200911005A

厚生労働科学研究費補助金

創薬基盤推進研究事業

# 多因子疾患モデルマウスの効率的樹立法の開発

平成２１年度　総括研究報告書

研究代表者　升井　伸治

平成２２（２０１０）年　3月

目次

Ⅰ.　総括研究報告
多因子疾患モデルマウスの効率的樹立法の開発　------------　1
升井　伸治
（資料）なし。

Ⅱ.　分担研究報告
分担者なし。

Ⅲ.　研究成果の刊行に関する一覧表　-------------------------　6

Ⅳ.　研究成果の刊行物・別刷　-------------------------　7

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総括研究報告書

# 多因子疾患モデルマウスの効率的樹立法の開発

研究代表者　升井　伸治　国立国際医療センター（研究所）　室長

疫学研究などで蓄積された遺伝学的データから、多因子疾患を再構築するシステムを意図し、任意の多重遺伝子ノックダウンマウスの高効率樹立法を開発する。具体的には、多種類のshRNAを発現するES細胞を樹立し、これを用いて作製したヘテロマウスを使って病態解析を行ってモデルケースとする。他の疾患モデルマウスと異なり、ヒトでの多因子疾患の病因をもつ材料を提供できる。

## A．研究目的

生活習慣病など多くの疾患は多因子疾患であることが知られるが、そのモデル動物はほとんど整備されていない。現在知られている多因子疾患モデル動物は、変異導入や選抜による自然発症モデル動物であり、表現型は似ているものの、病因が同一でないためにヒトの病態を正確に反映しているとは言い難い。一方で近年、遺伝学的データは膨大に蓄積され、多因子疾患の原因遺伝子群の同定作業は急速に進んでいる。今後は、同定した変異が病因となるかを決定する目的と、ヒト多因子疾患に即したモデルマウスを作成する目的で、多種類の遺伝子を機能阻害（SNPs の大半は機能抑制的と考えられる）して病態解析する必要があるだろう。そこで、本研究では多重ノックダウンシステムを開発し、多因子疾患の再構築システムとして提案する。

従来、遺伝子の機能阻害はノックアウト法などにより行われていたが、多種類を扱うには操作が煩雑に過ぎ、現実には多因子疾患を再構築することは不可能であった。本研究ではshRNAによるノックダウン法を用いるが、一般的手法であるゲノム上へのランダム挿入では、位置効果によって発現がばらつき多種類を一定レベルに発現できないこと、多数の遺伝子座で挿入突然変異が起きるため副作用が無視できないことが問題である。

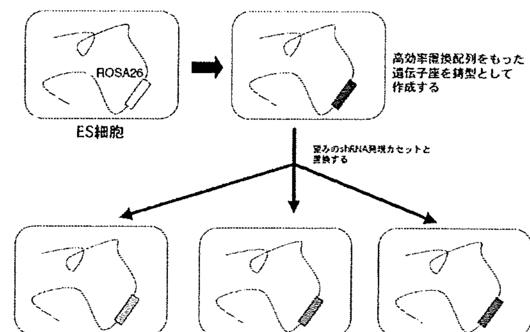


図１．本研究のスキーム

そこで、本研究では全組織で均一に発現

するROSA26遺伝子座に多種類のshRNA発現ユニットをノックインするシステムの開発を行う。そのための基盤技術として、これまでに私が ES 細胞において開発した、ROSA26上で発現カセットを高効率で置換できるシステムを用いる（図１；Nucl Acids Res.33:e43,2005）。２０年度までに２種ノックダウン、３種ノックダウンのシステムを構築できた。２１年度は、これらを用いたマウス樹立の試みと、SNPs解析の新しいシステム構築の試みを行った。

Ｂ．研究方法

マウスES細胞EBRTcH3およびBAg73C2はそれぞれ129/OlaおよびC57BL/6J由来であり、ジャームライントランスミッション能が広く確認されている ES 細胞株の派生株である。ゼラチンコートシャーレを用いて、フィーダー非存在下、LIF（ESGRO, Chemicon）1000U/ml、10%血清存在下で培養を行った。

遺伝子導入法は Lipofectoamin2000（Invitrogen）を用いたリポフェクション法にて行った。ノックダウン効率の検証として、SYBR Premix ExTaq（Takara）を用いた定量的RT-PCRを行い、リアルタイムPCR装置はMyiQリアルタイムPCR解析システム（BioRad）を用いた。

（倫理面への配慮）

マウスを用いた実験に関しては、厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針に従い実施する。また、遺伝子組み換え実験を行うが、P2レベルで申請は受理されている。

Ｃ．研究結果

i）２種ノックダウンシステム

２０年度までに、ROSA26遺伝子座では導入した発現ユニットの方向に依存せずに発現できること、および RNA ポリメラーゼ II 依存性プロモーターを互い違いに配置することで、異なる二種類の miRNA（生理的／内在性 shRNA）を発現できることを示した。RNAポリメラーゼIIである利点として、将来的に組織特異的プロモーターを利用した２種ノックダウンシステムへと応用できる。

解析例として、２型糖尿病関連遺伝子Hnf4aとTcf7l2(Nature 445:881-5,2007、これらのノックアウトマウスは胚性致死であるためノックダウンでのモデルマウスが必要とされる）のダブルノックダウンマウス作成を意図し、２０年度までに機能するshRNAを選定した。

２種ノックダウンシステムを搭載したES細胞クローンを２個取得し、ブラストシストへインジェクトすることでキメラマウスを２１匹得た。野生型マウスと交配し、ヘテロマウスの取得を試みたが、得られなかった。

ii)３種以上ノックダウンシステム

３種以上ノックダウンのために、RNAポリメラーゼIII系プロモーターを用いたノックダウンコンストラクト作成を行っ

た。上記 i)の RNA ポリメラーゼ II 転写系では、直列（タンデム）に連結した発現ユニットは転写干渉を起こして発現しないことが知られる。３種以上の転写ユニットを並べる場合、必ずどこかで直列に配置されてしまうことから、より多数の shRNA 同時発現のためにはタンデム連結でも転写干渉しないシステムが必要である。そこで、RNA ポリメラーゼ III 系プロモーターを採用した。MAR（Matrix Attachment Region; より確実なトランスジーン発現を保証するために挿入してある)近傍の SfiI サイトへ、ワンステップで４断片を順列特異的に導入できる（つまり望みの４種が入ったプラスミドを選択する必要が無い）システムを構築した。これにより、ノックダウンする遺伝子の種類数がｎ個に増加しても、ｎ回コンストラクション操作（１回のコンストラクションに１週間かかるとすると、ｎ週間かかることになる）を経ることなく迅速に多重ノックダウンコンストラクトが作成できる。

２０年度までにこのシステムによる Tcf7l2 と Hnf4a ノックダウンコンストラクトを作成終了し、これを ES 細胞へ導入した。２１年度はこれを用いてマウス作成を試みた。ブラストシストへインジェクトしてキメラマウス一匹を得た。これを野生型マウスと交配したが、ヘテロマウスは得られなかった。

２種ノックダウンシステムで用いた親株細胞 EBRTcH3 を、３種ノックダウンシステムでも用いた。染色体異常をもつ可能性を排除するため、染色体数解析を行ったが、異常は検出されなかった。

iii) 上記 ii)のシステムの場合、遺伝子抑制型の SNP には対応可能なのだが、遺伝子過剰発現型を反映した変異状態を作成できない。多種類のノックダウンコンストラクトとタンパク質が同時に発現できる新規システムの開発を意図し、原理的に新しいシステムの構築を行った。２０年度までに、直列につないだものでも両蛍光タンパク質の発現が確認している。２１年度には染色体に挿入した場合の発現量を解析した結果、大幅に減少することがわかった。したがって、一過性発現での使用を想定する必要がある。

そこで、試験管内（培養細胞）での複数 SNPs 相互作用の解析システムとしての応用を目指した。具体的には、複数遺伝子座の変異によって、複数の変異型タンパク質が存在するときに現れる表現型を、簡便に再現できるシステムの基盤技術としての応用を考えた（図３)。従来の複数遺伝子発現方法は、主にレトロウイルスなどを用いて一因子ずつばらばらに導入しており、細胞集団中において全因子が導入された細胞よりも導入されなかった細胞の方が多いため、効果の解析が困難である。多因子を同時に発現できる本システムを用いた場合、一過性発現の後の薬剤選択により、全因子を発現する細胞のみを選択でき、効果の解析が可能にな

る（図２）。

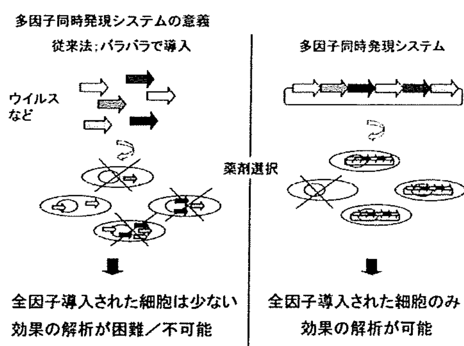


図２．多因子発現システムの意義

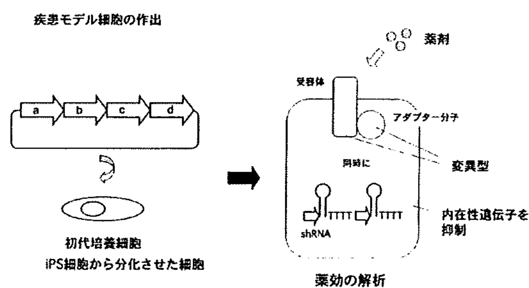


図３．疾患モデル細胞作出法として

本システムを用いて変異型タンパク質を同時に発現し、且つ内在性タンパク質を同時に抑制することで、SNPs 機能解析システムとしての使用が可能になると期待される。

内在性遺伝子の抑制のために、ES 細胞（BAg73C2）の Rosa26 遺伝子座に、２種・３種ノックダウンシステムで用いたカセット交換アレルを導入した（図４）。その結果、正しく導入された２クローンを得た。

