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Supplemental Information

An mTORC1-to-CDK1 Switch Maintains Autophagy Suppression during Mitosis

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Supplementary Figure Legends

Figure S1 (accompanying main figure 1): Autophagy initiation is repressed during mitosis (A) Montage from movie S1. HEK-293 cells expressing the tandem LC3 reporter mRFP-EGFP-LC3 were treated with paclitaxel for 4 hours prior to transfer to live-cell imaging stage. Representative images from before and one hour after addition of AZD8055 are shown (1 μM). Images from two fields of view are shown, with mitotically arrested cell indicated with arrow. **(B)** Asynchronous HEK293 GFP-ATG13 cells were either left untreated, treated with AZD8055 (1 μM), or incubated in starvation media for 2 hours. **(C)** Asynchronous HeLa cells were either left untreated, treated with AZD8055 (1 μM), or incubated in starvation media for 2 hours. Primary antibodies used: WIPI2 (green), p-S10 H3 (magenta). Scale bars: 20 μm

Figure S2 (accompanying Figure 2): Hyperphosphorylated Raptor is localised predominantly to the cytosol, not membrane, fraction. HeLa cells were treated with paclitaxel (t=16hrs; 50nM) and/ AZD8055 (t=2 hrs; 1 μM). Lysates were then fractionated into membrane and cytosol enriched fractions, with LAMP2 and ERK1/2 serving as the respective loading controls. Western blots are from a single biological replicate representative of three independent experiments.

Figure S3 (accompanying Figure 3): The EG5 inhibitor dimethylenastron also promotes hyperphosphorylation of autophagy regulators. (a) HCT116 cells were treated with Dimethylenastron (1 μM) for 16hrs. Two hours prior to lysis, cells were treated with either DMSO control or RO-3306 (2 μM). Western blots are from a single experiment representative of three independent experiments.

Figure S4 (accompanying Figure 4): Mitotic phosphorylation of autophagy regulators is observed across a diverse panel of cell lines. Cells were treated with Paclitaxel (50 nM) for 16hrs. Two hours prior to lysis cells were treated with either DMSO, RO-3306 (2 μM; CDK1 inhibitor), Trametinib (1 μM; MEK inhibitor) or AZD8055 (1 μM; mTOR inhibitor). Cell lines used were as follows: COLO205 (A), A549 (B), HEK293 (C), HT29 (D). Western blots are from a single experiment representative of three independent experiments.

Figure S5 (accompanying figure 4): Paclitaxel-induced hyperphosphorylation of autophagy regulators can be shown to be mTORC1 independent by multiple methodologies. (A) SW620 and 8055R cells were cultured in their respective media and treated with paclitaxel (16 hrs; 50 nM). **(B)** CO115 cells were transfected with indicated siRNa constructs (25nM) for 48 hours. **(C)** HCT116 cells were treated with Paclitaxel (50 nM) for 16 hrs. Two hours prior to lysis cells were treated with either DMSO or ZSTK474 (1μM; PI3K class I/II inhibitor) and had their media maintained or swapped for HBSS + 1% BSA. **(D)** HeLa cells stably expressing either wild-type (WT) or Δ30-TFEB-GFP were treated with AZD8055 (1μM) or incubated in starvation media for 2 hours. An antibody against Lamp2 was used for visualisation of lysosomes (magenta). **(E)** A representative mitotic WT-TFEB-GFP cell from the AZD8055 treatment arm of (D). Images are representative of the majority cells across two independent experiments. Scale bars: 20 μm **(F)** HeLa cells stably expressing either wild-type (WT) or Δ30-TFEB-GFP were treated with paclitaxel (50nM; t=16hrs) prior to mitotic shake-off. Adherent interphase-enriched (I) and suspension mitosis-enriched (M) fractions are indicated. Western blots are from a single experiment representative of three independent experiments.

