The anaphase promoting complex/cyclosome: a machine designed to destroy

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Abstract | The anaphase promoting complex/cyclosome (APC/C) is a ubiquitin ligase that has essential functions in and outside the eukaryotic cell cycle. It is the most complex molecular machine that is known to catalyse ubiquitylation reactions, and it contains more than a dozen subunits that assemble into a large 1.5-MDa complex. Recent discoveries have revealed an unexpected multitude of mechanisms that control APC/C activity, and have provided a first insight into how this unusual ubiquitin ligase recognizes its substrates.

Ubiquitin ligase (E3)
The third enzyme in a series — the first two are designated ubiquitin-activating (E1) and ubiquitin-conjugating (E2) — that is responsible for the ubiquitylation of target proteins. E3 enzymes provide platforms for binding E2 enzymes and specific substrates, thereby coordinating the ubiquitylation of selected substrates.

Polyubiquitin chains
Protein assemblies that are composed of several copies of the small protein ubiquitin. The ubiquitin residues are covalently attached to each other through isopeptide bonds.

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Cell proliferation in all eukaryotes depends strictly on the ubiquitin ligase (E3) activity of the anaphase promoting complex/cyclosome (APC/C). APC/C is a 1.5-MDa protein complex that is found in the nucleus of interphase cells, and that spreads throughout the cytoplasm and associates with parts of the spindle apparatus during mitosis. Without APC/C, cells cannot separate their sister chromatids in anaphase, they cannot exit from mitosis and divide into two daughter cells, and they cannot initiate the steps that are necessary for DNA replication later in S phase. APC/C seems to have similarly important functions in meiosis, and a number of observations imply that APC/C has also adopted new roles during the evolution of multicellular organisms in postmitotic differentiated cells (reviewed in REFS 1,2).

In all known cases, APC/C performs its various functions by assembling polyubiquitin chains on substrate proteins (Supplementary information S1 (table)), which targets these proteins for destruction by the 26S proteasome. Proteolysis is inherently associated with inactivation, which is what APC/C does in a number of cases in which its substrate protein is either a catalytic protein or an essential activator of such an enzyme. Prominent examples of these types of APC/C substrate are the mitosis-specific Aurora kinases and Polo-like kinase-1 (Plk1), and mitotic A- and B-type cyclins, the activating subunits of cyclin-dependent kinases-1 and -2 (Cdk1 and Cdk2). However, APC/C can also activate proteins, in this case, by targeting inhibitors of these proteins for destruction. Famous examples are securin, an inhibitor of the protease separase, and geminin, a protein that inactivates the replication factor CTD1.

In this article I discuss how APC/C might be able to selectively recognize its substrates, and review recent discoveries that have provided new insights into the essential mitotic functions of APC/C and its regulation during the cell cycle. But first I describe the APC/C complex and its cofactors, and their roles in APC/C-mediated ubiquitylation.

The APC/C complex and its cofactors

APC/C is composed of at least a dozen different subunits (TABLE 1), but it can only ubiquitylate substrates with the help of three cofactors, the ubiquitin-activating (E1) enzyme, a ubiquitin-conjugating (E2) enzyme and a co-activator protein.

APC/C cofactors. Like all E3 enzymes, APC/C uses ubiquitin residues that have been activated by E1 and then transferred to E2 enzymes. APC/C can collaborate with two E2 enzymes, UBCH5 and UBCH10 (which is also known as E2-C or UbcX)[3,4]. Although each of these is sufficient to support APC/C-mediated ubiquitylation reactions *in vitro*, UBCH10 orthologues in human cells, *Drosophila melanogaster* and fission yeast are essential for the initiation of anaphase, indicating that UBCH5 alone cannot fully support APC/C activity *in vivo*[5–7]. A possible exception is budding yeast, in which Ubc5 alone seems to be sufficient for APC/C function[8].

UBCH5 and UBCH10 associate with APC/C only transiently[9]. However, it is unknown whether the dynamic nature of this interaction is required for ubiquitylation reactions, as has been proposed to be the case for the ubiquitin ligase SCF[10], or whether the transient nature of APC/C–E2 interactions is simply a

Table 1 | **APC/C subunits and co-activators**

Vertebrates	*Drosophila melanogaster*	Budding yeast	Structural motifs	Functions
Subunits				
APC1/TSG24	Shattered	Apc1	RPN1 and RPN2 homology*	-
APC2	Morula	Apc2	Cullin homology	APC11 and DOC1 binding
CDC27/APC3	Makos	Cdc27	TPRs	CDH1 binding
APC4	-	Apc4	WD40 repeats	-
APC5	Ida	Apc5	TPRs	-
CDC16/APC6	-	Cdc16	TPRs	-
APC7	-	-	TPRs	-
CDC23/APC8	-	Cdc23	TPRs	-
DOC1/APC10	-	Doc1/Apc10	Doc domain	Substrate recognition, processivity
APC11	Lemming	Apc11	RING-H2 finger	E2 recruitment, E3 activity
CDC26	-	Cdc26	-	-
SWM1/APC13	-	Swm1/Apc13	-	-
-	-	Apc9	-	-
-	-	Mnd2	-	Ama1 inhibition
Co-activators				
CDC20/p55^{CDC}	Fizzy	Cdc20	C-box, WD40 repeats and IR-tail	Substrate recognition
CDH1 A–D	Fizzy-related	Cdh1/Hct1	C-box, WD40 repeats and IR-tail	Substrate recognition
-	Rap	-	C-box, WD40 repeats and IR-tail	Substrate recognition
-	-	Ama1	C-box, WD40 repeats and IR-tail	Substrate recognition
-	Cortex	-	C-box, WD40 repeats and IR-tail	Substrate recognition

*RPN1 and RPN2 are subunits of the 26S proteasome. Ama1, activator of meiotic APC/C protein-1; APC/C, anaphase promoting complex/cyclosome; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; Mnd2, meiotic nuclear division protein-2; swm/SWM, spore wall maturation; TPRs, tetratrico peptide repeats; TSG, testis-specific gene.

26S proteasome
A large multisubunit protease complex that selectively degrades multi-ubiquitylated proteins. It contains a 20S particle that carries the catalytic activity and two regulatory 19S particles.

Cyclin-dependent kinase
(Cdk). A protein kinase that has activity that depends on an association with a cyclin subunit. Cdks are essential for DNA replication and entry into mitosis.

Ubiquitin-activating (E1) enzyme
An enzyme that activates the C-terminal glycine residue of the small protein ubiquitin, allowing it to form a high-energy thioester bond to a specific cysteine residue of the E1. E1 then transfers this activated form of ubiquitin onto ubiquitin-conjugating (E2) enzymes.

Ubiquitin-conjugating (E2) enzyme
An enzyme that forms a thioester bond with a ubiquitin residue, which is transferred to the E2 enzyme from ubiquitin-activating (E1) enzyme. E2 uses the high energy from the thioester bond to generate an isopeptide bond between the ubiquitin residue and a lysine residue on a substrate protein.

SCF
A multisubunit ubiquitin ligase complex that is composed of two scaffolding subunits (cullin and Skp1), a RING-finger subunit that binds ubiquitin-conjugating (E2) enzymes and one of many F-box subunits that recruit substrates.

C-box
A sequence element (consensus DRF/YIPXR) that was first found in the N-terminal region of Cdc20. It is conserved in all known APC/C co-activators.

IR-tail
A sequence element (consensus IR) at the extreme C terminus of APC/C co-activators and the APC/C subunit Doc1.

reflection of the modularity of the ubiquitin system, in which a few E2 enzymes can interact with different E3 enzymes. Consistent with the possibility that the transient interaction is a reflection of the modularity of the ubiquitin system, UBCH5 is a highly promiscuous enzyme that can interact with several E3 enzymes, possibly in contrast to UBCH10, which so far is only known to support APC/C.

In addition to E2 enzymes, APC/C activity is also strictly dependent on one of several co-activator proteins that associate with APC/C during specific periods of the cell cycle. The best studied of these are Cdc20 and Cdh1, which are encoded by all known eukaryotic genomes, but additional meiosis-specific APC/C co-activators have been identified in yeast and *D. melanogaster* (TABLE 1). All of these proteins are characterized by the presence of sequence elements, known as the C-box[11] and the IR-tail[12,13], that mediate their binding to APC/C[11–13]. Also, all APC/C co-activators contain a C-terminal WD40 domain that is predicted to fold into a propeller-like structure, and that is now believed to recognize APC/C substrates by interacting with specific recognition elements in these substrates[14], called D-boxes[15] and KEN-boxes[16].

APC/C: a cullin–RING-finger ubiquitin ligase. APC/C is thought to be a distant relative of the ubiquitin ligase SCF because both contain subunits with cullin and RING-finger domains[17–19]. In the APC/C, the cullin domain of Apc2 is associated with the RING-finger domain of Apc11 (REFS 13,20), which in turn interacts with E2 enzymes[21,22]. Remarkably, Apc11 and the E2 enzyme UBCH5 alone can efficiently catalyse ubiquitylation reactions *in vitro*, albeit with reduced substrate specificity, indicating that other APC/C subunits are not absolutely essential for the E3 activity of APC/C[20–22]. There is no evidence that Apc11 participates directly in the transfer of ubiquitin residues onto substrates, and it is therefore generally believed that APC/C serves as a scaffold that brings E2 enzymes and substrates into close proximity (reviewed in REF. 23).

It remains a big mystery why APC/C is composed of many different subunits (12 have been identified in humans and 13 in budding yeast; TABLE 1), although it takes only four proteins to build a functional SCF (reviewed in REF. 24). Biochemical fractionation and scanning transmission electron microscopy (EM) experiments indicate that APC/C from animal cells has a mass of 1.4–1.5 MDa[25,26] (with a sedimentation

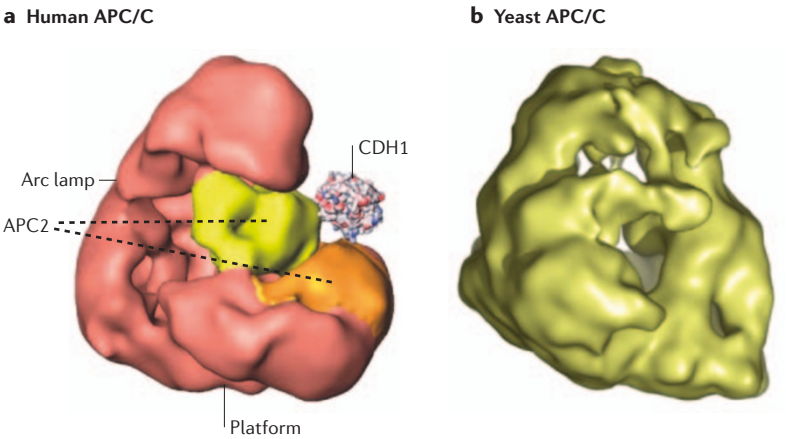


Figure 1 | **The three-dimensional structure of APC/C. a** | A three-dimensional (3D) model of human anaphase promoting complex/cyclosome (APC/C) obtained by cryo-negative staining electron microscopy (EM) and angular reconstitution. Human APC/C is composed of two large domains, known as 'platform' and 'arc lamp', that are flexible with respect to their relative positions to each other. Two candidate positions where the APC/C subunit APC2 might be located (as identified by antibody labelling) are shown, and a propeller domain similar to the one found in the co-activator CDH1 has been projected into the position where CDH1 is thought to bind. It is not known where CDC20 binds to APC/C. Reproduced with permission from REF. 26 © (2005) Elsevier. **b** | A 3D model of budding yeast APC/C obtained by cryo-EM and angular reconstitution. The models of human and yeast APC/C have similar triangular shapes, similar dimensions and both contain an internal cavity, but they differ in many structural details. Reproduced with permission from REF. 28 © (2005) Elsevier.

coefficient of 22S[27]), which places APC/C in the same size class as the ribosome, the 26S proteasome and chaperone complexes. Yeast APC/C is estimated to have a mass of 1.7 MDa[28] and can form even larger 36S dimers[12,167]. *In vitro*, the specific activity of these dimers is higher than that of monomers[28], which raises the interesting possibility that APC/C might function as a gigantic 3.4-MDa dimer *in vivo*.

APC/C architecture. The first structural insight into APC/C was obtained by cryo-EM of complexes purified from human cells, *Xenopus laevis* egg extracts[26,27] and budding yeast[28]. Three-dimensional modelling showed in all three cases that APC/C is an asymmetric triangular complex (200 by 230 Å in size) that is composed of an outer wall that encloses an internal cavity (FIG. 1). It has been speculated that substrate ubiquitylation might occur inside this cavity[27], but recent labelling experiments have shown that the co-activator CDH1 and the cullin domain of APC2 are located on the outside of the complex[26]. Because these proteins have been implicated in substrate recognition and ubiquitylation, respectively, it is now more plausible to think that ubiquitylation reactions occur on the outside. Vertebrate APC/C is composed of two large domains, known as 'platform' and 'arc lamp', that show a large degree of flexibility relative to each other. Interestingly, a change in their relative positions can also be observed when APC/C associates with CDH1, raising the possibility that co-activator binding might induce conformational changes in APC/C[26].

Topology of APC/C subunits. APC/C can be artificially dissociated into smaller subcomplexes[13,29,30], which has provided the first insights into the topology of its subunits (BOX 1). These studies have confirmed that Apc2 and Apc11 are essential for ubiquitylation activity[13,30], and have shown that yeast Apc2 also interacts with the small subunit Doc1 (REF. 30). However, in human cells, APC2 might not be the only binding partner of DOC1, because DOC1 binding is only reduced and not abolished if APC2 and APC11 are dissociated from human APC/C[13].

Like Doc1, Cdh1 seems to interact with more than one APC/C subunit. The TPR-domain protein Cdc27 binds to Cdh1 via its IR-tail[13,14,30], whereas Apc2 seems to interact with the C-box domain of Cdh1, either directly, or indirectly via an Apc2-binding partner, Apc11 or Doc1 (REF. 30). Interestingly, both Doc1 and Cdh1 have been implicated in substrate recognition and APC/C processivity, and their proximity to Apc2, Apc11 and the ubiquitin-charged E2 enzyme might therefore be important for catalysis of the ubiquitylation reaction.

Selective substrate recognition by APC/C

How are substrates recognized by APC/C? It has long been suspected that the essential role of co-activator proteins in APC/C activation might be to recruit substrates to APC/C, analogously to the role of adaptor proteins in SCF complexes. This view was supported by the observations that Cdc20 and Cdh1 can confer a limited degree of substrate specificity to APC/C[31–33], and that they can bind APC/C substrates, at least under certain conditions[11,34–38]. Also, the fact that Cdc20 and Cdh1 contain WD40 domains was consistent with this notion, because similar domains are found in the SCF adaptor proteins Cdc4 and β-Trcp, for which co-crystallization experiments have clearly shown that these domains bind substrates (reviewed in REF. 24).

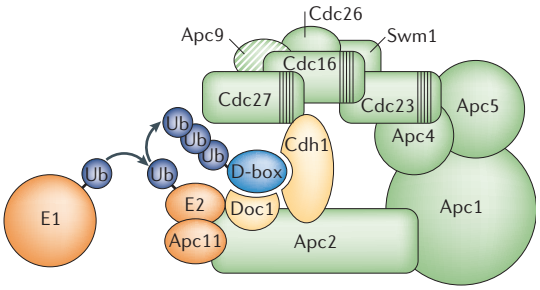
However, there were also observations and concerns that questioned the substrate–adaptor hypothesis. First, there was no agreement about which domains of the co-activators would bind to substrates[11,34–38], and, more importantly, there was no evidence that the detected association between substrates and co-activators was required for substrate ubiquitylation. The lack of evidence indicating that substrate–co-activator interactions are necessary for substrate ubiquitylation was a particular concern with regard to cyclins, because these proteins are not only substrates of APC/C but are also subunits of Cdks that are known to phosphorylate Cdc20 and Cdh1. Furthermore, it was discovered that the association of the yeast cyclin Clb2 with Cdh1 does not depend on the D-box domain of Clb2, although Clb2 degradation does[11], raising the question of whether the Clb2–Cdh1 interaction is required for Clb2 destruction. It was therefore also possible that cyclins would bind to Cdc20 and Cdh1 because the co-activators are Cdk1 substrates, and not vice versa. Last, it has been shown that cyclin B and the mitotic kinase Nek2A can bind to APC/C in *X. laevis* egg extracts in a manner that does not depend on Cdc20, the only co-activator that is present in significant amounts in these extracts[39,40], casting further doubt on the substrate–adaptor hypothesis.

WD40 domain
A propeller-shaped protein domain that is composed of sequence repeats that are ~40-amino-acid residues long and contain tryptophan (W) and aspartate (D) residues in conserved positions. In most cases, seven WD40 repeats fold into a seven-bladed propeller structure.

D-box
(Destruction-box). A sequence element (consensus RXXLXXXN) that was first discovered in the N terminus of mitotic cyclins that is required for their destruction. D-boxes can be recognized by APC/C^{Cdc20} and by APC/C^{Cdh1}.

KEN-box
A sequence element (consensus KEN) that is present in many APC/C substrates. KEN-boxes are preferentially, but not exclusively, recognized by APC/C^{Cdh1}.

Box 1 | **Model of how APC/C might recruit and ubiquitylate substrates**



Ubiquitin (Ub) is first activated and covalently bound through a thioester bond by the ubiquitin-activating (E1) enzyme and then transferred to the ubiquitin-conjugating (E2) enzyme with which the ubiquitin residue again forms a thioester bond. The ubiquitin-charged E2 enzyme interacts with anaphase promoting complex protein-11 (Apc11). This anaphase promoting complex/cyclosome (APC/C) subunit has ubiquitin ligase (E3) activity and promotes the transfer of the ubiquitin residue from the E2 enzyme to the substrate protein on which the C terminus of ubiquitin forms a covalent isopeptide bond with a lysine residue. In subsequent reactions, the attached ubiquitin can itself become ubiquitylated, resulting in the formation of a polyubiquitin chain. All proteins that are known to be involved in the catalysis of ubiquitylation reactions are shown in orange. Substrates are recruited to the APC/C if they contain a D-box or a KEN-box. Both of these sequences are recognized by an APC/C co-activator, such as Cdh1 or Cdc20. Cdh1 binds to APC/C by interacting with two subunits, Cdc27 and Apc2. Cdc27 is one of several TPR proteins that are present in the APC/C (TPR domains are shown as vertical stripes), and Apc2 is a scaffold subunit that binds to Apc11 via a cullin domain. The small globular protein Doc1 is required for processive ubiquitylation of substrates and might also interact with the D-box of substrates, although direct evidence for such an interaction is lacking. The APC/C subunits that are implicated in substrate recognition are shown in yellow. The topology of subunits is based on biochemical data in REFS 13,29,30. Note that this model illustrates subunit interactions but does not represent a structural map of where subunits are located in the three dimensional models that are shown in FIG. 1. Apc9 is hatched because so far it has only been detected in budding yeast APC/C. Human APC/C also contains another TPR subunit, APC7, and human DOC1 interacts not only with APC2 but also with another, unidentified subunit[13] (not shown here). Swm, spore wall maturation. Modified from REF. 30 © (2006) Cold Spring Harbor Laboratory Press.

How then does APC/C recognize its substrates? Several recent studies indicate that both co-activators and APC/C have important roles in this process. As in *X. laevis*[39], binding of substrates to APC/C can also be detected in budding yeast, but in this case the interaction depends on the presence of co-activators[12,41,42]. If, however, the small subunit Doc1 is removed from APC/C, the resulting APC/C$^{doc1\Delta}$ complex can no longer stably bind to substrates[12] and ubiquitylate them in a processive manner[9,43], despite the fact that Cdc20 and Cdh1 can still associate with APC/C[12]. These observations indicate that the co-activators are required but not sufficient for stable APC/C–substrate interactions, and that Doc1, and possibly other APC/C subunits, are also needed for this process. Similar observations have recently been reported for human APC/C, which can bind and ubiquitylate substrates more efficiently in the presence of Cdc20 than in its absence[44].

Co-activators, Doc1 and D-boxes: a 'ménage à trois'? How do co-activators and APC/C contribute to substrate recognition? In the case of co-activators, it has recently been found that the D-box of substrates directly contacts the WD40 domain of Cdh1 (REF. 14). Mutation of the WD40 domain compromises the capability of Cdh1 to bind substrates, and importantly, the resulting mutant APC/C^{Cdh1} complexes cannot ubiquitylate substrates in a processive manner[14]. These observations indicate that the binding of substrates to co-activators is indeed essential for efficient substrate ubiquitylation. A stoichiometric role for co-activators in substrate recruitment is also supported by the observations that APC/C, co-activator and substrate form a ternary complex[41,42], and that the amount of substrate that binds to APC/C is directly proportional to the amount of co-activator that is associated with APC/C[41].

The role of APC/C subunits in substrate recognition is more mysterious. Surprisingly, not only the interactions between substrates and co-activators but also those between substrates and APC/C seem to be D-box dependent[39,44], but the identity of the APC/C subunits that mediate these interactions is unknown. The best candidate so far is Doc1, the crystal structure of which has shown that this protein is composed of a compact globular domain with a surface that is predicted to interact with a ligand of unknown identity[45,46]. Mutational analyses have shown that this surface is essential for the capability of APC/C to ubiquitylate substrates in a processive manner[43]. Interestingly, domains that are homologous to Doc1 have also been identified in other ubiquitin ligases, implying that such 'Doc domains' might contribute to processive substrate ubiquitylation in other E3 enzymes[47,48]. In the case of APC/C, it is tempting to speculate that the unidentified ligand of Doc1 might be the substrate itself, but there are a number of other possibilities.

So far, it has been impossible to detect Doc1–substrate interactions. Indirect support for their existence, however, comes from the observation that the residual ubiquitylation activity of APC/C$^{doc1\Delta}$ is not further reduced by mutation of the D-box in substrates[43], which is consistent with the possibility that Doc1 contributes either directly or indirectly to the recognition of the D-box. The D-box might therefore interact with both the propeller domain of co-activators and with APC/C subunits such as Doc1, either sequentially or simultaneously, and therefore facilitate the formation of a ternary APC/C–co-activator–substrate complex (BOX 1). If correct, this arrangement would be strikingly similar to the domain structure of otherwise completely unrelated sugar-hydrolysing enzymes of the sialidase family. Also in

Cullin
A member of the cullin family of proteins. All cullins are subunits of SCF ubiquitin ligases or APC/C, and they bind to a RING-finger subunit via a conserved cullin domain.

RING finger
A small protein domain that binds two atoms of zinc (consensus $CXXCX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HXXCX_{(4-48)}CXXC$). Many RING-finger domains interact with ubiquitin-conjugating (E2) enzymes and have ubiquitin ligase (E3) activity.

TPR domain
(Tetratrico peptide repeat domain). A 34-amino-acid sequence repeat, clusters of which fold into a helical structure and mediate protein–protein interactions.

