The Necessity of Geminin
for Pluripotency and the Neural Lineage

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Here I declare that my doctoral thesis entitled “The Necessity of Geminin for Pluripotency and the Neural Lineage” was written independently with no other sources and aids than quoted.

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If you eliminate the impossible, whatever remains, however improbable, must be the truth.

Sherlock Holmes
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Abstract

Pluripotent mouse embryonic stem cells (ESCs) are in vitro derivatives of the early mouse embryo. They can self-renew infinitely, and have the ability to differentiate into embryonic lineages including the germ line. ESCs have the ability to give rise to all tissues of a mouse if added early in embryogenesis. They possess a specific transcriptional network, an “open” chromatin state and a rapid cell cycle. Our research group is interested to study the link between cell fate determination and cell cycle regulation, using the Geminin protein as an anchor. Geminin plays a central role in controlling the fidelity of DNA replication, and more recently additional functions in the regulation of proteins involved in patterning and differentiation were recognized. It is highly expressed in the early embryo and in pluripotent cells, and its genetic inactivation is lethal after the first few cell divisions.

We have generated ESCs, in which the Geminin gene can be inactivated by the addition of the small molecule tamoxifen. We found that Geminin is essential for self-renewal of pluripotent cells, and that the absence of Geminin causes reduction of pluripotency markers. The loss of Geminin altered the differentiation capacity of the ESCs; they could not give rise to the neural lineage anymore, but instead differentiated into the alternative mesendodermal lineage. Geminin turned out to be redundant in differentiated, somatic cells, but was necessary for their reprogramming to pluripotent cells. We could show that its function is targeted on the Sox2 gene, a key transcription factor of the pluripotency circuit, essential for the maintenance of pluripotency. The Sox2 enhancer requires Geminin for activity, and becomes epigenetically repressed in its absence. In conclusion, we introduce Geminin as a required factor for totipotency, pluripotency and the early neural lineage.
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<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpc</td>
<td>Days post coitum</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Epiblast</td>
</tr>
<tr>
<td>ESCs</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Inactivated fetal calf serum</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IMEM</td>
<td>Isocov's modified Eagle's medium</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>loxP</td>
<td>DNA recognition site for Cre</td>
</tr>
<tr>
<td>mAG</td>
<td>monomeric Azumi Green</td>
</tr>
<tr>
<td>ME</td>
<td>Mesendoderm</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroectoderm</td>
</tr>
<tr>
<td>o/n</td>
<td>Overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphat buffered saline</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb group</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Primitive endoderm</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>siRNA</td>
<td>Small interference RNA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethyleneethyldiamin</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
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</table>
1. Introduction

1.1 Early mouse development

The totipotent zygote undergoes cleavage divisions without increasing the overall size. Next the 8-cell stage morula undergoes an increase in the intracellular adhesion known as compaction. At this stage the cells are polarized, and they are not totipotent anymore. After compaction the embryo undergoes two more rounds of division to form the 32-cell stage. During these divisions surface cells are polarized while apolar cells are inside the morula. These distinct populations of the cells have different developmental fates: cells on the outside of the embryo give rise to the Trophoectoderm (TE) lineage\textsuperscript{1}, while inside cells contribute to the inner cell mass (ICM)\textsuperscript{1, 2}. Starting from this stage, a fluid filled cavity known as the blastocoel begins to form. Within the formation of the blastocoel the mouse embryo is known as the blastocyst (Fig. 1).

ICM segregates into two distinct lineages, the primitive endoderm (PE), and the epiblast (EPI). These cells are morphologically distinct; they have a specific spatial orientation in the blastocyst and they express a distinct set of transcription factors\textsuperscript{3-5}. Epiblast gives rise to the primitive streak, and gastrulation takes place. During gastrulation, epiblast differentiates to form a multilayered structure consisting of endoderm, mesoderm, ectoderm. These germ layers contribute to the formation of the entire adult organism. ICM and epiblast cells are known as pluripotent cells, referring to their ability to differentiate into any cell type found in the adult organism.
Figure 1. Early embryonic development.

The morphological changes and cell specification events take place from fertilization to gastrulation. The cell types in the embryos are color coded (adapted after<sup>6</sup>).
1.1.1 Transcriptional control of the early lineage determination

Although zygote contains a reservoir of transcription factors and an intrinsic regulatory network, during development differences in the cell polarity or exposure to morphogens cause changes in the internal transcription factor subset of the cells leading to their fate determination and specification. Early lineage determinations (specification of TE/ICM, segregation of EPI/PE and fate determination of neuroectoderm (NE)/mesendoderm (ME)) follow such a simple rule\(^7,^8\) (Fig. 2).

**TE/ICM specification:**

Trophoectoderm and ICM are the first alternative fates of the developing embryo. Caudal type homeobox 2 (Cdx2) transcription factor is expressed in all the cells in the eight-cell stage, but its expression becomes restricted to the outer layer of cells before blastocyst formation. It was shown that Cdx2 is necessary for the development of the TE\(^9,^10\) while ICM formation is dependent on the presence of another set of transcription factors known as pluripotency factors. Octamer 4 (Oct4)\(^11\), Nanog\(^12\), and SRY-box containing gene 2 (Sox2)\(^13\) play a pivotal role in the establishment of pluripotent ICM.

**EPI/PE segregation:**

GATA family transcription factors, especially Gata4 and Gata6, are expressed in the primitive endoderm. It was shown that the primitive endoderm lineage couldn’t form properly in the absence of Gata family transcription factors\(^14,^15\) and, Gata6 overexpression in ICM can result in a higher proportion of PE cells\(^16\). On the other hand, Nanog, the pluripotency promoting protein, contributes to the formation of epiblast\(^17\). Nanog deficiency causes loss of epiblast\(^18\). Therefore, it seems proper to say Gata factors and Nanog contribute to the formation of PE and EPI lineages respectively, while inhibiting the formation of the alternate lineage.
NE/ME fate:
Like other specification events, it seems that the formation of the mesendoderm and ectoderm from the primitive streak is regulated by two transcription factors belonging to the pluripotency factors. Oct4 and Sox2 are expressed in the pluripotent cells and their co-expression contributes to the maintenance of the pluripotent state\textsuperscript{19}. \textit{In vitro} studies of the mouse pluripotent embryonic stem cells revealed that Oct4 and Sox2 play pivotal role in the early commitment of the neuroectoderm or the mesendoderm lineage, respectively. If expressed alone, these factors can drive a lineage specific differentiation. Sox2 is necessary for the formation of the neuroectoderm, and it can suppress key regulatory genes of the mesendoderm, such as brachyury. On the other hand, Oct4 drives mesodermal specific gene expression and down-regulates neuroectoderm inducing genes\textsuperscript{20}.

![Figure 2. Transcriptional regulation of fate determination during the early mouse development.](image)

Schematic representation of the lineage commitment and its transcription factor regulation (Adapted after \textsuperscript{8,21}).
1.2 Embryonic Stem cells: Pluripotency *in vitro*

Mouse embryonic stem cells were the first pluripotent cells derived from the healthy mouse embryos\textsuperscript{22, 23}. These cells resemble pluripotent cells of the early epiblast in many aspects; they can be maintained in an undifferentiated state, and they have the ability to form all three germ layers of the mouse embryo. In addition to their pluripotentiality, these stem cells can self-renew infinitely. ESCs are traditionally cultured on the feeder layer of inactivated mouse fibroblasts in the presence of fetal bovine serum and leukemia inhibitory factor (LIF). LIF maintains the self-renewal of these cells by activating the signal transducer and activator of transcription 3 (Stat3) pathway\textsuperscript{24, 25}.

ESCs express alkaline phosphatase and specific surface markers such as SSEA1 and E-cadherin. Furthermore, female blastocyst derived ESC lines possess two active X-chromosomes representing a very primitive developmental state. In mice the method used to test the pluripotency of the ESCs is the ability to form chimeric animals upon injection into the blastocyst. These cells can contribute to all three germ layers: ectoderm, mesoderm and endoderm, in addition to the germ line. ESCs can be differentiated *in vitro* to any epiblast-derived lineage or cell type upon treatment with right signaling factors at the appropriate time. Therefore, these cells provide a strong tool to study differentiation particularly in the early inaccessible stages.

1.2.1 Transcriptional control of the ESCs

Mouse embryonic stem cells resemble the early blastocyst in terms of the expression of canonical pluripotency factors, such as Oct4, Sox2 and Nanog. Oct4, the well-studied transcription factor, is exclusively expressed in totipotent, pluripotent and germ cells. This protein is necessary for formation of pluripotent cells *in vivo*\textsuperscript{26} and *in vitro*\textsuperscript{27} and for the reprogramming of the somatic cells. Oct4 inactivation in ESCs leads to the exit from self-renewal and differentiation toward the trophectoderm\textsuperscript{28}, this finding is notable since it was shown that fate determination of trophoblastic cells takes place before the formation of pluripotent
cells. Additionally in appropriate conditions, Oct4 can drive the differentiation toward mesendoderm and endoderm\textsuperscript{8, 29, 30}. The interplay between Oct4 and Sox2 is a key regulatory mechanism in the establishment of the pluripotency. These two transcription factors can co-occupy and co-activate other genes in order to maintain the pluripotent state. Sox2 is expressed similar to Oct4 during development however, its inactivation results in a developmental defect in a later stage probably due to high amounts of maternal protein\textsuperscript{19}. The third member of the pluripotency network is Nanog. Nanog is necessary for establishment of the pluripotent state and its overexpression can maintain it even in the unfavorable culture conditions\textsuperscript{31, 32}. These proteins regulate the expression levels of each other and other core pluripotency genes such as Sall4, Hdac2, Sp1, Tcfp2l1, Essrb and Klf4. This regulatory network consists of protein complexes of these pluripotency core factors, which vary in composition and size\textsuperscript{30, 33, 34}. These transcription factor complexes are found in all pluripotent cells, and they collaboratively crosstalk and govern the pluripotent state.

1.2.2 Cell cycle of the pluripotent cells

Embryonic stem cells proliferate fast and infinitely and show unusual cell cycle features. Although the duration of the S and M phase are comparable to the somatic cells, these cells possess a shortened G2 and an extremely shortened G1 phase\textsuperscript{35-37}. In somatic cells many cell cycle regulators oscillate during the cell cycle and control the length of the gap phases (G1 and G2). In ESCs these proteins oscillate subtly compared to the somatic cells; enabling the cells to have a shorter gap phase and faster cell cycle\textsuperscript{38}.

Some studies have shown that upon cell cycle perturbations or depletion of some cell cycle regulators the pluripotency markers are still up-regulated\textsuperscript{39-41} while many more concluded that a fast abbreviated cell cycle is necessary for the pluripotency of the ESCs\textsuperscript{38, 39, 42-46}. In addition, some pluripotency core factors regulate the cell cycle in the ESCs\textsuperscript{47}. Causing a coordination of the cell cycle and differentiation. The differentiating cells start to lengthen their G1 phase while they
commit to somatic lineages and express differentiation markers\textsuperscript{48, 49}. It is still unclear whether differentiation causes the cells to slow down their cycle or the perturbation of the cell cycle leads to longer gap phases and higher transcription of the developmental genes.

1.2.3 Epigenetic regulation of the pluripotent state
Chromatin, chromosomal DNA packaged with histones, plays a pivotal role in regulating gene expression and fate determination. Chemical modification of the histones (e.g., acetylation, methylation, de-methylation, and ubiquitination), DNA methylation, action of DNA-binding proteins and chromatin-remodeling enzyme complexes can modify the chromatin structure and affect the gene expression. Embryonic stem cells are known to have a unique “open” chromatin state\textsuperscript{50} which results in global transcriptional hyperactivity\textsuperscript{51}. This globally open state is maintained by multiple mechanisms, which are in a tight interaction with pluripotency core genes. In short some pluripotency transcription factors facilitate the interaction of chromatin remodeling factors to the chromatin while some pre-bound chromatin complexes recruit the transcription factors to their site of action. A well-studied example is the SWI/SNF complex. This complex which is ubiquitously found in cells, contains a cell-specific protein composition\textsuperscript{52}. In ESCs the SWI/SNF complex is characterized by the presence of the core subunit Brg1, BAF155, and BAF60. In addition some Oct4-, Nanog-, and Sox2-associated proteins include components of this complex. This complex binds to pluripotency core genes and genes, which are regulated by pluripotency core genes. It was suggested that Brg1 binding to core pluripotency genes such as Sox2, Nanog or Oct4 are “tonically” repressed in order to fine-tune their expression to the desired level\textsuperscript{53}.

Although differentiation genes are repressed strongly in the ESCs, it was proposed that ESCs favor a transcriptionally “permissive” state. Differentiation genes are bivalently marked with two regulatory signals, the activating histone 3 lysine 4 tri-methylation (H3K4me3) and the inactivating histone 3 lysine 27 tri-
methylation (H3K27me3)\textsuperscript{54}. Presence of H3k27me3 causes the repression of the genes while presence of the activating signal (H3K4me3) facilitates the fast activation of the transcription upon removal of the inactivating mark. Therefore, in these cells the interplay between the differentiation and self renewal is reflected on the chromatin in a global manner (for more see\textsuperscript{53}).

Polycomb group proteins are responsible for the inactivation of the gene expression through methylation of the histone 3 lysine 27. These are two different multi-protein complexes, the polycomb repressive complex 1 (PRC1) and PRC2\textsuperscript{55}. PRC2 consists of four core proteins: EED, Suz12, Ezh2, and RbAp46/48 and catalyzes di- and tri-methylations of the histone k27 while PRC1 is recruited to tri-methylated H3K27 and catalyzed the mono-ubiquitination of the histone 2A. These complexes are responsible for the repression of the poised differentiation genes in ESCs and contribute to the maintenance of embryonic stem cell pluripotency\textsuperscript{56, 57} (for more see\textsuperscript{58}).

1.3 Reprogramming: “climbing the mount improbable”

In 1957 Conrad Hal Waddington suggested a simple metaphor to introduce aspects of developmental biology. He considered a developmentally potent cell (like an ESCs) as a ball, which is located on the summit of a hill with uneven slopes and valleys shaped by genes and epigenetic modification of the genes. During development the ball rolls down, and the slopes guide it into certain destination points in different valleys.

This simple model explains many aspects of development and differentiation. If any terminally differentiated cell is considered as a final point in the valleys, two important aspects of differentiation can be interpreted from this model: 1- during differentiation the cells give rise to progenies with lower differentiation potential (showing that the ball always goes down), and 2- the well-coordinated interaction of many factors is necessary to reach each destination (considering the fact that each slope is made by many smaller pieces of rocks (a metaphor for genes)
oriented in a way to favor rolling down). However, the question is if the terminally differentiated cell can climb the Waddington hill up again.

It was shown that reprogramming to a pluripotent state (Waddington summit) can be achieved by nuclear transplantation, cell fusion or direct reprogramming by expression of the exogenous factors\cite{59, 60}. Takahashi and Yamanaka (2006)\cite{61} demonstrated that the overexpression of merely four transcription factors (Oct4, Sox2, KLF4 and c-Myc) can convert a terminally differentiated fibroblast into a reprogrammed pluripotent cell known as induced pluripotent cell (iPS). These four factors initiate events, leading to cell proliferation and reactivation of endogenous pluripotency genes, which activates an auto-regulatory loop to maintain the pluripotent state. The generated iPSCs resemble ESCs in the pluripotency network expression and the ability to generate chimera and germ line transmission\cite{62-64}. Subsequently it turned out that reprogramming can be achieved through overexpression of alternative combinations of pluripotency transcription factors, chromatin remodeling complexes or substitution of factors with small molecules (for a more detailed discussion see\cite{53, 65, 66}).

Figure 3. Reprogramming of fibroblasts.

Schematic representation of some important events during the reprogramming (Adapted after\cite{67}).
Although reprogramming looks surprisingly simple, but detailed mechanisms involved in the generation of the iPSCs are still unclear. What is known is that any differentiated cell can be reprogrammed, but the reprogramming efficiency is varied and hard to determine. In a classical reprogramming experiment (mouse embryonic fibroblasts transduced with viral particles coding Oct4, Sox2, Klf4 and c-Myc) the efficiency is calculated as the number of the formed iPSC colonies out of 100 used mouse embryonic fibroblasts. However, the effect of cell proliferation/cell death and the possibility of deriving two colonies out of a single fibroblast has not been considered in these calculations.

