Brf1 Post-transcriptionally Regulates Pluripotency and Differentiation Responses Downstream of Erk MAP Kinase

Thesis by

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Abstract

FGF/Erk MAP Kinase Signaling is a central regulator of mouse embryonic stem cell (mESC) self-renewal, pluripotency and differentiation. However, the mechanistic connection between this signaling pathway activity and the gene circuits stabilizing mESCs *in vitro* remain unclear. Here we show that FGF signaling post-transcriptionally regulates the mESC transcription factor network by controlling the expression of Brf1 (*zfp36l1*), an AU-rich element mRNA binding protein. Changes in Brf1 level disrupts the expression of core pluripotency-associated genes and attenuates mESC self-renewal without inducing differentiation. These regulatory effects are mediated by rapid and direct destabilization of Brf1 targets, such as Nanog mRNA. Interestingly, enhancing Brf1 expression does not compromise mESC pluripotency, but does preferentially regulate differentiation to mesendoderm by accelerating the expression of primitive streak markers. Together, these studies demonstrate that FGF signals utilize targeted mRNA degradation by Brf1 to enable rapid post-transcriptional control of gene expression.

Acknowledgements

Everything comes in threes, as some would have you believe. Then again, the *Rule of Three* exists for a reason, and it's a rule that all Elowitzians obey. So here we go…

This work would not have been possible without the steadfast support of my advisor, Michael Elowitz. In all that I have done, he has been nothing but understanding, patient, honest, optimistic … and so much more. Through him, I discovered that science is an art, which demands both simplicity and beauty. I discovered that effective communication is just as important as a well-executed research plan. I discovered that making mistakes is part of the game, but that persistence and flexibility will get you through the challenges. And finally, I discovered the reasons for my personal commitment to science. I will always be eternally grateful for his mentorship.

This dissertation is the achievement of entire community, whose support guided my research efforts every step of the way. I want to thank: Kathrin Plath, Ellen Rothenberg and Angela Stathopolous for their advice, guidance and support as members of my Thesis Committee; Shelley Diamond, Diana Perez and Josh Verceles at the Caltech Flow Cytometry facility, for their technical support, advice and friendship; Igor Antoshechkin and Vijaya Kumar from the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech; Azim Surani for constructive feedback on our manuscript; and Angie Rizzino, for *fgf4-/-* mouse embryonic stem cells, a cell line that I could not have done without.

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Chapter 1

Introduction

Embryonic stem cells (ESCs) hold great promise for the development of novel therapeutic strategies (1). Owing to their capacity for multi-lineage differentiation, diseased or damaged tissues can be replaced outright, rather than relying on palliative care and the body's tolerance for wound healing (2). The discovery of ESCs thus heralds the age of regenerative medicine. ESCs can be used to rejuvenate non-regenerative tissues, such as the central nervous system, to correct deformities in tissue architecture and function (3). They can also serve as models for disease, and be modified for the treatment of genetic deficiencies or disorders (4, 5), to significantly improve a patient's quality of life. The development of ESC based strategies should provide safe and effective treatments for numerous pathologies.

However, while many potential medical applications for ESCs have been proposed, few have been implemented (6). In the decades following their discovery, the pace of clinical translation has lagged far behind the still basic science into what makes ESCs tick. In cell culture, ESCs are prone to differentiation, and research remains heavily focused on understanding the molecular basis of their pluripotency and self-renewal (7-9). To fully achieve the dream of regenerative medicine, we must first identify and control the regulatory factors which influence the biology and behavior of ESCs.

Here we focus on intercellular signaling and its regulation of pluripotency and self-renewal. Changes in signaling pathway activity strongly regulate the stability of ESC

cultures, but the mechanisms through which these regulatory effects are mediated have not been identified. Our goal was to characterize these mechanisms, and ascertain their functional and developmental relevance.

What are Embryonic Stem Cells?

ESCs are derived from the mammalian blastocyst, a pre-implantation embryonic structure composed of only 3 differentiated lineages: trophectoderm, primitive endoderm and epiblast (10, 11). The mammalian blastocyst is first hatched from the zona pellucida and attached to a bed of mouse embryonic fibroblasts *in vitro*. Under these conditions, epiblast cells grow out from the site of attachment and expand in great numbers, whereas extra-embryonic cells (trophectoderm and primitive endoderm) fail to proliferate. Stably propagating cultures of these self-renewing epiblast cells are called 'embryonic stem cells' because of their ability to differentiate into all somatic cell types including the germ cell lineage *in vitro* (10, 11). They can also be genetically modified using transgenic methods, and reintroduced into donor blastocysts to generate transgenic embryos, thus providing researchers with the technology to modify and propagate changes to the mammalian genome (12-14).

To circumvent the destruction of embryos, an alternative strategy was also developed to derive pluripotent cells. Terminally differentiated cells could be epigenetically reprogrammed to an ESC-like state by forced expression of pluripotency-associated transcription factors (Oct4, Sox2, Kl4 and Myc in mouse cells; Oct4, Sox2, Lin28 and Myc in human cells) (15). These reprogrammed cells exhibited many of the characteristics of genuine embryonic stem cells (16). However, because of their distinct

method of derivation, they were called ‘induced pluripotent stem cells’ (iPSCs). The derivation of patient specific iPSCs, which bypassed issues of immunological tolerance, further encouraged the development of personalized medical treatments. However, like ESCs, iPSCs are unstable cell types that frequently undergo spontaneous differentiation in suboptimal cell culture conditions.

The Role of Intercellular Signaling in Pluripotent Cells

ESC and iPSC cultures are stabilized by defined cell culture media formulations (17). The key constituents of these media formulations are factors that modulate signaling pathway activity. Activation or inhibition of specific signaling pathways can promote pluripotency and continued self-renewal, and reduce the incidence of spontaneous differentiation in cell culture. We describe each of these signaling pathways in greater detail, from a historical perspective, and discuss their possible mechanistic connection to pluripotency and self-renewal genes.

Leukemia Inhibitory Factor Signaling

The derivation of the first ESC lines required embryonic fibroblast feeder layers (10, 11). It was hypothesized that these feeder layers produced an unknown transacting signaling factor which stabilized ESCs in culture. Leukemia Inhibitory Factor (LIF) was identified in a screen for signaling ligands which could support the undifferentiated growth of mouse ESCs (18, 19). Supplementing ESC cultures with LIF eliminated the need for embryonic feeders.

LIF is part of the cytokine family of ligand/receptor systems, known to mediate developmental and homeostatic responses in the embryonic and adult mouse. As a cytokine, its regulation is often associated with the hematopoietic lineage, but it is known to be broadly expressed by other cell and tissue types. For example, LIF is secreted from the endometrium to promote an exit from embryonic diapause after periods of nutrient stress (20), and is essential for blastocyst implantation (21). LIF is also important for maintaining resident stem cell populations in the adult brain (22), inhibiting angiogenesis (23), promoting muscle regeneration (24), and suppressing inflammation associated pathologies (25).

LIF interacts with the heterodimeric cell surface receptor gp130/LIFR to activate the intracellular non-receptor tyrosine Janus kinase (JAK) (26) (Figure 1.1). Activated JAK then phosphorylates Stat protein second messengers, which shuttle to the nucleus to act as transcription factors. JAK kinase can also activate other second messenger pathways (27). For example, gp130/LIFR mediates a direct interaction with PI3 kinase, which regulates gene expression by activating Protein kinase B (PKB). Cytokine receptors can also associate with Ras via the adaptor protein SHC, which activates Erk MAP kinase signaling responses.

The activation of multiple second messenger pathways can have different effects on the maintenance of pluripotency and self-renewal. For example, upregulating Stat activity maintains pluripotency in the absence of LIF, indicating that the signaling pathway imparts its protective effects, in part, through Stat-mediated transactivation of pluripotency-associated genes (28). A constitutively active form of PKB can also support

mESC culture in the absence of LIF, as well (29). However, enhancing Erk MAP kinase signaling has been shown to rapidly induce mESC differentiation (30). These observations indicate that LIF is capable of activating numerous intracellular responses, which could have antagonistic functional and regulatory effects. However, with regards to LIF, it would appear that its ability to support pluripotency and self-renewal outweigh its destabilizing and differentiating effects.

Bone Morphogenic Protein Signaling

Even in the presence of LIF, mouse ESCs require serum, indicating additional extrinsic signaling requirements. It was known that culturing mouse ESCs without serum strongly promoted neural differentiation. Thus, this serum derived factor was expected to be anti-neurogenic. These observations led researchers to Bone Morphogenic Protein (BMP), which inhibits neural differentiation via Smad transcription factors, and is known to be present in serum.

BMPs are a subdivision of the Transforming Growth Factor-β (TGF-β) superfamily of ligands and receptors, first identified as potent inducers of bone formation, but are now known to mediate diverse developmental processes in the embryonic and adult mouse (31). BMP ligands are homodimers, but can exist as heterodimeric pairs. These signaling ligands nucleate cell surface receptors to form a heterotetrameric receptor complex composed of two Type I (BMPRIA/B, ALK1 or ALK2) and two Type II (BMPRII, ACVR-I or ACVR-II) receptors (Figure 1.2). BMP ligands can exhibit a preference for specific Type I and Type II receptor combinations, which influences the signaling activity

perceived by recipient cells. The formation of a heterotetrameric BMP receptor complex activates intracellular serine/threonine kinase domains to permit phosphorylation and activation of Smad (1/5/8) second messengers, which function as transcription factors (Figure 1.2).

The BMP pathway is thought to prevent mouse ESC differentiation by inducing the expression of Inhibitors of Differentiation (Id) proteins, which bind to and inhibit the activity of pro-neural basic Helix-loop-Helix transcription factors that are expressed in ESCs (32). However, it has also been shown to regulate the overall receptivity of mouse ESCs to differentiation, by controlling the expression of pluripotency associated factors, such as Cdh1 (33).

When ESCs were supplemented with LIF and BMP, self-renewal and pluripotency are maintained even in basal media (N2B27) (32). Thus, the discovery of LIF and BMP identified the minimal set of signaling pathway activities that were required to stabilize mouse ESCs in culture.

Discrepancies between embryonic and cell culture signaling requirements

Knockout phenotypes in the early mouse embryo suggested a possible role for Fibroblast Growth Factor (FGF) and Erk MAP kinase signaling as well (34). Loss of either FGF or Erk MAP kinase signaling compromised normal pre-implantation development by promoting epiblast at the expense of primitive endoderm (35, 36), while enhancing FGF or Erk MAP kinase signaling had the opposite effect (37). By contrast, LIF and BMP deficiencies had no effect on the development of pre-implantation mouse embryos.

How does the *in vivo* requirement for FGF and Erk MAP kinase relate to the *in vitro* dependence of mouse ESCs on LIF and BMP?

Researchers discovered that inhibition of FGF and its second messenger pathway Erk MAP kinase functionally replaced the requirement for LIF and BMP (38). LIF/Stat and BMP/Smad coincidentally regulated similar sets of downstream targets as FGF/Erk, and antagonized the differentiating effects of FGF/Erk MAP kinase signaling through indirect mechanisms (39). Maintaining the activity of exogenous signaling pathways (i.e. LIF and BMP) was therefore not required, as long as the activity of destabilizing signaling pathways (i.e. FGF/Erk) remained suppressed.

In agreement with these observations, using FGF/Erk MAP kinase signaling inhibitors enhanced the efficiency of ESC derivation from recalcitrant mouse strains (40, 41). FGF/Erk MAP kinase signaling inhibitors also permitted the first ever derivation of Rat ESCs, which cannot be supported by LIF and BMP alone (42). It was thus proposed that FGF/Erk MAP kinase was the key signaling pathway through which rodent ESCs are controlled (43).

Fibroblast Growth Factor Signaling

FGFs are secreted growth factors, which bind to FGF-specific receptor tyrosine kinases (FGFRs) (Figure 1.3). This interaction occurs in association with heparin sulfate proteoglycans (HSPGs), which coat the surface of cells and are often regarded as necessary cofactors for signaling. Upon receptor activation, docking proteins aggregate on the intracellular side of the receptor in a phosphorylation-dependent manner, to pass the activation signal to second messenger pathways.

The second messengers downstream of FGFR are intracellular kinase cascades (44) (Figure 1.3). For example, FGFRs frequently activate the Erk MAP kinase pathway via Ras, which is a Serine-Proline/Threonine-Proline directed kinase. Erk is known to regulate numerous cellular functions and activities such as cell division, DNA replication, gene expression and differentiation. FGFRs can also activate PI3 kinase directly. The activity of PI3 kinase promotes cell survival by inhibiting apoptosis, increasing the activity of nutrient sensing pathways and preventing differentiation via PKB.

The activation of both Erk MAP kinase and PI3 kinase signaling pathways could yield antagonistic functional and regulatory effects. Erk MAP kinase signaling promotes differentiation, whereas PI3 kinase maintains pluripotency and self-renewal. Interestingly, researchers determined that even though FGFRs have the capacity to signal through PI3 kinase, it does not appear to be a major regulator of this second messenger system in mouse ESCs (38). This observation could explain why disrupting FGF signaling primarily affects Erk MAP kinase signaling activity.

Erk MAP Kinase Signaling

The components of the Erk MAP kinase pathway are well conserved, consisting of three kinases linearly activated in a cascade, invariant across all eukaryotes (45). For mammalian cells, the Erk MAP kinase pathway receives inputs from Ras, a membrane localized GTPase, which associates with activated cell surface receptors, such as FGFR.

Erk is the terminal kinase in the phosphorylation cascade, and it is the major effector of the Erk MAP kinase signaling pathway (46). Erk binds and phosphorylates its protein targets to regulate their function, stability and/or cellular localization. Binding

and phosphorylation are discrete events, as docking and phosphorylation are mediated by separate protein domains. Although Erk post-translationally regulates its targets, it phosphorylates transcription factors and post-transcriptional regulatory proteins, such as components of the miRNA pathway and RNA binding proteins. Thus, Erk has the capacity to regulate genes at all levels of expression.

Interestingly, even though Erk binding and phosphorylation motifs have been extensively characterized *in vitro* (47), and the effects of signaling activation on mouse ESCs are well studied (38), the identification of real and functionally important targets which mediate these effects has been challenging. Erk can also activate other regulatory kinases such as S6K and RSK, which have different target specificities. As such, the mechanistic basis for how Erk MAP kinase signaling destabilizes pluripotency and self-renewal remains unclear.

Pluripotency and self-renewal are maintained by a transcription factor network

Rapid progress has been made in identifying key regulatory signaling pathways, such as FGF/Erk MAP kinase signaling. But the exact mechanisms through which these signaling pathways regulate the activity of pluripotency, self-renewal or differentiation factors have not been identified (48).

Pluripotency, self-renewal and differentiation are cellular properties which are regulated by a network of transcription factors. The core members of this transcriptional network include Oct4, Sox2, Nanog, Klf4, Nac1, Dax1 and Zfp281 which form a module that regulates developmental genes, and Myc and Rex1, which support proliferation and self-renewal (49). These transcription factors bind enhancer sequences

which drive the expression of hundreds of pluripotency and self-renewal associated genes and can, in certain instances, promote the repression of differentiation factors (50).

Compromising the expression of any core transcription factor can destabilize the ESC transcription factor network. These regulatory effects may also be dose-dependent. For example, enhancing Oct4 expression by 50% induces differentiation to mesendoderm, but downregulation of Oct4 by 50% causes primitive endoderm differentiation (51). In addition, controlling the expression of individual transcription factors can compensate for a deficiency in others. For example, enhancing Oct4 expression maintains pluripotency in cells devoid of Sox2 (52). Finally, sustained expression of specific factors can reinforce the expression of all others to maintain pluripotency, as is the case with Nanog (53).

These observations indicate that signaling-dependent control of any core transcription factor can strongly impact the stability of mouse ESCs (Figure 1.4).

The homeodomain protein Nanog is regulated by Erk MAP kinase signaling

FGF/Erk MAP kinase signaling strongly regulates the expression of Nanog (37). The homeodomain protein Nanog was first identified in a screen for transcription factors which, when overexpressed, could maintain pluripotency in the absence of LIF. Since then, it has been shown that Nanog maintains the most naïve state of pluripotency (54), and is absolutely required for germline differentiation (55). Knockdown or knockout of Nanog greatly destabilizes their culture and promotes rapid differentiation (55, 56). Although the regulation of Nanog has been extensively studied

at the promoter, mRNA and protein level, mechanisms that connect FGF/Erk MAP kinase signaling to its regulation have not been identified (48).

To better understand how FGF/Erk MAP kinase signaling regulates pluripotency and self-renewal gene circuits, we explored this specific regulatory relationship further, to uncover novel mechanistic connections and molecular players, which could have a broader role in the regulation of other key developmental transcription factors.

