Supplemental Figures (S1 – S22)

Supplemental Tables (S1 – S2)

Related Supplemental File, pertaining to:

- Additional replicates and full-view gels/blots
- Log-plot of cytotoxic plots in Figure 4
- Flow-cytometry analysis (related to Table S2)

Figure S1

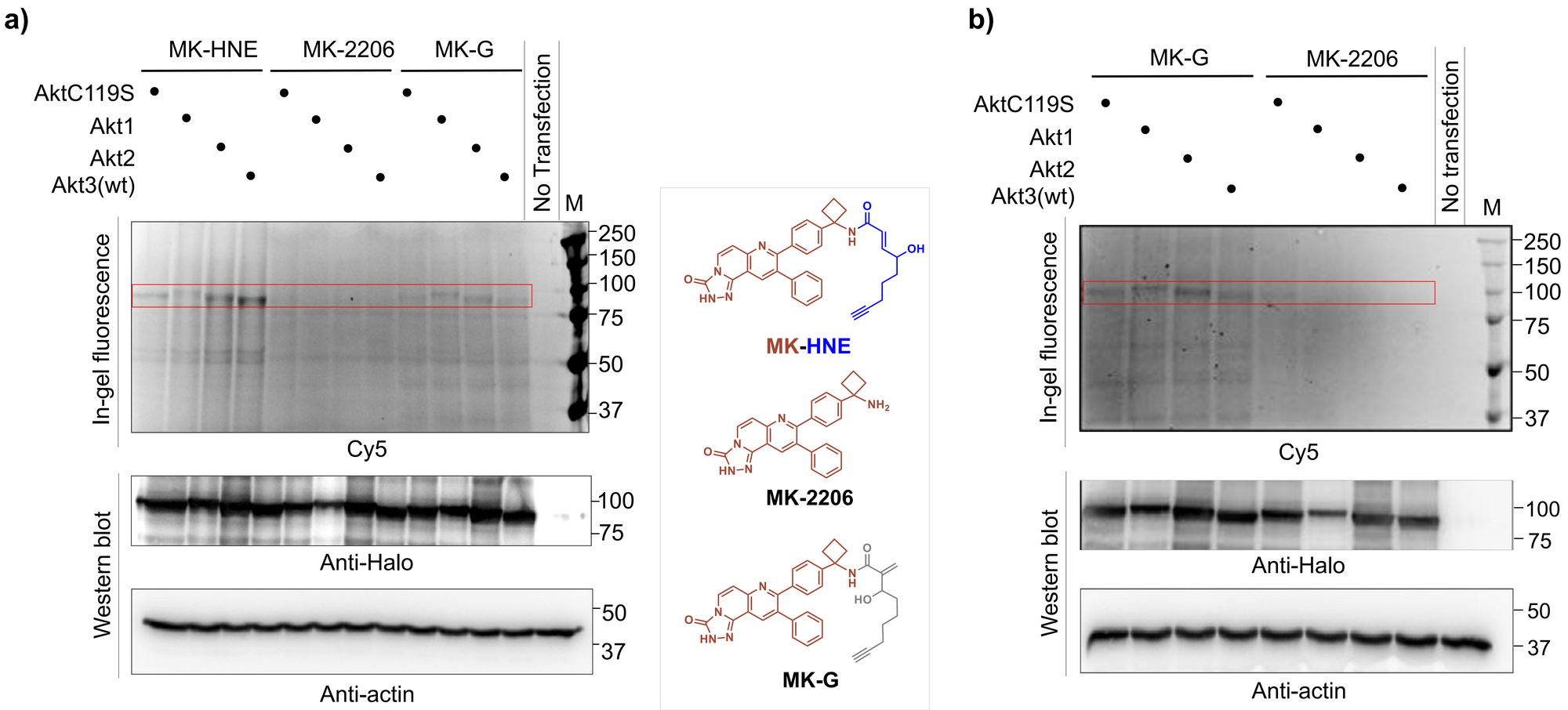
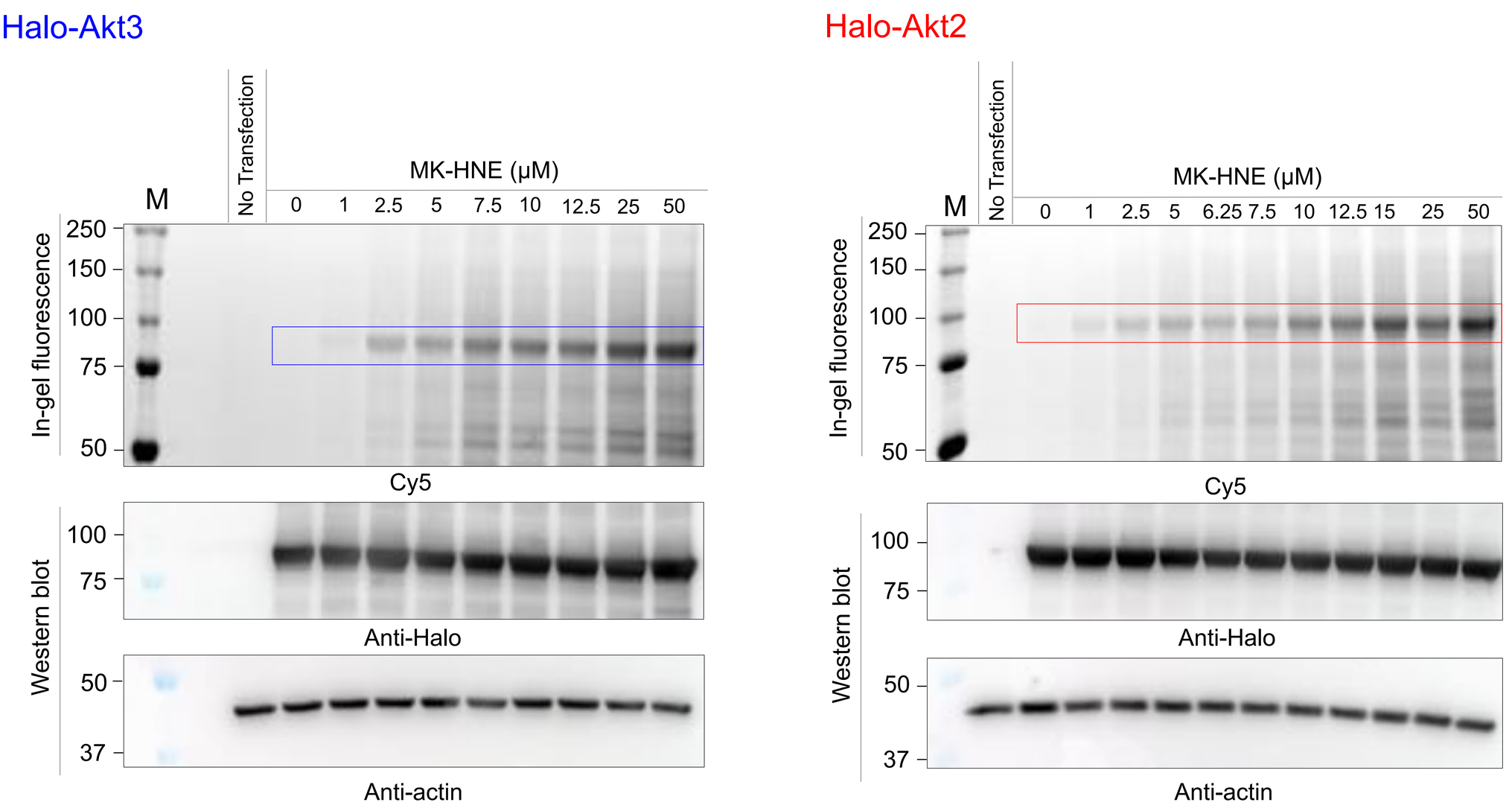

a)
MK-HNE
MK-2206
MK-G
No Transfection
M
AktC119S
Akt1
Akt2
Akt3(wt)
In-gel fluorescence
250
150
100
75
50
37
Cy5
Western blot
100
75
Anti-Halo
50
37
Anti-actin
MK-HNE
MK-2206
MK-G
b)
MK-G
MK-2206
No transfection
M
AktC119S
Akt1
Akt2
Akt3(wt)
In-gel fluorescence
250
150
100
75
50
37
Cy5
Western blot
100
75
Anti-Halo
50
37
Anti-actin

Figure S2


Halo-Akt3
Halo-Akt2
M
No Transfection
MK-HNE (μM)
0 1 2.5 5 7.5 10 12.5 25 50
0 1 2.5 5 6.25 7.5 10 12.5 15 25 50
In-gel fluorescence
250
150
100
75
50
Cy5
Western blot
100
75
Anti-Halo
50
37
Anti-actin

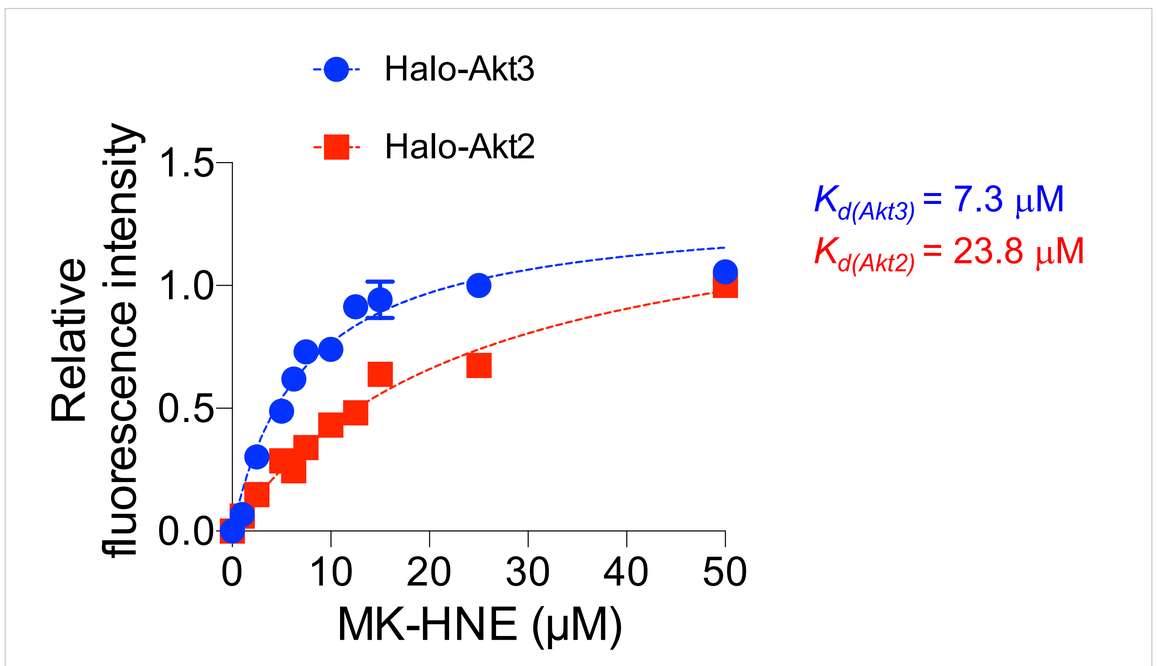

Halo-Akt3
Halo-Akt2
Relative fluorescence intensity
1.5
1.0
0.5
0.0
0
10
20
30
40
50
MK-HNE (μM)
$K_{d(Akt3)}$ = 7.3 μM
$K_{d(Akt2)}$ = 23.8 μM

Figure S3

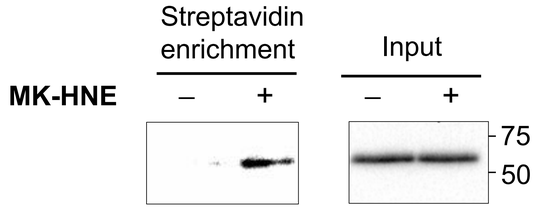
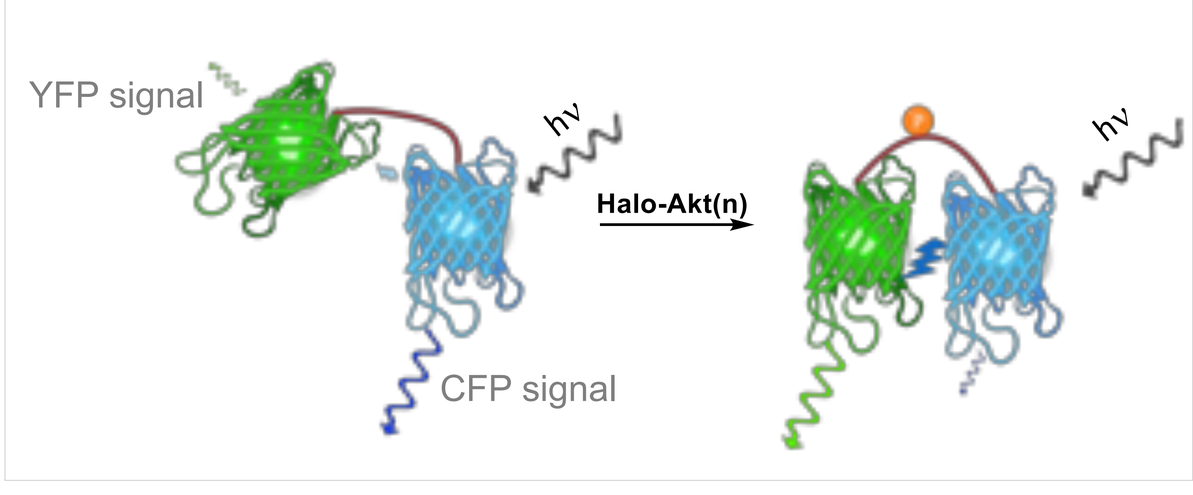


Figure S4


YFP signal
hν
Halo-Akt(n)
CFP signal
hν

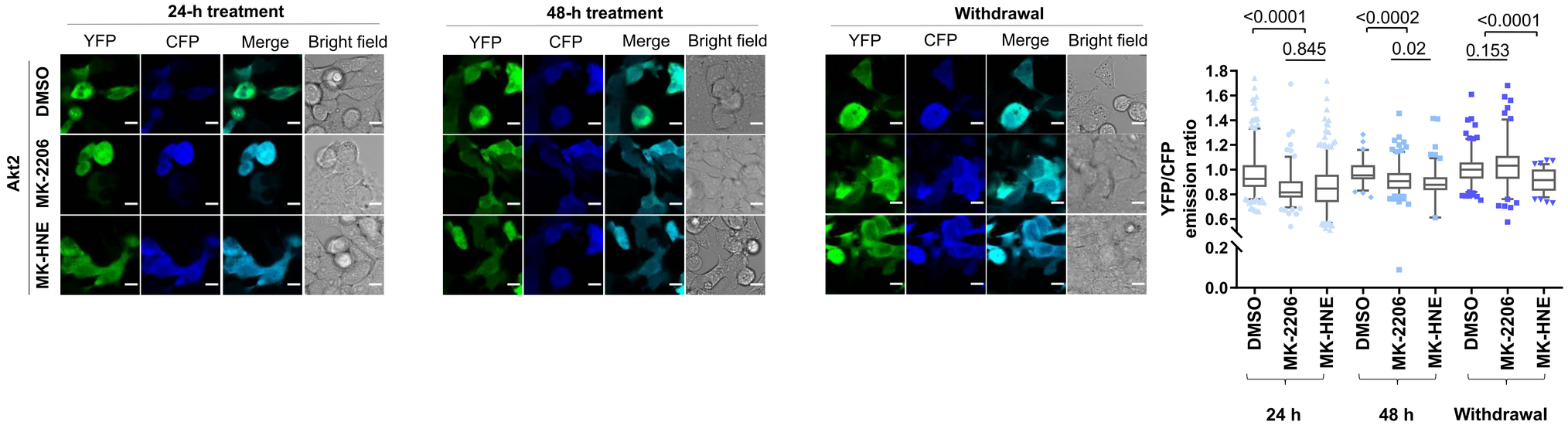
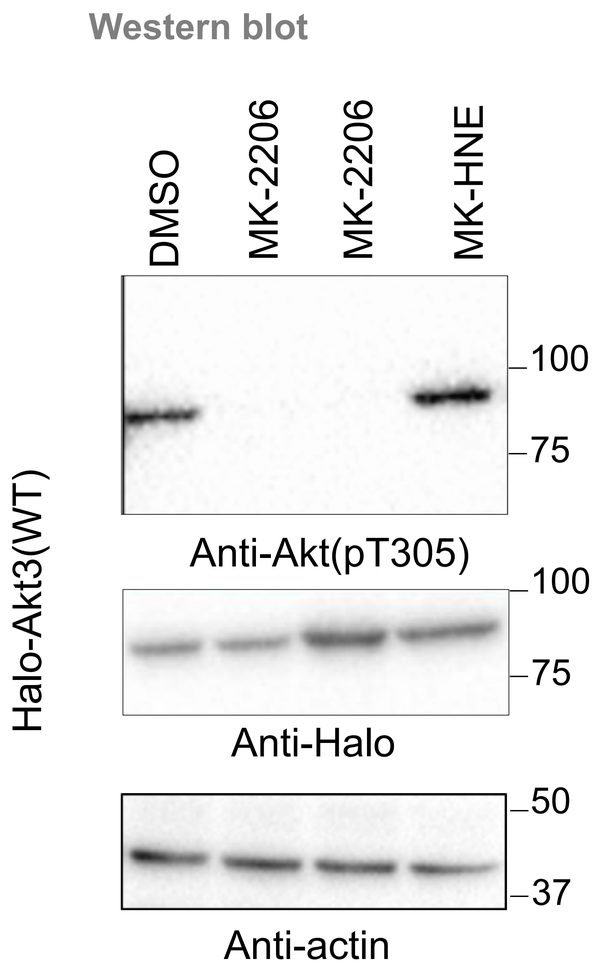
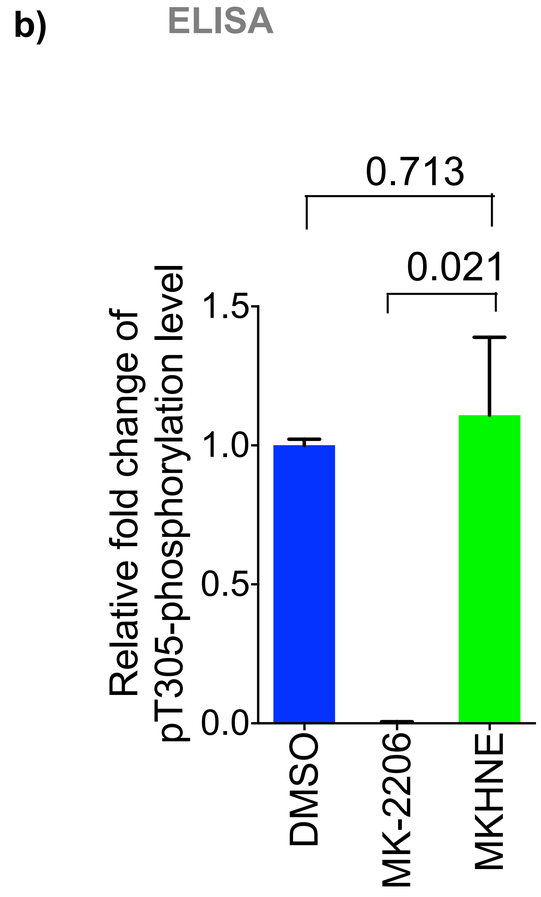

24-h treatment
48-h treatment
Withdrawal
YFP
CFP
Merge
Bright field
Akt2
DMSO
MK-2206
MK-HNE
<0.0001
0.845
<0.0002
0.02
<0.0001
0.153
YFP/CFP emission ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.2
0.0
DMSO
MK-2206
MK-HNE
24 h
48 h
Withdrawal

Figure S5


a)
Western blot
DMSO
MK-2206
MK-2206
MK-HNE
Halo-Akt3(WT)
100
75
Anti-Akt(pT305)
100
75
Anti-Halo
50
37
Anti-actin


b)
ELISA
0.713
0.021
Relative fold change of pT305-phosphorylation level
1.5
1.0
0.5
0.0
DMSO
MK-2206
MKHNE

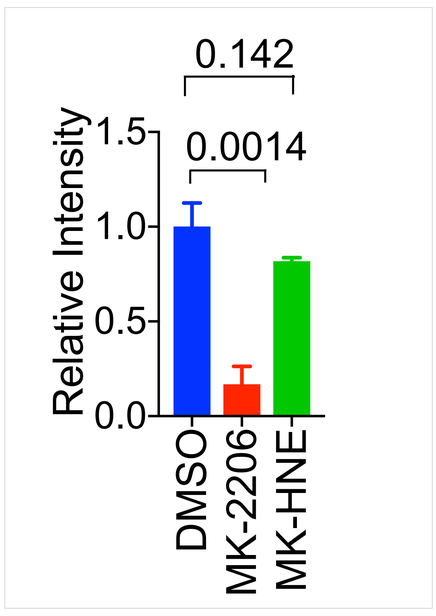

0.142
0.0014
Relative Intensity
1.5
1.0
0.5
0.0
DMSO
MK-2206
MK-HNE

Figure S6

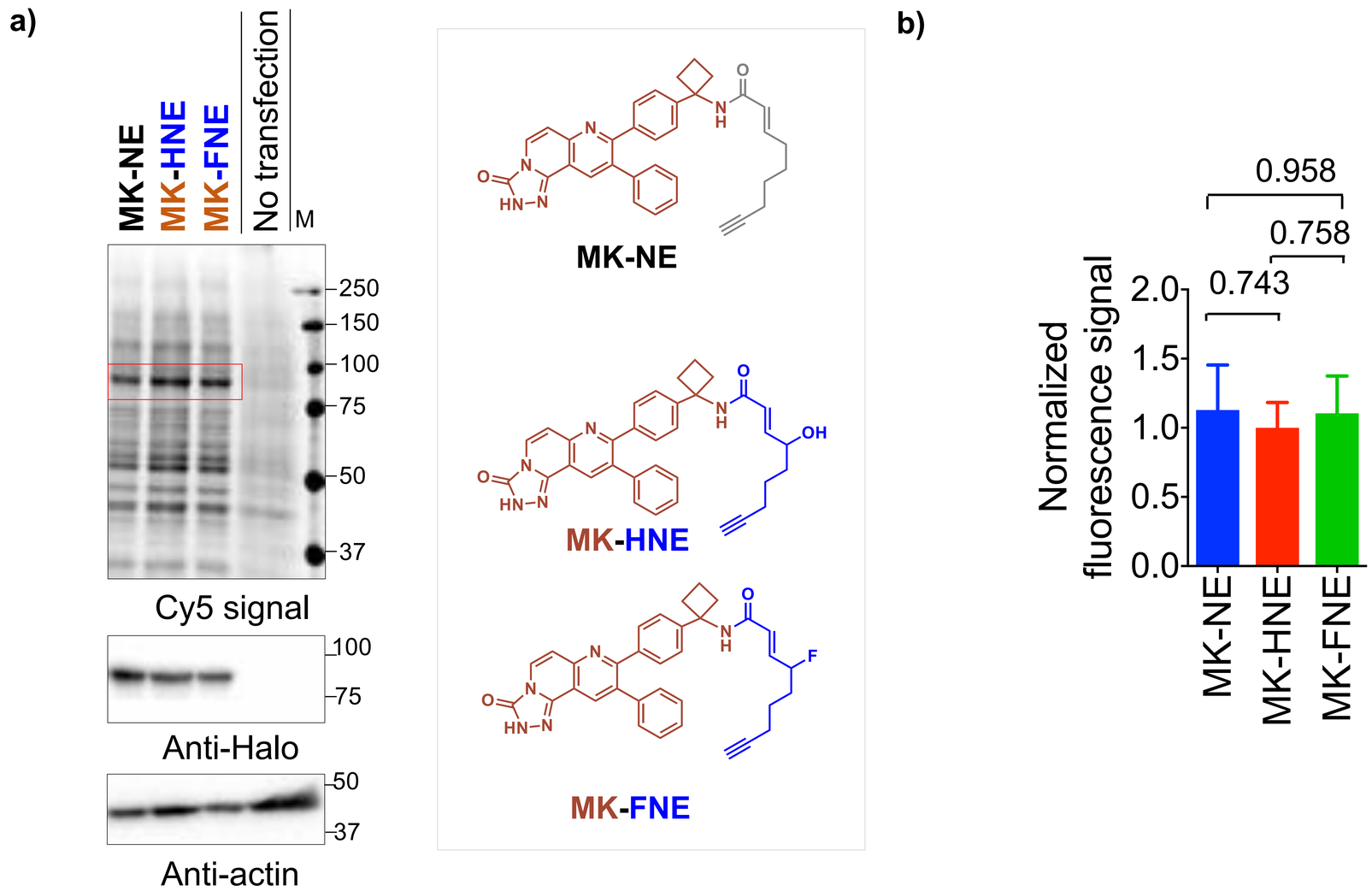
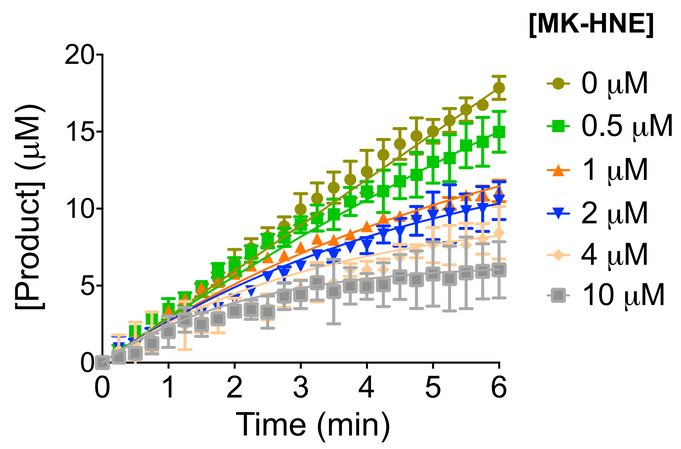
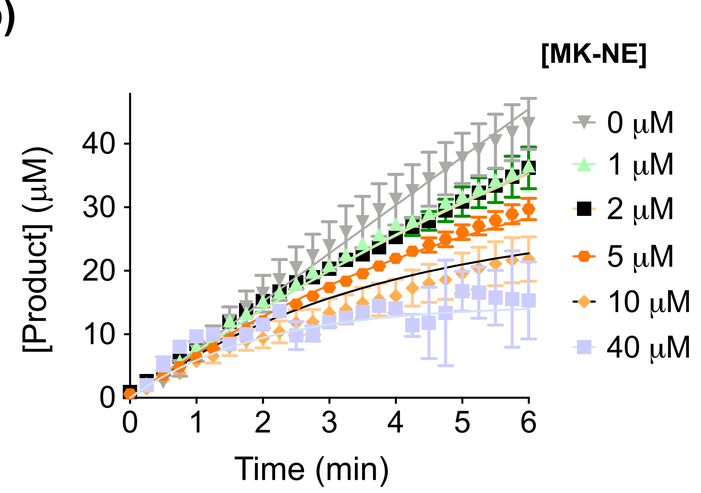

a)
MK-NE
MK-HNE
MK-FNE
No transfection
M
250
150
100
75
50
37
Cy5 signal
100
75
Anti-Halo
50
37
Anti-actin
MK-NE
MK-HNE
MK-FNE
b)
Normalized fluorescence signal
2.0
1.5
1.0
0.5
0.0
0.958
0.758
0.743
MK-NE
MK-HNE
MK-FNE

Figure S7

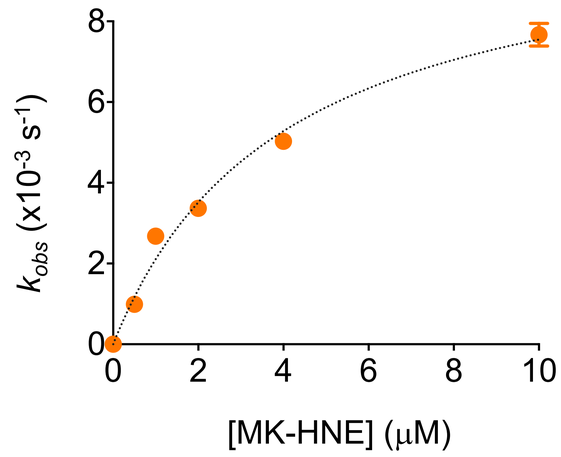
a)

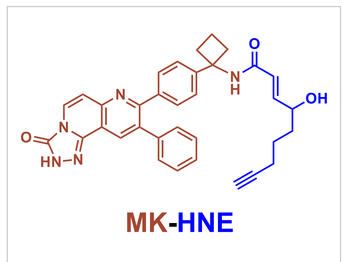


b)

