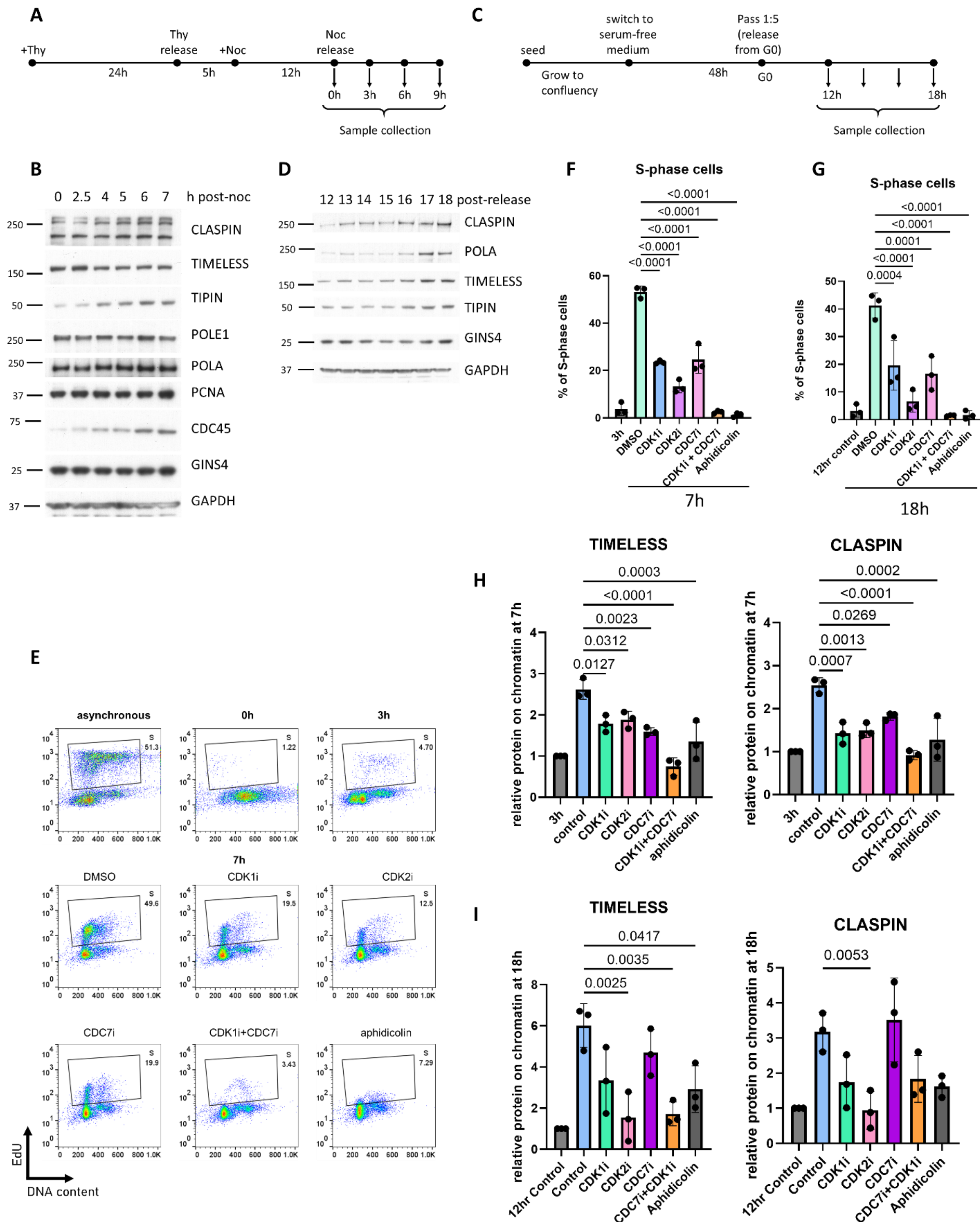

Figure S1. A. U2OS synchronization timeline using the double thymidine-nocodazole block and the timepoints used for sample collection. **B.** U2OS cells were synchronized as indicated on **A**. Samples were collected at the indicated timepoints post nocodazole release. Western blot analysis of the soluble fraction from the cells collected at the indicated timepoints is shown. **C.** RPE1-hTERT contact inhibition/serum starvation synchronization strategy. **D.** RPE1-hTERT cells were synchronized as indicated on **C**. Western blot analysis of the soluble fraction from the cells collected at the indicated timepoints is shown. **D-E.** U2OS, mAID2 clones O3 and L4 were synchronized as indicated on S1A. Inhibitors or DMSO was added at 3h post nocodazole release. 10 µM EdU was added for the last 30 min of treatment. Flow cytometry plots showing EdU incorporation and DNA content (7-AAD staining) (**D**) and quantifications (**E**). **G.** RPE1-hTERT cells were synchronized as indicated on **C**. Inhibitors or DMSO were added at 10h post release from G0. 10 µM EdU was added for the last 30 min of treatment. Quantification of EdU positive (S-

phase) cells are shown. H. Quantification of the experiment on **Fig.1E. I.** Quantification of the experiment on **Fig.1F.** Quantification of the flow cytometry and western blot data is shown as mean +/- SD from n = 3 independent experiments (**F-I).** One-way ANOVA was used for statistical analysis.

