PROBING THE FUNCTIONS OF Lin28 AND Jmjd6 IN MOUSE ES CELLS

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ABSTRACT

The unique identity embryonic stem cells (ESCs) are featured by their pluripotency and self-renewal ability. Oct4/Nanog transcriptional network and epigenetic regulation mainly control the ESC identity. However, there still are unknown factors which are required for ESC identity. Two proteins were studied in my project. The first one is a RNA-binding protein Lin28 which is used as a reprogramming factor. The second protein is a jumonji domain-containing factor Jmjd6 which was previously shown as a histone arginine demethylase.

In the first part, double knockdown *Lin28a* and *Lin28b* and subsequent gene expression microarray have indentified numerous potential Lin28 downstream genes in ESCs. My study has also revealed that Lin28 may regulate ESC self-renewal in both miRNA dependent and independent ways.

In the second part, our results demonstrate that Jmjd6 is indispensible for the maintenance of ESC pluripotency and there may be a feedback loop between Jmjd6 and the key transcription factor Oct4 /Sox2.

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LIST OF ABBREVIATIONS

αKG: α-ketoglutarate

BA: Basic aromatic

Bmp4: Bone mrophpgenetic protein 4

cDNA: Complementary DNA

Chd: Chromodomain-helicase-DNA-binding protein

ChIP: Chromatin immunoprecipitation

Csp: Cold-shock protein

DAPI: 4', 6-diamidino-2-phenylindole

DEPC: Diethyl pyrocarbonate

DMEM: Dulbecco's Modified Eagle Medium

Dnmts: DNA methyltransferases

ESCs: Embryonic stem cells

FBS: Fetal bovine serum

FDR: False discovery rate

GMEM: Glasgow Minimum Essential Medium

ICM: Inner cell mass

Id: Inhibitor of differentiation

iPSC: Induced pluripotent stem cell

JAK: Janus-associated tyrosine kinases

JHDM: JmjC-domain-containing histone demethylase

LB: Lysogeny broth

LIF: Leukaemia inhibitory factor

LIFR: Leukaemia inhibitory factor receptor

LSD1: Lysine specific demethylase

miRNA: microRNA

ncRNAs: Non-coding RNAs

PBS: Phosphate buffer saline

PcG: Polycomb-group

Pen/.Strp Penicillin/Streptomycin

PSR: Phosphatidylserine receptor

PVDF: Polyvinylidene fluoride

RA: Retinoic acid

RIP: RNA immunoprecipitation

RISC: RNA-induced silencing complex

RNAi: RNA interference

RRM: RNA-recognition motif

STAT: Signal transducer and activator of transcription

TSA: Trichostatin A

TUTase: Terminal uridylytransferase

Chapter 1

INTRODUCTION OF EMBRYONIC STEM CELLS

1.1 Embryonic stem cell (ESC)'s identity and regulation

1.1.1 Mammal development

During the development process, a totipotent zygote undergoes gradual differentiation into the pre-implantation after cavitation at the 16-cell morula stage. The first cell fate decision occurs in which a population of cells grow into the inner cell mass (ICM) while the rest cells end up with the trophectoderm that surrounds the ICM (Hanna et al., 2010; Ralston and Rossant, 2005). The ICM cells are considered pluripotent as they have the ability to give rise to three germ layers: ectoderm, mesoderm and endoderm but not the trophectoderm (Alison et al., 2002). The cells undergo further differentiation and some of them become multipotent adult stem cells which defines by the limited capacity to form a fixed repertoire of cell types depending on which organ in the body they are acquired from (Jaenisch and Young, 2008).

1.1.2 ESCs and their maintenance

Mouse embryonic stem cells (ESCs) are the in vitro equivalent of the ICM, established by in vitro cultures of mouse blastocytes (Evans and Kaufman, 1981). ESC distinguishes from its in vivo counterpart by having unlimited self-renewal capacity while they can be induced to differentiate into a variety of cell types (Ng and Surani, 2011). The propagation of mouse ES cells require the leukaemia inhibitory factor (LIF), which engages a heterodimeric receptor complex includes two cytokine receptors, LIF receptor (LIFR) and gp130 (Davis et al., 1993). It acts by activating Janus-associated tyrosine kinases (JAK) that phosphorylate the receptor chains (Narazaki et al., 1994; Stahl et al., 1994). In the down- stream of JAK is the signal

transducer and activator of transcription (STAT) proteins (Ihle, 1996). In ES cells, JAK kinases predominantly activate STAT3 by triggering its recruitment tyrosine phosphorylation and dimerization (Niwa et al., 1998). The STAT3 dimers then translocate to the nucleus, where they control the transcription of self-renewal genes (Burdon et al., 2002). However, LIF/Stat3 pathway alone is not sufficient to maintain ESCs as culturing of mESCs in serum-free medium supplemented with LIF still resulted in differentiation into neural cells (Ying et al., 2003). Bone morphogenetic protein 4 (Bmp4) , which was found to be the serum constituent that complements LIF, appear to be essential serum-derived factors by binding to BMP receptor (BMPR) and leading to phosphorylation of Smad proteins and activating expression of Inhibitor of differentiation (Id) proteins (Ying et al., 2003). Subsequently, Id proteins function as inhibitors of differentiation towards neural lineage. Therefore, LIF acts together with Bmp4 in this way to enhance the pluripotent state and self-renewal ability of mESCs.

1.1.3 **Epigenetic regulation of ESCs**

The term “epigenetics” was originally coined by Waddington to explain the means by which complex multicellular organisms are formed by differentiation of totipotent cells in the embryo (Waddington, 1942). Epigenetics was commonly defined as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” (Wu and Morris, 2001). Generally speaking, classic epigenetic regulations of gene expression include DNA methylation, transcription factors, and chromatin variation through modifications of DNA-binding proteins such as histones, DNA methylation as well as post-transcriptional regulation by non-coding RNAs (ncRNAs) (Goldberg et al., 2007).

1.1.4 **The regulation of transcription factors in pluripotency**

Three transcription factors Oct4, Sox2, and Nanog are the core regulators that specify ES cell identity (Avilion et al., 2003; Chambers et al., 2003a; Lee et al., 2004; Mitsui et al., 2003a; Nichols et al., 1998; Scholer et al., 1990). Encoded by the *Pou5f1* gene, Oct4 is a POU domain containing, octamer binding transcription factor (Okamoto et al., 1990) (Scholer et al., 1990) and is the most essential for early embryogenesis and for embryonic stem cell pluripotency. Its activity is crucial for the identity of the pluripotential founder cell population in the mammalian embryo (Nichols et al., 1998). Either deficient or over-expressed Oct4 in ESCs leads to differentiation towards trophectodermal lineage and mesodermal and endodermal lineages, including the primitive endoderm, respectively (Nichols et al., 1998; Niwa et al., 2000). Sox2 is a transcription factor with a HMG box DNA-binding domain that can heterodimerize with Oct4 on DNA and affect the expression of a number of genes involved in embryonic development such as FGF4 and ZFP206 (Yu et al., 2009; Yuan et al., 1995) . Like Oct4, Sox2 is also critical for early embryogenesis and for embryonic stem cell pluripotency, and Sox2-null ESCs differentiate into trophectoderm cells (Avilion et al., 2003; Masui et al., 2007). Nanog is a NK2 homeobox transcription factor that is highly expressed in the epiblast and ICM and absent from differentiated cells (Chambers et al., 2003b; Mitsui et al., 2003b). If Nanog was deleted, the ICM failed in developing to epiblast but to endoderm-like cells. Consistently, Nanog-deficient ES cells differentiated into endoderm lineage (Mitsui et al., 2003a), suggesting that Nanog is in the transcription factor hierarchy that defines ES cell identity. Genome-wide location analysis of Oct4, Sox2 and Nanog indicate that they co-target numerous genes, many of which encode developmentally important homeodomain transcription factors and regulators required in specialized regulatory circuits in ES cells (Boyer et al., 2005; Loh et al., 2006). Further studies have revealed that these three factors function to specify the ESC identity by activating transcription of regulating genes via recruitment of co-activators like p300, chromatin

remodelling complexes and the transcriptional machinery (Chen et al., 2008; Pardo et al., 2010; van den Berg et al., 2010; Young, 2011), and meanwhile repressing developmental genes by polycomb-group (PcG) complexes as well as other repressive complexes, such as NuRD, Sin3A and Pml complexes (Boyer et al., 2006; Lee et al., 2006; Liang et al., 2008; Pardo et al., 2010; van den Berg et al., 2010). In addition, they also bind to their own promoters in an auto-regulatory loop (Boyer et al., 2005). Marson's group integrated the core auto-regulation with downstream targets and revealed the importance of these regulation, as illustrated in the Figure 1 (Marson et al., 2008) . The autoregulatory circuitry may explain the mechanisms in which stem cell identity can be persistently maintained (Loh et al., 2006). And regulating Oct4, Sox2 and Nanog levels and function may affect the developmental potential of ES cells (Chambers et al., 2003a; Mitsui et al., 2003a; Nichols et al., 1998; Niwa et al., 2000).

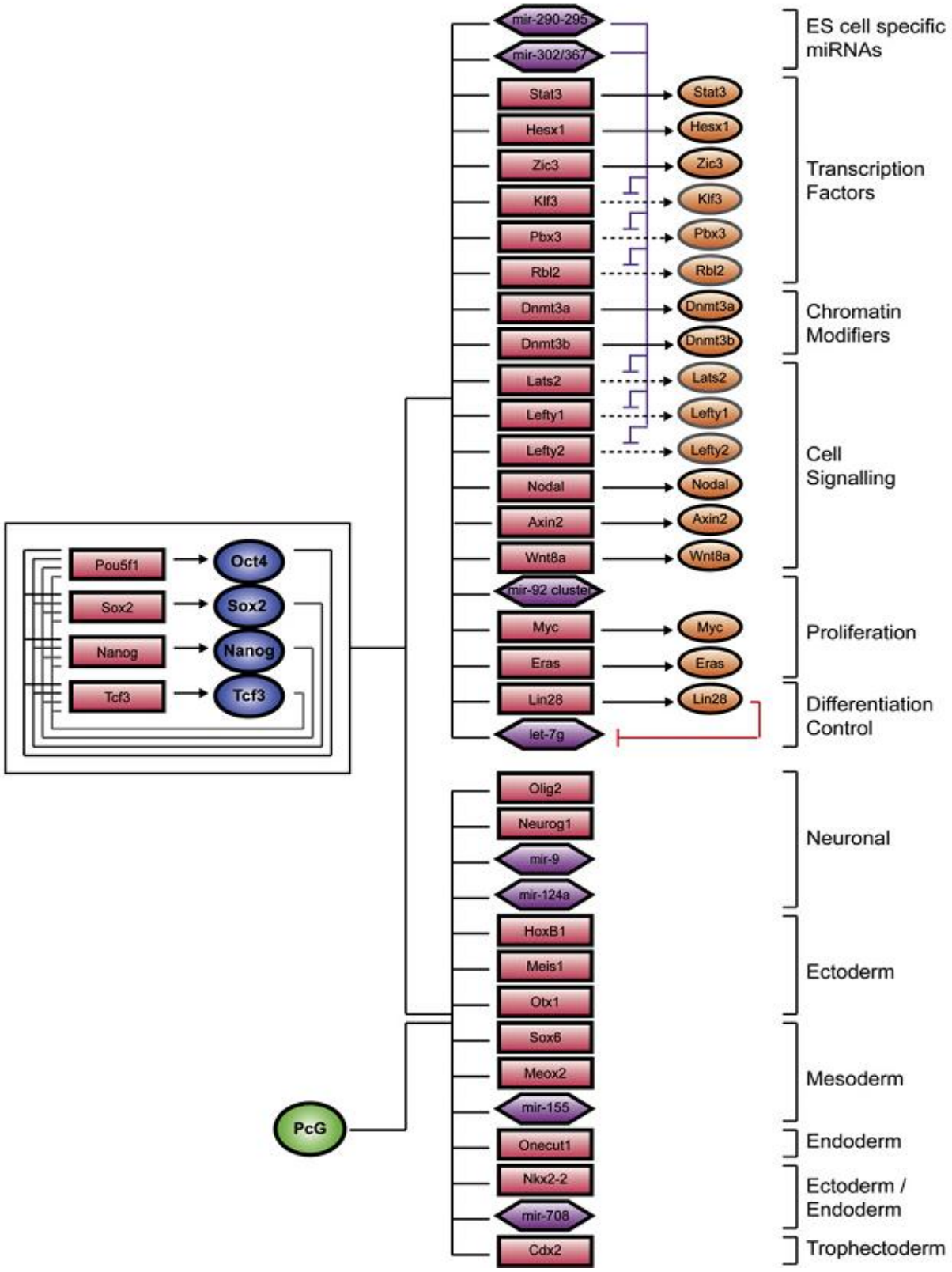


Figure 1. Multilevel regulatory network controlling ESC identity. Picture adapted from Marson et al., Cell 2008 (134):521-533.

1.1.5 **Chromatin modification in ESCs**

In mammalian cells, chromatin is a complex structure comprised of DNA and associated proteins, and transcriptional activity is intertwined with modifications to the chromatin structure. Thus, the complete transcriptional regulatory networks in ES cells integrate the assembly, disassembly, and covalent modifications of chromatin and DNA (Li et al., 2007a). As the basic subunit of chromatin, nucleosome consists of an octamer of 2 copies each of the core histone proteins including H2A, H2B, H3, and H4 (Luger et al., 1997). Linker histones such as H1 and its isoforms are involved in chromatin compaction and sit at the base of the nucleosome near the DNA entry and exit binding to the linker region of the DNA (Zhou et al., 1998). Posttranslational modifications occur in core histones such as acetylation, phosphorylation and methylation and the attachment of methyl groups occurs predominantly at specific lysine or arginine residues on histones H3 and H4 (Wood and Shilatifard, 2004). Normally, ESC chromatin is enriched in active marks (methylation of H3K4 and acetylation of H3 and H4) and deficient in silencing modifications (methylation of H3K9). Differentiation of ESCs is accomplished by global changes of histone modification pattern and transit to a less permissive chromatin state characterized by a decrease in H3K4me3 and an elevation of H3K9me (Meshorer et al., 2006). The so-called “bivalent domains” consisting of large regions of H3K27 methylation harboring smaller regions of H3K 4 methylation were identified as key developmental genes markers in embryonic stem cells, and histone methylation was shown to be notably weaker in differentiated cells. These may highlight the importance epigenetic regulation and suggest a novel chromatin-based mechanism for maintaining pluripotency (Bernstein et al., 2006).

1.1.6 **DNA methylation in ESCS**

DNA methylation is the addition or removal of a methyl group symmetrically on CpG dinucleotides. It is a stable and heritable mark that is involved in gene silencing, and is critical for development. In pluripotent ESCs, the pluripotency markers *Oct4*, *Nanog* and *Sox2* promoter regions are unmethylated to keep an active statue, and will undergo methylation upon differentiation (Lagarkova et al., 2006; Yeo et al., 2007). DNA methyltransferases (Dnmts) are a family of proteins related to the establishment and maintenance of DNA methylation. Dnmt3a and Dnmt3b are de novo methytranferases responsible for remethylation in postimplantation mouse embryos and in germ cells (Okano et al., 1999). In differentiating ESCs, the two enzymes directly interact and function synergistically to methylate the promoters of the *Oct4* and *Nanog* genes (Li et al., 2007b).Dnmt1 maintains DNA methylation at hemi-methylated DNA after DNA replication during cell divisions (Chen and Li, 2004). The mutants of *Dnmt3a* and *Dnmt3b* lead to loss of methylation and developmental defects while mutant of *Dnmt1* results in embryonic lethality and loss of majority methylation as well (Li et al., 1992; Li et al., 2007b). These studies show that global methylation may be indispensible for differentiation.

1.1.7 **MicroRNAs regulation in ESCs**

MicroRNA (miRNA) research is drawing increasing interest in stem cell field. miRNAs are small RNAs of ~22 nt that regulate target mRNAs through complementary base-pairing (Kim et al., 2009). miRNAs are generated mainly via two-step processing. In this canonical pathway, a newly transcript primary miRNA gene (pri-miRNA) is cropped into at least one hairpin structure precursor (pre-miRNA) and cleaved within the nucleus by an RNAseIII Drosa, co-assisted by DGCR8/Pasha (Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Lee

et al., 2003). Pre-miRNA is exported to the cytoplasm and gets processed by another RNAseIII, Dicer, to remove the "terminal loop region", transforming into a mature miRNA (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Lund et al., 2004). It is then cropped into the RNA-induced silencing complex (RISC) that contains the argonaute protein as the core component (Hammond et al., 2001; Mourelatos et al., 2002). Regulation of miRNA biogenesis can be achieved at multiple levels, including posttranscriptional level.

Numbers of miRNAs are predominantly expressed in ESCs or developmental embryonic tissues (Houbaviy et al., 2005; Houbaviy et al., 2003; Suh et al., 2004). Dicer-deficient mice lead to lethality early in development (Bernstein et al., 2003). Also, ESCs lacking miRNA-processing enzymes are defective in both differentiation and proliferation (Kanellopoulou, 2005; Murchison, 2005; Wang et al., 2007). A subset of miRNAs function to repress pluripotency by repressing transcription factors like Oct4, Sox2 and Klf4, or inducing differentiation by feedback regulations (Büssing et al., 2008; Xu et al., 2009b). In addition, genome-wide mappings of binding sites for key ES cell transcription factors were generated to incorporate miRNA gene regulation into the model of transcriptional regulatory circuitry of ES cells, which reveals highly overlapping occupancy of Oct4, Sox2, Nanog, and Tcf3 at miRNA promoters (Marson et al., 2008). All these imply miRNAs contribute to the control of early development and regulation of pluripotency.

