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Protein Kinases in Pluripotency – Beyond the Usual Suspects

Rosalia Fernandez-Alonso, Francisco Bustos, Charles A.C. Williams and Greg M. Findlay[*]

The MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, The University of Dundee, Dundee, DD1 5EH, UK
*to whom correspondence should be addressed; g.m.findlay@dundee.ac.uk

Abstract

Post-translational modification of proteins by phosphorylation plays a key role in regulating all aspects of eukaryotic biology. Embryonic Stem Cell (ESC) pluripotency, defined as the ability to differentiate into all cell types in the adult body, is no exception. Maintenance and dissolution of pluripotency is tightly controlled by phosphorylation. As a result, key signalling pathways that regulate pluripotency have been identified and their functions well characterised. Amongst the best studied are the FGF-ERK1/2 pathway, PI3K-AKT, the LIF-JAK-STAT3 axis, WNT-GSK3 signalling and the TGFβ/BMP family. However, these kinase pathways constitute only a small proportion of the protein kinase complement of pluripotent cells, and there is accumulating evidence that diverse phosphorylation systems modulate ESC pluripotency. Here, we review recent progress in understanding the overarching role of phosphorylation in mediating communication from the cellular environment, metabolism and cell cycle to the core pluripotency machinery.

Highlights:

- Pluripotency is a unique characteristic of Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs), and is tightly controlled by phosphorylation networks

- High-throughput technologies are being used to elucidate new pluripotency signalling pathways

- Environmental, metabolic, structural and stress-regulated phosphorylation networks are emerging as novel regulators of pluripotency.

Keywords: Pluripotency; Phosphorylation; Kinase; Embryonic Stem Cells; Signalling networks

Introduction

Pluripotency is a fundamental of metazoan biology, and is defined as the theoretical capability of a single cell to differentiate into any lineage in the developing organism [1]. The potential of pluripotent cells to form any tissue or organ in the body has pushed pluripotency research to the fore in the field of regenerative medicine. Pluripotent cells were initially isolated from developing embryos as Embryonic Stem Cells (ESCs; [2-4]). More recently, induced pluripotent stem cells (iPSCs) have been derived by developmentally reprogramming somatic cells, and these closely resemble ESCs at the molecular level [5-7].

Pluripotency comprises at least two molecularly distinct states, which differ according to species and developmental context [8]. Naïve pluripotency is a developmental "ground state" characteristic of cells from the pre-implantation mouse embryonic epiblast (mouse ESCs[mESCs]). Primed pluripotency is characteristic of post-implantation mouse epiblast stem cells (EpiSCs) and ESCs isolated from human embryos (hESCs), although human naïve pluripotent cells were recently derived from primed hESCs or human embryos [9-16] An intermediate state termed formative pluripotency was recently described [17], which represents the initial acquisition of developmental characteristics by naïve cells. Molecular distinctions and definitions of pluripotent states have been extensively reviewed elsewhere [18].

Pluripotency acquisition and maintenance is intrinsically linked to expression of a pluripotency gene regulatory network, particularly the core transcription factor triumvirate of OCT4/PO5F1, SOX2 and NANOG [19-21]. OCT4 is a "governor" of pluripotency, and is expressed in both naïve and primed pluripotent states via distinct enhancer elements [22]. OCT4 also controls lineage allocation following pluripotent exit [23]. SOX2 and NANOG are key regulators of naïve and primed pluripotency, although Nanog is expressed at a reduced level in primed cells. Other transcriptional regulators of pluripotency are differentially expressed in naïve and primed cells. The naïve state is marked by expression of the key NANOG effector *Esrrb*, Krueppel-like factors (*Klf2/4*), *Rex1*, *Fgf4* and *Nr0b1* [24]. As the term suggests, primed pluripotent cells express markers of lineage priming, including the de novo DNA methyltransferases *Dnmt3a/b* [25], the embryonic epiblast marker *Fgf5* and lineage specific transcription factors such as *Brachyury* [24].

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Pluripotency gene regulatory networks are under strict control of extrinsic and intrinsic signalling networks. Dynamic flow of signalling information between and within cells is dependent upon networks of reversible post-translational modifications, particularly protein phosphorylation [26]. Thus, pluripotent states can be accessed and stabilised by modulating activities of protein kinases. Specifically, cytokines and growth factors and selective protein kinases inhibitors can be exploited to manipulate pluripotency pathways. Pluripotent mESCs were initially captured [24] using a combination of Bone Morphogenetic Protein (BMP) to activate the SMAD1-Inhibitor of Differentiation (*Id*) pathway and Leukemia Inhibitory Factor (LIF) to activate JAK-STAT3 signalling. Accessing naïve "ground state" mESC pluripotency, however, requires inhibition of two protein kinases; MEK1/2, which phosphorylates and activates ERK1/2 MAP kinase, and GSK3, an antagonist of Wnt signalling [27, 28]. Primed pluripotency in human and mouse is supported by Fibroblast Growth Factor (FGF) and Activin [29-31], whilst human naïve pluripotency can be accessed using distinct combinations of growth factors and kinase inhibitors [9-16].

In this review, we train our focus away from these well-understood pluripotency signalling pathways to explore the role of emerging signalling networks and their impact upon maintenance and dissolution of pluripotent states. Furthermore, we shed light on exciting but poorly appreciated roles for cell cycle, environmental, metabolic, structural and stress-regulated phosphorylation networks in pluripotency regulation.