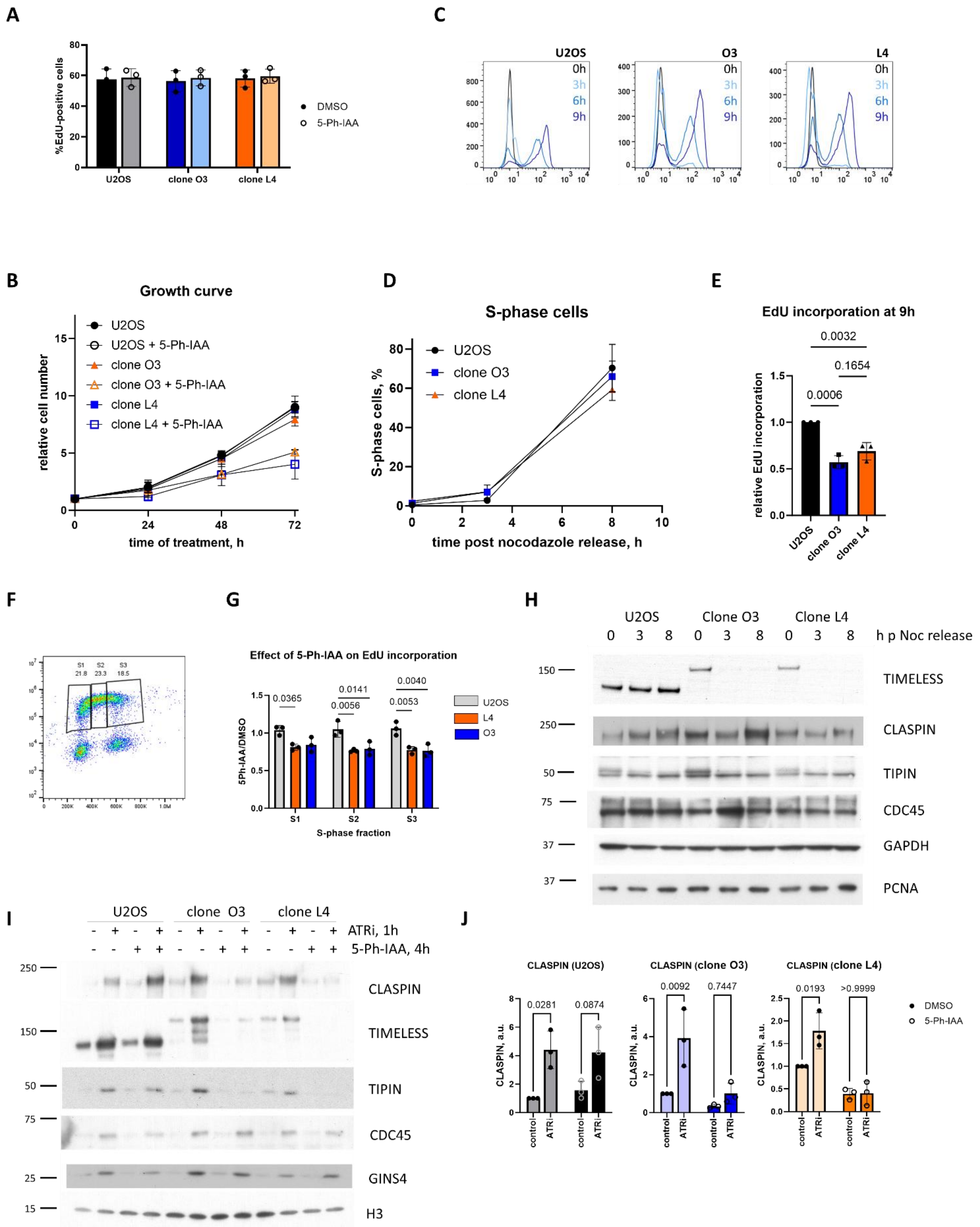

Figure S2. A. U2OS, clones O3 and L4 were treated for 16 h with 5-Ph-IAA. 10 µM EdU was added for the last 30 min of treatment. Quantification of the flow cytometry data is shown as mean +/-SD from n = 3 independent experiments. **B.** Equal numbers U2OS, clones O3 and L4 were seeded on 60 mm dishes and treated with DMSO or 5-Ph-IAA for 72 h. Cell numbers were counted every 24 h and growth curves were plotted. The data are depicted as mean +/- SD from n = 3 independent experiments. **C.** U2OS, clones O3 and L4 were synchronized using the double thymidine/nocodazole block, and treated as indicated on **Fig.S1A**. 10 µM

EdU was added for the last 30 min of treatment. EdU incorporation histograms are shown for synchronized untreated U2OS and clones O3 and L4 showing that TIM tagging did not affect the S-phase entry without TIMELESS depletion. **D-E.** U2OS, mAID2 clones O3 and L4 were synchronized using the double thymidine/nocodazole block, 5-Ph-IAA was added at the time of the release from nocodazole. 10 µM EdU was added for the last 30 min of treatment. Quantification of the number of S-phase cells and relative EdU incorporation from the flow cytometry data is shown as mean +/-SD from n = 3 independent experiments (**D-E**). **F-G.** U2OS, clones O3 and L4 were treated for 16 h with 5-Ph-IAA. 10 µM EdU was added for the last 30 min of treatment. Representative image with sub-S-phase gates is shown (**G**). Quantification of EdU incorporation for each sub-S-phase fraction is shown as mean +/-SD from n = 3 independent experiments. Two-way ANOVA was used for statistical analyses. **H**. U2OS, mAID2 clones O3 and L4 were synchronized using the double thymidine/nocodazole block, 5-Ph-IAA was added at the time of the release from nocodazole. Western blot analysis of the soluble fraction from the cells collected at the indicated timepoints is shown **I-J.**U2OS, clones O3 and L4 were treated for 4h with 5-Ph-IAA, as indicated. 5 µM ATRi was added to the indicated samples for 1 h, followed by cell lysis and the isolation of the nuclease-insoluble chromatin fraction. Western blots of the insoluble chromatin fraction are shown (**I**). Quantifications of protein loading from **I** are shown (**J**). Mean + SD from 3 independent experiments is shown. Two-way ANOVA was used for statistical analyses.

