

***Pou5f1 Post-translational Modifications
Modulate Gene Expression and Cell Fate***

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ABSTRACT

Embryonic stem cells (ESCs) are characterized by their unlimited capacity for self-renewal and the ability to contribute to every lineage of the developing embryo. The promoters of developmentally regulated loci within these cells are marked by coincident epigenetic modifications of gene activation and repression, termed bivalent domains. Trithorax group (TrxG) and Polycomb Group (PcG) proteins respectively place these epigenetic marks on chromatin and extensively colocalize with Oct4 in ESCs. Although it appears that these cells are poised and ready for differentiation, the switch that permits this transition is critically held in check. The derepression of bivalent domains upon knockdown of Oct4 or PcG underscores their respective roles in maintaining the pluripotent state through epigenetic regulation of chromatin structure. The mechanisms that facilitate the recruitment and retention of Oct4, TrxG, and PcG proteins at developmentally regulated loci to maintain the pluripotent state, however, remain unknown.

Oct4 may function as either a transcriptional activator or repressor. Prevailing thought holds that both of these activities are required to maintain the pluripotent state through activation of genes implicated in pluripotency and cell-cycle control with concomitant repression of genes required for differentiation and lineage-specific differentiation. More recent evidence however, suggests that the activator function of Oct4 may play a more critical role in maintaining the pluripotent state (Hammachi et al., 2012). The purpose of the studies described in this dissertation was to clarify the underlying mechanisms by which Oct4 functions in transcriptional activation and repression. By so doing, we wished to contextualize its role in pluripotent cells, and to provide insight into how changes in Oct4 function might account for its ability to facilitate cell fate transitions.

As a result of our studies we find that Oct4 function is dependent upon post-translational modifications (PTMs). We find through a combination of experimental approaches, including genome-wide microarray analysis, bioinformatics, chromatin immunoprecipitation, functional molecular, and biochemical analyses, that in the pluripotent state Oct4, Akt, and Hmgb2 participate in a regulatory feedback loop. Akt-mediated phosphorylation of Oct4 facilitates interaction with PcG recruiter Hmgb2. Consequently, Hmgb2 functions as a context dependent modulator of Akt and Oct4 function, promoting transcriptional poise at Oct4 bound loci. Sumoylation of Oct4 is then required to maintain Hmgb2 enrichment at repressed loci and to transmit the H3K27me3 mark in daughter progeny. The expression of Oct4 phosphorylation mutants however, leads to Akt inactivation and initiates the DNA Damage Checkpoint response. Our results suggest that this may subsequently facilitate chromatin reorganization and cell fate transitions. In summary, our results suggest that controlled modulation of Oct4, Akt, and Hmgb2 function is required to maintain pluripotency and for the faithful induction of transcriptional programs required for lineage specific differentiation.

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LIST OF ABBREVIATIONS

Ab	antibody
C-terminus	carboxy terminus
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CMM	cellular memory module
CTD	C-terminal domain (of RnapII)
CUT	cryptic unstable transcript
DAPI	4',6'-diamidino-2-phenylindole
DKO	double knockout
DMEM	Dulbecco's Modified Eagle's Media
DMP	Dimethyl pimelimidate
DNA	deoxy-ribonucleic acid
ESC	embryonic stem cell
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
HATs	histone acetyl transferases
hESC	human embryonic stem cell
HMTs	histone methyl transferases
ICM	inner cell mass
iPS cell	induced pluripotent stem cell
kDa	kilo-Dalton
MEF	murine embryonic fibroblast
mESC	murine embryonic stem cell
NEM	N-ethylmaleimide
N-terminus	amino terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PcG	Polycomb Group
PCR	polymerase chain reaction
PRC1	Polycomb repressor complex 1
PRC2	Polycomb repressor complex 2
PRE	Polycomb response element
PTM	post-translational modification
PVDF	polyvinylidene difluoride membrane
QRT-PCR	quantitative real-time PCR
RIPA Buffer	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RnapII	RNA polymerase II
RT	reverse transcriptase
R-point	restriction point
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sno	small nucleolar
Tc	Tetracycline
TDP	timing decision point
TEV	tobacco etch virus
TKO	triple knockout
TRD	transcriptional regulator domain
TrxG	Trithorax Group
TRE	Trithorax response element
UPS	Ubiquitin Proteasome System
UTR	untranslated region

CHAPTER 1

General Introduction

1. Introduction

Russian histologist Alexander Maximow first proposed that cellular diversity within mature organisms arose in a hierarchical manner from a primitive progenitor cell, or "stem" cell (Maximow, 2009). James Till and Ernest McCulloch formally proved this theory a half-century later. Their pioneering studies led to the identification of proliferating nodules, termed 'spleen colonies', which arose from bone marrow cells injected into irradiated mice (Becker et al., 1963; McCulloch and Till, 1960; Till and McCulloch, 1961). Further work demonstrated that the resultant number of colonies was directly proportional to the number of cells injected. This led to the hypothesis that such nodules were clonal, arising from stem cells within the marrow (Siminovitch, 1963). The new discipline of Stem Cell Biology grew from their initial endeavours (Siminovitch, 1963; Till et al., 1964).

Stem cells are now functionally defined according to their properties of self-renewal and potency. Developmentally, totipotent stem cells are at the apex of the stem cell hierarchy and are able to contribute to all cells of the developing blastocyst, both embryonic and extraembryonic. Pluripotent cells are able to contribute to all cells of the embryo proper, but do not contribute to placental derivatives. Multipotent cells are able to contribute to multiple tissue lineages but are more restricted than either of the former types. Recent approaches have attempted to classify stem cells, and hence, "stemness" according to the molecular repertoire of expressed genes (Ivanova et al., 2002; Ramalho-Santos et al., 2002), active signal transduction pathways (Armstrong et al., 2006; Brandenberger et al., 2004; Chen et al., 2008; Okita and Yamanaka, 2006; Vallier et al., 2009), cell cycle dynamics (Burdon et al., 2002; Neganova and Lako, 2008; White et al., 2005), genomic organization (Chambeyron and Bickmore, 2004; Ching et al., 2005; John et al., 2009; Luciani et al., 2006;

Zimber et al., 2004), or epigenetic state (Bernstein et al., 2006; Jenuwein, 2006; Jenuwein and Allis, 2001; Ringrose and Paro, 2007; Teitell and Mikkola, 2006).

Although much progress has been made in uncovering the basic features of particular stem cell types, perhaps current research has captured a partial picture--that of the stem cell's 'preferred state'. This existence is maintained by stringently regulated mechanisms that promote its stability (Efroni et al., 2008; LaCava et al., 2005; Szutorisz et al., 2006; Wyers et al., 2005) and is passed on to its cellular progeny in the absence of factors that would otherwise perturb it. Likewise, stem cells may represent a lasting steady state of gene expression halted in its differentiation pathway (Mikkers and Frisen, 2005). Consequently, it is conceivable that stemness is the quintessential state of dynamic equilibrium in which there is no excess, nor any lack. These cells possess rigorous control mechanisms which bring the cell back to its preferred state should transient fluctuations in gene expression occur. Imbalances in this dynamically regulated condition may lead to cell fate transitions or result in the diseased state if self-correcting mechanisms are not properly engaged.

1.1 Stem Cells

Stem cells are characterized by their unique ability to make identical copies of themselves (self-renew) and to become the highly specialized cell types within the adult organism (differentiate). Because of these properties they have tremendous potential to repair damaged tissue, or for use as a model system in drug development and basic discovery. More precise knowledge of the molecular mechanisms that regulate early developmental fate decisions is required to further their clinical use.

1.1.1 Embryonic, Adult, Induced, and Other Pluripotent Stem Cells

Stem cells have traditionally been divided into two categories: embryonic and adult. Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the developing blastocyst and are perpetually self-renewing and pluripotent. The pluripotent, primitive state of ESCs is partly maintained by the unique packaging of DNA in an open chromatin structure. Euchromatin is reinforced by the occurrence of tandem epigenetic marks (bivalent domains) of Polycomb Group (PcG) and Trithorax Group (TrxG) proteins. The PcG epigenetic modification (H3K27me3) maintains repression of genes required for differentiation and the coincident TrxG mark (H3K4me3) keeps these loci in a poised state. Pluripotency is contingent upon these appositional forces. A triad of transcriptional regulators, *Oct4*, *Sox2*, and *Nanog*, whose protein products bind to regulatory regions of the genome, also bound by PcG and TrxG proteins (Boyer et al., 2005; Boyer et al., 2006; Lee et al., 2006b; Loh et al., 2006), are required to maintain this state.

Adult stem cells reside in specialized cellular niches within the mature organism and are more limited in their differentiation potential. Like ESCs, adult stem cells contain bivalent epigenetic marks of gene activation and repression at genetic loci that may be expressed in their downstream progeny (Mikkelsen et al., 2007). Bivalent domains, however, do not mark genomic regions containing genes of alternate lineages. Instead, the transcriptional permissiveness of these domains has been resolved and they are marked by epigenetic modifications of gene silencing (H3K27me3, H3K9me3, and DNA methylation) (Simon and Kingston, 2009). This results in the dense packaging of these loci in heterochromatic regions of the genome, which is generally thought to block access of the transcriptional machinery.

A third category of stem cells, induced pluripotent stem cells (iPS), has recently been described (Takahashi and Yamanaka, 2006). During this process terminally differentiated somatic cells may be reprogrammed to an 'ES'-like state. Induced pluripotency requires the erasure and resetting of existing epigenetic marks to produce a cell that is marked by bivalent domains at developmentally regulated loci. This produces a primitive cell with endless options for its future fate.

While induced pluripotency makes possible the manufacture of 'customized stem cells' for therapeutic application, this also raises many questions. First, does reprogramming occur in the adult organism? If so, this would challenge our current mechanistic understanding of cancer etiology. Cancer can arise from aberrant differentiation of stem cells. Likewise, it also can result from the epigenetic reprogramming of a terminally differentiated cell. Although the outcome would be the same, effective therapeutic intervention used to treat each case might be entirely different. Second, what are the mechanisms by which reprogramming occurs? The relative infrequency and slow kinetics which have been described for this process (Li and Ding, 2010) underscores our partial knowledge about cell fate transitions. Finally, responsible use of these cells and elimination of medical complications in their therapeutic application (Amariglio et al., 2009) requires deeper understanding of the basic mechanisms by which these cells are generated. This is a critical issue since there are many similarities between iPSCs and cancer including involvement of key regulatory pathways (Trp53, c-Myc) and the generation of somatic mutations (Gore et al., 2011).

It has been demonstrated that protein-level expression of four transcription factors, *Oct4*, *Sox2*, *c-Myc*, and *Klf4*, are required for induced pluripotency in adult mice fibroblast cells (Takahashi and Yamanaka, 2006). Subsequent work revealed that different factors were

needed in various cell types (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007; Yu et al., 2007). This suggests that there may be differential requirements for the assembly of various activator and repressor complexes to achieve reprogramming from distinct chromatin states. It also intimates that full reprogramming might be compromised when required factors are unavailable.

The necessity or sufficiency of Oct4 (Kim et al., 2009a; Kim et al., 2009b) to induce pluripotency or to direct transdifferentiation (Szabo et al., 2010) provides strong evidence for the gene's role as a master transcriptional regulator. Sustained Nanog expression, however, is required for full reprogramming (Silva et al., 2009). Its overexpression also accelerates the kinetics of iPS generation (Hanna et al., 2009). This underscores the notion that factors which induced the stable and continued expression of Oct4, Nanog, and Sox2 would facilitate the generation of an ES-like state. The naïve chromatin state promoted by these "pluripotency" regulators might then be directed to its ultimate fate by the action of extrinsic factors.

The hypothesis that a hierarchy of chromatin states exists, even amongst pluripotent stem cells, is corroborated by the derivation of multiple types of pluripotent stem cells from both later staged embryos and adult organs. Their derivation depends on the expression of Oct4, Sox2, and Nanog. Such pluripotent stem cells include epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), embryonal carcinoma stem cells (McBurney, 1993), embryonic germ cells (Matsui et al., 1992), as well as the derivation of ES like cells from neonatal and adult testes (Guan et al., 2006; Kanatsu-Shinohara et al., 2004). Human ESCs are more similar to later stage EpiSCs based upon their growth factor requirements, gene expression profiles, morphology, and their ability to contribute to the formation of a chimeric mouse

(Rossant, 2008). Nevertheless, mESCs appear to represent the most primitive and restricted pluripotent cell type characterized to date.

1.1.2 Developmental Origins of Embryonic Stem Cells

Mammalian life begins with the union of haploid sperm and egg to form a totipotent zygote. This singular totipotent cell must then undergo successive rounds of proliferation and differentiation to replicate the body plan of the adult organism. Groundbreaking studies in developmental biology revealed that this is facilitated by the concerted activities of classic homeodomain, (Hox, Ubx, and Pax) and caudal (Cdx) homeodomain transcription factors, which regulate gene expression along the major body axes (Deschamps et al., 1999; MacDonald and Struhl, 1986; Mlodzik and Gehring, 1987; van der Hoeven et al., 1996). The cellular fate of particular regions along the axes may undergo homeotic transformation when mutations or dysregulated expression of these genes occur (Carrasco and Lopez, 1994). Such effects, however, are limited in a spatio-temporal fashion since transforming mutations occurred only if the cell had not undergone determination (Slack, 1983). Upon determination, future differentiation to the indicated fate would occur no matter the extracellular environment.

Guided initially by the expression of maternal effect genes (Eppig, 1996; Sorenson and Wassarman, 1976), embryonic genome activation does not occur until after the first cell division (Flach et al., 1982). Thereafter, a series of rapid, asynchronous cell divisions results in the first embryonic lineage specification that gives rise to two distinct populations of cells that form the blastocyst. The outer cells of the blastocyst are the trophoblast, called trophectoderm (TE). The inner cells form the epiblast, or inner cell mass (ICM). Generation

of each cell type is dependent upon the antagonistic effects of homeodomain transcription factors Cdx2 and Oct4. Knockout of either gene causes homeotic transformation and early embryonic lethality (Nichols et al., 1998; Niwa et al., 2000; Niwa et al., 2005). TE, which expresses Cdx2, forms the cells that comprise extraembryonic tissues required for placental and embryonic development (Ralston and Rossant, 2005; Rossant, 2007). The epiblast retains Oct4 expression and pluripotency, giving rise to all cells of the embryo proper. ESCs are derived from the epiblast (Figure 1).

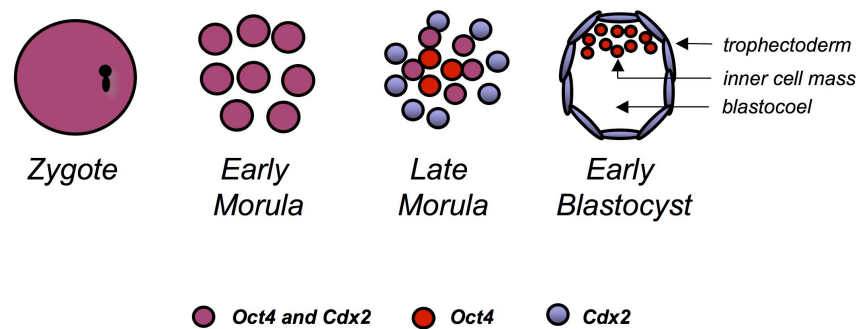


Figure 1. Developmental Origins of Embryonic Stem Cells. Embryonic development begins with the union of sperm and egg to form a totipotent zygote. At this stage, parental pronuclei (shown in black) remain physically separated until the first mitotic division (Donahue, 1972; Howlett and Bolton, 1985; Mayer et al., 2000). These early mitotic divisions occur within the oviduct, prior to implantation. Subsequently, these cells proliferate and undergo specification and commitment to trophoblast and epiblast lineages. This process depends upon the respective expression of *Cdx2* and *Oct4*. Notably, although initial formation of these cells is based upon antagonism, cooperation between epiblast (inner cell mass) and trophoblast (trophoblast) is required to maintain the integrity of each (Nichols et al., 1998; Strumpf et al., 2005). Embryonic stem cells are derived from the cells of the pluripotent inner cell mass at murine embryonic day 3.5 (E3.5). The epiblast then undergoes gastrulation to form the cell types comprising all three germ layers: mesoderm, ectoderm, and endoderm.

1.1.3 ES Genetic Regulatory Networks

Early embryonic development is attributed to the combined interactions of High Mobility Group (HMG) domain proteins and homeodomain transcription factors. HMG domain proteins comprise a large family of transcriptional regulators that include the classical HMG proteins such as *Hmga*, *Hmgb*, *Hmgn*, and *Tcf/Lef*, as well the *Sox* HMG domain proteins. The homeodomain family consists of the classic homeodomain proteins as well as the POU (Pit, Oct, Unc) homeodomain domain proteins. While both families are able to bind to DNA in a sequence-specific fashion, the ability of homeodomain transcription factors to recognize their cognate sequence is enhanced by interactions with partner HMG proteins. This cooperativity, coupled to the availability of specific family members in specific cellular contexts, dictates a combinatorial code in which competition for DNA binding sites, recruitment of various cofactors, and subsequent activation or repression of gene expression mediates cell fate decisions in early embryogenesis (Dailey, 2001). Extensive research reveals that maintenance of the self-renewing, pluripotent state is a direct reflection of the association between HMG domain protein Sox2 and homeodomain transcription factors Oct4 and Nanog.

Transcription factor *Sox2* (SRY HMG Box2) exists as a maternally expressed transcript whose expression is required for successful navigation of the first three embryonic lineages. Together with dimerization partner Oct4 (Remenyi et al., 2003), it maintains self-renewal of pluripotent cells (Avilion et al., 2003). *Sox2* knockout mice are embryonic lethal, at a developmentally later stage than *Oct4* knockouts (Nichols et al., 1998). This later lethality suggests that *Sox2* function in early embryonic development is dependent upon Oct4. Oct4, however, may possess *Sox2* independent functions that maintain viability even in the

absence of Sox2 expression. Recent studies have shown that Sox2 is not required for activation of Oct/Sox enhancer elements. It is required, however, for the sustained repression of Oct4 trophoblast targets (Masui et al., 2007) since its knockdown in established ES lines results in their differentiation to the trophoblast lineage (Li et al., 2007; Masui et al., 2007).

Sox2, by contrast, can play a supportive role in the generation of neural stem cells (NSCs). *Sox2* is highly expressed in neural progenitors and in the central nervous system (Ferri et al., 2004; Graham et al., 2003). Although it appears that *Sox2* expression is not essential for self-renewal or multipotency of NSCs (Miyagi et al., 2008), its function in this respect may not be apparent due to functional redundancy with other Sox factors (Miyagi et al., 2009; Zhao, 2004). Decreased proliferation and differentiation occurs upon conditional ablation of *Sox2* in retinal neural progenitors, where it is the sole Sox factor expressed (Taranova et al., 2006). A modest reduction in *Sox2* protein, however, results only in abnormal differentiation. This suggests that dosage effects of *Sox2* are critical to both maintain self-renewal and to modulate differentiation.

Homeodomain transcription factor *Nanog* was first identified by two laboratories exploring factors required for maintaining mESC pluripotency (Chambers et al., 2003; Mitsui et al., 2003). Both groups highlighted the function of *Nanog* overexpression to promote *Lif* independent self-renewal, which is normally required to promote the stem cell state by activation of Jak-Stat signaling (Matsuda et al., 1999; Niwa et al., 1998; Takeda et al., 1997). They discovered that *Nanog* mRNA is expressed in the inner cells of the compacted morula and in the inner cell mass. After implantation, however, its expression is downregulated. *Nanog* is also expressed in primordial germ cells (Chambers et al., 2003). *Nanog* knockout mice die at E5.5. These mice display ectopic expression of extraembryonic

endoderm lineage markers such as *Gata4* and *Gata6* (Mitsui et al., 2003). *Nanog* expression is not sufficient to maintain the pluripotent state in the absence of *Oct4*.

Pivotal high-throughput analyses have determined *Oct4* transcriptional targets in mESC and hESC. These efforts have revealed an unanticipated collaboration among *Oct4*, *Sox2*, and *Nanog* and have identified each as master regulators in a transcriptional hierarchy that regulates stem cell identity (Boyer et al., 2005; Loh et al., 2006; Vasiljeva et al., 2008). *Oct4*, *Sox2*, and *Nanog* colocalize in many regions of the genome in this network. Additional studies suggest that this central triad is integrated into an interconnected transcriptional network that controls the expression of downstream target genes through distinct molecular pathways (Ivanova et al., 2006; Matoba et al., 2006; Walker et al., 2007). This network regulates the activation of genes required for pluripotency and the concomitant repression of genes that are required for lineage commitment. This work reveals that composite binding is able to elicit either transcriptional activation or repression. However, the molecular mechanisms by which these differential effects occur are not yet clearly understood.

The function of developmentally regulated non-coding RNAs (ncRNAs), including microRNA (miRNA) (Bar et al., 2008; Marson et al., 2008) and large intergenic non-coding RNA (lincRNA) (Guttman et al., 2009) have recently been incorporated into the growing model of pluripotent stem cell function. ncRNAs form a transcriptional regulatory feedback loop with *Oct4*, *Sox2*, and *Nanog* (Sheik Mohamed et al., 2010; Tay et al., 2008a; Tay et al., 2008b; Xu et al., 2009). Recent evidence likewise suggests that *Oct4* and ncRNAs may play a critical role in the recruitment of PcG complexes to maintain gene repression in the pluripotent state. This includes the colocalization of ncRNA with *Oct4* and PcG, the

downregulation of ncRNA upon repression of Oct4, the association of ncRNAs with PcG proteins, and loss of PcG mediated repression upon knockdown of ncRNAs (Khalil et al., 2009).

1.2 Transcriptional Regulation

The emergence of lineage specific transcriptional programs is guided by the expression of master developmental control genes. These, in turn, are regulated by cascading signal transduction pathways, which impact their ability to form protein-protein (PPI) and protein-DNA (PDI) interactions. Although the interaction of these transcriptional complexes on chromatin is transient in steady state, this dynamism succumbs to the precise spatio-temporal assembly of complex supra-macromolecular assemblies upon differentiation. Then, localized changes in chromatin structure may facilitate changes in gene expression. Beyond this, however, three major questions remain. First, what is the signal that dictates this change in steady state gene expression and facilitates cell fate transitions? Second, how does the cell sense and transduce this signal? Third, what are the implications to chromatin structure and locus-specific regulation of gene expression? A thorough understanding of the molecular machinery that informs these decisions is necessary to address these questions.

1.2.1 The Transcriptional Machinery

The emergence of transcriptional programs required for lineage specific differentiation is dependent upon the production of protein encoding genes and non-coding RNAs by RNA Polymerase II (RnapII). RnapII mediated transcription can be divided into three distinct phases: initiation, elongation, and termination. Each phase is associated with a coordinate repertoire of regulators that are required for faithful induction of gene expression in response to extracellular cues.

RnapII initiation at core promoter DNA elements occurs in collaboration with the basal transcriptional machinery, which includes the general transcription factors (GTFs) TFIIA, B, D, E, F, and H. Together they comprise the preinitiation complex (PIC). The composition of TFIID is a prime example of the complexity of GTF complexes. This complex consists of the TATA binding protein (Tbp), Tbp associated factors (TAFs), as well as protein subunits containing chromatin remodeling and histone modifying activities (Davidson, 2003; Timmers and Tora, 2005). DNA binding transcription factors (TFs) regulate the differential assembly of these multi-subunit protein complexes on DNA. TFs likewise regulate their association with RnapII in a cell-specific fashion (Saunders et al., 2006). Finally, RnapII mediated transcription is further regulated by the Mediator Complex. This multi-protein assembly integrates input from co-activators and co-repressors to effect the recruitment and function of the PIC (Malik and Roeder, 2005).

Local destabilization of the DNA duplex prompts formation of an open DNA complex following RnapII recruitment (Tchernachenko et al., 2008). Several rounds of abortive transcript synthesis may occur from this open complex. However, several factors including retention of GTFs in the PIC at the promoter (Pokholok et al., 2002), the presence of

negative elongation factors DSIF and NELF (Wada et al., 1998; Yamaguchi et al., 1999), and contact with the nucleosome (Isben and Luse, 1992), stall RnapII at the promoter.

The transition from initiation to a productive elongation complex is regulated primarily by phosphorylation of its constituent members (Kobor and Greenblatt, 2002). The sequential phosphorylation of RnapII C-terminal domain (CTD) at serine 5 and serine 2 facilitates its release from the promoter and subsequent elongation into the coding region. Cdk7 contained in the TFIIF complex phosphorylates CTD at Serine 5. This prompts RnapII release from GTFs and the Mediator complex (Sogaard and Svejstrup, 2007; Yudovsky et al., 2000) and aids promoter clearance. Still, the transcriptional complex remains stalled at the 5' coding region of the gene in the absence of CTD Serine 2 phosphorylation. The Cdk9 subunit of P-Tefb catalyzes the phosphorylation of CTD Serine 2, DSIF, and NELF and allows elongation through the entire coding region and full transcriptional activation (Price, 2008). Multiple levels of phosphorylation may thus be required for the formation of a productive elongation complex. As well, regulation of P-Tefb recruitment to the RnapII holoenzyme is dependent upon PI3K/Akt signaling to release the Cdk9 subunit of P-Tefb from its repression complex (Contreras et al., 2007; Peterlin and Price, 2006).

The continued processivity of RnapII through chromatin is dependent upon complexes that coordinately regulate nucleosomal positioning. RnapII stalling, or transcriptional termination, may occur if chromatin structure is not dynamic. Chromatin is remodelled into transcriptionally permissive or repressive conformations by complexes that covalently modify histones, act in an ATP-dependent manner to reposition nucleosomes along DNA, facilitate histone exchange, or coordinate the disassembly and reassembly of the nucleosome. Several protein complexes perform this function. They include SWI/SNF,

ISWI, INO80, and M1-2/CHD FACT, and other nucleosome assembly proteins and histone chaperones (Armstrong, 2007; Belotserkovskaya and Reinberg, 2004; Cairns, 2005; Park and Luger, 2006), as well as members of the Trithorax (TrxG) and Polycomb (PcG) group of epigenetic regulators (Ringrose and Paro, 2004).

Recent studies in *Saccharomyces* suggest that two transcriptional termination pathways exist. CTD phosphorylation status and the type of transcript produced determines which pathway is selected. The first--the Torpedo model--depends upon CTD Ser2 phosphorylation to trigger transcriptional termination. This modification prompts 3' processing of the nascent transcript by polyadenylation and facilitates association of the nascent transcript with Rat1 (Xrn2). The nascent transcript is then degraded by this 5'-3' exoribonuclease which promotes release from RnapII and termination (West et al., 2004). An alternate to the Torpedo model implicates co-transcriptional cleavage and 3' to 5' processing by Rat1 and the exosome (Tollervey, 2004; West et al., 2004).

In the second pathway, termination of small nucleolar RNAs (SnoRNAs) and cryptic unstable transcripts (CUTs) is coupled to exosome mediated processing. Nrd1 is recruited to CTD, which is phosphorylated at Ser5, although it may also associate with CTD doubly phosphorylated at Ser2 and Ser5 (Vasiljeva et al., 2008). When Ser2 is dephosphorylated, however, Nrd1 mediates transcriptional termination and recruitment of the exosome (Gudipati et al., 2008). The exosome then acts to efficiently degrade produced transcripts unless they are otherwise protected. RNA binding proteins block exosome mediated degradation of SnoRNAs (Yang et al., 2005). By contrast, the absence of polyadenylation in CUTs prevents their exosome-mediated degradation (LaCava et al., 2005; Wyers et al., 2005). Recent evidence suggests that transcripts produced from CUTs also have functional

relevance in transcriptional regulation; CUT produced ncRNAs are able to modulate activity from their own promoter (Camblong et al., 2007).

The transition from initiation to productive elongation and the termination of RnapII mediated transcription is controlled by the phosphorylation-dependent modulation of RnapII function. CTD phosphorylation at Serine 2 in particular emerges as a key regulatory node in both processes. The role of Akt signaling in maintaining this mark suggests that decreased Akt activation may effect changes in RnapII mediated transcription. Likewise, if dynamic chromatin structure is not maintained, RnapII function is compromised. Akt signaling likely plays a role in this process.

1.2.2 Epigenetic Regulation of Gene Expression

The association of DNA with core histone proteins (two each of H2A, H2B, H3, and H4) forms the basic nucleosomal structure of chromatin in eukaryotes. A further layer of complexity to this ‘beads on a string’ model is provided by the higher order packaging of nucleosomal arrays into regions of euchromatin (open chromatin structure) and heterochromatin (compacted chromatin structure). This higher level of DNA organization has traditionally been perceived as a barrier to the basic cellular processes of DNA replication, transcription, and repair. The histone code hypothesis (Jenuwein and Allis, 2001) proposes that specific epigenetic modifications function to spatially organize regions of the genome. This allows rapid and efficient access to these regions during cell fate transitions. The regulation of this code is dependent upon the concerted action of Polycomb (PcG) and Trithorax (TrxG) group proteins.

PcG and TrxG group proteins are developmental-dynamic multi-protein complexes which play opposing roles in the regulation of gene expression. Their antagonistic effects upon Hox genes, for example, results in the modulation of segmentation genes that are required for early patterning events in *Drosophila* (Hueber and Lohmann, 2008). The effects of TrxG and PcG are mediated by their ability to facilitate post-translational modifications to histones, which impacts higher order chromatin structure. While TrxG recruitment may result in gene activation via tri-methylation of Histone 3 at lysine 4 (H3K4me3), PcG recruitment results in gene repression or silencing via deposition of H3K27me3 and H3K9me3. It is now believed that TrxG mediated gene expression is the default unless it is antagonized by PcG (Simon and Kingston, 2009).

PcG complexes can be divided into two separate classes: Polycomb Repressor Complex 2 (PRC2) and Polycomb Repressor Complex 1 (PRC1). Analysis of PcG protein complexes reveals that the core components and function of each are highly conserved across species (Ketel et al., 2005; Levine et al., 2002; Pasini et al., 2004). Multiple PRC2 and PRC1 complexes exist. The composition of each complex is modulated throughout development to perform specific functions (Kuzmichev et al., 2004; Kuzmichev et al., 2005; Otte and Kwaks, 2003; Plath et al., 2004). PRC2 members Ezh2, Suz12, and Eed function to initiate gene repression by placement of the H3K27me3 modification (Kuzmichev et al., 2004; Pasini et al., 2004; Vire et al., 2006). PRC1 functions to maintain the repressed state by using the H3K27me3 mark as a docking site which is read by Polycomb (Czermin et al., 2002). This results in the recruitment of H3K9me3 and DNA methyltransferase containing complexes to further promote a heritable chromatin structure termed cellular memory. An

overview of mammalian PcG PRC1 and PRC2 members and their respective functions can be found in Table 1.

Table 1. Polycomb Group Proteins

Protein	Drosophila Orthologue	Complex	Consensus Motif	Known Modifications Catalyzed	Function
Eed1-4	ESC; Extra sex combs	PRC2			Required for Ezh2 HMTase activity Alternate splice forms are differentially regulated upon differentiation. Interacts with Hdac, Hdac2, Histone H1, YY1 possible interaction with Itga4, Itgae, and Itgb7.
Ezh1	E(z) Enhancer of Zeste	PRC2			Expressed in adult and non-dividing cells.
Ezh2	E(z) Enhancer of Zeste	PRC2		H1K26me; H3K27me3; H3K9	Expressed in early embryonic development and in proliferating cells. Implicated in transcriptional repression of lineage regulatory genes in mESC (repressive mark of bivalent domain) Expression peaks at G1/S Interacts with Atrx, Sirt1, Hdac1, Hdac2, Dnmt1, 3a, and 3b
Suz12	Su(z)12 Suppressor of Zeste 12	PRC2		H3K27me3	Implicated in transcriptional repression of lineage regulatory genes in mESC Expression peaks at G1/S Interacts with Ezh2, Wdr77, and Histone H1
Ehmt2	G9a	PRC1		H3K27me3; H3K9me3; H3K9me1	Member of E2F6 complex expressed in G0 which includes Mga, Max, Tfdp1, Cbx3, Ehmt2, Euhmtase1, Ring1a, Ring1b, Pcgf6, L3mbtl2, and Yaf2.
Bmi1; Pcgf4	Psc; Posterior sex combs	PRC1			Member of PRC11 E3 ubiquitin ligase complex composed of Phc2, Bmi1, Ring1a, and Ring1b; required for E3 ubiquitin ligase activity of Ring2. Interacts with Pcgf2, Cbx2, Cbx4, Cbx8, Phc1-3, and Scmh1 and E4f1. Also member of Cul3 ubiquitin ligase complex required during mitotic division.
Cbx1	Hp1beta	PRC1			Chromodomain protein interacting with H3K9me; chromoshadow domain facilitates interaction with chromatin-associated non-histone proteins. Interacts with Suv39h1, Setdb1, Suv420h1-2. Not associated with mitotic chromocomes.
Cbx2	Pc; Polycomb	PRC1			Involved in chromatin compaction
Cbx4	Pc2	PRC1			SUMO E3 Ligase; Interacts with H3K9me3, Suv39h1, and Hipk2. Phosphorylated by Hipk2 upon DNA damage which promotes SUMOylation at K492 and enhances SUMO E3 ligase activity. Overexpression of Cbx4 leads to

					block at G2.
Cbx6		PRC1			Involved in chromatin compaction
Cbx7		PRC1			Interacts with Ring1a but not Bmi1, Eed, or Ezh2. Involved in repression of CDKN2A and regulation of cellular lifespan.
Cbx8		PRC1			Member of PRC1 class II complex composed of Pcgf2, Bmi1, Cbx2, Cbx4, Phc1-3, Schm1, Ring1a and Ring1b. Interacts with Mllt3.
L3mbtl2	CG16975				Member of the E2F6 complex observed at G0 composed of E2f6, Mga, Mac, Tfdp1, Cbx3, Ehmt2, Euhmtase1, Ring1a, Ring1b, Pcgf6, and Yaf2
Pcgf2; Rnf110	Psc	PRC1	GACTNGACT		Transcriptional repressor. Inhibits self-renewal of hematopoietic stem cells. Knockout studies indicate that Pcgf2 plays a role in negative regulation of chemokines, cytokines, and chemokine receptors in immune function.
Pcgf6; Rnf134		PRC1			Transcriptional repressor that may modulate levels of H3K4me3 by activation of Jarid1d histone demethylase. Phosphorylated during mitosis at S30 by Cdk7 the consequences of which are currently unknown.
Phc1	Ph; Polyhomeotic	PRC1			Member of PRC1 class II complex composed of Pcgf2, Bmi1, Cbx2, Cbx4, Phc1-3, Schm1, Ring1a and Ring1b. Also member of PRC1 E3 ubiquitin ligase complex composed of Phc2, Bmi1, Ring1a, and Ring1b
Phc2	Ph-p	PRC1			Member of PRC1 E3 ubiquitin ligase complex composed of Phc2, Bmi1, Ring1a, and Ring1b
Phc3		PRC1			Phosphorylated upon DNA damage by Atr or Atm.
Ring1; Ring1a	Ring	PRC1		H2AK119Ub	Member of RING finger family containing a RING (zinc finger DNA binding) domain. Associates with Bmi1, Eed1, Cbx4 in large nuclear domains. Ubiquitin E3 Ligase which may modulate Ring1b activity
Rnf2; Ring1b		PRC1		H2AK119Ub	Member of PRC1 E3 ubiquitin ligase complex composed of Phc2, Bmi1, Ring1a, and Ring1b. Contains ligase activity. Implicated in specification of anterior-posterior axis and cellular proliferation. Interacts with Hip2.
Scmh1	Scm; Sex combs on midleg	PRC1 PRC1			Interacts with Phc1 via sterile alpha motifs (SAM) contained in both proteins.
Setdb1	CG30426	PRC1		H3K9me2 H3K9me3	Interacts with Mbd1 and Akt. Recruited to chromatin via interaction with Caf-1 and targeted to histone H3 by Trim28 Facilitates trimethylation of H3K9me2 at S phase to silence

					euchromatic gene during replication-coupled chromatin assembly. Member of the Suvar3-9 family. Plays a role in the silencing of euchromatic genes. Interacts with MBD1 but only when MBD1 is not SUMOylated. Also interacts with Cbx1, Cbx5, Dnmt3a, Hdac1, Hdac2, Sin3a, Sin3b, and SUMO2.
Suv420h1		PRC1		H4K20	Targeted to histone H4 via interaction with Rb1 family members and functions in transcriptional repression and the establishment of constitutive heterochromatin. Interacts with Cbx1, 3, and 5, Rb1, Rb11, and Rb12.
Suv39h1		PRC1		H3K9me3	Plays a role in the establishment of constitutive heterochromatin at pericentric and telomeric regions. Targeted to histone H3 via interaction with Rb1 and may regulate switch for cell cycle exit and terminal differentiation in myogenic differentiation. Accumulates at centromeres during mitotic prometaphase but dissociates while in transition to meta- to anaphase.
Suv39h2		PRC1		H3K9me3	Associates with centromeric heterochromatin. Interacts with Smad5. Also interacts with Rb1 and is implicated in cell cycle regulation, transcriptional repression, and regulation of telomere length.
YY1	Pho;Pleiohomeotic	PRC1	CGCCATNTT		Transcriptional factor that is member of GLI-Kruppel class of zinc finger proteins. May act as activator or repressor of transcription.
YY2		PRC1	CGCCATNTT		Transcriptional activator or repressor that may antagonize function of YY1.

Cellular memory modules (CMMs) are regions of the genome that contain both PcG and TrxG response elements (PREs and TREs). The co-localization of PcG and TrxG proteins within *Drosophila* CMMs (Ringrose and Paro, 2004) indicates that these complexes may function in a concerted fashion to regulate gene expression in a cell-specific, developmental-specific context. The role of these proteins in mammalian cells is currently being clarified and the accumulated evidence suggests that they function in an analogous manner to their *Drosophila* homologues. However, due to the presence of multiple orthologues, the functional characterization of PcG and TrxG proteins in mammalian cells is far more complex. Finally, the relatively recent identification of histone-lysine demethylases, which remove the epigenetic modifications placed by PcG and TrxG proteins (Pedersen and Helin, 2010), suggests the existence of yet additional layers of transcriptional regulation for genes contained within CMMs.

An open structure (euchromatin) is observed in ESCs (Rasmussen, 2003). Differentiated cells, by contrast, contain opposing regions of euchromatin and heterochromatin, with silenced genes residing in heterochromatic regions of the genome (Rasmussen, 2003). The protruding tails of Histone H3, within the promoters of active genes in pluripotent cells, are post-translationally modified by acetylation (H3K9ac) and tri-methylation (H3K4me3). These marks delineate euchromatic regions. However, developmentally repressed loci are characterized by the presence of bivalent domains within the promoters of lineage restricted loci in pluripotent cells. These loci contain simultaneous marks of TrxG mediated H3K4me3 activation and PcG mediated H3K27me3 repression (Azuara et al., 2006; Bernstein et al., 2006). The expression of these loci is held in check even as they are poised for transcription.