1.1.8 **The establishment of iPSCs**

The dominant controlling status of epigenetic mechanisms in regulating ESCs makes the whole process seems reversible and it was soon proved by reprogramming with transferring the

nuclear of somatic cells into oocytes, or by fusion with ESCs (Cowan et al., 2005; Wilmut et al., 1997). Moreover, the re-generation of pluripotency can further be simplified with the induction of only four transcription factors (Takahashi and Yamanaka, 2006). These induced pluripotent stem cells (iPSCs) resemble ESCs in their morphology, key markers gene expression, chromatin configuration (Takahashi and Yamanaka, 2006). Furthermore, they fulfill requirements of all standard pluripotency assays, including in vitro differentiation into three germ layers, teratoma formation, chimera formation, germline transmission and tetraploid embryo complementation (Feng et al., 2009; Jaenisch and Young, 2008). Human fibroblasts were reprogrammed using a different combination of OCT4, SOX2, NANOG and LIN28 (Yu et al., 2007). Since then, various reprogramming ways were presented and the core factors were proved to be replaceable expect Oct4. Most interestingly, miRNA was also revealed to play an equally important role in reprogramming. Mir-302 was implicated in reprogramming that could convert human cancer lines to cells that resembled ESCs (Lin et al., 2008). Later on, expression of the *miR302/367* cluster was shown to reprogram rapidly and efficiently both mouse and human somatic cells to an iPSC state without a requirement for exogenous transcription factors. The resulting iPSCs exhibit characteristic gene expressions and functional properties of fully reprogrammed pluripotent cells. Moreover, the reprogramming efficiency is of >100-fold compared with OSKM, with approximately 10% of fibroblasts form iPSC colonies. The appearance of iPSC colonies and the activation of pluripotency markers also occur earlier by using the *miR-302/367* cluster than OSKM. On the other hand, the down-regulation of some miRNAs can be beneficial such as *let-7*, the expression of which antagonise with the ESC pluripotency, which will be elaborate later.

1.1.9 **Aims of our study**

As introduced above, the ESC identity is maintained by a complicated network in which numerous factors play roles. Here we aim to investigate the possible roles of two independent factors, Lin28 and Jmjd6 in regulating ESC identity.

Chapter 2.

PROBING THE FUNCTIONS OF LIN28 IN MOUSE ESCs

2.1 Lin28: structure and functions

2.1.1 Lin28's role in development

The heterochronic genes are the genetic hierarchy regulators that control the developmental sequence timing by specifying the temporal components of fates of cells in diverse tissues throughout the animal in the nematode C. elegans, which is used as a model for developmental sequence regulation (Ambros, 1989; Ambros and Horvitz, 1984). To make mutants of these heterochronic genes display development defects, where either precocious or retarded phenotypes observed as a result of certain cell fates occur abnormally early or late, respectively (Ambros and Horvitz, 1984). These mutants are influential as a majority of developmental events are affected, including cell division, cell cycles, sex determination and stage-specific terminal differentiation events (Ambros, 1989; Ambros and Horvitz, 1984). Lin28, a lineage timing regulator, is highly expressed in the first larval stage and down-regulated later that allow the transition to later stages. In the screen for heterochronic mutants in C.elegans, the mutant of *Lin28* result in precocious development, where many developmental events specific to the second larval stage (L2) were skipped and the subsequent events were brought forward accordingly. and premature developmental progression (Ambros and Horvitz, 1984; Moss, 1997). The lin-28 mutants were deformed and unable to lay eggs(Ambros and Horvitz, 1984; Euling and Ambros, 1996). These results demonstrate that Lin28 may govern the succession of cell fates in the larva.

2.1.2 The expression of Lin28

Mammals and other animals possess two *lin28*-like genes, *Lin28* (or *Lin28a*) and *Lin28b* (Balzer and Moss, 2007; Guo et al., 2006; Moss, 2003).Lin28 is highly-conserved protein and lin28 homologues have been identified in many diverse organisms. Lin28 is expressed in ESCs, spermatagonia and testis and some tissues and organs (Lee et al., 2005; Moss and Tang, 2003; Wang et al., 2001b; Yang and Moss, 2003). In developmental stages, Lin28 is strongly expressed throughout the whole embryo at E6.5, including the embryonic and extraembryonic ectoderm and endoderm (at protein level), and subsequently expressed in the ectoderm, endoderm and mesoderm at E7.5 (at protein level) (Moss and Tang, 2003; Yang and Moss, 2003). Lin28 expression is reduced during differentiation of ES cells. In adult primary myoblasts, Lin28 is barely detectable during proliferation, but dramatically up-regulated during terminal differentiation. Little expression is detected in resting muscle, but strongly up-regulated during regeneration of skeletal muscle fibers (Polesskaya et al., 2007).

2.1.3 **Lin28 as a RNA binding protein**

Both Lin28a and Lin28b protein contain a unique pairing of two RNA-binding domains: a cold-shock domain (CSD) and retroviral-type CCHC zinc knuckles, illustrated as in Figure 2 (Moss, 1997). Both domains are found in well-characterized RNA-binding proteins.

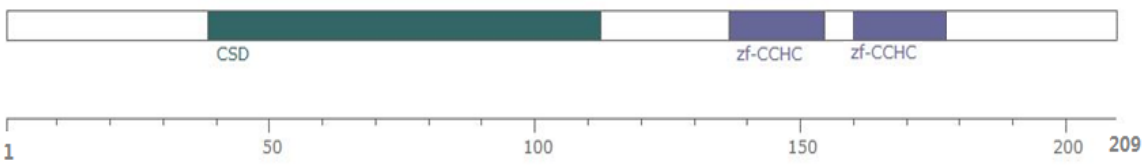


Figure 2. The CSD and CCHC domains in Lin28.

Found in vertebrates and invertebrates, the CSD is the characteristic domain of Y-box proteins, including FRGY2 of Xenopus and YB-1 of humans, with a conserved all-β-strand fold that contains a defined ssDNA/RNA-binding epitope that is able to bind both single-stranded DNA and RNA (Schindelin et al., 1994; Schnuchel et al., 1993). This domain is named after the prokaryotic cold-shock protein (Csp) and is a key component of the eukaryotic Y-box family of proteins, where it is coupled to auxiliary domains (Sommerville and Ladomery, 1996). It resembles the RNA-recognition motif (RRM) domain that exists in many RNA-binding proteins but is more conserved (Graumann and Marahiel, 1998; Manival et al., 2001). The CSD can confer sequence-specific RNA binding and interacts with additional RNA-binding domains called basic/aromatic (BA) islands (Wistow, 1990). The CCHC zinc finger exists in nucleocapsid proteins of retroviruses where it participates in genome recognition for packaging into virions (Gorelick et al., 1988). Each zinc finger motif is notified by characteristically spaced cysteine and histidine residues in the order CCHC (Moss, 1997). In addition, there is an immediately C-terminal in the zinc finger motifs that consists of a cluster of basic residues. Such clusters in RNA-binding proteins are important for interaction with RNA (De Rocquigny et al., 1993; Lazinski et al., 1989). Both of these domains have been studied for their structures and RNA-binding abilities (Ramboarina et al., 2004; Yu et al., 2003). However, Lin28a/Lin28b is the only animal protein to have both of them at the same time. This combination of structures sequence homologies suggests that *Lin28a/Lin28b* encodes an RNA-binding protein.

With the unique combination of these two RNA-binding domains, various targets of Lin28/Lin28b were increasingly found. Initial reports proposed that Lin28 protein may act to enhance the translation or stability of the *lin-14* mRNA (Arasu et al., 1991; Pepper et al., 2004; Seggerson et al., 2002). It is also found that LIN28 is associated with mRNAs and enhances translation of *Igf2* mRNA (Balzer and Moss, 2007; Polesskaya et al., 2007). However, the most notable target of both Lin28a and Lin28b would be the miRNA *let-7*, which has been proved to

be bound and blocked by Lin28a/Lin28b (Desjardins et al., 2012; Hagan et al., 2009; Heo et al., 2008; Heo et al., 2009; Lightfoot et al., 2011; Loughlin et al., 2012; Mayr et al., 2012; Nam et al., 2011; Piskounova et al., 2011; Viswanathan et al., 2008).

2.1.4 **One main target of Lin28**

The *lethal-7* (*let-7*) gene was initially discovered as a heterochronic switch gene that is essential for development in *C. elegans*. Loss of *let-7* gene activity in *C. elegans* causes reiteration of larval cell fates during the adult stage, whereas increased *let-7* gene dosage causes precocious expression of adult fates during larval stages (Reinhart et al., 2000; Sulston and Horvitz, 1977) . Afterwards, the highly conserved homologues of *let-7* family were found in other animals (Lagos-Quintana et al., 2003; Lee et al., 2007; Pasquinelli et al., 2003; Pasquinelli et al., 2000). *Let-7* plays roles in a set of biological processes, such as the regulation of stem-cell differentiation in *C.elegans*, neuromusculature development, limb development in mouse, and cell proliferation and differentiation (Lancman et al., 2005; Pasquinelli et al., 2000). Moreover, *let-7* was found to function as a tumor suppressor in many cancers in mammalians, where most or all *let-7* family members appear to be down-regulated (Esquela-Kerscher and Slack, 2006; Shi et al., 2008). *Let-7* also encodes a temporally regulated 21-nucleotide RNA that is complementary to elements in the 3' untranslated regions of lin28 genes in mammalians, rendering Lin28a/Lin28b become the main regulator to suppress its expression.

The posttranscriptional regulation of *let-7* by Lin-28a/Lin28b is required for normal development and contributes to the pluripotent state by preventing *let-7*-mediated differentiation of ESCs. The over-expression of Lin28 or repression of *let-7* promotes

reprogramming of fibroblasts to iPSCs both in human and mouse (Martinez and Gregory, 2010; Melton et al., 2010; Yu et al., 2007).

As described above, both Lin28a and Lin28b possess the two RNA-binding domains, CSD and CCHC, which are essential in binding to *let-7*. A recent study has demonstrated that, compared to the full-length protein, the isolated ZnF domains had four times less affinity for *let-7*, while 250 times less affinity for the isolated CSD (Desjardins et al., 2012). Lin28a binds to the terminal loop of *let-7* precursors and inhibit both pri-*let-7* processing by Drosha and Dicer (Piskounova et al., 2008; Viswanathan et al., 2008). A terminal uridylytransferase (TUTase), Zinc finger CCHC domain-containing protein 11 (Zcchc11) (also known as TUTase4 or TUT4), is recruited together with Lin28a to interact with *let-7*. Two conserved regions in the *let-7* terminal loop that recognized by Lin28a have been implicated: a single cytosine region and a 5'-GGAG-3' motif (Hagan et al., 2009; Heo et al., 2009). Mutation of these regions has shown lower affinity of Lin28a. In spite of the high degree of homology, the way Lin28b regulate *let-7* is distinct from Lin28a. Lin28b sub-locates in nucleus and binds to pri-*let-7* miRNAs to block processing by Microprocessor without recruiting Zcchc11.

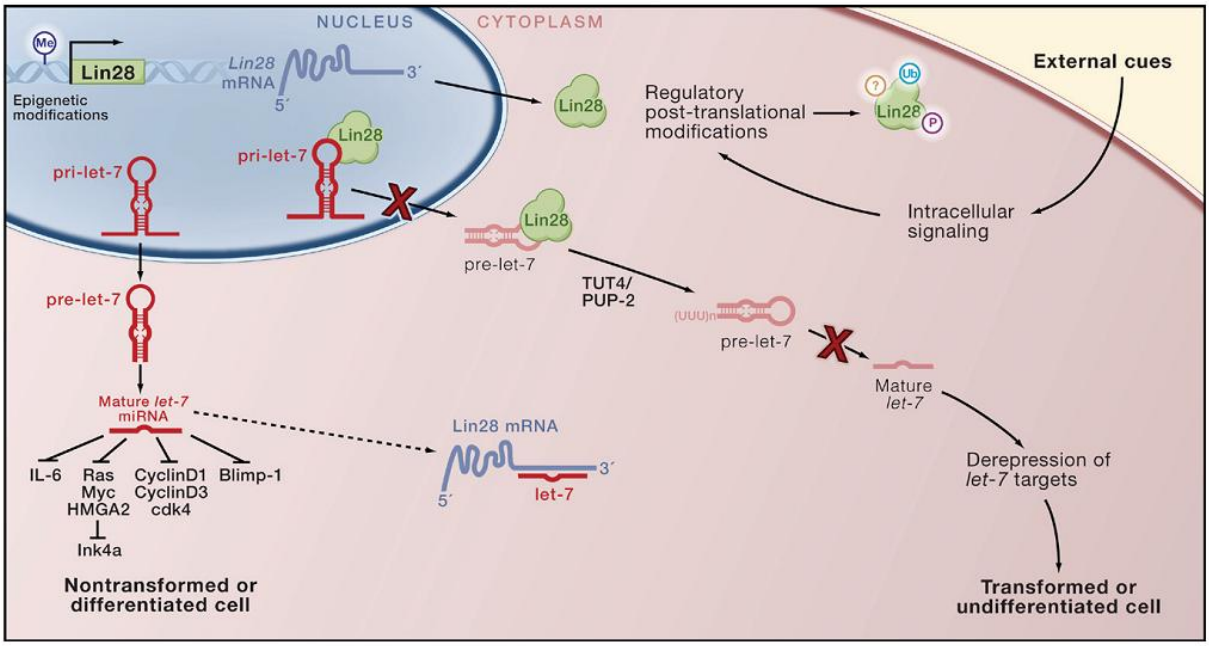


Figure 3. The Lin28 regulation to *let-7*. Picture adapted from Viswanathan et al., Science 2010 (320):97-100.

2.2 Materials and Methods

2.2.1 Cell culture

E14 mouse ES cells were cultured on 0.1% gelatin(Sigma)-coated plates and maintained in 5% CO2 incubator(Thermo) at 37 ^{o}C, using ESC medium containing 15% ES cell-qualified fetal bovine serum (FBS, Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acid (Invitrogen), 0.055 mM β-mercaptoethanol (Sigma-Aldrich), 1000 units/ml recombinant murine leukemia inhibitory factor (LIF)(Millipore), and topped up with Glasgow Minimum Essential Medium (GMEM) (Gibco Invitrogen). Medium was changed daily and passage was carried out once cells were about 80% confluent with following process: medium was removed and cells were washed twice with phosphate buffer saline (PBS), and then trypsinized using 0.05% trypsin for 5 min at 37 ^{o}C, and mechanical dissociation into single cells by pipetting. For retinoic acid (RA) treatment, LIF was removed from the medium and RA was added into the medium with 0.5 μM.

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)(Gibco Invitrogen) supplemented with 10% FBS, 60mM NaHCO3(Sigma), 1% Penicillin/Streptomycin (Pen/.Strep)(PAN Biotech GmbH) on non-gelatin-coated plates, Cell culture medium was changed every two days. Cells were passaged whenever necessary to maintain suitable confluency.

2.2.2 **Design of short hairpin RNA (shRNA) and generation of RNAi (RNA interference) plasmid**

shRNAs were designed from the software available from Eurofins MWG Operon website and those with the proper length (about 19-nucleotide) and high scores (>5) were chosen. The chosen sequence was integrated into a shRNA oligonucleotide containing a 9-nucleotide loop, with BglII and HindIII sticky ends for cloning, according to the template specified in the pSUPER RNAi System manual (OligoEngine). A BLAST search was done to ensure specificity of the shRNA primers for *Lin28a* and *Lin28b* respectively. The shRNA sequences for *Lin28a* and *Lin28b* were as follows (only upper sequences are listed here):

shRNA for *Lin28a*,

gatccccGAACATGCAGAAGCGAAGAttcaagagaTCTTCGCTTCTGCATGTTCttttta

shRNA for *Lin28b*,

gatccccGAAGTGCCATTACTGTCAGttcaagagaCTGACAGTAATGGCACTTCttttta

The forward and reverse oligonucleotides were annealed in annealing buffer (100 mM Tris-HCl, pH 8.0, 500 mM sodium chloride, 10 mM ethylenediaminetetraacetic acid [EDTA], 10 mM magnesium chloride) by immersing in boiling water for 5 min and left inside to cool overnight. The annealed oligonucleotides were ligated into pSUPER.puro vector predigested with BglII and HindIII (New England Biolabs) with T4 DNA ligase (New England Biolabs) and 1×T4 DNA ligase buffer (New England Biolabs).

The ligated products were then transformed into competent DH5α *Escherichia coli* cells after cooling on ice for 30 min, heat shock at 42^{o}C for 60 sec, cooling on ice for 2 min, and subsequent shaking in 1 ml of Lysogeny broth (LB) medium at 37 ^{o}C for 45 min before being evenly applied on ampicillin agar plates at 37 ^{o}C overnight. Single colonies were picked on the

next day and incubated overnight at 37 ^{o}C in 4 ml of LB medium containing ampicillin. Plasmid DNA was were extracted using the QIAprep Spin Miniprep Kit (Qiagen), and the plasmids with the presence of inserts was selected by digesting with restriction endonucleases EcoRI and XhoI (New England Biolabs) and comparing to a negative control (empty vector) upon 1% agarose gel electrophoresis. The sequencing PCR of selected plasmids were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences), using 10 pmol of primer and 300ng of DNA template and topped up to 10 µl working volume with nuclease-free water (Ambion). The samples were then sent for sequencing to confirm the accuracy of the inserts. Cycling parameters were set according to manufacturer's instructions for large DNA templates.

