Src Family Tyrosine Kinase Signaling in Mouse and Human Embryonic Stem Cells

by

Xiong Zhang

B.E., Xi'an Jiaotong University, 2007

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2013

UNIVERSITY OF PITTSBURGH

School of Medicine

This thesis was presented

by

Xiong Zhang

It was defended on

June 11th, 2013

and approved by

Thomas E. Smithgall, Ph.D., Major Advisor, Microbiology and Molecular Genetics

J. Richard Chaillet, Ph.D., Thesis Committee Chair, Microbiology and Mocular Genetics

Neil Hukriede, Ph.D., Developmental Biology

Gerald P. Schatten, Ph.D., Obstetrics, Gynecology and Reproductive Sciences

Alan Wells, M.D., D.M.Sc., Pathology

Src Family Tyrosine Kinase Signaling in Mouse and Human Embryonic Stem Cells

Xiong Zhang

University of Pittsburgh, 2013

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst stage embryo and are characterized by self-renewal and pluripotency. Previous work has implicated the Src family of protein-tyrosine kinases (SFKs) in the self-renewal and differentiation of mouse ES (mES) cells. These kinases display dynamic expression and activity changes during ES cell differentiation, suggesting distinct functions in the control of developmental fate. To test the hypothesis that c-Src and its closest phylogenetic relative, c-Yes, act in biological opposition to one another, I first showed that enforced expression of active c-Yes blocked ES cell differentiation to embryoid bodies by maintaining pluripotency gene expression. To determine the interplay of c-Src and c-Yes in mES cell fate determination, I employed a chemical genetics approach to generate c-Src and c-Yes mutants that are resistant to A-419259, a potent pyrrolopyrimidine inhibitor of the Src kinase family. This method allowed us to investigate individual kinase function in the presence of A-419259. I found that c-Src activity alone induces mES cell differentiation to the ectoderm and endoderm, while c-Yes inhibits this process. These studies show that even closely related kinases such as c-Src and c-Yes have unique and opposing functions in the same cell type.

While Src kinase signaling has been investigated in mES cells, the role of this kinase family in human ES (hES) cells is largely unknown. Using quantitative real-time RT-PCR, I determined the relative expression profile of individual SFK members in undifferentiated hES

cells vs. embryoid bodies derived from them. Like mES cells, hES cells express multiple SFK members with dynamic transcription changes during EB differentiation, indicating that individual members may play non-redundant roles. To assess the role of SFK activity in hES cells, I treated hES cell cultures with SFK inhibitors. SFK inhibition maintained hES cell colony morphology and expression of the pluripotency marker Tra-1-60 in differentiation medium. These observations support a role for Src family kinase signaling in the regulation of hES fate, and suggest that some parallels may exist in mouse and human ES cells for this intracellular signaling network.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENT

Looking back over my experience in graduate school, although there have been ups and downs, joys and sorrows; it has been a gorgeous journey. I have discovered that life is never a straight line, yet the beauty lies in the exploration out of the twists and turns. This journey would not have happened without those who guided me, accompanied me and supported me. I would like to offer my appreciation.

First, I acknowledge my utmost gratitude to my mentor, Dr. Thomas Smithgall, for his guidance, inspiration and support. Dr. Smithgall is an outstanding scientist, a thoughtful mentor and a generous person. No matter how busy he was, he always devoted time to guide me and generously offered new ideas when I faced challenges and seemly unsolvable problems. I was often motivated by his contagious enthusiasm and passion for science. Not only did Dr. Smithgall painstakingly edit and improve my writing, he also supported my exploration for my career goals according to my own interests.

I owe much gratitude to my dissertation committee members for constructive advice and constant support. I would like to thank Dr. Richard Chaillet for chairing my committee and his generosity. I would like to thank Dr. Neil Hukriede, for the commitment, sincere concern and assistance. I would like to thank Dr. Alan Wells, for his career suggestions and guidance. My deep appreciation goes to Dr. Gerald Schatten, for the generous support of the human ES cell

project; for allowing me to get hands-on experience with hES cell culture; and inviting me to participate in the stem cell course at Woods Hole.

I would like to also thank my collaborators from the Schatten group: Carrie Redinger, Dr. Chas Easley, Stacie Oliver, David Mcfarland, and Jody Mich-Basso. Human ES cell culture is difficult, yet your help and support made it much better and enjoyable!

Many warm thanks to the current and previous members of the Smithgall laboratory: Dr. Tony Meyn, Dr. Linda O'Reilly, Dr. Lori Emert-Sedlak, Dr. Jerrod Poe, Dr. Sabine Hellwig, Dr. Sherry Shu, Dr. Shoghag Panjarian, Dr. Teodora Pene-Dumitrescu, Dr. Jonathan Shaffer, Dr. Purushottam Narute, Dr. John Jeff Alvarado, Dr. Patty George, Jamie Moroco, Mark Weir, Prerna Grover, Sreya Tarafdar, Kathleen Makielski, Terri Robinson and Nageswara Alla. It has been a great pleasure to work with you all. Thank you for all the great suggestions, support and discussions. Special thanks to Tony and Linda for the guidance, help and suggestions on the stem cell project.

1.0 INTRODUCTION

1.1 EMBRYONIC STEM CELLS

Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of blastocyst stage embryo (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). The derivation of mouse ES (mES) cells is based on early research on teratomas and teratoma stem cells to establish the culture conditions and functional assays (Evans, 2011). ES cells are characterized by two properties: self-renewal, the ability to grow indefinitely without differentiation, and pluripotency, the developmental potential to generate all cell types from the embryo and adult body (Nichols and Smith, 2012). In culture, pluripotency is maintained by extrinsic growth factors, and expression of transcription factors. The transcription factors form an intricate network to control gene expression and maintain pluripotency. In conditions without renewal factors, ES cells differentiate, recapitulating the differentiation programs of the developing embryo (Murry and Keller, 2008). Human ES（hES）cells were derived 17 years after the establishment of mES cells. Although hES cells and mES cells have different culture conditions and signaling features, they express the same core transcription factors to maintain pluripotency (Thomson et al., 1998). Recent establishment of epiblast stem cells indicates that hES cells and mES cells may represent different states of pluripotent stem cells.

1.1.1 Mouse embryonic stem cells

Mouse embryonic stem (mES) cells are self-renewing, pluripotent cells derived from blastocyst stage mouse embryo (Evans and Kaufman, 1981; Martin, 1981). Early research on teratoma and embryonic carcinoma (EC) cells defined the feeder cell conditions required to maintain ES cells in culture, cell-surface markers for self-renewal, and functional assays for pluripotency. Later work established that mES cell pluripotency requires growth factors including leukemia inhibitory factor (LIF) and bone morphogenetic proteins (BMPs) (Evans, 2011). ES cells have the remarkable ability to contribute to embryo development and the germline, which allows genetic manipulation and production of transgenic animals. These features make ES cells a powerful tool to study development, genetics and disease (Evans, 2011).

1.1.1.1 Teratocarcinoma and embryonic carcinoma cells

The discovery and establishment of mES cells were largely based on the early research on teratocarinomas and the pluripotent cells they carry (Chambers and Smith, 2004; Solter, 2006). Teratoma, a rare tumor in humans, is composed of a mixture of cells from three different germ layers. The mouse strain 129 was found to have a high incidence of developing spontaneous teratomas. Teratocarcinoma, the malignant form of teratoma, contains undifferentiated stem cells that can form secondary tumors after transplantation into another host. These cells, termed embryonic carcinoma (EC) cells, were subsequently established for cell culture. In EC cell line derivation, Evans made the discovery that irradiated chick fibroblasts can be used as feeder layers to support undifferentiated growth of EC cells (Evans, 1972). In suspension culture without the feeder layer, EC cells differentiate to embryoid bodies, a ball-like structure

comprised of cell types derived from all three germ layers, similar to the early stages of embryonic development in vivo (Martin and Evans, 1975). In addition, alkaline phosphatase (Berstine et al., 1973) and stage-specific embryonic antigen 1(SSEA-1) (Solter and Knowles, 1978) were identified as the first biological markers for EC cells. When injected into developing blastocysts, rare EC cells can contribute to chimera mouse formation, including germ line transmission (Stewart and Mintz, 1981; Stewart and Mintz, 1982). The studies highlighted above, including the optimized culture conditions for EC cells with feeder fibroblast, in vitro EB formation assay, cell surface markers, and blastocyst injection of EC cells to assess developmental potential, laid a strong foundation for the discovery and study of ES cells.

1.1.1.2 Derivation of embryonic stem cells

In addition to spontaneous generation, teratomas can also be formed by transplanting pre-gastrulation stage embryonic cells into the testis or kidney capsules, which suggests that there might be a stem cell type that is equivalent to EC cells in the developing embryo. Evans & Kaufman and Martin independently derived pluripotent embryonic stem cells from the blastocyst stage of embryos (Evans and Kaufman, 1981; Martin, 1981). Evans and Kaufman induced embryo arrest (diapause) in pre-implantation stage embryos using ovariectomy and hormone treatment, and derived ES cells from explants of the intact diapause embryos with serum-containing medium and division-incompetent STO fibroblast feeders (Stromal cells derived from Sandoz inbred mouse (S), that are engineered to be resistant to 6-thioguanine(T) and ouabain(O)). Martin derived ES cell lines from the isolated inner cell mass (ICM) of pre-implantation embryos with EC cell-conditioned medium.

Traditionally, ES cells are derived from out-growth of intact blastocysts or isolated entire ICMs. ES cells have also been established from single, dissociated epiblast cells (Brook and Gardner, 1997), which convincingly demonstrated that ESCs are of epiblast origin at embryonic day 4.5 (E4.5). Mammalian embryonic development begins at the zygote, which undergoes three rounds of cleavage division to form the 8-cell embryo (E2.5; Figure 1). The blastomeres in the embryo then undergo compaction, gain polarity and form the morula. A series of subsequent divisions, accompanied by morphologic changes, result in a fluid-filled ball-like structure called blastocyst (E3.5), with the inner cell mass, a group of apolar cells, enclosed by an outer layer of polarized epithelial cells, trophectoderm. The segregation of trophectoderm and ICM is the hallmark of blastocyst formation, with ICM cells expressing the transcription factor Oct4 and trophectoderm cells expressing Cdx2. Subsequently, around E4.5, the ICM cells develop to form the epiblast and primitive endoderm, an epithelium between the epiblast and the blastocyst cavity (Stephenson et al., 2012). Mouse ES cells can be derived from E3.5 ICM cells, or E4.5 early epiblast cells (Nichols and Smith, 2012).

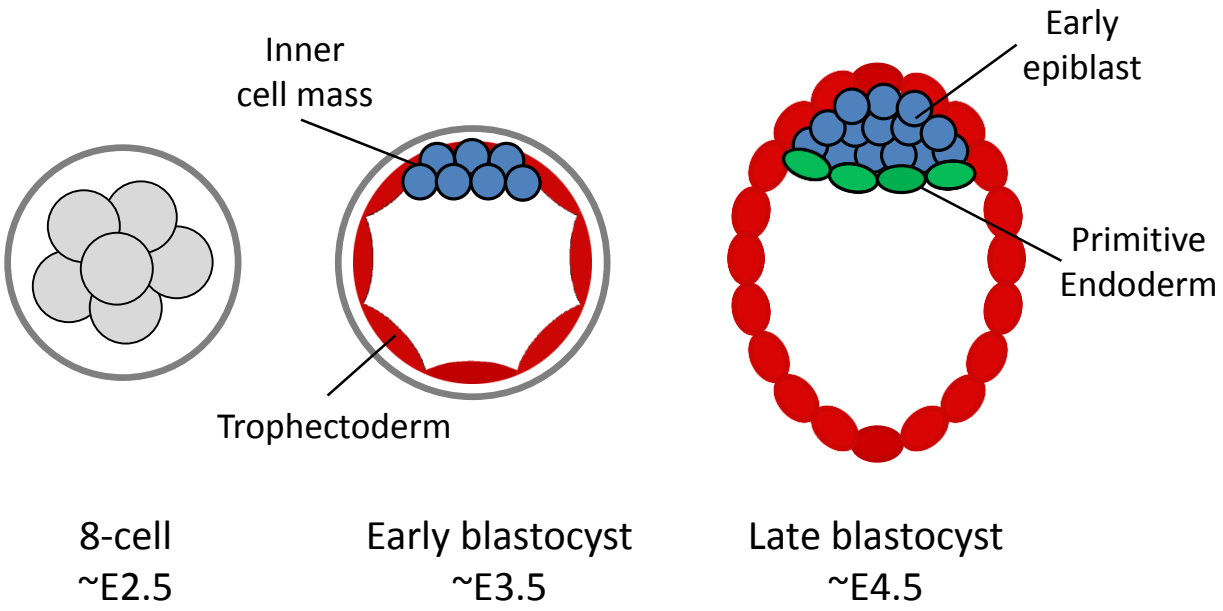

Figure 1. Early embryo development stages and ES cell derivation. The 8-cell stage embryo (left) undergoes compaction, polarization and several rounds of division, to form the early blastocyst at around E 3.5. The early blastocyst (middle) has trophectoderm, the outer polarized layer of epithelium, and the inner cell mass, a group of apolar cells clustered inside. By E4.5, as the blastocyst further develops, the ICM is segregated into the epiblast and primitive endoderm (right). The embryo is now ready to implant in the uterus. Adapted from (Stephenson et al., 2012)

1.1.1.3 ES cell culture conditions

Similar to EC cells, ES cells can be maintained in serum-containing medium with co-culture of feeder fibroblasts. STO mouse fibroblast cell-conditioned medium was shown to support EC cell growth and maintenance of pluripotency without feeders (Koopman and Cotton, 1984). Also, Buffalo rat liver (BRL) cell-conditioned medium can replace the fibroblast requirement for maintenance of ES cells (Smith and Hooper, 1987). These studies indicate that feeder cells produce a soluble factor to inhibit EC or ES cell differentiation. Subsequent research identified

the cytokine leukemia inhibitory factor (LIF) as the active component of conditioned medium that maintains the developmental potential of ES cells (Smith et al., 1988; Williams et al., 1988). In addition, bone morphogenetic proteins (BMPs), known anti-neuronal differentiation factors, are able to replace serum to support ES cell derivation and maintenance in combination with LIF. Thus, ES cell culture conditions have evolved from feeder and serum-containing medium, to feeder-free, LIF and serum containing medium, then to feeder-free, serum-free medium with LIF and BMPs.

More recently, the Smith group showed that ES cells can be maintained in a chemically-defined medium with two inhibitors—the MEK inhibitor PD0325901 and GSK3β inhibitor CHIR99021 (Ying et al., 2008; Silva et al., 2008). This so called '2i' medium, combined with LIF enabled the derivation of ES cell lines from traditionally refractory mouse strains like nonobese diabetic (NOD) mice (Hanna et al., 2009), or non-permissive species like the rat (Buehr et al., 2008).

1.1.1.4 ES cell properties: self-renewal and pluripotency

ES cells have two hallmark properties: Self-renewal and pluripotency. Self-renewal is the ability of stem cells to produce at least one daughter cell that has the same differentiation capacity as the parental cells. For ES cells, cell division can be symmetric, producing two daughter cells identical to the parental cell; or asymmetric, generating one stem cell and one differentiated cell (Smith, 2001). Pluripotency is the ability to differentiate to cell types from all three germ layers.

In vitro, ES cells can be maintained continuously without differentiation. Self-renewal is maintained with extracellular signals such as LIF and serum or BMPs, which enforces expression of core pluripotency transcription factors such as Oct4 and Nanog (discussed in the next section).

ES cells have a shortened cell cycle, with a short G1 phase and progression independent of the retinoblastoma (RB) protein (Burdon et al., 2002). Self-renewal can also be assessed by clonogenicity assay and expression of renewal markers such as alkaline phosphatase, SSEA-1 and transcription factors such as Oct4 and Nanog (Evans, 2011). However, ES cells are strictly a cell culture phenomenon: In vivo, the ICM cells only divide symmetrically for a short period of time and quickly differentiate to more developed lineages (Nichols and Smith, 2012).

Pluripotency can be assessed using a number of different functional criteria, with increasing stringency, as summarized in Table 1 (Jaenisch and Young, 2008). The most commonly used and least stringent test of pluripotency is embryoid body formation. Absent of support from feeder cells or LIF, ES cells are grown in suspension culture in liquid or methyl cellulose containing medium, where each single ES cell is able to divide to generate a ball-like structure containing all three germ layers, recapitulating the developmental events of the early embryo (Keller, 2005). The hallmark of pluripotency of ES cells is their ability to be incorporated into normal development when injected into developing blastocysts, producing chimeric mice and being able to contribute to the germ line. Germ line competency is the gold standard of pluripotency, enabling us to use gene-targeting to manipulate ES cells and create transgenic animals for study of developmental processes and to model disease (Capecchi, 2005). Tetraploid complementation is the most stringent test of pluripotency. In this approach, ES cells are injected into 4n host blastocysts. If the ES cells are truly pluripotent, then all of the somatic cells of the resulting embyo will be of ES cell origin since the host cells will only support the extraembryonic lineages.

Table 1. Functional assays to assess developmental potential of ES cells

Assay	Experimental Approach	Limitations
In vitro differentiation	Cultured cells are induced for differentiation via EB formation, or attached differentiation and are assayed for expression of specific linage markers.	No functional assay; multiple markers is required; hard to synchronize, heterogeneity complicates results.
Teratoma formation	Injection of ES cells into immuno-deficient mice to induce tumor formation, demonstrating the potential to generate differentiated cell types from all three germ layers	Normal development of cells is not tested.
Chimera formation	Injection of ES cells into developing blastocyst, to incorporate to the ICM and contribute to the normal development, generating chimeric mouse	Non-autonomous defects in test cells may be blinded by compensation from the host derived cells.
Germline competency	The ability of test cells to generate functional germ cells, and in the case of genetic manipulation, to generate transgenic animal.	Does not exclude epigenetic defects that might interfere with development.
Tetraploid complementa-tion	Injection of test cells to tetraploid host embryo. Since the 4n host can only contribute to extraembryonic tissues, the embryo is exclusively derived from the test cells.	Most Stringent test for pluripotency; the ability to form trophectoderm linages is not tested.

Adapted from (Jaenisch and Young, 2008)

1.1.2 Signaling networks regulating mES cell self-renewal and differentiation

Mouse ES cell pluripotency and differentiation are governed by a complex network of signaling pathways, which work in concert. Multiple intracellular kinase signaling pathways, including LIF-Jak-Stat3, BMP-SMAD-ID, MEK/Erk, PI3K-Akt and Wnt-GSK-β-Catenin pathways, play a dominant role in the regulation of ES cell fate (Liu et al., 2007). These extrinsic signals converge in an intrinsic transcription factor network controled by Oct4, Sox2 and Nanog to maintain pluripotency (Jaenisch and Young, 2008). In the absence of factors supporting pluripotency, ES cells differentiate to specific lineages, recapitulating the developmental process governed by morphogenesis related signals (Keller, 2005).

1.1.2.1 Cytokine and growth factor signaling pathways regulating self-renewal

Multiple intracellular kinase signaling pathways play a dominant role in the regulation of ES cell fate (Liu et al., 2007; Jaenisch and Young, 2008). At least five pathways are important for self-renewal (Figure 2). LIF signals through Janus-associated tyrosine kinases (Jaks) and signal transducer and activator of transcription 3 (STAT3), which in turn promotes expression of renewal factors including the POU domain transcription factor Oct4 (Okamoto et al., 1990) and the homeobox transcription factor Nanog (Niwa et al., 1998; Burdon et al., 1999a). The Mek/Erk signaling pathway antagonizes ES cell self-renewal (Burdon et al., 2002). Bone morphogenetic proteins (BMPs), which are serum components, activate transcription factors of the SMAD family and inhibit differentiation through induction of inhibitor of differentiation (ID) factors (Ying et al., 2003). Wnt proteins, which are also found in serum, signal through inhibition of the kinase GSK3β, leading to β-Catenin accumulation and pluripotency marker gene expression (Sato et al., 2004; Wray et al., 2011). In addition, the phosphatidylinositol 3'-kinase (PI3K) signaling pathway promotes ES cell self-renewal partly via regulation of Nanog expression (Paling et al., 2004; Storm et al., 2007).

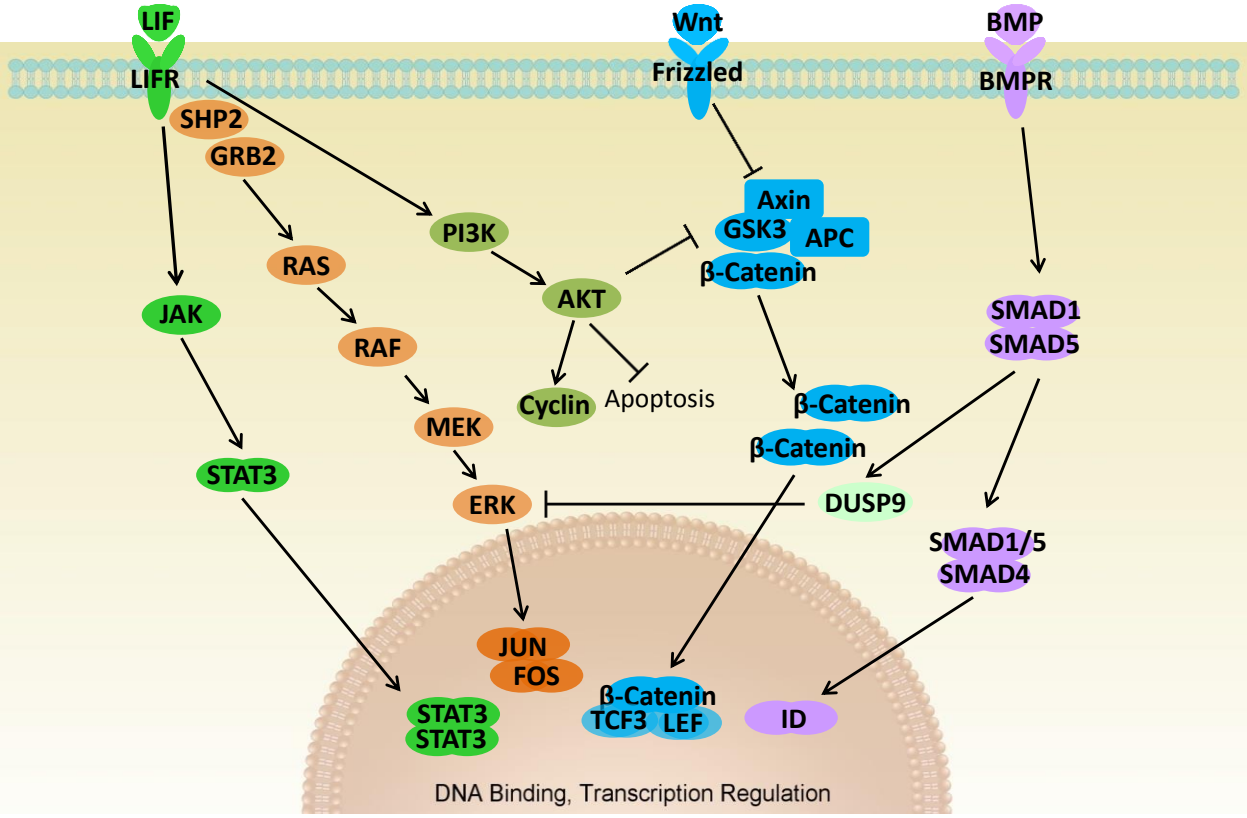

Figure 2. Key signaling pathways govern ES cell renewal: LIF-Jak-STAT3, Mek-Erk, BMP-SMAD-ID and Wnt-GSK-β-Catenin pathways. At least five pathways are important for self-renewal. LIF binds and activates the LIFR and GP130, which in turn activates three pathways. Firstly, GP130 signal through JAK to phosphorylate STAT3, which forms a dimer, translocates into the nucleus to regulate gene expression. GP130 also phosphorylates SHP2/Grb2, which in turn activates the Ras-Raf-Mek-Erk pathway, antagonizing ES cell self-renewal. A third pathway downstream of LIF involves the activation of PI3K-Akt pathway, which is important for cell-cycle progression, anti-apoptosis and self-renewal. BMP4, a component of serum, binds to its receptors, which form dimers and activate Smads. Smads then form a complex to regulate ID gene expression. Wnt binding to its receptor sequesters the destruction complex including GSK, APC and Axin, protecting β-Catenin from degradation. The accumulated β-Catenin then translocates into the nucleus and activates Wnt-regulated genes. Extensive crosstalk occurs between these signaling pathways. Shown in this figure include: Akt, part of PI3K pathway, inhibits GSK3 of the Wnt pathway; SMAD1/5, part of BMP pathway, inhibits Erk signaling through a phosphatase DUSP9 (see main text).

Mouse ES cells can be maintained and expanded in culture medium contain LIF and serum, and ES cells differentiate upon withdraw of LIF (Burdon et al., 2002). LIF induces the hetero-dimerization of LIF receptor and GP130 (Davis et al., 1993; Murakami et al., 1993), which in term activates JAKs (Stahl et al., 1994). JAKs phosphorylate tyrosine residues on GP130, which then engages and activates Src homology 2 (SH2) domain-containing signal transducer and activator of transcription 3 (STAT3). Upon activation, STAT3 forms a dimer, translocates into the nucleus and targets transcription activation (Niwa et al., 1998; Ihle, 2001). In addition, STAT3 activation is sufficient to prevent ES cell differentiation in the absence of LIF (Matsuda et al., 1999), while expression of an inhibitory form of STAT3 causes ES cell differentiation in the presence of LIF (Niwa et al., 1998), implicating this pathway in ES cell renewal.

LIF-LIFR-GP130 has another downstream signal, the Ras/mitogen-activated protein kinases (MAPK, or Erk) pathway, which is associated with ES cell differentiation. Activated GP130 recruits and phosphorylates the SH2 domain-containing protein-tyrosine phosphatase SHP2, which complexes with growth-factor-receptor-bound protein 2 (Grb2). SHP2 and Grb2 then activate the small GTPase Ras, which recruits the serine/threonine kinase Raf and subsequent activation of the MEK-Erk pathway downstream (Burdon et al., 2002). A chimeric receptor of GP130 without the SHP2 binding site failed to activate the Ras/MAPK pathway, and enhanced ES cell renewal by reducing the required LIF concentration. Chemical inhibition of this pathway by a MEK inhibitor had similar effect, indicating that the Ras/MAPK pathway antagonizes self-renewal (Burdon et al., 1999b). In addition, Erk2-null ES cells are refractory to differentiation, and FGF stimulation of the Erk pathway is required for ES cells differentiation

(Kunath et al., 2007; Stavridis et al., 2007). These studies defined the Ras/MAPK pathway as a differentiation signal.

BMPs are able to replace serum, in combination with LIF, to maintain moues ES cell self-renewal in culture (Ying et al., 2003). BMPs bind and activate transmembrane type I and type II receptors, which form a heterodimer, phosphorylate and activate receptor-regulated Smads (R-Smads: Smads 1, 5 and 8). Two of the activated R-Smads then form a trimeric complex with two-R-Smads and one Smad4, and translocate into the nucleus to regulate transcription (Massague and Chen, 2000; Massague et al., 2005; Feng and Derynck, 2005). In murine ES cells, the BMP-SMAD pathway induces expression of inhibitor of differentiation (ID) genes, which can inhibit neuronal differentiation (Ying et al., 2003). BMP4 was also shown to inhibit the Erk pathway (Qi et al., 2004). Recent research found that BMP4 attenuates Erk activity by the up-regulation of Erk-specific dual specificity phosphatase 9 (DUSP) via Smad1/5 and Smad4 (Li et al., 2012).

The phosphatidylinositol 3-kinase (PI3K) signaling pathway has been implicated in murine ES cell self-renewal (Takahashi et al., 2005). Activated by growth factor receptor kinases or by cell adhesion molecules, PI3K phosphorylates phosphatidylinositol-(4, 5)-biphosphate (PtdIns(4,5)P2) to generate second messenger PtdIns(3,4,5)P_3, which in turn activates Akt (also known as PKB), a serine/threonine kinase that phosphorylates substrates important for cell migration, proliferation and survival. PI3K/AKT signaling is directly counteracted by PTEN, a phosphatase and tumor suppressor, by dephosphorylating PtdIns (3,4,5)P_3 to PtdIns(4,5)P_2 (Cantley, 2002; Cully et al., 2006). PTEN-/- ES cells had an increased PtdIns(3,4,5)P3 level, elevated AKT activity, and an increased cell-growth rate (Sun et al., 1999). PI3K activity was associated with Cyclin D1 regulation and cell-cycle progression in ES

cells (Jirmanova et al., 2002). Chemical inhibition of PI3K or expression of a dominant-negative form of PI3K induced mES cell differentiation (Paling et al., 2004), while activation of PI3K signaling by constitutively active AKT maintained ES cell self-renewal without LIF (Watanabe et al., 2006). In addition, PI3K/AKT was shown to inhibit the MEK-ERK differentiation signal, and induce Nanog expression by inhibiting GSK-3 activity (Storm et al., 2007). More recently, PI3K was shown to regulate the transcription factor Tbx3, which in turn regulates Nanog expression in murine ES cells (Niwa et al., 2009).

Wnt-β-Catenin signaling is a key regulator of early development and embryonic stem cells (Clevers, 2006; Clevers and Nusse, 2012; Sokol, 2011). In the absence of Wnt, Axin serves as a scaffold, recruiting glycogen synthase kinase 3(GSK3), casein kinase 1 γ (CK1γ) and the adenomatosis polyposis coli (APC) protein to form a destruction complex, which phosphorylates β-Catenin to facilitate its ubiquitination by the E3 ligase β-TrCP and degradation by the proteasome. Wnt binds to a heterodimeric receptor composed of Frizzled and an LRP5/6 protein, to induce LRP5/6 phosphorylation, which in turn binds Axin and sequesters the destruction complex. β-Catenin is not degraded and accumulates in the cytoplasm, then translocates into the nucleus to regulate target gene expression with T-Cell Factors (TCF) transcription factors (Clevers and Nusse, 2012). In mES cells, Wnt signaling was implicated in maintenance of self-renewal and pluripotency: Loss of Wnt signaling is concomitant with ES cell differentiation (Anton et al., 2007), while null mutation of APC blocked differentiation and promoted self-renewal (Kielman et al., 2002). Genetic deletion or chemical inhibition of GSK3 promoted self-renewal in murine ES cells (Sato et al., 2004; Ying et al., 2008). Further, Wnt was able to prevent ES cell differentiation into epiblast stem cells and can facilitate ES cell renewal with LIF to replace other defined factors (ten et al., 2011). More recent reports show that β-Catenin

interacts with TCF3 to abrogate its repression of gene transcription (Wray et al., 2011) and by cooperate and enhance the transcriptional activation of Oct4 independent of TCF3 (Kelly et al., 2011).

To summarize, multiple signaling pathways regulate ES cell self-renewal and pluripotency. Of note, these signaling pathways form an intricate signaling network, and crosstalk with each other. For example, LIF activates JAK-Stat3, MEK-Erk, and PI3K pathways (Burdon et al., 1999a); both BMP-SMAD and PI3K pathways suppresses MEK-Erk pathway (Qi et al., 2004; Li et al., 2012; Storm et al., 2007); the PI3K pathway regulates Wnt signaling by inhibiting GSK3β (Storm et al., 2007); and Wnt stimulation upregulates STAT3 (Hao et al., 2006; Ogawa et al., 2006). Of note, ES cells was able to be maintained in chemically defined N2B27 medium with two inhibitors—the GSK3 inhibitor CHIR99021 and MEK inhibitor PD0325901 without LIF, BMP or serum (Ying et al., 2008; Silva et al., 2008). This indicates that ES cells can be maintained either by providing extrinsic signals (LIF, BMP/ Wnt) or through inhibition of innate, kinase-dependent differentiation mechanisms (Erk, GSK).

1.1.2.2 Transcription factor networks regulating pluripotency

The ES cell state is maintained by a unique transcription factor network, controlled by the core co-factors Oct4, Sox2 and Nanog (Niwa, 2007; Jaenisch and Young, 2008; Boyer et al., 2006; Ng and Surani, 2011).These core factors maintain stemness by regulating their own expression levels, activating and enhancing the expression of genes characteristic of ES cells, while repressing expression of genes related to differentiation and lineage commitment(Young, 2011)(Figure 3).

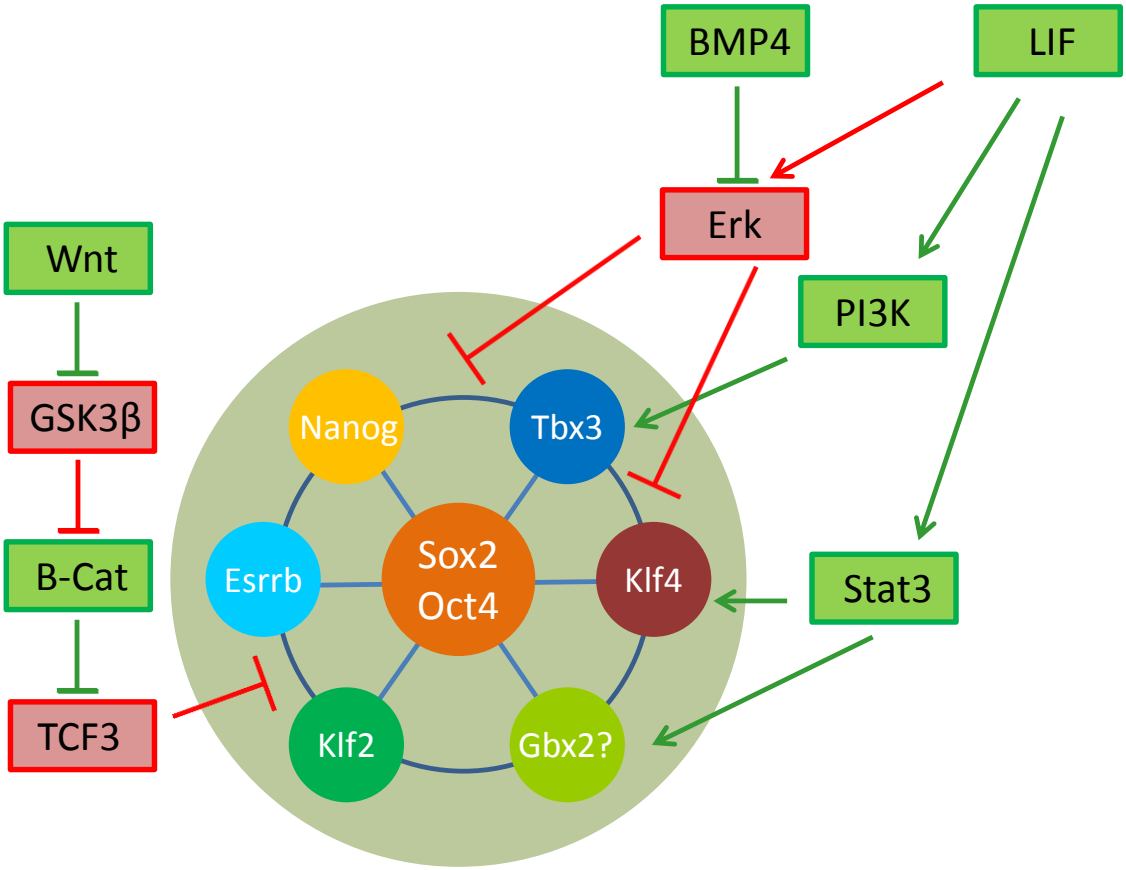

Figure 3. Core transcription factor network for pluripotency and interconnection with extrinsic stimuli. The core transcription factors are centered around Oct4 and Sox2, which are indispensable for self-renewal, surrounded by a circle of other validated facilitating factors including Nanog, Esrrb, Tbx3, Klf4, Klf2 and possibly Gbx2. The factors in the circle are individually dispensable, but collectively sustain self-renewal by cross-regulating each other, promoting self-renewal gene expression while suppressing differentiation-related genes. The rectangular boxes incorporate some key extrinsic signaling pathway components, with green boxes indicating renewal signals while red boxes indicating signals that antagonize self-renewal. Of note, Klf4 and Gbx2 are both activated by LIF-Stat3; Esrrb is preferentially regulated by Wnt /Gsk/Tcf3 signaling; and Tbx3 is upregulated by PI3K-Akt signaling. In contrast, Erk signaling has a negative effect on Nanog, Tbx3 and Klf4. Figure adapted from (Nichols and Smith, 2012), see main text for other references.

Oct4 (Oct3/4, encoded by *Pou5f1*) is a POU domain transcription factor expressed in the ICM and epiblast cells of early mouse embryos, and in pluripotent stem cells (Scholer et al.,

1990; Nichols et al., 1998). Oct4 is highly expressed in ES cells, and its expression quickly diminishes when ES cells differentiate. Oct4-deficient embryos can develop to the blastocyst stage, but the ICM cells are restricted to a trophectoderm fate (Nichols et al., 1998). In ES cells, Oct4 levels are tightly controlled: Acute repression of Oct4 induces trophectoderm differentiation; while overexpression causes ES cell differentiation to primitive endoderm and mesoderm (Niwa et al., 2000).

Sox2 (SRY-related HMG box 2) is an HMG-family protein that co-occupies many gene targets with Oct4. Sox2 also marks the pluripotent cells of the early embryo, although it is also expressed in early neuronal lineages. Expression of Sox2 diminishes when ES cells differentiate. Sox2-null embryos die shortly after implantation with no egg cylinder structure and failed epiblasts (Avilion et al., 2003). Conditional Sox2 knockouts show that Sox2-null ES cells differentiate to trophectoderm-like cells. However, those cells are rescued by enforced expression of Oct4 (Masui et al., 2007). These results indicate that Sox2 stabilizes ES cells in a pluripotent state by maintaining the requisite level of Oct4 expression.

Nanog, named by Austin Smith for the Celtic land of the ever-young, is a homeobox-containing transcription factor that is essential for stemness(Chambers et al., 2003; Mitsui et al., 2003). Nanog is highly expressed in the early embryo and pluripotent cells, and is down-regulated when cells differentiate. Deletion of Nanog causes early embryonic lethality, with failed epiblasts containing only primitive endoderm (Mitsui et al., 2003). Overexpression of Nanog drives ES cell self-renewal independently of LIF and Stat3 activation (Chambers et al., 2003). Nanog functions by promoting expression of pluripotency markers such as Oct4, Sox2, and Rex1; and by repressing expression of primitive ectoderm markers Gata4 and Gata6.

Accumulating evidence suggests that Oct4, Sox2 and Nanog form the core regulatory circuitry to control the ES cell pluripotent state (Boyer et al., 2005; Loh et al., 2006). These three factors bind each other at their own promoters, to positively regulate their own transcription, forming a positive-feedback autoregulatory loop. In addition, they often co-occupy their target genes, activating genes important for the ES cell state and pluripotency, while repressing genes related to lineage-specific differentiation. Oct4, Sox2 and Nanog can recruit multiple coactivators such as c-Myc, Stat3, Tbx3 and Klf4 to open up chromatin and activate gene expression. They can also repress linage-specific genes by recruiting chromatin regulators such as the histone methyltransferase SetDB1 and Polycomb group proteins, and by activating expression of repressive miRNAs (Jaenisch and Young, 2008; Young, 2011).

Rex1 (Zfp42) is a zinc-finger protein that is primarily expressed in the preimplantation embryo and ES cells, and downregulated when ES cell differentiate (Hosler et al., 1989; Rogers et al., 1991). Widely used as a marker for ES cells, Rex1 is regulated by Oct4 (Ben-Shushan et al., 1998). Rex1-/- ES cells had a greater susceptibility to retinoic acid-induced differentiation, indicating that Rex1 inhibits ES cell differentiation. However, ES cell derivation and normal embryonic development were unaffected by Rex1 knockout, demonstrating that Rex1 is dispensable for ES cell pluripotency (Scotland et al., 2009; Masui et al., 2008). It was therefore proposed that Rex1 is just a marker of pluripotency but its function is dispensable (Masui et al., 2008).

Developmental pluripotency-associated gene 4 (Dppa 4), a gene encoding a putative DNA-binding SAP domain protein, is exclusively expressed in mouse preimplantation embryos and pluripotent stem cells (Maldonado-Saldivia et al., 2007). Dppa4 overexpression does not support ES cell self-renewal, but partially inhibits differentiation. Knockdown of Dppa4 with

shRNA induced ES cell differentiation to primitive ectoderm. Further, Dppa4 was shown to localize to active chromatin to inhibit ES cell differentiation (Masaki et al., 2007). In addition, Dppa4 is a target gene of Oct4 and Sox2 (Chakravarthy et al., 2008). However, a gene knockout study showed that Dppa4 is dispensable for ES cell identity and germ cell development, but is essential for embryogenesis (Madan et al., 2009).

Estrogen-related receptor b (Esrrb) is an orphan nuclear receptor that is part of the pluripotency gene regulatory network (Loh et al., 2006; Ivanova et al., 2006). This protein is highly expressed in ES cells and the early embryo, and its downregulation causes ES cell differentiation. Recent research has shown that Esrrb is a pivotal target of Wnt/Gsk3/Tcf3 signaling, and Esrrb is downstream of and able to replace Gsk3 inhibition, in parallel to LIF-Stat3 signaling (Martello et al., 2012). Esrrb has also been shown to be a direct Nanog target gene, and is required for LIF-independent self-renewal following Nanog overexpression (Festuccia et al., 2012). Moreover, Essrb function requires its co-activator, Ncoa3, which bridges Esrrb to RNA polymerase II complexes and cooperates its gene regulatory effects with the Oct4-Sox2-Nanog core transcription factor circuitry (Percharde et al., 2012).

Tbx3 is a T-box transcription factor, which is important for pluripotency as RNAi down-regulation of this factor causes ES cell differentiation (Ivanova et al., 2006). Tbx3 was shown to be preferentially up-regulated by the PI3K-Akt pathway, down-regulated by the MAPK pathway and can stimulate Nanog expression. Overexpression of Tbx3 can maintain pluripotency independent of LIF (Niwa et al., 2009). In addition, Tbx3 can significantly improve the germline competency of induced pluripotent stem cells (discussed in section 1.1.3.5), by sharing downstream targets with Oct4, Sox2 and Nanog as well as other pluripotency-related genes and reprogramming factors (Han et al., 2010).

