

Cell cycle-dependent phosphorylation and regulation of cellular differentiation

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Abstract:

Embryogenesis requires an exquisite regulation of cell proliferation, cell cycle withdrawal and differentiation into a massively diverse range of cells at the correct time and place. Stem cells also remain to varying extents in different adult tissues, acting in tissue homeostasis and repair. Therefore, regulated proliferation and subsequent differentiation of stem and progenitor cells remains pivotal throughout life. Recent advances have characterised the cell cycle dynamics, epigenetics, transcriptome and proteome accompanying the transition from proliferation to differentiation, revealing multiple bidirectional interactions between the cell cycle machinery and factors driving differentiation. Here we focus on a direct mechanistic link involving phosphorylation of differentiation-associated transcription factors by cell cycle-associated Cyclin-dependent kinases. We discuss examples from the three embryonic germ layers to illustrate this regulatory mechanism that co-ordinates the balance between cell proliferation and differentiation.

Abbreviations list:

bHLH, basic-Helix-Loop-Helix; Cdk, Cyclin-dependent-kinase; Cdk_i, Cyclin-dependent-kinase-inhibitor; MRF, Muscle Regulatory Factor; Ngn2, Neurogenin2; Ngn3, Neurogenin3.

Main text:

Introduction:

The miracle of embryo development sees a single fertilised egg generate millions of specialised cells that form multiple complementary organ systems working together. Throughout the life of that organism, stem cells remain to varying extents in different tissues, acting in tissue homeostasis for example in the skin (1), gut (2), or bone marrow (3), or as a reserve for repair after injury in, for example, muscle (4) or liver (5). In contrast, the relative lack of neural stem cells is one factor that makes the nervous system so critically sensitive to damage. Thus, regulated proliferation and subsequent differentiation of stem and progenitor cells remains pivotal throughout life. Additionally, cellular reprogramming by directed differentiation of stem cells is emerging as a powerful tool in disease modelling and with

exciting potential in regenerative medicine (6). Conversely, inappropriate replication is a fundamental hallmark of cancer (7).

Terminal differentiation is usually coupled to exit from the cell cycle, but even early cell fate specification of pluripotent stem cells is tightly coordinated with cell cycle events (8). Indeed, a link between changes in cell cycle length/ dynamics and the onset of fate specification/ differentiation is well documented across pluripotent stem cells and multiple progenitor cell lineages (8-10). Recent advances have characterised the cell cycle dynamics, epigenetics, transcriptome and proteome accompanying this transition during development and in cellular reprogramming. This has uncovered multiple bidirectional and unexpected links between the machinery driving the cell cycle and factors driving differentiation. A full discussion of this is beyond the scope of this mini-review and readers are directed to detailed reviews in (11-13). Instead, we focus our discussion on a conserved and direct mechanistic link that antagonistically coordinates the cell cycle and differentiation through phosphorylation by Cyclin-dependent-kinases (Cdks).

Canonical roles of Cyclin-dependent kinases in the cell cycle:

The eukaryotic cell cycle consists of four sequential phases (Figure 1), with cell growth occurring in the G1 and G2 phases, DNA replication in the intervening S phase, and cell division and cytokinesis in M phase. Cells that pass the restriction point in G1 are committed to completing the cell cycle, but prior to this cells may withdraw to the quiescent G0 phase, or in response to various stresses, cells may alternatively become senescent with a permanent cell cycle arrest. Checkpoints occur during the cell cycle to ensure successful completion of critical events prior to progression; transitions are mediated by specific combinations of Cdks with their respective activating cyclin partners. Complex regulation of the cell cycle components occurs particularly at the levels of transcription, post-translational modification and protein degradation to ensure a unidirectional passage. The overall rate of cell cycle progression largely depends on the relative activity of Cyclins/Cdks driving the cell cycle forward versus Cyclin-dependent-kinase-inhibitors (Cdkis) of the Kip/Cip family (p21, p27, p53) and INK4 family (p15, p16, p18, p19) that inhibit the cell cycle. For reviews see (14, 15).

Traditional functions of Cyclin-Cdks include phosphorylation of a plethora of targets that drive cell cycle progression. However, there is also increasing evidence for non-traditional functions that are both kinase dependent and independent. These include additional roles in regulation of gene transcription, DNA damage repair, cell death, differentiation, and cell metabolism; reviewed in (13). Complementing the traditional kinase-dependent functions in promoting cell cycling, here we discuss Cdk-dependent phosphorylation events that concurrently inhibit differentiation.