Novel Pluripotency Signalling Pathways

The human protein kinome consists of 538 kinases ([32] and http://kinase.com/web/current/kinbase) and includes some of the most studied enzymes in biology. However, vast swathes of the kinome have not yet been investigated, and understudied kinases likely perform key functions in biological processes such as pluripotency maintenance and dissolution [33]. Indeed, total and phospho-proteome analysis indicates that at least 300-400 kinases are expressed in pluripotent mESCs (Jens Hukelman and G.M.F., unpublished data) and hESCs [34]. In this section, we discuss technologies which have been employed to uncover new pluripotency kinase signalling pathways.

Phosphoproteomic profiling is a powerful method to identify novel mechanisms by which phosphorylation modulates pluripotency. Initial studies focussed on identifying novel targets of well-understood signalling pathways (e.g. FGF2 in hESCs [35, 36]). However, unbiased comparison of the phosphoproteomes from pluripotent hESCs and those from differentiating or somatic cells has uncovered new phosphorylated targets relevant for pluripotency regulation. In this manner, multiple receptor tyrosine kinases with previously unappreciated roles in hESC pluripotency were identified [37]. Furthermore, kinase-substrate motif prediction analysis of differentially phosphorylated proteins indicates that distinct families of kinases are active in hESCs compared to somatic cells [38]. Importantly, this approach suggests novel roles for CDKs, Aurora, p38 and JNK kinases in pluripotency regulation [34], and reveals key hESC phosphorylation modules centred on CDK1/2 [39] and DNA methyltransferases [40]. Perhaps most excitingly, phosphoproteomic studies have the potential to reveal new pluripotency kinases, affirming phosphoproteomics as a core tool for systematic identification of novel mechanisms of pluripotency regulation.

Functional screening has also proven invaluable to uncover novel candidate pluripotency kinases. Global RNA interference screens have identified kinases which block mESC pluripotency maintenance [41] and reprogramming to pluripotency [42]. Interestingly, kinases such as TESK1 and LIMK2 identified in these studies have no previously described role in pluripotency or reprogramming. Future research will explore the molecular functions and regulation of these kinases in pluripotent cells. Similarly, cellular screening of kinase inhibitor libraries has elucidated new pluripotency pathways. A survey of selective kinase inhibitors uncovered a novel role for the ERK5 MAP kinase pathway in modulating mESC naïve-primed pluripotent transition [43]. In mESCs, ERK5 promotes expression of a key network of naive pluripotency factors, including *Klf2*, *Essrb*, and *Rex1* [43, 44], which suppresses transition of naïve cells to the primed state. Interestingly, both the ERK5 kinase and a C-terminal transcriptional activation domain are required for naïve maintenance [43]. ERK5 may therefore function in concert with transcription factors SP1 and MEF2 [45-47], which are required for *Klf2/4* expression in other developmental systems [44, 46, 48]. As with many emerging pluripotency pathways, identification of novel ERK5 substrates

would shed light on the mechanisms by which ERK5 controls pluripotency. In addition, identifying factors that specifically activate ERK5 may have utility in capturing naïve pluripotency and/or reprogramming somatic cells. In this regard, BMP, LIF and FGF activate ERK5 in mESCs and other cell types [44, 49, 50].

Identification of novel pluripotency kinase pathways thus represents an exciting niche within the ESC arena. Increasingly potent and selective tool compounds, including high-value collections from pharmaceutical companies and academic consortia [51, 52] in combination with genome editing technologies will provide unique opportunities to specifically disrupt kinase function in pluripotent cells [33]. Ultimately, we envision that pathways identified using these cutting-edge approaches will influence pluripotent stem cell-based clinical applications. In the meantime, a number of more prominent kinase signalling networks have relatively poorly understood roles in pluripotency regulation, which we now discuss.

Cell Cycle & DNA Damage Response Kinases in Pluripotency Regulation

Cell cycle progression is governed by a complex kinase network centred on the Cyclin Dependent Kinases (CDKs). Cell cycle phase is intrinsically linked to pluripotency maintenance and lineage commitment, with G1 phase providing a key window for developmental decision-making. A short G1 is deterministic for pluripotency maintenance [53], whilst lengthening G1 promotes differentiation [54], implying that kinases which govern cell cycle progression couple directly to the pluripotency machinery.

Cell Cycle Kinases and Pluripotency

Phosphoproteomic analysis pinpoints CDK1/2 as a pluripotency hub in hESCs [39], and several themes are now emerging with regard to the mechanisms by which CDKs modulate pluripotency. CDKs directly regulate phosphorylation and/or expression of pluripotency factors, or couple developmental decision-making to cell cycle phase. CDK activity can also pattern activation of signalling pathways which regulate pluripotency and differentiation.

In mESCs, CDK1 kinase activity is essential for pluripotency maintenance [55], which is underpinned at least in part by modulation of core pluripotency factors. CDK1 and OCT4 appear to cooperate in an unknown way to repress differentiation [56]. However, direct phosphorylation of

SOX2 by CDKs promotes pluripotency acquisition [57], whilst NANOG is phosphorylated by CDK1 in vitro [58]. Although the mechanisms and functional significance remain unclear, these studies set a precedent for direct modulation of pluripotency factors by CDK-Cyclin activities. Comprehensive analysis of CDK-dependent phosphorylation of core pluripotency transcription factors will determine the generality of this mechanism. In addition to direct phosphorylation of pluripotency factors, transcriptional regulation of pluripotency by CDK-Cyclins has been described. In hESCs, Cyclin D forms specific transcriptional complexes to control lineage specific genes [59], although this is independent of CDK kinase activity. CDK2 activity also promotes lineage specific gene expression in hESCs by phosphorylating and activating the histone methyl transferase MLL2 [60].

