Assessment of apoptosis and cell death

Apoptosis was monitored by annexin V-FITC staining and flow cytometry. Primary MM cell viability was determined by trypan blue exclusion. MM cell death was also monitored by MTT assay or by 7-AAD staining (0.5 μg/mL at 37°C for 30 min). To confirm apoptosis, MM cells were examined by TdT-mediated dUTP nick end labeling (TUNEL) staining using In Situ Cell Death Detective Kits (fluorescein; Roche, Penzberg, Germany) or IFC staining for caspase-3 activation using anti-cleaved (active) caspase-3 antibodies (Alexa Fluor® 488-conjugated antibody, Cell Signaling, CA) under fluorescence microscopy or flow cytometry as per the manufacturer's instructions.

RNA interference and stable transfection

RPMI8226 and U266 cells were transfected with RNAi oligonucleotides corresponding to Bim or Chk1. pSR-Bim and pSR-control constructs, encoding short hairpin RNA (shRNA) for Bim or scrambled sequence, were obtained as reported previously.[20] Constructs encoding Chk1 or non-targeting control shRNA were purchased from Upstate Biotechnology. Transfections were performed utilizing an Amaxa Nucleofector (Amaxa, Cologne, Germany) as per manufacturer's instructions. Clones were selected by puromycin (2 μg/mL for shBim) or by G418 (400 nM for shChk1).

Enrichment of G_0/G_1 MM cells by sorting smaller side-population (SSP) cells

To enrich G_0/G_1 cells in MM under regular culture conditions, viable smaller side population (SSP) cells were sorted by exclusion of Hoechst33342 staining of MM cells (H929 and RPMI8226) as described by Goodell et al (*Methods Mol Biol* 290:343–352, 2005). In brief, cells (1×10^6/ml) were incubated with Hoechst33342 (10 μg/ml) in DMEM buffer (with 10 mM Hepes and 2% FBS) for 60 min at 37°C, or cells were co-incubated with 50 μM verapamil, which blocks Hoechst efflux, as a control. The cells were then centrifuged and re-suspended in cold Hanks's balanced salt solution (HBSS) with 2 μg/ml propidium iodide (PI) to exclude dead cells. The sample was then put on ice before sorting. Gates were set for both the side population (SP) and low FSC population after gating out PI stained cells on a flow cytometer (BD FACSAria II Cell Sorter). Hoechst dye was excited with a UV laser at 350 nm and the signal measured with Hoechst blue and Hoechst red filters. These small SP cells were sorted by the cell sorter and collected in RPMI1640 medium (5% FBS for H929, and 3% FBS for RPMI8226 respectively) to retain the SP cells in G_0/G_1 during the treatment interval. The cell cycle profile was determined by PI staining immediately after sorting. Sorted cells then were treated with the AZD6244/AZD7762 regimen for 14 hr and stained with activated caspase-3 plus PI to determine the percentage of apoptotic cells within the G_0/G_1 phase population. In addition, the death of SP cells was also evaluated after treatment by trypan blue staining and microscopic observation.

Figure S1. Novel Chk1 and MEK1/2 inhibitors synergistically induce apoptosis in IL-6–dependent and –independent MM cells

(A) IL-6–dependent ANBL6 MM cells were exposed to 2.5 μM AZD6244 or 2.5 μM PD184352 (24 hr) followed by 50 nM AZD7762 (+ 48 hr), after which cells were subjected to Western blot analysis to monitor ERK1/2 phosphorylation, caspase-3 cleavage and PARP degradation. Each lane was loaded with 20 μg of protein; blots were stripped and re-probed with anti-tubulin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results. (B) IL-6–dependent KAS-6/1 MM cells were exposed to 2.5 μM AZD6244 or 2.5 μM PD184352 (24 hr) followed by 50 nM AZD7762 (+ 48 hr), after which the percentage of cell death was monitored by 7AAD staining and flow cytometry (* $P < 0.01$ and ** $P < 0.001$ vs. single agent treatment). (C–D) IL-6–independent 8226 (C) and U266 (D) MM cells were exposed to 5 μM AZD6244 (24 hr) followed by 300 nM AZD7762 (+ 48 hr), after which cell death was monitored by 7AAD staining and flow cytometry **(**** $P < 0.001$ vs single agent treatment). For panels B–D, values represent the means ± S.D. for three separate experiments performed in triplicate.