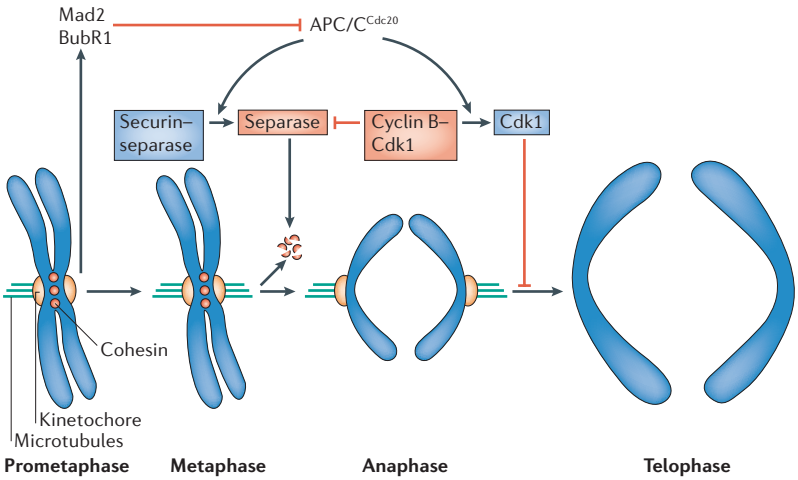


Figure 2 | **Regulation of anaphase and mitotic exit by APC/C^{Cdc20}.** During prometaphase, spindle-assembly-checkpoint proteins such as Mad2 and BubR1 are activated at kinetochores that are not (or not fully) attached with microtubules (indicated in green). Activated Mad2 and BubR1 inhibit the capability of anaphase promoting complex/cyclosome^{Cdc20} (APC/C^{Cdc20}) to ubiquitylate securin and cyclin B and thereby prevent anaphase and mitotic exit. In metaphase, when all kinetochores are attached to microtubules, APC/C^{Cdc20} ubiquitylates securin and cyclin B and thereby activates the protease separase and inactivates the cyclin-dependent kinase-1 (Cdk1). Separase then cleaves cohesin complexes (shown as red circles) that are holding sister chromatids together and thereby initiates sister-chromatid separation. Cdk1 inactivation leads to the dephosphorylation of Cdk1 substrates by protein phosphatases, and thereby enables exit from mitosis. In vertebrates, CDK1 inactivation also contributes to separase activation.

these enzymes, propeller structures that are similar to the ones found in APC/C co-activators and 'jelly-roll fold' domains that are structurally almost identical to Doc1 (REFS 45,46) collaborate in substrate binding and catalysis[49].

Essential functions of APC/C in mitosis

The capability of APC/C to selectively recognize its substrates at the correct time is essential for several key events in mitosis, the initiation of anaphase, exit from mitosis and the preparation for the next round of DNA replication.

Cyclin proteolysis. APC/C was initially discovered as a ubiquitin ligase that is essential for cyclin destruction in mitosis[25,50,51]. This process is initiated in metaphase[52] (that is, when all of the chromosomes have been attached to both poles of the mitotic spindle) and it is essential for Cdk1 inactivation and subsequent exit from mitosis (FIG. 2; REF. 53). The initiation of cyclin proteolysis depends on the form of APC/C that is associated with Cdc20 (APC/C^{Cdc20}). Although APC/C^{Cdc20} can attach ubiquitin residues to a number of different lysine residues in cyclin B in an apparently non-selective manner[54], Cdk1, the binding partner of cyclin, is spared from this modification and from the subsequent fate that cyclin experiences in the proteolytic channel of the 26S proteasome. However, once stripped off of cyclin B, Cdk1 is predicted to undergo a conformational change that prevents both ATP hydrolysis and access of protein substrates to the active site, resulting in the complete inactivation of Cdk1 (REF. 55). This situation allows protein phosphatases to dephosphorylate Cdk1 substrates, which is an essential prerequisite for disassembly of the mitotic spindle, chromosome decondensation, reformation of a nuclear envelope and formation of a cytokinetic furrow.

Cdk inactivation by cyclin proteolysis also has more far-reaching consequences for the subsequent cell cycle. A period of low Cdk activity during telophase and G1 is essential for the formation of pre-replicative complexes (pre-RCs) on origins of replication, on which DNA polymerases initiate DNA synthesis in S phase. Cdk activity inhibits the assembly of these complexes, and DNA replication is therefore strictly dependent on previous progression through mitosis when Cdk inactivation is initiated by APC/C^{Cdc20} (reviewed in REF. 56). This indirect S-phase-promoting role of APC/C is also part of the mechanism that restricts DNA replication to once per cell cycle. Because S phase depends not only on a period of low Cdk activity during which pre-RCs are assembled, but also on a subsequent phase of elevated Cdk activity during which DNA synthesis is initiated, every origin of replication can only be 'fired' once per cell cycle.

In budding yeast, cyclin degradation is the only essential S-phase-promoting function of APC/C, but, in insects and vertebrates, DNA replication also depends on the APC/C-mediated degradation of geminin, a protein that inhibits incorporation of the replication factor CDT1 into pre-RCs and thereby prevents the proper assembly of these complexes[57–60].

Sister-chromatid separation. The most well known and possibly most important function of APC/C, and the one that has lent the 'APC' its name, is its role in promoting anaphase (FIG. 2). To allow chromosome segregation, the cohesion that holds sister chromatids together first has to be dissolved. In metaphase, APC/C^{Cdc20} initiates this process by ubiquitylating securin, a small protein that functions both as a co-chaperone and as an inhibitor of the protease separase. Once activated, separase cleaves the Scc1 subunit of cohesin, a complex that holds sister chromatids together, and this cleavage therefore dissolves cohesion between sister chromatids (reviewed in REF. 61). Although securin destruction is essential for the activation of separase, budding yeast, cultured human cells and even mice can live without securin[62–65]. The implication is that there must be securin-independent mechanisms that control separase activity. One such mechanism might be the phosphorylation of separase and the subsequent stoichiometric association with Cdk1–cyclin B, which is sufficient for separase inhibition in *X. laevis* egg extracts[66,67]. APC/C^{Cdc20} might therefore contribute to separase activation by ubiquitylating both securin and cyclin B.

Life without APC/C. The genetic inactivation of APC/C has caused lethality in all species in which it has been investigated so far, ranging from fungi to mouse (REF. 68 and references therein). Because the expression of D-box mutants of securin and cyclin that cannot be recognized

Table 2 | **APC/C inhibitors**

Inhibitor	Species	Proposed to inhibit	Proposed mechanism	References
CDK1	Sc, Sp, Hs	APC/C^{CDH1} in S and G2 phase	CDH1 phosphorylation, causing dissociation from APC/C, and in Sc also nuclear export	72,76–79,154
Cdk1	Xl	APC/C^{Cdc20} in prometaphase (SAC)	Cdc20 phosphorylation, causing interaction with Mad2	155,156
SCF	Hs	APC/C^{CDH1} in S phase	CDH1 ubiquitylation/degradation	94
APC/C^{CDH1}	Sc, Hs	APC/C^{CDC20} in anaphase	CDC20 ubiquitylation/degradation	82–85
MAD2	Sc, Hs	APC/C^{CDC20} in prometaphase (SAC)	Inhibition of CDC20-substrate release	101–104,157
MAD2B*	Xl, Hs	APC/C^{CDH1} (and APC/C^{CDC20}?)	Inhibition of CDH1-substrate release	157,158
BUBR1	Hs	APC/C^{CDC20} in prometaphase (SAC)	Sequestration of CDC20	105–107
BUB1	Hs	APC/C^{CDC20} in prometaphase (SAC)	CDC20 phosphorylation	159
MAP kinase	Xl	APC/C^{Cdc20} in prometaphase (SAC)	Cdc20 phosphorylation	160
EMI1	Xl, Hs	APC/C^{CDC20} in prophase, APC/C^{CDH1} in S and G2 phase	Competitive inhibition of substrate binding to CDC20 and CDH1	90,91,118
RCA1	Dm	APC/C^{CDH1} in S and G2 phase	Unknown	88
XErp1/EMI2	Xl, Hs	APC/C^{CDC20} in meiosis II (CSF)	Unknown	139–141
Mes1	Sp	APC/C^{Cdc20} during meiosis I exit	Competitive inhibition of substrate binding to Cdc20	130
Mnd2	Sc	APC/C^{Ama1} in meiosis I	Unknown	126,127
RASSF1A	Hs	APC/C^{CDC20} in mitosis	CDC20 binding	161
Xnf7	Xl	APC/C^{Cdc20} in mitosis	Unknown, the E3 activity of Xnf7 is required	162
RAE1–NUP98	Mm	APC/C^{CDH1} in prometaphase (SAC)	Unknown, associates with APC/C^{CDH1}	163
Apoptin	CAV	APC/C^{CDC20} and APC/C^{CDH1}	APC1 binding, APC/C dissociation	164
Unknown	HCMV	APC/C^{CDH1} in G0 phase	Inhibition of the CDH1–APC/C interaction	165
E4orf4	HAV	APC/C^{CDC20}	Recruitment of PP2A to APC/C when E4orf4 is overexpressed in Sc	166

* MAD2B is also known as MAD2L2. APC/C, anaphase promoting complex/cyclosome; CAV, chicken anaemia virus; Cdk1/CDK1, cyclin-dependent kinase-1; Dm, *Drosophila melanogaster*; E3, ubiquitin ligase; EMI, early mitotic inhibitor; HAV, human adenovirus; HCMV, human cytomegalovirus; Hs, *Homo sapiens*; MAP, mitogen-activated protein; Mm, *Mus musculus*; Mnd2, meiotic nuclear division protein-2; RAE1, Rab escort protein-1; RCA1, regulator of cyclin A-1; SAC, spindle-assembly checkpoint; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Xl, *Xenopus laevis*; Xnf7, *Xenopus* nuclear factor-7.

by APC/C inhibits chromosome segregation and mitotic exit, respectively (reviewed in REF. 1), the role of APC/C in destroying securin and mitotic cyclins was generally assumed to be responsible for this lethality. However, many other proteins are also degraded in an APC/C-dependent manner (Supplementary information S1 (table)), and it is therefore possible that the stabilization of these proteins would also inhibit cell proliferation in APC/C mutants. This remains a possibility for most species, and in particular for multicellular organisms in which, for example, the degradation of geminin is required for DNA replication. However, recent studies indicate that the only essential functions of APC/C in budding yeast are indeed the degradation of securin and B-type cyclins. If the need to destroy these proteins is eliminated through genetic manipulation, yeast cells can proliferate in the absence of otherwise essential APC/C subunits[69] or its Cdc20 co-activator[70]. This is a remarkable result because it shows that the inactivation of other yeast APC/C substrates is either not essential, or that degradation is only one of several mechanisms that can inactivate these proteins.

This finding also has important practical implications because, for the first time, mutant forms of APC/C that lack essential subunits can now be generated and analysed[14,30,69]. Last, this finding lends support to the speculation that APC/C might have co-evolved either with Cdks to antagonize their activities, or with separase, to promote its activation in mitosis.

Regulation of APC/C in mitosis

Given that the inappropriate activation of APC/C could cause fatal errors in cell-cycle progression, APC/C-dependent degradation reactions are tightly controlled. Most of these mechanisms operate at the level of APC/C, often through APC/C-inhibiting proteins and enzymes (TABLE 2).

Activation of APC/C^{Cdc20} and APC/C^{Cdh1}. Like E2 enzymes, the co-activators Cdc20 and Cdh1 associate with APC/C only transiently, but in this case the interactions are tightly regulated, and the regulation of this association is one of the key mechanisms that determines when during the cell cycle APC/C is active (FIG. 3).

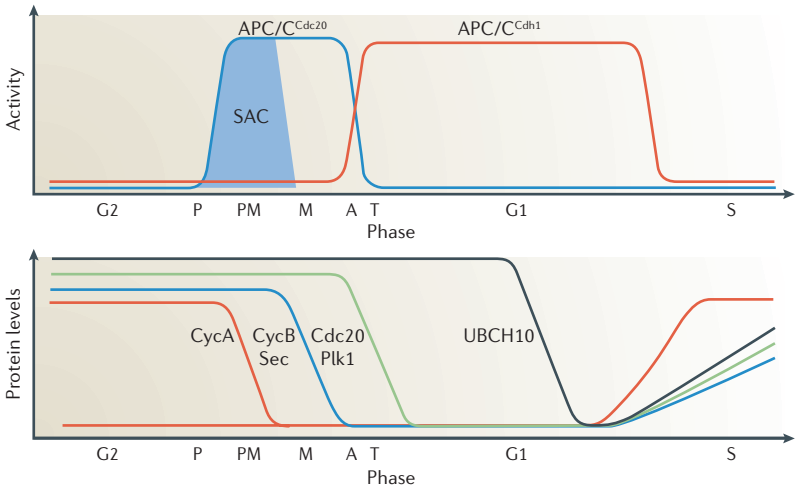


Figure 3 | **Activation of APC/C by Cdc20 and Cdh1 during the cell cycle.** Anaphase promoting complex/cyclosome^{Cdc20} (APC/C^{Cdc20}) is thought to be assembled in prophase (P) and initiates the degradation of cyclin A (CycA) already in prometaphase (PM). Proteolysis of cyclin B (CycB) and the separase inhibitor securin (Sec) also depends on APC/C^{Cdc20} but is delayed until metaphase (M) by the spindle-assembly checkpoint (SAC). During anaphase (A) and telophase (T), APC^{Cdh1} is activated, contributes to the degradation of securin and cyclin B, and mediates the destruction of additional substrates such as Polo-like kinase-1 (Plk1) and Cdc20, which leads to the inactivation of APC/C^{Cdc20}. In G1 phase, APC/C^{Cdh1} mediates the destruction of the ubiquitin-conjugating (E2) enzyme UBCH10, and thereby allows for the accumulation of cyclin A, which contributes to the inactivation of APC/C^{Cdh1} at the transition from G1 to S phase.

Cdc20 is already transcribed and translated during S and G2 phase, but it can only associate efficiently with APC/C in mitosis when several subunits of APC/C have been phosphorylated by mitotic kinases such as Cdk1 and Plk1 (REFS 71–75). By contrast, Cdh1 is prevented from efficient interaction with APC/C as long as Cdh1 is phosphorylated by different Cdks during the S and G2 phase and in the early stages of mitosis[72,76–79]. As a result, APC/C^{Cdc20} is active early in mitosis, whereas Cdh1 can only activate APC/C once APC/C^{Cdc20} has decreased the Cdk1 activity by initiating cyclin destruction, and when subsequently phosphates have been removed from Cdh1 by protein phosphatases such as Cdc14 in yeast[80].

These opposing effects of phosphorylation on APC/C^{Cdc20} and APC/C^{Cdh1} result in the switch from a high to a low Cdk state that is required for exit from mitosis and subsequent DNA replication. High Cdk1 activity in mitosis leads to the assembly of APC/C^{Cdc20}, which initiates cyclin proteolysis and decreases Cdk1 activity. This drop in Cdk1 activity promotes the formation of APC/C^{Cdh1}, which then maintains cyclin instability in G1 and enables a new round of DNA replication by permitting the assembly of pre-RCs.

Inactivation of APC/C^{Cdc20} and APC/C^{Cdh1}. APC/C^{Cdc20} is already inactivated during mitotic exit[81] (FIG. 3), presumably because APC/C dephosphorylation leads to the disassembly of APC/C^{Cdc20}, and because Cdc20 itself is a substrate of APC/C^{Cdh1} (REFS 82–85). By contrast, APC/C^{Cdh1} is inactivated later at the G1–S transition[72,76–79]. This inactivation is essential for the accumulation of APC/C substrates such as cyclins that are required for the initiation of DNA replication and subsequent entry into mitosis.

In budding yeast, the inactivation of APC/C^{Cdh1} depends on S-phase Cdks[85] that phosphorylate Cdh1 and thereby prevent its interaction with APC/C. The major cyclin that activates S-phase Cdks is Clb5, a protein that is targeted for destruction in mitosis by APC/C^{Cdc20} (REF. 70) but that does not seem to be a substrate for APC/C^{Cdh1} (REFS 31,32). It is therefore possible that the inactivation of APC/C^{Cdc20} by APC/C^{Cdh1} allows the accumulation of Clb5 during G1 phase, which then eventually leads to the inactivation of APC/C^{Cdh1} at the G1–S transition.

A similar situation might exist in *D. melanogaster* in which the overexpression of cyclin E, an S-phase cyclin that is neither a substrate of APC/C^{Cdc20} nor APC/C^{Cdh1}, is sufficient to stabilize APC/C substrates[86]. This is consistent with the possibility that cyclin E–Cdk2 inactivates APC/C^{Cdh1} by phosphorylating Cdh1. However, a second mechanism is required in *D. melanogaster* cells to keep APC/C^{Cdh1} inactive during the G2 phase. This mechanism depends on regulator of cyclin A-1 (Rca1), a protein that allows the accumulation of cyclin A during the G2 phase by inhibiting APC/C^{Cdh1} (REFS 87,88).

In vertebrates, the situation might be different (FIG. 4) because, in a purified system that is composed of human proteins, only cyclin A–CDK2 and not cyclin E–CDK2 was found to inhibit APC/C^{CDH1} (REF. 89), implying that the accumulation of cyclin A and not of cyclin E might be critical for the inactivation of APC/C^{CDH1}. However, cyclin A is itself a substrate of APC/C^{CDC20} and APC/C^{CDH1}, raising the question of how sufficient amounts of cyclin A can ever accumulate to inactivate APC/C^{CDH1}. Two mechanisms have recently been discovered that might be the solution to this problem (FIG. 4).

Vertebrate cells contain an orthologue of *D. melanogaster* RCA1, called early mitotic inhibitor-1 (EMI1), the expression of which is stimulated at the G1–S transition by the E2F transcription factor[90]. EMI1 inhibits APC/C^{CDH1} and can thereby allow the accumulation of APC/C substrates at the G1–S transition[90]. *In vitro*, EMI1 can competitively inhibit the binding of substrates to N-terminal fragments of CDC20 and CDH1 (REF. 91). However, it is not known whether this property explains the capability of EMI1 to inhibit APC/C^{CDH1} *in vivo*, because more recent evidence implies that substrates have to bind to the C-terminal WD40 domain of CDH1 to be ubiquitylated[14]. Although it is clear that EMI1 inhibits APC/C^{CDH1}, the precise mechanism of this inhibition is not yet fully understood.

A second model is based on the observations that cyclin A degradation critically depends on the levels of UBCH10 (REF. 92), and that UBCH10 itself is degraded in an APC/C^{Cdh1}-dependent manner during the G1 phase[7,92,93]. It has therefore been proposed that APC/C^{Cdh1} initiates its own inactivation by ubiquitylating UBCH10, which would lead to the stabilization of cyclin A and to the subsequent inhibition of APC/C^{Cdh1} by cyclin A–Cdk2 (REF. 92). In human cells and fission yeast, Cdh1 levels are also reduced in the S phase when Cdh1 is phosphorylated[72,78,79] due to the ubiquitylation by ubiquitin ligases of the SCF family[94].

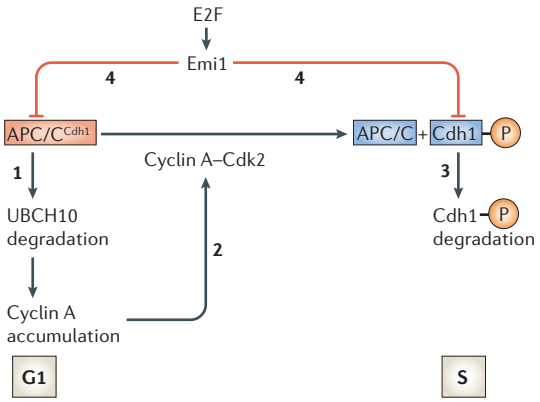


Figure 4 | **Inactivation of APC/C^{Cdh1} at the transition from G1 to S phase.** The inactivation of anaphase promoting complex/cyclosome^{Cdh1} (APC/C^{Cdh1}) at the end of G1 phase is important to allow the accumulation of proteins that are required for DNA replication and mitosis, such as cyclin A and cyclin B. Four different mechanisms have been proposed to contribute to this inactivation process in vertebrate cells. **1** | During the G1 phase, the APC/C-interacting ubiquitin-conjugating (E2) enzyme UBCH10 is itself degraded by APC/C^{Cdh1}. This process leads to the stabilization of those APC/C^{Cdh1} substrates that are ubiquitylated in a distributive manner, such as cyclin A[92,148]. **2** | Cyclin A activates cyclin-dependent kinase-2 (Cdk2), which in turn phosphorylates Cdh1 and thereby dissociates Cdh1 from APC/C[72,89]. **3** | Phosphorylated Cdh1 is ubiquitylated by SCF and thereby targeted for destruction by the 26S proteasome[94]. **4** | The transcription factor E2F activates the expression of early mitotic inhibitor-1 (Emi1), and Emi1 then inhibits the activity of APC/C^{Cdh1} (REF. 90). P, phosphate.

APC/C^{Cdc20} and the spindle-assembly checkpoint. The phosphorylation of APC/C subunits is already initiated at the beginning of mitosis in prophase, before spindle assembly has even started[75]. As APC/C phosphorylation promotes the binding of Cdc20, it is possible that APC/C^{Cdc20} is already assembled at this stage. Indeed, the destruction of several APC/C substrates such as cyclin A and Nek2A is initiated as soon as the nuclear envelope disintegrates at the transition from prophase to prometaphase (REFS 40,95–97 and references therein). If APC/C^{Cdc20} were able to initiate sister-chromatid separation and exit from mitosis already at this stage, there would not be enough time to bi-orient all the chromosomes on the mitotic spindle. To avoid this situation, the activity of APC/C^{Cdc20} is restrained by several mechanisms. The most important of these is the spindle-assembly checkpoint, which inhibits the capability of APC/C^{Cdc20} to initiate anaphase until all of the chromosomes have been bi-oriented (FIG. 2). Remarkably, the presence of a single kinetochore that is not attached to spindle microtubules is sufficient to delay anaphase[98,99], possibly by creating a diffusible signal that can inhibit APC/C^{Cdc20}. Furthermore, the spindle-assembly checkpoint can control APC/C^{Cdc20} in a substrate-specific manner, because it inhibits the capability of APC/C^{Cdc20} to ubiquitylate B-type cyclins and securin[52,81] without preventing the degradation of cyclin A and Nek2A[40,95–97].

Several proteins have been identified that are required for a functional spindle-assembly checkpoint and that are enriched on unattached kinetochores where checkpoint signalling is initiated (reviewed in REF. 100). Two of these proteins, called Mad2 and BubR1, have been shown to interact directly with APC/C^{Cdc20} *in vivo* and to inhibit its ubiquitylation activity *in vitro*[101–107]. Biochemical and structural studies have revealed that Mad2 has the remarkable ability to associate tightly, but in a mutually exclusive manner, with either Cdc20 or with another protein that is essential for checkpoint function, called Mad1 (REFS 108–110). When bound to Mad1, Mad2 is stably recruited to unattached kinetochores[108,111]. It has recently been proposed that this Mad1–Mad2 complex at kinetochores functions as a template for the assembly of Cdc20–Mad2 complexes[112]. According to this model, the Mad2 subunit of Mad1–Mad2 complexes would form dimers with diffusible Mad2 molecules and would somehow catalyse the tight association of diffusible Mad2 with Cdc20 (FIG. 5). It has been proposed that Mad2–Cdc20 complexes could then themselves function as templates for the formation of additional Cdc20–Mad2 complexes. Although speculative at the moment, this model is attractive because it could explain how the presumably weak checkpoint signal that is generated by a single unattached kinetochore could be amplified.

Although it is clear that Mad2 has an important and direct role in inhibiting Cdc20, it is not known whether Mad2 performs this function in isolation or as part of a larger protein complex. BubR1, the second checkpoint protein known to inhibit APC/C^{Cdc20}, and the related yeast protein Mad3, are part of such a complex[105–107,113]. This complex is present throughout the cell cycle and contains Cdc20, the checkpoint protein Bub3 and, according to some reports, also Mad2 (the complex that is composed of all four proteins is called mitotic checkpoint complex (MCC)[105]). Both MCC and recombinant, purified BubR1 are much more potent inhibitors of APC/C^{Cdc20} than purified Mad2 (REFS 105–107), but this observation could simply reflect the requirement for Mad1 in Mad2 activation.

