Chapter 15

Detection and Analysis of Cell Cycle-Associated APC/C-Mediated Cellular Ubiquitylation In Vitro and In Vivo

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Abstract

The anaphase-promoting complex or cyclosome (APC/C) is one of the major orchestrators of the cell division cycle in mammalian cells. The APC/C acts as a ubiquitin ligase that triggers sequential ubiquitylation of a significant number of substrates which will be eventually degraded by proteasomes during major transitions of the cell cycle. In this chapter, we present accessible methodologies to assess both in in vitro conditions and in cellular systems ubiquitylation reactions mediated by the APC/C. In addition, we also describe techniques to evidence the changes in protein stability provoked by modulation of the activity of the APC/C. Finally, specific methods to analyze interactors or posttranslational modifications of particular APC/C subunits are also discussed. Given the crucial role played by the APC/C in the regulation of the cell cycle, this review only focuses on its action and effects in actively proliferating cells.

Key words Anaphase-promoting complex or cyclosome (APC/C), Cell cycle, Ubiquitin, Ubiquitylation, Phosphorylation, Cdc27/APC3, Cdh1, Cdc20

1 Introduction

Progression through the cell division cycle in mammalian cells is an intricate process exquisitely regulated by posttranslational modifications such as phosphorylation and ubiquitylation [1, 2]. In particular, inactivation of essential cell cycle regulators is achieved through their ubiquitylation and proteasome-mediated degradation. Ubiquitylation mechanisms associated with proteolysis often involve the formation of a polyubiquitin chain covalently attached to the substrates. Those chains are generated via the repeated and sequential action of three types of enzymes called ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Chains of ubiquitin can be formed through lysines 6, 11, 27, 29, 33, 48, or 63 or at the amino terminal methionine of ubiquitin [3]. The anaphase-promoting complex or cyclosome (APC/C) is arguably the most important ubiquitin ligase

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(E3) acting during and orchestrating the mammalian cell cycle [4]. The APC/C is a giant multisubunit complex of about 1.2 MDa. The core of the APC/C is composed of at least 19 subunits (14 distinct polypeptides) in mammalian cells [5, 6]. In addition to its core complex, the APC/C also associates to substrate-binding/activatory regulatory subunits, namely Cdc20 or Cdh1. The APC/C belongs to the family of cullin-RING (Really Interesting New Gene) finger E3 ubiquitin ligases and it is capable of catalyzing ubiquitylation of a myriad of substrates that modulate major cell cycle processes including DNA replication, chromosomal segregation, progression through and exit from mitosis, maintenance of G1 phase of the cell cycle and cytokinesis [7, 8]. Most APC/C substrates are essential regulatory components of the cell cycle, such as geminin, cyclin A, securin, cyclin B1, Aurora A, and Polo-like kinase 1 (Plk-1).

The APC/C can mediate protein ubiquitylation with the help of several ubiquitin-conjugating enzymes: UbcH5A (Ube2D1), UbcH5B (Ube2D2/Ubc4), and in particular UbcH10 (Ube2C/UbcX/E2C/Vihar) are responsible for the initial or "priming" ubiquitylation activities of the APC/C, i.e., catalyzing the conjugation of the first ubiquitin moiety(ies) into putative substrates, while it has been found that another E2 termed Ube2S rather confers the APC/C ability to extend the length of the ubiquitin chains attached into its substrates, including generating branched ubiquitin chains [9–13].

Remarkably, the complexity of the APC/C also applies to the diverse topology of ubiquitin chains that can generate into its substrates. The APC/C is indeed capable of forming ubiquitin chains using lysines 11, 48, and 63 of ubiquitin although the emerging view related to the mammalian APC/C indicates that polyubiquitylation via lysine 11 is predominant at least during the cell cycle-related APC/C activation in conjunction with the activity of Ube2S [14].

The APC/C targets substrates possessing short linear recognition sequences such as the D-[RXXLXXXX(N)] and KEN-[KENXXX(N)] boxes using a mechanism involving a highly processive initial reaction followed by multiple encounters with the substrates and slower rates reactions that are favored by the presence of the "primed" ubiquitylated sites [15].