Klf4 is a member of the Kruppel-like family of conserved zinc-finger transcription factors, and one of the four 'Yamanaka factors' originally shown to reprogram fibroblasts to induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Klf4 is a direct transcriptional target of Stat3 and is activated by LIF-Jak-Stat3 signaling, with its overexpression sufficient to maintain pluripotency without LIF (Li et al., 2005; Niwa et al., 2009; Bourillot et al., 2009). Klf4 is negatively regulated by Erk phosphorylation, which promotes proteasome degradation of Klf4 (Kim et al., 2012). Klf4 was shown to directly regulate Nanog expression, and act upstream of this master pluripotency factor (Zhang et al., 2010). However, knockout of Klf4 does not have a phenotype. Through RNAi studies, Klf4 was found to be functionally redundant with its closely related factors Klf2 and Klf5, which also share many common targets with Nanog (Jiang et al., 2008). Unlike Klf4, which is regulated by LIF-Stat3 signaling, Klf2 is a direct target of Oct4, and can increase clonogenicity and maintain pluripotency independent of Stat3 (Hall et al., 2009). Thus, Klf2 and Klf4 are important transcription factors that transduce upstream signals to the core pluripotency circuitry.

Gbx2 is a homeobox gene that is implicated in pre-gastrulation development and mid/hindbrain development (Wassarman et al., 1997; Chapman et al., 1997). Gbx2 is highly expressed in the inner cell mass of the preimplantation embryo, and is down-regulated when ES cells differentiate (Chapman et al., 1997). Research has shown that Gbx2 is a direct target of LIF/Stat3 signaling, and when over-expressed, can sustain pluripotency independent of LIF. Gbx2 can also enhance reprograming efficiency, and alone can reprogram epiblast stem cells to ES cells (Tai and Ying, 2013). These findings, suggest Gbx2 is a marker of pluripotency and can integrate signaling cascades to the core pluripotency circuitry.

In summary, an intricate, self-regulated transcription factor network controls ES cell pluripotency (Figure 3). Oct4 and Sox2 sit in the center of this network and while irreplaceable, are dependent on other facilitating factors, including Nanog, Esrrb, Klf2, Klf4 and Tbx3. These facilitating factors are individually dispensable, but collectively sustain self-renew by relaying signals from upstream signaling cascades and by stabilizing the Oct4/Sox2-centered self-renewal signal (Nichols and Smith, 2012; Ivanova et al., 2006). Assessing the expression profile of this comprehensive set of transcription factors is a useful tool to study ES cell pluripotency and differentiation status. For example, EpiSCs express Oct4, Sox2 and Nanog, but not Klf2, Klf4, Rex1, and Gbx2; this can distinguish between naïve and primed pluripotency as described in more detail in the sections that follow.

1.1.2.3 Signaling and transcription factors in early development and ES cell differentiation

In vitro differentiation of ES cells includes EB formation, direct differentiation on ECM coated-plates, or co-culture with stromal cells. These in vitro differentiation protocols recapitulate some aspects of embryonic development, and the stage and lineage of development can be assessed by expression of specific lineage markers (Murry and Keller, 2008). Here I discuss the signaling and lineage markers for the early stages of mouse embryo development, most of which are conserved in humans as well.

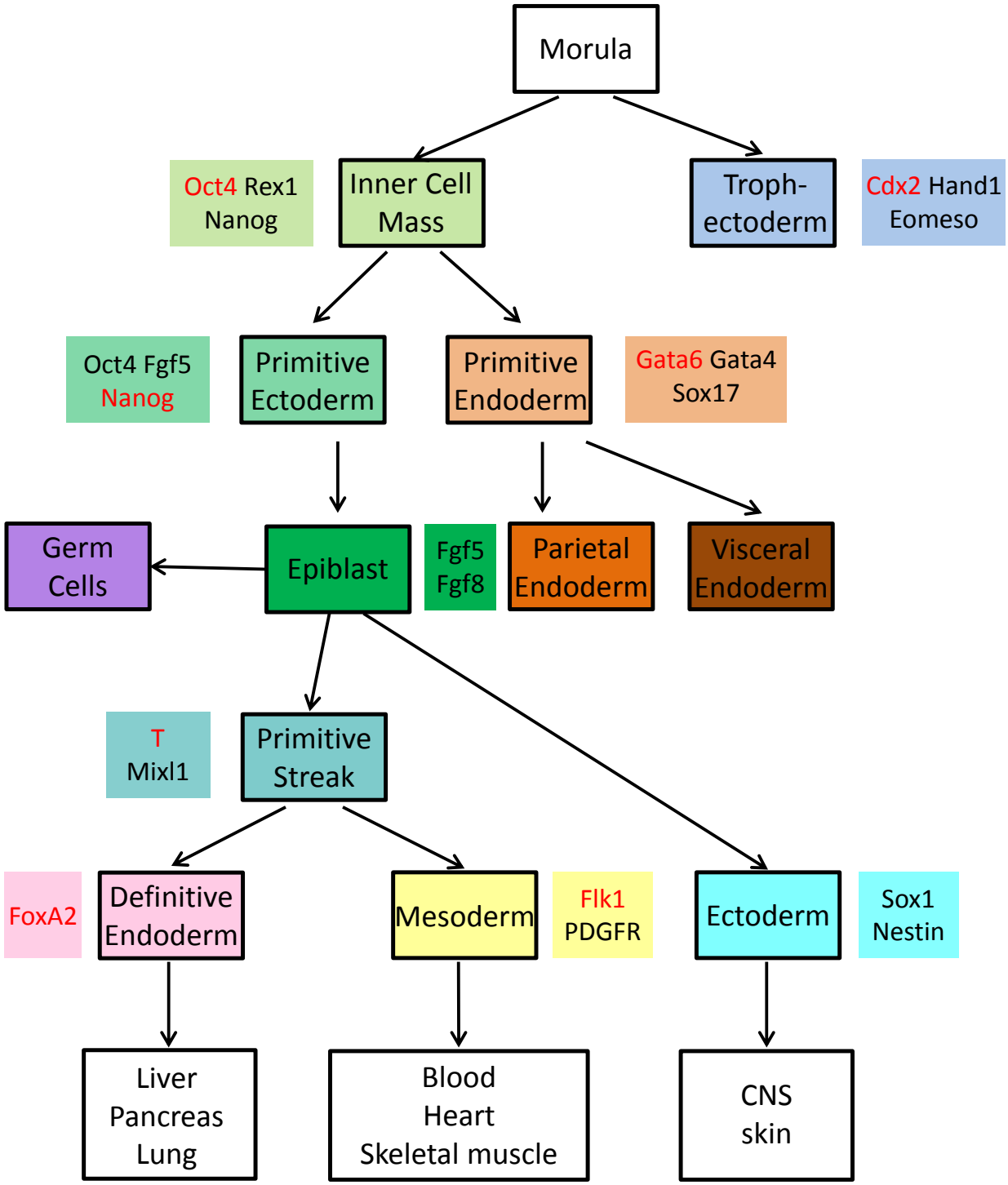

Figure 4. Embryo development lineages and ES cell differentiation markers. This figure depicts the scheme of early mouse development, featuring boxed specific developmental stages and lineages with their derivation relationship. The open box adjacent to each stage/lineage shows the identity of genes whose expression can be used to mark the specific lineage. Adapted from (Keller, 2005; Murry and Keller, 2008)

In the early blastocyst, two populations of cells are apparent: the outer polar trophectoderm cells, and the inner apolar ICM cells. The trophectoderm cells eventually give rise to trophoblast giant cells and the trophoblast layers of the placenta (Stephenson et al., 2012). In the outer cells, the transcription factor TEAD4 is activated, which upregulates the caudal-type homeobox transcription factor Cdx2 and T-box transcription factor Eomeso, driving cells towards the trophectoderm lineage. In the inner cells, the Hippo pathway is activated in response to cell-cell interaction, which turns TEAD4 off (Nishioka et al., 2009). Cells of the ICM express Oct4, which is essential for ICM identity. In the absence of Oct4, a blastocyst-like structure develops but ICM cells differentiate along the trophectoderm lineage (Nichols et al., 1998). Of note, trophoblast maintenance requires the inner cell mass, which secretes fibroblast growth factor 4 (Fgf4) to promote trophoblast proliferation (Nichols et al., 1998).

The early blastocyst then goes through a cavitation process, with trophectoderm cells transferring fluids into the blastocyst, forming the blastocoel cavity. The ICM then separates into two cell lineages—the epiblast (EPI, or primitive ectoderm) and primitive endoderm (PE). The epiblast will eventually form all germ layers of the embryo, while the primitive endoderm cells form the part of extraembryonic yolk sac (Stephenson et al., 2012). The segregation of EPI with PE depend on two transcription factors—Nanog and Gata6. These factors are initially co-expressed in early ICM cells, but gradually become mutually exclusive to determine these two lineages (Nichols and Smith, 2012). At E3.5 in developing embryo, individual ICM cells are heterogeneous, with cells expressing either Nanog or Gata6 in a mutually exclusive way. Lineage restriction starts when cells facing the blastcoel form the PE, and these Gata6-positive PE cells need a paracrine signal from Nanog-positive epiblast cells and the Grb2-Ras-MAPK

signaling pathway (Chazaud et al., 2006). While Fgf4 is expressed in epiblast cells, FGFR is expressed in the PE (Guo et al., 2010). PE formation is determined by sequential activation of the transcription factors Gata6, Sox17, Gata4 and Sox7 (Artus et al., 2011). The initial Gata6-positive PE cells are independent of Nanog-expressing cells, however, later expression of PE markers such as Sox17 and Gata4 depend on Fgf4 secreted from Nanog-positive cells (Frankenberg et al., 2011).

After segregation of the EPI and PE layers, the embryo is ready for implantation in the uterus. Upon implantation, the embryo forms the egg cylinder. During this process, the primitive endoderm further differentiates to the extraembryonic visceral endoderm and parietal endoderm. The epiblast cells transform from a cluster of compact cells to an epithelial layer, surrounded by the visceral endoderm (Stephenson et al., 2012). At this stage, the epiblast cells, or primitive ectoderm cells, express high levels of Fgf5 (which can serve as a marker for this lineage). EPL cells retain the ability to form EBs and teratomas. However, unlike ES cells, EPL cells express very low level of Rex1 and Gbx2, and cannot contribute to chimera formation (Pelton et al., 2002). Gastrulation follows, with a set of orchestrated morphogenetic movements, cell proliferation and differentiation to form the ectoderm, mesoderm and endoderm.

The formation of the primitive streak (PS) from epiblast cells marks the beginning of gastrulation, and then the epiblast cells undergo an epithelial to mesenchymal transition, egress through the PS, and develop into either mesoderm or definitive endoderm (Tam and Behringer, 1997). The gene Brachyury (T) (Kispert and Herrmann, 1994) and Mixl1 (Hart et al., 2002) are expressed throughout the PS and can serve as a marker for this transient structure. Foxa2 and Goosecoid (Gsc) are preferentially expressed in anterior PS regions, and HoxB1 and Evx1 in posterior regions (Murry and Keller, 2008). The formation of mesoderm and endoderm follow a

spatiotemporal development process. First, epiblast cells migrating through the posterior PS generate extraembryonic mesoderm that eventually develops to parts of the yolk sac. Subsequently, cells traversing more anterior parts of the PS give rise to mesoderm. Then, cells moving through the most anterior region of the PS form the definitive endoderm. Unlike mesoderm or endoderm, the ectoderm is formed from cells in the anterior epiblast that do not traverse the PS (Murry and Keller, 2008).

Germ layer specification is a complex process tightly controlled by signaling cascades including the Wnt, Nodal and BMP pathways (Murry and Keller, 2008). The induction of definitive endoderm is associated with high level of activin/Nodal signaling in the anterior region of the PS, and FoxA2 can be used as a marker along this lineage (Gadue et al., 2006). Mesoderm formation is characterized by the expression of Flk-1 and PDGFR (Kataoka et al., 1997). In contrast, cells of the ectoderm lineage develop from epiblast cells that do not traverse the PS. Interestingly, ectoderm is the "default" differentiation pathway when ES cells are cultured without serum or other signals that promote PS differentiation. Sox1 and Nestin can be used as markers for neuroectoderm. Differentiation along this lineage depends on FGF signaling, which is inhibited by BMPs (Ying and Smith, 2003; Ying et al., 2003).

In summary, embryonic development involves cell-cell interactions, spatiotemporal activation of signaling pathways, and expression of specific lineage-related transcription factors. ES cell differentiation also follows this development process. Based on the signaling pathways and transcription factors highlighted above, we can use sets of different lineage markers to track ES cell differentiation stages in vitro (Figure 4).

1.1.3 Human Embryonic Stem Cells

Because of species specific ES cell differences and non-optimal human embryo culture, initial attempts to derive human ES (hES) cells using mES cell culture conditions were unsuccessful. In fact, 17 years passed from the establishment of the first mES cell line to the derivation of the first hES cell lines (Yu and Thomson, 2008). Like mES cells, hES cells are pluripotent stem cells derived from inner cell mass of blastocyst stage human embryos produced by in vitro fertilization (Thomson et al., 1998). Although hES cells are of the same blastocyst origin as mES cells, the culture conditions, undifferentiated cell colony morphology, and extrinsic signaling pathways regulating their fate are very different. Nevertheless, they express the same core transcription factors to maintain pluripotency. The improvement in culture conditions for hES cell culture from feeder-based medium to chemically defined medium facilitated genetic manipulation of hES cells and signaling research. Recent establishment of epiblast stem cells argues that hES cells and mES cells may represent different states of pluripotent stem cells.

1.1.3.1 Human ES cell derivation and culture conditions

In the pioneering research of hES cell derivation by James Thomson's group, ICM cells were isolated by immunosurgery, and plated onto mitotically inactivated MEF cells in serum-containing medium for extended growth. Five hES cell lines were initially derived by this approach, with H1, H13 and H14 showing a normal XY karyotype; and H7 and H9 cell showing a normal XX karyotype. The hES cell lines had a high nucleus to cytoplasm ratio and high levels of telomerase activity; they expressed cell surfaces markers for pluripotency (alkaline phosphatase, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81); and when injected into severe combined

immunodeficient (SCID) mice, were able to form teratomas with cell lineages of all three germ layers (Thomson et al., 1998).

Subsequent work improved the culture conditions for hES cells. Knockout serum replacement and the addition of basic fibroblast growth factor (bFGF) was shown to support clonal derivation of hES cells from the original H9 cell line on feeder layers, although with very low efficiency (Amit et al., 2000). Feeder-free culture was then established by plating hES cells on Matrigel or laminin coated plates in medium conditioned by MEFs (Xu et al., 2001). At higher concentrations, bFGF allowed "truly" feeder-free culture of hES cells with unconditioned medium (Xu et al., 2005a; Levenstein et al., 2006). Noggin, an antagonist of BMP signaling, was shown to cooperate with bFGF to maintain pluripotency of hES cells (Xu et al., 2005b; Wang et al., 2005). Last but not the least, chemically defined culture medium was established for hES culture to facilitate clinical application (Lu et al., 2006; Yao et al., 2006; Ludwig et al., 2006).

One problem with hES cell culture is the poor survival of hES cells after cell dissociation, which hinders sub-cloning and gene-targeting research. Y-27632, a Rho-associated kinase (ROCK) inhibitor, was shown to protect dissociated hES cells from apoptosis, and facilitate sub-cloning (Watanabe et al., 2007). Further mechanistic studies elucidated the protective mechanism of ROCK inhibitor, where dissociated hES cells lose E-cadherin mediated cell-cell adhesion, which triggered ROCK-dependent hyper-activation of actin-myosin contraction and apoptosis (Chen et al., 2010; Ohgushi et al., 2010).

Since the initial derivation of hES cell lines, culture conditions have rapidly evolved from feeder and serum-based medium, to feeder-free, and finally chemically defined medium. The introduction of ROCK inhibitor facilitated clonal expansion and sub-cloning of hES cells. These

advances have greatly accelerated the pace of hES cell research and facilitated our understanding of the complex signaling networks that control their fate.

1.1.3.2 Human ES cell renewal and pluripotency, EB formation and teratoma formation

Like mES cells, hES cells have two important properties: self-renewal and pluripotency. Self-renew is the ability for prolonged proliferation without differentiation. This property can be assessed as extended passage with a normal karyotype, undifferentiated cell culture morphology, high activity of telomerase and alkaline phosphatase, expression of cell surface markers such as SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81, and expression of the pluripotency factors such as Oct4, Nanog, Sox2 and Rex1.

In hES cells, pluripotency is mainly assessed through embryoid body and teratoma formation. When cultured in suspension culture without feeder layers, hES cells spontaneously form aggregates called embryoid bodies as previously described for mES cells (Itskovitz-Eldor et al., 2000). EB formation is a convenient model for the study of human early embryonic development in vitro. EB formation can mimic, to some extent, the axis and polarity reorganization in development, and the temporal/sequential gene expression changes that occur during the stages of gastrulation and germ layer formation (Dvash et al., 2004). In addition, EB formation can serve as an initial step for subsequent directed differentiation into specific lineages with guidance from growth factors and small molecule inhibitors (Murry and Keller, 2008). The advantage of EB formation is that it provides a three-dimensional model that facilitates and mimics the complex cell to cell interactions during development. However, it is hard to precisely control this cell-cell microenvironment to synchronize EB formation. EBs are normally heterogeneous in size, which can complicate the interpretation of results (Zhu and Huangfu,

2013). More recently, the development of microwell culture to control of number of hES cells per cluster during embryoid body formation improved this limitation of the EB assay (Mohr et al., 2010).

Teratoma formation is a widely used assay for pluripotency in hES cells, and is considered the most stringent assay to demonstrate the differentiation potential of hESCs (Zhang et al., 2008). When transplanted into immunodeficient mice, hES cells give rise to teratomas with differentiated tumor tissues representing all three germ layers including neuronal tissues from ectoderm, muscle and blood lineages from mesoderm and gut epithelium and liver tissues from endoderm. The efficiency and quality of teratoma formation is determined by three factors: cell quality, cell number and injection site (Zhang et al., 2008). Teratoma formation is most efficient following subcutaneous or intramuscular implantation, with Matrigel enhancing the efficiency (Prokhorova et al., 2009; Hentze et al., 2009). Besides teratoma formation, hES cells also undergo random differentiation when cultured on gelatin-coated plates in serum-containing medium without bFGF. More importantly, defined differentiation conditions have been developed to drive hES cells toward specific lineages important for regenerative medicine, including retinal epithelium, pancreatic progenitor cells, β-cells, cardiomyocytes, and motor neurons [reviewed in (Zhu and Huangfu, 2013; Murry and Keller, 2008; Cohen and Melton, 2011)].

To summarize, self-renewal and pluripotency are the defining feature of hES cells. EB formation serves as a standard test for pluripotency and is a useful model for early embryogenesis, while teratoma formation serves as the gold-standard to test hES cell differentiation potential. Of note, the more stringent assays in mouse such as chimera formation and germline competency are not feasible in human for ethical reasons.

1.1.3.3 Growth factors and signaling pathways

The self-renewal of hES cells depends largely on the FGF and TGFβ/Nodal/Activin signaling pathways. This is very different from that of mES cells, which depend on LIF/Jak/Stat3 and BMP/SMAD/ID signaling. Below I discuss the regulation of FGF and TGFβ signaling in hES cells (Yu and Thomson, 2008).

FGF signaling is of pivotal importance for hES cell self-renewal. bFGF can maintain hES cell clonal growth on feeders, and can also support hES cell feeder-free growth at a high concentration. FGF has multiple signaling roles to maintain hES cell pluripotency. First, FGF signals through the FGF receptor tyrosine kinase and downstream Erk signaling to inhibit differentiation into extraembryonic lineages (Li et al., 2007; Dvorak et al., 2005). FGF-2 was also shown to activate both MEK/Erk and PI3K-Akt signaling pathways, which synergistically stimulate self-renewal, cell survival and adhesion (Eiselleova et al., 2009). Second, FGF can modulate TGFβ signaling, upregulating the expression of TGFβ ligands in both feeder cells and hES cells (Greber et al., 2007). In feeder-free culture, some hES cells self-differentiate into fibroblast cell-like supporting cells, creating their own "niche", which responds to FGF in similar manner as feeder cells (Bendall et al., 2007). Third, FGF signaling can inhibit neuronal induction in hES cells. FGF/Erk inhibition induced neuroectoderm differentiation in hES cells marked by Pax6 expression (Greber et al., 2011). Last but not the least, Fgf2 can sustain Nanog expression through the MEK-ERK pathway, which can switch the cell fate in the context of BMP4-induced differentiation (Yu et al., 2011).

TGFβ/Nodal/Activin is essential for hES cell self-renewal in culture (James et al., 2005; Vallier et al., 2005). In undifferentiated hES cells, the TGFβ/Nodal/Activin branch is activated

(with downstream signals transduced through SMAD2/3), while the BMP/GDF branch (SMAD1/5) is largely suppressed (James et al., 2005). Nodal was shown to inhibit hES cell differentiation to neuroectoderm (Vallier et al., 2004), and inhibition of Activin/Nodal signaling caused hES cell differentiation even in the presence of FGF (Vallier et al., 2005). In contrast, BMP caused hESCs to differentiate to trophectoderm in conditioned medium with bFGF (Xu et al., 2002). Subsequent investigation using defined medium found that both TGFβ-responsive SMADs (SMAD2/3) and BMP-SMADs (SMAD1/5) bind to the Nanog promoter, with SMAD2/3 being active in undifferentiated hESCs maintained by TGFβ and FGF. In the absence of growth factors, BMP-SMADs bind to the Nanog promoter and facilitate differentiation (Xu et al., 2008).

The FGF and TGFβ/Activin signaling pathways work synergistically to main hES cell pluripotency. FGF2 induces expression of key TGFβ pathway components including TGFβ1 and GREM1 while inhibiting BMP4 expression in both feeder cells and hES cells (Greber et al., 2007). Reciprocally, Activin A induces expression of bFGF to promote self-renewal and inhibit the BMP signal (Xiao et al., 2006). Thus, TGFβ/Nodal/Activin, in cooperation with bFGF maintains self-renewal, inhibits the BMP differentiation signal, and promotes proliferation and survival.

In addition, both Wnt and IGF (insulin-like growth factor) signaling have been implicated in the maintenance of hES cell pluripotency. BIO, a potent pharmacological inhibitor of GSK-3, was shown to support self-renewal of hES cells (Sato et al., 2004). IGF1 is secreted from "feeder-like" cells which spontaneously differentiate from hES cells to support "true" hES cells (Bendall et al., 2007). However, the role of Wnt signals in self-renewal was challenged when the Wnt/β-catenin signal was found to be insufficient to maintain pluripotency of hES cells, and

associated with differentiation instead (Dravid et al., 2005). IGF1 was also insufficient to support hES renewal in the absence of bFGF in chemically defined medium (Wang et al., 2009). These studies point to the heterogeneity of hES cell culture. Further research is needed to elucidate the roles of Wnt, IGF and other growth factor signaling pathways in hES cells.

1.1.3.4 Core transcription factor regulatory network

In mES cells, Oct4, Sox2 and Nanog form a core regulatory circuitry maintaining self-renewal and pluripotency (Jaenisch and Young, 2008). These three master transcription factors co-bind and regulate a large group of target genes, maintaining the expression of pluripotency genes while suppressing differentiation-related gene expression. Human ES cells express all three master regulators, suggesting that a similar core transcription factor network exists in hES cells (Boyer et al., 2005).

A genome-scale location analysis indicated that Oct4, Sox2 and Nanog co-occupy a large number of their targeted genes in hES cells (Boyer et al., 2005). Around 50% of Oct4 targeted promoters were co-bound by Sox2, while 90% of the genes co-bound by Oct4 and Sox2 were also targeted by Nanog, suggesting that these three factors function together in gene regulation. The genes targeted by all three core factors include both active and inactive genes. The active targeted genes include Oct4, Sox2 and Nanog themselves, other pluripotency-related transcription factors such as STAT3 and Dppa4, as well as components of the TGFβ and Wnt signaling pathways. The inactive genes include many transcription factors that are important in development and lineage specification, including Pax6 and NeuroD for ectoderm differentiation, Hand1 for mesoderm differentiation. This study concluded that Oct4, Sox2 and Nanog form an

auto-regulated, feed-forward circuitry to maintain self-renewal and pluripotency in hES cells, like in mES cells.

However, a recent study with shRNA knockdown and targeted overexpression revealed that in hES cells, Nanog, Oct4 and Sox2 have general and cell-line specific functions (Wang et al., 2012). Oct4 expression regulates and cooperates with BMP4 to specify hESC cell fate. High levels of Oct4 sustain self-renewal in the absence of BMP4 but induce mesendoderm differentiation in the presence of BMP. While low levels of Oct4 induce endoderm formation in the absence of BMP4, low levels of Oct4 specify extraembryonic differentiation in the presence of BMP4. Sox2 and Sox3 function redundantly in inhibiting mesendoderm differentiation, while Nanog mainly represses ectoderm differentiation. This study suggested a modular regulatory network for three master regulators in hES cells, in contrast to the co-regulatory circuitry in mES cells.

In summary, hES cells express the core transcription factors Oct4, Nanog, and Sox2, which regulate target gene expressions to promote self-renewal and pluripotency related genes while repressing differentiation related genes. Recent research indicates that instead of working as a trio like in mES cells, these three factors act as a modular regulatory network in hES cells, with each factors inhibiting a specific differentiation lineage.

1.1.3.5 New different states of pluripotent stem cells: iPS cells, mEpiSCs and naive human ES cells

While both are derived from ICM of the blastocyst stage embryo, mES cells and hES cells have different culture conditions and colony morphologies. Mouse ES cells depend on LIF-Jak-Stat3 signaling and BMP-SMAD-ID signaling for self-renewal. In contrast, hES cells depend on bFGF

and TGFβ/Activin signaling to remain in the undifferentiated state. LIF does not support self-renewal of hES cells, while BMP4 induces differentiation. While mES cells grow as domed colonies and can be passaged with single cell dissociation, hES cells grow as flattened colonies and can only be passaged in small cell clumps, with single cell dissociation causing hES cell death. Moreover, mES cells and hES cells express different cell-surface markers. For example, SSEA-1, a cell surface marker of pluripotency in mES cells, is a marker of differentiation in hES cells. Instead, hES cells express cell-surface SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, which are absent in mES cells. These and other properties of hES cells, when compared with mES cells, indicates that there are species-specific differences and that they represent different pluripotent states.

In 2006, the Yamanaka laboratory reported a revolutionary discovery that pluripotent cells, with similar properties to ES cells, can be generated from mouse fibroblasts by retrovirally introducing four genes—Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006). These cells, term induced pluripotent stem (iPS) cells, were tested extensively for pluripotency and passed the most stringent standard of tetraploid complementation (Stadtfeld and Hochedlinger, 2010). Human iPS (hiPS) cells were also generated with the same four factors (Takahashi et al., 2007) and later with different combinations (Yu et al., 2007). The hiPS cells are shown to be similar to established hES cells in terms of self-renewal and differentiation potential (Yamanaka, 2012).

The derivation of epiblast stem cells (EpiSCs) from postimplantation mouse embryos provides the evidence that hES cell-like stem cells can be derived from mouse embryos (Brons et al., 2007; Tesar et al., 2007). Mouse ES Cells and mEpiSCs represent two different states of pluripotency, termed naïve and primed states, respectively (Nichols and Smith, 2009)(Table 2).

Naïve pluripotent stem cells, exemplified by mES Cells, are responsive to LIF/Stat3 and BMP signaling, can be passaged with single cell dissociation and show clonal growth, have two active X chromosomes in female lines, and readily form chimeric mice with germline competence when injected into developing blastocysts. In contrast, primed pluripotent cells, such as EpiSCs, depend on bFGF and TGFβ/Activin signaling for maintenance, can only be passaged as clumps, have one X-chromosome inactivated in female lines, and rarely form chimeras when injected into host blastocysts but can form teratomas when injected into immunocompromised mice. Human ES cells share all those features with mouse EpiSCs that are distinct from mES cells. However, hES Cells are not identical to mEpiSCs, as hES Cells express the ICM marker Rex1 and hES Cell-specific transcription factors such as PRDM14 (Chia et al., 2010), which are absent in mEpiSCs.

Table 2. Comparison of naïve and primed pluripotent cell states

Property	Ground State	Primed State
Cultured cells	rodent ES Cells	rodent EpiSCs; hES Cells
Equivalent embryonic tissue	early epiblast	egg cylinder or embryonic disc
Response to Lif/Stat3	self-renewal	none
Response to Fgf/Erk	differentiation	self-renew
Response to 2i	self-renewal	differentiation/death
Response to BMP4	supported renewal	differentiation
pluripotency factors	Oct4, Nanog, Sox2, Klf2, Klf4	Oct4, Nanog, Sox2
colonly morphology	tight, domed colony	spread out, flattened
Clonogenicity	high	low
XX status in female lines	XaXa	XaXi
Blastocyst chimeras*	yes	no
Teratomas	yes	yes
Differentiation bias	none	variable

* Not applicable to human Adapted from (Nichols and Smith, 2009)

With the inhibition of GSK3 and Erk signaling pathways, and exogenous expression of reprogramming factors Klf4 or c-Myc, naïve ES cells were derived from previously refractory non-obese diabetic(NOD) mouse strain and from rat (Hanna et al., 2009; Buehr et al., 2008). These discoveries led to the derivation of mESC-like naïve hES cells (Hanna et al., 2010; Buecker et al., 2010). Naïve hES cells were achieved by growing hES cells or iPSCs with ectopic expression of Oct4, Klf4 and Klf2 in 2i medium with LIF, or by growing five-factor iPS cells under the same conditions. However, those naïve hES cells are very unstable, and require extended expression of reprogramming factors. These findings suggest that the naïve state of hES cells is intrinsically unstable, making hES cells least permissive to naïve state (Hanna et al., 2010).

To summarize, the derivation of mEpiSC lines and establishment of naïve hES cell lines, compared with traditional mESCs and hESCs, suggest that there are two states of pluripotency in both mouse and human. However, human pluripotent cells are refractory to culture in the naïve state, which may due to the species-specific genetic determinants.

1.2 SRC FAMILY NON-RECEPTOR TYROSINE KINASES

A major focus of my thesis research centered on the role of non-receptor protein-tyrosine kinase signaling pathways in the control of ES cell renewal and differentiation. Before reviewing previous work related to this theme, I will first provide a brief overview of the structure and function of the Src kinases, which constitute the largest family of non-receptor tyrosine kinases in the human kinome. The differential control of ES cell fate by individual members of the Src kinase family is at the center of my dissertation project.

The Src family of non-receptor tyrosine kinases was first discovered in the context of Rous Sarcoma Virus in the form of v-Src, which is an oncogene with the ability to transform cells in culture and rapidly induce large sarcomas in chickens (Martin, 2004). This viral oncogene was later found to be derived from a cellular gene, c-Src, which led to the oncogene hypothesis of cancer (Stehelin et al., 1976; Brugge and Erikson, 1977). There are 11 members of the Src-family kinase complement in the human genome (Manning et al., 2002), eight of which have been studied extensively in mammalian cells: Blk, Fgr, Fyn, Lck, Lyn, Hck, Src and Yes (Engen et al., 2008). In the mouse, Src, Fyn and Yes are ubiquitously expressed, while Lck, Lyn, Hck, Blk, and Fgr display more restricted expression to hematopoietic cells (Lowell and Soriano, 1996; Summy and Gallick, 2003a; Summy and Gallick, 2003b). Src family kinases are important in diverse signal transduction networks that govern cell proliferation, survival, motility and invasion (Engen et al., 2008; Parsons and Parsons, 1997). They serve as critical signal integrators or nodes, as their activity is regulated by a multitude of inputs upstream (growth factors, cytokines, hormones, cell attachment, antigens, to name a few) and they are wired to diverse signaling pathways downstream, including many that are linked to the regulation of ES cell growth and differentiation as described in the previous section.

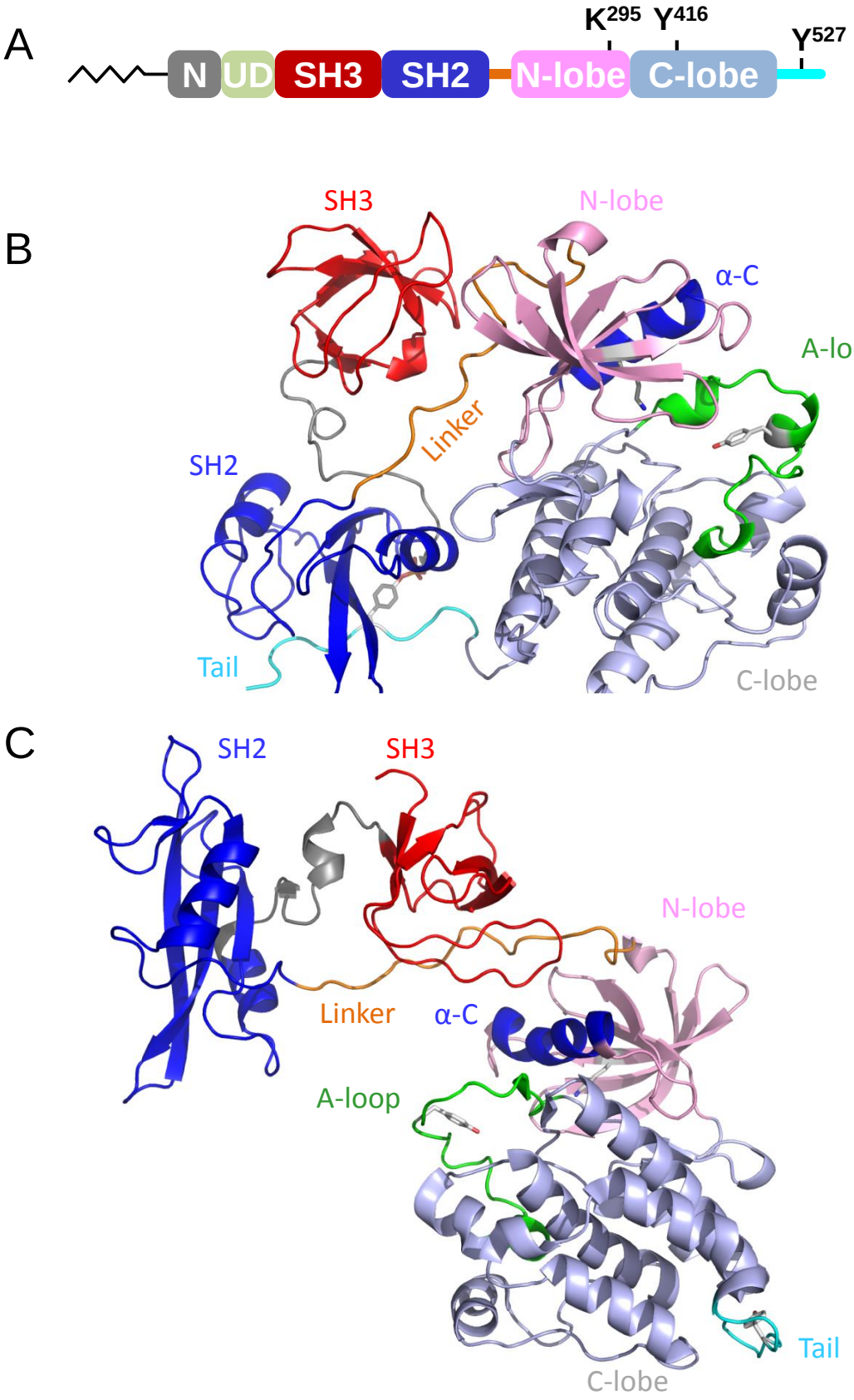
A
K295
Y416
Y527
N
UD
SH3
SH2
N-lobe
C-lobe
B
SH3
N-lobe
α-C
A-loop
Linker
SH2
Tail
C-lobe
C
SH2
SH3
N-lobe
Linker
α-C
A-loop
Tail
C-lobe

Figure 5. Src family tyrosine kinase domain organization and structure. This figure shows Src-family kinase (SFK) domain organization and structure. A.) Domain organization and key regulatory sites for SFKs include: an N-terminal myristoylation site (grey), a unique domain (light green), the SH3 domain (red), the SH3-SH2 connector (grey), the SH2 domain (blue), the SH2-kinase linker (orange); and the kinase domain including a smaller N-terminal lobe (pink) and a larger C-terminal lobe (light blue). The N-lobe has a critical lysine (Lys-295) which is essential for maintenance of the active conformation, while the C-lobe includes a conserved Tyr-416 in the activation loop. Phosphorylation of this tyrosine opens the kinase domain into the active state. The C-tail follows the kinase domain, with a critical conserved tyrosine residue at position 527. Phosphorylation of this tyrosine by Csk causes internal binding to SH2 and contributes to kinase downregulation. B.) X-ray crystal structure of Src, in the inactive conformation (Xu et al., 1999). Note the SH2 domain is engaged with phosphor-Tyr-527, while the SH3 domain is associated with SH2-kinase linker. These two interactions stabilize the kinase in the down-regulated conformation. The αC helix (blue) and the activation loop (green) are highlighted. (C) X-ray crystal structure of 'active' c-Src (Cowan-Jacob et al., 2005), which is not phosphorylated. Note that the SH2 domain is disengaged from the tail, while the SH3-linker interaction is retained. The kinase domain adopts an active conformation, with the αC helix turning toward the catalytic cleft and the activation loop adopting an open position to allow substrate binding.

1.2.1 Src family kinase structure

All Src-family kinases (SFKs) share the same arrangement of structural domains: a short N-terminal sequence for lipid modification, a unique domain characteristic of each individual kinase, Src homology 3 (SH3) and SH2 domains, a regulatory SH2-kinase linker, the tyrosine kinase (catalytic) domain and a C-terminal regulatory domain. (Figure 5A) The myristoylation of the first glycine in N-terminal sequence is required for membrane localization of SFKs and is essential for function (Lowell and Soriano, 1996). The unique domain is the only poorly conserved region within the kinase family, and may confer unique signaling properties on

individual members (more below). The SH3 domain binds to proline-rich sequences and contributes to substrate recruitment and also is essential for kinase activity regulation. The SH2 domain binds to phosphotyrosine-containing peptide sequences, functioning both in protein-protein interaction and kinase regulation. The kinase domain (also called the SH1 domain), with a bi-lobed structure comprised of N-terminal and C-terminal lobes, is responsible for phosphotransfer activity. X-ray crystallography has revealed that two intramolecular interactions are important for locking the kinase in inactive conformation: the SH3 domain interacts with the SH2-kinase linker, and the SH2 domain binds to phosphorylated tyrosine in the C-terminal regulatory tail. Disruption of either of these interactions is sufficient to activate the kinase (Tatosyan and Mizenina, 2000; Boggon and Eck, 2004; Ingley, 2008; Engen et al., 2008).

1.2.1.1 N-terminal region

The N-terminal domain (also called the SH4 domain) is a region with 15-17 amino acids which contains lipid-modification signals and is responsible for membrane anchorage of SFKs (Resh, 1994; Resh, 1999). In all SFKs, the glycine residue right after the initiator methionine is myristoylated cotranslationally and this modification is required for membrane binding. When v-Src Gly2 is mutated to Ala, membrane binding and cellular transformation are both abrogated. In the c-Src N-terminal domain, a module with three alternative lysine residues facilitates membrane binding in conjunction with myristate, and is also required for kinase function (Silverman et al., 1993). Most of the other SFKs (except c-Src and Blk) have a cysteine residue that can be reversibly palmitoylated. Only myristoylated proteins can be palmitoylated, which in turn can guide the kinase to membrane rafts. In response to different stimuli, reversible palmitoylation can change SFK membrane localization and signaling events (Resh, 1999). Thus,

the N-terminal domain modulates SFK membrane anchorage which is often essential for biological activity.

1.2.1.2 Unique domain

The unique domain, around 45-75 residues in length, follows the N-terminal domain and is the only non-conserved region among SFK members. The divergent unique domains are refractory to structural analysis and are absent from existing structures of SFKs. Although not required for intramolecular regulation in SFKs, the unique domain is thought to be involved in specific interactions with binding partners. It can also be phosphorylated at specific residues for kinase regulation. A well-studied example is the association of Lck with T-cell receptors CD4 and CD8α, which are essential for T-cell maturation (Rudd et al., 1988; Veillette et al., 1988). Structural analysis showed that two conserved cysteine residues in the Lck unique domain bind to two cysteine residues from CD4 or CD8α receptors by coordinating a Zn^{2+} ion (Kim et al., 2003). In addition, recent research showed that Src can be phosphorylated on Ser/Thr residues in the unique domain. These phosphorylation events induce a global electrostatic perturbation that releases c-Src from membrane, and changes subsequent signaling events (Perez et al., 2009). Hck can also be phosphorylated at a tyrosine in the unique domain, which contributes to its activation (Johnson et al., 2000). Moreover, in a study of kinase chimeras, replacing the v-Src N-terminal and unique domain with its c-Yes counterparts abrogated the ability of v-Src to transform chicken fibroblasts, indicating that the unique domain may confer signaling specificity between c-Src and c-Yes (Summy et al., 2003a). To summarize, the unique domain is the only specific region for each SFK protein. It can modulate specific molecular interactions, is regulated by phosphorylation, and may confer specificity among individual SFKs.

1.2.1.3 SH3 domain

The SH3 domain, about 60 amino acids in size, is necessary for SFK kinase regulation and interaction with adaptor proteins or substrates. Structural analysis revealed that the Src SH3 domain, like all SH3 domains, is a β-barrel comprised of five antiparallel β-strands and two prominent loops termed the RT-loop and n-Src loop (Musacchio et al., 1992). SH3 domains bind to target protein sequences that are rich in proline and other hydrophobic residues, especially proteins with the classic "PxxP" sequence. These sequences usually form a polyproline type II (PPII) helix which complexes with hydrophobic grooves on the surface of the SH3 domain (Musacchio et al., 1994). In SFK SH3 domains, PxxP-containing peptides can be docked in two opposite directions. Additional specificity and binding affinity are conferred by lysine or arginine residues N- or C- terminal to the PxxP motif (Feng et al., 1994). Class I ligands have an RxxPxxP motif and bind in an orientation opposite to that of Class II ligands which have an XpxxPxR motif (Lim et al., 1994). SH3 domains mediate both common and also specific binding of target protein to SFKs. For example, the Src, Fyn and Lyn SH3 domains have some common targets such as Shc, p62 and hnRNPK, but also have specific targets. In addition, extensive structural studies of many SH3 domains have revealed some atypical SH3 domain binding motifs. These non-consensus SH3 binding motifs do not always form a PPII helix, but utilize a negatively charged specificity pocket for binding (Saksela and Permi, 2012). An extreme case is the interactions between Fyn and the immune cell adaptor SAP, where a surface to surface interaction between Fyn SH3 domain and SAP SH2 domain exists, distinct from canonical PxxP-modulated biding (Latour et al., 2003; Chan et al., 2003).

