Role of CIZ1 in imprinted X inactivation

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

Raghunandan D R

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

Date: March, 2024

Under the guidance of

Supervisor: Prof. Dr. Srimonta Gayen,

Indian Institute of Science, Bangalore

From May 2023 to March 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled "**Role of CIZ1 in imprinted X inactivation**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Raghunandan D R** at the Indian Institute of Science under the supervision of **Prof. Dr. Srimonta Gayen**, Assistant Professor, Department of Developmental Biology and Genetics, during the academic year 2023-2024.

Srimonta Gayen

Prof. Dr. Srimonta Gayen

Committee:

Prof. Dr. Srimonta Gayen

Prof. Dr. Sanjeev Galande

Declaration

I hereby declare that the matter embodied in the report entitled "**Role of CIZ1 in imprinted X inactivation**" are the results of the work carried out by me at the Department of Developmental Biology and Genetics, Indian Institute of Science, Bangalore, under the supervision of **Prof. Dr. Srimonta Gayen**, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Raghunandan D R
20191145

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Abstract

X-chromosome inactivation (XCI) is a way of dosage compensation that occurs in female eutherian mammals. There are two modes of XCI: Imprinted XCI where the paternal X is silenced predominantly, and random XCI where one of the two X chromosomes is randomly inactivated. Xist, a lncRNA expressed from the inactive X (Xi), spreads throughout Xi and recruits various factors that establish epigenetic modifications to transcriptionally silence X-linked genes. Xist interacts with several RNA Binding Proteins that play roles in Xist-mediated gene regulation. One of these proteins is the Cip1-interacting Zinc finger protein 1 (CIZ1), a nuclear matrix protein enriched at the Xi. CIZ1 colocalizes with the Xist RNA and *ciz1* knockout leads to the disruption of Xist localization and loss of repressive marks. However, these functions are in the context of random XCI. In this project, we aimed to explore the possible role of CIZ1 in imprinted XCI. This study will provide insights into the evolutionary aspect of XCI and help in gaining knowledge about the conservation of mechanisms/pathways involved in the two types of XCI.

We strategized to obtain CRISPR-Cas9 mediated knockout of *ciz1* in XEN cells and study the Xist localization and X-linked gene expression. However, we observed the presence of CIZ1 despite a frameshift deletion. We are now working on shRNA mediated knockdown of CIZ1 to obtain maximal reduction of CIZ1 and investigate the dose dependence of CIZ1 in imprinted XCI, because the function of CIZ1 is dosage sensitive in random XCI. Currently, I am screening single-cell clones for CIZ1 knockdown.

Acknowledgments

I thank my supervisor Prof. Srimonta Gayen for providing the opportunity to work in the lab and providing inputs and ideas for the progress of the project. I thank my expert Prof. Sanjeev Galande for his time in my evaluation and providing suggestions. I thank Runumi Baro, Amritesh Sarkar, Avinchal Manhas, and Maniteja Arava for teaching some of the techniques and guiding me through different aspects of the project. I also thank Prof. Sundeep Kalantry for giving his thoughts and opinions on my project. I thank all my lab members in giving valuable suggestions on my project during the lab meetings and offering a wonderful work environment. I thank the central facility and the FACS facility members Mr. Munish and Mr. Bilal for single cell sorting. I thank my parents for always supporting me. Some of the figures were created with Biorender.com and Microsoft PowerPoint.

Contributions

Contributor name	Contributor role
Prof. Srimonta Gayen, Raghunandan D R	Conceptualization Ideas
Lab established protocols	Methodology
Hemant C Naik	Software
Raghunandan D R	Validation
-	Formal analysis
Raghunandan D R	Investigation
SG lab, SR lab, DBG Central facility, DBG FACS facility	Resources
-	Data Curation
Raghunandan D R	Writing - original draft preparation
Raghunandan D R, Prof. Srimonta Gayen	Writing - review and editing
Raghunandan D R	Visualization
Prof. Srimonta Gayen	Supervision
-	Project administration
Prof. Srimonta Gayen	Funding acquisition

Chapter 1 Introduction

1.1 Dosage compensation and X inactivation

Dosage compensation is a phenomenon that evolved with species that show sexual dimorphism and incur different sets of chromosomes that bring about the differences in the two sexes. Different species use different mechanisms of dosage compensation. For example, the X-linked gene expression in males is doubled relative to the females in Drosophila and conversely, the X-linked gene expression is halved in females compared to the males in C. elegans (Strome et al., 2014; Lucchesi and Kuroda, 2015). The idea of X chromosome inactivation (XCI) was first initiated with the finding of the Barr body in only the female cells and not in the male cells of mammals. This was accompanied with the observation that one of the X chromosomes in only mammalian females is condensed (Barr and bertram 1949). This along with phenotypic data from female mice and individuals with X-linked disorders served as a background for Mary Lyon's proposal of the idea of random XCI, in 1961 (Lyon, 1961; Lyon and Moore, 1998). In order for the X-linked gene expression to make up for the dosage with regard to the autosomal genes, Ohno further suggested the idea of upregulation of the X-linked genes in the active X chromosome in females as well as in males (Ohno, 1969). This was recently shown with evidences, by different groups (Lin et al., 2007; Lyu et al., 2022; Naik et al., 2022). X-chromosome inactivation is one of the ways of dosage compensation that occurs in eutherian female mammals predominantly brought out by epigenetic mechanisms (Brockdorff and Turner, 2015; Cantone and Fisher, 2017). There are two forms of XCI that are seen: i) Imprinted XCI, which is seen in marsupials and early mouse development prior to implantation and is characterized by the inactivation of the paternal X predominantly. However, because marsupials lack the presence of Xist RNA, the mechanisms by which these occur in the two species appear to be different (Huynh and Lee, 2003; Okamoto et al., 2004; Sado and Ferguson-Smith, 2005); and ii) Random XCI, in which one of the two X chromosomes is randomly inactivated and this can be seen in the post-implantation stages of mouse development, after the reactivation of the paternal inactive X, in the embryo proper and in humans (Mak et al., 2004). The two forms of XCI during mouse development is illustrated in Fig 1.

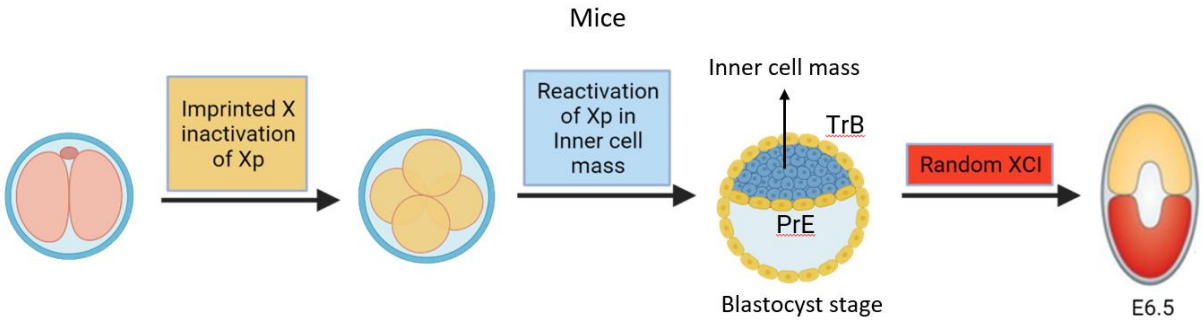


Fig 1. Two modes of XCI during early mouse development. Imprinted XCI occurs around the 4-cell stage and during the blastocyst stage, the paternal inactivated X is reactivated only in the ICM while other lineages maintain their paternal X inactive.

