## SUPPLEMENTARY FIGURE LEGENDS

**Figure S1. Inhibition of Cdk1 arrests cells in G2 with disengaged centrioles**

**A**. Quantification of centriole disengagement in pre-synchronised HeLa, U2OS and RPE1-hTERT cells with and without RO-3306 treatment. **B**. Flow cytometry of HeLa, U2OS and RPE1-hTERT cells that were asynchronous (AS), treated with aphidicolin alone for 16 hours, or pre-synchronised as in the Materials and Methods and treated with RO-3306 for 16 hours. **C**. Quantification of centriole disengagement in HeLa cells pre-synchronised with aphidicolin then treated with roscovitine for 16 hours. **D**. Quantification of centriole disengagement in mock, GAPDH and Cdk1 depleted HeLa cells. **E** and **F**. Western blot analysis with antibodies indicated from mock, GAPDH and Cdk1 depleted HeLa cells. Data in A, C and D show means ± sd; n=3, >100 cells counted.

**Figure S2. Plk1 is required for centriole disengagement during G2 arrest**

**A**. Immunofluorescence microscopy of pre-synchronised HeLa cells treated with RO-3306 and BI 2536 for 16 hours, and stained for γ-tubulin (red) and centrin (green). **B.** Immunofluorescence microscopy of HeLa cells transfected with myc-Plk1-T210D or myc-Plk1-K82R and treated with RO-3306 and MLN 8054 for 16 hours following pre-synchronisation. Cells were stained with antibodies against myc (green) and C-Nap1 (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.

**Figure S3. Plk1 can promote APC/C-independent centriole disengagement in G2 arrested cells**

**A**. HeLa cells were mock, GAPDH or securin depleted by RNAi whilst also, where indicated, transfected with myc-separase. After 12 hours, cells were synchronised, released and treated with RO-3306 alone or plus BI 2536. Cell lysates were prepared and Western blotted for securin, myc and α-tubulin. **B**. HeLa cells were mock-depleted or depleted of GAPDH or separase by RNAi and transfected with myc-separase-WT or RNAi-resistant separase (myc-separase-Res) as indicated. After 12 hours, cells were pre-synchronised with aphidicolin and then treated with RO-3306 for 16 hours. Cell lysates were prepared and Western blotted for myc, separase, GAPDH and α-tubulin. An N-terminal autocatalytic cleavage product of separase (<),

seen below the full-length myc-tagged separase (*), indicates activity of this construct in RO-3306 treated cells. **C**. HeLa cells were mock-depleted cells or cells depleted of Cdc20 or separase and transfected as indicated. After 12 hours, cells were synchronized and released without RO-3306 for 6 hours, or with RO-3306 for 16 hours. Cell lysates were prepared and Western blotted for Cdc20, separase, Plk1 and α-tubulin. **D**. HeLa cells were mock-depleted cells or cells depleted of Cdh1 or APC3 and transfected as indicated. After 12 hours, cells were synchronized and released without RO-3306 for 6 hours, or with RO-3306 for 16 hours. Cell lysates were prepared and Western blotted for APC3, Cdh1, Plk1 and α-tubulin.

**Figure S4. Disengagement in response to RO-3306 is not a consequence of DNA damage**

**A**. HeLa cells were synchronised by 16 hour aphidicolin treatment and 4 hour release then either left untreated or irradiated (5 Gy). Non-irradiated cells were fixed immediately, whilst irradiated samples were fixed 20 hours after IR treatment. Cells were stained for γH2AX. Scale bar, 10 μm. **B**. Immunofluorescence microscopy of HeLa cells treated with aphidicolin for 16 hours, released for 4 hours, then treated with RO-3306 for 16 hours, or treated with RO-3306 only for 16 hours. Cells were stained for γ-tubulin (green) and γH2AX (red). Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 μm. **C**. Quantification of γH2AX foci staining in HeLa cells following treatment as in B. **D**. Comparison of centriole disengagement in HeLa cells treated with RO-3306 for 16 hours or pre-synchronised with aphidicolin then treated with RO-3306 in those cells with or without γH2AX foci. Data in C and D are shown as mean +/- sd for three independent experiments in which at least 100 cells were counted.

