An APC/C inhibitor stabilizes cyclin B1 by prematurely terminating ubiquitination

Xing Zeng, Randall W. King

Supplementary Methods

Reagents

Tosyl-L-arginine methyl ester (TAME) was from Sigma (T4626, >98% by TLC). Acetyl-L-arginine methyl ester (AAME) was from BACHEM (E-1030). MG262 was from Boston Biochem (I-120, >95% by HPLC). *Xenopus* Cdc20 C-terminal peptide (sequence: TKKEKEKARSSKSIIHQSIR) was synthesized by Biomatik and purified to >95% (see HPLC and mass spec analysis results at the end of supplementary methods).

Preparation of *Xenopus* egg extract

Interphase *Xenopus* egg extract was prepared from eggs laid overnight according to the protocol of Murray[1] with the exception that eggs were activated with 2 μg/ml calcium ionophore (A23187, free acid form, Calbiochem) for 30 minutes prior to the crushing spin. Extract was frozen in liquid nitrogen and stored at -80 ºC. To make mitotic extract, MBP-cyclin B1Δ90 was added to interphase extract at 1 μM and incubated at 22°C for 30 min.

Luciferase assay

Luciferase assay was performed as described previously[2]. Brifely, a fusion protein consisting of the N-terminal domain of cyclin B1 and luciferase was added to mitotic extract at 10 μg/ml. The extract was incubated at 23°C and 3 μl samples were taken at 0, 30, 60, 90 and 120 min. The samples were mixed quickly with 30 μl of luciferin assay buffer (270 μM coenzyme A, 20 mM tricine, 3.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 530 μM ATP and 470 μM luciferin, pH 7.8) and the level of luminescence was measured on a Wallac 1420 multilabel counter.

Covalent coupling of Cdc27 antibody to protein A beads

For a single reaction, 5 µl protein A affiprep beads (Bio-Rad 156-0006) were washed with TBST (10 mM Tris, 150 mM NaCl and 0.01% Tween-20, pH 7.5) twice and incubated with 2 µg Cdc27 antibody (Santa Cruz, sc-9972, AF3.1) for 75 min at 4°C. The beads were washed with TBST for 10 min followed by two additional quick washes with TBST. Dimethyl pimelimidate (DMP, PIERCE, 21666) was freshly dissolved in 100 mM sodium tetraborate decahydrate, pH 9.0 at 20 mM. The beads were mixed with ten beads volume of DMP solution and incubated on a rotating wheel for 45 min in the dark at room temperature. The beads were then washed quickly with 200 mM Tris, pH 8.0 twice, followed by a final 1 h wash. Beads were then washed twice in TBST and twice in XB prior to APC immunoprecipitation.

PCR-amplification of Flag-tagged Cdc20/Cdh1 for *in vitro* translation

Flag-tagged WT Cdc20 was amplified from a pCS2-Cdc20 construct with a 5' primer CAACTCTATAATACGACTCACTATAGGGAACAGCCACCATGGACTACAAGGACGACGATGA CAAGGCACAGTTCGCGTTCGAGAG and a 3' primer TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGCGGATGCCTTGGTGGATG (the poly-T sequence was added to mimic the poly-A tail at the 3' end of the mRNA). Flag-tagged WT Cdc20ΔIR was amplified from the same construct with the same 5' primer but a different 3' primer TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGCCTTGGTGGATGAGGCTGC. Flag-tagged K-less Cdc20 was amplified from K-less Cdc20 construct[3] with a 5' primer CAACTCTATAATACGACTCACTATAGGGAACAGCCACCATGGACTACAAGGACGACGATGA CAAGGCCCAATTTGCCTTTGAATC and a 3' primer TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTACCTAATCCCCTGATGAATC.

Flag-tagged WT Cdh1 was amplified from a pET28a-Cdh1 construct with a 5' primer CAACTCTATAATACGACTCACTATAGGGAACAGCCACCATGGACTACAAGGACGACGATGA

CAAGATGGACCAGGATTATGAGAGAC and a 3' primer TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTATCGTATTCTCGTGAACAGG.

Construction of double HA-tagged Cdc20 mutants

All double HA-tagged Cdc20 mutants' DNAs were first generated by PCR and then digested with *Eco*RI and *Xho*I. A pCS2-WT Cdc20 construct was also digested with *Eco*RI and *Xho*I to separate the WT Cdc20 insert from the pCS2 vector. The Cdc20 mutants' DNAs were then ligated into the pCS2 vector to replace the original WT Cdc20. All mutants were confirmed by sequencing. Detailed information of PCR generation of Cdc20 mutants' DNAs is listed below.

$Cdc20^{1\text{-}76R}$: A 5' fragment was amplified from the K-less Cdc20 construct with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer CACTGCGATGGGGGATATAGCGATCGCCACCGGGCCTAGAGGGGGT. A 3' fragment was amplified from the pCS2-Cdc20 construct with a 5' primer CGCTATATCCCCCATCGCAGTG and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG. The 5' and the 3' fragments were then combined as templates and re-amplified with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG to yield the full length DNA of $Cdc20^{1\text{-}76R}$.

$Cdc20^{77\text{-}164R}$: A fragment covering 1-76aa of WT Cdc20 was amplified from the pCS2-Cdc20 construct with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer CCTGTGAGGAATGTACCTATCACCGCCAGGTTTGCTAGGAGT. A fragment covering 77-164aa of K-less Cdc20 was amplified from the K-less Cdc20 construct with a 5' primer GATAGGTACATTCCTCACAGG and a 3' primer CATTCCCAGAACTCCAATCCACCAAATTGAGATAGTAATCGTT. The two fragments were combined as templates and re-amplified with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer CATTCCCAGAACTCCAATCCACCAAATTGAGATAGTAATCGTT to yield a fragment covering 1-

76aa of WT Cdc20 and 77-164aa of K-less Cdc20 (5' fragment). A fragment covering 77-164aa of K-less Cdc20 was amplified from the K-less Cdc20 construct with a 5' primer GATAGGTACATTCCTCACAGG and a 3' primer CATTCCCAGAACTCCAATCCACCAAATTGAGATAGTAATCGTT. A fragment covering 165-499aa of WT Cdc20 was amplified from the pCS2-Cdc20 construct with a 5' primer GTGGATTGGAGTTCTGGGAATG and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG. The two fragments were combined as templates and re-amplified with a 5' primer GATAGGTACATTCCTCACAGG and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG to yield a fragment covering 77-164aa of K-less Cdc20 and 165-499aa of WT Cdc20 (3' fragment). The 5' and 3' fragments were combined as templates and re-amplified with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG to yield the full length DNA of $Cdc20^{77\text{-}164R}$.

$Cdc20^{165\text{-}499R}$: A 5' fragment was amplified from the pCS2-Cdc20 construct with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer CAAATTGAGATAGTAATCGTTTCGGATTTCAGGCGCATCCAG. A 3' fragment was amplified from the K-less Cdc20 construct with a 5' primer AACGATTACTATCTCAATTTG and a 3' primer ATGCCTCGAGCTACCTAATCCCCTGATGAATC. The 5' and the 3' fragments were combined as templates and re-amplified with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer ATGCCTCGAGCTACCTAATCCCCTGATGAATC to yield the full length DNA of $Cdc20^{165\text{-}499R}$.

$Cdc20^{1\text{-}164R}$: A 5' fragment was amplified from the K-less Cdc20 construct with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and a 3' primer CATTCCCAGAACTCCAATCCACCAAATTGAGATAGTAATCGTT. A 3' fragment was amplified from the pCS2-Cdc20 construct with a 5' primer GTGGATTGGAGTTCTGGGAATG and a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG. The 5' and the 3' fragments were combined as templates and re-amplified with a 5' primer ATGCGAATTCGATGGCCCAATTTGCCTTTGAATC and

a 3' primer ATGCCTCGAGTCAGCGGATGCCTTGGTGGATG to yield the full length DNA of $Cdc20^{1-164R}$.

Stripping Cdc20 from the APC by TAME treatment

APC was first immunoprecipitated from mitotic *Xenopus* extract and 200 μM TAME was added to the extract for 15 min to drive Cdc20 dissociation. The beads were then washed quickly with XB + 0.1% IGEPAL CA-630 4 times and then washed with XB + 0.1% IGEPAL CA-630 for 5 min × 2 while kept shaking vigorously.

APC depletion

For a single round of depletion, mitotic *Xenopus* extract was incubated with $1/10^{th}$ extract volume of Cdc27 antibody beads or (empty protein A beads as mock IP control) prepared as described above and incubated at 4°C on a rotator for 45 min.

Experiments comparing unmodified versus ubiquitinated cycB-NT

To generate ubiquitinated cycB-NT, an *in vitro* reconstituted ubiquitination assay was set up by mixing immunoprecipitated mitotic APC^{Cdc20} and ubiquitination reaction components containing 500 nM cycB-NT (detailed conditions are described in the main methods section). After 20 min, the supernatant was separated from the beads and subsequently used as the source of ubiquitinated cycB-NT. The same mixture of ubiquitination reaction components and 500 nM cycB-NT without incubation with the APC beads was used as the unmodified cycB-NT. To compare the ability of unmodified and ubiquitinated cycB-NT to promote Cdc20 binding to the APC, 10 μl of unmodified/ubiquitinated cycB-NT was mixed with 1 μl reticulocyte lysate containing *in vitro*-translated Cdc20. This cycB-NT/Cdc20 mixture was then supplemented with 10 μM MG132, 100 nM okadaic acid potassium salt, 5 μM ubiquitin vinyl sulfone (to inhibit proteasome, phosphatase and deubiquitinating enzyme activities in the reticulocyte lysate) and 20 μM UbcH10 C114S (to suppress further ubiquitination of cycB-NT once the mixture is added to the APC).

The mixture was then added to 5 µl beads loaded with APC stripped of Cdc20 by TAME-treatment and incubated on a shaker for 10 min. The beads were then separated from the supernatant and the distribution of Cdc20 was analyzed by anti-Flag Western blot. To compare the ability of the APC to further ubiquitinate unmodified and ubiquitinated cycB-NT, the same mixture was prepared as described with the exception that UbcH10 C114S was omitted. The mixture was incubated with APC stripped of Cdc20 by TAME-treatment and incubated on a shaker for 30 min. The products were analyzed by anti-HA Western blot.

Supplementary References:

1. Murray, A.W. Chapter 30 Cell Cycle Extracts. *Methods Cell Biol*. **36**, 581 (1991).
2. Verma, R., *et al*. Ubistatins inhibit proteasome-dependent degradation by binding the ubiquitin chain. *Science* **306**, 117-120 (2004).
3. Nilsson, J., Yekezare, M., Minshull, J. & Pines, J. The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat Cell Biol* **10**, 1411-1420 (2008).

BIOMATIK
Tel: 1-800-836-8089
Fax: 1-877-221-3515
custom@biomatik.com
www.biomatik.com

Certificate of Analysis

Date:	2011-10-11
Order Number:	SP1110779
Catalog Number:	265369
Peptide Name:	Cdc20 C-terminus(TR-20)
Sequence:	TKKEKEKARSSKSIIHQSIR
Lot Number:	P110921-ZJ265369
Molecular Formula:	$C_{100}H_{180}N_{34}O_{31}$
Molecular Weight:	2354.76
Quantity:	4.0mg
Reconstitution Condition Used In Purification::	H2O: Acetonitrile =4:1
Reconstitution Condition For Your Assay:	Refer to the Peptide Handling Guideline
TEST RESULTS	
Purity (by HPLC):	95.78%
	(95% Requested)
MS:	Consistent
Appearance:	White to off White Powder

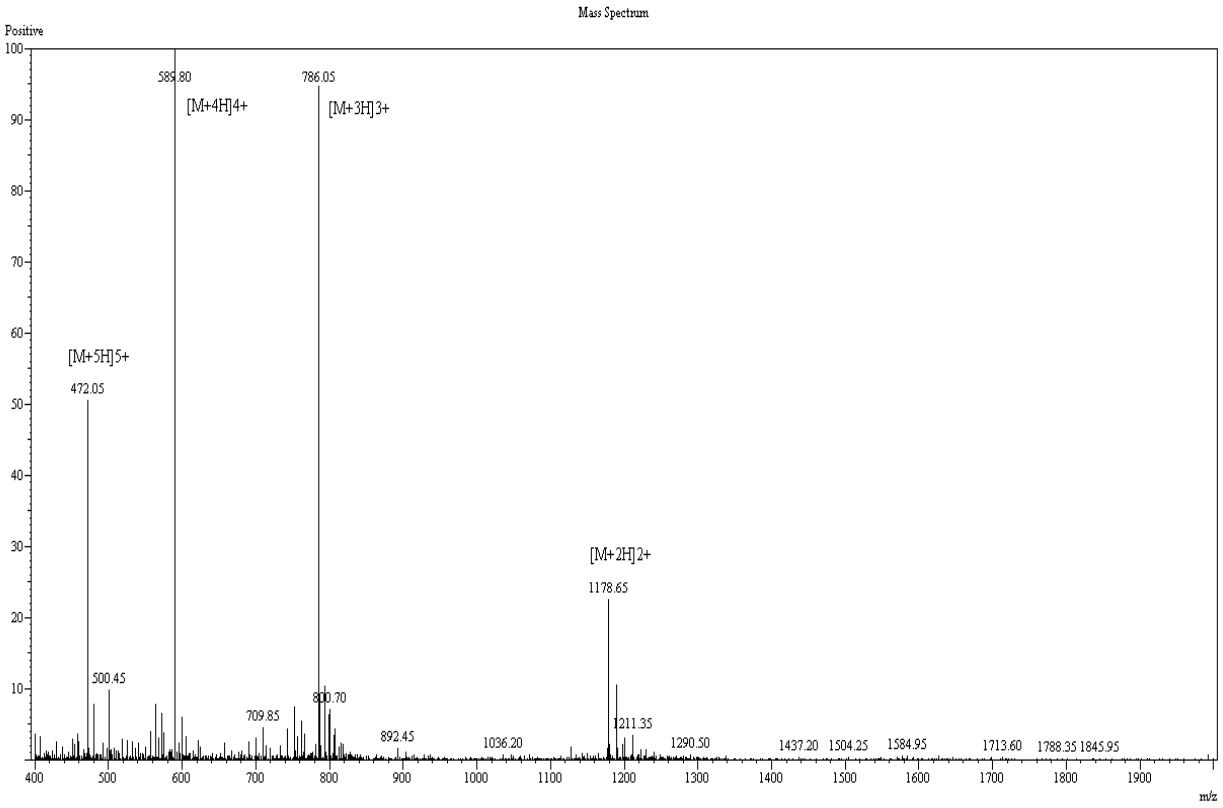
Storage Condition: -20°C Upon Arrival.
It is not recommended that peptides be stored in solution. The shelf life of peptides in solution is rather limited, especially for sequences containing Cysteine, Methionine, Tryptophan, Asparagine and Glutamine. Generally, the best approach is to reconstitute sufficient peptide for immediate usage only. For long-term storage please store at -20°C.