Cdk-dependent phosphorylation of transcriptional regulators can directly restrain differentiation:

The large superfamily of basic-Helix-Loop-Helix (bHLH) transcription factors are master regulators of many tissues during development, and the tissue-specific class II bHLH factors have conserved and well-established roles in directing cells out of the cell cycle and towards terminal differentiation in multiple lineages (16-21). These same factors have received significant attention in the field of regenerative medicine for their potent ability to convert fibroblasts and other cells into a range of cell types including neurons, (for example (22)). Phosphorylation represents one of the most rapid and reversible methods to alter transcription factor activity, potentially regulating nuclear localisation (for example NF-AT (23)), rate of proteolytic degradation (for example MyoD (24)) and/or efficiency of binding DNA and cofactors (for example Ets-1 (25) and Atonal (26)); for detailed review see (27). With this in mind, it is perhaps not surprising that there are numerous examples of critical developmental/reprogramming factors that undergo cell-cycle-dependent phosphorylation to influence their ability to drive differentiation. For example, Cdk1/2-mediated phosphorylation of Sox2 is required for optimal suppression of neuronal differentiation in proliferating neural stem cells (28). Further examples are summarised below, drawing from each of the three embryonic germ layers.

The neuro-ectoderm lineage:

Proneural bHLH proteins *Ascl1* and *Neurogenin2* (*Ngn2*) are considered master regulators of neurogenesis during development and reprogramming, with conserved functions from *Drosophila* to humans. By initiating cascades of downstream factors they coordinate

diverse processes such as cell cycle exit, neuronal commitment and subsequent differentiation and maturation (17). Expression of *Ascl1* and *Ngn2* is first detected in cycling progenitor cells and they additionally have a non-cell-autonomous role to promote progenitor maintenance in neighbouring cells via activation of Notch signalling (17). This model of Notch-mediated lateral inhibition is described from invertebrates to mammals, and has recently being adapted to reflect a more dynamic and oscillatory interaction between the proneurals and Notch component *Hes1* in progenitor cells, with sustained expression of proneurals and repression of *Hes1* accompanying differentiation (29). Furthermore, *Ascl1* has been shown to have cell-autonomous functions to directly promote proliferation in cycling progenitors through expression of key cell cycle components such as *E2F1*, *Skp2* and *Cdk2*. Global transcriptional profiles have identified distinct subsets of *Ascl1* target genes that are temporally regulated, with a second set of anti-proliferative targets becoming expressed after prolonged *Ascl1* expression (30).

Early work in *Xenopus* frog embryos characterised differential epigenetic availability of the promoters of *Ngn2* target genes. Proliferation-associated target genes such as Notch ligand *Delta* respond rapidly to lower levels of *Ngn2*; by comparison, differentiation-associated targets such as *NeuroD1* require higher levels of *Ngn2* for activation and have a greater dependence on histone acetyltransferase activity (31). It seems likely that more accessible chromatin around promoters and/or enhancers associated with readily transcribed proliferation targets need little or no proneural-induced chromatin remodelling to remain active. In contrast, *de novo* proneural-driven activation of genes associated with the transition to differentiation requires epigenetic remodelling that may be brought about by proneural factor recruitment of epigenetic remodellers. *Ngn2*, for instance, is able to associate with both p300/CBP histone acetyl transferases and SWI/SNF components, which can radically change chromatin accessibility and activation. Chromatin remodelling of promoters and/or enhancers by co-factor recruitment may necessitate more prolonged proneural protein association than that needed for gene activation at more accessible chromatin regions (32).

Several mechanisms may therefore underlie the switch between proliferation and differentiation modes for the proneural transcription factors. For example, progenitor-associated targets of *Ascl1* are enriched for CBF1/RBPj motifs, suggesting that *Ascl1* binding events may be regulated by components of the Notch signalling pathway (30). Alternatively, there may be changes in proneural protein regulation such as a switch in expression dynamics

that accompany the transition to differentiation (29), or a change in proneural protein structure/activity via post-translational modification (see below).

