NOVEL ROLES FOR MYC IN THE MAINTAINENCE OF PLURIPOTENCY

by

JAMES CLYDE CHAPPELL

(Under the Direction of Stephen Dalton)

ABSTRACT

Pluripotent stem cells (PSCs) are an important model system for studying early embryonic developmental processes of both normal and diseased organisms. Furthermore, investigating and revealing the mechanisms that govern PSC biology will be fundamental for their eventual use for therapeutic replacement of defective and/or damaged tissues.

Examination of the myelocytomatosis oncogene (MYC) family of transcription factors revealed regulation of a set of target genes that are critical for PSC maintenance, termed the dual-specificity phosphatases (DUSPs). The importance of MYC to PSC biology was previously attributed mainly to its role in activating genes involved in increased metabolism and proliferation. Here I demonstrated that the protein products of MYC genes activates expression of two DUSP family members, DUSP2 and DUSP7, which consequently inhibit activation of mitogen-activated protein kinase - extracellular signal-regulated kinases (MAPK/ERK) signaling, a key determinant of PSC differentiation. This finding demonstrates that the significance of MYC in PSC biology is more nuanced than activation of growth promoting genes.

INDEX WORDS:  Stem cells, myc, self-renewal, pluripotency, differentiation, Dusp, Erk, Map kinase signaling
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by

JAMES CLYDE CHAPPELL

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JAMES CLYDE CHAPPELL

Major Professor: Stephen Dalton
Committee: Stephen Hajduk
Shaying Zhao
Edward Kipreos

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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PLURIPOTENT STEM CELLS

Pluripotent stem cells (PSCs) are characterized by their ability to proliferate indefinitely and divide symmetrically into two identical daughter cells with each mitosis in \textit{in vitro} culture (Zwaka and Thomson 2005), a property designated as “self-renewal”. PSCs also have the ability to differentiate towards any of the three embryonic germ lineages that will go on to generate the mature systems of an adult organism, a property designated as “pluripotency”. PSCs are an ideal model system for recapitulating early \textit{in vivo} development, and therefore aid in interrogation of developmental mechanisms. They also have the enormous potential to serve as a therapeutic by replacing damaged or dysfunctional tissue resulting from disease or aging. Current PSC lines are predominantly established either from isolation of embryonic stem cells or from a directed de-differentiation process.

Embryonic Stem Cells

First derived in 1981 (Evans and Kaufman 1981; Martin 1981), embryonic stem cells (ESCs) are an \textit{in vitro} analog of cells found in the inner cell mass (ICM) of a blastocyst stage embryo. To establish ESC lines, ICM cells are isolated by
immunosurgery and cultured in appropriate signaling conditions. The resulting immortalized ESCs improved the capacity for studying early embryonic development compared to previous in vitro models, such as embryonal carcinoma (EC) cells, which have a restricted differentiation capacity (Martin 1980).

Advances in technology have increasing the efficiency of derivation and culturing of ESCs. It was determined that isolation of blastocysts following a period of delayed implantation, referred to as diapause, greatly improved the efficiency of ESC line generation (Brook and Gardner 1997). Furthermore, the necessity for co-culture with a layer of feeder cells is now obsolete; substituted for by gelatinization of culture dishes and the identification of individual growth factors that promote pluripotency and self-renewal.

**Human Embryonic Stem Cells and Epiblast Stem Cells**

Human embryonic stem cells (hESCs), first derived in the 1990s, are self-renewing and pluripotent, but exhibit many divergent properties when compared to murine ESCs (Thomson et al. 1995; Thomson et al. 1998). It has been demonstrated that these cells phenotypically resemble murine cells that are derived from the post-implantation stage of embryonic development, termed epiblast stem cells (epiSCs). EpiSCs and hESCs are more developmentally advanced, in terms of their spatiotemporal proximity to differentiation in vivo, and are considered to be in a “primed” state of pluripotency (Figure 1.1). These cell types require a distinctive cohort of growth factors, cooperation of Activin/Nodal
and FGF2 pathways (James et al. 2005; Vallier et al. 2005), to maintain a signaling environment conducive to pluripotency when compared to mESCs, which are considered “naïve” (Brons et al. 2007; Tesar et al. 2007). The ability to culture naïve human PSCs has only been recently achieved (Chan et al. 2013; Gafni et al. 2013; Takashima et al. 2014; Theunissen et al. 2014; Ware et al. 2014), and will allow researchers to investigate key species differences between developmentally equivalent cells.

**Induced Pluripotent Stem Cells**

In 2006, it was discovered that murine PSC lines could be established without the need for isolation from an embryo and without the technical complexity of reprogramming by the nuclear transfer technologies that were demonstrated over 50 years ago (Gurdon et al. 1958; Gurdon and Uehlinger 1966). Takahashi and Yamanaka demonstrated that enforcing expression of four key pluripotency-associated transcription factors, OCT4/SOX2/KLF4/MYC (OSKM), was sufficient to reprogram a small percentage of somatic cells to a pluripotent state (Takahashi and Yamanaka 2006). These induced pluripotent stem cells (iPSCs) were confirmed to be bona fide PSCs by their ability to generate germline chimaeras, the gold-standard assay for pluripotency (Bradley et al. 1984). This achievement opened the door for new methods of disease modeling, drug screening, and regenerative medicine. A considerable amount of research has since investigated the mechanisms involved in reprogramming, as well as methods for improving the otherwise inefficient process. One of the original four
reprogramming factors, c-MYC, is the focus of this dissertation, and its role in the reprogramming process is detailed in Chapter 2.

REGULATION OF PLURIPOTENCY BY SIGNALING PATHWAYS

Pluripotent stem cells (PSCs) rely on a number of key signaling pathways that direct them either to self-renew and proliferate, or to initiate differentiation towards a committed lineage. Traditionally PSCs were co-cultured with a feeder layer of fibroblasts, growth-arrested by treatment with chemicals or radiation, which provided the signaling environment conducive to self-renewal and pluripotency. Nowadays the most crucial signaling pathways responsible for PSC maintenance have been determined and advances in the culture systems have been made that render fibroblast feeder layers dispensable and provide a more defined culture environment for studying pluripotency mechanisms.

Leukemia Inhibitory Factor Signaling

Leukemia inhibitory factor (LIF) is a cytokine of the interleukin-6 (IL6) superfamily that is an integral component of self-renewal media. It binds and activates a specific cell surface receptor (LIFR) that heterodimerizes with a co-receptor gp130. This leads to activation of the Janus kinase (JAK) and the phospho-activation of the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) (Ernst et al. 1996; Niwa et al. 1998). Phosphorylated STAT3 then activates transcription of a cohort of target genes related to cell cycle control, immortalization and pluripotency (Figure 1.2)(Matsuda et al. 1999).
It was demonstrated that loss of inactivation of LIF or STAT3 in ESCs triggers differentiation but also that this result is negated by enforced expression of a key target of LIF/STAT3 signaling, c-MYC (Cartwright et al. 2005).

**Transforming Growth Factor Beta Signaling**

While LIF stimulates a signaling response necessary for self-renewal, it is not sufficient as the singular inhibitor of differentiation. Traditional self-renewal media requires supplementation with fetal calf serum (FCS), which has an undefined chemical composition and thus its precise effects on PSC maintenance are indeterminate. However, it has been demonstrated that PSC differentiation, caused by removal of FCS in culture conditions that promote neural lineages, is inhibited by supplementation with a recombinant transforming growth factor beta (TGFβ) protein, bone morphogenic protein 4 (BMP4)(Ying et al. 2003; Ying and Smith 2003).

BMP4 binds to its receptor dimer BMPR1/2, leading to phospho-activation of the SMAD proteins SMAD1, SMAD5, and SMAD8. SMAD1/5/8 forms a complex with SMAD4, enabling translocation to the nucleus, where a set of PSC maintenance genes is targeted for transcriptional activation. One such group of BMP4/SMAD target genes are the inhibitor of DNA binding (Id) genes, which promote PSC maintenance by inhibiting the binding of DNA by helix-loop-helix transcription factors that would otherwise activate transcription of differentiation genes (Figure 1.3)(Hollnagel et al. 1999). Another important BMP/SMAD target gene is dual-specificity phosphatase 9 (DUSP9), whose protein product can
inhibit phospho-activation of the differentiation promoting protein extracellular signal-regulated kinase (ERK) (Li et al. 2012). It is likely that DUSP9 cooperates with DUSP2 and DUSP7, which are stimulated by LIF signaling, to promote pluripotency as detailed in Chapter 3.

**Extracellular Signal-Regulated Kinase Signaling**

Activation of signaling through the fibroblast growth factor/mitogen-activated protein kinase/extracellular signal-regulated kinase (FGF/MAPK/ERK) pathway is a crucial stimulus of differentiation for mPSCs (Chen et al. 2006; Kunath et al. 2007). Mechanistically, ERK signaling begins with binding of extracellular FGF4 to its receptor, which initiates a phosphorylation cascade involving RAS, RAF, MEK, and ERK (Figure 1.4). ERK is anchored by MEK in the cytoplasm, but upon phosphorylation will translocate to the nucleus and accumulate. This will initiate differentiation by various mechanisms, including repression of NANOG transcription (Hamazaki et al. 2006).

The importance of the ERK signaling pathway for PSC biology has been highlighted in several ways. In one study, it was demonstrated that ESCs lacking either FGF4, or ERK2, fail to downregulate pluripotency markers, and fail to form any of the lineages of the three embryonic germ layers, when challenged by differentiating conditions (Kunath et al. 2007). Furthermore, modern PSC culture systems are reliant on either growth factors or small molecules that attenuate ERK phospho-activation, albeit by distinct mechanisms (Figure 1.5). The two-inhibitor (2i) culture system is effective at maintaining an undifferentiated state, in
part due to inhibition of ERK phosphorylation by inclusion of a MEK inhibitor, PD0325901 (Ying et al. 2008). Fetal calf serum (FCS)- LIF-based media also attenuates FGF4/ERK signaling, but relies on downstream, suppressive proteins (DUSPs) that are activated by TGFβ/BMP and LIF/STAT3 signaling.

**Dual Specificity Phosphatases**

Similar to other MAP kinases, ERK requires concurrent phosphorylation on two residues of a T–X–Y motif for activation (Boulton et al. 1991), and the duration and magnitude of that activation governs the biological consequences. Several members from the family of Dual Specificity Phosphatases (DUSPs) are capable of fine-tuning, or completely inhibiting ERK activity, by dephosphorylating either tyrosine or serine/threonine residues.

The DUSP family is sizable, with at least 61 different proteins encoded by the human genome, serving a variety of functional purposes in a wide array cell types (Patterson et al. 2009; Owens and Keyse 2007). Of greatest interest to this dissertation is the subfamily of 10 proteins that are MAP kinase specific regulators, referred to as MAP kinase phosphatases (MKPs). MKPs are structurally similar and are characterized by an N-terminal MAP kinase interaction motif (KIM), as well as a C-terminal catalytic domain with phosphatase activity. MKPs are further categorized by their subcellular location as either cytoplasmic, nuclear, or both and by their substrate specificity (Figure 1.6).

As described in the previous section, tight regulation of ERK signaling in PSCs is of upmost importance, and several DUSPs have been demonstrated to
play a role. Knockdown of DUSP5 in mESCs causes a reduction in pluripotency marker expression coincident with ERK activation, and negatively effects endoderm and mesoderm transcripts during an EB differentiation (Chen et al. 2011). Furthermore, DUSP9 was shown to be a downstream effector of BMP/SMAD signaling that is also critical for pluripotency (Li et al. 2012). Two others, DUSP2 and DUSP7, are targets of LIF signaling via MYC, as discussed in detail in Chapter 3.

**TRANSCRIPTION FACTOR NETWORKS MAINTAIN PSC IDENTITY**

The signaling environment of PSC culture is dictated by the presence of external growth factors, and/or small molecules, and establishes the intracellular networks that control PSC transcriptional programs and therefore cell fate. Recent studies have used chromatin immunoprecipitation following by DNA sequencing (ChIP-seq) to map the genome-wide locations of transcription factors and proposed a model for intracellular regulation of PSCs that involves functionally classifying transcription factors into three distinct regulatory modules (Figure 1.7) (Chen et al. 2008; Kim et al. 2008). The extent to which this model is accurate is still a matter of debate, but it provides a simplistic and illustrative framework for describing families of transcription factors and their role in PSC maintenance.