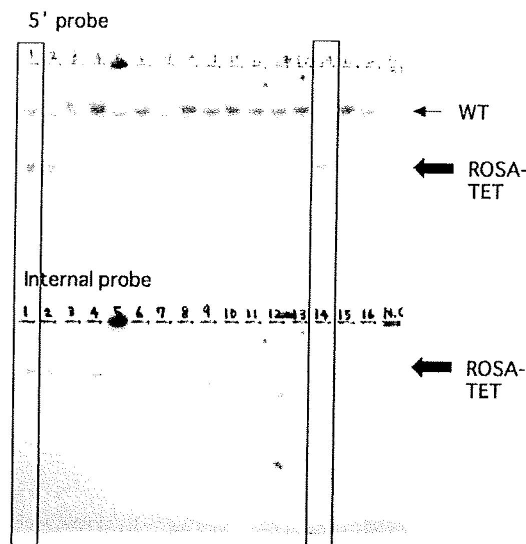


図４．カセット交換アレルの導入
BAgRTcH1, BAgRTcH14 細胞を取得した。

BAg73C2 細胞は、遺伝的背景が C57BL/6J 由来で、ノックアウト解析などで標準系統として使用されており、マウス個体の解析知見との照合が容易になるだろう。特に、129 系統と C57BL/6J 系統は代謝等の性質が大きく異なっていることが知られるため、使用する ES 細胞の系統はマウスのものと合致させておく必要があるだろう。将来的には両方の系統を比較することも必要になるだろう。

## D．考察

従来型プロモーターシステムを用いて、２種および３種遺伝子のノックダウンマウスを作成できるめどがついた。ジャームライントランスミッションが起きなかったが、フィーダーフリーES 細胞（操作

が簡便なシステムとして開発するためあえて用いた）はフィーダー依存性ES細胞と少し性質が異なるため、施設によってはジャームライントランスミッションが得られないケースも知られる。ES細胞からはクローンマウスが得られやすいことが知られているため、核移植を行ってヘテロマウスを直接得ることも対処手段の一つだろう。

C57BL/6J 由来の ES 細胞にカセット交換アレルを導入し、多因子発現システムとの組み合わせで試験管内 SNPs 機能解析システムとしての応用法を考案した。GWAS で得られた SNPs 一つ一つのマウスでの機能解析は C57BL/6J 系統で進められるだろう。一方、特定の細胞における薬効の解析などについては、試験管内での詳細な解析知見が必要とされるが、同系統であれば個体と細胞の知見の照合がスムーズに行える。今後は多因子発現システムを用いた SNPs 機能解析の例を提示することが必要だろう。一旦上手くいくとわかれば、ヒト iPS 細胞に応用するなど、広く使用されるようになるだろう。

E．結論

多重遺伝子ノックダウンマウスの効率的樹立法が確立されるめどがついた。多因子疾患の再構成モデルマウスの必要性を感じながらも作成できなかった多くの研究者が利用するようになるだろう。

大規模SNP解析で疾患との相関が発見された多数のSNPs／遺伝子については、それが機能的かどうかを簡便に検証する方法が必要であった。本システムを用いてマウス個体あるいは試験管内において簡便にSNPsの機能確認を行えば、複数のSNPsの相互作用から生じる表現型を多数同定できるだろう。

波及効果として、ヒト多因子疾患に即したモデルマウスが樹立されれば、薬剤のスクリーニングや治療法の開発に多大な貢献を果たすことが期待でき、国民の健康増進に寄与するだろう。

F．健康危険情報

なし。

G．研究発表

1．論文発表

Masui S. Pluripotency maintenance mechanism of embryonic stem cells and reprogramming. Int J Hematol. 91巻3号360-372, 2010

2．学会発表

なし。

H．知的財産権の出願・登録状況

1．特許取得

なし。

2．実用新案登録

なし。

3．その他

なし

別添５

# 研究成果の刊行に関する一覧表

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書　籍　名 | 出版社名 | 出版地 | 出版年 | ページ |
|---|---|---|---|---|---|---|---|
| | なし | | | | | | |
| | | | | | | | |
| | | | | | | | |

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| Masui S | Pluripotency maintenance mechanism of embryonic stem cells and reprogramming. | Int J Hematol. | 91巻3号 | 360-372 | 2010 |
| | | | | | |
| | | | | | |

Int J Hematol
DOI 10.1007/s12185-010-0517-9



# Pluripotency maintenance mechanism of embryonic stem cells and reprogramming

Shinji Masui

Received: 28 October 2009 / Accepted: 12 November 2009
© The Japanese Society of Hematology 2010

**Abstract** Embryonic stem (ES) cells are derived from blastocysts and are pluripotent. This pluripotency has attracted the interest of numerous researchers, both to expand our fundamental understanding of developmental biology and also because of potential applications in regenerative medicine. Systems biological studies have demonstrated that the pivotal transcription factors form a network. There they activate pluripotency-associated genes, including themselves, while repressing the developmentally regulated genes through co-occupation with various protein complexes. The chromatin structure characteristic of ES cells also contributes to the maintenance of the network. In this review, I focus on recent advances in our understanding of the transcriptional network that maintains pluripotency in mouse ES cells.

**Keywords** Pluripotency · ES cells · Transcriptional network

## 1 Introduction

Pluripotency is defined as the capability of a cell to differentiate into all the types of cells that make up an individual [1]. Tests for pluripotency include the formation of embryoid bodies in vitro that will differentiate into the three germ layers and in vivo contribution in chimeric mice and confirmation of germline transmission. Another test is teratoma formation, in which the cells are injected into nude mice to determine whether such injection leads to the development of tumors containing cells of all three germ layers. Embryonic stem (ES) cells, first reported in 1981 from the inner cell mass (ICM) of mouse E3.5 blastocysts [2, 3], can be stably cultured in vitro while maintaining pluripotency, and the properties of ES cells have allowed detailed analysis of the molecular mechanism maintaining pluripotency. Because the area is progressing rapidly, in this review, I focus on recent advances related to the transcriptional network that maintains pluripotency in mouse ES cells.

## 2 ES cells and other types of pluripotent stem cells

Before looking into the mechanism in detail, I briefly introduce other types of pluripotent stem cells that have recently been reported, and summarize their points of difference from mouse ES cells. Epiblast stem cells (EpiSCs) are derived from post-implantation embryos (E5.5–7.75), which contain the epiblast, a pluripotent derivative of the ICM [4, 5]. EpiSCs require fibroblast growth factor 2 (Fgf2, Mouse Genome Informatics, also known as bFgf) for growth. They have been shown to rely on Activin/nodal signaling, but do not show dependency on leukemia inhibitory factor (Lif) [4, 5], which is essential to maintain pluripotency in ES cells (see below). These features of EpiSCs are similar to those of human ES cells [6], and there may be a correspondence between the two.

Although EpiSCs can form teratomas, they provide only a very limited germline contribution in chimeric mice, and

S. Masui (✉)
Division of Molecular Biology and Cell Engineering, Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
e-mail: snjmsi@gmail.com

S. Masui
PRESTO, Japan Science and Technology Agency, Saitama, Japan

Published online: 16 February 2010

the gene expression pattern of EpiSCs is slightly different from that of ES cells, suggesting that the transcriptional network maintaining EpiSC pluripotency is regulated by a different mode [4, 5]. In fact, the expression of the central transcription factor gene *Oct3/4* is regulated by different regulatory regions in ES cells and EpiSCs (see below) [5]. Upon stimulation by Activin and Fgf2, ES cells can convert/differentiate to EpiSCs, while EpiSCs are unable to revert/dedifferentiate to ES cells with the addition of Lif. This suggests that EpiSCs are in a more advanced or committed developmental stage than are ES cells [7].

FAB-SCs, another form of pluripotent stem cell, are derived from blastocysts (E3.5) by culturing with three designated factors: Fgf2, Activin, and the GSK3$\beta$ inhibitor BIO, which is an agonist of wingless-related MMTV integration site (Wnt) signaling [8]. Although common molecular markers for pluripotency are expressed, FAB-SCs do not differentiate. However, upon stimulation by Lif and by bone morphogenetic protein 4 (*Bmp4*) and through cell–cell interaction including E-cadherin signaling, these cells can acquire differentiation capability similar to that of ES cells. This suggests that the FAB-SCs are in a "latent" state of pluripotency [8], which is probably maintained by somewhat different transcriptional network from that of the ES cells. To avoid confusion by integrating information derived from different types of pluripotent stem cells, in the following sections, I focus on mouse ES cells, and particularly on the transcriptional network in those cells, to understand the molecular mechanism maintaining pluripotency.

