Balancing potency and differentiation in mouse embryo-derived stem cells and *in vivo*

By

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Abstract

An inherent challenge for developing organisms is to maintain a critical balance between cell potency and differentiation. This is most obvious in embryonic stem (ES) cells where genetic, epigenetic and cell signalling pathways support ES cell ability to self-renew and to generate all embryonic lineages. Recently, a series of reports revealed how Polycomb-mediated repression might buffer the precocious expression of somatic lineage regulators in ES cells. Notably, these genes carry bivalent chromatin enriched in both repressive (H3K27me3) and permissive (H3K4me2) histone marks. They are targeted by the Polycomb repressive complexes PRC1 (Ring1B) and PRC2, which mediates H3K27me3 and assemble poised RNA polymerase II (RNAP II), conferring silencing of loci primed for future activation (or repression) upon ES cell differentiation.

During early development the transition from morula to blastocyst is the starting point for lineage segregation into the inner cell mass (ICM) and trophectoderm (TE). ES cells are derived from the ICM and are pluripotent. By contrast, TE-derived trophoblast stem (TS) cells are multipotent and contribute solely to placenta formation in vivo. To address whether ES cells epigenetic features are unique attributes of pluripotent cells in the early embryo, we compared the epigenetic status of key developmental genes in blastocyst-derived stem cells and in vivo. We provide direct evidence that bivalent histone markings operate in vivo from the eight-cell up to the blastocyst stage. Unexpectedly, we show that bivalent domains are retained at many somatic lineage regulators in extra-embryonic restricted cells. However, and in contrast to pluripotent cells, PRC1 (Ring1B) and poised RNAP are excluded from these PRC2-bound genes, consistent with a loss of gene expression priming. Instead, bivalent genes become selectively targeted by Suv39h1-mediated repression upon trophoblast lineage commitment. Collectively, our results suggest a mutually exclusive role for Ring1b and
Suv39h1 in specifying the fate of bivalent genes in a lineage-specific manner upon blastocyst formation and in stem cells.

The transcription factor Nanog is a key part of the core genetic network that sustains self-renewal in ES cells. In contrast, the Fgf/Erk signalling pathway primes ES cell for differentiation. How pluripotency-associated intrinsic factors may modulate the effect of extracellular pathways is yet to be investigated. We propose that Nanog can block autocrine Fgf/Erk responses while maintaining paracrine signalling in ES cells through direct repression and activation of Fgfr2 and Fgf4, respectively. Absence of Nanog notably enables an up-regulation of Fgfr2 expression and higher levels of active phosphorylated Erk1/2 forms. Moreover, we demonstrate that ectopic expression of constitutively active Fgfr2 in Nanog over-expressing cells is sufficient to partly bypass ES cell undifferentiated state, further highlighting the delicate balance between self-renewal and differentiation in pluripotent stem cells.
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Declaration of Originality

The work presented in this thesis was conducted by the author and any contribution from other members is referenced below.

Notably in Chapters 3 and 4, collection of mouse *in vivo* embryo samples and subsequent quantitative RT-PCR and C-ChIP analysis were carried out by Dr Olivia Alder. Ring1B ChIP analysis on embryonic and trophoblast stem cells was done by Dr Emily Brookes. MeDIP ChIP analysis on both embryonic and trophoblast stem cells was carried out by Dr Sandra Pinho. Suv39h1 RNAi treatment and quantitative RT-PCR analysis of trophoblast stem cells was performed by Dr Fabrice Lavial.
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<th>Full Form</th>
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<tbody>
<tr>
<td>μ</td>
<td>micro-</td>
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<tr>
<td>5mC</td>
<td>5’ methylcytosine</td>
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<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BS</td>
<td>Binding site</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>Celsius</td>
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<tr>
<td>c</td>
<td>Centi-</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>C-ChIP</td>
<td>Carrier chromatin immunoprecipitation</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>C(T)</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy-nucleoside triphosphate</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid Body</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Epi</td>
<td>Epiblast</td>
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<td>EpiSC</td>
<td>Epiblast stem cells</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorted</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MeDIP</td>
<td>Methylated DNA immunoprecipitation</td>
</tr>
<tr>
<td>Mef</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>nano-</td>
</tr>
<tr>
<td>N.D.</td>
<td>Not detectable</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>Ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P 40</td>
</tr>
<tr>
<td>NPCs</td>
<td>Neural progenitor cells</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate 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>PDVF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGC</td>
<td>Primordial germ cells</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repressive complex</td>
</tr>
<tr>
<td>PrE</td>
<td>Primitive endoderm</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio Immuno Precipiation Assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNAP II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>S.d.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>Ser5P</td>
<td>Phosphorylated serine position 5</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-Buffered Saline with 0.5% Tween20</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td>TET</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Tris</td>
<td>(hydroxymethyl)methyamine</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl Rhodamine Iso-Thiocyanate</td>
</tr>
<tr>
<td>TrxG</td>
<td>Trithorax group</td>
</tr>
<tr>
<td>TS cells</td>
<td>Trophoblast stem cells</td>
</tr>
<tr>
<td>TSL cells</td>
<td>Trophoblast stem-like cells</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>w/v</td>
<td>mass/volume</td>
</tr>
<tr>
<td>XEN</td>
<td>Extra-embryonic endoderm cells</td>
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</table>
Chapter 1 Introduction

Mammalian development is a remarkably regulated and co-ordinated process, which originates from a single cell. Formed from the fusion of two highly differentiated cells, sperm and egg, totipotent zygotic cells contain all that is necessary to enable the formation of a complex multicellular organism. Adult humans and mice are comprised of just over 200 specialized cell types ranging in function from skeletal muscle to sensory neuron. A fundamental and complex question for developmental biologists is how these genetically identical cells establish such diverse cell identities during development and how these are stably maintained.

An early proposal to explain the workings of development in 1892 suggested that unnecessary heritable elements (genes) were lost during cell specification (Weismann, 1892). So following this argument, a kidney cell would retain only the genetic information required for a kidney cell to function. In 1962, Gurdon and colleagues proved this theory to be inaccurate by showing that the nucleus from differentiated *Xenopus laevis* somatic cells could be transplanted into enucleated fertilized frog oocytes and these developed into, normal tadpoles and adult frogs (Gurdon, 1962). These experiments demonstrated that throughout progressive restriction of cell fate choice, each of our cells retain a complete set of genetic material and that any accumulated genetic changes are reversible. In recent years this has been further validated in mammals by the cloning of Dolly the sheep, cattle and mice from mature adult cells into enucleated oocytes (Gibelli et al., 1998; Hochedlinger and Jaenisch, 2002; Vignon et al., 1998; Wilmut et al., 1997). Therefore as we now know, differentiated states of functionally distinct cell types must arise from the selective expression of only subsets of genes encoded by the genome.
The milestone completion of the human genome project (Lander et al., 2001; McPherson et al., 2001) provided the DNA sequence that encodes all the information required to orchestrate the creation of an entire human organism. However, what regulator mechanisms are involved in decoding this information so as to establish and maintain distinct, reproducible, transcriptional programs during cell specification still requires elucidating. As will be discussed here the potency and differentiation of any given cell during development is controlled by a balanced amalgam of genetic, epigenetic and environmental influences.

1.1 Early mouse development

1.1.1 Mouse embryo pre-implantation development

The height of cell potency arises upon the fusion of the oocyte and sperm in the zygote. The newly formed zygote is a clearly totipotent cell within the mammalian life cycle with potential to create every cell type of a living organism as well as all the specialized extra-embryonic cells (Hemberger et al., 2009). Throughout the process of embryo development and beyond specific programmes of gene expression are established and progenitor cells progressively lose their developmental potential while acquiring different lineage-specific functions (Reik, 2007).

Due to ethical reasons there is limited access to human embryos and in vivo tissue, therefore, the steps leading to the earliest lineage decisions in mammals have primarily been studied in mice (Pera and Trounson, 2004). After fertilization, the mouse zygote undergoes three rounds of cell division during the first two days of development. At the eight-cell stage, the cells or blastomeres show no obvious morphological differences and maximize cell-to-cell contact to form the compact morula. Asymmetrical cell divisions at the morula stage along a basolateral cleavage plane accompanies the first differentiation event in the mouse embryo, which generates an internal cavity, the blastocoel and two visibly distinct cell populations at embryonic day 3.5 (E3.5) (Figure
1.1). The smaller inner cells form the inner cell mass (ICM) while the larger polarized outer cells become allocated to the extra-embryonic trophectoderm (TE) layer (Arnold and Robertson, 2009). Extensive lineage tracing and chimera studies in mice have shown that ICM cells are pluripotent, giving rise to all fetal tissues, while TE cells have a more restricted lineage potential (multipotent), with those overlying the ICM expanding to form progenitors contributing solely to placenta formation (Rossant, 2001; Takahashi and Yamanaka, 2006; Yamanaka et al., 2010). TE cells away from the ICM cease division but continue to endoreduplicate their DNA to form polyploid trophoblast giant cells. Around the E3.5 stage, the ICM of the implanting blastocyst segregates further into the epiblast (EPI) or primitive ectoderm covered by a second extra-embryonic lineage called the hypoblast or primitive endoderm (PrE), that lines the blastocoelic surface (Chazaud et al., 2006). EPI cells are also pluripotent, further developing into the embryo proper upon gastrulation. During this process the embryo forms the three primary germ layers (ectoderm, mesoderm and endoderm) as well as primordial germ cells (PGC). PrE cells are multipotent (like the TE) and primarily differentiate into the parietal and visceral endoderm layers that will line the yolk sac after implantation (Bielinska et al., 1999; Plusa et al., 2008; Rossant, 2001). Both the PrE and EPI cells at this stage are lineage restricted because they contribute only to their respective lineages in chimeric embryos (Gardner, 1982; Gardner, 1984; Gardner and Rossant, 1979). As well as facilitating maternal-fetal interactions to supply nutrients for and protecting the embryo within the uterus, both extra-embryonic TE and PrE lineages are important sources of signals for establishing correct anterior-posterior patterning prior to gastrulation (Rossant and Tam, 2009).

The germ cells that originate from the proximal epiblast at E6.25 and migrate to the developing gonads represent the end point of a differentiation cascade and can be considered highly specialized. Simultaneously, however, they have the capacity to return to totipotency (as a consequence of germ cell-specific epigenetic reprogramming) when
the gametes fuse together to form a new zygote and the life cycle continues (Hemberger et al., 2009).

**Figure 1.1: Schematic representation of the early stages mouse blastocyst development and lineage segregation.** Following fertilization, developmental progression from the eight-cell embryo sees a compaction step in which the cell boundaries of the early compacted morula are no longer visible. Around E3.5, the blastocyst gives rise to an external extra-embryonic layer, the trophectoderm (TE; green), surrounding an internal cavity, the blastocoel and a mosaic of cells, the inner cell mass (ICM; blue and purple). Approximately a day later the ICM further segregates into the lineage restricted primitive endoderm (PE; dark blue) and pluripotent primitive ectoderm or epiblast (Epi; light blue). The polar trophectoderm cells, overlying the ICM, continue to proliferate giving rise to the diploid extra-embryonic ectoderm (Exe; green) and ectoplacental cone (Epc; green) by E5.5. The mural trophectoderm cells (away from the ICM) stop dividing but continue to endoduplicate their DNA to form trophoblast giant cells and facilitate implantation. The PE cells in contact with the Epi differentiate into the visceral endoderm (VE; dark blue) and distal visceral endoderm (DVE; yellow). Those adjacent to the trophectoderm differentiate to become parietal endoderm (ParE; blue white gradient) cells, which migrate over the blastocoelic surface of the trophectodermal basement membrane. The Epi will further differentiated into three germ layers upon gastrulation. Embryo stages are not drawn to scale. (Adopted from Rossant and Tam, 2009).

### 1.1.2 Embryo-derived stem cells

Pluripotency is a transitory state of embryonic cells within the blastocyst that exists only for a brief time during development. However, recently it has become
possible to isolate ex vivo pluripotent embryonic stem (ES) cells from the blastocyst ICM and maintain them in culture under defined growth conditions. First isolated in 1981, mouse ES cells exhibit two remarkable features in culture (Evans and Kaufman, 1981; Martin, 1981). First, in the right conditions, they can be propagated indefinitely in a self-renewing state keeping their cell identity. This immortalized phenotype allows ES cell populations to be cultured and expanded over extended periods of time. The second feature is that, even after prolonged undifferentiated proliferation and/or clonal derivation, upon introduction back into a blastocyst, mouse ES cells retain their developmental potential to contribute to all three germ layers, as those naturally formed by the in vivo ICM (Martin, 1981). Human ES cell derivation in 1998 was considerably later primarily due to species-specific ES cell differences and suboptimal human embryo culture conditions (Thomson et al., 1998; Yu and Thomson, 2008). The differential potential of ES cells, particularly for human ES cells where cell transfer to form chimeras is not permitted, has also been demonstrated by injection into immunologically compromised adult mice where they form teratomas, which include cells derivative of all three embryonic layers. Since their initial derivation in the 1980s, mouse ES cells have also been derived from morula stage embryos and even from individual blastomeres of 2- to 8-cell stage embryos (Chung et al., 2006; Wakayama et al., 2007).

Stem cell lines have also been isolated from the two other lineages of the mouse blastocyst, the TE and PE. From these tissues researchers have successfully derived the trophoblast stem (TS) cells (Tanaka et al., 1998) and extra-embryonic endoderm (XEN) cells (Kunath et al., 2005). These progenitor cell lines share a similar stem cell characteristic: they can stably self-renew in culture without differentiating while faithfully mimicking the developmental properties of their associated lineage. In accordance with their different embryonic origins these stem cell lines are remarkably different in terms of their morphology, gene expression profiles and growth factors requirements for amplification (Rossant, 2008). Pluripotent mouse ES cells form
concave colonies maintained in culture media supplemented with leukemia inhibitory factor (LIF) and fetal calf serum (FCS). While epithelial-like TS cell colonies are either grown on a feeder layer of primary mouse embryonic fibroblasts (MEFs) or in MEF conditioned media, both with the addition of fibroblast growth factor 4 (Fgf4) and heparin to sustain cell growth (Tanaka et al., 1998). XEN cells on the other hand require exogenous Fgf for their derivation, but not for their ongoing maintenance (Kunath et al., 2005). ICM-derived ES cells contribute to all tissues of the chimera fetus, but poorly to the PE lineages and rarely to the TE lineage (Beddington and Robertson, 1989). TS cells solely contribute to the trophoblast lineages of the placenta (Tanaka et al., 1998) and XEN cells contribute exclusively to the visceral and parietal endoderm lineages (Kunath et al., 2005). Although the molecular mechanisms underlying lineage choice and maintenance are not fully understood, blastocyst-derived stem cells serve as a useful model system to understand this question (Niwa, 2010). Several lineage-specific transcription factors have unambiguously been identified as key regulators of cell identity in the early embryo. For instance, the individual and/or synergistic function of factors like Pou5f1 (a POU domain-containing factor also known as Oct4 encoded by Pou5f1)/Sox2/Nanog, Cdx2/Eomes/Gata3 and Gata6/Gata4/Sox7 have been shown to play central roles in maintaining ES, TS and XEN cells, respectively (Home et al., 2009; Ralston and Rossant, 2005; Rossant, 2008). Simple mis-expression of these transcription factors can convert ES cells into TS-like cells (Niwa et al., 2005) or XEN-like cells (Shimosato et al., 2007) which is discussed in greater detail in section 1.2.1.

Recently, two groups isolated epiblast stem cells (EpiSC) from the epiblast lineage at the E5.5-E6.5 post-implantation mouse embryo (Brons et al., 2007; Tesar et al., 2007). Though pluripotent, this cell line proliferates in the presences of Fgf and activin rather than LIF, that is required by ES cells. Similar to ES cells, EpiSCs are able to differentiate into all three germ layers in vitro and to form teratomas. In contrast to ES cells, EpiSCs rarely contribute to form mouse chimeras upon introduction into blastocysts (Brons et
1.2 Transcriptional control of pluripotency

1.2.1 Transcription factors and lineage identity in stem cells and the early embryo

Transcription factors are proteins that recognise specific DNA sequences and either participates in the activation or suppression of particular genes or gene families. The restricted expression of key transcription factors has been shown in recent years to strongly specify and/or maintain in vivo blastocyst lineages and their respective stem cell identities. Both Pou5f1 and the homeodomain-containing protein, Nanog have long been recognized as essential gatekeepers of pluripotency in the ICM and ES cells, mostly...
by repressing extra-embryonic cell fates (Figure 1.2) (Chambers et al., 2003; Chambers and Smith, 2004; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000). Their expression persists in the embryo proper until gastrulation where they become restricted to developing germ cells. *Pou5f1* seems to play a dual role in the ICM. Deletion of *Pou5f1* in mice results in embryonic lethality due to a failure to maintain the ICM, and the cells of this tissue divert towards the TE lineage (Nichols et al., 1998). Similarly, the *in vitro* deletion of Pou5f1 leads to the conversion of ES cells to TS-like cells (Niwa et al., 2000), indicating that *Pou5f1* is required to inhibit TE lineage specification. However, the precise levels of Pou5f1 appears to play an important role in regulating ES self-renewal as in these cells *Pou5f1* over-expression promoted differentiation towards an extra-embryonic PE or mesoderm cell fate (Niwa et al., 2000). Nanog also plays a critical role in maintaining pluripotency. Nanog-null embryos also initially produce pluripotent cells, but shortly after their specification these cells differentiate into the PE lineage (Chambers et al., 2003). Interestingly, Nanog-deficient mouse ES cells are still able to self-renew in culture, although they show an increased propensity to differentiate towards PrE-like cells and cannot contribute to mature germ cells (Chambers et al., 2003; Chambers et al., 2007). Further support for the role of Nanog in maintaining pluripotency came from the observation that Nanog over-expression is capable of conferring LIF-independent self-renewal in ES cells (Chambers et al., 2003). A third factor that has been shown to be a key regulator of pluripotency is the SOX-family transcription factor Sox2 which synergistically functions as a heterodimer with Pou5f1, stimulating the expression of self-renewal-associated factors and repressing differentiation-linked genes (Ambrosetti et al., 2000; Chew et al., 2005; Okumura-Nakanishi et al., 2005; Tomioka et al., 2002). The presence of maternally-derived Sox2 protein makes it difficult to study the function of Sox2 in early embryonic development, however after implantation, when the maternal contribution is largely lost, Sox2-null embryos generate solely TE and PE lineages (Avilion et al., 2003). The deletion of Sox2
in ES cells also leads to their conversion into TS-like cells (Masui et al., 2007). Recently, it was proposed that Sox2 might stabilize pluripotent ES cells by maintaining the required level of Pou5f1 expression (Masui et al., 2007).

Cdx2, the caudal-related homeodomain protein, is a key regulator of the TE lineage as demonstrated by the block in trophoblast differentiation after blastocyst formation in Cdx2-null mouse embryos (Strumpf et al., 2005). Surprisingly, Cdx2 is not necessary for the initial generation of the TE lineage, which might illustrate redundancy, most likely with the transcription factors TEA domain/transcription enhancer factor 4 (TEAD4) and Eomesodermin (Eomes) (Nishioka et al., 2008; Ralston and Rossant, 2008; Strumpf et al., 2005; Yagi et al., 2007). Interestingly, knockdown of Cdx2 by RNAi at the two-cell stage can generate a stronger phenotype than the zygotic mutant, leading to the proposal that a maternal pool of Cdx2 mRNA might also exist (Zernicka-Goetz et al., 2009). However, these findings have proven controversial as genetic depletion of maternal and zygotic Cdx2 did not affect TE specification (Wu et al., 2010). First detected in a non-uniform manner in the eight-cell stage embryo, Cdx2 becomes progressively restricted to the outer TE layer (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). In ES cells that usually do not differentiate towards the TE lineage, induced expression of Cdx2 promotes TE differentiation and the acquisition of TS cells properties (Niwa et al., 2005). It is noteworthy that this was achieved even in the sustained presence of Pou5f1, demonstrating that an imbalance between the two transcription factors is sufficient to influence cell identity. It has thus been proposed that Cdx2 functions to enhance the transcription of target loci associated with the TE lineage while suppressing the expression of pluripotent ICM linked Pou5f1 (Ralston and Rossant, 2005). Indeed mutual inhibition between Pou5f1 and Cdx2 coupled to the auto-regulative nature of the regulation of these factors is thought to amplify an initial small imbalance in the expression of these factors and result in their reciprocal expression pattern in the ICM and TE, respectively (Figure 1.2; (Dietrich and Hiiragi, 2007; Niwa et
Antagonism between key transcription factors for lineage decisions.

Cdx2 is proposed to promote TE (dark blue) fate commitment and maintenance in the early embryos, partly by repressing the ICM (light blue) gene *Pou5f1* after TE lineage formation. Reciprocal inhibition between Nanog and Gata6 is likely to drive the segregation between Epi (light blue) and PrE (orange) within the ICM.

The second blastocyst lineage choice, the segregation of the ICM into the pluripotent Epi and extra-embryonic PE by E4.5, is also influenced by essential transcription factors. The lineage transcription factors Nanog and Gata6, first form a mutually exclusive ‘salt and pepper’ mosaic pattern in the E3.5 ICM prior to sorting and relocation into the Gata6 positive PE epithelial layer covering the Nanog positive pluripotent Epi at E4.5 (Figure 1.1; Chazaud et al., 2006; Gerbe et al., 2008). As occurs with previously mentioned transcription factors, alterations in the expression levels of Nanog and Gata6 can lead to switching of cell phenotypes; loss of Nanog and overexpression of Gata6 drive mouse ES cells towards a XEN-like phenotype (Mitsui et al., 2003). Conversely, Gata6 null embryos form an ICM/epiblast, but no morphologically recognizable PE layer and thereafter experience embryonic lethality around E6.5 to E7.5 (Morrisey et al., 1998). A frequently used *in vitro* test of a factor’s influence over PrE formation is the derivation of round differentiated cell clumps called embryoid bodies (EBs). They form from ES cells cultured in suspension and characteristically develop an outer PE layer surrounding a mixture of differentiated cells from the three germ layers. These EBs can be generated from genetically manipulated or chemically treated ES cells.
allowing them to be used for functional assays. ES cells lacking Gata6 were unable to generate a distinguishable PrE-epithelial layer upon EB differentiation and failed to express genes encoding early or late endodermal markers (Morrisey et al., 1998). Overall, these studies highlight the importance of Gata6 for PrE emergence and maintenance during early mouse blastocyst development. Intriguingly, Nanog has been shown to directly repress Gata6 expression (Singh et al., 2007), while Gata6 overexpression induces a reduction in Nanog expression, supporting the presence of reciprocal inhibition between Nanog and Gata6 to promote ICM segregation into distinct Epi and PrE cell lineages (Figure 1.2 (Ralston and Rossant, 2005).

1.2.2 Core regulatory transcriptional networks

Research efforts have recently focused on gaining insights into the transcriptional networks regulating pluripotency. A transcriptional circuitry has been proposed that principally involves the collaboration of core transcription factors Pou5f1, Nanog and Sox2 to maintain pluripotency in ES cells (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008; Loh et al., 2006; Wang et al., 2006). Genome-wide analysis using chromatin immunoprecipitation (ChIP) to map the binding sites of Pou5f1, Nanog and Sox2 targets in mouse and human showed that these three factors co-occupy the promoters of a large number of developmental genes that are either transcriptionally activated or repressed (Boyer et al., 2005; Loh et al., 2006). Interestingly, among the targeted genes, these core factors were shown to positively regulate their own promoters, generating interconnected auto-regulatory loops. Other active targets include transcription factors, chromatin remodeling factors and components of signalling cascades identified as crucial for ES cell biology. Conversely, the Pou5f1/Sox2/Nanog core factors also contributed to the inactivation of targets encoding lineage-specific transcription factors whose absence helps prevent differentiation towards ectoderm, endoderm, mesoderm and extra-embryonic lineages. Other studies mainly focused on identifying ES cell self-
renewal factors by using RNAi and/or genome-wide transcriptome analysis uncovered Esrrb, Tbx3, Tcl1, Dppa4, Rif1, Dax1, Sall4 and Prdm14 as additional regulators of pluripotency, thus extending the complexity of the network that controls this process (Chia et al., 2010; Ivanova et al., 2006; Kim et al., 2008; Loh et al., 2006; Niakan et al., 2006; Sakaki-Yumoto et al., 2006). Interestingly, the discovery by Takahashi and Yamanaka that somatic cells can be reprogrammed to induced pluripotent stem (iPS) cells by ectopic expression of Pou5f1, Sox2, Klf4 and c-Myc, suggested that the proto-oncogene c-Myc and Klf-4 may also play a role in the regulatory circuitry of pluripotency (Takahashi and Yamanaka, 2006). How exactly these factors function to establish and/or maintain self-renewing, pluripotent cells still remains poorly understood.