**Core Pluripotency Factors**

The “core pluripotency” module centers around transcription factors that are seemingly indispensible for PSC maintenance. Oct4, Sox2, and Nanog each
target and regulate each other’s promoter regions forming a positive feedback loop (Boyer et al. 2005; Gagliardi et al. 2013). These proteins also activate expression of other genes that promote self-renewal (Festuccia et al. 2012). Nanog overexpression has been shown to inhibit differentiation when LIF/STAT3 signaling and/or BMP/SMAD is ablated (Chambers and Smith 2004).

The Polycomb Repressive Complexes

The Polycomb repressive complex (PRC) module is comprised of two Polycomb group protein (PcG) complexes that target and epigenetically repress a cohort of important developmental genes human and murine ESCs (Boyer et al. 2006; Lee et al. 2006; Leeb et al. 2010). The two multi-protein complexes, PRC1 and PRC2, can vary in composition and have distinct molecular mechanisms that account for their repressive abilities. PRC1 canonically includes an E3 ubiquitin-protein ligase, RING1 or RING2, which targets histone H2A for mono-ubiquitination on lysine 119 (H2AK119u1). This leads to compaction of nearby chromatin and inhibition of transcriptional by either blocking initiation or elongation by RNA polymerase II (Dellino et al. 2004; Zhou et al. 2008). Canonical mammalian PRC2 complexes contain an Enhancer of zeste (EZH) methyltransferase that is responsible for mono-, di-, and trimethylation of Histone H3 at lysine 27 (H3K27me3) that has been demonstrated to inhibit a competing mark, the gene-activating acetylation of the same lysine (Cao et al. 2002; Czermin et al. 2002; Fujioka et al. 2008). The exact mechanisms by which the PRC complexes are recruited, of which components are included, and under
which circumstances each plays a role are still being investigated, however their necessity for stem cell maintenance and proper differentiation is well accepted (Leeb et al. 2010; Endoh et al. 2012).

The MYC Transcription Network

The third regulatory module is centered on the MYC family (MYC) of transcription factors, namely c-MYC, N-MYC, and L-MYC. MYC activates transcription, along with an obligate binding partner MAX, by binding mainly to E-box (CACGTG) motifs at the proximal promoter regions of genes (Figure 1.8)(Blackwell et al. 1990; Blackwood and Eisenman 1991). MYC is well studied for its role in cancer, and its activity was originally discovered in retroviruses present in avian neoplasms (Mladenov et al. 1967; Graf and Beug 1978). Since then, numerous cancer types have been shown to exhibit copy number amplification, activating translocation, mutation of regulatory regions, or other deregulation of MYC that leads to increased metabolism and uncontrolled cell growth.

Of the three modules, the MYC-centered accounts for most of the functional similarities exhibited in cancer and PSCs. In both contexts, MYC interacts with a similar cohort of cofactors to activate a similar set of target genes. MYC/MAX complexes have been demonstrated to recruit a multitude of histone modifying enzymes such as histone acetyltransferases (HATs) that further promote the increased accessibility that characterizes PSC chromatin (Knoepfler et al. 2006). MYC also interacts with the basal transcriptional
machinery to convert stalled RNA polymerase 2 to a productive state (Figure 1.9)(Rahl et al. 2010). These activities describe the most studied role of MYC in PSCs, namely how genes involved in cell growth and metabolism are activated. In this dissertation, the focus is on new set of MYC target genes that complement these activities by inhibiting differentiation, rather than promoting self-renewal.
Figure 1.1. mESCs and EpiSCs are derived from different stages of embryonic development. mESCs are derived from the inner cell mass of a pre-implantation embryo. EpiSCs are derived from post-implantation epiblasts. (Adapted from Nichols and Smith 2009)
Figure 1.2. **MYC is a target of LIF signaling.** Leukemia inhibitory factor (LIF) binds the LIF receptor/gp130 on the cell surface, causing JAK to activate STAT3 by phosphorylation. MYC is a target of STAT3. ( Adapted from Arabadjiev et al. 2012)
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CHAPTER 2

ROLES FOR MYC IN THE ESTABLISHMENT AND MAINTAINENCE OF PLURIPOTENCY


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ABSTRACT

MYC and MYCN have been directly implicated in the transcriptional regulation of several thousand genes in pluripotent stem cells and possibly contribute to the activity of all transcribed genes. Control of transcription by a pause-release mechanism, recruitment of positive and negative epigenetic regulators and a general role in transcriptional amplification have all been implicated as part of the broad, overarching mechanism by which MYC controls stem cell biology. As would be anticipated from the regulation of so many genes, MYC is involved in a wide range of cellular processes including cell cycle control, metabolism, signal transduction, self-renewal, maintenance of pluripotency, and control of cell fate decisions. MYC transcription factors also have clear roles in cell reprogramming and establishment of the pluripotent state. The mechanism by which MYC accomplishes this is now being explored and promises to uncover unexpected facets of general MYC regulation that are likely to be applicable to cancer biology. In this chapter we review our current understanding of how MYC contributes to the maintenance and establishment of pluripotent cells and how it contributes to early embryonic development.
INTRODUCTION

Well before MYC was implicated in the establishment and maintenance of pluripotency, it was known as a potent oncogene with roles in transcriptional regulation of metabolism, differentiation, cell lifespan, cell cycle and cell size control. These functions are all generally relevant to the maintenance and establishment of pluripotent stem cells. Despite this however, defining the precise mechanism by which MYC functions has been problematic and has led to much confusion. The following discussion will focus on our current understanding of how MYC functions in early embryonic development, maintenance of stem cell identity and in somatic cell reprogramming.

MYC AND MYCN ARE FUNCTIONALLY REDUNDANT IN EARLY EMBRYONIC DEVELOPMENT

The MYC family of basic helix-loop-helix, leucine zipper transcription factors consists of MYC, MYCN and MYCL. DNA binding of MYC family members usually requires hetero-dimerization with MAX (Myc-associated factor X) through their respective leucine zipper domains (Blackwood and Eisenman 1991; Blackwell et al. 1993). Knockout studies in mice show that no single MYC family member, or MAX, is essential for early development and that MYCL is completely dispensable for all embryogenesis (Hatton et al. 1996; Shen-Li et al. 2000). Individual knockout of MYC, MYCN, or MAX in mice is inconsequential until around the midgestation stages of development. The expression of MYC and MYCN overlap considerably prior to gastrulation, explaining the absence of clear
developmental defects in single knockouts in pre- and early post-implantation embryos. At this time point, MAX expression becomes essential as knockout mice at ~5.5-6.5dpc cease development (Shen-Li et al. 2000). As primitive tissues begin to form the expression pattern of MYC and MYCN change. In mid-gestation development, when MYC and MYCN expression becomes non-overlapping, tissue-specific defects are observed following deletion of MYC or MYCN. MYC^{-/-} embryos for example, are growth retarded and die before ~10.5dpc with cardiac, neural and vascular abnormalities (Davis et al. 1993; Baudino et al. 2002). MYCN^{-/-} embryos die between 10.5-12.0 dpc with defects in several of the visceral organs including lung, heart, liver, gut and the peripheral and central nervous systems (Charron et al. 1992; Stanton et al. 1992; Sawai et al. 1993).

In a key experiment to evaluate the functional redundancy of MYC and MYCN in a developmental context, Malynn and co-workers engineered a mouse where MYC coding sequences were substituted with MYCN. Mice expressing MYCN under transcriptional control of the MYC locus grew to adult-hood, reproduced and generated fertile offspring with only mild skeletal muscle defects (Malynn et al. 2000). This report is often used as evidence to argue that MYC and MYCN are functionally redundant but it is possible that in disease pathogenesis such as cancer, the two family members may have non-equivalent functions (Kawauchi et al. 2012).

Several reports also implicate key roles for MYC family members in the maintenance, expansion and differentiation of stem cells populations including
pluripotent stem cells, hematopoietic stem cells and keratinocyte stem cells (Gandarillas and Watt 1997; Laurenti et al. 2008; Wilson et al. 2008). Embryonic stem cells (ESCs) isolated from mice lacking either MYC or MYCN are capable of long-term self-renewal and remain pluripotent (Malynn et al. 2000), consistent with other studies showing that MYC performs redundant roles in peri-implantation development. Simultaneous deletion of floxed MYC and MYCN alleles however, destabilize the pluripotent state resulting in differentiation towards endoderm and mesoderm (Smith et al. 2010; Varlakhanova et al. 2010). A similar phenomenon occurs in hematopoietic stem cells, where loss of either MYC or MYCN expression is largely inconsequential, whereas deficiency of both is lethal (Laurenti et al. 2008). In ESCs ectopic re-expression of MYC or MYCN restores pluripotency (Smith et al. 2010), supporting the idea that MYC and MYCN perform redundant roles in maintaining pluripotent stem cell identity.

**MYC PROMOTES THE MAINTENANCE OF PLURIPOTENT STEM CELLS**

Pluripotent stem cells (PSCs) divide symmetrically, generating two equivalent daughter stem cells at each mitosis (Zwaka and Thomson 2005). These cells retain the ability to self-renew over long periods of time and the capacity for differentiation towards multiple lineages associated with the three embryonic germ layers. Under feeder-free conditions, murine PSCs are typically cultured in the presence of an interleukin-6 cytokine family member, such as leukemia inhibitory factor (LIF). LIF serves to maintain pluripotency by binding gp130/LIF receptor heterodimers, resulting in phospho-activation of the STAT3 transcription
factor (Ernst et al. 1996; Niwa et al. 1998). STAT3 then activates target genes involved in cell cycle control, immortalization and suppression of differentiation. The first evidence pointing towards MYC playing a role in pluripotency came from the work of Cartwright and co-workers in 2005 (Cartwright et al. 2005). Here, MYC was shown to be a direct downstream transcriptional target of LIF/STAT3 signaling and that sustained MYC expression could alleviate the requirement for LIF over extended periods of time. Numerous regulatory proteins are responsible for modulating MYC degradation (Farrell and Sears 2014) and one notable feature of MYC protein in PSCs is its unusually long half-life. Pulse-chase experiments show that MYC stability in PSCs is comparable to oncogenic mutants, such as T58A. This enhanced stability requires canonical PI3K signaling to be active so that GSK3a activity is suppressed. Once LIF signaling is disrupted in mESCs, early differentiation is triggered by the collapse of PI3K activity, the activation of GSK3a and the degradation of MYC. The down-regulation of MYC and MYCN levels is dependent on loss of both STAT3-dependent transcriptional activation and enhanced MYC protein turnover. Coordination of these events is critical for the transition from the self-renewing, pluripotent state to that of an early, lineage-committed cell.