Perhaps unsurprisingly, CDK-Cyclin activities also control pluripotency via cell cycle phase, and as discussed previously, this may relate to G1 length [61]. Ng and colleagues describe a molecular mechanism underpinning G1 as a critical cell cycle phase during which mESC pluripotency is maintained or dissolved [62]. Elevated CDK-Cyclin B and ATM/ATR kinase activities during S and G2 cell cycle phases of the mESC cycle protect against pluripotent exit. However, in G1 these activities are less prominent, providing a window for differentiation [62].

CDK activity also modulates pluripotency more indirectly by patterning the signalling landscape. CDK4/6 phosphorylate SMAD2/3 at a distinct regulatory region to suppress TGFβ-SMAD2/3-dependent expression of lineage specific genes [63], indicating that CDK-Cyclin activities not only directly modulate expression and function of pluripotency genes, they also impact on interpretation of developmental signals. This may be an important mechanism by which CDKs coordinate pluripotency, ensuring that cells respond to developmental cues only within the appropriate cell cycle phase.

Finally, other cell cycle kinases have been uncovered as key pluripotency regulators. In mESCs, a kinome-wide RNAi screen showed that mitotic Aurora kinases regulate phosphorylation and degradation of p53 to promote pluripotency [41]. Furthermore, Aurora modulates OCT4 function in mESCs [64], exemplifying the increasingly important molecular connections between cell cycle protein kinases and pluripotency. ESCs have therefore evolved a variety of mechanisms

to tightly couple pluripotency and cell cycle phase, and these await further discovery and investigation.

DNA Damage Signalling in Pluripotent Cells

The unique ESC cell cycle, with its short G1 phase, subjects the genome to significant replication stress [65]. This can drive pluripotency exit [66], presumably to prevent potential transmission of mutations to future somatic lineages and the germline. As a result, the DNA Damage Response (DDR) is "hard-wired" to the pluripotency machinery to ensure that DNA is efficiently repaired in pluripotent cells [66]. As in somatic cells, the DDR centres on CHEK1/2 and ATM/ATR kinases. Activation of CHEK1 [67] and ATM [68] is required for reprogramming and iPSC genome stability, establishing the importance of DDR kinases in acquisition and maintenance of pluripotency. Interestingly, mESCs can activate ATM without inducing p53 [65] in order to mount an efficient DDR without promoting differentiation.

Although ESCs use the same conserved core DDR machinery as somatic cells, specific factors orchestrate DDR signalling to maintain ESC genome integrity. In mESCs, the pluripotency factor SALL4 recruits the Rad50/MRN DDR complex to specific genomic regions, which activates ATM to promote DNA repair [69]. Furthermore, mESCs specifically utilise the scaffold FILIA/KHDC3 to activate DDR kinases [70]. FILIA is phosphorylated following DNA damage, which activates ATM and CHEK2, promoting DNA repair and genome stability [70]. A major unresolved question concerns why ESCs utilise unique factors to coordinate the DDR.

Nutrition and Energy Sensing Kinases in Pluripotency

As exemplified by the cell cycle and DDR, fundamental cellular processes profoundly influence pluripotency acquisition and/or maintenance. Cellular nutrition and energy status continue along this theme (See Figure 1). As in other cell types, ESC nutrient responses are controlled by the PI3K-related protein kinase mechanistic Target Of Rapamycin (mTOR) [71]. mTOR is the kinase in two distinct complexes: mTORC1, defined by mTOR interaction with regulatory-associated protein of mTOR (RAPTOR) [72, 73], and mTORC2, defined by mTOR interaction with rapamycin insensitive companion of mTOR (RICTOR) [74]. In response to nutrients, mTORC1 phosphorylates and activates p70 S6 kinase 1 (which phosphorylates ribosomal S6 protein [75]) and eIF4E binding

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protein 1 (4E-BP1) [76]. mTORC1 integrates nutritional signals to enhance global and cap-dependent translation, ribosome biosynthesis, gene expression and autophagy [77, 78].

mTOR is a Central Regulator of ESC Pluripotency

Consistent with its role as a major nutrient sensor, mTOR is critical for ESC growth and proliferation [79], early embryonic development [80] and pluripotency [79, 81-85] in both mouse and human. In hESCs, mTORC1 activity is required for long-term pluripotency maintenance, and promotes expression of pluripotency factors OCT4, SOX2 and NANOG [85]. Furthermore, mTORC1 inhibition results in hESC differentiation to mesendoderm [83], suggesting an involvement of mTORC1 activity in pluripotency and restraining lineage specification.

mTOR complexes display highly specific signalling dynamics in pluripotent and differentiating cells. Although active in mouse and human ESCs, mTORC1&2 activity further increases during differentiation [81, 84, 86], which may require ERK1/2 activation of p90 RSK [81]. Interestingly, an mTOR-specific protein inhibitor, DEPTOR, acts to maintain mTOR kinase activity below a threshold level in both human and mouse ESCs [87]. DEPTOR suppression may thereby promote the increased mTORC1&2 activity observed upon ESC differentiation.

As expected from these studies, mTOR also displays complex signalling and expression dynamics during somatic cell reprogramming. In the mouse, mTORC1 and mTORC2 activities decrease during reprogramming via SOX2–dependent recruitment of the NuRD epigenetic remodelling complex, which shuts down the *mTOR* promoter [88]. Congruently, mTOR kinase inhibition enhances somatic cell reprogramming [76], although restoration of mTOR activity is critical for final steps of iPSC generation, presumably because mTOR is required for pluripotency maintenance [81, 84].

