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Insights & Perspectives

The cell cycle and differentiation as integrated processes: Cyclins and CDKs reciprocally regulate Sox and Notch to balance stem cell maintenance

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Abstract

Development and maintenance of diverse organ systems require context-specific regulation of stem cell behaviour. We hypothesize that this is achieved via reciprocal regulation between the cell cycle machinery and differentiation factors. This idea is supported by the parallel evolutionary emergence of differentiation pathways, cell cycle components and complex multicellularity. In addition, the activities of different cell cycle phases have been found to bias cells towards stem cell maintenance or differentiation. Finally, several direct mechanistic links between these two processes have been established. Here, we focus on interactions between cyclin-CDK complexes and differentiation regulators of the Notch pathway and Sox family of transcription factors within the context of pluripotent and neural stem cells. Thus, this hypothesis formalizes the links between these two processes as an integrated network. Since such factors are common to all stem cells, better understanding their interconnections will help to explain their behaviour in health and disease.

KEYWORDS
CDK, cell cycle, cyclin, differentiation, Notch, Sox, stem cell

INTRODUCTION

Stem cells are a diverse set of cells that underpin the development and maintenance of all organ systems.[1] These cells are defined by their ability to self-renew, proliferate and give rise to differentiated cell types. In humans, this definition can encompass cells from the fertilized egg to adult tissue resident stem cells,[2] but can also apply to several types of quiescent cells that gain stem cell attributes upon activation.[3,4] All multicellular life with definitive cell types must necessarily arise and be maintained by cells that exhibit these properties.[5]

In order to produce the diverse organ systems of higher animals, the regulation of stem cell proliferation, differentiation and cell fate specification must be tightly coupled in a context dependent manner.[6,7] The importance of this is exemplified by the variation in size and regenerative capacity of different organs, and underpinned by the expansion of cell cycle and differentiation regulators in the animal kingdom.[2] However, these processes have primarily been studied in isolation on the cell population level by looking at the effects of individual factors. This is not an ideal approach due to the cross regulation of the processes involved and the profound cellular changes inherent in cell division. For example, it is difficult to distinguish whether proteins inducing differentiation halt proliferation by directly affecting cell cycle regulator activity or via the cascade of events that occur during this cell process.[8,9] Fortunately, our understanding has recently been

Abbreviations: CDK, cyclin-dependent kinase; ESC, embryonic stem cell; IPSC, induced pluripotent stem cell; NSC, neural stem cell; G1, gap 1; S, synthesis; G2, gap 2; M, mitosis; bHLH, basic helix-loop-helix

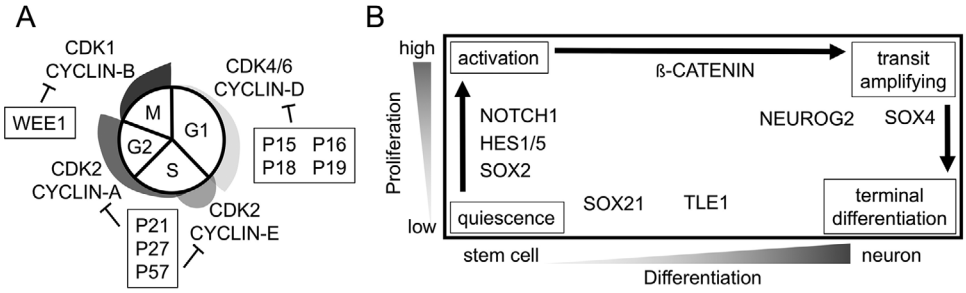

FIGURE 1 Summary of cell cycle and stem cell regulators. (A) Illustration of the activity (shaded forms) and regulators (boxes) of cyclin/CDK activity within different cell cycle phases. (B) Schematic of stem cell regulators discussed, and their approximate influences on NSC proliferation rate and differentiation state, overlaid by the activation of stem cells from quiescence to terminally differentiated cell types

improved by techniques providing single-cell resolution on cell states and cell-cycle phase dynamics.[10–12] Thus, it is now possible to appreciate the links between tightly intertwined pathways, such as those regulating the cell cycle and differentiation.

Cell cycle state regulates differentiation

In eukaryotes, proliferation is mediated by a highly conserved network of cyclins and their cyclin-dependent kinase (CDK) partners (Figure 1A, Box 1). To accomplish cell division, distinct cell cycle phase-specific cyclin-CDK complexes phosphorylate the myriad of cellular components that must act sequentially to achieve DNA replication and cytokinesis.[13] By altering the activity of these regulators, stem cell populations can maintain or expand their numbers to provide for the diverse needs of different organ systems.

The cell cycle begins as daughter cells enter the gap-1 phase (G1) following the division of a mother stem cell.[12] This transition involves the rapid decompaction and reorganization of chromatin based on the specific factors inherited by each cell.[41] These are often asymmetrically deposited in daughter cells, such that one progenitor is biased towards differentiation.[42] Such asymmetrically inherited factors can influence the length of each cell cycle phase, and this has also been demonstrated to influence differentiation outcomes.[43] For instance, G1-phase has been established as specifically permissive to differentiation signals, and lengthens as stem cells commit to specific lineages. This is because G1 provides a unique regulatory landscape for signalling events to influence differentiation, as DNA is unreplicated and chromatin is at its most relaxed.[44] In contrast, delaying G2- or M-phase has been shown to promote stem cell maintenance via the activities of the cell cycle regulators active within them.[10,45] Interestingly, several cell cycle regulators have been described to directly influence the expression of differentiation genes.[10,46] Thus, the cell cycle has a direct regulatory role in stem cell differentiation.

