

Supplementary Information

Sensitivity of Colorectal Cancer to Arginine Deprivation Therapy is Shaped by Differential Expression of Urea Cycle Enzymes.

Constantinos Alexandrou, Saif Sattar Al-Aqbi, Jennifer A Higgins, William Boyle, Ankur Karmokar, Catherine Andreadi, Jin-Li Luo, David A Moore, Maria Viskaduraki, Matthew Blades, Graeme I Murray, Lynne M Howells, Anne Thomas, Karen Brown, Paul N Cheng and Alessandro Rufini.

Supplementary Figure Legends

Supplementary Figure S1. A) Schematic of the urea cycle. Enzymes are in red and show the role of ASS1 and OTC in recycling citrulline and ornithine respectively. ASS1 argininosuccinate synthase 1; ASL, argininosuccinate lyase; OTC, ornithine transcarbamylase; CPS1, carbamoylphosphate synthase 1, ARG1, arginase 1. **B)** RKO cells were grown in arginine-free medium for 5 days. At day 5 cell culture medium was replenished with arginine (147.5 mg/L) and cells were counted for six additional days in the presence of arginine. Data are plotted as mean $\pm$ SEM.

Supplementary Figure S2. Western blot analysis of the mTOR downstream target 4E-BP1 and ribosomal S6 proteins in **A)** HCT116, **B)** RKO, **C)** SW480 and **D)** HT29 CRC cell lines following arginine deprivation for the indicated times. Both phosphorylated and total levels of the proteins are reported. Actin was used as endogenous loading control. Original western blots are reported in Supplementary Figure S15.

Supplementary Figure S3. Representative sequential images from individual mice fed with Control and Arginine-free diet over time. HCT116-Luc2 tumor xenografts were imaged at the indicated days. Prior to imaging in the IVIS imaging chamber, mice were injected subcutaneously with RediJect D-Luciferin (150 mg/kg).

Supplementary Figure S4. A) ASS1 and **B)** OTC immunohistochemical (DAB) staining scoring of representative TMA, utilizing the semi quantitative approach of H-score (0= negative, 1-100 = weak positive, 101-200 = positive and 201-300 strong positive) as obtained by automated software classification (right) and manual scoring (left). **C)** Cohen's Interobserver agreement between A) and B): kappa coefficient analysis kappa values between 0.41-0.60 indicate moderate agreement whereas values from 0.61-0.80 indicate substantial agreement.

Supplementary Figure S5. A) Meta-analysis of OTC expression in CRC datasets performed using the online tool CancerMA ($P < 0.001$). The Forest plot visualizes the meta-analysis results for OTC gene expression in the indicated colorectal cancer

datasets. Each dataset is illustrated by a square; the position on the x-axis representing the measure estimate of *OTC* gene expression (expressed as logarithm of “fold over control tissue”, lg2FC ratio), the size of the square being proportional to the weight of the study, and the horizontal line through it reflecting the confidence interval of the estimate. The diamond sign indicates the calculated average *OTC* lg2FC ratio and its confidence interval. *OTC* was found to be highly downregulated in all datasets analyzed. Colorectal_Ad, Colorectal adenoma; Colorectal_ADC, Colorectal adenocarcinoma; Colorectal_C, Colorectal cancer; Colorectal_Ep_C, Colorectal carcinoma, epithelial cells; Colorectal_Met, Colorectal cancer, metastatic; Colorectal_MRC, Colorectal cancer, metastatic recurrence; Colorectal_Muc_Ad, Colorectal adenoma, mucosa; Colorectal_Muc_C, Colorectal carcinoma, mucosa; Colorectal_PreAd, Precancerous adenoma of the colon. **B)** Table showing the downregulation of *OTC* expression (FC= fold over normal tissue control) and associated P values in the indicated dataset performed using the online tool Oncomine.

Supplementary Figure S6. A) Western blot analysis of ASS1 and c-Myc expression in the indicated cell lines treated with vehicle control or ADI-PEG20 (1 µg/mL) and rhArg1peg5000 (0.5 µg/mL) for the indicated times. Original western blots are reported in Supplementary Figure S15. **B)** and **C)** show linear correlation analysis of ASS1 and c-Myc expression levels in ADI-PEG20 and rhArg1peg5000 treated cells, respectively.

Supplementary Figure S7. Western blot analysis of protein expression of the urea cycle enzyme *OTC* in cells treated for the indicated time with rhArg1peg5000 (0.5 µg/mL). Actin was used as endogenous loading control. Original western blots are reported in Supplementary Figure S15.

Supplementary Figure S8. A) Western blot assessment of the apoptotic markers cleaved-PARP in CRC cell lines treated with rhArg1peg5000 for the indicated times. Original western blots are reported in Supplementary Figure S15. **B)** Quantification of cleaved PARP from three independent experiments, two-way ANOVA, *P<0.05; **P<0.01; ns=P>0.05. Data were normalized to actin loading control. Etoposide was used as positive control for apoptosis induction.

Supplementary Figure S9. Western blot analysis of mTOR pathway in the indicated cell lines. Cells were treated with vehicle control or rhArg1peg5000 (0.5 µg/mL).

Arginase effect was assessed in short (0h to 8h) and long incubation intervals (24h to 72h), detecting the phosphorylation status of the mTOR downstream targets 4E-BP1 and ribosomal S6 protein. Original western blots are reported in Supplementary Figure S15.

Supplementary Figure S10. Endpoint body weight of mice xenografted with **A)** RKO or **B)** SW480 cells and treated with vehicle control or rhArg1peg5000. Each dot represents one animal, mean $\pm$ SEM are also plotted. Data were analyzed using non-parametric Mann-Whitney test, $n=8$, ns = $P>0.05$.

Supplementary Figure S11. Western blot analysis of mTOR pathway in the indicated cell lines. Cells were treated with vehicle control or ADI-PEG20 (1 $\mu\text{g/mL}$) and assessed at the indicated time points to detect the phosphorylation status of the mTOR downstream targets 4E-BP1 and ribosomal S6 protein. Original western blots are reported in Supplementary Figure S15.

Supplementary Figure S12. A) Time course measurement of body weight of mice treated with vehicle control or ADI-PEG20. Each data point represent mean $\pm$ SEM. Data were analyzed with two-way ANOVA and Sidak's multiple comparison tests. $*P<0.05$. **B)** Endpoint body weight of mice xenografted with RKO cells and treated with vehicle control or ADI-PEG20. Each dot represents one animal, mean $\pm$ SEM are also plotted. Data were analyzed using non-parametric Mann-Whitney test, $n=8$, $**P<0.01$.

Supplementary Figure S13. Isobolograms obtained using the Chou-Talalay methods for combination studies of with ADI-PEG20 and 5-FU or oxaliplatin. Drugs were used at the indicated range of concentrations. The effect of treatment combination is shown over a range of ICs (IC_{50} to IC_{95}). The colored lines connect the IC_s values for the single drugs, the combinatorial concentrations for the equivalent ICs are shown as indicated in the legend. When the combinatorial concentration falls in proximity of the ICs line the two drugs act additively ($\text{CI}\sim 1$); combinations above and below the green indicates antagonism ($\text{CI}>1$) and synergism ($\text{CI}<1$), respectively. Data were analyzed using CompuSyn software.

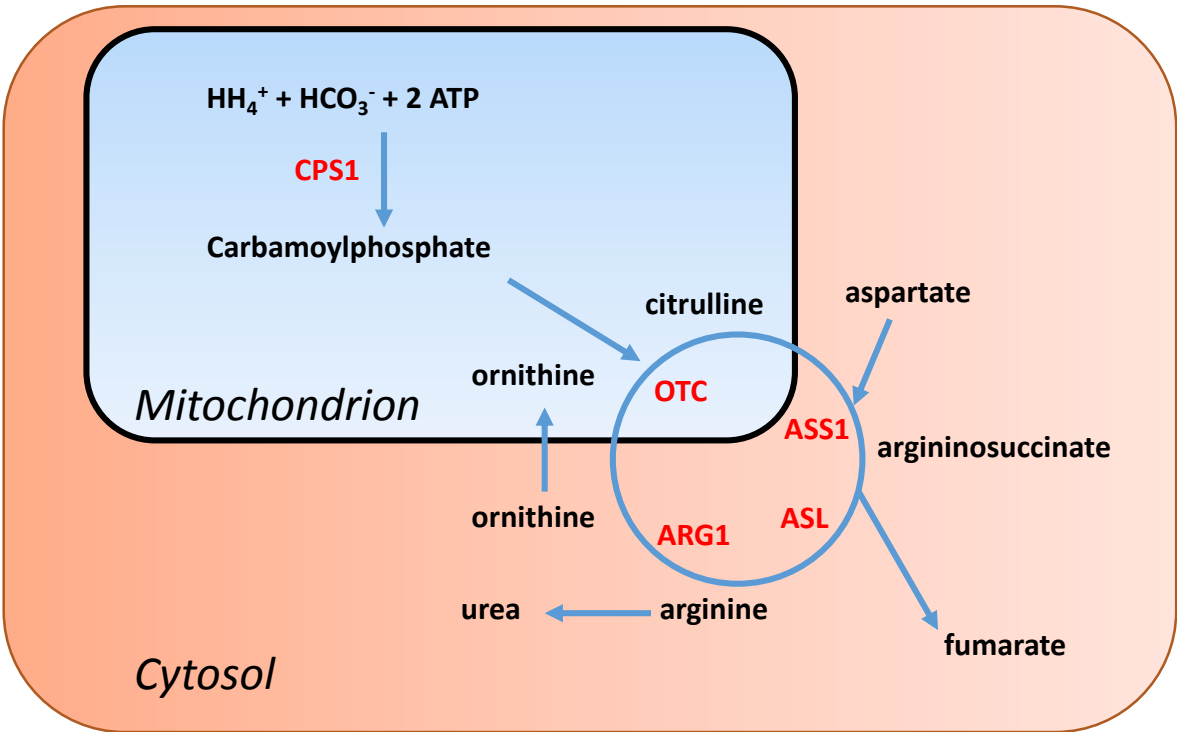
Supplemental Figure S14. Isobolograms obtained using the Chou-Talalay methods for combination studies of with rhArg1peg5000 and 5-FU or oxaliplatin. Drugs were used at the indicated range of concentrations. The effect of treatment combination is shown over a range of ICs (IC_{50} to IC_{95}). The colored lines connect the IC_s values for

the single drugs, the combinatorial concentrations for the equivalent ICs are shown as indicated in the legend. When the combinatorial concentration falls in proximity of the ICs line the two drugs act additively ($CI \sim 1$); combinations above and below the green indicates antagonism ($CI > 1$) and synergism ($CI < 1$), respectively. Data were analyzed using CompuSyn software.

Supplemental Figure S15. Original, uncropped western blots images corresponding to the cropped blots for the indicated figures.