## 3 Extracellular signaling that regulates self-renewal of ES cells

Several soluble factors have been identified that exert either positive or negative effects on ES cell self-renewal (cell proliferation while maintaining pluripotency) (Fig. 1). ES cells secrete an unidentified stem cell autocrine factor (SAF) to promote proliferation [9]. Presumably, this activity can be mimicked by high concentrations of adrenocorticotropic hormone (ACTH), which is known to induce proliferation in some types of cells. ACTH promotes proliferation in ES cells, probably through the inhibition of adenylyl cylase (AC) activity regulated by G-protein coupled receptors (GPCR). Alternatively, the SAF could be BNP known to be involved in cellular proliferation. In ES cells, BNP promotes proliferation through the activation of the E26 avian leukemia oncogene 1, 5′ domain (*Ets1*) and through repression of gamma-aminobutyric acid receptor A (*GABAaR*) [10], which negatively regulates proliferation by activating the phosphatidylinositol-3-OH kinase-related kinase (PIKK) family including ataxia telangiectasia mutated homolog (Atm) [11]. The above soluble factors might contribute to the characteristic cell cycle profile of ES cells, with few cells in the G1 phase and most of the cell population in the S phase. In this mode of cell cycle, a pivotal role is played by phosphatidylinositol-3-OH kinase-Akt (PI(3)K/Akt). That pathway is activated by Ras (Eras), a constitutively active form of Ras-family small GTPase that is expressed specifically in ES cells [12]. Several factors are essential in ES cell self-renewal, including T-cell lymphoma breakpoint 1 (Tcl1) [13–15] and myeloblastosis oncogene-like 2 (*Mybl2*, also known as *b-Myb*) [16–18], known to be involved in the regulation of the PI(3)K/Akt pathway (for details, see the excellent available reviews) [19, 20].

Lif, a member of the IL-6 family, is known to strongly promote self-renewal in ES cells [21]. Lif binds to leukemia inhibitory factor receptor (LIFR) to dimerize with interleukin 6 signal transducer (*Il6st*, also known as *gp130*), resulting in the phosphorylation of signal transducer and activator of transcription 3 (Stat3) via Janus kinase (Jak) activation [22]. Phosphorylated Stat3 dimerizes and translocates to the nucleus to activate a variety of downstream genes, including myelocytomatosis oncogene (*Myc*, also known as *c-Myc*) and Krüppel-like factor 4 (*Klf4*) [23, 24]. Repression of Stat3 results in differentiation [25], whereas artificial activation of Stat3 is sufficient to maintain pluripotency without Lif in the media [26]. In addition to *Stat3*, Lif signaling pathways are transduced to the PI(3)K/Akt pathway through which T-box3 (*Tbx3*) is activated, while activity is repressed by the mitogen-activated protein kinase (MAPK) pathway which is also activated by the Lif signal [24, 27]. Repression of *Tbx3* results in differentiation [28]. Forced expression of *Tbx3* blocks differentiation in the absence of Lif, by maintaining Nanog homeobox expression (*Nanog*, see below) [24].

In combination with the Lif-Stat3 pathway, the pluripotency of ES cells is modulated by transforming growth factor $\beta$ (TGF$\beta$) superfamily members. These include Bmp and Activin, which generally play diverse roles in cellular homeostasis [29]. In the ES cells, Bmp4 activates the MAD homolog 1 (Smad1). This upregulates the expression inhibitor of DNA-binding genes (*Id*), which suppress differentiation in combination with the Lif signal [30]. Activin/nodal signaling, which is known to have generally the opposite effect on Bmp4 [29], also contributes to promote the growth of ES cells [31]. In the latter study, Bmp4 signaling was shown to be dispensable for maintaining pluripotency. This finding, which appears contradictory, may reflect differences in the media components. That would affect the composition of the ES cell subpopulation based on the gene expression profile, as represented by the expression of the zinc-finger protein 42 gene (*Zfp42*, also known as *Rex1*) [32].

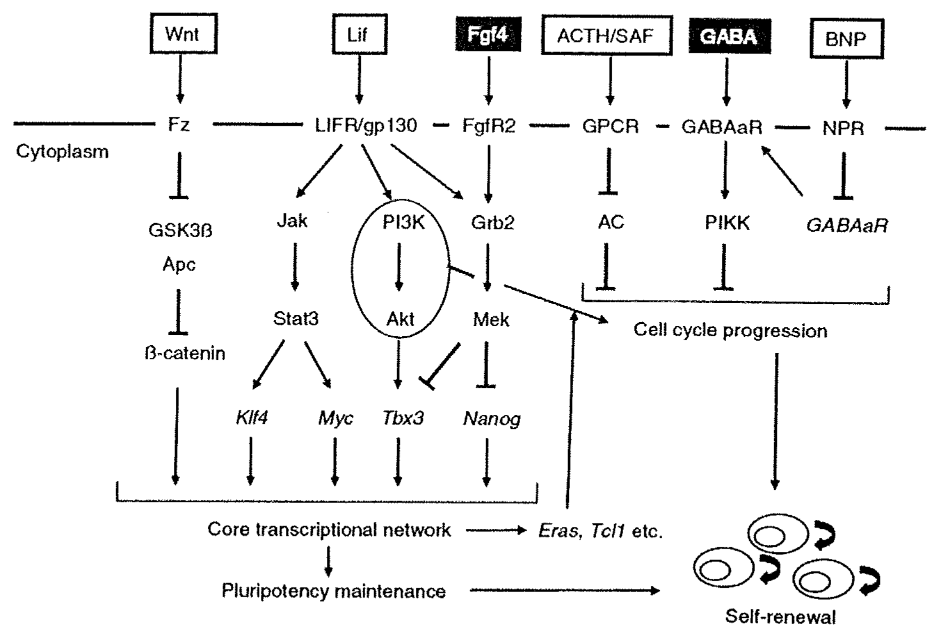


**Fig. 1** External signals contributing to self-renewal of ES cells. Wnt, Lif, and Fgf4 act to modulate the transcriptional network regulating pluripotency/differentiation, as well as to modulate cell cycle progression through the regulation of genes including *Eras* and *Tcl1*. The molecules shown in *white* and *black boxes* represent, respectively, positive and negative regulation (as a main function) for self-renewal. Popular terms for these factors have been used here for the ease of understanding

Wnt signaling also contributes to the maintenance of pluripotency. In the canonical Wnt pathway, the Wnt receptor Frizzled (Fzd) transduces the signal to glycogen synthase kinase 3$\beta$ (GSK3$\beta$) and adenomatosis polyposis coli (Apc). This enables catenin beta 1 (Ctnnb1, also known as $\beta$-catenin) to translocate into the nucleus to form the Ctnnb1/Tcf complex, which in turn activates the downstream genes [33]. Repression of *Apc* in ES cells causes resistance to differentiation in the media in the absence of Lif [34]. Administration of Wnt proteins, such as Wnt3a, Wnt5a and Wnt6, or BIO can contribute to maintaining the pluripotency of ES cells [35–37]. Inhibition of GSK3$\beta$, in combination with Fgf and Erk signaling inhibitors (see below), enhances the self-renewal of ES cells in a defined culture system [38]. In fact, in the presence of Wnt signaling, transcription factor 3 (Tcf3) activates the downstream genes that promote pluripotency maintenance by collaborating with the pivotal transcription factors *Oct3/4*, *Sox2* and *Nanog* (see below).

ES cells secrete fibroblast growth factor 4 (Fgf4) in an autocrine manner, which stimulates a Ras-Erk signaling cascade to induce differentiation. ES cells lacking *Fgf4* show resistance to differentiation [39]. The tyrosine kinase receptor of Fgf4 transduces the signal to Ras and Grb2, which are necessary to induce differentiation [40]. Loss of *Erk2* suppresses differentiation in ES cells [39]. Moreover, ES cells can be maintained in a pluripotent state in the absence of growth factors or cytokines through the inhibition of differentiation cues. This is done using inhibitors that target the Fgf receptor tyrosine kinases and the MAPK kinase 1/2 (Mek1/2, Erk activating enzymes) [38]. The downstream genes for Fgf signaling are largely unclear, as the target molecules of the activated Erk show considerable divergence, while *Tbx3* might be involved in the negatively regulated targets [24].

## 4 Transcription factors

As described above, extracellular signaling is transduced to the nucleus through transcription factors including Stat3 and Tcf3. Those factors listed below are part of the transcriptional network and play a central role in maintaining pluripotency.

*Oct3/4*, also known as *Pou5f1*, was initially described as either *Oct3* or *Oct4* by three different research groups [41–43], so the official designation includes both names. Oct3/4 consists of a POU domain as a DNA-binding domain, and two transactivation domains which lie on the N terminus and the C terminus, respectively [44]. The expression of *Oct3/4* is restricted in pluripotent cell lineages such as ICM and germ cells in vivo and undifferentiated ES cells in vitro, where it plays an indispensable role in maintaining pluripotency [41–43, 45, 46]. The regulatory regions of *Oct3/4* described to date consist of the proximal promoter (PP), proximal enhancer (PE), and distal enhancer (DE). DE activity is prominent in ICM, germ cells and ES cells, whereas in the post-implantation epiblast, EC cells and EpiSC, there is a decrease in DE activity and PE activity is dominant [5, 47]. DE activity depends on both the Oct–Sox motif and another motif bound by unknown ubiquitous factor(s) [48]. PE is activated by *Nr5a2* (also known as liver receptor homolog 1, *Lrh1*), and *Nr5a2* knockout embryos show loss of *Oct3/4* expression in the epiblast

[49]. PP is regulated by a variety of nuclear receptors. The activators include *Nr5a1* (also known as steroidogenic factor 1, *Sf1*) [50], *Rxrb* (retinoid × receptor beta) and *Nr5a2* [49, 51]. The repressive factors include *Nr2f1* (also known as chicken ovalbumin upstream promoter-transcription factors 1, *Coup-tfI*) [51], *Nr2f2* (also known as *Coup-tfII*) [51], and *Nr6a1* (also known as germ cell nuclear factor, *Gcnf*) [52]. *Nr6a1* repression in the embryo results in de-repressed *Oct3/4* expression in somatic lineages of that embryo, indicating that *Nr6a1* is necessary to restrict *Oct3/4* expression in pluripotent cell lineages [52].