Limited Usage and Warranty

This product is for laboratory research use only. Not for diagnostic or in vivo use. Not for human or drug use.
Biomatik warrants material of said quality at the time of delivery. It is the sole responsibility of the customers to determine the adequacy of all materials for any intended or specific purpose or use. Biomatik' sole obligation shall be the replacement of the material without charge or refund of purchase price. This warranty does not apply to a product which has been tampered with or altered in any way or which has been misused or damaged by accident or negligence. All claims must be received in writing (by fax or email) within 30 days upon successful delivery and failure to do so shall constitute a waiver by customers for any and all such claims.

Tel: 1-800-836-8089
Fax: 1-877-221-3515
custom@biomatik.com
www.biomatik.com

Mass Spectrometry Report



Sample Information

Date and Time	: 2011-9-28 10:28:36
User	: CHAO
Sample	: cdc20 C-terminus TR-20
Inj. Volume	: 1
MW	:2354.76
Lot No.	:P110921-ZJ265369

Probe:	ESI	Probe bias:	-3.5kv
Nebulizer Gas Flow:	1.5L/min	Detector:	1.0kv
DL:	-20.0v	T. Flow:	0.2ml/min
DL Temp:	250℃	B. conc:	50%H2O/50%ACN
Block Temp:	300℃		

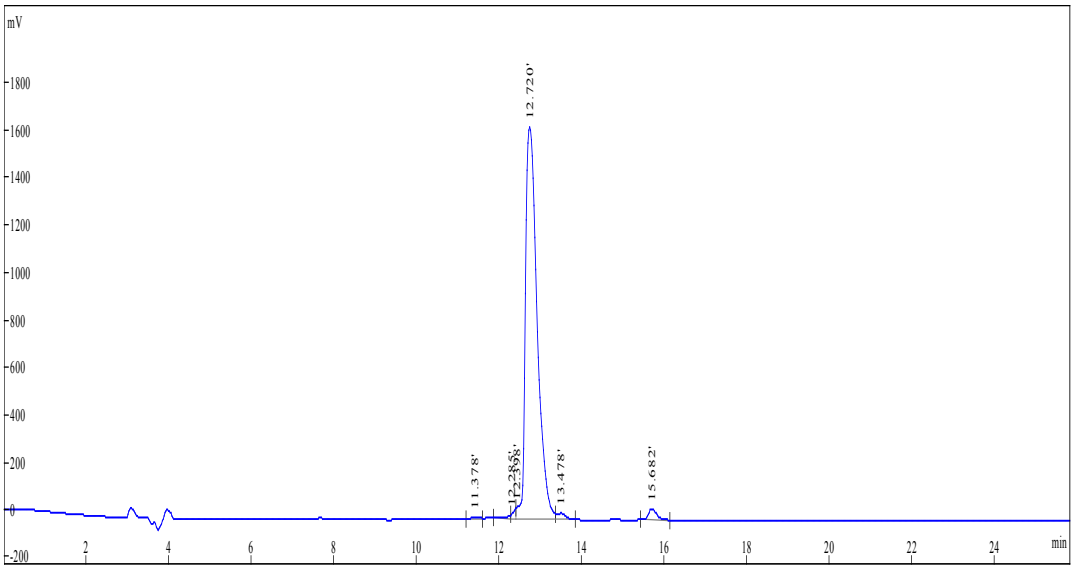
Tel: 1-800-836-8089
Fax: 1-877-221-3515
custom@biomatik.com
www.biomatik.com

HPLC Report

Peptide name : Cdc20 C-terminus(TR-20)
Lot number : P110921-ZJ265369
Column :4.6*250mn,VYDAC-C18
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient :

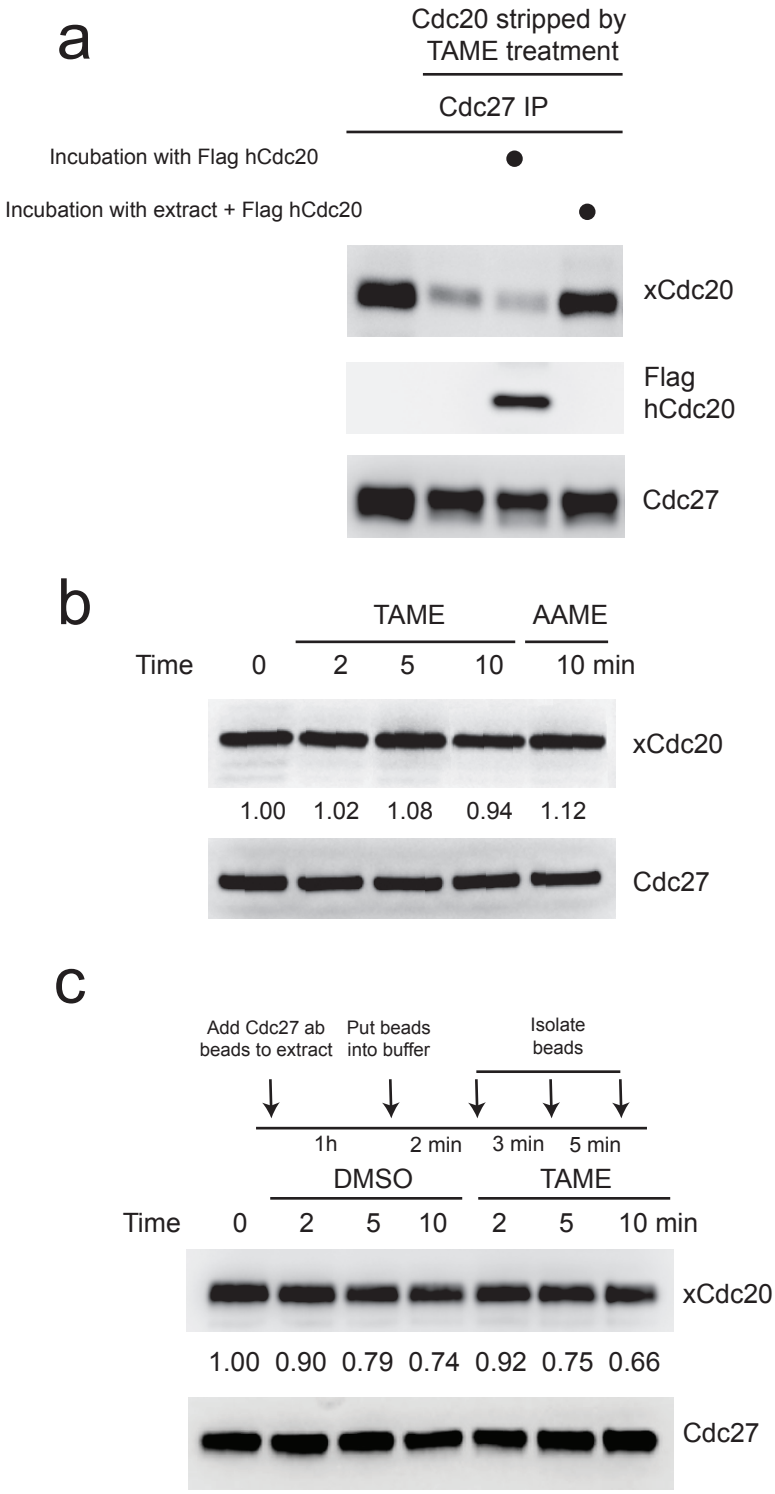
	A	B
0.01min	10%	90%
25min	35%	65%
25.01min	100%	0%
30min	Stop	

Flow rate :1.0ml/min
Wavelength :220nm
Volume :20ul

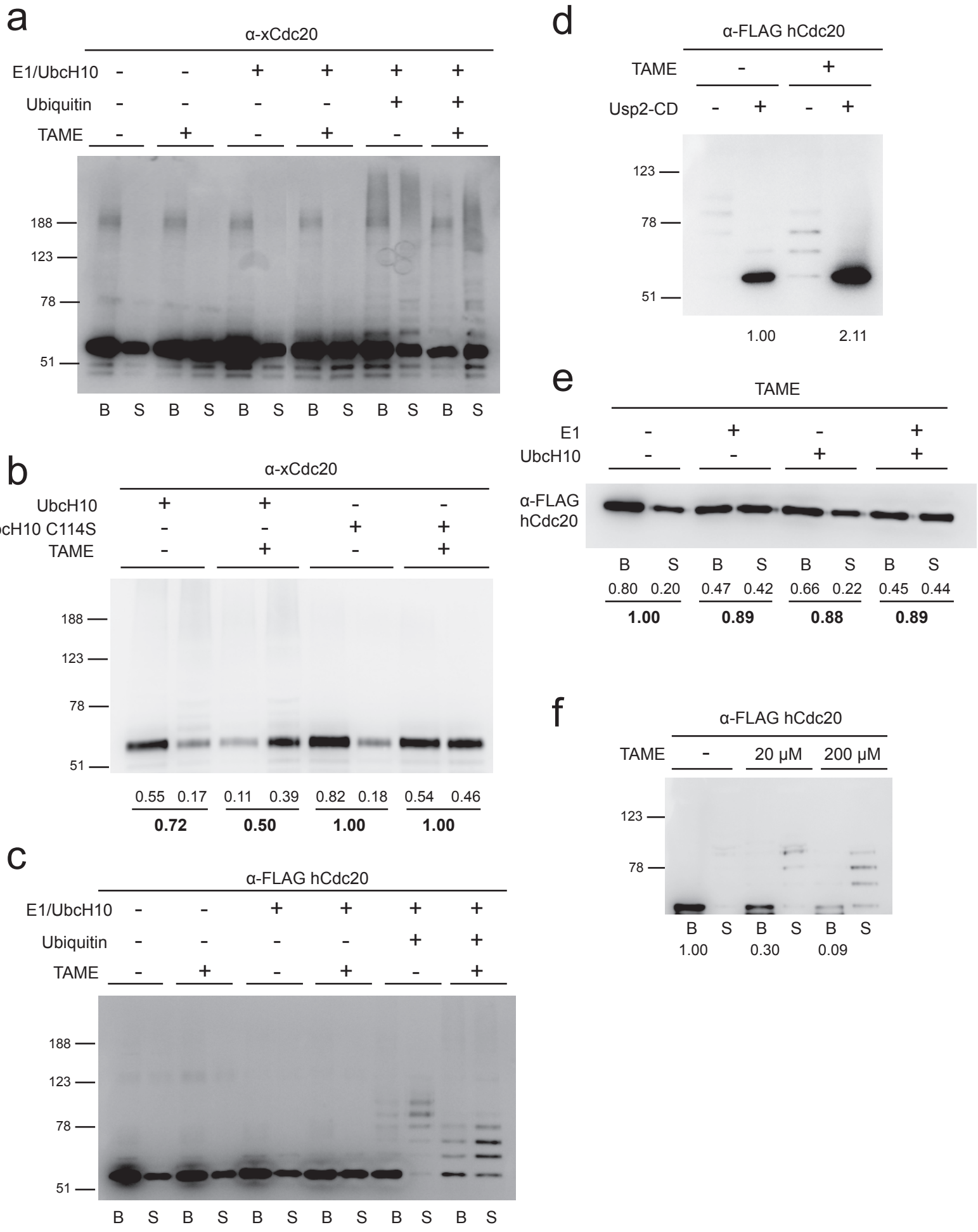


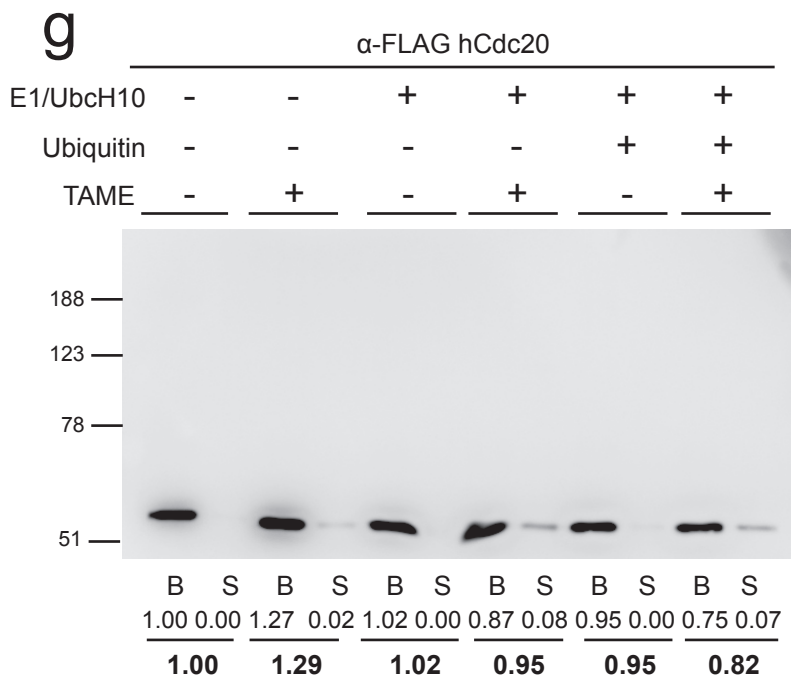
Rank	Time	Conc.	Area	Height
1	11.378	0.3039	103816	8673
2	12.285	0.3751	128149	16818
3	12.398	0.6651	227226	51557
4	12.720	95.78	32723034	1653348
5	13.478	1.002	342507	25256
6	15.682	1.881	642523	48287
Total		100	34167255	1803939