Figure S6 (accompanying Figure 5). HeLa cells also exhibit mTOR-independent hyperphosphorylation of autophagy regulators in response to paclitaxel. (A) Propidium iodide analysis of HT29 cells run in parallel with (Figure 5A). **(B)** HeLa cells were treated with

paclitaxel (t= 16hrs; 50 nM) and/ AZD8055 (t=2 hrs; 1 μM). Western blots were acquired using a fluorescent-based LiCOR Odyssey system and are from a single experiment representative of three independent experiments. **(C)** Panel demonstrates an example image (Untreated HAP1) of all the parameters utilised for analysis in (D). **(D)** Indicated cell lines were treated as indicated for two hours: paclitaxel (50nM), AZD8055 (1μM), HBSS + 1% BSA. Cells were then fixed and immunostained for p-ULK1 (S758) and p-H3 (S10). Representative P-ULK (S758) images from all cell lines and treatment conditions are shown. P-H3 (S10) positive cells are indicated by arrow for the other representative images. **(E)** Quantification of mean p-ULK1 (S758) intensity from (D) for different p-H3 (S10) sub-populations is shown. P-values calculated using Two-way Anova (Tukey). * $p<0.05$; ** $p<0.01$.

Figure S7 (accompanying Figure 6): Phosphorylation of ATG14 at S29, an ULK1 target site and critical autophagy initiation event, is repressed during mitosis.

(A) HCT116 cells were treated with paclitaxel (50nM; t=16hrs) and/or AZD8055 (1 μM; t=2hrs). (**B)** Quantification from fluorescent western blotting is provided. P-values calculated using One-way Anova (Tukey). Mean +/- S.D. across three independent experiments * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure S1 (related to Figure 1)