*Oct3/4* regulates a broad range of target genes. From a developmental perspective, the main target is caudal type homeo box 2 (*Cdx2*); repression of *Oct3/4* in ES cells results in differentiation into trophectoderm through upregulation of *Cdx2*, and forced expression of that gene induces differentiation into trophectoderm [53]. Oct3/4 has been known to activate downstream genes by binding to enhancers carrying the octamer–sox motif (Oct–Sox enhancer), for synergistic activation with the SRY-box containing gene 2 (Sox2).

*Sox2* expression is detected in pluripotent cell lineages and in the nervous system [54]. Sox2 consists of a DNA-binding HMG domain and a transactivation domain that can be divided into three subdomains [55]. The involvement of *Sox2* in pluripotency maintenance was first suggested by the occupation of an enhancer consisting of octamer and sox binding motifs (Oct–Sox enhancer) in the regulatory region of *Fgf4* [56]. Oct–Sox enhancers are found in the regulatory region of most of the genes that are specifically expressed in pluripotent stem cells, such as *Oct3/4*, *Sox2*, *Nanog*, *Utf1*, *Lefty* and *Fbx15* [48, 57–62]. Repression of *Sox2* in vivo will result in early embryonic lethality due to the failure of ICM maintenance, indicating that *Sox2* is necessary for the maintenance of pluripotent stem cells [54]. On the other hand, repression of *Sox2* does not affect the activity of the Oct–Sox enhancer in ES cells, suggesting that Sox2 and other Sox members expressed in these cells, including Sox4, Sox11 and Sox15, might be redundantly involved in Oct–Sox enhancer activation [63, 64]. Because the differentiation induced by repression of *Sox2* can be rescued by forced expression of *Oct3/4*, the primary role of *Sox2* in ES cell self-renewal is to maintain *Oct3/4* expression [64].

*Nanog*, named after Tir Na Nog (land of the ever young), was discovered through digital differential screening based on the expression patterns confined to ES cells [65], and by functional screening based on the capability to maintain pluripotency in the absence of Lif [66]. Nanog consists of three domains: a homeodomain similar to the NK-2 family, which acts as a DNA-binding domain, and the transactivation domains at the N-terminal and C-terminal, through which Nanog dimerizes to exert its full activity [67, 68]. Loss of *Nanog* in vivo results in early embryonic lethality [65]. However, *Nanog*-null ES cells can be established and still maintain pluripotency, albeit with an increasing tendency to differentiate, indicating that *Nanog* is not absolutely required to establish the pluripotency transcriptional network [69]. Nevertheless, because forced expression of *Nanog* increases the tendency toward undifferentiation under conditions that would normally favor differentiation, the function of *Nanog* can be seen as stabilization of pluripotency [69]. From a developmental point of view, one major role of *Nanog* might be the repression of GATA-binding protein 6 (*Gata6*), the forced expression of which induces differentiation into ExEn cells [70]. In the blastocyst, *Gata6* is first expressed in some cells of the ICM, most of which differentiate into extra-embryonic endoderm cells. Nanog expression and Gata6 expression are mutually exclusive, suggesting that *Nanog* suppresses differentiation into extraembryonic endoderm cells [71].

Klf4, containing C2H2-type zinc-finger motifs, regulates numerous processes including proliferation and differentiation in general [72]. In ES cells, Klf4 participates in the activation of an Oct–Sox enhancer in cooperation with Oct3/4 and Sox2 [62], and forced expression of *Klf4* maintain pluripotency in the absence of Lif. *Klf4* might regulate transition between different states of pluripotency. *Klf4* is strongly expressed in ES cells, but not in EpiSC, and forced expression of *Klf4* in EpiSC can convert/reprogram some (although not many) of these cells to ES cells [7]. The Klf4 function in pluripotency may be supported by redundant Klf family members. Although loss of *Klf4* does not seem to cause any defects in the pluripotency of ES cells or in the early embryo [62, 73], differentiation is induced by the simultaneous repression of *Klf2*, *Klf4*, and *Klf5*. This outcome suggests that these three molecules act redundantly to maintain pluripotency [74]. In fact, each of the three *Klf* factors in combination with *Oct3/4*, *Sox2*, and *Myc* can reprogram somatic cells into pluripotent stem cells (see below) [75]. On the other hand, *Klf4* and *Klf5* have slightly different functions in the proliferation of ES cells. *Klf4* suppresses that proliferation, while *Klf5* promotes it via the Tcl1–Akt pathway [15].

*Myc* is a potent oncogene, and is known to strongly promote proliferation [76]. In ES cells, *Myc* expression is regulated by Lif–Stat3 signaling. Stat3 activated by Lif signaling binds directly to the regulatory region of *Myc* to activate expression [23]. The sustained expression of a constitutive active form of Myc is sufficient to maintain pluripotency in the absence of Lif, whereas its dominant negative form antagonizes the maintenance of pluripotency and promotes differentiation [23].

The sal-like 4 (*Sall4*) gene belongs to the spalt family (C2H2 zinc-finger protein genes), conserved from

drosophila to human and playing a variety of important roles in development [77]. Repression of *Sall4* in the early embryo results in lethality due to ICM/epiblast defects [78, 79], although in rare cases ES cells can be established from the homozygote blastocysts [80]. Although *Sall4* may promote *Oct3/4* expression [81], *Sall4* might not be absolutely necessary for the maintenance of pluripotency, since *Sall4*-null ES cells are still pluripotent [79, 82]. In ES cells, repression of *Sall4* prolongs the G1 phase and thus retards proliferation [79, 82]. In the nucleus, *Sall4* localizes in the heterochromatin region, implying a genome-wide repressive function [79]. In fact, *Sall4* is involved in the NuRD complex (see below) to repress developmentally regulated genes including *Cdx2* [82, 83]. Collectively, *Sall4* promotes self-renewal of ES cells through promoting proliferation and repression of the target genes.

Estrogen-related receptor beta (*Esrrb*) has been known to play an essential role in placenta formation [84]. The *Esrrb* function is also necessary in ES cells, as differentiation is induced by the repression of *Esrrb* [28]. Esrrb directly interacts with Oct3/4 to activate various downstream genes, including *Nanog*, in which the regulatory region carries the Esrrb-binding site, the degenerated 9-bp estrogen-related receptor response element (ERRE). The expression of *Esrrb* is in turn regulated by *Oct3/4* and *Nanog*.

RE1 silencing transcription factor (*Rest*, also known as *NRSF*) is a transcriptional repressor that targets a group of neuronal genes by binding to the RE1 element in non-neural cells [85]. *Rest* may be essential for pluripotency maintenance in ES cells, as repression of *Rest* reduces the expression level of transcription factors such as *Oct3/4* and *Nanog*, derepressing the expression of several microRNAs including miR21 to suppress the pluripotency maintenance mechanism [86]. However, other researchers have reported that *Rest*-null ES cells can be established and that those cells express normal levels of pluripotency markers including *Oct3/4* and *Nanog* [87], hence, the contribution of *Rest* within the core transcriptional circuit is still controversial [88, 89].

*Tcf3* belongs to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, which binds to Wnt-response elements (WRE: C/T-C-T-T-T-G-A/T-A/T) via their HMG domain [90]. In the absence of Wnt signaling, or where such signaling is only present at low levels, TCF/LEFs acts as a component of a repressive complex, while in the presence of Wnt, TCF/LEF forms an activating complex with *Ctnnb1* to promote the expression of downstream genes [90]. This is the canonical Wnt signaling pathway, in which *Tcf3* positively or negatively regulates pluripotency in ES cells. *Tcf3* repression derepresses *Nanog*, producing resistance to differentiation, while stimulation of Wnt signaling upregulates the expression of genes for pivotal transcription factors such as *Oct3/4* and *Nanog* [91–94].

C2H2-type zinc-finger protein Zfx is involved in the proliferation of ES cells and hematopoietic stem cells [95]. In ES cells, repression of *Zfx* compromises proliferation while retaining differentiation capability. Forced expression of *Zfx* promotes ES cell proliferation by antagonizing differentiation through activation of *Tbx3* and *Tcl1*, both of which play essential roles in pluripotency maintenance and proliferation [95].

*Ronin*, whose products contain a DNA-binding THAP domain for epigenetic silencing of gene expression [96, 97], is expressed primarily during the earliest stages of embryonic development, and its absence is lethal at the peri-implantation stage in knockout embryos due to ICM defects [98]. *Ronin* repression in ES cells induces proliferation defects, while forced expression of *Ronin* allows ES cells to maintain pluripotency in the absence of Lif via an unknown pathway independent of *Stat3* [98]. Ronin also acts on pluripotency maintenance through a pathway independent of the core transcription factors, as forced expression of *Ronin* partially rescues the differentiation induced by *Oct3/4* repression, and the regulation of *Ronin* expression does not depend on *Oct3/4*, *Sox2* or *Nanog* (thus the designation *Ronin*, named after masterless Japanese samurai) [98]. Although the Ronin function is largely unclear, it involves the repression of global transcription, probably through the formation of a complex with HCF-1, a transcriptional regulator that has a variety of functions including transcriptional repression and cell proliferation [98, 99].