Extensive chromatin remodeling events are necessary for the reprogramming of the somatic cells. These remodelings would establish the dynamic, open state of the reprogramming and reactivate the pluripotency genes such as Nanog and Oct4. It was proposed that reactivation of the endogenous factors and re-establishment of the “open” chromatin state is the rate-limiting step defining the efficiency of reprogramming\textsuperscript{68}. 


1.4 Geminin

Geminin was initially characterized as a bi-functional molecule. A screen for recognition of proteins, which are regulated through a cell cycle dependent degradation, nominated Geminin as a cell cycle regulator, degraded by Anaphase Promoting Complex (APC) at the metaphase to anaphase transition during mitosis\textsuperscript{69}. Further investigations showed that Geminin plays a role in the regulation of DNA replication fidelity.

At the same time Geminin was identified in an expression screen for neural inducers in \textit{X. laevis}\textsuperscript{70}. It was shown that Geminin overexpression in embryos causes the expansion of neural plate due to the conversion of the ectodermal progenitors into the neural tissue instead of other cell types. In addition reducing Geminin expression results in loss of neural marker expression and formation of non-neural cells. Apparently this neuralizing effect of the Geminin is conserved between vertebrates and invertebrates such as Drosophila\textsuperscript{71}.

1.4.1 Geminin and CDT1, inhibition of re-replication

In order to maintain the integrity of the genome, each of the two daughter cells need to inherit one identical copy of the maternal genome. Therefore, a strict regulation of DNA duplication guarantees that each part of the DNA is precisely duplicated once and only once during each cell cycle. Origin licensing is one of the main mechanisms to monitor the replication integrity. During the G1 phase of a cell cycle origins of replication associate with pre-replication complexes (pre-RC). These complexes consist of the chromatin licensing and DNA replication factor 1 (Cdt1), the cell division cycle 6 homolog (Cdc6), the minichromosome maintenance complex (MCMs), and the Origin recognition complex (ORC). Together they “license” a origin of replication. During S phase, the licensed origins are recognized by the replication machinery while the relicensing of the origins is strongly inhibited. Geminin is one of several inhibitors of licensing. It needs to be absent in the G1 phase before it accumulates through the S/G2/M phases. During the S phase it binds to the released Cdt1, and inhibits its
rebinding to the origins, thus, preventing the re-launching of the duplicated origin\textsuperscript{72-74}. At the end of the M phase it is degraded by the APC/cyclosome (APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh1}) and it would not accumulate until late G1 when the APC\textsuperscript{Cdh1} is inactivated. The absence of Geminin during the G1 phase allows licensing to take place once more to prepare the genome for the next round of replication. In the late G1 Geminin levels exceed Cdt1 levels; thus, the licensing period ends before replication initiates. This mechanism ensures that every origin can fire only once per cell cycle, so that over-replication is prevented.

However, Geminin is not the only mechanism to prevent re-replication\textsuperscript{75}. In addition to Geminin, cyclin dependent kinases (CDKs) regulate the fidelity of DNA replication both directly and indirectly. CDKs down regulate the activity of the pre-RC components. Pre-RC components, ORC, Cdc6, and Cdt1, are recognized and phosphorylated by CDKs in a cell cycle regulated manner. Since all these phosphorylations prevent formation of the pre-RC, the deregulation of CDKs results in re-replication. In mammalian cells Cdk2 targets Cdt1 for destruction via the SCF\textsuperscript{Skp2} E3 ubiquitin ligase and the PCNA-dependent pathway (Cul4–Ddb1\textsuperscript{Cdt2} dependent destruction) during G2 and S phase\textsuperscript{76}. This mechanism restricts the availability of Cdt1 during S/G2 phases and contributes to the inhibition of relicensing. Thus, stabilization of Cdt1 can lead to re-replication. Cdk1 is critically necessary to block relicensing during G2 and M phases. It was shown that its inactivation will recruit pre-RC to DNA and activates APC\textsuperscript{Cdh1} leading to Geminin destruction.

In summary, a network of inhibitory pathways prevents pre-RC assembly. In every cell, these mechanisms cooperate to ensure that DNA is replicated once and only once per cell cycle\textsuperscript{75}. 
1.4.2 Geminin in cancer

In normal cells Geminin and Cdt1 levels are highly balanced and regulated in order to maintain the genomic integrity. Aberrations of this balance would cause genomic instability and DNA replication defects that predispose a cell to malignant transformations. Geminin expression is frequently deregulated in tumor cells. In addition many reports indicate that Geminin is a useful marker for prognosis prediction in colorectal cancer, pancreatic cancer, advanced intestinal-type gastric carcinoma, salivary gland carcinoma, oral squamous cell carcinoma, penile carcinoma, aggressive breast cancer and lung adenocarcinoma.

Furthermore, overexpression of Geminin in mammary epithelial cells causes the formation of aggressive tumors in immuno-deficient mice. The initiation of DNA replication in some cancer cells is solely regulated by the Geminin, whereas, non-cancer cells have additional regulatory mechanisms. Knockdown of Geminin in some cancer lines causes DNA re-replication and DNA damage, leading to apoptosis, but not in the cells derived from normal tissues. Additionally, normal levels of Geminin are necessary for the action of Topoisomerase type II alpha (TopoIIα) which acts in the termination of replication. Both depletion and overexpression of Geminin alters the action of TopoIIα causing genomic instability and replication defects.

In conclusion, Geminin expression is associated with some malignant transformations. Some transformed cells are susceptible to the loss of Geminin and undergo apoptosis in its absence. The possibility of killing some cancer cells by inhibition of Geminin activity, nominates Geminin as a worthy therapeutic target.
1.5 Geminin as a transcription modulator

Geminin has an at first sight unrelated function as a transcription or chromatin modulator. It is expressed in proliferating cells and may act in regulation of the transition from proliferation to differentiation. Accordingly, it was shown that Geminin is necessary for early development and maintenance of some differentiating lineages. Embryonic development shows a strong dose dependency to Geminin, suggesting the existence of several interaction partners, which can sense the different amounts of Geminin by competing for it.

1.5.1 Geminin in early development

Genetic ablation of Geminin in mouse results in pre-implantation mortality\(^{90, 91}\). Formation of the ICM is dependent on the Geminin in the early mouse embryo. Lack of Geminin induces endo-reduplication at the 8-cell stage leading to a developmental arrest. Remaining cells of the Geminin deficient embryo exhibit abnormal morphology and impaired cell-cell adhesion. These cells contain nuclei with abnormal shape and size, and they express trophoblastic markers. Therefore, Geminin deficient embryos lack the ability to form inner ICM. However, it is not clear whether Geminin inhibits the endo-reduplication or it regulates the balance between transcription factors necessary for formation of ICM and trophoblast (Oct4 and Cdx2) leading to a change in the cell fate.

1.5.2 Geminin and pluripotent cells

Geminin is highly expressed in the pluripotent cells\(^{35, 38, 92}\), and degraded in a cell cycle regulated manner\(^{38}\). In a recent study, Geminin was depleted in mouse ESCs and it resulted in a loss of stem cell identity and trophoblastic differentiation\(^{92}\). The same lab reports that Geminin, antagonizing SWI/SNF chromatin remodeling complex action, is necessary for the maintenance of pluripotency gene expression. However, it was extensively reported that ESC specific SWI/SNF complex is necessary for the maintenance of the pluripotent state and moderates the expression of the pluripotency genes\(^{93-96}\).
1.5.3 Geminin in neurogenesis

Geminin was initially found as a neuralizing factor\textsuperscript{70}. Partial interference with Geminin activity in Xenopus embryos resulted in a neural to epidermal cell fate change\textsuperscript{97}. In addition, Geminin is necessary to spatially restrict mesoderm, endoderm and non-neural ectoderm to their proper locations in the Xenopus embryo\textsuperscript{98}. It is necessary for neural fate determination of the ESCs, through establishment of a hyper-acetylated and open chromatin at neural genes\textsuperscript{99}. Geminin plays a role in neural fate acquisition through inhibition of the pro-neural basic helix-loop-helix (bHLH)-Brg1 interactions\textsuperscript{97}. Thus, Geminin is necessary to prevent premature differentiation and maintenance of the neural progenitors. It promotes a bivalent chromatin state at genes encoding neurogenesis transcription factors\textsuperscript{100}.

Geminin is highly expressed in Sox2\textsuperscript{+} neural progenitors of the central nervous system (CNS), and becomes down-regulated upon differentiation and cell specification\textsuperscript{101}. However, the role of Geminin in the development of the CNS is highly debated. Genetic inactivation of the Geminin in developing CNS was shown to increase the early born and decrease the late born neurons, without having an effect on the gliogenesis\textsuperscript{101}. However, other studies do not report any changes in the progression of the neurogenesis\textsuperscript{102, 103}.

In summary, Geminin is necessary for formation of the neural lineage, however, it is not necessary for further maturation of the cells. Its mechanism of action is not well understood, but it seems that Geminin interacts with chromatin remodeling complexes to form the neural lineage.

1.5.4 Geminin and hematopoietic system

Hematopoiesis system provides a well-studied system to evaluate the connection between cell differentiation and proliferation in the adult system. The blood cells are derived from the hematopoietic stem cells (HSCs), which are multipotent stem cells that give rise to all the blood cell types including myeloid (monocytes,
macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes and dendritic cells) and lymphoid lineages (T-cells, B-cells and NK-cells). Defects in the well-regulated differentiation and cell divisions of the cells can cause leukemia, myelo-proliferative disorders or marrow failure, but the factors and pathways, regulating this pattern, are not completely understood.

Geminin knockout in the hematopoietic stem cells severely perturbed the hematopoietic pattern. Stem and progenitor cell number are intact but erythrocyte production was abolished leading to severe anemia. On the other hand megakaryocyte production was enormously enhanced. *In vitro* cultured Gmnn⁻/⁻ megakaryocyte-erythrocyte precursors formed more megakaryocyte colonies while their ability to form erythroid colonies was lost. Additionally, their DNA content was normal. It was suggested that Geminin plays a role in fate determination of megakaryocyte-erythrocyte precursors by a replication-independent manner. Geminin can induce quiescence in HSCs through abrogation of their activity; therefore, its stability in HSCs is highly regulated.

1.5.5 Geminin and interaction partners

An increasing number of Geminin interaction partners was recently identified. In addition to CDT1, many new interactors with different functions support the role of Geminin as a transcription modulator. Among its interacting partners are basic transcription machinery, chromatin modulating factors (SWI/SNF complex and polycomb group proteins) and known homeodomain transcription factors (Hox and Six3).

**Interaction with basic transcription machinery:**

In order to start transcription the basic transcription machinery needs to be assembled near the promoter regions. Either TATA-box binding protein (Tbp) or TBP-like factor 1 (Tbpl1) is necessary to form this pre-initiation complex. The transcription starts with the recruitment of RNA polymerase II. During a screen for
Geminin interactors a novel protein was identified which binds also to Tbp and Tbpl1. This protein, Tipt (TATA-binding protein-like factor-interacting protein), was shown to activate transcription both from TATA-box-containing and from TATA-less promoters\textsuperscript{107}.

**Interaction with the chromatin remodeling factors:**

In certain developmental contexts Geminin interacts with members of the polycomb group proteins. The clustered Hox genes, encoding the homeodomain proteins of the hox family, are expressed in a well-regulated, spatiotemporal collinear manner along the anterior-posterior axis of the early embryo. It was shown that Geminin could bind to Hox genes in order to prevent their interaction with the DNA. In the same context Geminin interacted with the PcG protein, Scmh1 to regulate the transcription of the Hox genes during axial patterning\textsuperscript{108}. Geminin activity in restraining the commitment of the mesoderm, endoderm, and non-neural ectoderm depends upon the intact polycomb repressor functions\textsuperscript{98}. In addition Geminin can regulate the transition from neural precursors to neurons through its binding to the Brg1. During early induction of the neuroectoderm in chicken embryos, Geminin interacts with Brm, another core subunit of the SWI/SNF complex\textsuperscript{109}.

In summary, the functional diversity of Geminin interaction partners suggests a significant role for Geminin in coordination of cell cycle pace with the fate determination.
1.6 Aim of the thesis

This study has tried to shed light on the regulatory role of the Geminin in the embryonic stem cells by application of an inducible Geminin knockout ESC line (iGmnn ESC). The role of Geminin in self-renewal of the ESCs, their pluripotency and lineage commitment was of interest. Geminin’s effect was explored at a molecular level in order to gain an insight into its mechanism of action. In addition Geminin deficient somatic cells and their ability to reprogram into the pluripotent cells was investigated. In short, this study aimed to characterize the necessity of Geminin for pluripotency, neural induction and reprogramming of the somatic cells.
2 Results

2.1 Geminin protein is expressed in embryonic stem cells and is down regulated during differentiation

At first, the Geminin levels in the wild type MPI-II mouse embryonic stem cells (MPI-II ESCs) and their differentiated progenies were investigated. ESCs were differentiated as embryoid bodies (EBs), and after 5 days they were plated on the adhesive plates for further differentiation and analysis (Fig. 4A). Whole cell lysate protein analysis showed that undifferentiated ESCs expressed Geminin strongly but Geminin protein levels decreased upon EB differentiation (Fig. 4B). Data showed a down-regulation of the Oct4 mRNA, however, the levels of Geminin mRNA did not decrease (Fig. 4C). Further mRNA level quantification by quantitative RT-PCR (data not shown) revealed no significant difference in the amount of Geminin mRNA. In short, Geminin was expressed strongly in ESCs and its protein levels were decreased upon differentiation.

Figure 4. Geminin is down-regulated in mouse ESCs upon differentiation.

A) MPI-II ESCs were differentiated for 5 days as EBs followed by re-plating in adhesive culture plates for 4 more days to form the differentiated monolayer cultures. B) Whole cell lysates were harvested and analyzed by western blot. The amount of the loaded protein was controlled by α-tubulin amounts. C) ESCs, 5 days old EBs, and 9 days old Monolayer cultures (ML) were analyzed for Geminin and Oct4 mRNA by semi-quantitative RT-PCR. The amount of loaded mRNA was controlled by Gapdh amounts. Data represented in this figure was prepared in collaboration with Judith Schilling during her bachelor thesis project.
2.2 Geminin protein is expressed in embryonic stem cells and the neuroectodermal progenitors

In order to investigate the expression pattern of Geminin in different embryonic lineages such as ectoderm, mesoderm or endoderm, ESCs were differentiated to specific lineages (Fig. 5A). ESCs were plated in low density for 48 hours in the absence of LIF and serum in N2B27 medium and afterward they were differentiated for 36 more hours particularly toward mesendoderm (ME) or neuroectoderm (NE) by treatment with a Wnt agonist named chirion or Retinoic acid (RA). Expression patterns of Sox1, a neural specific transcription factor, and Brachyury, a mesendoderm specific transcription factor, serve as a control for the efficiency of differentiation. As shown in the Fig. 5B the majority of the cells differentiated toward ectoderm or mesendoderm. Further immunofluorescence analysis showed that pluripotency transcription factors Oct4 and Sox2 were present in the ES cells however, upon differentiation their presence was restricted only to a specific lineage; neuroectodermal progenitors expressed Sox2, while mesendodermal progenitors expressed Oct4 as reported previously. In order to investigate Geminin protein levels, whole cell lysates from the ESCs, NE and ME were analyzed by western blot (Fig. 5C). Nanog and Klf4 were used as specific markers for pluripotent cells. These two transcription factors were only observed in pluripotent undifferentiated ESCs and their absence in the mesendoderm and neuroectoderm confirms the absence of the pluripotent cells. Oct4 was higher in ESCs and ME and its levels decreased in the NE. Sox2 was high in ESCs; it was present in the NE and decreased strongly in the ME. Sox1 and Brachyury were found in a lineage-specific manner depicting a high differentiation specificity of the cultures. Immunoblot staining of Geminin revealed that it was present in ESCs and neuroectoderm progenitors, and was down regulated in mesendodermal progenitors (Fig. 5C).
Figure 5. Geminin is down-regulated in the mesendoderm, but not in the neuroectoderm.