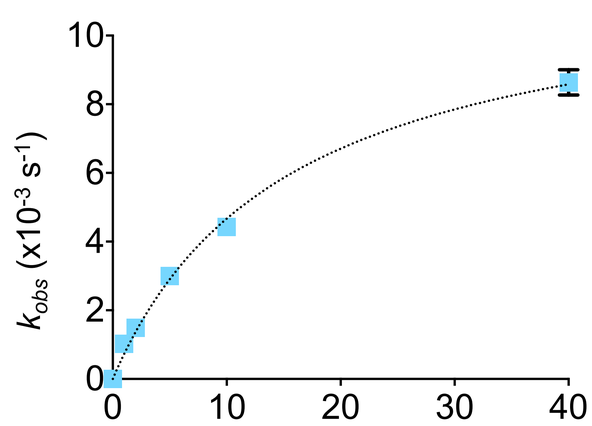


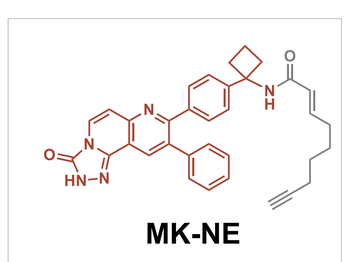
c)



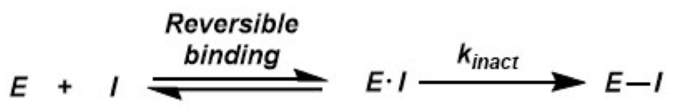


d)





e)



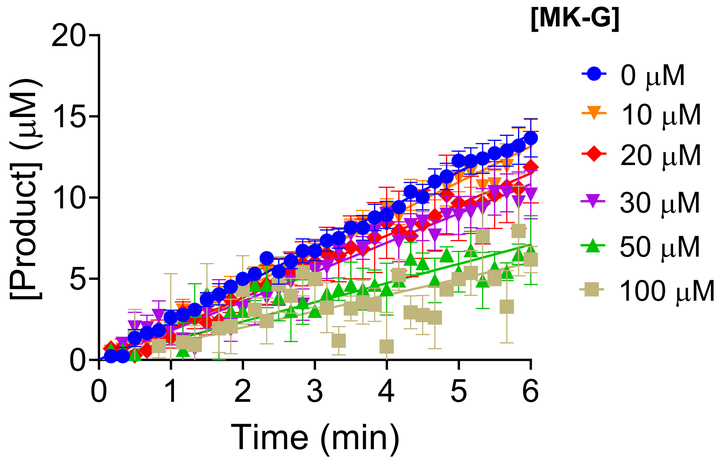
(i) $[P] = v_s t + \dfrac{v_i - v_s}{k_{obs}(1 - e^{-k_{obs}t})}$

(ii) $K_i^{app} = \dfrac{[E][I]}{[E \bullet I] + [E\text{–}I]}$; $k_{obs} = k_{inact} \dfrac{[I]^n}{(K_i^{app})^n + [I]^n}$

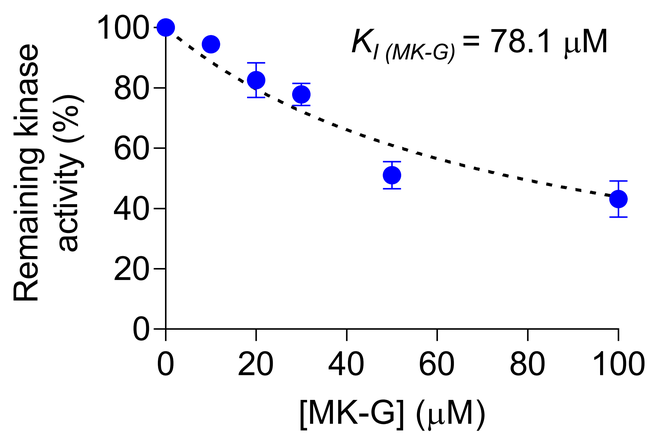
(iii) $k_{obs} = k_{inact} \dfrac{[I]}{K_i^{app} + [I]}$

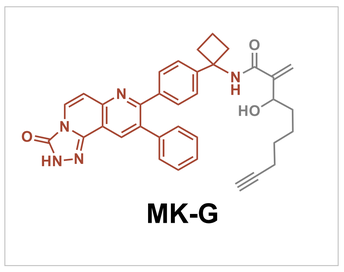
[*E*]: Free enzyme concentration; [*I*]: Inhibitor concentration; [*E*·*I*]: concentration of initial non-covalent enzyme-inhibitor complex; [*E*—*I*]: concentration of covalent enzyme-inhibitor complex; v_i: initial rate of product formation; v_s: rate of product formation at steady state; k_{obs}: rate constant for converting the initial active form of the enzyme to the inhibited form at steady state; K_i^{app}: apparent reversible and covalent binding kinetics between covalent inhibitor and enzyme; n: Hill coefficient for inhibitory cooperativeness of inhibitor upon binding to enzyme.

f)



g)





h)

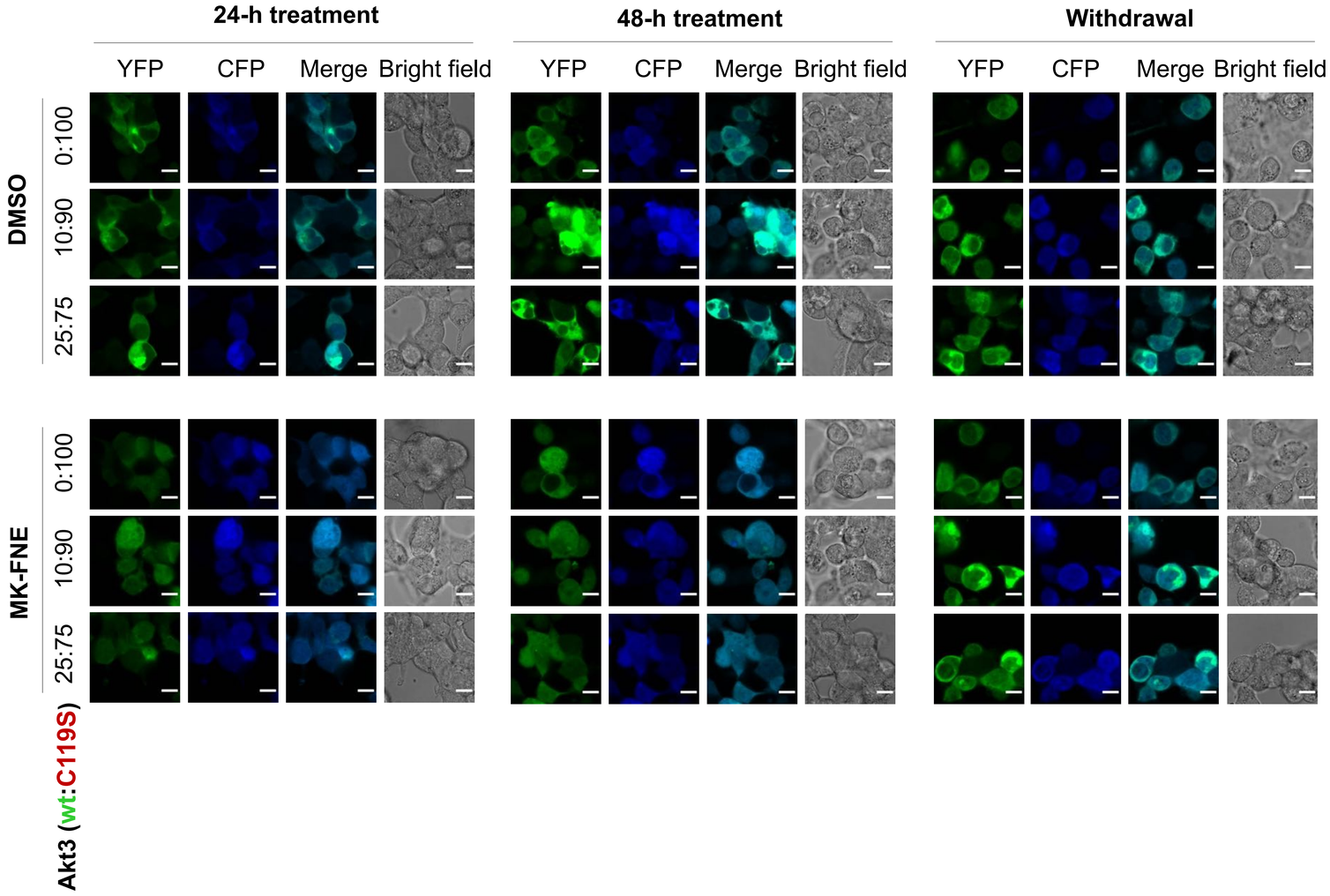
(iv) $[P] = kt$

(v) Remaining kinase activity(%) $= \dfrac{k_0 - k_I}{k_0} \times 100\%$

(vi) Remaining kinase activity(%) $= \dfrac{1}{1 + \dfrac{[I]}{K_I}} \times 100\%$

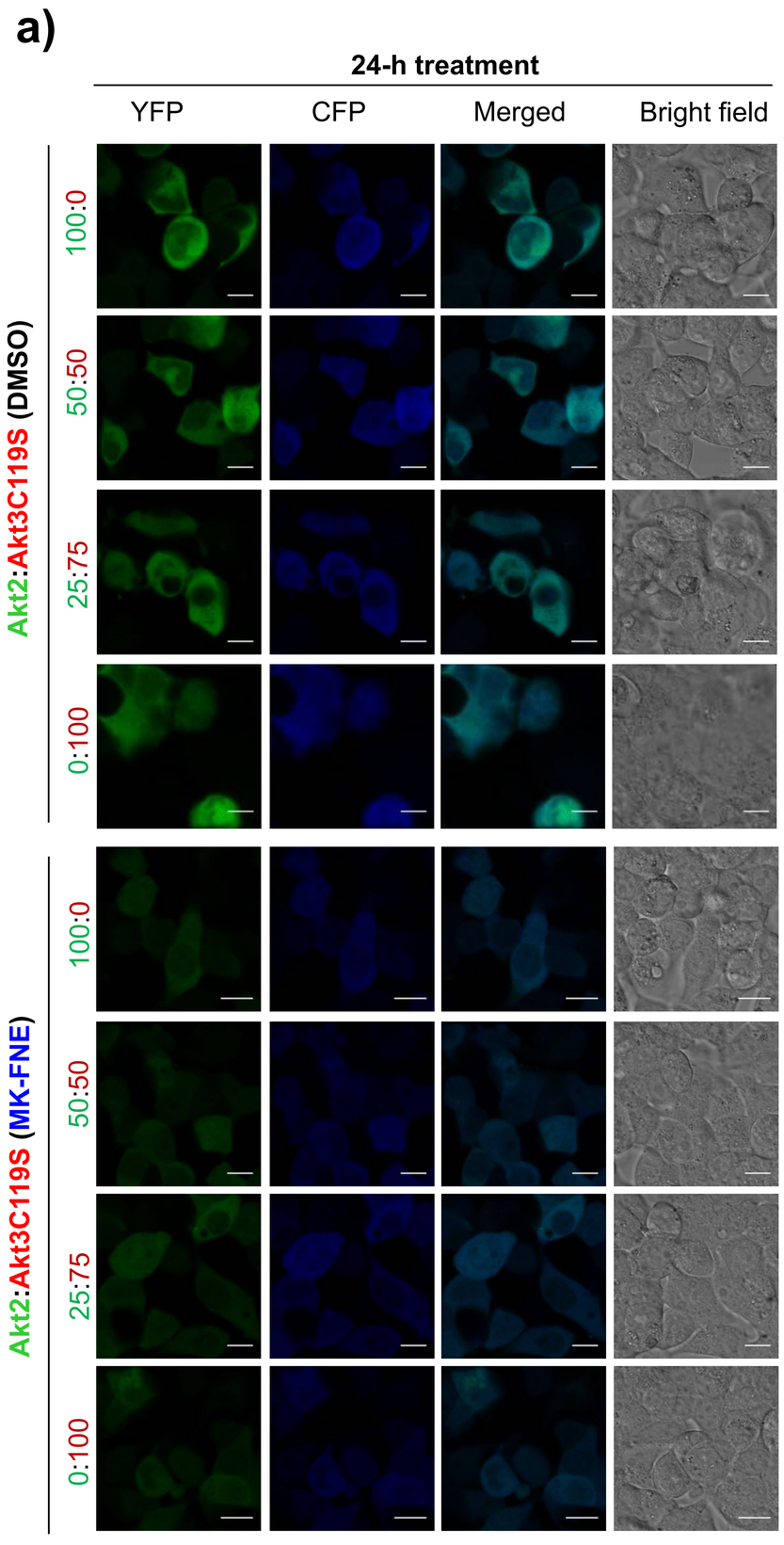
k_I: rate of product formation at the indicated inhibitor concentration; k_0: rate of product formation in the absence of inhibitor; [*I*]: inhibitor concentration; K_I: inhibitor constant

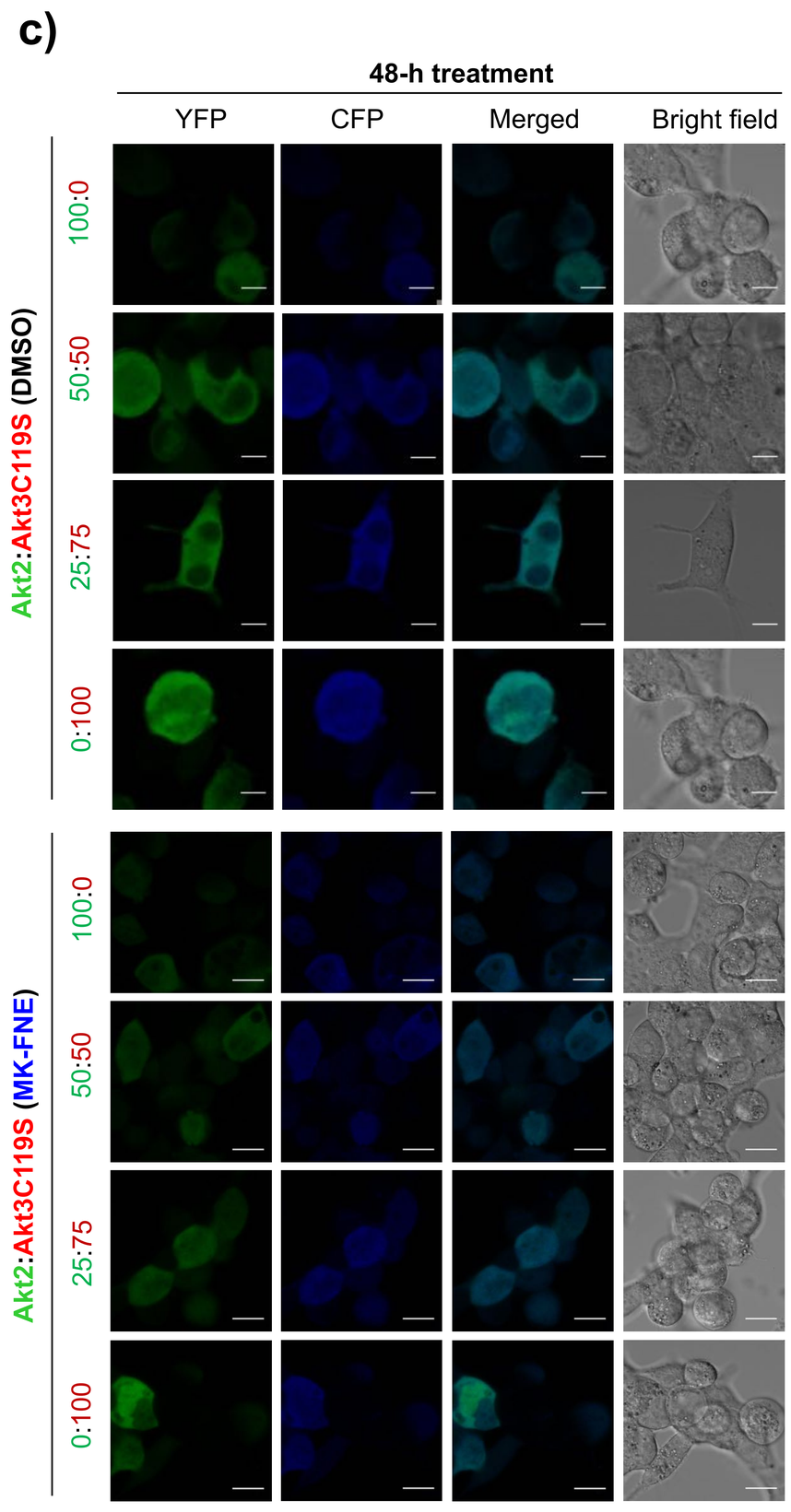
Figure S8

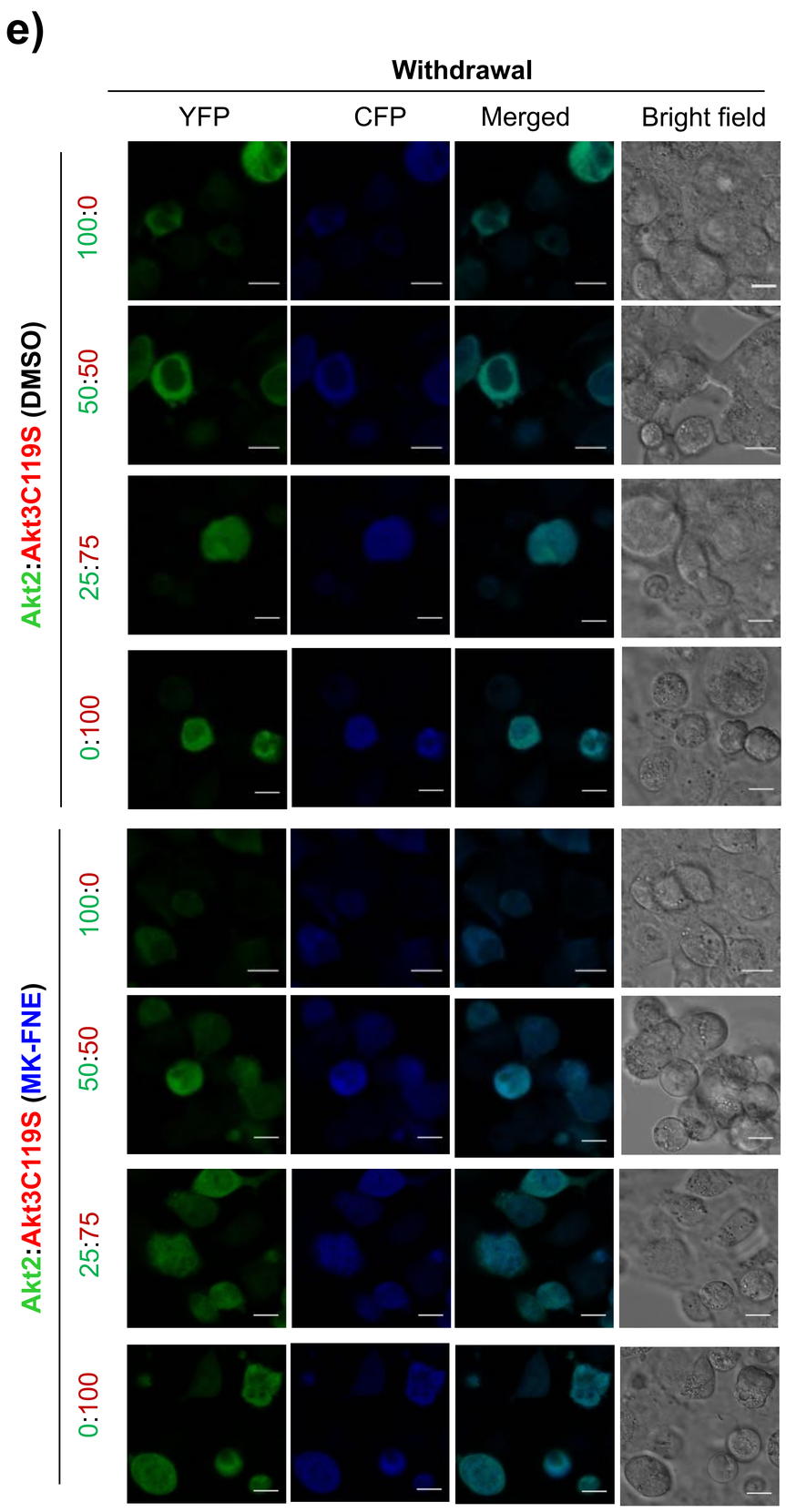


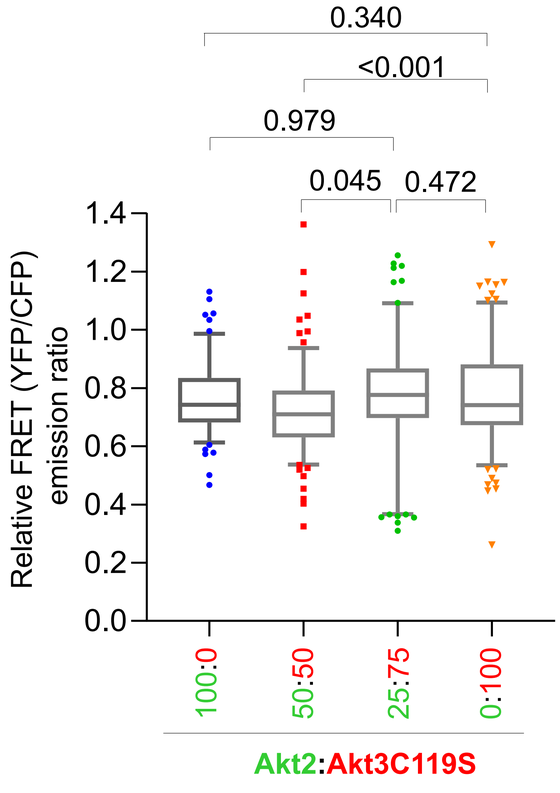
Relative FRET emission ratio for the indicated plasmid mixture	$= YFP/CFP_{\text{MK-FNE withdrawal}}/\langle YFP/CFP \rangle_{\text{DMSO withdrawal}}$

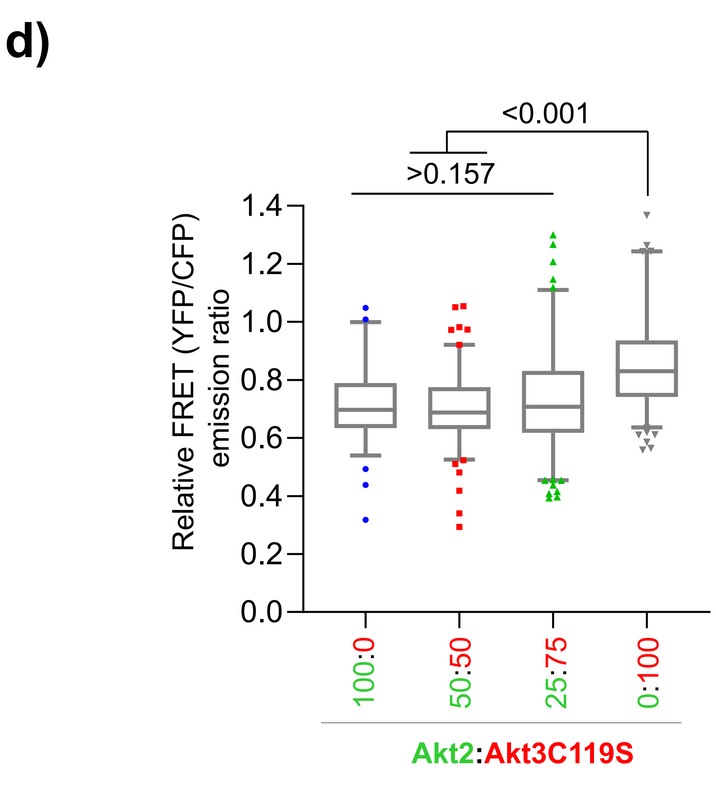
Figure S9

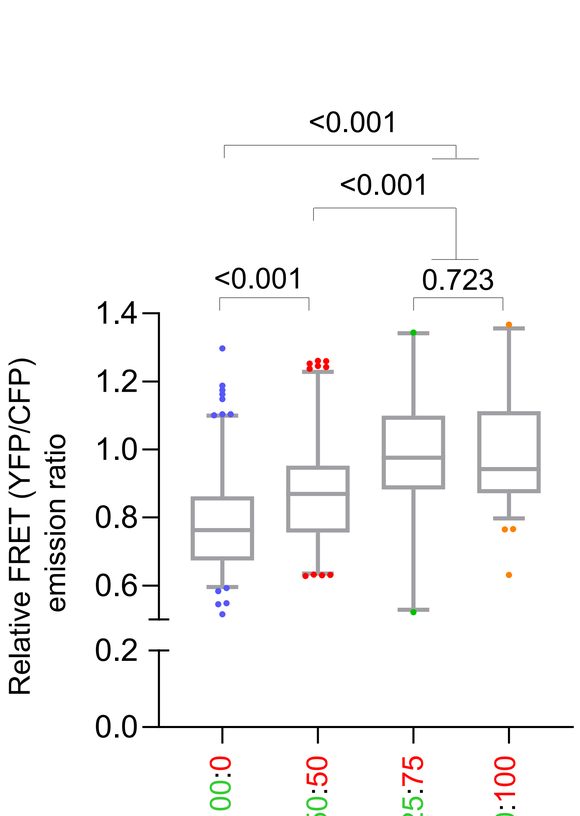












Relative FRET emission ratio for the stated plasmid mixture = $YFP/CFP_{MK\text{-}FNE}/\langle YFP/CFP\rangle_{DMSO}$

Figure S10

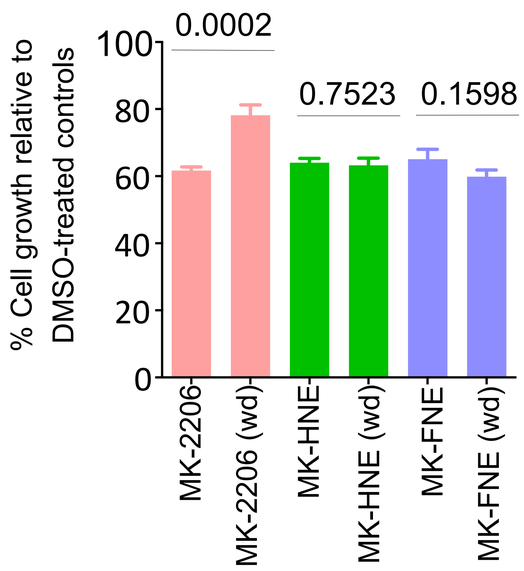

% Cell growth relative to DMSO-treated controls
100
80
60
40
20
0
0.0002
0.7523
0.1598
MK-2206
MK-2206 (wd)
MK-HNE
MK-HNE (wd)
MK-FNE
MK-FNE (wd)