## 5 Protein complexes regulating transcription

The functions of the transcription factors discussed above are controlled by associated co-activators and co-repressors (Fig. 2). Nanog physically interacts with transcription factors such as Oct3/4, Sall4, the dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X-chromosome gene 1 (Nr0b1, also known as Dax1), the BTB-domain containing protein related to *Drosophila* bric-a-brac/tramtrack (Nacc1) and Zfp281 (also known as the mouse homolog of human zinc-finger protein ZBP99), each of which are involved in distinct protein complexes contributing to transcriptional regulation by Nanog [100]. *Nacc1*, *Nr0b1*, and *Zfp281* are necessary to maintain pluripotency [100, 101]. Nacc1 and Sall1/4 interact with the nucleosome remodeling and histone deacetylation (NuRD) complex to repress the downstream genes [82, 83, 100, 102]. On the other hand, NuRD is not necessary for the self-renewal of ES cells, since repression of the gene for a NuRD component, methyl-CpG binding domain protein 3 (*Mbd3*), results in failure to commit to developmental lineages [103]. Nanog (and Oct3/4) interact with another

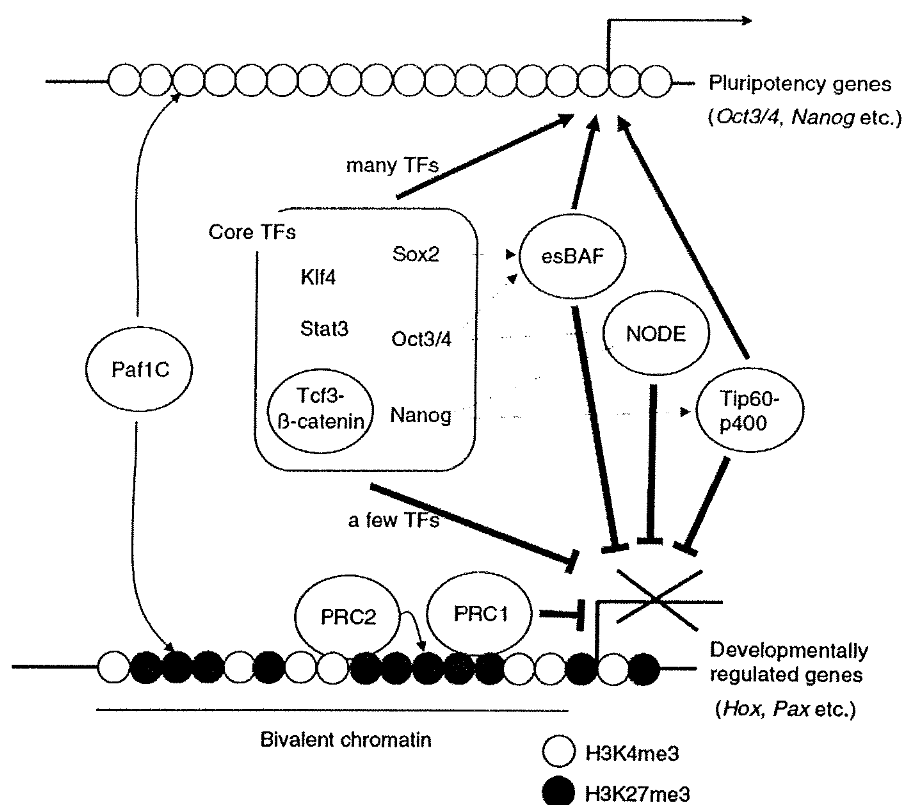


**Fig. 2** Transcriptional network maintaining pluripotency while keeping differentiation capability. Core transcription factors including Oct3/4, Sox2, and Nanog co-operated with various protein complexes to positively or negatively regulate the target genes. Many core transcription factors co-operate to activate genes for pluripotency, whereas in the developmentally regulated genes only a few of these factors are used for the repressing mechanism. This presumably includes the recruitment of PRC2, which is responsible for H3K27me3 modification where PRC1 is recruited to repress the target genes. *Curved arrows* indicate modification on histones. Protein complexes are shown in *circles*. *Thin broken lines* with and without *arrowheads* indicate association with and involvement with protein complexes, respectively. Transcriptional regulation is represented by *thick lines*. *TF* transcription factor

Hdac1/2- and Mta1/2-containing complex, NODE (for Nanog and Oct3/4 associated deacetylase), to co-occupy and repress Nanog-target genes [104]. Repression of *Mta1* derepresses the expression of developmentally regulated genes and induces ES cell differentiation [104].

Nanog and Oct3/4 co-occupy the downstream genes with Pol II-associating factor 1 complex (Paf1C) [105]. Paf1C is generally known to be involved in multiple processes, such as transcription initiation and elongation, transcript start site selection, and histone modification [106]. The components of Paf1C, including Ctr9, are expressed at higher levels in ES cells, and are downregulated during differentiation. Repression of Ctr9 causes expression changes similar to Oct3/4 or Nanog depletions, and induces differentiation, whereas forced expression of Ctr9 blocks ES cell differentiation. In ES cells, Paf1C binds to the promoters of pluripotency genes, where it is required to maintain a transcriptionally active chromatin structure through the maintenance of H3K4me3. On the other hand, in the lineage-control genes being repressed in ES cells, Paf1C is required not for the maintenance of H3K4me3, but instead for the maintenance of histone H3 lysine 27 trimethylation (H3K27me3, repressive chromatin mark), indicating that Paf1C supports pluripotency by maintaining the chromatin structure characteristic of ES cells (see below) [105].

*Nanog* recruits the Tip60-p400 histone acetyltransferase (HAT) and nucleosome remodeling complex (Tip60-p400) [107], which is normally involved in DNA repair and proliferation [108]. Localization of E1A-binding protein p400 (Ep400, also known as p400) to the promoters of both silent and active genes is dependent on histone H3 lysine 4 trimethylation (H3K4me3, active chromatin mark) and the presence of Nanog, since depletion of either Ash2l (a component of the H3K4me3 catalytic complex) or Nanog reduces Ep400 binding to target promoters. Repression of Ep400 deregulates the genes for developmental regulators, which significantly overlap with the downstream genes of *Nanog*, so that Tip60-p400 is necessary to maintain pluripotency. At the target promoters, Tip60-p400 acetylates histones including H4 for the proper regulation of gene expression [107].

ES cells express specific components of BAF (Brg/Brahma-associated factors), also called SWI/SNF ATP-dependent chromatin-remodeling complexes, to form esBAF [109]. The components of esBAF include Smarca4 (also known as Brg1) and Smarcc1 (also known as BAF155). The repression of these components induces differentiation in ES cells, indicating that esBAF is essential to maintain pluripotency. Although BAF complex is also found in differentiated cells, its composition in those cells differs from that in esBAF. Smarcc2 (also known as

BAF170) is found in the BAF complex in differentiated cells, but not in esBAF, and the forced expression of *Smarcc2* induces differentiation, suggesting that a specific subunit composition is required for pluripotency maintenance [109]. Smarca4 physically interacts with Oct3/4 and Sox2, and they co-occupy promoter regions of the pluripotency genes and developmentally regulated genes. Depending on the genetic context, esBAF functions as either a repressor or an activator in controlling the ES cell transcriptional circuit [109, 110].

## 6 Epigenetic factors

To initiate the differentiation process in precise timing, the developmentally regulated genes need to be poised for transcription in ES cells. Most transcriptionally silent developmentally regulated genes, such as the Hox family are repressed by polycomb repressor complexes (PRCs). In general, PRC2 catalyses trimethylation of H3K27, which is thought to provide a recruitment site for PRC1 (Fig. 2) [111]. In ES cells, PRC1 and PRC2 components co-occupy most of the target genes, where PRCs may be recruited by the core transcription factors such as *Oct3/4* [112, 113]. Repression of both *Ring1A* and *Ring1B*, the components of PRC1, results in complete differentiation of ES cells [112], whereas repression of either *Suz12* or *Eed*, the components of PRC2, derepresses the expression of the target genes and compromises differentiation capability [114, 115], indicating that PRCs are necessary for maintenance and execution of pluripotency [20]. Target regulation by PRCs involves the histone H2A variant H2AZ, which in general is implicated from yeast to human in many DNA-mediated processes, including gene regulation [116]. In ES cells, H2AZ and PRCs co-occupy the promoter regions of developmentally regulated genes, which are derepressed in the H2AZ-repressed ES cells, as seen with either *Suz12* or *Eed*, resulting in failure to undergo differentiation [117]. These target genes often have a "bivalent" chromatin structure, consisting of both H3K4me3 and H3K27me3 and poised for the initiation of expression [113, 117–119]. In fact, at the promoter regions, the bivalent gene RNA polymerase II (RNAP) complexes are assembled and phosphorylated on Ser 5, which marks inactive or poised genes, and are actually transcribed at low levels [120]. The mechanism of the repression involves histone H2A ubiquitination by PRC1, as repression of *Ring1A* and *Ring1B* results in the sequential loss of H2A ubiquitination, the release of poised RNAP, and subsequent gene derepression [120].

CpG methylation is also known to play important roles in epigenetic gene silencing and development. CpG DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, coordinately regulate CpG methylation in the genome [121]. In ES cells, CpG methylation is dispensable to the maintenance of pluripotency, as triple knockout ES cells (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) are capable of self-renewal [122]. However, a subset of developmentally regulated genes that are methylated at promoters in wild-type mES cells are derepressed in the triple knockout ES cells, while these genes lack either bivalent chromatin structure or association with PRC components or core transcription factors. These findings suggest that, within the promoter region of ES cells, CpG methylation represents a distinct epigenetic program that complements other regulatory mechanisms to ensure appropriate gene expression [123]. On the other hand, in distant regions (outside the promoters) such as the highly conserved non-coding elements (HCNE), which tend to be regulatory regions of developmentally regulated genes carrying a bivalent chromatin structure [118], the CpG methylation level is differentially regulated during cellular differentiation, probably regulating the accessibility of transcription factors to ensure the transcriptional network [124].

