MOLECULAR MECHANISMS OF EMBRYONIC STEM CELL PLURIPOTENCY:

TRANSCRIPTION, TELOMERE MAINTENANCE AND PROLIFERATION

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ABSTRACT

Somatic cell nuclear transfer and generation of induced pluripotent stem cells provide potential routes towards generation of patient specific embryonic stem (ES) cells. These procedures require induction of *Oct4* gene expression, high telomerase activity and specific cell proliferation, characteristics shared with cancer stem cells. The aim of this thesis is to gain further understanding of the molecular mechanisms that control these events.

In an attempt to identify factors involved in transcriptional regulation of the *Oct4*, the binding of SAF-A with the *Oct4* proximal promoter region in a LIF signalling dependent manner was established and subsequently demonstrated to be of functional importance for *Oct4* transcription. Further investigations revealed SAF-A in complex with proven affecters of *Oct4* transcription, Oct4 itself and Sox2, as well as with RNA polymerase II indicating that SAF-A could serve to bring together factors required for *Oct4* transcription and load them on the promoter. Moreover, SAF-A was found in a complex with the SWI/SNF-Brg1 chromatin remodelling protein in ES and differentiation induced cells. Functional assays revealed that dual depletion of SAF-A and Brg1 abolishes global transcription by RNA polymerase II indicating a fundamental role for the complex in RNA polymerase II mediated transcription.

The *Oct4* expression, as well as its transcriptional regulation were investigated in the biopsy samples from ovarian cancer patients. This investigation revealed reactivation of the *Oct4* expression independently of epigenetic regulation in biopsy samples from ovarian cancer patients. Further, these patients survived no more than 3.5 years from the diagnosis suggesting that Oct4 could be used as a prognostic factor of ovarian cancer mortality.

Telomere extension by telomerase is mediated by the shelterin complexes. The identification and biochemical characterization of the telomere shelterin complexes in *Xenopus* revealed conservation of their main functions in relation to human orthologs. Moreover, the temporal regulation of shelterin composition and subcomplex appearance was demonstrated during *Xenopus* embryonic development.

In screening for Tpt1 interacting factors in ES cells, Npm1 was found. The interaction occurred in a cell cycle dependent manner and subsequent functional assays proved its involvement in cell proliferation.

In conclusion, new insights regarding *Oct4* transcriptional regulation, telomere maintenance and ES cell proliferation are presented in this thesis.

Key words: embryonic stem cells, Oct4, SAF-A, Brg1, Tpt1, Npm1, transcriptional regulation, cell proliferation, shelterin

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Vizlin-Hodzic, D**., Johansson, H., Ryme, J., Simonsson, T., Simonsson, S. SAF-A has a role in transcriptional regulation of *Oct4* in ES cells through promoter binding.
Cellular reprogramming, *In press*. (2010)

II. **Vizlin-Hodzic, D.***, Ryme, J.*, Runnberg, R., Simonsson, S., Simonsson, T. SAF-A together with Brg1 is required for RNA polymerase II mediated transcription.
Submitted manuscript
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III. **Vizlin-Hodzic, D.**, Johansson, H., Jemt, E., Horvath, G., Simonsson, T., Simonsson, S. Oct4 as a Prognostic Biomarker of Ovarian Cancer.
Manuscript

IV. **Vizlin-Hodzic, D**., Ryme, J., Simonsson, S., Simonsson, T. Developmental studies of *Xenopus* shelterin complexes: the message to reset telomere length is already present in the egg.
FASEB J, 23; 2587-2594. (2009)

V. Johansson, H., **Vizlin-Hodzic, D.**, Simonsson, T., Simonsson, S. Translationally controlled tumor protein interacts with nucleophosmin during mitosis in ES cells.
Cell Cycle, 9; 2160-2169. (2010)

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ABBREVIATIONS

Brg1	Brahma related gene 1
BrdU	5-bromo-2-deoxyuridine
BMP4	bone morphogenetic protein 4
Cdk	cyclin dependent kinases
ChIP	chromatin immunoprecipitation
CTD	C-terminal domain
DE	distal enhancer
DNase I	deoxyribonuclease I
DNMT	DNA methyltransferase
EdU	5'-ethynyl-2'deoxyuridine
ES cells	embryonic stem cells
EU	5-ethynyl uridine
GCNF	germ cell nuclear factor
ICM	inner cell mass
Id	inhibitor of differentiation
IP	immunoprecipitation
iPS cells	induced pluripotent stem cells
JAK	Janus-associated tyrosine kinases
LIF	leukaemia inhibitory factor
MEF	mouse embryonic fibroblast
Ncl	nucleolin
Oct4	octamer binding transcription factor 4
PE	proximal enhancer
POT1	protection of the telomeres 1
PP	proximal promoter
RAP1	repressor activator protein
RARE	retinoic acid response element
RNA pol II	RNA polymerase II
SAF-A	scaffold attachment factor A
SCNT	somatic cell nuclear transfer
SF1	steroidogenic factor
STAT	signal transducer and activator of transcription
TERT	telomerase reverse transcriptase
TIN2	TRF1 interacting protein
TPP1	TIN2 and POT1 interacting protein
Tpt1	translationally controlled tumor protein
TRF	telomeric repeat-binding factor

INTRODUCTION

For the last three decades investigation of embryonic stem (ES) cells has resulted in better understanding of the molecular mechanisms involved in the differentiation process of ES cells to somatic cells. Under specific *in vitro* culture conditions, ES cells can proliferate indefinitely and are able to differentiate into almost all tissue specific cell lineages, if the appropriate extrinsic and intrinsic stimuli are provided. These properties make ES cells an attractive source for cell replacement therapy in the treatment of neurodegenerative diseases, blood disorders and diabetes. Prior to clinical significance, some problems still need to be overcome, like tumour formation and immunological rejection of the transplanted cells. To avoid the latter problem, the cloning of "sheep Dolly" in 1997 [1], more than 40 years after the first frogs were cloned [2], and recent generation of induced pluripotent stem (iPS) cells [3-6] have exposed the possibility to create patient specific ES-like cells whose differentiated progeny could be used in an autologous manner. During these reprogramming processes of somatic cells a unique transcriptional hierarchy and epigenetic state, high telomerase activity as well as a specific cell cycle of ES cells are induced. The aim of the current thesis is to gain further understanding of the molecular mechanisms that control these important events.

EMBRYONIC STEM CELLS

The generation of a new organism is initiated at the formation of a zygote by fertilization of an egg cell. The zygote undergoes cleavage and develops into a morula. The next important event in embryogenesis is characterized by the first specialization resulting in a formation of a hollow sphere of cells, termed a blastocyst. The outer layer of the blastocyst, the trophoblast, develops into extraembryonic tissues while the cells inside the sphere, termed the inner cell mass (ICM), are pluripotent, describing their capacity to specialize into all cell types and tissues.

In 1981, two groups demonstrated derivation of murine ES cells [7, 8] from the ICM (Figure 1). Almost 20 years later derivation of human ES cells using donated *in vitro* fertilized leftover embryos was reported [9].

The two main characteristics of ES cells are unlimited self-renewal and pluripotency, i.e capacity to differentiate into all cell types in the body. In mice, the most stringent test for pluripotency is injection of a labelled ES cell into a blastocyst resulting in the formation of a germ-line chimera [10]. Since this practice is not applicable in humans for ethical and practical reasons, the pluripotency of human as well as murine ES cells can be demonstrated either in response to specific stimuli *in vitro* or by teratoma formation following injection of ES cells in adult immunosuppressed mice [11]. In addition to self-renewal and pluripotency, ES cell characteristics include high nucleo-cytoplasmic ratio, prominent nucleoli, positive staining for alkaline phosphatase, rapid cell proliferation, high telomerase activity and expression of specific pluripotency markers.

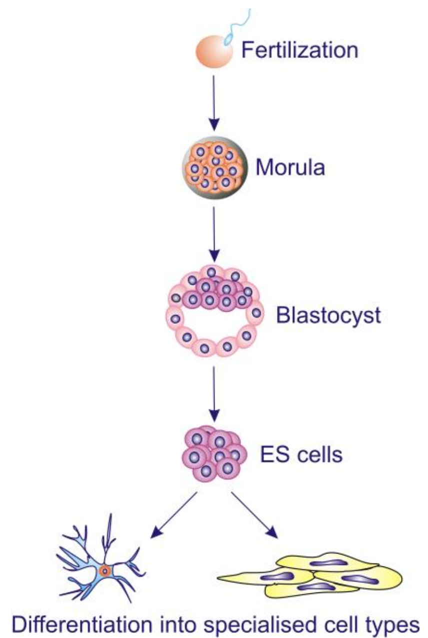

Figure 1. Derivation and developmental potentials of ES cells.

Applications of Embryonic Stem Cells

Given the possibility of forming a chimera, the derivation of murine ES cells has revolutionized the research of gene functions. Currently, the use of gene targeting to assess the gene functions in the living mouse is a routine procedure and can be performed with inducible systems allowing manipulation of gene expression at specific stages in specific cell populations [12]. In addition to developmental biology, ES cells provide a powerful tool in the areas of drug discovery and drug development [13] as well as for studying the underlying mechanisms of diseases [14, 15]. However, the expanding interest in ES cell research is in regard to their therapeutic potential for treatment of neurodegenerative diseases, blood disorders and diabetes.

REACQUISITION OF PLURIPOTENCY

An important first step to achieve the goal of ES cell based therapeutic approaches is the generation of patient specific ES cells. These autologous cells could be, after correction of genetic mutations, differentiated into required cell types or tissues and transplanted into the patient. However, specialized somatic cells are generally unable to reacquire the ES cell state due to their stable activation and repression of gene expression. These dramatic changes can be induced experimentally by nuclear reprogramming. Although, there are several potential techniques resulting in nuclear reprogramming, the focus here is on somatic cell nuclear transfer (SCNT) and induced pluripotent stem (iPS) cell generation (Figure 2).

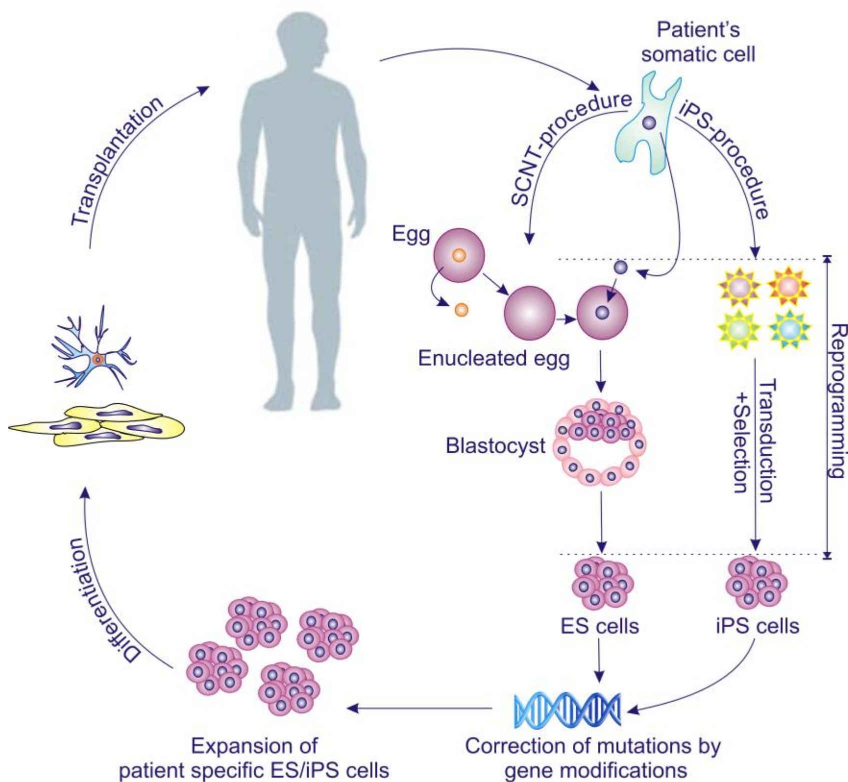

Figure 2. Schematic illustration of two possible procedures generating patient specific cells.

SOMATIC CELL NUCLEAR TRANSFER (SCNT)

In 1959, before ES cells were derived or any insights on regulators of pluripotency were available, it was successfully demonstrated that pluripotent state could be reacquired by somatic cell nuclear transfer (SCNT or cloning) in amphibians by John Gurdon [2]. During this procedure, the nucleus from a differentiated cell is transplanted into an enucleated egg cell and an embryo with identical DNA content to the donor is obtained (Figure 2). However, it took almost 40 years until SCNT was successfully used for generation of mammals [1]. The birth of the first normally developed mammal, the sheep Dolly, in addition to human ES cell generation exposed the possibility of therapeutic cloning which might be achieved by derivation of pluripotent cells from SCNT embryos, subsequent correction of genetic mutations, *in vitro* differentiation into homogeneous population of functional cells, and use for cell therapy (Figure 2). Since the birth of the sheep Dolly, SCNT was successfully performed in other species such as cow, mouse, goat, pig, cat, and rabbit [16]. Recently, it has also been shown that SCNT can produce human blastocyst stage embryos at an efficiency of 23% [17].