**Figure S5. Depletion of proteins in IR treated samples**

HeLa cells were mock, GAPDH, APC3, Cdc20, Cdh1, Separase or Emi1 depleted by RNAi whilst also, where indicated, transfected with myc-Plk1-T210D. After 12 hours, cells were synchronised, released for 4 hours and then irradiated. Cell lysates were prepared 20 hours post-IR and Western blotted for the proteins indicated.

**Figure S6. Mitotic consequences for cells released from RO-3306 arrest**

**A**. Immunofluorescence microscopy of metaphase cells following 1 hour washout from 16 hours RO-3306 treatment. Upper panels show DNA stained with Hoechst (blue in merge). Merge panels include γ-tubulin (green) and α-tubulin (red) antibody staining. **B**. Immunofluorescence microscopy of anaphase cells following 2 hour washout from 16 hours RO-3306 treatment. Images show DNA stained with DAPI. Merge panels include α-tubulin (red) antibody staining. **C**. Immunofluorescence microscopy of interphase cells following 20 hour washout from 16 hours RO-3306 treatment. Images show DNA stained with Hoechst. Merge panels include α-tubulin (red) antibody staining. Scale bars, 10 μm.

**Movie S1.** HeLa:α-tubulin-GFP cells were treated with aphidicolin for 16 hours before release into fresh media. After 4 hours, imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell undergoing a bipolar division.

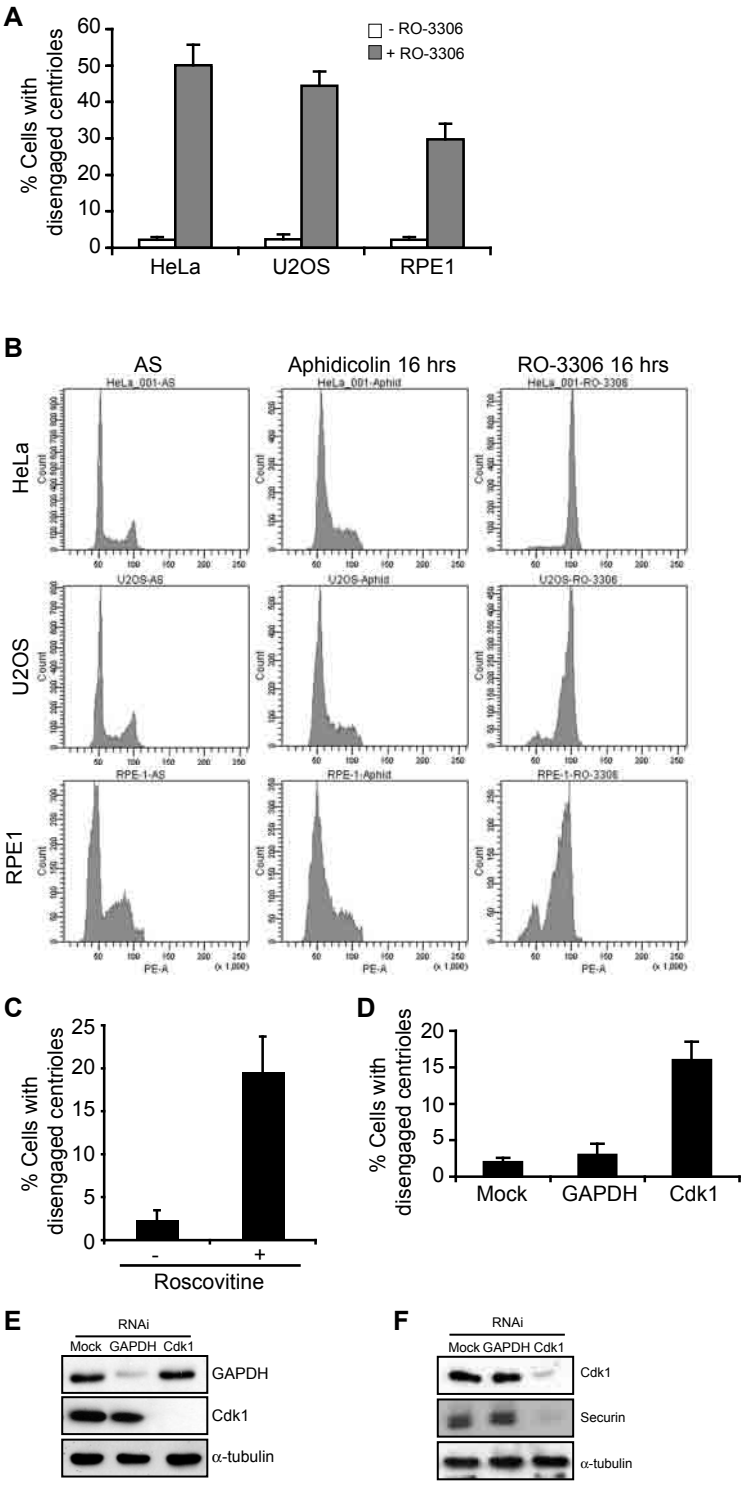
**Movie S2.** HeLa:α-tubulin-GFP cells were treated with aphidicolin for 16 hours before release into fresh media. After 4 hours, imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell undergoing a bipolar division.