In addition to their role as part of the pluripotency transcriptional circuit it is also clear that key individual members, like Pou5f1 and Sox2, also have lineage-specific roles and therefore can orchestrate germ layer fate decisions (Thomson et al., 2011). These observations led to the proposal that pluripotency, a transient state in vivo, is inherently unstable and that many transcription factors are in fact continually competing to specify differentiation towards a specific lineage, while simultaneously blocking commitment to mutually exclusive cell fates (Loh and Lim, 2011). For instance, it is thought that Pou5f1 specifically promotes mesodermal differentiation and represses neuroectodermal cell fate, while Sox2 does the opposite, and together, they repress differentiation towards either germ cell fate (Thomson et al 2011). The suggested cooperation between multiple regulators all working to inhibit each other’s preferred lineage choices while striving for dominance implies that these counteract one another and in this way create the careful balancing act of pluripotency.
1.2.3 Transcription factor heterogeneity of pluripotent cell populations

Until recently, mouse ES cells populations were thought to be homogeneous. Numerous studies now show that pluripotent ES cells in culture exist in different, inter-convertible subpopulations of cells and that this heterogeneity has significant consequences for their ability to self-renew and differentiate. The expression levels of Nanog, Rex1 (Zfp42; an ICM marker), Pecam1 (CD31; platelet/endothelial cell adhesion molecule 1) and Stella (an ICM and germ cell lineage marker) are all highly dynamic in mouse ES cells (Chambers et al., 2007; Furusawa et al., 2006; Furusawa et al., 2004; Hayashi et al., 2008; Singh et al., 2007; Toyooka et al., 2008). Recent work has suggested that even pluripotency-associated factors Pou5f1 and Sox2 factors are variably expressed in mouse ES cell populations, and that this variability creates distinct differentiation biases (Loh and Lim, 2011). Cell populations expressing, for instance, low levels of or lacking Nanog (10-20% of the total cell population) have an increased tendency to differentiate compared to ES cell populations with higher Nanog levels (80% of the overall culture population)(Chambers et al., 2007; Mitsui et al., 2003). Knock-in of the green fluorescent protein (GFP) gene into the Nanog locus established that the mosaic expression in ES cells is primarily determined at the transcriptional level, not related to the cell cycle and is reversible (Chambers et al., 2007). This data also supports the idea of a pluripotency platform where cross-inhibition between opposing fluctuating transcription factor circuits is required to inhibit a possible single lineage specification program and maintain self-renewal. Occasional spontaneous differentiation observed in mouse ES cell culture emphasizes that heterogeneous expression profiles might allow a ‘window of opportunity’ to respond to extrinsic signalling that can prompt differentiation (Graf and Stadtfeld, 2008). However, currently the underlying mechanisms for pluripotent ES cell heterogeneity are unclear. Interestingly, the balance between Nanog-low and Nanog-high ES cell subpopulations
was recently suggested to be epigenetically regulated by heterogeneously expressed polycomb repressive complex (PRC) component, Ezh2, directly repressed the Nanog promoter in Nanog-low ES cell populations (Villasante et al., 2011).

Transcription factors can also be mosaically expressed in vivo. Gata6 and Nanog are expressed in a mutually exclusive manner in the E3.5 ICM as demonstrated by immunohistochemistry and single-cell expression analysis (Chazaud et al., 2006; Kurimoto et al., 2006). Though whether this heterogeneity is highly dynamic in vivo still needs to be determined.

1.3 Cell signalling and pluripotency

1.3.1 Self-renewal signalling in pluripotent stem cells and the early mouse blastocyst

Intra- and extra-cellular signalling cues are crucial for progressive embryo development as well as preserving stem cells in culture. A viable, undifferentiated state of ES cells is promoted by culture media containing fetal calf serum (FCS) supplemented with a cytokine, the leukaemia inhibitory factor (LIF; (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003), to stimulate growth and self-renewal via the Janus kinase/signal transducers and activators of transcription (JAK/STAT3) signalling pathway (Niwa et al., 1998; Yoshida et al., 1994). LIF extrinsically stimulates this pathway by initiating heterodimerization of the LIF receptor with the gp130 membrane protein that leads to activation of JAK tyrosine kinase and subsequent recruitment and phosphorylation of STAT3 (Yoshida et al., 1994). STAT3 then dimerizes before translocating to the nucleus where it binds specific regulatory sequences to suppress differentiation in ES cells (Niwa et al., 1998; Raz et al., 1999). Removal of LIF or expression of the dominant negative STAT3 transgene promotes ES cell differentiation towards an array of specialized cell lineages (Nichols et al., 2001; Niwa et al., 1998), while the provision of constitutively active STAT3 renders mouse ES cells LIF-independent (Matsuda et al,
1999). However, LIF alone is incapable of maintaining ES self-renewal in serum-free media and it has been shown that it requires fetal calf serum (FCS), or the active ingredient in FCS, bone morphogenetic protein 4 (BMP4; a member of the TGF-β superfamily) to prevent differentiation (Ying et al., 2003). BMPs stimulate the phosphorylation of Smad1/5/8, which in turn induces the expression of inhibitor-of-differentiation (Id) proteins (Ying et al., 2003). In normal development, however, there is no evident need for LIF, gp130 or STAT3 prior to gastrulation, and the requirement of this pathway seems to be specific to embryos in diapause (Nichols et al., 2001; Takeda et al., 1997; Yoshida et al., 1996). This leaves open the question whether signalling cascades are required to promote self-renewal in vivo.

A further signalling pathway implicated in the regulation of pluripotency is the fibroblast growth factor/extracellular-signal-related kinase (Fgf/Erk) signalling cascade. Inhibition of this pathway has been shown to be sufficient to maintain pluripotency and remove the requirement for either FCS or BMP to maintain ES cell self-renewal (Burdon et al., 1999; Chen et al., 2006; Ying et al., 2008). The ERK signalling cascade is activated by fibroblast growth factor 4 (Fgf4) and is essential to render ES cells susceptible to further inducible lineage specific signals and ultimately differentiation (Kunath et al., 2007; Stavridis et al., 2007). As a consequence of this requirement, lack of Fgf4 or attenuation of downstream Fgf/Erk signalling impairs differentiation (Burdon et al., 1999; Kunath et al., 2007). This pathway inhibition combined with glycogen synthase kinase-3 beta (GSK3β) inhibition allows the maintenance of ES cells in a basal self-renewal state in serum free media (Ying et al., 2008). Similarly, reduction of Fgf/Erk signalling during blastocyst formation suppresses the development of the PrE and results in the entire ICM adopting an epiblast fate (Chazaud et al., 2006; Nichols et al., 2009; Yamanaka et al., 2010).

The canonical Wnt/β-catenin pathway has also been implicated in the maintenance of pluripotency (Sato et al., 2004; Ying et al., 2008) and nuclear
accumulation of β-catenin leads to activation of self-renewal regulators such as Pou5f1, Nanog and Stat3 (Hao et al., 2006). GSK3 inhibits the Wnt signalling pathway by phosphorylating β-catenin, enabling its ubiquitylation and subsequent proteolysis (Aberle et al., 1997; Rubinfeld et al., 1996). This suggested that the requirement for GSK3β inhibition along with Fgf/Erk signalling blockage to maintain pluripotency is via regulation of the Wnt/β-catenin pathway. However, surprisingly the role of GSK3 inhibition in maintaining pluripotency does not involve the regulation of β-catenin but rather the alleviation of Tcf3 repression via the Wnt pathway (Wray et al., 2011). Overall, it has been proposed that inhibition of both Fgf/Erk and GSK3β signalling cascades in ES cells with or without supplementary LIF supports a ‘ground’ self-renewal ES cell state (Ying et al., 2008).

1.3.2 Fgf/Erk signalling pathway

The important role of Fgf/Erk signalling in balancing the pluripotent versus differentiated state in ES cells has some apparent parallels in the developing mouse blastocyst. The significance of Fgf/Erk signalling in the early mouse embryo is illustrated by mutations in Fgf4, Fgfr2, Frs2a, Grb2 and Erk2, individual components of the cascade, all resulting in peri-implantation lethality (Arman et al., 1998; Chazaud et al., 2006; Cheng et al., 1998; Feldman et al., 1995; Hadari et al., 2001; Hatano et al., 2003; Saba-El-Leil et al., 2003). There are currently twenty-two known mammalian fibroblast growth factors (Fgfs) and four cell surface Fgf receptors, and alternative splicing of three of these can generate multiple receptor isoforms. Fgf4 is the principally transcribed ligand in the early mouse embryo and is the only one required pre-implantation (Feldman et al., 1995). First detected in the eight- to sixteen-stage morula, Fgf4 becomes restricted to the epiblast progenitors of the pluripotent ICM in the expanded blastocyst (Niswander and Martin, 1992; Rappolee et al., 1994). Interestingly, pluripotency-associated transcription factors Pou5f1 and Sox2 together directly enhance the
expression of Fgf4 (Yuan et al., 1995), which highlights the existence of regulatory relationships between transcription factors and cell signalling. The Fgf receptor that is predominantly expressed in the blastocyst is Fgfr2, which is restricted to the extra-embryonic TE and PE tissues (Haffner-Krausz et al., 1999; Orr-Urtreger et al., 1991). Fgfr2 is required for pre-implantation development (Arman et al., 1998) and given that Fgf receptors 1, 3 and 4 are all required in post gastrulation stages (Colvin et al., 1996; Deng et al., 1996; Deng et al., 1994; Weinstein et al., 1998; Yamaguchi et al., 1994) this indicates it is likely the specific receptor for Fgf4 in the early pre-implantation embryo.

The mutually exclusive ligand-receptor expression pattern that Fgf4/Fgfr2 show in the early E3.5 to E4.5 embryo suggests that ICM/epiblast-produced Fgf4 could influence the establishment and/or maintenance of both extra-embryonic lineages (Guo et al., 2010; Rossant and Cross, 2001; Wilder et al., 1997).

Assembly of active Fgf ligand and receptor complexes is facilitated by the availability of extracellular heparan sulfate proteoglycans (HSPG) that act as co-receptors during this process (Ornitz and Itoh, 2001). Fgf receptors comprise of an extra-cellular region with three immunoglobulin-like (Ig-like) domains, a single transmembrane helix and a split intracellular kinase domain. Alternative splicing of the third Ig domain of Fgf receptors 1 to 3 produces numerous Fgf receptor isoforms with different ligand specificity due to the variation in their recognition sites (Ornitz et al., 1996). Fgfr2 isoforms, IIIb and IIIc, both show binding affinity for Fgf4 as well as somewhat promiscuous binding of several other Fgfs. Binding of both the ligand and HSPG results in homo- or hetero-dimerization of their cognate Fgf receptors and subsequent autophosphorylation of intracellular domain tyrosine kinases of the Fgf receptor. Intriguingly, genetic mutation of an enzyme that is involved in proteoglycan side chain synthesis, UDP-glucose dehydrogenise, leads to an early embryonic phenotype similar to the lethality observed upon either Fgf8 or Fgfr1 targeted mutation (Garcia-Garcia and Anderson, 2003). Thus Fgf signalling activity is regulated at multiple
levels, including growth factor presence, receptor binding affinity and the availability of HSPG for interaction. Furthermore, gene disruption of *Kif16b*, a component of the KIF16B/Rab14 Golgi-to-endosome trafficking motor complex for Fgf receptors during early embryonic development, results in failed Epi and PrE lineage formation and mouse peri-implantation lethality (Ueno et al., 2011). This data emphasizes the importance of multiple mechanisms, like microtubule-based membrane trafficking of Fgf receptors, proteoglycan side chain synthesis and Fgf/Erk signalling, functioning all in the correct spatial and temporal sequence to promote early development.

Four main relatively ubiquitous pathways relay the activation signal downstream from the Fgf receptor and are pivotal mediators of lineage choice in ES cells and *in vivo*; these are the JAK/STAT, the phosphoinositide phospholipase C (PLCy), the phosphatidylinositol 3-kinase (PI3K) and the mitogen activated protein kinase (MAPK) ERK pathways (Figure 1.3; (Dailey et al., 2005). The recruitment and subsequent tyrosine phosphorylation of the docking protein, fibroblast growth factor receptor substrate 2 (Frs2), recruits the growth factor receptor-bound protein 2 (Grb2) and Src homology region 2 domain-containing phosphatase 2 (Shp2) to facilitate son of sevenless homology (Sos) activation of Ras by inducing the exchange of GDP for GTP. The activation of Ras then stimulates the MAPK Erk1/2 pathway via the Raf and Mek kinases, or the MAPK p38 and/or Jnk via the Rac and Mekk kinases. Generally, the Erk1/2 signalling cascade is associated with the mitogenic response, while p38 and Jnk kinases are usually linked with responses to stress and inflammation (Johnson and Lapadat, 2002). Spred, Sprouty, dual specificity phosphatases (DUSPs) and similar expression of Fgf (Sef) further modulate the Fgf/Erk signalling pathway at multiple levels as shown in Figure 1.3 (Furthauer et al., 2002; Groom et al., 1996; Kramer et al., 1999; Tsang et al., 2002; Wakioka et al., 2001). The formation of an Frs2, Grb2 and the docking protein Grb2-associated binding protein 1 (Gab1) complex initiates the activation of the PI3K/AKT pathway (Lamothe et al., 2004). PI3K phosphorylation of
PDK leads to the further activation of AKT, which stimulates factors that contribute to the protection against apoptosis (Schlessinger, 2000). AKT activation may also block the activity of GSK3, which has the potential to produce additional apoptosis signals (Jope and Johnson, 2004).

Activated tyrosines on the Fgf receptor intracellular domain form a docking site for Src homology (Sh2) domain containing PLCγ. Activation of PLCγ leads to hydrolysis of phosphatidylinositol-4, 5-diphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) and diaclglyceral (DAG). PIP3 induces the release of intracellular calcium (Ca+) from the endoplasmic reticulum, while DAG functions to activate calcium/calmodulin dependence protein kinases (PKC; Schlessinger, 2000), which can directly feedback to the MAPK pathway via Raf1. More recently, Fgf signalling has also been found to activate STAT factors of the JAK/STAT signal transduction pathway (Citores et al., 2007).

Figure 1.3: Schematic of the Fgf/Erk signalling pathway. Fgf ligand and heparan sulfate proteoglycans (HSPG) are required to activate Fgf receptors to stimulate intracellular protein signalling pathways. These include 1) JAK/STAT (Brown), 2) phosphoinositide phospholipase C (PLCγ; grey), 3) phosphatidylinositol 3-kinase (PI3K; green) and 4) the mitogen activated protein kinase (MAPK; blue) ERK pathway. Many of these culminate in signal transduction to the nucleus to regulate transcription. Spred, Sprouty and dual specificity phosphatases (DUSPs) function as negative feedback
mechanisms (orange) to reduce or remove Fgf-stimulated signalling. (Source: Lanner and Rossant, 2010)

1.3.3 Self-renewal signalling in extra-embryonic stem cells and in vivo

The importance of Fgf/Erk signalling is also observed in the emergence and maintenance of the extra-embryonic lineages. During mouse blastocyst development, a lack of Fgf/Erk signalling allows for TE specification, but causes defects in trophoblast differentiation (Lu et al., 2008) and in the proliferation of diploid trophoblast cells (Arman et al., 1998; Nichols et al., 1998; Tanaka et al., 1998). In contrast to ES cells, TS cells require Fgf4 and heparin for their derivation and maintenance in culture (Tanaka et al., 1998). Withdrawal of Fgf4 and MEFs in vitro induces TS cells to differentiate, predominantly forming trophoblast giant cells. On the other hand extra-embryonic XEN cells do not require exogenous Fgf-stimulated signalling for maintenance of their self-renewal (Kunath et al., 2005), but the PrE requires Fgf/Erk signalling for it specification. This is illustrated by the absence of PrE in embryos deficient for Grb2, a mediator of the Fgf/Erk pathway and upon Fgf/Erk pharmacological inhibition (Chazaud et al., 2006; Nichols et al., 2009), as well as by the fact that addition of exogenous Fgf drives the developing ICM solely towards a Gata6 expressing PrE cell fate (Yamanaka et al., 2010).

TS cells are derived from the section of the TE directly overlying the ICM known as the polar TE, but not from the mural TE. In vivo, TE cells in this position are stimulated to proliferate via signals from the underlying ICM, which produces and secretes essential Fgf4. Interestingly, Cdx2 is activated in TS cells via the Erk signalling cascade in response to Fgf4 and in turn promotes the expression of BMP4. It is proposed that in vivo TE cells produce and release BMP4 to sustain the underlying pluripotent ICM (Murohashi et al., 2010). This demonstrates the importance of environmental niche and the interdependence of each of the early lineages on one another for survival, patterning, and cell fate decisions.
1.4 Epigenetic regulation of pluripotency

1.4.1 Epigenetic gene regulation during early embryonic development

A term originally coined in 1942 by C. Waddington, epigenetics is currently thought of as the study of instructional modifications imposed on the DNA, without changing the underlying sequence, to provide additional levels of gene regulation and establish heritable transcriptional states (over cell division and/or from one generation to the next). The ability to acquire and inherit gene expression programs efficiently is vital for cell potency and differentiation. In eukaryotic cells, DNA is wrapped around core histone proteins and complexed with other chromosomal proteins to form chromatin (Figure 1.4). Chromatin structure can regulate gene expression by influencing the accessibility of regulatory and transcriptional proteins to their target sites. Ongoing research is now beginning to reveal key regulators including histone-modifying enzymes, chromatin-remodelling factors, DNA methyltransferases, structural chromatin proteins and non-coding RNAs (Hemberger et al., 2009; Jaenisch and Bird, 2003; Probst et al., 2009; Reik, 2007).
Figure 1.4: Epigenetic regulation of chromatin organisation. Schematic diagram showing the different levels of genome organisation and epigenetic regulation that impact on gene activity in eukaryotic cells. DNA can be covalently methylated (Me) at the carbon 5 of cytosine residues, providing a direct epigenetic mechanism of gene regulation. DNA (147 bp) is wrapped around compact octameric histone complexes containing two copies of each of the four core histones H2A, H2B, H3 and H4 (Luger et al., 1997) to form nucleosome structures (Kornberg and Thomas, 1974; Olins and Olins, 1974). These fold into several levels of condensed higher-order chromatin domains to be dynamically organised into functional nuclear compartments. Histones can also be post-translationally modified at specific amino acids on their histone tails (black wavy lines) for example, by acetylation (Ac, yellow), methylation (Me; red) phosphorylation (P; orange) and ubiquitylation (Ub; grey). Each covalent mark is thought to constitute a signal that read alone or in combination with other modifications either induces dynamic changes in chromatin structure or reinforces the current chromatin state by recruitment or blocking of regulatory proteins. Some modifications, like tri-methylation of lysine 4 of histone 3 (H3K4me3) or H3K9ac are generally linked with accessible, actively expressed chromatin, while others, like H3K27me3 or H3K9me3, are associated with inaccessible, repressed chromatin (Hublitz et al., 2009; Jenuwein and Allis, 2001). The nucleosome particles that comprise chromatin are represented as yellow cylinders and DNA as black lines. Together these mechanisms provide some molecular insight into
how the same genome can repeatedly produce a multitude of transcriptional profiles and subsequent cell types. (Adapted from: Probst et al., 2009; Sparmann and van Lohuizen, 2006)

Cell genomes are subjected to major epigenetic changes during mouse embryo pre-implantation development. DNA methylation is a repressive epigenetic mechanism in vertebrates often associated with stable, long-term silencing such as genomic imprinting and X-chromosome inactivation at the cytosine base of CpG dinucleotide dimers (notation used for cytosine and guanine nucleotides linked by a phosphate) (Bird, 2002; Suzuki and Bird, 2008). DNA methylation is carried out by the DNA methyltransferases Dnmt3A and Dnmt3B, and maintained by Dnmt1 via a copying mechanism that targets hemi-methylated DNA that ensures the inheritance of DNA methylation through cell division (Pradhan et al., 1999). Following fertilization of the oocyte, the densely packed paternal genome decondenses while being subject to active global DNA demethylation, with the exception of imprinted genes that are protected from the process, like occurs with the maternal genome (Reik et al., 2001). The maternal genome undergoes a passive decrease of repressive DNA methylation with each nuclear division, presumably due to nuclear depletion of the DNA methyltransferase Dnmt1 (Carlson et al., 1992). De novo DNA methylation levels begin to increase at the blastocyst stage (Santos et al., 2002). A possible purpose for the methylation reprogramming in pre-implantation embryos is for the parental genomes to lose their pre-existing epigenetic marks so that the genome can return to a totipotent state (Reik et al., 2001). Up until the 8-cell stage and prior DNA replication, an epigenetic asymmetry exists between maternal and paternal pericentric heterochromatin (densely packed, transcriptionally inactive chromatin region (Puschendorf et al., 2008). Maternal pericentric heterochromatin shows an intense staining for methyltransferase Suv39h1-mediated trimethylation of lysine 9 of histone 3 (H3K9me3) and repressive DNA methylation. While maternally provided Ring1B, a polycomb repressive complex (PRC)
component, and not Suv39h1-mediated H3K9me3, binds paternal pericentric heterochromatin and will also target maternal heterochromatin in the absence of Suv39h1. Thus revealing a hierarchy between repressive mechanisms (Puschendorf et al., 2008).

During early development, interplay among epigenetic modifiers and transcription factors exist to regulate cell fates. In particular, CARM1, a specific methyltransferase responsible for H3 arginine methylation, has been suggested to facilitates H3R17 and H3R26 methylation at Nanog and Sox2 promoters, enabling their up-regulation followed by cell contribution to the pluripotent ICM lineage (Torres-Padilla et al., 2007). Conversely, the H3K9 demethylase, Jmjd2c, functions to reverse the repressive H3K9me3 mark from the Nanog promoter and hence maintain its transcriptionally active state in ES cells (Loh et al., 2007). Thus an epigenetic balance must evolve to sustain pluripotency that maintains the active chromatin state of pluripotency-related genes and concurrently, keeps differentiation-promoting genes repressed. The histone H3K9 methyltransferase, ESET, is recruited in the ICM and ES cells to the TE-specific Cdx2 promoter by the Pou5f1 protein and epigenetically represses Cdx2 transcription, restricting cell specification towards the pluripotent ICM and away from the TE lineage (Yeap et al., 2009; Yuan et al., 2009). For differentiation to occur, pluripotency-linked factors must be repressed. For example, the Pou5f1 gene is first repressed by transcription factors and then by Ehmt1 and Ehmt2 methyltransferase complex-mediated repressive histone marks during ES cell differentiation. This silencing is ultimately reinforced by Dnmt3a and Dnmt3b DNA methylation of the Pou5f1 promoter (Epsztejn-Litman et al., 2008; Feldman et al., 2006).

1.4.2 ES cell chromatin environment

The chromatin characteristics of pluripotent ES cells are thought to reflect a generally ‘open’ and transcriptionally ‘permissive’ state (Meshorer and Misteli, 2006).
Notably their nuclei are approximately twice the volume of lineage-committed cells, indicative of an accessible nuclear environment (Faro-Trindade and Cook, 2006). In contrast, ES cell lineage specification leads to an increase in global levels of the repressive, heterochromatic mark H3K9me3 and a decrease in H3 and H4 acetylation leading to the accumulation of transcriptionally repressed heterochromatic regions (Boyer et al., 2006a; Lee et al., 2004). Interestingly, separate depletion of either histone demethylase, Jmjd1a or Jmjd2c, in ES cells by small interfering RNA (siRNA) induces differentiation with global increases in H3K9me2 and H3K9me3, respectively. This demonstrates a functional link between the requirement for low repressive mark levels due to histone demethylases and the maintenance of undifferentiated ES cells (Loh et al., 2007).

Another feature associated with gene-rich, expressed, accessible chromatin regions, carrying acetylated histones, is early DNA replication (Lin et al., 2003; Perry et al., 2004; Vogelauer et al., 2002). On the other hand, late DNA replication is an indicator of repressed, inaccessible chromatin regions such as constitutive heterochromatin and some facultative heterochromatin (Gilbert, 2002). In ES cells, the replication timing profile for many silent tissue-specific regulatory genes was during the early S-phase, reflecting a unique accessible chromatin state, while in more lineage-committed cells, they were late-replicating (multipotent hematopoietic stem cells and fully specialized T cells)(Azuara et al., 2006; Perry et al., 2004). For instance, neural-specifying loci, not expressed in either pluripotent ES or hematopoietic-restricted cell types, are early replicating in ES cells which retain the potential for neural differentiation and late replicating in hematopoietic-restricted cells, that lack neural potential (Azuara et al., 2006).

In pluripotent ES cells, major structural proteins, like HP1 and the linker histone H1, have been shown by fluorescence recovery after photobleaching (FRAP) experiments to be loosely bound to chromatin and exhibit highly dynamic exchange
rates (Meshorer et al., 2006). In comparison, differentiated cell chromatin generally has a more condensed, hypo-acetylated state showing decreased dynamics and tighter binding of H1, further suggesting that dynamic euchromatin environments contribute to the developmental plasticity of ES cells (Meshorer et al., 2006). This discovery was further supported by research on the chromatin-remodeling enzyme, Chd1, a chromodomain-containing protein that recognises H3K4me2/3. In Chd1-depleted ES cells, the usual rapid exchange of H1 observed in pluripotent ES cells is distinctly decreased and the cells reveal a decrease in differential potential towards PrE and mesoderm cell fates, while showing preference towards neural differentiation. Intriguingly, unlike with what happens with wild-type fibroblasts, attempts to reprogram Chd1-depleted fibroblast by forced expression of reprogramming-associated transcription factors Pou5f1, Sox2, Myc and Klf4, failed (Gaspar-Maia et al., 2009). These results emphasize the requirement for core transcriptional machinery and chromatin-modifying complexes to work in synergy to achieve and maintain a pluripotent state.

### 1.4.3 Poised chromatin signatures

Polycomb group (PcG) and Trithorax group (TrxG) proteins were discovered in *Drosophila melanogaster* as repressors and activators of Hox genes respectively (Schuettengruber et al., 2007). Two distinct PcG groups exist, PRC1 and PRC2. PRC2 comprises of Eed, Suz12 and lastly Ezh2, which catalyzes H3K27me3. The PRC1 complex contains Bmi1, Mel18, proteins of the Cbx family and E3 ubiquitin ligases Ring1A and Ring1B, which can monoubiquitylate H2AK119 (Bantignies and Cavalli, 2006; Pietersen and van Lohuizen, 2008; Ringrose and Paro, 2007; Simon and Kingston, 2009). Recently, ChIP studies in both mouse and human ES cells revealed components of the repressive-linked PcG complex, PRC2 (Suz12 and Eed), are present together with PRC2-mediated H3K27me3 at the promoters of many non-transcribed, developmentally genes, which are important for the government of lineage specification towards extra-embryonic and
somatic cell fates (Boyer et al., 2006b; Jorgensen et al., 2006; Lee et al., 2006). Concomitantly, two groups discovered that the promoters of many inactive, tissue-specific genes were enriched in ES cells simultaneously for seemingly opposing repressive H3K27me3 and active H3K4me2 or H3K4me3 modifications – so-called bivalent domains (Azuara et al., 2006; Bernstein et al., 2006). These target genes included Hox, Pax, Fox, Gata, Irx, Sox and Fgf gene families, often associated with developmental patterning during placental, neural and limb development. Together, PcG complexes and bivalent domains are suggested to hold regulatory genes silent yet ‘poised’ in pluripotent ES cells, for subsequent expression in response to developmental cues and a loss of pluripotency that is accompanied by resolution of the ‘bivalent’ mark (Figure 1.5; (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006b; Lee et al., 2006; Spivakov and Fisher, 2007)). Indeed, many developmental PcG target genes become lineage-inappropriately expressed upon genetic disruption of the PRC2 components Eed or Suz12 and consequent loss of H3K27 methylation in ES cells (Azuara et al., 2006; Boyer et al., 2006b; Lee et al., 2006). Furthermore, Eed-deficient ES cells, when compared to wild-type ES cells, are more prone to spontaneous differentiation, establishing a functional link between PcG protein binding, gene silencing and pluripotency.

