Cdk, cyclins and CKIs: roles beyond cell cycle regulation

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Summary
Cyclin-dependent kinases (Cdk) are serine/threonine kinases and their catalytic activities are modulated by interactions with cyclins and Cdk inhibitors (CKIs). Close cooperation between this trio is necessary for ensuring orderly progression through the cell cycle. In addition to their well-established function in cell cycle control, it is becoming increasingly apparent that mammalian Cdns, cyclins and CKIs play indispensable roles in processes such as transcription, epigenetic regulation, metabolism, stem cell self-renewal, neuronal functions and spermatogenesis. Even more remarkably, they can accomplish some of these tasks individually, without the need for Cdk/cyclin complex formation or kinase activity. In this Review, we discuss the latest revelations about Cdns, cyclins and CKIs with the goal of showcasing their functional diversity beyond cell cycle regulation and their impact on development and disease in mammals.

Key words: Cdk, Cyclin, CKI, Transcription, DNA damage repair, Proteolytic degradation, Epigenetic regulation, Metabolism, Stem cell self-renewal, Neuronal functions, Spermatogenesis

Introduction
Cyclin-dependent kinases (Cdns) contain a serine/threonine-specific catalytic core and they partner with regulatory subunits known as cyclins, which control kinase activity and substrate specificity. Cdk/cyclin complexes were first implicated in cell cycle control based on pioneering work in yeast, in which a single Cdk (Cdc28 in the budding yeast *Saccharomyces cerevisiae*; Cdc2 in the fission yeast *Schizosaccharomyces pombe*) was found to promote transitions between different cell cycle phases through its interactions with various cyclins (Beach et al., 1982; Evans et al., 1983; Nurse and Thuriaux, 1980; Nurse et al., 1976; Reed et al., 1982). Accordingly, Cdns are perceived as the engine that drives cell cycle progression whereas cyclins are considered to be the gears that are changed to aid the transition between cycle phases. The kinase activity of Cdk/cyclin complexes is tightly regulated by a plethora of Cdk inhibitors (CKIs), which serve as brakes to halt cell cycle progression under unfavorable conditions (Morgan, 2007).

In comparison to yeast, the mammalian cell cycle has evolved to include additional Cdns, such that the functions of a single Cdk in yeast is now divided among several mammalian Cdns. Although conceptually similar to the system in yeast, mammalian cells vary both Cdns and cyclins (instead of just the cyclin) during each phase of the cell cycle to ensure sequential progression through the cell cycle in an orderly fashion. This increased Cdk complexity is thought to satisfy the requirement for a more elaborate control over the proliferation of different cell types during the advancement from unicellular to complex multicellular organisms (Malumbres and Barbacid, 2009).

The advent of gene targeting in mice has spurred the interrogation of cell cycle regulation using genetics. When applied to the deletion of well-established cell cycle regulators, this approach has yielded unexpected results (Satyanarayana and Kaldis, 2009). For example, several groups have reported that interphase Cdns, which were deemed essential for mammalian cell cycle progression, are in fact dispensable in mice as their loss did not compromise viability but instead led to phenotypes in highly specialized cell types, including hematopoietic cells in *Cdks*−/− (Hu et al., 2009; Malumbres et al., 2004), endocrine cells in *Cdk4*−/− (Rane et al., 1999; Tsutsui et al., 1999) and meiotic germ cells in *Cdk2*−/− (Berthet et al., 2003; Ortega et al., 2003) mice. These findings highlighted the extent of functional redundancy in the regulation of cell cycle progression and uncovered novel tissue-specific functions for interphase Cdns, which are likely to be independent of their role in cell cycle control as closely related family members can readily assume vacancies in this aspect. Although in-depth characterization of the precise mechanism through which interphase Cdns maintain tissue homeostasis remains a challenging and important task for the future, the moonlighting of these classical regulators reveals the power of gene targeting in the identification of unique and non-redundant functions beyond cell cycle control.

Thus far, Cdk, cyclin and CKI family members have been implicated in transcription, DNA damage repair, proteolytic degradation, epigenetic regulation, metabolism, stem cell self-renewal, neuronal functions and spermatogenesis (Tables 1-3). In this Review, we aim to provide an update on how mammalian Cdns, cyclins and CKIs can influence these cellular and developmental processes beyond the cell cycle, with particular emphasis on how each of these processes can be accomplished through kinase-dependent or -independent mechanisms.

An overview of the Cdk, cyclin and CKI families
There are currently >20 members of the Cdk family (Malumbres et al., 2009), each characterized by a conserved catalytic core made up of an ATP-binding pocket, a PSTAIRE-like cyclin-binding domain and an activating T-loop motif (Fig. 1). Collectively, these features participate in Cdk activation, which involves the association with cyclins via the PSTAIRE helix to: (1) displace the T-loop and expose the substrate-binding interface; and (2) realign critical residues within the active site thereby priming it for the phospho-transfer reaction. Most Cdk family members also possess inhibitory (threonine 14, T14; tyrosine 15, Y15 in Cdk1) and activating (threonine 161, T161 in Cdk1) phosphorylation sites (Fig. 1). Phosphorylation at T14 and Y15 within the ATP-binding site by inhibitory kinases Wee1 and Myt1 interferes with proper ATP alignment, whereas T-loop phosphorylation at T161 by Cdk-activating kinases (CAKs) improves substrate binding and complex stability to enable full Cdk activation (Atherton-Fessler et al., 1993; Pavletich, 1999).
In contrast to the Cdk family, cyclins belong to a remarkably diverse group of proteins classified solely on the existence of a cyclin box that mediates binding to Cdk (Gopinathan et al., 2011). Sequence variations outside the cyclin box allows for differential regulation and functional diversity. Even though their name originated from the cell cycle-dependent fluctuations in expression levels, many of the newer members of the cyclin family in fact do not oscillate.

