



Review

Functional characterization of Anaphase Promoting Complex/Cyclosome (APC/C) E3 ubiquitin ligases in tumorigenesis



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ARTICLE INFO

Article history:

Received 16 November 2013

Received in revised form 9 February 2014

Accepted 12 February 2014

Available online 22 February 2014

Keywords:

APC/C E3 ligase

Ubiquitin ligase

Mouse model

Tumorigenesis

Ubiquitin-proteasome system

Cell cycle progression

ABSTRACT

The Anaphase Promoting Complex/Cyclosome (APC/C) is a multi-subunit E3 ubiquitin ligase that primarily governs cell cycle progression. APC/C is composed of at least 14 core subunits and recruits its substrates for ubiquitination via one of the two adaptor proteins, Cdc20 or Cdh1, in M or M/early G1 phase, respectively. Furthermore, recent studies have shed light on crucial functions for APC/C in maintaining genomic integrity, neuronal differentiation, cellular metabolism and tumorigenesis. To gain better insight into the *in vivo* physiological functions of APC/C in regulating various cellular processes, particularly development and tumorigenesis, a number of mouse models of APC/C core subunits, coactivators or inhibitors have been established and characterized. However, due to their essential role in cell cycle regulation, most of the germline knockout mice targeting the APC/C pathway are embryonic lethal, indicating the need for generating conditional knockout mouse models to assess the role in tumorigenesis for each APC/C signaling component in specific tissues. In this review, we will first provide a brief introduction of the ubiquitin-proteasome system (UPS) and the biochemical activities and cellular functions of the APC/C E3 ligase. We will then focus primarily on characterizing genetic mouse models used to understand the physiological roles of each APC/C signaling component in embryogenesis, cell proliferation, development and carcinogenesis. Finally, we discuss future research directions to further elucidate the physiological contributions of APC/C components during tumorigenesis and validate their potentials as a novel class of anti-cancer targets.

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Abbreviations: APC/C, Anaphase Promoting Complex/Cyclosome; AOM, azoxymethane; Bub3, budding uninhibited by benzimidazole protein 3; BubR1, bub1-related protein; Cdc20, cell division cycle 20; Cdh1, Cdc20 homologue protein 1; DMBA, 7,12-dimethylbenz- α -anthracene; Emi1, early mitotic inhibitor 1; FBW7, F-box and WD-40 repeat domain containing protein 7; H, hypomorphic allele; HECT, homologous to the E6-AP carboxyl terminus; KO, knockout; L-LTP, late-phase long-term potentiation; Mad2, mitotic arrest deficient protein 2; MCC, mitotic checkpoint complex; 4-OHT, 4-hydroxytamoxifen; OS, oligosyndactylism; Rassf1a, Ras association domain-containing family 1 isoform a; RING, really interesting new gene; SAC, spindle assembly checkpoint; SCF, Skp1-Cullin-1-F-box protein; Skp1, S-phase-kinase-associated protein 1; Tet-O, tetracycline-inducible overexpression; Tg, transgenic expression; TPA, 12-O-tetradecanoylphorbol-13-acetate; β -TRCP, β -transducin repeat-containing protein; Ub, ubiquitin; UPS, ubiquitin-proteasome system; ZBR, Zn-Binding Region

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1. Introduction

1.1. The ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system (UPS) plays a critical role in regulating numerous cellular pathways through controlling the abundance, activity and localization of an enormous variety of cellular proteins [1–4]. As shown in Fig. 1, a wealth of studies have demonstrated that protein ubiquitination-mediated degradation involves two discrete steps: the ubiquitination catalyzed by the sequential actions of the activating (E1), conjugating (E2), and ligase (E3) enzymes that leads to the covalent attachment of multiple ubiquitin molecules to a given substrate, and the subsequent degradation of the poly-ubiquitinated substrate by the 26S proteasome complex [5–7]. Although only a small

number of ubiquitin E1 and E2 enzymes have been identified and characterized, there are hundreds of E3 ubiquitin ligases in the human genome that determine substrate selectivity of the UPS through recruiting specific substrate proteins for targeted ubiquitination [8–10].

There are two major classes of E3 ligases in eukaryotes: the HECT (Homologous to the E6-AP Carboxyl Terminus) domain containing E3s and the RING (Really Interesting New Gene) domain containing E3s [9–11]. While HECT E3s form a transient, covalent linkage with ubiquitin at a conserved cysteine before transferring the ubiquitin molecule to its substrate, RING E3s catalyze the transfer of the ubiquitin molecule from the E2 enzyme to a substrate without direct substrate binding (Fig. 1) [11–14]. It is well established that ubiquitin transmits molecular signals in the cell through modifying substrate proteins by either mono-ubiquitination or a variety of poly-ubiquitin chains. Recent studies

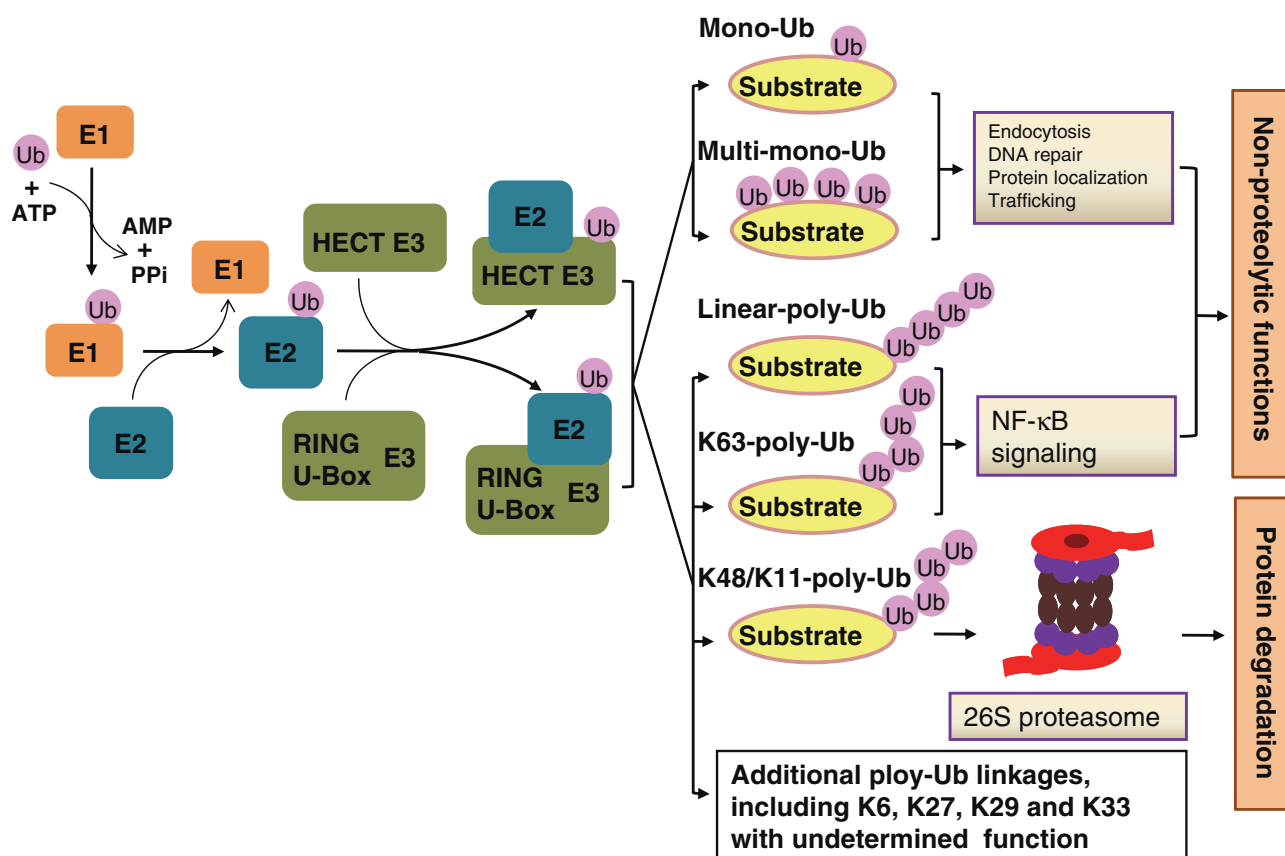


Fig. 1. A schematic illustration of the ubiquitin proteasome system. Ubiquitination is achieved by the sequential reaction of a conserved series of enzymes including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Ubiquitin is first activated and transferred to the E1 in an ATP-dependent manner; the activated ubiquitin is subsequently conjugated to the E2; then the E2-loaded ubiquitin is transferred to a substrate, catalyzed by an E3 ligase. The different models of ubiquitination and ubiquitin linkage exist, governing diverse biological outcomes: a given substrate could either be degraded if they are poly-ubiquitinated in a K48 or K11 linkage or modified with mono-ubiquitin, K63-linked, or other types of atypical line-linkage poly-ubiquitination to participate in various cellular functions including NF- κ B signaling, DNA repair or endocytosis.

revealed that ubiquitin chains can be assembled by covalently conjugating the C-terminal glycine with any of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) within the ubiquitin molecule, or by covalent attachment to the amino terminus, which is defined as linear linkage [15,16]. Mono-ubiquitination has been shown to play important roles in regulating many cellular processes, including endocytosis, trafficking, protein localization and DNA repair [17–19]. In terms of poly-ubiquitination events, of the eight different poly-ubiquitin linkages, the cellular roles of Lys48- and Lys63-linked poly-ubiquitin linkages have been extensively studied. Specifically, Lys48-linked poly-ubiquitin chains serve as a destruction tag to target substrates for degradation by the 26S proteasome [1,20,21]. By contrast, Lys63-linked poly-ubiquitination is generally not considered a destruction signal, but rather plays essential signaling roles by regulating NF- κ B signaling, receptor endocytosis and DNA repair processes [22–26]. In addition, the functions of other six atypical poly-ubiquitin linkages (K6, K11, K27, K29, K33, and linear linkage) are now beginning to be elucidated (Fig. 1) [15,27].

Recent work has demonstrated that SCF E3 ligases primarily promote Lys48-linked poly-ubiquitination through recruiting the initiating E2, UbcH5, to rapidly and efficiently establish the ubiquitination of a given substrate, followed by the second E2, Cdc34, to elongate the Lys48-linked poly-ubiquitin chains (Fig. 2, upper panel) [28]. In contrast, APC/C specifically assembles Lys11-linked chains as a degradation signal by utilizing two different E2s, UbcH10 (also named Ube2C) and Ube2S (Fig. 2, lower panel) [16,29–31]. Additionally, linear poly-ubiquitin chains also play a crucial role in NF- κ B activation [32,33]. Although the utilization of Lys6, Lys27, Lys29 and Lys33-linked poly-ubiquitin chains were reported, their functions remain poorly understood [15], which warrants further investigations to reveal their physiological roles and signature natures in various cellular processes.