Although the individual inactivation of MYC or MYCN has no discernable effect on pluripotency, their simultaneous loss destabilizes PSCs and results in differentiation towards primitive endoderm and mesoderm lineages (Smith et al. 2010). A key target of MYC in PSCs is the endoderm master regulator, GATA6. Transcriptional repression of GATA6 in PSCs requires MYC and following loss of
MYC activity, GATA6 levels increase and cells differentiate towards primitive endoderm. MYC also induces a number of miRNA targets that inhibit PSC differentiation (Lin et al. 2009a). One such target is the mir-17-92 cluster (Smith et al. 2010). These miRNAs maintain the rapid cell division cycle of PSCs by elevating the activity of cyclin-Cdk complexes and by limiting the activity of tumor suppressors, such as members of the retinoblastoma (RB) family and potentially p16INK4a. The MYC/MAX network has also been demonstrated to coordinate with BMP4 signaling in order to activate phosphatases that repress mitogen-activated protein kinase (MAPK) signaling (Figure 2.1) (Hishida et al. 2011; Li et al. 2012; Chappell et al. 2013). This is significant because activation of MAPK signaling by fibroblast growth factors (FGFs) is known to increase the susceptibility of PSCs to differentiation signals (Kunath et al. 2007). Loss of MYC or MAX in PSCs leads to the downregulation of ERK phosphatases, DUSP2 and DUSP7, stimulating MAPK signaling and resulting in differentiation (Chappell et al. 2013). The observation that differentiation can be blocked by a small-molecule inhibition of MEK provides an explanation for how PSCs are maintained by MAPK/MEK inhibition in LIF-depleted media (Ying et al. 2008). Under these conditions, self-renewal is not dependent on elevated MYC/MYCN levels (Marks et al. 2012) and implies that a major role for MYC/MYCN in PSCs is linked to its role as a negative regulator of MAPK signaling.
COMPLEXITIES OF MYC-DEPENDENT TRANSCRIPTIONAL REGULATION IN PLURIPOTENT STEM CELLS

MYC clearly plays a role in gene activation as part of its overall role in maintaining pluripotency, and this activating function is typically associated with its ability to hetero-dimerize with MAX at enhancer boxes (E-boxes; 5'-CACGTG-3') (Blackwood and Eisenman 1991). Recently, MYC has been proposed to regulate all actively transcribed genes in various cell types, including PSCs. In effect, this “universal amplifier” model implicates MYC as a non-linear amplifier of transcription for all active genes, whereby the amount of MYC bound near a gene’s transcription start site is correlated with transcriptional activity. Studies in PSCs indicate that MYC acts at transcription start sites by recruiting factors that release stalled RNA polymerase II complexes, promoting productive transcriptional elongation (Rahl et al. 2010; Lin et al. 2012; Nie et al. 2012)(Figure 2.2). While this activity is certainly important for enhancing cellular growth and proliferation, the issue of to what extent this activity accounts for the significance of MYC’s contribution to pluripotent biology is still unresolved.

Other studies suggest that MYC has important roles that are inadequately encompassed by the universal amplifier model. For example, MYC interacts with a diverse range of epigenetic modifiers to promote open, dynamic chromatin that is accessible to the transcription machinery (Gaspar-Maia et al. 2011; Orkin and Hochedlinger 2011). MYC interacts with TRRAP to recruit components of the GCN5 and NuA4 histone acetyltransferase (HAT) complexes to target genes (McMahon et al. 2000; Lin et al. 2009b). In some cases this function appears to
be important for maintenance of ESC identity, and for the establishment and
maintenance of euchromatic chromatin in PSCs by directing H3 and H4
acetylation (Frank et al. 2001; Cotterman et al. 2008)(Figure 2.2). Furthermore,
transcriptional repression is also an important MYC function unaccounted for by
the universal amplifier model. Besides the repression of GATA6 (Smith et al.
2010)(Figure 2.3), MYC also represses other developmentally important genes,
such as the HOX cluster, in collaboration with the POZ domain/Zn finger
transcription factor MIZ-1 (Varlakhanova et al. 2011). MYC also interacts with
components of the NuRD repressive complex such as HDAC1, RUVBL1,
RUVBL2 and also components of the CoREST complex such as LSD1 (Smith et
al. 2011). Interestingly, MYC has been shown in Drosophila to negatively auto-
regulate its own transcription through a polycomb-dependent mechanism (Khan
et al. 2009). It remains unclear if polycomb complexes broadly play a role in
MYC-mediated transcriptional repression, but interesting to note that ~10% of
MYC binding sites are co-occupied by the polycomb subunit SUZ12 and by the
H3K27 trimethyl mark (Lin et al. 2009b).

Over the last several years, ChIP-Chip and ChIP-Seq have been critical
for defining gene regulatory networks in PSCs that are controlled by core
pluripotency factors, such as OCT4 and NANOG (Chen et al. 2008; Kidder et al.
Similarly, these genome-wide binding studies have determined that MYC binds,
and possibly regulates, the transcription of at least 8,000 genes in PSCs (Smith
et al. 2011). Despite MYC being critical for pluripotency, it clearly regulates a set
of genes distinct from those regulated by core pluripotency factors. This set of genes termed the 'MYC module' suggests a somewhat contrasted role for MYC compared to the universal amplifier model described above (Chen et al. 2008; Kim et al. 2010). In this model, MYC functions to regulate only a subset of genes, many of which are distinctly important for the maintenance of self-renewal and pluripotency. Interestingly, the MYC module can also be identified in cancer cells, implying that MYCs role in pluripotency is related to its function in tumorigenicity (Kim et al. 2010; Rothenberg et al. 2010). Analysis of the MYC module reveals that MYC functions to regulate gene networks important for self-renewal such as growth, metabolism and cell cycle progression. Closer examination reveals that specific targets associated with pluripotency have been identified. For example, MYC binds and regulates the SOX2 gene (Lin et al. 2009b). This is significant because minor fluctuations in SOX2 expression have been shown to have a dramatic effect on PSC maintenance (Niakan et al. 2010). MYC also induces expression of the polycomb repressive complex components both directly, as in BMI1 (Guney et al. 2006), and indirectly as in EZH2 (Kaur and Cole 2013), which can then repress lineage-specifying genes downstream (Boyer et al. 2006). MYC also regulates self-renewal and pluripotency by transcriptionally activating microRNAs that inhibit differentiation and promote cell division (Lin et al. 2009a). One example is the mir-17-92 cluster that inhibits expression of cell cycle genes such as E2F1, CCND1 and RB2 (Smith et al. 2010).
ESTABLISHMENT OF PLURIPOTENCY

Shinya Yamanaka’s Nobel Prize winning work demonstrated that forced expression of four transcription factors, OCT4/SOX2/KLF4/MYC (OSKM), was sufficient to reprogram a differentiated somatic cell to an induced pluripotent stem cell (iPSC) state (Takahashi and Yamanaka 2006). This discovery complemented previous work that demonstrated the concept of reprogramming by nuclear transfer technologies (Gurdon et al. 1958; Gurdon and Uehlinger 1966) but in addition, created new opportunities for human disease modeling, drug screening and regenerative medicine. Many variations to Yamanaka's original approach have now been described (Buganim et al. 2012), including the substitution of MYC with other factors including the histone deacetylase (HDAC) inhibitors valproic acid (Huangfu et al. 2008) and trichostatin A (Araki et al. 2011). Presumably, these factors mimic the effects of MYC in the reprogramming process by promoting the formation of euchromatin. Reprogramming in the absence of ectopically expressed MYC however, is generally less efficient and delayed relative to when MYC-containing reprogramming cocktails are used (Nakagawa et al. 2008). As mentioned above, MYC can be omitted from reprogramming cocktails but questions have been raised as to the quality of reprogrammed cells generated under different conditions. One recent report provides evidence that MYC is essential to generate fully reprogrammed cells and that this is dependent on it ability to recruit HAT complexes to target genes (Araki et al. 2011). The use of wild-type MYC in clinical settings to generate PSCs is currently not favored because of its potential to deregulate cell
proliferation (Okita et al. 2007) but one group recently demonstrated that the ability of MYC to reprogram may be independent of its role in transformation (Nakagawa et al. 2010). They showed that utilizing MYCL, or similar MYC mutants leads to a decrease in tumorigenicity. This opens up the possibility that other forms of MYC may have utility, however because MYCL has been associated with some tumors, this phenomenon demands further investigation.

Of the four individual Yamanaka factors, MYC has the most potent effect on being able to establish a PSC like gene expression profile and appears to exert its effects early in the reprogramming process (Mikkelsen et al. 2008; Sridharan et al. 2009). A novel mechanism was recently described demonstrating that MYC functions as an enhancer of OSK binding to inaccessible chromatin in the early stages of reprogramming (Soufi et al. 2012). OSK serve as 'pioneer factors' by engaging enhancers of genes required for the establishment of pluripotency. MYC then binds modified enhancer boxes nearby and enhances the reprogramming process by a mechanism that is yet undetermined, but likely involves recruitment of chromatin-opening cofactors. At later stages of reprogramming, the combined effects of OKSM render chromatin more accessible around key pluripotency genes and co-localization of OSKM is lost as each factor resolves to their respective binding sites (Figure 2.4). This novel function in early reprogramming appears to be independent of transcriptional regulation, indicating that the universal amplifier model, discussed above, may be more applicable to MYC’s role in stem cell maintenance than to establishment of pluripotency. Interestingly, this permissive role of MYC may be similar to the way
in which aberrant overexpression can lead to transformation upon accumulation of other mutations in the context of tumorigenesis. More work needs to be done in order to fully understand the mechanics and detailed temporal aspects of reprogramming.

CONCLUSIONS
MYC clearly plays an essential role in the processes that underpin early embryonic development and pluripotent stem cell biology. Functions in promoting transcriptional activation of genes responsible for self-renewal and those imposing a differentiation blockade have been well-documented but exactly how these influence reprogramming at the molecular level remains an important problem. PSCs are likely to provide a critical biological platform for deciphering how MYC functions in a wide-range of biological contexts.

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Figure 2.1. External factors stimulate internal pathways, which cooperate to maintain PSCs. LIF/STAT signaling through MYC and/or BMP signaling through SMAD up-regulate dual-specificity phosphatases (DUSPs) to suppress FGF-stimulated ERK activity, thereby blocking differentiation.
Figure 2.2. LIF/STAT signaling stimulates transcription of MYC, which then heterodimerizes at E-boxes with MAX and amplifies transcription of active genes. MYC promotes productive transcriptional elongation by releasing stalled RNA polymerase II at transcription start sites. MYC promotes open, accessible chromatin by recruiting components of the MLL histone methyltransferase complex, such as GCN5, and by interacting with HATs, such as the NuA4 complex, which is important for PSC identity and maintenance of euchromatic chromatin.
Figure 2.3. MYC has been shown to act as a transcriptional repressor of developmentally important lineage specifiers, such as GATA6 and HOX genes, by interacting with MIZ-1 and components of the NuRD repressive complex such as HDAC1, RUVBL1, RUVBL2 and also components of the CoREST complex such as LSD1.
Figure 2.4. OSK act as 'pioneer factors' in reprogramming. MYC stabilizes and enhances binding of OSK to regulatory regions in the first 48 hours. This involves transient binding of MYC to non-canonical E-boxes, resulting in the reinforcement of OSK binding. This promotes euchromatin formation that renders promoters accessible in fully reprogrammed cells. MYC, OCT4, and KLF4 can then bind their respective target gene promoters at more proximal sites and then activate transcription. Unlike OKM, SOX2 remains enriched at distal enhancers.
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CHAPTER 3

MYC/MAX CONTROL ERK SIGNALING AND PLURIPOTENCY BY

REGULATION OF DUAL-SPECIFICITY PHOSPHATASES 2 AND 7


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ABSTRACT

Suppression of extracellular signal-regulated kinase (ERK) signaling is an absolute requirement for the maintenance of murine pluripotent stem cells (mPSCs) and requires the MYC-binding partner MAX. In this study, we define a mechanism for this by showing that MYC/MAX complexes suppress ERK activity by transcriptionally regulating two members of the dual-specificity phosphatase (DUSP) family. DUSPs function by binding and then inactivating ERK1,2 by dephosphorylating residues required for catalytic activity. MYC/MAX complexes achieve this by binding the promoters of DUSP2 and DUSP7, leading to their transcriptional activation, resulting in the attenuation of ERK activity. In the absence of MYC, ectopic DUSP2,7 expression severely delays differentiation, while loss of DUSP2,7 ectopically activates ERK, resulting in loss of pluripotency. These findings elucidate a novel regulatory role for MYC in PSC maintenance involving the stimulation of phosphatases that directly inhibit the MAPK/ERK signaling pathway. Moreover, it provides a mechanism for how leukemia inhibitory factor (LIF)/STAT3 signaling reaches across to the MAPK/ERK pathway through MYC and MAX to sustain pluripotency.
INTRODUCTION

The MYC family of transcription factors is comprised of three family members: MYC, MYCN, and MYCL. Roles for MYC and MYCN in maintenance of pluripotency and cell reprogramming (Takahashi and Yamanaka 2006; Nakagawa et al. 2010; Araki et al. 2011) have been firmly established, although mechanisms of MYC function remain unclear. Deletion of MYC and MYCN in murine pluripotent stem cells (mPSCs) results in loss of pluripotency and differentiation toward primitive endoderm (Smith et al. 2010; Varlakhanova et al. 2010). At the molecular level, MYC maintains pluripotency by directly activating cell cycle genes and repressing genes involved in cell fate specification, such as GATA6 (Smith et al. 2010). In conjunction with its heterodimeric binding partner, MAX, MYC regulates target genes by binding to regulatory motifs known as E-boxes (5′-CACGTG-3′) (Blackwell et al. 1990). Recently, MAX was shown to play an important role in maintaining PSCs by suppressing extracellular signal-regulated kinase 1,2 (ERK1,2) activity (Hishida et al. 2011). The mechanism for how MAX regulates ERK and whether this also involves MYC is unclear, however.