Random XCI is well studied in contrast to imprinted XCI. Following the establishment of random XCI, the inactivated X remains to be inactivated throughout subsequent mitotic cycles of the cell. The X inactive specific transcript (Xist), is a long non-coding RNA (lncRNA) that is upregulated during early differentiation, from the future inactive X (Xi). Xist RNA is a master regulator of XCI without which the female cells fail to initiate XCI (Brown et al., 1991; Borsani et al., 1991; Brockdorff et al., 1992; Brown et al., 1992; Brockdorff and Duthie, 1998; Kay, 1998). Xist RNA spreads throughout the Xi and recruits factors involved in chromatin remodelling to achieve transcriptional silencing. Interestingly, Xist RNA is transcribed and processed similar to other mRNAs. Xist is transcribed by RNA polymerase II and incurs many of the characteristics of an mRNA and yet escapes from being transported into the cytoplasm like the other mRNAs (Brockdorff et al., 1992; Brown et al., 1992; Melé et al., 2017). Notably, some genes on the inactive X chromosome manage to escape from silencing (Lyon, 1962; Carrel et al., 1999; Berletch et al., 2010). The escapee genes, their number and the level of their transcription may vary in different cell types (Yang et al., 2010). This phenomena in females, along with the presence of Y chromosome in males is believed to bring about the phenotypic and molecular differences between the two sexes. Long non-coding RNAs are known to associate with their proteins in order to carry out their function. Xist RNA consists of six conserved repeats wherein each of the repeats is dedicated to a particular function through associating with multiple RNA binding proteins (RBPs) as shown in Fig 1 (Chu et al., 2015).

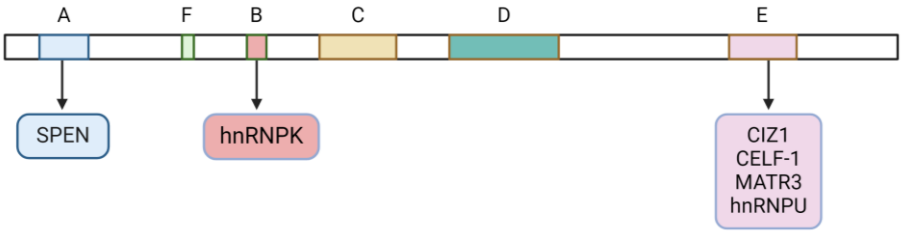


Fig 2. Xist RNA repeats and their associated proteins. Different repeats of Xist RNA contribute to a particular function by associating with a different set of proteins.

1.2 X chromosome inactivation

Since imprinted XCI is not very well studied, we will focus on the mechanisms of random XCI in the following.

1.2.1 Initiation and establishment of XCI

During early differentiation, monoallelic upregulation of Xist that occurs via RNF12, triggers the onset of random XCI. This requires the reduction in levels of pluripotency factors because differentiation and XCI go hand in hand and the pluripotency factors repress the Xist promoter in a way that RNF12 cannot upregulate Xist in the undifferentiated state (Jonkers et al., 2009; Barakat et al., 2011). RNF12 also indirectly plays a role in Xist upregulation because it is a ubiquitin ligase that helps in proteasomal degradation of REX1 because REX1 is shown to represses Xist expression (Gontan et al., 2012). Other loci such as Jpx and Ftp that are located in the Xist topologically associated domain (TAD), positively regulate Xist expression (Barakat et al., 2014). As the Xist upregulation is sensitive to RNF12 dosage, XCI is initiated only in cells that have more than one X chromosome and RNF12 dosage somehow helps in counting number of X chromosomes in the cell to help decide the number of X's to be inactivated. While RNF12 is demonstrated to be required for the initiation of imprinted XCI, there exists evidence suggesting that it might be dispensable for the initiation of random XCI in mice (Shin et al., 2010; Barakat et al., 2011). It still remains an open question as to what are the mechanisms involved for random upregulation of Xist in one of the two X chromosomes. According to a recent study, there might be a brief window of time during which Xist is expressed

biallelically before one of the two X chromosomes is randomly selected for inactivation (Valledor et al., 2023). Subsequently, before genomewide silencing takes place, the Xist RNA diffuses throughout the length of th e X chromosome in cis and aids in the compaction of the Xi (Żylicz et al., 2019) . The compaction of the Xi allows for a dense Xist RNA territory to be formed because Xist expression plateaus after a point to prevent its spreading to other nearby autosomal loci (Valledor et al., 2023). Additionally, there is evidence to support a role for Tsix in suppressing Xist expression from the future active X chromosome (Xa) (Lee et al., 1999). Xist RNA recruits the polycomb repressive complexes PRC1 and PRC2 to establish H2AK119ub1 and the H3K27me3 marks respectively (Plath et al., 2003; Silva et al., 2003; de Noples et al., 2004; Fang et al., 2004; Plath et al., 2004). While the H3K27me3 is observed in only dense Xist RNA territory, the H2AK119ub1 is seen in both the sparse and the dense Xist RNA territories (Valledor et al., 2023). This is due to the fact that high Xist RNA density helps in achieving higher histone deacetylase (HDAC) density, which is required for efficient deacetylation (De Andrade E Sousa et al., 2019; Valledor et al., 2023). Besides, establishment of H4K20me1 is recently reported to play a role in the compaction of the Xi (Tjalsma et al., 2021). hnRNPK is a protein that interacts with the B-repeats of Xist RNA (Nakamoto et al., 2020). It plays an important role by recruiting PRC1 complex to the Xi to establish H2AK119ub1 mark. PRC1 complex mediated H2AK119ub1 mark recruits PRC2 complex to the Xi (Almeida et al.; Pintacuda et al., 2017). It is debatable whether PRC1 or PRC2 is recruited first to the Xi and the corresponding histone marks established by it is further required for the PRC1 recruitment or vice versa. Both of the hypotheses are supported by existing evidences (Schoeftner et al., 2006; Tavares et al., 2012; Rose et al., 2016). There exist both dependent and independent components to establish both these marks and erasing either of the marks does not completely impair the establishment of the other (Colognori et al., 2019). A summary of how polycomb marks are established are shown in Fig 2.

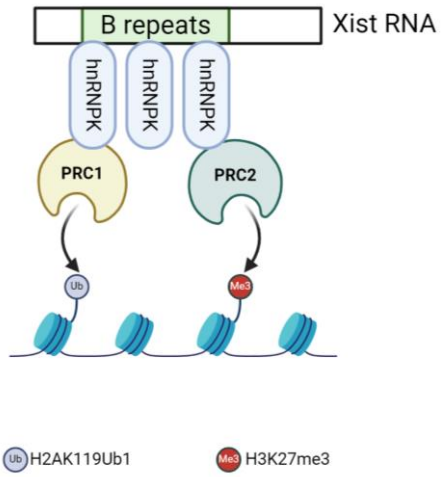


Fig 3. Establishment of Polycomb repressive histone marks. hnRNPK is recruited to the Xist B repeats. hnRNPK recruits the PRC1/2 complexes to the Xi. PRC1 complex deposits H2AK119Ub1 mark whereas PRC2 deposits the H3K27me3 repressive marks on histones.