For instance, while proliferating cell populations only pause in G1, cells can also remain in G1 indefinitely—termed G0. This silence can be the result of a stem cell entering quiescence or committing to terminal differentiation into a functional cell type.[47] The mechanisms that separate these two states are illustrative as to why the links between cell cycle and differentiation factors are so important. Both states downregulate core cell cycle factors such as CYCLIN-B1, -E2 and -A2 and rely on the activities of cyclin-dependent kinase inhibitors and P53.[47,48] However, this transcriptional profile is achieved and maintained by distinct mechanisms in these two cell states. While quiescent stem cells supress cell cycle genes via reversible transcriptional repression, differentiated cell types heterochromatinize the regions around these genes in order to permanently block their expression.[49,50] This difference is achieved by distinct factors that target appropriate epigenetic modifications to achieve cell cycle exit in each cell type.[51] Thus, the regulation of cell cycle states relies on specific transcriptional regulators.

Stem cell maintenance regulators guide cell cycle progression

For stem cells to generate the functional cells necessary for organ development and maintenance, they must carefully balance self-renewal and differentiation. To achieve this, different populations utilize distinct stem cell states and modes of proliferation to generate appropriate numbers of the differentiated cell types necessary for homeostasis.[2] Due to the diversity of organ systems in the body, this requires the activity of a complex network of inter- and intracellular regulatory pathways.

The variety of mechanisms utilized for organ maintenance and repair in different systems has only recently begun to be resolved. For instance, although the liver is among the most regenerative organs in the body, the identification of tissue resident stem cells has remained elusive.[26] It has recently been established that its ability to replace damaged tissue is dependent on the activation and proliferation of functional cells within the organ.[27] This has even been found to involve the transdifferentiation of one cell type into another, and thus illustrates the complex interplay between differentiation, proliferation and cell fate decisions.[28]

Most organs, such as the skin, intestine and brain, utilize a hierarchical mode of renewal whereby quiescent stem cells are periodically activated to proliferate and give rise to one stem cell and one transit amplifying cell.[29] Like stem cells, transit amplifying populations can self-renew; however, these cells are only competent to proliferate for a defined number of generations before they undergo terminal

Box 1: Cell cycle mechanisms

In proliferative cell populations, D-type cyclins (CYCLIN-D1, -D2 and -D3 in humans) cooperate with CDK4/6 to phosphorylate RB proteins during G1.[14,15] Phosphorylated RB loses its association with E2F transcription factors, releasing these to induce a cascade of transcriptional events that commit the cell to genome duplication.[16] Among the most prominent E2F targets include A- (CYCLIN-A1 and -A2)[17] and E-type (CYCLIN-E1 and -E2)[18] cyclins, which proceed to hyperphosphorylate RB via CDK2. This leads to further activation of E2F factors, which guide the cell into and through DNA-replication (S-phase). In order for CDK activity to promote cell cycle progression, it must first overcome the activity of CDK-inhibitors of the ARF/INK-family (P15, P16, P18 and P19), which primarily target CDK4/6 activity, and CIP/KIP-family (P21, P27 and P57), which also target CDK2 activity.[19] Even once a cell commits to DNA-replication the cell cycle can be halted by various sensors that monitor polymerase blockage and DNA damage. These sensors include the ATM/ATR kinases, which are upstream of phosphorylation cascades that induce CDK-inhibitors via CHK1/2 kinases and the P53 tumour suppressor transcription factor.[20]

Once a cell has completed DNA-replication, it enters the gap-2 (G2) phase of the cell cycle, which allows time for cell growth and homologous recombination mediated repair of any DNA damage incurred during replication.[21] In order to induce mitosis, levels of B-type cyclins (CYCLIN-B1, -B2 and -B3)[22] must reach a threshold sufficient to interact with CDK1.[23] CDK1 activity is inhibited by phosphorylated WEE1 kinase, which is also activated by DNA-damage via CHK1/2 kinases.[24] However, once a threshold is reached, CDK1/CYCLIN-B act in a positive feedback loop with CDC25 phosphatase and the functional effector of mitosis—PLK1 kinase, which represses WEE1 and the parallel M-phase inhibiting transcription factor MYT1.[25] This activity guides duplicated DNA through chromosome condensation and segregation, as well as cytokinesis.

differentiation.[29] This hierarchy allows long-lived stem cells to reduce their risk of incurring mutations during DNA-replication, while giving rise to the required number of differentiated cells.[30] In order to satisfy the specific demands of each organ system, extrinsic signalling pathways control the behaviour of intracellular pathways regulating stem cell maintenance and cell cycle progression in a context dependent manner.