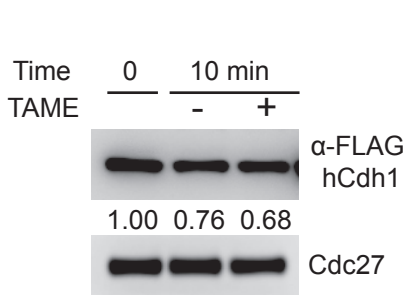
Supplementary Results

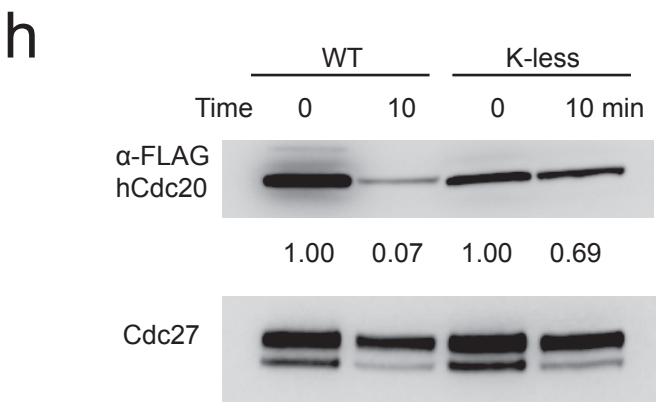


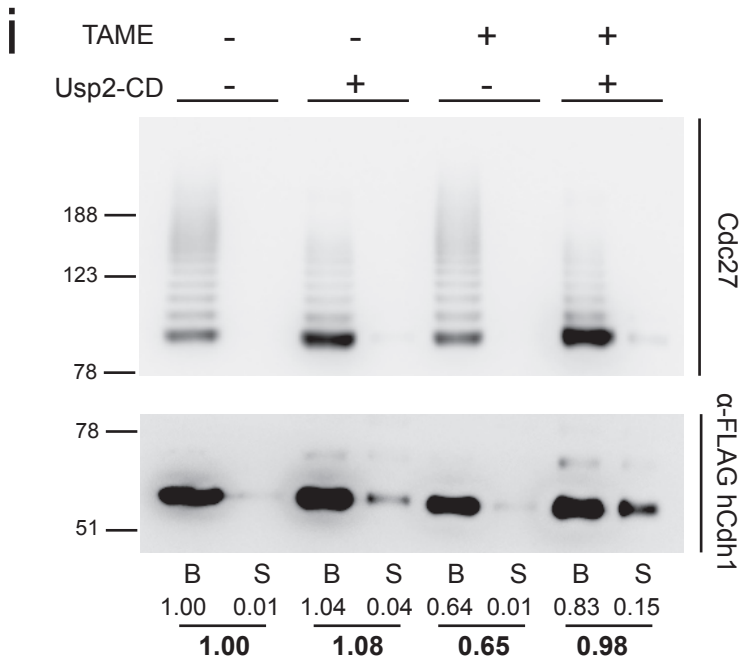
Supplementary Figure 1. (a) Endogenous Cdc20 outcompetes Flag-tagged human Cdc20 for APC binding. APC was immunoprecipitated from mitotic *Xenopus* extract treated with 200 μM TAME to remove pre-bound endogenous Cdc20. The beads were then incubated with either 1.3 μl reticulocyte lysate containing Flag-tagged Cdc20 or the same volume of reticulocyte lysate containing Cdc20 added into the extract (100 μl) for 10 min. The amount of bead-bound endogenous Cdc20, Flag-tagged Cdc20 and Cdc27 were analyzed by Western blot. (b) TAME does not induce rapid dissociation of Cdc20 from the APC in interphase *Xenopus* extract. APC^{Cdc20} was first immunoprecipitated from interphase extract with Cdc27 beads and 200 μM TAME or AAME (N-acetyl arginine methyl ester, an inactive derivative of TAME) was then added to the extract for indicated period of time. The beads were then isolated from the extract. The amounts of bead-bound Cdc20 and Cdc27 were analyzed by Western blot. (c) TAME does not induce rapid dissociation of Cdc20 from the APC in buffer. Immunoprecipitated APC^{Cdc20} was suspended in 200× bead volume of XB buffer -/+ TAME for the indicated period of time. Amounts of bead-bound Cdc20 and Cdc27 were analyzed by Western blot.


a
α-xCdc20
E1/UbcH10 - - + + + +
Ubiquitin - - - - + +
TAME - + - + - +
188
123
78
51
B S B S B S B S B S B S
b
α-xCdc20
UbcH10 + + - -
UbcH10 C114S - - + +
TAME - + - +
188
123
78
51
0.55 0.17 0.11 0.39 0.82 0.18 0.54 0.46
0.72 0.50 1.00 1.00
c
α-FLAG hCdc20
E1/UbcH10 - - + + + +
Ubiquitin - - - - + +
TAME - + - + - +
188
123
78
51
B S B S B S B S B S B S
d
α-FLAG hCdc20
TAME - +
Usp2-CD - + - +
123
78
51
1.00 2.11
e
TAME
E1 - + - +
UbcH10 - - + +
α-FLAG hCdc20
B S B S B S B S
0.80 0.20 0.47 0.42 0.66 0.22 0.45 0.44
1.00 0.89 0.88 0.89
f
α-FLAG hCdc20
TAME - 20 μM 200 μM
123
78
B S B S B S
1.00 0.30 0.09


g
α-FLAG hCdc20
E1/UbcH10 - - + + + +
Ubiquitin - - - - + +
TAME - + - + - +
188
123
78
51
B S B S B S B S B S B S
1.00 0.00 1.27 0.02 1.02 0.00 0.87 0.08 0.95 0.00 0.75 0.07
1.00 1.29 1.02 0.95 0.95 0.82

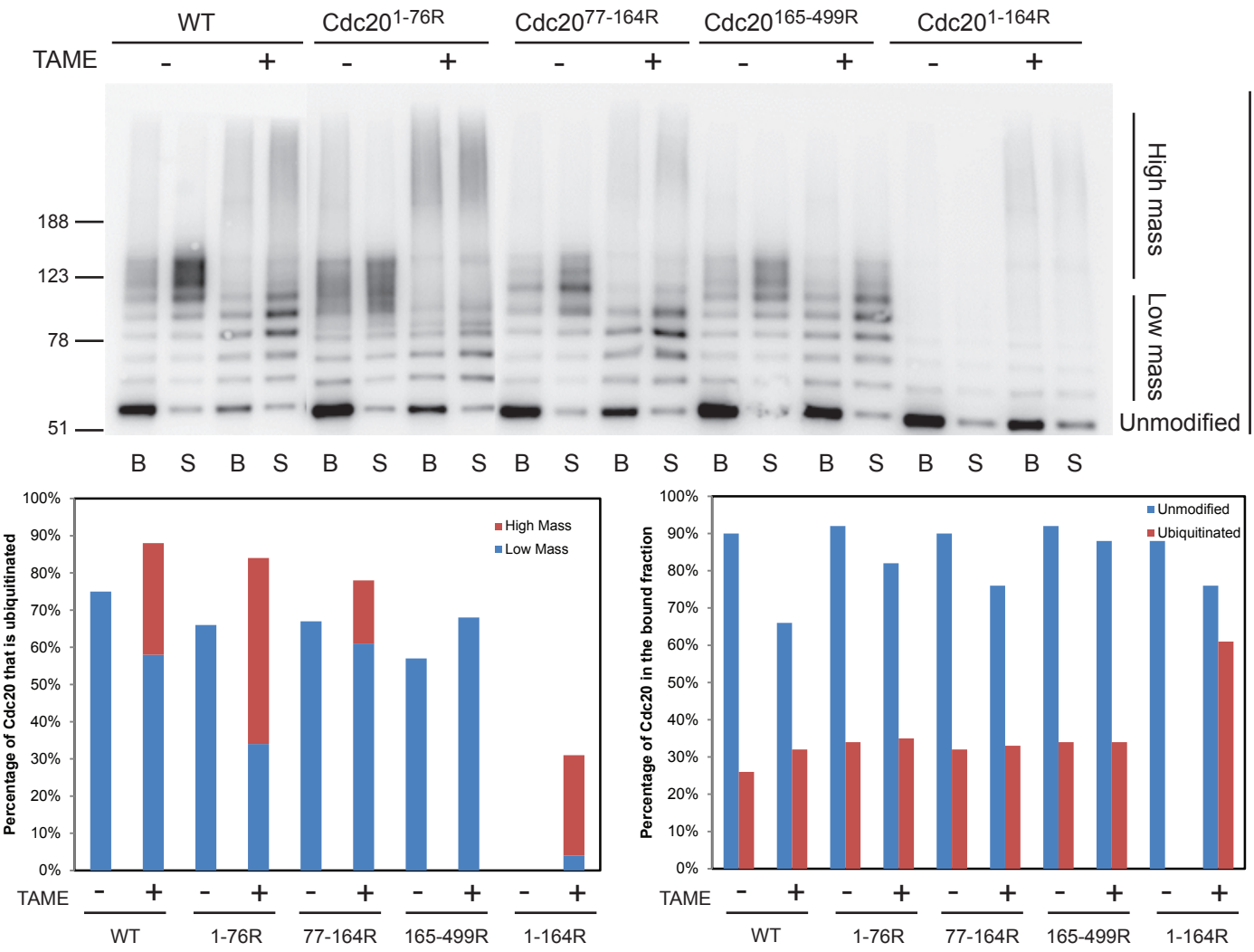

j
Time 0 10 min
TAME - +
α-FLAG hCdh1
1.00 0.76 0.68
Cdc27

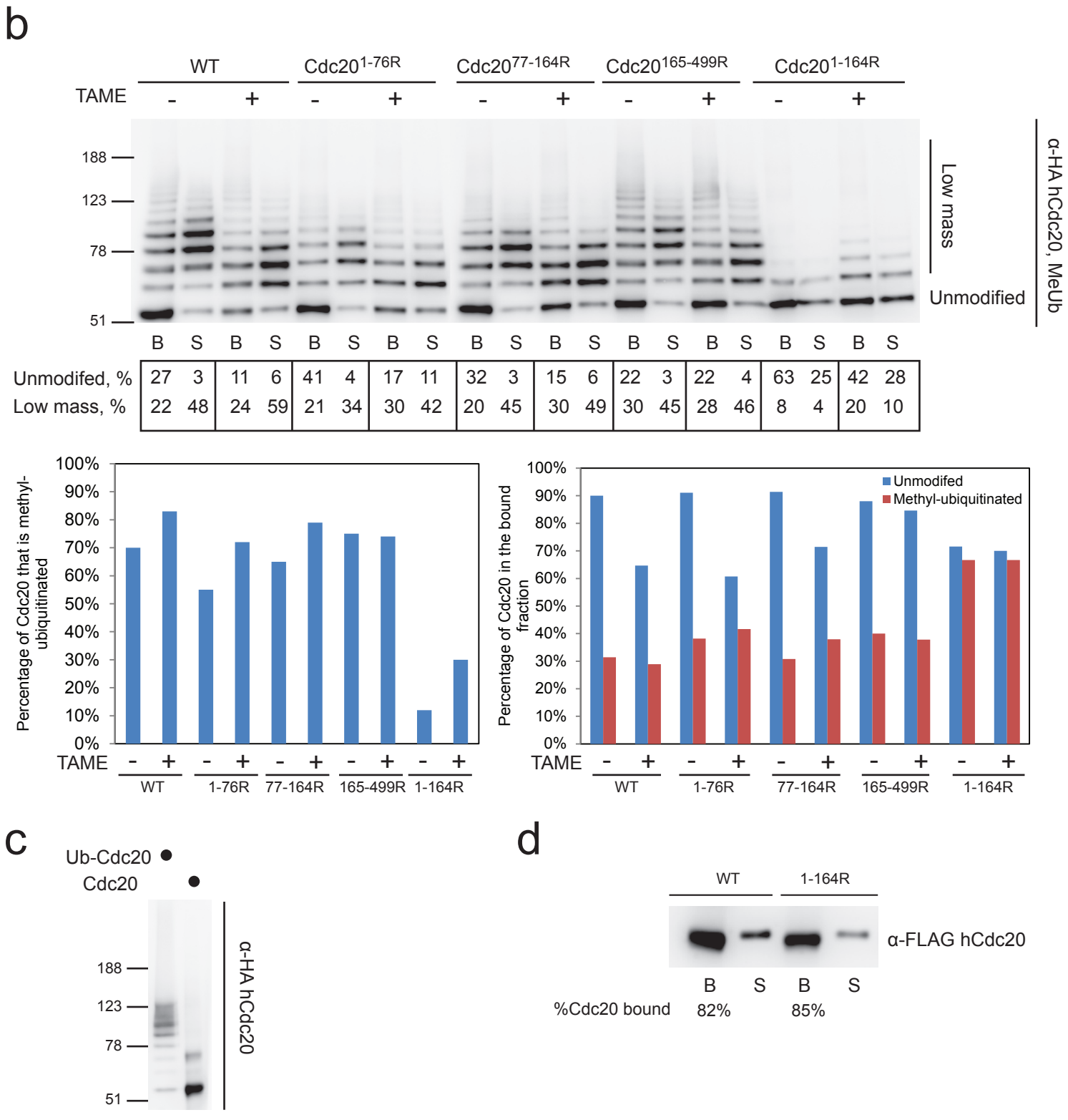

h
WT K-less
Time 0 10 0 10 min
α-FLAG hCdc20
1.00 0.07 1.00 0.69
Cdc27


i
TAME - - + +
Usp2-CD - + - +
188
123
78
Cdc27
78
51
α-FLAG hCdh1
B S B S B S B S
1.00 0.01 1.04 0.04 0.64 0.01 0.83 0.15
1.00 1.08 0.65 0.98