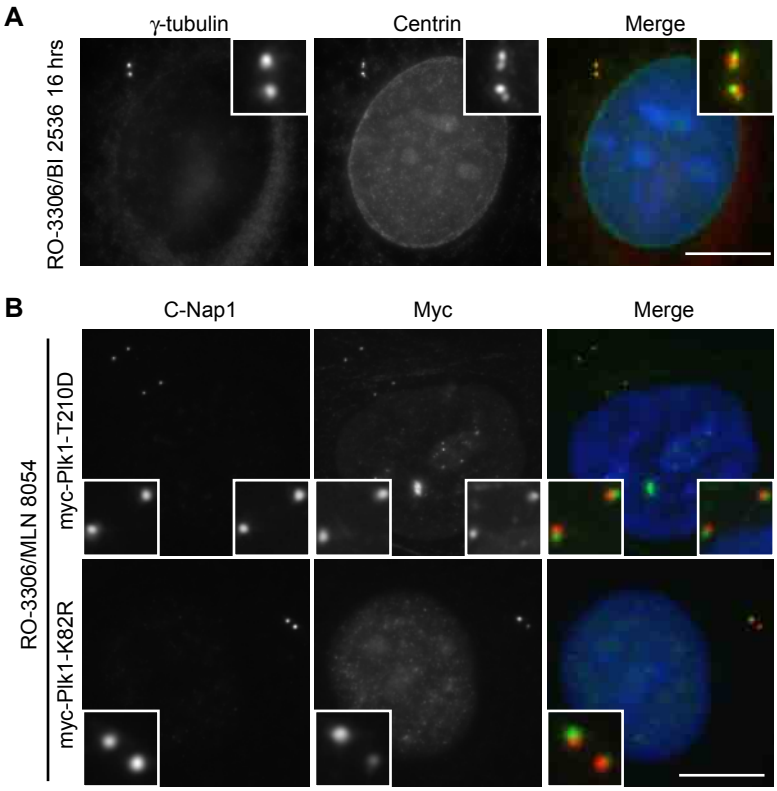
**Movie S3.** HeLa:α-tubulin-GFP cells were pre-synchronised and treated with RO-3306 for 16 hours. RO-3306 was washed out and imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell that initially forms a bipolar spindle, which then rapidly becomes tripolar, then tetrapolar. Eventually the two 'spare' poles move to one end of the cell as it undergoes a bipolar division.