The plasmid used in double knockdown trial was also constructed in pSUPER.puro. After plasmids contain Lin28a and Lin28b shRNAs were separately constructed, the shLin28a plasmid was then digested with XhoI and ClaI and the circular part was kept. Meanwhile, the shLin28b plasmid was digested with XhoI and BstBI, an isocaudarner of ClaI, and the linear part was collected and ligated to the circular Lin28a-containing plasmid, as shown in Figure 4. The combination of two shRNAs in one plasmid ensures the successful double knockdown.

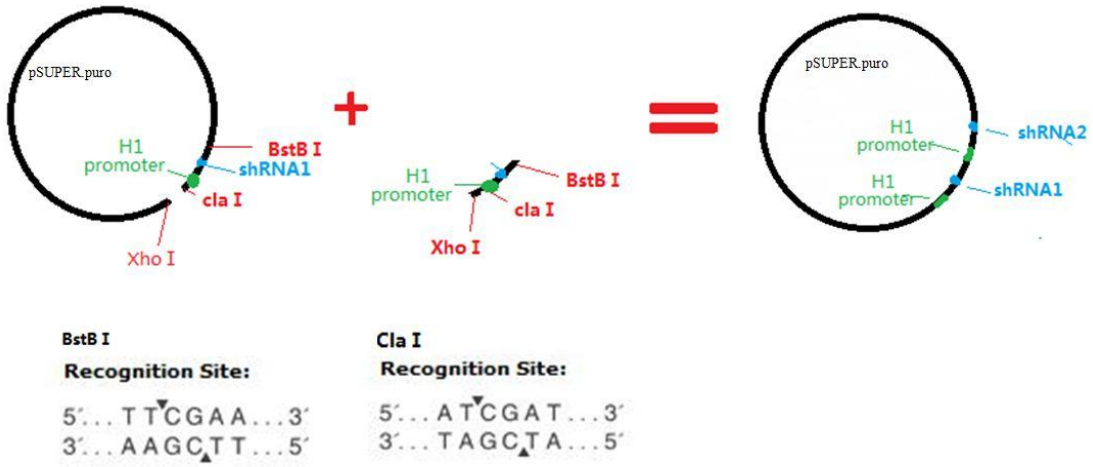


Figure 4. The construction of simultaneous knockdown plasmid.

2.2.3 Transfection and selection of transfected cells

Transfection of shRNA plasmids was carried out according to the standard steps in the product manual instructions. 10 μl Lipofectmine 2000 (Invireogen) was mixed with 250 μl Opti-MEM I Reduced Serum Medium (Invitrogen) for 5 min before its combination of the other mixture of 4 μg plasmid and 250 μl OptiMEM for 20 min. The final mixture was added into one well of a 6-well plate presented with 2 ml ESC medium drop by drop, with the plate swirled all the time. The transfected cells were incubated 14-16 h before selection.

Puromycin was diluted to 1.0 μg/ml in ESC medium to select transfected cells. Medium was changed every 24 h to maintain the antibiotic concentration. The cells were harvested after 72 h selection.

2.2.4 RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) and purified using RNeasy Mini Kit (Qiagen) according to the provided protocols. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water (Ambion). The Superscript III First-Strand Synthesis System with oligo (dT) priming primer (Invitrogen) were was used to convert mRNA to complementary DNA (cDNA).

The cDNA obtained from reverse transcription was diluted in a 1-in-20 ratio with nuclease-free water (Ambion). Forward and reverse primers (Appendix Supplementary Table 1) were pre-

mixed in a 1:1 proportion with 0.09 µl of the mixture being added into 10 µl of SYBR Green PCR Master Mix (Applied Biosciences), 8 µl of nuclease-free water (Ambion) and 2 µl of diluted cDNA for each well of a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems). The reaction plate was sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and spun down at 1,000 rpm for 2 min at 4 ^{o}C.Real-time PCR was conducted using the ABI Prism 7300 Real-Time PCR system (Applied Biosystems) at default parameters. Specificity of real-time PCR primers were verified by dissociation curve analysis and running PCR products in gel electrophoresis, for which only one band of the right size should be observed. Relative expression levels of target genes from sample cDNA were normalized to *β-actin* levels and reflected as a fold change compared to the control sample.

Let-7 miRNA used for qRT-PCR validation after being tested can be used in mouse liver cells were. Reverse transcription RCR was performed from 10 ng of total RNA sample with miRNA specific RT primers provided in the TaqMan® MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The 5S rRNA was used as the control and the primer was purchased from Sigma-Proligo. Final volume was topped up with DEPC water to 10µl.

Samples were mixed gently and spun down. The reaction mixture was incubated at 16 ^{o}C for 30 min. The reverse transcription was then carried out at 42 ^{o}C for 30 min followed by denaturation at 85 ^{o}C for 5 min. 1.5 µl of RT reaction product was then mixed with TaqMan® 2× Universal PCR Master Mix without AmpErase® UNG, miRNA-specific primer and probe mix from the TaqMan® MicroRNA Assays. The mixtures were incubated in a 96-well plate at 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C for 15 sec and 60 ^{o}C for 1 min, on an ABI Prism 7300 Real-Time PCR system (Applied Biosystems). The 5S primers and probe were designed and synthesized by Sigma-Proligo as followed:

5S forward: CGCCCGATCTCGTCTGAT;

5S reverse: GGTCTCCCATCCAAGTACTAACCA;

5S probe: TCGGAAGCTAAGCAGGGTCGGGC.

The 5S cDNA was diluted 500 times before real-time PCR. The PCR products were further analyzed with the Applied Biosystems 7300 System SDS software (Applied Biosystems).

Protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

ESC medium was removed and cells were washed twice with cold PBS (1st Base), after which the cells were scrapped in PBS and spun down at 3000rpm for 3 min at 4 ^{o}C. The cell pellet was subsequently washed two more times in PBS and resuspended in Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, 5 mM EDTA, pH 8.0, 0.002% bromophenol blue) with protease inhibitor cocktail (Roche) before heating at 95^{oC} for 5 min. The cell lysate was then centrifuged at 14,000 rpm for 15 min. The supernatant, which contains total protein, was harvested. Equal amounts of protein were loaded into each well of a 10% SDS polyacrylamide gel and ran in SDS-PAGE running buffer (25mM Tris, pH 8.3, 0.192 M glycine, 0.1% SDS) at 120V.

2.2.5 **Western Blot**

Proteins migrating differentially according to molecular weight on SDS-PAGE gel were transferred to a methanol-activated polyvinylidene fluoride (PVDF) membrane (Bio-Rad) by running at 320 mA for 1 h in Western Blot transfer buffer (25 mM Tris, pH 8.3, 0.192 M

glycine). Successively, the membrane was blocked using PBST (0.1% Tween-20 (Sigma) in PBS) with 5% skimmed milk for 1 h at room temperature. The membrane was probed with primary antibody (diluted in PBST with 5% skimmed milk) at room temperature for 1 h. The membrane was then washed thrice in 0.1% PBST for 10 min each, probed with secondary antibody conjugated to horseradish peroxidase (GE Healthcare) at room temperature for 45 min. After washing three times with 0.1% PBST, the membrane was incubated in Imobilon Western Chemiluminescent HRP Substrate (Millipore) for 5 min. The chemiluminescent signal was then detected using CL-Xposure Film (Thermo Scientific) in a dark room. Antibody incubation and washing steps were performed on a shaker at 70 rpm. Primary antibodies used were: rabbit anti-Lin28 (1:2000; 07-1385, Millipore), rabbit anti-Lin28b (1:1000; #4196, Cell signaling) mouse anti-β-actin (1:1000; sc-81178, Santa Cruz), goat anti-Oct4 (1: 2000; sc-8628, Santa Cruz), and rabbit serum anti-Nanog (1:1000).

2.2.6 **Gene expression microarray analysis**

RNA of Lin28 RNAi sample and control RNAi sample were harvested with TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit (QIAgen). Isolated RNA was converted to sense strand cDNA incorporated with dUTP using the Ambion WT Expression Kit (Applied Biosciences), followed by fragmentation and biotin labeling with the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix), and finally hybridized on the GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Log_2 transformed gene expression of knocked-down samples and control were compared. Genes that displayed significant fold change (>1.5 or <0.8) between the replicates were selected for further analysis. Cluster 3.0 was utilized to perform hierarchical clustering on selected genes (Eisen et al., 1998). Data was adjusted to center genes and array by

mean, before being clustered with the Euclidean distance similarity metric and Average linkage clustering method. The results were then visualized using Java Treeview (Eisen et al., 1998). Genes from individual clusters were isolated and subjected to Gene Ontology (GO) analysis using the Generic GO Term Finder (Boyle et al., 2004) on the MGI – *Mus musculus* database for biological processes that were enriched among the genes. Bonferoni correction for p-value was applied and false discovery rate (FDR) was calculated. Only GO terms with corrected p-values below the designated threshold (<0.01 for down-regulated genes, <0.02 for up-regulated genes in sample) were considered.

2.2.7 **Immunostaing**

ESCs were grown on coverslips placed in 24-well culture plates for a day before immunostaining. ESC medium was aspirated from wells and cells were washed twice with PBS. 4% paraformaldehyde (PFA) was used to fix cells for 20 min at room temperature. The cells were permeabilized with PBS plus 0.5% Triton X-100 for 20 min after PFA was removed. Next, the cells were washed with 0.1% PBS plus Tween-20 (PBST) for 5 min on a belly-dancer rotator. This was followed by 30 min incubation in 3% bovine serum albumin (BSA). After that the cells were probed with Lin28a (1:500,07-1385, Millipore) and Lin28b (1:500; #4196,Cell signaling) antibody respectively diluted in 3% BSA for 1 h. The cells were then washed with 0.1% PBST for 5 min for 4 times on the belly-dancer rotator before incubating with Alexa Fluor 568 goat anti-rabbit lgG antibody (Invitrogen) diluted to 1:1000 in 3% BSA for 30 min at room temperature. The cells were washed again with 0.1% PBST for 5 min on the belly-dancer rotator thrice. A drop of Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was placed on the microscope slide and the cover slip was sealed

with nail polish to keep the ES cells in contact with the mounting medium. Staining signal was then visualized.

2.2.8 **RNA immunoprecipitation (RIP)**

The RNA immunoprecipitation (RIP) was performed according to RNA-Binding Protein Immunoprecipitation Kit product manual (Millipore). ESCs were cultured in 10 cm plates and were scraped when about 80% confluent and washed with cold PBS twice. Complete RIP Lysis Buffer provided by kit was used to re-suspend the cells which were mixed homogeneously in the buffer by pipetting. Lysate was incubated on ice for 5 min to swell cells and then stored in -80 ^{o}C.

50 μl Magnetic beads were incubated with 5 μg Lin28 antibody (07-1385, Millipore) and IgG (12-370, Millipore) antibody respectively in 100 μl RIP Wash Buffer for 30 min after being washed twice with RIP Wash Buffer. Extra two washing were performed to minimize the background. The antibody-binding beads were then incubated with 900μl RIP Immunoprecipitation Buffer and 100 μl supernatant of newly thawed cell lysate prepared above in 4 ^{o}C overnight to undergo immunoprecipitation. Afterwards, the immunoprecipitated mixture was washed six times with RIP Wash Buffer.

The RNA-protein complex was then eluted in 150 μl Proteinase K Buffer at 55 ^{o}C for 30 min with shaking to digest the protein. Supernatant was collected together with 250 μl RIP Wash Buffer and RNA was precipitated with 50 μl Salt Solution1, 15 μl Salt Solution 2 ,5 μl precipitate Enhancer and 850 μl absolute ethanol after phenol-chloroform extraction. The mixture was kept at -80 ^{o}C overnight and was washed with 75% ethanol before reverse

transcription.

2.3 **Results**

2.3.1 **Lin28a decreases upon ESC differentiation**

The regeneration of pluripotency of differentiated cells upon transfection with *LIN28a* and other three factors suggest a possible involvement of Lin28a in pluripotency regulation in mouse ESCs. To determine the expression profile of *Lin28a* upon differentiation of ESCs, E14 mouse ESCs were induced to differentiate by culturing in retinoic acid (RA) and LIF withdrawal medium for periods of one day, two days and three days. LIF withdrawal medium was used here as LIF is an important constituent in ESC medium to maintain pluripotency in mESCs, without which the cells will differentiate (Nichols et al., 1996). Total RNA was extracted at the respective time points and real time-PCR was carried out to determine the levels of *Lin28a* mRNA relative to E14 cells cultured for the same time period in normal ES cell medium.

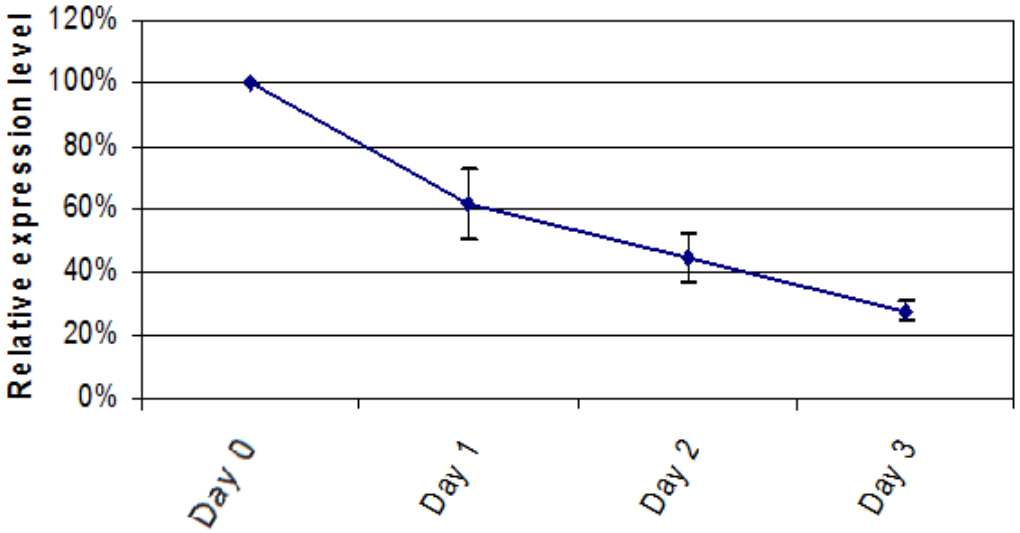


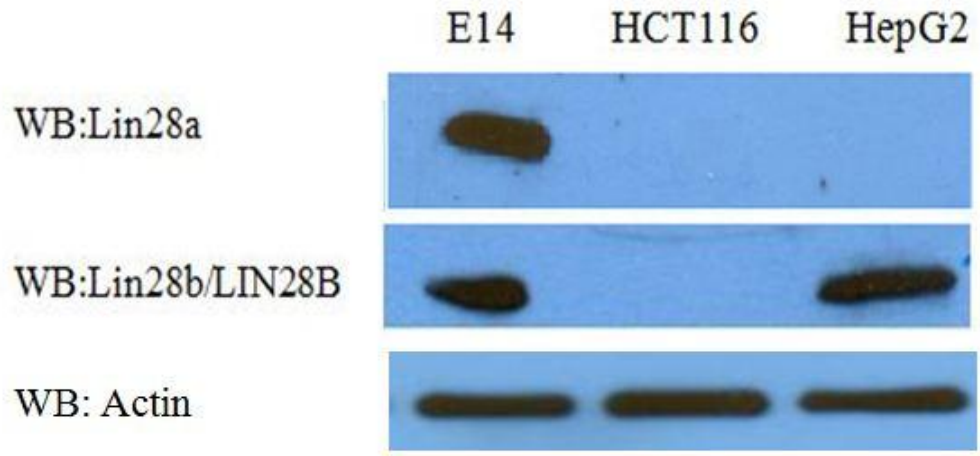
Figure 5. **Expression of *Lin28a* upon ESC differentiation.** mRNA of *Lin28a* was measured upon E14 cells were induced to differentiation by LIF removal and retinoic acid (RA) treatment.

As Figure 5 presented, the expression level of *Lin28a* showed a drastic decrease upon cell differentiation and only 30% left was by Day3. This result may suggest Lin28 is related to ESC pluripotency.

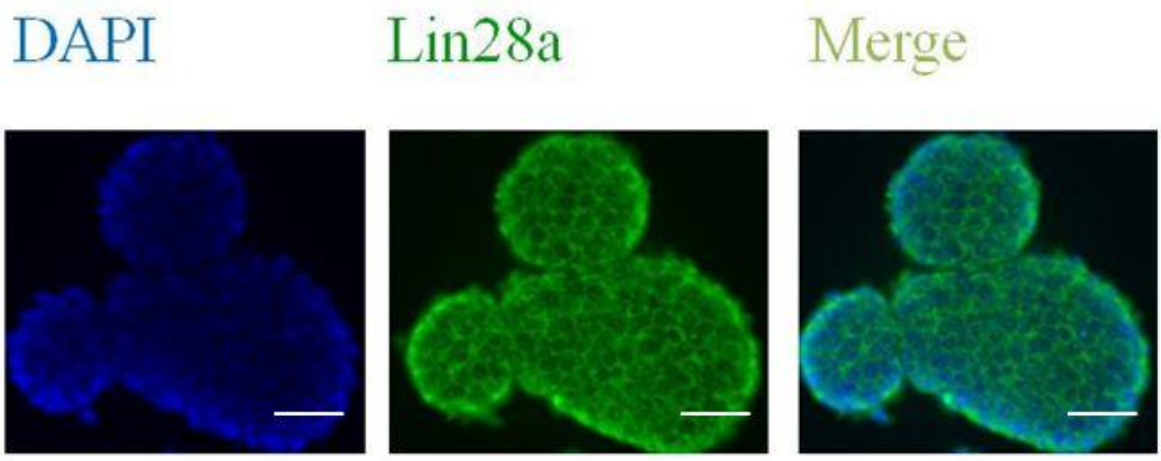
2.3.2 **Both Lin28a and Lin28b are expressed in E14 cells**

Although the paralogous proteins Lin28a and Lin28b have a high degree of sequence identity and conserved domains, the Lin28b inclines to be more involved in carcinoma than Lin28a (Guo et al., 2006; Helland et al., 2011; King et al., 2011; Permuth-Wey et al., 2011). Their distinct expressions in a variety of cells were revealed that they exert different mechanisms to function (Piskounova et al., 2011). Our western results supported by in situ hybridization and immunostaining results confirmed the expression of the two proteins. Both Lin28a and Lin28b were expressed in E14 cells, whereas HepG2 only express Lin28b. Neither Lin28a nor Lin28b was observed in HCT116 while both of their mRNAs were detected, suggesting that they might be blocked at translational level. Immunofluorescence assay was used to examine the subcellular localization of the endogenous Lin28A and Lin28B proteins (Figure 6A). Lin28A was predominately localized to the cytoplasm of E14 cells, while Lin28B was localized to specific foci in the nuclei of HepG2 and E14 cells.