Most reports therefore suggest that different thresholds of mTOR signalling specify distinct developmental identities. Consistent with this notion, exciting recent work shows that partial inhibition of mTORC1 activity maintains a state of "paused pluripotency" similar to diapause, a developmental arrest induced by nutrient starvation [89]. Why restrained mTOR activity is more compatible with pluripotency is not currently understood. However, mTOR drives global translation,

and mESCs have lower translation rates than differentiated cells [84, 86], which may buffer against spurious expression of lineage specific factors and differentiation.

Amino acid signalling in ESC pluripotency and self-renewal

A key function of the mTOR pathway is to mount cellular responses to nutrient availability, primarily amino acid levels. In contrast to somatic cells, where mTORC1 is activated in response to branched chain amino acids leucine and arginine [90], mESCs are exquisitely dependent upon threonine, and show little requirement for leucine/arginine for growth/proliferation [91]. Intriguingly, threonine reportedly regulates activity of both mTORC1&2 complexes in mESCs to maintain proliferation and *Oct4/Pou5f1* expression [92]. How threonine might control mTORC1&2 is not known, although evidence suggests this mechanism is distinct from the canonical branched chain amino acid sensing by RAG GTPases [93]. Thus, elucidating the molecular basis of this unique ESC nutrient sensing system remains an important unresolved question.

Kinase Signalling and Metabolic Programming in Pluripotency

As is the case with the nutrient sensing machinery, metabolic status and pluripotency are fundamentally intertwined. Distinct pluripotent states differentially utilise glycolytic and oxidative energy production systems [94], which plays a key role in pluripotency maintenance. Whilst naïve ESCs and somatic cells produce energy via mitochondrial oxidative phosphorylation, primed human and mouse ESCs obtain their ATP from glycolysis [95]. Transition between pluripotent states, pluripotency acquisition and differentiation therefore involves a metabolic conversion [94, 96-99], and mTOR plays several distinct roles in metabolic regulation in ESCs.

Autophagy/Mitophagy in Metabolic Switching and Pluripotency

mTOR supports glycolytic metabolism and pluripotency in primed hESCs and mouse EpiSCs [100], at least in part by promoting expression of pentose phosphate pathway (PPP) genes [101, 102]. In mESCs, mTOR also controls a unique GALECTIN-1 system for glucose uptake, which is essential for proliferation [103]. However, the best understood mTOR function in metabolic conversion from oxidative to glycolytic metabolism is via mitochondrial removal by the process of mitochondrial autophagy (mitophagy) [104]. Autophagy recycles proteins and organelles via formation of intracellular autophagosomes targeted for lysosomal degradation [105]. This is critical

for mESC pluripotency maintenance, as oxidative metabolism drives pluripotent exit [104]. Conversely, mESC differentiation increases mitochondrial synthesis to support oxidative metabolism [106]. In a similar vein, active mitophagy ensures that hESCs have relatively few mitochondria compared to somatic cells [7, 107], which restricts differentiation.

In addition to mTOR, autophagy/mitophagy is tightly regulated by the activities of several key protein kinases. A complex containing UNC51-like kinase-1 (ULK1) controls isolation membrane (phagophore) assembly, autophagosome nucleation and expansion [108]. ULK1 integrates opposing signals from AMP activated protein kinase (AMPK) and mTORC1 to control phagopore assembly. AMPK phosphorylates ULK1 to drive autophagy, whilst mTOR phosphorylates a distinct site on ULK1 to suppress autophagy [109, 110]. Elevated mTORC1 activity observed during differentiation thereby reduces autophagy in somatic cells [81].

Autophagy has been extensively studied during reprogramming to pluripotency. Canonical (ATG3-dependent [88]) and non-canonical autophagy pathways [111, 112] are activated during reprogramming to drive oxidative to glycolytic conversion [76]. AMPK induced autophagy is required for pluripotency acquisition in both mouse and human [76, 88, 113, 114], and pharmacological AMPK activation or mTOR inhibition enhances cell reprogramming by modulating autophagy [76]. Furthermore, increased *mTOR* transcription and mTOR kinase activity during the final stages of reprogramming drives autophagy, which aids pluripotency acquisition [88]. Specifically manipulating mitophagy and metabolic reprogramming using kinase inhibitors would add further valuable insight into the role of these processes in pluripotency.

Cellular and Environmental Stress Signalling in Pluripotency

Like nutrient and metabolic status, environmental stress profoundly impacts on pluripotency, although ESCs have high stress tolerance compared to differentiated cells [115, 116]. Responses to environmental stress are mediated by classical MAP kinase cascades [117] and other kinase signalling pathways. These pathways therefore perform key functions in pluripotent cells (See Figure 2).

Oxidative Stress

As discussed above, primed pluripotent ESCs rely primarily on glycolysis for ATP generation [99], which minimises oxidative stress generated by reactive oxygen species (ROS) and oxidative metabolism [118]. In hESCs, inhibition of oxidative phosphorylation enhances pluripotency [119], and culture in low oxygen/hypoxia improves pluripotency maintenance and reduces chromosomal abnormalities [120, 121]. Although acute oxidative stress does not directly impact on pluripotency gene expression [122], it can influence lineage choice during differentiation [41, 123, 124]. ESCs therefor appear to be metabolically programmed to avoid differentiation induced by oxidative stress.

