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MOLECULAR MECHANISMS OF MOUSE EMBRYONIC STEM
CELL DIFFERENTIATION

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MOLECULAR MECHANISMS OF MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

by

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Dedication

I dedicated this work to

my parents, who showed me the right way to live and encouraged to think independently,

for their endless love and support;

my beloved wife, Hae-Ywon, who always gave me wise advice whenever I need honest

opinion.

I appreciate all your love and support to finish my PhD.

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MOLECULAR MECHANISMS OF MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

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Mouse embryonic stem (ES) cells are pluripotent cells, meaning that they can give rise to all tissues in the body. This has catalyzed research in both early embryogenesis as a model system for mammalian development as well as regenerative medicine as a renewable source of unspecialized cells which can be converted into nearly any cell type required by a patient. ES cells have been an invaluable resource for advancing fundamental understanding of global transcriptional and epigenetic regulations, signaling pathways, and noncoding RNA in mammalian systems. However, the molecular mechanisms of how ES cells are differentiated remain much less understood.

Differentiation is a complex process involving actions of ES cell core factors, lineage specific regulators, epigenetic modifications, and chromatin remodelers. Thus, a single reporter-based screen would have been inappropriate to identify novel regulators of ES cell differentiation. To overcome the problems, we have developed a unique signature-based screen. This screen is capable of analyzing the expression of 48 genes simultaneously across dozens of different samples, and our gene list covers all three germ layers that arise during normal embryonic development, the trophectoderm, and epigenetic regulators of chromatin status. Our signature-based screen established several categories of genes based

on their comparative functions during the differentiation of ES cells. This will be a valuable information for other researchers interested in ES cell differentiation from various perspectives.

We have identified two novel regulators of ES cell differentiation – Yap1 and Rbpj. Yap1 is a transcriptional co-activator of Hippo signaling pathway. We disproved past misconceptions in the field about the role of Yap1 concerning its function in ES cell self-renewal, showing that like the inner cell mass, Yap1 is dispensable for long-term maintenance in culture. Conversely, we found that Yap1 is essential for proper ES cell differentiation. Rbpj is a transcriptional regulator of Notch signaling pathway. Consistent with previous observations of repressive role of Rbpj, Rbpj serves as a repressor of ES cell core factors in the absence of Notch signaling pathway. Repressive role of Rbpj is also required for proper differentiation of ES cells by silencing core factors.

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CHAPTER 1: INTRODUCTION

ESTABLISHMENT OF MOUSE EMBRYONIC STEM (ES) CELLS

In the 1960s, several studies reported the existence of a population of pluripotent cells in mice with the ability to differentiate into the three germ layers, endoderm, mesoderm, and ectoderm (Gardner, 1968; Kahan and Ephrussi, 1970; Pierce and Verney, 1961; Rosenthal et al., 1970). These pluripotent cells were initially identified in spontaneously occurring testicular teratomas in an inbred strain of mice (Stevens and Little, 1954). The teratomas contained a range of differentiated tissues that could be transplanted into other mice. Cells isolated from teratomas are called embryonic carcinoma (EC) cells, and they can grow in tissue culture. EC cells can contribute to generate chimeric mice when injected into a blastocyst, demonstrating their pluripotency, and they can be induced to differentiate *in vitro* through the formation of an embryonic body, which resembles early embryogenesis. EC cells can also form teratomas when injected back into mice, allowing scientists to observe embryonic development *in vivo*.

However, all EC cell lines have an abnormal karyotype, underscoring the need to identify pluripotent cells with normal karyotypes. In 1981, the British scientists M. J. Evans and M. H. Kaufman reported that they had successfully established pluripotent stem cells derived from the inner cell mass (ICM) of the developing blastocyst (Evans and Kaufman, 1981). The established cell lines, which were named EK cells after the initials of the discoverers, are karyotypically normal and exhibit essentially the same characteristics as EC cells, namely, the ability to form a teratocarcinoma *in vivo*, differentiation into an embryonic body *in vitro*, and contributing towards the generation of chimeric mice when injected back into the mouse blastocyst. EK cells, which are currently referred to as embryonic stem (ES) cells, possess the advantages of EC cells for studying early

development; however, they can also serve as a genetic tool to study individual genes *in vivo*. Shortly after the establishment of ES cells, chimeric mice containing disrupted genes or transgenes were generated to examine the functions of individual genes *in vivo* (Hooper et al., 1987; Kuehn et al., 1987).

IMPORTANCE OF STUDYING ES CELLS

The establishment of ES cell lines attracted the attention of scientific and clinical communities because of their two unique characteristics, self-renewal and pluripotency (reviewed in Young, 2011). Self-renewal is the process by which stem cells divide into daughter cells while maintaining the undifferentiated state, and pluripotency is the potential to differentiate into almost all cell types. Indefinite proliferation provides an unlimited source of normal cells that can be induced to differentiate into virtually all cell types. This is especially useful when specific cells are not vastly available or are difficult to culture *in vitro*. For example, testing the effect of drugs on heart cells is difficult because of the lack of a human heart cell line. However, ES cell-derived heart cells provide an effective platform to test and study the effects and toxicity of drugs on heart cells (He, 2003; Mummery, 2003). In addition, the pluripotency of ES cells is critical for the development of regenerative medicine technologies and to help understand early embryogenesis.

Development of regenerative medicine

Regenerative medicine is the process of creating functional cells or tissues to replace or repair tissues or organs damaged by age, disease, or injury. Several regenerative medicine-based therapies have received FDA approval and are commercially available. However, many of these therapies use tissues or cells derived from patients, which creates another wound, and their use is often delayed by the need to expand the cells in culture (Falanga and Sabolinski, 1999). ES cells are a suitable alternative because they provide an

ample source of cells that can differentiate into any type of cell. One notable example is a clinical trial by Advanced Cell Technology, in which human ES cell-derived retinal pigmented epithelial cells are used for the treatment of patients with Stargardt macular dystrophy (Schwartz et al., 2015). The preliminary results of the trial are promising, and no negative side effects were found. In addition, most phase 1 clinical trials of regenerative medicine passed the toxicity test, suggesting a bright future for this method (Alexey, 2015).

Insights into early embryogenesis

The pluripotency of ES cells provides developmental biologists with a model to study early embryonic development. Studying the differentiation of ES cells provides valuable information about early developmental stages that are difficult to access in the embryo. For example, ES cells were used to test the long-standing hypothesis that hematopoietic and endothelial lineages originate from a common progenitor (Haar and Ackerman, 1971). Studies show that the blood and endothelial lineages develop in close proximity at the same time in yolk sac blood islands, and immature hematopoietic and vascular cells share many gene expression patterns (Watt et al., 1995; Young et al., 1995). However, formal proof that a progenitor exists was first provided by the study of ES cell differentiation. Analysis of differentiated ES cells identified progenitor cells known as blast colony-forming cells (BL-CFCs), which can develop into hematopoietic and vascular progenitors (Choi et al., 1998; Nishikawa et al., 1998). A similar progenitor with the potential to develop into both hematopoietic and vascular lineages was later identified, demonstrating that the *in vitro* ES cell model can provide insight into early embryonic development (Huber et al., 2004).

On the other hand, the development of specific lineages from ES cells is often guided by the knowledge gained from embryonic development. The development of a

cardiac lineage from ES cells results in a heterogeneous population that needs to be subsequently purified to generate a homogenous cardiac cell population (Klug et al., 1996). Established knowledge of cardiomyocyte markers such as α-cardiac MHC in the developing embryo allows the generation of ES cells with selection markers under the control of a cardiomyocyte promoter. The selection of ES cells at the appropriate developmental stage enables the isolation of highly enriched cardiomyocyte populations (Zandstra et al., 2003). Understanding the differentiation mechanisms of ES cells leads to greater understanding of early developmental biology and vice versa.

Insights into gene regulation

Because of the initial limitation of establishing ES cell lines, most ES cell studies were conducted in a nearly identical genetic background. This situation creates a uniform environment for genomics studies, which allows the safe integration of diverse datasets of ES cell lines for systematic analyses. For this reason, mouse ES cells are among the most widely studied mammalian model systems in studies of the global interactions between DNA, proteins, and RNA. For example, binding regions of more than 100 DNA-binding proteins, 10 different histone modifications, DNase-sensitive regions, DNA methylation, and long-range chromosomal interactions have been mapped to mouse ES cells. This was achieved using massive-parallel high-throughput sequencing combined with various genomic tools such as chromatin immunoprecipitation (ChIP), chromatin interaction analysis by paired-end tag (ChIA-PET), assay for transposase-accessible chromatin (ATAC), and Hi-C (reviewed in Beck et al., 2015). Integration of these data provides an extended view of global gene regulatory mechanisms that were previously unknown and provides a useful starting point for understanding global gene regulation in other cell types and species. For example, integrated analysis of transcription factor occupancy identified

transcriptional networks in which various transcription factors occupied the same genomic targets in predictable patterns. Groups of genomic targets occupied by functionally similar transcription factors became known as modules (Kim et al., 2008). Integration of genome-wide DNA methylation patterns with extended transcriptional networks revealed different modes of gene regulation dependent on the presence of CpG islands (Beck et al., 2014). Comparison of ES cells and terminally differentiated cell types revealed that entry into productive elongation from a poised state is a general mechanism by which RNA polymerase regulates transcription upon differentiation (Min et al., 2011). The general idea of transcriptional regulation gained from the study of ES cells provides insight into other systems such as tumorigenesis (Kim et al., 2010), highlighting the advantages of studying ES cells to examine general gene regulatory mechanisms.

REGULATORY MECHANISMS OF ES CELL SELF-RENEWAL

Unlike terminally differentiated cells, ES cells can proliferate indefinitely while maintaining the differentiation potential. Various studies focused on elucidating the mechanisms underlying the self-renewal nature of ES cells. In 1998, Pou5f1, a member of the POU transcription factor family, was shown to be required for the maintenance of both the ICM of mouse embryos and ES cells (Nichols et al., 1998). In the mouse blastocyst, Pou5f1 is present exclusively in the ICM and is not detected in the trophectoderm, which becomes the placenta (Palmieri et al., 1994). The expression of Pou5f1 is high in ES cells and decreases upon differentiation. Pou5f1-deficient embryos undergo trophectoderm development but they are unable to form the ICM (Nichols et al., 1998). Furthermore, Pou5f1-deficient ES cells are unable to maintain self-renewal.

In addition to Pou5f1, several transcription factors including Nanog, Sox2, Klf4, and Esrrb play essential roles in the maintenance of the self-renewal of ES cells (Chambers et al., 2003; Jiang et al., 2008; Masui et al., 2007; Mitsunaga et al., 2004). These factors, which are referred to as core factors, form a transcriptional regulatory circuit, transcriptionally activating each other and regulate co-occupied downstream target genes. Polycomb repressive complex (PRC), which was initially discovered for its role in the regulation of drosophila development, is involved in the maintenance of ES cell self-renewal by inhibiting the expression of lineage-specific genes (Boyer et al., 2006).

One of the long-term goals of studying ES cells is to manipulate cell fate to produce the desired cell types. Although many intrinsic transcription factors can effectively determine cell fate, they are not readily available for biomedical purposes. Instead, researchers study signaling pathways that can be easily manipulated by changing media conditions and adding small molecules. Using signaling pathways to differentiate ES cells requires an understanding of how each signaling pathway is involved in cell specification and how specific pathways can be manipulated. Several pathways including LIF/Stat, Gsk3/Erk, and Wnt/β-catenin play a role in the regulation of pluripotency (Huang et al., 2015; Okita and Yamanaka, 2006). In this manuscript, we focus on describing the signaling pathways that are relevant to this study.

Transcriptional regulation

Screening to identify novel regulators of core factors

The initial discovery of a handful of core factors required for the maintenance of ES cells stimulated researchers to perform screens to identify novel regulators of self-renewal. Many of those screens used reporter systems containing promoters of well-known

core factors such as Pou5f1 or Zfp42 driving the GFP gene. Small-scale or genome-wide knockdown (KD) screens with various RNAi techniques including shRNA, siRNA, or esiRNA identified many factors that negatively regulate reporter levels upon depletion, thus controlling the self-renewal of ES cells (Ding et al., 2009; Hu et al., 2009; Ivanova et al., 2006). For instance, a shRNA-mediated screen with 70 candidate genes that are downregulated upon differentiation identified the novel transcription factors Esrrb, Tbx3, and Tcl1, which are required for the maintenance of ES cell self-renewal. ES cells with deficiencies in each factor grow slowly and show loss of alkaline phosphatase activity, morphology changes, and the induction of lineage-specific markers, indicating that these factors are indispensable for the maintenance of ES cells. However, there was an issue with reproducibility in these screening trials. In fact, the results of multiple genome scale RNAi screens overlap by only 8%, although fortunately, many of the unique hits in each screen were later validated in independent studies (Cinghu et al., 2014). This can be partially attributed to differences in the RNAi methods and reporter systems used in each screen trial. However, the lack of concordance also indicates that genome-wide screens have not reached saturation, and other critical factors remain to be discovered. In this regard, bioinformatics techniques have been applied to integrate published screening studies to identify novel regulators of ES cell self-renewal, which resulted in the identification of novel regulators such as Nucleolin (Cinghu et al., 2014). Candidate genes identified in bioinformatics studies are then tested by siRNA to examine their roles in the self-renewal of ES cells.

Similar screens were performed with human ES cells. Some of the important regulators present in mouse ES cells such as Nanog, Tcl1, Zic3, and Zscan10 were rediscovered in human screens, indicating that human and mouse ES cells may share

similar transcriptional networks (Chia et al., 2010). However, the screens also identified novel regulators such as PRDM1 and NFRKB. KD of PRDM14 in human ES cells downregulates ES cell-associated genes such as POU5F1, NANOG, SOX2, and DPPA4. Analysis of global binding patterns of PRDM14 revealed that PRDM14 shares many targets with core factors such as POU5F1, NANOG, SOX2, and the co-activator p300. In mice, Prdm14 plays a critical role in the establishment of germ cells, although it is not required for the maintenance of mouse ES cells or early embryogenesis (Ma et al., 2011). Further analysis of Prdm14 revealed that it is required for the derivation of ES cells from the blastocyst. It would be interesting to understand the different roles of Prdm14 in mouse and human ES cells. Despite the fact that all the above factors are required to maintain the self-renewal of ES cells, most of the screens failed to identify factors that may work independently of known core factors. Integrating genome-wide RNAi screens with multiplexed high-throughput sequencing may help uncover novel regulators of ES cell identity.

Transcriptional networks of core factors

Advances in ChIP-sequencing techniques led to the identification of global targets of DNA-binding proteins including transcription factors and chromatin remodelers (Johnson et al., 2007). The discovery of downstream targets of core factors such as Pou5f1, Nanog, and Sox2 indicates that core factors co-occupy many gene promoters (Kim et al., 2008; Loh et al., 2006). In addition, each core factor binds to its own regulatory locus and those of other core factors, leading to feed-forward regulation. Further analysis of their downstream targets revealed that multiple factor binding increases gene expression levels in ES cells, and their functions are enriched in stem cell maintenance and the regulation of transcription (Kim et al., 2008). One notable example of co-bound targets of core factors

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is Sall4, which is regulated by many core factors including Pou5f1, Nanog, Sox2, Zfp42, and Dax1 (Yuri et al., 2009). An independent study showed that Sall4 is exclusively expressed in ES cells, and Sall4-deficient mice die shortly after implantation, emphasizing its role in early development (Sakaki-Yumoto, 2006). Furthermore, depletion of Sall4 results in reduced proliferation both in the blastocyst and ES cells. Another study demonstrated that Sall4 binds to the promoter region of Pou5f1 and activates its expression (Yuri et al., 2009). Therefore, multiple core factor binding can serve as a robust indication of the functions of target genes.

This notion was recently revisited by Whyte et al (Whyte et al., 2013). They coined the term super-enhancer, which defines large genomic domains occupied by multiple master transcription factors. Genes regulated by super-enhancers are essential for cell identity. The list of super-enhancer-associated genes includes Pou5f1, Sox1, Nanog, Esrrb, nMyc, Sall4, Prdm14, Dppa5a, Tbx3, and Zfp42, which are well-known factors that regulate self-renewal of ES cells. However, whether super-enhancer-associated genes are also involved in ES cell differentiation remains unclear. Because many multiple core factor co-bound targets and super-enhancer-associated genes are active but do not disrupt the self-renewal of ES cells, it is intriguing to study their roles on the other characteristic of ES cells, differentiation potential.

Epigenetic regulation

The role of DNA methylation in ES cells

An intriguing phenomenon in biology is the fact that the same genomic information can somehow lead to the generation of more than 200 functionally different cell types in the human body. Aside from the contributions of transcription factors to cell identity, epigenetic regulation provides another layer of complexity to cell identity, in particular the

self-renewal capacity of ES cells. DNA methylation occurs at the cytosine of the CpG dinucleotide (reviewed in Bird, 2002). DNA methylation patterns vary in different cell types, ultimately controlling the expression of many tissue-specific genes. The importance of DNA methylation is reflected in studies of Dnmt, DNA methyltransferase knockout (KO) embryos. Dnmt3a and Dnmt3b single or double KO embryos display developmental defects such as embryonic lethality, premature death, and rostral neural tube defects (Okano et al., 1999). Dnmt3a and Dnmt3b double KO embryos show loss of *de novo* methylation, indicating the essential role of *de novo* methylation in embryogenesis. Similarly, deletion of Dnmt1, which is required for the maintenance of DNA methylation, also delays development and results in embryonic lethality (Li et al., 1992). Interestingly, Dnmt1 KO ES cells can proliferate and maintain self-renewal, suggesting that DNA methylation only affects the differentiation potential of ES cells. Further study of global methylation patterns in differentiating ES cells may help our understanding of how DNA methylation affects the pluripotency of ES cells.

The roles of histone modifications in ES cells

Covalent modification of histone tails with acetyl, methyl, phosphatidyl, or ubiquityl groups serves as an epigenetic marker in eukaryotes. These modifications provide a favorable environment for the activation or repression of nearby genes, with the specific effect governed by the combination of modifications. For example, H3K4me3 and H3K9ac are active histone marks, whereas H3K27me3 is a repressive mark (Bernstein et al., 2002; Kuzmichev et al., 2002; Liang et al., 2004). ES cells possess a unique chromatin structure characterized by a wide open chromatin architecture with active histone markers such as H3K4me3 and acetylation-enriched histones compared with the structure of differentiated cells. In addition, lineage-specific regulators in ES cells possess distinct histone

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modifications called 'bivalent markers' that contain both the H3K4me3 active mark and the H3K27me3 repressive mark (Bernstein et al., 2006; Rada-Iglesias et al., 2011). Bivalent-marked genes are generally inactive in ES cells, although they are rapidly induced upon differentiation. The H3K4me3 and H3K27me3 histone marks are generated by the Trithorax-group (TrxG) and PRC proteins, respectively. Deletion of Wdr5, the effector protein for H3K4me3, impairs the self-renewal of ES cells and somatic cell reprogramming (Ang et al., 2011). However, deletion of PRC2 subunits does not affect the self-renewal of ES cells, whereas it impairs differentiation, suggesting that the role of PRC is primarily the derepression of lineage-specific regulators upon differentiation.

Enhancers are DNA elements that act over a long range to activate transcription of target gene. Active enhancers are generally occupied by transcriptional co-activators (Blackwood and Kadonaga, 1998), but recent studies also identify several other marks such as p300 occupancy, H3K4me1, and H3K27ac (Creyghton et al., 2010; Rada-Iglesias et al., 2011, 2012). ES cells also possess poised enhancers marked by H3K27me3, which are rapidly activated upon differentiation. For proper differentiation of ES cells, enhancers associated by ES cell-specific genes need to be silenced. Kdm1a, a histone H3K4/K9 demethylase binds to active enhancer in ES cells and silences their activities by demethylating active histone marks (Whyte et al., 2012). Even though Kdm1a-depleted ES cells maintain self-renewal, the cells do not undergo proper differentiation due to the sustained levels of Nanog, Sox2, and Dppa5. The example of Kdm1a-deficient ES cells supports the idea that two unique characters of ES cells, self-renewal and differentiation potential can be functionally separable.

Signaling pathways

LIF/Stat pathway

Initially, ES cells were maintained on mitomycin C-inactivated STO feeder cells, a mouse embryonic fibroblast cell line (Evans and Kaufman, 1981). Because the ES cells were mixed with the feeder cell lines, it was important to identify factors secreted by the feeder cells that contributed to maintaining the undifferentiated state of ES cells. In 1988, leukemia inhibitory factor (LIF) was identified as a factor supporting the self-renewal of ES cells (Smith et al., 1988). Addition of recombinant LIF allows ES cells to expand without undergoing differentiation under feeder cell-free conditions. Furthermore, active Stat3, a downstream transcriptional regulator of the LIF/Stat signaling pathway, is sufficient to maintain the undifferentiated state of ES cells, supporting the importance of the LIF/Stat pathway for the self-renewal of ES cells (Matsuda et al., 1999). Active Stat3 induces pluripotency factor Klf4, which in turns activates Sox2 and Pou5f1, thus completing the signaling pathway from cytokine to ES cell self-renewal.

The self-renewal of human ES cells, on the other hand, cannot be maintained in the absence of feeder cells regardless of the presence of LIF (Thomson, 1998). However, LIF/Stat is active in human ES cells, as evidenced by the presence of the LIF receptors gp130 and LIFRβ and the activation and nuclear localization of Stat3 induced by LIF (Daheron et al., 2004; Kidder et al., 2008). There are several explanations for the different roles of the LIF/Stat pathway in mouse and human ES cells. Human ES cells could be in a different developmental stage than mouse ES cells. Human ES cells are more similar to mouse epiblast stem cells than mouse ES cells at multiple levels, such as culture conditions, expression profiles, and epigenetic status (Brons et al., 2007). In addition, the recent discovery of the 2i culture condition, which does not rely on LIF for the maintenance of ES cells, suggests that LIF/Stat3 acts to block ES cell differentiation rather than enhancing

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the self-renewal capacity (Ying et al., 2008). Further analysis of the roles of the LIF/Stat pathway is required to decipher the nature of the self-renewal of ES cells.

Activin/Nodal/TGFβ signaling pathway

The transforming growth factor (TGF)-β family consists of approximately 30 members that can be subdivided into several subgroups, including TGFβ, the activins, Nodal, bone morphogenetic proteins (BMPs), and growth/differentiation factors (GDFs) (reviwed in Heldin and Moustakas, 2016). These ligands activate heteromeric type I and type II receptors, which in turn activate Smad2 and Smad3 proteins by phosphorylation. Phospho-Smads interact with Smad4 and translocate to the nucleus to regulate a large number of downstream targets. The physiological outcomes of this pathway vary depending on cell type and the specific ligands, although they share common downstream effectors. For example, both Activin and TGFβ activate Smad2/3; however, mice deficient in each gene display different phenotypes. Activin A-deficient mice die within 24 h of birth, whereas TGFβ1 KO mice die at mid-gestation (Goumans and Mummery, 2000; Matzuk et al., 1995).

Since many of the TGFβ components affect mouse development only at post-implantation stages, it has been suggested that the TGFβ signaling pathway is not required for the establishment of pluripotency or the control of early cell fate, which mainly occur before implantation. However, other ligands in this pathway such as BMPs, GDFs, and Nodal play an important role in early mouse development. BMP signaling is required for many early developmental stages, including germ cell specification, differentiation of extra-embryonic tissues, and epiblast proliferation (Winnier et al., 1995). In addition, Nodal mutant embryos fail to gastrulate or form a primitive streak (PS) (Conlon et al., 1994). In contrast to the roles of Activin in *in vivo* early developmental biology, addition

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of recombinant Activin to differentiating ES cells activates mesodermal differentiation (Wiles and Johansson, 1999). Furthermore, TGFβ signaling is required for ES cell proliferation (Ogawa et al., 2006). Since the Activin/Nodal/TGFβ signaling pathway has different effects on *in vivo* early embryogenesis and *in vitro* differentiation of ES cells, in depth studies are required to elucidate the underlying mechanisms.

Hippo signaling pathway

The roles of the Hippo pathway in the development of ICM, the *in vivo* counterpart of ES cell, are well understood. The Hippo pathway is inactive in the outer layer of the embryo, which allows the nuclear translocation of Yap1 to activate downstream target genes, resulting in the differentiation of the outer layer cells into the trophectoderm (Nishioka et al., 2009). Yap1 is sequestered in the cytoplasm of the ICM of the blastocyst when the Hippo pathway is active. ES cells, which are derived from the ICM and grow as a colony, may also have an active Hippo pathway and cytoplasm-sequestered Yap1. However, Lian *et al*. argued that Yap1 regulates the self-renewal of ES cells, and depletion of Yap1 induces differentiation, which is not consistent with the functions of Yap1 in the ICM (Lian et al., 2010). A deeper understanding of the functions of Yap1 in ES cells is needed to clarify this issue.

Notch signaling pathway

Notch1 is expressed in ES cells but not in ICM (Hadland et al., 2004). Mesodermal differentiation studies show that transient activation of Notch1 blocks mesodermal differentiation, reducing the number of $Flk1^+$ mesodermal cells (Schroeder et al., 2006). Activated Notch1 further blocks the generation of cardiac muscle, and endothelial and hematopoietic cells (Schroeder et al., 2003). However, depletion of Rbpj, a downstream transcriptional regulator of the Notch pathway, promotes differentiation into a

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cardiomyocyte lineage. Taken together, these studies suggest that the Notch pathway plays a role in mesodermal differentiation. However, many questions remain unanswered, such as global target occupancy of Rbpj in ES cells, the effect of Notch signaling on core factors, and the effect of Notch inhibitors on ES cell differentiation.

Fgf4/Erk signaling pathway

In mouse ES cells, Fgf4 is the main stimulus activating the Erk1/2 signaling cascade (Kunath et al., 2007). Although inhibition of Fgf4 with the small molecule PD173074 or genetic ablation does not affect ES cell self-renewal, the differentiation potential of ES cells is markedly affected by blockage of Fgf4 signaling. Fgf4 KO or PD173074 treated ES cells are resistant to neuronal and mesodermal differentiation (Stavridis et al., 2007). The differentiation-inducing Fgf4 signaling pathway is active in self-renewing ES cells and is not inhibited by LIF, which promotes the self-renewal of ES cells (Ying et al., 2003b). Based on this observation, Ying *et al*. hypothesized that the self-renewal of ES cells can alternatively be achieved by inhibiting the active lineage commitment signal in ES cells. Indeed, ES cells can be maintained by inhibiting the Fgf4 and Gsk3 signaling pathways even without the addition of LIF (Ying et al., 2008). The 2i culture medium containing PD184362 and CHIR99021, which are inhibitors of Fgf4 and Gsk3, respectively, enables the culture of ES cells in a uniform and defined medium in a manner independent of serum and LIF. This indicates that ES cells have an active intrinsic circuitry that maintains the self-renewal of ES cells independently of extrinsic cytokines such as LIF or BMP. The presence of active differentiation pathways such as Fgf4 in self-renewing ES cells suggests that such signaling pathways may be one of the unidentified intrinsic regulators of pluripotency.

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REGULATION OF THE DIFFERENTIATION POTENTIAL OF ES CELLS

Many factors that are important for the self-renewal of ES cells have been identified in approximately 30 years of study after the establishment of ES cells. However, the mechanisms regulating the differentiation potential or pluripotency of ES cells are relatively less understood. The identification of transcription factors or signaling pathways involved in the exit from self-renewal or lineage specification is important for the application of ES cells to regenerative medicine and developmental biology. Upon differentiation, ES cells gradually lose pluripotency and become terminally differentiated cells. In correlation with decreased pluripotency, the expression of many active genes in ES cells also decreases, suggesting the existence of active regulators that control the pluripotency of ES cells.

Suppression of lineage-specific regulators by PRC

Most of the PRC downstream targets are lineage-specific regulators that need to be repressed to maintain the self-renewal of ES cells. Deletion of the PRC component Eed induces many PRC-bound lineage-specific regulators such as Gata4 and Gata6, although it does not disrupt the self-renewal of ES cells probably because of sustained core factors such as Pou5f1 and Nanog (Chamberlain et al., 2008). However, the differentiation potential is impaired in PRC-depleted ES cells. Suz12 deletion results in failure to form endodermal layers (Pasini et al., 2007), whereas Ezh2- or Eed-deficient ES cells display impaired mesoendodermal differentiation (Shen et al., 2008). Considering the repressive effects of PRC on lineage-specific regulators, it can be speculated that PRC deletion would enhance ES cell differentiation; however, it turns out that PRC also plays a role in silencing core factors upon differentiation (Li et al., 2010a; Walker et al., 2010). Therefore, it is important to understand how PRC inactivates core factors upon differentiation and why each PRC component selectively affects lineage specification.

Epigenetic plasticity of ES cells

The zygote, the only unequivocally totipotent cell, undergoes extensive paternal-specific demethylation (Mayer et al., 2000). Subsequent cell division in early embryonic development further reduces global DNA methylation up to the morula stage because of exclusion of Dnmt1 from the nucleus (Howell et al., 2001). *De novo* DNA methylation occurs before the blastocyst stage, which coincides with the very first cell fate decision (Santos et al., 2002). Since DNA methylation at promoter regions represses neighboring genes, global hypomethylation of totipotent cells may be one of the key features of pluripotency that allows any lineage-specific gene to be expressed upon differentiation. Consistent with the hypomethylation of the early embryo, global analysis of the DNA methylation patterns of ES cells and differentiated somatic cells shows that the genomes of ES cells are less methylated than those of somatic cells (Jackson et al., 2004). However, ES cells with demethylation caused by deletion of Dnmt1 or Dnmt3a and Dnmt3b display impaired erythrocyte and cardiomyocyte differentiation (Jackson et al., 2004). In addition, Dnmt3a and 3b double KO ES cells remain largely undifferentiated even under differentiation conditions. This could be due to the sustained levels of core factors that need to be silenced upon differentiation.

ES cells possess unique chromatin structures such as expansive regions of open chromatin, bivalent histone marks, and a hyperdynamic chromatin structure. Evidence from electron microscope, DNase I, MNase assay, and heterochromatin histone modification studies indicates that the genomes of ES cells are largely open and accessible to DNA-binding proteins compared with those of differentiated cells. In addition, most of the repressed lineage-specific regulators contain both activating H3K4me3 and repressive H3K27me3 histone marks, indicating that these genes are poised to be expressed upon ES cell differentiation. Many structural chromatin proteins such as HP1 and regular histones

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are more loosely bound to the chromatin of ES cells than to somatic cells (Meshorer et al., 2006). These data reflect the high level of epigenetic plasticity of ES cells, which might contribute to the pluripotency of ES cells.

Factors required for exit from pluripotency

Tcf3, a repressor of the Wnt signaling pathway, is one of the first factors required for the proper differentiation of ES cells. Since Tcf3 represses core factors, Tcf3-deficient ES cells do not undergo proper differentiation because of sustained levels of pluripotency genes. After the discovery of Tcf3, genome-wide KD studies showed that Tfe3 and Zfp706 are required for exit from self-renewal (Betschinger et al., 2013; Leeb et al., 2014). The regulatory mechanisms underlying differentiation of ES cells are to repress core factors, similar to that of Tcf3. Although ES cells deficient in these factors do not undergo proper differentiation because of high levels of core factors, whether they maintain differentiation potential remains unclear. Furthermore, the expression of these factors decreases upon differentiation, indicating that they may not be able to actively repress core factors in differentiating ES cells. Therefore, the identification of factors that affect pluripotency and/or the active downregulation of core factors is important to improve our understanding of the regulatory mechanisms underlying ES cell differentiation.