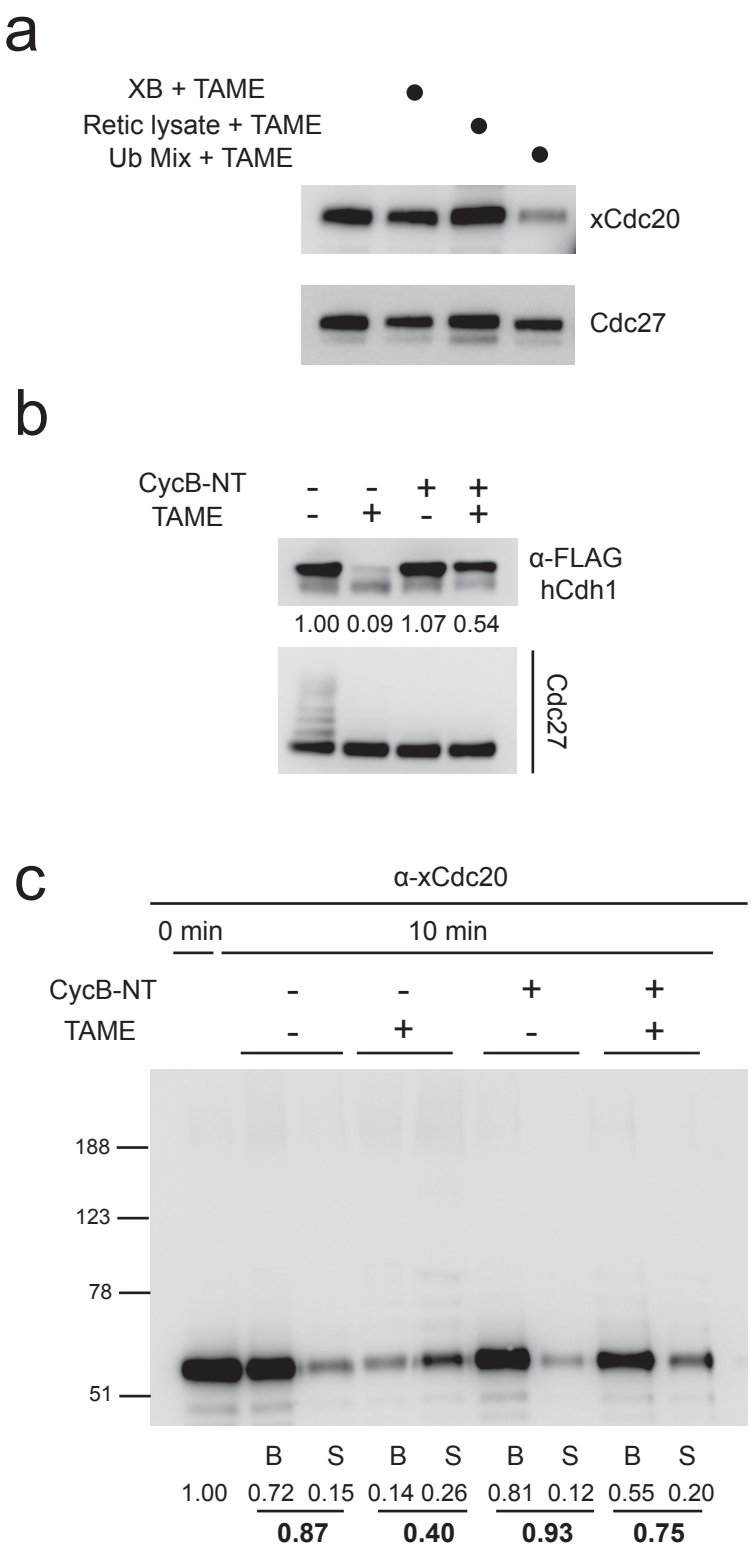
Supplementary Figure 2. (a) A longer exposure of Fig. 2a. (b) Ubiquitinitination of Cdc20 is critical for TAME-induced Cdc20 dissociation. Immunoprecipitated APC^{Cdc20} was incubated with E1 (250 nM), ubiquitin (150 μM), UbcH10 or the C114S mutant (2 μM) and TAME (200 μM) as indicated for 10 min. Cdc20 was analyzed by Western blot separately in the bead-bound (B) and the supernatant (S) fractions. (c) A longer exposure of Fig. 2b. (d) Deubiquitination of Flag-tagged Cdc20 confirms that TAME promotes Cdc20 ubiquitination. Supernatant from an *in vitro* Cdc20 ubiquitination assay +/-TAME that contained ubiquitinated Cdc20 was incubated with the catalytic domain of Usp2 and then analyzed by Western blot with a Flag antibody. Note the antibody-detectability of Cdc20 increases after deubiquitination. (e) E1 induces Cdc20 dissociation in the presence of TAME. Immunoprecipitated APC was high salt washed to remove endogenous Cdc20 from the APC and incubated with *in vitro*-translated human FLAG-tagged Cdc20. The beads were then incubated with 200 μM TAME and various components (250 nM E1 and 2 μM UbcH10) as indicated for 10 min. Cdc20 was analyzed by Western blot separately in the bead-bound (B) and the supernatant (S) fractions. (f) Lower concentration of TAME (20 μM) can induce Cdc20 ubiquitination and dissociation *in vitro*. The same assay as in Fig. 2b was repeated with 20 μM TAME. Numbers represent unmodified Cdc20 left at the end of the assay. (g) TAME does not induce ubiquitination and dissociation of $Cdc20^{K\text{-less}}$ in the presence of E1, UbcH10 and ubiquitin. The same assay was performed as in Fig. 2b, except APC was re-loaded with *in vitro*-translated human $Cdc20^{K\text{-less}}$. (h) TAME induces rapid dissociation of WT Cdc20 but not $Cdc20^{K\text{-less}}$ from the APC in mitotic extract. Immunoprecipitated APC^{Cdc20} was high salt washed to remove endogenous Cdc20 and re-loaded with either *in vitro*-translated WT or K-less human FLAG-tagged Cdc20. The beads were then suspended in mitotic extract -/+ 200 μM TAME. Bead-bound FLAG-tagged Cdc20 and Cdc27 were analyzed by Western blot. (i) APC^{Cdh1} undergoes Cdc27 auto-ubiquitination and is relatively resistant to TAME-induced Cdh1 auto-ubiquitination. Interphase APC was loaded with *in vitro*- translated Flag-tagged human Cdh1 and incubated with ubiquitination reaction components. The beads and the supernatants were separated and deubiquitinated with the catalytic domain of Usp2. Cdc27 and Cdh1 were then analyzed by Western blot. (j) Cdh1 is resistant to TAME-induced dissociation in interphase extract. APC^{Cdh1} beads were then suspended in interphase extract -/+ 200 μM TAME. Bead-bound Cdc27 and Cdh1 were analyzed by Western blot.

a


WT
Cdc20 1-76R
Cdc20 77-164R
Cdc20 165-499R
Cdc20 1-164R
TAME
- + - + - + - + - +
188
123
78
51
High mass
Low mass
Unmodified
α-HA hCdc20, WT Ub
B S B S B S B S B S B S B S B S B S B S
Percentage of Cdc20 that is ubiquitinated
High Mass
Low Mass
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
TAME
WT
1-76R
77-164R
165-499R
1-164R
Percentage of Cdc20 in the bound fraction
Unmodified
Ubiquitinated

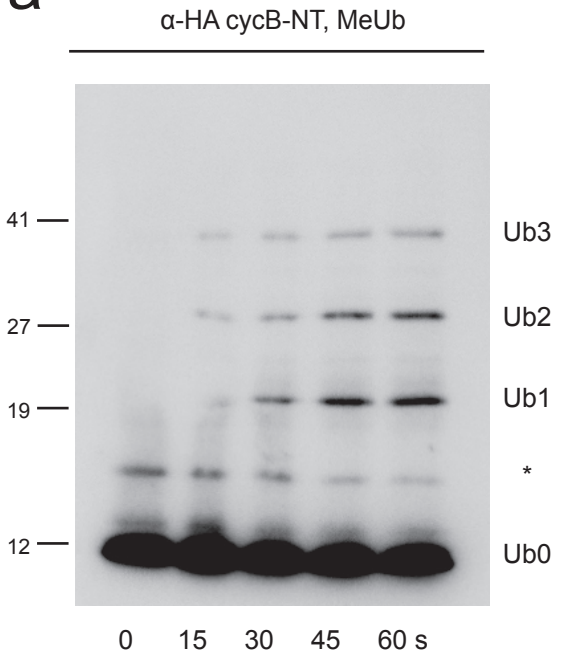


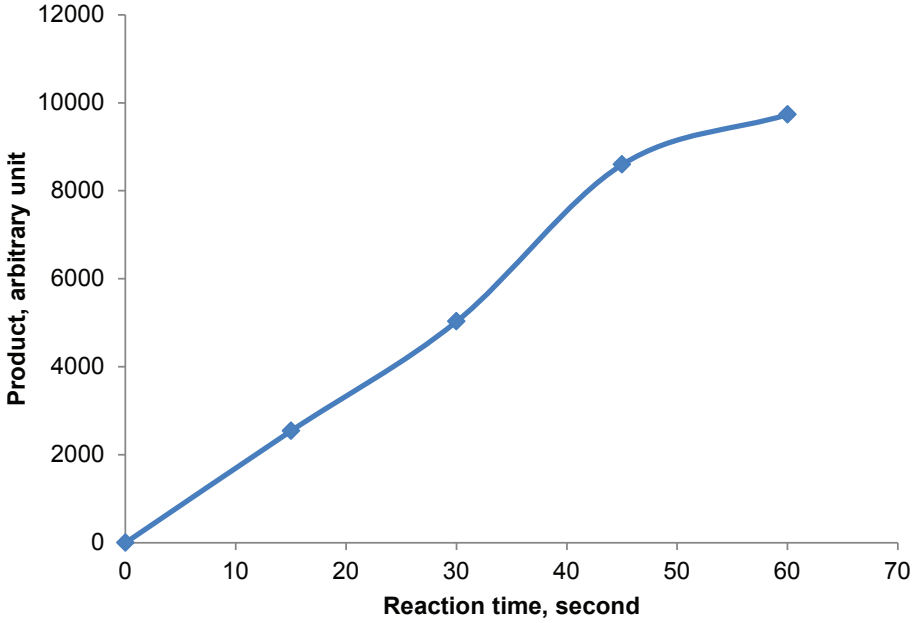
Supplementary Figure 3. (a) An independent repeat of the experiment in Fig. 3b (b) The same experiment as in (a) was repeated except with methylated ubiquitin. (c) Ubiquitinated and unmodified Cdc20 used in the competition assay in Fig. 3c. (d) WT Cdc20 and the 1-164R mutant display similar binding affinity for the APC. Immunoprecipicated APC loaded with either WT Cdc20 or the 1-164R mutant was incubated in buffer for 10 min. The beads and the supernatants were separated and the amount of Cdc20 present in these fractions was analyzed by Western blot. %Cdc20 bound was calculated as the fraction of bound Cdc20 signal relative to the total Cdc20 signal.



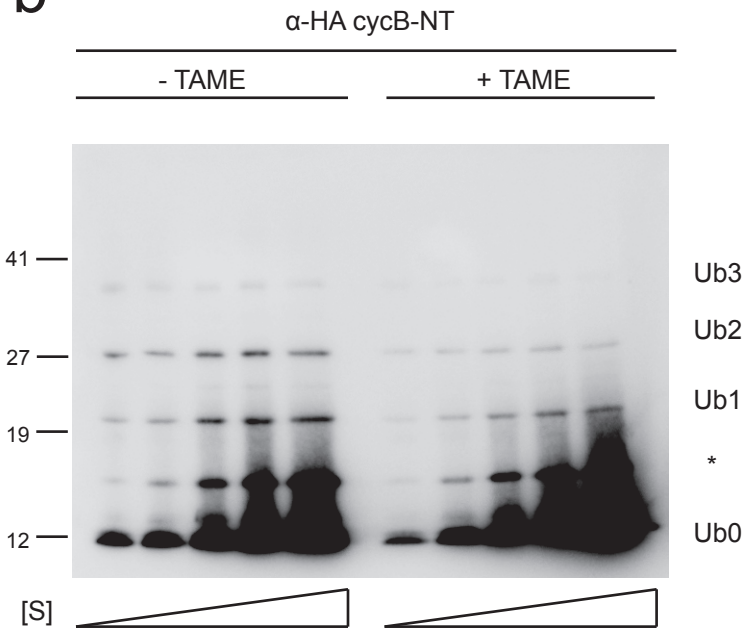
Supplementary Figure 4. (a) The reticulyte lysate system does not support TAME-induced Cdc20 dissociation. APC^{Cdc20} immunoprecipitated from mitotic Xenopus extract was suspended in buffer (XB), or reticulyte lysate, or the ubiquitination mixture (E1, UbcH10 and ubiquitin) along with 200 μM TAME for 10 min. The amount of bead bound Cdc20 was analyzed by Western blot. (b) TAME inhibits binding of free Cdh1 to the APC, which is suppressed by cycB-NT. The same experiment as in Fig. 4b was performed with *in vitro*-translated Cdh1. (c) Substrate suppresses Cdc20 ubiquitination. The same assay as in Fig. 4c was performed with immunoprecipitated APC^{Cdc20}.