Whereas most cyclins promote Cdk activity, CKIs restrain Cdk activity. CKIs are subdivided into two classes based on their structure and Cdk specificity. The Ink4 family members [p16INK4a (Cdkn2a), p15INK4b (Cdkn2b), p18INK4c (Cdkn2c) and p19INK4d]...
Cdk, cyclins and CKIs linked to transcription

Kinase-dependent transcriptional functions

The involvement of cell cycle regulators in transcription has been a long-standing affair and one of the best-characterized examples remains intimately linked to cell cycle control: the Rb/E2F pathway (Weinberg, 1995). In the hypophosphorylated state, the pocket proteins [retinoblastoma protein (Rb); also known as Rb1], p107 (Rbl1) and p130 (Rbl2)] bind to and sequester members of the E2F family of transcription factors (Dyson, 1998). Cdk4/6 and Cdk2, in association with their respective catalytic partners D- and E-type cyclins, are responsible for successively phosphorylating Rb, thereby alleviating its inhibition on E2F and allowing the activation of genes necessary for promoting S phase entry and DNA synthesis (Harbour and Dean, 2000; Trimarchi and Lees, 2002). By modulating the activity of G1 kinases, CKIs are also indirectly involved in regulating the expression of E2F-responsive genes.

Table 2. Established and emerging functions of cyclins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Established function</th>
<th>Kinase activity</th>
<th>Emerging function</th>
<th>Kinase activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A</td>
<td>Control of G1 phase of cell cycle in complex with Cdk4 or Cdk6; Rb/E2F transcription</td>
<td>Yes</td>
<td>FoxM1 and FoxK2 transcription in complex with Cdk2</td>
<td>Yes</td>
<td>Chen et al., 2009; Marais et al., 2010</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>Control of G1 phase of cell cycle in complex with Cdk1</td>
<td>Yes</td>
<td>FoxM1 and FoxK2 transcription in complex with Cdk1</td>
<td>Yes</td>
<td>Chen et al., 2009; Marais et al., 2010</td>
</tr>
<tr>
<td>Cyclin C</td>
<td>RNA Pol II transcription in complex with Cdk8</td>
<td>Yes</td>
<td>Wnt/β-catenin pathway in complex with Cdk8</td>
<td>Yes</td>
<td>Firestein et al., 2008</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>Control of G1 phase of cell cycle in complex with Cdk4 or Cdk6; Rb/E2F transcription</td>
<td>Yes</td>
<td>NF-Y, Stat, Creb2, Elk1, Znf423 and Cux1 transcription</td>
<td>No</td>
<td>Bienvenu et al., 2010</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Control of G1-S phase of cell cycle in complex with Cdk2; Rb/E2F transcription</td>
<td>Yes</td>
<td>Control of G1-S phase of cell cycle</td>
<td>No</td>
<td>HR-mediated DNA damage repair, Epigenetic regulation through MeD50, Inhibition of lipogenesis, Phosphorylation of neuronal function of Cdk5</td>
</tr>
<tr>
<td>Cyclin F</td>
<td>Cdk-activating kinase (CAK) activity</td>
<td>Yes</td>
<td>DNA damage response</td>
<td>No</td>
<td>ScF-mediated proteolysis</td>
</tr>
<tr>
<td>Cyclin H</td>
<td>RNA Pol II transcription in complex with CycH</td>
<td>Yes</td>
<td>RNA Pol II transcription in complex with Cdk12 and Cdk13</td>
<td>Yes</td>
<td>Bartkowiak et al., 2010; Blazek et al., 2011; Cheng et al., 2012</td>
</tr>
<tr>
<td>Cyclin K</td>
<td>RNA Pol II transcription in complex with Cdk9</td>
<td>Yes</td>
<td>DNA damage response in complex with Cdk9</td>
<td>Yes</td>
<td>Yu et al., 2010</td>
</tr>
<tr>
<td>Cyclin L</td>
<td>RNA Pol II transcription in complex with Cdk11</td>
<td>Yes</td>
<td>DNA damage response in complex with Cdk12</td>
<td>Yes</td>
<td>Blazek et al., 2011</td>
</tr>
<tr>
<td>Cyclin M</td>
<td>RNA Pol II transcription in complex with Cdk13</td>
<td>Yes</td>
<td>DNA damage response in complex with Cdk14</td>
<td>Yes</td>
<td>Davidson et al., 2009</td>
</tr>
<tr>
<td>Cyclin N</td>
<td>RNA Pol II transcription in complex with Cdk15</td>
<td>Yes</td>
<td>Synaptic trafficking and remodeling in complex with Cdk16</td>
<td>Yes</td>
<td>Ou et al., 2010; Park et al., 2011</td>
</tr>
<tr>
<td>Cyclin O</td>
<td>RNA Pol II transcription in complex with Cdk16</td>
<td>Yes</td>
<td>Spermatogenesis in complex with Cdk16</td>
<td>Yes</td>
<td>Mikolcevic et al., 2012</td>
</tr>
</tbody>
</table>

HR, homologous recombination; NHEJ, non-homologous end-joining; RNAPII, RNA polymerase II; ScF, Skp1-Cul1-F-box protein.
Table 3. Established and emerging functions of CKIs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Established function</th>
<th>Emerging function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>Inhibition of Cdk/cyclin complexes</td>
<td>NSC differentiation through silencing of Sox2 expression</td>
<td>Marques-Torrejon et al., 2013</td>
</tr>
<tr>
<td>p27</td>
<td>Inhibition of Cdk/cyclin complexes</td>
<td>Recruitment of transcriptional co-repressors</td>
<td>Pippa et al., 2012</td>
</tr>
<tr>
<td>p57</td>
<td>Inhibition Cdk/cyclin complexes</td>
<td>ESC differentiation through silencing of Sox2 expression</td>
<td>Li et al., 2012a</td>
</tr>
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ESC, embryonic stem cell; NSC, neural stem cell.