A) MPI-II ESCs were differentiated on gelatin-coated plates in the absence of serum for 48 hours and then exposed to RA or Chirion, in order to differentiate the ES cells to neuroectoderm (NE) and mesendoderm (ME), respectively. B) Undifferentiated ESCs, NE and ME were analyzed with immunofluorescence staining of pluripotency markers (Sox2 and Oct4), lineage specific markers (Sox1 and Brachyury). C) Western blot analysis of pluripotency markers (Klf4, Nanog, Sox2 and Oct4), lineage specific markers (Sox1 and Brachyury) and Geminin. Histone 2B levels were shown for control.
2.3 Geminin is degraded upon G1 entrance in the ESCs

Geminin is recognized by the degradation machinery of the somatic cells upon entry into G1. However, it was not clear if Geminin is degraded in a cell cycle-regulated manner in the pluripotent cells or not; therefore, it was interesting to study the dynamics of Geminin protein during the cell cycle of the ESCs. In order to visualize the cell cycle transitions in somatic cells, two sensor proteins were designed and applied. These sensor proteins included fluorescence proteins tagged with destruction sites of the proteins, which were degraded in some phases and present in the rest of the cell cycle\textsuperscript{110}. One of these sensors known as mAG-hGem (1/110) coded a chimeric protein composed of mAG (monomeric version of Azumi Green) and the 110 amino acids N-terminus of human Geminin containing its destruction box (Fig. 6A). It was transiently overexpressed in order to visualize Geminin degradation in wild type ESCs.

mAG-hGem fusion protein does not interfere with the cell cycle progression of the transfected cells however, it is recognized by destruction machinery of the cell causing elimination of the fluorescence signal. Therefore, it can be applied to visualize the phases in which the endogenous Geminin escapes degradation.

Total population of the cells was harvested and stained with propidium iodide in order to visualize their DNA content. Flow cytometric analysis of these cells revealed that more than 35% of the cells expressed Azumi Green, meaning that at least 35% of the population received the transfected mAG-hGem coding plasmid. Comparison of the whole population and Azumi Green positive fraction illustrated a change in the shape of the cell cycle distribution depicting a loss of the G1 phase, which resulted in a significant enrichment toward S/G2/M phase in the Azumi Green expressing fraction (Fig. 6B).
Figure 6. Down-regulation of mAG-hGem in G1 phase of the ESC cell cycle.

A) A fusion of human Geminin degradation site and a green fluorescent protein is used to visualize the cell cycle. B) ESCs were transfected with mAG-hGem were analyzed by flow cytometry in order to measure the red (DNA content) and green fluorescence content of the cells. Analysis showed that more than 35% of the cells were positive for green signal. The samples were gated for green fluorescence positivity and the DNA content of the positive population was compared against the DNA content of the whole population. DNA content distribution of one sample was shown on the left panel and the quantification of 3 independent experiments are demonstrated on the right panel.

However, mAG-hGem visualization did not exclude the possibility of in vivo inhibition of the Geminin degradation during cell cycle of the pluripotent cells. To exclude this possibility, the endogenous Geminin protein level in a synchronized ESC population was investigated. ESCs were synchronized in the M phase and after release into fresh medium cells were collected at different time points for further analysis (Fig. 7A). Flow cytometric analysis of the PI stained cells revealed an efficient synchronization. Cells were arrested in the M phase and the 80% of the cells entered the G1 phase after 90 min (Fig. 7B).

Whole cell lysate protein analysis revealed a decrease in the Geminin protein upon entry into G1 phase observed at the 90 min to 120 min after the release (Fig. 7C). This observation clearly indicated that degradation machinery of
pluripotent cells recognizes and degrades endogenous Geminin upon entrance into the G1 phase in pluripotent ESCs.

**Figure 7.** Endogenous Geminin is degraded upon G1 entrance in mESCs.

**A)** ESCs were cultured in feeder free cultures in ES-CM+2i medium, synchronized with 14 hours thymidine and 6 hours TN-16 treatment to accumulate in M phase. The cells were then released into the fresh medium and at the indicated time points after release the cells were harvested, trypsinized, fixed and stained with propidium iodide. Untreated asynchronous ESCs were used as control. **B)** The cell cycle distributions of cells were determined by flow cytometry. **C)** At the same time points whole cell lysates were collected for western blot analysis of Geminin controlled by levels of tubulin protein (async: asynchronous, sync: synchronized= 0 min).
2.4 Conditional inactivation of Geminin locus

As discussed previously, Geminin null embryos die at the 8-cell stage. To study the Geminin in pluripotent ESCs a conditional knockout strategy was applied. A conditional Geminin knockout mouse line was previously established, in which Gmnn exon 2 and 3 where flanked by loxP sites\textsuperscript{102}. This floxed allele is designed and depicted as "Gmnn\textsuperscript{fl}". Upon Cre expression in the same cell, the Gmnn\textsuperscript{fl} was excised to generate a Gmnn\textsuperscript{-}, unable to produce a functional potein (Fig. 8).

![Diagram of Geminin targeting construct](image)

**Figure 8.** Targeting strategy to generate Geminin conditional knockout allele.

Two LoxP sites were inserted in the first and third introns of Geminin genomic locus upon site-specific recombination. The floxed allele possesses exon 2 and 3 flanked by LoxP sites and upon Cre mediated recombination exons 2 and 3 are excised. Thus the remaining conditional knockout allele loses its ability to produce functional protein (adapted after\textsuperscript{102}).

The Gmnn\textsuperscript{fl/+} mice were mated to CMV-Cre\textsuperscript{+} transgenic animals and Gmnn\textsuperscript{fl/+} CMV-Cre\textsuperscript{+} progeny were further bred with wild type animals to obtain Gmnn\textsuperscript{-/+} progeny. The heterozygous animals were further mated to each other in order to obtain knockout animals. Genotyping of 131 born animals (16 litters) confirmed an embryonic lethality of the Geminin null embryos (Fig. 9). 33.5% (44/131) of the
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born animals were $Gmnn^+/-$ and 66.5% (87/131) were $Gmnn^-/-$ while no $Gmnn^-/-$ pups were born. These data clearly indicate that recombination leads to the nonfunctional knockout allele and the homozygous knockout mouse are embryonic lethal as reported previously\textsuperscript{91}.

![Diagram of genetic inactivation of Geminin leads to embryonic lethality.](image)

**Figure 9.** Genetic inactivation of Geminin leads to embryonic lethality.

Mating scheme for the generation of the $Gmnn^-/-$ mice.
2.5 Establishment of iGmnn ESCs

To investigate the role of Geminin in ESCs, the iGmnn ES cell line was established. Geminin transgenic mice were bred with the tamoxifen-inducible Cre recombinase coding strain\(^{111}\) (\(ER\text{-}Cre^+\)), in which, the recombination reaction can be triggered through administration of tamoxifen in vivo or 4-hydroxyl tamoxifen in vitro (Fig. 10A). Blastocysts were harvested and plated on the feeder-coated culture plates to outgrow to ESCs. The established ESC lines were genotyped and one line with the desired \(Gmmn^{fl/fl}\) \(ER\text{-}Cre^+\) genotype was selected. This line was named “iGmnn” and was further characterized to confirm its pluripotency. Morphology, alkaline phosphatase activity and the expression of pluripotency markers, Oct4, Sox2 and SSEA1 represented a pluripotent state (Fig. 10B). iGmnn ESCs were differentiated as EBs in order to investigate their differentiation potential (Fig. 10C). Immunofluorescence analysis of differentiated iGmnn ESCs revealed that they were able to give rise to all three germ layers upon EB differentiation shown by Sox1, Sox17 and Brachyury markers for the ectoderm, endoderm and mesoderm respectively (Fig. 10D). RT-PCR analysis of ESCs, 5 days old differentiated EBs, and 9 days old differentiated monolayer cultures showed a down regulation of the pluripotency markers Nanog and Zfp42 (also known as Rex1) and up-regulation of lineage specific markers such as Pax6 (ectoderm), Brachyury (mesoderm) and HNF4a (endoderm). Thus, iGmnn ESCs were able to differentiate into all three embryonic lineages.

Upon injection into 8-cell stage embryos, iGmnn ESCs were able to give rise to chimeric mice (7 chimeric animals out of 41 born animals, Fig. 10F), and the chimeric mice gave birth to iGmnn ES derived animals (3 out of 4 litters, Fig. 10G). Genotyping (data not shown) and agouti coat color of the pups showed that the iGmnn ESC are germ line competent. These characteristics demonstrated that the iGmnn ESCs are a fully pluripotent cell line, and can be used for further investigation of the role of Geminin in the ESCs.
Figure 10. iGmnn ESCs are pluripotent.

A) mating schema for the generation of the iGmnn ESCs. B) The established iGmnn ESCs were positively stained for alkaline phosphatase activity and they were immuno-stained for the pluripotency markers (Sox2, Oct4 and SSEA1). C) iGmnn ESCs were differentiated for 9 days as floating EBs in petri dishes followed by re-plating on tissue culture plates. D) Differentiated iGmnn ESCs were immuno-stained for lineage specific differentiation markers (Sox1: ectoderm marker, Brachyury: mesoderm marker and Sox17: endoderm marker). E) iGmnn ESCs, 5 days old EBs, and 9 days old monolayer were analyzed by RT-PCR for the expression of the pluripotency markers (Nanog and Zfp42) and differentiation markers (Pax6: ectoderm marker, Brachyury: mesoderm marker and HNF4α: endoderm marker). Gapdh was used to control the amount of loaded mRNA. F) iGmnn ESCs were able to give rise to chimeric mice (white with agouti patches) upon injection into C57Bl6/N blastocysts. G) An agouti animal (marked by arrowhead) is a progeny of an iGmnn ESC-derived chimeric female and a black male.
Next, the efficiency of the recombination of the floxed allele with tamoxifen was tested. The iGmnn ESCs, cultured in ES-CM, were treated with 4-hydroxyl tamoxifen. At different time points, the cells were harvested and analyzed for genomic recombination and the loss of Geminin protein. Genotyping PCR revealed 80% recombination as soon as 24 hours of tamoxifen exposure increasing by the length of the exposure (Fig. 11A). Western blot analysis of the whole cell protein extracts revealed 90% loss of Geminin protein after 48 h of tamoxifen treatment increasing to more than 95% at 96h (Fig. 11B).

Figure 11. iGmnn ESCs lose Geminin upon tamoxifen treatment.

A) iGmnn ESCs were cultured for 72 hours in ES-CM and were treated for different periods of time with tamoxifen. The genomic DNA was extracted and genotyped. Different combinations of primers in separate reactions were used to amplify the floxed and recombined knockout alleles. The same amount of genomic DNA was used for each reaction. B) iGmnn ESCs were cultured for 96 hours in ES-CM, and were treated for different time periods with tamoxifen. The whole cell lysates were harvested and analyzed by western blotting.
2.6 Geminin is necessary for the self-renewal of the ESCs

To investigate the role of the Geminin, the iGmnn ESC, cultured in ES-CM were treated with tamoxifen. After 48 h, the cells were trypsinized and plated on feeder-coated plates in ES-CM. After a few days, the formed colonies were subcloned and expanded. 21.3% of the formed colonies (29/136) were non-recombined $Gmnn^{fl/fl}$ and in 78.7% of them (107/131) one allele was recombined but no colony exhibited the complete recombination of both alleles (Fig. 12A).

![Diagram A](image)

**Figure 12.** Geminin is essential for the self-renewal of ESCs.

A) iGmnn ESCs were treated with tamoxifen and were trypsinized into single cells. The cells were grown on feeder-coated plates in order to give rise to single-cell derived colonies. These colonies were expanded and their genomic DNA was extracted. Genomic DNA samples from the grown ESC colonies were analyzed by genotyping PCR. B) Three partially recombined $Gmnn^{fl/+}$ colonies were re-exposed to tamoxifen and trypsinized into single cells. The cells were grown to give rise to single-cell derived colonies. These colonies were expanded and genotyped by PCR.
This led to the conclusion that Geminin is necessary for the self-renewal of the pluripotent ESCs. To exclude the possibility of incomplete recombination we treated 3 of heterozygous $Gmnn^{fl/-}$ formed colonies with tamoxifen once more. The cells were trypsinized onto single cells and the single cells were plated in order to form colonies. Genotyping of 59 new colonies revealed that these colonies were all $Gmnn^{fl/-}$ and no $Gmnn^{-/-}$ ESC lines were formed (Fig. 12B). These data indicated that although recombination efficiency is more than 80% after 48 h of tamoxifen treatment (Fig. 11) but the Geminin deficient cells are unable to self-renew and form colonies.

2.7 ESCs lose pluripotency markers upon loss of Geminin

Lack of Geminin causes ESCs to lose their self-renewal ability. However, it was unclear what happens to the Geminin deficient ESCs. Do they undergo a cell cycle arrest, or they differentiate and lose their pluripotent state?

The iGmnn ESC were treated with tamoxifen for 48 h (iGmnn/48hTx) in the ES-CM on the feeder layer (Fig. 13A). 48 h treatment is enough to efficiently recombine the genomic loci and abrogate the protein expression while the cells are not passaged or re-plated, thus the fate of the Geminin deficient cells can be determined. The iGmnn/48hTx cultures were stained for the alkaline phosphatase activity and were compared to the iGmnn cultures. Alkaline phosphatase activity is high in pluripotent cells, and it diminishes upon differentiation, therefore, it distinguishes the dome-shaped dark-blue undifferentiated from flattened light-blue differentiated colonies. After tamoxifen treatment, colonies were morphologically categorized either as differentiated (Fig. 13B: shown by white arrowheads) or ES-like undifferentiated (shown by black arrowheads). Quantification of colonies depicted a dramatic increase in the number of differentiated colonies after tamoxifen treatment. It seems that loss of Geminin triggers the differentiation of ESCs. Therefore, although the culture medium provides the signals necessary for the maintenance of the pluripotent state the recombined cells are not pluripotent anymore. In addition, after
tamoxifen treatment the number of formed colonies reduced slightly (Fig. 13B). Immunostaining of Geminin confirmed that there is a mutual relationship between Geminin expression and the ESC-like colony morphology. In the iGmnn/48hTx cultures the few remaining Geminin positive colonies showed a dome-shaped ES-like morphology and all the colonies with the flattened differentiated morphology were not stained for Geminin. Additionally, this staining revealed that the few remaining undifferentiated colonies in the tamoxifen treated cultures are the unrecombined colonies (Fig. 13C).

Quantitative RT-PCR analysis of the iGmnn/48hTx and iGmnn/72hTx ESCs showed a down-regulation of Geminin as measured by two different primer pairs. In addition, Geminin inactivation abolishes the expression of pluripotency markers such as Nanog, Zfp42, and Sox2 (Fig. 13D). Notably however, in the iGmnn/48hTx, Oct4 levels remained as high as its levels in pluripotent cells. Same results were observed when cells were stained for Oct4, Sox2 and Nanog protein (Fig. 13E). In contrast to the packed dome-shaped morphology of the pluripotent colonies the tamoxifen treated cells exhibit the flattened morphology. Colonies are flattened and dispersed, and the cells resemble differentiated cells. The cells are depleted from Sox2 and Nanog, however, they express Oct4 at a level similar to the untreated pluripotent cells.
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A

![Diagram showing ES-CM Tx process from iGmnn ESC to iGmnn/48hTx ESC](image)

B

![Bar graph showing percentage of differentiated and undifferentiated cells with Tx](image)

C

![Images comparing Gmnn and Tx](image)

D

![Graphs showing fold increase of Gmnn-1, Gmnn-2, Zfp42, Nanog, Oct4, and Sox2 with 48h and 72h Tx](image)

E

![Images comparing Nanog, Sox2, and DAPI under - and Tx conditions](image)
**Figure 13.** Geminin is essential for the pluripotent state of ESCs.

A) iGmnn ESCs were treated with tamoxifen for 48h (iGmnn/48hTx). B) iGmnn ESCs were stained for Alkaline Phosphatase activity. The colonies were quantified according to their AP staining and morphology. On the right side, the two bright field images of AP-stained ESC colonies show examples of undifferentiated (black arrow heads) and differentiated (white arrow heads) colonies. C) iGmnn ESCs immunostained for Geminin. D) iGmnn ESCs were harvested for RNA extraction and mRNA was analyzed by quantitative RT-PCR. E) iGmnn ESCs were treated with tamoxifen for 48h and immunostained for pluripotency markers. The white bar represents 100 μm.
2.8 Geminin knockout cells exhibit a slightly lengthened G1 phase

iGmnn/48hTx cells showed a differentiated morphology and loss of pluripotency transcription factor network. However, it was interesting to see if these cells undergo a cell cycle arrest or exhibit cell cycle perturbations. In order to investigate their cell cycle distribution, cells were cultured on gelatin-coated plates in the absence of feeder cells. After tamoxifen treatment the cells were trypsinized and harvested for flow cytometry. DNA content of the cells was visualized with propidium iodide and the cell cycle distributions of the cells were graphed (Fig. 14). Analysis of flow cytometry data detected a slight increase in the number of G1 phase cells (25% to 28%) at the expense of G2/M phase. However, there were no evidences for cell cycle arrest in iGmnn/48hTx cells. These cells proliferate slightly slower (Data not shown) which may indicate that their cell cycle is longer than iGmnn cells.