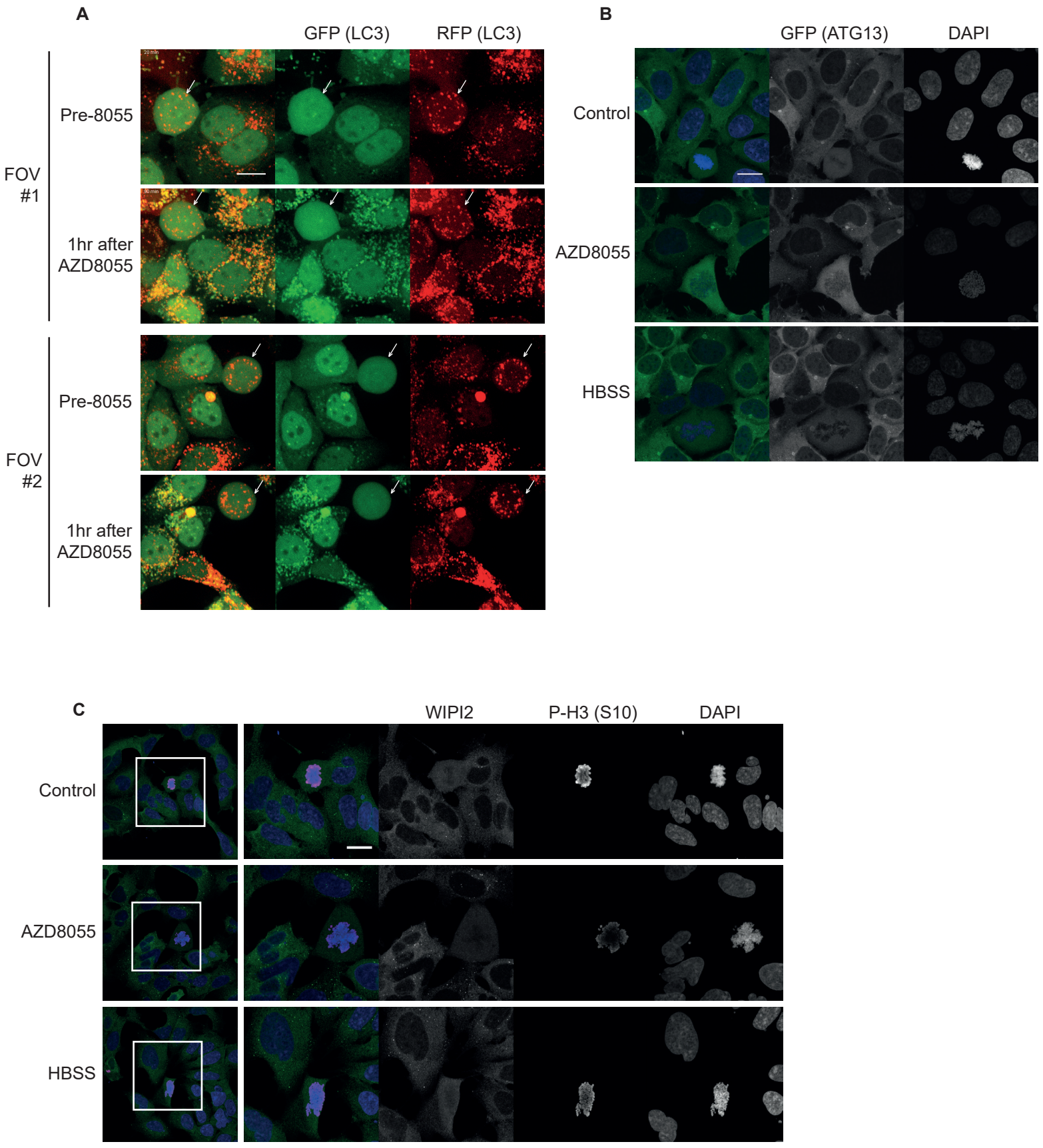


Figure S2 (related to Figure 2)

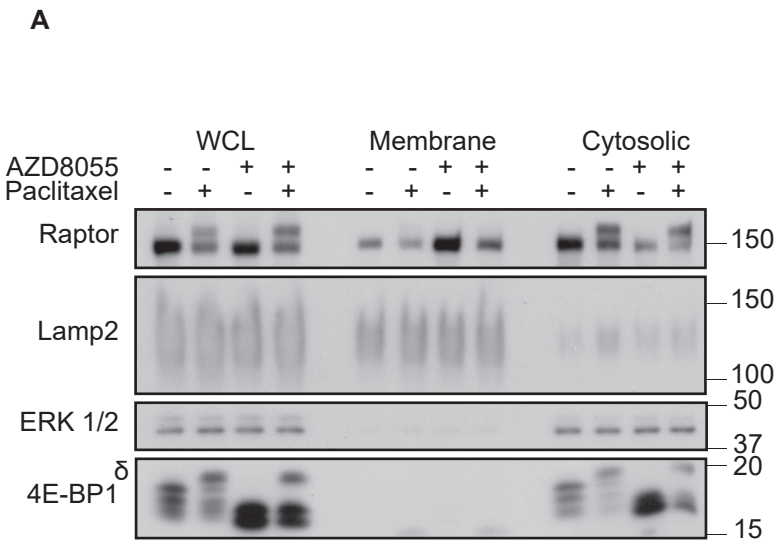


Figure S3 (related to Figure 3)

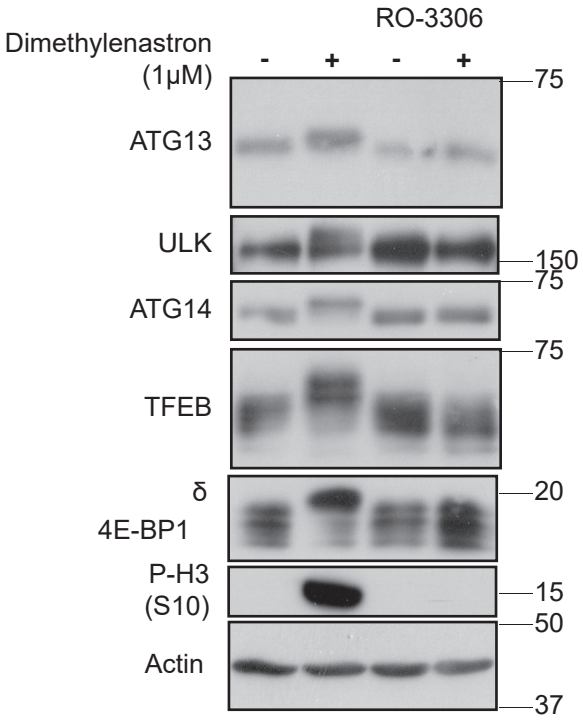


Figure S4 (related to Figure 4)

