Polyanions provide selective control of APC/C interactions with the activator subunit

Arda Mizrak1 & David O. Morgan1*

Transient interactions between the anaphase-promoting complex/cyclosome (APC/C) and its activator subunit Cdc20 or Cdh1 generate oscillations in ubiquitylation activity necessary to maintain the order of cell cycle events. Activator binds the APC/C with high affinity and exhibits negligible dissociation kinetics in vitro, and it is not clear how the rapid turnover of APC/C-activator complexes is achieved in vivo. Here, we describe a mechanism that controls APC/C-activator interactions based on the availability of substrates. We find that APC/C-activator dissociation is stimulated by abundant cellular polyanions such as nucleic acids and polyphosphate. Polyanions also interfere with substrate ubiquitylation. However, engagement with high-affinity substrate blocks the inhibitory effects of polyanions on activator binding and APC/C activity. We propose that this mechanism amplifies the effects of substrate affinity on APC/C function, stimulating processive ubiquitylation of high-affinity substrates and suppressing ubiquitylation of low-affinity substrates.
Regulatory systems in biology often depend on the transient formation of specific protein complexes. Dynamic interactions between protein partners must be achieved in the crowded and complex environment of the cell, where countless nonspecific collisions with other macromolecules can influence the rate of complex formation and disassembly. Numerous mechanisms are employed to enhance the specificity of protein interactions under these conditions, but our understanding of this problem is limited in part because studies of these systems often depend on analysis with purified components. Dynamic and specific protein–protein interactions lie at the heart of the regulatory system that governs progression through the cell division cycle. One of the key components of this system is a ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C), which triggers chromosome segregation and the completion of mitosis. The APC/C is activated at specific cell-cycle stages by transient interactions with an activator subunit. Despite their central role in ordering mitotic events, the mechanisms that govern APC/C–activator interactions remain poorly understood. The APC/C is a ubiquitin ligase (E3) of the RING family, which catalyzes the assembly of polyubiquitin chains on lysine side chains within specific substrates, leading to their degradation by the proteasome. The APC/C is composed of 13 subunits that form a stable 1.3 MDa core enzyme. Its activation requires association with an activator subunit, either Cdc20 or Cdh1, which acts as a substrate receptor and also stimulates enzymatic activity. The two activators act in sequence to promote degradation of a series of substrates. From prometaphase to anaphase of mitosis, the APC/C associates with Cdc20 to ubiquitylate securin and mitotic cyclins, driving sister-chromatid separation and the completion of anaphase. Cdc20 dissociates from the APC/C in late anaphase and is replaced by the second activator, Cdh1. Cdh1 maintains APC/C activity through late mitosis and during the subsequent G1, resulting in the sequential degradation of numerous Cdh1-specific substrates. When the cell enters the next cell cycle in late G1, Cdh1 dissociation inactivates the APC/C, thereby allowing cyclins and other APC/C targets to accumulate. Thus, transient association of the APC/C with different activator subunits organizes the timing of cell-cycle events.

Cdc20 and Cdh1 are structurally related proteins containing a seven-blade β-propeller WD40 domain that is the primary site of substrate binding. This domain is anchored to the APC/C by sequences in flanking N-terminal and C-terminal regions. The N-terminal segment includes highly conserved interaction motifs, such as the C-box, that are critical for association with the APC/C core. Upon binding the APC/C, the unstructured regions around the C-box form a helical bundle that interacts extensively with a cavity in the APC/C. Binding of activator to the APC/C also depends on a dipeptide IR motif at the activator C-terminus, which interacts with a binding groove on the Cdc27/Apc3 subunit of the APC/C. These N-terminal and C-terminal motifs are indispensable for activator binding, and mutation of either region abolishes APC/C-activator interactions.

APC/C substrates interact with the activator WD40 domain via short linear sequence motifs called degrons, of which the D box, KEN box, and ABBA motif are the most common. Cooperative binding of multiple degron sequences to the activator subunit provides the affinity required for efficient and processive ubiquitylation, and the affinity of degron binding is likely to influence the timing of degradation in vivo. Each of the three major degrons binds to a specific binding site on the activator WD40 domain. The KEN box and ABBA motif interact directly with the WD40 domain, while the D box interacts with both the WD40 domain and the adjacent core APC/C subunit Apc10/Doc1. Bivalent D box binding enhances APC/C–activator interactions by bridging the WD40 domain to the APC/C, providing additional interaction surfaces that stabilize the position of the activator on the APC/C.