Figure 14. Geminin deficient ESCs possess a lengthened cell cycle.

iGmnn ESCs were treated with tamoxifen for 48 h and prepared for flow cytometry of DNA content. The chart represents the cell cycle distribution of the cells.
2.9 Geminin is necessary for commitment to the neural lineage

Geminin was necessary for the self-renewal of the pluripotent cells and, lack of Geminin would cause the ESC to differentiate. However, it was interesting to know whether lack of Geminin would affect the differentiation of the ESC. In order to investigate this, the cells were plated on the gelatin-coated plates in differentiation medium, which allows the cells to differentiate and does not support the self-renewal of pluripotent cells. After 96 h the iGmnn/96hTx and iGmnn cells were immunostained for mesendoderm progenitor marker, Oct4, and the neuroectoderm progenitor marker, Sox2 (Fig. 15A).

![Figure 15](image)

**Figure 15.** Geminin deficient ESCs give rise to less Sox2 positive neuroectoderm progenitors.

A) iGmnn ESCs were differentiated in the differentiation medium and treated with tamoxifen for 4 days. B) The differentiated ESCs were immuno-stained for Sox2 and Oct4 and quantified. C) The differentiated ESCs were immuno-stained for differentiation markers and quantified (Sox1: neural lineage, Brachyury: mesoderm, Sox17: Endoderm, Gata4: primitive endoderm).

Quantification of the stained cells revealed that tamoxifen treatment caused a statistically significant (p-value=0.0152) increase in the number of the Oct4 positive cells accompanied with a significant (p-value=0.0109) decrease in the...
number of Sox2 positive cells (Fig. 15B). In conclusion, Geminin deficient ESCs lost their Sox2 expression but not their Oct4 expression. Thus these cells tend to differentiate to mesendoderm rather than neuroectoderm. Next, the differentiated cultures were stained for Sox1, Brachyury, Sox17 and Gata4, markers for neural lineage, mesendoderm, endoderm and primitive endoderm respectively. Correspondingly, an increase in the Brachyury positive cells but no change in the number of other cell types was observed (Fig. 15C).

Additionally the ability of the cells to differentiate specifically into neural lineage was tested. The cells were plated in a low density on gelatin-coated plates in a chemically defined default medium (DDM) and were differentiated for 12 days (ref). Low density would abrogate the autonomous signaling and absence of serum and particularly BMP factors would let the intrinsic pathways of neural induction to be activated. ESCs would first differentiate into neural progenitors, which later give rise to neuronal progenitors. After 12 days the neuronal progenitors were re-plated onto laminin/poly-L-ornithine plates in N2B27 medium to give rise to neurons, oligodendrocytes and astrocytes (Fig. 16A). Immunofluorescence staining of the cells illustrated this gradual commitment and specification in iGmnn ESCs (Fig. 16B). At day 0 the undifferentiated ESCs express oct4 and E-cadherin. During the course of differentiation the colonies expand and epiblast like cells form. These cells still express many pluripotency markers such as Oct4 and SSEA1. At day 5, early neural progenitors are already formed and can be detected with Pax6 and Sox1 expression. Later these neural progenitors give rise to neuronal progenitors and neurons, detected with nestin and Tuj1 expression. After the neurogenesis has taken place, several astrocytes marked by GFAP expression arise in the cultures.
Figure 16. Geminin is necessary for the differentiation of ESCs to the neural lineage.

A) iGmnn ESCs were cultured in feeder free conditions and were plated on gelatin-coated dishes in DDM medium for 12 days, and then re-plated into poly-L-lysine and laminin coated plates in N2B27 to further differentiate. Immunostaining of pluripotency markers (Oct4, E-cadherin, SSEA1), neural markers (Pax6, Nestin and Sox1), neuronal marker (Tuj1) and astrocyte marker (GFAP) at different time points is shown. B) iGmnn ESCs were differentiated for only 12 days as described. Whole cell protein was harvested every 48 h and immunoblotted for Geminin and Sox1 controlled by the amounts of α-Tubulin. C) iGmnn ESCs were differentiated to neural lineage in the presence and absence of tamoxifen for 4 days and stained for TUNEL activity. Genomic DNA was stained with DAPI.
Whole cell protein analysis of these differentiating cells (Fig. 16B) revealed that although during neuronal differentiation, ESCs lose their Geminin levels but the intermediate neural progenitors (D6-D8) still contained Geminin. To investigate the necessity of Geminin expression during neural differentiation, iGmnn ESCs were differentiated specifically toward neural lineage and were treated with tamoxifen. Analysis of the 4 days old differentiated neural progenitors revealed that upon tamoxifen treatment apoptosis is highly up-regulated causing a population-wide cell loss (Fig. 16C). In summary, Geminin deficient cells cannot give rise to neural lineage and undergo apoptosis in the neural specification conditions.

2.10 Geminin regulates Sox2 expression through chromatin remodeling complexes

Previous data indicated that Geminin deficient cells lose their Sox2 expression. To investigate this regulation, the epigenetic signature of Sox2 and Oct4 genes was analyzed by chromatin immunoprecipitations (ChIPs). Cells were cultured on gelatin-coated dishes and were treated with tamoxifen. Then the cross-linked chromatin was precipitated for known regulatory chromatin modifications such as histone 4 lysine 9 di-methylation (H4K9me2), histone 4 hyperacetylation (H4ac) and histone 3 lysine 27 tri-methylation (H3K27me3) and in addition anti-histone 3 antibody was used as a positive control for the chromatin immunoprecipitation. After precipitations the DNA was extracted and analyzed by qPCR, for the presence of DNA representing previously described Sox2 and Oct4 enhancers. Sox2 and Oct4 are mainly regulated through their enhancer sequences during early mouse development and in vitro differentiation. The extracted precipitated DNA was analyzed for the abundance of regulatory epigenetic signals on Sox2 and Oct4 stem cell regulated enhancer regions (Fig. 17A).
**Figure 17.** Chromatin immunoprecipitations on regulatory regions of Sox2 and Oct4.

A) Genomic loci of sox2 and Oct4 gene. Analyzed fragments of the DNA have been marked with red bars and capital letters (A-I)(SRR1: stem cell regulatory region 1\(^{112}\), SRR2: stem cell regulatory region 2\(^{112}\), DE: Oct4 distal enhancer region\(^{113}\), PE: Oct4 proximal enhancer region\(^{114}\)).

B) Precipitated DNA was analyzed by q-PCR for the regions A-I. The charts represent the relative changes in the modifications upon tamoxifen treatment (histone 4 lysine 9 di-methylation (H4K9me2), histone 4 hyperacetylation (H4ac) and histone 3 lysine 27 tri-methylation (H3K27me3), PRC2 complex (EZH2) and SWI/SNF complex (BRG1)).

The data indicated that although the total histone 3 shows an increase in all the analyzed regions the activating signal, histone4 acetylation, and the inactivating signal, Histone 43 lysine 4 di-methylation, did not significantly change upon tamoxifen treatment. However, H3k27me3 ChIP represented a different pattern. While abundances of Histone 3 lysine 27 tri-methylation was not significantly different on the Oct4 enhancer (regions H and I) and regions A-C and G of the Sox2 gene, it showed a significant increase on the Stem cell regulatory region 2 (SRR2) enhancer\(^{112}\) (regions D-F). Polycomb repressive complex 2 is responsible for tri-methylation of the 27th lysine residue of the histone 3. According to the increase in H3K27me3 modifications, the amount of bound Ezh2 protein, which is the catalytic subunit of the PRC2 complex, increased on the same regions especially on region F. PRC2 complex and SWI/SNF complex compete with each other on the pluripotency genes. Therefore, it was of interest to investigate the changes on the abundances of SWI/SNF complex on the enhancer regions of Sox2 and Oct4. Data indicated that Brg1, the core component of SWI/SNF complex dissociates from the SRR2 in tamoxifen treated cells.
2.11 Geminin is redundant in mouse embryonic fibroblasts

In order to study the role of Geminin in somatic cells, mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos. The conditional knockout mouse were bred to the tamoxifen inducible Cre line and Gmnn<sup>fl/+</sup> ER-Cre<sup>+</sup> progeny were bred to Gmnn<sup>fl/fl</sup> mice to give rise to embryos (Fig. 18A).

A) mating scheme for preparation of Gmnn<sup>fl/fl</sup> ER1-Cre and Gmnn<sup>fl/+</sup> ER-Cre embryos. B) Gmnn<sup>fl/fl</sup> ER1-Cre and Gmnn<sup>fl/+</sup> ER1-Cre MEFs were treated with tamoxifen for 48 hours. Genomic DNA was extracted and the efficiency of recombination was investigated by genotyping PCR with specific primers for each allele. Same amount of genomic DNA was used for each PCR reaction. C) Gmnn<sup>fl/fl</sup> ER1-Cre and Gmnn<sup>fl/+</sup> ER1-Cre MEFs were treated with tamoxifen for 48 hours. Whole cell lysate was run on the SDS-PAGE gels and Geminin was immunoblotted. The amount of loaded protein was controlled by Tubulin.

Figure 18. Inactivation of Geminin in mouse embryonic fibroblasts.
The established \( Gmnfl^{fl/fl} \) ER-Cre\(^+\) (fl/fl) and \( Gmnfl^{fl/+} \) ER-Cre\(^+\) (fl/+) MEF lines underwent recombination efficiently upon tamoxifen treatments as short as 48 h. The loss of Geminin in MEFs was investigated by genotyping PCR (Fig. 18B). In the absence of tamoxifen, trace amounts of knockout allele were detectable probably due to basal activity of inducible Cre recombinase. Upon 48 hours of tamoxifen treatment a decrease in the amount of floxed allele and an increase in the knockout alleles was evident. Western blot analysis of tamoxifen treated MEFs revealed down-regulation of the Geminin protein in \( fl/fl \) MEFs (Fig. 18C). Further analysis of these tamoxifen treated fibroblasts did not show any significant difference between the \( fl/fl \) and the \( fl/+ \) MEF cultures. Cells were stained for well-known cell cycle markers and the abundances of positive cells were calculated as percentage of the total population. Cyclin D1 is present during the G1 phase, cyclin A2 increases during S phase and G2 phase and cyclinB1 can be found in the nuclei during G2 and in the cytoplasm during M phase. \( fl/fl \) MEFs contained same number of positive cells for each marker depicting that Geminin knockout can not induce any cell cycle arrest in these cells (Fig. 19A). Ki67, a marker for proliferating cells, were found in the normal amounts in the \( fl/fl \) MEFs indicating that the knockout cells are proliferating with a rate comparable to the \( fl/+ \) cells (Fig. 19B). \( fl/fl \) cells possessed comparable number of cells undergoing mitosis, marked by phosphorylated histone 3 (pH3), and the same number of cells in the S phase labeled with a 4h Bromodeoxyuridine (BrdU) pulse (Fig. 19D). In addition, TUNEL staining indicated no significant increase in the apoptotic cells (Fig. 19E) and flow cytometric analysis of the PI stained cells revealed no cell cycle aberrations (Fig. 19F). Accordingly, the knockout MEFs proliferated with a rate comparable to \( fl/+ \) cells and contained no cell cycle aberrations.
Results

A) Cyclin D1 and Cyclin A2

B) Ki67

C) pH3 and BrdU

D) TUNEL

E) Cell cycle phase

[Images showing graphs and cellular images related to cyclin expression, cell cycle markers, and apoptosis]
Figure 19. Loss of Geminin does not cause cell cycle abberations or apoptosis in MEFs.

A) fl/+ and fl/fl MEFs were treated with tamoxifen for 48 h and immuno-stained for cyclins. Cells were counted and abundances were calculated relative to total number of the cells. B) fl/+ and fl/fl MEFs were treated with tamoxifen for 48 hours and immuno-stained for Ki67, a marker for proliferating cells. Cells were counted and abundances were calculated relative to total number of the cells. C) fl/+ and fl/fl MEFs were treated with tamoxifen for 48 h and immuno-stained for phosho-histone 3, the M phase marker. In addition to tamoxifen MEFs received a 4 h pulse of BrdU to label the cells in the S phase and were stained for BrdU in order to visualize the S phase. D) fl/+ and fl/fl MEFs were treated with tamoxifen for 48 h and stained for TUNEL (apoptosis marker). Treated cells were counted in each case and the percentage of positive cells is represented in the graph. E) fl/+ and fl/fl MEFs were treated with tamoxifen for 48 h, and analyzed with flow cytometry.

It appears that Geminin is redundant in MEFs and additional regulators such as cyclin A2 regulate the fidelity of DNA replication. Interestingly cyclin A2 knockout does not alter the cell cycle of the fibroblasts too. To investigate the cell cycle progression of the fibroblasts in the absence of both Geminin and cyclin A2, the MEFs were transfected with anti-cyclin A2 siRNA. It was hypothesized that this dual inactivation would challenge their ability to regulate the precision of their replication. Western blot analysis of transfected cells confirmed a high knockdown efficiency in MEFs transfected with cyclin A2 siRNA (Fig. 20A). Next fl/+ and fl/fl cells were transfected with anti-cyclin A2 siRNA and received tamoxifen treatment simultaneously. Flow cytometric analysis of the PI-stained knockout and control cells revealed no significant difference in the distribution of the cells in different phases of the cell cycle (Fig. 20B).
Figure 20. Concurrent loss of Geminin and cyclin A can be compensated in MEFs.

A) fl/+ MEFs were transfected with anti-cyclin A2 siRNA or control siRNA using lipofectamine. Cells were harvested; total protein was immune-blotted against cyclin A2 in order to measure the knockdown efficiency. B) fl/+ and fl/fl MEFs were treated with tamoxifen and transfected with anti cyclin A2 at the same time, and analyzed with flow cytometry.
2.12 Geminin is up-regulated during the reprogramming of MEFs to induced pluripotent stem cells (iPSCs)

MEFs can be converted into pluripotent cells and the resulting induced pluripotent stem cells (iPSCs) are indistinguishable from ESCs in many aspects. In order to explore the role of Geminin in reprogramming, transgenic inducible knockout MEFs were utilized. At first the efficiency of reprogramming was tested in our non-recombined MEFs. Retroviral particles containing Oct4 (O), Sox2 (S), Klf4 (K) and c-Myc (M) were prepared and MEFs were transduced with all four factors (OSKM) or combinations of three factors (OSK or OSM).

**Figure 21.** Mouse embryonic fibroblasts can undergo reprogramming upon overexpression of Oct4, Sox2, Klf4 and C-Myc. 
A) MEFs were reprogrammed with OSKM (Oct4, Sox2, Klf4 and C-Myc), OSK and OSM viral particles. Transduced plates were cultured for 3 more weeks in order to obtain reprogrammed induced pluripotent stem cells. B) 3 weeks old reprogrammed cultures were harvested for RNA extraction and the pluripotency markers, Nanog and Zfp42 mRNA levels were analyzed by quantitative PCR. C) 3 weeks old reprogrammed MEF cultures were stained for Alkaline Phosphatase activity.
48 hours after transduction cells were trypsinized and re-plated on the feeder-coated plates and cultured for 18-21 days in ES-CM medium (Fig. 21A). Reprogramming efficiencies of different factor combinations were compared by quantitative PCR analysis of two known pluripotency markers and by alkaline phosphatase (AP) staining of the cultures. Cells were harvested and 18 days after transduction, total RNA was extracted and cDNA was reverse transcribed. Relative abundances of nanog and Zfp42 were calculated using quantitative PCR. These two markers are up-regulated upon maintenance of the pluripotent state and, therefore, their levels reflect the efficiency of reprogramming and numbers of formed iPSCs. While OSKM-transduced cultures were most efficiently reprogrammed (100%), OSK led to about 60% efficiency of iPSCs production and OSM was capable to give rise to only 5-10% reprogrammed cells (Fig. 21B). Alkaline phosphatase staining of freshly fixed cultures gave similar results regarding the efficiency of reprogramming (Fig. 21C).