Direct differentiation of ES cells

The use of ES cells as a source in regenerative medicine requires the design of differentiation methods to efficiently generate the desired homogenous cell populations. However, the plasticity that allows ES cells to generate all three germ layers also makes them difficult to control. Lessons from developmental biology studies on early embryogenesis provide insight into the control of ES cell differentiation. For example, studies of gastrulation in mice identified the critical roles of BMP4, Nodal, and Wnt in PS

formation and the subsequent generation of the mesodermal lineage (Ng et al., 2005; Park et al., 2004; Wiles and Johansson, 1999). The expression of Brachyury is often used as a marker to monitor the formation of PS-like cells through *in vitro* differentiation of ES cells, as Brachyury is specifically expressed in the PS. Addition of BMP4 to the differentiation medium effectively generates a Brachyury-positive PS-like population and subsequently Flk-1-positive mesodermal cells. Furthermore, addition of Wnt accelerates the formation of a PS-like population and cardiac mesoderm development (Ueno et al., 2007). Activation of the Nodal pathway also induces differentiation into a PS-like population and the subsequent formation of endoderm or mesoderm (Kubo, 2004). However, further studies are required to achieve precise control of cell fate, as generating a homogeneous cell population is often an obstacle limiting the development of cell therapies.

AIMS OF THE RESEARCH PROJECT

ES cells have been extensively studied in both scientific and medical communities due to its unique characteristics – self-renewal and pluripotency. The information gained from the studies helps to understand various research areas such as early embryonic development, transcriptional and epigenetic regulation, reprogramming of cell fate, and direct differentiation. However, most of the current studies focus on the molecular mechanisms underlying self-renewal of ES cells and only a few studies have been conducted to decipher the nature of pluripotency. Since understanding and controlling differentiation of ES cell is key to developmental biology and regenerative medicine, we decided to identify novel regulators that govern the pluripotency.

In chapter 3, we have developed and validated signature based screen to identify novel regulators of ES cell differentiation. ES cell differentiation is a complex process that involves silencing of core factors, induction of three different germ layer specific

regulators, and re-distribution of epigenetic marks. Therefore screening regulators with single reporter system may not be able to cover the complicated differentiation process. Our signature-based screen allows to simultaneously monitor around 50 different gene expression levels so that we can measure the expressions of core factors, ectodermal, endodermal, mesodermal, and trophectodermal markers, and epigenetic regulators of more than 100 different samples. We first validated the screening system by comparing technical and biological repeats. After extensive optimization, we performed shRNA mediated KD screen to identify novel regulators of ES cell differentiation, separating them into distinct categories based on their overall functions.

In chapter 4, we have examined the molecular functions of Yap1, a transcriptional co-activator of Hippo signaling pathway, on ES cell differentiation. Since we have observed that cell density significantly affected differentiation of ES cells and Hippo signaling pathway is responsible for sensing and responding to cell-cell contact and cell densities, we hypothesized that Yap1 may regulate differentiation of ES cells. First, we tested whether Yap1, and its associated factors, such as Tead family proteins and Taz are required for self-renewal of ES cells. Then, we examined the activity and localization of Yap1 upon differentiation of ES cells. Lastly, we determined the functions of Yap1 on differentiation of ES cells.

In chapter 5, we have explored the roles of Pou5f1 repressors on ES cells differentiation. Until recently, only a few factors have been identified to repress Pou5f1 in ES cells. Tcf3 represses Pou5f1 and thus blocks proper differentiation of ES cells due to the sustained levels of core factors. Recently, we have reported that Tgif1 also represses Pou5f1 to counterbalance feed-forward activation of Pou5f1, thus maintaining optimal levels of Pou5f1. Misregulation of Tgif1 leads to abnormal differentiation of ES cells, suggesting the importance of Pou5f1 repressors on ES cell differentiation. To identify

Pou5f1 repressors, we have performed small-scale shRNA mediated screen with the candidate genes from published database. Then, we examined whether the positive hits are affecting the differentiation of ES cells.

CHAPTER 2: MATERIALS AND METHODS

CELL CULTURE

J1, E14, and CJ7 mouse ES cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco Ref. 11965) supplemented with 18% of fetal bovine serum (FBS), penicillin/streptomycin/L-glutamine (Gibco Ref. 10378), MEM nonessential amino acid (Gibco Ref. 11140), nucleosides (Millipore Cat. ES-008-D), 100uM β-mercaptoethanol (Sigma M3148), and 1000U/mL recombinant leukemia inhibitory factor (LIF, Millipore Cat. ESG1107). ES cells were cultured on 0.1% gelatin-coated plates at 37°C and 5% CO_2 and passaged every 2 days. HEK 293T cells were maintained in DMEM supplemented with 10% of FBS and penicillin/streptomycin/L-glutamine. To differentiate ES cells, cells were washed three times with the media without LIF and then incubated for 4 days while passaging every 2 days.

REAL TIME QUANTITATIVE PCR (RT-qPCR) ANALYSIS

Total RNA was isolated using the RNeasy plus mini kit (Qiagen Cat. 74134) and 500ng of RNAs were reverse transcribed using qScript cDNA SuperMix (Quanta Cat. 95048). RT-qPCRs were performed using 10 uL of PerfeCTa® SYBR® Green FastMix®, Low ROX™ (Quanta Cat. 95074), and 1uL of 5 X diluted cDNAs. RT-qPCR primers were designed to amplify the exon junction with amplicon size ~ 100 base pair. Primer sequences are listed in Appendix Table D-F. CT values of each primer were normalized against Gapdh using −ΔΔCt method to calculate fold change.

ShRNA LENTIVIRAL PRODUCTION AND INFECTION

HEK 293T cells were plated at ~6 X 10^6 cells per 100 mm^2 and then transfected with 6ug of pLKO.1 shRNA vector (Sigma), 4ug of pCMV-Δ8.9, and 2ug of VSVG plasmids using 30ul of Fugene 6 (Promega Ref. 2692), according to the manufacturer's

protocol. shRNA sequences are listed in Appendix Table A-C. After 24 hrs, HEK 293T medium was replaced with ES medium. 2 days after transfection, supernatant containing viral particles was collected and filtered through 0.45 um Supor® membrane (PALL Ref. 4654). ~2 X 10^5 ES cells were plated on 12-well plate with virus containing media supplemented with 10ug/mL polybrene (Millipore Cat. TR-1003-G). After one day of infection, cells are selected with appropriate antibiotics and passaged every two days. Cell morphology, AP staining, protein and mRNA levels were examined two passages after the infection.

WESTERN BLOT ANALYSIS

Cells were lysed with RIPA buffer (G-BIOSCIENCES Cat. 786-490) with the addition of PhosSTOP (Roche P.N. 04906845001) and 100x Halt™ Protease Inhibitor (Thermo Scientific P.N. 1860932). After three times of sonication with 30 sec interval, lyses were centrifuged at maximum speed for 10 min at 4°C. Supernatant was transferred to clean tube and Laemmli sample buffer (Biorad Cat. 161-0737) was added. Following antibodies were used for Western blot analyses: Yap1 (1:1000, Santa Cruz, SC-101199), p-Yap1 (1:000, Cell signaling 4911S), Pou5f1 (1:1000, Santa Cruz, SC-5279), Nanog (1:1000, Abcam, ab21624), Gapdh (1:2000, Santa Cruz, SC-166545), Actb (1:20000, Abcam, ab20272), Tead1 (1:1000, BD Biosciences, Cat. 610923), Tead3 (1:1000, Abcam, ab75192), and Tead4 (1:1000, Abnova, H00007004-M01).

FLUIDIGM

cDNAs were synthesized from 300ng of RNAs using qScript™ cDNA SuperMix (Quanta Cat# 95048-500) with total 20ul reaction. cDNAs were diluted 5-fold using Nuclease-Free water (Ambion AM9937) and 1.25ul cDNAs were preamplified using 2.5ul of 2X Taqman preamp Master Mix (Applied BioSystems Cat# 4391128), 0.5ul of 500nM

pooled primer mixture and 0.75ul of using Nuclease-Free water (Ambion AM9937). Preamplification program is 95°C for 10 min, 95°C for 15 s, 60°C for 4 min for various cycles. Subsequently, preamplified cDNAs were incubated with 0.4ul of Exonuclease I (New England Biolabs Cat# M0293), 0.2ul of Exonuclease I reaction buffer, 1.4ul of water in 37°C for 30 min and inactivated in 80°C for 15 min. Exonuclease treated samples were further diluted 5-fold using TE (TEKnova PN T0224) and gene expression were analyzed with 48.48 Dynamic Arrays on a BioMark System (Fluidigm).

CELL PROLIFERATION ASSAY

1 X 10^3 ES cells were plated on 96-well plate. Every 24 h, 10uL of Cell Counting Kit-8 (Dojindo CK04) was added to the media. Absorbance was measured using Infinite® M1000 PRO microplate reader (Tecan) at 450nm after 2hr incubation.

CORRELATION ANALYSES

To generate the gene expression correlation map, $\log_2$ (FPKM) values were used to calculate Pearson correlation coefficients. Clustering analysis and visualization of the data were done by Cluster 3.0 and Java Treeview, respectively (de Hoon et al., 2004; Page, 1996).

GENE ONTOLOGY ANALYSIS

For Gene Ontology (GO) analyses, differentially expressed genes were tested for enrichment of functional gene sets using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). Enrichment *P*-values were calculated using a modified Fisher Exact test. Similar GO terms are removed except the gene sets with the highest p-value.

GENERATION OF STABLE CELL LINES

pEF1α-FLBIO vector containing Yap1 full length cDNA was introduced into BirA expressing ES cells (control cells) by electroporation (Kim et al., 2009). Positive clones were selected under the selection of puromycin and geneticin. Multiple single colonies were picked 7-10 days later, and then Yap1 overexpressing clones were confirmed by western blot assay using anti-streptavidin-HRP (1:2000 dilution, Sigma-Aldrich Mfr. RPN1231), and anti-Yap1 antibodies (1:1000 dilution, Santa Cruz sc-101199). Yap1-KO cell lines were generated using CRISPR-Cas9 system following manufacturer's instructions (Life Technologies) (Cong et al., 2013; Mali et al., 2013). Briefly, GeneArt® CRISPR Nuclease Vector System (Life Technologies Cat. A21175) was used to edit genomic sequence in *Yap1* gene locus. Two different target sequences are TGCCGTCATGAACCCCAAGA and ATTGAAGAGCGCCTCCAAGT followed by CGG (PAM sequence). J1 ES cells were transfected with the cloned CRISPR-Cas9 nuclease construct using Lipofectamine® 2000 (Life Technologies Cat. 11668027) and incubated for 1 day. Transfected J1 ES cells were enriched by Dynabeads® CD4 Positive Isolation Kit (Life Technologies Cat. 11331D) and incubated for colonization. Each colony was picked, analyzed with Western blotting, and confirmed with sequencing for genomic editing at the target site.

IMMUNOFLUORESCENCE

~ 3 X 10^5/mL ES cells were plated on 0.1% gelatin pre-coated μ-Slide VI$^{0.4}$ (Ibidi Cat. 80606). Slides were fixed with 3.7% paraformaldehyde for 15 min at room temperature and permeabilized with 0.5% Triton X 100 for 10 min. Slides were then incubated with blocking solution (3% BSA and 1% normal horse serum in PBS) for 1 hr at room temperature, Yap1 primary antibody solution (1:200 dilution, Santa Cruz sc-101199) overnight at 4°C, and secondary antibody solution (1:1000 dilution) conjugated to Alexa

Fluor 488 for 1 hr at room temperature. Lastly, slides were mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes P36935) and imaged on a Zeiss 710 laser scanning confocal and structured illumination microscope.

LUCIFERASE REPORTER GENE ASSAY

For the luciferase reporter gene assay, $2.5X10^5$ J1 ES cells in each 24 well were co-transfected with 100 ng of the GTIIC vector or Pou5f1 enhancer luciferase vector (Dupont et al., 2011; Lee et al., 2015), and 5 ng of PGL4.75 vector containing a Renilla reporter gene as an internal control reporter using lipofectamine 3000 (Life Technologies, Cat. L3000008) and then cultivated for 24 hrs. To measure luciferase reporter gene activity, cells were washed 2 times with PBS, lysed, and the luciferase activities were measured using the Dual Luciferase® assay kit (Promega, E1910).

QUANTIFICATION OF IMMUNOFLUORESCENCE IMAGE

DAPI signal is used to distinguish nuclear portion from cytoplasmic portion of the cells. Total area of the cells is identified by pan-cadherin antibody. Intensity is calculated using ZEN software (ZEISS).

RNA SEQUENCING AND DATA PROCESSING

1ug of RNAs were used to generate illumina-compatible sequencing libraries using mRNA isolation kit (NEB, E7490L) and RNA library prep kit (NEB, E7530S) according to the manufacturer's protocol. Adapter ligation was done with sample-specific barcodes. RNA-seq libraries were sequenced using an Illumina NextSeq 500 machine. Single-end reads from RNA-seq were mapped onto the mouse genome assembly (mm9) using default setting of Tophat2. Transcript-level expression analysis was performed using Cuffdiff to calculate FPKM (fragments per kilobase of transcript per million mapped reads) (Trapnell et al., 2010).

CHAPTER 3: IDENTIFYING TRANSCRIPTION FACTORS REGULATING DIFFERENTIATION OF EMBRYONIC STEM CELLS

INTRODUCTION

Although numerous studies have been dedicated to characterizing genes that promote self-renewal in ES cells, comparatively fewer studies have attempted to characterize genes that enable and control ES cell differentiation. Additionally, the studies that have been performed to explore factors impacting ES cell differentiation have typically lacked adequate functional characterization of positive hits. For example, previous genome-wide KD studies of differentiating ES cells identified that Tcf3, Tfe3, and Zfp706 are required for exit from self-renewal in differentiating ES cells. Unfortunately, the authors did not further characterize their roles in differentiation potential or lineage specification into the three germ layers. To identify novel regulators responsible for proper differentiation of ES cells, we performed a small scale KD screen. Most of the candidate genes are robustly expressed in ES cells and diminished upon differentiation. However, KD of each of those factors did not significantly affect self-renewal of ES cells, which prompted us to hypothesize that some of them are involved in the regulation of other characteristics of ES cells, i.e. pluripotency or differentiation potential, rather than self-renewal.

To identify novel regulators responsible for maintaining and controlling differentiation potential of ES cells, we employed a unique signature-based KD screen rather than the traditional GFP-based reporter screens utilized in previous studies. Signature-based screen has been proven to be a powerful tool to identify molecules that can affect a whole pathway rather than a single component of the pathway (Hieronymus et

al., 2006; Shats et al., 2011). For example, a signature based screen identified a novel inhibitor of the androgen receptor (AR) signaling pathway which effectively blocks prostate cancer cell growth without leading to fatal drug resistance, which is the critical problem of known AR inhibitors. In this regard, signature-based screen on ES cell differentiation allows us to simultaneously monitor several different ES cell core factors as well as lineage specific regulators, identifying transcription factors that effectively regulates each lineage specification.

RESULTS

Genes bound by multiple core factors are actively expressed in ES cells and help maintain stemness

Previously we have shown that multiple transcription factor bound genes are more likely to be critical regulators of cell identity (Kim et al., 2008). Genes bound by 4 or more factors among 7 core factors (Pou5f1, Nanog, Sox2, Klf4, Nac1, Dax1, and Tcf3) are highly expressed in ES cells, and their expressions are decreased upon differentiation. These genes are enriched for H3K4me3 active histone marks and depleted for H3K27me3 repressive histone marks. In addition, they are generally enriched in developmental process. Some of these target genes were later re-discovered by other labs who explained their roles in ES cell identities. For example, Cbx1, a heterochromatin associated protein, is essential to maintain self-renewal and differentiation potential of ES cells (Mattout et al., 2015). In addition, Cited2-deficient ES cells display abnormal glucose metabolism and impaired differentiation during hypoxia (Li et al., 2014). However, many of these target

genes have not yet been fully characterized with regards to their roles in ES cell pluripotency.

Ankmy2	Ankrd10	Ankrd6	Anp32a	Asxl1	Bach1	Basp1
Bmp4	Brwd1	Cbx1	Cbx7	Cd38	Cdx1	Chd9
Cited2	Creb3	Dido1	Dnmt3a	E2f4	Eif4a2	Eno1
Epc2	Etv1	Etv4	Etv5	Evx1	Eya1	Fgfbp1
Fgfr2	Foxd3	Foxh1	Fubp3	Gbx2	Grhl3	Gsc
H2afx	H2afy2	Hes1	Hesx1	Hist1h1b	Hist1h2an	Hist1h2bb
Hist1h2bp	Hist1h3i	Hnrnpu	Hnrpdl	Hopx	Hoxb1	Hoxb13
Hoxb4	Id1	Jarid2	Kdm2b	Klf2	Klf9	Lmo4
Max	Med19	Mllt11	Mllt6	Msc	Msh6	Msx2
Mybl2	Mycn	Myst2	Mzf1	Nanog	Neurog1	Nfatc3
Nfe2l2	Nfkbia	Nkx2-2	Nolc1	Notch1	Notch4	Nr0b1
Nrarp	Olig2	Osr2	Otx2	Pax6	Pdlim1	Per2
Phc1	Phtf2	Plscr1	Pou5f1	Rai14	Rarg	Rax
Rbbp5	Rbbp7	Rbm14	Rest	Rfx4	Rlim	Rpap1
Rybp	Sall3	Sall4	Set	Sfrp1	Sox13	Sox2
Sp3	Spic	T	Tbl1xr1	Tbx20	Tbx3	Tcea1
Tcea3	Tcf7l1	Tfap2a	Tfap2c	Tfcp2l1	Tgif1	Tle3
Trib3	Trp53bp1	Txlng	Uba3	Uhrf2	Xrn2	Zfp13
Zfp146	Zfp148	Zfp219	Zfp280c	Zfp36l1	Zfp42	Zfp428
Zfp532	Zfp57	Zfp704	Zic2	Zic5	Zscan10	

Table 3.1 List of DNA binding domain containing genes co-occupied by 4 or more core factors. (Pou5f1, Nanog, Sox2, Klf4, Nac1, Dax1, and Tcf3)

First, we narrow down the gene list of previous study by selecting genes that contain DNA binding domain because cell identities are mainly determined by transcriptional regulators (Whyte et al., 2013) (Table 3.1). This list includes Pou5f1, Nanog, and Zfp42, which have already been shown to play essential roles in stemness. These positive hits help validate the list and show that the list is a credible research for exploring novel regulators

of ES cell identity. We further validated the list by checking their activity and known functions. In consistent with previous work, their expression levels are significantly higher than genes bound by fewer transcription factors and their roles are enriched in stem cell maintenance and embryonic development according to gene ontology (GO) analysis (Figure 3.1 A, B). With this list, we decide to perform KD screen to identify novel regulators of ES cell identity.
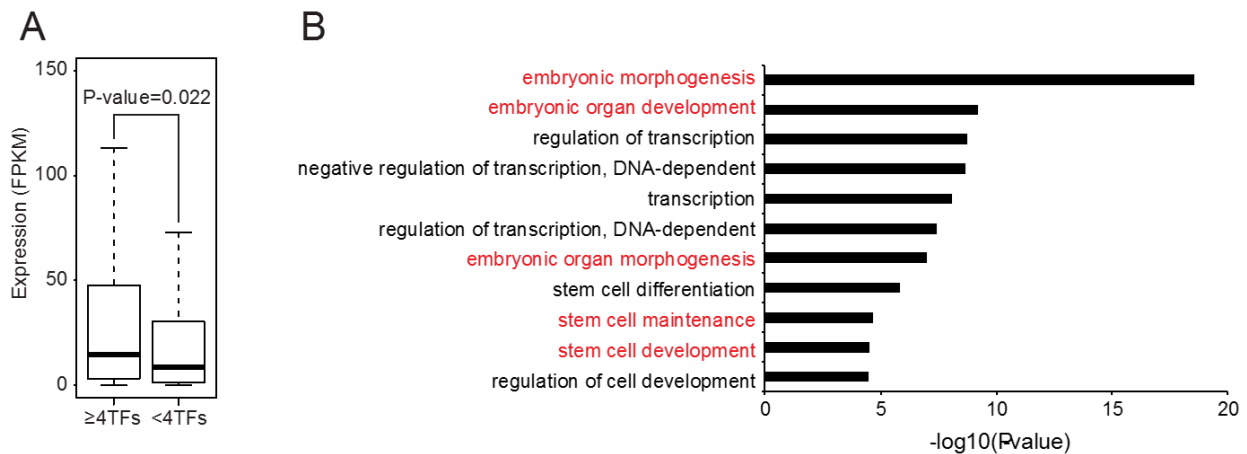
Figure 3.1 Common downstream targets of core factors are active and enriched in stem cell identities. (A) A box plot showing the distribution of expression levels of co-occupied targets of core factors. Occupied by 4 or more factors (left) or 3 or less factors (right). (B) Gene ontology (GO) analysis showing enriched biological process terms of common targets occupied by 4 or more core factors.

Signature-based screen to identify novel regulators of ES cell properties

Most of the previous screens utilized a single reporter system to discover novel regulators of ES cell properties (Ding et al., 2009; Ivanova et al., 2006). In particular, GFP reporter under the control of promoter of ES cell core factors has been used to identify regulators of self-renewal. Novel regulators that promote self-renewal should thus reduce GFP expression when the cells are depleted of the potential regulators. This simple readout was used as a proxy for stemness. Positive regulators of the GFP reporter were further analyzed to check whether they affect ES cell properties such as alkaline phosphatase

activity, round shape morphology, high proliferation rate, and core factor expression levels. This allowed researchers to confirm whether the hits were legitimate or not. Even though previous screens have identified several novel regulators of self-renewal of ES cells, they failed to find pluripotency or differentiation potential regulators that might act independently from core factors. In addition, single reporter system is not able to identify lineage specific regulators that are required for proper differentiation of ES cells to all three germ layers and trophectoderm, because differentiation into these four initial lineages is too complex to be measured with this approach.

To overcome the drawbacks of single core factor reporter system, we have utilized signature-based screen to simultaneously monitor about 50 pre-selected gene set. The gene set includes core factors (Pou5f1, Nanog, and Sox2), endoderm lineage regulators (Gata4, Gata6, and Sox17), mesoderm lineage regulators (T, Gsc, and Gata1), ectoderm lineage regulators (Nes, Gli2, and Fgf5), trophectoderm lineage regulators (Cdx2, Hand1, and Eomoes) (Table 3.2). To rule out the possibility of off-target effect of shRNAs, we have selected top 2 shRNAs from 5 different shRNAs targeting each candidate genes (Figure 3.2, Appendix Table A). Then we infected shRNA containing lentivirus to J1 ES cells and selected them with puromycin. shRNA expressing cells were split into self-renewing and differentiation condition media to examine the effect of candidate genes on ES cell properties. mRNA levels of pre-selected 50 genes were measured by Fluidigm from all the KD samples to identify novel regulators of ES cell properties.

Gene	Note	Gene	Note	Gene	Note
Ash2L	Pluripotency	Trp53	Pluripotency	Gsc	Mesoderm
Chd9	Pluripotency	Myc	Myc	Isl1	Mesoderm
Esrrb	Pluripotency	p400	Myc	T	Mesoderm
Fbxw7	Pluripotency	Tip60	Myc	Fgf5	Ectoderm
H2afx	Pluripotency	Trrap	Myc	Nestin	Ectoderm
Lin28	Pluripotency	Ezh2	PRC	Arid3a	Trophectoderm
LSD1	Pluripotency	Phc1	PRC	Cdx2	Trophectoderm
Nanog	Pluripotency	Rybp	PRC	Eomes	Trophectoderm
Pou5f1	Pluripotency	Suz12	PRC	Hand1	Trophectoderm
Rest	Pluripotency	Dkk3	Endoderm	Id2	Trophectoderm
Zfp42	Pluripotency	Gata4	Endoderm	Krt8	Trophectoderm
Sall4	Pluripotency	Gata6	Endoderm	Tead4	Trophectoderm
Sox2	Pluripotency	Sox17	Endoderm	Actb	Loading
Tcf3	Pluripotency	Bmp2	Mesoderm	Gapdh	Loading
Tgif1	Pluripotency	Bmp4	Mesoderm		
Tgif2	Pluripotency	Gata2	Mesoderm		

Table 3.2 Gene list used in signature based screen. Primer sequences are in Appendix Table E.
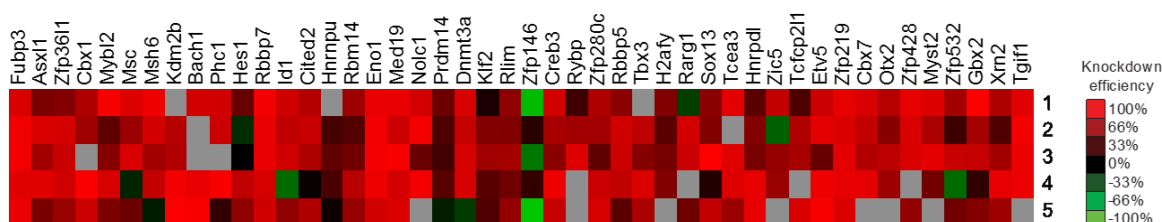
Figure 3.2 KD efficiency of shRNAs. Heatmap showing the KD efficiency of 46 candidate genes. Expression levels of each genes were measured by RT-qPCR then normalized by Gadph levels. Grey color indicates that ES cells infected with the lentivirus did not survive upon selection with puromycin.

Validation of signature based screen using Fluidigm

We first compared the result of Fluidigm with regular qPCR result (Figure 3.3). Overall results are quite similar to each other in terms of their direction of regulation. For

example, KD of Dmap1 or P400 decreases expression levels of Myc, Gata6, Sox17, Zfp42, and Nanog according to both methods. However, the overall heatmap is brighter in Fludigm compared to qPCR result, indicating that relative mRNA abundance is amplified in Fludigm. This might be due to the pre-amplification step in Fludigm. The differences in variation between Fludigm and qPCR results suggest that small differences in Fludigm may need to be validated by other methods such as qPCR.
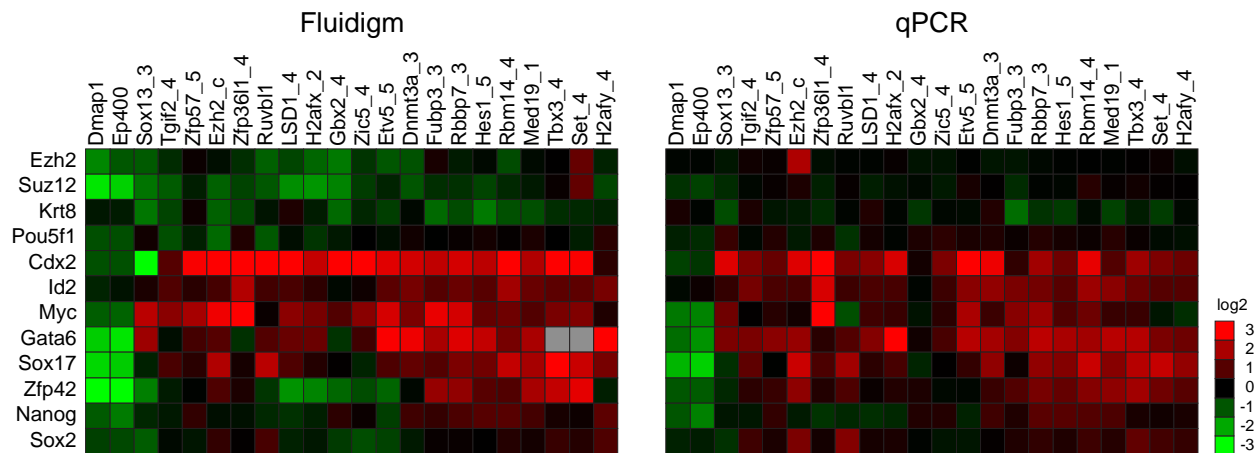
Figure 3.3 Comparison between Fludigm and qPCR data. Heatmap showing the mRNA levels of selected screen samples. Same cDNAs samples were used in both systems to validate the results of Fludigm with qPCR

Next, we examined whether Fludigm results are consistent between technical and biological repeats (Figure 3.4). 4 technical repeats of each cDNA samples were placed in a row to monitor the variation between repeats. Blocks of 4 repeats are easily observed in the heatmap, indicating that technical repeats from same cDNA sample are quite consistent. However, we observed strikingly different data between biological repeats in differentiation conditions. Detailed analysis of screening results reveals that some external conditions may affect differentiation of ES cells. First, infection efficiencies of lentiviruses carrying shRNAs are not even across the candidate genes. If cells are infected with a low titer of lentivirus, then only a few cells will get infected. Applying selection with antibiotics

will kill any uninfected cells. Thus, a tissue culture plate containing wells with an equal amount of starting cells but infected with different amounts of lentivirus will ultimately yield very uneven amounts of cells. Second, it is difficult to evenly seed the cells in small scale well. Cells may concentrate in the center of the well instead of forming an even distribution of spherical colonies, and this uneven distribution may ultimately affect gene expression. Lastly, cell densities affect differentiation efficiency. For example, the efficiency of neuronal differentiation of ES cells is high when seeding cells at low density (Ying et al., 2003b). Differentiation is already a heterogeneous process; if the density of the cells varies significantly across wells, each well may yield a different mix of the various differentiated lineages.
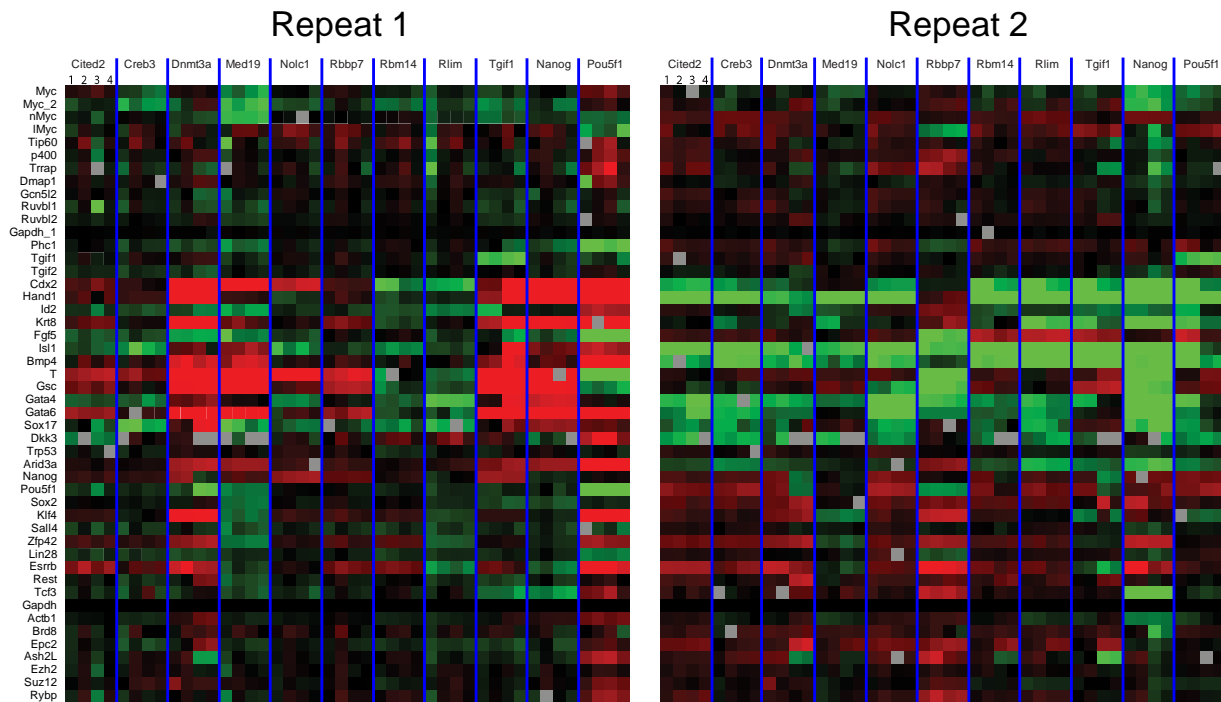
Figure 3.4 Comparison between biological and technical repeats. Heatmap showing the Fluidigm results of the selected gene KD samples in differentiation condition. Left and right panels are 2 biological repeats collected independently. Each panel contains 4 technical repeats of same cDNA samples. Sample and primer orders are consistent between panels. Each panels contains 4 technical repeats of each cDNA samples.