Finally, it is worth noting that a growing body of evidence indicates that the APC/C also plays important roles in highly differentiated and specialized cells such as neurons [16]. Methods to analyze and detect the activity of the APC/C in this particular cellular context will nonetheless not be discussed in this review.

2 Materials

2.1 In Vitro Analysis Methods of APC/C-Mediated Ubiquitylation

1. E1: Ube1 (*UBA1*) can be purchased from different sources including Boston Biochem or in-house produced from insect cells using a baculovirus-infection-based system.
2. E2: UbcH5A or B, UbcH10, Ube2S can be purchased from different sources such as Boston Biochem. They can also be produced in and purified from bacteria with different tags (6xHistidine, Glutathione S-transferase (GST), or Maltose-Binding Protein (MBP)).
3. E3: the APC/C can be immunoprecipitated from mammalian cells (as described in Subheading 3 below). A handful group of laboratories have been able to produce and purify recombinant APC/C using insect cells infected with baculovirus-based systems [17–21]. These protocols are however not discussed in this review.
4. Anti-Cdc27/APC3 antibodies: AF3.1 clone from Santa Cruz Biotechnology.
5. Cdc20: it can be produced and purified as a tagged full-length protein from insect cells. Alternatively, it can be in vitro transcribed-translated in rabbit reticulocyte lysates and employed to activate the APC/C as a programmed extract.
6. Cdh1: it can be produced and purified as a tagged full-length protein from insect cells. Alternatively, it can be in vitro transcribed-translated in rabbit reticulocyte lysates and employed to activate the APC/C as a programmed extract [22].
7. APC/C substrates: many substrates [geminin, securin, cyclin A, cyclin B1, the N-terminus of cyclin B1 (comprising the D-boxes), Cdc20 or Cdh1 themselves, Aurora A, or Plk-1] can be used to test the ubiquitylation activity of the APC/C. In this review, we propose to generate the substrates radiolabeled in rabbit reticulocyte extracts from a vector under the control of a T7 promoter. Alternatively, substrates can also be fluorescently labeled [21].
8. TnT® T7 Coupled Reticulocyte Lysate System (Promega).
9. L-[35S]-methionine (Perkin Elmer).
10. Micro Bio-spin 6 columns (Bio-Rad).
11. Adenosine triphosphate (ATP) (Sigma Aldrich).
12. Ubiquitin: can be purchased untagged or tagged from different sources or it can also be produced and purified from bacteria using pET or pGEX vectors.
13. Ubiquitylation buffer: 25 mM Tris–HCl (pH = 7.5), 50 mM NaCl and 10 mM $MgCl_2$ supplemented with an ATP regenerating system.

14. ATP regenerating system: 1.25 mM ATP, 1.25 mM $MgCl_2$, 1.9 mM Creatine Phosphate and 6.25 μg/ml Creatine Phosphokinase (components purchased from Sigma Aldrich).
15. Bovine serum albumin (BSA) (Sigma Aldrich).
16. Polyacrylamide gel electrophoresis equipment and ad-hoc SDS-PAGE solutions.
17. Gel staining solution: 10% acetic acid, 40% methanol and Coomassie blue (Carl Roth) in milli-Q graded water.
18. Gel destaining solution: 10% acetic acid and 40% methanol in milli-Q graded water.
19. Gel drying system.
20. Phosphorimaging screen (GE Healthcare).
21. Personal Molecular Imager™ (PMI™) system (Phosphorimager) (Bio-Rad).
22. Image Lab™ software or Quantity One 1-D analysis software (Bio-Rad).
23. Reagents for immunoblotting and detection using infrared technology.
24. Odyssey scanner and software (LI-COR Biosciences).
25. Quikchange Site Directed Mutagenesis kit (Agilent Technologies).