RnapII remains stalled at the promoter of bivalent domains. Genes within these domains are also bound by Oct4 and PcG (Boyer et al., 2006; Guenther et al., 2007; Lee et al., 2006b; Squazzo et al., 2006). Their derepression, however, occurs upon knockdown of Oct4 or PcG (Erhardt et al., 2003; Ivanova et al., 2006; Matoba et al., 2006). Taken together, these data suggest that the ability to block transcription in a locus-specific manner is critically regulated post-RnapII initiation at the level of the nucleosome. However, the mechanisms by which Oct4 and PcG retain this blockage at developmentally regulated loci and the signals that trigger its resolution during cell fate transitions remain unknown.

The mechanism of PcG recruitment to target loci remains an area of active study in the field of chromatin biology. Putative PREs, which span several hundred bases have been identified in *Drosophila* through extensive analysis of the cis regulatory elements bound by PcG (Decoville et al., 2001; Dejardin, 2005; Muller and Kassisi, 2006; Ringrose and Paro, 2007). A bioinformatics approach correctly identified PREs of the BX-C by using known consensus binding sites for Gaf, Zeste, and Pho. This led to the prediction of 167 putative PREs genome-wide (Ringrose et al., 2003). The approach, however, did not predict 94% of the PcG bound loci identified by ChIP (Ringrose and Paro, 2007). This disparity may have been caused by analytical stringency, potential looping of PcG complexes from the PRE to a site in trans, detection of transient interactions with weak PREs, or usage of alternate sequence specific binding proteins other than Gaf, Zeste, or Pho. A consensus binding site (G(A) motif; GAAAA) for *DSP1* (Human *Hmgb2* homologue) was identified (Dejardin, 2005) in a search for other sequence specific binders. DSP1 may function in PcG recruitment and in TrxG activation (Decoville et al., 2001; Rappailles et al., 2005; Salvaing et al., 2006). Refinement of PRE prediction algorithms, to include the G(A) motif, improved prediction

power (Fiedler and Rehmsmeier, 2006). This suggests that the G(A) motif may be instrumental in defining mammalian PREs. The association of DSP1 with both PcG and TrxG, moreover, may provide insight into the switching mechanism where PREs are converted to TREs (Bracken et al., 2006).

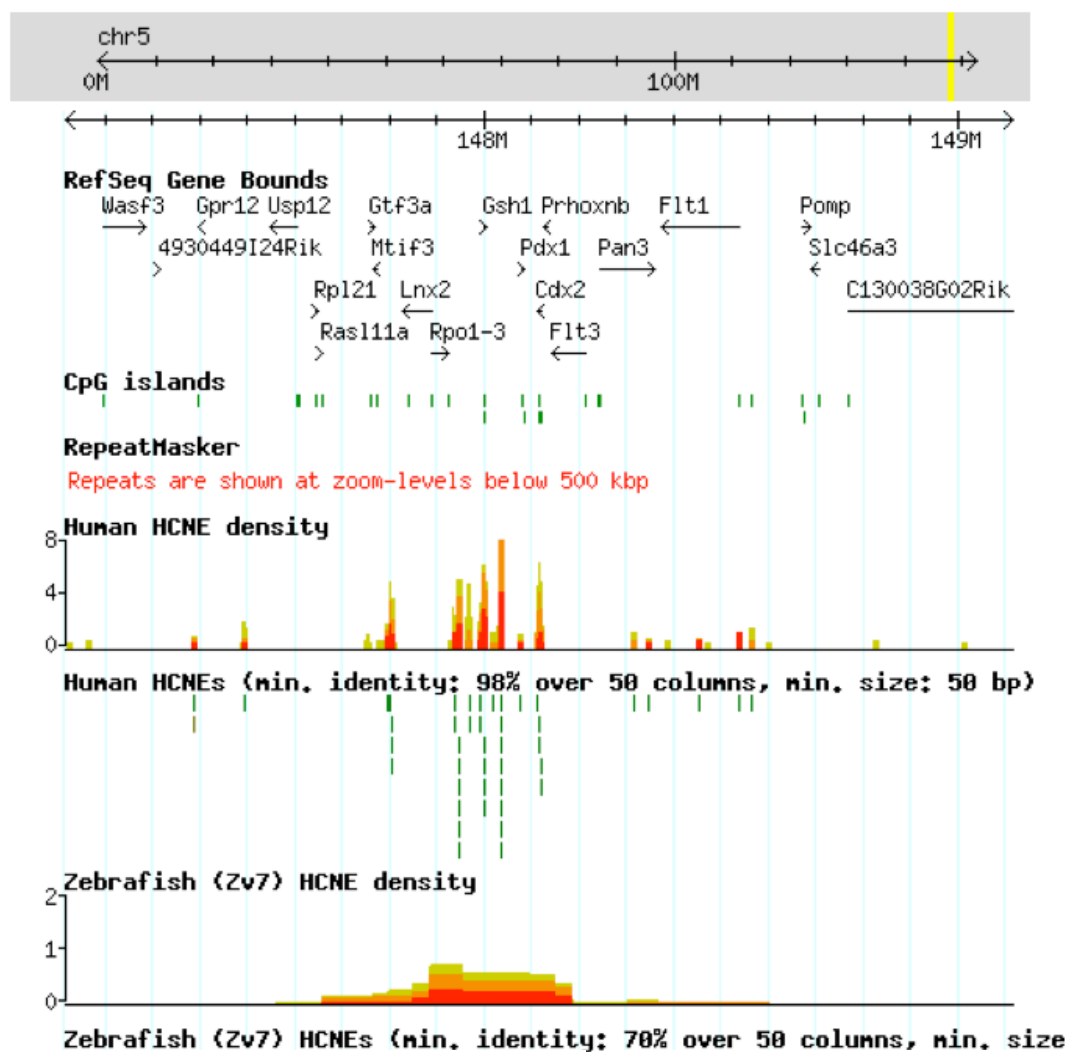
The identification and functional characterization of mammalian PREs and the mechanism of PcG recruitment are subjects of ongoing research. Their study has been hampered by the existence of multiple PcG orthologues as well as the broad distribution of PRC1 and PRC2 members on chromatin (Boyer et al., 2006; Ku et al., 2008; Lee et al., 2006b). Only two publications have identified and characterized *bona fide* PREs (Sing et al., 2009; Woo et al., 2010). Nevertheless, several sequence specific DNA binding candidates such as YY1, Oct4, Sox2, Nanog, and ncRNAs may function as PcG recruiters. Highly conserved non-coding elements (HCNEs), which contain ncRNAs, may also function as mammalian PREs.

Approximately 200 HCNE rich regions, displaying extensive cross-species conservation have been identified (Woolfe et al., 2006). HCNEs are enriched for: all Hox clusters; genes that encode for developmental regulators (Bernstein et al., 2006); miRNA; and lincRNA (Kikuta et al., 2007). Otherwise, these regions are gene-poor. A cursory analysis of DNA sequence within several HCNEs indicates that consensus binding motifs for Oct4 and Sox2 cluster with the G(A) motif recognized by Hmgb2 (Figure 2 and Ancora Database <http://ancora.genereg.net/cgi-bin/gbrowse/mm9/>) (Engstrom et al., 2008). The functional relevance of these sites remains to be established.

HCNEs, however, may not function as PREs for several reasons (Ringrose and Paro, 2007). First, analysis of Suz12 binding shows that only 8% of bound regions overlap with HCNEs. Second, genomic loci most strongly bound by PcG are not highly conserved.

Finally, there is no clear sequence consensus between the HCNEs examined and regions containing the highest levels of H3K27me3 enrichment.

A more thorough analysis using 3C, 4C, 5C or ChIP-loop (Gavrilov et al., 2009) may help to clarify these disparate findings. Experiments using these approaches may reveal that DNA elements are recruited in trans to facilitate chromatin looping and PcG repression (Sigrist and Pirrotta, 1997). This approach has been successful in establishing the role that long range chromatin looping between the promoter, intronic, and terminator regions plays in maintaining repression at the *Brca1* locus (Tan-Wong et al., 2008), which notably is an Oct4 target. Finally, these long-range interactions may not necessarily be facilitated by the formation of PcG complexes at PREs. A recent study performed in *Drosophila* suggests that chromatin looping and higher order (repressive) chromatin structure may be dependent upon the recruitment of chromatin insulator binding proteins (Li et al., 2011).

A***HCNEs within the Cdx2 Locus***

B

Organism	Genome assembly	Location	Size
Mouse	NCBI Build 37 (mm9)	chr5:148,111,299..148,111,417	119 bp
Human	NCBI Build 36 (hg18)	chr13:27,432,991..27,433,109	119 bp

Alignment	<i>POU_s</i>	<i>G(A) motif</i>	<i>Sox</i>
Mouse	TGCAGCGGGACA	ATGCGACTTTGAACCTCTAACCCTAAGCAAACA	ACCCTTGTGCTC
Human	TGCAGCGGGACAATGTCGACTTTGAACCTCTAACCCTAAGCAAACA	ACCCATGTGCTC	

Mouse	TTGCTCCACTGTCCAAACAGAGTTTATTGACAGGCGTTTCACGGCAACGCATAGCAACC		
Human	TTGCTCCACTGTCCAAACAGGGTTTATTGACAGGCGTTTCACGGCAACGCATAGCAACC		

Similarity: 116 / 119 identical columns = 97.48%

Mouse	NCBI Build 37 (mm9)	chr5:148,115,927..148,116,017	91 bp
Human	NCBI Build 36 (hg18)	chr13:27,438,271..27,438,361	91 bp

Alignment	<i>G(A) motif</i>	<i>Oct</i>	<i>Sox</i>
Mouse	GACAAGGCGATCTTATCAAA	GAAGCGCTTTCAATGCGCTTAATAACA	ACCGCATTCTGT
Human	GACAAGGCGATCTTATCAAA	GAAGCGCCTTTCAATGCGCTTAATAACA	ACCACATTCTGT

Mouse	TTGAATTACCACTACTGAGCAAATGGCGGCC		
Human	TTGAATTACCACTACTGAGCAAATGGCGGCC		

Similarity: 89 / 91 identical columns = 97.80%

Mouse	NCBI Build 37 (mm9)	chr5:148,119,055..148,119,109	55 bp
Human	NCBI Build 36 (hg18)	chr13:27,441,638..27,441,691	54 bp

Alignment	
Mouse	GAGCTTCCTCCTTCCAACCTGGGTGCACCTTAACCTCCAATCACAGGTTCAAAGA
Human	GAGCTTCCTCCTTCCAACCT-GGTGCACCTTAACCTCCAATCACAGGTTCAAAGA

Similarity: 54 / 55 identical columns = 98.18%

Figure 2. Identification of Clustered Oct, Sox, and G(A) Motifs within the *Cdx2* Locus. Mining of the Ancora Database (<http://ancora.genereg.net/cgi-bin/gbrowse/mm9/>) reveals the presence of multiple clustered consensus sites for Oct, Sox, and Hmgb2 (DSP1; G(A) motif) within HCNEs at the *Cdx2* locus. The HCNE at 148,111,299 - 148,111,417 is 3' to the coding region, the HCNE at 148,115,927-148,116,017 occurs in intron 1, and the HCNE at 148,119,055-148,119,109 occurs within the promoter, just 75 bases upstream from another clustered Oct/Sox/Hmgb2 element. A cursory analysis also reveals the existence of clustered sites within HCNEs at *Pax7*, *Hoxb1*, *Rest*, *Brca1*, and *Hand1* loci. Functional analyses such as luciferase based reporter systems employing these cis-regulatory elements and analysis of expression following perturbations to Hmgb2 recruitment will be required to establish the functional significance of the G(A) motif in mammalian PcG recruitment and transcriptional regulation.

1.2.3 Transcriptional Regulation and The DNA Damage Response

The mechanisms that regulate the transcriptional machinery are linked to the DNA damage response. First, RnapII stalling and transcriptional arrest are observed at sites of DNA damage (Tornaletti, 2005). Then, following arrest, an ordered progression of modified histones and histone variants emerge at these sites (Heo et al., 2008). This progression is dependent upon histone chaperone activity and chromatin remodeling complexes required for elongation of RnapII through chromatin (de Koning et al., 2007). Moreover, the spatio-temporal assembly of protein factories that are required for DNA repair and cell-cycle progression are recruited to these modified loci before RnapII mediated transcription resumes (Harrison and Haber, 2006; Lisby and Rothstein, 2004). This response is initiated by cell cycle PTM-dependent changes in PcG and TrxG gene function, which impact histone modifications.

Monoubiquitination of histone H2A at lysine 119 (H2AK119ubn) by PRC1 (Kapetanaki et al., 2006) is the earliest epigenetic change observed at sites of DNA damage (Bergink et al., 2006; Celeste et al., 2003). Silencing may be a direct effect of H2AK119ubn or may occur via trans-histone cross-talk with H3K4me3 (Briggs et al., 2002; Fingerhahn et al., 2008; Yang and Seto, 2008). Although H2AK119ubn antagonizes Mll3 directed H3K4me3 and inhibits RnapII initiation, it does not impact H3K27me3 or H3K9me3 enrichment (Cao et al., 2005; Nakagawa et al., 2008; Wang et al., 2004). The removal of the H3K27me3 mark, moreover, is associated with upregulation of DNA repair genes and loss of PcG silencing (Agger et al., 2007) and requires the action of Kdm6a (Utx) or Kdm6b (Jmjd3) (Pedersen and Helin, 2010). Notably, both Kdm6a and Kdm6b have been implicated in the DNA damage response (Cho et al., 2007; Martinelli et al., 2011).

Trans-histone crosstalk suggests that the presence of specific histone modifications may either regulate or reflect a temporal progression of events that are required for cellular based DNA transactions. The G2 DNA Damage checkpoint prior to mitotic division emerges as a critical regulator of PcG function and gene expression in this paradigm. For example, regulation of H2Ak119ubn is cell cycle dependent: it is observed during G1 and S and is required for Hox gene silencing (Cao et al., 2005; Wang et al., 2004). Unless H2Ak119 is deubiquitinated, however, the cell cycle remains blocked at G2 (Joo et al., 2007). Furthermore, extensive analysis by mass spectrometry in HeLa cells reveals that other histone post-translational modifications are regulated during G2/M. Global decreases in H3K27me1-3, H3K36me1-3, increased H4K20me1-2, and exclusive phosphorylation of H4S1, H3S10, H3S28, H3.2T3, and H3.3S31 occur during this phase of the cell cycle (Bonenfant et al., 2007).

The ability of many PcG, TrxG, and SWI/SNF chromatin remodeling complex members to mediate histone modifications is phosphorylation dependent (Niessen et al., 2009). Similarly, Akt-mediated phosphorylation generally results in the displacement of these complexes from the chromatin template (Cha et al., 2005; Foster et al., 2006; Huang and Chen, 2005). Akt signaling apparently promotes the pluripotent state by maintaining a dynamic flux of these complexes. As ES cells undergo differentiation, however, these complexes exhibit decreased mobility (Ren et al., 2008).

An overview of Akt targets implicated in the DNA damage response and transcriptional regulation is provided in Table 2. These combined studies suggest that Akt signaling plays a major role in maintaining self-renewal via inhibition of apoptosis, maintaining dynamic chromatin structure, cell cycle checkpoint abrogation, attenuation of PRC2 and SWI/SNF

function, and inactivation of Gsk3 β . Evaluation of the substrates negatively modulated by Akt indicates that loss of Akt signaling promotes G2/M arrest, apoptosis, and chromatin remodeling. This intimates that decreased Akt signaling during G2/M may be the trigger that facilitates the DNA damage response and, thereby, cell fate transitions. PcG is not, however, maintained on chromatin during G2/M. How the H3K27me3 mark is passed on to cellular progeny after mitotic cell division has yet to be clarified.

Table 2. Akt Phosphorylation Modulates Key Proteins Implicated in Transcriptional Regulation and Cell Cycle Progression.

Protein	Function	Kinase-Residue	Implication	Reference
Chk1	Cell Cycle Checkpoint Regulator	Akt-Ser280 Atr-Ser345	Inhibits Checkpoint Function Cell cycle arrest at G2/M	(King et al., 2004)
Trp53	DNA binding transcription factor; classical tumor suppressor gene	Atr Ser15 Gsk3 β -Ser315	Cytoplasmic localization and proteasomal degradation due to Mdm2 mediated ubiquitination Rapid nuclear localization to Rad51, Mre11, and Blm DNA repair foci Repression of Nanog Expression	(Boehme et al., 2008) (Restle et al., 2005) (Lakin et al., 1999) (Lin et al., 2005b; Watcharasit et al., 2002)
Mdm2	E3 Ubiquitin Ligase	Akt-Ser166 Akt-Ser188 Atr-Ser395	Nuclear localization to facilitate ubiquitination of targets such as Trp53 Loss of nuclear function	(Feng et al., 2004; Ogawara et al., 2002; Zhou et al., 2001) (Maya et al., 2001)
Brca1	Transcriptional regulator implicated in DNA repair and tumor suppression. Associates with RnapII and histone deacetylases. E3 Ubiquitin ligase	Akt-Thr509 Atr-Ser1423 Atr-Ser1524	Cytoplasmic localization Nuclear localization and activation of Casp3 which facilitates Nanog cleavage and differentiation	(Altiok et al., 1999) (Fujita et al., 2008; Martin and Ouchi, 2005)
Ikbkb	Kinase that acts as an inhibitor of I κ B	Akt-S180	Activates Ikbkb to promote degradation of I κ B and promotes transcriptional activity of NF κ B to stimulate transcription of 'anti' apoptotic genes such as Bcl2 and IAP family members	(Barre and Perkins, 2007)
Gsk3β	Kinase that regulates β -Catenin-TCF/Lef signaling	Akt-Ser9	Causes inactivation of Gsk3 β which is required for active Wnt, HH, Notch, and Bmp signaling	(Kaytor and Orr, 2002)
Bad	Apoptotic Pathway	Akt-Ser99	Results in cytoplasmic sequestration and inactivation of Bad by 14-3-3 proteins. Inability to activate genes implicated in apoptosis.	(Sastry et al., 2006)
Casp9	Apoptotic Pathway	Akt-Ser196	Inhibition of apoptosome formation	(Cardone et al., 1998)
Hexim1	Member of 7SK/Hexim1/2 P-Tefb Repression Complex	Akt-Thr270 Akt-Ser278	Phosphorylation of Hexim 1 releases P-Tefb (Cdk9 subunit) to facilitate transcriptional activation	(Contreras et al., 2007)

Ezh2	Polycomb Repressor Complex 2 member Facilitates modification of H3K27me3	Akt-Ser21	Decreases DNA binding and inhibits methyltransferase activity	(Cha et al., 2005)
Baf155 Smarcc1	SWI/SNF Chromatin Remodeling Complex; member of TFIID GTF and MLL H3K4 methyltransferase complex; Embryonic lethal	Akt-Thr302 Akt-Ser319 Akt-Ser328	Specific function of Smarcc1 phosphorylation is currently unknown however, phosphorylation of SWI/SNF is generally accepted to impair binding to chromatin and hence inactivate chromatin remodeling. This has been observed to occur specifically at G2/M.	(Foster et al., 2006) (Simone, 2006)
CBP/p300	Histone Acetyltransferase that facilitates acetylation of multiple lysine residues on Histone H3 and H4	Akt-Ser1834	Stimulates acetyltransferase activity; Enhances transcription of target genes	(Huang and Chen, 2005)

Pioneering studies suggest that the H3K27me3 mark remains on chromatin once established (Hansen et al., 2008). This model, however, discounts the role of Utx and Jmjd3 in H3K27me3 demethylation following DNA damage (Miller et al., 2010). The Hansen model also precludes the possibility of further changes in chromatin state, including the generation of iPS cells. It also questions the continued requirement for PRC2 in the differentiated state. Therefore, since PRC2 and PRC1 are displaced from mitotic chromosomes (Aoto et al., 2008; Miyagishima et al., 2003), this suggests that a PcG-independent mechanism, which maintains the H3K27me3 mark throughout G2/M exists. This mechanism would function during G2/M to pass on cellular memory to the daughter cell following mitotic cell division.

PcG recruiter *Hmgb2* is a prime candidate to maintain the H3K27me3 mark throughout G2/M. *Hmgb2* is a ubiquitously expressed protein with the highest levels of expression occurring in early development (Štros et al., 2007). *Hmgb2* participates in transcriptional regulation through direct binding and modulation of chromatin structure like other HMG family members (Yoshida, 1987). These changes impact accessibility of transcription factors and the transcriptional machinery to the chromatin template (Tremethick and Molloy, 1986, 1988). However, unlike PRC2 and PRC1, *Hmgb2* associates with mitotic chromosomes (Pallier et al., 2003).

Hmgb2 is a member of the SET Complex consisting of *Set*, *Hmgb2*, *Hmgb1*, *Nme1*, *pp32*, and *Ape1* (Chakravarti and Hong, 2003; Fan et al., 2002). Post-translational modification of *Hmgb2* impacts its function in transcriptional regulation and the DNA Damage Response. The presence of the acidic C-terminal tail promotes transcriptional repression. Proteolytic cleavage, however, results in acetylation, nuclear translocation, and its increased function as

a transcriptional activator (Pasheva et al., 2004; Ugrinova et al., 2009). Proteolytic cleavage also promotes Hmgb2 function in the DNA damage response. This, in turn, results in caspase independent and dependent DNA damage (Krynetskaia et al., 2009) (Kalinowska-Herok and Widlak, 2008).

The emergent data intimates that concerted mechanisms, which function at the transcriptional, post-transcriptional, translational, and post-translational levels, maintain pluripotency. This state is modulated by multiple feedback and feed-forward loops that maintain balanced levels of pluripotency regulators *Oct4*, *Sox2*, and *Nanog*. These regulatory networks establish a very dynamic state that maintains active repression of lineage restricted loci through guarded epigenetic vigilance. It is responsive to slight changes and preserves dynamic equilibrium by PTM-dependent mechanisms. Deeper knowledge of the interplay between extracellular signaling cascades and the mechanisms which regulate cell fate transitions will illuminate our understanding of the pluripotent state. Figure 3 provides an overview of known key regulators of the pluripotent state.

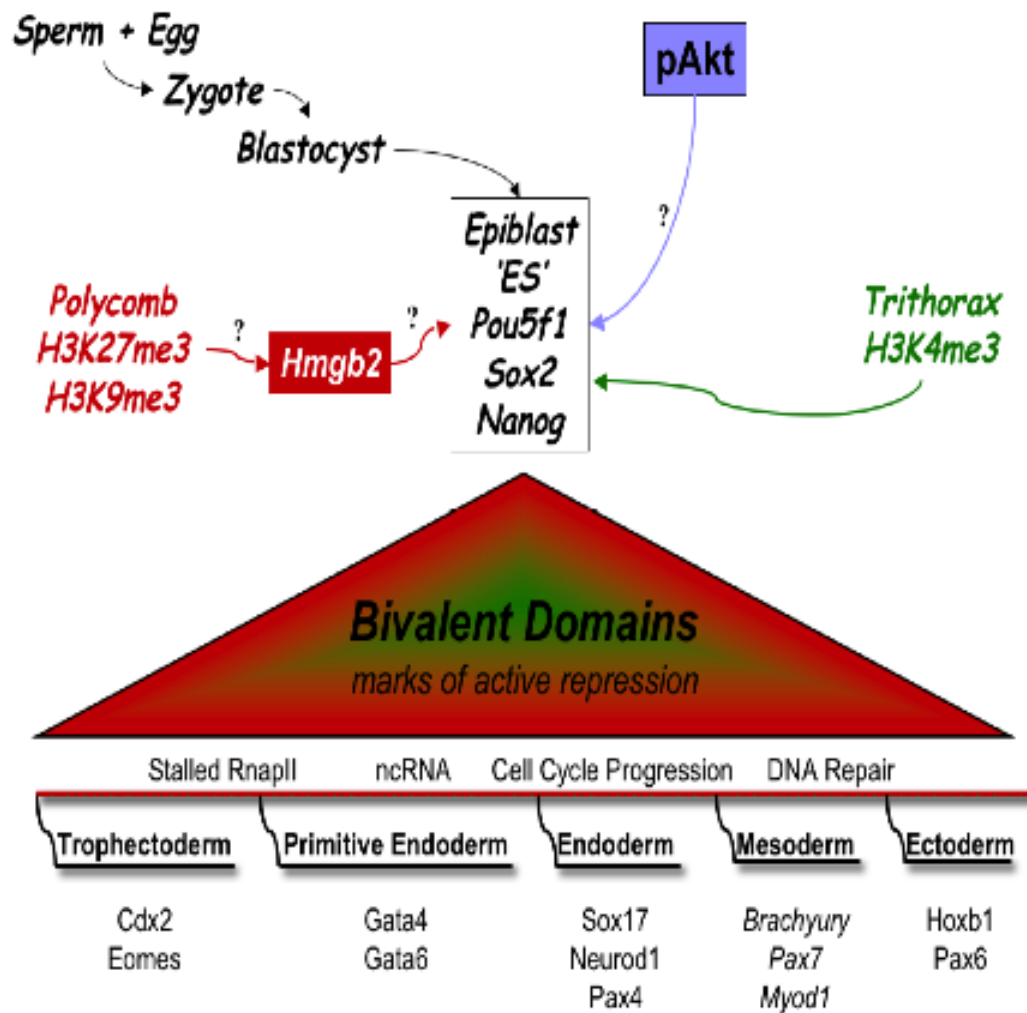


Figure 3. Transcriptional Control of Development. Cellular phenotype is dictated by the combinatorial expression of regulatory genes that drive lineage specific differentiation. These transcription factors coordinately promote differentiation to the prescribed fate through activation of genes required for phenotypic function with concomitant inhibition of alternate differentiation pathways. Although once thought that each cellular state would be dictated by regulators expressed uniquely in each lineage it now appears that the spatio-temporal activation of specific signal transduction cascades may impact the combinatorial interactions amongst the TFs, noncoding RNAs and essential cofactors expressed in each state. This, combined with the availability of DNA binding sites in each distinct cellular state, is required for the expression of lineage specific transcriptional programs. The role of pluripotency regulators Oct4, Sox2 and Nanog to maintain repression of developmentally regulated genes such as Cdx2, Gata4, Sox17, Pax6, and MyoD is critical to successful navigation of embryonic milestones. Further research is required to fully integrate PcG recruiter Hmgb2 and Akt into the growing network of factors which maintain the pluripotent state.

1.3 *Oct4*

The role of Oct4 as master regulator of the pluripotent state has been confirmed by twenty years of accumulated research. Despite the extensive publications dedicated to Oct4, the molecular mechanisms by which it is able to function as both a transcriptional activator and repressor are not understood. A review of recent literature regarding Oct4 expression, regulation, and protein interactions may clarify its important role in stem cell function.

1.3.1 Oct4 Expression

Oct4 has been described as the ‘gatekeeper in early mammalian development’ (Pesce, 2001). Present in the early embryo up to the four-cell stage as a maternally expressed transcript, Oct4 expression is initially seen in all cells of the developing morula until the first embryonic lineage specification (Palmieri et al., 1994). Oct4 is required for the formation of a pluripotent inner cell mass. Homozygous *Oct4* mutant mice die at E3.5. because the pluripotent inner cell mass undergoes a homeotic transformation to trophectoderm (Nichols et al., 1998).

Normal physiological expression of Oct4 maintains the pluripotent state. Deviations in its expression, however, lead to differentiation (Niwa et al., 2000). Then, transient upregulation of Oct4 is required for the formation of primitive endoderm/mesoderm (Pesce and Scholer, 2001). Later stage expression of Oct4 is observed throughout the epiblast and the primitive streak until the end of gastrulation at approximately E8.5. Finally, Oct4 expression is restricted to the primordial germ cells (Boiani and Scholer, 2005). This expression pattern of Oct4 suggests the existence of a totipotency cycle which is maintained by Oct4 expression (Pesce, 2001). Its absence leads to differentiation.

The requirement for Oct4 beyond early embryonic development has been described in non-mammalian species where it maintains ectodermal (Burgess, 2003; Catena, 2004) and endodermal (Lunde et al., 2004; Reim et al., 2004) progenitor cells. Reim et al. have shown that *Spiel-ohne-grenzen/Pou2* (Spg), the Oct4 orthologue in *Danio rerio* is required for formation of ectodermal, mesodermal, and endodermal derivatives (Lunde et al., 2004; Reim and Brand, 2002, 2006; Reim et al., 2004). Furthermore, morpholino knockdown of Spg reveals dual functions for this protein as it first maintains the pluripotent state, and then activates lineage restricted loci in later stage development (Burgess, 2003).

Since Oct4 expression has been observed in adult somatic stem cells (Kucia et al., 2006; Tai et al., 2005) its role in later stage development is now under investigation. Oct4 expression is observed in cancer (Chambers and Smith, 2004; Monk, 2001) and its overexpression may lead to the transformed state (Gidekel et al., 2003) by blocking progenitor cell differentiation (Hochedlinger et al., 2005). However, others believe that these results may be attributed to aberrant detection of Oct4 processed pseudogenes or the use of non-specific antibodies (Liedtke et al., 2007). A second study revealed that lack of Oct4 expression did not compromise regenerative capacity nor restoration of cellular homeostasis in multiple models of tissue damage/regeneration (Lengner et al., 2007). Yet, the idea persists that Oct4 expression continues past early embryonic development since an ES transcriptional program promotes the transformed state (Ben-Porath et al., 2008; Ohm et al., 2007; Santagata et al., 2007).

1.3.2 Domain Structure and Regulation of Oct4

Oct4 is a member of the homeodomain superfamily that consists of classical homeodomain proteins such as Ubx, Hox proteins, and POU homeodomain transcription factors. Characterized by a homeobox domain (POU_H) conserved within all members of this superfamily, Oct4 also contains a POU specific domain (POU_s) that is connected to POU_H by a flexible linker region. Oct4 binds a specific DNA motif, called the octamer motif, containing an invariant ATGC recognized by POU_s and the permissive AAAT by POU_H. POU_H is not able to bind DNA with high affinity, but half-site binding is assisted through cooperativity with POU_s (Verrijzer et al., 1990) and partner HMG proteins (Dailey, 2001). The linker promotes flexible DNA binding in order and orientation of POU_s and POU_H on the chromatin template. While the POU_s domain is structurally similar to several prokaryotic repressor proteins, POU_H is similar to the classic homeodomain proteins (Herr and Cleary, 1995).

The *Oct4* genomic locus (chromosome 17 in *mus musculus*) spans approximately 5 kb and contains 5 exons which code for a protein of 352 amino acids. Early studies revealed the existence of a proximal promoter (PP), distal enhancer (DE), and proximal enhancer (PE) (Yeom et al., 1996) which regulate cell-type specific expression of Oct4. Lrh-1 (Nr5a2) binding at the PE and DE maintains Oct4 expression within the epiblast (Gu et al., 2005). Subsequently, Gcnf (Nr6a1) mediates Oct4 repression in somatic cells by binding to the PP (Fuhrmann et al., 2001). The presence of retinoic acid response elements (RAREs) contained within the PE and DE suggests that differential usage of these elements may occur in a cell type specific context. Sf1 binding to the RAREs promotes Oct4 expression in embryonal cancer cells (EC) (Barnea and Bergman, 2000). By contrast, retinoic acid mediated

differentiation of mESC and EC reveals a shift in occupancy from Sf1 (Nr5a1) to Gcnf, which promotes Oct4 downregulation (Fuhrmann et al., 2001). Consistently, upon GCNF knockout, Oct4 repression and proper formation of the anterior-posterior axis is not observed (Chung et al., 2001). This homeotic transformation is attributed to abrogated Mbd3 and Mbd2 recruitment and *de novo* promoter methylation (Gu et al., 2006). Consensus about Oct4 downregulation in somatic cells has yet to be attained. In Gu et al., Oct4 downregulation was observed despite the loss of promoter methylation. In a second study, however, knockout of *de novo* DNA methylases Dnmt3a and 3b abrogated Oct4 downregulation that occurred upon differentiation (Jackson et al., 2004). More recent evidence implicates Ehmt2 (G9A) in Oct4 inactivation during somatic cell differentiation (Feldman et al., 2006).

Oct4 protein is divided between its DNA binding and transcriptional regulatory domains. The former, which is also its protein interaction domain, is contained within its central POU_S and POU_H domains. The latter are located within its N- and C-terminal regions (Dailey, 2001). Domain swapping experiments have revealed non-equivalent roles for the trans-activating functions of Oct4 N- and C-terminus (Niwa et al., 2002). Moreover, the ability of the C-terminus to illicit a transcriptional response is dependent upon the composite POU domain, which may be regulated in a phosphorylation dependent manner (Brehm et al., 1997). Although alternate splicing has not been described in mouse, human Oct4 protein is alternately spliced with two variant forms Oct3a and Oct3b. The latter is a truncated version, that lacks the N-terminus (Takeda et al., 1992). Despite the described functional distinctions for these two variants (Atlasi et al., 2008; Cauffman et al., 2006), their expression has

recently been attributed to the aberrant detection of processed pseudogenes (Liedtke et al., 2007).

Oct4 function is dose dependent (Niwa et al., 2000). Its protein level expression is affected by ubiquitination and sumoylation (Wei et al., 2007; Xu et al., 2004; Zhang et al., 2007). Ubiquitination of Oct4 by E3 Ubiquitin Ligase Wwp2 results in decreased stability and targeting for degradation via the 26S Proteasome (Xu et al., 2004). The ubiquitination site has not been identified. C-terminal fusion of Oct4 with Ubiquitin or overexpression of Wwp2, however, resulted in decreased transcriptional response from an artificial reporter.

In contrast to the decreased stability of Oct4 observed upon ubiquitination, Oct4 sumoylation at K118 results in increased stability and DNA binding (Wei et al., 2007; Zhang et al., 2007). Sumoylation generally acts to negatively modulate transcription factor function (Gill, 2005; Holmstrom et al., 2003). Oct4 sumoylation, however, may function to activate or repress specific target genes. Co-expression of the SUMO1 (K118R) mutant form of Oct4 resulted in decreased transactivation from artificial reporters containing the PORE and MORE octamer binding motifs in HEK 293 cells (Wei et al., 2007). Increased activity was found in a similar assay using an endogenous enhancer element (Tsuruzoe et al., 2006).

Taken together, these results suggest that ubiquitination and sumoylation of Oct4 may play important regulatory functions in maintaining self-renewal and pluripotency. Figure 3 summarizes a PTM dependent model of Oct4 function in modulating its cellular localization and stability, protein-protein interactions, protein-DNA interactions, and the ability to activate specific repertoires of downstream target genes. More thorough knowledge about the PTM-dependent regulation of Oct4 function will clarify the factors that maintain the pluripotent state and how they are modulated to permit lineage specific differentiation.

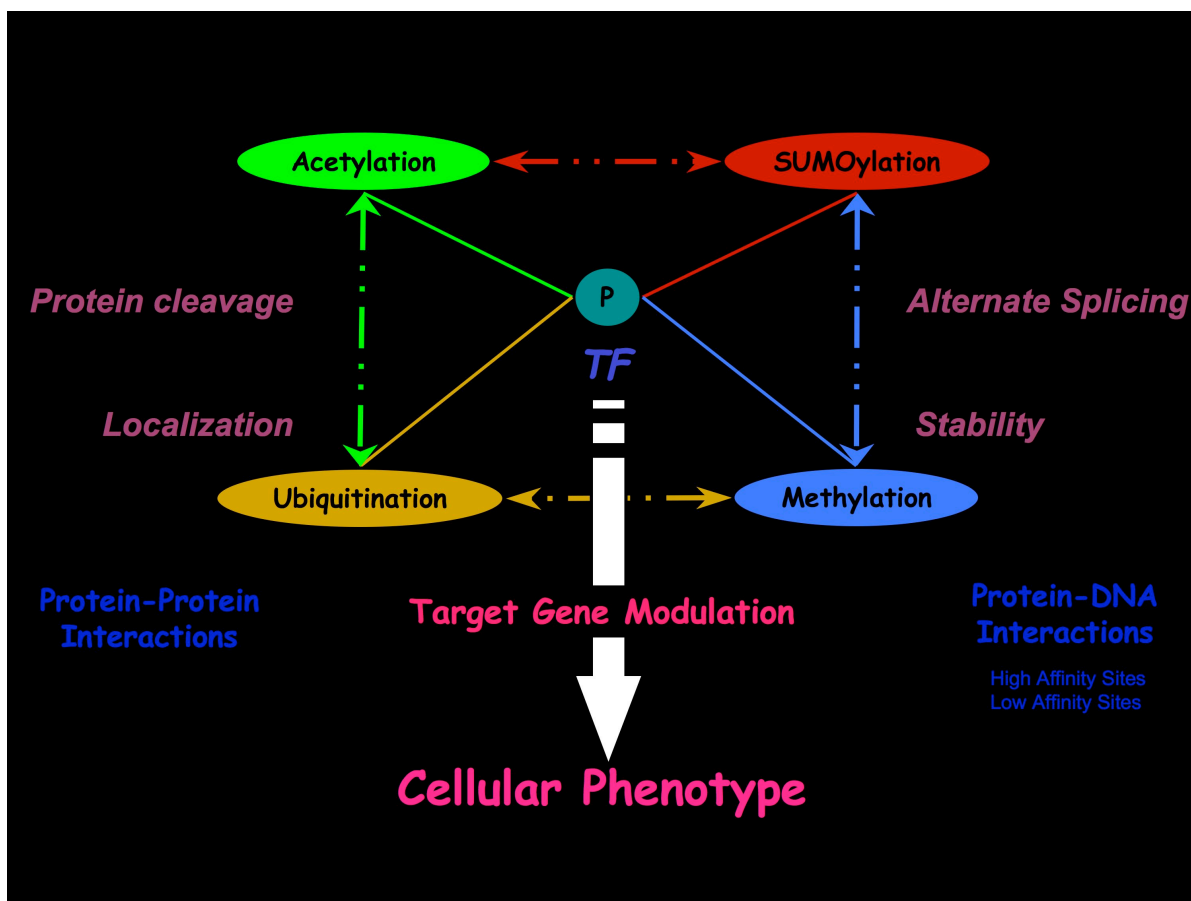


Figure 4. The Complexity of Transcriptional Regulation. The emerging view is that transcriptional control is modulated by the presence of post-translationally modified (PTM) forms of transcription factors (TFs). Similar to the histone code in which specific histone modifications may either facilitate or inhibit subsequent modifications their may also exist a corresponding PTM code that impacts TF stability, localization, protein-protein interactions and protein-DNA interactions and ultimately modulates its ability to regulate a specific repertoire downstream targets (Anensen et al., 2006; Chuikov et al., 2004; Kang et al., 2006; Knights et al., 2006; Papouli et al., 2005). From a systems perspective this swift, unambiguous, yet reversible mode of transcriptional regulation would provide the cell with a means to rapidly respond to cues that would facilitate progression to an altered state of gene expression. A comprehensive understanding of the role of Oct4 PTMs in light of other post-transcriptional and post-translational processing events will provide insight into the modulation of gene expression and cellular phenotype. Solid lines and broken arrows indicate that each PTM may be impacted by prior PTMs that serve a priming function to either promote or inhibit subsequent post-translational modifications.