Numerous intrinsic and extrinsic regulatory pathways have been shown to influence stem cell proliferation and differentiation. For instance, FOXOs are present in yeast and have a well described role in the repression of D-type Cyclins in the hematopoietic system.[31] However, this hypothesis focuses on the well described interactions between cyclins/CDKs, the Notch and Wnt signalling pathways, as well as the Sox family of transcription factors (Figure 1B, Box 2). Active Notch signalling is an essential pathway for maintaining the stem cell pool within a differentiating population and is mediated by cell-cell contact.[32] In contrast, the Wnt-pathway has been best characterized as an important regulator of stem cell proliferation and amplification, and is mediated by soluble Wnt protein binding to receptors on the cell surface.[33] As with the myriad of other signalling pathways, contact- and morphogen-mediated mechanisms allow each organ to precisely control their own homeostasis. However, in order to do so, cell surface signalling events must be translated into changes in gene regulation via interactions with specific transcription factors.[34,35]

The transduction of cell surface signalling most often results in the conversion of pathway specific transcription factors into gene activating complexes.[31,35] However, such transcription factors are often ubiquitously expressed, and thus rely on specifically expressed partner transcription factors to execute organ specific processes.[6,7] This allows these partner factors to modify the assembly of transcription regulating complexes. For instance, both the Notch and Wnt-pathways have been shown to act in coordination with members of the Sox-family of transcription factors, such that different Sox factors can modulate their activities.[52,53] This is because different Sox family members have been found to interact with the DNA binding transducers of each signalling pathway. Importantly, such transcriptional complexes underpin organ specific cell cycle activities and cell fate potentials.[6,7,11]

Within this essay, we will discuss known interactions between stem cell regulatory networks and the cell cycle machinery. We hypothesize that cell cycle factors are integral regulators of stem cell maintenance, with reciprocal feedback from stem cell pathways into proliferation. We establish the relevance of these interactions by discussing the overlapping evolutionary expansion and direct mechanistic links of factors regulating these two stem cell processes. A better understanding of these processes—as a unified framework—will be necessary to design interventions into the many diseases that result from stem cell disfunction.

MULTICELLULARITY, CELL CYCLE REGULATORS AND STEM CELL FACTORS EVOLVED IN PARALLEL

Although unicellular life is thought to have arisen rapidly on earth, it was not for billions of years after this that multicellular life was able to establish itself.[54] This timeline illustrates the complexity of preconditions that first needed to be met in order to allow for the founding of a stem cell pool that could differentiate into specialized, cooperative cell types.[55] The factors discussed here are involved in balancing stem cell self-renewal and the generation of terminally differentiated progeny. To see how these cell cycle and differentiation factors may have evolved as co-dependent networks, it is informative to examine the timing of each factor's appearance and diversification within the eukaryotic lineage. Thus, here we will contrast the repertoire of these factors in budding yeast,[56] choanoflagellates,[57] trichoplax[58]

Box 2: Stem cell maintenance regulators

Notch signalling

When a cell commits to differentiation, it upregulates Delta or Jagged cell surface ligands, which induce the intracellular protease cleavage of Notch receptors in neighbouring stem cell membranes.[32] The released Notch intracellular domain (NICD) then transits to the nucleus, where it activates gene expression via interaction with RBPJ transcription factors.[32] Notch promotes stem cell maintenance partly by activating Hes basic helix-loop-helix (bHLH) Group E transcription factors. In turn, these act by repressing the expression of differentiation inducing proneural bHLH Group A proteins, such as NEUROG2.[34]

Wnt signalling

The Wnt pathway is activated by the binding of Wnt-ligands to Lrp and Frz-receptors at the cell surface. This allows Dvl phosphoprotein-mediated inhibition of a multiprotein complex, which otherwise phosphorylates and targets the soluble form of the β-CATENIN adhesion molecule for degradation.[33] When soluble β-CATENIN accumulates, it translocates to the nucleus and activates genes promoting proliferation, such as CYCLIN-D1 and MYC. This is mediated via an interaction with Tcf/Lef family transcription factors, which bind to Tle co-repressors in the absence of active Wnt-signalling.[35]

Sox transcription factors

The Sox family of transcription factors are HMG-box factors expressed within all cell lineages. They are named for their relationship to human sex determining SRY and bind DNA via the minor groove. These factors include the SoxB1 genes (SOX1, 2 and 3), which potently maintain stem cell proliferation and multipotent characteristics.[36] In contrast, the SoxB2 (SOX14 and 21),[37] SoxD (SOX5, 6 and 13)[38] and SoxE (SOX8, 9 and 10)[39] groups repress proliferation and promote lineage specific cell identities, while the SoxC group (SOX4, 11 and 12) induces proliferation and terminal differentiation within various lineages.[40,8]

and humans. We will focus on these species because they each represent essential stages of genetic novelty and diversification leading to the evolution of functional stem cells. However, to fully understand this process would require a deeper look into other sequenced genomes, such as those of amoeba,[59] fission yeast,[60] sponges (porifera)[61] and jellyfish (cnidarians).[62]

To begin, we discuss budding yeast, which are among the most well characterized eurkaryotes. They exhibit complex behaviours including colony formation, while having no distinct cell types.[63] Similarly, choanoflagellates can utilize solitary or colonial living strategies, but display more distinct phenotypes and gene expression patterns in each state.[64] In contrast, trichoplax are bonafide multicellular organisms with several differentiated cell types.[65] These traits are amplified in human biology, and lead to our current view of stem cell maintenance and regulation.[2] Based on these comparisons, one intriguing possibility is that the benefits of multicellularity provided evolutionary pressure for the diversification of stem cell transcription factors and the cell cycle machinery (Figure 2).