To gain more insight into the molecular mechanisms of APC/C–activator interactions, we analyze the dissociation dynamics of activators in vitro. Using biochemical methods, we show that nucleic acids and other polyanions in cell lysates are able to rapidly dissociate activators from the APC/C. Interestingly, substrate D box interactions between activator and the core APC/C reduce activator dissociation by polyanions, providing a mechanism that enhances activator binding when the enzyme is occupied with high-affinity substrate.

**Results**

Cell lysates dissociate Cdh1 and Cdc20 from the APC/C. The transient binding of activators to the APC/C during the cell cycle indicates that the activator dissociation rate in the cell must be sufficiently high to allow loss of activator from the APC/C over short time scales of minutes (Fig. 1a). To investigate the dynamics of activator binding, we developed an assay to measure activator dissociation from budding yeast APC/C in vitro (Fig. 1b). We used translation in vitro to prepare 35S-labeled activator proteins, which were incubated with APC/C that had been immunopurified on magnetic beads from asynchronous cells carrying TAP-tagged APC/C subunit Cdc16. Following extensive washing and dilution, the amount of bound activator was then measured over time to estimate an activator dissociation rate (Fig. 1b).

Cdh1 and Cdc20 both exhibited negligible dissociation in 30 min (Fig. 1c, left panels), suggesting that activators bind the APC/C with high affinity. These results are consistent with the extensive binding surfaces seen in structural studies of APC/C-activator complexes, but they are not consistent with the rapid dissociation that occurs in vivo. We hypothesized that active mechanisms might exist in the cell to promote activator dissociation. Indeed, we found that incubation of APC/C with Cdc16 or Cdc20 with whole lysates of budding yeast cells promoted activator dissociation (Fig. 1c, right panels). This effect was not due to disassembly of the APC/C or proteolysis of the APC/C or activator (Supplementary Fig. 1a, b). Similar results were obtained with APC/C that was tagged at another APC/C subunit, Ap,1 suggesting that activator dissociation is not due to the instability of the tagged Cdc16 subunit (Supplementary Fig. 1c). Dissociation rate depended on lysate concentration, and robust activator dissociation was achieved with cell lysates containing over 1 mg/ml of protein (Fig. 1d).

Cdc20 and Cdh1 activate the APC/C at different stages of the cell cycle. To test whether dissociation activity is temporally regulated in a similar fashion, we compared lysates from cells arrested with mating pheromone (G1 arrest) or microtubule-depolymerizing drug nocodazole (metaphase arrest). Dissociation was promoted equally by these two lysates (Supplementary Fig. 1d), suggesting that the dissociation activity does not change during the cell cycle.

ATP hydrolysis is the major energy source for protein remodelers, which are capable of pulling subunits of protein complexes apart. To investigate whether activator dissociation in cell lysates is ATP-dependent, we used gel filtration to remove molecules smaller than 5 kDa from cell lysates. Depleted lysates showed no significant dissociation activity, but activity was restored by addition of 5 mM ATP (Fig. 1e). ATP alone did not promote activator dissociation (Fig. 1e, left panels). Addition of AMP-PNP also restored activity to depleted lysates (Fig. 1e), indicating that ATP hydrolysis is not required for the activity.

Stimulation of the dissociation activity by AMP-PNP ruled out canonical chaperone systems, such as Hsp70, Hsp90, and Hsp104, which require ATP hydrolysis to carry out their functions. The lack of a requirement for ATP hydrolysis also ruled out Cdh1
phosphorylation, which is known to inhibit its binding to the APC/C [10]. We further narrowed the possible candidates by estimating the size of the activity by gel filtration chromatography. The activity eluted with a broad peak of 80–100 kDa (Supplementary Fig. 2a). This molecular size eliminated the possibility that activator dissociation was due to large molecular machines, such as the proteasome or the chaperonin CCT, both of which are thought to influence activator binding to the APC/C [40–43].