1.3.3 The Oct4 Regulome

Although abundant genome-wide localization data indicates that Oct4, PcG, and TrxG proteins colocalize on chromatin, Oct4 interaction with either protein family has yet to be established. Characterization of the Oct4 regulome (the regulatory components--including genes, mRNAs, ncRNA, proteins, and metabolites--with which Oct4 interacts in specific cellular states) in steady-state gene expression and during cell fate transitions would point to the mechanism of their locus specific recruitment and the epigenetic mechanisms by which Oct4 differentially regulates transcription. Oct4 and Nanog are known to interact and associate with multiple transcriptional co-repressor complexes (Liang et al., 2008b; Wang et al., 2006). These findings confirm the role of Oct4 in maintaining active gene repression at developmentally regulated loci. They are not sufficient, however, to explain the differential modulation of Oct4 bound loci.

The interaction between Oct4 and Pml may clarify the dichotomous function of Oct4 in transcriptional regulation. It can also serve to connect Oct4 function to the DNA damage response. Pml nuclear bodies function in a wide variety of cellular processes including transcriptional regulation, DNA repair, apoptosis, and cell-cycle regulation (Ruggero et al., 2000; Salomoni and Pandolfi, 2002). Pml nuclear bodies are sumoylation and SIM dependent (Muller et al., 1998; Nacerddine et al., 2005; Shen et al., 2006). The spatio-temporal organization of these nuclear structures serve as depots to compartmentalize nuclear proteins implicated in chromatin remodeling at the G2/M DNA Damage checkpoint (Luciani et al., 2006). Akt and its inactivating phosphatase Pp2a are also contained within Pml Nbs. Pml^{-/-} cells, however, display diffuse nuclear accumulation of activated Akt and increased tumorigenesis (Trottman et al., 2006). The initiating events that lead to the DNA

damage response and chromatin remodeling within Pml Nbs and the potential involvement of Oct4 are currently under study.

1.4 Hypothesis and Specific Aims

Oct4 participates in both activation and repression of its target genes to maintain pluripotency. We hypothesize that Oct4 transcriptional function is dependent upon post-translational modification by Akt-mediated phosphorylation and sumoylation. Furthermore, we propose that sequential placement of PTMs upon Oct4 is required for temporal activation of distinct classes of transcriptional targets with which it subsequently interacts to maintain the dynamic pluripotent state. First, Oct4 sumoylation maintains active repression at several bivalent domains and promotes cell cycle progression. Second, dynamic Akt-mediated Oct4 phosphorylation promotes interaction with Hmgb2 to pass on epigenetic memory to cellular progeny. The inability to sequentially modify Oct4, however, results in decreased Akt signaling and initiation of the DNA damage response, which may lead to cell fate transitions.

Objective 1

Based on the assumption that coregulation often implies participation in similar biological processes, an extensive and varied population of stem cells and their differentiated derivatives were transcriptionally profiled. Analysis of the data was performed to find genes whose transcript level expression was correlated to Oct4 was performed to gain insight into factors required for Oct4 function.

Objective 2

Oct4 participates in both the transcriptional activation and repression of its target genes to maintain the pluripotent state. Recent high-throughput proteomics approaches have identified non-equivalent roles for the N- and C-terminal transcriptional regulatory domains

(TRD) of Oct4. The N-terminal domain interacts with transcriptional co-repressors, whereas the C-terminal TRD interacts with co-activators, chromatin remodellers, and genes implicated in the response to DNA damage. Identification of consensus sites for Akt and SUMO suggest that Oct4 function may be modulated by post-translational modifications. These may subsequently impact protein interactions and binding to specific cassettes of transcriptional targets. Functional genomics and biochemical analyses to support the hypothesis that Oct4 post-translational modifications modulate protein-DNA and protein-protein interactions were therefore performed.

CHAPTER 2

Oct4 targets regulatory nodes to modulate stem cell function

Purpose

Although several previous studies comparing transcriptional profiles of various stem cell populations have sought to harmonize our understanding of stem cell function, these studies revealed very few genes which were commonly expressed, and called into question the notion that a molecular signature common to all stem cell types existed.

In this study, which was conducted as part of the Stem Cell Genomics Project, we transcriptionally profiled an extensive and varied array of stem cells and their differentiated derivatives. Analysis of the resulting data was then performed to find genes whose transcript level expression was correlated to Oct4 in an attempt to define the transcriptional modules in which Oct4 participated and potentially regulated. As a result of the analysis a model was proposed whereby Oct4 functions to regulate genes implicated in chromatin structure, nuclear architecture, DNA repair, apoptosis, and cell cycle control to modulate cell fate decisions. This analysis has served as a roadmap for subsequent studies which have attempted to delineate the impact of Oct4 post-translational modifications vis-à-vis protein complex formation, cellular localization, chromatin state, and gene expression in self-renewal and lineage commitment.

Contribution of Co-authors

Pearl Campbell and Michael Rudnicki conceived and designed the experiments.

Pearl Campbell performed all of the experimental work.

Pearl Campbell, Miguel Navarro-Andrade, and Carol Perez Iratxeta analyzed the data.

Carol Perez Iratxeta contributed analysis tools.

Oct4 Targets Regulatory Nodes to Modulate Stem Cell Function

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and Michael A. Rudnicki^{1,2,3}

Keywords: Embryonic stem cells, Oct4, transcriptome, self-renewal, pluripotency, differentiation, cancer, Polycomb Group, Trithorax Group, chromatin

Running title: Oct4 and Stem Cell Function

Abstract

Stem cells are characterized by two defining features; the ability to self-renew and to differentiate into highly specialized cell types. The POU homeodomain transcription factor *Oct4* (*Pou5f1*) is an essential mediator of the embryonic stem cell state and has been implicated in lineage specific differentiation, adult stem cell identity, and cancer. Recent description of the regulatory networks which maintain 'ES' have highlighted a dual role for *Oct4* in the transcriptional activation of genes required to maintain self-renewal and pluripotency while concomitantly repressing genes which facilitate lineage specific differentiation. However, the molecular mechanism by which *Oct4* mediates differential activation or repression at these loci to either maintain stem cell identity or facilitate the emergence of alternate transcriptional programs required for the realization of lineage remains to be elucidated.

To further investigate *Oct4* function we employed gene expression profiling together with a robust statistical analysis to identify genes highly correlated to *Oct4*. Gene Ontology analysis to categorize overrepresented genes has led to the identification of themes which may prove essential to stem cell identity including chromatin structure, nuclear architecture, cell cycle control, DNA repair, and apoptosis. Our experiments have identified previously unappreciated roles for *Oct4* for firstly, regulating chromatin structure in a state consistent with self-renewal and pluripotency, and secondly, facilitating the expression of genes that keeps the cell poised to respond to cues that lead to differentiation. Together, these data define the mechanism by which *Oct4* orchestrates cellular regulatory pathways to enforce the stem cell state and provides important insight into stem cell function and cancer.

Introduction

Embryonic Stem Cells (ESCs) are derived from the inner cell mass of the pre-implantation embryo and are characterized by their unlimited capacity for self-renewal and their ability to contribute to all cell lineages. The successful derivation and culture of human ESCs (hESCs) (Trounson and Pera, 2001) has opened the possibility of their use for generating cells for transplant, for tissue engineering or for drug development and testing. Importantly, full exploitation of the potential of hESCs will require the complete understanding of the function of the genetic factors that specify stem cell identity and regulate their commitment towards specific differentiated cell lineages. However, the transcriptional networks and molecular mechanisms that regulate the formation, self-renewal, and differentiation of hESC and mouse ESC (mESC) remain at best poorly understood.

Oct4 (*Pou5f1*), a POU-homeodomain transcription factor, plays a central role in self-renewal, pluripotency, and lineage commitment. Initially expressed as a maternal transcript, *Oct4* is required for the formation of a pluripotent inner cell mass (Nichols et al., 1998). Moreover, strict control of *Oct4* expression is necessary to maintain ESC identity. Alterations in *Oct4* expression promote differentiation and leads to the specification of ectodermal (Shimozaki et al., 2003), endodermal (Reim et al., 2004), or mesodermal (Niwa et al., 2000) primitive progenitors. Furthermore, *Oct4* has been shown to promote tumor growth in a dose dependent manner (Gidekel et al., 2003) and epithelial dysplasia by interfering with progenitor cell differentiation (Hochedlinger et al., 2005), is expressed in various human tumors (Jin, 1999; Monk, 2001) and adult stem cells (Tai et al., 2005) thus extending the role of *Oct4* from embryo to adult.

Recent identification of *Oct4* transcriptional targets in ESCs has revealed an unanticipated collaboration between *Oct4*, Sox2, and Nanog and provides a starting framework of the core transcriptional circuitry which maintains 'ES' through coordination of a series of feedback and feedforward loops (Boyer et al., 2005; Loh et al., 2006). Furthermore, several signaling pathways including LIF/JAK/STAT, BMP, WNT, PI3K, MAPK/ERK, TGF β and Notch (Batlle E, 2002; Boiani and Scholer, 2005; Burdon et al., 2002; Chickarmane et al., 2006) have been shown to modulate stem cell function. Several key questions however still remain unresolved as a result of these studies. Firstly, what are the regulatory mechanisms that maintain self-renewal and pluripotency? Conversely, what are the molecular inputs that drive differentiation? Finally, and most importantly, can we deduce the essential themes that characterize stem cell function and thereby utilize this knowledge to gain insight into normal developmental processes to predict the consequences of aberrations to these processes that ultimately lead to human disease?

To address these questions and further elucidate the factors that mediate stem cell function, we undertook an analysis to identify genes whose expression is correlated to *Oct4*. With the understanding that coexpression of genes may imply coregulation and participation in similar biological processes (Zhang et al., 2004), we sought to identify genes which were correlated to *Oct4* transcript expression in a wide variety of stem/progenitor populations which were analyzed by Affymetrix GeneChip technology as part of the Stem Cell Genomics Project (Perez-Iratxeta et al., 2005). We hypothesized that by using *Oct4* as a marker gene for self-renewal, pluripotency, and early lineage commitment, this analysis would lead to the identification of 1) Genes that are central to stem cell identity; 2) *Oct4* target genes; and 3) Genes that modulate *Oct4* function. Although several previous studies

have sought to harmonize our understanding of ‘stemness’ (Ivanova et al., 2002; Ramalho-Santos et al., 2002) it has been suggested that rather than the capacity for self-renewal and differentiation, the unique defining feature of a stem cell is that it represents a lasting steady-state of gene expression suspended in its differentiation pathway, yet maintaining the ability to respond to niche induced signals to carry out the indicated program of cellular specialization (Mikkers and Frisen, 2005). Insight into the juncture between cell extrinsic and intrinsic factors described above will provide an enhanced understanding of the molecular mechanisms which confer stem cells with this ability.

Lineage commitment can be described as a process whereby the unlimited ability for self-renewal and potency are gradually restricted as a cell progresses from one steady state of gene expression to the next. Recently attributed to stochastic events which increase the likelihood of a specific developmental outcome (Arias and Hayward, 2006), this view is in direct opposition to determinism, which precludes the processing of molecular cues emanating from the cellular niche. In juxtaposition to both the stochastic and deterministic models of development is the view that cellular commitment is facilitated by a hierarchy of transcriptional regulatory networks (Lee et al., 2002) which exert precise biological control by combinatorial interactions at the protein-protein, and protein-DNA level. The function of these networks is highly responsive to molecular inputs, allowing the rapid processing and relay of information required for either maintenance of a specific cellular state, or progression to an altered steady state. Importantly, our data suggests that Oct4 maintains stem cell identity by targeting key regulatory genes which play critical roles in determining cell fate.

Results and Discussion

Oct4 Correlation Analysis

A set of 45 murine samples collected as part of the *Stem Cell Genomics Project* and deposited in *StemBase* (<http://www.StemBase.ca/>) (Perez-Iratxeta et al., 2005) were selected to form the basis of this analysis (Supplemental Table S1). A wide variety of samples comprising adult and embryonic stem cells and their differentiated derivatives were collected in biological triplicate and hybridized to the Affymetrix MOE430 GeneChip Set for a total of 270 GeneChips. Following normalization, scaling, and filtering of the data the standard Pearson correlation coefficient (ρ) between every probeset which passed the filter, to the *Oct4* probeset was computed. A probeset was considered correlated to *Oct4* if $|\rho| \geq 0.75$. This computation was repeated 10,000 times with random subsets consisting of 65% to 70% of the data. Probesets that were correlated to the *Oct4* associated probe in at least 40% of the trials were retained for further analysis (Supplemental Table S2).

The stringency of our correlation analysis is set by two parameters; $|\rho| \geq 0.75$ and the percentage of trials in which this value for ρ is met or exceeded. In setting these parameters our aim was to prioritize genes for analysis which may have either represented *Oct4* targets or genes which were implicated in self-renewal, pluripotency, or early lineage commitment. The values were pragmatic in nature; chosen as such to produce a reasonable number of genes which could be analyzed in a coherent fashion, possibly being able to provide a snapshot as it were of ‘stemness’. The use of more or less stringent parameters would result in the identification of fewer or more genes. Of note, cursory examination of the cutoffs used reveals that should we have increased the percentage of trials for which $|\rho|$

≥ 0.75 from 40% to 50% we would not have identified at least two previously identified Oct4 targets; Sox2 (49%) and Cdyl (40%) (Boyer et al., 2005; Loh et al., 2006).

As a result of this analysis 1299 probesets (1155 unique transcripts) were found to be correlated to *Oct4*. Seventy-five probesets (69 transcripts) were negatively correlated, while 1224 probesets (1086 transcripts) were positively correlated. The validity of this method for the identification of genes related to stem cell identity is assured by the presence of genes which have previously defined roles in ESCs such as *Utf1*, *Fgf4*, *Nanog*, and *Sox2* which were correlated to Oct4 in 100%, 99%, 97% and 49% of the trials respectively. Comparison of the transcript expression levels of *Oct4* and correlated *Nanog*, *Sox2*, *Tdrd7*, *Mef2a*, and uncorrelated *Myog* across all samples utilized in this analysis demonstrates the range of *Oct4* expression in these samples and also lends meaning at a biological level to the statistical analysis performed (Figure 1).

GO Categorization of Oct4 Correlated Genes

To gain insight into the functions of *Oct4* correlated genes, GOstat analysis (Beissbarth and Speed, 2004) was performed. As a result of this analysis a number of gene ontology (GO) categories were found to be correlated to Oct4 expression. Many over-represented terms were related to transcription and DNA replication (nucleic acid binding, DNA helicase, nucleolus), RNA processing (rRNA processing, spliceosome complex, and RNA splicing), and cellular localization (nucleolus and Cajal body). Many under-represented terms were related to inter-cellular communication (cell communication, receptor activity, signal transduction). A complete output from GOstat is provided (Supplemental Table S3) with a graphical display summarizing the most significant results (Figure 2).

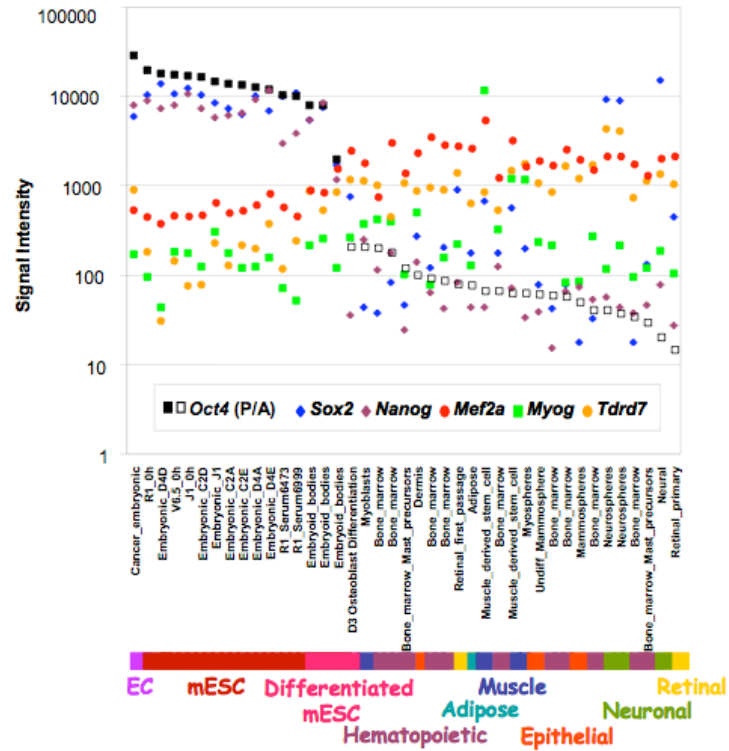


Figure 1. Oct4 Correlation Analysis. ESC, EC, myogenic, neuronal, retinal, and hematopoietic stem cells and their differentiated derivatives underwent Affymetrix gene expression profiling as part of the Stem Cell Genomics Project. A set of 45 samples were profiled in biological triplicate and hybridized to the MOE430 GeneChip set. Mean intensity values for each biological triplicate are plotted in log scale on the Y-axis, with an approximate cutoff of 1000 demarcating detection status of each gene represented. Transcript expression levels of genes positively (*Nanog*, *Sox2*), negatively (*Tdrd7* and *Mef2a*), and not (*Myog*) correlated to *Oct4* are displayed. Detection calls of 'Present' for *Oct4* are depicted by solid black squares. 'Absent' calls are represented by open black squares.

Because this method of analysis is highly dependent upon the GO categories associated with a specific gene, the use of alternate GO databases can result in divergent findings. Moreover, such analyses are limited by the availability of databases which possess accurate annotations that keep pace with current research.

To overcome these limitations, further refinement of GO classifications for the *Oct4* correlated genes was performed by manual curation of a wide variety of databases such as *NetAffx*, *GeneCards*, *Ensembl*, *Stanford Source*, *Bioinformatics Harvester*, and *PubMed* (Supplemental Table S2). This analysis revealed that the categories transcriptional regulation, intracellular signaling, mRNA splicing, cell cycle, DNA repair, and chromatin were highly represented within the positively *Oct4* correlated genes. Categories highly represented within the negatively correlated genes included transcriptional regulation, protein modification, transport, intracellular signaling, and apoptosis. A summary of these findings is provided in Figure 2 with representative genes in highly enriched categories provided in Table 1. Of note, these findings are highly consistent with a previously published GO analysis performed following *Oct4* knockdown in hESC (Babaie et al., 2007).

Target Gene Validation

To validate our premise that this analysis would lead to the identification of *Oct4* direct transcriptional targets, we performed a screen scanning the genomic region from 2 kb upstream of the transcriptional start site to 2 kb downstream from the 3-prime end of the transcribed region of the correlated genes for the presence of neighboring *Oct4* and dimerization partner *Sox2* binding sites (Supplemental Material and Methods). As a result of this analysis 392 genes were found to possess at least one putative composite binding site

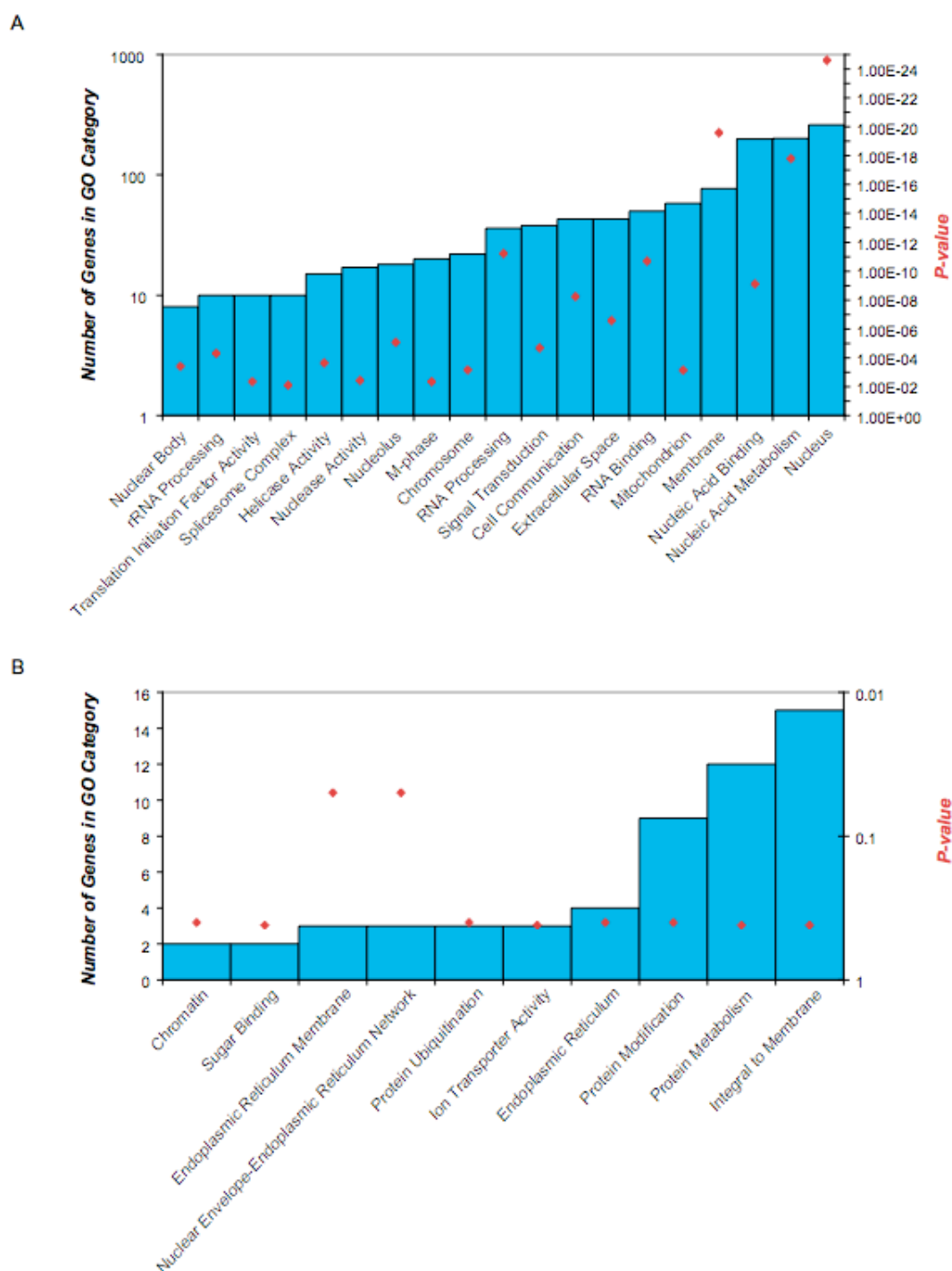


Figure 2. GOstat functional classification of *Oct4* correlated genes. GOstat analysis reveals highly enriched Gene Ontology (GO) categories for *Oct4* positively (A) and negatively (B) correlated genes. Number of genes in each category depicted along with P-value assigned in GoState. Only non-redundant GO categories with the most abundant number of genes contained are depicted.

Table 1. Categories of Genes Identified as Oct4 Correlated.

Chromatin Structure	Nuclear Architecture	DNA Repair	Apoptosis	Cell Cycle Control
Arid1a	Pml	Blm	Aatf	Anapc10
Arid5b	Pum1	Brca1	Api5	Bub1
Ash1l	Coil	Chk1	Aven	Ccna2
Ash2l	Ncl	Ddb1	Bag4	Ccnb1
Cdyl	Mep50	Fancd2	Ciapi1	Ccnb2
Rest	Nup54	Lig1	Commd10	Ccne1
Jarid1b	Nup160	Lig3	Gtse1	Ccnf
Jarid2	Gemin4	Mre11a	Opa1	Chfr
Nasp	Gemin5	Msh2	Siva	Cdk5rap3
Phc1	Sfrs2	Parp1	Spin1	Cul2
Rnf134	Snrpn	Rad17	<i>Bin1</i>	D14Abb1e
Setdb1	Snrpa	Rad51	<i>Blp1</i>	Gstp1
Suz12	Snrpa1	Trp53	<i>Serpinb9</i>	Igf2bp1
<i>Bmi1</i>	Snurf	Xrcc5	<i>Sh3glb1</i>	Jarid1b
<i>Phc3</i>	Sf3b14	<i>Tdrd7</i>	<i>Casp6</i>	Nipp1

Highly represented Gene Ontology categories as identified by manual curation of databases such as *NetAffX*, *GeneCards*, *Ensembl*, *Stanford Source*, and *Bioinformatics Harvester* and *PubMed*. Representative genes in each category are provided. Positively correlated genes are displayed in normal font. Negatively correlated genes are displayed in bold italics.

(Supplemental Table S4) with several genes such as *Oct1/Pou2f1*, *Smyd3*, and *Ranbp17* containing multiple (17, 16, and 14 respectively) putative sites, which may reflect a requirement for strict regulatory control of these genes throughout development. Although one might predict that genes containing multiple binding sites would show a higher degree of correlation to *Oct4*, a very cursory analysis of the data reveals that this is in fact not the case. Genes containing 1 *Oct4/Sox2* binding site (and % correlation) are: *Lig3* (+62), *Kctd3* (+91), *Bin1* (-41), *Bmi1* (-55), *Nasp* (99 and 79-two probesets). Genes containing from 5 to 10 sites include: *Insig2* (-61), *Ipo11* (+92), *Myst4* (+94), *Nr6a1* (52 and 53) and *Strbp* (52). Genes with greater than 10 sites are: *Pou2f1* (+50), *Ranbp17* (+99), and *Smyd3* (+45).

Validation of 28 of these loci by chromatin immunoprecipitation (ChIP) followed by quantitative real-time PCR (QRT-PCR) confirmed the identification of 26 *Oct4* direct transcriptional targets (Figure 3; Supplemental Table S6). Notably, since the completion of our studies, these findings have been confirmed by several groups (Babaie et al., 2007; Boyer et al., 2005; Ivanova et al., 2002; Loh et al., 2006; Matoba et al., 2006).

Further examination of these directly regulated target genes in the context of the correlated gene-list reveals important insights into how *Oct4* regulates pivotal pathways involved in controlling pluripotency, self-renewal and early lineage commitment.

Oct4 Correlated Genes are Implicated in Chromatin Regulation

Recent experiments indicate that chromatin organization is dynamic and is subject to regulatory mechanisms that enforce the transcriptional potential of the genome during cellular commitment and differentiation. Chromatin is remodeled into transcriptionally permissive or repressive conformations by complexes that covalently modify histones, act in

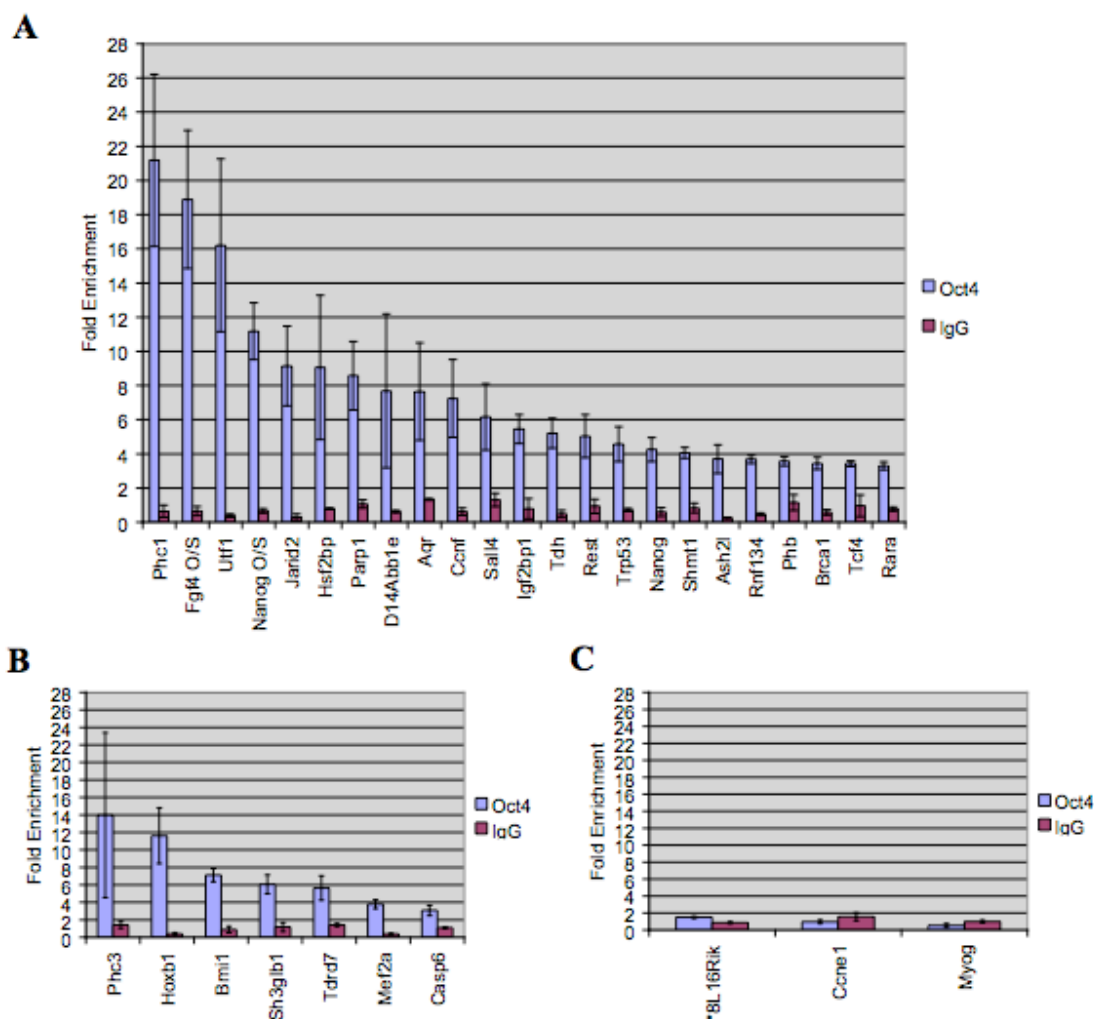


Figure 3. Validation of Oct4 targets. Chromatin immunoprecipitation (ChIP) assays were performed with Oct4 and IgG antibody and no antibody as a negative control followed by Quantitative Real-time PCR analysis (ChIP/QRT-PCR) for putative positively regulated Oct4 target (A), negatively regulated Oct4 target, and non-validated (C) genes. *BL16Rik represents 1110008L16Rik. Results are from two independent ChIP assays, with duplicate QRT-PCR assessment for each. Error bars denote standard error of the mean (SEM).

an ATP-dependent manner to reposition nucleosomes along DNA, or facilitate histone exchange. Several complexes have been identified including SWI/SNF, ISWI, INO80, and M1-2/CHD, and Trithorax group (TrxG), and Polycomb group (PcG) proteins which mediate chromatin remodeling by facilitating epigenetic modification of histone tails to activate or repress gene expression, respectively (Cairns, 2005).

Thirty-five genes implicated in chromatin remodeling were correlated to Oct4. Putative positive target genes include SWI/SNF members Smarcc1, AT rich interactive domain (Swi1 like) containing proteins (ARID domains) Arid1a, Arid5b, Jarid1b and Jarid2, which was confirmed as a direct Oct4 target. Notably, these ARID domain containing proteins, a subset of the Jumonji C family, have recently been associated with histone demethylase activity (Klose et al., 2006). Several other genes containing MYST, SET, and CHROMO, and BROMO domains, which facilitate or recognize specific histone modifications, were also identified.

Rest has been implicated in the repression of neuronal specific genes via its ability to recruit cofactors such as histone deacetylases (HDACs), Corest, Sin3, and Mecp2 (Huang et al., 1999). The identification of Rest as a direct Oct4 target, in light of its role in maintaining chromatin plasticity throughout neurogenesis, (Ballas et al., 2005) provides a mechanistic understanding of Oct4's role in promoting neural differentiation (Shimozaki et al., 2003). Ironically, Rest has recently been described as both a tumor suppressor (Westbrook et al., 2005) and an oncogene (Lawinger et al., 2000). The identification of Corest and Mecp2 as respectively positively and negatively correlated to Oct4 may provide insight into the dynamic nature of Rest co-repressor complexes throughout development that could explain these seeming incongruities. Furthermore, this hypothesis is supported by the

recent description of the changing Rest-regulon in the progression from embryonic stem cells to neural stem cells (NSC) to differentiated neurons (Sun et al., 2005)

Importantly, several members of the TrxG and PcG of transcription factors such as Ash1l, Suz12, Ash2l, Phc1, and Rnf134, Bmi1, and Phc3 were correlated to Oct4, with the five latter genes validated as Oct4 targets. Diverse functions for PcG and TrxG genes in cancer, cell cycle control, and stem cell function have recently been described (Lee et al., 2006b; Lessard and Sauvageau, 2003; Valk-Lingbeek et al., 2004). The direct transcriptional regulation of several members of these complexes places Oct4 central to the coordination of these activities. The localization of Suz12, a member of Polycomb Repressor Complex 2 (PRC2) at many Oct4 repressed loci in ESC (Lee et al., 2006b; Squazzo et al., 2006) provides indication that Oct4-Polycomb interaction may play a significant role in the active repression of lineage. Furthermore, knock-down or overexpression of Oct4 has been shown to result in perturbed expression of several members of PcG and TrxG that we have identified as Oct4 targets and has led to loss of the pluripotent state (Ivanova et al., 2006; Matoba et al., 2006). A comparison of the results of this study to the previous studies can be found in Table 2. Taken together, these data provide strong support for Oct4's role in maintaining chromatin structure in mESC via regulation of and interaction with a unique constellation of PcG and TrxG complexes.

The negative correlation between *Bmi1* and *Oct4* was surprising in light of its role in maintaining hematopoietic and neuronal stem cells (HSCs, NSCs). Although necessary for self-renewal of HSCs and NSCs, expression of *Bmi1*, which leads to chromatin condensation and stable gene silencing (Francis et al., 2004) may be inconsistent with self-renewal in pluripotent cells. Pluripotency involves the ability to repress genes whose expression would

Table 2. Cross-Study Comparison of Oct4 Target Genes.

Gene Symbol	Campbell et al.	Loh et al.	Boyer et al.	Ivanova et al.	Matoba et al.
	ChIP-PCR in mESC	ChIP-PET in mESC	ChIP-ChIP in hESC	Perturbed expression following Oct4 shRNA	Perturbed Expression following manipulation of Oct4 expression (up or down)
Phc1	✓	✓	-	✓	✓
Fgf4	✓	-	-	✓	✓
Utf1	✓	-	-	✓	✓
Nanog O/S	✓	✓	✓	-	✓
Jarid2	✓	-	✓	-	✓
Hsf2bp	✓	-	-	✓	✓
Parp1	✓	-	-	-	✓
D14Abb1e	✓	-	-	-	✓
Aqr	✓	-	-	-	-
Ccnf	✓	-	-	-	✓
Sall4	✓	-	-	-	✓
Igf2bp1	✓	-	-	-	✓
Tdh	✓	-	-	✓	✓
Rest	✓	✓	✓	✓	✓
Trp53	✓	-	-	-	✓
Nanog	✓	-	-	-	✓
Shmt1	✓	-	-	-	✓
Ash2l	✓	-	-	-	✓
Rnf134	✓	-	-	-	-
Phb	✓	-	-	-	✓
Brca1	✓	-	-	-	✓
Tcf4	✓	-	✓	-	✓
Rara	✓	-	-	✓	✓
Phc3	✓	-	-	-	✓
Hoxb1	✓	✓	✓	-	✓
Bmi1	✓	-	-	-	✓
Sh3glb1	✓	-	-	✓	-
Tdrd7	✓	-	-	-	-
Mef2a	✓	-	-	-	-
Casp6	✓	-	-	✓	✓

Comparison of validated Oct4 targets to previous studies employing ChIP-Pet, ChIP-ChIP and expression analysis following Oct4 knockdown or overexpression. Discordant findings in the ChIP based approaches may be explained by the use of promoter based chips or stringency of analysis. Although shRNA knockdown of Oct4 reveals few genes that are predicted to be bona fide Oct4 targets that are identified in common, comparison to the dataset in Matoba et al. (Matoba et al., 2006) reveals that expression of most of the targets identified in this study are in fact perturbed upon up or downregulation of Oct4. Discordant findings between this study and Matoba et al. may be impacted by the temporal nature of Oct4 regulation of these target genes as has been described previously for the Rest regulon (Sun et al., 2005).

result in a loss of potential while retaining the ability to reawaken these transcriptional programs upon differentiation. Therefore, while transcriptional repression is necessary in both pluripotent cells and their differentiated progeny, the means to accomplish it may, of necessity, be entirely different.

PcGs exist as developmentally regulated multi-subunit complexes (Kuzmichev et al., 2004). Therefore it is predicted that alterations in the balance of PcG members would have profound implications for maintenance of the stem cell state. If, as anticipated above, inappropriate upregulation of *Bmi1* (and/or *Phc3*) leads to the repression of genes that are required for pluripotency, this may ultimately be manifested in a cell's inability to differentiate and may provide a partial explanation for the oncogenic roles of these proteins. Conversely, it is postulated that downregulation of other PcG members such as *Phc1* would result in the de-repression of genes required for differentiation which would compromise self-renewal (Kim et al., 2004a).

Cell Cycle Control in Stem Cells Requires Inactivation of pRb for Self-Renewal, Activation for Differentiation

Carefully regulated execution of cell cycle progression is accomplished in stem cells by a unique constellation of genes which impact self-renewal and lineage commitment. Activation of intracellular signaling pathways such as *PI3K*, *Ras/Raf*, and *Jak/Stat* by molecular cues emanating from the stem cell niche mediate phosphorylation events which control the activity of cyclin/CDK complexes and culminate in the modulation of genes (such as *pRb* and *Trp53*) that are implicated in cell cycle checkpoint, cell cycle exit, and differentiation (Benevolenskaya et al., 2005).

Assessment of GO terms revealed that 38 cell-cycle related genes were positively correlated to *Oct4* including *Cdc25a*, *Gspt1*, *Ppp1r8*, *Ccnb2*, *Ccne1*, *Ccna2*, *Ccnb1*, and *Ccnf*. Validated *Oct4* target *Ccnf* is implicated in cell cycle control at the G1/S and G2/M checkpoints and has recently been associated with the maintenance of *pRb* in a hyperphosphorylated, inactive state (Sissons et al., 2004). The role of *Ccnf* in this process may in part be due to the E3 ubiquitin ligase domain of *Ccnf* to mediate the degradation of phosphatases such as *Pp1* involved in the sequential activation of *pRb* through G1/S and G2/M (Tamrakar et al., 2000). Conversely, the significantly *Oct4* correlated (92%) *Pp1* negative regulatory subunit *Nipp1* (*Ppp1r8*) may facilitate the functional inactivation of *pRb*. This hypothesis is consistent with the requirement of *Nipp1* in early embryonic development (Van Eynde et al., 2004) and points toward a potential role for *Nipp1* in tumorigenesis (Broceno et al., 2002). In addition to its role in cell cycle control is also involved in mRNA splicing, and transcriptional repression through interactions with the PcG complexes making it an important putative *Oct4* target, capable of integrating the diverse functions of cell cycle control, alternate splicing, chromatin structure, and transcriptional regulation (Van Eynde et al., 2004).