Regulation of MAPK/ERK is complex, and the biological outcomes of signaling through this pathway are tightly controlled by its duration, magnitude, and subcellular localization (Caunt et al. 2008). Activation of ERK by MEK-dependent phosphorylation can be countered by the activity of dual-specificity phosphatases (DUSPs) that bind and inhibit ERK activity. This phosphatase family is important for regulating spatial and temporal aspects of MAPK/ERK.
signaling in a wide range of cell types (Caunt et al. 2008; Patterson et al. 2009; Owens and Keyse 2007). A key requirement for mPSC self-renewal is that ERK activity be suppressed (Kunath et al. 2007). Although the mechanism by which ERK antagonizes self-renewal pathways has not been defined in mPSCs, it is clear that its activity must be maintained below a threshold level for maintenance of the pluripotent state. FGF4/ERK autocrine signaling is believed to prime PSCs for differentiation, but under self-renewal conditions, this pathway is suppressed by an as yet uncharacterized mechanism (Lanner et al. 2010). When these signaling barriers are removed, however, autocrine FGF4 signaling drives MAPK/ERK activity, resulting in differentiation (Kunath et al. 2007).

Understanding how signals converge on ERK to restrict its activity is critical for a complete understanding of how PSCs transition to a differentiated state. The observation that MAX restricts ERK activity in PSCs (Hishida et al. 2011) offers a potential answer to this general question, but it remains unclear how MAX blocks full ERK activation. In this study, we show that MYC and MAX transcriptionally regulate two members of the DUSP family that serve to suppress ERK activity (Dowd et al. 1998; Theodosiou and Ashworth 2002). These findings establish critical new roles for MYC and MAX in pluripotency through regulation of the ERK pathway and establish a broader signaling network linking leukemia inhibitory factor (LIF)/STAT3 to MAPK/ERK.
RESULTS

MYC suppresses ERK activity in murine pluripotent cells

Since previous studies established that loss of MAX leads to increased ERK activity and loss of pluripotency (Hishida et al. 2011), we asked whether this was also true following loss of MYC and MYCN. This was tested using a mPSC line carrying floxed alleles of MYC and MYCN (Smith et al. 2010) and a transgene expressing a Cre recombinase– glucocorticoid receptor fusion (CreGR). Addition of the glucocorticoid dexamethasone (Dex) translocates the CreGR fusion to the nucleus, where it efficiently deletes floxed MYC and MYCN alleles, as indicated by the loss of MYC and MYCN protein within 24 h (Fig. 3.1A). This coincided with the activation of ERK; up-regulation of endoderm transcript markers such as GATA6, GATA4, SOX17, and SOX7; and loss of alkaline phosphatase (AP) activity (Fig. 3.1A-C). All of these responses were blocked by addition of the MEK inhibitor UO126. We also independently confirmed that loss of MAX increases ERK activity (Supplemental Fig. 3.1). Ectopic expression of a MYC–estrogen receptor fusion protein (MYC-ER) under control of the estrogen analog 4-hydroxytamoxifen (4OHT) blocked the activation of ERK in MYC- and MYCN-deleted cells (Fig. 3.1D) and maintained GATA6 transcripts at levels comparable with the wild-type control (Fig. 3.1E). These data indicate that MYC and MAX maintain pluripotent cells by suppressing ERK activity. This provides an explanation for how mPSCs can be maintained in MEK/GSK3 inhibitor (2i)-based media where MYC expression is reduced (Ying et al. 2008; Marks et al. 2012).
MYC regulates transcription of DUSP2 and DUSP7

To explain these observations mechanistically, we searched for MYC target genes in the MYC Cancer Gene: MYC Target Gene Database (http://www.myccancergene.org/site/myctargetdb.asp) that could potentially regulate ERK activity. Direct regulation of ERK by MYC was ruled out because the ERK1,2 protein remained constant irrespective of MYC status (Fig. 3.1A). Of the known MYC target genes that can be potentially regulated by MYC, the most notable were members of the DUSP gene family (DUSP2/7). Consistent with this possibility, the DUSP7 protein is down-regulated in parallel to MYC and MYCN during murine embryonic stem cell (mESC) differentiation, just prior to the activation of ERK (Fig. 3.2A). We note a delay in the activation of ERK following the decline of DUSP7 levels; this suggests that low levels of DUSP continue to inhibit ERK during the early stages of differentiation. Along with OCT4 and NANOG, DUSP2/7 transcripts also decline following LIF withdrawal, while FGF5 transcript levels increase (Fig. 3.2B). DUSP7 protein is made under all conditions tested (FCS/LIF, 2i, and BPM4+LIF), whereas DUSP2 and another protein implicated in ERK regulation, DUSP9 (Li et al. 2012), show condition-specific expression patterns (Supplemental Fig. 3.2).

To investigate whether MYC regulates DUSP2/7 transcript levels, MYC and MYCN were deleted by addition of Dex to the double-floxed MYC and MYCN PSCs. Consistent with this possibility, loss of MYC and MYCN led to decreased levels of DUSP2/7 mRNA (Fig. 3.2C). A second experiment was then performed in which MYC activity was further elevated in wild-type R1 ESCs using an
inducible MYC-ER transgene. This showed that addition of 4OHT further elevated DUSP2/7 transcripts. Results in Figure 2, C and D, therefore indicate that MYC regulates DUSP2/7. To examine this in further detail, we asked whether DUSP2/7 could be reactivated in cells that lost expression due to MYC deletion. This involved MYC deletion (+Dex) for 3 d and ectopic MYC-ER expression from day 2 onward. This experiment shows that following loss of endogenous MYC and MYCN, DUSP2/7 mRNA levels decrease, but this can be rescued by ectopic MYC expression (MYC-ER+4OHT) (Fig. 3.2E). These results indicate that MYC regulates DUSP2/7 in PSCs.

The possibility that DUSP2/7 are directly regulated by MYC/MAX was suggested by the presence of E-boxes in their respective promoter regions (Fig. 3.2F,G). To establish whether MYC/MAX regulate DUSP2/7 directly, we first performed chromatin immunoprecipitation (ChIP) analysis on the promoter regions of both genes. This analysis showed significant enrichment of MAX, MYC, and MYCN binding in proximal promoter regions for the two genes (Fig. 3.2F,G). In addition to this, ectopic expression of MYC-ER (+4OHT) increased DUSP2/7 promoter activity above already elevated levels, as shown by luciferase reporter assays (Supplemental Fig. 3.3), and loss of MYC activity significantly reduced MYC, MYCN, and MAX binding to the DUSP2,7 promoters (Supplemental Fig. 3.4). These data further support the model that MYC/MAX regulates DUSP2,7 at the level of transcription and that down-regulation of MYC underpins decreased DUSP expression and ERK activation during differentiation.


**DUSP2/7 are required for MYC-dependent suppression of ERK**

The data presented so far point to a mechanism in which MYC promotes pluripotency by suppressing ERK activity through a DUSP2/7-dependent mechanism. To establish whether DUSP7 activity is sufficient to suppress ERK activation in PSCs, we generated a MYC and MYCN floxed ESC line expressing DUSP7 under the control of a tetracycline (Tet)-inducible promoter (Fig. 3.3A). In the presence of endogenous MYC and MYCN (CreGFP), ERK activity and differentiation markers GATA6, GATA4, and SOX17 remain low but are elevated following loss of MYC and MYCN due to CreGFP expression (Fig. 3.3B,C). Tet-induced expression of DUSP7, however, completely blocked the up-regulation of phospho-ERK and GATA6, GATA4, and SOX17 transcripts, indicating that DUSP7 is a major effector of MYC-dependent MAPK signaling in PSCs.

We then asked whether DUSP7 could maintain characteristics of pluripotent cells following LIF withdrawal. Within 4 days of LIF withdrawal, the percentage of AP-positive colonies dropped from almost 90% to <2%. In contrast, enforced expression of DUSP7 following addition of Tet maintained the percentage of AP-positive colonies at ~35% (Fig. 3.3D,E). Together, these results show that DUSP7 can suppress ERK activation in the absence of MYC and can partially substitute for LIF in promoting pluripotency.

**DUSP2 and DUSP7 are required for maintenance of pluripotency**

To establish whether *DUSP2,7* are required for PSC maintenance, we transduced mESCs with lentiviral constructs expressing shRNAs that knocked

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down DUSP2 by >80% and DUSP7 by >90% (Fig. 3.4A). Transduction of both lentiviruses caused a distinct morphology change to mESC colonies, indicative of differentiation (Fig. 3.4B). This was associated with increased ERK activity, a reduction in NANOG protein (Fig. 3.4C), and up-regulation of the endoderm marker FOXA2 (Fig. 3.4D). Similar effects were observed with different shRNAs targeting DUSP2 and DUSP7 (Supplemental Fig. 3.5), indicating that the effects were specific to knockdown of DUSPs. To test the requirement for DUSP2/7 in pluripotency, LacZ+ mESCs were transduced with DUSP2 and DUSP7 shRNAs and then injected into blastocyst stage embryos to evaluate embryonic contribution. Thirteen days after the introduction of blastocysts into recipient females, embryos were evaluated for developmental contribution by control (GFP) or DUSP2,7 shRNA transduced mESCs (Fig. 3.4E). LacZ+ cells transduced with GFP shRNA lentivirus broadly contributed to embryonic tissues (67%), whereas DUSP2 and DUSP7 knockdown cells did so at a greatly reduced frequency (22%). Overall, these data show that DUSP2/7 are required for maintenance of pluripotency.

**Binding of DUSP7 to ERK is required for self-renewal of pluripotent cells**

Commercially available antibodies for DUSP7 do not immunoprecipitate its target efficiently, so we transfected cells with a construct expressing epitope (HA)-tagged DUSP7 to investigate interactions between DUSP and ERK. Reciprocal immunoprecipitations between DUSP7 and ERK in whole-cell lysates showed specific interactions (Fig. 3.5A,B), confirming that DUSP7 interacts with ERK in
mESCs. To evaluate the role of DUSP7 binding to ERK further, we expressed a Tet-regulated hypermorphic allele of ERK2(D319N) that is defective in binding DUSP proteins (Chu et al. 1996). Under conditions that normally promote mESC self-renewal (+LIF), expression of ERK2^{D319N} following addition of Tet resulted in reduction of NANOG protein (Fig. 3.5B) and a significant reduction in the percentage of AP-positive colonies (Fig. 3.5C,D). Together, these data indicate that ERK becomes activated in pluripotent cells when it is refractory to inhibition by DUSP7. Under these conditions, PSC self-renewal is significantly compromised.
DISCUSSION

A recent study describing MYC as a general transcriptional amplifier in mice indicates that it serves as a global regulator of active genes (Nie et al. 2012). Our data are not inconsistent with these findings that some of its functions in self-renewal can be substituted for by only a small number of genes. Deletion of MYC and MYCN in mESCs promotes endoderm differentiation (Smith et al. 2010), but this can be abrogated by ectopically expressing one of its key targets, DUSP7. Taking this into account, it is intriguing that mESCs can be maintained with significantly reduced MYC levels under conditions where ERK signaling is inhibited (Hishida et al. 2011; Marks et al. 2012). It seems that ERK signaling also negatively impacts reprogramming because the MEK inhibitor PD035901 enhances induced PSC (iPSC) generation efficiency (Lin et al. 2009). This observation predicts that MYC could be substituted by MEK inhibitor or DUSP knockdown in reprogramming cocktails.

MYC suppresses ERK activity via DUSP2/7

Evidence supporting the significance of MYC and MAX in PSC biology has rapidly accumulated, but its important role in regulation of ERK activity has not been previously characterized at the mechanistic level. The mechanisms associated with pluripotency and/or self-renewal in which MYC has been implicated are diverse and include promotion of rapid cell cycle progression, increased rates of metabolism and cell growth, and direct repression of important lineage-specifying genes. In this study, we determined a previously unidentified
role for MYC and MAX where together they maintain pluripotency by suppressing ERK through a DUSP-dependent mechanism (3.27).