1.2. Multi-subunit RING finger ubiquitin E3 ligases

The RING-finger-type ubiquitin E3 ligases are thought to make up the largest family of E3 ubiquitin ligases and have been linked to cell cycle control and human diseases, especially tumorigenesis [7,9]. Two multi-subunit RING finger ubiquitin ligase families, the Skp1-Cullin-1-F-box protein (SCF) and APC/C, are crucial for cell cycle progression through proteasome-dependent degradation of key cell cycle regulators such as cyclins and CDK inhibitors [7,12,34]. The SCF E3 ligase complexes consist of an adaptor protein, Skp1 (S-phase-kinase-associated protein 1), the scaffold protein Cullin-1, a RING-finger protein RBX1 or RBX2 (also known as ROC1/2), and an F-box protein that determines substrate specificity by recruiting proteins for SCF-mediated degradation [21,35,36]. Although approximately 69 F-box proteins have been identified in the human genome, only three F-box proteins, S-phase kinase-associated protein 2 (Skp2), F-box and WD-40 repeat domain containing protein 7 (FBW7), and β -transducin repeat-containing protein (β -TRCP) are well studied and thought to exert oncogenic (for Skp2), tumor suppressor (for FBW7) or context-dependent (for β -TRCP) roles, respectively [35,37]. Importantly, the oncogenic protein Skp2 promotes cell cycle progression during S and G2 phases largely through targeted degradation of negative cell cycle regulators such as p27, p21, p57 and FOXO1, most of which are characterized tumor suppressors [7,38]. On the other hand, the tumor suppressor protein FBW7 mediates the ubiquitination and degradation of oncogenic proteins that positively regulate the cell cycle, including cyclin E, c-Myc, c-Jun, and Notch [7,21,39]. The role of β -TRCP as an oncogene or tumor suppressor is possibly tissue-specific or cellular context-dependent [7,38]. In different tissues or cell types, β -TRCP may function as either a tumor suppressor or an oncogene in a context-dependent manner, in part through recognition and degradation of cell cycle regulators such as Cdc25A, Emi-1/2, and Wee1 [40].

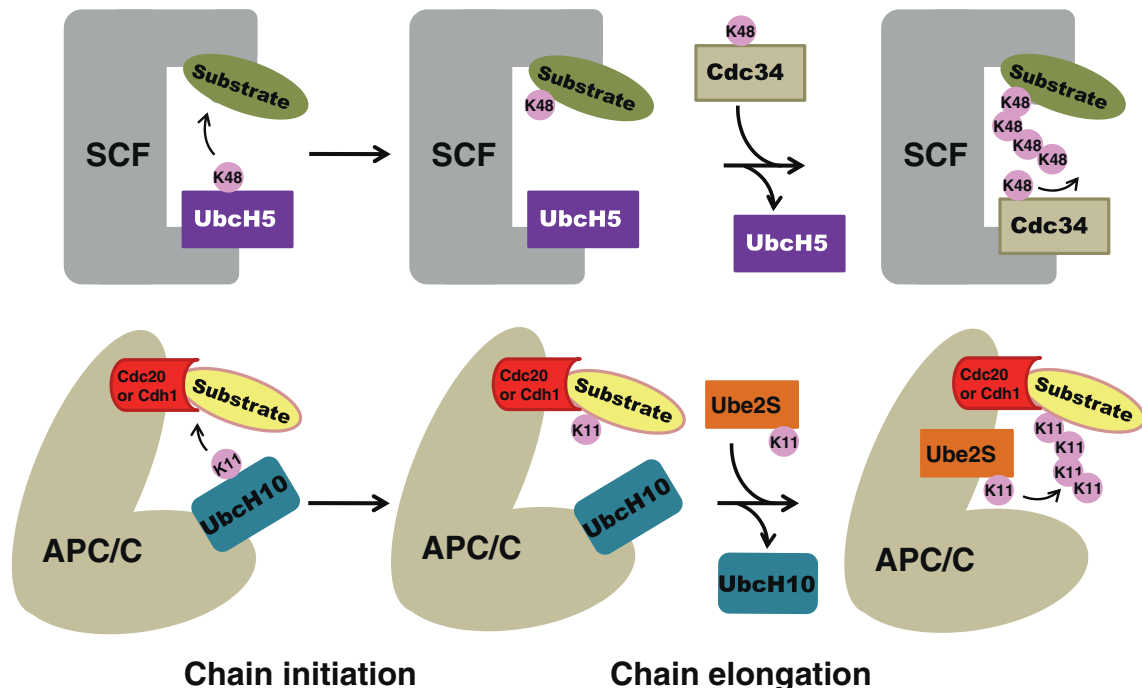


Fig. 2. The molecular mechanisms of poly-ubiquitin chain formation by SCF and APC/C ligases using initiating and elongating E2s. SCF types of E3 ligases mainly promote K48-linkage poly-ubiquitination. In doing so, SCF E3 ligases facilitate the first E2, UbcH5, to rapidly and efficiently initiate the ubiquitination of a given substrate. Subsequently, the second E2, Cdc34, functions in promoting the formation of K48-linkage poly-ubiquitin chains (upper panel). On the other hand, the initiation of K11-linked ubiquitin chain is catalyzed by the first E2, UbcH10, which directly binds the APC/C catalytic sub-complex. After adding the first ubiquitin moiety to the substrate, the second E2, Ube2S, is required for the processive elongation of K11-linked ubiquitin chains (lower panel).

1.3. Components of the APC/C ubiquitin E3 ligase complex

Although the functional components of the APC/C consist of a RING finger protein APC11, a Cullin-like subunit APC2, and an interchangeable substrate recognition subunit, which is generally similar to the architecture of the SCF complex, the complexity and diversity of subunits and functions are strikingly different between APC/C and SCF [41]. To date, at least 14 different proteins including APC1/TSG24, APC2, APC3/Cdc27, APC4, APC5, APC6/Cdc6, APC7, APC8/Cdc23, APC10/Doc1, APC11, APC13/SWM1, APC15/Mnd2, APC16 and Cdc26, as well as the co-activator subunit (Cdc20 or Cdh1) have been identified to functionally assemble into a holoenzyme complex with a combined molecular mass of approximately 1.5 megadaltons [42–45]. Early studies of the APC/C were difficult due to the large size and complex nature of the APC/C holoenzyme. However, recent studies using structural, genetic and biochemical approaches have begun to reveal the general architecture and the molecular mechanism by which APC/C recognizes, and mediates ubiquitination, of its substrates [46–50].

According to recently resolved crystal structures, the APC/C complex consists of three sub-complexes [43,46,50,51]: a scaffolding sub-complex, a catalytic and substrate recognition sub-complex, and a tetrapeptide repeat (TPR) arm (Fig. 3). The scaffolding sub-complex is composed of APC1, APC4 and APC5; the catalytic sub-complex contains APC2, APC11 (the RING finger protein) and APC10/Doc1; and the TPR arm consisting of APC3/Cdc27, APC6/Cdc16 and APC8/Cdc23, which provides binding sites for the scaffolding subunit and one of the co-activators (Cdc20 or Cdh1). In addition, other factors including Cdc26, APC13/Swm1, and the newly identified APC16 may also play a role in stabilizing the TPR arm. Interestingly, the Barford group has recently demonstrated that the APC/C core subunit APC10/Doc1 might also contribute to substrate recruitment via recognition of D-boxes within substrate proteins [52], further highlighting the complexity of different roles of APC/C subunits in governing APC/C-dependent ubiquitination of substrates. Nevertheless, the molecular mechanisms of how these APC/C subunits assemble under physiological conditions, and whether

there are additional components of the APC/C remain to be further characterized.

1.4. Functions of the APC/C E3 ligase in cell cycle control

The APC/C has critical roles in regulating cell cycle progression, but has also recently been implicated in the regulation of other essential cellular processes including genomic stability, apoptosis, metabolism and development through degradation of specific substrates. Regulation of cell cycle progression by the APC/C occurs primarily through the temporal coordination of two co-activators, Cdc20 or Cdh1, which form either the APC/C^{Cdc20} or APC/C^{Cdh1} E3 ligase complex. Specifically, APC/C^{Cdc20} primarily controls the metaphase to anaphase transition and mitotic exit, while APC/C^{Cdh1} is primarily active during the end of mitotic exit and early G1 phase.

During the G2 phase of the cell cycle, leading up to mitosis, Cdc20 is phosphorylated by Cdk1 and other mitotic kinases, which activates APC/C^{Cdc20} in part by promoting the interaction of Cdc20 with the APC/C core complex [53,54]. However, during conditions of aberrant mitotic events, such as misaligned spindles or improperly attached kinetochores on sister chromatids, the E3 ligase activity of APC/C^{Cdc20} is largely inhibited by the spindle assembly checkpoint (SAC) mainly through sequestering Cdc20 from the APC/C core complex by the mitotic checkpoint complex (MCC) consisting of MAD2, BUBR1/Mad3 and BUB3 [55–60]. Furthermore, several groups have shown that unattached kinetochores provide a catalytic platform for the formation of MCC, where Mad1, Bub1, Mad2 and essential mitotic-checkpoint proteins initially bind with the unattached kinetochores and serve to recruit Cdc20 to unattached kinetochores, which inhibit the activity of APC/C^{Cdc20} [61–64]. Additionally, other SAC components including MAD1, BUB1 and multipolar spindle-1 (MPS1) are required to amplify the SAC signals and enhance the rate of MCC formation [65–69]. When the checkpoint requirement is satisfied after all sister chromatids are attached to the bipolar spindle, the inhibition towards APC/C^{Cdc20} is diminished [55,56,58–60,70]. Active APC/C^{Cdc20} is then able to promote the ubiquitination and destruction of Securin, an inhibitor of Separase (Table 1) [71,72]. Separase, a protease that cleaves the Cohesin complexes, becomes active and triggers sister chromatid segregation. Further degradation of Cyclin B by APC/C^{Cdc20} reduces Cdk1 activity, which eventually gives a “GO” signal for anaphase to commence (Table 1). As cells exit mitosis, phosphorylation of Cdh1, which negatively regulates APC/C^{Cdh1} E3 ligase activity [73–75], is reduced due to the decline in Cdk1 kinase activity, thereby replacing Cdc20 with Cdh1 within the APC/C complex [34,45].