Besides these important roles in substrate recruitment, SH3 domains are also critical for regulation of Src-family kinase activity. Structures of the inactive forms of c-Src and Hck

showed that the SH3 domain binds intramolecularly to a PPII helix formed by the SH2-kinase linker (Xu et al., 1997; Sicheri et al., 1997; Xu et al., 1999; Sicheri et al., 1997). This interaction pushes the SH3 domain to the back of the N-lobe of the kinase domain and stabilizes the kinase in the inactive conformation (shown in Figure 5).

1.2.1.4 SH2 domain

The SH2 domain, around 100 residues in length, is another non-catalytic domain important for SFK substrate recruitment, localization and kinase regulation (Pawson, 2004). Structurally, SH2 domains contain a central antiparallel β-sheet flanked by two alpha-helices. These secondary elements form two peptide binding pockets: One accommodates the phosphotyrosine (pTyr) with a conserved arginine residue in the base of the pocket; the other recognizes residues C-terminal to the pTyr, providing a specificity determinant (Waksman et al., 1993; Songyang et al., 1993). Phosphopeptide library screening showed that SFK SH2 domains preferentially bind to a pYEEI motif, with the pTyr and isoleucine residues occupying the two pockets, respectively (Songyang et al., 1993). While the pTyr-binding pockets of SH2 domains are highly conserved and almost invariant, the C-terminal recognition site is more divergent and can confer specificity among different SH2 domains and corresponding pTyr-containing binding sequences (Pawson and Nash, 2000). By interacting with pTyr-containing peptide sequences, SH2 domains relay protein tyrosine kinase signaling to a network of intracellular pathways that regulate many cellular functions. More recently, a fluorescence polarization-based screen of 50 SH2 domains and 192 physiological pTyr peptides demonstrated that the specificity is based on SH2 domain recognition of permissive amino acid residues that enhance binding and non-permissive residues that prohibit binding near the essential pTyr. Thus, subtle differences in the context of peptide ligands can be distinguished by different SH2 domains (Liu et al., 2010).

In addition to substrate binding and modulating signaling partners, the SH2 domain is also essential for stabilizing SFKs in the inactive conformation. In the downregulated structure of c-Src, the SH2 domain binds to the kinase tail with a highly conserved tyrosine residue. This intramolecular interaction locks the kinase in an inactive conformation, with the help from the SH3-linker interaction (Xu et al., 1997; Sicheri et al., 1997; Xu et al., 1999) (Figure 5).

1.2.1.5 Tyrosine kinase domain

The tyrosine kinase domain, comprised of an N-terminal lobe (N-lobe) and a C-terminal lobe (C-lobe), is the most conserved region among all SFK members and is structurally conserved in all protein tyrosine kinases. The smaller N-lobe includes five β-strands and a single α-helix (α-C-helix). The larger C-lobe is mainly composed of α-helices with only a few β strands. A short, flexible hinge connects the N-lobe with C-lobe, and catalytic activity occurs in the cleft between the two lobes (Boggon and Eck, 2004). The active site is formed by the glycine-rich loop and C-helix from the N-lobe, and the catalytic and activation loops from the C-lobe. Kinase activity is controlled by the conformation and relative position of these elements, which are modulated by phosphorylation and by interaction with regulatory domains. The catalytic loop (residues 385-403 in c-Src) contains highly conserved Asp and Asn residues, which are important for coordinating the ATP-Mg^{++} complex. The activation loop (residues 404-432 in c-Src) is of central importance to kinase activity, with phosphorylation of the highly conserved Tyr-416 required for full activation.

Several kinase domain elements interact and stabilize the inactive conformation. In the N-terminal end of activation loop, Asp404 forms an ionic interaction with Lys-295, disrupting the Lys-295 and Glu-310 interaction which is required for the active state. This Asp-404 and Lys-295 interaction induces the movement of Phe-424 toward steric clash with the α-C-helix of

the N-lobe, forcing the α-C-helix away from the catalytic cleft (Schindler et al., 1999). In addition, the residues 413-418 of the activation loop form a short α-helix (A-loop helix), which turns Tyr-416 to the catalytic center of the kinase domain. Tyr-416 forms hydrogen bonds with Arg-385 and Arg-386, is buried into a hydrophobic pocket and protected from auto phosphorylation. Thus, the activation loop occludes the catalytic domain and prevents the binding of peptide substrates (Xu et al., 1999). Moreover, the non-catalytic conformation of the α-C-helix is stabilized by interactions between the inward-facing surface of the α-C-helix with the N-terminal end of activation loop, a salt bridge between Glu-310 of the αC-helix and Arg-409, and by insertion of Trp-260 into the α-C-helix (Xu et al., 1999; Sicheri et al., 1997).

In the active conformation, activation loop Tyr-416 is phosphorylated. This phosphorylation induces the activation loop to move away from the catalytic cleft and form a conformation suitable for substrate binding. This conformation is stabilized by the interaction between pTyr-416, Arg-385 and Arg-409 in Src (Yamaguchi and Hendrickson, 1996; Cowan-Jacob et al., 2005). The position change of the activation loop and Asp-404 allows the α-C-helix to turn towards the catalytic cleft and into its active orientation. This liberates Glu-310 in α-C-helix, which forms a salt bridge with Lys-295, an interaction both critical for ATP binding and stabilization of the active αC helix conformation (Xu et al., 1999). Note that this Lys:Glu salt bridge is a highly conserved feature of active kinase domain conformations, and substitution of the Lys residue is a common approach to creating an inactive kinase mutant.

At the end of the kinase domain, a C-terminal tail (residues 521-535 in c-Src) contains a tyrosine residue (Tyr-527) that is important for intramolecular regulation. The highly-conserved Tyr-527 is phosphorylated by regulatory kinases C-terminal Src kinase (Csk) and Csk-homologous kinase (Chk) (Ingley, 2008; Roskoski, Jr., 2005). This phosphorylation event creates

an SH2-binding site and induces intramolecular binding of the tail to SH2 domain, locking the kinase in an inactive conformation (Xu et al., 1999; Boggon and Eck, 2004). In v-Src, the tail sequence containing the conserved tyrosine is replaced by an unrelated 12-residue fragment derived from the virus. The classic SH2-pTyr tail interaction is compromised, making the kinase constitutively active (Cooper et al., 1986).

1.2.2 SFK regulation

The activity of Src family tyrosine kinases is tightly controlled by intramolecular interactions, phosphorylation, and engagement with binding partners, all of which are discussed below.

1.2.2.1 Intramolecular regulation

Src family kinase activity is tightly regulated through elegant intramolecular interactions by three key components, which are called “the latch, the clamp and the switch” (Harrison, 2003). The short C-terminal tail containing Tyr-527 is the “latch”. Phosphorylation of Tyr-527 by Csk and Chk enables the tail to interact with the SH2 domain, forming the latch and locking the kinase domain in an inactive form (Figure 5B). In addition, the SH3 domain interacts with the SH2-kinase linker through a left-handed PPII helix formed by three proline residues in the linker. This interaction attaches SH3 domain to the back of the N-lobe of the kinase domain. The SH3 and SH2 domains assemble to form a “clamp” and cooperate to stabilize the downregulated conformation of the kinase. The SH3-SH2 clamp packs to the back of the kinase domain in opposition to the catalytic cleft, indirectly prohibiting the catalytic cleft from opening by displacing the α-C helix and stabilizing the inactive state (Roskoski, Jr., 2004). Either dephosphorylation of pTyr-527 or displacement of SH3-SH2 dissociates the clamp and leads to a

constitutively active Src (Young et al., 2001). The switch is the activation loop in the kinase domain, which can switch between active and inactive conformations as a function of Tyr-416 phosphorylation as described above.

Neither the SH2-pTyr-527 interaction nor and SH3-linker interaction is of optimal affinity: The C-terminal tail has pTyr527 binding to the first SH2 pocket, but has no leucine or isoleucine in pY+3 position to occupy the second binding pocket. The SH2-kinase domain linker sequence does not fit a classical PxxP consensus sequence for high-affinity SH3 domain binding. This flexibility allows binding partners with higher affinity to outcompete these lower affinity intramoleular interactions, disrupt the assembled, inactive conformation, and expose the Tyr-416 for autophosphorylation and switch on the kinase (Xu et al., 1999). Based on the intramolecular interaction described above, SFKs can be activated by: unlatching with pTyr527 dephosphorylation and displacement of the tail from SH2 domain, unclamping by SH3 and SH2 ligand binding and switching the enzyme on by phosphorylation on the pY416 (Harrison, 2003). The examples of these three mechanisms are discussed below.

1.2.2.2 Regulation by phosphorylation and dephosphorylation

As described in the previous section, Src is tightly regulated by Csk-mediated phosphorylation of Tyr-527 in the C-terminal tail. Purified Src proteins are mostly phosphorylated at Tyr-527 and had their SH2 domain engaged with the tail to adopt the inactive conformation, with only 2% tail dephosphorylated and released from the SH2 domain (Wang et al., 2002). In vivo, 90-95% of Src is Tyr-527 phosphorylated in basal conditions (Roskoski, Jr., 2005). Mutation of the C-terminal tyrosine to phenylalanine renders SFKs constitutively active and able to transform Rat2 fibroblasts (Cooper et al., 1986). Csk homology kinase (Chk) also catalyzes the phosphorylation of Tyr-527 in SFKs. Csk is expressed in all mammalian cells,

while Chk is expressed in a more restricted pattern (Roskoski, Jr., 2004). Homozygous knockout of Csk results in embryonic lethality, with an elevation of overall SFK activity, suggesting that Csk is a master regulator of all Src Family kinases (Imamoto and Soriano, 1993). These studies also illustrate the importance of tight control of SFK activity to early embryogenesis.

Phosphorylation of Tyr-416 in the activation loop is the hallmark of SFK activation, which can override the inhibitory effect of pTyr527 and SH2-tail interaction (Sun et al., 1998). Tyr-416 phosphorylation is believed to occur mainly via intermolecular auto-phosphorylation (Martin, 2001). Src family members are also able to phosphorylate one another (Ingley, 2008). When Tyr-416 is phosphorylated, the kinase is stabilized in an active conformation. Thus, the pTyr-416 is required for full activation. Phosphospecific antibodies that recognize pTyr-416 in the context of the activation loop are useful tools for the assessment of SFK activity in cells.

Besides auto-phosphorylation at Tyr-416 and phosphorylation by Csk and Chk at Tyr-527, Src and SFKs can be phosphorylated at Tyr-213 in SH2 domain, and several serine and threonine residues (reviewed in (Roskoski, Jr., 2005)). The platelet-derived growth factor receptor (PDGFR) can phosphorylated Src at Tyr-213, which upregulates kinase activity and can override negative regulation by pTyr-527 (Stover et al., 1996). In addition, CDK1/cdc2 kinase, an enzyme important for cell cycle transition, can phosphorylate Src at Thr34, Thr46 and Ser72, all of which activates Src (Shenoy et al., 1992).

SFK activity is also regulated by dephosphorylation by phosphotyrosine phosphatases. While dephosphorylation of pTyr-527 is involved in SFK activation, that of pTyr-416 is associated with kinase inactivation and activity control. For the C-terminal tail pTyr-527, the phosphatases PEP (proline-enriched tyrosine phosphatase), SHP1 (tandem SH2 domain-containing protein-tyrosine phosphatase) SHP2 and CD45 (transmembrane receptor like tyrosine

phosphatase) have all been shown to dephosphorylate pTyr-527 and upregulate Src activity in different cell types (reviewed in (Roskoski, Jr., 2005; Ingley, 2008)). Several of these phosphatases can also dephosphorylate pTyr416 in the activation loop, including CD45, SHP1 and PEP. PEP was shown to dephosphorylate pTyr416 to regulate Src activity. Further, PEP can recruit Csk to phosphorylated Tyr527 of Src, further down-regulating the kinase (Cloutier and Veillette, 1996).

1.2.2.3 Regulation by engagement with binding partners

In the assembled, inactive state of Src, the low affinity of SH2-tail and SH3-linker interactions allows binding partners with higher affinity to outcompete the intramoleular interactions, and subsequently activate the kinase. Substantial evidence has accumulated regarding the activation of SFKs by its SH2 and SH3 domain binding partners (Boggon and Eck, 2004). For example, the PDGFR can recruit and activate Src by binding to the Src SH2 domain through membrane proximate pTyr residues on the receptor (Alonso et al., 1995). Nef, an HIV accessory protein, binds and activates the macrophage Src-family member Hck. Nef contains a PxxPxR motif preconfigured in a PPII conformation that binds to the Hck SH3 domain with high activity (Lee et al., 1996). This SH3-dependent activation of Hck by Nef overrides the inhibitory tail-SH2 interaction (Lerner and Smithgall, 2002). In addition, Csk binding protein (Cbp) has been identified as an adaptor for SFK signaling (Kawabuchi et al., 2000; Ingley, 2008). Cbp contains a SH3 binding sequence, and multiple tyrosines that can engage many SFK molecules. Cbp can recruit and activate SFK through SH3 binding. The activated SFKs then phosphorylated multiple tyrosine residues on Cbp. These pTyr residues recruit more SFK molecules by binding to their SH2 domains, facilitating trans-autophosphorylation in SFK Tyr-416 and signal amplification. Conversely, Cbp also binds to Csk to mediate SFK inactivation. Cbp can also

recruit the suppressor of cytokine signaling 1 (SOCS1) protein by binding to its SH2 domain, which is involved in ubiquitination and degradation of SFKs (Ingley, 2008). Of note, while the regulatory domain can alter kinase activity by binding substrates, the state of the kinase domain can also affect substrate binding affinities of the regulatory domains. For example, binding of high affinity inhibitors in the kinase domain of Src can reduce substrate binding by its SH2 and SH3 domains (Krishnamurty et al., 2013).

In addition to binding with SH2 or SH3 domain with classical interactions, substrates can also bind other domains to activate SFKs. A recent study show that integrin β3 primes and binds Src, by binding the RGT sequence within the N-Src loop of the SH3 domain (Xiao et al., 2013). Also, as discussed in the previous section, CD4 and CD8α receptors activate Lck through interaction with its unique domain (Kim et al., 2003).

1.2.3 SFK functions

1.2.3.1 Phylogenetic relationship of Src family members

SFKs have been implicated in the regulation of many biological processes such as cell migration, proliferation, differentiation and survival (Thomas and Brugge, 1997). In mammalian cells, eight 'classical' Src family members are expressed, including c-Src, c-Yes, Fyn, Fgr, Hck, Lck, Lyn and Blk (Yrk is only expressed in chickens and is not included). Three additional members with very similar sequences to the classical SFKs include Brk, Frk and Srm (Manning et al., 2002). C-Src, Fyn and c-Yes are expressed ubiquitously; Fgr, Hck, Lck, Lyn and Blk are expressed primarily in hematopoietic cells; while Frk, Brk and Srm are expressed in epithelial cells (Table 3) (Thomas and Brugge, 1997; Lowell and Soriano, 1996). Many of the SFKs are expressed as different isoforms due to alternative splicing. For example, Src has two neuronal

isoforms, while Fyn has a hematopoietic isoform Fyn(T) and a brain isoform Fyn(B) which is also expressed in other tissues.

Individual kinases may be expressed at elevated levels in specific cell types. For example, c-Src is expressed at higher levels in neurons, osteoclasts and platelets (Soriano et al., 1991), while c-Yes is highly expressed in brain, fibroblasts, endothelial cells and platelets (Stein et al., 1994). In addition, most cells express multiple SFK members, which have both redundant and distinct functions. A SFK member may have specific subcellular localizations and carry out specific functions.

Based on sequence alignment and homology analysis, classic SFKs can be divided into two sub-families. The Src-A family includes Src, Yes, Fyn and Fgr while the Src-B family includes Blk, Hck, Lck and Lyn. Across all family members, Src and Yes share the most homology and are most closely related in the SFK phylogenetic tree (Robinson et al., 2000).

Table 3. Summary of expression patterns and mutant phenotypes of SFK members

Gene	Protein Product	Expression pattern	Phenotype of Single mutant
c-src	$p60^{c\text{-}src}$ (3 isoforms)	ubiquitous, high in brain, platelets and osteoclasts	osteopetrosis, defective osteoclast
c-yes	$p62^{c\text{-}yes}$	ubiquitous, high in brain, fibroblasts, endothelial cells and platelets	None observed
fyn	$p59^{fyn(B)}$ $p59^{fyn(T)}$	brain isoform: brain, fibroblasts, endothelial cells and keritinocytes; thymocyte form: T-cells, B-cells.	impaired hippocampus development, LTP defect, bad memory; mild defect in thymocyte signaling
hck	$p59^{hck}$, $p61^{hck}$	myeloid cells	mild defects in macrophage function
lck	$p56^{lck}$	T-cells, NK cells	blocked T-cell development; defective TCR signaling
lyn	$p53^{lyn}$, $p56^{lyn}$	brain, B-cells and myeloid cells	defect in B-cell function, autoimmunity
c-fgr	$p58^{c\text{-}fgr}$	myeloid cells, mature B-cells	None observed
blk	$p56^{blk}$	B-cells	None observed
frk	$p57^{frk}$	Epithelial cells, kidney, liver, intestine	None observed

Adapted from (Lowell and Soriano, 1996), except Frk (Chandrasekharan et al., 2002) .

1.2.3.2 SFK knockout phenotype and implication for function

Src family kinases have been implicated in many biological processes such as cell adhesion, migration, proliferation and survival. SFK members are activated by adhesion receptors like integrins, by receptor tyrosine kinases including the PDGFR, EGFR and IGFR, and by cytokine receptors including the IL-2 receptor. Active SFKs can activate and cooperate with the FAK, Ras-Raf-MAPK and PI3K pathways to reorganize the cytoskeleton, to promote cell cycle progression and to prevent apoptosis (Thomas and Brugge, 1997). These signaling pathways are interconnected, and involve multiple SFK members. Since cells usually express multiple SFKs, the same upstream signal can act on multiple SFK members, which function redundantly. Many studies of SFK function are based on v-Src, the constitutively active, viral form of the kinase. Although the v-Src function is valuable to predict the physiological function of c-Src, it is not ideal since SFKs are usually turned on transiently to function. Therefore, genetic studies using knockout mice are of great value to study the redundant and unique signaling in SFKs. A summary of phenotypes for individual SFK knockout mice is summarized in Table 3 (Lowell and Soriano, 1996).

The first SFK disruption study in mice was reported for Src (Soriano et al., 1991). Despite the high expression of Src in brain, platelets and osteoclasts, only osteoclast function was defective in Src-null mice. Lack of Src caused osteopetrosis, and most of the homozygous mice died several weeks after birth. Subsequent studies showed that osteoclasts require Src activity downstream of integrin signaling to function (Miyazaki et al., 2004). Fyn disruption mainly resulted in neuronal defects, with defective hippocampal development and long-term potentiation (Grant et al., 1992). Fyn mutation also had a mild effect on thymocytes by disrupting their T-cell receptor (TCR) signaling (Stein et al., 1992). Remarkably, no phenotype

was observed in mice with disrupted c-Yes, which has a very similar gene expression profile to c-Src and Fyn (Stein et al., 1994). In the same study, compound knockouts of Src, Yes and Fyn were created by crossing the single mutant animals. Most of the Src/Fyn or Src/Yes double mutants died perinatally, while a substantial portion of the Fyn/Yes double mutants were viable. Histopathological studies of the rare, viable Src/Fyn or Src/Yes double mutant mice failed to reveal abnormalities other than the osteopetrosis caused by the Src mutation. The surviving Fyn/Yes double mutant mice developed immune complex glomerulonephritis, a renal defect (Stein et al., 1994). In another study involving Hck/Src double mutant mice, two thirds died at birth, and the rest developed a more severe form of osteopetrosis compared with Src mutant only, indicating that Hck can partially compensate for the absence of Src (Lowell et al., 1996).

In contrast to the relatively mild phenotype of the single and double SFK knockout mice, triple disruption of Src, Yes and Fyn simultaneously results in embryonic lethality. This triple knockout leads to embryonic death at E9.5 and is associated with severe developmental defects. For example, the embryos are not "turned", with inverted germ layer orientation (Klinghoffer et al., 1999). In addition, disruption of Csk, a master regulator of SFK activity, also had a severe phenotype (Nada et al., 1993; Imamoto and Soriano, 1993). Embryos homozygous with Csk mutant die between E9 and E10, with complex neural tube and other defects. In Csk knockout embryos, overall SFK activity is greatly enhanced with increased phosphorylation of substrate proteins.

Gene disruption studies, highlighted above, suggest that SFKs are essential for embryo development but also may function redundantly. Single or double deletions of Src family members have a relatively mild phenotype, while triple deletion of Src, Yes, and Fyn, or deletion of Csk to upregulate all SFK activity, causes severe defects and embryonic lethality. Although

the knockout studies reveal some unique functions for individual members (Lck in T-cell development and Src in osteoclasts, for example), functional redundancy and compensation is predominant. Since cells express multiple SFKs, the function of one or two SFK members can be substituted with other members expressed. The surrogate SFK members can be induced for higher expression, higher activity, or change cellular their localization as possible mechanisms of compensation. For example, Src has increased solubility in the brain of Fyn knockout mice, supporting this notion (Stein et al., 1994).

1.2.4 SFK signaling

Src family tyrosine kinases play critical roles in many cell functions by coupling with upstream receptors and cell-adhesion signaling components. SFKs can be activated by integrin and other adhesion receptors, receptor tyrosine kinases, cytokine receptors, G-protein coupled receptors and immune response receptors (Thomas and Brugge, 1997; Parsons and Parsons, 2004). Upon activation, SFKs phosphorylate substrates in multiple locations of the cell including the cytosol, peri-plasma membrane, cellular compartments, or cell adhesion sites. SFK-mediated phosphorylation either directly affects substrate kinase activity, or creates additional binding sites for more SH2 domain-containing signaling molecules (Martin, 2001). Here, I emphasize SFK signaling with receptor tyrosine kinases and cell adhesion receptors. These interactions with signaling partners have been implicated in cancer biology, and similar interactions and functions are also conserved in ES cells.

1.2.4.1 Signaling with receptor tyrosine kinases

SFKs can be activated by many receptor tyrosine kinases, including the platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) and insulin-like growth factor-1 receptor (IGF1R). Activated receptor tyrosine kinases have been propose to engage SFKs by binding of p-Tyr residues on the active receptor with the SH2 domain of SFKs, initiating the activation of SFKs through a regulatory domain displacement model as described above (Bromann et al., 2004). SFKs can then activate multiple signaling pathways by recruiting and phosphorylating substrates directly, or by creating binding sites in docking proteins. These events ultimately lead to many biological effects, including changes in the cytoskeleton and in gene expression (Figure 6).

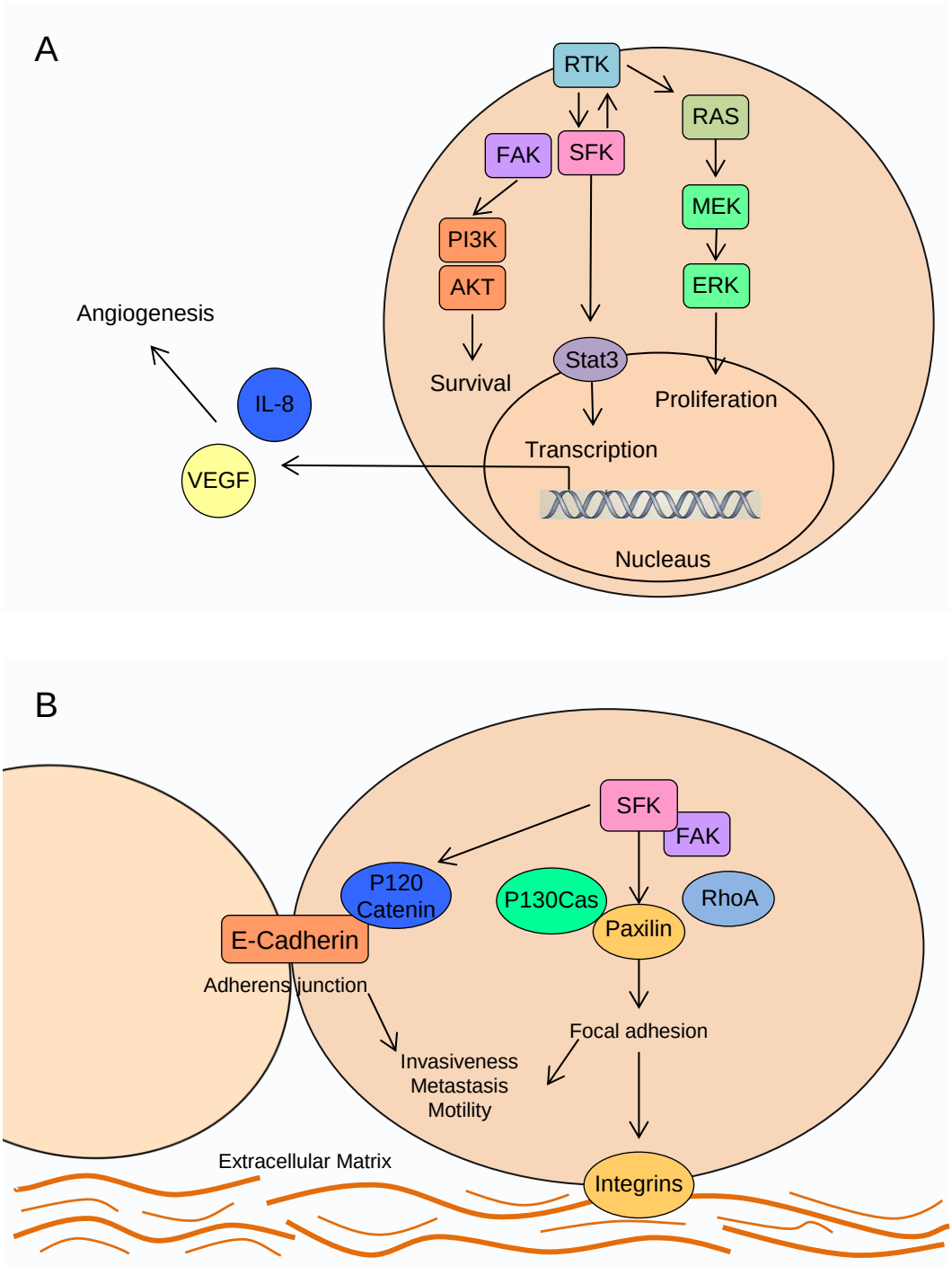

Figure 6. SFK signaling pathways and function. SFKs interact and cooperate with RTKs to activate downstream signals important for cell survival, proliferation and angiogenesis (A). SFKs activate and cooperate with the PI3K/AKT pathway to promote survival and with the Ras/Mek/Erk pathway to promote proliferation. By acting on transcription factors such as Stat3, SFKs can also promote transcription and secretion of growth factors that promote angiogenesis. SFKs are of great importance in regulation of cell adhesion, migration and invasion (B). Activated SFKs can phosphorylate p120 catenin to disrupt adherens junctions. By association with FAK, SFKs target downstream effectors including Paxillin, $p130^{cas}$ and RhoA, to regulate focal adhesions and cytoskeletal organization. Adapted from (Kim et al., 2009).

SFKs can activate PI3K by phosphorylation, or up-regulate PI3K signaling by inhibiting its antagonist—PTEN. PI3K/Akt signaling in turn regulates translation initiation, prevents apoptosis and promotes cell survival. In addition, Src can activate the Mek/Erk pathway by recruiting the Grb2-Sos complex or Shc with docking proteins. The Mek/Erk pathway can then promote proliferation and regulate gene expression. In addition, SFKs can phosphorylate Stat3, which in turn regulates the expression of transcription factor Myc, contributing to the Myc regulation of cell growth and proliferation (Kim et al., 2009; Martin, 2001) (Figure 6).

1.2.4.2 Signaling with integrin and focal adhesion kinase

Both focal adhesions and adherens junctions are necessary for cell attachment, motility, and are important for cell proliferation, survival and differentiation. Focal adhesions are the interactions of cells with the extracellular matrix (ECM) via integrins or intracellular transmembrane receptors, while adherens junctions are direct cell-cell connections regulated by cadherin-catenin mediator complexes. SFKs play an important role in these important adhesive events which also have a central role in cancer progression (Kim et al., 2009; Ishizawar and Parsons, 2004; Guarino, 2010).

Integrins can sense changes in the cellular environment, triggering signaling pathways to adapt the cytoskeletal organization accordingly. SFKs transmit the integrin-dependent signal. SFKs are directly activated by integrin β, and through engagement with focal adhesion kinase (FAK), to modulate downstream signals including RhoA and p120 catenin (Huveneers and Danen, 2009). c-Src and FAK form a complex, with the SH2 domain of Src binding to pTyr397 of FAK. This binding facilitates Src phosphorylation of FAK on multiple tyrosine sites to

enhance FAK activity. Active Src-FAK complexes phosphorylate paxillin and p130Cas, important modulators of cell migration. The Src-FAK comples also activates matrix metalloproteinases (MMP) 2 and 9, which also contribute to cell migration and invasion. Src also phosphorylates p120 catenin to disrupt its association with E-cadherin, resulting in enhanced cell migration (Kim et al., 2009). In addition, active Src drives the activation of multiple small G-proteins, including the Rho-family GTPases Rac, Cdc42 and Rho, to regulate adhesion and cytoskeleton organization. In summary, SFKs have important roles in regulating cell adhesion and migration, which are implicated in cancer progression and invasion. In addition, coordinated cellular migration and cell:cell contacts are critical to early development, which may explain the dramatic impact of Csk-knockout on embryogenesis.

1.3 SFK SIGNALING IN MURINE ES CELLS

As summarized in the previous section, SFKs are regulated by many upstream signals and have a prominent role in the coordinated regulation of cell adhesion, proliferation, growth and survival. Loss of regulation of these pathways can contribute to the pathogenesis of cancer and also interfere with early embryogenesis. Previous work from our group and others has shown that SFKs also play important roles in embryonic stem cell self-renewal and differentiation.

1.3.1 SFK expression and function in mES cells and EBs

Several years ago, our group made the unexpected discovery that seven of the eight classical SFKs—Src, Yes, Fyn, Hck, Lck, Lyn and Fgr—are expressed in mES cells. We also found that when ES cells were induced to differentiate into EBs, Hck and Lck were quickly silenced transcriptionally, while Src, Fyn expression was retained (Meyn, III et al., 2005). Around the same time, the Melton lab reported that c-Yes activity was down-regulated during EB differentiation, although its expression was maintained at the transcript level (Anneren et al., 2004). These results indicate that SFK members are differentially regulated in ES cells and EBs, suggesting that individual SFKs may play discrete, non-redundant roles in mES cells. To study SFK activity and function in ES cells, small molecule inhibitors were introduced (Meyn, III et al., 2005). While partial inhibition of endogenous SFK activity with the ATP-competitive inhibitors SKI-1 and PP2 induced differentiation of ES cells in the presence of LIF, suppression of all SFK activity using higher concentrations of these inhibitors or with the more potent inhibitor A-419259 blocked differentiation in the absence of LIF (Meyn, III et al., 2005). The specificity of these inhibitors against individual SFK members was also explored in this study using in vitro kinase assays. Interestingly, c-Yes and Hck were found to be more sensitive to SKI-1 treatment than c-Src and Fyn, suggesting that selective inhibition of Hck and Yes with low inhibitor concentrations may account for the differentiation observed in ES cells. These observations led to signaling model in which SFKs play non-redundant and opposing roles in ES cells (Figure 7A), with Hck and Yes promoting renewal while Fyn and Src control differentiation (Meyn, III et al., 2005).

This model is also supported by other studies linking both Hck and Yes to ES cell renewal. Early studies showed that Hck activity increased following LIF treatment of ES cells.

Hck was shown to physically associate with gp130, and expression of an active tail mutant of Hck reduced the LIF requirement for ES cell self-renewal (Ernst et al., 1994). Consistent with these observations, transcription of Hck was rapidly silenced as ES cells differentiate to EBs (Meyn, III et al., 2005). Other work linked c-Yes to ES cell renewal. Unlike Hck, c-Yes is expressed both in pluripotent ES cells and in differentiated EBs (Anneren et al., 2004). While c-Yes kinase is active in ES cells, where it is regulated by both LIF and serum, its activity is downregulated during differentiation to EBs. RNAi-mediated knockdown of c-Yes expression reduces expression of the renewal marker Nanog while increasing expression of the differentiation marker, GCNF, supporting an inhibitory role for c-Yes in ES cell differentiation. Transcription of c-Yes in ES cells is regulated by the pluripotency factor Oct4, further supporting a role for c-Yes in renewal (Zhang et al., 2007). In contrast to Hck and c-Yes, c-Src and Fyn expression and activity persists when ES cells differentiate, indicating that these two kinases may function in differentiation (Meyn, III et al., 2005). Using a chemical genetics approach, our group found that c-Src activity alone is sufficient to induce differentiation of ES cells to primitive ectoderm (Meyn, III and Smithgall, 2009), further supporting Src as a differentiation related kinase. Details of this approach are described in the next section.

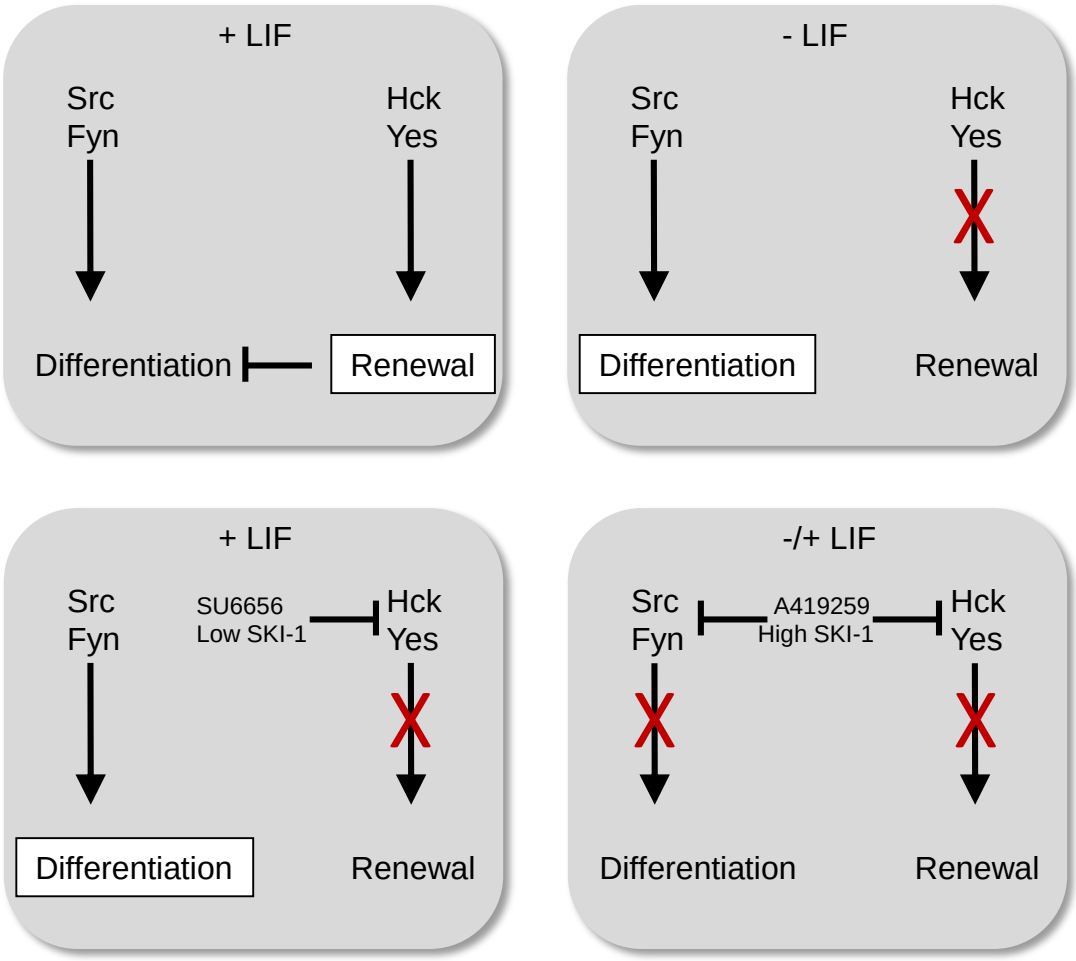

Figure 7. Model for the regulation of mES cell fate by Src family kinases. SFK members regulate two distinct signaling pathways: Src and Fyn induce differentiation; while Hck and Yes promote self-renewal. Top left: In cycling ES cells, Hck and Yes in the renewal pathway is maintained by cytokine LIF. This renewal pathway dominates over the rival differentiation pathway. ES cells maintain pluripotency and expand. Top right: Under differentiation conditions, LIF removal causes loss of Hck and Yes activity, and the renewal pathway is shut down and ES cells differentiate as the default condition. Bottom left: The SFK inhibitor SU6656, which shows some selectivity towards c-Yes, as well as low concentrations of SKI-1 preferentially inhibit Hck and Yes, causing differentiation in the presence of LIF. Bottom right: Complete inhibition of SFKs in ES cells blocks both the pathways for renewal and differentiation. These cells proliferate slowly and maintain pluripotency in the presence or absence of LIF. Adapted from (Meyn, III et al., 2005).

1.3.2 Chemical genetics approaches to study the individual functions of SFK members

Knockout studies imply that SFKs mainly function redundantly. However, in ES cell fate determination, there are non-redundant and even opposing roles in Yes/Hck versus Src/Fyn. To

elucidate the mechanism, it is necessary to investigate the individual role of each SFK member in ES cells. One approach is use RNA interference to knockdown one member at a time. For example, RNAi knockdown of Yes caused ES cells to lose renewal marker expression and an increase differentiation (Anneren et al., 2004). However, the knockdown approach suffers from the same limitation as knockout mice, in that functional compensation may occur during the selection process required to establish the knock-down cell population. In addition, the knockdown approach, like the genetic knockouts and in contrast to pharmacological inhibition, eliminates the kinase protein entirely. Another approach is the use of selective kinase inhibitors. However, because of the highly conserved nature of Src-family kinase domains, truly selective ATP-competitive inhibitors for individual SFKs are not currently available and may be impossible to develop. An alternative is to use a chemical genetics approach—combining small molecule kinase inhibitors with genetic mutations to achieve selective inhibition or resistance for a specific kinase (Bishop et al., 2000).

The crystal structure of Hck in complex with SFK inhibitor PP1, revealed the structural basis of PP1 specificity against SFKs (Schindler et al., 1999). In the inactive conformation, the SFK has a hydrophobic cavity with an opening toward the ATP binding pocket. As an ATP competitive inhibitor, PP1 binds to the ATP pocket, occupying the site normally bound by the adenine base of ATP. Several SFK residues are critical for PP1 binding, including Thr-338, Glu-339, Met-341 and Lys-295, all of which form hydrogen bonds with PP1. These hydrogen bonds orient PP1 to a position with its 3-substituted pheny group sticking into the hydrophobic pocket. Accessibility of inhibitors to this hydrophobic pocket confers specificity, and is controlled by a critical residue Thr-338, often referred to as the “gatekeeper” residue (Liu et al., 1999). Alignment of the kinase domains of PP1-sensitive SFKs with those of PP1-resistant kinases such

as PKA and Jak2 reveals that the PP1-resistant kinases have methionine instead of threonine in the gatekeeper position (Schindler et al., 1999). This larger methionine residue creates steric clash with the inhibitor, and limits inhibitor accessibility to the hydrophobic pocket. Therefore, mutation of the gatekeeper Thr to a bulkier residue such as methionine can confer inhibitor resistance to SFKs. Note also that the amino acid occupying the gatekeeper position is not highly conserved across different kinase families, and can be substituted experimentally without compromising kinase activity.

Using this chemical genetics approach, our group designed gatekeeper mutants of all SFK members expressed in ES cells to be resistant to the broad-spectrum (non-selective) SFK inhibitors PP2 and A419259 (Meyn, III and Smithgall, 2009). These mutants were introduced into mES cells with recombinant retroviruses. This combination of inhibitor resistant (IR) mutants with inhibitors allowed the investigation of individual SFK function in ES cells. In the presence of 1 μM of A419259, which was previously shown to inhibit all endogenous SFK activity, ES cells formed small rounded colonies that were unable to differentiate. In contrast, the ES cells expressing Src-IR mutants adopted a flattened morphology even in the presence of LIF. Compared with untreated cells, inhibitor-treated Src-IR cells showed reduced Gbx2 and Rex1 expression and an increased level of Fgf5 expression, resembling primitive ectoderm-like cells. This study showed that Src activity alone is sufficient to drive murine ES cells towards primitive ectoderm differentiation (Meyn, III and Smithgall, 2009).

1.3.3 A role for Src family kinase c-Yes in ES cell regulation

In contrast to c-Src as a differentiation-related kinase, c-Yes has been related to self-renewal in ES cells. c-Yes is highly expressed in both human and mouse ES cells, and is activated by both

serum and LIF (Anneren et al., 2004). LIF can induce interaction between gp130 and the Yes SH2 domain, suggesting a possible mechanism for Yes activation following LIF stimulation (Tamm et al., 2011). EB differentiation correlated with down-regulation of c-Yes activity in both human and mouse ES cells. Knockdown of c-Yes with RNAi induced ES cell differentiation, with reduced expression of Oct4 and Nanog (Anneren et al., 2004). Moreover, Oct4 was shown to bind to the Yes promoter and to upregulate Yes expression (Zhang et al., 2007). These studies all support c-Yes as a renewal-related gene in mouse ES cells.

More recent work showed that active c-Yes controls the TEAD2 transcription factor through the Yes-associated protein, YAP. Active YAP-TEAD2 complexes bind to DNA in the nucleus and promote transcription of renewal related genes, including the master regulators Oct4 and Nanog (Tamm et al., 2011). Yes, Yap and Tead2 are all highly expressed in ES cells, with expression down-regulated in response to differentiation. Active c-Yes phosphorylates YAP and activates YAP-TEAD2 dependent transcription. In addition, activation of this pathway promotes the expression of Oct4 and Nanog, and its suppression induces ES cell differentiation. Moreover, suppression of TEAD2 function increased the expression of endoderm specific genes, indicating that this pathway might suppress endoderm differentiation in ES cells.