LIF is generally considered to be a pluripotency maintenance factor through its ability to bind LIF receptor/ gp130 heterodimers by transducing signals through the activation of STAT3 (Niwa et al. 1998). A second aspect of LIF signaling involves the gp130-dependent phosphorylation of SHP-2 that activates ERK and, paradoxically, serves to antagonize pluripotency maintenance networks (Burdon et al. 1999). An important target of STAT3 in PSCs is MYC, a transcription factor with known but poorly defined roles in the maintenance of PSCs (Cartwright et al. 2005). In this study, we propose a model in which LIF controls ERK activity by using MYC as a downstream effector to activate DUSP transcription (see Fig. 3.6). An unexpected outcome of this is the realization that LIF/ gp130 has the potential to simultaneously activate and represses ERK activity through separate signaling pathways. In the context of self-renewal, its role in activation of the STAT3/MYC/DUSP pathway is critical. In the scenario where LIF is withdrawn, MYC levels decrease, leading to loss of DUSP activity. In the absence of restraining signals, FGF4-dependent autocrine signals are then free to elevate ERK activity, and differentiation then follows (Lanner et al. 2010). It is unlikely that DUSPs are the only class of phosphatases that have the capacity to dephosphorylate ERK. In other cell types and in other signaling scenarios, DUSP-independent modulation of ERK by protein phosphatases are likely to be important.
ERK suppression in different signaling contexts

The experiments that we present here were performed in traditional media containing fetal calf serum supplemented with LIF. Under these conditions, MYC, MYCN, DUSP2, and DUSP7 are expressed at high levels. Another study recently reported that in LIF media supplemented with BMP4, self-renewal was promoted by Smad-dependent transcriptional activation of DUSP9 (Li et al. 2012). Under these conditions, the BMP4–SMAD and LIF–STAT3-MYC pathways synergistically cosuppress ERK activity by promoting DUSP9 and DUSP2/7 expression, respectively. This indicates that multiple signaling inputs can simultaneously converge on ERK to suppress its activity. DUSP7 was the only DUSP to be robustly expressed under a wide range of conditions, including LIF/FCS, LIF/BMP, and 2i. DUSP9 expression, for example, was restricted to BMP/LIF conditions and is therefore unlikely to play a major role in ERK regulation in 2i or traditional LIF/FCS conditions.

In total, the results presented provide new insight into how the LIF–STAT3 signaling pathway promotes self-renewal. An important aspect of this mechanism involves new roles for MYC in the modulation of MAPK/ERK signaling through DUSP effector molecules. Although different DUSP family members may collaborate and be regulated themselves by different signaling pathways, DUSP7 seems to be the most broadly used under a range of culture conditions. Our data, together with the previously known role of ERK in regulation of MYC (Yeh et al. 2004), suggest that a positive feedback loop may exist where MYC stimulates its own activity through regulation of DUSP. This is the first study showing a role for
MYC in the modulation of cell signaling—a mechanism that has clear implications for its role in tumor development. It will be interesting to establish whether MYC/MAX regulate DUSP activity in other cell types or whether this regulation is restricted to PSCs.

MATERIALS AND METHODS

Cell culture and blastocyst injections

R1 mESCs, MYC^{fl/fl} MYCN^{fl/fl} miPSCs (Smith et al. 2010), MAX^{−/−} mESCs (Hishida et al. 2011), and LacZ^{+} miPSCs (Smith et al. 2010) were cultured in LIF and fetal calf serum supplemented medium on gelatin-coated dishes. The following reagents were used at the concentrations indicated: Dex (20 mM), U0126 (20 mM), doxycycline (1 mg/mL), and 4OHT (100 nM). The BMP4/LIF culture condition was used as described previously (Ying et al. 2003). The 2i culture condition was used as described previously (Ying et al. 2008). Differentiation was performed in attachment or suspension culture in medium lacking LIF or knockout serum replacement (KSR). Blastocyst injections and embryo analysis were performed as described previously (Cartwright et al. 2005).

Lentiviral transduction for shRNA knockdown

The shRNA plasmids for DUSP2 (TRCN0000028961), DUSP7 (TRCN0000080729), and the GFP control (RHS4459) were purchased from Open Biosystems. To make lentivirus, shRNA plasmids and Trans-lenti shRNA packaging plasmids were transfected into H293T cells according to the kit
manual (Open Biosystems, TLP4615). After determining the virus titer, mESCs were transduced at a multiplicity of infection of 5:1. Puromycin selection (1 mg/mL) for 4 d was applied to select cells with stable viral integration. Quantitative PCR (qPCR) and Western blot were used to assess the knockdown of targets.

**Tet-inducible expression**

DUSP2-HA, DUSP7-HA, and ERK2(D319N) fragments were cloned from cDNA clones (Origene) and placed under the control of a Tet-inducible promoter from pLVX-tight-puro (Clontech, PT3996-5). In the absence of Tet-express, pLVX-tight-puro provides very low background expression, whereas addition of Tet-express strongly transactivates target genes. Expression constructs were transfected into mESCs using Lipofectamine 2000 and stable cell lines established with 1 mg/mL puromycin selection. To induce target gene expression, 1 x 10^5 Tet-inducible cells were plated in six-well plates. The next day, Tet-express- transducible protein (Clontech, #631178) was added for 1 h in serum-free medium to induce gene expression. Cells were allowed to grow in complete medium for an additional 12–24 h before assay for gene induction.

**Transcript expression assays and luciferase assays**

RNA was isolated with the RNeasy mini kit (Qiagen). cDNA libraries were prepared from RNA using the iScript reverse transcription kit (Bio-Rad). Transcript levels were assayed by RT-qPCR using TaqMan assays (Applied
Biosystems) as described by Smith et al. (2010). Transcript levels were normalized to GAPDH and performed in triplicate. Luciferase assays were performed with the Dual Luciferase Reporter kit (Promega) according to instructions and analyzed on a Synergy 2 plate reader (BioTek). Unless stated, P-values relating to changes in transcript levels were <0.01.

**Immunoblotting, immunostaining, and ChIP**

For immunoblotting, immunostaining, and ChIP experiments, we used commercially available antibodies to MYC (Santa Cruz Biotechnology, sc-764), phospho-ERK1/2 (Cell Signaling, 9102), ERK1/2 (Cell Signaling, 9102), MYCN (Calbiochem, OP13), MAX (Santa Cruz Biotechnology, sc-197), OCT4 (Santa Cruz Biotechnology, sc-5279), DUSP7 (Santa Cruz Biotechnology, sc-47667), ERK1/2 (Santa Cruz Biotechnology, sc-135900), NANOG (CosmoBio, REC-RCAB0002PF), rabbit IGG (Abcam, ab46540), and FOXA2 (Millipore, 07-633).

The following primer sets for ChIP-qPCR were designed using Primer3 (Rozen and Skaletsky 2000): DUSP2 promoter F (59-GGACAATTTCTG ACGGTTG-39) and R (59-CCATTTTCTCCCCAGGTTT-39), DUSP2 control F (59-AACCATGTCTAGGGGTGTGC-39) and R (59-GGACTGAGGAGTGGAGAGTG-39), DUSP7 promoter F (59- GCCAACACAGAGCAGGAGA-39) and R (59-AGAGCCAGTC TTCCCTCCTC-39), and DUSP7 control F (59-GGACTGAGG GCTTGAGAGGT GAG-39) and R (59-CTTAGCAAGCAGCAGT GAG-39). AP staining was carried out with an AP staining kit (Sigma). Immunoblotting,
immunostaining, AP staining, and ChIP experiments were as described previously (Smith et al. 2010).

**ACKNOWLEDGEMENTS**

This work was supported by grants to S.D. from the National Institute of Child Health and Human Development (HD049647) and the National Institute for General Medical Sciences (GM75334).
Figure 3.1. **MYC regulates ERK activity in mPSCs.** (A) Whole-cell lysates from MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} CreGR cells grown without treatment or with Dex for 1 d and 2 d +/- U0126 (UO) were immunoblotted and probed with antibodies as indicated. (B) Expression of MYC and primitive endoderm marker transcripts were assayed from MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} CreGR cells grown +/- 2 d of Dex treatment +/- UO126. Fold changes were normalized to MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} CreGR cells that were not exposed to Dex. Error bars represent standard deviation. (C) MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} CreGR cells grown +/- 2 d of Dex treatment +/- UO126 were stained for AP activity and then scored as being AP+ or AP- (N = 195). Bar, 100 µm. (D) Whole-cell lysates from MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} CreGR MYC-ER cells grown with no treatment and with Dex for 2 d, 3 d, and 3 d + 24 h 4OHT were immunoblotted and probed with antibodies as indicated. (E) Expression of the indicated transcripts was determined from the conditions in D, and fold changes were normalized to the “no treatment” condition.
Figure 3.2. MYC transcriptionally regulates DUSP2 and DUSP7. (A) Whole-cell lysates from R1 mESCs grown in differentiating conditions (-LIF) were collected over 5 d in 24 h increments and then immunoblotted and probed with antibodies as indicated. (B) Transcript levels were assayed in R1 ESCs and 4 d following withdrawal of LIF. Fold changes in transcript levels were determined in triplicate after normalization to untreated cells. Error bars represent standard deviation. (C) DUSP2 and DUSP7 transcript levels were assayed in MYC^{fl/fl} MYCN^{fl/fl} or MYC-/- MYCN-/- -deleted cells. CreGR expression was induced by addition of Dex (+Dex) for 48 h. (D) Wild-type R1 mESCs carrying a MYC-ER transgene were induced by addition of 4OHT for 24 h. Transcript levels were assayed as in C. (E) DUSP2 and DUSP7 transcript levels were assayed from MYC^{fl/fl} MYCN^{fl/fl} CreGR MYC-ER cells without or after Dex treatment for 2 d, 3 d, and 3 d + 24 h 4OHT. Fold changes were normalized to untreated cells. (F,G) MYC/MAX specifically bind to the promoter region of DUSP2 (F) and DUSP7 (G). ChIP-immunoprecipitated DNA was amplified by quantitative PCR (qPCR) with primers spanning the DUSP2,7 promoters. Schematic representations of DUSP2,7 are shown with potential MYC-binding sites (E-boxes) indicated by triangles. All E-boxes are canonical with the sequence 5'-CACGTG-3'. (*) P < 0.05. All other P-values are <0.01. “Control” regions are located 2 kb upstream of the transcription start sites of DUSP2 and DUSP7 and do not contain an E-box.
Figure 3.3. Down-regulation of MYC, MYCN, DUSP2, and DUSP7 correspond to an increase in ERK activity and differentiation. (A) Tet regulation of HA-DUSP7 ESC line grown in the absence (-) or presence (+) of Tet activator protein. After 24 h induction (+Tet), cells were harvested and subjected to immunoblot analysis, probing with HA monoclonal antibody to detect DUSP7. Load control, HA cross-reacting, nonspecific protein was used as a load control. (B) MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} cells containing Tet-inducible DUSP7 were transfected with a CreGFP vector +/- Tet and then sorted for GFP 48 h post-transfection. Whole-cell lysates were then immunoblotted and probed with antibodies as indicated. (C) Transcript levels from B were assayed in triplicate. Fold changes were normalized to untreated MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} cells. Error bars represent standard deviation. (D) Tet-inducible DUSP7 ESCs were plated on 24-well plates. After LIF withdrawal, Tet-express was added to induce DUSP7 expression. Cells were allowed to grow in differentiating condition for 4 d +/- Tet express and then stained for AP activity. (E) The percentage of AP-positive colonies under each condition is shown. All assays were performed in triplicate (N = 164).
Figure 3.4. DUSP2/7 expression is necessary for maintenance of mPSCs.

