A Reappraisal of Enrichment of Cell Cycle Phases in Synchronized Estrogen Receptor-Positive Cell Models Derived from Breast Adenocarcinomas

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Supplementary Figures S1-S12

Fig. S1

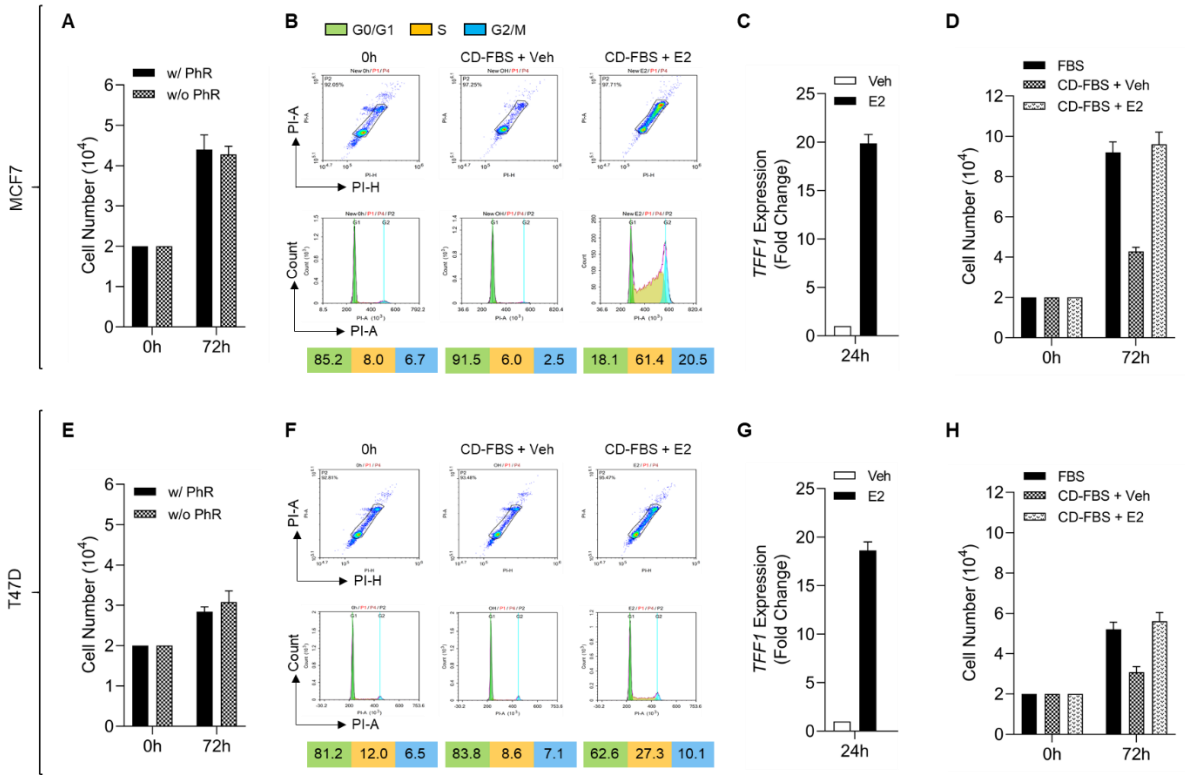

Fig. S1. Effects of phenol red (PhR), CD treatment of FBS, and E2 replacement on growth characteristics of MCF7 and T47D cells. To examine the effect of PhR on the growth of MCF7 or T47D cells, cells were plated in DMEM or RPMI 1640 tissue culture medium, respectively, without (w/o) or with (w) PhR supplemented with 10% CD-FBS in 6-well culture plates at 20,000 cells/well. Cells were grown for 72 h changing the media at 48 h. Cells were then collected by trypsinization and (**A** & **E**) counted with a hemocytometer. (**B-C** & **F-G**) To examine the effects of CD-FBS on the accumulation of cells at G0/G1, (**B-C**) MCF7 or (**F-G**) T47D cells were grown in DMEM or RPMI 1640 medium, respectively, supplemented with 10% CD-FBS in 6-well tissue culture plates for 72 h. Cells were either collected by trypsinization (0h) or treated without (0.01% ethanol, Veh) or with 10^{-9} M E2 for 24 h. (**B** & **F**) A fraction of the collected cells was subjected to flow cytometry, (**C** & **G**) and the remaining fraction was subjected to RNA isolation followed by qPCR for the expression of *TFF1* using the expression of *RPL0* for normalization. G0/G1, S, and G2/M indicate cell cycle phases. (**D** & **H**) To assess the effect of E2 replacement on growth characteristics of (**D**) MCF7 or (**H**) T47D cells, cells grown in media supplemented with 10% CD-FBS for 72 h were incubated in the respective media either supplemented with 10% FBS, or with 10% CD-FBS without (0.01% EtOH) or with 10^{-9} M E2 for 72 h. Cells were then collected by trypsinization and counted with a hemocytometer.

Fig. S2

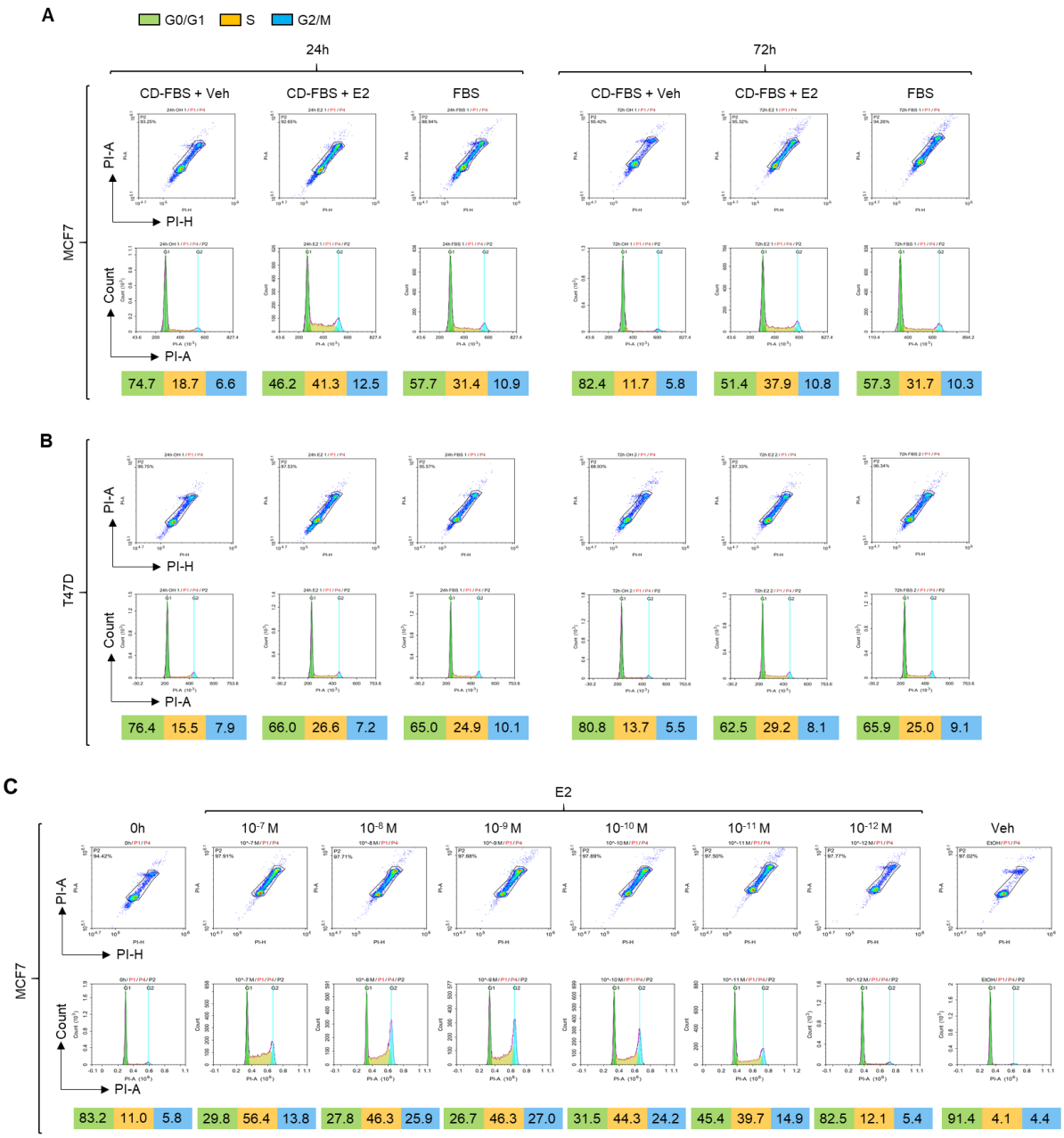

Fig. S2. Effects of CD-FBS on the accumulation of MCF7 or T47D cells at G0/G1, and effects of various concentrations of E2 on cycle progression of MCF7 cells synchronized at G0/G1. (**A**) MCF7 or (**B**) T47D cells were grown in DMEM or RPMI 1640 medium, respectively, supplemented with either 10% FBS or 10% CD-FBS containing 0.01% ethanol as vehicle control (Veh), or 10^{-9} M E2 in 6-well tissue culture plates up to 24 or 72 h. Collected cells were subjected to flow cytometry analyses. (**C**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72h were either collected by trypsinization (0h) or treated without (0.01% ethanol, Veh) or with various concentrations of (10^{-12} to 10^{-7} M) E2 for 24 h in the same medium. Cells were then collected and subjected to flow cytometry analysis. G0/G1, S, and G2/M indicate cell cycle phases. Representative images with cell cycle phases from the same experiment are presented.