Figure S2. G_0G_1-enriched MM cells display increased susceptibility to Chk1 inhibitors

(A) H929 MM cells were cultured in 0.1% or 10% FBS-containing medium for the indicated intervals, after which cell proliferation was monitored by cell counts using a Coulter Counter. (B) After cultured in 0.1% or 10% FBS medium for 16 hr, H929 cells were exposed (6 hr) to 150 nM UCN-01, 300 nM AZD7762, or 5 nM taxol and then subjected to Western blot analysis to monitor cleavage of caspase-3 and PARP. (C) H929 cells were incubated in 0.1% vs 10% FBS for 48 hr, after which they were exposed to 300 nM AZD7762 or 150 nM UCN-01 for 24 hr and subjected to cell cycle analysis. Arrows indicate apoptotic (sub-G_1) cells. (D) U226 cells stably transfected with Chk1 shRNA (clone C4 and E7) and scrambled sequence shRNA were cultured in medium containing 0.05% or 10% FBS for 48 hr, after which Western blot analysis was performed to monitor cleavage of caspase-3 and PARP. For (B) and (D), each lane was loaded with 20 μg of protein; blots were stripped and re-probed with anti-tubulin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results.

Figure S3. Chk1/MEK1/2 inhibition induces caspase-3 activation in statin$^+$ (G_0G_1) H929 and primary CD138$^+$ MM cells

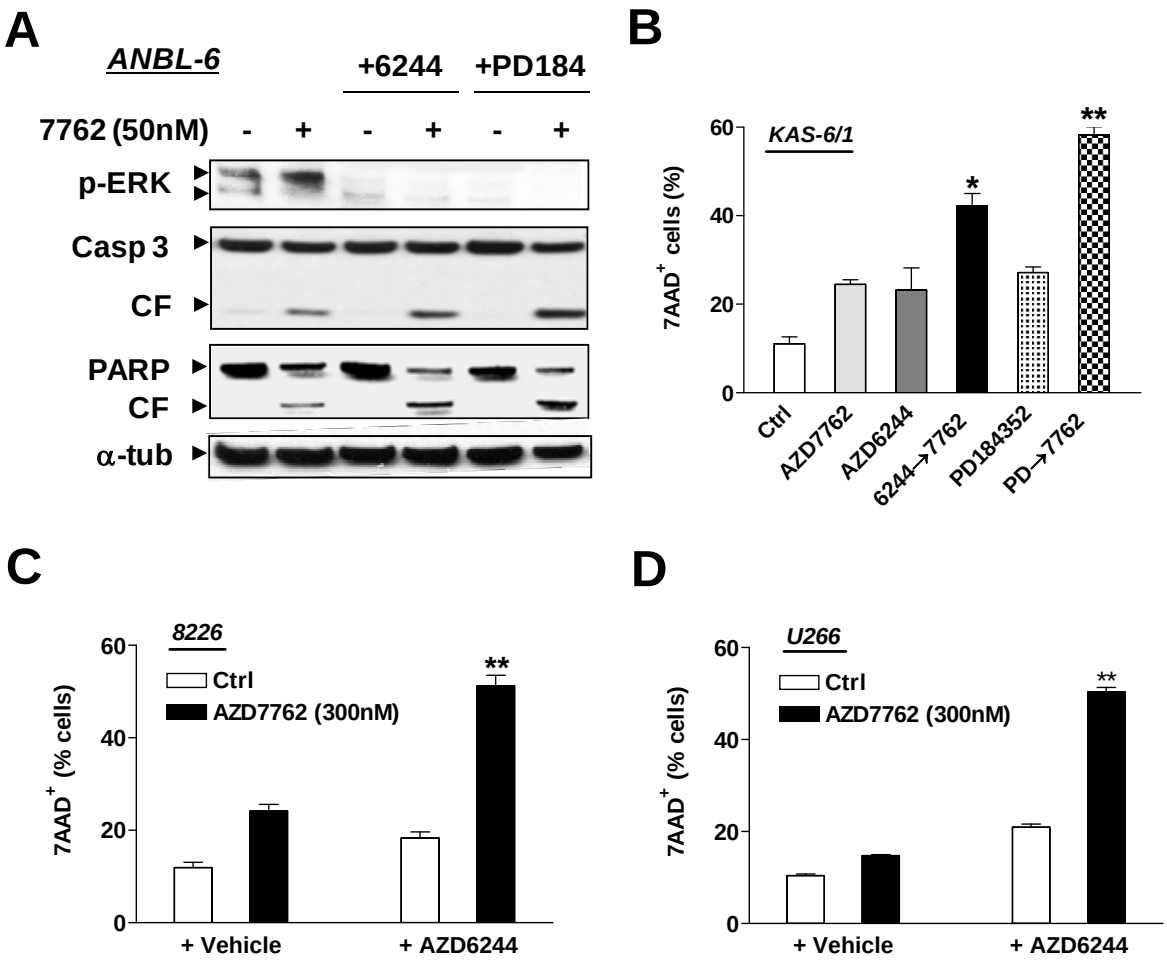
(A) H929 MM cells were cultured in either 0.1% or 10% FBS medium for 90 hr, after which Western blot analysis was performed to monitor expression of statin, a marker for quiescent (G_0) cells. Each lane was loaded with 20 μg of protein; blots were stripped and re-probed with anti-actin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results. (B–C) These cells (B) and primary CD138$^+$ MM cells (C) were immunofluorescently stained with antibodies against statin followed by AF 599-conjugated secondary antibody, and counterstained with DAPI. Mouse IgG was used to replace primary antibody as a negative control to confirm the specificity of staining. Images were captured at 60×/1.40 under oil and merged as indicated. Red fluorescence for statin is localized on the nuclear membrane. (D) G_0G_1-enriched H929 cells were treated with 5 μM PD184352 (24 hr) and 300 nM CEP3891 (+ 18 hr). (E) Primary CD138$^+$ MM cells were exposed to 5 μM PD184352 and 500 nM CEP3891 for 24hr. For panels (D) and (E), after treatment, cells were stained with antibodies against statin (red fluorescence) and cleaved caspase-3 (green fluorescence), and counterstained with DAPI (blue fluorescence). Images were captured at 60×/1.40 under oil and merged as indicated.

