
Figures and figure supplements

Analysis of pulsed cisplatin signalling dynamics identifies effectors of resistance in lung adenocarcinoma

Jordan F Hastings et al

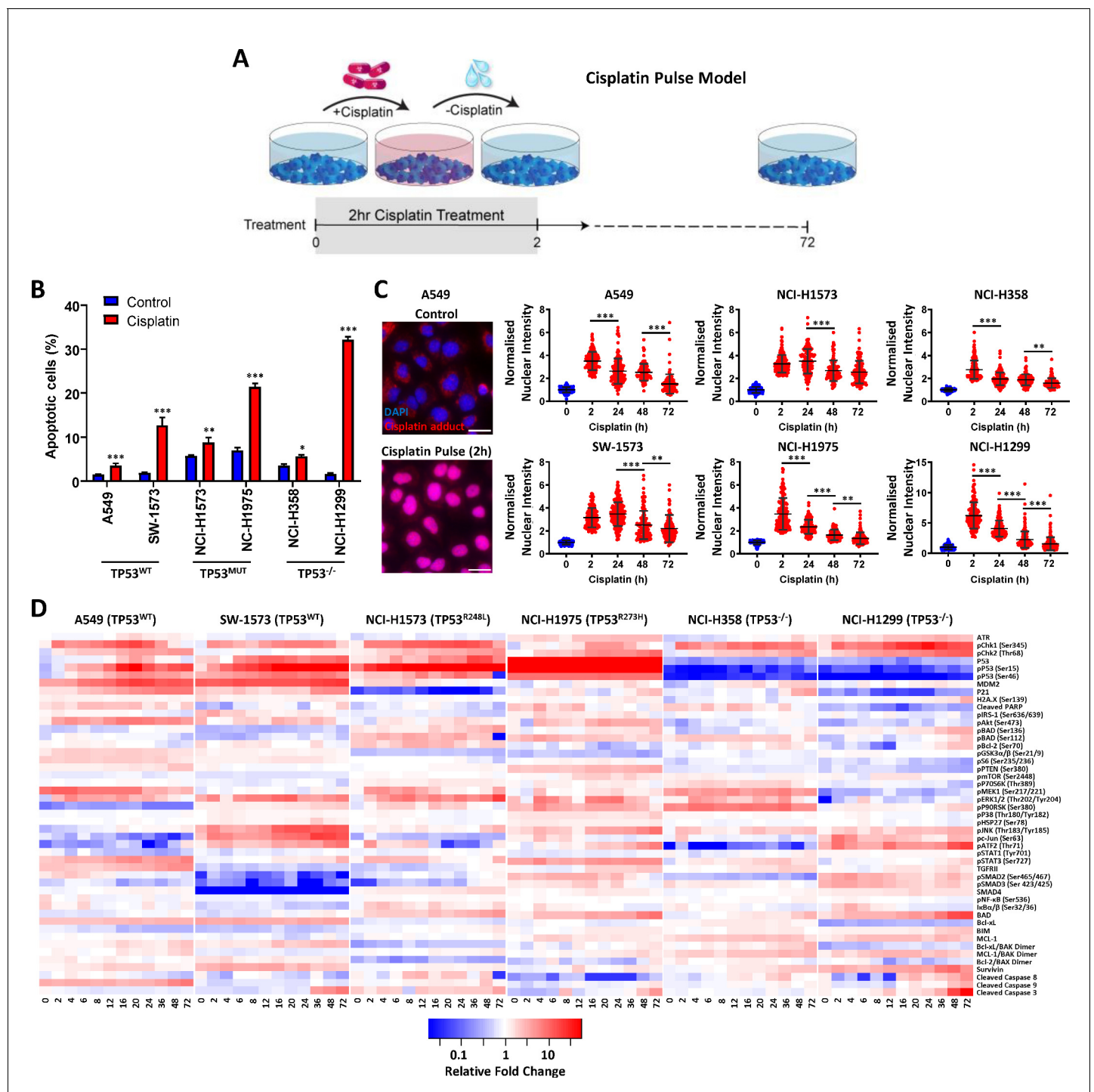


Figure 1. Multiplexed analysis of cisplatin-induced signalling. (A) Schematic of the cisplatin pulse model (5 µg/mL, 2 hr) and continuous pulse model (5 µg/mL, 72 hr). (B) Apoptosis measured by propidium iodide staining for the sub-G1 population, performed 72 hr following a cisplatin pulse across a panel of lung adenocarcinoma cell lines, as indicated ($n = 3$, mean \pm SD). Statistical significance was determined by t-test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (C) Representative images of anti-cisplatin antibody staining in A549 cells following a cisplatin pulse, and quantification of nuclear cisplatin-DNA adducts across the cell line panel ($n \geq 100$, mean \pm SD). Nuclear staining intensity was normalized to background, cytoplasmic staining within each cell line. Statistical significance was determined by one-way ANOVA (*** $p < 0.001$, ** $p < 0.01$). All treatment conditions (red) are significantly different from control (blue), $p < 0.001$. (D) Multiplexed analysis of DNA damage, apoptosis and signalling pathways following a cisplatin pulse across a panel of lung adenocarcinoma cell lines, as indicated ($n = 3$, mean).

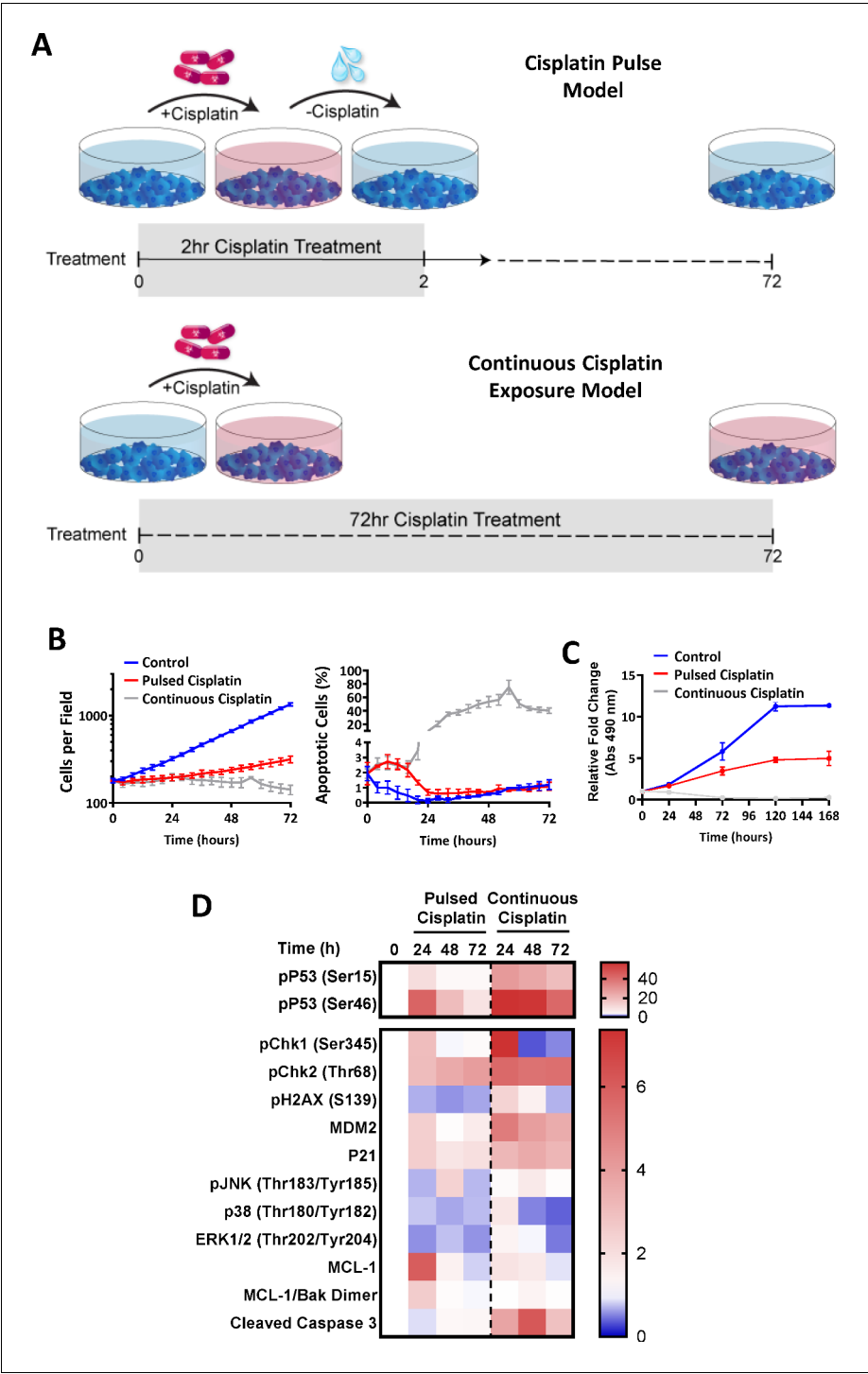


Figure 1—figure supplement 1. Continuous versus pulsed cisplatin treatment of A549 cells. (A) Schematic of the cisplatin pulse model (5 $\mu\text{g}/\text{mL}$, 2 hr) and continuous pulse model (5 $\mu\text{g}/\text{mL}$, 72 hr). (B) Live-cell imaging of A549 cells treated either continuously, or with a cisplatin pulse. Apoptotic cells were identified by uptake of propidium iodide (mean \pm SD). (C) MTS Proliferation assay performed on A549 cells treated either continuously, or with a cisplatin pulse (mean \pm SD, $n = 6$). (D) Multiplexed analysis of key DNA damage, apoptosis and signalling proteins in A549 cells treated either continuously, or with a cisplatin pulse ($n = 3$, mean).