Cell densities affect differentiation efficiency of ES cells

To investigate the effect of cell densities on gene expression patterns of ES cells, we designed an experiment in which ES cells are serially diluted in self-renewing and differentiation conditions. Consistent with previous observations that cell-cell contact inhibits Myc expression, ES cells at high cell density significantly reduced Myc mRNA levels more than 100-fold compared to the cells at low density (Figure 3.5A) (Lee et al., 1995). However, most of other tested genes are not significantly altered in self-renewing conditions (Figure 3.5B). On the other hand, many of the lineage specific regulators exhibit enormous changes in differentiation condition (Figure 3.5C). Expressions of all three germ layers as well as trophectoderm lineage are not increased at high cell density even when the cells are in differentiation media. In consistent with the idea that core factors and lineage specific regulators are repressing each other, some core factors including Zfp42, Esrrb, and Dax1 show opposite trend to lineage specific regulators at various cell densities.
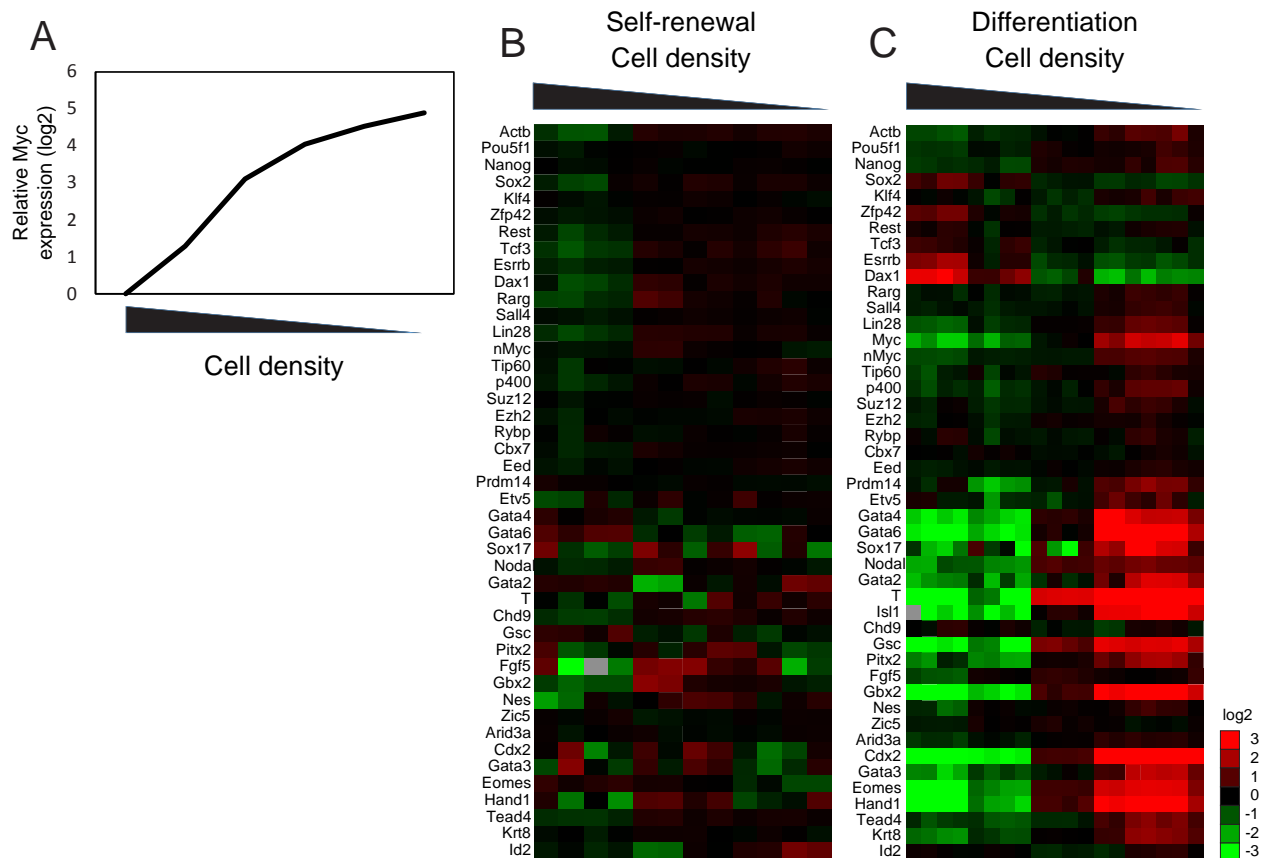
Figure 3.5 Cell density affects gene expressions in ES cells. (A) Relative Myc expression levels of serial diluted ES cells in self-renewing condition. Gene expression levels of serial diluted ES cells in self-renewing (A) and differentiation (B) condition measured by Fluidigm

Knockdown screen to identify novel regulators of ES cell self-renewal and differentiation potential

Since most of the candidate genes are highly expressed in ES cells, we hypothesized that these genes may regulate self-renewal or differentiation potential of ES cells. We have performed KD screen with 134 shRNAs comprising 78 genes in self-renewing and differentiation conditions and simultaneously measured expression levels of 45 genes (Figure 3.6). To minimize the effect of cell density on gene expression profile, we prepared highly concentrated virus and tested their individual infection efficiency to confirm that they could infect most of the cells in each well. Each well of infected cells were carefully

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monitored to have similar cell density and even distribution. As expected, KD of well-known core factors such as Pou5f1, Prdm14, and Nanog decreases overall core factor levels in self-renewing ES cells (Figure 3.6A). Consistent with previous reports, KD of Myc-related factors such as Tip60, Dmap, Ep400, and Trrap induces some of lineage specific regulators such as Sox17, Bmp2, Isl1, and T. Although they are the subunits of histone acetyltransferase complex, which mainly activates targets through the acetylation of histone tail, they suppress lineage specific regulators in ES cells, possibly through the interaction with Hdac6 (Chen et al., 2013; Fazzio et al., 2008). We also identified novel regulators such as Myst2, Mybl2, Zfp428, Zic5, and Cbx7. KD of those genes induces lineage specific regulators in ES cells without altering core factor expressions. It would be interesting to examine whether these genes are associated with PRC which inhibits lineage specific regulators in self-renewing ES cells. However, aside from those few factors, the majority of the other candidates do not significantly alter core factor expression, lineage specific regulator expressions, cell morphology, or proliferation rate upon KD, suggesting that they are not required for self-renewal of ES cells.

Next, we asked whether these genes affect differentiation potential of ES cells by checking gene expression signature in differentiation condition (Figure 3.6B). Surprisingly, we noticed that expression levels of tested genes are significantly altered compared to self-renewing condition, indicating that KD of candidate genes may have greater impact on differentiation than maintenance of ES cells. The expression profile can be grouped into three distinct clusters of genes based on their functions. Cluster 1 contains many of well-known ES cell core factors such as Pou5f1, Nanog, and Prdm14. Upon KD of these factors in cluster 1, expression levels of core factors are down-regulated while many of lineage specific regulators are induced. This means that suppression of these genes in ES cells stimulates differentiation. Interestingly, cluster 1 contains novel regulators such

as Xrn2, Zic5, and Bach1 that affect core factor expressions only in differentiation condition. These data suggest that these factors are either inactive or possess functions unrelated to transcriptional regulation while ES cells are in self-renewing conditions, but their function changes after the exit from self-renewal to promote differentiation. Meanwhile, knocking down genes in cluster 2 leads to higher expression of histone acetylase and PRC complexes, and reduced induction of lineage specific regulators. Since PRC and histone acetylase complexes are known repressors of lineage specific regulators, our data is consistent with previous observation. We noticed that ectoderm lineage regulators such as Fgf5, Nestin, and Hobx1 expressions are rather increased, whereas other lineage specific regulators are decreased. This may indicate that KD of these genes favors one lineage over another during differentiation. However, it has not been studied whether PRC and histone acetylase complexes differentially regulate each lineage upon differentiation. It might be interesting to study whether these complexes differentially regulate each lineage specifications *in vitro* differentiation and *in vivo* embryonic development. Since KD of genes in cluster 2 induces PRC and histone acetylase complexes, further studies of the genes in this cluster will also help to reveal upstream regulators of the complexes. Lastly, KD of genes in cluster 3 results in significant inhibition of lineage specific regulators, accompanied by induction of core factors. This implies that these genes are important for up-regulation of lineage markers as well as down-regulation of core factors during differentiation. Cluster 3 includes Tcf3, a well-known core factor repressor, indicating that genes in cluster 3 may be required for the exit from self-renewal. In contrast to cluster 2, where ectoderm-specific lineage markers were favored over other lineages, upon KD of the genes in cluster 3, lineage markers from all three lineages including the trophectoderm failed to be induced. Early embryonic development requires very precise lineage specification; each cell in the ICM must ultimately differentiate into

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the proper lineage in a consistent manner for the fetus to grow properly. These genes may be essential for that process and reveal insights about the nature of pluripotency itself.

Signature-based KD screen with 78 genes provides rough idea on their functions in self-renewal and differentiation of ES cells. However, the novel regulators discovered in this study may need to be confirmed by other methods such as CRISPR/Cas9 knockout, siRNA, overexpression (OE), and functional assays. Investigation of the roles of tested genes in direct differentiation into specific lineages may also provide important information on regenerative medicine and developmental biology. The results also prove the advantage of the signature-based screen over single reporter system by discovering novel regulators that function independent of well-known core factors.
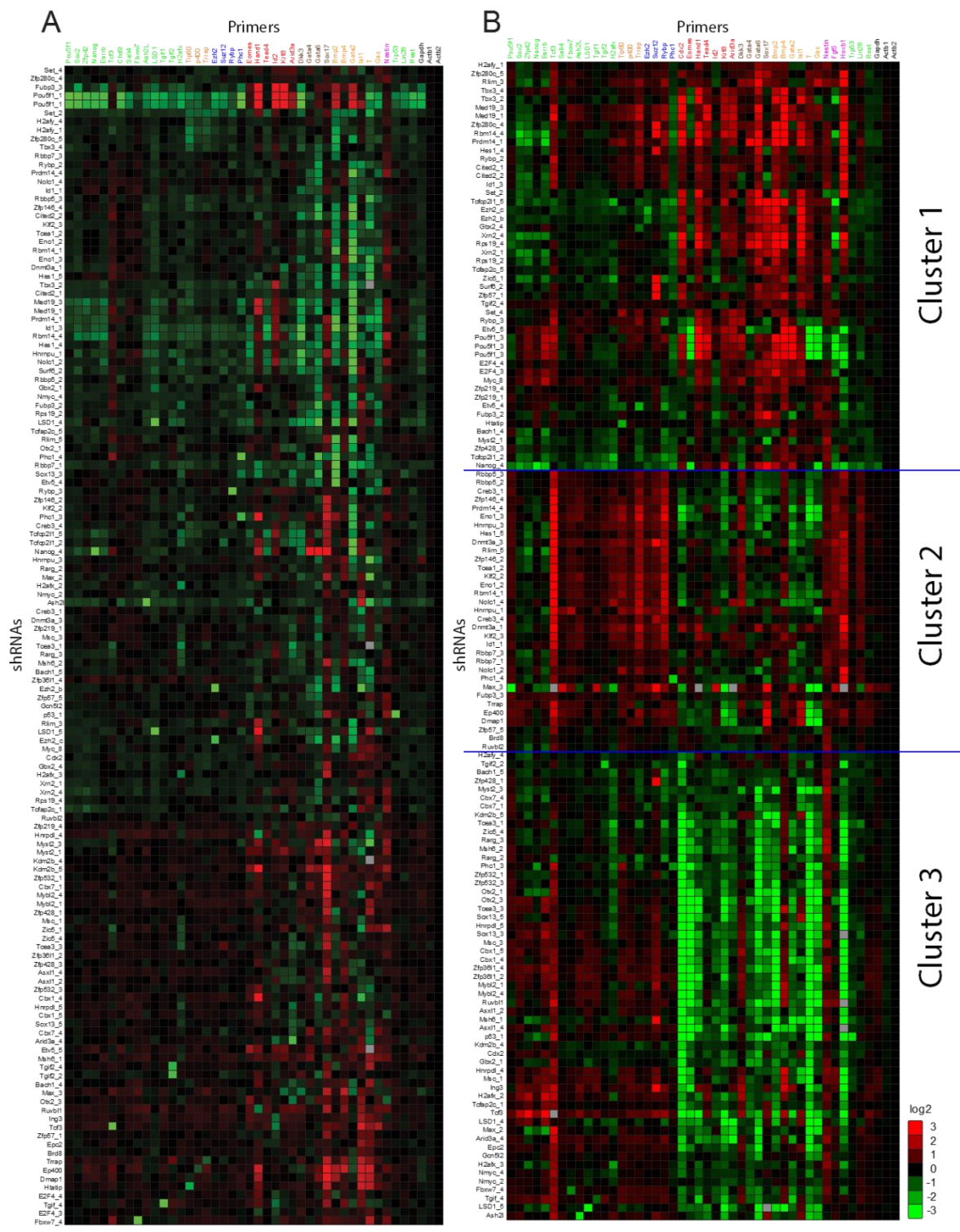
Figure 3.6 Signature based KD screen. Heatmaps showing the relative gene expression levels upon KD of each genes in self-renewing. (A) and differentiation (B) condition. Each columns indicates tested primers of different category – core factors (green), histone acetylase (orange), PRC (blue), trophectoderm (red), endoderm (dark brown), mesoderm (light brown), ectoderm (purple), loading control (black). Each rows indicate KD samples.

DISCUSSION

In summary, we performed small scale KD screen to identify novel regulators of ES cell characteristics. Compared to screens performed by other groups, we discovered numerous candidates that significantly disturbed ES cell differentiation potential without substantially affecting expression of core factors. This observation suggests that signature-based screen can be a powerful tool to identify novel regulators of differentiation, which can be missed by single reporter system. Since ES cell has potential to be differentiated into all three germ layers as well as trophectoderm, simultaneous observation of several marker genes will be helpful to identify regulators of each lineages at once. In addition, when studying differentiation of ES cells external conditions must be carefully controlled to obtain consistent results. We have shown that cell density significantly affects differentiation of ES cells. Using siRNA or CRISPR-Cas9 may help to reduce cell density variations which can be caused by lentiviral efficiency. Culturing cells carefully to maintain an even density and consistent colony size can also improve reproducibility of differentiation.

While analyzing the screen data we observed interesting gene expression patterns of lineage specific regulators. Whereas many of endodermal and mesodermal markers such as Gata6 and T display similar regulatory patterns across most of the samples, ectodermal markers such as Fgf5 and Nestin exhibit opposite gene expression patterns to other lineage specific regulators implying that there might be inhibitory mechanisms between ectoderm and other lineages. For example, Tet proteins regulate the balance between neuroectoderm and mesoderm by inhibiting Wnt signaling pathway (Li et al., 2016). Identifying factors that disrupt the inhibition of lineages may help to understand the underlying molecular mechanisms of embryonic development.

Since differentiation of ES cells is significantly affected by cell densities it is important to examine the mechanisms by which signaling pathway is involved in this process. Hippo signaling pathway senses cell-cell contact by transmembrane cadherin protein and activate downstream transcriptional activator Yap1 when cell density is low (Mo et al., 2014). However the roles of Yap1 on differentiation of ES cell has not been studied extensively, which prompted us to investigate the functions of Yap1 on ES cell identity.

CHAPTER 4: YAP1 IS DISPENSABLE FOR SELF-RENEWAL BUT REQUIRED FOR PROPER DIFFERENTIATION OF MOUSE EMBRYONIC STEM (ES) CELLS

INTRODUCTION

The Hippo signaling pathway, modulated by cell density and cell-cell contact, is implicated in diverse cellular processes including cell proliferation (Huang et al., 2005; Kim and Koh, 2011; Mori et al., 2014; Schlegelmilch et al., 2011; Silvis et al., 2011), apoptosis (Huang et al., 2005; Lee and Yonehara, 2012), and organ size control (Camargo et al., 2007; Zhao et al., 2007). Yap1, a transcriptional co-activator of the Hippo pathway, is known to play a crucial role in the segregation of inner cell mass (ICM) and trophectoderm (TE) during early embryogenesis (Cockburn et al., 2013; Hirate et al., 2013; Nishioka et al., 2009; Rayon et al., 2014; Wicklow et al., 2014). While Yap1 resides in the nucleus of trophectodermal cells and functions as a critical co-activator for TE development, it is mainly sequestered in the cytoplasm of the ICM as a phosphorylated inactive form due to active Hippo signaling (Nishioka et al., 2009). However, the role of Yap1 in ICM is still elusive (Cockburn et al., 2013; Nishioka et al., 2009). In addition to Yap1, Taz and Tead family members are also crucial players in the Hippo pathway. Taz, a homologue of Yap1, shares redundant functions with Yap1, such as controlling cell proliferation and sensing mechanical stress (Dupont et al., 2011; Imajo et al., 2014). Tead proteins play important roles in TE differentiation during early embryogenesis. They form a complex with Yap1, and are known to activate their downstream target genes (Li et al., 2010b; Ota and Sasaki, 2008).

Notably, there are two different observations on the roles of Yap1 in embryonic stem (ES) cells. Some recent studies have suggested that Yap1 plays an important role in the maintenance of mouse ES cells as an active factor in the nucleus (Lian et al., 2010; Tamm et al., 2011). These works showed KD of Yap1 promotes differentiation of ES cells while OE of Yap1 not only enhances self-renewal but also inhibits differentiation of ES cells even under neuronal differentiation conditions (Lian et al., 2010). However, the study showing nuclear-localized Yap1 in ES cells is somewhat contradictory to the function of the Hippo signaling since mouse ES cells grow as tightly packed colonies. It has been suggested that high cell density or cell-cell contact activates the Hippo signaling and subsequent sequestration of Yap1 in the cytoplasm of various cell lines such as HaCaT and NIH-3T3 (Kim and Koh, 2011; Mori et al., 2014; Varelas et al., 2010; Zhao et al., 2007). Accordingly, another recent study has claimed that both Yap1 and Taz are dispensable for the self-renewal of ES cells in 2i (Gsk3β and Mek inhibitors) culture condition (Azzolin et al., 2014). In this case, Yap1- and Taz-depleted ES cells maintained undifferentiated state under differentiation-promoting culture conditions (Azzolin et al., 2014). Consistent with this observation, studies of neuronal differentiation from ES cells have shown that high cell density, which activates the Hippo signaling and sequesters Yap1 in the cytoplasm, blocks differentiation of ES cells (Tropepe et al., 2001; Ying et al., 2003a), suggesting that nuclear localization of Yap1 might be important in normal differentiation of ES cells.

In the current study, we show that Yap1 is dispensable for the maintenance of ES cells but critical in their differentiation. Additional testing of Yap1-associated factors including Tead family proteins and Taz also supports the dispensability of Yap1 for the self-renewal of ES cells. In line with gradual up-regulation of Yap1 level upon differentiation of ES cells, OE of Yap1 in ES cells enhances nuclear abundance of Yap1 accompanied by induction of various lineage-specific marker genes. On the contrary,

Yap1-depleted ES cells showed impaired differentiation. Taken together, our data demonstrate a critical role of Yap1 in normal differentiation rather than self-renewal of ES cells.

RESULTS AND DISCUSSION

Yap1 is dispensable for self-renewal of mouse ES cells

Previous studies reported the requirement of Yap1 in the maintenance of mouse ES cells by showing that KD of Yap1 promotes differentiation of ES cells (Lian et al., 2010; Tamm et al., 2011). Conversely, another study claimed that double KD of Yap1 and Taz in 2i media does not disrupt self-renewal of ES cells (Azzolin et al., 2014). To decipher the roles of Yap1 in self-renewal and pluripotency of ES cells, we first performed KD of Yap1 using lentivirus-delivered shRNAs in J1 mouse ES cells (Figure 4.1A). In contrast to the previous reports (Lian et al., 2010; Tamm et al., 2011), we found that even with >85% of Yap1 KD, ES cells maintain normal colony morphology with high alkaline phosphatase
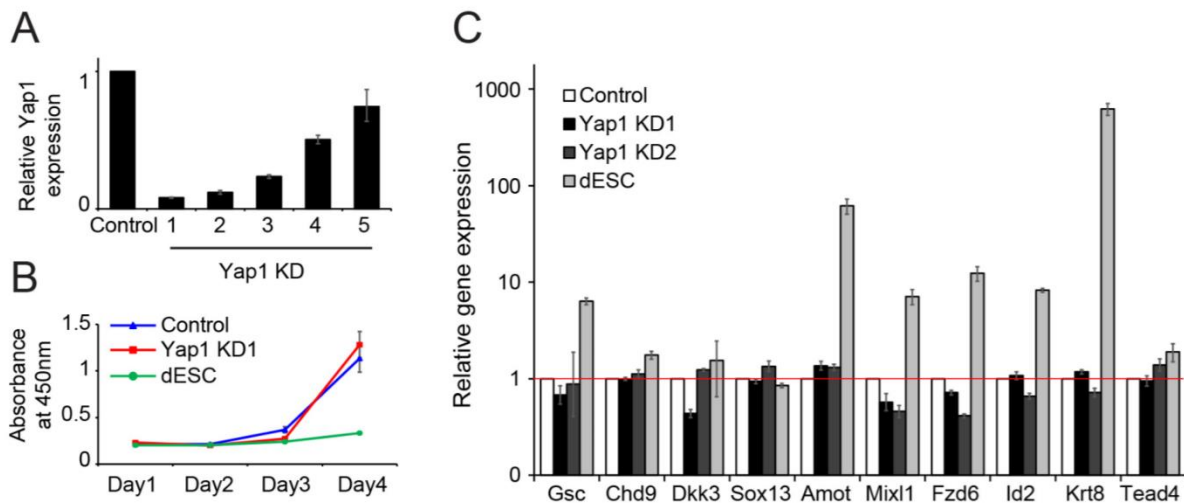
Figure 4.1. Yap1 is dispensable for self-renewal of J1 mouse ES cells. (A) Yap1 mRNA levels measured by RT-qPCR upon shRNA-based KD. Five different shRNA sequences were tested and shRNAs 1 and 2 (KD1 and KD2) were used for further studies. (B) Cell proliferation rates of Yap1 KD cells and control cells. (C) mRNA levels of lineage-specific marker genes upon KD of Yap1. Differentiating ES cells (dESC) were used as control cells.

(AP) activity, whereas ES cells with KD of Pou5f1 undergo differentiation accompanied by loss of AP activity, as expected (Figure 4.2A). We additionally found that these Yap1-depleted ES cells show comparable proliferation rate to that of control ES cells (Figure 4.1B). In agreement with these observations, overall expression levels of ES cell core pluripotency factors, such as Pou5f1 and Nanog, as well as several lineage specific regulators were not significantly altered upon KD of Yap1 (Figures 4.2B-D, and 4.1C). We validated our observation by testing two additional ES cell lines (E14 and CJ7), and confirmed that KD of Yap1 does not significantly affect the features of normal self-renewing ES cells (Figure 4.3A-F).
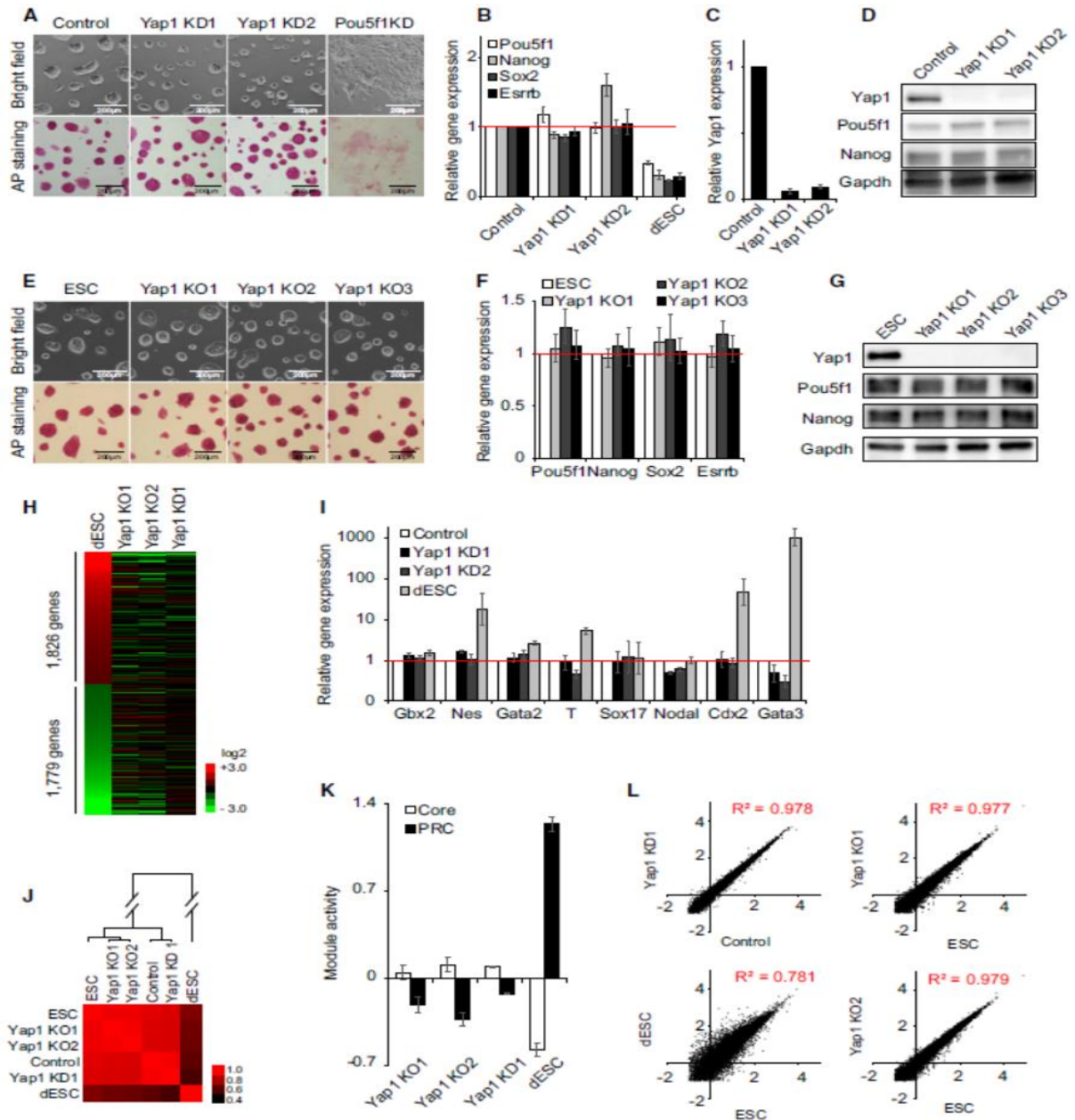
Figure 4.2. Yap1 is dispensable for self-renewal of J1 mouse ES cells. (A) Colony morphology and alkaline phosphatase (AP) activity of ES cells upon KD of Yap1 and Pou5f1. KD1 and KD2 indicate two different shRNA sequences tested. All the following cell morphology and AP staining pictures were taken two passages (4 days) after lentivirus infection unless otherwise stated. Legend continued on next page.

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(B-C) mRNA expression levels of Pou5f1, Nanog, Sox2, Esrrb (B), and Yap1 (C) upon KD of Yap1. All the following mRNA samples were harvested 4 days after lentivirus infection while passaged every 2 days unless otherwise stated. Data are represented as mean ± SD. (D) Protein levels of Yap1, Pou5f1 and Nanog upon KD of Yap1. All the following protein samples were harvested 4 days after lentivirus infection while passaged every 2 days unless otherwise stated. (E) Colony morphology and AP activity of mouse embryonic stem cells (ESC) and three Yap1 KO clones (KO1-KO3). (F) mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb upon KO of Yap1. Data are represented as mean ± SD. (G) Protein levels of Yap1, Pou5f1 and Nanog in Yap1 KO clones. (H) A heatmap showing relative mRNA expression levels of 3,605 genes differentially expressed (> 2-fold) between ES cells and differentiating ES cells (dESC). Genes were sorted by the fold changes of gene expression between dESC and ES cells. Corresponding gene expression profiles obtained from Yap1 KO1, Yap1 KO2, and Yap1 KD cells are also shown. (I) mRNA expression levels of lineage-specific marker genes upon KD of Yap1. dESC were used as control cells. (J) A heatmap showing Pearson correlation coefficients of gene expression profiles obtained from ESC, control virus infected ES cells (Control), dESC, Yap1 KD cells, and Yap1 KO cells. (K) Relative average module activities (Core and PRC) in Yap1 KD1 cells, KO cells and dESC. Module activities were normalized by the data obtained in ES cells. Data are represented as mean ± SEM. (L) Scatter plots showing $\log_{10}$ (FPKM) values of genes in Yap1 KD1 cells and Control (upper left panel), dESC and ESC (bottom left panel), and Yap1 KO cells and ES cells (right two panels). Pearson's correlation coefficients (R^2) are indicated. 'FPKM' indicates Fragments Per Kilobase of transcript per Million fragments mapped.
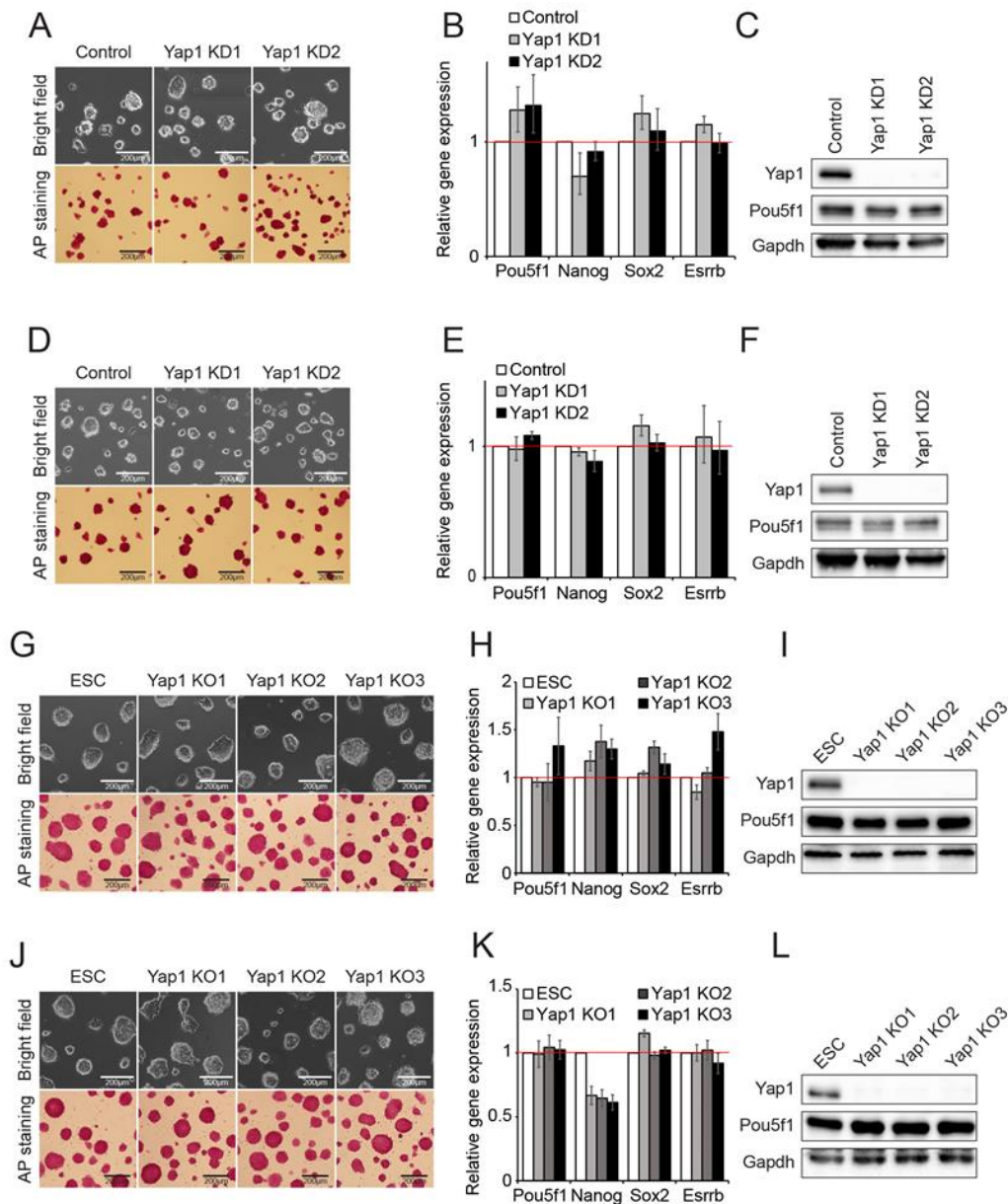
Figure 4.3. Yap1 is dispensable for self-renewal of E14 and CJ7 mouse ES cells. (A-C) Data from CJ7 ES cells. Colony morphology and AP activity of Yap1 KD cells (A), mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb in Yap1 KD cells (B), protein expression levels of Yap1, Pou5f1, and Gapdh were measured in Yap1 KD cells (C). (D-F) Data from E14 ES cells. Colony morphology and AP activity of Yap1 KD cells (D), mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb in Yap1 KD cells (E), protein expression levels of Yap1, Pou5f1, and Gapdh were measured in Yap1 KD cells (F). (G-I) Data from CJ7 ES cells (ESC). Colony morphology and AP activity of Yap1 KO clones (G), mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb in Yap1 KO clones (H), protein expression levels of Yap1, Pou5f1, and Gapdh were measured in Yap1 KO clones (I). (J-L) Data from E14 ES cells. Colony morphology and AP activity of Yap1 KO clones (J), mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb in Yap1 KO clones (K), protein expression levels of Yap1, Pou5f1, and Gapdh were measured in Yap1 KO clones (L).