Oxidative stress activates the ERK1/2, c-Jun N-terminal kinase (JNK) and p38 MAP kinase signalling cascades, the phosphoinositide 3-kinase (PI(3)K)/AKT pathway and the nuclear factor (NF)-κB signalling pathways [125]. ERK1/2 and PI(3)K/AKT activation occurs largely by protein tyrosine phosphatase inhibition and receptor tyrosine kinase phosphorylation [125], whilst the p38 and JNK MAP kinase cascades are activated by apoptosis signal-regulating kinase 1 (ASK1). The redox regulatory protein thioredoxin (TRX) normally inhibits ASK1, but oxidative stress dissociates the TRX–ASK1 complex, facilitating JNK and p38 phosphorylation and activation [126, 127]. NF-κB is also activated by several stress pathways, including IκB kinase (IKK)α/β and ERK1/2 [128].

In mESCs, NF-κB transcription promotes differentiation [129], and NANOG maintains pluripotency at least in part by suppressing NF-κB signalling [130]. NF-κB transcription is also activated during hESC differentiation [131], although intriguingly, the key NF-κB kinase activator IKKα/β promotes OCT4 and NANOG expression in mESCs [132], suggesting that the role of the NF-κB signalling pathway in pluripotency is not yet fully understood. ERK1/2, JNK and p38 MAP kinase signalling pathways also promote mESC pluripotent exit [133-135], although whether these are oxidative stress-specific responses to remains to be determined. Furthermore, as we discuss below, the role of MAP kinases in pluripotency may depend on both cellular context and type of stress response. Nevertheless, these data indicate that signalling responses to oxidative stress have a central function in pluripotency maintenance and gene expression.

Hyperosmotic Stress

Hyperosmotic stress occurs when external osmolarity exceeds the intracellular physiological range, and has been linked to pluripotency regulation. The WNK1-4 family kinases have emerged as key modulators of the cellular response to hyperosmotic conditions by regulating ion transport [136, 137]. However, investigation of WNK kinase functions in pluripotent cells awaits development of selective tools. To date, most studies into kinase signalling in hyperosmotic stress responses focus on MAP kinase signalling pathways p38 and JNK.

In contrast to oxidative stress, hyperosmotic activation of p38 actively promotes pluripotency [138] and facilitates somatic reprogramming to iPSCs [138, 139]. During reprogramming, p38 specifically promotes de-methylation of pluripotency gene promoters [138] via an undefined mechanism. In addition, p38 kinase inhibitors specifically suppress OCT4 transcriptional activity under hyperosmotic conditions [140]. In contrast, JNK activity promotes pluripotent exit following acute hyperosmotic stress by suppressing OCT4 expression [141] and phosphorylating KLF4 to suppress transcriptional activity [142]. Therefore, a somewhat confusing picture of pathway-specific stress responses in pluripotent cells is emerging. Dissecting roles of individual kinases under specific stress conditions using selective kinase inhibitors should provide clarity to this field.

Signalling Responses to other Environmental and Cellular Stresses

A further stress exerted on pluripotent cells is shear stress (SS), caused by fluid movement over the cell surface. First reported in endothelial cells [143], SS occurs in response to load-bearing or surface laminar flow [144], and can be lethal in preimplantation embryos [145]. ESCs reportedly mount differential responses depending on strength and duration of SS. In pluripotent mESCs, SS stabilises expression of *Oct4* and the naïve pluripotency marker *Rex1* [146]. In contrast, acute SS promotes ectodermal fate in differentiating mESCs, whilst prolonged SS results in mesendoderm specification [147]. Thus, it may be desirable to exploit culture conditions that generate a certain threshold of SS to efficiently propagate naïve pluripotent cells.

SS signals are primarily transduced by mechanosensors such as integrins, receptor tyrosine kinases (particularly VEGFR2), G-protein coupled receptors, ion channels and intercellular junction proteins [148-152]. These trigger kinase signalling cascades which modulate transcription

of SS response genes. In particular, the ERK5 pathway, which promotes naïve pluripotency in mESCs [43], plays a key role in SS responses. ERK5 is thought to mediate SS responses via KLF2 induction [153] or NRF2 activation [154], and therefore may be the missing link that connects SS signals to the core pluripotency machinery.

Finally, Endoplasmic Reticulum (ER) homoeostasis and the unfolded protein response (UPR) is crucial for cell viability and is regulated by protein kinases IRE1 and PERK [155]. In ESCs, ER stress promotes VEGF expression and vascular differentiation [156], and inhibiting ER stress reduces VEGF expression and supports mESC pluripotency [157, 158]. However, hESCs have a relatively active UPR [159], suggesting that pluripotent cells nevertheless rely on a threshold level of UPR for survival and/or to maintain pluripotency. Thus, a key question is establishing how ESCs effectively balance the UPR to to prevent spurious differentiation.

Mechanical and structural signalling in pluripotency

As we have seen, eliciting appropriate stress responses is critical to maintain pluripotency and survival at the level of individual cells. However, metazoan development also requires that cells sense the topography of their immediate environment. As a result, intercellular forces and mechanical signals from the Extra-Cellular Matrix (ECM) profoundly influence pluripotency (See Figure 3). Understanding the molecular pathways involved in ESC mechanotransduction will be invaluable to develop effective cell culture strategies to exploit pluripotent cells for tissue regeneration [160].