Figure S1

A



B

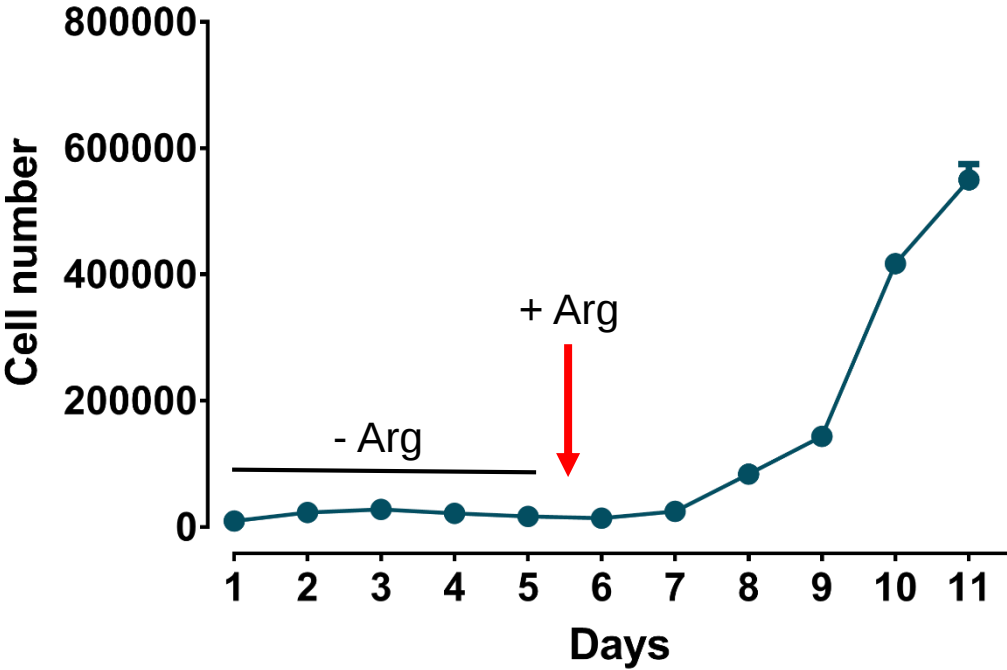


Figure S2

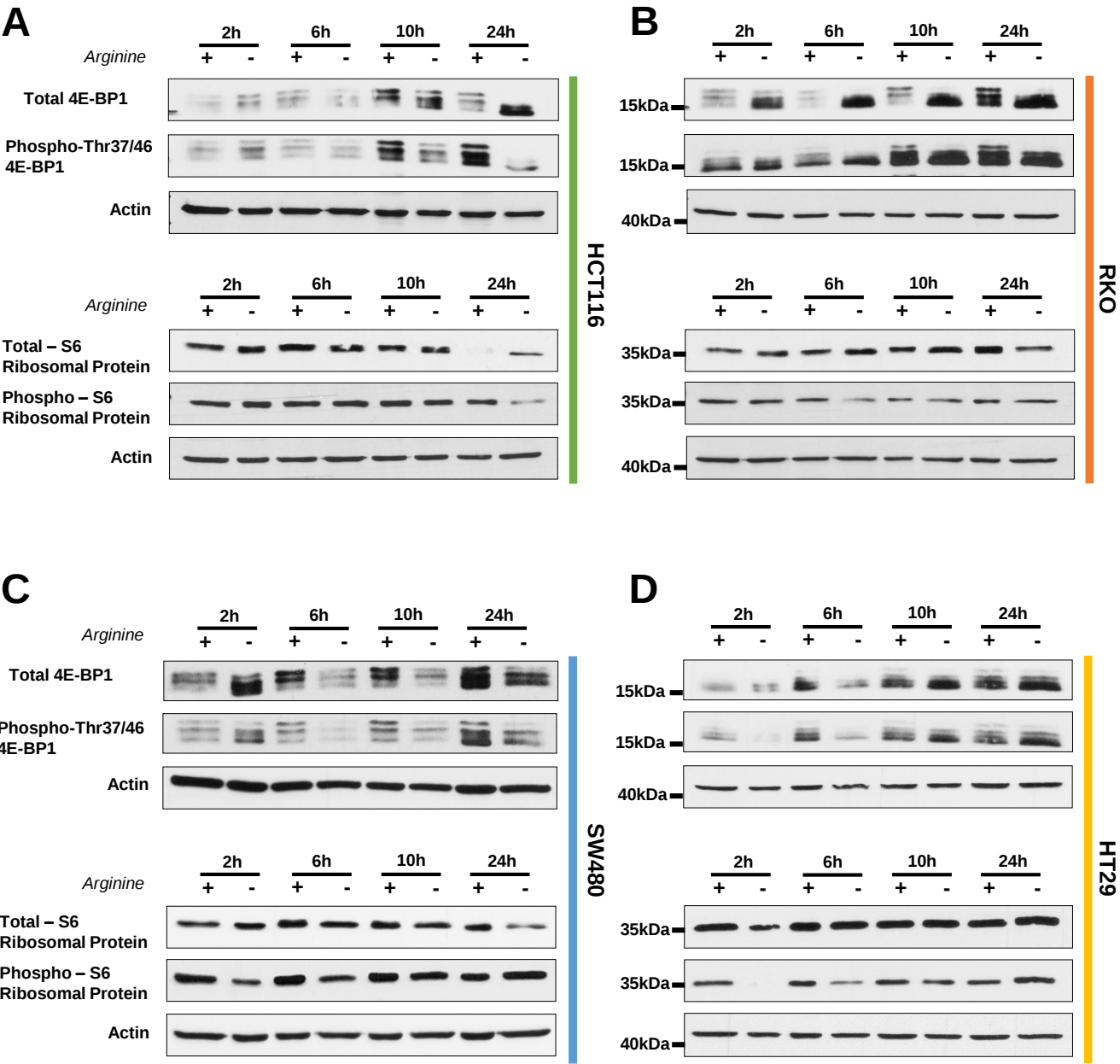


Figure S3

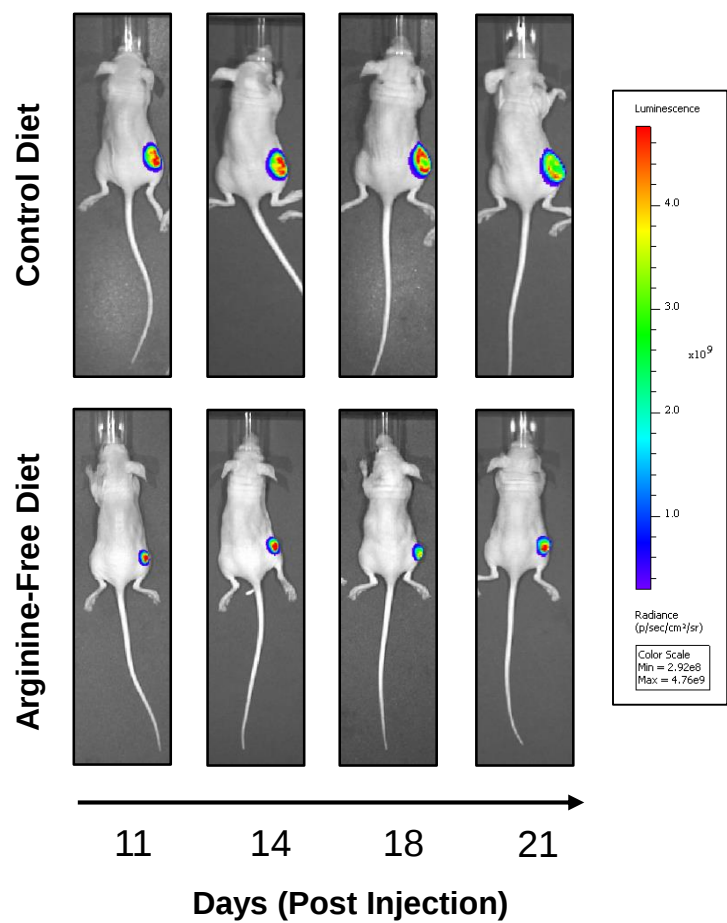
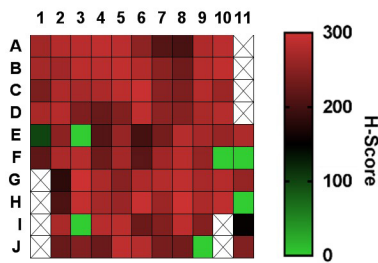


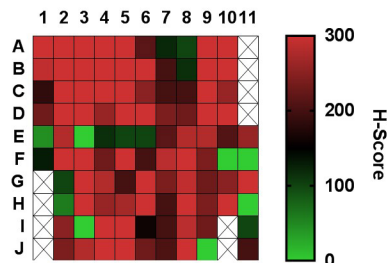
Figure S4

A

ASS1 Expression / TMA scoring - Aperio ImageScope

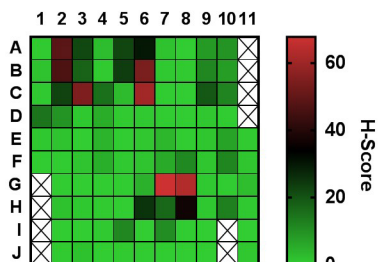


ASS1 Expression / TMA scoring - Pathologist

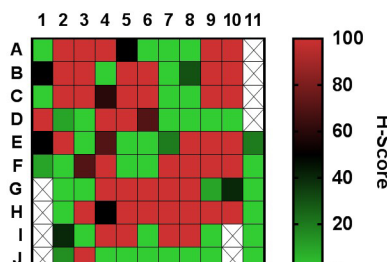


B

OTC Expression / TMA scoring - Aperio ImageScope

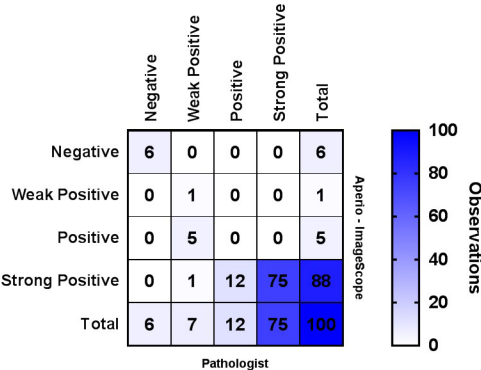


OTC Expression / TMA scoring - Pathologist



C

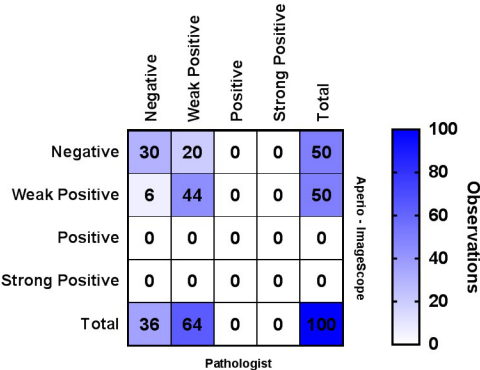
Cohen's kappa coefficient - ASS1 Scoring



Kappa = 0.454
Strenght of agreement : **moderate**

Weighted kappa = 0.686
Strenght of agreement : **good**

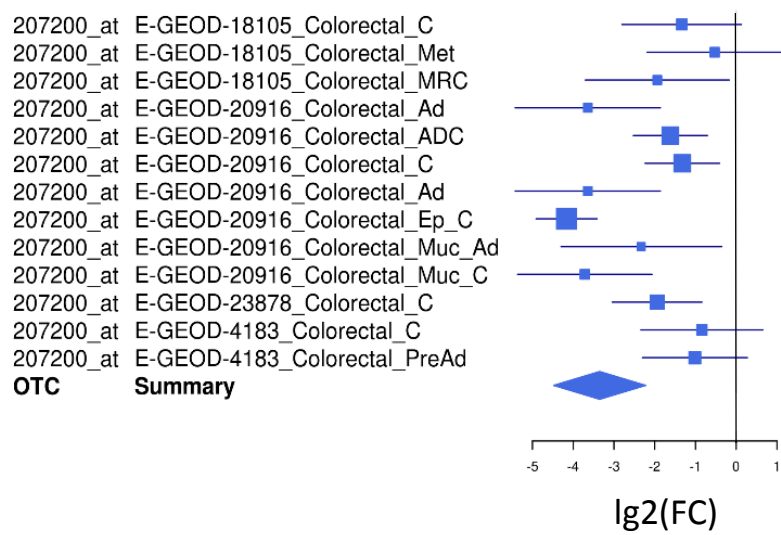
Cohen's kappa coefficient - OTC Scoring



Kappa = 0.480
Strenght of agreement : **moderate**

Figure S5

A

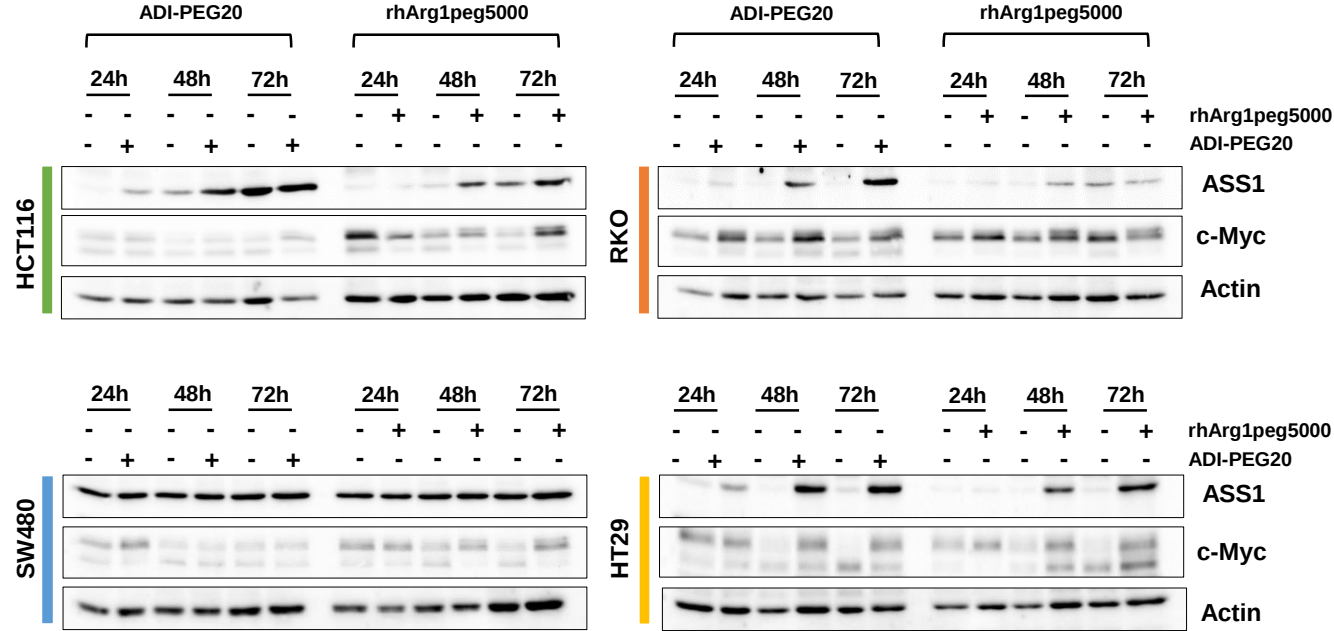


B

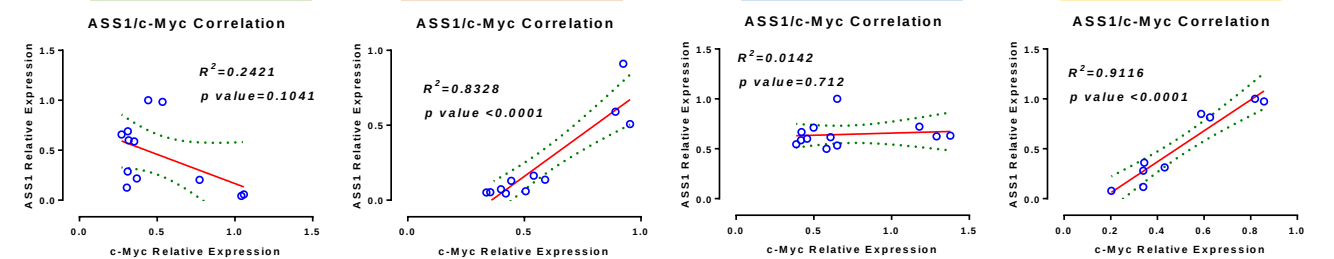
Dataset	FC	P value
Hong	-9.176	6.16E-9
TCGA	-7.730	2.93E-23
Ki	-3.082	1.57E-5
Kaiser	-1.899	7.48E-8
Gaedcke	-1.661	1.12E-5