Based upon this analysis it is predicted that alterations in the expression of *Oct4* correlated genes such as *Ccnf* or *Nipp1* that impact the functional status of *pRb* (or *pRb* family member *p107*) would have profound consequences. Inactivation of *pRb* is required for self-renewal; activation of *pRb* is obligatory for cell cycle exit and differentiation. An imbalance in either of these processes, possibly emanating from dysregulated signaling from the stem cell niche or mutations in the key regulators would lead to unrestrained cellular proliferation.

Genes Involved in Apoptosis and DNA Repair are Correlated to Oct4 and are Implicated in Stem Cell Differentiation

Prevailing thought holds that the initial stages of apoptosis involve the caspase mediated induction of DNA strand breaks and either the recruitment of DNA repair genes that act in concert to halt cell cycle progression and restore genomic stability or, if the damage is not able to be repaired, in further cleavage of DNA, nuclear blebbing, and other processes which have been elegantly and thoroughly described elsewhere that culminate in programmed cell death. Both apoptosis and DNA repair are regulated by several multi-component complexes with the roles of *Trp53*, *Brcal*, and *pRb* being central to their coordination (Bartek and Lukas, 2001).

Analysis of the *Oct4* gene list revealed an important emerging theme; mechanisms to actively repress apoptotic pathways are involved in maintaining the stem cell state. Twenty-five apoptotic genes were positively correlated to *Oct4*, the majority of which, including *Aatf*, *Api5*, *Aven*, *Bag4*, *Commd10*, *Nipa*, and *Opal*, function to inhibit apoptosis. In addition, *Bin1*, *Blp1*, *Serpinb9*, *Sh3glb1*, and *Casp6*, all apoptosis inducing genes, were found to be negatively correlated to *Oct4*, with *Sh3glb1* and *Casp6* confirmed as targets.

Thirty genes implicated in DNA damage and repair, were positively correlated to *Oct4*. Members of the *Brcal* associated surveillance complex (BASC) including *Brcal*, *Msh2*, *Mre11a*, *Rad51*, *Blm*, *Chek1*, as well as *Parp1*, *Trp53*, *Fancd2*, *Tdrd7*, and *Xrcc5*, were included. The validation of *Trp53*, *Tdrd7*, *Brcal*, and *Parp1* as direct *Oct4* targets strengthens the importance of this group of genes in stem cell function.

The high frequency at which apoptotic genes were negatively correlated to *Oct4* and anti-apoptotic genes were positively correlated to *Oct4* implies that ‘anti-apoptosis’ is an important theme for maintaining the stem cell state. Conversely, this may also suggest that

genes which modulate the initial response to aberrant chromatin structure, apoptosis, and DNA repair, may play important roles in lineage commitment. This notion is consistent with the role of tudor domain containing proteins (such as *Tdrd7*) in DNA damage response (Stucki and Jackson, 2004), *Casp3* in skeletal muscle differentiation (Fernando et al., 2002), and the roles of *Parp1* (Hemberger et al., 2003), *Trp53* (Lin et al., 2005b), and *Brca1* (Furuta et al., 2005) to modulate differentiation. Interestingly, a relationship between DNA damage repair, chromatin remodeling (Morrison et al., 2004), and histone deacetylation (Fernandez-Capetillo and Nussenzweig, 2004), all previously implicated in cellular differentiation, has recently been described. Moreover, knowledge of the normal developmental functions of these genes in cellular differentiation provides mechanistic insight into how these genes, when mutated, lead to cancer.

Nuclear Architecture in Stem Cells Reinforces Their Defining Characteristics

The nucleus is the site of many processes that profoundly impact cellular phenotype including transcription, mRNA splicing, and DNA replication and repair. Research has revealed that in fact control of these activities is coordinated in a dynamic, spatio-temporal manner. The presence of specific nuclear structures (nuclear bodies; NBs), whose function is to concentrate key regulatory molecules, mainly to loci of actively transcribed genes, facilitates this coordination (Zimmer et al., 2004).

As a result of this analysis several key molecules whose presence is indicative of NBs were observed. *Pml* and *Coil* (Cajal Bodies and PML Bodies), *Gemin4* and *Gemin5* (Gems), *Nup35*, *43*, *54*, *98*, *133*, *160*, *188* (Nuclear Pore Complex), *Ncl* and *Nolc1* (Nucleolus) and 46 genes implicated in RNA metabolism (Splicing Speckles, Spliceosome, Exosome, and

Cajal Bodies) were positively correlated to Oct4. Several genes implicated in nuclear transport such as *Ipo11*, *Kpna1*, *Tnpo2* and *3*, *Xpot*, *Gle1l*, *Xpo5* and *6* and direct Oct4 targets *Igf2bp1* and *Phb* were also positively correlated.

The incidence of nuclear bodies is incremental with cellular proliferative capacity and their localization is predominately to transcriptionally active regions of chromatin, although the mechanisms that direct their localization are largely unknown. Based upon the high degree of correlation of *Oct4* to constituents of NBs, it is conceivable that *Oct4* target binding may function to modulate the accessibility of local chromatin to these structures and thereby enforce the transcriptional potential of specific genetic loci in early development. The identification of *Hoxb1* as a negatively regulated *Oct4* target is consistent with this hypothesis in light of the recent finding that in ESCs *Hoxb1*, although not expressed, is poised at the surface of its chromosome territory. In the initial stages of differentiation *Hoxb1* is transcriptionally activated which results in chromatin decondensation and reorientation of this locus to the nuclear centre (Chambeyron and Bickmore, 2004). Together, these findings lead us to predict that *Oct4* binding functions not only in the transcriptional repression of genes that would otherwise facilitate lineage commitment, but also presents a means whereby these loci are organized spatially within the nucleus so as to be poised for activation given the appropriate cue.

In addition to the normal physiological roles for NBs described above, they also play key roles in the response to DNA damage, DNA repair, apoptosis, and senescence. Loss of regulation in the recruitment and coordination of key genes contained in these structures (*Trp53*, *Pml*, *Brca1*, *Blm*, etc.) would be predicted to have profound implications in the ability of a cell to respond to signals that would lead to differentiation. Such dysregulation is

associated with the accumulation of NBs at sites of DNA damage (DNA damage induced foci) and is implicated in several types of cancer such as acute promyelocytic leukemia (*Pml-Rara* translocation) and Bloom's Syndrome (Zimber et al., 2004).

Conclusions

Through the use of gene expression data compiled from a vast collection of adult and embryonic stem cells and their differentiated derivatives we have performed a robust statistical analytic method to identify genes that are correlated to *Oct4*. Although several previous studies have mapped transcriptional targets of *Oct4*, we believe that this study provides further insight into the transcriptional regulatory networks, factors, and cofactors that modulate stem cell function. Importantly, our experiments have revealed hitherto unappreciated roles for *Oct4* for firstly, regulating chromatin structure in a state consistent with self-renewal and pluripotency, and secondly, facilitating the expression of genes that keeps the cell poised to respond to cues that lead to differentiation. Furthermore, our analyses has led to the elucidation of themes that are essential for maintaining 'ES' including permissive chromatin structure, nuclear architecture, cell cycle control, apoptosis, and DNA repair. Finally, we have identified 26 direct Oct4 transcriptional targets which may represent candidate regulatory nodes by which cell fate decisions could be directed to facilitate the use of hESCs in therapeutic and regenerative medicine (Figure 4A and Table S2).

The Oct4 Transcriptional Regulatory Network

The expression of *Oct4* in various forms of human cancer (Jin, 1999; Monk, 2001) and a recently described role for *Oct4* in adult stem cells (Tai et al., 2005) and the expansion of epithelial progenitor cells (Hochedlinger et al., 2005) supports the theory that cancer is a disease of stem cells. This theory postulates that cancers arise in stem cells or early committed progenitors (Sell and Pierce, 1994) due to their inability to differentiate in a regulated fashion. *Oct4* directly regulates the transcription of genes such as *Trp53*, *Brcal*, *Parp1*, and *Bmi1* which play a central role in a cell's proclivity to undergo transformation, apoptosis, senescence, and now differentiation.

The process of development and the commitment to differentiate is guided by the ordered expression and repression of genes required to enforce specific transcriptional programs. Knowledge of the emerging Oct4 transcriptional regulatory network provides a means whereby we can begin to understand the molecular mechanisms that guide these processes and gain insight into aberrations that lead to disease. While the stem cell state is guarded by highly dynamic, complex, and interrelated mechanisms which impact the repertoire, location, and functional state of expressed genes, lineage commitment can be described as a process whereby the unlimited ability for self-renewal and potency are gradually restricted as a cell progresses from one steady state of gene expression to the next. These diametrically opposed states are mediated by a contrasted balance of forces that impact chromatin structure, nuclear architecture, cell cycle, DNA repair, and apoptosis (Figure 4B and C). Further examination of the interactions among the genes identified as a result of this study will provide a more thorough understanding of the pressures that guide cell fate. Critically, only by understanding the normal developmental function of a gene can we begin to

understand the role that it may play in disease. Importantly, our experiments have defined how *Oct4*, as the master regulator of embryonic stem cell function, plays a central role in regulating key genes in pivotal pathways involved in controlling pluripotency, self-renewal and differentiation.

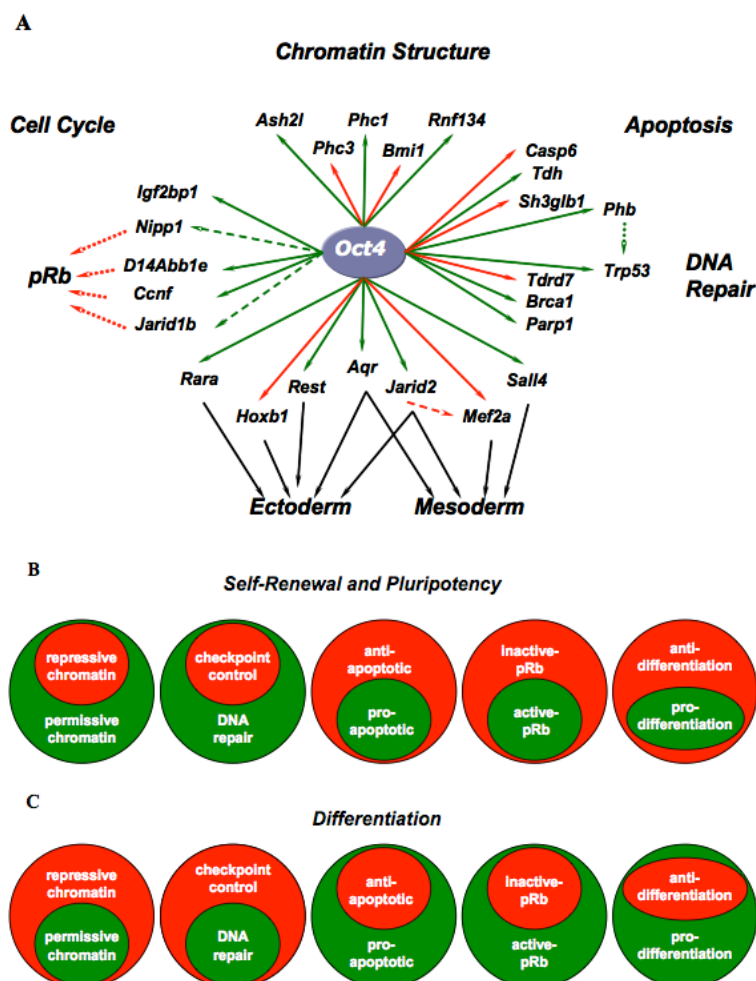


Figure 4. The Oct4 transcriptional regulatory network. Validated Oct4 targets (A) are indicated by solid red or green lines. Red and green indicate negative and positive regulation, respectively for all cases. Dashed lines emanating from Oct4 indicate putatively regulated genes. Solid black lines represent potential regulatory nodes that could facilitate the directed differentiation of ESCs. The pressures that preserve stem cell function and modulate early lineage commitment are diametrically opposed. While Oct4 acts to maintain self-renewal and pluripotency in the undifferentiated 'ES' state by its modulation of genes that act to maintain permissive chromatin structure, DNA repair, anti-apoptosis, and inactive *pRb* (B), in differentiation the balance of these forces is altered to favour repressive chromatin structure, DNA checkpoint control, apoptosis, and active pRB which facilitate cellular commitment (C).

Materials and methods

Stem Cell Culture and Isolation

The samples included in this study were obtained from various members of the Stem Cell Network in support of The Stem Cell Genomics Project. Full descriptions of the origin and experimental conditions used to derive each sample can be obtained from StemBase; (<http://www.scgp.ca:8080/StemBase>).

Target Labeling and Hybridization

Total RNA (10 ug or 10-50 ng) was labeled as per manufacturer's suggested methods (Affymetrix, Santa Clara, California, USA). Briefly, following first strand and second strand cDNA synthesis, samples underwent a single round (10 ug starting material) or two rounds (10-50 ng starting material) of linear amplification using a T7 based *in vitro* transcription (IVT) kit (MegascriptT7, Ambion). During the final round of IVT, biotinylated nucleotides were incorporated into the nascent strand (Enzo Biotech, Farmington, Connecticut, USA) to produce the labeled target cRNA. Ten micrograms of cRNA were fragmented to reduce complexity and hybridized overnight to the MOE 430 GeneChip Set, according to standard protocol. The GeneChips were then washed and stained with Streptavidin R-Phycoerythrin (SAPE). Signal amplification was accomplished by subsequent staining with biotinylated anti-streptavidin, followed by an additional incubation with SAPE. Scanning and absolute analysis was performed in MAS 5.0 to generate the experiment (.exp), raw image (.dat), intensity (.cel) and absolute analysis (.chp) files. All samples were scaled to a target intensity of 1500 during analysis.

Correlation Analysis

Normalized expression values for each probeset were obtained from MAS 5.0 (<http://www.affymetrix.com/products/software/specific/mas.affx>) and the mean expression value for each set of biological triplicates was calculated. The data were scaled by normalizing to the trimmed mean for all probesets in the chips (98%). Probesets that had a consensus detection call of present (P) in more than 7% and less than 93% of the samples were included in the analysis. The standard Pearson correlation coefficient (ρ) between every probeset which passed the filter, to the *Oct4* probeset (1417945_at) was computed. A probeset is considered correlated to *Oct4* if the absolute value of ρ is greater than or equal to 0.75. This computation was repeated 10,000 times with random subsets consisting of 65% to 70% of the data. Probesets that were correlated in at least 40% of the trials were retained for further analysis.

GOSTat Analysis

GOSTat (<http://gostat.wehi.edu.au/>) was used to examine selected sets of probesets for over- and under-representation of GO terms, using MGI (<http://www.informatics.jax.org/mgihome/>) as GO to gene association database, and using false discovery rate correction. This method is sensitive to the GO annotations attached to the genes related to the probes, thus the result might change if another database (e.g. GOA) is used.

Binding Site Analysis

The genomic region from 2 kb upstream of the transcriptional start site to 2 kb downstream from the 3-prime end of the transcribed region of the correlated genes was scanned for the presence of neighboring *Oct4* (ATGCAAAT) and *Sox2* (AACAAAG) binding sites. Global analysis of the *Oct4* correlated gene-list was performed in a conservative fashion based upon POU/HMG/DNA ternary complex assembly as determined by crystal structure assessment of *Fgf4* and *Utf1* (Remenyi et al., 2003). First, the two components of the *Oct4* binding site, namely the POU specific domain (POU_S) and the POU homeodomain (POU_H) were forced to be consecutive in the sequence while independently in any direction, and in any of the two strands. A perfect match was required for POU_S (ATGC), and one mismatch was allowed at any of the four positions of POU_H (AAAT). Second, we defined the *Sox2* binding site as either AACAAAG, which corresponds to the predominant pattern, or the observed variations AACAAAT, or AACAAATG, in any direction or strand. The maximum distance between *Oct4* and *Sox2* binding was constrained to 3 nucleotides.

Manual assessment of binding sites for a subset of the *Oct4* correlated genes as well as developmentally important regulators *Hoxb1* and *Tcf4* was performed in a less restrictive fashion. POU_S was held invariant while the POU_H (AAAT) was allowed to vary by one mismatch in any of the four nucleotide positions. Target sequence identification for the two POU domains relative to each other and to the *Sox2* site were not restricted in order, orientation, or strand. Finally, as has been observed for *Oct4/Sox2* cooperative binding on *Opn* (Botquin et al., 1998), the distances between the *Oct4* and *Sox2* binding sites was relaxed and allowed to span up to 100 nucleotides.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NJ, USA). Briefly, 5×10^6 J1 ESCs were cross-linked with 1% formaldehyde for 15 minutes at room temperature. Cells were washed three times in ice-cold PBS with protease inhibitors and lysed in buffer provided to which protease inhibitors were also added. The lysates were sonicated to an average size of 1500 bp and 250 ug of input chromatin was used for each assay. Immunoprecipitation was performed overnight at 4°C with *Oct4* antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California) and IgG as a negative control.

Quantitative Real-time PCR

Quantitative PCR was performed using primers that flanked the regions containing putative *Oct4* and *Sox2* binding sites with the MX4000 (Stratagene, La Jolla, California, USA) using iQ SYBR Green Supermix (BioRad, Hercules, California.). The following cycling parameters were employed: 96° 10 minutes, followed by 40 cycles of 96°C for 30 seconds, 57°C for 1 minute, and 72°C for 45 seconds. Primer sequences for each amplicon are described in Supplemental Table S2. Each result represents two independent ChIP assays with duplicate QRT-PCR analyses performed on each target gene for each assay. 100% amplification efficiency is assumed based on $\Delta\Delta C_t$ values of ~ 3.3 between each point of a 10-fold serial dilution curve performed for a subset of the amplicons. A 2-fold enrichment therefore represents the minimum threshold for confirmation as an *Oct4* target. Error bars denotes the Standard Error of the Mean. Subsequent to QRT-PCR analysis, each amplicon underwent DNA sequence analysis on an ABI 3730 to confirm identity.

Acknowledgements

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Supplemental Material

Table S1 provides samples used for *Oct4* correlation analysis.

Table S2 designates primer Sequences for *Oct4* target validation by ChIP/QRT-PCR.

Table S3 provides annotation of *Oct4* targets.

Table S4 provides a summary of *Oct4* Correlated Genes with Probeset ID, gene symbol, gene name, direction and percentage of correlation, chromosomal location, summary GO category used for Figure 2 and GO biological process were listed when known. Table S4 can be found at:

<http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0000553#s4>

Table S5 provides results of GoStat Analysis which includes GO identifier, GO category, included genes, and p-values. Table S5 can be found at:

<http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0000553#s4>

Table S6 contains *Oct4/Sox2* putative binding site analysis with Gene symbol, RefSeq or Ensembl ID, putative binding sequence, and location in transcript enumerated. Table S6 can be found at:

<http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0000553#s4>

Table S1. Samples used for Oct4 Bootstrapping Analysis

Sample ID	Sample Name	Sample ID	Sample Name
<i>128</i>	J1 ES	<i>271</i>	Neurospheres
<i>242</i>	J1 ES	<i>198</i>	Neurospheres
<i>249</i>	J1 14D EB	<i>272</i>	Neurospheres P107-/-
<i>206</i>	R1 ES	<i>255</i>	Mammospheres
<i>217</i>	R1 14D EB	<i>256</i>	Mammospheres, 6D Differentiation
<i>153</i>	V6.5 ES	<i>269</i>	Myospheres
<i>175</i>	V6.5 14D EB	<i>274</i>	Myospheres Sca1+
<i>169</i>	D4 ES	<i>270</i>	Myospheres 7D Differentiation
<i>167</i>	D4 ES Attached	<i>199</i>	Adipose Spheres
<i>168</i>	D4 ES Detached	<i>200</i>	Dermis Spheres
<i>166</i>	C2 ES	<i>147</i>	BM Sca1+CD45+
<i>164</i>	C2 ES Attached	<i>233</i>	BM Sca1-CD45-
<i>165</i>	C2 ES Detached	<i>234</i>	BM Sca1-CD45-
<i>219</i>	R1 Oct4 GFP Serum 6999	<i>235</i>	BM Sca1-CD45-
<i>220</i>	R1 Oct4 GFP Serum 6473	<i>294</i>	BM Lin-Sca1+cKit+
<i>132</i>	P19 EC 2D Aggregated	<i>295</i>	BM Lin-Sca1-cKit+
<i>129</i>	P19 EC 1D Monolayer	<i>296</i>	BM Lin-Sca1-cKit-
<i>130</i>	P19 EC 1D Aggregated	<i>291</i>	BM Lin-Sca1+cKit-
<i>131</i>	P19 EC 2D Monolayer	<i>293</i>	BM Lin-
<i>196</i>	D3 30D Osteoblast Differentiation	<i>292</i>	BM Total Population
<i>240</i>	Retinal Spheres Primary	<i>236</i>	BM Mast Cell Precursors
<i>232</i>	Retinal Spheres First Passage	<i>237</i>	BM Mature Mast Cells
		<i>137</i>	C2C12 Myoblasts

Table S2. Primer Sequences for Oct4 Target Validation by ChIP/QRT-PCR

Target	Forward Primer	Reverse Primer
<i>Aqr</i>	5'-TTCAGAGGACTCCACCACAGGA-3'	5'-CGTTGGGATGGCTACCTTTCTC-3'
<i>Ash2l</i>	5'-TGGGGAGGACTGTGGAAGGACT-3'	5'-TGGAACACAGCCACTGTGCTCT-3'
<i>Bmi1</i>	5'-ACCTAAATTTGTACAGTCCC-3'	5'-CATTGGTAACTTTTGCTTC-3'
<i>Brcal</i>	5'-ATGCACTGAACTCCAAGCAGGT-3'	5'-CAGCCTAGGCTGTGAAGCAAGA-3'
<i>Casp6</i>	5'-GATCCAGCCGAGCAGTACAAGA-3'	5'-CCTGTGAAAGGGCTTAGCAGTG-3'
<i>Ccnf</i>	5'-GGGGAACCCCTCACACTGTCTCT-3'	5'-CTTCCAGAGGCTACCAAAAAGCA-3'
<i>D14Abbl1e</i>	5'-AAGCTGATGGTGGCCTCCTAA-3'	5'-CCTACGTAAGCTAATGCT-3'
<i>Hoxb1</i>	5'-CCCTCCCTCTGGTCCCTTCTTT-3'	5'-GGGCACATGTGATCTCTC-3'
<i>Hsf2bp</i>	5'-ACTGGGCGTCAGGACAGAATG-3'	5'-TCCCATGTGCCCATAGTTTCC-3'
<i>Igf2bp1</i>	5'-GGCCCTAGATCTTGAATGAGG-3'	5'-CAGCACGTGGAAAACACCAAAA-3'
<i>Jarid2</i>	5'-TGTTTGGTTTCATTTCCC-3'	5'-CTATAATGTGCCCCACAA-3'
<i>Mef2a</i>	5'-TGAAGACCCAGAACCCATACCC-3'	5'-GAGGCTCTTCCCCACTTTCTTC-3'
<i>Nanog</i>	5'-TGTTTGTAGTGTGGGTATGGGCC-3'	5'-TGTGGTCCCTCCTCTTTC-3'
<i>Parp1</i>	5'-CTGAAGCTGTCATCTTGGTGCC-3'	5'-GGCATTCTGCCTAGCACTCAGT-3'
<i>Phb</i>	5'-ATGCACTATCCATTGCGCCA-3'	5'-GGGATTAAAGCGTCCGCCA-3'
<i>Phc1</i>	5'-ACCTCAGCCATAATTAGTTG-3'	5'-AAACCCCTTCACCTCTCC-3'
<i>Phc3</i>	5'-CGTATCCATTATGTATGTGCT-3'	5'-TTACAAAAAGCCAATGGTGTA-3'
<i>Rara</i>	5'-GTCAACAGAGGACACAGTGGCC-3'	5'-TGCAATCCCTCTGCCTCAGC-3'
<i>Rest</i>	5'-TGGTTGGAATTCTGCTCTG-3'	5'-TCCTGGAAGTCACTGGGATT-3'
<i>Rnf134</i>	5'-TATGAGTGCTGTTCCATA-3'	5'-CTTGTTGTTTCTCCTTTG-3'
<i>Sall4</i>	5'-TAAGGGATCCTGTCTGGCACAC-3'	5'-TTGCAAGCTCTGGGAAATGACT-3'
<i>Sh3glb1</i>	5'-TCTAATCAGACTCATTTATGGG-3'	5'-CCGCAGCAATCTAAATCT-3'
<i>Shmt1</i>	5'-ATCAGAGCCACCTGGAAACAGA-3'	5'-GCTGCTGGATGCTTAAAGAAGC-3'
<i>Tcf4</i>	5'-ATGACAATCCAGAGAGCAGAAG-3'	5'-GCTCGATGACTCTATCCGTGTAA-3'
<i>Tdh</i>	5'-GCAGTTATTCTTCTCCAGCGG-3'	5'-GCTTGAGGGGAAGAGTGCAATT-3'
<i>Tdrd7</i>	5'-AGAGGGAGTGCTTCGTTTCA-3'	5'-GCCATTAAAGGCTGCTCACAAC-3'
<i>Trp53</i>	5'-GCAACTTCTAGAAACCCTGGGG-3'	5'-TTGGGAAATGGAGGCCTGG-3'
<i>Myog</i>	5'-CCGTCCGTCCAAGACAACCC-3'	5'-CCCCCTCTAAGCTGTTGC-3'
<i>Ccne1</i>	5'-TCGCCTGCAGGAGAGGATGA-3'	5'-CGTGGACCCCTGCTCTTTCATC-3'
<i>1110008L16Rik</i>	5'-CATGCAGGGAAATGGAGCC-3'	5'-TCACTTCCCTGCAGCTGTCC-3'
<i>Utf1</i>	5'-CTCAGTTTGAATACTCTGGGCC-3'	5'-GAGGACCTTGGAATTTAGCT-3'
<i>Fgf4 (Kuroda et al., 2005)</i>	5'-AGACTTCTGAGCAACCTCCCGAA-3'	5'-CAACTGTCTTCTCCCCAACACTCT-3'
<i>Nanog (Kuroda et al., 2005)</i>	5'-GTCTTTAGATCAGAGGATGCCCC-3'	5'-CTACCCACCCCCTATTCTCCCA-3'

Table S3. Annotation of Oct4 Targets

Target	Correlation to Oct4	Function	Potential or Known Role in Stem Cells, Development and/or Disease
<i>Aqr</i>	+90%	RNA dependent RNA polymerase	Expressed in mesoderm, neural crest, and neuroepithelium. Gene trap insertion mutants (not null mutants however) are normal in viability and fertility (Sam et al., 1998). Role for <i>Aqr</i> in RNAi in mammalian oocytes discounted (Stein et al., 2003).
<i>Ash2l</i>	+99%	Transcriptional Activation	Trithorax group protein downregulated in megakaryocyte but not in erythroid differentiation. Highly expressed in leukemic cell lines (Wang et al., 2001).
<i>Bmi1</i>	-55%	Transcriptional Repression	Component of Polycomb Repressive Complex 1 (PRC1)/Maintenance complex implicated in self-renewal of neural (Molofsky et al., 2003) and hematopoietic (Lessard and Sauvageau, 2003) stem cells.
<i>Brca1</i>	+69%	DNA Damage Response	Part of the BASC Complex responsible for cell cycle checkpoint in response to DNA strand breaks. Mutations in <i>Brca1</i> are responsible for at least 80% of inherited breast and ovarian cancers (BOC) (Wang et al., 2000).
<i>Casp6</i>	-69%	Induction of Apoptosis	Effector Caspase, cleaves <i>Parp1</i> in vitro. Loss of expression in gastric cancer (Yoo et al., 2004).
<i>Ccnf</i>	+100%	Regulation of Cell Cycle	Homozygous mutants are embryonic lethal, MEF mutants display cell cycle defects with impaired cell cycle reentry from quiescence (Tetzlaff et al., 2004).
<i>D14Abb1e</i>	+100%	Unknown	<i>Retinblastoma-associated protein 140 (Rap140)</i> Tumor Antigen expressed in Cutaneous T-cell Lymphoma, Leukemia, and Melanoma (Eichmuller et al., 2001).
<i>Hsf2bp</i>	+100%	Transcriptional Regulation	Modulates transcriptional activity of <i>Heat Shock Factor 2</i> in testis (Yoshima et al., 1998).
<i>Hoxb1</i>	---	Transcriptional Regulation	Developmentally important homeobox transcription factor. Implicated in neuronal development (Arenkiel et al., 2004).
<i>Igf2bp1</i>	+99%	Nucleic Acid Binding	High expression in embryonic development and in <i>CD34+</i> cord blood samples. Re-expression in 5'-azacytidine

			treated adult <i>CD34+</i> BM samples. Expressed in breast cancer (Ioannidis et al., 2005).
<i>Jarid2</i>	+100%	Chromatin Remodelling	Transcriptional repressor required for neural tube formation and normal heart development (Takeuchi et al., 1999).
<i>Mef2a</i>	-49%	Transcriptional Regulation	Activates transcription of muscle specific genes. Mutations in <i>Mef2a</i> associated with Coronary artery disease (Bhagavatula et al., 2004).
<i>Parp1</i>	+100%	DNA Repair	Mediates <i>Nad+</i> dependent transcriptional repression of chromatin (Kim et al., 2004b).
<i>Phb</i>	+98%	DNA Metabolism	Induces transcriptional activation of <i>Trp53</i> . Mutations in <i>Phb</i> associated with sporadic breast cancer (Fusaro et al., 2003; Manjeshwar et al., 2003).
<i>Phc1</i>	+100%	Transcriptional Repression	Component of Polycomb Repressive Complex 1 (PRC1)/Maintenance complex. Implicated in self-renewal of hematopoietic stem cells (Ohta et al., 2002) and cardiac morphogenesis (Shirai et al., 2002).
<i>Phc3</i>	-40%	Transcriptional Repression	Component of Polycomb Repressive Complex 1 (PRC1)/Maintenance complex.
<i>Rara</i>	+49%	Transcriptional Regulation	Responsive to morphogen Retinoic Acid. Controls cell function by direct regulation of gene expression. Implicated in Acute Promyelocytic Leukemia due to translocation with PML (de The et al., 1991).
<i>Rest</i>	+76%	Transcriptional Repression	Maintains neural stem cells in undifferentiated state (Ballas et al., 2005). Modulates chromatin plasticity of neuronal precursors (Ballas et al., 2005). Putative tumor suppressor in mammary epithelia (Westbrook et al., 2005).
<i>Rnf134</i>	+100%	Transcriptional Repression	Polycomb group protein, interacts with <i>Rnf2</i> (Akasaka et al., 2002).
<i>Sall4</i>	+99%	Transcriptional Regulation	Spalt transcription factor implicated in Okihiro Syndrome, which is phenotypically characterized by forearm malformations. Recently identified role in <i>Xenopus</i> limb development and regeneration (Borozdin et al., 2004; Neff et al., 2005).

<i>Sh3glb1</i>	-78%	Induction of Apoptosis	<i>Bax interacting factor (Bif1)</i> is a proapoptotic factor necessary for the regulation of outer mitochondrial membrane morphology (Karbowski et al., 2004).
<i>Shmt1</i>	+99%	Glycine hydroxymethyl transferase activity	Polymorphisms in <i>Shmt1</i> implicated in Acute Lymphocytic Leukemia (Skibola et al., 2002).
<i>Tcf4</i>	---	Transcriptional Regulation	bHLH transcription factor involved in repression of brain specific Fgf1 (Liu et al., 1998).
<i>Tdh</i>	+100%	l-threonine dehydrogenase activity	Aberrantly methylated in breast cancer (Miyamoto et al., 2005).
<i>Tdrd7</i>	-95%	Nucleic Acid Binding	Tudor domain containing protein which interacts with Pictaire 2 to facilitate terminal differentiation of neurons. Tudor domains play a role in diverse cellular functions such as response to DNA strand breaks (Hirose et al., 2000; Stucki and Jackson, 2004).
<i>Trp53</i>	+90%	DNA Damage Response; Transcriptional Regulation	Cell cycle regulation with divergent roles in growth arrest or apoptosis dependent upon cellular context. Mutated or inactivated in approximately 60% of human tumors. Recent role described for Trp53 in the differentiation of ESCs via transcriptional repression of Nanog (Lin et al., 2005b).

CHAPTER 3

An Akt-Oct4-Hmgb2 Feedback Loop Regulates Pluripotency

Purpose

Oct4 is a key regulator of the transcriptional network that establishes and maintains the pluripotent state. Upon differentiation, however, alterations in external stimuli impact the architecture of this network and results in the spatio-temporal expression of genes required for tri-germlayer formation. Since Oct4 function is required in pluripotency and differentiation, our goal was to characterize changes to the Oct4 regulatory network that occurred during early differentiation. We hypothesized that this might lead to an enhanced mechanistic understanding of how cell fate transitions occur.

As a result of these studies we found that Akt promoted Oct4 phosphorylation and stability. This maintained interaction with Akt, Hmgb2 and other members of the SET complex such as Nme1 and Set. Upon differentiation, when Akt was inactive, loss of Oct4 phosphorylation resulted in their decreased interaction. A functional genomics approach was then employed to determine the relevance of Oct4 interaction with Hmgb2 in light of its described role as a Polycomb Group recruiter in *Drosophila*. Hmgb2 colocalized with Oct4 at repressed loci but its knockdown resulted in decreased Akt signaling and decreased expression of Oct4 transcriptional targets. Loss of Oct4, Hmgb2, and H3K27me3 enrichment and increased transcriptional activity at bivalent domains were observed only upon loss of Oct4 sumoylation, which also resulted in establishment of G1 checkpoint function. Expression at these loci however, required Nme1 function. Taken together, our results suggest that Oct4 and the SET complex participate in a PTM-dependent feed forward loop that promotes Akt signaling and transcriptional poise at developmentally regulated loci.

Contribution of Co-authors

Pearl Campbell conceived and designed the experiments.

Pearl Campbell performed all of the experimental work and analysis.

An *Akt-Oct4-Hmgb2* Feedback Loop Regulates Pluripotency

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Running Title: The Oct4-Akt-Hmgb2 Regulatory Loop

Keywords: *Oct4*, *Akt*, *Hmgb2*, bivalent domains, transcriptional repression, pluripotency, SET Complex

Abstract

In pluripotent stem cells, bivalent domains mark the promoters of developmentally regulated loci. Histones in these chromatin regions contain coincident epigenetic modifications of gene activation and repression. How these marks are transmitted to maintain the pluripotent state in daughter progeny remains poorly understood. Our study demonstrates that Oct4 post-translational modifications form a positive feedback loop, which promote Akt activation and interaction with Hmgb2. This preserves the H3K27me3 modification in daughter progeny and defers DNA damage processing. However, if Oct4 is not phosphorylated, a negative feedback loop is formed that inactivates Akt and initiates the DNA damage response. Oct4 sumoylation is subsequently required to promote G1/S progression and transmission of the repressive H3K27me3 mark following DNA replication. Therefore, post-translational modifications regulate the ability of Oct4 to direct the spatio-temporal formation of activating and repressing complexes to orchestrate chromatin plasticity and pluripotency.

Introduction

Embryonic stem cells (ESCs) are marked by the presence of bivalent domains at the promoters of developmentally regulated loci (Bernstein et al., 2006). These domains contain coincident epigenetic modifications of Trithorax Group (TrxG) gene activation (H3K4me3) and Polycomb Group (PcG) repression (H3K27me3). Although RNA Polymerase II (RnapII) initiates at the promoters of these repressed loci, formation of a productive transcriptional elongation complex does not follow (Guenther et al., 2007). Their de-repression upon downregulation of Oct4 (Ivanova et al., 2006; Matoba et al., 2006) or PcG (Boyer et al., 2006; Erhardt et al., 2003; Walker et al., 2010) suggests that bivalent domains contain the regulatory machinery required to inform subsequent stages of cellular differentiation. Their expression, however, is held in check by Oct4-PcG regulation of chromatin structure.

ESCs may self-renew, engage in a transitory arrest in cell cycle progression, differentiate, or die, in the course of every cell division. Intrinsic and extrinsic signals direct these choices by fine-tuning stochastic processes in operation during steady state. Active signal transduction cascades then impact the cell's ability to engage transcriptional programs that lead to phenotypic change (Arias and Hayward, 2006). G1 and G2 DNA damage checkpoints are critical nodes during this process. Since Akt signaling regulates these checkpoints, it plays a fundamental role in maintaining ESC pluripotency and modulating cell fate decisions (Chang et al., 2003; Kimura and Nakano, 2009; Watanabe et al., 2006). Nevertheless, preservation of the H3K27me3 mark on chromatin throughout G2/M is sufficient to transmit PcG repression in the daughter cell (Hansen et al., 2008). The stable inheritance of this mark and its relationship to Akt signaling, however, remains poorly

understood since PcG proteins do not associate with mitotic chromosomes (Aoto et al., 2008).

In this study, we investigated the role of Oct4 phosphorylation and sumoylation in maintaining the pluripotent state. We found that Akt-mediated phosphorylation of Oct4 created a positive feedback loop, which facilitated interaction with Hmgb2 and abrogated checkpoint function. When Oct4 is not phosphorylated, however, a negative feedback loop resulted in Akt inactivation and initiation of the DNA damage response. Despite the observed upregulation of Trp53 and Cdkn1a, Oct4 sumoylation resulted in decreased G1 checkpoint function. This maintained H3K27me3 repression at most developmentally regulated loci. Oct4 sumoylation, therefore, presented a physical barrier to RnapII passage and maintained plasticity at remodeled sites. Such plasticity was required to mark sites that may be activated until the next chromatin state becomes fixed. Consequently, Oct4 sumoylation functions to propagate the H3K27me3 mark in cellular progeny.

Results

Oct4 protein expression is dependent upon PI3K/Akt signaling

Oct4 is a key regulator of the pluripotency transcriptional network, which upholds pluripotency by two decisive mechanisms. First, it maintains repression of developmentally regulated loci and differentiation inducing genes. Second, *Oct4* and its concerted regulatory network impact the expression of genes that are required for cell cycle progression and cellular survival. Upon differentiation, however, alterations in external stimuli result in the coordinate regulation of gene expression programs that promotes tri-germ layer formation and further development of the embryo. Since *Oct4* is required in both pluripotency and differentiation (Thomson et al., 2011), our goal was to characterize early changes in the *Oct4* transcriptional regulatory network which might provide mechanistic understanding of its divergent roles in these processes.

To gain insight into the factors responsible for *Oct4* function, we first assessed its expression in pluripotent murine ESCs (mESCs; ES) and during the initial stages of differentiation. The strategy selected was LIF withdrawal, decreased serum stimulation, and growth as aggregates called embryoid bodies (EBs). For our studies, this model system was preferred since it recapitulates many aspects of *in vivo* differentiation within the developing blastocyst (Doetschman et al., 1985; Shen and Leder, 1992). In mESCs, *Oct4* protein may exist as a doublet (Fig. 1A). During early differentiation (at six to twelve hours), however, a transient increase in *Oct4* protein was observed. Afterward, *Oct4* protein expression gradually decreased. In contrast to the changes observed in *Oct4* protein, relatively stable expression of its transcript was observed throughout the time-course (Fig. 1B). This

discrepancy suggested that Oct4 stability might be affected at the post-transcriptional and/or post-translational level.