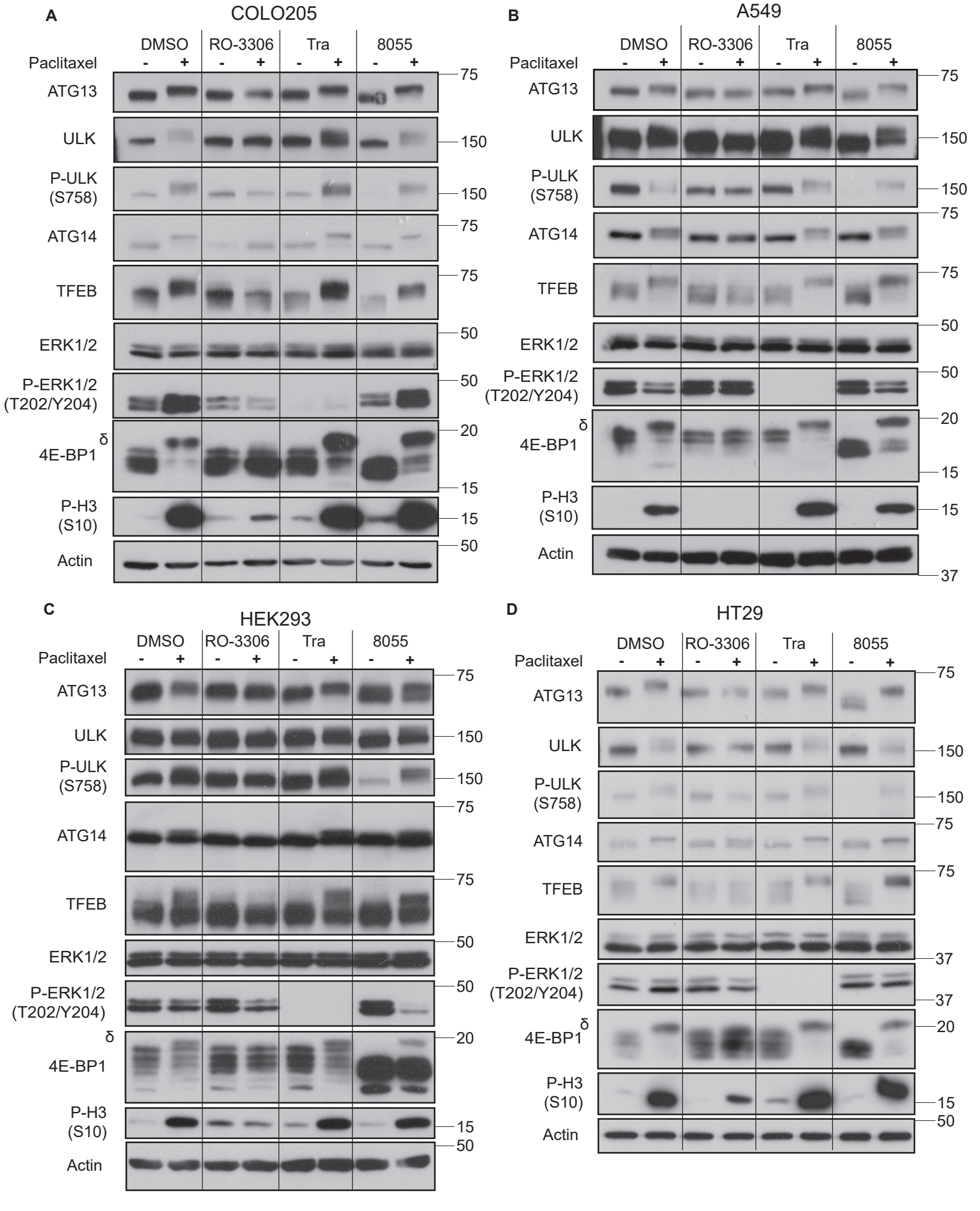


Figure S5 (related to Figure 4)