Arrows indicate cells exhibiting co-localization of statin and cleaved caspase-3. Results are representative of 3 separate experiments.

Figure S4. Chk1/MEK1/2 inhibition induces γH2A.X expression in statin$^+$ (G_0G_1) H929 cells

H929 cells cultured in 0.1% FBS medium for 64 hr were exposed to 5 μM PD184352 (24 hr) and 300 nM CEP3891 (+ 6 hr). Cells were then immunofluorescently stained with antibodies against statin (red fluorescence) and AF 488-conjugated phospho-H2A.X (Ser139, γH2A.X, green fluorescence), and counterstained with DAPI (blue fluorescence). Images were captured at60×/1.40 under oil and then merged as indicated. Arrows indicate cells exhibiting co-localization of statin and γH2A.X.

Figure S1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+ AZD6244
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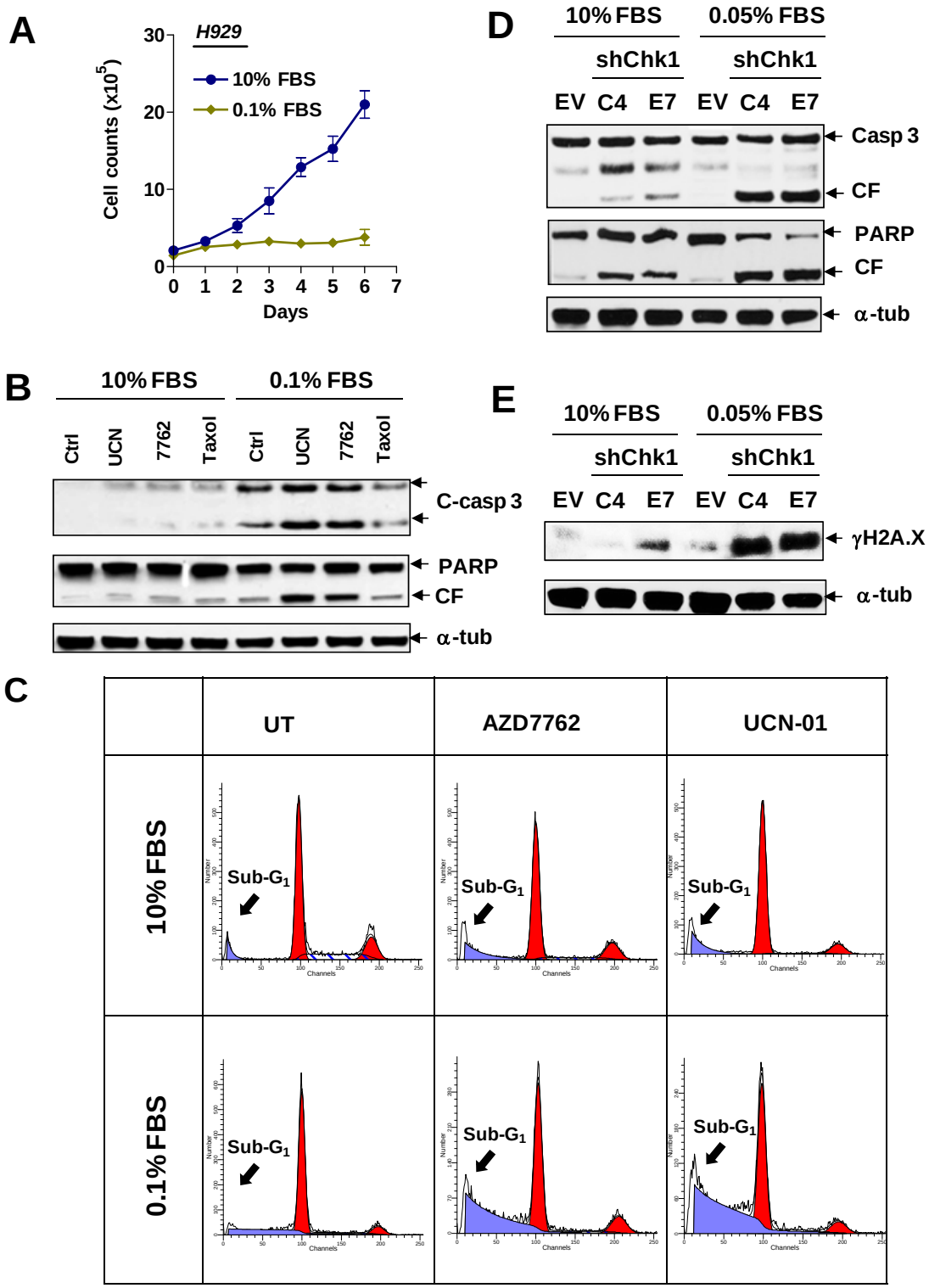
+ Vehicle
+ AZD6244