**Fig. 1** A biochemical activity in yeast cell lysates dissociates activators from the APC/C. a Cells expressing TAP-tagged Cdc16 were arrested at G1 with 1 μg/ml α-factor for 3 h and released into YPD. APC/C was immunoprecipitated at the indicated time points and western blotted for Cdh1 (upper) and Cdc20 (lower). Uncropped western blots are provided in the Source Data file. b This schematic illustrates our APC/C-activator-binding assay. cdh1Δ APC/C (TAP-tagged on the Cdc16 subunit) was immunopurified on magnetic beads and incubated with 35S-Cdc20 or 35S-Cdh1 produced by translation in vitro. After removing unbound activators by washing, buffer or yeast lysate (2.5 mg/ml protein concentration) was added in the presence of 5 mM ATP. At various times, APC/C was pulled down from the reaction mix and the amount of bound activator determined by SDS-PAGE and PhosphorImager analysis. Due to the extreme dilution of APC/C-activator complexes on the beads, rebinding of dissociated activator is expected to be negligible under these conditions. c As described in panel b, we measured dissociation of radiolabeled Cdc20 (top) or Cdh1 (bottom) from immobilized APC/C over the indicated time course (at 25 °C). Buffer control contains lysis buffer and 5 mM ATP. Fraction remaining was measured by calculating the ratio of activator signal at indicated time points to zero time point signal. Results were plotted and fitted to an exponential one phase decay equation in GraphPad Prism. Similar results were observed in several independent experiments. Source data are provided in the Source Data file. d Cdh1 dissociation reactions were performed as in panel c in the presence of serially diluted yeast lysate, supplemented with 5 mM ATP. Remaining activator after 45 min is plotted as a function of protein concentration. Data indicate means (±SEM) from three independent experiments. Source data are provided in the Source Data file. e Yeast lysates were subjected to gel filtration in lysis buffer to remove molecules smaller than ~5 kDa. Desalted lysates were supplemented with buffer (black), 5 mM ATP (red), or 5 mM AMP-PNP (blue) prior to the Cdh1 dissociation reaction. These results are representative of multiple experiments. Source data are provided in the Source Data file.
Characterization of the activator dissociation activity. We attempted to isolate the dissociation activity from yeast lysates using a variety of chromatographic methods and other purification approaches. During the course of these studies, we discovered that the activity is resistant to boiling. Incubation of cell lysates at 95 °C for 10 min led to precipitation of most proteins in the lysate, but the dissociation activity remained in solution (Fig. 2a). We then incorporated heat treatment as a step in an effective purification scheme, as follows (Fig. 2b). We applied cell lysate to a hydroxyapatite column, from which the activity could be eluted with 100 mM phosphate. The eluate was boiled, and the supernatant was dialyzed and re-applied to the hydroxyapatite column. The activity no longer bound to the column but was instead collected in the flow-through fraction (Fig. 2c). This method resulted in a high yield of activity and a high degree of purity. The flow-through fraction of this preparation was used for further characterization of the activity.

This fraction contained abundant activity despite having a very low protein concentration, leading us to question whether the dissociation activity is composed of protein or other macromolecules. Indeed, activity remained after treatment with the general protease Proteinase K (Fig. 2d), which led to the degradation of all detectable protein in the preparation (Supplementary Fig. 2b). However, treatment with a general nuclease, Benzonase, diminished the activity (Fig. 2d). Purification and analysis of the nucleic acids in the preparation revealed that the major nucleic acid species were about 80 nucleotides in length, and these species disappeared after RNase A treatment (Fig. 2e). We then purified the predominant RNA species and found that they removed Cdh1 from the APC/C in the presence of ATP, and RNase A treatment abolished the activity (Fig. 2f). These results argued that the RNA component is necessary and sufficient to dissociate activator subunit from the APC/C in an ATP-dependent manner.

Polyanions promote activator dissociation. To further understand what types of RNA molecules provide the dissociation activity, we cloned and sequenced the major RNA species in our active preparation, revealing that the major RNAs in the preparation were tRNAs and rRNA fragments (see the "Methods" section). tRNA^Gln
and tRNA<sub>Ser</sub> were particularly abundant in the sample. To test their activity directly, tRNA<sup>Gln</sup> and tRNA<sup>Ser</sup> were transcribed in vitro and gel purified. Both species removed Cdh1 from the APC/C in the presence of ATP, with half-maximal dissociation occurring at concentrations of 0.7 and 0.9 μM, respectively (Fig. 3a).

To determine if the activity was specific for tRNA or could be seen with any RNA species, we transcribed a pool of random RNA sequences of the same length as the glutamine tRNA used above. These RNA molecules removed activator from the APC/C at similar concentrations as tRNA (0.9 μM, Fig. 3a), suggesting that activator dissociation could be achieved by a variety of RNA sequences. To determine if this mechanism is a general function of nucleic acids, we synthesized two random, complementary 75-base single-stranded DNA oligonucleotides. Both sequences dissociated the activator subunit from the APC/C even more efficiently than RNA, with half-maximal concentrations of 0.2 and 0.6 μM (Fig. 3b). These results suggest that nucleic acids, independent of their sequence, are capable of promoting dissociation of activators from the APC/C.