Figure S11

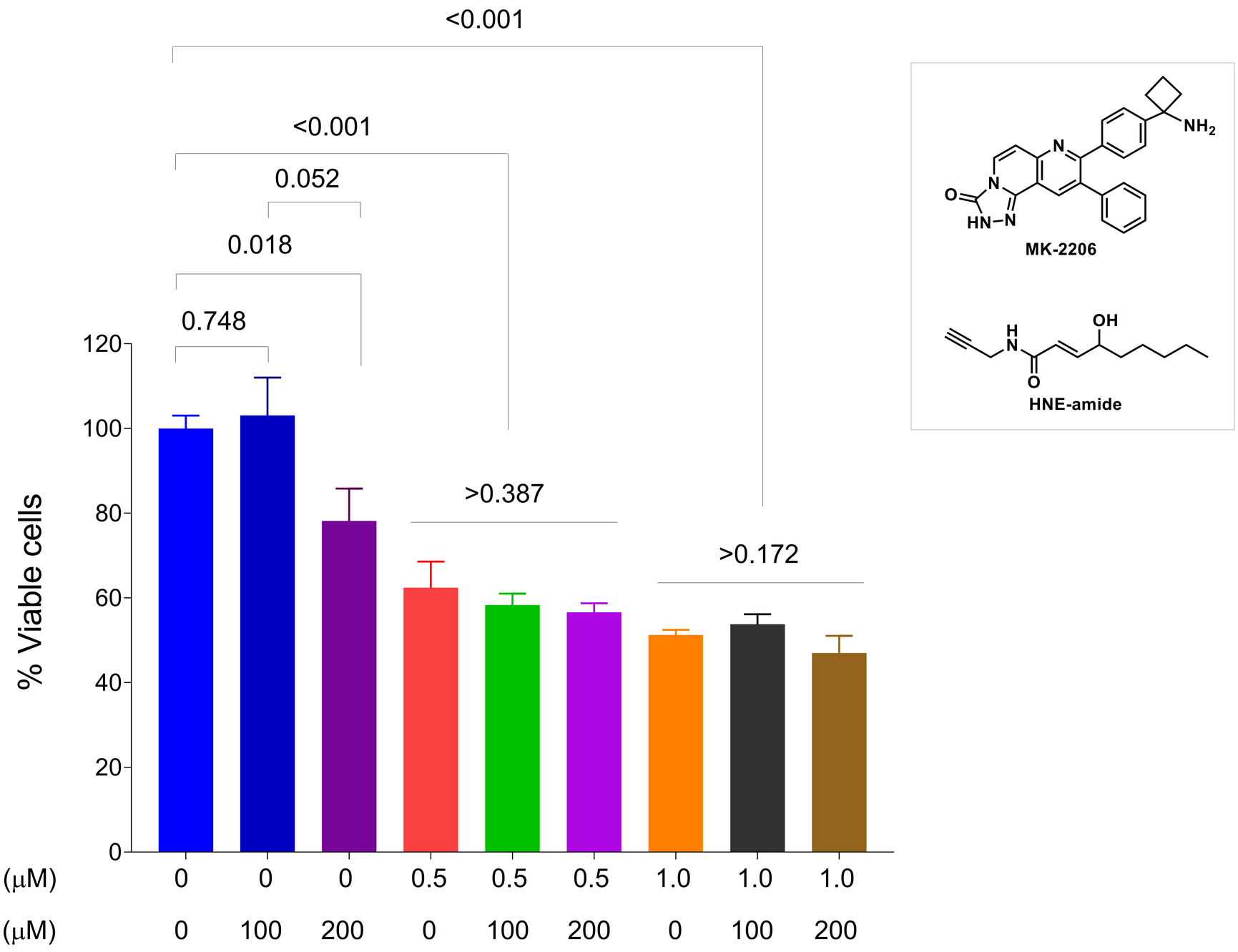
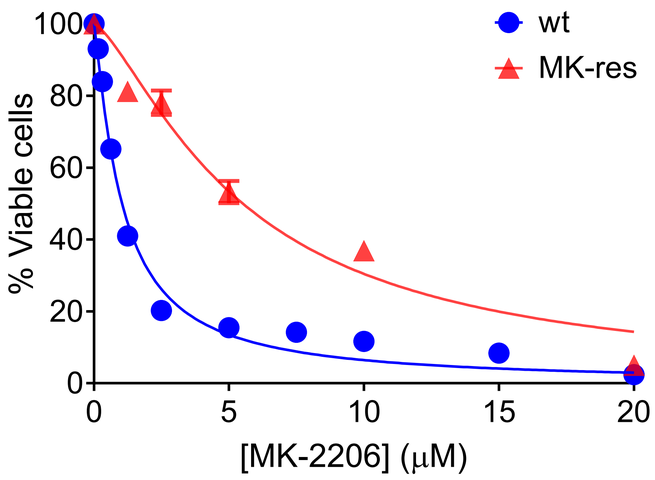
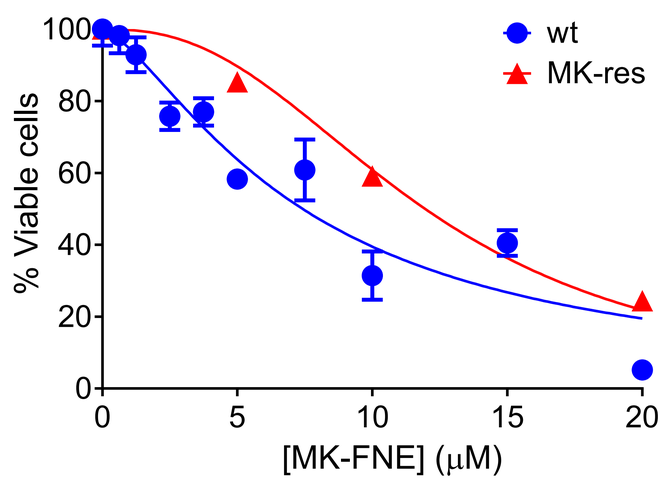

<0.001
<0.001
0.052
0.018
0.748
>0.387
>0.172
% Viable cells
120
100
80
60
40
20
0
[MK-2206] (μM)
0 0 0 0.5 0.5 0.5 1.0 1.0 1.0
[HNE-amide] (μM)
0 100 200 0 100 200 0 100 200
MK-2206
HNE-amide

Figure S12

a)

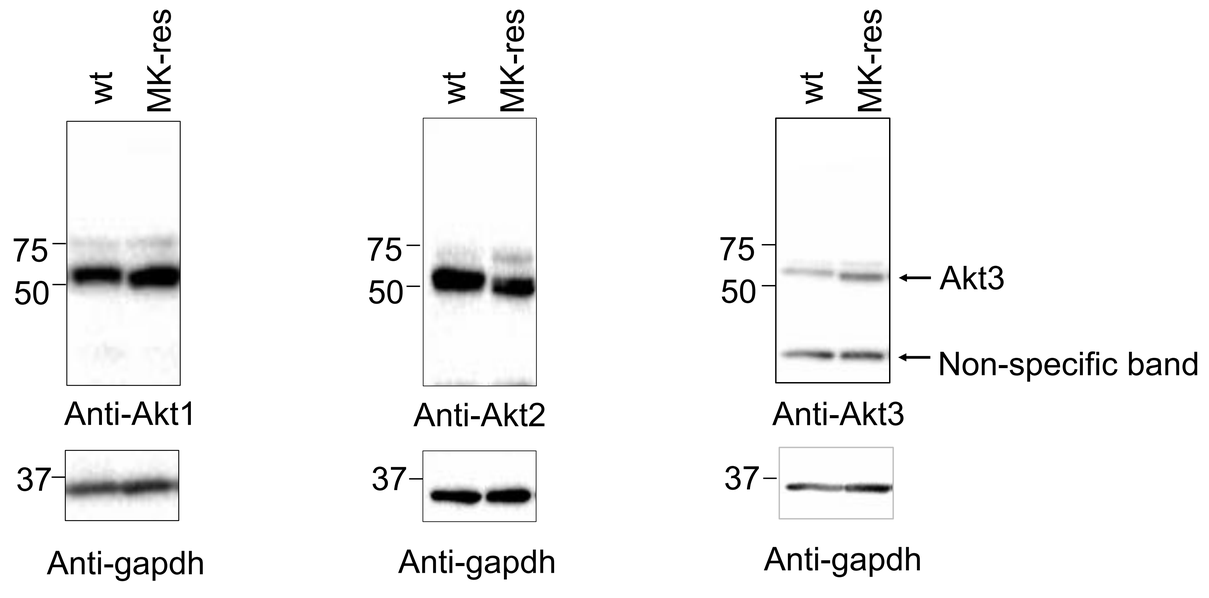


Fold increase in **MK-2206** resistance: 5.3



Fold increase in **MK-FNE** resistance: 1.6

b)



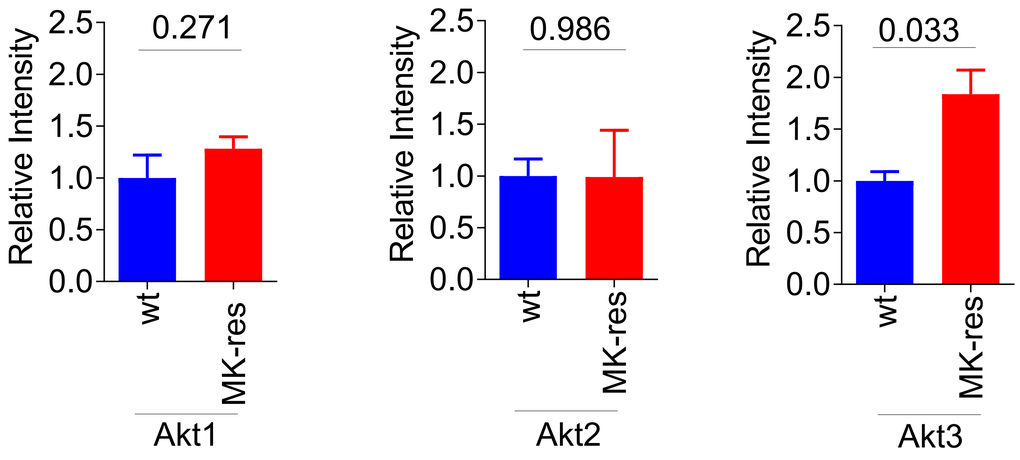
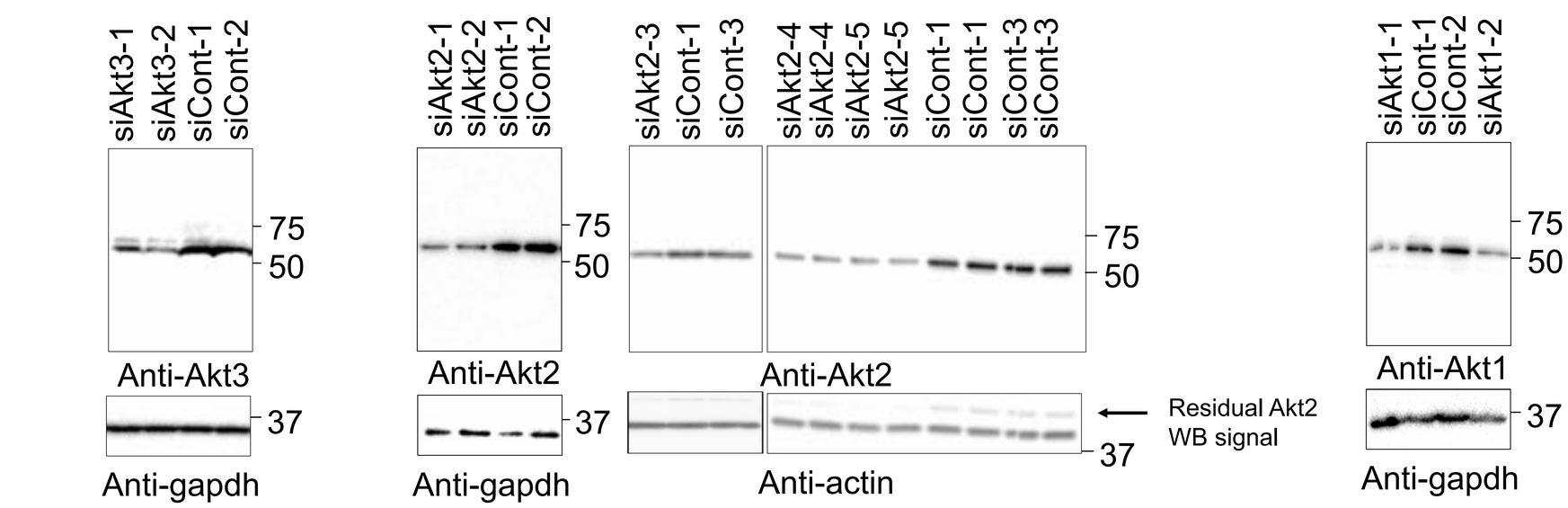


Figure S13


siAkt3-1
siAkt3-2
siCont-1
siCont-2
75
50
Anti-Akt3
37
Anti-gapdh
siAkt2-1
siAkt2-2
siCont-1
siCont-2
siAkt2-3
siCont-1
siCont-3
siAkt2-4
siAkt2-4
siAkt2-5
siAkt2-5
siCont-1
siCont-1
siCont-3
siCont-3
75
50
Anti-Akt2
37
Anti-gapdh
75
50
Anti-Akt2
37
Anti-actin
Residual Akt2
WB signal
siAkt1-1
siCont-1
siCont-2
siAkt1-2
75
50
Anti-Akt1
37
Anti-gapdh

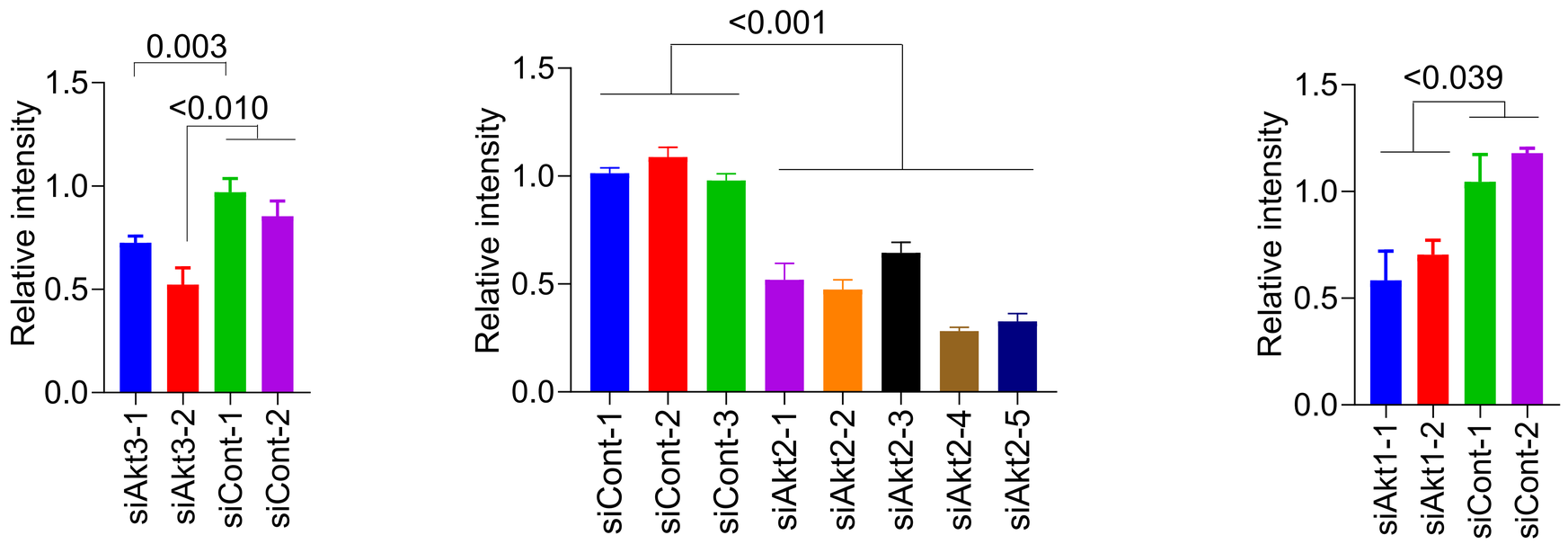

0.003
<0.010
Relative intensity
1.5
1.0
0.5
0.0
siAkt3-1
siAkt3-2
siCont-1
siCont-2
<0.001
Relative intensity
1.5
1.0
0.5
0.0
siCont-1
siCont-2
siCont-3
siAkt2-1
siAkt2-2
siAkt2-3
siAkt2-4
siAkt2-5
<0.039
Relative intensity
1.5
1.0
0.5
0.0
siAkt1-1
siAkt1-2
siCont-1
siCont-2

Figure S14

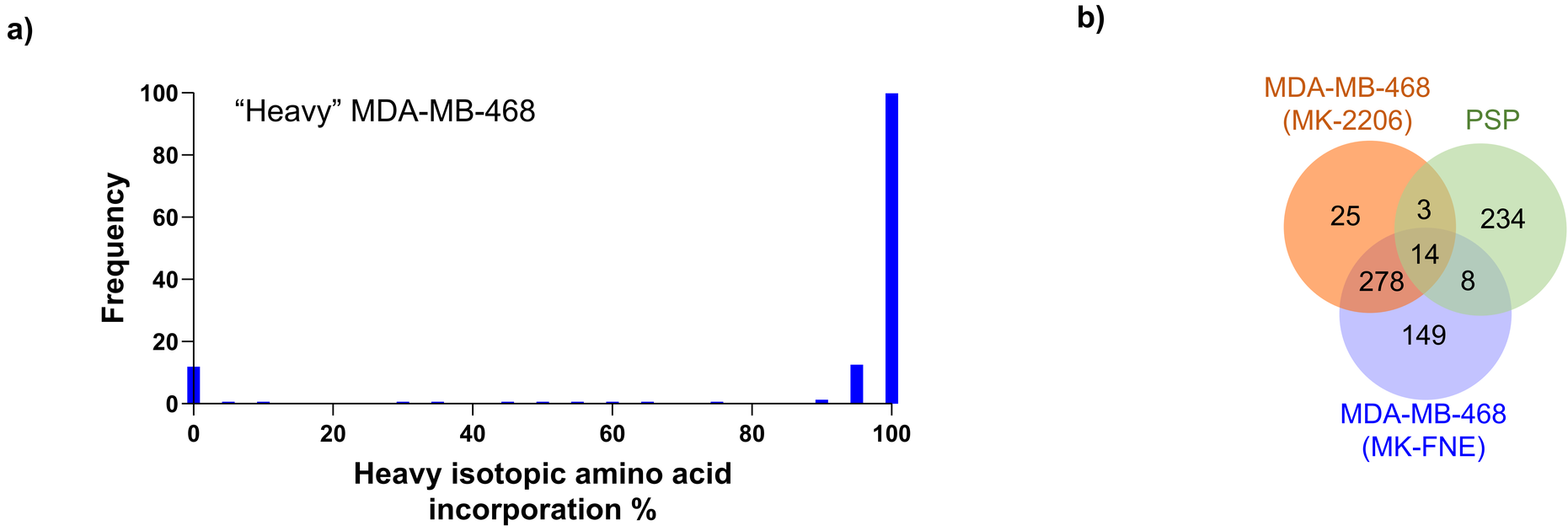

a)
“Heavy” MDA-MB-468
Frequency
100
80
60
40
20
0
0
20
40
60
80
100
Heavy isotopic amino acid incorporation %
b)
MDA-MB-468 (MK-2206)
PSP
MDA-MB-468 (MK-FNE)
25
3
234
14
278
8
149

Figure S15

KIFC1

64	TSHP**RVP**S**S**LTTVPQ[a]	77
90	KTGP**RCS**T**T**AIATGL	103
150	LK**RCRER**T**TQ**T**T**LDQE	163
215	ELEE**RLS**T**T**QEGLVQ	228
250	EKER**RLQ**T**T**SEAALS	263
269	VASL**RQE**T**T**VAQAAL[a]	282
352	RSDE**RRG**T**T**LSGAPA[a]	365
533	ERSS**RSH**S**S**VFQLQI	546
651	LNSL**RFA**S**S**KVNQCV	664

Grsf1

55	AVPT**RSY**S**S**QESKTT[a]	68
227	DYRG**RRK**T**T**GEAYVQ	240

JUP

47	EACG**RQY**T**T**LKKTTT[a]	60
199	LDTA**RCT**T**T**SILHNL	212
229	PALV**RML**S**S**PVESV	242
316	VQIM**RNY**S**S**YEKLLW	329
373	LWTL**RNL**S**S**DVATKQ	386
456	VCAL**RHL**T**T**SRHPEA[a]	469
578	MEIF**RLN**T**T**IPLFVQ	591

Figure S16

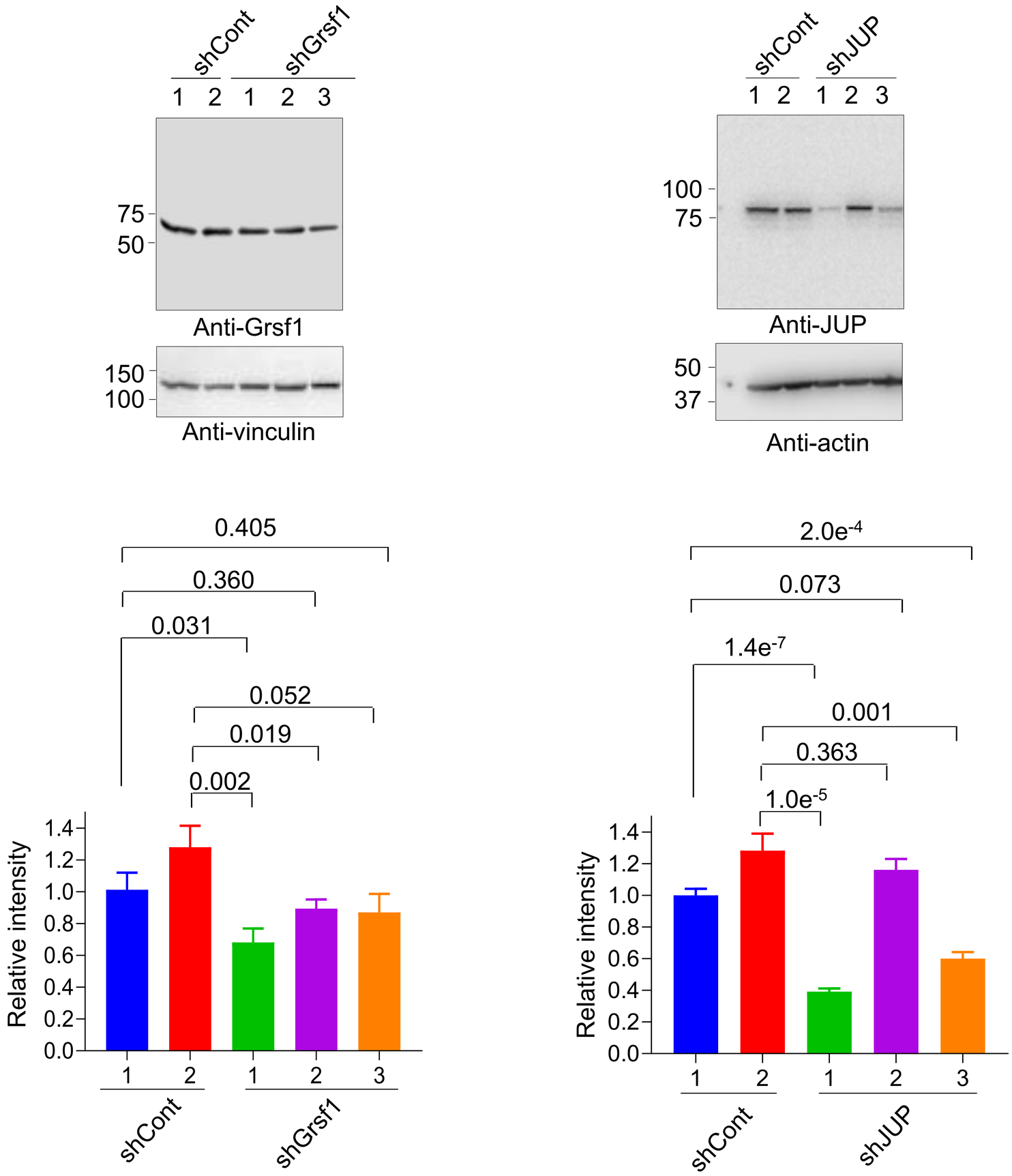
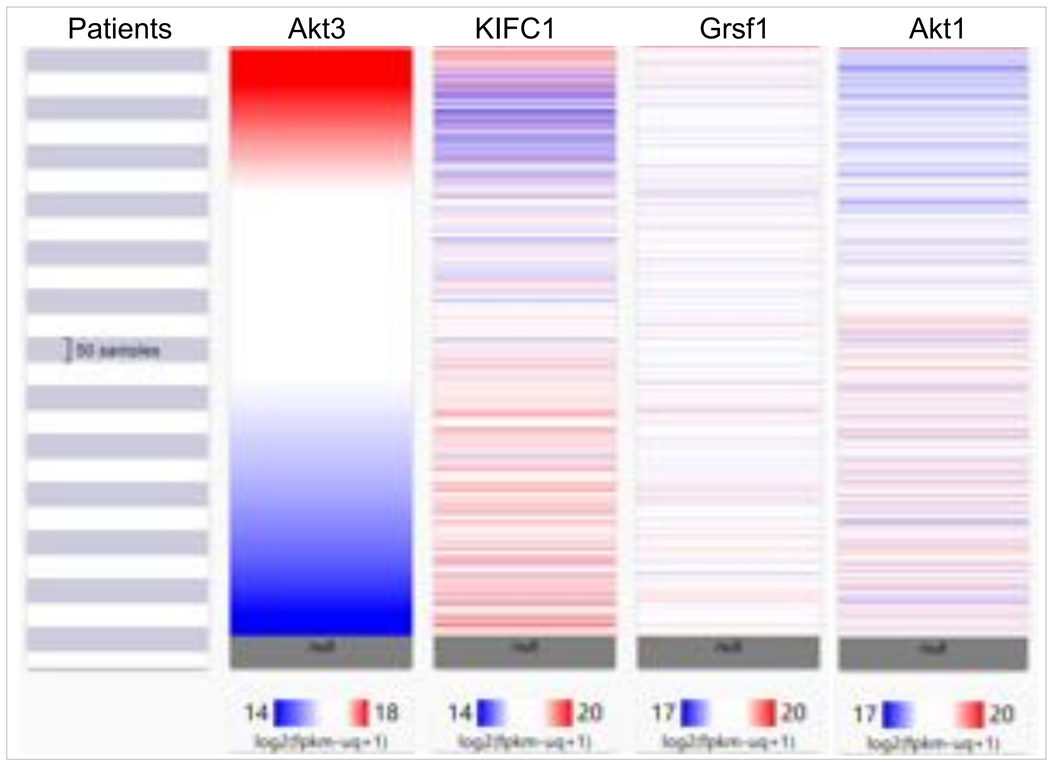
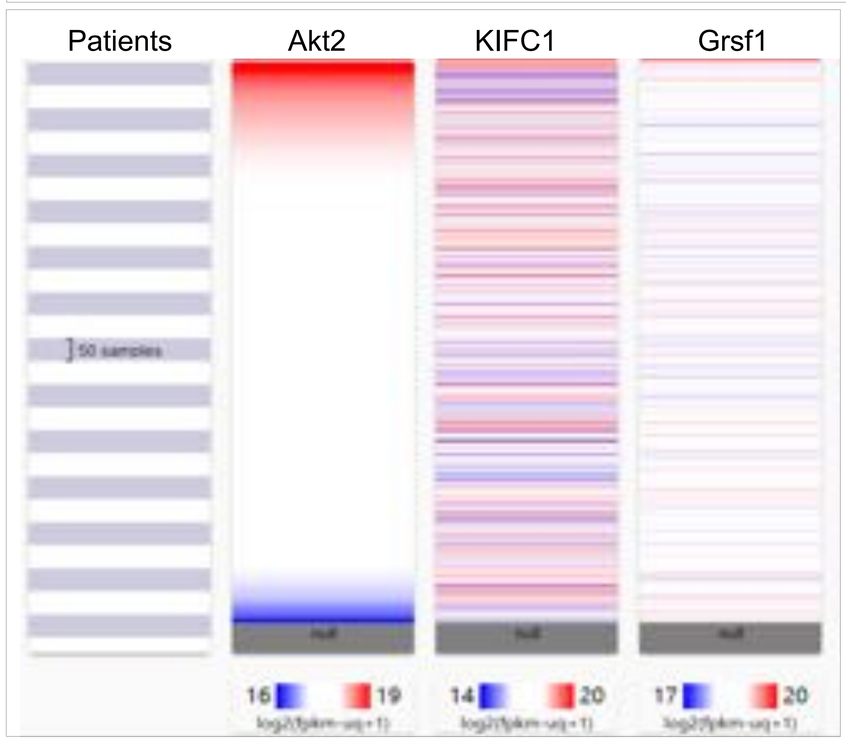
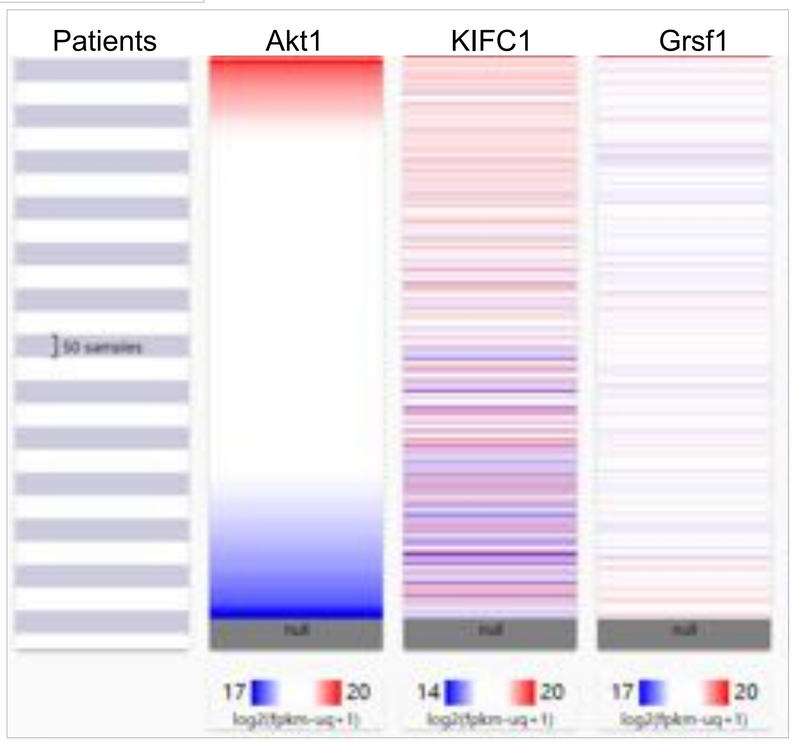

shCont
shGrsf1
1 2 1 2 3
75
50
Anti-Grsf1
150
100
Anti-vinculin
0.405
0.360
0.031
0.052
0.019
0.002
Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 1 2 3
shCont
shGrsf1
shCont
shJUP
1 2 1 2 3
100
75
Anti-JUP
50
37
Anti-actin
2.0e-4
0.073
1.4e-7
0.001
0.363
1.0e-5
Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 1 2 3
shCont
shJUP