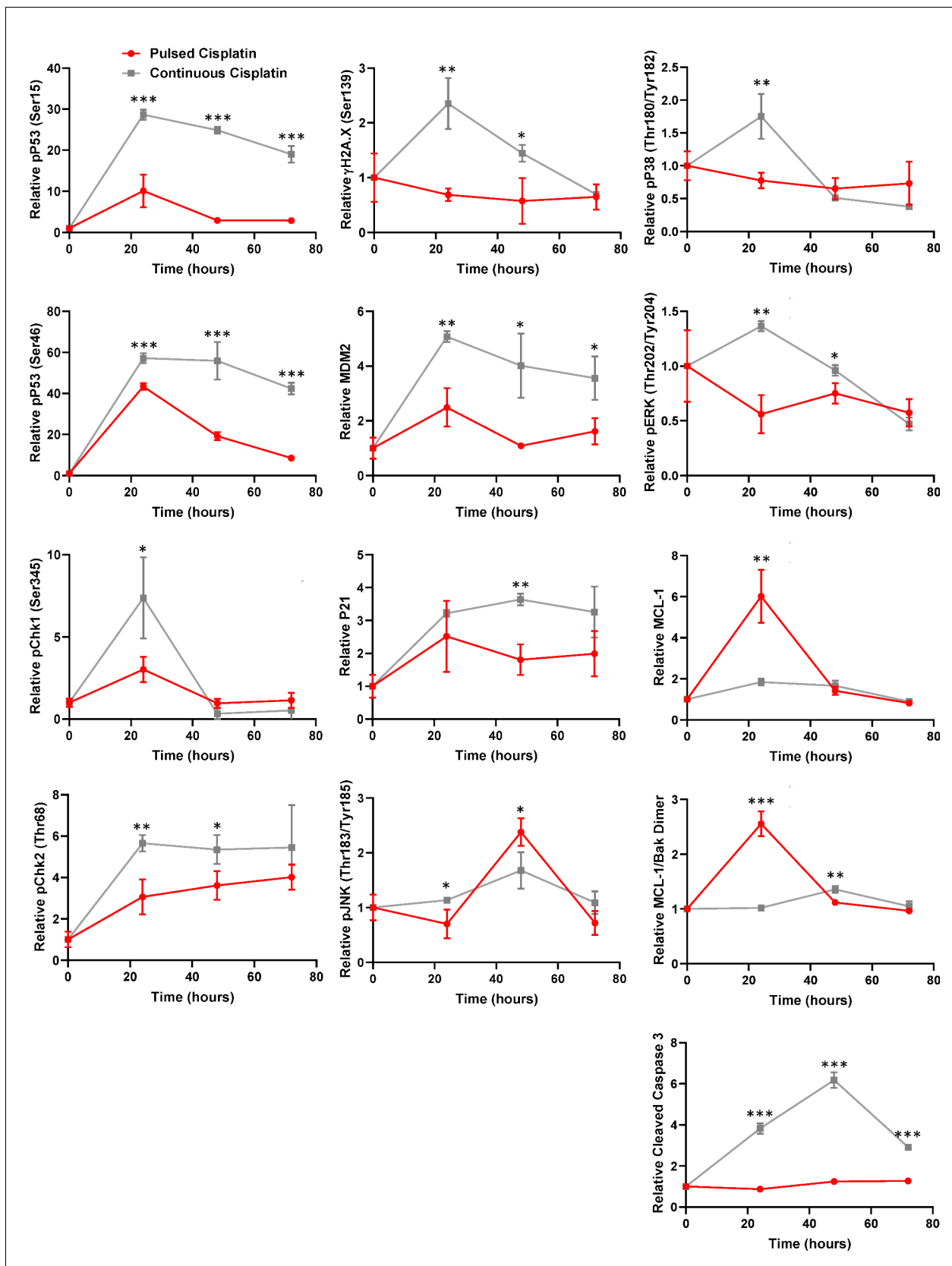


Figure 1—figure supplement 2. Continuous versus pulsed cisplatin treatment of A549 cells. Raw data for the timecourse, multiplex analysis of DNA damage response proteins following continuous cisplatin treatment (grey) or a cisplatin pulse (red) (5 μ g/mL, 2 hr) in A549 cells. Statistical significance was determined by Student's t-test ($n = 3$, mean \pm SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

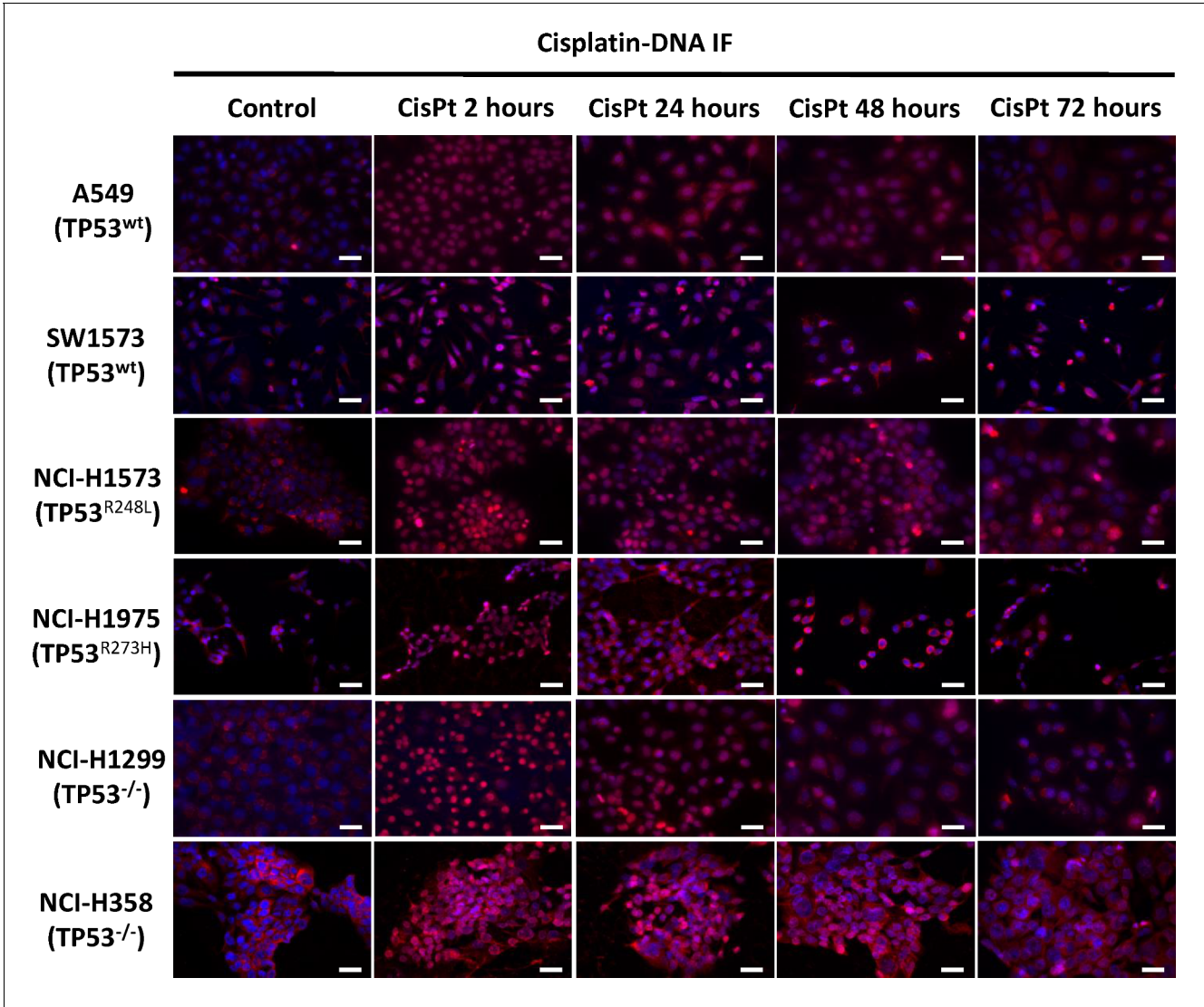


Figure 1—figure supplement 3. Imaging of cisplatin-DNA adducts. Representative images of anti-cisplatin antibody staining across the cell line panel following a cisplatin pulse (5 µg/mL, 2 hr). Scale bar: 40 µm.