Geminin levels were determined in wild type MEFs, MPI-II ESCs and one established iPSC line (iPSC-37). Whole cell lysates were immuno-blotted for Geminin and an internal control, Tubulin (Fig. 22). Data represents a high elevation (more than 20 times) of Geminin protein in the pluripotent cells.

**Figure 22.** Geminin is up-regulated in the pluripotent cells.

Western blot analysis of MEFs, MPI-II ESCs and iPSC-37 shows up-regulation of Geminin in pluripotent cells. Geminin is present in fibroblasts in a low level, however, it can be visualized (Fig. 18C).
2.13 Geminin is indispensable for reprogramming

The fact that Geminin levels increases in the reprogrammed cells, suggested a role for Geminin in the reprogramming of the somatic fibroblasts. Although Geminin is not necessary for the fibroblast cells, its augmentation on the way toward pluripotent cells may indicate a pivotal role in the reprogramming of the MEF cells. To study that, the established fl/fl and fl/+ MEF lines were treated with tamoxifen and transduced with OSKM coding viral particles as depicted in Fig. 23.

![Figure 23. Geminin is necessary for the reprogramming.](image)

**A)** fl/+ and fl/fl MEFs were reprogrammed with OSKM (Oct4, Sox2, Klf4 and C-Myc) viral particles in the presence of tamoxifen. Transduced plates were stained for alkaline phosphatase after 20 days. Data indicates a reduction of colony number in reprogrammed fl/fl cultures. **B)** Reprogrammed fl/fl MEFs were sub-cloned and genotyped. Lack of -/- colonies (1/83) suggested a role for Geminin in the reprogramming of MEFs. The only homozygous knockout line showed a differentiated morphology and failed to grow further.
MEFs received tamoxifen and after 48 hours were transduced in the presence of tamoxifen. At day 2 after transduction, cells were re-plated on feeder-coated plates and tamoxifen treatment was continued until day 7. AP staining of plates at 20 days after transduction revealed a strong decrease in the number of stained colonies in fl/fl cultures (Fig. 23), which were picked and expanded individually. Genotyping of formed colonies showed the presence of Gmnn^{fl/fl} and Gmnn^{fl/+} colonies but no Gmnn^{-/-} colonies were observed (Fig. 23). Notably, the only Gmnn^{-/-} cell line that was cloned, showed differentiated morphology and delayed growth kinetics, and failed to passage further. These data clearly reveal a vital role of Geminin for the induction of pluripotency. However, it remained unclear whether Geminin was necessary for the induction of reprogramming or maintenance of already induced cells.

**Figure 24.** Geminin is necessary for the maintenance of reprogramming.

**A)** Gmnn^{fl/+} ER1-Cre and Gmnn^{fl/fl} ER1-Cre MEFs were reprogrammed with OSKM (Oct4, Sox2, Klf4 and C-Myc) viral particles in the presence of tamoxifen. **B)** Reprogrammed plates were stained for alkaline phosphatase at different time points.
To investigate this question, inducible knockout and control cells were treated with tamoxifen and reprogrammed as described and then were investigated at different time points (Fig. 23A). The time course alkaline phosphatase staining illustrated that in the absence of Geminin the initial steps of reprogramming are intact, but the number of AP positive colonies is reduced in the \textit{fl/fl} plates on day 13 and later (Fig. 24B). This observation suggests that Geminin deficient cells can be induced towards pluripotency, but they cannot maintain their pluripotent state.

In order to visualize the proliferating cells, iPSC cultures were treated with BrdU on day 7, 11 and 15 after transduction, were fixed on the next day, and the BrdU positive cells were visualized and quantified (Fig. 25B). The graph represents the relative number of BrdU positive cells in \textit{fl/+} and \textit{fl/fl} cultures. At day 8, the number of BrdU positive cells is less among the fl/fl cells however, due to the high standard error the difference is not significant (p-value= 0.7313). At day 12, the difference between the number of the positive cells in different cultures increased but the differences are not statistically significant. High standard error illustrates a high heterogeneity among different colonies. A few days later, at day 16, the difference between the two cell lines became more evident and statistically significant (p-value=0.0232). Thus, it was concluded that at earlier stages there is no difference between the numbers of proliferating cells. However, as reprogramming advances, in the \textit{fl/fl} cells the number of proliferating cells reduces and the difference becomes evident at later stages. This observation is consistent with aberrant maintenance of the reprogramming in Geminin deficient cells.
Figure 25. Geminin deficient cells fail to maintain their proliferation.

**A** fl/fl and fl/+ MEFs were reprogrammed with OSKM (Oct4, Sox2, Klf4 and C-Myc) viral particles in the presence of tamoxifen. At different time points, the transduced plates were treated with BrdU and stained for it. **B** The graph represents the number of BrdU incorporated cell per counted field relative to the control cells.

### 2.14 Geminin cannot substitute any reprogramming factor

Geminin is necessary for the maintenance of reprogramming, however, would it be able to induce reprogramming or enhance the induction of the reprogramming? To study that, the established OG2 MEF line was reprogrammed with different combinations of reprogramming factors and Geminin. OG2 MEFs contained a green fluorescent protein (GFP) reporter under the control of distal enhancer element of the Oct4 gene, which would express GFP upon reprogramming. This reporter would provide the possibility to visualize, quantify or sort the reprogrammed cells. MEFs were transduced with OSKM or different combinations of 3 factors (SKM, OKM, OSM and OSK) or different combinations of 3 factors and Geminin (GSKM, OGKM, OSGM and OSKG). The transduced cultures were stained for alkaline phosphatase activity after 14 days (Fig. 64). Comparison of AP staining showed a slight decrease in reprogramming efficiency upon removal of C-Myc factor and a stronger effect upon removal of Klf4, Sox2 and Oct4. In addition, data demonstrates that addition of Geminin to reprogramming cocktail cannot enhance the reprogramming and Geminin cannot substitute any of the reprogramming factors.
Figure 26. Geminin cannot substitute any reprogramming factor.

OG2 MEFs were reprogrammed with viral particles containing different combinations of reprogramming factors and Geminin (O: Oct4, S: Sox2, K: Klf4, M: C-Myc and G: Geminin). Transduced plates were stained for alkaline phosphatase 14 days after transduction.
3. Discussion

3.1 Replication and cell cycle regulation by Geminin

3.1.1 Geminin is degraded during the cell cycle of ESCs
The cell cycle of ESCs is tightly bound to the undifferentiated state of these cells. It was reported that the majority of the regulatory factors including cyclins, Cdc6, and Geminin are rather constitutively expressed during the cell cycle of ES cells than being restricted to a specific phase\textsuperscript{35}. It has been speculated that Geminin levels are high in all the phases of the cell cycle in ESCs, resulted by the inhibition of its recognition by the degradation machinery due to the presence of Emi, the inhibitor of the APC/C complex\textsuperscript{92}. However, studies conducted under more efficient synchronization methods have illustrated a dynamically degrading Geminin in ESCs like other somatic cells\textsuperscript{38}. Geminin and many other cell cycle regulatory proteins are degraded in a cell cycle-regulated manner; but, since the G1 phase of the ESCs is short in length, a homogenously synchronized cell population is necessary to observe it.

mAG-hGem construct codes a green fluorescence protein, Azumi green, fused to the destruction box of the Geminin. When the degradation machinery of the cell recognizes the Geminin destruction box, this fusion protein is degraded and no green fluorescence can be detected. Over-expression of mAG-hGem in ESCs resulted in an Azumi green positive population residing only in S/G2/M phases. This population lacked the cells in the G1 phase of the cell cycle, thus Geminin degradation box must be detected prior to entry into G1 and during the G1 phase. These data clearly illustrates a recognition and degradation of Geminin’s destruction box. However, it is possible that \textit{in vivo}, the endogenous full-length protein is protected against degradation due to the presence of the inhibitors which bind only to the full-length protein.
Finally, investigation of the endogenous protein in a synchronized cell population confirmed the degradation of the Geminin in ESCs. The homogenously synchronized population of the ESCs showed a decrease in the endogenous Geminin upon entrance into the G1 phase. Although the efficiency of synchronization was about 85%, it was possible to observe significant decrease in the level of the endogenous protein. This observation depicted that unlike previous reports\textsuperscript{35, 92}, the degradation machinery of the cells degrades the endogenous Geminin protein.

3.1.2 Replication and cell cycle regulation in the absence of Geminin

In rapidly proliferating cells such as cleavage stage embryos, pre-implantation embryos and cancer cells, Cdt1 activity is the rate-limiting factor for the origin licensing\textsuperscript{115}. Geminin binds and inactivates Cdt1 while protecting it from ubiquitination and degradation. Therefore in fast proliferating cells such as ES cells, geminin deficiency would result in a loss of Cdt1\textsuperscript{116}. In two knockdown studies\textsuperscript{116, 117}, no re-replication was observed while in contrast Yang and colleagues observed nuclei enlargement in ESCs\textsuperscript{118}. This discrepancy could result from different residual levels of geminin after siRNA depletion. In our ESCs the inactivation of the geminin gene did not lead to re-replication, but resulted in a slightly longer cell cycle. Geminin down-regulation may have caused in a slower cell cycle, and this change led to the loss of the pluripotent identity. It is widely accepted that a fast, abbreviated cell cycle is necessary for the pluripotency of the ESCs\textsuperscript{115, 119, 120}. Noteworthy, it was shown that upon cell cycle perturbations or depletion of some cell cycle regulators the pluripotency markers are still up regulated debating the connection between the fast cell cycle and the pluripotent identity\textsuperscript{121}. On the other hand an induction of differentiation and reduction in the pluripotency gene expression, can lengthen the cell cycle of the ESCs\textsuperscript{36, 46, 119}. In summary, we interpret the observed increased length of the cell cycle after geminin knockout as a consequence of differentiation of ESCs.
3.2 Geminin is required for pluripotency

3.2.1 Geminin is down regulated during differentiation

Geminin is expressed in the mouse ESCs and its protein level decreases upon their differentiation as EBs (Fig. 4)\(^{35}\). However, mRNA level is not reduced as fast as the protein level is down regulated. This may indicate a post-transcriptional regulation of Geminin expression in differentiating cells. On the other hand, it may reflect the changes in the cell cycle of the differentiating ESCs. In an asynchronized ESC population only 10-15% of the cells are in the G1 phase, while, differentiation is coupled to an increase in the length of the G1 phase resulting in a population of the cells with more than 50% G1 phase cells. Knowing the fact that during the cell cycle Geminin is only found in the S/G2/early M phases, and it is absent in the late M/G1 phase. It can be concluded that in a differentiating population the ratio of the Geminin expressing to the total cells decreases. Thus, this dramatic change in the cell cycle can alone cause a reduction in the total Geminin content of the cells even if there is no change in the expression of the Geminin mRNA.

Geminin was known as a neuralizing factor, expressed in the Sox2\(^+\) cells of the neural lineage (reviewed in the introduction). In this study, Geminin is expressed in the neuroectoderm (Fig. 5), and during neural induction but not in the later stages where neurogenesis takes place (Fig. 16B). However, upon differentiation to mesendoderm Geminin level decreases (Fig. 5). Presence of Geminin protein in neural lineage is in accordance with the former observation that Geminin decreases during spontaneous differentiation of ESCs. The majority of differentiating cells gives rise to mesoderm and endoderm rather than ectoderm. Therefore, a reduction of Geminin in meso- and endoderm progenitors is sufficient to cause a reduction in the total amount of geminin in the whole population of differentiating EBs. In summary, Geminin is expressed in
pluripotent cells and neuroectoderm and it is down-regulated in the differentiating mesendoderm progenitors.

3.2.2 Geminin expression is tightly bound to the pluripotent state

Geminin-deficient embryos arrest their development at 8-cell stage and never give rise to the ICM, suggesting that Geminin is necessary for the totipotency\textsuperscript{90, 91}. Additionally, it was shown that while 8-cell embryos can give rise to ESCs, the Geminin-deficient 8-cell morulas couldn’t give rise to ESCs if cultured \textit{in vitro}\textsuperscript{91}. Therefore, Geminin is necessary for the derivation of the pluripotent ESCs.

Gmnn\textsuperscript{fl/fl} ER-Cre ESCs (iGmnn ESCs), which were established in this study, carry both alleles of Geminin gene flanked by loxP sites and express a tamoxifen inducible Cre recombinase transgene\textsuperscript{111, 122}. The genotype of these cells, provides the possibility of Geminin genetic inactivation upon administration of a small chemical named tamoxifen. Protein analysis clearly depicted that these cells lose more than 90% of their Geminin protein as early as 48h after induction. Thus a minority stays unrecombined or partially recombined; therefore, the iGmnn/48hTx cells are a heterogeneous population consisting of Gmnn\textsuperscript{fl/fl}, Gmnn\textsuperscript{fl/-}, and Gmnn\textsuperscript{-/-} cells. These cells provide a suitable system to study the role of Geminin in pluripotent cells. Up to now, these cells are the only available system to study the events following the Geminin knockout in ESCs.

In this study, after tamoxifen treatment of iGmnn ESCs, these cells were plated as single cells in order to give rise to pluripotent colonies. Genotyping of the formed colonies clearly indicated an absence of the Gmnn\textsuperscript{--} ESCs (Fig. 12). This experiment indicates a pivotal role for Geminin in the maintenance of the pluripotent state. Therefore, Geminin is not only necessary for the derivation of ESCs but it is necessary for the self-renewal of pluripotent ES cells.

iGmnn/48hTx ESCs give rise to less colonies and the majority of the colonies exhibited a flattened differentiated colony morphology. Additionally the cells down-regulated their pluripotency marker expression, such as Sox2, Nanog and Zfp42 expression but Oct4 levels retained as high as undifferentiated cells. The
expression of differentiation markers such as Sox1, Brachyury or Sox17 was not significantly changed. Cdx2, the trophectoderm marker was not expressed and the cells did not contain p-cadherin (unpublished data). The expression of Gata6 was slightly up-regulated but not Gata4 (unpublished data).

Although there are no other genetic inactivation studies on Geminin in ESCs, but there are a few reports characterizing the ESCs treated with anti Geminin siRNA\textsuperscript{92,123} or inducible shRNA\textsuperscript{99} exhibiting a fundamental discrepancy among their findings. The first study reports a role for Geminin in the pluripotent state of the cells, showing a down regulation of Sox2 and loss of pluripotent cell morphology upon treatment of the ESCs with anti-Geminin siRNA\textsuperscript{123}. Geminin is shown to contribute to the expression of Oct4, Sox2, and Nanog, maintaining the pluripotency\textsuperscript{92}. Same study reports an expression of the trophoblastic lineage markers such as Troma1 and p-cadherin coupled to increase in the size of the nuclei in ESCs treated with siRNA against Geminin. However, they fail to detect early trophoblastic markers such as Cdx2 or Tead4 in their cells. A third study, characterizes an ES line expressing an inducible shRNA against Geminin\textsuperscript{99}. In the context of pluripotent state these study reveals a slight decrease in the number of formed colonies upon Geminin depletion. However, they fail to detect any changes in the pluripotency governing transcription factors such as Nanog.

The discrepancy among these studies can be resulted from the fact that although they have down-regulated the Geminin levels but its expression is not completely diminished. In addition the first two studies, are characterizing a heterogeneous population, transfected with siRNA. In these populations there are differences in the remaining Geminin levels of each cell compared to the others and the Geminin levels can be recovered upon degradation of the siRNA. Additionally not all the studies have investigated the same criteria of the cells. To sum it up, this study is the only genetic inactivation study providing a population of ESCs, in which the majority of the cells are completely depleted from Geminin.

This study has led to the conclusion that Geminin contributes to the stabilization of the pluripotent state by modulating the expression of pluripotency genes such
as Sox2 and Nanog rather than regulating the cell cycle progression of the cells. However, a more detailed analysis of the iGmnn/48hTx cells’ transcriptome can shed light on the Geminin effect entirely.