Figure S2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Taxol
C-casp 3
PARP
CF
α-tub
C
UT
AZD7762
UCN-01
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CF
PARP
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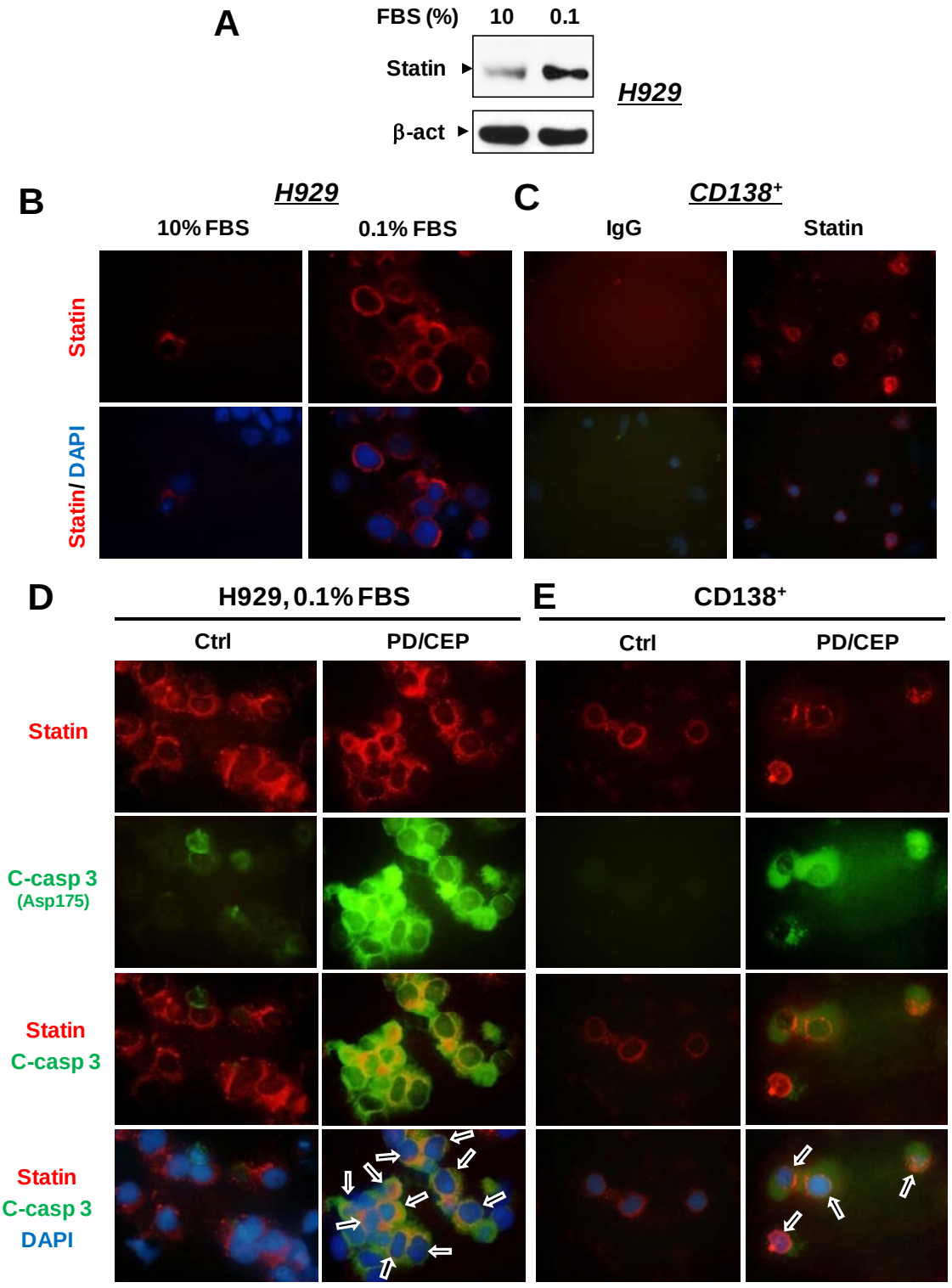
E
γH2A.X