Although the kinase-dependent transcriptional control of G1/S transition is well documented, corresponding events mediating the switch from G2 into M phase are just beginning to emerge. FoxM1 is a member of the forkhead box (Fox) superfamily of transcription factors (Hannenhalli and Kaestner, 2009; Myatt and Lam, 2007), target genes of which include essential regulators of mitosis and components of the spindle assembly checkpoint (Laouikil et al., 2005; Sadasivam et al., 2012; Wonsley and Folliette, 2005). The transcriptional activity of FoxM1 is kept silent during most phases of the cell cycle, as its N-terminal repressor domain (RD) interacts with and abolishes the function of its C-terminal transactivation domain (TAD). During the G2 phase of the cell cycle, this auto-

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Fig. 1. Alignment of the kinase core of Cdk family proteins. Important motifs are highlighted in green boxes, including the ATP-binding domain, the cyclin-binding domain (PSTAIRE in Cdk1) and the residues demarcating the start and end of the T-loop. Regulatory phosphorylation sites are highlighted in purple boxes, including the inhibitory threonine and tyrosine residues in the ATP-binding domain (T14 and Y15 in Cdk1) and an activating threonine residue in the T-loop (T161 in Cdk1). Non-conserved residues are colored pink. The extent of conservation is represented by the height of the black bar beneath each residue. Mouse protein sequences used in this alignment are from Cdk1 (NP_031685), Cdk1 v1 (NP_094326), Cdk1 v2 (NP_058036), Cdk2 (NP_031694), Cdk4 (NP_034000), Cdk5 (NP_034003), Cdk6 (NP_034004), Cdk8 (NP_705827), Cdk9 (NP_570393), Cdk10 v1 (NP_919428), Cdk10 v2 (NP_919426), Cdk11 (NP_031687), Cdk12 v1 (NP_001103096), Cdk12 v2 (NP_001103098), Cdk12 v3 (NP_081228), Cdk13 v1 (NP_001074527), Cdk13 v2 (NP_081394), Cdk14 (NP_035204), Cdk15 (NP_001028545), Cdk16 (NP_001028547), Cdk17 (NP_666351), Cdk18 (NP_032821), Cdk19 v1 (NP_001161776), Cdk19 v2 (NP_937807). Note: only the kinase domain is shown; N- and C-terminal extensions are excluded.
The regulation of RNA polymerase II (RNA Pol II)-based transcription by members of the Cdk and cyclin families has been well described. The carboxy-terminal domain (CTD) of RNA Pol II contains multiple heptapeptide repeats that can be targeted by Cdk/cyclin complexes, with Cdk1 being the first to be identified (Cisek and Corden, 1989). Progressive changes in the phosphorylation status of the CTD play a crucial role in the timing of its polymerase activity and the sequential recruitment of various co-regulators. Following a long history of reports about Cdk/cyclin complexes with catalytic activity towards the CTD (Fig. 3), newly annotated members of the Cdk and cyclin families continue to join the ranks in the control of RNA Pol II-based transcription. Specifically, it was recently demonstrated that cyclin K partners with Cdk12 and Cdk13 to mediate phosphorylation of the CTD (Bartkowiak et al., 2010; Blazek et al., 2011; Cheng et al., 2012). Collectively, it should be appreciated that the control of RNA Pol II-based transcription is analogous to the regulation of the cell cycle, whereby a series of Cdk/cyclin complexes, activities of which are restricted during each phase of the transcription cycle, is required to achieve the dynamic patterns of phosphorylation marks on the CTD and drive the step-wise progression from pre-initiation, initiation, elongation to termination (Fig. 3). A better understanding of how Cdk/cyclin complexes trigger each transition and how the CTD code is deciphered into productive events during RNA synthesis will be the aim of future investigations. Unlike cell cycle regulation, which is plagued by extensive compensatory mechanisms, Cdk and cyclin members involved in transcriptional control appear to be non-redundant as their ablation usually results in embryonic lethality; this applies to Cdk7 (Ganuz et al., 2012), Cdk8 (Westering et al., 2007), Cdk11 (Li et al., 2004), cyclin H (Patel and Simon, 2010), cyclin T2 (Kohoutek et al., 2009) and cyclin K (Blazek et al., 2011).

In addition to the regulation of global gene expression, Cdk/cyclin complexes have been implicated in specific transcriptional pathways, the most notable of which is the Wnt/β-catenin signaling cascade (Fig. 4). Wnt signaling controls a multitude of developmental processes and, unsurprisingly, aberrant pathway activity has been linked to various diseases. The most common manifestation of de-regulated Wnt signaling is colorectal cancer, in which loss-of-function mutations in the APC tumor suppressor gene are prevalent, leading to hyperactivation of β-catenin (Bienz and Clevers, 2000). Therefore, suppressing the Wnt pathway became an attractive route for therapeutic intervention (Anastas and Moon, 2013). An RNAi screen to identify modifiers of β-catenin transcriptional activity and colon cancer cell proliferation pinpointed CDK8 as a key player and demonstrated its copy number amplification in a substantial fraction of colorectal cancer, in which loss-of-function mutations in the APC tumor suppressor gene are prevalent, leading to hyperactivation of β-catenin (Bienz and Clevers, 2000). Therefore, suppressing the Wnt pathway became an attractive route for therapeutic intervention (Anastas and Moon, 2013). An RNAi screen to identify modifiers of β-catenin transcriptional activity and colon cancer cell proliferation pinpointed CDK8 as a key player and demonstrated its copy number amplification in a substantial fraction of colorectal cancers (Fierstein et al., 2008). Although the precise mechanism by which Cdk8 potentiates β-catenin-mediated transcription remains poorly understood, its kinase activity was demonstrated to be essential, and its role as part of the ‘Mediator complex’ together with cyclin C, MED12 and MED13 (Knuesel et al., 2009) was suggested to be involved.