For neither Mad2 nor BubR1 it is understood how they inhibit Cdc20. Mad2 can be found in association with APC/C^{Cdc20}, implying that Mad2 does not prevent binding of Cdc20 to APC/C[101,102,114,115]. For recombinant BubR1, it has been shown that its binding to Cdc20 can prevent the association of Cdc20 with APC/C[106,107]. However, it is not known whether this is the physiological mode of the function of BubR1, because activation of the spindle-assembly checkpoint also leads to association of BubR1 with APC/C[105,116,117], which is not what would be predicted if the primary function of BubR1 was to sequester Cdc20 away from APC/C.

Inhibition of APC/CCdc20 by Emi1. In *X. laevis* egg extracts and in purified systems, Emi1 cannot only inhibit APC/C^{Cdh1}, but also APC/C^{Cdc20} (REFS 91,118). By contrast, the *D. melanogaster* orthologue RCA1 seems to antagonize only APC/C^{CDH1} (REF. 88). At the beginning of mitosis, Emi1 itself is targeted for degradation by Plk1, which creates phospho-sites on Emi1 that are recognized by SCF$^{\beta TrCP}$ (REFS 119,120). It has been proposed

Checkpoint
A surveillance mechanism that delays progression through the cell cycle if processes such as DNA replication and spindle assembly have not been completed.

Kinetochore
A large proteinacous structure that assembles on centromeric DNA, binds the plus ends of microtubules and thereby connects chromosomes with spindle poles.

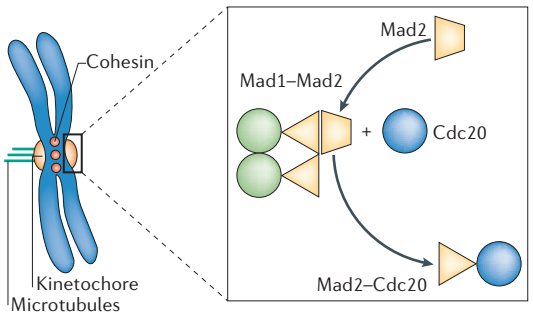


Figure 5 | **Activation of Mad2 at unattached kinetochores.** Mad2 is an inhibitor of anaphase promoting complex/cyclosome^{Cdc20} (APC/C^{Cdc20}) that is activated at kinetochores that are not (or not fully) attached to spindle microtubules. Mad2 exists in two different states, in an 'open' conformation (yellow rhomboid) in the cytosol, and in a 'closed' conformation (yellow triangle) that is recruited to kinetochores via the spindle-assembly-checkpoint protein Mad1 (green circle). The template model of Mad2 activation[112] proposes that the open Mad2 forms a conformational heterodimer with the closed Mad2 at kinetochores. This interaction somehow converts the open Mad2 molecule into a closed form that associates with Cdc20, analogously to how prion proteins propagate conformational changes through oligomerization. How Mad2 inhibits the capability of Cdc20 to activate APC/C remains unknown.

that Emi1 inhibits APC/C^{Cdc20} at the beginning of mitosis in prophase, at a time when the spindle-assembly checkpoint might not yet be active, until Emi1 itself is destroyed. This hypothesis could explain why cyclin A destruction is only initiated in prometaphase, although APC/C^{Cdc20} might already be activated by Cdk1 in prophase. The notion that Emi1 destruction is required for the timely activation of APC/C^{Cdc20} is supported by the observations that the degradation of cyclin A and other APC/C substrates is delayed in mouse cells that lack the SCF adaptor protein βTrCP, in which Emi1 cannot be degraded[121], and that cells in which non-degradable Emi1 mutants are expressed are delayed in prometaphase[122]. However, cells from which Plk1 has been depleted initiate cyclin A degradation with normal kinetics[123,124]. It remains to be seen whether Emi1 is targeted for destruction by other kinases or residual amounts of Plk1 in these cells, or whether Emi1 destruction is not essential for cyclin A proteolysis, which would be similar to the situation that is observed for RCA1 in *D. melanogaster*.

Regulation of APC/C in meiotic and endo cycles

In mitotic cell cycles, ploidy is maintained by strictly alternating DNA replication with chromosome segregation, but variations of this scheme are frequently used to generate cells with altered ploidy. In meiosis, two rounds of chromosome segregation without an intervening S phase lead to the formation of haploid germ cells, and in endoreduplication (endo) cycles, multiple rounds of S phase cause the generation of polyploid cells. In these situations, APC/C activity is regulated by additional mechanisms.

Inhibition of APC/C^{Ama1} by Cdk1 and Mnd2 in meiosis I. When budding yeast cells enter meiosis, a third co-activator, activator of meiotic APC/C protein-1 (Ama1), is expressed as well as Cdc20 and Cdh1 (REF. 125). APC/C^{Ama1} is required for sporulation and it also contributes to the degradation of the securin Pds1 and the cyclin Clb5 in anaphase of meiosis I[126]. Because Pds1 and Clb5 degradation leads to the loss of sister-chromatid cohesion and exit from meiosis, respectively, APC/C^{Ama1} is inhibited during the early stages of meiosis I by at least two mechanisms that prevent the precocious initiation of these events. During metaphase, Cdk1 contributes to the inhibition of Ama1, perhaps by directly phosphorylating Ama1. Accordingly, Cdk1 inactivation by APC/C^{Cdc20}-mediated cyclin destruction is required for the activation of APC/C^{Ama1} in anaphase I[126].

Furthermore, the inhibition of APC/C^{Ama1} during pre-meiotic S phase and prophase is strictly dependent on meiotic nuclear division protein-2 (Mnd2) (REFS 126,127), a protein that has only been identified in yeasts so far. Mnd2 is a stoichiometric subunit of APC/C both in mitotic and meiotic cells[128,129], although it is only essential for viability in the meiotic cells, in which Ama1 is expressed. Remarkably, Mnd2 only prevents the activation of APC/C by Ama1, but not the activation by Cdc20 or Cdh1 (REF. 126). How Mnd2 performs this function is unknown, as is the mechanism by which Mnd2 is inactivated at the onset of anaphase I so that APC/C^{Ama1} can become active.

Inhibition of APC/C^{Cdc20} by Mes1 in meiosis I–II. To ensure that meiosis I is followed by meiosis II, and not by S phase, cells must exit meiosis I without completely inactivating Cdks. Otherwise, pre-RCs could be assembled that would permit another round of DNA replication. In fission yeast, it has recently been shown that the small protein Mes1 functions as an inhibitor of APC/C^{Cdc20} and prevents the complete degradation of cyclins during exit from meiosis I[130]. Interestingly, Mes1 binds to the WD40 domain of Cdc20, as does the mitotic cyclin Cdc13, implying that Mes1 modulates APC/C activity by competing with cyclins for Cdc20 binding[130].

Inhibition of APC/C^{Cdc20} by CSF in meiosis II. In contrast to meiosis I, meiosis II is usually followed by another S phase, but in diploid organisms this round of DNA replication must not be initiated until two haploid germ cells have fused to form a zygote. Before fertilization, vertebrate eggs are therefore arrested in metaphase of meiosis II by an activity known as cytostatic factor (CSF)[131]. CSF inhibits APC/C[132] and thereby prevents anaphase and exit from meiosis II. The establishment, but not the maintenance, of CSF activity depends on cyclin E–Cdk2 and on a signalling pathway that involves the kinase Mos (reviewed in REF. 133). In *X. laevis* eggs, the spindle-assembly checkpoint proteins Bub1, Mad1 and Mad2 are also required for the establishment of CSF, but, surprisingly, only Mad1 is needed for its maintenance[134,135].

A second protein that was initially thought to be essential for CSF activity is Emi1 (REF. 136), but more recent studies have shown that Emi1 is unstable in meiosis II, as it is in mitotic cells, and it is therefore presumably not responsible for the inhibition of APC/C^{Cdc20} in CSF-arrested eggs[137,138]. However, a protein related to Emi1, known as XErp1 or Emi2, is stable during meiosis II until fertilization occurs and is required to maintain the CSF arrest[139–141]. Similar to Emi1, XErp1 is targeted for degradation by Plk1, but unlike Emi1, XErp1 can only be recognized by Plk1 once XErp1 has been phosphorylated by calmodulin-dependent kinase II[138,142]. This kinase is activated by a transient increase in intracellular calcium levels that occurs during fertilization, and the degradation of XErp1 and the subsequent activation of APC/C^{Cdc20} can therefore only occur once fertilization has occurred.

Role of APC/C in endo cycles. Endo cycles can lead to the formation of polytene chromosomes in which several sister chromatids remain connected, or these cycles can lead to the formation of polyploid cells. In *D. melanogaster*, ovarian nurse cells first generate polytene chromosomes but later separate their sister chromatids and thereby become polyploid. This transition from polyteny to polyploidy is affected by mutations in Morula, the *D. melanogaster* orthologue of the APC/C subunit Apc2 (REF. 143). In Morula mutants, nurse cells undergo several endo cycles, but then accumulate cyclin B, enter mitosis and remain arrested instead of transiting to the polyploid state. These observations imply that in some endo cycles APC/C activity is required to suppress entry into mitosis. Whether APC/C is constitutively active in endo cycles, or whether it has to be activated at the polyteny–polyploidy transition, remains unknown.

Substrate and spatial regulation of APC/C

APC/C activity is largely controlled by co-activators and inhibitors, but a number of observations indicate that, in addition, APC/C-mediated ubiquitylation reactions are regulated at the substrate level or by restraining these reactions to specific locations in the cell.

Regulation of APC/C substrates by phosphorylation. In most cases, APC/C seems to ubiquitylate its substrates independently of their post-translational modification state (reviewed in REF. 144). By contrast, SCF ubiquitin ligases can recognize many of their substrates only if they have been phosphorylated or hydroxylated (reviewed in REF. 24). However, it has recently been discovered that phosphorylation can protect substrates from APC/C. When human cells re-enter the cell cycle from quiescence, the replication factor CDC6 is phosphorylated by cyclin E–CDK2. The phosphorylated sites are located directly adjacent to the D-box of CDC6, and therefore prevent recognition of CDC6 by APC/C^{CDH1} (REF. 145). This mechanism allows CDC6 to accumulate before other APC/C substrates, so that CDC6 can initiate pre-RC formation before geminin and cyclin A would prevent this process. Similarly, substrate phosphorylation has been proposed to delay the APC/C-dependent degradation of Aurora-A until the end of mitosis[146].

Quiescence
The physiological state of cells that are not in the cell cycle.

Intrinsic regulation of APC/C^{Cdh1} by substrate ordering. Similar to CDC6, cyclin A accumulates earlier than other APC/C substrates at the G1–S transition[92], when cyclin A is thought to contribute to the inactivation of APC/C^{Cdh1} (REF. 89). This preferential stabilization of cyclin A as well as the phenomenon that Cdc20, Plk1 and Aurora-A are degraded in a sequential order during mitotic exit[147] have recently been attributed to kinetic differences in the ubiquitylation of these substrates[148]. According to this model, 'early' substrates are ubiquitylated in a processive manner; that is, substrates only require a single APC/C-binding event to obtain a ubiquitin chain. By contrast, 'late' substrates are modified in a distributive fashion; that is, these proteins repeatedly shuttle on and off the APC/C before a polyubiquitin chain has been assembled on them[148]. Distributive substrates would therefore be susceptible to de-ubiquitylation by de-ubiquitylating enzymes and to competition by more processive substrates, and these substrates can therefore be degraded later than processive substrates. How processively a protein is ubiquitylated is at least in part dependent on the identity of its D-box, and can therefore be an intrinsic property of substrates. Cyclin A is a distributive substrate, and this might explain why it cannot be degraded any more at the end of G1 when UBCH10 levels drop[148].

Spatial regulation of APC/C. In mitotic animal cells, a large fraction of APC/C is present in a soluble form in the cytoplasm, but APC/C and CDC20 can also be detected on different parts of the spindle apparatus. APC/C is enriched on unattached kinetochores[149], which raises the interesting possibility that APC/C might transiently interact with kinetochores to associate with inhibitory spindle-assembly-checkpoint proteins.

APC/C is also present on centrosomes and spindle microtubules[75,150], as is cyclin B[52]. In human cells, centrosomal cyclin B molecules are degraded before cytoplasmic cyclin B[52], and in syncytial *D. melanogaster* embryos, cyclin B is only degraded in close vicinity to mitotic spindles, whereas the bulk of cyclin B remains stable until the Cdh1 orthologue Fizzy-related is expressed later in development[151,152]. Interestingly, *D. melanogaster* mutants in which centrosomes dissociate from mitotic spindles can still degrade cyclin B on the detached centrosomes but no longer on the spindle[153]. It is therefore possible that APC/C^{CDC20} is locally activated at centrosomes by CDK1 and PLK1 (which are both enriched there), and that cyclin B and possibly other APC/C substrates have to move to this site to be ubiquitylated.

The discovery of APC/C: ten years on

During the decade since APC/C was discovered[25,50,51,150] we have learnt that there are more APC/C subunits, substrates and regulatory mechanisms than anyone could have predicted. Some of these groups might continue to grow in size, but the main future challenge will be to understand how all of these molecules and mechanisms actually work. Obtaining insight into how APC/C recognizes, ubiquitylates and discharges its substrates, and how

these steps are controlled, will require various approaches: quantitative *in vivo* imaging of the interactions between APC/C and its regulators and substrates in space and time; kinetic and quantitative biochemical analyses of the reactions that are mediated by APC/C; and solving the structures of APC/C-pathway components at both the atomic and the macromolecular levels by applying crystallography, NMR and high-resolution EM. These molecular approaches will have to be complemented by rigorous *in vivo* tests of our current ideas. Are all of the proposed inhibitors (TABLE 2) important for controlling APC/C activity under physiological conditions, and if not, which ones are? Reverse loss-of-function genetics will be essential to answer this important question.

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Spatiotemporal regulation of the anaphase-promoting complex in mitosis

Sushama Sivakumar and Gary J. Gorbsky

Abstract | The appropriate timing of events that lead to chromosome segregation during mitosis and cytokinesis is essential to prevent aneuploidy, and defects in these processes can contribute to tumorigenesis. Key mitotic regulators are controlled through ubiquitylation and proteasome-mediated degradation. The APC/C (anaphase-promoting complex; also known as the cyclosome) is an E3 ubiquitin ligase that has a crucial function in the regulation of the mitotic cell cycle, particularly at the onset of anaphase and during mitotic exit. Co-activator proteins, inhibitor proteins, protein kinases and phosphatases interact with the APC/C to temporally and spatially control its activity and thus ensure accurate timing of mitotic events.

Monoubiquitylation
The addition of a single ubiquitin to a target protein.

Cell cycle transitions are driven by oscillations in the activity of cyclin-dependent kinases (CDKs). These oscillations in CDK activity are often controlled by the production and degradation of cyclins, which bind to and activate CDKs. In higher eukaryotes, there are approximately 20 different CDKs and CDK-related proteins (all of which are serine/threonine protein kinases) and 4 major cyclin classes; different combinations of CDKs and cyclins regulate cell-phase-specific events such as DNA replication and mitosis[1]. The abundance of cyclins and other cell cycle regulators (such as CDK inhibitors (CKIs)) oscillates during the cell cycle as a result of controlled expression and timely proteolysis mediated by the ubiquitin–proteasome pathway[2], and this drives the forward progression of the cell cycle.

The E3 ubiquitin ligase APC/C (anaphase-promoting complex; also known as the cyclosome) controls the order of events that ensures accurate chromosome segregation during mitosis, thus contributing to the maintenance of genomic integrity. Activity of the APC/C during mitotic progression is modulated in time and space by complex and multilayered regulatory events that include co-activator binding, post-translational modification, inhibition by the spindle checkpoint (also termed the spindle assembly checkpoint or mitotic checkpoint) and compartmentalization in subcellular locations. These events regulate the activity of the APC/C to eventually promote the rapid and irreversible transition to anaphase and mitotic exit.

This Review focuses on the spatiotemporal regulatory pathways that govern APC/C function in mitosis. Substantial recent advances in defining the structure of the APC/C, its associations with E2 enzymes, and the complex spatiotemporal regulation of its activators and inhibitors make this an opportune time to summarize our current understanding.

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The APC/C ubiquitylation pathway

Ubiquitin–proteasome pathways involve the covalent attachment of multiple ubiquitin molecules to protein substrates that are targeted for degradation by the 26S proteasome complex[3]. The attachment of ubiquitin to target proteins is a three-step process catalysed by at least three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3)[4]. Ubiquitin (a small 8 kDa protein) is transferred to E1 in an ATP-dependent manner. This activated ubiquitin is then transferred to the E2 enzyme, and the E3 ligase catalyses the binding of ubiquitin to a lysine on target proteins. Binding of further ubiquitin molecules to either one of seven lysine residues of ubiquitin or its amino terminus results in the formation of polyubiquitin chains[5]. Monoubiquitylation can affect protein localization or protein–protein interactions[6]. Polyubiquitin chains linked through different ubiquitin lysines have distinct structures and influence the fate of the modified protein differently. K11- and K48-linked chains target proteins for proteasomal degradation, whereas K63-linked chains typically facilitate protein–protein interactions that are required for signalling. Polyubiquitin chains linked through K6, K27, K29 and K33 also exist, but these are less well understood[4,7–9].

The human genome encodes two E1 enzymes, at least 35 E2 enzymes and ~600 E3 enzymes. Members of the cullin–RING family of E3 ligases have key roles in many aspects of cell cycle control[10]. Of these, the APC/C plays a prominent part, as it controls progression into, through and out of mitosis by mediating degradation of key regulators at precise times. Although the APC/C is often described as becoming 'activated' at the metaphase–anaphase transition, this is an oversimplification. The APC/C is active throughout mitosis and much of the rest of the cell cycle. Under exquisitely fine regulation, it is able to show strongest targeting of specific substrates at specific points during mitotic progression (FIG. 1). We discuss below the many aspects of this regulation.

Structure of the APC/C. In 1995, the APC/C was discovered as a mitosis-specific E3 ubiquitin ligase in clam[11], *Xenopus laevis*[12] and budding yeast[13]. In recent years, much progress has been made in understanding the structural organization of the APC/C by using insect cell expression systems to reconstitute the multisubunit E3 ligase with or without its regulators[14–19].

The vertebrate 1.22 MDa APC/C is composed of 14 different protein subunits (19 subunits in total, as 5 subunits are present in 2 copies) (FIG. 2; TABLE 1). The complex is largely organized into three structural domains, called the platform, the catalytic core and the tetratricopeptide repeat (TPR) lobe (also known as the 'arc lamp' owing to its overall shape)[14,15,17,18,20,21]. The platform subcomplex forms a base to join the other subunits of the APC/C. The catalytic core subcomplex on its own cannot efficiently recruit substrates but, along with an E2 enzyme, it can provide low ubiquitylation activity to the APC/C. The TPR lobe consists of several structurally related proteins with multiple TPRs. Three other subunits, the TPR accessory factors, stabilize the APC/C subunits in the TPR lobe[14,22]. The subunits in the TPR lobe account for more than 80% of the mass of the APC/C, exist as homodimers and are required to provide important scaffolding functions to the APC/C[15,23]. Furthermore, these subunits coordinate assembly of the APC/C and mediate important interactions with regulatory proteins that modulate APC/C activity. Importantly, this region of the APC/C also interacts with an inhibitory complex called the mitotic checkpoint complex (MCC), which has a key role in regulating mitotic progression[15,23]. Together, this multisubunit E3 ubiquitin ligase cooperates with at least two E2 enzymes and one of two co-activator proteins, CDC20 or CDC20 homologue 1 (CDH1; also known as FZR1) in all eukaryotes, to recruit and ubiquitylate substrates for proteasomal degradation during mitosis.

E2 enzymes of the APC/C. In yeast and human cells, distinct E2 enzymes collaborate with the APC/C to initiate and then elongate ubiquitin chains. In yeast, Ubc1 and Ubc4 can both catalyse ubiquitin chain initiation and elongation in conjunction with the APC/C. However, Ubc4 functions preferentially in chain initiation, whereas Ubc1 favours chain elongation[24,25]. In higher eukaryotes, including vertebrates, ubiquitin-conjugating enzyme E2C (UBE2C; also known as UBCH10 and UBCX) links the first ubiquitin to substrates by binding to the RING domain of APC11 (REFS 26,27). At least *in vitro*, another initiating E2 enzyme, UBE2D (also known as UBCH5), can also fulfil this role[27–32]. Chain elongation is catalysed by UBE2S, which binds to a distinct surface of APC11 and also binds via its carboxy terminus to other components of the catalytic core and platform[26,31,33–35].

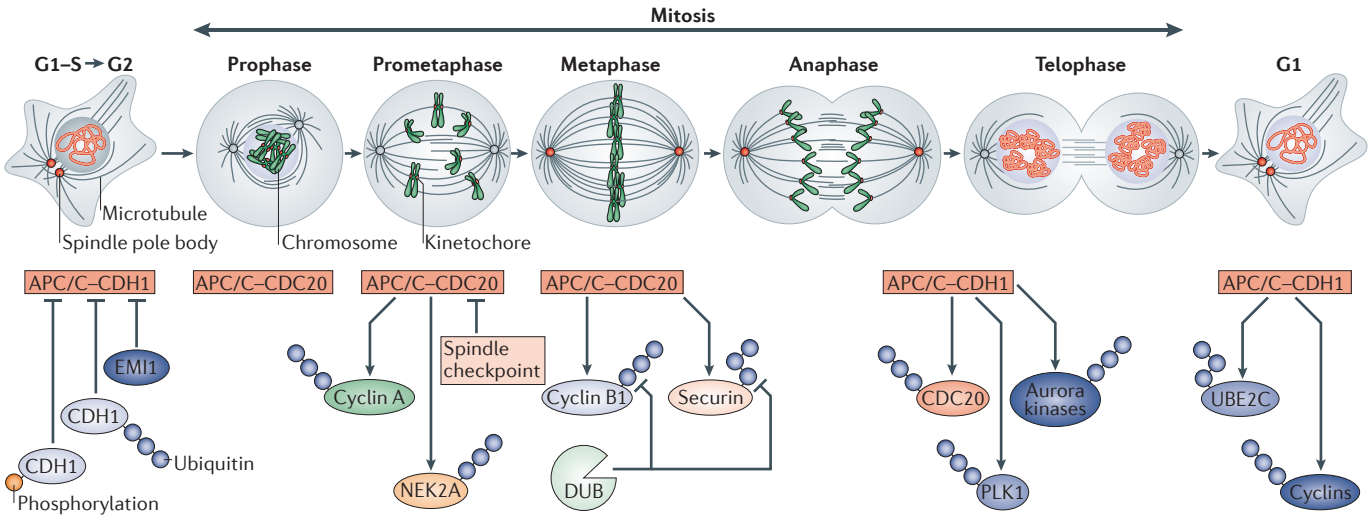


Figure 1 | **Ordered degradation of APC/C substrates.** The APC/C (anaphase-promoting complex; also known as the cyclosome) ubiquitylates proteins, marking their degradation at specific times and driving forward the progression of the cell cycle. APC/C–CDC20 ubiquitylates substrates during early and mid-mitosis, whereas APC/C–CDH1 (CDC20 homologue 1) ubiquitylates substrates after anaphase onset, during mitotic exit and in G1 phase. APC/C–CDC20 ubiquitylates cyclin A and NIMA-related kinase 2A (NEK2A) in prometaphase. During prometaphase APC/C–CDC20 activity towards late substrates, securin and cyclin B1, is suppressed by the spindle checkpoint. At metaphase, the spindle checkpoint is silenced, and ubiquitylation of securin and cyclin B1 is maximized. At mitotic exit, APC/C–CDH1 ubiquitylates CDC20, Aurora kinases and Polo-like kinase 1 (PLK1). At the G1–S transition, APC/C–CDH1 is inactivated by a combination of binding to the APC/C inhibitor early mitotic inhibitor 1 (EMI1), degradation of ubiquitin-conjugating enzyme E2C (UBE2C), CDH1 phosphorylation, and ubiquitylation and degradation of CDH1. DUB, deubiquitylating enzyme.