(A) R1 mESCs were infected with lentiviruses expressing shRNAs for DUSP2 (TRCN0000028960), DUSP7 (TRCN0000080728), DUSP2 and DUSP7, or GFP (control). Four days after lentiviral transduction, target knockdown was assayed in triplicate by qPCR. Fold changes in transcripts were determined after normalization to GFP knockdown cells. Error bars represent standard deviation. (B) Colony morphology after DUSP2/7 and GFP knockdown (4 d). Bar, 100 µm. (C) Whole-cell lysates were collected after DUSP2/7 and GFP shRNA knockdown. Samples were immunoblotted and probed with antibodies as indicated. (D) Immunofluorescent staining using FOXA2 antibody after DUSP2/7 and GFP knockdown. Bar, 50 µm. (E) R1 mESCs marked by β-galactosidase activity were infected with lentiviruses expressing shRNAs for DUSP2/7 or GFP control and injected into blastocyst stage C57BL/6 embryos. After transfer into recipient females, embryos were allowed to develop until embryonic day 14.5 (E14.5). LacZ staining was then performed on fixed, whole embryos. The number of blastocysts injected, the number of chimeras generated, and the percentage of chimeras generated are indicated (control, N = 21; DUSP2/7, N = 18). The percentage of embryo contribution for control and DUSP2/7 knockdown cells is graphed on the right. (*) P < 0.05.
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IB: HA
IB: ERK

B

Tet: - +

NANOG
ERK
CDK2

C


% AP colonies

D

ERK2.D319N

- Tet + Tet

*
Figure 3.5. DUSP7 directly interacts with ERK in PSCs. (A) HA-DUSP7-expressing ESC line was established by transfecting a Tet-inducible HA-DUSP7 expression plasmid into R1 ESCs. After 1 d of Tet-express induction, cell lysate was made for coimmunoprecipitation (IP) experiments. Reciprocal immunoprecipitation of DUSP7 and ERK was detected using ERK1/2 and HA antibodies. (B) R1 mESCs containing Tet-inducible ERK2(D319N) were cultured +/-Tet for 2 d. Whole-cell lysates were then immunoblotted and probed with antibodies as indicated. (C) Tet-inducible ERK2(D319N) cells were cultured +/-Tet for 2 d, followed by -Tet for 3 d, and then stained for AP. (D) The percentage of AP-positive colonies under each condition was determined from triplicate experiments. Error bars represent standard deviation (N = 74). (*) P < 0.05. All other P-values are <0.01.
Figure 3.6. A model for ERK regulation by MYC/MAX complexes in murine pluripotent cells. MYC/MAX activates transcription of DUSP2 and DUSP7 in their E-box-containing promoters. The protein products are characterized by phosphatase activity necessary for dephosphorylation of phospho-ERK. Suppressing ERK activity is necessary for maintenance of a pluripotent state and is necessary in the presence of autocrine FGF signaling.
Supplementary Figure 3.1. MAX−/− cells were maintained in the absence of Dox with LIF to maintain expression of a Tet-regulated transgene (Tet-OFF) in the null MAX background. Cells were then grown with (+) or without (-) Dox for 6 d in the presence of LIF and then harvested. Whole cell lysates were then immunoblotted, probing with antibodies as indicated.
Supplementary Figure 3.2. Whole cell lysates from mESCs cultured under the conditions indicated (BMP4/LIF, 2i or FCS/LIF) were subject to immunoblot analysis and probed with antibodies for Dusp2,7,9 and pan-Erk.
**Supplementary Figure 3.3.** MycER cells were transfected with the DUSP2 or DUSP7 promoter-luciferase reporter constructs, treated +/- 4OHT for 48 h and collected for luciferase activity determination. Assays were performed in triplicate and were normalized against a renilla luciferase control. Error bars represent standard deviation. * denotes $P < 0.05$. All other $P$-values are $<0.01$. 

Supplementary Figure 3.4. c-MYC<sup>fl/fl</sup> N-MYC<sup>fl/fl</sup> CreGR cells were grown without treatment or with Dex for 2 days. c- N-myc and Max ChIP-immunoprecipitated DNA was amplified by qPCR with primers spanning E-boxes in the DUSP2,7 promoters and there respective controls. 'Control' regions are 2kb upstream of the DUSP2 and DUSP7 transcription start sites and do not contain Myc binding sites (E-boxes).
Supplementary Figure 3.5. (A) R1 mESCs were infected with lentiviruses expressing shRNAs (independent of those used in Figure 4) for Dusp2 (TRCN00000289610), Dusp7 (TRCN0000080729), Dusp2 and 7 or GFP (control). Fours days after lentiviral transduction, target knock-down was assayed in triplicate by Q-PCR. Fold-changes in transcript levels were determined after normalization to GFP knock-down cells. Error bars represent standard deviation. (B) Whole cell lysates were collected after Dusp2/7 and GFP shRNA knock-down. Samples were immunoblotted and probed with antibodies as indicated. (C) Colony morphology after Dusp2/7 and GFP knock-down. Micron bar, 100 µm.
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Dowd S, Sneddon AA, Keyse SM. 1998. Isolation of the human genes encoding the pyst1 and Pyst2 phosphatases: Characterisation of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP


CHAPTER 4

DETAILED EXPERIMENTAL PROCEDURES

MATERIALS AND METHODS

Alkaline Phosphatase Staining

Alkaline phosphatase staining was carried out with an Alkaline Phosphatase staining kit (Sigma 86R-1KT) according to the manufacture's instructions. Fixative solution was prepared: 1.25 mL citrate, 3.25 mL acetone, 0.4 mL 37% formaldehyde. Alkaline dye solution was prepared: 100 µL sodium nitrate, 100 µL alkaline solution, leave for two minutes, add to 4.5mL ddH2O, add 100µL Naphthol. Cells were stained on the dish: wash with PBS, add fixative solution for 30 seconds, wash with PBS for 45 seconds, add alkaline dye for 15 minutes at room temperature in the dark, wash with PBS, photograph.

Antibodies

Antibodies used in these studies are listed in Table 4.1.

Blastocyst Injections

Blastocyst injections and embryo analysis were performed in order to determine the necessity of DUSP expression for early embryonic development. R1 mESCs expressing β-GALACTOSIDASE (Smith et al. 2010) were infected with
lentiviruses expressing shRNAs for DUSP2/7 or a GFP control and injected into blastocyst stage C57BL/6 embryos. After transfer into recipient females, the embryos were allowed to develop until embryonic day 14.5 (E14.5). Whole embryos were fixed and stained for LacZ. Mouse work was done in compliance with the University of Georgia animal use and ethics guidelines.

**Cell Cycle Analysis**

Propidium iodide staining followed by flow cytometry was used to determine DNA content. Cells were fixed in 70% ethanol and stained with propidium iodide (50 mg/ml), RNase A (200 mg/ml), and BSA (100 mg/ml) in PBS for 30 min at 37°C. Flow cytometry was performed using the Beckman Coulter CyAn instrument and results were analyzed using FlowJo (http://www.flowjo.com/).

**Cell Lines**

The following murine pluripotent stem cell (PSC) lines were used in these studies: R1 murine embryonic stem cells (ESCs) (Nagy et al. 1993), MYC\textsuperscript{fl/fl} MYCN\textsuperscript{fl/fl} murine induced pluripotent stem cells (iPSCs) (Smith et al. 2010), MAX\textsuperscript{−/−} mESCs (Hishida et al. 2011), and LacZ+ murine iPSCs (Smith et al. 2010).

**Chromatin Immunoprecipitation**

Cells were collected as 10 million cell aliquots in 1 mL of PBS. DNA/protein complexes were cross-linked by addition of 27.5 µL of 37% formaldehyde and 10-minute incubation. Formaldehyde was quenched by addition of 50 µL of 2.5 M...
glycine and 5 minute incubation. Following centrifugation for 30 seconds at 2000 x g, and removal of supernatant, pellets were flash frozen in liquid nitrogen for later use.

Cells were lysed by addition of 5 mL LB1 (Table 4.2) and rocking at 4°C for 10 minutes. Following centrifugation for 5 minutes at 1350 x g at 4°C, supernatant was discarded. 5 mL of LB2 (Table 4.2) were added to the pellets and rocked at 4°C for 10 minutes. Following centrifugation for 5 minutes at 1350 x g at 4°C, supernatant was discarded. Pellets were resuspended in 1 mL of LB3 (Table 4.2) and DNA was sonicated to a size of ~200-1000 base pairs using a Covaris S220. Sonicated lysate was saved at -80°C for later use.

Protein-G magnetic beads (Life Technologies #10003D) were incubated with an antibody overnight at 4°C to form antibody-bead complexes. Antibody bead complexes were then incubated overnight at 4°C with sonicated lysates to immunoprecipitate chromatin. Following immunoprecipitation, chromatin was washed 3 times with RIPA buffer (Table 4.2), washed 1 time with TE containing 50mM NaCl, and eluted from the beads by addition of 210 µL elution buffer (Table 4.2) and 15 minute incubation at 65°C. DNA-protein cross-links were reversed by overnight incubation at 65°C.

RNA was degraded by addition of 200 µL TE and 4 µL of 20 mg/mL RNaseA and incubation for 2 hours at 37°C. Protein was degraded by addition of 7 µL of CaCl₂ and 4 µL of 20 mg/mL proteinase K and incubation for 30 minutes at 55°C. DNA was purified by phenol:chloroform extraction and ethanol precipitation. ChIP-DNA was assayed by QPCR using Sybr Green chemistry and
primer sets designed using Primer3 (Rozen and Skaletsky 2000), listed in Table 4.3.

**Co-Immunoprecipitation Assay**

Antibodies were conjugated to protein G that is covalently coupled to magnetic beads (Dynabeads, Life Technologies #10003) by overnight incubation at 4°C. Protein complexes were extracted from freshly collected cell pellets using a lysis buffer consisting of the following: 10mM Hepes, 10mM potassium chloride (KCl), 0.1mM EDTA, 0.1mM EGTA, 1.6% nonyl phenoxypolyethoxylethanol (NP-40), 1mM dithiothreitol, and protease inhibitors (Roche 11873580001). These lysates were then incubated overnight with the antibody-protein G-bead mixture. Immunoprecipitated protein was then immunoblotted for proteins of interest.

**Culture of mPSCs**

Murine PSCs were cultured in feeder-free conditions on gelatin-coated tissue culture grade plastic dishes (Thermo Scientific) in a constant environment of 37°C and 5% CO₂. Serum-based culture medium contained Dulbecco’s Modified Eagle Medium (DMEM; Cellgro 10-013) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals S10295), 10% knockout serum replacement (KSR; Life Technologies 10828-028), 0.1mM β-mercaptoethanol (Life Technologies; 21985-023), 100U/mL penicillin-streptomycin (Cellgro 30-002-CI), and 1000 units/mL recombinant leukemia inhibitory factor (LIF, Millipore ESG1107), and was changed daily. The cells were passaged every three or four
days, when culture dishes became confluent, by washing with Dulbecco’s phosphate buffered saline (DPBS, Cellgro 21-031) followed by a 3 minute cell detachment step with 0.25% Trypsin-EDTA (Sigma T4049) at 37°C, followed by generation of a single cell suspension by pipetting. The trypsin was then inactivated by addition of an equal volume of culture medium. The cell density was determined using a hemocytometer. Following centrifugation at 1000 rpm for 4 minutes, the cells were resuspended at a density of $1.5 \times 10^4$ cells/mL and plated in a new tissue culture dish.

The Bone Morphogenic Protein 4/Leukemia Inhibitory Factor (BMP4/LIF) culture condition (Ying and Smith 2003) contained a mixture of Dulbecco’s Modified Eagle Medium, Ham’s F-12 (DMEM/F-12 50-50 Cellgro 10-090-CV), and Neurobasal medium (Invitrogen 21103-049) supplemented with 0.1mM β-mercaptoethanol (Life Technologies; 21985-023), 100U/mL penicillin-streptomycin (Cellgro 30-002-CI), 50 µg/mL bovine serum albumin (BSA, Sigma A7906), 10ng/mL BMP4 (R&D Systems 314-BP), 1000 units/mL recombinant LIF (Millipore ESG1107), and was changed daily.

The 2i culture media (Ying et al. 2008) used consisted of an identical formula to the BMP4/LIF culture media, however BMP4 was replaced by supplementation with a Map Kinase Kinase (MEK) inhibitor (PD-0325901, Sigma PZ0162) and a glycogen-synthase kinase 3 (GSK3) inhibitor (CHIR99021, LC labs C-6556).