Apart from modulating the transcriptional activity of β-catenin in the nucleus, cell cycle regulators can also exert their influence over Wnt signal transduction remotely at the cell surface (Fig. 4). This is made possible by the recent discovery of Cdk14/cyclin Y complexes, which are anchored to the plasma membrane (Jiang et al., 2009). Membrane tethering is dependent on an N-terminal myristoylation motif on cyclin Y and is responsible for bringing the catalytic domain of Cdk14 in close proximity to its substrate, the Wnt co-receptor Lrp6 (Davidson and Niehrs, 2010; Davidson et al.,...
and and cyclin T (cycT) are subunits of the positive transcription elongation factor b (P-TEFb), which promotes the extension of the pre-mRNA transcript by:

(1) phosphorylating the CTD to engage its RNA polymerizing activity (Fu et al., 1999; Peng et al., 1998). Cdk11/cyclin L (cycL) interacts with a variety of elongation factors to facilitate transcription elongation, including El1, TFIIF, FACT (Trembley et al., 2002). In addition, Cdk11/cyclin L is involved in RNA processing co-transcriptionally through its association with and phosphorylation of factors responsible for pre-mRNA splicing, such as SC35 (Srfs2) and 9G8 (Srfs7) (Dickinson et al., 2002; Hu et al., 2003; Loyer et al., 1998). Cdk7/cyclin H in complex with the RING finger protein Mat1 (Mnat1) are components of TFIIH, which phosphorylates (P) the C-terminal domain (CTD) of RNA Pol II to induce promoter clearance and the transition from initiation to elongation during transcription (Serizawa et al., 1995; Shiekhattar et al., 1995).

Fig. 3. Cdk/cyclin complexes regulate RNA Pol II-based transcription. RNA Pol II (RNAPII) forms part of the pre-initiation complex (PIC) responsible for gene transcription in eukaryotes. Other members of PIC include the general transcription factor complexes TFIIB,-D,-E,-F and -H. Cdk7/cyclin H (cycH) in complex with the RING finger protein Mat1 (Mnat1) are components of TFIIH, which phosphorylates (P) the C-terminal domain (CTD) of RNA Pol II to induce promoter clearance and the transition from initiation to elongation during transcription (Serizawa et al., 1995; Shiekhattar et al., 1995). The phosphorylated CTD serves as a platform for the recruitment of enzymes that catalyze the addition of a methylguanosine cap to the 5' end of the emerging transcript. Cdk8 and cyclin C (cycC), together with Med12 and Med13, are part of the Mediator complex, which functions mainly as a transcriptional repressor by: (1) phosphorylating the CTD to preclude its recruitment to promoter DNA and inhibit the assembly of the PIC (Hengartner et al., 1998; Rickert et al., 1999); and (2) phosphorylating cyclin H to negatively regulate the activity of TFIIH on the CTD (Akoulitchev et al., 2000). Cdk9 and cyclin T (cycT) are subunits of the positive transcription elongation factor b (P-TEFb), which promotes the extension of the pre-mRNA transcript by: (1) phosphorylating negative elongation factor (NELF) and DRB sensitivity inducing factor (DSIF) to release the stalling of the elongation complex; and (2) phosphorylating the CTD to engage its RNA polymerizing activity (Fu et al., 1999; Peng et al., 1998). Cdk11/cyclin L (cycL) interacts with a variety of elongation factors to facilitate transcription elongation, including El1, TFIIF, FACT (Trembley et al., 2002). In addition, Cdk11/cyclin L is involved in RNA processing co-transcriptionally through its association with and phosphorylation of factors responsible for pre-mRNA splicing, such as SC35 (Srfs2) and 9G8 (Srfs7) (Dickinson et al., 2002; Hu et al., 2003; Loyer et al., 2008; Loyer et al., 1998).
induce ETS2-mediated transcription of c-RAF (RAF1). This resulted in the activation of an alternative mitogen-activated protein kinase (MAPK) pathway that allowed tumor cells to circumvent their reliance on ERα signaling and continue dividing even in the presence of tamoxifen. The authors proceeded to highlight the clinical relevance of this finding by demonstrating that breast cancer patients with ERα-positive tumors that express low levels of CDK10 (owing to methylation and silencing of the CDK10 promoter) display higher occurrence of relapse and poorer overall survival. Together with data from other groups (Leman et al., 2009; Yu et al., 2012; Zhong et al., 2012), there is now compelling evidence to suggest that Cdk10 might function as a tumor suppressor in normal cells by inhibiting the oncogenic potential of its interacting partner Ets2. Whether Cdk10 has other physiological roles in addition to the suppression of Ets2 transactivation activity remains to be determined.

Numerous studies have also suggested a transcriptional role for cyclin D1 (reviewed by Coqueret, 2002). Most of these were postulations derived from in vitro assays and cell culture experiments. However, elegant work to define the complete repertoire of cyclin D1-interacting partners in vivo has now firmly secured the status of cyclin D1 as a regulator of transcription (Bienvenu et al., 2010). Using Flag- and hemagglutinin (HA)-tagged cyclin D1 knock-in mice, pull-downs were performed in selected cellular compartments and binding proteins were identified by mass spectrometry. Among the interactors was a significant representation of transcriptional regulators in addition to the expected cell cycle partners. To address a possible transcriptional role for cyclin D1, chromatin immunoprecipitation coupled with DNA microarray analysis (ChIP-chip) was employed for the genome-wide mapping of DNA binding sites. Remarkably, cyclin D1 was found to be associated with >900 promoter regions that collectively bear DNA-recognition motifs for transcription factors Nfy, Stat (Soat1), Creb2 (Atf2), Elk1, Znf423 and Cux1. Physical interaction between cyclin D1 and each of these transcription factors was later established and suggested to be essential for bringing cyclin D1 to gene promoters in a sequence-specific manner. Clearly, cyclin D1 plays a key role in the regulation of transcription and this was exemplified in the development of the retina, where cyclin D1 associates with the upstream regulatory element of the Notch1 gene. At this genomic locus, cyclin D1 is poised for the recruitment of chromatin-modifying enzymes such as the CREB binding protein (CBP; Crebbp) where its histone acetyltransferase activity is subsequently required for the activation of Notch1 expression. More importantly, this transcriptional jurisdiction over the Notch1 gene is proven to be the underlying cause of retina defects in mice with germline deletion of cyclin D1 (Fantl et al., 1995; Sicinski et al., 1995), as the phenotype can be rescued by re-introducing the constitutively active intracellular domain of Notch1. This study illustrates how the cell cycle regulatory role of cyclin D1 can be easily compensated by closely related family members, but the transcriptional role of cyclin D1 in specific tissues is exclusive and independent of its association with Cdkks. In addition to the retina, cyclin D1 displays non-redundant functions in mammary glands (Fantl et al., 1995; Sicinski et al., 1995) and it would be interesting to determine whether similar modulation of transcriptional programs takes place in this tissue.