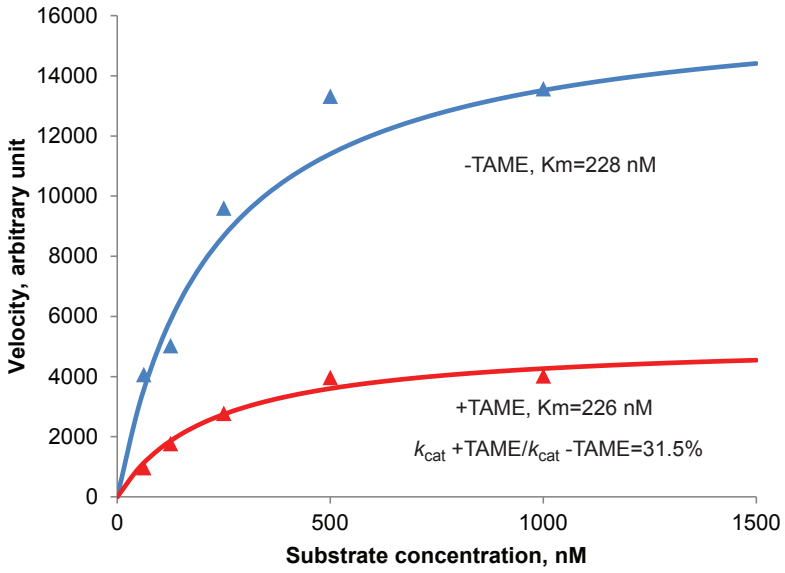
a


α-HA cycB-NT, MeUb
41
27
19
12
Ub3
Ub2
Ub1
*
Ub0
0 15 30 45 60 s

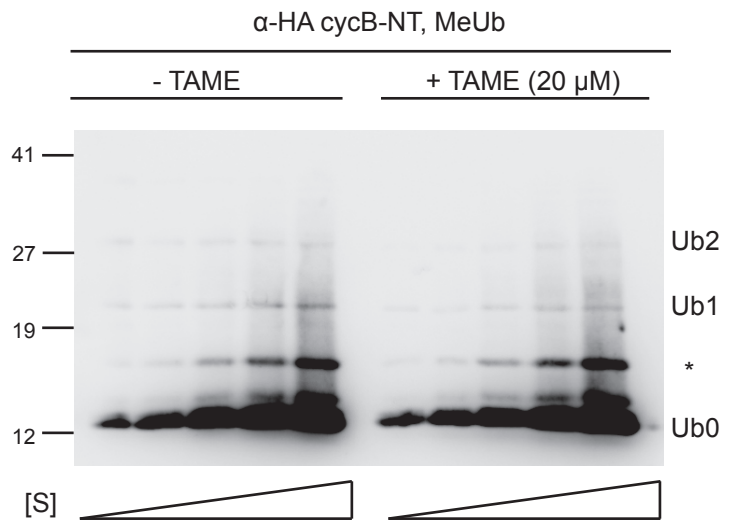

Product, arbitrary unit
12000
10000
8000
6000
4000
2000
0
0 10 20 30 40 50 60 70
Reaction time, second

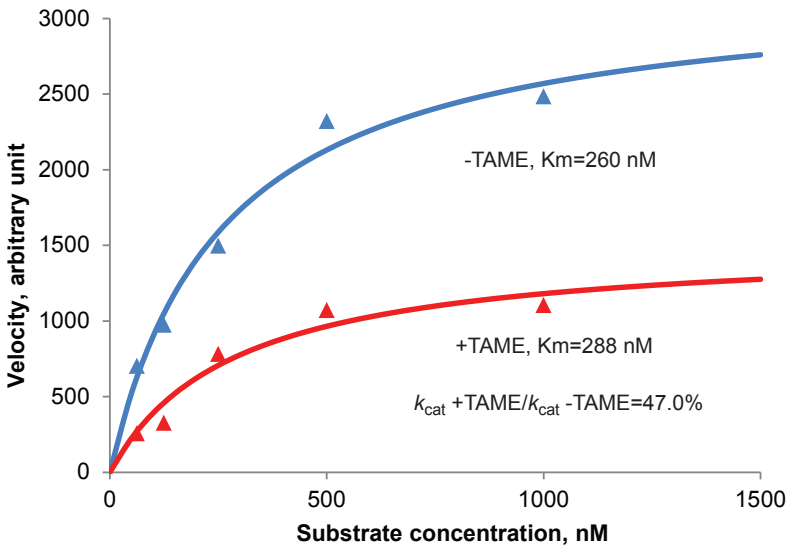
b


α-HA cycB-NT
- TAME
+ TAME
41
27
19
12
Ub3
Ub2
Ub1
*
Ub0
[S]

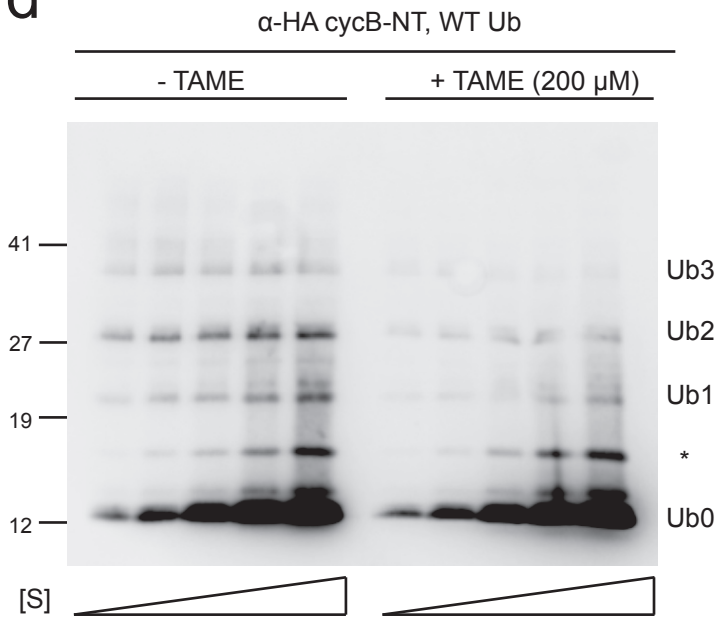

Velocity, arbitrary unit
16000
14000
12000
10000
8000
6000
4000
2000
0
0 500 1000 1500
Substrate concentration, nM
-TAME, Km=228 nM
+TAME, Km=226 nM
kcat +TAME/kcat -TAME=31.5%

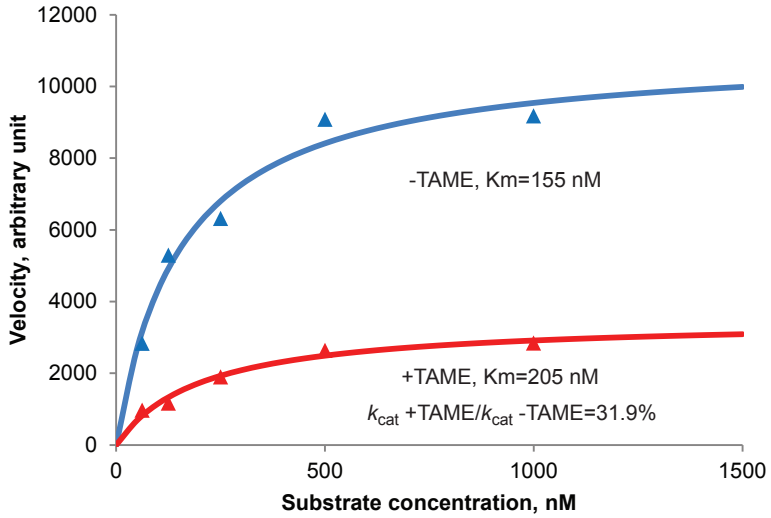
c


α-HA cycB-NT, MeUb
- TAME
+ TAME (20 μM)
41
27
19
12
Ub2
Ub1
*
Ub0
[S]

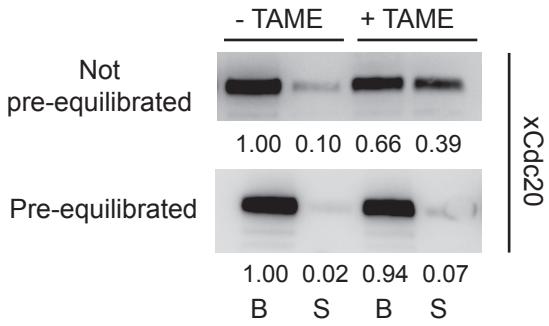

Velocity, arbitrary unit
3000
2500
2000
1500
1000
500
0
0 500 1000 1500
Substrate concentration, nM
-TAME, Km=260 nM
+TAME, Km=288 nM
kcat +TAME/kcat -TAME=47.0%

d

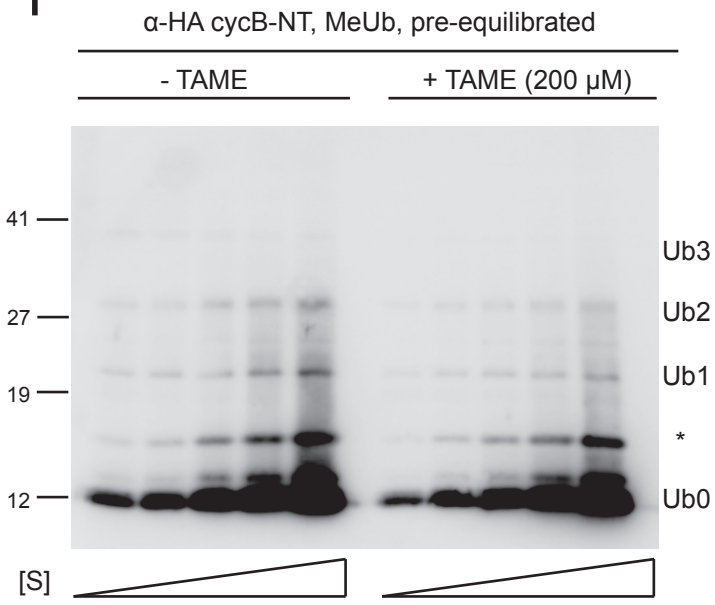

α-HA cycB-NT, WT Ub
- TAME
+ TAME (200 μM)
41
27
19
12
Ub3
Ub2
Ub1
*
Ub0
[S]

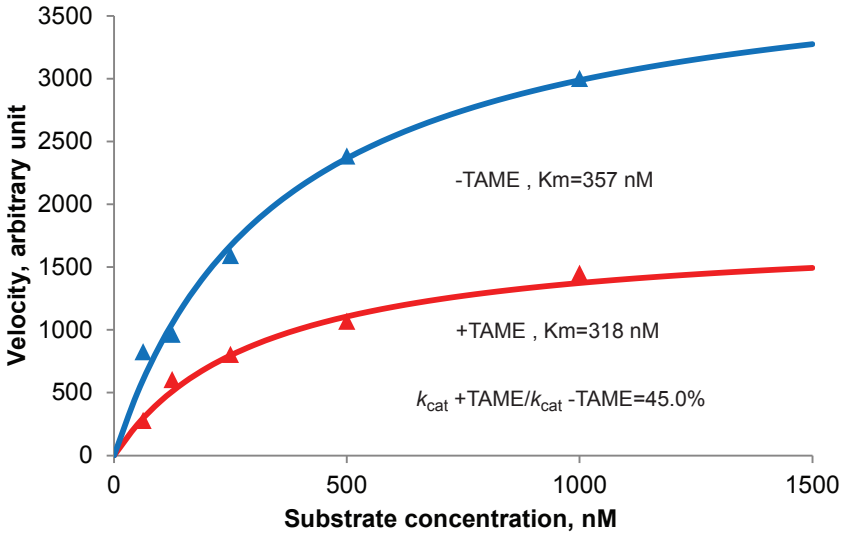

12000
10000
8000
6000
4000
2000
0
Velocity, arbitrary unit
-TAME, Km=155 nM
+TAME, Km=205 nM
k_{cat} +TAME/k_{cat} -TAME=31.9%
0
500
1000
1500
Substrate concentration, nM

e

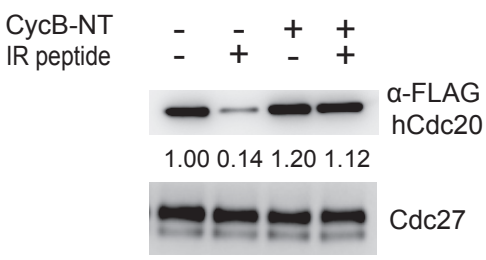

- TAME
+ TAME
Not
pre-equilibrated
1.00 0.10 0.66 0.39
Pre-equilibrated
1.00 0.02 0.94 0.07
B S B S
xCdc20