In addition to regulating the degradation of Cyclin B1 and Securin during the metaphase to anaphase transition, the APC/C targets a large array of substrates for ubiquitin-mediated degradation (see Table 1). For instance, activation of APC/C^{Cdh1} promotes the degradation of Cdc20 [88,89], Plk1 [137], Aurora A [76], Aurora B [77,78] and Tpx2 [77], which ensures a low kinase activity environment to pave the road for mitotic exit. Interestingly, it was recently reported that the APC/C regulates spindle formation through promoting the degradation of four spindle-binding proteins Bard1, Hmnr, HURP and NuSAP [82]. Furthermore, the stress-activated kinase JNK [118], HEC1 [114] and EYA1 [106] were also identified to be ubiquitin substrates of APC/C^{Cdh1} during the transition from mitosis to the G1 phase. Unlike the major role of APC/C^{Cdc20} in promoting the metaphase to anaphase transition, APC/C^{Cdh1} plays a pivotal role in governing cell cycle progression through the G1 phase by sustaining low Cdk activity by mediating the degradation of mitotic cyclins [158], Cdc25A [90], Skp2 [96,149] and Cks1 [96]. In addition, APC/C^{Cdh1} may control the timely transition from the G1 phase to S phase by controlling the destruction of the replication regulators Geminin [110] and Cdc6 [87], as well as its own E2, UbcH10 [156], which leads to the stabilization of Cyclin A and inactivation of APC/C^{Cdh1}. Therefore, APC/C^{Cdh1} may be a key regulator to govern

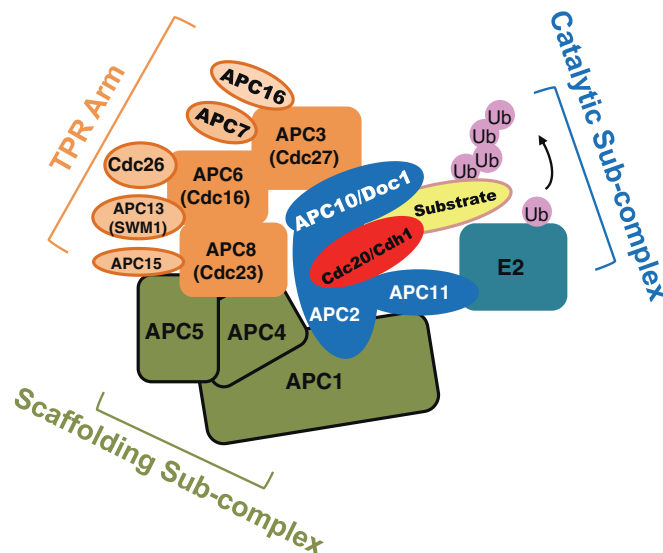


Fig. 3. A schematic illustration of the APC/C ubiquitin E3 ligase complex. The APC/C holoenzyme can be divided into three functional sub-complexes: the scaffolding sub-complex, the catalytic sub-complex, and the TPR arm. The scaffolding sub-complex (consisting of APC1, APC4, and APC5) connects the TPR arm to the catalytic subunit. APC3/Cdc27, APC6/Cdc16, and APC8/Cdc23 are assembled to the TPR arm, along with accessory proteins including APC7, APC13/SWM1, APC16, and Cdc26 that play a role in stabilizing the TPR arm. The catalytic sub-complex contains APC2, a Cullin family related protein, the RING finger protein APC11, APC10/Doc1, and one of the substrate recruiting and catalytic coactivators, Cdc20 or Cdh1.

the length of the G1 phase in part by directing the timely loading of pre-RCs at origins of DNA replication in S phase.

1.5. Cell-cycle independent functions of APC/C E3 ligase

In addition to regulation of cell cycle progress, recent studies have begun to reveal cell-cycle-independent functions of APC/C, including regulation of cell differentiation, genomic integrity, developmental processes and the nervous system [159–162]. As illustrated in Table 1, many regulators of DNA damage repair and genomic stability such as Claspin [97,98], UPS1 [157] and Rad17 [142] were recently characterized as *bona fide* Cdh1 substrates. Furthermore, the identification of Mcl-1 [123] as a Cdc20 substrate as well as G9a and GLP [113] as Cdh1 substrates expands APC/C functionality into regulating cellular apoptosis and senescence. In addition, APC/C also participates in other cell cycle-independent functions including regulating cellular metabolism [112], cell mobility [140] and gene transcription [104,105,128] through degradation of specific substrates. However, further biochemical and mouse modeling studies are required to validate a physiological role and pinpoint the underlying molecular mechanisms for APC/C^{Cdh1} in these cellular processes.

Emerging evidence implicates APC/C in the differentiation and function of the nervous system in part through governing the ubiquitination and degradation of neuron-specific substrates (Table 1). Specifically, APC/C^{Cdh1} was found to control axon growth and patterning in the process of normal brain development [163]. Subsequent studies reported that mechanistically, APC/C^{Cdh1} regulates neuronal development through targeting two axon growth-promoting factors, Id2 and SnO, for degradation [116,148]. Subsequent studies revealed that APC/C^{Cdc20} regulates dendrite morphogenesis and presynaptic differentiation through degradation of the transcription factors Id1 [115] and NeuroD2 [132], respectively. Further studies showed that synaptic plasticity, synaptic size and the bioenergetic and antioxidant status of neurons are controlled by APC/C^{Cdh1}-mediated degradation of GluR1 [111], Liprin- α [121,122] and Pfkfb3 [138]. Although several aspects of how the APC/C regulates the nervous system have been uncovered at the cellular level, it remains largely unclear how at the organismal level, APC/C deficiency could affect neuronal function, including mammalian learning and memory [164], and whether APC/C functions in neurological and psychiatric disorders.

1.6. Regulations of APC/C activity

In addition to critical roles for APC/C in many cellular processes described above through promoting targeted degradation of a cohort of substrates, APC/C and its associated E3 ligase activity are tightly controlled by multiple means such as phosphorylation, inhibitor binding, subcellular localization, and destabilization of its subunits or activators. Specifically, during early stage of mitosis, phosphorylation of APC/C subunits including scaffolding proteins APC1 and TPR proteins (APC6/Cdc16, APC8/Cdc23, APC3/Cdc27 and APC7) by Cdk1 and Plk1, recruits Cdc20 to the APC core complex to form an active APC/C^{Cdc20} holoenzyme [53,54,165]. Additionally, phosphorylation of co-activators Cdc20 or Cdh1 provides another layer of regulation of APC/C activity. Although phosphorylation of Cdc20 by mitotic kinases largely activates APC/C^{Cdc20} [53,54], APC/C^{Cdc20} E3 ligase activity, on the other hand, is inhibited by Cdk1, Bub1, and MAPKs during the spindle checkpoint [166–168]. Furthermore, Cdk-mediated phosphorylation of Cdh1 prevents its binding to the APC/C core complex and inactivates APC/C^{Cdh1} from late G1 to mitotic exit [53,73,169]. Furthermore, phosphorylation of APC/C substrates has been shown to protect them from APC/C-mediated destruction. For example, phosphorylation of Cdc6 by Cdk2/Cyclin E during S phase blocks its binding to Cdh1, protecting Cdc6 from APC/C^{Cdh1}-mediated ubiquitination and degradation [170]. Similarly, Skp2 escapes Cdh1-mediated degradation when phosphorylated by Akt [171,172].

Interestingly, several endogenous APC/C inhibitors (as shown in Table 4) have been found to restrain APC/C activity through direct interaction. Among these inhibitors, SAC components Mad2, BubR1 and Bub3 were discovered through genetic screens in the budding yeast *Saccharomyces cerevisiae* by two independent groups [173,174]. Notably, several key SAC components were found enriched at the unattached kinetochores during mitosis, suggesting a central role of SAC components in regulating spindle formation and mitotic progression [175–177]. The observation that Cyclin B and Securin were quickly degraded after the last sister chromatid connected to the kinetochore further strengthened the physiological link between SAC and mitotic control [102,178]. Further studies showed a direct interaction between Cdc20 and major SAC components including Mad2, BubR1 and Bub3 to form the mitotic checkpoint complex (MCC), which in turn prevents the association of Cdc20 with APC/C core complex [179–184]. Han et al. has recently demonstrated that Mad2 binding to Cdc20 could open Cdc20 intra-molecular association and this open conformation of Cdc20 is subsequently capable of binding the N-terminus of BubR1, which produces the functional mitotic checkpoint-derived inhibitor to selectively block ubiquitination of Cyclin B and Securin by APC/C^{Cdc20} [185].

In addition to the SAC-mediated regulation of APC/C activity during the metaphase to anaphase transition, the Ras association domain-containing family 1 isoform a (Rassf1a) inhibits APC/C^{Cdc20} during mitotic entry through its D-box motifs that display high-affinity binding towards Cdc20 [186,187]. However, both MCC and Rassf1a only inhibit APC/C^{Cdc20} activity, whereas the F-box protein Emi1 (also called FBXO5) not only suppresses APC/C^{Cdc20} during S and G2 phases [188], but also suppresses APC/C^{Cdh1} at the G1-S transition [189,190]. Mechanistically, Emi1 contains a Zn-Binding Region (ZBR) and a conserved D-box, both of which contribute to the inhibition of APC/C activity through binding to the APC/C core complex and its coactivators Cdc20 or Cdh1 [189]. Emi1 binds APC/C coactivators via its D-box with high affinity, preventing the recruitment of APC/C substrates to the APC/C core complex, while the ZBR domain directly suppresses APC/C E3 ligase activity by associating with the APC/C core subunits [188,189]. In this scenario, Emi1 functions as an APC/C “pseudo-substrate” to block APC/C's access to other substrates.

Unlike Emi1, the Emi1 homolog Emi2 inhibits APC/C to regulate meiotic cell division [191–193]. An RL tail motif at the C-terminus of Emi1/Emi2 was identified to function as a docking site for the APC/C, thereby promoting the interactions of the D-box and the ZBR domains of Emi1/Emi2 with APC/C to inhibit APC/C activity [194]. The “pseudo-substrate” theme was adopted by the Mad2 homolog, Mad2b (also named Mad2L2), which mirrors Emi1/Emi2 in inhibiting both activities of APC/C^{Cdc20} and APC/C^{Cdh1} through association with Cdc20 and Cdh1 [195,196]. Additionally, pseudo-substrate mechanisms were also identified in yeast proteins Acm1 (APC/C^{Cdh1} modulator 1) [197–199], Mes1 [124,200], and Mad3p [201]. For example, the budding yeast protein Acm1, which contains two D-box motifs and a KEN-box motif, interacts with the WD40 domain of Cdh1 to inactivate APC/C^{Cdh1} by preventing interaction of Cdh1 with its target substrates [197–199]. Similarly, the fission yeast protein Mes1, which also contains D-box and KEN-box motifs, inhibits the E3 ligase activity of the APC/C through a pseudo-substrate mechanism [124,200]. Moreover, Mad3p in *S. cerevisiae* functions as a pseudo-substrate inhibitor of APC/C^{Cdc20} through competition with APC/C substrates for Cdc20 binding [201].

