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REVIEW ARTICLE

Emerging roles of metazoan cell cycle regulators as coordinators of the cell cycle and differentiation

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In multicellular organisms, cell proliferation must be tightly coordinated with other developmental processes to form functional tissues and organs. Despite significant advances in our understanding of how the cell cycle is controlled by conserved cell-cycle regulators (CCRs), how the cell cycle is coordinated with cell differentiation in metazoan organisms and how CCRs contribute to this process remain poorly understood. Here, we review the emerging roles of metazoan CCRs as intracellular proliferation-differentiation coordinators in multicellular organisms. We illustrate how major CCRs regulate cellular events that are required for cell fate acquisition and subsequent differentiation. To this end, CCRs employ diverse mechanisms, some of which are separable from those underpinning the conventional cell-cycle-regulatory functions of CCRs. By controlling cell-type-specific specification/differentiation processes alongside the progression of the cell cycle, CCRs enable spatiotemporal coupling between differentiation and cell proliferation in various developmental contexts *in vivo*. We discuss the significance and implications of this underappreciated role of metazoan CCRs for development, disease and evolution.

Keywords: APC/C; CDKs; cell cycle; cell fate; CKIs; coordination; development; differentiation; progenitor cells; stem cell

Growth and differentiation are the two major processes in the development of multicellular organisms. Through growth, the organism augments its body mass, mainly by increasing cell number by passage through the cell cycle. Through differentiation, the organism generates a variety of cell types with distinct forms and functions that make up tissues and organs. For the proper development and homeostasis of metazoan organisms, these two processes must be tightly coordinated in time and space.

Through decades of intense research on the molecular mechanisms that control the cell cycle, the complete set of proteins that regulate key cell-cycle events has been identified [1] (Fig. 1). Remarkably, these proteins, which are commonly known as 'cell-cycle regulators' (CCRs), are found to be structurally and functionally conserved from simple unicellular organisms, such as yeast, to complex multicellular organisms, such as humans. As CCRs ultimately dictate cell proliferation by controlling the cell cycle, understanding how CCRs function and are regulated *in vivo* is key to deciphering the mechanisms that ensure the crucial coordination between cell proliferation and differentiation in metazoans.

Investigations using various multicellular models have confirmed the critical importance of CCRs for

Abbreviations
APC/C, anaphase-promoting complex/cyclosome; Ark, Aurora-related kinase; CCR, cell-cycle regulator; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; Plk, Polo-like kinase; SCF, Skp1-cullin1-F-box; TF, transcription factor.

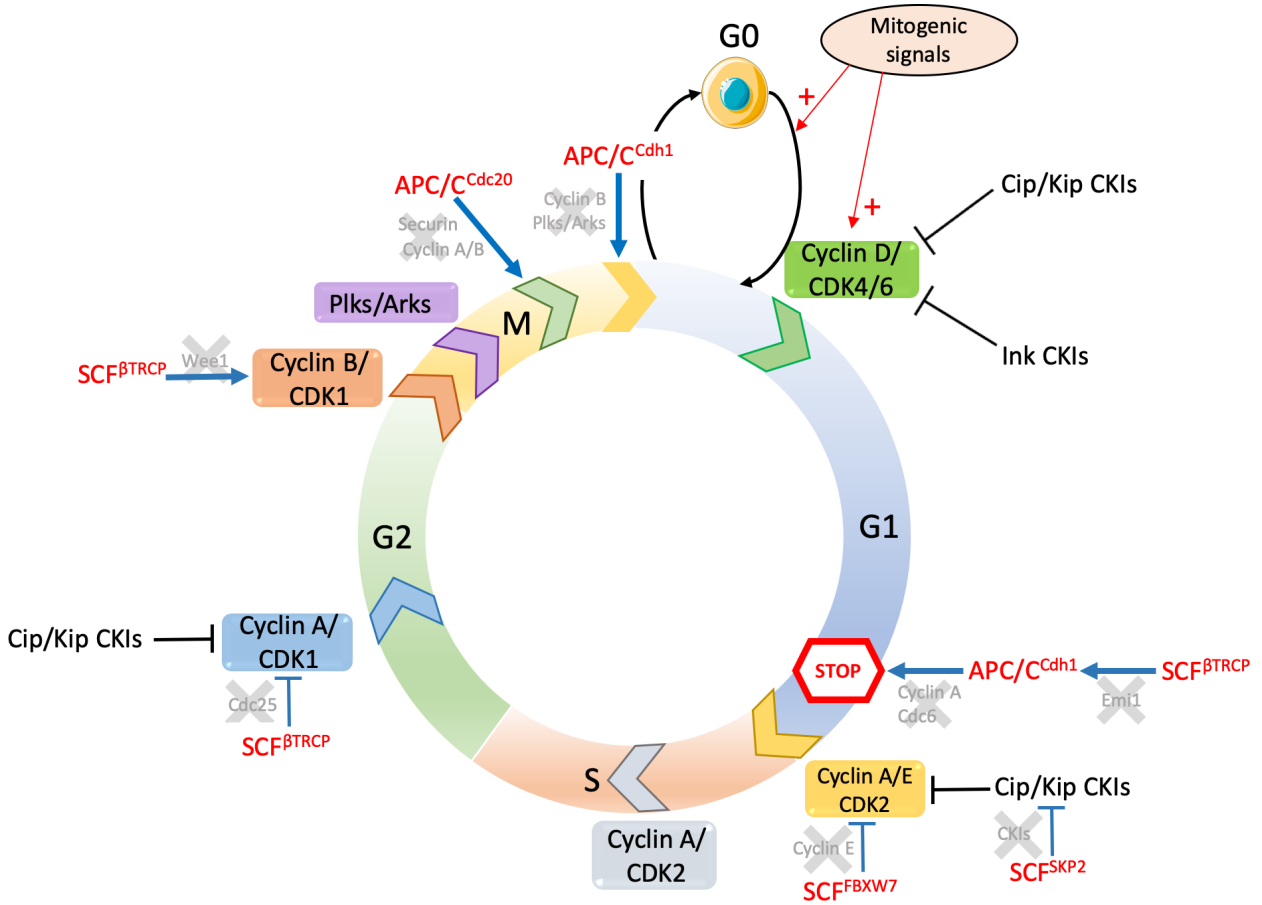

Fig. 1. The functions of the major CCRs in cell-cycle regulation. Cell-cycle progression is tightly controlled by a set of proteins that can either promote (cell-cycle engines) or inhibit (cell-cycle brakes) this process. Among the cell-cycle engines, the CDKs are known to phosphorylate a large number of proteins during specific windows of the cell cycle to promote its progression. CDK activity depends on their association with a cyclin, which accumulates during specific phases of the cell cycle. Thus, upon G1 entry, CDK4/6 associates with Cyclin D to control G1 progression. Next, Cyclin E begins to accumulate from the middle of G1 phase and interacts with CDK2 to promote the G1-to-S transition and DNA replication. Correct progression through S phase is ensured by the Cyclin A/CDK2 complex then, during G2, Cyclin A associates with CDK1 until the transition to M phase. Finally, progression through mitosis is promoted by the Cyclin B/CDK1 complex, and the mitotic kinases, Plks and Aurora A/B (Arks). The kinase activity of CDKs can be inhibited by two groups of CKIs: the Cip/Kip CKIs inhibiting several CDKs and the Ink CKIs inhibiting CDK4/6. CKIs ensure that cells do not enter any cell-cycle phases prematurely or with DNA damage. Although CKIs seem to be dispensable for normal cell-cycle progression, they are essential during development. The fourth group of CCRs is the one formed by the ubiquitin ligase complexes. Several ubiquitin ligase complexes are required to induce the degradation of CCRs during specific windows of the cell cycle, acting as either engines or brakes according to the protein targeted for degradation. On one hand, the APC/C complex associates with its co-activator Cdc20 to direct several mitotic events, including chromosome segregation and mitotic exit through Securin and mitotic Cyclin degradation, respectively. Then, APC/C binds Cdh1 from late M to the end of G1 phase to regulate G0 and G1 lengths. On the other hand, the SCF ubiquitin ligase associates with several regulatory subunits to ubiquitinate critical CCRs from the G1-to-S transition to the beginning of mitosis, including Cyclin E, the phosphatase Cdc25 and Cip/Kip CKIs. Blue arrows indicate proteasomal degradation. Red arrows indicate activation.

the control of cell proliferation *in vivo*. During development as well as in adulthood, CCRs stimulate cell proliferation for tissue growth while terminating it upon the induction of differentiation and cell quiescence. The functions of CCRs are also crucial for setting cell division rates by modifying the length of each cell-cycle phase, and for switching cell-cycle modes, for example from the mitotic cycle to meiosis or the endoreplication cycle (also called the endocycle). Importantly, several CCRs have been identified as direct targets of various transcription factors (TFs) or signalling pathways that play pivotal roles in the regulation of tissue development and homeostasis of metazoan organisms. Transcriptional regulation of CCRs

enables the integration of cell-cycle control into the developmental programme and hence contributes to the coordination between cell proliferation and differentiation in metazoans [2].