3.2.3 Geminin expression is necessary for neural lineage commitment

We concluded from our data that Geminin depletion causes the ESCs to lose their Sox2 expression, relinquish their pluripotency network and differentiate while they still express considerable amount of Oct4. It was shown that both Oct4 and Sox2 contribute to the pluripotent state through maintaining the expression of each other and of other pluripotency factors. Oct4 is required for the mesendodermal lineage, which is suppressed by Sox2. Additionally, Sox2 is required for the neuroectodermal lineage, which is suppressed by Oct4.

In order to investigate the ability of the iGmnn ESCs to differentiate into different lineages these cells have been differentiated spontaneously toward all three lineages. Accordingly, Geminin depletion decreased the number of Sox2 positive cells and increased the number of Oct4 positive cells in the differentiating ESCs. Additionally, iGmnn ESCs were differentiated into the neural lineage in a stringent condition, which selects neural progenitors and does not support the survival of mesendoderm lineage. Notably, Geminin-deficient cells committed apoptosis and failed to differentiate into the neural lineage.

Many studies have suggested a transcription-modulating role for Geminin during the neural induction and formation of neuronal progenitors. Kroll and colleagues showed a similar phenotype in the ESCs expressing an inducible shRNA against Geminin. They showed that Geminin depletion impairs the ability of the ES cells to form neural lineage, while its overexpression promoted neural fate even in the presence of the mesendoderm inducing signals. In addition it is shown before that Geminin regulates the expression of the Sox2 gene in chicken neurogenesis however, the interaction partner, involved in the regulation, does not have a homolog in the other organisms. Collectively the data
Discussion 66

presented here indicates an essential role of Geminin for the expression of Sox2. Both genes are essential for the pluripotency and the neural lineage.

3.2.4 Geminin is necessary for the maintenance of reprogramming

Gmnn fl/fl MEFs did not show any significant phenotypes. Early passages of tamoxifen treated MEFs neither showed a cell cycle arrest nor a significant difference in proliferation or apoptosis. This data is in accordance with former studies done in the immortalized fibroblasts\textsuperscript{124}. As discussed in section 1.4.1 cells have different mechanisms to safeguard the fidelity of their DNA expression. Down-regulation of one of these mechanisms can be compensated with up-regulation of other, redundant pathways. For example MEFs can tolerate the lack of Cyclin A2 through up-regulation of their Cyclin E\textsuperscript{42}. In summary, these results provided evidence for a redundant role of Geminin in mouse embryonic fibroblasts.

The importance of Geminin for pluripotency suggests that it is also essential for the induction of the pluripotency. Indeed Geminin deficient fibroblasts cannot form iPSCs, however, Gmnn\textsuperscript{fl/fl} cultures exhibit a similar cell number and proliferation rate until around 13 days after transduction and they start to fail at later stages. It was shown that after transduction of OSKM, the cell proliferation rate increase massively, the cells which escape the cell cycle arrest caused by entrance of reprogramming factors, proliferate and become the progenitors of iPSCs. A few days later an extensive remodeling of the epigenetic marks is necessary to re-set the epigenetic state into an Open state and to allow the endogenous pluripotency transcription factors to be expressed and access their target in the genome in order to maintain the pluripotent state\textsuperscript{125} (for more see\textsuperscript{65}).

It was shown that pluripotency transcription factors such as Oct4 and Nanog are indispensable from reprogramming and their genetic inactivation would not allow any iPSC to form\textsuperscript{18,125}. Therefore, the re-expression of the endogenous genes is a rate-limiting step in the maintenance of the reprogrammed state and mature iPSCs. In this study, a role for Geminin in the maintenance of Sox2 expression
was suggested; therefore, this is of high interest to investigate the dynamics of endogenous Sox2 expression in these cells. Geminin deficient pre-iPS cells fail to maintain their pluripotent state due to failure in re-establishing their endogenous Sox2 expression (preliminary data). In addition it predicts that Geminin expression is only necessary at later stages of reprogramming, when the endogenous Geminin protein is enough for maintenance of reprogramming. Thus addition of Geminin to the viral cocktail would not affect the reprogramming.

To sum up, Geminin is redundant in MEFs but the absence of Geminin constitutes a major barrier for re-maintaining the pluripotency in vitro most probably due to an inability to re-express the endogenous Sox2.

3.3 Geminin regulates the Sox2 expression through modulating its epigenetic signature

In different developmental contexts, Geminin was shown to interact with SWI/SNF complex\(^97,\)\(^{109}\) and Polycomb group proteins\(^98\) in order to contribute to the maintenance of the neural genes and inhibition of the mesendoderm lineage. In addition it was shown that Geminin is associated to the hyper-acetylated chromatin and regulates the neural lineage commitment through maintaining the hyper-acetylated state\(^99\). In this study, Geminin depletion has no effect neither on the histone 4 hyper-acetylation nor on the histone 3 lysine 4 di-methylations of the Sox2 enhancers or any other tested region, including the oct4 enhancer. Rather in the absence of Geminin, the inactivating signal, histone 3 lysine 27 trimethylations increased on the SRR2 enhancer of the Sox2 gene, followed by an enrichment of the catalytic subunit of the PRC2 complex. In exchange, the presence of the SWI/SNF complex was diminished on the SRR2 enhancer of the Sox2 gene.

This finding can be summarized in a model (Fig. 27). In the presence of Geminin, Brg1 is recruited to \(\text{SRR2}\) enhancer and therefore, \(\text{Sox2}\) gene is expressed. In the absence of Geminin the activating SWI/SNF complex is dissociated, and
PRC2, the inactivating competitor complex, occupies the enhancer region causing accumulation of the inactivating signals and loss of Sox2 expression. This model is in agreement with former observations. SWI/SNF complex is associated with pluripotency genes and pluripotency factors target genes, and contributes to the fine-tuning of their expression, and upon Brg1 depletion, the SWI/SNF binding sites are occupied by PRC2 complex.

Geminin is specifically controlling the binding of the SWI/SNF complex to the Sox2 enhancer region and its absence is not affecting the other pluripotency gene, Oct4. It is of high interest to decipher the molecular details of this gene-specific regulation. Geminin may recruit the SWI/SNF complex to the Sox2 enhancer. However, the direct interaction between Geminin and Brg1 was reported only in an in vitro system. Many immunoprecipitation of Geminin and its overexpressed tagged version was performed and the precipitated samples were analyzed by mass spectrometry (unpublished data). Except Cdt1, which was precipitating with Geminin in all samples, no other SWI/SNF or Polycomb member was detected. In addition, co-immunoprecipitation experiment with Brg1 And Geminin in ESCs did not show any direct interaction (unpublished data). In addition to explore the possibility of Geminin interaction with chromatin, or its enrichment at the SRR2 site, many chromatin immunoprecipitations in the presence of protein-protein cross-linkers was done. However, none of these experiments was able to confirm an association of Geminin with chromatin.

On the other hand, Geminin may contribute to the inhibition of the PRC2 complex, however, no direct evidence was observed for such an interaction in ESCs. Notably, in ESCs PRC2 and PRC1 complexes are actively binding and silencing the developmental genes even in the presence of Geminin. Noteworthy, it is known that these protein complexes can have different compositions. Therefore, further investigations are necessary to exclude the possibility that a different composition of PRC2 complex is present on the Sox2 enhancer.

In conclusion, it has been shown that Geminin maintains the expression of Sox2. Therefore, it seems necessary to investigate if the rescue of Sox2 expression can
retrieve the pluripotent state and the ability to differentiate into the neural lineage in iGmnn/Tx ESCs.

**Figure 27.** Geminin regulates Sox2 through regulation of the epigenetic signature of SRR2.

A model summarizing the findings of chromatin immunoprecipitation depicts a change in the active state of SRR2 enhancer to an inactive state.

### 3.4 Geminin is indispensable to toti-pluri-neural lineages

Previous studies \(^{90, 91, 99}\) and this study depict a lineage-specific role for Geminin. Geminin is present and necessary in the totipotent cells and its loss abrogates the totipotency. Geminin is present in pluripotent cells and its loss caused an exit from the pluripotent state and finally Geminin was necessary for the commitment into neural lineage. Therefore, Geminin functions as a key factor for the formation of the totipotent-pluripotent-neural lineage during the development. This study nominates Sox2 as the key target gene which expression is necessary for the
establishment of this axis (Fig. 28) and predicts that Geminin is necessary for the maintenance of epiblast stem cells\textsuperscript{127, 128}, the in vitro equivalent of the pluripotent late epiblast.

Figure 28. Geminin safeguards the toti-pluri-neural fate determination in the embryonic development.

Geminin (shown in yellow) is expressed in totipotent, pluripotent and neuroectoderm cells together with Sox2, and required for these lineages.

This study defined a transcription-modulating role for Geminin in the lineage commitments of the early embryo. However, Geminin was known for its role in the inhibition of re-replication and as discussed extensively before, in non-embryonic tissues the Geminin expression marks the proliferating cells. In addition Geminin was reported to interact and regulate many transcription factors, chromatin remodeling complexes and the basic transcription machinery. Finally it seems that Geminin interacts with many complexes simultaneously.
These findings suggest a complex mechanism for Geminin’s action in regulation of the embryonic lineage commitment.

Geminin is only present in the multi-cellular organisms and no Geminin homolog was found in uni-cellular organisms. This differences cause the metazoan cells to have an extra mechanism for regulation of their DNA replication fidelity compared to yeast or other unicellular eukaryotes. For that reason it was speculated that in higher eukaryotes the presence of a more than one mechanisms has provided the possibility for Geminin to acquire additional roles during the regulation in the embryonic development. It seems more probable to consider a gene, which for the first time appears in the multicellular organisms to be involved with the most pivotal aspect of multi-cellularity, the cell specifications. Therefore, it can be hypothesized that Geminin has evolved as a transcription modulator, which was regulated during the cell cycle, in order to facilitate the coordination of proliferation and differentiation. In this respect, Geminin acquired the DNA re-replication inhibition as a secondary role, which may guarantee a higher regulation on the fidelity of genomic replication during a fast cell cycle.
4. Materials and Methods

4.1 Cell Biology

4.1.1 Cell lines

Table 1. Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type ES cells (MPI-II, 129Sv strain, XY karyotype)</td>
<td></td>
</tr>
<tr>
<td>Plat-E cells</td>
<td>130</td>
</tr>
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</table>

4.1.2 Mouse lines

Table 2. Mouse lines used in this study

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type B6N (C57Bl6/N)</td>
<td>-</td>
</tr>
<tr>
<td>Geminin conditional knockout</td>
<td>102</td>
</tr>
<tr>
<td>ER-Cre</td>
<td>111</td>
</tr>
<tr>
<td>CMV-Cre</td>
<td>131</td>
</tr>
<tr>
<td>Oct4-GFP</td>
<td>132</td>
</tr>
</tbody>
</table>

4.1.3 Isolation of mouse embryonic fibroblasts

Appropriate matings were set up and females were checked every day. Vaginal plug positive females were considered to be pregnant and staged as embryonic day 0.5 (E0.5). Pregnant females were euthanized by the CO₂ exposure 13 days after copulation (E13.5) followed by cervical dislocation. Uterus was dissected out and transferred to a 50-ml tube containing 20-30 ml cold sterile PBS. In a laminar flow cabinet, uterus was transferred into a 10 cm tissue culture dish containing 10 ml sterile PBS. The uterus and yolk sacs were cut to expose the
embryos. After the embryo were cut out, each fetus were transferred individually in a new dish containing 10 ml sterile PBS. The limbs, liver, heart, tail and the head (brain) were cut. Tail or limb biopsies were used for DNA extraction followed by genotyping. Each embryo (the remaining trunk) was transferred to a well of a 6-well plate. 2-3 ml 0.25% Trypsin/EDTA (GIBCO) was added to each embryo and the embryos were incubated at 37 °C for 10-15 minutes. Afterwards embryos were disrupted, using two forceps until only small cell clusters remained. MEF culture medium (DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAN-Biotech)) was added to each sample and pipetted vigorously and repeatedly up and down to break up the digested tissues into a cell suspension. Samples were transferred into 15 ml tubes and the total volume was adjusted to 10 ml per sample by addition of medium. The samples were placed for 2-3 min under the flow cabinet to let the cell clumps sediment in order to get rid of the bigger clumps. The cell suspensions were pipetted into culture dishes containing 15 ml fresh medium. Then, the cells were cultured at 37 °C under 5% CO₂ concentration. On the next day, the MEF cells were categorized based on the genotyping results; they were washed with PBS and trypsinized with 4 ml 0.25% Trypsin/EDTA. Cells with the same genotype were pooled; the cells were counted and were frozen as 10^6 cells/vial (for more details on cell passage, freezing and revival check the following sections).

4.1.4 Cell passage and freeze
The cells were passaged as soon as they were reaching 70-90% confluency. The cells were washed with pre-warmed PBS. 0.25% Trypsin/EDTA (Invitrogen) solution was equally distributed onto the washed cells, and the cells were incubated at 37 °C for about 5 minutes. The dish was shaken until all the cells became floating. Culture medium (containing FBS) was added to stop the trypsin digestion, pipetted up and down for several times to dissociate the cells, and then transferred into a 15-ml Falcon tube. Centrifugation was carried out at 1,000 rpm for 5 minutes to pellet the cells. The cell pellet was dissociated by tapping the
falcon tube gently 4-5 times. For passage, the cell pellet was re-suspended in appropriate amount of medium necessary for 3-4 culture plates. To freeze, the cell pellet was re-suspended in 3 ml culture medium. 3 ml 2x freezing medium (DMEM (GIBCO) supplemented with 16%DMSO and 25%FBS) was added and mixed and immediately 1.5 ml cell suspension was transferred into each cryotube. The cryotubes were transferred to a Cryo-safe™ cooler (Bellart products) containing isopropanol and were frozen at -80°C. the cells were kept at -80°C for a week to 2 months. For longer storage the cells were transferred in liquid nitrogen at least 1 day after they were frozen.

4.1.5 Cell revival
Cells from liquid nitrogen or -80 °C freezer were thawed at 37 °C as quickly as possible and were transferred gently into a 15 ml Falcon tube containing 3-5 ml pre-warmed culture medium. Cells were collected by centrifugation at 1000 rpm for 5 min. appropriate amount of fresh medium was added to the cell pellet and mixed well in order to break down cell clumps. The cells were transferred into the desired culture plate. The cells were cultured at 37 °C in 5% CO₂.

4.1.6 Gelatin coating of culture plates
Plates were coated with gelatin (0.2% in PBS) for 10 min. Gelatin was removed before use. Glass surfaces needed a longer coating time (1-2 h). For plating embryonic stem cells coating time was increased to 2 h (for culture plates) until overnight (for glass surfaces) in order to achieve better results.

4.1.7 Preparation of mitotically inactivated feeder cells
B6J derived MEFs from liquid nitrogen or -80 °C freezer were revived. As soon as the cells became confluent, the cultures were passaged 1:4-1:8. As soon as the passaged fibroblasts reached the 90-100% confluency, the cells were inactivated by treatment with Mitomycin-C (10mg/ml) for 2.5 h at 37 °C. Afterwards the cells were washed with PBS twice and trypsinized. The
trypsinized cells were either re-plated on gelatin-coated plates as feeder layers for further usage or frozen.

4.1.8 Embryonic stem cell derivation

Female mice were induced for superovulation. Appropriate matings were set up and females were checked for the vaginal plug on the next day. Vaginal plug positive females were considered to be pregnant and staged as embryonic day 0.5 (E0.5). Pregnant females were euthanized by cervical dislocation 3 days after copulation (E3.5). The uteri were cut out and blastocysts were flushed in ES-CM (embryonic stem cells conventional medium: Knockout™ DMEM (GIBCO) supplemented with 20% FBS (PAN-biotech), 1 mM β- mercaptoethanol (Sigma-Aldrich), 2 mM L-Glutamine (GIBCO), 1% non essential amino acids (GIBCO), 1 mM Sodium Pyruvate (GIBCO) and 1000 u/ml LIF (Invitrogen)).