2.2 In Vivo Analysis Methods of APC/C-Mediated Ubiquitylation

1. Nocodazole (Sigma Aldrich).
2. Thymidine (Sigma Aldrich).
3. Cycloheximide (Sigma Aldrich).
4. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.5 mM KH_2PO_4.
5. Lysis Buffer: 50 mM Tris–HCl (pH = 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT and 10% glycerol, supplemented with a proteases-phosphatases inhibitors cocktail (Roche Diagnostics).
6. Nitrogen decompression buffer: 20 mM Tris–HCl (pH = 7.5), 5 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT, supplemented with a proteases-phosphatases inhibitors cocktail (Roche Diagnostics).
7. Protein A/G PLUS-agarose (Santa Cruz Biotechnology).
8. Protein A sepharose beads (Bio-Rad).
9. Protein G agarose resin (Roche Diagnostics).
10. Proteasome inhibitor MG-132 (Selleckchem).
11. Ubiquitin aldehyde (Boston Biochem).
12. 4× SDS sample buffer: 250 mM Tris–HCl (pH = 6.7), 8% SDS, 40% glycerol, 0.4 M dithiothreitol (it can be replaced by β-mercaptoethanol) and 0.02% bromophenol blue.

13. Emi1: vector to transfect mammalian cells.
14. proTAME (tosyl-L-arginine methyl ester) (Boston Biochem).
15. Human cell lines: HeLa S3, HEK-293T, HFF-1 or other cells of interest (ATCC).
16. Tissue culture reagents, materials and media.
17. Immunoprecipitation buffer: 20 mM Tris–HCl (pH = 8.0), 150 mM NaCl, 1% NP-40 and 2 mM EDTA, supplemented with a proteases-phosphatases inhibitors cocktail (Roche Diagnostics) and MG-132 (at a final concentration of 50 μM)
18. Bacterially expressed and purified recombinant proteins (GST or 6xHistidine-tagged).
19. Binding buffer: 50 mM Tris–HCl (pH = 7.5), 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 5 mM EDTA and 2 mM $MgCl_2$, supplemented with a proteases-phosphatases inhibitors cocktail (Roche Diagnostics).
20. Nickel Xpure agarose resin (Bio-Connect).
21. DNA mini-prep and Maxi-prep purification kits.

3 Methods

3.1 General Methods

3.1.1 In Vitro Production of Unlabeled or [^{35}S]-Methionine-Labeled Proteins

Purified plasmids preparations (at least DNA mini-prep quality graded) encoding known substrates of the APC/C (see Materials) are transcribed-translated in vitro in the presence of L-[^{35}S]-methionine in rabbit reticulocyte lysates following manufacturer's instructions, then loaded on pre-equilibrated (20 mM Tris–HCl, pH = 7.4) Micro Bio-spin 6 columns in order to eliminate non-incorporated radioactive methionine.

3.1.2 Cell Cycle Synchronization Protocols

To properly assess the effects of the APC/C in the ubiquitin-triggered degradation of substrates along the cell cycle, mastering common methods used to synchronize cell lines during their division cycle is required. There are several specialized reviews which have addressed specific methodologies to synchronize cells along the cell cycle [23]. Those methodologies are cell-type-specific and in all cases need to be verified either by fluorescence-activated cell sorting (FACS) monitoring DNA content (by propidium iodide labeling for example) or by microscopy or biochemical analyses (enzymatic assays and/or Western-blotting) visualizing known cell cycle markers [22, 24–26]. Here, we will briefly summarize the most widely used and simple methods of cell cycle synchronization:

Nocodazole Block

Cells are grown in the presence of 330 nM nocodazole for 18 h. After that incubation, a significant fraction of cells will arrest in prometaphase. Release from the arrest can be accomplished by 1–2

washes (depending on the attachment displayed by the cells) with PBS followed by growth in fresh medium.

Double-Thymidine Block

Cells are grown in the presence of 2 mM thymidine for 18 h, washed twice with PBS and released into thymidine-free media for 6–8 h, and finally grown again for 12 h in the presence of 2 mM thymidine. Under these conditions, cells are arrested in late G1 phase. A final release can be accomplished by 1–2 washes with PBS followed by growth in fresh medium.