Fig. S3

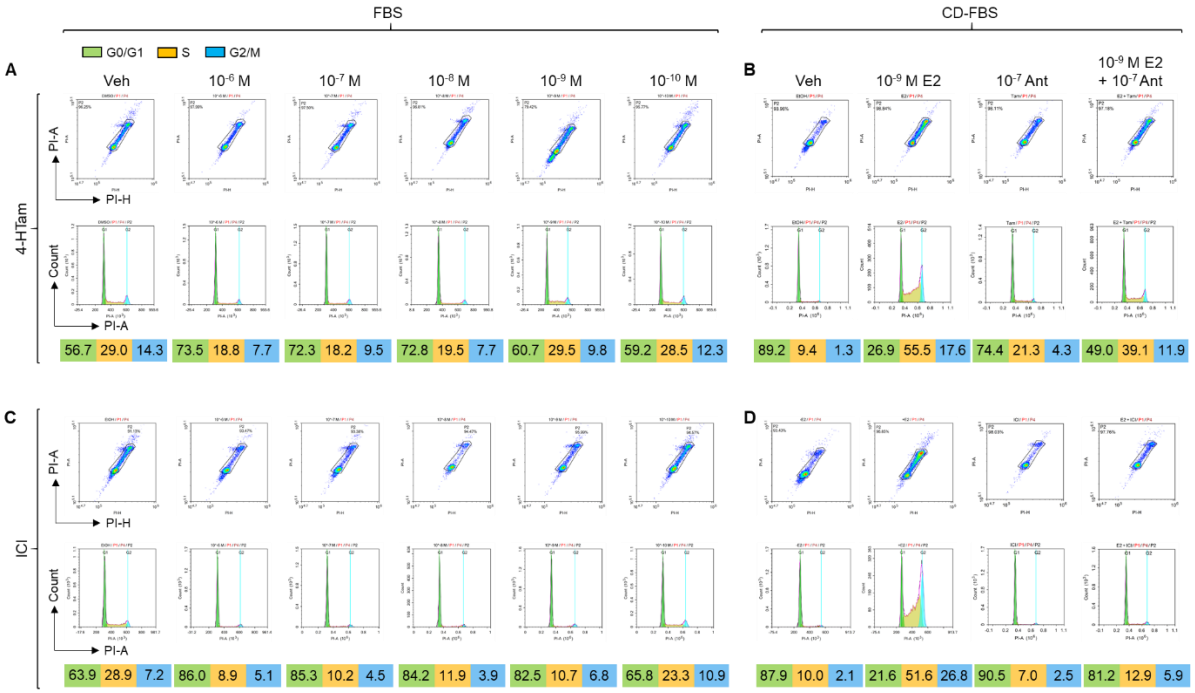

Fig. S3. Effects of 4-hydroxytamoxifen, 4-HTam, or Imperial Chemical Industries 182780, ICI, on cycle phase distribution of MCF7 cells. (A & **C)** Cells maintained in DMEM medium supplemented with 10% FBS were incubated in fresh media containing 0.0002% DMSO or 0.01% ethanol as the vehicle (Veh) control respectively, or various concentrations, 10^{-10} to 10^{-6} M (**A**) 4-HTam or (**C**) ICI for 24 h. Cells were collected by trypsinization and subjected to flow cytometry. (**B** & **D**) MCF7 cells maintained in DMEM medium supplemented with 10% CD-FBS for 72 h to synchronize cells at G0/G1 were incubated in the same medium without (Veh) or with E2 (10^{-9} M) and/or (**B**) 4-HTam (10^{-7} M) or (**D**) ICI (10^{-7} M) for 24h. Cells were then collected and subjected to flow cytometry analysis. G0/G1, S, and G2/M indicate cell cycle phases. Representative images with cell cycle phases from the same experiment are presented.

Fig. S4

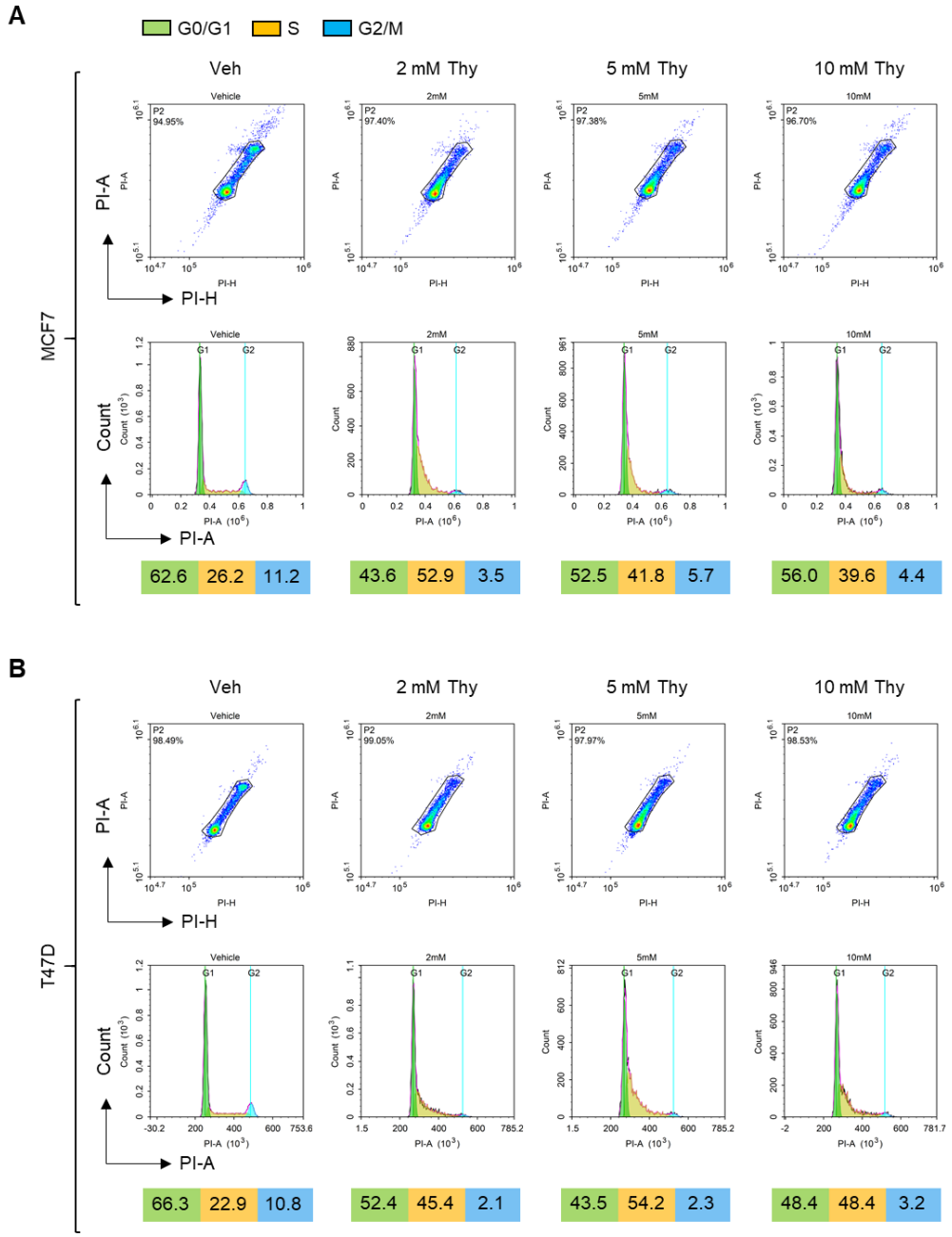

Fig. S4. Effects of various concentrations of thymidine (Thy) on cycle synchronization of MCF7 and T47D cells. (**A**) MCF7 or (**B**) T47D cells maintained in DMEM or RPMI 1640 medium, respectively, supplemented with 10% FBS were incubated in fresh media with vehicle control (Veh) or various concentrations (2, 5, or 10 mM) of thymidine for 14 hours. Cells were then released from thymidine treatment by washing the cells with 1x PBS and re-incubating them in their respective medium containing 10% FBS without thymidine for 12 hours. The cells were subsequently washed and incubated with the same medium containing 10% FBS and vehicle control (Veh) or 2, 5, or 10 mM thymidine for 22 hours. Cells were collected with trypsinization and subjected to flow cytometry. G0/G1, S, and G2/M indicate cell cycle phases. Representative images from the same experiment are shown.