SPEN protein interacts with the SMRT/NCoR complexes and it consists of RNA Recognition Motifs (RRMs) (Shi et al., 2001; Ariyoshi and Schwabe, 2003). It plays a role in silencing of Xi linked genes. SPEN functions by activating HDAC3 that is present within the SMRT/NCoR complex (Guenther et al., 2001; Dossin et al., 2020). SPEN is recruited to the Xi by interacting with the Xist A repeat region (Monfort et al., 2015). They are recruited to active promoters and enhancers and they silence genes via exclusion of RNA Pol II and HDAC 3 (McHugh et al., 2015; Żylicz et al., 2019; Dossin et al., 2020). Xist nucleates protein gradients to form supramolecular complexes (SMACs) that can include multiple copies of the same protein. SPEN consists of an intrinsically disordered region (IDR) that helps in its accumulation in the SMACs (Markaki et al., 2021; Jachowicz et al., 2022). This along with the compaction of the Xi to bring more genes closer to the Xist locus resolves this problem of small number of Xist puncta relative to the number of chromosome-wide loci to be silenced (Markaki et al., 2021). Interestingly, these SMACs are also present at the Xist locus and they possibly regulate the expression of Xist in order to restrict Xist RNA to the Xi (Jachowicz et al., 2022). After silencing of the Xi, SPEN disengages from the Xi chromatin (Dossin et al., 2020). Despite all these mechanisms of silencing, the repressed state of the Xi is not quite stable and therefore, there is a possibility of reactivation of the Xi. Hence, a maintenance

component is necessary so that the repressed state is propagated through cell divisions.

1.2.2 Maintenance of XCI

The cells have adopted two strategies to ensure the maintenance of the repressed state of Xi: 1) The conventional H2A molecules are replaced with a variant macro H2A [97,99,100,101,102] (Coztanzi et al., 1998; Mermoud et al., 1999; Rasmussen et al., 2000; Chadwick and Willard, 2002; Pasque et al., 2011) and 2) DNA methylation is established on the promoters CpG islands and this methylation can be propagated by different DNMTs through mitosis (Norris et al., 1991; Sado et al., 2000; Weber et al., 2007; Gendrel et al., 2012; Yagi et al., 2020). Henceforth, the role of Xist becomes partly dispensable for the maintenance of XCI because the knockout of Xist does after this point does not lead to widespread reactivation of Xi (Arava et al.). There exist many factors that ensure for the localization of Xist RNA to the Xi. This is important to maintain the repressive marks such as H3K27me3. CIZ1 and other proteins such as PTBP1, MATR3, CELF1 and hnRNPU interact with the E repeats on the Xist RNA (Pandya-Jones et al., 2020). They function to anchor the Xist RNA onto the Xi and they also help in the maintenance of the H3K27me3 repressive mark. Xist RNA dispersal is observed in the absence of these proteins without significant changes in Xist expression (Hasegawa et al., 2010; Ridings Figueroa et al., 2017; Sunwoo et al., 2017; Pandya-Jones et al., 2020). This suggests that they help in anchoring the Xist RNA to the Xi. The E-repeats of Xist seems to play the major role in anchoring the Xist to the Xi as shown in Fig 4. Though there is a decrease or absence of the H3K27me3 repressive mark on the Xi post Xist dispersal, there is no widespread reactivation of the Xi (Ridings-Figueroa et al., 2017). Collectively, the evidences suggest that there are several factors with many of them having redundant partners that function in the maintenance phase of XCI. This results in the immense stability of XCI wherein deleting one or two factors does not lead to widespread reactivation, which makes it difficult to decipher the function of each factor contributing in the process. This is reviewed extensively elsewhere (Keniry and Blewitt, 2023). Therefore, though there is a lot known about the mechanisms of initiation of XCI, little is known about the mechanisms by which the Xi is maintained in the inactive state due to the above-mentioned difficulties in studying the same.

1.3 CDKN1A interacting zinc finger protein 1 (CIZ1)

CIZ1 is a nuclear matrix protein which is highly conserved among the vertebrates, which was first characterized to promote DNA replication (Coverley et al., 2005). It was originally identified as an interactor of p21 or CDKN1A in a yeast two hybrid screen (Mitsui et al., 1999). Ciz1 gene is present on chromosome 2 in mice and it consists of 17 exons. There are at least 22 different isoforms characterized that arise due to alternative splicing. It consists of multiple domains for different functions as shown in Fig 3: 1) Two disordered domains at the N-terminus that interacts with RNAs (Sofi et al., 2022), 2) CDK phosphorylation sites and an RXL motif that interacts with cyclins and cyclin dependent kinases (CDKs) during DNA replication (Ainscough et al., 2007), 3) Zinc finger domains that help in interacting with DNA (Copeland et al., 2015), 4) a nuclear localization signals that allows it to enter the nucleus, 5) a matrin3 homology domain that allow for dimerization of CIZ1 and it acts as an interaction interface with RNAs (Turvey et al.). The N terminal region is called the replication domain as it plays a role in replication and the C terminal region is called the anchoring domain as it helps in anchorage to the nuclear matrix (Ridings Figueroa et al., 2017). CIZ1 has been linked to several types of cancers due to aberrant splicing (Greaves et al., 2012; Turvey et al.). Recently it has been shown that CIZ1 knockout (KO) in female mice leads to a lymphoproliferative disorder with complete penetrance during adulthood (Ridings-Figueroa et al., 2017). However, it is worth noting that CIZ1 KO mice (both males and females) do survive till adulthood and do not show premature lethality (Ridings-Figueroa et al., 2017). These evidences and the fact that CIZ1 was found to interact with Xist RNA in a proteomic screen led to the interest in exploring the role of CIZ1 in XCI (Chu et al., 2015).

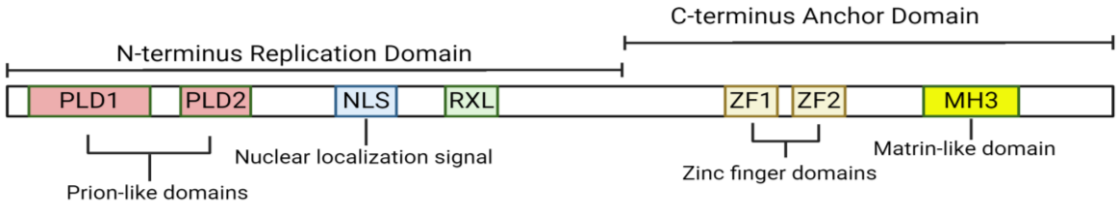


Fig 4. CIZ1 protein. Different regions and domains of CIZ1 are depicted in this figure. N-572 represents the replication domain and C-275 represents the Anchor domain.