The Cip/Kip family of CKIs (p21Cip1, p27Kip1 and p57Kip2) represents another group of proteins that have deviated from their role in cell cycle control to become regulators of transcription. They bind directly to components of the transcriptional machinery and, analogous to the interaction with Cdk/cyclin complexes, this association is usually inhibitory. p21 is known to interact with a range of transcription factors involved in various biological processes (reviewed by Besson et al., 2008; Dotto, 2000). Specifically, its direct association with and inhibition of E2F proteins complements its effect on Cdk/cyclin complexes to augment the repression of E2F-responsive genes and induce efficient cell cycle arrest (Delavaux and La Thangue, 1999; Devgan et al., 2005; Dimri et al., 1996). p27 also participates in a number of cellular functions through its ability to localize at multiple gene promoters with p130-E2F4 and enhance the recruitment of transcriptional co-repressors such as Sin3A and histone deacetylases (Pippa et al., 2012). Although members of the Cip/Kip family modulate the expression of numerous genes, their influence over cell fate when stationed at genes involved in self-renewal or differentiation is perhaps the most significant impact of Cip/Kip-dependent transcription (see later section). Besides transcription, Cip/Kip proteins display essential roles in the regulation of apoptosis and actin cytoskeletal dynamics (Besson et al., 2008), topics that are not covered here owing to space constraints. However, it is important to point out that these effects are also attributed to the suppression of key components in the respective pathways. Therefore, even though Cip/Kip proteins were originally described as inhibitors of Cdk/cyclin complexes, they should really be regarded as general repressors...
within the cell. This unique ability to sequester a wide diversity of proteins is probably due to their conformational flexibility, which renders them extremely malleable and capable of fitting snugly with the targets they are bound to (Adkins and Lumb, 2002; Esteve et al., 2003; Lacy et al., 2004; Russo et al., 1996). In future studies, deciphering the regulatory mechanisms that control the specificity and availability of Cip/Kip proteins will enable us to understand better their involvement in normal development as well as in diseases.

**Cdk5, cyclins and CKIs involved in DNA damage repair**

The cell cycle is adorned with DNA damage checkpoints that halt cell cycle progression in response to DNA damage so that DNA repair can be initiated and faithful transmission of genetic information can occur. The DNA replication checkpoint ensures that the genome is accurately duplicated before progression into mitosis, and the spindle assembly checkpoint delays anaphase onset until all chromosomes are properly aligned. Components of these checkpoints act on cell cycle regulators to elicit cell cycle arrest as part of the DNA damage response (DDR). However, recent studies have suggested that members of the Cdk and cyclin families can modulate the DNA repair machinery and contribute to the maintenance of genome integrity (Fig. 5). For example, cyclin E1 accumulates at stalled replication forks to prevent the dissociation of Cdc6 and promote the activation of Chk1 (Chek1), which initiates the replication stress signaling cascade (Lu et al., 2009). Cyclin D1 localizes to DNA double-strand breaks (DSBs) to induce the recruitment of Rad51, which activates homologous recombination (HR)-mediated DNA repair (Jirawatnotai et al., 2011; Li et al., 2010). In addition to HR, DSBs can be repaired by the error-prone non-homologous end-joining (NHEJ). Although Cdk kinase activity is dispensable for the function of cyclin D1 in HR, it is necessary for the commitment to HR over NHEJ. By phosphorylating yeast Sae2 and Dna2, Cdk1 triggers DNA-end resection, which is the initial step in HR and therefore participates in the pre-selection of DNA repair pathways (Chen et al., 2011; Huertas et al., 2008). The cell cycle-dependent fluctuations in Cdk1-associated kinase activity might thus explain why HR, which requires identical sister chromatids to be present as template to guide repair, is restricted to G2/M whereas NHEJ operates in G1. The functional significance of cell cycle regulators in the control of DNA repair is further underscored by the discovery that post-mitotic neurons transit from G0 to G1 in order to activate the NHEJ repair machinery. Cell cycle re-entry is mediated by Cdk3/cyclin-C-dependent phosphorylation of Rb, which is sufficient for progression through early G1 but not for entry into S phase, a move that would have induced apoptosis (Tomashevski et al., 2010). As neurons are long-lived and thus under prolonged insult by reactive oxygen species, an efficient system for the repair of DNA lesions is particularly important for survival and normal functioning in these cells. It would be interesting to determine how neurons safeguard their genome integrity through DNA repair but at the same time avoid getting
killed from cell cycle activation, as failure in either mechanism can lead to tumor initiation or neurodegeneration, respectively.

Transcriptional regulators of the Cdk and cyclin families are also involved in DNA repair. Cdk9/cyclin K interacts with Atr, Atrip and claspin and reduces the breakdown of stalled replication forks by limiting the amount of single-stranded DNA (Yu et al., 2010). Cdk12/cyclin K controls the expression of several DDR genes (Blazek et al., 2011). Consistent with its broad role in the maintenance of genome stability, dysregulation of CDK12 has been detected in various tumors. For example, CDK12 is one of the most frequently mutated genes in ovarian cancer, a disease driven by defective HR (Bell et al., 2011). As crippling mutations were concentrated in the kinase domain, the kinase activity of Cdk12 is assumed to be important for the suppression of malignant transformation. The identification of Cdk12/cyclin K substrates that function in the transcriptional activation of DDR genes will remain an important task for the future.