1.4 HYPOTHESIS AND SPECIFIC AIMS

1.4.1 Hypothesis

Previous work, highlighted above, has implicated c-Yes in the regulation of ES cell pluripotency. Whether or not c-Yes kinase activity alone is sufficient to maintain this essential property of ES

cells has not been explored. Since c-Yes activity is downregulated in response to EB formation, and RNAi knockdown of c-Yes induced differentiation in the presence of LIF (Anneren et al., 2004), I hypothesize that enforced expression of c-Yes may be sufficient to drive self-renewal and/or block EB differentiation of mES cells. In addition, c-Src, the closest phylogenetic relative of c-Yes, induces differentiation (Meyn, III and Smithgall, 2009), in direct opposition to c-Yes. Whether c-Yes is dominant over c-Src, or vice versa is still unknown. In the case of transcription factors, pluripotent factors are dominant over differentiation factors; it might be the same case in this pair of opposing kinases. Therefore, I hypothesize that the pro-renewal, or anti-differentiation signal of c-Yes, is dominant over the differentiation-inducing signal of c-Src.

Finally, while previous research clearly defined the importance of SFKs in mES cells, their roles in hES cells are largely unknown. Some evidence suggests that although the growth factor conditions for hES cell growth are disparate from those of mES cells, the downstream signaling pathways might be similar. SFKs may represent one of those downstream pathways. I hypothesize that like mES cells, hES cells express multiple SFK members and SFKs may also play divergent roles in hES cells self-renewal and differentiation.

1.4.2 Specific Aims

1.4.2.1 Aim 1: Investigate the contribution of c-Yes to the growth and self-renewal of murine ES cells and test the opposing roles of c-Src and c-Yes in ES cell differentiation.

In mES cells, c-Yes has been implicated in proliferation and self-renewal. To better understand the regulation of c-Yes in mES cell self-renew and differentiation, I first showed that c-Yes transcript levels were unchanged during ES cell differentiation, while its kinase activity was downregulated. To determine whether downregulation of c-Yes kinase activity is required

for differentiation, I introduced wild-type, inactive and active mutants of c-Yes into mES cells using recombinant retroviral vectors. I found that enforced expression of active c-Yes did not change undifferentiated colony morphology or marker expression in the presence of LIF, but blocked ES cell differentiation to embryoid bodies. This differentiation block was not observed with kinase-defective c-Yes, indicating a requirement for kinase activity. Further, qPCR analysis showed that mES cells expressing active forms of c-Yes continued to express pluripotency markers under conditions for EB formation, suggesting that c-Yes signals for renewal override the normal differentiation program.

Despite their close phylogenetic similarity, c-Yes and c-Src appear to have opposing roles in ES cell differentiation. To explore the interplay of c-Src and c-Yes in ES cell renewal and differentiation, I employed a chemical genetics approach in engineering c-Src and c-Yes mutants that are resistant to SFK inhibitor A-419259. Previous studies have shown that A-419259 treatment blocks all SFK activity in ES cells, preventing differentiation while maintaining pluripotency. Expression of inhibitor-resistant (IR) c-Src alone rescues the A-419259 differentiation block, resulting in primitive-ectoderm differentiation (Meyn, III and Smithgall, 2009). Unlike the Src-IR cells, inhibitor treatment of mES cells expressing Yes-IR did not show changes in lineage marker expression indicative of differentiation. This observation is consistent with the proposed anti-differentiation function of c-Yes in ES cells. Next, I tested whether the c-Yes signal for anti-differentiation or the c-Src signal for pro-differentiation is dominant by expressing both IR-mutants in the same ES cell population. I expanded our previous study to show that c-Src induced ES cell differentiation to both primitive ectoderm and endoderm, and promoted epithelial-mesenchymal transition (EMT). Remarkably, c-Yes activity suppressed c-Src mediated endoderm differentiation and EMT. Together, these results define c-

Yes as a potent blocker of ES cell differentiation that can antagonize the primitive endoderm differentiation induced by c-Src; and show even closely related kinases such as c-Src and c-Yes have unique and opposing functions in the same cell type.

1.4.2.2 Aim 2: Study SFK signaling in human ES cell self-renewal and differentiation.

While our findings in Aim 1 have clearly defined the importance of Src family kinases in mES cells, their roles in hES cells are less clear. To investigate SFK signaling in hES cells, I first used RT-PCR and qPCR to determine the relative expression profile of SFKs in hES cells vs. EBs derived from them. Expression of six Src-family kinases (Fyn, c-Yes, c-Src, Lyn, Lck and Hck) was detected in the hES cell lines H1 and H9. During embryoid body formation, Fyn and Src transcript levels increased slightly, while c-Yes and Lyn levels remained unchanged. Conversely, Lck and Hck expression levels dropped dramatically. To explore if SFK activity is required for hES cell maintenance and differentiation, I treated hES cells with the pan-SFK inhibitor A-419259, and found that the treated cells retained the morphology of pluripotent colonies and continued to express the pluripotency marker TRA-1-60 under culture conditions for differentiation. Taken together, these results support an important role for Src family kinase signaling in the regulation of hES cell fate, and suggest unique functions for individual SFKs in hES cells as observed previously in mES cells.

2.0 THE C-YES TYROSINE KINASE IS A POTENT SUPPRESSOR OF ES CELL DIFFERENTIATION AND ANTAGONIZES THE ACTION OF ITS CLOSEST PHYLOGENETIC RELATIVE, C-SRC

2.1 ABSTRACT

ES cells are derived from the inner cell mass of the blastocyst stage embryo and are characterized by self-renewal and pluripotency. Previous work has implicated the Src family of protein-tyrosine kinases in the self-renewal and differentiation of mES cells. These kinases display dynamic expression and activity changes during ES cell differentiation, suggesting distinct functions in the control of developmental fate. Here we used ES cells to test the hypothesis that c-Src and its closest phylogenetic relative, c-Yes, act in biological opposition to one another despite their strong homology. We first showed that enforced expression of active c-Yes blocked ES cell differentiation to embryoid bodies by maintaining pluripotency gene expression. To explore the interplay of c-Src and c-Yes in ES cell renewal and differentiation, we employed a chemical genetics approach. We engineered c-Src and c-Yes mutants that are resistant to A-419259, a potent pyrrolopyrimidine inhibitor of the Src kinase family. Previous studies have shown that A-419259 treatment blocks all Src-family kinase activity in ES cells, preventing differentiation while maintaining pluripotency. Expression of inhibitor-resistant c-Src but not c-Yes rescued the A-419259 differentiation block, resulting in a cell population with

properties of both primitive ectoderm and endoderm. Remarkably, when inhibitor-resistant c-Src and c-Yes were expressed together in ES cells, c-Yes activity suppressed c-Src mediated differentiation. These studies show that even closely related kinases such as c-Src and c-Yes have unique and opposing functions in the same cell type. Selective agonists or inhibitors of c-Src vs. c-Yes activity may allow more precise pharmacological manipulation of ES cell fate and have broader applications in other biological systems which express multiple Src family members such as tumor cells.

2.2 INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass of the developing blastocyst (Evans and Kaufman, 1981; Martin, 1981). ES cells are characterized by self-renewal, the ability to multiply indefinitely without differentiation (Suda et al., 1987), and pluripotency, the developmental potential to generate cell types from all three germ layers (Bradley et al., 1984; Beddington and Robertson, 1989). In the absence of feeder cell layers, ES cells can be maintained in an undifferentiated state by culturing them in serum-based medium supplemented with the cytokine, leukemia inhibitory factor (LIF) (Smith et al., 1988) or in defined medium in the presence of LIF and bone morphogenetic proteins (BMPs) (Ying et al., 2003). In the absence of LIF, ES cells differentiate to embryoid bodies (EBs) when cultured under non-adherent conditions. EB formation mimics the earliest stages of embryonic development, giving rise to all three germ layers (Keller, 1995; Keller, 2005).

Multiple intracellular kinase signaling pathways play a dominant role in the regulation of ES cell fate (Liu et al., 2007; Jaenisch and Young, 2008), with at least four pathways important for self-renewal. LIF signals through Janus kinases (JAKs) and signal transducer and activator of transcription 3 (STAT3). This pathway promotes expression of renewal factors including the POU domain transcription factor, Oct4 (Okamoto et al., 1990), and the homeobox transcription factor, Nanog (Niwa et al., 1998; Burdon et al., 1999a). Bone morphogenetic proteins (BMPs), which are serum components, activate transcription factors of the SMAD family and inhibit differentiation through induction of inhibitor of differentiation (ID) factors (Ying et al., 2003). Wnt proteins, which are also found in serum, inhibit glycogen synthase kinase-3β activity, leading to β-Catenin accumulation and pluripotency marker gene expression (Sato et al., 2004; Wray et al., 2011). In addition, the phosphatidylinositol 3'-kinase (PI3K) signaling pathway promotes ES cell self-renewal partly via regulation of Nanog expression (Paling et al., 2004; Storm et al., 2007).

Previous work has implicated the Src family of non-receptor protein tyrosine kinases in self-renewal and differentiation of murine ES cells as well(Anneren et al., 2004; Meyn, III et al., 2005). Seven of the eight mammalian Src family members are expressed in murine ES cells, and several family members are active in cycling ES cells cultured in the presence of LIF and serum (c-Src, c-Yes, Fyn, and Hck). Accumulating evidence supports the hypothesis that individual members of this kinase family may play distinct roles in regulating ES cell fate. For example, early studies showed that expression of an active mutant of the Src-family kinase Hck reduces the LIF requirement for ES cell self-renewal, implicating Hck in the suppression of differentiation (Ernst et al., 1994). More recent studies from our group showed that transcription of Hck is rapidly silenced as ES cells differentiate to EBs, consistent with this idea (Meyn, III et

al., 2005). In contrast to Hck, c-Src is expressed in both ES cells and differentiated EBs. Moreover, when c-Src remains active in the absence of all other Src-family kinase activity, it is sufficient to induce differentiation of ES cells (Meyn, III and Smithgall, 2009).

Other work has linked c-Yes, the closest phylogenetic relative of c-Src, to the suppression of ES cell differentiation. Like c-Src, c-Yes is expressed in both pluripotent ES cells and in differentiated EBs (Anneren et al., 2004). While the c-Yes kinase is active in self-renewing ES cells, where it is regulated by both LIF and serum, its activity is downregulated during differentiation. RNAi-mediated knockdown of c-Yes expression reduces expression of the renewal factor Nanog while increasing expression of the differentiation marker, GCNF. Transcription of c-Yes in ES cells is regulated by the pluripotency factor Oct4, supporting a role for c-Yes in self-renewal (Zhang et al., 2007). Recent work shows that active c-Yes controls the TEAD2 transcription factor through the Yes-associated protein, YAP (Tamm et al., 2011). Active YAP-TEAD2 complexes bind Oct4 promoters, supporting a positive feedback loop between c-Yes and Oct4 in self-renewal.

In this study we examined the biological interplay of c-Yes and c-Src, closely homologous kinases previously shown to act in direct opposition to one another in ES cells. First, we expressed c-Yes in mES cells using a retroviral vector system that drives low-level protein expression in transduced ES cell populations (Meyn, III and Smithgall, 2009). EB maturation was completely blocked in ES cells expressing active c-Yes, while EBs formed by ES cells expressing a kinase-inactive c-Yes mutant were unaffected. EBs that formed from the c-Yes-transduced ES cell population expressed both pluripotency and differentiation markers, suggesting that c-Yes kinase activity prevents differentiation by maintaining expression of the self-renewal program. Using a chemical genetics approach that permits only c-Yes and c-Src

signaling in ES cells in the absence of all other SFK signaling, we found that c-Yes interfered with the induction of differentiation previously observed with c-Src in this system (Meyn, III and Smithgall, 2009). In addition, we found that c-Yes also suppressed the induction of the epithelial-mesenchymal transition (EMT) by c-Src. Together, these observations show that despite their high degree of sequence similarity, c-Yes and c-Src have opposing roles in the regulation of ES cell fate. Our results have important implications not only for the regulation of ES cell differentiation, but many other cellular contexts in which multiple members of this kinase family are expressed.

2.3 RESULTS

2.3.1 Downregulation of c-Yes kinase activity during differentiation of ES cells to EBs

To monitor changes in c-Yes expression and activity during ES cell self-renewal and differentiation, we compared endogenous c-Yes RNA levels, protein abundance and kinase activity in self-renewing ES cells vs. EBs following 6 days in differentiation culture (6-day EBs). For these experiments, we used the mES cell line D3, which readily forms EBs when plated under non-adherent conditions in the absence of LIF (Figure 8A) (Meyn, III et al., 2005). Total RNA was isolated from self-renewing ES cells and 6-day EBs, followed by quantitative real-time RT-PCR (qPCR) analysis of c-Yes and c-Src transcript levels as well as markers of self-renewal and differentiation. Differentiation to EBs resulted in negligible changes in the expression of c-

Yes and c-Src (Figure 8B). Expression of the self-renewal markers Oct4 and Nanog were significantly down-regulated while the lineage-specific differentiation markers Gata4 (endoderm), Fgf5 (ectoderm) and T (mesoderm) were up-regulated in the 6-day EBs, consistent with the onset of differentiation in the developing EBs.

To investigate c-Yes protein stability and activity as a function of differentiation, c-Yes was immunoprecipitated from ES cells and 6-day EBs, followed by immunoblotting with a phosphospecific antibody against the c-Yes activation loop tyrosine. As shown in Figure 8C, activation loop tyrosine phosphorylation was greatly diminished in EBs relative to ES cells despite equivalent recovery of c-Yes protein. Immunoblots also showed a dramatic reduction in the level of the pluripotency marker, Oct4, consistent with differentiation. This result shows that c-Yes kinase activity is downregulated during EB formation, consistent with a role for this Src-family member in maintenance of pluripotency as originally proposed by Anneren et al. (Anneren et al., 2004).

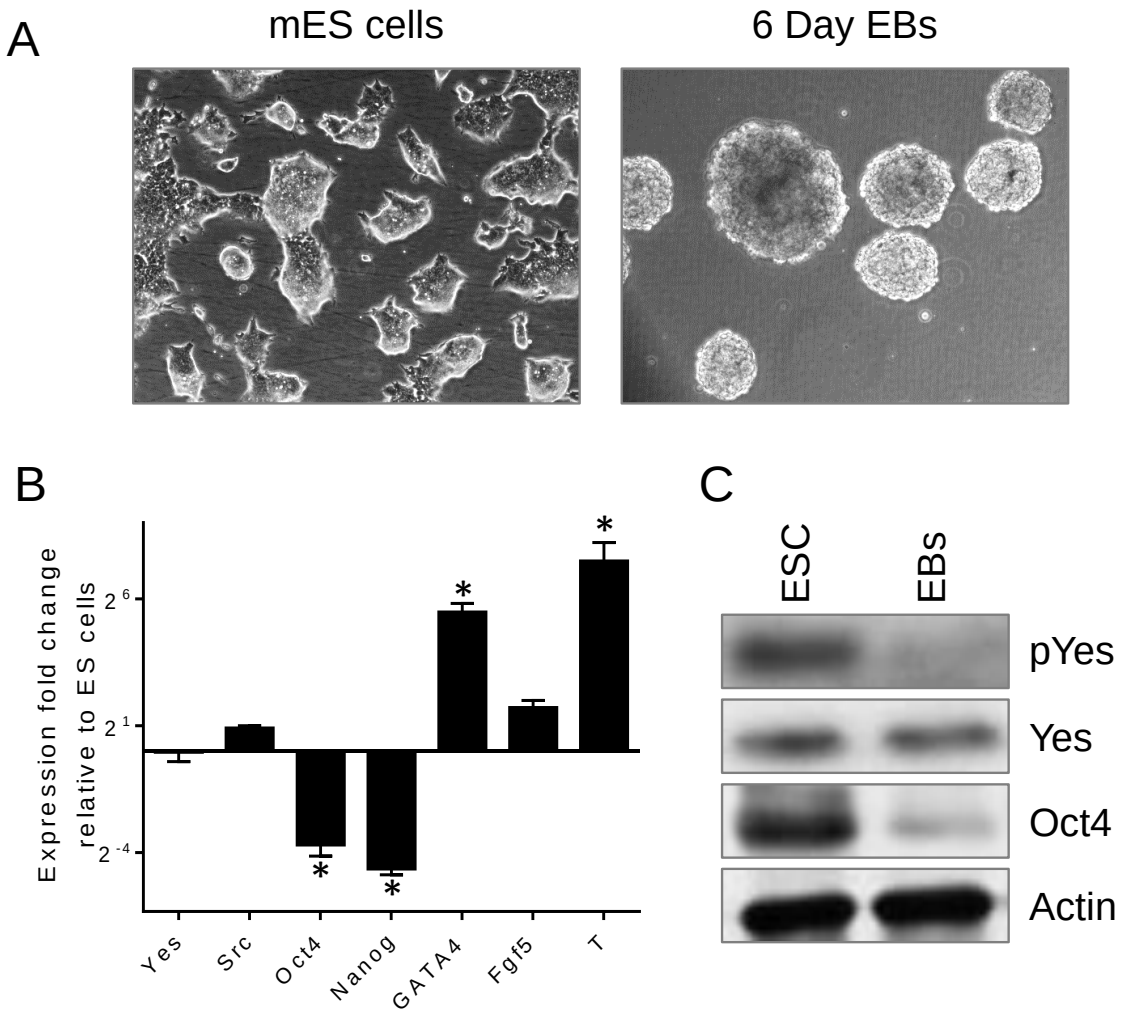

Figure 8. Downregulation of c-Yes kinase activity during EB formation. A) Bright-field images of the mES cell line D3 cultured under conditions for self-renewal (left) or differentiation to embryoid bodies (EBs) for 6 days (right). Magnification, 100X. B) Src-family kinase and ES cell marker gene expression was determined by qPCR in self-renewing ES cells and 6-day EBs. This panel of markers includes the self-renewal regulators Oct4 and Nanog as well as the differentiation genes Gata4 (endoderm), Fgf5 (ectoderm/epiblast) and T (mesoderm). Analyses were conducted using three biological replicates and the results are expressed as the average of the Log2 fold-change in each marker level in EBs relative to ES cells ± S.E.M. (*$P < 0.05$, Pair-wise Fixed Reallocation Randomization Test.) C) Lysates were prepared from ES cells and 6-day EBs, and endogenous c-Yes protein was immunoprecipitated and blotted with a phosphospecific antibody for the active form of the kinase (pYes) as well as the c-Yes protein. Cell lysates were also blotted for Oct4 as marker of ES cell self-renewal status and actin as a loading control.

2.3.2 Validation of c-Yes retroviral expression constructs in Rat2 cells

This Results presented in the previous section support a role for c-Yes as a positive regulator of ES cell pluripotency and suggest that downregulation of c-Yes kinase activity may be required for ES cells to exit the self-renewal program. To test this hypothesis, we engineered a series of c-Yes retroviral expression constructs based on a murine stem cell virus (MSCV) promoter which we have previously shown to remain active in both self-renewing ES cells and differentiated EBs (Meyn, III and Smithgall, 2009). In addition, the c-Yes cDNA clones were coupled to a G418 selection marker via an internal ribosome entry site (IRES) to ensure stable expression in the transduced cell populations.

Like all Src kinases, c-Yes consists of an N-terminal unique region, followed by SH3 and SH2 domains, the kinase domain, and a negative regulatory tail (Engen et al., 2008). To create an inactive mutant of c-Yes, we substituted Lys295 (numbering based on the c-Src crystal structure (Xu et al., 1999)) in the kinase domain with Arg. In addition, we created a constitutively active form of c-Yes by replacement of the negative regulatory tail tyrosine (Tyr527) with phenylalanine (Figure 9A). To validate the retroviral expression vectors carrying wild-type and mutant forms of c-Yes, we used Rat2 fibroblasts as a model system. These cells provide a convenient assay that relates Src-family kinase activity with biological function (transformed colony formation (Pene-Dumitrescu et al., 2008; Pene-Dumitrescu and Smithgall, 2010)).

Rat2 fibroblasts were infected with recombinant retroviruses carrying wild-type, kinase-dead and kinase-active forms of c-Yes, followed by G418 selection. Each of the transduced Rat2 cell populations was then plated in soft agar colony assays for anchorage independent growth as a measure of c-Yes biological function. As shown in Figures 9B and 9C, cells expressing the active mutant of c-Yes produced a large number of transformed colonies, while no colonies were

observed with cells expressing wild-type c-Yes or the kinase-dead mutant. Expression of c-Yes from the retroviral vector was confirmed by RT-PCR with total RNA isolated from each cell population and a primer pair specific for the c-Yes transgene (Figure 9D).

To assay for c-Yes kinase activity in each of the Rat2 cell populations, c-Yes was immunoprecipitated followed by immunoblotting for protein recovery and activation loop tyrosine phosphorylation. Figure 9E shows a small increase in the quantity of c-Yes protein recovered from cells expressing the c-Yes cDNAs relative to the vector controls. The tail mutant of c-Yes reacted strongly with the phosphospecific antibody, consistent with elevated kinase activity and transforming function. In contrast, c-Yes recovered from cells expressing the wild-type protein showed only a modest increase in reactivity with the activation loop antibody relative to endogenous c-Yes. This result demonstrates that the level of wild-type c-Yes over-expression achieved with the MSCV-based retrovirus was not sufficient to cause kinase upregulation, consistent with the lack of transformed colony formation.

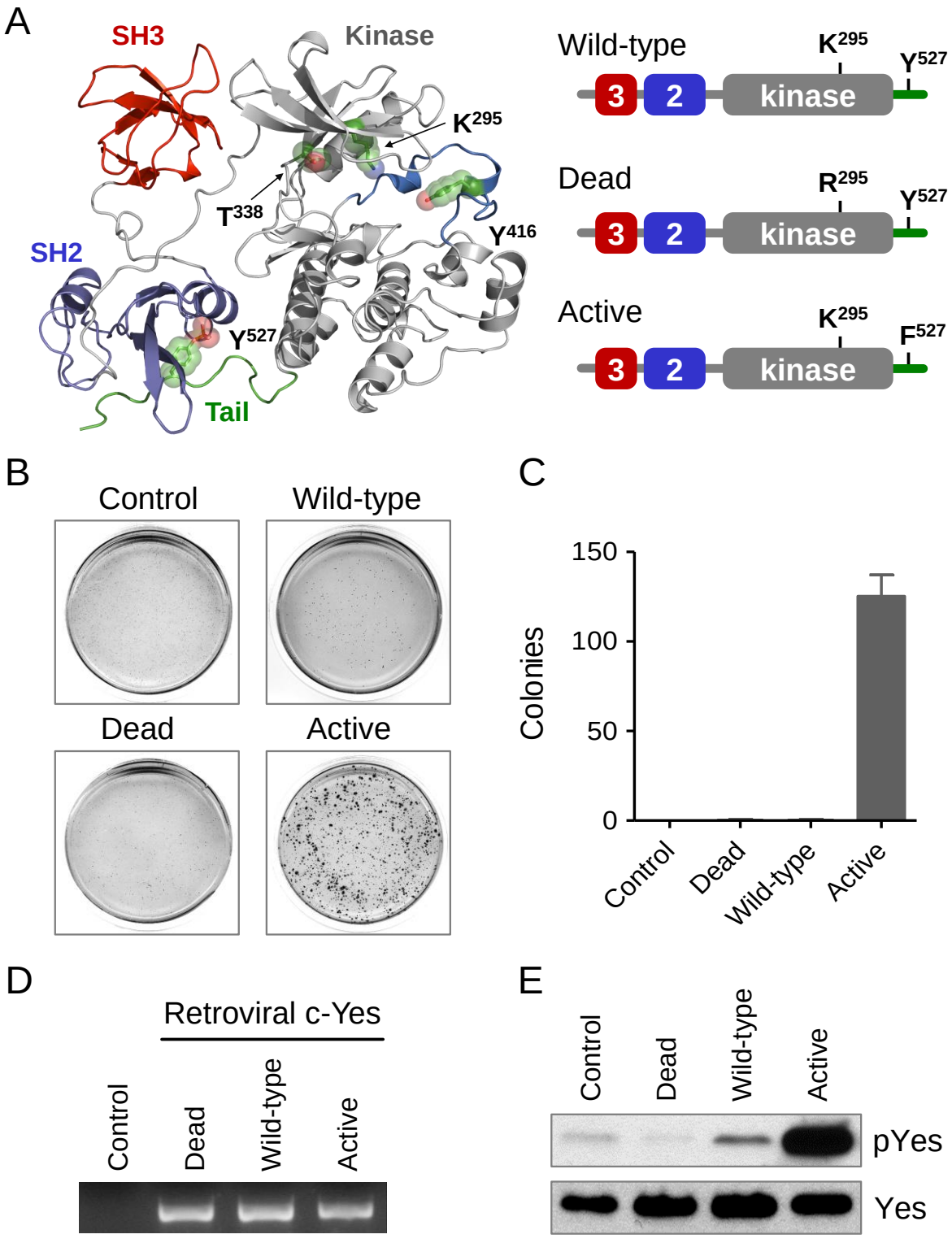

Figure 9. Validation of c-Yes retroviral expression constructs in Rat2 cells. A) Src-family kinase structure and c-Yes mutants used in this study. *Left*: A model of downregulated c-Src is shown on the left (PDB: 2SRC) and like c-Yes consists of an N-terminal unique region (not shown), followed by SH3 and SH2 domains, the kinase domain, and a negative regulatory tail. Key residues include the gatekeeper threonine (T338), the active site lysine (K295),

the activation loop tyrosine (Tyr416), and the tail tyrosine (Y527). *Right*: Substitution of Lys295 in the kinase domain with arginine renders the kinase inactive (Dead), while replacement of the tail tyrosine (Y527) with phenylalanine produces a constitutively active mutant (Active). B, C) The wild-type and mutant forms of c-Yes shown in part A were introduced into Rat2 fibroblasts using recombinant retroviruses. Following selection with G418, control and c-Yes-transduced fibroblasts were cultured in soft-agar colony assays for the transformed phenotype. Images of representative plates are shown in part B, and the average number of transformed colonies formed from each cell population is shown in Part C ± S.D.; n=3. D) RT-PCR indicates virus-specific c-Yes expression. RNA was isolated from each of the four Rat2 cell populations shown in part B, and RT-PCR was performed with a primer pair specific for the c-Yes transgene. A scanned image of the resulting agarose gel shows the expected 735 base-pair product. E) Kinase activity. c-Yes proteins were immunoprecipitated from each of the Rat2 cell populations and blotted with a phosphospecific antibody for the active form of the kinase (pYes) as well as the c-Yes protein.

2.3.3 Low-level retroviral expression of c-Yes in ES cells does not affect undifferentiated colony morphology or marker expression.

To test the effect of these c-Yes constructs on self-renewal, cultures of ES cells were transduced with recombinant retroviruses carrying wild-type, kinase-active and kinase-dead forms of c-Yes as described in the preceding section. Following selection with G418, each ES cell population formed undifferentiated colonies indistinguishable from control cells transduced with the empty vector (Figure 10A). RT-PCR was performed using total RNA isolated from each cell population and a primer pair specific for the c-Yes transgene. Figure 10B shows that the c-Yes retroviral transgene is expressed in each case but was not observed in RNA isolated from control cells transduced with the empty retroviral vector. The presence of the kinase domain mutations was confirmed in the RT-PCR products from the transduced cell lines by DNA sequence analysis (data not shown). The relative growth rates of the c-Yes-transduced ES cell populations were indistinguishable from that of the control cells (data not shown). Finally, qPCR analysis showed that the expression levels of the pluripotency markers Rex1, Nanog and Oct4, as well as the differentiation markers Fgf5 (ectoderm) and T (mesoderm) were essentially unchanged in each of the c-Yes-transduced ES cell populations relative to control ES cells (Figure 10C). Taken

together, these results indicate that retroviral transduction with c-Yes expression vectors did not effect, de novo, the regulation of self-renewal in cycling mES cells.

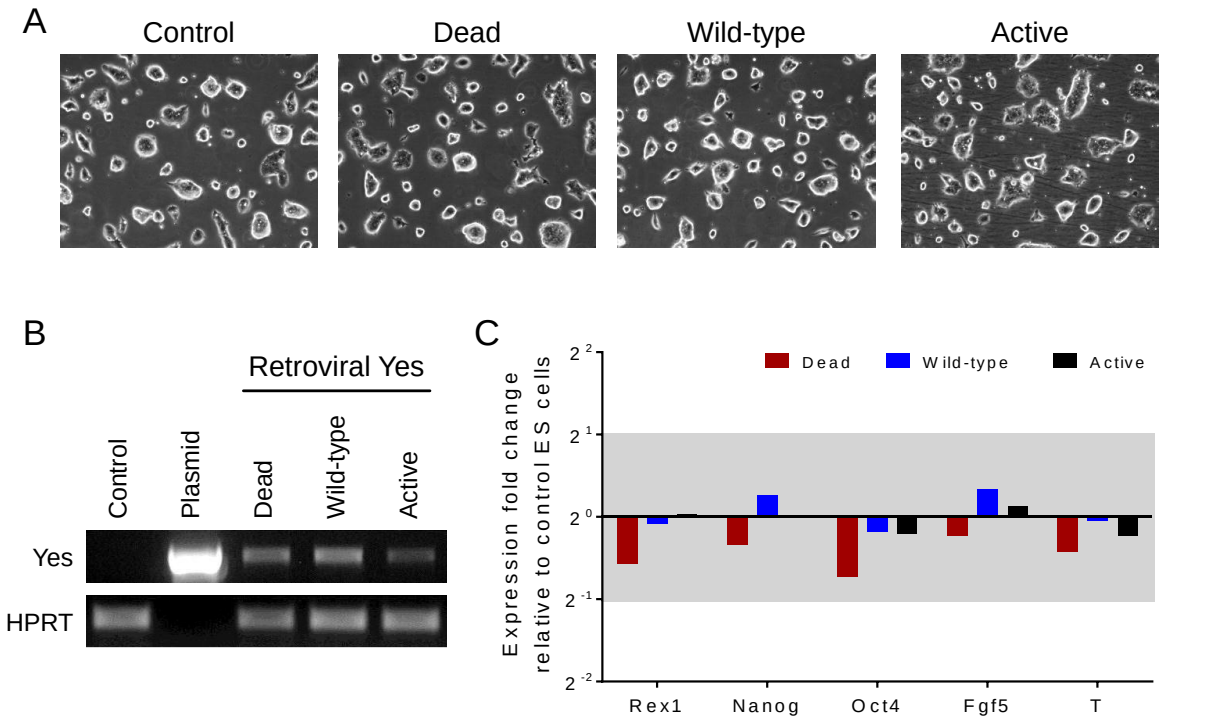

Figure 10. Low-level retroviral expression of c-Yes in mES cells does not affect colony morphology or pluripotency marker expression. A) Cultures of mES cells were transduced with the wild-type, kinase-active and kinase-dead c-Yes retroviruses shown in Figure 2, or with an empty vector as negative control. Infected cell populations were selected with G418, and retained undifferentiated colony morphology when cultured in presence of LIF and serum. Magnification, 100X. B) RT-PCR indicates virus-specific c-Yes expression. RNA was isolated from each of the ES cell populations shown in part A, and RT-PCR was performed with a primer pair specific for the c-Yes transgene. A scanned image of the resulting agarose gel shows the expected 735 base-pair product. The retroviral plasmid with the c-Yes insert served as the amplification template in a positive control reaction (*Plasmid*). C) ES cell marker gene expression. Quantitative RT-PCR analysis was performed on each of the c-Yes-transduced cell populations as well as the negative control cells for expression of the pluripotency markers Rex1, Oct4, and Nanog as well as the differentiation markers Fgf5 (ectoderm) and T (mesoderm). Results are expressed as fold change of each c-Yes transduced cell population relative to the vector control ES cells. All five markers examined varied by less than two-fold in each of the transduced cell populations (shaded grey area). This experiment was repeated twice with comparable results; a representative example is shown.

2.3.4 ES cells expressing active c-Yes fail to form EBs.

As described in the preceding section, ectopic expression of wild type c-Yes, kinase dead c-Yes, or constitutively active c-Yes in ES cells did not affect undifferentiated colony morphology, selected pluripotency and differentiation marker expression (Figure 11) or the rate of cell proliferation (data not shown) under conditions of self-renewal. These observations are consistent with previous knock down experiments that support a role for c-Yes in the maintenance of mES cell self-renewal(Anneren et al., 2004; Tamm et al., 2011). One prediction of this hypothesis is that the presence of unregulated c-Yes activity during EB formation will interfere with the loss of pluripotency that accompanies differentiation. To test this idea, ES cells expressing wild-type, kinase dead, or constitutively active c-Yes were plated in suspension culture without LIF to induce EB formation along with vector control cells. As shown in Figure 4, control ES cells and cells expressing kinase-dead c-Yes formed spherical clusters of cells after 6 days, consistent with normal differentiation to EBs. In contrast, ES cells expressing either wild-type c-Yes or the kinase-active mutant grew as much smaller irregularly shaped groups of cells. To allow for a quantitative size comparison, the cultures were stained with DAPI and EB size was estimated from the area of two-dimensional projections of confocal images. As shown in Figure 11, this size analysis revealed that the EBs derived from ES cells expressing the wild-type or active forms of c-Yes were significantly smaller ($p < 0.05$) than those derived from control ES cells or cells expressing kinase-dead c-Yes. Furthermore, these results show that the small increase in c-Yes activity resulting from expression of the wild type c-Yes construct is sufficient to interrupt normal EB development (See Figure 9E). This suggests that endogenous c-Yes activity must be tightly regulated to ensure normal ES cell differentiation.

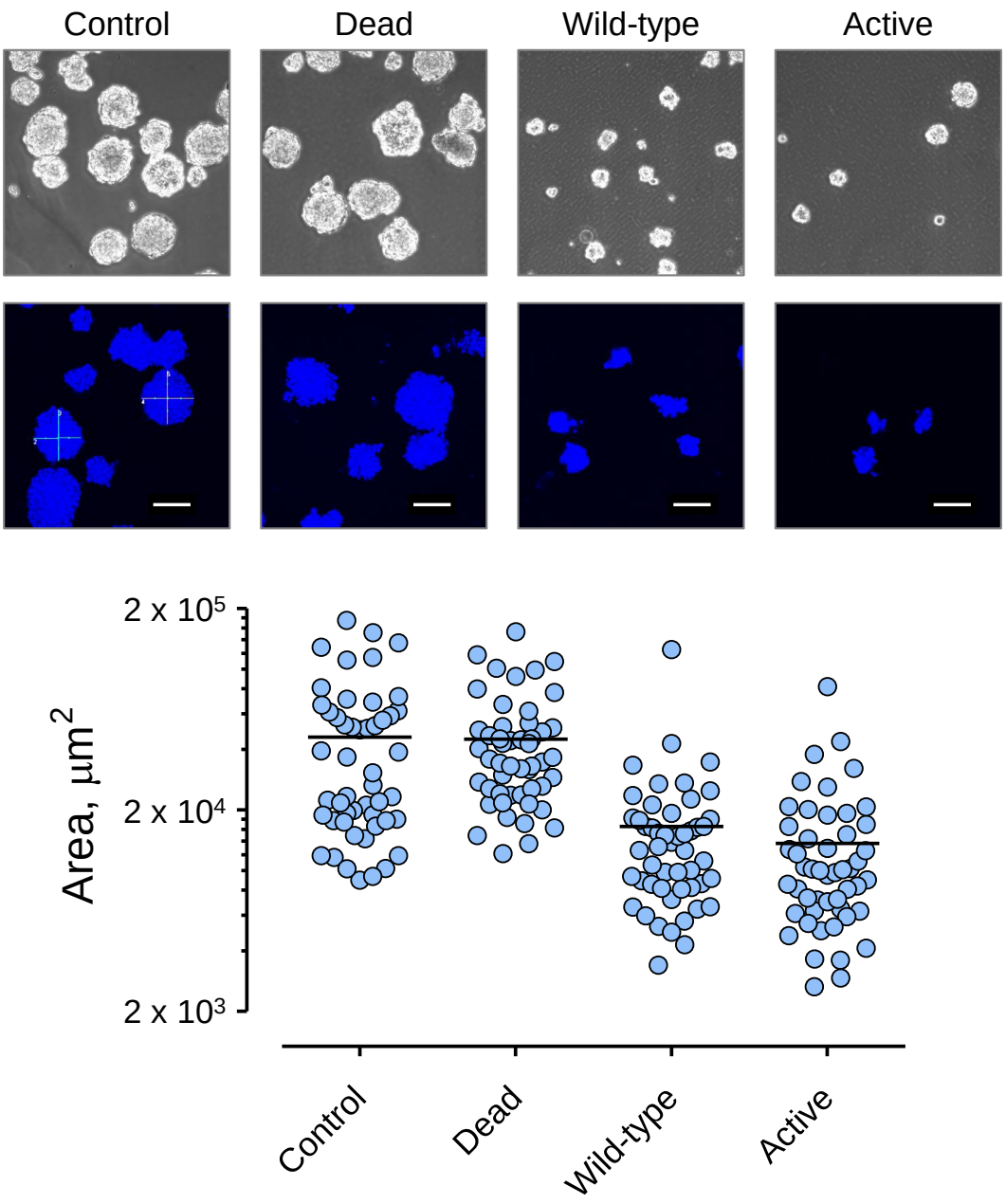

Figure 11. Mouse ES cells expressing active c-Yes kinases fail to form EBs. ES cell populations expressing wild-type, active and dead forms of c-Yes as well as the vector control were plated in EB formation assays, and representative images were taken 6 days later (top row). EBs were stained with DAPI and imaged by confocal microscopy (bottom row; scale bar 100 µm). EB size was estimated from the area of a 2D projection of these confocal images using the Olympus FV10-ASW 2.1 software. Size estimates for 50 EBs derived from each mES cell population are shown with the median size indicated by the black bar. This experiment was repeated twice with comparable results; a representative example is shown. Statistical comparisons (unpaired Student's t-tests) showed no difference in the average sizes of the EBs formed from the control vs. kinase-dead cell populations or between the wild-type vs. active c-Yes populations. However, both the wild-type and active c-Yes cell populations formed EBs statistically smaller than the control population ($p < 0.0001$).

2.3.5 ES cells expressing active c-Yes kinases express both pluripotency and differentiation markers during EB formation.

ES cells transduced with active c-Yes were significantly impaired in their ability to form EBs of similar size to those derived from control cells. We hypothesized that this defect in EB development may result from a failure of these cultures to properly execute differentiation pathways. To investigate this possibility, pluripotency and differentiation marker expression profiles were compared in 6-day EBs formed from each of the undifferentiated ES cell populations. As shown in Figure 12A, levels of the pluripotency markers Rex1, Dppa4, Esrrb, Klf2, Klf4, Oct4 and Nanog were significantly higher in EBs derived from ES cells expressing either wild-type or the active mutant of c-Yes compared with those from control ES cells and from cells expressing kinase-dead c-Yes. These results link c-Yes kinase activity to the expression of genes directly involved in self-renewal. The failure of these cells to downregulate pluripotency gene expression may account for their failure to form EBs.

We next explored changes in differentiation marker expression in the EBs derived from the same four ES cell populations. As shown in Figure 12B, Sox17/Gata4 (endoderm), Pax6, Fgf5 (ectoderm), Fgf8 (epiblast) and T (mesoderm) were expressed at similar levels in all four cell populations following six days of EB culture conditions. These observations support the idea that c-Yes kinase activity does not directly inhibit differentiation marker expression but instead maintains the expression of self-renewal genes which in turn prevents normal EB development. Similar results were seen with cells expressing either the wild-type or the constitutively active c-Yes proteins, providing further evidence that even a small increase in c-Yes kinase activity is sufficient to disrupt mES cell differentiation. Finally, the similarity of the results obtained with

the control cells and cells expressing kinase-dead c-Yes confirms that regulation of the self-renewal program by c-Yes is dependent on kinase activity.

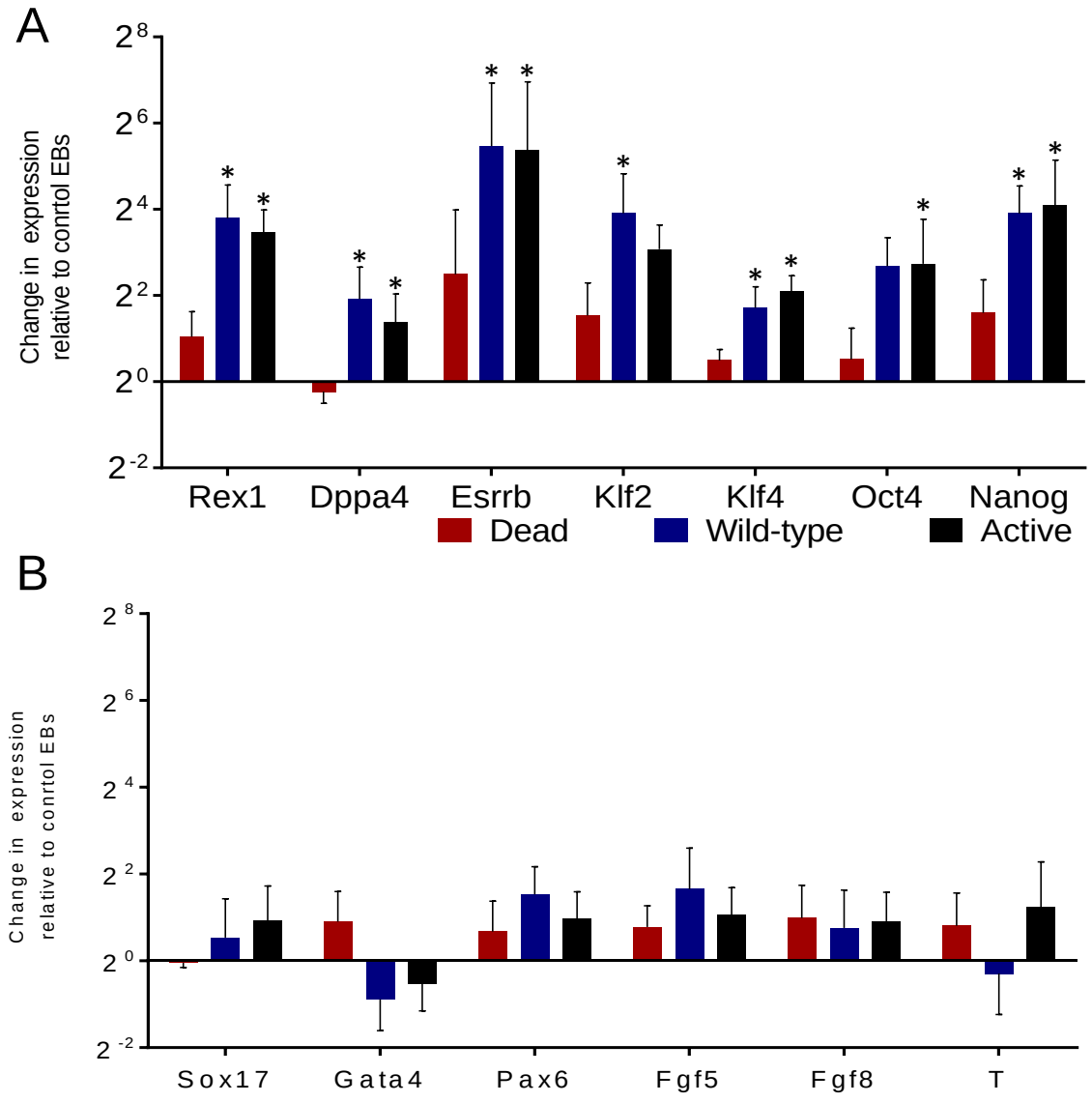

Figure 12. EBs formed from mES cells expressing active c-Yes Kinases retain pluripotency marker expression. ES cell populations expressing wild-type, active and dead forms of c-Yes as well as the vector control were cultured under conditions for EB formation. Total RNA was extracted from 6-day EBs followed by qPCR analysis with primers specific for the pluripotency (A) and differentiation (B) markers shown. The results are expressed as the average of the fold-change in each marker level in EBs derived from c-Yes transduced ES cells relative to control EBs ± S.E.M. (n=3, *P < 0.05, Pair-wise Fixed Reallocation Randomization Test.) The lineages represented by each of the differentiation markers are as follows: Sox17 and GATA4, endoderm; Pax6, ectoderm; Fgf5, Fgf8, ectoderm/epiblast; T, mesoderm.