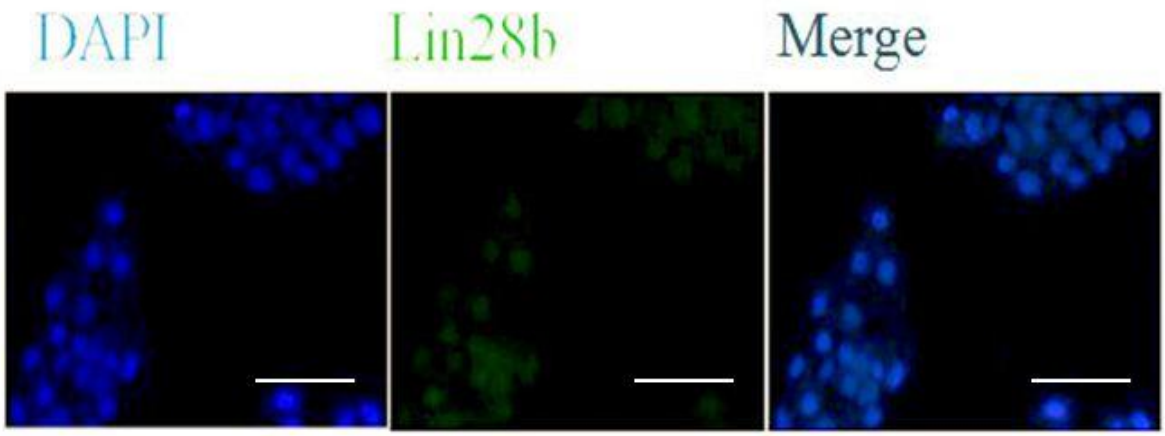
6A


E14
HCT116
HepG2
WB:Lin28a
WB:Lin28b/LIN28B
WB: Actin

6B


DAPI
Lin28a
Merge

6C



6D

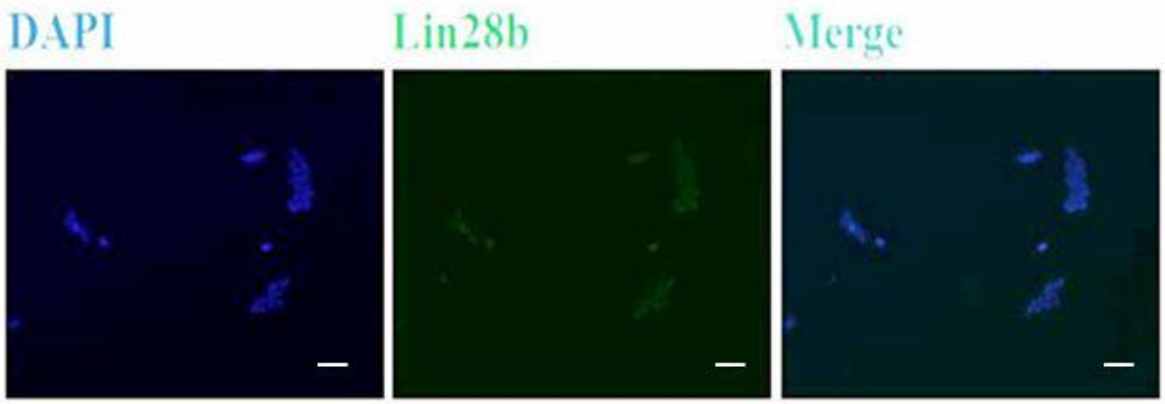


Figure 6. Expression of Lin28a and Lin28b/LIN28B in mouse ESCs and human cancer cells. (A) Protein of Lin28a and Lin28b/LIN28B from E14, HCT116, HepG2 cells determined by Western blot. (B-D)Immunofluorescence detection of Lin28a in E14, Lin28b in E14 and LIN28B in HepG2, respectively. DAPI was used to indicate the nucleus. Scale bars represent 50 μm.

2.3.3 **Global gene level changes indicate Lin28 is essential for ESC in self-renewal.**

To investigate the Lin28's role in ESC, shRNAs of *Lin28a* and *Lin28b* were designed and transfected in ESC line E14 to knockdown *Lin28a* and *Lin28b* respectively. In addition, a simultaneous knockdown of these two genes was performed. As seen in Figure 7, double knockdown were more efficient than individual knockdown. This would probably result from the highly conserved sequence identity between Lin28a and Lin28b that makes it possible that one protein may complement in the absence of the other. Therefore, it is necessary to knock down these two genes simultaneously.

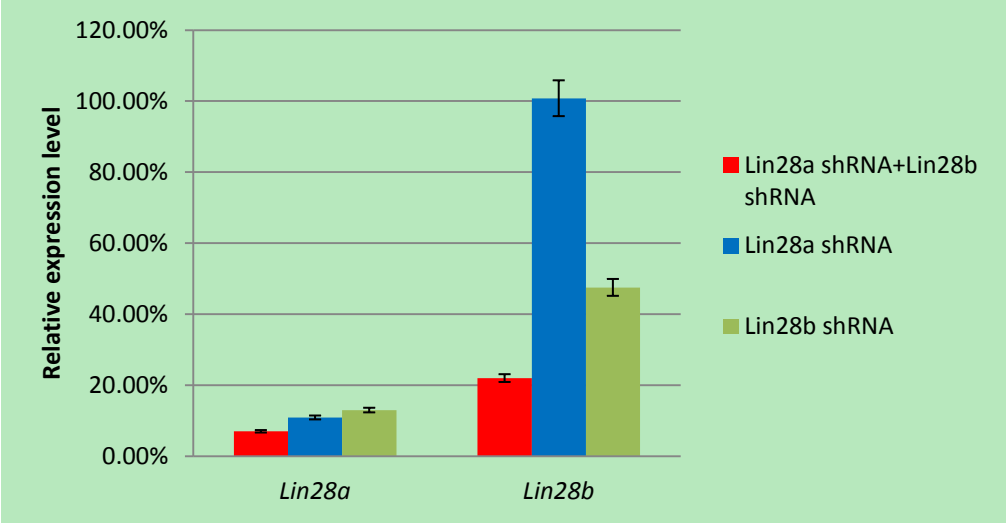
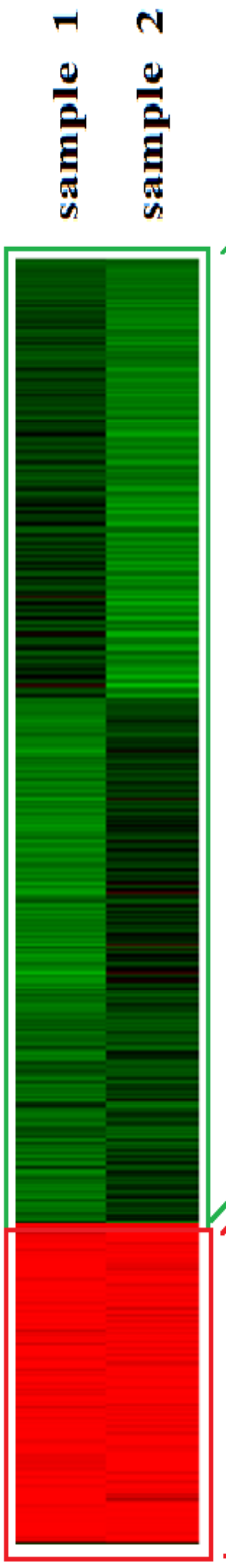


Figure 7. Effects of *Lin28a, Lin28b* single and double knock-down on their own expression levels. E14 cells were transfected with m*Lin28a* and m*Lin28b* shRNAs, both independently and simultaneously. Cells were selected for 72 hours, before total RNA was extracted to test for the knockdown efficiency.

Transcriptome from *Lin28* double-knocked-down cells were compared to control cells transfected with empty pSUPER.puro vector, we found that 2182 genes were significantly different between the two, of which 671 were up-regulated (> 1.5-fold) and 1511 down-regulated (<0.7-fold). We then randomly selected many genes from the list of up-regulated and down-regulated genes to do validation. Primers used for validation can be found in

Supplementary Table 8. Although the precise values of the fold change between the real time-PCR results and the microarray results differed, in general, the trend of up-regulation and down-regulation of the tested genes was consistent between both sets of data (Figure 8 BC). This implies that the direction of change of the genes can be inferred from the microarray data reliably. Hence, both up-regulated and down-regulated genes were separately examined for Gene Ontology terms related to biological processes that were enriched within the two groups.

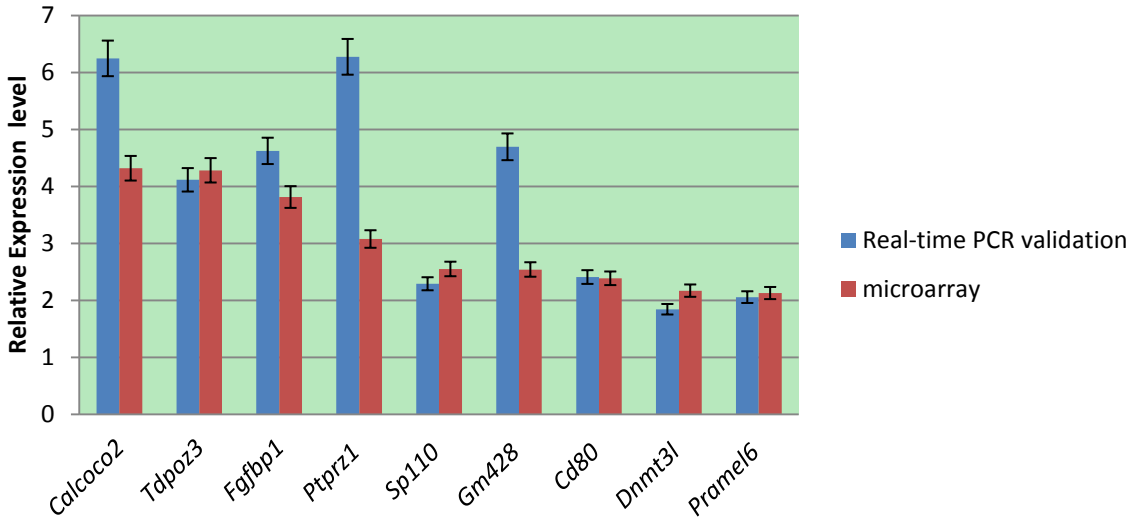
A



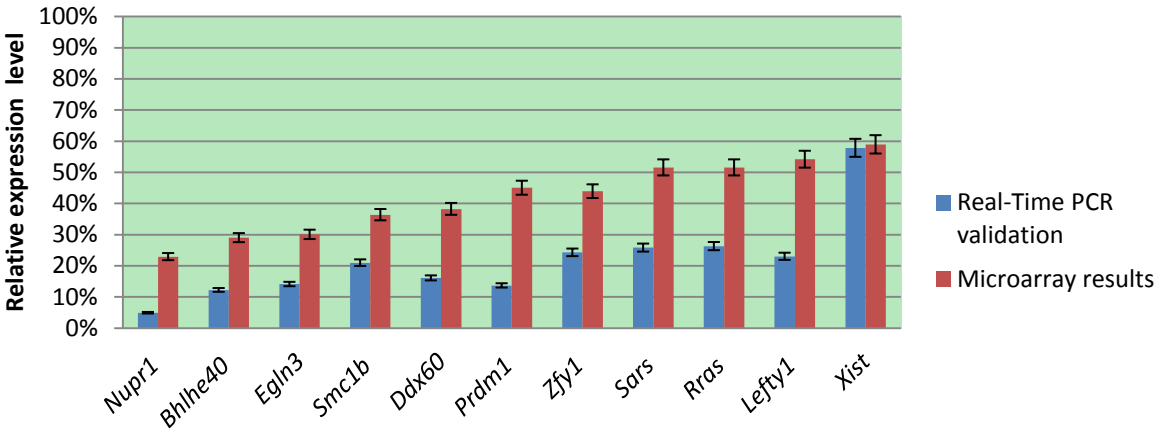
Enriched GO terms(down-regulated),p-value<0.01:
GO:0019752 [6]: carboxylic acid metabolism
GO:0006082 [5]: organic acid metabolism
GO:0043038 [7]: amino acid activation
GO:0043039 [8]: tRNA aminoacylation
GO:0006418 [8]: tRNA aminoacylation for protein translation
GO:0009451 [6]: RNA modification
GO:0006400 [7]: tRNA modification
GO:0006520 [6]: amino acid metabolism
GO:0006066 [5]: alcohol metabolism
GO:0005975 [5]: carbohydrate metabolism
GO:0006952 [5]: defense response
GO:0006629 [5]: lipid metabolism
GO:0006519 [5]: amino acid and derivative metabolism
GO:0009607 [4]: response to biotic stimulus
GO:0006096 [8]: glycolysis
GO:0015849 [5]: organic acid transport
GO:0046942 [6]: carboxylic acid transport
GO:0044255 [5]: cellular lipid metabolism
GO:0009308 [5]: amine metabolism
GO:0006092 [7]: main pathways of carbohydrate metabolism
GO:0042632 [4]: cholesterol homeostasis
GO:0000238 [11]: zygotene
GO:0007129 [12]: synapsis
GO:0016125 [6]: sterol metabolism
GO:0006399 [6]: tRNA metabolism

Enriched GO terms(up-regulated),p-value<0.01:
GO:0050896 [3]: response to stimulus
GO:0007608 [7]: perception of smell
GO:0009605 [4]: response to external stimulus
GO:0009581 [5]: detection of external stimulus
GO:0007600 [5]: sensory perception
GO:0007606 [6]: sensory perception of chemical stimulus
GO:0007186 [6]: G-protein coupled receptor protein signaling pathway
GO:0006730 [5]: one-carbon compound metabolism
GO:0007275 [2]: development
GO:0000050 [5]: urea cycle
GO:0050874 [3]: organismal physiological process
GO:0001525 [6]: angiogenesis
GO:0000051 [4]: urea cycle intermediate metabolism
GO:0006525 [5]: arginine metabolism
GO:0001558 [4]: regulation of cell growth
GO:0050877 [4]: neurophysiological process
GO:0009887 [4]: organogenesis
GO:0048514 [5]: blood vessel morphogenesis
GO:0048513 [3]: organ development
GO:0006349 [4]: imprinting
GO:0009653 [3]: morphogenesis
GO:0016049 [4]: cell growth
GO:0001568 [4]: blood vessel development
GO:0008361 [5]: regulation of cell size

B


Relative Expression level
7
6
5
4
3
2
1
0
Calcoco2
Tdpoz3
Fgfbp1
Ptprz1
Sp110
Gm428
Cd80
Dnmt3l
Pramel6
Real-time PCR validation
microarray

C


Relative expression level
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Nupr1
Bhlhe40
Egln3
Smc1b
Ddx60
Prdm1
Zfy1
Sars
Rras
Lefty1
Xist
Real-Time PCR validation
Microarray results

D

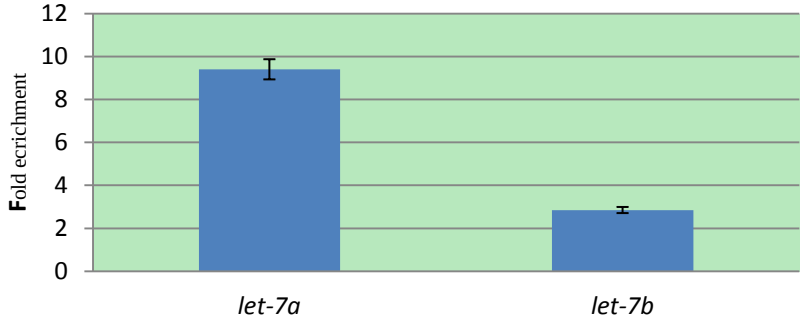

Fold ecrichment
12
10
8
6
4
2
0
let-7a
let-7b

Figure 8. Global gene expression change via microarray analysis on *Lin28a* and *Lin28b* simultaneous knockdown cells. (A) Up-regulated genes (red) and down-regulated genes (green) were subjected to Gene Ontology search for enriched biological process terms. Selected enriched terms are shown to reduce redundancy. (B) Validation of microarray for up-regulated genes. (C) Validation of microarray for down-regulated genes. (D)Detection of *let-7* miRNA expression.