To rule out the possibility of off-target effects and incomplete depletion due to shRNA-mediated KD strategies, we additionally established Yap1 KO ES cell lines harboring premature stop codons on both alleles by CRISPR-Cas9 based genome editing strategies (Cong et al., 2013; Mali et al., 2013). Consistent with the KD results, Yap1 KO ES cells sustained self-renewing status and showed normal ES colony morphology, high AP activity, and comparable levels of pluripotency-related genes to those of wild-type ES cells (Figures 4.2E-G and 4.3G-L). Yap1 KO ES cells were able to maintain self-renewal for more than a month in culture (Fig 4.4A-C). Taken together, these results indicate that Yap1 is dispensable for self-renewal of mouse ES cells.
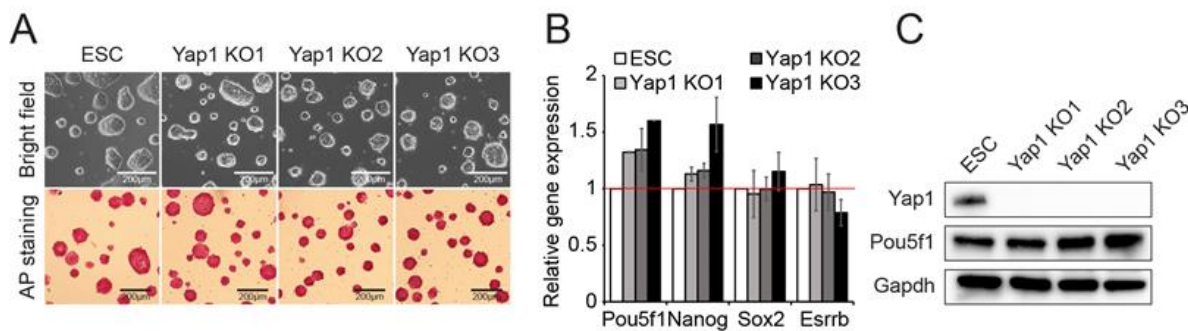
Figure 4.4 Yap1-depleted ES cells can maintain self-renewal for more than a month in culture. (A) Colony morphology and AP activity of Yap1 KO clones cultured for more than a month. (B) mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb in Yap1 KO clones shown in (A). (C) Protein expression levels of Yap1, Pou5f1, and Gapdh were measured in Yap1 KO clones shown in (A).

To further validate the dispensability of Yap1 in self-renewal of ES cells, we sought to monitor the global gene expression profiles of normal, differentiating, Yap1 KD, and Yap1 KO ES cells using RNA-seq approaches. As expected, comparison of expression profiles between ES and differentiating ES cells revealed many differentially expressed genes (DEGs) (Figure 4.2H). However, expression levels of these genes were not altered significantly upon KD or KO of Yap1 (Figure 4.2H) which was further confirmed by RT-

qPCR (Figure 4.2I). Overall, these results indicate that the depletion of Yap1 does not trigger differentiation of ES cells.

Unlike differentiating ES cells, a hierarchical clustering of global expression data revealed that Yap1 KD and Yap1 KO ES cells were clustered together with normal and control ES cells, indicating that Yap1-deficient ES cells have similar expression profiles to those of normal ES cells (Figure 4.2J). We also investigated the activity of previously defined functional modules in ES cells (Core and PRC) (Kim et al., 2010). Module activity is defined as an averaged expression of all genes in each module. Briefly, the Core module includes core pluripotency factors such as Pou5f1, Nanog, and Sox2, most of which are highly expressed in self-renewing ES cells. On the other hand, the PRC module includes many lineage-specific regulators, such as Fgf5, Bmp4, and Hand1, most of which are repressed in ES cells. Since differentiation of ES cells decreases the activity of Core module but increases the activity of PRC module (Kim et al., 2010), we sought to examine module activities upon KD or KO of Yap1 to test if cells maintain self-renewal. As shown in Fig 1K, ES cells with depletion of Yap1 did not show down-regulation of Core module activity or up-regulation of PRC module activity, suggesting that Yap1-depleted ES cells largely maintain self-renewing and undifferentiated states. Further correlation analyses verified that the global expression patterns of Yap1-depleted ES cells showed higher correlation with those of control ES cells ($R^2 = 0.978$ for Yap1 KD, $R^2 = 0.977$ for Yap1 KO1, and $R^2 = 0.979$ for Yap1 KO2) than differentiating ES cells ($R^2 = 0.781$) (Figure 4.2L). Collectively, these data provide strong evidence that the depletion of Yap1 does not significantly alter the self-renewal of ES cells.

Taz and Tead family proteins are not required for self-renewal of ES cells, and do not compensate Yap1 functions in Yap1-depleted ES cells

Taz is homologous to Yap1 and has similar functions to Yap1, such as regulation of proliferation and activation of TE lineage markers (Home et al., 2012; Imajo et al., 2014). To rule out the possibility of compensation by Taz in Yap1 KD ES cells, we performed both single and double KD of Yap1 and Taz using shRNAs under drug selections (Blasticidin and Puromycin, respectively). J1 ES cells with depletion of both Yap1 and Taz (>85% of KD for each) maintained typical colony morphology as well as high AP activity (Figure 4.5A, B). Additionally, the levels of pluripotency markers, such as Pou5f1 and Nanog as well as various lineage markers were not significantly affected by either single or double KD of Yap1 and Taz (Figure 4.5C, D), indicating that the dispensability of Yap1 in self-renewing ES cells is not due to the compensatory effect of Taz.

Since Yap1 is known to require Tead family proteins to activate its downstream target genes in NIH-3T3 and MCF10A cell lines (Li et al., 2010b; Zhao et al., 2008, 2009), we investigated whether Tead proteins are also dispensable for the maintenance of ES cells. To do so, we first performed KD of Tead2 in ES cells. In contrast to the previous report (Tamm et al., 2011), we did not observe any significant alteration of cell morphology or reduced AP activity upon down-regulation of Tead2 (at least >90% of KD in mRNA levels) (Figure 4.6A-B). In accordance with the colony morphology, Tead2 KD ES cells expressed similar levels of pluripotency genes compared to wild-type ES cells (Figure 4.6C-D). These results were confirmed by generation of three independent Tead2 KO ES cell clones using CRISPR-Cas9 strategies. These Tead2 KO clones also maintained self-renewal without differentiation (Figure 4.6E-G). We additionally conducted triple KD of Tead1/3/4 with triple drug selection (at least >80% of KD for each), and did not observe any significant

alteration of cell morphology or AP activity which is in contrast to the previous report (Lian et al., 2010) (Figure 4.5E-F). Similar to the results obtained from the KD of Yap1, triple Tead KD ES cells expressed comparable levels of pluripotency genes shown in wild-type ES cells without any significant activation of lineage-specific regulators (Figure 4.5G-I). The results were further validated by double KD of Tead1/3 in Tead4 KO cells. ES cells with Tead4 KO and Tead1/3 KD also maintained self-renewal (Figure 4.6H-J). Collectively, our data suggest that both Yap1 and Yap1-associated proteins such as Taz and Tead are not required for self-renewal of ES cells.

Yap1 is induced and translocated into the nucleus upon differentiation of ES cells

In order to investigate the roles of Yap1 in differentiation of ES cells, we examined the expression level of Yap1 in self-renewing mouse ES cells as well as upon differentiation of ES cells. Analysis of published mRNA expression data obtained upon time-course differentiation of embryoid body (EB) (Hailesellasse Sene et al., 2007) revealed that Yap1 is moderately expressed in ES cells while its expression gradually increases upon differentiation (Figure 4.7A). We differentiated mouse J1 ES cells by the withdrawal of leukemia inhibitory factor (LIF) in the culture media and examined the level of Yap1. Consistent with the results from the EB differentiation, both mRNA and protein levels of Yap1 were moderately increased upon spontaneous differentiation (Figure 4.7B, C).

Since active Hippo signaling leads to phosphorylation and cytoplasmic sequestration of Yap1, thereby blocking Yap1's function as a transcriptional coactivator (Cockburn et al., 2013; Nishioka et al., 2009; Zhao et al., 2007), we examined the levels of phospho-Yap1 and its subsequent localization in both self-renewing and differentiating ES cells.
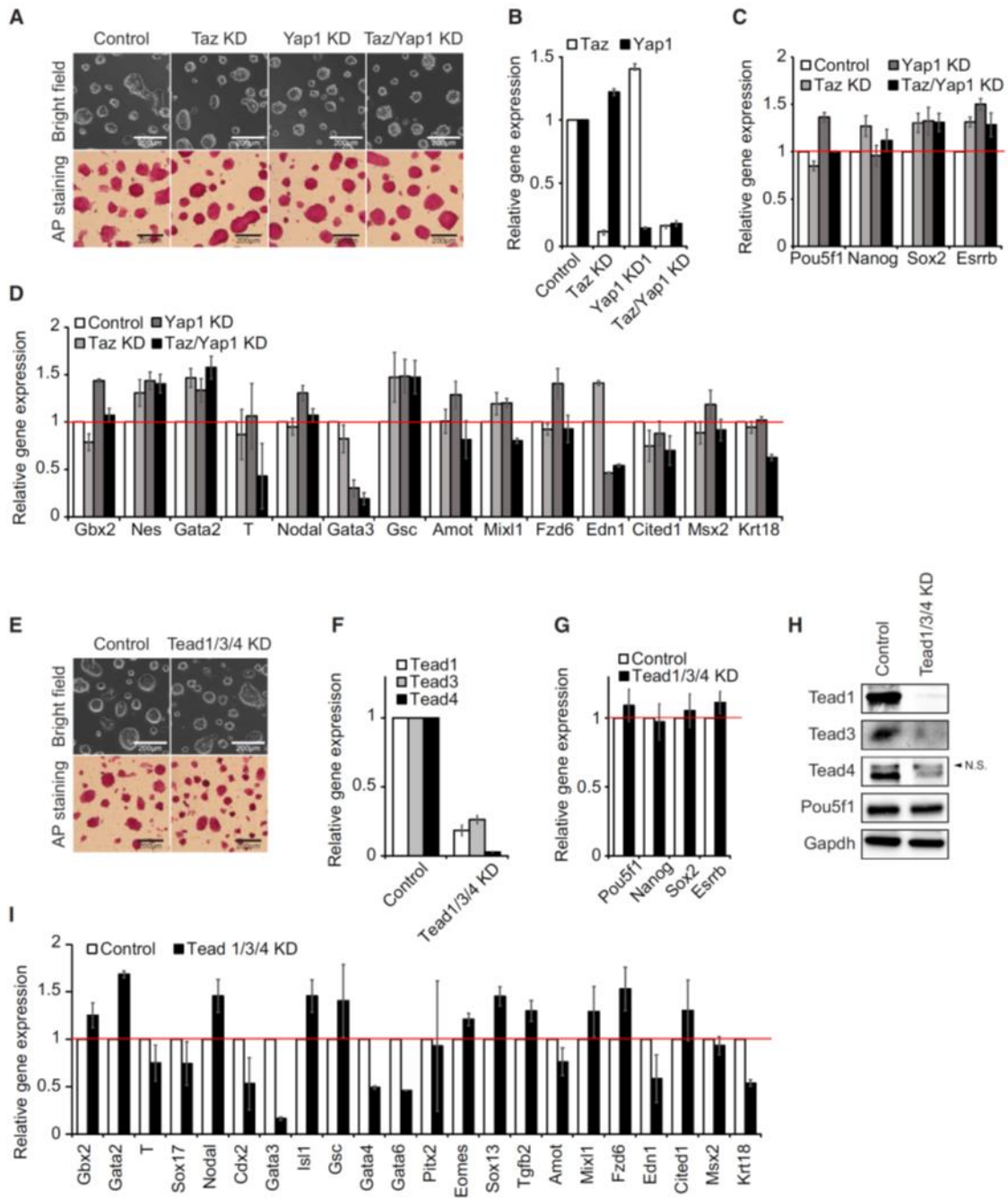
Figure 4.5 Taz and Tead family proteins are not required for the self-renewal of J1 mouse ES cells. (A) Colony morphology and AP activity of ES cells upon KD of Yap1 and Taz. (B-D) mRNA expression levels of Yap1 and Taz (B), Pou5f1, Nanog, Sox2, and Esrrb (C), and lineage-specific marker genes upon KD of Yap1 and Taz. Data are represented as mean ± SD. (E) Colony morphology and AP activity of ES cells upon KD of Tead 1/3/4. (F-G) mRNA expression levels of Tead1, Tead3, and Tead4 (F) and Pou5f1, Nanog, Sox2, and Esrrb (G) upon KD of Tead 1/3/4. Data are represented as mean ± SD. (H) Protein levels of Tead1, Tead3, Tead4, and Pou5f1 upon KD of Tead 1/3/4. (I) mRNA expression levels of lineage-specific marker genes upon KD of Tead 1/3/4.
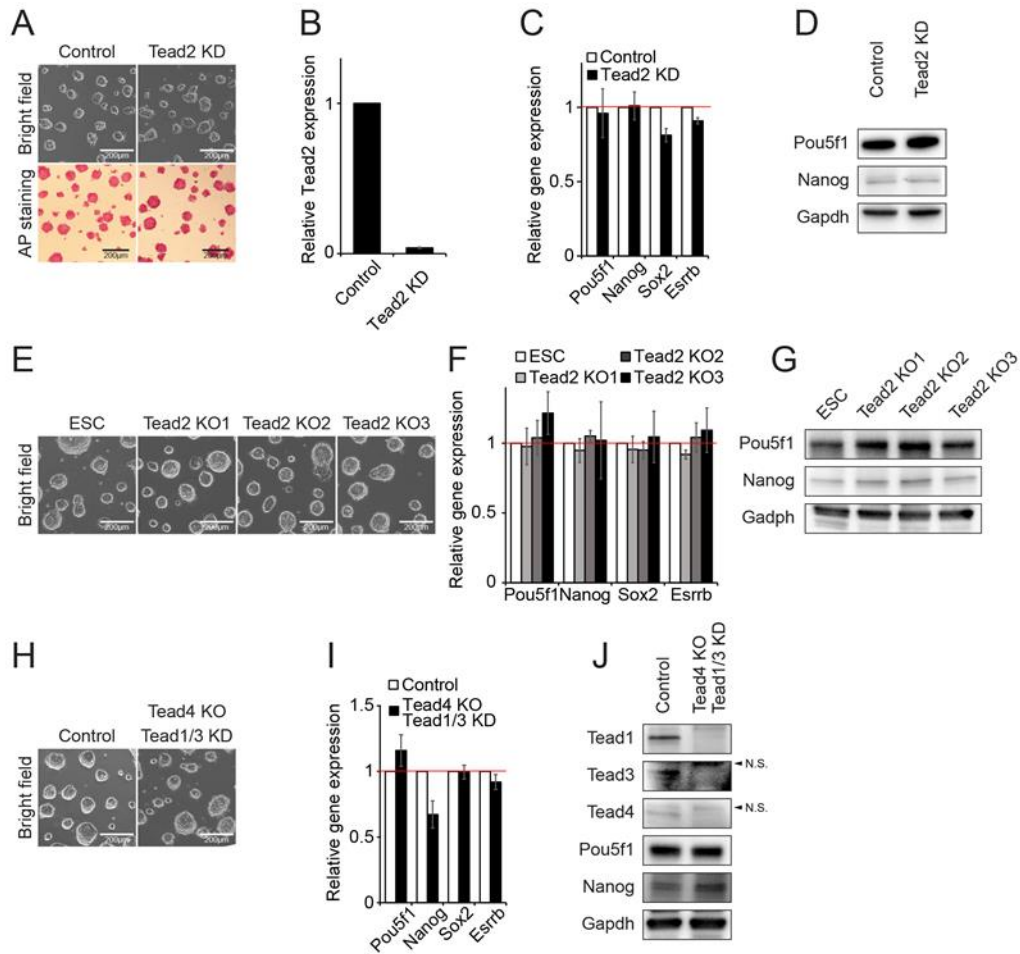
Figure 4.6 Tead family proteins are not required for the self-renewal of ES cells. (A) Colony morphology and AP activity of Tead2 KD ES cells. (B) mRNA levels of Tead2 measured by RT-qPCR upon shRNA-based KD. (C) mRNA expression levels of Pou5f1, Nanog, Sox2, and Esrrb upon KD of Tead2. Data are represented as mean ± SD. (D) Protein levels of Pou5f1 and Nanog upon KD of Tead2. (E) Colony morphology of three Tead2 KO clones (KO1-KO3) and control ES cells. (F) mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb upon KO of Tead2. Data are represented as mean ± SD. (G) Protein levels of Pou5f1 and Nanog in Tead2 KO clones. (H) Colony morphology of ES cells upon KO of Tead4 and KD of Tead1/3. (I) mRNA levels of Pou5f1, Nanog, Sox2, and Esrrb upon KO of Tead4 and KD of Tead1/3. Data are represented as mean ± SD. (J) Protein levels of Pou5f1 and Nanog in Tead4 KO and Tead1/3 KD ES cells.

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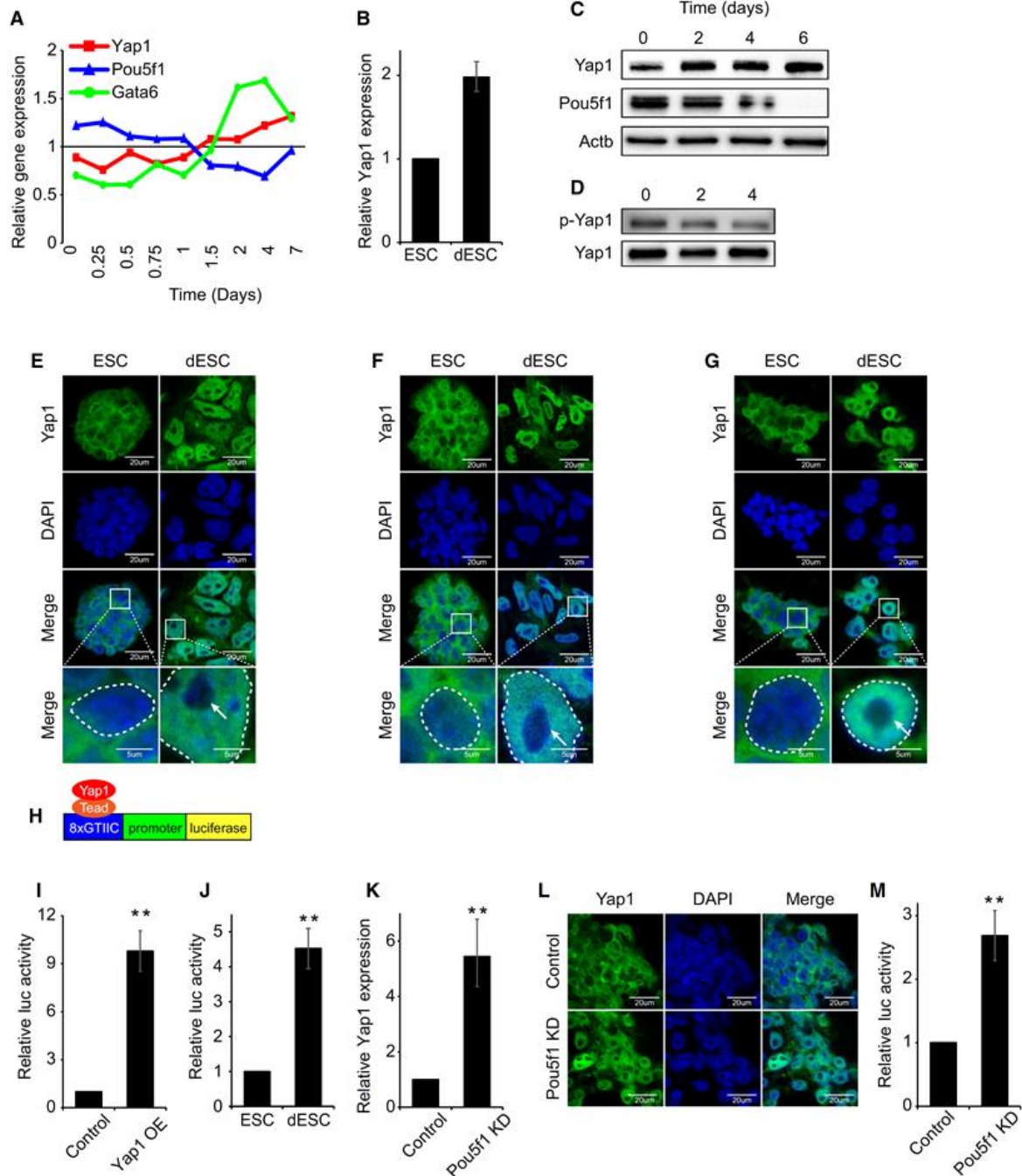
Figure 4.7. Yap1 is up-regulated and translocated into nucleus during ES cell differentiation. (A) Relative mRNA levels of Yap1, Pou5f1 and Gata6 during time-course embryoid body (EB) differentiation. Gene expression data were obtained from GSE3749. Pou5f1 and Gata6 serve as representative ES cell marker and lineage-specific marker, respectively. (B) Relative Yap1 mRNA levels in ES cells (ESC) and differentiating ES cells (dESC) (LIF withdrawal for 4 days) and data are represented as mean ± SD. To differentiate ES cells, cells were incubated in LIF withdrawn medium for 4 days. Both ESC and dESC were passaged every 2 days. (C) Protein levels of Yap1 and Pou5f1 during time-course differentiation upon LIF withdrawal. Legend continued on next page

(D) Phospho-Yap1 levels during time-course differentiation. Samples were normalized by total Yap1 level. (E-G) Immunofluorescence (IF) images depicting localization of Yap1 in J1 (D), CJ7 (E), and E14 (F) mouse ESC (top) and dESC (bottom). The white arrow indicates nucleolus. Bottom panels represent higher magnification of the above panels. Dashed circle indicates nucleus border. (H) A schematic diagram depicting a Yap1 responsive luciferase reporter (8xGTIIC) construct. (I) Luciferase reporter assay using Yap1 responsive luciferase reporter (8xGTIIC) upon transient OE Yap1 in ES cells. *P*-values were calculated using Student's t-test. Two asterisks indicate P<0.01. 'Control' indicates ES cells infected with control virus not expressing any specific shRNA sequence and all RT-qPCR data are represented as mean ± SD (I-K). (J) Relative activity of Yap1 responsive luciferase reporter gene in ESC and dESC. *P*-values were calculated using Student's t-test. (K) Relative Yap1 mRNA levels in Control and Pou5f1 KD ES cells. (L) IF images depicting localization of Yap1 in Control and Pou5f1 KD ES cells. (M) Relative activity of Yap1 responsive luciferase reporter gene upon Pou5f1 KD in ES cells. *P*-values were calculated using Student's t-test.

Western blot analysis showed that Yap1 is highly phosphorylated in self-renewing ES cells but the level of phospho-Yap1 is reduced in differentiating ES cells (Figure 4.7D). Given the fact that phospho-Yap1 is sequestered in the cytoplasm (Cockburn et al., 2013; Nishioka et al., 2009; Zhao et al., 2007), we examined Yap1 localization by immunofluorescence (IF). Consistent with hyper-phosphorylation of Yap1 in ES cells, IF results revealed that Yap1 resides primarily in the cytoplasm of self-renewing ES cells (Figure 4.7E-G). However, upon differentiation of multiple mouse ES cell lines we tested (J1, CJ7, and E14), Yap1 was translocated into the nucleus (Figures 4.7E-G and 4.8A-D). Cytoplasmic Yap1 in ES cells could be attributed to compact ES cell colonies with active Hippo signaling (Zhao et al., 2007), while lower cell density of differentiating ES cells growing in a monolayer leads to inactive Hippo signaling, resulting in the nuclear localization of Yap1.
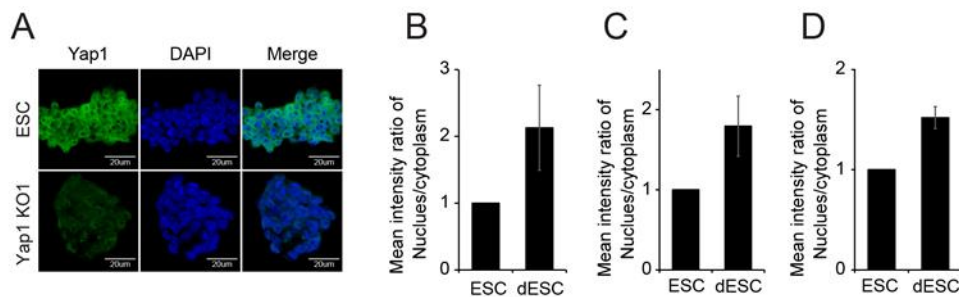
Figure 4.8. Yap1 is translocated into the nucleus upon differentiation of ES cells. (A) Immunofluorescence (IF) images depicting Yap1 signals in J1 ES cells (ESC) and Yap1 KO clone. (B-D) Quantification of relative Yap1 localization between ESC and differentiating ES cells (dESC) from three different cell lines: J1 (B), CJ7 (C), and E14 (D). See material and methods for detailed quantification method.

We further investigated the activity of nuclear Yap1 using a synthetic Yap1-responsive luciferase (8xGTIIC) construct as previously designed for the measurement of Yap1 transcriptional activity in mechanical stress condition (Figure 4.7H) (Dupont et al., 2011; Mahoney et al., 2005; Tamm et al., 2011; Xiao et al., 1991). The luciferase construct

contains repeated Yap1-Tead binding motifs (8 times) in front of the minimal cTNT promoter followed by a luciferase reporter gene (Dupont et al., 2011; Farrance et al., 1992; Mahoney et al., 2005). As shown in Figure 4.7I and J, we observed a significant increase in luciferase activity in both ES cells with transient OE of Yap1 and in differentiating ES cells compared to the reporter activity in self-renewing ES cells, indicating the increased level of nuclear Yap1 either by OE of Yap1 or ES cell differentiation promotes transcription of the reporter gene. An induced level and nuclear localization of Yap1 were also confirmed along with the increased Yap1 activity in Pou5f1 KD ES cells undergoing TE differentiation (Figure 4.7K-M).

Yap1 is required for normal differentiation of ES cells

As we observed increased expression levels and nuclear localization of Yap1 in differentiating ES cells (Figure 4.7), we hypothesized that Yap1 may have critical roles in differentiation of ES cells. To address this, we tested differentiation potential of Yap1 KD ES cells. Upon 3 days of differentiation, completely differentiated and mono-layered cellular morphology with reduced AP activity were observed in control ES cells. However, Yap1 KD cells maintained typical colony morphology with high AP activity comparable to that of self-renewing ES cells even after 2-3 days of differentiation (Figure 4.9A). We further found that expression levels of some pluripotency factors such as Sox2 and Esrrb were relatively highly maintained in Yap1-depleted cells upon differentiation, although the expression of other core factors, Pou5f1 and Nanog, were decreased similar to their levels in control cells upon differentiation (Figure 4.10A). Moreover, up-regulation of various lineage-specific markers, such as Nes, T, Gsc, Gata6, Cdx2, and Gata3 was significantly impaired during differentiation of Yap1-depleted cells (Figure 4.10B), suggesting that the depletion of Yap1 affects differentiation potential of ES cells.

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In order to get further insight into the roles of Yap1 in global transcriptional regulation during differentiation, we analyzed gene expression profiles obtained from RNA-seq of normal ES cells and Yap1-depleted ES cells before and after differentiation. As shown in Figure 4.9B, gene expression patterns of DEGs (Yap1 KD ES cells/wild-type ES cells) upon differentiation showed an inverse correlation with the expression patterns of wild-type differentiating cells over self-renewing ES cells. The heatmap results clearly revealed that Yap1-depleted ES cells are not properly differentiated. Additional analyses of the Core and PRC module activity consistently indicated that Yap1 depletion causes stronger Core module activity with weaker PRC module activity during differentiation, indicating that KD of Yap1 delayed or impaired proper differentiation of ES cells (Figure 4.9C). Gene ontology (GO) term analysis of the list of genes that were not properly induced in Yap1-depleted cells compared to wild-type ES cells upon differentiation also revealed that these genes are implicated in various development-related processes, such as blood vessel development, chordate embryonic development, and *in utero* embryonic development (Figure 4.10C). All these collectively demonstrate that the adequate level of Yap1 is critical in normal differentiation of ES cells.