Yeast

Yeast, such as *Saccharomyces cerevisiae*, represent a distant relative of all animal species, with which they shared a common ancestor approximately a billion years ago. They exhibit all definitive eukaryotic traits, and though they can show functional differentiation within a colony, they display no distinct cell types.[63] Yeast lines have the ability to self-renew clonally, but they cannot be termed as stem cells since their progeny are unable to undergo terminal differentiation. However, they do have a tightly regulated cell cycle with checkpoints before DNA-synthesis and mitosis, as well as the ability to enter a G0 state of hibernation.[66]

While yeast lack the transcription factor families and pathway actors associated with stem cell regulation,[67] some have ancestral DNA-binding domains shared widely by eukaryotes, such as those of Sox [68] and bHLH [69] transcription factors. Similarly, they have only one clear CDK homologue, CDK1, which is able to licence all stages of the cell cycle via interactions with the cyclin homologues CLN and CLB.[70] Thus, the yeast genome provides the basic repertoire of factors utilized in the expansion of cell cycle regulation and stem cell pathways observed in higher animals.[71]

Choanoflagellates

Choanoflagellates, such as *Salpingoeca rosetta* and *Monosiga brevicollis*, are believed to be the closest living ancestor of metazoan animals, having diverged approximately 600 million years ago.[72] Free living unicellular individuals are known to congregate and live as colonies under certain conditions.[64] Interestingly, within these colonies, individual choanoflagellates have been observed to dramatically change their morphology and upregulate structural proteins that are conserved in animals.[72] As these distinct cell behaviours are reversible, these organisms still lack stem cells, but as with yeast, they are also capable of entering a hibernating quiescent state in stressed environments.[73]

The ability of choanoflagellates to behave differently under colonial conditions coincides with the appearance of Sox/Tcf [57] and RBPJ transcription factors as well as the Notch receptor,[74] which were long believed to be metazoan specific. Although the homology between *Monosiga brevicollis* and metazoan Sox/Tcf, RBPJ and Notch are low,

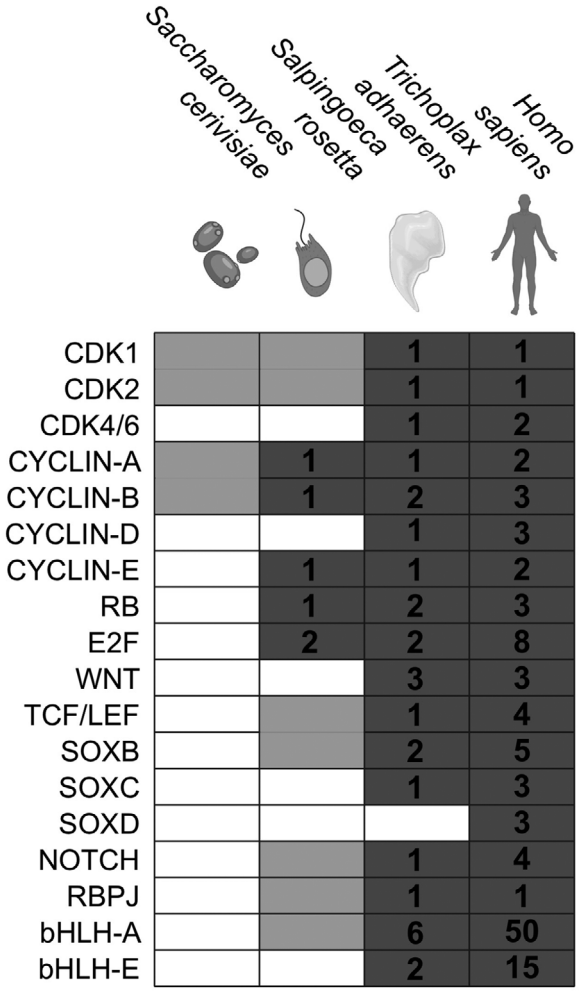

FIGURE 2 Evolution of cell cycle and stem cell regulators. Presence (dark grey) or absence (white) of genes and gene families discussed here are indicated. Light grey denotes the presence of a gene with domain homology to the group indicated. Fused boxes show when the role of two separate human genes are performed by a single gene. Numbers inset show the number of individual genes present within a gene family in each organism

they show sufficient homology to suggest that they share a common ancestor.[57] Similarly, choanoflagellates contain a more metazoan complement of cell cycle regulators than yeast, as they express identifiable homologues of CDK1/2 and cyclins A, B and E.[70] Moreover, the essential cell cycle regulating transcription factor P53 also appears for the first time within this lineage.[57] Thus, although not diversified, many of the key protein domains utilized in animal stem cell regulation are present in choanoflagellates.