2.3.6 Design of c-Yes gatekeeper mutants resistant to the broad spectrum Src-family kinase inhibitor, A-419259.

Selective kinase inhibitors represent valuable probes for biological function. Unlike genetic knock-outs or RNAi-based approaches, inhibitor treatment has immediate biological impact, and does not allow the system to respond with compensatory changes in gene expression. However, isoform-selective inhibitors of c-Yes or other members of the Src-kinase family are currently unavailable, due to the close sequence and structural similarity of the individual family members. To circumvent this issue for c-Yes in ES cells, we turned to a chemical genetics approach previously developed in our laboratory to demonstrate a role for c-Src kinase activity in ES cell differentiation to primitive ectoderm (Meyn, III and Smithgall, 2009). This method paired the broad-spectrum Src-family kinase inhibitor A-419259 with a c-Src variant engineered to be resistant to this compound. Introduction of the inhibitor-resistant (Src-IR) mutant into ES cells had no effect in the absence of inhibitor treatment. However, ES cells expressing Src-IR differentiated upon addition of A-419259, indicating that c-Src kinase activity, in the absence of all endogenous SFK activity, was sufficient to induce this differentiation response.

To create analogous IR variants of c-Yes, we substituted the threonine residue at the c-Yes kinase domain gatekeeper position (T338) with methionine (Figure 13A) in the context of the tail-activated (Y527F) form of c-Yes described above. Tail-activated mutants of c-Yes with wild-type and IR kinase domains were then expressed in Rat2 cells, followed by soft-agar colony assays in the presence of A-419259. Figure 13B shows that both populations of Rat2 cells formed transformed colonies. Colony formation by Rat2 cells expressing tail-activated c-Yes with a wild-type kinase domain was very sensitive to A-419259 treatment, with more than a 90% reduction in colony number at an inhibitor concentration of 100 nM. In contrast, colony

formation by Rat2 cells expressing the tail-activated c-Yes mutant with the IR kinase domain was unaffected by A-419259 treatment even at a concentration of 1 μM. To correlate transforming activity with kinase function, c-Yes proteins were immunoprecipitated from the transformed cell populations and immunoblotted with a phosphospecific antibody to the c-Yes activation loop tyrosine as described earlier. As shown in Figure 13C, c-Yes from cells transformed by active Yes with a wild-type kinase domain was sensitive to A-419259 treatment, while c-Yes-IR remained active at all concentrations of A-419259 tested, consistent with colony formation data. These experiments show that methionine substitution of the c-Yes gatekeeper threonine results in inhibitor resistance in the same manner as observed previously with c-Src.

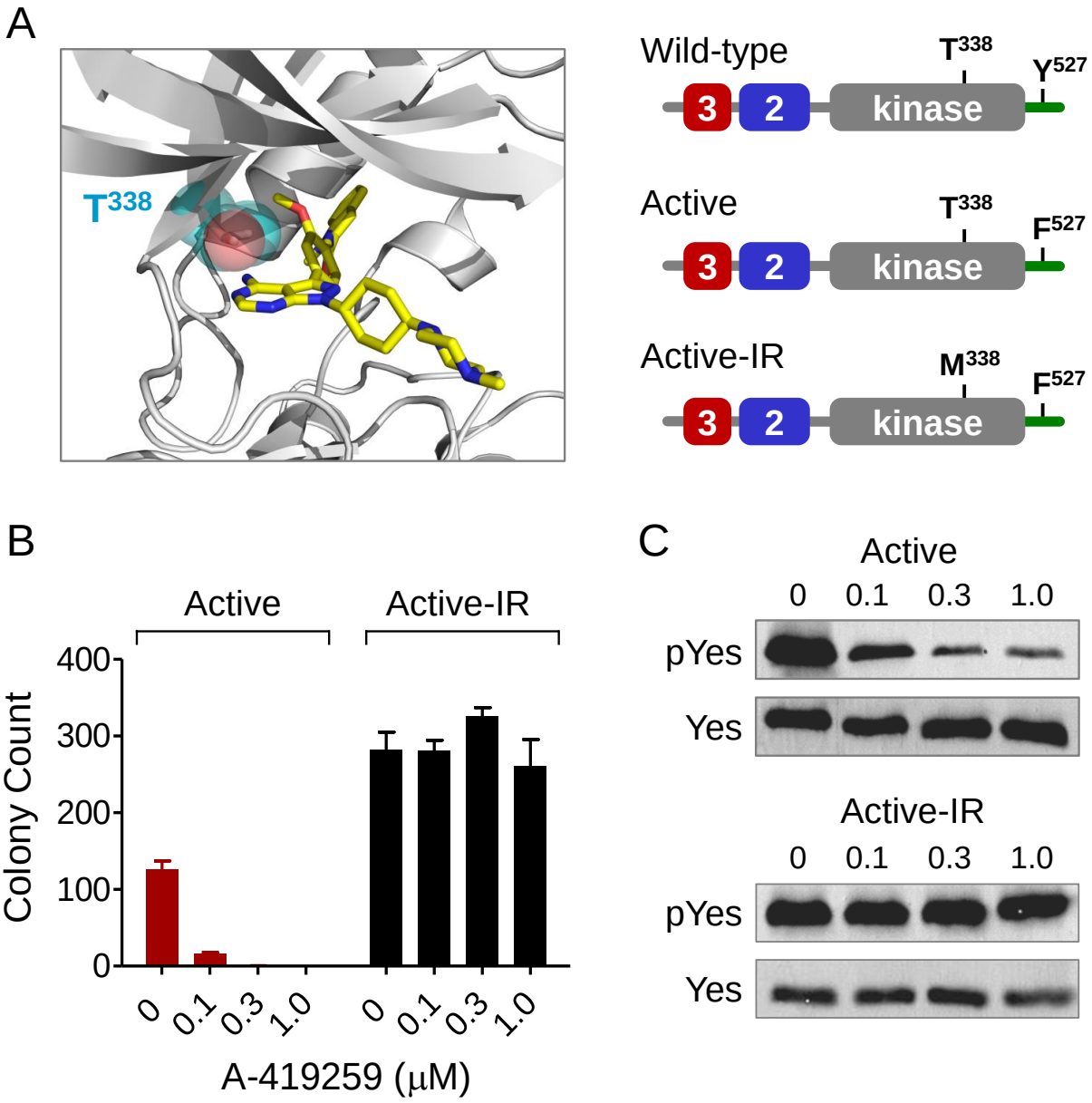

Figure 13. Validation of inhibitor-resistant mutants of c-Yes in Rat-2 fibroblasts. *A) Right:* Close-up view of the active site of the Src-family kinase Hck highlighting the juxtaposition of the kinase domain gatekeeper threonine (T338) and an analog of the broad spectrum Src-family kinase inhibitor, A-419259 (model rendered using the PDB file 2C0I; inhibitor carbon atoms in yellow). For fibroblast transformation experiments, the gatekeeper threonine was replaced with methionine and combined with an activating tail mutation to create the Active-IR mutant shown on the left. B) Rat2 fibroblasts were infected with recombinant retroviruses expressing tail-activated mutants of c-Yes with wild-type and IR kinase domains and assayed for soft-agar colony formation in the presence of the A-419259 at the concentrations shown. Transformed colonies were counted 12 days later from triplicate assays and the data are shown as the average colony count ± S.D. C) Rat2 cells expressing the Active and Active-IR forms of c-Yes were treated in the presence or absence of A-419259 for 48 hours. c-Yes proteins were immunoprecipitated from cell lysates and blotted with a phosphospecific antibody (pYes) and for c-Yes protein recovery.

2.3.7 Stable expression of inhibitor-resistant c-Yes (Yes-IR) in ES cells does not affect self-renewal marker expression.

We next infected ES cells with the wild-type or IR c-Yes retroviral vectors or with an empty vector as a control, followed by selection with G418. In the absence of A-419259 treatment, all three ES cell populations grew as colonies characteristic of undifferentiated ES cells (Figure 14A). In the presence of A-419259, cells expressing wild-type c-Yes as well as the vector control cells formed small tight colonies devoid of flattened, differentiated cells, consistent with our previous results with ES cells grown in the presence of this inhibitor(Meyn, III and Smithgall, 2009; Meyn, III et al., 2005). In contrast, the Yes-IR ES cells formed larger colonies compared to control ES cells, with some flattened colony morphology when cultured in the presence of the inhibitor. Active c-Yes was readily detected in Yes-IR expressing cells treated with A-419259, but not in control cells or cells expressing Yes-WT, verifying that Yes-IR remains active in A-419259 treated cells (Figure 14B). We next examined the effect of inhibitor treatment on the expression of self-renewal and differentiation markers in Yes-IR cells (Figure 14C). No significant expression changes were observed following qPCR analysis of the pluripotency markers Rex1, Nanog and Oct4 or the differentiation markers T (mesoderm) and Fgf5 (ectoderm). These experiments suggest that under conditions where c-Yes is the lone active Src family member present, no major changes in ES cell pluripotency or differentiation occur.

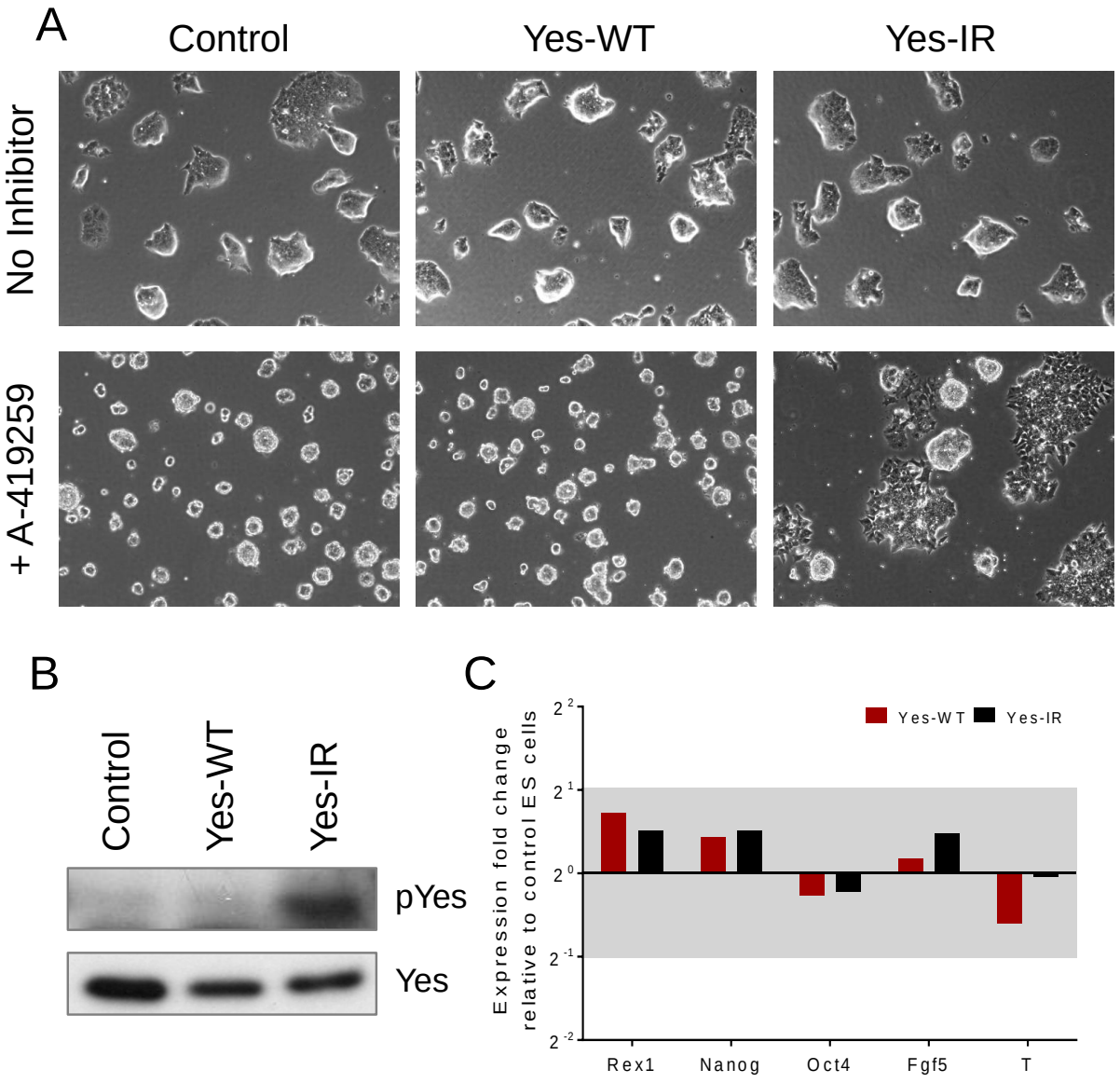

Figure 14. Stable expression of inhibitor-resistant c-Yes in ES cells. A) ES cells were infected with recombinant retroviruses carrying wild-type c-Yes (Yes-WT), the IR mutant (Yes-IR), or an empty vector (Control) and selected with G418. The infected ES cell populations were cultured under self-renewal conditions with LIF and in the presence or absence of A-419259 (1 µM) for 48 hours. Representative bright-field images are shown. Magnification; 100X. B) Kinase activity. Each of the ES cell populations from part A were treated with A-419259 (1 µM) for 48 hours. c-Yes proteins were immunoprecipitated from cell lysates and blotted with a phosphospecific antibody (pYes) and for c-Yes protein recovery. C) Src-family kinase inhibitor treatment does not influence expression of self-renewal or lineage markers in Yes-IR cells. ES cells expressing wild-type c-Yes (Yes-WT), the IR mutant (Yes-IR), as well as the vector control cells were grown in the presence of LIF and A-419259 (1 µM) for 4 days. RNA was then extracted and expression of Rex1, Nanog, Oct4, Fgf5 and T was determined by qPCR. Results are expressed as fold change between the ES cell populations expressing the wild-type or IR forms of c-Yes and the control ES cells. All five markers examined varied by less than two-fold in each of the transduced cell populations (shaded grey area). This experiment was repeated twice with comparable results; a representative example is shown.

2.3.8 Differentiation of ES cells driven by c-Src is antagonized by c-Yes.

Previous work from our group demonstrated that c-Src activity alone is sufficient to drive differentiation of ES cells using an analogous inhibitor-resistant allele of c-Src and A-419259 (Meyn, III and Smithgall, 2009). In contrast, work presented here demonstrates that despite close structural homology to c-Src, c-Yes activity inhibits rather than promotes ES cell differentiation. To determine which of these two opposing signals is epistatic to the other, we introduced the IR forms of both c-Yes and c-Src into ES cells. This was accomplished using c-Yes-IRES-Puro and c-Src-IRES-Neo retroviral vectors, followed by double selection with puromycin and neomycin. When cultured under standard conditions in the absence of A-419259, the resulting four cell populations grew with similar undifferentiated colony morphology (Figure 15A). Ectopic expression of the c-Yes and c-Src transgenes was confirmed by RT-PCR with vector-specific primers (Figure 15B). The presence of the c-Src and C-yes gatekeeper mutations (T338M) was confirmed in the PCR products from the transduced cell lines by nucleotide sequencing (data not shown). Selected pluripotency and differentiation markers were also examined by qPCR (Figure 15C). No changes in marker expression were observed in cells expressing c-Src-IR, c-Yes-IR or both IR mutants in the absence of A-419259 treatment.

We next examined the effect of A-419259 treatment on cell morphology, pluripotency and differentiation marker expression in ES cells expressing Src-IR or Yes-IR either alone or in combination. Cells were cultured in the presence of A-419259 and LIF for 4 days and images of the resulting colonies are shown in Figure 16A. With A-419259 treatment, control ES cells formed tight, small colonies, while colonies formed from the c-Yes-IR cells showed a more flattened morphology as described earlier. In contrast, cells expressing c-Src-IR or both inhibitor-resistant alleles grew as flat sheets in the presence of the inhibitor. We then assayed

relative expression levels of pluripotency (Tbx3, Rex1, Nanog, Oct4 and Gbx2) and differentiation markers for endoderm (Sox17, Gata4), ectoderm (Fgf5, Sox1, Nestin), mesoderm (T), epiblast (Fgf8) and trophectoderm (Hand1) by qPCR. The data are presented in Figures 16B and C as changes in expression relative to the control cell population. Inhibitor treatment of ES cells expressing c-Src-IR alone resulted in the upregulation of Gbx2, Sox17, Gata4, Gata6, Fgf5 and Hand1 expression, indicative of differentiation to endoderm and trophectoderm in addition to primitive ectoderm-like cells as reported previously by our group (Meyn, III and Smithgall, 2009). This finding suggests that c-Src selectively regulates a braoder range of differentiation responses that originally reported. In contrast to c-Src, the c-Yes-IR cells did not express any of these differentiation markers in response to A-419259 treatment. This observation is consistent with the idea that c-Yes signals help to maintain ES cells in an undifferentiated state.

Expression of both inhibitor resistant alleles in the same cell population revealed dominance of c-Yes over c-Src in terms of a subset of differentiation marker expression. As shown in Figure 16C, induction of the endoderm markers Sox17, Gata4 and Gata6 by c-Src was significantly repressed in cells expressing IR alleles of both c-Src and c-Yes vs. c-Src alone. In contrast, expression of Gbx2, Fgf5 and Hand1 remained the same when both IR alleles were expressed, raising the possibility that other renewal-related SFKs (e.g., Hck) may influence these pathways. This observation demonstrates that the c-Yes anti-differentiation signal is epistatic to the c-Src signal for endoderm differentiation, despite the close structural homology of these two Src family kinases.

Recent studies have shown that c-Src promotes the EMT (Li et al., 2011), a process essential for lineage specification during development (Thiery et al., 2009). To investigate a possible connection of the EMT to Src-induced differentiation of ES cells, we assayed the

relative expression levels of EMT markers previously linked to c-Src, including Igf2, SIP1, Ncad, Snail1, Twist1 and Eomes. We also assessed changes in the expression of matrix metalloproteinases (MMPs), which play an important role in the EMT. As shown in Figure 16D, A-419259 treatment of ES cells expressing c-Src-IR alone resulted in up-regulation of all of these EMT markers, consistent with a role of the EMT in c-Src-mediated differentiation of ES cells. In contrast, c-Yes-IR cells did not express any of the EMT markers tested in response to A419259 treatment. Interestingly, co-expression of c-Yes-IR suppressed c-Src-mediated induction of Snail1, Twist1 and MMP14 expression, with a partial reversal of MMP9 expression in response to inhibitor treatment of ES cells expressing both IR alleles. These results show that c-Src activity alone is sufficient to promote EMT marker expression and differentiation, while c-Yes activity inhibits both c-Src mediated EMT and endodermal differentiation.

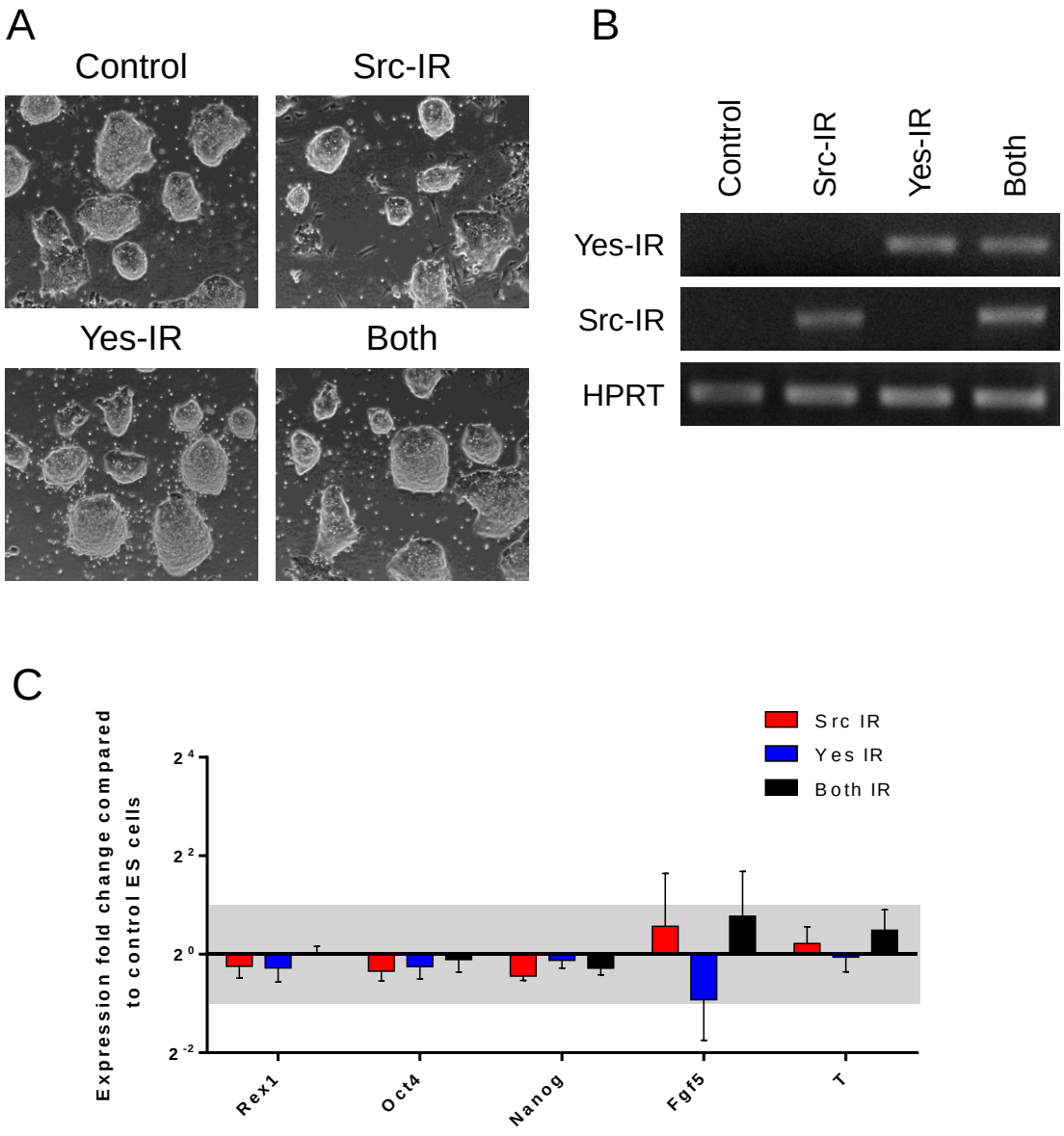

Figure 15. Co-expression of inhibitor-resistant mutants of c-Yes and c-Src in ES cells does not affect self-renewal or differentiation marker expression in the absence of A-419259 treatment. A) ES cell populations expressing the IR mutants of c-Src (Src-IR) or c-Yes (Yes-IR) either alone or in combination (Both) were cultured under self-renewal conditions with LIF for 48 hours. Cells transduced with the corresponding empty retroviral vectors served as the negative control. Representative bright-field images are shown. Magnification; 100X. B) Vector-derived Yes-IR and Src-IR expression was confirmed by RT-PCR with vector-specific primers. A scanned image of the resulting agarose gel shows the expected 819 base-pair product for Src-IR and the 604 base-pair product for Yes-IR. A fragment of the HPRT sequence was amplified as a positive control. C) RNA was extracted from the ES cell populations in part A and expression of the pluripotency markers Rex1, Nanog and Oct4, and the differentiation markers Fgf5 and T was determined by qPCR. The analysis was performed in triplicate, and results are expressed as the fold change relative to the control ES cell population ± S.E.M. All five markers examined varied by less than two-fold in each of the transduced cell populations (shaded grey area).

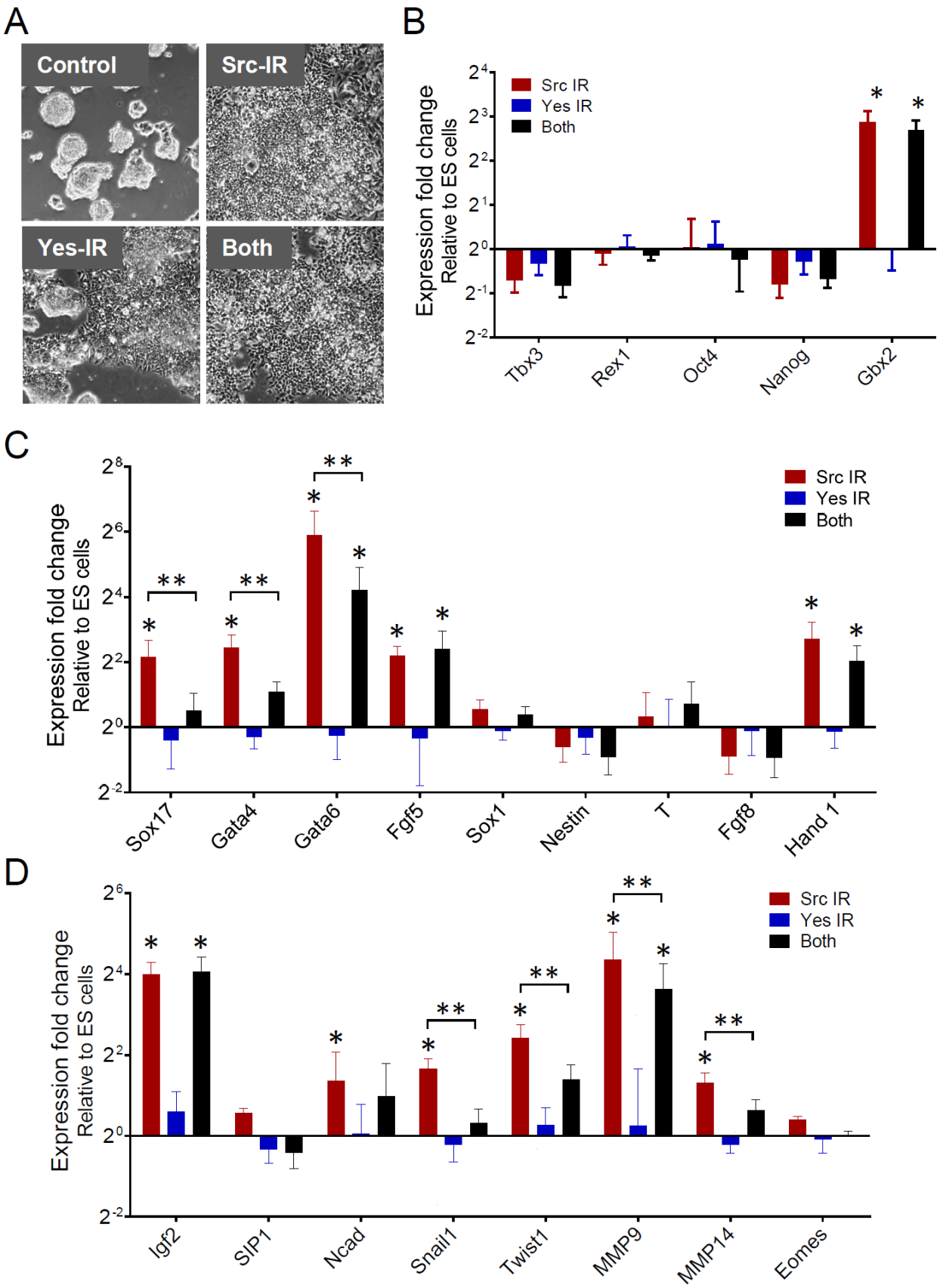
A
Control
Src-IR
Yes-IR
Both
B
Expression fold change
Relative to ES cells
Src IR
Yes IR
Both
Tbx3
Rex1
Oct4
Nanog
Gbx2
C
Sox17
Gata4
Gata6
Fgf5
Sox1
Nestin
T
Fgf8
Hand 1
D
Igf2
SIP1
Ncad
Snail1
Twist1
MMP9
MMP14
Eomes

Figure 16. Differentiation of mES cells driven by c-Src is antagonized by c-Yes. A) ES cell populations expressing inhibitor-resistant mutants of c-Src (Src-IR) or c-Yes (Yes-IR) either alone or in combination (Both) were cultured in the presence of LIF and A-419259 (1 μM). Cells transduced with the corresponding empty retroviral vectors served as the negative control. Representative bright-field images were recorded 4 days later. Magnification; 100X. B, C, D) RNA was extracted from the A-419259-treated ES cell populations shown in part A and qPCR analysis was performed with primers specific for markers of pluripotency (B), differentiation (C) and EMT (D). The analysis was performed on four replicates from two independently derived sets of ES cell populations, and results are expressed as the fold change relative to the control ES cell population ± S.E.M. (*$P < 0.05$ compared with control ES cells; **$P < 0.05$ compared with Src-IR cells; Pairwise Fixed Reallocation Randomization Test.) Note that expression of Src-IR alone induced differentiation in the presence of A-419259 treatment, with up-regulation of the endoderm markers Sox17, Gata4 and Gata6, the primitive ectoderm marker Fgf5, the trophectoderm marker Hand1, and the EMT markers Igf2, Snail1, Twist1, MMP9 and MMP14. Endoderm differentiation marker expression was blocked in cells co-expressing both Src-IR and Yes-IR and in the presence of the inhibitor. This antagonistic effect of Yes-IR correlates with the down-regulation of EMT markers Snail1, Twist1, MMP9 and MMP14.

2.4 DISCUSSION

Work presented here shows that the Src-family kinase c-Yes generates a potent anti-differentiation signal in mES cells. Enforced expression of either wild-type or an active form of c-Yes at modest levels completely inhibited differentiation of ES cells to EBs. This suppressive effect was not observed with kinase-defective c-Yes, indicating a requirement for c-Yes kinase activity. Interestingly, expression of both wild-type c-Yes as well as a kinase-active 'tail' mutant inhibited EB formation to the same extent, showing that ES cells are very sensitive to the c-Yes anti-differentiation signal. Our observations are consistent with prior studies showing that c-Yes kinase activity is stimulated by the self-renewal cytokine LIF and that RNAi-mediated knockdown of c-Yes induces ES cell differentiation (Anneren et al., 2004). Although c-Yes kinase activity interferes with EB formation, it cannot sustain self-renewal following LIF withdrawal. Indeed, ES cells expressing the active forms of c-Yes undergo morphological differentiation when cultured under adherent conditions in the absence of LIF. Furthermore, qPCR analysis showed that the small EBs formed from ES/c-Yes cells express the same

differentiation markers as control EBs (Figure 12). However, unlike control EBs, these cell clusters also continued to express pluripotency factors, including Oct4 and Nanog. Thus the presence of active c-Yes appears to prevent EB formation by interfering with the repression of pluripotency genes as opposed to blocking the differentiation program.

Previous studies have shown that individual members of the Src kinase family display distinct patterns of expression and activity during ES cell self-renewal and differentiation (see Introduction). Seven of the eight mammalian Src family members are expressed simultaneously in self-renewing mES cells (Meyn, III et al., 2005), making investigation of their individual contributions to self-renewal and differentiation a challenge. To address this problem, we developed a chemical genetics approach based on the broad-spectrum Src-family kinase inhibitor, A-419259. Treatment of ES cells with this inhibitor blocks all endogenous Src-family kinase activity in ES cells, locking them in an undifferentiated state (Meyn, III et al., 2005). Expression of a mutant of c-Src with engineered resistance to this inhibitor caused the ES cells to differentiate to primitive ectoderm-like cells (Meyn, III and Smithgall, 2009). This chemical genetics approach allowed us to demonstrate a role for c-Src in the earliest stages of ES cell differentiation for the first time. In the present study, we expanded our marker analysis and found c-Src activity alone is able to induce ES cell differentiation to cells with characteristics of both primitive ectoderm and endoderm, as exemplified by Fgf5, Sox17, Gata4 and Gata6 expression. Moreover, we discovered that c-Src activity alone drives the expression of EMT markers, consistent with a previous report of Src-mediated EMT during induction of ES cell differentiation through the Calcineurin-NFAT pathway (Li et al., 2011).

Here we also describe an analogous 'gatekeeper' mutant of c-Yes that is resistant to A-419259. Unlike c-Src, ES cells expressing this c-Yes mutant did not differentiate in response to

inhibitor treatment, and instead retained the same pluripotency gene expression pattern as control ES cells. Interestingly, when inhibitor-resistant mutants of both c-Yes and c-Src were co-expressed in ES cells, the presence of c-Yes activity prevented the induction of both endoderm and EMT marker expression by c-Src. These observations support a previous model of the regulation of endogenous c-Src and c-Yes kinase activity during ES cell renewal and differentiation (Meyn, III et al., 2005). In presence of LIF, both c-Yes and c-Src are active, but the presence of active c-Yes overrides the c-Src signal for differentiation. Upon LIF withdrawal, c-Yes activity is shut off, allowing active c-Src to drive differentiation. More broadly, our work suggests that although ES cells can be maintained in a perpetual state of self-renewal, they are poised to differentiate. Other studies have established that key differentiation genes are transcriptionally initiated in self-renewing ES cells, but are silenced by the master transcriptional regulators Oct4, Nanog and Sox2 (Jaenisch and Young, 2008). The ability of c-Yes kinase activity to prevent the transcriptional silencing of these master regulators of self-renewal as shown here may explain its potent suppressive effect on ES cell differentiation.

Our result is surprising given that c-Src, c-Yes and Fyn have been shown to play redundant roles and are able to compensate for one another in knockout mouse models (Stein et al., 1994; Lowell and Soriano, 1996; Klinghoffer et al., 1999). Although c-Yes and c-Src are similar in structure and are regulated by shared upstream signals, they are also have specific signaling roles (Sancier et al., 2011). The chemical genetics approach used here may be advantageous over gene knockdown in that it employs an inhibitor that is fast acting and reversible, ruling out the effects of compensation. Taking advantage of this system, we are investigating downstream targets that are differentially affected by Src-IR and Yes-IR. Our initial experiments suggest that SFK signaling may cross-talk with the Wnt signaling pathway.

Preliminary experiments show that c-Src may phosphorylate and activate GSK3β, thereby promoting β-catenin degradation. This outcome is consistent with a role for c-Src in differentiation, as β-catenin promotes renewal. In contrast, other work suggests that c-Yes may have a protective effect on β-catenin degradation. In HT29 colon cancer cell lines, c-Yes activity was associated with β-catenin localization and β-catenin target gene expression (Sancier et al., 2011).

In addition, SFKs have been shown to be required for integrin-FAK signaling and important for cytoskeletal organization, cell shape and adhesion (Klinghoffer et al., 1999). Here we show that A-419259 causes ES cells to adopt a small and tight morphology, devoid of flattened, differentiated cells. This indicates that SFK activity is important for flattened cellular morphology. However, either Yes-IR or Src-IR expression rescued this phenotype, indicating the redundant role of c-Src and c-Yes in maintaining cell shape. This might due to SFK interaction with the cytoskeletal and cell-cell interaction proteins. Since differentiation is associated with flattened cellular morphology, pan-SFK inhibition eliminates flattened cells and promotes self-renewal. Interestingly, SFK inhibition is found to promote direct reprograming of somatic cells to iPS cells by replacing Sox2 (Staerk et al., 2011).

In conclusion, we report that low-level, sustained expression of active c-Yes maintains ES cell renewal marker expression following LIF withdrawal, resulting in a profound block to EB formation. Using a chemical genetics approach, we demonstrated that the c-Yes signal favoring self-renewal is indirect opposition to the c-Src differentiation signal, despite the close structural homology between these kinases. A long-standing tenet of the Src-family kinase field is that individual family members have similar, if not redundant, biological functions. Our results clearly demonstrate that this is not always the case, and provide an important caveat to the use of

broad-spectrum, small molecule inhibitors of the entire Src kinase family to make conclusions about the biological activity of individual family members or the family as a whole. Finally, our results provide a strong rationale for the development of selective compounds to control c-Src vs. c-Yes activity; such compounds may allow more precise pharmacological manipulation of ES cell renewal and differentiation. Indeed, a recent unbiased chemical library screen identified broad-spectrum Src-family kinase inhibitors as potent enhancers of somatic cell reprogramming to an ES cell-like state (iPS cells) (Staerk et al., 2011). Our work predicts that a Src-selective inhibitor (or a c-Yes agonist) may provide an even greater enhancement in reprogramming efficiency.

2.5 MATERIALS AND METHODS

2.5.1 Cell culture

The mES cell line D3 was obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 1000 U/ml LIF as described previously (Meyn, III et al., 2005). Rat2 fibroblasts were obtained from the ATCC and maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimitotic as described elsewhere (Pene-Dumitrescu and Smithgall, 2010). For inhibitor treatment, ES cells (10^6) were plated on gelatin-coated 60 mm plates for 24 h. A-419259 (Santa Cruz Biotechnology) was added to the culture medium to a final

concentration of 1 µM using DMSO as the carrier solvent (0.1% final) (Meyn, III and Smithgall, 2009).

2.5.2 Retroviral transduction of mES cells and Rat2 fibroblasts

A cDNA encoding murine c-Yes was purchased from Thermo Scientific in the mammalian expression vector pSPORT6. Kinase-dead (Lys295 to Arg), active (Yes-Tyr527 to Phe; Yes-YF), and inhibitor-resistant (Thr338 to Met; Yes-IR) mutants of c-Yes were generated by site-directed mutagenesis (QuickChange XL method; Stratagene). Residues are numbered according to the crystal structure of c-Src (PDB: 2SRC) (Xu et al., 1999). The c-Yes cDNAs were subcloned into the retroviral expression vectors, pMSCV-IRES-Neo and pMSCV-IRES-Puro (Clontech) using the In-Fusion cloning method (Clontech). Analogous retroviral expression vectors for c-Src have been described elsewhere (Meyn, III and Smithgall, 2009). Retroviral stocks were produced by co-transfecting 293T cells with the retroviral vectors and an ectropic packaging plasmid and the resulting viral supernatants were used to infect ES cells as described (Cherry et al., 2000; Meyn, III and Smithgall, 2009). Transduced ES cell populations were selected and maintained with 250 µg/ml G418 or 1.5 µg/ml puromycin or both for double selection. Rat2 cells were infected as described previously (Pene-Dumitrescu and Smithgall, 2010), followed by selection with 800 µg/ml G418 for 14 days and maintenance in 400 µg/ml G418.

2.5.3 Embryoid body formation

Embryoid bodies were cultured as previously described (Meyn, III et al., 2005). Briefly, pluripotent ES cell colonies were trypsinized to single cells and plated at low density (5000 cells/ml) without LIF in 10 cm bacterial grade petri dishes to prevent attachment. EBs were fixed in 4% paraformaldehyde 6 d later, permeabilized with 0.1% Triton-X-100 in PBS, and stained with DAPI (Sigma). EBs were imaged by confocal microscopy and their size was estimated from the area of a 2D projection of the confocal images using the Olympus FV10-ASW 2.1 software as previously described (O'Reilly et al., 2011).

2.5.4 RT-PCR analysis

Total RNA was isolated from ES cell cultures with the RNeasy Plus Mini Kit (Qiagen) and cDNA was synthesized from 2 µg total RNA with a random decamer as primer according to the manufacturers' protocols (Ambion). For RT-PCR to confirm c-Yes and c-Src transgene expression, one-twentieth of each RT reaction was used in a 50 µl PCR reaction, with primers (5 µM) specific for detection of transcripts from the transduced retroviruses. PCR reaction products were resolved on 1% agarose gels and stained with ethidium bromide. For quantitative RT-PCR (qPCR) analysis, 1 µl of a 1:50 dilution of the cDNA reaction, primers (5 µM) and RT2 SYBR green qPCR master mix (Qiagen) were used for each reaction. Primer sets were obtained from Qiagen (Quantitative Primer Assays), and results were normalized using GAPDH as the reference gene. Quantitative PCR data were analyzed using the pairwise Fixed Reallocation Randomization Test and the REST 2009 software (Pfaffl et al., 2002). Primer efficiency was set

to a value of 1 and 5,000 iterations were used to calculate changes in expression and statistical significance. Results are reported as fold change in expression ± S.E.M.

2.5.5 Immunoprecipitation and immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer supplemented with phosphatase and protease inhibitors as described (Meyn, III et al., 2005). Cell lysates were clarified by centrifugation and 1 mg of protein extract was incubated with 1 µg of a c-Yes antibody (BD Biosciences, Cat. # BD610375) for 1 h. Protein G-Sepharose (20 µl of a 50% w/v slurry; Invitrogen, 10-1243) was added followed by incubation for an additional 3 h. Samples were washed three times with RIPA buffer, mixed with SDS-PAGE loading buffer, heated to 95 °C for 5 min and separated on SDS polyacrylamide gels. Electrophoresis samples were prepared in a similar fashion for cell lysates, followed by SDS-PAGE and membrane transfer.