Figures

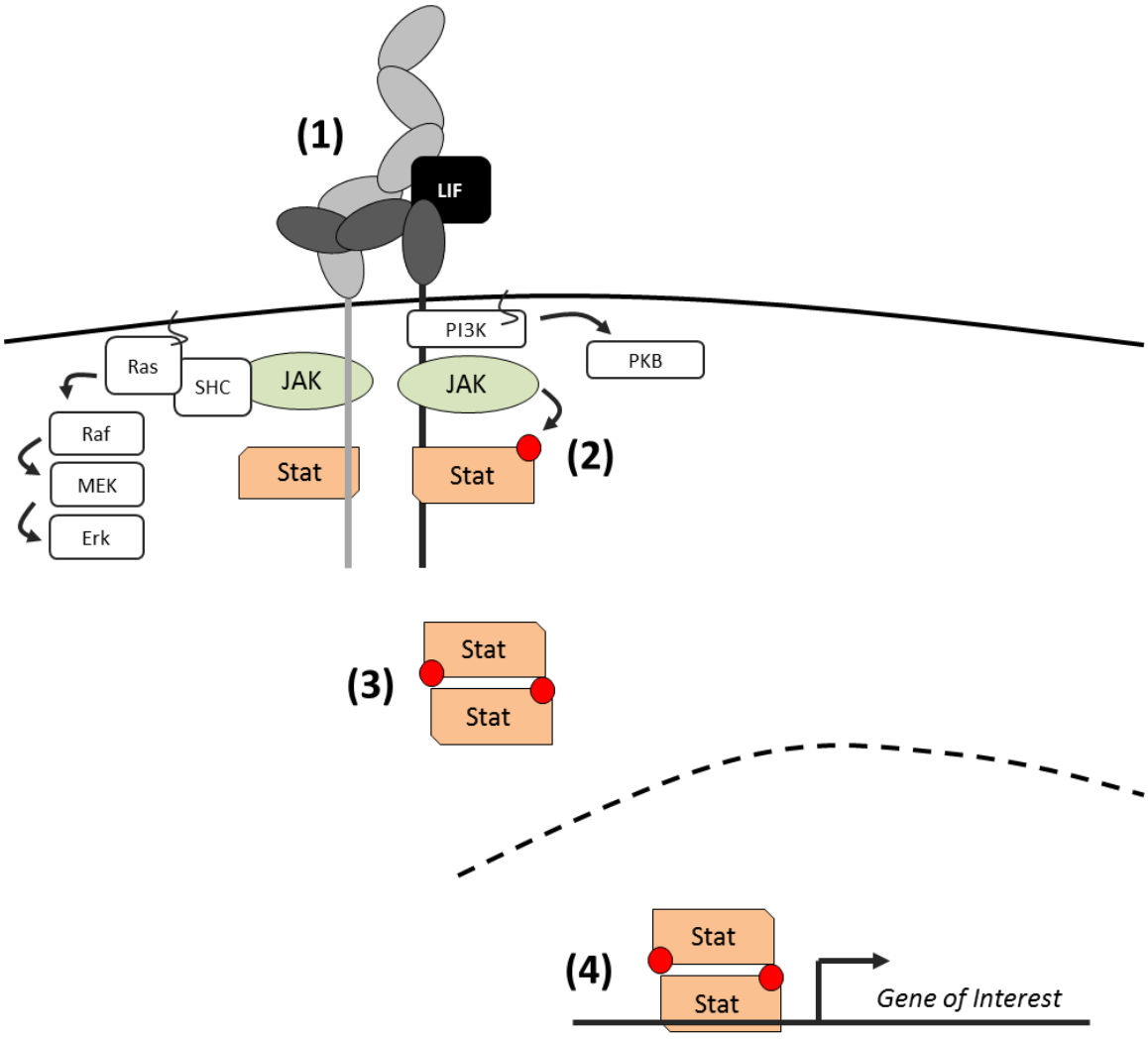


Figure 1.1 – *The LIF/JAK Signaling Pathway.* (1) LIF brings together gp130 and LIFR surface receptors to form a heterodimeric receptor complex that activates JAK kinase (green). (2) JAK kinase can phosphorylate Stat (red), (3) which can then forms dimers, (4) and shuttle to the nucleus as a transcription factor. JAK can also activate PI3 kinase and Erk MAP kinase second messenger pathways.

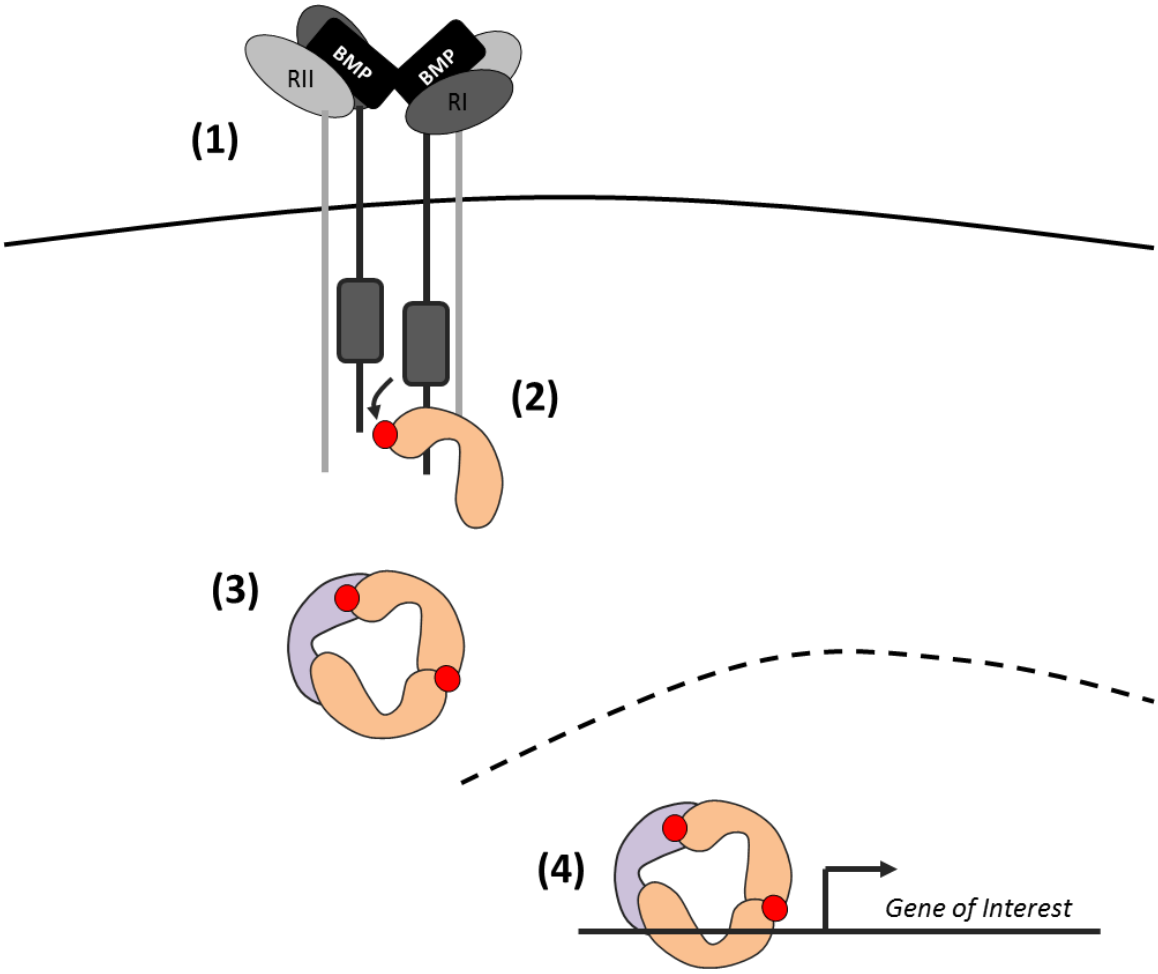


Figure 1.2 – *The BMP Signaling Pathway.* (1) BMP homodimers and heterodimers nucleate four surface receptors (two Type I and two Type II receptors). (2) Type I receptors within the heterotetrameric receptor complex can phosphorylate the C-terminal domain of Smad1/5/8 proteins, (3) to permit their trimerization with co-Smads (purple). (4) Activated Smad trimers shuttle to the nucleus to modulate transcription.

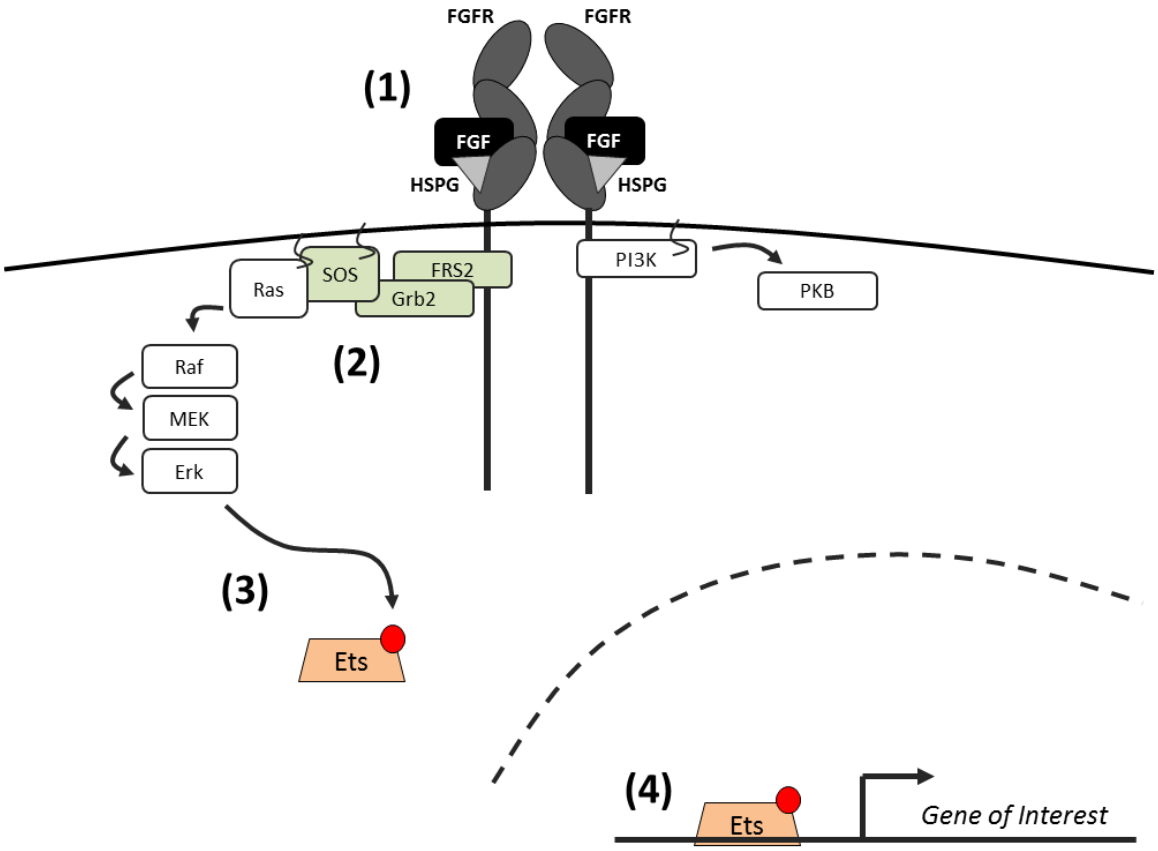


Figure 1.3 – *The FGF Signaling Pathway.* (1) FGF in association with heparin sulfate proteoglycans permit homodimerization of FGFR. Homodimerization of cell surface receptors greatly enhances their intrinsic tyrosine kinase activity, (2) leading to the phosphorylation of several adaptor proteins (green) which dock the intracellular domain. (3) Adaptor proteins channel the activation signal to Erk MAP kinase and PI3 kinase signaling pathways. (4) Terminal kinases within these pathways phosphorylate transcription factors, among other possible protein targets.

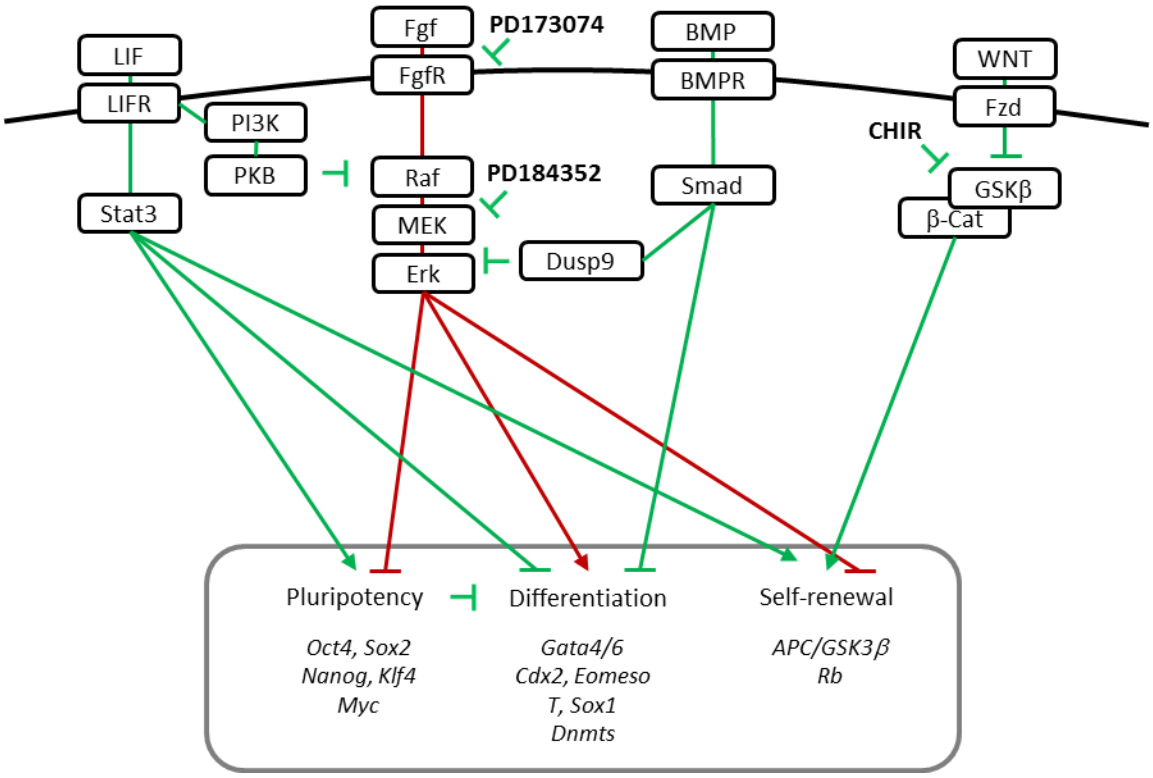


Figure 1.4 – *Signaling pathways regulating mouse ESCs*. LIF/Stat, FGF, Erk MAP kinase, BMP/Smad and WNT/β-Catenin signaling influence the pluripotency, self-renewal and differentiation in mouse ESCs. They can have stabilizing (green lines) or destabilizing (red lines) effects. Signaling pathways are stimulated by providing exogenous signaling ligand, such as for LIF and BMP. Alternatively, signaling pathway activity can also be modulated using pharmacological inhibitors, such as CHIR (targets GSK3β), PD173074 (targets FGF receptors) and PD184352 (targets MEK1/2). The mechanistic connection between these signaling pathways and the molecular regulators of pluripotency, self-renewal and differentiation (*italicized*) remains unclear.

Chapter 2

FGF/Erk MAP kinase signaling utilizes post-transcriptional regulatory mechanisms in mouse ESCs.

(This chapter was adapted, in part, from (57))

Introduction

Mouse ESCs are trapped in a unique developmental state which allows them to proliferate without differentiating. This developmental state is stabilized by transcription factors, of which an increasing number have been shown to play indispensable roles. A core network of less than 20 factors is thought to support the most critical functions by driving the expression of pluripotency-associated genes, and activating repressors of differentiation cues or markers.

Intercellular signaling regulates this transcription factor network via second messenger proteins, which can have stabilizing or destabilizing effects. For example, FGF/Erk MAP kinase signaling is known to destabilize mESC pluripotency and self-renewal, but it may do so through numerous transcriptional, post-transcriptional and post-translational mechanisms. FGF/Erk MAP kinase signaling is a well-characterized signaling pathway, but very little is known about how it is mechanistically connected to the regulation of pluripotency genes.

How can candidate mechanisms be identified? Among the most well-characterized pluripotency factors, Nanog expression is known to be strongly regulated by FGF/Erk MAP kinase signaling (10). The anti-correlation between FGF/Erk MAP kinase

signaling activity and Nanog expression is well-documented *in vitro* and *in vivo* (37). To connect FGF/Erk MAP kinase signaling to pluripotency and differentiation gene circuits, we explored the dynamics of this regulatory relationship further to uncover novel mechanistic connections and their molecular players.

Materials and Methods

Nanog Reporter Cell Line

We used mESCs carrying a *histone2B-yfp* reporter driven by the endogenous *nanog* promoter [v6.5 mESCs, Strain: (C57BL/6 x 129S4/SvJae), F1] to explore the connection between Nanog expression and FGF or Erk MAP kinase signaling (Figure 2.1). Stable integration of the *histone2B-yfp* reporter into the *nanog* locus inactivated one of the *nanog* alleles, which reduced overall Nanog expression levels in these cells. However, these reporter cell lines are haplo-sufficient and remain pluripotent (58).

Immunohistochemistry and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Timecourses

To readout changes in protein level in response to FGFR or MEK1/2 inhibition, with 200nM PD173074 or 5uM CI-1040, respectively, we immunostained inhibitor treated reporter cell lines with a polyclonal Nanog antibody (Bethyl Labs, A300-397A, 1:1000). Cultures were harvested with 0.25% Trypsin-EDTA, dispersed into single cell suspensions and added to an equal volume of 4% Formaldehyde. Cells were pelleted, resuspended in 10% FBS in PBS to block, gently pelleted again, and then stained with

primary and secondary antibodies at 4°C, overnight. Before flow cytometry analysis, cells were pelleted and resuspended in 2.5 mg/ml BSA in 1xHBSS. All samples, stained and unstained, were analyzed with a Miltenyi Biotech VYB flow cytometer. Compensation and background correction were applied post-acquisition.