1.4 CIZ1 and other nuclear matrix proteins in random XCI

The accumulation of CIZ1 at the prospective Xi is observed as early as day 1 of mouse embryonic stem cells (mESCs) differentiation [ridings]. CIZ1 colocalizes with Xist RNA and H3K27me3 mark on the Xi and there is a cell cycle dependent foci of CIZ1 that disappears during the anaphase similar to that of Xist RNA (Ridings Figueroa et al., 2017; Sunwoo et al., 2017). CIZ1 is associated with RNA as well as nuclear matrix protein at the Xi (Ridings-Figueroa et al., 2017). The absence of CIZ1 and other nuclear matrix proteins results in decreased H3K27me3 enrichment at the Xi (Ridings-Figueroa et al., 2017; Pandya-Jones et al., 2020). The effect of CIZ1 seems to be dose-dependent because overexpression of CIZ1 also leads to Xist RNA dispersal similar to what is observed in the case of CIZ1 KO (Sunwoo et al., 2017). However, this leads to only subtle changes in X-linked gene expression from the Xi (Ridings-Figueroa et al., 2017). Interestingly, the repressive marks that were reduced or absent in CIZ1 null cells, reappear after long term culturing of the cells (Stewart et al., 2019). This is due to the increase in abundance of the lighter isoform of EZH2 which is the catalytic subunit of the PRC2 complex and the loss of spatial restriction of the enzyme as shown in Fig 5 (Stewart et al., 2019). CIZ1 consists of prion like domains at the N-terminal end that helps in its assembly at the Xi and maintains the specificity to interact with Xist RNA while preventing promiscuous interactions with other RNAs (Sofi et al., 2022).

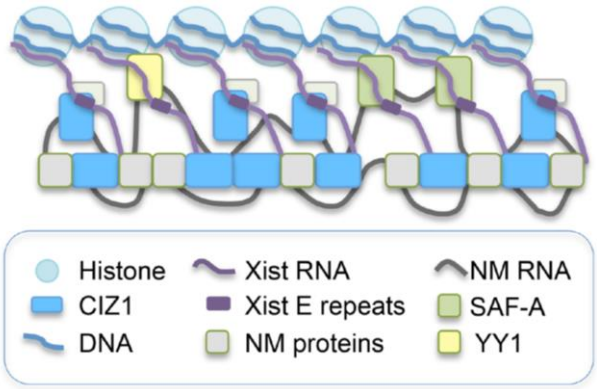


Fig 5. Xist localization in the maintenance phase of XCI. Several factors that bind primarily to the Xist E repeats, function in localizing Xist RNA to the Xi and absence of these results in Xist RNA dispersal in the nucleus (Ridings-Figueroa *et al.*, 2017).

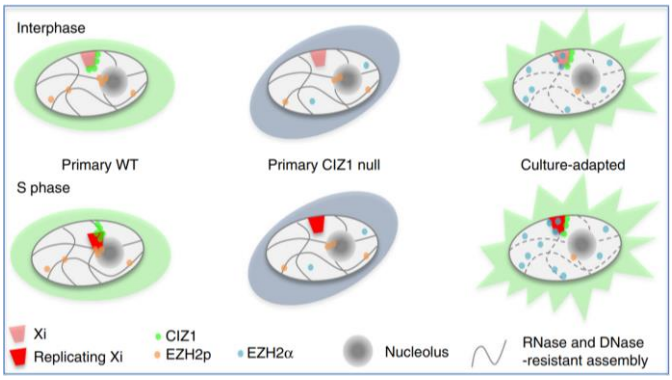


Fig 6. Model for the mechanism by which repressive marks reappear on the Xi. The switch from heavier isoform to the lighter isoform of EZH2 plays a role in the reappearance of repressive marks in CIZ1 null cells (Stewart *et al.,* 2019).

Scaffold attachment factor (SAF-A) or hnRNPU is a matrix protein that consists of an RNA binding RGG domain and a DNA binding domain (Kipp et al., 2000; Helbig and Fackelmayer, 2003). However, SAF-A or CIZ1 is not recruited to the X chromosome in the undifferentiated state despite basal level expression of Xist, which suggests that the chromatin state might play a role in recruitment of these proteins (Pullirsch et al., 2010). Knockout of SAF-A but not CIZ1, leads to embryonic lethality which suggests that CIZ1 either might have redundant factors present during early mice development that compensate for the loss of CIZ1 or CIZ1 may not be required for imprinted XCI (Ridings-Figueroa et al., 2017). Interestingly, SAF-A KO leads to dispersed Xist RNA but CIZ1 colocalizes with the dispersed Xist RNA (Sunwoo et al., 2017). Therefore, CIZ1 and hnRNPU depend on each other and probably work in the same pathway to anchor Xist to the Xi. Synthetically tethering CELF-1, MATR3 or PTBP1 but not CIZ1 to Xist RNA that does not have intact E repeat region, rescues the Xist dispersal phenotype (Pandya-Jones et al., 2020). In many of the somatic cell types in humans, it is observed that hnRNPU is not necessary for Xist localization (Kolpa et al., 2016). Similarly, CIZ1 also seems to have a cell-type specific role in anchoring Xist to the Xi (Ridings-Figueroa et al., 2017). All these functions of CIZ1 were observed in the context of random XCI and its roles in imprinted XCI remains to be explored. It has also been previously shown in our lab that CIZ1 enriches at the Xi in the context of imprinted XCI as well (Majumdar et al.).

1.5 Aim and objectives of the study

The aim of this project is to explore the role of CIZ1 in imprinted X inactivation. The significance of this project is that this will provide insights into the evolutionary aspects of XCI. This will help understand which of the pathways are conserved or different between the two modes of XCI. Lastly, it will also help provide insights whether there is any context specific role of CIZ1. The objectives to achieve the goal of the project are divided as follows:

1. To generate Ciz1 KO in extra embryonic stem (XEN) cells

2. To study Xist localization in CIZ1 KO XEN cells

3. To investigate X-linked gene expression in Ciz1 KO XEN cells

Chapter 2 Materials and Methods

2.1 Cell lines and maintenance

2.1.1 Extraembryonic endodermal stem (XEN) cells

These cells are obtained from the primitive endoderm (PrE) of the mouse blastocyst, which is an extra embryonic lineage, before implantation. These cells grow by adhering to the surface of the culture plates. These cells undergo imprinted X inactivation this is our model system for the study. These are hybrid lines derived from two mouse strain where the maternal alleles are of the Mus musculus molossinus (JF1) strain and paternal alleles are of Mus musculus musculus (S129) strain. This is shown in Fig 6. This allows us to perform allele-specific analysis post high-throughput sequencing due to the presence of single nucleotide polymorphisms. The media used for culturing these cells consists of Dulbecco's Modified Eagle Medium (DMEM, Himedia, Cat #AL007A) that is supplemented with 20% Foetal Bovine Serum (FBS, GIBCO, Cat #10270-106), 3 mM L-Glutamine, penicillin/streptomycin, 100 µM non-essential amino acids and 100 µM β-mercaptoethanol. 0.2% gelatin was used to coat the cell culture dishes and the cells were grown at 37 C with 5% CO_2. While passaging, the cells were detached from the surface using 0.05% trypsin EDTA for 3 minutes followed by diluting the trypsin with exhaust media and spinning the cells at 2k rpm for 5 minutes or 3k rpm for 3 minutes. The trypsin-exhaust media is removed and the cells are resuspended in fresh media and plated uniformly according to the desired ratio.