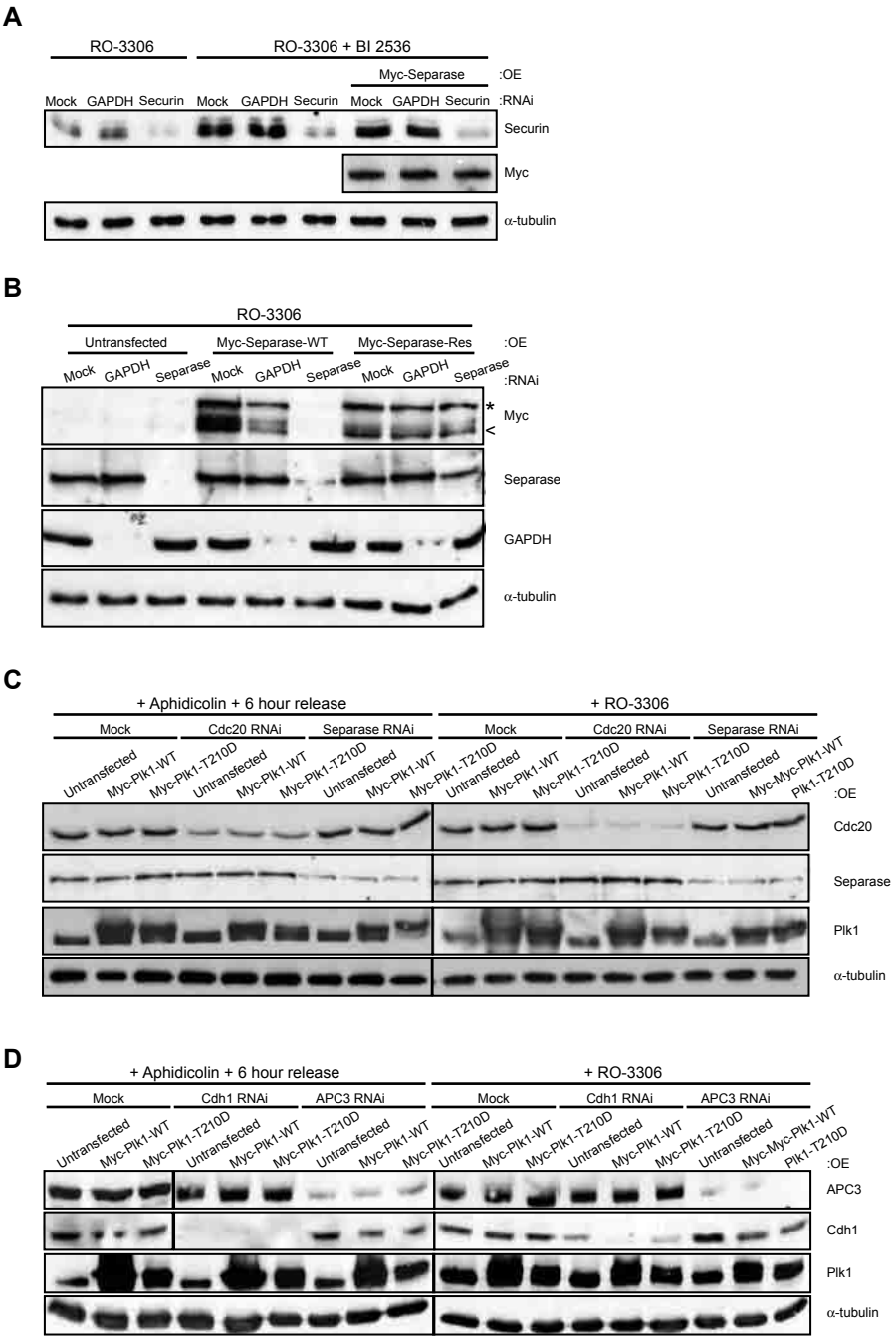
**Movie S4.** HeLa:α-tubulin-GFP cells were pre-synchronised and treated with RO-3306 for 16 hours. RO-3306 was washed out and imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell that initially looks like it will have two spindle poles but forms a tripolar spindle, which becomes tetrapolar, before the cell undergoes a tripolar division.