However, accumulating evidence points towards an additional mechanism that also contributes to coordinating these two processes, which involves unanticipated metazoan-specific roles for CCRs. In addition to cell proliferation defects and over-proliferation phenotypes, animals lacking normal CCR functions exhibited a wide variety of tissue-specific phenotypes that appeared unrelated to misregulations of the cell cycle. Analyses of these phenotypes have led to the discovery of novel functions of CCRs in the regulation of fate specification and differentiation of various types of metazoan cells. Hereafter, we refer to the roles of CCRs in non-cell-cycle events such as the regulation of developmental TFs and cell signalling, cellular specification/differentiation and asymmetric cell division, as 'non-canonical' functions of CCRs. Of note, these functions are not thoroughly understood and most of them are not shared by homologous CCRs in unicellular organisms. Combining some of these non-canonical functions with their conserved cell-cycle-related functions, metazoan CCRs spatiotemporally couple cell-type-specific specification/differentiation processes to cell-cycle events, thereby acting as the intracellular coordinators between cell proliferation and differentiation. Despite its potential significance for metazoan biology, this emerging role of metazoan CCRs has not yet received due recognition.

Here, we review the current knowledge of the roles of metazoan CCRs as the intracellular proliferation-differentiation coordinators, featuring their non-canonical functions in the control of cellular specification/differentiation. Due to space constraints, we focus on four groups of CCRs: cyclin-dependent kinases (CDKs), mitotic kinases, CDK inhibitors (CKIs) and ubiquitin ligases anaphase-promoting complex/cyclosome (APC/C) and Skp1-Cullin1-F-box (SCF), which constitute the 'core' cell-cycle machinery (Fig. 1). Nonetheless, most of the concepts discussed herein are likely applicable to other CCRs. We discuss only those coordination mechanisms in which CCRs regulate cell-type-specific differentiation processes and the cell-cycle progression by employing both their non-canonical and cell-cycle-regulating functions in the same cell population. We do not mention any mechanisms in which CCRs affect differentiation through their cell-cycle functions, for example by changing the length of a certain cell-cycle phase or by converting cell-cycle modes. Similarly, the role of cell death and senescence, which may also be induced by some CCRs, in cellular differentiation is not discussed either, although these events take significant part in development and tissue homeostasis [3–5]. Readers interested in these topics should refer to other reviews, including the two reviews in this special issue [6,7,8].

Cell-cycle engines

Protein phosphorylation is the main modification involved in the control of the eukaryotic cell cycle. The conserved family of serine-threonine kinases CDKs, bound to regulatory subunit cyclins, phosphorylates a large number of proteins to modify their activities, stabilities and interactions during specific windows of the cell cycle. Through these phosphorylation events, CDKs drive cell-cycle progression and, therefore, are often referred to as 'cell-cycle engines' [1] (Fig. 1). Another set of kinases commonly called mitotic kinases, such as Polo-like kinases (Plks) and Aurora-related kinases (Arks), also cooperates with CDKs preferentially during mitosis and regulates various mitotic events to ensure accurate chromosome segregation and cell division (Fig. 1).

The active roles of CDKs in cellular processes beyond cell-cycle control have been widely acknowledged. In particular, CDKs are known to play a crucial role in the regulation of general transcription. CDK1 phosphorylates components of the general transcription complex globally to suppress transcription during mitosis [9]. Moreover, some CDK family members, such as CDK7 and CDK9, function exclusively in transcription control [10]. However, besides these roles in the control of general transcription, accumulating evidence suggests that metazoan CDKs, as well as mitotic kinases, also regulate cell-type-specific transcription, thereby impacting cell fate specification and differentiation in various metazoan cells. In addition, recent studies have also indicated various post-transcriptional mechanisms by which these cell-cycle kinases influence cell fate choice upon the induction of differentiation. Notably, some of these functions do not even require the enzymatic activity of these kinases, unlike their functions in cell-cycle regulation.

Below, we will first introduce evidence for the regulations of cell-type-specific specification or differentiation events by non-canonical functions of the main cell-cycle-regulating CDK members, CDK1, CDK2 and CDK4/6, and two mitotic kinases, Plk1 and Aurora A, and discuss how these cell-cycle engines combine these functions and the positive regulation of the cell cycle to couple cell specification/differentiation processes to cell division.

CDK1

CDK1 (also known as CDC2) is undoubtedly the most well-known CCR, which binds A-type and B-type cyclins and phosphorylates numerous proteins, including other CCRs, during the cell cycle (Fig. 1). Importantly, CDK1 is the sole CDK member whose function is absolutely essential for the proliferation of all eukaryotic cells ever examined [11]. CDK1 activity is normally rate-limiting and required for early mitotic events, including mitotic entry (the G2-to-M transition). However, in the absence of other CDKs, CDK1 can compensate for the loss of their activities and also drive the G1-to-S phase transition [11,12].

The first evidence of the role of CDK1 in cell differentiation came from a study on the *Drosophila* CDK1 mutant allele $cdc2^{E51Q}$ [13]. In wild-type *Drosophila* neuroblasts (NBs), a number of proteins, including a cell fate determinant Numb, localise asymmetrically, accumulating on the apical cortex during mitosis [14]. However, in $cdc2^{E51Q}$ mutant NBs, these proteins are distributed uniformly throughout the cell cortex during mitosis [13]. In addition, these NBs cannot align the mitotic spindle with the apicobasal axis, unlike the wild-type [13]. As these mutant NBs are able to complete cell division, this observation suggests that CDK1 regulates cell fate determinant localisation and spindle orientation in asymmetrically dividing NBs, independently of its cell-cycle function.

In line with this finding, subsequent studies in the early-stage *Caenorhabditis elegans* embryo also uncovered multiple functions of CDK1 in the regulation of asymmetric cell division. In four-cell-stage embryos, the endomesoderm (EMS) blastomere divides asymmetrically along the anterior-posterior axis, generating two daughter cells that are destined for different cell fates [15]. When the EMS enters mitosis, WRM-1, a worm β-catenin homologue, accumulates specifically on the anterior domain of the cell cortex and orients the mitotic spindle along the anterior-posterior axis [15]. It was shown that CDK1 directly phosphorylates WRM-1 and promotes its dissociation from the posterior cortex, which allows the accumulation of WRM-1 on the apical cortex [15]. CDK1 also regulates the asymmetric distribution of cell fate determinants in the earlier one-cell-stage worm embryo by inducing the degradation of OMA-1 [16,17]. OMA-1 is a zinc finger RNA-binding protein that is necessary for oocyte maturation and needs to be degraded during the first mitosis of the zygote to allow the asymmetric cytoplasmic accumulation of cell fate determinants including PIE-1 [17]. It was shown that hypomorph CDK1 mutations or depletion of Cyclin B3 stabilises OMA-1 and causes the transformation of the C blastomere, which produces skin and muscle, to the EMS blastomere, resulting in the formation of extraintestinal tissue [16]. Moreover, the precise temporal control of CDK1 activity is also critical for the successful completion of asymmetric division of the *C. elegans* embryo. It was shown that the CDK1 inactivation during late mitosis is required for displacement of the mitotic spindle towards one side of the cortex, to produce differently sized daughter cells after cytokinesis [18]. Importantly, during asymmetric division of the mouse oocyte, the CDK1 inactivation during anaphase is required for the spindle movement towards the cortex; in addition, CDK1 inactivation also induces F-actin polymerisation in the cytoplasm of the oocyte, which is required for spindle protrusion into the plasma membrane and the subsequent formation of the polar body [19]. These results strongly suggest that CDK1 also regulates asymmetric division of the oocyte in mammals.

These observations underscore the pivotal role of metazoan CDK1 in asymmetric cell division: CDK1 promotes various subcellular events that are specifically required for the asymmetric division of each cell type while driving mitotic progression, thereby coordinating cell fate specification and cell division in these cells (Fig. 2). In all the examples above, the mutations or the inactivation of CDK1 appears not to block mitotic progression, suggesting that the functions of CDK1 to regulate the asymmetric division-specific processes are functionally independent of its cell-cycle function [13,15,16]. However, molecular mechanisms underlying these functions of CDK1 in asymmetric division, including its targets, remain largely unknown, and data for the differentiation function of CDK1 are still scarce, particularly in vertebrates. This lack of evidence may be due to the technical difficulties inevitably associated with studying the function of a gene essential for cell viability, such as CDK1. Alternatively, compared with other CCRs, CDK1 may be highly specialised in cell-cycle control because of its absolute requirement for cell proliferation.