The therapeutic potential [18-20] and the equivalency between ES cells derived from natural and SCNT embryos [21, 22] have been demonstrated in mouse model. However, the success of SCNT is not complete. The vast majority of embryos reconstructed by nuclear transfer in animals either die before birth or produce unhealthy offspring. In addition to donor cell cycle stage and developmental stage of donor cells, the faulty epigenetic reprogramming has been proposed as the major cause of developmental failure and abnormal phenotypes in these animals [23]. Another limitation of SCNT is the requirement of donor oocytes resulting in ethical concerns. Moreover, human ES cells have not been derived by SCNT which is essential for proposed therapeutic treatments. Despite these limitations, SCNT is the most efficient nuclear reprogramming method to generate blastocyst embryos from which ES cells can be established.

INDUCED PLURIPOTENT STEM CELLS (IPS CELLS)

In 2006, it was demonstrated that pluripotency can be reacquired in mouse fibroblasts by retrovirus-mediated introduction of the four transcription factors Oct4, Sox2, Klf4 and c-Myc (Figure 2) [3]. These cells were termed induced pluripotent stem (iPS) cells. Subsequently, human iPS cells were successfully

generated by using the same set of factors, as used earlier in mouse model, as well as other factors [4-6, 24, 25].

The demonstrated reprogramming without the requirement for oocytes represents the major advantage of iPS cell generation in comparison to SCNT. In addition, the therapeutic potential of iPS cells in combination with genetic repair has already been successfully shown in mouse models of sickle cell anemia, Parkinson's disease, Duchenne muscular dystrophy (DMD) and hemophilia A [26-29]. However, there are some limitations regarding therapeutic applications of these cells such as use of oncogenes i.e. c-Myc, Oct4, Sox2 and Klf4 [30-32] as well as retroviruses in their initial generation. Subsequent investigations demonstrated that iPS cells can be generated without c-Myc with reduced reprogramming efficiency [5, 25, 33] and apart from Oct4 all other transcription factors have been successfully replaced by another member of the same protein family [34]. New strategies involving non-integrating vectors [35-39], excisable integrating vectors [40-43] and direct delivery of four recombinant reprogramming proteins [44] have successfully been employed for generation of transgene-free iPS cells. Other limitations of iPS cell generation such as low effectiveness and slow reprogramming process are limitations that remain.

REGULATORS OF PLURIPOTENCY

For the purpose of developing ES cell based therapeutic approaches as well as understanding SCNT and iPS cell generation, the thorough knowledge of molecular mechanisms that underlie the pluripotency and self-renewal of ES cells is required. Below, some of the present knowledge regarding these mechanisms will be discussed.

EXTRINSIC REGULATORS OF PLURIPOTENCY

Murine ES cells were established and maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) [7, 8]. Subsequent studies identified leukaemia inhibitory factor (LIF) as a MEF-secreted component having major impact on pluripotency maintenance of ES cells [45, 46]. In the absence of LIF signalling these cells differentiate into primitive endoderm and mesoderm [47].

LIF is a member of IL-6 cytokine family. It binds to its receptor (LIFR) which recruits gp130 to form a high affinity heterodimer complex. Formation of LIFR-gp130 heterodimers leads to the rapid activation of Janus-associated

tyrosine kinases (JAKs) followed by phosphorylation, dimerization and thereby activation of signal transducer and activation of transcription (STAT). Specifically, the ability of LIF to maintain ES cell state in the presence of serum is dependent upon activation of STAT3 [48, 49]. It has also been demonstrated that bone morphogenetic protein (BMP4) can replace the requirement for serum but not that of LIF in maintaining undifferentiated state of ES cells. BMP4 acts by inducing inhibitor of differentiation (Id) pathway to block neural differentiation [50]. In conclusion, LIF has a pivotal role in maintaining undifferentiated state of murine ES cells independently on culturing conditions.

The regulation of human ES cell lines differs from that of the mouse (Figure 3). It has been reported that LIF signalling is not sufficient to maintain self-renewal [9, 51, 52] while BMP4 induces differentiation of human ES cells to trophoblast [53]. The central importance for pluripotency and self-renewal of human ES cells is regarded bFGF and activin signalling [54, 55]. Differences in characteristic signalling pathways between murine and human ES cells might be dedicated to different developmental stages of embryos from which the cells are derived.

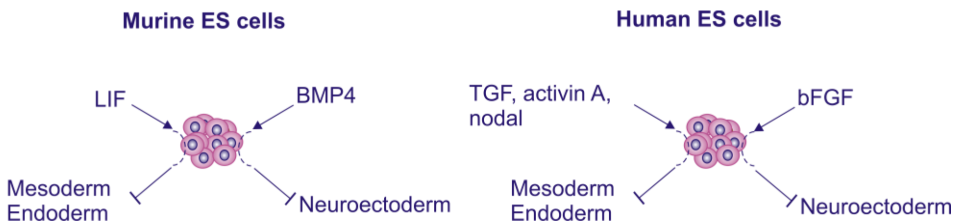

Figure 3. Extrinsic regulators of pluripotency in ES cells.

INTRINSIC REGULATORS OF PLURIPOTENCY

Besides these extrinsic factors, there are intrinsic factors, discussed in this thesis, that have pivotal role for specifying the undifferentiated state of both murine and human ES cells. One of these is a unique transcriptional hierarchy characterized partially by transcription factor Oct4 and specific epigenetic marks. ES cells also display high levels of telomerase activity and *TERT* expression, both of which are rapidly down-regulated during differentiation [56] and are much lower or absent in somatic cells. Further, the cell cycle of ES cells is very specific [57]. Therefore, high telomerase activity or the expression of

TERT and an ES specific cell cycle can be regarded as other markers of undifferentiated ES cells (Figure 4). Each of these will be discussed below.

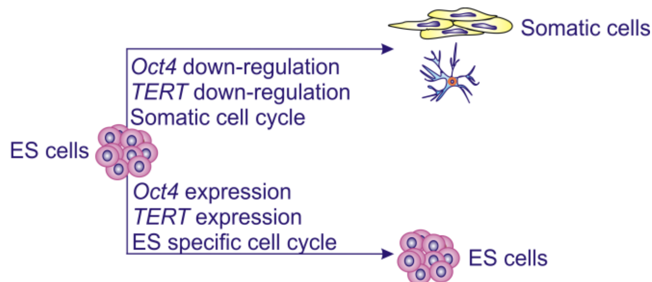

Figure 4. Intrinsic regulators of pluripotency in ES cells.

Transcriptional Regulation

The completion of the sequencing of the human genome provided a base to a new era in biology and medicine. However, the identification of the DNA sequence is of restricted importance since the character and developmental stage of a cell is defined by its constituent proteins, which are the result of specific patterns of gene expression. Thus, each developmental stage is characterized by its respective gene expression profile and a knowledge regarding regulation of the gene expression in ES cells is of central importance for gaining insight in pluripotency maintenance.

Gene expression is a multistep process involving epigenetic events, transcription, RNA processing, RNA export and translation. Epigenetic events are involved in modifications of DNA, i.e. DNA methylation, and chromatin remodelling which are altered during differentiation of ES cells.

In the mammalian genome, DNA methylation occurs on the cytosine residues in the context of CpG dinucleotides and is generally associated with stable transcriptional repression of particular genes. DNA methylation levels change during early mouse development. Shortly after fertilization, there is a subsequent wave of active paternal DNA demethylation. The maternal genome is also demethylated but in a replication dependent manner [58]. The patterns of DNA methylation are initially established during the blastocyst stage of embryonic development by DNMT3A and DNMT3B [58-60] in a process called *de novo* methylation. These epigenetic marks are reproduced during

successive rounds of mitosis by DNMT1 [61]. Human ES cells were demonstrated to possess a unique DNA methylation pattern in comparison to differentiated and cancer cells supporting a concept of DNA methylation contributing to the undifferentiated state of ES cells [62-64].

ES cells are also known to have a greater proportion of their genome as less condensed euchromatin with acethylated H3 and H4 histones as well as tri-methylated histone H3 at lysine which are generally associated with transcriptional activity [65, 66]. Further, ES cell chromatin is characterized by simultaneous presence of both activating and repressive histone modifications at lineage-specific genes suggesting presence of silent but primed state of activation [66, 67] which probably promotes ES cell plasticity. During differentiation, repressive histone modifications are erased from activated lineage-specific promoters whereas activating histone modifications are erased from promoters that remain silent. These covalent modifications of histone amino termini are affected by the activity of chromatin remodelling enzymes such as histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyl transferases (HMT) and histone demethylases.

The other category of chromatin remodelling enzymes utilizes the hydrolysis of ATP to disrupt contacts between histones and DNA resulting in alterations of nucleosome conformation, position and higher order chromatin structure [68]. This is achieved either by sliding the nucleosomes [69] or by inducing a DNA twist in the absence of histone movement [70]. The well characterized family of ATP dependent chromatin remodelling complexes is SWItch/Sucrose NonFermentable (SWI/SNF). SWI/SNF complexes have been implicated in regulation of pluripotency [71, 72] and posses a catalytic subunit that preferentially interacts with acetylated histones [73]. In mammals, the SWI/SNF complexes consist of approximately 10 subunits and ATPases enzymatic activity is achieved by either Brahma (Brm) or Brahma related gene 1 (Brg1). Despite the fact that Brm and Brg1share a high degree of amino acid sequence identity, only Brg1 has been proven important during early embryonic development. *Brg1* gene knock-out has been demonstrated lethal at the blastocyst stage of development [74] and maternally derived Brg1 has been reported required for zygotic genome activation [75].

These mechanisms represent the final effect in the transcriptional hierarchy mediated by binding of sequence specific transcription factors to accessible DNA regulatory sequences situated upstream of the transcription initiation sites i.e. promoter regions. The activity of transcription factors is regulated by

numerous signal transduction pathways and is one of the most important steps in the control of pluripotency. Three transcription factors i.e. Oct4, Sox2 and Nanog, have been reported central for the transcriptional regulatory hierarchy that specifies ES cell identity. Recently, the identification of the common target sites of these transcription factors by chromatin immunoprecipitation (ChIP) assay in combination with genome-wide localization analysis has suggested the existence of a regulatory network that maintains pluripotency [76, 77].

These experiments together with Oct4's necessity in iPS cell generation [34] highlight it as the most important transcription factor for maintenance and reacquisition of pluripotency. Below, some of the properties and present knowledge regarding transcriptional regulation of *Oct4* gene will be discussed.

OCT4

Oct4 (also referred to as Oct3, Pou5f1, Oct3/4, Oct-4, NF-A3) is one of the most important transcription factors during embryogenesis regulating either positively or negatively expression of a broad range of target genes [78]. It is a member of POU domain family of octamer binding proteins consisting of POU specific (POUs) and POU homeo (POUh) domains which are connected via a linker. These domains make specific contact with DNA through a helix-turn-helix structure and recognize a consensus octamer motif ATGCAAAT [79].

The *Oct4* expression profile follows a strict developmentally regulated pattern and is involved in the maintenance of an undifferentiated, pluripotent embryonic cell state during the first and second lineage determinations in the early mouse embryo [80]. In line with its embryonic expression pattern, *Oct4* is expressed in ES, embryonic carcinoma (EC) and embryonic germ (EG) cells [81, 82].

Oct4 has proven essential during early mouse development. Mouse embryos lacking *Oct4* die due to a defective ICM consisting of only trophoectoderm [81]. Further, the critical level of Oct4 is required to maintain pluripotency of ES cells [83]; a twofold increase in *Oct4* expression causes differentiation into primitive endoderm and mesoderm lineage also generated upon withdrawal of LIF [47], whereas a reduction of *Oct4* to less than 50% triggers differentiation into trophectoderm correlating with the phenotype of *Oct4* deficient embryos [81]. Ectopic *Oct4* expression has been observed in a variety of tumours such as ovarian, prostate and gastric tumours [30, 84-86]. Thus, the failure to maintain Oct4 levels within narrow limits can disrupt normal development and contribute to tumour development.

Oct4 regulation

The expression of *Oct4* is controlled by specific upstream regulatory sequences (Figure 5). *Oct4* gene expression is driven by a TATA-less minimal promoter (proximal promoter) which is located within the first 250 bp of the transcription initiation site. In addition to the proximal promoter (PP), two enhancer regions are important for expression of the *Oct4* gene. The proximal enhancer (PE) is required for *Oct4* expression in the epiblast, while the distal enhancer (DE) region drives expression in the morula, ICM and primordial germ cells [87]. Comparison between the upstream sequences of human, bovine and mouse *Oct4* promoters revealed four conserved regions. Within these regions, there is a number of important nucleotide sequences where factors involved in gene regulation can bind. In the proximal promoter, a putative Sp1/Sp3, steroidogenic factor (SF-1), Retinoic Acid Response Element (RARE) and 1A-like (or CTCF) binding sites have been proposed (Figure 5) [88, 89].