Genome-wide ChIP-sequencing (ChIP-seq) analysis of pluripotent mouse ES cells revealed a strong positive correlation between high CpG containing promoters (HCPs) and bivalently marked loci when compared to more lineage-restricted neural progenitor cells (NPCs) and Mefs, indicating that CpG poor regions must be subject to alternative modes of regulation (Mikkelsen et al., 2007). Over 2500 HCPs were bivalently marked in self-renewing ES cells, while a reduced prevalence of approximately only 1000 and 200 promoters remained detectably bivalent in Mefs and NPCs, respectively (Bernstein et al., 2006; Mikkelsen et al., 2007). Apart from some exceptions, the majority of the opposing modifications were resolved in the more lineage restricted cell types, with lineage-
appropriate genes becoming activated and depleted for repressive H3K27me3, while lineage-inappropriate genes lost permissive histone acetylation and H3K4me3 (Figure 1.5;(Bernstein et al., 2006; Mikkelsen et al., 2007). These observations are consistent with the notion of poised chromatin structures.

Figure 1.5: Schematic diagram of bivalent chromatin domain resolution upon lineage commitment from ES cells. In self-renewing, pluripotent ES cells, many transcriptionally silenced, developmental genes are co-occupied by the surprising combination of permissive (H3K4me2) and repressive (H3K27me3) epigenetic marks. Upon ES cell differentiation, the ‘bivalent’ chromatin marks are generally reported to be resolved to solely H3K4me2 or H3K27me3 enrichment or neither, in accordance with their transcriptional status. A subset of loci (silent or weakly induced), however, may retain a bivalent chromatin configuration in lineage-committed cells.

The idea of gene ‘priming’ was further supported when RNA polymerase II (RNAP) complexes were shown to assemble at bivalently marked genes in ES cells in a ‘poised’ configuration for transcriptional activation (Brookes and Pombo, 2009; Guenther et al., 2007; Stock et al., 2007). Traditionally, gene transcription requires the multi-subunit pre-initiation complex (PIC), formed by TFIID which recognizes promoters and is stabilized by TFIIB during recruitment of hypo-phosphorylated RNAP II and other
general transcription factors. The mediator, a large coactivator complex, acts as a bridge between upstream activator proteins, recruited RNAP II and general transcription factors. Intrinsic helicase activity separates the DNA at promoters allowing access to a single stranded template and formation of the first phosphodiester bond. Mammalian RNAP II's largest subunit RPB1 contains an unusual C-terminal domain (CTD) consisting of 52 copies of a heptad consensus sequence (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7), which is subjected to targeted phosphorylation (Buratowski, 2003; Corden et al., 1985). Phosphorylation of the RNAP II on Ser5 residues by TFIIH induces release from mediator interaction at the promoter (Komarnitsky et al., 2000). Post promoter escape, RNAP II proceeds to intrinsic pausing sites where it can be halted by negative factors (Core and Lis, 2008). Further phosphorylation by P-TEFb on Ser2 CTD residues corresponds to the formation of a stable elongation complex (Marshall et al., 1996). Termination of transcription is likely coupled with mRNA cleavage and polyadenylation, while RNAP II is subject to dephosphorylation and recycled (Krishnamurthy et al., 2004; Mandal et al., 2002; Richard and Manley, 2009).

In pluripotent ES cells, RNAP II is predominantly detected by ChIP using specific antibodies in a Ser5 CTD phosphorylated form (Ser5P) and not in Ser2P or hypophosphorylated conformations (8WG16) at PRC1 and PRC2 occupied, bivalently marked genes (Brookes and Pombo, 2009; Stock et al., 2007). The absence of the transcriptional elongation-linked Ser2P RNAP at 'poised' promoters reflects the low levels of full-length and spliced transcripts produced. This 'poised' RNAP configuration is enforced by PRC1-mediated H2A monoubiquitination at lysine 119, which is crucial for safeguarding ES cell pluripotency (Wang et al., 2004). This was demonstrated when induced depletion of PRC1 catalytic component Ring1A/B caused a decrease in H2A ubiquitination levels, higher lineage-specific transcript levels and the detection of hypophosphorylated RNAP II (Jorgensen et al., 2006; Stock et al., 2007). Interestingly, transcriptional priming of bivalent genes was also found dependent on the individual
PRC2 component Jarid2. ES cells lacking Jarid2, in contrast to other PcG repressive complex mutants, did not inappropriately express PRC2 targeted genes, but instead failed to recruit PRC1 and RNAP II, which abrogated any correct lineage-specific gene expression upon differentiation (Landeira et al., 2010). This indicates the crucial requirement of transcriptional priming at bivalent gene for both the pluripotent state of ES cells and subsequent cell fate decisions.

Further genome-wide analysis suggests that two distinct groups of bivalent domains exist, one that has co-occupancy with both PRC1 and PRC2 (40%) and another that is targeted solely by PRC2 (60%) (Ku et al., 2008). Promoters bound exclusively by PRC2 are poorly conserved, more likely to loss H3K27me3 upon differentiation and largely encode membrane proteins or genes of current unknown function. In contrast, PRC1-bound bivalent domains occupy promoters that are evolutionarily conserved and within large H3K27me3-rich regions containing the majority of key developmental regulator genes. Inducing Ring1A/B knockout in ES cells demonstrated a 32% up-regulation of PRC1 and PRC2 bound bivalent loci after 48 hours treatment while only 16% of those occupied solely by PRC2 were up-regulated (Ku et al., 2008).

A significant subset of PcG-bound, silent, bivalently marked loci that encode key developmental regulators is also co-occupied by the core transcription factors Pou5f1, Nanog and Sox2 (Boyer et al., 2005; Boyer et al., 2006b; Lee et al., 2006; Loh et al., 2006). These epigenetic and genetic factors as illustrated in the above sections are important regulators of pluripotency in ES cells. However, other factors are likely required as only about half of the identified PcG targeted, bivalent loci are bound by these three transcription factors (Lee et al., 2006). Deciphering how pluripotency is achieved and maintained remains an important scientific challenge. Continuous studies are uncovering numerous mechanisms including transcriptional networks, extrinsic signals and chromatin structures that are required in the balance to maintain ES cells in a self-renewing state, but still primed for potential lineage commitment as shown in Figure
Figure 1.6: Key regulators controlling the pluripotency state. Fundamental regulators that influence the ES cell pluripotent state include chromatin structures, the transcription factor network and extrinsic signal cues towards and against cell differentiation. (Adapted from Chi and Bernstein, 2009).
1.5 Aims of this study

Gene expression programmes must be tightly controlled to govern cell identity and lineage choice. An inherent challenge for developing organisms is to maintain a critical balance between stable and flexible gene regulation. This is most obvious in pluripotent ES cells, which are functionally characterized by their ability to self-renew and to generate all somatic lineages when induced. In this study we will focus on mammalian pre-implantation development. Using embryo-derived stem cell models and \textit{in vivo} studies, we aim to advance understanding of how pluripotency is safeguarded in the ICM while surrounding cells undergo TE lineage restriction.

We will exploit a novel chromatin immunoprecipitation approach designed for small cell samples (Carrier ChIP) to address whether the epigenetic features of ES cells are also seen \textit{in vivo} in the developing embryo. This will also imply examining how and when bivalent histone markings are established at key developmental silent genes and subsequently erased or modulated upon lineage commitment. In addition, we will determine whether additional repressive epigenetic mechanisms operate in TE-committed cells to silence embryonic-associated genes both \textit{in vivo} and \textit{in vitro} and explore the process of chromatin remodelling upon TE cell fate acquisition using an \textit{in vitro} ES to TS-like cell conversion system.

The transcription factor Nanog is a key part of the core genetic network that sustains self-renewal in ES cells. In contrast, the Fgf/Erk signalling pathway primes ES cells for differentiation. In a second ongoing project, we will take advantage of a panel of genetically modified ES cell lines for Nanog to explore how this pluripotency-associated factor may modulate the effect of the extracellular Fgf/Erk pathway. Using a combination of ChIP and luciferase reporter assays, we will examine whether Nanog can regulate autocrine Fgf/Erk responses by directly controlling the expression of its
specific mediators in ES cells, *Fgfr2* and *Fgf4*. We will next explore the effect of *Fgfr2* forced expression on balancing self-renewal and differentiation in ES cells.
Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

**E14Tg2a**  Hprt deficient mouse ES cell line derived from Lesch-Nyhan embryos (Hooper et al., 1987); feeder-independent; 129/Ola genetic background.

**EF4**  Genetically engineered Nanog over-expressing mouse E14Tg2a ES cells line carrying a *loxP* flanked Nanog transgene (Chambers et al., 2003); feeder-independent; a gift from I. Chambers (MRC Centre for Regenerative Medicine, Edinburgh, UK)

**Mef**  Mouse embryonic fibroblasts isolated from wild type CF-1 embryos (E13.5).

**RCN(t)**  Genetically engineered E14Tg2a ES cells lines carrying a tamoxifen inducible Cre-ERT2 recombinase (Schneider, 1972) in its ROSA26 locus to establish RC cells. Transfection of RC cells with CAGloxPNanog-IRESpacP-loxP-eGFPpA produced RCN cells (Chambers et al., 2007). Deletion of the Nanog transgene, denoted by the suffix (t), was achieved by culturing cells in 1μM 4-OH-Tamoxifen; feeder-independent; a gift from I. Chambers (MRC Centre for Regenerative Medicine, Edinburgh, UK)

**RCNβH**  Nanog<sup>flx</sup>/<sup>flx</sup> mouse ES cell line genetically engineered from RCN ES cells subjected to sequential gene targeting to integrate βgeo into one Nanog locus and *hph* into the second endogenous Nanog locus (Chambers et al., 2007); feeder-independent; a gift from I. Chambers (MRC Centre for Regenerative Medicine, Edinburgh, UK)

**RCNβH(t)**  Nanog<sup>−/−</sup> mouse ES cell line generated by deletion of the Nanog transgene, denoted by the suffix (t), achieved by culturing RCNβH cells in 1μM 4-
OH-tamoxifen (Chambers et al., 2007). Maintained in the constant presence of 25μg/ml Hygromycin B (Roche); feeder-independent; a gift from I. Chambers (MRC Centre for Regenerative Medicine, Edinburgh, UK)

S2
Drosophila melanogaster cell line derived from a primary culture of late stage (20-24 hrs old) embryos (Schneider, 1972).

TNGA
Derived from E14Tg2A mouse ES cells where a Nanog allele was targeted by a GFP-ires-pacP cassette (Chambers et al., 2007).

TS-B1
Mouse TS cell line (Mak et al., 2002); feeder dependent; a kind gift from N. Brockdorff, Developmental Epigenetics Group, University of Oxford.

ZHBTc4
Genetically engineered mouse E14Tg2a ES cell line carrying a tetracycline-regulatable Pou5f1 transgene replacing endogenous Pou5f1 alleles (Niwa et al., 2000); feeder-independent; 129/Ola genetic background; a kind gift from A. Smith (Wellcome Trust Centre for Stem Cell Research, University of Cambridge).

2.1.2 Primary Antibodies

β-actin
Rabbit polyclonal anti-β-actin antibody (ab8227; Abcam). Used for WB at 1:5000 dilution.

β-tubulin
Mouse monoclonal anti-β-tubulin antibody (05-661; Millipore). Used for WB at 1:10,000 dilution.

Erk1/2
Rabbit monoclonal anti-Erk1/2 antibody (4695; Cell signaling). Used for WB at 1:2000 dilution.

Fgfr1
Rabbit polyclonal anti-Fgfr1 (C-15) antibody (Sc-121, Santa Cruz). Used for WB at 1:1000 dilution.

Fgfr2
Rabbit polyclonal anti-Fgfr2 (C-17) antibody (Sc-122, Santa Cruz). Used for WB at 1:500 dilution and IF at 1:200 dilution.
Gata4  Rabbit polyclonal anti-Gata4 antibody (Sc-9053, Santa Cruz). Used for IF at 1:200 dilution.

Gata6  Rabbit polyclonal anti-Gata6 antibody (Sc-9055, Santa Cruz). Used for IF at 1:200 dilution.

IgG  Rabbit anti-IgG (whole molecule) fraction of antiserum antibody (M-7023; Sigma Aldrich Company Ltd., Gillingham, UK). Used for ChIP at 5-10 μg/IP.

Methyl-C  Mouse monoclonal anti-5-methylcytidine (33D3; Eurogenetec, Belgium). Used for methylated DNA immunoprecipitation (MeDIP) at 10 μl/reaction dilution.

Nanog  Rabbit polyclonal anti-mouse Nanog (REC-RCAB0002P-F; Cosmo Bio Co., Ltd., Japan) used for IF at 1:500, FACS at 1 μg/1x106 cells, WB at 1:1000 dilution and ChIP at 10ug/IP.

Oct4  Goat polyclonal anti-Oct4 (N19 sc-8628; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Used for IF at 1:100, FACS at 1 μg/1x106 cells and WB at 1:1000 dilution.

Phallodin  TRITC conjugated anti-phallodin antibody. (P1951; Sigma). Used for IF at 1:100 dilution.


Phospho-Fgfr  Mouse monoclonal anti-phospho Fgfr (T653/T654) (3476; Cell signaling). Used for WB at 1:1000 dilution.

Ring1B  Courtesy of Haruhiko Koseki. Used for ChIP at 10ug/IP.

Sox2  Rabbit polyclonal anti-mouse Sox2 (ab15830; Abcam). Used for western blot at 1:1500 dilution.

Suv39h1  Mouse monoclonal anti-Suv39h1 (ab12405; Abcam). Used for ChIP at 10ug/IP.
**Suz12** Rabbit polyclonal anti-mouse Suz12 (pAb-029-050; Diagenode SA, Liège, Belgium). Used for WB at 1:1000 dilution and ChIP at 10μg/IP.

**H3** Rabbit polyclonal anti-histone 3 carboxy terminal (ab1791; Abcam Ltd., Cambridge, UK). Used for WB at 1:5000 dilution and ChIP at 5μg/IP.

**H3K27me3** Rabbit polyclonal anti-histone 3 lysine 27 trimethylation (07-449; Upstate). Used for WB at 1:2500 dilution and ChIP at 5μg/IP.

**H3K4me2** Rabbit polyclonal anti-histone 3 lysine 4 dimethylation (07-030; Upstate). Used for WB at 1:5000 dilution and ChIP at 5μg/IP.

**H3K4me3** Rabbit polyclonal anti-histone 3 lysine 4 trimethylation (ab8580; Abcam). Used for WB at 1:2500 dilution and ChIP at 5μg/IP.

**H3K9me3** Rabbit polyclonal anti-histone 3 lysine 9 trimethylation (07-442; Upstate). Used for WB at 1.2500 dilution and ChIP at 5μg/IP.

### 2.1.3 Secondary Antibodies

**Alexa 488** Goat anti-mouse IgG (A11029; Molecular Probes Invitrogen, Ltd., Paisley, UK). Used for IF at 1:500.

**Alexa 488** Goat anti-rabbit IgG (A11034; Molecular Probes). Used for IF at 1:500.

**Alexa 488** Donkey anti-goat IgG (A-11055; Molecular Probes). Used for IF at 1:500.

**Alexa 568** Goat anti-mouse IgG (A11031; Molecular Probes). Used for IF at 1:500 dilution

**Alexa 568** Goat anti-rabbit IgG (A11036; Molecular Probes). Used for IF at 1:500 dilution

**FITC** Goat anti-mouse IgM (F9259; Sigma). Used for IF at 1:200 and FACS at 1:500 dilution.

**HRP-conjugated** Donkey anti-goat IgG HRP (sc-2020, Santa Cruz). Used for WB at 1:2000 dilution.

HRP-conjugated Goat anti-rabbit IgG HRP (sc-2004, Santa Cruz). Used for WB at 1:5000 dilution.

2.2 Methods

2.2.1 Cell culture

All tissue culture reagents were from Invitrogen (Invitrogen Ltd, Paisley, UK), unless stated otherwise. Mouse embryonic stem (ES) cells were grown and maintained in an undifferentiated state in Glasgow Modified Essential Medium (GMEM)-BHK21 medium supplemented with 15% (v/v) foetal calf serum (FCS; Globeoharm, Esher, UK), non-essential amino acids (1x), sodium pyruvate (1mM), sodium bicarbonate (0.075%), penicillin/streptomycin (10μg/ml), L-glutamine (292μg/ml), 2-mercaptoethanol (0.1mM) and recombinant leukemia inhibitory factor (Lif; made in-house) on 0.1% gelatin-coated surfaces (Sigma) unless otherwise stated. All mammalian cells were cultured at 37°C and 5% (v/v) CO₂ and routinely dissociated with trypsin-EDTA. Doxycycline (1 μg/ml, Sigma), 4-hydroxy-tamoxifen (1 μM, Sigma), cycloheximide (0.1 μg/ml, Sigma) and human recombinant fibroblast growth factor (Fgf4; R&D systems 25ng/ml) and heparin (Sigma, 1μg/ml) were added to the media when indicated.

TS-B1 cells were cultured either on mitotically inactivated mouse embryonic fibroblast (MEF) or in the presence of 30% Roswell Park Memorial Institute (RPMI) 1640 medium and 70% media Mef conditioned by supplemented with 20% FCS, sodium pyruvate (1mM), 2-mercaptoethanol (0.1mM), L-glutamine (292μg/ml), penicillin/streptomycin (100U/ml) and human recombinant fibroblast growth factor (Fgf4; R&D systems 25ng/ml) and heparin (Sigma, 1μg/ml). TS-B1 cells (Mak et al., 2002) were a kind gift from N. Brockdorff, Developmental Epigenetics Group, University of Oxford. MEFs were derived from E12.5 embryos and cultured in high-glucose
Dulbecco's modified medium (DMEM) supplemented with 10% foetal calf serum (FCS; Globepharm, Esher, UK), L-glutamine (2mM) and antibiotics, grown until 80-85% confluence, dissociated with trypsin-EDTA before γ-irradiation and frozen for future use in 10% dimethyl sulfoxide (DMSO), 90% FCS. Conditioned media was prepared by incubating 10ml TS medium with 2 x 10^6 cells 40 Gy irradiated MEFs on 100 mm plates for at least 72 hrs. Media was filtered (0.2 μm) before storing at -20. To induce TS differentiation (TS-like), ZHBTc4 ES cells were seeded at low density (1 x 10^3/ 10 cm^2) on irradiated MEF feeders, treated with doxycycline (Dox; Sigma) at 1μg/ml and provision of 37ng/ml Fgf4 and heparin (1μg/ml) propagation in culture selected for cells that underwent conversion giving rise to a pure population. *Drosophila Melanogaster* S2 cells were maintained in Schneider's Medium supplemented with 10% heat-inactivated FCS and penicillin/streptomycin at room temperature.

2.2.2 Embryo collection and dissection

Pre-pubescent CBAB6 F1 females were super-ovulated by intrauterine injections of PMSG (5U Folligon®) followed 42 hrs later by HCG (5U Chorulon®) and mating overnight with CBA/B6 F1 males. To obtain blastocysts morulae were flushed from uteri at embryonic day E2.5 in EmbryoMax® M-2 (Millipore) and cultured overnight to form blastocysts in 30μl droplets of equilibrated EmbryoMax® M-16 (Millipore) under mineral oil. TE samples were acquired using Leica manual manipulators and a holding pipette to immobilize the embryo and micro-dissection blades (Bioniche) to physically dissect away mural TE on a Nikon Diaphot with DIC optics. Individual ICMs were isolated by immunosurgery on a Nikon SMZ dissection microscope, using rabbit anti-mouse serum, guinea-pig complement and acidified Tyrode’s solutions (Sigma) and a finely pulled pipette as previously described (O'Neill et al., 2006). To obtain 8-cell embryos uteri were flushed at E2.0 and compacted morulae were collected at E2.5 and snap frozen before ChIP analysis. (Protocol carried out by O. Alder)
2.2.3 Chromatin immunoprecipitation (ChIP) analysis

2.2.3.1 Histone modifications ChIP

This protocol for histone modifications has been previously described in Azuara et al, 2006. All reagents used were from Sigma unless stated otherwise. Approximately 1x10^7 exponentially growing cells were harvested, washed twice in PBS and cross-linked with 1% PFA for 10 min at room temperature. After quenching of PFA with glycine at a final concentration of 125 mM for an additional 10 min at room temperature, the cells were washed twice with ice-cold PBS containing protease inhibitor cocktail (Roche) and lysed in 1 ml ice-cold lysis buffer [50 mM Tris-HCL pH 8.1, 10 mM EDTA pH 8.0, 1% SDS]. All buffers were supplemented with 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail. Using a Bioruptor™ 200 (Diagenode), the chromatin was sonicated on ice to an average size of 300 to 500 bp running the settings: output-high, 30 sec on, 30 sec off, for 15 min and analysed on 1.5% agarose gels. Insoluble proteins were discarded after centrifugation of the lysate at 15,000 rpm for 15 min at 18°C and DNA concentration quantified by spectrophotometry (NanoDrop® ND-1000, Wilmington, USA). 140 μg of fragmented chromatin was diluted 1/10 in dilution buffer [1% Triton X-100, 2 mM EDTA pH 8.0, 150 mM NaCl, 20 mM Tris-HCL pH 8.1] with protease inhibitors (Roche) and incubated with 30 μl of blocked protein A-sepharose beads at 4°C for 2hrs, on a spinning wheel, to pre-clear the solution of any non-specific binding. Protein A-sepharose beads were blocked overnight at 4°C in dilution buffer supplemented with 10 μl of BSA (New England Biolabs, 10 mg/ml) and 4 μl salmon sperm DNA (10 mg/ml), on a spinning wheel and then resuspended in dilution and lysis buffer in a 10:1 ratio. Pre-cleared chromatin was then immunoprecipitated with 5 μg of primary antibody for histone modifications, a negative anti-IgG control and an anti-H3 total antibody for data normalization, overnight at 4°C on a spinning wheel. A list of antibodies used for ChIP is shown in section 2.1.2. Immuno-complexes were collected by adding 30 μl of blocked
beads once again and left immunoprecipitating 2 hrs on a spinning wheel, at 4°C. Unbound chromatin was removed by washing beads 4 times in 1 ml of ice-cold wash buffer [20 mM Tris-HCl pH 8.1, 2 mM EDTA pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100] and once in final high salt wash buffer [20 mM Tris-HCl pH 8.1, 2 mM EDTA pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100] on ice. After adding elution buffer [1% SDS, 0.1 M NaHCO3] to elute the immuno-complexes from the beads, these were treated with 100μg/ml RNase and 500μg/ml proteinase K, 2 hrs at 37°C followed by 6 hrs of incubation at 65°C to reverse the cross-links. Finally, the DNA was sequentially extracted with phenol/chloroform/Isoamylalcohol and precipitated in 50% isopropanol containing 5 mM NaAc and 20 μg of glycogen carrier (Glycoblue, Ambion). After purification, DNA was resuspended in 80 μL of TE. Quantification of precipitated DNA was performed using qRT-PCR amplification (see section 2.2.5.3) and the primers listed in Appendix I: Table I-2. Histone modification levels were normalised against total H3 detected, and the ratio of modified-H3 to total H3 was denoted as "relative abundance".

2.2.3.2 RNA polymerase and chromatin modifiers ChIP

This protocol was previously described in detail by Stock et al., 2007 with minor modifications. Briefly, cells were treated directly with 1% formaldehyde for 10 min at 37°C and quenched with 125 mM glycine. Cells were washed in ice-cold PBS and lysed at 4°C for 10 min in swelling buffer [25 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl and 0.1% NP-40]. All buffers were supplemented with 5 mM NaF, 2 mM Na3VO4, 1 mM PMSF, and protease inhibitor cocktail. Cells were scraped from plates, nuclei extracted by homogenization, resuspended in sonication buffer [50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate and 0.1% SDS] and sonicated to produce chromatin fragments less than 1.6 kb in length. 600 μg of solubilised chromatin was pre-cleared with blocked Protein-A (rabbit antibodies) or Protein-G-sepharose beads (mouse antibodies) and incubated overnight at 4°C with primary antibody on a
rotating wheel. A list of antibodies used for ChIP as well as quantities and suppliers is shown in section 2.1.2. Antibody-chromatin complexes were collected with blocked beads for 3 hrs at 4°C before sequential washes: 1x sonication buffer, 1x sonication buffer containing 500 mM NaCl, 1x with 20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40 and 0.5% Na-deoxycholate, and 2x TE buffer. Immunoprecipitated chromatin was eluted and DNA recovered by phenol/chloroform/Isoamylalcohol extraction and ethanol precipitation as previously described in section 2.2.3.1. Final DNA concentrations were determined by PicoGreen fluorimetry (Molecular Probes, Invitrogen) and the amount of DNA precipitated by each antibody was normalized against the total input material. Quantification of precipitated DNA was performed using qRT-PCR amplification and the primers listed in Appendix I: Table I-2.

2.2.3.3 Sequential ChIP analysis

The same method was followed as in the histone modification ChIP protocol except that chromatin was eluted in a solution of 50mM Tris-HCl (pH 7.5), 10mM EDTA, 1% SDS and heated for 10 min at 65°C. Eluted chromatin was diluted 10-fold with dilution buffer (see above) and subjected to a second round of immunoprecipitation. Samples were first immunoprecipitated with tri-methyl H3K27 antibodies and sequentially immunoprecipitated with di-methyl H3K4 antibodies or vice versa and anti-IgG antibody as a control as well as total H3 antibody for normalisation.