Fig. S5

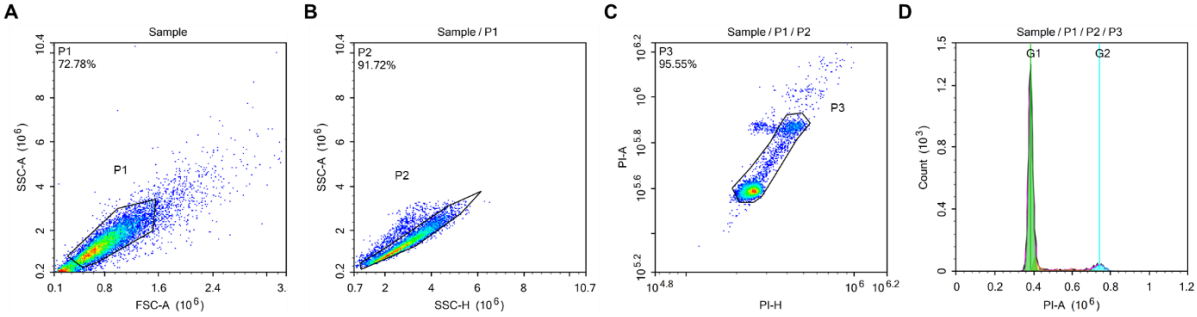

Fig. S5. The gating strategy for flow cytometry analysis. (**A**) The total cell population is selected in the dot plot showing the forward scatter area (FSC-A) vs. the side scatter area (SSC-A) of all acquired data points, yielding child population P1. (**B**) Single cells are selected using the dot plot showing side scatter height (SSC-H) vs. side scatter area (SSC-A) of population P1, yielding child population P2. (**C**) The dot plot showing propidium iodide height (PI-H) vs. propidium iodide area (PI-A) of population P2 is then used to select the PI-stained population marked as P3. (**D**) The child population P3 is used to analyze the cell cycle phase distribution of the population in the cell cycle plot, which gives the percent distribution of the cells in each cell cycle phase.

Fig. S6

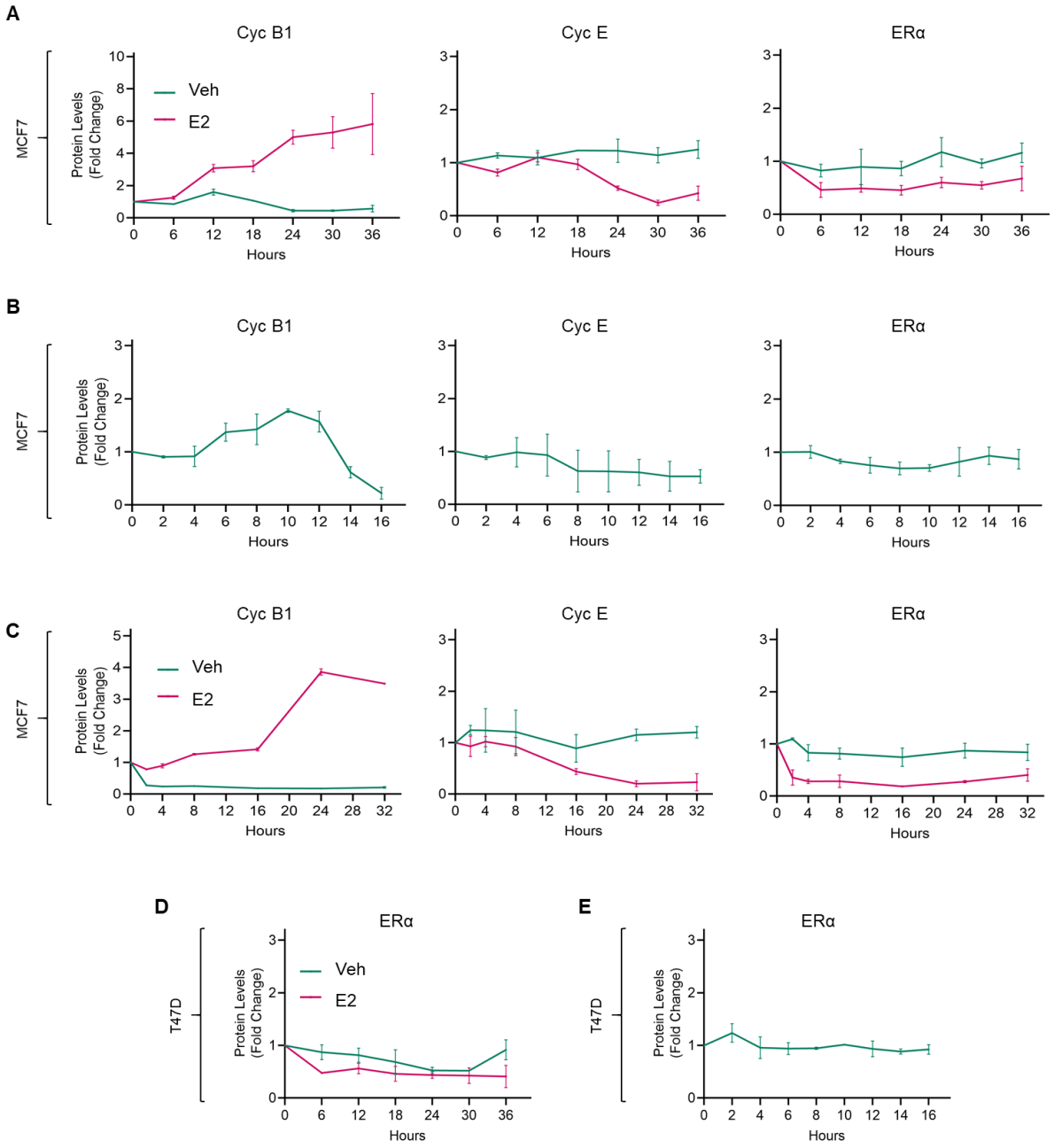

Fig. S6. Densitometric analyses of protein levels relative to that of HDAC1. (**A**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were subsequently maintained in the same medium containing 0.01% ethanol (EtOH) as vehicle control or 10^{-9} M E2 for 6 h intervals up to 36 h. At the termination, cells were collected with trypsinization, and a fraction of the cells were processed for WB analysis using an antibody for Cyclin B1 (Cyc B1), Cyclin E (Cyc E), ERα, or HDAC1. Densitometric analyses of protein levels relative to HDAC1 from the same experiment conducted two independent times are indicated in the graphs. (**B**) MCF7 cells grown in DMEM medium supplemented with 10% FBS were synchronized at the G1/S transition with 2 mM thymidine and released (RDTB) with fresh DMEM medium supplemented with 10% FBS (0 h). Cells were collected with trypsinization at 2 h intervals for 16 h following release and a fraction of the cells was processed for WB analysis using an antibody for Cyclin B1 (Cyc B1), Cyclin E (Cyc E), ERα, or HDAC1. Densitometric analyses of protein levels relative to HDAC1 from the same experiment conducted two independent times are indicated in the graphs. (**C**) MCF7 cells

grown in DMEM medium supplemented with 10% FBS for 72 h were then synchronized in G0/G1 with 2 mM thymidine and released (RDTB) with DMEM medium containing 10% CD-FBS in the absence (0.01% EtOH) or presence of 10^{-9} M E2 for 2-4 h intervals up to 32 h. At the termination at each time point, cells were collected with trypsinization, and a fraction of the cells was processed for WB analysis using an antibody for Cyclin B1 (Cyc B1), Cyclin E (Cyc E), ERα, or HDAC1. Densitometric analyses of protein levels relative to HDAC1 from the same experiment conducted two independent times are indicated in the graphs. (**D**) T47D cells grown in RPMI 1640 medium supplemented with 10% CD-FBS for 72 h were subsequently maintained in the same medium containing 0.01% ethanol (EtOH) as vehicle control or 10^{-9} M E2 for 6 h intervals up to 36 h. At the termination, cells were collected with trypsinization, and a fraction of the cells was processed for WB analysis. Densitometric analyses of ERα protein levels normalized to HDAC1 levels within the same experiment, are shown in the graph for two independent experiments. (**E**) T47D cells grown in RPMI 1640 medium supplemented with 10% FBS were synchronized at the G1/S transition with 2 mM thymidine and released (RDTB) with fresh RPMI 1640 medium supplemented with 10% FBS (0 h). Cells were collected with trypsinization at 2 h intervals for 16 h following release and a fraction of the cells was processed for WB analysis. Densitometric analyses of ERα protein levels normalized to HDAC1 levels within the same experiment, are shown in the graph for two independent experiments.