2.1.2 Transformed Human Embryonic Kidney (HEK293T) cells

These are transformed human cells. They were used to prepare viral particles for the shRNA mediated knockdown experiment. These cells grow by adhering to the surface of the culture plates. The media used for culturing these cells consists of DMEM, 20% Foetal Bovine Serum, penicillin/streptomycin. 0.2% gelatin was used to coat the surface of the cell culture dishes and the cells were grown at 37 C with 5% CO_2. While passaging, the cells were detached from the surface using 0.05% trypsin EDTA for 30 seconds followed by diluting the trypsin with exhaust media and spinning the cells at 2k rpm for 5 minutes or 3k rpm for 3 minutes. The trypsin-

exhaust media is removed and the cells are resuspended in fresh media and plated uniformly according to the desired ratio.

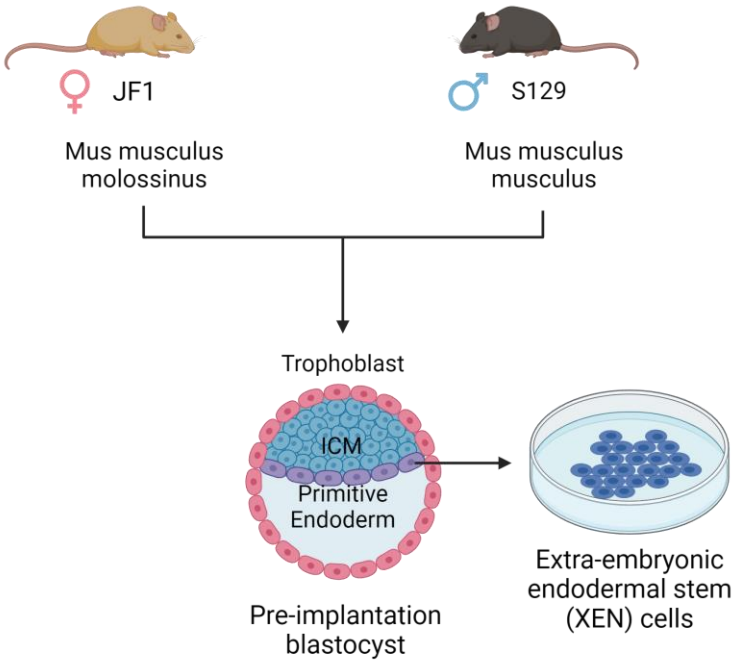


Fig 7. XEN cells as a model for imprinted X inactivation. XEN cells are derived from the PrE of the pre-implantation blastocyst. These cells are in the maintenance phase of imprinted XCI.

2.2 Generation of CIZ1 KO XEN cells

2.2.1 gRNA design and cloning

The guide RNAs used in this study were obtained from Rodermund et al., 2021. These were designed to delete around a 10.66 kb region of the ciz1 gene from exon 5 until the last exon. This segment consists of most parts of CIZ1 including the nuclear localization signals (NLS) present on exon 7. The gRNA sequence oligos were ordered such that they additionally consist of BbsI restriction site overhangs. The gRNA sequence had a CACCG at its 5' end where CACC represents the BbsI site and G helps increase transcription efficiency from the U6 promoter. The complementary oligo had a AAAC at the 5' end that is the restriction site and a C at the 3' end that complements the added G in the other strand. The plasmid vector used for cloning is the pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene, Cat #62988). Since the XEN cells were obtained from a hybrid strain of mice, we also checked for SNPs at the location of the two gRNA sequences and confirmed that there was no SNPs.

The gRNA oligos were annealed with their complementary oligos in an annealing buffer that consists of 10 mM Tris HCl (pH=8), 100 mM NaCl and 1 mM EDTA. 15 µl each of 100 mM oligos were used in a total of 75 µl reaction. The reaction mixture was incubated in a thermal cycler in the following order of temperatures: 92 C for 2 min, 72 C for 2 min, 55 C for 2 min and 37 C for 2 min. 1 ug of the PX459 vector was digested with BbsI restriction enzyme for 3 hours at 37 C, in a 50 µl reaction. The digestion was confirmed by running the digested and undigested plasmid control on an agarose gel. The digested plasmid was gel purified. A ligation reaction was set up with 50 ng of the digested plasmid and annealed oligos using T4 ligase, at 16 C overnight with a total reaction volume of 20 µl.

Competent DH5 alpha cells was prepared and the transformation efficiency of the cells using PUC18 plasmid was approximately $6x10^5$ colony forming units (CFU). The ligated vector was transformed into the competent cells and incubated overnight. Single colonies were picked, cultured in LB broth and plasmids were extracted from the cells. The cloning was confirmed by restriction digestion and sanger sequencing. The plasmids with inserts do not get digested and hence do not show a single band like in the case of control plasmids without the insert. Glycerol stocks were prepared from the remaining LB culture. The plasmid vectors were also sent for Sanger sequencing using a primer at the U6 promoter to confirm that the gRNA sequence has indeed been cloned into the vector.

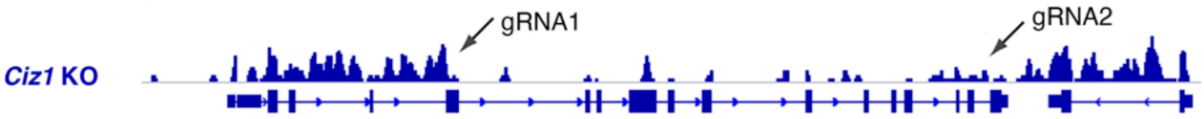


Fig 8. CIZ1 KO strategy. The positions of the gRNAs used are shown as described in Rodermund et al., 2021. gRNA1 targets exon 5 and gRNA2 targets the last exon region.

Description	Sequence (5'-3')
CIZ1_sgRNA 1	CAGCCTTACACCACCCCAGA
CIZ1_sgRNA 2	CAGGGCGTTGCGGGCGTTGA

Table 1 gRNA sequences used for CIZ1 knockout as mentioned in Rodermund et al., 2021.

The limitation is that there is a possibility that a truncated protein is formed that consists of the disordered regions and hence these if not degraded, can accumulate

in the cytoplasm since they do not have the NLS signals. Expasy tool was used for insilico translation of mRNA (Gasteiger et al., 2003).

2.2.2 Transfection and puromycin selection

4 µg of each of the two gRNAs containing vectors and 20 µl of turbofect (Thermofisher, Cat #R0531) were diluted to a total volume of 1 ml opti-MEM (Invitrogen, Cat #11058021). The components were mixed, briefly vortexed and incubated at room temperature for 20 min. The XEN cells were previously plated on a 10 cm plate such that the they are around 60% confluent at the time of transfection. The transfection mix is then put dropwise onto the cells with the growth media. The media was changed around 9 hours post transfection. 3 µg/ml concentration of puromycin (Takara, #631305) was used to select for the cells that have taken up the vectors. Wildtype cells were also treated with puromycin to confirm that the puromycin is working. This selection was done from 36-96 hours post transfection.