Trichoplax

Together with cnidaria and porifera, the placazoan *Trichoplax adhaerens* represents one of the most basal branches of the metazoan lineage.[58] Their six established cell types include two types of epithelial cells, lipophils, gland cells, crystal cells and fibre cells.[65] Although they can regenerate and reproduce sexually, no definitive stem cell has been identified.[75] Several cell types have been proposed as stem cells based on their gene expression patterns and necessity for regeneration, but our view of tissue resident stem cells in vertebrates may not be applicable to these animals.[65] For instance, poriferan stem cells have been well characterized and found to be characterized by epithelial to mesenchymal transition and transdifferentiation of specific epithelial cell types.[76] To simultaneously perform different stem cell activities, such as quiescence, proliferation and differentiation, *Trichoplax* requires a dynamic tool kit of cell cycle and cell identity regulators.

Placazoan cell type diversity is underpinned by the appearance and diversification of multiple transcriptional regulators within their genome.[58] These include five Sox protein subfamilies (B1, B2, C, E and F), which span all of the phases of differentiation and numerous cell types in vertebrates.[68] Moreover, these can be distinguished from a definable Tcf gene, which is complemented by the primary effectors and regulators of Wnt signalling in vertebrates: Wnt, Frz, β-catenin, Gsk3, Axin and Dvl.[58] The Notch pathway exhibits a similar burst in diversification to include definitive Notch, Delta and RBPJ signal transducers,[74] as well as over 25 bHLH transcription factors representing all of the subfamilies present in vertebrates.[69] Importantly, this diversification of transcriptional regulators occurred in parallel with the appearance of the CDK4/6 kinase family and their D-type cyclin regulators.[70] Interestingly, the E2F pathway and CYCLIN-D1 are present in both plants and animals, while CDK4/6 are novel to multicellular animals, suggesting evolutionary novelty and specific gene retention within the metazoan lineage.[70] Thus, in addition to the regulatory factors already in place, the genetic diversification that occurred at the base of the metazoan lineage laid the foundation for the expansion of the animal kingdom.

Humans

Homo sapiens support and develop hundreds of cell types and trillions of functional cells from a single totipotent stem cell. In addition, dozens of subtypes of tissue resident stem cells are maintained throughout an individual's life to ensure the functionality of the organs they reside in [77]. Dependence on the organ, tissue repair and rejuvenation can involve the processes of cellular quiescence, activation, self-renewal, transit amplification, terminal differentiation and even de- or transdifferentiation.[78] All of these involve a deep coordination between the regulation of cell cycle activity and differentiation.

The human genome contains 20 Sox,[68] four Tcf/Lef,[79] and 118 bHLH transcription factors.[69] These factors provide the context for individual cells to interpret the external signals, such as those of Notch and Wnt, that guide their cell cycle activity and cell fate commitment. This diversity is also exhibited in the cell cycle, which is controlled by 20 CDK, 29 cyclin and 7 CDK inhibitor genes, along with their myriad of interacting proteins.[70] It is likely that this expanded number of regulators is required to ensure the fidelity of cell cycle and differentiation processes within the different organ contexts of our bodies.

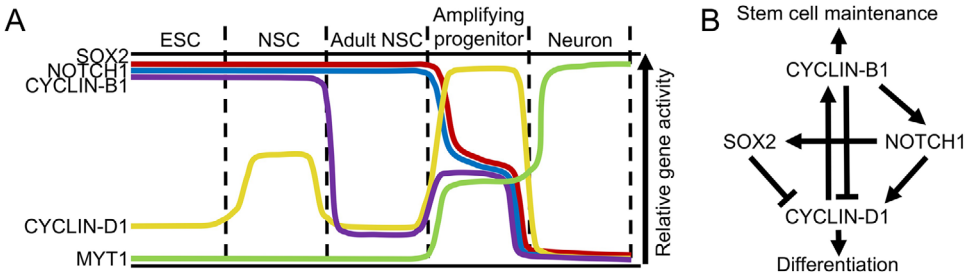

FIGURE 3 Cell cycle and stem cell regulatory relationships during neurogenesis. (A) Relative transcript levels of *SOX2*, *NOTCH1*, *CYCLIN-B1*, *CYCLIN-D1* and *MYT1* expressed by different cell populations in the neuronal lineage. (B) Established transcriptional relationships between SOX2, NOTCH1, CYCLIN-B1 and CYCLIN-D1 within NSCs

DIRECT MECHANISTIC LINKS BETWEEN DIFFERENTIATION AND CELL CYCLE REGULATION

Although not usually thought of as interacting pathways, the cell cycle and stem cell commitment machinery show reciprocal regulation at numerous levels. Experiments on these processes have been difficult to interpret depending on the time point and marker used for analysis.[80] This is because even opposing effects can produce similar results, such as that quiescence and terminal differentiation both halt proliferation. In contrast, transit amplifying progenitors are committed to differentiation, but show a high rate of proliferation.[81] An example of the challenges faced in experimenting on these systems is observed in the over expression of SOX2 and NEUROG2 in the developing cortex. After 48 h both manipulations produce few proliferating cells, but only NEUROG2 upregulates terminal differentiation markers such as MYT1.[11] Moreover, at 24 h NEUROG2 electroporated cells are rapidly proliferating, while those over expressing SOX2 have already slowed their rate of proliferation (Figure 3).[11] Thus, in order to understand how these interlinked processes regulate one another, mechanistic insight is essential for the appropriate interpretation of results. Here, we summarize the known mechanistic links between specific cell cycle and differentiation factors by addressing the maintenance and differentiation of embryonic stem cells (ESCs) and neural stem cells (NSCs).