Cell-Cell Interactions: Signaling Downstream of Cadherins in Pluripotency

The mammalian genome encodes more than 100 cadherins [161], which are key components of adherens junctions. E-cadherin (CDH1) is a master regulator of mESC and hESC biology [162], and underpins the compact mESC morphology [163]. A key signalling function of CDH1 in mESCs is to support activation of the LIF signalling pathway. $Cdh1^{-/-}$ mESCs respond poorly to LIF stimulation [164-166], displaying reduced STAT3 activation and pluripotency gene expression [167]. CDH1 forms a ternary complex with LIF receptor and gp130, and this interaction is required for efficient LIF signalling [164]. Interestingly, this system is stabilised by a positive feedback loop,

whereby the STAT3 target *Klf4* activates the *Cdh1* promoter [168], promoting cadherin-based cell–cell adhesion and LIF signalling to maintain pluripotency.

In primed ESCs, the cadherin picture becomes more complex. CDH1 depletion converts naive mESCs into primed EpiSCs, whereupon N-cadherin (CDH2) is upregulated [167] to support pluripotency in this context [169]. However, CDH1 retains a critical signalling role in primed cells, by potentiating ACTIVIN-SMAD2/3 signalling and *Nanog* expression in EpiSCs [165], and PI3K-AKT signalling and *NANOG* and *OCT4* expression in hESCs [170]. Therefore, cadherins have clear signalling functions in both naïve and primed ESCs, and these are required for pluripotency maintenance.

Cell-ECM Interactions: Signalling Downstream of Integrins in Pluripotency

Integrins are heterodimeric transmembrane receptors which couple the extracellular matrix (ECM) to intracellular signalling networks via cytoskeletal adaptor proteins [171]. Although integrin engagement with ECM substrates supports self-renewal and pluripotency of hESCs [172-176], the mechanisms are poorly understood in comparison to the cadherins. Nevertheless, we explore the mechanisms by which integrins signal to the core pluripotency gene regulatory network.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase activated by focal adhesion formation and integrin activation. FAK activation generates phosphotyrosine docking sites for adaptor/scaffolding proteins and signalling molecules [177], which include Src Family Kinases (SFKs), the scaffold p130CAS, the GRB2 adaptor and PI3K. These activate downstream signalling pathways including ERK1/2 and AKT [178]. In hESCs, integrin signalling to FAK supports pluripotency, and FAK inhibition results in differentiation and anoikis [179]. Integrin activation of FAK activates PI3K-AKT and MDM2 to suppress p53 activity [179], which supports cell survival and pluripotency [180, 181]. However, FAK is also activated following hESC differentiation and inactivated during reprogramming of fibroblasts to iPSCs [182]. Furthermore, mESC pluripotency maintenance inversely correlates with integrin activation [183]. Therefore, there is contradictory evidence regarding the role of integrin signalling in pluripotency, which will only be resolved by developing strategies to disentangle the pleiotropic functions of integrins and FAK in ESCs.

SRC family kinases (SFKs) are membrane associated non-receptor tyrosine kinases, which transduce signals from integrins and other cell surface receptors to the actin cytoskeleton [184]. There are eight mammalian SFKs [185], many of which are expressed in mESCs. Paradoxically, inhibition of all SFKs in mESCs promotes pluripotency [186], whilst inhibition of specific isoforms can induce differentiation [187]. This conundrum was elegantly tackled using inhibitor resistant SFK mutants, which showed that selective c-SRC activation induces primitive endoderm differentiation [188]. In contrast, specific YES knockdown suppresses *Nanog* and *Oct4*, inducing mESC differentiation [189]. Interestingly, the differentiation-promoting activity of c-SRC is antagonised by YES in mESCs [189], and hESCs [190], indicating that these kinases may directly inhibit or compete with each other.

To further complicate matters, SFKs isoforms are activated by multiple stimuli. In addition to activation by integrins and receptor tyrosine kinases such as FGFRs [35], YES and HCK are activated by LIF in mESCs via recruitment to gp130 [186, 191-193], consistent with a role in pluripotency signalling. YES kinase activity is suppressed following mESC differentiation [189], whilst HCK activation reduces the LIF requirement for mESC self-renewal [191]. Additionally, specific SFK functions may be underpinned by distinct developmental expression profiles. OCT4 positively regulates *Yes* expression in mESCs [194], ensuring that YES is abundant and activated in pluripotent cells. YES specifically phosphorylates YAP, a transcription factor inhibited by the Hippo pathway (see section below), which drives YAP-TEAD dependent *Oct4* transcription and mESC pluripotency [192]. Accumulating evidence therefore suggests that c-SRC drives differentiation, whilst YES and HCK function to maintain pluripotency. However, lack of specific tools to study SFKs have hindered efforts to identify the molecular mechanisms underpinning their functional specificities in ESCs.

Hippo Signalling Connects Cellular Mechanics to the Pluripotency Machinery

A final key cellular mechanosensing system centres around the conserved Hippo kinase signalling pathway, which coordinates cell growth, proliferation and fate in response to cell–cell contact and polarity. The core Hippo pathway consists of the MST1/2 (Hpo in Drosophila) and LATS1/2 (Wts) kinases, which form a cascade that phosphorylates YAP (Yki) and TAZ transcriptional cofactors.

YAP phosphorylation promotes 14-3-3 binding and prevents YAP/TAZ accumulation in the nucleus [195, 196]. Hippo activation therefore restricts YAP/TAZ dependent transcription of genes required for cell growth and proliferation, and is emerging as a major regulator of pluripotency [197].