2.2.3 PCR screening

Post selection, the dead cells were removed and cells are allowed to grow. Single cells are then sorted into a 96 well plate. The single cells cloned are allowed to grow. Crude genomic DNA is extracted from the clones. Cells were pelleted and the excess media was removed. A mix of tail lysis buffer and proteinase K was used to resuspend the cells and this mix was incubated at 65 C overnight. The mixture was then heated at 95 C for 10 min to inactivate the proteinase K and stored at 4 C. The primers for PCR screening were used from Rodermund et al., 2021 and they were designed such that the forward primer is upstream of the gRNA cut site in exon 5 and the reverse primer lies downstream of the cut site (This sequence will not be present in case there is a deletion of the 10 kb segment). Therefore, the amplicon is amplified only in the case where wildtype sequence is present or in the case of heterozygous deletion and not in the homozygous deletion. The PCR conditions were standardized using WT cells. The melting temperatures (T_m) of the primers were calculated insilico using Sci-tools and five temperatures around T_m-3 C were chosen for standardizing the annealing temperature. The PCR reactions were carried out using rTaq DNA polymerase. The annealing temperature which gives an

intense band for desired amplicon and minimum intensities for non-specific amplicons was chosen. The PCR products were bromophenol blue dye in a 2% agarose gel. The PCR was run with a WT sample as a positive control to compare with the clones and with just ultrapure water as a negative control to check for DNA contamination in the PCR reagents. In case there was no band observed, we went on to do another PCR with established primers in the lab that amplify a region on another chromosome to confirm that the DNA is intact. The amplicons of probable clones were cut from the gel and purified using the Abzyme gel purification kit and the purified DNA was sent for Sanger sequencing using the forward primer that was used for PCR amplification. The positive clones obtained at this stage were further verified for absence of protein through western blot.

Description	Sequence (5'-3')
CIZ1_WT-PCR_FP	CTCCGTGCTTTCAATGTGAC
CIZ1_WT-PCR_RP	ACTACTCACCTTGCGATTGG

Table 2 PCR primers used to screen for CIZ1 knockout as mentioned in Rodermund et al., 2021.

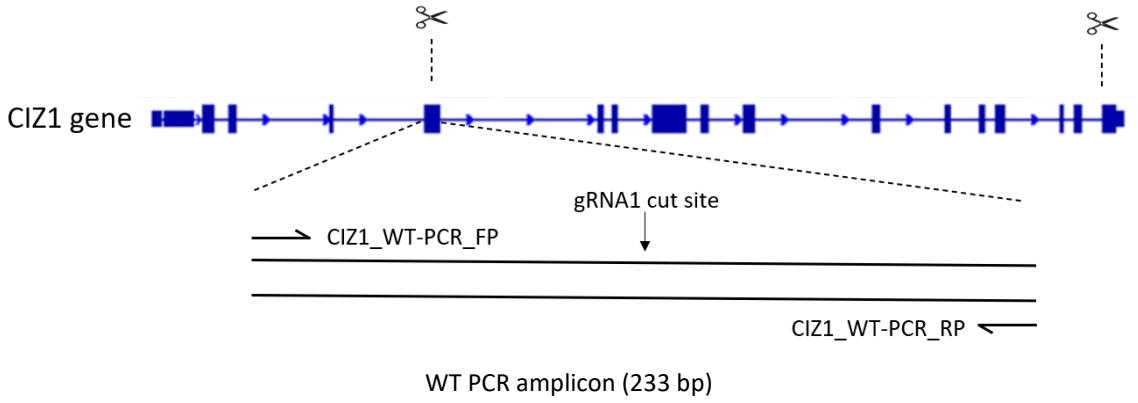


Fig 9. CIZ1 KO PCR screening strategy. The wildtype PCR strategy to screen for CIZ1 knockouts is depicted as described in Rodermund et al., 2021. A 233 bp amplicon band will be observed on an agarose gel for clones with WT genotype and this amplicon will not be present in homozygous CIZ1 KO clones.

2.2.4 Protein extraction and western blot

The positive clones from the PCR screening were validated for the absence of protein using western blot. Cells were plated in 35 mm culture dish and once the plates were confluent, the cells were used for protein preparation. Cells were pelleted at room temperature and the culture media was removed. They were given 3x PBS wash at 4 C at 5000 rpm for 5 minutes. A cell lysis buffer with 3% protease inhibitor cocktail (PIC) was prepared. PBS was removed almost completely and 110 µl of the lysis buffer was added to the cells and resuspended. 40 µl aliquots were stored at -80 C. This approximately gives the same amount of protein as the protein was prepared with approximately the same number of cells and all the proteins required for the western blot were prepared in the same batch to eliminate any batch effects.

The western blot conditions were first standardized for effective probing for CIZ1. The proteins were thawed in ice. 10 µl of 5x SDS loading dye (1% v/v of 1M Tris-base pH 6.8, 40% Glycerol, 5% β-mercaptoethanol, 12.5% w/v SDS, 0.1% w/v Commasive blue G250) was added and the tubes were heated at 95 C for 10 minutes for denaturation. These denatured proteins were loaded into 10% SDS PAGE. Wet transfer was done using PVDF membranes at 90 V for 2 hours. 5% milk powder in 0.1 % TBST was used for blocking for 1 hour. The primary antibody for CIZ1 was of rabbit origin (Abclonal, Cat #A17349) and the one for beta-actin was of mouse origin. They were incubated at 4 C overnight. The washes were done using 1x TBS three times for 6 minutes each. The blots were developed using ECL and were imaged using Biorad chemi doc.

2.3 Generation of CIZ1 knockdown XEN cells

2.3.1 shRNA design and plasmid extraction

We chose an shRNA which was already present in a library against human CIZ1. This was obtained commercially at the Indian Institute of Science. We checked for sequence match and we chose the shRNA which matched the mouse CIZ1 sequence completely and the one which had minimal off target effects. It targets a region in exon 9 that is present in all the desired isoforms of CIZ1 that play a role in XCI. These were cloned using a pLKO.1 vector into bacterial and we received the

bacterial plates. Single colonies were cultured and plasmids were extracted from the cultures.

Description	Sequence (5'-3')
CIZ1_shRNA	CCGGCCTCCAGTTCTTCTGCTACATCTCGAGATGTAGCAGAAGAACTGGAGGTTTTTTG

Table 3 shRNA sequence used for CIZ1 knockdown

2.3.2 Preparation of lentiviral particles

The shRNA was present in the pLKO.1 vector that consists of a 5' to 3' long terminal repeat sequence that includes the shRNA sequence that will be packaged in the viral particles. This along with two other helper plasmids: 1) psPAX2 which helps in packaging of the viral particles and 2) pMD2.G that consists of genes that code for the envelope proteins; were used for transfection. We used around 1.4 µg of pMD2.G vector, 2.5 µg of psPAX2 vector and 2.5 µg of pLKO.1 vector for transfection with turbofect and it was done similar to the transfection procedure mentioned above. These were transfected into HEK293T cells at around 70-80% confluency with XEN media, in a 60 mm plate. The media was changed post 12 hours of transfection and the cells were left for 48 hours for virus preparation.

2.3.3 Transduction and screening

The exhaust media that contains the viral particles was taken and filtered using 0.45 um filter to remove any dead cells that may have come along. 12 µg/ml of polybrene was added to the filtered exhaust media. This along with 500 ul of fresh XEN media were added to healthy WT XEN cells that were plated in a 60 mm dish with a confluency of 60%. The media was changed post 12 hours of transduction to allow for integration of the shRNA construct into the genome of the XEN cells. Around 48 hours post media change, puromycin selection was done at 3 µg/ml concentration for a total of 60 hours.