Thymidine-Nocodazole Block

Cells are treated for 18–24 h with media containing 2 mM thymidine. After two washes and a release into fresh media for 6 h, cells are treated with 330 nM nocodazole for 10–12 h. Using this protocol, a population of cells in G1 can be obtained by washing 1–2 times with PBS and releasing the nocodazole-arrested cells into fresh media for 3 h.

3.1.3 Cells Extracts Preparation

From each of the protocols described above, it is possible to produce cell lysates that will allow to: (1) semi-purify the APC/C in order to perform in vitro ubiquitylation assays; or (2) perform in vitro degradation assays monitoring the capacity of the APC/C to instigate ubiquitin-mediated substrates degradation.

In the case of cells synchronized using a thymidine-nocodazole block for example, cells are first synchronized at prometaphase after the consecutive treatment, then washed (1–2 times with PBS) and finally released for 3 h into fresh media. After the short release, a significant fraction of cells are found in G1 phase of the cell cycle. Extracts from cells at G1 phase can be used to obtain a semi-purified and activatable APC/C. After cell synchronization, cells are subsequently harvested and lysed in a buffer containing 50 mM Tris–HCl (pH = 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, and 10% glycerol, supplemented with proteases-phosphatases inhibitors cocktail (*see* **Note 1** for an alternative lysis/cell disruption method).

3.2 APC/C-Catalyzed In Vitro Substrate Ubiquitylation

The in vitro APC/C-mediated ubiquitylation assay uses ^{35}S-methionine radiolabeled proteins as putative substrates and semi-purified APC/C from extracts of G1-synchronized HeLa S3 cells.

1. One ml of lysate from G1-synchronized cells in a single Eppendorf tube is used in order to perform ten ubiquitylation reactions.
2. One ml of lysate from G1-synchronized cells is incubated with 10 μg of antibodies raised against human Cdc27/APC3 during 4 h at 4 °C with constant rotation.
3. The mixture is incubated with beads capable of binding the anti-Cdc27/APC3 antibodies during 2 h at 4 °C with constant rotation (*see* **Note 2**).

4. The mixture is then centrifuged at 4 °C for 5 min at 1,600 × *g*.
5. Precipitates are washed at least three times with the appropriate buffer used to prepare the lysate but without detergents.
6. Ubiquitylation reactions are started by mixing in a maximum volume of 20 µl the following reagents: 5–10 µl of beads containing the immunoprecipitated and semi-purified APC/C, 1–2 µl of in vitro transcribed-translated and radiolabeled substrate, 100 nM Ube1 (E1), 2 µM UbcH10/Ube2C (and/or 0.1 µM Ube2S) depending on the E2 of interest, 20 mM ATP, 1.5 mg/ml ubiquitin, 10 mM DTT and 2 mg/ml BSA in a ubiquitylation buffer including 25 mM Tris–HCl (pH = 7.5), 50 mM NaCl and 10 mM $MgCl_2$, supplemented with an ATP regenerating system (1.25 mM ATP, 1.25 mM $MgCl_2$, 1.9 mM Creatine Phosphate and 6.25 µg/ml Creatine Phosphokinase) (see Note 3).
7. In the case of elution of the APC/C from the antibody-beads reagent, a total of 20 nM of semi-purified APC/C is used per reaction. Elution of the APC/C can be eventually performed using a competing peptide (see Note 4).
8. According to the particular APC/C which wants to be analyzed, the reaction must be supplemented with 1–2 nM of recombinant Cdc20 or Cdh1. Alternatively, 1–2 µl of rabbit reticulocyte extracts (depending on the efficiency of accumulation of the translated protein in the extract) programmed to in vitro transcribe-translate unlabeled Cdc20 or Cdh1 can also be employed.
9. The reaction is incubated at 30 °C with gentle shaking if the APC/C was not eluted from the agarose or sepharose beads after immunoprecipitation. It is recommended to perform a kinetic involving several time points (0, 15, 30, 60, 90, and 120 min) when attempting for the first time an APC/C-instigated in vitro ubiquitylation reaction.
10. The ubiquitylation reaction is stopped by addition of 4× sample buffer.
11. Samples are boiled at 95 °C for 5 min and centrifuged at 1,600 × *g* for 1 min.
12. Samples are loaded in an SDS-PAGE (we typically use 10–12 % Laemmli gels) and separated by applying a current of 160 V constant.
13. After electrophoresis, the gel is immediately dried on top of a Whatman paper with the help of a gel drying system (Bio-Rad).
14. Dried gels are exposed overnight using a Phosphorimaging screen (GE Healthcare Life Sciences) and scanned in a Phosphorimager (Bio-Rad).
15. Signal can be appropriately visualized and quantified with the help of ad-hoc software (such as Quantity One and/or Image Lab).