1.7. APC/C-independent functions of Cdc20 and Cdh1

Although the major functions of Cdc20 and Cdh1 are to regulate cell cycle progression in an APC/C-dependent manner, APC/C-independent roles have also been characterized. In this regard, the Reed group showed that Cdc20 has APC/C-independent function in controlling mitosis in budding yeast [202]. Notably, they found that Cdc20 is critical for spindle elongation and premature chromosome segregation in *Mec1* or *Rad53* mutant cells that lack the DNA damage checkpoint,

Table 1
Summary of known substrates of APC/C.

Substrates	Functions/signaling pathways of substrates	Coactivators: Cdc20/Cdh1 ^a	References
Aurora A	Mitotic serine/threonine kinases regulating cell cycle progression	Cdh1	[76]
Aurora B	Serine/threonine kinase controlling spindle assembly, chromosome alignment and segregation	Cdh1	[77,78]
ASK (Dbf4)	Activator of S-phase kinase	Cdh1	[79]
Anillin	An actin-binding protein required for cytokinesis	Cdh1	[80]
Acm1	Cdh1 inhibitor in budding yeast	Cdh1/Cdc20	[81]
Bard1	A subunit of the Brca1-Bard1 tumor suppressor controlling spindle pole formation	Cdh1/Cdc20	[82]
BRSK2	An AMP-activated protein kinase (AMPK)-related kinase	Cdh1	[83]
Bub1	Serine–threonine kinase that plays multiple roles in chromosome segregation and spindle checkpoint	Cdh1	[84]
Clb2	Perturbs MAPK pathway signaling in yeast	Cdh1	[85]
Cdc5	Polo kinase Cdc5, a key factor in controlling Cdc14 localization	Cdh1	[86]
Cdc6	Governs the initiation of eukaryotic DNA replication	Cdh1	[87]
Cdc7	Regulates DNA replication and DNA repair	Cdh1	[79]
Cdc20	APC/C coactivator, recruiting substrates for APC/C-dependent degradation in early mitosis	Cdh1	[88,89]
Cdc25A	Tyrosine protein phosphatase induces mitotic progression by dephosphorylating CDK1 and stimulating its kinase activity	Cdh1	[90]
Cdt1	Cooperates with CDC6 to initiate DNA replication	Cdh1	[91]
Cenp-F	Required for kinetochore function and chromosome segregation in mitosis	Cdc20	[92]
Cik1	Association with the kinesin Kar3 to control both the mitotic spindle and nuclear fusion during mating	Cdh1	[93]
CKAP2	A novel microtubule-associated protein that is frequently upregulated in various malignancies	Cdh1	[94,95]
Cks1	The cofactor of Skp2 involving in G1/S transition	Cdh1	[96]
Claspin	Activates Chk1 and regulating DNA damage repair	Cdh1	[97,98]
Conductin	Inhibitor of the Wnt signaling pathway	Cdc20	[99]
Cyclin A	Controls S phase and G2/M transition	Cdc20/Cdh1	[100,101]
Cyclin B	Activates Cdk1 and controls the G2/M transition	Cdc20/Cdh1	[102]
Ect2	GDP/GTP exchange factor regulates RhoA at mitosis.	Cdh1	[103]
E2F1	Eukaryotic transcription factor to govern G1/S transition and apoptosis	Cdc20/Cdh1	[104]
E2F3	Eukaryotic transcription factor and amplified in various human tumors	Cdh1	[105]
EYA1	Controls cell proliferation, survival and M-to-G1 transition	Cdh1	[106]
FAN1	FANCD2-associated nuclease 1, previously known as KIAA1018, is required for cellular resistance against DNA inter-strand crosslinking (ICL) agents	Cdh1	[107]
Fin1	A spindle-stabilizing protein in yeast	Cdh1	[108]
FoxM1	The forkhead box M1 (FoxM1) is a transcription factor that activates expression of the cell cycle genes required for both S and M phase progression	Cdh1	[109]
Geminin	Inhibits DNA replication by preventing the incorporation of MCM complex into pre-replication complex (pre-RC).	Cdh1	[110]
GluR1	Regulates homeostatic plasticity	Cdh1	[111]
GLS1	Metabolizes glutamine to glutamate	Cdh1	[112]
GLP	Regulates histone H3K9 methylation and senescence	Cdh1	[113]
G9a	Regulates histone H3K9 methylation and senescence	Cdh1	[113]
HEC1	Controls kinetochore microtubule dynamics and mitotic exit	Cdh1	[114]
Hmmr	Regulates the localization of Tpx2 at the spindle pole	Cdh1/Cdc20	[82]
Hsl1	Involves in the morphogenesis checkpoint and MAPK pathway signaling in yeast	Cdh1	[85]
HURP	Nucleates and crosslinks microtubules in the vicinity of chromatin	Cdh1/Cdc20	[82]
Id1	Inhibits dendrite growth	Cdc20	[115]
Id2	Promotes axon growth	Cdh1	[116]
IQGAP	Promotes actomyosin-ring-independent cytokinesis at least in part by activation of Cyk3p in yeast	Cdh1	[117]
JNK	Controls cell survival, differentiation, and exit from mitosis	Cdh1	[118]
Kid	Also called KIF22, a Kinesin-like protein involved in spindle formation and the movements of chromosomes during mitosis and meiosis	Cdh1	[119]
Kif18A	Microtubule-depolymerizing kinesin that plays a role in chromosome congression	Cdc20	[120]
Liprin-α	Regulates synaptic size	Cdh1	[121,122]
Mcl-1	Anti-apoptotic protein	Cdc20	[123]
Mes1	A substrate of the APC/C as well as an inhibitor in fission yeast	Cdc20	[124]
MgcRacGAP	An important regulator of the Rho family GTPases-RhoA, Rac1, and Cdc42; indispensable in cytokinesis and cell cycle progression	Cdh1	[125]
MOAP-1	Modulator of apoptosis protein 1 (MOAP-1), an enhancer of Bax activation induced by DNA damage	Cdh1	[126]
Mps1	Dual specificity protein kinase with key roles in regulating the spindle assembly checkpoint and chromosome-microtubule attachments	Cdh1/Cdc20	[127]
Myf5	Muscle transcription factor	Cdh1	[128]
NEDL2	A HECT type ubiquitin ligase that enhances p73 transcriptional activity and degrades ATR kinase in lamin misexpressed cells	Cdh1	[129]
Nek2A	Regulate centrosome separation and spindle formation	Cdc20	[130,131]
NeuroD2	Neurogenic differentiation factor 2 inhibits presynaptic differentiation	Cdc20	[132]
NIPA	An F-box like protein that targets nuclear Cyclin B1 for degradation	Cdh1	[133]
Nlp	A key regulator in centrosome maturation that contributes to chromosome segregation and cytokinesis	Cdh1/Cdc29	[134]
Nrm1	Transcriptional activation of MBF in yeast	Cdh1	[135]
NuSAP	Nucleates and crosslinks microtubules in the vicinity of chromatin	Cdh1/Cdc20	[82]
PHF8	A demethylase PHF8 activates gene transcription primarily by demethylating histon H3 and H4	Cdc20	[136]
Plk1	A serine/threonine-protein kinase that activates MPF and assembles the mitotic spindle	Cdh1	[137]
Prkfb3	A key enzyme for regulating glycolysis	Cdh1	[138]
PR-Set7	Protein methyltransferase playing an essential role in mammalian cell cycle progression	Cdh1	[138,139]
p190	Controls Rho activity and cell mobility	Cdh1	[140]
p21 ^{Cip1}	Inhibits cyclin-dependent kinase activity	Cdc20	[141]
Rad17	Activates DNA damage checkpoint	Cdh1	[142]
RAP80	Recruits BRCA1 to DNA damage sites in the DNA damage-induced ubiquitin signaling pathway	Cdh1/Cdc20	[143]
RCS1	A mitotic regulator that controls the metaphase-to-anaphase transition	Cdh1	[144]
REV1	Y-family polymerase specialized for replicating across DNA lesions at the stalled replication fork	Cdc20	[145]
Securin	Inhibits separase activity	Cdc20/Cdh1	[71,72]

Table 1 (continued)

Substrates	Functions/signaling pathways of substrates	Coactivators: Cdc20/Cdh1 ^a	References
Sgo1	Shugoshin 1 (Sgo1) protects centromeric sister-chromatid cohesion in early mitosis, thus preventing premature sister-chromatid separation	Cdh1	[146]
Six1	An important mediator of normal development	Cdh1	[147]
SnoN	Inhibits TGF- β signaling and promote axon growth	Cdh1	[148]
Skp2	F-box protein that promotes the degradation of Cdk inhibitors p27 ^{Kip1} and p21 ^{Cip1}	Cdh1	[96,149]
Sp100	PML-NB scaffold protein, which localizes to nuclear particles during interphase and disperses from them during mitosis, participates in viral resistance, transcriptional regulation, and apoptosis	Cdc20	[150]
TACC3	Transforming acidic coiled-coil protein 3 (TACC3) is important for regulating mitotic spindle assembly and chromosome segregation	Cdh1	[151]
TK1	Thymidine kinase regulates dTTP production and genomic stability	Cdh1	[152,153]
TMPK	Thymidylate kinase regulates dTTP production and genomic stability	Cdh1	[152]
Tpx2	Regulates spindle assembly	Cdh1	[77]
TRB3	Endoplasmic reticulum (ER) stress-inducible protein, is induced by CHOP and ATF4 to regulate their function and ER stress-induced cell death	Cdh1	[154]
TRRAP	Histone acetyltransferase complex component	Cdh1/Cdc20	[155]
UbcH10	E2 enzyme and essential factor of APC/C	Cdh1	[156]
UPS1	Ubiquitin carboxyl-terminal hydrolase 1 regulates DNA repair and genomic stability	Cdh1	[157]
Yhp1	Transcriptional activation of Mcm1 in yeast	Cdh1	[135]

^a Please note that the listed substrate as an APC/C^{Cdc20} and/or an APC/C^{Cdh1} target is based upon the original reports. Further validations are required to systematically determine whether any identified given substrate is a dual substrate for APC/C^{Cdc20} and APC/C^{Cdh1}, or a unique substrate for APC/C^{Cdc20} or APC/C^{Cdh1}.

whereas the APC/C core components, Cdc16, Cdc23 and APC2, are not required for this process [202]. However, APC/C-independent functions for Cdc20 in other higher eukaryotic organisms remains to be described.