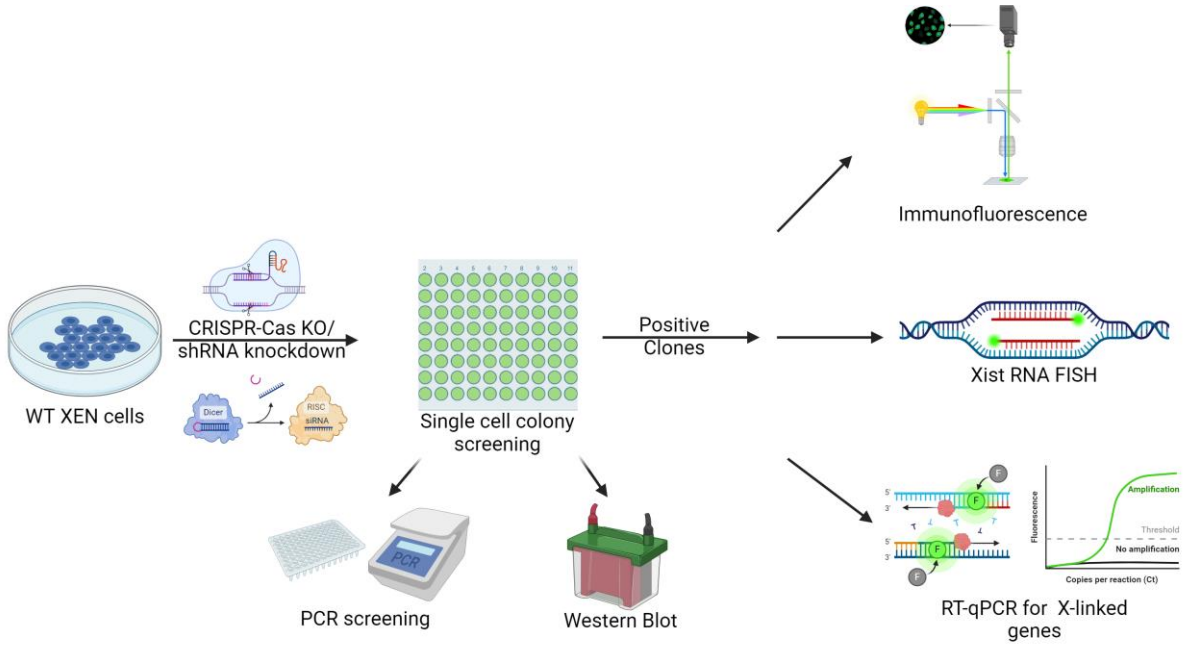


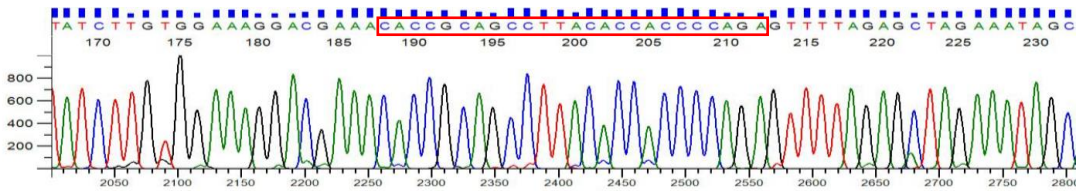
Fig 10. Workflow of the project. Firstly, we generate a KO or a knockdown for CIZ1 in XEN cells. Secondly, we test for Xist localization in CIZ1 KO or knockdown cells and investigate the inactivation status of Xi linked genes using RT-qPCR.

Chapter 3 Results and Discussion

3.1 Three positive clones were obtained by PCR screening

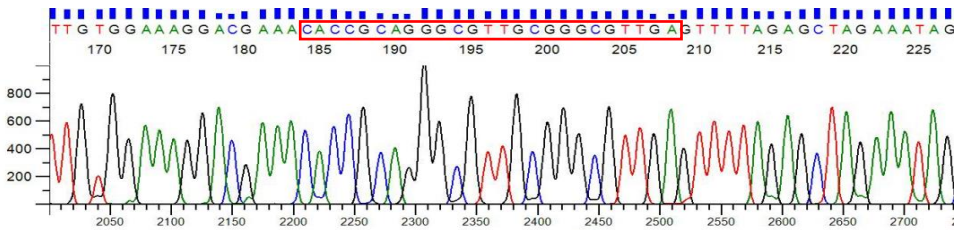
The gRNAs were cloned into the PX459 vector as confirmed by Sanger sequencing as shown in Fig 8, with clean peaks without noise. During the PCR screening, we obtained four single cell clones that produced bands that appeared slightly below the WT amplicon size as shown in Fig 9a. We were interested in these clones and thought that these could be the desired clones because an article published by Sunwoo et al. 2017, shows that a 16 bp deletion in the exon 5 of ciz1 genes leads to a frameshift that results in absence of CIZ1 protein. This is because exon 5 is present in at least all the known desired spliced variants that we wanted to eliminate. Therefore, we purified the amplicon and performed Sanger sequencing. We aligned the WT amplicon sequence with the clone amplicon sequence using the T-coffee alignment tool (Notredame et al., 2000) and we found that there was a frameshift deletion in all the expected clones as shown in Fig 9b. The sequence that previously resulted in the absence of CIZ1 protein also is shown in Fig 9b. The number of bases deleted varied across the clones and we observe that two of the three clones have the exact same deletion as previously published. A total of 15 single cell clones were screened. We sequenced the amplicons in duplicates to confirm the deletion.

a



gRNA 1

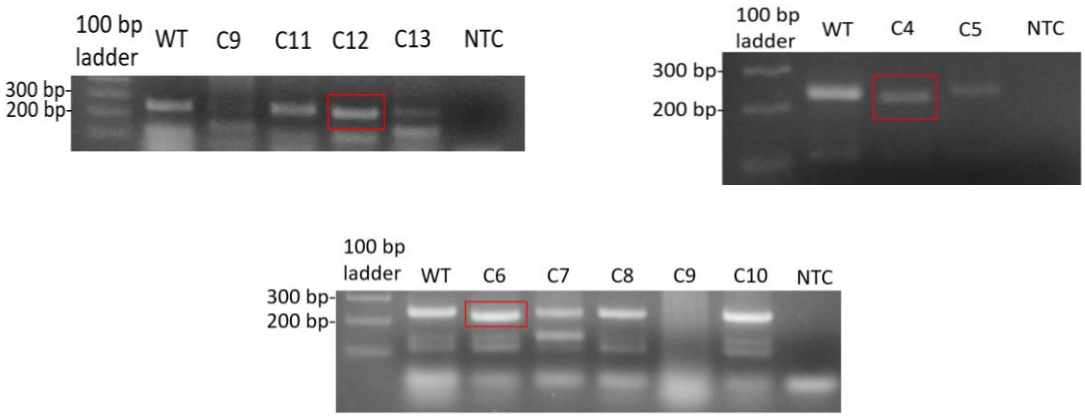
b



gRNA 2

Fig 11. Sequencing results of gRNA cloning. The sanger sequencing of a) gRNA 1 and b) gRNA 2 are shown. The peaks are clean with no noise and the gRNA sequences are highlighted in the red box.

a



b

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WT          CAGCCTTACACCACCCCAGATGGTCACCCCAAATCTGCAGCAGTT
C4          CAGCCTTA----------------CACCACACATCTGCAGCAGTT
C6          CAGCCTTT----------------AACCCCAAATCTGCAGCAGTT
C12         CAGCCTTACACC------------ACCCCAAATCTGCAGCAGTT
CIZ1_KO     CAGCCTTA----------------CACCCCAAATCTGCAGCAGTT

cons        *******      ***  **  **********
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Fig 12. Three positive clones were obtained from the PCR screening. a) Shows the agarose gel images of the PCRs performed on the clones and the bands that appear a little lower than the WT and that were sent for sequencing are highlighted in a red box. b) Shows the alignment of the sequences of WT, the three clones that incur a frameshift deletion in exon 5 and the sequence that previously yielded the absence of CIZ1 protein. CIZ1_KO represents the frameshift deletion that resulted in no protein previously, in mESCs.