**Cdns, cyclins and CKIs regulating proteolytic degradation**

Orderly cell cycle transitions are made possible by the cyclical synthesis and destruction of cyclins. The periodic expression of cyclins is achieved by the cell cycle-dependent activation of the transcription factors E2F and FoxM1, whereas the oscillating proteolysis of cyclins is mediated through the concerted actions of two E3 ubiquitin ligase families: the Skp1-Cul1-F-box protein (SCF) complex, which operates from late G1 to early M phase, and the anaphase-promoting complex/cyclosome (APC/C), which functions at anaphase until the end of G1 phase (Bassermann et al., 2013; Nakayama and Nakayama, 2006). Direct involvement of cell cycle regulators in the ubiquitin-proteasome machinery had not been reported until a recent breakthrough in efforts to assign a biological role to cyclin F identified it as an authentic F-box protein. Cell cycle-dependent fluctuations in cyclin F levels cause corresponding changes in the activity of SCF^Cyclin F_. Because the cyclin box forms the substrate recognition module, cyclin F recruits substrates to SCF for ubiquitination in a manner analogous to cyclins bringing substrates to Cdk for phosphorylation (Fig. 6) (D’Angiolella et al., 2013). Unlike other F-box proteins, which require prior phosphorylation to bind substrates, this distinctive mode of substrate recognition enables cyclin F to target a different subset of proteins. CP110 (CCP110), a protein involved in centrosome duplication, interacts with cyclin F. Timely ubiquitin-mediated proteolysis of CP110 by SCF^Cyclin F_ is crucial for the maintenance of centrosome homeostasis and mitotic fidelity (D’Angiolella et al., 2010). Ribonucleotide reductase family member 2 (RRM2) is also a substrate of SCF^Cyclin F_ (D’Angiolella et al., 2012). RRM2 is a subunit of ribonucleotide reductase (RNR), which catalyzes the conversion of ribonucleotides to deoxyribonucleotides (dNTPs) that are used for DNA synthesis during replication and repair. Balanced pools of dNTPs are important to prevent misincorporation during DNA synthesis, whereas elevated amounts of dNTPs are required to satisfy increased demands during DNA repair. By carefully modulating the availability of RRM2 in accordance with cell cycle progression and genotoxic stress levels, cyclin F-mediated degradation of RRM2 aids in the preservation of genome integrity and the execution of DNA repair. In summary, the scenario presented here illustrates how the periodic expression of a cyclin member can be exploited in a cell-cycle independent system, the ubiquitin-proteasome pathway, to achieve similar fluctuations in activity.

**Cdns, cyclins and CKIs linked to epigenetic regulation**

The versatile members of the Cdk and cyclin families have now extended their foothold into epigenetic regulation (Fig. 7). Enhancer of zeste homolog 2 (EZH2), a member of the Polycomb-group (PcG) family, is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which plays a key role in global transcriptional gene silencing through the addition of the repressive histone H3 lysine 27 trimethylation (H3K27me3) mark. CDK1- and CDK2-dependent phosphorylation of EZH2 at threonine 350 (T350) positively regulates its methyltransferase activity and augments its suppression of target loci, which consist of genes involved in lineage specification (Chen et al., 2010). The net effect of this modification is increased cell proliferation, which is consistent with the role of Cdk/cyclin complexes in driving cell cycle progression. Ezh2-T350 phosphorylation by Cdns was also validated in a separate study and is suggested to promote the binding of Ezh2 to Hotair and Xist, non-coding RNAs responsible for bringing PRC2 to target loci (Kaneko et al., 2010). Because the kinase activity of Cdk1 and Cdk2 peaks at S-M phase, the enhancement of the methyltransferase activity of Ezh2 during this period of the cell cycle ensures that H3K27me3 is incorporated into newly synthesized histones after S phase and is inherited by daughter cells during M phase (Zeng et al., 2011).

By contrast, there are reports claiming that Cdk-dependent phosphorylation of Ezh2 on a different residue (T487 in mouse) produced the exact opposite effect and either disrupted the binding of Ezh2 to other PRC2 components such as Suz12 and Eed (Wei et al., 2011) or targeted it for ubiquitin-mediated degradation (Wu and Zhang, 2011). The end result is a decline in H3K27 trimethylation, de-repression of Ezh2 target genes, and induction of differentiation. Phosphorylation at T487 might form part of a negative-feedback...
of glucose to form glycogen (Tudhope et al., 2012). However, phosphorylation of a separate threonine residue (T350) promotes the binding of EzH2 to the noncoding RNAs (ncRNAs) Hotair and Xist and facilitates trimethylation of histone H3 lysine 27 (H3K27). Cdk4/cyclin D (cycD)-dependent phosphorylation of Mep50 serves to activate its interactor Prmt5 and the dimethylation of histone H3 arginine 8 (H3R8) and H4 arginine 3 (HR3). In addition to histone modification, DNA methylation at CpG dinucleotides is increased by Cdk1-, Cdk2- and Cdk5-mediated phosphorylation of Dnmt1. Me, methyl group.

**Cdk, cyclins and CKIs controlling stem cell self-renewal**

Cell cycle control and stem cell self-renewal are two closely related processes. It is well-established that pluripotent embryonic stem cells (ESCs) possess a distinctive mode of cell cycle regulation characterized by rapidly alternating rounds of S and M phases that are interspersed by short gap phases (Becker et al., 2006; Burdon et al., 2002; Singh and Dalton, 2009). This property enables them to undergo the massive expansions in cell number necessary in early embryogenesis. As development proceeds, a gradual decline in the overall rate of cell cycle progression (which is mainly attributed to a lengthening of G1) accompanies the acquisition of more restricted cell fates in committed progenitors, ultimately culminating in complete cell cycle withdrawal as post-mitotic cells are generated. Considering the correlation between cell cycle kinetics and stem cell identity, it was perhaps not too surprising when it was first reported that cell cycle regulators actively participate in the specification of cell fate. This is particularly well studied in the context of neurodevelopment, in which an increase in G1 duration caused by chemical inhibition of Cdk kinase activity (Calegari and Huttner, 2003) or germline loss of G1 kinases (Lim and Kaldis, 2012) was sufficient to trigger premature neuron formation in neural stem cells (NSCs). As such, G1 lengthening was purported as a cause, rather than a consequence, of neuronal differentiation. There is now substantial evidence supporting a direct involvement of cell cycle regulators in the determination of division outcome, i.e. proliferation versus differentiation. However, it remains unclear how prolonging G1 induces differentiation mechanistically, other than the hypothesis that because G1 is the period of the cell cycle in which cells are exposed to extrinsic differentiating stimuli, spending more time in G1 should arguably lead to an accumulation of cell fate determinants to levels sufficient for them to exert an effect (Dehay and Kennedy, 2007; Götz and Huttner, 2005). Although this has been a compelling explanation thus far, recent studies are beginning to shed light on how changes in Cdk activity can modify intrinsic cell factors to influence cell fate.