2.2.3.4 Carrier ChIP analysis

This protocol was developed and described by (O’Neill et al., 2006) with minor modifications included. D. melanogaster S2 cells (5x10^7 cells/ml) were mixed with 40 eight-cell embryos, 20 morula, 10 ICM, 10 TE or TS cell samples and washed in NB buffer [15 mM Tris-HCL, pH 7.4, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 0.1 mM EGTA, 0.5 mM 2- mercaptoethanol] supplemented with 5 mM sodium butyrate. All buffers were supplemented with 5 mM NaF, 2 mM Na3VO4, 1 mM PMSF, and protease inhibitor.
cocktail. Samples were resuspended in NB buffer 0.5% Tween-40 and stirred for 1 hr at 4°C. Nuclei were released by homogenization in a Dounce all-glass homogenizer until approximately 80% intact nuclei were observed under the microscope. Nuclei were pelleted, resuspended in NB buffer plus 5% (v/v) sucrose, pelleted and resuspended in digestion buffer [50 mM Tris-HCl pH 7.4, 0.32 M sucrose, 4 mM MgCl2, 1 mM CaCl2]. DNA concentration was measured, 5U of micrococcal nuclease (GE Healthcare) per 2.5 μg of chromatin was added and samples incubated at 18°C for a maximum of 5 min. Samples were spun at 1,800g, first supernatant removed and stored at 4°C. The pellet was resuspended in 500 μl-1 ml lysis buffer [2 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, 5 mM sodium butyrate, 0.4 mM glycine] and dialyzed overnight at 4°C against lysis buffer before collection of second supernatant and pellet samples. Supernatants and pellet were analyzed by agarose gel electrophoresis. If the chromatin comprised of mainly mono, di and tri nucleosomes, supernatants were combined and used for ChIP. Samples were incubated in [50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 20 mM Na butyrate, 5 mM Na2EDTA] with primary antibody on a rotating wheel at 5 rpm overnight at 4°C. Blocked protein A sepharose (GE healthcare) was added to immobilize chromatin-antibody complexes samples rotated for 3 hrs at room temperature. The supernatant of centrifuged samples were kept on ice; reflecting the unbound fraction of input chromatin. Bound fractions were washed in progressively stringent low to high salt buffers [50-150mM NaCl]. Elution was carried out for 30 min at room temperature [50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 20 mM Na butyrate, 5 mM Na2EDTA, 1% SDS]. 0.2mg/ml Proteinase K was added to both unbound and bound fractions and incubated at 50°C for 2 hrs. DNA was isolated using a Qiaquick gel extraction kit (Qiagen) and resuspended in 50-60μl TE. DNA concentrations were determined by PicoGreen fluorimetry (Molecular Probes, Invitrogen) and unbound fractions were further diluted in TE to match bound concentrations before analysis by quantitative real-time PCR (qRT-PCR; section 2.2.5.3) and expressed as relative enrichment (bound/unbound
The C-ChIP primers listed in Appendix I: Table I-2.

2.2.4 Methylated DNA immunoprecipitation analysis (MeDIP)

MeDIP was carried out using genomic DNA from ES, TS and TSL as previously described (Weber et al., 2005). Genomic DNA was isolated from cells by overnight incubation in 500 μl of Lysis buffer [200 mM NaCl, 100 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.2% SDS, 250 μg proteinase K] at 55°C and subsequent phenol/chlorophorm/isoamylalcohol and chlorophorm/isoamylalcohol extractions. DNA was then precipitated with 50% isopropanol, followed by 70% ethanol wash, and resuspension in TE with 20 μg/ml RNAse A. DNA concentration was determined using a NanoDrop ND-1000. Genomic DNA was sonicated (VC 130 PB, Sonics & Materials, Inc) to produce random fragments ranging in size from 300 to 1000 bp. 5 μg of fragmented DNA was used for the MeDIP assay. After denaturation for 10 min at 95°C and cooling down on ice for 2 min, samples were immunoprecipitated for 2 hours at 4°C with 10 μl of Methyl-C antibody in a final volume of 500 μl of immunoprecipitation buffer [10 mM Na-Phosphate pH 7.0, 0.14 M NaCl, and 0.05% Triton X-100] with constant rotation. Chromatin-antibody complexes were then precipitated with 30 μl of Dynabeads® with M-280 sheep antibody against mouse IgG (Dynal Biotech, Oslo, Norway) for 2 hrs at 4°C and washed three times with 700 ul of immunoprecipitation buffer. Beads were then treated with 70 μg of proteinase K for 3 hours at 50°C and methylated DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation at -20°C and resuspended in 60 μl of TE buffer. The abundance of methylated DNA was determined by qRT-PCR amplification with 25 ng of input DNA and 2 μl of the immunoprecipitated methylated DNA and expressed as relative enrichment (fold over input). (Protocol carried out by S. Pinho)
2.2.5 RNA interference

Lentivirus vector containing a short-hairpin RNA (shRNA) targeting mouse Suv39h1 mRNA was cloned in pLKO.1 (Sigma) by F. Lavial. The sequence used to successfully knockdown expression of Suv39h1 is as follows: 5′-GCCTTTGTACTCAGGAAAG-3′ (TRCN97439). Recombinant lentiviruses were kindly generated by A. Chandrashekar (Imperial College London, UK) using a three-plasmid system in 293T cells as previously described (Kutner et al., 2009). Virus-containing culture supernatants were collected after 24 and 48 hours of transfection, pooled, concentrated and used for infection. Control and Suv39h1-knockdown TS cells were collected at 5 days post-infection for subsequent expression and chromatin analysis. (Protocol carried out by F. Lavial)

2.2.6 Reverse transcription polymerase chain reaction (RT-PCR)

2.2.6.1 RNA extraction and cDNA synthesis

RNA extraction was performed using RNeasy protect mini kit (Qiagen, Valencia, Ca) and residual DNA was eliminated using the RNase-free DNase kit (Qiagen) according to manufacturer’s instructions. RNA was then reverse transcribed using Superscript™ First-Strand Synthesis system (Invitrogen). 1-2 μg of total RNA was supplemented with 1 μl of 10 mM dNTP mix (Invitrogen) and 1 μl of oligo (dT)12-18 (Invitrogen) and diluted in Rnase free water to a final volume of 11 μl. The mixture was incubated at 65°C for 5 min and put on ice for 1 min, when 1 μl of 0.1 M DTT, 4 μl of 5X first strand buffer, 1 μl of RnaseOUT (Invitrogen) were added before being further incubated at 42°C for 2 min and 1 μl of 200 U/μl Superscript II was added. A reaction mixture without the enzyme was also set up as a control (designated “-RT”). The mixture was incubated at 42°C for 1 hr and at 70°C for 15 min to stop the reaction. 181ul of Rnase-free water was added and cDNAs of interest were then detected by quantitative real-time PCR.
2.2.6.2 Primer design and testing for quantitative PCR

ChIP primers were designed with the Primer Express software (Applied Biosystems, Warrington, UK) and expression primers were designed with PerlPrimer software (Marshall, 2004) using sequences from the mouse and human database from ENSEMBL (http://www.ensembl.org/index.html). The following design criteria were used: amplicon size of 100-200 bp, GC content of 40-60%, melting temperature 55-65°C. All primer pairs were tested for predicted products within the mouse and human genome using in silico PCR tools such as PUNS (http://okeylabimac.med.utoronto.ca/PUNS) or the UCSC genome browser (http://genome.ucsc.edu). Primers yielding a single predicted PCR product of the correct size were ordered from Sigma Genosys (the primers sequences are shown in their 5’ to 3’ orientation). The efficiency of amplification for each primer pair was tested by qRT-PCR with sequential 2-fold dilutions of genomic DNA or cDNA (Pfaffl, 2001). Primers yielding poor linear fits of the C(T) versus logarithm of concentration (R^2<0.98) or efficiencies lower than 1.8 or greater than 2.2 were discarded. When species specificity was required (i.e. analysis of C-ChIP samples), primers were designed to specifically amplify the mouse sequences but not the Drosophila orthologs. All primers are listed in Appendix I: Table I-1 and 2.

2.2.6.3 Real-time quantitative PCR analysis (qRT-PCR)

Real-time PCR analysis was carried out in 30μl reaction volume with Jumpstart Syber-Green PCR Mastermix (Sigma), 300nM primers and 2ul of template. A reaction without DNA was included to control for the formation of primer dimers and each measurement was performed in duplicate. PCR reactions were performed on a DNA Engine OpticonTM using Opticon Monitor 3 software (MJ Research Inc., Waltham, MA), running the following program: an initial denaturating step at 95°C for 15 min, 40 cycles
of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec at which point the fluorescence was read at 72°C, 75°C, 78°C and 83°C. The melting curve was determined from 70°C to 90°C, at 0.2°C intervals. The analysis of the qRT-PCR data was performed with the Opticon Monitor 3 software and the relative abundance of sequences was calculated using the ΔΔC(T) method (Pfaffl, 2001). When the amplification efficiency is close to 2, the relative amount of PCR products between reactions 1 and 2 can be calculated as 2-ΔC(T)1/2-ΔC(T)2, being C(T) the threshold cycle at which fluorescence due to PCR products becomes detectable above background. Gene expression data was normalized to the average of two housekeeping genes as previously described (Vandesompele et al., 2002). Primer sequences used in this study for gene expression and ChIP analysis can be found in Appendix I.

2.2.7 Genomic PCR

2.2.7.1 Genomic DNA extraction

Genomic DNA was isolated from cells by overnight incubation in 500 μl of lysis buffer (200 mM NaCl, 100 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.2% SDS, 250 μg Proteinase K) at 55°C and subsequent phenol/chlorophorm/isoamylalcohol and chlorophorm/isoamylalcohol extractions. DNA was then precipitated with isopropanol (50%), followed by 70% ethanol wash, and resuspension in Tris-EDTA (TE; 10 mM Tris-HCl pH 8.0, 1 mM EDTA). DNA concentration was quantified by spectrophotometry.

2.2.7.2 Genomic PCR

PCR analysis was performed in 50 μl using 0.4 μM primers, 1.25 U of HotStarTaq™ DNA polymerase (203205, Qiagen, Valencia, CA) with the following program: 95°C for 15 min, then cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. PCR products were separated by electrophoresis on 1% agarose gels and visualised by ethidium bromide staining.
2.2.8 Cloning and DNA delivery into ES cells

2.2.8.1 Engineering of luciferase reporter constructs

Enzymes and buffers were from New England Biolabs (New England Biolabs Ltd, Herts, UK) unless otherwise stated. Mouse Nanog binding sites determined at Fgf4 and Fgfr2 loci were amplified from mouse ES cell genomic DNA by PCR with primers: Fgf4 5'-CCCTGGTGACTAAGCTGGAG 3' and 5'-CAATTCCGAGCACCTAAGGA 3' and Fgfr2 5'-ACGGCACCCATTATGTGTTC 3' and 5'-GCCTCTCTACCCCTGTTCAA 3'. The PCR products were run on 1% agarose gels with the 2-log DNA ladder (0.1-10kb) (NEB) and bands of correct size were purified using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. PCR products were A tailed by incubation with 5U Taq polymerase (Invitrogen) and 0.2mM dATP for 20 min at 70°C before ligation into the pGEM-T Easy vector (Promega). 50ng of vector and a three-molar excess of insert were ligated with 1 μl (400U) of T4 DNA ligase overnight at 4°C. 5μl of DNA was then added to 25μl of competent TAM-1 cells (Active Motif), incubated on ice for 15 min, 30 sec at 37°C and 5 min back on ice. Transformed cells were then incubated for 1 hr in 250μl Lysogeny Broth (LB) broth at 37°C with agitation before being plated onto LB agar supplemented with 50 μg/μl of ampicillin and incubated overnight at 37°C. Individual colonies were picked into LB broth with 50 μg/μl of ampicillin and incubated overnight at 37°C with agitation. DNA was extracted from exponentially growing cultures with the Miniprep or Midipreps kits (Qiagen) according to manufacturer's instructions and final DNA diluted in double distilled water and sequence verified (MRC Clinical Science Centre sequencing facility). To generate Firefly-luciferase reporter constructs, the inserts carrying the Nanog binding sites of Fgf4 and Fgfr2 were cleaved out of pGEM-T Easy by their Sacl sites and ligated into the Sacl site of pGL3-Promoter vector (Promega) using the procedure described above. Correct sequence orientation was verified by
restriction enzyme digestion and sequencing (MRC Clinical Science Centre sequencing facility).

2.2.8.2 Mutagenesis

To generate luciferase constructs with mutated Nanog binding sites QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was used as per the manufacturer’s instructions. Briefly, the Nanog binding sites within the luciferase constructs were mutated using the primers: Fgf4 5’-GGGAGACTTCTGAGCAACCTCCCAGGCCACTTTATGGAAGGCTACAG 3’ and 5’-TCCCATAAAGTGGGCTCGAGGTTGCTCAG 3’ and Fgfr2 5’-TCCACCCGACCCGACTCTTGTTGTGGTGG 3’ and 5’-TCACCGCACGGGCGGTTGGCCGTGG 3’ which converted the ATTA binding sequence to GGCC (underlined). Primers were designed using QuickChange Primer Design Program (http://www.stratagene.com/qcprimerdesign; Strategene). PCR was performed in 50μl with 50ng template, 250ng primers and 2.5U PfuTurbo DNA polymerase (Stratagene) with the following program: 95°C for 30 sec, then 18 cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 5min and 20 sec. Original template was DpnI digested at 37°C before transformation into competent TAM-1 cells (Active Motif).

2.2.8.3 DNA delivery into ES cells

DNA delivery into ES cells was performed using lipofectamine 2000 (Invitrogen). 1x10⁶ ES cells were plated in each well of a 6-well plate and 24 h later cells were transfected. Following the manufacturer’s guidelines the DNA:Lipofectamine complexes were prepared at a ratio of 1:3 (μg:μl). 4 μg DNA were diluted in 400 μl of OptiMEM (Invitrogen) while lipofectamine was also diluted 1:40 in OptiMEM. After incubating for 5 min both solutions were combined and left at room temperature for 20 min. The mixture was then added to the cells and incubated at 37°C overnight. Finally, the transfection mix was replaced for 2 ml ES cell culture medium plus LIF. To form stable
Fgfr expressing ES cells clones from pcDNA3.1 vectors, selection was applied 24 hrs after transfection (G418, 400 μg/ml; Invitrogen) and resistant ES cell colonies were manually isolated after 10 days.

2.2.9 Luciferase reporter activity assay

ES cells were plated in 96-well plates at 2x10^4 per well and Lipofectamine™ 2000 was used to cotransfect 20ng of supercoiled luciferase reporter plasmids with 10ng control Renilla reported plasmid (pCMV-renilla) for normalizing transfection efficiency into the ES cells. Cells were lysed 48 hrs after transfection with Steadyliteplus (PerkinElmer) for 20 min at room temperature in the dark as per the manufacturer's instructions. The luciferase and renilla activities were measured in a Luminometer (Victor2™ 1420; PerkinElmer™) using a Dual-luciferase assay system (Promega).

2.2.10 Immunofluorescence (IF) analysis and alkaline phosphatase activity

Cells seeded on glass coverslips pre-coated with 0.1% gelatin were washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Fixed samples were washed in PBS and, for intracellular staining, permeabilized with 0.4% Triton X-100 in blocking solution [0.5% bovine serum albumin (BSA), 0.1% FCS, in PBS] for 30 min. Samples were incubated sequentially in blocking solution for a further 30 min and in primary antibody (see section 2.1.2) diluted in blocking solution for overnight at 4°C in a humid chamber. Coverslips were subsequently washed in PBS before being incubated with secondary antibodies coupled with appropriated fluorophores (Molecular Probes; see section 2.1.3) diluted in blocking solution for 1 hr in a humid chamber. Finally, cells were washed twice in PBS for 5 min and mounted in Vectashield (Vector) with DAPI (0.1 μg/ml). Samples were visualised using a TCS SP5 Leica laser-scanning confocal microscope. Images were processed using Leica Confocal
software and Adobe Photoshop CS2. Microscope settings and laser power were kept constant between the controls and samples.

For alkaline phosphatase activity assays, ES cells were stained using an alkaline phosphatase assay kit (Sigma) according to manufacturer's instructions. Images of alkaline phosphatase activity staining were collected using a Nikon E600 microscope.

### 2.2.11 Colony formation assay

Wild-type (E14Tg2a) and Nanog over-expressing (EF4) ES cells were seeded at a low density ($1.8 \times 10^3$/well) in a 6-well plate in ES cell culture media. ES cells were transfected overnight with control (pcDNA3.1), Fgfr2-myc tagged Fgfr2 (Fgfr2-myc) or constitutively active Fgfr2 (Fgfr2-ca) containing pcDNA3.1 vectors (a kind gift from A. Mansukhani; Mangasarian et al., 1997; Mansukhani et al., 2000; Mansukhani et al., 1992) using Lipofectamine 2000 (Invitrogen). Following day the transfected ES cells were placed under neomycin selection in ES cell media plus FCS with or without LIF for 9 days, changing the media each day to remove any endogenously produced LIF. Formed colonies were fixed, stained for alkaline phosphatase activity as per the manufacturer’s instructions and the number of undifferentiated, mixed or differentiated colonies was scored from each condition. A colony with greater than 90% staining corresponded to an undifferentiated state, 90%–20% was called mixed and less than 20% staining was denoted as differentiated.

### 2.2.12 Western blotting

Whole cell extracts were obtained by direct lysis of cells with Radio Immuno Precipitation Assay (RIPA) buffer [150 mM NaCl, 50 mM Tris pH 8.0, 0.5% Deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40 plus protease inhibitors] on ice for 30 min. Samples were centrifuged at 14,000 rpm for 20 min at 4°C and supernatant collected and stored at -80°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the Bio-Rad minigel system. Protein
solutions were quantified using the Bradford assay (Thermo Fisher Scientific) and readings taken from Optimax tunable microplate reader (Molecular devices). Protein samples were diluted 1:1 with Laemmli sample buffer (BioRad) with 5% β-mercaptoethanol and heated at 95°C for 5 min. 5 -50 μg of protein sample and the benchmark pre-stained protein ladder (Fermentas) were loaded on an acrylamide (Bio-Rad) stacking gel [5% (w/v) acrylamide, 0.125M Tris (pH 6.8), 0.1% (w/v) N,N,N',N'-tetramethylethylenediamine] and separated in a 10% acrylamide resolving gel [10% (w/v) acrylamide, 0.4 M Tris (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine] using Tris-glycine electrophoresis buffer [1.5% (w/v) Tris, 7.2% glycine, 0.5% (w/v) SDS] for 1.5 hrs at 120V. Resolved acrylamide gels were blotted onto methanol activated polyvinylidene fluoride (PDVF) membranes (Chemicon/Milipore) in transfer buffer [48 mM Trizma base, 39 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol] using the trans-blot semi-dry electrophoretic transfer system (Bio-Rad) for 1 hr at 20V. The membranes were incubated in blocking buffer [5% (w/v) fat free milk powder (Sigma), Tris-Buffered Saline plus 0.5% Tween20 (TBS-T)] for 1 hr at room temperature, followed by primary antibody incubation diluted in blocking buffer in TBS-T buffer, overnight at 4°C, with agitation. After washing 3 times for 10 min in TBS-T, blots were incubating with the appropriate HRP-conjugated secondary antibody (Santa Cruz) in blocking buffer, for 1 hr at room temperature. Excess secondary antibody was removed by washing the membrane for 10 min 3 times in TBS-T buffer. Blot membranes were treated by enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, UK) following manufacturer’s instructions for HRP-conjugated antibody detection before being exposed to X-ray films (CL-XPosure Film, Thermo Scientific, UK) and using the OPTIMAX IGP developer. A list of primary and secondary antibodies used for western blot as well as quantities and suppliers are show in sections 2.1.2 and 2.1.3.
Chapter 3 Analysis of bivalent chromatin signatures in stem cells and in vivo.

Pluripotent ES cell identity is stably maintained in culture by flexible, epigenetic silencing that prevents premature expression of critical developmental genes that are in a primed state of expression. Such mechanisms include Polycomb-mediated repression by PRC1 and PRC2 complexes (Boyer et al., 2006b; Lee et al., 2006), co-existence of repressive H3K27me3 and permissive H3K4me2 histone marks at key regulatory loci (Azuara et al., 2006; Bernstein et al., 2006; Pan et al., 2007; Zhao et al., 2007) and assembly of a ‘poised’ RNA polymerase II complex that is phosphorylated on the CTD serine 5 residue (Brookes and Pombo, 2009; Guenther et al., 2007; Stock et al., 2007). While these data show that ‘poised’ bivalent domains exist in pluripotent stem cells, it is still unclear whether bivalent histone marks are present in the developing mouse blastocyst. Here we asked whether bivalent chromatin signatures occur at key developmental promoters in the pluripotent ICM as well as the more lineage-restricted multipotent TE and investigated the potential acquisition of these marks prior to blastocyst formation (Figure 3.1).

Figure 3.1: Stages of mouse pre-implantation embryo development. The diagram illustrates the early steps at which carrier ChIP was carried out until blastocyst formation at E3.5. ES and TS cells can be derived from the ICM and TE, respectively.
3.1 Bivalent histone marking operates *in vivo* at key developmental genes in the early mouse embryo.

The study of the epigenetic mechanisms involved in differentiation and development *in vivo* is challenging due to the techniques available and the limited amount of material from samples like pre-implantation embryo tissue (Figure 3.1) or biopsies. Chromatin immunoprecipitation or ChIP is used for determining the genomic and locus specific distribution of proteins and their modifications. For ChIP assays a large number of cells, roughly $10^6$-$10^7$ cells are ideally required. The recent derivation of stem cells like ES and TS cells from the ICM and TE respectively, has made these quantities attainable and has proved an invaluable tool for assessing regulatory mechanisms of pluripotency. Expanded in culture, pluripotent ES cells have the potential to differentiate into multiple cell lineages as well as providing germline transmission (Evans and Kaufman, 1981; Martin, 1981). In spite of this, the questions that remains is if cultured stem cells are a suitable model to study the epigenetic mechanisms involved in development *in vivo*.

Efforts by a number of groups in recent years have modified existing ChIP protocols and reduced the necessary starting material to 100-1000 cells (Acevedo et al., 2007; Dahl and Collas, 2008; O’Neill et al., 2006). By combining native ChIP, *Drosophila* SL2 cells as a source of ‘carrier’ chromatin and PCR, Laura O’Neill and Matthew VerMilyea developed carrier-ChIP (C-ChIP). The underlying principle being that millions of evolutionarily distinct *Drosophila* S2 carrier cells can protect precious small cell samples from significant losses when pooled together for chromatin preparation and immunoprecipitation. The authors found that the epigenetic profile of key regulatory genes including *Nanog*, *Pou5f1* and *Cdx2* determined by C-ChIP, correlated between mouse ES samples of ~1000 cells and pooled dissected ICMs, consisting of ~100 cells, but as expected differed to that of isolated TE samples (O’Neill et al., 2006). We took advantage of the novel C-ChIP method to examine the existence, formation and
resolution of bivalent histone domains at critical developmental genes *in vivo* before and after lineage segregation in the early mouse blastocyst (Figure 3.1).

All embryo samples were flushed from the oviducts of superovulated pre-pubescent CBAB6 F1 females after mating with CBAB6 F1 males. Eight-cell stage embryos were collected on embryonic day E2.0 and compacted morula stage embryos were collected E2.5. Both samples were snap frozen for subsequent expression and chromatin experiments. To obtain ICM and TE samples, E2.5 morula were cultured overnight and then selected based on morphology for the early blastocyst stage. ICM samples were isolated by an immunosurgery technique established in 1975 (Solter & Knowles, 1975). First, blastocysts are treated with rabbit anti-mouse serum, which cannot penetrate through the outer trophoblast layer and therefore only coats the nascent blastocyst. Upon transfer into guinea pig complement, the TE is selectively lysed and an uncontaminated ICM sample is released as seen in Figure 3.2A. A fine holding pipette immobilizing blastocysts and a micromanipulator apparatus enabled pure TE samples to be mechanically separated, using a micro-dissection blade, and collected. As shown in Figure 3.2B, the C-ChIP protocol begins by mixing small pools of ex vivo embryo samples (10-12 ICM/TE samples, 20 morula or 40 eight-cell stage embryos) with *Drosophila* SL2 cells before nuclei and chromatin preparation. Micrococcal nuclease digestion prepared the native chromatin fragments before subsequent immunoprecipitation using H3K27me3 or H3K4me2 specific antibodies or nonspecific precipitation monitored by control IgG antibodies. Precipitated DNA sequences constituted the bound fraction and the corresponding supernatant formed the unbound fraction. DNA was further quantified by picogreen fluorometry and unbound fractions diluted to match that of the bound samples, before PCR analysis.
Figure 3.2: Collection of Inner Cell Mass and Trophoderm samples from early blastocysts and schematic summary of Carrier-ChIP protocol. (A) Image of a typical early mouse blastocyst selected for experiments. ICM samples were prepared by immunosurgery; selective, complement-induced lysis of trophoblast cells enabled them to be washed away for collection of a relatively pure ICM sample (top). Mural trophoderm was separated by manual dissection; blastocysts were retained by a holding pipette and part of the TE cut away from ICM to ensure no contamination of the sample for C-ChIP analysis (bottom). (B) A diagrammatic summary of the carrier ChIP (C-ChIP) procedure.

To obtain the relative enrichment of histone modifications present only from mouse and not from Drosophila DNA by qRT-PCR analysis, species-specific primers were designed. Primers were monitored for amplification efficiency in low template conditions (Figure 3.3).
Figure 3.3: Characterization of efficient, species-specific primers for C-ChIP analysis by quantitative PCR amplification. (A) Efficiency plots of Pou5f1 and Cdx2 primers illustrate primer performance across serial dilutions of mouse genomic DNA (gDNA) at a low template range. (B) Amplification plots in log scale show the detected fluorescence against number of cycles (C(T) value) in PCR reactions containing mouse only (M), C-ChIP samples (IP) or Drosophila melanogaster (D) only DNA. (C) Melting curves depict primers specificity, both Pou5f1 and Cdx2 primer amplicons from mural DNA only and C-ChIP samples are consistent while Drosophila DNA only samples show a distinguished pattern.