G2 and M-phase promote ESC pluripotency maintenance

There is an extensive body of work examining cell cycle regulation and pluripotency maintenance in ESCs.[82] The pluripotency network of transcription factors has been mapped in detail, resulting in the discovery of induced pluripotent stem cells (IPSCs) via the forced expression of SOX2, OCT4, KLF4 and NANOG.[83,84] However, as these factors can be substituted by other proteins in IPSC formation, pluripotency is suggested to emerge once a series of overlapping transcriptional networks are engaged [85]. ESCs have a unique cell cycle, with an unregulated G1/S-phase checkpoint that does not require D- or E-type cyclins in order to licence DNA replication.[86] This abridged G1-phase relies on constitutive CDK2 activity, which allows ESCs to proceed directly into S-phase following cell division.[87] This rapid cycling has been found to be an intrinsic trait of pluripotency and sign of successful IPSC reprogramming.[88] Thus, the regulation of pluripotency maintenance has been directly linked to an active cell cycle state. However, the mechanisms behind this have only recently begun to be understood.

In a recent study looking for early signs of ESC differentiation, the authors used an RNA interference screen to identify factors important for pluripotency maintenance. This revealed regulators of S-, G2- and M-phases to be essential for the maintenance of pluripotency. Interestingly, the specific factors identified included DNA-damage repair pathway genes, such as ATM/ATR, CHK2 and P53 kinases, as well as CDK1.[45] Although CDK1 had not previously been implicated in stem cell maintenance, it is known to be both necessary and sufficient for progression of the ESC cell cycle, and was recently shown to direct the dissociation of OCT4 from chromatin during M-phase (Figure 4A).[89,90] Moreover, the direct link between S- and G2-phases and pluripotency is exemplified by the ability of CDK2 to potentiate SOX2 induction of pluripotency in IPSCs. Interestingly, the SOX2 phosphorylation resulting from this interaction was also demonstrated to occur in neural progenitors, suggesting a commonly employed mechanism.[91] Thus, cell cycle regulation appears to play a key role in the induction and maintenance of the pluripotent state.

CYCLIN-D1 promotes ESC lineage commitment

As stated above, the G1-phase of the cell cycle has previously been found to be a key period of competence for ESC commit to specific lineages.[44] During this stage, specific factors can become dominant and induce differentiation toward specific fates, such as SOX2 in neuroectoderm and OCT4 in mesoendoderm commitment.[92] Notably, Notch signalling has been shown to be required for ESC differentiation towards all embryonic germ layer fates.[93] Once this occurs, cell's G1- and G2-phases lengthen as CDK2 activity is downregulated and the standard regulatory checkpoints are instituted in a lineage specific manner.[94,95] This lengthening of G1 continues throughout development, as stem cells transit through pluripotent and multipotent states, before entering quiescence.[96]

Although not highly expressed in pluripotent cells, D-type cyclins have been shown to be upregulated and play a key role during ESC lineage commitment (Figure 4B).[97] As in other systems, CYCLIN-D1