3.3 APC/C-Triggered In Vitro Substrate Degradation

In order to evidence cell cycle-dependent degradation mediated by the APC/C using mammalian extracts in vitro, the following procedure can be utilized:

1. Concentrated lysates (at least 10 μg/μl) are prepared from cell cycle-synchronized cells at the different phases of the cell cycle (as described above) (*see* **Note 5**).
2. Functional cell extracts are supplemented with an ATP regenerating system, 10 μM ubiquitin and 0.1 mg/ml cycloheximide to block protein synthesis in the extract.
3. Five μl of concentrated extracts are mixed with a 1/10 volume of radiolabeled substrate from a reticulocyte lysate in vitro transcription-translation reaction.
4. Extracts can eventually be supplemented with Cdc20 or Cdh1 (wild-type or mutant proteins).
5. Incubation is performed at 30 °C according to a kinetic including several time points between 0 and 3 h. The reaction is scaled-up accordingly to the number of time points.
6. Fractions are analyzed by SDS-PAGE.
7. Gels are fixed and stained with a solution containing 10% acetic acid, 40% methanol, and Coomassie blue staining. Destaining can be achieved by incubating the gels in the same solution in the absence of Coomassie blue.
8. Stained gels showing equal loading of total extracts are dried in Whatman papers with the help of a gel drying system.
9. Dried gels are visualized and analyzed with the help of a Phosphorimager.
10. Rates of degradation for a particular substrate can be obtained by fitting the intensity of the visualized bands to an exponential decay function, and eventually subtracting the rate of nonspecific degradation in the presence of 50 μM MG-132 to inhibit cellular proteasomes.
11. In order to confirm that the degradation observed for a particular substrate in the concentrated cell extracts is due to the APC/C, several experimental conditions can be tested: (a) addition of recombinant Emi1 (produced and purified from bacteria) to the extract should block degradation [27, 28]; (b) mutagenesis of linear motifs present in the substrates (such as D- or KEN-boxes) which are able to directly interact with the substrate adaptors/activators of the APC/C (Cdc20 and/or Cdh1) or potentially other subunits of the APC/C core, should also abrogate degradation [29, 30]; (c) blockade of the APC/C by chemical inhibitors such as TAME should also decrease or abrogate rates of substrates degradation [31]; and finally (d) extracts lacking appropriate levels of Cdc20 or Cdh1 should be less efficient instigating ubiquitylation and proteasomal degradation of APC/C substrates (*see* **Note 6**).

3.4 APC/C-Instigated In Vivo Substrate Ubiquitylation

Although most methodological approaches related to the study of the APC/C often utilize in vitro experiments in which the complexity of the cellular systems is reduced, it could also be beneficial to employ a cell line system in order to quickly be able to screen mutations performed at the level of the substrate but also at the level of the conjugating ubiquitins. Indeed, in this protocol, we propose the use of hemagglutinin (HA)-tagged ubiquitin either as a wild-type construct or mutated in one, several, or all possible residues (1 methionine and 7 lysines) used in ubiquitin to form a polymeric chain. On the other hand, the putative APC/C substrate will be also tagged (with either a FLAG- or a myc-tag) allowing the analysis of ubiquitylation of substrates in cells by a simple immunoprecipitation procedure as follows:

1. Tissue culture cell lines (such as HeLa S3, HEK-293T, or HFF-1) are grown in ad-hoc culture media. Typically, at least 1×10 cm dish of confluent cells are needed for each immunoprecipitation.
2. Cells are first washed once with PBS and harvested by trypsinization and centrifugation at room temperature at $1{,}600 \times g$ for 5 min.
3. Cell pellets are washed once with PBS and subsequently kept on ice.
4. Lysis is performed in immunoprecipitation buffer (20 mM Tris–HCl, pH = 8.0; 150 mM NaCl; 1% Nonidet P-40 (NP-40) and 2 mM EDTA) supplemented with a proteases-phosphatases inhibitors cocktail (Roche) and 50 μM MG-132 in order to enhance visualization of ubiquitylated substrates. Lysis is performed on ice for not more than 20 min in a volume of ~1 ml per 10 cm dish of confluent cells.
5. Lysates are centrifuged at $16{,}000 \times g$ for 20 min at 4 °C. The supernatant is transferred to pre-chilled Eppendorf tubes while pellets are either discarded or saved at −80 °C.
6. Cleared supernatants are incubated in the presence of ~1 μg antibodies directed against the tag of the APC/C substrate of interest. We routinely use anti-c-myc antibodies chemically conjugated to agarose beads (SC-40 AC from Santa Cruz Biotechnology) or anti-FLAG M2 affinity gel (Sigma Aldrich) in order to immunoprecipitate myc- or FLAG-tagged substrates, respectively. Incubation is performed for 6 h at 4 °C with constant rotation.
7. Immunoprecipitates are collected via centrifugation at $1{,}600 \times g$ for 5 min at 4 °C, washed three times with immunoprecipitation buffer and one time with PBS at 4 °C.
8. The experiment is stopped by adding 20 μl of 2× sample buffer to the immunoprecipitated beads slurry.

9. Samples are boiled for 5 min at 95 °C and analyzed by SDS-PAGE and Western-blotting.
10. We suggest to perform first a Western-blot using an anti-HA polyclonal (rabbit) antibody in order to detect putative ubiquitylated proteins that will migrate at higher molecular weights compared to the weight of the unmodified substrate. By using antibodies raised in different species for both immunoprecipitation and Western-blotting, the idea is to reduce to a bare minimum the detection of the heavy and light chains of the immunoglobulins from the immunoprecipitating antibodies. The chemically conjugated commercially available antibody-beads (usually) display reduced background in this particular configuration. Subsequently, a re-blot should be performed using anti-tag substrate or anti-substrate antibodies in order to confirm immunoprecipitation of the right target. This is of crucial importance especially in cases when ubiquitylation patterns will be qualitatively and/or quantitatively compared under different experimental conditions such as mutations in the substrate and/or the conjugating ubiquitins.
11. Western-blotting is performed following classical methodologies. In our experience, we favor detection of bands using infrared fluorescently labeled secondary antibodies with the help of an Odyssey device from LI-COR Biosciences.
12. If ubiquitylation of a substrate is detected, inhibition of the APC/C by proTAME or by downregulating APC/C subunits can be done in order to confirm specificity of the reaction.

3.5 APC/C-Mediated In Vivo Substrate Degradation

The following protocol relies on the rationale that tempering with the protein levels of different key subunits of the APC/C should influence the cellular's ability to control the stability of a putative substrate of the APC/C via its proteasomal-mediated degradation.

Given the cell cycle-dependent activation of the APC/C, it is plausible that some of the effects observed will be better evidenced when cells are synchronized at specific cell cycle stages employing the protocols mentioned above.

3.5.1 Downregulation and Overexpression of APC/C Co-activators

Cdh1

Downregulation of Cdh1 can be achieved by using the pSUPER-Cdh1 construct that encodes for a synthetic siRNA against Cdh1: 5′-UGAGAAGUCUCCCAGUCAGTT-3′. The pSUPER empty plasmid is used as a negative control [22]. On the other hand, overexpression of Cdh1 can be accomplished with the help of the pCMV-myc-Cdh1 vector that expresses a myc-tagged Cdh1 protein in cells [22].