Hippo signalling is activated by mechanical inputs from adherens junctions, tight junctions, apical–basal polarity complexes and the actin cytoskeleton [198]. F-actin stabilization results in YAP/TAZ activation, whilst F-actin disruption drives Hippo activation and inactivation of YAP/TAZ [199]. G-protein coupled receptors (GPCRs) also respond to mechanical signals to modulate RHO family GTPase activation and actin dynamics [200]. In hESCs, the guanine nucleotide exchange factor (GEF) AKAP-LBC activates RHOA signalling by modulating actin microfilament organization, and this is required to sustain nuclear localisation of YAP/TAZ [201].

YAP and TEAD cofactors are highly expressed in ESCs [202], and along with TAZ are required for maintenance of mouse and human pluripotency [203-205]. Elevated YAP/TAZ activity also maintains and expands tissue-specific stem cell compartments [206-213]. Overexpression of YAP along with OCT4, SOX2 and KLF4 drives reprogramming of mouse fibroblasts to pluripotency [205]. Conversely, activation of Hippo signalling is a barrier to reprogramming [214, 215], although LATS2 appears to suppress reprogramming by inhibiting TAZ but not YAP [214]. Nevertheless, The YAP/TAZ/TEAD transcriptional module is a crucial determinant of pluripotency, and its function is directly opposed by the Hippo signalling pathway.

Mechanistically, YAP/TAZ/TEAD drive mESC pluripotency by directly inducing *Oct4* and *Nanog* expression [192]. However, YAP also patterns and integrates signals from other pluripotency signalling pathways. Indeed, YAP may promote naïve pluripotency in part by suppressing differentiation-inducing effects of GSK3 inhibition in hESCs [216]. As discussed previously, YAP phosphorylation by the SFK YES increases TEAD2 transcriptional activity at the *Oct4* and *Nanog* promoters in mESCs [192]. In addition, cross-talk between Hippo and TGFβ/BMP signalling also promotes pluripotency. In hESCs, TAZ associates with SMAD2/3 to maintain nuclear localisation and potentiate *OCT4* and *NANOG* expression in response to TGFβ [203]. Furthermore, Beyer et al. identified a regulatory complex composed of TAZ/YAP with TEADs with SMAD2/3 and OCT4 (termed TSO). TSO acts to suppress expression of differentiation markers

whilst supporting expression of core pluripotency genes, thereby maintaining pluripotency [217]. Additionally, in mESCs, YAP has been shown to function in the BMP pathway via SMAD1-dependent recruitment to BMP responsive enhancers to block neural differentiation [204]. Therefore, the Hippo signalling pathway and its YAP/TAZ/TEAD transcriptional module integrate diverse signals to couple the physical environment to transcriptional regulation of pluripotency.

Perspectives

Biological function has been ascribed to a relatively small fraction of the kinome, and evidence suggests that understudied kinases and signalling pathways play critical roles in key biological processes. In this review, we explore newly uncovered pluripotency kinases and more established kinase signalling pathways with emerging roles in pluripotency regulation. A general theme is that environmental and cellular conditions modulate diverse kinase networks, which profoundly impact upon expression and function of pluripotency factors. These molecular connections ensure that pluripotency is either maintained or dissolved depending on the cellular environment. Although much progress has been made, a more complete understanding of how cell cycle, DNA damage signalling, metabolism, stress and mechanical factors modulate pluripotency is required to exploit these processes in pluripotent cell technologies. Furthermore, recent advances in our understanding of distinct pluripotent states requires that we investigate kinase functions in pluripotent cells within the framework of the naïve-formative-primed pluripotency paradigm.

A significant future challenge will be to elucidate key mechanisms and substrates by which protein kinases control pluripotency. In this regard, phosphoproteomic studies and "functional kinomics" enable novel phosphorylation networks to be comprehensively mapped and interrogated. We propose that kinase signalling pathways identified using these unbiased approaches will elaborate the most exciting new molecular targets to be exploited in pluripotent stem cell applications.

Acknowledgments

Figure legends

Figure 1: Metabolism and energetic signaling in pluripotency maintenance, acquisition and exit. (Left) Primed mESC, hESC and iPSC primarily utilise glycolytic metabolism. Under these metabolic conditions, AMPK signaling phosphorylates and activates ULK1 to stimulate mitophagy. Pluripotency factors such as OCT4, SOX2 and NANOG are highly expressed, and SOX2 recruits NuRD repressor complex to restrict mTOR expression. However, mTOR signaling supports pluripotency and protein translation. (Right) Differentiation induces a metabolic switch to mitochondrial oxidative respiration based metabolism. mTOR expression and activation increase to elevate global translation. High mTOR activity promotes ULK1 inhibitory phosphorylation, which suppresses mitophagy allowing mitochondrial accumulation.

Figure 2: Multiple stress signalling pathways modulate pluripotency and differentiation.

Oxidative Stress signalling promotes expression of lineage specific factors by activating multiple signalling pathways. Reactive Oxygen Species (ROS) produced by oxidative metabolism promotes activation of ERK1/2 via inhibition of protein tyrosine phosphatases (PTPs). ROS also results in dissociation of TRX and ASK1 to enable JNK1 and p38 activation. These pathways are thought to promote pluripotent exit and differentiation in response to ROS. Hypoxic conditions also increase ROS production and Endoplasmic Reticulum (ER) stress, which induces Unfolded Protein Response (UPR) genes, VEGF induction and differentiation. Hyperosmotic activation of p38 promotes Oct4 expression, whilst JNK inhibits KLF4 and OCT4. In contrast, shear stress (SS) promotes expression of pluripotency factors including OCT4 and REX1. The ERK5 signalling pathway may be a key mechanism to integrate SS signals with the core pluripotency gene regulatory network.