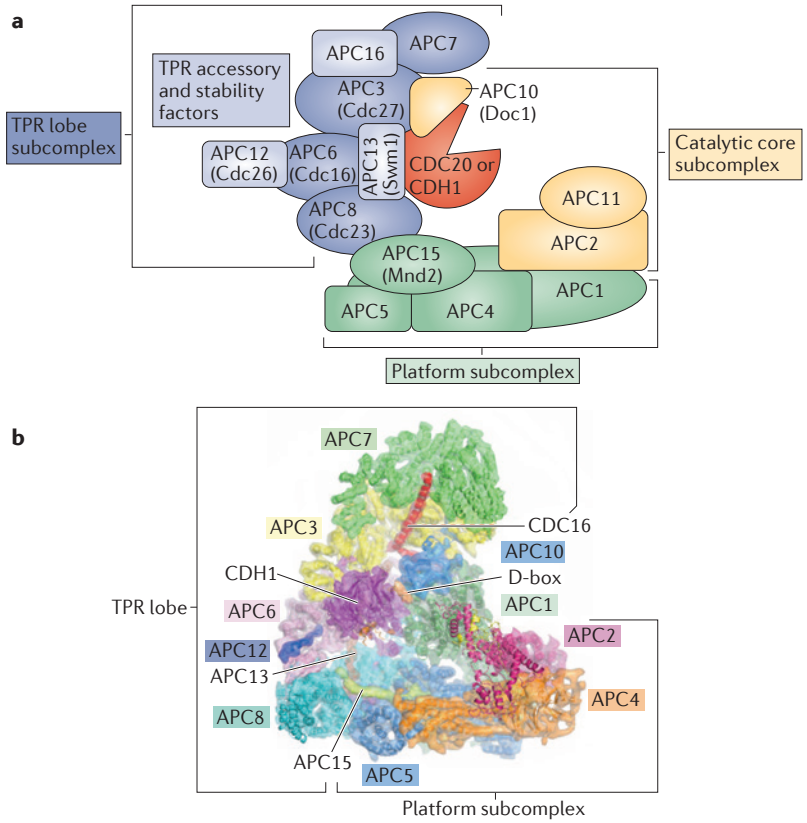


Figure 2 | **Structural organization of the APC/C.** **a** | The subunits of the APC/C (anaphase-promoting complex; also known as the cyclosome) can be largely organized into three subcomplexes: the platform (APC1, APC4, APC5 and APC15), the catalytic core (APC2, APC11 and APC10) and the tetratricopeptide repeat (TPR) lobe (APC8, APC6, APC3 and APC7) subcomplex[14]. APC/C subunit nomenclature used in yeast is shown in parentheses and TABLE 1. The APC1 subunit in the platform is the largest APC/C subunit and acts to bridge the other subcomplexes: the catalytic core and the TPR lobe[19,20]. APC2 acts as a scaffold for the catalytic core. APC11 potentiates the interaction with ubiqutin-conjugating enzymes (E2 enzymes), and APC10 forms part of the substrate-binding pocket[68,69]. The TPR lobe has multiple subunits that form homodimers and provide important scaffolding functions to the APC/C. Accessory proteins stabilize subunits in the TPR lobe: APC12 stabilizes APC6; APC13 interacts with the TPRs of APC3, APC6 and APC8; and APC16 interacts with the TPRs of APC3 and APC7 subunits[14]. Although most subunits exist as monomers, APC3, APC6, APC7, APC8 and APC12 are present as dimers. **b** | Cryo-electron microscopy reconstruction of the human APC/C–CDH1 (CDC20 homologue 1) complex depicts the location of the individual subunits along with their underlying secondary structures. Part **b** reproduced from REF. 14, Nature Publishing Group.

Although the use of two E2 enzymes is conserved throughout evolution, the linkage specificity of polyubiquitin is less conserved, and how specific linkages affect cell cycle progression in each species remains an active area of investigation. Budding yeast APC/C modifies substrates with K48-linked ubiquitin chains[25]. By contrast, in higher eukaryotes, such as *X. laevis* and humans, the APC/C primarily generates K11-linked chains or mixed K11- and K48-linked chains, which are both recognized and degraded by the 26S proteasome[7,30–33,36]. Recently, it has been shown that UBE2S can build branched ubiquitin chains by adding multiple K11-linked ubiquitins to existing ubiquitin chains linked though K48. These branched ubiquitin chains allow efficient recognition by the proteasome and can promote substrate degradation when APC/C activity is limiting[37].

Deubiquitylating enzymes (DUBs), which counteract APC/C-mediated ubiquitylation, have also been found to have important roles in mitotic control[38]. The DUBs shorten ubiquitin chain length, thereby regulating the order and timing of substrate degradation. For example, the DUB ubiquitin-specific protease 37 (USP37) removes polyubiquitin chains on cyclin A at the G1–S transition. This allows entry into the S phase[39]. The precise roles of DUBs in mitosis require further study.

Co-activators of the APC/C. The APC/C is largely inactive without one of its co-activators, CDC20 or CDH1. Their C termini contain a WD40 domain that forms a binding platform to recruit APC/C substrates[40,41]. In addition, CDC20 and CDH1 promote ubiquitylation by enhancing the interaction of the APC/C with E2-ubiquitin[14,26,35,42]. CDH1 and possibly CDC20 bind to the subunits APC3 and APC8 through interaction with TPR motifs[14].

Although structurally related, CDC20 and CDH1 activate the APC/C at different times. CDC20 associates with the phosphorylated APC/C in early mitosis and leads to the degradation of prometaphase and metaphase substrates[41,43–48]. Later, during anaphase and into G1 phase, CDC20 is replaced by CDH1. CDK1, BUB1 and MAPK phosphorylate CDC20 on multiple residues. Phosphorylation of some residues inhibit, whereas others stimulate, APC/C activity[21,45,49–53]. Phosphorylation of CDH1 by CDK1 inhibits its association with the APC/C until mid to late anaphase[45,54–56]. At that time, decreasing CDK1 activity and increased phosphatase activity results in dephosphorylation of CDH1, which then binds to and activates the APC/C, thereby causing substrate degradation in late mitosis and during G1 phase. It was also shown that CDH1 is sequestered in mitosis by mitotic arrest deficient 2-like protein 2 (MAD2L2); degradation of this protein during anaphase frees CDH1 to bind to and activate the APC/C[57].

Recent structural studies have provided valuable insights into the APC/C–CDH1–substrate–E2 enzyme complex[14,15,17,18]. The catalytic module of the APC/C, which consists of APC2–APC11, was found to be flexible[14]. Interestingly, the platform subunits of the APC/C were displaced upon co-activator–substrate binding. Co-activator binding disrupts the interaction between APC8 and APC1, which causes a downward displacement of APC8 and other platform subunits and concomitantly pushes the catalytic module (APC2 C-terminal domain APC11) upwards[14]. This change in conformation possibly increases the catalytic activity of the APC/C by bringing the initiating E2-ubiquitin close to the substrate[14,15,17] (FIG. 3a). The co-activator CDC20 binds to the C-terminal region (called the C-terminal peptide (CTP)) of UBE2S, which might aid in recruiting UBE2S to the APC/C[35]. The UBE2S CTP could then be passed to the APC2–APC4 region of the platform, towards which it shows strong affinity[26]. At a site on the APC/C that is distinct from the chain initiation site that functions through UBE2C, the UBE2S–platform interaction generates a site for ubiquitin chain elongation. This region of the APC/C also interacts with specific residues on the terminal ubiquitin of the growing chain to position it as an acceptor for the addition of the next ubiquitin[35].

Table 1 | **Subunits of the APC/C**

Budding yeast protein	Vertebrate protein	Stoichiometry	Functions	Refs
Platform subcomplex				
Apc1	APC1	1	Scaffolding	14
Apc4	APC4	1	Scaffolding; required for binding to UBE2S	35
Apc5	APC5	1	Scaffolding	14
Mnd2	APC15	1	Promotes CDC20 ubiquitylation and thus mediates disassembly of mitotic checkpoint complex	20, 127, 128
Catalytic core subcomplex				
Apc2	APC2	1	Catalytic; required for binding to UBE2S	26
Apc11	APC11	1	Catalytic; binds to initiating E2 enzyme; interacts with and activates elongating E2; recruits acceptor ubiquitin	26,35
Doc1	APC10	1	Part of degron (D-box) receptor	66,67, 69,71, 72
TPR lobe subcomplex				
Cdc27	APC3	2	Scaffolding; binds to APC10 and CDH1 or CDC20	14, 23,72
Cdc16	APC6	2	Scaffolding	14,22, 23,200
Not present	APC7	2	Scaffolding	14,23
Cdc23	APC8	2	Scaffolding; binds to CDC20	14,23
TPR accessory and stability factors				
Cdc26	APC12	2	Stabilizes APC6	14,22
Swm1	APC13	1	Stabilizes APC3, APC6 and APC8	14
–	APC16	1	Stabilizes APC3 and APC7	14

APC/C, anaphase-promoting complex (also known as the cyclosome); CDH1, CDC20 homologue 1; TPR, tetratricopeptide repeat; UBE2S, ubiquitin-conjugating enzyme E2S.

Substrate recognition sequences. Substrates have degradation sequences — known as degrons — through which they bind specifically to the APC/C–co-activator complex. Most substrates have a 9-residue D-box (RXXLXXI/VXN)[58–61] and/or a KEN-box (KENXXXN/D)[62–64]. The degrons interact with two distinct regions on the WD40 domain of co-activators[21,40,65]. D-box substrates bind to a bipartite receptor formed by APC10 and the lateral surface of the co-activator WD40 domain. APC10 enhances substrate binding and the processivity of the ubiquitylation reaction[62,65–72]. The KEN-box degrons interact with a region on the surface of the co-activator WD40 domain[62,73].

Although these degrons are required, they are not sufficient, which suggests that substrates contain additional non-conserved sequences that are required for binding to the APC/C–co-activator complex[56,62]. These additional recognition sites might be important for fine-tuning the timing of substrate degradation during the progression of mitosis[74]. There are other distantly related APC/C degron motifs, such as the O-box in ORC1 (similar to the D-box), the G-box in *X. laevis* kinesin-like DNA-binding protein (kid; similar to the KEN-box), the A-box found in Aurora kinase, the CRY-box in CDC20, and less clearly defined degrons in claspin and Iqg1 (REFS 36,75).

The timing of substrate degradation during mitosis is important to regulate proper mitotic progression. Regulators can modulate APC/C activity but, in addition, the substrates themselves are post-translationally modified to regulate their precise timing of degradation. For example, in vertebrates, phosphorylation of CDC6 (a licensing factor for DNA replication) prevents recognition by APC/C; phosphorylation of securin enhances ubiquitylation by APC/C, and phosphorylation of S-phase kinase-associated protein 2 (SKP2) causes reduced binding to CDH1 (REFS 36,76–78). Furthermore, acetylation of the spindle checkpoint protein BUBR1 (also known as BUB1β) at a lysine residue close to its KEN-box inhibits ubiquitylation, thereby inhibiting its degradation[36,79]. Localization of substrates is also important. APC/C substrates that promote mitotic spindle assembly are concentrated on spindle microtubules and are thus protected from degradation[80]. Therefore, substrates are post-translationally modified or differentially localized to regulate the timing of their degradation in mitosis.

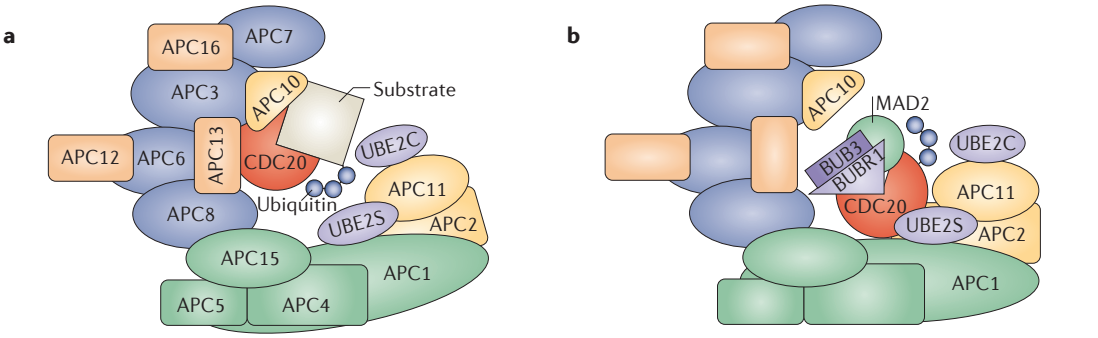
Regulation of the APC/C in early mitosis

Although the most prominent roles of the APC/C after full activation are induction of anaphase onset and mitotic exit, it is regulated to be active towards distinct substrates even in early mitosis. This early activity has important consequences for mitotic progression.

Phosphorylation and subcellular localization of the APC/C. Phosphorylated APC/C can be detected in the prophase nucleus by immunofluorescence[46]. The APC/C is phosphorylated at approximately 34 sites located on multiple subunits, and some of these phosphorylation events enhance binding of the co-activator CDC20 (REFS 46,47). Phosphorylation is predominantly catalysed by cyclin B1–CDK1, the efficiency of which is increased when CDK1 is bound to its small accessory subunit CKS[44,46,47,81,82]. The CKS proteins are conserved through evolution, bind to CDK1 and CDK2 *in vitro*, and can allow binding to a previously phosphorylated CDK consensus site through an anion-binding site[83,84]. Thus, a cyclin–CDK–CKS complex can phosphorylate one site on a substrate and remain bound, continuing to phosphorylate other nearby CDK sites. CDK1-mediated APC3 phosphorylation decreases when CKS proteins are depleted from mitotic *X. laevis* egg extracts[81,83]. Moreover, phosphorylated APC/C binds to a CKS affinity column[85], and CKS mutants in different organisms arrest in mitosis with elevated levels of mitotic cyclins[44,83,86,87].

The complex composed of cyclin B1, CDK1 and CKS is the primary but not the only kinase that phosphorylates and activates the APC/C in mitosis. Some studies

Securin
A protein inhibitor of the protease separase.



Mitotic APC/C: active towards D-box and Ken-box substrates

Mitotic APC/C bound to MCC: inhibits recruitment of D-box and Ken-box substrates but active in ubiquitylating CDC20

Figure 3 | **Conformational changes during APC/C activation and inactivation.** The APC/C (anaphase-promoting complex; also known as the cyclosome) undergoes conformational changes upon co-activator and substrate binding to bring the E2-ubiquitin close to the substrate, and this conformational activation is inhibited by the mitotic checkpoint complex (MCC). **a** | Conformational activation of the APC/C upon co-activator and substrate binding is shown. Co-activator binding disrupts the interaction between APC8 and APC1, causing a downward shift of the platform that is accompanied by an upward shift of the catalytic module (APC2–APC11). This might bring the E2-ubiquitin close to the substrate and potentiate attachment of the initiating ubiquitin[14,129]. CDC20 is also required for the activity of the chain-elongating ubiquitin-conjugating enzyme UBE2S[35]. A distinct region on APC2, near the APC2–APC4 junction, is required to bind to UBE2S[26]. The APC/C also tethers the distal molecule of an emerging ubiquitin conjugate close to UBE2S, thereby potentiating efficient ubiquitin chain elongation. **b** | APC/C bound to the MCC is shown. The MCC components inhibit the recruitment of late mitotic substrates that rely on recognition though D-box and KEN-box motifs, and hence inhibit APC/C activity towards these substrates. Mitotic arrest deficient 2-like protein 1 (MAD2) and BUBR1 bind to CDC20 and prevent its ability to recruit substrates. CDC20 as part of the MCC is also pushed downwards towards platform subunits and prevented from forming the D-box co-receptor with APC10 (REF. 15). This position of CDC20 might also facilitate its own ubiquitylation and subsequent degradation during active spindle checkpoint signalling.

have suggested that Polo-like kinase 1 (PLK1) activates APC/C in mitosis, although others have indicated that inhibiting PLK1 activity does not prevent APC/C activation[46,88]. Although specific functions for individual phosphorylation sites have not been mapped, phosphorylation is likely to affect the structure, localization and APC/C binding to activators, substrates or inhibitors during mitosis[46,89,90].

Related to and perhaps controlled by phosphorylation, localization of the APC/C to different cellular compartments is likely to be important in mitotic progression but has received considerably less attention than other aspects of APC/C regulation. The concentration of APC/C and differences in its phosphorylation could give rise to spatial regulation of APC/C at different subcellular locations. The APC/C has been reported to concentrate at centrosomes, microtubules, chromosomes and kinetochores during mitosis[89–94].

The APC/C inhibitor protein early mitotic inhibitor 1 (EMI1; also known as FBXO5) plays a major part during interphase to inhibit APC/C activity and allow accumulation of mitotic cyclins for mitotic entry. Most EMI1 is degraded through SKP1–cullin-1–F-box (SCF)-mediated ubiquitylation in the early M phase, but a small pool persists and concentrates at spindle poles via its interaction with nuclear mitotic apparatus protein (NUMA) and the dynein–dynactin complex. This complex then produces a concentrated pool of APC/C at the spindle poles. Retention of this APC/C at spindle poles requires the activity of protein phosphatase 2A (PP2A), which maintains this population of APC/C in a hypophosphorylated state. This contrasts with the bulk of cytoplasmic APC/C, which is highly phosphorylated in mitotic cells before anaphase. It was hypothesized that inhibition of the APC/C at spindle poles by EMI1 blocks hypophosphorylation local cyclin degradation, hence promoting high activity of CDK1 at spindle poles to enhance spindle assembly[89,92,94,95].

Our recent study showed that the amount of hypophosphorylated APC/C bound to mitotic chromosomes increases as cells progress to metaphase[90]. However, unexpectedly and in contrast to the predicted low activity of APC/C at centrosomes, the hypophosphorylated APC/C associated with mitotic chromosomes showed significantly higher ubiquitin ligase activity than did APC/C in the bulk mitotic cytoplasm[90]. Although these studies highlight a possible relationship between subcellular control of APC/C activity and the localization of protein kinase and phosphatase activities, they only begin to 'skim the surface' of phosphoregulation. The roles of the phosphorylations at specific sites on APC/C subunits and their dynamic changes during mitosis remain unexplored, and many questions and ambiguities remain. For example, despite the reported concentration of hypophosphorylated APC/C at spindle poles and chromosomes, an antibody made against a specific phosphorylated residue on APC1 (phospho-S355) was reported to concentrate specifically at spindle poles and unattached kinetochores[46,93]. This suggests that considerable underlying complexity associated with spatial regulation of APC/C activity in mitosis remains to be investigated.

Nuclear mitotic apparatus protein
(NUMA). A protein that partners with dynein in the assembly and maintenance of spindle poles.

Dynein–dynactin complex
A microtubule motor complex involved in the transport of spindle checkpoint proteins from kinetochores to the spindle pole.

The spindle checkpoint. At metaphase, when the last chromosome bi-orients on the mitotic spindle, APC/C-mediated ubiquitylation of securin and cyclin B1 — which are anaphase targets — is accelerated, and these proteins are rapidly degraded, resulting in chromatid separation and mitotic exit. An evolutionarily conserved mechanism called the spindle checkpoint inhibits the activity of APC/C–CDC20 until all chromosomes are bi-oriented on the mitotic spindle and are under mechanical tension from kinetochore–microtubule interactions (FIG. 1). The many protein interactions and kinase activities that catalyse spindle checkpoint signalling at kinetochores of unattached chromosomes are not discussed in detail here but have been discussed in several recent reviews[96–99].

Prometaphase substrates of APC/C–CDC20. Although the spindle checkpoint strongly inhibits the ubiquitylation of securin and cyclin B1, certain APC/C targets are efficiently degraded in prometaphase, or when the checkpoint is fully activated by arresting cells in mitosis with microtubule drugs. Within minutes of nuclear envelope breakdown, cyclin B1–CDK1 activity reaches maximal levels, and APC/C–CDC20 ubiquitylates prometaphase substrates such as cyclin A and NIMA-related kinase 2A (NEK2A; also known as NEK2), thereby targeting them for degradation by the 26S proteasome[100–104] (FIG. 1). In normal dividing cells, 80% of cyclin A and more than 50% of NEK2A are degraded before metaphase[100]. How prometaphase targets are ubiquitylated in the presence of an active spindle checkpoint is an active area of study. The primary mechanism seems to be the ability of prometaphase targets to use alternatives to the canonical D-box and KEN-box motifs to bind to the APC/C. Once these alternative substrates are modified with an initial ubiquitin moiety, elongation of the chains is carried out through UBE2S. Importantly, UBE2S activity is apparently not inhibited by spindle checkpoint signalling[35]. This allows the APC/C to elongate chains on substrates that do not require canonical D-box or KEN-box interaction with the APC/C.

Cyclin A and NEK2A can bind to the APC/C in multiple ways to promote their degradation in prometaphase. Cyclin A is bound to CDC20 in G2 phase and early mitosis. Immediately after nuclear envelope breakdown, cyclin A is targeted to the APC/C by the CKS subunit of its CDK partner, which then promotes cyclin A degradation[83,105]. Similarly, in yeast the degradation of the S phase cyclin Clb5 in early mitosis depends on its interaction with Cdk1–Cks1 and an N-terminal Cdc20-binding region[106]. Degradation of NEK2A depends on an exposed C-terminal methionine-arginine (MR) dipeptide tail. This MR tail facilitates direct binding of NEK2A to the APC/C even in the absence of CDC20. Thus, CDC20 is required for degradation of NEK2A but not for the recruitment of NEK2A to the APC/C[103,104,107].

APC/C activity and mitotic duration. Rapid degradation of securin and cyclin B1 occurs after spindle checkpoint inactivation. However, describing the APC/C as 'activated' at the metaphase–anaphase transition is an oversimplification, as the APC/C also degrades early mitotic substrates cyclin A and NEK2A[108]. Additionally, APC/C activity mediates slow degradation of cyclin B1 in prometaphase. This is countered by cyclin B1 production during mitosis[109]. Continued cyclin B1 synthesis is required to maintain cells in mitotic arrest induced with microtubule drugs[109,110]. Indeed, some evidence suggests that the cyclin B1 gene is transcribed during mitosis and that this transcription is required to sustain a mitotic arrest induced with microtubule drugs[109]. The gradual degradation of cyclin B1 might account for 'mitotic slippage' in which cells escape out of mitotic arrest induced by microtubule drugs[108,111–113]. The balance between synthesis and degradation is likely to differ among species and cell types, resulting in variation in the duration of mitotic arrest exhibited by different cells in the presence of microtubule inhibitors[113]. The type and concentration of microtubule drugs also influence the strength of spindle checkpoint signalling, thus affecting APC/C activity and the rate of degradation[108]. Although prometaphase APC/C targets are degraded in cells that are arrested in mitosis with microtubule drugs, the rate of their degradation is decreased by strong checkpoint activation. Cells treated with high concentrations of microtubule-depolymerizing drugs such as nocodazole have maximal checkpoint signalling. In cells treated with low concentrations of nocodazole or in cells treated with microtubule stabilizers such as Taxol, in which microtubules or small fragments persist and associate with kinetochores, checkpoint signals are weaker[108,114]. In addition, the presence of intact microtubules might sequester substrates or promote the transport of the APC/C to favourable subcellular locations (for example, to chromosomes) for activation[80,90].