The above experiments were performed with single-stranded nucleic acid polymers. Compared to double-stranded species, single-stranded nucleic acids exhibit more flexibility and heterogeneity in secondary structure. Slight variations in the dissociation activity of the sequences we tested (particularly differences between two complementary DNA oligonucleotides) suggest that nucleic acid structure and flexibility could play a role in disruption of APC/C-activator interactions. We explored this issue further by annealing the two complementary 75-nucleotide DNA oligonucleotides to reduce their flexibility and structural complexity. This double-stranded DNA dissociated activators less efficiently than either single strand (1 μM, Fig. 3c). Thus, efficient dissociation might be enhanced by the flexibility or secondary structures of single-stranded nucleic acids.

We also tested the effect of nucleic acid length on activator dissociation. A 75-base single-stranded DNA molecule was more than five-fold (half-maximal concentration 0.16 μM) more potent in activator dissociation than a 50-base oligonucleotide (1.8 μM). A 25-base oligonucleotide had little effect even at high concentrations (Fig. 3d). The higher activity of longer polymers could result from their greater flexibility and secondary structures. Activator dissociation might also depend on nucleic acid interactions with multiple distant sites on the APC/C and/or activator.

Given that both DNA and RNA can efficiently remove activators from the APC/C independent of nucleotide sequence, we next investigated if the negatively charged phosphate backbone of nucleic acids is responsible for activator dissociation. We tested the activity of polyphosphate, long chains of phosphate molecules that are found at high concentrations in cells from
every branch of life\textsuperscript{44}. In addition to providing energy storage, polyphosphate is believed to alter protein structure and stability through extensive electrostatic interactions with protein surfaces\textsuperscript{45}. We found that a mixed length polyphosphate preparation (45–160 phosphates per chain) was capable of dissociating activators, while similar concentrations of orthophosphate had no effect (Fig. 3c). Polyphosphate concentrations required for activator dissociation were higher than those observed with nucleic acids, even if nucleic acid concentration is expressed in terms of total phosphate concentration. We suspect that this reduced potency is due to heterogeneity in polyphosphate chain length. Taken together, our results show that biological polymers containing long chains of negatively charged phosphates are potent catalysts of APC/C-activator dissociation.

ATP promotes activator dissociation by sequestering Mg\textsuperscript{2+}. Nucleic acids require ATP at millimolar concentrations for efficient activator dissociation (Supplementary Fig. 3a). ATP alone, even at high concentrations, is not sufficient to dissociate activators (Supplementary Fig. 3a), and its hydrolysis is not necessary (Fig. 1e). To explore the role of ATP further, we tested whether other nucleoside phosphates could substitute for ATP. The purified RNA preparation from the flow-through fraction of Fig. 2b displayed dissociation activity when supplemented with 3 mM ATP, AMP-PNP or GTP, and less effectively with ADP, but not with AMP (Fig. 4a). We hypothesized that dissociation might simply require molecules containing two adjacent phosphates. Consistent with this possibility, we found that inorganic pyrophosphate and tri-phosphate promoted the ability of DNA to dissociate the activator (Fig. 4b).

Phosphates on nucleic acids, polyphosphate chains, and free ATP interact with divalent cations such as Mg\textsuperscript{2+}. If polymeric negative charges are critical for the ability of nucleic acids and polyphosphate to dissociate activators, then magnesium ions could neutralize this charge and thereby block activator dissociation. Our dissociation reactions contain 2.5 mM Mg\textsuperscript{2+} ions, and we therefore hypothesized that millimolar concentrations of ATP promote activator dissociation by sequestering these ions. Consistent with this hypothesis, 2.5 mM Mg\textsuperscript{2+} inhibited DNA-dependent or polyphosphate-dependent activator dissociation in the absence of ATP (Fig. 4c–f; Supplementary Fig. 3b). In the absence of Mg\textsuperscript{2+}, addition of ATP did not increase polyphosphate-dependent Cdh1 dissociation and only slightly enhanced DNA-dependent dissociation (Fig. 4d, f). In reactions containing both ATP and Mg\textsuperscript{2+}, we observed that the inhibitory effect of Mg\textsuperscript{2+} occurred only when it was in molar excess of ATP (Fig. 4g, Supplementary Fig. 3c). Interestingly, equimolar ATP did not completely abrogate the inhibitory effects of Mg\textsuperscript{2+} on polyphosphate-dependent Cdh1 dissociation (Fig. 4e, f). One plausible explanation is that polyphosphate, due to the close spacing of its phosphates, has a high affinity for Mg\textsuperscript{2+} that is similar to that of ATP, and ATP is therefore a less effective competitor for Mg\textsuperscript{2+} binding. In sum, our findings suggest that phosphate residues on ATP sequester Mg\textsuperscript{2+} ions and thereby enhance the negative charge of phosphate-containing polymers, which is required for activator dissociation.