Figure S17

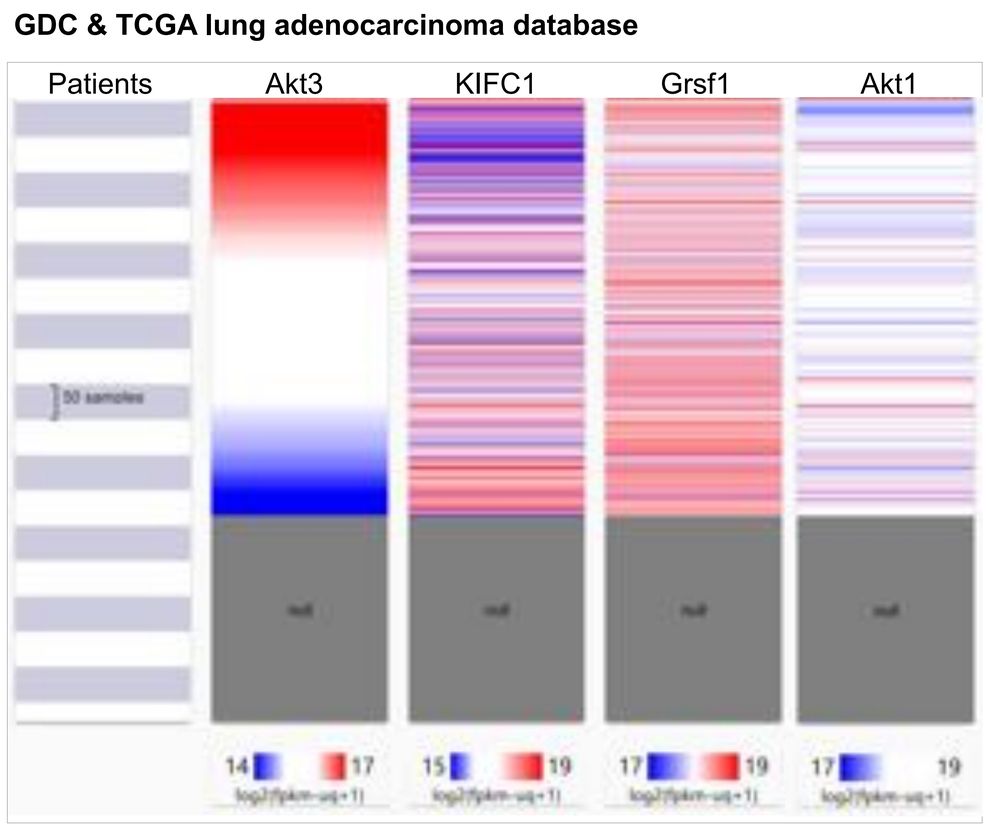


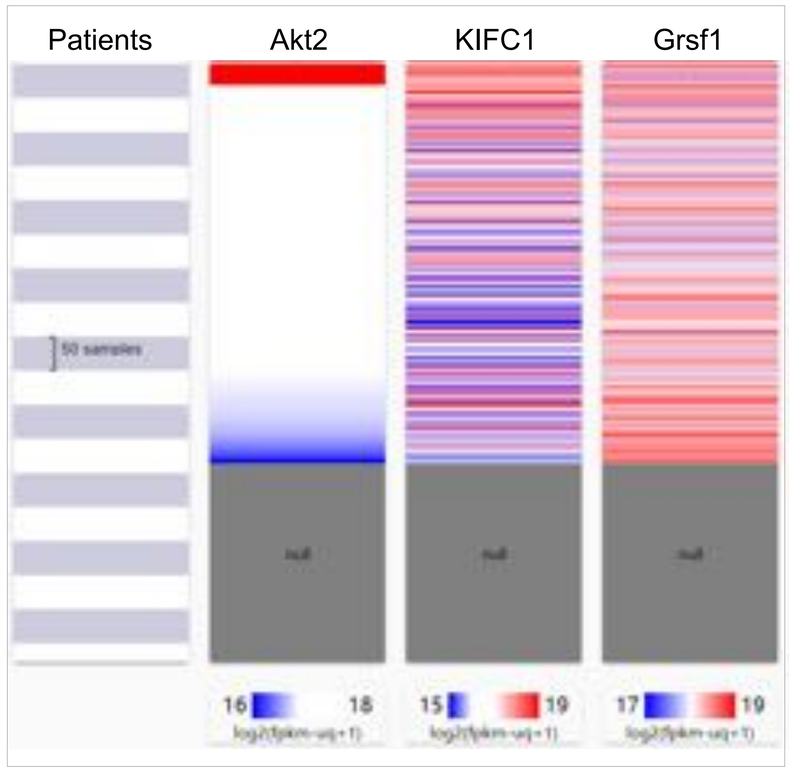


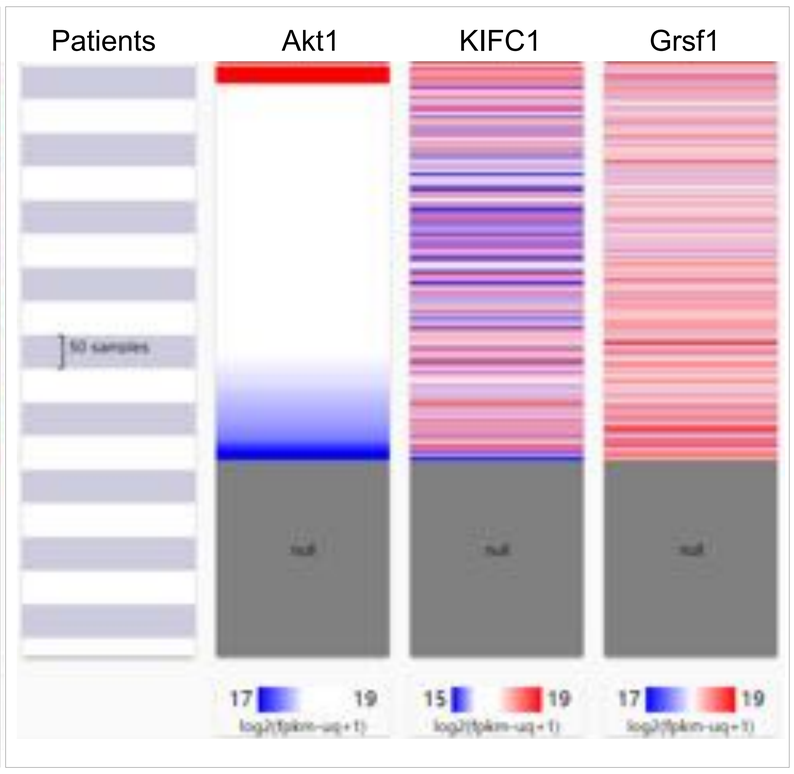


	Akt3			Akt2		Akt1	
	KIFC1	Grsf1	Akt1	KIFC1	Grsf1	KIFC1	Grsf1
***r*-value**	-0.3196	-0.2150	-0.3302	0.02945	-0.08708	0.2209	-0.05116
***P*-value (two tailed)**	<0.0001	<0.0001	<0.0001	0.3046 (ns)	0.0024	<0.0001	0.0744 (ns)

Figure S18



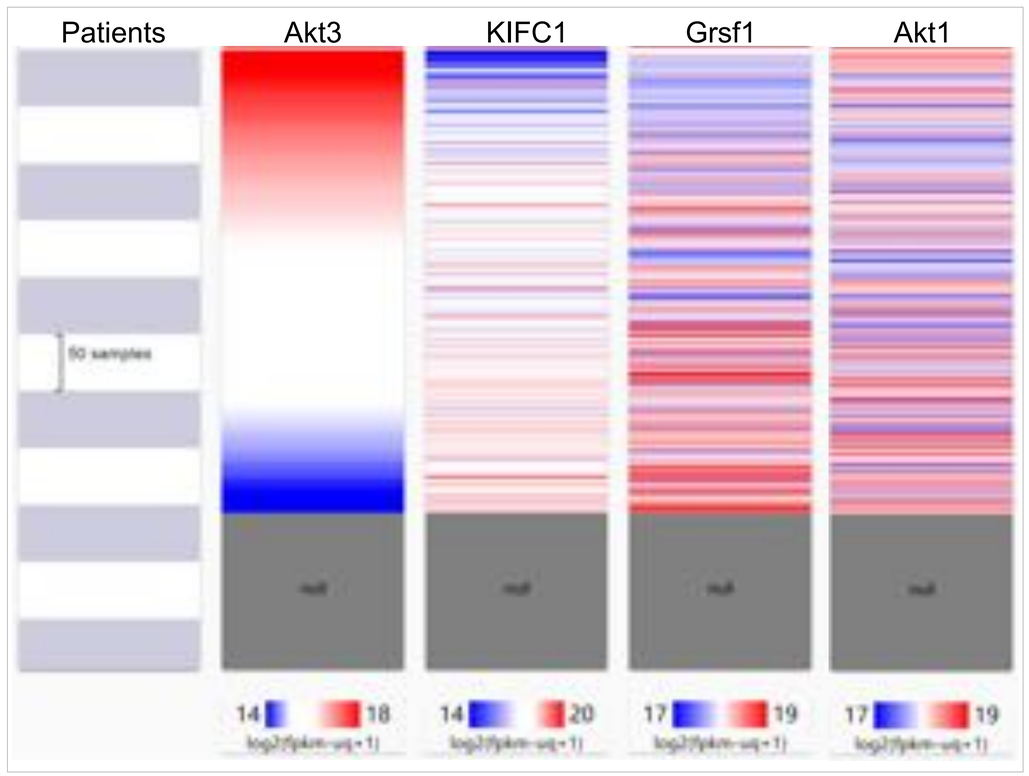


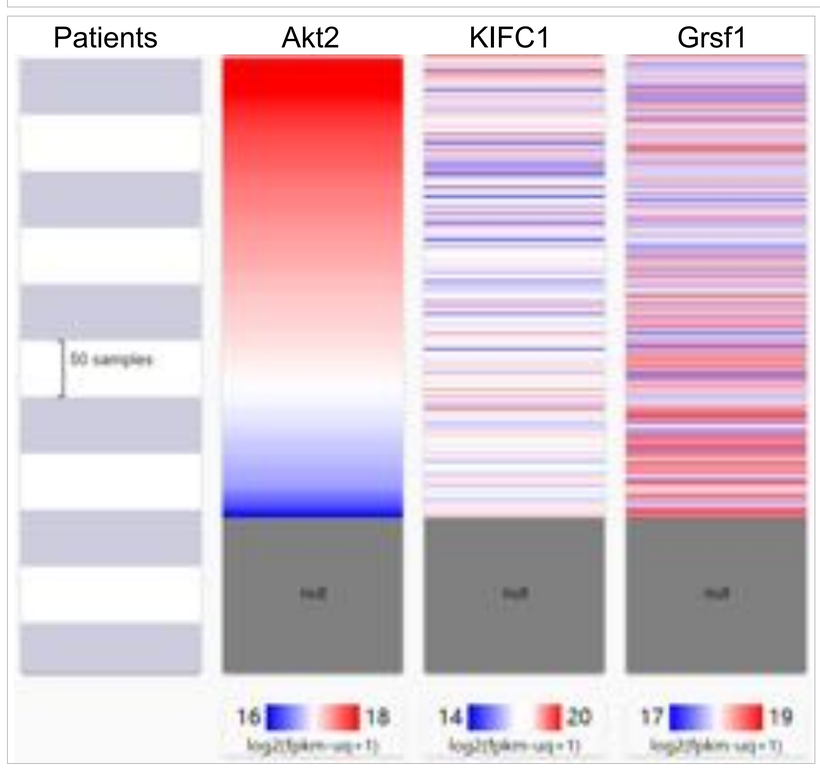


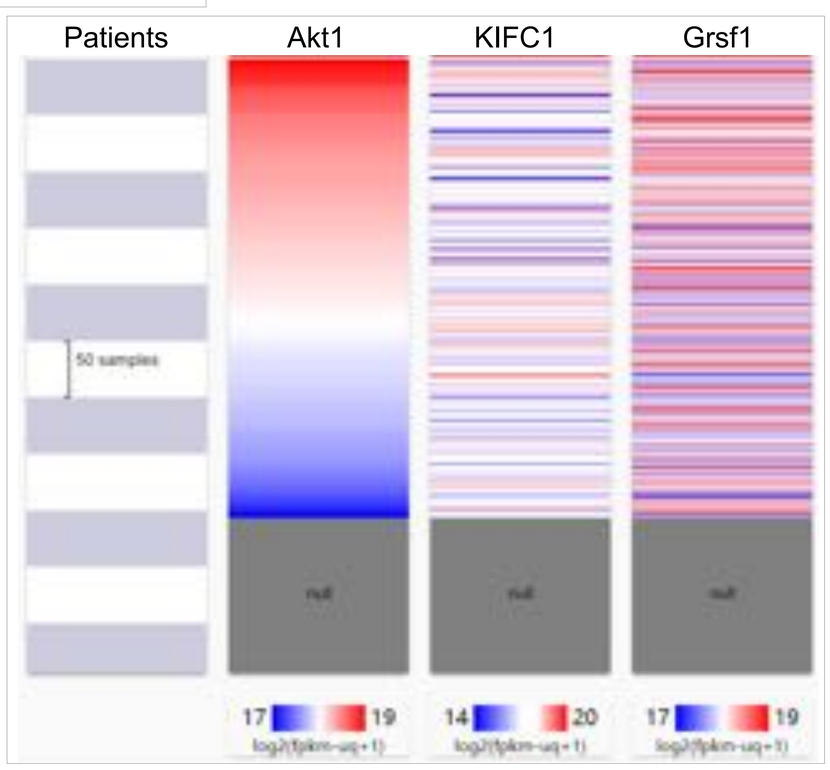
	Akt3			Akt2		Akt1	
	KIFC1	Grsf1	Akt1	KIFC1	Grsf1	KIFC1	Grsf1
***r*-value**	-0.2356	-0.1428	-0.1802	0.3050	-0.1134	0.07164	-0.1547
***P*-value (two tailed)**	<0.0001	0.0005	<0.0001	<0.0001	0.0060	0.0834 (ns)	0.0002

Figure S19

GDC & TCGA stomach cancer database



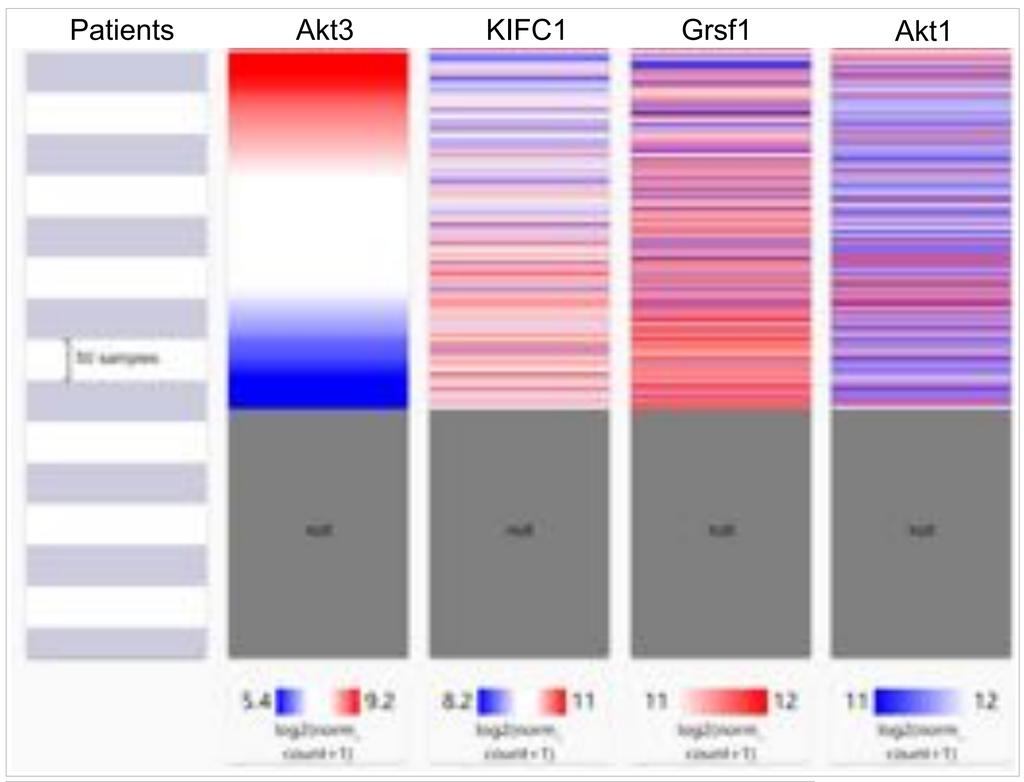


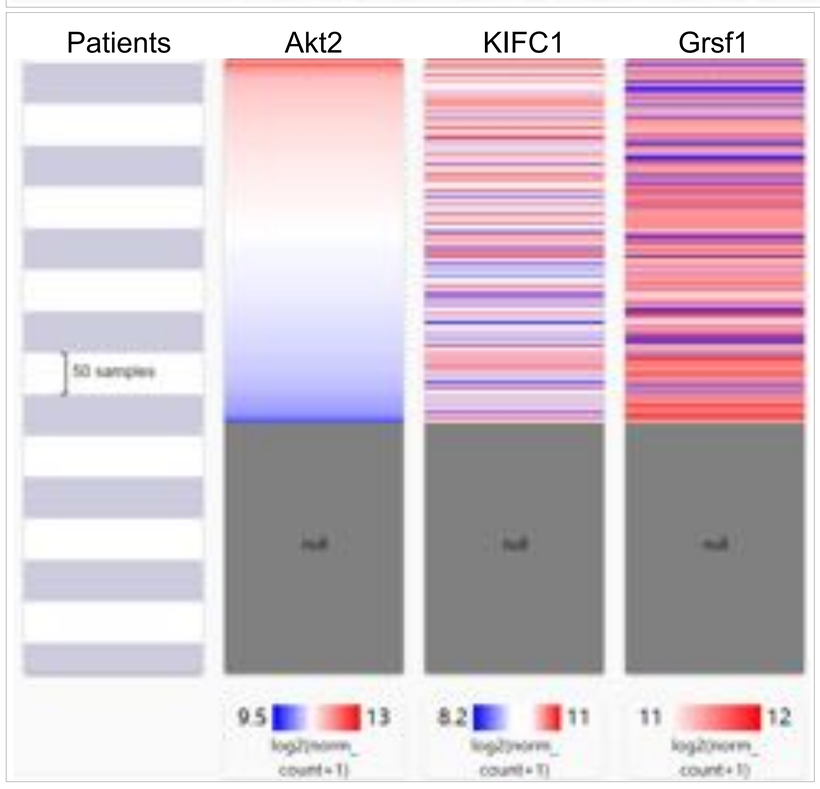


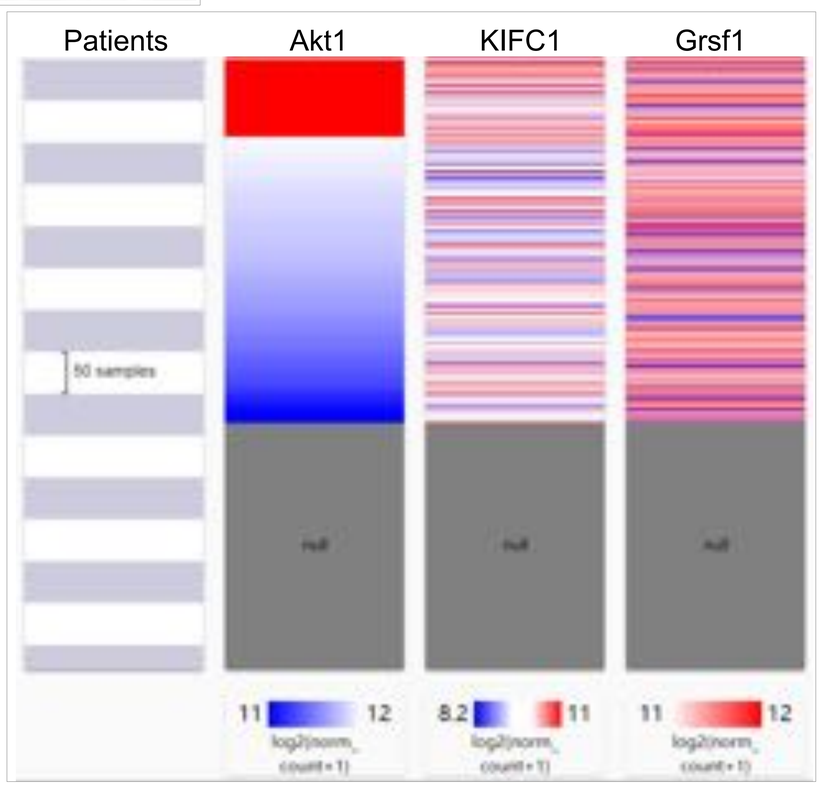
	Akt3			Akt2		Akt1	
	KIFC1	Grsf1	Akt1	KIFC1	Grsf1	KIFC1	Grsf1
***r*-value**	-0.6038	-0.3894	-0.07261	-0.01795	-0.2237	0.02342	0.1099
***P*-value (two tailed)**	<0.0001	<0.0001	0.1437 (ns)	0.7181 (ns)	<0.0001	0.6375 (ns)	0.0267

Figure S20

TCGA colon and rectal cancer database

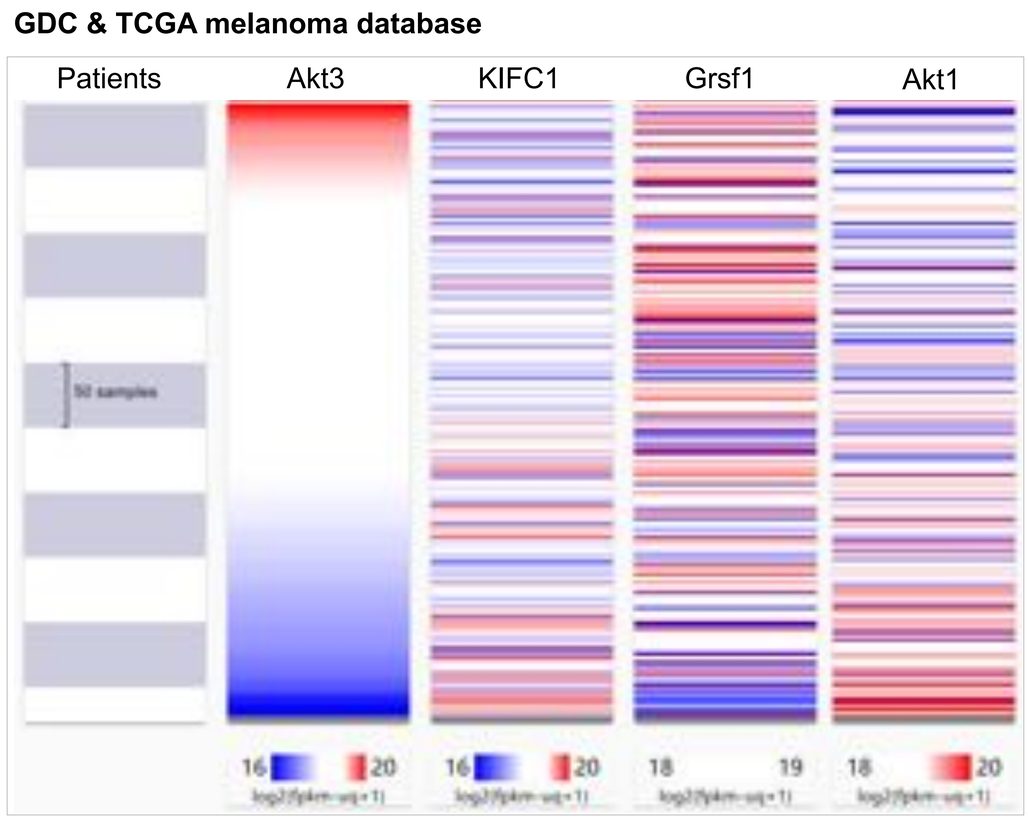


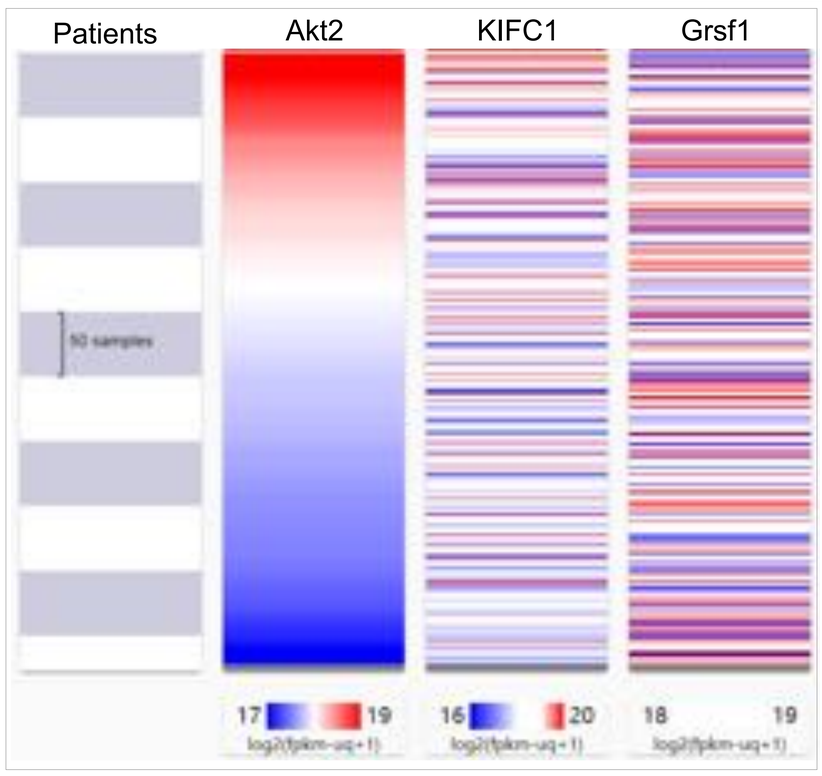


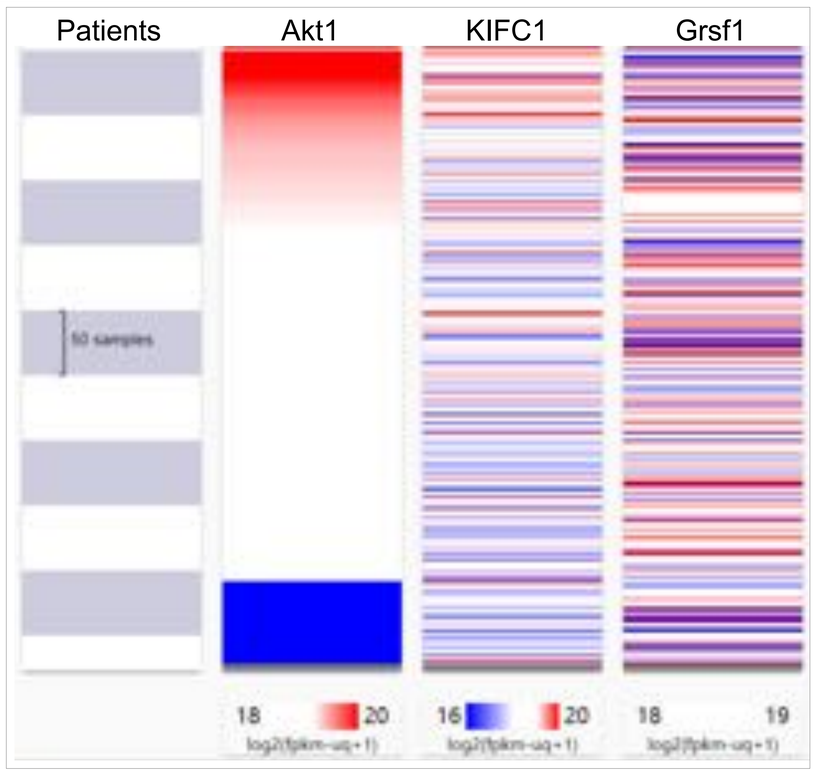


	Akt3			Akt2		Akt1	
	KIFC1	Grsf1	Akt1	KIFC1	Grsf1	KIFC1	Grsf1
***r*-value**	-0.3640	-0.3467	0.08688	0.1553	-0.2343	0.07418	0.03202
***P*-value (two tailed)**	<0.0001	<0.0001	0.0706 (ns)	0.0012	<0.0001	0.1228 (ns)	0.5058 (ns)