Figure S3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H929
B
H929
10% FBS
0.1% FBS
Statin
Statin/ DAPI
C
CD138+
IgG
Statin
D
H929, 0.1% FBS
Ctrl
PD/CEP
Statin
C-casp 3
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C-casp 3
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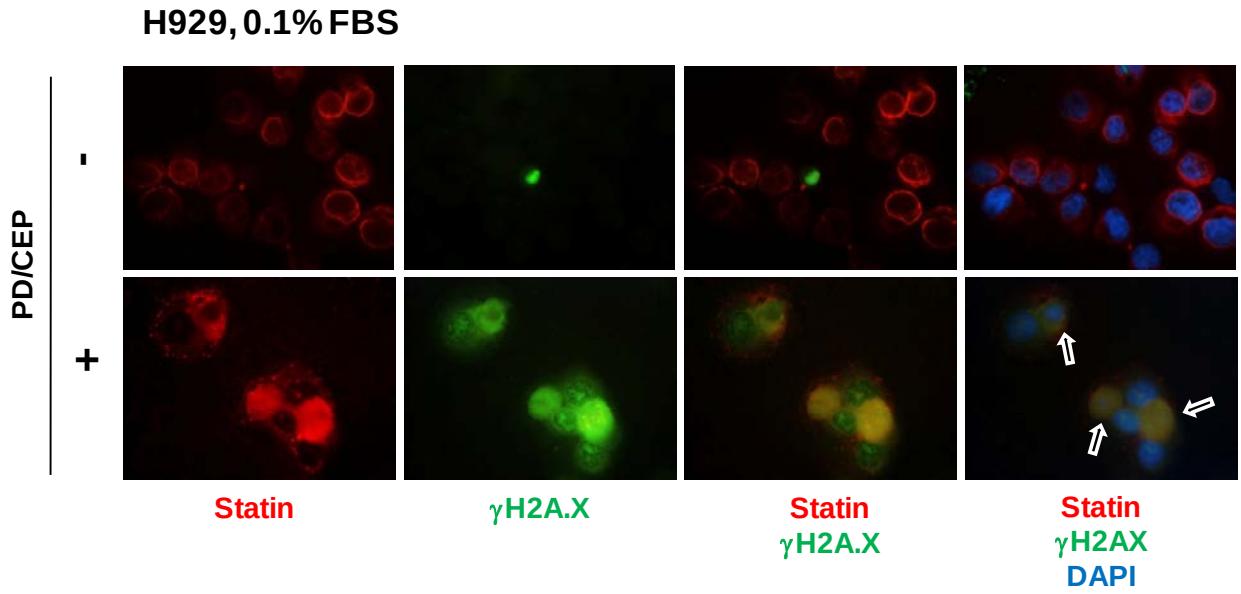
CD138+
Ctrl
PD/CEP

Figure S4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PD/CEP
-
+
Statin
γH2A.X
Statin
γH2A.X
Statin
γH2AX
DAPI