Inhibition of the APC/C by the MCC. The primary components of the spindle checkpoint include MAD1 (mitotic arrest deficient 1-like protein 1), MAD2, BUBR1 (Mad3 in yeast), BUB1, BUB3 and monopolar spindle protein 1 (MPS1) (reviewed in REFS 96–99). MAD1–MAD2 heterodimers at unattached kinetochores catalyse a conformational change in an additional MAD2 (to form closed MAD2 or C-MAD2) that allows it to bind to and inhibit CDC20 (REF. 115). Robust inhibition also requires the binding of C-MAD2–CDC20 to BUBR1 and BUB3 (REFS 116,117). This complex of spindle checkpoint proteins — MAD2–CDC20–BUBR1–BUB3 — forms the MCC[118–122].

The crystal structure of the fission yeast MCC provided valuable information about interactions within the MCC components[73]. BUBR1 was found to interact through multiple residues with both C-MAD2 and CDC20. BUBR1 has two KEN-boxes, one in the N terminus and another in the C terminus. The N-terminal KEN-box of BUBR1 binds to CDC20 and MAD2, thereby promoting assembly of the MCC. The C-terminal KEN-box is not required for the MCC–APC/C interaction but is required to inhibit substrate recruitment to the APC/C[123]. It was initially proposed that the C-terminal KEN-box might bind to a second copy of CDC20 (REF. 21), and a recent experimental study supports that model[124].

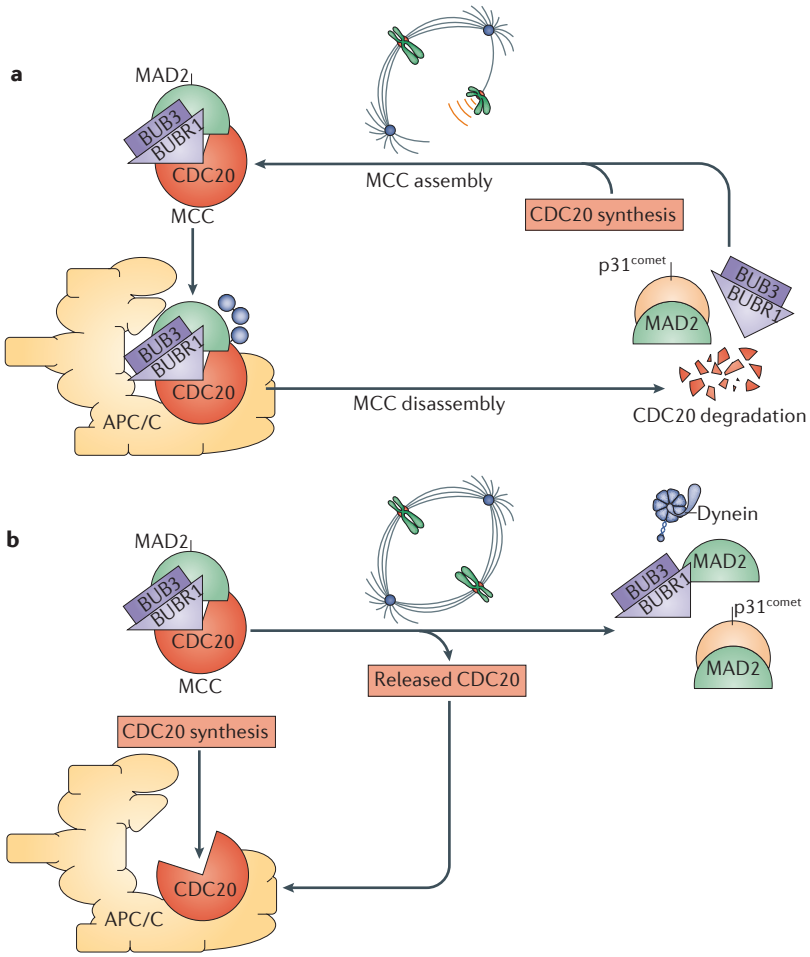


Figure 4 | **MCC turnover during mitosis. a** | In the presence of unattached kinetochores, mitotic arrest deficient 2-like protein 1 (MAD2), BUBR1, BUB3 and CDC20 interact to form a diffusible mitotic checkpoint complex (MCC) that binds to and inhibits the APC/C (anaphase-promoting complex; also known as the cyclosome). The APC/C ubiquitylates and promotes the degradation of its co-activator CDC20. CDC20 is continuously synthesized during mitosis. In the continued presence of unattached kinetochores, spindle checkpoint proteins MAD2 and BUBR1–BUB3 can be recycled to bind to newly synthesized CDC20, form the MCC and inhibit the APC/C. **b** | Once all sister kinetochores achieve bipolar attachment to the spindle and are under mechanical tension, MCC formation is inhibited and MCC disassembly dominates. CDC20 released from the MCC and freshly synthesized CDC20 generate the APC/C–CDC20 complex with high activity towards late mitotic substrates[20,127,128]. Several mechanisms contribute to the loss of MCC activity. MCC catalysis at kinetochores is inhibited by the transport of several checkpoint components, including MAD2 and BUBR1 from kinetochores by the minus-end-directed motor protein dynein[96,154–156]. p31^{comet} competes with BUBR1 for binding to MAD2 and prevents conformational activation of MAD2 (REF. 141). MCC disassembly allows APC/C activation, leading to ubiquitylation and degradation of securin and cyclin B1 for anaphase onset and mitotic exit.

MCC binding to the APC/C sterically hinders APC/C activity by disrupting the substrate-binding site and preventing substrate recruitment (FIG. 3b). MAD2 on its own competes with the APC/C for the same binding site of CDC20 and can thus inhibit the association of CDC20 with the APC/C[125,126]. MCC binding positions CDC20 downwards towards the APC/C platform, thus disrupting the D-box receptor formed between CDC20 and APC10 (REFS 15,21,73). This position of CDC20 might also promote its own ubiquitylation in an APC15-dependent manner[20,127,128]. The N-terminal KEN motif of BUBR1 also binds to and blocks the KEN-box receptor on the surface of the CDC20 WD40 domain. A second CDC20 molecule can bind to the MCC through the D-box and C-terminal KEN-box of BUBR1, and this interaction seems to be important for maximal checkpoint signalling[124]. Last, the interactions of the MCC with the catalytic core of the APC/C also partially impair the binding or function of UBE2C (REFS 15,129).

MCC turnover in mitosis. Several recent studies have indicated that continuous turnover of the MCC is an essential component for generating a system that can respond rapidly to the cessation of spindle checkpoint signalling after chromosome bi-orientation (FIG. 4). Metaphase is normally very transient, and delays at metaphase can lead to cohesion fatigue whereby spindle-pulling forces induce asynchronous chromatid segregation without mitotic exit[130,131]. Both free MCC and MCC bound to the APC/C have to be disassembled to fully activate the APC/C after spindle checkpoint inactivation[132]. Although not completely understood, continuous assembly and disassembly of the MCC during mitosis seems to prime the cell for rapid and strong APC/C-mediated degradation of anaphase targets, securin and cyclin B1, once checkpoint signalling is switched off.

CDC20 synthesis and degradation. During mitosis, CDC20 associated with the APC/C is continuously ubiquitylated and degraded. This is balanced by the continuous synthesis of the protein, hence ensuring constant steady-state levels of CDC20 during prometaphase[133]. APC15, a subunit of the platform subcomplex of APC/C, is required for CDC20 ubiquitylation and degradation[20,127,128]. Initially, it was suggested that degradation of CDC20 in prometaphase might be a mechanism to limit its accumulation and hence prevent premature APC/C activation in the presence of unattached kinetochores[134–136]. More recent evidence suggests that continued synthesis and degradation of CDC20 has a key role in rapidly increasing APC/C activity to ubiquitylate anaphase targets when the spindle checkpoint is silenced[20,111,127,128]. CDC20 synthesis and degradation is intimately connected to continued generation of the MCC at unattached kinetochores and disassembly of the MCC in the cytoplasm (FIG. 4a). Free MCC is in excess of MCC bound to APC/C–CDC20 (REFS 124,132). During CDC20 degradation and MCC turnover, this excess free MCC might rapidly bind to APC/C–CDC20, thereby promoting strong inhibition of APC/C activity in the presence of unattached kinetochores[132]. Inhibition of CDC20 degradation or APC/C activity causes metaphase arrest, which is subsequently followed by cohesion fatigue; this suggests that APC/C activity is required to silence the spindle checkpoint[110,137,138]. Cohesion fatigue has been subsequently shown to reactivate the spindle checkpoint, which suggests that inhibition of APC/C activation at metaphase can cause reactivation of the spindle checkpoint[139]. MCC turnover is thus required for rapid anaphase onset and mitotic exit after checkpoint silencing[136] (FIG. 4b). Last, although APC15 is required

for CDC20 ubiquitylation, it is not required for APC/C–CDC20 or APC/C–CDH1 activity towards the mitotic substrates securin or cyclin B1 (REFS 20,127,128).

p31^{comet} promotes MCC release from the APC/C. Another key component in MCC turnover is p31^{comet}, a protein that is required for normal mitotic progression in vertebrates, but homologues have not been identified in lower eukaryotes. p31^{comet} is a MAD2 paralogue that forms a dimer with C-MAD2 (REFS 140–143). p31^{comet} structurally mimics MAD2 and competes with BUBR1 for MAD2 binding. Its binding to MAD2 prevents conformational activation of MAD2 (REF. 141). Depletion of p31^{comet} stabilizes the MCC, inhibits full activation of the APC/C and delays mitotic exit[111,144–146]. Depletion of p31^{comet} also inhibits CDC20 degradation during prometaphase and increases the amount of MAD2 in the MCC[111,147]. Conversely, overexpression of p31^{comet} overrides a spindle checkpoint-mediated arrest[147]. Using mitotic extracts from mammalian cells, it was found that p31^{comet}-mediated MCC disassembly required hydrolysis of the β-γ bond of ATP[148,149]. Recently, the AAA-ATPase thyroid hormone receptor interactor 13 (TRIP13), which binds to p31^{comet}, was found to be required for MCC disassembly[150]. TRIP13 and p31^{comet} together release MCC from the APC/C, promote MCC disassembly and inactivate the spindle checkpoint[150].

The p31^{comet} protein binds to unattached kinetochores, and its activity might be modulated by the strength of checkpoint signalling[145]. Strong checkpoint signalling that results from high concentrations of microtubule depolymerizers such as nocodazole leads to unattached kinetochores and higher levels of MAD2 associated with the MCC. By comparison, in cells treated with a microtubule stabilizer such as Taxol, there is some microtubule association with kinetochore, and this results in a weaker checkpoint signal. As MAD2 is often present at substoichiometric levels in MCC compared to the BUBR1, controversy remains about whether the complete MCC is the key APC/C inhibitor or whether the MCC is an intermediary in the formation of a BUBR1–BUB3–CDC20 complex (known as BBC), which then serves as the primary inhibitor[116,123,147,151]. Levels of MAD2 in the MCC seem to correlate with the strength of checkpoint signalling[116,147,151], which suggests that the complete MCC, containing MAD2, is the more potent APC/C inhibitor. Finally, the protein CUE domain-containing protein 2 (CUEDC2) has been implicated in releasing MAD2 from the APC/C[152]. Interestingly, depletion of either p31^{comet} or CUEDC2 results in transient delays at metaphase, but these cells generally progress to anaphase. One explanation for this is that these proteins or others that have not yet been discovered have redundant essential roles in promoting anaphase onset. Alternatively, these proteins might have evolved in higher eukaryotes to 'fine-tune' or amplify signals to promote anaphase onset after chromosome alignment at metaphase.

Silencing the spindle checkpoint. Bipolar attachment of spindle microtubules and the mechanical tension they impart on kinetochores result in molecular changes that quell checkpoint signalling. However, loss of microtubule attachment in metaphase cells can reactivate the checkpoint. By severing microtubule attachments with a focused laser, it was determined that the 'point of no return' after which the spindle checkpoint cannot be reactivated is approximately 5 minutes before anaphase onset in HeLa cells[153]. Several mechanisms participate in checkpoint silencing. Some checkpoint signalling proteins, including MAD1, MAD2, MPS1 and BUBR1, are depleted from the kinetochore and moved to the spindle poles through the action of the minus-end-directed microtubule motor dynein[96,154–156]. In metazoans, this dynein-mediated protein 'stripping' dampens spindle checkpoint signalling catalysed at kinetochores[96] (FIG. 4b).

Other proteins specifically accumulate in higher amounts at kinetochores of chromosomes as they achieve bipolar attachment and reach metaphase. One of these is PP1, the activity of which is required for checkpoint silencing[157]. Reversible protein phosphorylation is a key regulatory mechanism of spindle checkpoint signalling[97,99,157]. The kinases BUB1, MPS1 and Aurora B promote checkpoint signalling (reviewed in REFS 97–99) (FIG. 5). Aurora B kinase is also involved in destabilizing kinetochore–microtubule attachments, which results in checkpoint activation. Another element that accumulates at metaphase kinetochores is the spindle and kinetochore-associated (SKA) complex. The SKA complex has both microtubule- and kinetochore-binding properties[158,159]. Depletion of the SKA complex generates a sustained metaphase arrest that eventually results in cohesion fatigue, where chromatids are pulled apart by spindle forces without anaphase onset[160]. How the SKA complex promotes the metaphase–anaphase transition is not completely understood, but it seems to function, at least in part, by promoting APC/C accumulation on metaphase chromosomes[161].

APC/C at the metaphase–anaphase transition

Spindle checkpoint silencing causes the cessation of kinetochore-based MCC assembly. Newly synthesized CDC20 and/or free CDC20 released by MCC disassembly rapidly amplifies APC/C activity targeting securin and cyclin B1 for proteasomal degradation (FIG. 1). Securin degradation liberates the protease separase, which cleaves the RAD21 component of the cohesin complex and allows synchronous chromatid separation in anaphase. Cyclin B1 degradation results in CDK1 inactivation. Reversal of the CDK1 phosphorylation cascade by cellular phosphatases (such as PP1 and PP2A) induces cytokinesis and mitotic exit (FIG. 5). There is strong evidence for positive feedback in CDK1 inactivation during mitotic exit. Even when cells are arrested with high concentrations of microtubule inhibitors, the application of drugs that inhibit CDK1 rapidly induces many of the events associated with mitotic exit, including degradation of cyclin B1 (REF. 162).

Changes in phosphorylation during anaphase and mitotic exit are likely to be key regulators of the APC/C. Binding of CDC20 to the APC/C is controlled, at least in part, by the removal of inhibitory phosphorylations[50]. During anaphase, dephosphorylation of CDH1 and

Cohesin complex
A protein complex that holds replicated sister chromatids together before anaphase.

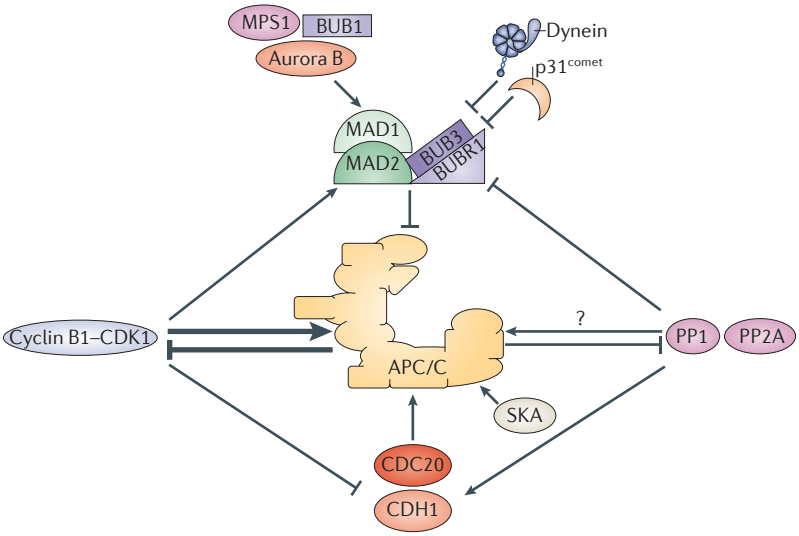


Figure 5 | **Positive and negative modulators control rapid changes in APC/C activity.** Mitotic progression is primarily regulated through two main activators, cyclin-dependent kinase 1 (CDK1) and the APC/C (anaphase-promoting complex; also known as the cyclosome). These work through a feedback mechanism whereby CDK1-mediated activation of APC/C ultimately induces the degradation of cyclin B1 and CDK1 inactivation (thick black arrows). APC/C activity is further modulated by a host of other components that are themselves regulated by post-translational modification and by subcellular localization, particularly at kinetochores. The resulting regulatory networks control APC/C activity and allow the APC/C to respond to rapid changes in kinetochore attachment and detachment. The spindle checkpoint proteins mitotic arrest deficient 1-like protein 1 (MAD1), MAD2, BUBR1 and BUB3 inhibit APC/C activity. These spindle checkpoint proteins are themselves activated by the mitotic protein kinases monopolar spindle protein 1 (MPS1), BUB1, Aurora B, cyclin B1 and CDK1, and inhibited by $p31^{comet}$, protein phosphatases (PP1 and PP2A) and dynein. These regulators affect the localization or activity of the spindle checkpoint proteins. Although the spindle checkpoint inhibits the APC/C, regulators of the spindle checkpoint also directly modulate APC/C activity. This results in complex regulatory networks that 'fine-tune' APC/C activity during mitosis. In addition, some proteins have roles in both inhibiting and promoting APC/C activity. For example, CDK1 has inhibitory roles in phosphorylating CDC20, CDC20 homologue 1 (CDH1) and spindle checkpoint proteins. At the same time, CDK1 phosphorylation enhances APC/C–CDC20 activity. The interplay of these regulators and the existence of subcellular pools of APC/C that differ in post-translational modification and inhibitor or activator binding is likely to have important roles in the dynamic regulation of APC/C activity during progressive stages of the cell cycle. SKA, spindle and kinetochore-associated.

degradation of the CDH1-binding protein MAD2L2 allows CDH1 to bind to and activate the APC/C[45,54,55,57,163]. APC/C–CDH1 recognizes substrates such as CDC20, Polo and Aurora kinases, UBE2C and geminin (FIG. 1). Although APC/C–CDH1 mediates degradation of these substrates at anaphase, it might not be essential, as depletion of CDH1 stabilizes Aurora A and Aurora B but does not affect the degradation of PLK1, geminin and CDC20 (although CDC20 is degraded more slowly)[164–166]. Mitotic exit is thus largely unaffected when CDH1 is deleted in budding yeast, fission yeast[167], *Drosophila melanogaster*[168] or depleted from mammalian cells[36,169,170]. CDC20 might persist and compensate for CDH1 in its absence. Finally, many APC/C subunits are highly phosphorylated in mitosis. Most of these phosphorylations are removed during anaphase and mitotic exit. Whether sites are dephosphorylated in a specific order to regulate mitotic exit remains uncertain.

Subcellular compartmentalization of APC/C activity. Although studies have focused on temporal control of APC/C activity, evidence suggests that APC/C within certain cellular compartments might be differentially regulated. Interestingly, pools of APC/C associated with spindle poles and chromosomes are hypophosphorylated compared to the bulk APC/C in the mitotic cytoplasm. In the case of the spindle pole pool, it is hypothesized that the APC/C is specifically inactivated[95]. In the case of the chromosome-associated pool, the APC/C was assayed and found to be more active than the cytoplasmic pool[161].

Indirect evidence suggests that APC/C-mediated degradation is compartmentalized, and cyclin B1 degradation might be spatially regulated. In syncytial *D. melanogaster* embryos, cyclin B1–GFP staining is lost first from the spindle poles, which suggests that degradation begins there, whereas in human cells it is lost simultaneously from the spindle poles and chromosomes[171,172]. Securin degradation is also spatially controlled. The majority of securin protein seems to be free and phosphorylated in the cytoplasm, and only a small dephosphorylated pool binds to and inhibits separase on chromosomes. PP2A dephosphorylates the securin bound to separase[78]. Upon full activation of APC/C–CDC20 at anaphase onset, the bulk of the free cytoplasmic phosphorylated securin is degraded before the small pool of securin bound to separase on chromatin[173]. Autocleaved separase is thought to inhibit CDK1 on chromosomes after cohesion cleavage to further repress CDK1 activity and hence initiate rapid poleward movement of sister chromatids[173,174].

Regulation of the APC/C in interphase

After anaphase onset and mitotic exit, the two main substrates of the APC/C–CDH1 are S phase and mitotic cyclins, the levels of which are kept low to prevent cell cycle entry until a cell commits to another round of division. In the absence of CDH1, mammalian cells accumulate cyclin A early and begin DNA replication prematurely[36].

Post-translational modification. APC/C–CDH1 must be inactivated for cells to re-enter the cell cycle and begin DNA replication. This is thought to occur by a combination of cyclin–CDK-mediated phosphorylation and inhibitor binding. G1-phase cyclin E–CDK or cyclin A–CDK complexes inactivate CDH1 by phosphorylation and prevent it from binding to the APC/C[36,54,55]. APC/C–CDH1 inactivation can also occur by degradation of its E2 enzyme, UBE2C. By ubiquitylating UBE2C and mediating its degradation, APC/C–CDH1 inactivates itself[175]. Finally, CDH1 can be auto-ubiquitylated by the APC/C at the end of G1 phase to target itself for degradation and allow cell cycle re-entry[10,176].

Inhibitor binding. Inactivation of APC/C–CDH1 can also occur through binding of inhibitors. In budding yeast, Acm1 (APC/Cdh1 modulator 1) has been identified as an inhibitor of APC/C–Cdh1 (REF. 177). Similarly, Rca1 (F-box protein regulator of Cyclin A) in *D. melanogaster*[178] and EMI1 in vertebrates also function as inhibitors of APC/C–CDH1 (REF. 179). In budding yeast, Acm1 acts as a pseudosubstrate by competing with other substrates for

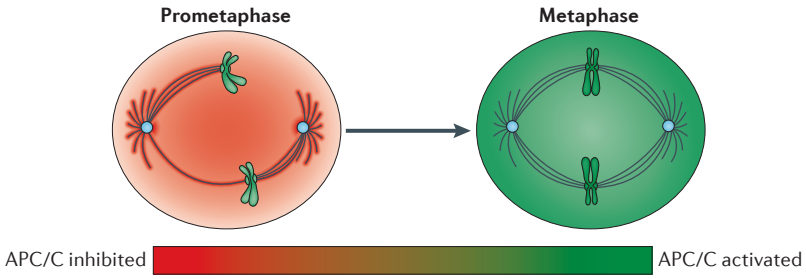


Figure 6 | **Hypothesis for the spatiotemporal regulation of the APC/C in mitosis.** In prometaphase, APC/C (anaphase-promoting complex; also known as the cyclosome) activity is inhibited towards late mitotic substrates to prevent anaphase onset and mitotic exit until all kinetochores are bi-oriented on the mitotic spindle and properly attached to microtubules. During mitosis, subcellular localization of the APC/C and its substrates might have important roles in mitotic progression. Some APC/C is concentrated at centrosomes where it is bound and potentially inhibited by binding to a protein complex containing early mitotic inhibitor 1 (EMI1), nuclear mitotic apparatus protein (NUMA) and dynein–dynactin[94]. Spindle assembly factors are localized to microtubules and thereby protected from APC/C-mediated degradation until completion of spindle formation[80]. The spindle checkpoint generates the diffusible mitotic checkpoint complex (MCC) catalysed at unattached kinetochores to inhibit the soluble cytosolic APC/C (the intensity of the red colour denotes the degree of APC/C inhibition; green indicates APC/C activation). A small pool of active APC/C–CDC20 might remain associated with chromosomes in prometaphase, potentially escaping checkpoint inhibition and contributing to the basal cyclin B1 degradation seen in cells arrested in mitosis with microtubule drugs. Upon proper microtubule attachment at metaphase, active APC/C–CDC20 further accumulates on chromosomes, and this is dependent on the spindle and kinetochore-associated (SKA) complex[90]. Loss of inhibition by spindle checkpoint proteins generates globally strong APC/C activity throughout the cytoplasm. Final activation of APC/C might occur on chromosomes to allow rapid cohesin cleavage and synchronous anaphase chromatid separation[173]. Therefore, APC/C activity is regulated spatiotemporally to control proper mitotic progression.