An intriguing and convenient method to coordinate the cell cycle and differentiation would be through the direct regulation of bHLH transcription factors by cdk-dependent phosphorylation. Single regulatory phospho-sites are known to fine-tune proneural activity in a variety of contexts, for example in Ngn2-directed motor neuron specification downstream of GSK3 β (33). However, proneural proteins such as Ngn2 and Ascl1 have also been shown to be quantitatively sensitive to cdk activity via a multi-site phosphorylation model, whereby the level of proneural activity is directly regulated by the level of kinase activity and therefore the number of sites that are phosphorylated (34, 35). Ngn2 is phosphorylated on up to nine serine-proline sites by Cdk1/2 (34). This multi-site phosphorylation limits the ability of Ngn2 to drive expression of differentiation-associated targets such as NeuroD1, while phosphorylation has little effect on expression of proliferation-associated genes such as Delta (34, 36). The extent of Ngn2 phosphorylation parallels the exposure to cdk activity and negatively correlates with DNA binding and transcriptional output from the NeuroD1 promoter (34). Experimentally preventing phosphorylation with serine to alanine substitutions in a phospho-mutant Ngn2 promotes neuronal differentiation through increased protein stability and enhanced DNA binding affinity (34, 36). Both of these effects may contribute to a prolonged promoter/enhancer dwell time required for epigenetic remodelling of “closed” promoters at differentiation-associated genes.

Consistent with this multi-site phospho-regulatory model being conserved amongst proneural proteins, a corresponding “rheostat” response, where the number of phosphorylation events at least semi-quantitatively regulates transcriptional activity, is also described for NeuroD4 (37). Furthermore, Ascl1 is phosphorylated on multiple sites by Cdk2, and the respective serine to alanine phospho-mutant Ascl1 has superior ability to drive trans-differentiation of mammalian fibroblast cells to neurons, highlighting the biomedical application of manipulating proneural protein activity (35).

In this way, proneural protein phospho-status may impact on downstream target expression due to different subsets of genes having a different chromatin accessibility; pro-proliferative genes are readily activated while pro-differentiation genes require more extensive epigenetic modification that can only be brought about by un(der)phosphorylated proneural

factors, while further feedbacks may also be involved (Figure 2). The epigenetic barrier to differentiation can be further enforced by Cdk1/2-mediated phosphorylation of EZH2, the catalytic subunit of polycomb repressor complex 2 (PCR2). Phosphorylation at threonine 350 enhances silencing of developmental regulators of the Hox, Fox and Sox family, possibly through enhanced chromatin recruitment via non-coding RNAs (38, 39).

The mesoderm lineage:

Analogous to the proneural proteins in neurogenesis, the formation of functional skeletal muscle is orchestrated through the expression of a conserved family of Muscle Regulatory Factors (MRFs), namely MyoD, Myf5, myogenin and MRF4; all of which are able to induce myogenic conversion when introduced into fibroblasts (21). MyoD and Myf5 are expressed in proliferating myoblasts and MyoD is recognised as a critical component involved in the balance between proliferation and differentiation in these cells (40). MyoD has complex physical and regulatory interactions with cell cycle machinery including Cdk4 and retinoblastoma protein to directly inhibit cell cycle progression. Conversely, Cyclin-D1/Cdk4 can inhibit the myogenic activity of MyoD in a kinase independent manner (41).

Early experiments to explore phospho-regulation of MyoD focused on proliferating myoblasts or fibroblasts transfected with MyoD *in vitro*, demonstrating cyclic fluctuations in MyoD protein driven by Cdk-mediated phosphorylation. Serine 200 is phosphorylated by Cdk2 during late G1 to mediate protein destabilisation prior to S phase, and serines 5 and 200 are phosphorylated by Cyclin-B/Cdk1 in late G2/M to ensure MyoD is released from condensed chromosomes during mitosis (24, 42-45). In other mesodermal lineages, a similar cell-cycle-dependent protein fluctuation is described for Runx2, a bone and cartilage-specific transcription factor expressed in proliferating osteoblasts and chondrocytes. Cyclin-D1/Cdk4 phosphorylates Runx2 on serine 472, promoting ubiquitination and proteasomal degradation that limit Runx2 ability to upregulate the Cdk1 p27Kip1 and thus limiting cell cycle exit and differentiation (46).