Ectopic expression of Yap1 in ES cells is sufficient to induce up-regulation of lineage marker genes

To further test roles of Yap1 in ES cell differentiation, we performed OE of Yap1 in ES cells. Yap1 mainly resides in the cytoplasm of self-renewing ES cells. While OE of Yap1 increases both nuclear and cytoplasmic Yap1 levels, we detected more nuclear Yap1 in Yap1 OE cells, indicating that exogenous Yap1 can translocate into the nucleus and act on its target genes (Figure 4.10D-E). Yap1 OE cells also showed flattened morphology similar to that of differentiating ES cells with reduced AP activity even in the presence of LIF (Figure 4.9D). We further examined global gene expression profiles of Yap1 OE cells,

and a clustering analysis showed that the DEGs upon OE of Yap1 (Yap1 OE/ES cells) are highly similar to the DEGs of differentiating ES cells over self-renewing ES cells (Figure 4.9E). Consistently, the activity of the Core module was significantly decreased upon OE of Yap1, while the PRC module activity was dramatically increased (Figures 4.9F and 4.10F). These results suggest that OE of Yap1 is sufficient to trigger ES cell differentiation. Additional GO term analysis revealed that genes up-regulated upon OE of Yap1 are significantly enriched in developmental processes, such as chordate embryonic development, skeletal system development, and embryonic organ development (Figure 4.9G), further demonstrating that the ectopic expression of Yap1 promotes differentiation of ES cells.
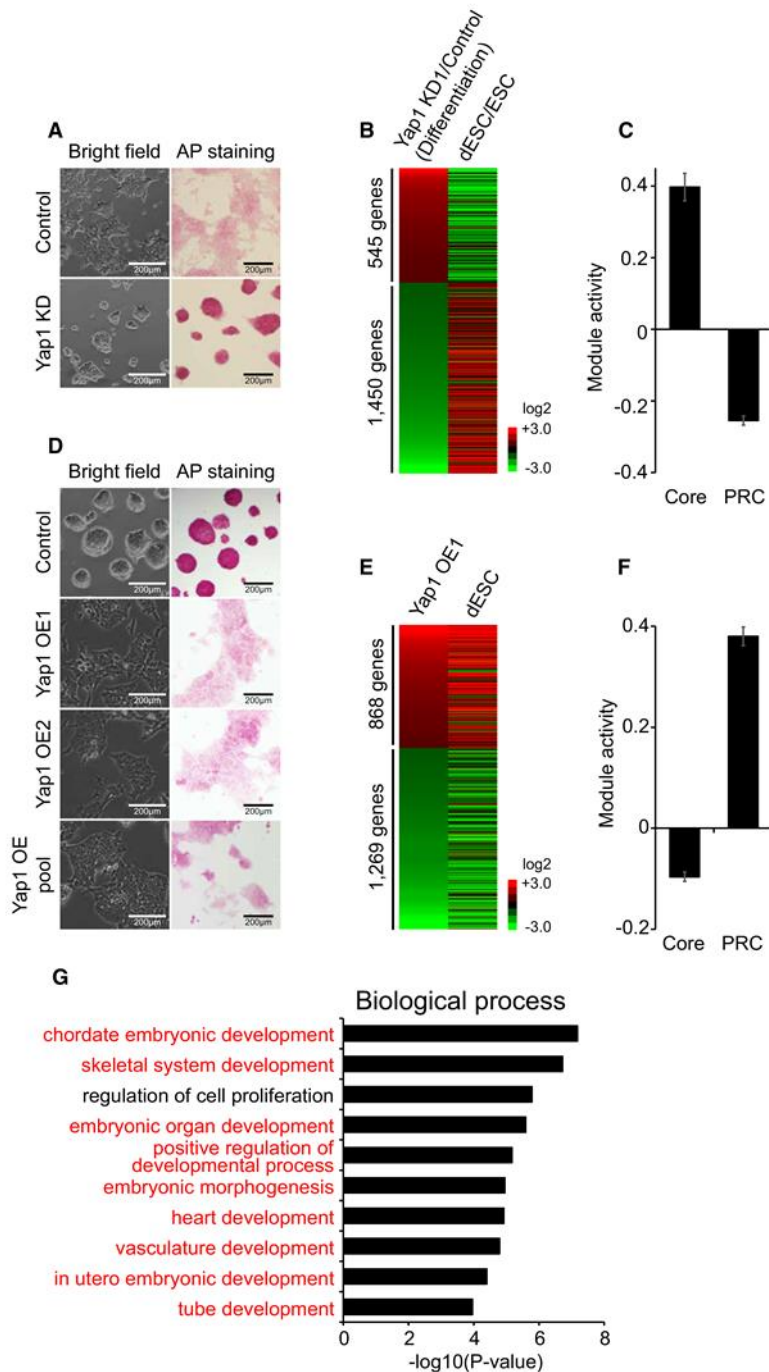
Figure 4.9 Yap1 is required for differentiation of ES cells. (A) Colony morphology and AP activity of Control and Yap1 KD ES cells upon differentiation. Morphology and AP staining pictures were taken 2 days after differentiation. (B) A heatmap showing relative mRNA expression levels of 1,995 genes differentially expressed (> 2-fold) between Yap1 KD ES cells and Control upon 4 days of differentiation. Genes were sorted by the fold changes of gene expression between Yap1 KD ES cells and Control (first column). Corresponding gene expression profiles obtained from differentiating ES cells (dESC) are shown in the second column. Legend continued on next page.

(C) Relative average module activities (Core and PRC modules) between Yap1 KD ES cells and Control upon differentiation. Data are represented as mean ± SEM. (D) Colony morphology and AP activity in Yap1 OE cells. Two different Yap1 OE clones (OE1 and OE2) and pool of Yap1 OE (OE pool) were used. Cell morphology and AP staining pictures were taken 3 weeks after electroporation. (E) A heatmap showing relative mRNA expression levels of 2,137 genes differentially expressed (>2-fold) between Yap1 OE ES cells and control ES cells. Genes were sorted by the fold changes of gene expression between Yap1 OE ES cells and control ES cells (first column) and corresponding gene expression profiles obtained from dESC are shown. (F) Relative average module activities (Core and PRC modules) between Yap1 OE cells and control cells are shown. Data are represented as mean ± SEM. (G) Genes up-regulated in Yap1 OE cells were tested using David 6.7. Significantly enriched gene ontology (GO) terms (biological functions) are shown. Developmental process-related GO terms are highlighted in red.
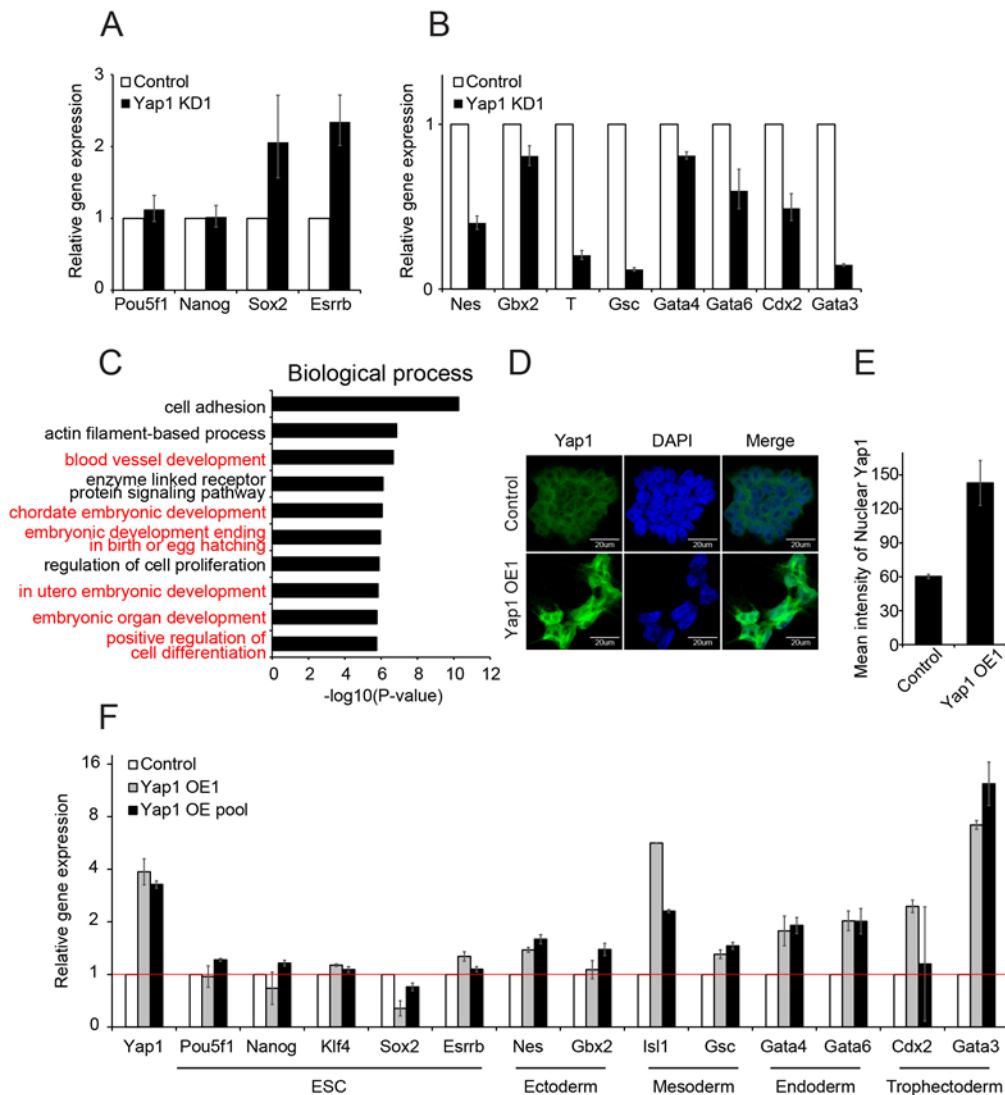
Figure 4.10 Alteration of Yap1 affects differentiation of ES cells. (A-B) mRNA levels of ES cell core factors (A) and lineage-specific markers (B) in Yap1 KD1 cells upon 4 days of differentiation. White bars indicate the levels of genes tested in control virus infected ES cells (Control) upon differentiation. (C) Bar graphs showing significantly enriched gene ontology (GO) terms (biological functions). GO analysis of genes down-regulated in Yap1 KD cells upon differentiation was performed using David 6.7 tools. Developmental process-related terms are highlighted in red. (D) IF images showing localization of Yap1 in control and Yap1 OE cells. (E) Quantification of nuclear Yap1 in control and Yap1 OE cells. (F) mRNA levels of ES cell (ESC) core factors and lineage-specific marker genes in Yap1 OE cells and Yap1 OE pool.

Unlike the well-established functions of the Hippo signaling pathway in the first cell fate decision, the roles of Yap1 in ES cells, ICM, and during differentiation of ES cells or ICM are still not well understood. Here, we reveal that Yap1, a transcriptional effector of Hippo pathway, is a crucial factor implicated in differentiation rather than self-renewal of ES cells. In contrast to the previous reports (Lian et al., 2010; Tamm et al., 2011), a depletion of Yap1 does not show any significant effect on the maintenance of multiple ES cells we tested. This is consistent with the dispensability of Yap1 for maintaining the stemness of ES cells grown under the 2i condition (Azzolin et al., 2014). In agreement with the dispensability of Yap1 in ES cells, other key effectors of the Hippo pathway (Tead family proteins and Taz) do not compensate for depletion of Yap1, and are also not necessary for the maintenance of ES cells, implying that the transcriptional effectors of the Hippo pathway are at least dispensable for self-renewal of mouse ES cells. In addition, we show that Yap1 is mainly sequestered in the cytoplasm of self-renewing ES cells, while it localizes in the nucleus upon differentiation. The predominant nuclear localization of Yap1 in differentiating ES cells may be due to inactive Hippo signaling in the cells growing with lower density. Consistently, OE of Yap1 in ES cells triggers nuclear localization of Yap1 and induces differentiation along with activation of diverse lineage markers. Our global expression analyses further suggest that Yap1 may promote differentiation by activating differentiation-related genes rather than repressing pluripotency-related genes, which is consistent with the observation of Yap1 KO embryo, which dies around E10 due to the defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation (Morin-Kensicki et al., 2006). Noticeably, a recent study done in human ES cells suggested that YAP1 represses mesendoderm differentiation (Beyer et al., 2013), possibly due to the differences in signaling pathways between human and mouse ES cells (Amit et al., 2000; Nichols et al., 2001). In-depth investigation of the impaired regulation of three lineage-

specific genes as well as TE-related genes in Yap1 KD ES cells upon differentiation may provide further insights into the functions of Yap1 in early embryogenesis. Together, our data establish that Yap1 is a critical regulator for proper differentiation but dispensable for self-renewal of ES cells.

CHAPTER 5: THE ROLES OF POU5F1 REPRESSORS ON EMBRYONIC STEM CELLS DIFFERENTIATION

INTRODUCTION

Core transcription factors such as Pou5f1, Sox2, and Nanog maintain pluripotency of ES cells by activating themselves as well as other core transcription factors (reviewed in Orkin, 2005). The maintenance of specific and precise levels of core factors is critical for the pluripotency of ES cells. For example, slight increase of Pou5f1 differentiates ES cells into primitive endoderm and mesoderm (Niwa et al., 2000). Thus, there must be negative regulators that tone down any over-activation of core factors by this autoregulatory and feedforward loop. However, only a few repressors that repress core factors in ES cells have successfully been identified so far. Tcf3 and Tgif1 are two notable examples (Lee et al., 2015; Pereira et al., 2006). Tcf3, transcriptional regulator of Wnt signaling pathway, co-occupies downstream target genes with Pou5f1 and Nanog, thus being an integral member of core pluripotency network (Cole et al., 2008). However, unlike other core factors which form a positive feedforward loop, Tcf3 represses Pou5f1 and Nanog and sustains optimal core factor levels to maintain the pluripotency of ES cells (Pereira et al., 2006). Recently, we reported that Tgif1, the terminal transcriptional repressor of TGFβ signaling pathway, also counterbalances the activity of core factors (Lee et al., 2015). Similar to Tcf3, Tgif1 co-occupies downstream targets with other core factors but represses their activities.

The precise level of Pou5f1 expression determines not only whether ES cell self-renewal can be successfully maintained, but also whether ES cells can successfully differentiate. Elevated levels of Pou5f1 impair the differentiation potential of ES cells and embryonic carcinoma cells by activating core factors and inhibiting lineage specific

regulators. Conversely, depletion of repressors of Pou5f1 seems to have similar physiological effects as OE of Pou5f1. ES cells in which Tcf3 has been depleted, for example, are resistant to differentiation when incubated in LIF withdrawal differentiation media and maintain round shape morphology and high alkaline phosphatase activity (Pereira et al., 2006). In line with this, several lineage specific regulators failed to become induced in Tcf3 KO ES cells during differentiation. Aside from attaining a greater understanding of gene regulation before and after self-renewal, studying Pou5f1 repressors has also provided insight into mammalian development. In agreement with *in vitro* differentiation assays where Tcf3-depleted ES cells do not express mesodermal marker, Tcf3 KO mouse display neonatal death due to a lack of B cells in fetal liver (Merrill, 2003).

Despite the importance of Pouf51 repressors in differentiation of ES cells, only a few such factors have been identified so far. Furthermore, most of the screens in ES cells are focused on identifying activators of core factors, which may be implicated in the regulation of ES cell self-renewal rather than pluripotency. Here, we report that our shRNA-mediated screen identifies Rbpj, Gli2, and Taf6 as negative regulators of Pou5f1 in ES cells. In addition, depletion of Rbpj, downstream effector of Notch signaling pathway, delays differentiation by sustaining expression of core factors and inhibiting induction of lineage specific regulators.

Small scale shRNA mediated screen identifies Pou5f1 repressors in ES cells

Previously, Ding et al., performed genome wide RNAi screen to identify Pou5f1 modulators in ES cells (Ding et al., 2009). Their primary goal was to identify genes essential for the maintenance of ES cell identity. From the screen, KD of 21 genes with esiRNA reduced Pou5f1 promoter driven GFP levels greater than 2 times of standard deviation. The list includes two of Pol II-associating factor 1 complex (Paf1C), which is essential for the maintenance of ES cells. However, there are also 32 genes that induce Pou5f1 expression more than standard deviation upon esiRNA mediated KD (Figure 5.1). We further narrow down the list by filtering out low expression levels in ES cells. Finally, we only selected genes that have known potential repressor function (Table 5.1). This list contains the previously identified Pou5f1 repressor, Tgif1, validating its potential to find other Pou5f1 repressors. In addition, some of the candidates have been reported to have repressive roles in other cellular contexts, but their roles in ES cells have not yet been well characterized. To rule out the possibility that induction of Pou5f1 is due to any off-target effects of shRNA, we tested 5 different shRNA targeting different regions of each genes
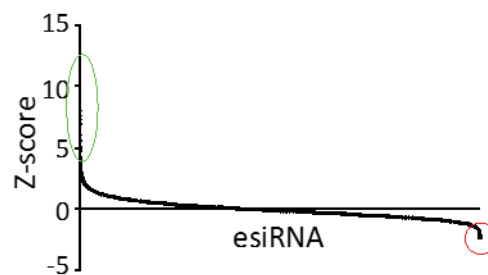
Figure 5.1 Z-score of esiRNA mediated Pou5f1 promoter reporter assay. Data adapted from Ding et al., 2009. GFP was placed under the control of a Pou5f1 promoter. Hits in the green circle correspond to genes that cause reduced fluorescence upon knockdown. These genes therefore sustained Pou5f1 expression, and their reduction caused a drop in Pou5f1 expression (and therefore the GFP reporter). Hits in the red circle correspond to genes that cause increased fluorescence upon knockdown. Ergo, these genes likely repressed Pou5f1 expression, and their reduction caused an increase in Pou5f1 expression.

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(Appendix Table C). Each lentivirus containing shRNA was used to infect J1 mouse ES cells. Then, we measured Pou5f1 levels by RT-qPCR (Figure 5.2). Initial screen identifies Rbpj, Taf6, Gli2, and Med26 as Pou5f1 repressors that induce Pou5f1 upon KD by each shRNAs. However, core factor repression by Med26 was not reproducible in a subsequent validation with top 2 shRNAs (Figure 5.3A), narrowing down final candidate repressors to Rbpj, Taf6, and Gli2.

Gli2	Trip4	Taf1b
Rai14	Zfp13	Mycbp
Sirt6	Med26	Taf6
Phf12	Taf12	Nr6a1
Rbpj	Gli1	Zfp198
Mphospho8	Trip13	Bcl3

Table 5.1 List of candidate Pou5f1 repressors

Rbpj is a transcriptional regulator belonging to the Notch signaling pathway. Notch signaling pathway is activated upon binding with its ligand, which is a transmembrane protein of neighboring cells. Upon activation, the intracellular domain of Notch is cleaved by γ-secretase and then translocates into the nucleus to bind with Rbpj and activate downstream target genes. Though traditionally studied as an activator, in the absence of
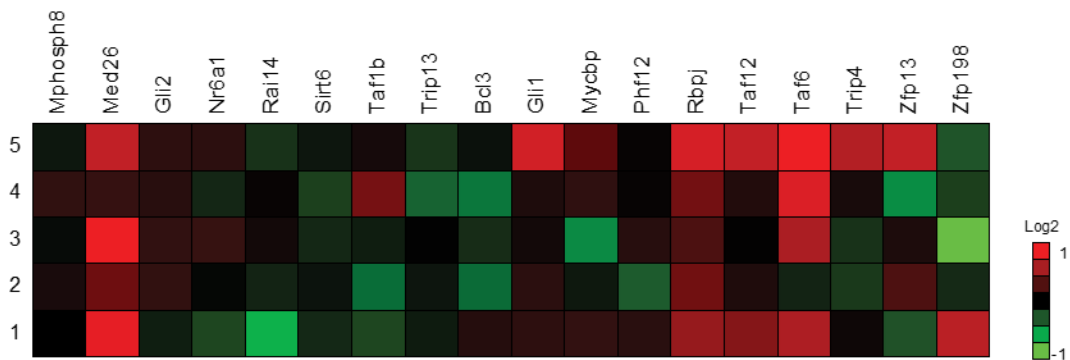
Figure 5.2 shRNA mediated screen to identify Pou5f1 repressor. Heatmap shows relative Pou5f1 levels upon KD of each genes with 5 different shRNAs

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the Notch intracellular domain, Rbpj can function as a transcriptional repressor (Blaess, 2006). In somatic stem cells, activation of Notch signaling increases cell proliferation, but its role in ES cells have not been studied yet. Gli2 is a transcriptional effector of HH signaling pathway. The binding of HH ligand to its receptor causes maturation of Gli2 to its activator form, while the absence of the ligand causes it to be proteolytically processed into its repressive form (Pan et al., 2006). Although Gli2 is mainly processed into activator, the existence of a repressive form Gli2 may also have significant effects on downstream gene expression. It has been reported that Gli2 binds to Pou5f1 in ES cells and increases proliferation rate of ES cells but it is not yet clear whether Gli2 functions as an activator or repressor in ES cells (Li et al., 2013). Taf6 is a subunit of basal transcription factor TFIID. Interestingly, its level is high in ES cells compared to other somatic cells such as NIH 3T3 or fibroblast cell line (Pijnappel et al., 2013). TFIID has been known to play an important role in the pluripotent transcriptional circuitry but the functions of its individual subunits have not been explored yet.
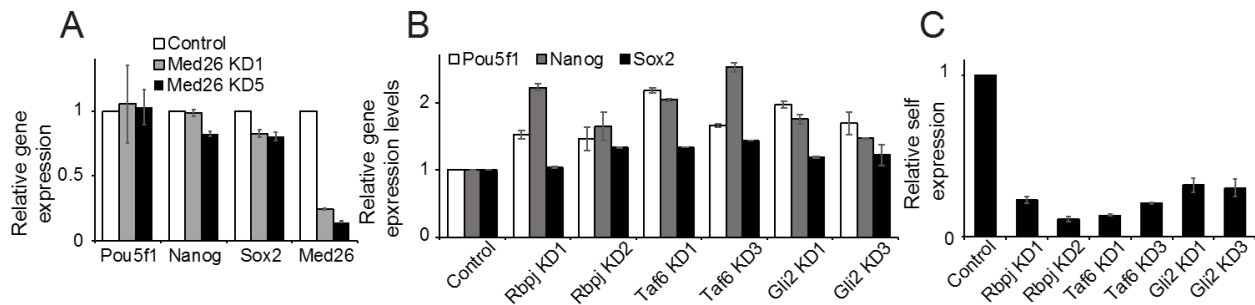
Figure 5.3 The effect of candidate repressors on core factors. (A)RT-qPCR results showing the relative expression levels of core factors and Med26 upon KD of Med26. (B)RT-qPCR results showing the relative expression levels of Pou5f1, Nanog, and Sox2 upon depletion of Rbpj, Taf6, and Gli2 with top 2 shRNAs. (A) KD efficiency of each shRNAs measured by RT-qPCR.

Rbpj, Taf6, and Gli2 repress core factors

Core factors such as Pou5f1, Nanog, and Sox2 form an autoregulatory and feedforward loop by activating their own expression as well as that of other core factors (Cole et al., 2008). In agreement with the interconnected positive regulation of core factors, Tgif1, a known Pou5f1 repressor, not only down-regulates Pou5f1, but also represses multiple other core factors such as Nanog, Sox2, and Tbx3. We investigated whether candidate Pou5f1 repressors can also suppress Nanog and Sox2. KD of Rbpj, Taf6, or Gli2 with top 2 shRNAs induces all the tested core factors such as Pou5f1, Nanog, and Sox2, indicating that these factors affect the autoregulatory loop of core factors (Figure 5.3B, C). Interestingly, even though we have initially screened the genes that repress Pou5f1 levels, KD of Rbpj, Taf6, or Gli2 induces Nanog levels more than Pou5f1. This might be because copy numbers of Pou5f1 mRNA (~1000 RPKM) is significantly higher than Nanog (~200 RPKM). It also suggests that Pou5f1 level is tightly regulated in ES cells so that it is resistant to any changes. Since core factors are co-regulating each other, lower variation of Pou5f1 levels are indirect consequence of Nanog repression of the candidate repressors.

We further validated repressive roles of Rbpj, Taf6, and Gli2 by luciferase assays, which measures Pou5f1 enhancer activity. After infection of ES cells with lentivirus containing shRNA against Rpbj, Taf6, or Gli2, luciferase vector containing the enhancer loci of Pou5f1 as well as the Renilla luciferase internal control were co-transfected into the cells, and luminescence was measured to examine the activity of Pou5f1 enhancer (Figure 5.4A) (Lee et al., 2015). Consistent with Pou5f1 mRNA levels, induction of Pou5f1 enhancer activities upon KD of repressors are relatively minor. We also confirmed that Pou5f1 protein level increased after KD of these factors, supporting the repressive roles of

Rbpj, Taf6, and Gli2 (Figure 5.4B). Collectively, we conclude that KD of Rbpj, Taf6, or Gli2 induces Pou5f1 and other associated core factors.
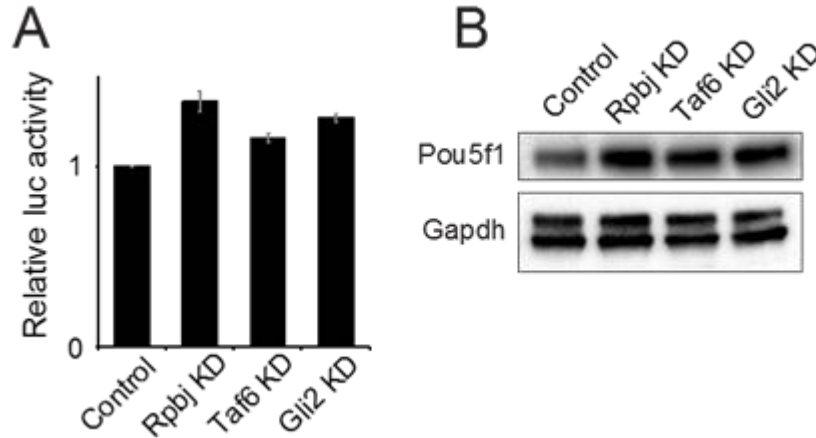
Figure 5.4 The effect of candidate repressors on Pou5f1. (A) Relative activity of Pou5f1 enhancer containing luciferase. Firefly activities are normalized by Renilla luciferase activities. (B) Western blot analysis showing Pou5f1 protein levels upon KD of each candidate repressors.

Depletion of Rbpj impairs ES cell differentiation

Since depletion of Tcf3 delays differentiation of ES cells due to lack of proper core factor downregulation (Pereira et al., 2006), we hypothesized that depletion of Rbpj, Taf6, or Gli2 may also inhibit proper differentiation. After shRNA-mediated KD of Rbpj, Taf6, or Gli2, ES cells were incubated in LIF withdrawn media to induce spontaneous differentiation. After 4 days, cell morphology as well as AP activity were examined to analyze the differentiation status of each samples (Figure 5.5A). As expected, control ES cells display flattened and spiky morphology and weak AP activities, indicating that they are undergoing differentiation. However, Rbpj KD ES cells maintain round shape morphology and high AP activity comparable to that of self-renewing ES cells. Similar to self-renewing condition, Rbpj KD ES cells in differentiation condition have high Pou5f1 levels compared to differentiated control ES cells, suggesting that Rbpj is critical for

repressing Pou5f1 to increase differentiation efficiency after the exit from self-renewal. Additionally, the expression levels of other core factors such as Nanog, Sox2, Klf, and Esrrb are significantly higher after Rbpj KD compared to differentiated control ES cells. Along with sustained level of core factors, lineage specific regulators are not properly induced, meaning that Rbpj KD cells had a severe differentiation defect. The results suggest that repression of core factors via Rbpj is essential for differentiating into the three germ layers after withdrawal of LIF. Surprisingly, KD of Taf6 and Gli2, did not affect the differentiation efficiency of ES cells. This might be because Taf6 and Gli2's functions in repression of Pou5f1 are redundant with other regulators and the subsequent induction of Pou5f1 upon KD of Taf6 or Gli2 is not enough to inhibit differentiation (Figures 5.4B, 5.5B). In contrast, Rbpj may be a more crucial repressor of Pou5f1, so much so that KD of Rbpj is sufficient to disrupt ES cell pluripotency. It would be worthwhile to study whether there is a threshold of core factor expression that inhibits proper differentiation of ES cells, and whether Rbpj alone (but not Taf6 or Gli2) is sufficient to reduce Pou5f1 expression beneath this threshold to enable proper differentiation.

Inhibition of Notch1 synergizes with KD of Rbpj in self-renewing ES cells, but does not further affect differentiation of ES cells

Notch1 is a transmembrane protein of Notch signaling pathway. Upon binding with the ligand, Notch1 intracellular domain (NICD) is cleaved by γ-secretase and translocates into the nucleus (reviewed in Artavanis Tsakonas et al., 1999). Then, it binds to Rbpj to activate downstream target genes. In ES cells, activation of Notch by transient OE of NICD inhibits early mesodermal differentiation (Schroeder et al., 2006). On the other hand, inhibition of Notch1 by small molecule N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT), induces intestinal differentiation when treated with Wnt activator BIO (Ogaki et al., 2013). However, it has not been studied

whether Rbpj is involved in the Notch-mediated differentiation of ES cells. To examine the genetic interaction of Notch1 and Rbpj in ES cells, we first administered DAPT to control and Rbpj KD ES cells. Interestingly, inhibition of Notch by DAPT further increases Pou5f1 and Nanog expression levels in Rbpj KD ES cells, indicating a synergistic effect (Figure 5.6A). The result supports the idea that Rbpj can function as a repressor when it is not bound by NICD, specifically as a repressor of core factor genes (Borggrefe and Oswald, 2009). Then, we tested the effect of DAPT on ES cells in differentiating conditions. Similar to treatment of ES cells in self-renewing conditions, DAPT alone was insufficient to increase expression of core factors, but in stark contrast, it did not seem to synergize with KD of Rbpj in negative affecting their expression (Figure 5.6B). In contrast, DAPT treatment alone slightly inhibited the induction of a few lineage markers such as Cdx2 and T, but KD of Rbpj had a far greater effect on lineage marker expression and DAPT did not show any synergistic effect (Figure 5.6B). These data seem to suggest that inhibition of Notch1 has a greater effect in self-renewing compared to differentiating ES cells, and that the presence of Rbpj takes precedence over the inhibition of Notch in supporting differentiation. Additional studies may want to address the roles of other Notch signaling pathway members as well as the NICD-independent vs. NICD-dependent gene regulatory functions of Rbpj in differentiating ES cells. Furthermore, it would be intriguing to figure out why Notch1 inhibition induces intestinal differentiation but does not seem to affect the efficiency of pan differentiation in ES cells.

DISCUSSION

Since ES cell core factors form a positive feedforward loop but excessive expression of these core factors compromises ES cell identity, there must be other factors which repress these core factors and thereby maintain a moderate level of expression (Niwa

et al., 2000). Until recently, only a few factors have been identified that repress Pou5f1 and thereby regulate ES cell pluripotency (Cole et al., 2008; Lee et al., 2015; Merrill, 2003). Here, we report that Rbpj, Taf6, and Gli2 repress core factors in ES cells. Lentiviral shRNA-mediated KD of any of these genes increases expression of core factors such as Pou5f1 and Nanog at the RNA and protein levels.