In parallel, we tracked mRNA level changes. Total RNA was harvested with QIAzol reagent (Qiagen, 79306), after which cDNAs were amplified and analyzed using qRT-PCR. A single qPCR reaction was composed of 0.5ul of cDNA, primers or primers with probes, and qPCR reaction mix (diluted to a final volume of 10ul). For qPCR experiments using TaqMan/hydrolysis probes (5' Dye: FAM, 3' Quencher: Zen/Iowa Black FQ) or primers, cDNAs were profiled with SsoFast Supermix Reagent (Bio-Rad) using the manufacturer recommended protocol. In brief, we employed a 2-step thermocycling protocol (an initial 30 second 95°C melt, followed by 40 cycles of 5 second 95°C melt and 10 second 60°C anneal/extend).

Two-dimension (2D) gel electrophoresis

Two-dimension (2D) gel electrophoresis was used to detect phosphorylated residues. Cells were harvested and lysed in detergent-free lysis buffer (8M Urea, 200mM NaCl and 40mM HEPES pH 8.0) at a concentration of $2x10^6$ cells per 100ul. Lysates were supplemented with Complete Protease Inhibitor (Roche) and Phosphatase Inhibitor Cocktail I and II (Sigma) when appropriate. Samples were separated on a pH gradient (pH 3.5 to pH 10) followed by molecular weight separation via SDS-PAGE. Samples treated with calf intestinal phosphatase (CIP) were first dialyzed a thousand-fold using

Amicon Ultra Centrifugal Filter Units (3kDa) to remove Urea and inhibitors. Samples were then incubated with 10 Units of CIP for 3hrs at 37°C in NEB Buffer "3" before separation by 2D-gel electrophoresis.

In vitro Phosphorylation Assay

Direct phosphorylation by Erk MAP kinase was screened *in vitro*. A total of 5ug of NiNTA purified, recombinant produced Nanog/Myc-6His, was incubated with 200ng Human Erk1 (Cell Signaling Technologies) in Kinase Buffer (25mM Tris-HCl, 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na_3VO_4, 10mM $MgCl_2$) supplemented with 100uM ATP, [γ-32P]. Radioactive labeling was screened using a phosphorimager.

Nanog mRNA half-life Measurement

To measure changes in Nanog mRNA half-life, mESC cultures were co-transfected with a reverse Tet (rTet) expression plasmid (PGK-H2B-mCherry/T2A/rTet) and either CMV(2xTetO)-H2B-YFP or CMV(2xTetO)-Nanog (with or without AREs) at 19:1 ratio by mass. Culture media was then transitioned to media containing FGF4 and Heparin (10ng/ml and 10ug/ml respectively) or PBS to determine the regulatory effect of ERK MAP kinase signaling in *fgf4-/- cells*. To stop transcription from the CMV-TO promoter, Doxycycline was added to obtain a final concentration of 1ug/ml which permits binding of rTet to Tet operator sequences. Changes in the abundance of H2B-YFP or Nanog mRNA relative to Gapdh, Sdha and Tbp housekeeping genes was measured using qRT-PCR. To distinguish from endogenous Nanog mRNA, we developed a primer set that

specifically recognized the 5'UTR of Nanog expressed only from the CMV(2xTetO) promoter.

Results and Discussion

We used pharmacological inhibitors of signaling to disrupt the regulation of Nanog expression (Figure 2.2.A). We discovered that in response to FGFR inhibition, Nanog protein levels increased in two distinct stages: An initial response was observed within 5 hours, with a second, more gradual increase occurring over the next 20 hours (Figure 2.2.B, blue solid line). Interestingly, the initial rapid increase in Nanog protein (≤ 5hrs) could not be explained by a corresponding increase in transcription, as YFP fluorescence increased only after a short delay (Figure 2.2.B, blue dotted line). We also observed the same two-stage response to MEK1/2 inhibition (Figure 2.2.B, red solid and dotted lines). These results indicate that in addition to transcriptionally regulating Nanog expression, FGF and Erk MAP kinase signaling independently regulate Nanog protein levels on a much faster timescale.

Previous work has shown that a *cis*-regulatory element in the proximal *nanog* promoter is required for downregulating Nanog transcription by FGF/Erk MAP kinase signaling (59) (Figure 2.3). This region of the *nanog* promoter contains a transcriptional enhancer, composed of DNA sequences surrounding a conserved Oct4/Sox2 binding motif (60). Ablating this Oct4/Sox2 enhancer compromises the transcriptional regulation of Nanog by FGF/Erk MAP kinase signaling (59). Since Oct4 and Sox2 are known to nucleate many pluripotency associated transcription factors, FGF/Erk MAP kinase signaling thus mediates its repression by first downregulating one or more of these

activating transcription factors (Figure 2.3) (49). Nanog has been shown to auto-regulate its own transcription. Post-translational regulation of Nanog could compromise enhancer function to permit signaling dependent control over the *nanog* promoter, and could explain why the transcriptional response to FGFR and MEK1/2 inhibitors is delayed relative to protein level changes.

Erk may influence Nanog protein stability directly via phosphorylation (Figure 2.4.A). We determined that Nanog protein is phosphorylated (Figure 2.4.B), and multiply phosphorylated Nanog accumulates when the proteasome is inhibited (Figure 2.4.C). However, Nanog is not phosphorylated by Erk MAP kinase (Figure 2.5). These data do not discount the possible role of other unidentified kinases, which operate downstream of Erk1/2 activation, and for which consensus binding motifs have not been identified.

In the absence of a direct post-translational mechanism that could account for protein level changes, another possible explanation is that regulation of Nanog mRNA leads to corresponding changes in Nanog protein. Erk MAP kinase signaling might regulate the biogenesis of microRNAs (61), which would then regulate Nanog mRNA translation and stability directly. Recent studies implicate several miRNAs in the maintenance of self-renewal and differentiation (62, 63), and a few have been shown to target Nanog mRNA (64).

To explore this possibility, we profiled changes in mature miRNA expression in *fgf4*$^{-/-}$ cells after brief stimulation with HrFgf4/Heparin using microarrays. Differentiation associated miRNAs were upregulated after 5 hours of FGF signaling activation (Figure

2.6). Notably, miR-296-3p expression was upregulated ~2-fold by FGF, which has been shown in previous studies to directly regulate Nanog expression (64).

FGF signaling reduces Nanog mRNA levels (Figure 2.7). We discovered that transfecting *fgf4-/-* mESCs with an antago-miR against miR-296-3p could not block the downregulation of Nanog mRNA by FGF4/Heparin (Figure 2.7). Furthermore, transient knockdown of Dicer and/or Drosha expression using siRNAs could not alter Nanog or YFP mRNA levels after 36 hours (Figure 2.8). These data indicate that microRNAs may not play a central role in the regulation of Nanog expression.

Interestingly, the response of Nanog mRNA to FGF or Erk MAP kinase signaling inhibitors was similarly rapid. Using qRT-PCR, we compared Nanog transcript levels to Histone2B-yfp mRNA levels. Here, we also observed a two-stage response to the inhibition of FGFR or MEK1/2, with changes in mRNA occurring well before changes in transcription, and on a similar timescale as protein level changes (compare Figure 2.2.B and 2.9.B). We note strong similarities between Nanog mRNA and protein level changes (compare Figure 2.2.B and 2.9.B). It is possible that changes in protein levels simply reflect changes in mRNA abundance.

We hypothesized that FGF/Erk MAP kinase signaling rapidly activated or enhanced the expression of an RNA binding protein, which then post-transcriptionally regulated Nanog mRNA. We inspected the Nanog mRNA sequence for protein binding motifs and discovered several AU-rich elements (AREs) in the 3'UTR (Figure 2.10.A). AU-rich element RNA binding proteins (AUBPs) have been shown to regulate mRNA stability and translational efficiency in other cellular contexts (65). To determine whether

Nanog's AREs are post-transcriptionally regulated by AUBPs in response to FGF/Erk MAP kinase signaling (Figure 2.10.B), we measured changes in the Nanog mRNA half-life in the presence or absence of AREs, and in the presence or absence of FGF signaling. Using *fgf4-/-* mESCs, we determined that the half-life of Nanog mRNA is 2.5 ± 0.4 hours (± s.e.m.) without FGF4/Heparin and 1.5 ± 0.3 hours (± s.e.m.) with Fgf4/Heparin (Figure 2.10.C, left). Removing all 3'UTR ARE sites [ΔARE (1,2,3)] eliminated the post-transcriptional regulation of Nanog mRNA by FGF signaling (Figure 2.10.C, middle). A negative control mRNA (H2B-YFP) which does not have AREs is also insensitive to changes in FGF signaling (Figure 2.10.C, right).

Together, these data show that Nanog's AREs are post-transcriptionally regulated by FGF signaling, highlighting the potential regulatory significance of AUBPs as signaling intermediates and as regulators of development.

Figures

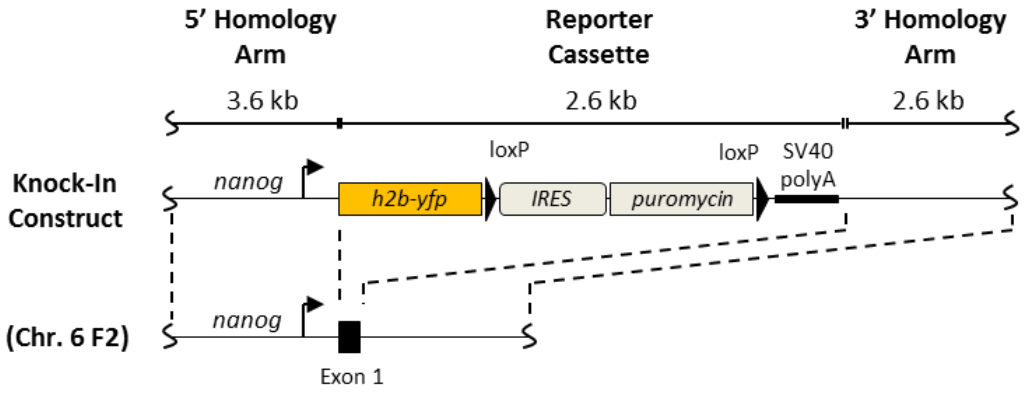


Figure 2.1 – Fluorescent reporter of Nanog transcription. A diagram of a *histone2b-IRES-puromycin*-SV40pA knock-in cassette used to replace exon1 of the *nanog* gene.

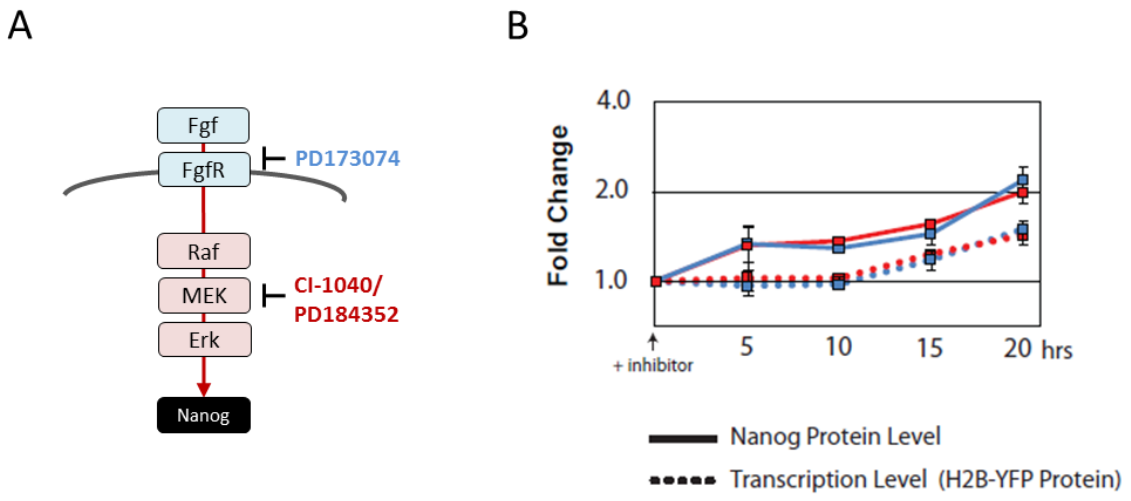


Figure 2.2 – ***FGF/Erk MAP kinase signaling regulates Nanog protein expression faster than Nanog transcription*****.** (A) Cartoon of the FGF/Erk MAP kinase signaling pathway and its connection to Nanog. This regulatory connection was explored using FGFR and MEK1/2 inhibitors, PD173074 (100ng/ml) and CI-1040 (5uM), respectively. (B) The effect of FGF or MEK inhibition on Nanog protein levels and transcription over a 20 hour time course [± SEM, n=4]. Values show fold change in concentration relative to an untreated negative control (DMSO only).

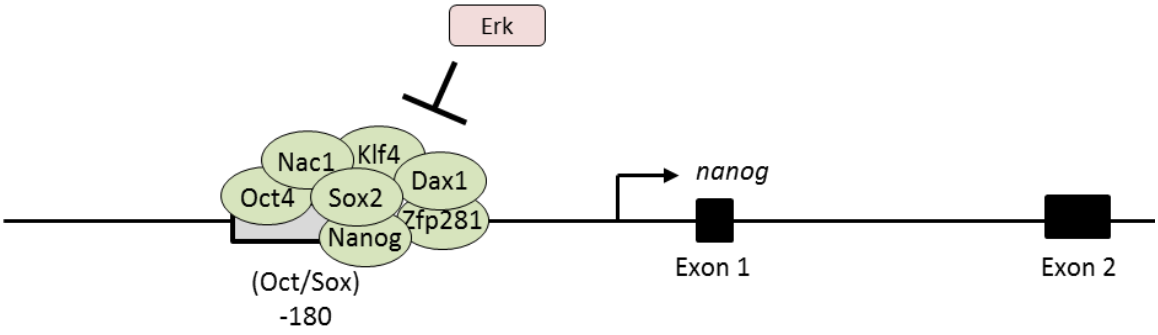


Figure 2.3 – FGF regulates the Nanog proximal promoter. FGF regulates factors which bind the Oct/Sox enhancer, approximately -180 from the transcription start site, to downregulate Nanog transcription.

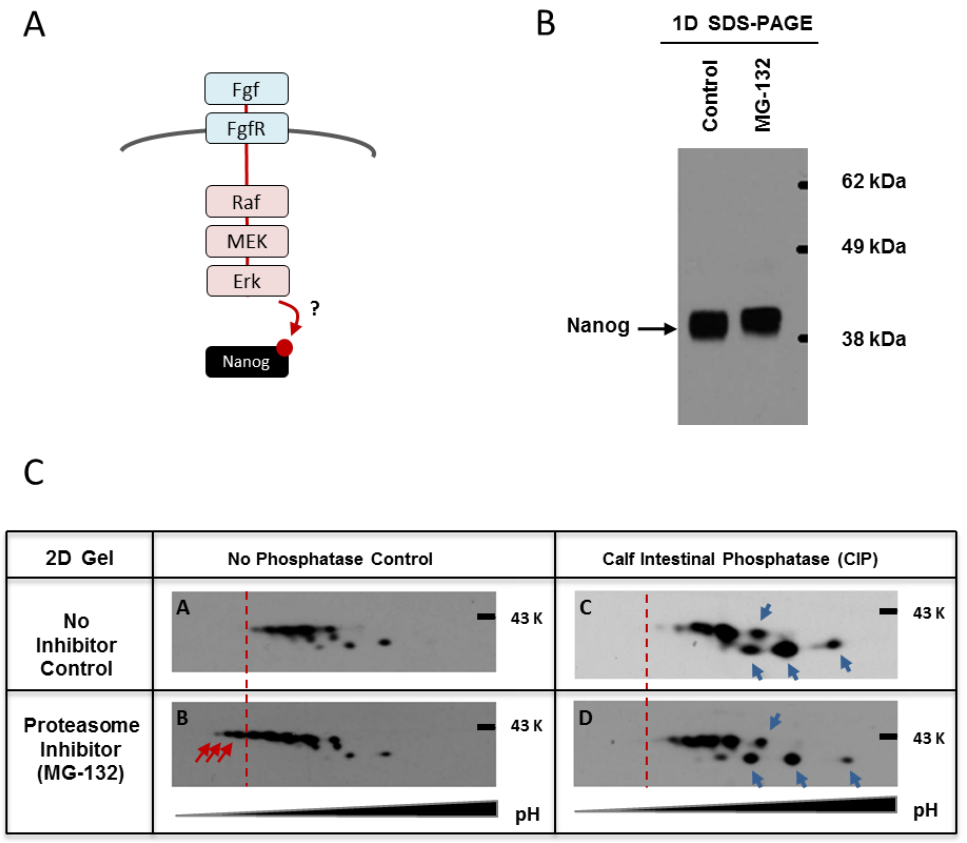


Figure 2.4 – Nanog protein is phosphorylated. (A) Erk could phosphorylate Nanog protein directly. (B) 1-dimension SDS-PAGE blotting for endogenous Nanog in the presence and absence of proteasome inhibitor (MG-132). Note the slight change in electrophoretic mobility after proteasome inhibition. (C) Each panel shows 2-dimension western blot for Nanog under the indicated conditions: Panels B and D include proteasome inhibitor. Panels C and D include Calf Intestinal Phosphatase treatment. Red arrows show that Nanog accumulates acidic post-translational modifications when mESC cultures are grown with proteasome inhibitor. Blue arrows indicate the accumulation of less acidic forms of Nanog protein after protein lysates are treated with phosphatase, which confirms the existence of phospho-modifications.