Figure S21



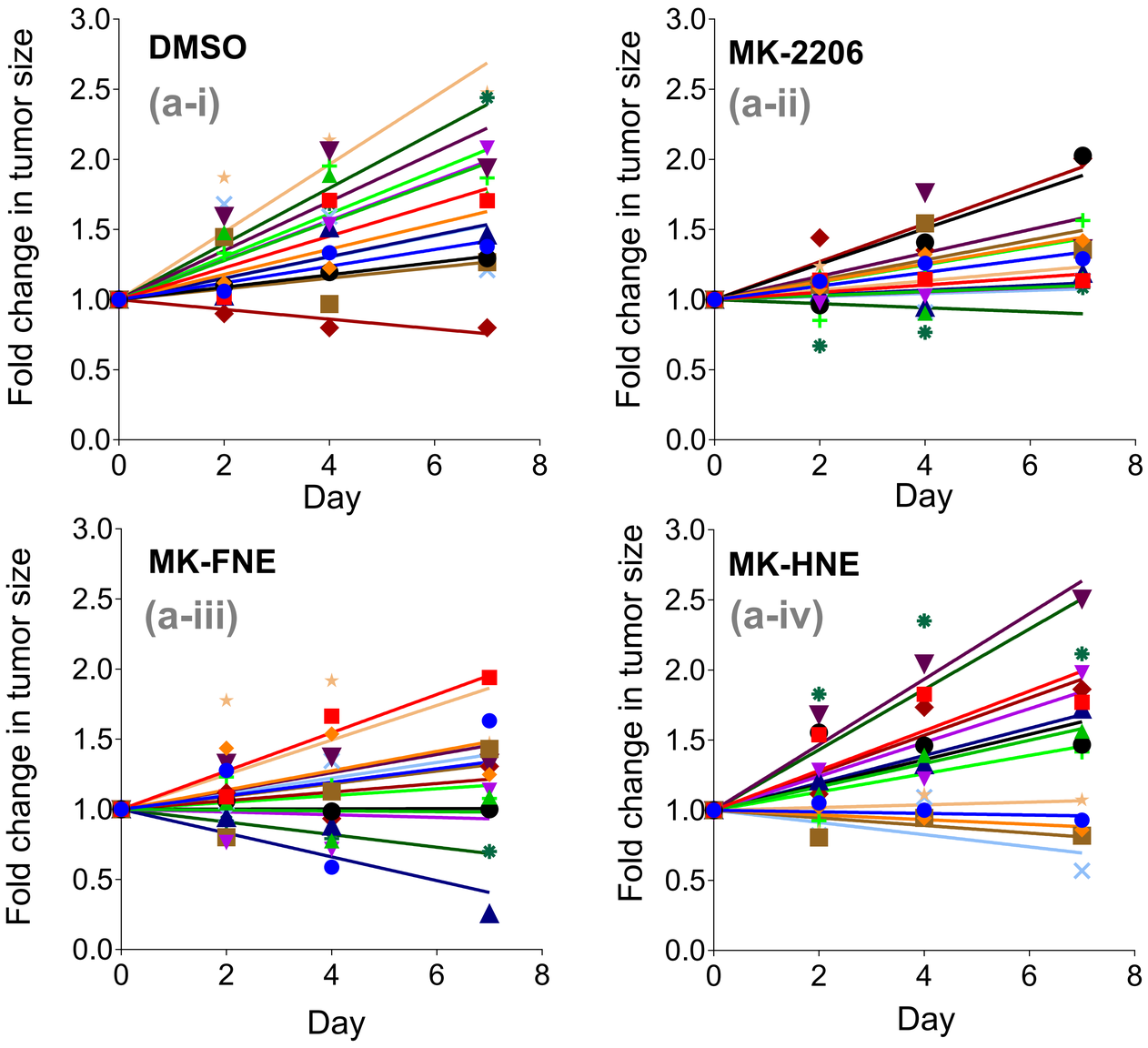




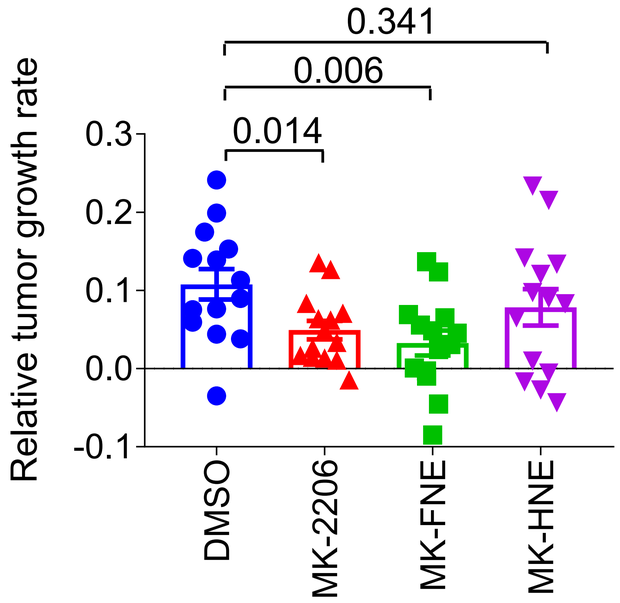
	Akt3			Akt2		Akt1	
	KIFC1	Grsf1	Akt1	KIFC1	Grsf1	KIFC1	Grsf1
***r*-value**	-0.1696	0.2069	-0.3300	0.1751	-0.04888	0.2569	-0.07167
***P*-value (two tailed)**	0.0002	<0.0001	<0.0001	0.0001	0.2892 (ns)	<0.0001	0.1200 (ns)

Figure S22

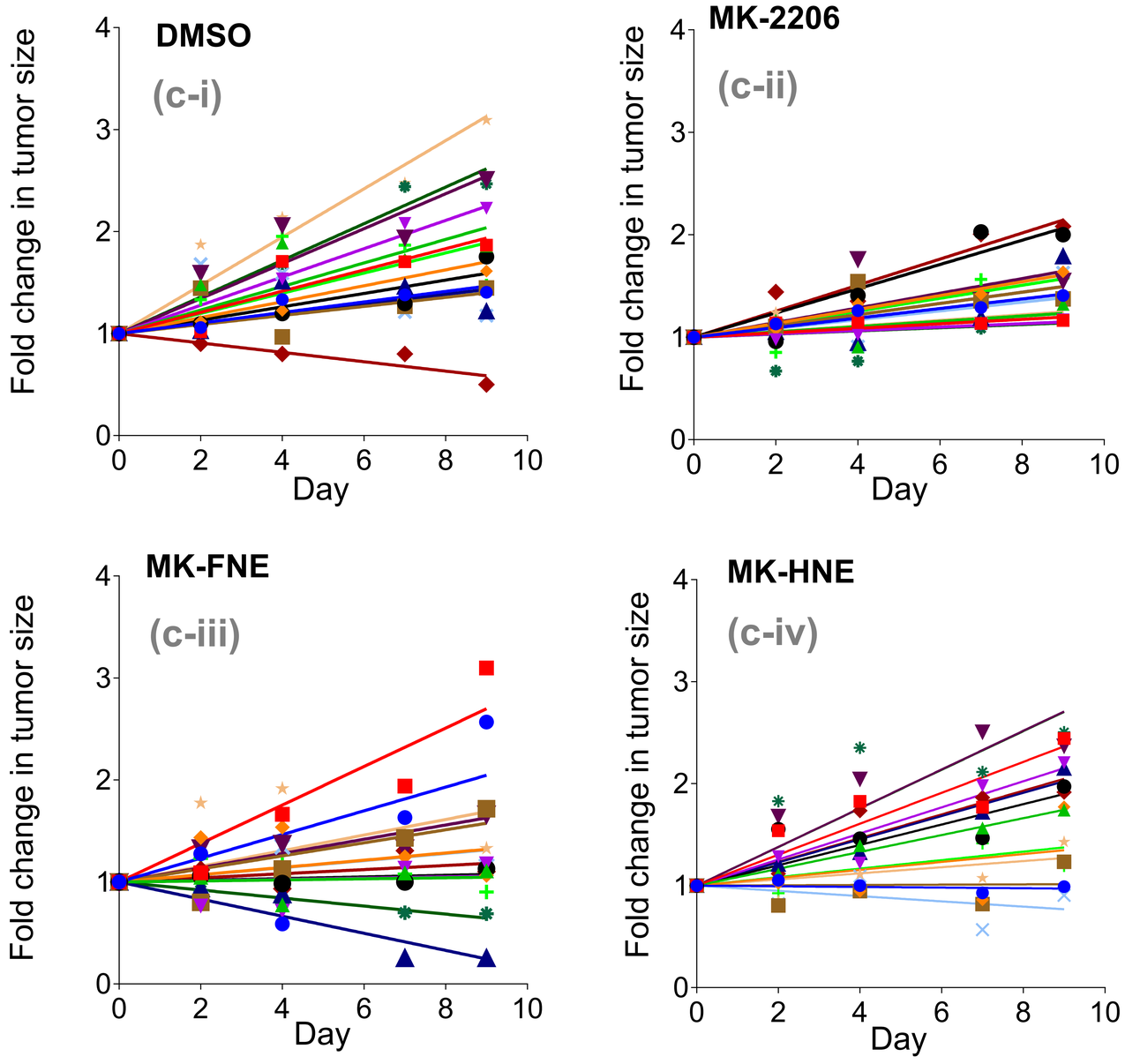
a)



b)

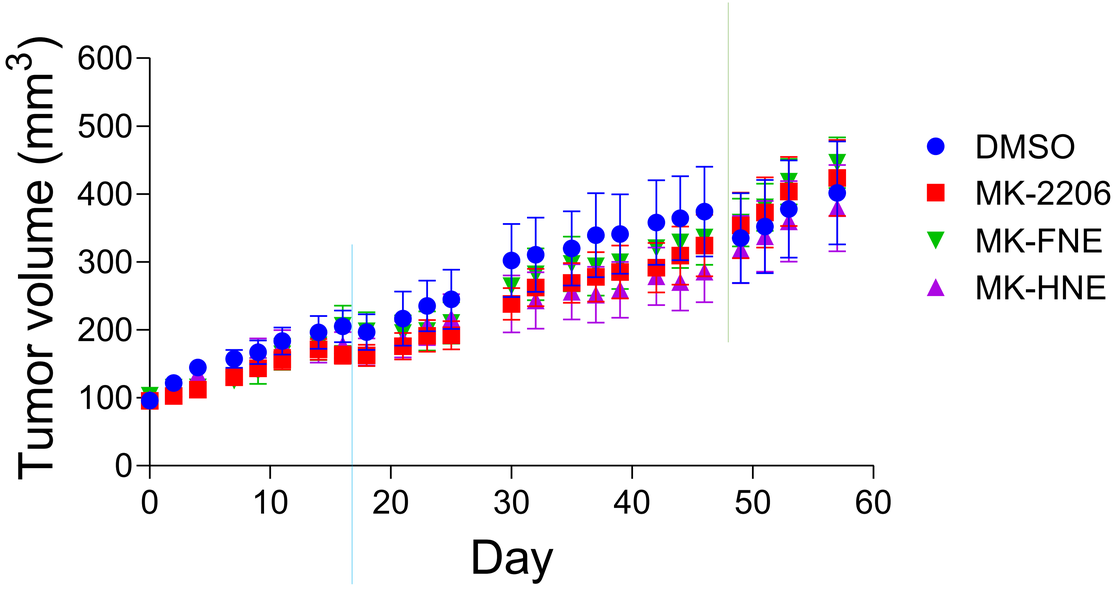


c)



d)

One unexpected death found in DMSO treatment group on Day-49.



(i) Final dosing regimen on Day-17
(ii) Two mice in each treatment group were euthanized for immunohistology and other studies on Day-18

f)

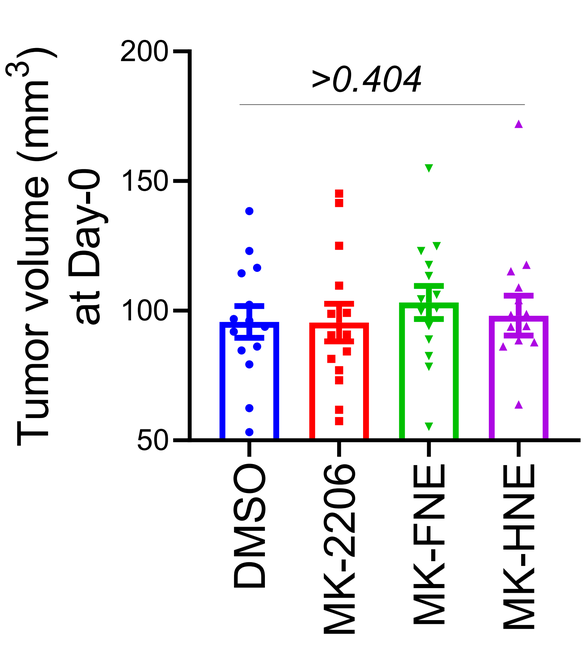


Table S1

Summary of the extrapolated EC_{50}s from cytotoxicity plots of **MK-FNE** and **MK-2206** against MDA-MB-468 cells transfected with Akt-isoform-targeting siRNA (n = 8, mean ± s.e.m). See also **Figure 4b**. The selectivity of **MK-FNE** is calculated according to the indicated equation.

	siCont-1	siCont-2	siCont-3	siAkt1-1	siAkt1-2	siAkt2-1	siAkt2-2	siAkt2-3	siAkt2-4	siAkt2-5	siAkt3-1	siAkt3-2
EC_{50} (MK-2206)	2.2 ± 0.2	3.3 ± 0.2	3.1 ± 0.4	1.1 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	4.0 ± 0.4	1.7 ± 0.3	2.8 ± 0.4	3.0 ± 0.5	1.1 ± 0.1	1.3 ± 0.1
EC_{50} (MK-FNE)	8.7 ± 0.7	10.0 ± 1.0	9.7 ± 2.5	8.2 ± 0.7	2.3 ± 0.4	4.7 ± 0.7	4.9 ± 1.4	7.1 ± 1.0	7.3 ± 1.0	8.1 ± 1.7	1.0 ± 0.3	1.2 ± 0.2
Fold increase in Selectivity	–0.1	0.1	0.1	–0.5	–0.6	–0.2	1.8	-0.2	0.3	0.3	2.9	2.7

Equation:

$$Fold\ increase\ in\ selectivity = \frac{\left[\frac{<(EC_{50})_{siCont}>}{(EC_{50})_{siAkt(n)}}\right]_{MK-FNE}}{\left[\frac{<(EC_{50})_{siCont}>}{(EC_{50})_{siAkt(n)}}\right]_{MK-2206}} - 1$$

Where $<(EC_{50})_{siCont}>$ designates the average of a given compound's EC_{50}s against the two control knockdown lines; $(EC_{50})_{siAkt(n)}$ is the given compound's EC_{50} against the indicated Akt isoform-specific knockdown line by siRNA.

Table S2

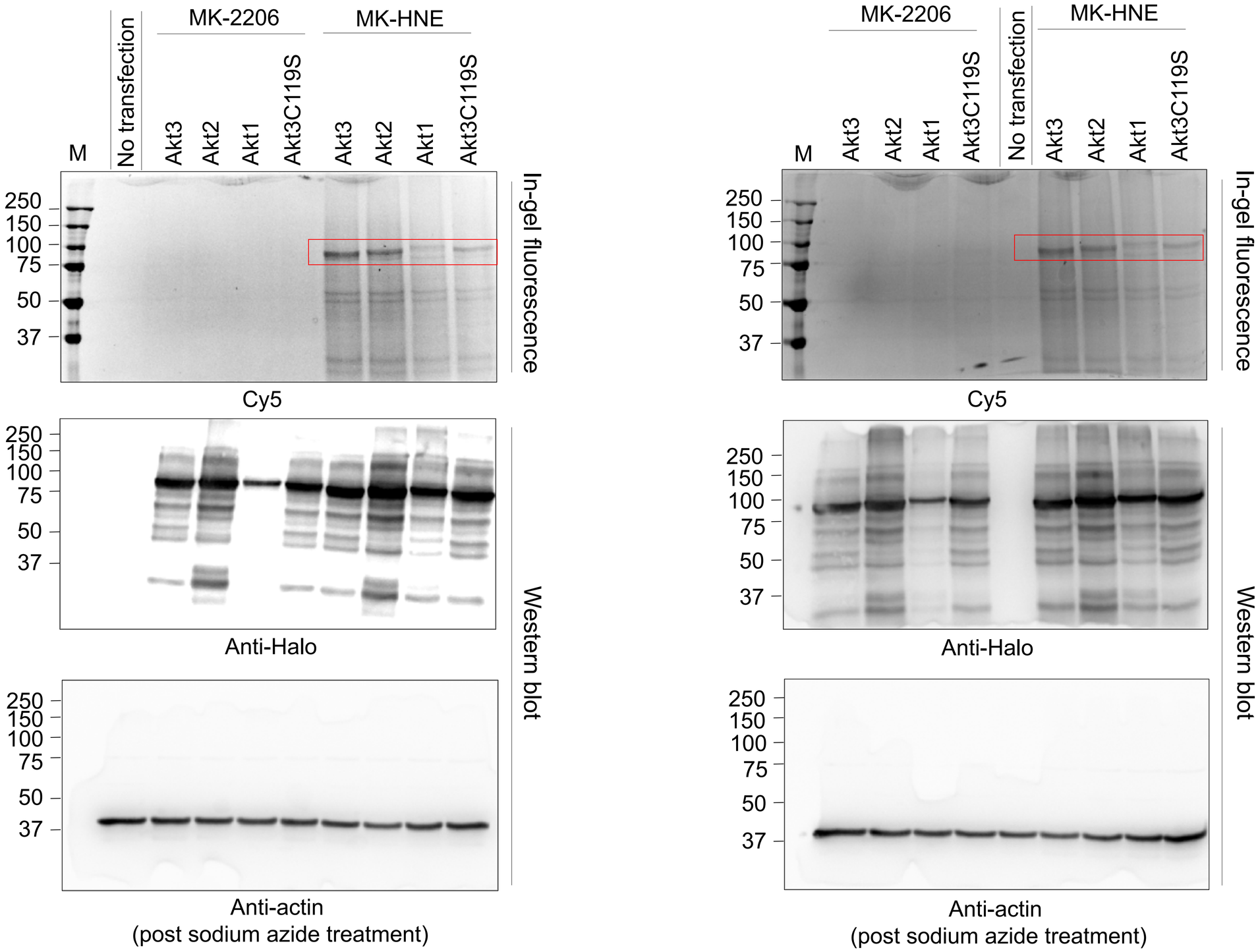
Cell cycle analysis

	DMSO	MK-2206		MK-HNE		MK-FNE		GDC0068	GSK-690693
		EC_{60}	EC_{80}	EC_{60}	EC_{80}	EC_{60}	EC_{80}	EC_{60}	EC_{60}
G1/G0	39.2	47.1	59.9	43.7	44.3	46.4	52.4	52.6	47.7
G2/M	32.4	27.7	20.4	26.1	23.0	28.6	28.2	28.3	30.0
S	22.2	23.7	18.6	27.4	22.1	23.0	16.6	12.1	14.3
subG0	0.8	0.5	0.7	1.3	9.7	0.9	1.8	1.6	0.9

Related Manuscript File

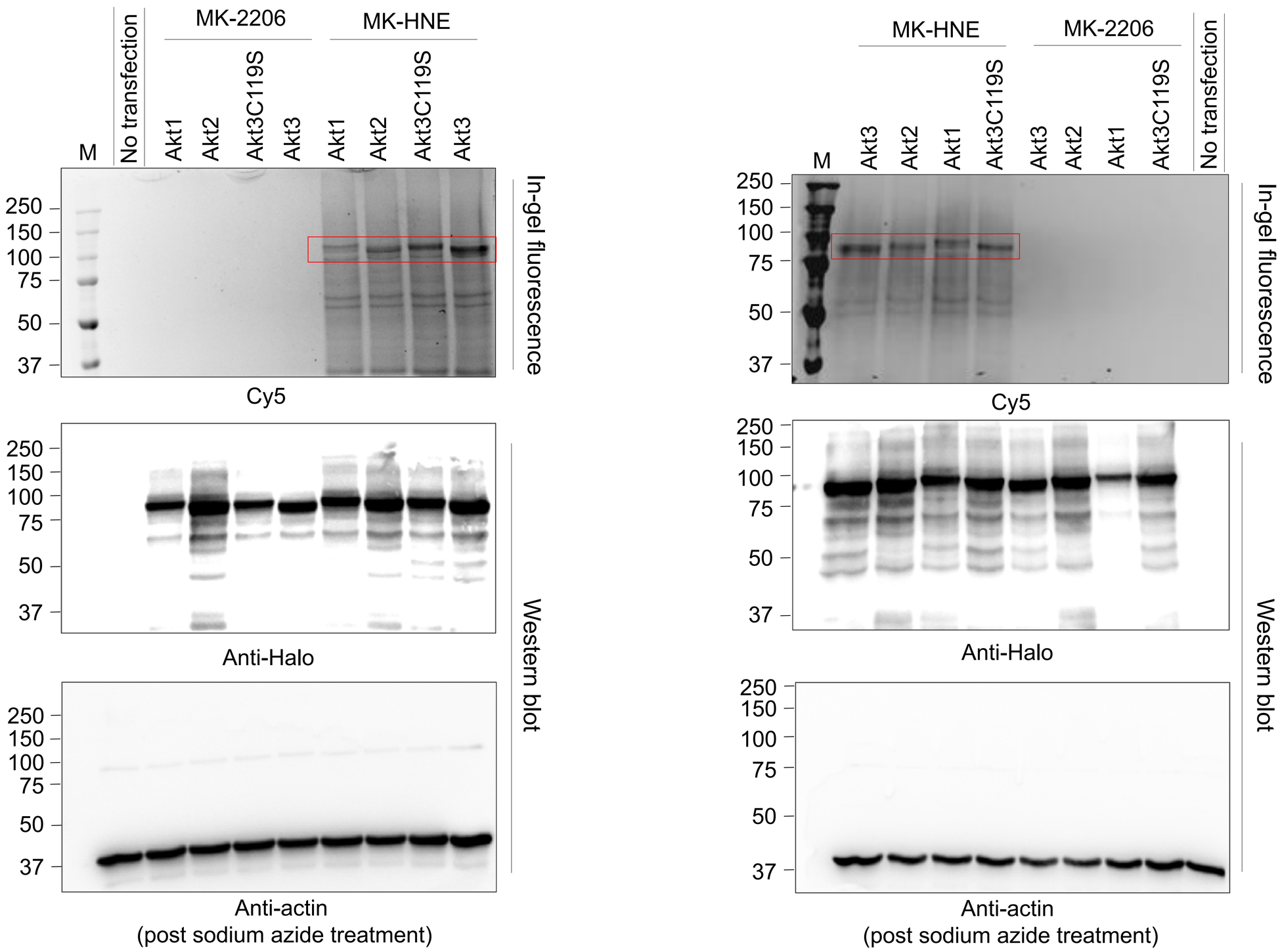
- Additional replicates and full-view gels (pp 2-19)
- Log-plot of cytotoxic plots in Figure 4 (pp 20)
- Flow-cytometry analysis (pp 21-23) (related to Supplementary Table S2)

Representative replicates related to data in Main Figure 1b-c



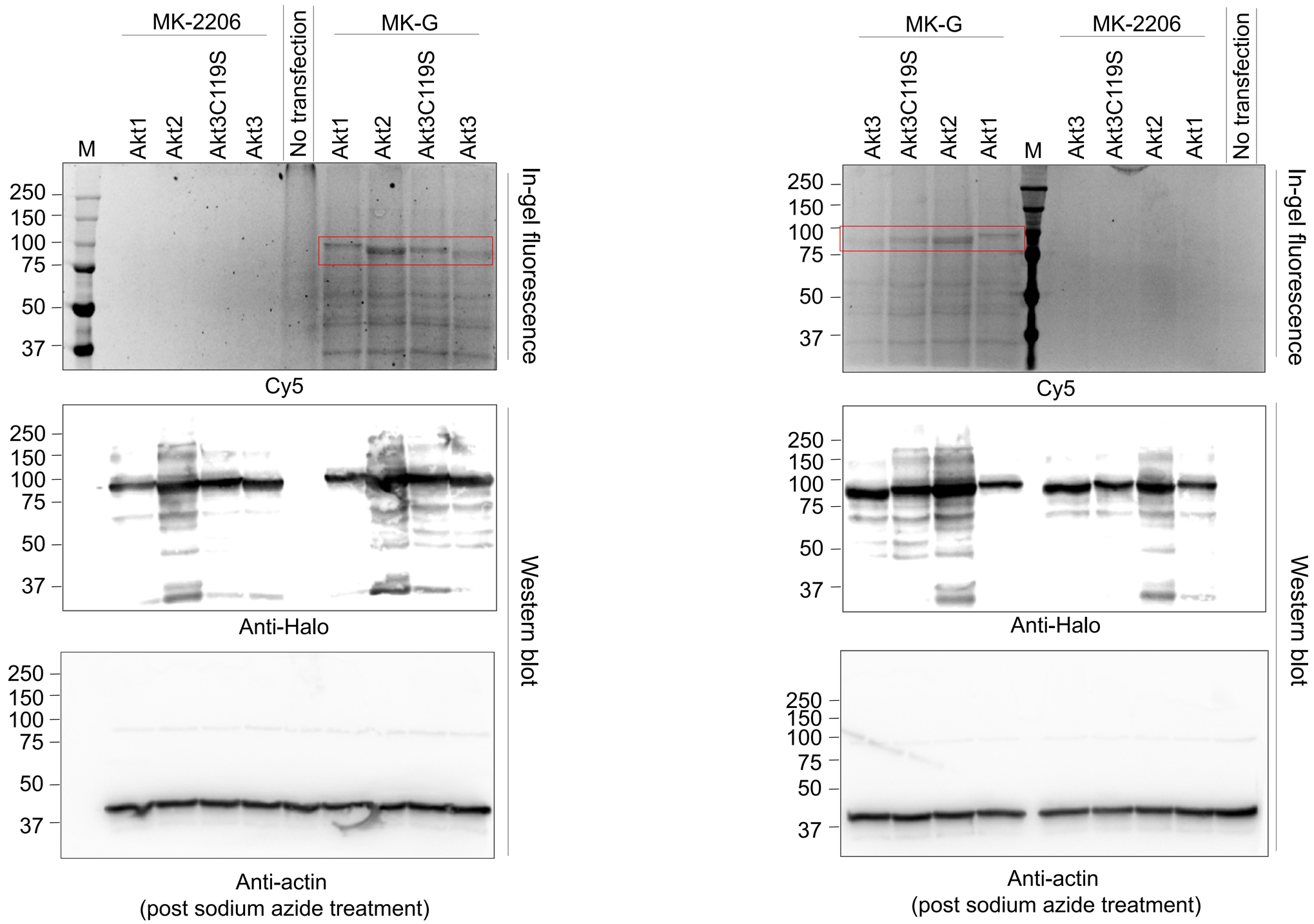
Note: Rectangular box indicates the Cy5 signal of Halo-Akt(n) used in the quantitation