Differentiations were performed in attachment or suspension culture in the serum-based medium lacking LIF and KSR.
**Drugs and Small Molecules**

Dexamethasone (Sigma D4902) was used at a concentration of 20 µM to induce translocation of a CreGR fusion protein to the nucleus of \( MYC^{fl/fl} \) \( MYCN^{fl/fl} \) miPSCs and subsequent deletion of floxed MYC alleles. U0126 (Cell Signaling #9903) was used at a concentration of 20 µM to inhibit phosphorylation of extracellular signal-regulated kinases (ERK1/2) by MAP kinase kinase (MEK1/2). Doxycycline (Sigma 44577) was used at a concentration of 1 µg/mL to induce expression of an exogenous HA-Dusp7 vector. 4-Hydroxytamoxifen (4-OHT; Sigma H7904) was used at a concentration of 100 nM to induce translocation of a MycER fusion protein to the nucleus of R1 mESCs.

**Fluorescence Activated Cell Sorting**

Fluorescence activated cell sorting (FACS) was performed on a MoFlo (Beckman Coulter) in biological triplicate.

**Immunofluorescent Staining Analysis**

Cells were washed with PBS and fixed with 4% PFA for 10 minutes at room temperature. Fixed cells were incubated with primary antibodies overnight at 4°C. The following day, cells were washed and incubated with secondary antibodies for 1 hour at room temperature. The cells were then washed with PBS containing DAPI and imaged.
Inducible Expression of **DUSP & ERK**

HA-DUSP2, HA-DUSP7, and ERK2(D319N) were sub-cloned from cDNA clones (Origene) and placed under the control of a Tet-inducible promoter from a pLVX-tight-puromycin expression vector (Clontech, PT3996-5). Following transfection of the expression vector, and several days of 1 mg/mL puromycin selection, stable cell lines were established in which the absence of Tet-express provides very low background expression. Tet-express protein (Clontech, #631178) was added daily for one hour in serum-free media in order to strongly trans-activate expression of the target gene. Following induction, the cells were allowed to grow for the specified time period prior to assaying for gene induction.

Inducible Expression of **MYC**

In order to efficiently overexpress Myc, a cell line was generated that stably expressed a Myc-estrogen receptor fusion protein (mycER). This involved transfection of a vector into R1 mES cells that contained the DNA sequence for *MYC*, a modified estrogen receptor, and a geneticin (G418) resistance gene, under the control of the pCAG promoter. Following G418 selection, a cell line was established that stably expressed the mycER in the cell cytoplasm. Tamoxifen (4-OHT) was added to the cell culture at a concentration of 100 nM in order to induce nuclear translocation of the mycER, and thus Myc overexpression.
**Inducible Inactivation of MYC and MYCN**

Induced pluripotent stem cells (iPSCs) were generated previously from a mouse containing \( MYC^{fl/fl} \) and \( MYCN^{fl/fl} \) floxed alleles (Smith et al. 2010). Previously, these floxed alleles could be deleted from the genome by transient transfection of a CreGFP expression vector, followed by sorting for GFP positive cells, yielding double knockout cells. In order to improve the efficiency of this process, a cell line was generated that stably expressed a CreGFP-glucocorticoid receptor fusion protein (CreGR). This involved sub-cloning of the CreGFP from a commercially available plasmid (Addgene plasmid #13776) into a pCAG-glucocorticoid vector that also expressed a puromycin resistance cassette. This fusion protein vector was transfected into miPS cells containing the \( MYC^{fl/fl} \) & \( MYCN^{fl/fl} \) floxed alleles and selection by puromycin led to the establishment of a stable cell line. Dexamethasone (Sigma D4902) was then used at a concentration of 20 µM to induce translocation of the CreGR fusion protein to the nucleus of the \( MYC^{fl/fl} \ MYCN^{fl/fl} \) miPSCs leading to efficient deletion of floxed MYC alleles.

**Luciferase Assays**

Luciferase assays were performed with the Dual Luciferase Reporter kit (Promega E1910) according to instructions and analyzed on a Synergy 2 plate reader (BioTek).
RNA Isolation and Semi-quantitative RT-PCR analysis

RNA was isolated with the RNeasy mini kit (Qiagen 74104). cDNA libraries were prepared from RNA using the iScript cDNA synthesis kit (Bio-Rad #170-8891). Transcript levels were assayed by RT-qPCR using TaqMan assays (Applied Biosystems), were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh), analyzed using the ddCT method, and performed in triplicate. Applied Biosystems TaqMan assays used in these studies are listed in Table 4.2.

Transfection of mPSCs

200,000 cells were plated overnight in a 35mm gelatin coated tissue culture dish. The following morning 10 micrograms of DNA were incubated with 10 microliters of Lipofectamine 2000 (Life Technologies #11668-027) for 30 minutes at room temperature. This mixture was added to the culture dishes containing fresh media and grown at 37°C for approximately 8-12 hours, at which point fresh media was added. If necessary, the appropriate drug selection was added approximately 36-48 hours after transfection.

Western Blot Analysis

Between 1 x 10^6 and 1x10^7 cells were collected and used to prepare extracts for western blotting. Cells were washed once with DPBS, detached using trypsin EDTA, quenched using serum-containing medium, centrifuged, and flash-frozen as pellets. Lysates were prepared in a modified RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS) supplemented with
protease inhibitor (Roche, cat# 04693116001), phosphatase inhibitor (Calbiochem #524624), and DTT (Millipore #233155). Lysate was isolated by centrifugation at 4°C at 20,800g for 10 minutes. The concentration of the supernatant was determined using the Bradford assay kit (Bio-Rad #500-0006), and then it was taken to a new tube and combined with an equal volume of Laemmli buffer (Bio-Rad #161-0737). Between 10-30 µg of lysate, alongside a protein molecular weight ladder (Kaleidoscope Molecular Weight Marker, Bio-Rad #161-0375), was loaded per lane of an 8%-15% Tris-HCl gel and run at a constant 200 volts for approximately 30 minutes.

REFERENCES


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</tr>
<tr>
<td>DUSP7(Pyst2)</td>
<td>Santa Cruz</td>
<td>sc-47667</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>EGFR</td>
<td>Santa Cruz</td>
<td>sc-120</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>ERα (G-20)</td>
<td>Santa Cruz</td>
<td>sc-544</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>ERα (MC-20)</td>
<td>Santa Cruz</td>
<td>sc-542</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>FLAG</td>
<td>Sigma</td>
<td>F1804</td>
<td>1 µg/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>FLAG</td>
<td>Sigma</td>
<td>F7425</td>
<td>800 ng/µL</td>
<td>1:1000</td>
</tr>
<tr>
<td>FoxA2</td>
<td>Millipore</td>
<td>07-633</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Gata4</td>
<td>R&amp;D</td>
<td>AF2606</td>
<td>200 ng/µL</td>
<td>1 µg/mL</td>
</tr>
<tr>
<td>Gata4</td>
<td>Santa Cruz</td>
<td>sc1237</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Geminin (C16)</td>
<td>Santa Cruz</td>
<td>sc-8448</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>H3K27ac</td>
<td>Abcam</td>
<td>ab4729</td>
<td>1 µg/µL</td>
<td>x</td>
</tr>
<tr>
<td>H3K27ac</td>
<td>Abcam</td>
<td>ab4729</td>
<td>900 ng/µL</td>
<td>x</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Abcam</td>
<td>ab8580</td>
<td>200 ng/µL</td>
<td>x</td>
</tr>
<tr>
<td>HA- tag</td>
<td>Sigma</td>
<td>H3663</td>
<td>1 µg/µL</td>
<td>1:2000</td>
</tr>
<tr>
<td>HA- tag (6E2)</td>
<td>Cell Signaling</td>
<td>2367</td>
<td>x</td>
<td>1:1000</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Abcam</td>
<td>ab8898</td>
<td>1 µg/µL</td>
<td>1:1000</td>
</tr>
<tr>
<td>IgG (rabbit)</td>
<td>Abcam</td>
<td>ab46540</td>
<td>1 µg/µL</td>
<td>x</td>
</tr>
<tr>
<td>Lsd1</td>
<td>Upstate</td>
<td>05-939</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>Abcam</td>
<td>ab53570</td>
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<td>x</td>
</tr>
<tr>
<td>Max (C-17)</td>
<td>Santa Cruz</td>
<td>sc-197</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
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</table>

Table 4.1a. Antibodies used in these studies.
<table>
<thead>
<tr>
<th>Anti-</th>
<th>Company</th>
<th>Catalog #</th>
<th>Concentration</th>
<th>Western</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcm2 (BM28)</td>
<td>BD Biosciences</td>
<td>610700 (1)</td>
<td>x</td>
<td>1:500</td>
</tr>
<tr>
<td>Mcm4 (G-7)</td>
<td>Santa Cruz</td>
<td>sc-28317</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>Mcm5 (H-300)</td>
<td>Santa Cruz</td>
<td>sc-22780</td>
<td>200 ng/µL</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mcm6 (D-12)</td>
<td>Santa Cruz</td>
<td>sc-55577</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>Mcm7 (141.2)</td>
<td>Santa Cruz</td>
<td>sc-9966</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>Med12</td>
<td>Bethyl</td>
<td>A300-774A</td>
<td>1 µg/µL</td>
<td>x</td>
</tr>
<tr>
<td>Mll1</td>
<td>Bethyl</td>
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<td>1 µg/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>Mll2</td>
<td>Sigma</td>
<td>SAB1402978</td>
<td>x</td>
<td>1:500</td>
</tr>
<tr>
<td>Myc</td>
<td>Cell Signaling</td>
<td>9402</td>
<td>x</td>
<td>1:1000</td>
</tr>
<tr>
<td>Myc (N262)</td>
<td>Santa Cruz</td>
<td>sc-764</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Myc (C-20)</td>
<td>Santa Cruz</td>
<td>sc-790</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Mycn</td>
<td>Calbiochem</td>
<td>OP13</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Mycn (C-19)</td>
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<td>sc791</td>
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<td>1:200</td>
</tr>
<tr>
<td>Nanog</td>
<td>CosmoBio</td>
<td>RCAB0002PF</td>
<td>200 ng/µL</td>
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</tr>
<tr>
<td>Oct3/4 (C-10)</td>
<td>Santa Cruz</td>
<td>sc5279</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Oct3/4 (H-134)</td>
<td>Santa Cruz</td>
<td>sc9081</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Oct3/4 (N-19)</td>
<td>Santa Cruz</td>
<td>sc8628</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>P-44/42 Mapk (P-Erk1/2)</td>
<td>Cell Signaling</td>
<td>9101</td>
<td>x</td>
<td>1:500</td>
</tr>
<tr>
<td>P- BetaCatenin</td>
<td>Cell Signaling</td>
<td>9561</td>
<td>x</td>
<td>1:1000</td>
</tr>
<tr>
<td>P-GSK3B</td>
<td>Cell Signaling</td>
<td>9323</td>
<td>x</td>
<td>1:1000</td>
</tr>
<tr>
<td>P-S6 Ribosomal</td>
<td>Cell Signaling</td>
<td>2211S</td>
<td>x</td>
<td>1:1000</td>
</tr>
<tr>
<td>p107</td>
<td>Santa Cruz</td>
<td>sc318</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>p130</td>
<td>Santa Cruz</td>
<td>sc317</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>p16</td>
<td>Santa Cruz</td>
<td>sc1207</td>
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<td>NO</td>
</tr>
<tr>
<td>p21 (C19)</td>
<td>Santa Cruz</td>
<td>sc-397</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>p27 (M-197)</td>
<td>Santa Cruz</td>
<td>sc-776</td>
<td>200 ng/µL</td>
<td>1:500</td>
</tr>
<tr>
<td>p44/42 Mapk (Erk1/2)</td>
<td>Cell Signaling</td>
<td>9102</td>
<td>x</td>
<td>1:500</td>
</tr>
<tr>
<td>p44/42 Mapk (Erk1/2)</td>
<td>Santa Cruz</td>
<td>sc-135900</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Pecam1(CD31)</td>
<td>BD Pharamingen</td>
<td>558736</td>
<td>500 ng/µL</td>
<td>1:1000</td>
</tr>
<tr>
<td>Pol II N-term</td>
<td>Santa Cruz</td>
<td>sc-899</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
<tr>
<td>Smc1a</td>
<td>Bethyl</td>
<td>A300-055A</td>
<td>1 µg/µL</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sox17 (V-20)</td>
<td>Santa Cruz</td>
<td>sc17356</td>
<td>200 ng/µL</td>
<td>1:200</td>
</tr>
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</table>