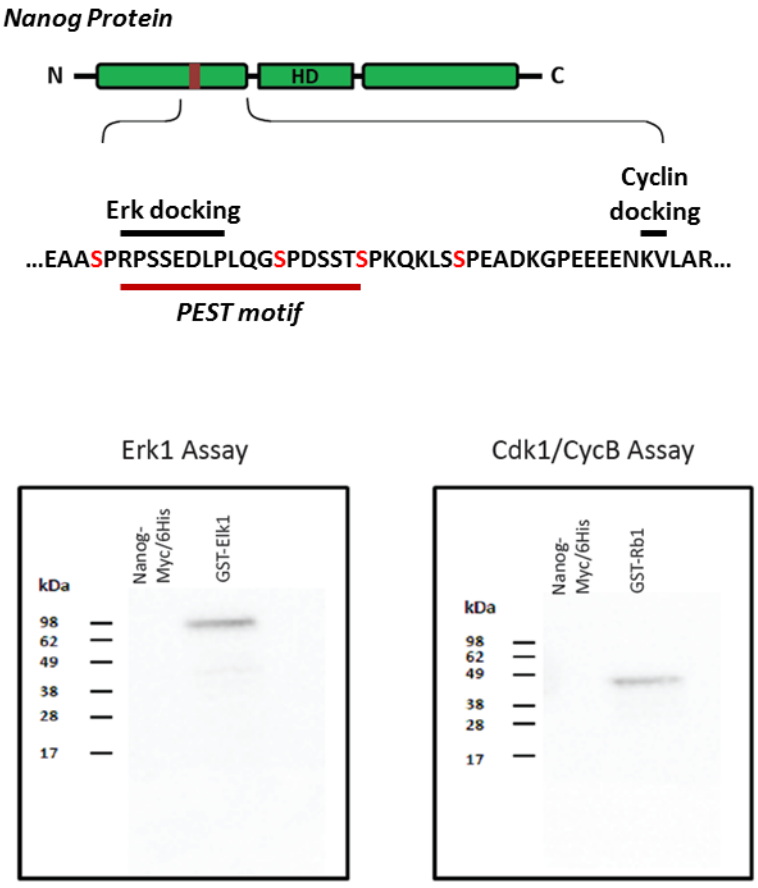


Figure 2.5 – Nanog protein is not phosphorylated by Erk. Nanog protein contains kinase docking sites (Erk and Cyclin) and phosphorylation motifs overlapping a PEST domain, which could link phosphorylation events to protein destabilization. However, recombinant Nanog-Myc/6His was not phosphorylated by Erk1 or Cdk1/CycB *in vitro.* By contrast, the Erk1 and Cdk1/CycB targets, GST-Elk1 and GST-Rb1, respectively, could be labeled with γ-32P-ATP.

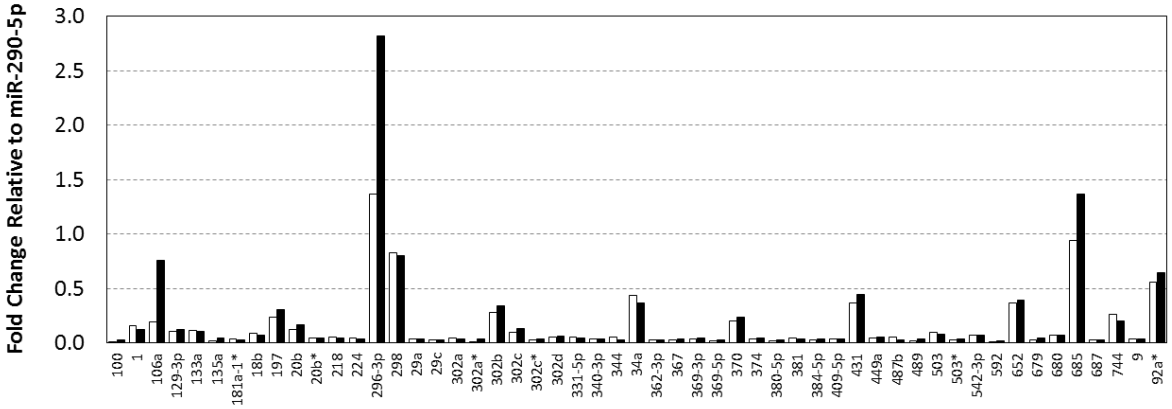


Figure 2.6 – FGF upregulates miR-296-3p expression. Mature miRNA expression was profiled in the presence (black bars) or absence (white bars) of FGF4/Heparin (5 hours) in *fgf4-/-* R1 mESCs. Plotted is the expression of known differentiation associated miRNAs, relative to miR-290-5p (positive control). Note the ~2-fold change in miR-296-3p, a miRNA that has been shown to post-transcriptionally regulate Nanog mRNA (64).

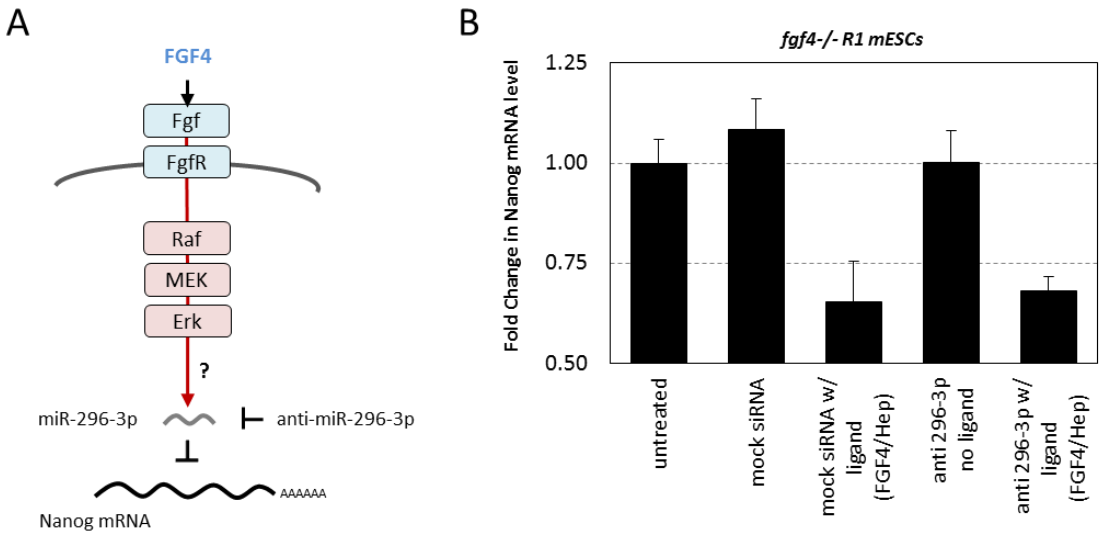


Figure 2.7 – Anti-miR-296-3p cannot block FGF-dependent regulation of Nanog. (A) miR-296-3p may act as a regulatory intermediate of FGF/Erk MAP kinase signaling. (B) Transfecting *fgf4-/-* R1 mESCs with anti-miR-296-3p [10nM] cannot prevent the downregulation of Nanog mRNA by FGF4/Heparin (5 hours).

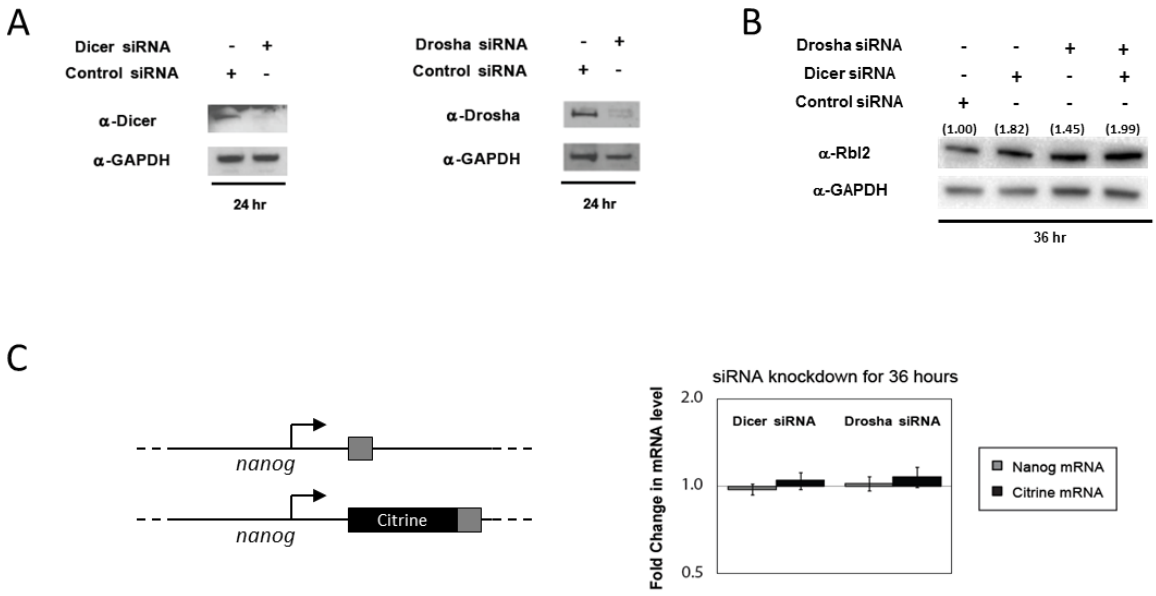


Figure 2.8 – miRNAs do not regulate Nanog mRNA and transcription levels. (A) Western blots showing successful knockdown of Dicer and Drosha after 24 hours. (B) Rbl2 is upregulated in response to Dicer and Drosha knockdown after 36 hours (positive control). (C) Dicer or Drosha knockdown does not affect Nanog mRNA or transcription levels in the Nanog reporter cell line [± SEM, n=4].

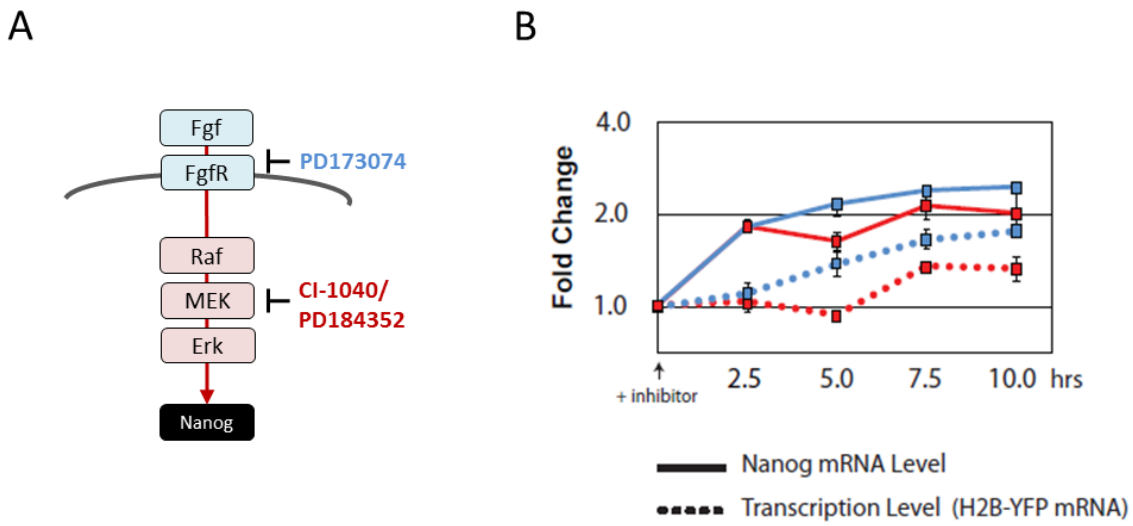


Figure 2.10 – FGF/Erk MAP kinase signaling regulates Nanog mRNA expression faster than Nanog transcription. (A) Cartoon of the FGF/Erk MAP kinase signaling pathway and its connection to Nanog. This regulatory connection was explored using FGFR and MEK1/2 inhibitors, PD173074 (100ng/ml) and CI-1040 (5uM), respectively. (B) The effect of FGFR and MEK1/2 inhibition on Nanog mRNA levels and transcription over a 10-hour time-course [± SEM, n=4]. Note the rapid change in protein and mRNA level and slower transcriptional response [± SEM, n=3].

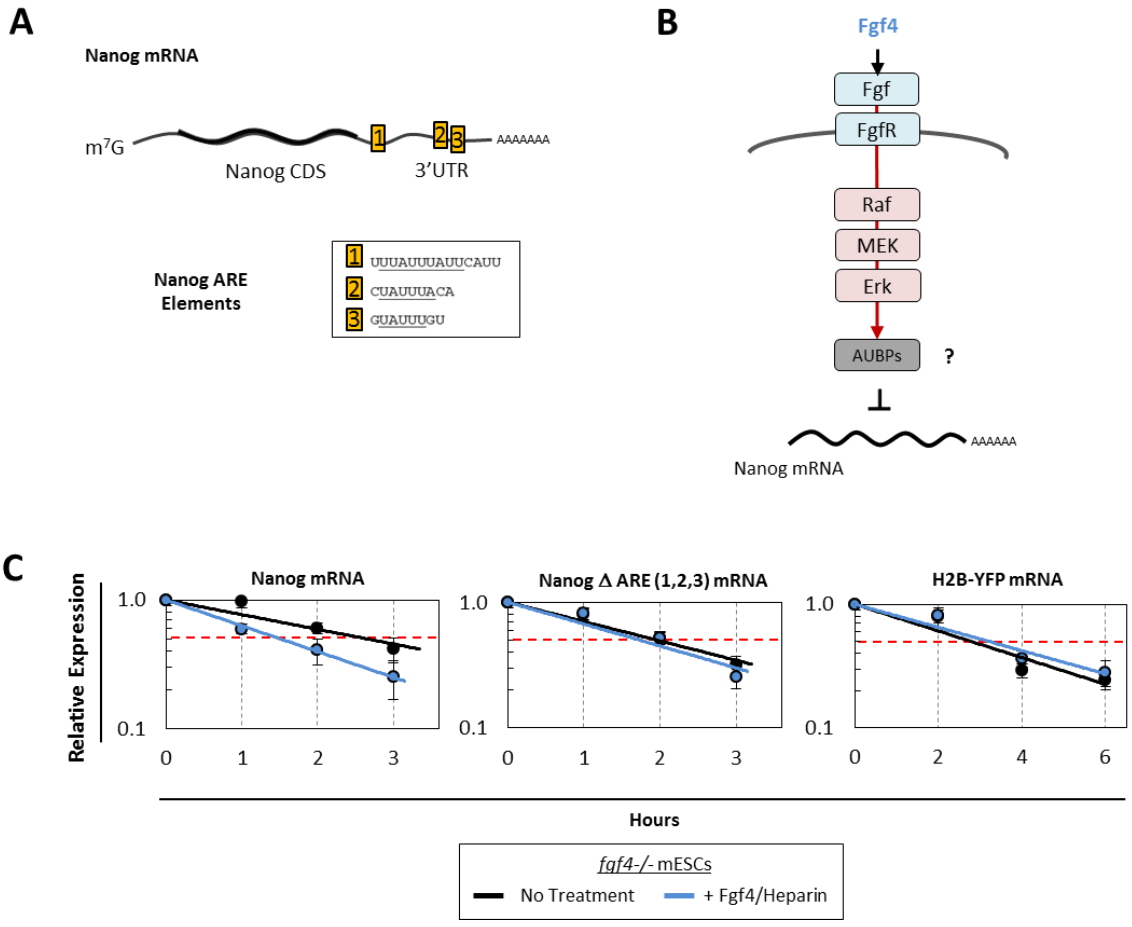


Figure 2.10 – Nanog is regulated by AU-rich element RNA binding proteins. (A) Illustration indicating the relative location and sequence of AU-rich elements in the 3'UTR of Nanog. (B) AUBPs may act as regulatory intermediates of FGF/Erk MAP kinase signaling. (C) Changes in mRNA half-life were profiled in the presence (blue data points) or absence (black data points) of FGF signaling in *fgf4-/-* mESCs for wild-type Nanog mRNA (top), mutant Nanog mRNA with deleted AREs (ΔARE (1,2,3)) (middle), and H2B-YFP mRNA (bottom) [± SEM, n = 4]. Red dashed lines indicate half the initial mRNA concentration.

Chapter 3

FGF/Erk MAP kinase signaling regulates the expression of Brf1, an AU-rich element RNA binding protein.

(This chapter was adapted, in part, from (57))

Introduction

AU-rich element mRNA binding proteins (AUBPs) represent an important class of regulators required for the proper development of embryonic and adult tissues in the mouse (66), but whether they have developmentally important roles in mouse embryonic stem cells (mESCs) remains unclear. A recent proteomic survey identified more than 500 mRNA binding proteins, several of which are AUBPs (67). Independent of the microRNA pathway, AUBPs are known regulators of splicing, mRNA stability, translational efficiency and RNA transport (68), and could provide an additional layer of developmental regulation that complements other pluripotency and self-renewal mechanisms. AUBPs are essential in many developmental systems, such as during hematopoiesis, neurogenesis, germ cell commitment and placental morphogenesis (69-71). Their absence or misregulation can be lethal, and often promotes disease progression (72-74). Despite growing interest in the many functions of AUBPs, their regulation and function in mESCs remains poorly understood.