Consistent with previous observations of Tcf3-deficient ES cells, which are resistant to differentiation, KD of Rbpj delays proper differentiation of ES cells. Identifying global targets of Rbpj will help to understand how Rbpj regulates differentiation. Comparing the binding loci of Rbpj with other core factor repressors such as Tcf3 and Tgif1 will provide an idea whether Rbpj shares regulatory mechanisms with Tcf3 and Tgif1. Previous report of global binding sites of Rbpj in mouse myogenic cells demonstrate that Rbpj binding sites can be divided into constant and inducible regions (Castel et al., 2013). They further showed that the inducible binding sites largely overlap with NICD binding sites. However, it is not yet clear whether binding of Rbpj can repress its targets. Identifying sites that are occupied by Rbpj but not by NICD will help to understand repressive roles of Rbpj. In addition, it is important to know whether binding patterns of Rbpj changes upon differentiation. Although we have shown that several lineage specific regulators are not properly induced in Rbpj-depleted differentiating ES cells, it is not clear whether it is caused by direct inhibition of sustained Pou5f1. Rbpj has been known to directly activate a neuronal marker Hes1 (Castel et al., 2013), implying that Rbpj may directly activate lineage specific regulators independent of Pou5f1.

Since Rbpj is a transcriptional regulator of Notch signaling pathway, we examined the effect of Notch signaling pathway on the roles of Rbpj in ES cells. Treatment with Notch1 inhibitor synergistically increased expression of core factors in self-renewing, but not differentiating conditions. This suggests that Notch1 is active in self-renewing

condition so that inhibition of Notch1 has synergistic effect with Rbpj KD. On the other hand, ineffectiveness of the Notch inhibitor in differentiating ES cells implies that Notch1 becomes inactive in this condition so that inhibitor may have lost its target. This is consistent with previous report that Rbpj acts as a repressor in the absence of NICD (Li et al., 2012). It is important to examine the roles of other Notch signaling components such as Notch ligands, γ-secretase, Notch family proteins to understand general roles of Notch signaling on ES cell differentiation.

Surprisingly, KD of each of these repressors does increase Pou5f1 levels high enough to induce premature differentiation to primitive endoderm. Previous report showed that Pou5f1 must be increased at least 50% to induce differentiation (Niwa et al., 2000), indicating that other Pou5f1 repressors may be able to partially compensate for the depletion of any of these repressors. Even though we are able to identify three repressors of Pou5f1 in ES cells, depletion of each of them does not strongly perturb Pou5f1 mRNA levels due to the tight intrinsic regulation of Pou5f1 levels to maintain ES cell identity(Kellner and Kikyo, 2010). As a future direction it might be advantageous to use other core factors such as Zfp42 or Esrrb, which may have a wider range of variation in expression, as a reporter gene to identify repressors of core factors.
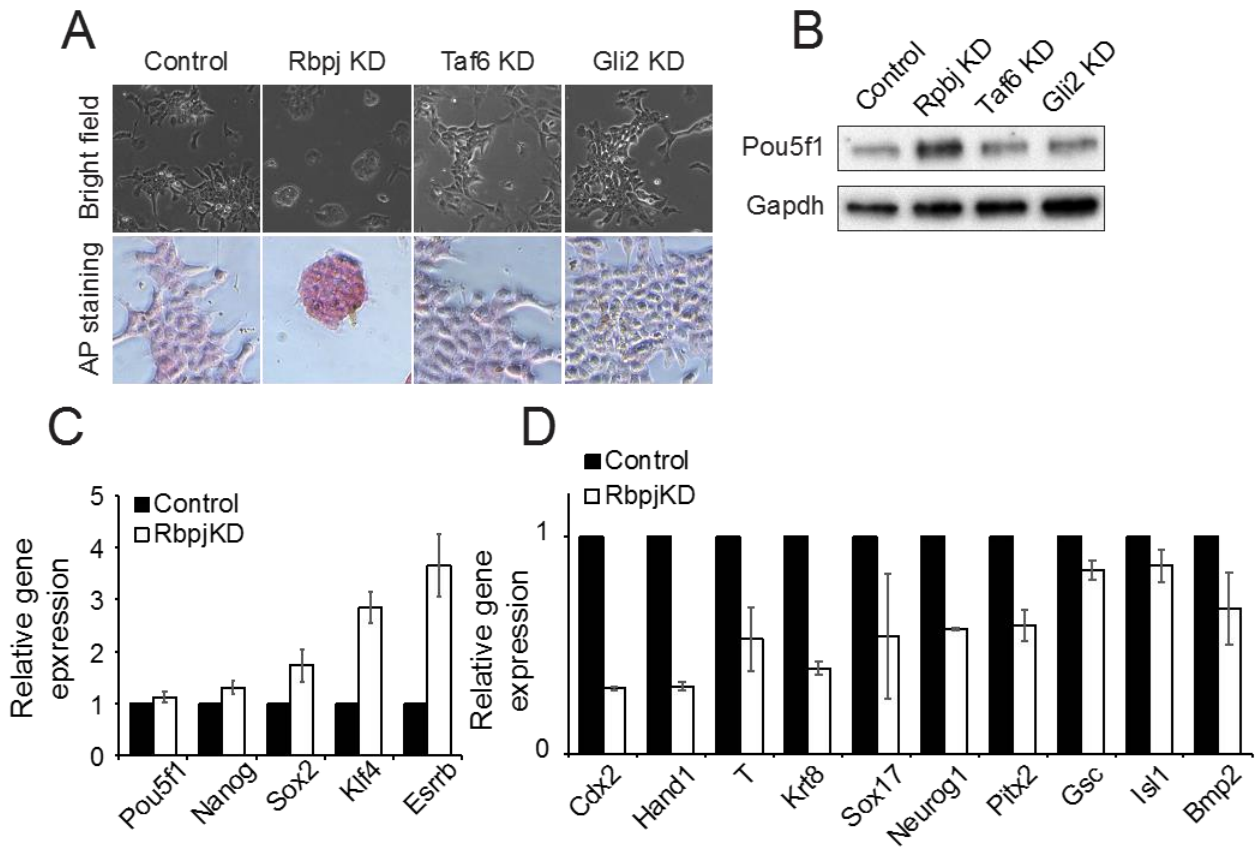
Figure 5.5 KD of Rbpj impairs differentiation of ES cells. (A) Colony morphology and AP activity of differentiating ES cells. ES cells infected with each shRNAs are induced differentiation by withdrawal of LIF. Colony morphology and AP activity pictures were taken 4 days (2 passages) after differentiation. (B)Western blot analysis showing Pou5f1 protein levels of (A). Core factors (C) and lineage specific regulator (D) expression levels upon KD of Rbpj in differentiation condition
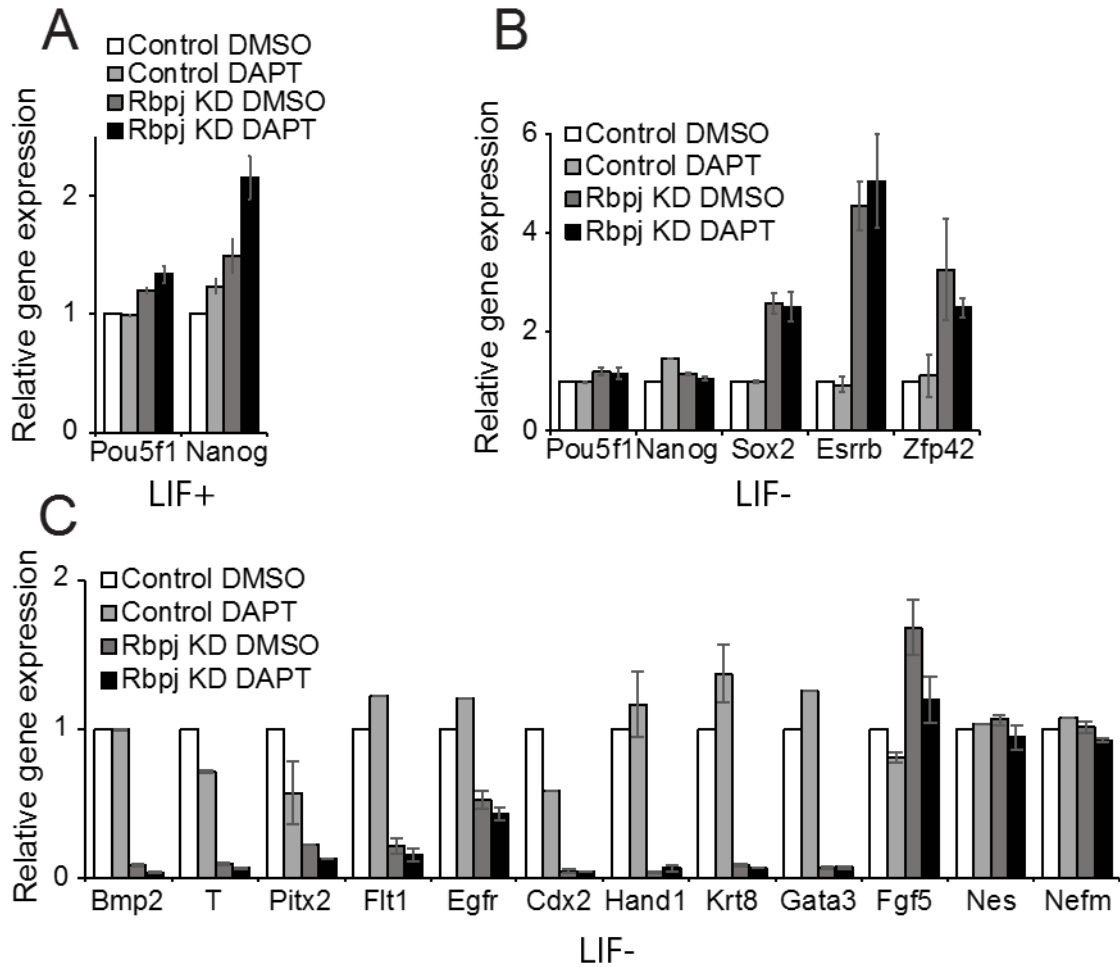
Figure 5.6 Inhibition of Notch synergizes with Rbpj KD in self-renewing, but not differentiating conditions. (A)RT-qPCR data showing Pou5f1 and Nanog mRNA levels upon treatment of DAPT in control and Rbpj KD self-renewing ES cells. RT-qPCR data showing core factors (B) and lineage specific regulators (C) upon treatment of DAPT in control and Rbpj KD differentiating ES cells

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

During my doctoral studies, we have investigated regulatory mechanisms of mouse ES cell differentiation. To this end, we have employed a broad range of cellular and biological approaches, along with high-throughput genomics approach. With this, we have found several novel regulators of ES cell differentiation and broadened our understanding of how transcription factors and signaling pathways regulate differentiation.

First, we developed a signature-based screen that can simultaneously monitor the expression of 48 genes that are essential for ES cell identity and differentiation, and we used this screen to analyze more than 100 KD samples. We quantified mRNA levels of several different classes of genes, including core factors, PRC, 3 lineage specific regulators, and trophectoderm, all of which strongly influence the transcriptional and epigenetic landscape of ES cells. Technical, but not biological, replicates were highly consistent with one another. We have found that cell densities significantly affect differentiation efficiency of ES cells, and surprisingly used this obstacle to develop a project that analyzed the role of density in differentiation. We optimized the screen to maintain even cell density and distribution even after antibiotic selection by concentrating lentivirus titer. As a result of the screen, candidate genes, many of which remain poorly characterized in the literature, are clustered into 3 distinct groups. We discovered that many genes can affect differentiation potential without disrupting self-renewal of ES cells suggesting that self-renewal and pluripotency are functionally separable. Our screen serves as a fruitful resource for future investigators who are interested in genes that affect ES cell differentiation via transcriptional regulation. Further studies are required to understand how each genes affect differentiation of each lineages.

Even though we have optimized the signature-based screen, there are several points that need to be considered for subsequent rounds of screening in different contexts. First, the list of primers must be carefully chosen. Some of the well-known lineage specific regulators of developmental biology are not suitable for monitoring differentiation of ES cells. For example, Gata4 and Gata6, well-known endodermal markers of embryonic development, are not significantly induced upon LIF withdrawn-mediated early differentiation. Even though Pou5f1 protein levels rapidly decrease upon differentiation, Pou5f1 mRNA remains high in early differentiation of ES cells. Using primer sets that are rapidly changing upon differentiation can enhance the credibility of the screen system. Second, pre-amplification cycles must be carefully chosen based on the primer sets. Since this signature-based screen use two rounds of PCR reactions, the signals can be rapidly saturated. Lastly, since cell densities significantly affect the differentiation efficiency, we recommend to avoid any RNAi methods that rely on drug selection and maximize KD efficiency without the use of selection. Maintaining an even cell number and distribution is the key to generate reproducible data. Transient transfection of siRNA or CRISPR/Cas9 can be a suitable alternative to shRNA mediated KD.

In order to unravel the molecular mechanisms of density-mediated differentiation regulation, we studied Yap1, a transcriptional co-activator of Hippo signaling pathway, which senses cell densities and regulates cellular process accordingly. We discovered that Yap1 is essential for proper ES cell differentiation thanks to its co-activator activities in concert with its binding partners, the Tead family proteins. In stark contrast to previous observation, we have shown that Yap1 is completely dispensable for self-renewal of ES cells. Normally, the Hippo pathway sequesters Yap1 and renders it inactive in response to high cell density. Since ES cells form a compact colony with high cell density and become

flattened upon differentiation, we reasoned that Hippo signaling pathway would be active in ES cells, thus inactivating Yap1, and deactivated upon differentiation, thus leading to the liberation of Yap1. Indeed, we have observed that Yap1 is induced and translocated into the nucleus upon differentiation. Consistent with the activity and localization of Yap1, Yap1-depleted ES cells fail to differentiate, and ectopic OE of Yap1 is sufficient to force ES cells to exit self-renewal and undergo spontaneous differentiation.

Since Yap1 is a co-activator, its biological activity depends on its ability to bind to proteins that can then bind to DNA. Despite the crucial roles of Yap1 for differentiation, we do not yet know the direct chromosomal targets and the binding partners of Yap1 in differentiating ES cells. ChIP-seq will identify global targets of Yap1, which will likely include lineage markers and other genes that are capable of moderating the vast transcriptional changes that occur during changes in cell identity. In addition to Tead family proteins, which are essential for Yap1's transcriptional regulatory activities, it is important to understand whether upstream Hippo signaling pathway components such Lats, Mats kinases and Cadherin proteins are also involved in differentiation of ES cells. While studying the functions of Yap1 on ES cell differentiation, we observed that Yap1-depleted ES cells undergo significant cell death upon differentiation. Since Yap1 is a well-known oncogene that protects the cell from apoptosis and it has been shown that low levels of apoptosis are critical for efficient differentiation, understanding how Yap1 negatively controls apoptosis during ES cell differentiation and how this influences lineage specification may be an informative future endeavor. Finally we also need to explore how Yap1 and Hippo signaling pathways translate cell density signals into inhibition of differentiation. It is known that Yap1 influences Wnt signaling in other cellular contexts. Thus, density may indirectly affect numerous other signaling pathways due to its inhibitory effects on Yap1 activation through the Hippo pathway.

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Lastly, we studied the roles of Pou5f1 repressors on ES cell differentiation. Even though maintaining an optimal level of Pou5f1 is critical for preserving ES cell identity and preventing inappropriate differentiation, only few repressors have been identified until now. Using a shRNA-mediated screen, we have identified three novel Pou5f1 repressors in ES cells. KD of Rbpj, Taf6, and Gli2, positive hits of the screen, increases the expression of not only Pou5f1, but also other core factors such as Nanog and Sox2. This is critical, because these core factors orchestrate global gene expression programs in ES cells, but it was previously unclear what upstream factors control them. Among these three candidate repressors, depletion of Rbpj caused ES cells to fail to differentiate normally and maintain stemness even in the absence of LIF due to heightened expression of Pou5f1. However, the other two candidate repressors were dispensable for differentiation. This implies that there might be a critical threshold of Pou5f1 levels that allow to maintain normal differentiation of ES cells. In addition, there may be unknown targets that are regulated by only Rbpj but not by Taf6 or Gli2, which help to repress core factors. Since Rbpj is a transcriptional regulator of Notch signaling pathway, we asked how Notch1 affects Rbpj-mediated differentiation. It is known that activation of Notch1 leads to the liberation of the NICD fragment, which can bind to Rbpj and cause it to function as an activator, rather than as a repressor. We inhibited Notch1 and found that in self-renewing ES cells, combined inhibition of Notch1 and KD of Rbpj synergized to activate expression of core factors. However, inhibition of Notch1 does not exhibit any synergistic effect with Rbpj KD in differentiating ES cells, implying that Notch1 may become inactive upon differentiation. Overall, my doctoral research has led to advances in our understanding of pluripotency and its upstream regulators, and this knowledge can be applied to technologies that demand precise regulation of cell fate, including medicine and stem cell therapy.

APPENDIX

Appendix Table A. shRNA used in chapter 3

Gene	Clone	sequence
Fubp3	1	CCGGCATAGGAAGGAACGGAGAAATCTCGAGATTTCTCCGTTCCTTCCTATGTTTTTTG
Fubp3	2	CCGGCATCACTGGAGACCCATTTAACTCGAGTTAAATGGGTCTCCAGTGATGTTTTTTG
Fubp3	3	CCGGGCCTGGCTTTCACAACGATATCTCGAGATATCGTTGTGAAAGCCAGGCTTTTTG
Fubp3	4	CCGGGACCCTAACCTGCGGATATTTCTCGAGAAATATCCGCAGGTTAGGGTCTTTTTG
Fubp3	5	CCGGACTAAACATTGCTGGTAATTTCTCGAGAAATTACCAGCAATGTTTAGTTTTTTG
Asxl1	1	CCGGGTGGAAAGCTGTGGGTCTAATCTCGAGATTAGACCCACAGCTTTCCACTTTTTG
Asxl1	2	CCGGGTCGGCCCACTTACCAGATATCTCGAGATATCTGGTAAGTGGGCCGACTTTTTG
Asxl1	3	CCGGTGCCACCCACCCATCATATTTCTCGAGAAATATGATGGGTGGGTGGCATTTTTG
Asxl1	4	CCGGTCAGCGGCAGTGCACTCAATACTCGAGTATTGAGTGCACTGCCGCTGATTTTTG
Asxl1	5	CCGGGTGCCACAGGCCTACTCATAACTCGAGTTATGAGTAGGCCTGTGGCACTTTTTG
Zfp36l1	1	CCGGCCACATACAATATCTGTGTAACTCGAGTTACACAGATATTGTATGTGGTTTTTG
Zfp36l1	2	CCGGCCGCTGCCACTTCATTCATAACTCGAGTTATGAATGAAGTGGCAGCGGTTTTTG
Zfp36l1	3	CCGGAGACATTCCGCTCGCAGATTTCTCGAGAAATCTGCGAGCGGAATGTCTTTTTTG
Zfp36l1	4	CCGGCAGCTTCTCAGCAGCCTTAAGCTCGAGCTTAAGGCTGCTGAGAAGCTGTTTTTG
Zfp36l1	5	CCGGTGTGCCGTCCCTTCGAAGAAACTCGAGTTTCTTCGAAGGGACGGCACATTTTTG
Cbx1	1	CCGGCCTGACCTTATTGCTGAGTTTCTCGAGAAACTCAGCAATAAGGTCAGGTTTTTG
Cbx1	2	CCGGTCTTCTAAAGTGGAAGGGTTTCTCGAGAAACCCTTCCACTTTAGAAGATTTTTG
Cbx1	3	CCGGCAGAGCGGATTATTGGAGCTACTCGAGTAGCTCCAATAATCCGCTCTGTTTTTG
Cbx1	4	CCGGGAGTTTCTACAGTCACAGAAACTCGAGTTTCTGTGACTGTAGAAACTCTTTTTG
Cbx1	5	CCGGGAGGTACTAGAAGAAGAGGAACTCGAGTTCCTCTTCTTCTAGTACCTCTTTTTG
Mybl2	1	CCGGCCAGAATCTCTCAAGCGTGAACTCGAGTTCACGCTTGAGAGATTCTGGTTTTTG
Mybl2	2	CCGGGTCAAGAAGTATGGCACCAAACTCGAGTTTGGTGCCATACTTCTTGACTTTTTG
Mybl2	3	CCGGGCTCTCGACATTATGGATGAACTCGAGTTCATCCATAATGTCGAGAGCTTTTTG
Mybl2	4	CCGGGAGACAACAGATGTAAGGTTACTCGAGTAACCTTACATCTGTTGTCTCTTTTTG
Mybl2	5	CCGGTCTGCTAAAGAACTCGGACATCTCGAGATGTCCGAGTTCTTTAGCAGATTTTTG
Msc	1	CCGGGTAACCAAAGAAAGCCCAGTTCTCGAGAACTGGGCTTTCTTTGGTTACTTTTTG
Msc	2	CCGGGCTTTGTGGAACTTCCGCTTACTCGAGTAAGCGGAAGTTCCACAAAGCTTTTTG
Msc	3	CCGGCGACATTTCTTCTGCGGAAGACTCGAGTCTTCCGCAGAAGAAATGTCGTTTTTG
Msc	4	CCGGCGCGGGAGGATGCAAGAGGAACTCGAGTTCCTCTTGCATCCTCCCGCGTTTTTG
Msc	5	CCGGCCAACAGGCTTTGTGGAACTTCTCGAGAAGTTCCACAAAGCCTGTTGGTTTTTG
Msh6	1	CCGGGTGGTAAATTCAGACAACATTCTCGAGAATGTTGTCTGAATTTACCACTTTTTG
Msh6	2	CCGGCCCGTAATCTACCTGAAGAATCTCGAGATTCTTCAGGTAGATTACGGGTTTTTG
Msh6	3	CCGGGCTGGCCTTGATTAACGGATTCTCGAGAATCCGTTAATCAAGGCCAGCTTTTTG

Msh6	4	CCGGGCAGCTCAAGTCTCAGAACTTCTCGAGAAGTTCTGAGACTTGAGCTGCTTTTTG
Msh6	5	CCGGGCAGGGTTTGACTCTGATTATCTCGAGATAATCAGAGTCAAACCCTGCTTTTTG
Kdm2b	1	CCGGGCGTAGTCCACCTCGTGTTATCTCGAGATAACACGAGGTGGACTACGCTTTTTG
Kdm2b	2	CCGGTAGAGTCCCTTCCGGAGAATACTCGAGTATTCTCCGGAAGGGACTCTATTTTTG
Kdm2b	3	CCGGGTTGGATCCATGCGGTTTATACTCGAGTATAAACCGCATGGATCCAACTTTTTG
Kdm2b	4	CCGGGCGGCTCATTATTCGCCATATCTCGAGATATGGCGAATAATGAGCCGCTTTTTG
Kdm2b	5	CCGGCGCTGTGGAAATATCTGTCATCTCGAGATGACAGATATTTCCACAGCGTTTTTG
Bach1	1	CCGGGCTCCTTCAAAGTCCAGCATTCTCGAGAATGCTGGACTTTGAAGGAGCTTTTTG
Bach1	2	CCGGGCGTACACAATATCGAGGAATCTCGAGATTCCTCGATATTGTGTACGCTTTTTG
Bach1	3	CCGGTATAGAATGACCAGGTAATAACTCGAGTTATTACCTGGTCATTCTATATTTTTG
Bach1	4	CCGGGAAATTGGAAACTACGATTATCTCGAGATAATCGTAGTTTCCAATTTCTTTTTG
Bach1	5	CCGGGCTCGACTGTATCCATGACATCTCGAGATGTCATGGATACAGTCGAGCTTTTTG
Phc1	1	CCGGGCTTATTAGCTCAGCCACATACTCGAGTATGTGGCTGAGCTAATAAGCTTTTT
Phc1	2	CCGGCCTCCAAACAGTGATCTAGTACTCGAGTACTAGATCACTGTTTGGAGGTTTTT
Phc1	3	CCGGGCCTGGCTGTTCAGGTTATAACTCGAGTTATAACCTGAACAGCCAGGCTTTTTG
Phc1	4	CCGGCACCTGAACCAACCTCTAAACCTCGAGGTTTAGAGGTTGGTTCAGGTGTTTTTG
Phc1	5	CCGGGCAACCTAATGCGGCTCAATACTCGAGTATTGAGCCGCATTAGGTTGCTTTTT
Hes1	1	CCGGTCTCTTCTGACGGACACTAAACTCGAGTTTAGTGTCCGTCAGAAGAGATTTTTG
Hes1	2	CCGGGAGGCGAAGGGCAAGAATAAACTCGAGTTTATTCTTGCCCTTCGCCTCTTTTTG
Hes1	3	CCGGTCAACACGACACCGGACAAACCTCGAGGTTTGTCCGGTGTCGTGTTGATTTTTG
Hes1	4	CCGGGAAAGATAGCTCCCGGCATTCCTCGAGGAATGCCGGGAGCTATCTTTCTTTTTG
Hes1	5	CCGGTGACCCAGATCAACGCCATGACTCGAGTCATGGCGTTGATCTGGGTCATTTTTG
Rbbp7	1	CCGGCCTTTGATTCAACTGTCATTTCTCGAGAAATGACAGTTGAATCAAAGGTTTTTG
Rbbp7	2	CCGGGCGTGTCATCAACGAAGAGTACTCGAGTACTCTTCGTTGATGACACGCTTTTTG
Rbbp7	3	CCGGGAGAAGTGAATCGTGCTCGTTCTCGAGAACGAGCACGATTCACTTCTCTTTTTG
Rbbp7	4	CCGGCCGTTTCTGTATGACCTGGTTCTCGAGAACCAGGTCATACAGAAACGGTTTTTG
Rbbp7	5	CCGGCGCCTGAATGTGTGGGATTTACTCGAGTAAATCCCACACATTCAGGCGTTTTTG
Id1	1	CCGGGCAGCATGTAATCGACTACATCTCGAGATGTAGTCGATTACATGCTGCTTTTTG
Id1	2	CCGGGCATGTGTTCCAGCCGACGATCTCGAGATCGTCGGCTGGAACACATGCTTTTTG
Id1	3	CCGGGAGCTGAACTCGGAGTCTGAACTCGAGTTCAGACTCCGAGTTCAGCTCTTTTTG
Id1	4	CCGGGCGAGGTGGTACTTGGTCTGTCTCGAGACAGACCAAGTACCACCTCGCTTTTTG
Id1	5	CCGGCGGCTGCTACTCACGCCTCAACTCGAGTTGAGGCGTGAGTAGCAGCCGTTTTTG
Cited2	1	CCGGCCTTAGTGATAGAAATGGGTTCTCGAGAACCCATTTCTATCACTAAGGTTTTTG
Cited2	2	CCGGGAAGCTCAACAACCAGTATTTCTCGAGAAATACTGGTTGTTGAGCTTCTTTTTG
Cited2	3	CCGGCCACCAGATGAACGGGACAAACTCGAGTTTGTCCCGTTCATCTGGTGGTTTTTG
Cited2	4	CCGGGCACGCCTTCAACGCCCTCATCTCGAGATGAGGGCGTTGAAGGCGTGCTTTTTG
Cited2	5	CCGGGTCAGCTGTTGACTCGGTTAACTCGAGTTAACCGAGTCAACAGCTGACTTTTTG
Hnrnpu	1	CCGGCCTGGGAAATACAACATTCTTCTCGAGAAGAATGTTGTATTTCCCAGGTTTTTG

Hnrnpu	2	CCGGGCGAGGAAATAATCGTGGCTACTCGAGTAGCCACGATTATTTCCTCGCTTTTTG
Hnrnpu	3	CCGGCCATAACTGTGCAGTTGAATTCTCGAGAATTCAACTGCACAGTTATGGTTTTTG
Hnrnpu	4	CCGGCAGTGGTTTGTCTTGATACTTCTCGAGAAGTATCAAGACAAACCACTGTTTTTG
Hnrnpu	5	CCGGGCAATAAGAATAAGAGTGGCACTCGAGTGCCACTCTTATTCTTATTGCTTTTTG
Rbm14	1	CCGGGCGCGATACTTAAGGTTGTTTCTCGAGAAACAACCTTAAGTATCGCGCTTTTTG
Rbm14	2	CCGGGCAATGTATCGGCTGCATGTACTCGAGTACATGCAGCCGATACATTGCTTTTTG
Rbm14	3	CCGGCGCTATTCGGGCTCCTATAATCTCGAGATTATAGGAGCCCGAATAGCGTTTTTG
Rbm14	4	CCGGTGCAGCTTCTTCGCTTAATTCCTCGAGGAATTAAGCGAAGAAGCTGCATTTTTG
Rbm14	5	CCGGTATGGTTCCGACCGGCGTTTACTCGAGTAAACGCCGGTCGGAACCATATTTTTG
Eno1	1	CCGGCAATCATGTGATTGGTCTGAACTCGAGTTCAGACCAATCACATGATTGTTTTTG
Eno1	2	CCGGCGGCACAGAGAATAAATCTAACTCGAGTTAGATTTATTCTCTGTGCCGTTTTTG
Eno1	3	CCGGCCCGGCTTTCAATGTGATCAACTCGAGTTGATCACATTGAAAGCCGGGTTTTTG
Eno1	4	CCGGCCTAACATCCTGGAGAACAAACTCGAGTTTGTTCTCCAGGATGTTAGGTTTTTG
Eno1	5	CCGGCTGGTTAGCAAGAAAGTGAATCTCGAGATTCACTTTCTTGCTAACCAGTTTTTG
Med19	1	CCGGTGACGGGCAGCACCAATTTAACTCGAGTTAAATTGGTGCTGCCCGTCATTTTTG
Med19	2	CCGGACCTGGAGCAGGCCTATAATACTCGAGTATTATAGGCCTGCTCCAGGTTTTTG
Med19	3	CCGGCAGTGTCGTCTGATGCATATTCTCGAGAATATGCATCAGACGACACTGTTTTTG
Med19	4	CCGGTGAGCCACAATCACGGATATACTCGAGTATATCCGTGATTGTGGCTCATTTTTG
Med19	5	CCGGTTCTACCTGATGCGGGAATTGCTCGAGCAATTCCCGCATCAGGTAGAATTTTTG
Nolc1	1	CCGGGCAGTGACAACTCCTAAGCAACTCGAGTTGCTTAGGAGTTGTCACTGCTTTTTG
Nolc1	2	CCGGGCAGTGACGATTCTGATTCAACTCGAGTTGAATCAGAATCGTCACTGCTTTTTG
Nolc1	3	CCGGCAAAGGTGAAGTTACAGTCAACTCGAGTTGACTGTAACTTCACCTTTGTTTTTG
Nolc1	4	CCGGGATTCCAGTGAAGAGGAGAAACTCGAGTTTCTCCTCTTCACTGGAATCTTTTTG
Nolc1	5	CCGGCAAGCCAGTCAGTACAACCAACTCGAGTTGGTTGTACTGACTGGCTTGTTTTTG
Prdm14	1	CCGGGCCGTAGCTCCTGCGATATTTCTCGAGAAATATCGCAGGAGCTACGGCTTTTTG
Prdm14	2	CCGGACCTTGAATTACAGGATTAAGCTCGAGCTTAATCCTGTAATTCAAGGTTTTTG
Prdm14	3	CCGGACAATCTGCCCTGGTACAAATCTCGAGATTTGTACCAGGGCAGATTGTTTTTG
Prdm14	4	CCGGTTAAGTCGTCCCAGTCAATATCTCGAGATATTGACTGGGACGACTTAATTTTTG
Prdm14	5	CCGGACTCCCGAAGTACCACGATTTCTCGAGAAATCGTGGTACTTCGGGAGTTTTTG
Dnmt3a	1	CCGGCCAGATGTTCTTTGCCAATAACTCGAGTTATTGGCAAAGAACATCTGGTTTTTG
Dnmt3a	2	CCGGGCAGACCAACATCGAATCCATCTCGAGATGGATTCGATGTTGGTCTGCTTTTTG
Dnmt3a	3	CCGGCCACCAGGTCAAACTCTATAACTCGAGTTATAGAGTTTGACCTGGTGGTTTTTG
Dnmt3a	4	CCGGCCAGAACTGTAAGAACTGCTTCTCGAGAAGCAGTTCTTACAGTTCTGGTTTTTG
Dnmt3a	5	CCGGCCCGTGATGATTGACGCCAAACTCGAGTTTGGCGTCAATCATCACGGGTTTTTG
Klf2	1	CCGGCCTAAACAACGTGTTGGACTTCTCGAGAAGTCCAACACGTTGTTTAGGTTTTTG
Klf2	2	CCGGCCTTATCATTGCAACTGGGAACTCGAGTTCCCAGTTGCAATGATAAGGTTTTTG
Klf2	3	CCGGGACCGATTGTATTTCTATAAGCTCGAGCTTATAGAAATACAATCGGTCTTTTTG
Klf2	4	CCGGGACCCTTTCAGTGCCACTTGTCTCGAGACAAGTGGCACTGAAAGGGTCTTTTTG