Figure S6

A



B



C

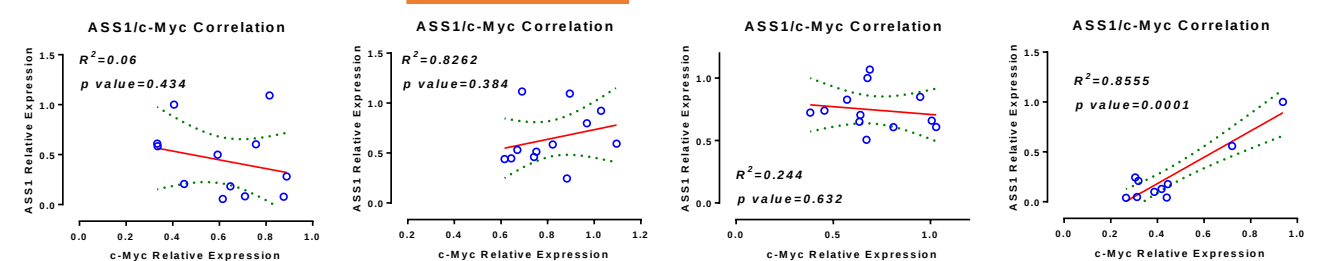


Figure S7

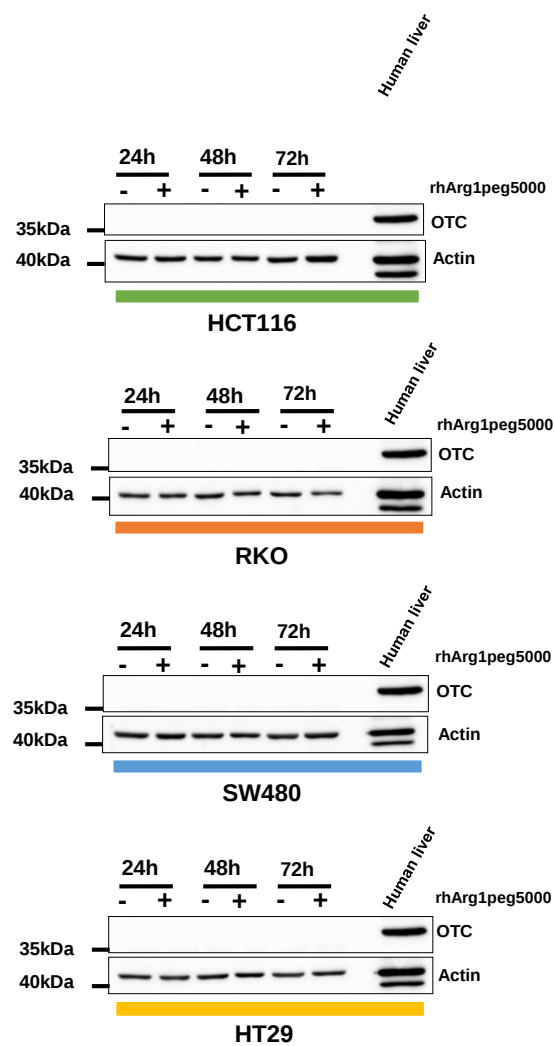


Figure S8

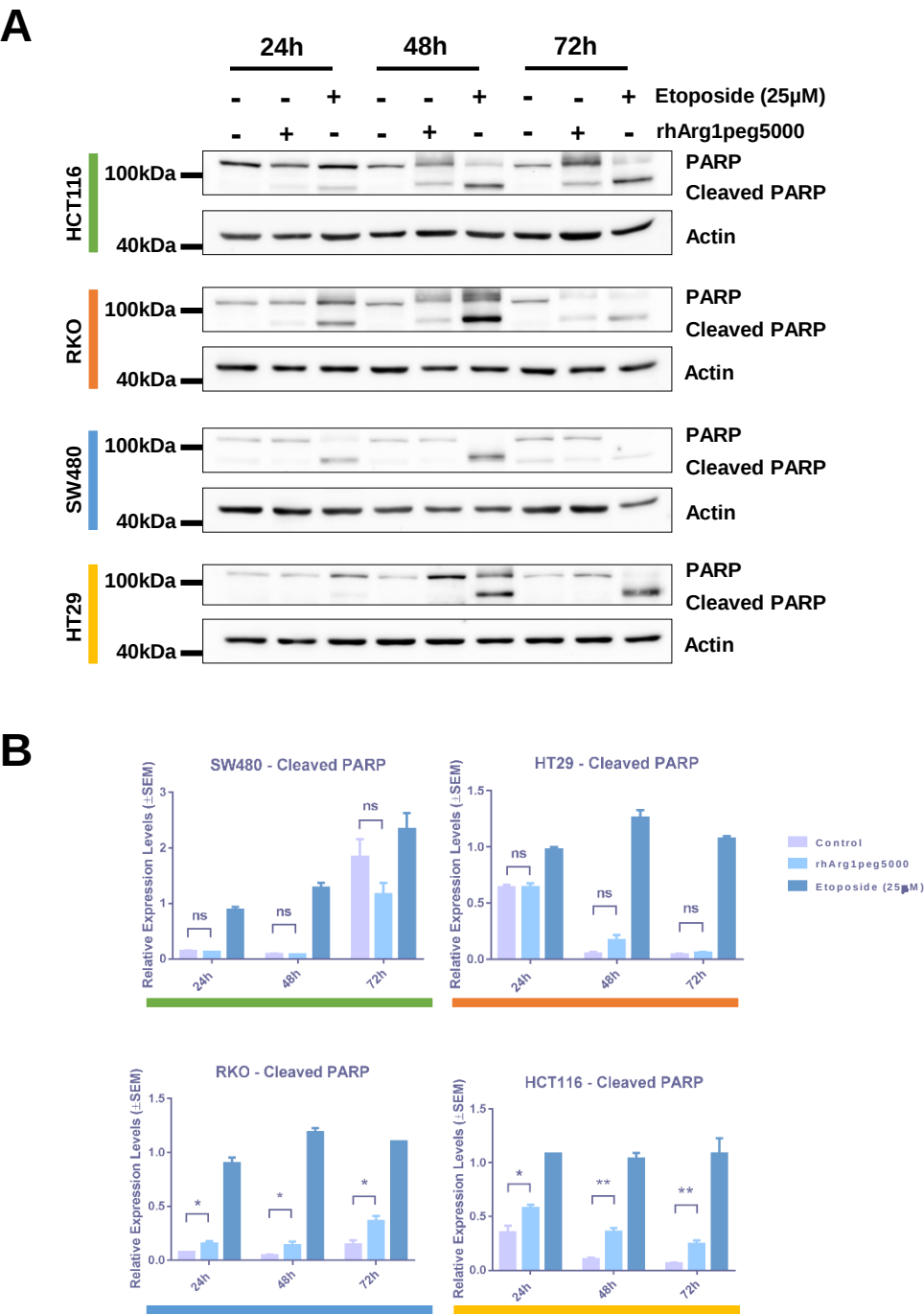


Figure S9

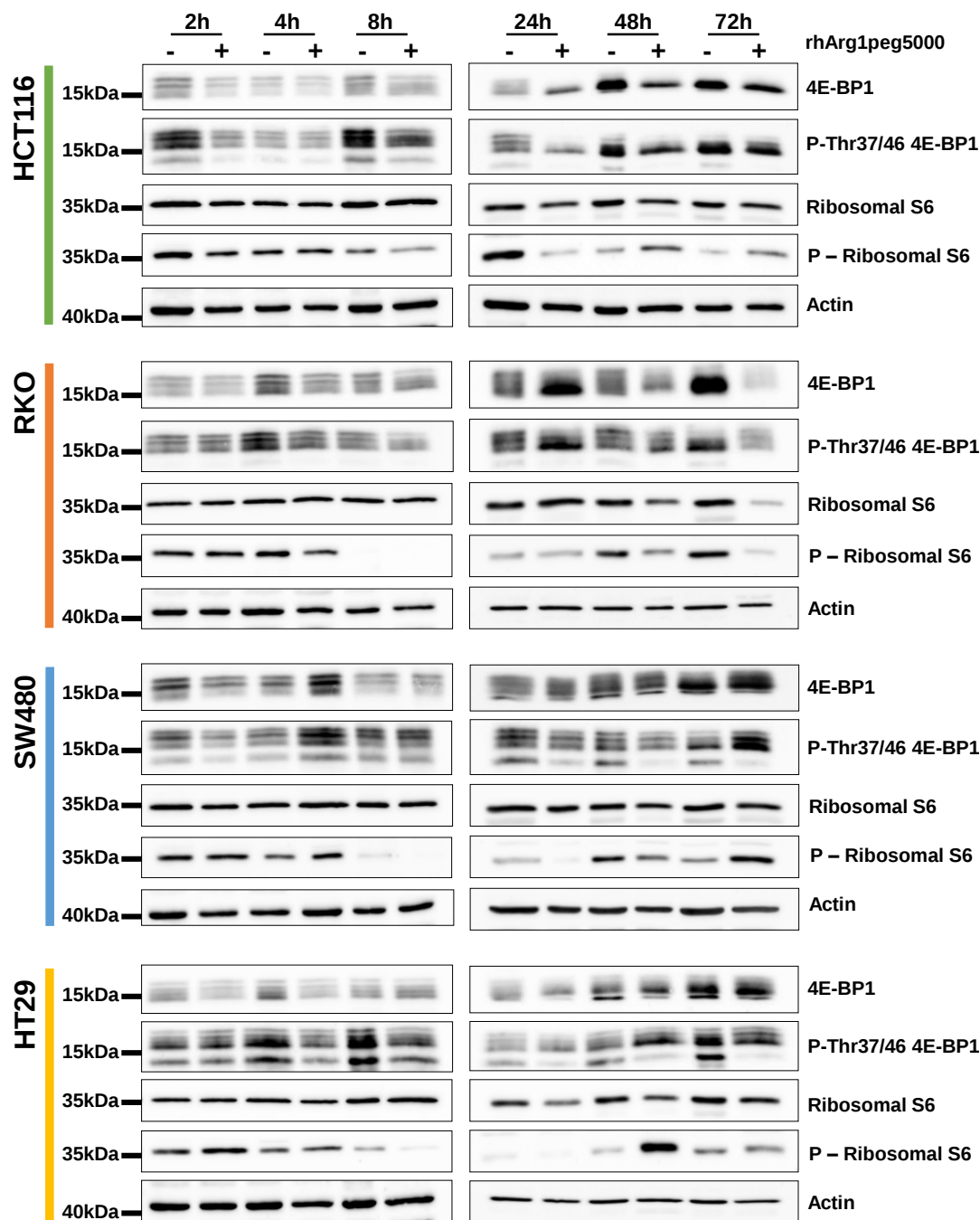


Figure S10

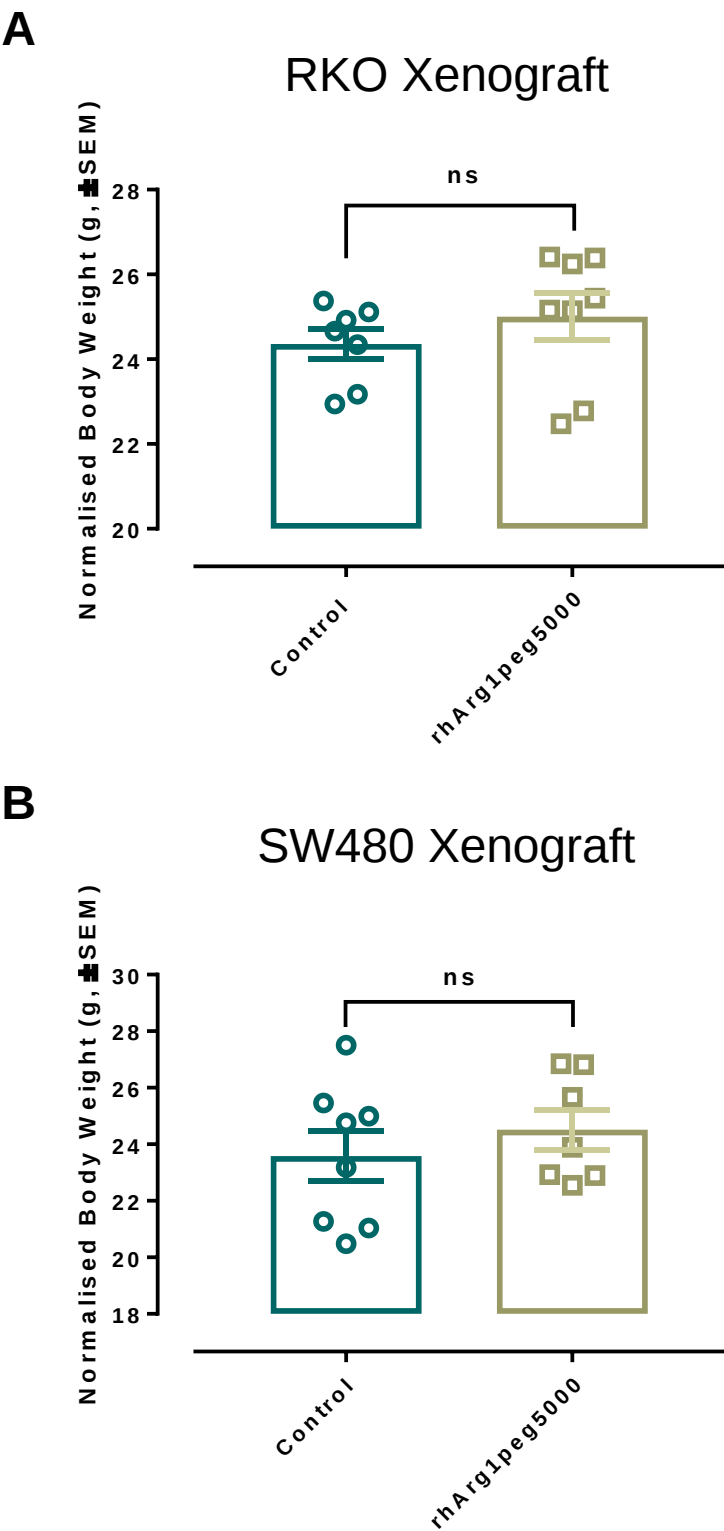


Figure S11

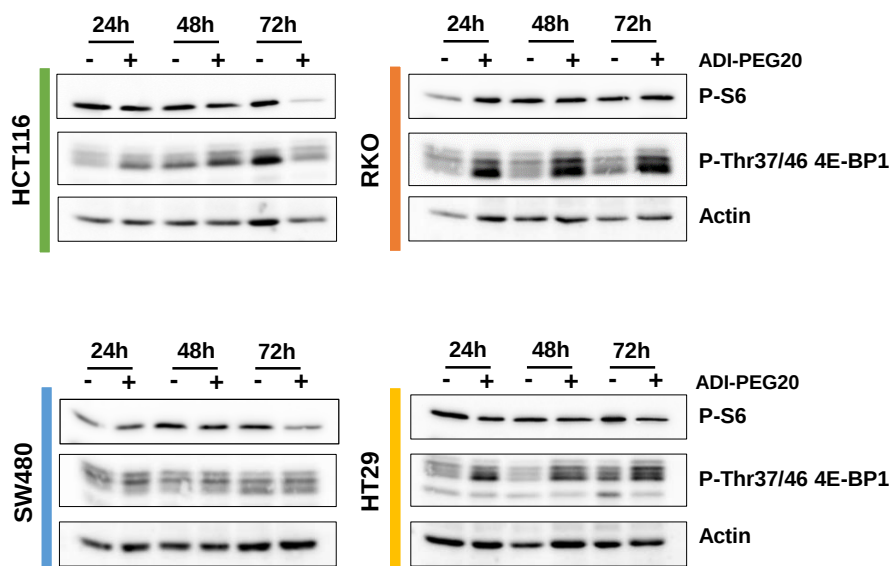
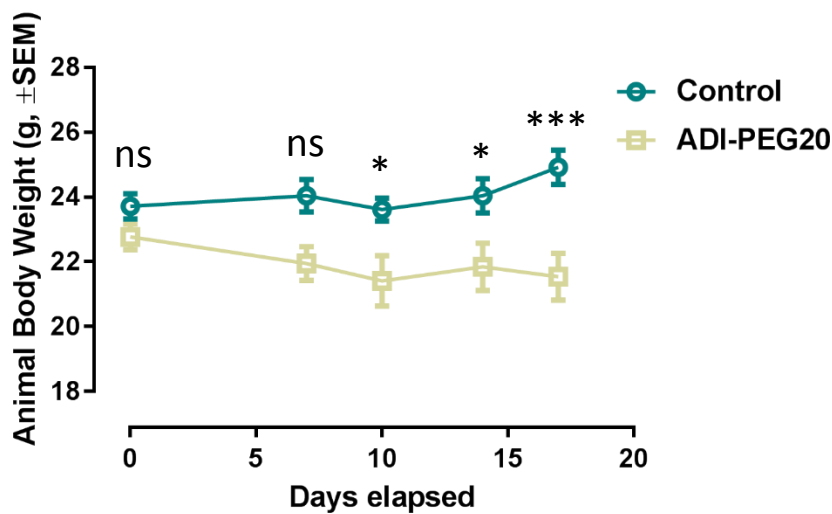