To explore whether H3K27me3 and H3K4me2 are both enriched at key developmental genes in vivo, we assessed the promoters of Sox1 and Gata4, two examples of genes that are bivalently marked in ES cells (Azuara et al., 2006; Bernstein et al., 2006; Santos et al., 2010; Stock et al., 2007). The promoter regions of Pou5f1 and Cdx2 were also examined, as they are known markers of the ICM/ES cells and TE/TS cells, respectively. mRNA expression analysis of these candidate genes, to verify their transcriptional status, revealed that neither Sox1 nor Gata4 mRNA levels were detectable from the eight-cell to the blastocyst stage (Figure 3.4). In contrast, Pou5f1 transcripts were detected at a low level at the eight-cell stage and at higher levels in the
compacted morula as well as the ICM and TE of the newly formed blastocyst. As expected, Cdx2 was primarily expressed in the TE.

![Figure 3.4: mRNA expression levels of candidate loci before and after blastocyst lineage segregation.](image)

**Figure 3.4: mRNA expression levels of candidate loci before and after blastocyst lineage segregation.** Quantitative RT-PCR analysis of Pou5f1, Cdx2, Sox1 and Gata4 during one typical series of eight-cell (light grey), morula (dark grey), ICM (green) and TE (orange) sample preparations. Values shown are normalized to S17 and L19. N.D., not detectable.

The results of the epigenetic analysis are presented as ratios of the bound and unbound fraction values displaying the relative enrichment levels of modified histones at the selected promoters. Figure 3.5 shows that both Sox1 and Gata4 promoters have high enrichment for H3K27me3 and H3K4me2 in all four embryo samples. This enrichment is also observed in the nascent TE sample and indicates that bivalent chromatin domains exist at the early stages of in vivo development. This is consistent with the lack of mRNA expression detected from these loci. Pou5f1 was highly enriched for permissive H3K4me2 in comparison to H3K27me3 levels in all four embryo samples. The ICM, in which Pou5f1 is predominantly expressed, showed the highest levels of H3K4me2 and the nascent TE, where expression is still detected, also showed significant H3K4me2 levels. Interestingly, a slight enrichment of H3K27me3 at Pou5f1 was determined in the TE, which may suggest Pou5f1 epigenetic silencing is beginning to
occur in a subset of cells within the TE layer, consistent with continuing blastocyst maturation. *Cdx2*, in contrast, showed a more dynamic histone profile with enrichment for both H3K4me2 and H3K27me3 in the early eight-cell embryo and ICM, where this gene is expressed at low or variable levels. In contrast to this *Cdx2* is depleted of H3K27me3 marks by the formation of TE-committed cells in which the locus is strongly expressed. Together these data show that bivalent chromatin signatures associated with gene silencing exist in the early mouse embryo from the eight-cell stage up until blastocyst formation. Most strikingly, both permissive and repressive marks were detected at lineage-inappropriate genes in the newly developed TE.

Figure 3.5: *In vivo* C-ChIP examination of bivalent domains at selected promoters in early mouse embryos prior to and after blastocyst lineage segregation. C-ChIP and qRT-PCR were used to assess enrichment levels of H3K27me3 and H3K4me2 at the promoters of *Sox1, Gata4, Pou5f1* and *Cdx2* loci in 8-cell (light grey) compacted morula (dark grey), ICM (green) and TE (orange) samples. Denoted by the dotted lines are pre-immune IgG controls showing only background, unspecific precipitation levels.
Antibody-bound and unbound chromatin fractions were amplified in parallel by qRT-PCR using the same quantity of DNA. Enrichments are expressed as bound/unbound ratios. Error bars represent s.d. of three independent experiments using triplicate PCR amplifications for each gene.

3.2 Bivalent domains mark silent, lineage inappropriate genes in trophoblast stem cells.

The C-ChIP analysis showed that, as occurs in ES cells, during blastocyst formation the inner cell mass harbours bivalent chromatin signatures at the promoters of key select, developmental genes. This suggests that ES cells are a valuable tool for further understanding in vivo epigenetic mechanisms. The second observation, that bivalent histone marks also exist at lineage-inappropriate genes in the extra-embryonic TE layer, the tissue destined to form the placenta, is intriguing, as it appears at odds with prevailing view that bivalent domains become resolved at activated or repressed loci upon differentiation. We therefore investigated further bivalent histone domains in TE-restricted trophoblast stem (TS) cells.

In this analysis we compared E14Tg2a-derived ES cells line denoted ZHBTC4 (kindly supplied by H. Niwa) with a previously established extra-embryonic restricted TS cell line B1 kindly provided by N. Brockdorff (Mak et al., 2002; Niwa et al., 2000). The two cell lines display different morphology and require different growth culture conditions to remain in a proliferating, undifferentiated state. Namely, TS self-renewal is sustained by the provision of fibroblast growth factor 4 (Fgf4), heparin and primary mouse embryonic fibroblasts or conditioned TS media while pluripotent ES cells are maintained by Leukemia inhibitory factor (LIF) and fetal calf serum (FCS) and grown on 0.1% gelatin-coated plates.

For this analysis we focused on previously identified bivalent genes with CpG-rich promoters in ES cells (Azuara et al 2006; Bernstein et al 2006) and which were recently shown to be joint PRC2 and PRC1 targets (Ku et al 2008). The chosen list of genes (Table 1) that exhibit similar properties within their promoter region include a
variety of transcriptional regulators ($Kdr$, $Gata4$, $Hoxa7$, $Atoh1$, $Mixl1$, $Pax3$, $Sox1$ and $Sox7$) typically found in a number of different somatic lineages, as well as TS-associated markers ($Cdx2$, $Fgfr2$ and $Hand1$). As summarized in Table 1, genetic inactivation of most of these selected loci result in embryonic lethality.
Table 1: Names of the selected gene for candidate-based epigenetic analysis in ES and TS cells. Gene symbols, their encoded protein, lineage affiliation, associated in vivo gene inactivation phenotypes and key references. Gene symbols are stated as reported within Mouse Genome Informatics (Bult et al., 2008). * Lineage affiliation reflects tissues in which genes are predominantly expressed in accordance with UniGene cDNA libraries (Wheeler et al., 2003).
To verify and extend our previous *in vivo* observation, the occurrence of bivalent marking at several lineage-inappropriate genes was assessing in multipotent TS cells, using ChIP assays. In the ChIP protocol, chromatin was cross-linked by the addition of formaldehyde to proliferating cells, as opposed to native chromatin required for carrier ChIP. ES and TS cell lines were expanded and treated with 1% formaldehyde to preserve any specific protein-DNA interactions present at that time. Extracted chromatin was then fragmented by sonication into pieces averaging between 300-500bp before immunoprecipitation using specific antibodies against H3K27me3, H3K4me2, H3 C-terminal and negative control IgG antibodies. Treatment of ChIP samples with high temperatures and enzymatic digestion destroyed all immunoprecipitated proteins and enabled the associated DNA to be purified for analysis. qRT-PCR primers were designed to target and amplify up to 600bp upstream of each gene's transcription start site (Appendix I: Table I-2). The abundance of modified histones was monitored and potential differences in nucleosome density were accounted for by normalisation to corresponding levels of H3 abundance and expressed as relative enrichment (percentage over H3). Background levels of unspecific antibody binding and DNA precipitation were assessed by the inclusion of an IgG control.

Corroborating previously published data (Azuara et al., 2006; Bernstein et al., 2006), Figure 3.6A (left panel) shows significant levels of enrichment for both repressive H3K27 and permissive H3K4 methylation marks in ES cells at the selected panel of silent developmental genes (*Cdx2, Fgfr2, Hand1, Kdr, Pax3, Atoh1, Sox1, Hoxa7, Mixl1, Gata4* and *Sox7*). The actively transcribed control gene Actin showed, in contrast, enrichment for H3K4me3 and low levels of H3K27me3 as expected. In contrast to this the negative control intergenic region (Inter.) showed little enrichment for either histone modification (Figure 3.6A). In TS cells, all the silent gene promoters analyzed, *Atoh1, Sox1, Hoxa7, Mixl1, Gata4* and *Sox7* showed high levels of repressive H3K27me3 as well
as permissive H3K4me2 (right panel, Figure 3.6A), reflecting the results of the C-ChIP on isolated TE samples. Five of the candidate genes (Cdx2, Fgfr2, Hand1, Kdr and Pax3) were enriched for permissive H3K4me3 while only low levels of H3K27me3 were observed at these loci, consistent with their active transcriptional status in TS cells. The epigenetic profile of both positive and negative control genes, Actin and Inter., respectively, was similar in TS and ES cells.

Figure 3.6: Comparison of H3K27me3 and H3K4me2 enrichment levels at selected promoter loci promoters between ES and TS cells. (A) Relative enrichment of H3K27me3 and H3K4me2 at a selected panel of promoters and controls in ZHBTc4-ES (green bars) and B1-TS (orange bars) cells. Background enrichment levels assessed by IgG antibodies are shown as grey bars. Error bars represent s.d. of three independent
experiments. Expression status is shown as an aid and indicates whether genes are actively transcribed (+) or not (-). (B) Sequential ChIP of same gene promoter panel using first anti-H3K27me3 followed by anti-H3K4me2 antibodies on either ES or TS cells. Enrichments are shown relative to total H3. Error bars represent s.d. of three independent experiments.

To verify that both H3K27 and H3K4 methylation are simultaneously present on the same chromosomal allele in TS and ES cells, and thus exclude the possibility of differential loci marking within the cell population, we performed sequential ChIP, an approach previously used in ES cells (Figure 3.6B, Bernstein et al., 2006). Fragmented chromatin from TS cells (and ES cells as controls) was immunoprecipitated first with H3K27me3 antibody and second with H3K4me2 antibody. Consequently only chromatin that concomitantly carried both kinds of modification was retained in the final precipitate. Promoters occupied by only H3K27me3 or H3K4me3 were lost. In TS cells, using qRT-PCR analysis we found that after sequential ChIP each promoter associated with bivalent domains (Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7) was significantly enriched when compared to the control genes that were enriched solely for H3K27 or H3K4 methylation (Right panel; Figure 3.6B). Concurring with previously published data, selected developmental regulators were all depicted as bivalent in ES cells (Left panel; Figure 3.6B). The above result suggests that the bivalent domains accurately represent the epigenetic state on single key regulatory alleles in ES and TS cells and confirms the conventional ChIP data. Altogether, this data indicates that loss of developmental potency is not necessarily accompanied by the loss of bivalent histone marks at many silent, lineage-inappropriate genes in TS cells. Moreover, experiments carried out by O. Alder (Alder et al., 2010; data not shown) determined that high levels of H3K4 methylation were retained at these loci upon TS cell differentiation and placenta formation, indicating that during trophoblast lineage commitment loss of
developmental potency is not necessarily accompanied by loss of this histone modification.

Sequential ChIP was also carried out vice versa with an H3K4me2 targeting antibody used in the first incubation followed by H3K27me3 specific antibody for the sequential immunoprecipitate. However, high background levels were detected in the control genes, most likely due to the higher affinity of H3K4me2 antibody for its target. This higher affinity would then cause the elution conditions to be insufficient to remove the H3K4me3 antibody from targeted DNA. The higher background led to the amplification of active gene regions, like Cdx2 in TS cells, despite no previous indication of H3K27me3 occupancy at this site (Figure 3.7)

Figure 3.7: Bivalent domains mark key embryonic regulators in extra-embryonic TS cells. Sequential ChIP firstly using anti-H3K4me2 and secondly anti-H3K27me3 antibodies was performed on ZHBTc4-ES and TS-B1 cells. Enrichment at selected promoters is expressed relative to H3. Data shown is from a single experiment. Expression status is indicated below gene names; actively transcribed genes are marked (+).

3.3 Different chromatin environments at bivalently marked loci in TS cells compared to ES cells.

The existence of bivalent histone configurations at key developmental loci is usually associated with 'priming' for future expression or repression. The function of
these domains at lineage-inappropriate genes in extra-embryonic committed TS and in vivo TE cells is unclear. To explore further the chromatin status of bivalent loci in TS cells, we assessed the extra-embryonic restricted stem cells for additional features of poised chromatin that were previously identified in pluripotent ES cells. All candidate loci are targets of both PRC2 and PRC1 complexes in ES cells as mentioned previously (Ku et al., 2008). PRC2 comprises of three major components, Eed, Suz12 and Ezh2, which catalyses H3K27 tri-methylation. Deletion of either Suz12 or Eed disrupts the PRC2 complex and leads to the ‘chaotic’ up-regulation of many bivalently marked genes (Azuara et al., 2006; Boyer et al., 2006b). PRC1 has been shown to consolidate silencing by reinforcing PRC2 repression. Proteins of the Cbx family with affinity for H3K27 methylation are part of the PRC1 complex along with Bmi1 and Mel18 (Bantignies and Cavalli, 2006; Fischle et al., 2003; Min et al., 2003; Pietersen and van Lohuizen, 2008; Ringrose and Paro, 2007; Sauvageau and Sauvageau, 2010; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007; Simon and Kingston, 2009). PRC1 also contains the E3 ubiquitin ligases Ring1A and Ring1B that mono-ubiquitylate histone H2A lysine 119. A functional link was established between PRC1 and RNA Polymerase II (RNAP II) in ES cells. Conditional deletion of Ring1A/B revealed that Ring1B restrains RNAP II generating a poised conformation and preventing untimely expression of genes marked by bivalent chromatin signatures (Stock et al., 2007; Zhou et al., 2009).

PRC2 and PRC1 binding was assessed in TS cells by ChIP using antibodies against Suz12 and Ring1B, respectively. As expected, the loss of H3K27me3 at genes in TS cells was also confirmed by lack of Suz12 binding. As shown in Figure 3.8A (upper panels), substantial enrichment for Suz12 binding was consistently seen at all bivalently marked genes both in ES and TS cells, although relatively lower levels were seen in TS cells. In distinct contrast to ES cells, none of these silent, PRC2-bound genes were significantly enriched for the PRC1 component, Ring1B, relative to expressed genes in TS cells (lower panels; Figure 3.8A). The chromatin profiles of control loci remained
unchanged in both cell populations. Western blot analysis revealed that the different patterns of Suz12 and Ring1B binding did not correlate with changes in protein levels in the two cell types (Figure 3.8B). Immunofluorescence verified the presence of Ring1B in TS cell by showing co-localization of Ring1B and Suz12 on the inactive X chromosome of female cells, in accordance with what has been shown before (Figure 3.8C; de Napoles et al., 2004; Fang et al., 2004). The absence of PRC1-binding at these bivalently marked genes in TS cells, but not in ES cells suggests that these genes may lie in different chromatin environments in extra-embryonic and embryonic tissues.

Figure 3.8: Differential enrichment of Polycomb repressive complex component Ring1B at bivalently marked developmental loci in ES and TS cells. (A) Binding of PRC2 and PRC1 components, Suz12 and Ring1B, respectively, was assessed by ChIP and qRT-PCR in ES and TS cells at a selected panel of key developmental loci. Enrichment is
expressed relative to ChIP input. Expression status is shown as an aid to show whether genes are actively transcribed (+) or not (-). Data shown is representative of three individual experiments, error bars represent s.d. The Ring1B ChIP experiment was carried in collaboration with E. Brookes (B) Western blot analysis with anti-Suz12, anti-Ring1B and anti-tubulin (loading control) antibodies in ES and TS cells. (C) Immunofluorescence analysis in female B1-TS cells, showing co-localization of Suz12 and Ring1B on inactive X chromosomes in trophoblast cells (white arrows); scale bar represents 10μm.

As mentioned previously PRC1 and poised RNAP II are functionally linked in ES cells. Together they are thought to prime bivalent genes for future activation or repression upon differentiation while preventing their inappropriate expression. In eukaryotes the largest RNAP II subunit has a C-terminal repeat domain (CTD) consisting of 52 copies of the heptad consensus repeat sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (Corden, 1990). Hypophosphorylated RNAP II is recruited to promoters where the CTD is subject to hyperphosphorylation, a state traditionally associated with the production of full-length, spliced transcripts. Both Ser-2 and Ser-5 residues of the heptad repeat are common targets of phosphorylation (P), the transcription pre-initiation complex associates with Ser-5P at the 5’ end of genes, while Ser-2P correlates with transcript elongation in the coding region (Brookes and Pombo, 2009; Stock et al., 2007). In ES cells bivalent genes were found to assemble RNAP II complexes preferentially phosphorylated on Ser-5 residues though, unlike inducible genes such as those belonging to the heat shock family, little hypophosphorylated RNAP II could be detected (Guenther et al., 2007; Stock et al., 2007). Unexpectedly RNAP II Ser5P was also detected in the coding region of these genes but little evidence of elongation linked Ser2P was found. This unusual configuration of RNAP II at bivalent genes has been described as poised at associated promoters. Release of RNAP II from the poised conformation and up-regulation of target gene expression in ES cells (from primed to overt transcription) was induced by conditional deletion of Ring1A/B (Endoh et al.,
To assess whether silent promoters occupied by opposing histone modifications in TS cells are also subjected to transcriptional priming, the recruitment and conformation of RNAP II were investigated by ChIP using previously validated antibodies against either hypo- phosphorylated (8WG16) or Ser5-phosphorylated (Ser5P; 4H8) RNAP II configurations (Brookes and Pombo, 2009; Stock et al., 2007; Xie et al., 2006; Xie and Pombo, 2006). In agreement with previous reports, bivalent genes and active controls (Actin and Hprt) were found highly enriched for Ser5P RNAP II at their promoter regions compared to the non-transcribed Intergenic region in ES cells (left panel; Figure 3.9). A poised RNAP II conformation was discriminated from transcriptional active RNAP II by measuring the level of 8WG16 binding, which was evident at actively transcribed controls, but as reported was virtually absent at bivalent promoters in ES cells (Guenther et al., 2007; Stock et al., 2007). In TS cells, Ser5P and 8WG16 dually assembled at expressed genes (Cdx2, Fgfr2, Hand1, Kdr and Pax3) as well as active controls (Actin and Hprt), as expected (right panel; Figure 1.8). RNAP II complexes (Ser5P/8WG16) were, in contrast, absent or barely detectable at the promoters of Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7 in TS cells. Altogether these data indicate that RNAP II complexes and PRC1 do not occupy lineage-inappropriate promoters in TE-restricted stem cells. This is consistent with a loss of gene priming for transcription.
Figure 3.9: Poised RNA polymerase II occupancy and conformation is undetected at bivalent promoters in TS cells. Abundance of Ser5 phosphorylated (Ser5P) or hypo-phosphorylated (8WG16) RNAP II at bivalent, active and intergenic loci in ZHTc4-ES (green bars) and B1-TS (orange bars) cells. Enrichment was assessed by ChIP and qRT-PCR and is expressed relative to ChIP input. Expression status is shown as an aid to indicates whether mRNA is actively produced (+) or not (-). Error bars represent s.d. of three independent experiments.

3.4 Conclusion

Bivalent chromatin domains, recently identified in ES cells, are thought to provide pluripotent cells with the flexibility to differentiate into all three germ layers while preventing premature pluripotency loss. By C-ChIP we demonstrate for the first time that bivalent chromatin domains exist in vivo in the early mouse embryo suggesting that these are biologically significant structures that function to regulate genes during embryonic development (Figure 3.5). Identification of similar signatures from the eight-cell stage embryo indicates such tight transcriptional control may operate in the embryo before ICM formation. Within the morula it is essential that a subset of cells commit to an extra-embryonic fate ensuring embryo implantation and future development of the
embryo proper. Surprisingly, we found that cells which undergo TE lineage-restriction, and associated stem cells, also carry bivalent chromatin signatures at lineage-inappropriate genes (Figure 3.5 and 3.6). Further examination of the chromatin environment at these loci in ES and TS cells interestingly revealed a loss of Ring1B and RNAP II binding in extra-embryonic cells consistent with loss of gene priming. This data questions the unmitigated association between bivalent chromatin domains, ‘priming’ for future expression and bivalent modification resolution upon differentiation. Priming may be a unique hallmark of pluripotent cells in the early pre-implantation embryo. This also opens up the fascinating question of how PRC1 recruitment and poised RNAP II occupancy are controlled during development. To understand how different chromatin environments are established at bivalent genes in ES and TS cells, we next embarked on a series of experiments using a previously established system in which ES cells can be induced to acquire a TS cell fate.
Chapter 4 Dynamic chromatin environment changes at bivalent genes upon trophectoderm lineage commitment in vitro and in vivo.

The classical pluripotency factor Pou5f1 (also known as Oct4) is regarded as a core regulator of cell lineage specification in the early embryo. The Pou5f1 gene was discovered in 1990 (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990b) and encodes a POU domain containing transcription factor. Pou5f1 is maternally expressed in the unfertilized egg and all blastomeres of the morula before becoming restricted to the pluripotent compartment at the late blastocyst stage (Nichols et al., 1998; Palmieri et al., 1994; Scholer et al., 1990a; Scholer et al., 1989). At this E4.0-E4.5 stage, Pou5f1 is not detected in the blastocyst's extra-embryonic trophectoderm due to inhibition by the transcription factor Cdx2 that is highly expressed in this tissue (Niwa et al., 2005). In Pou5f1-deficient mice the ICM develops normally but cannot be maintained resulting in pluripotent cells diverting towards TE differentiation and ultimately peri-implantation lethality (Nichols et al., 1998). When Pou5f1-expressing ES cells are injected into developing embryos to form chimeras they contribute almost exclusively to embryonic tissues, while Cdx2-expressing TS cells contributed solely to the extra-embryonic tissue (Tanaka et al., 1998). This contribution bias led many stem cell biologists to conclude that ES cells possessed limited ability to differentiate into extra-embryonic tissue cells. However, recent studies have demonstrated that, subject to manipulation, ES cells can indeed be pushed towards an extra-embryonic trophoblast cell fate both in vitro and in vivo (Lu et al., 2008; Niwa et al., 2000; Niwa et al., 2005; Schenke-Layland et al., 2007). For example, in the ZHBTc4 ES cell line Pou5f1 can be conditionally inactivated, and when this occurs ES cells convert into TS cells (Niwa et al., 2000). In the following experiments we took advantage of this in vitro system to further analyse the relationship
between embryonic versus extra-embryonic cell identity and targeted chromatin changes.

### 4.1 ZHBTc4-derived TS-like cell line characterization.

In recent years Hitoshi Niwa and colleagues have explored the relationship between Pou5f1, Cdx2 and lineage identity by genetically manipulating blastocyst-derived stem cells. A tet transactivator and Doxycycline (Dox)-responsive *Pou5f1* transgene were first introduced into an E14Tg2a ES cell line. Endogenous *Pou5f1* alleles were subsequently disrupted by incorporation of internal ribosome entry sites (IRES) to create ZHBTc4 ES cells (Niwa et al., 2000). Immunofluorescence analysis indicated that Pou5f1 protein was efficiently lost upon Dox treatment throughout ZHBTc4 ES cells populations after 24 hours of Dox treatment (Figure 4.1; Niwa et al., 2000). In response to this loss of Pou5f1 protein, Cdx2 positive cells steadily increased throughout the course of treatment reaching 40% of the population by 72 hours (Alder O; personal communication). These observations corroborate with the transcriptional inhibition function of Pou5f1 on the Cdx2 promoter (Niwa et al., 2005). Other ES cell associated proteins such as Nanog and Sox2 persisted in a high proportion of cells at 24 hours after treatment but gradually decreases over time (Alder O; personal communication). Simultaneously, and in accordance with previously published results (Niwa et al., 2000), Pou5f1 depletion in ZHBTc4 ES cells led not only to the induction of Cdx2 expression, but also to an increase in the expression levels of other trophectoderm markers such as Fgfr2 and Hand1 after 48 hours Dox-treatment (Alder O; personal communication).
Figure 4.1: \textit{Pou5f1} repression in ZHBTc4 cells and subsequent derivation of TS-like cells by provision to TS cells culture conditions. (A) Doxycycline (Dox) response of ZHBTc4 ES cells was assessed by immunofluorescence staining for Pou5f1 and TE-associated Cdx2 before and after 24 hours of Dox treatment (ESDox). Scale bars represent 10μm. (B) Schematic of \textit{Pou5f1} repression and \textit{Cdx2} up-regulation induced by Dox treatment of ZHBTc4 ES cells. The provision of inactivated primary embryonic fibroblasts in the presence of Fgf4 and heparin promoted the derivation of TS-like (TSL) cells. (C) Bright field images compare the morphology of ZHBTc4-derived TS-like (TSL) cells cultured in TS cell derivation conditions combined with treated with Dox for 9 days and traditionally derived TS-B1 (TS) colonies. (D) Immunofluorescence analysis for TS-associated marker Cdx2 shows homogenous staining patterns between TSL cells and TS-B1 cells. Note that no Cdx2 signal was detected in untreated ZHBTc4-ES cells, as expected (data not shown). Scale bar represents 10 μm.

To address the relationship between Pou5f1, lineage identity and chromatin profiles, Dox-treated ZHBTc4 ES cell colonies were gradually induced to convert into TS-like (TSL) cells using an established protocol (Niwa et al., 2000). Briefly, for TSL derivation, ES cells plated at low density on mouse embryonic fibroblasts were Dox-treated to cause \textit{Pou5f1} shutdown and at the same time were provided with conditions...
permissive for TS cell growth (high levels of Fgf4 and heparin). Over 16 days the morphology of treated cells altered from ES colonies to those with a uniformly flattened morphology, similar to that of the blastocyst-derived B1 TS cell line (Figure 4.1C). At this stage, ZHBTc4-derived TSL cells showed a similar pattern of Cdx2 staining to the wild type TS cell line (85-90% cell positive), while Pou5f1 protein was undetectable in either culture (Figure 4.1D). TS-like cells could be serially passaged and were propagated in culture for up to 6 weeks. Together this data proved the ZHBTc4 line to be a reliable ES cell system in which to perform further experiments.

4.2 ‘Poised’ chromatin profile alterations upon Pou5f1 inactivation and TE lineage commitment.