In addition to regulation in genic regions, transcription in ES cells is regulated at a genome-wide level. Intergenic sequences are broadly transcribed to express large intervening non-coding RNAs (lincRNAs), evolutionally conserved multi-exonic RNAs [125]. There are more than a thousand lincRNAs among different cell types. In ES cells, the core transcription factors regulate the expression of some lincRNAs, which play a role in pluripotency maintenance and differentiation [125]. Moreover, the synthesis of global mRNA is enhanced in ES cells. Transcriptional hyperactivity is accomplished by the elevated expression of chromatin-remodeling genes, such as *Smarca4* and the general transcription machinery including TATA box-binding protein (*Tbp*) [126]. In fact, normally silent repeat regions are active in ES cells, and tissue-specific genes are sporadically expressed at low levels [126]. The mechanism for regulation of these normally repressed regions may involve the proteasome system. Inhibition of proteasome activity by either chemical (MG132) or siRNA targeting to 26S proteasome components (such as ß4) increases the binding of transcription factors and RNAP in regulatory regions of normally repressed genes in ES cells, resulting in the activation of cryptic promoters in intergenic regions. This suggests that these factors restrict permissive transcriptional activity while keeping the genes in a potentiated state, ready for activation at later stages via assembly of the 26S proteasome [127].

This hyperactive transcription in ES cells might be supported by the hyperdynamic chromatic structure, which is potentially permissive of transcription. There is loose binding of the heterochromatin component HP1, the linker histone H1, and core histones, and these factors display

highly dynamic movement in ES cells as compared to differentiated cells [128]. Repression of histone cell cycle regulation defective homolog A (Hira), the nucleosome assembly factor, results in elevated levels of unbound histones, and differentiation is accelerated. In contrast, differentiation arrest is displayed in ES cells expressing either mutant H1 with increased chromatin-binding capacity or shRNA targeting *Chd1* that is required for maintain euchromatin, indicating that the hyperdynamic chromatin structure is essential to pluripotency maintenance [128, 129]. Widespread in ES cells, transcription becomes restricted as differentiation proceeds. This may involve the formation of large organized chromatin K9 modifications (LOCKs), the histone H3 lysine 9 dimethylation (H3K9me2, repressive histone mark) enriched regions encompassing up to several mega bases [130]. The formation of LOCKs is a function of euchromatic histone lysine *N*-methyltransferase 2 (Ehmt2, also known as G9a), which catalyzes the methylation of H3K9 [130, 131].

## 7 Regulations at post-transcriptional level

Translation of transcripts is regulated in ES cells. At the global level, ribosome loading on transcripts increases during differentiation, thereby enhancing translational efficiency through signaling pathways including the mammalian target of rapamycin (mTOR; a downstream effecter for PI3K/Akt-mediated regulation). This suggests that the protein synthesis capacity in undifferentiated ES cells is poised to allow rapid elevation of translation rate in response to differentiation signals [132]. At specific mRNA levels, the expression of the core transcription factor proteins is regulated by microRNA (miRNA). The expression of *miR-134*, *miR-296* and *miR-470* is upregulated upon differentiation induction, to target the coding sequence of *Oct3/4*, *Sox2*, and *Nanog* mRNA leading to the downregulation of the protein (and/or mRNA) levels, probably to ensure the progress of the differentiation process [133]. The expression of these developmentally regulated miRNAs is repressed by PRCs and the core transcription factors, as seen in the transcription factors that are developmentally regulated. The core transcription factors also promote a subset of miRNA expression, such as *miR-290* and *miR-302*, through which they fine tune the expression level of components of pluripotency maintenance and proliferation [134]. The developmentally regulated miRNAs include let-7, which regulates a diverse process including differentiation and proliferation through translational regulation of the various target mRNAs [135]. The processing/maturation of let-7 is blocked by *Lin28* [136, 137], which is capable of establishing human induced pluripotent stem (iPS) cells in conjunction with *Oct3/4*, *Sox2*, and *Nanog* [138], suggesting that let-7 negatively regulates pluripotency maintenance.

The activity of *Oct3/4* and *Sox2* is regulated by the nuclear import pathway. Nuclear proteins are known to be selectively imported into the nucleus by transport factors, such as karyopherin (importin) [139]. In ES cells, *Oct3/4* is selectively imported into the nucleus by *Kpna1* (karyopherin alpha 1), whereas *Xpo4* (exportin 4), another member of Ran-regulated nuclear transport receptors including karyopherin, facilitates nuclear import of *Sox2*, contributing to pluripotency maintenance [140].

The pluripotency machinery consisting of the core transcription factors should disappear precisely on schedule during the course of differentiation to ensure the establishment of a transcriptional network of differentiated cells. Upon induction of differentiation, the activity of Caspase-3 is upregulated to cleave the substrates including *Nanog* and presumably *Ronin* [98, 141]. Forced expression of the constitutive active *Casp3* (the gene of Caspase-3) induces differentiation, whereas Casp3 knockout ES cells become refractory to differentiation stimulus, indicating that the regulation of caspase activity is essential in both pluripotency maintenance and initiation of differentiation [141].

## 8 Transcriptional network to maintain pluripotency

How do these factors maintain pluripotency in the system as a whole? Genome-wide studies have revealed that *Oct3/4*, *Sox2*, *Nanog*, *Klf4*, *Tcf3*, and *Stat3* tend to co-occupy the target genes associated with cellular differentiation status, whereas genes involved in the proliferation process seem to be co-occupied by a distinct group of transcription factors including *Zfx* and *Myc* [92, 142, 143]. With greater binding of the transcription factors categorized in the former group, the target genomic regions tend to carry enriched H3K4me3 to function as an ES cell-specific enhancer, possibly corresponding to those substances previously identified as Oct–Sox enhancers, while binding of a single transcription factor marks the repressive transcriptional state, H3K27me3 (Fig. 2) [142, 143]. This mechanism may involve recruitment of PRCs and/or several other protein complexes that contribute to activating/repressing activities of the core transcription factors as described above. The expression of these epigenetic factors, including PRC components and histone modification enzymes, is regulated by the core transcription factors to self-stabilize an entire molecular network consisting of transcriptome and epigenome. The example studied in detail includes *Jmjd1a* and *Jmjd2c*, histone H3K9me2 and H3K9me3 (repressive marks) demethylase genes, whose expression is regulated by *Oct3/4*, thereby promoting the expression of the target genes including *Tcl1* and *Nanog*, respectively [13, 14].

## 9 Reprogramming

To date, several different approaches can be applied to the reprogramming of somatic cells to a pluripotent state [144, 145]. Briefly, a somatic cell nucleus transferred to an unfertilized egg acquires pluripotency and develops into a blastocyst, allowing cloned ES cells to be established. Alternatively, somatic cells can be reprogrammed by cell–cell hybridization (cell fusion). The disadvantages of these techniques are the destruction of the embryo (i.e. ethical problem) and the tetraploid karyotype of the fused cells (i.e. risk problem in transplantation), respectively. Because these problems can be avoided using iPS cells, most recent reprogramming studies have utilized iPS cell systems.

In iPS induction, *Oct3/4*, *Sox2*, *Klf4*, and *Myc* (4 factors) are simultaneously introduced into somatic cells, and the reprogrammed cells are selected by marker gene expression and/or morphology [146]. Retroviruses were initially used to introduce the four factors, but the resulting iPS cells showed numerous insertions in the chromosome, raising safety issues. Currently, iPS cells can be established by a DNA-free method, in which the proteins of the four factors are tagged with an arginine stretch that confers membrane permeability [147, 148]. The cell types in which successful reprogramming have been reported include fibroblasts, primary hepatocytes, and completely differentiated B lymphocytes, suggesting that all the cell types in the body can be reprogrammed using the iPS technique [149–151]. The progression of reprogramming in iPS induction is relatively slow when compared with other techniques. In cell fusion, the upregulation of *Oct3/4* in completely differentiated cells is observed within 2 days [152], whereas in the iPS process this upregulation is first detectable 16 days after induction [153].

The molecular mechanism of this reprogramming is not clear at present. The efficiency of iPS establishment is low, less than a few percent of cells treated [154], indicating that in most cells the reprogramming is aborted even in the presence of the four factors. The function of Myc for iPS induction is dispensable, although it enhances the efficiency of the iPS establishment, probably through repressing the expression of differentiated cell-specific genes while promoting binding of Oct3/4, Sox2, and Klf4 (OSK) to their target genes [75, 155]. In fact, in partially reprogrammed cells OSK does not bind to the target genes (which are thus not expressed), suggesting that the cellular environment ensuring access of these factors to the target genes is rate limiting in reprogramming [155]. In addition, administration of chemical inhibitors targeting epigenetic factors that are associated with transcriptional repression is effective for enhancing iPS cell induction. These inhibitors include BIX-01294 (G9a inhibitor) [156], AZA (5-aza-cytidine, Dnmts inhibitor) [154, 157], VPA (valproic acid, Hdac inhibitor) [158], and TSA (trichostatin A, Hdac inhibitor) [158], and such findings suggest that target accessibility accompanying the global transcriptional activation seen in ES cells is critical to the initiation of the pluripotency transcriptional network. During iPS induction, OSK activity is enhanced by the transcription factors known to co-regulate with OSK, which include Esrrb, Sall4 and Tcf3 (via Wnt signaling), since forced expression of those factors can enhance the efficiency of iPS induction [80, 142, 159–161].