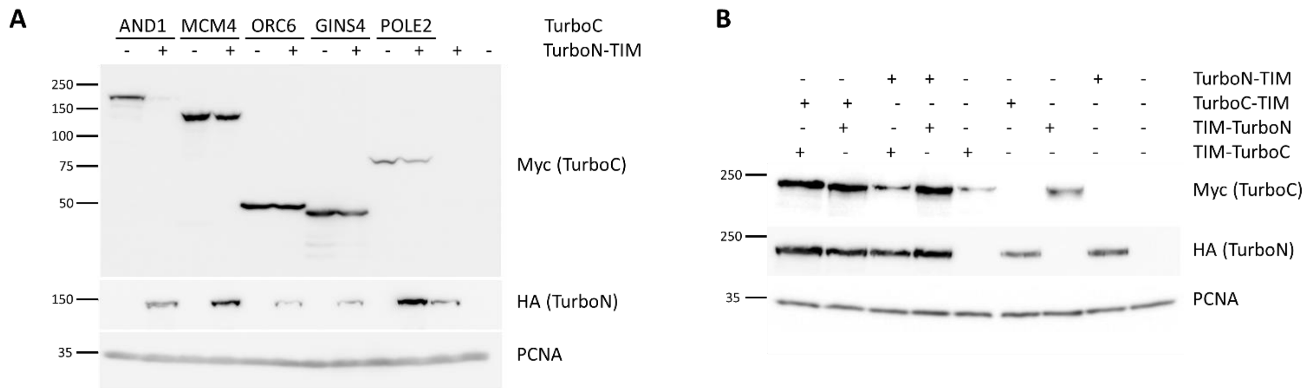

Figure S3: A-B. HEK293FT cells were transfected with the plasmids expressing indicated TurboN- or TurboC-tagged proteins. 48h after transfection cells were treated with 50 µM biotin for 1 hour and lysed. Cell lysates were analyzed by western blot using antibodies against indicated proteins.