Polyanions enhance APC/C substrate selectivity. The APC/C ubiquitylates its targets only when bound by an activator, so dissociation of activators by polyanions should reduce APC/C activity. We tested this possibility by measuring APC/C ubiquitylation activity toward radiolabeled Pds1/securin in vitro. As predicted, incubation of APC/C\textsuperscript{Cdh1} with single-stranded DNA prior to the ubiquitylation reaction reduced Pds1 ubiquitylation (Fig. 5a).

To better understand APC/C activity in the presence of DNA, we simultaneously measured Pds1 ubiquitylation and activator dissociation at various DNA concentrations. Even after just 2.5 min of incubation with DNA, Pds1 ubiquitylation by APC/C\textsuperscript{Cdh1} was almost completely inhibited at DNA concentrations of 1 μM or higher (Fig. 5b, c), despite the fact that a significant amount of Cdh1 remained bound to the APC/C (Fig. 5b, d). After 30 min, ubiquitylation activity remained inhibited and was accompanied by more Cdh1 dissociation (Fig. 5c, d). These results revealed that inhibition of APC/C activity by polyanions is not due simply to activator dissociation. Instead, they suggest that inhibition occurs in two steps: rapid inhibition of substrate ubiquitylation followed by a slower process of activator dissociation.

We next analyzed the effect of DNA on the ubiquitylation of a selection of substrates, using Cdh1 or Cdc20 as activator. We tested three substrates: Pds1 and S-phase cyclin Clb5, both well-established substrates of APC/C\textsuperscript{Cdc20} in metaphase; and Hsl1, a protein that is ubiquitylated primarily by APC/C\textsuperscript{Cdh1} in late mitosis but is also an excellent substrate for APC/C\textsuperscript{Cdc20} in prophase\textsuperscript{15,37}. As before, we observed that APC/C\textsuperscript{Cdh1} activation toward Pds1 was potently inhibited by single-stranded DNA, with a half-maximal DNA concentration of 0.2 μM (Fig. 5e). Interestingly, Pds1 ubiquitylation by APC/C\textsuperscript{Cdc20} was relatively resistant to inhibition (Fig. 5e). Results with the S-phase cyclin Clb5 displayed a similar trend, with greater inhibition of activity with APC/C\textsuperscript{Cdh1} and resistance to DNA with APC/C\textsuperscript{Cdc20} (Fig. 5f). Finally, DNA only slightly reduced ubiquitylation of Hsl1 by APC/C\textsuperscript{Cdc20} and had no effect on Cdh1-dependent activity (Fig. 5g).

The three substrates we tested are likely to have different affinities for different APC/C-activator complexes. Substrate affinity enhances processivity of the ubiquitylation reaction: a slower dissociation rate (and thus higher affinity) generally results in a greater number of ubiquitins attached during a single binding event\textsuperscript{31–33}. Pds1 and Clb5 are both more processively modified by APC/C\textsuperscript{Cdc20} than APC/C\textsuperscript{Cdh1} (ref. 26) (Fig. 5c, f), suggesting that they have greater affinity for Cdc20 than Cdh1. Hsl1 is an unusual substrate that is modified with high processivity with either activator (Fig. 5g), and it is known to contain an exceptionally high-affinity D box that has been used extensively in structural studies of the APC/C\textsuperscript{3,15,46,47}. Together, these results suggest that DNA is a less effective inhibitor of reactions with substrates that bind with high affinity.

D box binding inhibits polyanion-mediated dissociation. Our evidence that high-affinity substrate ubiquitylation is resistant to inhibition by DNA led us to test the possibility that high-affinity substrate binding also blocks the effect of DNA on activator dissociation. We purified a fragment of Hsl1 (aa 667–872) that contains the D box and the KEN box and tested the effect of Hsl1 binding on single-stranded DNA-mediated dissociation. Hsl1 blocked DNA-driven dissociation of Cdh1 from the APC/C (Fig. 6a).

The D box sequence is known to bridge the WD40 domain of the activator subunit to the core APC/C subunit Apc10, thereby enhancing activator binding to the APC/C\textsuperscript{20,32–37}. Consistent with this idea, we found that a peptide containing only the Hsl1 D box was able to block the effects of DNA and polyphosphate on activator dissociation (Fig. 6b; Supplementary Fig. 4a), while mutant D box or KEN box peptides had no effect (Fig. 6b).

Our previous results suggested that polyanionic inhibition of APC/C\textsuperscript{Cdh1} activity toward Pds1 or Clb5 (Fig. 5e, f) results because their D boxes exhibit lower affinity for Cdh1 and their binding to the APC/C is therefore more easily disrupted by polyanions. We explored this possibility further by comparing the ability of Hsl1 and Pds1 D boxes, at varying concentrations, to
block activator dissociation by DNA. The Hsl1 D box peptide inhibited most activator dissociation at 0.4–4 μM, whereas equivalent inhibition of activator dissociation required 10–50-fold higher concentrations of the Pds1 D box peptide (Supplementary Fig. 4b, c). Similar differences were observed when we tested the effects of different D box concentrations on activator dissociation over a range of DNA concentrations (Fig. 6c, d). These results support the possibility that Hsl1 and Pds1 have different affinities for APC/C<sup>Cdh1</sup>, resulting in differing resistance to the effects of DNA. Also consistent with this possibility, we found that a Pds1 fragment (1-110aa) containing the D box effectively inhibited the dissociation of Cdc20 (Supplementary Fig. 4d). Together with our studies of ubiquitylation reactions in Fig. 5, these results argue that a high-affinity D box interaction holds the activator in a position that blocks the effects of polyanions on substrate ubiquitylation and activator dissociation.
Discussion

Precise temporal control of APC/C activity during the cell cycle relies on short-lived interactions between the APC/C and the activator subunit. Our studies suggest that the transient binding of activator to the APC/C is unlikely to result from spontaneous dissociation, which is negligible in vitro. Instead, rapid activator dissociation depends on abundant cellular polyanions, such as DNA, RNA, and polyphosphate, which also inhibit APC/C activity. The inhibitory effects of polyanions are blocked by strong substrate binding, providing a mechanism to selectively enhance ubiquitylation of high-affinity substrates.

There is growing evidence that negatively charged polymeric biomolecules influence the structural dynamics of proteins in the cell. Nucleic acids and polyphosphate exhibit chaperone-like properties, accelerate protein folding, and serve as anti-aggregation agents by solubilizing a variety of protein aggregates in vitro. Long stretches of phosphate residues also inhibit polymerization of multimeric proteins and stabilize soluble structures. Our work reveals that polyanions also influence the dynamics of APC/C-activator interactions, specifically disrupting APC/C-activator binding, while leaving the multi-subunit APC/C core intact.
We found that submicromolar concentrations of RNA and DNA promote APC/C-activator dissociation, while polyphosphate acts at submillimolar concentrations. These concentrations are far lower than RNA and polyphosphate concentrations inside the cell. tRNA alone can be found in cells at a concentration of ~200 μM, while polyphosphate concentration is estimated to be as high as 100 mM (refs 44,53). Even if these molecules are partially neutralized by positively charged ions, such as Mg^{2+} and other charged macromolecules in the cell, it seems likely that there are sufficient quantities of various negatively charged polymers to exceed the low concentrations needed in vitro to promote APC/C inhibition and activator dissociation.

The binding of activator to the APC/C is known to be regulated by multiple mechanisms. The best understood is phosphorylation of Cdh1 by cyclin-dependent kinases (Cdks), which is known to reduce Cdh1 binding to APC/C in the cell10,12,54. Several Cdh1 phosphorylation sites are found in N-terminal regions that mediate APC/C binding. These regions become less accessible to Cdks upon APC/C binding, suggesting that phosphorylation is likely to block association of free Cdh1 rather than promoting dissociation. Activator binding to the APC/C is also controlled in part by activator ubiquitylation. Cdc20 and Cdh1 levels decrease as a result of APC/C-mediated autoubiquitylation and degradation in late mitosis and G1, respectively55. Cdc20 autoubiquitylation is also involved in disruption of the mitotic checkpoint complex that blocks APC/C-mediated mitotic inhibition during activation of the spindle assembly checkpoint41-43,56,57. However, there is no indication that activator phosphorylation or ubiquitylation is required for the polyanion-mediated activator dissociation we observed in cell lysates or with purified components.

In addition to providing insights into the control of APC/C-activator interactions, we uncovered clues about APC/C-substrate binding. APC/C substrates contain combinations of one to three degrons, of which the D box is particularly critical because of its role in linking the activator to the APC/C core. The amino acid sequences of the D box and other degrons vary in different substrates, and it is likely that these sequences evolved to be accommodated by different APC/C-activator complexes. Substrate affinity for each of the two activators determines the substrate residence time, which helps determine the processivity of ubiquitylation and the timing of substrate degradation in vivo41. Our evidence suggests that substrate affinity also controls the ability of polyubiquitins to inhibit APC/C activity: the binding of high-affinity substrates results in resistance to the effects of polyubiquitins, while low-affinity substrates are less resistant. Thus, differences in enzymatic activity with substrates of different affinities are amplified in the presence of polyubiquitins, providing a selectivity filter that promotes ubiquitylation of high-affinity targets while suppressing background activity toward low-affinity substrates. Furthermore, the ability of substrate binding to stabilize activator binding could enhance APC/C-activator function at cell-cycle stages when abundant substrates are present.

In the absence of substrate, the activator is attached with high affinity to the APC/C via flexible N- and C-terminal tethers, such that the globular WD40 domain does not interact with the Apc10 subunit and remains mobile15,21. D box binding bridges the gap between activator and Apc10, providing a third site of activator binding. How do polyubiquitins promote activator dissociation? Any proposed mechanism must account for the observation that D box binding blocks the effects of DNA on both the ubiquitylation reaction and activator binding. One speculative model (Fig. 6e) is that some part of the long polyubiquitin polymer interacts with the APC/C at sites adjacent to the activator that are exposed in the absence of a substrate. Polyubiquitins might push the activator WD40 domain away from Apc10, preventing D box binding (and inhibiting ubiquitylation), while at the same time promoting activator dissociation by disrupting binding sites for the activator termini. In this scenario, high-affinity D box binding would overcome the inhibitory effects of polyubiquitins by locking the activator against Apc10.

According to this model, polyanions are essentially competitive inhibitors of D box binding. Confirmation of this idea will require the development of quantitative methods for the measurement of substrate-binding affinities at varying nucleic acid or polyphosphate concentrations. The model also predicts that D box binding should block the inhibitory interactions between polyubiquitins and the APC/C. Interestingly, we found that increasing D box concentrations did not affect the half-maximal inhibitory DNA concentration (Fig. 6c, d), suggesting that substrate does not have much impact on polyubiquitin binding. However, it is likely that long polyubiquitin molecules interact with multiple distant sites on the APC/C, and any local effects of D box binding might not result in a significant loss of overall polyubiquitin affinity (Fig. 6e).

With combined concentrations exceeding tens of millimolar, nucleic acids, polyphosphate chains, ATP and other charged macromolecules are in frequent close contact with proteins inside the cell. Transient interactions between polyubiquitins and protein surfaces may create further crowding in the molecular microenvironment around proteins, while also altering the structural and functional dynamics of protein complexes and other macromolecules. Emerging roles of ATP beyond energy provision suggest that the electrostatic microenvironment around biomolecules has an impact on biochemical outputs58. Our understanding of polyubiquitin biology is
limited, but further studies could help us discover and characterize other examples of non-canonical molecular interactions that modulate complex enzyme functions.

**Methods**

**Synchronization and western blotting.** All yeast strains were derivatives of W303. For western blotting of activators during the cell cycle, yeast cells carrying TAP-tagged Cdc16 (Strain yAM4: Cdc16-TAP::KanMX, MATα) were grown overnight and diluted the next morning. When cells reached OD 0.3, cells were synchronized at 30 °C for 3 h with 1 μg/ml α-factor. Following release from the arrest by washing, cells were harvested at various times and lysed by bead beating in lysis buffer as described in the next section. TAP-tagged APC/C was immunoprecipitated with magnetic IgG-coupled magnetic beads (Invitrogen #14301) at 4 °C. The APC/C was separated by 10% SDS-PAGE. Gels were dried on Whatman paper, exposed to a phosphor screen (FujiFilm BAS-2500), and scanned to obtain a high-resolution image. The image was then subjected to quantitative analysis using ImageJ software. The Source Data file contains the details of the experiments and the raw data used to generate the figures. Uncropped autoradiographs and source data are provided in the Source Data file.