Collectively, the mechanism of reprogramming in iPS induction can be hypothesized as follows. Upon introduction of the four factors, the endogenous genes for the transcription factors necessary to pluripotency are primed and gradually induced to express through the regulatory region targeted by OSK. Subsequently, the transcriptional circuit begins to self-stabilize via increasing expression of the endogenous core transcription factors, through a positive-feedback loop, while repressing the developmentally regulated genes through recruitment of epigenetic factors and various protein complexes such as Paf1C and NODE. Once the stable transcriptional network is established/self-stabilized, exogenous cDNA expression is no longer necessary, and iPS cells indistinguishable from ES cells can be obtained after selection based on the expression of endogenous *Oct3/4* and/or *Nanog*. This outcome is the result of concerted action by a group of molecules that play a central role in pluripotency.

## 10 Conclusion

Pluripotency of ES cells is externally regulated through several molecules, including Wnt and Lif, whose signaling pathway activates transcription factor genes such as *Klf4* and *Nanog* in the nucleus. The core transcription factors, including Oct3/4, Sox2, and Klf4 positively self-regulate while also repressing developmentally regulated genes by co-occupation with a variety of protein complexes. Introduction of *Oct3/4*, *Sox2*, and *Klf4* into the somatic cells gradually reconstitutes the above transcriptional network with the aid of Myc and epigenetic modifiers, which might allow the regulatory regions of the target genes more access to these transcription factors.

**Acknowledgments** I thank Dr. Mitsuhiro Endoh for his helpful suggestions. This work was supported in part by the PRESTO program of the Japan Science and Technology Agency and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. This work is also supported in part by the Grants-in-Aid for Scientific research from the Ministry of Health, Labor, Welfare of Japan, and by that of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References


1. Solter D. From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genet. 2006;7:319–27.
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. 1981;78:7634–8.
3. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154–6.
4. Brons IG, Smithers LE, Trotter MW, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature. 2007;448:191–5.
5. Tesar PJ, Chenoweth JG, Brook FA, et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature. 2007;448:196–9.
6. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
7. Guo G, Yang J, Nichols J, et al. Klf4 reverts developmentally programmed restriction of ground state pluripotency. Development. 2009;136:1063–9.
8. Chou YF, Chen HH, Eijpe M, et al. The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. Cell. 2008;135:449–61.
9. Ogawa K, Matsui H, Ohtsuka S, Niwa H. A novel mechanism for regulating clonal propagation of mouse ES cells. Genes Cells. 2004;9:471–7.
10. Abdelalim EM, Tooyama I. BNP signaling is crucial for embryonic stem cell proliferation. PLoS ONE. 2009;4:e5341.
11. Andang M, Hjerling-Leffler J, Moliner A, et al. Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation. Nature. 2008;451:460–4.
12. Takahashi K, Mitsui K, Yamanaka S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature. 2003;423:541–5.
13. Matoba R, Niwa H, Masui S, et al. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. PLoS ONE. 2006;1:e26.
14. Loh YH, Zhang W, Chen X, George J, Ng HH. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev. 2007;21:2545–57.
15. Ema M, Mori D, Niwa H, et al. Kruppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs. Cell Stem Cell. 2008;3:555–67.
16. Tanaka Y, Patestos NP, Maekawa T, Ishii S. B-*myb* is required for inner cell mass formation at an early stage of development. J Biol Chem. 1999;274:28067–70.
17. Iwai N, Kitajima K, Sakai K, Kimura T, Nakano T. Alteration of cell adhesion and cell cycle properties of ES cells by an inducible dominant interfering Myb mutant. Oncogene. 2001;20:1425–34.
18. Tarasov KV, Tarasova YS, et al. B-MYB is essential for normal cell cycle progression and chromosomal stability of embryonic stem cells. PLoS ONE. 2008;3:e2478.
19. Takahashi K, Murakami M, Yamanaka S. Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. Biochem Soc Trans. 2005;33:1522–5.
20. Niwa H. How is pluripotency determined and maintained? Development. 2007;134:635–46.
21. Smith AG, Heath JK, Donaldson DD, et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature. 1988;336:688–90.
22. Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol. 2002; 12:432–8.
23. Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development. 2005;132:885–96.
24. Niwa H, Ogawa K, Shimosato D, Adachi K. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature. 2009;460:118–22.
25. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 1998;12:2048–60.
26. Matsuda T, Nakamura T, Nakao K, et al. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J. 1999;18:4261–9.
27. Paling NR, Wheadon H, Bone HK, Welham MJ. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J Biol Chem. 2004;279:48063–70.
28. Ivanova N, Dobrin R, Lu R, et al. Dissecting self-renewal in stem cells with RNA interference. Nature. 2006;442:533–8.
29. Wu MY, Hill CS. Tgf-beta superfamily signaling in embryonic development and homeostasis. Dev Cell. 2009;16:329–43.
30. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell. 2003;115: 281–92.
31. Ogawa K, Saito A, Matsui H, et al. Activin-nodal signaling is involved in propagation of mouse embryonic stem cells. J Cell Sci. 2007;120:55–65.
32. Toyooka Y, Shimosato D, Murakami K, Takahashi K, Niwa H. Identification and characterization of subpopulations in undifferentiated ES cell culture. Development. 2008;135:909–18.
33. Willert K, Jones KA. Wnt signaling: is the party in the nucleus? Genes Dev. 2006;20:1394–404.
34. Kielman MF, Rindapaa M, Gaspar C, et al. *Apc* modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. Nat Genet. 2002;32:594–605.
35. Hao J, Li TG, Qi X, Zhao DF, Zhao GQ. WNT/beta-catenin pathway up-regulates Stat3 and converges on LIF to prevent differentiation of mouse embryonic stem cells. Dev Biol. 2006;290:81–91.
36. Ogawa K, Nishinakamura R, Iwamatsu Y, Shimosato D, Niwa H. Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells. Biochem Biophys Res Commun. 2006;343:159–66.
37. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med. 2004;10:55–63.
38. Ying QL, Wray J, Nichols J, et al. The ground state of embryonic stem cell self-renewal. Nature. 2008;453:519–23.
39. Kunath T, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development. 2007;134:2895–902.
40. Cheng AM, Saxton TM, Sakai R, et al. Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell. 1998;95:793–803.
41. Rosner MH, Vigano MA, Ozato K, et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature. 1990;345:686–92.
42. Scholer HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P. New type of POU domain in germ line-specific protein Oct-4. Nature. 1990;344:435–9.
43. Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M, Hamada H. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell. 1990;60:461–72.