For immunoblotting, proteins were transferred to PVDF membranes and probed with antibodies to the c-Yes activation loop, the c-Yes protein, Oct4, and actin as a loading control. The following primary antibodies were used at a 1:1000 dilution: active Src pY418 (Invitrogen 44660G), rabbit anti-Yes (Cell Signaling Technology, AB13954), mouse anti-Oct4 (Santa Cruz, SC-5279), and mouse anti-Actin (Millipore, MAB1501).

2.5.6 Rat2 cell soft-agar assay

Oncogenic transformation of Rat2 cells was assessed as colony forming activity as described previously (Pene-Dumitrescu and Smithgall, 2010). In brief, Rat2 cells transduced with various retroviral expression vectors were assayed in triplicate in 35 mm Petri dishes (BD Biosciences) using SeaPlaque agarose (Lonza). The bottom layer of 0.5% agarose was prepared in growth medium (1.0 ml) containing the DMSO carrier solvent alone (0.2%) or twice the final concentration of A-419259 where indicated. The top layer (1.0 ml) consisted of 0.3% agarose in growth medium contain 10,000 Rat2 fibroblasts. Colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) ten days later and counted from scanned images using Quantity One colony counting software (BioRad). For experiments with the Src-family kinase inhibitor, A-419259, the compound was added to the bottom agarose layer at twice the final concentration with DMSO as carrier solvent at 0.1% final concentration.

3.0 SRC FAMIY TYROSINE KINASE SIGNALING IS IMPORTANT FOR HUMAN EMBRYONIC STEM CELL DIFFERENTIATION

3.1 ABSTRACT

Embryonic stem (ES) cells are characterized by pluripotency, the developmental potential to generate cell lineages derived from all three primary germ layers. In the past decade, great progress has been made on the cell culture conditions, transcription factor programs and intracellular signaling pathways that control murine and human ES cells. Although both are derived from the inner cell mass of the blastocyst stage embryo, human ES (hES) cells and mouse ES (mES) cells have distinct culture conditions, and respond to different receptor tyrosine kinase signaling pathways. For example, FGF signaling is important to maintain hES cell pluripotency, yet induces differentiation in mES cells. Previous work from our group has implicated the Src family of non-receptor protein-tyrosine kinases in mES cell self-renewal and differentiation. Multiple members of the Src kinase family are expressed in mES cells, and individual family members appear to play distinct roles in regulating their developmental fate. Both Hck and Yes are important in self-renewal, while c-Src activity alone is sufficient to induce differentiation. While these findings implicate Src kinase signaling in mES cell renewal and differentiation, the role of this kinase family in hES cells is largely unknown. Here, I explored Src family kinase signaling in hES cells. First, quantitative real-time RT-PCR was used to

determine the relative expression profile of individual Src family members in undifferentiated hES cells vs. EBs derived from them. Of the eleven Src-related kinases in the human genome, Fyn, c-Yes, c-Src, Lyn, Lck and Hck were expressed in H1, H7 and H9 hES cells, while Fgr, Blk, Srm, Brk, and Frk transcripts were not detected. Of these, c-Yes, Lyn, and Hck transcript levels remained constant in self-renewing hES cells and differentiated EBs, while c-Src and Fyn showed a modest increase in expression as a function of differentiation. In contrast, Lck expression levels dropped dramatically as a function of EB differentiation. In addition, to assess the role of Src family kinase activity hES cells, we treated hES cell cultures with inhibitors specific for the Src kinase family, including Src Kinase Inhibitor 1, PP2 and A-419259. Untreated control hES cultures grown in mTeSR1 medium formed typical pluripotent colonies circumscribed by differentiated cells. Transfer of hES cells to differentiation medium resulted in flattened colony morphology that correlated with a loss of cell-surface staining for the hES cell pluripotency marker, Tra-1-60. Dramatically, hES cells maintained in the presence of 1 µM A-419259 retained the morphology of domed, pluripotent colonies and maintained TRA-1-60 expression in differentiation medium. Similar morphological changes were observed in SKI-1 treated cells. Taken together, these observations support a role for Src family kinase signaling in the regulation of hES fate, and suggest that some parallels may exist in mouse and hES cells for this intracellular signaling network.

3.2 INTRODUCTION

Human ES (hES) cells are pluripotent stem cells derived from the inner cell mass of blastocyst stage human embryos produced by in vitro fertilization (Thomson et al., 1998). Although hES

cells are of the same blastocyst origin as mES cells, they respond to different growth factors for maintenance in culture and depend on distinct receptor tyrosine kinase signaling pathways. For example, hES cells depend on bFGF and TGFβ/Activin signaling to maintain the undifferentiated state. While factors essential for mES cell renewal, LIF and BMPs, have no effect, or induce differentiation in hES cells, respectively (Yu and Thomson, 2008). FGF signals through the FGF receptor to activate MEK/Erk signaling to inhibit differentiation and PI3K-Akt signaling to promote survival (Dvorak et al., 2005; Li et al., 2007). In addition, the TGFβ/Nodal/Activin signaling inhibits neuronal differentiation, and works synergistically with FGF to maintain hES cell pluripotency (Vallier et al., 2005). Despite these and other differences between mES and hES cells, the transcription factors governing pluripotency are similar, with both mES and hES cells expressing the master pluripotency factors, Oct4, Nanog and Sox2 (Boyer et al., 2005). Moreover, hES cells are pluripotent, as they can from embryoid bodies in vitro and teratomas in vivo when injected into immunodeficient mice. While the growth factor conditions, receptor kinase signaling, and transcription factor networks have been investigated in hES cells, the signaling pathways downstream of receptor tyrosine kinases have not been fully explored.

As critical signal integrators, the Src family of non-receptor tyrosine kinases has been linked to many receptor tyrosine kinases including the FGFR to regulate cell adhesion, proliferation, growth and survival (Thomas and Brugge, 1997). There are 11 Src-related kinases in the human genome (Manning et al., 2002), eight of which have been studied extensively in mammalian cells: Blk, Fgr, Fyn, Lck, Lyn, Hck, c-Src and c-Yes. In the mouse, c-Src, Fyn and c-Yes are ubiquitously expressed, while Lck, Lyn, Hck, Blk and Fgr display more restricted expression to hematopoietic cells (Lowell and Soriano, 1996). Surprisingly, at least seven

members of the Src kinase family are expressed in mES cells, and individual family members appear to play distinct roles in regulating their developmental fate (Meyn, III et al., 2005). Expression of Hck is rapidly silenced as mES cells differentiate to embryoid bodies (EBs), suggesting a role in self-renewal. In contrast, c-Src activity alone is sufficient to induce differentiation of mES cells to primitive ectoderm (Meyn, III et al., 2005; Meyn, III and Smithgall, 2009). Other studies have shown that both Hck and c-Yes are important for mES cell self-renewal downstream of LIF (Ernst et al., 1994; Anneren et al., 2004; Tamm et al., 2011). While these findings implicate Src kinase signaling in mES cell renewal and differentiation, the role of this kinase family in hES cells is largely unknown.

To explore whether SFK signaling is conserved in hES cell renewal and pluripotency, I first showed that three hES cell lines, H1, H7 and H9, all express comparable levels of the SFK members c-Src, c-Yes, Fyn, Lck, Lyn and Hck. Next, I investigated changes in expression levels of each SFK during differentiation of hES cells to EBs. As hES cells differentiate, c-Src and Fyn transcript levels increased slightly, while Lck expression dropped dramatically. To assess the role of Src family kinase activity in hES cells, I treated hES cell cultures with the SFK-specific inhibitors. Human ES cells treated with the potent pyrrolopyrimidine SFK inhibitor A-419259, previously shown to block all endogenous SFK activity in mES cells, retained the morphology of pluripotent colonies and continued to express the cell-surface renewal marker TRA-1-60 under culture conditions for differentiation. These observations support a role for SFK signaling in the regulation of hES cell fate, and suggest that unique function for individual SFKs may exist in hES cells as observed previously in mES cells.

3.3 RESULTS

3.3.1 Human ES cells express multiple Src family kinases

Of the eleven Src-related kinases in the human genome, only c-Yes has been studied in hES cells. c-Yes kinase activity is downregulated as hES cells differentiate to EBs, while the c-Yes protein is expressed in both hES cells and differentiated EBs (Anneren et al., 2004). To study SFK signaling in hES cells, we first determined which SFK members are expressed in hES cells using RT-PCR. Human ES cell lines (H1, H7 and H9) were maintained in chemically defined mTeSR medium, under-feeder free conditions on Matrigel-coated plates (Ludwig et al., 2006). The cells grow as slightly domed colonies with some random differentiation at the edge of each colony (Figure 17A). RT-PCR analysis revealed that H1 hES cells express six SFKs, including c-Src, c-Yes, Fyn, Lck, Hck and Lyn. Of these, c-Src, c-Yes, Fyn and Lck are most highly expressed (Figure 17B). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and pluripotency marker, Pou-domain transcription factor Oct4 were used as positive control.

We next determined the relative expression levels of these six SFKs in comparison to the pluripotency markers Oct4 and Nanog, in the H1, H7 and H9 hES cell lines using quantitative real-time PCR (qPCR, Figure 17C). Compared with H1 hES cells, both H7 and H9 cells expressed comparable levels of the pluripotency marker Oct4 as well as the SFKs Fyn, c-Src, c-Yes, Lck and Lyn, with less than two-fold differences in relative expression levels. We also observed that H7 cells express higher levels of the pluripotency marker Nanog and the SFK Hck compared with H1 cells, while H9 cells express lower levels of Nanog, indicative of heterogeneity between these hES cell lines. In summary, these results show that hES cells

express multiple SFK members, and expression levels of SFKs c-Src, c-Yes, Fyn and Lck are largely consistent among the three hES cell lines tested.

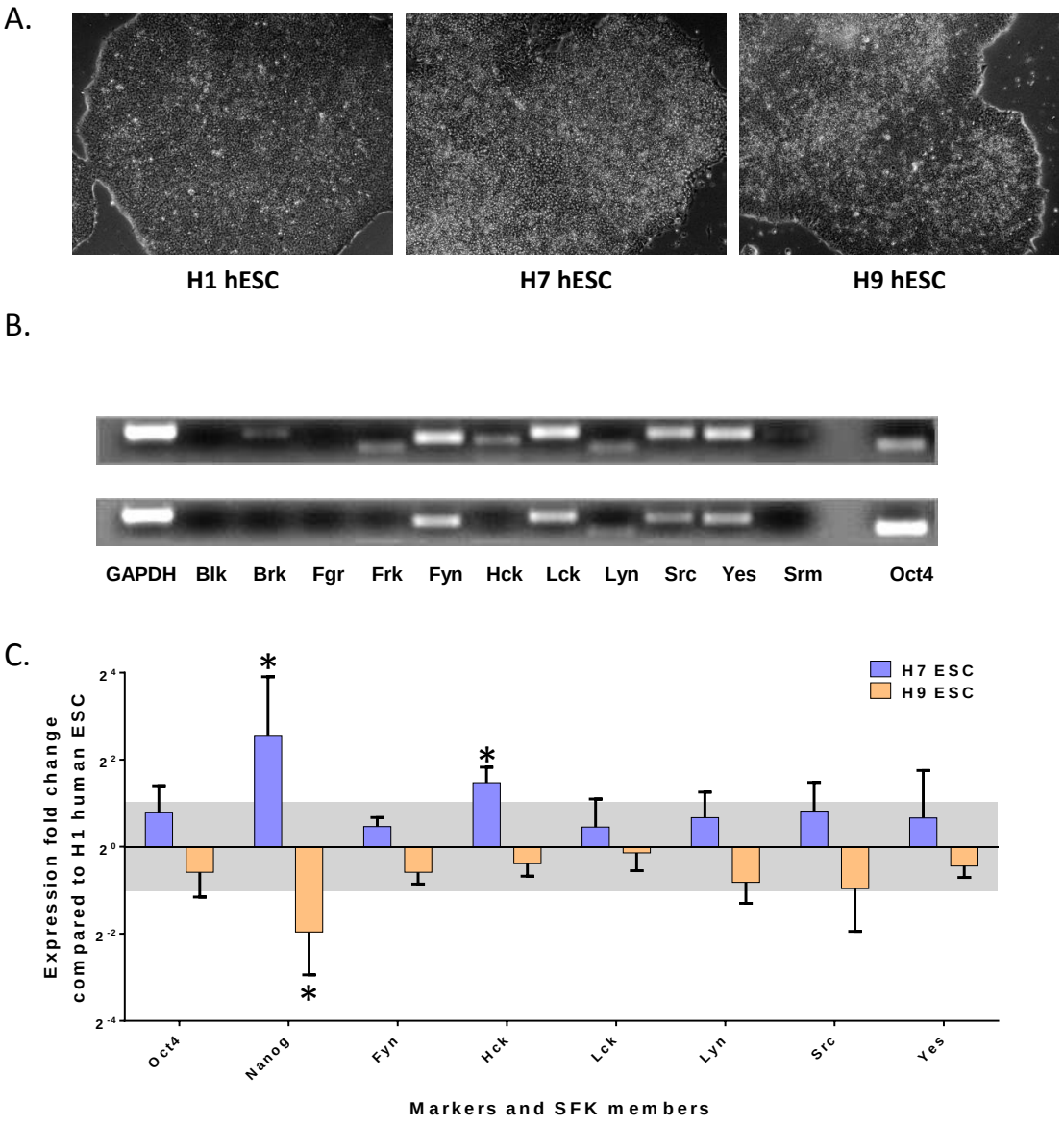

Figure 17. Src family kinase expression in hES Cells (A) Human ES cell lines (H1, H7 and H9) were maintained under feeder-free conditions on Matrigel-coated plates using mTeSR1 medium. Cell culture images were taken 5 days after passage, before harvesting for RNA isolation (magnification: 100×). (B) SFK expression was determined by RT-PCR using specific primers for each kinase with RNA isolated from H1 cells. Fyn, c-Yes, c-Src, Lyn, Lck and Hck were detected in H1 cells. (C) Relative SFK and Oct4 expression levels were determined by Q-PCR in H1, H7 and H9 cells. For each hES cell line, this experiment was repeated three times in different passages of cells. The results are presented as average expression fold change compared to that in H1 hES cells ± S.E.M (*P < 0.05, Pairwise Fixed Reallocation Randomization Test), with GAPDH as internal control. Oct4 and most of the SFK expression varied by less than two-fold (shaded grey area).

3.3.2 Src family kinase expression during human embryoid body differentiation

Embryoid body (EB) formation is a convenient model to assess the pluripotency of hES cells. When cultured in suspension culture without feeder layers, hES cells spontaneously form aggregates or EBs as previously described for mES cells (Itskovitz-Eldor et al., 2000). Developing EBs turn on expression of differentiation markers, contain cell types characterizing all three germ layers, and mimic the early developmental stages of gastrulation and germ layer formation (Murry and Keller, 2008).

Previous research from our group found that SFK members have dynamic changes of expression in response to murine ES cell differentiation (Meyn, III et al., 2005). To investigate whether SFK members are also differentially regulated during the differentiation of hES cells into EBs, I initiated EB formation from cultured H1, H7 and H9 cells. Aggrewell microplates were used to initiate formation of EBs that are homogenous in size and cell number (2000 hES cells/ EB), to allow direct and reproducible comparison (Mohr et al., 2010). H1 and H9 EBs were maintained in a commercial differentiation medium (Aggrewell medium, Stemcell technologies), while H7 EBs were maintained in hES cell differentiation medium. EBs were harvested 3, 6 and 12 days after EB initiation. Total RNA was extracted from EBs derived from all three hES cell lines, followed by qRT-PCR analysis of SFK expression as well as markers of self-renewal and differentiation (Figures 18, 19 and 20).

In all three hES cell lines tested, Lck expression levels were downregulated in response to EB differentiation, suggesting that Lck might be important for hES cell renewal. In contrast to Lck, c-Src and Fyn expression were increased approximately 2-fold in 12 day EBs compared with corresponding hES cells, indicating that c-Src and Fyn might be related to differentiation. In

addition, the expression levels of pluripotency markers Oct4 and Nanog were consistently downregulated, suggesting that the human EBs were differentiated.

In addition we observed some cell-line specific expression changes. In H1 hES cells (Figure 18), both Lyn and Hck were downregulated in 12 day EBs compared with renewing H1 ES cells, while only Sox1 was upregulated among the differentiation markers analyzed. The differentiation markers showed a trend of increased expression in response to EB formation. However, the heterogeneous nature of hES H1 cell line may express high levels of these differentiation markers as well, masking the increase in response to EB formation. In H9 hES cells (Figure 19), Hck expression levels were down-regulated, while most differentiation markers analyzed were upregulated during EB differentiation. In contrast, in H7 hES cells (Figure 20), expression of Hck was increased in response to EB differentiation, while the differentiation markers T and Gata4 were both upregulated. These results indicate that hES cell lines are heterogeneous with respect to differentiation marker and SFK expression changes during EB differentiation.

Despite the heterogeneity mentioned above, Lck expression was consistently downregulated while c-Src and Fyn expression were up-regulated during EB differentiation of H1, H7 and H9 hES cells. These dynamic changes in individual SFK expression support distinct functions for SFKs in the regulation of hES cell self-renewal and differentiation, similar to what we have already defined in murine ES cells.

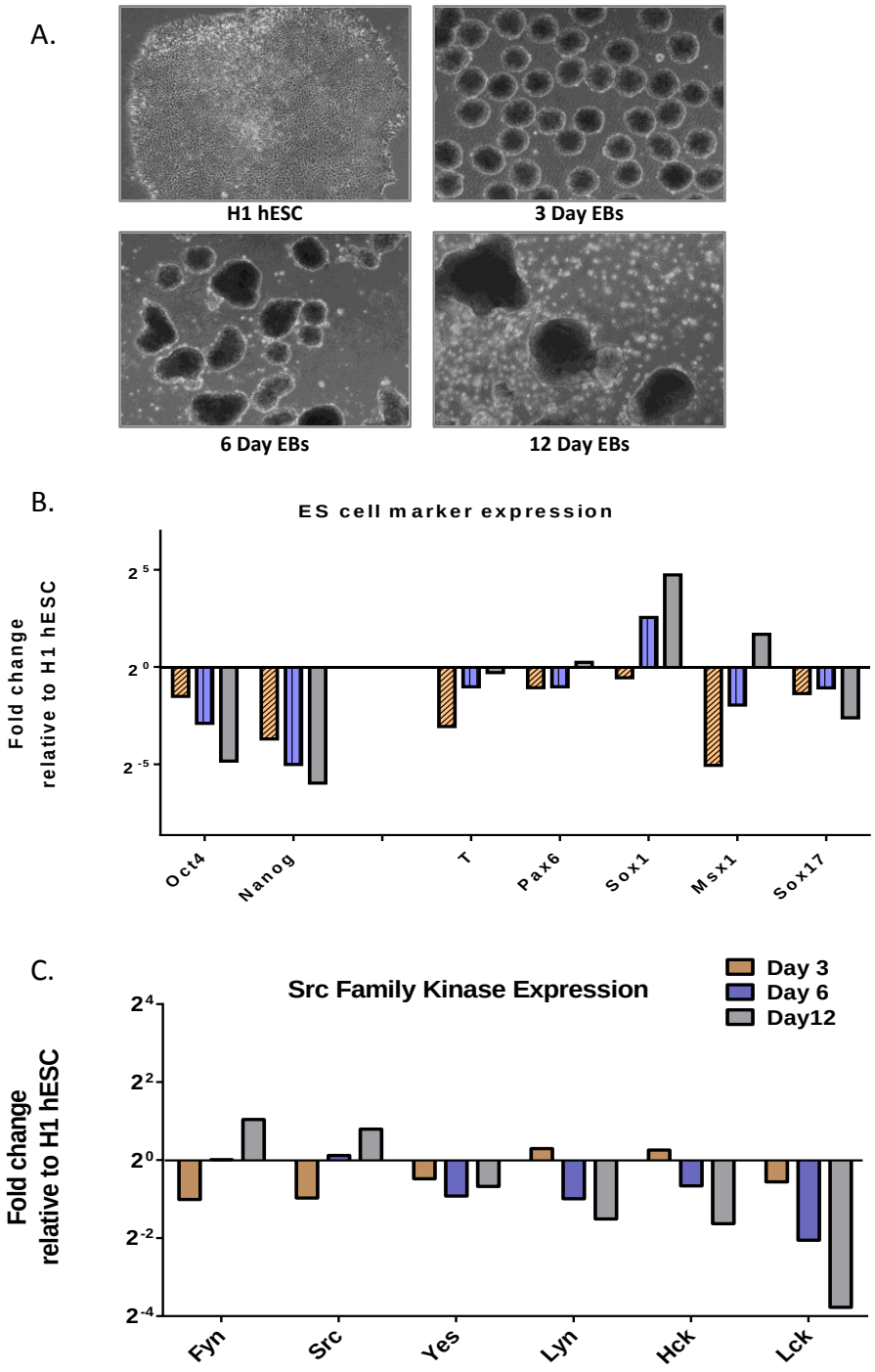

Figure 18. Src family kinase expression during EB formation from H1 Cells. A.) EB formation was initiated from H1 ES cells using Aggrewell plates (StemCell Technologies), with 2000 cells/EB. EBs were maintained in Aggrewell medium in suspension culture. EB images were taken 3, 6 and 12 days later and before harvest for RNA and qPCR analysis (Magnification 100×). B.) RNA was extracted from 3 day, 6 day and 12 day EBs. Expression of self-renewal (Oct4, Nanog) and differentiation markers (T, mesoderm; Pax6, Sox1, Msx1, ectoderm; and Sox17, endoderm) was determined by Q-PCR relative to control hES cells maintained in mTeSR medium. C.) Expression of the SFKs Fyn, c-Src, c-Yes, Lyn, Hck and Lck was determined by Q-PCR. This experiment was performed twice with similar results; one representative set of data is shown.

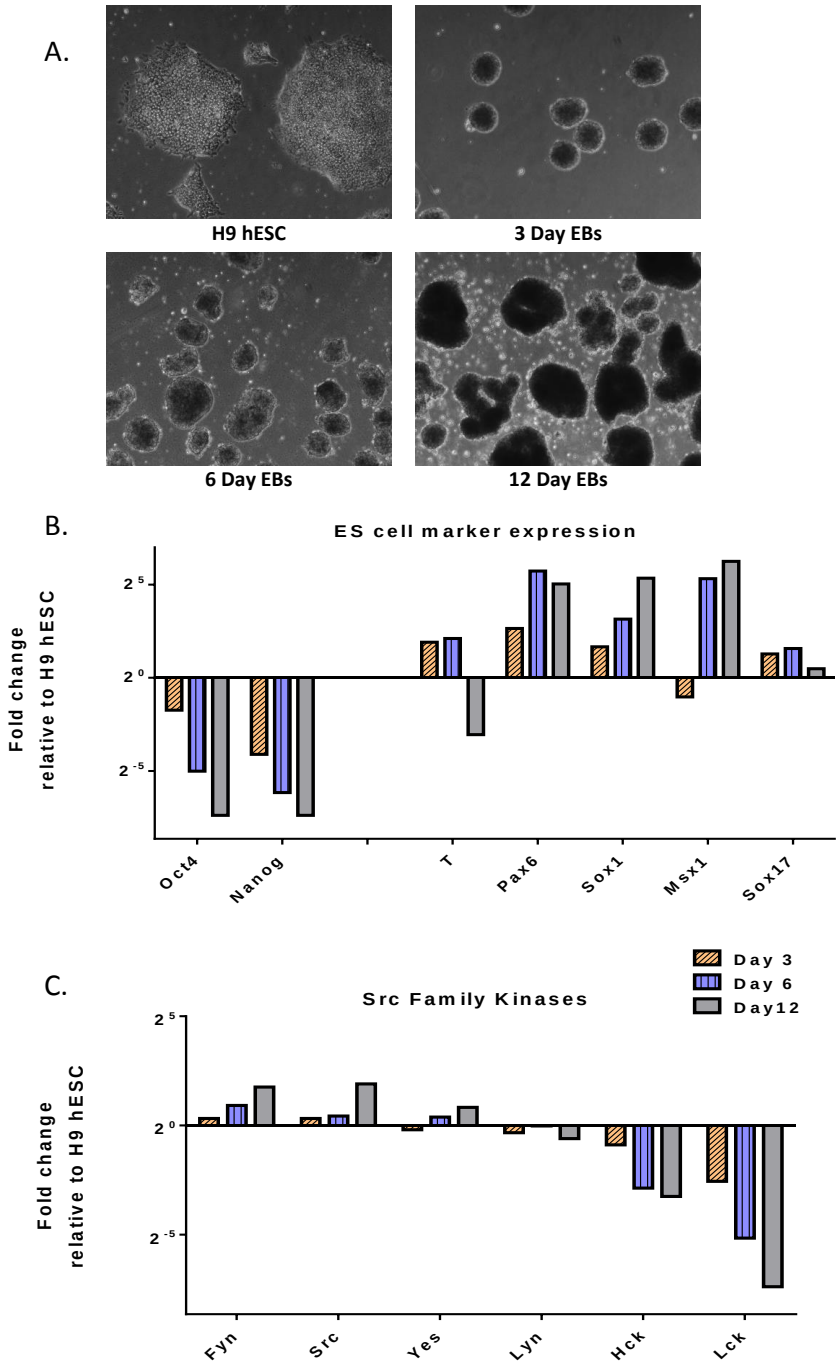

Figure 19. Src family kinase expression during EB formation from H9 cells. A.) EB formation was initiated from H9 ES cells using Aggrewell plates (StemCell Technologies), with 2000 cells/EB. EBs were maintained in Aggrewell medium in suspension culture. EB images were taken 3, 6 and 12 days later and before harvest for RNA and qPCR analysis (Magnification 100×). B.) RNA was extracted from 3 day, 6 day and 12 day EBs. Expression of self-renewal markers (Oct4, Nanog) and differentiation markers (T, mesoderm; Pax6, Sox1, Msx1, ectoderm; and Sox17, endoderm) was determined by Q-PCR relative to control hES cells maintained in mTeSR medium. C.) Expression of SFKs Fyn, c-Src, c-Yes, Lyn, Hck and Lck was determined by Q-PCR. This experiment was performed twice with similar results, and one representative set of data is shown.

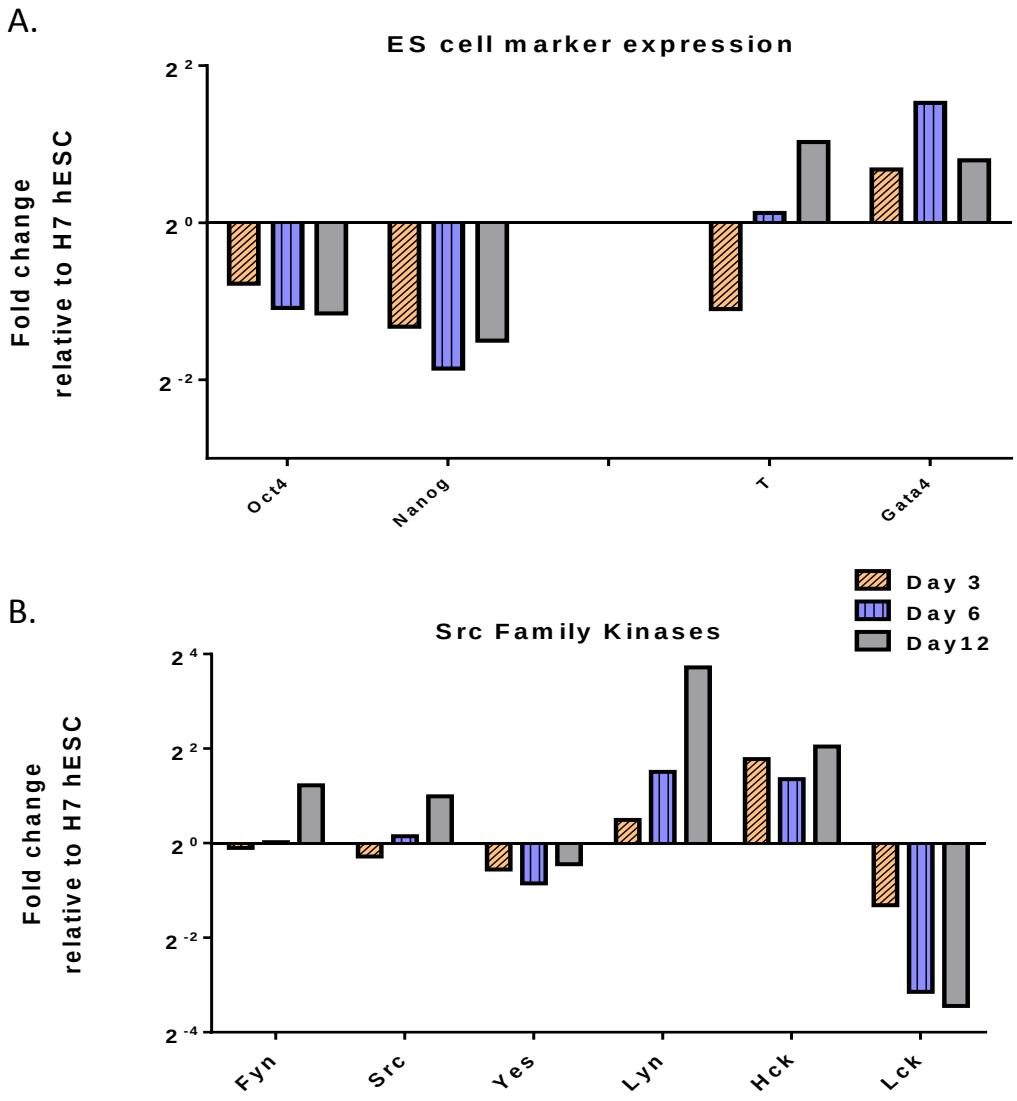

Figure 20. Src family kinase expression during EB formation from H7 cells. EB formation was initiated from H7 ES cells using Aggrewell plates (StemCell Technologies), with 2000 cells/EB. EBs were maintained in hESC differentiation medium in suspension culture. A.) RNA was extracted from 3 day, 6 day and 12 day EBs. Expression of self-renewal markers (Oct4, Nanog) and differentiation markers (T, mesoderm and Gata4, endoderm) was determined by Q-PCR relative to control hES cells maintained in mTeSR medium. B.) Expression of SFKs Fyn, c-Src, c-Yes, Lyn, Hck and Lck was determined by Q-PCR. This experiment was performed twice with similar results, and one representative set of data is shown.

3.3.3 Src family kinase inhibition blocks hES cell differentiation

The dynamic changes in SFK expression that occur during EB formation suggest that the activity of this kinase family might be important in early development. To test whether the SFK activity is required for hES cell maintenance and differentiation, we treated hES cells in mTeSR renewal medium or differentiation medium with small molecule inhibitors that are specific against the Src family as a whole. Three different SFK inhibitors were used: Src Kinase Inhibitor-I (SKI-1), PP2 and A-419259. Previous studies from our lab with these three inhibitors showed that partial suppression of overall SFK activity with lower concentrations of these inhibitors led to mES cell differentiation, while complete suppression of all SFK activity suppressed mES cell growth and delayed differentiation (Meyn, III et al., 2005). Using the inhibitor concentrations determined in the previous study with mES cells, we tested whether the same effects are observed in hES cells. H1 hES cells were passaged onto Matrigel-coated plates and grown in mTeSR medium for three days. The culture medium was then replaced with differentiation medium (DM) or fresh renewal medium (mTeSR) with or without the SFK inhibitors for an additional three days. The morphology of the resulting hES cell cultures was then recorded.

SKI-1, a 4-anilinoquinazoline with moderate potency against SFKs, was first examined. As shown in Figure 21A, untreated control H1 cells formed slightly domed, pluripotent colonies circumscribed by some differentiated cells in mTeSR medium, while transfer of hES cells to differentiation medium resulted in flattened colony morphology. Incubation with SKI-1 at a concentration of 5 μM had no effect on colony morphology in cells either in mTeSR or in differentiation medium. However, treatment of hES cells with a higher concentration (10 μM) of SKI-1 resulted in maintenance of typical undifferentiated hES cell colony morphology, despite

the switch to differentiation medium. These results suggest that inhibition of SFK activity with SKI-I blocked hES cell differentiation.

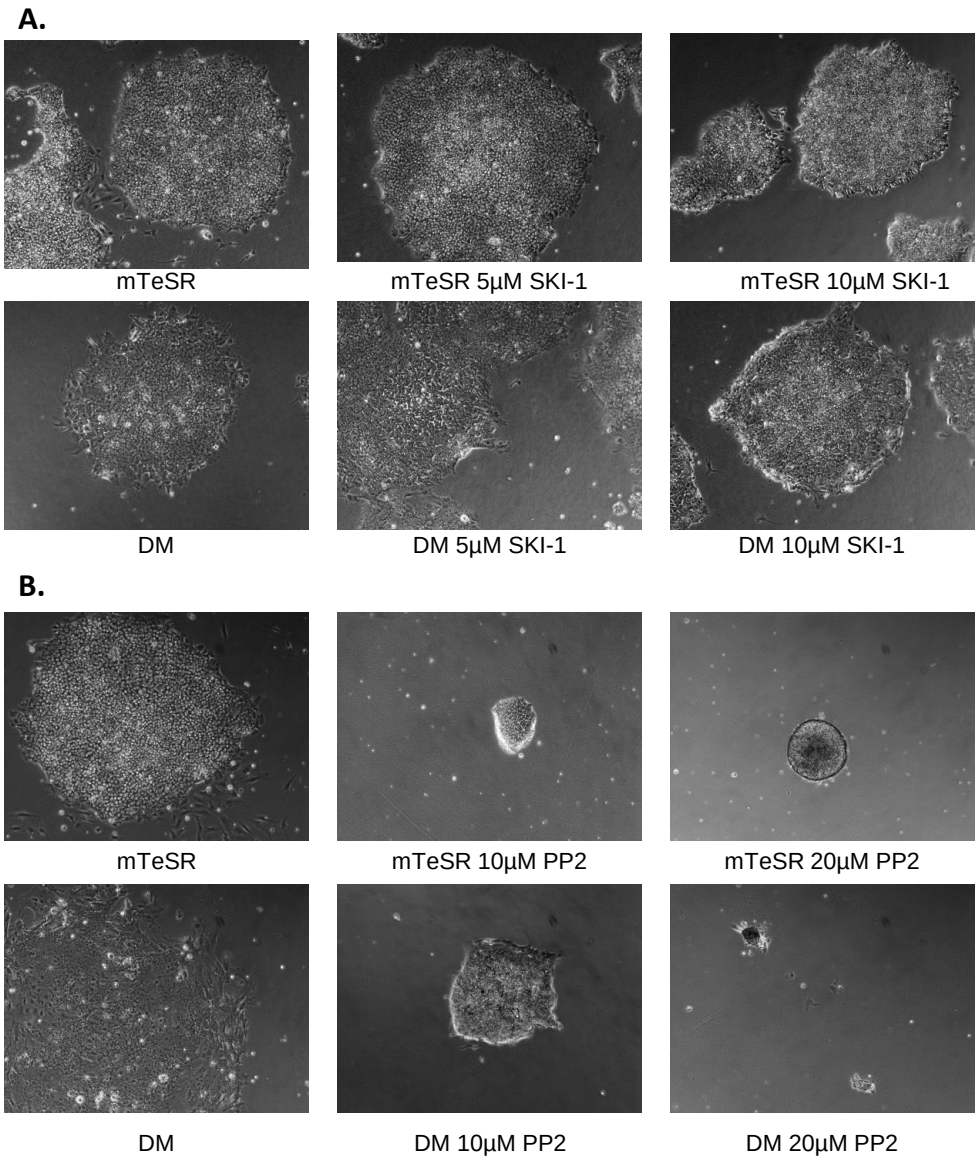

Figure 21. Inhibition of SFK activity with SKI-1 and PP2 in hES cells. H1hES cells were maintained in mTeSR medium or switched to differentiation medium (DM; DMEM/F-12 with 5% knockout serum replacement and 15% Fetal Bovine Serum) with or without the SFK inhibitor SKI-1 (A) and PP2 (B) at the concentrations indicated. Images were recorded three days later (Magnification 100×).

PP2, a pyrazolo-pyrimidine SFK inhibitor, was tested next. As shown in Figure 21B, PP2 treatment at 10 μM maintained hES cell colony morphology in differentiation medium. However, PP2 also reduced the overall number and size of the attached hES cell colonies in both mTeSR and differentiation medium, suggesting that PP2 may be toxic to hES cells at this

concentration. Indeed, at a higher PP2 concentration (20 μM), very few colonies of hES cells remained, and most colonies rounded up and detached.

A-419259, a SFK inhibitor with more specificity and higher potency than PP2, was also tested on the H1 line of hES cells (Figure 22A). A-419259 treatment had no effect on the colony morphology of hES cells in mTeSR medium. However, when hES cells were switched to differentiation medium in the presence of 1 μM A-419259, they retained the morphology of domed, pluripotent colonies. This effect was not observed when the inhibitor concentration was lowered to 0.3 μM,suggesting a threshold effect. The same effect of A-419259 treatment on hES cell differentiation was also observed with the H7 line of hES cells, as shown in Figure 22 C.

We next investigated whether the observed effect of A-419259 treatment on hES cell colony morphology correlated with inhibition of endogenous SFK activity. For this experiment, self-renewing H1 hES cells were treated overnight with A-419259 at 1 μM, followed by immunoblotting of cell extracts with the pY418 phosphospecific antibody to detect active SFKs. As shown in Figure 22B, constitutive SFK activity was readily detected in untreated hES cells, while 1 μM of A419259 completely blocked SFK activity.

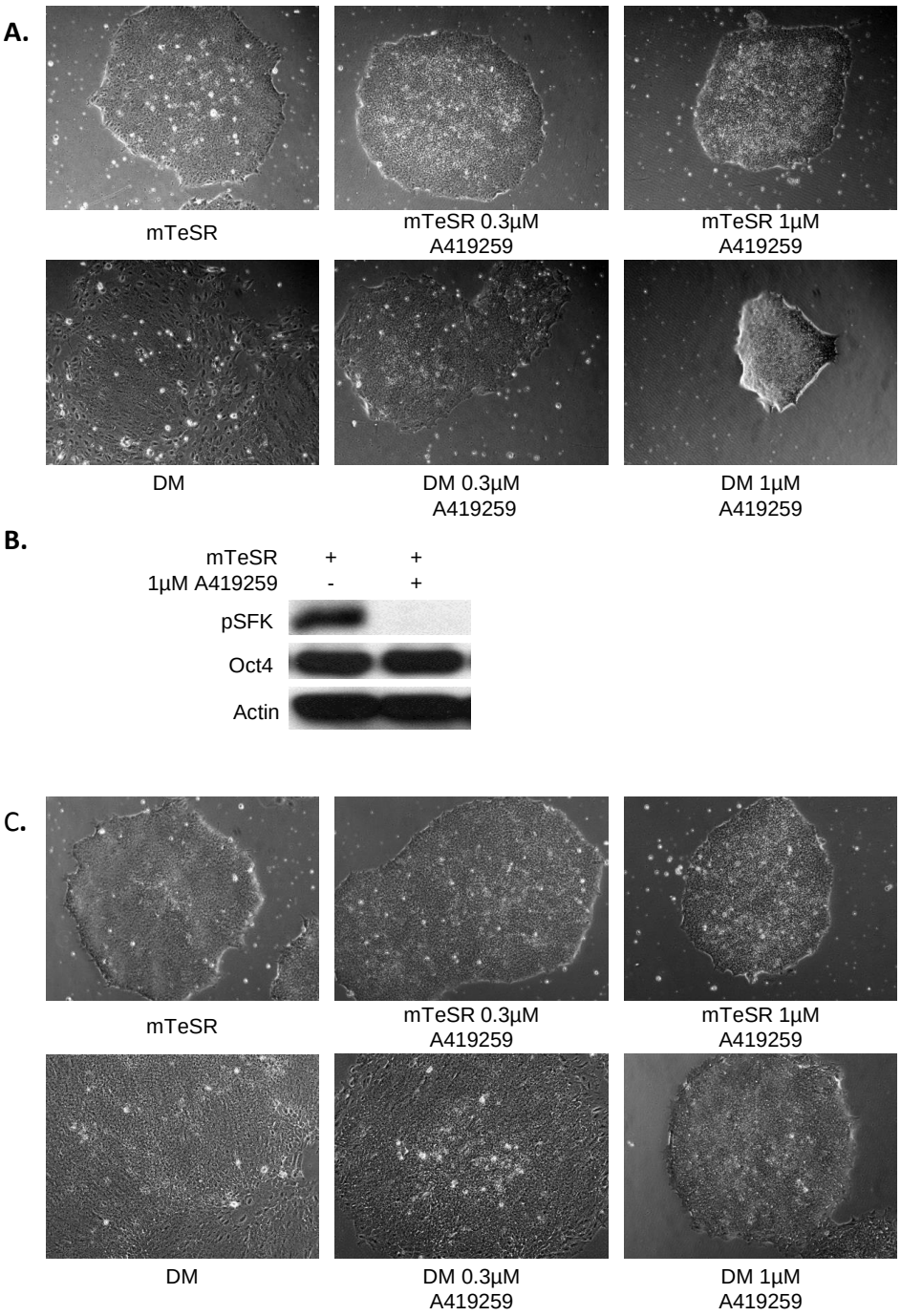

Figure 22. Global SFK inhibition with A-419259 blocks hES cell differentiation. A) H1 hES cells were grown in mTeSR renewal medium or switched to differentiation medium with or without the pan-SFK inhibitor, A-419259. Note that inhibitor treatment results in the maintenance of colonies with rounded, domed morphology characteristic of undifferentiated hES cells. (B) H1 hES cells growing in mTeSR medium were treated with or without A-419259 at a concentration of 1 μM overnight. Global SFK activity was determined by probing cell lysates with phosphospecific antibodies directed against the activation loop phosphotyrosine as a measure of kinase activity. Oct4 and Actin protein levels in the cell lysates were also examined as control. C) H7 hES cells were treated with A-419259 under the same conditions as in part A.