Klf2	5	CCGGCCTAAACAACGTGTTGGACTTCTCGAGAAGTCCAACACGTTGTTTAGGTTTTTTG
Rlim	1	CCGGGCCCTGAAGAATGGACCATATCTCGAGATATGGTCCATTCTTCAGGGCTTTTTG
Rlim	2	CCGGGCAGAATCTTAAATACTGGATCTCGAGATCCAGTATTTAAGATTCTGCTTTTTG
Rlim	3	CCGGCGAGAGTAGCTCAGAGATGTTCTCGAGAACATCTCTGAGCTACTCTCGTTTTTG
Rlim	4	CCGGCCTCCAACCATAGTTCTTGATCTCGAGATCAAGAACTATGGTTGGAGGTTTTTG
Rlim	5	CCGGGAGTACTATCAGGATTCCTATCTCGAGATAGGAATCCTGATAGTACTCTTTTTG
Zfp146	1	CCGGGCATTTCCATAGTAGAGAGAACTCGAGTTCTCTCTACTATGGAAATGCTTTTTG
Zfp146	2	CCGGTCAGCCACAAATCGAATCTTACTCGAGTAAGATTCGATTTGTGGCTGATTTTTG
Zfp146	3	CCGGAGCACGAGCATTTCCATAGTACTCGAGTACTATGGAAATGCTCGTGCTTTTTG
Zfp146	4	CCGGAGCTCATCTCTAACCGTGCATCTCGAGATGCACGGTTAGAGATGAGCTTTTTG
Zfp146	5	CCGGCGTCGCTGATTGTGCATGTGACTCGAGTCACATGCACAATCAGCGACGTTTTTG
Creb3	1	CCGGGCGGAGGAAGATTCGTAACAACTCGAGTTGTTACGAATCTTCCTCCGCTTTTTG
Creb3	2	CCGGGCTAGAGGAAAGCGGAGATTTCTCGAGAAATCTCCGCTTTCCTCTAGCTTTTTG
Creb3	3	CCGGCAGCCCTTCTTTGGTCATCTTCTCGAGAAGATGACCAAAGAAGGGCTGTTTTTG
Creb3	4	CCGGACAGGAGATGTCTAGGCTGATCTCGAGATCAGCCTAGACATCTCCTGTTTTTG
Creb3	5	CCGGCGAGGAAGAGAAGAAGCTCTTCTCGAGAAGAGCTTCTTCTTCCTCGTTTTTG
Rybp	1	CCGGCCACCGAGTTTGTAGTGCTTACTCGAGTAAGCACTACAAACTCGGTGGTTTTTG
Rybp	2	CCGGCCAGGAAACCTCGCATCAATTCTCGAGAATTGATGCGAGGTTTCCTGGTTTTTG
Rybp	3	CCGGCAGCAGTGAATGATGAATCTTCTCGAGAAGATTCATCATTCACTGCTGTTTTTG
Rybp	4	CCGGGCATACAGTCTGCTAACGCTACTCGAGTAGCGTTAGCAGACTGTATGCTTTTTG
Rybp	5	CCGGGCATACAGTCTGCTAACGCTACTCGAGTAGCGTTAGCAGACTGTATGCTTTTTG
Zfp280c	1	CCGGGCTCGCATTATCCAGCTCTTTCTCGAGAAAGAGCTGGATAATGCGAGCTTTTTG
Zfp280c	2	CCGGCCCTTATATGAATCACTACATCTCGAGATGTAGTGATTCATATAAGGGTTTTTG
Zfp280c	3	CCGGCGACCCAAGATATACCGTCTTCTCGAGAAGACGGTATATCTTGGGTCGTTTTTG
Zfp280c	4	CCGGCCGTAACTTCTGAATCCGCTTCTCGAGAAGCGGATTCAGAAGTTACGGTTTTTG
Zfp280c	5	CCGGCCCGAAATGCAGACTACAATTCTCGAGAATTGTAGTCTGCATTTCGGGTTTTTG
Rbbp5	1	CCGGCGTTTGAATAATCATGGCTTTCTCGAGAAAGCCATGATTATTCAAACGTTTTTG
Rbbp5	2	CCGGGCTCTATTGTATTTACCCATTCTCGAGAATGGGTAAATACAATAGAGCTTTTTG
Rbbp5	3	CCGGCCGGATTGTTATTTGGGATTTCTCGAGAAATCCCAAATAACAATCCGGTTTTTG
Rbbp5	4	CCGGCCGACCCATCATAGCTTCTATCTCGAGATAGAAGCTATGATGGGTCGGTTTTTG
Rbbp5	5	CCGGGCCTTCTGTAGCAGTGATGAACTCGAGTTCATCACTGCTACAGAAGGCTTTTTG
Tbx3	1	CCGGCCTCCTAAACAAACTAACAAACTCGAGTTTGTTAGTTTGTTTAGGAGGTTTTTG
Tbx3	2	CCGGGCGAATGTTCCCTCCGTTTAACTCGAGTTAAACGGAGGGAACATTCGCTTTTTG
Tbx3	3	CCGGCGGCCAAGATTTCCACCACTACTCGAGTAGTGGTGGAAATCTTGGCCGTTTTTG
Tbx3	4	CCGGGAAACAGAATTCATCGCCGTTCTCGAGAACGGCGATGAATTCTGTTTCTTTTTG
Tbx3	5	CCGGGCTGACGACTGTCGATATAAACTCGAGTTTATATCGACAGTCGTCAGCTTTTTG
H2afy2	1	CCGGGTTCACAGTTTCTGTGTCTTTCTCGAGAAAGACACAGAAACTGTGAACTTTTTG
H2afy2	2	CCGGGCGATAAAGAAGGAACATCAACTCGAGTTGATGTTCCTTCTTTATCGCTTTTTG

H2afy2	3	CCGGCCAGAGTGACATCAGCCATATCTCGAGATATGGCTGATGTCACTCTGGTTTTTG
H2afy2	4	CCGGCGATAAAGAAGGAACATCAAACTCGAGTTTGATGTTCCTTCTTTATCGTTTTTG
H2afy2	5	CCGGGCTACTGAAAGGAGTGACTATCTCGAGATAGTCACTCCTTTCAGTAGCTTTTTG
Pou5f1	1	CCGGGCCGACAACAATGAGAACCTTCTCGAGAAGGTTCTCATTGTTGTCGGCTTTTT
Ash2l	4	CCGGGCCGGACACCTACAAAGATAACTCGAGTTATCTTTGTAGGTGTCCGGCTTTTTG
Dmap1	5	CCGGTGCTTTGCTTTACTCTGACAACTCGAGTTGTCAGAGTAAAGCAAAGCATTTTTG
Htatip	4	CCGGCCTCCTATCCTACCGAAGTTACTCGAGTAACTTCGGTAGGATAGGAGGTTTTTG
Gcn5l2	1	CCGGGCTACCTACAAAGTCAATTATCTCGAGATAATTGACTTTGTAGGTAGCTTTTTG
Trrap	3	CCGGCGCTGAACTTTACTCTCTGTTCTCGAGAACAGAGAGTAAAGTTCAGCGTTTTTG
Tcf3	5	CCGGCGGGACTCCAAGTAGTAATGACTCGAGTCATTACTACTTGGAGTCCCGTTTTTG
Ep400	5	CCGGCGGAAGAATCTCAATGGCATACTCGAGTATGCCATTGAGATTCTTCCGTTTTTG
Myc	4	CCGGCATCCTATGTTGCGGTCGCTACTCGAGTAGCGACCGCAACATAGGATGTTTTTG
Nanog	4	CCGGGCCAACCTGTACTATGTTTAACTCGAGTTAAACATAGTACAGGTTGGCTTTTTG
p53	1	CCGGCCGACCTATCCTTACCATCATCTCGAGATGATGGTAAGGATAGGTCGGTTTTT
Ruvbl2	2	CCGGCCGAGAACAGATCAATGCAAACTCGAGTTTGCATTGATCTGTTCTCGGTTTTTG
Ruvbl1	3	CCGGGCCACAGAGTTTGACCTTGAACTCGAGTTCAAGGTCAAACTCTGTGGCTTTTTG
Ing3	1	CCGGCGATTGGACTTACGACCCAAACTCGAGTTTGGGTCGTAAGTCCAATCGTTTTTG
Brd8	2	CCGGCCACACTTGATCTTAGTCAAACTCGAGTTTGACTAAGATCAAGTGTGGTTTTTG
Epc2	2	CCGGCCTGTCAATGTGCATATCAATCTCGAGATTGATATGCACATTGACAGGTTTTTG
Tgif	4	CCGGGATGGCAAGAGATGCATTATTCTCGAGAATAATGCATCTCTTGCCATCTTTTTTGAAT
Fbxw7	4	CCGGCGCATAGTTAGTGGTTCTGATCTCGAGATCAGAACCACTAACTATGCGTTTTT
Arid3a	4	CCGGTGAGGGAGATAGGCATTTGATCTCGAGATCAAATGCCTATCTCCCTCATTTTTG
LSD1	4	CCGGGCTACATCTTACCTTAGTCATCTCGAGATGACTAAGGTAAGATGTAGCTTTTTG
LSD1	5	CCGGCCACGAGTCAAACCTTTATTTCTCGAGAAATAAAGGTTTGACTCGTGGTTTTTG
E2F4	3	CCGGCATCGGTCTGATCGAGAAGAACTCGAGTTCTTCTCGATCAGACCGATGTTTTTG
E2F4	4	CCGGCCACGATTACATCTACAACCTCTCGAGAGGTTGTAGATGTAATCGTGGTTTTTG
Zfp57	1	CCGGCCTGACATTTGTCGGAAGCAACTCGAGTTGCTTCCGACAAATGTCAGGTTTTTG
Zfp57	5	CCGGCCAACACACTCAGAATGCAAACTCGAGTTTGCATTCTGAGTGTGTTGGTTTTTG
Max	2	CCGGGCAACAGAGTATATCCAGTATCTCGAGATACTGGATATACTCTGTTGCTTTTTG
Max	3	CCGGCCCAAATCCTAGACAAAGCAACTCGAGTTGCTTTGTCTAGGATTTGGGTTTTTG
Tcfap2c	1	CCGGCGCAGTGCAGAATTATATCAACTCGAGTTGATATAATTCTGCACTGCGTTTTTG
Tcfap2c	5	CCGGGCAGCTACTTACCAGTGGTATCTCGAGATACCACTGGTAAGTAGCTGCTTTTTG
H2afx	2	CCGGCGAGTACCTCACTGCCGAGATCTCGAGATCTCGGCAGTGAGGTACTCGTTTTTG
H2afx	3	CCGGGCTCGAGTACCTCACTGCCGACTCGAGTCGGCAGTGAGGTACTCGAGCTTTTTG
Nmyc	2	CCGGCCTCACTCCTAATCCGGTCATCTCGAGATGACCGGATTAGGAGTGAGGTTTTTG
Nmyc	4	CCGGGCAGCAGCAGTTGCTAAAGAACTCGAGTTCTTTAGCAACTGCTGCTGCTTTTTG
Rps19	2	CCGGCCCATATGATGAGAACTGGTTCTCGAGAACCAGTTCTCATCATATGGGTTTTTG
Rps19	4	CCGGCGGTGTCAGACCCAGCCATTTCTCGAGAAATGGCTGGGTCTGACACCGTTTTTG

Surf6	2	CCGGCAGGAACAGAAAGCTATGGATCTCGAGATCCATAGCTTTCTGTTCCTGTTTTTTG
Gbx2	1	CCGGGGATGTGGATTACAGCTCAGATCTCGAGATCTGAGCTGTAATCCACATCTTTTTG
Gbx2	4	CCGGCGAGTCAAAGGTGGAAGATGACTCGAGTCATCTTCCACCTTTGACTCGTTTTTG
Xrn2	1	CCGGCCAGAGGATAACGTCAGGTTACTCGAGTAACCTGACGTTATCCTCTGGTTTTTG
Xrn2	4	CCGGCAACGATACTACAAGAACAAACTCGAGTTTGTTCTTGTAGTATCGTTGTTTTTG
Tgif2	2	CCGGGCAGATATGTAACTGGTTCATCTCGAGATGAACCAGTTACATATCTGCTTTTTG
Tgif2	4	CCGGGCTGCAGATATGTAACTGGTTCTCGAGAACCAGTTACATATCTGCAGCTTTTTG
Ezh2	b	CCGGGCACAAGTCATCCCGTTAAAGCTCGAGCTTTAACGGGATGACTTGTGCTTTTTG
Ezh2	c	CCGGGCGTATAAAGACACCACCTAACTCGAGTTAGGTGGTGTCTTTATACGCTTTTTG
Cdx2	1	CCGGGTTTCACTTTAGTCGATACATCTCGAGATGTATCGACTAAAGTGAAACTTTTTG
Rarg	1	CCGGAGGGAGCAGAAAGGGCTATAACTCGAGTTATAGCCCTTTCTGCTCCCTTTTTT
Rarg	2	CCGGGCTCAGCATTGCCGACCAGATCTCGAGATCTGGTCGGCAATGCTGAGCTTTTT
Rarg	3	CCGGCAATGACAAGTCTTCTGGCTACTCGAGTAGCCAGAAGACTTGTCATTGTTTTT
Rarg	4	CCGGCATTTGAGATGCTGAGCCCTACTCGAGTAGGGCTCAGCATCTCAAATGTTTTT
Rarg	5	CCGGCGGGTCTATAAGCCATGCTTTCTCGAGAAAGCATGGCTTATAGACCCGTTTTT
Sox13	1	CCGGCCTGGTTGTATCCCAAGGAAACTCGAGTTTCCTTGGGATACAACCAGGTTTTTG
Sox13	2	CCGGGCTTTACCTATTCAGCCCATTCTCGAGAATGGGCTGAATAGGTAAAGCTTTTTG
Sox13	3	CCGGGCTGATTCAACAGCAGCACAACTCGAGTTGTGCTGCTGTTGAATCAGCTTTTTG
Sox13	4	CCGGCACTTGTAGATACATTCCCAACTCGAGTTGGGAATGTATCTACAAGTGTTTTTG
Sox13	5	CCGGGCACTTGTAGATACATTCCCACTCGAGTGGGAATGTATCTACAAGTGCTTTTTG
Tcea3	1	CCGGGCCTCAGAAATAGAAGACCATCTCGAGATGGTCTTCTATTTCTGAGGCTTTTTG
Tcea3	2	CCGGCCATATCTATCAAGAACTCAACTCGAGTTGAGTTCTTGATAGATATGGTTTTTG
Tcea3	3	CCGGGCGGTGTAGCAAGTGCAAGAACTCGAGTTCTTGCACTTGCTACACCGCTTTTTG
Tcea3	4	CCGGGAAGCTGAACAGTTGCCAGATCTCGAGATCTGGCAACTGTTCAGCTTCTTTTTG
Tcea3	5	CCGGCTCAGAAATAGAAGACCATATCTCGAGATATGGTCTTCTATTTCTGAGTTTTTG
Hnrpdl	1	CCGGGCGTAAAGATTAAAGTGGAAACTCGAGTTTCCACTTTAATCTTTACGCTTTTTG
Hnrpdl	2	CCGGGCTATGATTATACTGGGTATACTCGAGTATACCCAGTATAATCATAGCTTTTTG
Hnrpdl	3	CCGGCCAGAACAATTACCAGCCCTACTCGAGTAGGGCTGGTAATTGTTCTGGTTTTTG
Hnrpdl	4	CCGGGAGCCAGTAAAGAAACTGTTACTCGAGTAACAGTTTCTTTACTGGCTCTTTTTG
Hnrpdl	5	CCGGGAGCCAGTAAAGAAACTGTTACTCGAGTAACAGTTTCTTTACTGGCTCTTTTTG
Zic5	1	CCGGCCATGTCCTAACCTGAAACAACTCGAGTTGTTTCAGGTTAGGACATGGTTTTTG
Zic5	2	CCGGGCTTCAGAATATGACAGGCTTCTCGAGAAGCCTGTCATATTCTGAAGCTTTTTG
Zic5	3	CCGGGATCGAAAGAAACACTCCCATCTCGAGATGGGAGTGTTTCTTTCGATCTTTTTG
Zic5	4	CCGGGTAAGATTCGAGGCTGTGATACTCGAGTATCACAGCCTCGAATCTTACTTTTTG
Zic5	5	CCGGCCTGAAGTCATGCGGACGATACTCGAGTATCGTCCGCATGACTTCAGGTTTTTG
Tcfcp2l1	1	CCGGCCCGCCTTTGAATCACTAATTCTCGAGAATTAGTGATTCAAAGGCGGGTTTTTG
Tcfcp2l1	2	CCGGGCACTGTATCAGCACGGAATTCTCGAGAATTCCGTGCTGATACAGTGCTTTTTG
Tcfcp2l1	3	CCGGACCTTAACATACCTCAATCAACTCGAGTTGATTGAGGTATGTTAAGGTTTTTTG

Tcfcp2l1	4	CCGGCGTGGTGGTAAGCAATGAGATCTCGAGATCTCATTGCTTACCACCACGTTTTTG
Tcfcp2l1	5	CCGGCGGCTCAAGAGAAGGAGAAATCTCGAGATTTCTCCTTCTCTTGAGCCGTTTTTG
Etv5	1	CCGGGCGACCTTTGATTGACAGAAACTCGAGTTTCTGTCAATCAAAGGTCGCTTTTTG
Etv5	2	CCGGACAACTATTGTGCCTACGATACTCGAGTATCGTAGGCACAATAGTTGTTTTTTG
Etv5	3	CCGGCAGTCTGATAACTTGGTGCTTCTCGAGAAGCACCAAGTTATCAGACTGTTTTTG
Etv5	4	CCGGAGGAAGTTTGTGGACACAGATCTCGAGATCTGTGTCCACAAACTTCCTTTTTG
Etv5	5	CCGGAGCTTGCCCTTTGAGTATTATCTCGAGATAATACTCAAAGGGCAAGCTTTTTG
Zfp219	1	CCGGCTGGTTTCTCAAGGGTCACATCTCGAGATGTGACCCTTGAGAAACCAGTTTTTG
Zfp219	2	CCGGCTCAAGTATCACCTTCAGCGTCTCGAGACGCTGAAGGTGATACTTGAGTTTTTG
Zfp219	3	CCGGGCGGCTACATCTACGCCTGAACTCGAGTTCAGGCGTAGATGTAGCCGCTTTTTG
Zfp219	4	CCGGCTGGTTTCTCAAGGGTCACATCTCGAGATGTGACCCTTGAGAAACCAGTTTTTG
Zfp219	5	CCGGGCGGCTACATCTACGCCTGAACTCGAGTTCAGGCGTAGATGTAGCCGCTTTTTG
Cbx7	1	CCGGCCTGAATGTATTGGGAGGAATCTCGAGATTCCTCCCAATACATTCAGGTTTTTG
Cbx7	2	CCGGCCTCAAGTGAAGTTACCGTGACTCGAGTCACGGTAACTTCACTTGAGGTTTTTG
Cbx7	3	CCGGGCAAAGTTGAATATCTGGTGACTCGAGTCACCAGATATTCAACTTTGCTTTTTG
Cbx7	4	CCGGCGTGACTGACATCACCGCCAACTCGAGTTGGCGGTGATGTCAGTCACGTTTTTG
Cbx7	5	CCGGCGGAAGGGCAAAGTTGAATATCTCGAGATATTCAACTTTGCCCTTCCGTTTTTG
Otx2	1	CCGGCCACTGATTGCTTGGATTATACTCGAGTATAATCCAAGCAATCAGTGGTTTTTTG
Otx2	2	CCGGGCATGGACTGTGGATCTTATTCTCGAGAATAAGATCCACAGTCCATGCTTTTTTG
Otx2	3	CCGGGCTGACTGCTTGGATTATAAACTCGAGTTTATAATCCAAGCAGTCAGCTTTTTG
Otx2	4	CCGGCCTGATTTGCAAATGATTGATCTCGAGATCAATCATTTGCAAATCAGGTTTTTG
Zfp428	1	CCGGCTATAAGGTGAAACAGCGAATCTCGAGATTCGCTGTTTCACCTTATAGTTTTTTG
Zfp428	2	CCGGCGAATATGACCCTGGCTATAACTCGAGTTATAGCCAGGGTCATATTCGTTTTTTG
Zfp428	3	CCGGGAGGAAGATGATGAAGACCTTCTCGAGAAGGTCTTCATCATCTTCCTCTTTTTG
Zfp428	4	CCGGGCTATAAGGTGAAACAGCGAACTCGAGTTCGCTGTTTCACCTTATAGCTTTTTG
Zfp428	5	CCGGCTATAAGGTGAAACAGCGAATCTCGAGATTCGCTGTTTCACCTTATAGTTTTTTG
Myst2	1	CCGGCCCATGCTTTCCTTTGTATTTCTCGAGAAATACAAAGGAAAGCATGGGTTTTTG
Myst2	2	CCGGGCCCTTCAGATGCTCAAGTATCTCGAGATACTTGAGCATCTGAAGGGCTTTTTG
Myst2	3	CCGGCGAAAGCTACAATTTCAACATCTCGAGATGTTGAAATTGTAGCTTTCGTTTTTG
Myst2	4	CCGGGACCTGATAGATGAGTGGATACTCGAGTATCCACTCATCTATCAGGTCTTTTTG
Myst2	5	CCGGCCTGACAAGTGAATATGACTTCTCGAGAAGTCATATTCACTTGTCAGGTTTTTG
Zfp532	1	CCGGAGCATTTGACATACCAGATATCTCGAGATATCTGGTATGTCAAATGCTTTTTTG
Zfp532	2	CCGGGATCAAGGACCCTGATGTAAACTCGAGTTTACATCAGGGTCCTTGATCTTTTTG
Zfp532	3	CCGGGCCAAATGTTCTGGTACTTAACTCGAGTTAAGTACCAGAACATTTGGCTTTTTG
Zfp532	4	CCGGCGGTGAAAGCCACGGTCATATCTCGAGATATGACCGTGGCTTTCACCGTTTTTG
Zfp532	5	CCGGACATCAGGCCACACGGATAAACTCGAGTTTATCCGTGTGGCCTGATGTTTTTTG
Tgif	1	CCGGCCATTTCATTCCTGCGTAGTTCTCGAGAACTACGCAGGAATGAAATGGTTTTTG
Tgif	2	CCGGGATCCAAATCAGTTCACGATTCTCGAGAATCGTGAACTGATTTGGATCTTTTTG

Tgif	3	CCGGAGTACAGATGTACCGCAAATACTCGAGTATTTGCGGTACATCTGTACTTTTTTG
Tgif	4	CCGGGGATGGCAAGAGATGCATTATTCTCGAGAATAATGCATCTCTTGCCATCTTTTTTGAAT
Tgif	5	CCGGATTTCAGAAGCTAGCTCTATTCTCGAGAATAGAGCTAGCTTCTGAAATTTTTTG
Gbx2	1	CCGGGATGTGGATTACAGCTCAGATCTCGAGATCTGAGCTGTAATCCACATCTTTTTG
Gbx2	2	CCGGTCACGTTAGCAGGTTCGCTATCTCGAGATAGCGAACCTGCTAACGTGATTTTTG
Gbx2	3	CCGGGCCTGGTCAGACTGCTCATAACTCGAGTTATGAGCAGTCTGACCAGGCTTTTTG
Gbx2	4	CCGGCGAGTCAAAGGTGGAAGATGACTCGAGTCATCTTCCACCTTTGACTCGTTTTTG
Gbx2	5	CCGGCGGACTGCCTTCACCAGCGAACTCGAGTTCGCTGGTGAAGGCAGTCCGTTTTTG
Xrn2	1	CCGGCCAGAGGATAACGTCAGGTTACTCGAGTAACCTGACGTTATCCTCTGGTTTTTG
Xrn2	2	CCGGCCATTCCATTATGCACCATTTCTCGAGAAATGGTGCATAATGGAATGGTTTTTG
Xrn2	3	CCGGCCAAATGATGTGGAGTTTGATCTCGAGATCAAACTCCACATCATTTGGTTTTTG
Xrn2	4	CCGGCAACGATACTACAAGAACAAACTCGAGTTTGTTCTTGTAGTATCGTTGTTTTTG
Xrn2	5	CCGGGCTATTACATAGCTGATCGTTCTCGAGAACGATCAGCTATGTAATAGCTTTTTG
Tcea1	2	CCGGGAATATTCCTCCTGATCTATTCTCGAGAATAGATCAGGAGGAATATTCTTTTTG
Set	2	CCGGCCCGACATGGATGATGAAGAACTCGAGTTCTTCATCATCCATGTCGGGTTTTTG
Set	4	CCGGGAGAGCTTCTTTACCTGGTTTCTCGAGAAACCAGGTAAAGAAGCTCTCTTTTTG

Appendix Table B. shRNA used in chapter 4

Gene	Clone	sequence
Yap1	1	CCGGGAAGCGCTGAGTTCCGAAATCCTCGAGGATTTCGGAACTCAGCGCTTCTTTTTG
Yap1	2	CCGGTGAGAACAATGACAACCAATACTCGAGTATTGGTTGTCATTGTTCTCATTTTTG
Yap1	3	CCGGACTTGGAGGCGCTCTTCAATGCTCGAGCATTGAAGAGCGCCTCCAAGTTTTTG
Yap1	4	CCGGCGGTTGAAACAACAGGAATTACTCGAGTAATTCCTGTTGTTTCAACCGTTTTTG
Yap1	5	CCGGTCCAACCAGCAGCAGCAAATACTCGAGTATTTGCTGCTGCTGGTTGGATTTTTG
Tead1	1	CCGGCAGAAGGAAATCTCGTGATTTCTCGAGAAATCACGAGATTTCCTTCTGTTTTTG
Tead2	1	CCGGCTCGAAGGAAATCGAGAGAAACTCGAGTTTCTCTCGATTTCCTTCGAGTTTTTG
Tead3	1	CCGGGGTGCGAGTACATGATCAATTTCTCGAGAAATTGATCATGTACTCGCACTTTTTG
Tead4	1	CCGGGCTGAAACACTTACCCGAGAACTCGAGTTCTCGGGTAAGTGTTTCAGCTTTTTG
Taz	1	CCGGCCTGCATTTCTGTGGCAGATACTCGAGTATCTGCCACAGAAATGCAGGTTTTTG

Appendix Table C. shRNA used in chapter 5

Gene	Clone	sequence
Mphospho8	1	CCGGCCTACCTAGTTGTAAACTTAACTCGAGTTAAGTTTACAACTAGGTAGGTTTTTG

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Mphospho8	2	CCGGGCCGGATTTCTCAACAGATTTCTCGAGAAATCTGTTGAGAAATCCGGCTTTTTG
Mphospho8	3	CCGGGCCAAGGTTAAGTTGCTAATACTCGAGTATTAGCAACTTAACCTTGGCTTTTTG
Mphospho8	4	CCGGGCGAGGGAGGTAAGAATCTTTCTCGAGAAAGATTCTTACCTCCCTCGCTTTTTG
Mphospho8	5	CCGGCACCTCTGAAATAATCGGTTTCTCGAGAAACCGATTATTTCAGAGGTGTTTTTG
Med26	1	CCGGGCTACCAGTTTACTAACGATTCTCGAGAATCGTTAGTAAACTGGTAGCTTTTTG
Med26	2	CCGGTGTTAAACCTGTGCGGTTAAACTCGAGTTTAACCGCACAGGTTTAACATTTTTG
Med26	3	CCGGCGCACCACTTTATGGCTGAATCTCGAGATTCAGCCATAAAGTGGTGCGTTTTTG
Med26	4	CCGGGCGCTTGAACATTCTGCCTTACTCGAGTAAGGCAGAATGTTCAAGCGCTTTTTG
Med26	5	CCGGCCTGAAGAATCGCAACGACATCTCGAGATGTCGTTGCGATTCTTCAGGTTTTTG
Gli2	1	GTACCGGTATGTTTACCCGCTCCTATTTCTCGAGAAATAGGAGCGGGTAAACATATTTTTTG
Gli2	2	CCGGCACCAACCCTTCAGACTATTACTCGAGTAATAGTCTGAAGGGGTTGGTGTTTTTG
Gli2	3	CCGGTGTGGAGGACTGCCTACATATCTCGAGATATGTAGGCAGTCCTCCACATTTTTG
Gli2	4	CCGGTCGACCTACAACGCATGATTCCTCGAGGAATCATGCGTTGTAGGTCGATTTTTG
Gli2	5	CCGGTATCTCCTTGATACGACTTTCCTCGAGGAAAGTCGTATCAAGGAGATATTTTTG
Nr6a1	1	CCGGGCATTGGACCAGTCCAGATATCTCGAGATATCTGGACTGGTCCAATGCTTTTT
Nr6a1	2	CCGGCCAGATGATCGAGCTGAACAACTCGAGTTGTTCAGCTCGATCATCTGGTTTTT
Nr6a1	3	CCGGGCCTCCACATTATCAATACATCTCGAGATGTATTGATAATGTGGAGGCTTTTT
Nr6a1	4	CCGGCCAGTAGGTCTGTGGAACTAACTCGAGTTAGTTCCACAGACCTACTGGTTTTT
Nr6a1	5	CCGGCCAAACCGCTTTCCTGATCTTCTCGAGAAGATCAGGAAAGCGGTTTGGTTTTT
Rai14	1	CCGGCGAACACTGTGGACGCCTTAACTCGAGTTAAGGCGTCCACAGTGTTCGTTTTTG
Rai14	2	CCGGACTGCTCTGTTATCGAGAATACTCGAGTATTCTCGATAACAGAGCAGTTTTTTG
Rai14	3	CCGGGAAACTGAAGGACACGCTAAACTCGAGTTTAGCGTGTCCTTCAGTTTCTTTTTG
Rai14	4	CCGGAGGATTTGCCACGGGACTTAACTCGAGTTAAGTCCCGTGGCAAATCCTTTTTTG
Rai14	5	CCGGATCACGGCGCAGATGTCAATTCTCGAGAATTGACATCTGCGCCGTGATTTTTG
Sirt6	1	CCGGCATGTCCAACACAGCTCCTTTCTCGAGAAAGGAGCTGTGTTGGACATGTTTTTG
Sirt6	2	CCGGGCATGTTTCGTATAAGTCCAACTCGAGTTGGACTTATACGAAACATGCTTTTTG
Sirt6	3	CCGGGTTTGACACCACCTTCGAGAACTCGAGTTCTCGAAGGTGGTGTCAAACTTTTTG
Sirt6	4	CCGGTCCCAAGTGTAAGACGCAGTACTCGAGTACTGCGTCTTACACTTGGGATTTTTG
Sirt6	5	CCGGAGAGGAATGTCCCAAGTGTAACTCGAGTTACACTTGGGACATTCCTCTTTTTTG
Taf1b	1	CCGGCGATAAGTCCGTCGCATATAACTCGAGTTATATGCGACGGACTTATCGTTTTTG
Taf1b	2	GTACCGGACGTTTGATCCTATAGCTAAACTCGAGTTTAGCTATAGGATCAAACGTTTTTTTTG
Taf1b	3	CCGGAGGATCATATTCCGTACATAACTCGAGTTATGTACGGAATATGATCCTTTTTTG
Taf1b	4	CCGGGACTATGAAGACATCTATAAACTCGAGTTTATAGATGTCTTCATAGTCTTTTTG
Taf1b	5	GTACCGGTTAACCTGTCAAGTAGTAAAGCTCGAGCTTTACTACTTGACAGGTTAATTTTTTG
Trip13	1	CCGGCCGAGTAGTCAATGCTGTGTTCTCGAGAACACAGCATTGACTACTCGGTTTTT
Trip13	2	CCGGCGTACTCTTCTCAGACAAGAACTCGAGTTCTTGTCTGAGAAGAGTACGTTTTT
Trip13	3	CCGGGAAGATATAAAGTCGAGTGTTCTCGAGAACACTCGACTTTATATCTTCTTTTT