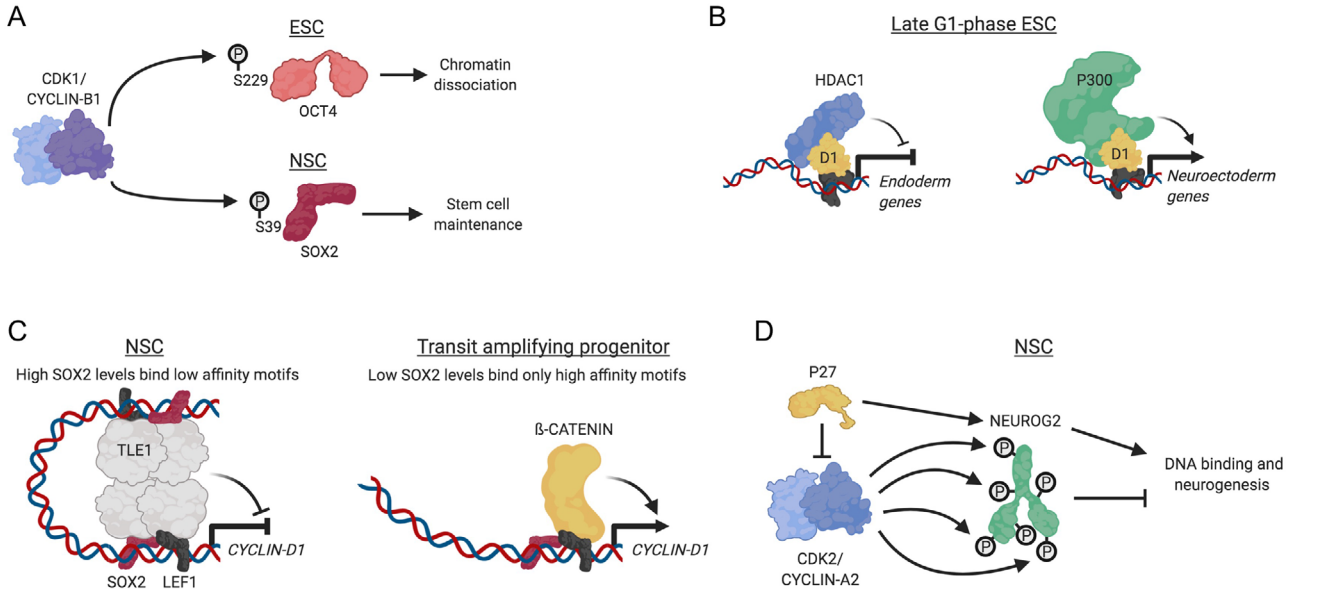

FIGURE 4 Mechanistic links between cell cycle and stem cell regulators. (A) CDK1/CYCLIN-B1 activity phosphorylates serine 229 on OCT4 in ESCs, which promotes its dissociation from chromatin during M-phase. In NSCs, this complex phosphorylates serine 39 of SOX2 to potentiate its role in stem cell maintenance. (B) When CYCLIN-D1 has built up in late G1-phase ESCs, it binds to specific transcription factors on endodermal and neuroectodermal gene promoters. On endodermal genes, it recruits HDAC1 and represses their expression, while on neuroectodermal genes it recruits the histone acetyltransferase P300 to activate transcription. (C) SOX2 is expressed at high levels in NSCs, which permits binding to low affinity Sox-sites in the *CYCLIN-D1* promoter. In cooperation with TCF/LEF transcription factors, this promotes recruitment of TLE1 co-repressors to block *CYCLIN-D1* expression. SOX2 levels decrease when NSCs commit to differentiation and enter a transit amplifying state, which allows the recruitment of *β*-CATENIN and activation of *CYCLIN-D1* transcription. (D) In NSCs, CDK2/CYCLIN-A2 phosphorylate NEUROG2 protein at multiple sites, which prevents it from binding DNA and activating neurogenesis. The CDK inhibitor P27 also stabilizes NEUROG2 protein and promotes its activity in a CDK/cyclin independent manner

has been shown to act downstream of Wnt-signalling to enact subtle, but instructive differences in ESC G1-phase length.[98] As such, D-type cyclins have been shown to regulate TGF-*β* signalling in order to permit commitment to endoderm early in G1-phase and neuroectoderm at later stages.[99] Interestingly, the ability of CYCLIN -D1 to regulate gene activity has been demonstrated to be at least partially mediated by direct transcriptional regulation of target genes.[100] Though the consequences of CYCLIN -D1 binding are context dependent, its genomic targets have been suggested to be determined by binding with various transcription factors, including SP1 and E2Fs.[101] Such CYCLIN-D1 complexes have been shown to directly promote a neuroectodermal fate, further demonstrating that the links between cell cycle regulation and cell fate commitment act in a lineage specific manor (Figure 4B).[97]

SOX2, NOTCH1 and CYCLIN-B1 constitute a NSC maintenance network

As the brain develops, NSCs progressively slow their proliferation and eventually enter a quiescent state in the adult brain. This change in proliferation rate is paralleled by the sequential generation of different neural cell types. For instance, in the developing cortex stem cells first give rise to deep, then upper layer neurons, followed by astrocytes and oligodendrocytes.[102,103] Slow proliferation relieves these long lived cells from the stresses of cell division, and thus NSCs that will be maintained throughout life are already identifiable in the embryo by their proliferation rate.[104]

Such slowly proliferating stem cells were found to rely on P57 for their maintenance into adulthood, which agrees with previously published data showing P57 to repress neuronal differentiation.[104,105] Moreover, we have shown that high levels of SOX2 maintain neural stem cells in a slowly proliferating state by suppressing CYCLIN-D1 transcription directly at its promoter in a dose-dependent fashion. As the most targeted site in the NSC genome, this was found to associate via an interaction with Tcf/Lef transcription factors on DNA, which stabilized SOX2 binding to off-consensus binding sites and promoted the formation of TLE1 corepressor complexes at the expense of *β*-catenin activator recruitment (Figure 4C).[35] Interestingly, SOX2 activity in neural stem cells has been found to be potentiated by CDK1 phosphorylation at S39, but not by CDK4/6 (Figure 4A).[106] This fits with our more recent results demonstrating that M-phase genes, including CDK1, demarcate cortical NSCs that are maintained and give rise to later born lineages. As such, we found that the overexpression of CYCLIN-B1 delays differentiation via the upregulation of the Notch pathway and genes promoting symmetrical stem cell divisions.[10] In fact, the homologue of CDK1 was found to be necessary for accurate protein distribution during cytokinesis in *Drosophila* neuroblasts almost 20 years ago.[107] Together, these results demonstrate that core M-phase regulators play an essential role in NSC maintenance.

CYCLIN-D1 and bHLH factors promote NSC differentiation

NSC commitment to terminal neural differentiation most often involves the generation of transit amplifying progenitors.[29] In contrast to slow cycling stem cells, transit amplifying progenitors are characterized by their rapid turnover and short G1-phase in most systems.[2] While amplifying progenitors can undergo many cell divisions, particularly in primates, they cannot be maintained as stem cells and all of their progeny will eventually differentiate.[11,108] Thus, there is an intimate relationship between G1-phase regulation and stem cell's commitment to differentiation during brain expansion.

