Motif #39155), diluted in blocking solution supplemented with 20mM RVC and 1U/µl RNase inhibitor (RiboLock, Thermo Fisher Scientific), were added to the cells for 60 minutes at RT. Following three washes with PBS-T, fluorophore labelled secondary antibodies (Jackson Immuno Research: Alexa Fluor® 488 AffiniPure Donkey α-Mouse IgG (H+L), #715-545-150; Alexa Fluor® 488 AffiniPure Donkey α-Rabbit IgG (H+L), #711-545-152; Alexa Fluor® 647 AffiniPure Donkey α-Mouse IgG(H+L), #715-605-150), in blocking solution/RVC/RNase inhibitor, were applied at a dilution of 1:1000 and incubated for 60 minutes at room temperature in the dark. Slides were washed twice with PBS-T and once with PBS. Slides are post-fixed for ten minutes with 4% paraformaldehyde in PBS at room temperature, washed once in PBS followed by a wash in 2xSSC. After nearly complete drying of the samples the FISH protocol applies from the point of adding the probe to the slides.

**VISUALISATION OF IMMUNOFLUORESCENCE AND FISH**

Samples were analysed on a ZEISS Axio Observer Z.1 fluorescence microscope equipped with a Hamamatsu OrcaFlash 4.0 camera and a Plan Apochromat 100x/1.46 oil DIC objective. Images were processed using ZEISS Zen Pro 2.0 software and figures were prepared using ImageJ/Fiji and Adobe Photoshop to crop pictures and adjust brightness and contrast.

**IN VIVO EXPERIMENTS**

**REGULATIONS AND BACKGROUND**

All *in vivo* experiments were performed under the license ZH152/17 in accordance to the standards and regulations of the *Kantonale Ethikkommission Zürich*. Breeding, maintenance, timed matings and plug checks were carried out at the ETH Phenomics
Center Mouse Facility. Experiments were mainly performed by Charles-Etienne Dumeau, a trained embryologist. B6.CDK8tm1(fl/fl)Eucomm mice were a kind gift of Professor Markus Stoffel and B6.Cg-Tg(Sox2-cre)1Amc/J 103 mice were kindly provided by Professor Jennifer Nichols.

**PHENOTYPIC ASSESSMENT**

Embryonic day 12.5 embryos were collected according to regulations and under supervision of Charles-Etienne Dumeau. Pictures were taken at the same magnification using an Olympus MVX10 stereo microscope mounted with an Olympus DP73 camera.

**EMBRYONIC STEM CELL DERIVATION**

ESC were derived solely by Charles-Etienne Dumeau according to published procedures 133.

**GENOTYPING**

DNA of embryos and derived ESC is performed as described above. Primer used for genotyping PCR can be found in Table 6.

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<tr>
<th>GENE</th>
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<td>Interleukin2</td>
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*Table 6*: PCR Primer used for genotyping. Purchased from Microsynth
**Transcriptomic Analysis**

RNA was extracted as described above and handed over to the Functional Genomics Center Zürich for sequencing. Polyadenylated RNA was enriched using Oligo(dT) beads and libraries were prepared using an Illumina TruSeq Stranded mRNA kit. Libraries were sequenced on an Illumina HiSeq 4000 machine, creating 125base single end reads. For analysis of the obtained data I used the commercial software CLC Genomics Workbench 11 (QIAGEN) licenced to the ETH. Adapters were not trimmed and the reads were aligned to the Ensembl reference genome (GRCm38.94). Reads were normalised using the Transcripts per Kilobase Million (TPM) method, which normalises for gene length before normalising for sequencing depth, thereby allowing direct comparison of TPM values between samples. The obtained TPM values are used for the representation of transcriptomic changes plotted in Figure 10A I-III, which represent the ratio of TPM values of induced samples over uninduced samples for (I) and (II) and the ratio of ΔCdk8 over WT in uninduced conditions for (III). TPM values are also used as input for the heatmap in Figure 10B, which was created using the online tool heatmapper.ca. Statistically significant differentially expressed genes were obtained by comparing the data set of interest against a control group, meaning treated against non-treated for differentially regulated X-linked genes in Figure 10 or ΔCdk8 non-treated against WT non-treated for general transcriptional changes depicted in Figure 14A-E, using a Wald test. Confounding factors have not been taken into consideration. The threshold for significance was set to an absolute fold change bigger than 2 and an FDR p-value smaller than 0.01.
APPENDICES
APPENDICES