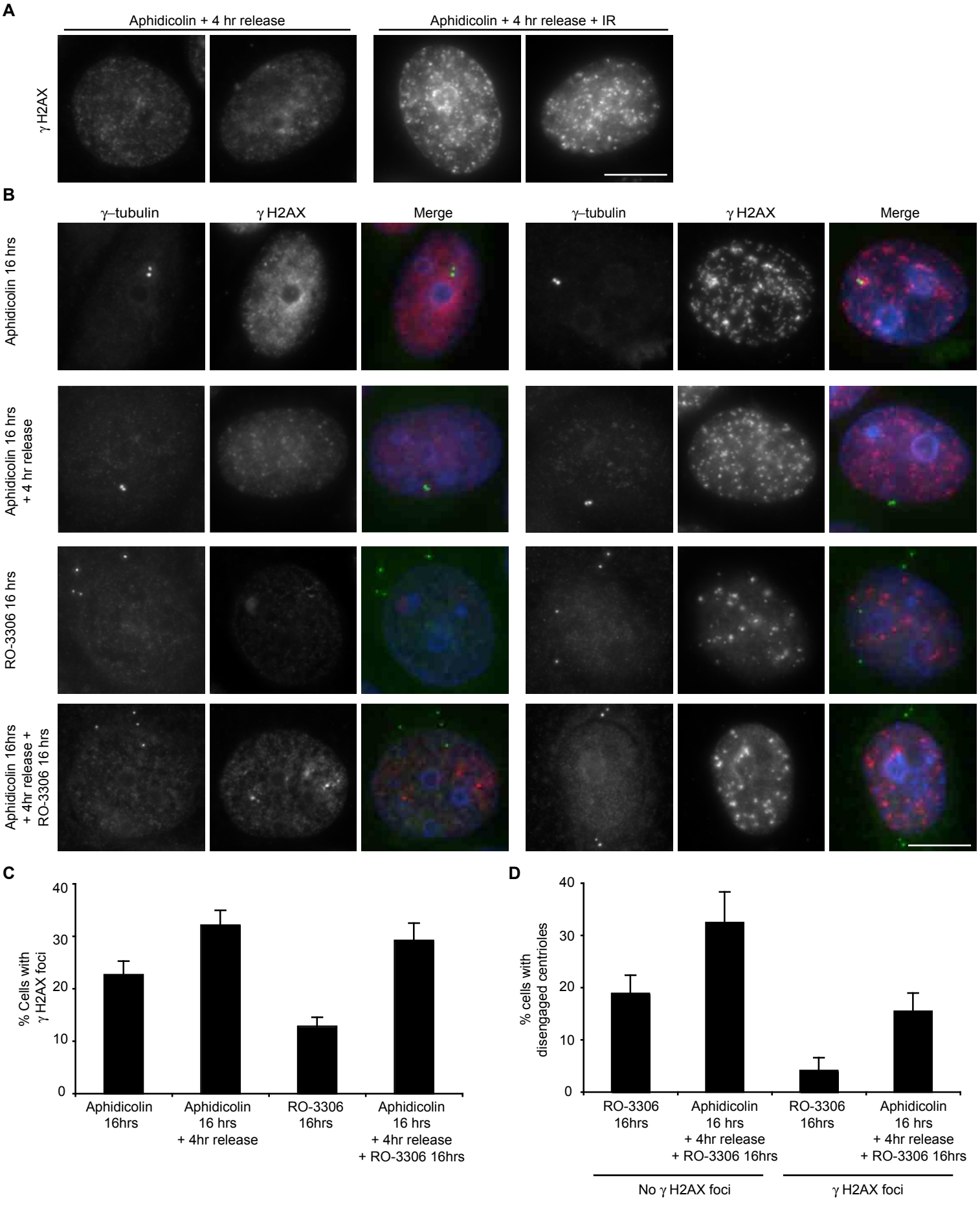
**Movie S5.** HeLa:α-tubulin-GFP cells were pre-synchronised and treated with RO-3306 for 40 hours. RO-3306 was washed out and imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell initially forming a tetrapolar spindle, but later undergoing a tripolar division.

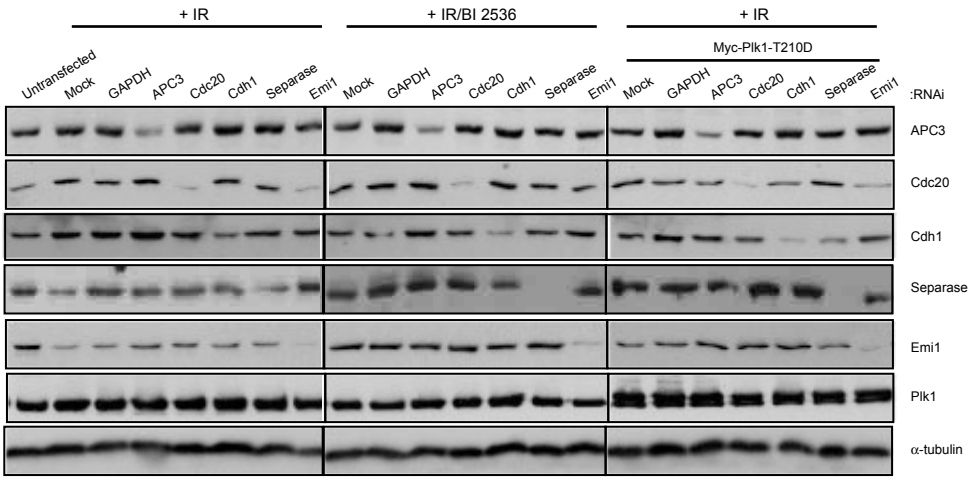
**Movie S6.** HeLa:α-tubulin-GFP cells were pre-synchronised and treated with RO-3306 for 40 hours. RO-3306 was washed out and imaging commenced with a z-stack acquired every 5 minutes. Movie is a maximum intensity projection of a cell initially forming a tetrapolar spindle, but later undergoing a tripolar division.