Cdh1 binding, thereby inhibiting their recruitment to the APC/C[180,181]. In anaphase, APC/C–Cdc20-mediated degradation of Acm1 activates APC/C–Cdh1 and, at the end of G1 phase, accumulation of Acm1 probably inactivates APC/C–Cdh1 (REF. 181). In vertebrate cells, EMI1 levels rise during the S phase and decline at mitotic entry. *In vitro* EMI1 inhibits both APC/C–CDC20 and APC/C–CDH1, and *in vivo* EMI1 overexpression has been shown to result in the accumulation of APC/C substrates[179,182].

Structural evidence shows that EMI1 inhibits the APC/C in ways similar to the MCC[183]. The C terminus of EMI1 binds to multiple sites on APC/C–CDH1 to block the substrate-binding site[183]. EMI2 (also known as ERP1) is a protein closely related to EMI1 that functions in oocyte meiosis to inhibit APC/C activity. After ovulation and before fertilization, oocytes are arrested at the metaphase stage of meiosis II. EMI2 as a component of cytostatic factor mediates this arrest[184–186]. EMI2 is also necessary for the early mitotic divisions of *Xenopus* embryos[187]. Both EMI1 and EMI2 inhibit ubiquitin chain elongation by UBE2S. The EMI proteins have a functionally similar C-terminal tail through which they compete with UBE2S for APC/C binding[183,188–190]. Depletion of EMI1 leads to premature activation of APC/C during G2 phase and destabilization of geminin and cyclin A[191,192]. When EMI1 does not inactivate APC/C–CDH1, cells re-replicate their genomes and become polyploid[36].

At mitotic entry EMI1 is ubiquitylated and degraded by the SCF–β-TRCP (β-transducin repeat-containing protein) ubiquitin ligase[193–196]. Expression of a non-degradable form of EMI1 does not prevent APC/C activation[88,192,197,198], which suggests that other mechanisms also allow the APC/C to escape inhibition by EMI1 during mitotic entry. A good candidate is CDK1-mediated phosphorylation, as phosphorylated EMI1 seems unable to bind to and inhibit the APC/C efficiently[199].

Conclusions and current questions

The APC/C serves as a central control node that regulates transitions in mitosis and at other points in the cell cycle. It is subject to multiple activators and inhibitors that tune its activity and specificity to individual substrates. The temporal management of the APC/C by its regulators is well documented. More evidence for spatial regulation at the subcellular level is beginning to appear. To ensure proper mitotic progression, the APC/C is positively regulated by mitotic protein kinases and co-activators, and negatively regulated by the spindle checkpoint and inhibitors (FIG. 5). Modulators of the APC/C ensure that the substrates are ubiquitylated and degraded at precise times in the appropriate sequence to ensure accurate chromatid segregation.

The localization of the APC/C or its substrates to mitotic organelles might aid in regulation of its activity during mitosis (FIG. 6). The APC/C accumulates on chromosomes as cells reach metaphase, and the chromosome-associated APC/C pool has a higher ubiquitylation activity[90]. At metaphase, it has been observed that motor proteins on microtubules transport spindle checkpoint proteins away from the kinetochore. Therefore, after proper microtubule attachment, inhibitors of the APC/C are hauled away from the kinetochore, whereas the APC/C itself is accumulating on chromosomes. Final activation of the APC/C might occur on chromosomes to closely link cohesin cleavage to synchronous chromatid separation at anaphase[78,173] (FIG. 6). The compartmentalization of APC/C to chromosomes might be important for its final activation. It is possible that an active pool of APC/C is partitioned away from the cytosolic APC/C that is inhibited by the spindle checkpoint proteins. During MCC turnover, this active and primed pool of APC/C–CDC20 might be responsible for the basal level of cyclin B1 degradation in cells arrested in mitosis by microtubule poisons. An active pool of APC/C–CDC20 might also catalyse the rapid degradation of cyclin B1 at metaphase upon spindle checkpoint inactivation. Moreover, localization to microtubules protects certain substrates from APC/C-mediated degradation[80] whereas, until metaphase, APC/C on centrosomes is anchored and potentially kept inactive by the EMI1–NUMA–dynein–dynactin complex[94] (FIG. 6). An important challenge in the future will be to understand how APC/C localized at specific compartments affects mitotic progression. It is possible that endogenous inhibitors and activators of the APC/C regulate the ligase differentially in subcellular compartments, and tracking APC/C activities at the subcellular level will be challenging but important in understanding its control over cell cycle transitions.

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Phosphatases: providing safe passage through mitotic exit

Claudia Wurzenberger and Daniel W. Gerlich

Abstract | The mitosis-to-interphase transition involves dramatic cellular reorganization from a state that supports chromosome segregation to a state that complies with all functions of an interphase cell. This process, termed mitotic exit, depends on the removal of mitotic phosphorylations from a broad range of substrates. Mitotic exit regulation involves inactivation of mitotic kinases and activation of counteracting protein phosphatases. The key mitotic exit phosphatase in budding yeast, Cdc14, is now well understood. By contrast, in animal cells, it is now emerging that mitotic exit relies on distinct regulatory networks, including the protein phosphatases PP1 and PP2A.

Nuclear envelope
Two membranes surrounding the cell nucleus, of which the outer membrane is continuous with the endoplasmic reticulum. The nuclear envelope in higher eukaryotes also contains a lamina adjacent to the inner nuclear membrane.

Mitotic spindle
An assembly of centrosomes, microtubules and chromosomes that supports chromosome segregation.

Kinetochores
Multiprotein structures that assemble at the centromere and mediate attachment of chromosomes to microtubules of the mitotic spindle.

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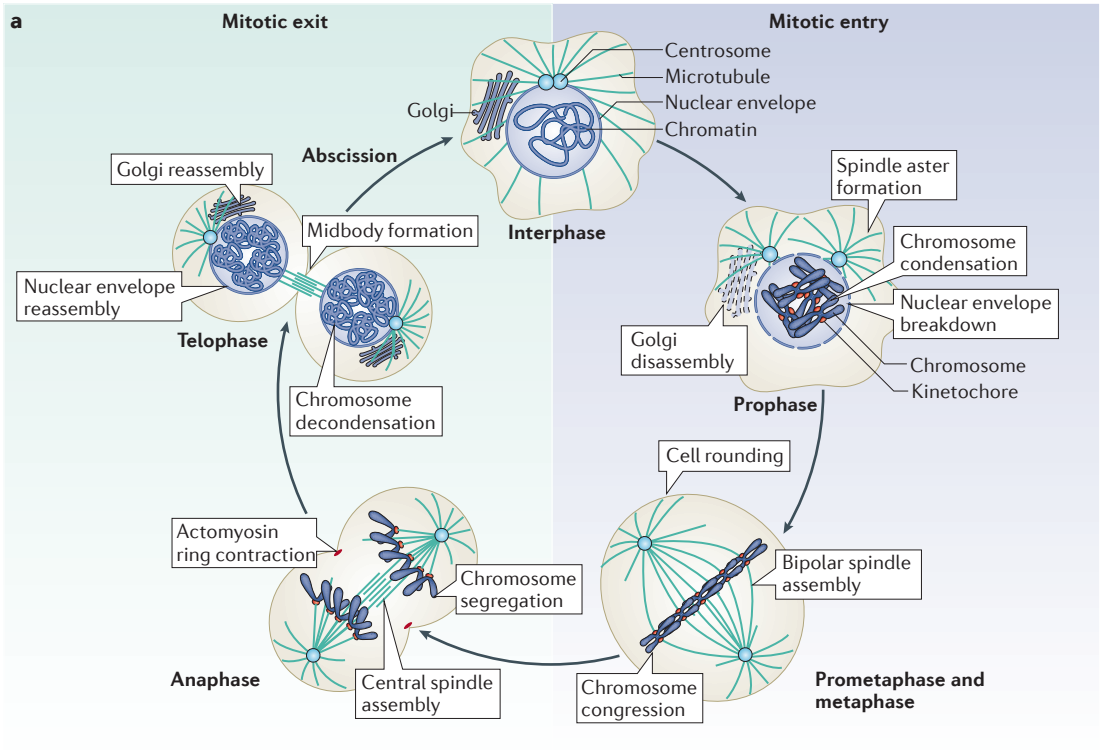
When entering mitosis, animal cells undergo extensive structural reorganization, including cell rounding[1], nuclear envelope breakdown (reviewed in REF. 2), chromosome condensation (reviewed in REF. 3) and assembly of the mitotic spindle (reviewed in REF. 4) (FIG. 1). These structural changes enable attachment of cytoplasmic microtubules to kinetochores and movement of individual sister chromatids to opposite poles of the cell. Furthermore, intracellular organelles, such as the endoplasmic reticulum[5] and the Golgi apparatus[6], change their organization to reduce spatial interference with the mitotic spindle and to facilitate their partitioning into emerging daughter cells during cytokinesis.

These cellular reorganization events at the interphase-to-mitosis transition depend on the activation of various mitotic kinases, most importantly a protein complex of cyclin-dependent kinase 1 (CDK1) and cyclin B (a complex that is also known as maturation-promoting factor (MPF)), and members of the Aurora and Polo-like kinase (PLK) families. These mitotic Ser/Thr protein kinases mediate cellular reorganization through a spatially and temporally confined pattern of phosphorylation (FIG. 1). A wide range of mitosis-specific phosphorylation events has been detected on a large number of substrates[7–12]. However, the link between individual phosphorylation events and specific cellular processes is often unclear.

High CDK1–cyclin B activity promotes mitotic progression until all chromosomes are aligned at the metaphase plate of the mitotic spindle. At these early stages of mitosis, CDK1 is already preparing for its own inactivation by phosphorylating the APC/C (anaphase-promoting complex, also known as the cyclosome). This enables the APC/C to bind its co-activator CDC20 to form an E3 ubiquitin ligase that later targets many mitotic proteins, including cyclin B, for degradation by the 26S proteasome by the addition of ubiquitin (reviewed in REFS 13,14). APC/C^{CDC20} is kept inactive by the spindle assembly checkpoint until all chromosomes attach to microtubules originating from opposite spindle poles (reviewed in REF. 15). Once this is achieved, the inhibitory signal from the spindle assembly checkpoint is alleviated, committing the cell to exit mitosis. Mitotic exit comprises all events that occur after 'satisfaction' of the spindle assembly checkpoint, including chromosome segregation, cytokinesis and reassembly of interphase cell structures. This is regulated through degradation of key mitotic factors and removal of phosphorylations from mitotic substrates.

The rise in APC/C^{CDC20} activity initiates mitotic exit by targeting several mitotic determinants for degradation, resulting in the formation and separation of two interphase daughter cells. Of particular importance is the degradation of securin, which normally inhibits the protease separase (also known as separin). Removal of securin therefore allows separase to cleave the sister chromatid cohesion 1 (SCC1) subunit of the cohesin complex to initiate chromosome segregation. Another key mitotic exit event is the APC/C^{CDC20}-induced proteasomal destruction of cyclin B, which inactivates mitotic CDK1 (reviewed in REF. 16). During late anaphase, low CDK1 activity allows binding of the APC/C to a second co-activator, CDC20 homologue 1 (CDH1), which replaces CDC20 to broaden APC/C substrate specificity, for example towards CDC20, Aurora kinases and PLK1 (REFS 17–21).



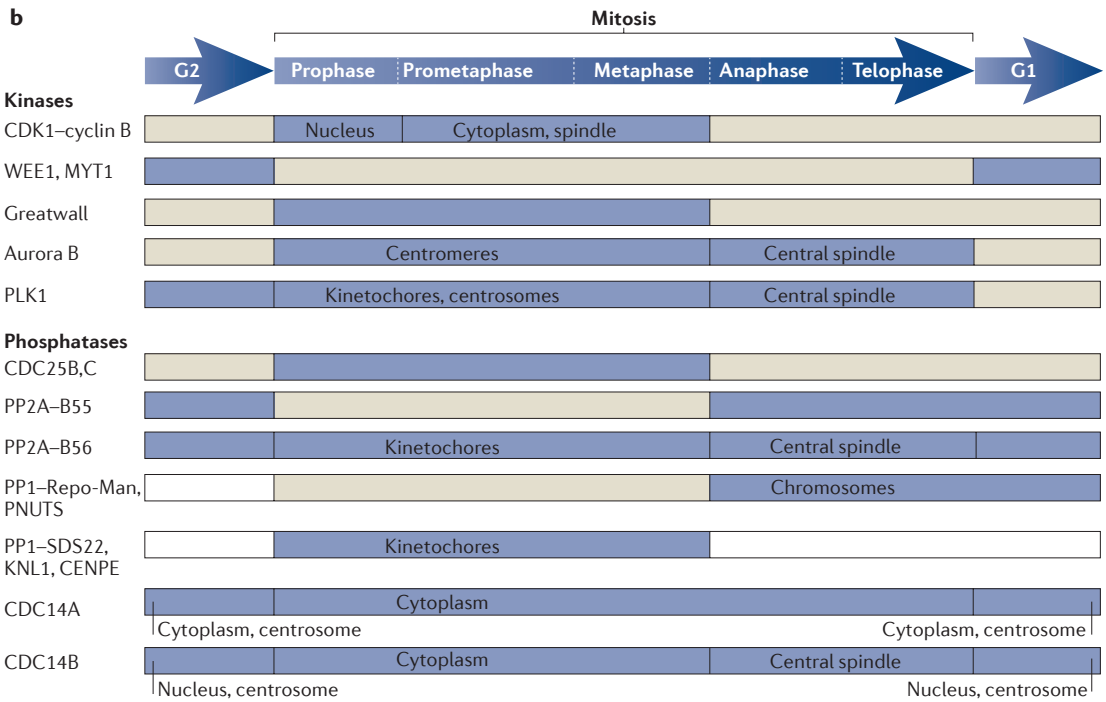


Figure 1 | **Cellular reorganization during mitotic progression and the activity of important mitotic kinases and phosphatases. a** | Cellular reorganization during mitotic entry and exit. Schematic of centrosomes, chromatin, microtubules, nuclear envelope and Golgi for a cell progressing through mitosis. Attachment of all chromosomes to opposite spindle poles 'satisfies' the spindle assembly checkpoint and initiates mitotic exit. Key events that occur at each stage of the cell cycle are indicated. **b** | Localization and activity of mitotic kinases and phosphatases during mitosis. The blue bars indicate stages of the cell cycle during which each protein or complex shows high kinase or phosphatase activity; if applicable, its cellular localization is shown. For example, active cyclin-dependent kinase 1 (CDK1)–cyclin B localizes to the nucleus during prophase and then to the cytoplasm and the spindle during prometaphase and metaphase. Stages of the cell cycle during which the kinase or phosphatase is inactive are represented by beige bars. The white bars indicate unknown activity. CENPE, centromere-associated protein E; MYT1, membrane-associated Tyr/Thr kinase 1; PLK1, Polo-like kinase 1; PNUTS, phosphatase 1 nuclear targeting subunit.

APC/C
(Anaphase-promoting complex, also known as the cyclosome). A large E3 ubiquitin ligase protein complex that targets mitotic cyclins and securin for 26S proteasome-mediated proteolysis. CDC20 or CDC20 homologue 1 (CDH1) are alternative APC/C co-activators that determine substrate specificity.

E3 ubiquitin ligase
An enzyme that, in conjunction with an E2 ubiquitin-conjugating enzyme, covalently attaches ubiquitin to a Lys residue on target proteins.

26S proteasome
A large protein complex that degrades Lys48-linked polyubiquitylated proteins by proteolysis.

Ubiquitin
A 76-amino-acid regulatory protein that can be covalently linked to target proteins by E3 ubiquitin ligases. Chains of ubiquitin linked by a Lys48 residue target proteins for 26S proteasome-mediated destruction.

Spindle assembly checkpoint
A signalling network that inhibits the activity of the APC/C (anaphase-promoting complex, also known as the cyclosome) and its co-activator CDC20 in the presence of unattached or tension-less kinetochores.

Separase
A protease that cleaves cohesin complexes at the metaphase-to-anaphase transition to enable chromosome segregation. In budding yeast, separase further inhibits protein phosphatase PP2A–CDC55 independently of its catalytic activity.

Cohesin
A protein complex that mediates cohesion between replicated sister chromatids.

Early models of cell cycle regulation attributed temporal waves of CDK1 substrate phosphorylation mostly to oscillatory kinase activities. It is now clear, however, that both kinase and phosphatase regulation have important functions in shifting the balance of cell cycle-dependent phosphorylations on a broad range of substrates. Despite low substrate specificity and constitutive activity of catalytic phosphatase subunits, their association with a large range of regulatory subunits targets phosphatases with high specificity towards diverse substrates or intracellular locations (BOX 1). The crucial and evolutionarily conserved role of the dual-specificity phosphatase CDC25 in mitotic entry is well established. However, it seems that mitotic exit phosphatases differ between eukaryotic organisms, despite these species having evolutionarily highly conserved mitotic kinases (reviewed in REFS 22–25).

The most important mitotic exit phosphatase in budding yeast is the well-characterized dual-specificity phosphatase Cdc14. The identity and regulation of mitotic exit phosphatases in animal cells is still not fully resolved, but many recent studies highlight the importance of Ser/Thr protein phosphatases of the PP1 and PP2A families (BOX 1).

In this Review, we discuss recent advances in the identification and characterization of the phosphatases that counteract mitotic kinases, most importantly CDK1, Aurora kinases and PLK1. We outline the current model for Cdc14 regulation and function in budding yeast and then focus on animal cells, discussing the regulatory networks of mitotic exit phosphatases and how mutual control of kinases and phosphatases governs the assembly of interphase organelles. We also highlight new technologies that have facilitated the study of mitotic exit phosphatases and outline the potential of mitotic exit as a target for anticancer therapy.

Building new interphase cells

One of the best-understood examples of structural reorganization that is driven by mitotic phosphoregulation is the breakdown and reassembly of the nuclear envelope in vertebrate cells (reviewed in REF. 2). It has long been known that nuclear envelope breakdown involves CDK1-dependent phosphorylation of lamin proteins, which leads to the disassembly of the nuclear lamina, a filamentous protein meshwork underlying the nuclear membranes[26–28]. Similarly, phosphorylation of nucleoporins mediates disassembly of nuclear pore complexes[29]. During mitotic exit, PP1 (REFS 30,31) and PP2A[32] are required for timely nuclear envelope reassembly, but it is not known whether their effects are directly at the level of lamin and/or nucleoporin dephosphorylation.

Reassembly of functional nuclei after mitosis also requires chromatin decondensation, which depends on PP1 and its regulatory subunits Repo-Man (recruits PP1 onto mitotic chromatin at anaphase protein; also known as CDCA2) and PNUTS (phosphatase 1 nuclear targeting subunit), which are targeted to chromatin[33,34]. Targeting of a Repo-Man–PP1γ complex to chromatin occurs during early anaphase[35], and this complex mediates dephosphorylation of a Thr3 residue on histone H3 (REF. 36). However, whether this substrate site is relevant for the regulation of chromatin condensation is not known. PNUTS accumulates on chromatin at a later stage, after nuclear envelope reformation[33], and thus may contribute to a distinct step of chromatin decondensation.

Another example of how substrate phosphorylation controls cellular reorganization during the progression through mitosis is the disassembly and reassembly of the Golgi apparatus, which is driven by mitotic phosphorylation of Golgi stacking proteins and the matrix protein 130 kDa *cis*-Golgi matrix (GM130)[37]. Dephosphorylation of GM130 induces Golgi reassembly during mitotic exit and depends on the ubiquitously localized phosphatase PP2A in complex with its regulatory subunit B55α (also known as Bα, PR55α and PPP2R2A)[32,38].

The mitotic spindle undergoes extensive reorganization during late stages of mitosis by forming a central spindle that comprises antiparallel bundles of microtubules. This involves protein regulator of cytokinesis 1 (PRC1), a microtubule-bundling protein, and the central spindlin complex, which is composed of the kinesin motor protein mitotic kinesin-like protein 1 (MKLP1; also known as KIF23) and the RHO-specific GTPase-activating protein (GAP) male germ cell RACGAP (MGCRACGAP; also known as RACGAP1). Before anaphase, CDK1-dependent phosphorylation of PRC1 and MKLP1 prevents their association with microtubules of the central spindle and inhibits their microtubule-bundling activity[39–41]. Removal of these phosphorylations during anaphase leads to central spindle formation and cleavage furrow ingression, thus ensuring temporal coordination of cytokinesis with chromosome segregation. A similar mechanism regulates transfer of the chromosomal passenger complex, which is another important component of the central spindle. Removal of a CDK1-dependent phosphorylation on one of its components, inner centromere protein (INCENP), mediates relocalization of the chromosomal passenger complex from centromeres to microtubules at the metaphase-to-anaphase transition[42,43]. In budding yeast, Cdc14 reverts these phosphorylation events on Sli15 (the yeast homologue of INCENP)[43,44], but the specific phosphatase (or phosphatases) that regulates this in animal cells remains to be identified.

Counteracting CDK1–cyclin B

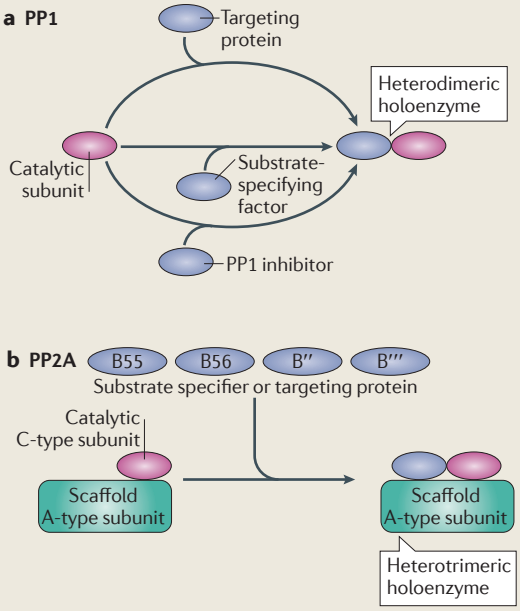
The shift in CDK1-dependent substrate phosphorylation during cell cycle progression involves regulation at the kinase and the phosphatase levels. Whereas phosphatase regulation has long been known to govern CDK1 activation during mitotic entry in yeast and animal cells[22,23,25,45–47], the role of regulated phosphatases during mitotic exit has emerged only over the past few years. In budding yeast, genetic studies revealed that the release of Cdc14 from the nucleolus is a key event that promotes the dephosphorylation of Cdk1 (which in budding yeast is also known as Cdc28) substrate and thereby mitotic exit[23,24,48–50]. The lack of phenotypes observed after depletion or genetic deletion of Cdc14 homologues in other species, however, has challenged

Dual-specificity phosphatase
A phosphatase that removes phosphates from Ser/Thr and Tyr.

Chromosomal passenger complex
A complex of Aurora B kinase and its regulatory cofactors, inner centromere protein (INCENP), borealin and survivin. It is activated on centromeres during early mitosis and is then transferred to the central spindle at anaphase onset.

Nucleolus
A non-membrane-bounded nuclear structure at which ribosomal gene transcription and pre-ribosome assembly occurs.

Box 1 | **Phosphatase specificity through regulatory subunits**



Animal genomes encode a large number of highly specific protein kinases, among which the Ser/Thr kinases are the most abundant; for example, 426 out of 518 genes encoding protein kinases in humans encode Ser/Thr kinases)[177]. The number of genes encoding catalytic subunits for Ser/Thr phosphatases is much smaller (~40 genes)[124]. These subunits also have much lower specificity towards diverse substrates *in vitro* than typical Ser/Thr kinases (reviewed in REFS 95,98,123) and therefore associate with a large range of regulatory subunits that modulate substrate specificity, intracellular localization and overall activity of the holoenzyme (reviewed in REFS 95,97,98,122–124). The most abundant phosphatases in animals belong to the PP1 and PP2A families. These are members of the PPP superfamily of protein phosphatases, which also includes calcineurin, PP4, PP5, PP6 and PP7.