**Activator binding and dissociation.** A yeast strain carrying Cdc16-TAP and lacking Cdh1 (Strain DOM1226: cdh1::LEU2, Cdc16-TAP::HIS, MATα) was harvested at OD₆₀₀ = 1 and flash frozen in liquid nitrogen. Cell pellets were lysed by bead beating in lysis buffer (25 mM HEPES pH 7.4, 150 mM KOAc, 2.5 mM MgCl₂, 10% Glycerol, 0.5% Triton X-100, 1 mM PMSF, 1 mM DTT, and EDTA-free protease inhibitor mix) and centrifuged at 20,000×g for 15 min at 4 °C. The APC/C was immunoprecipitated with IgG-coupled magnetic beads (Invitrogen #14301) at 4 °C. Cdc20 and Cdh1 were translated in vitro using the Tnt Quick-Coupled reticulocyte lysate system (Promega #L1170) and [35S]-Methionine (Perkin Elmer #NEC709A001MC). APC/C on beads was incubated with reticulocyte lysate containing Cdc20 or Cdh1 for 30 min at 25 °C, followed by washing three times with lysis buffer to remove unbound activator.赖氨酸 dissociation reactions were performed at 25 °C by incubating APC/C with DNA + Hsl1 D-box peptide or DNA + Hsl1 KEN box peptide (green). Reactions were performed in the presence of 1.5 μM 75-mer ssDNA and 3 mM ATP. Uncropped autoradiographs and source data are provided in the Source Data file. Activator binding and dissociation was measured with 1.5 μM 75-base ssDNA and 3 mM ATP. Uncropped autoradiographs and source data are provided in the Source Data file.
Phosphor Screen and scanned in a Typhoon 9400 Imager. Images were analyzed using ImageQuant (GE Healthcare). Fraction of Cdc20 and Cdh1 remaining on the APC/C was calculated to relative to the zero timepoint or buffer control. In Fig. 3b, APC/C was immunopurified from lysates of a strain lacking Cdh1 and carrying GFP-tagged Apcl and TAP-tagged Cdc6 (Strain NYH14: clde1:LEU2, Cdc16-TAP/HIS, Apcl1-gfp-GaRA3, MATa). The Hsl1 fragment used in Fig. 6a was expressed in insect cells and purified using a Strep-tag-purification column. Degron peptides used in Fig. 6b were purchased from CPC Scientific. Peptide sequences are listed in Supplementary Table 1.

Partial purification of dissociation activity. Wild-type yeast cells were collected at OD600 = 1.0, washed twice with PBS, and resuspended in water and flash frozen in liquid nitrogen. Cells were lysed using a coffee grinder in a buffer containing 25 mM HEPES pH 7.6, 25 mM KOAc, 2.5 mM MgCl2, 10% glycerol, 0.5% N-lauryl sarcosine, and protease inhibitor mix. Lysate was cleared by centrifugation at 100,000 x g for 1 h and filtered through a 0.22 mm cellulose filter. Cleared lysate was applied to a Bio-Rad CHT type II ceramic hydroxyapatite column using a peristaltic pump. The column was washed with 15 column volumes of buffer HA-A (25 mM HEPES 7.6, 25 mM KOAc, 10% glycerol) containing 2.5 mM MgCl2. Samples were eluted with a step phosphate gradient using seven column volumes of the same buffer supplemented with 50, 100, 250, or 500 mM PO43=.

RNA preparation and sequencing. RNA species in the flow-through and elution fractions were concentrated, buffer-exchanged into Buffer HA-A with 2.5 mM MgCl2 and tested for activator dissociation in the presence of 3 mM ATP. The activity was collected in the ow-through fraction. This fraction was dialyzed into Buffer HA-A overnight and concentrated 10-fold for further characterization in ow-through and elution fractions. This fraction was dialyzed into Buffer HA-A overnight and concentrated 10-fold for further characterization in dissociation assays. The activity in this fraction was stable for more than 2 weeks at 4°C with no significant loss of activity.

Nucleic acid preparation for dissociation reactions. Sequences used in dissociation assays are listed in Supplementary Table 1. RNA sequences were transcribed in vitro36 using T7 RNA Polymerase (NEB M0425). Chemically synthesized DNA oligonucleotides or transcribed RNA species were separated on a 10% TBE-urea polyacrylamide gel. RNA bands were excised, ethanol precipitated, and resuspended in Buffer HA-A without MgCl2.

APC/C ubiquitylation assay. Yeast cells carrying Cdc6-TAP and lacking Cdh1 were collected at OD600 = 1, flash frozen, and lysed using beat beating in lysis buffer. APC/C was immunopurified on IgG beads and activated by in vitro-translated Cdc20 or Cdh1. Cdc6 and Cdh1 used in Fig. 5e-g were expressed in insect cells using the baculovirus system and purified using Tag-purification methods. For APC/C substrates, Pds1, Hsl1 fragment (aa 667–800, 250, or 500 mM PO43=, concentrated. The elution activity was collected in the ow-through fraction. This fraction was dialyzed into Buffer HA-A overnight and concentrated 10-fold for further characterization in dissociation assays. The activity in this fraction was stable for more than 2 weeks at 4°C with no significant loss of activity.

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A.M. conceived the project, performed the experiments, and wrote the paper, with guidance from D.O.M.

Competing interests
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Correspondence and requests for materials should be addressed to D.O.M.

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