For the down-regulated genes, many terms related to RNA metabolisms especially tRNA, including the term "tRNA metabolism" itself, were discovered to be enriched (Figure 8A), indicating the Lin28's role as a RNA regulation protein. Given that the tRNAs are the main participants in the translation process, this result also support the previous observation that Lin28 functions as a translation enhancer (Peng et al., 2011b) by activating the tRNA metabolisms, which has never been studied before. The gene *Zcchc11* (Zinc finger CCHC domain-containing protein 11) is in the down-regulation gene list, which is interesting because the protein Zcchc11 was reported to be recruited together with Lin28 in uridylating the pre-miRNA, which then fails to be processed by Dicer and thus gets degraded by nuclease (s). The down-regulation of Zcchc11 may possibly imply the weakened ability to inhibit miRNA and it was proved by the increased fold of miRNA *let-7*a and *let-7*b in a miRNA real time -PCR (Figure 8D). Correspondently, a set of *let-7* targets like *c-Myc*, *Hmga2*, *K-Ras* level decreased. Besides, Lin28 seemed to closely relate to glucose metabolism by including several relevant terms such as "glycolysis", "glucose metabolism", "glucose catabolism", "hexose metabolism". In fact, a recent study has revealed the Lin28/*let-7* axis is an important modulators of glucose metabolism through interactions with the insulin-PI3K-mTOR pathway and T2D-associated genes identified in GWAS (Zhu et al., 2011). It would be interesting to investigate additional mechanisms and feedback that may exist.

Up-regulated genes yielded enrichment for biological process terms mainly related to development and cell growth. There were as many as 35 annotated genes involved in the term "development" and 22 for term "organogenesis", and the number was at the top of all terms, hinting the essential role of Lin28 in development. Among them were some genes related to embryogenesis, gametogenesis, placentation and development of specific tissues. Besides, some genes notified for their roles in chromatin modification were also been up-regulated. Three DNA (cytosine-5) -methyltransferases (*Dnmt3a*, *Dnmt3b* and *Dnmtl*) that are required for genome-wide de novo methylation and is essential for the establishment of DNA methylation patterns during development were up-regulated (Okano et al., 1999).

Unexpectedly, although varieties of development related genes were influenced by *Lin28* double knockdown, none of the pluripotency marks--*Oct4*, *Nanog*, *Sox2* was down-regulated. In consistent with this, nearly all the lineage markers (ectoderm, mesoderm, endoderm, trophectoderm) sustained their mRNA level. Therefore, Lin28 may not indispensable in regulating pluripotency, at least in mRNA level.

Western blot was also performed to test whether pluripotency markers were affected in protein level. Cell lysate was denatured and protein among was quantified to 400μg protein loaded in to each well, both the double knockdown sample and control respectively. As shown in Figure 9, there is no significant difference in protein level of both Oct4 and Nanog.

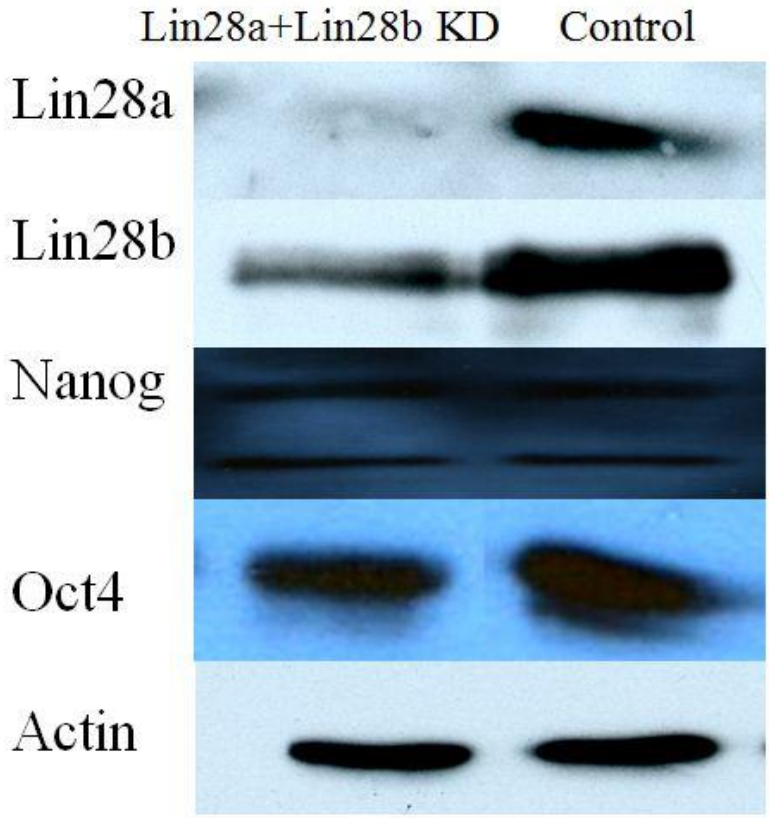


Figure 9. Western blot of Lin28a and Lin28b simultaneous knockdown in E14.

2.3.4 **Exploring other RNA targets of Lin28**

The two RNA-binding domains make it possible for Lin28 to have more RNA targets. And it has been reported that Lin28 may modulate cell proliferation by enhancing the translation of various cell-cycle regulators in mouse ESCs (Xu et al., 2009a). The cyclin family are a subset of proteins in the transition of cell cycles in association with cyclin-dependent kinase(Cdk)s. The enrichment of these cyclins indicates Lin28 may affect cell proliferation by binding to them. Hence RNA immunoprecipitation (RIP) was performed to pull down the RNA-protein complex and the sample would be sent for RNA-sequencing afterwards. However, as in our

result, the enrichment folds for the cyclins were not as high as published data (Figure 10). It is possible the RIP protocol needs a further optimization.

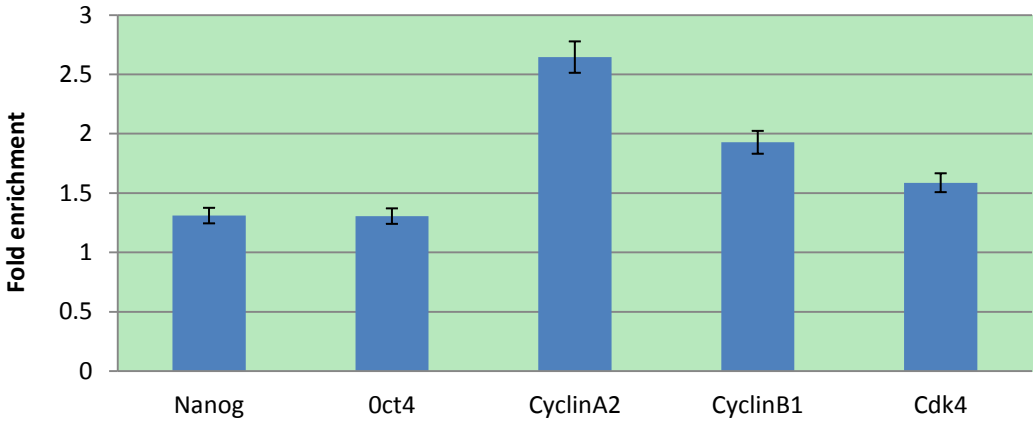


Figure 10. Enrichment fold change of pluripotency markers and cell cycle regulators separated from Lin28-RNA complexes. Data was normalized with *actin* and compared with IgG RIP.

2.4 Discussion

2.4.1 **Lin28 may regulate the self-renewal of ESCs both in miRNA dependent and independent ways**

Here we demonstrate that both Lin28's mammalian homologs, Lin28a and Lin28b, though with distinct subcellular locations, are both expressed in E14 cells. In fact, in spite of the high similarity, these two homologs seem to have their own preferred targets. Lin28a is predominately located in cytoplasm while Lin28b is expressed exclusively in nucleus. They both bind to pre-*let-7* but in different ways. Lin28b was firstly found in human hepatocellular carcinoma and was more related to oncogenesis according to the previous studies (Guo et al., 2006; Helland et al., 2011; King et al., 2011; Permuth-Wey et al., 2011; Yuan et al., 2012). In a screen trial of several human cancer cell lines, Lin28a and Lin28b were found mutually exclusive as no co-expression of them was observed (Piskounova et al., 2011). The mechanism of how cells choose to express one of them is not clear yet. However this probably implies Lin28 is dispensable in cancer cells. By comparison, ESCs in both human and mouse express these two together, reaffirming the essential role of Lin28 in ESCs.

It is commonly considered that most of Lin28's functions are linked to its miRNA target, *let-7*. Contrary to Lin28, the expression of *let-7* is rarely detected in undifferentiated ESCs. But it increases drastically upon differentiation and is maintained at a certain level afterwards, as introduced above. In fact, *let-7* targets several oncogenes like *c-Myc, K-Ras, cyclin D1* and *IL6* and its loss has been linked to oncogenes (Iliopoulos et al., 2009; Kumar et al., 2007; Roush and Slack, 2008). A regulatory circuit made up of NF-κB, Lin28b, *let-7* and IL6 was found in most cancer cells links inflammation to cellular transformation and is important for transformation and cancer cells growth (Iliopoulos et al., 2009). The majority of cancer cells

tested showed the characteristics of the inflammatory regulatory circuit, namely Lin28b overepxpression, *let-7* downregulation, and high levels of IL6. The accumulation of IL6 in this circuit is required for sufficient binding to the IL6 receptor to cause phosphorylation and nuclear entry of the STAT3 transcription factor, which then promote transformation (Niu et al., 2002). Moreover, the knock down of *Lin28b* by siRNA together with inhibition of NF-κB, which is a Lin28b activator, showed a significant suppression of tumor growth in all treated mice, proving the essential role of Lin28b in cancer cells by repressing *let-7* (Iliopoulos et al., 2009).

Interestingly, though STAT3 contributes to the maintenance of ESCs, the mRNA level of neither *STAT3* nor *IL6* was not affected by *Lin28a* and *Lin28b* double knockdown and the evocable up-regulation of *let-7* according to our result. However, other vital targets of *let-7*, including *c-Myc*, *K-Ras* and *HMGA2*, were down–regulated significantly. All of these targets were found to promote stem cells self-renewal but not pluripotency maintenance. In addition, though *let-7*'s expression is antagonistic with the cell's stemness, there is no direct evidence that *let-7* can cause differentiation in mammalians. In fact, in reprogramming human somatic cells using four factors including LIN28, it is discovered that LIN28 is not absolutely required for the initial reprogramming, nor is it subsequently required for the stable expansion of reprogrammed cell, although LIN28 can influence the frequency of reprogramming (Yu et al., 2007). Moreover, in our microarray result, none of the pluripotency markers was down-regulated. These suggest that Lin28 is more necessary in regulating self-renewal of ESCs than in maintenance of pluripotency.

Several reports also indicate that Lin28 can affect protein levels by working as transcription enhancers to regulate mRNA, where Lin28 function independent of *let-7*. Qiu and Peng reported that Lin28 can associate with Oct4 mRNA in human embryonic cells and directly

promote Oct4 translation (Peng et al., 2011a; Qiu et al., 2009). However, the western blot result upon Lin28a/Lin28b double knockdown in our mouse ESCs did not show any significant decrease, which may result from the different mechanisms between human and mouse. Meanwhile, both our RIP and the Xu group's result showed an increase in cyclins that regulate cell proliferation, though not as strong as them (Xu et al., 2009a). Again our results suggest Lin28 tends to regulate ESCs self-renewal rather than pluripotency.

However, it is still to be revealed whether Lin28 has other critical RNA targets. The molecular basis for interaction of *let-7* with Lin28 has been well studied. The CSD domain recognizes the sequence consensus of NGNGAYNNN (Y=pyrimidine;N=any base) and inserts into the loop at one end of the stem-loop structure in pre-*let-7*. The CCHCx2 domain recognizes a GGAG motif at the other end. The sequence and distance of these domains are variable (Nam et al., 2011). The identification of recognition site may shed light on the common characteristics of Lin28's targets. The Lin28a protein has been purified and is to be used in the future to find more possible targets.

In conclusion, the double knockdown *Lin28a* and *Lin28b* and subsequent gene expression microarray have indentified numerous potential Lin28 downstream genes in ESCs. Our study has also revealed that Lin28 may regulate ESC self-renewal in both miRNA dependent and independent ways.

2.5 **Future work**

We have shown here that Lin28 may affect the ESC self-renewal according to the microarray result. However, though various genes undergo increased or decreased fold change, the mechanism behind this variation is not clear. Hence further study will be required to unravel the underlying mechanisms.

In addition, Lin28 may have other RNA targets. To discover the potential targets, RIP is to be optimized and the resulting sample will be sent for high-throughput RNA sequencing. The identification of whole genome Lin28 targets will provide more insights into Lin28 functions in ESCs.

Chapter 3

PROBING THE FUNCTIONS OF JMJD6 IN MOUSE ESCs

3.1 **Jmjd6: structure and function**

Originally identified as phosphatidylserine receptor (PSR), JmjC domain-containing protein 6 (Jmjd6) is ubiquitously expressed throughout embryogenesis and in adult tissues, although at different levels (Cikala et al., 2004; Fadok et al., 2000). PSR was shown to be involved in recognition and engulfment of apoptotic cells (Fadok et al., 2000; Hong et al., 2004; Kunisaki et al., 2004; Li et al., 2003; Wang et al., 2003). However, a few other studies using $PSR^{-/-}$ cells drew opposite conclusions since $PSR^{-/-}$ cells are fully competent in clearing apoptotic cells (Bose et al., 2004; Cikala et al., 2004; Cui et al., 2004; Mitchell et al., 2002). Interestingly, PSR-null mice died at the perinatal stage (Bose et al., 2004; Kunisaki et al., 2004; Li et al., 2003) . Moreover, PSR disruption leads to many developmental defects such as lung defects (Li et al., 2003); severe anaemia (Kunisaki et al., 2004); growth retardation and developmental defects of some organs (Bose et al., 2004). These suggest that PSR is involved in multiple biological processes although more studies are required to interpret the underlying molecular mechanisms. Jmjd6 was revealed with its recruitment of a JmjC domain, a motif belongs to the superfamily of the 2-oxoglutarate and Fe (II) dependent oxygenase , and conserved from *S. cerevisiae* to human. Jmjd6 functions as a dioxygenase that demethylates histone H3 at arginine 2 and histone H4 at arginine3 in both biochemical and HeLa cell-based assays (Chang et al., 2007). A histone H3 arginine 2 methylation antagonizes histone H3 lysine 4 methylation and inhibits the binding of MLL1 and WDR5 to the H3K4me marks in different organisms (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008; Kunisaki et al., 2004).This highlights the importance of Jmjd6 in chromatin modification and gene regulation. More recently, Webby et al revealed that Jmjd6 serves as a dioxygenase to catalyse the lysyl-5-hydroxylation of a RNA splicing-associated factor U2AF65 in HEK 293 cells (Webby et al.,

2009). Furthermore, Jmjd6 interacts with many proteins which are connected with RNA metabolism, showing that Jmjd6 has multiple substrates besides histone (Webby et al., 2009). Taken together, these studies show that Jmjd6 participates in diverse cellular processes probably via histone modification and RNA metabolism, and thus may have played roles in ESCs.

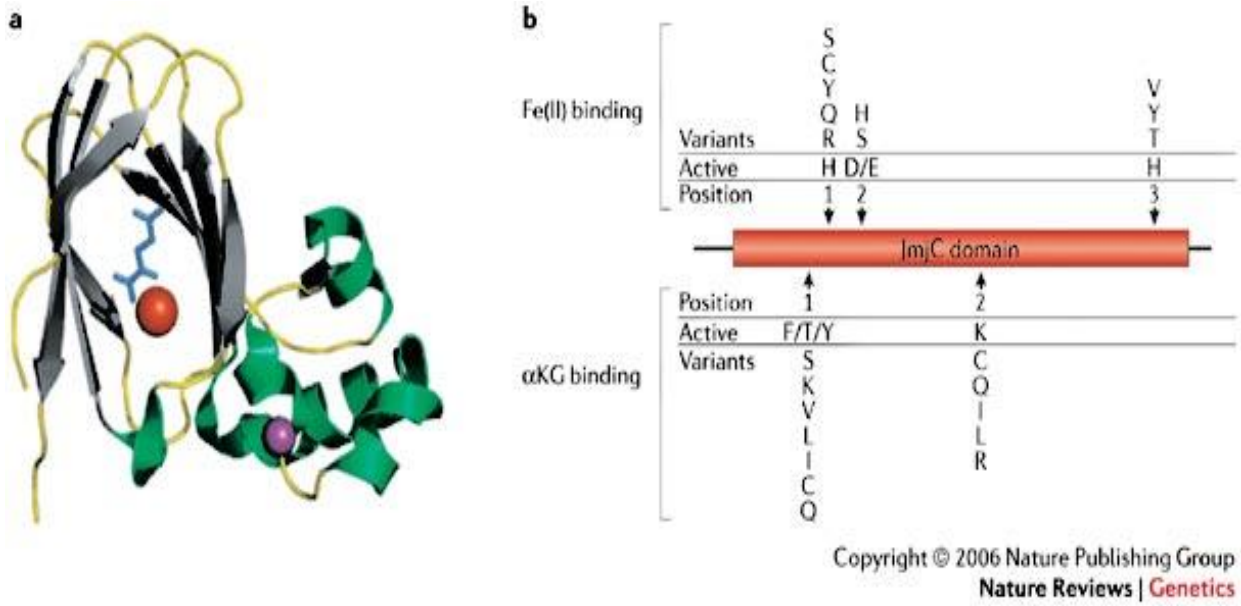


Figure 11. The JmjC domain structure (Adapted from Robert et al., 2006). (a) The 3D image structure of the JmjC domain of JHMD3A/JMJD2A. The grey sheets are the eight β-sheets of the cofactor-coordinating pocket, red ball represents the Fe (II) ion and αKG with blue. Green is the α-helical region combined with the zinc ion and purple is the zinc molecule. (b) A schematic illustration of the JmjC domain. It demonstrates the location of the Fe (II)-binding and αKG-binding residues.