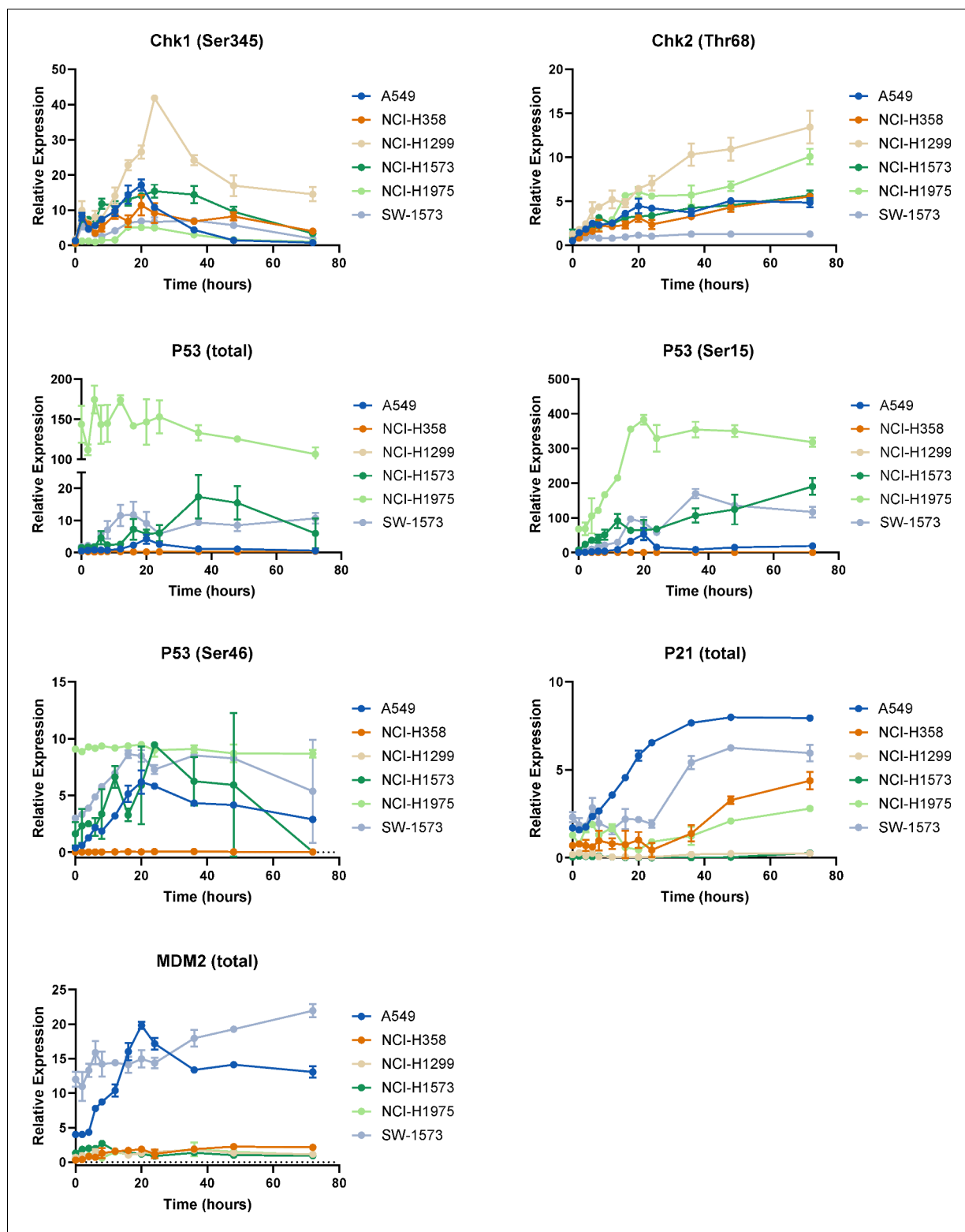


Figure 1—figure supplement 4. p53 pathway dynamics. Raw data for the timecourse, multiplex analysis of DNA damage response proteins following a cisplatin pulse (5 $\mu\text{g/mL}$, 2 hr) across a panel of cell lines, as indicated ($n = 3$, mean \pm SD).

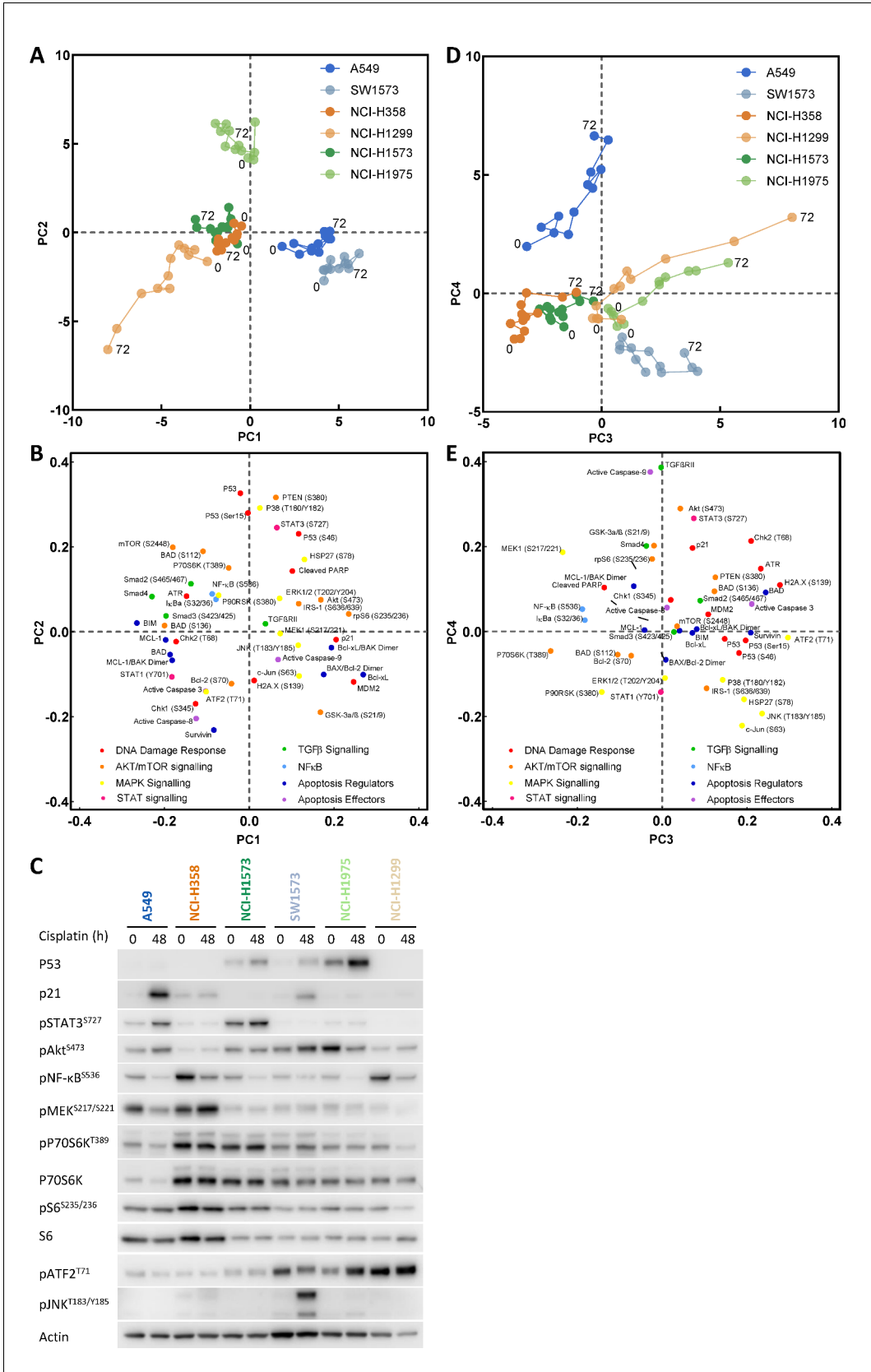


Figure 2. Principal component analysis. (A) Visualisation of principal component 1 (PC1) against component 2 (PC2) for the principal component analysis of cisplatin induced signalling across the cell line panel. (B) Distribution of the analytes according to their weighting within PC1 and PC2. (C) Figure 2 continued on next page

Figure 2 continued

Western blotting for selected analytes across the cell panel prior to, and 48 hr post a cisplatin pulse. (D) Visualisation of principal component 3 (PC3) against component 4 (PC4) for the principal component analysis of cisplatin induced signalling across the cell line panel. (E) Distribution of the analytes according to their weighting within PC3 and PC4.