Several reports point to a role of CDK1 in the regulation of cell differentiation through epigenetic control in human cells. It was shown that CDK1 directly phosphorylates EZH2 methyltransferase to inhibit its enzymatic activity as well as its assembly into the Polycomb-Repressive Complex 2 and that in an *in vitro* osteogenesis model, CDK1 promotes the differentiation of mesenchymal stem cells into osteogenic lineages concurrently with the derepression of EZH2 target genes [20]. However, there are controversies in the CDK1 phosphorylation sites of EZH2 as well as the effects of CDK1-dependent phosphorylation on

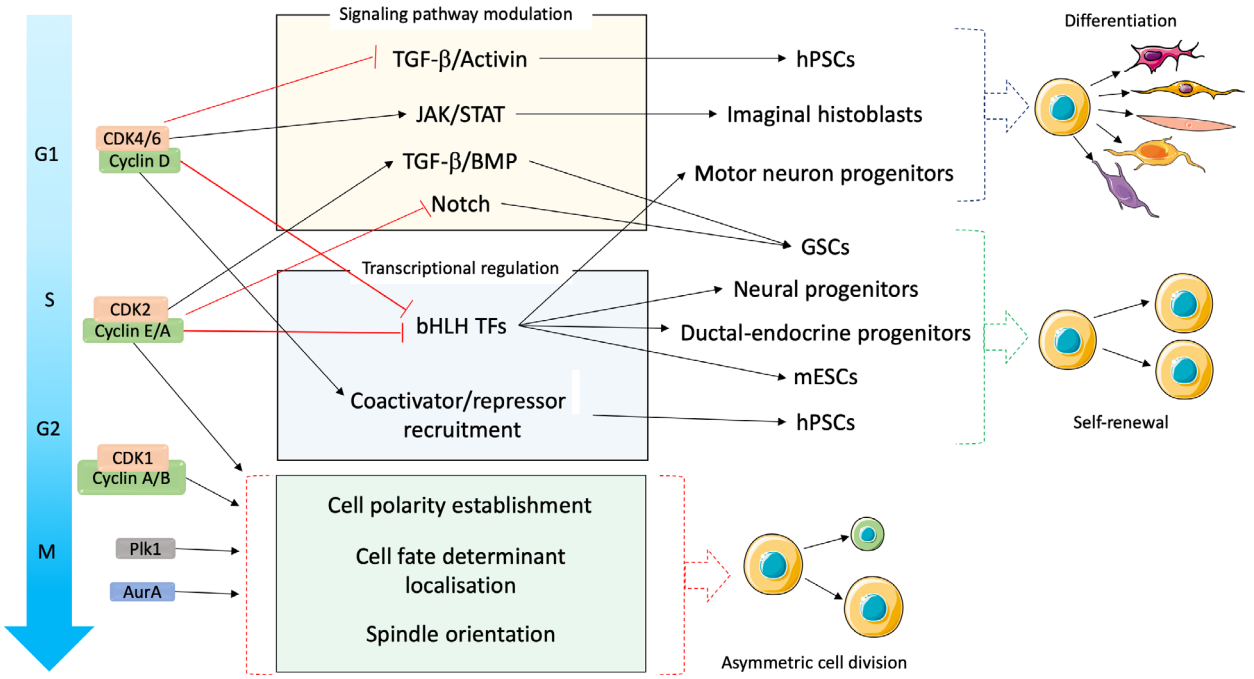

Fig. 2. The roles of cell-cycle engines in coupling the regulation of cell fate determination and cellular differentiation with cell-cycle progression. Through transcription-regulating functions, CDKs can impact the differentiation of various cell types both positively and negatively. The G1 complex Cyclin D/CDK4-6 inhibits myoblast, granulocyte and endoderm differentiation but promotes retinal and neuroectoderm differentiation. Although CDK2 associated with either Cyclin E or Cyclin A mainly acts as an inhibitor of cell differentiation, it is a positive regulator of asymmetric cell division by regulating the localization of cell fate determinants. A few reports point towards the positive role of CDK1 in osteogenic differentiation. So far, CDK1 has been shown to promote asymmetric cell division through the regulation of both cell fate determinant localisation and spindle orientation. Finally, the mitotic kinases Aurora A (AurA) and Plk1 impact cell fate mainly through the regulation of asymmetric cell division and the control of the mitotic spindle orientation by phosphorylating various target proteins.

EZH2 activity [20–22]. Clarifying the role of CDK1-dependent phosphorylation of EZH2 functions *in vivo* may provide additional evidence for the role of CDK1 in the proliferation-differentiation coordination in vertebrates.

CDK2

CDK2 and its partner cyclins, Cyclin E and Cyclin A, are expressed in virtually all proliferating cells; in the late G1 phase, Cyclin E and Cyclin A accumulate in the cell and activate CDK2 to initiate DNA replication [1,23] (Fig. 1). It has been shown that CDK2 activity is rate-limiting for the G1/S transition in mouse embryonic fibroblasts (MEFs) [24–26]. CDK2 is also essential for *Drosophila* embryogenesis [27], supporting the essential role of CDK2 for cell proliferation in metazoans. However, surprisingly, in mice CDK2 and Cyclin E are largely dispensable for embryogenesis and the proliferation of many cell types [24–26]. This is owing to the compensatory functions of other CDKs and cyclins [11,28,29], exemplifying the high degree of functional redundancy between different forms of CDK complexes in mammals.

Besides the roles in cell proliferation control, CDK2-Cyclin A/E complexes have been found to possess the additional molecular function to regulate gene expression. CDK2 physically interacts with various transcription regulators and modulates their transcriptional activities through phosphorylation [30-41]. Studies in *in vivo* and *in vitro* differentiation systems have shown that, by regulating cell-type-specific TFs through phosphorylation, CDK2 impacts the differentiation of various progenitor cells. In vertebrates, CDK2 inhibits the differentiation of neural progenitors by regulating the proneural basic helix-loop-helix (bHLH)-type TF Neurogenin2 (Ngn2); *Xenopus* CDK2 (possibly also CDK1) directly phosphorylates Ngn2 and inhibits its DNA-binding activity, and hence its capacity to induce proneural genes [42]. Similarly, recent studies demonstrated that CDK2 also phosphorylates the proendocrine bHLH TF Neurogenin3

(Ngn3) on multiple sites, which destabilises Ngn3 and suppresses its transcriptional activity [43,44]. In mouse and human pancreas development models, an unphosphorylatable mutant of Ngn3 promotes the differentiation of ductal-endocrine progenitors into the endocrine lineage more robustly than the wild-type Ngn3 protein [43]. These data underpin the key roles of CDK2 in promoting self-renewal of different types of progenitor cells by suppressing the transcriptional activities of lineage-specific bHLH TFs while promoting the G1/S transition in these progenitors (Fig. 2).

Besides the regulation of transcription regulators, CDK2 has been shown in invertebrates to impact cell fate choice through two additional types of regulation. First, like CDK1, CDK2 also promotes asymmetric cell division (Fig. 2). In *Drosophila* embryos, CDK2-Cyclin E is required for the asymmetric division of the thoracic NB, NB6-4t [45]. A mutation in *cyclin E* causes a conversion of the division mode of NB6-4t from asymmetric to symmetric, resulting in the formation of two glial precursors instead of one neuron and one glia; conversely, overexpression of Cyclin E in the abdominal NB NB6-4a, which normally divides symmetrically, transforms its division from symmetric to asymmetric [45]. CDK2-Cyclin E also promotes asymmetric cell division of *C. elegans* one-cell embryo. Prior to the first mitotic division of this embryo, centrosomes migrate towards the posterior cortex of the embryo, where they provide a local signal to the cortex to initiate the polarisation process [46]. It was shown that CDK2-Cyclin E accumulates at the migrating centrosomes and recruits SPD-2 and SPD-5 proteins, which is necessary for the centrosomes to induce the cortical polarisation [46]. Depletion of CDK2 or Cyclin E causes the loss of the cortical polarity, resulting in defective asymmetric division of the embryo [46]. Although these studies did not identify the direct phosphorylation targets of Cdk2-Cyclin E, the mutant NBs and the embryos with CDK2 or Cyclin E depletion were able to initiate DNA replication and complete cell division [45,46]. Thus, given the requirement of Cdk2 activity for the division of these cells, Cdk2-Cyclin E is likely to promote asymmetric division through the mechanisms that are independent of its cell-cycle functions.