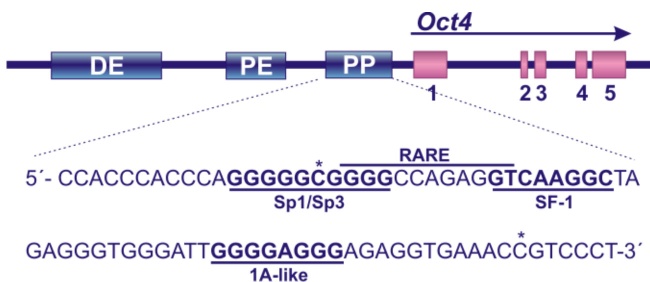

Figure 5. Schematic illustration of *Oct4* gene regulatory regions.

Previous studies have shown that the Sp1/Sp3 [90] transcription factor and several members of the nuclear receptor family, including GCNF [91], LRH-1 [92], SF-1 [93], RAR/RXR and COUP TF I/II [94], may be implicated in *Oct4* expression by binding to its proximal promoter region. Of these, GCNF is the best validated because *Oct4* expression in GCNF deficient embryos is not repressed efficiently in somatic cells indicating that GCNF is the repressor of *Oct4* [91].

In addition to DNA-binding transcription factors which regulate expression of *Oct4* genes, its transcription is also regulated by DNA methylation due to the CG-rich promoter region. CpG sites in *Oct4* promoter are unmethylated in ES cells and become methylated in somatic cells in which *Oct4* is not expressed. Thus, to reactivate *Oct4* properly in cloned embryos, somatic cell nuclei may need to undergo extensive demethylation of the *Oct4* promoter during nuclear

reprogramming [95]. When somatic nuclei were injected to oocytes from *Xenopus laevis*, *Oct4* transcription was reactivated [96, 97]. It was shown that oocytes have an activity that can demethylate repressed genes and that this may be an essential part of the nuclear reprogramming process [97]. Gadd45a was recently identified as participating in the DNA demethylation activity in *Xenopus laevis*, indicating that active demethylation occurs by a DNA repair mechanism [98].

ChIP assays revealed that histones binding to *Oct4* enhancer/promoter region are hyperacetylated, but hypomethylated, in ES cells. The primary chromatin remodelling determinants on *Oct4* and *Nanog* are acetylation of H3K9 and demethylation of dimethylated H3K9 during reprogramming by embryonic carcinoma (EC) cell extracts [99].

Post-translational modifications, such as sumoylation [100] and ubiquitination [101], are also known to modify the activity of Oct4.

TELOMERE MAINTENANCE

Telomeres are unique DNA-protein structures constituting the final 5-20 kb of all human and 10-80 kb of all mouse chromosomes ending in a 100-200 nucleotide 3'-single stranded overhangs [102, 103]. Telomeres play an essential role in the control of genomic stability by allowing cells to distinguish natural chromosome ends from damaged DNA and protecting chromosomes against degradation and fusion [104, 105].

In most human cells, telomeres shorten during successive rounds of mitosis due to the incomplete replication of linear DNA molecules and the absence of elongating mechanisms [106]. As an exception, cell types that proliferate indefinitely including ES [54] and cancer cells maintain their telomeres at a constant length. In such cells, the enzyme telomerase adds TTAGGG repeats to chromosome ends and thereby maintains the telomere length [107-109]. Telomerase is a ribonucleoprotein enzyme that contains two core components, telomerase reverse transcriptase (TERT) and telomerase RNA (TR). The RNA component serves as an integral template for *de novo* synthesis of telomeric DNA.

The elongation by telomerase depends on the conformation of the telomeric DNA [110, 111]. It has been shown that telomeric overhangs can fold back and anneal with the double stranded complementary sequence forming T-loop which can facilitate formation of a higher order structure. This process is modulated by shelterin complex which is also proposed to regulate telomere

protection [112]. Shelterin complex consists of six proteins: TRF1, TRF2, TIN2, RAP1, POT1 and TPP1 (Figure 6).

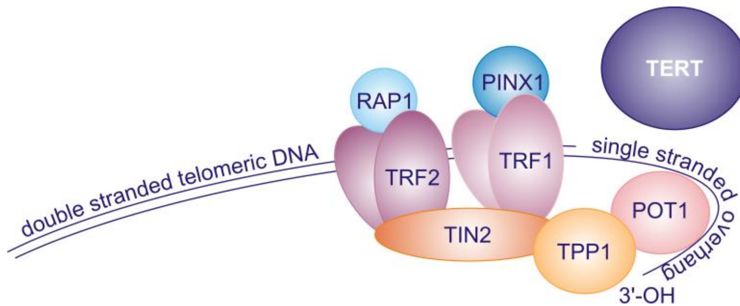

Figure 6. Schematic of shelterin complex on telomeric DNA.

TRF1 [113] and TRF2 [114, 115] are homodimeric proteins that bind double-stranded telomeric DNA via the C-terminal Myb domain [113]. The difference between these proteins is demonstrated in their N-terminus; TRF1 has an acidic N-terminus while TRF2 has an alkaline N-terminus [115]. TRF1 and TRF2 regulate formation of the T-loop and thereby indirectly the telomere extension by giving telomerase access to the telomeres. However, TRF1 and TRF2 do not directly associate with each other, but interact with other components of shelterin complex such as TIN2 [116] and RAP1 [117].

The TRF1 interacting nuclear protein (TIN2) [116, 118] is a linchpin in the telomeric complex. The N- terminus of TIN2 binds TRF2 while its C-terminus binds TRF1.

The telomeric single-stranded 3’ overhangs are directly bound by the protection of the telomeres 1 (POT1) protein via an oligosaccharide or oligonucleotide binding (OB) domain [119, 120]. POT1 interacts indirectly with TRF1 and TRF2 via TIN2 and TPP1, and thereby affects synthesis of telomeric DNA by telomerase [121-126]. TPP1 (TIN2 and POT1 interacting protein) bridges the interaction between POT1 and TIN2. It is also referred as PTOP (POT1 and TIN2 organizing protein), PIP1 (POT1 interacting protein) and TINT1 (TIN2 interacting protein). TPP1 contains a functional nuclear localising signal and localizes to both the cytoplasm and the nucleus, where it binds to POT1 and TIN2 and regulates assembly of the shelterin complex [127].

The repressor activator protein 1 (RAP1) contains BRCT, Myb, a coiled-coil and RCT domains. The Myb domain has no detectable DNA binding activity. The RCT domain is responsible for its association with TRF2 [117].

In addition to their established roles in cellular aging, stem cell biology and cancer [128-130], telomeres have recently been proposed functionally important in epigenetic gene regulation and vertebrate embryonic development [131, 132]. In animals generated by SCNT, telomere length in somatic cells has been found to be comparable with that in age-matched normally fertilized animals suggesting that the enucleated oocyte has the ability to reset the telomere length of the donor somatic cell by the elongation of telomeres [133-135].

Cell Cycle

All cells reproduce by duplicating their genetic content and segregating copies precisely into two genetically identical daughter cells during a cell cycle. However, the cell cycle of differentiated somatic and ES cells differ in structural and consequently temporal perspectives. Generally, the cell cycle of somatic cells is composed of S phase and M phase which are separated by gap phases, G1 and G2, allowing cell cycle progression to be regulated by various intracellular and extracellular signals. Unlike to somatic cells, ES cells divide very rapidly owing to a truncated G1 phase. Murine and human ES cells transit the cell cycle once every 8-12h and 15-30h, respectively [57, 136]. This ES cell capacity reflects unusually rapid proliferative rates of the cells that they originate from.

The cell cycle progression is controlled by the control system consisting of cyclin-dependent kinases (Cdks) [137] in complex with cyclins. The cyclins introduce conformational changes and partial activation of Cdks. Cdk-cyclin complexes are required for proper transition from one cell cycle phase to the next and therefore have to be activated at precise points of the cell cycle. The activation of Cdk-cyclin complexes is controlled at multiple levels, including complex assembly, regulation of cyclin levels, post-translational modifications of the Cdk subunit, Cdk complex localization and by modulation of Cdk inhibitor (CKI) levels.

In somatic cells, passage from G1 into S phase normally requires Cdk4 and 6 as well as cyclins D and E. ES cell division is driven by modest Cdk6-cyclin D and constitutively high Cdk2-cyclin E and Cdk2-cyclin A levels. The activity of only Cdk1-cyclin B is regulated during the cell cycle phases of ES cells [57] (Figure 7). Further, in somatic cells there are checkpoints which are missing in

the ES cell cycle. These permit an arrest in cell cycle progression if previous events have not been completed and should be seen as accessory systems that have been added to provide a more sophisticated form of regulation.

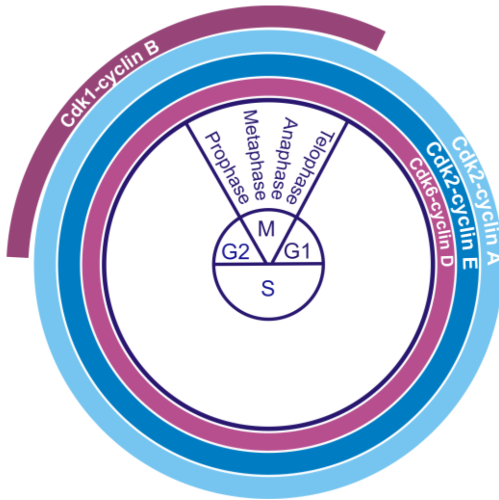

Figure 7. Schematics representing ES cell specific cell cycle control systems.

Cancer Stem Cells

It has been established that ES and cancer cells share several molecular properties including self-renewal and differentiation capacity. Evidence demonstrate that many pathways that are generally associated with cancer also are implicated in regulation of ES cells. Thus, the concept of cancer stem cells have evolved hypothesizing presence of small population of immortalized adult stem cells that have been dedifferentiated. These cells have been detected in leukaemia, brain tumours, breast cancer and pancreatic cancer [138-143]. The concept of the cancer stem cell is further strengthened by previously mentioned observation that *Oct4* is reactivated in a variety of tumours [84-86].

ASPECTS ON METHODOLOGY

IDENTIFICATION OF PROTEIN BINDING DNA SITES

EMSA (PAPER IV)

Electrophoretic mobility shift assay (EMSA) is a method to study the interaction of a protein with a DNA sequence. It works on the basis that the interaction of an *in vitro* translated protein (or protein present in the extract) with DNA retards the mobility of the DNA by non-denaturing polyacrylamide gel electrophoresis (PAGE). The major advantages of EMSA are high specificity and simplicity of the procedure.

In experiments described in paper IV EMSA was employed to characterize the binding of *in vitro* translated shelterin proteins to the telomeric DNA sequence.

DNASE I FOOTPRINTING (PAPER I)

DNase I footprinting technique has been a very useful method in gaining information about the location of a protein binding site in the DNA sequence. In footprinting experiments the interaction with a protein protects the DNA in the binding region from the cleavage of an endonuclease such as deoxyribonuclease I (DNase I). When this approach is used in combination with dideoxy sequencing analysis, the DNA sequence to which the protein is bound is resolved. This method is highly specific and is often used despite being technically more difficult than EMSA.

In experiments described in paper I, we aimed to identify differential protection of the non-methylated and methylated *Oct4* regulatory region due to the differentiation status of cells used for extract preparation.

IDENTIFICATION OF DNA ASSOCIATED PROTEINS

DNA AFFINITY CHROMATOGRAPHY (PAPER I)

DNA affinity chromatography is a technique used for the purification of DNA binding proteins. Various supports such as Sepharose, cellulose and silica can be used for coupling of non-specific or specific DNA by several coupling chemistries.

In experiments described in paper I, the footprint region was covalently coupled to the cyanogen bromide-activated Sepharose®4B with the major

advantage of low non-specific protein binding [144]. The DNA associated proteins from extracts prepared from ES and differentiation induced cells were enriched and by nano-LC-FT-ICR mass spectrometry analysis identified.

VERIFICATION OF DNA-PROTEIN INTERACTIONS

CHROMATIN IMMUNOPRECIPITATION (PAPER I)

The aforementioned techniques provide information about DNA-protein binding specificities *in vitro*. However, it is essential to gain information about binding of particular protein to specific gene regulatory regions in the context of a cellular system. Chromatin immunoprecipitation (ChIP) is a method that has been used in this purpose. The crosslinked protein-DNA complexes are sonicated into small fragments and immunoprecipitated. Following immunoprecipitation, crosslinking is reversed, proteins are removed and the DNA is recovered. The DNA is then screened by PCR to determine if specific gene regulatory region was bound by the protein of interest.

Sequential chromatin immunoprecipitation (Re-ChIP) is used to address whether two proteins can simultaneously co-occupy a stretch of DNA in cells. The protein–DNA complexes are formaldehyde-crosslinked and subjected to two sequential immunoprecipitations with antibodies of different specificity.

In paper I binding of the identified protein and its simultaneous co-occupancy with RNA polymerase II to different *Oct4* regulatory regions during two different conditions, i.e. pluripotency and induced differentiation, were investigated. Optimisation of a crosslinking and sonication steps were performed. Two control samples were included in each experimental setup; the input sample indicating presence and amount of chromatin used in the ChIP reaction and no antibody sample indicating the amount of background signal. The quantification of immunoprecipitated DNA by ChIP and Re-ChIP was performed by Real-Time and conventional PCR, respectively.