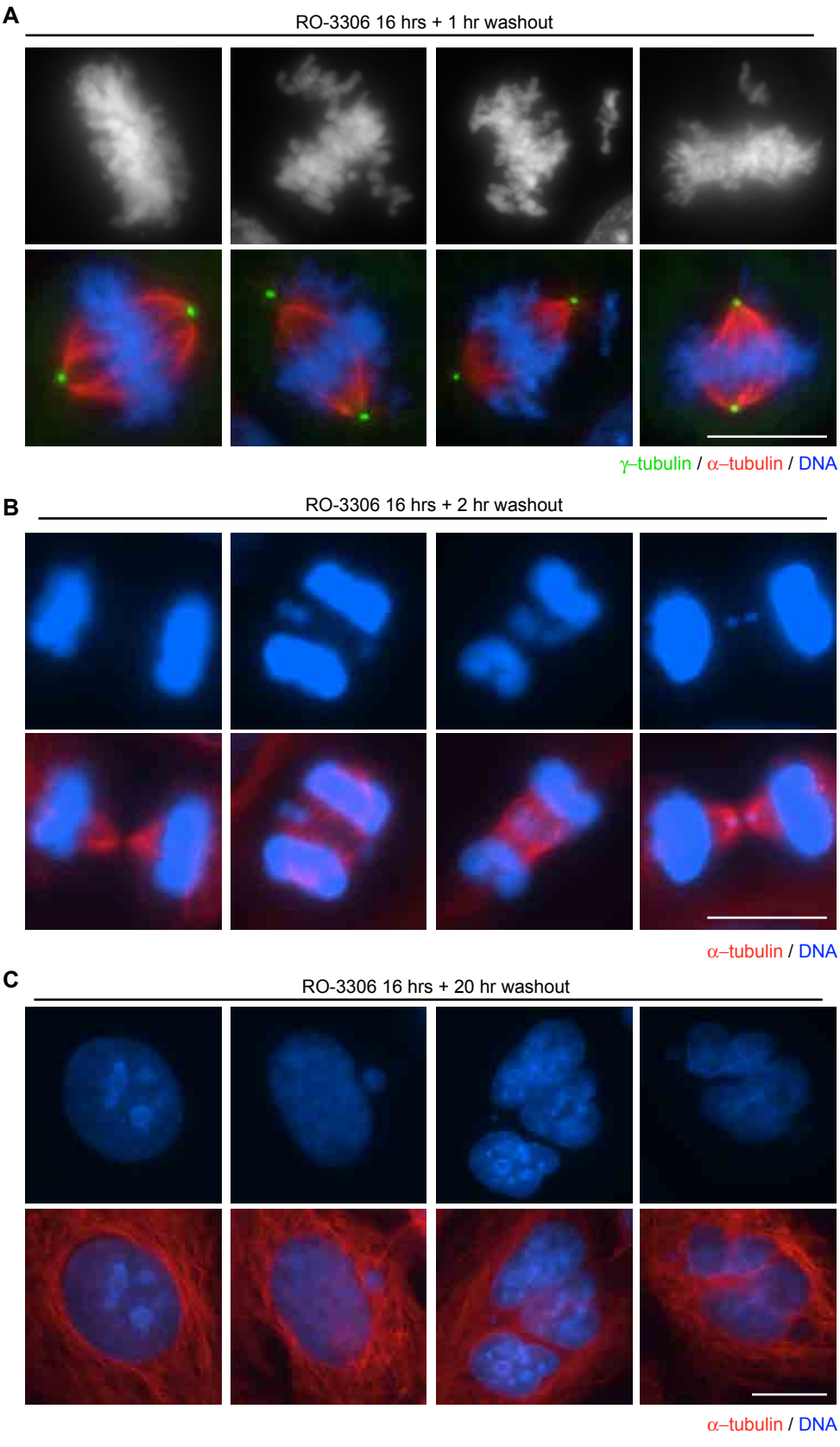

A
% Cells with disengaged centrioles
60
50
40
30
20
10
0
- RO-3306
+ RO-3306
HeLa
U2OS
RPE1
B
AS
Aphidicolin 16 hrs
RO-3306 16 hrs
HeLa
U2OS
RPE1
HeLa_001-AS
HeLa_001-Aphid
HeLa_001-RO-3306
U2OS-AS
U2OS-Aphid
U2OS-RO-3306
RPE-1-AS
RPE-1-Aphid
RPE-1-RO-3306
Count
PE-A
C
% Cells with disengaged centrioles
25
20
15
10
5
0
-
+
Roscovitine
D
% Cells with disengaged centrioles
20
15
10
5
0
Mock
GAPDH
Cdk1
E
RNAi
Mock GAPDH Cdk1
GAPDH
Cdk1
α-tubulin
F
RNAi
Mock GAPDH Cdk1
Cdk1
Securin
α-tubulin


A
γ-tubulin
Centrin
Merge
RO-3306/BI 2536 16 hrs
B
C-Nap1
Myc
Merge
RO-3306/MLN 8054
myc-Plk1-T210D
myc-Plk1-K82R


A
RO-3306
RO-3306 + BI 2536
Myc-Separase
:OE
Mock GAPDH Securin Mock GAPDH Securin Mock GAPDH Securin
:RNAi
Securin
Myc
α-tubulin
B
RO-3306
Untransfected
Myc-Separase-WT
Myc-Separase-Res
:OE
Mock GAPDH Separase Mock GAPDH Separase Mock GAPDH Separase
:RNAi
Myc
Separase
GAPDH
α-tubulin
C
+ Aphidicolin + 6 hour release
+ RO-3306
Mock
Cdc20 RNAi
Separase RNAi
Untransfected
Myc-Plk1-WT
Myc-Plk1-T210D
Myc-Myc-Plk1-WT
:OE
Cdc20