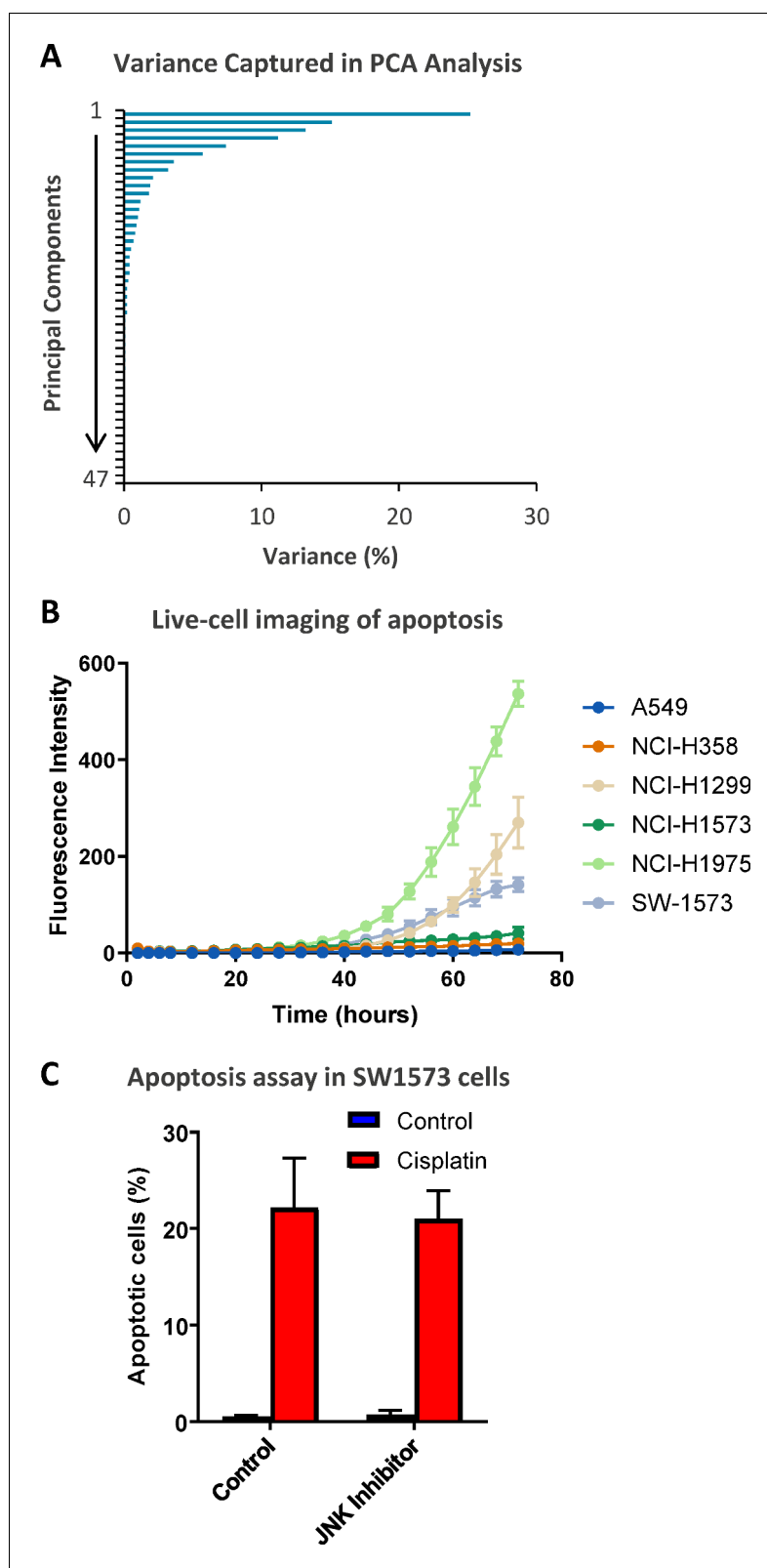


Figure 2—figure supplement 1. Extended analysis of the PCA data. (A) The variance captured within each principal component of the principal component analysis (PCA), presented in **Figure 2**. (B) Live-cell imaging of apoptosis across the cell lines indicated on the Incucyte platform using a fluorescent caspase substrate (1 μ M) ($n = 3$, mean \pm SD). (C) Apoptosis measured by propidium iodide staining for the sub-G1 population in SW1573 cells. Figure 2—figure supplement 1 continued on next page

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cells, performed 72 hr following a cisplatin pulse (5 μ g/mL, 2 hr) with the addition of a JNK inhibitor (JNK VIII, 20 μ M) as indicated ($n = 3$, mean \pm SD).

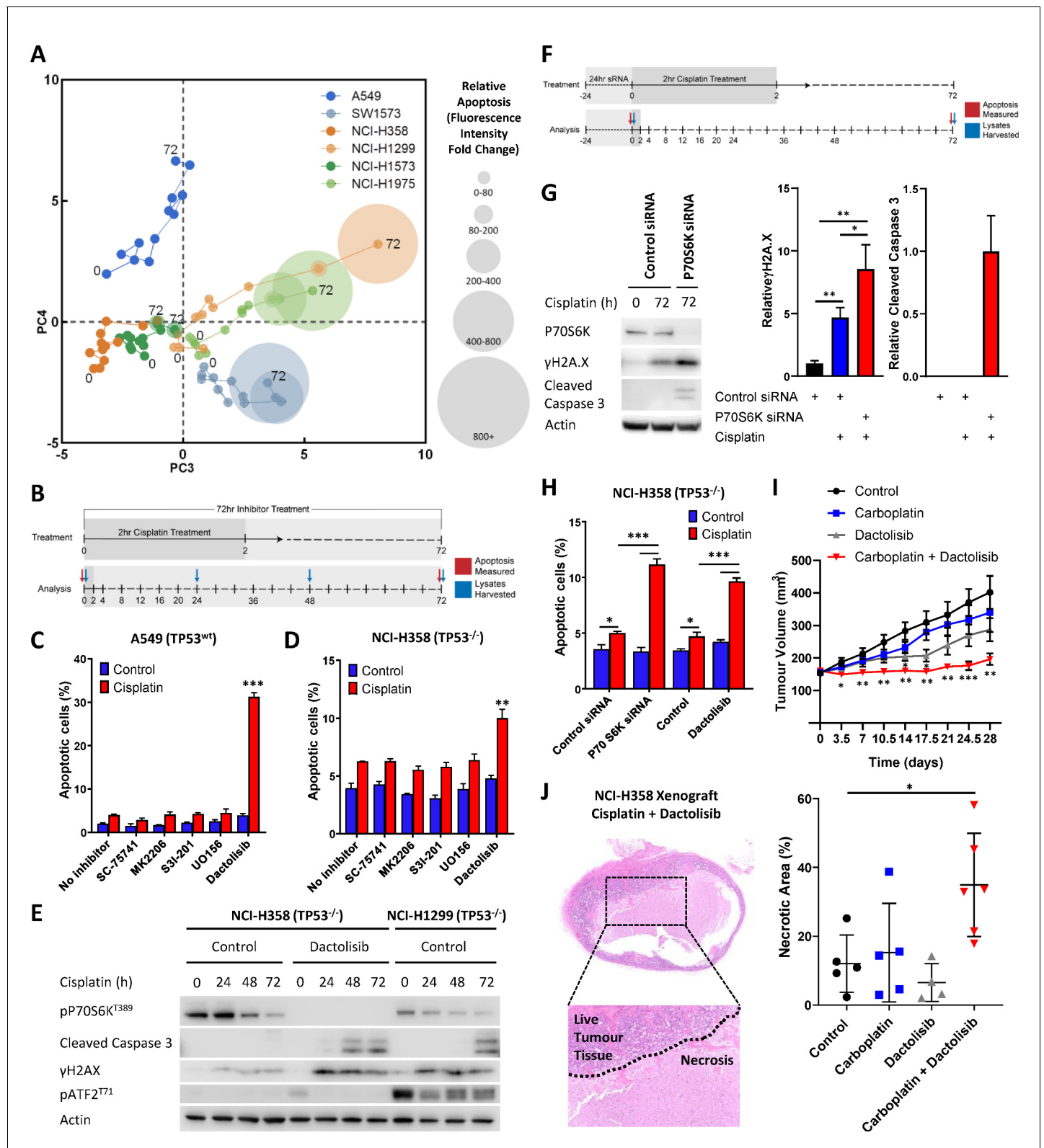


Figure 3. Visualising and targeting mechanisms of cisplatin resistance. (A) An overlay of real-time imaging of apoptosis following a cisplatin pulse, performed on the Incucyte platform using a fluorescent caspase substrate (1 μ M), on top of the PCA plot of component 3 against component 4. (B) Schematic of the cisplatin pulse model, with the addition of small molecule inhibitors, and outline of sample collection for apoptosis assays and western blotting. (C,D) Apoptosis measured by propidium iodide staining for the sub-G1 population in A549 and NCI-H358 cells, performed 72 hr following a cisplatin pulse. (E) Western blot analysis of pP70S6K^{T389}, Cleaved Caspase 3, γ H2AX, pATF2^{T71}, and Actin in NCI-H358 (TP53^{-/-}) and NCI-H1299 (TP53^{-/-}) cells treated with cisplatin and dactolisib. (F) Timeline of treatment and analysis. (G) Western blot and bar graphs showing relative H2A.X and cleaved caspase 3 levels in cells treated with control siRNA, P70S6K siRNA, and cisplatin. (H) Apoptosis measured by propidium iodide staining in NCI-H358 (TP53^{-/-}) cells treated with control, cisplatin, or dactolisib. (I) Tumour volume over time in xenografts treated with control, carboplatin, dactolisib, or carboplatin + dactolisib. (J) Necrotic area in xenografts treated with control, carboplatin, dactolisib, or carboplatin + dactolisib.