Fig. S7

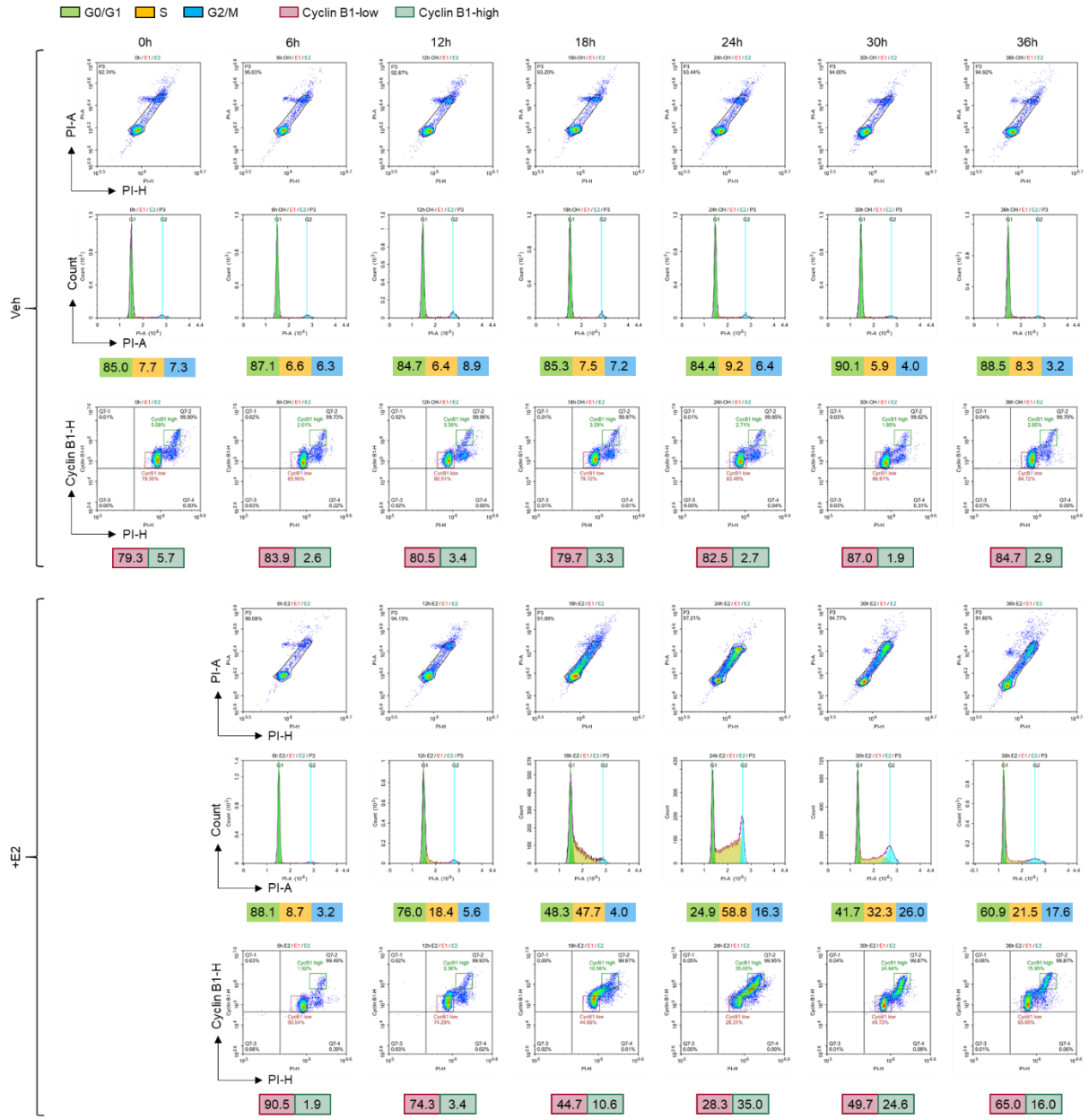

Fig. S7. Bivariate analysis of Cyclin B1 synthesis alongside the total DNA content through the cell cycle progression of MCF7 cells synchronized at G0/G1 by hormone withdrawal. MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were subsequently maintained in the same medium containing 0.01% ethanol (EtOH) as vehicle control or 10^{-9} M E2 for 6 h intervals up to 36 h. At the termination, cells were collected with trypsinization and fixed with ice-cold 70% ethanol. The cells were then subjected to intracellular staining using Cyclin B1 antibody followed by Alexa Fluor-488 Goat anti-mouse secondary antibody, and subsequently subjected to propidium iodide staining for flow cytometry analysis. Representative images from the same experiment conducted two independent times are shown. G0/G1, S, and G2/M indicate cell cycle phases. The percentage of Cyclin B1-low (red boxes) and Cyclin B1-high (light green boxes) populations are shown.

Fig. S8

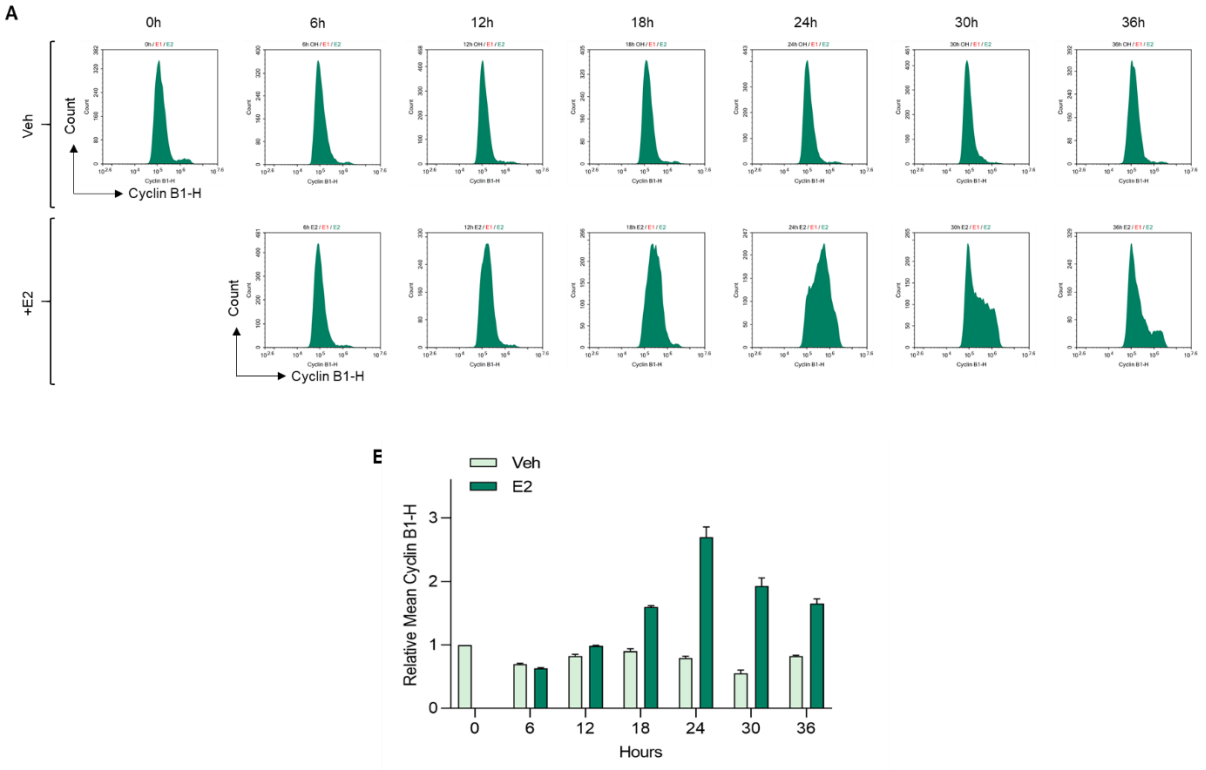

Fig. S8. Quantification of Cyclin B1 synthesis assessed with flow cytometry through the cell cycle progression of MCF7 cells synchronized at G0/G1 by hormone withdrawal. MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were subsequently maintained in the same medium containing 0.01% ethanol (EtOH) as vehicle control or 10^{-9} M E2 for 6 h intervals up to 36 h. At the termination, cells were collected with trypsinization and fixed with ice-cold 70% ethanol. The cells were then subjected to intracellular staining using Cyclin B1 antibody followed by Alexa Fluor-488 Goat anti-mouse secondary antibody, and subsequently subjected to propidium iodide staining for flow cytometry analysis. (**A**) Representative images of Cyclin B1-H histograms from the same experiment conducted two independent times are shown. (**B**) A bar graph of two independent biological replicates represents the mean Cyclin B1-H signal at the given time points relative to 0 h.