To investigate dynamic changes in the epigenetic profile occurring at key developmental regulators during conversion from a pluripotent ES cell to TS-like cell fate we used the ZHBTc4 in vitro cell system. Chromatin was collected from ZHBTc4 ES cells grown in proliferative conditions (ES), after 48 hours of Dox treatment (ES\textsuperscript{Dox}), after conversion towards TS-like cells (TSL) and compared to blastocyst-derived TS cells (TS). Enrichment levels of H3K27me3 and H3K4me2 were examined at the same selection of candidate genes as in the previous chapter. Alterations in selected gene expression levels were monitored after 24 and 48 hours of Dox treatment in ES culture conditions and after TSL derivation.

After 48 hours of Dox treatment and Pou5f1 shutdown the enrichment of H3K27me3 was notably reduced at the Fgfr2 promoter, while the levels of the bivalent modifications remained unchanged at other selected developmental regulators (ES\textsuperscript{Dox}; Figure 4.2). This included no detectable loss of H3K27me3 at the induced loci Cdx2, Hand1 and Pax3 (Figure 4.2 and 4.3). Potentially this is due to previously observed heterogeneity of ZHBTc4 ES cell culture after 2 days of Dox treatment. H3K27me3 marks were, however, lost in TSL cells at all active TE-associated genes (Cdx2, Fgfr2, Hand1, Kdr and Pax3) that retained high levels of H3K4me2 marks. This is consistent with the
resolution of bivalent chromatin structures into active, H3K4me2 enriched configurations at induced genes upon TS-like cell derivation. In contrast, H3K27me3 marks remained abundant at un-induced lineage-inappropriate loci (Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7), alongside the active H3K4me2 histone modification in TSL cells, as seen in TS cells (Figure 4.2 and 4.3). These results emphasize that forced Pou5f1 protein loss and provision of TS cell conditions induces bivalent signature resolution at actively transcribed genes while silent loci retained both repressive and permissive histone modifications even after conversion towards TE lineage commitment. Crucially the epigenetic and transcriptional profile of TS-like cells was revealed to closely resemble that of traditionally derived TS cells.
Figure 4.2: Bivalent chromatin marks are maintained at un-induced loci upon Pou5f1 shutdown and subsequent TS-like cell derivation from ZHBTc4-ES cells. Enrichment for both H3K27me3 and H3K4me3 modifications relative to detected H3 were examined at a panel of key developmental genes using ChIP and qPCR in ZHBTc4-ES cells after 0 (ES) and 48 hours (ES\textsuperscript{Dox}) Dox treatment, TS-like (TSL) cells and B1-TS (TS) cells. Background levels (from control IgG antibodies) were shown as grey bars. Data shown is from three independent experiments and error bars represent s.d. Brackets highlight silent developmental genes that retain bivalent chromatin domains in TS cells.
Figure 4.3: Kinetics of candidate gene expression in ZHBTc4-ES cells following Pou5f1 shutdown and trophectoderm lineage commitment. Expression levels of selected genes were assessed by qRT-PCR in ZHBTc4-ES cells after 0 (white), 24 (light grey) and 48 hours (dark grey) of doxycycline (Dox) treatment to induce Pou5f1 repression, in ZHBTc4-derived TS-like cells (light black) and B1-TS (black) cells. Data was normalized to housekeeping genes Actin and L19 and expressed relative to untreated ZHBTc4-ES cells (ES=1). Data shown represents the mean from three independent experiments and error bars represent s.d. Brackets highlight transcriptionally silent loci.

Separate genome-wide ChIP studies in metazoan ES cell lines have mapped shared repressed target genes for polycomb group members and transcription factors Pou5f1, Sox2 and/or Nanog (Boyer et al., 2005; Boyer et al., 2006b; Lee et al., 2006; Loh et al., 2006; Sharov et al., 2008). Interestingly, analysis of ES cells that lose Pou5f1 expression and of those that are deficient in Ring1A and Ring1B indicate that these cell lines have very similar mRNA expression profiles (Endoh et al., 2008). The observation that in ZHBTc4 ES cells Pou5f1 shutdown causes to a rapid and global loss of PRC1 and
PRC2 engagement at target loci led the authors to suggest that a functional link exists between the polycomb complex component Ring1A/B and the core transcription factor Pou5f1 (Endoh et al., 2008).

To assess the polycomb group complex status at key developmental regulators during ES conversion towards the TE cell lineage we examined the impact of Pou5f1 protein depletion (ES\textsuperscript{Dox}) on Suz12-PRC2 and Ring1B-PRC1 occupancy by ChIP. The enrichment levels were compared to that in untreated ES cells and conventionally-derived TS cells. In agreement with the findings of Endoh et al., genes up-regulated after Dox treatment of ZHBTc4 ES cells showed a decrease in both Suz12 and Ring1B enrichment relative to untreated ES cells (Figure 4.4A). This confirms that an inverse correlation exists between polycomb complex occupancy and active transcribed genes. In contrast to this, inactive genes like Atoh1, Sox1 and Hoxa7 retained an abundance of Suz12 and Ring1B binding in ES\textsuperscript{Dox} cells, indicating that their targeting is not completely Pou5f1-dependent in these cells. Notably, active and inactive genes can be distinguished more clearly by the patterns of Suz12 occupancy in ES\textsuperscript{Dox} cells than by the H3K27me3 levels at the same stage of Dox treatment (as shown in Figure 4.2), possibly reflecting a passive loss of this histone mark after Pou5f1 shutdown and during cell division. Upon TS-like derivation, genes previously found to be marked by H3K27me3 in TSL cells were also targeted by Suz12 (Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7). Ring1B enrichment on the other hand was depleted to levels comparable to background IgG signals at all candidate loci in TS-like cells and reiterated the situation in blastocyst-derived TS cells.
Figure 4.4: Dynamic loss of PRC1 occupancy and transcriptional gene priming of embryonic developmental regulators upon trophoderm lineage commitment. (A, B) C-ChIP and qPCR were used to assess the abundance of PRC2 (Suz12) and PRC1 (Ring1B) (A) as well as Ser5P RNAP and 8WG16 RNAP II complexes (B) in untreated (ES), Dox-treated (ESDox) ZHBTc4-ES cells for 48 hours, TS-like cells (TSL) and traditionally-derived B1-TS (TS) cells. Background IgG antibody control levels are shown as grey bars. Data shown is representative of three independent experiments and error bars represent s.d. Brackets highlight silent developmental genes that retain bivalent chromatin domains in TS cells.
RNAP II in a poised conformation has previously been shown to co-localize with Ring1B and be enforced by PRC1-mediated H2A ubiquitination in ES cells (Stock et al., 2007). After finding a decrease in Ring1B enrichment at bivalently marked loci upon TE lineage commitment, we further examined the recruitment and conformation of RNAP II during Pou5f1 depletion and TS-like derivation. ChIP was carried out using antibodies against either Ser5-phosphorylated (Ser5P; 4H8) or hypo-phosphorylated (8WG16) RNAP II. As shown in Figure 4.4B, actively transcribed genes are enriched for both Ser5P and 8WG16 RNAP II at their promoter regions. In contrast to this, poised RNAP II is distinguished by high enrichment for Ser5P RNAP II and low levels of 8WG16 binding in ES cells. Inactive loci lack an abundance of either RNAP II phosphorylated state as shown at the control Intergenic region. The pattern of enrichment in the ES\textsuperscript{Dox} population is less clear, activated genes (Cdx2, Fgfr2, Hand1, Kdr and Pax3) seem to lose Ser5P RNAP II binding, indicating that it may be necessary to remove poised RNAP II before a normal cycle of transcription can occur. As observed previously when assessing H3K27me3 binding, Fgfr2 responds early as a Pou5f1 target and consistent with this interpretation, Fgfr2 rapidly gains higher levels of 8WG16 after 48 hours Dox treatment. Interestingly, Ser5P enrichment is still evident at un-induced Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7 in ES\textsuperscript{Dox} cells, as previously seen for Ring1B. This indicates that Pou5f1 protein alone is not responsible for keeping genes poised for future expression in ES cells. Genes induced upon TS-like derivation (Cdx2, Fgfr2, Hand1, Kdr and Pax3) showed enrichment for both RNAP II complexes, with similar levels detected in extra-embryonic lineage committed TS cells. In contrast, RNAP II complexes were virtually absent at the inactive promoters of bivalently marked genes (Atoh1, Sox1, Hoxa7, Mixl1, Gata4 and Sox7) in TSL cells, as seen in TS cells.

Variation in the epigenetic and transcriptional control of key developmental genes in ES and TS cells was demonstrated in the previous chapter. Although bivalent
domains were observed in both cell types, PRC1 and poised RNAP II were notably absent from lineage-inappropriate genes in extra-embryonic restricted cells, indicative of a loss of gene priming for transcription. By taking advantage of the ZHBTc4 ES cell system we showed that such distinctions are dependent on lineage identity. Conversion of ES cells into TSL cells induced chromatin remodeling at previously poised loci to mimic the situation at activate and silent genes in traditionally-derived TS cells. Interestingly when examining the direct impact of Pou5f1 shutdown (ES\textsuperscript{Dox}) we found that PRC1’s Ring1B and poised RNAP II were retained at some key developmental genes after 48 hours, suggesting that gene priming in ES cells is not solely dependent on this core pluripotency transcription factor.

4.3 Selective induction of the H3K9 tri-methyltransferase Suv39h1 upon trophoblast lineage commitment.

The generation of silent, 'unprimed' promoters lacking both Ring1B and RNAP II recruitment at bivalently modified genes in TSL and TS cells compared to ES cells suggests that other repressive mechanisms might operate to regulate these loci in extra-embryonic tissues. Pou5f1 protein plays a key role in specifying gene expression patterns and subsequently embryonic versus extra-embryonic lineage identity. As a consequence of this, its loss will significantly change these transcription patterns and likely modify the network of epigenetic regulators in ES cells to that present in TSL cells. This may facilitate the chromatin remodeling that we observe at key developmental genes. To further assess the relationship between lineage identity and targeted chromatin changes, we surveyed the relative mRNA levels by qRT-PCR of a cohort of chromatin modifiers in ES cells, ES\textsuperscript{Dox} cultures, TSL cells and blastocyst-derived TS cells (Figure 4.5A).
Figure 4.5 Gene expression profile changes of selected chromatin regulators upon Pou5f1 repression and TS-like cell derivation. (A) qRT-PCR analysis of selected chromatin regulators expression in ZHBTc4-ES cells after 24 and 48 hours of the addition of Dox (ES\textsuperscript{Dox}) in the presence of LIF, and in ZHBTc4-derived TSL (TSL) cells and B1-TS (TS) cells. Values shown are normalized to Actin and L19 and are expressed relative to untreated ZHBTc4-ES cells (ES=1). Up-regulation (mean±s.d.>2) and down-regulation (mean±s.d.<0.5) are colored in red and blue, respectively. Data shown represents 3 independent experiments. (B) Histograms showing the expression pattern of Cdx2, Pou5f1, Suv39h1, Jmjd2c, Suv39h2, Ehmt1, G9a and Eset upon Pou5f1 shutdown and TS-like cell derivation. Error bars represent s.d. of three independent experiments. (C) Expression levels of Cdx2, Suv39h1, Suv39h2 and G9a, as assessed by qRT-PCR, in morula (grey), dissected ICM (green) and TE (orange) sample preparations. Values shown are normalised to S17 and L19 mRNA levels and are expressed relative to morula samples (morula=1). Error bars represent s.d. of three independent experiments. Note that Ehmt1 and Eset transcript levels were below detection in all three samples under the same experimental conditions (data not shown). N.D., not detectable.
An overview of this expression analysis is shown in Figure 4.5 and raw data is provided in Appendix II: Table II-1. Using untreated ES cells as a reference, all genes that become down-regulated were denoted in blue and loci with a greater than 2-fold expression increase were highlighted in red. Early alterations in chromatin modifier composition induced directly by Pou5f1 shutdown can be traced in Dox treated ZHBTC4 cells (ES\textsuperscript{Dox}) while changes linked with extra-embryonic lineage specification are referred to those seen in TSL and TS cells when normalizing to untreated ES cells (ES cells = 1). Note that the expression profile of TSL cells shows remarkable similarity to traditionally-derived TS cells with only two exceptions, LSD1 and MLBR (Figure 4.5A).

Consistent with previous studies, significantly reduced expression of PRC2 component *Eed* and little change in the expression of its partners, *Ezh2* and *Suz12* was observed in ES\textsuperscript{Dox}, TSL and TS cells when compared to ES cells (Mak et al., 2004; Matoba et al., 2006). The expression of several PRC1 subunits chromdomain containing protein-Cbx7, polyhomeotic gene-Phc1 and Ring1A were also down-regulated upon ES cell conversion into TSL cells yet other PRC1 components including Cbx8 and Phc2 were significantly up-regulated upon Pou5f1 protein depletion and TS-like cell derivation compared to untreated ES cells. The large number of potential PRC1 subunits in mammalian cells and their dramatic transcriptional variation over ES to TS cell conversion might reflect the numerous polycomb complex forms that can exist (Otte and Kwaks, 2003). Consistently, differential recruitment of Phc1 and Phc2 to the inactive X chromosome has previously been demonstrated in ES and TS cells, respectively (de Napoles et al., 2004). Fewer changes were observed among the H3K4 methyltransferases Mixed Lineage Leukaemia 1 (*MLL1*) and *MLL2*, although *MLL3* expression was down-regulated in TSL and TS cells. The first identified demethylase, H3 K4 or K9 demethylase *LSD1*, showed reduced transcript levels in TS cells but not in Pou5f1 depleted ES cells or upon TS-like cell derivation.
Remarkably, we observed a pronounced imbalance in the expression levels of histone H3K9 demethylases and histone H3K9 methyltransferases between embryonic versus extra-embryonic stem cells types. Notably, the H3K9 histone demethylase class that distinctly decreased in expression over TE commitment were the Jumonji C domain-containing proteins *Jmjd1a* and *Jmjd2c*, which catalyse lysine demethylation on histone tails through an oxidative reaction. These recently identified H3K9 tri/di-demethylases are highly transcribed in undifferentiated ES cells and steadily down-regulated upon *Pou5f1* loss (Cloos et al., 2006; Loh et al., 2007; Wissmann et al., 2007).

On the other hand, the mRNA levels of all known H3K9 methyltransferases including *Ehmt1*, *G9a*, *Suv39h1* and *Suv39h2* were up-regulated to different extents in ES<sub>Dox</sub>, TSL, and TS cells as compared to ES cells with the exception of *Eset* whose expression remained unchanged. In particular *Suv39h1* H3K9 tri-methyltransferase transcript levels was significantly increased (30-fold) upon TS-like derivation and in TS cells compared to untreated ES cells, an increase in expression that was equivalent to the increase observed for *Cdx2* (Figure 4.5B). In comparison, *Suv39h1*, *Ehmt1* and *G9a* displayed a relatively modest expression increase (4- to 6-fold) when compared to in vitro TE lineage formation from ZHBTc4 cells. These results indicate a potential key role for *Suv39h1* during the emergence or maintenance of a TS-like cell fate. Importantly, this role is supported in vivo by the selective induction of *Suv39h1* in contrast to *Suv39h2* or *G9a* in dissected TE samples relative to ICM or morula cells (Figure 4.5C). More experiments were carried out to determine whether this dramatic change in the ratio of H3K9 demethylases to methylases might effect the epigenetic regulation of bivalently marked regulators in ES and TS cells.

### 4.4 H3K9 tri-methylation specifies epigenetic asymmetry at bivalent genes between ICM ad TE lineages.

Upon TE lineage commitment key developmental, yet lineage-inappropriate, genes lose Ring1B binding and show no detectable assembly of poised RNAP II. We have
determined a concomitant up-regulation in H3K9 methyltransferase *Suv39h1* mRNA levels. A previous study demonstrated that Ring1B and Suv39h1 bind in a mutually exclusive fashion in the early zygote (Puschendorf et al., 2008). Ring1B showed an affinity for paternal heterochromatin, which lacks H3K9me3 and HP1β, while Suv39h1 bound preferentially maternal heterochromatin. Induced depletion of H3K9me3 and Suv39h1 resulted in Ring1B binding to and acting as a repressor on both paternal and maternal heterochromatin, which illustrates a hierarchy where dominant Suv39h1 appears to block Ring1B targeting.

To focus on whether silent, bivalent genes may be directly targeted by Suv39h1 during TE lineage specification in the absence of Ring1B binding, we assessed the enrichment of H3K9me3 modifications and Suv39h1 occupancy at selected developmental regulators and control (*Actin* and γ–*satellite*) loci in ES, ES\textsubscript{Dox}, TSL and TS cells (Figure 4.6A and B). As expected, genes that are activated upon *Pou5f1* shutdown and TS-like cell derivation, that retain high levels of H3K4me2 and lose H3K27me3 and Suz12 enrichment (*Cdx2, Fgfr2, Hand1, Kdr* and *Pax3*), showed little acquisition of repressive H3K9me3 (see Appendix II: Figure II-1). Consistent with this, gene promoters that are inactive during TE lineage commitment attained H3K9me3 at bivalent chromatin domains (*Atoh1, Sox1, Hoxa7, Mixl1, Gata4* and *Sox7*) as well as Suv39h1 recruitment (Figure 4.6A and B). It’s noteworthy that in contrast to TSL cells, H3K9me3 is barely enriched in ES\textsubscript{Dox} cells, whereas Ring1B and Ser5P RNAP II were only transiently retained at un-induced loci (see Appendix II: Figure II-1). This suggests that the appearance of H3K9me3 and loss of poised chromatin features are likely to be coordinated. In all four populations an abundance of H3K9me3 bound at constitutive heterochromatin (γ-satellite repeats) while actively expressed *Actin* showed low enrichment.
Figure 4.6: H3K9me3, Suv39h1 and de novo DNA methylation occupy lineage-inappropriate genes upon trophectoderm lineage cell commitment. (A,B,C) The abundance of H3K9me3 (A) and Suv39h1 (B) was assessed by ChIP and 5-methyl cytosine DNA (5mC) by MeDIP (C) at candidate gene promoters and controls in ZHBTc4-ES after 0 (green; ES) and 48 hours (light green; ES\textsuperscript{Dox}) of Dox treatment to induce Pouf51 repression, ZHBTc4-derived TS-like (light orange; TSL) and B1-TS (orange; TS) cells. Background levels (from control IgG antibodies) are shown as grey bars. Enrichment is expressed relative to H3 or input. Error bars represent s.d. of three independent experiments. MeDIP was carried in collaboration with S. Pinho. Brackets highlight silent developmental genes that retain bivalent chromatin domains in TS cells.
Suv39h1-mediated H3K9me3 is typically associated with gene silencing and commonly interacts with HP1 to repress transcription by stabilising heterochromatin subdomains. Chromatin structures such as these enable DNMTs to distinguish transcriptionally repressed states from active loci, as these enzymes have little intrinsic target specificity beyond a tendency to methylate CpG dinucleotides (Fujita et al., 2003; Fuks et al., 2003). To explore whether further layers of repression target key developmental genes marked by H3K9me3, DNA methylation levels were analysed by methylated DNA immunoprecipitation (MeDIP) analysis (where methylated DNA fragments from genomic DNA are isolated via a 5-methylcytosine (5mC) specific antibody).

As expected, repressive DNA methylation was virtually absent from bivalently marked developmental regulator loci in untreated ES cells (Figure 4.6C). Similarly, little DNA methylation was detected in Pou5f1 depleted ES\textsuperscript{Dox} populations after 48 hours Dox treatment, apart from that detected at the positive control heterochromatin γ–satellite repeat region. Up-regulated and actively transcribed genes, Cdx2, Fgfr2, Hand1, Kdr and Pax3 in TSL and TS cells, respectively, that are only enriched for H3K4me3 and not H3K27me3, revealed DNA methylation levels comparable to the active control locus, Actin. However, a strong correlation was observed between silent genes selectively targeted by Suv39h1 and H3K9me3 and those enriched for de novo DNA methylation in TSL and TS cell populations (Sox1, Hoxa7, Mixl1, Gata4 and Sox7). These results suggest that upon loss of Ring1B binding during TE lineage commitment, combined repressive marks such as H3K9me3 and associated DNA methylation cooperate to stably ‘lock-in’ bivalent lineage-inappropriate genes in a repressed state.

In mammalian cells H3K9 methylation can be mediated by a number of different histone methyltransferases including Suv39h1, Suv39h2, G9a, Ehmt1 and Eset. To verify that selective targeting by Suv39h1 mediates the subsequent acquisition of substantial H3K9me3 levels upon the loss of pluripotency during TE emergence, we carried out
Suv39h1 knockdown by RNAi in TS cells (Figure 4.7A and B). Briefly, proliferating TS cells were infected with lentivirus carrying a vector containing a short-hairpin RNA (shRNA) targeting specifically mouse Suv39h1. Expression analysis confirmed the knock-down of Suv39h1 with no significant impact on the expression of other H3K9 methyltransferases (Suv39h2, G9a, Ehmt1 and Eset) (Figure 4.7A). Knockdown of this histone methyltransferase led to a decline of H3K9me3 levels at the bivalently marked loci (Sox1, Gata4 and Sox7) as assessed in these experiments by C-ChIP and qRT-PCR analysis (Figure 4.7B), thereby confirming the unambiguous dependency of H3K9 methylation on Suv39h1.

The existing presence of trivalent chromatin domains enriched for H3K4me2, H3K27me3 and H3K9me3 was further confirmed at silent, lineage-inappropriate loci in TS cells by a series of sequential ChIP experiments (Figure 4.7C). Enrichments of both H3K9me3 and H3K27me3 (top panel; Figure 4.7C) were discovered at bivalently marked lineage-inappropriate genes in TS cells, which were also found to carry an abundance of H3K9 and H3K4 methylation at their promoter regions (bottom panel; Figure 4.7C).
Figure 4.7: Suv39h1-mediated H3K9me3 targets bivalent genes in the trophectoderm cell lineage in vitro and in vivo. (A) Expression levels of Suv39h1, Suv39h2, G9a, Ehmt1 and Eset were assessed by qRT-PCR in B1-TS cells after knockdown using Suv39h1 shRNA construct. Values shown are normalized to S17 and L19 mRNA levels and are expressed relative to control cells. Error bars represent s.d. of three independent experiments. (B) Histogram showing quantification by C-ChIP of H3K4me2 (green), H3K27me3 (blue) and H3K9me3 (red) levels at the silent promoters of Sox1, Gata4 and Sox7 in Suv39h1-knockdown TS cells relative to control cells after normalizing against their respective unbound fractions and IgG controls. Error bars represent s.d. of three independent experiments. Expression status (-) is shown as an aid. (C) Sequential ChIP using first anti-H3K9me3 and then anti-H3K27me3 (upper panel), or anti-H3K9me3 followed by anti-H3K4me2 (bottom panel) antibodies in B1-TS cells. Enrichment is expressed relative to H3. Error bars represent s.d. of three independent experiments. (D) Quantification by C-ChIP of H3K9me3 enrichment levels at the promoters of Sox1, Gata4 and Cdx2 in compacted morula (grey), ICM (green) and TE (orange). Unspecific precipitation was monitored by control IgG antibodies; background levels are denoted by dotted lines. Error bars represent s.d. of three independent experiments.
To verify that the addition of repressive H3K9me3 marks at bivalent lineage-inappropriate genes in TS cells is also occurring in vivo, we examined the abundance of Suv39h1-mediated H3K9me3 at *Sox1*, *Gata4* and *Cdx2* promoters before (morula) and after mouse blastocyst development (ICM and TE) by C-ChIP and qRT-PCR analysis (Figure 4.7D). Actively transcribed *Cdx2* showed little enrichment for H3K9me3 in the morula, ICM or TE, as expected. In contrast to this, both *Sox1* and *Gata4* promoter regions harbored in addition to the previously determined H3K27me3 and H3K4me2 marks (see Figure 3.5), high levels of H3K9me3 in the TE (Figure 4.7D). Moreover, low enrichment levels for H3K9me3 in compacted morula suggest that the acquisition of this repressive modification might be a late event associated with a loss of lineage plasticity upon trophoblast cell fate consolidation. Overall, these results demonstrate that alternative repressive strategies operate in the early embryo to regulate key developmental genes in a lineage specific manner.

### 4.5 Conclusion

Bivalent chromatin marks are thought to provide flexible silencing of key developmental regulators that could potentially be required later on during mammalian development. Here, we revealed, however, that bivalent chromatin domains are present at the promoters of key developmental regulators in the blastocyst TE and derived TS populations that are not required for placenta formation. We showed that both PRC1 (Ring1B) and poised RNAP II complexes – two hallmarks of gene priming in pluripotent cells – are dynamically removed from bivalent gene promoters upon pluripotency loss and ES cell conversion into TSL cells. Instead, these loci become targeted by the repressive Suv39h1-mediated H3K9me3 and DNA methylation while retaining high levels of methylated H3K4. This creates a trivalent histone conformation (H3K9me3-H3K27me3-H3K4me2) at silent lineage-inappropriate genes, as seen in TSL and TS cell cultures, and most importantly, in the lineage-restricted TE in vivo. Interestingly, this
epigenetic signature was also maintained in the developing *in vivo* placenta (Alder O, personal communication). The additional layers of repressive H3K9 methylation and DNA modifications are proposed to provide a cellular ‘fail-safe’ mechanism which stably silences embryonic-associated loci in TE committed cells and consolidates lineage choice, protecting against reprogramming. Simultaneously, as suggested here, a safeguard mechanism such as Ring1B, must exist in the early embryonic pluripotent tissue to actively protect poised promoter regions from early or inappropriate H3K9 methylation and *de novo* DNA methylation. This highlights the precarious balance between pluripotency and differentiation during development, and in our culture dishes, that is modulated in part by opposing epigenetic mechanisms.
Chapter 5 Analysis of Nanog buffering effect on the Fgf/Erk cell signalling pathway in ES cell cultures.