Figure 3 continued

cisplatin pulse with the addition of small molecule inhibitors (1 μ M) as indicated ($n = 3$, mean \pm SD). Statistical significance was determined by t-test (** $p < 0.001$, ** $p < 0.01$). (E) Western blotting on lysates from NCI-H358 and NCI-H1299 cells following a cisplatin pulse, with the addition of dactolisib (1 μ M), as indicated. (F) Schematic of the cisplatin pulse model, with the addition of siRNA pre-treatment, and outline of sample collection for apoptosis assays and western blotting. (G) Western blotting on lysates from NCI-H358 cells, treated with P70S6K or control siRNA, as indicated, prior to and following a cisplatin pulse ($n = 3$, mean \pm SD). Statistical significance was determined by one-way ANOVA (** $p < 0.01$, * $p < 0.05$). (H) Apoptosis measured by propidium iodide staining for the sub-G1 population in NCI-H358 cells, treated with P70S6K, control siRNA or dactolisib, as indicated, performed 72 hr following a cisplatin pulse ($n = 3$, mean \pm SD). Statistical significance was determined by one-way ANOVA (** $p < 0.001$, * $p < 0.05$). (I) Tumour growth in nude mice bearing NCI-H358 xenografts with continuous treatment of vehicle control or dactolisib (45 mg/kg) prior to, and following a single dose of carboplatin (60 mg/kg) ($n \geq 4$, mean \pm SEM). Statistical significance was determined by one-way ANOVA at each time point (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (J) Quantification of necrosis in NCI-H358 xenografts following the treatment described in (I) ($n \geq 4$, mean \pm SD). Statistical significance was determined by one-way ANOVA * $p < 0.05$).

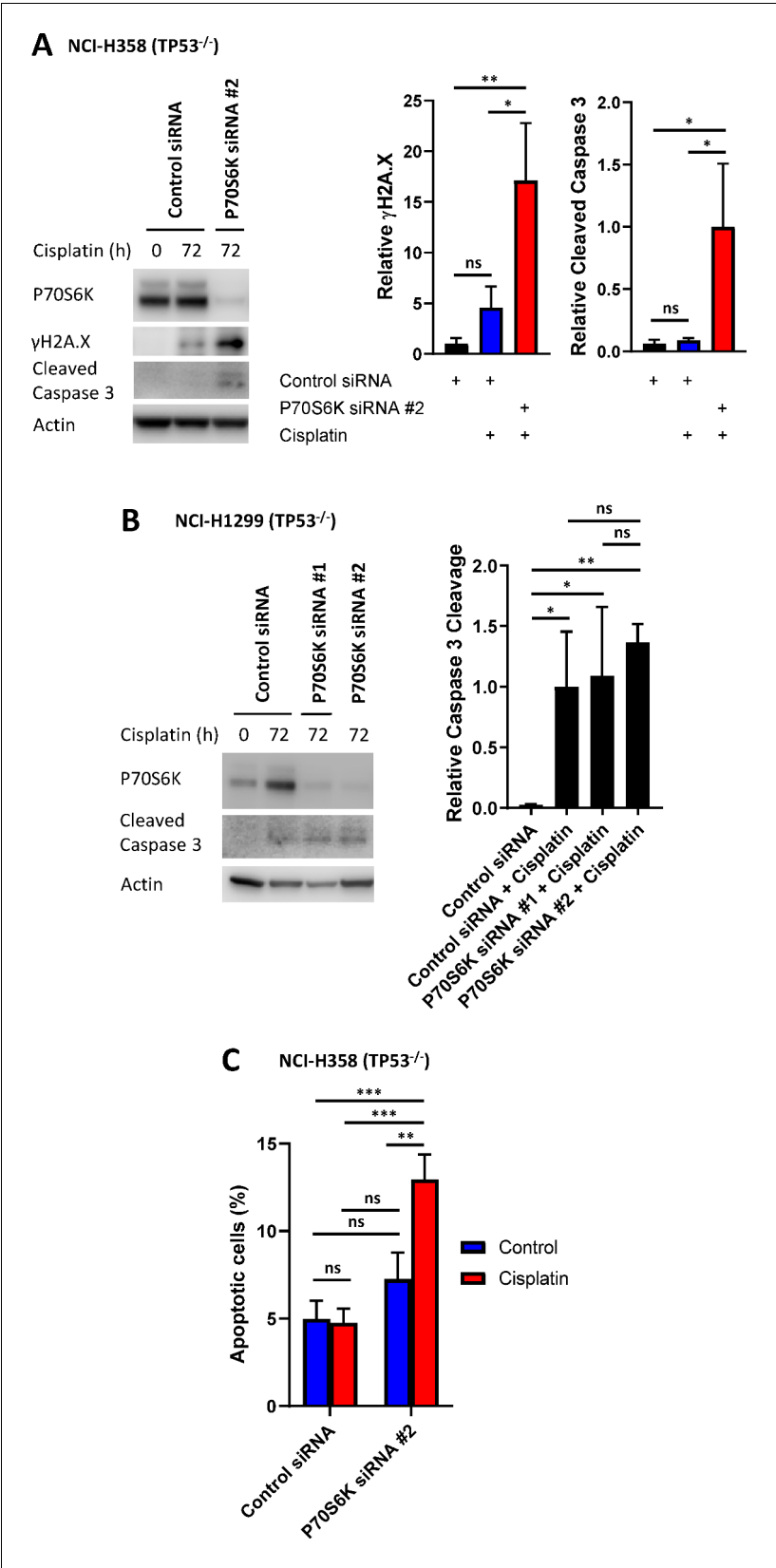


Figure 3—figure supplement 1. P70S6K knockdown in NCI-H358 and NCI-H1299 cells. (A) Western blotting on lysates from NCI-H358 cells, treated with P70S6K or control siRNA, as indicated, prior to and following a cisplatin

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Figure 3—figure supplement 1 continued

pulse (5 $\mu\text{g/mL}$, 2 hr). Quantification was performed on three individual replicates ($n = 3$, mean \pm SD). Statistical significance was determined by one-way ANOVA (** $p < 0.01$, * $p < 0.05$). (B) Western blotting on lysates from NCI-H1299 cells, treated with P70S6K or control siRNA, as indicated, prior to and following a cisplatin pulse (5 $\mu\text{g/mL}$, 2 hr). Quantification was performed on three individual replicates ($n = 3$, mean \pm SD). Statistical significance was determined by one-way ANOVA (** $p < 0.01$, * $p < 0.05$). (C) Apoptosis measured by propidium iodide staining for the sub-G1 population in NCI-H358 cells, treated with P70S6K or control siRNA, as indicated, performed 72 hr following a cisplatin pulse ($n = 3$, mean \pm SD). Statistical significance was determined by one-way ANOVA (** $p < 0.001$, * $p < 0.05$).

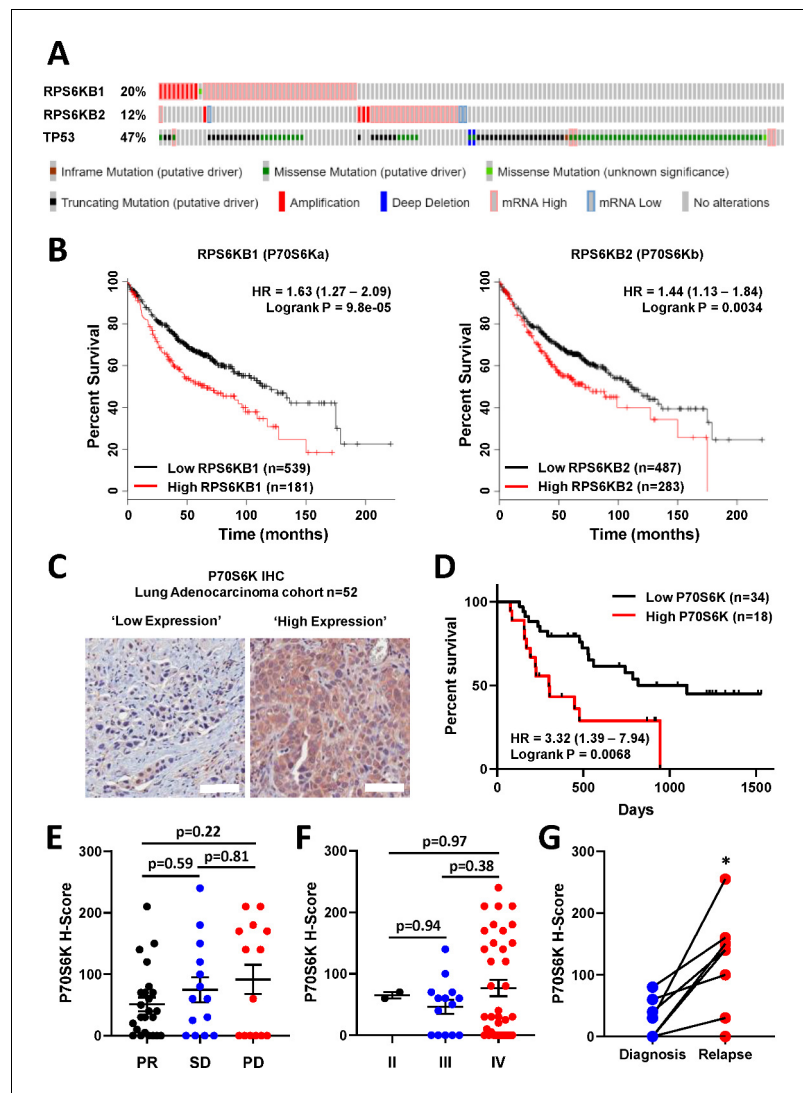


Figure 4. P70S6K in lung adenocarcinoma. (A) The frequency of somatic mutations and mRNA over-expression for the *RPS6KB1*, *RPS6KB2* and *TP53* genes in a publicly available cohort of lung adenocarcinoma patients (cBioPortal). (B) The association between *RPS6KB1* and *RPS6KB2* mRNA expression and overall patient survival in a publicly available patient cohort (KM Plotter). Statistical significance was determined by log rank test. (C) Representative images of P70S6K immuno-histochemistry staining from a cohort of 52 lung adenocarcinoma patients (Scale bar = 100 μ M). (D) Survival analysis based upon P70S6K staining in this cohort. Statistical significance was determined by log rank test. (E) The association between tumour P70S6K staining intensity (H-Score) and patients that underwent a partial response (PR), or presented with stable disease (SD) or progressive disease (PD). Statistical significance was determined by one-way ANOVA, (F) The association between tumour P70S6K staining intensity and tumour stage. Statistical significance was determined by one-way ANOVA. (G) P70S6K staining in eight matched patient samples from diagnosis and relapse (n = 8). Statistical significance was determined by t-test (* $p < 0.05$).