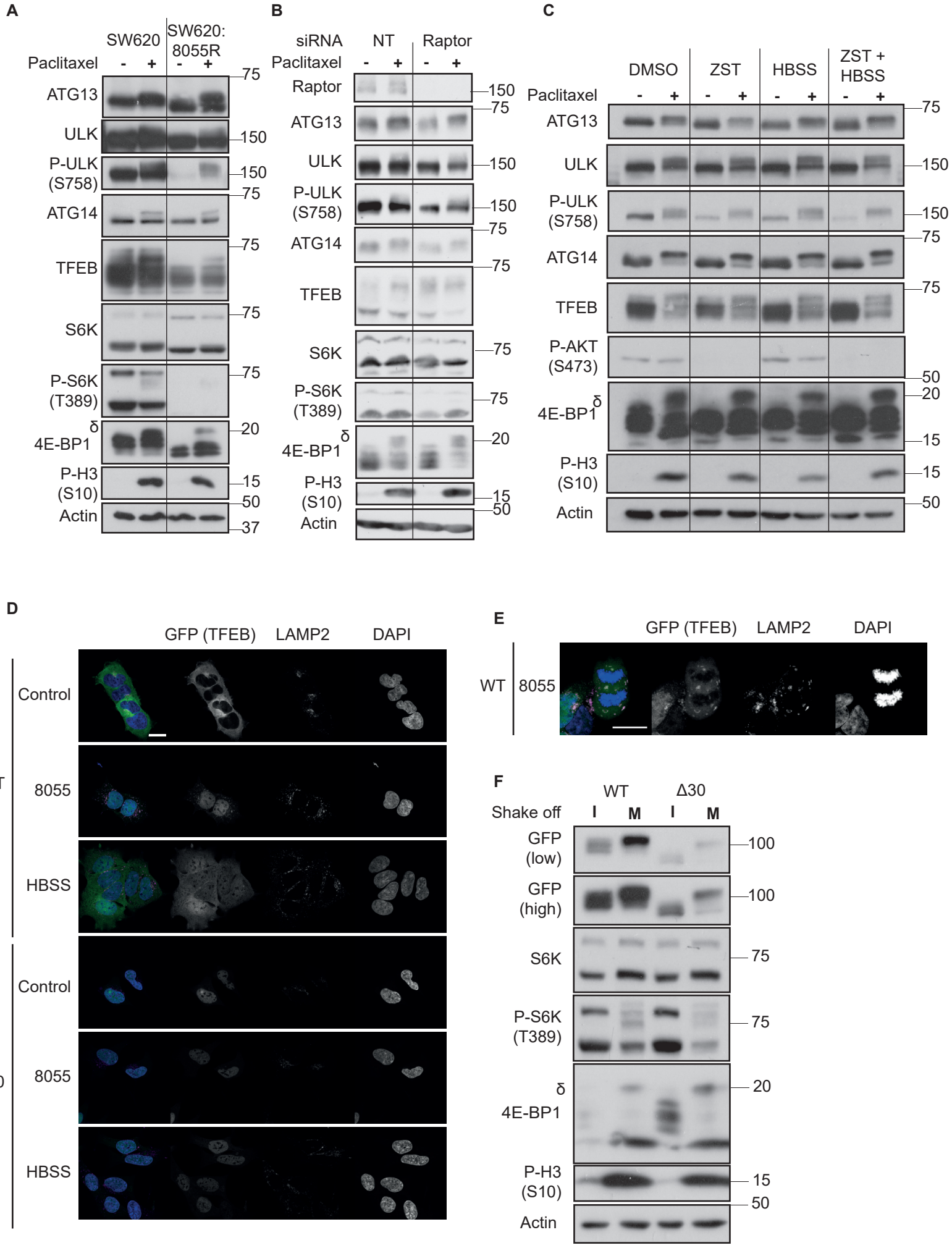


Figure S6 (Related to Figure 5)