DETECTION OF cDNA AND PROTEIN LEVELS

QUANTITATIVE REVERSE TRANSCRIPTASE PCR (PAPER I, II, IV, V)

Reverse transcriptase polymerase chain reaction (RT-PCR) is used in paper I, II, IV and V to amplify, detect and quantitatively determine specific RNA-transcripts by reverse transcription of RNA to cDNA and subsequent Real-Time PCR using SYBR Green dye.

In described experiments two step RT-PCR and standard curve methods were used to estimate mRNA levels.

IMMUNOBLOTTING (PAPER I, II, III, V)

Immunoblotting, also known as Western blot, is a commonly used technique that allows detection of the presence, relative amount and molecular weight of a specific antigen separated by the size on SDS polyacrylamide gel followed by transfer to a synthetic membrane and subsequent detection by the antigen specific antibody. Optimisation of a transfer and blocking efficiency as well as antibody concentration are important for obtaining high quality immunoblotting.

IMMUNOFLUORESCENCE (PAPER I, II, III, V)

Immunofluorescence is commonly used antibody-based method to detect presence and localization of a specific antigen in cells. One of the main difficulties with immunofluorescence is overcoming non-specific background fluorescence. Optimisation of fixation times, pre-treatment with blocking agents, concentration of both primary and secondary antibodies are important for obtaining high quality immunostaining.

In experiments described in paper I, II, III and V immunofluorescence was followed by confocal microscopy, an important tool to visualize presence and localization of the specific antigen in the cell. In comparison to conventional wide-field optical microscopy it offers several advantages including the ability to choose focal plane, to eliminate or reduce out-of-focus background fluorescence as well as to collect serial sections from thick samples. In addition, in our experiments we sequentially scanned various fluorophores to reduce possible crossover and bleed-through which can be a significant problem with simultaneous multiple-wavelength excitations.

IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS

IN VITRO BINDING PROTEIN ASSAY (PAPER V)

In vitro binding protein assay is used to identify protein-protein interactions. It is based on coupling of an *in vitro* translated protein to the support and purification of its interacting partners by incubation with protein extract.

In paper V *in vitro* translated protein of interest was coupled to the cyanogen bromide-activated Sepharose®4B and its interacting partners were purified from extracts prepared from ES cells. Nano-LC-FT-ICR mass spectrometry analysis was used to identify the purified proteins.

CO-IMMUNOPRECIPITATION (PAPER I, II, V)

Co-immunoprecipitation (Co-IP) involves immunoprecipitation of intact protein-protein complexes by an antibody that targets a known protein that is believed to be a member of a larger protein complex. Generally co-IP is followed by immunoblotting to detect the proteins in the complex. The approach is usually used for epitope-tagged proteins. However, in papers I, II and V endogenous complexes between proteins are detected. Each experimental setup contained a IgG control for comparison.

IN SITU PROXIMITY LIGATION ASSAY (PAPER I, II, V)

In situ proximity ligation (*in situ* PLA) assay is a method to investigate protein-protein complexes and to visualize their spatial and temporal changes occurring naturally or as a cause of different cellular treatments [145]. The *in situ* PLA is based on recognition of proteins by pairs of antibodies raised in different species. A specie specific secondary antibodies with attached DNA strands participate in ligation, replication and sequence visualizing reactions. The major advantages of this method are the ability to visualize and simultaneously quantitatively determine spatial and temporal localization of the endogenous protein complexes within the cells and to detect even few protein-protein complexes. However, the specificity and sensitivity of the method is dependent on the accuracy of the primary antibodies.

In papers I and II the *in situ* PLA was employed to investigate differentiation mediated changes in protein complexes. In these experiments ES cells were induced to differentiate by either withdrawal of LIF or addition of retinoic acid from/to culturing media. To analyze the involvement of different kinases on

investigated protein-protein complexes in paper V, ES cells were treated with two different Plk1 inhibitors (BI2536 or wortmannin) and one Cdk-cyclin inhibitor (purvalanol A) prior to *in situ* PLA analysis. Control samples such as an internal control for individual comparison, i.e. one treated and one non-treated sample, as well as technical and biological controls were included in each experimental setup to eliminate experimental variances from different experiments.

PROTEIN FUNCTION

TRANSFECTION (PAPER I, II, V)

To study the effect of knock-down or over-expression of specific gene genetically modified cells have to be generated. The generally used method to introduce exogenous gene material into cells, such as siRNA, shRNA or transgene vectors, is transfection which is not effective in ES cells.

In papers I, II and V we accessed high efficiency in delivering siRNA, SureSilencing™ shRNA and pEPI-eGFP-vector into ES cells by liposome-based transfection with HiPerfect and Lipofectamine LTX (Invitrogen) four hours post seeding [146], respectively. Twenty four hours post transfection, selection for shRNA transfected cells was started by adding optimized concentration of puromycin. The effect of gene knock-down was detected by either Real-Time RT-PCR, immunoblotting or immunofluorescence. In over-expression experiments the autonomously replicating pEPI-eGFP vector was used. The effect of gene over-expression was detected by immunofluorescence.

CELL PROLIFERATION (PAPER V)

Cell proliferation assay was used in paper V to examine the number of cells still being able to synthesize new DNA following RNA interference. This was examined using a novel alternative to BrdU, a thymidine analog 5'-ethynyl-2'deoxyuridine (EdU, Click-iT™ EdU Imaging Kit, Invitrogen) that is incorporated efficiently into DNA for measuring DNA synthesis [147]. In comparison to BrdU, the advantage of EdU is that it does not require DNA denaturation since it uses small molecules for detection and consequently not affect the additional antibody staining.

GLOBAL TRANSCRIPTION (PAPER I, II)

Global transcription assay was used in paper I and II to examine the number of cells still being able to transcribe following RNA interference. This was examined using a novel alternative to both radioactive nucleoside labelling and BrU, an uridine analog 5-ethynyl uridine (EU, Click-iT®RNA Imaging Kit, Invitrogen) that incorporates efficiently into nascent RNA [148]. In comparison to radioactive nucleoside labelling and BrU incorporation, EU allows spatial determination of transcripts in high-resolution and faster whole-mount staining of large organs and tissue fragments.

DETECTION OF DNA METHYLATION

BISULFITE SEQUENCING (PAPER III)

Bisulfite sequencing involves the treatment of genomic DNA with sodium bisulfite which deaminates unmethylated cytosines converting them into uraciles. Methylated cytosines are not changed. The sodium bisulfite treatment is followed by PCR and sequencing. By comparison of the sequences from sodium bisulfite untreated and treated DNA, the unmethylated and methylated sites are revealed.

The advantage of this method is that it obtains single base-pair resolution for specific regions. However, it is dependent on highly efficient bisulfite conversion.

AIMS

Methodologies to reprogram somatic cells into patient specific pluripotent cells, which could potentially be used in drug discovery and cell replacement therapies, are currently advancing. Previous studies have revealed a unique transcriptional hierarchy and epigenetic state, high telomerase activity as well as specific cell cycle to be of pivotal importance for the pluripotency of ES cells and the success of reprogramming. The overall aim of this thesis has been to gain further understanding of the molecular mechanisms that control these important events in ES cells.

More specifically, the aims of the included papers were:

- To gain further understanding regarding the transcriptional activation of pluripotency marker *Oct4* in ES cells (Paper I)

- To find novel interacting partners to identified *Oct4* regulator (Paper II)

- To investigate the oncogenic properties of *Oct4* in patients suffering from ovarian cancer (Paper III)

- To identify and characterize the telomere shelterin complexes in *Xenopus laevis* and *tropicalis*, two commonly used model organisms for developmental biology studies (Paper IV)

- To gain further understanding regarding Tpt1 which is suggested important for the effectiveness of SCNT (Paper V)

RESULTS AND DISCUSSION

PAPER I - SAF-A HAS A ROLE IN TRANSCRIPTIONAL REGULATION OF *OCT4* IN ES CELLS

Oct4 expression has been proven essential for the formation of the ICM from which ES cells originate [81] and for successful iPS cell creation [34]. However, molecular details regarding *Oct4* transcriptional activation in ES cells are not completely understood.

ENDOGENOUS SAF-A BINDS *OCT4* PROXIMAL PROMOTER

In an attempt to identify factor/factors involved in transcriptional regulation of *Oct4*, we first screened for sequence specific binding differences at the evolutionary conserved *Oct4* proximal promoter region using *in vitro* DNase I footprinting. Protein extracts prepared from ES and by LIF withdrawal differentiation induced cells were used. The most prominent difference in protection, based on differentiation mediated by LIF withdrawal, was observed in the 1A-like region which has been shown important for active demethylation of the *Oct4* proximal promoter and reactivation of the *Oct4* gene during SCNT in *Xenopus* oocytes [97]. By using DNA affinity chromatography followed by SDS-PAGE, DNA binding of a 120 kDa factor from exclusively ES cell extract was revealed. Since these findings were in accordance with our *in vitro* DNase I footprinting results, the 120 kDa band was excised from the gel and identified by nano-LC-FT-ICR mass spectrometry analysis to be SAF-A, also referred to as hnRNP U. SAF-A is an abundant nuclear protein which contains an arginine/glycine-rich region (RGG box) located in the C-terminus and SAP motif in the N-termimus responsible for its binding to RNA and single/double stranded DNA, respectively [149, 150]. We found it a good candidate due to its proposed involvement in gene specific transcriptional regulation of *apolipoprotein D*, *Bmal1* and developmentally regulated *Shh* and *Klf2* genes [151-154]. Moreover, no viable SAF-A knock-out mice have been reported, however hypomorphic mutations in the noncoding region of SAF-A resulted in post-implantation lethality at E6.5 [155], suggesting its vital importance during early development and contribution to a variety of essential biological functions.

Since the assays employed, i.e. DNase I footprinting and DNA affinity chromatography, gave information regarding *in vitro* binding of proteins to regulatory region, we sought to investigate proposed presence of SAF-A at the *Oct4* promoter in ES cells. ChIP followed by Real-Time PCR was performed and demonstrated preferential presence of SAF-A at the *Oct4* proximal promoter in ES cells while induction of differentiation by LIF withdrawal resulted in a decline of SAF-A binding. The similar pattern of SAF-A binding was revealed also at the *Nanog* promoter. These results strengthen the notion of SAF-A's presence at the *Oct4* proximal promoter in ES cells and are in accordance with aforementioned reports suggesting association of SAF-A with elements in the promoter region of developmentally regulated *Shh* and *Klf2* genes [152, 153].

FUNCTIONAL IMPORTANCE OF SAF-A

The next issue addressed regarded the function of SAF-A in ES cells. To explore SAF-A's role on global transcription, RNA interference mediated decreases of SAF-A in combination with incorporation of EU and immunofluorescence were used. SAF-A depletion had temporal impact on global transcription. SAF-A depletion for 48 hours had no effect on the proportion of transcriptionally active ES cells while prolonged depletion for a total of 72 hours decreased the number of transcriptionally active cells by 57% in comparison to the control.

These results in addition to recent reports that SAF-A might interact with the CTD of RNA polymerase II (RNA pol II) [156, 157] intrigued us to examine a possible interaction between SAF-A and RNA pol II in ES cells. *In situ* PLA and co-immunoprecipitation experiments revealed that endogenous SAF-A could be found in complex with endogenous RNA pol II independently of CTD phosphorylation, mRNA and induced differentiation. These results suggest two distinct but not mutually exclusive roles for SAF-A: the complex between SAF-A and pCTD-RNA pol II reflects transcriptional elongation events and the complex between SAF-A and npCTD-RNA pol II reflects transcriptional initiation events.

Next, we investigated the simultaneous binding of SAF-A and RNA pol II at two specific *Oct4* regions, one corresponding to transcriptional initiation and the other to transcriptional elongation. Sequential ChIP revealed presence of SAF-A:RNA pol II complex at the *Oct4* proximal promoter but not at the *Oct4*

intron region during pluripotent conditions. These results suggest that SAF-A is involved specifically in the transcriptional initiation of *Oct4* gene.

Further, the specific effect of SAF-A on *Oct4* transcription was measured by Real-Time RT-PCR as well as immunofluorescence in control and SAF-A depleted ES cells. SAF-A depletion for 48 hours was accompanied with decreased Oct4 mRNA and protein levels. At this time point SAF-A depletion did not affect global transcription and therefore the effect on *Oct4* expression can be considered specific. To strengthen the impact of our findings, rescue experiments were performed demonstrating that the ectopic expression of human SAF-A could rescue the SAF-A knock-down phenotype.

SAF-A depletion for 96 hours resulted in decreased mRNA levels of housekeeping genes correlating with results from the global transcription assay. Although decreased global transcription at this time point was observed, *Brachyury* was up-regulated suggesting a loss of pluripotency and directed differentiation along the mesodermal pathway. In accordance, the morphological changes of ES cells were observed in SAF-A depleted cells. The observed differentiation along the mesodermal pathway is not in accordance with previous reports indicating correlation of *Oct4* down-regulation with differentiation of ES cells into trophectoderm [83]. Notably, the absence of LIF signalling induces differentiation of murine ES cells into the mesodermal pathway [47] which we observe when SAF-A is depleted in presence of LIF signalling. Thus, our RNA interference experiments suggest connection between LIF signalling and SAF-A's role in transcriptional regulation of *Oct4* which is in accordance with our initial experiments.