REFERENCES


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation (A-Z)</th>
<th>Meaning</th>
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<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
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<tr>
<td>APC&lt;sub&gt;Min&lt;/sub&gt;</td>
<td>Adenomatous polyposis coli multiple intestinal neoplasia</td>
</tr>
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<td>Bex4</td>
<td>Brain expression X-linked 4</td>
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<td>BMP</td>
<td>Bone morphogenic protein</td>
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<td>Colorectal carcinoma</td>
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<td>Cre</td>
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<td>CCCTC binding factor</td>
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<td>Carboxy terminal domain</td>
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<td>Cyclin C</td>
</tr>
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<td>Doxycycline</td>
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</tr>
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IFNγ  Interferon γ
iXCI  Imprinted X-chromosome inactivation
Jarid2  Jumonji, AT rich interactive domain 2
Jpx  Just proximal to Xist
Lbr  Lamin-B receptor
Lef1  Lymphoid enhancer-binding factor 1
IncRNA  Long non-coding RNA
m6A  N6-methyladenosine
macroH2A  Macro histone 2A
Mecp2  Methyl-CpG-binding protein 2
Med12/L  Mediator subunit 12/like
Med13/L  Mediator subunit 13/like
Mettl3  Methyltransferase like 3
Myc  Myelocytomatosis
Nicd  Notch intracellular domain
Oct4  Octamer binding transcription factor 4
PAR  Pseudo autosomal region
Pcgf3/5  Polycomb group Ring finger protein 3/5
PCR  Polymerase chain reaction
Pdk3  Pyruvate dehydrogenase kinase 3
Pgk1  Phosphoglycerate kinase 1
Pls3  Plastin 3
Pol II  DNA dependent RNA Polymerase II
PrC1/2  Polycomb repressive complex 1/2
Prdm14  PR domain zinc finger protein 14
qRT-PCR  Quantitative reverse transcription polymerase chain reaction
R26nlsrtTA  Nuclear localisation signal reverse tetracycline transactivator at the ROSA26 locus
RA  Retinoic acid
Rbm15/B  RNA binding protein 15/B
Rex1  Reduced expression protein 1
Ring1  Ring finger protein 1
Rlim  Ring Finger Protein, LIM Domain Interacting
RNA FISH  RNA Fluorescence in situ hybridisation
RNAseq  RNA sequencing
Rnf12/Rlim  Ring finger protein 12
Rrm2  Ribonucleoside diphosphate reductase subunit M2
rXCI  Random X-chromosome inactivation
S727  Serine at position 727 of the protein
SEC  Super elongation complex
Ser2/5  Serine 2 / Serine 5 of Pol II CTD
Smad1  SMA (small phenotype, c. elegans) + MAD (mothers against decapentaplegic, drosophila)
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Smchd1</td>
<td>Structural maintenance of chromosomes flexible hinge domain containing 1</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sry box 2</td>
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<tr>
<td>Spen</td>
<td>Split end</td>
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<tr>
<td>Sry</td>
<td>Sex-determining region Y</td>
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<tr>
<td>Stat1/3</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TetON</td>
<td>Tetracycline controlled transcriptional activation</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<tr>
<td>TFII-I/H</td>
<td>Transcription factor II – I/H</td>
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<tr>
<td>TG</td>
<td>Transgene</td>
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<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<tr>
<td>TPM</td>
<td>Transcripts per kilobase million</td>
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<tr>
<td>Tsix</td>
<td>Reverse of Xist</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>Wtap</td>
<td>Wilms tumour suppressor gene associated protein</td>
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<tr>
<td>X^A</td>
<td>Active X-chromosome</td>
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<tr>
<td>XCI</td>
<td>X-chromosome inactivation</td>
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<tr>
<td>Xi</td>
<td>Inactive X-Chromosome</td>
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<td>XIC</td>
<td>X-chromosome inactivation centre</td>
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<tr>
<td>Xist</td>
<td>X-inactive specific transcript</td>
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<td>Xist^{TX/TX}</td>
<td>Homozygous tetracycline inducible Xist allele</td>
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<td>X^M</td>
<td>Maternal X-chromosome</td>
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<tr>
<td>X^P</td>
<td>Paternal X-chromosome</td>
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<tr>
<td>Yap</td>
<td>Yes associated protein</td>
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<td>Ythc1</td>
<td>YTH domain containing protein 1</td>
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<tr>
<td>ΔATP</td>
<td>Deletion of the amino acid crucial for ATP binding</td>
</tr>
<tr>
<td>ΔCdk8</td>
<td>Loss of Cdk8 function</td>
</tr>
</tbody>
</table>
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