Representative replicates related to data in Main Figure 1b-c



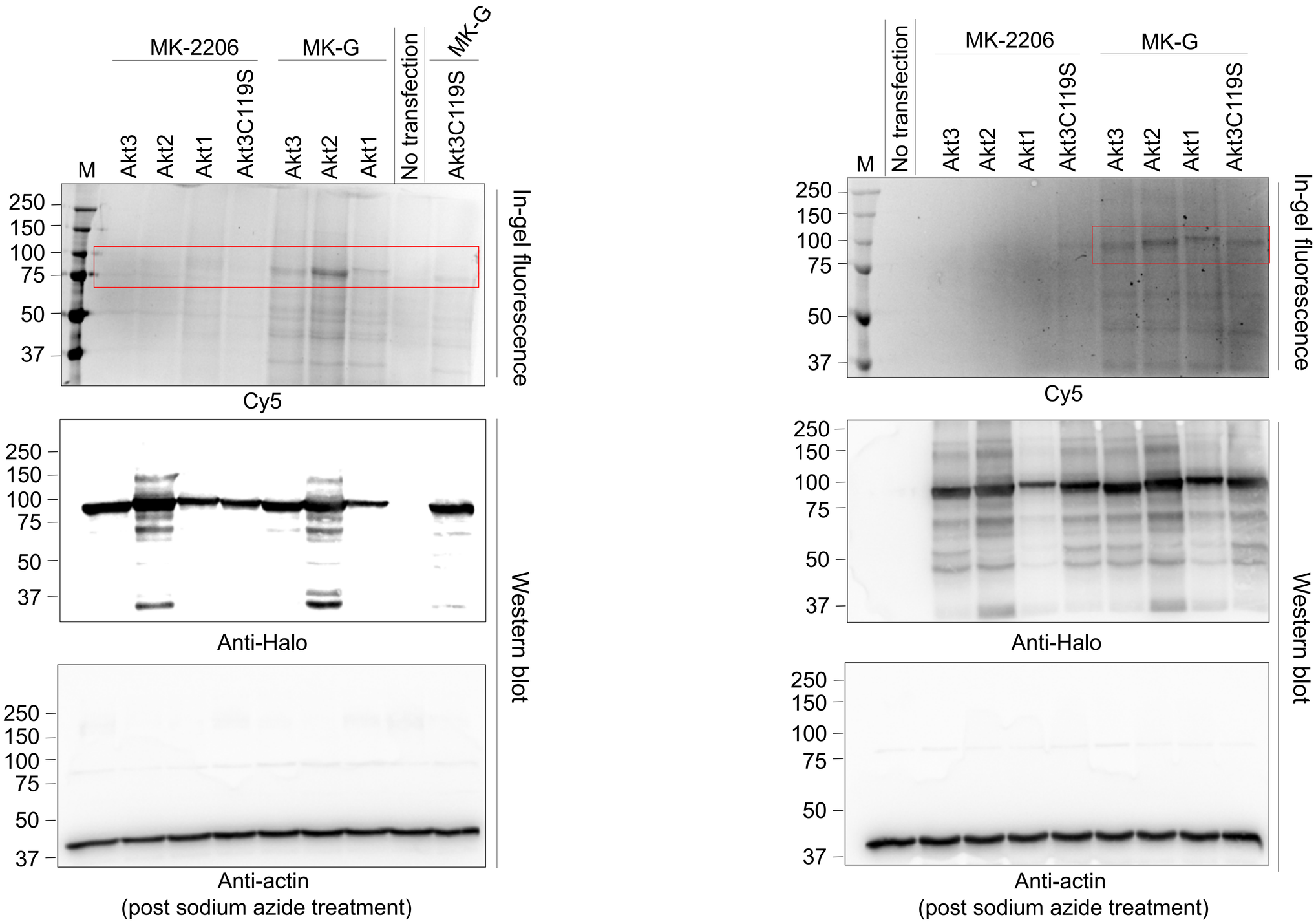
Note: Rectangular box indicates the Cy5 signal of Halo-Akt(n) used in the quantitation

Representative replicates related to data in Supplemental Figure S1



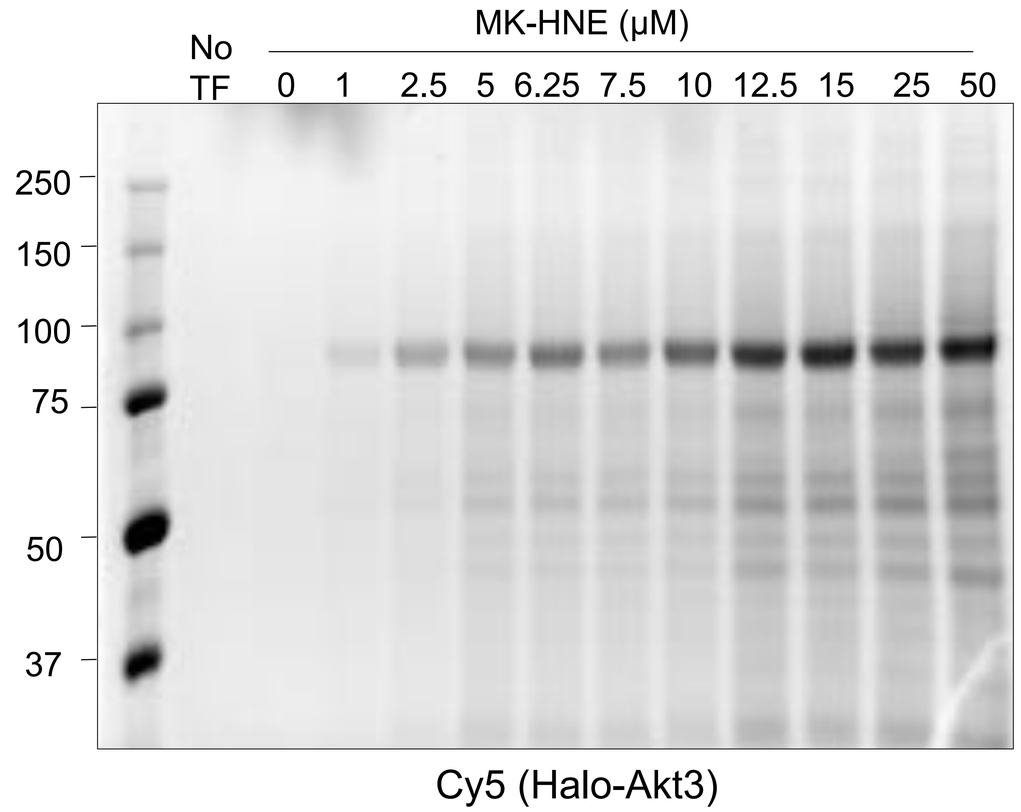
Note: Rectangular box indicates the Cy5 signal of Halo-Akt(n) used in the quantitation

Representative replicates related to data in Supplemental Figure S1 - continued

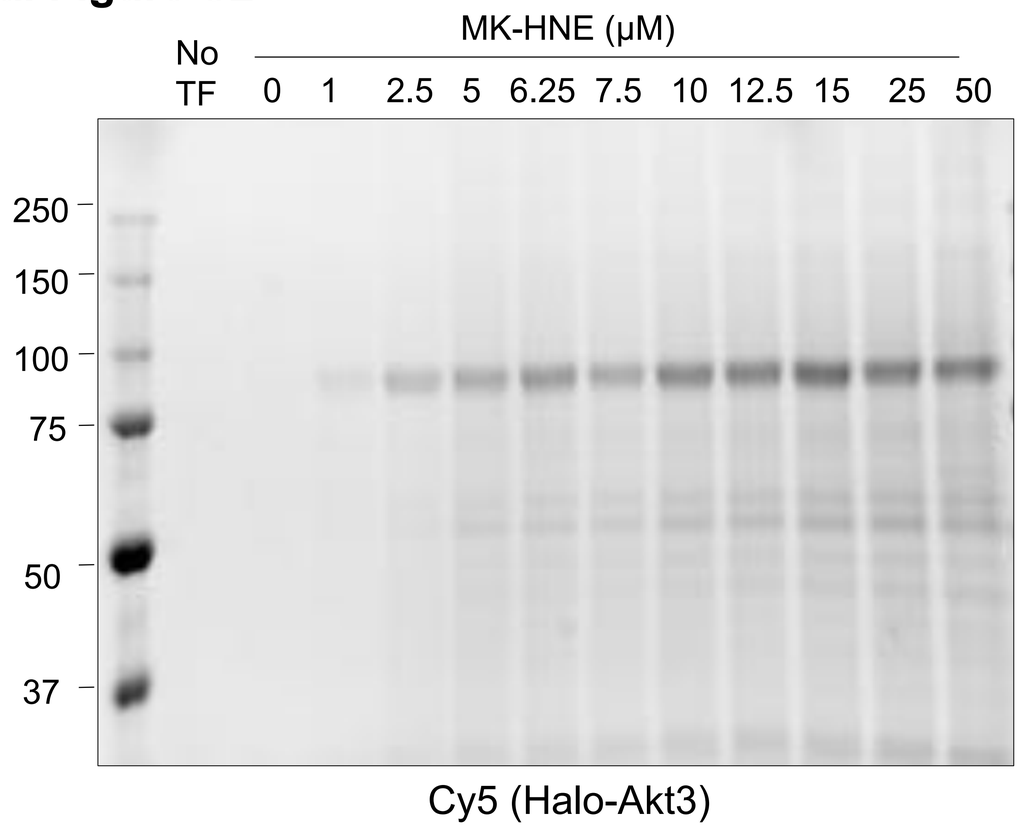


Note: Rectangular box indicates the Cy5 signal of Halo-Akt(n) used in the quantitation

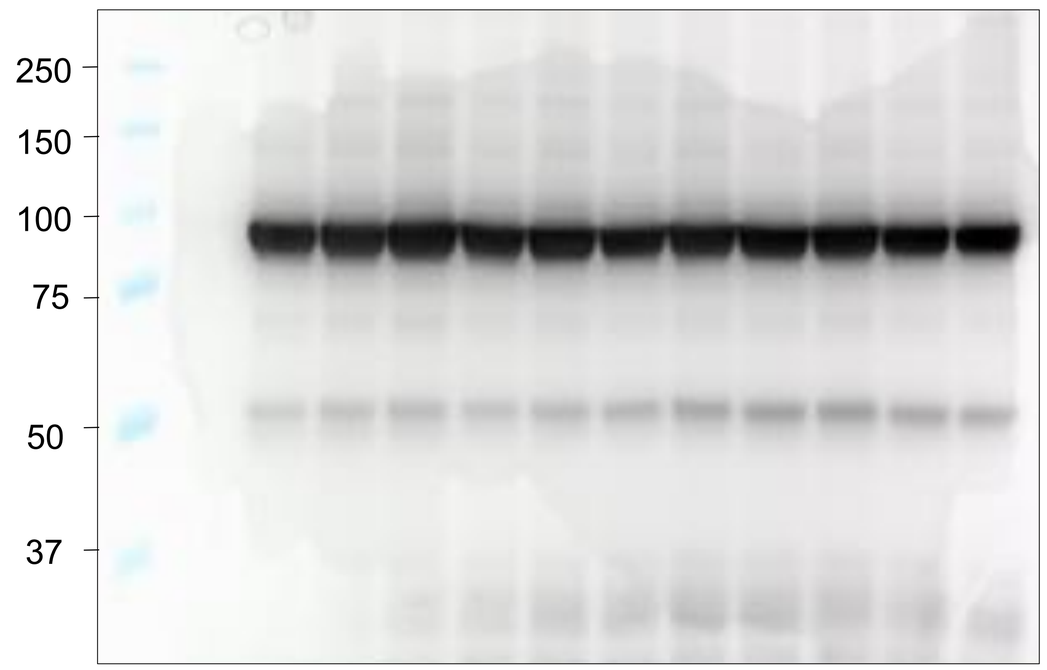
Representative replicates related to data in Supplemental Figure S2



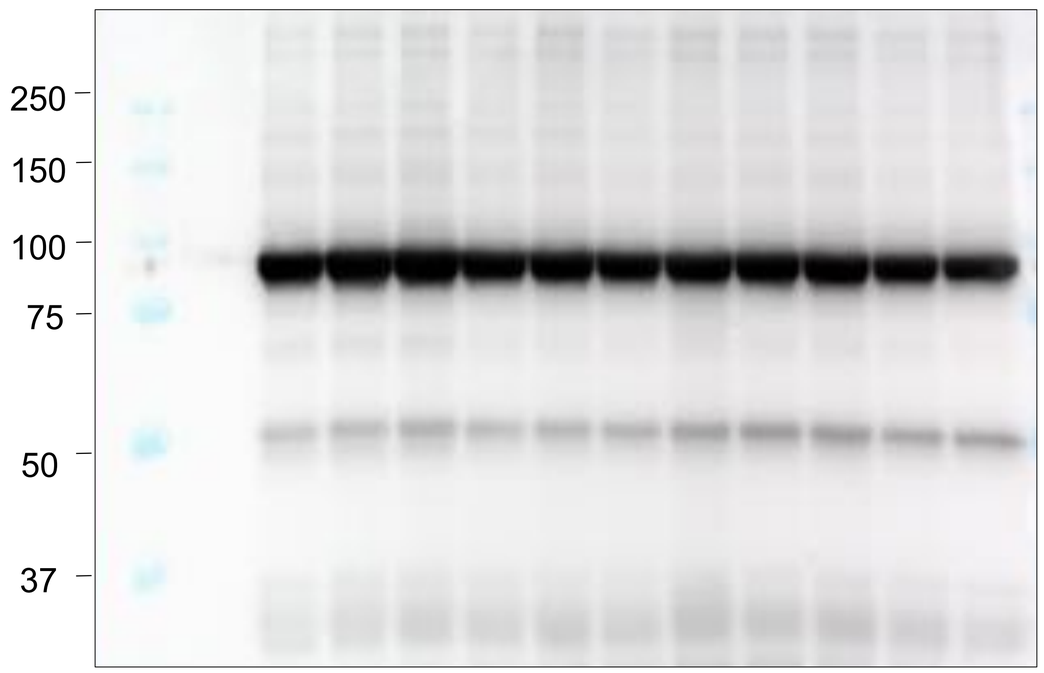
Cy5 (Halo-Akt3)



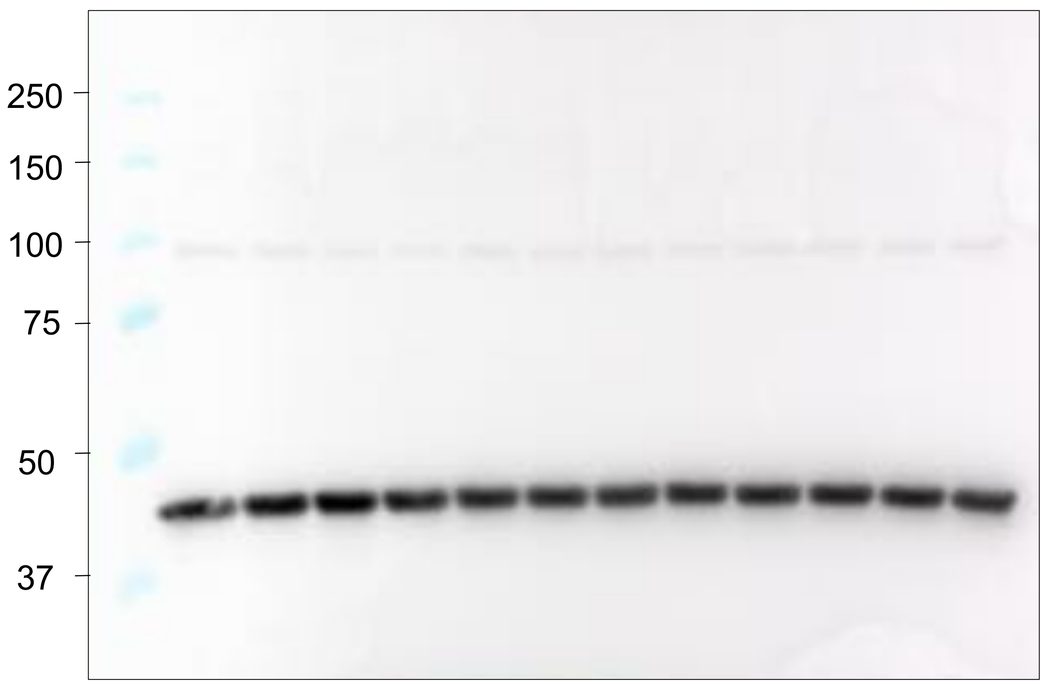
Cy5 (Halo-Akt3)



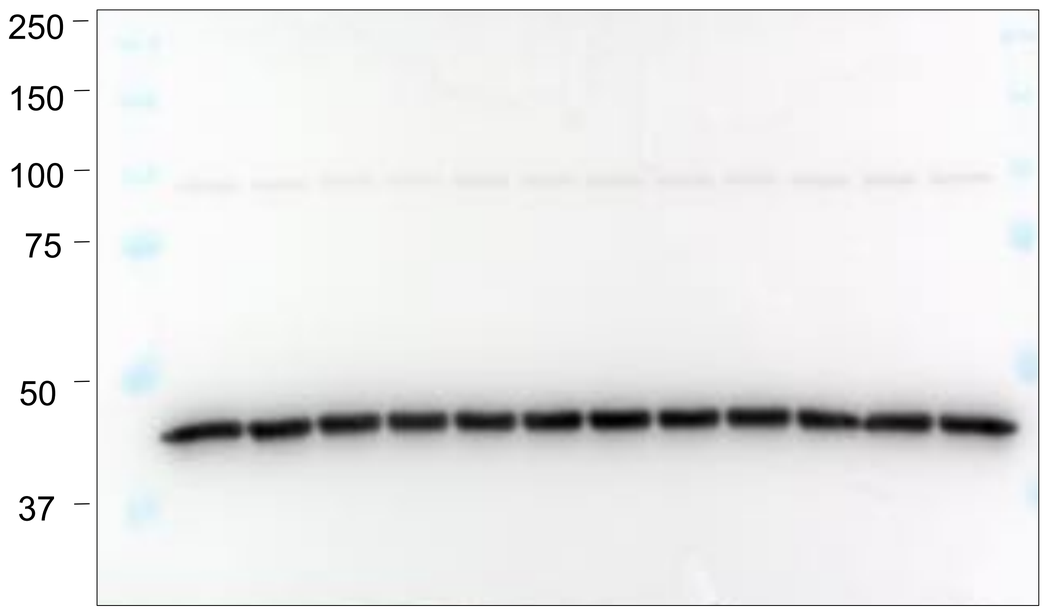
anti-Halo



anti-Halo

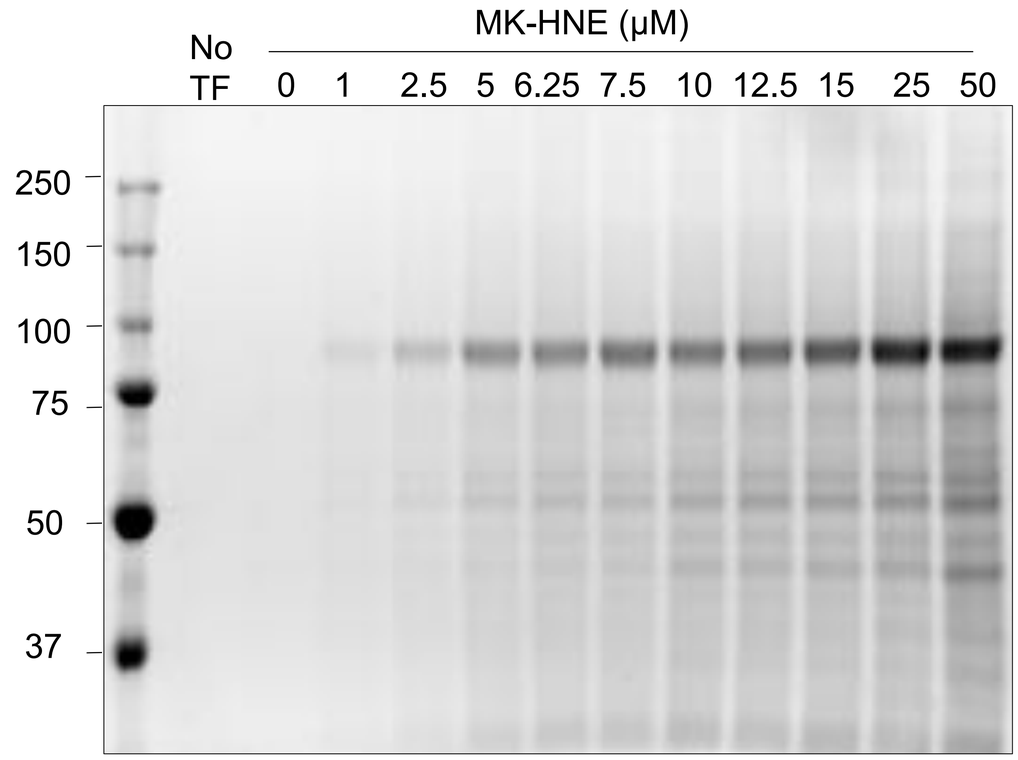


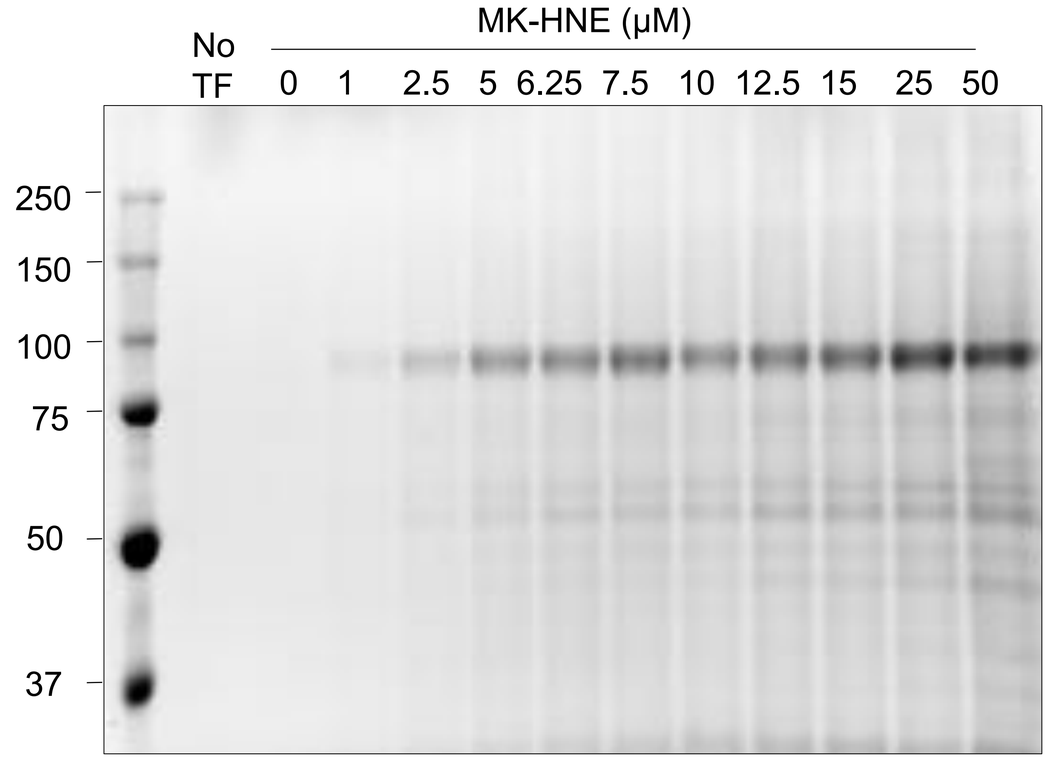
anti-actin

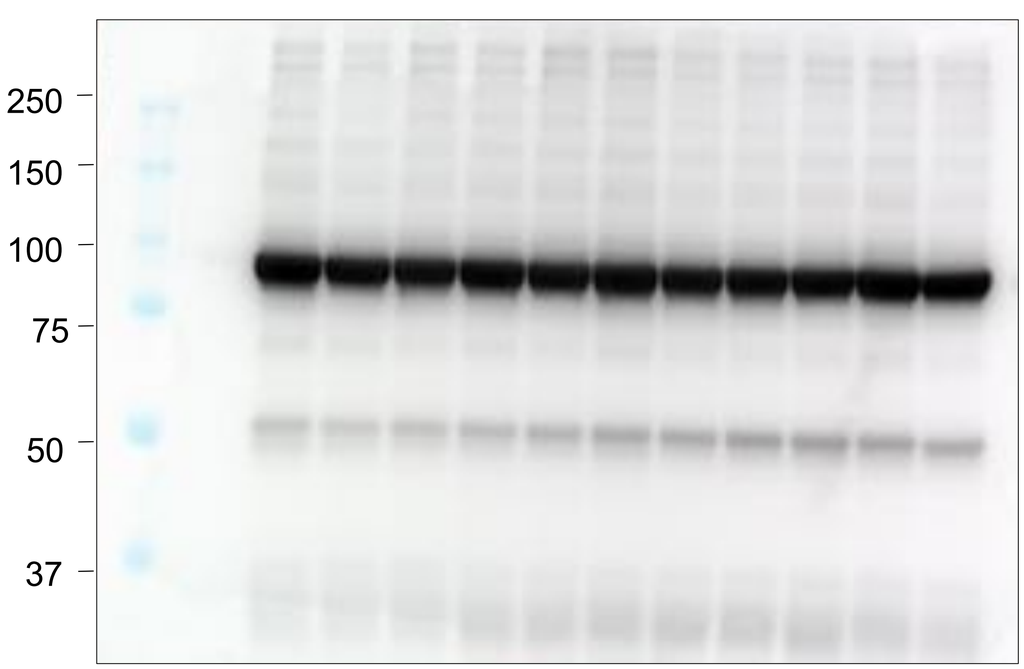


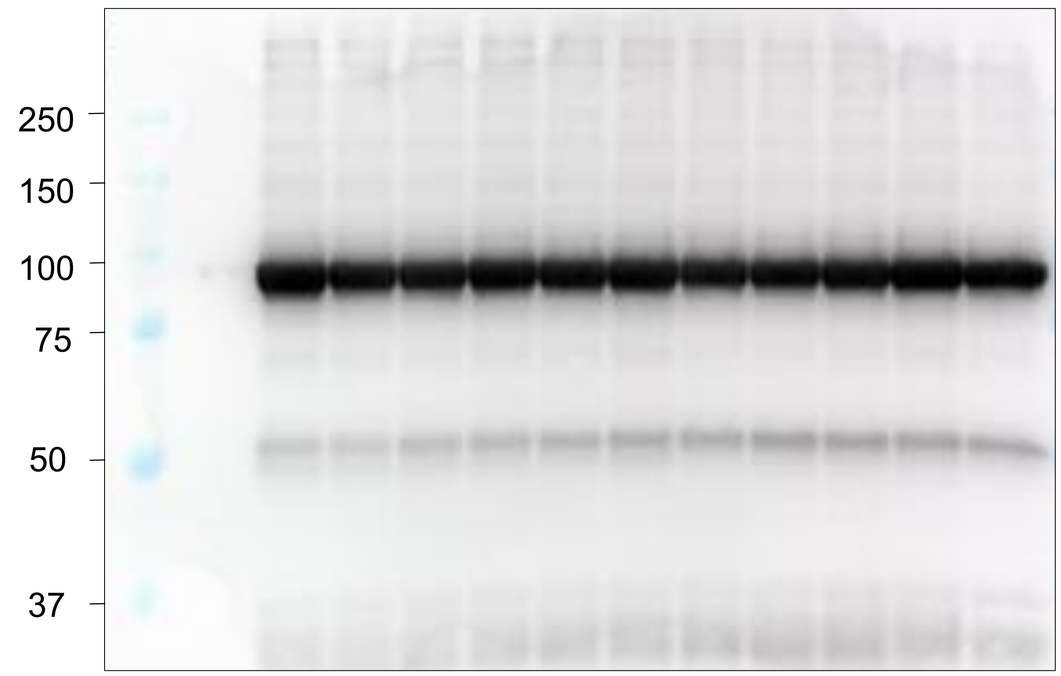
anti-actin

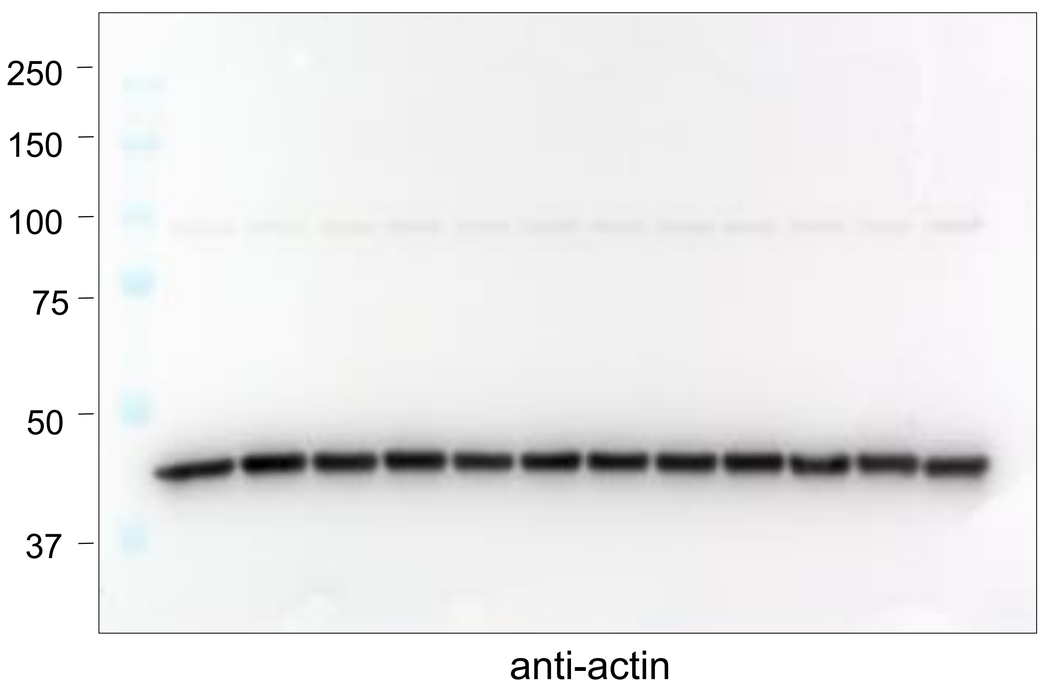
Representative replicates related to data in Supplemental Figure S2