Following ICM/TE lineage segregation, the ICM further develops and separates into the pluripotent epiblast and extra-embryonic primitive endoderm (PrE) that will go on to differentiate into all three germ layers of the embryo and the yolk sac, respectively. Several studies showed that these blastocyst lineages are promoted by a number of key transcription factors both in vivo and in lineage-derived stem cells. Frequently used as lineage markers, the transcription factor Nanog is associated with the pluripotent ICM/epiblast and derived ES cells, while Gata6 is linked with PrE formation and derived XEN cells. After being co-expressed in all blastomeres, Nanog and Gata6 start to show a mutually exclusive ‘salt-and-pepper’ expression pattern in the E3.5 ICM, before compartmentalization into two distinct expression domains that strictly delineate the epiblast and PE in the E4.5 blastocyst (Chazaud et al., 2006; Dietrich and Hiiragi, 2007; Plusa et al., 2008). Interestingly, Nanog is also mosaically expressed in cultured ES cells with FCS and LIF. ES cells expressing low levels of Nanog were found to have a higher propensity to differentiate, while Nanog over-expression enables ES cells to self-renew in a LIF-independent manner (Chambers et al., 2003; Chambers et al., 2007).

Research by genetic inactivation and chemical inhibition revealed the importance of the Fgf/Erk signalling pathway in promoting differentiation in ES cells (Kunath et al., 2007; Stavridis et al., 2007). For instance, ES cells null for Fgf4, a major stimulant of the Erk pathway, showed restricted ability to commit to neural and mesoderm differentiation, a phenotype rescued by the addition of exogenous Fgf4 protein (Kunath et al., 2007). Interestingly, Fgf/Erk signalling inhibition using a specific Mek inhibitor induced a homogeneous Nanog expression pattern in ES cells, while concomitant addition of GSK3 inhibitor (also known as 2i conditions; Mek and GSK3...
inhibitors) enabled ES cells to propagate in the absence of FCS and LIF (Ying et al., 2008). Similarly, ex vivo culture of an eight-cell stage embryo in 2i conditions led to the formation of an ICM completely composed of Nanog-positive cells with no detectable Gata6-positive PrE cells (Nichols et al., 2009). Janet Rossant’s lab further revealed how modulating the levels of Fgf/Erk signalling (using a specific Mek inhibitor or by increasing amount of exogenous Fgf) can bias ICM cell’s fate towards either the epiblast or PrE, respectively. Intriguingly, the fate of the cells could be reversed towards the alternative cell lineage by switching the culture conditions at E3.25 during blastocyst maturation, but not after E3.75, delineating cell fate flexibility within the early blastoscyt (Yamanaka et al., 2010). Taken together, these studies suggest a critical role for the Fgf/Erk signalling cascade in balancing pluripotency versus differentiation both in vivo and in stem cells. Here we explore how the pluripotency promoting factor Nanog might modulate the extracellular activated Fgf/Erk pathway to maintain ES cell self-renewal in vitro.

5.1 Absence of Nanog in ES cells correlates with an increase in active phospho-Erk1/2 levels.

Nanog is part of the core regulatory circuitry in ES cells (Boyer et al., 2005), promotes ES cell self-renewal (Chambers et al., 2007) and mediates the acquisition of pluripotency upon somatic cell reprogramming in vitro (Silva et al., 2009). To determine whether different levels of Nanog can modulate Fgf/Erk cell signalling responses in ES cells, we took advantage of previously characterized genetically modified ES cell lines for Nanog expression (kindly provided by I. Chambers; Chambers et al., 2003; Chambers et al., 2007). Western blot analysis confirms the varying Nanog protein levels in Figure 5.1A; proliferating Nanog-null cells (RCNβH(t)) were here compared to control RCN(t) ES cells while Nanog over-expressing cells (EF4) were compared to wild-type E14Tg2a ES cells. All four ES cells populations were maintained in ES media culture conditions with FCS and LIF unless otherwise stated. Remarkably, we observed an increase in the
levels of active phosphorylated Erk1/2 (phospho-Erk1/2) forms in RCNβH(t) cells as compared to control cell basal levels (Figure 5.1A). Over-expression of Nanog did not, however, induce any detectable difference in phospho-Erk1/2 levels, which coincides with previously published data (Chambers et al., 2003).

To explore this further, we next examined whether the levels of phospho-Erk1/2 forms could also be dynamically altered upon addition of exogenous Fgf4 in Nanog-null ES cells as compared to control ES cells. Data showed a consistent increase in active phospho-Erk1/2 levels within 15 min from basal levels after the addition of exogenous Fgf4 protein in RCNβH(t) cells, but notably not in Nanog expressing RCN(t) ES cells (Figure 5.1B). These experiments were conducted with heat-treated FCS to degrade any residing growth factor activity. Importantly, the proteoglycan, heparin, was simultaneously added with Fgf4 as it is required to catalyze potential binding between Fgfs and their specific receptors (Ornitz et al., 1992). Altogether, these results point at a possible role for Nanog in buffering Fgf/Erk cell signalling responses in ES cells.

**Figure 5.1: Interplay between Nanog and Fgf/Erk cell signalling activity in ES cells.**
(A) Western blotting with anti-Nanog antibody confirmed the varying levels of Nanog protein present in RCN(t) (control), RCNβH(t) (null), E14Tg2a (control) and EF4 (overexpressing) mouse ES cell lines. Western blotting with anti-phospho-Erk1/2 and anti-
Erk1/2 antibodies revealed an inverse correlation between the levels of phospho-Erk1/2 and Nanog protein in mouse ES cells. Equivalent protein loading is shown with mouse Actin detection. (B) Western blotting with anti-phospho-Erk1/2 and anti-Erk1/2 antibody showed that the addition of exogenous Fgf4 (50ng/ml) and heparin (1ug/ml) triggers an increase in phospho-Erk1/2 levels in RCNβH(t), but not RCN(t) ES cells maintained in ES culture conditions with heat-treated FCS and LIF. Equivalent protein loading is shown with total Erk1/2 detection. Data are representative of at least two independent experiments.

5.2 The Fibroblast growth factor receptor 2 is inversely expressed to Nanog in ES cells.

To investigate how Nanog may mediate its buffering effect on the Fgf/Erk cell signalling pathway in ES cells, we carried out quantitative RT-PCR analysis on Nanog control (RCN(t) and E14Tg2a), Nanog null (RCNβH(t)) and over-expressing (EF4) cell lines with a particular focus on Erk signalling mediators. As previously reported, we confirm that all cell populations analyzed constantly expressed high mRNA levels for several pluripotency-associated genes including Pou5f1, Sox2, Klf4 and Essrb (Chambers et al., 2003; Chambers et al., 2007). This is in agreement with an undifferentiated ES cell state despite the different Nanog transcript levels (Figure 5.2A). Interestingly, fibroblast growth factor receptor 2 (Fgfr2) was the only Fgfr family member whose expression seemed inversely correlated to Nanog (Figure 5.2B and C). Detected at very low levels in the control cells, Fgfr2 expression was markedly up-regulated in RCNβH(t) and repressed in EF4 ES cells. This was confirmed by looking at the mRNA levels of two Fgfr2 isoforms with high affinity for the ligand Fgf4, Fgfr2IIIdb and Fgfr2IIIC (Figure 5.2B). In contrast, analysis of Fgfr1, Fgfr3 and Fgfr4 expression patterns in the same ES cell lines did not reveal any similar pattern, suggesting that Nanog may specifically regulate the expression of Fgfr2 in pluripotent ES cells. Note that Fgf4 mRNA levels were also altered in RCNβH(t) Nanog-null cells, but not in EF4 (Figure 5.2D). Earlier research has shown that Pou5f1 and Sox2 directly regulate Fgf4 (Yuan et al., 1995). Our results suggest that Nanog may also activate this gene in cooperation with Pou5f1 and Sox2.
Altogether, these results demonstrate an inverse expression pattern for *Fgfr2* and *Nanog*, suggesting that Nanog might regulate the Erk cell signalling pathway in ES cells through repression of *Fgfr2*, an upstream mediator.

**Figure 5.2: Gene expression profile of Nanog-null and Nanog over-expressing ES cells.** Quantitative RT-PCR analysis of gene expression in control RCN(t) and E14Tg2a (white bars) compared to Nanog null RCNβH(t) cells (grey bars) and Nanog over-expressing EF4 cells (black bars). Data are normalized to *L19* and *S17* and expressed relative to control RCN(t) or E14Tg2a cells. Error bars represent the s.d. of three independent experiments.
5.3 Dynamic loss of Nanog results in a rapid up-regulation of Fgfr2 expression.

To investigate whether Nanog dynamically regulates the expression of Fgfr2, we used a previously established conditional Nanog-null (RCNβH) ES cell line in which tamoxifen (Tx) treatment induces the loss of Nanog expression (Chambers et al., 2007). Accordingly, Nanog was virtually found undetectable both at the mRNA and protein levels within 24 hours upon addition of Tx (Figure 5.3A and B). In contrast, Pou5f1 and Sox2 expression was maintained or slightly increased over 5 days of Tx treatment. Importantly, we observed that Fgfr2 was promptly induced following Nanog loss (2-3 days after Tx addition; Figure 5.3A and B), alongside Gata6, a direct gene target of Nanog in ES cells (Singh et al., 2007). In contrast, Gata4, as well as two other PrE-associated markers, Dab2 and Pdgfra, were induced later (4-5 days after Tx treatment). Fgf4 expression was also noticeably down-regulated upon Nanog depletion, indicating its steady expression may depend on all three core pluripotency factors Pou5f1, Sox2 and Nanog. Collectively, our data indicate that Nanog tightly regulates the expression of Fgfr2 and Fgf4 in ES cells and highlight Fgfr2 as a hallmark of differentiation upon Nanog loss.
Figure 5.3: Gene expression analysis upon Nanog deletion in ES cells. (A) Histograms showing the expression pattern of Nanog, Pou5f1, Sox2, Fgfr2, Gata6, Gata4, Fgf4, Dab2 and Pdgfra upon tamoxifen treatment (1 μM) of RCNβH ES cell samples, collected at different times (0 to 5 days). Data are normalized to L19 and S17. Error bars represent the s.d. of three independent experiments. (B) Western blotting with anti-Nanog, anti-Fgfr2 and anti-Pou5f1 antibodies revealed a steady increase in Fgfr2 protein levels upon Nanog depletion. Equivalent protein loading is shown with β-tubulin detection.
5.4 Fgfr2 is variably expressed within undifferentiated ES cells

Until recently, mouse ES cells cultures were perceived as homogeneous cell populations expressing similar levels of pluripotency-associated factors like Pou5f1 and Sox2 to sustain survival and self-renewal. Nanog expression has since been shown to fluctuate in undifferentiated ES cell cultures, providing an opportunity for differentiation (Chambers et al., 2007; Singh et al., 2007). As Fgfr2 induction was found to be an early event following Nanog disappearance, we next investigated whether Fgfr2 expression could be mosaic and mutually exclusive with Nanog in ES cells. Here, we took advantage of an established Nanog/GFP knock-in (TNGA) ES cell line with eGFP inserted at the Nanog AUG codon, which allows discrimination between Nanog-high and Nanog-low ES cell subpopulations according to GFP intensity as detected by flow cytometry. Accordingly, TNGA cells were sorted into either GFP high or GFP low cell subpopulations (Figure 5.4; Appendix II: Figure II-2). GFP expression variation was confirmed by quantitative RT-PCR analysis across populations with Pou5f1 and Sox2 mRNA steady levels highlighting the undifferentiated state of the cells (Figure 5.4B). Similar Pou5f1 protein levels were also detected by western blotting, in contrast to GFP and Nanog (Figure 5.4C). Note, however, the presence of residual low levels of Nanog protein found in GFP low populations, possibly due to differences in GFP and Nanog half-lives (Figure 5.4C). Fgf4 mRNA levels also remained steady between undifferentiated ES cells subpopulations, as seen for Pou5f1 and Sox2 in these experiments. Importantly, Fgfr2 expression was higher in the GFP low ES cell subpopulations, like Gata6 (Figure 5.4B). This corroborates with the early induction of Fgfr2 and Gata6 upon Nanog loss in ES cells (Figure 5.3) and demonstrates that Fgfr2 is also variably expressed amongst ES cells, with higher levels detected in GFP/Nanog low, primed ES cells.
Figure 5.4: Increased levels of *Fgfr2* expression in GFP/Nanog low ES cell subpopulations. (A) GFP sorting profile of GFP/Nanog knock-in TNGA ES cells grown in ES culture media with FCS and LIF. (B) Quantitative RT-PCR analysis of *GFP, Pou5f1, Sox2, Fgfr2, Gata6* and *Fgf4* in sorted GFP\textsuperscript{high} or GFP\textsuperscript{low} TNGA cells. Data are normalized to *L19* and *S17*. Error bars represent the s.d. of three independent experiments. (C) Western blotting with anti-GFP, anti-Nanog and anti-Pou5f1 antibodies in sorted GFP\textsuperscript{high} or GFP\textsuperscript{low} TNGA cells. Equivalent protein loading is shown by mouse β–tubulin detection.
5.5 Nanog directly targets Fgfr2 and Fgf4 gene regulatory regions.

To address whether Nanog regulation of Fgfr2 and Fgf4 was direct, we screened both loci as well as 5kb upstream of their transcription start sites (TSS) for potential Nanog binding sites using the previously determined consensus sequence (C/G)(G/A)(C/G)C(G/C)ATTAN(G/C) (Mitsui et al., 2003). DNA sequence regions matching 6 to 10 nucleotides of the Nanog consensus sequence were identified as putative binding sites (Appendix II: Figure II-3). Figure 5.5A schematically highlights the location of 3 potential Nanog binding sites (BS) found at the Fgf4 gene region and 4 at the Fgfr2 loci. Nanog binding was next assessed at these sites by ChIP assay on fixed, sonicated E14Tg2a (wild-type) ES cell chromatin, showing high enrichments within the Fgf4 3’ untranslated region (UTR; Fgf4_BS3) and downstream of the Fgfr2 TSS (Fgfr2_BS4; Figure 5.5B). Nanog binding was further confirmed in EF4 Nanog over-expressing cells but undetected in the absence of Nanog in RCNβH(t) cells, as expected (Figure 5.5C). Gata6 specific PCR primers were used as a control in these experiments (Singh et al., 2007).
Figure 5.5: ChIP analysis of putative Nanog targets regions across *Fgfr2* and *Fgf4* loci. (A) Schematic representation of putative Nanog binding sites in *Fgfr2* and *Fgf4* gene regions. Nanog binding site (BS) indicated in red. (B) ChIP analysis with anti-Nanog antibody and quantitative RT-PCR in undifferentiated E14Tg2a ES cells confirm the direct binding of Nanog to *Fgfr2 5’ UTR region* and *Fgf4 3’ UTR region*, as assessed up to +/- 450 bp from the consensus sequences. (C) Nanog ChIP and quantitative RT-PCR in E14Tg2a (wild-type), EF4 Nanog over-expressing and RCNβH(t) Nanog null ES cells. Gata6 primers were used as a positive control. Dotted line represents the average IgG background. Enrichments are expressed relative to the ChIP input. Error bars represent s.d. of three independent experiments.
5.6 Nanog directly represses Fgfr2 and promotes Fgf4 expression.

In order to assess whether Nanog has a direct effect on Fgfr2 and Fgf4 expression, we used luciferase reporter assays. A 200-bp DNA fragment carrying Fgfr2 BS4 and a 250-bp fragment containing Fgf4 BS3 were individually subcloned into pGL3-Promoter luciferase vectors (Promega), creating Fgfr2-Luc and Fgf4-Luc constructs, respectively (Figure 5.6A). Luciferase reporter activities were assessed by transient transfection in control RCN(t) and Nanog null RCNβH(t) ES cells maintained in ES media. Note that both cell populations express similar levels of Pou5f1 and Sox2 proteins in the presence or absence of Nanog (Figure 5.6B). Consistent with the repressive action of Nanog on Fgfr2, the absence of Nanog enabled higher levels of Fgfr2-Luc luciferase activity in RCNβH(t) ES cells compared to control ES cells (p < 0.05; Two-way ANOVA; Figure 5.6C). Conversely, Fgf4-Luc reported activity was notably higher by approximately 3-fold in the presence of Nanog (p < 0.001; Two-way ANOVA). To verify that Nanog action is mediated through the identified consensus site binding, Fgfr2-Luc and Fgf4-Luc constructs were mutated at Fgfr2 BS4 and Fgf4 BS3, respectively (ATTA to GGCC; Fgfr2mut-Luc and Fgf4mut-Luc) and transfected in RCN(t) and RCNβH(t) ES cells. As expected, higher levels of Fgfr2mut-Luc luciferase activity were similarly observed in the presence or absence of Nanog compared to Fgfr2-Luc in the presence of Nanog. In contrast, the mutation in Nanog BS3 of Fgf4 was sufficient to completely abolish the transcription factor's enhancing effect. Collectively, these data show that Nanog directly represses the expression of Fgfr2, while enhancing that of its cognate ligand, Fgf4.
Figure 5.6: Luciferase activity of Fgfr2-luc and Fgf4-luc reporter in Nanog null and control ES cells. (A) Schematic diagram of original and mutated constructs. (B) Western blotting with anti-Nanog, anti-Pou5f1 and anti-Sox2 antibodies confirms equal protein levels of Pou5f1 and Sox2 in ES cells with or without Nanog. Equivalent protein loading is shown with Actin detection. (C) Luciferase activity of Fgfr2 and Fgf4 regulatory regions as assessed in Nanog expressing RCN(t) and null RCNβH(t) ES cells. All data are made relative to control pGL3 vector activity. Luciferase units are normalized to renilla. Error bars represent the s.d. of three independent experiments. Two-way ANOVA followed by Bonferroni post-test was used to calculate statistical significance. * p < 0.05, *** p < 0.001 versus RCN(t) within Fgfr2-luc and Fgf4-luc, respectively.

5.7 Conclusion

Nanog is amongst the core transcriptional factors that maintain ES cell self-renewal. In contrast, the Fgf/Erk cell signalling pathway promotes ES cell differentiation and thus must be tightly regulated in proliferating ES cell cultures. Here we show that Nanog can buffer the effect of autocrine Fgf/Erk responses in ES cells by directly repressing the expression of its upstream mediator Fgfr2. Nanog concurrently sustains
high levels of Fgf4 production, most likely in a cooperative manner with Pou5f1 and Sox2 (Yuan et al., 1995). We find that the absence of Nanog in ES cells correlates with increased Fgfr2 expression and enables higher levels of active phosphorylated Erk1/2 forms in the presence of Fgf4. The maintenance of these Nanog-null ES cells in a self-renewing state, however, indicates that other layers of control must be present to sustain ES cell pluripotency. However, conditional deletion of Nanog in Tx-inducible RCNβH ES cells revealed that Fgfr2 is an early hallmark of differentiation. Moreover, Fgfr2 was found variably expressed amongst undifferentiated ES cells with its highest levels being detected in primed, Nanog low ES cells subpopulations. Taken together, our results reveal how Nanog may directly modulate the Fgf/Erk signalling pathway in ES cells through Fgfr2 gene repression and thus maintain a critical balance between self-renewal and differentiation. Next we assess the impact of Fgfr2 forced expression on Nanog promoted ES cell self-renewal by using a colony forming assay.
Chapter 6 Validation of a role for Fgfr2 in balancing self-renewal and differentiation in ES cells.

Over the years Fgfr2 isoforms have been found to play key roles in later developmental processes like bone growth, lung morphogenesis and limb bud formation (Arman et al., 1998; Arman et al., 1999; De Moerlooze et al., 2000; Hajihosseini et al., 2001; Xu et al., 1998). Fgfr2 is a transmembrane spanning receptor with three immunoglobulin-like domains, which show specific ligand affinity, and a cytoplasmic tyrosine kinase domain. Fgfr2 appears to be the predominantly expressed receptor for the blastocyst, although it is restricted to the extra-embryonic trophoblast and primitive endoderm lineages (Arman et al., 1998), where Nanog is not simultaneously expressed.

In Fgfr2 null mice, both ICM and trophectoderm form normally, however, defective PrE differentiation was observed, ultimately resulting in peri-implantation lethality (Arman et al., 1998). In contrast, ICM outgrowth derived from Nanog null blastocysts fully differentiate into parietal endoderm-like cells, consistent with a lack of epiblast in vivo (Mitsui et al., 2003). This indicates critical roles for Nanog and Fgfr2 in promoting pluripotency and PrE formation, respectively. Interestingly, Nanog null ES cells, though able to maintain LIF-dependent self-renewal in culture, also show a propensity towards extra-embryonic endoderm differentiation (Chambers et al., 2003; Mitsui et al., 2003).

To date, the effect of ectopically expressing Fgfr2 in ES cells has not been studied. By doing so in wild-type and Nanog over-expressing ES cells, we will assess the interplay between Nanog and Fgfr2 activity in balancing pluripotency and differentiation in ES cells.
6.1 Transient Fgfr2 expression can partly bypass Nanog promoted self-renewal ability and favour differentiation in ES cells.

A cohort of core transcription factors proved essential to stably maintain ES cell identity by cooperatively regulating the activation and repression of pluripotency and differentiation promoting genes, respectively (Boyer et al 2005; Loh et al 2006). We previously found (see section 5.6) that the homeobox transcription factor Nanog directly represses the expression of Fgf activated receptor, Fgfr2, in ES cells. To further validate the functional significance of this observation, we directly tested the impact of over-expressing Fgfr2 on ES cell self-renewal ability. We used pcDNA3.1 expression vectors carrying a neomycin selection gene with inserted myc-tagged mouse Fgfr2 (Fgfr2-myc), a C342Y point mutated constitutively active Fgfr2 (Fgfr2-ca) or Fgfr1 under the control of a CMV promoter (kindly provided by A. Mansukhani; (Mangasarian et al., 1997; Mansukhani et al., 1992). Western blot analysis was carried out to confirm the constitutively active status of Fgfr2-ca construct using anti-phospho-Fgfr antibody (Figure 6.1). As expected, similar levels of phosphorylated Fgfr were observed in Fgfr2-ca transiently transfected ES cells treated with or without FCS, demonstrating the ligand-independent activity of the mutated Fgfr2 construct. In contrast, however, variable levels of active phospho-Erk1/2 were detected in Fgfr2-ca transfectants in the presence or absence of FCS, as previously reported (Mangasarian et al., 1997). Steady Pou5f1 and Nanog levels confirmed the undifferentiated state of all transfected ES cells populations analysed. Control cell sample transfected with Fgfr1 is used to validate the specificity of the anti-Fgfr2 antibody in these experiments (Figure 6.1).
Figure 6.1: Characterization of Fgfr2 constructs. Western blotting with anti-phospho-Fgfr, anti-Fgfr2 and anti-Fgfr1 antibodies in wild-type E14Tg2a ES cells transiently transfected (48 hours) with empty (pcDNA3.1), myc-tagged Fgfr2 (Fgfr2-myc), constitutively active Fgfr2 (Fgfr2-ca) and Fgfr1 (Fgfr1) pcDNA3.1 expression constructs. Cells were transfected in ES self-renewing, proliferative media conditions supplemented with LIF and FCS [+\] and maintained in the same conditions unless otherwise stated ([-\], withdrawal of FCS 12 hours before sample collection). Western blotting with anti-Nanog and anti-Pou5f1 confirm the undifferentiated state of transiently transfected E14Tg2a ES cell samples. Anti-phospho-Erk1/2 and anti-total Erk1/2 antibody probing show a decrease in phospho-Erk1/2 levels across all samples upon FCS withdrawal. Equivalent sample loading is represented by similar β-tubulin protein levels.

The effect of Fgfr2 ectopic expression on ES cell self-renewal was measured using combined colony forming and alkaline phosphatase activity assays. Following over-night transfection with control (pcDNA3.1), Fgfr2-myc and Fgfr2-ca constructs, wild-type (E14Tg2a) and Nanog over-expressing (EF4) ES cells were plated at low density under neomycin selection with or without LIF in ES media plus FCS. After 9 days, plates were fixed, stained for alkaline phosphatase activity and the number of undifferentiated, mixed or differentiated colonies scored for each condition as illustrated in Figure 6.2A. Provision of LIF and FCS supports the formation of
undifferentiated colonies. In contrast, removal of LIF enables an increased proportion of differentiated colonies in wild-type ES cells (see control E14Tg2a transfectants; Figure 6.2B, left panel). We found that Fgfr2 forced expression further promotes differentiation in E14Tg2a cells, with a shift from about 45% to 90% in the proportion of differentiated colonies in Fgfr2-myc and Fgfr2-ca transfectants as compared to control transfectants. Though the proportion of E14Tg2a undifferentiated colonies increased with the addition of LIF as expected, there was about 20% lower frequency in both Fgfr2 E14Tg2a transfectants as compared to control cells. Nanog over-expression in ES cells (EF4) is known to confer resistance to differentiation (Chambers et al., 2003). Accordingly, Fgfr2-myc and Fgfr2-ca EF4 transfectants without LIF only yielded a mild increase in differentiated and mixed colonies. However, EF4 transfectants in proliferative LIF conditions revealed a notable decrease in the proportion of undifferentiated colonies (from about 76% to 31%) and concomitant increase in mixed colonies (from about 20% to 50%) in Fgfr2 transfectants as compared to control cells. This indicates that Fgfr2 ectopic expression can at least partly bypass the Nanog regulated self-renewal ability of EF4 cells. Fgf receptors are known to stimulate downstream signals via a variety of pathways including the Jak/Stat, PLCγ, PI3K and Erk cascades (Dailey et al., 2005). To verify Fgfr2 acts via the Fgf/Erk cell signalling pathway, we repeated the above experiments with the addition of the specific Mek inhibitor, PD0325901. As shown in Figure 6.2C, Erk inhibition abrogated Fgfr2-induced differentiation in all tranfectants treated with PD0325901 as compared to untreated cells (see Figure 6.2B). Taken together, our results demonstrate that modulating Fgfr2 expression levels can shift the balance from self-renewal to differentiation via the Fgf/Erk signalling cascade in ES cells.
Figure 6.2: Fgfr2 forced expression compromises Nanog promoted ES cells self-renewal ability via the Fgf/Erk signalling pathway. (A) Examples of undifferentiated (top), mixed (middle) and differentiated (bottom) ES cell colonies as revealed by alkaline phosphatase staining. (B) Low seeded E14Tg2a (left) and EF4 (right) ES cells were transfected with empty (pcDNA3.1), myc-tagged Fgfr2 (Fgfr2-myc) or constitutively active Fgfr2 (Fgfr2-ca) expression vectors. After neomycin selection for 9 days, colonies were fixed, stained for alkaline phosphatase activity and counted. Dark blue, undifferentiated colonies; light blue, mixed colonies; white, differentiated colonies. (C) Same as (B) but with the addition of the specific Mek inhibitor, PD0325901 (1μM), upon neomycin selection. Data are representative of three independent experiments.