Cdc20

Downregulation of Cdc20 can be successfully achieved using siRNA reagents commercialized by Dharmacon (GE Healthcare) such as the ON-TARGETplus CDC20 siRNA SMART pool. On

the other hand, overexpression of Cdc20 can be accomplished with the help of the pCMV-myc-Cdc20 vector expressing a myc-tagged Cdc20 protein in cells [22].

3.5.2 Downregulation of APC/C Subunits

Depletion of specific APC/C subunits can be performed with 100 pmol siGENOME Smartpool siRNA commercialized by Dharmacon (GE Healthcare) per well of a 6-well dish using lipofectamine RNAiMax (Life Technologies) as transfection reagent. Efficiency of the siRNAs targeting the subunits APC1, APC3, APC4, APC5, APC6, and APC8 of the APC/C have been recently reported [32].

Although downregulation or overexpression of subunits of the APC/C can readily alter the steady-state levels of the APC/C substrates, it is nevertheless highly recommended to perform careful calculations of the substrates half-life in the presence of cycloheximide during a kinetic of several hours including an adequate number of time points.

Overexpression of APC/C subunits can be achieved by transfecting cells with lipofectamine 3000 (Life Technologies) using untagged or tagged plasmids. Expression plasmids for the different APC/C subunits can be purchased from sources such as GeneCopoeia or OriGene.

3.5.3 Biochemical Inhibition of the APC/C

In order to block APC/C-instigated degradation in cells, several inhibitors can be used:

1. Emi1, a protein that is capable of inhibiting the APC/C, can be overexpressed in cells using the pCS2+-myc-Emi1 vector (generated by Peter K. Jackson, Genentech).
2. Overexpression of peptides comprising tandems of D-box motifs can also be used as a way to block APC/C-mediated ubiquitylation and proteasomal degradation of APC/C substrates in cells.
3. More recently exciting advances have been achieved by the group of Randall W. King in their efforts to chemically inhibit the APC/C. Currently, proTAME is a permeable prodrug that is converted inside the cells into its active compound (TAME) and that is capable of prematurely stopping efficient ubiquitylation of APC/C substrates. The drug is commercially available. More recently, King's group has reported another inhibitor (named APC inhibitor or apcin) that binds to Cdc20 and disrupts the ubiquitylation of D-box containing substrates [33, 34].

3.6 Binding Assays Using APC/C Subunits

In the following protocol, the APC/C subunits can be used either as baits or as putative interaction partners for virtually any other protein of interest:

Bacterially expressed and purified recombinant proteins, either GST- or 6xHistidine-tagged (1–5 μg) are pre-bound to 10 μl of glutathione sepharose beads (GE Healthcare) or Nickel-NTA

agarose beads (Qiagen) respectively, by incubating for 2 h at 4 °C on a rotating-wheel, followed by three washes with binding buffer (50 mM Tris–HCl, pH = 7.5; 150 mM NaCl; 0.5% NP-40; 5 mM EGTA; 5 mM EDTA; and 2 mM $MgCl_2$, supplemented with a proteases-phosphatases inhibitors cocktail (Roche)). Bait-bead complexes are then mixed with 5–10 μl of reticulocyte lysate (which has been previously programmed to express the protein of interest, labeled with ^{35}S-methionine) in a total volume of around 750 μl of binding buffer. After incubation for 4–6 h at 4 °C on a rotating-wheel, the beads are gently centrifuged, washed three times with binding buffer and resuspended in 25 μl of 2× sample buffer. The beads are finally boiled for 5 min and subjected to SDS-PAGE, followed by Coomassie staining of the gel in order to verify equal amounts of recombinant pulled-down proteins in every sample. Gels are finally dried and visualized/analyzed with the help of a Phosphorimager (Bio-Rad).

3.7 Regulation of APC/C Subunits by Posttranslational Modifications

Many subunits of the APC/C have been reported to be phosphorylated by several kinases. In particular, the substrate adaptors/activators Cdc20 and Cdh1 are the target of a complex regulation of their function by multisite phosphorylation [22].