The second type of regulation by which CDK2-Cyclin E impacts differentiation is through modulation of cell responsiveness to extracellular signals (Fig. 2). It was shown that *Drosophila* CDK2-Cyclin E promotes the maintenance of female germline stem cells (fGSCs) by stimulating decapentaplegic (Dpp) signalling *Drosophila* tumour necrosis factor [TGF]-β/BMP signalling) in the fGSCs [47]. The fGSC clones carrying *cdk2* or *cyclin E* mutations become arrested in the G1 phase, but also frequently undergo premature differentiation, due to their reduced response to Dpp ligands secreted from the somatic cells in the stem niche [47]. *C. elegans* CDK2-Cyclin E also regulates GSC maintenance through the modulation of another extracellular signalling pathway, the Notch pathway [48,49]. It is known that, in the *C. elegans* gonad, Notch ligands presented by the distal tip cells promote the proliferation of GSCs and prevent their premature differentiation [50–52]. As GSCs move away from the distal tip, Notch signalling is attenuated due to the translational repression of the Notch receptor by the GLD-1 protein [53]. It was shown that Cyclin E is highly expressed in the GSC population near the distal tip and promotes GLD-1 degradation, thereby maintaining high responsiveness of the GSCs to Notch ligands for their self-renewal [48,49]. Interestingly, once accumulated in GSCs, GLD-1 also represses Cyclin E translation, thereby inducing the robust commitment of the GSCs to the meiotic programme through a positive feedback loop [54]. These results strongly suggest that CDK2-Cyclin E promotes stem cell self-renewal by increasing cell responses to various extracellular signals (Fig. 2). However, these studies did not examine the effects of a cell-cycle arrest on GSC differentiation. Thus, the possibility that CDK2-Cyclin E may promote self-renewal of the GSCs through acceleration of the cell cycle cannot be ruled out.

In essence, while promoting cell proliferation through the induction of DNA replication, metazoan CDK2 complexes positively and negatively regulate cell differentiation through multiple modes of regulation, such as regulation of TFs, asymmetric cell division and regulation of signal responses, in different cell types, including neural progenitors, GSCs and early-stage embryos (Fig. 2). Thus, metazoan CDK2 complexes may function to couple the choice between symmetric or asymmetric cell division to the cell's commitment to a new round of the cell cycle. Further studies are needed to uncover molecular details of the cell-type-specific functions of CDK2 complexes and to assess the importance of temporal coupling between cell specification events and S phase progression.

CDK4/6

Unlike CDK1 and CDK2 complexes, CDK4/6-Cyclin D is a metazoan-specific CDK complex. Early studies in cultured mammalian cells established a well-accepted model of the subcellular event leading to the entry into a new round of the cell cycle [55]. In this

model, CDK4/6-Cyclin D is assigned to the central role in initiating this process; during the early G1 (or late G0) phase, it phosphorylates the retinoblastoma (pRb) transcription repressor, which releases E2F1 TF to induce the expression of genes required for DNA replication and mitosis [55]. However, subsequent studies unexpectedly discovered that CDK4/6-Cyclin D is mostly dispensable for cell proliferation *in vivo* [56–59]. Mice lacking CDK4, CDK6 or Cyclin D only develop defects in a small number of tissues, and MEFs derived from these animals proliferate at a rate comparable to wild-type MEFs, only showing a delay in S phase initiation after serum starvation [56–59]. Thus, CDK4/6-Cyclin D is not universally required for cell-cycle control and rather is likely to be a cell-type-dependent CCR, which regulates cell-cycle progression only in specific cell populations or microenvironments. It is noteworthy that *Drosophila* CDK4-Cyclin D has been proposed to promote cell-cycle progression indirectly, by accelerating cell growth rate [57,60,61]; the growth-promoting activity of CDK4-Cyclin D may also be conserved in the human counterpart [62].

Although the precise role of CDK4/6-Cyclin D in cell-cycle progression remains obscure, a substantial number of reports have demonstrated the profound role of CDK4/6-Cyclin D in gene regulation. It has been shown that CDK4/6-Cyclin D directly phosphorylates various TFs and components of transcriptional coactivator/corepressor complexes to regulate their activities and/or protein stability [37,41,63–65]. Surprisingly, unlike CDK2-Cyclin A/E, some of these transcription-regulating functions of CDK4/6-Cyclin D do not require its kinase activity or even complex formation. For example, CDK4 inhibits androgen-mediated transactivation by competing with the androgen receptor for the binding site on the histone acetyltransferase, p300/CREB binding protein-associated factor (PCAF) [66,67]. The mutant form of Cyclin D1 that cannot bind CDK4/6 represses transcriptional activation by the proneural bHLH TF NeuroD [68], whereas Cyclin D directly binds both the oestrogen receptor and PCAF, and mediates their complex formation to stimulate oestrogen-induced transcription [69]. Furthermore, the studies using a *cyclin D* knock-in mouse demonstrated that Cyclin D, as a monomer, acts as a cell-type-specific transcriptional regulator: it binds gene promoter regions in a tissue-specific manner and modulates developmentally regulated gene expression [70,71].

Further studies of the role of CDK4/6-Cyclin D in various specific cell types have illustrated the ability of CDK4/6-Cyclin D to control the differentiation of these cells through transcription regulation. In an *in vitro* myogenesis model, CDK4-Cyclin D inhibits myogenic differentiation by repressing the transactivation activity of the myogenic bHLH protein MyoD through both phosphorylation-dependent and phosphorylation-independent mechanisms [72–76]. In mouse embryonic stem cells (mESCs), CDK6-Cyclin D cooperates with CDK2-Cyclin E to maintain pluripotency by stabilising the pluripotency factors through phosphorylation; mESCs lacking both Cyclin D and Cyclin E gradually lose pluripotency while proliferating at a normal rate [77]. The critical role of the transcription-regulating functions of CDK4/6-Cyclin D in differentiation has also been confirmed by *in vivo* studies. It was shown that *Drosophila* CDK4 mutant embryos fail to express some segmentation genes such as *even-skipped*, exhibiting a maternal effect phenotype of the loss of abdominal segments, similar to JAK/STAT pathway mutants; CDK4-Cyclin D binds STAT92E and stimulates its transcriptional activity [78]. Also, in the developing chicken spinal cord, Cyclin D1 promotes the differentiation of motor neuron progenitors, which appears to be caused by the upregulation of expression of the pro-neurogenic bHLH TF Hes6 by Cyclin D1, independently of its cell-cycle function [79]. Note that this proneural function of Cyclin D1 stands in contrast to its role in the developing mouse cerebral cortex, where CDK4-Cyclin D is thought to inhibit the differentiation of neural progenitors by accelerating the G1-to-S transition [80], suggesting that CDK4/6-Cyclin D may differentially regulate neural differentiation through either a cell-cycle-dependent or a cell-cycle-independent mechanism, depending on the type of neural progenitors.

This growing body of evidence supports the critical role of CDK4/6-Cyclin D in the regulation of cellular specification/differentiation through transcriptional regulation in metazoan organisms. A key question concerning the non-canonical functions of CDK4/6-Cyclin D is how these functions of CDK4/6-Cyclin D are related to its cell-cycle function, which seems to be also cell-type-dependent: do non-canonical and cell-cycle functions of CDK4/6-Cyclin D coincide in some cells, or do they work totally independently, operating in distinct cell populations? Several pieces of evidence argue that the two types of functions of CDK4/6-Cyclin D indeed cooperate in certain cell populations. It was shown that Cyclin D1 knockout (KO) mice exhibit a decrease in retinal cell number due to the reduced proliferation rate of retinal precursor cells, which indicates that CDK4/6-Cyclin D is rate-limiting for cell-cycle progression in these precursors [59,81]. Importantly, in addition to the cell number reduction, the mutant retina also exhibits defects in cell fate

specification, which are partially restored by the forced activation of Notch signalling [56,59,81]. It was previously shown that in the developing retina, Cyclin D1 accumulates in the upstream regulatory region of the *Notch1* gene to stimulate its expression, independently of CDK4/6 [70]. Thus, the cell-cycle function and the transcription regulation function of CDK4/6-Cyclin D may collaborate in the mouse retinal precursors to coordinate the proliferation and specification of these cells. Recent studies of human pluripotent stem cells (hPSCs) provide another example of cooperation. Triple knockdown of Cyclin D1, D2 and D3 blocked the proliferation of hPSCs, indicative of the essential role of CDK4/6-Cyclin D in cell-cycle progression in hPSCs [82]. It was shown that CDK4/6-Cyclin D also regulates the choice of cell fate upon differentiation induction in hPSCs. The hPSCs differentially responded to the same differentiation-inducing signal in different cell-cycle phases: the hPSCs preferentially committed to the endodermal or mesodermal lineage in the early G1 phase and to the ectodermal lineage in the late G1 phase [82]. CDK4/6-Cyclin D regulates this differential response of hPSCs through two distinct mechanisms: first, CDK4/6-Cyclin D promotes the nuclear export of Smad2 and Smad3 through direct phosphorylation to inhibit TGF-β/Activin signalling, which is inhibits ectoderm differentiation [82]. Second, independently of CDK4/6, Cyclin D1 binds and recruits coactivator/corepressor complexes onto specific genome regions to induce neuroectoderm genes and to repress endoderm genes [83]. Thus, CDK4/6-Cyclin D appears to coordinate G1 phase progression and cellular responsiveness to external signals in hPSCs by combining its cell-cycle and non-cell-cycle functions. However, both in hPSCs and mouse retinal precursors, the importance of the coupling between cell-cycle progression and the transcriptional events remains unclear.