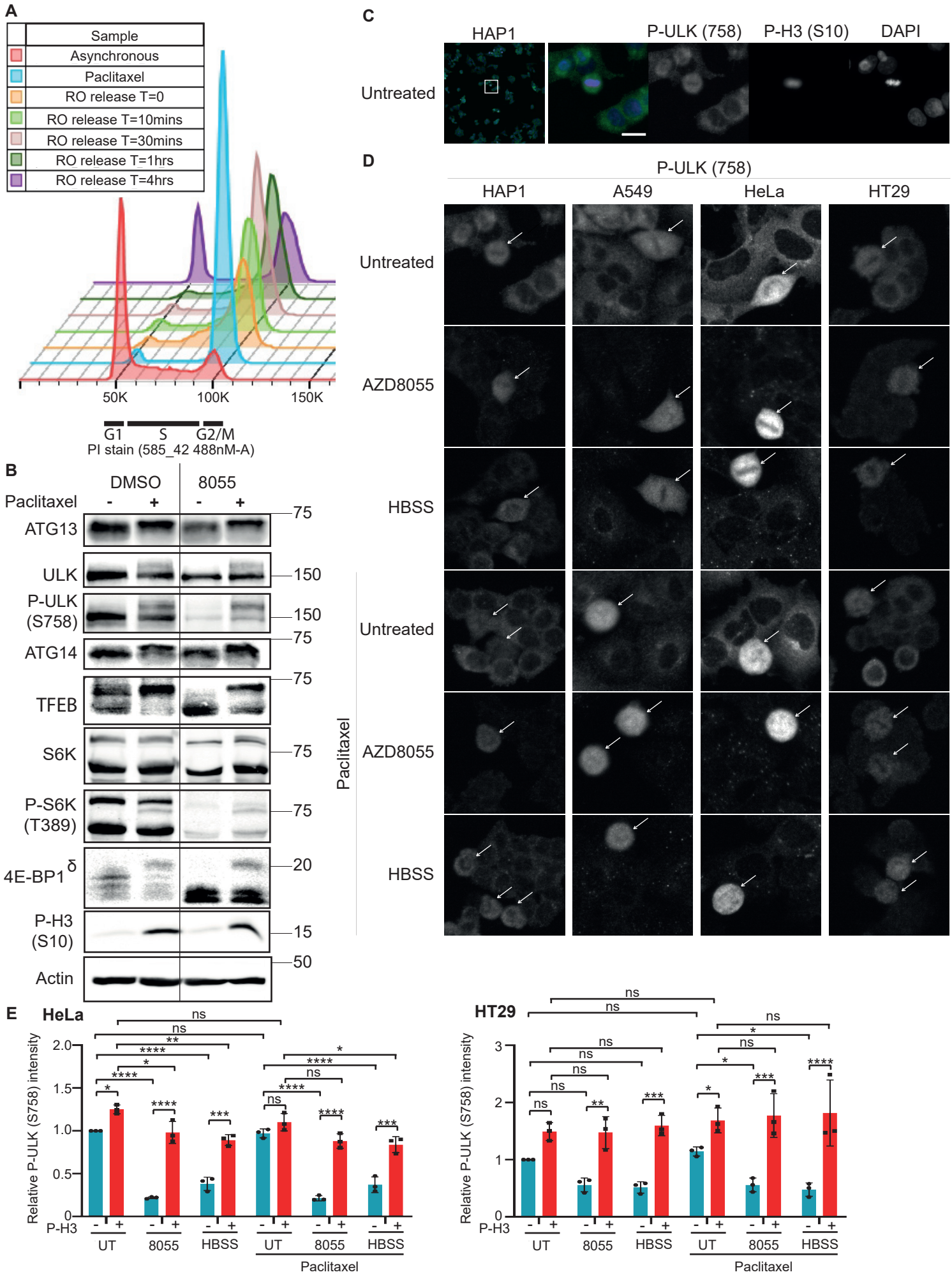
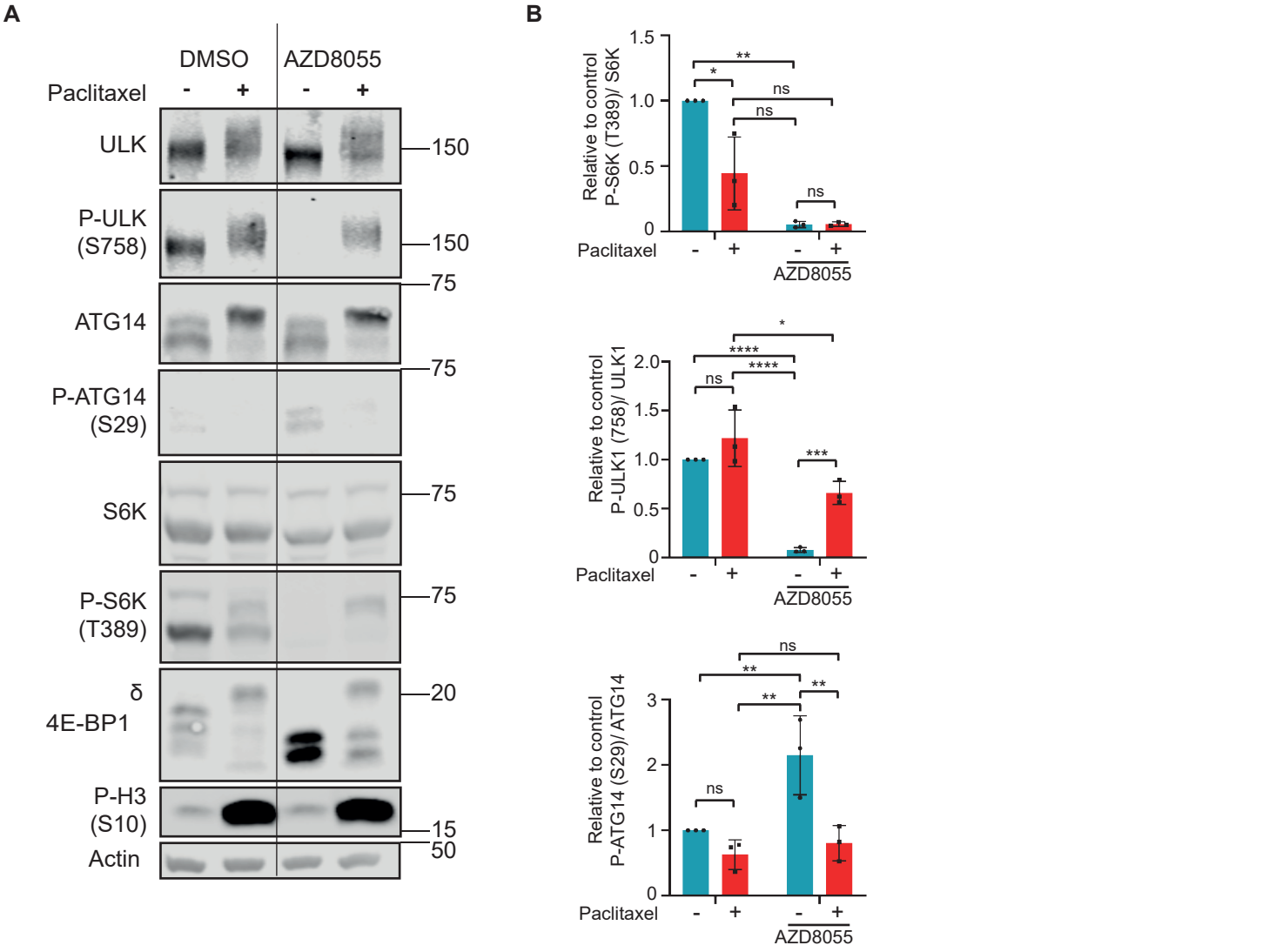