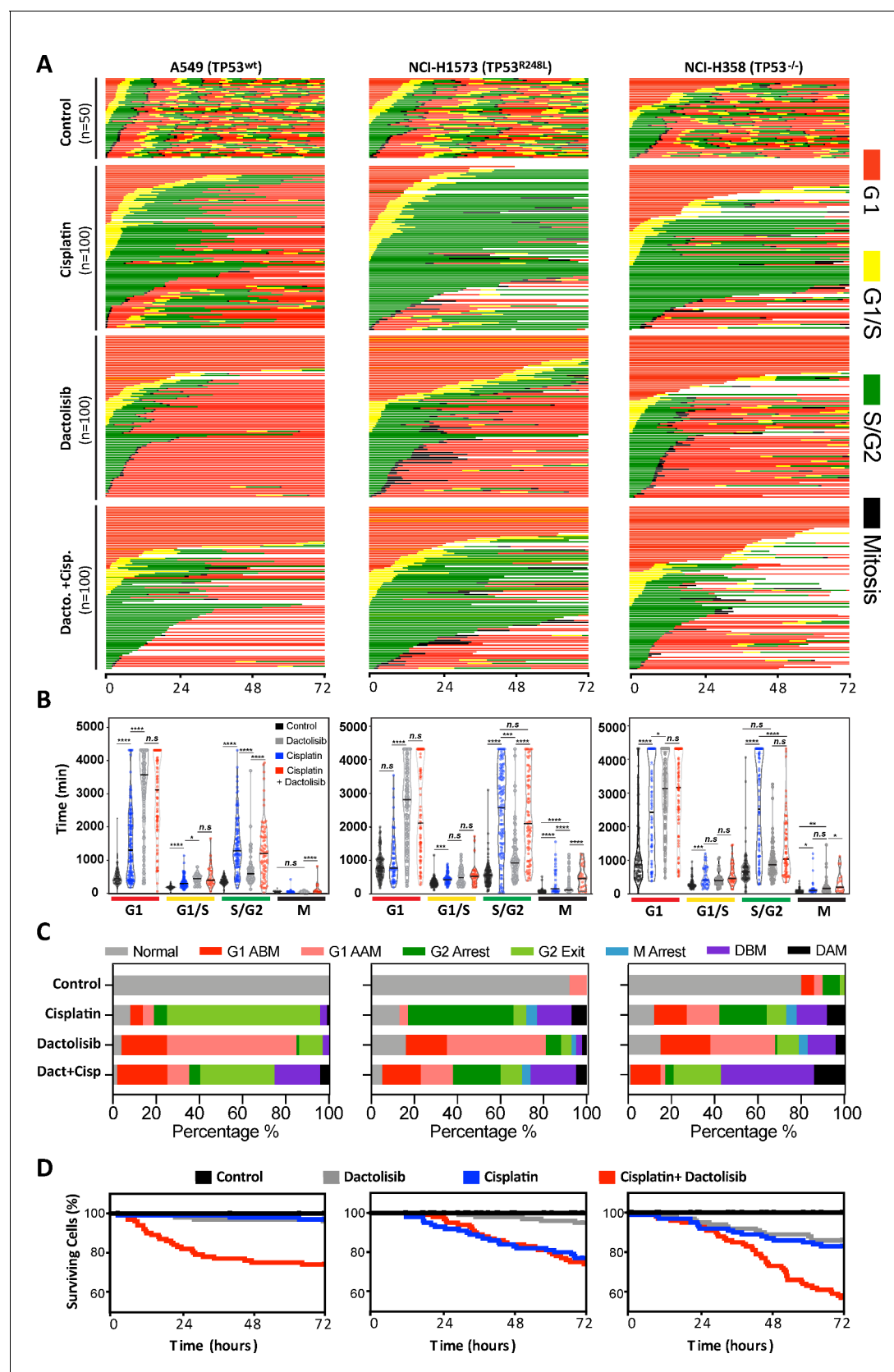


Figure 5. FUCCI analysis of cell cycle progression. (A) Live-cell imaging of the FUCCI biosensor proteins mVenus-hGeminin(1/110) and mCherry-hCdt1 (30/120), stably expressed by the A549, NCI-H1573 and NCI-H358 cell lines. Images were taken every 20 min for 72 hr under control conditions, or

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Figure 5 continued

following a cisplatin pulse (5 $\mu\text{g}/\text{mL}$, 2 hr) in the presence or absence of dactolisib (1 μM). (B) Quantification of the length of each cell cycle phase under each treatment condition ($n = 17\text{--}175$, mean \pm SD). Statistical significance was determined by one-way ANOVA (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (C) Quantification of fate of each cell; including G1 arrest before mitosis (G1 ABM), G1 arrest after mitosis (G1 AAM), death before mitosis (DBM) and death after mitosis (DAM). (D) Survival curves indicating the proportion of viable cells over time under each treatment condition.

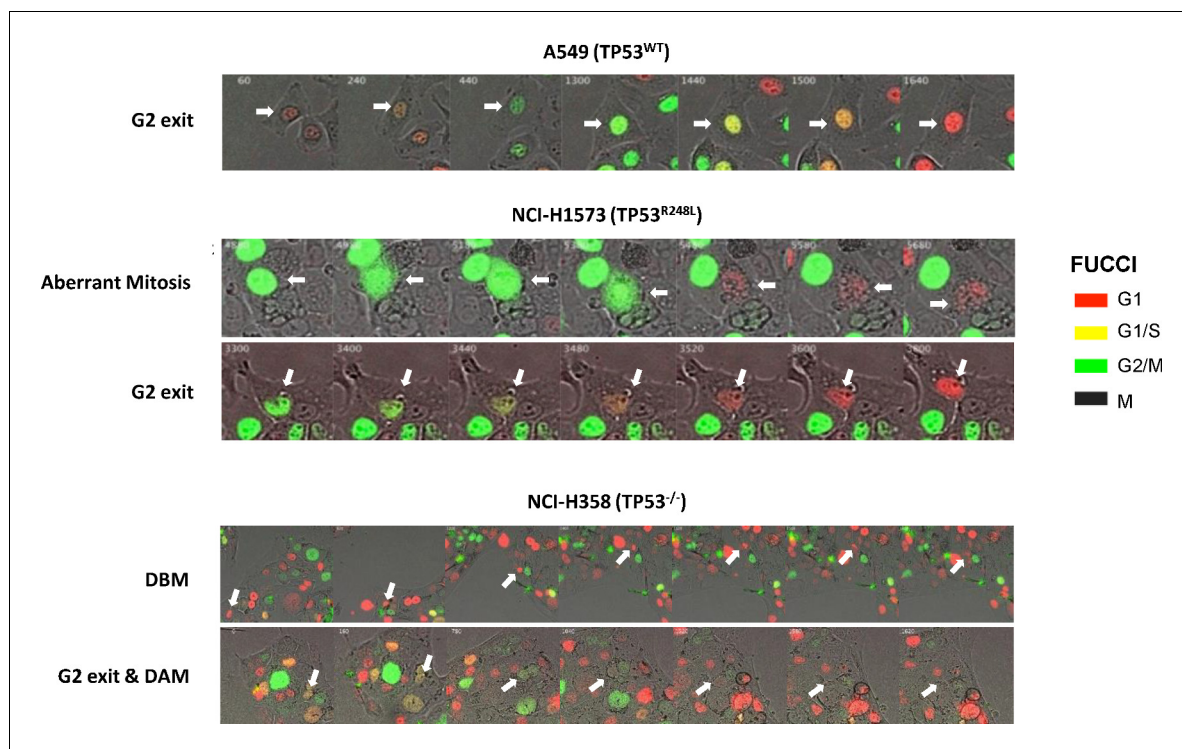


Figure 5—figure supplement 1. Representative images of cells expressing the FUCCI biosensor, undergoing an aberrant mitosis, G2 exit, Death before Mitosis (DBM) and Death after Mitosis (DAM) following a cisplatin pulse (5 µg/mL, 2 hr).