PP1 typically functions as a heterodimeric complex containing one of four isoforms of the catalytic subunit — PP1α, PP1β, PP1γ1 or PP1γ2 (a testis-specific alternative splice variant of PP1γ) — and one of many different regulatory subunits that can either inhibit PP1, restrict its substrate specificity or target an active complex to specific intracellular locations (see the figure, part **a**). ~180 human PP1-interacting proteins have been identified so far (reviewed in REFS 122,123). Some regulatory subunits form heterotrimeric PP1 complexes[178,179].

PP2A typically functions as a heterotrimeric complex containing a structural scaffold A-type subunit (also known as PR65), a catalytic C-type subunit and a regulatory B-type subunit (see the figure, part **b**). The human genome encodes two closely related isoforms of the catalytic subunit (PP2Aα (also known as PPP2CA) and PP2Aβ (also known as PPP2CB)) and of the scaffolding subunit (PR65α (also known as PPP2R1A) and PR65β (also known as PPP2R1B)). It also encodes at least 15 isoforms of the regulatory subunit, which are categorized into four divergent subfamilies: B55 (also known as B, PR55 and PPP2R2A), B56 (also known as B′, PR61 and PPP2R5), B″ (also known as PR48, PR72, PR130 and PPP2R3) and B‴ (also known as PR93, SG2NA, PR110 and striatin) (reviewed in REFS 97,98). The catalytic C-type subunit forms a stable core dimer with a scaffold A-type subunit, and subsequent association of a B-type regulatory subunit then increases substrate specificity of the heterotrimeric complex. Potentially, more than 70 heterotrimeric PP2A complexes can be generated by combining these A-, B- and C-type subunits.

Crystal structures of PP1 and PP2A holoenzymes have revealed that regulatory subunits contribute to substrate specificity by directly binding to the substrate and by changing the accessibility of the catalytic pocket[180–182]. Many regulatory subunits further confer access to substrates by tethering the phosphatase complex to specific subcellular locations[95,97,98,122–124]. Aside from association with diverse regulatory subunits, phosphatase complexes may be further regulated by phosphorylation, methylation or ubiquitylation[32,34,54,118–120,127].

the idea that this phosphatase might have a universal role. Through several recent studies in various species, phosphatases of the PP1 and PP2A families have come into focus as CDK1-counteracting phosphatases in animal cells.

CDK1–cyclin B activity is regulated by several mechanisms, including transcriptional control, phosphorylation and intracellular localization (reviewed in REF. 51). The formation of CDK1–cyclin B complexes initiates during interphase through increased synthesis of cyclin B. However, before mitotic entry, the WEE1 and myelin transcription factor 1 (MYT1) kinases restrain the activity of CDK1–cyclin B by inhibitory phosphorylation at Thr14 and Tyr15 on CDK1. On entry into mitosis, the conserved dual-specificity phosphatase CDC25 removes these phosphorylations, assisted by the inhibition of WEE1 and MYT1. CDC25 (which in mammals has three potentially redundant isoforms, CDC25A, CDC25B and CDC25C) is itself regulated on many levels. During interphase, CDC25 is kept inactive by associating with 14-3-3 proteins[52,53] and PP2A bound to the regulatory subunit B56 (also known as B′, PR61 and PPP2R5)[54]. Dissociation of PP2A allows constitutively active CDK2 to partially activate CDC25, which promotes the dissociation of 14-3-3 proteins and further CDC25 activation through the removal of inhibitory phosphorylations by PP1 (REFS 55,56). Partially active CDK1 then contributes both to the activation of CDC25 and inhibition of WEE1 and MYT1 by direct phosphorylation, thus forming two amplification loops that establish switch-like and sustained activation of CDK1–cyclin B[57,58].

After bipolar attachment of all chromosomes to the mitotic spindle, APC/C^{CDC20} targets cyclin B for destruction by the proteasome. In animal cells, this leads to almost complete CDK1 inactivation during early anaphase[18,59]. In budding yeast, APC/C^{Cdc20}-induced degradation of the B-type cyclin Clb2 is significantly slower, leading to a reduction of Cdk1 activity to only about 50% during anaphase[60,61]. Cdc28 inactivation for mitotic exit thus depends on additional mechanisms, involving stabilization and accumulation

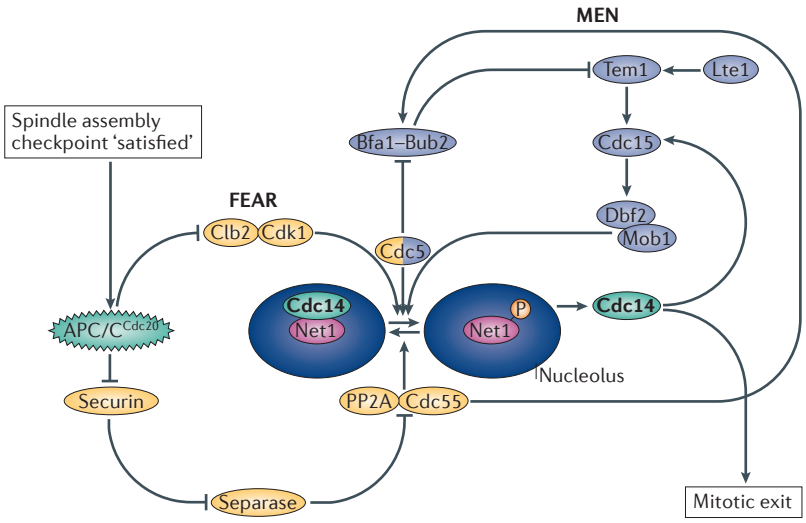


Figure 2 | **Regulation of Cdc14 activity during mitotic exit in budding yeast.** The Cdc14 early anaphase release (FEAR; yellow) and mitotic exit network (MEN; light blue) regulatory networks activate Cdc14 for budding yeast mitotic exit. Release of Cdc14 from nucleolar sequestration is mediated by phosphorylation of its inhibitor Net1. The FEAR network leads to Net1 hyperphosphorylation by cyclin-dependent kinase 1 (Cdk1)–Clb2 and Cdc5 (a Polo-like kinase). On 'satisfaction' of the spindle assembly checkpoint, the APC/C (anaphase-promoting complex, also known as the cyclosome) and its co-activator Cdc20 are activated, which leads to proteasomal degradation of securin. This relieves separase inhibition, which can then bind PP2A–Cdc55 and inhibit its dephosphorylation of Net1. The MEN network sustains Cdc14 release by a pathway involving the small GTPase Tem1 — which is in turn regulated by the guanine nucleotide exchange factor (GEF) low temperature essential 1 (Lte1) and the GTPase-activating protein (GAP) Bfa1–Bub2 — and several downstream kinases, including Cdc15 and MPS1 binder 1 (Mob1)–Dbf2, that further promote phosphorylation of Net1 and allow the release of Cdc14 from the nucleolus.

of the Cdk1 inhibitor protein Sic1 and activation of a second APC/C co-activator, Cdh1, which promotes complete destruction of Clb2 during late telophase (reviewed in REFS 23,24,50).

The inactivation of CDK1 alone is not sufficient to drive mitotic exit, as CDK1 substrate dephosphorylation also depends on phosphatase activation in all organisms studied so far. In budding yeast, the main mitotic exit phosphatase is Cdc14, which mediates both completion of Cdk1 inactivation, by upregulating Sic1 and Cdh1, and dephosphorylation of Cdk1 substrates[23,24,48–50]. Other organisms, however, seem to rely on distinct mitotic exit phosphatases, despite the presence of genes that are homologous to *CDC14* (REFS 22,25,62–70). Because the regulation and function of Cdc14 in budding yeast has been reviewed extensively elsewhere[23,24,50], we outline its role in budding yeast only briefly here and then concentrate on the phosphatases that regulate mitotic exit in animal cells.

Cdc14 control in budding yeast. Activation of the dual-specificity phosphatase Cdc14 is a key event in promoting several hallmarks of mitotic exit in budding yeast, including chromosome segregation, spindle elongation and late cytokinetic events. Aside from its function in directly inactivating mitotic Cdk1, Cdc14 also removes phosphorylations from Cdk1 substrates, including spindle regulators, such as Ase1, the chromosomal passenger complex component Sli15 and the APC/C co-activator Cdh1 (REFS 23,24,43,48,50,71,72).

Two regulatory networks — the Cdc14 early anaphase release (FEAR) network and the mitotic exit network (MEN) — function sequentially during anaphase to control the activity of Cdc14 in budding yeast (reviewed in REFS 23,24,50) (FIG. 2). Both networks regulate Cdc14 through its cell cycle-dependent association with the inhibitor Net1, which sequesters Cdc14 in the nucleolus.

The FEAR network mediates the initial activation of Cdc14 during early anaphase, when Cdk1 activity is still high[73–78]. This network is activated by APC/C^{Cdc20}-induced proteasomal degradation of securin (also known as Pds1 in budding yeast), leading to the activation of separase (also known as Esp1 in budding yeast). After its release from securin, separase inhibits the phosphatase PP2A–Cdc55 by direct binding, independently of its proteolytic activity[75,79]. This drop in PP2A–Cdc55 phosphatase activity allows Cdk1 and the Polo-like kinase Cdc5 to phosphorylate Net1, which then releases active Cdc14 from the nucleolus into the nucleoplasm. At this stage, Cdc14 promotes anaphase spindle elongation, by dephosphorylating the spindle midzone proteins anaphase spindle elongation 1 (Ase1) and filaments in-between nuclei 1 (Fin1)[72,80,81], and segregation of ribosomal DNA, by a mechanism that depends on condensin and Aurora B kinase[82,83]. The activation of Cdc14 by the FEAR network, however, is only transient; this is because decreasing Cdk1 activity during anaphase progression cannot sustain high levels of Net1 phosphorylation, and rising Cdc14 activity might also promote its own nucleolar sequestration[75,84].

Cdc14 activation is therefore sustained by the MEN pathway. A key component of this pathway is the Ras-like small GTPase Tem1, which is positively and negatively regulated by the guanine nucleotide exchange factor (GEF) low temperature essential 1 (Lte1) and the bipartite GAP Bfa1–Bub2, respectively (reviewed in REFS 23,24,50). Tem1 activation depends on correct spindle orientation and elongation, as this brings spindle pole-localized Tem1 in proximity to its activator, Lte1, which concentrates at the bud cell cortex. Further contributing to the regulation of Tem1, the inhibitory effect of Bfa1–Bub2 is confined to the mother cell by the spindle-positioning checkpoint (reviewed in REFS 85,86). Tem1 then activates two kinases, Cdc15 and MPS1 binder 1 (Mob1)–Dbf2, which either directly or indirectly increase Net1 phosphorylation, leading to sustained activation of Cdc14 and complete reversal of mitotic Cdk1 substrate phosphorylation. The MEN pathway also induces the release of Cdc14 to the cytoplasm through phosphorylation of a nuclear localization signal on Cdc14 (REFS 87,88), thereby promoting its effects on additional substrates.

The general requirement of Cdc14 for mitotic exit does not seem to be conserved in organisms other than budding yeast (reviewed in REF. 67). The fission yeast Cdc14 homologue, Cdc14-like phosphatase 1 (Clp1; also known as Flp1), contributes to the control of cytokinesis,

Box 2 | **Tools to study mitotic exit phosphatases**

Mitotic progression can be studied biochemically in extracts of synchronized cells, which enables measurement of protein abundance and changes in phosphorylation using specific antibodies on western blots. Cell cycle progression can be reconstituted *in vitro* using embryonic extracts, for example of *Xenopus laevis*[105], which enables precise temporal control of perturbations, such as immunodepletion or addition of purified components.

Because of the stochastic nature of chromosome attachment and the resulting variability in mitotic exit timing, bulk measurements in extracts should be complemented by single-cell measurements, ideally in live cells. Protein degradation can be efficiently assayed in live cells using APC/C (anaphase-promoting complex, also known as the cyclosome) substrates tagged to green fluorescent protein[17,18,21,59,183–186]. Phosphorylation events can be visualized in live cells using fluorescence resonance energy transfer (FRET)-based biosensors that change FRET efficiency based on a conformational change that occurs in response to phosphorylation at a kinase substrate site[187]. FRET-based biosensors have been generated for Aurora B[165], Polo-like kinase 1 (PLK1)[91,165] and cyclin-dependent kinase 1 (CDK1)[188]. By fusion to other proteins or targeting domains, these FRET-based biosensors can be targeted to distinct subcellular sites, such as chromatin, kinetochores or microtubules[161,165,189], which can allow analysis of phosphorylation events at particular locations.

The complex and interconnected regulatory networks proposed for mitotic exit are difficult to analyse based on experimental models alone. Mathematical modelling and simulation of how networks behave upon perturbation can allow one to make predictions that are suitable for quantitative experimental testing. This approach has been successfully used to reveal how the wiring of mitotic regulatory networks establishes switch-like responses and irreversible transitions[172,176,190–193].

but is not required for any other aspects of mitotic exit or Cdk1 inactivation[63,69]. In the nematode *Caenorhabditis elegans,* RNA interference (RNAi) depletion or mutation of CDC-14 also leads to cytokinesis defects, but not to any other defects in mitotic exit[64,68].

Three homologues of yeast Cdc14 (CDC14A, CDC14B, and CDC14C) have been identified in vertebrates. Although human CDC14B can functionally rescue a *CDC14* deletion in budding yeast[89], RNAi-mediated single depletion, genetic deletion or overexpression of human CDC14 isoforms do not delay mitotic exit progression in human cells[32,62,65–67,70]. Human CDC14A dephosphorylates and activates the APC/C co-activator CDH1 *in vitro*[90,91], but the relevance of this remains unclear given the lack of a clear effect on mitotic exit. However, numerous studies suggest functions of vertebrate CDC14 that are unrelated to mitotic exit, including roles in the DNA damage checkpoint[92], DNA repair[66] and centrosome duplication and function[65,70,93].

Given the diverse phenotypes that are observed when CDC14 is perturbed in different organisms, it seems unlikely that the key functions of Cdc14 in mitotic exit in budding yeast are evolutionarily conserved. However, definitive proof that CDC14 is not generally required for vertebrate mitotic exit has not yet been established, as mutants in which all vertebrate CDC14 isoforms are deleted have not been generated.

PP2A–B55 in animal mitosis. CDK1-counteracting phosphatases that are distinct from CDC14 have been identified in animal cells. The regulatory networks that these phosphatases form are much more complex than initially thought. Different phosphatases of the PP1 and PP2A families contribute to the reversal of mitotic CDK1-mediated phosphorylation events, but potential redundancy and variances in the isoforms that are expressed in different experimental systems have hampered the identification of a unified regulatory mechanism.

PP2A protein complexes are phosphatases that are abundant in cells and are involved in many processes, including cell growth, differentiation, apoptosis, cell motility, the DNA damage response and cell cycle progression (reviewed in REFS 94–96). In their active form, they are composed of one catalytic subunit, one scaffold subunit and one of the many regulatory subunits that provide substrate specificity (BOX 1). About 15 regulatory subunits in vertebrates have been classified into four different subunit families — B55, B56, B″ (also known as PR48, PR72, PR130 and PPP2R3) and B‴ (also known as PR93, SG2NA, PR110 and striatin)[97,98]. Members of the B55 subunit family have been revealed as the regulatory subunits that act in PP2A complexes to counteract CDK1 during mitotic exit in animal cells.

In vitro, B55-type regulatory subunits confer specificity of PP2A complexes towards a CDK1 substrate consensus sequence (Ser-Pro or Thr-Pro), in contrast to the regulatory subunits of other phosphatase subfamilies[99–101]. This suggests that PP2A–B55 might be important for mitotic exit in animal cells. Consistent with this, *Drosophila melanogaster* larval neuroblasts with mutated B55 show perturbed chromosome segregation[102]. However, this phenotype could also result from defects in preceding cell cycle events, for example erroneous DNA replication or improper chromosome attachment to the mitotic spindle. Evidence that PP2A–B55 indeed functions in mitotic exit was obtained by biochemical analysis of *Xenopus laevis* embryonic extracts[103,104] and by image-based RNAi screening in human cells[32].

Cell cycle progression can be faithfully recapitulated *in vitro* using cytosolic extracts from *X. laevis* oocytes, providing a powerful system for experimental manipulation[105] (BOX 2). In a set of immunodepletion experiments targeting various PP1 and PP2A subunits, the regulatory PP2A subunit B55δ was particularly important for timely dephosphorylation of various cdk1 substrates during mitotic exit[103,104]. These biochemical assays in *X. laevis* embryonic extracts did not address whether cellular reorganization events during mitotic exit were disrupted by loss of B55δ.

In a complementary approach, postmitotic nuclear reassembly was scored in an RNAi screen targeting a genome-wide set of phosphatases in human tissue culture cells[32]. The only phosphatase identified in this screen was a protein complex of PP2A containing the B55α regulatory subunit. RNAi-mediated depletion of B55α delayed postmitotic reformation of the nuclear envelope and the Golgi apparatus, as well as disassembly of the mitotic spindle and chromosome decondensation. An assay using chemical inhibition of CDK1 to induce mitotic exit further indicated that PP2A–B55α contributes to CDK1 substrate dephosphorylation, rather than regulation of CDK1 itself[32].

Why mitotic exit in *X. laevis* relies mostly on b55δ, whereas human tissue culture cells apparently depend only on B55α, is not known. In mouse fibroblasts, RNAi-mediated depletion of either B55α or B55δ delayed mitotic exit, indicating that both regulatory subunits contribute to mitotic exit[106]. Notably, the *D. melanogaster* genome encodes only one B55 subunit[102]. The different phenotypes revealed in different model systems could reflect variable expression levels of the respective isoforms, yielding detectable phenotypes only when the predominantly expressed isoforms are targeted. Genetic studies will be needed to overcome experimental limitations in these depletion techniques and to determine the relative contribution of different B55-type isoforms to mitotic exit.

In contrast to the animal cell PP2A–B55α, its budding yeast homologue, $PP2A^{Cdc55}$, is a negative regulator of mitotic exit and counteracts the phosphorylation of the Cdc14 inhibitor Net1 in the FEAR and MEN pathways[75,79] (FIG. 2). Resolving this apparent evolutionary diversity in phosphatase regulation in the context of generally highly conserved cell cycle control will be an important challenge in future studies.

Cell cycle regulation of PP2A–B55. Despite a long history of experimental research, the regulation of PP2A is still poorly understood. Cell cycle-dependent control of PP2A–B55 may involve association with inhibitory subunits and mitosis-specific post-translational modifications on the B55 subunit in question.

Inactivation of PP2A during mitosis was initially observed in *X. laevis* embryonic extracts[45–47,103] and was later confirmed by studies using human tissue culture cells[32,107]. A key player in the cell cycle-dependent regulation of PP2A–B55 is Greatwall kinase (the human homologue of which is also known as microtubule-associated Ser/Thr kinase-like (MASTL)[108,109]). *D. melanogaster* neuroblasts that express mutant Greatwall, or cultured *D. melanogaster* cells in which Greatwall has been depleted by RNAi, show defects in prophase chromosome condensation, nuclear envelope breakdown, chromosome congression and spindle morphology, indicating that Greatwall might promote mitotic entry[110,111]. This has been subsequently confirmed in *X. laevis* embryonic extracts, in which Greatwall is required for mitotic entry and maintenance of the mitotic state[104,112–115].

Greatwall regulates PP2A–B55 indirectly, through phosphorylation of two small regulatory proteins, α-endosulphine (ensa) and cyclic AMP-regulated phosphoprotein 19 (arpp19), which then bind and inhibit PP2A–B55 (REFS 116,117). This promotes the mitotic state in two ways (FIG. 3a). First, PP2A–B55 inhibition increases the net phosphorylation on various cdk1 substrates, owing to reduced counteracting dephosphorylation of these substrates[114]. Second, Greatwall activates cdk1 as part of a regulatory feedback loop that removes inhibitory Tyr14 and Thr15 phosphorylation from cdk1 (REFS 104,112–115). In this autoregulatory loop, the inhibition of PP2A–B55 by ensa and arpp19 via Greatwall has been proposed to increase the levels of activating phosphorylation events on cdc25 phosphatase and inhibitory phosphorylation events on wee1 and myt1. This model is supported by observations in human and mouse cells suggesting that the CDK1–Greatwall–PP2A–B55 network is evolutionarily conserved in mammalian cells[106,108,109].

ensa and arpp19 do not inhibit any of several other tested PP2A complexes containing regulatory subunits of other subfamilies[117], indicating that Greatwall specifically regulates cdk-counteracting PP2A–B55 complexes, rather than generally inactivating PP2A. This enables other PP2A complexes to perform their mitotic functions even in the presence of high Greatwall activity; for example, the role of PP2A–B56 in the protection of centromeric cohesion until anaphase onset would be unaffected[118–121].

PP2A–B55 may be regulated by additional mechanisms, including post-translational modifications and association with other subunits (FIG. 3a). Mass-spectrometric analysis of PP2A–B55α purified from human tissue culture cells reveals several cell cycle-regulated phosphorylation sites, with Ser167 phosphorylation on B55α being particularly abundant during mitosis[32]. A phospho-mimicking Ser167Glu mutant of B55α binds less efficiently to the PP2A core dimer (the catalytic and scaffold subunits), suggesting that formation of a functional heterotrimeric PP2A complex may be controlled by phosphorylation of the regulatory subunit. Interestingly, Ser167 is part of a CDK1 substrate motif (Ser-Pro-X-Arg), which implies potential feedback between CDK1 and PP2A–B55α. However, this hypothesis and the functional relevance of this phosphorylation for cell cycle progression have not yet been investigated. Furthermore, the scaffold subunit of PP2A physically and functionally interacts with the nuclear import factor importin β1 during mitosis, which may be part of another uncharacterized PP2A regulatory mechanism[32].

The discovery of the mechanisms by which PP2A is regulated provides a starting point for elucidating how mitotic exit regulatory networks are wired in vertebrates. The relative importance of the proposed regulatory mechanisms in distinct model systems remains to be experimentally dissected, and the potential redundancy between different regulatory PP2A isoforms adds to the complexity of mitotic exit regulation in animal cells.

PP1 in animal cells. PP1 has been proposed as another phosphatase that contributes to CDK1 substrate dephosphorylation and mitotic exit progression in animal cells. PP1 is one of the most abundant cellular phosphatases and regulates a range of processes, such as glycogen metabolism, transcription, cell polarity, vesicle trafficking, the DNA damage response and cell cycle progression (reviewed in REFS 98,122). The PP1 catalytic subunits (which are encoded by three genes in mammalian cells, *PP1A*, *PP1B* (also known as *PP1D*), and *PP1G*) form stable dimeric complexes with a huge range of regulatory subunits that determine substrate specificity, subcellular localization or phosphatase activity (reviewed in REFS 95,98,122–124) (BOX 1).