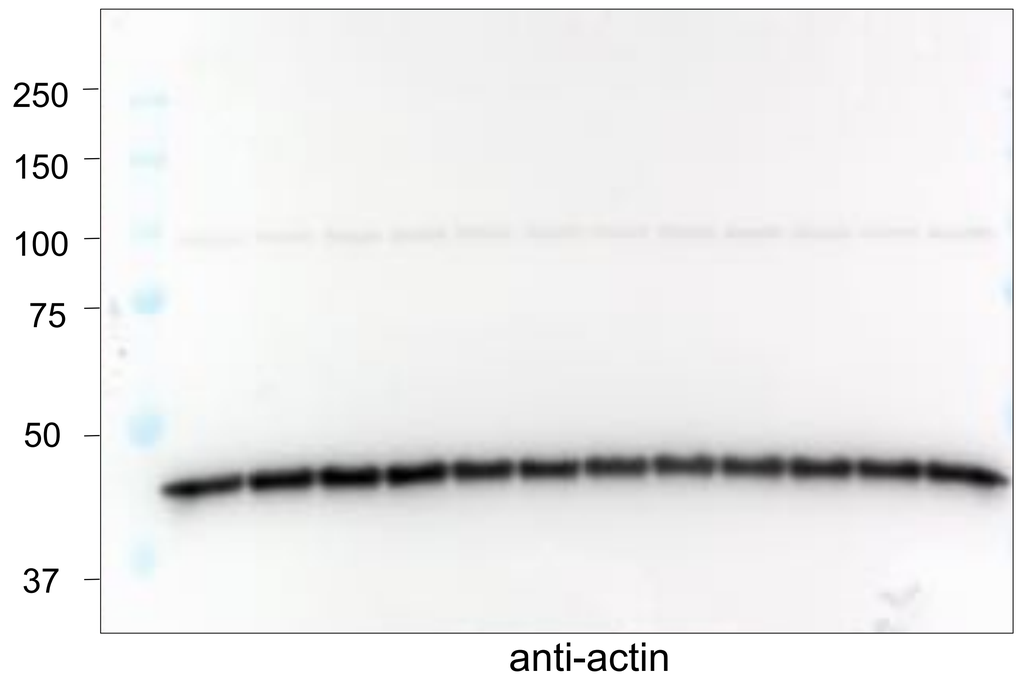




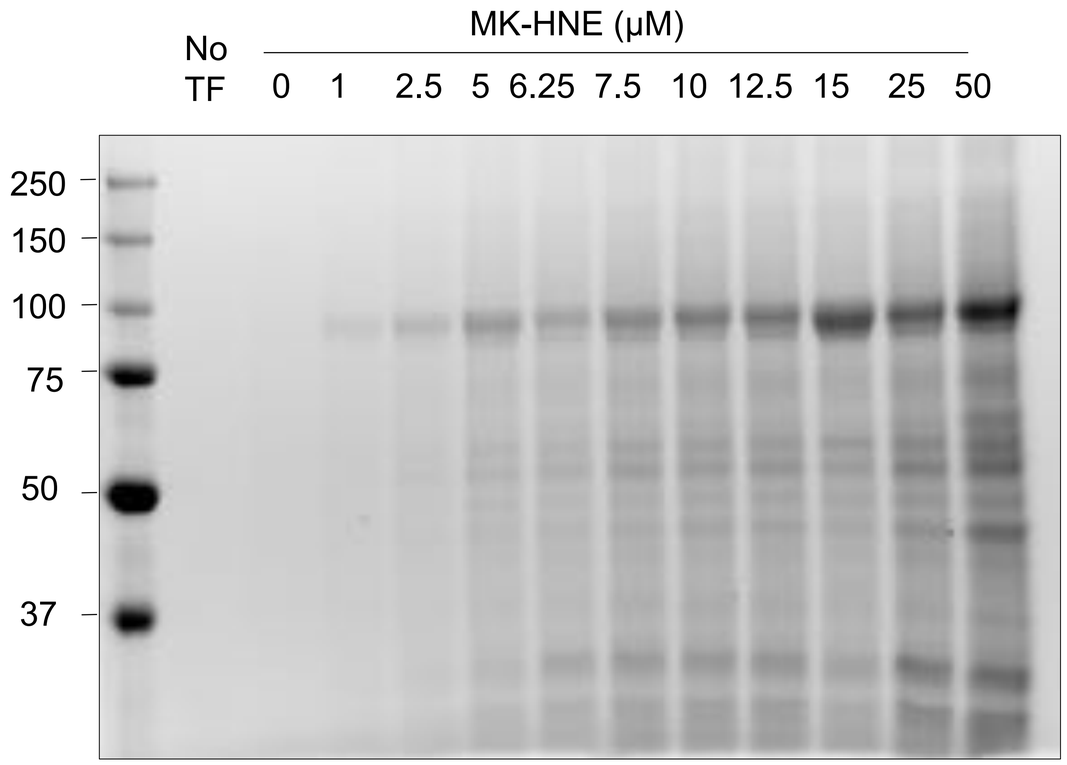


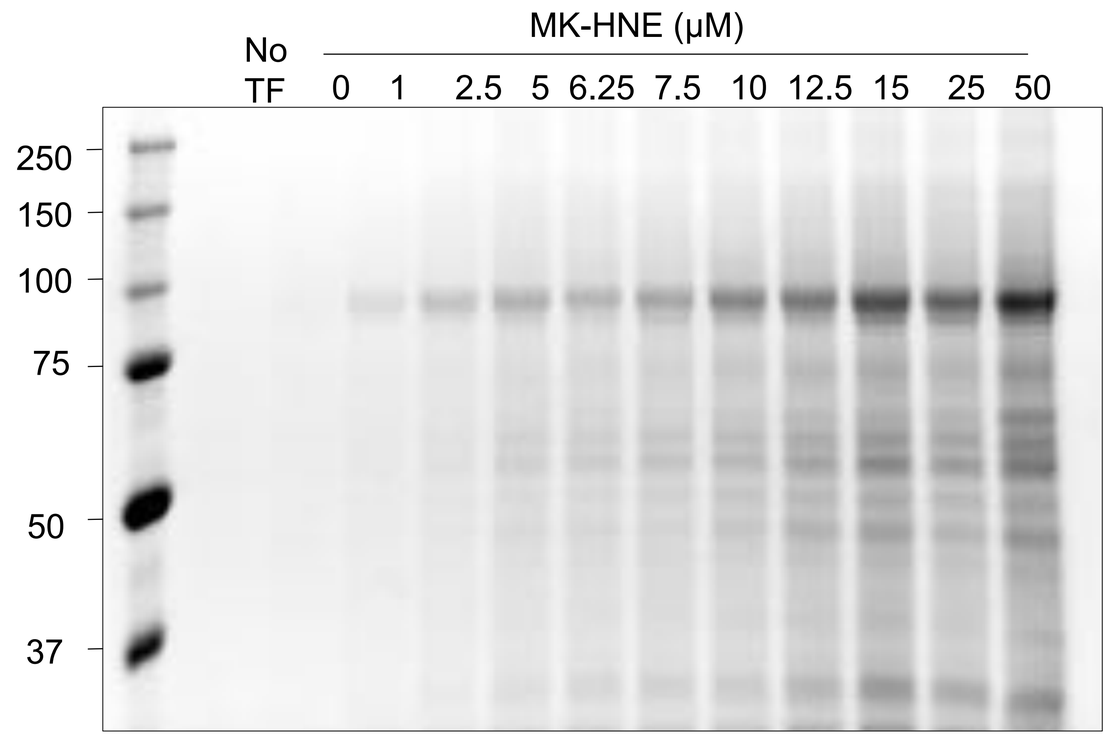


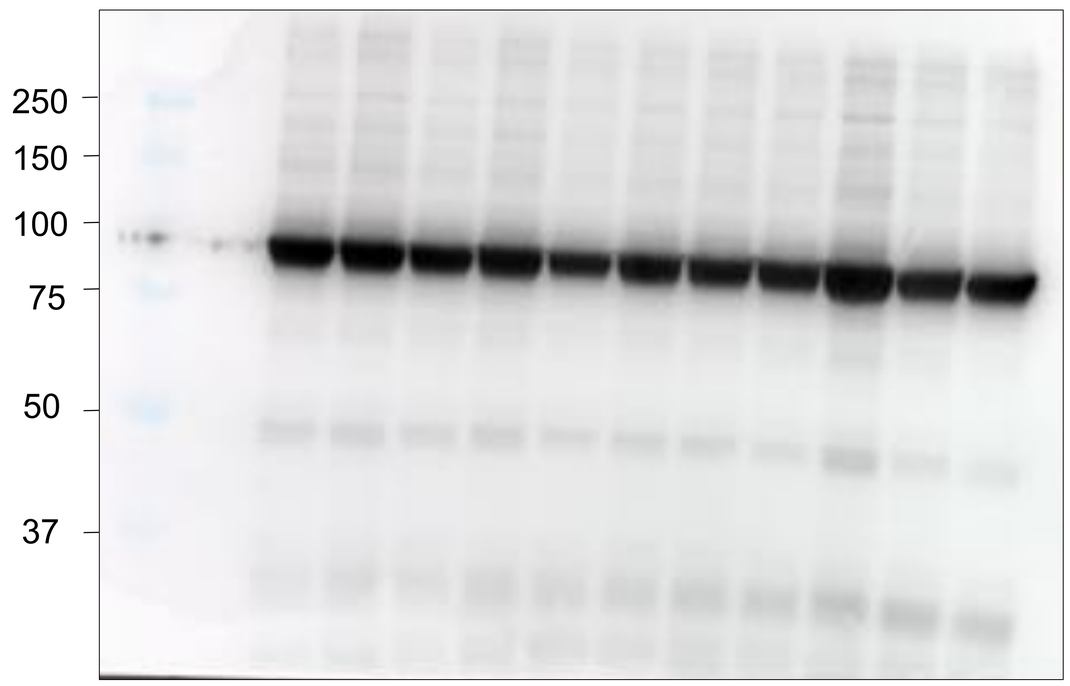


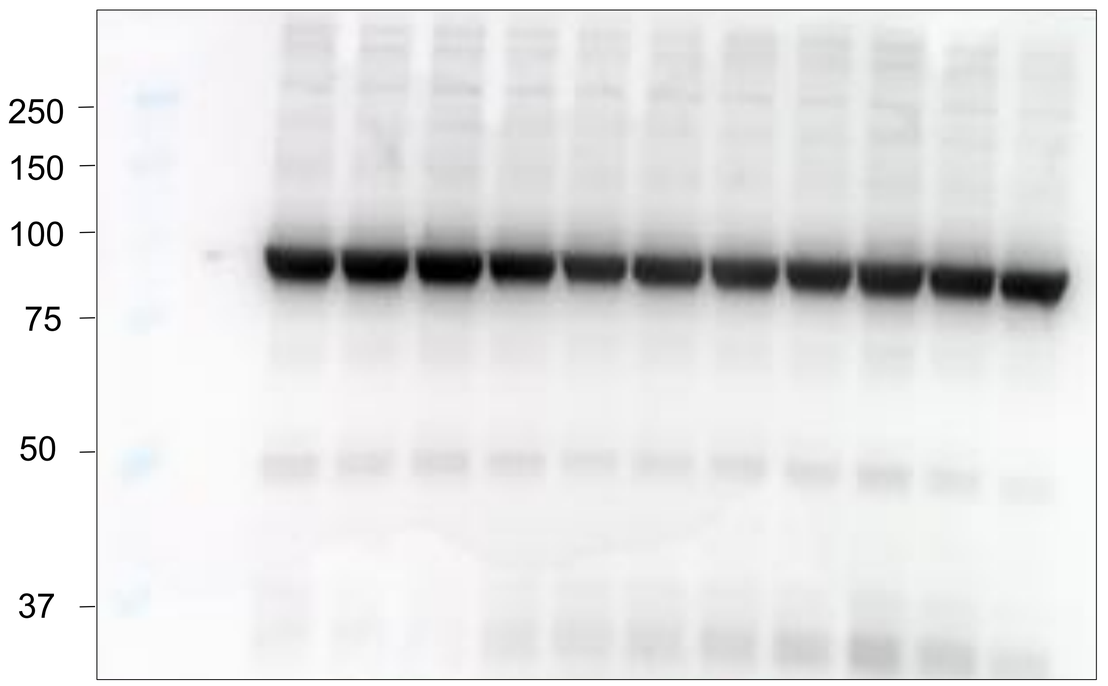


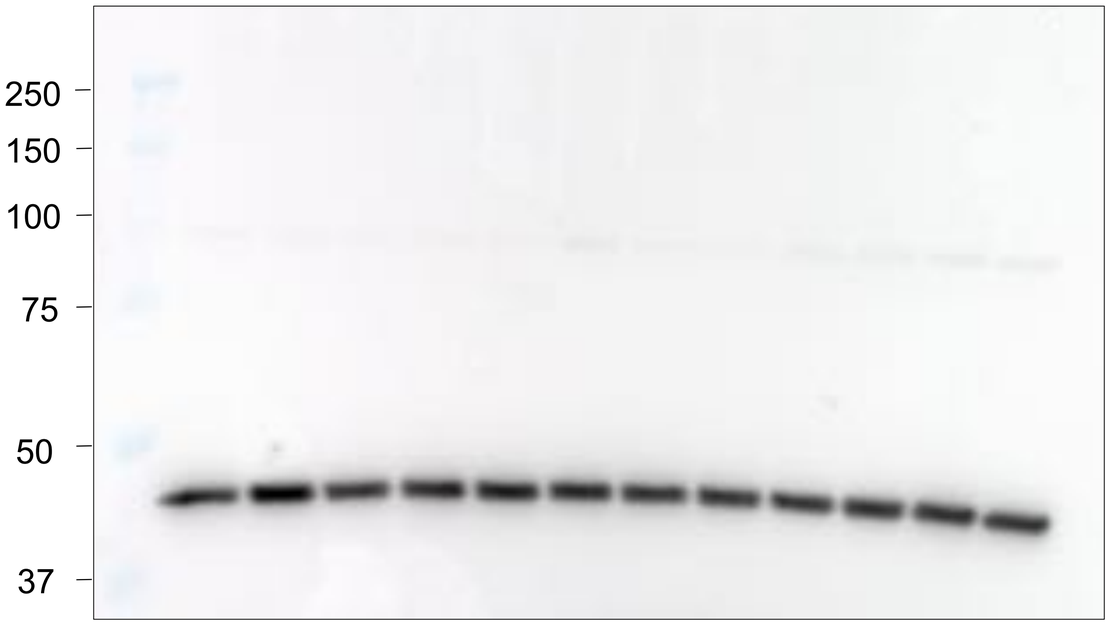
Representative replicates related to data in Supplemental Figure S2



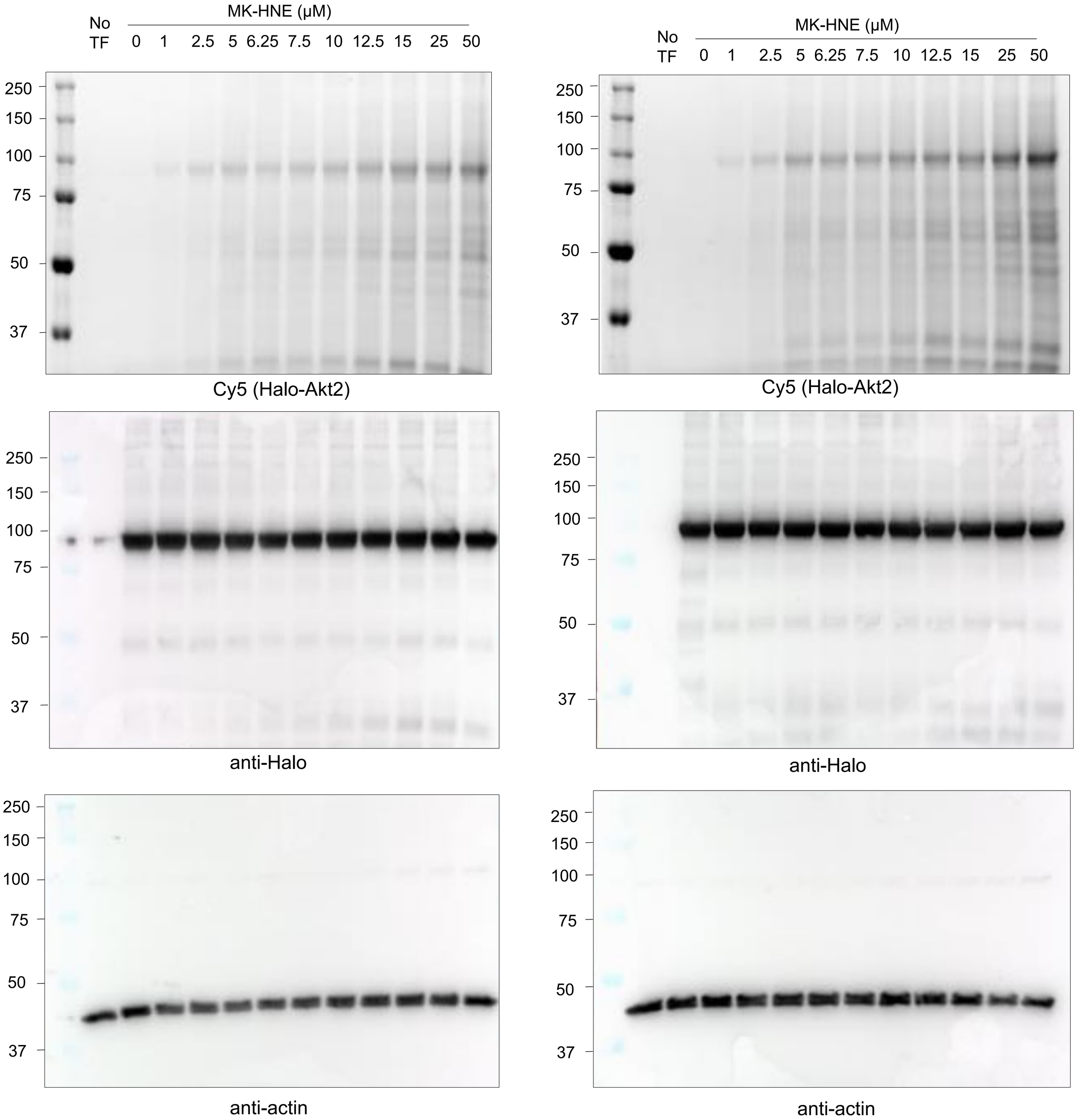






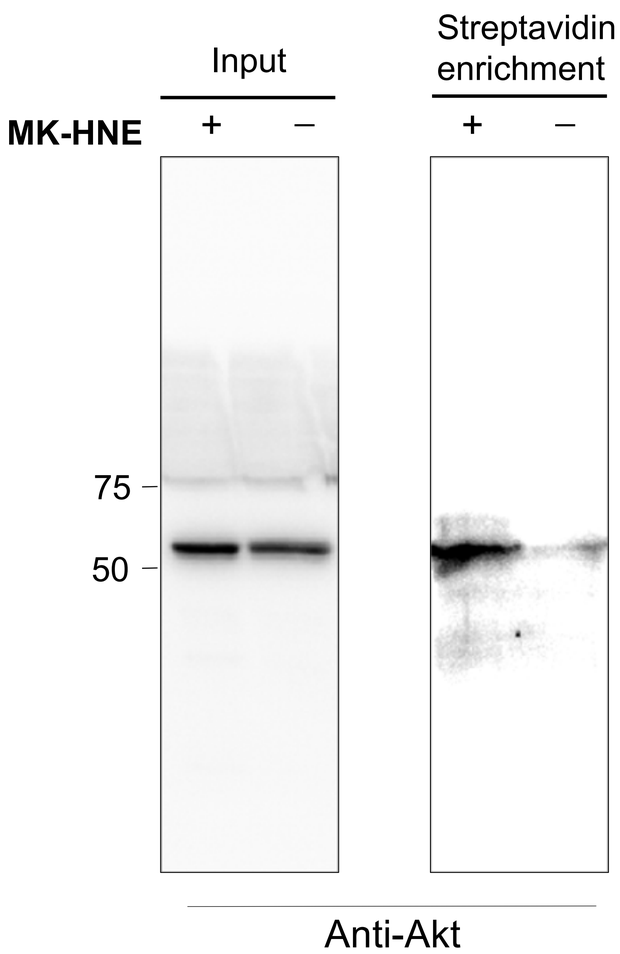


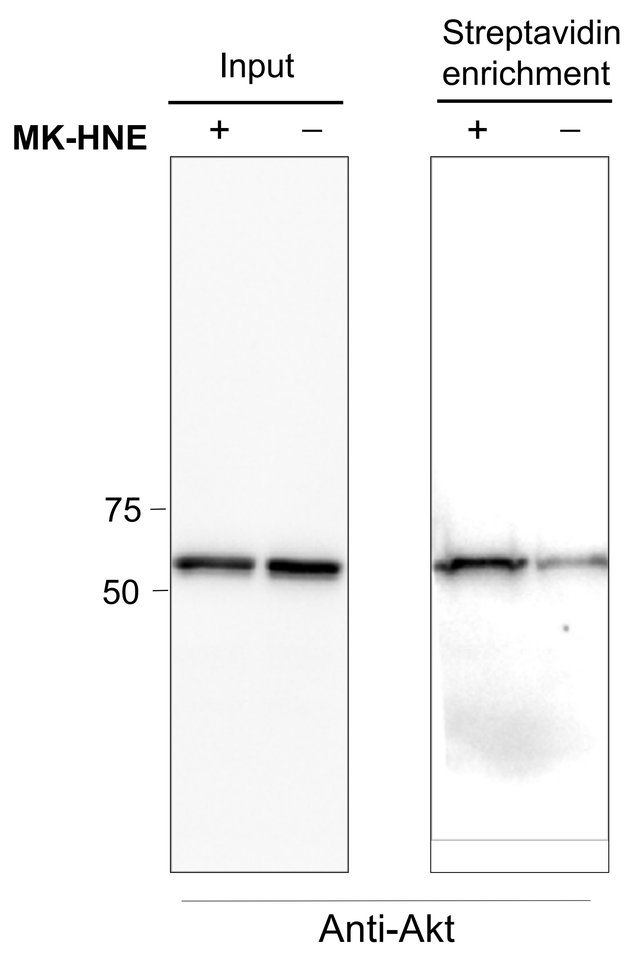
Representative replicates related to data in Supplemental Figure S2



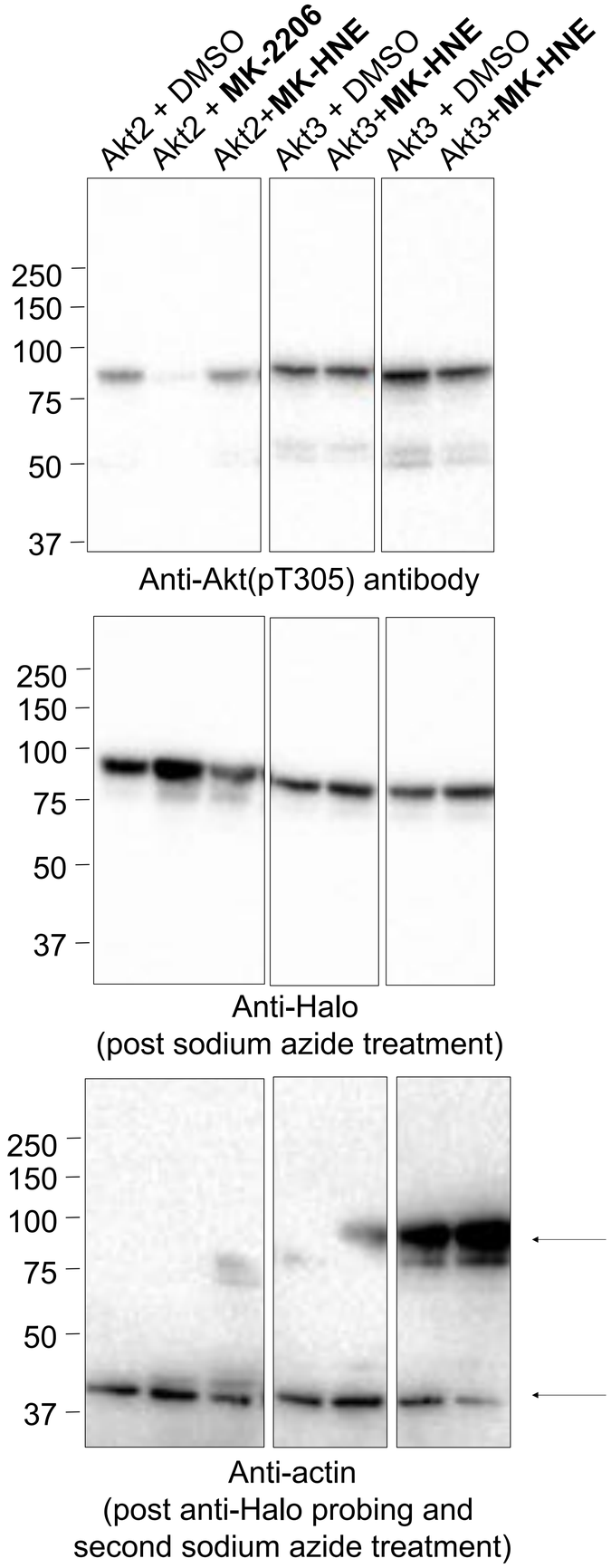
Representative replicates related to data in Supplemental Figure S3

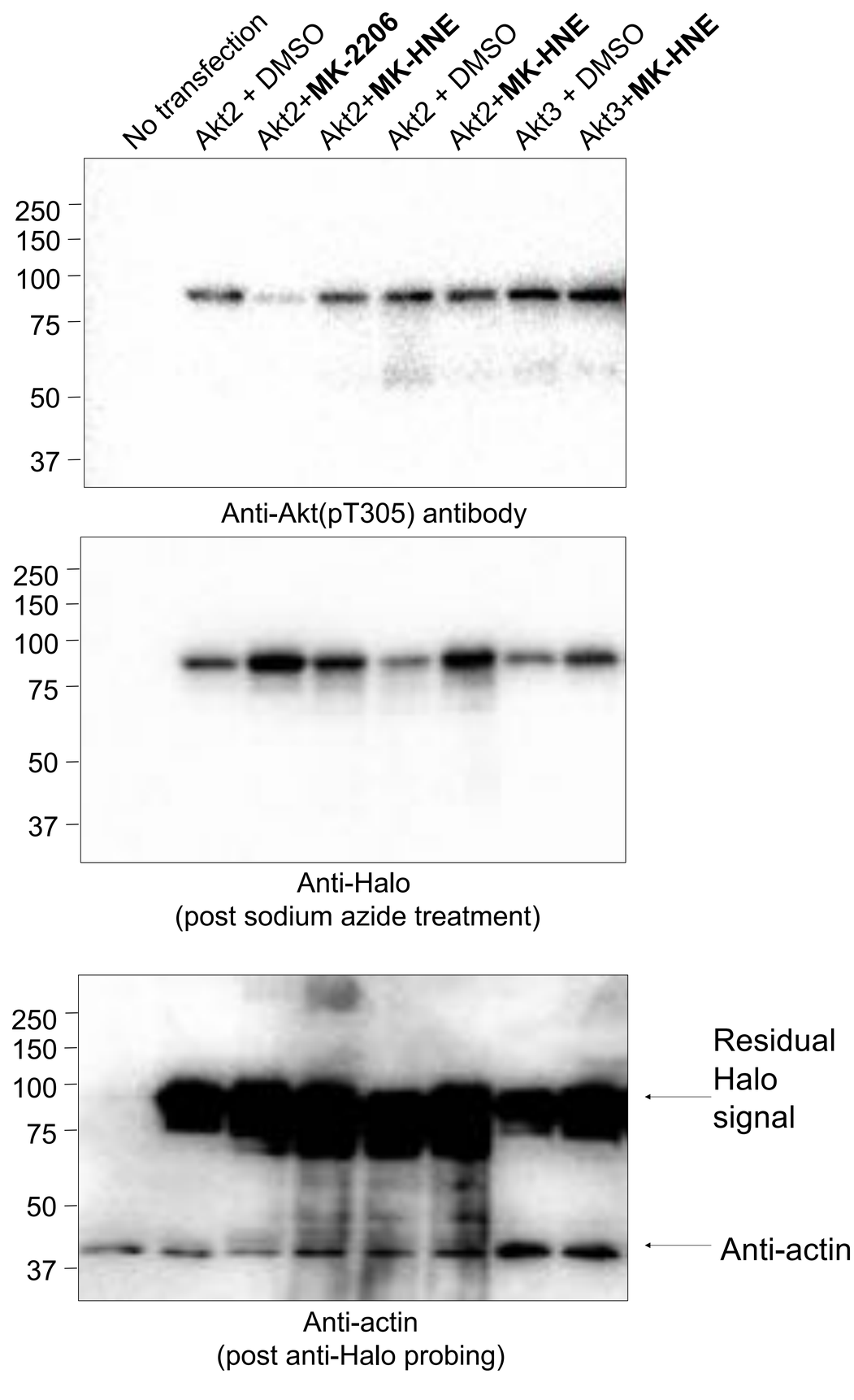
Endogenous Akt pull-down using MK-HNE (5 μM)