Figure S12

A



B

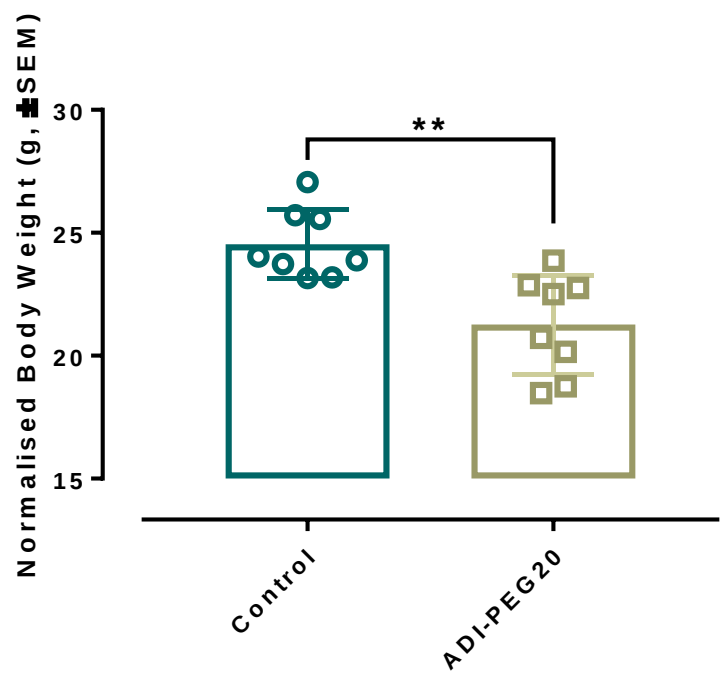


Figure S13

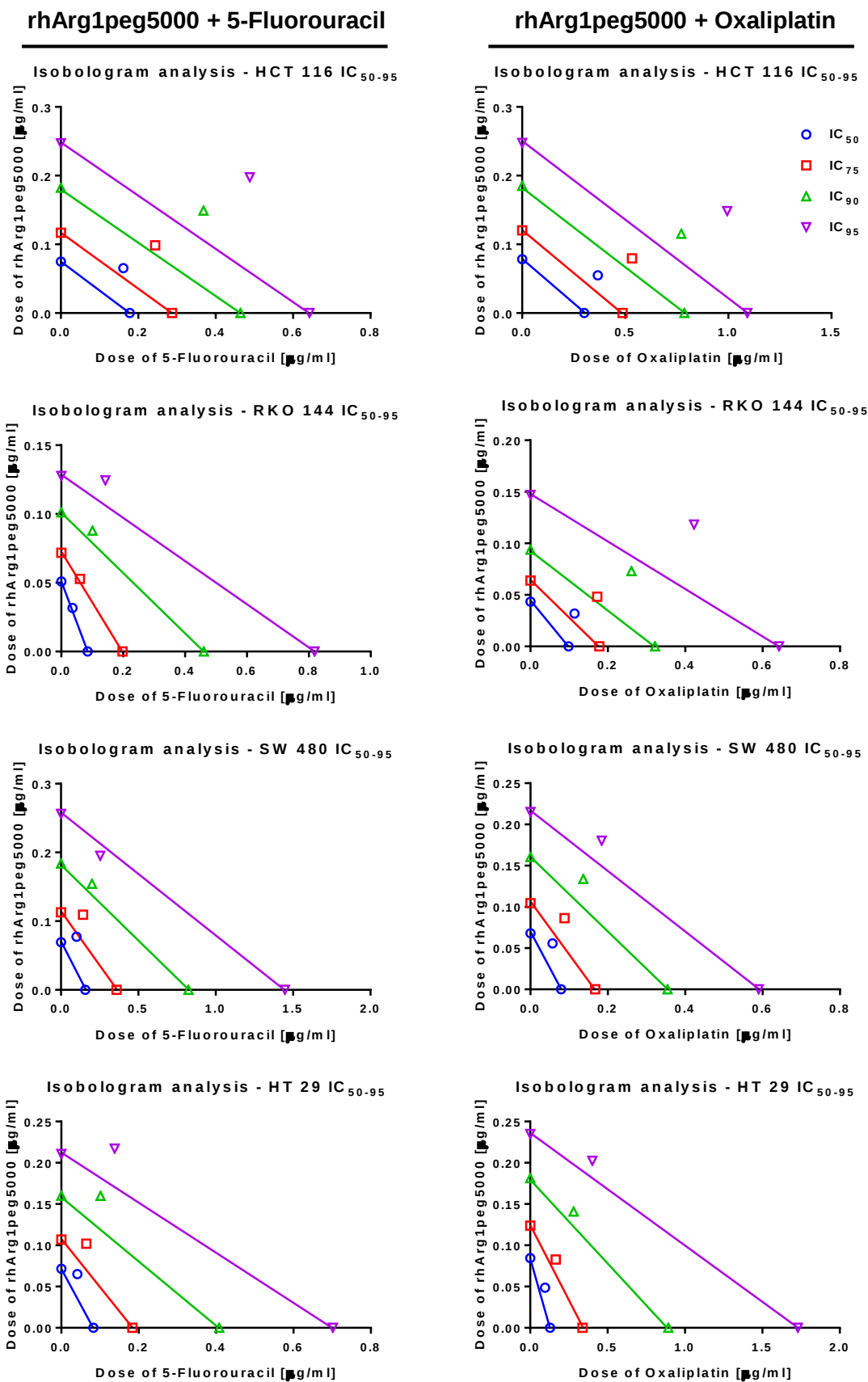


Figure S14

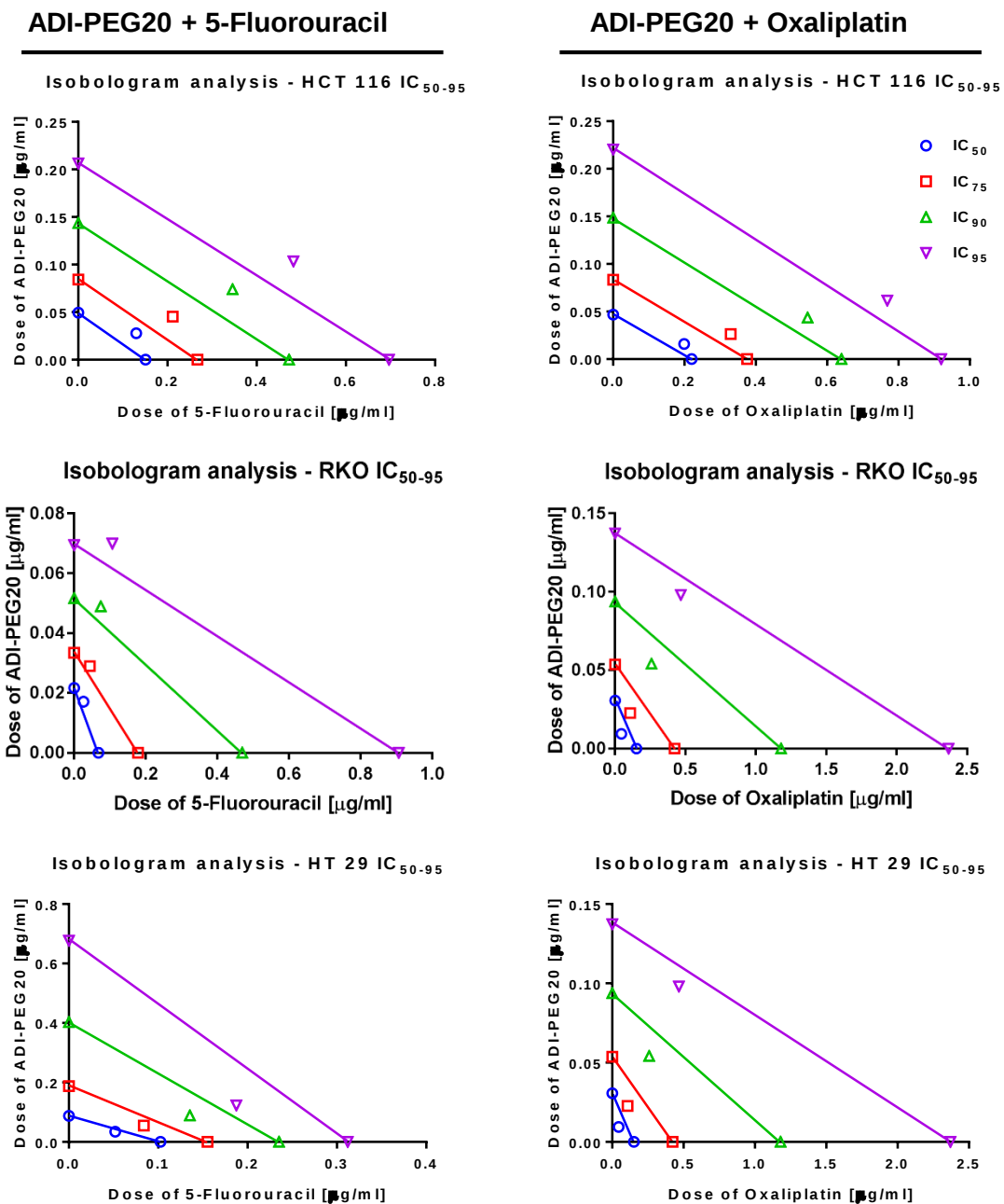


Figure S15

Western blots relative to Figure 1

Cyclin D1

Actin

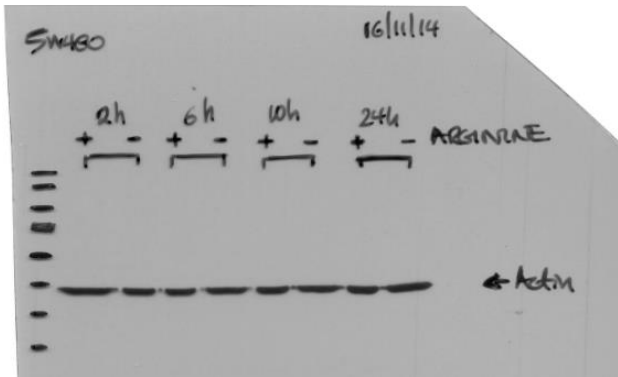
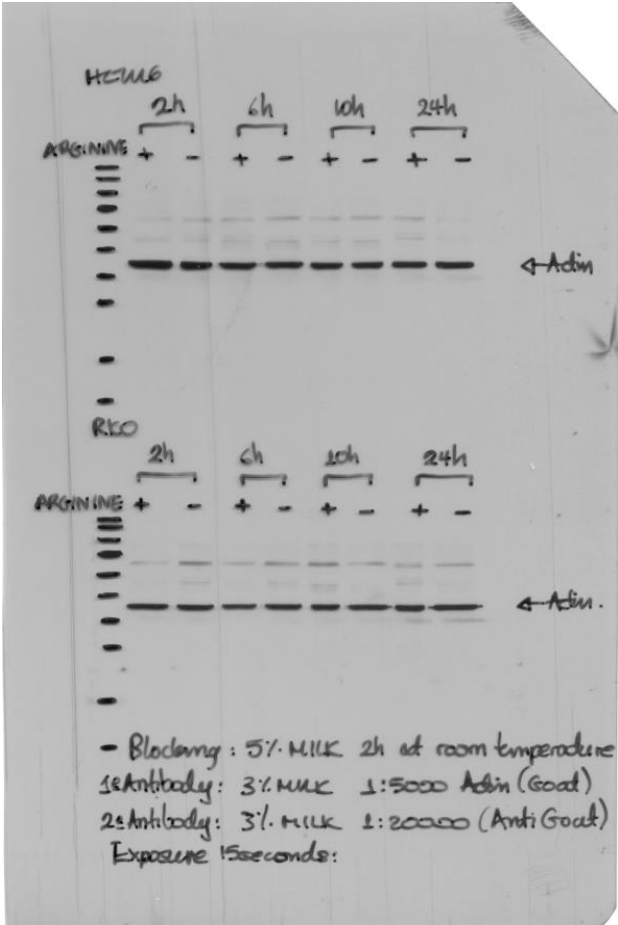
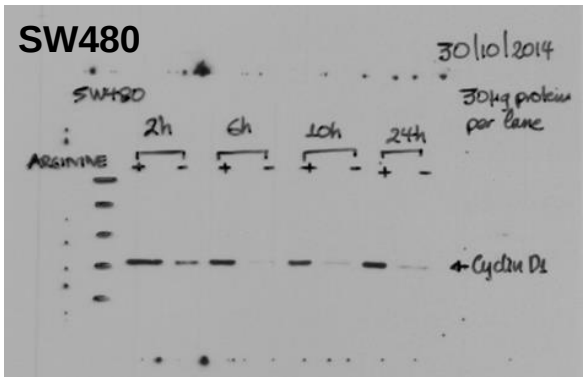
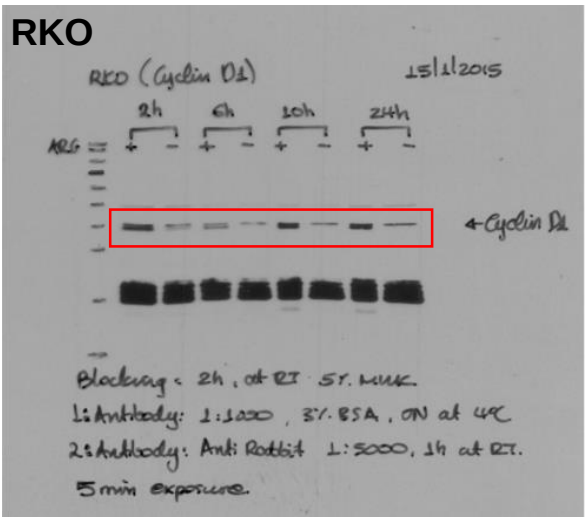
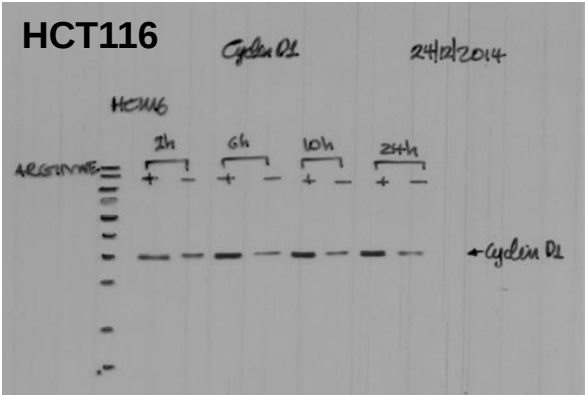