SAF-A IS IN COMPLEX WITH OCT4 AND SOX2

Given that RNA pol II association factor, Paf1, affects *Oct4* expression by the interaction with Oct4 protein itself [158, 159] we explored if SAF-A also exists in close proximity to endogenous Oct4. *In situ* PLA revealed endogenous SAF-A:Oct4 complexes in ES cells. These results are in accordance with report suggesting SAF-A in complex with Brn-4, which belongs to the same protein family as Oct4 [160].

Since Oct4 and Sox2 have been previously reported to interact with the *Oct4* enhancer region and thereby regulate *Oct4* expression [161, 162], we investigated whether SAF-A can also be found in complex with Sox2. *In situ* PLA revealed endogenous SAF-A:Sox2 complexes in ES cells. These findings

might suggest that SAF-A serves to bring together factors required for *Oct4* expression in ES cells and load them on the promoter together with RNA pol II. The suggestion is strengthened by the fact that the number of SAF-A:Oct4 complexes decreased approximately by 70 % upon induction of differentiation by LIF withdrawal for two days, although Oct4 levels were not significantly reduced during this time period. The number of SAF-A:Sox2 complexes decreased to the same extent as well, suggesting that any of these lost interactions could be considered as a candidate for controlling SAF-A dissociation from the *Oct4* proximal promoter.

The next question addressed the dissociation of SAF-A from the *Oct4* proximal promoter as well as the dissociation of SAF-A:Oct4 and SAF-A:Sox2 complexes observed upon early differentiation mediated by LIF withdrawal. Given that Oct4 protein levels were not significantly affected at this stage of differentiation, we reasoned that the observed dissociations could be explained by a decrease in SAF-A protein levels. In contrary to our expectations, induced differentiation did not decrease either SAF-A mRNA or protein levels. Thus, dissociation of SAF-A from the *Oct4* proximal promoter as well as dissociation of SAF-A:Oct4 and SAF-A:Sox2 complexes and consequently *Oct4* down-regulation cannot be ascribed simply to a decrease in SAF-A levels.

SAF-A IS IN COMPLEX WITH STAT3

Given that pluripotency of ES cells is dependent on LIF signalling which results in STAT3 activation [48] and that SAF-A is not quantitatively affected by differentiation induced by LIF withdrawal, we next explored the possible interaction between SAF-A and STAT3 in ES cells. *In situ* PLA revealed presence of endogenous SAF-A:STAT3 complexes. Further, the quantity of complexes decreased by differentiation induced by LIF withdrawal. These observations might provide a link in a chain of interactions ranging from extrinsic stimuli by LIF to an intrinsic response which possibly results in *Oct4* transcriptional initiation. However, these observations do not explain either dissociation of SAF-A from the *Oct4* proximal promoter or dissociation of SAF-A:Oct4 and SAF-A:Sox2 complexes mediated by LIF withdrawal since we and others [163] have not detected STAT3 associated with the *Oct4* promoter.

Our discoveries allow us to propose a model for *Oct4* transcriptional initiation. This explains how signalling by extrinsic LIF proceeds via STAT3, which translocates into the nucleus [48, 49] and could interact with SAF-A. SAF-A binds the *Oct4* promoter through to some unknown factors. The

transcription factors Oct4 and Sox2 associate with SAF-A. Since Oct4 and Sox2 have been found to interact preferentially with the enhancer region of the *Oct4* promoter [161, 162] it is likely that the complex with SAF-A, Oct4 and Sox2 links enhancer with the proximal promoter region. SAF-A next recruits npCTD-RNA pol II to the transcription start site to initiate *Oct4* transcription. Upon early differentiation mediated by LIF withdrawal for two days, SAF-A association with the *Oct4* promoter is reduced followed by blocked transcriptional initiation of *Oct4*.

FUTURE INVESTIGATION

The remaining question addresses the molecular event responsible for SAF-A dissociation. The finding that SAF-A levels do not decrease after LIF withdrawal but rather increase suggests that the promoter release is not controlled directly by the level of SAF-A. It is possible that in the ES cells a modification of SAF-A rather than its mere presence/absence, may be a critical parameter for the dissociation of SAF-A from the *Oct4* promoter. The approach that could be used to reveal this important issue is immunoprecipitation of endogenous SAF-A from ES and differentiation induced cells followed by nano-LC-FT-ICR mass spectrometry analysis, respectively. However, one must consider protein digestion by other enzymes than trypsin since SAF-A digestion by trypsin generates either very short or long peptides which are not optimal for successful nano-LC-FT-ICR mass spectrometry analysis.

Paper II - SAF-A together with Brg1 is Required for RNA Polymerase II Mediated Transcription

In addition to the gene specific regulation by SAF-A, it has also been demonstrated to interact with affecters of epigenetic marks, histone acetyl transferases [164, 165] to relax chromatin structure. These findings indicate that SAF-A participates in various aspects of transcriptional regulation by interacting with a wide range of nucleic acids and proteins.

Given the complexity of the transcriptional regulation by SAF-A, we hypothesized that other epigenetic modifiers of nucleosomal structure can be found in complex with SAF-A. Therefore we addressed whether SAF-A could be detected in association with Brg1, a catalytic subunit of SWI/SNF ATP dependent chromatin remodelling complexes, which is reported to preferentially interact with acetylated histones [73].

SAF-A is Associated with Brg1 in General Manner

The spatial distribution of endogenous SAF-A and Brg1 was analysed first. We found SAF-A and Brg1 localized to the nucleus of ES cells and co-localization could be observed by using ImageJ software. Next, co-immunoprecipitation was performed demonstrating endogenous Brg1 in the complex with endogenous SAF-A. Consistent with co-localization and co-immunoprecipitation experiments, the *in situ* PLA visualized SAF-A:Brg1 complexes in the nucleus of ES cells. These findings indicate that endogenous SAF-A and Brg1 are a part of the same complex in ES cells. When ES cell differentiation was induced, by either LIF withdrawal or addition of retinoic acid, the endogenous SAF-A:Brg1 complexes remained intact indicating general nature of the complex.

SAF-A:Brg1 Complexes are Involved in RNA Polymerase II Mediated Transcription

To investigate function of SAF-A:Brg1 complex in ES cells RNA interference experiments were performed. The effect of decreased SAF-A and Brg1 protein levels on each other's transcription was first assayed. Depletion of one of the proteins for 48 hours did not have any effect on expression of the other. However, prolonged depletion to a total of 96 hours resulted in decreased levels of total mRNA as well as mRNA levels of housekeeping genes.

These findings prompted us to investigate the involvement of SAF-A and Brg1 alone and as a complex on global transcription in ES cells. The effect of decreased protein levels on global transcription was detected by EU incorporation followed by immunofluorescence. SAF-A depletion for 48 hours had no significant effect on the proportion of transcriptionally active ES cells while prolonged depletion for a total of 72 hours decreased it by 57% in comparison to the control. The depletion of Brg1 affected global transcription also in a temporal manner. Brg1 depletion for 48 hours decreased the proportion of transcriptionally active ES cells by 26% while prolonged depletion for a total of 72 hours decreased it by 54% in comparison to the shRNA control. Interestingly, the dual depletion of SAF-A and Brg1 for 48 and 72 hours decreased the proportion of transcriptionally active ES cells by 42% and 92% in comparison to the control, respectively. During indicated time period incorporation of EU in nucleoli could be observed indicating that RNA pol I transcription machinery remained unaffected. Thus, prolonged silencing of the proteins to a total of 72 h highlights the pivotal role of the observed complex in RNA pol II mediated transcription.

SAF-A:BRG1 COMPLEX IS PARTIALLY ASSOCIATED WITH RNA

Both SWI/SNF complexes and SAF-A have been proposed to be involved in RNA processing [166-168]. To determine whether the association of SAF-A with Brg1 is mediated by RNA, the total extracts prepared from ES cells were treated with RNase A followed by co-immunoprecipitation. The association of SAF-A with Brg1 was decreased by the RNase A treatment although a part of the association was resistant to the treatment. These results suggest that there are two types of SAF-A:Brg1 association; one independent and the other dependent on the presence of RNA.

Given that both SAF-A and Brg1 have been involved in modification of chromatin structure [68, 164, 165], are reported to interact with RNA pol II [156, 157, 169] and remain all along the transcribing gene as a component of growing RNP particles [168], the observed SAF-A:Brg1 complex could be required for many intermediate steps of transcription or link these together.

FUTURE INVESTIGATION

Given that observed SAF-A:Brg1 complex is of fundamental role for RNA pol II mediated transcription, it would be very interesting to investigate whether the SAF-A:Brg1 association is direct and which protein domains are responsible for possible direct association. In this regard, SAF-A and Brg1 constructs should be made, proteins expressed and tested for direct association. However, it should be mentioned that SAF-A is a very unstable protein and cannot be expressed in bacteria.

Another interesting issue that has not been addressed is whether the phenotype observed by the depletion of one of the proteins can be rescued by simultaneous over-expressing of the other protein.

PAPER III - OCT4 AS A PROGNOSTIC BIOMARKER OF OVARIAN CANCER

Ovarian cancer is the most lethal gynaecologic cancer. Current ovarian cancer treatments are inefficient at targeting the cells that sustain tumour growth. Therefore it is very important to understand the molecular origins of the ovarian cancer. It has been suggested that tumors arise from cancer stem cells with capacity for self-renewal and differentiation, two characteristics shared with ES cells. Given that ectopic *Oct4* activation has been suggested as a candidate biomarker for cancer stem cells [30, 84, 170], we explored possible reactivation of *Oct4* gene in patients suffering from ovarian cancer.

OCT4 IS REACTIVATED IN PATIENTS SUFFERING FROM OVARIAN CANCER

To investigate if *Oct4* was reactivated in ovarian cancer, Oct4 protein levels in the biopsy samples from 20 ovarian cancer patients were analyzed. Western blot revealed detectable levels of Oct4 protein in biopsy samples from eight patients which is in agreement with recent report demonstrating the presence of Oct4 in biopsy samples from ovarian cancer patients [86]. Notably, we found that patients with *Oct4* expressed survived no more than 3.5 years from the diagnosis suggesting that Oct4 could be used as a prognostic factor of mortality in women with ovarian cancer.

OCT4 PROMOTER IS NOT DEMETHYLATED IN OVARIAN CANCER TISSUE

DNA demethylation has previously been reported to be a prerequisite for the reactivation of *Oct4* [97]. Therefore, two regions of the *Oct4* promoter, i.e. PP and DE, were analysed with respect to CpG methylation. These analyses revealed that ovarian cancer tissue is a very heterogeneous cell population in respect to DNA methylation. In contrary to our expectations, the degree of *Oct4* promoter methylation was significantly higher in deceased ovarian cancer patients (80.4%) than in those that had been successfully treated (66.9%). Similar observation has been reported for promoter region of the catalytic subunit of telomerase (*TERT*) which is reactivated in cancer cell lines [171]. Alternatively, our data may indicate that *Oct4* is reactivated through a promoter switch, which permits *Oct4* expression irrespective of methylation status.

In summary, our investigation now implicates reactivation of the stem cell marker *Oct4* in ovarian cancer, and thus indirectly adds support to the concept of ovarian cancer stem cells.

FUTURE INVESTIGATION

It should be marked that method used, i.e. bisulfite sequencing, gives insight of DNA methylation at the allele resolution in a single cell. Given that ovarian cancer tissue is a very heterogeneous cell population and that the great part of the cells probably do not express *Oct4*, additional methods should be used prior bisulfite sequencing to gain more information about correlation of *Oct4* reactivation and *Oct4* promoter DNA methylation. One possibility is to employ laser based microdissection (LSM) for isolation of Oct4 positive cells from ovarian cancer tissue followed by bisulfite sequencing.

Paper IV - Developmental Studies of Xenopus Shelterin Complexes

The recent discovery proposing the importance of telomeres in epigenetic gene regulation and vertebrate embryonic development calls for the establishment of model organisms to study shelterin and telomere function under normal developmental conditions. Therefore, the aim of this project was to identify and characterize the telomere shelterin complexes in *Xenopus laevis* and *tropicalis* which are commonly used model organisms for developmental biology studies.

Identified *Xenopus Laevis* and *Tropicalis* Shelterin Proteins Interact with Telomeric DNA

Possible orthologs of human shelterin components were identified from *Xenopus laevis* and *Xenopus tropicalis* cDNA libraries. A clustalW alignment of amino acid sequences showed high homology (50-90% and 51-88%; Table1) between human (h)/ *Xenopus laevis* (Xl) and human (h)/ Xenopus *tropicalis* (Xt) ortologs, respectively. The identity was shown to be between 19-50% and 25-50% (Table1), respectively.

Human	***Xenopus laevis***		***Xenopus tropicalis***	
shelterin protein	**Homology(%)**	**Identity(%)**	**Homology(%)**	**Identity(%)**
hTRF1	85	34	81	32
hTRF2	90	36	88	36
hPOT1	67	50	67	50
hTIN2	66	26	65	25
hRAP1	77	32	79	33
hTPP1	50	19	51	20
hPINX1	78.4	48.5	79	50.3

Table 1. Homology and identity between human and *Xenopus laevis*/ *tropicalis* orthologs.