The expression and activity of AUBPs is known to be regulated by growth factor signaling in many cellular contexts (75, 76). In mESCs, the FGF/Erk MAP kinase signaling pathway is a central regulator of self-renewal, pluripotency and differentiation (43, 48).

Although much is known about the developmental effects of FGF/Erk MAP kinase signaling inhibition or activation (30, 38), the regulatory mechanisms employed downstream of Erk1/2 often remain unclear. Various transcriptional, post-transcriptional and post-translational mechanisms are engaged to regulate target genes (44). As part of this signaling cascade, AUBPs could mediate rapid signaling-dependent responses, but this potential role has not been investigated.

Materials and Methods

We measured changes in AUBP mRNA level using qRT-PCR. As described previously, total RNA was harvested with QIAzol reagent (Qiagen), after which cDNAs were amplified and analyzed using qRT-PCR. Further information regarding qRT-PCR reaction conditions and reagents can be found in the Supplementary Methods section.

For Western blot analysis, cells were harvested and lysed with SDS Loading Buffer (1% SDS, 10% glycerol, 0.01% bromphenol blue and 60mM Tris pH 6.8) at a concentration of $1x10^6$ cells per 100ul. To profile nuclear and cytoplasmic protein fractions, harvested cell pellets were first processed with NE-PER Nuclear Protein Extraction Kit, before the addition of SDS Loading Buffer.

Results and Discussion

AUBP expression in mESCs has been documented by several groups (67, 74, 77). The known sensitivity of AUBPs to growth factors suggested that these proteins could be regulated by FGF/Erk MAP kinase signaling (76, 78). To explore this potential regulatory connection, we first profiled the transcriptome of E14 mESCs using high-

throughput sequencing to determine which AUBPs are actively expressed (Figure 3.1.A). We identified several classes of AUBPs, including: (1) members of the Zfp36 protein family [TTP (*zfp36*), Brf1 (*zfp36l1*) and Brf2 (*zfp36l2*)], which are known to play critical roles during hematopoiesis by destabilizing Cytokine and Notch-Delta signaling-associated mRNAs, (2) members of the Hu protein family [HuR (*elavl1*), HuB (*elavl2*)], which stabilize their mRNA targets and are known to actively regulate germ cell development, and (3) Auf1 (*hnrnpd*), which can stabilize or destabilize mRNA and modulate inflammation in the adult mouse (65).

To determine whether any of the detected AUBPs was regulated by FGF/Erk MAP kinase signaling, we measured changes in their expression in response to pharmacological inhibitors of MEK1/2. We discovered that TTP and Brf1 responded strongly to MEK1/2 inhibition, with mRNA levels downregulated greater than 2-fold after 5 and 10 hours (Figure 3.1.B). Brf2, Auf1 and KHSRP mRNA levels were also slightly downregulated. Interestingly, three out of five of these responding genes are members of the Zfp36 protein family (Figure 3.1.B, red bracket).

We explored the regulatory connection between Zfp36 AUBPs and FGF/Erk MAP kinase signaling further by measuring how TTP, Brf1 and Brf2 responded to short and long periods of MEK1/2 inhibition (Figure 3.2.A). Incubation with MEK1/2 inhibitor resulted in a rapid reduction in TTP, Brf1 and Brf2 mRNA level as gauged by RT-qPCR (Figure 3.2.B). Brf1, Brf2, and to a lesser extent, TTP mRNA level changes were significant within 1 hour of inhibitor treatment (Figure 3.3.A), and continued to decrease after 7.5 hours of inhibition (Figure 3.2.B). However, after 10 hours, TTP and Brf2 mRNA

expression recovered, while Brf1 expression remained suppressed (Figure 3.2.B). Since the pharmacological inhibitor provides continuous suppression of Erk MAP kinase signaling (Figure 3.3.B), these data indicate that TTP and Brf2 mRNA respond only transiently (t < 10 hours) to changes in Erk MAP kinase signaling, whereas Brf1 mRNA maintains a sustained response to the level of Erk MAP kinase signaling.

Protein level changes were also rapid, with a 30% reduction in TTP and a 50% reduction in Brf1 within 1.5 hours of inhibitor treatment (Figure 3.2.C and 3.3.C). However, whereas Brf1 protein levels continued to fall for the remainder of the time course, reaching ~10-fold less protein by 30 hours, TTP protein levels recovered and increased above DMSO treated controls (Figure 3.2.C). These data indicate that the regulation of TTP protein becomes distinct from the regulation of TTP mRNA at later times (compare Figure 3.2.B and 3.2.C). We note that in other cellular contexts, direct phosphorylation of TTP protein by Erk1/2 has been shown to reduce its stability (75). Furthermore, Zfp36 AUBPs also contain AU-rich sequences in their own mRNAs, which enable direct auto- and cross-regulation (Figure 3.3.D). These mechanisms could explain why TTP protein and mRNA levels respond differently after prolonged MEK1/2 inhibition. In contrast, Brf2 protein levels were much less affected, dropping slightly at 10 hours, and then recovering at later time points. Thus, at both the mRNA and protein levels, Brf2 responds more weakly to these perturbations (Figure 3.2.C). These results indicate that AUBP levels respond to changes in Erk MAP kinase signaling with different kinetics.

To further validate these findings, we checked whether upregulating FGF signaling could produce opposite results to inhibition. We added FGF4/Heparin to *fgf4-/-* R1 mESCs (strain FD6), in order to activate Erk MAP kinase signaling (Figure 3.2.D) (79). TTP, Brf1 and Brf2 mRNA levels increased within 5 hours of ligand addition, with similar changes at the protein level (Figure 3.2.E and 3.2.F). These changes occurred specifically within the cytoplasmic compartment, consistent with a role for these AUBPs in regulating targeted degradation of mature mRNAs (Figure 3.2.F). Together, these results indicate Zfp36 protein expression responds rapidly to both increases and decreases in FGF/Erk MAP kinase signaling activity, leading to both transient (TTP, Brf2) and sustained (Brf1) regulatory responses.

Among the Zfp36 AUBPs, FGF/Erk MAP kinase signaling most strongly regulated the expression of Brf1 in pluripotent conditions (Figure 3.2). During differentiation, Brf1 expression is similarly dynamic, and continued to be regulated by FGF/Erk MAP kinase signaling (Figure 3.4.A). The ~2-fold reduction in Brf1 expression over 4 days of LIF withdrawal (Figure 3.4.A) tracked concomitant changes in FGF4 expression (Figure 3.4.B), and the downregulation of the Erk MAP kinase target genes Spred2 and Spry2 (Figure 3.4.B). Could Brf1 mechanistically link FGF/Erk MAP kinase signaling to the post-transcriptional regulation of Nanog mRNA?

Figures

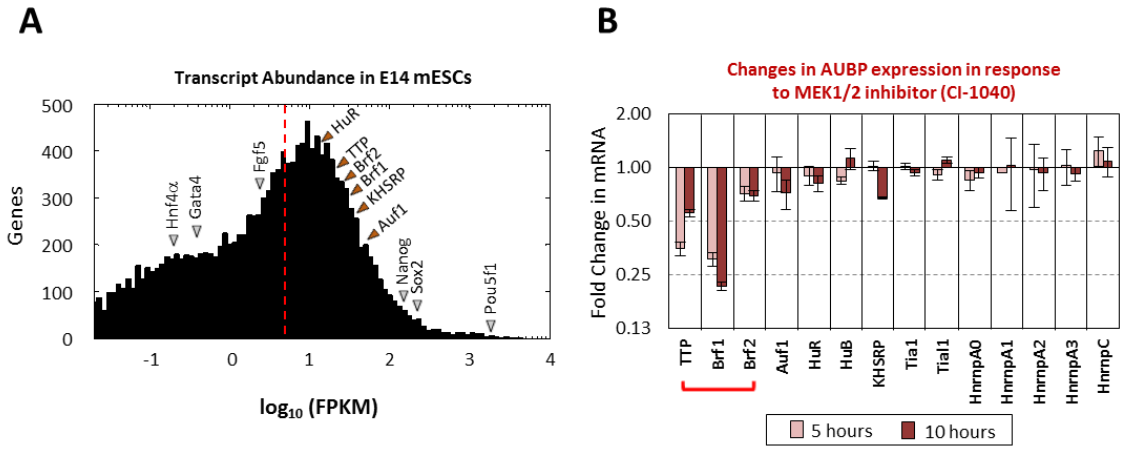


Figure 3.1 – Erk MAP kinase signaling regulates AUBP expression in mESCs. (A) Distribution of average transcript abundances (FPKM) from two total RNA biological replicates. Highlighted are notable mESC pluripotency and differentiation genes (gray triangles), which were used to distinguish actively expressed genes (mean FPKM ≥ 5, 7,194 genes) from the gene expression background (mean FPKM < 5, 11,119 genes). FPKM = 5 denoted by red dashed line. Well-characterized and actively expressed AUPBs are highlighted (orange triangles). (B) Profiling changes in AUBP expression using RT-qPCR in response to a pharmacological inhibitor of MEK1/2 (CI-1040/PD184352) after 5 and 10 hours.

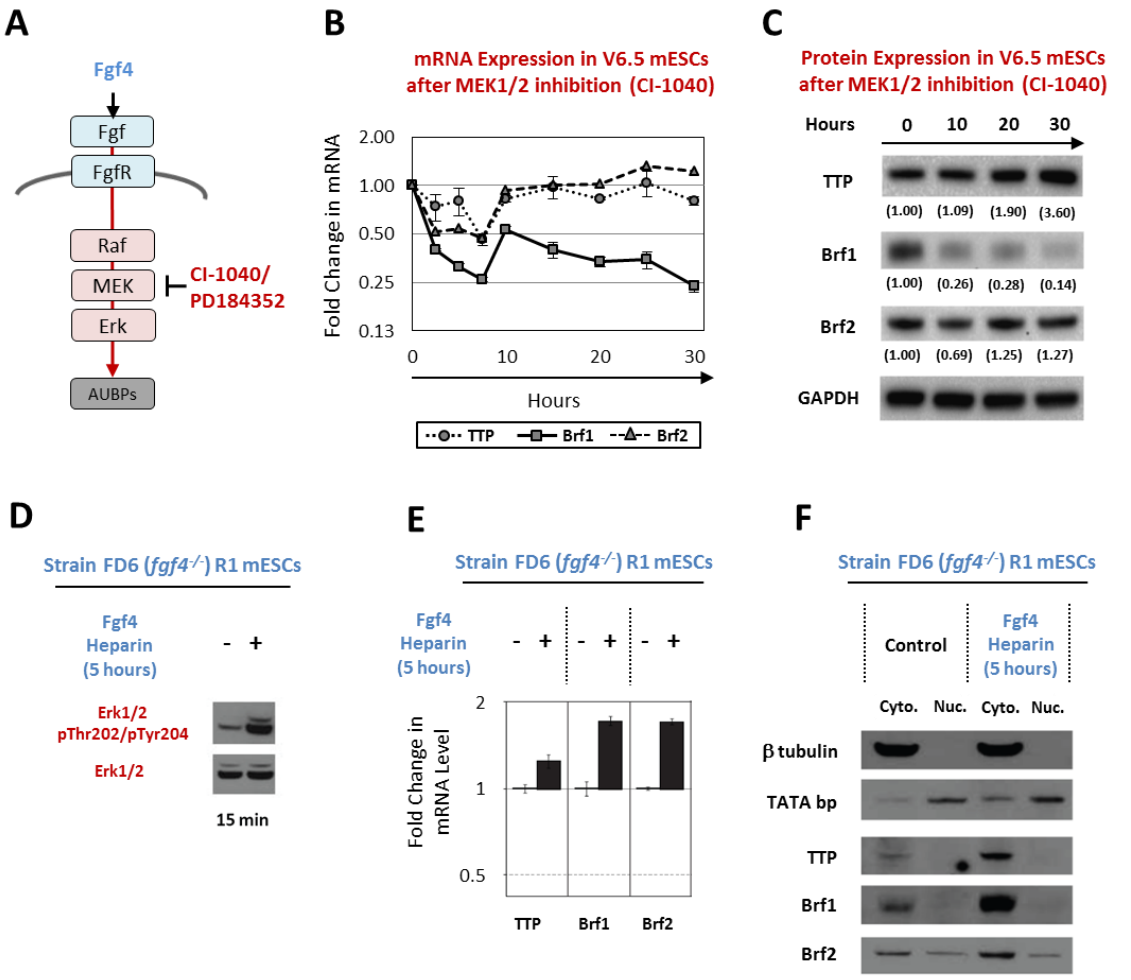


Figure 3.2 - FGF/Erk regulates the expression of Zfp36 RNA-binding proteins. (A) Cartoon depicting the potential relationship between FGF/Erk MAP kinase signaling and AUBP expression. Whereas the addition of Fgf4/Heparin activates the FGF/Erk MAP kinase pathway, culturing cells with 5 uM PD184352/CI-1040 inhibits Erk1/2 activation. (B) A 30 hour RT-qPCR time course of TTP, Brf1, and Brf2 mRNA level changes in response to MEK1/2 pharmacological inhibitors [± SEM; n=3 for all time points], and (C) corresponding Western blots. (D) Western blot staining for Erk1/2 and phospho-Erk1/2 (Thr202/Tyr204) showing pathway activation in cells stimulated with 10ng/ml FGF4 + 10ug/ml Heparin for 15 minutes. (E) Changes in TTP, Brf1 and Brf2 after 5 hour

incubation with or without FGF4/Heparin as indicated [± SEM; n = 3]. (F) Western blot profiling of changes in TTP, Brf1 and Brf2 protein level in fgf4-/- mESCs after stimulation with FGF4/Heparin for 5 hours. To compare changes in intracellular localization, proteins were harvested sequentially as cytoplasmic [cyto] or nuclear [nuc] fractions, with β-tubulin and TATA binding protein serving as localization controls.

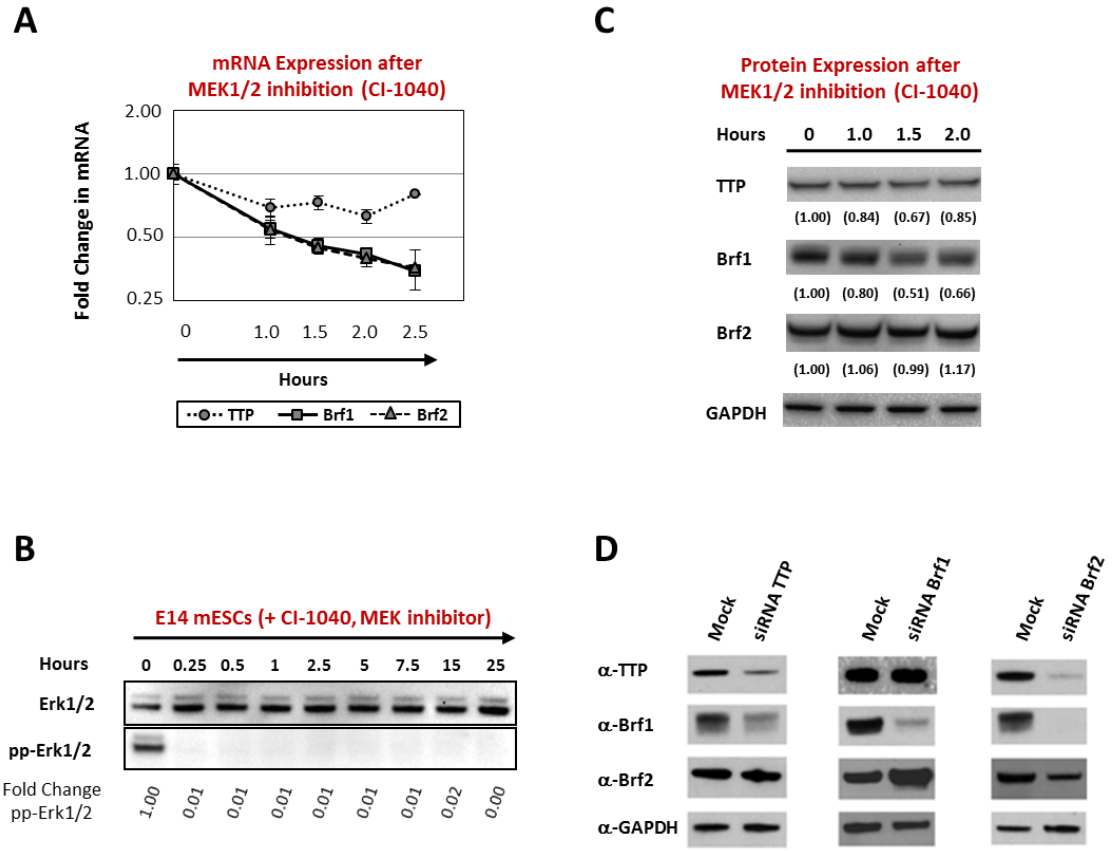


Figure 3.3 – Rapid changes in Zfp36 expression at the mRNA and protein level. (A) A 2.5 hour qRT-PCR time course of TTP, Brf1, and Brf2 mRNA level changes in response to MEK1/2 pharmacological inhibitors [± SEM; n=3 for t ≤ 2hrs; n=2 for t = 2.5hrs]. (B) Pharmacological inhibition of MEK1/2 with 5 uM PD184352/CI-1040 inhibits Erk1/2 activation for >24 hours. (C) Representative Western blots of TTP, Brf1 and Brf2 protein levels for a 2.5 hour time course. (D) Zfp36 AUBPs contain AREs within their own 3'UTRs, and have the capacity to directly auto- and cross-regulate their own expression. siRNA knockdown of individual Zfp36 AUBPs (10nM final siRNA concentration) leads to corresponding protein level changes in the remaining Zfp36 AUBPs. Protein levels were assayed 36 hours after siRNA transfection by Western blot.