Figure S15

Western blots relative to Figure 2

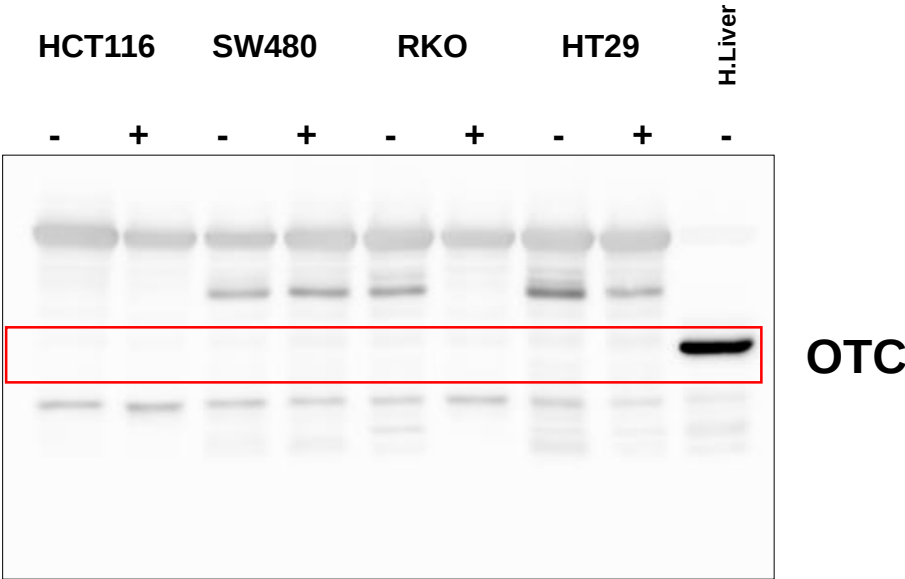
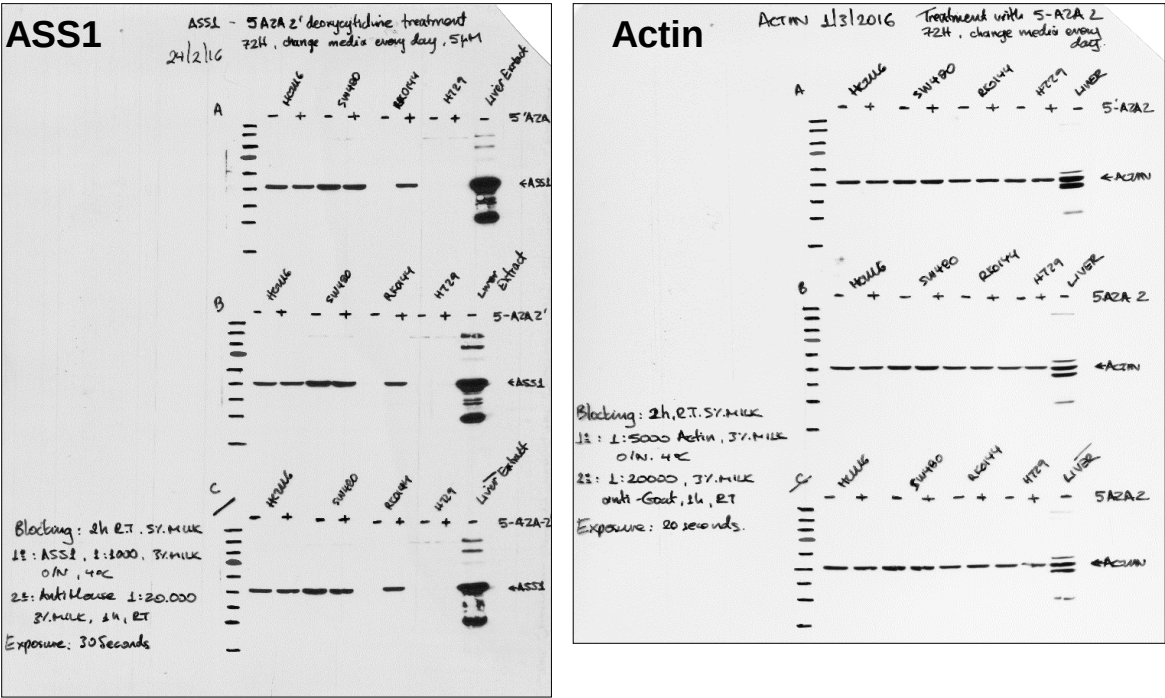