In summary, while promoting cell-cycle progression in a cell-type-dependent manner, CDK4/6-Cyclin D, as a whole complex and as individual components, also regulates cell specification/differentiation through transcription regulation (Fig. 2). Considering that the established cell-cycle function of CDK4/6-Cyclin D is also mediated by transcription control (regulation of a transcription repressor pRb), it is tempting to speculate that, during evolution, CDK4/6-Cyclin D might have initially emerged as a CDK specialised in the regulation of cell-type-specific transcription and might have later extended the transcriptional function to the regulation of the cell cycle or cell growth. In this respect, future studies may aim to decipher molecular mechanisms underlying cell-type-specific functions of CDK4/6-Cyclin D, as well as upstream regulatory mechanisms that orchestrate these cell-type-specific functions. Given that CDK4/6-Cyclin D is the first CCR that has been clinically validated as an anti-cancer drug target [84], a further understanding of its *in vivo* functions is crucial to gain insight into the roles of CCRs in tumour development as well as their potentials as anti-cancer targets.

Mitotic kinases, Plk1 and Aurora A

During the cell cycle, mitotic kinases Plk1 and Arks regulate a variety of mitotic events, including spindle assembly, chromosome segregation and cytokinesis, which involve reorganisation of subcellular structures such as chromosomes, cytoskeletons and organelles [1] (Fig. 1). Mitotic kinases orchestrate these events through protein phosphorylation, functioning in parallel with, or downstream of, CDKs [1], often by phosphorylating additional sites on the proteins that have been phosphorylated ('primed') by CDKs [85]. In addition to these cell-cycle functions, mitotic kinases have also been implicated in a wide range of cellular processes that coincide with cell division to influence the fates of newly generated daughter cells. Mounting evidence, mainly from studies using invertebrate models, highlights the regulation of asymmetric cell division as a common route through which mitotic kinases couple cell fate decision to the passage through mitosis (Fig. 2).

Mitotic kinases control multiple aspects of asymmetric cell division. First, Plk1 and Aurora A control the localisation of cell fate determinant proteins, in most cases by directly phosphorylating the fate determinants or their adaptor proteins. For example, *Drosophila* larval NBs carrying hypomorph mutations in *polo* (*Drosophila* Plk1 orthologue) or *Aurora A*, fail correctly to localise several cell fate determinants on the cell cortex during mitosis to asymmetrically segregate them into the two daughter cells; consequently, the mutant NBs continue self-renewing, eventually forming brain tumours [86–89]. It was shown that Polo phosphorylates the Partner of Numb protein, an adaptor protein for the Notch signalling inhibitor Numb, to localise Numb at the basal cortex during mitosis, whereas Aurora A phosphorylates Par6 to promote assembly of the PAR complex, which in turn dissociates Numb from the anterior cortex [86,89,90]. Aurora A also regulates Numb localisation through Par6 phosphorylation in *Drosophila* sensory organ precursors [89,90]. Thus, in these progenitors, Plk1 and Aurora A affect daughter cell fate by facilitating unequal segregation of Numb into two daughter cells, which leads to

differential Notch signalling activation between the two daughters. Interestingly, it was shown that human Plk1 also binds and phosphorylates the human orthologue of Numb [91,92], raising the possibility that Plk1 may also regulate Numb segregation in human cells. In *C. elegans* one-cell embryos, Plk1 was shown to promote the formation of a polarised gradient of cell fate determinants in the cytoplasm (cytoplasmic polarity) during mitosis [93,98]. Upon entry into mitosis, Plk1 binds the RNA-binding protein MEX-5 preferentially to accumulate in the anterior cytoplasm of the embryo; Plk1 then phosphorylates a germplasm protein, POS-1, which increases the intracellular mobility of POS-1 in the anterior cytoplasm and creates a permissive environment for its retention only in the posterior cytoplasm [93]. A failure to establish the polarised POS-1 gradient disrupts subsequent embryonic development [93].

Second, Plk1 and Aurora A also direct the orientation of the mitotic spindle during asymmetric cell division. The spindle orientation is critical for daughter cell fate, as it ensures accurate partitioning of cellular components into the two daughter cells and also positions each of the daughter cells in the proper local environment after division. It was shown that *Drosophila Polo* and *Aurora A* mutant NBs not only mislocalise fate determinants but also fail to align the spindle with the apicobasal axis [87,88]. Importantly, this function is clearly separable from the established mitotic functions of Polo and Aurora A; the mutant NBs still form a bipolar spindle and complete cell division [86–88]. There is evidence that this function may be conserved in vertebrates. It was shown that in the developing mouse cortex, Plk1 inhibition causes spindle misorientation in neural progenitors [95], and that in the adult mouse mammary epithelium, Aurora A regulates spindle orientation of mammary stem cells through Notch signalling activity [96]. However, the direct phosphorylation substrates of Plk1 and Aurora A that are responsible for the spindle misorientation phenotypes have not been identified. In *Drosophila* wing and ovarian follicular epithelial cells, phosphorylation of the Lethal(2) giant larvae (Lgl) protein by Aurora A is required to align the mitotic spindle along the epithelium during symmetric division of these cells. However, Lgl phosphorylation is not required for spindle orientation in NBs [97,98]. Thus, mitotic kinases are likely to regulate spindle orientation through different substrates in different cell types.

Lastly, mitotic kinases also promote asymmetric cell division by setting up cell polarity, the event that is a prerequisite for asymmetric cell division. During the oocyte-to-embryo transition in *C. elegans*, Plk1 and Aurora A collaborate to establish the first polarity in the embryo through three distinct steps. First, during oocyte maturation Plk1 and Aurora A inhibit precocious cortical accumulation of the PAR complex to suppress premature symmetry breaking [99–101]; Plk1 phosphorylates a PAR complex subunit Par3, which suppresses its oligomerisation as well as the assembly of the PAR complex [101]. Second, soon after the embryo enters mitosis, the mitotic kinases localise at the centrosomes to trigger the initial symmetry-breaking event, downstream of the aforementioned function of CDK2-Cyclin E [46]; after centrosomes reach the proximity of the posterior cortex, Aurora A is then released from the centrosomes into the cytoplasm and locally inhibits the actomyosin assembly at the posterior cortex, which creates a polarised cortical actin flow that is required for the establishment of cortical polarity [99,102]. Finally, after the initial polarity formation, Aurora A then spreads through the entire cytoplasm and globally inhibits cortical actomyosin assembly, which prevents a spontaneous polarity reversal caused by a centrosome-independent cue [99,102].

Although evidence in vertebrate is still limited, the substantial body of evidence outlined above has established mitotic kinases as the main coordinators between mitotic progression and subcellular events required for asymmetric cell division (Fig. 2). Given that their main role in cell-cycle regulation is also to couple structural reorganisation of various subcellular structures to the progression of mitosis, it might be reasonable to assume that mitotic kinases have extended this coupling function to various subcellular processes that are specific to metazoan cells over the course of evolution. Plk1 and Aurora A have also been shown to phosphorylate various TFs, epigenetic regulators and signalling pathway components [103–106]. It is important to determine the roles of these phosphorylation events in cell specification/differentiation and how these events coincide with mitosis.

Of note, mitotic kinases also play a role in some non-mitotic cells. It was shown that Plk1 regulates transcriptional repression and intracellular transport in the maturing *Drosophila* oocyte [107,108] and that Aurora A stimulates neurite extension through phosphorylation of Par3 and NDEL1 in mammalian neurons [109,110]. In postmitotic mammalian cells, Plk1 and Aurora A also regulate assembly of the primary cilium, a metazoan-specific organelle that is involved in cell motility, cell polarity, signal transduction and mechanosensing [111–113]. In dividing cell, the kinase activity and expression of Plk1 and Aurora A are normally downregulated during interphase [1]. Thus, additional cell-type-specific regulatory mechanisms should

exist in the non-mitotic cells to uncouple the functions of the mitotic kinases from the cell cycle. Subcellular localisation appears to be one of these mechanisms; in the *C. elegans* oocyte, Aurora A accumulates at the cortex, colocalising with cortical microtubules, which is not observed in dividing cells [99].

Cell-cycle brakes and ubiquitin ligases

During the cell cycle, CKIs bind and inhibit the kinase activities of CDKs and two Cullin-RING type ubiquitin ligase complexes, APC/C and SCF, destabilise various CCRs, including cyclins and mitotic kinases, through ubiquitin-mediated proteolysis (Fig. 1). Thus, CKIs and the ubiquitin ligases often, if not in all situations, act as negative regulators of cell-cycle progression, hence 'cell-cycle brakes'. Cellular differentiation commonly accompanies a semi-permanent cell-cycle arrest ('cell-cycle exit'), generally in the G1 or G0 phase, or slowing down of the cell cycle. In these situations, the negative CCRs often regulate cellular events that promote cell differentiation while decelerating the cell cycle, thereby coordinating cellular differentiation with cell-cycle exit or cell-cycle lengthening (Fig. 3).