Likewise, Cdh1 also possesses APC/C-independent functions. Our group found that Cdh1 regulates the activity of the transcription factor E2F1 through competitive interaction with the hypo-phosphorylated form of Rb during the G1 phase when Cdh1 is APC/C-free [97]. Furthermore, we demonstrated that Cdh1 could regulate osteoblast differentiation in an APC/C-independent manner through disrupting Smurf1 auto-inhibition, therefore controlling the MEKK2-JNK signaling pathway downstream of Smurf1 [203]. More importantly, we found that the Δ C-box-Cdh1, a Cdh1 mutant that is incapable of binding to APC/C and degrading Cdh1 substrates, could still promote the E3 ligase activity of Smurf1. Taken together, these findings support the notion that APC/C-free Cdh1 may possess additional functions beyond its canonical APC/C-dependent roles in cell cycle progression. Given the fact that all these studies were conducted in in vitro cell-culture settings, the physiological relevance of these cell-cycle independent functions of Cdc20 and Cdh1 still awaits confirmation in mouse model studies.

2. The roles of APC/C in tumorigenesis – revealed by mouse models

A number of genetically engineered mouse models have been developed to study the physiological functions of APC/C core subunits, coactivators, and inhibitors in the past 18 years. Here, we summarize these mouse models and present a comprehensive and updated overview of how these various models provide fundamental insights into the physiological functions of APC/C in vivo. Moreover, we will discuss possible future research directions with regard to the APC/C signaling pathway and establishment of additional mouse models to elucidate molecular mechanisms and potential therapeutic applications in the treatment of human diseases, with emphasis on both cancer and neurological diseases.

2.1. APC/C core subunits

2.1.1. Anaphase-promoting complex subunit 2 (APC2)

Although 14 core proteins of the APC/C have been identified so far, KO mouse models have only been established for the two catalytic subunit proteins, *Apc2* and *Apc10/Doc1* (Table 2). APC2 forms a minimal catalytic core of APC/C with the RING finger domain protein APC11. Although the catalytic core does not have substrate specificity, it appears that APC2 is important for the APC/C activity [204,205]. To further understand the physiological significance of APC2 in vivo, the Nasmyth group established an *Apc2* KO mouse model in 2004 and found that loss of *Apc2* led to early embryonic lethality at the E6.5 stage [206]. Notably, conditional inactivation of APC2 in quiescent hepatocytes of adult livers caused re-entry into the cell cycle without any obvious external stimulus and arrested at metaphase [206]. Analysis of APC/C substrates in *Apc2*-deficient hepatocytes demonstrated elevated expression of several APC/C substrates such as Cyclin D1, Cyclin A2, Cyclin B1, Securin and Plk [206]. These results reinforce the notion that APC2 plays a vital role in cell cycle progression and early embryogenesis through regulating the levels of key cell cycle regulators such as mitotic Cyclins and Securin.

Additionally, the expression of *Apc2* is predominant in neurons within the adult mouse brain, suggesting that APC2 has important functions in neuronal cells [212]. In support of this notion, higher expression of several APC/C core subunits and the coactivator Cdh1 were found in post-mitotic, terminally differentiated neurons [213]. In order to identify the physiological function of APC2 in the nervous system, an *Apc2*-conditional KO mouse was generated in 2011 to specifically deplete *Apc2* in excitatory forebrain neurons [214]. Interestingly, although mice depletion of *Apc2* in excitatory neurons were viable and had no difference in basal anxiety, motor-coordination, or explorative depressive-like behavior, their abilities in forming spatial memories and extinction of fear memories were severely impaired [214]. Furthermore, deregulated APC/C activity by downregulation of Cdh1 in post-mitotic neurons has also been implicated in neurodegenerative

Table 2

Mouse phenotypes upon knockout of the core subunits of APC/C ubiquitin E3 ligase complex.

APC/C core subunits	Mouse models	Major phenotypes	References
APC2	<i>Apc2</i> ^{-/-}	Embryonic lethality before E6.5	[206]
	Conditional <i>Apc2</i> ^{-/-} (Liver)	Liver failure and premature death	[206]
	Conditional <i>Apc2</i> ^{-/-} (Excitatory neuron)	Impaired cognitive function	[207]
APC10/Doc1	<i>Apc10</i> ^{-/-}	Early and recessive embryonic lethality	[208,209]
	<i>Apc10</i> ^{+/-}	Showed fusion of the second and third digits on all four limbs	[210,211]

Keys: +, wild type allele; -, null allele.

diseases, such as Alzheimer's disease [215], urging for the generation of a Cdh1 conditional KO mouse in neuronal tissues to further explore the role of Cdh1 in memory, learning or neurodegenerative diseases.

2.1.2. Anaphase-promoting complex subunit 10 (APC10)

A mouse model carrying the *Apc10/Doc1*-null mutation was generated as early as the 1960s [208]. At that time, the *Apc10/Doc1* was named *Oligosyndactylism* (OS) based on the phenotype of heterozygotic mice with fusion of the second and third digits on all four limbs [208,216]. However, homozygotes of the OS mutation caused an early and recessive embryonic lethality due to the inability of mutant embryonic cells to complete mitosis. Further analysis showed that the OS mutation prevented the transition from metaphase to anaphase at the blastocyst stage, due to enhanced stability of APC/C substrates such as Cyclin B and Securin [209]. Subsequent structural and expression analyses of the genomic region around the OS mutation led to the finding that the anaphase-promoting complex core component *Apc10/Doc1* was disrupted, providing an explanation for the mitotic arrest phenotype of the radiation-induced OS mutant [210,211]. Although expression of APC10/Doc1 was also detected in tissues that mostly contain differentiated cells, such as adult brain [213,215], tissue-specific KO mouse models have yet to be established. Hence, conditional KO mouse models are required for further analysis of tissue-specific roles of APC10/Doc1 in human diseases such as cancer or neurological disorders.

2.2. APC/C co-activators

The two co-activators of the APC/C, Cdc20 and Cdh1 (see Table 3), have been relatively well studied compared to other APC/C components. Increasing evidence has demonstrated that Cdc20 is frequently overexpressed in human cancers including lung cancer [217], pancreatic ductal adenocarcinoma [218], cervical cancer [219], glioblastoma [220], oral cancer [221] and epithelial ovarian cancer [222]. Moreover, depletion of Cdc20 inhibits tumor growth [223]. These results suggest that Cdc20 expression is positively correlated with human cancer or tumor growth. Contrary to Cdc20, Cdh1 is often found to be downregulated or lost in many human cancers [98,224,225] and targeted depletion of *Cdh1* predisposes mice to tumorigenesis [226]. However, it has also been reported that Cdh1 is overexpressed in some tumor types through immunohistochemical staining of tissue microarrays [227]. To address the physiologic functions of Cdc20 and Cdh1, several genetically modified mouse models have been generated as summarized below.

2.2.1. Cell division cycle 20 (Cdc20)

In 2007, Li et al. demonstrated that loss of *Cdc20* through the gene-trapping method caused mouse embryonic lethality at the two-cell stage due to metaphase arrest [228]. Further analysis showed that the metaphase arrest depended on the function of Securin as the metaphase arrest phenotype was abolished in *Cdc20* and Securin double mutant

embryos [228]. Subsequently, a tamoxifen inducible conditional *Cdc20* KO mouse (*Cdc20*^{-lox}/RERT^{+/Cre}) was generated by the Malumbres group in 2010, in which they found that depletion of *Cdc20* in adult mice with a 4-hydroxytamoxifen (4-OHT) supplemented diet specifically caused abundant metaphase arrest in proliferative tissues such as the intestine and testis [223]. More importantly, to elucidate the role for Cdc20 in vivo, skin tumors were induced in mice with the two-stage carcinogenesis protocol using 7,12-dimethylbenz- α -anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), and 4-OHT was then topically applied to the papillomas. Strikingly, compared with control mice, tumor growth was completely inhibited in *Cdc20*^{-/-}/RERT^{+/Cre} mice [223]. Further histological analysis revealed that depletion of Cdc20 in skin tumors resulted in arrest of tumor cells in metaphase-like stages, accompanied by induction of cellular apoptosis [223]. Hence, this study indicates that developing specific inhibitors for Cdc20 may represent a potential novel therapeutic approach for the treatment of human cancers with overexpressed Cdc20 [232].

It has also been reported that Cdc20 also plays a crucial role during meiosis. Yin et al. found that Cdc20 is required for the anaphase onset of meiosis I but not meiosis II in mouse oocytes [233]. Through microinjecting anti-Cdc20 antibody at different meiosis stages to block the function of Cdc20 in mouse oocytes, they found that only when anti-Cdc20 antibody was injected at the pre-metaphase I did they see a majority of oocytes arrest at the metaphase I stage [233]. Furthermore, to understand the physiological role of Cdc20 in meiosis and fertility, the van Deursen group generated a series of mutant mouse strains with graded reduction in Cdc20 expression levels to avoid embryonic lethality, as observed in *Cdc20* KO mice [229]. These hypomorphic mice, with lower Cdc20 expression, appeared to be healthy and had normal lifespan, folliculogenesis and fertilization rates. However, the fertility of female mice was significantly reduced. Mechanistically, they uncovered that a large portion of these embryos died after the first few embryonic divisions because of chromosome misalignment during meiosis I in oocytes [229]. These results demonstrated that Cdc20 levels might be essential for differentiation of primary oocytes. However, it remains to be determined whether Cdc20 insufficiency affects human female infertility.

2.2.2. Cdc20 homologue protein 1 (Cdh1, also called Fizzy-related protein 1, Fzr1)

Notably, expression of Cdh1 is reduced in several tumor cell lines [234] and tumor tissues, including prostate, ovary, breast, colon, liver and brain [98,224,225]. Concomitant with downregulation of Cdh1 expression, several APC/C^{Cdh1} targets, such as Cyclin B, Aurora A, Aurora B, Tpx2, Cdc6 and Cdc20, are frequently upregulated in human cancer tissue samples [235]. On the other hand, Lehman et al. demonstrated that Cdh1 was overexpressed in certain tumor types [227].

Several genetically engineered mouse models were generated to identify the physiologic functions of Cdh1 in vivo. The Malumbres group generated a *Cdh1* KO mouse model, and found that whole-body

Table 3
Major physiological functions of the APC/C co-activators.