3.2 **Material and methods**

3.2.1 **Construction of over-expression plasmids**

PCR primers for Jmjd6 over-expression were designed using the coding sequence of mouse Jmjd6 with addition of a BglII and MulI restriction sites at the 5' and 3' end, respectively. The sequences of the PCR primers are:

Forward: 5'- atataAGATCTatgaaccacaagagcaagaag-3'

Reverse: 5' -tatatACGCGTtcacctggaggagctgcgctct-3'

PCR amplification was performed using Expand High Fidelity PCR System (Roche), with wild-type E14 cDNA as template. The PCR product was cloned into pPyCAGIP (Chambers et al., 2003). The presence of inserts was selected by digesting with restriction endonucleases BglII and MulI (New England Biolabs) followed by 1% agarose gel electrophoresis. The sequencing PCR for selected plasmids was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences), using 10 pmol of primer and 300ng of DNA template and topped up to 10μl working volume with nuclease-free water (Ambion). The samples were then sent for sequencing to confirm the accuracy of the inserts. Cycling parameters were set according to manufacturer's instructions for large DNA templates.

Design of short hairpin RNA (shRNA) and generation of RNAi (RNA interference) plasmid

The *Jmjd6* shRNA was designed the same way as described for *Lin28*, The shRNA sequences for *jmjd6* were as follows (only upper is listed here)

shRNA for Jmjd6,

gatccccAATGAAACCCTTTACCTAttcaagagaTAGGTAAAGGGTTTCATTGT tttta

3.2.2 **Chromatin immunoprecipitation (ChIP) assay and ChIP-sequencing**

ES cells grown in 245 mm x 245 mm square culture dishes (Corning) were cross-linked with 1% formaldehyde for 10 min at room temperature on a shaker at150 rpm. The formaldehyde was quenched by shaking for another 5 min at room temperature after adding 0.2 M glycine. Cells were then washed twice with cold PBS, and harvested by scrapping and centrifuging at 3000rpm for 15 min at 4^{o}C. The cell pellet was further washed with cold PBS before being lysed in SDS cell lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100) containing protease inhibitor cocktail (Roche) by re-suspension and rotation at 4^{o}C for 15 min. The cell nuclei were isolated upon centrifugation at 2,000 rpm for 10 min at 4^{o}C, and the nuclei pellet was lysed in nuclear lysis buffer (50 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]-KOH, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS) with protease inhibitor cocktail by rotating for 18 min at 4^{o}C. The chromatin was then extracted by spinning down at 20,000 rpm for 30 min at 4^{o}C. The chromatin pellet was then washed twice with ChIP buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail, by rotating at 4^{o}C for 15 minutes each.

Sonication was performed to break down the cDNA into proper size (about 500 bp) on the Vibra-Cell VCX750 (Sonics). The glass beads were removed by centrifuging at 20,000 rpm for 45 min at 4^{o}C and collecting the supernatant. Size of the sonicated chromatin was determined by de-crosslinking input DNA (100 µl chromatin extract, 90 µl TE buffer [10 mM Tris-HCl, pH 8.0, 1mM EDTA], 30 µl pronase, 200 µl ChIP elution buffer [50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS]) at 42^{o}C for 2 h followed by 67^{o}C for 6 h, precipitated out via phenol-

chloroform extraction, and resolved through agarose gel electrophoresis. Average chromatin size was about 300 to 500 base pairs.

Dynabeads Protein G (Invitrogen) beads were washed with ChIP buffer twice (5 min rotation at room temperature, centrifugation at 2,000 rpm for 1 min, each) before being coated with rabbit anti-Jmjd6 antibody or rabbit anti-mouse IgG antibody (Abcam) as control by incubating for 2 hours at room temperature. The coated beads were then added to pre-cleared chromatin extract and rotated overnight at 4^{o}C. After incubation, the beads were washed thrice with ChIP buffer, once with ChIP buffer plus 0.35 M NaCl, once with ChIP wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate), and finally eluted in ChIP elution buffer (5mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) while agitating at 1,400 rpm at 69^{o}C for 45 min. The immunoprecipitated chromatin was then de-crosslinked and isolated as above.

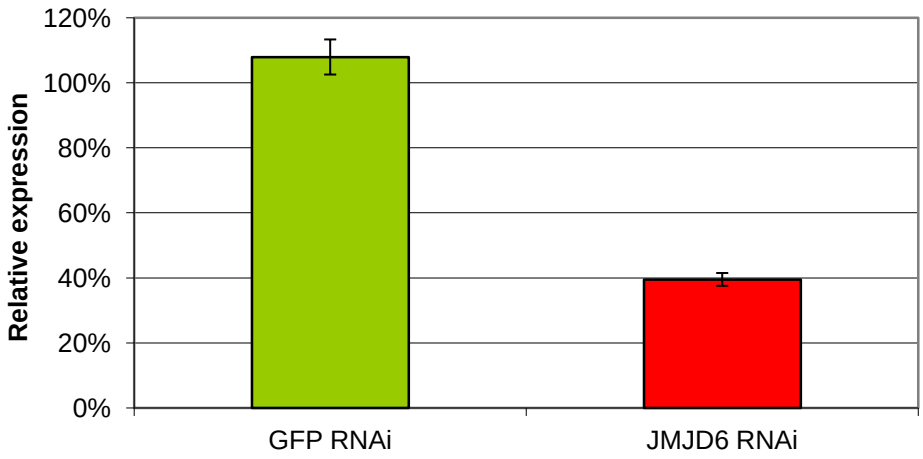
3.3 **Results**

3.3.1 **Knockdown of *Jmjd6* leads to ESC differentiation**

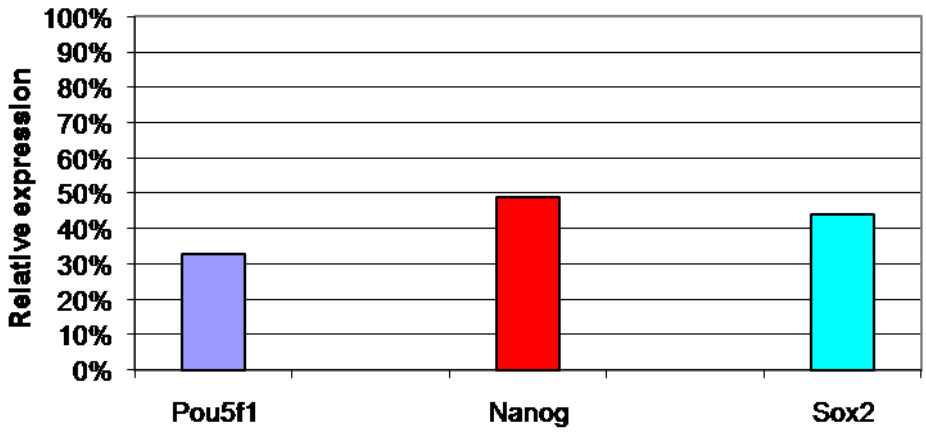
To test Jmjd6 function in ES cells, RNA interference (RNAi) was used to knockdown *Jmjd6* transcript levels using constructs expressing *Jmjd6* shRNAs. Down-regulation of Jmjd6 (Figure 12A) consistently led to significant reduction of the transcription factors Oct4, Sox2 and Nanog which are critical for maintaining ES cell pluripotency and suppressing their differentiation (Figure 12B). This suggests Jmjd6 expression is required to maintain expression of key transcription factors necessary for ES pluripotency. Indeed, ES cells in which Jmjd6 was down-regulated adopted a variety of morphologies characteristic of differentiating cells (Figure 12C).

Thus it was expected that reduced expression of these genes would result in increased expression of marker genes for specific differentiated lineages. Indeed, qRT- PCR of transcripts following *Jmjd6* RNAi revealed the anticipated expression of such germ layer markers. Expression of three endoderm markers, *Gata6*, *Sox17*, and *FoxA2* expression increased by 2, 3.8 and 4.9 fold respectively; the mesoderm marker *Nkx2.5* increased 2 fold while others showed no significant change in expression level. Expression levels of three ectoderm markers showed no significant change (Figure 12D). Together these results indicate that concomitant with the loss of expression of pluripotency transcription factors, ES cells initiate differentiation into endoderm following Jmjd6 down-regulation.

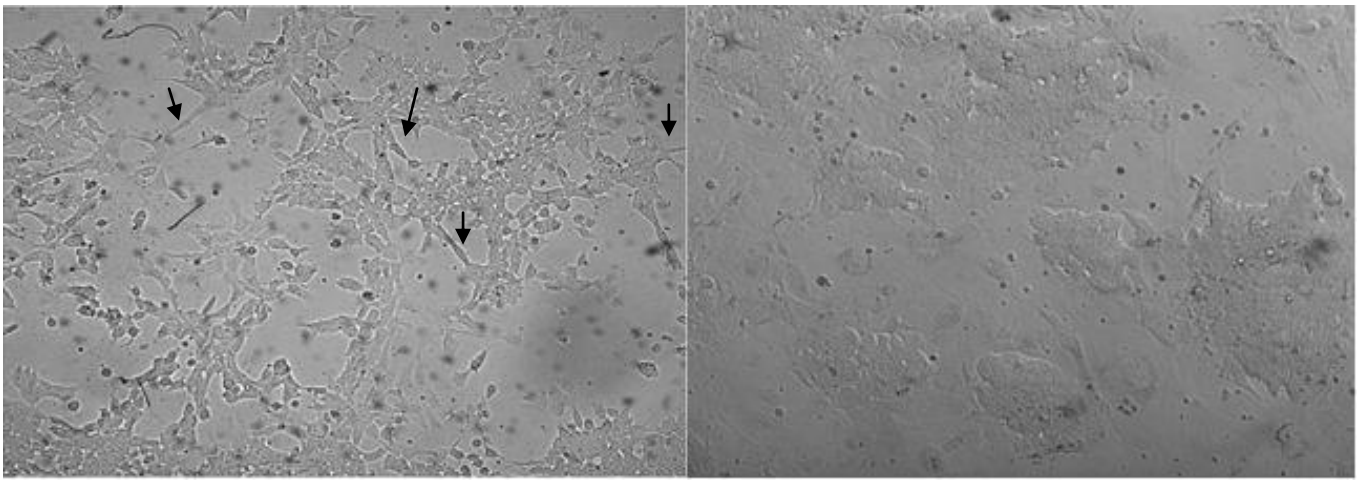
12A


120%
100%
80%
60%
40%
20%
0%
Relative expression
GFP RNAi
JMJD6 RNAi

12B


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative expression
Pou5f1
Nanog
Sox2

12**C**



12D

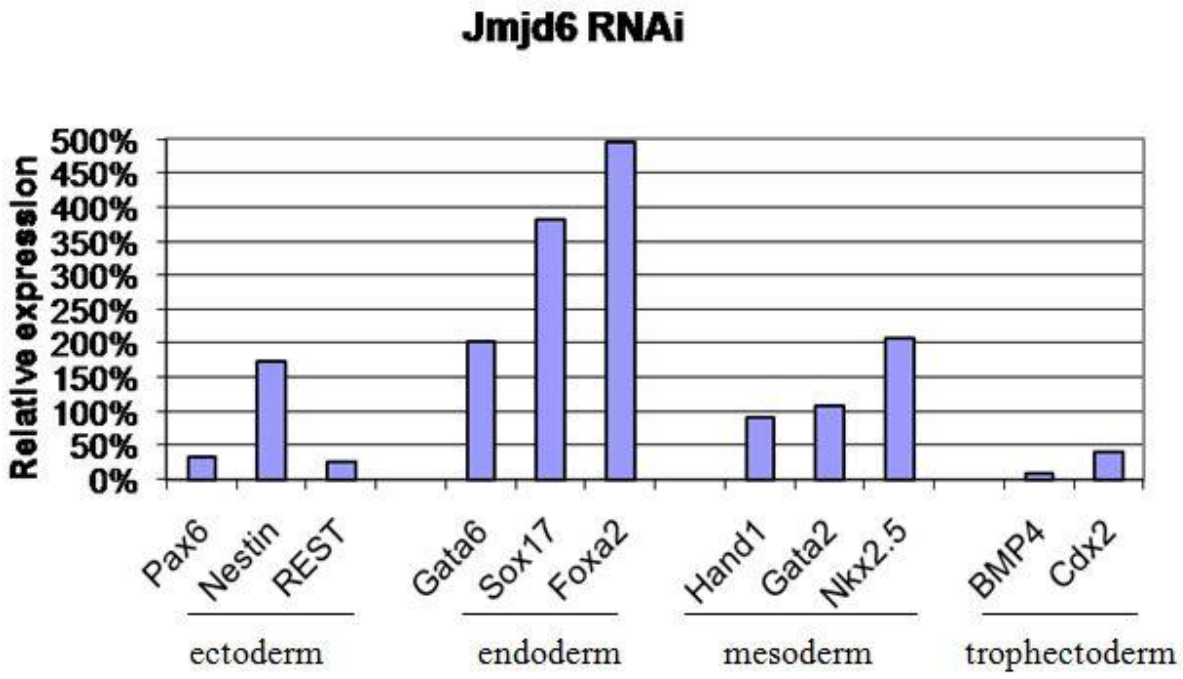
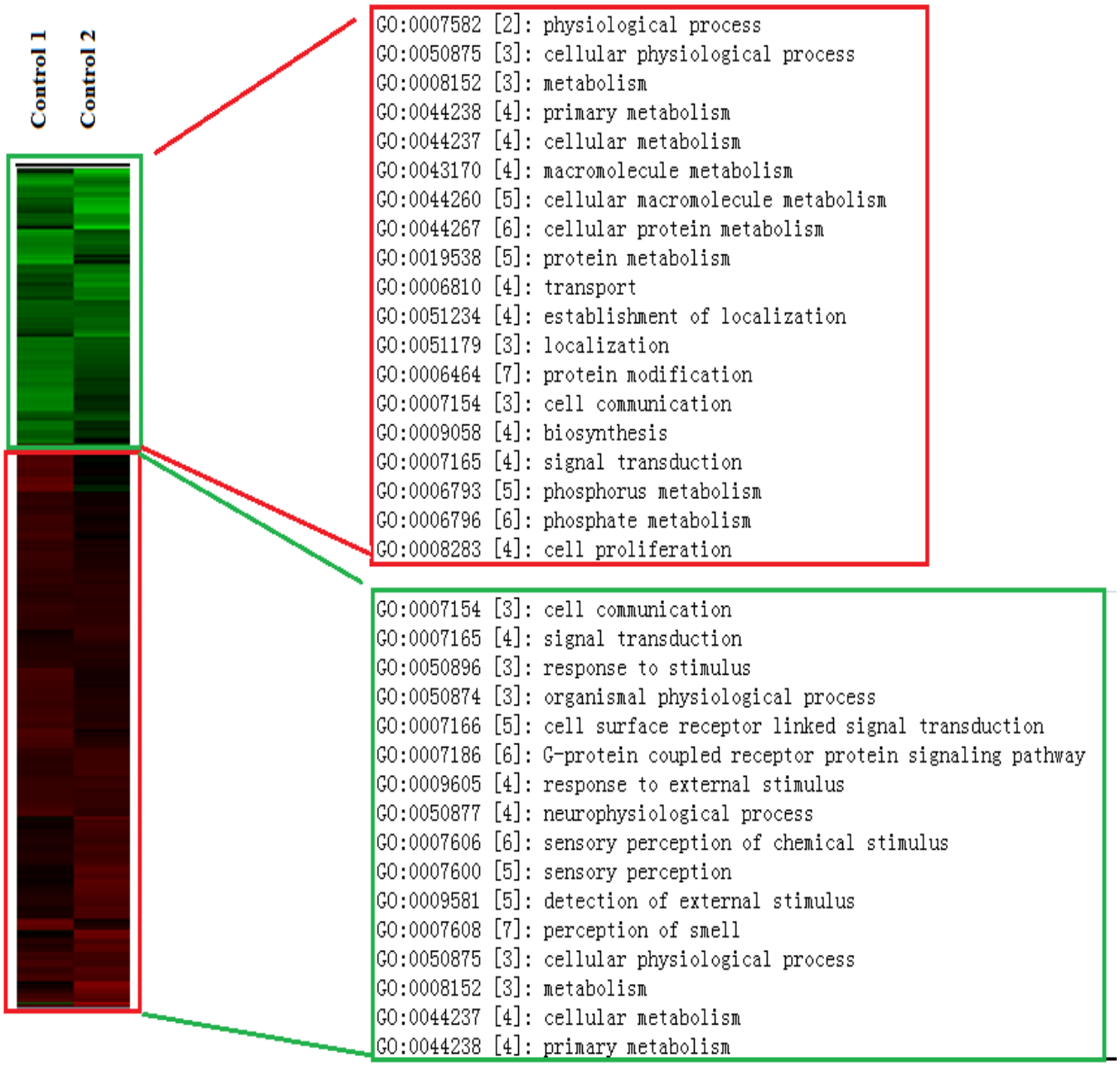


Figure 12. **Jmjd6 depletion directs ES cells to differentiation.** (A) Transfection of *Jmjd6* shRNA but not *GFP* shRNA (negative control) downregulates Jmjd6 level. RNA was extracted

3 days after transfection and quantified by real-time PCR. The indicated transcript levels are plotted as percentages relative to those following transfection of empty vector control. Sample was assayed in duplicate and normalized to endogenous *β–actin*. (B) Jmjd6 knockdown results in decreased *Nanog, Sox2* and *Oct4* mRNA levels (C) Cell differentiation following Jmjd6 depletion, compared by WT ES cells. Note stellate cells after *Jmjd6* RNAi. (D) Jmjd6 depletion directs ES cells to express indicated ectodermal, endodermal (endo), mesodermal (meso) and trophectodermal (Tro) marker genes assessed by real-time PCR.