The sequence analysis revealed that Xt, as reported Xl, TRF1 contain conserved C-terminal MYB/homeodomain and N-terminal domains which are required for interaction with telomeric DNA and formation of homodimers, respectively. These results are in accordance with EMSA demonstrating that *Xenopus* TRF1, just like hTRF1, binds double stranded telomeric DNA with high affinity and specificity *in vitro* and simultaneously forms a complex with TIN2. The acidic N-terminus is missing in XtTRF1 as it previously has been reported for XlTRF1

[172]. This domain has been demonstrated to be responsible for interactions with the shelterin accessory factor Tankyrase in humans [173]. Because Tankyrase regulates binding of TRF1 to the telomeric DNA in humans, the missing acidic N-terminus in *Xenopus* TRF1 likely has implications for TRF1 regulation by Tankyrase.

The sequence analysis revealed that XtTRF2, like previously identified XlTRF2 [174], shares several features of hTRF2. The C-terminal MYB/homeodomain is conserved. The hTRF1 and hTRF2 MYB/homeodomains have similar affinities for telomeric DNA [175]. The hTRF2 is reported to form a highly specific complex with telomeric DNA when in complex with RAP1 [112, 117]. The region of hTRF2 that is responsible for interactions with RAP1 is conserved in *Xenopus* as demonstrated by sequence analysis. In addition, *Xenopus* RAP1 contains a putative MYB domain and C-terminal TRF2 interacting domain. EMSAs reveals that *Xenopus* RAP1-TRF2 complex has significantly higher specificity for double stranded telomeric DNA than TRF2 on its own. Moreover, the sequence analysis revealed that *Xenopus* TRF2 lacks the basic N-terminus. The functional significance of this domain has not been elucidated.

Further, the sequence analysis revealed that the only detected difference between the human and *Xenopus* POT1 orthologs is that the central linker peptide, which connects the two N-terminal DNA-binding OB folds to the C-terminal protein-interacting OB-fold, is slightly longer in hPOT1. This peptide linker is believed to allow the DNA binding module of POT1 to move relative to its protein-interacting module, so that shelterin can modulate the structure and accessibility of the single-stranded telomeric G-overhang. The binding properties, i.e. high affinity and specificity, of *Xenopus* POT1 to single stranded telomeric DNA [121] were confirmed *in vitro* by EMSA.

The identified *Xenopus* TIN2 lacks conserved domains, just like hTIN2 ortholog. However, the hTIN2 binding domains of hTRF1 (aa 256–276) [118] and hTRF2 (aa 1–220) [176, 177] correspond to the highly conserved regions in *Xenopus* orthologs.

The sequence analysis of TPP1 revealed that fixed secondary structure as well as regions responsible for interactions with POT1 are highly conserved between human and *Xenopus* TPP1.

These studies suggest that identified *Xenopus* shelterin proteins have conserved main domains and functions of their human orthologs.

Xenopus Shelterin Genes are Temporaly Regulated during Development

The shelterin gene mRNA levels during *Xenopus* embryogenesis were quantified for developmental stages between blastula and gastrula. The expression of all shelterin components, as well as the shelterin accessory factor *PINX1* and telomerase, were changed during embryogenesis. The expression of all shelterin genes decreased, but there was no apparent overall covariance between their expression profiles. The expression profile of telomerase was different from the expression profiles of the shelterin genes. However, subgroups of shelterin genes exhibited covariance. The *POT1*, *TPP1* and *TIN2* genes formed a shelterin subgroup whose expression profiles were very similar. The expression profile of *TRF1* was very similar to that of *PINX1*, whereas the *TRF2* and *RAP1* genes formed the third shelterin subgroup. These results indicate that the composition of shelterin and the formation of its subcomplexes appear to be temporally regulated during *Xenopus* embryonic development.

Future Investigation

Our results demonstration high telomerase and shelterin gene expression during early embryogenesis may reflect a telomere length-resetting mechanism reported for iPS cells [178] and for animals cloned through SCNT [133-135]. Since *Xenopus* shelterin proteins are now identified and SCNT in *Xenopus* is relatively effective, this important issue should be explored by quantitative determination of shelterin gene mRNA levels during SCNT in *Xenopus*.

PAPER V - TPT1:NPM1 COMPLEX IS INVOLVED IN CELL PROLIFERATION OF ES CELLS

Given that Tpt1, also referred as TCTP, HRF, Fortilin or P23, has been proven important for embryonic development [179] and found to affect *Oct4* expression in *Xenopus* SCNT [180] as well as the outcome of SCNT in bovine [181], we addressed functional importance of this protein in ES cells.

TPT1 SUBCELLULAR LOCALIZATION CHANGES DURING DIFFERENTIATION

Tpt1 is highly conserved and abundant eukaryotic protein. Conflicting results have been reported regarding the cellular localization of Tpt1 [182, 183]. Using western blot and immunofluorescence analysis, we detected Tpt1 localized to both cytoplasm and nucleus in ES cells. When differentiation was induced, by either LIF withdrawal or addition of RA, preferential cytoplasmic localization and decreased protein levels of Tpt1 were detected. These data are in agreement with the previous reports suggesting high Tpt1 levels in ES cells [184-186] which are decreasing during neuronal lineage commitment [187].

TPT1 INTERACTS WITH NPM1 IN A CELL CYCLE DEPENDENT MANNER

To explore the function of Tpt1 in ES cells, we first screened for novel Tpt1 interaction partners using recombinant Tpt1 protein covalently linked to cyanogen bromide activated sepharose beads. The beads were incubated with ES cell extract. Potential Tpt1 binding factors were eluted with increasing ionic strength and fractionated by SDS-PAGE. Bands of interest were excised from the gel and by nano-LC-FT-IRC mass spectrometry analysis identified. The band corresponding to 38 kDa in the 0.7 M NaCl elute was identified to be Npm1, also referred as B23, NO38 or numatrin, which has been reported expressed in high levels in both murine and human ES cells [184, 185].

Immunofluorescence analysis in combination with BioPix iQ 2.0 software revealed the significantly higher co-localization of Tpt1 and Npm1 in mitotic in comparison to interphase ES cells. These results were confirmed by *in situ* PLA. Additionally, Npm1 was co-immunoprecipitated using anti-Tpt1, further strengthening the observed results that endogenous Tpt1 and Npm1 interact in ES cells.

Tpt1 and Npm1 Interact Independently of Plk1 Phosphorylation

Both Tpt1 and Npm1 have previously been demonstrated to be phosphorylated by Plk1 during mitosis [188, 189]. To investigate whether the Tpt1:Npm1 interaction is regulated by Plk1 phosphorylation, ES cells were treated with two different Plk1 inhibitors, wortmannin [190] and BI2536 [191], which arrest cell cycle progression at metaphase and prophase, respectively. While wortmannin treatment resulted in weaker intensity and decreased quantity of PLA signals, the BI2536 treatment did not affect either quantity or intensity of PLA signals. These contradictory results could have several possible explanations. BI2536 is a specific Plk1 inhibitor, while wortmannin also inhibits several other kinases. The observation that BI2536 have no effect on Tpt1:Npm1 interaction might therefore indicate that other kinases than Plk1 regulate Tpt1:Npm1 interaction. The alternative explanation might be that Plk1 indeed is involved in regulation but that it occurs in later stages of mitosis. Overall, these results reveal that the Tpt1: Npm1 interaction is not dependent on Plk1 mediated phosphorylation, at least not during the early stages of mitosis.

Tpt1:Npm1 Complexes are Involved in ES Cell Proliferation

To investigate the impact of Tpt1 and Npm1 alone or as a complex on cell proliferation, ES cells were manipulated by either over-expression or depletion of indicated proteins followed by EdU proliferation assay and confocal microscopy.

Npm1 over-expression resulted in increased proliferation in ES cells (14.7%). In contrary, increased levels of Tpt1 had decreasing effect on ES cell proliferation (15.9%). This is in accordance with earlier studies in other cellular systems reporting slow growing cells as a result of Tpt1 over-expression [192]. Interestingly, dual over-expression of Tpt1 and Npm1 stabilized proliferation to normal ES cell rate.

Depletion of Tpt1 and Npm1 alone for 48 and 72 hours resulted in significant decrease in cell proliferation. Moreover, ES cells depleted for both Tpt1 and Npm1 exhibited yet larger reduction in proliferation in comparison to cells depleted for Tpt1 and Npm1 alone.

Our discoveries propose a role for the Tpt1:Npm1 complex in ES cell proliferation and are in accordance with previous reports suggesting the role of Tpt1 and Npm1 in cell proliferation in different cellular systems [188, 192-196].

The over-expression experiments indicate that Tpt1 and Npm1 do not have redundant roles and therefore both proteins are of great importance in regulating ES cell proliferation. The observation that over-expression as well as depletion of Tpt1 decreases ES cell proliferation indicates that there is a critical level of the protein that is pivotal for this event to occur which is also a possible explanation regarding its importance for cloning.

FUTURE INVESTIGATION

Given that observed Tpt1:Npm1 interaction is important for proper ES cell proliferation and probably is conserved in cancer cells, it is very important to identify protein domains and subsequently investigate the mechanisms responsible for the observed interaction.

CONCLUDING REMARKS

- SAF-A was found to bind preferentially to the *Oct4* proximal promoter in ES cells. Functional assays revealed that depletion of SAF-A affects Oct4 mRNA and protein levels. Further, SAF-A was found in the complex with a previously proven affecters of *Oct4* expression, Oct4 and Sox2, in a LIF signalling dependent manner as well as with RNA pol II suggesting that SAF-A serves to bring together factors required for *Oct4* expression and load them on the promoter together with RNA pol II in ES cells.

- SAF-A was found in complex with the SWI/SNF-Brg1 chromatin remodelling protein in ES and differentiation induced cells. Functional assays revealed that dual depletion of SAF-A and Brg1 in ES cells abolishes global transcription by RNA polymerase II indicating a fundamental role for the SAF-A/Brg1 complex in RNA polymerase II mediated transcription.

- Ectopic *Oct4* expression was found in the biopsy samples from ovarian cancer patients who survived for no more than 3.5 years from the diagnosis suggesting that Oct4 could be used as a prognostic factor of mortality in women with ovarian cancer. Further, observed *Oct4* reactivation was not dependent on DNA demethylation of the investigated *Oct4* regulatory regions.

- *Xenopus* shelterin proteins as well as the shelterin accessory factor were identified and the conservation of main functions in relation to their human orthologs was demonstrated by *in vitro* expression and biochemical characterization. Moreover, the temporal regulation of shelterin composition and subcomplex appearance was demonstrated during *Xenopus* embryonic development.

- Tpt1 was found to interact with Npm1 in a cell cycle dependent manner, with a significant peak during mitosis. Functional assays revealed that dual depletion of Tpt1 and Npm1 results in larger decrease in cell proliferation than their combined individual decreases.

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REFERENCES

1. Wilmut, I., et al., *Viable offspring derived from fetal and adult mammalian cells.* Nature, 1997. **385**(6619): p. 810-813.
2. Gurdon, J.B., *The use of Xenopus oocytes and embryos as a route towards cell replacement.* J Biosci, 2005. **30**(1): p. 11-14.
3. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-676.
4. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-872.
5. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-1920.
6. Park, I.H., et al., *Reprogramming of human somatic cells to pluripotency with defined factors.* Nature, 2008. **451**(7175): p. 141-146.
7. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-7638.
8. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-156.
9. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-1147.
10. Bradley, A., et al., *Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines.* Nature, 1984. **309**(5965): p. 255-256.
11. Martin, G.R., *Teratocarcinomas and mammalian embryogenesis.* Science, 1980. **209**(4458): p. 768-776.
12. Capecchi, M.R., *Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century.* Nat Rev Genet, 2005. **6**(6): p. 507-512.
13. Sartipy, P., et al., *The application of human embryonic stem cell technologies to drug discovery.* Drug Discov Today, 2007. **12**(17-18): p. 688-699.
14. Yamashita, H., et al., *Embryonic stem cell-derived neuron models of Parkinson's disease exhibit delayed neuronal death.* J Neurochem, 2006. **98**(1): p. 45-56.
15. Di Giorgio, F.P., et al., *Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model.* Nat Neurosci, 2007. **10**(5): p. 608-614.
16. Wilmut, I., et al., *Somatic cell nuclear transfer.* Nature, 2002. **419**(6907): p. 583-586.