APC/C co-activators	Mouse models	Major phenotypes	Putative roles in tumorigenesis	References
Cdc20	<i>Cdc20</i> ^{-/-}	Embryonic lethality at the two-cell stage	Cancer target	[228]
	<i>Cdc20</i> ^{-/-} /Securin ^{-/-}	Unable to rescue the embryonic lethality and the sister chromatid separation defects		[228]
	Conditional <i>Cdc20</i> ^{-/-}	Caused abundant metaphase arrests in proliferative tissues and inhibited the growth of tumors in vivo		[223]
Cdh1	<i>Cdc20</i> ^{-H}	Females produce either no or very few offsprings	Context-dependent	[229]
	<i>Cdh1</i> ^{-/-}	Embryonic lethality at around E9.5–E10.5 stage; Poorly developed placenta		[164,226]
	<i>Cdh1</i> ^{+/-}	Showed a decrease in survival by 25 months of age; Elevated spontaneous epithelial tumor incidence; Learning/memory defects		[164,226]
	Conditional <i>Cdh1</i> ^{-/-}	Deficiency of Cdh1 in the developing nervous system results in hypoplastic brain, hydrocephalus, replicative stress and p53-mediated apoptotic death		[230,231]

Keys: +, wild type allele; -, null allele; H, hypomorphic allele.

depletion of *Cdh1* led to embryonic lethality at E9.5–E10.5 in part due to defective endo-reduplication of trophoblast cells and placental mal-function [226], which was confirmed by another independent *Cdh1* knockout mouse using a gene-trap method [164]. *Cdh1* heterozygous mice showed a decrease in survival and were more susceptible to develop epithelial tumors, such as mammary adenocarcinomas and fibroadenomas [226], indicating that *Cdh1* may function as a haploinsufficient tumor suppressor. In addition, *Cdh1* may be required for learning and memory, which was impaired in *Cdh1* heterozygous mice with defective late-phase long-term potentiation (L-LTP) in the hippocampus [164], while the underlying molecular basis remains unclear. Recent reports demonstrated that tissue-specific genetic ablation of *Cdh1* in the developing nervous system results in p53-mediated apoptotic death [230,231]. Hence, the role of *Cdh1* in tumorigenesis might be context-dependent or tissue-specific. Therefore, it will be important to develop additional tissue-specific conditional *Cdh1* KO mouse models to fully understand the role of *Cdh1* in tissue development and tumorigenesis.

2.3. APC/C inhibitors

As one of the master regulators of cell cycle progression and possibly genomic stability, the activity of the APC/C is tightly controlled by various mechanisms including endogenous inhibitory proteins (Table 4). These naturally occurring inhibitors have been evolutionarily developed to maintain ligase activity of APC/C in check, ensuring precise cell cycle progression and chromosomal stability. The failure in these inhibitory mechanisms contributes to the development of human diseases, especially cancer [236,237]. Various mouse models have been established in order to identify the physiological functions of these APC/C inhibitors in vivo, which are summarized in Table 4.

2.3.1. Mitotic arrest deficient protein 2 (*Mad2*)

Among various APC/C endogenous inhibitors, three SAC proteins *Mad2*, *Mad3/BubR1*, and *Bub3* have been reported to play central roles in controlling APC/C E3 ligase activity. Specifically, *Mad2* inhibits

APC/C^{Cdc20} through binding to *Cdc20* to prohibit its association with the APC/C core complex. However, optimal inhibition requires the additional association of *Cdc20* with *Mad3/BubR1* and *Bub3*, leading to assembly of the mitotic checkpoint complex (MCC) [181]. The MCC ensures accurate chromosome segregation and stability by blocking APC/C^{Cdc20} E3 ligase activity, thus preventing the degradation of Cyclin B and Securin and delaying the transition from metaphase to anaphase until all chromosomes have successfully created bipolar attachments to the mitotic spindles [55,59,256].

To understand the physiological functions of these APC/C inhibitors in vivo, several mouse models have been established thus far. Dobles et al. reported that *Mad2* KO resulted in mouse embryonic lethality in utero at about E6.5–E7.5 and cells derived from *Mad2*-null embryos at E5.5 are unable to arrest in mitosis in response to nocodazole-induced spindle disruption [238]. Furthermore, loss of *Mad2* led to chromosome missegregation coupled with a decrease in the number of mitotic cells and increased apoptotic cell death in the majority of embryos at about E6.5–E7.5 [238]. Subsequently, the Benezra group demonstrated that deletion of one *Mad2* allele caused defective mitotic checkpoint in both human cancer cells and murine embryonic fibroblasts [239]. More importantly, *Mad2*^{+/-} mice were prone to developing papillary lung adenocarcinomas with very long latencies, indicating a tumor suppressor role for *Mad2* [239]. In keeping with this finding, Chi et al. demonstrated that *Mad2* and *p53* deficiencies cooperated to promote tumorigenesis as *Mad2*^{+/-}/*p53*^{+/-} mice displayed increased cancer incidence and tumor burden than their *p53*^{+/-} counterparts [240]. Consistently, loss of heterozygosity around the 4q27 region containing *Mad2* was identified in several tumor types [257–259].

On the other hand, *Mad2* has also been found to be overexpressed in many human cancers, such as malignant lymphoma [260], lung cancer [261–263], hepatocellular carcinoma [264,265], colorectal carcinoma [266], soft tissue sarcoma [267] and gastric cancer [268]. To elucidate the consequence of *Mad2* overexpression, the Benezra group generated a transgenic mouse model with conditional overexpression of *Mad2* using a tetracycline-inducible system [241] and demonstrated that *Mad2* overexpression led to a wide range of neoplasia, including lung

Table 4
Major physiological functions of the characterized endogenous APC/C inhibitors.

Inhibitors	Mouse models	Major phenotypes	Role in cancer	References
<i>Mad2</i>	Knockout (KO)	<i>Mad2</i> ^{-/-} <i>Mad2</i> ^{+/-} <i>Mad2</i> ^{+/-} / <i>p53</i> ^{+/-}	Embryonic lethality around E6.5–E7.5 Prone to develop spontaneously lung tumors Higher tumor incidence relative to single mutant mice	Context-dependent [238] [239] [240]
	Transgenic (Tg)	<i>Tet-O Mad2</i> <i>Tet-O Mad2 and KRas</i>	Displayed a wide range of neoplasias Accelerating lung tumorigenesis and tumor relapse after KRas withdrawal	[241] [242]
	Knockout (KO)	<i>BubR1</i> ^{-/-} <i>BubR1</i> ^{+/-}	Embryonic lethality beyond E8.5 Prone to develop tumors after AOM treatment, and showed abnormal megakaryopoiesis	Emerging role as a tumor suppressor [243] [243,244]
		<i>BubR1</i> ^{H/H}	Prone to lung tumors after DMBA treatment; Early onset of aging phenotypes, and exhibited accelerated age-related gliosis phenotype	[245–247]
		<i>BubR1</i> ^{+/-} / <i>Apc</i> ^{Min/+} <i>BubR1</i> ^{H/H} / <i>p16</i> ^{INK4a-/-} <i>BubR1</i> ^{H/H} / <i>p19</i> ^{Arf-/-}	Showed a 10-fold increase in colon tumors and a 50 % decrease in small intestine tumors relative to <i>adenomatous polyposis coli</i> (<i>Apc</i>) ^{Min/+} mice Showed increased lung tumors relative to <i>p16</i> ^{INK4a-/-} mice; Attenuated age-related phenotypes Accelerated aging process, whereas no significant difference in survival curves relative to <i>p19</i> ^{Arf-/-} mice	[248] [249] [249]
<i>Bub3</i>	Transgenic (Tg)	Overexpression <i>BubR1</i> transgenic mice	Delays age-related deterioration and aneuploidy, extends lifespan, and reduces tumorigenesis	[250]
		<i>Bub3</i> ^{-/-} <i>Bub3</i> ^{+/-}	Embryonic lethality around E8.5 No obvious abnormalities in development or fertility; No cancer predisposition but higher tumor incidence after DMBA treatment	Emerging role as a tumor suppressor [251,252] [251,252]
		<i>Bub3</i> ^{+/-} / <i>p53</i> ^{+/-}	No substantial differences in the rates of survival or tumorigenesis relative to <i>Bub3</i> ^{+/-} / <i>p53</i> ^{+/-} mice	[253]
		<i>Bub3</i> ^{+/-} / <i>Rb1</i> ^{+/-} <i>Bub3</i> ^{+/-} / <i>Rae1</i> ^{+/-}	No significant differences in the rates of survival or tumorigenesis relative to <i>Bub3</i> ^{+/-} / <i>Rb1</i> ^{+/-} mice Higher incidence of tumor formation compared with single heterozygous mice after DMBA treatment; Displayed early aging-associated phenotypes	[253] [254]
		<i>Emi1</i> ^{-/-}	Embryonic lethality before implantation; Defective cell proliferation and mitotic progression; Multipolar spindle and misaligned chromosomes	To be determined [255]

Keys: +, wild type allele; -, null allele; Tet-O, tetracycline-inducible overexpression; H, hypomorphic allele; AOM, azoxymethane; DMBA, 7,12-dimethylbenz- α -anthracene.

adenomas, hepatocellular carcinomas, lymphomas, and fibrosarcomas [241]. Moreover, induced expression of Mad2 resulted in stabilization of the APC/C substrates Securin and Cyclin B, delaying exit from mitosis and generating a greater aneuploid/polyploidy cell population [241]. These results suggest that Mad2 overexpression may exert oncogenic potentials by generating a hyperactive mitotic checkpoint to override the critical role of the APC/C in the control of cell cycle and genomic stability. Consistently, a recent study suggested that Mad2-induced chromosome instability also led to lung tumor relapse in transgenic mice with tetracycline-inducible overexpression of KRAS and Mad2 [242]. These data demonstrated that aberrant overexpression of Mad2 correlates with tumorigenesis in part due to chromosome missegregation. Generation of conditional KO and Tg mice for *Mad2* would be useful to further define whether Mad2 exerts causally either a tumor suppressor or an oncogenic role in different tissue settings, and if so, what is the exact molecular mechanism(s) by which Mad2 regulates tumorigenesis.