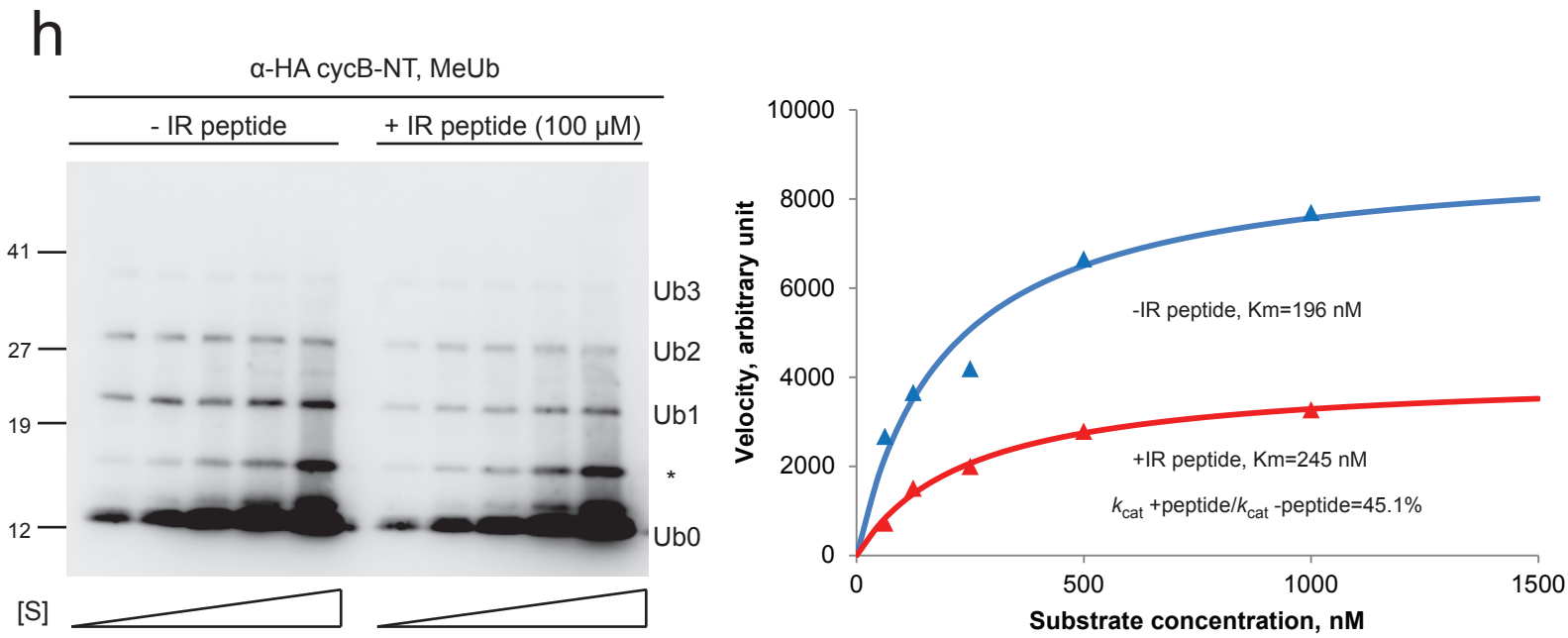
f


α-HA cycB-NT, MeUb, pre-equilibrated
- TAME
+ TAME (200 μM)
41
27
19
12
Ub3
Ub2
Ub1
*
Ub0
[S]

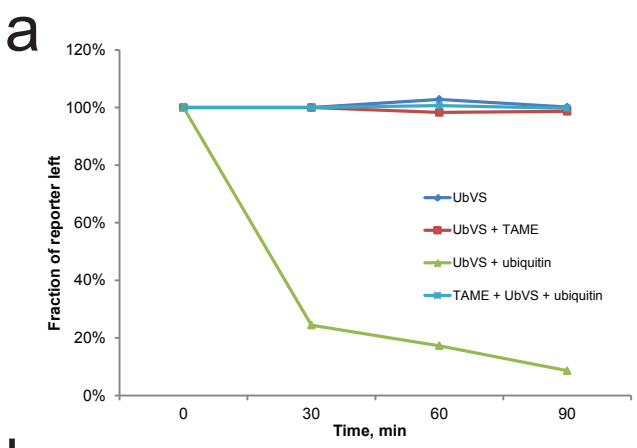

3500
3000
2500
2000
1500
1000
500
0
Velocity, arbitrary unit
-TAME , Km=357 nM
+TAME , Km=318 nM
k_{cat} +TAME/k_{cat} -TAME=45.0%
0
500
1000
1500
Substrate concentration, nM

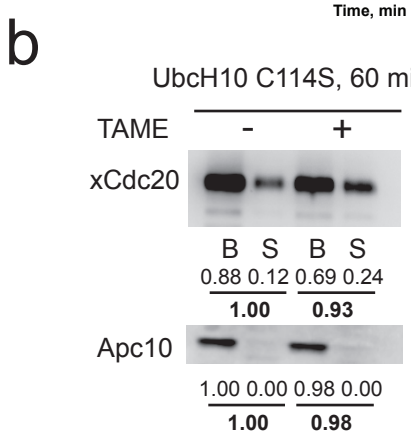
g

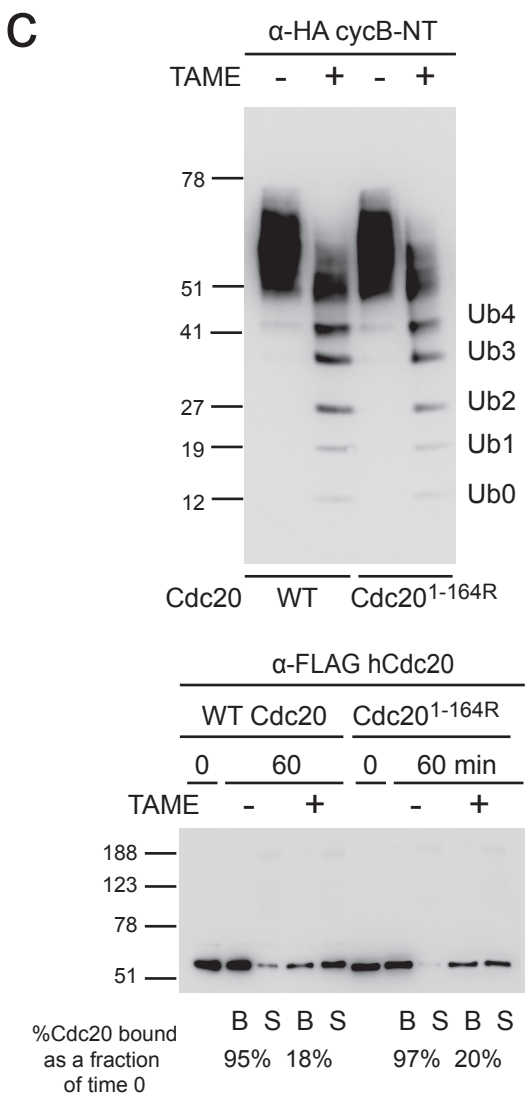

CycB-NT
IR peptide
- - + +
- + - +
α-FLAG
hCdc20
1.00 0.14 1.20 1.12
Cdc27

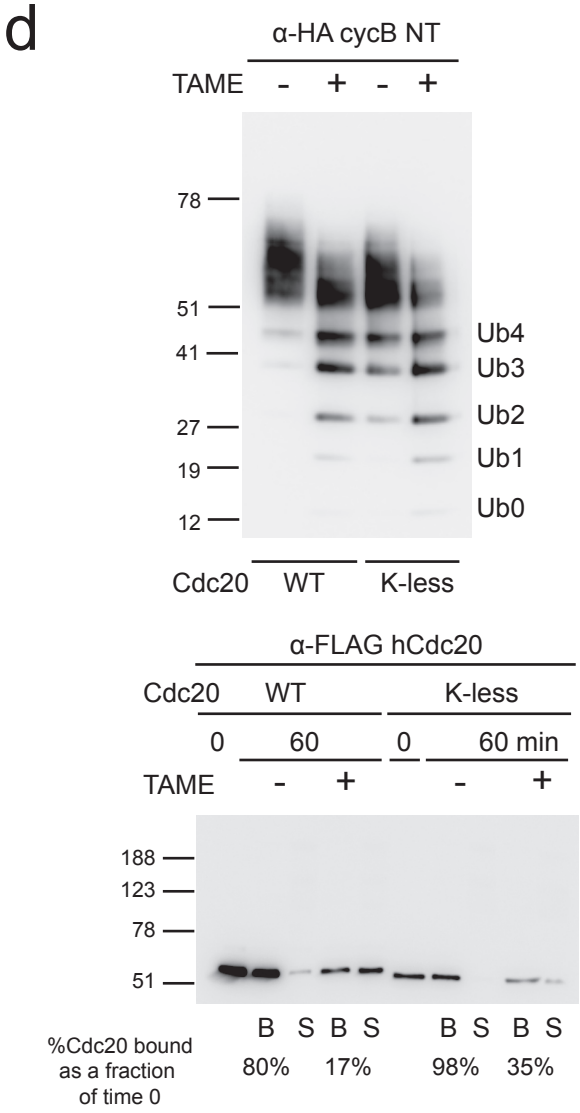


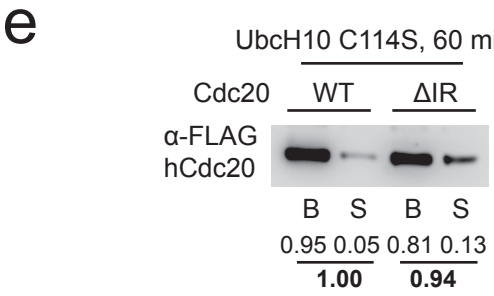
Supplementary Figure 5. (a) Characterization of the initial velocity of ubiquitination of cycB-NT by APC^{Cdc20}. A reconstituted ubiquitination assay was set up with a fixed cycB-NT concentration of 62.5 nM. The reaction was stopped at indicated time points and the products were detected by anti-HA blot. Band intensity of the mono-, di- and tri-ubiquitinated substrate was quantitated and plotted against reaction time. * An SDS-resistant aggregated form of substrate from the prep. (b) TAME reduces the k_{cat} but does not affect the K_m of APC^{Cdc20}. A reconstituted ubiquitination assay was performed with immuopurified APC, different concentrations of cycB-NT (from left to right: 62.5, 125, 250, 500 and 1000 nM) and methylated ubiquitin. The reaction was stopped at 30 seconds and the products were detected by anti-HA blot. Band intensity of the mono-, di- and tri-ubiquitinated substrate was quantitated and plotted against substrate concentration. Data were fit to a hyperbolic curve by nonlinear regression. (c) TAME reduces the k_{cat} but does not affect the K_m of APC^{Cdc20}. The same assay as in (b) was repeated witha a lower concentration of TAME (20 μM). (d) TAME has the same effect on the k_{cat} and the K_m of APC^{Cdc20} in a reaction with WT ubiquitin. The same experiment as in (b) was repeated with WT ubiquitin. (e) Pre-equilibration with cycB-NT maintains Cdc20 binding to the APC in the presence of TAME. APC^{Cdc20} (2.5 μl beads) was pre-washed with TAME-containing reaction buffer followed by the addition of ubiquitination reaction components with 1000 nM cycB-NT (20 μl). At 30s, the beads were separated from the supernatant and the distribution of Cdc20 was analyzed by Western blot. Alternatively, APC^{Cdc20} (2.5 μl) was first washed with TAME-containing buffer and pre-equilibrated with 125 nM cycB-NT (10 μl). The reaction was started by adding 10 μl 2x ubiquitination reaction components to achieve a final cycB-NT concentration of 62.5 nM. At 30s, the beads were separated from the supernatant and the distribution of Cdc20 was analyzed by Western blot. (f) TAME reduces the k_{cat} but does not affect the K_m of APC^{Cdc20} under the pre-equilibration condition. The same assay as in (b) was repeated with the pre-equilibration protocol as in (e). (g) A Cdc20 IR peptide inhibits the binding of free Cdc20 to the APC, which can be suppressed by cycB-NT. The same experiment in Fig. 4b was repeated with an IR peptide derived from *Xenopus* Cdc20. (h) The Cdc20 IR peptide reduces the k_{cat} but has little effect on the K_m of APC^{Cdc20}. The same experiment as in (b) was repeated with the Cdc20 IR peptide.

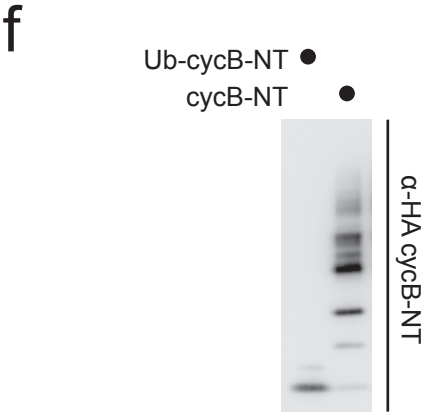

a
120%
100%
80%
60%
40%
20%
0%
Fraction of reporter left
0
30
60
90
Time, min
UbVS
UbVS + TAME
UbVS + ubiquitin
TAME + UbVS + ubiquitin


b
UbcH10 C114S, 60 min
TAME - +
xCdc20
B S B S
0.88 0.12 0.69 0.24
1.00 0.93
Apc10
1.00 0.00 0.98 0.00
1.00 0.98

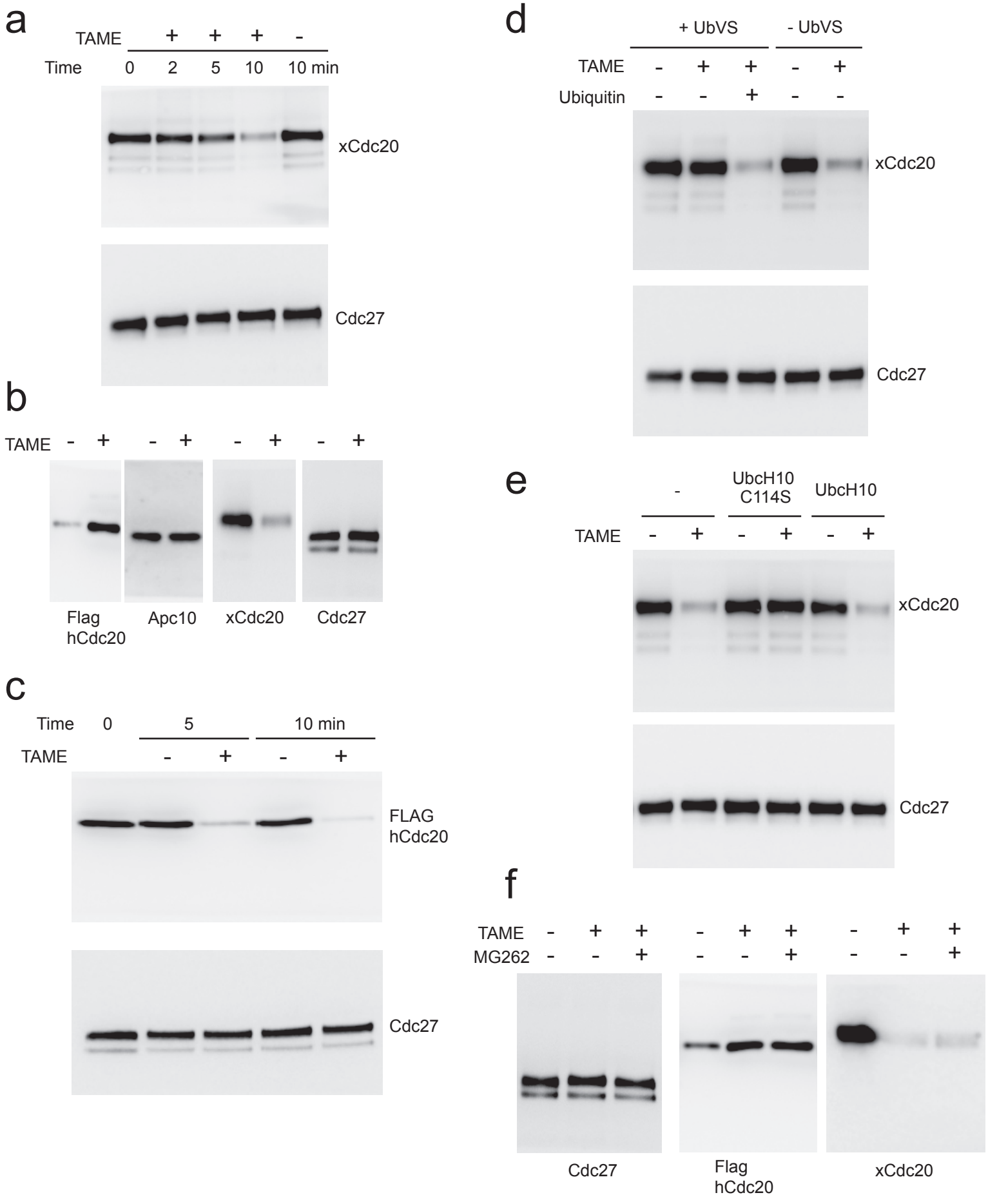

c
α-HA cycB-NT
TAME - + - +
78
51
41
27
19
12
Ub4
Ub3
Ub2
Ub1
Ub0
Cdc20 WT Cdc20 1-164R
α-FLAG hCdc20
WT Cdc20 Cdc20 1-164R
0 60 0 60 min
TAME - + - +
188
123
78
51
%Cdc20 bound as a fraction of time 0
B S B S B S B S
95% 18% 97% 20%