Figure S15

Western blots relative to Figure 3

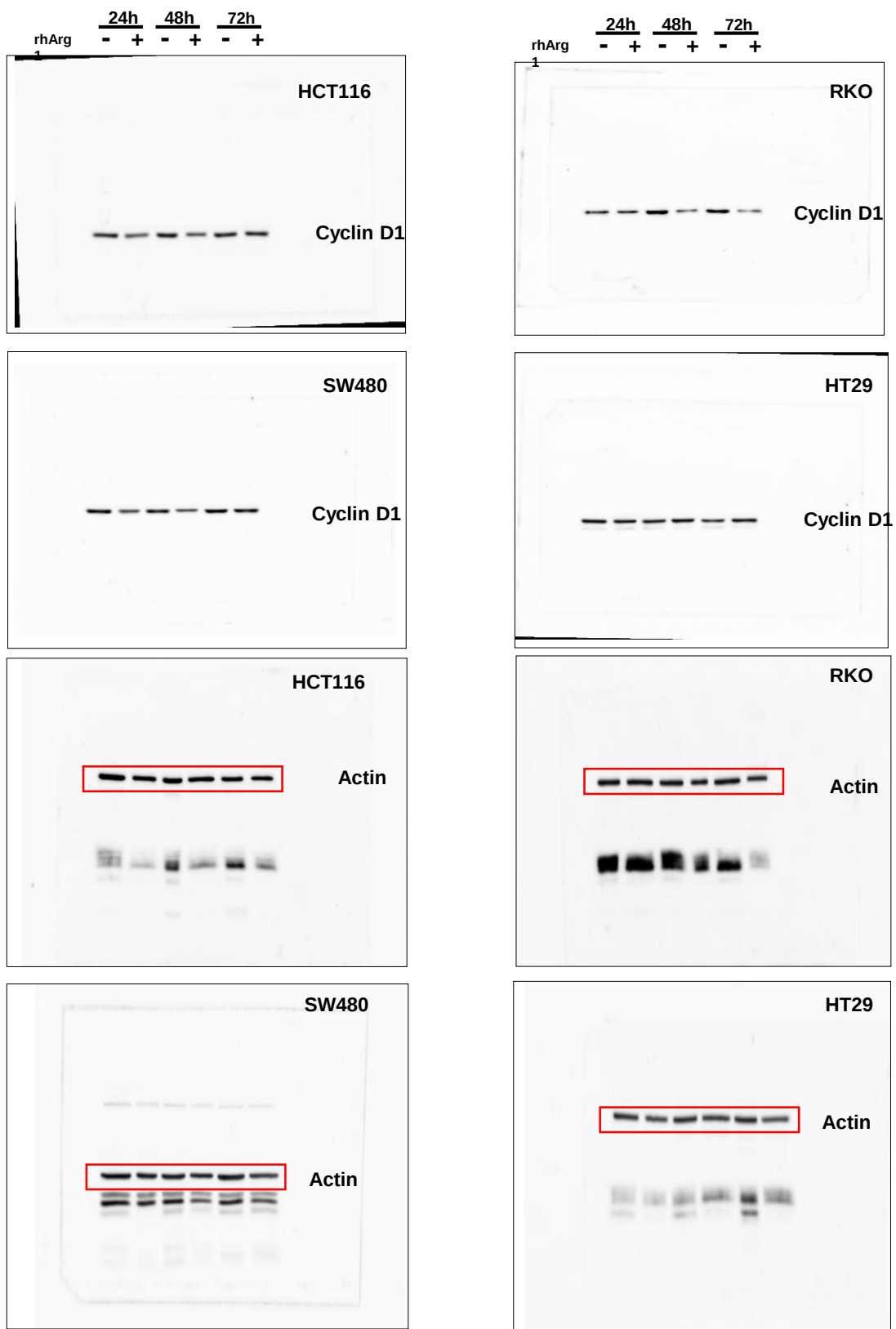


Figure S15

Western blots relative to Figure 3

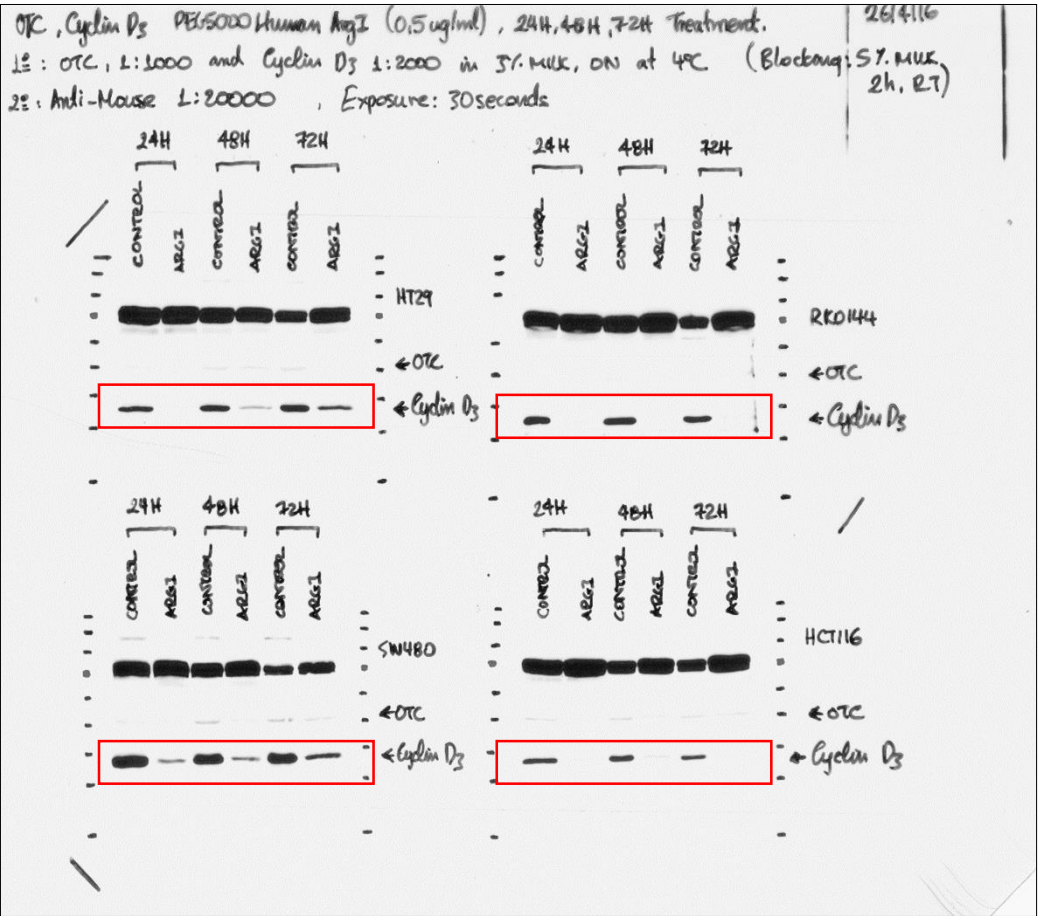


Figure S15

Western blots relative to Figure 4 –RKO xenografts

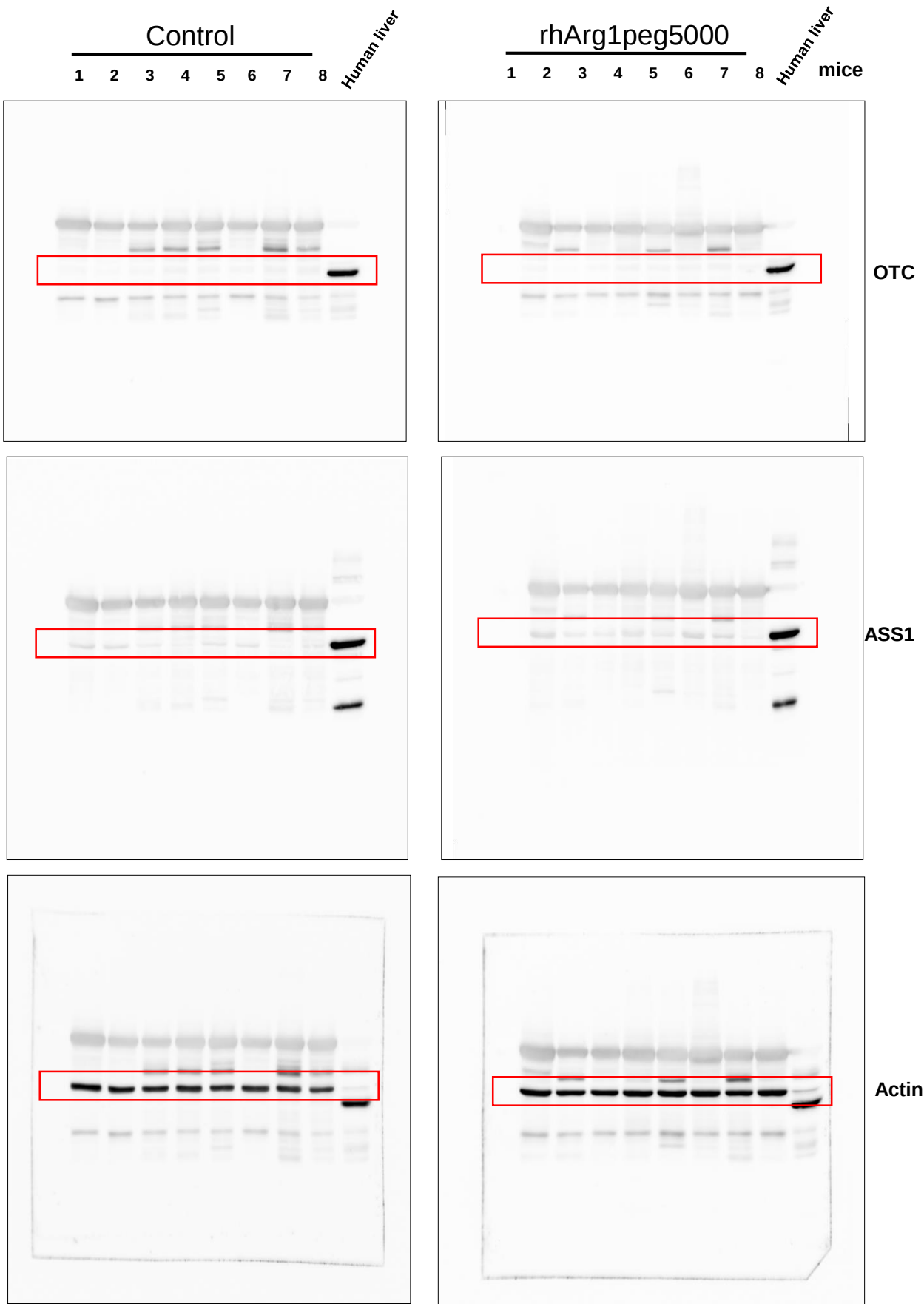


Figure S15

Western blots relative to Figure 4 –SW480 xenografts

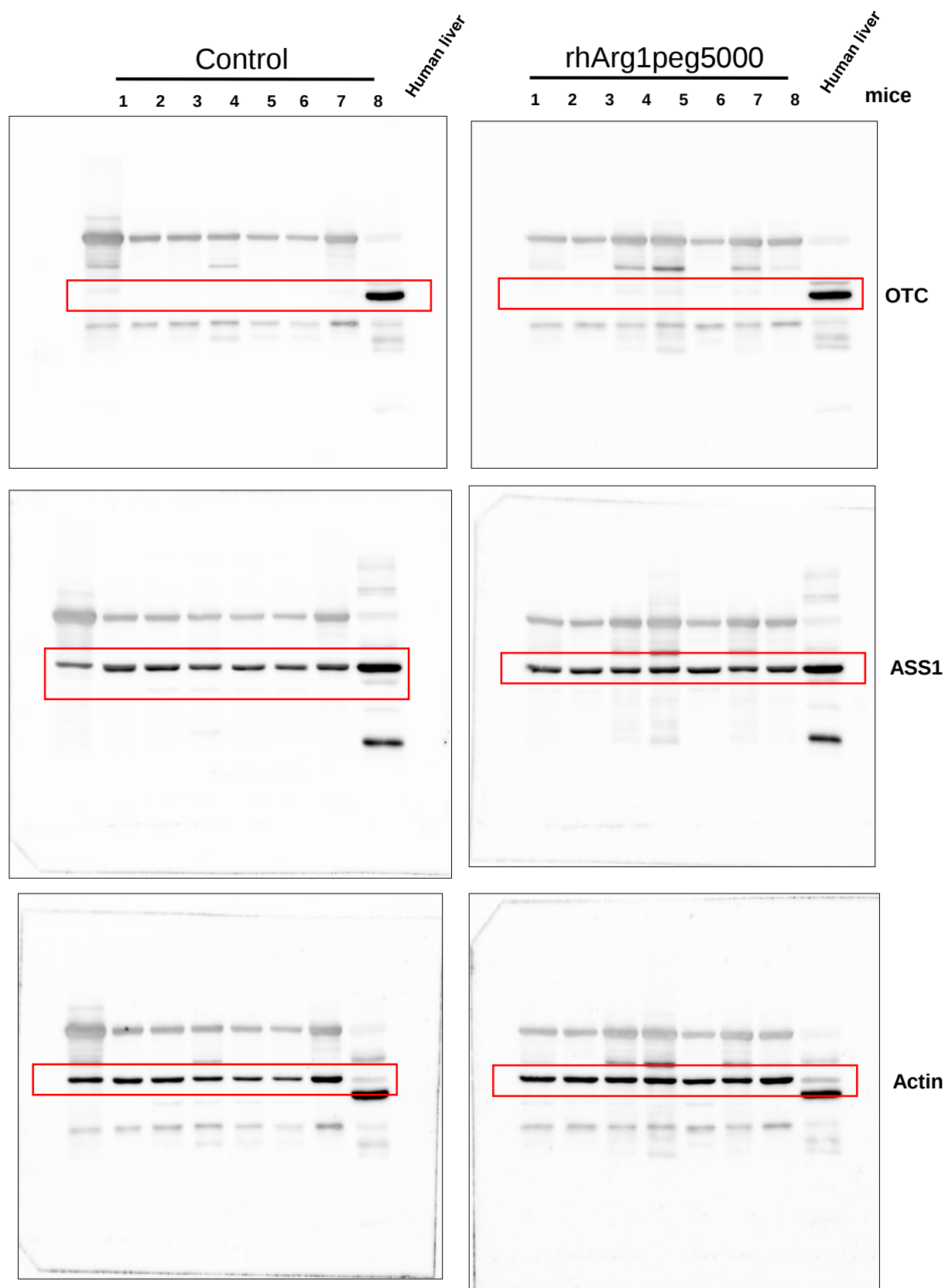


Figure S15

Western blots relative to Figure 5

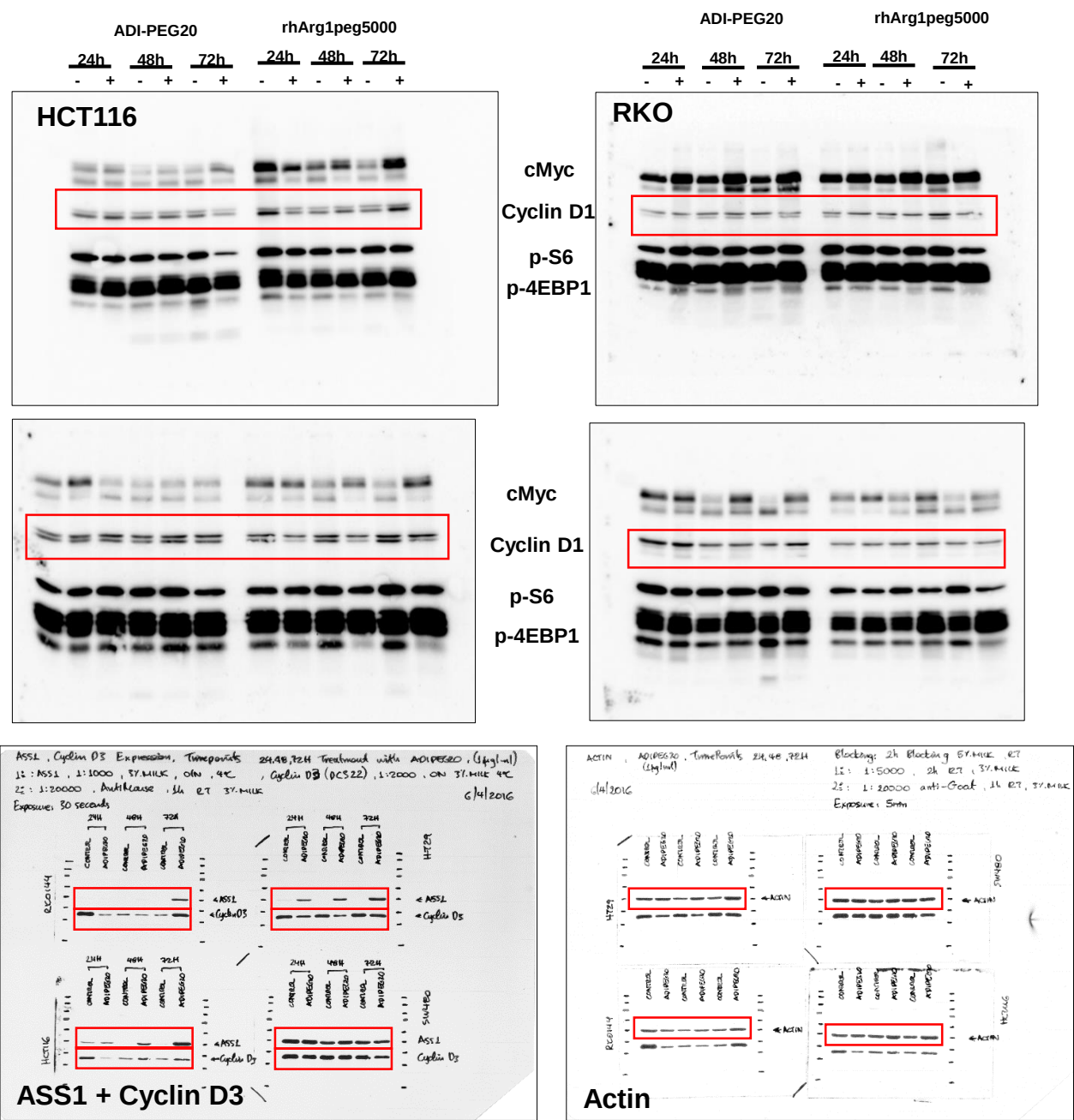


Figure S15

Western blots relative to Figure 6 –RKO xenografts

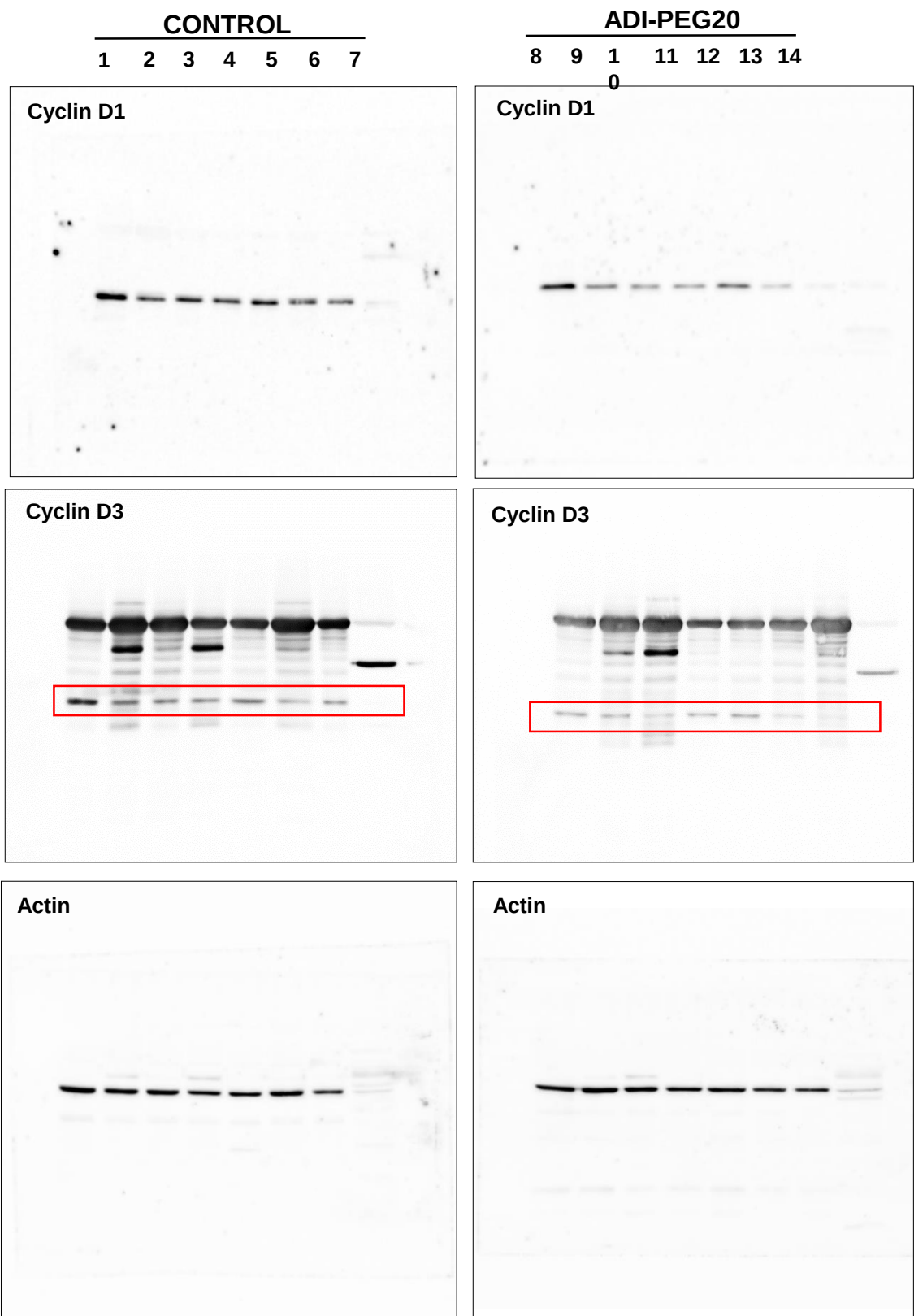


Figure S15

Western blots relative to Figure S2



Figure S15

Western blots relative to Figure S2



Figure S15 Western blots relative to Figure S6

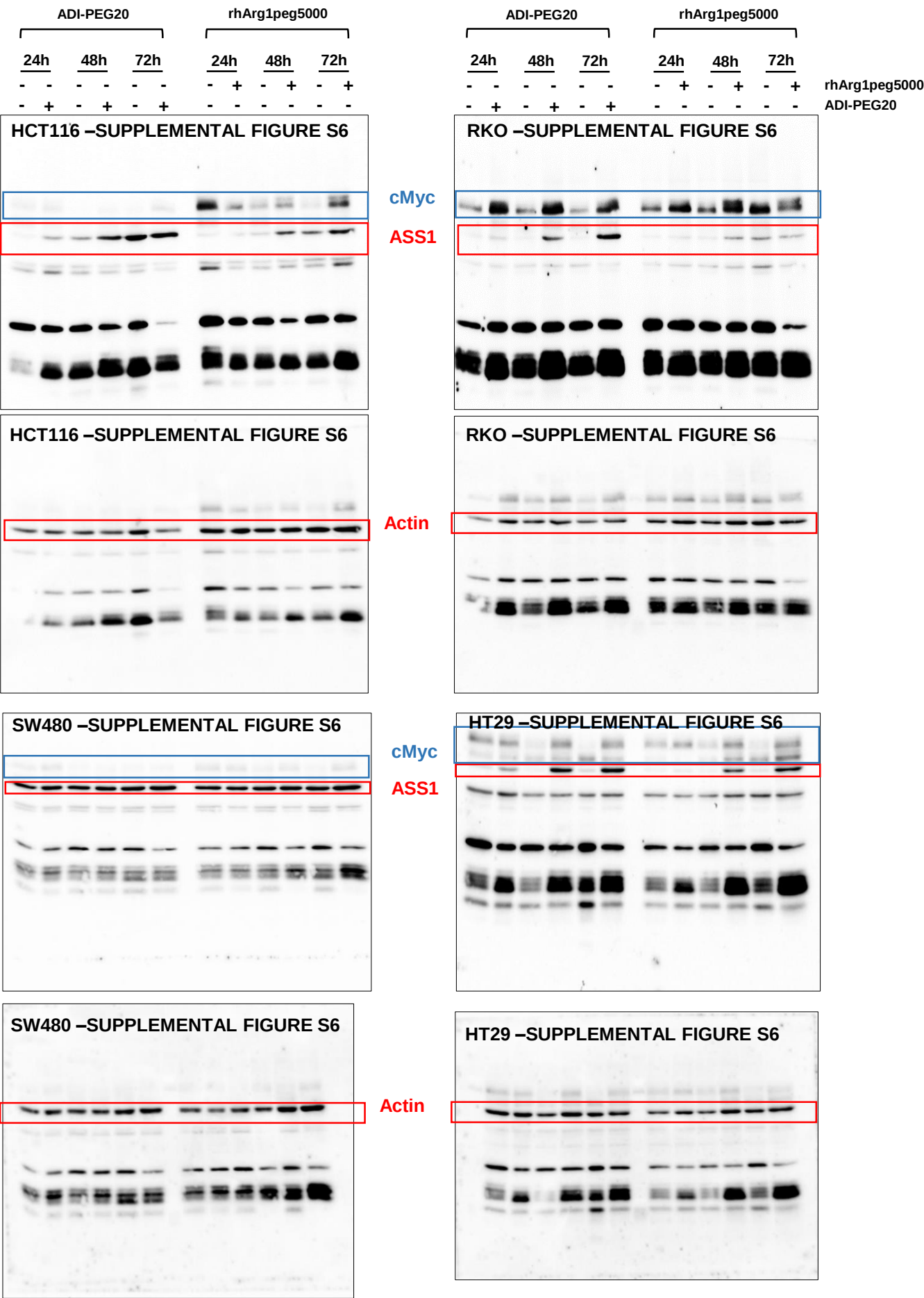


Figure S15

Western blots relative to Figure S7



Figure S15