MyoD also contains other conserved proline directed kinase sites and these are phosphorylated in an *in vivo* model of myogenesis in *Xenopus* embryos, although specific kinases responsible were not investigated (47). This multi-site phosphorylation model is highly

reminiscent of that described for the proneural proteins, with phospho-status influencing both protein stability and chromatin association. Yet, distinct from the proneural model where the number but not the precise location of available phospho-sites is key, in MyoD, the regulatory sites are confined to the C terminus of the protein and with a substantial role for phosphorylation of serine 200. This may reflect the complexity of transcriptional and epigenetic functions of MyoD that are being revealed by genome wide analysis, but nevertheless demonstrate a mechanistically conserved mode of regulation in both nerve and muscle (47).

In addition to the bHLH Muscle Regulator Factors, skeletal muscle differentiation is promoted by the MEF2 proteins that bind A/T-rich sequences in the regulatory regions of many muscle specific genes. MEF2D is expressed with MyoD in proliferating myoblasts and MEF2C is upregulated downstream of MyoD, modulated by a network of coactivators and repressors. Although the precise mechanism is not clear, Cyclin-D/Cdk4 kinase activity inhibits MEF2C function through altered nuclear subdomain localisation. Thus, this is a second Cdk-dependent mechanism to inhibit myogenic differentiation in parallel to that operating with the muscle regulatory bHLH factors (48).

Taken together, within the mesoderm lineage there is a recurrent theme of Cdk-dependent phosphorylation altering protein stability, and in mesoderm, Cdk4 appears to have a particularly prominent role. Consistent with this, Cyclin-D1/Cdk4 also inhibits differentiation of cardiomyocytes via proteasome-mediated degradation of key transcription factor GATA4 (49).

The endoderm lineage:

While neural and muscle tissue have long been studied for the association between cell cycle and differentiation, investigations in the pancreas have been more recent and generally focus on specification of the endocrine lineage (50-53). Endocrine differentiation is driven by the activity of the bHLH transcription factor Neurogenin3 (Ngn3), and similar to the apparently contrasting roles of Ascl1 in driving both proliferation and differentiation target genes (30), Ngn3 also exhibits this paradox. There is a high degree of heterogeneity in Ngn3 protein expression in endocrine progenitors and it is the level of Ngn3 protein that may be critical for determining the balance between proliferation and differentiation; low levels have been

proposed to maintain progenitor proliferation, while high levels of Ngn3 promote endocrine differentiation, conclusions supported by data from hypomorphic Ngn3 mice (54). Interestingly, the transition between low-to-high Ngn3 states has been linked to variations in cell cycle length and to the activity of Cyclin/Cdks (50-53). In particular, lengthening of the G1 phase of the cell cycle is essential for proper induction of Ngn3 and initiation of endocrine cell differentiation (51). Moreover, as with other master regulators of neurogenesis (Ngn2 and Ascl1) and myogenesis (MyoD), Ngn3 undergoes Cdk-mediated phosphorylation on multiple sites (50). While all pairs of Cyclin/Cdks are capable of phosphorylating Ngn3 in an *in vitro* kinase assay (50), only selective inhibition of Cdk1/2 and not of Cdk4/6 dephosphorylated Ngn3 in a cell line, suggesting that Cdk1/2 might play a more prominent role in Ngn3 phosphorylation (50, 51). This phosphorylation primes Ngn3 for degradation and reduces its ability to drive endocrine differentiation. Consistent with this, a phospho-mutant form of Ngn3 that cannot undergo this mode of regulation drives formation of an increased number of endocrine cells in the pancreas *in vivo* (50), as does the ablation of the ubiquitin ligase Fbxw7, which regulates Ngn3 degradation in a phosphorylation-dependent manner (55).

Combining data defining the cell cycle parameters of endocrine progenitors and their correlation with Ngn3 expression (51, 53, 56), a feed-forward loop of Ngn3 regulation is suggested. In this model, while Cdk-mediated phosphorylation of Ngn3 maintains Ngn3 in an unstable form that promotes pancreatic progenitor proliferation, the accumulation of the underphosphorylated and stabilised forms of Ngn3 begins the process of cell cycle exit and differentiation. Ngn3 activates Cdk inhibitors like Cdkn1a (p21), as well as responding to them by becoming progressively more de-phosphorylated and more stable. Activation of this loop is what marks firm commitment to differentiation. (50-52).