d
α-HA cycB NT
TAME - + - +
78
51
41
27
19
12
Ub4
Ub3
Ub2
Ub1
Ub0
Cdc20 WT K-less
α-FLAG hCdc20
Cdc20 WT K-less
0 60 0 60 min
TAME - + - +
188
123
78
51
%Cdc20 bound as a fraction of time 0
B S B S B S B S
80% 17% 98% 35%


e
UbcH10 C114S, 60 min
Cdc20 WT ΔIR
α-FLAG hCdc20
B S B S
0.95 0.05 0.81 0.13
1.00 0.94

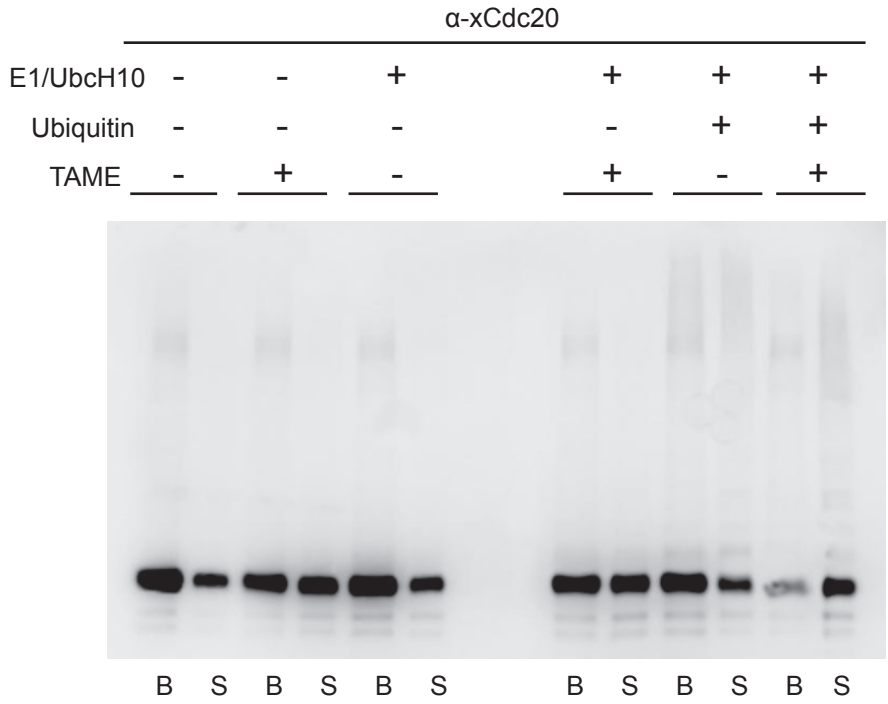

f
Ub-cycB-NT
cycB-NT
α-HA cycB-NT

Supplementary Figure 6. (a) TAME stabilizes the luciferase reporter in UbVS-treated extract. Mitotic extract was treated with 20 μM ubiquitin vinyl sulfone (UbVS) for 30 min (for the +TAME condition, the extract was pre-treated with 200 μM TAME for 10 min before UbVS). The luciferase reporter was added with or without 20 μM ubiquitin as indicated. (b) Substrate maintains Cdc20 binding to the APC in the presence of TAME, E1, UbcH10 C114S and ubiquitin. Immunoprecipitated APC^{Cdc20} was incubated with 500 nM cycB NT, 250 nM E1, 2 μM UbcH10 C114S, 150 μM ubiquitin and 200 μM TAME as indicated for 60 min. Cdc20 and Apc10 levels were analyzed separately in the bound (B) and the supernatant (S) fractions. (c) TAME reduces cycB-NT ubiquitination level in a reaction with $APC^{Cdc201\text{-}164R}$. The same assay as in Fig. 5b was run with APC loaded with WT Cdc20 or $Cdc20^{1\text{-}164R}$ for 60 min. (d) TAME reduces cycB-NT ubiquitination level in a reaction with $APC^{Cdc20K\text{-}less}$. The same assay as in Fig. 5b was run with APC loaded with WT Cdc20 or $Cdc20^{K\text{-}less}$ for 60 min. (e) Substrate maintains Cdc20ΔIR binding to the APC in the presence of E1, UbcH10 C114S and ubiquitin. APC loaded with WT Cdc20 or the ΔIR mutant was incubated with 500 nM cycB-NT, 250 nM E1, 2 μM UbcH10 C114S and 150 μM ubiquitin for 60 min. Cdc20 and Apc10 levels were analyzed separately in the bound (B) and the supernatant (S) fractions. (f) Unmodified and ubiquitinated cycB-NT used in the Cdc20 binding assay in Fig. 5e.

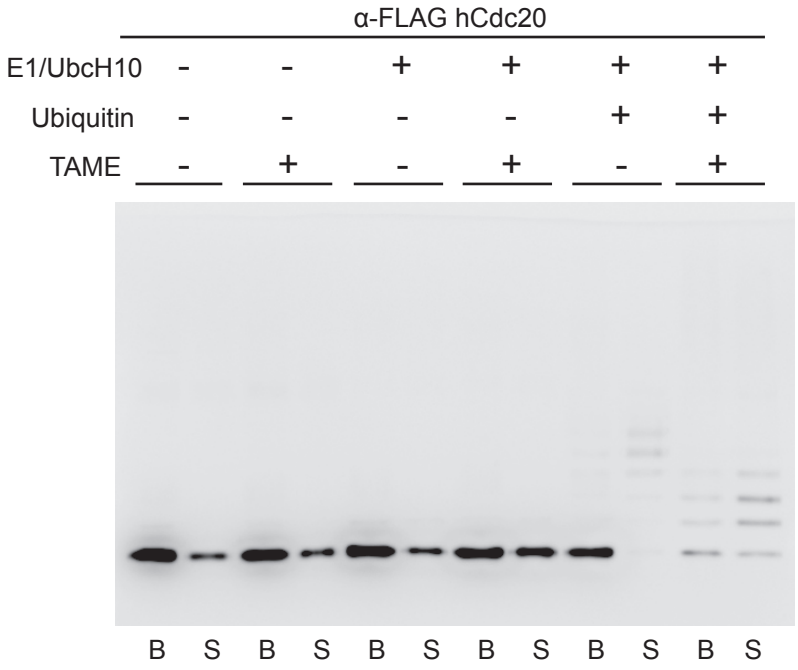


Supplementary Figure 7. Full gel images of Figure 1a to g.

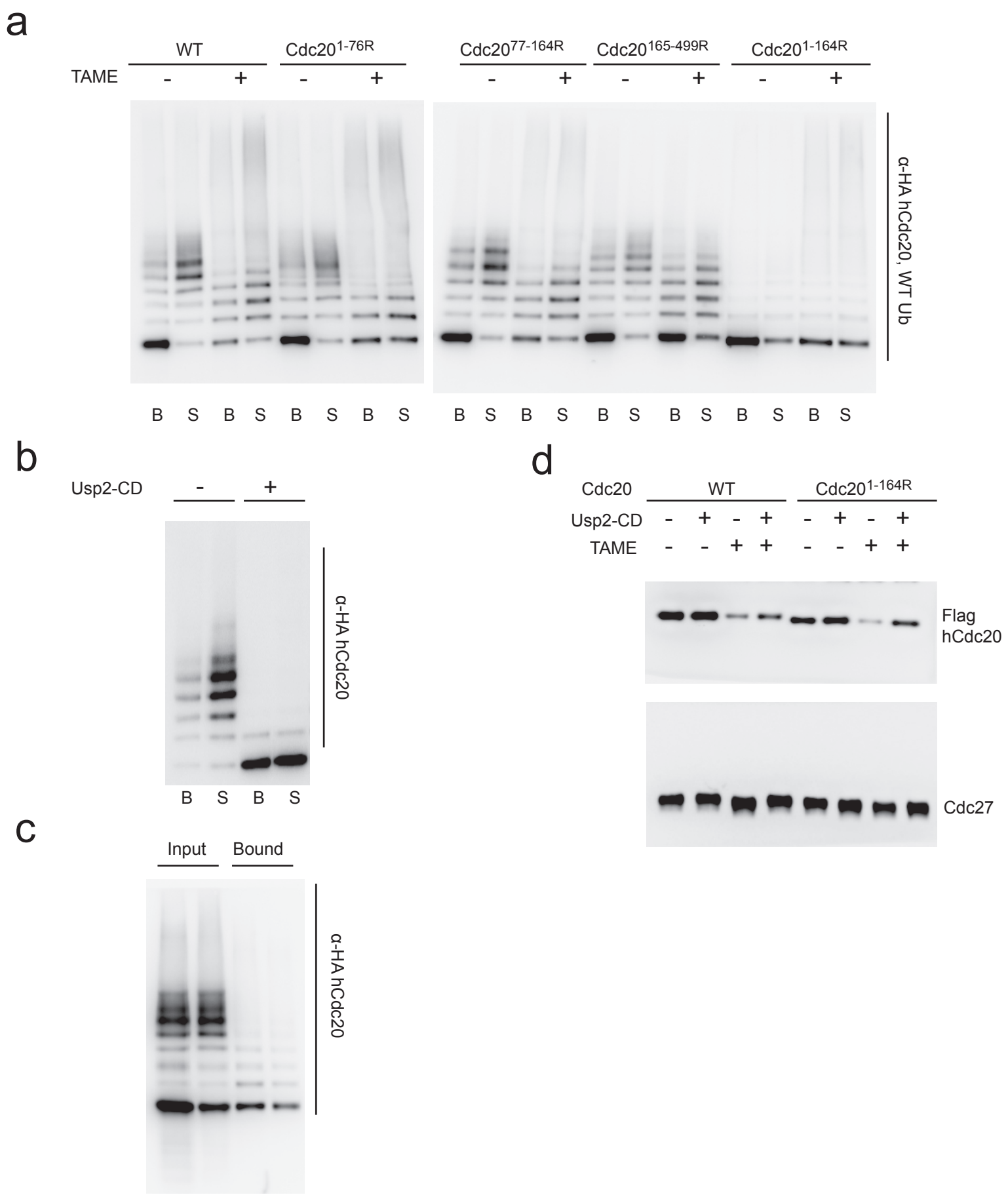
a



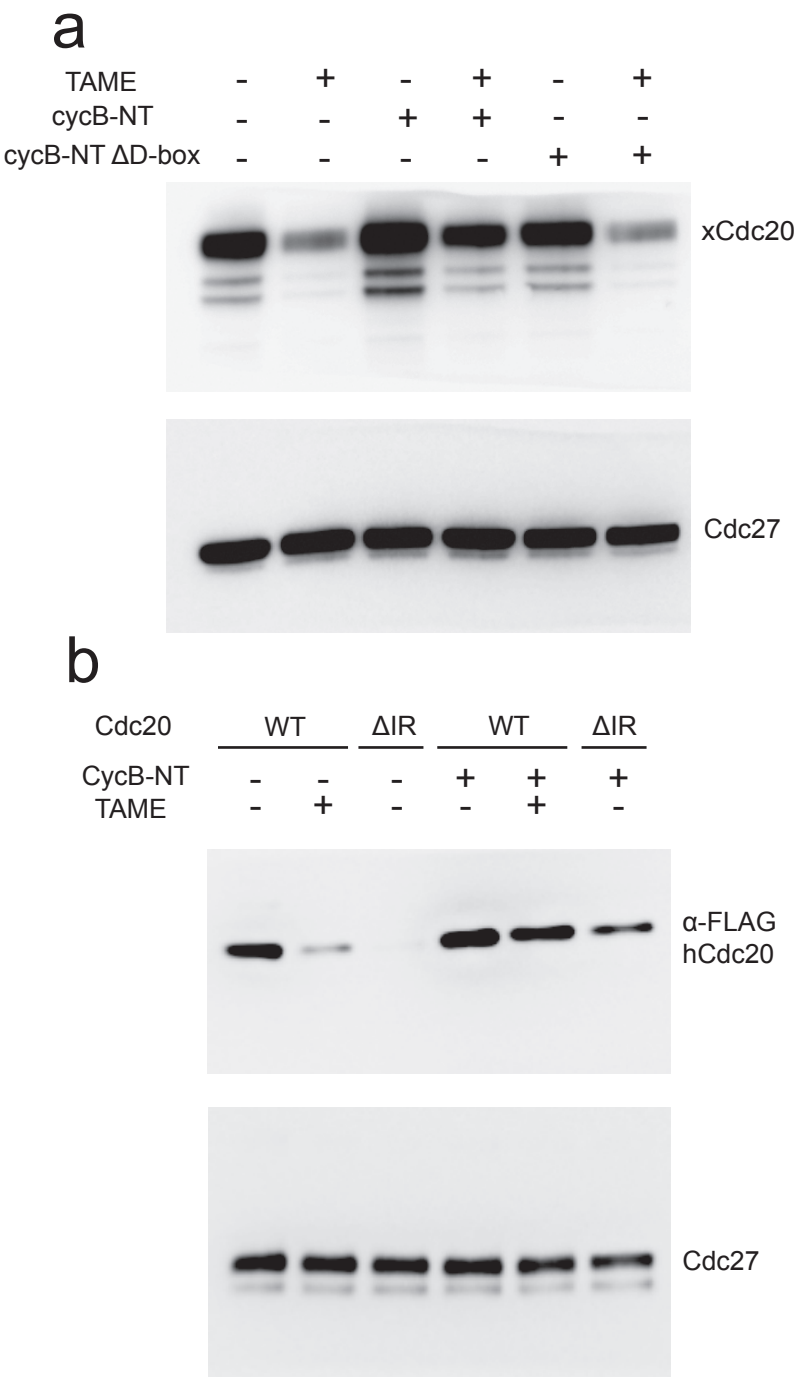
b



Supplementary Figure 8. Full gel images of Figure 2a to b.