Pluripotent cells depend upon activation of several signal transduction cascades to maintain their undifferentiated state (Burdon et al., 2002; Vallier et al., 2009). In particular, activation of PI3K/Akt signaling appears to be a key regulatory node in maintaining the signaling networks required for pluripotency since it fosters cell cycle progression and cellular survival in these primitive cells. Activation of Akt occurs by phosphorylation at two sites, Threonine 308 (Thr308) and Serine 473 (Ser473). We therefore examined Akt signaling in the differentiation time-course previously described, by detection of these modified residues. A moderate level of Akt phosphorylation was observed in mESCs, (Fig. 1C). Following differentiation however, decreased phosphorylation at both Thr308 and Ser473 was observed. A transient increase in pAkt Thr308 was observed at eighteen hours. By twenty-four hours, however, Akt signaling was virtually inactive and remained so even after forty-eight hours.

Akt activation status paralleled the amount of Oct4 protein observed in our time-course. We therefore hypothesized that it might act to sustain Oct4 expression. To assess the impact of PI3K/Akt signaling upon Oct4 protein, we used LY294002 (LY), a potent inhibitor of this pathway. Treatment of mESCs with LY resulted in decreased Oct4 protein, especially the lower band of the doublet (Fig. 1D). Inhibition of the 26S Proteasome with MG132, however, resulted in increased detection of the upper band of the Oct4 doublet. Combined treatment with LY and MG132 resulted in increased detection of both upper and lower bands of the doublet. Taken together, this data suggested that Akt signaling is required for Oct4 protein stability. Since LY may also target other signaling pathways at similar concentrations

(Searl and Silinsky, 2005), we directly assessed the impact of Akt upon Oct4 protein by treatment with Akti1/2, a potent and specific Akt inhibitor (DeFeo-Jones et al., 2005). The mESCs were treated with increasing amounts of Akti1/2. This resulted in a dose-dependent reduction of Akt phosphorylation at Thr308 and Ser473 (Fig. 1E). A slight decrease in phosphorylation of two Akt targets, GSK3 α and GSK3 β were also observed, although expression of their total protein amounts remained relatively stable. Markedly decreased detection of Oct4 was also observed at the highest levels of Akti1/2 treatment. Taken together, our data suggests that Oct4 protein expression is dependent upon Akt signaling. Moreover, since inhibition of the 26S Proteasome by MG132 resulted in increased Oct4 protein, our data may suggest that Akt signaling either directly or indirectly impacts Oct4 protein stability. However, since Akt signaling is also implicated in translational control (Ruggero and Sonenberg, 2005) it is possible that Akt inhibition may impact translation of Oct4 protein. The increased detection of Oct4 protein observed in the LY + MG132 treatment condition, however, favours the former hypothesis.

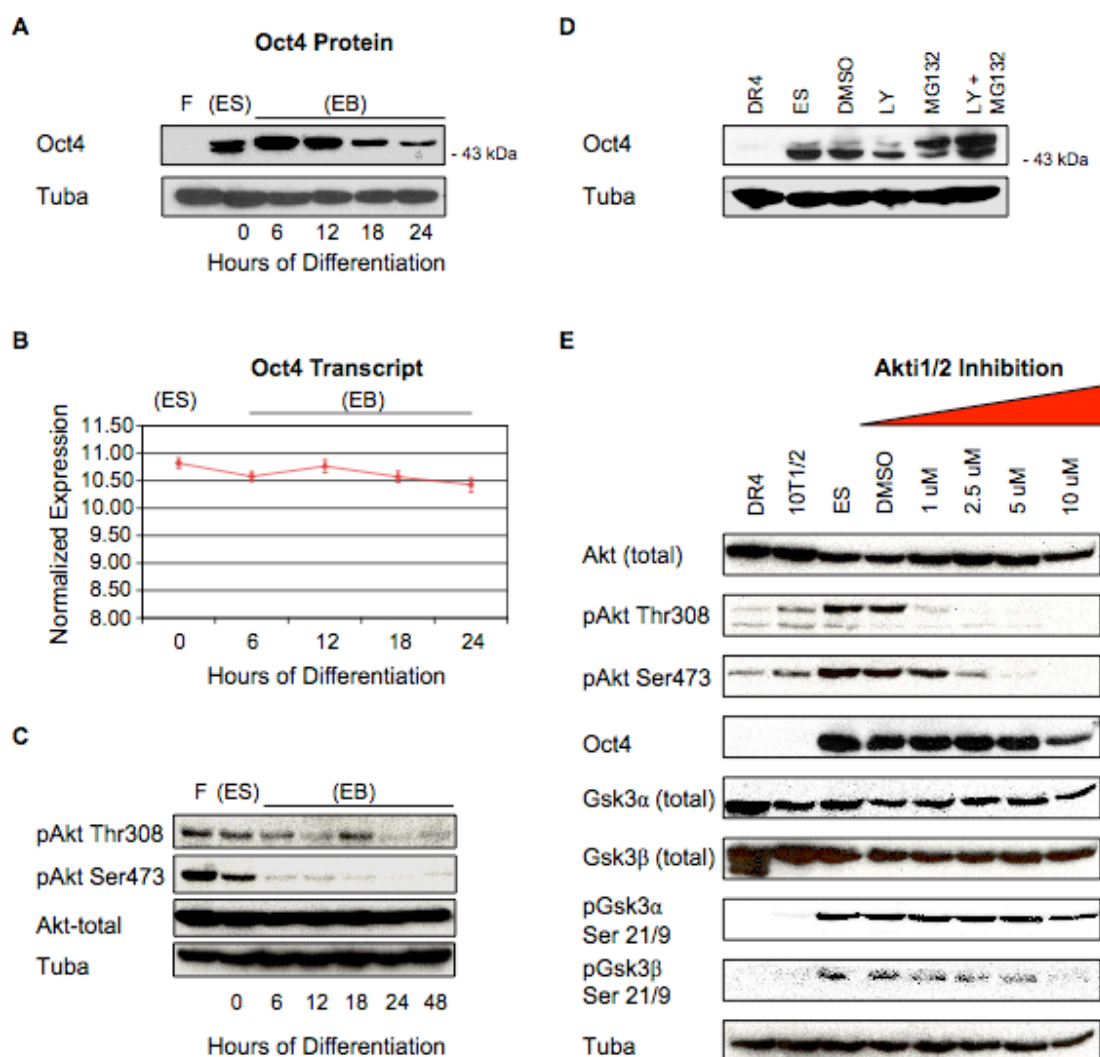


Figure 1. Oct4 protein expression is dependent upon PI3K/Akt signaling.

(A) Western analysis of Oct4 protein in mESCs (ES) and in early differentiation as embryoid bodies (EBs). The lane designated 'F' is protein lysate from 10T1/2 fibroblasts here and throughout. Tuba is shown as a loading control here and throughout. (B) Quantiles normalized Oct4 transcript expression from J1 mESC differentiation time-course analyzed by Affymetrix GeneChip. Normalized values represent the mean of three biological replicates $\pm$ SEM. (C) Western analysis of Akt and phosphorylated forms of Akt detected in ES-EB differentiation time-course. (D) Western analysis of Oct4 protein in ES, control (DMSO), 10 μ M LY (LY294002), 10 μ M MG132, and combined 10 μ M LY and MG132 treated for one hour with the indicated inhibitors. (E) Western analysis of the indicated proteins in mESCs (ES) and following treatment with specific Akt inhibitor Akt1/2 at the indicated concentrations for 4 hours. (The lane designated DR4 is protein lysate from mitotically inactivated DR4 fibroblasts, used as a feeder layer in mESC culture.) Oct4 protein levels are greatly decreased following Akt inhibition. Only slightly decreased phosphorylated Gsk3 is observed.

Akt phosphorylates Oct4

Analysis of Oct4 protein domain structure reveals that it contains a consensus Akt phosphorylation site. The motif spans the flexible linker region and extends into the POU_H (Fig. 2A). The extensive cross-species conservation implies a regulatory function for this domain. Since our results suggested that Akt signaling stabilized Oct4 protein we next determined whether Oct4 is a direct Akt phosphorylation target, by immunoprecipitation-kinase (IP-kinase) assays. First, 10T1/2 fibroblasts were exposed to Ro-31-8220, an Akt agonist (Therese et al., 2006). This resulted in heightened Akt activation (Fig. 2B). Next, the activated Akt was immunoprecipitated from the lysates using a pAkt Ser473 antibody. On-bead kinase assays were first performed using Gsk3 β , a well-known Akt target (Fig. 2C). The phosphorylated form of Gsk3 β was detected with a pGsk3 β Ser21/9 antibody following exposure of the recombinant protein to the immunoprecipitate. Phosphorylation of Gsk3 β , however, was not observed in the sample that was not exposed to Akt. Having confirmed the functional activity of our lysates, we next performed a replicate on-bead IP-Kinase assay using recombinantly produced C-terminal 6x His-TEV-3x FLAG epitope-tagged (CTAP) wild-type and T228A mutant forms of Oct4. The wild-type construct retained the consensus Akt motif. The T228A construct, however was phospho-deficient since the acceptor Thr residue was mutated (Fig. 2D). Following the *in vitro* kinase assay, the samples were detected with an anti-FLAG antibody to assess Oct4 expression. Specific antibodies to phosphorylated Oct4 T228 are not available. Therefore, we utilized the pAkt Substrate antibody to indicate the phosphorylated form of Oct4. This antibody detects proteins containing Akt consensus motifs, but only when they are phosphorylated. The pAkt substrate antibody detected wild-type Oct4 after exposure to activated Akt. It did not however,

visualize the T228A mutant form or the wild-type version not exposed to the activated Akt. Our results strongly suggested that Oct4 is a direct Akt phosphorylation target and that T228 is the site of phosphorylation.

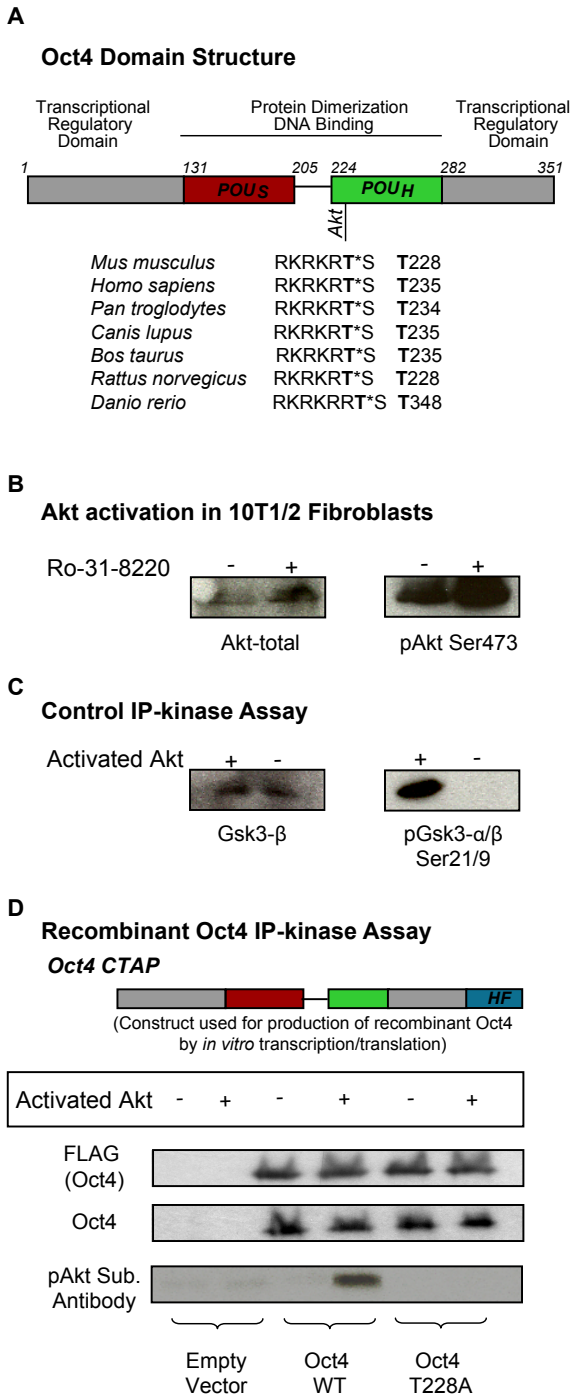


Figure 2. Oct4 is a direct phosphorylation target of Akt.

(A) Schematic of Oct4 protein domain structure. POU_S and POU_H denote the POU-specific and POU-homeo domains respectively. Together these domains, and the flexible linker region (denoted by straight line between POU_S and POU_H), comprise the protein dimerization and DNA binding domains of Oct4. A consensus Akt motif spans part of the linker region and the first 5 amino acids of the POU_H. T228 is the consensus Akt phosphorylation site in mouse. This site exhibits extensive cross-species conservation. (B) Western analysis of lysates from 10T1/2 fibroblasts treated with Akt

activator Ro-31-8220 detected with anti-total Akt and pAkt Ser473 to indicate Akt activation status. (C) Control IP-kinase assay using a pAkt Ser473 antibody to immunoprecipitate activated Akt. The immunoprecipitate was exposed to recombinant Gsk3 β protein, a well-known Akt target. Subsequently, an on-bead in-vitro kinase assay was performed. Western analysis using an anti-phospho Gsk3 α/β antibody was used to detect the phosphorylated form of Gsk3 following exposure to activated Akt. (D) IP-kinase assay performed as above except using recombinant Oct4 wild-type and T228A mutant recombinantly produced by *in vitro* transcription/translation of the Oct4 CTAP epitope tagged construct shown. The CTAP vector contains a 6x His -TEV - 3x FLAG epitope tag. An anti-FLAG epitope tag antibody detects the recombinantly produced Oct4. The anti-pAkt Substrate antibody (pAkt Sub.) detects wild-type, but not the Oct4 T228A mutant following the on-bead IP-kinase assay.

Oct4 interacts with PcG recruiter Hmgb2 in an Akt-dependent manner

Our results suggested that Oct4 protein expression was dependent upon Akt signaling. The increased Oct4 expression observed at six hours, however, was at variance with the decrease in Akt activation status observed (Fig. 1C). To resolve these discrepant findings and further substantiate the link between Akt signaling and Oct4 protein expression we first examined Oct4 phosphorylation status at three time-points. Immunoprecipitation of endogenous Oct4 protein followed by detection with the pAkt substrate antibody revealed that low-levels of Akt-phosphorylated Oct4 were present in mESCs (Fig. 3A, upper panel). At six hours of differentiation, however Akt-mediated phosphorylation of Oct4 slightly increased. By twenty-four hours of differentiation, phosphorylated Oct4 was not detected. The pattern of Oct4 phosphorylation was also reflected in the amount of total Oct4 protein observed (Fig. 3A, middle panel and Fig. 1A). By twenty-four hours of differentiation a vast reduction in global phosphorylated Akt substrates was detected by the pAkt Substrate antibody.

The observed increase in phosphorylated Oct4 could indicate that Akt-mediated phosphorylation of Oct4 increased during early differentiation. This might suggest a transient increase in Akt phosphorylation prior to the six-hour time-point. To clarify, we assessed expression of Akt and its phosphorylated forms over an abbreviated three-hour time-course. Consistent with our hypothesis, increased levels of activated Akt were observed from thirty minutes to three hours of differentiation (Fig. 3B). These data suggests that the increase in Oct4 protein expression is due to heightened Akt signaling resulting in increased phosphorylation of Oct4. However, this does not rule out the existence of other contributing factors, including decreased dephosphorylation of either Oct4 or Akt.

In a search for genes implicated in Oct4 function, we observed that Hmgb2 mRNA levels were more highly correlated to Oct4 than was its interaction partner Sox2 (83% vs. 49%) (Campbell et al., 2007). Since Hmgb2 expression is dependent upon PI3K/Akt signaling (Le et al., 2005), this suggested that Hmgb2 might be more fully integrated into the pluripotency transcriptional regulatory network than previously appreciated. Expression of Oct4, Sox2, and Hmgb2 transcript in the differentiation time-course was relatively stable as compared to two other Oct4 correlated genes, Tdgfl and Smn1 (Fig. 3C). Expression of these genes was respectively down and upregulated at six hours of differentiation, consistent with our previous analysis (Sene et al., 2007).

Hmgb2 is a Polycomb Group (PcG) recruiter in early *Drosophila* development where it is observed in large multi-protein complexes, along with Trithorax Group (TrxG) proteins (Ringrose and Paro, 2004). We hypothesized that Hmgb2 might perform an analogous role in mammalian development. Since PcG and TrxG proteins generally lack sequence specific binding, recruitment of Hmgb2 to its G(A) motif (Dejardin, 2005) might direct these complexes to specific genomic loci. Moreover, Hmgb2 interaction with Oct4 could provide a developmental context for coordination of TrxG and PcG function in pluripotent cells. An interaction between Oct4 and Hmgb2 has previously been described (Butteroni et al., 2000). Their relationship in mESCs, however, and its potential role in maintaining the pluripotency transcriptional network has not been explored. Therefore, we examined this interaction throughout our differentiation time-course to clarify its potential role in pluripotency.

IP-Westerns revealed that Oct4 interacted with two major forms of Hmgb2 in mESCs, exhibiting migration at 29 and 72 kDa (Fig. 3D). Since Hmgb2 may be extensively post-translationally modified (Zhang and Wang, 2010), this suggested that Oct4 may interact with

differentially modified forms of Hmgb2 in mESC. At six hours of differentiation, however, the interaction between Oct4 and Hmgb2 markedly decreased. By twenty-four hours, interaction was not observed. Reciprocal IP-Westerns performed in mESCs and at twenty-four hours of differentiation were performed to confirm these results. Pull-down of endogenous Hmgb2 and Akt confirmed that Oct4 interacted with each in mESCs (Fig. 3E). In differentiation, however their interaction was not observed. The Oct4 antibody also detected proteins IP'd by the pAkt Substrate antibody in mESCs, but not in EBs. Since a band with the same migration pattern was obtained when the reciprocal IP-Western was performed (Fig. 3A), this suggested that levels of phosphorylated Oct4 decreased during differentiation.

In contrast to its role in PcG recruitment during early *Drosophila* development, Hmgb2 has also been described as an integrator of diverse cellular pathways implicated in pluripotent stem cell function including transcriptional regulation, DNA damage response, chromatin structure, and cell cycle regulation. Characterization of Hmgb2 has suggested that its role within the SET Complex, consisting of Hmgb2, Set, Nme1, Ape1, and pp32, may provide insight into its multi-faceted function (Lieberman and Fan, 2003). Since Oct4 was correlated to Set in our previous study (Fig. 3C), we next determined whether it might participate in this complex. IP-Westerns revealed that Oct4 interacted with Set and Nme1 in pluripotent mESCs (Fig. 3F). Their interaction however, was not observed upon differentiation. Taken together, our data suggested that Akt signaling promoted Oct4 interaction with Hmgb2 and its interactors.

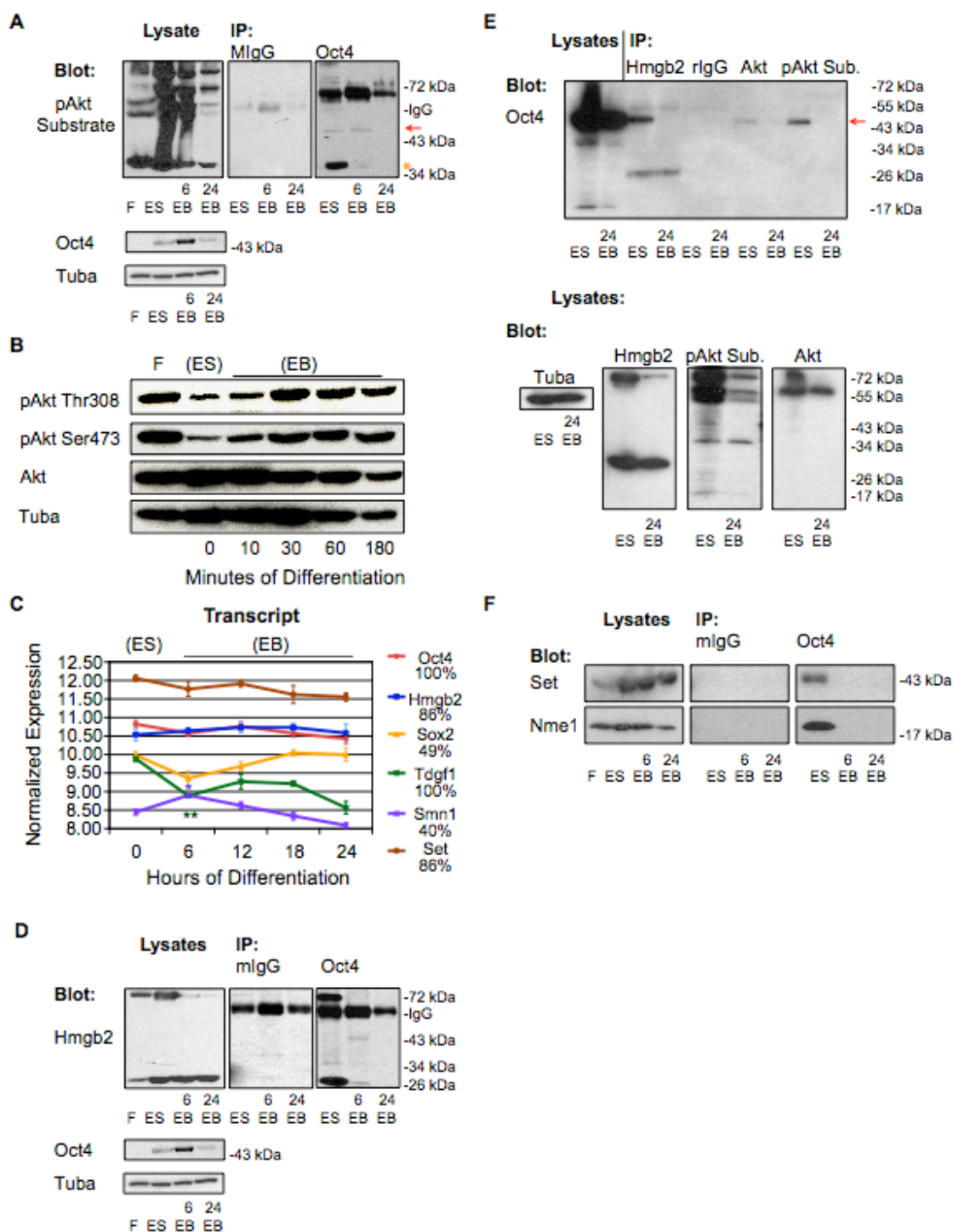


Figure 3. Akt signaling promotes Oct4 interaction with Hmgb2 and the SET Complex

(A) Endogenous Oct4 protein was IP'd from J1 mESC (ES), 6 and 24 hour EB lysates. Western analysis with the pAkt Substrate antibody detected 45 kDa and 72 kDa bands, consistent with the

molecular weight of Oct4 and sumoylated-Oct4 (Wei et al., 2007). The 45 kDa band shows increased detection after 6 hours of differentiation. The 72 kDa band is increased at 24 hours. The 34 kDa band detected (*) may be phosphorylated Sox2 (Jeong et al., 2010). This band decreases upon differentiation. (B) Expression of total and activated Akt (pAKT Thr308 and pAkt Ser473) in 10T1/2 fibroblasts and an abbreviated J1 mESC-EB differentiation time-course. (C) Quantiles normalized transcript level expression of Oct4 and correlated genes from J1 mESC differentiation time-course from Affymetrix GeneChip data. Normalized values represent the mean of three biological replicates $\pm$ SEM. Numbers in parentheses indicates degree of correlation to Oct4 from our previous study. (D) Endogenous Oct4 protein was IP'd from J1 mESC (ES), 6 and 24 hour EB lysates and analyzed using an anti-Hmgb2 antibody. Robust Oct4 interaction with Hmgb2 is observed in ES but is markedly decreased at 6 hours of differentiation. Interaction is not detected at 24 hours of differentiation. (E) Endogenous proteins were IP'd from J1 mESC, and 24 hour EB lysates with anti-Hmgb2, anti-Akt, anti-pAkt Substrate and control rIgG antibodies. These were analyzed by Western blot using an anti-Oct4 antibody. Oct4 phosphorylation and interaction with Hmgb2 and Akt is detected in mESCs but not in 24 hour EBs. Global decreases in phosphorylated Akt substrates occur during differentiation except for the 34 kDa band. Compare to Figures 3A and 3D. (F) Oct4 interacts with SET complex members Set and Nme1 in pluripotent mESCs but not in differentiating EBs by IP-Western.

Hmgb2 modulates Akt signaling and Oct4 transcriptional targets

Since Hmgb2 is implicated in PcG recruitment, we predicted that Hmgb2 would bind at Oct4 repressed loci. Two Oct4 targets were selected for our initial assessment: *Phc1* and *Hoxb1*. *Hoxb1* is a repressed Oct4 target, bound by PRC2 (Boyer et al., 2006), and marked by a bivalent domain in mESCs (Bernstein et al., 2006). The *Hoxb1* autoregulatory enhancer (ARE) regulates its expression in the fourth rhombomere of the hindbrain (Bell et al., 1999). The ARE contains clustered Oct, Sox, and G(A) binding motifs (Fig. 4A). In contrast, *Phc1* is a member of the PcG Repressor Complex 1 (PRC1), an expressed gene in mESC, lacking the repressive H3K27me3 histone modification enriched at bivalent domains. Its expression is required to maintain pluripotency (Walker et al., 2007). Sequential chromatin immunoprecipitation (ChIP) assays confirmed that Oct4 and Hmgb2 co-localized at the *Hoxb1* ARE in mESCs (Fig. 4B). By contrast, only Oct4 enrichment was seen at the *Phc1* promoter.

Since Oct4 and Hmgb2 colocalization at a bivalent Oct4 target was observed, we next wanted to assess the functional import of Hmgb2 upon regulation of Oct4 target genes. Knockdown of Hmgb2 was performed using two siRNA molecules previously shown to be effective in mouse embryonic fibroblasts (Yan et al., 2010). Decreased transcript expression of Hmgb2 was observed with both siRNAs in mESCs. The efficiency of siRNA #2 was much higher, resulting in greater than 90% knockdown of Hmgb2 mRNA (Fig. 4C). Knockdown of Hmgb2 notably, and contrary to our hypothesis, resulted in reduced expression of most Oct4 regulated genes including pluripotency regulators *Oct4*, *Sox2*, *Nanog*, bivalent domain genes *Hoxb1* and *Pax7*, and PcG members *Phc1*, as well as DNA damage response genes *Parp1*, *Brca1*, and *Trp53*. By contrast, cell cycle regulator *Cdkn1a*

was slightly upregulated. Hmgb2 protein was decreased in the knockdown cells, as was Oct4 protein (Fig. 4D). Importantly, expression of total Akt, activated Akt, and global phosphorylated Akt substrates were also decreased upon Hmgb2 knockdown (Fig. 4E), further substantiating the link between Oct4, Akt, and Hmgb2.

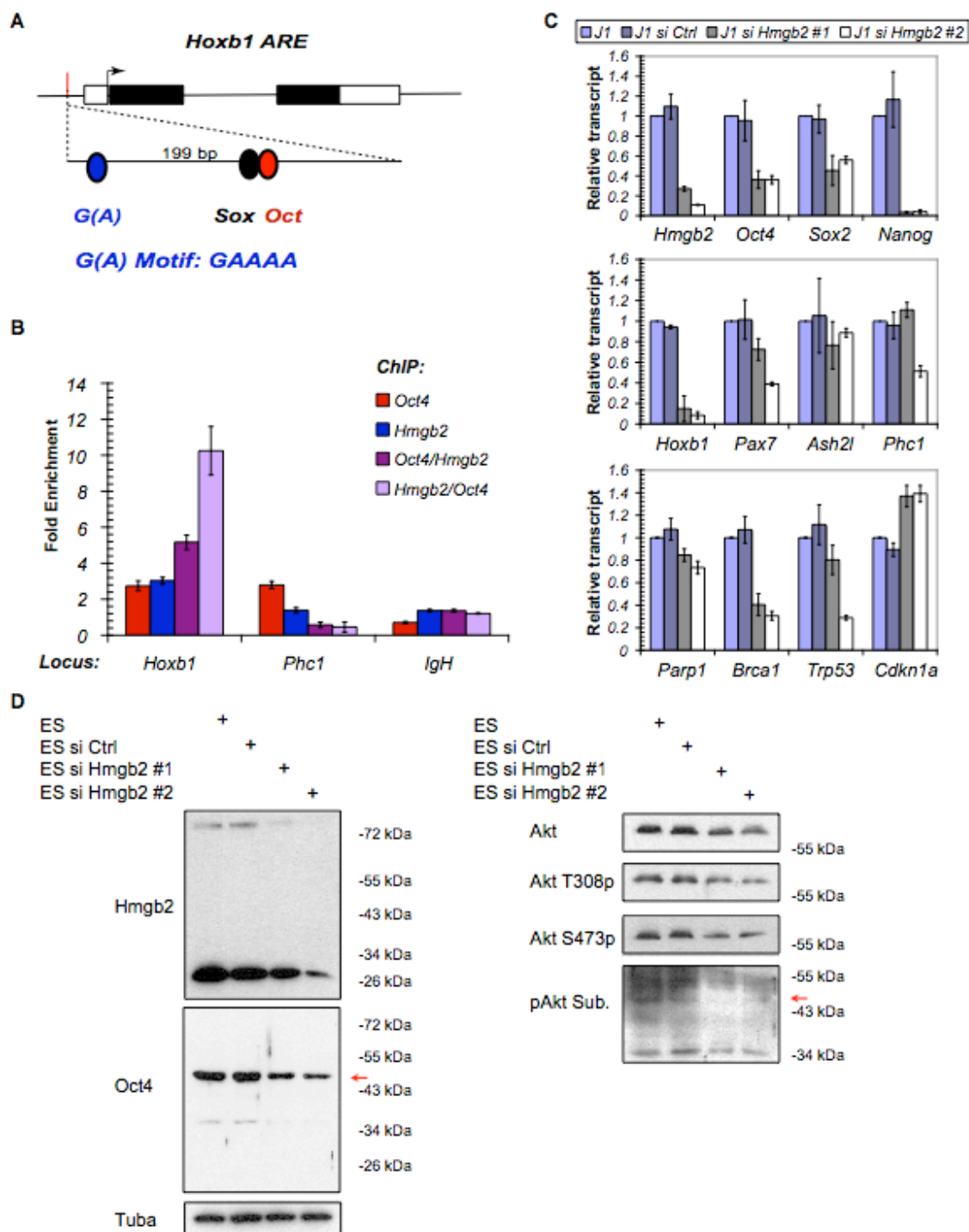


Figure 4. Hmgb2 modulates Akt signaling and Oct4 transcriptional targets.

(A) Schematic of the *Hoxb1* auto-regulatory enhancer (ARE) depicting clustered Oct, Sox, and G(A) motifs. (B) Enrichment and co-localization of Oct4 and Hmgb2 by chromatin immunoprecipitation (ChIP) and sequential ChIP-QPCR at endogenous *Hoxb1*, *Phc1*, and *IgH* loci in J1 mESC. The order

of ChIP assessment is indicated by the legend: Oct4/Hmgb2 is Oct4 for the first ChIP, Hmgb2 for the second. Fold enrichment is normalized to enrichment of negative control (rlgG) ChIP or sequential ChIP and input. Values represent the mean of biological duplicates analyzed in duplicate (n=4) $\pm$ SEM. (C) QRT-PCR analysis of Oct4 transcriptional targets following siRNA mediated knockdown of Hmgb2 in J1 mESC. Relative expression is calculated by normalization to Gapdh and expression levels obtained in J1 mESCs. Values represent the mean of biological duplicates analyzed in replicate (n=4) $\pm$ SEM. (D) Western analysis showing protein level expression of the indicated proteins following Hmgb2 knockdown. Expression of Oct4, Akt, phosphorylated Akt, and global phosphorylated Akt substrates are markedly decreased in the knock-down cells.

Oct4 phosphorylation at T228 modulates transcriptional cassettes implicated in cell cycle regulation and the response to DNA damage

Taken together, our data suggested that Oct4, Akt, and Hmgb2 participate in an interconnected gene regulatory network that maintained Akt activation, as well as expression of Oct4 transcriptional targets. However, since Hmgb2 knockdown did not result in the upregulation of bivalent domains, we hypothesized that Hmgb2 might be implicated in both activation and repression of Oct4 transcriptional targets. Previous studies have intimated that Hmgb2 may participate in context-dependent activation or repression of its target genes (Decoville et al., 2001). Therefore, we next more clearly examined the role of Oct4 post-translational modifications in providing this framework.

The *in vivo* relevance of Oct4 phosphorylation was first assessed. To this end, pooled stable mESC sublines expressing Oct4 wild-type and Akt phospho-mutant epitope-tagged constructs were generated. The T228A mutant is an Akt phospho-deficient, while the T228D is a phosphomimetic that acts as if it were constitutively phosphorylated. Analysis of the stable clones demonstrated transcript expression of the Oct4 construct. Moreover, the exogenously supplied transcript did not impact levels of pluripotency regulators Sox2 or Nanog (Fig. 5A). Total Oct4 transcript, however, appeared to be slightly downregulated in the wild-type expressing subline, consistent with previously published results that Oct4 represses its own expression (Pan et al., 2006).

Western analysis of lysates from the sublines indicated expression of the exogenously supplied Oct4 by the FLAG epitope tag antibody (Fig. 5B). Total Oct4 protein was also assessed. Increased higher molecular weight forms, consistent with sumoylated forms of Oct4, were observed in each Akt phospho-mutant subline. Despite these changes in Oct4

protein expression, consistent levels of Ssea1 expression and alkaline phosphatase staining (Supplemental Fig. S1) indicated that these cells were pluripotent. Western blotting with Ki67 and PcnA also suggested that the stable clones maintained a highly proliferative state.

Transcript analysis of Oct4 targets in these sublines revealed a profound perturbation to genes implicated in cell cycle regulation and DNA damage repair (Fig 5C). In this case, cell cycle regulator *Cdkn1a* was downregulated. By contrast, *Parp1* and *Brca1* were upregulated. *Ccnf*, which is implicated in DNA damage checkpoint (Fung et al., 2002), was also upregulated in the T228A and T228D mutants. Oct4 bivalent domain target genes such as *Hoxb1*, *Neurod1*, *Pax7*, *Myod1*, and *Cdx2*, however, displayed no change in expression.

Protein expression analysis was generally in agreement with the transcript data, confirming that the stable mutant clones initiated a DNA damage response (Fig. 5D). Upregulated expression of Trp53, Hmgb2, Parp1, and Brca1 was evident. Increased detection of global Sumo1 and Sumo2/3 conjugates may suggest that the higher molecular weight forms of Hmgb2, Trp53, and Parp1 is due to their modification by Sumo (Wrighton, 2010). Increased Brca1 and γ H2ax S129p, which recruit repair proteins to sites of DNA damage, were also observed. The T228A mutant expressing sublines in particular, appeared to have activated a very strong stress response as was indicated by the very high levels of Brca1 (Starita and Parvin, 2003) and Ser15 phosphorylated Trp53 (Shieh et al., 1997). Akt T308p staining indicated heightened Akt activation in the Oct4 wild-type clones. The T228D mutants retained similar levels of activated Akt as the untransfected or empty vector control mESCs. In the T228A mutants, Akt was detected primarily in its inactive form. This reinforced our hypothesis that Akt signaling through Oct4 provided protection from DNA damage induced cellular stress.

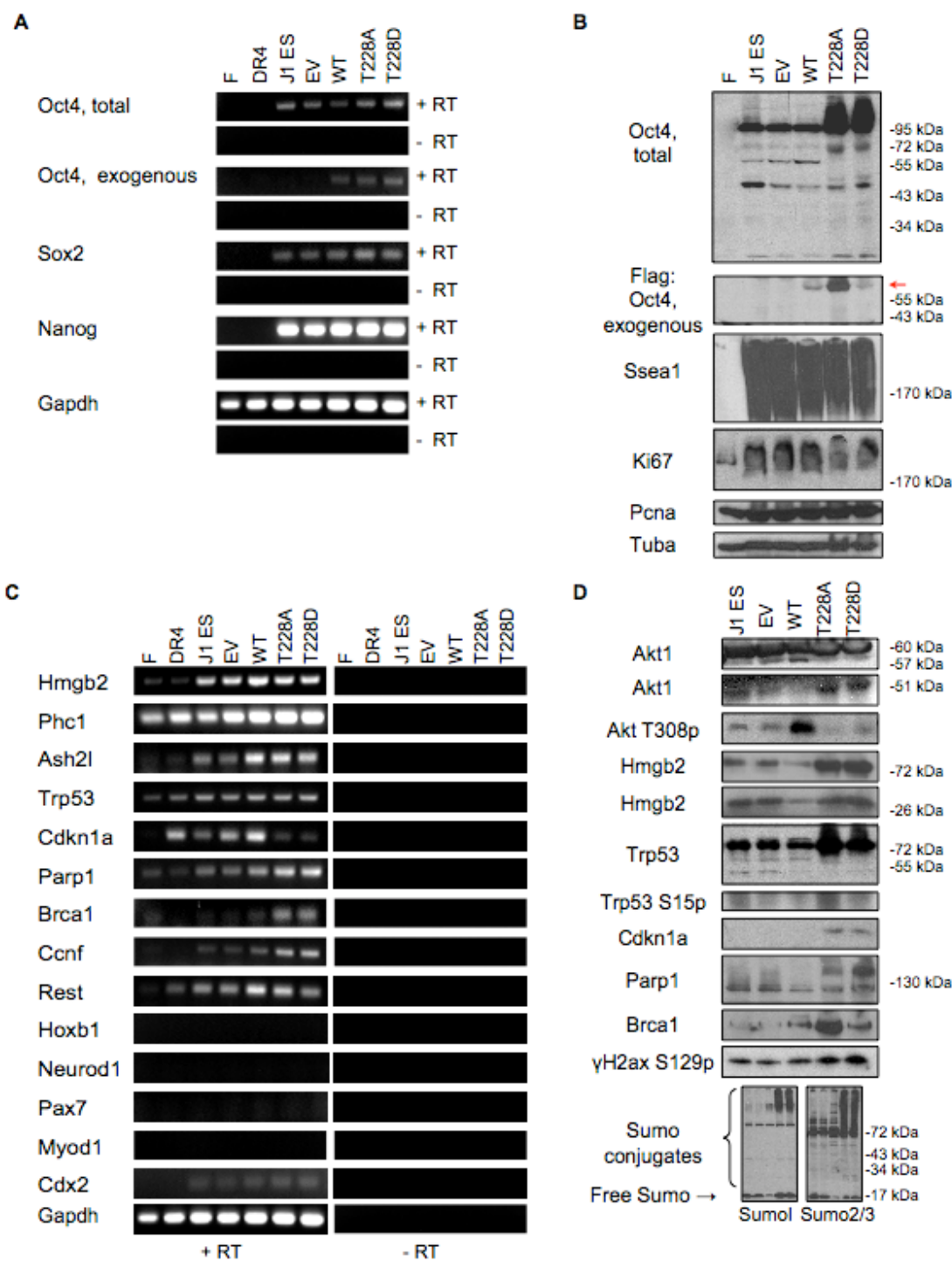


Figure 5. Oct4 phosphorylation modulates Hmgb2 and Akt.