2.3.2. Bub1-related protein (BubR1)

The mitotic checkpoint protein BubR1 (also named Mad3) functions as an endogenous inhibitor of APC/C^{Cdc20} via assembling a complex with Mad2 and Bub3 [181]. Subsequent studies demonstrated that the N-terminal domain of BubR1 is essential for binding the Mad2-Cdc20 complex with APC/C in a KEN box-dependent manner [269,270]. BubR1 inhibits APC/C activity during interphase and causes the accumulation of Cyclin B prior to onset of mitosis. Similar to Mad2 KO, complete loss of *BubR1* in mice led to an embryonic lethality around E8.5 in utero partly due to extensive apoptosis [243]. However, *BubR1*^{+/-} mice, unlike *Mad2*^{+/-} mice, exhibited more splenomegaly and extramedullary megakaryocytes, which were coupled with a decrease in erythroid progenitors in bone marrow [243]. An increased number of megakaryocytes, the major cell type with a polyploid DNA content, strongly indicated a slippage of mitotic arrest and abnormal mitotic exit in the *BubR1*^{+/-} cells [243]. Furthermore, *BubR1*^{+/-} mice were more prone to tumorigenesis induced by azoxymethane (AOM) treatment, suggesting a tumor suppressor role for BubR1 [243].

To further define the role of BubR1 in tumorigenesis, the van Deursen group generated a series of mice with gradual reduction of BubR1 expression by using the wild-type (*BubR1*⁺), knockout (*BubR1*⁻), and hypomorphic (*BubR1*^H) alleles [245]. They confirmed that *BubR1*^{-/-} mice died during embryonic development, while *BubR1*^{-H} mice died a few hours after birth. The *BubR1*^{H/H} mice had normal phenotype at birth, but showed slow postnatal growth, infertility, and early onset of aging phenotypes, including short lifespan, loss of subdermal adipose tissue, cataract formation, severe spinal kyphosis and impaired wound healing [245]. Further analysis demonstrated that *BubR1*^{H/H} MEFs had lower Cyclin B/Cdk1 kinase activity than wild-type MEFs after prolonged exposure to nocodazole, indicative of a spindle checkpoint defect [245]. Moreover, *BubR1*^{H/H} mice exhibited an accelerated age-related gliosis and aging-associated vascular phenotypes [246,247]. Although very few spontaneous tumors were observed in *BubR1*^{+/-} and *BubR1*^{H/H} mice, *BubR1*^{+/-} mice were prone to rapid development of lung and colon adenocarcinomas after exposure to the carcinogen, dimethylbenzanthrene (DMBA) [244]. Furthermore, the incidence of colonic tumors were increased about ten fold in *BubR1*^{+/-}/*Apc*^{Min/+} compound mice, which is accompanied by mitotic slippage in the presence of nocodazole and premature separation of sister chromatids [248].

Interestingly, although expression levels of the tumor suppressors p16^{Ink4a} and p19^{Arf} were increased with aging in different tissues [271,272], inactivation of these two tumor suppressors in *BubR1*^{H/H} mice exhibited opposing phenotypes [249]. Inactivation of p16^{Ink4a} attenuated the development of age-related pathological phenotypes, whereas p19^{Arf} inactivation accelerated the process of aging in the *BubR1*^{H/H} mice [249]. Moreover, *BubR1* insufficiency had synergistic effects on tumorigenesis in lung epithelial cells with loss of p16^{Ink4a}, but did not synergize to drive tumorigenesis with p19^{Arf} loss [249].

Recently, Baker et al. reported that *BubR1* overexpression protects against aneuploidy, extends healthy lifespan and confers resistance to tumorigenesis [250]. These results from both KO and Tg *BubR1* mice models demonstrated that by inhibiting APC/C, BubR1 plays an essential role in regulating chromosome stability and suppresses cancer development. However, it remains unclear why there is a synergistic effect of BubR1 insufficiency with loss of the p16^{Ink4a} tumor suppressor, but not with the loss of the p19^{Arf} tumor suppressor, and why this synergistic effect is only observed in certain tissues such as lung epithelial cells, but not in other cell types [249]. Thus, generation of conditional *BubR1* KO or Tg mouse models as well as the compound mouse models with deletion of related genes along the BubR1 pathway would be essential to elucidate the molecular mechanisms underlying the tumor suppressor role of BubR1 in various tissue settings.

2.3.3. Budding uninhibited by benzimidazole protein 3 (Bub3)

Bub3, another conserved component of the mitotic spindle assembly complex, also plays an essential role in inhibiting APC/C E3 ligase activity. To determine the physiological roles of Bub3 in vivo, Kalitsis et al. generated *Bub3* KO mouse in 2000 [251]. *Bub3*^{-/-} mice showed embryonic lethality and died around E6.5–E7.5 in utero. An abundance of mitotic errors from E4.5–E6.5 embryos, such as the formation of micronuclei, chromatin bridging, lagging chromosomes, and grossly abnormal nuclear morphology were also observed [251]. However, *Bub3*^{+/-} mice had no obvious abnormalities in development or fertility compared to wild-type mice [251]. Moreover, there were no significant differences in the rates of survival or tumorigenesis between *Bub3*^{+/-}/*p53*^{+/-} and *Bub3*^{+/-}/*p53*^{+/-} or between *Bub3*^{+/-}/*Rb1*^{+/-} and *Bub3*^{+/-}/*Rb1*^{+/-} mice [253]. Consistently, in another independent study, *Bub3*-null mice were embryonic lethal while heterozygous *Bub3* mice were viable and showed mitotic checkpoint defects with greater rates of premature sister chromatid separation and chromosome missegregation [252]. Importantly, although *Bub3*^{+/-} mice did not spontaneously develop tumors, they were more prone to carcinogen-induced lung tumorigenesis [252]. Furthermore, mice double haploinsufficient for *Bub3* and *Rae1* (*Bub3*^{+/-}/*Rae1*^{+/-}) led to higher incidences of tumor formation after dimethylbenzanthrene (DMBA) treatment compared with single haploinsufficient mice [254]. Like *BubR1*^{H/H} mice [245], *Bub3*^{+/-}/*Rae1*^{+/-} mice displayed premature aging-associated phenotypes, including cataract formation, lordokyphosis, and significantly reduced dermal thickness and subdermal adipose tissue, and aged faster than *BubR1*^{H/H} mice [254]. Hence, these findings indicate that activation of the senescence pathway might be the cause of early onset of aging-associated phenotypes in mice with defects in mitotic checkpoint genes [254].

2.3.4. Early mitotic inhibitor 1 (Emi1)

Emi1 plays a crucial role in regulating the G1 to S phase transition and mitotic progression through inhibiting the E3 ligase activity of APC/C^{Cdh1}. It has been reported that Emi1 expression is upregulated at the beginning of interphase and is degraded in early mitosis just prior to degradation of Cyclin A by APC/C^{Cdc20} [188,190]. Furthermore, the Pagano and Jackson laboratories demonstrated that SCF^{β-TRCP1} could target Emi1 for degradation, which was required for the timely activation of APC/C^{Cdc20} in early mitosis [273]. Moreover, the Pagano group generated *β-Trcp1* KO mice and found that Emi1 and mitotic cyclins were accumulated in *β-Trcp1*^{-/-} MEFs [273]. Another independent report from the Jackson group revealed that degradation of Emi1 by SCF^{β-TRCP1} was required for APC/C^{Cdc20} activation in early mitosis. Moreover, failure of β-TRCP1-mediated Emi1 destruction caused the stabilization of many APC/C substrates and subsequent mitotic catastrophe, such as blockage of prometaphase, chromosome missegregation, and centrosome amplification [273]. Importantly, both groups found that the DSGXS motif within Emi1, and phosphorylation of the two serine residues in this motif, was required for recruiting the SCF^{β-TRCP} ubiquitin ligase to degrade Emi1 [273]. Subsequent studies from the Jackson

and Herskho groups further identified polo-like kinase 1 (Plk1) as the primary kinase to phosphorylate the serine residues in the DSGxxS motif of Emi1, therefore ear-marking Emi1 for ubiquitination and degradation by SCF^{β-TRCP} [274,275].

To determine the physiological functions of Emi1 in vivo, Lee et al. generated *Emi1*-deficient mice using the gene-targeting technique [255]. They demonstrated that *Emi1*^{-/-} embryos died during the pre-implantation stage in part due to defects in mitotic progression, including multipolar spindles formation and chromosomal missegregation [255]. Expectedly, reduced levels of APC/C substrates such as Cyclin A were observed in the resulting *Emi1*-deficient embryos [255]. Embryonic lethality upon germline deletion of *Emi1* prevents the study of its role in tumorigenesis. Thus, establishment of a conditional *Emi1* KO mouse model is essential to clarify the physiological function of Emi1 in mitotic regulation and tumorigenesis in a tissue-specific manner.

3. Conclusion and future perspectives

The APC/C ubiquitin E3 ligase complex, consisting of at least 14 core proteins, is the most complicated member of the RING finger ubiquitin E3 ligase family and plays a prominent role in controlling cell cycle progression [9,41]. From the initial identification of mitotic cyclins as APC/C substrates in 1995, we have gained a deeper understanding of the biochemical features and the biological functions of the APC/C E3 ligases, as well as their implications in human diseases through a large array of studies using a combination of biochemical, structural and genetic approaches. In this review, we have updated the growing list of the identified ubiquitin substrates for APC/C (see Table 1). More importantly, we have summarized the studies using genetically modified mouse models through transgenic expression and germline inactivation of the components of the APC/C core subunits, coactivators and inhibitors (Tables 2–4). Moreover, we have also discussed the respective functions of these genes, although very limited, under in vivo physiological conditions. To achieve a thorough understanding of the physiological functions of APC/C E3 ligases and their potential contributions to human diseases, we proposed the following directions for future study of these very important E3s.

First, although APC/C is composed of at least 14 core proteins, only two knockout mouse models have been established with targeted inactivation of *Apc2* and *Apc10/Doc1*, respectively. To address why the APC/C complex requires so many subunits to function as an ubiquitin E3 ligase, a long-standing question in the APC/C research field, it will be important to investigate the physiological functions of each APC/C subunit. Therefore, a systematic generation of genetic knockout mouse models targeting the remaining APC/C components will be a critical approach to reveal the role for individual APC/C subunits. Given the fact that germline knockout of either APC2 or APC10 causes embryonic lethality, a tissue-specific conditional knockout strategy should be used to generate these specific APC/C component-KO mice. Given that deletions and point mutations of several core subunits of APC/C, such as APC3/Cdc27, APC6/Cdc16 and APC8/Cdc23, are reported in colon cancer cell lines and human tumor samples [276], it will be of great interest to determine the causal role of these subunits by generating and characterizing colon specific deletion mouse models for colon tumorigenesis.