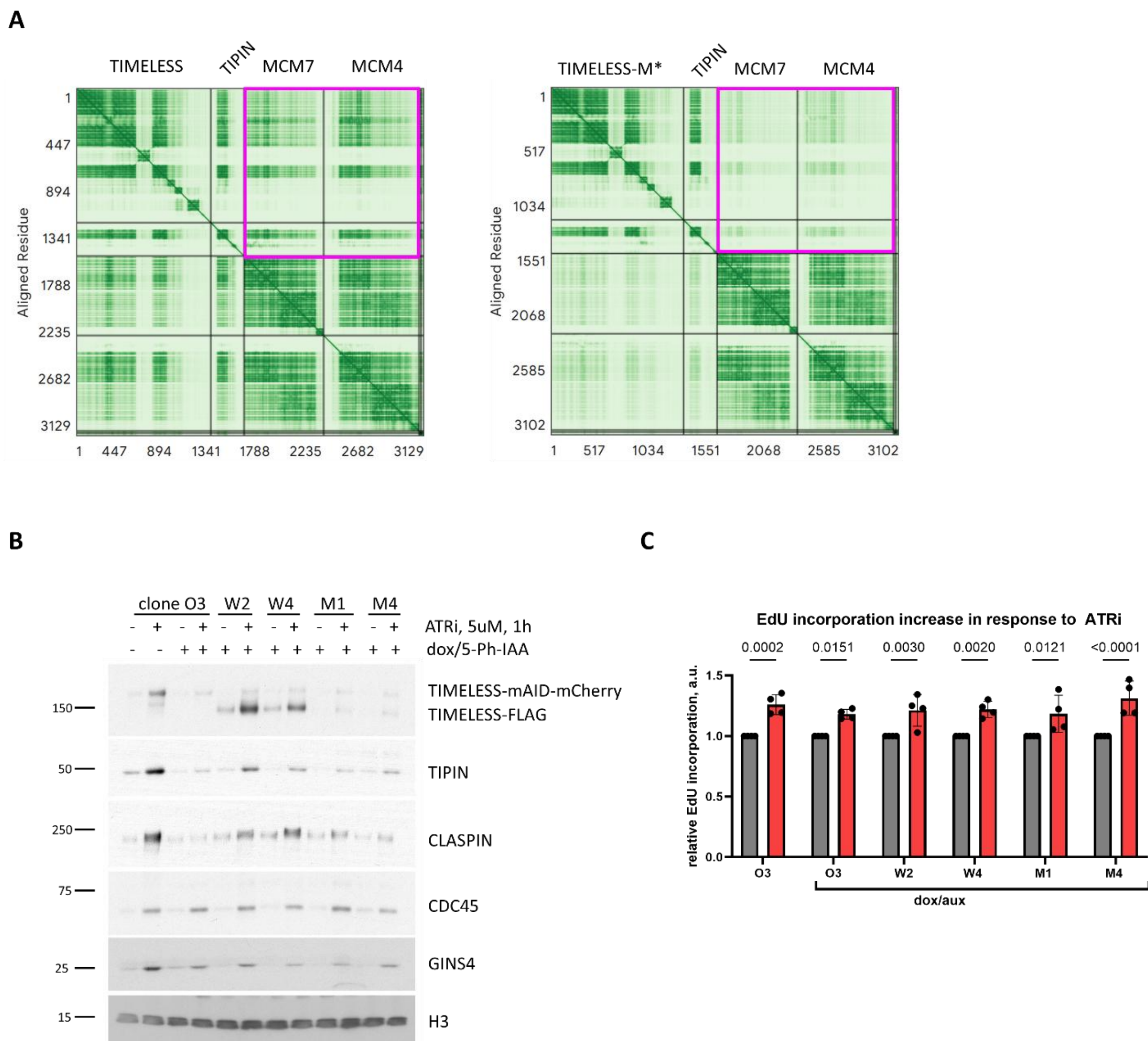

Figure S4. A. TIPIN, MCM7 and MCM4 are modeled in Alphafold3 with TIMELESS WT or TIMELESS-M*. The intensity of green indicated the predicted error. Magenta frame indicates the relative position confidence between TIMELESS and MCM subunits. **B-C.** mAID2 clone O3, and clones based on O3 expressing WT TIMELESS (W2 and W4) or TIMELESS M* (M1 and M4), were treated with doxycycline and 5Ph-IAA for 16h. ATRi or DMSO was added for 1h. Western blot analyses of nuclease-insoluble chromatin fractions are shown **(B).