Fig. S9

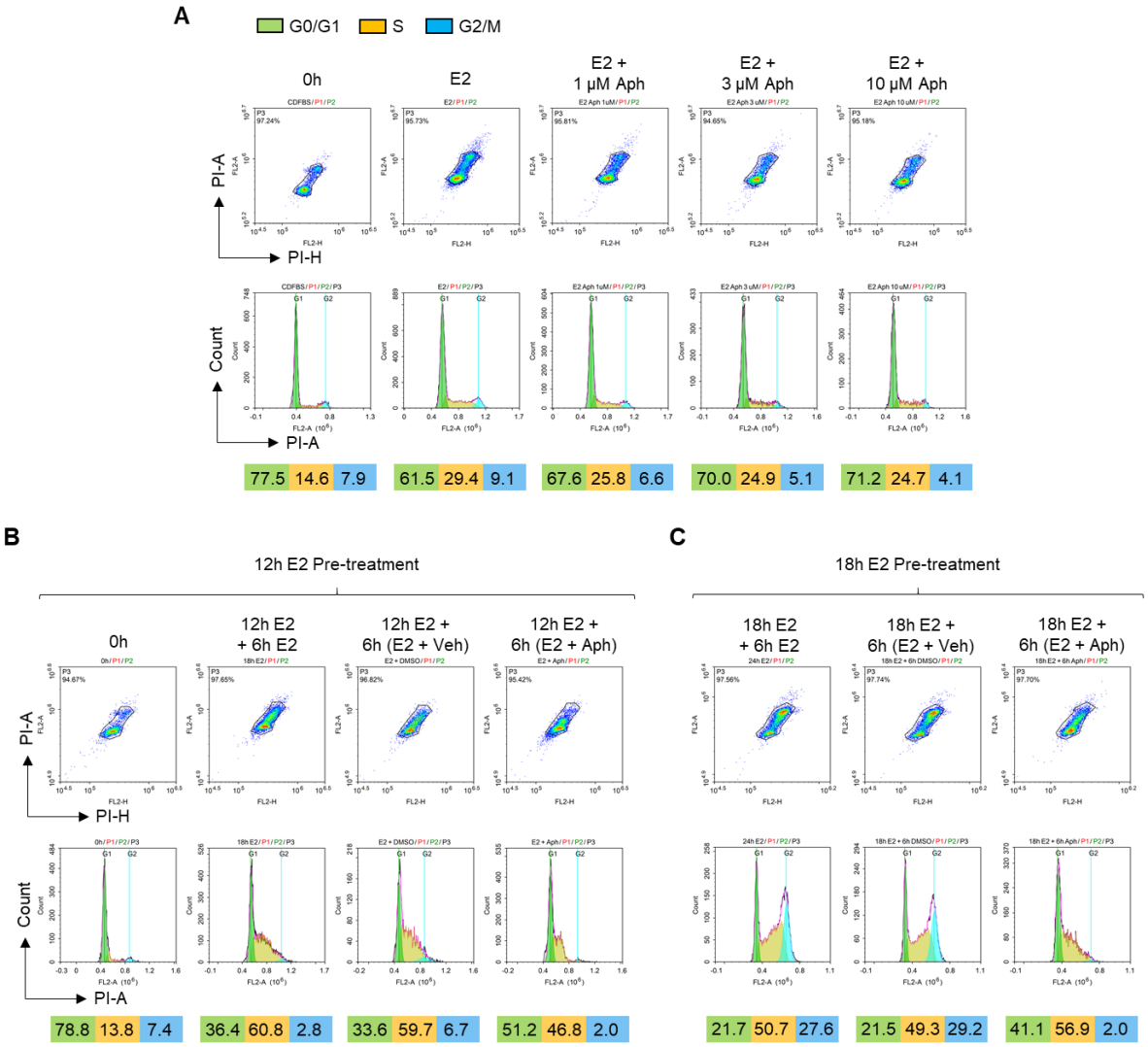

Fig. S9. Determination of the optimal dose and time for the enrichment of the S phase by aphidicolin in synchronized MCF7 cells present at different stages of the S phase induced by the E2 treatment. (**A**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were either collected by trypsinization (0h) or treated in the same medium with 10^{-9} M E2 alone or together with 1, 3, or 10 μM aphidicolin (Aph) for 24 h. (**B-C**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were either collected by trypsinization (0h) or treated in the same medium with 10^{-9} M E2 for (**B**) 12 h, or (**C**) 18 h. Cells were then incubated in the same medium without (0.01% DMSO as the vehicle control for aphidicolin) or with 10 μM aphidicolin in the absence or presence of 10^{-9} M E2 for an additional 6 h. At the termination, cells were collected by trypsinization and subjected to flow cytometry. Representative images with cell cycle phases from the same experiment are presented.

Fig. S10

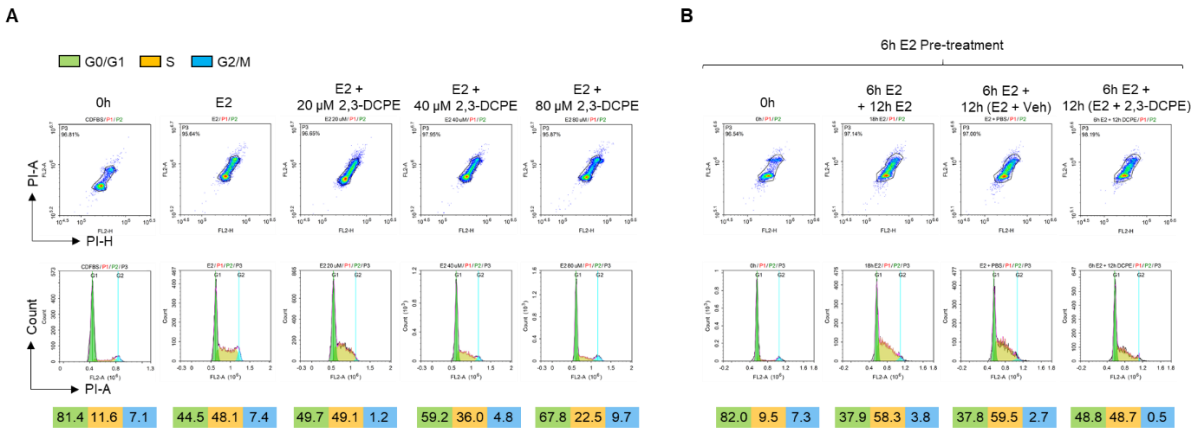

Fig. S10. Determination of the optimal dose and time for the enrichment of the S phase by 2,3-DCPE in synchronized MCF7 cells. (**A**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were either collected by trypsinization (0h) or treated in the same medium with 10^{-9} M E2 alone or together with 20, 40, or 80 µM 2,3-DCPE for 24 h. (**B**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were either collected by trypsinization (0h) or treated in the same medium with 10^{-9} M E2 for 6 h, and subsequently without (0.01% PBS as the vehicle control for 2,3-DCEP) or with 20 µM 2,3-DCEP in the absence or presence of 10^{-9} M E2 for an additional 12 h. At the termination, cells were collected by trypsinization and subjected to flow cytometry. Representative images with cell cycle phases from the same experiment are presented.

Fig. S11

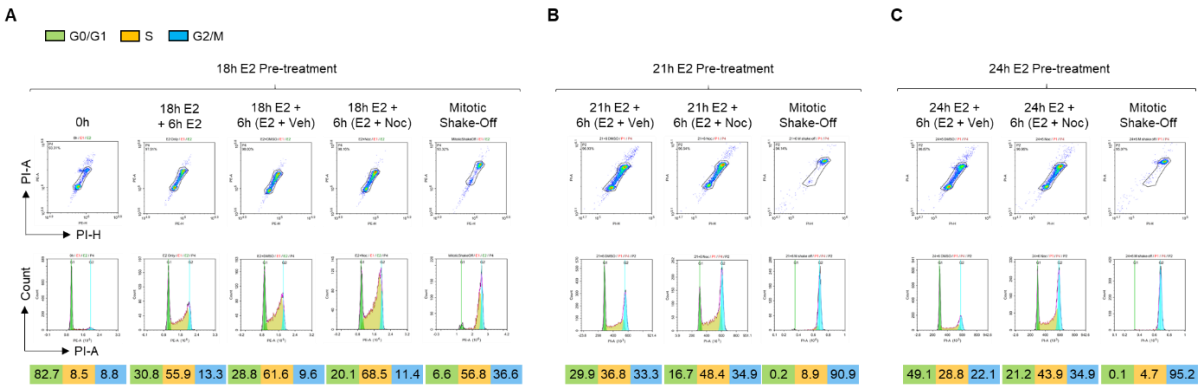

Fig. S11. Assessing the optimal time for the enrichment of the G2/M or M phase by nocodazole (Noc) in synchronized MCF7 cells present at different stages of the S phase in response to the E2 treatment. (**A-C**) MCF7 cells grown in DMEM medium supplemented with 10% CD-FBS for 72 h were either collected by trypsinization (0h) or treated in the same medium with 10^{-9} M E2 for (**A**) 18 h, (**B**) 21 h, or (**C**) 24 h. Cells were re-incubated in the fresh medium containing 10^{-9} M E2 and/or DMSO, as vehicle control for nocodazole, or 0.3 µM nocodazole (Noc) for an additional 6 h. At the termination, cells were subjected to trypsinization or mitotic shake-off for collection. The phase distribution of cells was assessed with the flow cytometer. Representative images with cell cycle phases from the same experiment are presented.

Fig. S12

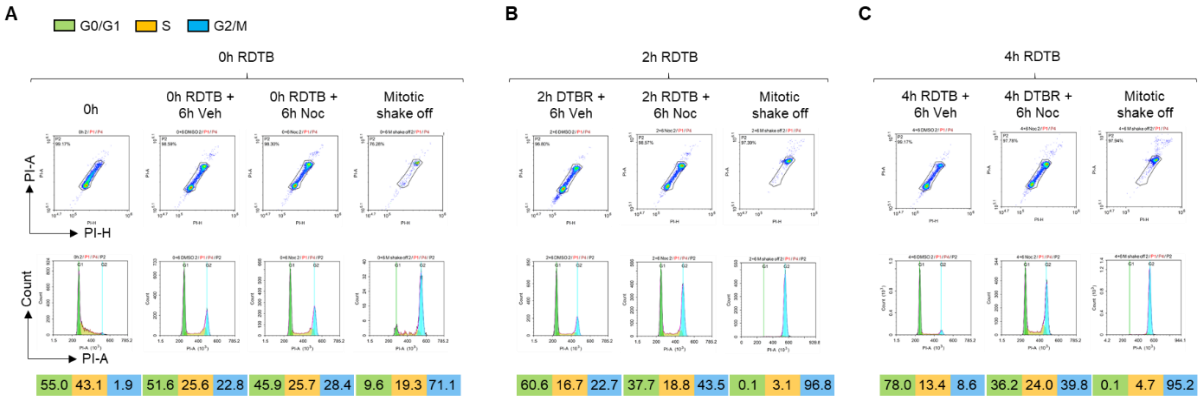

Fig. S12. Examination of the optimal time for the enrichment of the G2/M or M phase by nocodazole (Noc) following the release of synchronized T47D cells from double thymidine block (RDTB). (**A-C**) T47D cells grown in RPMI 1640 medium supplemented with 10% FBS were synchronized at the G1/S transition with 2 mM thymidine and released with fresh RPMI 1640 medium supplemented with 10% FBS. At the time of release (**A**) 0 h, or (**B**) 2 h, or (**C**) 4 h after the release, the cells were treated without (0.01% DMSO) or with 0.3 µM nocodazole (Noc) for an additional 6 h. At the termination, cells were subjected to trypsinization or mitotic shake-off followed by flow cytometry for the phase distribution. Representative images with cell cycle phases from the same experiment are shown.

Supplementary Information, Uncropped Images for Figures 1, 2, 3, 4, 6, 7, and 8

Figure 1 – Uncropped Images

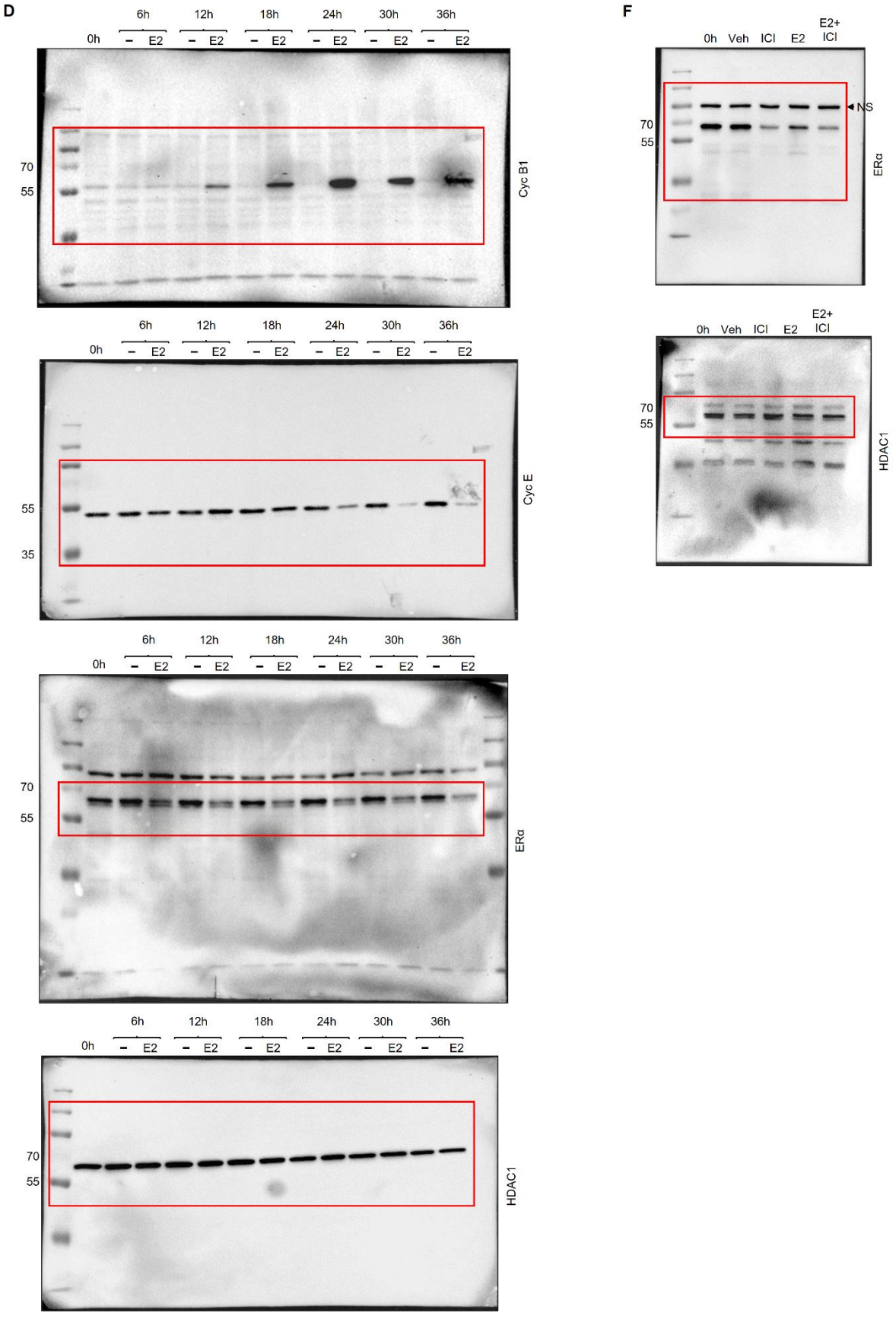

Figure 2 – Uncropped Images

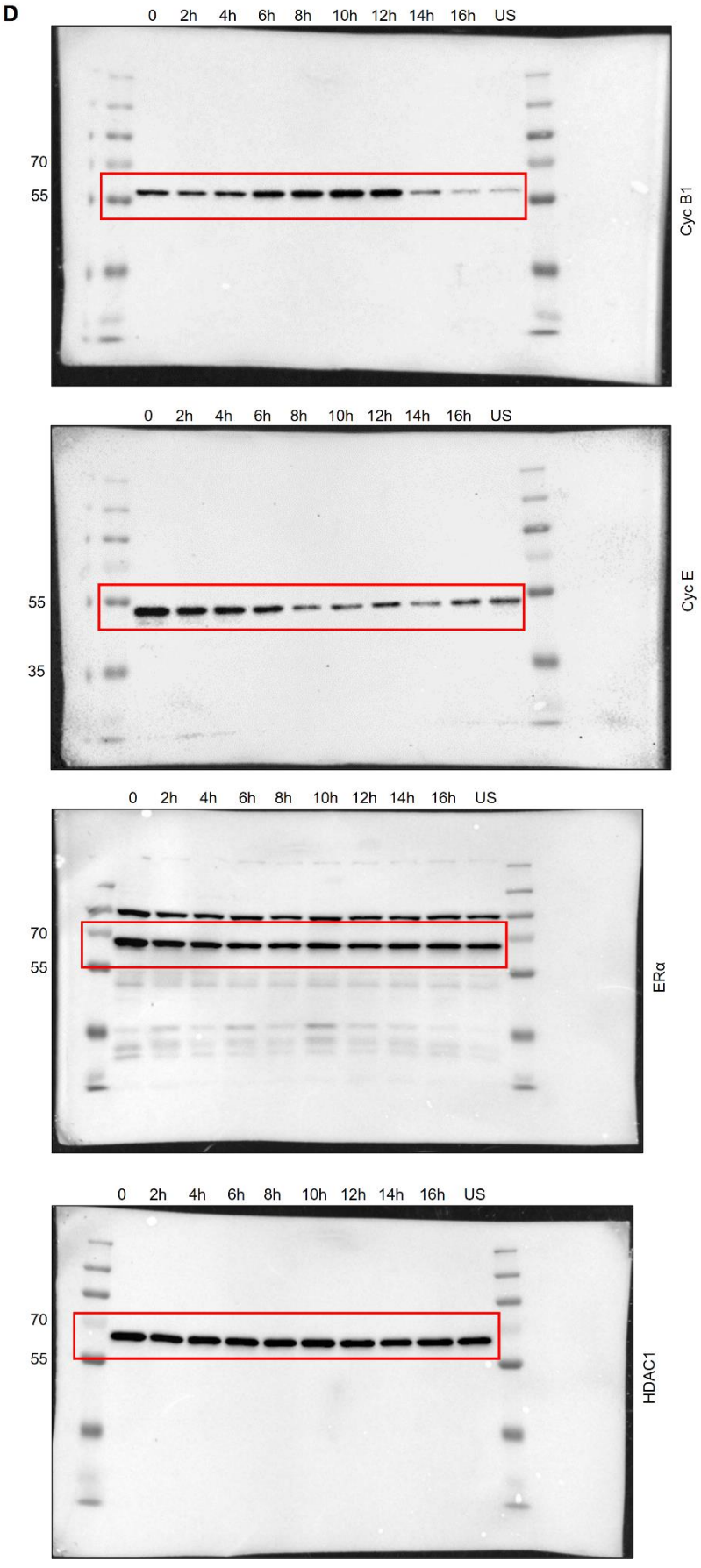

Figure 3 – Uncropped Images

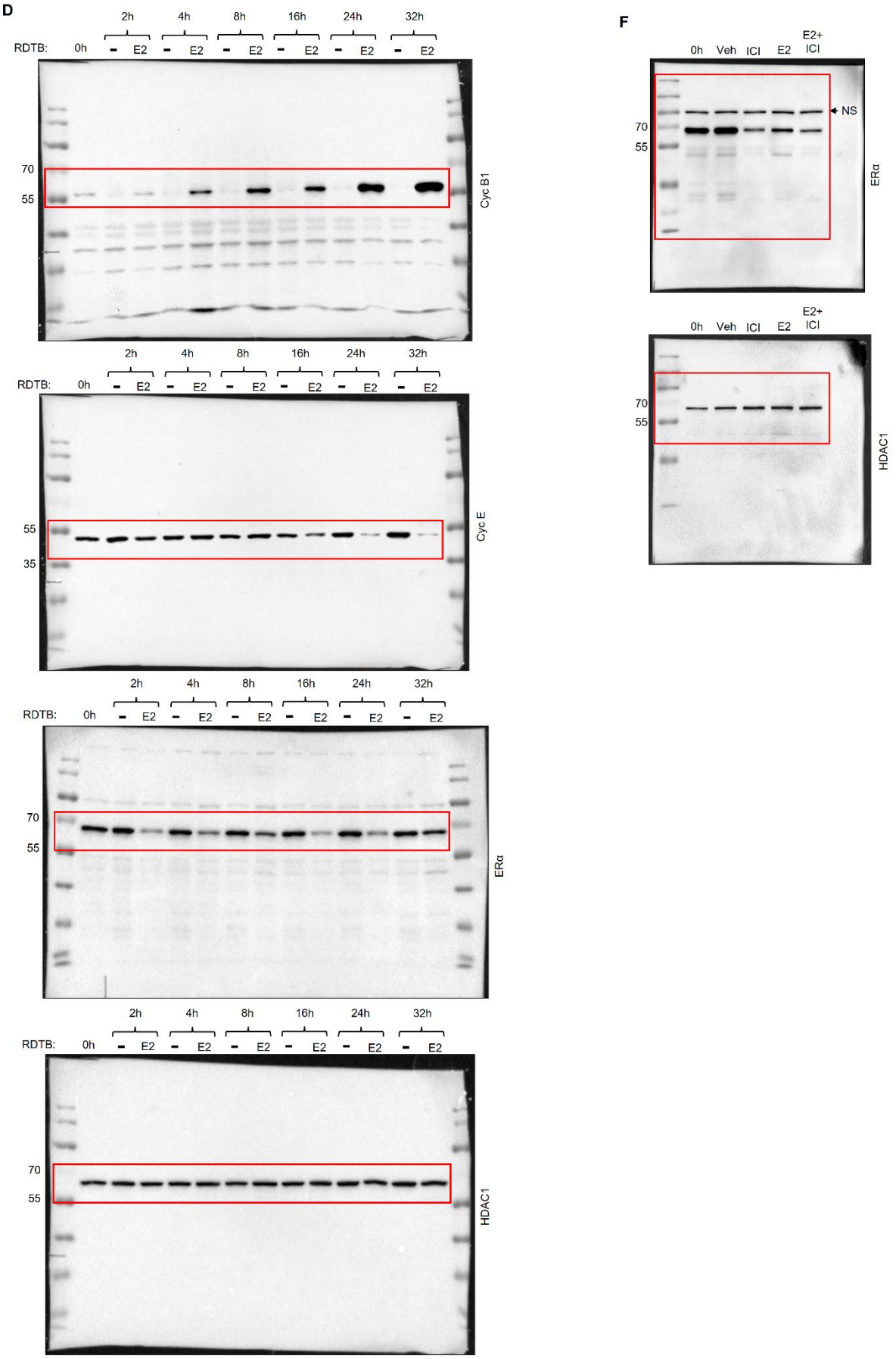