The blastocysts were immediately transferred to a laminar flow cabinet and plated on feeder-coated 35-mm culture plates (4-6 blastocyst on each plate). The medium was changed after 2 days and once more 2 days later. 5-6 days after plating the blastocysts were grown into out-growths containing ESCs. Outgrowths were cut with 20-μl pipette tips and transferred into 50-μl ES trypsin/EDTA (8g/l NaCl, 0.4g/l KCl, 0.1g/l Na2HPO4, 1g/l Glucose, 3g/l Tris Base and 2.5g/l Trypsin) and incubated for 3 min at 37 °C. Afterwards the trypsin was stopped with 100- μl ES-CM followed by pipetting up and down vigorously in order to create shearing force to dissociate the cells. The cell suspension from each blastocyst was transferred to each well of a 24-well plate coated with feeders and containing ES-CM. The single cells formed colonies and reached 90% confluency after 2-3 days. After reaching confluency the cells were trypsinized and divided into three equal volumes, of which two volumes were used for freezing and one for genomic DNA extraction.
4.1.9 ES cells maintenance and passage
Mouse ES cells were routinely maintained on gelatin-coated, feeder-coated 35-mm plates and fed daily with ES-CM or any other specified medium. ESCs were passaged every 2-3 days depending on the level of confluency. To passage the cultures were washed with PBS and trypsinized by ES trypsin/EDTA for 3 min until the colonies detached from the culture plates. Then trypsin activity was stopped by the addition of culture medium. The cell supernatant was vigorously pipetted up and down using 2-ml pipettes, transferred to a Falcon tube and centrifuged for 5 min at 1000 rpm. The pellet was reconstituted in medium and the cells were re-plated on desired plates in desired abundance.

4.1.10 Tamoxifen (4-hydroxyl tamoxifen) treatment of the cells
100 mg 4-hydroxil tamoxifen (Sigma-Aldrich) was dissolved in 10 ml ethanol (Merck) at 37 °C for 1 hour. The solution was filter-sterilized, aliquoted and stored at -20 °C. Frozen tamoxifen solutions were used up to three months after the preparation. Fibroblast growth medium was supplemented with 100-500 nM tamoxifen and ES-CM medium was supplemented with 1 μM tamoxifen for 48 hours or longer.

4.1.11 Feeder layer free culture of ESCs
Embryonic stem cells were plated on gelatin-coated plates in ES-CM medium as single cells. Cells were harvested or passaged 3-4 days after plating.

4.1.12 Sub-cloning of the ES Cells
The formed ES clones were cut with 20-μl pipette tips and transferred into 50-μl drops of ES trypsin/EDTA and incubated for 3 min at 37 °C. Afterwards the trypsin was stopped with 100 μl ES-CM followed by pipetting up and down vigorously in order to create shearing force to dissociate the cells. The cell suspension from each clone was transferred to each well of a 24-well plate coated with feeders and containing ES-CM. The single cells formed clones and
reached confluency after 2-3 days. Afterwards, the cells were trypsinized and divided into three equal volumes, 2 used for freezing and one used for genomic DNA extraction.

4.1.13 ES differentiation

ESCs were differentiated using four different methods:

**Embryoid Body differentiation**

ESCs were trypsinized into single cells and were counted using a hemocytometer (Neobar). 2-8 x 10^6 cells were diluted in 20 ml differentiation medium: IMDM (GIBCO) supplemented with 20% FBS (PANbiotech), 450 nM monothioglycerol (Sigma-Aldrich), 2 mM L-Glutamine (GIBCO) and 1% non essential amino acids (GIBCO). Cell suspension was transferred into a 15-cm bacterial grade culture plate and cultured further for 5 days. After 24 hours the cells formed aggregates. Medium was changed every 2 days. After 5 days, the EBs were collected, centrifuged and medium was discarded. EBs were washed with PBS once and treated with 2 ml 0.25% trypsin/EDTA for 5 minutes. The aggregates were dissociated by pipetting and the trypsin was inactivated by addition of 18 ml differentiation medium. The cell solution was transferred to gelatin-coated culture plates or glass chamber slides. The cells were cultured for 4 additional days in differentiation medium.

**Monolayer differentiation**

ESCs were trypsinized into single cells and were counted using a hemocytometer (Neobar). 1.5 x 10^4 cells/cm^2 were diluted in appropriate amount of differentiation medium. Cell suspension was transferred into gelatin coated culture plates or glass chamber slides. These plates were cultured further for 4 days.
Generation of cortical neurons from ESCs\textsuperscript{134}

ESCs were differentiated into neural and neuronal progenitors, neurons as well as astrocytes as described\textsuperscript{134}.

Differentiation toward neuroectoderm and mesendoderm progenitors\textsuperscript{9}

ESCs were trypsinized into single cells and were counted using a hemocytometer (Neoban). $1.5 \times 10^4$ cells/cm$^2$ were diluted in appropriate amount of N2B27 medium\textsuperscript{135} (50\% DMEM/F12 (GIBCO) supplemented with 1 mM β-mercaptoethanol (Sigma-Aldrich), 2 mM L-Glutamine (GIBCO), 1\% non essential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), 50 µg/ml bovine serum albumin fraction V (Invitrogen) and N2 supplement (Invitrogen) mixed with 50\% Neurobasal medium (GIBCO) supplemented with 2 mM L-Glutamine (GIBCO) and B27 supplement without retinoic acid (Invitrogen)).

Cell suspension was transferred into a gelatin coated culture plates or glass chamber slides and the cells were cultured for 48 hours. After 48 hours the medium was changed to N2B27 medium supplemented with 3 mM Chirion (CHIR99021, StemGent) for mesendodermal differentiation or 500 nM RA (retinoic acid, Sigma-Aldrich) for neuroectodermal differentiation. For differentiation experiments, cells were immunostained 36 hours after addition of the differentiation signal. ESCs were propagated in N2B27 media supplemented with LIF and 2i: 3 mM Chirion and 100 nM MEK1/2 inhibitor III PD0325901 (Calbiochem).

4.1.14 Transfection of plasmid DNA

ESCs were plated in gelatin-coated plates in ES-CM until they reached 60\% confluency. The cells were transfected with mAG-hGem(1/110)pcDNA3 plasmid\textsuperscript{110} and Lipofectamine™ 2000 (Invitrogen) according to manufacturer’s instructions.
4.1.15 Transfection of siRNA

MEFs were trypsinized into single cells. The siRNA (cyclin A siRNA (sc-29283, Santa Cruz Biotechnologies) or non-targeting siRNA (Dharmacon) and Lipofectamine™ 2000 (Invitrogen) were mixed according to manufacturer’s instructions. After the second incubation time the siRNA/lipofectamine mixture was mixed with the cell suspension and transferred to gelatin-coated plates. The cells were cultured for 2 more days before analysis.

4.1.16 Reprogramming

PlatE cells (Platinum-E retroviral packaging cell line, Ecotrophic) were maintained in DMEM (GIBCO) supplemented with 10% FBS, 1 μg/ml Puromycin and 10 μg/ml Blasticidin on gelatin-coated plates. For production of viral particles the cells have been plated at 6 x 10^6 cells/10-cm plate without antibiotics (Blasticidin and Puromycin) overnight and on the next day each 10-cm plate was transfected with 27 μl Fugene 6™ (Roche, Promega) and 9 μg of one of the pMXs plasmids: pMXs-Oct3/4 (Addgene-plasmid 13366), pMXs-Sox2 (Addgene-plasmid 13367), pMXs-Klf4 (Addgene-plasmid 13370), pMXs-c-Myc (Addgene-plasmid 13375), pMXs-Gmnn (Provided by Dr. K. Boese). Transfection was done according to instruction provided by Fugene 6™’s manufacturer. The cells were cultured overnight and the medium was changed on the next day. 24 hours later the medium was collected from culture plates and filtered with a 0.45 μm cellulose filter (Millipore). 4 μg/ml Polybrene (Sigma-Aldrich) was added to the viral particle containing medium and it was used for transduction of fibroblasts.

One day before transduction the early passage primary fibroblasts (passages 1-3) were plated at 5 x 10^5 cells/10-cm plate in MEF medium. On the transduction day, the viral particle-containing medium was used to transduce the MEFs. In order to obtain OSKM (Oct4, Sox2, Klf4 and c-Myc) viral particle containing medium, equal amounts of each supernatant was mixed together. Three-factor reprogramming (OSK, OSM, SKM and OKM) was done with combining one part MEF medium supplemented with 4 μg/ml Polybrene to three parts viral particle
containing mediums. 2-3 days after transduction the cells were plated on feeder-coated plates in ES-CM at 1-2 x 10^4 cell/1 ml medium. Medium was changed every 1-2 days depending on the cell proliferation rate (every 2 days at the beginning and from day 10 onwards on a daily basis).

4.1.17 Visualization of alkaline phosphatase activity

The culture plates containing ESCs or reprogrammed MEFs were washed once with PBS and were fixed in 4% paraformaldehyde in PBS for 30 min. Next the plates were washed three times with PBS and once with alkaline phosphatase buffer (100 mM Tris.HCl pH 9.5, 100 mM NaCl and 50 mM MgCl_2). Next, the cells were stained in blue by exposure to NBT/BCIP working solution (2% NBT/BCIP enzyme substrate (Roche) in alkaline phosphatase buffer) for 10-30 minutes in dark. Afterwards the solution was discarded and the plates were washed once with PBS and kept in PBS at 4 °C. The colonies were imaged under bright field microscope. The plates were imaged using a digital camera (Canon).

4.1.18 Immunofluorescence analysis of cultured cells

For immunofluorescence analysis of the expressed proteins or protein modifications, the cells were plated on glass chamber slides (Thermo-Fischer) or glass coverslips (gelatin-coated or feeder-coated depending on the cell type or the experiment). The cells were transferred to the bench and they were stained as below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Treatment</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4% (w/v) Paraformaldehyde in PBS</td>
<td>15-20</td>
</tr>
<tr>
<td>2,3,4</td>
<td>PBS</td>
<td>3 x 5</td>
</tr>
<tr>
<td>5</td>
<td>PBS-0.5% Triton X100</td>
<td>5</td>
</tr>
<tr>
<td>6,7</td>
<td>PBT (0.1% tween in PBS)</td>
<td>2 x 5</td>
</tr>
<tr>
<td>8</td>
<td>10% FBS in PBT</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>Primary antibody in 10% FBS in PBT</td>
<td>Overnight at 4° C</td>
</tr>
<tr>
<td>10,11,12</td>
<td>PBT</td>
<td>3 x 5</td>
</tr>
</tbody>
</table>
Secondary fluorescence conjugated antibody

PBT

After last PBT washing, the glass surface was washed once more with ddH₂O and mounted with VECTASHIELD mounting medium with DAPI (Vector laboratories, Inc.). The sample was applied to a BX-60 fluorescence microscopy (Olympus) or a Leica TCS SP5 confocal microscope.

Table 3. Antibodies used for immunofluorescence analysis

<table>
<thead>
<tr>
<th>Primary and secondary antibodies</th>
<th>Manufacturer</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Brachyury</td>
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<td>Roche</td>
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<tr>
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<td>Santa Cruz Biotechnologies</td>
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<td>Santa Cruz Biotechnologies</td>
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<tr>
<td>Ki67</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
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<td>Nanog</td>
<td>ABGent, Cosmobio</td>
<td>1:100</td>
</tr>
<tr>
<td>Nestin</td>
<td>BD Bioscience</td>
<td>1:100</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>BD Bioscience</td>
<td>1:200</td>
</tr>
<tr>
<td>Pax6</td>
<td>DSHB</td>
<td>1:100</td>
</tr>
<tr>
<td>Phospho-histone 3 (pH3)</td>
<td>Cell signaling</td>
<td>1:200</td>
</tr>
<tr>
<td>Sox1</td>
<td>R&amp;D</td>
<td>1:100</td>
</tr>
<tr>
<td>Sox2</td>
<td>Santa Cruz Biotechnologies</td>
<td>1:100</td>
</tr>
<tr>
<td>Sox17</td>
<td>R&amp;D</td>
<td>1:100</td>
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<tr>
<td>SSEA1</td>
<td>Santa Cruz Biotechnologies</td>
<td>1:400</td>
</tr>
<tr>
<td>Tuj1</td>
<td>Covance</td>
<td>1:100</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-mouse F(ab')</td>
<td>Molecular Probes</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
4.1.19 Synchronization of ESCs

Synchronization was done according to this publication\textsuperscript{35} with some modifications. Mouse embryonic stem cells were cultured on gelatin coated plates in ES-CM medium supplemented with 2i. After 48 hours the cultures became 60% confluent. The medium was changed to pre-warmed ES-CM supplemented freshly with 2.5 mM thymidine (Sigma) and the cells were incubated for 12 hours. Next, the medium was changed to pre-warmed ES-CM supplemented with 150 ng/ml TN-16 (Enzo life sciences) and incubated for 7 hours. Later, the medium was changed to pre-warmed fresh ES-CM in order to let the cells to release from the cell cycle arrest. The cells were harvested at different time points for analysis of the cell cycle and Geminin protein levels.

4.1.20 Flow cytometric analysis of the cell cycle

Cells were trypsinized into single cells; the cell suspension was centrifuged in order to obtain the cells. The pellet was washed twice with 10 ml ice cold PBS. The cells were pelleted each time by 1,000 rpm centrifugation at 4°C for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml ice cold PBS. The falcon tubes were already cooled on ice and filled with 3 ml 100% ethanol pre-cooled at -20°C. The cells were aspirated into 1 ml syringe with a 20 gauge needle attached, and were injected into the cold ethanol with pressure. The cells were incubated at least 1 hour on ice before the analysis, and can be stored for weeks at -20°C.

Shortly before the analysis, the cells were spun down at 500g (1,400 rpm) in a
Heraeus centrifuge for 5 minutes at 4 °C. The ethanol was carefully removed immediately after the centrifugation, and the pellet was let to dry by putting the tube over head on a tissue paper (2 to 5 minutes). The rest of the ethanol was removed from the walls of the tube with tissue paper. The cells were re-suspended in 425 μl PBS. The re-suspended cells were vortexed to break the cell clumps. RNase A (Roche) dissolved in 10 mM Tris, pH 7.5 was incubated for 15 minutes at 95 °C in order to inactivate the DNases, aliquoted and frozen at -20 °C. 50 μl of 1 mg/ml RNase A was added to each sample, followed by 25 μl of 1 mg/ml propidium iodide (Sigma-Aldrich). The staining was performed for 30 minutes at RT in the dark. All the measurements must be performed within the next 3 hours upon staining.

The cells were analyzed for cell cycle distribution using FACSCalibur™ Flow Cytometer (BD biosciences). The samples were vortexed immediately before measurement. The collected data were analyzed using the FlowJo software (Tree Star Inc.). The program determined control values and the indistinct peaks were located according to the control values.

4.1.21 Terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL) assay
The apoptotic cells were detected using the ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Millipore). Percentage of apoptotic cells was determined by counting the number of the TUNEL positive cells in comparison to DAPI positive nuclei.

4.1.22 Annexin V staining
In ESCs apoptosis was detected by The Annexin V-Cy3™ apoptosis detection kit (Sigma-Aldrich) and fluorescence microscopy according to manufacturer’s instructions.
4.1.23 BrdU staining
In order to visualize the replicating cells in the S-phase of the cell cycle, the cells have been incubated with BrdU 2 hours to overnight. 140 mg of BrdU (sigma) was dissolved in 877 μl PBS at 37 °C. This solution was aliquoted and frozen at -20 °C and was diluted 1 to 100 in fresh medium before treatment of the cells. The cultures were stained for incorporated BrdU and DAPI.

4.1.24 Quantification and statistical analysis of immunofluorescence staining
Stained cultures were quantified manually or by ImageJ software (NIH). Statistical analysis of data was done with an on-line tool (http://www.graphpad.com/quickcalcs).
4.2 Protein Biochemistry

4.2.1 Protein Extraction
The cells were washed with PBS and appropriate amounts of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, NaDOC, 1% SDS) supplemented freshly with protease inhibitor cocktail (Roche), was added to the plates. The plates were incubated for 5 min on ice and the cell lysate was scraped into Eppendorf tubes and were mixed with 4x laemmli buffer.