6.2 Generation of stable Fgfr2 over-expressing ES cell clones.

We next asked whether Fgfr2 could be constitutively expressed in wild-type and/or Nanog over-expressing ES cell lines. E14Tg2a and EF4 cells were seeded at low density prior to transfection with the Fgfr2-myc and Fgfr2-ca constructs, as well as Fgfr1 and empty pcDNA2.1 constructs used as controls in these experiments. Cells were then placed under neomycin selection for 10 days. Generated clones were pooled and
analysed by western blotting two and four passages post selection. As shown in Figure 6.3, Fgfr2-ca expressing clones could be generated from both E14Tg2a and EF4 backgrounds. Fgfr2-ca expression was however rapidly extinguished in wild-type clones (over 4 passages), most likely due to Fgfr2-mediated pro-differentiation effect as demonstrated above (Figure 6.2).

**Figure 6.3: Establishing stable Fgfr2 over-expressing ES cell lines.** Western blotting with anti-Fgfr2 and anti-Fgfr1 (control) antibodies on pooled E14Tg2 and EF4 ES clones transfected with empty (pcDNA3.1), myc-tagged Fgfr2 (Fgfr2-myc), constitutively active Fgfr2 (Fgfr2-ca) or Fgfr1 (control) constructs after 2 passages and 4 passages post neomycin selection in proliferative conditions (LIF and FCS). Equivalent sample loading is represented by similar β-actin protein levels.

In contrast, Fgfr2 over-expressing EF4 cell clones (EF4\textsuperscript{Fgfr2-ca}) could be maintained and propagated under neomycin selection, forming typical undifferentiated colonies that stained positive for alkaline phosphatase activity, as seen in control transfected EF4 cells (EF4\textsuperscript{pcDNA3.1}; Figure 6.4A). Similar Nanog and Pou5f1 protein levels between EF4\textsuperscript{Fgfr2-ca} and EF4\textsuperscript{pcDNA3.1} cells further indicate the undifferentiated state of the newly established clones, as assessed by western blot and immunofluorescence staining (Figure 6.4 B and C).
Figure 6.4: Characterization of stable Fgfr2 over-expressing EF4 ES cell line. (A) Phase-contrast images of ES cell colonies (top panel) and alkaline phosphatase activity staining (bottom panel) of both pcDNA3.1 (EF4pcDNA3.1) and Fgfr2-ca (EF4Fgfr2-ca) stably expressing EF4 ES cell lines. (B) Western blotting with anti-Fgfr2, anti-Nanog and anti-Pou5f1 antibodies on EF4pcDNA3.1 and EF4Fgfr2-ca ES cell populations. Equivalent protein loading is shown with Actin detection. (C) Immunofluorescence staining with anti-Fgfr2 and anti-Nanog antibodies of EF4pcDNA3.1 and EF4Fgfr2-ca ES colonies. Anti-DAPI and anti-phalloidin counter staining highlight cell nuclear and membrane regions, respectively. Scale bar, 15μm.
We previously showed that the core pluripotency factor Nanog directly represses the transcription of Fgfr2 in ES cells (see section 5.6). Here we demonstrate that forced expression of wild-type (Fgfr2-myc) and constitutively active Fgfr2 (Fgfr2-ca) impedes on ES cell self-renewal ability as assessed using colony forming assays (Figure 6.2). Notably, the ability of Fgfr2 to promote differentiation was observed in both wild-type and Nanog-over-expressing ES cells and appears to be mediated via the Fgf/Erk cell signalling pathway. Although the involvement of other Fgfr2-associated downstream signalling pathways cannot be formally ruled out, our results indicate that Fgfr2-stimulated signalling is potent enough to partly bypass ES cell self-renewal status supported by Nanog; an effect completely abrogated in the presence of the Mek inhibitor, PD0325901. Interestingly though, we were able to stably establish a ES cell line that constitutively over-expresses both Nanog and Fgfr2 (Figure 6.3 and 6.4). This concomitant with high levels of Nanog protein being capable of buffering the differentiation-inducing effects of Fgfr2 in proliferative LIF culture conditions and further highlights the delicate balance between self-renewal and differentiation operating in ES cells. In contrast, our attempt to constitutively express Fgfr2 in wild-type ES cells proved unsuccessful. The fluctuating expression of Nanog in wild-type ES cells may indeed open opportunities for Fgfr2-stimulated cell signalling to prime Nanog-low cells for differentiation and thus gradually shift the balance from self-renewal to differentiation in Fgfr2-expressing colonies. While Nanog is shown to repress many components promoting lineage-restricted differentiation (Loh et al., 2006), it is worth noting that the forced expression of Fgfr2 is sufficient to destabilise ES cell self-renewing state and promote differentiation, further demonstrating the critical importance of modulating the Fgf/Erk signalling cascade to preserve ES cell identity.

ES cells can be induced to aggregate and form embryoid bodies (EBs) in which PrE cells emerge and sort to the border of the structure, forming an organised cell layer
that stains positive for the two PrE associated markers, Gata6 and Gata4 (Coucouvanis and Martin, 1995; Rula et al., 2007). As previously reported though, Nanog over-expression can severely impair PrE formation in vitro (Figure 6.5A and B; F. Lavial, unpublished data; (Hamazaki et al., 2004). It will be interesting to investigate whether Fgfr2 expression can also bypass Nanog-mediated blockage of PrE differentiation upon EB formation.

Figure 6.5: Nanog expression influences embryoid body PrE formation. (A) Quantitative RT-PCR analysis of Gata6, Gata4 and Sox17 in wild-type E14Tg2a and Nanog over-expressing EF4 ES cell lines during EB formation at days 0, 3 and 5. Data represents the mean and s.d. of three independent experiments normalized to L19 and S17. (B) Immunostaining analysis with anti-gata6 and anti-gata4 antibodies on fixed day 5 EBs from wild-type control E14Tg2a and Nanog over-expressing EF4 cell lines. Nuclear staining using DAPI. Scale bar is 20μm. (Data kindly provided by F. Lavial). These data confirm that the constitutive presence of Nanog can significantly impair PrE formation as assessed upon EB formation in vitro.
Chapter 7 Discussion

7.1 Specifying epigenetic asymmetry at key developmental genes between embryonic and extra-embryonic lineages in the early embryo and in stem cells.

In 2006, several studies revealed how PcG-mediated gene repression might provide flexible, epigenetic silencing of loci primed for future activation (or repression) upon ES cell differentiation (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006b; Lee et al., 2006). Whether ES cell epigenetic features also exist in vivo and to what extent they are involved in delineating pluripotency in the early embryo remained to be established. In this study, we provide in vivo evidence that bivalent chromatin domains are present from eight-cell embryos up to the blastocyst stage at silent gene promoters, as assessed by carrier ChIP assay (Alder et al. 2010). This suggests that transcriptional gene priming may be established at the chromatin level early during pre-implantation development and so prior to blastocyst lineage segregation. Unexpectedly, we find that bivalent histone markings, as well as being attributes of emerging pluripotent cells within the ICM, are also retained at lineage-inappropriate genes in TE-restricted cells. Closer analysis revealed, however, that bivalently marked genes in TE-derived stem cells are not occupied by PRC1 (Ring1B) and poised RNAP II complexes, consistent with a loss of gene priming for transcription. Instead, we demonstrate that alternative repressive epigenetic mechanisms operate at these loci in the trophoblast lineages, as assessed both in vitro and in vivo. First, we establish that bivalent, lineage-inappropriate genes become selectively targeted by repressive Suv39h1-mediated H3K9me3 and de novo DNA methylation upon ES cell conversion into TS-like cells, as seen in TS cells. Moreover, we verified that Suv39h1-mediated H3K9 methylation is also acquired in vivo at silent loci in the TE lineage, but not in the ICM upon blastocyst formation. Taken together, our findings suggest a mutually exclusive role for Ring1B and
Suv39h1 in regulating the fate of bivalent genes during pre-implantation embryo development and in stem cells.

This leads us to propose a model where by PRC1 (Ring1B) and poised RNAP II - two hallmarks of gene priming in pluripotent cells - are dynamically removed from PRC2-bound, bivalently marked promoters upon TE cell fate acquisition and Suv39h1 recruitment. In this model, H3K9me3 and downstream DNA methylation are thought to reinforce or stabilise the repressed state of Suv39h1-bound loci upon Ring1B loss. The absence of Ring1B at these promoters in lineage-restricted TS cells does not, however, exclude that other PRC1-like complexes might be recruited. The composition of PcG complexes is known to be dynamic during development (Schuettengruber and Cavalli, 2009; Otte and Kwaks, 2003; Schuettengruber and Cavalli, 2009) as illustrated by the reverse expression pattern of Phc homologs Phc1 and Phc2 in ES and TS cells (Figure 7.1) and the differential recruitment of these two proteins to the inactive X chromosome in the two stem cell types (de Napoles et al., 2004).

**Figure 7.1: Proposed model for the formation of distinct chromatin states at PRC2 bound, bivalent loci *in vivo* upon blastocyst lineage segregation.** The first lineage segregation event in mammalian pre-implantation development occurs during the transition from morula to blastocyst. A primed state in pluripotent cells is associated with Suz12 (PRC2) bound, bivalently marked (H3K4me2 and H3K27me3) loci also being occupied by Ring1B (PRC1) and poised RNAP II. In contrast, a transcriptionally repressed state is linked to a loss of PRC1 and RNAP II upon TE cell fate commitment and the acquisition of Suv39h1-mediated H3K9me3 and DNA methylation (5mC).
Interestingly, while Suv39h1 and Suv39h2 are usually associated with the formation and function of constitutive heterochromatin in eukaryotic cells, differential loading of Ring1B and Suv39h onto heterochromatin was previously observed in the newly formed zygote, specifying epigenetic asymmetry between parental genomes (Arney et al., 2002; Probst et al., 2007; Puschendorf et al., 2008). In that case, Suv39h1-mediated H3K9me3 is enriched on the maternal genome, while Ring1B selectively targets paternal genome; a pattern maintained through cell divisions up to the eight-cell stage (Puschendorf et al., 2008). Furthermore, in the absence of Suv39h1, Ring1B becomes associated with the zygotic maternal genome depleted of H3K9me3, thus revealing a strict hierarchy between repressive mechanisms in the early embryo. Whether similar hierarchies or mechanistic relationships operate at bivalently marked genes upon TE cell fate specification and chromatin remodelling (from a primed to repressed) is an interesting view that would need to be formally demonstrated. While Suv39h1 depletion by RNAi in TS cells proved sufficient to decrease the levels of detectable H3K9me3, we have yet to assess whether this impacts on the recruitment of Ring1B to bivalently marked loci. Conversely, preliminary attempts to over-express Suv39h1 in proliferating ES cells indicated that high levels of Suv39h1 might be detrimental to ES cell proliferation and/or survival (F. Lavial, unpublished observation). This is consistent with previously reported in vivo transgenic analysis demonstrating low frequency of Suv39h1 over-expressing founder mice with retarded growth and mixed penetrance (Czvitkovich et al., 2001). Simultaneously altering the culture conditions, to TS culture media for example, while over-expressing Suv39h1 in ES cells could enable us to examine further whether Suv39h1 recruitment directly induces the release of Ring1B from bivalently marked promoters.

A key role for Ring1B in regulating developmental potential is consistent with previous data both in vitro and in vivo. In ES cells, conditional knockdown of Ring1A/B revealed how PRC1 can reinforce the ‘poised’ conformation of RNAP II bound to silent,
key developmental loci that are required later on during development (Stock et al., 2007; van der Stoop et al., 2008). In vivo, Ring1B mutant mice form morphologically normal blastocysts, yet their development becomes severely compromised soon after implantation (Puschendorf et al., 2008; Voncken et al., 2003). Interestingly, the loss of Ring1B binding at bivalent genes in TS cells is reflected by a loss of poised RNAP II, reiterating the existence of a unique mechanistic relationship between these two complexes (Alder et al., 2010). The mechanisms underlying PRC1 and RNAP II targeting at primed genes in emerging pluripotent cell remains largely unknown, however. It is worth noting that pluripotency and transcriptional priming may also be safeguarded through active protection from H3K9 and/or DNA methylation. In support of this hypothesis, the H3K9 demethylase Jmjd2c was reported to be highly expressed in early cleavage stage embryos as well as in ES cells but not in TS cells (Alder et al., 2010; Wang et al., 2010). In contrast, Suv39h1 expression gradually increases during pre-implantation development (Puschendorf et al., 2008) and becomes restricted to the TE lineage upon blastocyst formation as well as in TS cells (Alder et al., 2010).

An intriguing aspect of this study is the addition of repressive H3K9me3 and DNA methylation at promoters that retain high levels of H3K4 methylation in TS cells; a combination of histone modifications also reported in embryonic carcinoma cells (Ohm et al., 2007). This indicates that a loss of H3K4 methylation might not be a 'prerequisite' for enabling a repressed state at bivalent genes that are not activated upon loss of pluripotency, in striking contrast to prevailing views (Azuara et al., 2006; Bernstein et al., 2006; Meissner et al., 2008; Mikkelsen et al., 2007; Mohn et al., 2008; Roh et al., 2006). Our findings reveal a novel mechanism by which 'bivalency' and gene priming can be resolved by an additional layer of repression, Suv39h1-mediated H3K9me3 and the formation of a trivalent histone conformation (H3K9me3, H3K4me2 and H3K27me3) at silent, embryonic-associated genes in extra-embryonic restricted cells as seen both in vivo and in TS cells (Alder et al., 2010). Although the co-occupancy of all
these histone modifications has been verified by sequential ChIP in TS cells, the inhibitory effect of H3K4 methylation towards H3K9 methylation suggest that all three histone modification may not be occurring on the same histone tail (Wang et al., 2001). Interestingly, a recent genome-wide analysis found that while H3K4me3 distribution remained overall unchanged between ES, TS and XEN cells, the size of H3K27me3 domains was significantly reduced in extra-embryonic compared to embryonic stem cells (Rugg-Gunn et al., 2010). In this study, higher enrichment levels for repressive H3K9me3 at silent, H3K4me3 marked promoters were also detect in both TS cells and in vivo but not in pluripotent cells in agreement with the establishment of lineage-specific histone modifications during development. Whether a subset of genes has a trivalent histone domain as defined in our study was not addressed however.

Recently, genome-wide studies uncovered the occurrence of trivalent histone configurations at a subset of genes encoding early somatic as well as trophectoderm markers in pluripotent ES cells (Bilodeau et al., 2009; Yuan et al., 2009). This indicates that ‘trivalency’ might not be a unique attribute of the trophectoderm lineage. In pluripotent cells, however, many promoters enriched for H3K9me3 are targeted by a different member of the H3K9 methyltransferase family, Eset (also known as Setdb1), thereby highlighting the individual roles and/or target specificity of Suv39h1 and Eset in stem cells and early development. Whether Eset-bound promoters also lack Ring1B and poised RNAP II binding remains to be clarified.

Since first discovered in 2006, bivalent chromatin signatures have been found in different species and cell types (Alder et al., 2010; Barski et al., 2007; Bernstein et al., 2006; Lindeman et al., 2010; Pan et al., 2007; Vastenhouw et al., 2010). Notably, these opposing histone marks are also present, although at a lower frequency than in ES cells, in mesenchymal stem cells, hematopoietic or neural stem cells and progenitors as well as T cells, and tend to resolve upon differentiation to ‘univalency’ (Collas, 2010; Cui et al., 2009; Mazzarella et al., 2011; Mikkelsen et al., 2007; Mohn et al., 2008; Roh et al.,
Dynamic changes of target genes between pluripotent and progenitor cells have however been reported and suggest that distinct sets of developmental regulatory loci are primed and rapidly activated upon differentiation cues in different cell contexts. Note that, the presence of bivalent histone markings (H3K4 and H3K27 methylation) per se does not always equate to transcriptional priming as seen in TS cells. Further assessment for the presence of PRC1 and ‘poised’ RNAP II complexes or alternative repressive hallmarks is likely to be required to fully establish the chromatin status of developmental genes during embryogenesis.

In summary, this study reveals an important role for PRC1 (Ring1B) and Suv39h1 (H3K9me3) in delineating distinct chromatin states at key developmental genes during embryonic versus extra-embryonic lineage commitments in the early embryo and in stem cells.

7.2 Analysis of the interplay between the key transcription factor Nanog and the Fgf/Erk cell signalling pathway in ES cells.

The importance of Fgf/Erk cell signalling during early embryo development and particularly PrE emergence arises from the peri-implantation lethality of Fgf4, Fgfr2, Frs2a, Grb2 and Erk2 mutants, all mediators of this pathway (Arman et al., 1998; Chazaud et al., 2006; Cheng et al., 1998; Feldman et al., 1995; Hadari et al., 2001; Hatano et al., 2003; Saba-El-Leil et al., 2003). More recently, two independent studies suggest that autocrine Fgf/Erk stimulation primes ES cells for differentiation (Kunath et al., 2007; Stavridis et al., 2007). In this ongoing study, we propose that the heterogeneous expression of the core pluripotency factor Nanog could enable paracrine Fgf/Erk responses in a subset of ES cells at any given time in culture. As ES cells fluctuate between Nanog-high and Nanog-low states, we find that the Erk signalling receptor, Fgfr2, is also variably expressed, with highest levels observed in primed Nanog-low cells. Similarly, Nanog-null ES cells, which are more prone to differentiation (Chambers et al., 2003), highly express Fgfr2 and show increased levels of active phosphorylated
Erk1/2 form as compared to Nanog expressing cells in the presence of Fgf4. ChIP and luciferase reporter assays further revealed that Fgfr2 is directly and tightly repressed by Nanog, providing an explanation of how this pluripotency-associated factor can modulate or buffer the effect of the Fgf/Erk signalling pathway within self-renewing ES cell cultures. While Fgf4 mRNA can be detected in both Nanog-high and Nanog-low ES cells, we find that Nanog directly activates and sustains high levels of Fgf4 expression, most likely in combination with Oct4 and Sox2 (Yuan et al., 1995); this shared regulation may account for the gradual down-regulation of Fgf4 expression upon loss of Nanog in ES cells (see section 5.3). Taken together, our results suggest that Fgf4 could be steadily expressed in Nanog-high ES cells, which in turn activates Fgf/Erk cell signalling in neighbouring Nanog-low, Fgfr2 expressing ES cells, hence dynamically generating a subpopulation of cells primed for differentiation (Figure 7.2).

**Figure 7.2: Proposed model for the regulation of distinct self-renewing and primed states within ES cell cultures.** ES cell cultures express fluctuating levels of the pluripotency-linked transcription factor Nanog. ES cells can transit from a Nanog-high, self-renewing state to a Nanog-low, primed state and vice versa. Variations in Nanog expression enable specification of the primed state by promoting Fgf4 expression in Nanog-high cells while releasing the repression of Fgfr2 (and other loci like Gata6) in Nanog-low cells that can respond to Fgf4-induced signalling via the Erk pathway in a
paracrine manner. Nanog-mediated regulation of Fgfr2 and Fgf4 expression may underlie epiblast/PrE segregation in the developing blastocyst.

Heterogeneous and fluctuating expression has also been reported in ES cell populations for several other pluripotency markers including Stella and Rex1 (Hayashi et al., 2008; Payer et al., 2006; Toyooka et al., 2008). Based on their differential gene expression patterns at least three ES cells states of been suggested - self-renewing unprimed cells (Nanog/Stella/Rex1-positive), cells primed for germ layer differentiation (Nanog/Stella/Rex1-negative and Brachyury/Fgf5-positive) and cells primed for PrE differentiation (Nanog-negative and Gata6/Hex1-positive)(Lanner and Rossant, 2010; Singh et al., 2007). Whether Fgfr2 is expressed across the entire Nanog-low cell compartment or selectively delineates a subpopulation of primed ES cells – e.g. PrE primed cells – remains to be clarified. Interestingly, an inverse correlation between Fgfr2 and Fgf4 expression has been previously reported by single cell quantitative RT-PCR analysis in PrE and Epi progenitor cells of the segregating ICM, respectively (Guo et al., 2010; Kurimoto et al., 2006). Here we hypothesis that our in vitro observations could extend to the in vivo developing embryo with Nanog playing a pivotal role in regulating the expression of these two Fgf/Erk cell signalling mediators. Hence, it would be of interest to assess whether the deletion of Nanog in vivo also results in the release of Fgfr2 expression. Interestingly, Nanog depletion by electroporation of Nanog shRNA construct in vivo is sufficient to induce the expression of Gata6 (C. Chazaud; personal communication). Whether Fgfr2 induction would precede or result from Gata6 expression could also be assessed in these experiments.

Another interesting aspect of this study is the finding that ectopic expression of Fgfr2 can partly bypass Nanog promoted ES cell self-renewal ability. While Nanog over-expressing ES cells stably maintain an undifferentiated state even in the absence of LIF (Chambers et al. 2003), we observe an increased incidence of differentiation upon forced expression of Fgfr2 in Nanog high ES cells. This was however abrogated in the presence
of a Mek inhibitor, confirming that the pro-differentiation effect of Fgfr2 is mediated through the Erk signalling pathway. Suppression of Fgfr2 expression could therefore be one mechanism by which Nanog prevents self-renewing cells from differentiating.

It would be of interest to further explore the impact of forced Fgfr2 expression on the differentiation potential of Nanog over-expressing cells using embryoid body (EB) formation as a model system. Interestingly, constitutive expression of Nanog impairs PrE formation in EBs with only a few mis-located Gata6 positive cells being detected (Hamazaki et al., 2004; see Figure 6.5) Whether Fgfr2 over-expression can rescue this phenotype can be addressed using our newly generated Fgfr2 and Nanog over-expressing cell line.

Currently, the exact mechanism by which Fgfr2-mediated Fgf/Erk cell signalling supports differentiation remains largely unknown. Interestingly the MAPK pathway was shown to support Th2 development by stabilizing Gata protein in naïve CD4 T cells (Yamashita et al., 2005). It would be interesting to assess whether Fgf/Erk signalling operates a similar manner by stabilizing Gata6 protein and such facilitating the emergence of PrE lineage. Other factors working either in synergy with or against Fgf4/Fgfr2/Erk signalling are also likely to be important for lineage priming and commitment. For instance, Pou5f1 was shown to influence Erk signalling by repressing the expression of a serine/threonine kinase, Stk40, which can activate the signalling pathway and induce PrE differentiation in ES cells (Li et al., 2010).

In summary, our findings demonstrate that Nanog directly activates Fgf4 expression while suppressing expression of Fgfr2, which plays an active role in promoting ES cell differentiation through the Fgf/Erk signalling pathway. This provides a novel mechanistic insight into how Nanog can critically modulate Fgf/Erk signalling in ES cells.


proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell. 7:663-676.


reprograming properties of pluripotent and extra-embryonic stem cells implicate chromatin remodelling as an important early event in the developing mouse embryo. *Epigenetics Chromatin*. 3:1.


### Appendix I: Primer Sequences

Table 1-1: Primer sequences for the amplification of mouse transcripts by qRT-PCR analysis.

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Table I-2: Primer sequences for the amplification of mouse ChIP, sequential ChIP and C-ChIP temple by qRT-PCR.

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## Appendix II: Complimentary Data Sets

Table II-1: Expression levels of selected chromatin modifying complexes upon *Pou5f1* repression and TS-like cell derivation.

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The expression of selected chromatin modifying complexes was compared using qRT-PCR in ZHBTC4-ES, ZHBTC4 after 24 and 48 hrs of Dox treatment (ES\textsuperscript{Dox}), in ZHBTC4-derived TSL and B1-TS cells (left panel). Values shown are normalized to Actin and L19 and are relative to untreated ES cells (ES=1, left panel). Data and corresponding s.d. results from 3 independent experiments.
Figure II-1: Targeted chromatin changes at key developmental genes upon Pou5f1 shutdown and TSL cell derivation. ChIP analysis of untreated (ES) and Dox treated ZHBTC4 ES (ESDox) cells, ZHBTC4-derived TS-like (TSL) cells and traditionally-derived TS-B1 (TS) cells at selected panel of loci. Relative enrichment of H3K27me3, H3K4me2 and H3K9me3 are shown relative to H3. Binding patterns of DNA methylation (5mC), PRC2 (Suz12), PRC1 (Ring1B), Ser5P and hypophosphorylated RNAP II (8WG16) are shown relative to input. Background levels (from control IgG antibodies) are shown as grey bars. Data shown is representative of 3 experiments, error bars represent s.d.
Brackets demarcate silent developmental genes that retain bivalent chromatin domains in TS cells. Asterisk indicates the use of an alternative control loci for H3K9me3 ChIP, γ-sats were analyzed in place of the intergenic control regions and function here as a positive control locus.

Figure II-2: Increased levels of Fgfr2 expression in GFP/Nanog low ES cell subpopulations. Quantitative RT-PCR analysis of GFP, Pou5f1, Sox2, Fgf4, Fgfr2 and Gata6 in sorted GFP high or GFP low TNGA ES cells from three independent experiments and the mean are shown. Error bars represent the s.d. of the three independent experiments. Data are normalized to L19 and S17.
Figure II-3: Putative Nanog target regions across \textit{Fgfr2} and \textit{Fgf4} loci. (A) Schematic representation of putative Nanog binding sites in Fgf4 and Fgfr2 gene regions. Nanog binding site (BS) indicated in red. (B) DNA sequence, matching score to consensus sequence, location from predicted TSS, sense, primer sequences and size of amplicon produced from primers for putative Nanog target regions across \textit{Fgfr2} and \textit{Fgf4} loci. Transcription start site, TSS.
Publications

Parts of the work presented here have been published as follows: