Supplemental information

Molecular mechanisms of APC/C release from spindle assembly checkpoint inhibition by APC/C SUMOylation

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Figure S1

A

S  M

98-  49-  APC3

49-  Actin

S  M

16-  H3S10

49-  Actin

B

WT  GL2  Ubc9  Ubc9b

98-  49-  17-  49-

APC4-(SUMO-2)1  APC4

Actin

Ubc9

Cycling cells

C

2

Input  Pulldown

U2OS  U2OS-His10-SUMO2  U2OS

AS  STLC  AS  STLC  AS  STLC

191  91

191  91

APC4

SUMO2/3

Ponceau S

3

Input  Pulldown

U2OS  U2OS-His10-SUMO2  U2OS

AS  STLC  AS  STLC  AS  STLC

191  91

191  91

APC4

SUMO2/3

Ponceau S

D

U2OS  HIS10-SUMO2

% of MPM2 positive cells

AS  STLC

E

RNAi

GL2  Ubc9  RanBP2

98-  49-  17-  14-

APC4-(SUMO-2)2  APC4

Actin

Ubc9

H3S10p

Mitotically arrested cells
Figure S1. STLC arrested cells are efficiently arrested in mitosis and RanBP2 depletion abolishes APC/C SUMOylation bands in vivo. Related to figure 1.

(A) Cell cycle synchronization control for Figure 1A. Immunoblotting against APC3 (left panel) or phosphorylated Ser10 of histone H3 (right panel) shows that cells are efficiently arrested in mitosis with STLC. Lower panel: loading control.

(B) Cycling cells were analysed for APC4 SUMOylation. Ubc9 was depleted for 48 h using 20 nM siRNA after which the cells were harvested and analysed by immunoblotting against APC4. Loading was controlled by immunoblotting against actin and depletion was confirmed by immunoblotting against Ubc9.

(C) Repeats of experiments in Figure 1C. Method is described in Figure 1C and in Methods.

(D) Cell cycle synchronization of experiments in Figure 1C was verified by FACS analysis of DNA content and MPM2 (Mitotic Phosphoprotein Marker 2, anti-Cdk phosphorylated proteins) expression and shown in FACS plots (y-axis MPM2 positive cells, x-axis DNA content). Quantifications are shown in a bar graph. Data represent mean with one standard deviation (n=2).

(E) SUMOylated APC4 bands disappeared in cells treated with siRNAs against RanBP2 or Ubc9 but not in control cells, suggesting that APC/C SUMOylation depends on RanBP2. Proteins were depleted for 48h after which STLC was added and cells were left for additional 16 h before harvesting. 20 nM siRNA was used to deplete Ubc9 and 40 nM to deplete RanBP2. APC4 SUMOylation was detected by immunoblotting against APC4 and RNAi efficiency was assessed by immunoblotting against Ubc9 and RanBP2. Immunoblotting against phosphorylated histone H3 Ser10 (H3S10p) confirmed that the difference in APC4 SUMOylation is not due to cells being at different stages of the cell cycle.
Figure S2. Analysis of effects of the N-terminal 6xHis tag of SUMO-2 on the SUMOylation reaction and SUMO isoforms loading controls. Related to figure 2.

(A) A SUMOylation assay showed that APC/C SUMOylation by SUMO-2 and by a SUMO-2 mutant where all Lys were replaced with Arg (SUMO-2钾) both produced two additional SUMOylation bands above APC4. The SUMOylation assay was performed as described in Methods and APC4 was detected by immunoblotting with antibodies against APC4.

(B) De-SUMOylation assay shows that SENP2 can remove SUMO from the APC/C. De-SUMOylation was performed with either 0.5 µM APC/C or 0.5 µM SUMOylated APC/C and 0.5 µM SENP2-catalytic domain as indicated, in a buffer containing 25 mM Tris (pH 8), 150 mM NaCl, 0.1% Tween-20 and 2mM DTT in a total reaction volume of 20 µL. APC4 and SUMO-2/3 were detected by immunoblotting with antibodies against APC4 and SUMO-2/3, respectively.

(C) SDS-PAGE gel of SUMO-1 and SUMO-2 loading controls used for Figure 2A stained with Coomassie Blue.

(D) APC/C SUMOylation by the 6xHis-SUMO-2 isoform is much faster than by 6xHis-SUMO-1 isoform. The SUMOylation assay was performed as described in Methods and APC4 was detected by immunoblotting with antibodies against APC4.

(E) SDS-PAGE gel of 6xHis-SUMO-1 and 6xHis-SUMO-2 loading controls used for Figure S2D stained with Coomassie Blue.

(F) APC/C SUMOylation by unmodified SUMO-2 isoform is much faster than by 6xHis-SUMO-2. The SUMOylation assay was performed as described in Methods and APC4 and SUMO-2/3 were detected by immunoblotting with antibodies against APC4 and SUMO-2/3, respectively.

(G) SDS-PAGE gel of SUMO-2 and 6xHis-SUMO-2 loading controls used for Figure S2F stained with Coomassie Blue.

(H) APC4 phosphorylation state summary at S777 and S779 before and after lambda phosphatase treatment as determined by mass spectrometry peptide analysis.

(I) SDS-PAGE gel of SUMO-2钾 and SUMO-2 loading controls used for Figure 2D stained with Coomassie Blue.

(J) SDS-PAGE gel of SUMO-1钾, SUMO-2钾 and SUMO-2 loading controls used for Figure 2E stained with Coomassie Blue.
Figure S3

A. SDS-PAGE of SUMO-APC/C gel filtration (GF) elution profile.

B. Typical micrograph.

C. 2D classes.

D. Typical micrograph.

E. WHB domain with additional density.

F. 1. All good particles after 2D classification
   1,002,696 particles
   27% 42% 15% 16%
   701,885 particles
   137,954 particles

2. 3D classification
   16% Bad particles
   27% Good particles
   15% Noisy particles
   32% Good particles

3. Bayesian polishing
   3.78 Å
   701,885 particles

4. Focused 3D classification without alignment after signal subtraction
   WHB/PC10/APC3
   3.49 Å
   12 iter.
   20% High occupancy
   Revert to full particles and refine

5. Revert to full particles and refine
   Masks for Multi Body Refinement
   3.78 Å
   137,954 particles

6. Body 2 with the best density
   3.78 Å
Figure S3. Biochemical and structural characterization of *in vitro* SUMOylated APC/C. Related to figure 3.

(A) Coomassie blue stained SDS-PAGE gel showing gel filtration (GF) fractions of *in vitro* SUMOylated APC/C.

(B) Representative cryo-electron micrograph.

(C) Typical 2D classes used for structure determination.

(D) Gold-standard Fourier Shell Correlation (FSC) curves of all SUMOylated APC/C reconstructions in this study.

(E) SUMOylated APC/C map colored by local resolution estimation.

(F) Three-dimensional classification scheme for SUMOylated APC/C structure determination, resulting in classes with APC2\(^{WHB}\) domain density being clearly visible. All particles after 2D classification were refined together to give an initial model (1), which were 3D classified into four classes (2). The two best 3D classes were selected after visual examination, combined and refined together. Bayesian polishing was performed on these best particles with CTF refinement in RELION 3.0 (3). APC2\(^{WHB}\), APC10, APC3 and APC1 PC repeats were then masked and the signal from the rest of the APC/C was subtracted, and focused 3D classification without alignment was performed to give three classes (4), only one of which had high occupancy of APC2\(^{WHB}\) (5). Full particles with high APC2\(^{WHB}\) occupancy were then refined and the APC/C was split into three bodies for multi body refinement (6), which gave a final map with a well-resolved APC2\(^{WHB}\).
Figure S4

Superposition of SUMOylated apoAPC/C map with structure of the APC/C<sub>Ca28</sub> model

Difference map between apoAPC/C and SUMOylated apoAPC/C

1. 701,885 particles
   All good particles after bayesian polishing
   (Supplementary figure 5C)
   Focused 3D classification without alignment after signal subtraction
   ~30%
   ~20%
   Defined density at APC2/4 interface
   ~50%

2. 165,281 particles
   All good particles after bayesian polishing
   Focused 3D classification without alignment after signal subtraction
   ~30%
   ~20%
   Defined density at APC2/4 interface
   ~50%

3. 350,938 particles
   550,938 particles
   Focused 3D classification without alignment after signal subtraction
   ~30%
   ~20%
   Defined density at APC2/4 interface
   ~50%

4. Focused 3D classification without alignment after signal subtraction
   ~30%
   ~20%
   Defined density at APC2/4 interface
   ~50%

5. Focused 3D classification without alignment after signal subtraction
   ~30%
   ~20%
   Defined density at APC2/4 interface
   ~50%

Revert to full particles and refine

Additional density
Figure S4. Details of $\text{APC2}^{\text{WHB}}$ domain interaction with the rest of the APC/C. Related to figure 3.

(A) Coomassie Blue stained SDS-PAGE gel of SUMOylated $\text{APC/C}^{\text{WHB}}$ that was used for structure determination.

(B) $\text{APC2}^{\text{WHB}}$ binds directly to amino acids 140-150 (140-150) loop of APC10.

(C) Repositioned $\text{APC2}^{\text{WHB}}$ domain has only modest overlap with Cdc20. Superposition of SUMOylated apo$\text{APC/C}$ map onto the $\text{APC/C}^{\text{Cdc20,Hsl1}}$ structure (PDB ID 5G04) visualized in Chimera. APC10 – forest green; blue – Cdc20; D-box of Hsl1 – yellow; 144-150 loop – red.

(D) Model of the SUMOylated $\text{APC/C}$ with repositioned $\text{APC2}^{\text{WHB}}$ domain is overlayed with the difference map between SUMOylated apo$\text{APC/C}$ and apo$\text{APC/C}$. Clear positive differences are visible for SUMO-2, $\text{APC2}^{\text{WHB}}$ (below APC10) and within $\text{APC2}^{\text{CTD}}$ and cullin repeats.

(E) Three-dimensional classification scheme for SUMOylated $\text{APC/C}$ structure determination, resulting in class with clear density at the $\text{APC2-APC4}$ interface, assigned to SUMO-2. The processing was continued after Bayesian polishing described and shown in Figure S3F (1). APC4 and APC2 were masked and the signal was subtracted from the rest of the $\text{APC/C}$ and focused 3D classification without alignment was performed to give three classes (2). apo$\text{APC/C}$ (no density), partially activated $\text{APC/C}$ were excluded and only class with additional density was taken for subsequent processing. $\text{APC/C}$ was refined again (3) and a tight mask around APC4 WD40 domain and APC2 were used for another round of focused 3D classification without alignment (4). A single class with defined density at $\text{APC2-APC4}$ interface was taken and refined to give a final map (5).
Figure S5

A. wt apoAPC/C interaction with SUMO-2

B. Elution fractions

C. wt apoAPC/C SUMO2 only

D. wt apoAPC/C-SUMO2 pre-incubated

E. SUMOylated APC/C class from supplementary figure 6E, highlighting SUMO2 (in pink)

F. Superposition of EMDB 10518 and SUMO2 density at APC2-4 interface

Superposition of EMDB 10518 and SUMO2 density at APC2-4 interface
Figure S5. SUMO-2 directly binds to APC/C and APC/C\(^{\Delta SIM}\). Related to figure 4.

(A) Analytical gel filtration chromatogram of SUMO-2 and APC/C, and SUMO-2 pre-incubated with the APC/C. APC/C at 1 µM was incubated with 60 µM SUMO-2 on ice for 30 min and then samples were run on a Superose 6 gel filtration column.

(B) Immunoblotting of peak fractions against SUMO-2 shows that SUMO-2 eluted in earlier fractions only when it was pre-incubated with the APC/C. The samples were analysed by immunoblotting against SUMO-2.

(C) Analytical gel filtration chromatogram of SUMO-2 and APC/C\(^{\Delta SIM}\), and SUMO-2 pre-incubated with APC/C\(^{\Delta SIM}\). APC/C\(^{\Delta SIM}\) at 1 µM was incubated with 60 µM SUMO-2 on ice for 30 min and then samples were run on a Superose 6 gel filtration column.

(D) SDS PAGE gel of purified APC/C\(^{\Delta SIM}\) compared to SUMOylated APC/C\(^{\Delta SIM}\) which was used for structural work. The gel was stained with Coomassie blue.

(E) Comparison of the APC/C in complex with Nek2A (Alfieri et al., 2020), which exhibits APC2\(^{WHB}\) domain rearrangement to the APC2-4 interface, and SUMO2 density as observed in classes described in Figure S4E. EMDB 10518 was used to assess the APC/C-Nek2A complex; APC2\(^{WHB}\) is coloured green. SUMO2 is coloured pink.

(F) The SUMOylation reaction proceeded faster when the APC2\(^{WHB}\) was removed. SUMOylation reaction was performed as described in Methods.
Figure S6

A

B

C

D

E

1: Normalised value for P-APC/C
2: Activity of SUMOylated P-APC/C relative to non-SUMOylated P-APC/C

Relative activity of SUMOylated P-APC/C

1.00

0.50

0.00

1

2
Figure S6. Ubiquitination assays. Related to figure 5.

(A) Coomassie stained SDS-PAGE gel showing different APC/C samples used in ubiquitination assays in Figure 5A, C, E.

(B) Titration of E2 enzymes used in the ubiquitination assay. The assays were performed for 10 min using Hsl1 as a substrate with indicated amounts of UbcH10 and UbcH5 without Ube2S enzymes. Ubiquitination was detected by immunoblotting against the His-tag of ubiquitin.

(C) Comparison of SUMOylated and non-SUMOylated APC/C ubiquitination activity towards Kif18B using UbcH10 as the sole E2 enzyme. The reactions were incubated for the time indicated in the Figure and ubiquitination was detected by immunoblotting against the His-tag of ubiquitin. The assays were performed at least in triplicate.

(D) Comparison of wild-type phosphorylated APC/C activity and phosphorylated APC/CΔSIM towards securin in the ubiquitination assay. UbcH10 was used as the sole E2 enzyme. Ubiquitination was detected by immunoblotting against securin. The assays were performed at least in triplicate.

(E) Comparison of SUMOylated and non-SUMOylated phosphorylated APC/CΔSIM ubiquitination activity towards securin using UbcH10 as the sole E2 enzyme. Ubiquitination was detected by immunoblotting against securin. The assays were performed at least in triplicate and quantified as described in Methods. The error bar represents mean with one standard deviation of the ratio of the SUMOylated/non-SUMOylated APC/C activity. One star indicates a significance smaller than 0.05. Significance was calculated using an unpaired Student’s t-test.
**Figure S7**

A. Superposition of SUMOylated apoAPC/C map with structure of the APC/C\(^{\text{MCC-Closed}}\) complex in closed conformation

B. Structure of the APC/C\(^{\text{MCC-Closed}}\) complex shows close contacts between BubR1 and 144-150 loop of APC10

C. Superposition of SUMOylated apoAPC/C map with structure of the APC/C\(^{\text{MCC-Open}}\) complex in open conformation

D. All absorption curves from MCC/Cdc20 reconstitution experiments plotted on the same coordinates

E. Absorption difference between APC/C and APC/C pre-incubated with MCC/Cdc20

F. Loading controls and elution fractions

- **APC/C sample**
  - P, SP
  - + MCC/Cdc20

- **Absorption difference plots**
  - P-aAPC/C
  - P-APC/C with MCC/Cdc20
  - P-(SUMO-2)\(^2\)-APC/C
  - P-(SUMO-2)\(^2\)-APC/C with MCC/Cdc20
  - MCC/Cdc20 alone

- **Elution fractions**
  - 98-
  - 49-
  - 38-
  - 17-
Figure S7. Comparison of SUMOylated apoAPC/C structure with MCC-APC/C\textsuperscript{Cdc20} structure. Related to figure 6.

(A) Superposition of the SUMOylated apoAPC/C map onto APC/C\textsuperscript{MCC-Closed} structure (PDB ID 5LCW) shows a clear clash between APC2\textsuperscript{WHB} and BubR1 and the tilted Cdc20\textsuperscript{A}. APC1 – dark blue; Cdc20 – light blue; BubR1 – yellow, APC10 – forest green.