3.3.2 **Global gene level changes indicate Jmjd6 is essential for ESC in maintenance of pluripotency .**

The whole genome cDNA microarray hybridization was performed using *Jmjd6* knockdown sample and the data was normalized to empty pSUPER.puro vector. The level of 1031 genes changed significantly, of which 370 were up-regulated (> 1.5-fold) and 661 were down-regulated (<0.7-fold). Validation was done by randomly selecting genes from the list of up-regulated and down-regulated genes, after which real time -PCR was carried out to examine the levels of the chosen genes in *Jmjd6* knock-down cells relative to control cells. Although the precise values of the fold change between the qRT-PCR results and the microarray results differed, in general, the trend of up-regulation and down-regulation of the tested genes was consistent between both sets of data (Figure 13A B). This implies that the direction of change of the genes can be inferred from the microarray data reliably. As such, both up-regulated and down-regulated genes were separately interrogated for Gene Ontology terms related to biological processes that were enriched within the two groups (Figure 13 A; Appendix Table 6,7).


Control 1
Control 2
GO:0007582 [2]: physiological process
GO:0050875 [3]: cellular physiological process
GO:0008152 [3]: metabolism
GO:0044238 [4]: primary metabolism
GO:0044237 [4]: cellular metabolism
GO:0043170 [4]: macromolecule metabolism
GO:0044260 [5]: cellular macromolecule metabolism
GO:0044267 [6]: cellular protein metabolism
GO:0019538 [5]: protein metabolism
GO:0006810 [4]: transport
GO:0051234 [4]: establishment of localization
GO:0051179 [3]: localization
GO:0006464 [7]: protein modification
GO:0007154 [3]: cell communication
GO:0009058 [4]: biosynthesis
GO:0007165 [4]: signal transduction
GO:0006793 [5]: phosphorus metabolism
GO:0006796 [6]: phosphate metabolism
GO:0008283 [4]: cell proliferation
GO:0007154 [3]: cell communication
GO:0007165 [4]: signal transduction
GO:0050896 [3]: response to stimulus
GO:0050874 [3]: organismal physiological process
GO:0007166 [5]: cell surface receptor linked signal transduction
GO:0007186 [6]: G-protein coupled receptor protein signaling pathway
GO:0009605 [4]: response to external stimulus
GO:0050877 [4]: neurophysiological process
GO:0007606 [6]: sensory perception of chemical stimulus
GO:0007600 [5]: sensory perception
GO:0009581 [5]: detection of external stimulus
GO:0007608 [7]: perception of smell
GO:0050875 [3]: cellular physiological process
GO:0008152 [3]: metabolism
GO:0044237 [4]: cellular metabolism
GO:0044238 [4]: primary metabolism

B

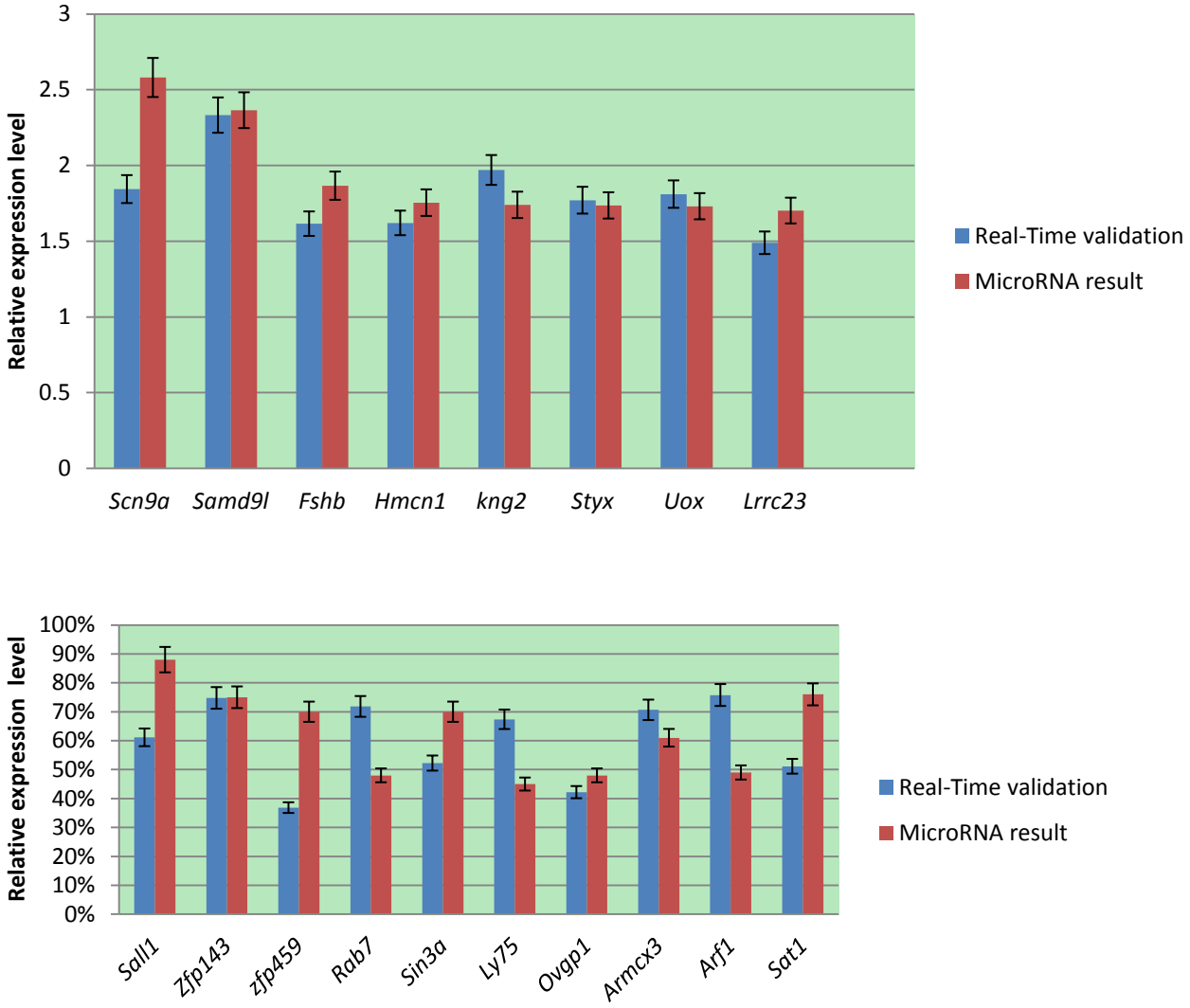


Figure 13. Global gene expression changes via microarray analysis on *Jmjd6*-knocked-down cells. (A) Up-regulated genes (green) and down-regulated genes (red) were subjected to Gene Ontology search for enriched biological process terms. Selected enriched terms are shown to reduce redundancy. (B) Validation of microarray data to ensure that microarray data were reliable.

The GO items for up-regulated genes implicated that Jmjd6 was involved in various metabolisms including primary metabolism, cellular metabolism, macromolecule metabolism,

cellular macromolecule metabolism, cellular protein metabolism, protein metabolism as well as the term metabolism itself.

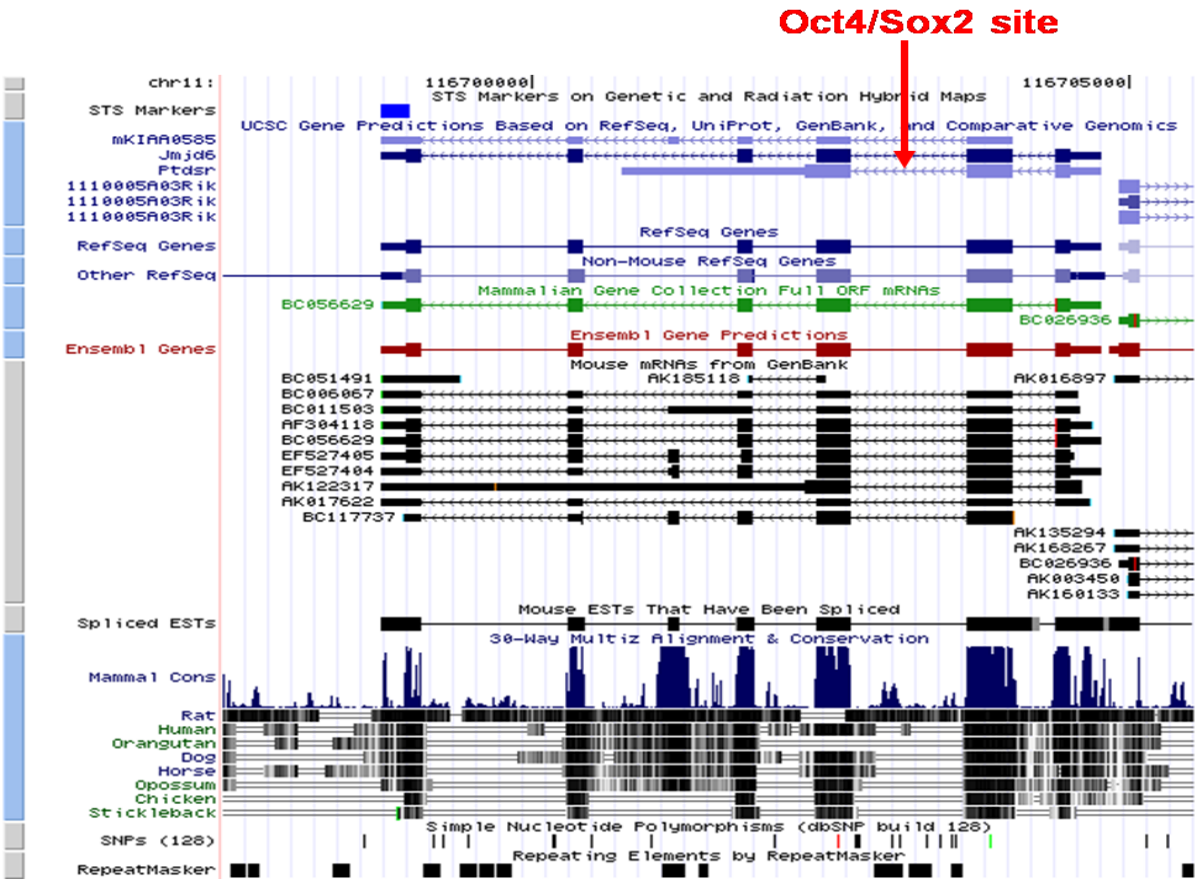
As respect to the down-regulated genes, there are some aspects worthy notifying. Some of them are related to metabolisms, supporting an active role of Jmjd6 in metabolism. Though never been reported, the massive down-regulated (62) olfaction genes may imply a novel role of Jmjd6 in regulation perception of smell. Moreover, Jmjd6 seems to play a role in every aspect of epigenetic regulations as a set of genes critical in histone modification, DNA methylation, chromatin-remodeling as well as miRNA metabolism were down-regulated. *Glp*, *G9a*, *Suv39h1*, *Suv39h2*, *Suz12*, *Setdb1*, which are essential for repressive H3K9 methylation, were found to be down-regulated in mRNA level (Dodge et al., 2004; Pasini et al., 2004; Peters et al., 2001; Tachibana et al., 2002; Tachibana et al., 2005). Similarly, the mRNA level of *Whsc1l1*, *Ezh2* that are responsible for H3K27 methylation decreased (Angrand et al., 2001; O'Carroll et al., 2001). Interestingly, the genes that regulate the active methylation of H3K4 were also found declined such as the nearly whole family of MLL (mixed-lineage leukemia) family (*Mll1*, *Mll3*, *Mll5*) and Whsc1l1 (Angrand et al., 2001; Goo et al., 2003; Heuser et al., 2009; Miller et al., 2001). On the other hand, the HDAC family that responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4), including *Hdac2*, *Hdac3*, *Hdac5*, *Hdac6*, were down-regulated as well. Furthermore, nearly all members of chromodomain-helicase-DNA-binding protein (Chd) family, which are closely related to ESC pluripotency by maintaining open chromatin or by facilitating histone deacetylation, were down-regulated (Gaspar-Maia et al., 2009; Tong et al., 1998). Besides, the genes critical for DNA (or RNA) methylation (like *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Setd6*, *Setd8*, *Rnmt*, *Tfb1m*) and genes involved in chromatin remodeling (*Lsh*, *Srg3*, *Atrx*) were down-regulated upon *Jmjd6* knockdown (Garrick et al., 2006; Geiman and Muegge, 2000; Kim et al., 2001; Levy et al., 2011; Okano et al., 1999; Pillutla et al., 1998; Xiao et al., 2005). The effect even includes the

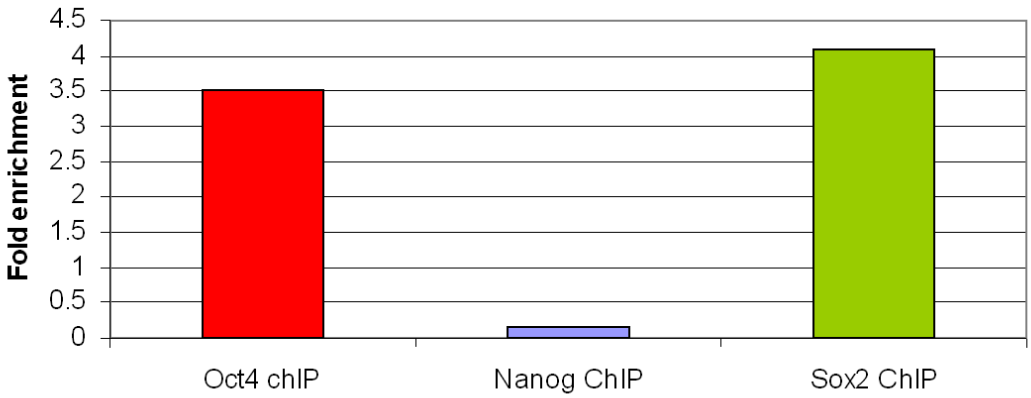
miRNA metabolism. Dicer, which is essential in processing a mature miRNA, was down-regulated (Hutvágner et al., 2001). These down-regulations in different epigenetic regulation mechanisms demonstrate a drastic change in ESC status, which is more likely to demonstrate the cell differentiation upon the knockdown of *Jmjd6*.

3.3.3 **Oct4 and sox2 bind to *Jmjd6* intron 2.**

The transcription factors Oct4, Sox2 and Nanog are master proteins that maintain ES cell identity and pluripotency. Since Jmjd6 appears to be a pluripotency factors, we asked whether Oct4, Sox2 and Nanog bind to the *Jmjd6* genomic site. ChIP experiments were performed and the ChIP DNA was assayed using quantitative PCR. As expected, we found that Oct4 and Sox2, but not Nanog, bind to intron 2 region of *Jmjd6*. Oct4 ChIP and Sox2 ChIP revealed 3.5 and 4.2 enrichment fold respectively, indicating that Oct4 and Sox2 interacts with this *Jmjd6* intronic enhancer (Figure 14A B).

14A





14B

Figure 14. Oct4/Sox2 binding site within *Jmjd6* gene and qRT-PCR of fold changes of pluripotency factors. Oct4 and Sox2 but not Nanog bind to *Jmjd6* genomic site. (14A) Oct4/Sox2 binding region is shown in genome browser. (14B) Oct4, Sox2 ChIP and Nanog ChIP DNA was assayed by real-time PCR using primers located in Jmjd6 intron 2. Fold

enrichments were calculated from the apparent IP efficiency (ratio of ChIP enriched DNA over input) and normalized to the level at a control region defined as 1.0 for a given extract from a specific cell line.

3.3.4 **ES cells over-expressing Jmjd6 express higher-level of Nanog**

To test whether *Jmjd6* over-expression helps sustain the pluripotent state, transformed ES cell lines were constructed stably expressing 26-fold elevated levels of Jmjd61 (Figure 15). The resulting ES cell line had typical ES cell morphologies (not shown), were AP positive (not shown). The expression level of *Oct4* in these lines was comparable to control ES cells whereas *Nanog* was elevated to 2.3 fold (Figure 15), suggesting that Jmjd6 overexpression may help ES cells to resist differentiation. Interestingly, the Prmt6 level was downregulated dramatically to 16% in the Jmjd6 overexpressing cells. Given that Prmt6 methylates histone H3 at arginine 2 but Jmjd6 demethylates at the same site, it would be of interest studying the relationship between the two enzymes.