(A) RT-PCR analysis of pooled stable J1 mESC clones expressing the indicated Oct4-CTAP epitope tagged constructs. Exogenous expression of wild-type or mutant Oct4 does not appear to impact

expression of Sox2 or Nanog. Expression of total Oct4 appears to be slightly downregulated in the Oct4 wild-type expressing cells. (B) Western analysis of replicate samples as in A, above. Expression of exogenous and total Oct4 protein is detected in the stable clones. Red arrow denotes the exogenously expressed Oct4. Constant level detection by anti-Ki67 and anti-Pcna antibodies suggests that the cells remain in cycle. Ssea1 expression is used as a surrogate marker to indicate pluripotency. (C) RT-PCR analysis detects altered expression profiles for Oct4 transcriptional targets implicated in DNA damage and cell cycle in the wild-type, phospho-deficient (T228A) and phosphomimetic (T228D) transfected cells. Expression of bivalent domain containing genes such as *Hoxb1*, *Neurod1*, *Pax7*, *Myod1*, and *Cdx2* however, remain unchanged. (D) Western analysis of the stable clones with the indicated antibodies that exogenous expression of wild-type Oct4 leads to increased activated Akt (pAkt Thr308). Expression of either T228 mutant form however, leads to increased expression of DNA damage response genes. Increased detection of Sumo1 and Sumo2/3 modified conjugates are also detected in the mutant expressing cells.

Sumoylated Oct4 maintains cell cycle progression and a bivalent chromatin structure

Since Oct4 phosphorylation status did not impact the expression of bivalent domain containing transcriptional targets, we next examined the role of Oct4 sumoylation in maintaining their repression. Post-translational modification by Sumo1 is generally dependent upon the recognition of a Sumo consensus motif -- Ψ KXE-- contained within target substrates (Sampson et al., 2001). Ψ represents a hydrophobic amino acid, whereas X represents any amino acid. Sumoylation of Oct4 at K118 has previously been described (Tsuruzoe et al., 2006; Wei et al., 2007; Zhang et al., 2007). However, Oct4 contains two consensus Sumo motifs (VKLE--K118 and CKSE--K215). Since sumoylation is implicated in transcriptional repression (Gill, 2005), a series of mutant constructs was built to test the hypothesis that sumoylation of Oct4 maintains PcG repression. The K118R and K215R mutants abrogated not only sumoylation, but also every other potential PTM which may occur at the indicated lysine residue. The V117A and C214A constructs however, abrogated PTM by sumoylation only since the hydrophobic residues required as part of the consensus Sumo motif were mutated while the lysine residues remained intact. If sumoylation were required for Oct4 function as a transcriptional repressor, then increased transcriptional activity from Oct4 regulated loci, with a concomitant decrease in H3K27me₃, would be expected upon expression of the mutants.

Assays using the *Oct4* constructs, co-transfected with the *Hoxb1* ARE luciferase reporter into mESCs (Figure 6A), demonstrated that wild-type *Oct4* decreased activity from the luciferase reporter (Fig. 6B). The opposite however, was observed when any mutant form was substituted. The lysates from these experiments were analyzed by Western (Supplemental Fig. S2). Similar results to those obtained in mESC were obtained with a

heterologous system. Co-transfection of *Hmgb2* and wild-type *Oct4* with the *Hoxb1* reporter in fibroblasts resulted in decreased activity when compared to transfection with *Oct4* alone; the combination of *Hmgb2* with the K118R mutant produced a significant increase (Supplemental Fig. S3A-C).

Notably, a synergistic transcriptional effect was observed when *Oct4* and *Hmgb2* were co-transfected into fibroblasts. The wild-type and K118R mutant did not elicit a differential transcriptional response on their own. Co-transfection of wild-type *Oct4* with *Hmgb2*, however, resulted in increased repression. Similarly, the ability of the K118R mutant to elicit a positive transcriptional response was enhanced in the presence of *Hmgb2*. Taken together, these results may suggest that *Oct4* function as either a transcriptional activator or repressor depends upon *Hmgb2*. *Hmgb2* may thus be a limiting cofactor for *Oct4* in fibroblasts. Conversely, it appeared that *Oct4* sumoylation provided a context-dependent cue for *Hmgb2* function as either a transcriptional activator or repressor.

ChIP assays were next performed to examine the effects of *Oct4* sumoylation upon regulation of the endogenous *Hoxb1* locus in mESC. Decreased *Oct4*, *Hmgb2*, and H3K27me3 enrichment were observed at the *Hoxb1* ARE upon expression of the K118R mutant whereas, wild-type *Oct4* expression resulted in their increased enrichment (Fig 6C-D). Consistently, increased *Hoxb1* transcript expression was observed in the K118R mutant cells (Fig. 6E; the legend is provided in 6F).

Several other *Oct4* target genes were examined to substantiate the sumoylation-dependent role of *Oct4* in maintaining transcriptional repression. Comparable results were found for *Nanog*, *Sox2*, *Cdx2*, *Hand1*, *Gata4*, *Pax7*, *Mef2a*, *Rest*, *Ash2l*, and *Phc3* (Supplementary Figs. S4 and S5). *Eomes*, *Gata6*, and *Myod1*, however, were not regulated in an equivalent

manner. By contrast, despite the observed increase in activity from the *Phc1* luciferase reporter, the endogenous *Phc1* locus was negatively modulated by expression of the K118R mutant (Supplemental Fig. S6A-B). Since the luciferase reporter only contained regulatory elements from the *Phc1* promoter, it was conceivable that other regulatory elements, either in cis, or in trans, might be responsible for these discordant findings.

To clarify, additional primers tiling along the *Phc1* transcript were designed (Supplemental Fig. S6C). RT-PCR analysis with these primers indicated that low level expression of *Phc1* transcript was observed in the K118R mutant sublines but only up to exon 2. Intron 2 of *Phc1* notably contains adjacent POU_s and G(A) motifs (Supplemental Fig. S6D). ChIP primers were designed to tile down this putative regulatory region of *Phc1* to assess Oct4, Hmgb2, RnapII, and H3K36me3 enrichment. ChIP assays confirmed increased Oct4 and Hmgb2 enrichment surrounding the putative +2337 regulatory element in the K118R mutant subline (Supplemental Fig. S6E). As well, RnapII appeared to accumulate in the mutant cells despite the decreased expression, suggesting that a pause in polymerase activity was induced at this locus. We next assessed H3K36me3 enrichment tiling down the *Phc1* locus. Generally, H3K36me3 marks loci that are actively undergoing transcription (Morris et al., 2005). It is also implicated in alternative splicing, transcriptional repression, and DNA repair (Wagner and Carpenter, 2012). Tiling ChIP-PCR assays confirmed that H3K36me3 extended into the *Phc1* locus in the K118R mutant subline (Supplemental Fig. S6G). Finally, increased H2AK119Ubn enrichment was observed in the mutant expressing cells, suggesting that a DNA damage response had been initiated at this locus, since H2AK119Ubn is one of the earliest epigenetic changes observed during DNA damage (Bergink et al., 2006; Celeste et al., 2003).

A more extended examination of the *Cdx2*, *Hoxb1*, and *Phc1* loci was performed in the K118R mutant subline. This analysis revealed alterations in PcG, TrxG, and RnapII enrichment (Supplementary Fig. S7A-C). Taken together, our results suggested that sumoylation of Oct4 is a key mechanism to maintain a stalled RnapII and transcriptional repression in mESCs. However, since all bivalent domains were not de-repressed in the Sumo mutant expressing cells, this indicated that additional factors mediated repression at Oct4 bound loci.

DNA damage is implicated in activation of cell cycle checkpoints (Kastan and Bartek, 2004). Embryonic stem cells, however do not undergo the typical cell cycle regulation observed in more differentiated cells, and are devoid of checkpoint function (Burdon et al., 2002). Nonetheless, upon differentiation cell cycle checkpoints become active (White et al., 2005). Since an early marker indicative of DNA damage was observed in the K118R mutant subline, we examined cell cycle in wild-type and Sumo mutant mESC sublines.

Enumeration of DAPI stained nuclei in the mESC sublines revealed a significant decrease in mitotic index in the Oct4 wild-type expressing cells (Fig. 6G). By contrast, the K118R mutant subline displayed a significant increase. Inspection of DAPI stained K118R mutant clones revealed that mitotic delays occurred at prometaphase and at the anaphase to telophase transition (Supplementary Fig. S8).

To more clearly attribute the cell cycle impediments to sumoylation of Oct4 at K118, an additional construct was employed. The V117A mutant exchanged the hydrophobic residue at the -1 position for alanine, which is very weakly hydrophobic. While the K118R mutant would result in loss of any post-translational modification at K118, the V117A mutant would impact only sumoylation at K118. Analysis of asynchronous mESC sublines expressing

wild-type, K118R, or V117A mutant Oct4 constructs exhibited significant alterations in their cell cycle profiles (Figure 6H). Wild-type expressing clones exhibited increased S-phase population (82.74% vs. 76.02% $p \leq 5 \times 10^{-4}$). The K118R mutant expressing cells had an elevated G2/M population (18.25% vs. 0.2025% $p \leq 5 \times 10^{-6}$). The V117A clones exhibited a highly statistically significant G1 population (95.77% vs. 20.13% $p \leq 5 \times 10^{-7}$).

Our results suggested that both G1 and G2 cell cycle checkpoints were activated by stable expression of the Oct4 mutants. The APC/C regulates mitotic spindle assembly checkpoints and is also implicated in G1 cell cycle progression (Sudo et al., 2001). The altered protein level expression of APC/C substrates Ccnb1, Cdkn1a, Ccne1, and p55Cdc in the mutant expressing cells (Fig. 6I) is therefore consistent with the delayed cell cycle progression phenotypes observed in these cells.

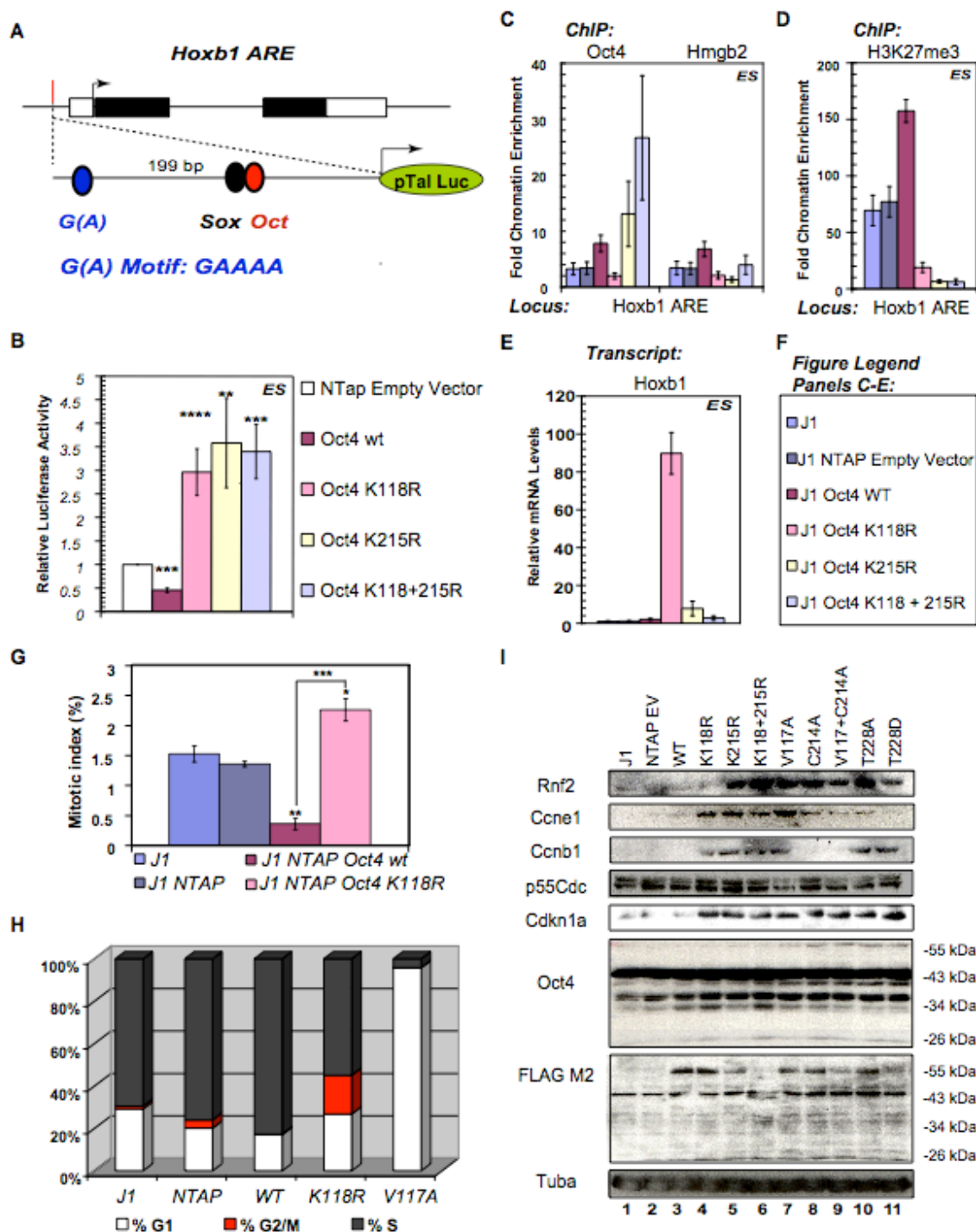


Figure 6. Oct4 sumoylation is required to maintain Hmgb2 recruitment and a bivalent chromatin structure in mESCs.

(A) Schematic diagram of the *Hoxb1* auto-regulatory enhancer (ARE) depicting clustered Oct, Sox, and G(A) motifs. This regulatory region was cloned upstream of a luciferase reporter. (B) Luciferase assays performed in J1 mESCs transiently expressing the indicated Oct4 wild-type and mutant

constructs with the *Hoxb1* luciferase reporter. Results are normalized to empty vector and internal Renilla Luciferase control. Mean $\pm$ SEM are displayed for 9 independent replicates. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ by two tailed Student's t-test. (C) Enrichment of Oct4, Hmgb2, and H3K27me3 (D) at the *Hoxb1* ARE in J1 mESCs and pooled, stable sublines expressing Oct4 wild-type or the indicated mutant constructs by ChIP. Fold enrichment is normalized to enrichment of negative control (rlgG) ChIP and input. Values represents the mean of biological duplicates analyzed in replicate ($n=4$) $\pm$ SEM. (E) Relative expression of *Hoxb1* in J1 mESCs and pooled, stable clones expressing Oct4 wild-type or the indicated mutant constructs by QRT-PCR. Relative expression following knockdown was calculated by normalization to *Gapdh* and J1 mESC. Values represent the mean of biological duplicates analyzed in replicate ($n=4$) $\pm$ SEM. (F) Figure legend for panels C-E.

(G) Enumeration of mitotic figures in wild-type J1 mESCs and stable pooled clones of NTAP empty vector, wild-type, and K118R mutant Oct4. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.001$, $n=4$. (H) Cell cycle distribution of asynchronous wild-type J1 mESC or pooled stable clones of J1 mESC overexpressing the indicated constructs analyzed by FACS on a DakoCytomation MoFlo. The resultant data were analyzed in Modfit to assess G1, S, and G2/M populations. Values displayed are representative of 4 replicate experiments. (I) Western analysis of J1 mESCs and pooled stable J1 mESC clones expressing the indicated wild-type and mutant NTAP constructs. Altered detection of cell cycle regulatory APC/C substrates *Ccne1*, *Ccnb1*, *p53Cdc*, and *Cdkn1a* is observed in the Oct4 mutant expressing clones.

Nme1 functions in transcriptional activation of Oct4 bound loci

To integrate SET complex function into our expanding gene regulatory network, we examined the role of Nme1 in modulating expression of Oct4 transcriptional targets. Hmgb2 colocalized with Oct4 at the *Hoxb1* ARE, a repressed locus in mESC (Fig. 4B). Hmgb2 enrichment, however, was not observed at the *Phc1* promoter, which is expressed in mESC. By contrast, Nme1 enrichment was observed only at expressed loci. Its enrichment at the *Hoxb1* and *Phc1* loci respectively increased and decreased following ectopic expression of the K118R mutant form of Oct4 (Fig. 7A).

The pattern of Nme1 enrichment observed at expressed loci suggested that it might promote expression of Oct4 target loci. Since Set inhibits Nme1 function when they are in association, it was possible that the Oct4-SET Complex interaction restrained Nme1 function required for transcriptional activation of Oct4 targets in mESC. To test this hypothesis we performed siRNA mediated knockdown of Nme1 in mESCs and in those transfected with the Oct4 K118R mutant. Expression of the wild-type and K118R mutant Oct4 constructs resulted respectively in a two-fold-decrease and increase in *Hoxb1* transcript (Fig. 7B). A 50% knockdown of Nme1 transcript was observed in the cells that were co-transfected with the K118R mutant construct along with the Nme1 siRNA. In contrast to the upregulation of *Hoxb1* observed when the mutant construct alone was transfected, even partial knockdown of Nme1 was sufficient to maintain repression.

(A) ChIP-PCR assays were performed as previously described to evaluate enrichment of Nme1 at the Hoxb1 ARE and Phc1 promoter in the pooled stable Oct4 wild-type and K118R mutant sublines. (B) Quantitative Real-Time PCR to assess the effects of transient Nme1 knockdown upon Oct4 K118R mutant expression in J1 mESC. Relative mRNA levels were calculated by normalization to Gapdh and endogenous levels in J1s. Each data point is calculated as the mean of two independent transfections with each undergoing PCR twice (n=4). Error is $\pm$ SEM. (C) Our model. Please refer to the discussion for a complete description.

Discussion

Understanding the molecular mechanisms that regulate the pluripotent state has generated great interest in the scientific community. It has direct implications to the fields of developmental biology, stem cell biology and regenerative medicine, epigenetic reprogramming, and cancer research. Our model (Fig. 7C) is the first to link Akt signaling, PTM-dependent Oct4 function, transcriptional regulation, and the DNA damage response to both pluripotency and differentiation. The results suggest that Oct4 Akt-mediated phosphorylation and sumoylation act by complementary mechanisms to facilitate interaction with the SET complex. This, in turn, maintains a bivalent chromatin structure at developmentally regulated loci, cell cycle progression, and the pluripotent state. Our study advances the emerging hypothesis that the above-mentioned formerly isolated disciplines are integrally related at a mechanistic level.

Specifically, our results suggest that an Oct4-Akt-Hmgb2 regulatory loop maintains the primitive, pluripotent state. We first observed that Oct4 interacted with Hmgb2 in the undifferentiated state (Fig. 3D). The nature and strength of their association declined during differentiation, however, and appeared to parallel Akt activation status (Fig. 1C and 3A and D). Expression of wild-type Oct4 in mESCs enhanced expression of Hmgb2 transcript (Fig. 6C) and resulted in increased Akt activation (Fig. 6D). Hmgb2 knockdown led to decreased Oct4 transcript and protein as well as decreased Akt activation (Fig. 6C-D). Conversely, Akt signaling was found to impact Oct4 stability (Fig. 1D-E). Taken together, it appears that this feed forward loop functions to maintain finely tuned levels of Oct4, Hmgb2 and Akt. Our data, and the data of others, suggests that this loop would maintain expression of other pluripotency regulators vital for cycle progression, heightened DNA repair mechanisms,

chromatin stability, and inhibition of apoptosis. These characteristics are all hallmarks of the pluripotent state.

The molecular mechanisms that promote Akt activation in pluripotent cells, and its role in facilitating cell fate transitions, are not well understood. PI3K/Akt signaling is critical to the survival of cells from the preimplantation blastocyst (O'Neill, 2008; Riley et al., 2005) and to their *in vitro* survival and expansion as pluripotent ESCs (Watanabe et al., 2006). Recent studies have linked Akt function to Nanog (Kim et al., 2010) and Sox2 (Jeong et al., 2010) regulation of pluripotency. Akt signaling, however, may either promote or inhibit cell fate transitions. This pleiotropic effect has been observed in differentiation of pluripotent cells to committed progenitors (Bang et al., 2001; Lopez-Carballo et al., 2002), as well as in the induction of pluripotency from terminally differentiated cells (Nakamura et al., 2008). Taken together, these studies suggest that dosage effects, as well as chromatin context, may be critical to Akt function.

Oct4 and Hmgb2 emerge as critical determinants of Akt function as a result of our studies. Previously, Oct4 sumoylation has been shown to enhance its stability (Wei et al., 2007). Our results extend these preliminary findings, since we observe that Oct4 dimerization is enhanced upon sumoylation (Supplemental Fig. S9). This suggests that Oct4 dimerization promotes its stability. Interaction with Hmgb2 was observed only with wild-type but not with either of the T228 Akt phospho-mutant forms (Supplemental Fig. S10). This may also suggest that Hmgb2 interacts with an Oct4 dimer. Since over-expression of wild-type Oct4 in mESCs resulted in maximal Akt activation, this further intimates that Oct4 dimerization is required to maintain interaction with Hmgb2 and Akt, thus promoting Akt activation at specific genomic loci. However, rather than mediating PcG-repression only, it appears that

Hmgb2 functions to maintain a permissive chromatin environment at Oct4 bound loci (Fig. 4). Following mitotic cell division, repression of these permissive domains, however, may require sumoylation to maintain Oct4 dimerization. Since Oct4 differentially interacts with transcriptional repressors and genes implicated in DNA damage and chromatin remodeling respectively through its N- and C- termini (Liang et al., 2008b; Pardo et al., 2010; van den Berg et al., 2010; Wang et al., 2006), this may suggest that dimerization is required to restrain the function of Oct4 C-terminal interactors, many of which are notably inhibited by Akt-mediated phosphorylation.

Akt functionally links cell cycle progression, DNA damage processing, and the negotiation of cell fate transitions through Oct4 and the SET Complex. Their coordinate modulation is feasible since Oct4 transcriptionally regulates the expression of genes implicated in these processes. As well, nucleosomal assembly protein Set, for which the SET Complex is named, regulates cell cycle transition at G1/S and G2/M by respective control of Cdkn1a (Estanyol et al., 1999) and Cyclin B-Cdk1 (Canela et al., 2003). Importantly, Set is also implicated in Akt signaling since it is an inhibitor of the Akt phosphatase, Pp2a (Li et al., 1996). Oct4 sumoylation and interaction with Set therefore, provides insight into how cell cycle progression is maintained even in the presence of DNA damage, since their interaction may prevent Akt dephosphorylation and inactivation (Kandel et al., 2002).

Oct4 phosphorylation activates Akt, defers DNA damage processing, and overrides cellular checkpoints. This understanding is crucial since full activation of the DNA damage response is required for stem cell differentiation (Fernando et al., 2005; Fernando et al., 2002; Fujita et al., 2008; Sherman et al., 2011). Tight regulation of this response may be globally implicated in cell fate decisions, including induced pluripotency, since DNA

damage accumulates during the generation of induced pluripotent stem cells. Notably, the inactivation of Trp53 and Cdkn1a, which may be mediated by activated Akt, enhances iPSC generation (Blanpain et al., 2011; Gore et al., 2011; Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009). Taken together, this data implicates the Oct-Akt-Hmgb2 loop in maintaining a primitive chromatin state by maintaining a balanced combinatorial epiproteomic signature at target loci. Likewise, regulation of Oct4 phosphorylation and sumoylation may also play an important role in facilitating cell fate transitions promoting either differentiation or cellular reprogramming in a context-dependent manner.

In sum, our data suggests that the Akt-Oct4-Hmgb2 regulatory loop functions to coordinate stem cell competence and specification. Active Akt signaling facilitates a dynamic exchange of PcG and TrxG proteins from the chromatin template (Niessen et al., 2009). As mESCs undergo differentiation, however, these complexes exhibit decreased mobility (Ren et al., 2008). Our results demonstrate that Oct4 interacts with Hmgb2, Akt, and other SET Complex members in the pluripotent state. This intimates that Oct4 recruitment of activated Akt to specific genomic loci may maintain this dynamic flux. In this manner, Oct4 and Hmgb2 maintain a permissive chromatin structure that remains competent to act upon external stimuli. We hypothesize that Oct4 sumoylation marks these permissive sites for repression to specify cellular fate and full activation of gene expression upon differentiation.

Further research is required to fully understand the upstream mechanisms, which facilitate Oct4 interaction with Akt, Hmgb2, and the SET Complex. As well, the identity of Sumo proteases implicated in Oct4 de-sumoylation and the phosphatase responsible for Oct4 dephosphorylation remain to be clarified. Like Oct4, Akt, Hmgb2 and the SET Complex, it

is anticipated that these molecules will play key roles in the pluripotency gene regulatory network. Knowledge of their function will greatly enhance our understanding of the spatio-temporal regulation of Oct4 function through choice of interaction partners, cellular localization, and chromatin binding. Therefore, identification of these factors will be the next step for a comprehensive understanding of the molecular mechanisms underpinning pluripotency, cell fate transitions, cellular reprogramming, and cancer.

Materials and methods

All experiments described in this study were performed in triplicate unless noted otherwise.

Cell culture

Undifferentiated J1 mESCs, embryoid bodies (EBs), and 10T1/2 fibroblasts were cultured as previously described (Campbell et al., 2007).

Small chemical modulation of Akt signaling

J1 mESCs were cultured for 48 hours, treated with 10 uM LY294002 (Cell Signaling Technologies) plus or minus 10 uM MG132 (Sigma) for 1 hour at 37° C, and then harvested as indicated. Akt1/2 (Calbiochem) inhibition was performed with the indicated concentration plus or minus 10 uM MG132 (Sigma). Ro-31-8220 (Sigma) was used at a final concentration of 10 uM for 1 hour.

Cell transfections, Western blots, and IP-Westerns

Cells were transfected with the indicated constructs using Lipofectamine 2000 Transfection Reagent (Invitrogen). Forty-eight hours post-transfection (or at the indicated time) cell lysates were prepared in RIPA buffer (Sigma) supplemented with protease inhibitors (Roche Complete Mini), phosphatase inhibitors (Sigma Phosphatase Inhibitor Cocktails 1 and 2), and 100 mM N-ethylmaleimide (NEM, Sigma) to inhibit endogenous enzymes. Stable clones were selected by culture in the appropriate antibiotic (NTAP constructs require puromycin at 1 ug/ml) and prepared as above.

Immunoprecipitation assays were performed as follows. Cleared lysates were precipitated overnight at 4°C with the indicated antibody and collected by incubation with Protein A/G Agarose (Pierce). The immunoprecipitates were washed five times with supplemented RIPA Buffer, denatured, and then run on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 2.5% Blotto (2.5% dry milk, 0.5% TWEEN-20 in TBS) and incubated with the indicated primary antibody. Appropriate secondary antibodies conjugated to HRP (BioRad) were used for ECL Detection (Amersham).

siRNA knockdown

Hmgb2 siRNA #1 and #2 were originally purchased from Dharmacon (Yan et al., 2010). Control scrambled siRNA #1 was purchased from Ambion (Applied Biosystems). Each siRNA was used at a final concentration of 40 nM and transfected into J1 mESCs in 24-well plates using Lipofectamine 2000 (Invitrogen).

GeneChip Analysis

Affymetrix gene expression profiling was performed in biological triplicate for J1 mESCs and following 6, 12, 18, and 24 hours of differentiation as EBs as previously described (Campbell et al., 2007). The associated files for analysis can be found at (<http://www.scgp.ca:8080/StemBase>) or within the GEO repository: GSM86112, GSM86114, GSM86116, GSM86118, GSM86120, GSM86122, GSM86124, GSM86126, 'GSM86128, GSM86294, GSM86296, GSM86298, GSM86300, GSM86302, GSM86304

Normalized expression was calculated in R.2.13 BioC 2.8 (Bioconductor). The data was pre-processed using the `expresso` () function using RMA background correction, Quantiles normalization (Bolstad et al., 2003), perfect match only correction, and median polish. Normalized expression represents the mean of three biological replicates $\pm$ SEM.

Immunofluorescence

J1 mESCs were plated onto an 8-well chamber slide and adhered overnight at 37°C. Following the indicated treatment cells were fixed in 3.7% paraformaldehyde and blocked for 30 minutes in 5% normal goat serum/ 0.3% Triton X-100 prepared in PBS. Primary antibodies were prepared at 0.2 ug/ml in blocking buffer, applied to cells, and incubated overnight at 4°C. Where no primary antibody is indicated, the cells were incubated in blocking buffer only. Secondary antibodies were prepared at 1:2000 dilution (Goat anti Mouse ALEXA 546 and Goat anti Rabbit ALEXA 488; Molecular Probes) in 0.3% Triton X-100 prepared in PBS with 0.5 ug/ml DAPI added. Cells were incubated for 1 hour in the dark then washed and a chamber slip was applied with aqueous anti-fade mounting media (Dako). Imaging was performed on a Zeiss AxioScope. All images are at 20X power and are representative of triplicate experiments.

Cell cycle analysis

Cells were harvested, fixed by dropwise addition of 100% ice cold EtOH to final concentration of 70% and incubated on ice for one hour. They were then centrifuged and washed with Dulbecco's Phosphate Buffered Saline (DPBS).

Cell pellets were resuspended in 500 ul of DPBS to which 5 ul of 20 mg/ml RNase A was

added following incubation at 37°C for one hour. Ten microliters of 1 mg/ml propidium iodide (PI) was added and tubes were kept in dark at 4°C for at least 3 hours to overnight. Prior to analysis, cells were washed with DPBS. They were resuspended to final 250ul volume in DPBS and analyzed on a MoFlo (DakoCytomation) to obtain DNA content. The acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc.) to assess cell cycle distributions.

ChIP and Sequential ChIP

ChIP assays were performed according to the method of Millipore (Formerly Upstate) as previously described (Campbell et al., 2007) with slight modifications. Briefly, 1×10^7 cells were cross-linked with 1% formaldehyde for 15 minutes at room temperature. The cells were washed 3X with ice cold Dulbecco's PBS to which protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (Sigma) were added. They were then lysed in the kit-provided buffer containing the same protease and phosphatase inhibitors and sonicated to average fragments sizes of 0.5 kbp. Two-hundred and fifty ng of input chromatin was used for each ChIP with 10% amount of input chromatin being reserved as input DNA. A total of 3 ug of the designated antibody was used for each ChIP. Immune-complexes were captured with Protein A or Protein G agarose (Millipore). Following washes and elution with the provided buffers, the cross-links were reversed, the DNA purified, and finally resuspended in 10 mM Tris pH 8.0.

Sequential ChIPs were performed as above, except elution volume for the first ChIP was decreased by one-half (250 ul total) prior to dilution with Millipore ChIP dilution buffer to

reduce SDS concentration before continuing with the second round of ChIP which was performed as per usual.

Antibodies used for ChIP in this study: Oct4 (sc-9081; Santa Cruz Biotechnology), Hmgb2 (ab61973, 67282; Abcam), Hmgb1 (ab18256; Abcam), Ezh2 (18-7395; Zymed), Ehmt2 (ab40548; Abcam), RnapII, 8WG16 (MMS-126R; Covance), RnapII Ser2p (MMS-129R; Covance), RnapII Ser5p (MMS-134R; Covance), H3K36me3 (ab9050; Abcam), H2K27me3 (ab6002; Abcam), Rnf2 (sc-9766; Santa Cruz Biotechnology), H2AK119ubn1 (05-678; Millipore), Ash2l (A300-489A; Bethyl Laboratories), H3K4me3 (ab8580, Abcam) FLAG M2 (F1804; Sigma).

ChIP-QPCR

ChIP-QPCR was performed in an MX4000 (Stratagene) using iQ SYBR Green Supermix (BioRad). Fold enrichment was calculated by the $\Delta\Delta C_t$ method given as: (C_t of Specific Antibody - C_t of Input) - (C_t of Species control IgG - C_t of Input) with fold enrichment equal to $2^{-\Delta\Delta C_t}$. ChIP assays using matched species normal control IgG were used as negative controls to calculate fold enrichment. Results represent Mean $\pm$ SEM from two independent experiments with PCR from each performed in duplicate. Upon initial use of primer sets, amplicons were sequence validated to ensure the correct products were obtained. Primer sequences can be found in the Supplemental Material.

Luciferase assays

Luciferase assays were performed using the Dual-Luciferase Reporter system (Promega) per manufacturer's recommended protocol. Two micrograms of DNA were used for each

transfection (per each well of p24 plate) using empty vector to maintain equal amounts as necessary. Ten nanograms of pGK Renilla Luciferase vector was also included in each transfection and served as an internal normalization control. Relative luciferase activity was calculated by normalization to internal pGK Renilla Luciferase and empty vector controls. Results represent the Mean $\pm$ SEM for nine independent experiments.

RNA isolation and Quantitative RT-PCR.

Total RNA was isolated using the RNeasy MiniKit (Qiagen) according to manufacturer's recommended protocol. After DNase treatment (Promega) first-strand cDNA was synthesized from 2 μ g total RNA in a 20 μ l reaction volume with Oligo-dT primers using SuperScript First-Strand Synthesis (Invitrogen). Real-time PCR was performed using SYBR Green Mix (SuperArray) in an MX4000 (Stratagene). Results represent Mean $\pm$ SEM from two independent experiments with PCR from each performed in duplicate. Relative quantitation was determined by comparative C_t method normalizing to *Gapdh* and values obtained in undifferentiated J1 mESC. Upon initial use of primer sets, amplicons were sequence validated to ensure the correct products were obtained. Primer sequences can be found in the Supplemental Material.

in vitro Transcription/Translation

Recombinant Oct4 wild-type and T228A proteins were produced with the TnT Coupled Reticulocyte Lysate Systems (Promega) according to manufacturer's recommended procedure.

IP-Kinase assays

Assays were performed using *in vitro* transcribed/translated Oct4 proteins with the Non-radioactive Akt Kinase Assay Kit (Cell Signaling Technology) according to manufacturer's recommended protocol.

Alkaline Phosphatase staining

Following stable selection of the Oct4 constructs in J1 mESCs the cells were plated in 24 well plates. Alkaline phosphatase staining was performed according to the manufacturer's (Stemgent) recommended protocol. Quantiation was performed on 4 independent replicates. Values represent the Mean $\pm$ SEM.

Acknowledgements

The authors would like to acknowledge the expert technical assistance provided by StemCore Laboratories at the Sprott Centre for Stem Cell Research. We also thank Drs. Nan Yan and Judy Lieberman for the gift of the Hmgb2 siRNA reagents and Dr. William Stanford for critical review of this manuscript. This work was supported from grants from Genome Canada, the Ontario Genomics Institute, the Stem Cell Network, and the Canadian Institutes of Health Research.

SUPPLEMENTARY INFORMATION

Campbell and Rudnicki

An *Akt-Oct4-Hmgb2* Feedback Loop Regulates Pluripotency

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Supplemental materials and methods

ChIP-qPCR primers used in this study

Locus	Forward Primer	Reverse Primer
Hoxb1 ARE	5'CCCTCCCTCTGGTCCCTTCTTT3'	5'GGGCACATGTGATCTCTC3'
Phc1 -4100	5'ACCTCAGCCATAATTAGTTG3'	5'AAACCCTTTCACCTCTCC3'
Phc1 -137	5'-CTTTGTGGAGTTACTGTGATTTTG-3'	5'-CATACAGGGACATCTGTGCTATCT-3'
Phc1 +2337	5'-TAATGTTTCCCTGATTGTATTTGG-3'	5'-GTCCAACACAGCATCAAATGA-3'
Cdx2	5'GAATATTCAGCTGTGATCTGGT3'	5'ACACAGACACCAATGGCTG3'
Pax7 (intron 7)	5'TTTAAAATGAGTCTTGCTGGACC3'	5'CTCTTTTACACACCATTTCATCTTA3'
Mef2a	5'TGAAGACCCAGAACCCATACCC3'	5'GAGGCTCTCCCCACTTTCTTC3'

QRT-PCR primers used in this study

Gene	Forward Primer	Reverse Primer
Oct4, endogenous	5'-TTGGGCTAGAGAAGGATGTGGTT-3'	5'-GGAAAAGGGACTGAGTAGAGTGTGG-3'
Oct4, exogenous. Primers are located in exon 4 (Forward and in the C-terminal FLAG epitope tag (Reverse).	5'-CCAATCAGCTTGGGCTAGAG-3'	5'-CCGTCATGGTCTTTGTAGTCG-3'
Sox2	5'-GAGTGGAACTTTTGTCCGAGA-3'	5'-GAAGCGTGTACTTATCCTTCTTCAT-3'
Nanog	5'-ATGAGTGTGGGTCTTCTGG-3'	5'-GCTTCCTGGCAAGGACCT-3'
Hoxb1	5'-CCATATCCTCCGCCGAG-3'	5'-CGGACTGGTCAGAGGCATC-3'
Neurod1	5'-CAGAAGGCAAGGTGTCCCGAG-3'	5'-CCGCTCTCGCTGTATGATTTGGT-3'
Cdx2	5'-GCCAGCGGCGAAACCTGT-3'	5'-TGCCTCTCGGAGAGCCCA-3'
Eomes	5'-CTTCCGCGCCACGTCTAC-3'	5'-TTGTAGTGGCGGTGGGG-3'
Hand1	5'-CCCCTCTTCCGTCCTTAC-3'	5'-CTGCGAGTGGTCACACTGAT-3'
Cdh3	5'-GCCAGGACTCTGAAGTTTGC-3'	5'-CAAGTTCAAGCCCTGAGAGG-3'
Gata4	5'-TTTGATGACTTCTCAGAAGGCA-3'	5'-CATTACATACAGGCTCACCTC-3'
Gata6	5'-GAGCTGGTGCTACCAAGAGG-3'	5'-TGCAAAAGCCCATCTCTTCT-3'
Pax7	5'-TCCTCTTCTTCCCGAACTT-3'	5'-GTGCCGATATCAGGAGACTG-3'
Myod1	5'-CGTCCAGCAACCCGAACC-3'	5'-GCGCACCGCCTCACTGTA-3'
Rest	5'-GTGCGAACTCACACAGGAGA-3'	5'-AAGAGGTTTAGGCCCCGTTGT-3'
Ash2l	5'-GAAATGTGCCTTAGTGCCTTG-3'	5'-CAAGTCATTTTCCAGGTCTCTG-3'
Phc3	5'-GCAGTACCACCACCACCACCAT-3'	5'-CGCGTACATCTGCTGCAGGTAC-3'
Parp1	5'-TGAGAACTCGGAGGCAAGT -3'	5'-CACCTTGCTCTTCTTGGAG-3'
Hmgb2	5'-TGTCTCTGTACGCCTTCTTC -3'	5'-CCTCTCATCTTCTGGTTTCG-3'
Bra1	5'-CCCTCAAGAAGCTGGAGATG-3'	5'-GCTGTAATGAGCTGGCATGA-3'
Trp53	5'-CGGGTGGAAGGAAATTTGTA-3'	5'-CTTCTGTACGGCGGTCTCTC-3'
Ccnf	5'-ACCTCGTACCCTGTGTCCTG-3'	5'-TCTGTCCTCCTGAAGGCTGT-3'
Rest	5'-GTGCGAACTCACACAGGAGA-3'	5'-AAGAGGTTTAGGCCCCGTTGT-3'

Gapdh 5'-GTATTGGGCGCCTGGTCACC-3' 5'GTGGGGTCTCGCTCCTGGAA-3'

Vector Construction

Expression plasmids

Total RNA was isolated from J1 mESC using the RNeasy MiniKit (Qiagen) according to manufacturer's recommended protocol. After DNase treatment (Promega) first-strand cDNA was synthesized from 2 ug total RNA in a 20 ul reaction volume with Oligo-dT primers using SuperScript First-Strand Synthesis (Invitrogen). Full-length cDNA sequences were amplified using tailed primers as indicated below to enable subsequent cloning into pCDNA3, pBrit-CTap, pCan-HA1, pCan-MYC1 (cloned as BamHI/EcoRI fragment) or pBrit-NTap (cloned as an EcoRI/BamHI fragment) vectors. Hmgb2 was cloned into pCDNA3, pCan-Myc1 or pCMV-Tag5a (cloned as a BamHI/XhoI fragment). The Stop codon was removed for C-terminal fusion protein expression as appropriate. The Myc, HA, and CTAP/NTAP (6x His-TEV-3x FLAG) epitope tags have molecular weights of 1333.64, 1477.7, and 6514.76 Da, respectively. Primers for amplification of full-length cDNA (stop included):

Gene	Forward Primer	Reverse Primer
Oct4	5'ATGGCTGGACACCTGGCT3'	5'TCAGTTTGAATGCATGGGAGAGC3'
Hmgb2	5'ATGGGCAAGGGTGACCCCAAC3'	5'TCATCTTCATCCTCCTCTCTTC3'

Site-directed mutagenesis was performed on constructs with the primers indicated below to mutate the indicated sites. Compound mutants were made sequentially with the indicated primers. Truncation mutants were cloned as BamHI/EcoRI fragments using tailed primers.