*Table 4.1b. Antibodies used in these studies. (Continued)*
<table>
<thead>
<tr>
<th><strong>Lysis Buffer 1 (LB1)</strong></th>
<th>Stock For 500mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Hepes-KOH, ph 7.5</td>
<td>25 mL</td>
<td>50 mM</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>14 mL</td>
<td>140 mM</td>
</tr>
<tr>
<td>0.5M EDTA, ph 8</td>
<td>1 mL</td>
<td>1 mM</td>
</tr>
<tr>
<td>50% glycerol</td>
<td>100 mL</td>
<td>10%</td>
</tr>
<tr>
<td>10% NP-40</td>
<td>25 mL</td>
<td>0.50%</td>
</tr>
<tr>
<td>10% Tx-100</td>
<td>12.5 mL</td>
<td>0.25%</td>
</tr>
<tr>
<td>ddH2O</td>
<td>322.5 mL</td>
<td>x</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Lysis Buffer 2 (LB2)</strong></th>
<th>Stock For 500mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, ph 8</td>
<td>5 mL</td>
<td>10 mM</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>20 mL</td>
<td>200 mM</td>
</tr>
<tr>
<td>0.5M EDTA, ph 8</td>
<td>1 mL</td>
<td>1 mM</td>
</tr>
<tr>
<td>0.5M EGTA, ph 8</td>
<td>500 µL</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ddH2O</td>
<td>473.5 mL</td>
<td>x</td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>Lysis Buffer 3 (LB3)</strong></th>
<th>Stock For 50mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, ph 8</td>
<td>500 µL</td>
<td>10 mM</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>1 mL</td>
<td>100 mM</td>
</tr>
<tr>
<td>0.5M EDTA, ph 8</td>
<td>100 µL</td>
<td>1 mM</td>
</tr>
<tr>
<td>0.5M EGTA, ph 8</td>
<td>50 µL</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>10% Na-Deoxycholate</td>
<td>500 µL</td>
<td>0.10%</td>
</tr>
<tr>
<td>20% N-lauroylsarcosine</td>
<td>1.25 mL</td>
<td>0.50%</td>
</tr>
<tr>
<td>ddH2O</td>
<td>46.6 mL</td>
<td>x</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Wash Buffer (RIPA)</strong></th>
<th>Stock For 500mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Hepes-KOH, ph 7.5</td>
<td>25 mL</td>
<td>50 mM</td>
</tr>
<tr>
<td>5M LiCl</td>
<td>50 mL</td>
<td>500 mM</td>
</tr>
<tr>
<td>0.5M EDTA, ph 8</td>
<td>1 mL</td>
<td>1 mM</td>
</tr>
<tr>
<td>10% NP-40</td>
<td>50 mL</td>
<td>1%</td>
</tr>
<tr>
<td>10% Na-Deoxycholate</td>
<td>35 mL</td>
<td>0.70%</td>
</tr>
<tr>
<td>ddH2O</td>
<td>339 mL</td>
<td>x</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Elution Buffer</strong></th>
<th>Stock For 500mL</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, ph 8</td>
<td>2.5 mL</td>
<td>50 mM</td>
</tr>
<tr>
<td>0.5M EDTA, ph 8</td>
<td>1 mL</td>
<td>10 mM</td>
</tr>
<tr>
<td>10% SDS</td>
<td>5 mL</td>
<td>1%</td>
</tr>
<tr>
<td>ddH2O</td>
<td>41.5 mL</td>
<td>x</td>
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Table 4.2. Buffer compositions for chromatin immunoprecipitation.
<table>
<thead>
<tr>
<th>Target</th>
<th>Catalog Number</th>
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</thead>
<tbody>
<tr>
<td>DUSP2 promoter Forward</td>
<td>5'-GGACAATTTCCCTGACGGTTG-3'</td>
</tr>
<tr>
<td>DUSP2 promoter Reverse</td>
<td>5'-CCATTTTCTCCAGGGTTTT-3'</td>
</tr>
<tr>
<td>DUSP2 control Forward</td>
<td>5'-AACCATGTCTAGGGGTGTC-3'</td>
</tr>
<tr>
<td>DUSP2 control Reverse</td>
<td>5'-CAGCACCAATTGACGAGGAG-3'</td>
</tr>
<tr>
<td>DUSP7 promoter Forward</td>
<td>5'-GCCTAACACAGAGGAG-3'</td>
</tr>
<tr>
<td>DUSP7 promoter Reverse</td>
<td>5'-AGAGCCGAATTCAGCGAGA-3'</td>
</tr>
<tr>
<td>DUSP7 control Forward</td>
<td>5'-GGACTGAGGAGCAGGAG-3'</td>
</tr>
<tr>
<td>DUSP7 control Reverse</td>
<td>5'-CTTAGCAAGGAGGAG-3'</td>
</tr>
</tbody>
</table>

**Table 4.3. Sybr Green primers used in these studies.**
<table>
<thead>
<tr>
<th>Target</th>
<th>Catalog Number</th>
<th>Target</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurka</td>
<td>Mm01248177_m1</td>
<td>Gata6</td>
<td>Mm00802636_m1</td>
</tr>
<tr>
<td>Aurkb</td>
<td>Mm01718146_g1</td>
<td>Hist1H4K</td>
<td>Mm03017776_g1</td>
</tr>
<tr>
<td>Cdca2</td>
<td>Mm00558459_m1</td>
<td>Hist2H2BE</td>
<td>Mm00558749_s1</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Mm00772472_m1</td>
<td>HoxB4</td>
<td>Mm00657964_m1</td>
</tr>
<tr>
<td>Cdk5r2</td>
<td>Mm00438150_s1</td>
<td>HoxD8</td>
<td>Mm04207478_g1</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>Mm00432448_m1</td>
<td>Klf4</td>
<td>Mm00516104_m1</td>
</tr>
<tr>
<td>Cdkn2b</td>
<td>Mm00483241_m1</td>
<td>Max</td>
<td>Mm00484802_g1</td>
</tr>
<tr>
<td>Cdx2</td>
<td>Mm01212280_m1</td>
<td>Med12</td>
<td>Mm00804032_m1</td>
</tr>
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<td>Cer1</td>
<td>Mm00515474_m1</td>
<td>Med22</td>
<td>Mm00600035_m1</td>
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<tr>
<td>Ctf</td>
<td>Mm00484027_m1</td>
<td>Myc</td>
<td>Mm00487803_m1</td>
</tr>
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<td>cyclin A1</td>
<td>Mm00432337_m1</td>
<td>Mycl1</td>
<td>Mm00493155_m1</td>
</tr>
<tr>
<td>cyclin E1</td>
<td>Mm00432367_m1</td>
<td>Mycn</td>
<td>Mm00476449_m1</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>Mm00432881_m1</td>
<td>Nanog</td>
<td>Mm02019550_s1</td>
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<td>Dppa3</td>
<td>Mm00515474_m1</td>
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<td>Mm00446859_m1</td>
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<td>Pax6</td>
<td>Mm00443081_m1</td>
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<td>Pou3f1</td>
<td>Mm00843534_s1</td>
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<tr>
<td>Dusp2</td>
<td>Mm00839675_g1</td>
<td>Pou5f1</td>
<td>Mm03053917_g1</td>
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<tr>
<td>Dusp6</td>
<td>Mm00518185_m1</td>
<td>Rex1 (Zfp42)</td>
<td>Mm01194089_g1</td>
</tr>
<tr>
<td>Dusp7</td>
<td>Mm01232570_m1</td>
<td>Rnf2(Ring1b)</td>
<td>Mm00803321_m1</td>
</tr>
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<td>Dusp9</td>
<td>Mm00512648_g1</td>
<td>Rm1</td>
<td>Mm00485870_m1</td>
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<td>Eed</td>
<td>Mm00496951_m1</td>
<td>Sall1</td>
<td>Mm00491266_m1</td>
</tr>
<tr>
<td>Eomes</td>
<td>Mm01351985_m1</td>
<td>Smc1a</td>
<td>Mm00490624_m1</td>
</tr>
<tr>
<td>Esrrb</td>
<td>Mm00446859_m1</td>
<td>Sox17</td>
<td>Mm00488363_m1</td>
</tr>
<tr>
<td>Fgf5</td>
<td>Mm00438919_m1</td>
<td>Sox2</td>
<td>Mm03053810_s1</td>
</tr>
<tr>
<td>FoxA2</td>
<td>Mm00776876_m1</td>
<td>Sox7</td>
<td>Mm00776876_m1</td>
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<td>FoxD4</td>
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<td>Mm00436877_m1</td>
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<td>Gata4</td>
<td>Mm00484689_m1</td>
<td>Zic2</td>
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</tbody>
</table>

Table 4.4. Applied Biosystems Taqman probes used in these studies.
It has long been accepted that MYC’s main role is to bind E-box motifs in the proximal promoters of genes in order to activate transcription. While this premise remains mostly unchallenged, recent investigation of global binding shows that expression of relatively high levels of MYC allows “invasion” of genomic loci where it does not necessarily play a role in regulating transcription. This has led to considerable debate regarding what constitutes a bona fide MYC target gene, and dispute regarding its mechanism of action. One hypothesis is that MYC is recruited to every active gene, where it then amplifies transcriptional output. The other hypothesis is that MYC has a discrete set of target genes mostly involved in metabolism and cell growth, and that upregulation of these target genes is responsible for the indirect consequence of global transcriptional amplification. Due to a high level of MYC expression compared to somatic cells, and the identification of non-regulatory MYC binding sites, this debate is particularly relevant to pluripotent stem cell (PSC) biology.

In this dissertation we identified two genuine MYC target genes, DUSP2 and DUSP7, which do not promote cell growth but rather inhibit differentiation signals. Overexpression of MYC led to upregulation of DUSP transcripts and depletion of MYC led to their downregulation. Direct binding and activation of
DUSP promoters was confirmed by chromatin immunoprecipitation and luciferase reporter assays, respectively. The depletion of MYC permitted activation of ERK signaling and thus differentiation, but could be abrogated by addition of a MEK inhibitor, or by ectopic expression of DUSP7. This demonstrated that activation of DUSP is an important MYC activity, as it blocks differentiation. It also revealed a mechanism to explain an observation made by Hishida and colleagues that MAX expression inhibits ERK in PSCs (Hishida et al. 2011). Furthermore, we showed that DUSP activity is critical for early development in vivo by demonstrating a reduced capacity of DUSP deficient PSCs to contribute to chimeric embryos. This further demonstrated the importance of MYC for maintaining PSCs, beyond the well-studied role of promoting cell growth.

While our work has established the significance of MYC and DUSPs in naïve PSCs, it is an open question what role they play in other stages of pluripotency. MYC expression is significantly lower in mPSCs grown in 2i culture (Marks et al. 2012), and we show that overall DUSP levels are reduced as well (Chapter 3). This could potentially be the result of downregulation of a feedback loop. Previous studies have shown that phosphorylation of serine 62 by ERK stabilizes MYC protein and increases its transcriptional activity (Lutterbach and Hann 1994; Sears et al. 1999). In 2i media, however, ERK is kept inactive by the presence of a MEK inhibitor. Inactive ERK may therefore lead to less stable, and less active MYC, and therefore less DUSP gene transcription. This explanation is appealing for two reasons; it accounts for reduced MYC and DUSP expression,
and it is rational to conclude that DUSP activity is superfluous in this circumstance where its target does not exist due to small-molecule inhibition.

In primed pluripotent cells, i.e. murine epiSCs or human ESCs, an increased level of sustained FGF/ERK signaling activation, in cooperation with Activin A signaling, is necessary for self-renewal (Vallier et al. 2005). However ERK levels must still be tightly regulated, as its unrestricted activation also leads to differentiation (Na et al. 2010). It remains to be determined whether the balance of ERK activation in primed cells is dependent on a DUSP dependent mechanism. Furthermore, because MYC activity is typically reduced in terminally differentiated tissues, it will be interesting to determine if DUSPs identified in this study play a role in later stages of development.

REFERENCES