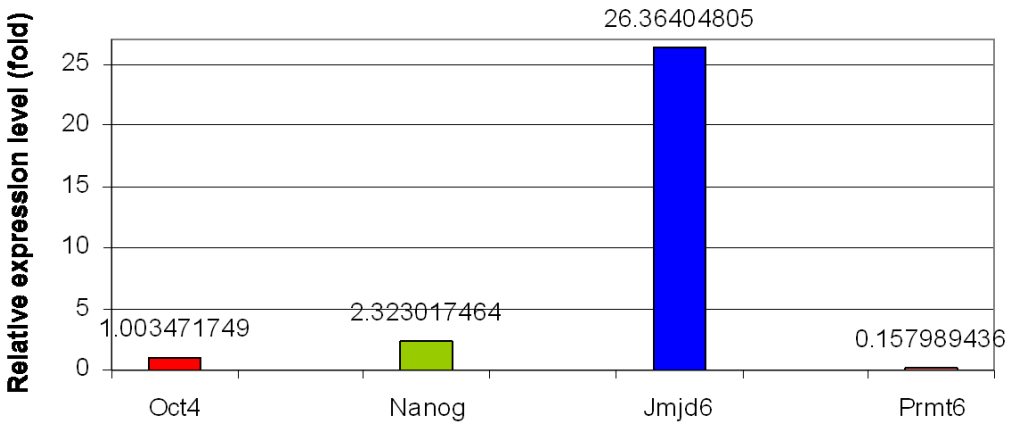


Figure 15. Jmjd6 overexpressing cells upregulate Nanog level. RNA level of Jmjd6 overexpressing cells were compared with that of wild type ES cells. Fold changes are shown

3.4 Discussion

3.4.1 The possible mechanism underlying Jmjd6 regulation of pluripotency

JmjC-containing protein is one of major demethylase enzyme classes that catalyse lysine demethylation of histones through an oxidative reaction that requires iron Fe (Ⅱ) and α-ketoglutarate (αKG) as cofactors (Tsukada et al., 2006). Compared to Lysine specific demethylase 1 (LSD1), which can only remove mono and dimethyl lysine modifications, the JmjC-domain-containing histone demethylases (JHDMs) can remove all three histone lysine-methylation states (Klose et al., 2006a). Result from JmjC domain's regulation in histone demethylase activity, Jumonji family proteins play a key role during embryogenesis and carcinogenesis through the regulation of chromatin structure and gene expression (Klose et al., 2006a; Takeuchi et al., 2006). The majority members of the JmjC-domain-containing family are related to histone methylation, like JHDM1 to reverse H3K36, JHDM2A to reverse H3K9, JHDM3 and JMJD2A-D to demethylase H3K9 and H3K36 and Jmjd3 to reverse H3K27 (possibly) (Boyer et al., 2006; Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006b; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Moreover, Jmjd1a and Jmjd2c have been shown to be bound by Oct4 and encoded for H3K9 demethylase that regulate the expression of pluropotency markers such as Nanog and Tcl1 in mouse ESCs (Loh et al., 2007). Another comparative genomics analyses revealed the binding sites for Oct4, AP-1, and bHLH transcription factors within the promoter region locate 5' to exon 1B of human JMJD1C gene and were conserved in chimpanzee, cow, mouse and rat JMJD1C orthologs, indicating that POU5F1-mediated expression of JMJD1C histone demethylase is implicated in the reactivation of silenced genes in undifferentiated ES cells, pancreatic islet, and diffuse-type gastric cancer (Katoh, 2007).

Here we demonstrate as a member of JmjC-domain-containing protein family, Jmjd6 may also contribute to the maintenance of pluripotency of ESCs. This conclusion can be supported mainly by three aspects in our results, namely the down-regulation of pluripotency markers and up-regulation linage markers, the epigenetic status change towards differentiate cells and the binding of Oct4 and Sox2 to Jmjd6's intronic region. The specific mechanism about how Jmjd6 acts in the network remains to be elucidated. Nevertheless, models for transcriptional activation and repression can be inferred from other JmjC-domain-containing proteins. It is interesting that Oct4 binds to the intronic enhancer of *Jmjd6* and the *Oct4* mRNA level decreased upon *Jmjd6* knockdown, which may implicate the possible regulation circuit between Jmjd6 and Oct4. Furthermore, as reported previously, methylation at H3 arginine 2 was found to abrogate trimethylation of H3K4 and methylation of histone H4 at arginine 3 (Kirmizis et al., 2007; Wang et al., 2001a). The loss of Jmjd6 leads to the reduction in H3R2 demethylation, which may in turn impede the active H3K4 methylation that finally decreases the pluripotency markers like Oct4 and Sox2. In addition, the decline of Oct4 and Sox2 expression in return lessens the Jmjd6 as the weakened binding strength, thus forming the feed-back loop, as illustrated in Figure 16. The massive decrease of methylases of H3K4 and the deacelases as well as the decline of Chd family may support conclusion.

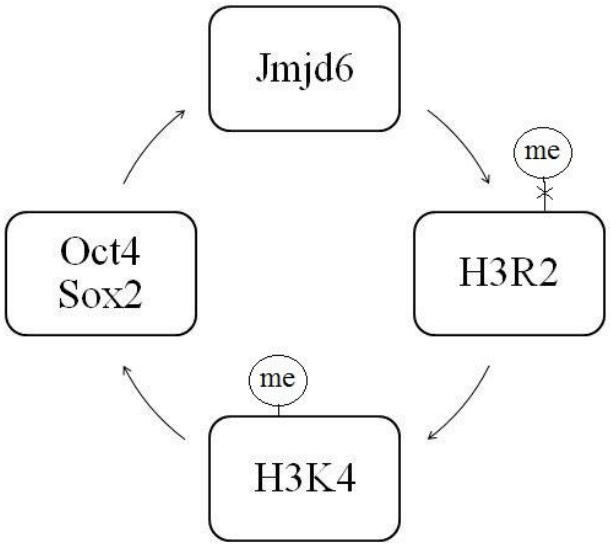


Figure 16. The proposed regulation loop among pluripotency markers, Jmjd6, H3R2 demethylation and the methylation of H3K4.

The reversible process of epigenetic regulation makes it an attractive tool for researchers to master and control the process to some extent. Hence, to understand the whole epigenetic landscape may shed light on the total developmental process. Here we propose two critical genes that epigenetically regulate ESC identity either in self-renewal or pluripotency, indicating the significant role of various epigenetic ways for maintenance of ESC identity. We provided a good research model by which one single gene can affect ESC in more than one aspect. Lin28, which function by either miRNA-dependent or independent ways, contributes to the ESC self-renewal. Jmjd6, a H3R2 demethylase, seems to maintain pluripotency by modifying histone and regulating transcription factor activity. These results also confirm the complex regulation network in ESC identity and may offer some new insights into epigenetic regulation in ESCs.

3.5 **Future work**

Here we demonstrate that Jmjd6 may regulate ESC pluripotency. To fully understand how Jmjd6 regulates ESC identity, ChIP-seq will be performed to identify Jmjd6 direct targets. We will combine ChIP-seq results with gene expression microarray data so as to unravel the mechanism of Jmjd6 regulation of ESC identity.

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APPENDICES

Supplementary Table 1. Sequences of primer pairs for *Lin28a, Lin28b, Jmjd6* and pluripotency genes, used for qPCR in *Lin28a ,Lin28b* and *Jmjd6* knock-down assay.

Gene	**Forward (5' - 3')**	**Reverse (5' - 3')**
β-actin	ACCAACTGGGACGACATGGAGAA	TACGACCAGAGGCATACAGGGAC
Lin28a	GGGGCCGGCATCTGTAAGTGGTTCA	CAGTGACACGGATGGATTCCAGACCCTTG
Lin28b	AAAAATCCCCCAAAGGCCTTGAGTCAATA	ATCATCCTGGACTCTTCTTCTCGCACAGT
Jmjd6	CGACTGGACCCGGCACAACTACTACGAGA	CGGACCAGCCCTCTTGTGCATTGAG
Pou5f1	TTGGGCTAGAGAAGGATGTGGTT	GGAAAAGGGACTGAGTAGAGTGTGG
Nanog	GGTTGAAGACTAGCAATGGTCTGA	TGCAATGGATGCTGGGATACTC
Sox2	CCAGGAGAACCCCAAGATGCACAACT	AAGCCTCCGGGAAGCGTGTACTTATCCTT

Supplementary Table 2. Sequences of primer pairs for germ layer markers and lineage markers, used for qPCR in *Lin28a , Lin28b and Jmjd6* knock-down assay and embryoid body assay.

Gene	**Forward (5' - 3')**	**Reverse (5' - 3')**
Pax6	GGGCGCAGACGGCATGTATGATAAA	AGTCGCATCTGAGCTTCATCCGAGTCTTC
Nestin	AGAGGAAGAGCAGCAAGGCCATGAC	TCCCTGACTCTGCTCCTTCTTCTTCAT
Rest	CCCTTCCGTTGTAAGCCATGCCAGTATGA	TGGTGCTTCAGGTGTGCCGTGTAGTGAT
Gata6	TGTGCAATGCATGCGGTCTCTACAGCA	TTCATAGCAAGTGGTCGAGGCACCC
Sox17	TGAAAGGCGAGGTGGTGGCGAGTAG	CAACGCCTTCCAAGACTTGCCTAGCATCT
Foxa2	CCTACGCCAACATGAACTCGATGA	GTAGAAAGGGAAGAGGTCCATGATCCACT
Hand1	CCTGCCCAAACGAAAAGGCTCAGGACCCA A	CGACCGCCATCCGTCTTTTTGAGTTCAGCC
Gata2	GGCCTCTTCTTCTGCAGGGGGTAGTGTAG	GCACATAGGAGGGATAGGTGGGTATCGG
Nkx2-5	GAAGGCAGTGGAGCTGGACAAAGCCGAGA	GGAACCAGATCTTGACCTGCGTGGACGTG
Bmp4	GTTCCTGGACACCTCATCACACGACTACT	GTAACGATCGGCTGATTCTGACATGCT
Cdx2	CGCAGAACTTTGTCAGTCCTCCGCAGTACC	GTATTCGGCGGGGCTGCTGTAGCCCATAG C

Supplementary 3 Sequences of primer pairs for cell cycle regulators, used for qPCR in Lin28 RIP.

Gene	**Forward (5' - 3')**	**Reverse (5' - 3')**
CyclinA2	GCTCAAGACTCGACGGGTTGC	GCTGCATTAAAAGCCAGGGCATC
CyclinB1	TCCCTCGGTGGGATTCAAGTGC	CAGGAGTGGCGCCTTGGTATGG
Cdk4	GTACGGCTGATGGATGTCTGTGCTACTTC	CAGGCCGCTTAGAAACTGACGCATTAG

Supplementary Table 4. Top 20 up-regulated genes upon *Lin28a* and *Lin28b* simultaneous knock-down, from microarray data.

Ref_Seq	Gene Symbol	Log2 Fold Change
NM_029755	*Calcoco2*	4.317535
NM_207271	*Tdpoz3*	4.281335
NM_009258	*Spink3*	3.874857
NM_008009	*Fgfbp1*	3.812489
NM_001163172	*Tmem92-ps*	3.719574
NM_009705	*Arg2*	3.573322
NM_001081306	*Ptprz1*	3.075732
NM_029755	*Calcoco2*	3.029686
NM_001081324	*Neto2*	2.970331
NM_010156	*Samd9l*	2.935079
NM_001142734	*Gm8994*	2.827043
NM_175271	*Lpar4*	2.792043
NM_177913	*A430089I19Rik*	2.771816
NM_177913	*A430089I19Rik*	2.771816
NM_177913	*A430089I19Rik*	2.771816
NM_001034101	*Gm13119*	2.76148
NM_177913	*A430089I19Rik*	2.75267
NM_001113736	*Gm13040*	2.742504
NM_177913	*A430089I19Rik*	2.723691
NM_177187	*D5Ertd577e*	2.674249

Supplementary Table 5. Top 20 down-regulated genes upon *Lin28a* and *Lin28b* simultaneous knock-down, from microarray data.

Ref_Seq	Gene Symbol	Log2 Fold Change
NM_145833	*Lin28a*	0.202096593
NM_019738	*Nupr1*	0.229213843
NM_011498	*Bhlhe40*	0.29008016
NM_028133	*Egln3*	0.300623711
NM_019877	*Copz2*	0.310522728
NM_026929	*Chac1*	0.314977186
NM_178404	*Zc3h6*	0.316930571
NM_013703	*Vldlr*	0.321999412
NM_011990	*Slc7a11*	0.322809283
NM_010243	*Fut9*	0.343306791
NM_009127	*Scd1*	0.355756133
NM_080470	*Smc1b*	0.363772573
NM_146017	*Gabrp*	0.365494522
NM_001081215	*Ddx60*	0.382403751
NM_147041	*Olfr57*	0.384129932
NM_018861	*Slc1a4*	0.386891248
NM_001081027	*Kcnt2*	0.389124158
NM_173866	*Gpt2*	0.393736736
NM_029197	*4930528F23Rik*	0.394764418
NM_177420	*Psat1*	0.421104729

Supplementary Table 6. Top 20 up-regulated genes upon *Jmjd6* simultaneous knock-down, from microarray data.

Ref_Seq	Gene Symbol	Log2 Fold Change
NM_001160386	*Dnahc7b*	2.543609
NM_146294	*Olfr1167*	2.439289
NM_001011790	*Olfr1382*	2.412542
NM_009245	*Serpina1c*	2.390137
XM_975226	*Naaladl2*	2.322925
NM_011190	*Psme2*	2.249713
NM_001011757	*Olfr663*	2.221278
NM_146566	*Olfr830*	2.165318
NM_181754	*Gpr141*	2.145285
ENSMUST00000103356	*Gm1418*	2.103497
NM_029755	*Calcoco2*	2.093128
ENSMUST00000098824	*Gm10683*	2.05128
NM_010156	*Samd9l*	2.04904
BC083121	*5430413K10Rik*	2.043052
NM_011002	*Olfr59*	2.041484
NM_153093	*AF366264*	2.037979
ENSMUST00000103653	*Gm16591*	2.024393
NM_029122	*Iqca*	2.019524
AY053573	*Rdh18-ps*	1.999047
NM_001105184	*Vmn2r71*	1.992955

Supplementary Table 7. Top 20 down-regulated genes upon *Jmjd6* simultaneous knock-down, from microarray data.

Ref_Seq	Gene Symbol	Log2 Fold Change
NM_033398	*Jmjd6*	0.300942
NR_033621	*Olfr856-ps1*	0.30312
NM_009127	*Scd1*	0.399691
NM_029870	*A930001N09Rik*	0.408027
NM_013825	*Ly75*	0.435951
NM_028430	*Ppil6*	0.436083
NM_027870	*Armcx3*	0.439023
NM_025799	*Fuca2*	0.443059
NM_007696	*Ovgp1*	0.443424
NM_029001	*Elovl7*	0.456033
NM_001162917	*Dennd4a*	0.469317
NM_177677	*Dnajc5g*	0.470971
NM_027870	*Armcx3*	0.480057
NM_001162917	*Dennd4a*	0.484761
NM_172597	*Txndc16*	0.485575
NM_001162917	*Dennd4a*	0.48783
NM_178404	*Zc3h6*	0.49216
NR_028528	*Snord57*	0.495453
NM_010439	*Hmgb1*	0.496941
NM_153526	*Insig1*	0.49743

Supplementary Table 8. Sequences of primer pairs used for qPCR in microarray validation.

Gene	**Forward (5' - 3')**	**Reverse (5' - 3')**
calcoco2	TGAAAAGTTCATCCCTCGACG	TCCCCGGACTAAACCATCTTC
Tdpoz3	TTTCATTGCCATTCATGCCTGT	GCTGGCTAGATTCTAAGACCACA
Fgfbp1	ACTCCACAGCCTCATCCT	CTGCTCCTCCTCAGTCACAG
Ptprz1	GCTTTGATGCGGACAGATTTTC	GGAGGGGATGTCAATGATCCA
Sp110	GTGAACATCGCCTATGCCATC	CCGGAGGTTGACCTTGCTG
Gm428	ACCCAACTGACTGCCTTA	TCTGCTTTCTCCTTCCTG
Cd80	ACCCCCAACATAACTGAGTCT	TTCCAACCAAGAGAAGCGAGG
Dnmt3l	GCTCTAAGACCCTTGAAACCTTG	GTCGGTTCACTTTGACTTCGTA
Pramel6	ATGAGTGTTGACTCCCTACCC	GTGAAGGCTACCTTGAACATCTC
Nupr1	CCCTTCCCAGCAACCTCTAAA	TCTTGGTCCGACCTTTCCGA
Bhlhe40	ACGGAGACCTGTCAGGGATG	GGCAGTTTGTAAGTTTCCTTGC
Egln3	AGGCAATGGTGGCTTGCTATC	GCGTCCCAATTCTTATTCAGGT
Smc1b	ACAAAGACGTGCATGGATTGC	CCCTCATGGTTATCAATTCCAGC
Ddx60	CTTGGGTCAGAGTTGCCCATA	GCATAAAGACGAAGGATGTCAGA
Prdm1	CTTCTCTTGGAAAAACGTGTGGG	TCATATCAGCGTCCTCCATGT
Zfy1	CAGATCAGAGCACTAGCATTCG	CTGGCAGTGACATTCTGGTCT
Sars	CGGGTGGATAAAGGAGGGGA	TGCCCGAAATCTGCATCGTC
Rras	GGGGCAAGAGGAATTTGGTG	GGTCCTTGACTCTGAGGATCT
Lefty1	CAAGACCCTTTCAGGACACC	CCATCCCTTCCACATCAGC
Xist	CCATACCCTCATACCCTA	TCTCTCAAACCACCACAC

Supplementary Table 9. Sequences of primer pairs used for qPCR in microarray validation.

Gene	**Forward (5' - 3')**	**Reverse (5' - 3')**
Sall1	CTCAACATTTCCAATCCGACCC	GGCATCCTTGCTCTTAGTGGG
Zfp143	GGCCATGCTACTCGGGTAAC	TGTGCCTTCTGTTGATCTCCT
Zfp459	GCAAAGACAAGGCTCTACTGG	GGAGTGATTCACAATTAGTGGGC
Rab7	AGGCTTGGTGCTACAGGAAAA	CTTGGCCCGGTCATTCTTGT
Sin3a	AGTGTCAACGTGGTCGAGAG	ATGCAGACGCTTCTTGCTTAC
Ly75	CCTACGGGAGACCTTGTGAAT	AGCAACTTCCAATCTGCTCATT
Ovgp1	TACTGCCTACAAACTGGTGTGC	TGCGTACAAAGAAAGGGGTCC
Armcx3	CTGGAGCCTGCTATTGCATTT	TCAGACCAGTCATTATACCTGGC
Arf1	TGGGCGAAATTGTGACCACC	TCCACTACGAAGATCAAGCCT
Sat1	GAGAACACCCCTTCTACCACT	GCCTCTGTAATCACTCATCACGA