(B) BubR1 of the MCC and APC2\textsuperscript{WHB} bind to the same 144-150 loop of APC10. 144-150 loop of APC10 is coloured red.

(C) Superposition of the SUMOylated apoAPC/C map onto the MCC-APC/C\textsuperscript{Cdc20-Open} structure (Alfieri et al., 2016) a minor clash between APC2\textsuperscript{WHB} and BubR1 or Cdc20\textsuperscript{A}. APC1 – dark blue; Cdc20 – light blue; BubR1 – yellow, APC10 – forest green.

(D) Chromatograms of all the samples plotted on the same coordinates without any modification. The fractions used for immunoblotting are indicated above the chromatograms.

(E) A plot of absorption difference at 280 nm between APC/C samples and APC/C samples pre-incubated with MCC and Cdc20. Chromatograms shown in Figure S7D were used to calculate the absorption differences.

(F) Left panel: immunoblotting against APC2 and BubR1, an MCC component, shows equal loading of all reactants for the reconstitution experiments, P: phosphorylated APC/C, SP: phosphorylated SUMOylated APC/C, -: sample without APC/C (i.e. MCC-Cdc20 alone). Right panel: immunoblotting against APC2 shows that equal amounts of APC/C are present in peak fractions, whereas immunoblotting against MCC components, BubR1, Bub3 and Mad2 shows reduced amount of MCC in the SUMOylated APC/C sample. The peak fractions taken for the immunoblotting are shown in Figure S7D.
### Data collection

*In vitro SUMOylated APC/C*

| Parameter                          | Value                                      |
|-----------------------------------|--------------------------------------------|
| **EM**                            | **FEI Titan Krios, 300 kV**                |
| **Detector**                      | **Falcon 3, counting mode**                |
| **Pixel size (Å)**                | 1.07-1.085                                 |
| **Magnification**                 | 75,000x                                    |
| **Defocus range (µm)**            | 2-3                                        |
| **Electron exposure (e−/Å²)**     | 0.5-0.6                                    |
| **Number of micrographs**         | 5,587                                      |

### Reconstruction

| Table                        | SUMOylated apoAPC/C with repositioned APC2\textsuperscript{WHB} | Body 2 with repositioned APC2\textsuperscript{WHB} after Multi Body refinement |
|------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------------|
| **Particles used for final reconstruction** | 137,954                                                        | 137,954                                                                       |
| **Software**                 | Relion 3.0                                                      | Relion 3.0                                                                     |
| **Applied symmetry**         | C1                                                              | C1                                                                             |
| **Accuracy of rotations (degrees)** | 1.359                                                          | 3.71                                                                          |
| **Accuracy of translations (pixels)** | 0.924                                                          | 1.554                                                                         |
| **B-factor applied**         | -30                                                             | -30                                                                            |
| **Final resolution (Å)**      | 3.78                                                            | 3.78                                                                          |
| **FSC threshold**             | 0.143                                                           | 0.143                                                                         |

### Refinement

| Table                        | SUMOylated apoAPC/C with repositioned APC2\textsuperscript{WHB} |
|------------------------------|-------------------------------------------------------------------|
| **Initial model used**       | 5G05                                                              |
| **Map sharpening B factor (Å²)** | -30                                                              |
| **Model composition**        |                                                                  |
| **Chains**                   | 23                                                                |
| **Non-hydrogen atoms**       | 63,827                                                            |
| **Protein residues**         | 8,109                                                             |
| **B factors (Å²)**           |                                                                  |
| **Protein**                  | 135.53                                                            |
| **R.m.s. deviations**        |                                                                  |
| **Bond lengths (Å)**         | 0.005                                                             |
| **Bond angles (°)**          | 0.990                                                             |
| **Validation**               |                                                                  |
| **MolProbity score**         | 2.86                                                              |
| **Clashscore**               | 14.04                                                             |
| **Rotamer outliers (%)**     | 9.39                                                              |
| **Ramachandran plot**        |                                                                  |
| **Favored (%)**              | 92.66                                                             |
| **Allowed (%)**              | 7.13                                                              |
| **Disallowed (%)**           | 0.21                                                              |

**Table S1**
Table S1. CryoEM data collection, processing information and refinement statistics. Related to Figure 3.