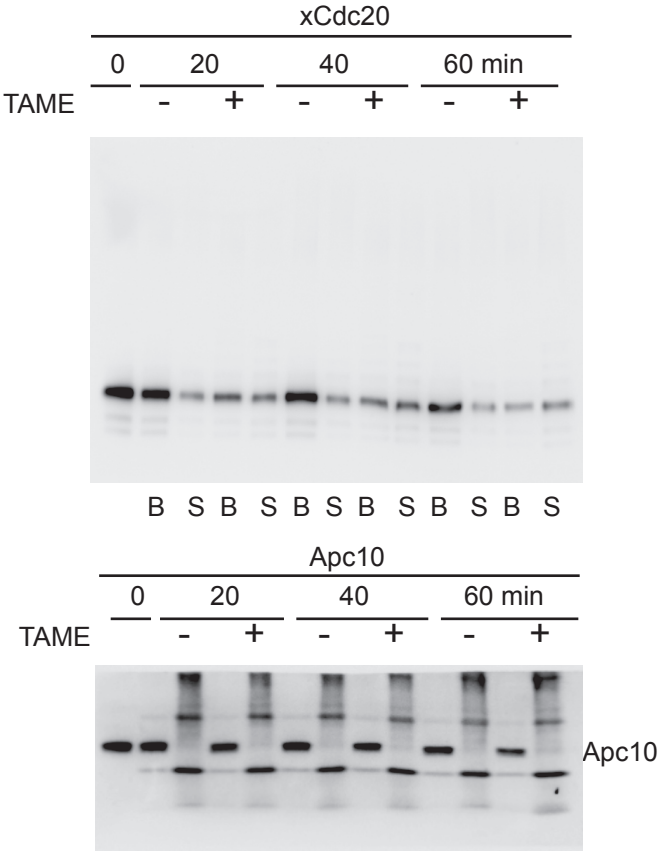
Supplementary Figure 9. Full gel images of Figure 3b to e.



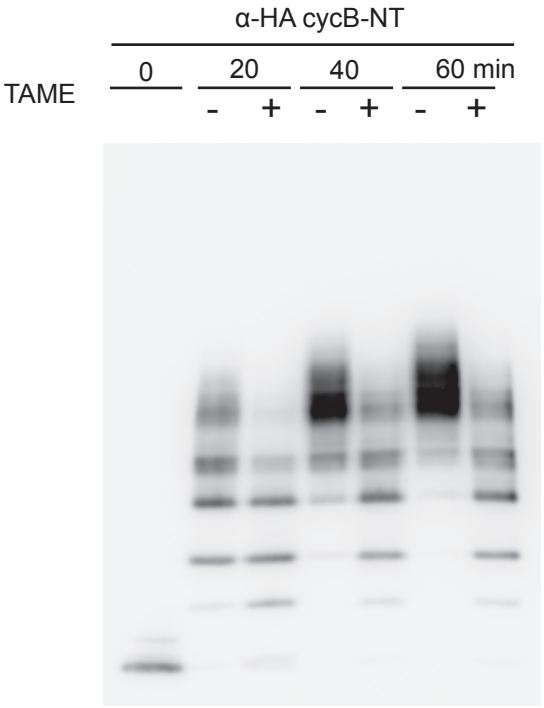
Supplementary Figure 10. Full gel images of Figure 4a to c.

a

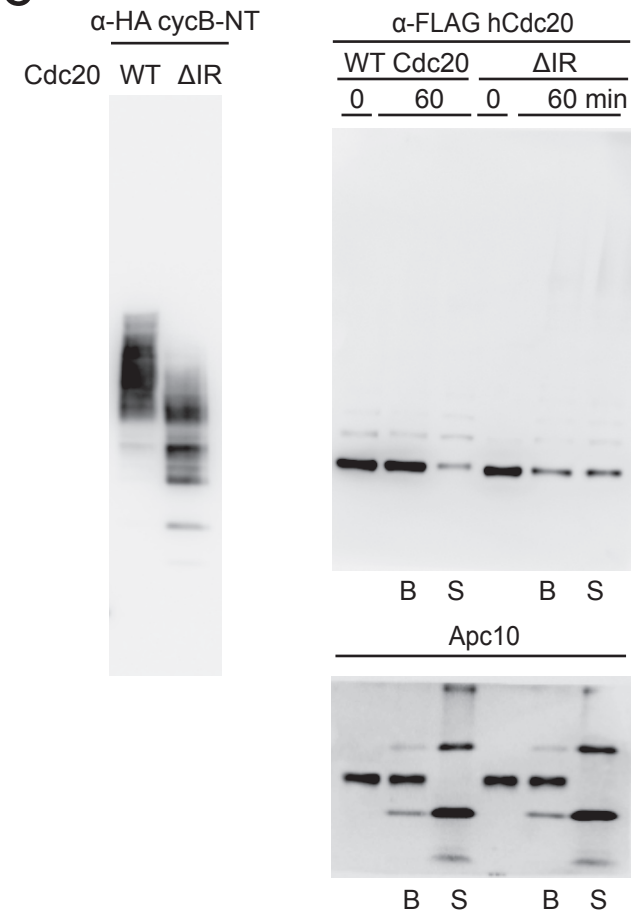

α-HA cycB-NT
No UbVS
UbVS + ubiquitin
TAME
- + - +

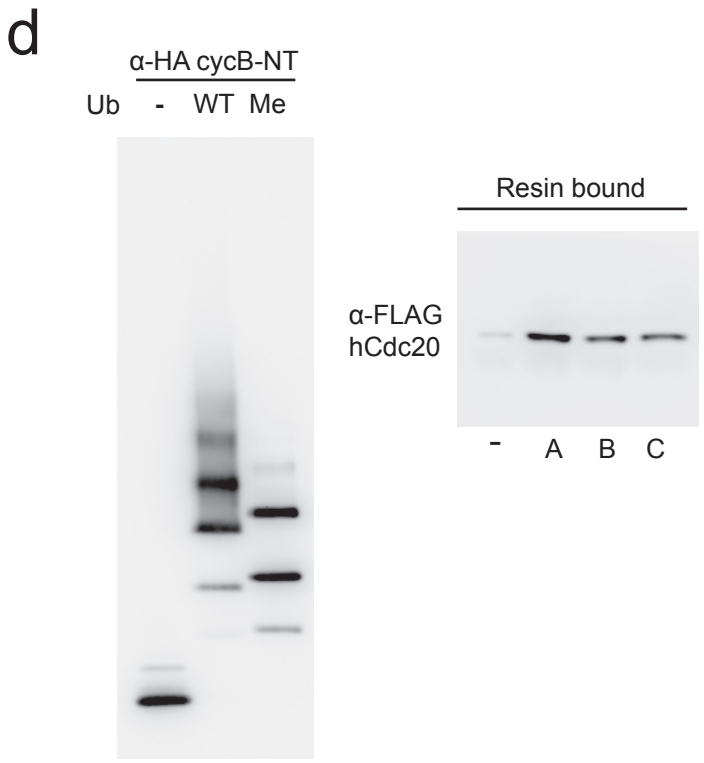

xCdc20
0 20 40 60 min
TAME
- + - + - +
B S B S B S B S B S B S
Apc10
0 20 40 60 min
TAME
- + - + - +
Apc10

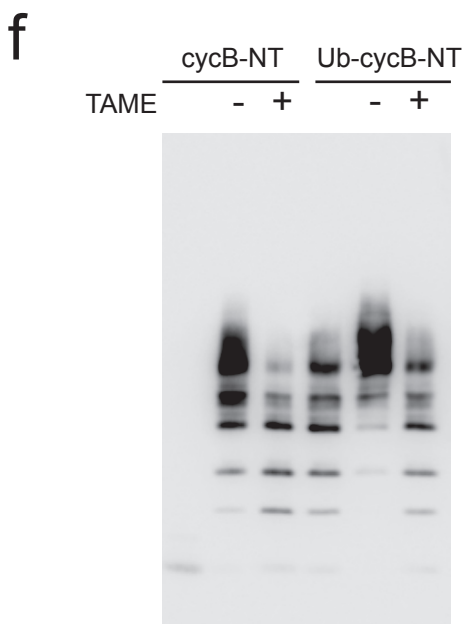
b

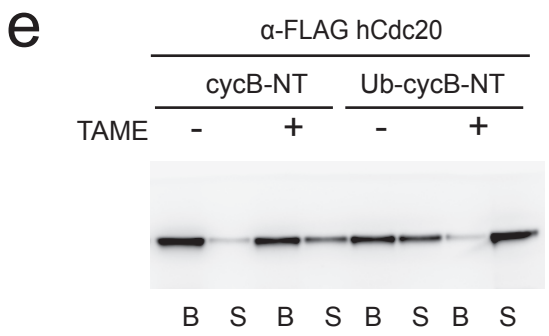

α-HA cycB-NT
0 20 40 60 min
TAME
- + - + - +

c


α-HA cycB-NT
Cdc20 WT ΔIR
α-FLAG hCdc20
WT Cdc20 ΔIR
0 60 0 60 min
B S B S
Apc10
B S B S

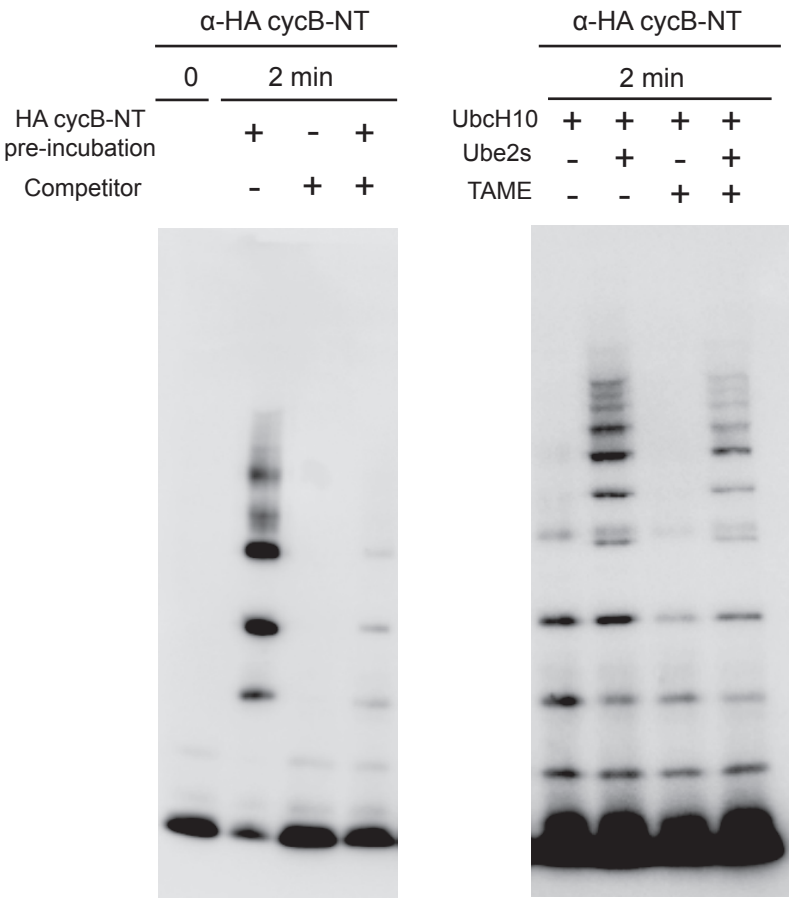




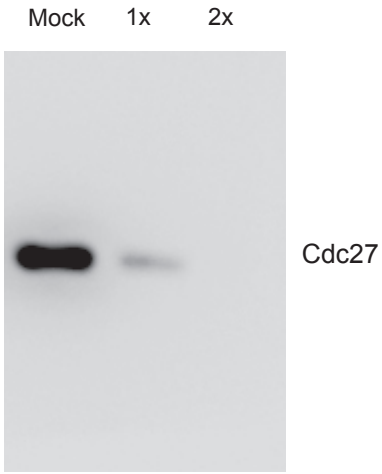


Supplementary Figure 11. Full gel images of Figure 5a to f.

a



b



Supplementary Figure 12. Full gel images of Figure 6a and c.