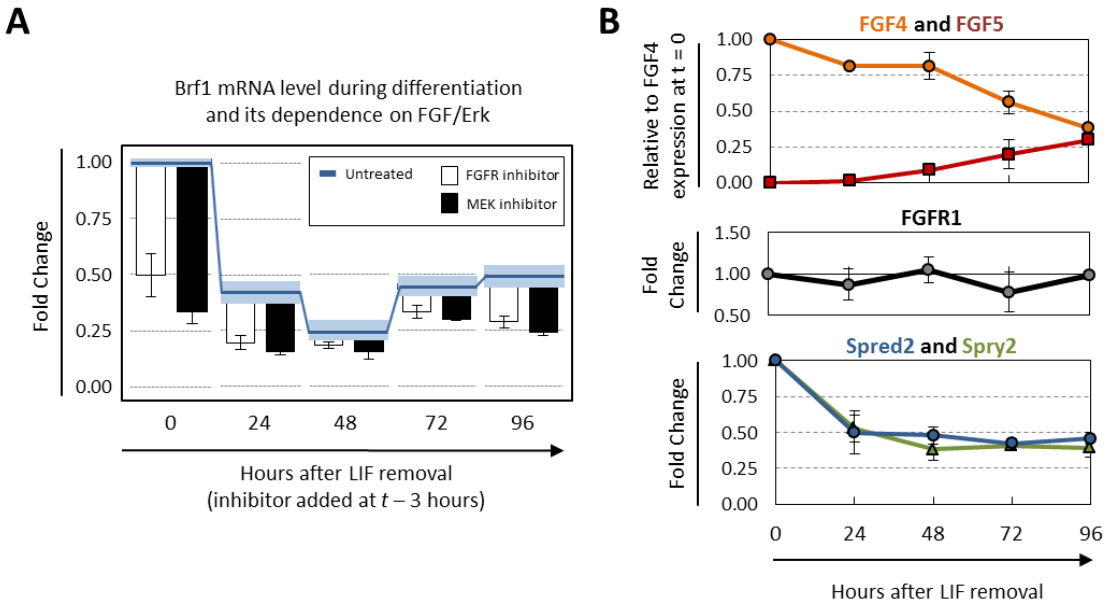


Figure 3.4 – Brf1 tracks changes in FGF/Erk during differentiation. (A) Changes in Brf1 expression after LIF removal (blue line; light blue bounding boxes at each time point represent ± SEM, n = 3), and its dependence on FGF or Erk MAP kinase signaling (white and black boxes, 3 hour inhibitor treatment) [± SEM, n = 3]. (B) Changes in FGF4, FGF5, FGFR1, Spred2 and Spry2 after LIF removal [± SD, n = 2].

Chapter 4

Brf1 binds many pluripotency associated genes *in vitro.*

(This chapter was adapted, in part, from (57))

Introduction

Several AUBP families exist and have the capacity to bind numerous mRNA targets. AUBPs interact with mRNAs via a core (-UUAUUUAUU-) motif located in the 3'UTR (80). This sequence is common, and can be found in the 3'UTR of 6% of mouse protein coding genes (Table 4.1). However, depending on the local sequence context surrounding the binding site, variation in this minimal motif could exist, to increase or decrease the association of AUBPs with target mRNAs. Previous work has already shown the existence of non-canonical AREs that mediate AUBP binding and regulation (81). The sequence determinants that permit regulation of target mRNAs have not been fully characterized.

Transcriptome-wide identification of Brf1 targets has not been undertaken in any developmental or cellular system. To better understand the functional and developmental significance of Brf1 in mESCs, and to further characterize its mechanistic connection to Nanog, we performed a number of different immunoprecipitation studies which assayed for direct protein-to-RNA binding.

Materials and Methods

RNA immunoprecipitation sequencing (RIPseq) Procedure and Analysis

To identify Brf1 mRNA targets, we conducted RNA immunoprecipitation sequencing (RIPseq) (Figure 4.1.A). Briefly, cytoplasmic extract from approximately $1x10^7$ E14 mESCs was distributed equally among 2 samples and 2 controls. For sample reactions, 5ug of anti-Brf1/2 antibody was used for 50ul of magnetic protein A/G beads. For control reactions, 5ug of rabbit IgG with no immunoreactivity was used for 50ul of magnetic protein A/G beads. After stringency washes and proteinase K digestion, RNA was isolated using Qiazol reagent.

RIP-purified RNAs and total RNA from E14 mESCs were prepared for sequencing using a TruSeq RNA Sample Prep kit. RNAs were fragmented to generate lengths of approximately 200 nucleotides, reverse transcribed with random hexameric primers to generate double stranded DNA, blunted, adenylated and ligated to Illumina sequencing adapters (150bp). DNA fragments were gel separated and all fragments running at 350bp were extracted and amplified. Amplified DNA fragments were then sequenced using an Illumina HiSeq2000.

Raw sequencing reads were trimmed (of 13nts from 5' end) prior to Bowtie mapping using a mouse transcript annotation containing only protein coding genes (18,313 genes), derived from the NCBI37/mm9 genome build. Mapping statistics were generated using eXpress (82). For enrichment analysis, fragments per kilobase exon per million mapped reads (FPKM) were used as a measure of transcript abundance (83).

We computed a statistic ($E_{RIP,n}$) that represents the degree to which the abundance of the n^{th} transcript is enriched by antibody mediated RIP. This statistic uses differences in a transcript's abundance after Brf1/2 antibody RIP (mean $FPKM_{BRF,n}$)

compared to rabbit IgG RIP (mean $FPKM_{IgG,n}$), normalized by their initial abundance in total RNA before RIP (mean $FPKM_{Total,n}$). This difference was further normalized by a penalty factor (P), which accounts for a transcript's tendency to be non-specifically purified, and is thus a saturating function of transcript abundance in the IgG control experiment:

$$E_{RIP,n} = \frac{(A_{BRF,n} - A_{IgG,n})}{P}$$

Where,

$$A_{BRF,n} = \frac{\text{mean FPKM}_{\text{BRF},n}}{\text{mean FPKM}_{\text{Total},n}}$$

$$A_{IgG,n} = \frac{\text{mean FPKM}_{\text{IgG},n}}{\text{mean FPKM}_{\text{Total},n}}$$

$$P = \text{median}\left(A_{IgG}\right) + A_{IgG,n}$$

Although $A_{IgG,n}$ captures the level of non-specific association of a transcript with assay components (i.e., protein A/G beads, rabbit IgG, etc.), non-specific association of transcripts with immunoprecipitated RBP/RNA complexes could not be independently quantified, and could contribute a background to these $E_{RIP,n}$ values.

AUBP Pull-down Assay

To isolate AUBPs from cytoplasmic extracts *in vitro*, IL-2 and Nanog RNAs were produced *in vitro* using T7 Ampliscribe (Epicentre Technologies). These RNAs were then hybridized to biotin-DNA oligonucleotides at their 3' end. 200 pmoles of IL-2 or Nanog RNA was used for 250 pmoles of biotin-DNA oligonucleotide. Hybridization reactions were added to 1mg streptavidin magnetic agarose beads (New England Biolabs). Crude cytoplasmic extracts used for protein pull down assays were obtained using NE-PER reagents (Thermo Scientific). RNA/DNA-bead conjugates were incubated in crude cytoplasmic extract for 1hr at 4°C, washed 5x with Binding Buffer [20mM Tris-HCl pH 7.5, 50mM KCl, 1mM EDTA, 1mM DTT, 0.5% Triton-X 100 with RNAse inhibitors] and incubated in High Salt Elution Buffer [Binding Buffer + 1M NaCl] to collect RNA-bound protein fractions. Protein pull downs were analyzed via Western blot.

Full description of high-throughput sequencing datasets can be found in (57).

Results and Discussion

We computed a statistic, denoted E_{RIP} for each actively expressed transcript purified via RIPseq. E_{RIP} represents the amount of mRNA co-precipitated with Brf1 protein over non-specific background levels. Genes with high E_{RIP} values were more likely to have AREs in their 3'UTR (Figure 4.1.B). For example, considering the transcripts most enriched by Brf1 immunoprecipitation (positive outliers, $E_{RIP} > 1.226$, 418 genes), 25.1% contained the minimal full consensus ARE and 60.0% contained the minimal partial consensus ARE. These percentages represent a 3 to 4-fold increase in ARE abundance relative to their frequency among all protein coding genes (Figure 4.1.B).

Moreover, several of the most highly enriched target genes were previously characterized as direct targets of Zfp36 proteins (e.g., Ier3, Mllt11 and Pim3), including Zfp36 proteins themselves (84, 85). Interestingly, based on our definition of the minimal ARE element, many highly enriched target genes do not contain consensus AREs. However, the existence of non-canonical (though still poorly characterized) AU-rich sequences has been documented, and could explain the enrichment of these mRNAs (81). Thus, the RIPseq assay can selectively enrich for mRNAs containing AU-rich elements.

Several pluripotency-associated factors were detected in the Brf1-RIP fraction, potentially explaining the developmental effects of Brf1 overexpression. For example, the core pluripotency regulators Nanog (E_{RIP} = 0.58) and Klf2 (E_{RIP} = 7.15) were both enriched, and were within the top quartile of enriched targets. Nanog broadly inhibits mESC differentiation, and its expression is reduced as cells lose pluripotency and commit to extra-embryonic and somatic cell lineages in culture (53, 86). Klf2, along with Klf4 and Klf5, inhibits mesendoderm differentiation. Knockdown of Klf factors up-regulates primitive streak markers, as well as Cdx2, a gene expressed in trophectoderm and extra-embryonic mesoderm (87). Also consistent with a role for Brf1 in promoting mesendoderm, the pluripotency factors Kdm4c (E_{RIP} = 0.63) and Zfp143 (E_{RIP} = 0.99) were enriched in the RIPseq assay. Knockdown of the lysine methyl-transferase Kdm4c is known to upregulate mesendoderm and extra-embryonic mesoderm markers (88). Zfp143 coordinates with Oct4 to transcriptionally activate Nanog. siRNA knockdown of Zfp143 rapidly initiates differentiation and promotes the expression of Fgf5, Cdx2 and

Cdh3, which are expressed in trophectoderm and cells that commit to extra-embryonic mesoderm (89). Understanding the regulation of these mRNAs may provide mechanistic insights into the Brf1-dependent control of gene expression, as well as its developmental effects in mESCs.

To corroborate these RIPseq results, we assayed for direct binding of Brf1 to an enriched mRNA, in this case, Nanog (Figure 4.2.A). Previous work has shown that Nanog is strongly regulated by FGF/Erk MAP kinase signaling (36), and these effects are mediated, in part, by direct regulation of the Nanog promoter (59). Post-transcriptional regulation by Brf1 would provide an alternative mechanism to repress Nanog expression.

We conducted an AUBP pull-down assay using RNA as bait (Figure 4.2.B, left). Wild-type RNA and variants with ARE sequence mutations were expressed in vitro and hybridized at their 3' ends to DNA oligonucleotides coupled to magnetic microbeads. These RNA-microbead conjugates were then incubated with crude cytoplasmic protein extracts, and all proteins capable of binding hybridized RNAs were magnetically isolated and purified for further analysis.

We used the 3'UTR sequence of IL-2 as a positive control, since it contains clusters of ARE sequences that are bound and regulated by Zfp36 AUBPs (90). Western blots showed that Brf1 could be purified from mESC lysates using a conjugated wild-type IL-2 sequence, but not using a mutant IL-2 lacking known AREs (Figure 4.2.B, right). Two Brf1 bands of different sizes were detected, possibly indicating the purification of different post-translationally modified forms of Brf1.

We next repeated the assay using Nanog mRNA as bait. The 3'UTR of Nanog mRNA is approximately 1kb, and contains three potential ARE elements: one full consensus (Site 1 in Figure 4.2.A) and two partial non-consensus sequences (Sites 2 and 3 in Figure 4.2.A). Western blotting of these protein pull-downs indicated that Brf1 bound to wild-type Nanog mRNA (Figure 4.2.B, right). Mutating the full-consensus ARE (Site 1) significantly reduced Brf1 binding. Removing the remaining two partial non-consensus AREs (Sites 2 and 3) did not appear to further reduce the Brf1 signal. In contrast, the presence or absence of AREs did not affect the binding of other RNA binding proteins. For example, addition of an androgen receptor 3'UTR sequence, which contains a poly(C) RNA binding protein 1 (PCBP1) site, to Nanog mRNA permitted isolation of PCBP1 protein (Figure 4.2.C). This binding was not affected by the presence or absence of Nanog's AREs. In contrast, a Nanog mRNA containing only a 120 nucleotide polyA without a PCBP1 site did not bind PCBP1 protein. Together, these results confirm that Brf1 binds specifically to Nanog mRNA in an ARE-dependent manner.

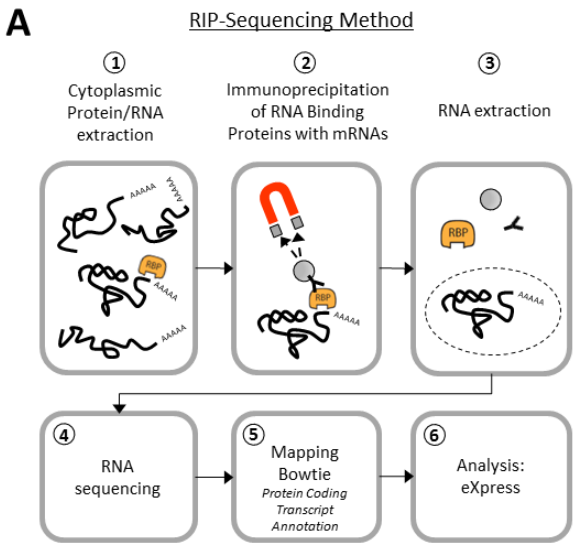
Regulation of these mRNA targets indicates that changes in Brf1 expression have the potential to broadly affect the pluripotency transcription factor network to influence the biology and behavior of mESCs.

Tables

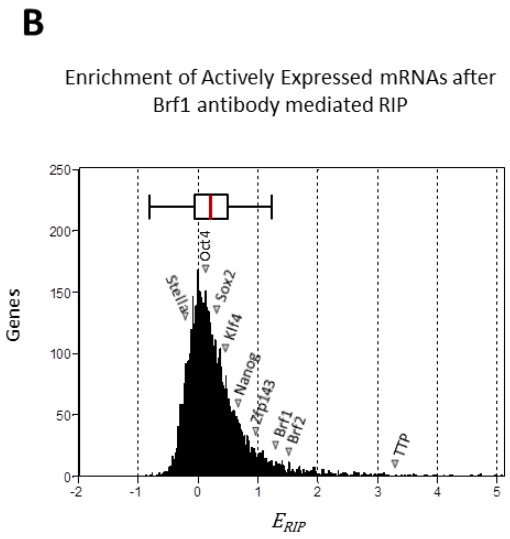
Motif	% of Annotation	Consensus
-ATTTATTTATTTATTTATTTA-	0.08%	Full
-ATTTATTTATTTATTTA-	0.2%	Full
-ATTTATTTATTTA-	0.5%	Full
-ATTTATTTA-	4.5%	Full
-TTATTTATT-	6.0%	Full (minimal)
-TATTTAT-	22.8%	Partial (minimal)
-CTATTTATT-	2.2%	Partial
-TTATTTATC-	1.0%	Partial
-CTATTTATC-	0.5%	Partial

Table 4.1 – Frequency of ARE motifs among mouse protein coding genes.

Figures



	Raw Sequencing Reads	Uniquely Mapped Reads
Total RNA 1	41,504,591	31,195,095
Total RNA 2	31,255,823	22,623,281
Brf1/2 RIP Sample 1	37,909,362	7,075,298
Brf1/2 RIP Sample 2	27,965,675	5,157,037
Rabbit IgG RIP Control 1	23,262,834	2,244,609
Rabbit IgG RIP Control 2	38,725,737	3,910,793



Percentile (x) of E_{RIP} Values	Full ARE	Partial ARE	genes
x ≥ 89 %tile (Positive Outliers)	25.1%	60.0%	418
x ≥ 75 %tile (Top Quartile)	13.8%	38.9%	1798
75 %tile ≥ x ≥ 25 %tile	5.8%	18.8%	3597
x ≤ 25 %tile (Bottom Quartile)	6.7%	20.5%	1799

Figure 4.1 – Brf1 binds other pluripotency-associated mRNAs. (A) Method used for RNA-RBP immunoprecipitation and RNA sequencing (RIPseq). Sequencing statistics for two total RNA samples, two Brf1/2 antibody RIP derived samples and two rabbit IgG RIP controls are presented. (B) Distribution of ERIP values for actively expressed genes (n = 7,194 genes). The location of several notable pluripotency associated transcripts is highlighted. Box Plot Statistics: median (red line, ERIP = 0.17), lower quartile boundary (ERIP = -0.04), upper quartile boundary (ERIP = 0.47) and statistical outliers (median ± 1.5 × [upper quartile – lower quartile]). Table: Frequency of full ARE motifs (-UUAUUUAUU-) and partial ARE motifs (-UAUUUAU-) among genes classified as ERIP outliers or in different ERIP quartiles.