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Trip13	4	CCGGGACACAGAACTAAAGGCTAAACTCGAGTTTAGCCTTTAGTTCTGTGTCTTTTT
Trip13	5	CCGGGACAAACAGTTTGAGGAGAAACTCGAGTTTCTCCTCAAACTGTTTGTCTTTTT
Bcl3	1	CCGGCCTCAAGAACTGTCACAATGACTCGAGTCATTGTGACAGTTCTTGAGGTTTTTG
Bcl3	2	CCGGACGTGAACGCTCAGATGTATTCTCGAGAATACATCTGAGCGTTCACGTTTTTTG
Bcl3	3	CCGGCCCGAAAGATGCTCCTGGCTTCTCGAGAAGCCAGGAGCATCTTTCGGGTTTTTG
Bcl3	4	CCGGCCTTTACTACCAGGGACCTTTCTCGAGAAAGGTCCCTGGTAGTAAAGGTTTTTG
Bcl3	5	CCGGCGCAATTATGAAGGGCTCACTCTCGAGAGTGAGCCCTTCATAATTGCGTTTTTG
Gli1	1	CCGGCCTATCCTGATCCACCAGAAACTCGAGTTTCTGGTGGATCAGGATAGGTTTTTG
Gli1	2	CCGGCCTGTGTACCACATGACTCTACTCGAGTAGAGTCATGTGGTACACAGGTTTTTG
Gli1	3	CCGGCCACATCAACAGTGAGCATATCTCGAGATATGCTCACTGTTGATGTGGTTTTTG
Gli1	4	CCGGCGACTTGAGCATTATGGACAACTCGAGTTGTCCATAATGCTCAAGTCGTTTTTG
Gli1	5	CCGGGCATGGGAACAGAAGGACTTTCTCGAGAAAGTCCTTCTGTTCCCATGCTTTTTG
Mycbp	1	CCGGCCTAGAATTGGCAGAAATGAACTCGAGTTCATTTCTGCCAATTCTAGGTTTTTG
Mycbp	2	CCGGGAATAGCTTGTATAGTATATGCTCGAGCATATACTATACAAGCTATTCTTTTTG
Mycbp	3	CCGGGAGAAATATGAAGCTACTGTACTCGAGTACAGTAGCTTCATATTTCTCTTTTTG
Mycbp	4	CCGGCCTTATATGAAGAACCAGAGACTCGAGTCTCTGGTTCTTCATATAAGGTTTTTG
Mycbp	5	CCGGCCTTATATGAAGAACCAGAGACTCGAGTCTCTGGTTCTTCATATAAGGTTTTTG
Phf12	1	CCGGCCTGAACCGAATCCACAAGAACTCGAGTTCTTGTGGATTCGGTTCAGGTTTTTG
Phf12	2	CCGGCGGAGAAATCGAGATAAATATCTCGAGATATTTATCTCGATTTCTCCGTTTTTG
Phf12	3	CCGGCCAACTCACTTCGAGCATTTACTCGAGTAAATGCTCGAAGTGAGTTGGTTTTTG
Phf12	4	CCGGGCTACTGTGTATATGGACAAACTCGAGTTTGTCCATATACACAGTAGCTTTTTG
Phf12	5	CCGGCCCAAGCAGTATTGTTGCCAACTCGAGTTGGCAACAATACTGCTTGGGTTTTTG
Rbpj	1	CCGGCTGTATCACAACTCCACAAATCTCGAGATTTGTGGAGTTGTGATACAGTTTTTG
Rbpj	2	CCGGGCAGACTCATTGGGCTACATTCTCGAGAATGTAGCCCAATGAGTCTGCTTTTTG
Rbpj	3	CCGGCCAGACAGTTAGTACCAGGTACTCGAGTACCTGGTACTAACTGTCTGGTTTTTG
Rbpj	4	CCGGCCAGTGACTTTGGTCCGAAATCTCGAGATTTCGGACCAAAGTCACTGGTTTTTG
Rbpj	5	CCGGCCCTGTGCGTTTATTGGAATACTCGAGTATTCCAATAAACGCACAGGGTTTTTG
Taf12	1	CCGGCCAGGTATTGACCAAGAAGAACTCGAGTTCTTCTTGGTCAATACCTGGTTTTTG
Taf12	2	CCGGGCTACAGATCGCTGATGATTTCTCGAGAAATCATCAGCGATCTGTAGCTTTTTG
Taf12	3	CCGGGTAAGAGAAGTAGATCCTAATCTCGAGATTAGGATCTACTTCTCTTACTTTTTG
Taf12	4	CCGGGCTTTACAGAGAAGCATATATCTCGAGATATATGCTTCTCTGTAAAGCTTTTTG
Taf12	5	CCGGTGTACCACAGAGGCTCACAAACTCGAGTTTGTGAGCCTCTGTGGTACATTTTTG
Taf6	1	CCGGCCCGAATCACTAAGACCTTTACTCGAGTAAAGGTCTTAGTGATTCGGGTTTTTG
Taf6	2	CCGGCTGAGCAACATCGACCGTATTCTCGAGAATACGGTCGATGTTGCTCAGTTTTTG
Taf6	3	CCGGCCAGTGACATTGACTATGCATCTCGAGATGCATAGTCAATGTCACTGGTTTTTG
Taf6	4	CCGGCCAGCAGTAATGACCTGCATTCTCGAGAATGCAGGTCATTACTGCTGGTTTTTG
Taf6	5	CCGGGTGTGAATGTAGTTCAGAATACTCGAGTATTCTGAACTACATTCACACTTTTTG

Trip4	1	CCGGCGTGGGAAAGATGTGGAGTTTCTCGAGAAACTCCACATCTTTCCCACGTTTTTG
Trip4	2	CCGGCGAGAATATGTGACAGACCTTCTCGAGAAGGTCTGTCACATATTCTCGTTTTTG
Trip4	3	CCGGCCCAAGTTATTGACGATGAATCTCGAGATTCATCGTCAATAACTTGGGTTTTTG
Trip4	4	CCGGCCTGGATGTCAGCGAGGAAATCTCGAGATTTCCTCGCTGACATCCAGGTTTTTG
Trip4	5	CCGGGTGGAGTTTCCAAATGACTATCTCGAGATAGTCATTTGGAAACTCCACTTTTTG
Zfp13	1	CCGGCCTGAGAGAGAGCAGTGAGAACTCGAGTTCTCACTGCTCTCTCTCAGGTTTTTG
Zfp13	2	CCGGCGCTCCAATCTCATCGCACATCTCGAGATGTGCGATGAGATTGGAGCGTTTTTG
Zfp13	3	CCGGTCCAATCTCATCGCACATAATCTCGAGATTATGTGCGATGAGATTGGATTTTTG
Zfp13	4	CCGGCCAGGTAATGTGCAACACTGTCTCGAGACAGTGTTGCACATTACCTGGTTTTTG
Zfp13	5	CCGGTCAGGAGTCAACACACATCAACTCGAGTTGATGTGTGTTGACTCCTGATTTTTG
Zfp198	1	CCGGGCTCCAAAGAAACTCTGTGTTCTCGAGAACACAGAGTTTCTTTGGAGCTTTTTG
Zfp198	2	CCGGGCGTGGAAACATTGGGTCAAACTCGAGTTTGACCCAATGTTTCCACGCTTTTTG
Zfp198	3	CCGGCCAGTAACTTTGGTGTGAATACTCGAGTATTCACACCAAAGTTACTGGTTTTTG
Zfp198	4	CCGGGCATAGTTACATATTGCGAATCTCGAGATTCGCAATATGTAACTATGCTTTTTG
Zfp198	5	CCGGGCTGAGCTTAACTATGGGTTACTCGAGTAACCCATAGTTAAGCTCAGCTTTTTG

Appendix Table D. RT-qPCR primers used in chapter 3

Gene	Forward	Reverse
Tip60	CGAAAGCTTTTCTATGTCCATTACA	TCTTGGGAAATTGGATCTTCTTTA
p400	TTTACAAGCGTATCTTAGGCAGAAT	GTGCTCATCGTTGATAACTTCAGAT
Trrap	GATTTCTTACCTTTCTCCAAGATGG	GGAATCCTGTGGAGTATCTCAAGTA
Myc	ACTACGACTCCGTACAGCCCTAT	TTTCTTCCAGATATCCTCACTGG
Fbxw7	AGACTTCATCTCCTTGCTTCCTAA	AAATTCTCCAGTATCGACAAGTCTG
Ash2L	TGGGATATGTACAAAATGGTTCAC	GAAAGTAGGTATTCCCACTGTGATG
Ezh2	GGGAGAGAACAATGATAAAGAAGAAG	ATTCTCAGGAGGTTCAATATTTGG
Suz12	GGAAATGGAAGAATGTCCAATAAGT	TAATGTAGGTCCCTGAGAAAATGTC
Rybp	AAGAAAAACACCAACAAGAAAACAA	GTCTTTGTTGTAGCGTTAGCAGACT
Phc1	CTACAGCCTCCTCTCCTGTTGT	CTCTCCTCCTCAGACTCAGCTTT
Tgif1	GAACACAGATACAACGCCTATCC	CGTTGATGAACCAGTTACAGACC
Tgif2	AGTCTCTCTGGACAGACCAACCT	AGATGGTGAACTGATTAGGGTCTTT
Arid3a	AGGTTATCAACAAGAAACTGTGGAG	TACTTCATGTACTGTGTCCGAAGTG
LSD1	CGGGCCAAGGTAGAATACAG	GGGGAAGCTTCTTTTCCTTC
H2afx	AGTACCTCACTGCCGAGATCC	AGCTTGTTGAGCTCCTCGTC
Nestin	AGGACCAGGTGCTTGAGAGA	TTCGAGAGATTCGAGGGAGA

Bmp2	CGCTTCTTCTTCAATTTAAGTTCTG	AACTACTGTTTCCCAAAGCTTCCT
Bmp4	GAGTTTCCATCACGAAGAACATCT	AGGAGATCACCTCATTCTCTGG
Sall4	AAAACTGCCTGTTTCCTAGTGG	AAGGTCTTTGGGTCTTGGAATAA
Zfp42	CAGTCCAGAATACCAGAGTGGAA	ACTCTAGGTATCCGTCAGGGAAG
Lin28	CAGAAGCGAAGATCCAAAGG	GATGCTTTGGCAAAAGTGG
Rest	ACTTCTCAGAAAGTGAAGGAGAAGG	GGTTCTACAGCACTTAGCTCCAA
Tcf3	TCATGTTGTATATGAAGGAGATGAGG	CTGTTCTTCTCTTGACAGGTTGTG
Gapdh	AAATTCAACGGCACAGTCAAG	CACCCCATTTGATGTTAGTGG
Actb_1	CACCACACCTTCTACAATGAGC	AACATGATCTGGGTCATCTTTTC
Actb_2	GATCTGGCACCACACCTTCTACAATG	CGTACATGGCTGGGGTGTTGAAG
Dmap1	CTTTACTCTGACAAAAAGGATGCAC	AGTAAAAGGCATCCATTTCCAA
Gcn5l2	GAATGTGTCAGAGGACGAGATTAAC	ATAGACTTGTTTGGTGTCTGTGTCC
Ep400	TTTACAAGCGTATCTTAGGCAGAAT	GTGCTCATCGTTGATAACTTCAGAT
p53	GTCATCTTTTGTCCCTTCTCAAAA	AGAGTACGTGCACATAACAGACTTG
Ruvbl2	ATGAGCAAGACTGAGGCTCTAAC	GTCAATCTGGATCTCTACCACCTC
Ruvbl1	AGGAGACTAAGGAGGTTTATGAAGG	TATGATCACATGGCTGATAGTTTTG
Ing3	AAGAGCAAAAACAACACCAAGTCT	ACTACAGTTGAAGACGATGAACACA
Brd8	AAGGAGAAGTGGTAGAAACTGTTGA	TCTCTCCTGTGTCTCCTTTATGACT
Epc2	CTCTACCAACAATGATCCTTACGTT	CTCAGCTTCAGCATCTTTTCATAA
E2F4	AGCTCAAGGCAGAGATCGAG	TGTTCTGGACGTCCTCAGTG
Zfp57	ACCTGACATTTGTCGGAAGC	TGGTCCACCTTTGAATACCC
Max	GGCAGAATGCTCTTCTGGAG	GCGTTGGTGTAGAGGCTGTT
Tcfap2c	CCACGTCGAAGTACAAAGTAACTG	TTTTTGGACTTTGCTCTTCTGAG
Nmyc	ACTCAGATGATGAGGATGACGAG	CACAGTGATCGTGAAAGTGGTTA
Rps19	ATAAAGAGCTTGCCCCATATGAT	ATCTTGGTCATGGAACCAACC
Surf6	AGGAATCAGGGCTGATCTTTAAC	GTGTCAGGTTCCCTTTCACTTT
Xrn2	AAAGATTCCAGTTGATGCCAGTA	TGGTTTGTCTTCAGGATGAGTG
Fubp3	AGGAACTTCTTATCCCTGCATCT	TGGATCATGACCATTTTCACAC
Asxl1	ATGCTACATTCCAACTCAAGAGG	CATCCACTGTAGCTGCATTTCTA
Zfp36l1	AGGGTAACAAGATGCTCAACTACAG	GTTCTGATGGAACTTGGAGCTG
Cbx1	TTCTCAGATGAGGACAACACTTG	GCCTCCCTCTGACTTATCTGTCT
Mybl2	GGACTCTTGTAACAGCCTCACC	CTCCAGGGTATCCTGTTTGTTC
Msc	GACACCAAGCTTTCCAAACTG	GTGCACATAGCTGTCCTCGTAG
Msh6	TTTGGGCTAAGATGGAAGGTTAC	TGTACATGAACACGGACAGATTT
Rarg	GTCTACAATCGGTGGAGACACAG	GTAGCCAGAAGACTTGTCATTGC
Tcea3	ACTGCTCAGACAAGGAAGTGGT	CTCTTTCTTCTCTTTCTCCTTTGGT
Hnrpdl	GGACATGAATGAGTACAGCAACA	AGCTCAAGCCTCCAATAAACATT

Zic5	TTGACGGTTGTGATAGGAAGTTT	ATCACAGCCTCGAATCTTACAGT
Tcfcp2l1	GGTGAAGCTACATGAAGAGACCTTA	TATTTCGTGTTCAGATCTTGGAAGT
Etv5	TGGAGAAAAGTGCCTCTACAACT	GGGAAATAGGGAATTCTGATGG
Zfp219	GCATCACTTAAAAGTGCATCTCC	GTGACGCTGAAGGTGATACTTGA
Cbx7	AAGGGCAAAGTTGAATATCTGGT	CTCTCTCCTCCTTCTCCTCGTAG
Otx2	AAGTGAGTTCAGAGAGTGGAACAAG	CTCCAGATAGACACTGGAGCACT
Zfp428	GGAGGAAGATGATGAAGACCTTT	CACCTTATAGCCAGGGTCATATTC
Myst2	AGCATACAGACAGTTCAGAGAGTGA	ATTTTTAGTTTCTTGGGAACTCTGG
Zfp532	ACCAGTGGACAACAAATGAAGAA	TAAACTTTCCTGATGCCTTTGTG
Kdm2b	GAAGGCCCAGAAGAGAAGAAA	TTTTGGATCTCATGGTTGAGC
Bach1	GCAGAAGGAGTAAGAACAGAATCG	CAGCAAGCTCTCCTTTTCACTTT
Hes1	GAAGGGCAAGAATAAATGAAAGTCT	TTCCAGAATGTCTGCCTTCTCTA
Rbbp7	GTAAAATTGGAGAAGAACAATCAGC	ATTCCAGCTGAAGTCAGAAATCTT
Id1	TCCTGCAGCATGTAATCGAC	GATCGTCGGCTGGAACAC
Cited2	CAGGTTTAACAACTCCCAGTTCAT	GTAGGGGTGATGGTTGAAATACTG
Hnrnpu	GACACAGAAGAAGGCAGAAGTAGAG	TTCAACATAGGTTATTTCATCAAAGC
Rbm14	GCTGCATGTACAAGTCAGGAAT	CTTCCTTCTCCATGTGAACAAAC
Eno1	CCTAGAACTCCGAGACAATGATAAG	ATTCACTTTCTTGCTAACCAGAGC
Med19	GTGGCTCTTTTAATCCAATCACA	TGCTTATGCTTATTCTTCTTCTTGG
Nolc1	CAGTGACCAAGAAGGCTAAGAAA	CAGCCTTCTGTGTGGGAAGT
Prdm14	GCCTCCGGATCCATATTCTT	TGCTTGTTCAGGCTGGAAG
Dnmt3a	CCTACTACATCAGCAAACGGAAA	GTTCTCTTCCACAGCATTCATTACT
Klf2	CATACTTGCAGCTACACCAACTG	CCAGTTGCAATGATAAGGCTTC
Rlim	AGAAGTCAAATGGATCGCTTG	CCCAGCAAATTGTTGTCTCTC
Tcea1	CTTGTCGATCACACTCTGGACTT	AAAACCCACCTGAAGACACTTG
Zfp146	CAGGCGATAAGCCTTACGAG	CGTAGGGTTTCTCACCCGTAT
Creb3	TCCATTCTCCATGATCACAACTAC	CAGTATCAGCCTAGACATCTCCTGT
Zfp280c	AGCCAGAAGACTCACACCACTT	CCAGGTCTCGTTGTTCTGCT
Rbbp5	AGGCGAGGGGAATATATTTATACAG	GTATTGCTTGTCCCAGTTGTTACTC
Tbx3	AGTACTTTTCGAACCTACCTGTTCC	GCAAAGGGATTGTTGTCTATTTTTA
H2afy	TCAAGTACAGGATCAGCGTAGGT	CTATCCGTGCCTTCTTGTTGTC
Set	GACGCAGGTGCTGATGAGT	GCCTCTCCTTCTTCATCATCC
Chd9	CAAAGAGCAGCACTCTCAAAAA	GACTGCCTTTCACTTCTTCTTCA
Esrrb	TAAAAAGCCATTGACTAAGATCGTC	CAATTCACAGAGAGTGGTCAGG
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Pou5f1	TCTGGAGACCATGTTTCTGAAGT	TACAGAACCATACTCGAACCACAT
Sox2	GCGGAGTGGAAACTTTTGTC	TATTTATAATCCGGGTGCTCCT
Trp53	GTCATCTTTTGTCCCTTCTCAAAA	AGAGTACGTGCACATAACAGACTTG

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Dkk3	GAAGTTCACAAGATAACCAACAACC	AATGATACATTCATGGCTCCTCTT
Gata4	TTCTCAGAAGGCAGAGAGTGTGT	ATGCCGTTCATCTTGTGATAGAG
Gata6	GACGGCACCGGTCATTACC	ACAGTTGGCACAGGACAGTCC
Sox17	CTAAGCAAGATGCTAGGCAAGTCT	GTACTTGTAGTTGGGGTGGTCCT
Gata2	GCCTCTACCACAAGATGAATGG	GTCTGACAATTTGCACAACAGG
Gsc	AGAAGGTGGAGGTCTGGTTTAAG	GAGGACGTCTTGTTCCACTTCT
Isl1	GGGATGGGAAAACCTACTGTAAAAGAGA	GTCGTTCTTGCTGAAGCCTATGCTG
T	CTTCAAGGAGCTAACTAACGAGATG	GTCCAGCAAGAAAGAGTACATGG
Fgf5	CAAAGTCAATGGCTCCCACGAA	CTACAATCCCCTGAGACACACAGC
Cdx2	GCGAAACCTGTGCGAGTGGATG	CGGTATTTGTCTTTTGTCCTGGTTTTCA
Eomes	ATAAGATGTACGTTCACCCAGAATC	GCACCTTTGTTATTGGTGAGTTTTA
Hand1	CCTTCAAGGCTGAACTCAAAAA	GCGCCCTTTAATCCTCTTCT
Id2	ATCACCAGAGACCTGGACAGAAC	GCTATCATTCGACATAAGCTCAGA
Krt8	AGAACATGAGCATTCATACGAAGA	GAGCTCATTCCGTAGCTGAAG
Tead4	CTATGACAAGTTCCCAGAGAAGAAG	GTCATCGATGTTGGTATTGAGGT

Appendix Table E. RT-qPCR primers used in chapter 4

Gene	Forward	Reverse
Amot	GTGTCATAGCTGGTGTTAGGAGAGT	GGAAGAGAATGTGATGAGACACTTT
Cdx2	GCGAAACCTGTGCGAGTGGATG	CGGTATTTGTCTTTTGTCCTGGTTTTCA
Chd9	CAAAGAGCAGCACTCTCAAAAA	GACTGCCTTTCACTTCTTCTTCA
Cited1	AGTAGGCCAGAGAGTTCATCTCC	TCTGAAATGCCAACTATGTCGAG
Edn1	CAACCTTCGTAGTTTCCTTCCTT	CAGAAAAGCTTAAAAGACTCCAAGA
Eomes	ATAAGATGTACGTTCACCCAGAATC	GCACCTTTGTTATTGGTGAGTTTTA
Esrrb	TAAAAAGCCATTGACTAAGATCGTC	CAATTCACAGAGAGTGGTCAGG
Fgf5	CAAAGTCAATGGCTCCCACGAA	CTACAATCCCCTGAGACACACAGC
Fzd6	ACAGTGTGGCAATCTGTTACATTC	GTCGGAAATTGTGTGAGAAAATAGT
Gata2	GCCTCTACCACAAGATGAATGG	GTCTGACAATTTGCACAACAGG
Gata3	TGGGCTGTACTACAAGCTTCATAA	CTTTTTCGATTTGCTAGACATCTTC
Gata4	TTCTCAGAAGGCAGAGAGTGTGT	ATGCCGTTCATCTTGTGATAGAG
Gata6	GACGGCACCGGTCATTACC	ACAGTTGGCACAGGACAGTCC
Gbx2	AAGACGAGTCAAAGGTGGAAGAT	CAGTCTGACCAGGCAAATTGT
Gsc	AGAAGGTGGAGGTCTGGTTTAAG	GAGGACGTCTTGTTCCACTTCT
Hand1	CCTTCAAGGCTGAACTCAAAAA	GCGCCCTTTAATCCTCTTCT

Id2	ATCACCAGAGACCTGGACAGAAC	GCTATCATTCGACATAAGCTCAGA
Isl1	GGGATGGGAAAACCTACTGTAAAAGAGA	GTCGTTCTTGCTGAAGCCTATGCTG
Klf4	GTGCAGCTTGCAGCAGTAAC	AGCGAGTTGGAAAGGATAAAGTC
Krt18	TTTGCGAATTCTGTGGACAA	GCGCATGGCTAGTTCTGTCT
Krt8	AGAACATGAGCATTCATACGAAGA	GAGCTCATTCCGTAGCTGAAG
Msx2	CTCTTGCAGTCTTTTCGCCTTAG	CTCTTGCAGTCTTTTCGCCTTAG
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Nes	AGGACCAGGTGCTTGAGAGA	TTCGAGAGATTCGAGGGAGA
Nodal	CATGTTGAGCCTCTACCGAGA	AAGAGAAGTCAAACGTGAAAGTCC
Pitx2	CTTGGACTCCTCCAAACATAGACT	CACATCCTCATTCTTTCCTTGCT
Pou5f1	TCTGGAGACCATGTTTCTGAAGT	TACAGAACCATACTCGAACCACAT
Sox13	AGTGACTGGAAGGAGAGGTTTCT	CAAAAGCTGGAGTTCCTTCTCAG
Sox17	CTAAGCAAGATGCTAGGCAAGTCT	GTACTTGTAGTTGGGGTGGTCCT
Sox2	GCGGAGTGGAAACTTTTGTC	TATTTATAATCCGGGTGCTCCT
T	CTTCAAGGAGCTAACTAACGAGATG	GTCCAGCAAGAAAGAGTACATGG
Taz	ACCAACAGTAGCTCAGATCCTTTC	AGGAAGTCTTCTGGAGTTGTGG
Tead1	TTGATAGCCAGATACATCAAACTCA	TTTCTCGAACTTTCTTTCTTGCTAA
Tead2	GTCAGATGAGGGCAAGATGTATG	AAAACCTGAATATGGCTGGAGAC
Tead3	AAAACCAGGACAAGAAAACAGGT	GAGAGCTTTGTCCTTGGAGACTT
Tead4	CTATGACAAGTTCCCAGAGAAGAAG	GTCATCGATGTTGGTATTGAGGT
Tgfb2	CTGCTTTAGAAATGTGCAGGATAAT	CACAGAAGTTAGCATTGTACCCTTT
Yap1	GAGCAAGCCATGACTCAGGA	TCTGGAGACCATGTTTCTGAAGT

Appendix Table F. RT-qPCR primers used in chapter 5

Gene	Forward	Reverse
Mphosph8	AGCAGTCAGGAAAGATATTCAGAGA	TGGTGAAATATCTTCTTTTGTGTCA
Med26	GTGTTGGAAGTGATCTCCAGTCT	AGCTCCTCATTCTTGGTTTTCTT
Gli2	AGAGAGAGAAGCTCAAGTCACTGAA	GTATTGTTGAAGTTTTCCAGGACAG
Nr6a1	ACAGGAAGGCTATCAGAGAAGATG	CCAGACATGATTCTTTCAATTTCTT
Rai14	TGCAGGAATTCAAAACCTTCTATTA	CCACTTCGTTCTGAGCTTATTTTAG
Sirt6	TGTTTGTTTGTTTGTTTGTTTGTTT	CTAGGGAGTCCTGGTCTCAAAAA
Taf1b	TAAGCTTTTTCCAGAAGAGATGAAA	ATATGGCAAGTTCAATCATGTTTTT
Trip13	TACTGGAAAAACATCCCTTTGTAAG	TAGAAAATAGGCTGTGGCTGTTTAT
Bcl3	CTTTACTACCAGGGACCTTTGATG	CATATCATGGAGTAGGGGTGAGTAG
Gli1	TGAGCCTGAGTCTGTGTATGAGA	ATATGCTCACTGTTGATGTGGTG

Mycbp	TGGTAGCCTTATATGAAGAACCAGA	ATTCTAGGCGAAGCAGCTCTATT
Phf12	AACTGACCTGTACCACTGCACTAC	TATGGTCTAACTCATGCTGTGTCC
Rbpj	CTCCACAAATGTGCATTTTACCT	TATTTTGTTCTTTTGGACATGGAGT
Taf12	TTGACCAAGAAGAAATTACAAGACC	CTCTCAATAAAATCATCAGCGATCT
Taf6	AGTTAAAGAATGTGGAGCCACTGTA	TTCCTTCTCTTCATAGAAGTACAGCTC
Trip4	CGCAAAATTGTTTCTATGTATCCTT	TTAAAGTGCTAAAACAGAAGGCACT
Zfp13	CAGAGATGTACTACAGGGGAAAAAC	TAGTCCTTATCGATTTTGTGTCCTC
Zfp198	GAAACAGTAAATTTCTCTGGCGTTA	GTTGCAGGTAACACATCTTAATCCT
Neurog1	GCTTCAGAAGACTTCACCTATGG	CTAGTGGTATGGGATGAAACAGG
Flt1	TCTACCAAATCATGTTGGATTGCTG	ATGTAATCTTTCCCATCCTGTTGGA
Egfr	TCGAGGACTCCCCTCTTGAGT	AGGCGTCTTCTTTGACACGG
Nefm	AGAATACCAGGATCTCCTTAACGTC	CTGAAAATGTGCTAAATCTGGTCTC
Pou5f1	TCTGGAGACCATGTTTCTGAAGT	TACAGAACCATACTCGAACCACAT
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Sox2	GCGGAGTGGAAACTTTTGTC	TATTTATAATCCGGGTGCTCCT
Klf4	GTGCAGCTTGCAGCAGTAAC	AGCGAGTTGGAAAGGATAAAGTC
Esrrb	TAAAAAGCCATTGACTAAGATCGTC	CAATTCACAGAGAGTGGTCAGG
Zfp42	CAGTCCAGAATACCAGAGTGGAA	ACTCTAGGTATCCGTCAGGGAAG
Cdx2	GCGAAACCTGTGCGAGTGGATG	CGGTATTTGTCTTTTGTCCTGGTTTTCA
Hand1	CCTTCAAGGCTGAACTCAAAAA	GCGCCCTTTAATCCTCTTCT
T	CTTCAAGGAGCTAACTAACGAGATG	GTCCAGCAAGAAAGAGTACATGG
Krt8	AGAACATGAGCATTCATACGAAGA	GAGCTCATTCCGTAGCTGAAG
Sox17	CTAAGCAAGATGCTAGGCAAGTCT	GTACTTGTAGTTGGGGTGGTCCT
Pitx2	CTTGGACTCCTCCAAACATAGACT	CACATCCTCATTCTTTCCTTGCT
Gsc	AGAAGGTGGAGGTCTGGTTTAAG	GAGGACGTCTTGTTCCACTTCT
Isl1	GGGATGGGAAAACCTACTGTAAAAGAGA	GTCGTTCTTGCTGAAGCCTATGCTG
Bmp2	CGCTTCTTCTTCAATTTAAGTTCTG	AACTACTGTTTCCCAAAGCTTCCT
Gata3	TGGGCTGTACTACAAGCTTCATAA	CTTTTTCGATTGCTAGACATCTTC
Fgf5	CAAAGTCAATGGCTCCCACGAA	CTACAATCCCCTGAGACACACAGC
Nes	AGGACCAGGTGCTTGAGAGA	TTCGAGAGATTCGAGGGAGA

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