CDK inhibitors

There are two families of CKIs: the Kip/Cip family CKIs inhibit multiple CDKs, whereas the INK family CKIs primarily inhibit CDK4/6 (Fig. 1) [114]. In mammals, there are three Kip/Cip family members - p21Cip1, p27Kip1 and p57Kip2 - and four Ink4 family members, p16INK4a, p15INK4b, p18INK4c and p19INK4d. In contrast, far fewer CKIs exist in invertebrates: one or two Kip/Cip family CKIs and no Ink4 family CKIs.

In unicellular organisms, CKIs are essential for the proper G1-to-S transition, preventing the premature initiation of DNA replication [115,116]. In contrast, in some metazoan cells, CKIs are dispensable for normal kinetics of the cell cycle. Triple CKI KO MEFs, which lack all of the Cip/Kip family CKIs, proliferate at a rate comparable to that of wild-type cells [117]. p21Cip1 is only required for the induction of G1 arrest in the presence of severe DNA damage [118,119]. Nonetheless, the functions of CKIs have been shown to be crucial for proper cell proliferation control *in vivo*. Both in vertebrates and invertebrates, mutants lacking all the Cip/Kip CKIs are embryonic lethal, exhibiting over-proliferation of various types of cells

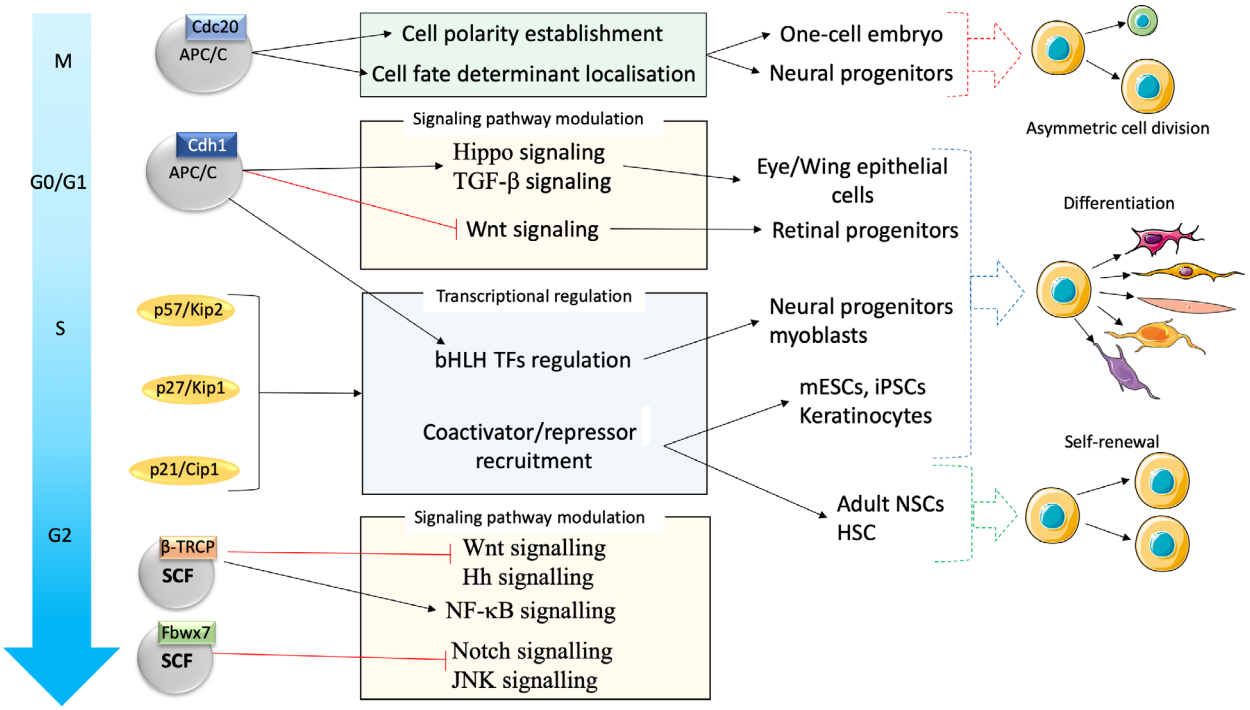

Fig. 3. The roles of cell-cycle brakes in coupling the regulation of cell fate determination and cellular differentiation with cell-cycle arrest. The CKIs seem to promote differentiation mainly by modulating gene expression through their interaction with several transcriptional regulators, independently of their cell-cycle functions. The two ubiquitin ligase complexes, APC/C and SCF, modulate various signalling pathways through inhibition or proteasomal degradation of specific targets. Thus, the cell-cycle brakes can impact on cellular differentiation.

[116,119–122]. Although single *p27Kip1* KO mice can survive up to adulthood, the KO pups display excessive cell proliferation and overgrowth of numerous tissues [124–126]. Other CKIs, including INK4 family proteins, also function in various adult tissues, restricting cell division rates and proliferative potential of tissue stem cells, or maintaining the quiescent state of terminally differentiated cells [127–133].

In addition to these cell-cycle-related phenotypes, animals defective in CKI functions also exhibit a wide range of tissue-specific developmental phenotypes that are not easily attributable to the deregulation of the cell cycle. For example, *p57Kip2* KO mouse embryos display differentiation defects in kidneys, chondrocytes, muscles and cleft palates [134,135]. Subsequent analyses of these tissue-specific phenotypes have revealed the critical roles of metazoan CKIs in regulating differentiation in a broad range of cell types [114]. It was shown that the *Xenopus laevis* p27Kip1 orthologue Xic1 induces cell-cycle exit in retinal and neural progenitors and it also promotes their differentiation into Müller glial fate and early neuronal fate, respectively [136,137]. Detailed dissection of the Xic1 functions resulted in the first clear demonstration of the differentiation-promoting activity of a Cip/Kip family CKI that is molecularly separable from its CKI activity; a mutant human p21Cip1 protein that was defective in CDK inhibition was able to trigger progenitor differentiation, whereas mutant forms of Xic1 that retained CKI activity were not [136,137].

Mechanistically, a large part of cell-cycle-independent functions of CKIs seem to be mediated by the capacities of CKIs to interact physically with various transcription regulators. It has been shown that, through interaction with TFs, including E2F family TFs, CKIs are recruited to specific gene promoter regions and inhibit the transcriptional activities of the TFs, or facilitate or impede interactions of the TFs with coactivator/corepressor complexes [138–144] (Fig. 3). In various progenitor cells, CKIs also bind and upregulate lineage-specific bHLH TFs to promote differentiation of these progenitors upon the differentiation induction, while inducing cell-cycle arrest in these cells. It was shown that, in the developing mouse cerebral cortex, p27Kip1 promotes the differentiation of neural progenitors by binding the neurogenic bHLH TF Ngn2; the interaction stabilises Ngn2 and enhances its transcriptional activity to induce the expression of proneural genes [145]. During myogenesis, p57Kip2 interacts with the myogenic bHLH TF MyoD to induce muscle-specific genes [146]. An analogous bHLH TF-mediated mechanism is likely to underlie the aforementioned differentiation-promoting function of Xic1 [136,137].

CDK inhibitors also function as transcription repressors to restrict the self-renewing capacities of various stem cells or to promote their commitment to a certain fate. In mammalian keratinocytes and haematopoietic stem cells (HSCs), it was shown that loss of p21Cip1 results in an increased stem cell population [131,147]. In keratinocytes, p21Cip1 localises at the *Wnt4* gene promoter and represses *Wnt4* transcription, which promotes self-renewal of the stem cell pool [144,148]. In addition, in mESCs and induced pluripotent stem cells, upon the induction of differentiation p27Kip1 is recruited to the enhancer sequence of the pluripotency factor gene *Sox2* and recruits a corepressor complex to suppress *Sox2* expression [143,149]. Importantly, the regulation of *SOX2* transcription may be one of the main functions of Cip/Kip family CKIs in mice; major phenotypes in *p27Kip1* KO mouse embryos, such as body overgrowth, retinal defects and pituitary hyperplasia, are rescued merely by removing one copy of the *SOX2* gene [149].

Furthermore, although it may seem counterintuitive, CKIs can also inhibit cell differentiation. It was shown that in subependymal neural stem cells (SNSCs) in the adult mouse brain, depletion of p21Cip1 causes the premature differentiation of SNSCs into astrocytes, due to abnormal activation of TGF-β/BMP signalling in SNSCs [150]. This TGF-β/BMP activation is caused by the loss of the p21Cip1 function to repress the expression of the TGF-β/BMP ligand Bmp2 in SNSCs [150]. In addition, p21Cip1 also promotes the self-renewing capacity of SNSCs by repressing SOX2 expression through direct binding to a *SOX2* enhancer [127,151]. The p21Cip1 depletion causes premature exhaustion of SNSCs due to excessive expression of SOX2 [127,151]; SOX2 overexpression induces replicative stress in SCSCs, although the underlying mechanism is unclear [151]. Similarly, p21Cip1 depletion also triggers a premature loss of a self-renewing capacity of mouse HSCs after initial expansion of the HSC pool [131]. Thus, in various adult stem cells, CKIs function to prevent their premature differentiation and/or their premature exhaustion, while maintaining their slow proliferation rates.