17. French, A.J., et al., *Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts.* Stem Cells, 2008. **26**(2): p. 485-493.
18. Barberi, T., et al., *Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice.* Nat Biotechnol, 2003. **21**(10): p. 1200-1207.
19. Rideout, W.M., 3rd, et al., *Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy.* Cell, 2002. **109**(1): p. 17-27.
20. Tabar, V., et al., *Therapeutic cloning in individual parkinsonian mice.* Nat Med, 2008. **14**(4): p. 379-381.
21. Wakayama, S., et al., *Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts.* Stem Cells, 2006. **24**(9): p. 2023-2033.
22. Brambrink, T., et al., *ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 933-938.
23. Cezar, G.G., *Epigenetic reprogramming of cloned animals.* Cloning Stem Cells, 2003. **5**(3): p. 165-180.
24. Lowry, W.E., et al., *Generation of human induced pluripotent stem cells from dermal fibroblasts.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 2883-2888.
25. Nakagawa, M., et al., *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts.* Nat Biotechnol, 2008. **26**(1): p. 101-106.
26. Hanna, J., et al., *Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin.* Science, 2007. **318**(5858): p. 1920-1923.
27. Wernig, M., et al., *Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease.* Proc Natl Acad Sci U S A, 2008. **105**(15): p. 5856-5861.
28. Kazuki, Y., et al., *Complete genetic correction of ips cells from Duchenne muscular dystrophy.* Mol Ther, 2010. **18**(2): p. 386-393.
29. Xu, D., et al., *Phenotypic correction of murine hemophilia A using an iPS cell-based therapy.* Proc Natl Acad Sci U S A, 2009. **106**(3): p. 808-813.
30. Gidekel, S., et al., *Oct-3/4 is a dose-dependent oncogenic fate determinant.* Cancer Cell, 2003. **4**(5): p. 361-370.
31. Wei, D., et al., *Emerging role of KLF4 in human gastrointestinal cancer.* Carcinogenesis, 2006. **27**(1): p. 23-31.
32. Bass, A.J., et al., *SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas.* Nat Genet, 2009. **41**(11): p. 1238-1242.

33. Wernig, M., et al., *c-Myc is dispensable for direct reprogramming of mouse fibroblasts.* Cell Stem Cell, 2008. **2**(1): p. 10-12.
34. Yamanaka, S., *A fresh look at iPS cells.* Cell, 2009. **137**(1): p. 13-17.
35. Okita, K., et al., *Generation of mouse induced pluripotent stem cells without viral vectors.* Science, 2008. **322**(5903): p. 949-953.
36. Gonzalez, F., et al., *Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector.* Proc Natl Acad Sci U S A, 2009. **106**(22): p. 8918-8922.
37. Stadtfeld, M., et al., *Induced pluripotent stem cells generated without viral integration.* Science, 2008. **322**(5903): p. 945-949.
38. Yu, J., et al., *Human induced pluripotent stem cells free of vector and transgene sequences.* Science, 2009. **324**(5928): p. 797-801.
39. Jia, F., et al., *A nonviral minicircle vector for deriving human iPS cells.* Nat Methods, 2010. **7**(3): p. 197-199.
40. Yusa, K., et al., *Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon.* Nat Methods, 2009. **6**(5): p. 363-369.
41. Woltjen, K., et al., *piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells.* Nature, 2009. **458**(7239): p. 766-770.
42. Soldner, F., et al., *Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors.* Cell, 2009. **136**(5): p. 964-977.
43. Chang, C.W., et al., *Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells.* Stem Cells, 2009. **27**(5): p. 1042-1049.
44. Zhou, H., et al., *Generation of induced pluripotent stem cells using recombinant proteins.* Cell Stem Cell, 2009. **4**(5): p. 381-384.
45. Smith, A.G., et al., *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides.* Nature, 1988. **336**(6200): p. 688-690.
46. Williams, R.L., et al., *Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells.* Nature, 1988. **336**(6200): p. 684-687.
47. Niwa, H., *Molecular mechanism to maintain stem cell renewal of ES cells.* Cell Struct Funct, 2001. **26**(3): p. 137-148.
48. Niwa, H., et al., *Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3.* Genes Dev, 1998. **12**(13): p. 2048-2060.
49. Matsuda, T., et al., *STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells.* EMBO J, 1999. **18**(15): p. 4261-4269.

50. Ying, Q.L., et al., *BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3.* Cell, 2003. **115**(3): p. 281-292.
51. Daheron, L., et al., *LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells.* Stem Cells, 2004. **22**(5): p. 770-778.
52. Reubinoff, B.E., et al., *Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.* Nat Biotechnol, 2000. **18**(4): p. 399-404.
53. Xu, R.H., et al., *BMP4 initiates human embryonic stem cell differentiation to trophoblast.* Nat Biotechnol, 2002. **20**(12): p. 1261-1264.
54. Amit, M., et al., *Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture.* Dev Biol, 2000. **227**(2): p. 271-278.
55. Beattie, G.M., et al., *Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers.* Stem Cells, 2005. **23**(4): p. 489-495.
56. Armstrong, L., et al., *mTert expression correlates with telomerase activity during the differentiation of murine embryonic stem cells.* Mech Dev, 2000. **97**(1-2): p. 109-116.
57. Stead, E., et al., *Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities.* Oncogene, 2002. **21**(54): p. 8320-8333.
58. Wu, S.C. and Y. Zhang, *Active DNA demethylation: many roads lead to Rome.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 607-620.
59. Okano, M., S. Xie, and E. Li, *Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases.* Nat Genet, 1998. **19**(3): p. 219-220.
60. Okano, M., et al., *DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development.* Cell, 1999. **99**(3): p. 247-257.
61. Hermann, A., R. Goyal, and A. Jeltsch, *The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites.* J Biol Chem, 2004. **279**(46): p. 48350-48359.
62. Bibikova, M., et al., *Human embryonic stem cells have a unique epigenetic signature.* Genome Res, 2006. **16**(9): p. 1075-1083.
63. Fouse, S.D., et al., *Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation.* Cell Stem Cell, 2008. **2**(2): p. 160-169.

64. Meissner, A., et al., *Genome-scale DNA methylation maps of pluripotent and differentiated cells.* Nature, 2008. **454**(7205): p. 766-770.
65. Lee, J.H., S.R. Hart, and D.G. Skalnik, *Histone deacetylase activity is required for embryonic stem cell differentiation.* Genesis, 2004. **38**(1): p. 32-38.
66. Azuara, V., et al., *Chromatin signatures of pluripotent cell lines.* Nat Cell Biol, 2006. **8**(5): p. 532-538.
67. Bernstein, B.E., et al., *A bivalent chromatin structure marks key developmental genes in embryonic stem cells.* Cell, 2006. **125**(2): p. 315-326.
68. Havas, K., I. Whitehouse, and T. Owen-Hughes, *ATP-dependent chromatin remodeling activities.* Cell Mol Life Sci, 2001. **58**(5-6): p. 673-682.
69. Lomvardas, S. and D. Thanos, *Nucleosome sliding via TBP DNA binding in vivo.* Cell, 2001. **106**(6): p. 685-696.
70. Gavin, I., P.J. Horn, and C.L. Peterson, *SWI/SNF chromatin remodeling requires changes in DNA topology.* Mol Cell, 2001. **7**(1): p. 97-104.
71. Ho, L., et al., *An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5181-5186.
72. Kidder, B.L., S. Palmer, and J.G. Knott, *SWI/SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells.* Stem Cells, 2009. **27**(2): p. 317-328.
73. Shen, W., et al., *Solution structure of human Brg1 bromodomain and its specific binding to acetylated histone tails.* Biochemistry, 2007. **46**(8): p. 2100-2110.
74. Bultman, S., et al., *A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes.* Mol Cell, 2000. **6**(6): p. 1287-1295.
75. Bultman, S.J., et al., *Maternal BRG1 regulates zygotic genome activation in the mouse.* Genes Dev, 2006. **20**(13): p. 1744-1754.
76. Boyer, L.A., et al., *Core transcriptional regulatory circuitry in human embryonic stem cells.* Cell, 2005. **122**(6): p. 947-956.
77. Zhou, Q., et al., *A gene regulatory network in mouse embryonic stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(42): p. 16438-16443.
78. Babaie, Y., et al., *Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells.* Stem Cells, 2007. **25**(2): p. 500-510.
79. Falkner, F.G. and H.G. Zachau, *Correct transcription of an immunoglobulin kappa gene requires an upstream fragment*

containing conserved sequence elements. Nature, 1984. **310**(5972): p. 71-74.
80. Pesce, M. and H.R. Scholer, *Oct-4: gatekeeper in the beginnings of mammalian development.* Stem Cells, 2001. **19**(4): p. 271-278.
81. Nichols, J., et al., *Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4.* Cell, 1998. **95**(3): p. 379-391.
82. Rosner, M.H., et al., *A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo.* Nature, 1990. **345**(6277): p. 686-692.
83. Niwa, H., J. Miyazaki, and A.G. Smith, *Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells.* Nat Genet, 2000. **24**(4): p. 372-376.
84. Looijenga, L.H., et al., *POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors.* Cancer Res, 2003. **63**(9): p. 2244-2250.
85. Chen, Z., et al., *Oct4, a novel marker for human gastric cancer.* J Surg Oncol, 2009. **99**(7): p. 414-419.
86. Peng, S., N.J. Maihle, and Y. Huang, *Pluripotency factors Lin28 and Oct4 identify a sub-population of stem cell-like cells in ovarian cancer.* Oncogene, 2010. **29**(14): p. 2153-2159.
87. Yeom, Y.I., et al., *Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells.* Development, 1996. **122**(3): p. 881-894.
88. Nordhoff, V., et al., *Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences.* Mamm Genome, 2001. **12**(4): p. 309-317.
89. Minucci, S., et al., *Retinoic acid-mediated down-regulation of Oct3/4 coincides with the loss of promoter occupancy in vivo.* Embo J, 1996. **15**(4): p. 888-899.
90. Pesce, M., et al., *Binding of Sp1 and Sp3 transcription factors to the Oct-4 gene promoter.* Cell Mol Biol (Noisy-le-grand), 1999. **45**(5): p. 709-716.
91. Gu, P., et al., *Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation.* Mol Cell Biol, 2005. **25**(19): p. 8507-8519.
92. Gu, P., et al., *Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development.* Mol Cell Biol, 2005. **25**(9): p. 3492-3505.
93. Yang, H.M., et al., *Transcriptional regulation of human Oct4 by steroidogenic factor-1.* J Cell Biochem, 2007. **101**(5): p. 1198-1209.

94. Schoorlemmer, J., et al., *Characterization of a negative retinoic acid response element in the murine Oct4 promoter.* Mol Cell Biol, 1994. **14**(2): p. 1122-1136.
95. Yamazaki, Y., et al., *Gradual DNA demethylation of the Oct4 promoter in cloned mouse embryos.* Mol Reprod Dev, 2006. **73**(2): p. 180-188.
96. Byrne, J.A., et al., *Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes.* Curr Biol, 2003. **13**(14): p. 1206-1213.
97. Simonsson, S. and J. Gurdon, *DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei.* Nat Cell Biol, 2004. **6**(10): p. 984-990.
98. Barreto, G., et al., *Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation.* Nature, 2007. **445**(7128): p. 671-675.
99. Freberg, C.T., et al., *Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract.* Mol Biol Cell, 2007. **18**(5): p. 1543-1553.
100. Wei, F., H.R. Scholer, and M.L. Atchison, *Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation.* J Biol Chem, 2007. **282**(29): p. 21551-21560.
101. Xu, H.M., et al., *Wwp2, an E3 ubiquitin ligase that targets transcription factor Oct-4 for ubiquitination.* J Biol Chem, 2004. **279**(22): p. 23495-23503.
102. Zijlmans, J.M., et al., *Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats.* Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7423-7428.
103. Hiyama, E. and K. Hiyama, *Telomere and telomerase in stem cells.* Br J Cancer, 2007. **96**(7): p. 1020-1024.
104. McClintock, B., *The Production of Homozygous Deficient Tissues with Mutant Characteristics by Means of the Aberrant Mitotic Behavior of Ring-Shaped Chromosomes.* Genetics, 1938. **23**(4): p. 315-376.
105. McClintock, B., *The Behavior in Successive Nuclear Divisions of a Chromosome Broken at Meiosis.* Proc Natl Acad Sci U S A, 1939. **25**(8): p. 405-416.
106. Greider, C.W., *Telomere length regulation.* Annu Rev Biochem, 1996. **65**: p. 337-365.
107. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-413.
108. Morin, G.B., *The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats.* Cell, 1989. **59**(3): p. 521-529.

109. Yu, G.L., et al., *In vivo alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs.* Nature, 1990. **344**(6262): p. 126-132.
110. Simonsson, T., *A substrate for telomerase.* Trends Biochem Sci, 2003. **28**(12): p. 632-638.
111. Smogorzewska, A. and T. de Lange, *Regulation of telomerase by telomeric proteins.* Annu Rev Biochem, 2004. **73**: p. 177-208.
112. de Lange, T., *Shelterin: the protein complex that shapes and safeguards human telomeres.* Genes Dev, 2005. **19**(18): p. 2100-2110.
113. Chong, L., et al., *A human telomeric protein.* Science, 1995. **270**(5242): p. 1663-1667.
114. Bilaud, T., et al., *Telomeric localization of TRF2, a novel human telobox protein.* Nat Genet, 1997. **17**(2): p. 236-239.
115. Broccoli, D., et al., *Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2.* Nat Genet, 1997. **17**(2): p. 231-235.
116. Chen, Y., et al., *A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins.* Science, 2008. **319**(5866): p. 1092-1096.
117. Li, B., S. Oestreich, and T. de Lange, *Identification of human Rap1: implications for telomere evolution.* Cell, 2000. **101**(5): p. 471-483.
118. Kim, S.H., P. Kaminker, and J. Campisi, *TIN2, a new regulator of telomere length in human cells.* Nat Genet, 1999. **23**(4): p. 405-412.
119. Baumann, P. and T.R. Cech, *Pot1, the putative telomere end-binding protein in fission yeast and humans.* Science, 2001. **292**(5519): p. 1171-1175.
120. Lei, M., E.R. Podell, and T.R. Cech, *Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection.* Nat Struct Mol Biol, 2004. **11**(12): p. 1223-1229.
121. Wang, F., et al., *The POT1-TPP1 telomere complex is a telomerase processivity factor.* Nature, 2007. **445**(7127): p. 506-510.
122. Hockemeyer, D., et al., *Telomere protection by mammalian Pot1 requires interaction with Tpp1.* Nat Struct Mol Biol, 2007. **14**(8): p. 754-761.
123. Liu, D., et al., *PTOP interacts with POT1 and regulates its localization to telomeres.* Nat Cell Biol, 2004. **6**(7): p. 673-680.
124. Xin, H., et al., *TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase.* Nature, 2007. **445**(7127): p. 559-562.
125. Ye, J.Z., et al., *POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex.* Genes Dev, 2004. **18**(14): p. 1649-1654.