Separase
Plk1
α-tubulin
D
+ Aphidicolin + 6 hour release
+ RO-3306
Mock
Cdh1 RNAi
APC3 RNAi
Untransfected
Myc-Plk1-WT
Myc-Plk1-T210D
Myc-Myc-Plk1-WT
:OE
APC3
Cdh1
Plk1
α-tubulin


A
Aphidicolin + 4 hr release
Aphidicolin + 4 hr release + IR
γ H2AX
B
γ–tubulin
γ H2AX
Merge
γ–tubulin
γ H2AX
Merge
Aphidicolin 16 hrs
Aphidicolin 16 hrs + 4 hr release
RO-3306 16 hrs
Aphidicolin 16hrs + 4hr release + RO-3306 16 hrs
C
% Cells with γ H2AX foci
0
10
20
30
40
Aphidicolin 16hrs
Aphidicolin 16 hrs + 4hr release
RO-3306 16hrs
Aphidicolin 16 hrs + 4hr release + RO-3306 16hrs
D
% cells with disengaged centrioles
0
10
20
30
40
RO-3306 16hrs
Aphidicolin 16 hrs + 4hr release + RO-3306 16hrs
RO-3306 16hrs
Aphidicolin 16 hrs + 4hr release + RO-3306 16hrs
No γ H2AX foci
γ H2AX foci


+ IR
+ IR/BI 2536
+ IR
Myc-Plk1-T210D
Untransfected
Mock
GAPDH
APC3
Cdc20
Cdh1
Separase
Emi1
:RNAi
APC3
Cdc20
Cdh1
Separase
Emi1
Plk1
α-tubulin


A
RO-3306 16 hrs + 1 hr washout
γ–tubulin / α–tubulin / DNA
B
RO-3306 16 hrs + 2 hr washout
α–tubulin / DNA
C
RO-3306 16 hrs + 20 hr washout
α–tubulin / DNA