Mutation	Forward Primer	Reverse Primer
Oct4 K118R	5'CCCAATGCCGTGAGGTTGGAGA3'	5'CACCTTCTCCAACCTCACGGCA3'

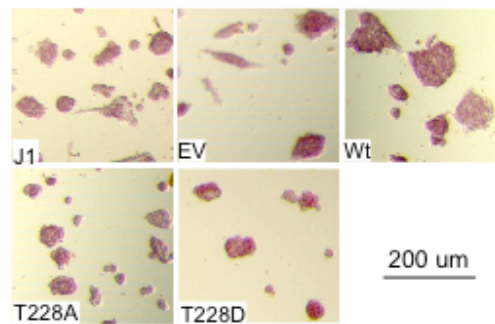
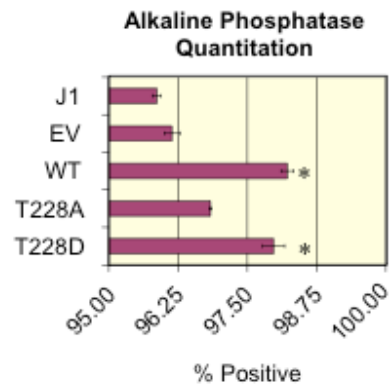
Oct4 K215R	5'TCAGGAGATATGCAGATCGGAG3'	5'AGGGTCTCCGATCTGCATATCT3'
Oct4 V117A	5'CCCAATGCCGCGAAGTTGGAGA3'	5'CACCTTCTCCAACCTCGCGGCA3'
Oct4 C214A	5'TCAGGAGATAGCCAAATCGGAG3'	5'AGGGTCTCCGATTTGGCTATCT3'
Oct4 T228A	5'GAAGAGAAAGCGAACTAGCATTGAGAAC3'	5'GTTCTCAATGCTAGTTCGCTTTCTCTTC3'
Oct4 T228D	5'GAAAGCGAGATAGCATTGAG3'	5'ATGCTATCTCGCTTTCTCTTC3'
Oct4 aa1-278	5'ATGGCTGGACACCTGGCT3'	5'TTAGCCCTTCTGGCGCCGGTTACA
Oct4 aa224-352	5'ATGAAGAGAAAGCGAACTAGCATT	5'GTTTGAATGCATGGGAGAGC3'
Hmgb2 K114R	5'GATCAGAATTGAACACCCAGGC3'	5'GTTCAATTCTGATCTTTGGGCG3'

Luciferase reporter constructs

Inserts for reporter constructs were amplified from J1 mESC genomic DNA using MluI/BglII tailed primers to enable cloning into pTal-Luciferase Vector (Clontech). Primer sequences are as indicated below:

Locus	Forward Primer	Reverse Primer
Hoxb1ARE	5'AAAAACGCGTAAAGAGAGGCTGAGGGAGAGAA3'	5'AAAAAGATCTGGTCCCCGGTTACAAAGT3'
Cdx2	5'AAAAACGCGTTGGGGGCGCAACCTGGA3'	5'AAAAAGATCTAAAGTTCTGCGGAGCCAG-3
Ash2l	5'AAAAACGCGTTGGGGAGGACTGTGGAAGGACT3'	5'AAAAAGATCTTGGAACACAGCCACTGTGCTCT3'
Phc3	5'AAAAACGCGTCGTATCCATTTATGTATGTGCT3'	5'AAAAAGATCTTTACAAAAAGCCAATGGTGTA3'

Supplemental figures

A**B**

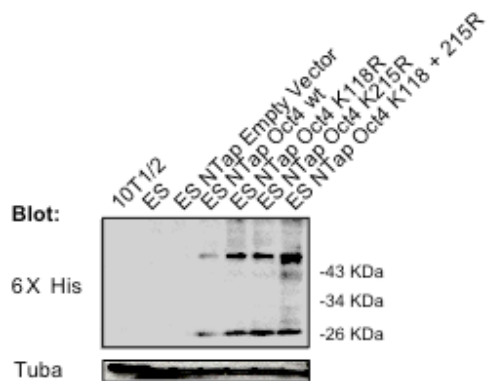
Supplemental Figure S1. Alkaline Phosphatase staining in Oct4 over-expressing mESC sublines.

(A) Representative alkaline phosphatase staining of mESC colonies. Size of each colony is indicated.

(B) Quantitation of the alkaline phosphatase stained cells. Numbers represent the Mean ± SEM, n=4.

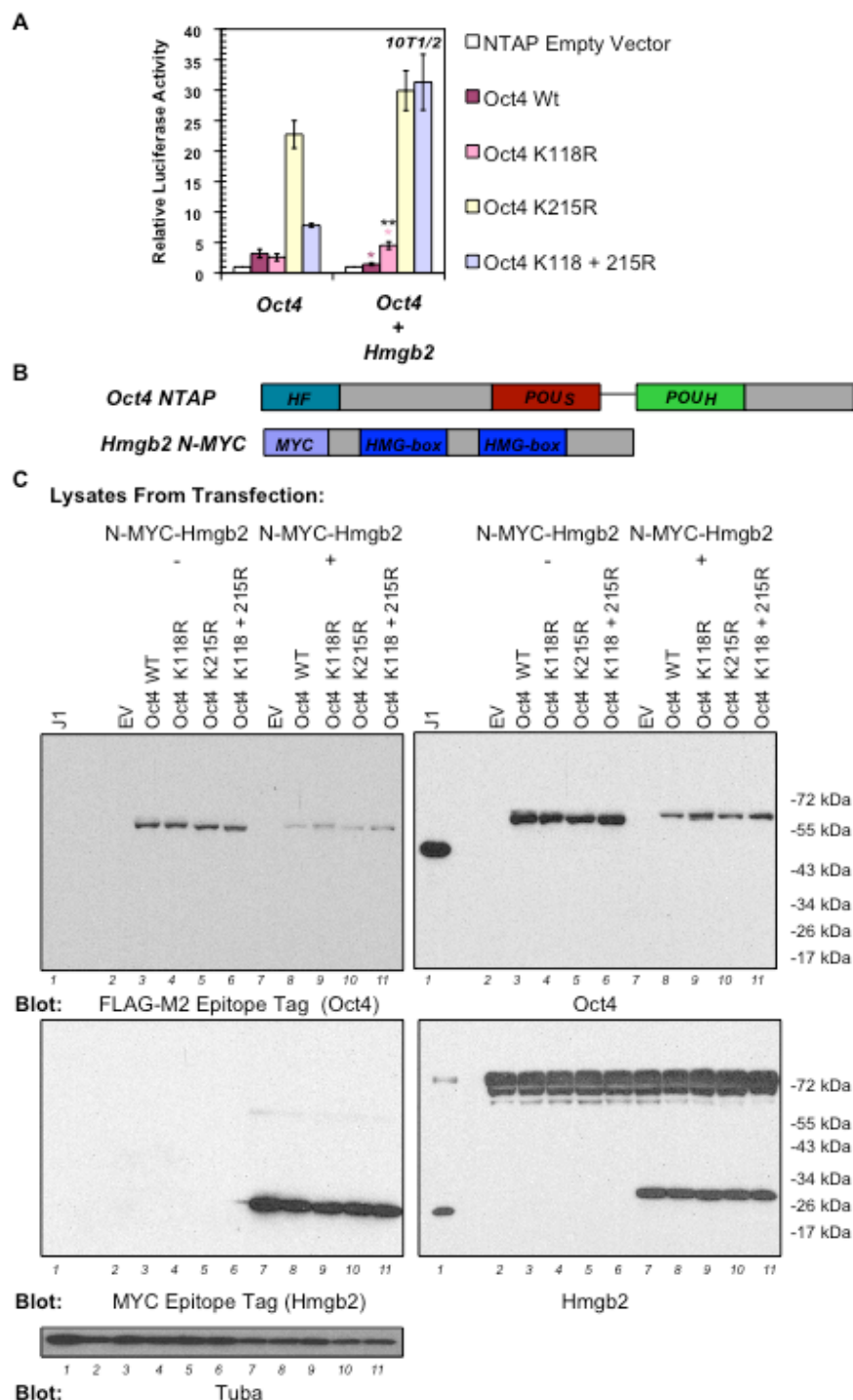
Statistical significance: * $p \leq 0.05$ by two tailed Student's t-test.

Lysates from luciferase assays performed in mESCs



Supplemental Figure S2. Western Analysis of luciferase lysates.

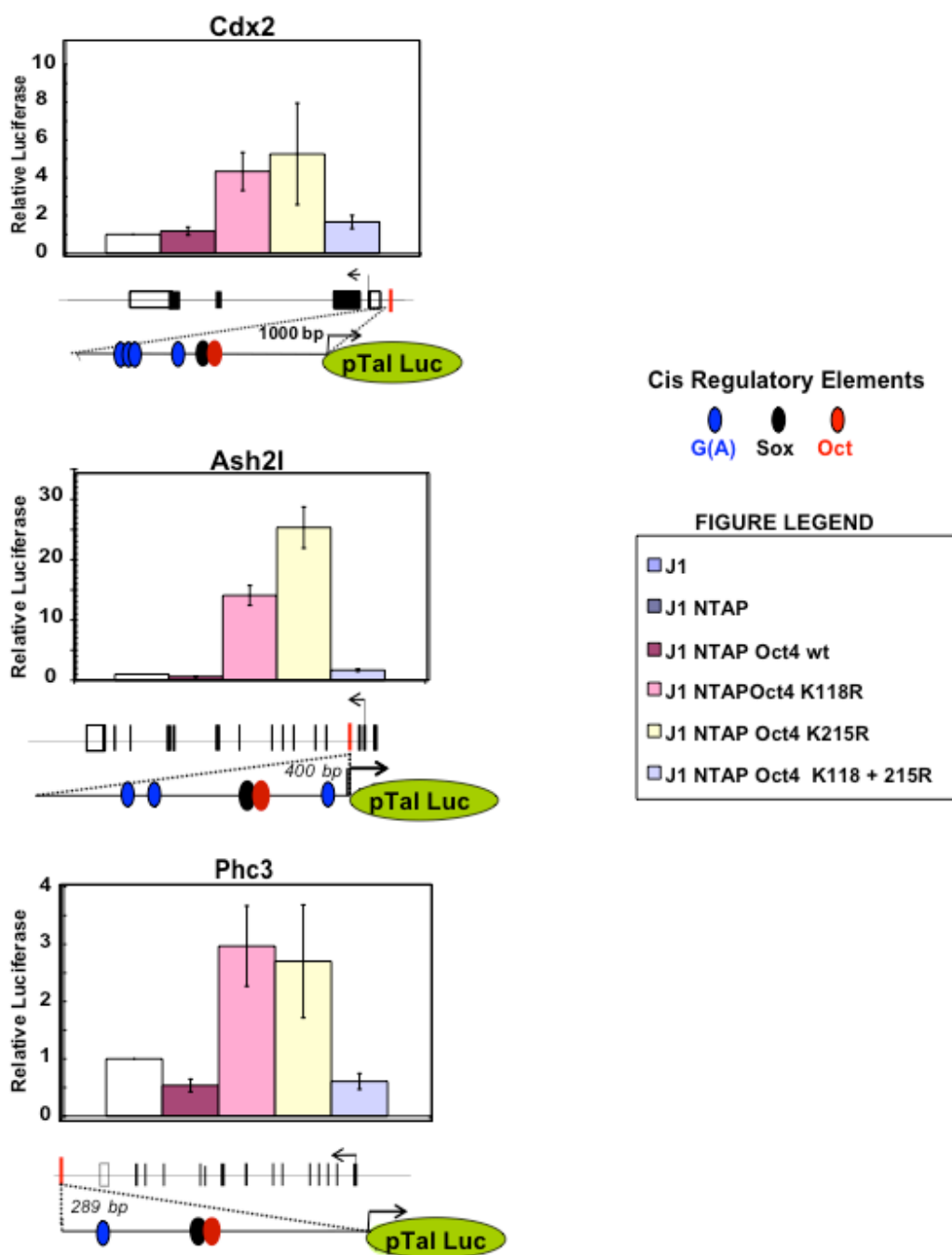
Western analysis of mESCs lysates from luciferase assay described in Figure 6B.



Supplemental Figure S3. Transcriptional synergy between Oct4 and Hmgb2 in 10T1/2 fibroblasts.

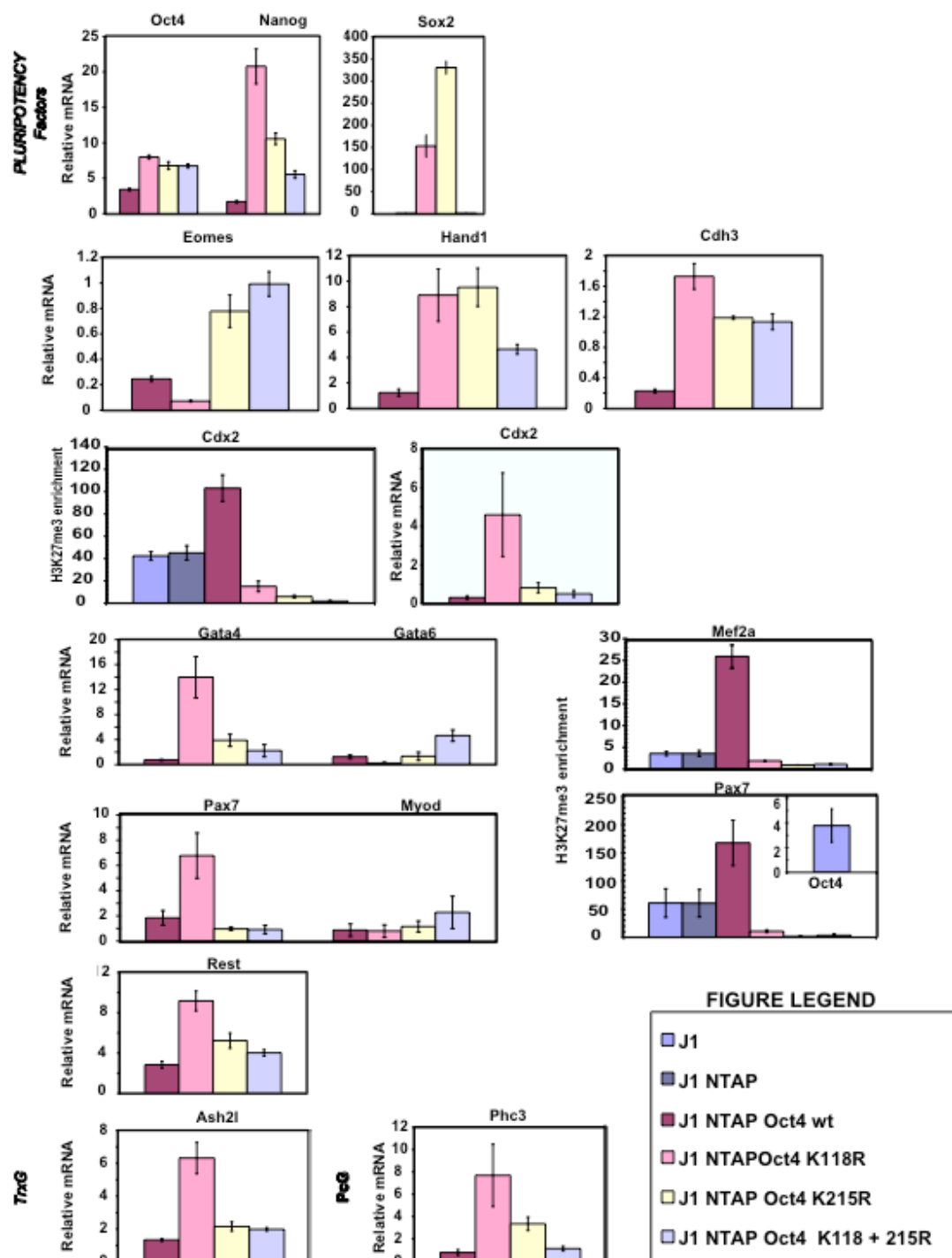
(A) Luciferase assays as described in Figure 6B, except performed in 10T1/2 fibroblasts. Data depicted are Mean $\pm$ SEM, n=9. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.005$, by Student's t-test. * Compared to Oct4 wild-type only (without Hmgb2) transfection. * Compared to Oct4 K118R only. **

Compared to wild-type Oct4 co-transfected with Hmgb2. (B), Schematic of Oct4 and Hmgb2 constructs employed in luciferase assays. NTAP denotes N-terminal 6X His-TEV-3X-FLAG epitope tagged Oct4 construct (Oct4 NTAP). DNA binding POU-specific domain (POU_S) and POU-homeobox domain (POU_H) are indicated in the red and green boxes, respectively. Hmgb2 construct contains an N-terminal MYC epitope tag (N-MYC). The two DNA binding HMG boxes of Hmgb2 are shown in blue. (C) Western blots of lysates from luciferase assays. Note doublet band upon co-transfection of Hmgb2 and the Oct4 K118R mutant and 60 kDa band detected by the anti-MYC epitope tag antibody directed against Hmgb2.



Supplemental Figure S4. De-repression of Oct4 targets in Sumo mutant expressing mESCs.

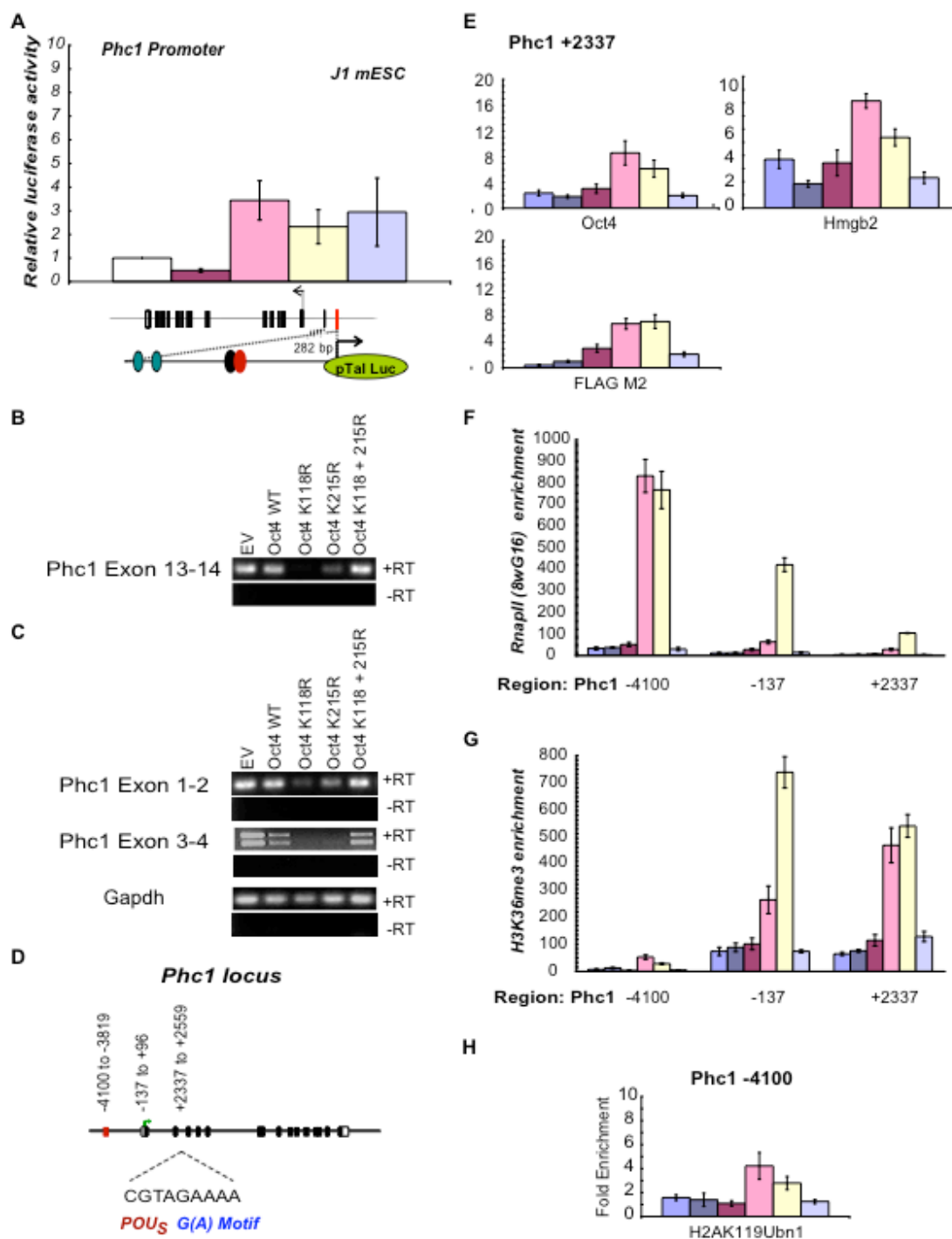
Luciferase assays were performed in J1 mESCs as previously described. The indicated Oct4 wild-type or mutant construct was co-transfected along with reporters for Cdx2, Ash2l, or Phc3. Schematic below each result indicates promoter/enhancer region within the genomic context of each transcript as well as the location of Oct, Sox, and G(A) motifs. Figure legend provides key to graphs.



Supplemental Figure S5. Oct4 SUMOylation at K118 mediates transcriptional repression in mESC.

Pooled stable mESC sublines expressing the indicated constructs were harvested for QRT-PCR and ChIP-QPCR analysis to ascertain the effect of Oct4 wild-type and mutant over-expression to the

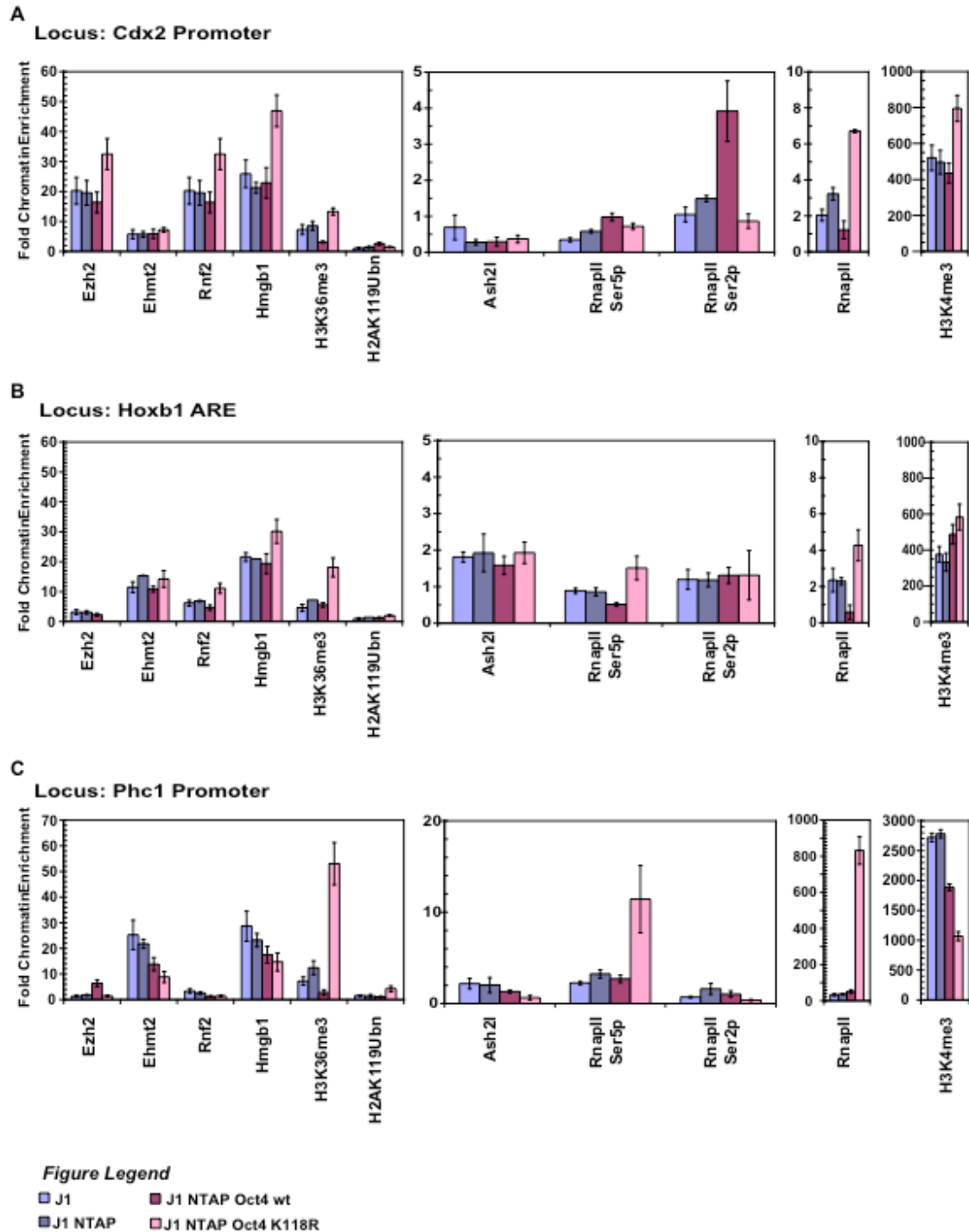
indicated transcriptional target in undifferentiated J1 mESCs. All analyses were performed as previously described.



Supplemental Figure S6. Oct4 K118R mutant and Hmgb2 block RnapII at *Phc1* locus.

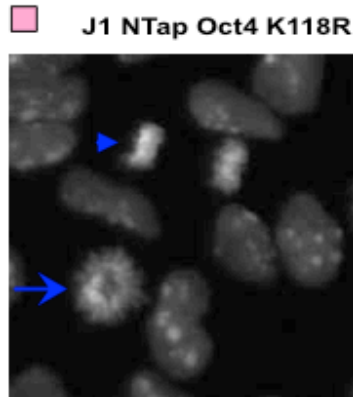
(A) Luciferase assays performed in J1 mESCs as previously described using *Phc1* promoter luciferase reporter. Lower schematic represents genomic context of region used in reporter and indicates location of Oct, Sox, and G(A) motifs. Legend for all panels can be found in Supplementary

(B) RT-PCR analysis of Phc1 expression in stably expressing Oct4 wild-type and mutant clones in mESC using primers located in Exons 13 and 14. (C) RT-PCR analysis of Phc1 transcript level expression in J1 mESC clones stably expressing the indicated Oct4 N-Tap construct using primers tiling down Phc1 transcript. (D) Diagram of Phc1 exon structure with location of ChIP-qPCR primers indicated. POU₅ and G(A) motifs identified at +2337, located in intron 2. (E) Increased enrichment of Oct4 (Oct4 specific and FLAG epitope tag antibody) and Hmgb2 are observed at the Phc1 +2337 regulatory element. (F) Tiling ChIP-qPCR at Phc1 locus shows increased RnapII at promoter in the K118R mutants. (G) H3K36me3 extends into coding region. (H) Increased enrichment of H2A K119Ubn1 suggests initiation of the DNA damage response at this locus. ChIP-PCR shows increased Oct4 and Hmgb2 enrichment at the +2337 region of Phc1. Data suggests that loss of Oct4 sumoylation mediates RnapII block and repression of Phc1 through increased binding of Oct4 and Hmgb2 at Oct/G(A) motif at +2337. All analysis performed as previously described.



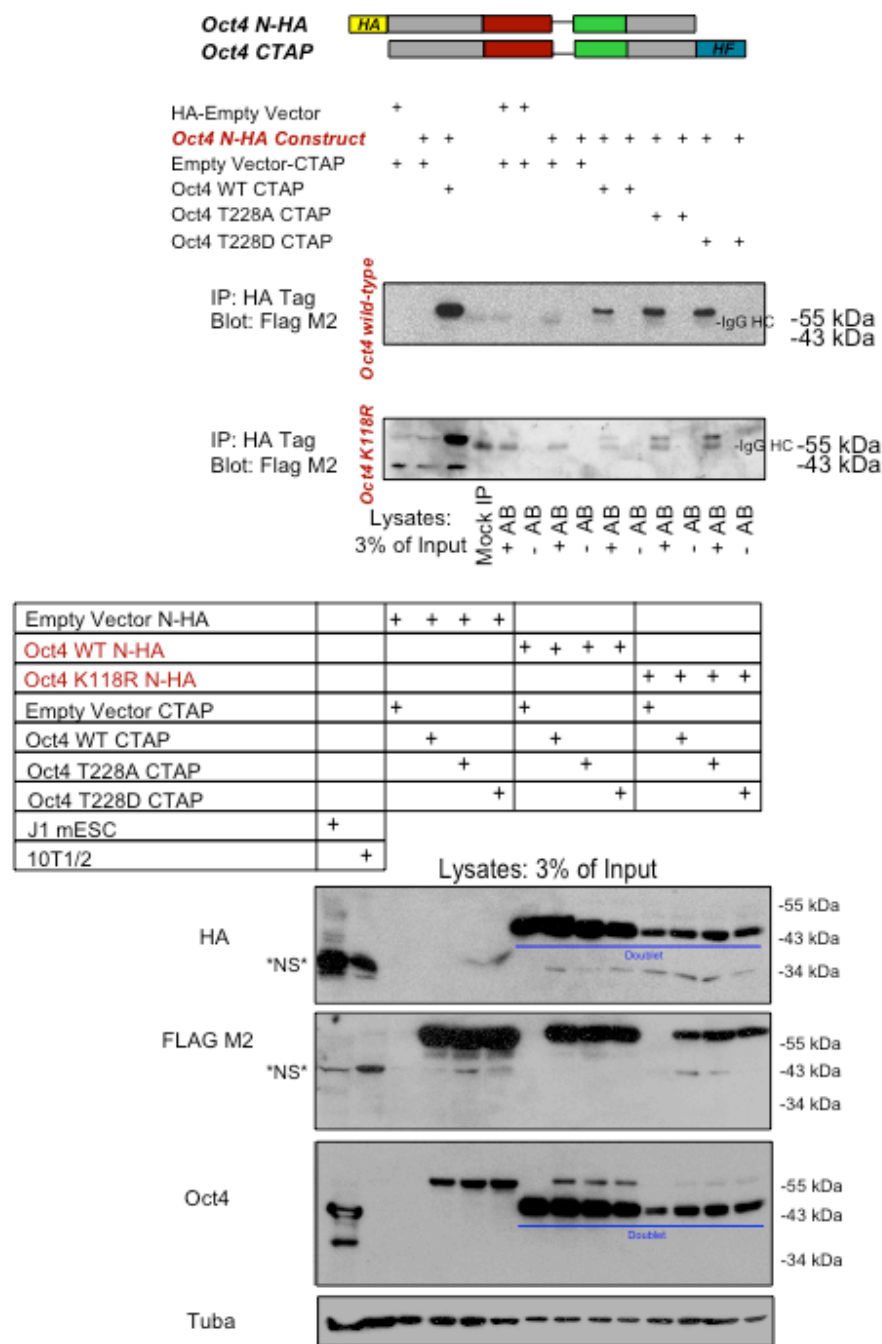
Supplemental Figure S7. Altered enrichment of transcriptional regulators and histone modifications in Oct4 K118R mutant sublines.

Extended analysis of chromatin occupancy at the Cdx2 promoter, Hoxb1 ARE, and Phc1 promoter by ChIP in J1 mESCs and pooled, stable clones expressing Oct4 wild-type or the indicated mutant constructs by ChIP. The analysis was performed as previously described.



Supplemental Figure S8. Oct4 K118R mutant impedes cell cycle progression.

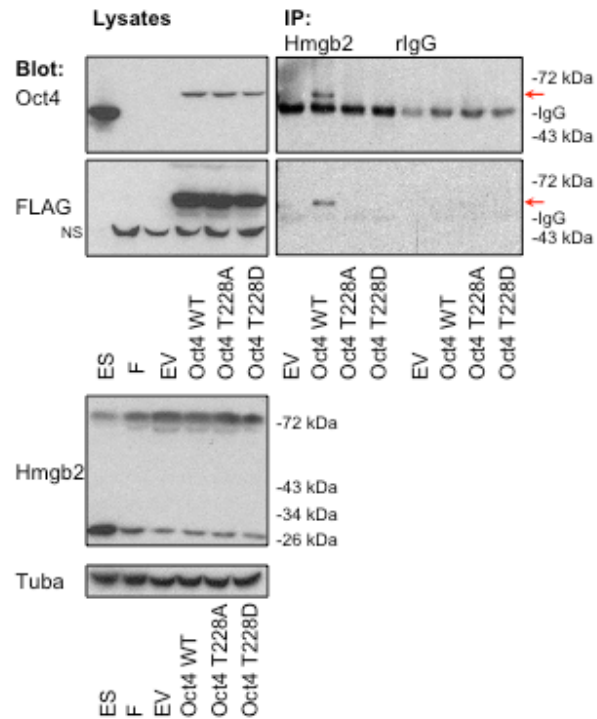
Representative DAPI staining of pooled stable clones of J1 mESC over-expressing the Oct4 K118R used for enumeration of mitotic figures. Blue arrow denotes representative cell blocked at prometaphase. Blue arrowhead denotes representative cell blocked at anaphase to telophase transition. DAPI staining was performed in 4 independent experiments.



Supplemental Figure S9. Oct4 sumoylation is required for dimerization.

(A) Schematic depiction of N-terminal HA and C-terminal 6x His-TEV-3x FLAG epitope tagged constructs used in this experiment (Upper panel). Dimerization of Oct4 requires preservation of the K118 Sumo consensus site. 10T1/2 fibroblasts were transiently transfected with the indicated wild-type or mutant constructs. The FLAG antibody readily detected the immunoprecipitate from the anti-HA antibody when the wild-type N-HA Oct4 construct was used, but this was greatly reduced when

the K118R mutant was co-transfected. Lysates from the transient transfections are shown below. Mock IP represents antibody pulldown from RIPA buffer only.



Supplemental Figure S10. Hmgb2 interacts with wild-type Oct4.

Transient expression of the indicated Oct4 wild-type and T228 mutant construct in 10T1/2 fibroblasts. Lysates were harvested 28 hours post-transfection. Hmgb2 interaction with Oct4 was observed for wild-type but not for either the T228A or T228D mutants.

CHAPTER 4

Discussion

4.1 Overview of Major Findings

While each cell within the developing embryo contains the same genome, gene expression and cellular phenotype are determined by the presence of specific histone modifications at distinct genomic loci. TrxG and PcG proteins place these marks upon chromatin. The successful navigation of developmental milestones is dependent upon the antagonistic effects of both protein families to ensure proper embryonic patterning (Bantignies and Cavalli, 2006). PcG and TrxG also function in later developmental stages to maintain the health and homeostasis of the mature organism (Barber and Rastegar, 2010).

Understanding the mechanisms that maintain PcG expression and facilitate their locus-specific recruitment enhances insight into the etiology of diseases caused by transcriptional dysregulation. PcG maintains homeostasis within the adult organism by controlling the balance between stem cell self-renewal and differentiation until the cell's final fate is achieved. This function is linked to its modulation of cell cycle dynamics (Orford and Scadden, 2008). First, PcG prevents premature expression of genes required for later stages of differentiation and the ectopic expression of alternate lineage genes in more committed progenitor cells (Ezhkova et al., 2009). Second, it maintains primitive stem cell and committed progenitor populations by averting stem cell exhaustion (Antonchuck et al., 1999; Kamminga et al., 2006; Lessard and Sauvageau, 2003; Lessard et al., 1999). Finally, PcG blocks the expression of genes required for cell cycle reentry upon terminal differentiation (Blais et al., 2007; Boyer et al., 2006; Bruggeman et al., 2005; Ezhkova et al., 2009; Kamminga et al., 2006; Lee et al., 2006b; Lessard and Sauvageau, 2003; Ohta et al., 2002; Zencak et al., 2005). PcG dysregulation has been closely associated with several

differentiation-defective disorders of neuronal, hematopoietic, and myogenic origin (Gabellini et al., 2002; Gil et al., 2005; Valk-Lingbeek et al., 2004).

The research presented in this thesis clarifies the mechanisms by which Oct4 participates in PcG repression, thereby maintaining a bivalent chromatin structure at developmentally regulated loci in pluripotent cells. The results of studies performed to identify genes that were correlated to Oct4 transcript level expression were presented in Chapter 2. GO classification of these genes revealed that they represented pivotal regulators in cellular processes such as cell cycle, DNA repair, apoptosis, chromatin structure, and nuclear architecture. This analysis, moreover, led to the development of a model (Chapter 2 Figure 4), defining the molecular mechanisms that guide self-renewal and differentiation. Our study suggests that heightened DNA repair, abrogation of cell cycle checkpoints, and permissive chromatin organization maintain the pluripotent state. By contrast, differentiation requires cell cycle checkpoint activation, apoptosis, and repressive chromatin organization.