The ability of CYCLIN-D1 and CDK4/6 to upregulate intermediate progenitor markers and induce differentiation has been demonstrated in the embryonic brain.[11,97] A similar effect was seen in the developing spinal cord, where CYCLIN-D1 was shown to act via the upregulation of HES6, which has the opposite function to HES1/5.[109] However, this appears to be a stage specific effect, as similar experiments in the adult hippocampus result in expansion of both stem cell and transit amplifying populations.[110] Although only examined for A- and B-type cyclins, NEUROG2 activity was shown to be negatively regulated by CDK1/2-dependent phosphorylation at multiple sites, which blocks its ability to bind DNA (Figure 4D).[111] In agreement with this, P27 has been shown to promote neuronal differentiation by stabilizing NEUROG2 protein through multi-site phosphorylation (Figure 4D).[46] Finally, mathematical modelling of HES1 and NEUROG2 activities during G1 has been used to suggest how both can inhibit S-phase entry via distinct mechanisms to slow and halt the cell cycle, respectively.[112] Thus, stem cells and terminally differentiated cell types both actively inhibit cell cycle re-entry, while transit amplifying cells actively promote proliferation, by linking these processes via distinct mechanisms.

CONCLUSION

Here, we hypothesize that the genetic circuits regulating the cell cycle and differentiation are interdependent to the point that they should be addressed in parallel. We suggest that these potent molecular pathways have co-evolved within the animal lineage and allowed for the advent of terminal differentiation, as well as the diversification of stem cell identities necessary for complex animal life. Although numerous specific interactions have already been identified, we believe there are likely to be many that are yet to be revealed at all stages of development.

The evolution and diversification of many gene families occurred in the earliest metazoan lineages, driven by the survival advantages derived from multicellularity.[55] It is possible that this co-emergence of genes was promoted by the reciprocal regulatory relationships formed between these novel factors. Importantly, the ability to terminally differentiate is only made possible by multicellularity, when other cells in an organism are able to assume the role of tissue and lineage maintenance.

Although many direct interactions have been identified between cell cycle regulators and stem cell pathways, these have yet to be encompassed by an overarching model. It is tempting to suggest that the cell cycle provides a core mechanism for balancing differentiation and self-renewal via the asymmetric distribution of differentiation factors during cytokinesis.[107] However, the pathways governing proliferation and differentiation have been shown to act within the context of specific cell states.[7,11] Recent results suggest that the regulatory networks downstream of CDK1, CDK4/6, Sox transcription factors and the Notch pathway are key in this regard.[10]

Genome wide analyses have forced cell biologists to appreciate branched and recursive feedback loops as the mediators of complex cell processes such as the cell cycle and stem cell maintenance. However, we argue that the degree of crosstalk between these overarching processes remain underappreciated and should be further explored. Importantly, a deeper understanding of such synergies could have implications for the development of treatments for stem cell diseases, such as cancer, by helping to predict indirect intracellular targets and cellular responses.

FUTURE DIRECTIONS

As shown above, there are examples of direct interactions between cell cycle regulators and differentiation pathways. Expanding on these findings to uncover novel interactions in more diverse organ systems should be a priority. Using these results as a guide, we would suggest future experimental effort be focused on better understanding the roles of CDK1 and CDK4/6 in differentiation, as well as SOX2 and NOTCH1 in controlling the cell cycle:

1. In order to confirm the conservation and necessity of roles already described for these factors, functional experiments in basal metazoans will be necessary. There are several organisms of interest. Choanoflagellates [113] and trichoplax [114] are transfectable, while Hydra cnidarians have recently been genetically modified.[115] Moreover, Hydra developmental expression patterns and chromatin landscapes have recently been characterized on the single cell level, which would aid in the interpretation of results.[116] Thus, it would be interesting to transfect choanoflagellates and trichoplax, or engineer Hydra lines to overexpress or knockdown the homologues or human versions of the key factors listed above. Analysing such experiments using single cell RNA-sequencing, would allow the assessment of their roles in development, tissue regeneration and the stem cell compartment.
2. Although CDK activity is known to target many proteins, it remains poorly characterized outside of the canonical cell cycle targets. To identify kinase targets that could have an effect on stem cell maintenance, we would recommend applying liquid chromatography with tandem mass spectrometry on cells following the acute overexpression and knockdown of CDK1 and CDK4/6.[117] In order to ensure that these experiments could lead to generalizable conclusions, we would recommend performing these experiments in ESCs as well as

more lineage restricted stem cells, such as embryonic cortical and adult hippocampal NSCs.

3. One intriguing, yet underexplored function of CDK4/6 is its ability to bind chromatin and regulate transcription. If this function were to be generalized to other CDKs, it would fundamentally alter our interpretation of how cell cycle activity is mediated.[100,101] Thus, it would be fascinating to performing ChIP-sequencing experiments targeting CDK1 and CDK4/6 in both ESCs and the developing mouse cortex. Furthermore, performing RNA-sequencing on CDK knockdown and overexpression experiments in parallel would provide solid evidence of the roles CDKs play in transcriptional regulation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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