Because the switch to an alternative cell type during differentiation requires drastic alterations in gene expression, cell cycle regulators are consistently suggested to target transcription factors as an effective means to evoke such global changes in transcriptional programs (Fig. 8). For example, positive regulators of cell cycle progression can either activate self-renewal factors or inhibit differentiation factors to maintain stemness. Cdk1 was reported to pair with Oct4 (Pou5f1), a transcription factor crucial for the establishment of pluripotency in ESCs, to repress Cdx2 expression and prevent differentiation into the trophectoderm lineage (Li et al., 2012b). In NSCs, Cdk kinase activity is required for the multi-site phosphorylation of Neurogenin2 (Ngn2; Neurog2), a proneural basic helix-loop-helix (bHLH) transcription factor; this reduces the affinity of Ngn2 for E box DNA in a dose-dependent manner and inhibits the expression of neurogenic genes (Ali et al., 2011). The presence of several consensus sequences for
Cdk phosphorylation on Ngn2 is particularly interesting as collectively they form a means of detecting the level of Cdk kinase activity in order to balance neural progenitor maintenance and neuronal differentiation in accordance with cell cycle length (Hindley and Philpott, 2012). In myoblasts, Cdk-dependent phosphorylation of MyoD (Myod1), a bHLH transcription factor involved in myogenic differentiation, enhances its turnover through ubiquitin-mediated degradation and promotes the maintenance of a proliferative state (Song et al., 1998).

In contrast to Cdks and cyclins, negative regulators of cell cycle progression activate differentiation factors or inhibit self-renewal factors to induce differentiation. For example, p27 also impinges upon Ngn2 in NSCs but, contrary to the impairment of function associated with the phosphorylation by Cdk/cyclin complexes, p27 interacts with Ngn2 to stabilize it and consequently allow it to enhance the expression of proneural genes required for neurogenesis (Nguyen et al., 2006). The effects of Cdk phosphorylation on MyoD can similarly be counteracted by association with p57, which in turn promotes the accumulation of MyoD and the transactivation of muscle-specific genes (Reynaud et al., 2000). Two separate studies have recently shown that the binding of p21 and p27 to the enhancer of Sox2, which encodes an HMG-box transcription factor essential for the maintenance of stem cell identity, is key to its transcriptional silencing so that differentiation can be initiated in NSCs and ESCs (Li et al., 2012a; Marqués-Torrejón et al., 2013). Taking into consideration the extensive involvement of Cdks, cyclins and CKIs in the specification of cell fate (Fig. 8), the longstanding view that cell cycle regulation revolves around the coordination of events required for the duplication of a cell (e.g., DNA replication, mitosis and cytokinesis) should only be applied to unicellular organisms, in which the outcome of cell division is purely the production of two identical daughter cells. In multicellular organisms, the decision to divide has to be integrated with external environmental cues and internal cellular status to define the type of daughter cells generated during cell division. In these instances, cell cycle regulators are endowed with additional responsibilities that will ensure the timely production of appropriate cell types during the course of development. This is probably why higher organisms have acquired additional cell cycle members such that each can be specialized for eliciting particular responses in specific organs. In time, sophisticated analysis on an organismal level is bound to uncover additional links between the cell cycle and self-renewal/differentiation machineries.

Cdk, cyclins and CKIs in neuronal function

Cdk5 is an unconventional member of the Cdk family that has long been implicated in various aspects of neuronal function, including neuronal migration, axon guidance, and synaptic transmission (reviewed by Su and Tsai, 2011). Consistent with its importance in post-mitotic neurons, Cdk5 partners with the neuro-specific proteins p35 and p39 (Cdk5r2) to activate its kinase activity, rather than with cyclins, which are usually expressed only in dividing cells. Calpain-mediated cleavage of p35 (to p25) and the subsequent hyperactivation of Cdk5 were found to be associated with neuronal death in several neurodegenerative diseases (Patrick et al., 1999). This fueled an intensive search for targets of Cdk5 that are affected under these pathological conditions. Two recently characterized substrates are apurinic/apyrimidinic endonuclease 1 (Apel; Apex1) and endophilin B1 (EndoB1; Sh3glb1) (Fig. 9). Cdk5-dependent phosphorylation of Apel reduces its ability to function in base excision repair and causes death of neurons following excessive DNA damage (Huang et al., 2010). Cdk5-dependent phosphorylation of EndoB1 also results in neuronal loss through the induction of autophagy and the accumulation of autophagosomes (Wong et al., 2011). With each new addition to the ever-growing list of Cdk5 substrates, we gain a little more insight into the pathogenesis of neurological disorders associated with deregulated Cdk5 and a better appreciation of the magnitude of the involvement of Cdk5 in the maintenance of proper neuronal function.