Figure S7 (Related to Figure 6)



	Treatment	P-H3 (S10)			
		Negative		Positive	
		Cells	Puncta	Cells	Puncta
Figure 1B quantification (ATG13) exp1	Untreated	91	152	17	4
	AZD8055	73	957	18	21
	HBSS	118	802	25	28
Figure 1B quantification (ATG13) exp2	Untreated	142	375	29	21
	AZD8055	143	1558	29	26
	HBSS	110	732	32	24
Figure 1B quantification (ATG13) exp3	Untreated	108	146	25	9
	AZD8055	91	949	30	23
	HBSS	93	573	22	16
Figure 1E quantification (WIPI2) exp1	Untreated	64	211	12	3
	AZD8055	116	1602	14	4
	HBSS	59	499	12	2
Figure 1E quantification (WIPI2) exp2	Untreated	63	18	13	1
	AZD8055	69	648	14	3
	HBSS	65	718	15	7
Figure 1E quantification (WIPI2) exp3	Untreated	85	136	13	2
	AZD8055	85	993	15	3
	HBSS	129	1587	18	14

Table S1 (Related to Figure 1): Markers of the omegasome are strongly reduced during mitosis.

Cell and puncta counts (Imaris automated spot counting) for the experiments in Figure 1B and Figure 1E are shown

Phosphopeptide	Site
ATG13 (194-282)	
TPPIMGIIIDHFVDRPYPSS**S**PMHPCNYR	S224
TAGEDTGVIYPSVEDSQEVCTTSFST**S**PPSQLSSSR	S259 (known mTOR site)
ULK1 (706-827)	
AGGTSSPSPVVFTVG**S**PPSGSTPPQGPR	S758 (known mTOR site)
AGGTSSPSPVVFTVGSPPSGS**T**PPQGPR	T764
MFSAGPTG**S**ASSSAR	S781
ATG14 (348-470)	
NLMYLV**S**PSSEHLGR	S383 (known mTOR site)
SGPFEVR	S392
VSDEETDLGTDWENLP**S**PR	S440 (known mTOR site)
FCDIPSQSVEVSQSQSTQA**S**PPIASSSA	S462

Table S2 (Related to Figure 6): Sites in ATG13, ULK1 and ATG14 phosphorylated by CCNB1-CDK1 *in vitro*

HAP1 cells were treated with paclitaxel (50nM; t=16 hrs) prior to lysis and subsequent immunoprecipitation of cyclin B1 (or bead-only control). The immunoprecipitated CDK1 was then incubated with indicated GST-tagged protein fragments for 15 minutes at 30^{o}C. GST-tagged fragments were then digested with trypsin and analysed by mass spectrometry as outlined in the methods. Sites phosphorylated by CCNB1-CDK1 in both of two independent experiments are identified in Red underline.