To study phosphorylation of Cdc20 and/or Cdh1, in vitro kinase reactions can be performed with *bona fide* kinases (obtained as recombinant proteins or immunoprecipitated from cells) and recombinantly purified Cdc20 or Cdh1. Given the difficulties to produce those adaptors from bacteria, they should be either produced using the baculovirus-insect cells system or as chopped tagged proteins in bacterial systems. We have successfully produced mutant proteins of Cdh1 (deleted of its N- or C-terminus) in bacteria by tagging them with an MBP solubility-helping tag (encoded by the *MalE* gene). Those proteins were purified by standard protocols using amylase magnetic beads (New England Biolabs).

In vitro kinase assays can be performed in the so-called histone H1 kinase buffer (50 mM Tris–HCl pH = 7.5; 20 mM EGTA; 10 mM $MgCl_2$; 1 mM β-glycerophosphate; and 1 mM DTT) that especially works for kinases of the CDK family but that can be adopted (or adapted) for other kinases.

3.7.1 In Vitro Kinase Assays

1. Mix the kinase reaction in pre-chilled Eppendorf tubes on ice. A typical reaction includes in a total volume of 10–20 μl: 1–2 μg of the phosphorylation reaction substrate, 50 μM of unlabeled ATP, the kinase of interest (in suspension or bound to beads after immunoprecipitation), 0.1–1 μl of 32P-γ-ATP from a 10 μCi/μl stock and a sufficient volume of kinase buffer.
2. Incubate the reaction at 30 °C during 30–60 min with mild shaking if beads are present in the reaction.
3. Stop the reaction by adding 4× sample buffer.

4. Boil the samples at 95 °C for 5 min.
5. Subject the reaction to SDS-PAGE analysis (see Note 7).
6. Stain and destain the gel in order to visualize the reaction.
7. Dry the gel, visualize and analyze with the help of a Phosphorimager. The time of exposure using a Phosphorimaging plate can be gauged based on the emission detected with a Geiger counter. Time of exposure can vary from minutes to days.

4 Notes

1. Alternatively, in order to better preserve enzymatic activities from the extracts, harvested cells can be preferably disrupted by nitrogen decompression using a buffer composed of 20 mM Tris–HCl (pH = 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT, supplemented of a proteases-phosphatases inhibitors cocktail.
2. We have successfully used protein A/G agarose beads (Santa Cruz Biotechnology), protein A sepharose beads (Bio-Rad), or protein G agarose resins (Roche). The incubation with the beads (an amount equivalent to 50–100 μl of compacted slurry) is performed during 2 h at 4 °C with constant rotation. It is important that enough volume of mixture is present in the Eppendorf tube to guarantee a proper mixing of the beads during the incubation.
3. Accessorily, 50 μM MG-132 (a proteasome inhibitor) and 3 μM Ubiquitin aldehyde (Ubal, a deubiquitinating enzymes inhibitor) can be used depending on the particular experimental condition considered in order to detect enhanced substrate ubiquitylation.
4. In the case of the AF3.1 antibody (Santa Cruz Biotechnology), the competing peptide corresponds to amino acids 814–823 of human Cdc27/APC3.
5. In order to detect APC/C-Cdc20-mediated ubiquitylation and subsequent proteasomal degradation, extracts from cells transitioning during metaphase-anaphase are required. On the other hand, in order to detect APC/C-Cdh1-mediated ubiquitylation and subsequent proteasomal degradation, extracts from cells exiting mitosis and in early G1 phase are needed.
6. Concentrated extracts can be immunodepleted from APC/C subunits by sequential rounds of immunoprecipitation. Alternatively, relevant subunits of the APC/C involved in substrates' modification can be initially downregulated in the cells by means of shRNA or siRNA technologies.
7. Of note, the non-incorporated 32P-γ-ATP migrates slightly ahead than the bromophenol blue present in the sample buffer

forming the migration front of the gel. If the gel is long enough, the non-incorporated 32P-γ-ATP can be kept inside the gel and discarded as a solid waste, avoiding contamination of the gel running buffer and other laboratory plastic ware.

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