Western blots relative to Figure S8

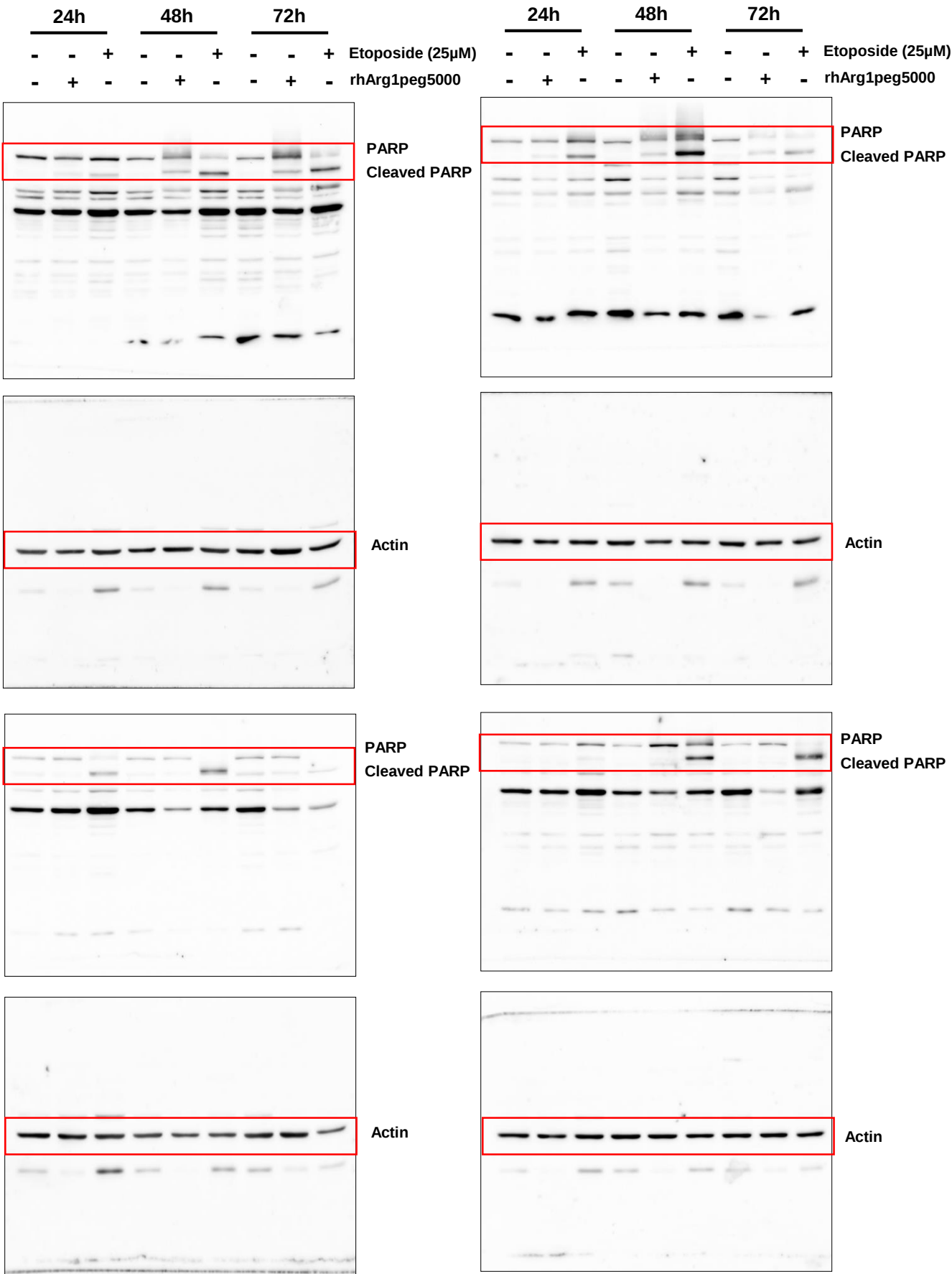


Figure S15

Western blots relative to Figure S9-HCT116

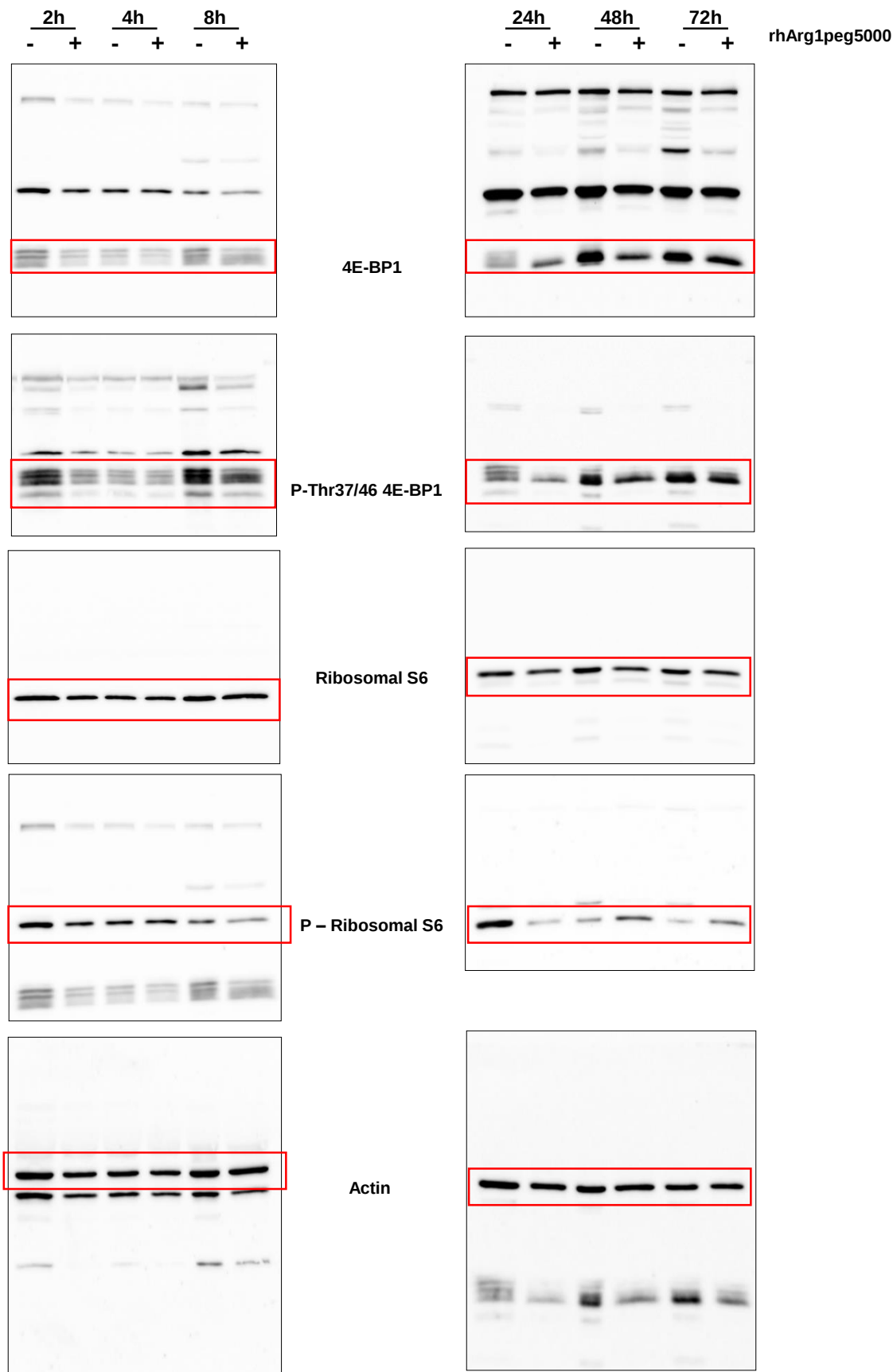


Figure S15

Western blots relative to Figure S9-RKO

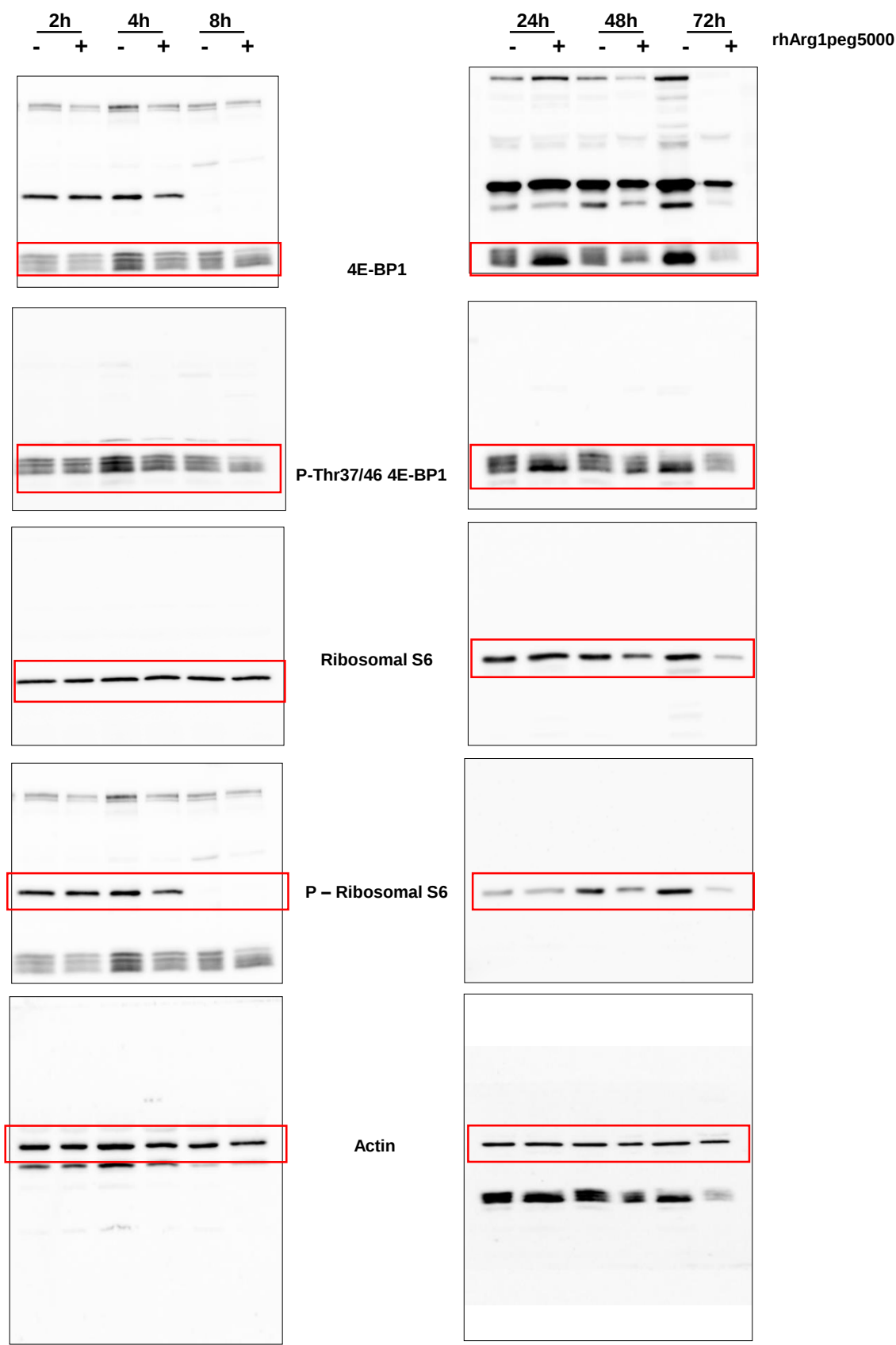


Figure S15

Western blots relative to Figure S9-SW480

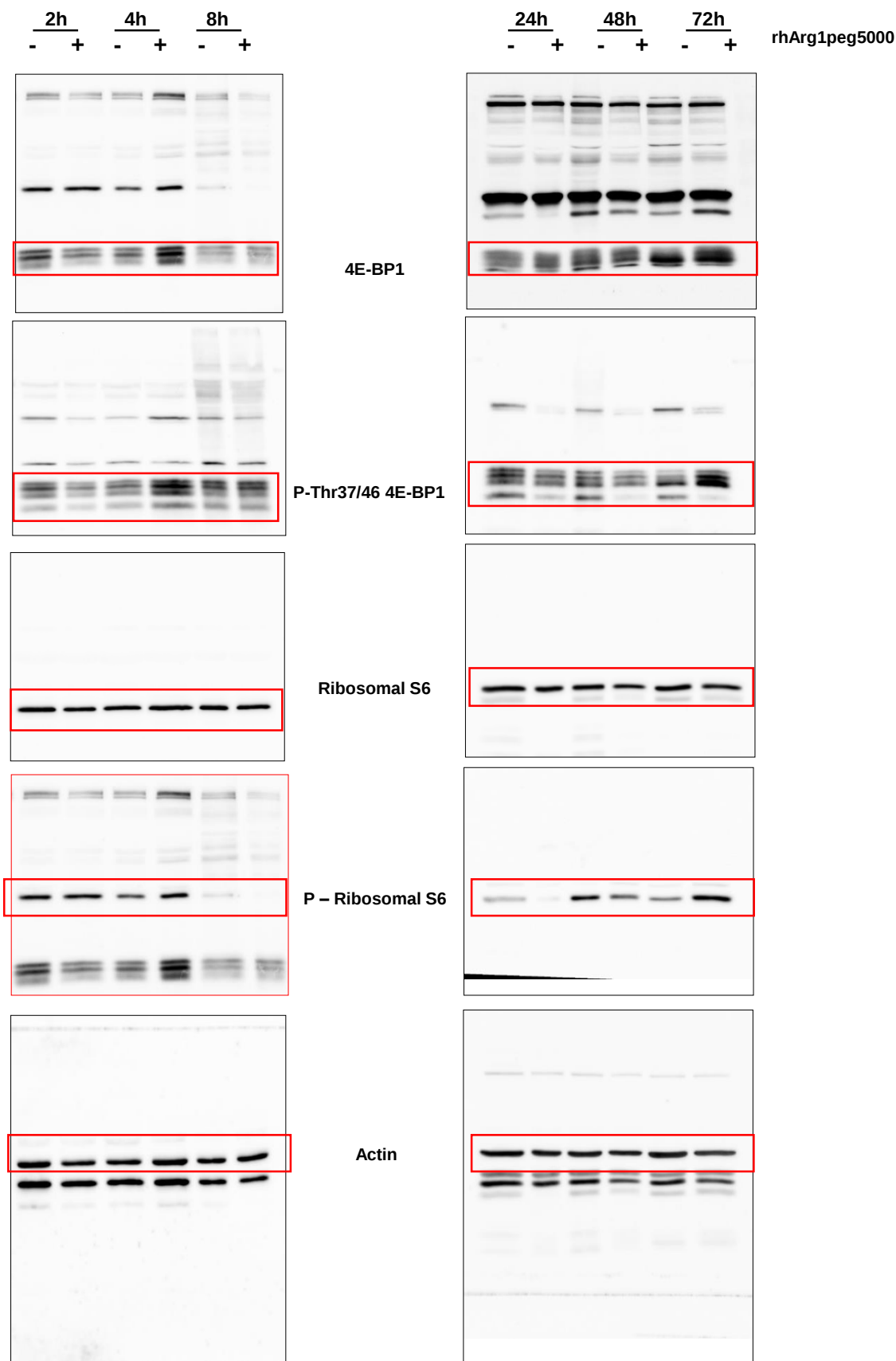


Figure S15

Western blots relative to Figure S9-HT29

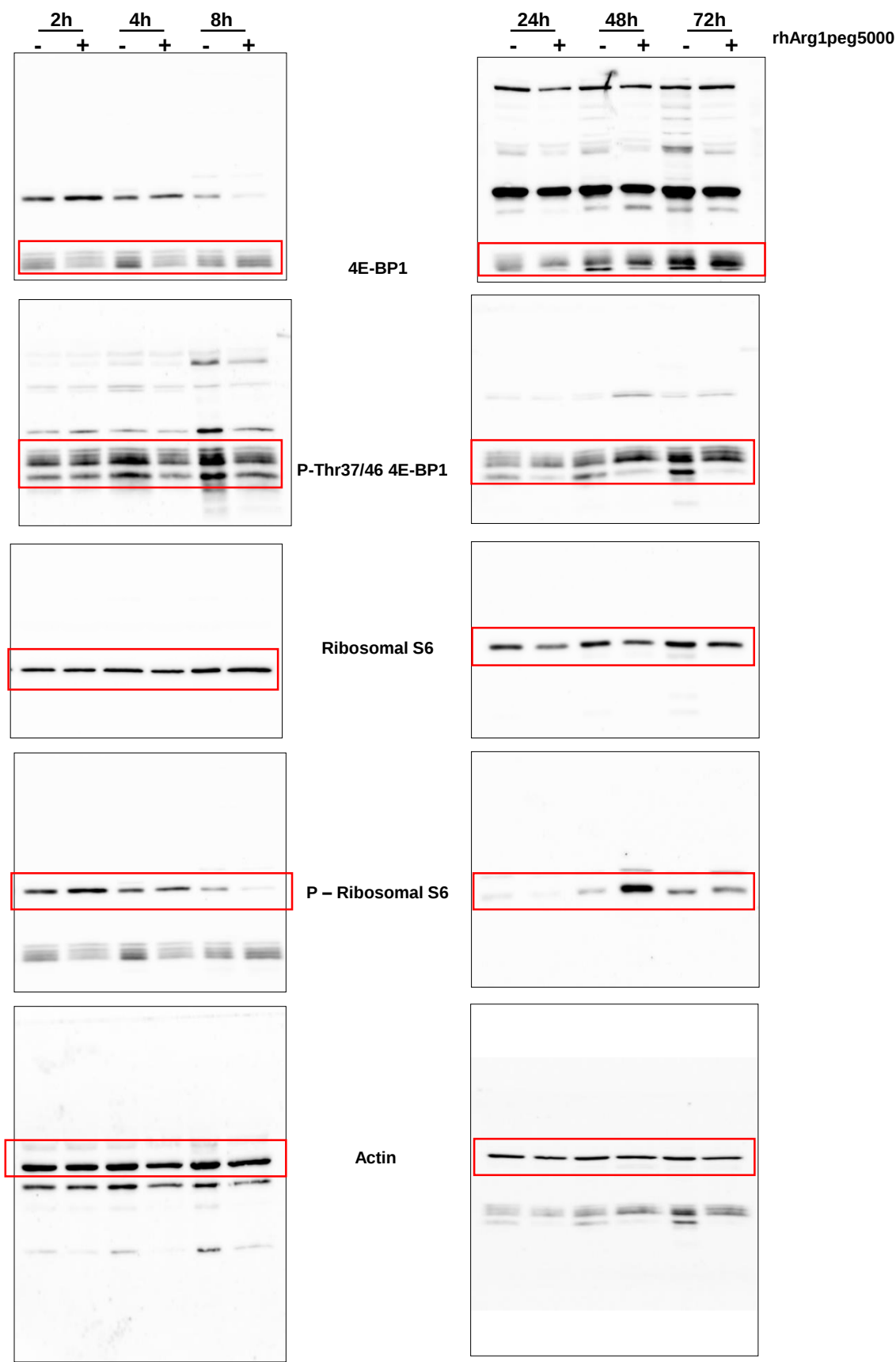
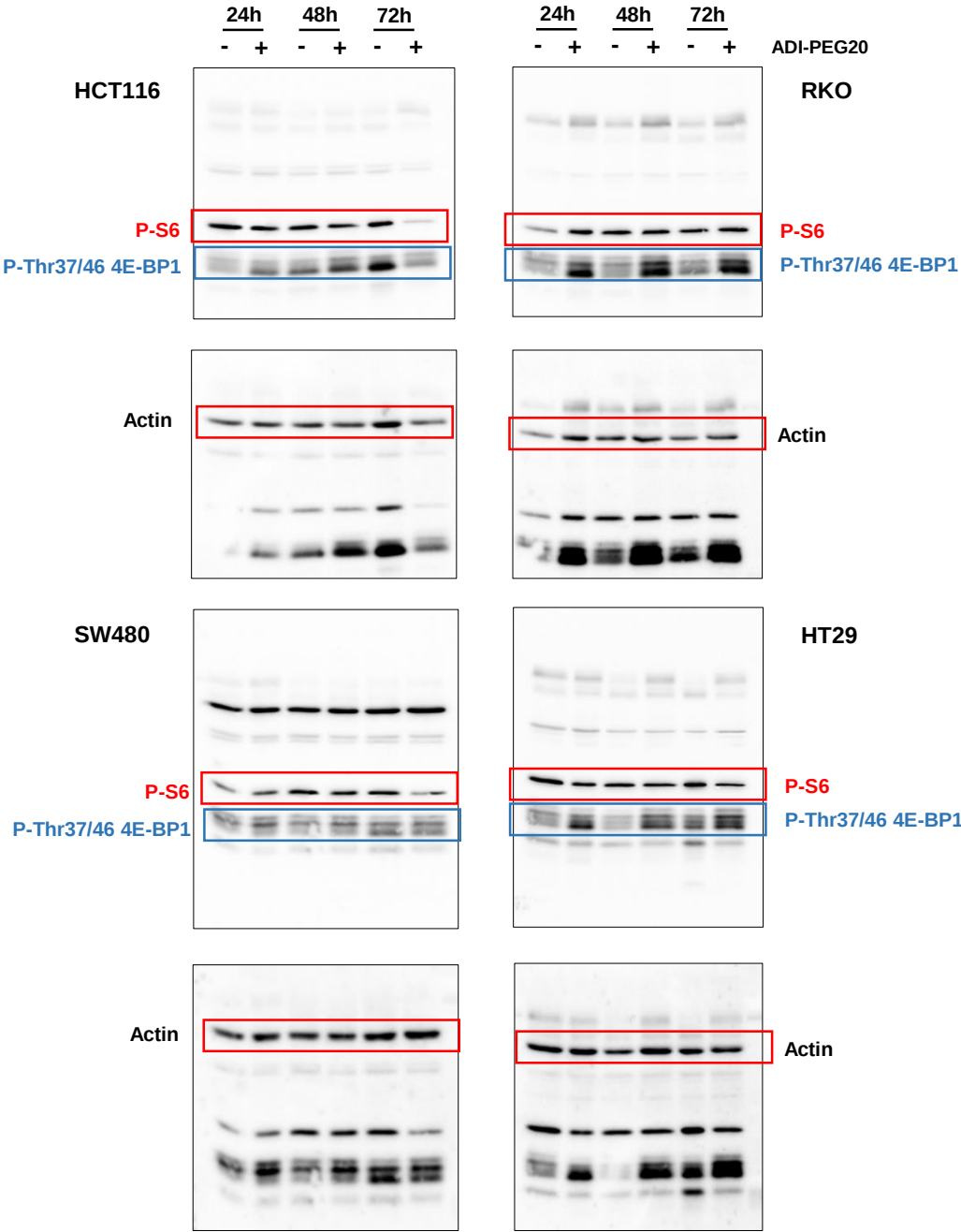


Figure S15

Western blots relative to Figure S11



Characteristic	Number patients	Percentage (%)	Relationship with survival
Sex			
Male	340	52.3	$\chi^2=0.027$, $p=0.870$
Female	310	47.7	
Age			
<70	305	46.9	$\chi^2=29.213$, $p<0.001$
≥70	345	53.1	
Tumour site			
Proximal colon	261	40.2	Proximal v distal, $\chi^2=8.418$, $p=0.004$