Moreover, when we analysed the in-frame codons that appeared downstream of the deletion, there was a premature stop codon that appeared in exon 5 itself. There were no mixed peaks that was observed in the Sanger sequencing chromatogram downstream of the deletion which indicates that the deletion is probably homozygous. Here, we cannot eliminate the fact that there might be a heterozygous deletion where in one of the alleles has undergone the whole 10 kb deletion whereas the other allele had the 16 bp deletion. This is more likely to have occurred because it is quite a rare event that the same number of bases have been deleted in both the alleles upon Cas9 mediated double strand breaks. Nevertheless, in both the cases we expect a truncated or a misfolded protein to be formed. This made us to wonder whether there was a mutation that occurred in one of the alleles at the gRNA binding site because this heterozygous mutation in four clones seemed again unlikely. This might be the only possible reason if there was a heterozygous deletion because the

presence of SNPs was already checked beforehand. These four positive clones were further used to confirm the absence of protein using western blot.

3.2 CIZ1 protein was present in all the positive clones

Though we had obtained the deletions as required to prevent expression of functional protein, we could observe the presence of protein at the expected sizes in the western blot as shown in Fig 10. We were baffled by this result and we went on to troubleshoot. Firstly, we considered WT contamination in the clones. But, the intensity of the bands was similar to that of the WT ones. We also repeated the experiment with the same set of clones and also with new vials that were not cultured with WT cells at any point in time and there was no other cell culture work going on at the time of preserving those vials, in the hood that I was working in. We could still observe the bands for all the isoforms that we did not desire. Therefore, handling issues and contamination can be ruled out.

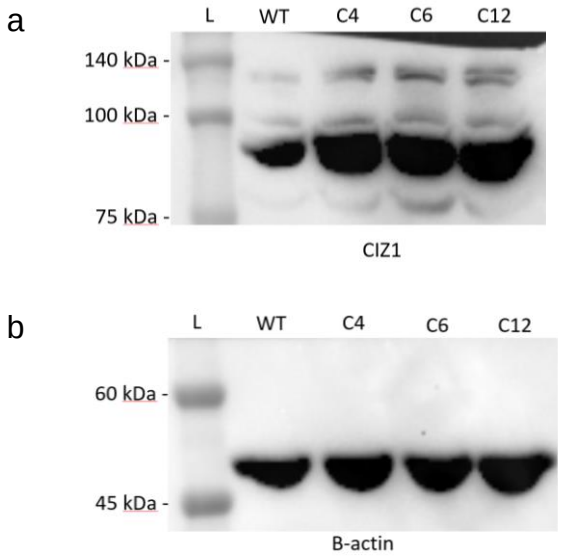

Fig 13. All the clones showed presence of CIZ1 protein. a) Shows the blot probed with CIZ1. The weight that is observed for CIZ1 is around 90-100 kDa and one band around 130 kDa and b) shows the blot probed with beta actin and the molecular weight of beta actin is around 42 kDa.

Next, we checked for antibody binding sites because it is possible that a truncated protein is expressed and that the antibody is binding to the truncated protein. The antibody was obtained by using a part of human CIZ1 peptide sequence and was

shown to be cross reactive against mouse CIZ1 as well, as mentioned in the datasheet. The immunogen peptide sequence was aligned with the mouse CIZ1 protein sequence to find the analogous immunogen sequence in mice. It was found that this immunogen sequence is encoded by the DNA sequence that is present downstream of the frameshift deletion in exon 5, in exon 7. The mRNA of the desired variants of ciz1 with the frameshift deletions obtained in the clones and these were translated insilico using the expassy tool as shown in Fig 11 (Gasteiger et al., 2003).

Fig 14. A truncated protein is made by translating the mRNA after deletion insilico. a) Shows the translation of one of the transcripts of WT CIZ1 using expassy tool. b) Shows the translation of the transcript generated post the frameshift deletion in clone 4. Clone 6 has one amino acid substitution and clone 12 has an extra amino acid compared to clone 4.

Further, the proteins that could form in the frame of WT CIZ1 with alternate start sites were aligned with the immunogen sequence. This showed a 37% identity between the two. But, if the antibody binds to these sequences, they would appear at a lower molecular weight because these proteins are significantly smaller than the WT isoforms. This was not the case and hence we moved on to the final possibility that probably the bands obtained were all non-specific which is mostly unlikely because all the bands including the ones for the isoforms appear at the expected sizes. The best way to test this out was by performing the western blot with a

negative control that is using a CIZ1 knockout cells itself. Because this was not available with us as is the case most of the time, we could not perform this experiment to confirm the non-specificity of the antibody. We also checked for SNPs or random mutations at the premature stop codon site. But we found that there were no SNPs at the stop codon site which is clear while aligning the NCBI database sequence with the WT or the clone amplicon sequences. Finally, it is also unlikely that the knockout of CIZ1 is lethal to the XEN cells because it has been shown that CIZ1 homozygous KO mice do survive till adulthood. The only explanation that we can give for this result is that probably there was a point mutation at the location of one of the PCR screening primers or both that occurred in one of the two alleles, in the parental vial during replication. This in turn resulted in the amplicon from only one of the two alleles which mislead us into thinking that the deletion has occurred in both the alleles whereas in reality, the intact protein was being made from the allele that did not get amplified with the primers. We thought about this very recently and we will test this soon. However, this requires further investigation by using a different set of PCR primers around the same region to verify the same. We also cannot rule out the non-specificity of the antibody because it is of human origin and it has been observed in our lab previously that it binds quite well to endogenous proteins. So, there is a little chance that the protein may not be very specific to CIZ1 although the bands appear at relevant positions. The best way to test this would be to have a negative control which is CIZ1 KO itself. Currently, we do not have them and we can probably test on the CIZ1 KO cells generated previously by other labs in order to confirm the specificity of the antibody. In parallel to screening the clones using different set of primers, we started with shRNA mediated knockdown of CIZ1.

3.3 shRNA mediated knockdown of CIZ1

It has been shown that the effect of CIZ1 in Xist anchorage is dose-dependent and the overexpression of CIZ1 results in the Xist dispersal phenotype similar to that observed with CIZ1 KO (Sunwoo et al., 2017). Hence, we performed the shRNA mediated knockdown of CIZ1. We prepared the lentiviral particles using HEK cells and transfected them into XEN cells. We sorted single cells into 96 well cell culture plate and currently, I am screening the single-cell clones for CIZ1 knockdown using western blot. If we obtain a knockdown, we will move on to doing immunofluorescence of CIZ1 to validate the knockdown and Xist RNA FISH to study

the Xist localization in CIZ1 knockdown cells. In case there is Xist dispersal, we would further perform RT-qPCR and RNA-seq experiments to study the reactivation status of genes in the Xi.

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