Second, it remains largely unclear mechanistically why the two APC/C coactivators display different roles in tumorigenesis. The possible explanations might be: (i) substrates with oncogenic or tumor suppressor functions are not shared between the Cdc20 and Cdh1. This is supported by the fact that APC/C^{Cdc20} typically degrades substrates with D-boxes [277], while APC/C^{Cdh1} could degrade a wider range of substrates containing the D-box [277], KEN-box [278], A-box [76,279], O-box [280], CRY box [281] or GxEN box [282]. Notably, many substrates of APC/C^{Cdh1}, including Plk1, Aurora A, Skp2, and UbcH10, were upregulated in human cancers [283]. Although Skp2 [96,149] and UbcH10 [156,282] have been characterized as specific APC/C^{Cdh1} substrates, it remains elusive whether elevated Plk1 and Aurora A

expressions are mainly due to defects in APC/C^{Cdh1} and/or APC/C^{Cdc20} activities. Therefore, future studies should be directed to distinguish the overlapping or unique substrates of APC/C^{Cdh1} versus APC/C^{Cdc20}. (ii) APC/C^{Cdh1} and APC/C^{Cdc20} activities are present at different cell cycle phases. APC/C^{Cdh1} is active in late M/early-mid G1 while APC/C^{Cdc20} is active in early-mid M phase, which might differentially affect tumorigenesis. (iii) Although current studies are limited, similar to observations made in Akt1 versus Akt2 [284], the different behaviors of Cdh1 versus Cdc20 in tumorigenesis might be derived from their tissue-specific expression. It is highly possible that different expression levels of Cdc20 and Cdh1 in different tissues or organs may affect their cellular functions. (iv) Another possibility for the different tumorigenic effects of Cdc20 and Cdh1 is that biochemically, Cdh1 can promote the degradation of Cdc20, indicating that an opposite role for Cdh1 and Cdc20 in tumorigenesis could be due to excess degradation of Cdc20 by Cdh1.

Notably, Cdc20 expression is positively correlated with human cancer in clinical samples and depletion of Cdc20 inhibits tumor growth, whereas a possible tumor suppressor role for Cdh1 is observed in different, but not all human cancers. Hence, designing a specific inhibitor targeting of APC/C^{Cdc20}, rather than a pan-APC/C inhibitor such as pro-TAME [280,285] should be more selective in killing cancer cells with Cdc20 overexpression (Fig. 4).

Third, recent studies demonstrated that for the Cullin-Ring class of E3 ligases (CRL), conjugation of the ubiquitin-like molecule NEDD8 to the Cullin subunit is critical for the E3 ligase complex to adopt catalytically active conformations [286]. Therefore, the E3 ligase activities of all CRLs could be positively regulated by neddylation, and MLN4924, a specific inhibitor of the neddylation process, has been actively pursued and proven effective as a novel class of anti-cancer agents in many preclinical studies [287]. However, due to its enormous size, thus making biochemical characterization difficult, it remains largely unclear how the APC/C complex is assembled and whether the assembly and the integrity of APC/C is also under tight regulation via various post-translational modifications. At the present time, it is known that APC/C activities could be regulated by both phosphorylation [53,54,165] and acetylation [277,288]. Future studies should be directed to determine if the core components of APC/C are modified by ubiquitin-like molecules, such as SUMO, NEDD8, and ISG5, or by other mechanisms including, but

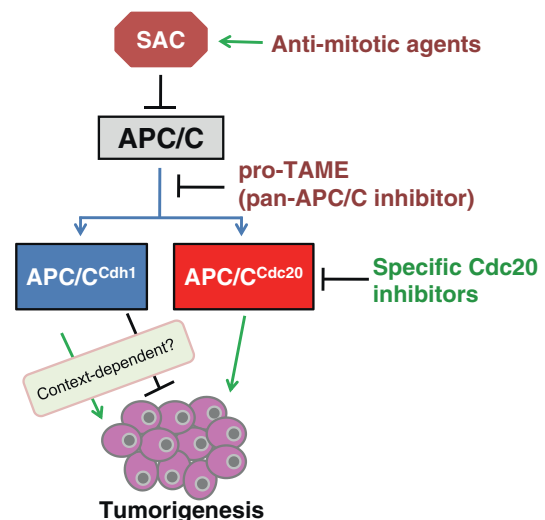


Fig. 4. Developing specific Cdc20 inhibitors, but not pan-APC/C inhibitors, as potential therapeutic target for cancer treatment. Recent studies demonstrated Cdc20 expression is positively correlated with human cancer and depletion of Cdc20 inhibits tumor growth, whereas a possible tumor suppressor role for Cdh1 is observed in different, but not all human cancers. Hence, it is necessary to develop specific Cdc20 inhibitors, rather than the pan-APC/C inhibitors, such as pro-TAME and the anti-mitotic agent, for selective killing of cancer cells.

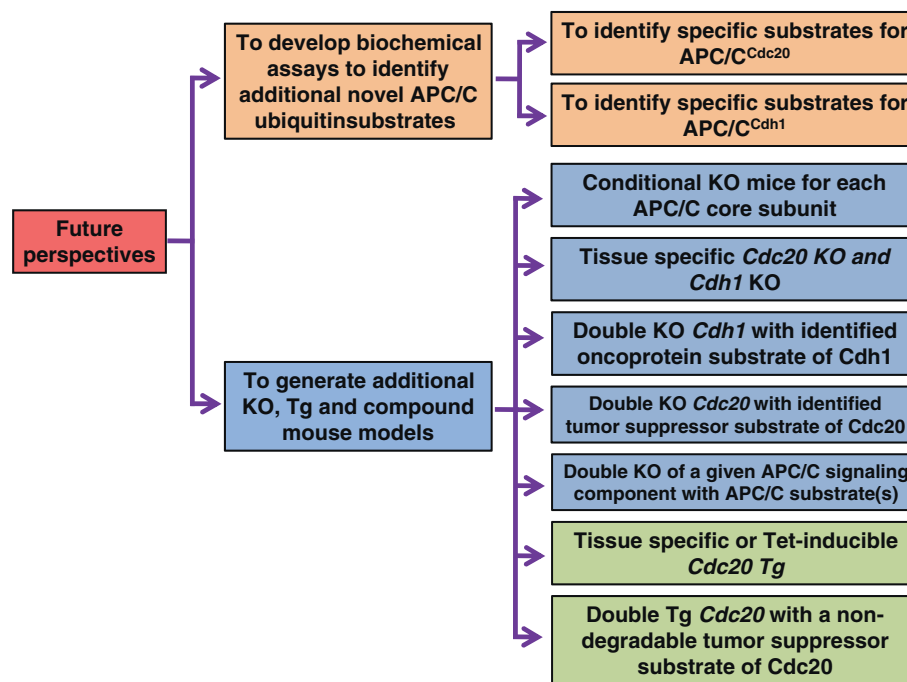


Fig. 5. Future perspectives to determine the physiological roles for APC/C E3 ligases in tumorigenesis. The current biochemical and genetic data are insufficient to define the exact role for each APC/C signaling component in tumorigenesis, and cannot fully explain the molecular mechanisms underlying the opposing roles of Cdc20 versus Cdh1 in cancer. Hence, future researches using a combined array of various biochemical and genetic tools are required for a better understanding of APC/C function at the biochemical and physiological levels. Biochemically, identification and characterization of additional substrates for APC/C^{Cdh1} and APC/C^{Cdc20} through a combination of biochemical and cell biology methods, will aid the further understanding of their respective roles in tumorigenesis. Genetically, extensive efforts should be made to systematically generate conditional KO mice for each APC/C core complex component, as well as to generate inducible Tg mice models for both Cdc20 and Cdh1 in an attempt to thoroughly define their role in tumorigenesis in various tissues. More importantly, in order to pinpoint the critical downstream effects mediating the tumorigenic role for Cdh1, Cdc20 or any given APC/C components, compound KO mouse models should be generated to examine whether additional depletion of a downstream signaling component could rescue the observed tumorigenic phenotype, thereby offering a causal relationship and mechanistic insight.

not limited to, Tyr-phosphorylation, Lysine or Arginine methylation or Proline-hydroxylation, and to further elucidate the underlying molecular mechanisms that control the modification-dependent switch-OFF or switch-ON of APC/C activities.

Finally, extensive efforts should be made using KO mouse models to determine molecular mechanism of each component of APC/C E3 in tumorigenesis. For example, it will be mechanistically insightful through the determination of whether tumorigenesis triggered by *Cdh1* deletion could be rescued by simultaneous deletion of its downstream target such as Skp2 [96,149] (Fig. 5). Furthermore, it warrants further studies regarding whether conditional expression of a non-degradable Cdc20 substrate, most likely acting as a tumor suppressor, will retard the growth of certain types of tumors with Cdc20 overexpression. Additionally, although cell culture-based studies have clearly shown an important role for various APC/C inhibitors including Mad2, BubR1, Bub3 and Emi1 in causing chromosomal instability, and various mouse models suggest their involvement in tumorigenesis, no causal relationship has been firmly established as to whether hyper-active APC/C^{Cdc20} is the primary driver of tumorigenesis. To rigorously examine this concept, various APC/C inhibitor KO mouse models should be crossed with *Cdc20* or *Apc2* heterozygous mice to examine whether reduction in APC/C^{Cdc20} could alter the process of tumorigenesis. Moreover, results derived from such studies could likely provide the rationale for the molecular basis in developing specific Cdc20 inhibitors to treat cancers, especially those with defective MCC activities.

Protein degradation by the ubiquitin proteasome system regulates many key cellular processes, and consistent with this, a significant number of genes are devoted to this basic biochemical activity. The APC/C is one of many ubiquitin E3 ligase complexes assembled in the cell where it has a deep-rooted function in regulating cell cycle progression. Although significant advances have been made thus far in the function, regulation, and consequences of aberrant activity of the APC/C, there

are still many important questions to be addressed including the role of APC/C^{Cdc20} and APC/C^{Cdh1} in tumorigenesis. No doubt that through utilization of advanced cell biological techniques and mouse modeling, a greater understanding of the APC/C will be established, and novel therapeutics targeting the APC/C will be developed for treatment of human diseases such as cancer and neurodegenerative disorders.

Conflict of interest

The authors declare that they have no conflict of interest. Correspondence and requests for materials should be addressed to W.W. (ww2@bidmc.harvard.edu).

Acknowledgements

We sincerely apologize to all those colleagues whose important work was not cited in this paper due to space limitations. We thank Pengda Liu, Hiroyuki Inuzuka, Brian North, Alan W Lau, Shavali Shaik, Adriana Tron and other members of the Wei laboratory for the critical reading and discussion of the manuscript. W.W. is an ACS research scholar and a LLS research scholar. This work was supported in part by the NIH grants to W.W. (GM089763, GM094777 and CA177910) and Y.S. (CA 171277).

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