A greater understanding of the regulation of Ngn3 differentiation activity is highly relevant to cellular reprogramming and beta-cell regeneration for diabetes. In support of the feed-forward model, Cdk inhibitors have been successfully used to improve the efficiency of β -cell differentiation from human embryonic stem cells (51), induced pluripotent stem cells (57), mouse embryonic explants (57) and in mouse adult pancreas during injury-mediated regeneration (57). However, the complexities of inter-related factors influencing differentiation have yet to be unravelled. For example, Cdk4 and Cdk2 knockout mice exhibit a decreased number of islets and develop diabetes (58, 59), consistent with the classical Cdk role in promoting cell cycle and β -cell proliferation. However, the primary event upon Cdk2 depletion is impaired β -cell function and may reflect a loss of Cdk2-mediated phosphorylation of Foxo1

that is required to maintain β -cell differentiation (60). Dissecting this complex regulatory network will better inform reprogramming strategies and therapeutic options to restore beta cell function. Phosphorylation of proneural proteins by Cdks in other endodermal tissues such as gut may also regulate the differentiation programme (Philpott, Winton, In Press), although there is little work in this area at present.

Conclusions and perspectives:

Across all three embryonic germ layers there are multiple examples of Cdk-dependent phosphorylation events that directly inhibit differentiation, while Cdks concurrently promote progression through the cell cycle. Phosphorylation may occur on specific regulatory sites of differentiation factors (for example (38, 46)), or it may occur on multiple sites on these factors, where the number rather than location of phosphorylation events is often critical (34, 37). Phosphorylation may be affected by multiple Cyclin/Cdks or by specific Cyclin-Cdk complexes, and specificity for distinct Cyclin/Cdks may in part underlie the importance of specific cell cycle phases for commitment to different cell lineages.

While the effects of phosphorylation vary depending on the target protein, certain common themes emerge, such as regulation of protein stability through ubiquitin-mediated degradation and regulation of DNA binding affinity. It is possible that single regulatory phospho-sites mediate context-dependent functions while multi-site phosphorylation provides a more general rheostat mechanism for regulating temporal selection of target genes. Many of these “differentiation” factors have additional cell- and non-cell-autonomous roles in promoting progenitor maintenance, while the different subsets of target genes activated require different levels of epigenetic remodelling prior to activation (31). As such, differentiation-associated genes are highly sensitive to the level of transcription factor protein and its chromatin affinity, both of which can be reduced by Cdk-dependent phosphorylation (12).

Embryogenesis requires exquisite regulation of progenitor cell proliferation, cell cycle withdrawal and differentiation to allow generation of a massively diverse range of cells at the correct time and place. Understanding the protein networks and mechanisms that orchestrate these complex developmental programmes is also highly relevant beyond the field of developmental biology. For example, certain types of cancers, particularly childhood cancers such as neuroblastoma, are now being considered as disorders of differentiation, and

understanding the derangements in these tumours could open new therapeutic options (61). Additionally, rapid advances are being made in the field of cellular reprogramming for regenerative medicine, providing new human-derived and patient specific *in vitro* models for disease modelling, drug screening and potential cell replacement therapies. Moreover, lineage specific differentiation factors such as bHLH proteins are critical components of reprogramming protocols and manipulation of their activity to enhance differentiation has been successfully used to improve conversion efficiency and cellular maturity (35). Thus, a greater understanding of the mechanisms that inter-link cell proliferation and differentiation during development can have far-reaching implications in many fields of biology and medicine.

Declarations of interest:

The authors declare no competing financial interests.

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Author contribution statement:

RA wrote the endoderm section and prepared Figure 2, while LH wrote the rest of the manuscript and prepared Figure 1. AP reviewed, discussed and edited the manuscript.

Figure titles and legends:

Figure 1: The eukaryotic cell cycle.

The cell cycle consists of sequential phases with unidirectional passage and check-points to ensure successful completion of a phase before the next transition. Progression through the cycle is driven by specific combinations of Cyclin-dependent-kinases along with their activating Cyclin partners, shown adjacent to their approximate position in the cycle. Two families of Cyclin-dependent-kinase inhibitors act as braking mechanisms; members of the INK4 family inhibit Cdk4/6 in G1 phase and members of the Cip/Kip family have more widespread activity throughout the cell cycle.