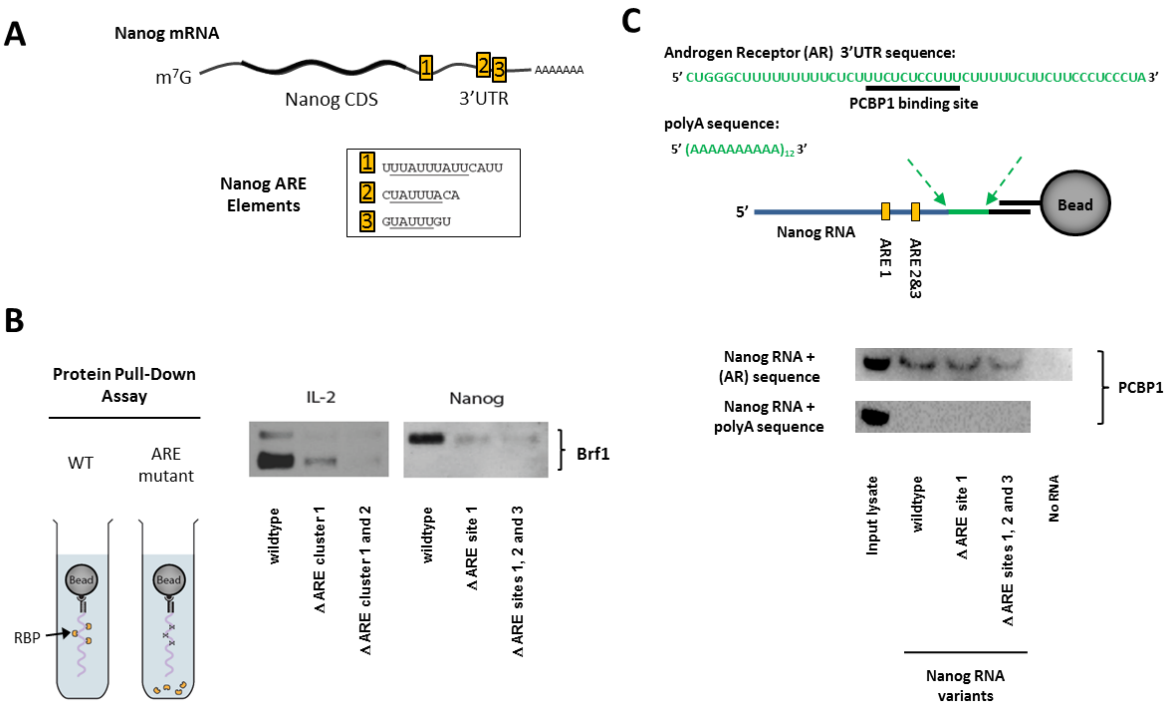


Figure 4.2 – Nanog is bound and regulated by Brf1. (A) Cartoon illustration of IL-2 and Nanog mRNA indicating the relative location and sequence of AU-rich elements in the 3'UTR. ARE mutants were generated by changing the core (-ATTTA-) motif to (-AGGGA-). (B) Left: Schematic diagram of the assay used to extract RBPs from crude protein extracts. Beads are conjugated to mRNAs with or without AU-rich elements. Right: Western blot for Brf1 protein, present in IL-2 and Nanog mRNA protein isolates. ARE site mutations reduced the extraction of Brf1 from crude protein lysates for both IL-2 sequence control and Nanog mRNA. (C) A Western blot for poly(C) RNA binding protein 1 (PCBP1) was performed as an RBP recovery control. We appended a 3'UTR sequence from the androgen receptor (AR) gene which is known to bind PCBP1 (site underlined) to the 3' end of wild-type and ARE mutant versions of Nanog (cartoon, top). We show that the presence or absence of ARE sequences does not inhibit the binding of PCBP1 to its target sequence. Moreover, the absence of the AR derived 3'UTR sequence prevents association with PCBP1 (as assayed using a polyA sequence of 120 nucleotides).

Chapter 5

Brf1 post-transcriptionally regulates Nanog in response to FGF/Erk MAP kinase.

(This chapter was adapted, in part, from (57))

Introduction

We showed that FGF/Erk MAP kinase signaling strongly regulates the expression of Brf1, and that Brf1 binds Nanog mRNA *in vitro*. However, it remains unclear whether FGF signaling uses Brf1 to regulate Nanog mRNA expression. Although we have shown that post-transcriptional regulation of Nanog mRNA is ARE dependent, FGF could regulate Nanog mRNA stability through other uncharacterized AUBPs. To test whether Brf1 mediates FGF/Erk MAP kinase signaling-dependent regulation, we conducted an epistasis assay.

Materials and Methods

Mock or Brf1 siRNAs were transfected into *fgf4-/-* R1 mESCs. After 36 hours, changes in Brf1 or Nanog mRNA level, in the presence or absence of FGF4/Heparin, were measured using qRT-PCR.

Results and Discussion

Relative to siRNA control (Figure 5.1, first column), Brf1 siRNAs caused a slight (~10%) downregulation of Brf1 and a corresponding increase in Nanog mRNA levels in *fgf4-/-* R1 mESCs (Figure 5.1, second column). These data indicate that Brf1 is expressed, albeit at a lower level, even when FGF signaling is absent (Figure 5.2) and that

alternative pathways support its basal expression. However, FGF signaling remains the dominant regulator of Erk MAP kinase in mESCs (Figure 5.2), and Erk MAP kinase appears to be the main driver of Brf1 expression (Figure 5.1).

Addition of FGF4/Heparin ligand increased Brf1 and decreased Nanog by greater than 2-fold within 5 hours (Figure 5.1, third column). In agreement with its role as a regulatory intermediate, the presence of Brf1 siRNAs reduced this regulation, yielding a smaller upregulation of Brf1 and a smaller downregulation of Nanog (Figure 5.1). We note that the inability of Brf1 siRNAs to fully block the downregulation of Nanog can be partly explained by limited knockdown efficiency, but could also reflect Brf1-independent regulatory mechanisms. In the absence of a more stringent test of epistasis (i.e. *zfp36l1-/-* mESCs), these data are sufficient to demonstrate Brf1's role as a regulatory intermediate of the FGF signaling pathway.

Figures

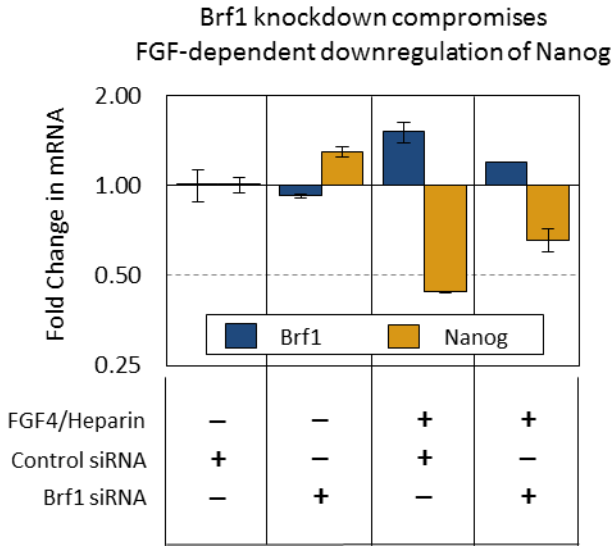


Figure 5.1 – Brf1 regulates Nanog in response to FGF. Transfection of Brf1 siRNAs can compromise the regulation of Nanog by FGF4/Heparin in fgf4-/- mESCs [± SD, n = 2].

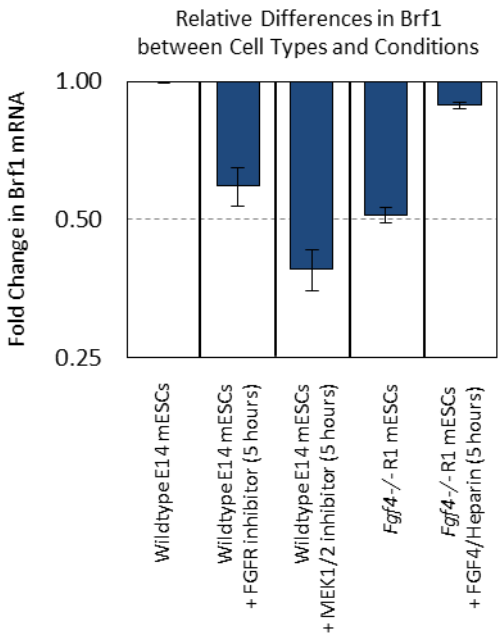


Figure 5.2 – Brf1 is strongly regulated by FGF/Erk MAP kinase signaling. Shown are relative differences in Brf1 expression level among different cell types and cell culture conditions, as specified.

Chapter 6

Changes in Brf1 expression affect mouse ESC self-renewal and developmental potential.

(This chapter was adapted, in part, from (57))

Introduction

FGF/Erk MAP kinase signaling destabilizes the expression of pluripotency factors, and is required for mESC differentiation. Our RIPseq data connects Brf1 to the regulation of numerous pluripotency factors. As a mediator of FGF/Erk MAP kinase signaling, changes in Brf1 expression may strongly regulate self-renewal, pluripotency or differentiation responses.

Materials and Methods

To determine the functional effect of Brf1 expression on pluripotent and differentiating cells, we perturbed Brf1 expression using siRNAs, which produced a ~4-fold decrease in Brf1 protein relative to wild-type. We also created stable transgene-mediated overexpression cell lines, which increased Brf1 protein levels ~4-fold above wild-type levels (Figure 6.1.A). For transgene expression in mESCs, clones expressing H2B-YFP ($Brf1^{1x}$) or Brf1-T2A-H2B-YFP ($Brf1^{4x}$) were derived for these studies. Of these, one control $Brf1^{1x}$ clone that expressed wild-type levels of Brf1 protein, and one $Brf1^{4x}$ clone expressing ~4-fold more Brf1 protein, was chosen for further analysis.

Results and Discussion

To quantify changes in mESC self-renewal brought about by Brf1 overexpression, we co-cultured YFP(+) Brf1^{4x} clones with wild-type YFP(-) E14 mESCs. In this assay, any change in self-renewal ability manifests as changes in relative proliferation rate, and hence, a change in the YFP(+)/YFP(-) ratio (91). Compared to Brf1^{1x}, co-cultures with Brf1^{4x} exhibited a significant proliferation defect, with the YFP(+)/YFP(-) ratio reduced by ~20% every 48 hours (Figure 6.1.B). In these cells, the expression of several core pluripotency genes is altered (Figure 6.1.C). However, most remain pluripotent in conditions with LIF plus serum. Removing LIF rapidly initiates differentiation, and during the first 48 hours, Brf1 siRNA knockdown in Brf1^{1x} or Brf1 overexpression in Brf1^{4x} produced only modest effects on the rate at which some markers of pluripotency were downregulated (Figure 6.1.D).

In contrast to the mild effect of Brf1 expression on the downregulation of pluripotency factors (Figure 6.1.D), the upregulation of differentiation markers is strongly affected by Brf1 (Figure 6.2). After 3 days of LIF withdrawal, we observed a striking bias in gene expression when comparing Brf1^{4x} to Brf1^{1x} cultures. LIF withdrawal generally promotes mesoderm differentiation (92). Indeed, Brachyury (T) was upregulated in differentiating Brf1^{1x} and Brf1^{4x} cultures. However, Brachyury expression was 100-fold greater in the Brf1^{4x} cell line (Figure 6.3.A). Transfecting Brf1^{1x} cultures with Brf1 siRNAs produced the opposite effect, downregulating Brachyury expression approximately 4-fold relative to untreated controls (Figure 6.3.A). Furthermore, siRNAs against Brf1 could attenuate the upregulation of Brachyury in Brf1^{4x}, indicating that this regulation resulted specifically from Brf1 overexpression. In agreement with these

results, flow cytometry profiling indicated that a larger fraction of Brf1^{4x} cells expressed Brachyury protein by 84 hours compared to Brf1^{1x} (Figure 6.3.C). These findings were further supported by the upregulation of mesendodermal markers Goosecoid (Gsc), Mixl1, and Wnt3A (Figure 6.3.A), indicating that Brf1 accelerated commitment to mesendodermal fates. Ectoderm markers (Nodal, Fgf5 and Gbx) were not affected, whereas extra-embryonic and definitive endoderm markers (Gata6, Hnf4a and FoxA2) showed weaker basal expression levels that responded differentially to Brf1 (Figure 6.3.A).

Brf1 expression did not influence neural differentiation. Because serum inhibits neural differentiation (32), we cultured cells in N2B27 serum-free media without LIF and BMP4. After 3 days in this media, most markers of differentiation appeared to be unaffected by Brf1 (Figure 6.3.B). For example, Sox1 mRNA and protein was readily detected, but its expression levels were similar in Brf1^{1x} and Brf1^{4x} cultures (Figure 6.3.B and 6.3.C). Interestingly, even in N2B27, the basal expression of Brachyury was upregulated in a Brf1-dependent manner. Thus, Brf1 appears to mainly affect mesendodermal differentiation pathways.

Figures

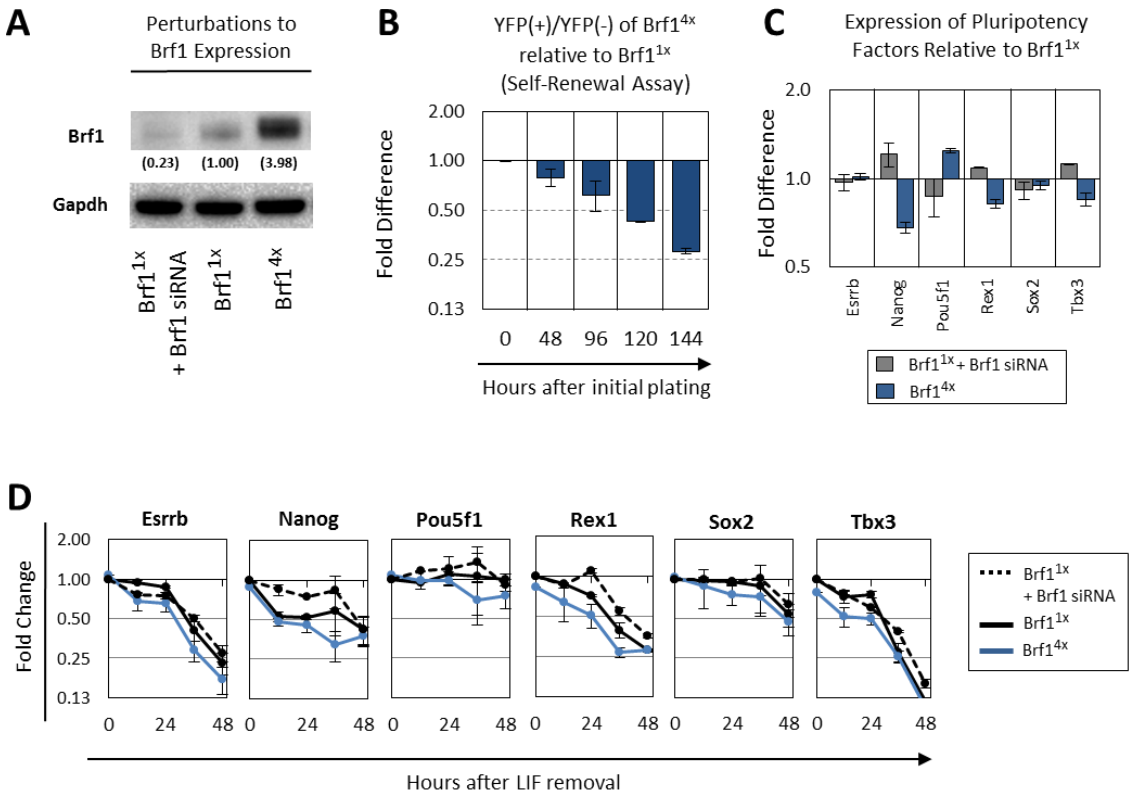


Figure 6.1 – Brf1 expression compromises mouse ESC self-renewal. (A) Brf1 expression was profiled in E14 carrying a CMV-H2B-YFP expression cassette (Brf1^{1x}), Brf1^{1x} treated with Brf1 siRNAs, and E14 carrying a CMV-Brf1/T2A/H2B-YFP expression cassette (Brf1^{4x}). A Western blot of Brf1 protein shows that siRNA knockdown reduced Brf1 expression by 75% after 36 hours, and stable CMV driven Brf1 expression increases Brf1 protein levels by 4-fold relative to Brf1^{1x}. Chemiluminscent intensities were normalized to Gapdh signal as loading control. (C) Changes in self-renewal of Brf1^{4x} relative to Brf1^{1x}. Differences in proliferation rate were gauged by changes in the ratio of YFP(+) transgene expressing cells to YFP(-) wild-type cells. For t = 48 and 96 hours [± SEM, n = 4]. For t = 120 and 144 hours [± SEM, n = 3]. (D) Changes in the expression of core

pluripotency genes via RT-qPCR after Brf1 siRNA knockdown in $Brf1^{1x}$ (gray bars) or enhanced Brf1 expression in $Brf1^{4x}$ (blue bars) over 36 hours [± SEM, n = 2]. (F) Profiling changes in the expression of core pluripotency genes via RT-qPCR during the early stages of differentiation (15% Serum, without LIF) in $Brf1^{1x}$ treated with Brf1 siRNAs (dashed black line), $Brf1^{1x}$ (solid black line) and $Brf1^{4x}$ (solid blue line) [± SEM, n = 2].