4.2.2 Western blot analysis
The glass plates, spacers and comb were washed with ethanol, air-dried and fashioned into the gel cassette. The separation gel’s ingredients were mixed (table below) and poured into the cassette to 0.5 cm below the tip of the combs and covered with 0.5ml of isopropanol to prevent contact with air before polymerization. After polymerization the isopropanol was discarded. The stacking gel’s components were mixed (table below) and poured into the cassette over the separation gel and the comb was inserted into the space between glass plates. After the polymerization of the stacking gel, the comb was removed and the gel was inserted into the tank filled with electrophoresis buffer (table below). 25 -40 µl of each sample were boiled for 5 min and loaded on 15% poly acrylamide gels using a Hamilton syringe. 10 µl of benchmark™ protein marker (Invitrogen) was loaded in one well of the gel and the gel was run with 22 mA for 1.5 -2 hours. The protein was transferred on nitrocellulose membrane. The membrane was blocked with blocking buffer, stained with appropriate primary antibody diluted in blocking buffer overnight at 4 °C. The membrane was washed with PBS-T tree times and incubated with the HRP-conjugated secondary antibody diluted in the blotting buffer for 2 hours at room temperature. It was washed with PBS-T and the signal was detected using SuperSignal® West Femto Maximum Sensitivity Substrate
(Thermo), SuperSignal® West pico Maximum Sensitivity Substrate (Thermo) or homemade-ECL (table below).

**Table 4. Buffers and solutions used in western blotting analysis**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Buffer composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA Buffer</td>
<td>50mM Tris (pH7.4), 1% NP40, 0.25% NaPO₃, 150mM NaCl, 1mM EDTA</td>
</tr>
<tr>
<td>Laemmli Buffer (4x)</td>
<td>250 mM Tris pH 6.8, 40% glycerol, 0.04% bromophenol Blue, 4% 2-mercaptoethanol, 8% SDS, 0.05 M DTT</td>
</tr>
<tr>
<td>Stacking Gel Buffer (4x)</td>
<td>0.5 M Tris, 0.4% SDS, pH 6.8</td>
</tr>
<tr>
<td>Separation Gel Buffer (4x)</td>
<td>1.5 M Tris, 0.4% SDS, pH 8.8</td>
</tr>
<tr>
<td>Stacking gel components (5%):</td>
<td>1.25 ml 4x stacking buffer, 830 µl acrylamide 30%, 2.92 ml ddH₂O, 30 µl APS 10% (w/v), 10 µl TEMED</td>
</tr>
<tr>
<td>Separation gel components (15%):</td>
<td>2.5 ml 4x separation buffer, 5ml acrylamide 30%, 2.4ml ddH₂O, 100µl APS 10% (w/v), 20µl TEMED</td>
</tr>
<tr>
<td>Electrophoresis Buffer</td>
<td>12.5 mM Tris, 96 mM glycine, 0.05% SDS</td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>48 mM Trisbase, 3.9 mM glycine, 0.037% SDS, 20% methanol</td>
</tr>
<tr>
<td>PBS-T</td>
<td>0.1% Tween 20 in PBS</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>5% w/v milk powder in PBS</td>
</tr>
<tr>
<td>Homemade ECL</td>
<td>100mM glycine pH 10 (with NaOH), 0.4mM luminol (Sigma-Aldrich), 8mM 4-iodophenol (Sigma-Aldrich), 0.12% (w/w) hydrogen peroxide in water</td>
</tr>
</tbody>
</table>
**Table 5.** Antibodies used in western blotting analysis

<table>
<thead>
<tr>
<th>Primary and secondary antibodies</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachyury</td>
<td>R&amp;D</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cyclin A2</td>
<td>Santa Cruz Biotechnologies</td>
<td>1:1000</td>
</tr>
<tr>
<td>Geminin (FL209)</td>
<td>Santa Cruz Biotechnologies</td>
<td>1:500</td>
</tr>
<tr>
<td>Histone 2B</td>
<td>Active motif</td>
<td>1:1000</td>
</tr>
<tr>
<td>Klf4</td>
<td>ABGent</td>
<td>1:1000</td>
</tr>
<tr>
<td>Nanog</td>
<td>ABGent, Cosmobio, Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>BD Bioscience</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sox1</td>
<td>R&amp;D</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sox2</td>
<td>Santa Cruz Biotechnologies</td>
<td>1:1000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Cell signaling</td>
<td>1:2000</td>
</tr>
<tr>
<td>Goat-anti-rabbit-HRP</td>
<td>Covance</td>
<td>1:10000</td>
</tr>
<tr>
<td>Goat-anti-mouse-HRP</td>
<td>Dianova</td>
<td>1:10000</td>
</tr>
<tr>
<td>Rabbit-anti-goat-HRP</td>
<td>Abcam</td>
<td>1:10000</td>
</tr>
</tbody>
</table>
4.3 Molecular Biology

4.3.1 DNA isolation from mouse

The tail-tips or tissue was incubated overnight at 55 °C with lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Triton-X100) supplemented with proteinase K (Roche). DNA was precipitated after lysis with isopropanol and washed with 70% ethanol. DNA extraction from the blood was conducted with DNeasy™ blood and tissue kit (Qiagen).

4.3.2 DNA isolation from cell culture

The mammalian cells were incubated with 0.5 -1 ml of DNA lysis buffer containing freshly added Proteinase K at the 37 °C room for 10-15 minutes followed by 2-3 hours incubation at 55°C. The lysis product was spun down at 13,000 rpm for 5 minutes and the supernatant was mixed with isopropanol. The precipitated pellet was washed with 70% ethanol, air dried, and dissolved in ddH₂O.

4.3.3 Genotyping PCR

For genotyping of mice, the GoTaq DNA Polymerase (Promega) was used. All the reactions contained 10 pmol of forward and reverse primers (Sigma), and 20 mM of dNTPs (Genecraft). The PCR was done using a Mastercycler® Gradient (Eppendorf). All the primers and the PCR program were designed previously 102.

<table>
<thead>
<tr>
<th>PCR Program for Genotyping:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-65°C</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
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</table>
Table 6. Genotyping primers

<table>
<thead>
<tr>
<th>Genotyping Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gmnn allele common forward</td>
<td>GAAAAGCGACAGGCAGTTGAG</td>
</tr>
<tr>
<td>Gmnn wild type allele reverse</td>
<td>GTCCCAAGGAGAACGCTGAAG</td>
</tr>
<tr>
<td>Gmnn Floxed allele reverse</td>
<td>CAGCGCATCGCCTTCTATC</td>
</tr>
<tr>
<td>Gmnn Knockout allele reverse</td>
<td>GTACCAGGAACTGAGCTGAG</td>
</tr>
<tr>
<td>Cre forward</td>
<td>ATGCTTCTGTCCGTTTGCCG</td>
</tr>
<tr>
<td>Cre reverse</td>
<td>CCTGTTTTGCACGTTCAACC</td>
</tr>
</tbody>
</table>

4.3.4 DNA electrophoresis

0.8 -1.5 % (w/v) Agarose was dissolved in the TBE buffer in order to prepare the gels. Electrophoresis was conducted according to previously established protocols\textsuperscript{102}.

4.3.5 RNA extraction

Cells were washed with PBS twice and were scraped in RNAlater™ buffer (Qiagen). For extraction cells were centrifuged and the pellet were lysed in RLT buffer supplemented with β-mercaptoethanol according to the instruction provided for RNeasy™ mini kit (Qiagen). RNA extraction was performed with RNeasy™ mini kit (Qiagen) and extracted RNA was treated with RNase free DNase (Qiagen). The concentration of the extracted RNA was measured and RNA samples were frozen as 5 -10 μl aliquots.

4.3.6 Reverse transcription of mRNA

1-2 μg of each RNA sample was used to prepare cDNA. Reverse transcription was conducted with the Omniscript™ kit (Qiagen), random primers (Promega), oligo(dT) Primers (Promega) and RNasin™ RNase inhibitor (Promega) according to the instruction provided by manufacturers. Negative samples were prepared with addition of all required components except reverse transcriptase (provided in the Omniscript™ kit).
4.3.7 Semi-quantitative analysis of gene expression

For the amplification of cDNA the GoTaq DNA Polymerase (Promega) was used. All the reactions contained 10 pmol of forward and reverse primers and 20 mM of dNTPs (Genecraft) and the PCR was done using a MastercyclerR Gradient (Eppendorf).

### Table 7. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geminin</td>
<td>forward GCA GAG AAA ATG AGT TGC CAA</td>
<td>Provided by Dr. A. Klimke</td>
</tr>
<tr>
<td></td>
<td>reverse ACT CAG CCT CTC GAT TAC C</td>
<td></td>
</tr>
<tr>
<td>Oct4</td>
<td>forward CTG AGG GCC AGG CAG GAG GAG</td>
<td>Provided by Dr. A. Klimke</td>
</tr>
<tr>
<td></td>
<td>reverse CTG TAG GGA GGG CTT CGG GAC TT</td>
<td></td>
</tr>
<tr>
<td>Pax6</td>
<td>forward TCA CAG CGG AGT GAA TCA G</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse CCC AAG CAA AGA TGG AAG</td>
<td></td>
</tr>
<tr>
<td>Nanog</td>
<td>forward CAC CCA CCC ATG CTA GTC TT</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse ACC CTC AAA CTC CTG GTC CT</td>
<td></td>
</tr>
<tr>
<td>Zfp42</td>
<td>forward TGT CCT CAG GCT GGG TAG TC</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>reverse TGA TTT TCT GCC GTA TGC AA</td>
<td></td>
</tr>
<tr>
<td>Brachyury</td>
<td>forward CGC TGT GAC TGC CTA CCA GAATG</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse GAG AGA GAG CGA GCC TCC AAA C</td>
<td></td>
</tr>
<tr>
<td>HNF4α</td>
<td>forward CTT CCT TCT TCA TGC CAG</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse ACA CGT CCC CAT CTG AAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward CCA TGT TTG TGA TGG GTG TGAACC</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse TGT GAG GGA GAT GCT CAG TGTTGG</td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td>forward TCA CTG TGC CTG AAC TTA CC</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse GGA ACA TAG CCG TAA ACT GC</td>
<td></td>
</tr>
</tbody>
</table>
4.3.8 Quantitative analysis of gene expression (qPCR)

mRNA of interest was quantified by real time quantitative PCR using SYBER Green. Reactions were held in triplicates and each reaction contained 25 ng cDNA, 10 μl KAPA SYBR™ FAST qPCR Master Mix (Qiagen) and 4 pmol of each primer in ddH₂O. The PCR was done using a Realplex² thermocycler (Eppendorf). The relative abundances of the mRNA was calculated based on the $\Delta\Delta$CT method and the melting curve analysis was performed in order to make sure that a unique product is amplified. Negative samples were analyzed as single reactions in order to rule out the possibility of contaminations.

Table 8. qPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geminin1</td>
<td>forward GCA GAG AAA ATG AGT TGC CAA</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>reverse ACT CAG CCT CTC GAT TAC C</td>
<td></td>
</tr>
<tr>
<td>Geminin2</td>
<td>forward ACGCTGAAGATGATCCAGCCTTCT</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>reverse TAGCTGGTCATCCCAAAGCTTCTT</td>
<td></td>
</tr>
<tr>
<td>Oct4</td>
<td>forward CTG AGG GCC AGG CAG GAG CAC GAG</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>reverse CTG TAG GGA GGG CTT CGG GAC TT</td>
<td></td>
</tr>
<tr>
<td>Nanog</td>
<td>forward CAC CCA CCC ATG CTA GTC TT</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>reverse ACC CTC AAA CTC CTG GTC CT</td>
<td></td>
</tr>
<tr>
<td>Zfp42</td>
<td>forward TGT CCT CAG GCT GGG TAG TC</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>reverse TGA TTT TCT GCC GTA TGC AA</td>
<td></td>
</tr>
<tr>
<td>Sox2-tot</td>
<td>forward GGT TAC CTC TTC CTC CCA CTC CAG</td>
<td>Provided by Dr. K. Boese</td>
</tr>
<tr>
<td></td>
<td>reverse TCA CAT GTG CGA CAG GGG CAG</td>
<td></td>
</tr>
<tr>
<td>Sox2-endo</td>
<td>forward TAG AGC TAG ACT CCG GGC GA TGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reverse TTG CCT TAA ACA AGA CCA CGA AA</td>
<td></td>
</tr>
<tr>
<td>Ubiquitin C</td>
<td>forward AGGTCAACACAGGAAGACAGACGTA</td>
<td><a href="http://www.rtprimerdb.org">www.rtprimerdb.org</a></td>
</tr>
<tr>
<td></td>
<td>reverse TCACACCCAAGAAACAAAGCACA</td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>forward GTC CTG TGG CCA TCT GCC TA</td>
<td><a href="http://www.rtprimerdb.org">www.rtprimerdb.org</a></td>
</tr>
<tr>
<td></td>
<td>reverse GGG ACG CAG CAA CTG ACA TT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward CCA TGT TTG TGA TGG GTG TGAACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reverse TGT GAG GGA GAT GCT CAG TGTGG</td>
<td></td>
</tr>
</tbody>
</table>
4.3.9 Chromatin immuno-precipitation (ChIP)

ESCs were washed with PBS and the chromatin was cross-linked by exposure to 1% formaldehyde (Pierce) for 10 min. The cross-linked chromatin was harvested in lysis buffer and sonicated using a Bioruptor® XL sonicator (Diagenode) for 35 min (30 sec on/off) at 4 °C. Sonicated chromatin was examined for the size of the sonicated fragments by reverse cross-linking and electrophoresis. The sonicated chromatin was diluted with dilution buffer and pre-cleared using Protein A/G Agarose beads (Santa Cruz Biotechnologies). After pre-clearing the beads were separated with centrifugation, the samples were divided in different Eppendorf tubes and 10% of the sample volumes were kept at 4 °C as “input samples”. 1-2 μg of primary antibodies were added to each Eppendorf tube and were incubated on the shaker at 4 °C overnight. Protein A/G Agarose beads (Santa Cruz Biotechnologies) were added to antibody-chromatin complexes for 2 hours at 4°C. Beads were washed once with Low Salt Immune Complex Wash Buffer, once with High Salt Immune Complex Wash Buffer, once with LiCl Immune Complex Wash Buffer and twice with TE Buffer. DNA was eluted from the beads and the samples were reverse cross-linked and treated with RNase and proteinase K. DNA was extracted with the PCR purification kit (Qiagen). Further information regarding the buffer compositions, detailed steps and troubleshooting can be found in the EZ ChIP™, Chromatin Immuno-precipitation Kit’s Instruction Manual (Upstate, Millipore). Used Antibodies are listed in the tables below.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone 3</td>
<td>Abcam</td>
</tr>
<tr>
<td>Histone 3 lysine 4 trimethylation</td>
<td>Active motif</td>
</tr>
<tr>
<td>Histone 3 lysine 27 trimethylation</td>
<td>Active motif</td>
</tr>
<tr>
<td>Histone 4 hyperacetylation</td>
<td>Millipore</td>
</tr>
<tr>
<td>EZH2</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>BRG1</td>
<td>Santa Cruz Biotechnologies</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Santa Cruz Biotechnologies</td>
</tr>
</tbody>
</table>
4.3.10 Quantitative analysis of ChIP

Extracted precipitated DNA was analyzed by qPCR as explained before. The relative fold enrichment was calculated with $\Delta\Delta CT$ method\textsuperscript{137} primer specific efficiencies (AB: antibody precipitated sample, Tx: tamoxifen treated, control: untreated cells).

$$\text{Fold enrichment} = \frac{\text{primer efficiency}}{\frac{Ct(\text{input,Tx})-Ct(\text{AB,Tx})}{Ct(\text{input,control})-Ct(\text{AB,control})}}$$

Primer-specific efficiencies (1.90 -2) were calculated from the standard curve made by measurements of the serial dilutions of the input samples.

**Table 10.** Primers for the detection of Sox2 and Oct4 genomic regions used in ChIP analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Forward CATAGCGTGTCAGTGATCTCC</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>Reverse GCTTCCAAACCCATCCTTACAG</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Forward CCCTCCTCTCTCTAATCTCCTTATGG</td>
<td>109</td>
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<tr>
<td></td>
<td>Reverse AACTCTCATAGCCCCTAACTGTC</td>
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</tr>
<tr>
<td>C</td>
<td>Forward GAGTTCCAGCTTTGCGTTTG</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>Reverse TTGTCCAGGCCCTTTTCTTAG</td>
<td></td>
</tr>
<tr>
<td>D</td>
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<tr>
<td></td>
<td>Reverse CCCTTCCAGCTCCTTACCC</td>
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<td>E</td>
<td>Forward GAGCTGAGTCGGGTCAATTA</td>
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<td>Reverse AGGCTGAGTCGGGTCAATTA</td>
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<tr>
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<tr>
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<td>Reverse ATCTCTCTGGCCCTCTCCCAT</td>
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Curriculum Vitae

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Publication