Figure 2: Model illustrating the potential consequences of multi-site phosphorylation on bHLH transcription factor activation during the transition from proliferation to differentiation.

When the cell cycle is active, phosphorylated bHLH proteins have rapid protein degradation and weaker DNA binding that is sufficient to activate only proliferation-association target genes with open chromatin at the promoter/enhancer. As the cell cycle lengthens and slows, Cyclin-Cdk activity declines and the bHLH proteins become progressively de-phosphorylated. The un(der)phosphorylated bHLH transcription factors have increased stability and enhanced chromatin binding that is required to bring about necessary epigenetic changes for activation of differentiation genes.

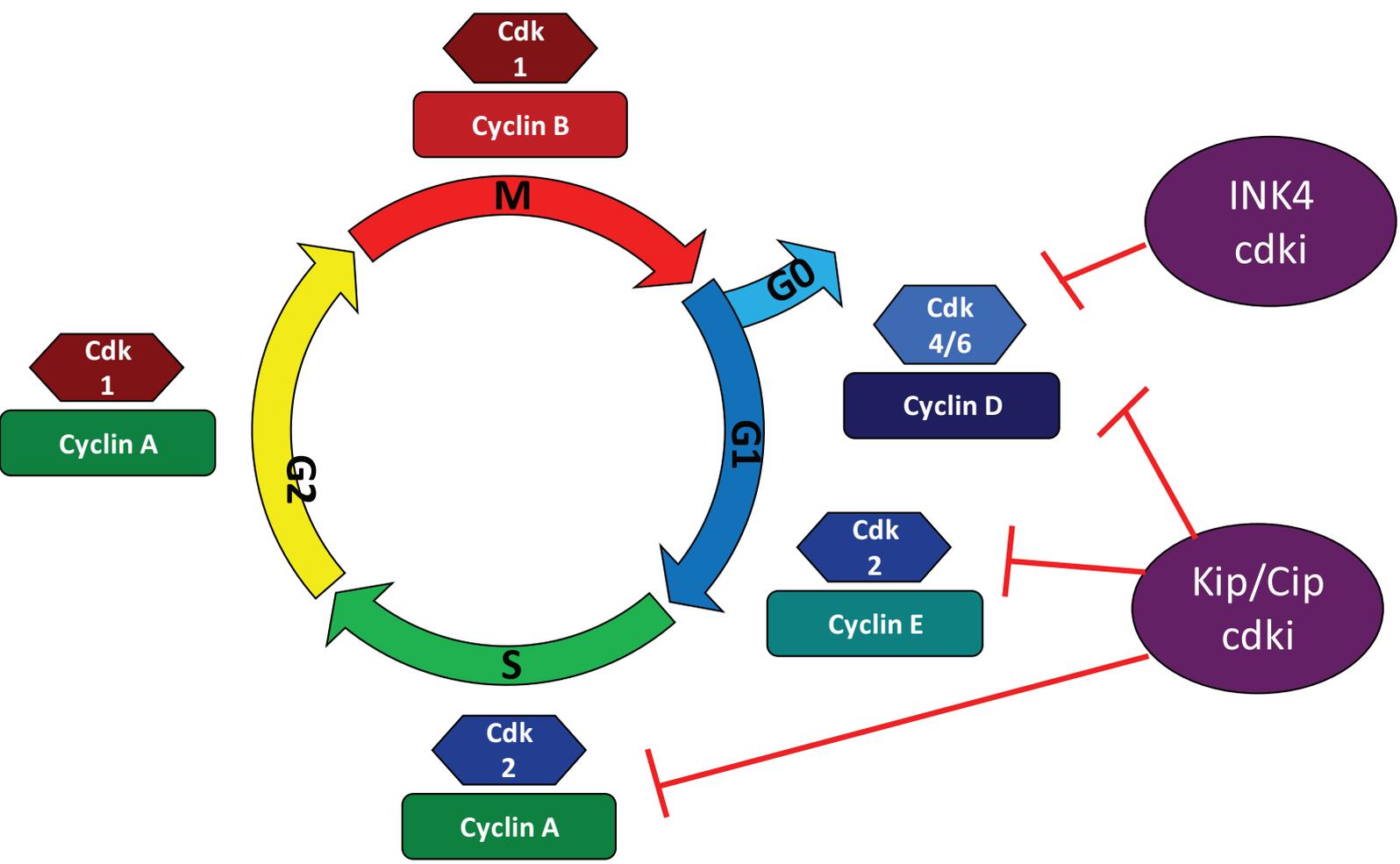
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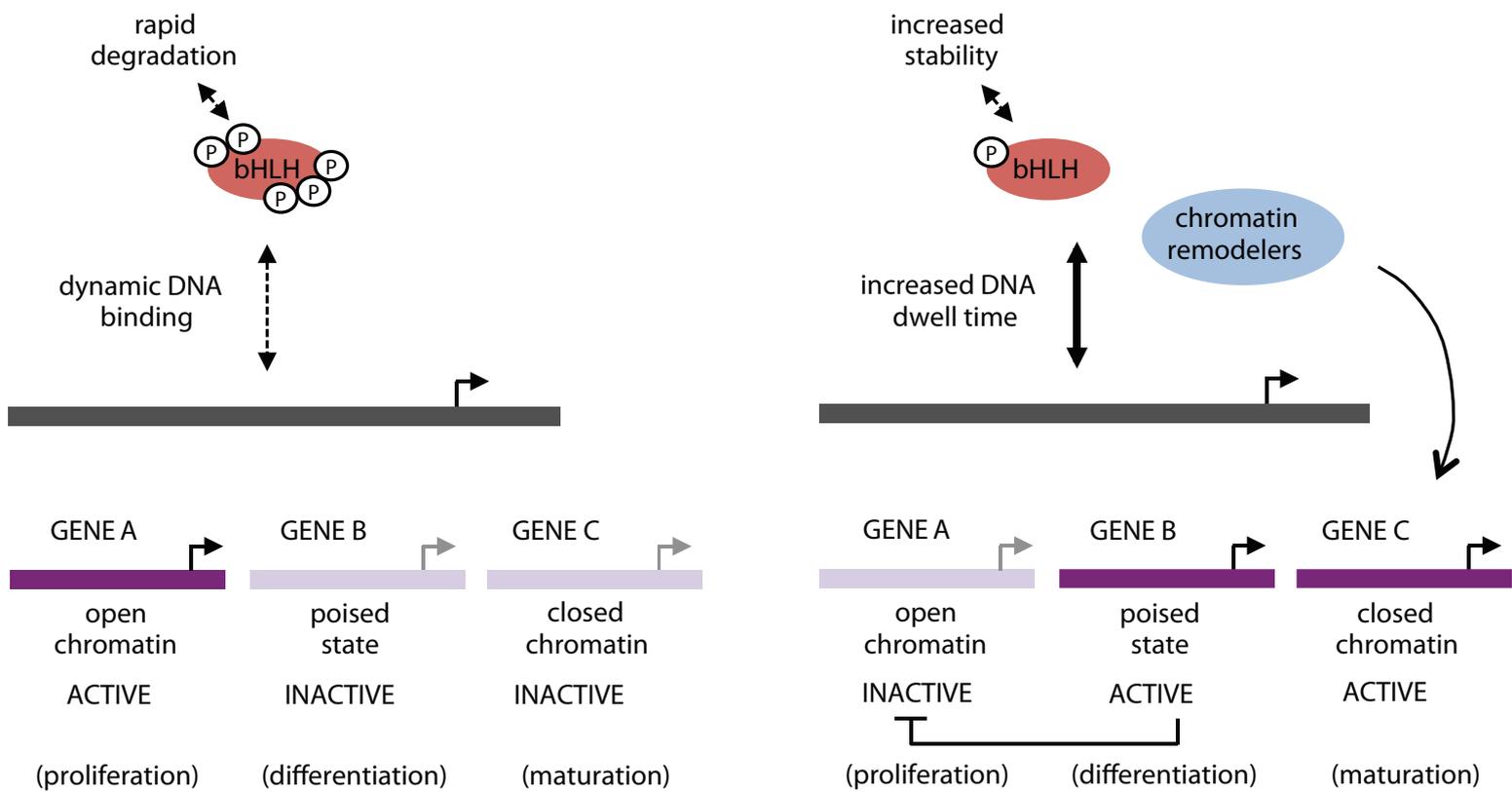
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CDK kinase activity

Cdk inhibitor levels

Developmental time