Taken together, these studies indicate that CKIs both positively and negatively regulate cell differentiation by directly modulating the expression of various genes. Through these transcriptional functions, CKIs may spatiotemporally couple cell fate specification to cell-cycle exit in progenitor cells or may link self-renewal to the extended cell cycle in adult stem cells (Fig. 3). It is crucial to clarify how the transcriptional

functions and the negative regulation of the cell cycle by CKIs cooperate to ensure the balance between proliferation and differentiation in these cell types. Somewhat surprisingly, there is no evidence for the involvement of non-cell-cycle functions of invertebrate CKIs in development. However, in both vertebrates and invertebrates, CKIs regulate actin cytoskeleton through the control of RhoGTPases [152]. In mice, p21Kip1 was shown to regulate neural extension through the regulation of RhoA activity [145]. Given the critical role of cytoskeleton in a wide range of developmental processes, including cell migration, cell polarity and morphogenesis, the cytoskeletal function of CKIs may also play a role in invertebrate development.

APC/C and SCF ubiquitin ligases

Alongside phosphorylation, ubiquitination is the central protein modification to cell-cycle control, mediating timely proteolysis through the ubiquitin-proteasome pathway. The irreversible nature of proteolysis is key to the unidirectionality of the cell cycle, ensuring the 'only once per cell cycle' occurrence of the replication and segregation of the chromosomes. The structurally related complexes APC/C and SCF are the two major ubiquitin ligases involved in cell-cycle control, and act as engines and brakes for cell-cycle progression, depending on the cell-cycle stages and on which substrate receptor subunit assembles into the complexes (Fig. 1) [153].

APC/C

APC/C binds one of the two regulatory subunits, CDH1 (also known as Fizzy-related or Fzr1) and CDC20 (also known as Fizzy), which activate APC/C in specific windows of the cell cycle and also confer distinct substrate specificities [153]. The interphase-specific subunit CDH1 binds APC/C from late mitosis to the end of the G1 phase. Studies using cultured cells and animal models suggest that APC/C^{CDH1} mainly functions to prevent the G1-to-S transition and thus acts as a critical determinant of the length of the G1 (and G0) phase (Fig. 1). In cycling human cells, CDH1 depletion shortens the G1 phase by accelerating S phase entry, which often causes DNA damage [154]. In many metazoan organisms, CDH1 is not expressed during early embryonic stages when cells divide rapidly; its expression levels gradually increase concurrently with the elongation of the G1 phase [155]. In various progenitor cells, APC/C^{CDH1} is required for the G1 phase lengthening upon the onset of differentiation [156]. APC/C^{CDH1} is also suggested to be involved in maintenance of G0 arrest in some terminally differentiated cells [156,157].

APC/C^{CDH1} is one of the few CCRs that are thought to be active during the G1/G0 phase. Therefore, potential roles of APC/C^{CDH1} in terminal differentiation and tissue-specific functions have been intensively investigated in various postmitotic cells, particularly in neurons [156,158,159]. In *Drosophila* and *C. elegans* presynaptic neurons, APC/C^{CDH1} controls the number of presynaptic boutons by regulating the levels of liprin-α and the glutamate receptor [160,161]. In mouse and rat neurons, APC/C^{CDH1} degrades the inhibitor of bHLH TFs, the Id2 protein, to regulate the axonal morphology and neural activity [162]. Importantly, in rat neurons, APC/C^{CDH1} activity is also necessary to maintain the G0 phase arrest of the neurons by keeping Cyclin B levels low [157]. Thus, APC/C^{CDH1} may couple terminal differentiation and cell-type-specific functions to G0 arrest in various terminally differentiated cells.

Besides these functions, recent reports have revealed the roles of APC/C^{CDH1} in the cell fate decision process in progenitor cells [156]. In the developing *Drosophila* eye, it was shown that APC/C^{CDH1} influences the cell fate choice of retinal progenitors by modulating the activity of a major extracellular signalling pathway, the Wnt signalling pathway [163]. In the eye primordium (the eye imaginal disc), Wnt signalling prevents undifferentiated progenitors from committing to the retinal fate and instead promotes their commitment to the head cuticle fate [164]. It was shown that partial depletion of APC/C subunits in the progenitor cells blocks their differentiation into photoreceptor neurons by inducing ectopic activation of Wnt signalling [163]. It was previously shown that APC/C^{CDH1} induces G1 arrest in the same progenitor population prior to the initiation of differentiation [165]. Thus, APC/C^{CDH1} couples the suppression of Wnt signalling to G1 arrest during *Drosophila* retinal differentiation.

Mounting evidence suggests that the regulation of intra- and extracellular signalling pathways may be one of the major mechanisms by which APC/C^{CDH1} influences cell fate determination and differentiation (Fig. 3). In the above example, the regulation of Wnt signalling is mediated by APC/C^{CDH1}-dependent degradation of the evolutionally conserved kinase NimA-related kinase 2 (Nek2) [163,166], which phosphorylates the conserved Wnt pathway component Dishevelled (Dsh) to stimulate Wnt signalling activity [169]. It was shown that APC/C^{CDH1} also uses the same Nek2-Dsh pathway and modulates the non-

canonical Wnt pathway, the Wnt/planar cell polarity (PCP) pathway, to influence epithelial cell polarity in the late stage of *Drosophila* eye development and in developing wing epithelia [168]. Importantly, the human Nek2 homologue has also been shown to be targeted by APC/C [169] and to phosphorylate human Dsh protein in human cells [170]. It was previously reported that in *Xenopus* embryos, morpholinos against APC/C subunits cause phenotypes that are reminiscent of those observed upon the deregulation of the Wnt/PCP pathway [171]. Taken together, these data strongly suggest that APC/C^{CDH1} also modulates Wnt pathways in vertebrates. Moreover, it was shown in human cells and *Drosophila* tissues that APC/C^{CDH1} regulates the transcriptional activity of the TGF-β signalling pathway through the degradation of the negative modulator SnoN [172–174]. APC/C^{CDH1} also degrades LATS kinases (Warts in *Drosophila*) in a cell-cycle-dependent manner and modulates the Hippo pathway [175].

APC/C^{CDH1} may also regulate cell fate decisions of neural progenitors through protein degradation in the developing mouse cortex. It was shown that CDH1 promotes the differentiation of cortical neural progenitors [176,177] and that APC/C^{CDH1} degrades a number of proteins that are expressed specifically in the neural progenitors [178–181]. However, in these progenitors, CDH1 also controls the length of the G1 phase upon the induction of differentiation [176,177]. It was shown that lengthening the G1 phase by other means, for example by inhibiting CDK4-Cyclin D, also induces the differentiation of the neural progenitors [6]. Therefore, it needs to be clarified whether APC/C^{CDH1} promotes the differentiation of neural progenitors through its cell-cycle and/or cell-cycle-independent functions.

The mitotic form of APC/C, APC/C^{CDC20}, normally acts as a positive regulator of the cell cycle. During the cell cycle, CDC20 is normally expressed in G2 and M phases and supports the activity of APC/C to direct critical mitotic events, such as spindle formation, sister chromatid separation and mitotic exit [182] (Fig. 1). Although to a much lesser extent than APC/C^{CDH1}, APC/C^{CDC20} has also been implicated in cell differentiation. It was shown that APC/C^{CDC20} regulates asymmetric cell division of one-cell-stage *C. elegans* embryos and *Drosophila* larval NBs: CDC20 is required for the establishment of cell polarity in the *C. elegans* embryo [183], and *Drosophila* larval NBs carrying mutations in APC/C subunits fail to localise cell fate determinants on the basal cortex during mitosis [184] (Fig. 2). Thus, similar to other mitotic regulators, APC/C^{CDC20} may couple asymmetric division-specific events to mitotic progression.

Although CDC20 is normally not transcribed during the G1 or G0 phase, there are some exceptions where CDC20 is expressed in postmitotic cells and regulates cellular events specific to the cell types. It was shown that in postnatal rodent neurons, CDC20 promotes the formation and growth of dendrites through the degradation of the Id1 protein [185]. CDC20 was also found to be expressed in postmitotic ependymal cells in the developing mouse brain and to regulate the formation of multicilia during ependymal cell differentiation [186]. Unique regulatory mechanisms specific to these postmitotic cells appear to allow uncoupling of these cell-type-specific functions of CDC20 from its cell-cycle functions. In the rat granule neurons, CDC20 was found to be enriched at centrosomes, which is normally only observed during mitosis; this centrosomal localisation is critical for the function of APC/C^{CDC20} in regulating dendrite growth [185]. In the multiciliating ependymal cells, CDC20B, a vertebrate-specific CDC20 homologue that has no assigned cell-cycle functions, is specifically expressed and cooperates with CDC20 in the multiciliation process [187].