Although it is generally believed that Cdk5 does not bind to members of the cyclin family, a recent study suggests that Cdk5 can still live up to its name as a cyclin-dependent kinase and pair with cyclins if they are made available in the terminally differentiated neurons. Using Flag- and HA-tagged cyclin E1 knock-in mice, high levels of cytoplasmic cyclin E1 were detected in association with Cdk5 in non-proliferating cells of the adult...
Fig. 9. Cell cycle regulators in control of neuronal function. Cdk5 in complex with p35 is tethered to the cell membrane, and their associated kinase activity is crucial for maintaining normal neuron physiology. Aberrant cleavage of p35 results in: (1) the formation of p25, a truncated form with a longer half-life than p35; and (2) the loss of membrane targeting, thereby allowing Cdk5 to gain access to nuclear substrates. Recent studies have identified two novel substrates phosphorylated by hyperactive Cdk5/p25: apurinic/apyrimidinic endonuclease 1 (Apel) and endophilin B1 (EndoB1). Phosphorylation (P) of Apel inhibits DNA repair whereas phosphorylation of EndoB1 induces the formation of autophagosomes. Both are responsible for increased neuronal death in neurodegenerative diseases. Cdk5 also partners with cyclin E (cycE) but this interaction sequesters Cdk5 away from its activator p35. Cdk5/p35 and Cdk14/cyclin Y (cycY) complexes also play important roles in presynaptic vesicle trafficking as well as in synaptic elimination and formation during synaptic remodeling. Note that the length of the axon is greatly reduced for simplicity (dashed lines).

Cdns, cyclins and CKIs regulating spermatogenesis

The importance of cell cycle regulators in the control of spermatogenesis has been revealed as many mutant mice lacking components of the cell cycle machinery are sterile. These include cyclin A1 (Liu et al., 1998), Cdk2 (Berthet et al., 2003; Ortega et al., 2003) and Cdk4 (Rane et al., 1999; Tsutsui et al., 1999) knockouts. However, the precise mechanism underlying their non-redundancy in meiosis and the events leading up to the formation of mature spermatozoa has largely remained a mystery. A glimpse of light in this darkness was offered by the meticulous characterization of the role of Cdk16/cyclin Y in the terminal differentiation steps of spermatogenesis (Mikolcevic et al., 2012). Cdk16 knockout mice are sterile and, although the testis contained all the cell types at different stages of spermatogenesis, closer examination of the spermatozoa revealed multiple abnormalities, including dyskinesia, aberration in annulus structure, and malformed sperm heads. Collectively, these defects impair the function of the spermatozoa and contribute to infertility. Hopefully, a growing understanding of how cell cycle regulators participate in male germ cell development will spur the formulation of more effective therapies for the treatment of reproductive dysfunction in humans.

Conclusions

It is now evident that Cdns, cyclins and CKIs are more than just regulators of the cell cycle. They are multifaceted proteins with important functions in processes that are distinct from the main events in cell division. However, rather than labeling these as 'cell cycle-independent roles', it should be appreciated that the majority of these emerging functions are closely intertwined with the cell cycle. For example, cell cycle regulators modify transcription to achieve differential expression of gene clusters appropriate for the proliferative status of the cell; they pre-select DNA repair mechanisms to utilize the most appropriate form of repair in accordance with the period of the cell cycle; they control degradation to ensure timely destruction of cell cycle proteins; they activate methyltransferases to impart epigenetic marks onto newly synthesized histones and DNA; they vary metabolic pathways to

Brain (Odajima et al., 2011). However, partnership with cyclin E1 is inhibitory as it sequesters Cdk5 away from its authentic activators p35 and p39. Additionally, ablation of cyclin E1 derepresses Cdk5 and causes impaired synapse function and memory deficits in mice. These results reveal an unexpected role for cyclin E1 as a Cdk5 antagonist, and highlight its cell cycle-independent function in the formation of synaptic circuits and memories. Together with a previous report demonstrating a kinase-independent role of cyclin E1 in the loading of mini-chromosome maintenance (MCM) proteins during DNA replication origin licensing (Geng et al., 2007), there is now convincing evidence supporting a function for cyclin E1 beyond cell cycle regulation.

Abundant expression of Cdk16, a newly identified member of the Cdk family, was also detected in post-mitotic brain cells (Besset et al., 1999). Together with its regulatory subunit cyclin Y, Cdk16 is important for polarized trafficking of presynaptic vesicles and synapse elimination during neural circuit re-wiring in nematodes (Ou et al., 2010; Park et al., 2011) (Fig. 9). Whether these findings are translatable to mammalian neurons awaits further investigation. Based on both studies, the effects of Cdk16/cyclin Y on synapse function are either parallel or complementary to those elicited by Cdk5/p35. It is interesting to note that Cdk5 and Cdk16 can be targeted to the plasma membrane via the N-myristoylation of their activators p35 and cyclin Y, respectively, implying that membrane tethering might be key to their neuronal function. In conclusion, it appears that the restricted expression pattern of Cdns and cyclins in non-proliferating tissues is often indicative of a physiological role beyond cell cycle regulation.

Although the classical cell cycle regulators have been neglected in the analysis of post-mitotic neurons, it is important to point out a caveat: the view that cell cycle regulation in a non-dividing cell is meaningless may no longer be justified. In fact, studies have suggested that mature neurons are in a constant struggle to keep their cell cycle in check and negligence in this surveillance often leads to death of neurons following cell cycle re-initiation (Herrup and Yang, 2007). Further probing into how control of the cell cycle affects neuronal survival could potentially place cell cycle regulators at the center of neurodegenerative disorders.
supply the necessary energy level for driving cell cycle events; and they target self-renewal or differentiation factors to dictate the outcome of cell division in stem cells. In systems that are not directly cell cycle-related, the characteristic fluctuation in the activities of cell cycle regulators can be reused for different purposes. For example, the changing activities of Cdk/cyclin complexes are valuable to the attainment of orderly progression through the transcription cycle mediated by RNA Pol II. In view of the tremendous amount of new information generated in recent years, the study of cell cycle regulators is certainly a far cry from being a mature field and the continuous pursuit towards understanding the complete repertoire of their physiological functions is bound to unveil many more surprises along the way.

Acknowledgements
We apologize to researchers whose work laid the foundation for topics covered here but are not cited owing to space constraints.

Funding
The Kaldis laboratory is funded by the Biomedical Research Council of A*STAR (Agency for Science, Technology and Research), Singapore.

Competing interests statement
The authors declare no competing financial interests.

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