The PTM-dependent role of Oct4 in safeguarding pluripotency was examined in Chapter 3. Two key insights guided this inquiry. First, High Mobility Group transcription factor Hmgb2, which functions as a PcG recruiter in *Drosophila*, was correlated to Oct4 in our first study. Its relationship to Oct4 was much stronger than was that of Sox2. Since Hmgb2 interacts with Oct4 in EC (Butteroni et al., 2000), and plays a role in transcriptional regulation and the DNA damage response (Lieberman and Fan, 2003), we theorized that this interaction in mESC might influence gene expression and cell fate decisions. Second, we noted that the themes identified in the initial study also represented key regulatory nodes in the Akt signaling pathway. Since Oct4 contained an Akt consensus site, we proposed that it might function as a transducer of Akt signaling.

Experiments described in Chapter 3 revealed that Oct4 phosphorylation and sumoylation are required to maintain the pluripotent state. The inability to modify Oct4 established cell cycle checkpoint function. The loss of Oct4 sumoylation resulted in G1 checkpoint activation. Consequently, decreased recruitment of Hmgb2 to chromatin and the resolution of bivalent domains were observed. A regulatory feedback loop, in which Akt inactivation initiates the DNA damage response, is established whenever Oct4 cannot be phosphorylated. Examination of cells expressing Akt-mutant versions of Oct4 highlighted the vital role of Oct4 phosphorylation in facilitating interaction with Hmgb2. This association was essential to maintain transcriptional poise at developmentally regulated loci. These particular studies suggest that Oct4, Akt, and Hmgb2 participate in a gene regulatory loop, required for two hallmarks of the pluripotent state: active Akt signaling and a permissive chromatin environment. It appears that Oct4 sumoylation however, is required to maintain the H3K27me3 mark at bivalent domains and to promote cell cycle progression. In this manner, chromatin plasticity is retained and thereby permits subsequent stages of differentiation in response to external cues.

4.2 Implications

While it is now generally recognized that Oct4 performs a crucial role in promoting pluripotency, the underlying mechanisms that regulate this state, the initial inputs that trigger differentiation, and the subsequent changes that drive cell fate transitions remain poorly understood. Long-standing research has suggested that Oct4 may function as either a transcriptional activator or a repressor. It is generally accepted that both of these activities are required to maintain pluripotency by activation of genes required for pluripotency and repression of those required for differentiation (Boyer et al., 2005; Boyer et al., 2006; Chen et al., 2008; Loh and Lim, 2011; Pasini et al., 2010). Contrasting evidence however, suggests that the activating function of Oct4 is more critical to the maintenance and induction of pluripotency (Hammachi et al., 2012).

Several studies have indicated that post-translational modification (PTM) of Oct4 by phosphorylation may regulate its function in pluripotent stem cells (Phanstiel et al., 2011; Saxe et al., 2009; Swaney et al., 2009). A more recent study, furthermore, has identified 14 unique phosphorylation sites (11 novel sites) within Oct4 (Brumbaugh et al., 2012). The Brumbaugh study extends our understanding of Oct4 function through functional characterization of two critical residues, T234 and S235 (T228 and S229 in mouse) by mutation analysis. Electrophoretic mobility shift assays described in the paper suggest that constitutive co-phosphorylation of these residues decrease Oct4 binding to chromatin and also decrease the reprogramming efficiency of Oct4 as compared to wild-type. Although the upstream kinases responsible for phosphorylation of these residues were not identified, Oct4 phosphorylation by ERK2 was confirmed at several residues.

The research presented by Brumbaugh et. al., however leaves several questions regarding how Oct4 regulates pluripotency unanswered. First, is there a relationship between phosphorylation of Oct4 at T234 and S235 (and the other identified ERK2 phosphorylation sites)? Although mutations at both T234 and S235 to mimic constitutive phosphorylation at these sites resulted in decreased DNA binding and reprogramming efficiency, mutation of each single site did not produce similar results. This may suggest a cumulative effect as proposed by the authors. It however, may also suggest that dimerized forms of differentially phosphorylated Oct4 protein may assemble on chromatin more readily and/or more stably. The study also lacks in vivo data which demonstrates the biological relevance of these sites in pluripotent stem cells or how altered signaling cascades during cell fate transitions may impact Oct4 phosphorylation and its PTM-dependent function in each of these states.

The studies presented in this thesis suggest that context-dependent regulation of Oct4 function is crucial to our understanding of the pluripotent state. First, a dynamic PTM-dependent feedback loop between Akt, Oct4, and Hmgb2 maintains transcriptional poise at bivalent domains, self-renewal, and pluripotency. Second, the inability to phosphorylate Oct4 results in decreased Akt signaling. This initiates the G2/M DNA damage response as indicated by elevated levels of γ H2AX, Brca1, and Cdkn1a. Global increases in Sumo1 and Sumo2/3 conjugates and high molecular weight forms of Trp53 and Parp1, consistent with their modification and enhanced function during the DNA damage response (Polo and Jackson, 2011; Vlachostergios et al., 2009; Wrighton, 2010), are also detected in the Oct4 Akt-mutant expressing cells. Finally, the faithful emergence of lineage specific transcriptional programs requires Oct4 phosphorylation and sumoylation. Although Oct4 phosphorylation maintains interaction with Hmgb2 during mitotic progression, its

sumoylation facilitates G1/S progression and maintains chromatin plasticity through recruitment of co-repressor complexes, which may act to restrain the function of Oct4 C-terminal interactors. Together, these modifications maintain repression of developmentally regulated loci while promoting their transcriptional poise until full activation of lineage specific gene expression programs occurs during differentiation. Our model (Figure 1) is the first to link definitively Akt signaling, PTM-dependent Oct4 function, transcriptional regulation, and the DNA damage response to both pluripotency and the initiation of differentiation.

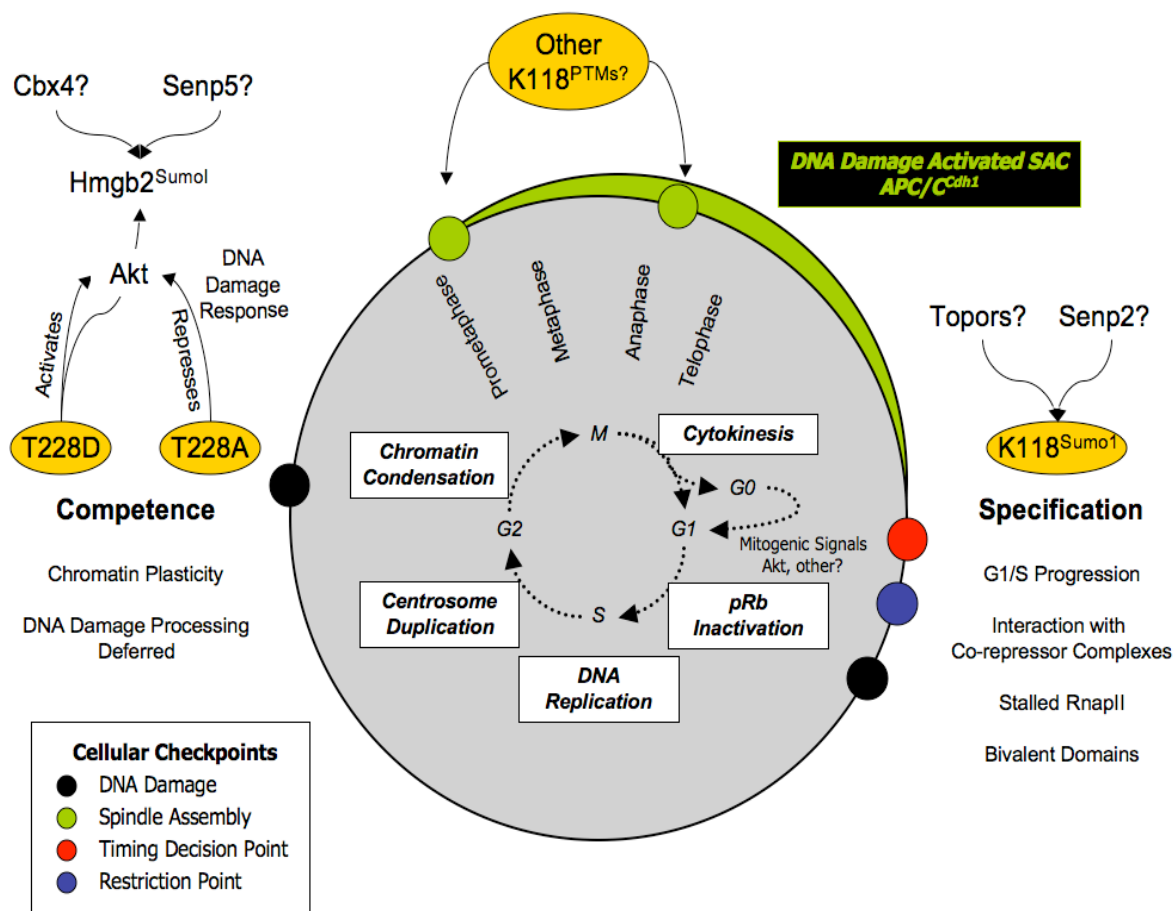


Figure 1. The Akt-Oct4-Hmgb2 Axis Regulates Cellular Checkpoint Function, Gene Expression, and Cell Fate Transitions. Please refer to text for thorough discussion.

4.2.1 An Oct4-Akt-Hmgb2 Gene Regulatory Loop Maintains the Primitive Stem Cell State

Oct4 directs the expression of PcG proteins and transmission of the H3K27me3 mark in a PTM-dependent manner. This indicates that dynamic PI3K/Akt signaling through Oct4 plays a central role in maintaining the stem/progenitor state (Figure 2). The PI3K/Akt signaling pathway is one of the most dysregulated pathways in cancer (Altomare and Testa, 2005; Bader et al., 2005). Whereas mutations in this pathway have been implicated in many types of cancers (Vivanco and Sawyers, 2002), epigenetic mechanisms also force its constitutive activation and are implicated in a wide-range of tumor types, including pediatric Wilms tumour (Ravenel and al., 2001), colorectal cancer (Cui et al., 2003), and rhabdomyosarcoma (Merlino and Helman, 1999).

Epigenetic-based transcriptional dysregulation often precedes somatic mutations, which are acquired at later stages to promote the survival and expansion of the epigenetic progenitor. While many think that this founding cell may result from aberrant adult somatic stem cell differentiation, the reprogramming of terminally differentiated somatic cells (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007) suggests that cancer may also arise from a once terminally differentiated cell. Recent studies implicating a stem cell like chromatin state in cancer (Kondo et al., 2008; Ohm et al., 2007) reinforces the hypothesis that epigenetic silencing mechanisms play a major role in both processes.

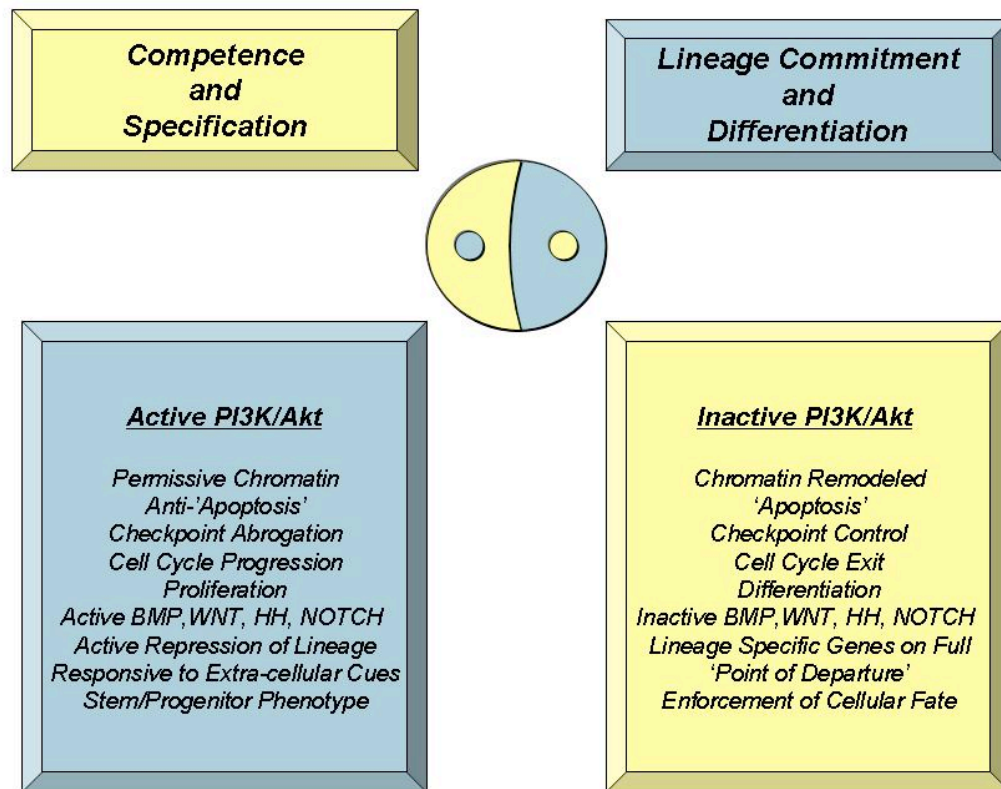


Figure 2. PI3K/Akt Signaling Through Oct4 Maintains the Stem/Progenitor State. Please refer to text for full discussion.

The role of Akt signaling in maintaining the stem/progenitor state and in facilitating cell fate transitions is complex. Low level Akt signaling maintains cellular quiescence with cells predominantly in G0/G1. Its increased activation, however, activates adult stem cells and promotes S-Phase entry (Chabanon et al., 2008; Gallia et al., 2009; Kimura and Nakano, 2009; Kobiela et al., 2007; Kuang et al., 2008; Zhang et al., 2006). Akt signaling may inhibit differentiation (Bang et al., 2001) by maintaining neural progenitor self-renewal in a dose-dependent fashion (Sinor and Lillien, 2004). By contrast, increased Akt activation may also promote neuronal differentiation (Lopez-Carballo et al., 2002). This effect is partly due to its Trp53-dependent anti-apoptotic protective function of neural progenitors (Eves et al., 1998; Tedeschi and Di Giovanni, 2009).

The effects of Akt signaling in induced pluripotency are similarly pleiotropic. PI3K/Akt signaling promotes de-differentiation of primordial germ cells (PGCs) by inactivating Trp53 (Kimura and Nakano, 2011). Akt upregulation also supports differentiation of mESCs to PGCs (Yamano et al., 2010). These cells maintain their plasticity, however, and can return to the pluripotent state by culture in mESC conditions. The absolute requirement for Akt signaling in induced pluripotency is unclear because it may promote or inhibit nuclear reprogramming. This depends upon the chromatin context (Nakamura et al., 2008). Therefore, it appears that dosage effects and other as yet to be defined factors are critical to Akt function. Furthermore, the level of Akt signaling may define several metastable chromatin states that retain the ability to respond to other signal transduction pathways. Despite the discrepancies about Akt function in facilitating cell fate transitions, Trp53-Cdkn1a activation status appears to be a key regulatory node in modulating its function (Hanna et al., 2009; Marion et al., 2009) and maintaining chromatin plasticity. At the

molecular level, moreover, it appears that activation of Akt signaling impacts two central pathways which oppose pluripotency: Gsk3 and Mapk (Lee et al., 2006a; Loh and Lim, 2011; Qi et al., 2004; Ying et al., 2008; Yuan et al., 2011; Zhang and Liu, 2002).

4.2.2 Oct4 PTMs Abrogate Cellular Checkpoints and Defer DNA Damage Processing

Akt-mediated phosphorylation and sumoylation of Oct4 create a positive feedback loop, which facilitates cell cycle progression and abrogates cellular checkpoints (Figure 1). These, in turn, maintain chromatin plasticity by deferring DNA damage processing through activation of Akt and inactivation of Trp53. Just as Akt signaling and Trp53-Cdkn1a inactivation play a role in mESC pluripotency and induced pluripotency, they are also critical regulators of preimplantation development (Gross et al., 2005; Jin et al., 2009; Niwa, 2007; Riley et al., 2005). The deferral of DNA damage processing by active Akt signaling may also cause the mutations observed during the generation of iPS cells (Blanpain et al., 2011; Gore et al., 2011; Hussein et al., 2011). It remains to be seen, however, if these mutations become fixed, and lead to permanent deleterious effects, or are resolved upon differentiation.

G2/M and G1/S checkpoints signify transitional periods during the cell cycle. These phases represent critical junctures when DNA damage may be corrected prior to either mitotic cell division or DNA replication. Both checkpoints require Trp53 activation to establish and maintain the repair response (Giono and Manfredi, 2006; Siliciano et al., 1997). In mESCs, however, Trp53 is maintained primarily in its inactive, unphosphorylated state that abrogates checkpoint function (Aladjem et al., 1998; Burdon et al., 2002). Despite

this inactivation, mESCs do possess a functional DNA repair machinery (Chuykin et al., 2008) and, when compared to somatic cells, exhibit heightened repair (Hong et al., 2007).

Our data indicates that Hmgb2 expression and Akt-mediated phosphorylation of Oct4 maintain active Akt signaling. This, in turn, promotes cell cycle progression and suppresses G2 checkpoint function, even in the presence of DNA damage (Kandel et al., 2002; Xu et al., 2010). Notably, these cells are refractory to apoptosis. The lack of this modification, however, results in Akt inactivation and Brca1 upregulation, which impacts cell cycle progression at G2/M and the expression of downstream genes required to coordinate the DNA damage response (MacLachlan et al., 2000).

4.2.3 Oct4 Sumoylation Transmits H3K27me3 to Maintain Chromatin Plasticity in the Daughter Cell

In the absence of Akt signaling, Oct4 sumoylation is required to maintain cell cycle progression, as well as H3K27me3 at remodeled sites. Therefore, we propose that Oct4 sumoylation presents a physical barrier to RnapII passage and maintains chromatin plasticity: perhaps through interaction with co-repressor complexes. This plasticity is required to mark sites that may be activated until the next chromatin state becomes fixed. Thus, Oct4 sumoylation is the mechanism that propagates the H3K27me3 mark upon mitotic cell division.

Oct4 sumoylation at G1 results in increased H3K27me3 enrichment at many developmentally regulated loci and G1/S progression. In addition to the DNA damage checkpoint, the cell also undergoes one additional checkpoint during G1/S transition: restriction point (R-point). R-point delimits cell passage into S phase and whether DNA replication will occur. Nutrient supply affects this decision. Insufficient nutrients, which may

result from serum deprivation, inhibit protein synthesis and results in the cell entering a quiescent state called G0. R-point is regulated by replication timing decision point (TDP) (Gilbert, 2010) during which the origins of DNA replication are set. Since the inability to sumoylate Oct4 induces G1 checkpoint and gene expression, this suggests that such modification may play a role in regulating R-point, G1/G0 transition, or TDP. Oct4 sumoylation status could also impact changes in transcriptional programs required during nucleosomal disassembly or reassembly (Williams and Tyler, 2007) when paternal histone are copied to the nascent strand.

While histone marks are inherited following DNA replication, the mechanisms underlying this process remain unclear. Nucleosomal disassembly occurs ahead of the replication fork, and nucleosomal reassembly occurs after its passage. Then, histones are deposited onto DNA in a replication-coupled manner (Perry et al., 1993) as the replication fork progresses. Evidence suggests that histones H3 and H4 may remain associated with DNA during replication fork passage (Gruss et al., 1993). Whether H3/H4 associates as a dimer or a tetramer, however, is still in question (Xu and Zhu, 2010) and is an important consideration for propagation of the H3K27me3 mark.

H3K27me3 can be stably inherited for many cell divisions (Hansen et al., 2008; Margueron et al., 2009). Perhaps this parentally marked histone functions as a template to transfer epigenetic modifications to newly synthesized histones when they are incorporated into the nascent DNA strand (Francis, 2009). The discrepant data may yet be resolved by observing H3K27me3 inheritance in self-renewing versus differentiating cells. Our results suggest that the H3K27me3 mark is faithfully inherited in both DNA strands only when Oct4 is sumoylated. The G1 checkpoint established upon loss of Oct4 sumoylation implies

that this pause may be necessary to allow chromatin reorganization to reset replication origins in the differentiating cell (Perry et al., 2004). Oct4 sumoylation consequently, may act within the replication timing decision point to maintain H3K27me3 within chromatin and enforce the existing chromatin state.

The spatial organization of chromatin within the nucleus is a reflection of the expression state of the genes contained within specific genomic loci. Chromatin organization may also facilitate access of the regulatory machinery required during cell fate transitions to specific genomic loci. In mESCs, repressed genes are marked by H3K27me3 in their promoter regions and are compartmentalized at the nuclear periphery (Galy et al., 2000). Upon differentiation, however, this repressive mark is removed and the locus relocates to the nuclear centre (Chambeyron and Bickmore, 2004). Localization of genes at the nuclear periphery, in close proximity to Pml nuclear bodies (NBs) and to the nuclear pore, however, does not invariably lead to repression. Rather, the ability to activate or repress these genes is dependent upon promoter sequence and Pml NB composition (Block et al., 2006).

Oct4 interacts with Pml (Liang et al., 2008a). Pml Nbs function to facilitate cell fate transitions as predetermined processing sites for damaged DNA (Boe et al., 2006). Pml NBs are cytoplasmically localized during M and G1 (Jul-Larsen et al., 2009). Pml acts to stabilize and concentrate DNA repair genes at nuclear pores in a SUMO1 dependent manner at G2/M, upon cellular stress, or upon DNA damage (Nagai et al., 2011; Saitoh et al., 2006; Xu et al., 2003; Zhao et al., 2009). Importantly, SUMO1-dependent recruitment of DNA repair genes has been shown to facilitate chromatin remodeling within Pml-NBs (Luciani et al., 2006). Taken together, our data suggests that Oct4 dephosphorylation and Akt inactivation would promote chromatin remodeling in Pml NBs. This remodeling event would subsequently be

enforced at the next G1/S transition only if Oct4 is not sumoylated and the H3K27me3 mark is not inherited.

4.3 Limitations of the Dissertation Research

As with all research, this dissertation is not without limitations. Critical examination of the scientific methodology employed and results described within this work is required to fully understand its impact and contribution to the study of transcriptional regulation in pluripotency. Some of these limitations may provide direction for future research, as outlined in section 4.4. We will therefore next explore the major limitations of the findings described in Chapters 2 and 3 of this thesis.

In Chapter 2 transcript level gene co-expression data was examined in a wide array of stem cell populations and their differentiated derivatives. A bioinformatics approach was then used to identify genes whose expression tracked with Oct4 transcript. The rationale was that this approach could lead to increased understanding of Oct4 function, and in particular, the transcriptional modules in which Oct4 participated to maintain the pluripotent state. The impact of this study was hampered by three major limitations in the methodological approach employed for validation of data. First, the ChIP-seq assays described in our study interrogated only a small subset of the genes correlated to Oct4 in this study. More comprehensive approaches, such as ChIP on Chip or ChIP-Seq, would have provided much stronger evidence for Oct4 binding and its putative impact on expression of the bound genes. Second, binding of Oct4 at specific loci does not necessarily suggest transcriptional regulation. Oct4 knockdown or over-expression studies would be required to substantiate this claim which were not performed in this study. Third, this work did not validate the role of any of the individual genes, or the enriched GO categories identified with respect to their involvement in pluripotency or differentiation. Nonetheless, many studies either cited in the original PlosOne publications or within the body of this thesis have subsequently validated

much of our data and point to the contribution of this work to pluripotent stem cell biology in expanding the repertoire of genes and gene function implicated in maintaining this state.

Chapter 3 subsequently attempted to characterize changes in the Oct4 regulome, which might lead to an enhanced mechanistic understanding of the factors that maintain pluripotency and those which facilitate cell fate transitions. The studies in this chapter focused upon Akt signaling and PTM-dependent regulation of Oct4 function. The interpretation of our data was limited by several shortcomings both technical and methodological in nature. First, the data presented in Chapter 3 did not provide additional evidence of Oct4 sumoylation, nor did it seek to describe its relationship to Akt-mediated phosphorylation or other Oct4 PTMs. Since both Oct4 sumoylation and Akt-mediated phosphorylation have both been described as a stabilizing force to Oct4 protein (Lin et al., 2012; Wei et al., 2007) and this work, it will be important to determine whether they act individually or in a cumulative manner in future studies. Next, the IP-kinase assay data presented in Chapter 3, Figure 2 of this work did not definitively prove that Oct4 was a direct Akt substrate in ESCs. However when combined with the findings in two subsequent publications (Brumbaugh et al., 2012; Lin et al., 2012) that provided mass spectrometry data in hESCs and IP-kinase data employing recombinantly produced Akt, our data corroborates these findings and also suggests the importance of the loss of Oct4 phosphorylation at T228 (in mouse) in facilitating cell fate transitions. Finally, Chapter 3 lacked *in vivo* data, which would be important to substantiate the biological role of Oct4 PTMs during early development. In particular, the assessment of Akt signaling, Oct4 phosphorylation and interaction with Hmgb2 and the SET complex within pre-implantation blastocysts would help to further dissect the role of this feedback regulatory network during early development.

As well, the development of Oct4 knock-in PTM mutant models (either global or tissue specific expression) would further help to assess their regulatory role in Oct4 function during normal development and in disease. These models would be exceptionally useful in dissecting out the epigenetic and transcriptional deregulation that is a hallmark of cancer. They may also provide insight into the initial events that lead to the transformed state as opposed to those which underly cancer progression and the concomitant cellular heterogeneity that renders cancer refractory to current treatment regimens.

4.4 Future Directions

Continued study of the link between signal transduction and epigenetic regulation of gene expression will provide insight into how their dysregulation causes disease. This knowledge will provide a more rational basis for the treatment and eventual cure of differentiation defective diseases such as neurodegenerative disorders, myopathies, and cancer. We have highlighted an important role for Akt signaling through Oct4 in maintaining the primitive stem cell state in this study. Although our observations have been made based upon global modulation of Oct4, insight into locus specific regulation of Oct4 function will be of paramount importance for an enhanced understanding of self-renewal, differentiation, cellular reprogramming, and the diseased state.

4.4.1 The Role of Oct4 and Hmgb2 in PcG Recruitment

Several questions arise from our research into the role of Oct4 interaction with Hmgb2. The answers to these questions may further illuminate the process of cell fate transitions. First, does Hmgb2 play a role in facilitating chromatin looping during mitotic progression? This question could easily be addressed through a 3C approach and would clarify the mechanism whereby specific genomic loci are made accessible to Pml Nbs to facilitate chromatin remodeling. Based on our model (Figure 1) we predict that Hmgb2 is retained at loci only where Oct4 remained phosphorylated. As a result, the looped out regions would not contact Pml Nbs and would not undergo remodeling. Second, does Hmgb2 directly function as a PcG recruiter, or is its role indirect, perhaps through interaction with Oct4 or other proteins? Our results indicate that Oct4 and Hmgb2 together modulate the expression of PcG and TrxG proteins. Since their interaction and co-recruitment to chromatin is also required to

maintain bivalent domains, this suggests that Oct4 and Hmgb2 participate in PcG recruitment. More direct evidence, demonstrating Hmgb2 interaction with Phc1 or other PcG members, would delineate the role of Hmgb2 as a PcG recruiter. Study of the Hmgb2 interactome in the presence or absence of Oct4 expression would clarify whether Hmgb2 functions alone in this respect, or whether interaction with Oct4 is required to direct Hmgb2 to specific loci. High-throughput genomics and proteomics approaches like ChIP-Seq and mass spectrometry that are subsequently validated via ChIP-PCR and immunoprecipitation will address these questions. Finally, is the G(A) motif, recognized by Hmgb2, required for PRE function? This question could be answered through complementary *in vitro* and *in vivo* approaches. Luciferase-based assays, using Oct4 target loci, plus and minus the G(A) motif would determine if loss of this motif mediates heightened activation of the reporter. *In vivo* approaches studying the impact of G(A) motif deletion will help to elucidate the functional relevance of this motif in maintaining PcG repression and averting homeotic transformation during early development (in mice). The Hoxb1 locus would be a priority candidate for this type of study.

4.4.2 What lies Upstream of Oct4 and Hmgb2 PTMs?

It will also be important to determine the factors that lie upstream of Oct4 and Hmgb2 sumoylation. Likewise, understanding how they, in turn, are regulated may provide clues into the spatio-temporal function of Hmgb2 and Oct4. For example, our data suggests that Hmgb2 sumoylation plays a role in mitotic chromatin condensation. A potential candidate for Hmgb2 symoylation is E3 Ligase Cbx4, whose overexpression leads to a block at G2/M (Dahiya et al., 2001). Cbx4 has also been found to regulate the recruitment of Bmi1, other

PcG members, and DNA repair proteins to sites of DNA damage (Gieni et al., 2011; Ismail et al., 2010; Ismail et al., 2012), suggesting that Cbx4 mediated sumoylation is required for repair of DNA damage. We also propose that Senp5 is a contender for catalyzing Hmgb2 desumoylation since it localizes to the nuclear periphery prior to nuclear envelope breakdown (Zunino et al., 2009). Although Hmgb2 sumoylation may direct chromatin looping to maintain the repressed state, its desumoylation would be required for nuclear envelope breakdown and mitotic progression. Standard molecular approaches such as knockdown, overexpression, and *in vitro* sumoylation assays would help to clarify the potential roles of Senp5 and Cbx4 upon Hmgb2 function.

Identification of candidate regulators for Oct4 sumoylation may be intractable since it interacts with sumoylated proteins, including Ubc9. In addition, certain SIM containing sumo substrates may not require an E3 SUMO ligase; association with Ubc9, the sole E2 SUMO ligase may be sufficient to effect sumoylation (Jakobs et al., 2007). Nevertheless, since global increases in Sumo conjugates and initiation of the DNA Damage Response was observed in our Oct4 Akt-mutant sublines, DNA damage regulated Topors (Lin et al., 2005a) represents a likely candidate for modification of Oct4. We also predict that Sumo protease Senp2, which facilitates G1/S progression (Chiu et al., 2008), may function in Oct4 de-sumoylation. Topors and Senp2 might therefore act as toggle switches to first negotiate TDP, by mediating Oct4 sumoylation, and then G1 DNA damage checkpoint, by removal of this modification since overexpression of Topors may lead to G1 arrest (Saleem et al., 2004; Yang et al., 2010). As well, in *Drosophila*, dTopors has been identified as a chromatin insulator (Capelson and Corces, 2005), suggesting that site-dependent regulation of Topors function may mediate long-range chromatin looping to facilitate a repressive chromatin state

at sites of DNA damage (Comet et al., 2011). Similar approaches as those taken for Hmgb2 can also be utilized to clarify the potential role of Topors and Senp2 upon Oct4 sumoylation. Likewise, they can also be used to explore whether Oct4 may be marked at K118 by other modifications, the temporal progression of these events, their *in vivo* functional relevance, and whether specific modifications may prohibit or facilitate subsequent modifications.

4.4.3 The Role of Oct4 in a Sustained DNA Damage Response

The role of Oct4 beyond the initiation of the DNA damage response remains unknown. Our results indicate that Trp53, Parp1, Brca1, Cdkn1a, γ H2Ax, and other genes implicated in the response to DNA damage are upregulated in cells expressing Akt phospho-mutant versions of Oct4. It is known that Oct4 also interacts with many of these genes. Future studies aimed at dissecting their PTM-dependent recruitment to DNA repair foci, perhaps via interaction with Oct4's SIM is warranted.

Insight into the PTM-dependent ability of Oct4 to abrogate cellular checkpoints despite the increase in Cdkn1a protein level expression may come from continued study of its function within the context of the SET complex. Specifically, Oct4 interaction with Set and Hmgb2 may provide insight into abrogation of the G2/M, G1/S observed in the Oct4 mutant expressing cells. For example, overexpression of Set blocks G2/M transition by inhibiting Cyclin b-cdk1 activity (Canela et al., 2003). Set also inhibits Pp2a, which regulates Akt phosphorylation and controls mitotic exit (Forester et al., 2007). Therefore, we hypothesize that Oct4 phosphorylation would promote interaction with Set and sumoylated Hmgb2. Upon Hmgb2 desumoylation or Oct4 dephosphorylation, however, the complex would destabilize, Set's inhibition of Pp2a would be released, and the checkpoint would be

successfully negotiated. Investigation of the potential role of Pp2a as the phosphatase for Oct4 T228p would also be warranted in light of this hypothesis. Set also inhibits Cdkn1a modulation of Cyclin E/Cdk2 complexes and promotes G1/S transition (Estanyol et al., 1999). Cdkn1a inactivation, by Oct4-Set, and abrogation of G1 checkpoint may be related to SAC abrogation (Chan et al., 2008).

The relationship between Oct4 and Nme1 facilitating cell fate transitions warrants further attention. SET Complex member Nme1 is a DNase which facilitates single strand (ssDNA) nicking (Zhao et al., 2007). mESCs routinely exhibit non-induced ssDNA breaks and display γ H2AX foci during G2/M (Chuykin et al., 2008). However, these ssDNA breaks are usually resolved and do not necessarily lead to a sustained DNA damage response or to apoptotic cell death. This suggests therefore that ssDNA breaks may function normally during G2/M progression in the absence of further genomic insult. Consequently, Oct4 may recruit Nme1 to target specific genomic loci for ssDNA nicking to facilitate normal mitotic progression.

Atr activation and recruitment to stalled replication forks in a γ H2AX-dependent manner in S-phase cells in the presence of ssDNA breaks is an initial step in activation of a "full blown" DNA damage response (Ward et al., 2004). Since Oct4 sumoylation promotes G1 transition, and Senp2 overexpression blocks cell-cycle progression at G1/S, we predict that concerted sumoylation and de-sumoylation of Oct4 is required to avert replication fork stalling at bivalent domain containing loci, which have a stalled RnapII. The inability to de-sumoylate Oct4, however, may result in transcription and replication fork collision. The activation of SET complex members Anp32a and Tnfsf13 leads to induction of dsDNA breaks via apoptosome formation and Caspase mediated activation of Dfffb (Lieberman and Fan, 2003). This apoptotic SET sub-complex is inhibited, however, through sustained

interaction with Hmgb2 and Set. Consequently, we would hypothesize that Oct4 interaction with Hmgb2 and Set would hinder dsDNA breaks and chromatin remodeling at G2/M, leading to differentiation. Continued ChIP-Seq, ChIP-PCR, gene expression, and interaction studies in cells expressing Oct4 wild-type and mutant constructs will illuminate the complex interactions occurring at Oct4 regulated loci during cell cycle progression and cell fate transitions.

4.4.4 Oct4/SET Complex Function in Cell Fate Transitions

DNA damage must be repaired following checkpoint activation prior to cell-cycle resumption. Just as Oct4 initiates the response to DNA damage, we hypothesize that it may also function in processing and resolution of the accrued lesions. The sumoylation interaction motif (SIM; motif found to facilitate interaction with sumoylated proteins) contained within the POU_H domain of Oct4 may function to recruit sumoylated proteins to Pml Nbs. It is known that the spatio-temporal recruitment of several DNA repair proteins, which are transcriptionally regulated by and with which Oct4 interacts (Campbell et al., 2007; Pardo et al., 2010), are required for successful repair of dsDNA breaks (Hemberger et al., 2003; Heo et al., 2008; MacLachlan et al., 2000; Wang et al., 2000). Moreover, high molecular weight conjugates of several DNA repair genes and global upregulation of Sumo conjugates are observed in Oct4 Akt mutant expressing cells. These findings highlight the potential role of Oct4 phosphorylation status and its SIM in facilitating the formation of non-covalent Ubc9 complexes during the DNA damage response (Prudden et al., 2011). ChIP-Seq analysis of Oct4 SIM deletion mutants, combined with gene expression profiling, will clarify whether this motif is required in recruitment of Sumo modified targets to or from

damaged sites. It will also reveal the impact of interactions mediated through Oct4's SIM to transcript level expression. Recent studies indicating that several PcG members are sumoylated (Riising et al., 2008), further underscores the potential importance of Oct4 SIM in facilitating their recruitment to facilitate transcriptional change following the damage response.

Hmgb2 recruitment to repressed sites decreases as the gene undergoes activation, whereas enrichment of Hmgb1 increases (Supplementary Fig. 5). Although the association of Hmgb1 and Hmgb2 with chromatin is usually transient (Bianchi and Agresti, 2005), Hmgb1 binding is greatly enhanced upon histone 4 (H4) deacetylation in apoptotic cells (Scaffidi et al., 2002). Hmgb1 possesses a distinct ability to recognize unusual DNA structures such as broken or looped DNA where it interacts with Trp53 (Jaouen et al., 2005; Stros et al., 2004) and interferes with formation of the PIC (Sutrias-Grau et al., 1999). Hmgb1, however, also is able to resolve aberrant DNA structures which leads to release of transcriptional arrest (Waga et al., 1990). Consequently, we propose that cell fate transitions are mediated by the exchange of Hmgb1 and Hmgb2 binding at Oct4 targets.

Future studies employing knockdown and overexpression of Hmgb1 and Hmgb2 will allow us to assess the functional relevance of their exchange during cell fate transitions. Moreover, ChIP-Seq analysis of their enrichment at Oct4 targets in cells expressing wild-type and mutant constructs, combined with gene expression profiling, will allow us to assess the impact of these genes upon changes in transcriptional programs. Hmgb1/2^{-/-} double knockout (DKO) mice succumb embryonically (Bianchi and Agresti, 2005). Single knockouts of *Hmgb1* or *Hmgb2* are however, viable. *Hmgb1*^{-/-} mice succumb perinatally due to immune dysfunction. By contrast, *Hmgb2*^{-/-} mice display reduced fertility in males

due to increased apoptosis of germ cells in the seminiferous tubules and immotile spermatozoa (Ronfani et al., 2001). In light of its recently described role as a cytokine (Degryse and de Virgilio, 2003) which is active in stem cell recruitment (Palumbo and Bianchi, 2004), and its ability to substitute for Sox2 in preimplantation embryonic development (Xie et al., 2010), Hmgb1 may provide a link between niche induced signaling and inflammatory responses (De Santa et al., 2007) associated with PcG silencing. Moreover, it will be of utmost interest to assess the feasibility of iPS cell generation in *Hmgb2*^{-/-}, *Hmgb1*^{-/-}, or combined *Hmgb1/2*^{-/-} somatic cells.

4.5 Final Thoughts

Oct4 remains at the forefront of a regulatory network that maintains embryonic stem cell self-renewal and facilitates cell fate transitions. In response to dynamic Akt signaling, transcriptional poise is maintained at developmentally restricted loci in an Oct4 and Hmgb2 PTM-dependent manner. Our studies suggest that Oct4 modulates the expression of genes with which it subsequently interacts to promote cell cycle progression, self-renewal, and pluripotency. Activation of the DNA damage response and cell fate transitions may ensue in the absence of a functional Akt-Oct4-Hmgb2 regulatory feedback loop. Successful resolution of the transition will result in altered gene expression programs and changes in cellular phenotype. Inefficient DNA repair, however, may lead to the diseased state. Further understanding of the PTM-dependent impact of Akt, Oct4, and Hmgb2 in this process will help gauge the effectiveness of chemotherapeutic strategies and provide novel targets for the application of newly developed or existing agents (Lee et al., 2009; Yang et al., 2009).

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