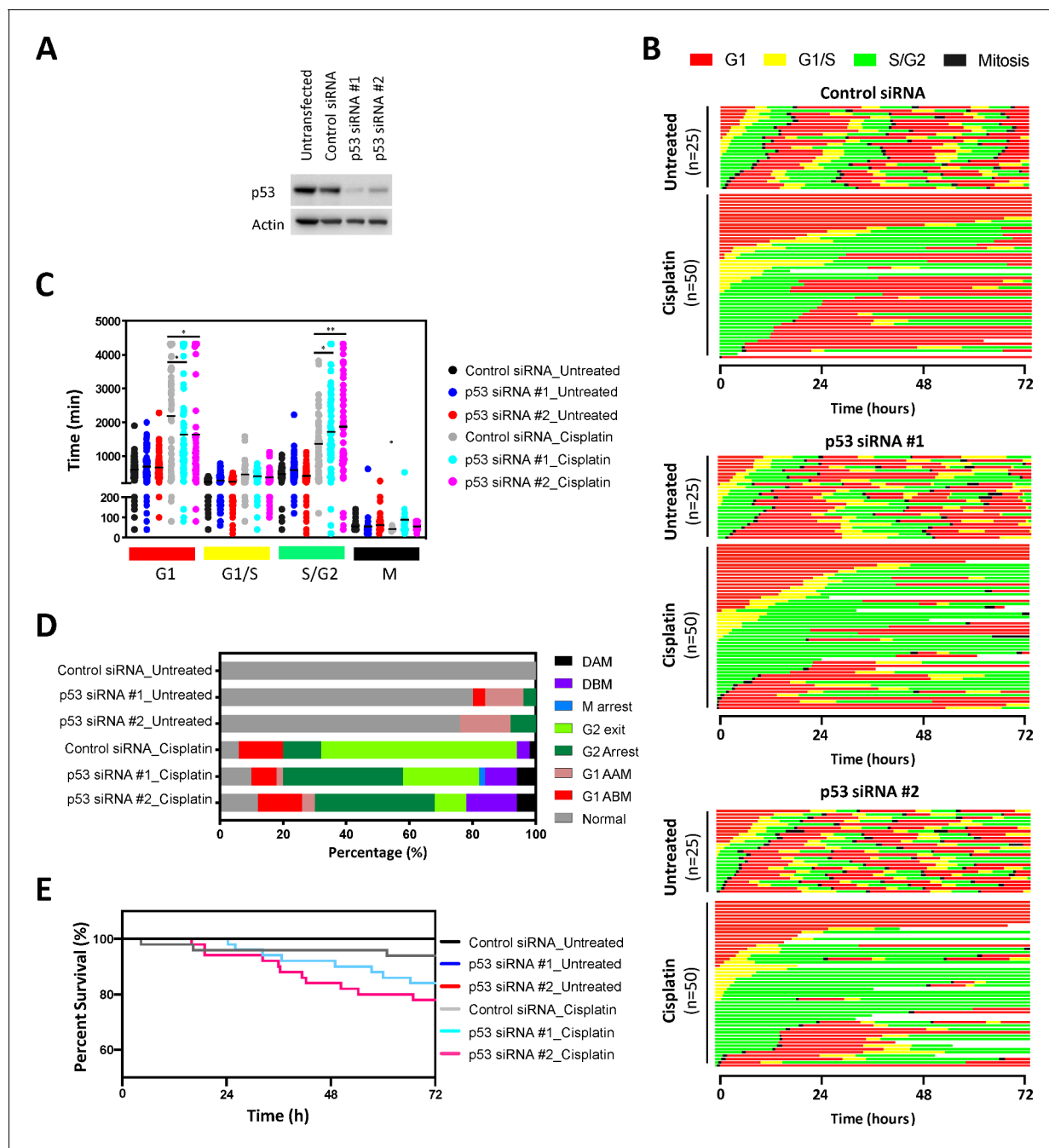


Figure 5—figure supplement 2. FUCCI analysis following p53 knockdown. (A) Western blot confirming specific knockdown of p53 with two different siRNAs. (B) Live-cell imaging of the FUCCI biosensor proteins mVenus-hGeminin(1/110) and mCherry-hCdt1(30/120), stably expressed by the A549 cell line following 48 hr treatment with control or p53 targeting siRNA, as indicated. Images were taken every 20 min for 72 hr under control conditions, or following a cisplatin pulse (5 μ g/mL, 2 hr). (C) Quantification of the length of each cell cycle phase under each treatment condition. Statistical significance was determined by one-way ANOVA (**** p <0.0001, *** p <0.001, ** p <0.01, * p <0.05). (D) Quantification of fate of each cell; including G1 arrest before mitosis (G1 ABM), G1 arrest after mitosis (G1 AAM), death before mitosis (DBM) and death after mitosis (DAM). (E) Survival curves indicating the proportion of viable cells over time under each treatment condition.

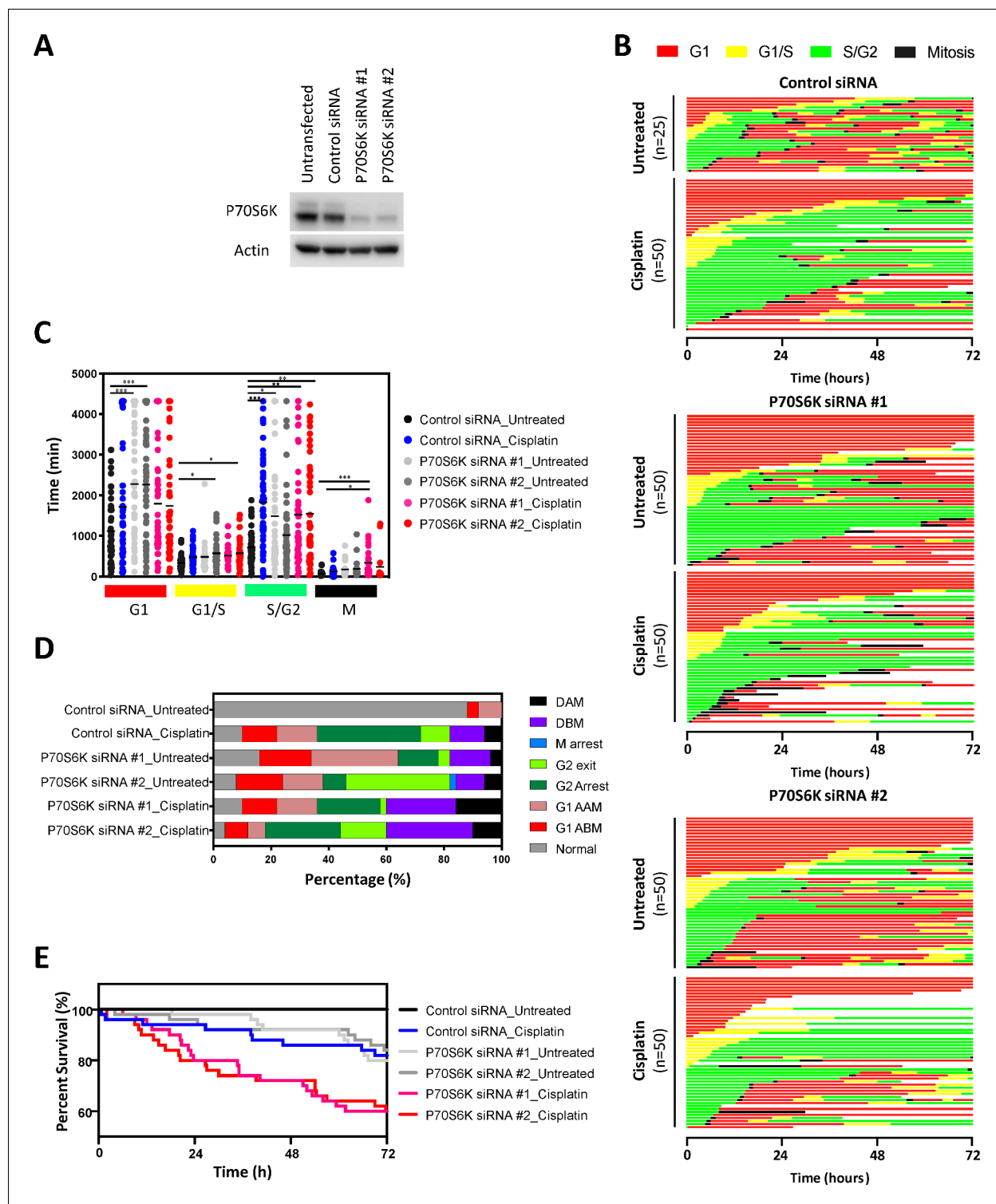


Figure 5—figure supplement 3. Fucci analysis following P70S6K knockdown. (A) Western blot confirming specific knockdown of P70S6K with two different siRNAs. (B) Live-cell imaging of the Fucci biosensor proteins mVenus-hGeminin(1/110) and mCherry-hCdt1(30/120), stably expressed by the NCI-H358 cell line following 48 hr treatment with control or P70S6K targeting siRNA, as indicated. Images were taken every 20 min for 72 hr under control conditions, or following a cisplatin pulse (5 $\mu\text{g}/\text{mL}$, 2 hr) in the presence or absence of dactolisib (1 μM). (C) Quantification of the length of each cell cycle phase under each treatment condition ($n = 17\text{--}175$, mean \pm SD). Statistical significance was determined by one-way ANOVA (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (D) Quantification of fate of each cell; including G1 arrest before mitosis (G1 ABM), G1 arrest after mitosis (G1 AAM), death before mitosis (DBM) and death after mitosis (DAM). (E) Survival curves indicating the proportion of viable cells over time under each treatment condition.