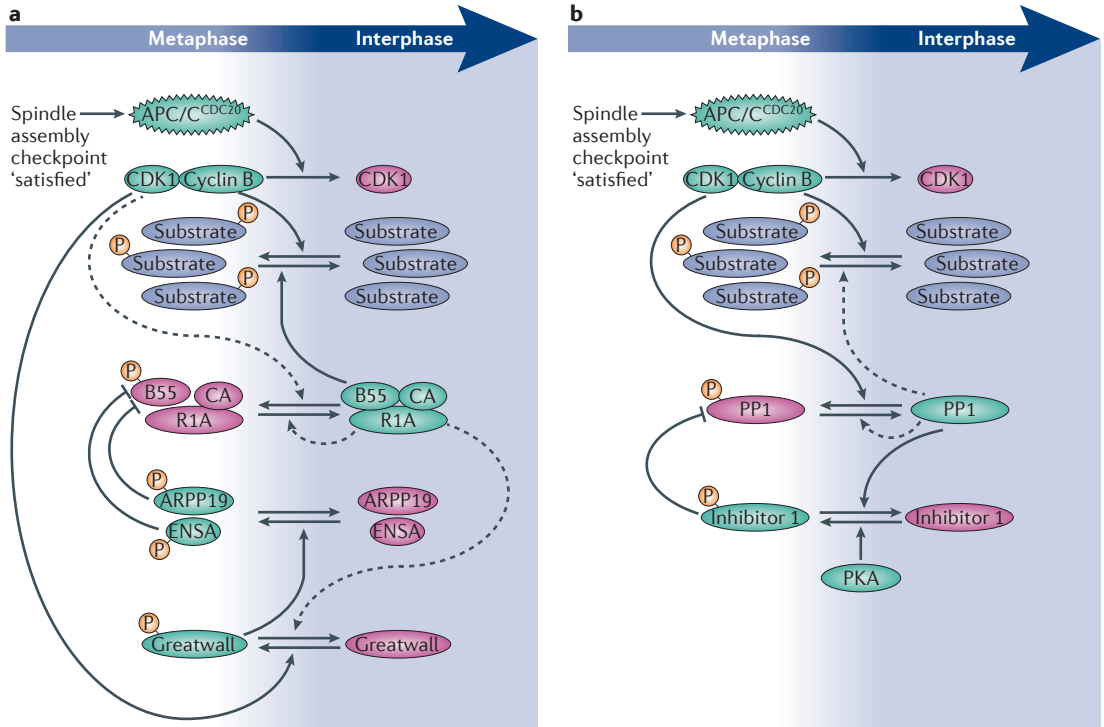


Figure 3 | **Model for regulatory networks of PP2A and PP1 during vertebrate mitotic exit.** Green indicates high kinase or phosphatase activity, whereas pink indicates low activity. The equilibrium of the reactions shown shifts towards the right as cells progress through mitotic exit to re-establish interphase. Dashed lines indicate potential feedback regulation that has not yet been experimentally validated. **a** | Regulation of protein phosphatase PP2A–B55, shown here as a heterotrimeric complex consisting of the catalytic subunit CA, the regulatory subunit B55 and the scaffolding subunit R1A. Phosphorylation levels of cyclin-dependent kinase 1 (CDK1) substrates are governed by the balance of CDK1 and PP2A–B55 activities. On 'satisfaction' of the spindle assembly checkpoint, activation of the APC/C (anaphase-promoting complex, also known as the cyclosome) and its co-activator CDC20 leads to CDK1 inactivation, thereby decreasing the activity of Greatwall kinase. This results in decreased phosphorylation of the Greatwall substrates α-endosulphine (ENSA) and cyclic AMP-regulated phosphoprotein 19 (ARPP19), relieving their inhibition of PP2A–B55. **b** | Regulation of PP1. During mitosis, PP1 activity is restrained by binding to inhibitor 1, which has been phosphorylated by protein kinase A (PKA). High CDK1 activity leads to inhibition of PP1 by phosphorylation. Inactivation of CDK1 promotes (auto)dephosphorylation of PP1 and inhibitor 1.

In *D. melanogaster*, mutation or RNAi-mediated depletion of PP1 induces mitotic defects, including abnormal anaphase spindles and chromosome mis-segregation[125,126]. A direct role for PP1 in mitotic exit has been proposed, based on the delayed dephosphorylation of diverse cdk1 substrates that is observed after immunodepletion of PP1 in *X. laevis* egg extracts or addition of recombinant PP1-specific protein inhibitor 1 (REF. 127). Furthermore, RNAi-mediated depletion of inhibitor 1 in mouse fibroblasts leads to premature dephosphorylation of CDK1 substrates[106].

However, two other laboratories have found only minor effects on cdk1 substrate dephosphorylation after immunodepletion of PP1 or addition of other PP1 inhibitors to *X. laevis* embryonic extracts[103,104]. Moreover, no catalytic or regulatory PP1 subunits were identified in an RNAi screen for mitotic exit regulators in human cells[32]. Nevertheless, these studies do not totally rule out a contribution of PP1 to mitotic exit, owing to the technical limitations inherent to immunodepletion or RNAi experiments, and potential redundancy between different PP1 catalytic subunits (BOX 1).

PP1 is an attractive candidate for a mitotic exit phosphatase because *X. laevis* PP1, as well as the human catalytic subunits PP1α and PP1γ1, are negatively regulated by CDK1-dependent phosphorylation on a conserved Thr320 residue[127–129] (FIG. 3b). Reduced CDK1 activity at the onset of mitotic exit triggers rapid PP1 activation through a positive feedback loop, involving auto-dephosphorylation of the inhibitory Thr320 on PP1, as well as PP1-mediated dephosphorylation of inhibitor 1, leading to its dissociation from PP1 (REF. 127). Thr320 is part of a CDK1 substrate motif that is conserved in the human PP1β subunit and in PP1 in other organisms, suggesting a conserved mechanism for mutual regulation of CDK1 and PP1.

It is unlikely that PP1 directly dephosphorylates CDK1 substrates, given that fractionation experiments of mammalian cells or *X. laevis* embryonic extracts identified phosphatase activities directed against CDK-phosphorylated sites co-purifying with PP2A but not with PP1 (REFS 100,130). PP1 could instead contribute to the activation of PP2A–B55, potentially as part of the Greatwall–ENSA–ARPP19 pathway, but this model has not been tested experimentally.

Calcineurin and meiotic exit. As with mitosis, increased CDK1 substrate phosphorylation drives entry into meiosis. However, reversal of these phosphorylation events during meiotic exit depends on a phosphatase that is distinct from the mitotic exit phosphatases. Neither PP1 nor PP2A seems to contribute to meiotic exit[127,131], whereas the Ca^{2+}-dependent phosphatase calcineurin (also known as PP2B and PPP3C) is required for the exit from metaphase of meiosis II both in *X. laevis* egg extracts[131,132] and *D. melanogaster* oocytes[133]. The dependence of oocyte meiotic exit on calcineurin probably reflects an adaptation to the rise in intracellular Ca^{2+} concentration that is induced by fertilization, which triggers exit from a prolonged arrest in metaphase II. The Ca^{2+} signal also activates the APC/C by a meiosis-specific mechanism via calcium- and calmodulin-dependent kinase II (CaMKII)[134,135]. Together, these two pathways enable a prolonged arrest in meiosis II, as well as a rapid and sharp transition to a fertilized egg.

Keeping other mitotic kinases in check

Many mitotic processes involve phosphoregulation by kinases other than CDK1. Of particular importance for late mitotic events are the Ser/Thr protein kinases Aurora B and PLK1, which control chromosome alignment, anaphase spindle reorganization and cytokinesis (reviewed in REFS 136,137). The various functions of Aurora B and PLK1 are largely specified by dynamic changes in their intracellular localization — both kinases first associate with centromeres during early mitosis and then relocate to the central spindle during anaphase (reviewed in REFS 136,137) (FIG. 1b). In addition, the complex spatiotemporal patterns of Aurora B and PLK1 substrate phosphorylation are fine-tuned through the action of localized phosphatases.

Phosphatases counteracting Aurora B kinase. Aurora B kinase is the enzymatic subunit of the chromosomal passenger complex, which also contains three non-enzymatic subunits: INCENP, survivin and borealin. These non-enzymatic subunits regulate Aurora B activity and specify its intracellular localization and function to control mitotic chromosome structure, mitotic spindle assembly, correction of erroneous kinetochore–microtubule attachments, cleavage furrow ingression and cytokinetic abscission[15,136,138,139].

Several mechanisms contribute to the regulation of the chromosomal passenger complex: phosphorylation on the T-loop of Aurora B[140] and clustering of the chromosomal passenger complex on chromatin[141] are essential for its kinase activity, and dephosphorylation of INCENP controls the translocation of the chromosomal passenger complex from centromeres to the central spindle at anaphase onset[42,43]. Removal of the chromosomal passenger complex from anaphase chromosomes further depends on Aurora B ubiquitylation by the E3 ubiquitin ligase cullin 3 (REFS 142,143), the CDC48 (also known as p97) system[144] and interaction with the kinesin MKLP2 (REF. 145). Finally, the APC/C^{CDH1}–proteasome pathway inactivates Aurora B by degradation after mitosis[20].

Genetic rescue experiments in budding yeast and *C. elegans* have led to the identification of PP1 as an Aurora B-counteracting phosphatase[146–148], and this was subsequently confirmed in *X. laevis* egg extracts[149,150] and human tissue culture cells[151]. PP1 counteracts the action of Aurora B at distinct intracellular sites (FIGS 1,4a), to which it is directed by association with various targeting factors; for example, PP1 is localized to kinetochores by centromere-associated protein E (CENPE), KNL1 and SDS22 (REFS 152–155) (BOX 1).

In budding and fission yeast, PP1 (known as Glc7 in budding yeast and Dis2 in fission yeast) is a main opponent of Aurora B during regulation of the mitotic spindle assembly checkpoint. Aurora B orthologues (increase-in-ploidy 1 (Ipl1) in budding yeast and Aurora-related kinase 1 (Ark1) in fission yeast) contribute to the maintenance of mitotic spindle assembly checkpoint signalling[156,157], whereas PP1 is required to silence the spindle assembly checkpoint after correct attachment of all chromosomes[158,159]. Whether PP1 controls the spindle assembly checkpoint at the level of Aurora substrate dephosphorylation, or indirectly through regulation of other kinases, is not known.

In human cells, PP1 controls the attachment of spindle microtubules to kinetochores during metaphase by counteracting Aurora B-mediated phosphorylation of outer kinetochore components and microtubule-destabilizing factors[153,160] (FIG. 4a). The kinetochore protein KNL1, the PP1 regulatory subunit SDS22 and the kinetochore motor protein CENPE target PP1 to kinetochores through direct binding[152–154]. Kinetochore-localized PP1 counteracts Aurora B effects on substrate phosphorylation[153] and also reduces activating phosphorylation of Aurora B on its T-loop[154]. Conversely, active Aurora B inhibits PP1 targeting to kinetochores by phosphorylation of the PP1-binding motif in KNL1 (REF. 153) and CENPE[152]. The mutual control between Aurora B and PP1 can establish a switch-like change in substrate phosphorylation at the outer kinetochore, when mechanical tension physically separates Aurora B at the inner centromere from PP1 and its substrates at the outer kinetochore, thus enabling a fast response to errors in chromosome attachment[153,160,161] (FIG. 4a). Targeting of Aurora B to centromeres further depends on priming phosphorylation of histone H3 Thr3 by haspin kinase[162,163], which is counteracted by Repo-Man–PP1γ[36].

PP1 also opposes Aurora B phosphorylation on chromosome arms, at a conserved Ser10 phosphorylation site on histone H3 (REFS 149,151,164). This may be regulated by the PP1 protein inhibitor 2 (REFS 149,151). At anaphase onset, PP1γ is targeted to chromatin by the removal of inhibitory CDK1 phosphorylations from the regulatory subunit Repo-Man[34,35]. This chromatin-targeted pool of PP1 then contributes to the regulation of mitotic chromosome decondensation[34] and is an interesting candidate for the general removal of Aurora B-dependent phosphorylations from chromatin components, although the only substrate identified so far is Thr3 on histone H3 (REF. 36).

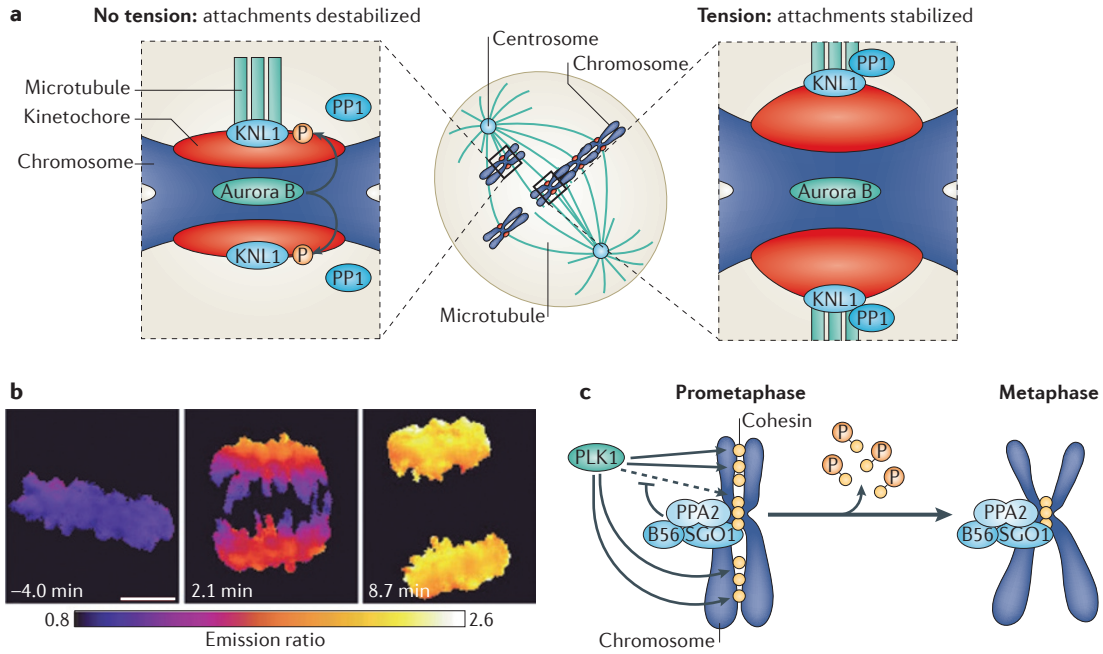


Figure 4 | **Spatial control of phosphorylation patterns by phosphatases counteracting Aurora B and PLK1.** **a** | Model for Aurora B and protein phosphatase PP1 function in sensing tension at the centromere and kinetochore. A metaphase cell is shown in the centre, in which some chromosomes are attached to microtubules from only one spindle pole (left inset) and others have correctly aligned along the metaphase plate and are under tension (right inset). On kinetochores that are not under tension, Aurora B at the inner centromere is in close proximity to the outer kinetochore, so it can phosphorylate outer kinetochore components, such as KNL1, to suppress PP1 binding. High Aurora B activity and low PP1 activity at the outer kinetochore destabilize microtubule attachment through phosphorylation of additional substrates, such as NDC80. Attachment of both sister kinetochores to opposite spindle poles stretches the kinetochore and centromere and thereby physically separates Aurora B from substrates at the kinetochore. This leads to dephosphorylation of KNL1 by residual PP1 activity, which is further accelerated by PP1 that then binds to unphosphorylated KNL1. **b** | Spatial gradient of Aurora B substrate phosphorylation on chromatin during anaphase. Aurora B substrate phosphorylation was visualized in a live human cell by a fluorescence resonance energy transfer (FRET)-based biosensor targeted to chromatin by fusion to histone H2B. A low emission ratio (purple) indicates high phosphorylation levels. As chromosomes undergo segregation, there is a decrease in phosphorylation of Aurora B substrates (yellow). Time is in minutes relative to anaphase onset (t = 0 min). **c** | Model for how PP2A–B56 counteracts Polo-like kinase 1 (PLK1) to protect centromeric cohesion in animal cells. During prophase and prometaphase, PLK1 phosphorylates the SA2 subunit of cohesin to promote dissociation of cohesin complexes from chromosome arms. At centromeres, shugoshin 1 (SGO1) recruits PP2A–B56 to counteract PLK1 phosphorylation (dashed line), thus maintaining a pool of centromere-bound cohesin until anaphase onset. Images in part **b** are reproduced, with permission, from REF. 165 © (2008) Macmillan Publishers Ltd. All rights reserved.

The development of fluorescence resonance energy transfer (FRET)-based phosphorylation biosensors (BOX 2) has further revealed that Aurora B substrate dephosphorylation on anaphase chromosomes proceeds in a striking spatiotemporal pattern[165] (FIG. 4b). Segregation of chromosomes away from Aurora B at the central spindle coincides with the removal of Aurora B phosphorylations on chromatin substrates. Dephosphorylation occurs along a gradient in which high phosphorylation is present on chromatin regions close to the central spindle and low phosphorylation is observed on chromatin towards the cell cortex. This indicates that there may be a diffusible component to Aurora B kinase activity and/or a spatial gradient of counteracting phosphatase activity. PP1 may be a good candidate for such a phosphatase, either as a cytoplasmic complex containing the α-isoform or β-isoform of the catalytic subunit, or as the γ-isoform, which is targeted to chromosomes[155].

Phosphatases reverting PLK1 phosphorylation. PLK1 is another mitotic Ser/Thr kinase that dynamically changes its localization during different stages of cell division (FIG. 1b). PLK1 controls entry into mitosis, centrosome maturation, sister chromatid cohesion, activation of the APC/C and cytokinesis (reviewed in REFS 137,166).

PLK1 substrate recognition depends on binding of its Polo-box domain to substrates that have been primed through phosphorylation by other kinases, such as CDK1, or by PLK1 itself. Thus, phosphatases can directly counteract PLK1 at the substrate site or remove priming phosphorylations to decrease PLK1 binding affinity to substrates. Phosphatases that counteract PLK1 during mitotic exit have not yet been identified, but the regulation of centromeric cohesion during prometaphase involves opposing activities of PLK1 and PP2A (FIG. 4c).

In mammalian cells, PLK1 promotes dissociation of cohesin from chromosome arms by phosphorylating the cohesin subunit SA2 during prometaphase[167].

Box 3 | **Mitotic exit as a target for cancer therapy**

Several classes of cancer therapeutics target dividing cells by disrupting the mitotic spindle, which first leads to a spindle assembly checkpoint-mediated mitotic arrest, and then often to mitotic cell death[194]. In some cases, however, cancer cells adapt to these spindle poisons and escape mitotic arrest by a process termed mitotic slippage[195–197]. Mitotic slippage proceeds despite the presence of kinetochore-localized spindle assembly checkpoint proteins, such as MAD2 (also known as MAD2L1) and BUBR1 (also known as BUB1β), and involves slow cyclin B degradation that is due to low residual APC/C (anaphase-promoting complex, also known as the cyclosome) activity[198], which is only partially counteracted by mitotic re-synthesis of cyclin B[199,200]. Pathways that induce mitotic cell death rather than mitotic slippage may also compete with each other[195,196], but a detailed dissection of the underlying regulatory networks is complicated, owing to large variability in how different cell types respond to antimitotic drugs[195].

Recent studies have tested the potential of inhibiting mitotic exit rather than activating the spindle assembly checkpoint to induce cell death and prevent tumour cell escape by mitotic slippage. Inhibition of the APC/C, using either small-molecule inhibitors or RNA interference-mediated depletion of its co-activator CDC20, arrested and killed cells in mitosis more efficiently than classical spindle poisons[201,202]. Furthermore, genetic deletion of *Cdc20* induced tumour regression in a mouse model[106]. The emerging relevance of mitotic exit phosphatases may be exploited in the development of future cancer therapies, which will aim at selective inhibition of phosphatase holoenzymes by targeting regulatory subunits that are relevant for mitotic exit.

At centromeric regions, the protein shugoshin 1 (SGO1; also known as SGOL1) recruits PP2A–B56 to protect SA2 against PLK1-mediated phosphorylation and thereby maintains a pool of persistent cohesin[118–121]. In addition to regulating centromere-localized PP2A–B56, PP1 generally constrains the activity of PLK1 to suppress premature loss of cohesion[168,169]. Thus, a balance of kinase and phosphatase activities establishes chromosomal patterns of cohesion along the metaphase chromosome axis.

At later stages of mitosis, PLK1 translocates from kinetochores to the anaphase central spindle by binding to the microtubule-bundling protein PRC1 (REFS 40,170). Before anaphase onset, phosphorylation of PRC1 by CDK1 inhibits PRC1 binding to PLK1. Only when CDK1 activity decreases during anaphase can PLK1 create its own binding site by phosphorylating PRC1 at a site adjacent to the CDK1 phosphorylation site[40]. However, the phosphatase that removes the CDK1-mediated phosphorylation from PRC1 has not yet been identified.

Although Aurora B and PLK1 are similarly localized at the anaphase central spindle, dephosphorylation of PLK1 substrates does not occur along a gradient, as is observed for Aurora B substrates[165]. This could be explained by distinct diffusible properties of the two kinases, or by the existence of distinct phosphatases that dephosphorylate Aurora B and PLK1 substrates.

Kinetic framework of mitotic exit

As with other cell cycle transitions, mitotic exit needs to progress irreversibly. Initially, this irreversibility was attributed to APC/C^{CDC20}-induced proteasomal degradation of cyclin B[171]. Subsequent studies, however, showed that phosphoregulation can also ensure that mitotic exit is unidirectional[172–175]. This ability is due to the positive feedback and double-negative feedback loops that regulate the activity of CDK1 and its counteracting phosphatases (FIGS 2,3), which establish switch-like transition kinetics and unidirectionality[174]. The complex wiring of regulatory networks during mitotic exit complicates intuitive analysis and predictions of how the network will behave when perturbed. Mathematical models can overcome this limitation by simulating perturbation conditions, and can then be subsequently validated experimentally (BOX 2). Mathematical modelling has revealed, for example, the importance of feedback loops that regulate the Cdk1 inhibitor protein Sic1 to ensure irreversible progression through mitotic exit in budding yeast[172], and the relevance of feedback loops that rapidly activate separase to promote synchronized sister chromatid segregation[176]. Quantitative testing of hypotheses using computer models may provide an opportunity to dissect the daunting complexity of the many phosphatases and isoforms of their regulatory subunits that orchestrate mitotic exit in metazoans.

Conclusions and outlook

It has become clear that temporally and spatially regulated mitotic kinases and phosphatases together shape the waves of substrate phosphorylation events that drive mitotic entry and exit. In budding yeast, the Cdc14 phosphatase mediates Cdk1 inactivation and dephosphorylation of Cdk1 substrates. By contrast, mitotic exit in animal cells is independent of CDC14 and instead relies on phosphatases of the PP1 and PP2A families.

Failure to progress normally through mitotic exit can induce cell death, which may be exploited to kill hyperproliferating cancer cells (BOX 3). A profound understanding of mitotic exit regulation could thus set the stage for new therapeutic strategies against cancer. In this context, regulatory subunits of mitotic exit phosphatases, such as B55, provide interesting candidates for the development of new pharmacological inhibitors that selectively target specific phosphatase holoenzymes.

Animal genomes encode multiple isoforms of catalytic and regulatory phosphatase subunits, which has impeded the characterization of mitotic exit phosphatases. Additionally, the number of regulatory phosphatase subunits greatly expanded during evolution, so that many mammalian genes, such as those encoding Repo-Man or PNUTS, do not have apparent orthologues in lower eukaryotes. No experimental perturbation in animal cells has so far been able to establish a permanent block in mitotic exit progression, suggesting that undiscovered phosphatases may function with at least partial redundancy to those phosphatases characterized so far. However, there is now firm evidence that PP2A–B55 has a key role in dephosphorylating CDK1 substrates during mitotic exit, and we are beginning to unravel the regulatory networks that keep PP2A–B55 inactive during early mitotic stages.

Research so far has mainly focused on the identification and functional characterization of CDK1-counteracting phosphatases. Equally important for our understanding of how cells reorganize during mitotic exit is the identification of relevant substrate phosphorylation sites and the mechanisms governing their temporally and spatially ordered dephosphorylation. New cell biological tools, such as FRET-based phosphorylation biosensors, combined with proteomics, should enable us to tackle this complex problem.

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