Figure 4 – Uncropped Images

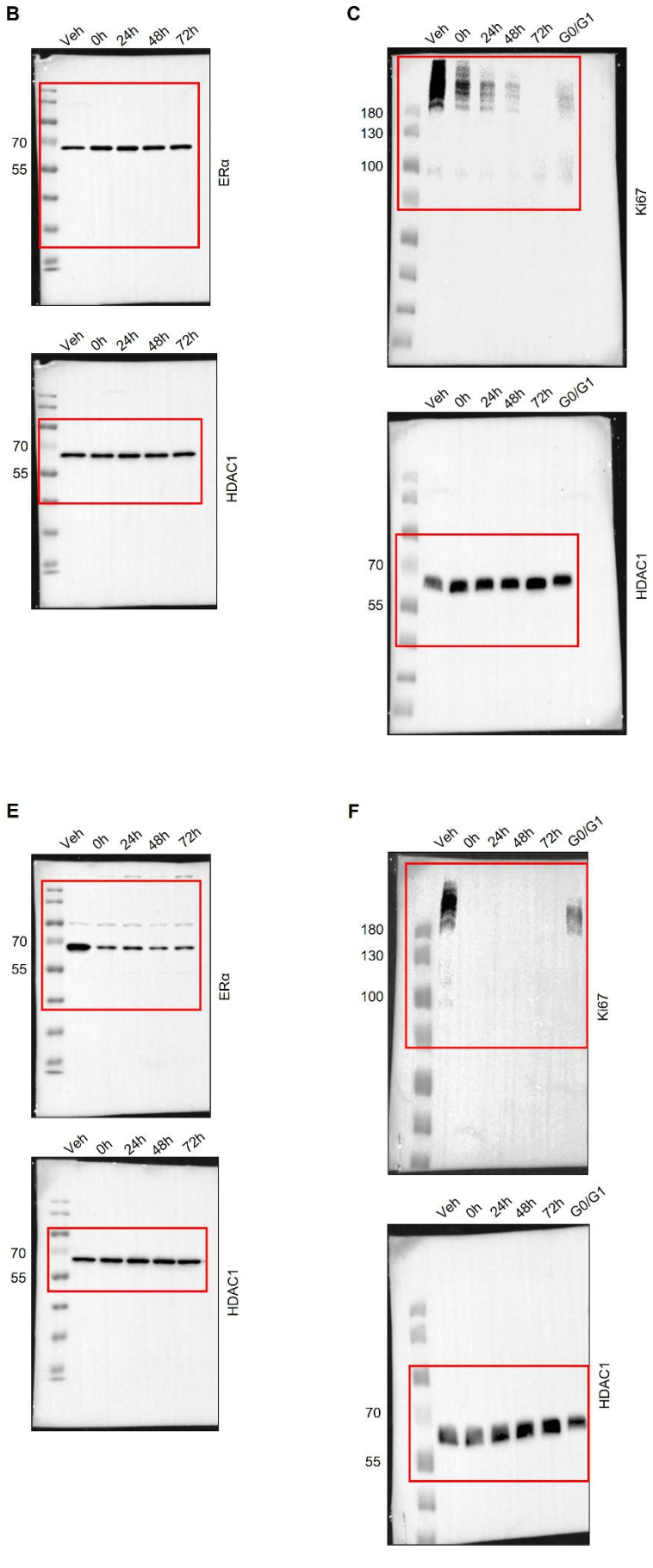

Figure 6 – Uncropped Images

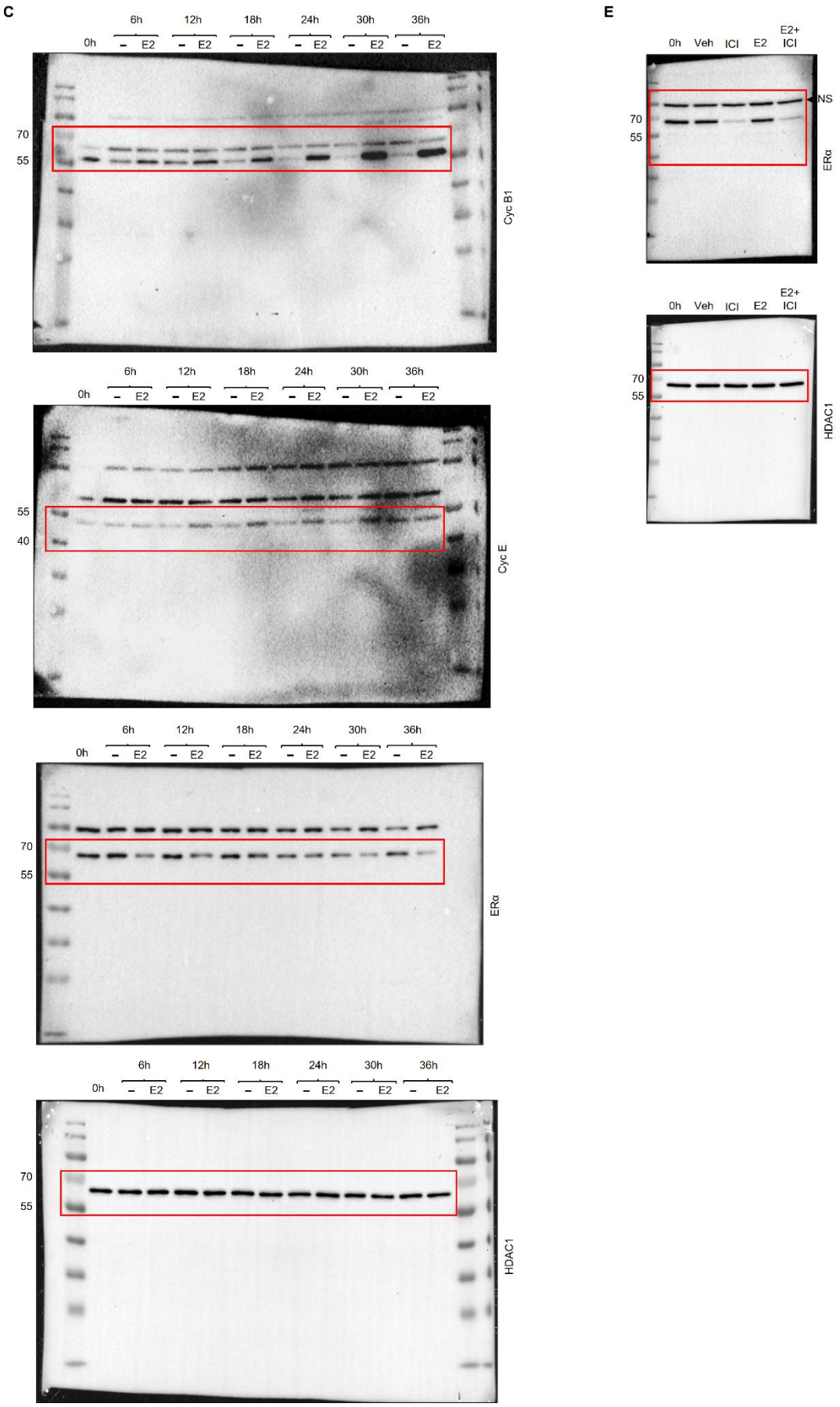

Figure 7 – Uncropped Images

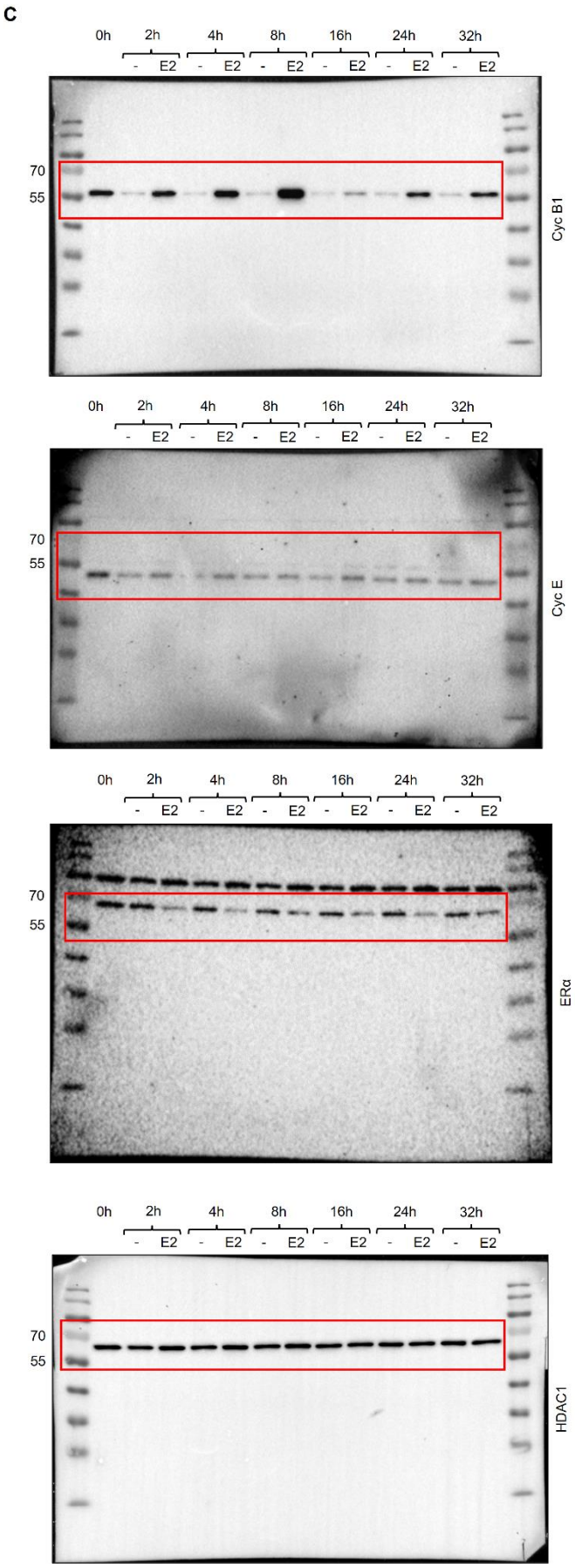

Figure 8 – Uncropped Images

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