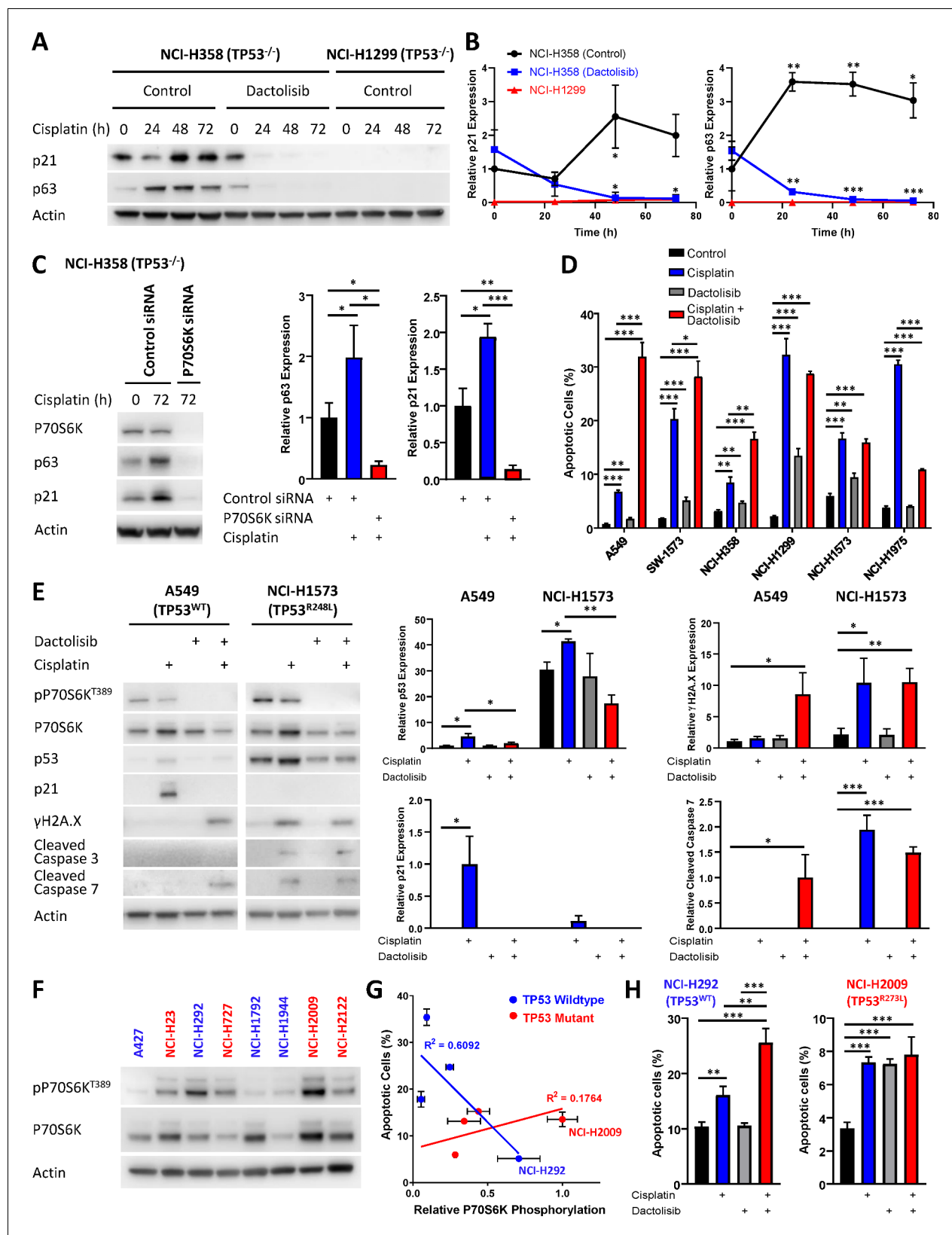


Figure 6. Response to dactolisib is dependent upon *TP53* mutation status. (A) Western blotting on lysates from NCI-H358 and NCI-H1299 cells following a cisplatin pulse, with the addition of dactolisib (1 μ M), as indicated. (B) Quantification of effects of dactolisib upon cisplatin induced p63 and p21 expression ($n = 3$, mean \pm SD). (C) Western blotting on lysates from NCI-H358 cells, treated with P70S6K or control siRNA, as indicated, prior to cisplatin treatment. (D) Quantification of effects of dactolisib upon cisplatin induced apoptosis. (E) Western blotting on lysates from A549 and NCI-H1573 cells, treated with dactolisib and cisplatin, as indicated. (F) Western blotting on lysates from various cell lines, treated with dactolisib and cisplatin, as indicated. (G) Quantification of effects of dactolisib upon cisplatin induced apoptosis. (H) Quantification of effects of dactolisib upon cisplatin induced apoptosis. Figure 6 continued on next page

Figure 6 continued

and following a cisplatin pulse ($n = 3$, mean \pm SD). (D) Apoptosis measured by propidium iodide staining for the sub-G1 population performed 72 hr following a cisplatin pulse with the addition of dactolisib ($1 \mu\text{M}$) as indicated ($n = 3$, mean \pm SD). (E) Western blotting on lysates from A549 and NCI-H1573 cells, 48 hr following a cisplatin pulse, with the addition of dactolisib ($1 \mu\text{M}$), as indicated ($n = 3$, mean \pm SD). (F) Western blotting across a second panel of lung adenocarcinoma cell lines. (G) Correlation between P70S6K phosphorylation and apoptosis, as measured by propidium iodide staining for the sub-G1 population, performed 72 hr following a cisplatin pulse ($n = 3$, mean \pm SD). For all panels the statistical significance was determined by one-way ANOVA (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

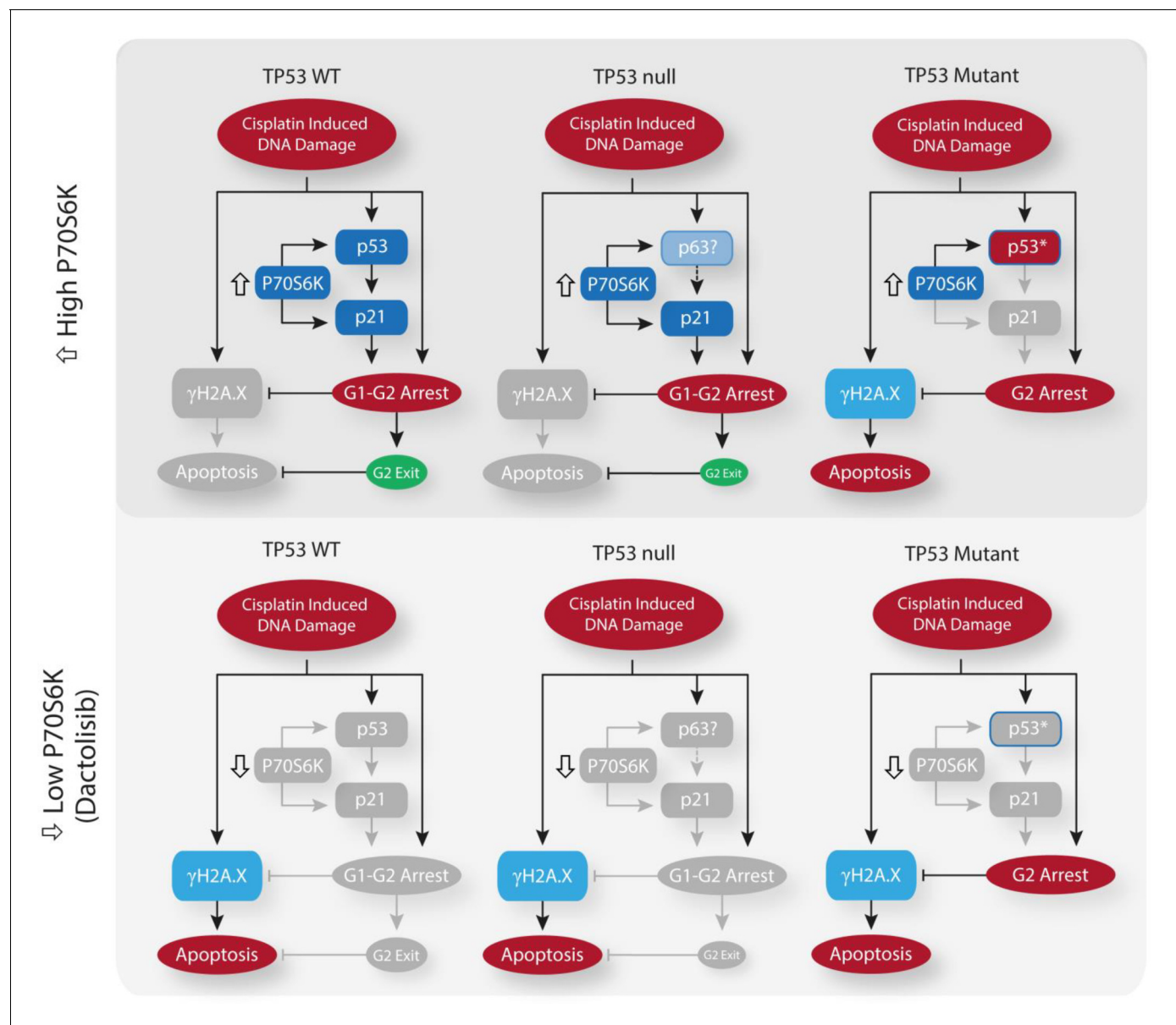


Figure 7. Schematic outlining the effect of cisplatin on cell cycle arrest, DNA damage and apoptosis across the spectrum of TP53 mutation states, and the influence of P70S6K expression levels or inhibition upon these processes.