Figure 3: Mechanical and structural signaling in pluripotency.

(1) E-cadherin (CDH1) is a key component of adherens junctions and forms a ternary complex with the LIF receptor and gp130 in mESCs. This complex is required for efficient JAK-STAT3 signalling and pluripotency factor expression. The STAT3 target KLF4 activates *Cdh1* expression generating a positive feedback loop to stabilise naïve pluripotency. (2) Integrins couple the extracellular matrix (ECM) to intracellular signaling. Integrins activate Focal Adhesion Kinase (FAK) to support pluripotency via activation of PI3K-AKT and MDM2. Downstream, the Src family kinase (SFK) YES phosphorylates the transcription factor YAP to promote pluripotency. (3) The Hippo pathway provides a further link between cellular mechanics and pluripotency. The Ser/Thr kinases MST1/2 and LATS1/2 form a kinase cascade that phosphorylates YAP (at a site distinct from that phosphorylated by YES), which promotes 14-3-3 binding and cytoplasmic retention. Nuclear YAP/TAZ/TEAD promotes transcription of pluripotency genes including *Oct4*.

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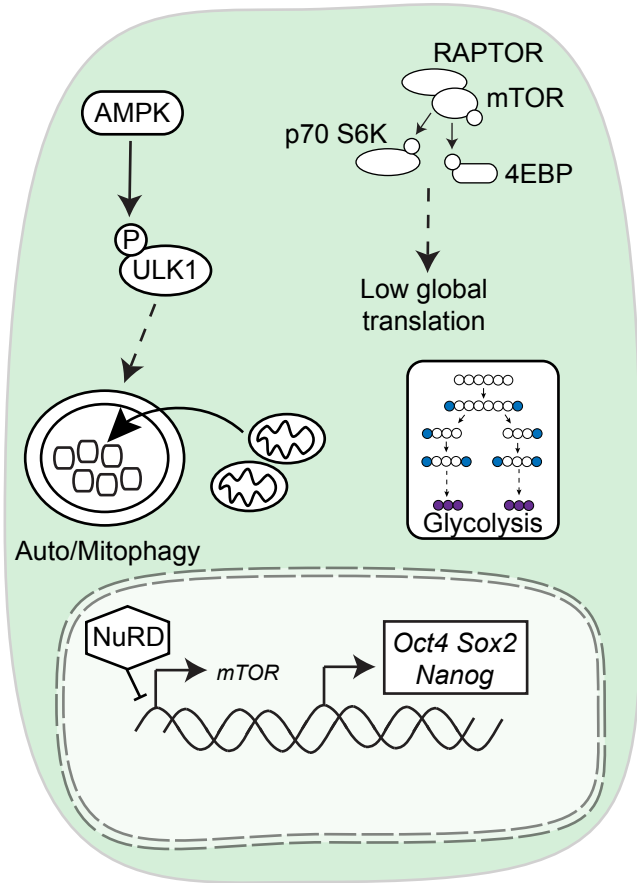[166] Soncin F, Ward CM. The function of e-cadherin in stem cell pluripotency and self-renewal. Genes. 2011;2:229-59.

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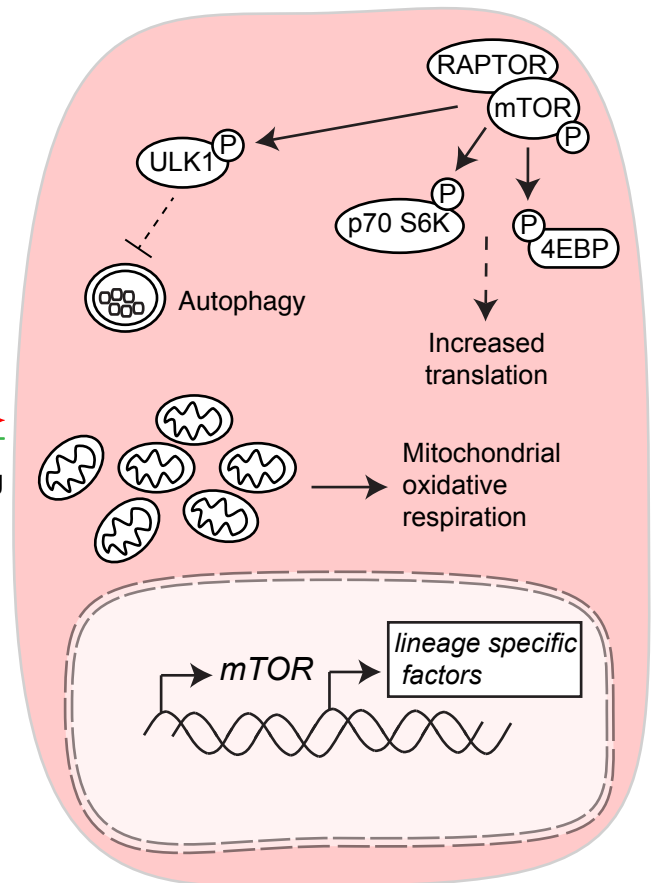
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**Primed ESC
(Glycolytic Metabolism)**

**Differentiated Cell
(Oxidative Metabolism)**

Fernandez-Alonso et al, 2016 Figure 1

Fernandez-Alonso et al, 2016 Figure 2

Fernandez-Alonso et al, 2016 Figure 3