44. Niwa H, Masui S, Chambers I, Smith AG, Miyazaki J. Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. Mol Cell Biol. 2002;22:1526–36.
45. Palmieri SL, Peter W, Hess H, Scholer HR. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev Biol. 1994;166:259–67.
46. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998;95:379–91.
47. Yeom YI, Fuhrmann G, Ovitt CE, et al. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development. 1996;122:881–94.
48. Okumura-Nakanishi S, Saito M, Niwa H, Ishikawa F. Oct-3/4 and Sox2 regulate *Oct-3/4* gene in embryonic stem cells. J Biol Chem. 2005;280:5307–17.
49. Gu P, Goodwin B, Chung AC, et al. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. Mol Cell Biol. 2005;25:3492–505.
50. Barnea E, Bergman Y. Synergy of SF1 and RAR in activation of *Oct-3/4* promoter. J Biol Chem. 2000;275:6608–19.
51. Ben-Shushan E, Sharir H, Pikarsky E, Bergman Y. A dynamic balance between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and retinoic acid receptor:retinoid X receptor heterodimers regulates *Oct-3/4* expression in embryonal carcinoma cells. Mol Cell Biol. 1995;15:1034–48.
52. Fuhrmann G, Chung AC, Jackson KJ, et al. Mouse germline restriction of *Oct4* expression by germ cell nuclear factor. Dev Cell. 2001;1:377–87.
53. Niwa H, Toyooka Y, Shimosato D, et al. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell. 2005;123:917–29.
54. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 2003;17:126–40.
55. Ambrosetti DC, Scholer HR, Dailey L, Basilico C. Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. J Biol Chem. 2000;275:23387–97.
56. Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev. 1995;9:2635–45.
57. Nishimoto M, Fukushima A, Okuda A, Muramatsu M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol. 1999;19:5453–65.
58. Tomioka M, Nishimoto M, Miyagi S, et al. Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic Acids Res. 2002;30:3202–13.
59. Tokuzawa Y, Kaiho E, Maruyama M, et al. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol. 2003;23:2699–708.
60. Rodda DJ, Chew JL, Lim LH, et al. Transcriptional regulation of Nanog by OCT4 and SOX2. J Biol Chem. 2005;280:24731–7.
61. Kuroda T, Tada M, Kubota H, et al. Octamer and Sox elements are required for transcriptional *cis* regulation of *Nanog* gene expression. Mol Cell Biol. 2005;25:2475–85.
62. Nakatake Y, Fukui N, Iwamatsu Y, et al. Klf4 cooperates with Oct3/4 and Sox2 to activate the *Lefty1* core promoter in embryonic stem cells. Mol Cell Biol. 2006;26:7772–82.
63. Maruyama M, Ichisaka T, Nakagawa M, Yamanaka S. Differential roles for Sox15 and Sox2 in transcriptional control in mouse embryonic stem cells. J Biol Chem. 2005;280:24371–9.
64. Masui S, Nakatake Y, Toyooka Y, et al. Pluripotency governed by *Sox2* via regulation of *Oct3/4* expression in mouse embryonic stem cells. Nat Cell Biol. 2007;9:625–35.
65. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003;113:631–42.
66. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. 2003;113:643–55.
67. Wang J, Levasseur DN, Orkin SH. Requirement of Nanog dimerization for stem cell self-renewal and pluripotency. Proc Natl Acad Sci USA. 2008;105:6326–31.
68. Pan GJ, Pei DQ. Identification of two distinct transactivation domains in the pluripotency sustaining factor Nanog. Cell Res. 2003;13:499–502.
69. Chambers I, Silva J, Colby D, et al. Nanog safeguards pluripotency and mediates germline development. Nature. 2007;450:1230–4.
70. Fujikura J, Yamato E, Yonemura S, et al. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev. 2002;16:784–9.
71. Chazaud C, Yamanaka Y, Pawson T, Rossant J. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev Cell. 2006;10:615–24.
72. McConnell BB, Ghaleb AM, Nandan MO, Yang VW. The diverse functions of Kruppel-like factors 4 and 5 in epithelial biology and pathobiology. Bioessays. 2007;29:549–57.
73. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet. 1999;22:356–60.
74. Jiang J, Chan YS, Loh YH, et al. A core Klf circuitry regulates self-renewal of embryonic stem cells. Nat Cell Biol. 2008;10:353–60.
75. Nakagawa M, Koyanagi M, Tanabe K, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol. 2008;26:101–6.
76. Cole MD, Cowling VH. Transcription-independent functions of MYC: regulation of translation and DNA replication. Nat Rev Mol Cell Biol. 2008;9:810–5.
77. de Celis JF, Barrio R. Regulation and function of Spalt proteins during animal development. Int J Dev Biol. 2009;53:1385–98.
78. Elling U, Klasen C, Eisenberger T, Anlag K, Treier M. Murine inner cell mass-derived lineages depend on Sall4 function. Proc Natl Acad Sci USA. 2006;103:16319–24.
79. Sakaki-Yumoto M, Kobayashi C, Sato A, et al. The murine homolog of *SALL4*, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with *Sall1* in anorectal, heart, brain and kidney development. Development. 2006;133:3005–13.
80. Tsubooka N, Ichisaka T, Okita K, Takahashi K, Nakagawa M, Yamanaka S. Roles of Sall4 in the generation of pluripotent stem cells from blastocysts and fibroblasts. Genes Cells. 2009;14:683–94.
81. Zhang J, Tam WL, Tong GQ, et al. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of *Pou5f1*. Nat Cell Biol. 2006;8:1114–23.
82. Yuri S, Fujimura S, Nimura K, et al. *Sall4* is essential for stabilization, but not for pluripotency, of embryonic stem cells by repressing aberrant trophectoderm gene expression. Stem cells. 2009;27:796–805.
83. Lu J, Jeong H, Kong N, et al. Stem cell factor SALL4 represses the transcriptions of PTEN and SALL1 through an epigenetic repressor complex. PLoS ONE. 2009;4:e5577.

84. Luo J, Sladek R, Bader JA, Matthyssen A, Rossant J, Giguere V. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. Nature. 1997;388:778–82.
85. Hermanson O. Stem cells have different needs for REST. PLoS Biol. 2008;6:e271.
86. Singh SK, Kagalwala MN, Parker-Thornburg J, Adams H, Majumder S. REST maintains self-renewal and pluripotency of embryonic stem cells. Nature. 2008;453:223–7.
87. Jorgensen HF, Terry A, Beretta C, et al. REST selectively represses a subset of RE1-containing neuronal genes in mouse embryonic stem cells. Development. 2009;136:715–21.
88. Buckley NJ, Johnson R, Sun YM, Stanton LW. Is REST a regulator of pluripotency? Nature. 2009;457:E5–6 (discussion E7).
89. Jorgensen HF, Chen ZF, Merkenschlager M, Fisher AG. Is REST required for ESC pluripotency? Nature. 2009;457:E4–5 (discussion E7).
90. Hoppler S, Kavanagh CL. Wnt signalling: variety at the core. J Cell Sci. 2007;120:385–93.
91. Pereira L, Yi F, Merrill BJ. Repression of *Nanog* gene transcription by Tcf3 limits embryonic stem cell self-renewal. Mol Cell Biol. 2006;26:7479–91.
92. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev. 2008;22:746–55.
93. Yi F, Pereira L, Merrill BJ. Tcf3 functions as a steady-state limiter of transcriptional programs of mouse embryonic stem cell self-renewal. Stem cells. 2008;26:1951–60.
94. Tam WL, Lim CY, Han J, et al. T cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. Stem cells. 2008;26:2019–31.
95. Galan-Caridad JM, Harel S, Arenzana TL, et al. Zfx controls the self-renewal of embryonic and hematopoietic stem cells. Cell. 2007;129:345–57.
96. Macfarlan T, Kutney S, Altman B, Montross R, Yu J, Chakravarti D. Human THAP7 is a chromatin-associated, histone tail-binding protein that represses transcription via recruitment of HDAC3 and nuclear hormone receptor corepressor. J Biol Chem. 2005;280:7346–58.
97. Clouaire T, Roussigne M, Ecochard V, Mathe C, Amalric F, Girard JP. The THAP domain of THAP1 is a large C2CH module with zinc-dependent sequence-specific DNA-binding activity. Proc Natl Acad Sci USA. 2005;102:6907–12.
98. Dejosez M, Krumenacker JS, Zitur LJ, et al. Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. Cell. 2008;133:1162–74.
99. Wysocka J, Myers MP, Laherty CD, Eisenman RN, Herr W. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev. 2003;17:896–911.
100. Wang J, Rao S, Chu J, et al. A protein interaction network for pluripotency of embryonic stem cells. Nature. 2006;444:364–8.
101. Niakan KK, Davis EC, Clipsham RC, et al. Novel role for the orphan nuclear receptor Dax1 in embryogenesis, different from steroidogenesis. Mol Genet Metab. 2006;88:261–71.
102. Lauberth SM, Rauchman M. A conserved 12-amino acid motif in Sall1 recruits the nucleosome remodeling and deacetylase corepressor complex. J Biol Chem. 2006;281:23922–31.
103. Kaji K, Caballero IM, MacLeod R, Nichols J, Wilson VA, Hendrich B. The NuRD component Mbd3 is required for pluripotency of embryonic stem cells. Nat Cell Biol. 2006;8:285–92.
104. Liang J, Wan M, Zhang Y, et al. Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nat Cell Biol. 2008;10:731–9.
105. Ding L, Paszkowski-Rogacz M, Nitzsche A, et al. A genome-scale RNAi screen for Oct4 modulators defines a role of the Paf1 complex for embryonic stem cell identity. Cell Stem Cell. 2009;4:403–15.
106. Warner MH, Roinick KL, Arndt KM. Rtf1 is a multifunctional component of the Paf1 complex that regulates gene expression by directing cotranscriptional histone modification. Mol Cell Biol. 2007;27:6103–15.
107. Fazzio TG, Huff JT, Panning B. An RNAi screen of chromatin proteins identifies Tip60–p400 as a regulator of embryonic stem cell identity. Cell. 2008;134:162–74.
108. Tyteca S, Vandromme M, Legube G, Chevillard-Briet M, Trouche D. Tip60 and p400 are both required for UV-induced apoptosis but play antagonistic roles in cell cycle progression. EMBO J. 2006;25:1680–9.
109. Ho L, Ronan JL, Wu J, et al. An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency. Proc Natl Acad Sci USA. 2009;106:5181–6.
110. Ho L, Jothi R, Ronan JL, Cui K, Zhao K, Crabtree GR. An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. Proc Natl Acad Sci USA. 2009;106:5187–91.
111. Kohler C, Villar CB. Programming of gene expression by polycomb group proteins. Trends Cell Biol. 2008;18:236–43.
112. Endoh M, Endo TA, Endoh T, et al. Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development. 2008;135:1513–24.
113. Boyer LA, Plath K, Zeitlinger J, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature. 2006;441:349–53.
114. Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol. 2007;27:3769–79.
115. Chamberlain SJ, Yee D, Magnuson T. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem cells. 2008;26:1496–505.
116. Draker R, Cheung P. Transcriptional and epigenetic functions of histone variant H2A.Z. Biochem Cell Biol. 2009;87:19–25.
117. Creyghton MP, Markoulaki S, Levine SS, et al. H2AZ is enriched at polycomb complex target genes in ES cells and is necessary for lineage commitment. Cell. 2008;135:649–61.
118. Bernstein BE, Mikkelsen TS, Xie X, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006;125:315–26.
119. Azuara V, Perry P, Sauer S, et al. Chromatin signatures of pluripotent cell lines. Nat Cell Biol. 2006;8:532–8.
120. Stock JK, Giadrossi S, Casanova M, et al. Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol. 2007;9:1428–35.
121. Bogdanovic O, Veenstra GJ. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosome 2009;118:549–65.
122. Tsumura A, Hayakawa T, Kumaki Y, et al. Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. Genes Cells. 2006;11:805–14.
123. Fouse SD, Shen Y, Pellegrini M, et al. Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell. 2008;2:160–9.
124. Meissner A, Mikkelsen TS, Gu H, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature. 2008;454:766–70.
125. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009;458:223–7.