3.3.4 Human ES cells maintain Tra-1-60 expression following SFK inhibition

Human ES cells express several markers for pluripotency, including cell surface markers SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81, as well as nuclear pluripotency factors such as Oct4, Nanog and Sox2 (Thomson et al., 1998; Boyer et al., 2005). To determine whether SFK inhibition has an effect in marker expression in hES cells, we determined Tra-1-60 expression in H7 cells following treatment with A-419259. Tra-1-60 is a cell-surface protein that has been recognized as one of the most stringent markers for hES cell pluripotency (Chan et al., 2009).

H1 hES cells were passaged and grown in mTeSR renewal medium for 3 days, followed by continued growth on mTeSR or differentiation medium (DM) in the presence or absence of 1 μM A-419259 for 3 additional days. Cells were then fixed and stained for cell-surface marker Tra-1-60 (Figure 23A). Untreated hESC cultures maintain typical, round hES cell morphology and stained strongly with Tra-1-60 in mTeSR medium. The switch to DM caused the cells to adopt the flattened colony morphology and lose Tra-1-60 expression, indicative of differentiation. However, hES cells maintained in 1 μM A-419259 retained the morphology of domed, pluripotent colonies and continued to express TRA-1-60 despite the switch to differentiation culture conditions. Treatment of the H7 line of hES cells with A-419259 produced and identical result, as shown in Figure 23B.

Together, these experiments show that global inhibition of endogenous SFK activity in hES cells with A-419259 is sufficient to sustain pluripotent hES cell colony morphology and Tra-1-60 expression under culture conditions for differentiation.

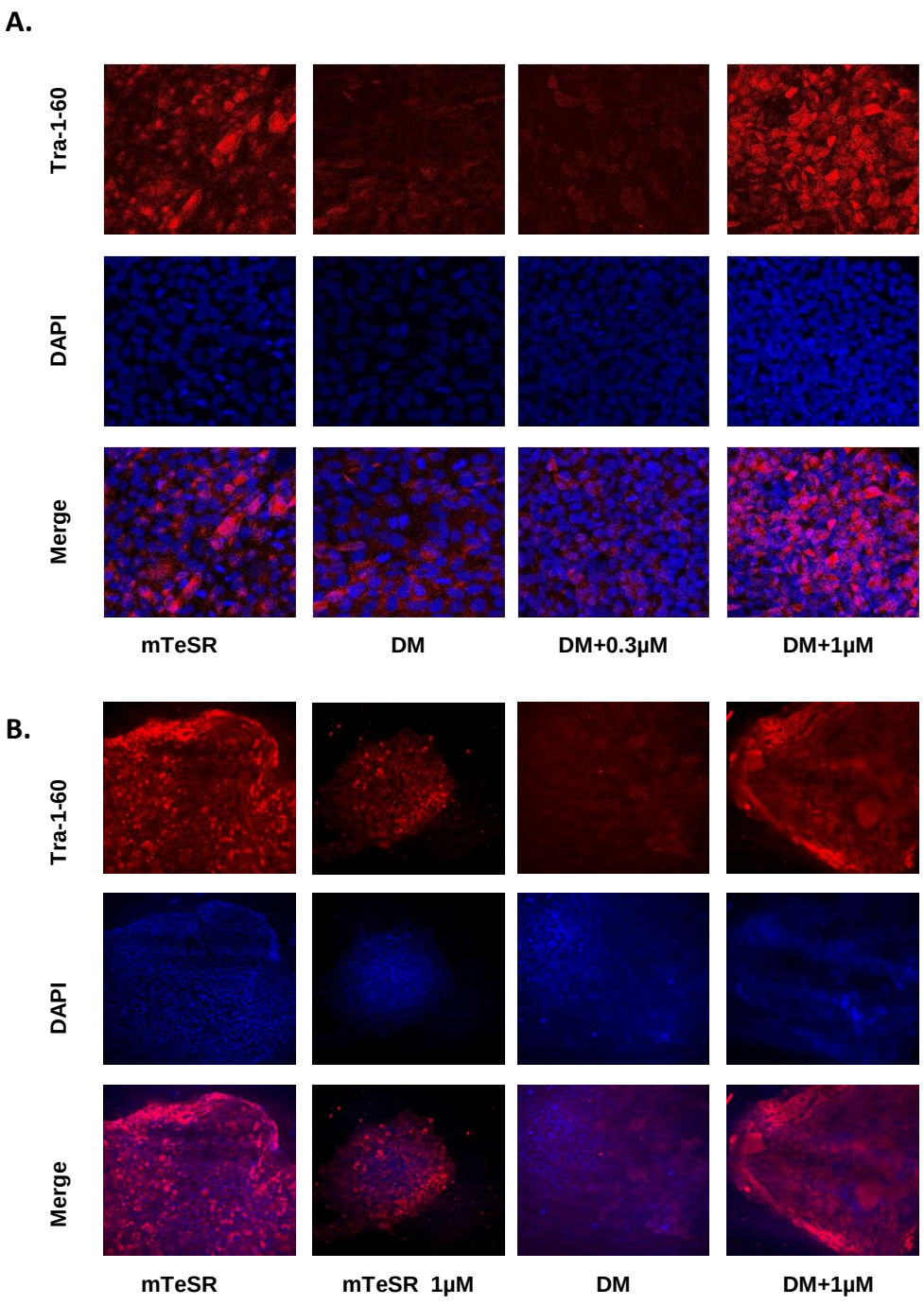

Figure 23. Both H1 and H7 hES cells retain Tra-1-60 expression following SFK inhibition. A.) H1 hES cells were grown on mTeSR medium for 3 days, then switched to either mTeSR medium or differentiation medium (DM; DMEM/F-12 with 5% KSR, 15% FBS) with 0.3 or 1 μM A419259 or left untreated. Cells were fixed and stained for the cell-surface pluripotency marker Tra-1-60 three days later (Magnification 400×). Untreated hESC cultures stain strongly with Tra-1-60, while the switch to DM causes the cells to adopt flattened colony morphology and lose Tra-1-60 expression. However, hES cells maintained in 1 μM A-419259 retained the morphology of domed, pluripotent colonies and continued to express TRA-1-60 in DM. B.) H7 hES cells were grown on mTeSR medium for 3 days, then switched to either mTeSR medium or differentiation medium with 1 μM A419259 or left untreated. Cells were fixed and stained for the Tra-1-60 3 days later (Magnification 100×).

3.4 DISCUSSION

In this study, we found that hES cells express multiple SFK members with c-Src, c-Yes, Fyn and Lck most highly expressed at the transcript level. SFK members showed dynamic changes in expression levels during the differentiation process of hES cells to EBs: Lck expression dropped dramatically as hES cells differentiate while c-Src and Fyn expression were up-regulated slightly during this process. These expression changes suggest that Lck may be associated with self-renewal, while c-Src and Fyn may be related to differentiation. In mES cells, both c-Src and Fyn kinases are expressed. c-Src activity alone induces mES cell to differentiate into primitive ectoderm (Meyn, III and Smithgall, 2009). c-Src may play similar role in hES cell as well.

Human ES cells express a high level of Lck, which is surprising given that Lck expression is restricted to T-lymphocytes in adults. Lck has also been identified as a highly expressed gene in a microarray study comparing gene expression profiles in pluripotent hES cells with their differentiated counterparts (Cao et al., 2008). In addition, in a global phosphoproteomic study of hES cells, Lck was identified as a signaling protein with more phosphorylation sites in pluripotent hES cells than in differentiated hES cell derivatives (Brill et al., 2009). These results are consistent with our findings, showing that Lck is regulated through transcriptional regulation as well as phosphorylation, implicating this kinase in hES cell self-renewal and/or suppression of differentiation. In addition to Lck, another SFK member, Hck, was shown to be highly expressed in mES cells and was rapidly down-regulated in mouse EBs. However, we only detected a low levels of Hck transcripts in hES cells. Interestingly, two pluripotent states have recently been proposed: the naïve and primed states (Nichols and Smith,

2009) (see Introduction). Mouse EpiSCs and hES cells exemplify primed pluripotency while mES cells exemplify naïve pluripotency. We have preliminary results suggesting that Hck is highly expressed in mES cells but down-regulated in moues EpiSCs. Thus, Hck may be a marker of naïve pluripotency, which explains why it is strongly expressed in mES cells yet only expressed in hES cells at very low levels.

We also show that global inhibition of SFK activity blocks hES cell differentiation, implicating SFK signaling in hES cell differentiation. This is similar to what we observed in mES cells. Treatment of hES cells with the pan-SFK inhibitors PP2, SKI-1 and A-419259 all suppressed hES cell differentiation and maintained hES cell colony morphology in differentiation medium. Moreover, hES cells treated with A-419259, the most potent and selective of these inhibitors, continued to express Tra-1-60 under culture conditions for differentiation, consistent with the maintenance of undifferentiated colony morphology.

Finally, we found that the renewal marker Nanog and the Src-family member Hck are differentially expressed among different hES cell lines. Also, in the process of EB formation, some SFK members such as Hck and Lyn, and some differentiation markers such as Pax6 and Msx1 are differentially up-regulated or down-regulated. These observations demonstrate the heterogeneity between hES cell lines. Human ES cell lines have functional and molecular heterogeneity, with cell lines requiring different pluripotency regulators and showing differentiation bias (Wang et al., 2012; Bock et al., 2011). This cell line-specific heterogeneity may be associated with the intrinsically unstable state of "primed" pluripotency (Nichols and Smith, 2009). For future studies, it would be interesting to isolate purer hES populations from these lines for gene expression analysis.

In summary, hES cells express multiple SFK members, and individual members are dynamically regulated in different ways during EB differentiation of hES cells, indicating that individual SFK members may play distinct role in hES cell self-renewal and differentiation. Global inhibition of SFK activity blocked hES cell differentiation, and maintained pluripotency marker expression. Our results support a role for SFK signaling in the regulation of hES fate, and suggest that some parallels may exist in mouse and hES cells for this intracellular signaling network.

3.5 MATERIALS AND METHODS

3.5.1 Cell culture

Human ES cell culture. H1, H7 and H9 hES cells (WiCell WA01, WA07, WA09) were maintained in feeder-free culture conditions with mTeSR medium (Stemcell Technologies) on 6-well plates coated with hESC-qualified matrigel (BD Biosciences). Medium was changed daily, and cells were passaged every 6 days using Dispase (Stemcell Technologies) as per the manufacturer's instructions.

EB formation. ES cells were dissociated into single cells with Accutase (Stemcell Technologies), pelleted and re-suspended in mTeSR medium containing 10 μM of the ROCK inhibitor Y27632. Viable cells were counted using Trypan Blue (Invitrogen) and equal numbers of cells were loaded into each well of AggreWell plates (Stemcell Technologies). For each hES cell line used, each EB was started with aggregates of 2000 cells. EB aggregation of dissociated ES cells were then initiated as per manufacturer's instructions. EBs were harvested 24 hours

later, re-suspended in Aggrewell medium (Stemcell Technologies) or differentiation medium (described below) , and maintained on ultra-low attachment plates (Corning).

Inhibitor treatment. ES cells were passaged onto Matrigel-coated 6-well plates and maintained in mTeSR medium for three days. Culture medium was then changed to differentiation medium or mTeSR with or without different concentrations of SFK inhibitors for an additional three days. Differentiation medium was composed of 5% knockout serum replacement, 15% fetal bovine serum, 1% non-essential amino acid, 1% L-glutamine, and 1% Pen/Strep in DMEM/F-12 medium (Life Technologies). SKI-1 and PP2 were purchased from Calbiochem while A-419259 was purchased from Santa Cruz Biotechnology.

3.5.2 RT-PCR

RNA isolation, RT-PCR, quantitative real-time RT-PCR, and data analysis were performed as described in section 2.5.4. Human EBs were harvested 3, 9 and 12 days after initiation and were washed using a cell strainer (BD Biosciences) to remove single cells before lysis.

3.5.3 Protein Blots

Cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer supplemented with phosphatase and protease inhibitors as described (Meyn, III et al., 2005). Cell lysates were clarified by centrifugation and equal amounts of total protein were mixed with SDS-loading buffer, heated and separated on SDS polyacrylamide gels. Proteins were transferred to PVDF membranes and probed with antibodies to the SFK activation loop, Oct4, and actin as a loading control. The following primary antibodies were used at a 1:1000 dilution: active Src

pY418 (Invitrogen 44660G), mouse anti-Oct4 (Santa Cruz, SC-5279), and mouse anti-Actin (Millipore, MAB1501).

3.5.4 Immunocytochemistry and fluorescence microscopy

Cells were plated onto Matrigel-coated coverslips in 6-well plates and maintained in mTeSR medium for three days. The cell culture medium was then switched to differentiation medium or mTeSR with or without inhibitors for three additional days. Cells were then washed with PBS, fixed in 4% paraformaldehyde (Sigma) for 15 minutes, and blocked with PBS containing 5% bovine serum albumin (BSA) (Fisher Sicientific) and 5% normal goat serum (Invitrogen) for 1 hour at room temperature. Cells were then incubated with primary antibodies overnight at 4 ^{o}C in blocking buffer, followed by 3 washes in PBS. Secondary antibody (Invitrogen, 1:1000) incubation was performed at 37 ^{o}C for 1 hour followed by 3 washes. Samples are then mounted using mounting medium containing DAPI (Vector Laboratories). The primary antibody used was mouse anti-Tra-1-60 1:200 (Santa Cruz, SC-21705).

4.0 OVERALL DISCUSSION

4.1 SUMMARY OF FINDINGS AND SIGNIFICANCE

Embryonic stem cells have to choose between two fates in every cell cycle, to renew, or to differentiate. This fate determination program is intrinsic to stem cells and is a tightly controlled process. While the growth factor conditions and transcription factor networks governing stem cell fates have been studied extensively, the signaling pathways connecting these extrinsic signals to intrinsic gene transcription programs are not fully explored. Src family tyrosine kinases are such modules relaying extrinsic signals to biological functions, and have been implicated in mES cell fate determination. Here, I tested the hypothesis that c-Src and its closest phylogenetic relative, c-Yes, act in biological opposition to one another in the context of ES cell fate determination. I first discovered that enforced expression of active c-Yes blocked ES cell differentiation to embryoid bodies by maintaining pluripotency gene expression. To determine the interplay of c-Src and c-Yes in mES cell fate determination, I employed a chemical genetics approach to investigate individual kinase function. I found that c-Src activity alone induces mES cell differentiation to the ectoderm and endoderm, while c-Yes inhibits the endoderm differentiation, acting in direct opposite to c-Src. These studies are the first to directly show that even closely related kinases such as c-Src and c-Yes have unique and opposing functions in the same cell type. In addition, my work shows that c-Src-induced differentiation correlates with

upregulation of EMT-related genes, incorporating the c-Src signal, EMT and ES cell differentiation. Elucidating the connection between these mechanisms and developing specific small molecule modulators of c-Src activity vs. c-Yes or vice versa may promote cellular programing or differentiation to specific lineages. My second aim investigated the largely unknown role of SFKs in human ES (hES) cells. I determined the relative expression profile of individual Src family members in undifferentiated hES cells vs. embryoid bodies derived from them. We found that hES cells express multiple SFK members, some of which display dynamic transcription changes during EB differentiation, indicating that individual members may play non-redundant roles. To assess the role of Src family kinase activity hES cells, I treated hES cell cultures with a panel of chemically distinct SFK inhibitors. SFK inhibition maintained human ES cell colony morphology and expression of the pluripotency marker Tra-1-60 in differentiation medium. These observations support a role for Src family kinase signaling in the regulation of hES fate, and suggest that some parallels may exist in mouse and human ES cells for this intracellular signaling network. My research lays a foundation for further investigation of SFK signaling in hES cells, which may shed light on hES cell maintenance and differentiation, essential steps for regenerative medicine.

4.1.1 c-Yes is a potent anti-differentiation signal and acts in direct opposition to c-Src

In the first chapter, I showed that c-Yes is a potent suppressor of mES cell differentiation. Enforced expression of either wild-type or an active mutant of c-Yes at modest levels blocked ES cell differentiation to embryoid bodies. This suppressive effect requires kinase activity, since it is not observed with kinase-defective c-Yes. Interestingly, EB formation is very sensitive to c-Yes signaling, as both wild-type c-Yes and a kinase-active c-Yes mutant inhibited this process,

resulting in the formation of only small cell clusters. In addition, we found that c-Yes activity is associated with the maintenance of pluripotency marker expression. Unlike control EBs, where pluripotency factors are all down-regulated, the small cell clusters formed from ES/c-Yes cells continue to express pluripotency factors, such as Oct4, Nanog, Klf4 and Esrrb. This finding is consistent with the reported role of c-Yes as a kinase associated with mES cell self-renewal (Anneren et al., 2004), where c-Yes is downstream of LIF and signals through Yes associated protein (YAP) and transcription factor TEAD2 to activate Nanog and Oct4 expression (Tamm et al., 2011).

Next, to study the role of Src family kinases individually, we employed a chemical genetics approach and designed inhibitor resistant (IR) mutants of c-Src and c-Yes. Treatment of ES cells with SFK inhibitor A-419259 blocks all endogenous Src-family kinase activity in ES cells, locking them in an undifferentiated state (Meyn, III et al., 2005). Expression of IR mutants in mES cells allowed us to study the role of c-Src or c-Yes activity individually, in the presence of inhibitor concentrations that block all endogenous SFK activity. Expression of c-Yes IR mutant did not rescue the differentiation block associated with A-419259 treatment, but pluripotency marker expression was retained. This is consistent with the role of c-Yes as a renewal-related kinase. In contrast, inhibitor-treated cells expressing Src-IR mutants adopted a flattened, differentiated morphology, consistent with our previous finding that that c-Src activity alone promotes ES cell differentiation to primitive ectoderm-like cells (Meyn, III and Smithgall, 2009).

By expanding the qPCR marker analysis, I showed that c-Src activity alone induces mES cell differentiation into both primitive ectoderm and endoderm lineages, as exemplified by Fgf5, Sox17, Gata4 and Gata6 expression. Further, I found c-Src is a potent activator of the epithelial-

mesenchymal transition (EMT), with significant upregulation of EMT related genes such as Snail1, Twist1, Igf2, MMP9 and MMP14. The activation of EMT-associated genes is concomitant with the up-regulation of cell-lineage differentiation markers. The EMT regulates multiple important tissue remodeling processes in early embryonic development and has also been implicated in cancer invasion and metastasis (Thiery et al., 2009). EMT has also been associated with differentiation in both mouse and human ES cells (Eastham et al., 2007; Spencer et al., 2007). Conversely, inhibition of this process promoted the nuclear reprograming of mouse fibroblast cells to induced pluripotent stem cells (iPSCs) (Li et al., 2011). In addition, the EMT is proposed to be an essential and early step for lineage commitment in ES cells, since elevation of EMT markers preceded activation of lineage markers in response to differentiation signals (Li et al., 2011). Our results directly link c-Src kinase activity with the EMT, implicating this fundamental process in c-Src-induced differentiation of mES cells.

The mechanism of how c-Src activity regulates EMT progression is still unknown. EMT is a developmental program tightly controlled by the TGFβ/Nodal/BMP, Wnt/β-Catenin, Notch and FGF signaling pathways (Yang and Weinberg, 2008; Thiery et al., 2009). Src may modulate the EMT downstream of these growth factors. In addition, c-Src has been implicated in cell adhesion, migration and cytoskeletal reorganization—key processes associated with the EMT as well. For example, c-Src phosphorylates p120 catenin to disrupt its association with E-cadherin; c-Src couples with FAK to activate MMP2 and MMP9 and promote migration; and c-Src activates the Rho-family GTPases Rac, Cdc42 and Rho, to regulate adhesion and the F-actin cytoskeleton (Guarino, 2010; Kim et al., 2009). Interestingly, functional loss of E-cadherin in epithelial cells is a hallmark for the EMT, while both MMPs and Rho-family GTPases are major players in EMT (Yang and Weinberg, 2008). These findings indicate that c-Src may play a major

role in the EMT process, by cooperating with and modulating multiple proteins associated with cell-cell adhesion, cell-matrix adhesion and migration.

Moreover, we made the discovery that c-Yes acts in direct opposition to c-Src. We found that the c-Yes signal suppresses c-Src-induced differentiation and the EMT. Remarkably, when both c-Yes and c-Src IR mutants were introduced into the same cell population and treated with the inhibitor, c-Yes activity prevented the induction of endoderm differentiation and EMT by c-Src. Of note, suppression of endoderm differentiation might be a characteristic of the Yes-YAP-TEAD2 signaling pathway. A significant increase in endoderm-specific gene expression was observed when TEAD2 activity is downregulated in mES cells (Tamm et al., 2011), consistent with our findings described here.

Our observations help to define c-Yes as a suppressor of differentiation. We show that c-Yes activity is sufficient to maintain expression of the core transcription factors that govern pluripotency, and is dominant over the differentiation signals induced by c-Src. These findings support a previous model of the regulation of endogenous c-Src and c-Yes kinase activity during ES cell renewal and differentiation (Meyn, III et al., 2005). In the presence of LIF, both c-Yes and c-Src are active, but the presence of active c-Yes overrides the c-Src signal for differentiation. Upon LIF withdrawal, c-Yes activity is shut off, allowing active c-Src to drive differentiation. More broadly, our work suggests that although ES cells can be maintained in a perpetual state of self-renewal, they are poised to differentiate. However, our data also show that c-Yes activity alone cannot sustain ES cell self-renewal. This is point is supported by at least three lines of evidence. 1) ES cells expressing active c-Yes adopted a flattened, differentiated morphology in the absence of LIF in adherent culture. 2) Although ES cells expressing active c-Yes only form abnormal, small aggregates under culture conditions for EB formation,

differentiation markers were expressed. 3) Complete inhibition of SFK activity does not result in differentiation; rather, the cells remain pluripotent (Meyn, III et al., 2005). Taken together, these results show that c-Yes is an important suppressor of differentiation but is not sufficient to maintain ES cell self-renewal. In addition, our results provide a strong rationale for the development of selective compounds to control c-Src vs. c-Yes activity; such compounds may allow more precise pharmacological manipulation of ES cell renewal and differentiation. Indeed, a recent unbiased chemical library screen identified broad-spectrum Src-family kinase inhibitors as potent enhancers of somatic cell reprogramming to an ES cell-like state (iPS cells) (Staerk et al., 2011). Our work predicts that a Src-selective inhibitor (or a c-Yes agonist) may provide an even greater enhancement in reprogramming efficiency.

A long-standing tenet of the Src-family kinase field is that individual family members have similar, if not redundant, biological functions. Our results clearly demonstrate that this is not always the case, and provide an important caveat to the use of broad-spectrum, small molecule inhibitors of the entire Src kinase family to make conclusions about the biological activity of individual family members or the family as a whole. However, the mechanisms of how these two closely related kinases act in opposition to one another are still not clear. Several factors might account for this difference: 1) c-Src and c-Yes may bind different adaptor or substrate proteins through their SH3 or SH2 domains or even through their unique domains. For example, c-Yes protects and maintains tight junctions by interacting with occludins; in contrast, c-Src activity is associated with occludin downregulation and tight junction dissociation by activating Raf-1 (Summy et al., 2003b). 2) c-Yes and c-Src may have different sub-cellular localizations. c-Yes can be both myristoylated and palmitoylated, which target it to lipid rafts,

while c-Src has no palmitoylation site (Thomas and Brugge, 1997). This localization difference may also account for recruitment of distinct binding partners.

In summary, we defined c-Yes as a potent suppressor of mES cell differentiation, showing that low-level, sustained expression of active c-Yes maintains ES cell renewal marker expression following LIF withdrawal, resulting in a profound block to EB formation. In addition, using a chemical genetics approach, we discovered that c-Yes acts in direct opposition to c-Src, to inhibit c-Src-induced endoderm differentiation and EMT marker expression. These findings reveal a key regulatory mechanism in the dynamic control of murine ES cell pluripotency and differentiation. More broadly, our results are the first to show that closely related kinases like c-Src and c-Yes are not necessarily functioning redundantly, but may act in direct opposition to each other in the same cell type.

4.1.2 SFK signaling is important for human ES cell differentiation

While our group and others have implicated SFK signaling in mES cells, the role of this kinase family is not clear in human ES cells. In my second aim, I explored Src family kinase signaling in hES cells.

We found that human ES cells express multiple SFK members: Of the eleven Src-related kinases in the human genome, Fyn, c-Yes, c-Src, Lyn, Lck and Hck were expressed in H1, H7 and H9 hES cells, while Fgr, Blk, Srm, Brk, and Frk transcripts were not detected. During hES cell differentiation into EBs, c-Yes, Lyn, and Hck transcript levels remained constant in self-renewing hES cells and differentiated EBs, while c-Src and Fyn had a modest increase in expression. In contrast, Lck expression levels dropped dramatically as a function of EB differentiation. The dynamic expression changes of SFK members during hES cell differentiation

into EBs indicate that they play different roles in this process. Lck, which is expressed only in the T-cell compartment in adults, is highly expressed in human ES cells, yet down-regulated during differentiation. This suggests that Lck might be important for the self-renew program. On the other hand, c-Src and Fyn, which are highly expressed in hES cells, are further upregulated during differentiation, suggesting that they may be involved in this process.

Our surprising finding that Lck is strongly expressed in human ES cells and is transcriptionally silenced during differentiation to EBs supports a unique function in the control of hES cell renewal. This idea is supported by previous gene expression profiling studies showing that Lck transcript levels are high in renewing hES cells but down-regulated in differentiated cells, and a phosphoproteomic analysis showing Lck proteins are preferentially phosphorylated at Tyr-179 in undifferentiated H1 cells (Brill et al., 2009; Cao et al., 2008). However, the questions of how Lck activity regulates the hES cell self-renewal program is still unknown. SFKs are involved in multiple signaling pathways, including receptor tyrosine kinase signaling and integrin modulated cell-adhesion signaling (Thomas and Brugge, 1997; Ingley, 2008). In human ES cells, SFK may signal downstream of FGF/FGFR, and cross talk with MEK/Erk, PI3K/Akt and Wnt/β-Catenin signaling pathways, all of which have been implicated in hES cell self-renew and differentiation.

In addition, we report that SFK inhibition maintains hES cell colony morphology and expression of the pluripotency marker Tra-1-60. To assess the role of Src family kinase activity hES cells, we treated hES cell cultures with inhibitors specific for the Src kinase family, including Src Kinase Inhibitor 1 (SKI-1), PP2 and A-419259. Untreated control hES cultures grown in mTeSR1 medium formed typical pluripotent colonies circumscribed by differentiated cells. Transfer of hES cells to differentiation medium resulted in flattened colony morphology

that correlated with a loss of cell-surface staining for the hES cell pluripotency marker, Tra-1-60. Dramatically, hES cells maintained in the presence of 1 μM A-419259 retained the morphology of domed, pluripotent colonies and maintained TRA-1-60 expression in differentiation medium. Similar morphological changes were observed in SKI-1 treated cells.

These inhibitor studies suggest an important role for SFKs in hES cell differentiation. Based on our previous work in mES cells, we speculate the c-Src may have an important role in the differentiation process. As suggested in Chapter 2, c-Src induces EMT and differentiation in murine ES cells. Since EMT is also correlates with human ES cell differentiation (Eastham et al., 2007), Src may induce EMT and promote hES cell differentiation as well. While we were able to show that A-419259 treatment inhibits all endogenous SFK activity in hES cells in a manner very similar to mES cells, we were not able to identify the specific family members that are constitutively active in hES cells. Future studies may approach this problem using the chemical genetic strategy that was successfully applied to mES cells.

In summary, the observations reported in chapter 3 support a role for Src family kinase signaling in the regulation of hES fate, and suggest that some parallels may exist in mouse and human ES cells for this intracellular signaling network. This is the first report to comprehensively investigating SFKs in human ES cells, and these results will help elucidate the mechanism of this important family of kinases in human ES cells.

4.2 FUTURE DIRECTIONS

4.2.1 Identify signaling pathways downstream of c-Src and c-Yes that account for the different stem cell fates associated with SFK signaling

My thesis research led to the surprising discovery that c-Src and c-Yes directly oppose each other in mES cells. The signaling pathways downstream of c-Src that are responsible for ES cell differentiation are not known. While c-Yes has been shown to phosphorylate YAP and activate YAP-TEAD2-dependent transcription (Tamm et al., 2011), other pathways may also contribute to the role of c-Yes as suppressor of differentiation in mES cells. The opposing roles of c-Yes and c-Src may be related to the distinct signaling partners they recruit, or their subcellular localizations. The SH2 and SH3 domains of c-Src and c-Yes may have different specificities for binding partners. For example, the c-Src SH3 domain readily binds actin filament associated protein (AFAP) while the c-Yes SH3 domain does not (Summy et al., 2003b). This may partially explain why c-Yes fails to control actin filament rearrangement, and thus cannot compensate for c-Src function in osteoclast cells from c-Src knockout mice (Soriano et al., 1991; Summy et al., 2003b). Although not able to bind AFAP, c-Yes SH3 domain can uniquely modulate interaction with YAP (Clump et al., 2005).

To narrow down the signaling molecules most closely related to ES cell fate determination, we could combine the Src-IR or Yes-IR mutants with SH3 and SH2 null mutants, to probe which function domain mutation abrogates the biological function. For example, Src-IR could be combined with mutations that inactivate the function of the Src SH3 domain. The resulting Src-IR/SH3 mutant could then be expressed in ES cells, and tested for differentiation in response to A-419259 treatment as described in Chapter 2. If the Src-IR/SH3 null mutant fails to

induce ES cell differentiation, then SH3-binding partners may be involved in this biological process. Subsequently, Src SH3 domain can be immobilized and used as baits for capture of SH3 binding partners from ES cell extracts, followed by identification through standard MS-based proteomics. This technology has been successfully used by our group to identify a circadian rhythm protein, Timeless, as a substrate of Src that is essential for ES cell differentiation to EBs (O'Reilly et al., 2011).

To interrogate signaling pathways downstream of c-Src that induce ES cell differentiation, we could use Src-IR in combination with A-419259 as a model system for small molecule screening. Since a number of signaling pathways active in ES cells might act downstream of Src, small molecule inhibitors of these pathways can be introduced, and those inhibitors which abrogate Src-induced differentiation could be selected for further biochemical analysis. Candidate pathways include the MEK/Erk, PI3K/AKT, FAK, Wnt/β-Catenin and EMT signaling pathways. As described in earlier sections, each of these pathways has been linked to c-Src in other biological contexts.

The ultimate tool to identify downstream signaling pathways involves the phosphoproteomics approach of stable isotope labeling of amino acids in cell culture (SILAC) (Pimienta et al., 2009). Control ES cells with all SFK activity blocked by A-419259 will be grown in normal medium, while Src-IR ES cells with A419259 treatment will be grown in heavy medium with all arginine labeled with ^{13}C. Therefore, all proteins from the Src-IR cells will generate heavier peptides than their counterparts in control cells. These two cell populations can then be combined in equal ratios, trypsinized, and analyzed together by Liquid Chromatography-Mass Spectrometry. In the resulting mass spectra, a pair of light and heavy peptides will show up together as a doublet because of the isotope labeling. The peak intensities in those doublets will

reflect the abundance of the actual protein. Proteins giving rise to mass peaks with high intensity in Src-IR cells but low intensity in control cells will be implicated in Src mediated differentiation. A pilot experiment was performed in our lab, and we identified several target proteins that were differentially phosphorylated in cells as a function of A-419259 treatment for complete SFK inhibition. These targets included key components of the Wnt/β-Catenin pathway, the pluripotency factor Sox2 and zinc-finger transcription factor Sal-like protein 4 (SAL4).

Another interesting question is how c-Src activity promotes the EMT in the context of ES cells. ES cell colonies expressing Src-IR cells undergo a dramatic morphological change following A-419259 treatment, adopting a flattened morphology. One could speculate that part of this morphological shift is due to the EMT, as cell:cell contacts are lost and the cells become more autonomous. It would be interesting to investigate how c-Src activity connects with the hallmark EMT transcription factors such as Snail1 and Twist, which may contribute to these morphological changes. This will shed light on the connection between the EMT and ES cell differentiation. The EMT has also been implicated in cancer progression, and c-Src-induced changes related to the EMT in ES cells may also have a crucial role in cancer metastasis.

4.2.2 Further study of SFK signaling in human ES cells

We found that Src family kinase signaling is important in the regulation of hES fate, and that some parallels exist between mouse and human ES cells for this intracellular signaling network. Further studies to elucidate SFK signaling in hES cells are crucial.

First, are SFKs responsive to hES cell growth factors? Since Lck has been identified as a self-renewal related kinase, it would be important to determine if it is responsive to bFGF, and

activated by FGFR, because this growth factor is a key regulator of hES cell pluripotency. Also, the downstream effectors of SFKs are not clear in human ES cells.

In addition, what will happen to hES cells overexpressing SFKs? Can overexpression of Lck block human ES cell differentiation, in a manner similar to that observed with c-Yes in mouse ES cells? Conversely, can overexpression of c-Src or Fyn induce differentiation? We could also combine kinase active mutants, or employ the chemical genetics approach to study specific SFK signaling in hES cells.

Another interesting point relates to the absence of Hck expression in hES cells despite the fact that it is highly expressed in renewing mES cells but undergoes rapid transcriptional silenceing as mES cells differentiate. These observations suggest that Hck expression and activity may be specifically associated with naïve as opposed to primed pluripotency. A comparative analysis of SFK expression and activity profiles in mEpiSCs vs. mESCs, and in hESCs vs. naïve hESCs, would help to clarify this important issue.

Moreover, we hypothesize that c-Src and Fyn may be involved in hES cell differentiation. Previous studies have shown that the EMT is associated with hESC differentiation (Eastham et al., 2007), and in our mES cell study, c-Src activates EMT-associated gene expression. Both c-Src and Fyn may have similar function in hES cells, and parallel pathways may exist in human ES cells.

One major difficulty of hES cell research is the heterogeneity of cell culture. Unlike mES cells, hES cultures represent a mixture of pluripotent and differentiated cell types, even under self-renewal conditions, which complicates the analysis of fate marker expression. Also, hES cells are sensitive to single cell dissociation, which hinders clonal passage and genetic manipulation. The newly developed naïve human ES cells culture and human ES cell lines with

selective reporters for pluripotency (for example, Oct4-GFP hESCs) may facilitate future signaling studies with SFKs (Hanna et al., 2010; Buehr et al., 2008).

4.3 CLOSING REMARKS

ES cells have the remarkable property of self-renewal and pluripotency, the ability to generate all cell types of the adult body. ES cells provide an in vitro model system of cell differentiation that mimics early embryonic development. When combined with genetic manipulation, mES cells provide an unprecedented gateway to in vivo models in the form of transgenic animals (Evans, 2011). The development of human ES cells (Thomson et al., 1998), and the newer human induced pluripotent stem cells (Takahashi et al., 2007; Yu et al., 2007) opened a new era for disease modeling and hold great potential for cell therapy, regenerative medicine, and drug discovery. Cell fate determination is of central importance to stem cell biology and the development of stem cell therapy. Multiple signaling pathways contribute to fate determination of stem cells. In this dissertation reseach project, I provided new evidence that Src-family tyrosine kinases are important for the growth and fate specification of both human and mouse embryonic stem cells. In mES cells, I provided direct evidence that c-Yes is a potent inhibitor of differentiation and opposes the action of c-Src to drive differentiation. This result provides a strong challenge to the idea that individual Src family members play redundant biological roles. In addition, this study suggests that selective inhibitors of individual members of the Src family may promote reprograming efficiency, or drive differentiation to specific lineages. Moreover, our results connected stem cell differentiation, Src activity, with the fundamental developmental program of the EMT. Since the EMT is also associated with cancer progression and the formation of cancer stem cells, my results in the ES cell context may also have significance for tumor biology.

BIBLIOGRAPHY

Alonso,G., Koegl,M., Mazurenko,N., and Courtneidge,S.A. (1995). Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. J. Biol. Chem. *270*, 9840-9848.

Amit,M., Carpenter,M.K., Inokuma,M.S., Chiu,C.P., Harris,C.P., Waknitz,M.A., Itskovitz-Eldor,J., and Thomson,J.A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol. *227*, 271-278.

Anneren,C., Cowan,C.A., and Melton,D.A. (2004). The Src family of tyrosine kinases is important for embryonic stem cell self-renewal. J. Biol. Chem. *279*, 31590-31598.

Anton,R., Kestler,H.A., and Kuhl,M. (2007). Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells. FEBS Lett. *581*, 5247-5254.

Artus,J., Piliszek,A., and Hadjantonakis,A.K. (2011). The primitive endoderm lineage of the mouse blastocyst: sequential transcription factor activation and regulation of differentiation by Sox17. Dev. Biol. *350*, 393-404.

Avilion,A.A., Nicolis,S.K., Pevny,L.H., Perez,L., Vivian,N., and Lovell-Badge,R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. *17*, 126-140.

Beddington,R.S. and Robertson,E.J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development *105*, 733-737.

Ben-Shushan,E., Thompson,J.R., Gudas,L.J., and Bergman,Y. (1998). Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. Mol. Cell Biol. *18*, 1866-1878.

Bendall,S.C., Stewart,M.H., Menendez,P., George,D., Vijayaragavan,K., Werbowetski-Ogilvie,T., Ramos-Mejia,V., Rouleau,A., Yang,J., Bosse,M., Lajoie,G., and Bhatia,M. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature *448*, 1015-1021.

Berstine,E.G., Hooper,M.L., Grandchamp,S., and Ephrussi,B. (1973). Alkaline phosphatase activity in mouse teratoma. Proc. Natl. Acad. Sci. U. S. A *70*, 3899-3903.

Bishop,A.C., Ubersax,J.A., Petsch,D.T., Matheos,D.P., Gray,N.S., Blethrow,J., Shimizu,E., Tsien,J.Z., Schultz,P.G., Rose,M.D., Wood,J.L., Morgan,D.O., and Shokat,K.M. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395-401.

Bock,C., Kiskinis,E., Verstappen,G., Gu,H., Boulting,G., Smith,Z.D., Ziller,M., Croft,G.F., Amoroso,M.W., Oakley,D.H., Gnirke,A., Eggan,K., and Meissner,A. (2011). Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell *144*, 439-452.

Boggon,T.J. and Eck,M.J. (2004). Structure and regulation of Src family kinases. Oncogene *23*, 7918-7927.

Bourillot,P.Y., Aksoy,I., Schreiber,V., Wianny,F., Schulz,H., Hummel,O., Hubner,N., and Savatier,P. (2009). Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. Stem Cells *27*, 1760-1771.

Boyer,L.A., Lee,T.I., Cole,M.F., Johnstone,S.E., Levine,S.S., Zucker,J.P., Guenther,M.G., Kumar,R.M., Murray,H.L., Jenner,R.G., Gifford,D.K., Melton,D.A., Jaenisch,R., and Young,R.A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947-956.

Boyer,L.A., Mathur,D., and Jaenisch,R. (2006). Molecular control of pluripotency. Curr. Opin. Genet. Dev. *16*, 455-462.

Bradley,A., Evans,M., Kaufman,M.H., and Robertson,E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature *309*, 255-256.

Brill,L.M., Xiong,W., Lee,K.B., Ficarro,S.B., Crain,A., Xu,Y., Terskikh,A., Snyder,E.Y., and Ding,S. (2009). Phosphoproteomic analysis of human embryonic stem cells. Cell Stem Cell *5*, 204-213.

Bromann,P.A., Korkaya,H., and Courtneidge,S.A. (2004). The interplay between Src family kinases and receptor tyrosine kinases. Oncogene *23*, 7957-7968.

Brons,I.G., Smithers,L.E., Trotter,M.W., Rugg-Gunn,P., Sun,B., Chuva de Sousa Lopes SM, Howlett,S.K., Clarkson,A., Ahrlund-Richter,L., Pedersen,R.A., and Vallier,L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191-195.

Brook,F.A. and Gardner,R.L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. Proc. Natl. Acad. Sci. U. S. A *94*, 5709-5712.

Brugge,J.S. and Erikson,R.L. (1977). Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature *269*, 346-348.

Buecker,C., Chen,H.H., Polo,J.M., Daheron,L., Bu,L., Barakat,T.S., Okwieka,P., Porter,A., Gribnau,J., Hochedlinger,K., and Geijsen,N. (2010). A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. Cell Stem Cell *6*, 535-546.

Buehr,M., Meek,S., Blair,K., Yang,J., Ure,J., Silva,J., McLay,R., Hall,J., Ying,Q.L., and Smith,A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. Cell *135*, 1287-1298.

Burdon,T., Chambers,I., Stracey,C., Niwa,H., and Smith,A. (1999a). Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. Cells Tissues. Organs *165*, 131-143.

Burdon,T., Smith,A., and Savatier,P. (2002). Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol. *12*, 432-438.

Burdon,T., Stracey,C., Chambers,I., Nichols,J., and Smith,A. (1999b). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev. Biol. *210*, 30-43.

Cantley,L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Cao,F., Wagner,R.A., Wilson,K.D., Xie,X., Fu,J.D., Drukker,M., Lee,A., Li,R.A., Gambhir,S.S., Weissman,I.L., Robbins,R.C., and Wu,J.C. (2008). Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. PLoS. One. *3*, e3474.

Capecchi,M.R. (2005). Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. *6*, 507-512.

Chakravarthy,H., Boer,B., Desler,M., Mallanna,S.K., McKeithan,T.W., and Rizzino,A. (2008). Identification of DPPA4 and other genes as putative Sox2:Oct-3/4 target genes using a combination of in silico analysis and transcription-based assays. J. Cell Physiol *216*, 651-662.

Chambers,I., Colby,D., Robertson,M., Nichols,J., Lee,S., Tweedie,S., and Smith,A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chambers,I. and Smith,A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150-7160.

Chan,B., Lanyi,A., Song,H.K., Griesbach,J., Simarro-Grande,M., Poy,F., Howie,D., Sumegi,J., Terhorst,C., and Eck,M.J. (2003). SAP couples Fyn to SLAM immune receptors. Nat. Cell Biol. *5*, 155-160.

Chan,E.M., Ratanasirintrawoot,S., Park,I.H., Manos,P.D., Loh,Y.H., Huo,H., Miller,J.D., Hartung,O., Rho,J., Ince,T.A., Daley,G.Q., and Schlaeger,T.M. (2009). Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. Nat. Biotechnol. *27*, 1033-1037.

Chandrasekharan,S., Qiu,T.H., Alkharouf,N., Brantley,K., Mitchell,J.B., and Liu,E.T. (2002). Characterization of mice deficient in the Src family nonreceptor tyrosine kinase Frk/rak. Mol. Cell Biol. *22*, 5235-5247.