SCF complexes

Compared with APC/C, SCF has a much larger number of regulatory subunits that possess distinct substrate specificities (F-box proteins, 69 different F-box genes present in the human genome) and is involved in the regulation of a myriad of biological processes [153]. Among the F-box proteins, β-TRCP1/2 (also known as Fbxw1/11), Fbxw7 (or CDC4) and Skp2 (or Fbxl1) are particularly important for cell-cycle control, targeting degradation-critical CCRs, such as cyclin D/E, Cip/Kip family CKIs, CDC25 and Emi1 (Fig. 1) [153].

The involvement of the SCF complexes in cellular processes besides cell-cycle regulation has been widely acknowledged [188]. In particular, SCF plays key roles in the regulation of major developmental signalling pathways. $SCF^{β\text{-}TRCP}$ has been shown to be a critical regulator of signal transduction of two conserved signalling pathways, Wnt and Hedgehog (Hh). In the absence of Wnt ligands, $SCF^{β\text{-}TRCP}$ ubiquitinates phosphorylated β-catenin to induce its degradation and turns off downstream signal transduction of the canonical Wnt signalling pathway [189,190]. Similarly, $SCF^{β\text{-}TRCP}$ also shuts off Hh signalling by targeting the essential pathway component Ci (Gli in mammals): in the absence of Hh ligands, $SCF^{β\text{-}TRCP}$ ubiquitinates Ci to trigger its proteolytic cleavage, and the resultant truncated form acts as a transcriptional repressor that inhibits the expression of target genes [189,191,192].

$SCF^{\beta\text{-}TRCP}$ is also involved in the activation of NF-κB signalling by targeting the NF-κB inhibitor, IκB, for degradation [192]. Another form of SCF, SCF^{Fbxw7}, also modulates the activities of major signalling pathways: SCF^{Fbxw7} destabilises Notch and c-Jun proteins, components of Notch and JNK signal transduction cascades, respectively [194–197]. In addition to signalling components, the SCF complexes also target various tissue-specific TFs, including SNAIL and DMRT1 [198–200].

Through these regulatory activities, $SCF^{\beta\text{-}TRCP}$, SCF^{Fbxw7} and SCF^{Skp2} engage in various developmental processes alongside cell-cycle regulation (Fig. 3). *Drosophila* mutations in *slimb* (*Drosophila* β-TRCP orthologue) disrupt development due to ectopic activation of Hh and Wnt signalling [189]. Mice lacking one of the two β-TRCP paralogues, β-TRCP2, die during early embryogenesis, and conditional KO of both the β-TRCP paralogues severely disrupts testicular development, which is partially rescued by co-depletion of the Snail1 TF [199]. *Fbxw7* KO mice exhibit defects in the development of the brain, yolk sac and heart chamber, which are attributable to the deregulation of Notch and/or c-Jun pathways [196,201].

However, although the SCF complexes control both cell cycle and cell differentiation, unlike other CCRs, they may contribute little to the proliferation-differentiation coordination in normal conditions. This is because the rate-limiting step in substrate ubiquitination by SCF complexes is generally not the activation of the complexes but rather post-translational modifications (e.g. phosphorylation, glycosylation) of substrates, which allow recognition of these substrates by F-box proteins and are regulated by other factors [153]. Therefore, cell-cycle and non-cell-cycle functions of the SCF complexes are mostly temporally uncoupled. Nevertheless, it should be noted that misregulations of the SCF complexes, by mutation, overexpression or other means, can simultaneously affect cell-cycle events and differentiation processes that they regulate. A number of F-box proteins including β-TRCP, Fbxw7 and Skp2 have been identified as oncogenes or tumour suppressors in various types of human cancer [202,203]. Cell-cycle and non-cell-cycle functions of the SCF complexes may collaborate in tissue-specific tumorigenic and carcinogenic mechanisms.

Discussion and concluding remarks

Precise coordination between cell proliferation and cellular differentiation is of paramount importance for the development of multicellular organisms. However, the mechanisms behind this coordination remain largely elusive. In the classical view, it is believed that cell proliferation and cellular differentiation are independently regulated by distinct sets of molecules and that the coordination between the two processes is achieved by transcriptional regulations of CCR and differentiation factor genes by common upstream developmental signalling or TFs. However, this classical model falls short in explaining numerous *in vivo* observations where cell proliferation and differentiation appear to be spatiotemporally tightly coupled and often interdependent. As discussed above, it has become evident that various CCRs, the central players in the control of cell proliferation in all eukaryotic cells, possess additional capacities to affect the specification or differentiation of specific cell populations or to directly regulate the activities of TFs and signalling pathway components. By combining these non-canonical functions with the conserved cell-cycle functions, CCRs spatiotemporally couple cell fate determination and differentiation processes to cell-cycle progression in various types of cells, thereby coordinating cell proliferation and differentiation in metazoan organisms (Figs 2 and 3).

It has been hypothesised that the length of a certain cell-cycle phase (particularly, G1 phase) may influence the choice of cell fate in various stem cells and progenitors [6,7]. This hypothesis predicts that elongation of a cell-cycle phase may provide sufficient time for cell-type-specific TFs to bind and act on the chromatin. However, empirical evidence for this model has not been provided. It is worth pointing out that most of the original studies used overexpression or depletion of certain CCRs to induce the change of the kinetics of the cell cycle (for example, Lange *et al.* [80]). Thus, it is possible that non-canonical functions of these CCRs, instead of the cell-cycle change, may account for the observed effect on cell fate specification. It was recently shown that, in mice and human pancreas development models, forced elongation of the G1 phase by CDK2 inhibition induces differentiation in endocrine progenitor cells [44]. However, in the same progenitor population it was also shown that CDK2 phosphorylates the proendocrine Ngn3 to induce its degradation, thereby inhibiting the differentiation of endocrine progenitors [43,44]. It is crucial to determine whether the cell-cycle change *per se* impacts the differentiation of the progenitors/stem cells, or whether temporal coupling of the G1-to-S transition to the regulation of pro-differentiation factors by the intracellular coordination function of CCRs may be critical in these cells.

There are many unanswered questions concerning the non-canonical functions of CCRs. First, compared

with their highly conserved functions of cell-cycle regulation, the differentiation functions of metazoan CCRs appear highly diverse; how are the activities of CCRs regulated *in vivo* to enable these cell-type-specific functions? One mechanism appears to be the regulation of their substrates or binding partners: proteins that CCRs act on are expressed only in specific cell populations. Alternatively, CCRs may target proteins whose functions are context-dependent, for example, a component of an extracellular signalling pathway. Another mechanism may be cell-type-specific regulations of CCRs: CCRs are recruited to specific subcellular compartments, operate independently of their regular binding partner(s) or are regulated by activators or inhibitors that are expressed exclusively in specific cell types. In the last case, non-canonical functions of CCRs can be totally uncoupled from their cell-cycle functions. Cell-type-specific regulatory mechanisms of CCRs are an important subject of future study.

Another question is how CCRs have gained divergent functions during evolution. There are potential advantages in having the additional capacities of CCRs to regulate cellular differentiation for metazoan organisms. For example, the capacity of CCRs directly to regulate differentiation factors enables the regulation of the function of these factors within a single cell cycle, providing high temporal resolution to cell specification and/or differentiation processes. In addition, combined with the regulation of CCRs by developmental pathways, the regulation of differentiation by CCRs may allow formation of feedback mechanisms between CCRs and developmental pathways. Such feedback mechanisms may contribute to long-term maintenance of the self-renewal capacity of stem cells and cellular quiescence of terminally differentiated cells, as well as to tumour suppression, by creating the bi-stable states of the undifferentiated mitotic state and the differentiated postmitotic state. Considering these potential advantages, the majority of non-cell-cycle functions of CCRs are likely to be evolutionally conserved. Various non-cell-cycle functions have been characterised only in invertebrate CCRs and it is important to examine the presence of analogous functions in their vertebrate homologues.

Non-canonical functions of CCRs also hold important clinical implications. Abnormal cell-cycle regulation is a hallmark of cancer. Mutations or overexpression of CCRs may promote cancer development not only through cell-cycle deregulations but also through the cell-type-specific functions, which may explain enrichment of certain mutations/amplifications of CCR genes in specific cancer types. On the other hand, it is possible to use the non-canonical functions of CCRs in our favour. For example, it was recently shown that the high efficacy of CDK4/6 inhibitors against breast cancer is mediated by non-cell-cycle functions of CDK4/6 to promote anti-tumour immunity [204]. Thus, better understanding of *in vivo* functions of CCRs may also be critical to gain insight into tissue-specific carcinogenic mechanisms as well as to find a new therapeutic avenue against cancer.

The biggest challenge in investigating the functions of CCRs *in vivo* is their essential roles in cell viability. However, the recent advances in genome editing, *in vitro* tissue culture, advanced imaging methods and single-cell analysis techniques enable more detailed, time- and space-resolved examination of CCR functions than ever. We therefore optimistically envisage rapid progress in our understanding of the *in vivo* function of CCRs in the coming years.

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