126. Loayza, D. and T. De Lange, *POT1 as a terminal transducer of TRF1 telomere length control.* Nature, 2003. **423**(6943): p. 1013-1018.
127. Chen, L.Y., D. Liu, and Z. Songyang, *Telomere maintenance through spatial control of telomeric proteins.* Mol Cell Biol, 2007. **27**(16): p. 5898-5909.
128. Blasco, M.A., *Telomere length, stem cells and aging.* Nat Chem Biol, 2007. **3**(10): p. 640-649.
129. Collado, M., M.A. Blasco, and M. Serrano, *Cellular senescence in cancer and aging.* Cell, 2007. **130**(2): p. 223-233.
130. Finkel, T., M. Serrano, and M.A. Blasco, *The common biology of cancer and ageing.* Nature, 2007. **448**(7155): p. 767-774.
131. Blasco, M.A., *The epigenetic regulation of mammalian telomeres.* Nat Rev Genet, 2007. **8**(4): p. 299-309.
132. Schoeftner, S. and M.A. Blasco, *Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II.* Nat Cell Biol, 2008. **10**(2): p. 228-236.
133. Betts, D., et al., *Reprogramming of telomerase activity and rebuilding of telomere length in cloned cattle.* Proc Natl Acad Sci U S A, 2001. **98**(3): p. 1077-1082.
134. Lanza, R.P., et al., *Extension of cell life-span and telomere length in animals cloned from senescent somatic cells.* Science, 2000. **288**(5466): p. 665-669.
135. Tian, X.C., J. Xu, and X. Yang, *Normal telomere lengths found in cloned cattle.* Nat Genet, 2000. **26**(3): p. 272-273.
136. Savatier, P., et al., *Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells.* Oncogene, 1996. **12**(2): p. 309-322.
137. Nigg, E.A., *Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle.* Bioessays, 1995. **17**(6): p. 471-480.
138. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-3988.
139. Hermann, P.C., et al., *Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer.* Cell Stem Cell, 2007. **1**(3): p. 313-323.
140. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-648.
141. Li, C., et al., *Identification of pancreatic cancer stem cells.* Cancer Res, 2007. **67**(3): p. 1030-1037.
142. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer Res, 2003. **63**(18): p. 5821-5828.

143. Singh, S.K., et al., *Identification of human brain tumour initiating cells.* Nature, 2004. **432**(7015): p. 396-401.
144. Kadonaga, J.T. and R. Tjian, *Affinity purification of sequence-specific DNA binding proteins.* Proc Natl Acad Sci U S A, 1986. **83**(16): p. 5889-5893.
145. Soderberg, O., et al., *Direct observation of individual endogenous protein complexes in situ by proximity ligation.* Nat Methods, 2006. **3**(12): p. 995-1000.
146. Chen, S., et al., *Establishing efficient siRNA knockdown in mouse embryonic stem cells.* Biotechnol Lett, 2007. **29**(2): p. 261-265.
147. Chehrehasa, F., et al., *EdU, a new thymidine analogue for labelling proliferating cells in the nervous system.* J Neurosci Methods, 2009. **177**(1): p. 122-130.
148. Jao, C.Y. and A. Salic, *Exploring RNA transcription and turnover in vivo by using click chemistry.* Proc Natl Acad Sci U S A, 2008. **105**(41): p. 15779-15784.
149. Gohring, F., et al., *The novel SAR-binding domain of scaffold attachment factor A (SAF-A) is a target in apoptotic nuclear breakdown.* The EMBO journal, 1997. **16**(24): p. 7361-7371.
150. Gohring, F. and F.O. Fackelmayer, *The scaffold/matrix attachment region binding protein hnRNP-U (SAF-A) is directly bound to chromosomal DNA in vivo: a chemical cross-linking study.* Biochemistry, 1997. **36**(27): p. 8276-8283.
151. Levros, L.C., Jr., et al., *Characterization of nuclear factors modulating the apolipoprotein D promoter during growth arrest: Implication of PARP-1, APEX-1 and ERK1/2 catalytic activities.* Biochim Biophys Acta, 2010. **1803**(9): p. 1062-1071.
152. Zhao, J., et al., *HnRNP U mediates the long-range regulation of Shh expression during limb development.* Hum Mol Genet, 2009. **18**(16): p. 3090-3097.
153. Ahmad, N. and J.B. Lingrel, *Kruppel-like factor 2 transcriptional regulation involves heterogeneous nuclear ribonucleoproteins and acetyltransferases.* Biochemistry, 2005. **44**(16): p. 6276-6285.
154. Onishi, Y., et al., *Rhythmic SAF-A binding underlies circadian transcription of the Bmal1 gene.* Mol Cell Biol, 2008. **28**(10): p. 3477-3488.
155. Roshon, M.J. and H.E. Ruley, *Hypomorphic mutation in hnRNP U results in post-implantation lethality.* Transgenic Res, 2005. **14**(2): p. 179-192.
156. Kim, M.K. and V.M. Nikodem, *hnRNP U inhibits carboxy-terminal domain phosphorylation by TFIIH and represses RNA polymerase II elongation.* Mol Cell Biol, 1999. **19**(10): p. 6833-6844.

157. Kukalev, A., et al., *Actin and hnRNP U cooperate for productive transcription by RNA polymerase II.* Nat Struct Mol Biol, 2005. **12**(3): p. 238-244.
158. Ding, L., et al., *A genome-scale RNAi screen for Oct4 modulators defines a role of the Paf1 complex for embryonic stem cell identity.* Cell Stem Cell, 2009. **4**(5): p. 403-415.
159. Ponnusamy, M.P., et al., *RNA Polymerase II Associated Factor 1/PD2 Maintains Self-Renewal by Its Interaction with Oct3/4 in Mouse Embryonic Stem Cells.* Stem Cells, 2009. **27**(12): p. 3001-3011.
160. Malik, K.F., et al., *The class III POU factor Brn-4 interacts with other class III POU factors and the heterogeneous nuclear ribonucleoprotein U.* Brain Res Mol Brain Res, 1997. **45**(1): p. 99-107.
161. Chew, J.L., et al., *Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells.* Mol Cell Biol, 2005. **25**(14): p. 6031-6046.
162. Okumura-Nakanishi, S., et al., *Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells.* J Biol Chem, 2005. **280**(7): p. 5307-5317.
163. Kidder, B.L., J. Yang, and S. Palmer, *Stat3 and c-Myc genome-wide promoter occupancy in embryonic stem cells.* PLoS One, 2008. **3**(12): p. e3932.
164. Martens, J.H., et al., *Scaffold/matrix attachment region elements interact with a p300-scaffold attachment factor A complex and are bound by acetylated nucleosomes.* Mol Cell Biol, 2002. **22**(8): p. 2598-2606.
165. Obrdlik, A., et al., *The histone acetyltransferase PCAF associates with actin and hnRNP U for RNA polymerase II transcription.* Mol Cell Biol, 2008. **28**(20): p. 6342-6357.
166. Dreyfuss, G., S.A. Adam, and Y.D. Choi, *Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription.* Mol Cell Biol, 1984. **4**(3): p. 415-423.
167. Dellaire, G., et al., *Mammalian PRP4 kinase copurifies and interacts with components of both the U5 snRNP and the N-CoR deacetylase complexes.* Mol Cell Biol, 2002. **22**(14): p. 5141-5156.
168. Tyagi, A., et al., *SWI/SNF associates with nascent pre-mRNPs and regulates alternative pre-mRNA processing.* PLoS Genet, 2009. **5**(5): p. e1000470.
169. Neish, A.S., et al., *Factors associated with the mammalian RNA polymerase II holoenzyme.* Nucleic Acids Res, 1998. **26**(3): p. 847-853.

170. Cheng, L., et al., *OCT4: a novel biomarker for dysgerminoma of the ovary.* Am J Surg Pathol, 2004. **28**(10): p. 1341-1346.
171. Zinn, R.L., et al., *hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site.* Cancer Res, 2007. **67**(1): p. 194-201.
172. Crumet, N., et al., *A truncated acidic domain in Xenopus TRF1.* Gene, 2006. **369**: p. 20-26.
173. Smith, S., et al., *Tankyrase, a poly(ADP-ribose) polymerase at human telomeres.* Science, 1998. **282**(5393): p. 1484-1487.
174. Nishiyama, A., et al., *Cell-cycle-dependent Xenopus TRF1 recruitment to telomere chromatin regulated by Polo-like kinase.* EMBO J, 2006. **25**(3): p. 575-584.
175. Court, R., et al., *How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: a view from high-resolution crystal structures.* EMBO Rep, 2005. **6**(1): p. 39-45.
176. Kim, S.H., et al., *TIN2 mediates functions of TRF2 at human telomeres.* J Biol Chem, 2004. **279**(42): p. 43799-43804.
177. Ye, J.Z., et al., *TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres.* J Biol Chem, 2004. **279**(45): p. 47264-47271.
178. Marion, R.M., et al., *Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells.* Cell Stem Cell, 2009. **4**(2): p. 141-154.
179. Koide, Y., et al., *Embryonic lethality of fortilin-null mutant mice by BMP-pathway overactivation.* Biochim Biophys Acta, 2009. **1790**(5): p. 326-338.
180. Koziol, M.J., N. Garrett, and J.B. Gurdon, *Tpt1 activates transcription of oct4 and nanog in transplanted somatic nuclei.* Curr Biol, 2007. **17**(9): p. 801-807.
181. Tani, T., et al., *Bovine oocytes with the potential to reprogram somatic cell nuclei have a unique 23-kDa protein, phosphorylated transcriptionally controlled tumor protein (TCTP).* Cloning Stem Cells, 2007. **9**(2): p. 267-280.
182. Li, F., D. Zhang, and K. Fujise, *Characterization of fortilin, a novel antiapoptotic protein.* J Biol Chem, 2001. **276**(50): p. 47542-47549.
183. Zhang, D., et al., *Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and Fortilin. The potential role of MCL1 as a fortilin chaperone.* J Biol Chem, 2002. **277**(40): p. 37430-37438.
184. Elliott, S.T., et al., *Two-dimensional gel electrophoresis database of murine R1 embryonic stem cells.* Proteomics, 2004. **4**(12): p. 3813-3832.

185. Richards, M., et al., *The transcriptome profile of human embryonic stem cells as defined by SAGE.* Stem Cells, 2004. **22**(1): p. 51-64.
186. Baharvand, H., et al., *Identification of mouse embryonic stem cell-associated proteins.* J Proteome Res, 2008. **7**(1): p. 412-423.
187. Wang, D. and L. Gao, *Proteomic analysis of neural differentiation of mouse embryonic stem cells.* Proteomics, 2005. **5**(17): p. 4414-4426.
188. Yarm, F.R., *Plk phosphorylation regulates the microtubule-stabilizing protein TCTP.* Mol Cell Biol, 2002. **22**(17): p. 6209-6221.
189. Zhang, H., et al., *B23/nucleophosmin serine 4 phosphorylation mediates mitotic functions of polo-like kinase 1.* J Biol Chem, 2004. **279**(34): p. 35726-35734.
190. Liu, Y., et al., *Polo-like kinases inhibited by wortmannin. Labeling site and downstream effects.* J Biol Chem, 2007. **282**(4): p. 2505-2511.
191. Lenart, P., et al., *The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1.* Curr Biol, 2007. **17**(4): p. 304-315.
192. Gachet, Y., et al., *The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1257-1271.
193. Jiang, P.S. and B.Y. Yung, *Down-regulation of nucleophosmin/B23 mRNA delays the entry of cells into mitosis.* Biochem Biophys Res Commun, 1999. **257**(3): p. 865-870.
194. Brady, S.N., et al., *Nucleophosmin protein expression level, but not threonine 198 phosphorylation, is essential in growth and proliferation.* Oncogene, 2009. **28**(36): p. 3209-3220.
195. Li, J., et al., *Nucleophosmin regulates cell cycle progression and stress response in hematopoietic stem/progenitor cells.* J Biol Chem, 2006. **281**(24): p. 16536-16545.
196. Qing, Y., et al., *Role of Npm1 in proliferation, apoptosis and differentiation of neural stem cells.* J Neurol Sci, 2008. **266**(1-2): p. 131-137.