Chapman,G., Remiszewski,J.L., Webb,G.C., Schulz,T.C., Bottema,C.D., and Rathjen,P.D. (1997). The mouse homeobox gene, Gbx2: genomic organization and expression in pluripotent cells in vitro and in vivo. Genomics *46*, 223-233.

Chazaud,C., Yamanaka,Y., Pawson,T., and Rossant,J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev. Cell *10*, 615-624.

Chen,G., Hou,Z., Gulbranson,D.R., and Thomson,J.A. (2010). Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell *7*, 240-248.

Cherry,S.R., Biniszkiewicz,D., van,P.L., Baltimore,D., and Jaenisch,R. (2000). Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol. Cell Biol. *20*, 7419-7426.

Chia,N.Y., Chan,Y.S., Feng,B., Lu,X., Orlov,Y.L., Moreau,D., Kumar,P., Yang,L., Jiang,J., Lau,M.S., Huss,M., Soh,B.S., Kraus,P., Li,P., Lufkin,T., Lim,B., Clarke,N.D., Bard,F., and Ng,H.H. (2010). A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. Nature *468*, 316-320.

Clevers,H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469-480.

Clevers,H. and Nusse,R. (2012). Wnt/beta-catenin signaling and disease. Cell *149*, 1192-1205.

Cloutier,J.F. and Veillette,A. (1996). Association of inhibitory tyrosine protein kinase p50csk with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells. EMBO J. *15*, 4909-4918.

Clump,D.A., Qazi,I.H., Sudol,M., and Flynn,D.C. (2005). c-Yes response to growth factor activation. Growth Factors *23*, 263-272.

Cohen,D.E. and Melton,D. (2011). Turning straw into gold: directing cell fate for regenerative medicine. Nat. Rev. Genet. *12*, 243-252.

Cooper,J.A., Gould,K.L., Cartwright,C.A., and Hunter,T. (1986). Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science *231*, 1431-1434.

Cowan-Jacob,S.W., Fendrich,G., Manley,P.W., Jahnke,W., Fabbro,D., Liebetanz,J., and Meyer,T. (2005). The Crystal Structure of a c-Src Complex in an Active Conformation Suggests Possible Steps in c-Src Activation. Structure. (Camb.) *13*, 861-871.

Cully,M., You,H., Levine,A.J., and Mak,T.W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat. Rev. Cancer *6*, 184-192.

Davis,S., Aldrich,T.H., Stahl,N., Pan,L., Taga,T., Kishimoto,T., Ip,N.Y., and Yancopoulos,G.D. (1993). LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science *260*, 1805-1808.

Dravid,G., Ye,Z., Hammond,H., Chen,G., Pyle,A., Donovan,P., Yu,X., and Cheng,L. (2005). Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells *23*, 1489-1501.

Dvash,T., Mayshar,Y., Darr,H., McElhaney,M., Barker,D., Yanuka,O., Kotkow,K.J., Rubin,L.L., Benvenisty,N., and Eiges,R. (2004). Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. Hum. Reprod. *19*, 2875-2883.

Dvorak,P., Dvorakova,D., Koskova,S., Vodinska,M., Najvirtova,M., Krekac,D., and Hampl,A. (2005). Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells *23*, 1200-1211.

Eastham,A.M., Spencer,H., Soncin,F., Ritson,S., Merry,C.L., Stern,P.L., and Ward,C.M. (2007). Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. Cancer Res. *67*, 11254-11262.

Engen,J.R., Wales,T.E., Hochrein,J.M., Meyn,M.A., III, Banu,O.S., Bahar,I., and Smithgall,T.E. (2008). Structure and dynamic regulation of Src-family kinases. Cell Mol. Life Sci. *65*, 3058-3073.

Ernst,M., Gearing,D.P., and Dunn,A.R. (1994). Functional and biochemical association of Hck with the LIF/IL- 6 receptor signal transducing subunit gp130 in embryonic stem cells. EMBO J. *13*, 1574-1584.

Evans,M. (2011). Discovering pluripotency: 30 years of mouse embryonic stem cells. Nat. Rev. Mol. Cell Biol. *12*, 680-686.

Evans,M.J. (1972). The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells. J. Embryol. Exp. Morphol. *28*, 163-176.

Evans,M.J. and Kaufman,M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.

Feng,S., Chen,J.K., Yu,H., Simon,J.A., and Schreiber,S.L. (1994). Two binding orientations for peptides to the Src SH3 domain: Development of a general model for SH3-ligand interactions. Science *266*, 1241-1247.

Feng,X.H. and Derynck,R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu. Rev. Cell Dev. Biol. *21*, 659-693.

Festuccia,N., Osorno,R., Halbritter,F., Karwacki-Neisius,V., Navarro,P., Colby,D., Wong,F., Yates,A., Tomlinson,S.R., and Chambers,I. (2012). Esrrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cells. Cell Stem Cell *11*, 477-490.

Frankenberg,S., Gerbe,F., Bessonnard,S., Belville,C., Pouchin,P., Bardot,O., and Chazaud,C. (2011). Primitive endoderm differentiates via a three-step mechanism involving Nanog and RTK signaling. Dev. Cell *21*, 1005-1013.

Gadue,P., Huber,T.L., Paddison,P.J., and Keller,G.M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A *103*, 16806-16811.

Grant,S.G., O'Dell,T.J., Karl,K.A., Stein,P.L., Soriano,P., and Kandel,E.R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science *258*, 1903-1910.

Greber,B., Coulon,P., Zhang,M., Moritz,S., Frank,S., Muller-Molina,A.J., Arauzo-Bravo,M.J., Han,D.W., Pape,H.C., and Scholer,H.R. (2011). FGF signalling inhibits neural induction in human embryonic stem cells. EMBO J. *30*, 4874-4884.

Greber,B., Lehrach,H., and Adjaye,J. (2007). Fibroblast growth factor 2 modulates transforming growth factor beta signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. Stem Cells *25*, 455-464.

Guarino,M. (2010). Src signaling in cancer invasion. J. Cell Physiol *223*, 14-26.

Guo,G., Huss,M., Tong,G.Q., Wang,C., Li,S.L., Clarke,N.D., and Robson,P. (2010). Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Dev. Cell *18*, 675-685.

Hall,J., Guo,G., Wray,J., Eyres,I., Nichols,J., Grotewold,L., Morfopoulou,S., Humphreys,P., Mansfield,W., Walker,R., Tomlinson,S., and Smith,A. (2009). Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. Cell Stem Cell *5*, 597-609.

Han,J., Yuan,P., Yang,H., Zhang,J., Soh,B.S., Li,P., Lim,S.L., Cao,S., Tay,J., Orlov,Y.L., Lufkin,T., Ng,H.H., Tam,W.L., and Lim,B. (2010). Tbx3 improves the germ-line competency of induced pluripotent stem cells. Nature *463*, 1096-1100.

Hanna,J., Cheng,A.W., Saha,K., Kim,J., Lengner,C.J., Soldner,F., Cassady,J.P., Muffat,J., Carey,B.W., and Jaenisch,R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. U. S. A *107*, 9222-9227.

Hanna,J., Markoulaki,S., Mitalipova,M., Cheng,A.W., Cassady,J.P., Staerk,J., Carey,B.W., Lengner,C.J., Foreman,R., Love,J., Gao,Q., Kim,J., and Jaenisch,R. (2009). Metastable pluripotent states in NOD-mouse-derived ESCs. Cell Stem Cell *4*, 513-524.

Hao,J., Li,T.G., Qi,X., Zhao,D.F., and Zhao,G.Q. (2006). WNT/beta-catenin pathway up-regulates Stat3 and converges on LIF to prevent differentiation of mouse embryonic stem cells. Dev. Biol. *290*, 81-91.

Harrison,S.C. (2003). Variation on an Src-like theme. Cell *112*, 737-740.

Hart,A.H., Hartley,L., Sourris,K., Stadler,E.S., Li,R., Stanley,E.G., Tam,P.P., Elefanty,A.G., and Robb,L. (2002). Mixl1 is required for axial mesendoderm morphogenesis and patterning in the murine embryo. Development *129*, 3597-3608.

Hentze,H., Soong,P.L., Wang,S.T., Phillips,B.W., Putti,T.C., and Dunn,N.R. (2009). Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. Stem Cell Res. *2*, 198-210.

Hosler,B.A., LaRosa,G.J., Grippo,J.F., and Gudas,L.J. (1989). Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. Mol. Cell Biol. *9*, 5623-5629.

Huveneers,S. and Danen,E.H. (2009). Adhesion signaling - crosstalk between integrins, Src and Rho. J. Cell Sci. *122*, 1059-1069.

Ihle,J.N. (2001). The Stat family in cytokine signaling. Curr. Opin. Cell Biol. *13*, 211-217.

Imamoto,A. and Soriano,P. (1993). Disruption of the *csk* gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell *73*, 1117-1124.

Ingley,E. (2008). Src family kinases: regulation of their activities, levels and identification of new pathways. Biochim. Biophys. Acta *1784*, 56-65.

Ishizawar,R. and Parsons,S.J. (2004). c-Src and cooperating partners in human cancer. Cancer Cell *6*, 209-214.

Itskovitz-Eldor,J., Schuldiner,M., Karsenti,D., Eden,A., Yanuka,O., Amit,M., Soreq,H., and Benvenisty,N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol. Med. *6*, 88-95.

Ivanova,N., Dobrin,R., Lu,R., Kotenko,I., Levorse,J., DeCoste,C., Schafer,X., Lun,Y., and Lemischka,I.R. (2006). Dissecting self-renewal in stem cells with RNA interference. Nature *442*, 533-538.

Jaenisch,R. and Young,R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567-582.

James,D., Levine,A.J., Besser,D., and Hemmati-Brivanlou,A. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development *132*, 1273-1282.

Jiang,J., Chan,Y.S., Loh,Y.H., Cai,J., Tong,G.Q., Lim,C.A., Robson,P., Zhong,S., and Ng,H.H. (2008). A core Klf circuitry regulates self-renewal of embryonic stem cells. Nat. Cell Biol. *10*, 353-360.

Jirmanova,L., Afanassieff,M., Gobert-Gosse,S., Markossian,S., and Savatier,P. (2002). Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene *21*, 5515-5528.

Johnson,T.M., Williamson,N.A., Scholz,G., Jaworowski,A., Wettenhall,R.E., Dunn,A.R., and Cheng,H.C. (2000). Modulation of the catalytic activity of the Src family tyrosine kinase Hck by autophosphorylation at a novel site in the unique domain. J. Biol. Chem. *275*, 33353-33364.

Kataoka,H., Takakura,N., Nishikawa,S., Tsuchida,K., Kodama,H., Kunisada,T., Risau,W., Kita,T., and Nishikawa,S.I. (1997). Expressions of PDGF receptor alpha, c-Kit and Flk1 genes

clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells. Dev. Growth Differ. *39*, 729-740.

Kawabuchi,M., Satomi,Y., Takao,T., Shimonishi,Y., Nada,S., Nagai,K., Tarakhovsky,A., and Okada,M. (2000). Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature *404*, 999-1003.

Keller,G. (2005). Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. Genes and Development *19*, 1129-1155.

Keller,G.M. (1995). In vitro differentiation of embryonic stem cells. Curr. Opin. Cell Biol. *7*, 862-869.

Kelly,K.F., Ng,D.Y., Jayakumaran,G., Wood,G.A., Koide,H., and Doble,B.W. (2011). beta-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. Cell Stem Cell *8*, 214-227.

Kielman,M.F., Rindapaa,M., Gaspar,C., van,P.N., Breukel,C., van,L.S., Taketo,M.M., Roberts,S., Smits,R., and Fodde,R. (2002). Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. Nat. Genet. *32*, 594-605.

Kim,L.C., Song,L., and Haura,E.B. (2009). Src kinases as therapeutic targets for cancer. Nat. Rev. Clin. Oncol. *6*, 587-595.

Kim,M.O., Kim,S.H., Cho,Y.Y., Nadas,J., Jeong,C.H., Yao,K., Kim,D.J., Yu,D.H., Keum,Y.S., Lee,K.Y., Huang,Z., Bode,A.M., and Dong,Z. (2012). ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. Nat. Struct. Mol. Biol. *19*, 283-290.

Kim,P.W., Sun,Z.Y., Blacklow,S.C., Wagner,G., and Eck,M.J. (2003). A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. Science *301*, 1725-1728.

Kispert,A. and Herrmann,B.G. (1994). Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. Dev. Biol. *161*, 179-193.

Klinghoffer,R.A., Sachsenmaier,C., Cooper,J.A., and Soriano,P. (1999). Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J. *18*, 2459-2471.

Koopman,P. and Cotton,R.G. (1984). A factor produced by feeder cells which inhibits embryonal carcinoma cell differentiation. Characterization and partial purification. Exp. Cell Res. *154*, 233-242.

Krishnamurty,R., Brigham,J.L., Leonard,S.E., Ranjitkar,P., Larson,E.T., Dale,E.J., Merritt,E.A., and Maly,D.J. (2013). Active site profiling reveals coupling between domains in SRC-family kinases. Nat. Chem. Biol. *9*, 43-50.

Kunath,T., Saba-El-Leil,M.K., Almousailleakh,M., Wray,J., Meloche,S., and Smith,A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development *134*, 2895-2902.

Latour,S., Roncagalli,R., Chen,R., Bakinowski,M., Shi,X., Schwartzberg,P.L., Davidson,D., and Veillette,A. (2003). Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. Nat. Cell Biol. *5*, 149-154.

Lee,C.-H., Saksela,K., Mirza,U.A., Chait,B.T., and Kuriyan,J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell *85*, 931-942.

Lerner,E.C. and Smithgall,T.E. (2002). SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat. Struct. Biol. *9*, 365-369.

Levenstein,M.E., Ludwig,T.E., Xu,R.H., Llanas,R.A., VanDenHeuvel-Kramer,K., Manning,D., and Thomson,J.A. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells *24*, 568-574.

Li,J., Wang,G., Wang,C., Zhao,Y., Zhang,H., Tan,Z., Song,Z., Ding,M., and Deng,H. (2007). MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. Differentiation *75*, 299-307.

Li,X., Zhu,L., Yang,A., Lin,J., Tang,F., Jin,S., Wei,Z., Li,J., and Jin,Y. (2011). Calcineurin-NFAT signaling critically regulates early lineage specification in mouse embryonic stem cells and embryos. Cell Stem Cell *8*, 46-58.

Li,Y., McClintick,J., Zhong,L., Edenberg,H.J., Yoder,M.C., and Chan,R.J. (2005). Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. Blood *105*, 635-637.

Li,Z., Fei,T., Zhang,J., Zhu,G., Wang,L., Lu,D., Chi,X., Teng,Y., Hou,N., Yang,X., Zhang,H., Han,J.D., and Chen,Y.G. (2012). BMP4 Signaling Acts via dual-specificity phosphatase 9 to control ERK activity in mouse embryonic stem cells. Cell Stem Cell *10*, 171-182.

Lim,W.A., Richards,F.M., and Fox,R.O. (1994). Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. Nature *372*, 375-379.

Liu,B.A., Jablonowski,K., Shah,E.E., Engelmann,B.W., Jones,R.B., and Nash,P.D. (2010). SH2 domains recognize contextual peptide sequence information to determine selectivity. Mol. Cell Proteomics. *9*, 2391-2404.

Liu,N., Lu,M., Tian,X., and Han,Z. (2007). Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells. J. Cell Physiol *211*, 279-286.

Liu,Y., Bishop,A., Witucki,L., Kraybill,B., Shimizu,E., Tsien,J., Ubersax,J., Blethrow,J., Morgan,D.O., and Shokat,K.M. (1999). Structural basis for selective inhibition of Src family kinases by PP1. Chem. Biol. *6*, 671-678.

Loh,Y.H., Wu,Q., Chew,J.L., Vega,V.B., Zhang,W., Chen,X., Bourque,G., George,J., Leong,B., Liu,J., Wong,K.Y., Sung,K.W., Lee,C.W., Zhao,X.D., Chiu,K.P., Lipovich,L., Kuznetsov,V.A., Robson,P., Stanton,L.W., Wei,C.L., Ruan,Y., Lim,B., and Ng,H.H. (2006). The Oct4 and Nanog

transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. *38*, 431-440.

Lowell,C.A., Niwa,M., Soriano,P., and Varmus,H.E. (1996). Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis. Blood *87*, 1780-1792.

Lowell,C.A. and Soriano,P. (1996). Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev. *10*, 1845-1857.

Lu,J., Hou,R., Booth,C.J., Yang,S.H., and Snyder,M. (2006). Defined culture conditions of human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A *103*, 5688-5693.

Ludwig,T.E., Levenstein,M.E., Jones,J.M., Berggren,W.T., Mitchen,E.R., Frane,J.L., Crandall,L.J., Daigh,C.A., Conard,K.R., Piekarczyk,M.S., Llanas,R.A., and Thomson,J.A. (2006). Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. *24*, 185-187.

Madan,B., Madan,V., Weber,O., Tropel,P., Blum,C., Kieffer,E., Viville,S., and Fehling,H.J. (2009). The pluripotency-associated gene Dppa4 is dispensable for embryonic stem cell identity and germ cell development but essential for embryogenesis. Mol. Cell Biol. *29*, 3186-3203.

Maldonado-Saldivia,J., van den Bergen,J., Krouskos,M., Gilchrist,M., Lee,C., Li,R., Sinclair,A.H., Surani,M.A., and Western,P.S. (2007). Dppa2 and Dppa4 are closely linked SAP motif genes restricted to pluripotent cells and the germ line. Stem Cells *25*, 19-28.

Manning,G., Whyte,D.B., Martinez,R., Hunter,T., and Sudarsanam,S. (2002). The protein kinase complement of the human genome. Science *298*, 1912-1934.

Martello,G., Sugimoto,T., Diamanti,E., Joshi,A., Hannah,R., Ohtsuka,S., Gottgens,B., Niwa,H., and Smith,A. (2012). Esrrb is a pivotal target of the Gsk3/Tcf3 axis regulating embryonic stem cell self-renewal. Cell Stem Cell *11*, 491-504.

Martin,G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A *78*, 7634-7638.

Martin,G.R. and Evans,M.J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc. Natl. Acad. Sci. U. S. A *72*, 1441-1445.

Martin,G.S. (2001). The hunting of the Src. Nat. Rev. Mol. Cell Biol. *2*, 467-475.

Martin,G.S. (2004). The road to Src. Oncogene *23*, 7910-7917.

Masaki,H., Nishida,T., Kitajima,S., Asahina,K., and Teraoka,H. (2007). Developmental pluripotency-associated 4 (DPPA4) localized in active chromatin inhibits mouse embryonic stem cell differentiation into a primitive ectoderm lineage. J. Biol. Chem. *282*, 33034-33042.

Massague,J. and Chen,Y.G. (2000). Controlling TGF-beta signaling. Genes Dev. *14*, 627-644.

Massague,J., Seoane,J., and Wotton,D. (2005). Smad transcription factors. Genes Dev. *19*, 2783-2810.

Masui,S., Nakatake,Y., Toyooka,Y., Shimosato,D., Yagi,R., Takahashi,K., Okochi,H., Okuda,A., Matoba,R., Sharov,A.A., Ko,M.S., and Niwa,H. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat. Cell Biol. *9*, 625-635.

Masui,S., Ohtsuka,S., Yagi,R., Takahashi,K., Ko,M.S., and Niwa,H. (2008). Rex1/Zfp42 is dispensable for pluripotency in mouse ES cells. BMC. Dev. Biol. *8*, 45.

Matsuda,T., Nakamura,T., Nakao,K., Arai,T., Katsuki,M., Heike,T., and Yokota,T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J. *18*, 4261-4269.

Meyn,M.A., III, Schreiner,S.J., Dumitrescu,T.P., Nau,G.J., and Smithgall,T.E. (2005). SRC family kinase activity is required for murine embryonic stem cell growth and differentiation. Mol. Pharmacol. *68*, 1320-1330.

Meyn,M.A., III and Smithgall,T.E. (2009). Chemical genetics identifies c-Src as an activator of primitive ectoderm formation in murine embryonic stem cells. Sci. Signal. *2*, ra64.

Mitsui,K., Tokuzawa,Y., Itoh,H., Segawa,K., Murakami,M., Takahashi,K., Maruyama,M., Maeda,M., and Yamanaka,S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.

Miyazaki,T., Sanjay,A., Neff,L., Tanaka,S., Horne,W.C., and Baron,R. (2004). Src kinase activity is essential for osteoclast function. J. Biol. Chem. *279*, 17660-17666.

Mohr,J.C., Zhang,J., Azarin,S.M., Soerens,A.G., de Pablo,J.J., Thomson,J.A., Lyons,G.E., Palecek,S.P., and Kamp,T.J. (2010). The microwell control of embryoid body size in order to regulate cardiac differentiation of human embryonic stem cells. Biomaterials *31*, 1885-1893.

Murakami,M., Hibi,M., Nakagawa,N., Nakagawa,T., Yasukawa,K., Yamanishi,K., Taga,T., and Kishimoto,T. (1993). IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science *260*, 1808-1810.

Murry,C.E. and Keller,G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell *132*, 661-680.

Musacchio,A., Noble,M., Pauptit,R., Wierenga,R., and Saraste,M. (1992). Crystal structure of a Src-homology 3 (SH3) domain. Nature *359*, 851-855.

Musacchio,A., Saraste,M., and Wilmanns,M. (1994). High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. Nat. Struct. Biol. *1*, 546-551.

Nada,S., Yagi,T., Takeda,H., Tokunaga,T., Nakagawa,H., Ikawa,Y., Okada,M., and Aizawa,S. (1993). Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell *73*, 1125-1135.

Ng,H.H. and Surani,M.A. (2011). The transcriptional and signalling networks of pluripotency. Nat. Cell Biol. *13*, 490-496.

Nichols,J. and Smith,A. (2009). Naive and primed pluripotent states. Cell Stem Cell *4*, 487-492.

Nichols,J. and Smith,A. (2012). Pluripotency in the embryo and in culture. Cold Spring Harb. Perspect. Biol. *4*, a008128.

Nichols,J., Zevnik,B., Anastassiadis,K., Niwa,H., Klewe-Nebenius,D., Chambers,I., Scholer,H., and Smith,A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Nishioka,N., Inoue,K., Adachi,K., Kiyonari,H., Ota,M., Ralston,A., Yabuta,N., Hirahara,S., Stephenson,R.O., Ogonuki,N., Makita,R., Kurihara,H., Morin-Kensicki,E.M., Nojima,H., Rossant,J., Nakao,K., Niwa,H., and Sasaki,H. (2009). The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev. Cell *16*, 398-410.

Niwa,H. (2007). How is pluripotency determined and maintained? Development *134*, 635-646.

Niwa,H., Burdon,T., Chambers,I., and Smith,A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. *12*, 2048-2060.

Niwa,H., Miyazaki,J., and Smith,A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372-376.

Niwa,H., Ogawa,K., Shimosato,D., and Adachi,K. (2009). A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature *460*, 118-122.

O'Reilly,L.P., Watkins,S.C., and Smithgall,T.E. (2011). An unexpected role for the clock protein timeless in developmental apoptosis. PLoS. One. *6*, e17157.

Ogawa,K., Nishinakamura,R., Iwamatsu,Y., Shimosato,D., and Niwa,H. (2006). Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells. Biochem. Biophys. Res. Commun. *343*, 159-166.

Ohgushi,M., Matsumura,M., Eiraku,M., Murakami,K., Aramaki,T., Nishiyama,A., Muguruma,K., Nakano,T., Suga,H., Ueno,M., Ishizaki,T., Suemori,H., Narumiya,S., Niwa,H., and Sasai,Y. (2010). Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. Cell Stem Cell *7*, 225-239.

Okamoto,K., Okazawa,H., Okuda,A., Sakai,M., Muramatsu,M., and Hamada,H. (1990). A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell *60*, 461-472.

Paling,N.R., Wheadon,H., Bone,H.K., and Welham,M.J. (2004). Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J. Biol. Chem. *279*, 48063-48070.

Parsons,J.T. and Parsons,S.J. (1997). Src family protein tyrosine kinases: Cooperating with growth factor and adhesion signaling pathways. Curr. Opin. Cell Biol. *9*, 187-192.

Pawson,T. (2004). Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell *116*, 191-203.

Pawson,T. and Nash,P. (2000). Protein-protein interactions define specificity in signal transduction. Genes Dev. *14*, 1027-1047.

Pelton,T.A., Sharma,S., Schulz,T.C., Rathjen,J., and Rathjen,P.D. (2002). Transient pluripotent cell populations during primitive ectoderm formation: correlation of in vivo and in vitro pluripotent cell development. J. Cell Sci. *115*, 329-339.

Pene-Dumitrescu,T., Peterson,L.F., Donato,N.J., and Smithgall,T.E. (2008). An inhibitor-resistant mutant of Hck protects CML cells against the antiproliferative and apoptotic effects of the broad-spectrum Src family kinase inhibitor A-419259. Oncogene *27*, 7055-7069.

Pene-Dumitrescu,T. and Smithgall,T.E. (2010). Expression of a Src family kinase in chronic myelogenous leukemia cells induces resistance to imatinib in a kinase-dependent manner. J. Biol. Chem. *285*, 21446-21457.

Percharde,M., Lavial,F., Ng,J.H., Kumar,V., Tomaz,R.A., Martin,N., Yeo,J.C., Gil,J., Prabhakar,S., Ng,H.H., Parker,M.G., and Azuara,V. (2012). Ncoa3 functions as an essential Esrrb coactivator to sustain embryonic stem cell self-renewal and reprogramming. Genes Dev. *26*, 2286-2298.

Perez,Y., Gairi,M., Pons,M., and Bernado,P. (2009). Structural characterization of the natively unfolded N-terminal domain of human c-Src kinase: insights into the role of phosphorylation of the unique domain. J. Mol. Biol. *391*, 136-148.

Pfaffl,M.W., Horgan,G.W., and Dempfle,L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. *30*, e36.

Pimienta,G., Chaerkady,R., and Pandey,A. (2009). SILAC for global phosphoproteomic analysis. Methods Mol. Biol. *527*, 107-16, x.

Prokhorova,T.A., Harkness,L.M., Frandsen,U., Ditzel,N., Schroder,H.D., Burns,J.S., and Kassem,M. (2009). Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. Stem Cells Dev. *18*, 47-54.

Qi,X., Li,T.G., Hao,J., Hu,J., Wang,J., Simmons,H., Miura,S., Mishina,Y., and Zhao,G.Q. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc. Natl. Acad. Sci. U. S. A *101*, 6027-6032.

Resh,M.D. (1994). Myristylation and palmitylation of Src family members: The fats of the matter. Cell *76*, 411-413.

Resh,M.D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim. Biophys. Acta *1451*, 1-16.

Robinson,D.R., Wu,Y.M., and Lin,S.F. (2000). The protein tyrosine kinase family of the human genome. Oncogene *19*, 5548-5557.

Rogers,M.B., Hosler,B.A., and Gudas,L.J. (1991). Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. Development *113*, 815-824.

Roskoski,R., Jr. (2004). Src protein-tyrosine kinase structure and regulation. Biochem. Biophys. Res. Commun. *324*, 1155-1164.

Roskoski,R., Jr. (2005). Src kinase regulation by phosphorylation and dephosphorylation. Biochem. Biophys. Res. Commun. *331*, 1-14.

Rudd,C.E., Trevillyan,J.M., Dasgupta,J.D., Wong,L.L., and Schlossman,S.F. (1988). The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. U. S. A *85*, 5190-5194.

Saksela,K. and Permi,P. (2012). SH3 domain ligand binding: What's the consensus and where's the specificity? FEBS Lett. *586*, 2609-2614.

Sancier,F., Dumont,A., Sirvent,A., Paquay de,P.L., Edmonds,T., David,G., Jan,M., de,M.C., Coge,F., Leonce,S., Burbridge,M., Bruno,A., Boutin,J.A., Lockhart,B., Roche,S., and Cruzalegui,F. (2011). Specific oncogenic activity of the Src-family tyrosine kinase c-Yes in colon carcinoma cells. PLoS. One. *6*, e17237.

Sato,N., Meijer,L., Skaltsounis,L., Greengard,P., and Brivanlou,A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. *10*, 55-63.

Schindler,T., Sicheri,F., Pico,A., Gazit,A., Levitzki,A., and Kuriyan,J. (1999). Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. Mol. Cell *3*, 639-648.

Scholer,H.R., Ruppert,S., Suzuki,N., Chowdhury,K., and Gruss,P. (1990). New type of POU domain in germ line-specific protein Oct-4. Nature *344*, 435-439.

Scotland,K.B., Chen,S., Sylvester,R., and Gudas,L.J. (2009). Analysis of Rex1 (zfp42) function in embryonic stem cell differentiation. Dev. Dyn. *238*, 1863-1877.

Shenoy,S., Chackalaparampil,I., Bagrodia,S., Lin,P.H., and Shalloway,D. (1992). Role of p34cdc2-mediated phosphorylations in two-step activation of pp60c-src during mitosis. Proc. Natl. Acad. Sci. U. S. A *89*, 7237-7241.

Sicheri,F., Moarefi,I., and Kuriyan,J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature *385*, 602-609.

Silva,J., Barrandon,O., Nichols,J., Kawaguchi,J., Theunissen,T.W., and Smith,A. (2008). Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS. Biol. *6*, e253.

Silverman,L., Sudol,M., and Resh,M.D. (1993). Members of the src family of nonreceptor tyrosine kinases share a common mechanism for membrane binding. Cell Growth Differ. *4*, 475-482.

Smith,A.G. (2001). Embryo-derived stem cells: of mice and men. Annu. Rev. Cell Dev. Biol. *17*, 435-462.

Smith,A.G., Heath,J.K., Donaldson,D.D., Wong,G.G., Moreau,J., Stahl,M., and Rogers,D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature *336*, 688-690.

Smith,A.G. and Hooper,M.L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev. Biol. *121*, 1-9.

Sokol,S.Y. (2011). Maintaining embryonic stem cell pluripotency with Wnt signaling. Development *138*, 4341-4350.

Solter,D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat. Rev. Genet. *7*, 319-327.

Solter,D. and Knowles,B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc. Natl. Acad. Sci. U. S. A *75*, 5565-5569.

Songyang,Z., Shoelson,S.E., Chaudhuri,M., Gish,G., Pawson,T., Haser,W.G., King,F., Roberts,T., Ratnofsky,S., Lechleider,R.J., Neel,B.G., Birge,R.B., Fajardo,J.E., Chou,M.M., Hanafusa,H., Schaffhausen,B., and Cantley,L.C. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.

Soriano,P., Montgomery,C., Geske,R., and Bradley,A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell *64*, 693-702.

Spencer,H.L., Eastham,A.M., Merry,C.L., Southgate,T.D., Perez-Campo,F., Soncin,F., Ritson,S., Kemler,R., Stern,P.L., and Ward,C.M. (2007). E-cadherin inhibits cell surface localization of the pro-migratory 5T4 oncofetal antigen in mouse embryonic stem cells. Mol. Biol. Cell *18*, 2838-2851.

Stadtfeld,M. and Hochedlinger,K. (2010). Induced pluripotency: history, mechanisms, and applications. Genes Dev. *24*, 2239-2263.

Staerk,J., Lyssiotis,C.A., Medeiro,L.A., Bollong,M., Foreman,R.K., Zhu,S., Garcia,M., Gao,Q., Bouchez,L.C., Lairson,L.L., Charette,B.D., Supekova,L., Janes,J., Brinker,A., Cho,C.Y., Jaenisch,R., and Schultz,P.G. (2011). Pan-Src Family Kinase Inhibitors Replace Sox2 during the Direct Reprogramming of Somatic Cells. Angew. Chem. Int. Ed Engl.

Stahl,N., Boulton,T.G., Farruggella,T., Ip,N.Y., Davis,S., Witthuhn,B.A., Quelle,F.W., Silvennoinen,O., Barbieri,G., Pellegrini,S., and . (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science *263*, 92-95.

Stavridis,M.P., Lunn,J.S., Collins,B.J., and Storey,K.G. (2007). A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. Development *134*, 2889-2894.

Stehelin,D., Varmus,H.E., Bishop,J.M., and Vogt,P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature *260*, 170-173.

Stein,P.L., Lee,H.M., Rich,S., and Soriano,P. (1992). pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741-750.

Stein,P.L., Vogel,H., and Soriano,P. (1994). Combined deficiencies of Src, Fyn, and Yes tyrosine kinases in mutant mice. Genes Dev. *8*, 1999-2007.

Stephenson,R.O., Rossant,J., and Tam,P.P. (2012). Intercellular interactions, position, and polarity in establishing blastocyst cell lineages and embryonic axes. Cold Spring Harb. Perspect. Biol. *4*.

Stewart,T.A. and Mintz,B. (1981). Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells. Proc. Natl. Acad. Sci. U. S. A *78*, 6314-6318.

Stewart,T.A. and Mintz,B. (1982). Recurrent germ-line transmission of the teratocarcinoma genome from the METT-1 culture line to progeny in vivo. J. Exp. Zool. *224*, 465-469.

Storm,M.P., Bone,H.K., Beck,C.G., Bourillot,P.Y., Schreiber,V., Damiano,T., Nelson,A., Savatier,P., and Welham,M.J. (2007). Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. J. Biol. Chem. *282*, 6265-6273.

Stover,D.R., Furet,P., and Lydon,N.B. (1996). Modulation of the SH2 binding specificity and kinase activity of Src by tyrosine phosphorylation within its SH2 domain. J. Biol. Chem. *271*, 12481-12487.

Suda,Y., Suzuki,M., Ikawa,Y., and Aizawa,S. (1987). Mouse embryonic stem cells exhibit indefinite proliferative potential. J. Cell Physiol *133*, 197-201.

Summy,J.M. and Gallick,G.E. (2003a). Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev. *22*, 337-358.

Summy,J.M. and Gallick,G.E. (2003b). Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev. *22*, 337-358.

Summy,J.M., Qian,Y., Jiang,B.H., Guappone-Koay,A., Gatesman,A., Shi,X., and Flynn,D.C. (2003a). The SH4-Unique-SH3-SH2 domains dictate specificity in signaling that differentiate c-Yes from c-Src. J. Cell Sci. *116*, 2585-2598.

Summy,J.M., Sudol,M., Eck,M.J., Monteiro,A.N., Gatesman,A., and Flynn,D.C. (2003b). Specificity in signaling by c-Yes. Front Biosci. *8*, s185-s205.

Sun,G., Sharma,A.K., and Budde,R.J. (1998). Autophosphorylation of Src and Yes blocks their inactivation by Csk phosphorylation. Oncogene *17*, 1587-1595.

Sun,H., Lesche,R., Li,D.M., Liliental,J., Zhang,H., Gao,J., Gavrilova,N., Mueller,B., Liu,X., and Wu,H. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc. Natl. Acad. Sci. U. S. A *96*, 6199-6204.

Tai,C.I. and Ying,Q.L. (2013). Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state. J. Cell Sci. *126*, 1093-1098.

Takahashi,K., Murakami,M., and Yamanaka,S. (2005). Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. Biochem. Soc. Trans. *33*, 1522-1525.

Takahashi,K., Tanabe,K., Ohnuki,M., Narita,M., Ichisaka,T., Tomoda,K., and Yamanaka,S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861-872.

Takahashi,K. and Yamanaka,S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Tam,P.P. and Behringer,R.R. (1997). Mouse gastrulation: the formation of a mammalian body plan. Mech. Dev. *68*, 3-25.

Tamm,C., Bower,N., and Anneren,C. (2011). Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF. J. Cell Sci. *124*, 1136-1144.

Tatosyan,A.G. and Mizenina,O.A. (2000). Kinases of the Src family: structure and functions. Biochemistry (Mosc.) *65*, 49-58.

ten,B.D., Kurek,D., Blauwkamp,T., Koole,W., Maas,A., Eroglu,E., Siu,R.K., and Nusse,R. (2011). Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nat. Cell Biol. *13*, 1070-1075.

Tesar,P.J., Chenoweth,J.G., Brook,F.A., Davies,T.J., Evans,E.P., Mack,D.L., Gardner,R.L., and McKay,R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.

Thiery,J.P., Acloque,H., Huang,R.Y., and Nieto,M.A. (2009). Epithelial-mesenchymal transitions in development and disease. Cell *139*, 871-890.

Thomas,S.M. and Brugge,J.S. (1997). Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. *13*, 513-609.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Vallier,L., Alexander,M., and Pedersen,R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. *118*, 4495-4509.

Vallier,L., Reynolds,D., and Pedersen,R.A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. *275*, 403-421.

Veillette,A., Bookman,M.A., Horak,E.M., and Bolen,J.B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell *55*, 301-308.

Waksman,G., Shoelson,S.E., Pant,N., Cowburn,D., and Kuriyan,J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: Crystal structures of the complexed and peptide-free forms. Cell *72*, 779-790.

Wang,D., Esselman,W.J., and Cole,P.A. (2002). Substrate conformational restriction and CD45-catalyzed dephosphorylation of tail tyrosine-phosphorylated Src protein. J. Biol. Chem. *277*, 40428-40433.

Wang,G., Zhang,H., Zhao,Y., Li,J., Cai,J., Wang,P., Meng,S., Feng,J., Miao,C., Ding,M., Li,D., and Deng,H. (2005). Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochem. Biophys. Res. Commun. *330*, 934-942.

Wang,X., Lin,G., Martins-Taylor,K., Zeng,H., and Xu,R.H. (2009). Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells. J. Biol. Chem. *284*, 34054-34064.

Wang,Z., Oron,E., Nelson,B., Razis,S., and Ivanova,N. (2012). Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell *10*, 440-454.

Wassarman,K.M., Lewandoski,M., Campbell,K., Joyner,A.L., Rubenstein,J.L., Martinez,S., and Martin,G.R. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development *124*, 2923-2934.

Watanabe,K., Ueno,M., Kamiya,D., Nishiyama,A., Matsumura,M., Wataya,T., Takahashi,J.B., Nishikawa,S., Nishikawa,S., Muguruma,K., and Sasai,Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. *25*, 681-686.

Watanabe,S., Umehara,H., Murayama,K., Okabe,M., Kimura,T., and Nakano,T. (2006). Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene *25*, 2697-2707.

Williams,R.L., Hilton,D.J., Pease,S., Willson,T.A., Stewart,C.L., Gearing,D.P., Wagner,E.F., Metcalf,D., Nicola,N.A., and Gough,N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature *336*, 684-687.

Wray,J., Kalkan,T., Gomez-Lopez,S., Eckardt,D., Cook,A., Kemler,R., and Smith,A. (2011). Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. Nat. Cell Biol. *13*, 838-845.

Xiao,L., Yuan,X., and Sharkis,S.J. (2006). Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. Stem Cells *24*, 1476-1486.

Xiao,R., Xi,X.D., Chen,Z., Chen,S.J., and Meng,G. (2013). Structural framework of c-Src activation by integrin beta3. Blood *121*, 700-706.

Xu,C., Inokuma,M.S., Denham,J., Golds,K., Kundu,P., Gold,J.D., and Carpenter,M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. *19*, 971-974.

Xu,C., Rosler,E., Jiang,J., Lebkowski,J.S., Gold,J.D., O'Sullivan,C., Delavan-Boorsma,K., Mok,M., Bronstein,A., and Carpenter,M.K. (2005a). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells *23*, 315-323.

Xu,R.H., Chen,X., Li,D.S., Li,R., Addicks,G.C., Glennon,C., Zwaka,T.P., and Thomson,J.A. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat. Biotechnol. *20*, 1261-1264.

Xu,R.H., Peck,R.M., Li,D.S., Feng,X., Ludwig,T., and Thomson,J.A. (2005b). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat. Methods *2*, 185-190.

Xu,R.H., Sampsell-Barron,T.L., Gu,F., Root,S., Peck,R.M., Pan,G., Yu,J., Antosiewicz-Bourget,J., Tian,S., Stewart,R., and Thomson,J.A. (2008). NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell *3*, 196-206.

Xu,W., Doshi,A., Lei,M., Eck,M.J., and Harrison,S.C. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol. Cell *3*, 629-638.

Xu,W., Harrison,S.C., and Eck,M.J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

Yamaguchi,H. and Hendrickson,W.A. (1996). Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature *384*, 484-489.

Yamanaka,S. (2012). Induced pluripotent stem cells: past, present, and future. Cell Stem Cell *10*, 678-684.

Yang,J. and Weinberg,R.A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell *14*, 818-829.

Yao,S., Chen,S., Clark,J., Hao,E., Beattie,G.M., Hayek,A., and Ding,S. (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc. Natl. Acad. Sci. U. S. A *103*, 6907-6912.

Ying,Q.L., Nichols,J., Chambers,I., and Smith,A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell *115*, 281-292.

Ying,Q.L. and Smith,A.G. (2003). Defined conditions for neural commitment and differentiation. Methods Enzymol. *365*, 327-341.

Ying,Q.L., Wray,J., Nichols,J., Batlle-Morera,L., Doble,B., Woodgett,J., Cohen,P., and Smith,A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519-523.

Young,M.A., Gonfloni,S., Superti-Furga,G., Roux,B., and Kuriyan,J. (2001). Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell *105*, 115-126.

Young,R.A. (2011). Control of the embryonic stem cell state. Cell *144*, 940-954.

Yu,J. and Thomson,J.A. (2008). Pluripotent stem cell lines. Genes Dev. *22*, 1987-1997.

Yu,J., Vodyanik,M.A., Smuga-Otto,K., ntosiewicz-Bourget,J., Frane,J.L., Tian,S., Nie,J., Jonsdottir,G.A., Ruotti,V., Stewart,R., Slukvin,I.I., and Thomson,J.A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.

Yu,P., Pan,G., Yu,J., and Thomson,J.A. (2011). FGF2 sustains NANOG and switches the outcome of BMP4-induced human embryonic stem cell differentiation. Cell Stem Cell *8*, 326-334.

Zhang,P., Andrianakos,R., Yang,Y., Liu,C., and Lu,W. (2010). Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. J. Biol. Chem. *285*, 9180-9189.

Zhang,W.Y., de Almeida,P.E., and Wu,J.C. (2008). Teratoma formation: A tool for monitoring pluripotency in stem cell research.

Zhang,Z., Liao,B., Xu,M., and Jin,Y. (2007). Post-translational modification of POU domain transcription factor Oct-4 by SUMO-1. FASEB J. *21*, 3042-3051.

Zhu,Z. and Huangfu,D. (2013). Human pluripotent stem cells: an emerging model in developmental biology. Development *140*, 705-717.