** After a 30 min EdU pulse, quantification of EdU incorporation by FACS is shown as mean +/- SD from n = 3 independent experiments. Two-way ANOVA was used for statistical analyses (**C**).

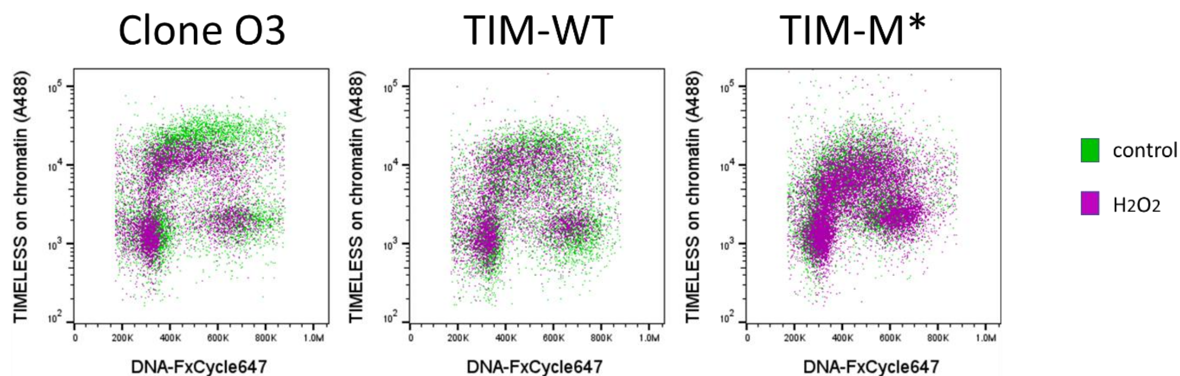

Figure S5. A. Clones based on O3 expressing WT TIMELESS (W2) or TIMELESS M* (M1), were treated with 10ng/ml doxycycline for 48h and 5Ph-IAA for 16h, mAID2 clone O3 was treated with DMSO for 16h. 50µM H_2O_2 was added to the cells for 30 min before harvest. After CSK extraction and fixation, cells were stained with anti-TIMELESS antibodies and a DNA dye. Representative Flow cytometry plots are shown.