Distal colon	245	37.7	Distal v rectal, $\chi^2=0.906$, $p=0.341$
Rectum	144	22.2	Colon v rectum, $\chi^2=0.098$, $p=0.754$
Tumour differentiation			
Well/moderate	600	92.3	$\chi^2=0.976$, $p=0.323$
Poor	50	7.7	
Extra-mural venous invasion			
Present	140	21.5	$\chi^2=100.946$, $p<0.001$
Absent	510	78.5	
Mismatch repair protein status			
Deficient	96	15.2	$\chi^2=2.848$, $p=0.091$
Proficient	536	84.8	
pT stage			
T1	30	4.6	T1 v T2, $\chi^2=0.382$, $p=0.536$
T2	114	17.5	T2 v T3, $\chi^2=24.739$, $p<0.001$
T3	411	63.2	T3 v T4, $\chi^2=30.159$, $p<0.001$
T4	95	14.6	
pN stage			
N0	364	56	N0 v N1, $\chi^2=54.071$, $p<0.001$
N1	177	27.2	N1 v N2, $\chi^2=17.636$, $p<0.001$
N2	109	16.8	
Dukes stage			
A	120	18.5	A v B, $\chi^2=5.059$, $p=0.025$
B	244	37.5	B v C, $\chi^2=65.510$, $p<0.001$
C	286	44	

Supplemental Table 1. Clinico-pathological characteristics of patients in the TMA cohort.