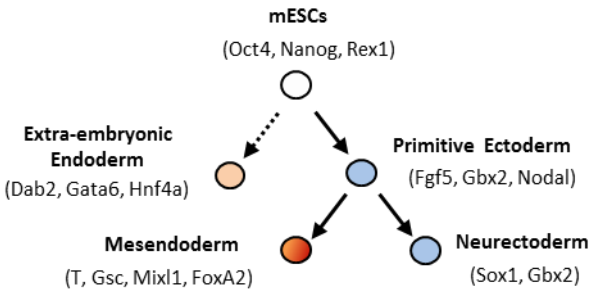


Figure 6.2 – Developmental potential of mouse ESCs.

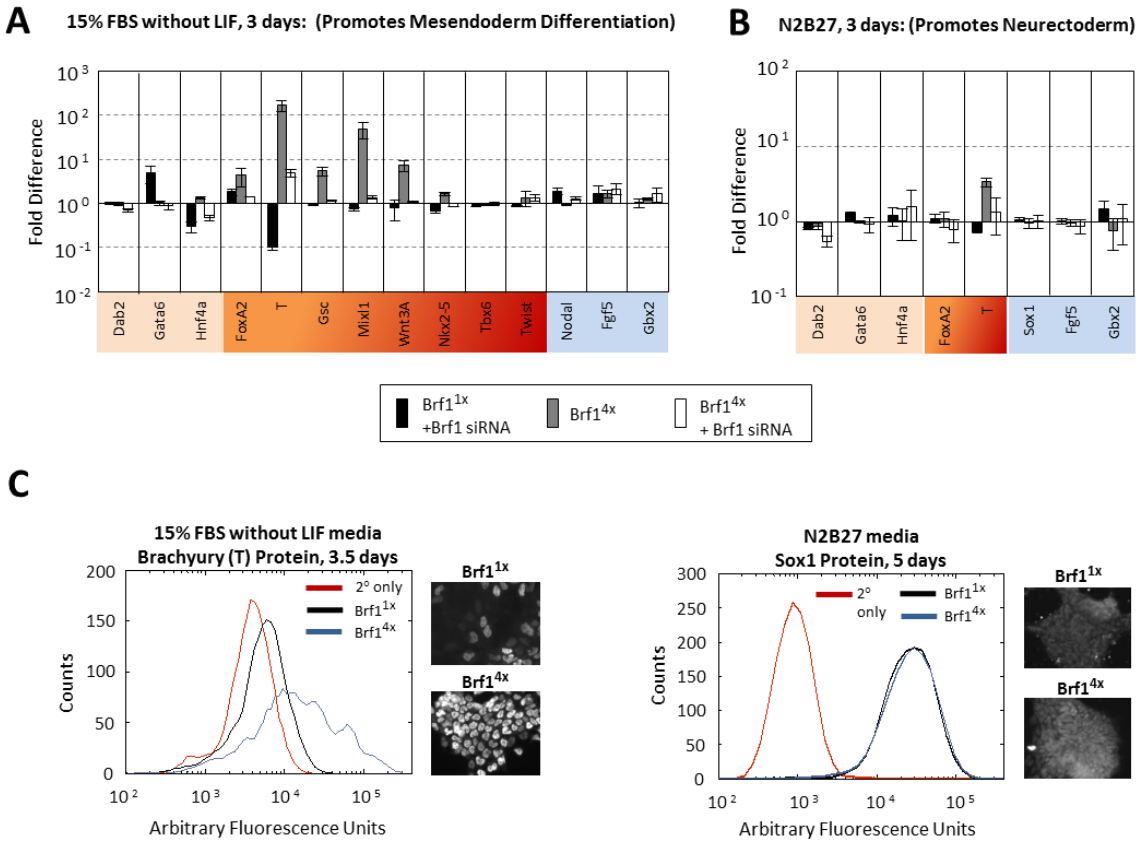


Figure 6.3 – Brf1 expression promotes mesendoderm differentiation. (A) Brf1^{1x} and Brf1^{4x} were cultured in mesoderm differentiation media (15% Serum without LIF) for 3 days. Fold difference in the expression of several lineage specific differentiation markers in Brf1^{4x} relative to Brf1^{1x} after 3 days of differentiation [± SEM, n = 3]. (B) Brf1^{1x} and Brf1^{4x} were cultured in neuroectoderm differentiation media (N2B27) for 3 days. Fold difference in the expression of several lineage specific differentiation markers in CMV-Brf1 E14 relative to Control E14 after 3 days of differentiation [± SEM, n = 3]. (C) (Right) Brachyury immunostain and associated flow cytometry data after 3.5 days of differentiation (n = 2000 cells). (Left) Sox1 immunostain and associated flow cytometry data after 5 days of differentiation (n = 1500 cells).

Concluding Remarks

AUBPs are critical regulators of developmental gene expression which respond to inter- and intracellular signals. Some specific examples of this regulation include the repression of Notch1 in response to PI3K/Erk MAP kinase signals during T-cell development (93), repression of TNFalpha in response to p38/Erk MAP kinase signals during inflammation (94), and the stabilization of p21 in response to ATR/ATM kinase activation after DNA damage (95). Here we demonstrate AUBPs mechanistically connect FGF/Erk MAP kinase signaling to the regulation of pluripotency, self-renewal and differentiation in mESCs. More specifically, control over Brf1 expression rapidly regulates the expression of key pluripotency-associated genes, reduces the capacity to self-renew, and enhances mesendoderm differentiation upon LIF withdrawal.

Why has Brf1 been selected for implementing FGF-dependent cellular responses in mESCs? One possibility is that, owing to its rapid transcriptional response to FGF signaling and its short protein and mRNA half-life (~1.5 hours and ~1 hour, respectively), Brf1 is capable of tracking dynamic changes in Erk MAP kinase activity. Brf1 directly affects mRNA abundance, and provides stem cell populations with a mechanism to quickly respond to changes in FGF signaling, without necessarily altering underlying transcriptional states. However, the benefit of these dynamical properties to the biology of pluripotent or differentiating mESCs still remains unclear. Brf1 also provides a regulation that is similar to miRNAs, which also provide mRNA-level repression while maintaining flexibility in target selection. Interestingly, Zfp36 AUBPs and miRNAs are known to cooperate in regulating some mRNA targets (96), indicating a potential point

of convergence between these two regulatory mechanisms. Future work will address why this system is particularly well adapted to serve as a regulator in mESCs, and as a mediator of FGF/Erk MAP kinase signaling.

Whether Brf1 plays a similar role in the embryo is unclear. We note that the regulatory effects of Brf1 in cell culture mimic the developmental response to FGF/Erk MAP kinase signaling at different stages of embryonic development. For example, FGF4 signaling promotes extra-embryonic endoderm differentiation in the inner cell mass partly by destabilizing the expression of pluripotency genes (97). Brf1 may participate in this process by repressing Nanog and other pluripotency regulators. At a slightly later developmental stage, and one that is more relevant to mESCs, both FGF4 and FGF8 are required for mesoderm induction (98, 99). The expression of Brachyury, a regulator of mesoderm morphogenesis, is enhanced by FGF4 and FGF8 signaling through unknown mechanisms (100). Our results show that Brf1 expression during differentiation in mESCs similarly enhances Brachyury and other primitive streak markers. This regulatory connection could explain why Brf1 knockout mice also exhibit the same gross defects in chorio-allantoic fusion, neural tube closure and placental organization at mid-gestation (E11) as FGFR2 knockouts, an indication of shared regulation and function (74, 101). Although the regulation of FGF/Erk MAP kinase signaling likely differs between the embryonic and cell culture context, these observations implicate Brf1 as an important FGF/Erk MAP kinase-inducible regulator of development in both systems.

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Appendix A

Supplemental Materials and Methods

Cell Lines and Cell Culture

fgf4-/- mESCs (strain FD6) were a kind gift ofrom Dr. Angie Rizzino, University of Nebraska Medical Center.

Cultures were routinely passaged in complete ES culture medium (15% FBS, 1000U/ml LIF, NEAA, Sodium Pyruvate and βME in DMEM) in the absence of feeders. Mesoderm differentiation media: 15% FBS, NEAA, Sodium Pyruvate and βME in DMEM (92); Neurectodermal differentiation media: N2B27 serum free media (102).

Reagents, Antibodies, Signaling Inhibitors and siRNAs

Qiazol reagent (Qiagen); iScript Kit (Bio-Rad); SsoFast Probes Supermix (Bio-Rad)

Mouse monoclonal (L34F12) anti-p44/p42 (Cell Signal, Cat# 4696, 1:2000); Rabbit monoclonal (D13.14.4E) anti-phospho-p44/p42 (Cell Signal, Cat# 4370, 1:2000); Mouse anti-TBP (Abcam, Cat# ab818, 1:1000); Rabbit anti-Beta Tubulin (Abcam, Cat# ab6046, 1:1000); Rabbit polyclonal anti-Brf1/2 (Cell Signal, Cat# 2119, 1:1000); Rabbit anti-Zfp36 (Protein Tech Group, Cat# 12737-1-AP, 1:500); Rabbit anti-hnRNP E1 (Cell Signal, Cat# 8534, 1:500); Goat anti-Brachyury (R & D Systems, Cat# AF2085, 1:200); Rabbit anti-Sox1 (GeneTex, Cat# GTX62974, 1:200); Mouse anti-Gapdh (Abcam, Cat# ab8245, 1:5000)

CI-1040 (also known as PD184352, Axon, 5uM); PD173074 (Sigma, 100ng/ml)

TTP siRNA (Santa Cruz Biotechnology, 10nM); Brf1 siRNA (Santa Cruz Biotechnology, 10nM); Brf2 siRNA (Santa Cruz Biotechnology, 10nM); All Stars Negative Control siRNA (Qiagen, 10nM)

High Throughput Sequencing Data

Raw sequencing data discussed in this publication was deposited in the NCBI Gene Expression Omnibus (Accession Number: GSE40104).

Appendix B

Supplemental Information

Primers and Probe Characterization:

Gene Name	Accession	Primers and Probe	Slope/Efficiency/R^2
Citrine (YFP)		Primer1: 5'-CCACCTTCGGCTACGGCCTGA-3' Primer2: 5'-GCCATGATATAGACGTTGTGG-3'	3.24/116%/0.998
Dab2	NM_023118.5	Primer1: 5'-TGTACTTTGTGGGTTCTGTCC-3' Primer2: 5'-GGTTGTGCTTGTTTCTACTTCG-3' Probe: 5'-CTGCTTGCCTTCCCGTCATGTCTAA-3'	3.59/90%/0.999
Esrrβ	NM_011934.4	Primer1: 5'-CCTTTACTATCTGTGCCTGGTC-3' Primer2: 5'-AGTGCTTCTCTTTGGTGCTG-3' Probe: 5'-ACACGTCTGTCATCCTTGCCTGC-3'	3.27/102%/0.998
Fgf5	NM_010203.4	Primer1: 5'-TGACTGGAATGAGTGCATCTG-3' Primer2: 5'-GGGTTTGGAATTTGGGTTGAG-3' Probe: 5'-ATTAAGCTCCTGGGTCGCAAGGG-3'	3.40/97%/1.000
FoxA2	NM_010446.2	Primer1: 5'-GATGTACGAGTAGGGAGGTTTG-3' Primer2: 5'-AACATGAACTCGATGAGCCC-3' Probe: 5'-CCAAGACATACCGACGCAGCTACA-3'	3.21/105%/0.999
Gapdh	NM_008084.2	Primer1: 5'-CTCCACGACATACTCAGCAC-3' Primer2: 5'-CCACTCACGGCAAATTCAAC-3' Probe: 5'-AGGAGCGAGACCCCACTAACATCA-3'	3.38/97%/0.999
Gata6	NM_010258.3	Primer1: 5'-AGCAAGATGAATGGCCTCAG-3' Primer2: 5'-CTCACCCTCAGCATTTCTACG-3' Probe: 5'-CAACTGTCACACCACAACCACTACCT-3'	3.35/99%/1.000
Gbx2	NM_010262.3	Primer1: 5'-GCAGTGTTTTGAAAGGGATAGG-3' Primer2: 5'-TGTTTGTCCTTGTGTCTCCTG-3' Probe: 5'-TTTGGGCACGTATGGGAAGGTGG-3'	3.63/89%/0.999
Hnf4α	NM_008261.2	Primer1: 5'-GGGCAGGAGAAGGATAAGAAAG-3' Primer2: 5'-GCAAAGCCATCAAGAGTCAAC-3' Probe: 5'-TGGGAAGCTACAGTCAAGGTGCATT-3'	3.68/87%/0.996
Hprt	NM_013556.2	Primer1: 5'-GCCCCAAAATGGTTAAGGTTG-3' Primer2: 5'-AACAAAGTCTGGCCTGTATCC-3' Probe: 5'-CTTGCTGGTGAAAAGGACCTCTCGAA-3'	3.36/98%/0.999
Nanog	NM_028016.2	Primer1: 5'-CAAAGGATGAAGTGCAAGCG-3' Primer2: 5'-CCAGATGCGTTCACCAGATAG-3' Probe: 5'-CAGCACCAGTGGAGTATCCCAGC-3'	3.35/99%/0.999

Nodal	NM_013611.4	Primer1: 5’-TTCACCGTCATTCCTTCTCAG-3’ Primer2: 5’-GATGCCAACACTTTTCTGCTC-3’ Probe: 5’-ACACCTGCTTTTCCAGTGCCCT-3’	3.38/98%/1.000
Oct4	NM_013633.3	Primer1: 5’-CACTCTACTCAGTCCCTTTTCC-3’ Primer2: 5’-GTTCTCTTGTCTACCTCCCTTG-3’ Probe: 5’-TTTCCCTCTGTTCCCGTCACTGC-3’	3.34/99%/1.000
Rex1	NM_009556.3	Primer1: 5’-ACATCCTAACCCACGCAAAG-3’ Primer2: 5’-CATTAAGACTACCCAGCCTGAG-3’ Probe: 5’-TGTCTCCACCTTCAGCATTTCTTCCC-3’	3.17/107%/1.000
Sdha	NM_023281.1	Primer1: 5’-AGTGGGCTGTCTTCCTTAAC-3’ Primer2: 5’-GGATTGCTTCTGTTTGCTTGG-3’ Probe: 5’-TGGGCATGTCTCTGAGGGATTGG-3’	3.21/105%/1.000
Sox1	NM_009233.3	Primer1: 5’-TCTTTCCTGTGGTTCTGCC-3’ Primer2: 5’-GAAATCAAAGGCACGCTGTC-3’ Probe: 5’-TTGTCCCTATCCTTGGCCTTGTCC-3’	3.79/84%/0.999
Sox2	NM_011443.3	Primer1: 5’-CCAATCCCATCCAAATTAACGC-3’ Primer2: 5’-CTATACATGGTCCGATTCCCC-3’ Probe: 5’-CCGCCCTCAGGTTTTCTCTGTACAA-3’	3.78/84%/0.999
T	NM_009309.2	Primer1: 5’-GCTGGAAATATGTGAACGGG-3’ Primer2: 5’-GTTGGTGAGTTTGACTTTGCTG-3’ Probe: 5’-AAAATTGGGCGAGTCTGGGTGGA-3’	3.87/81%/1.000
Tbp	NM_013684.3	Primer1: 5’-TGATTGCTGTACTGAGGCTG-3’ Primer2: 5’-CTTACGGCACAGGACTTACTC-3’ Probe: 5’-ACTGTTGGTGTTCTGAATAGGCTGTGG-3’	3.24/104%/0.998
Tbx3	NM_011535.2	Primer1: 5’-TCCCATTATCCTCAACCTTGC-3’ Primer2: 5’-CACACGAAGCCCTCTACAAG-3’ Probe: 5’-CCATAGCTGCCCCTTTTACCCCA-3’	3.48/94%/0.998
Zfp36 (TTP)	NM_011756.4	Primer1: 5’-CCCTGTCCTCTTGTTCCTTTTC-3’ Primer2: 5’-TGGTTAGGGTCTCTTCGAGTC-3’ Probe: 5’-CTTTCCCCTTCTGCCTTCTCTGCT-3’	3.14/108%/1.000
Zfp36l1 (Brf1)	NM_007564.5	Primer1: 5’-GCACACTCTTCCTCTTCCTTATAG-3’ Primer2: 5’-AGGCAAGATTAGTCAACAGGG-3’ Probe: 5’-ACCTTTTACTTCCCAGCCCGAACC-3’	3.23/104%/0.997
Zfp36l2 (Brf2)	NM_001001806.2	Primer1: 5’-TCGCCCGTTATTCATCTTGG-3’ Primer2: 5’-CGTAGAAGGGTGACAGAAGTG-3’ Probe: 5’-AAGCGTGGAGGTTGGGAGGT-3’	3.17/107%/1.000
½ life tag		Primer1: 5’- CTGTTTTGACCTCCATAGAAGAC-3’ Primer2: 5’- AGATTGAGGATGCTGAGCGTC-3’	3.32/100%/0.999