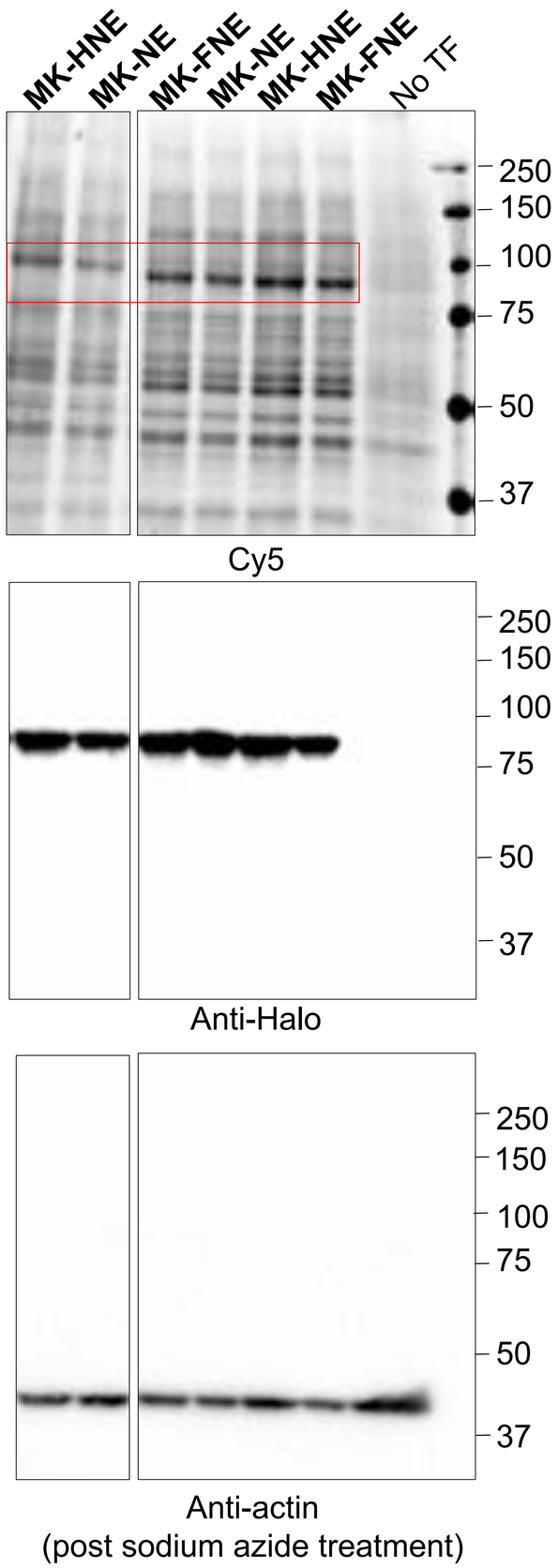


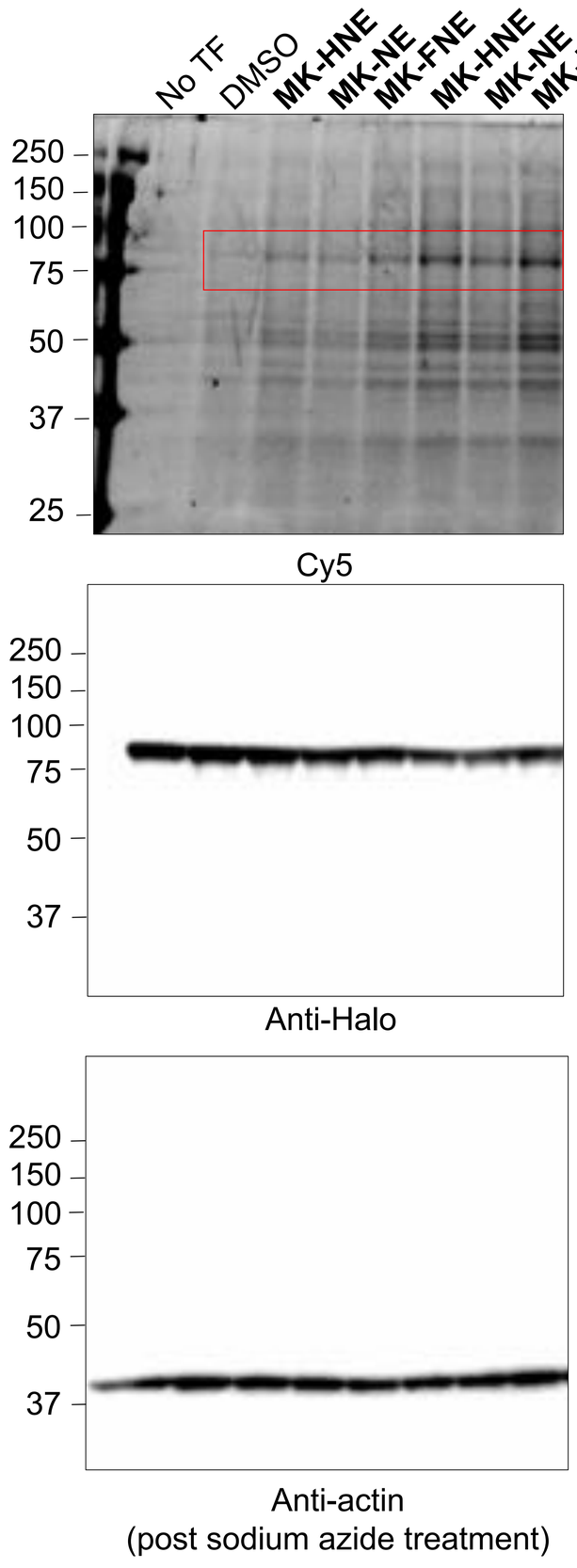
Representative replicates related to data in Supplemental Figure S5





Representative replicates related to data in Supplemental Figure S6

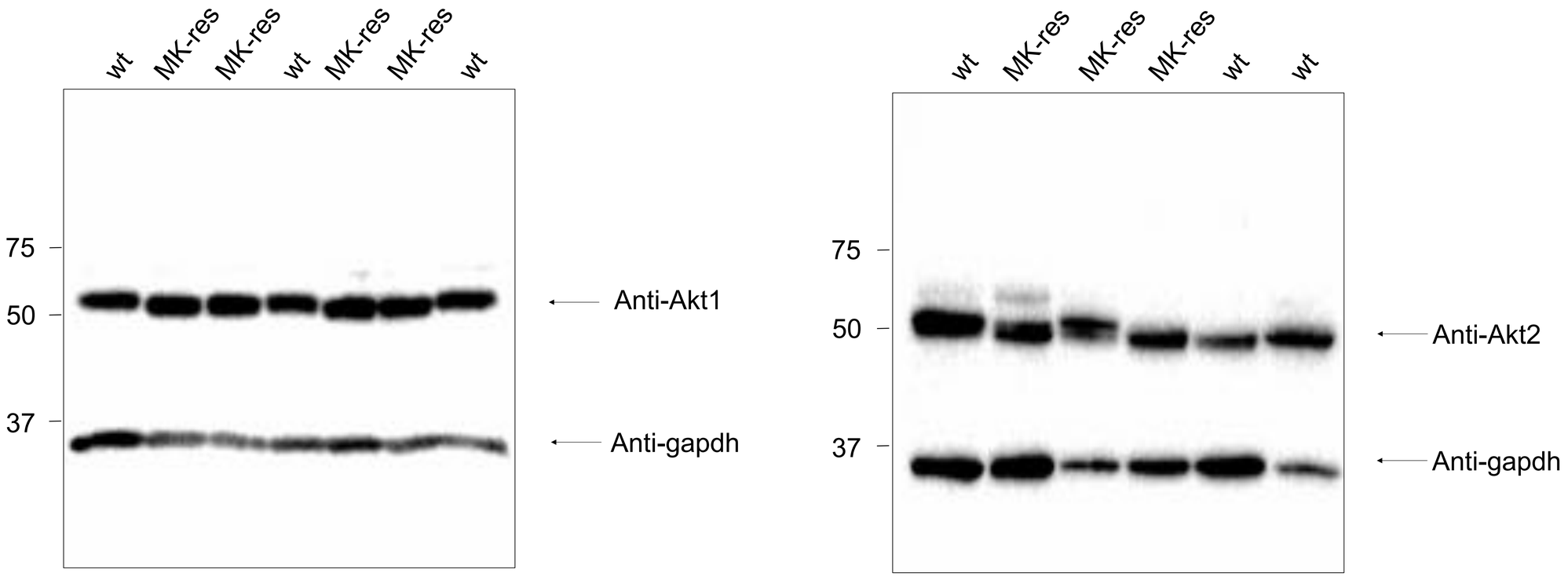




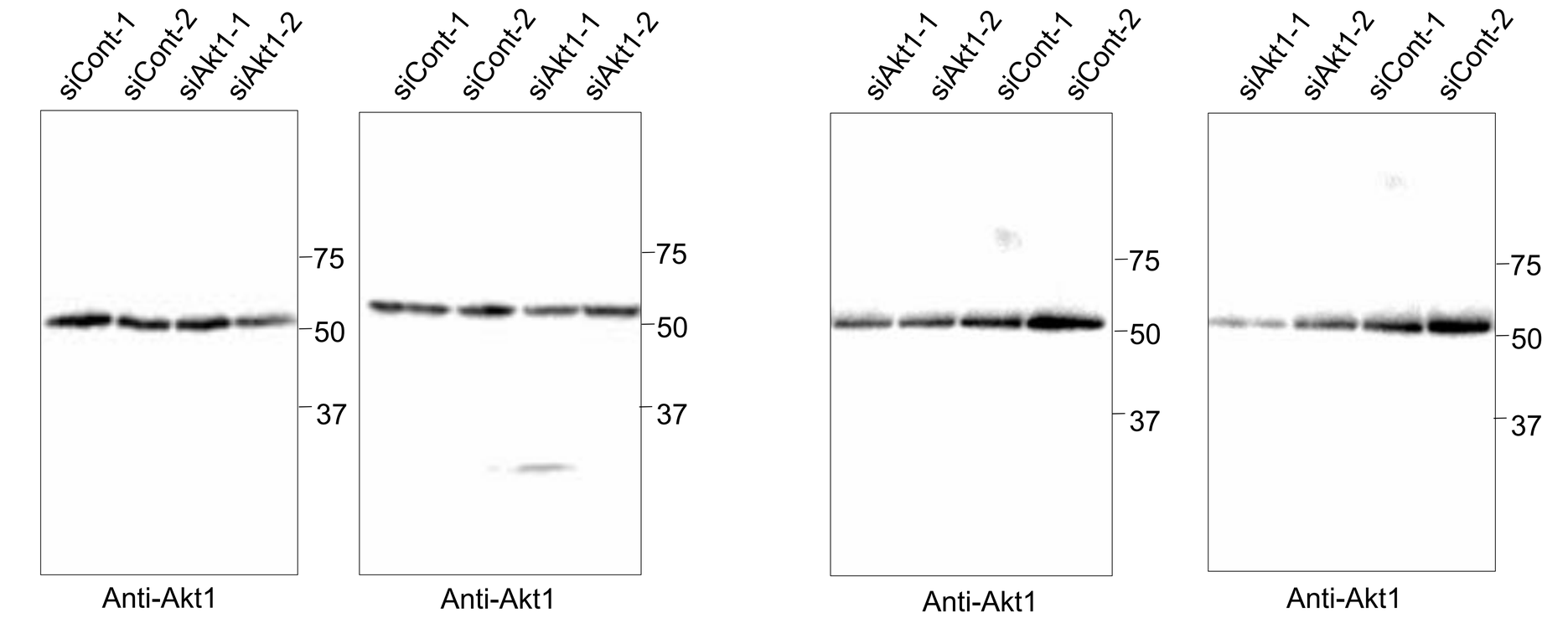
Note: Rectangular box indicates the Cy5 signal of Halo-Akt(n) used in the quantitation

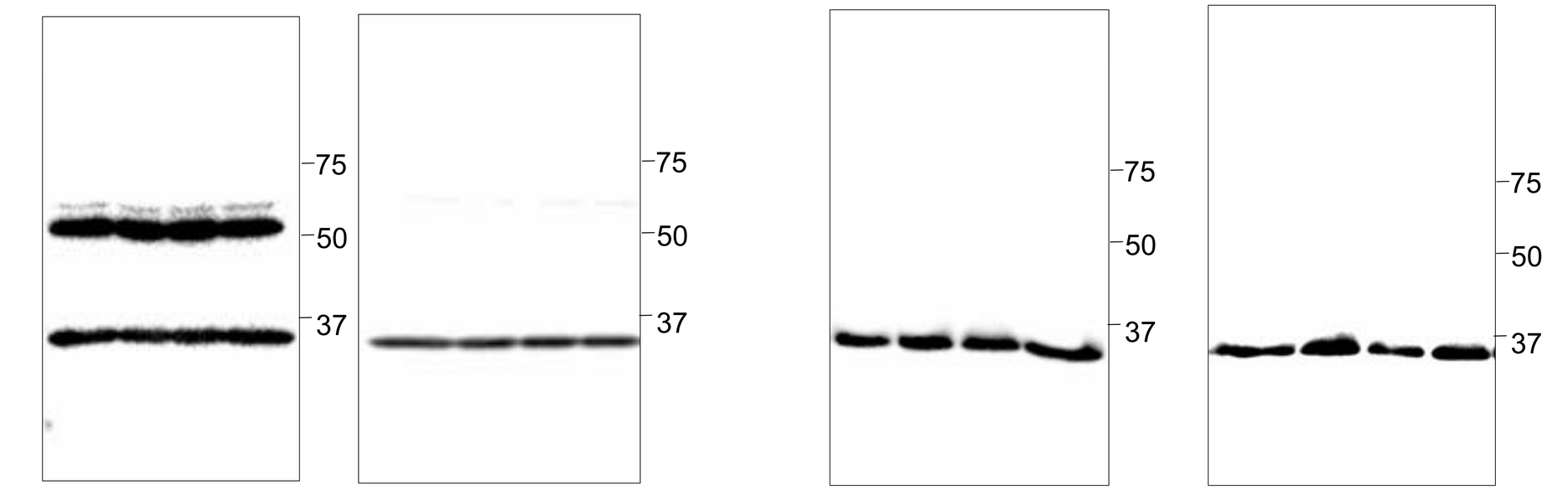
Representative replicates and full-view blots related to data in Supplemental Figure S10b

MK-res (MK-2206-resistant cells)

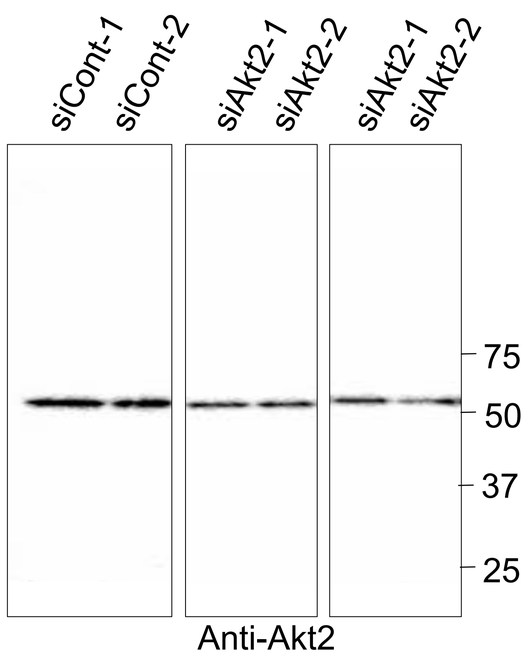


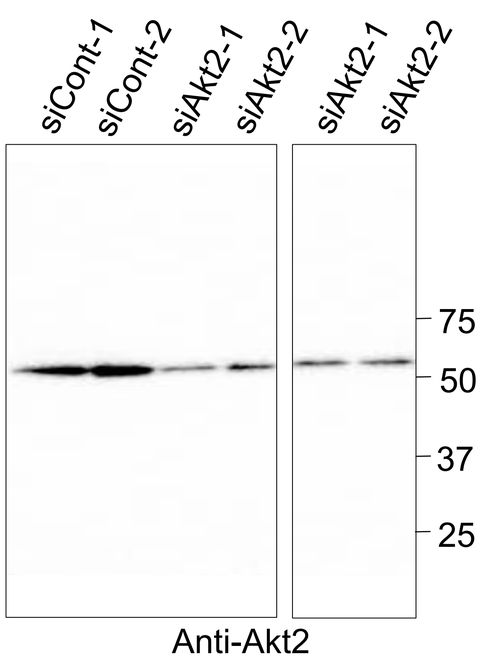
Representative replicates related to data in Supplemental Figure S11

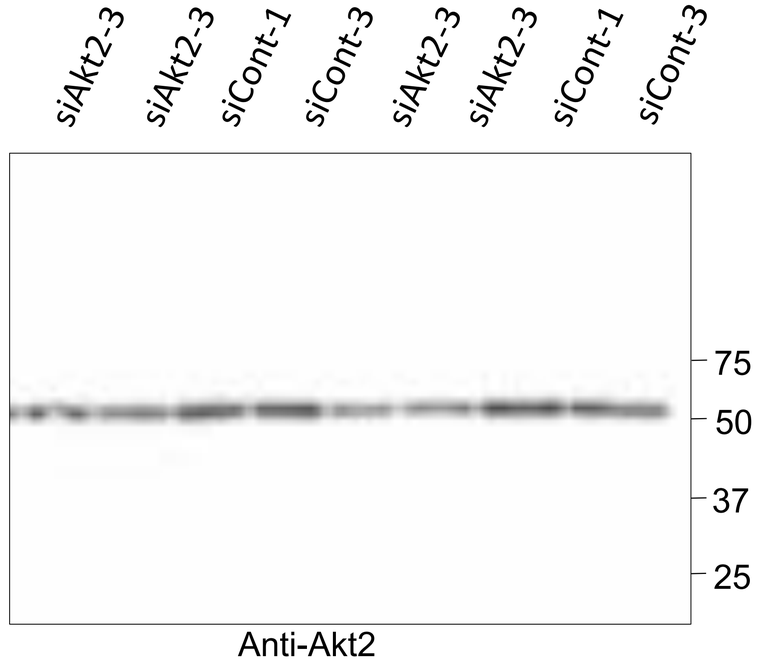


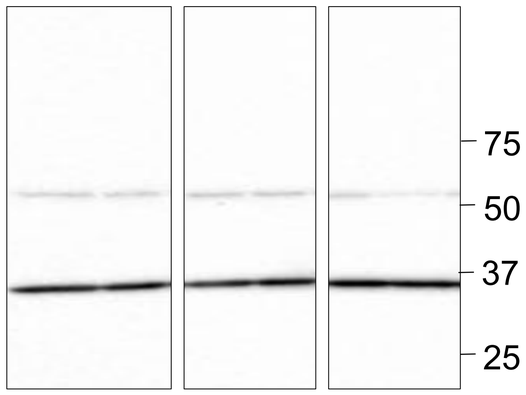


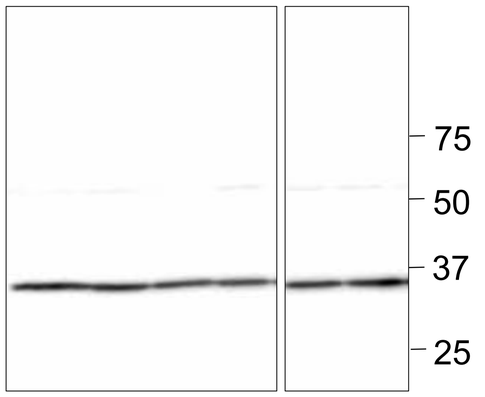
Representative replicates related to data in Supplemental Figure S11 - continued

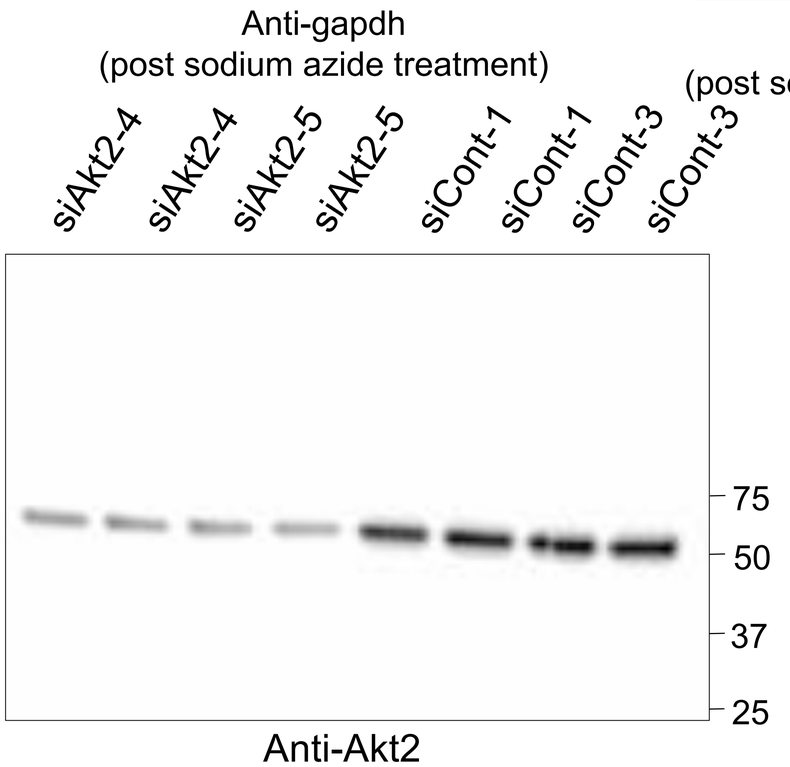


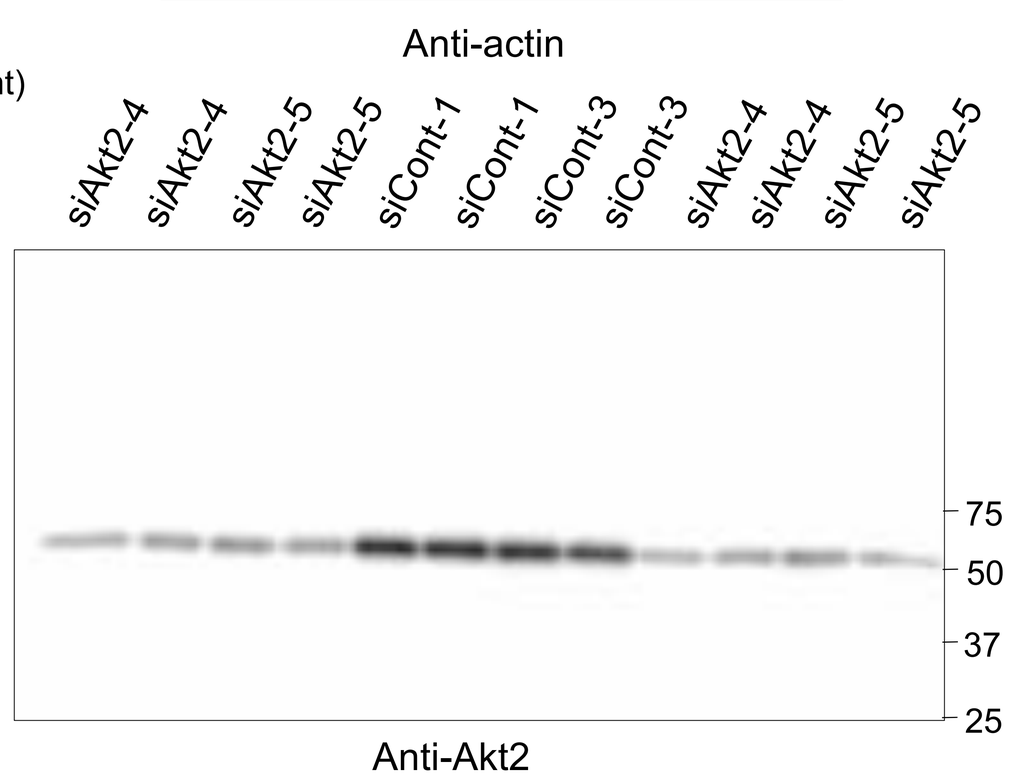


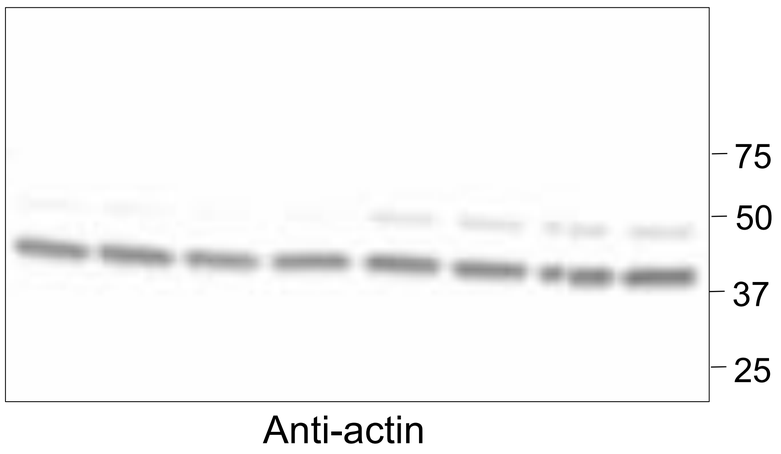


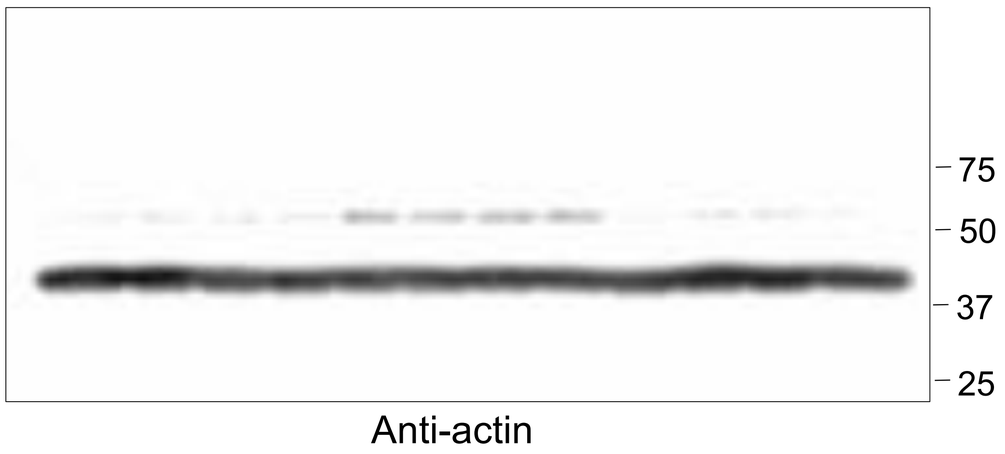




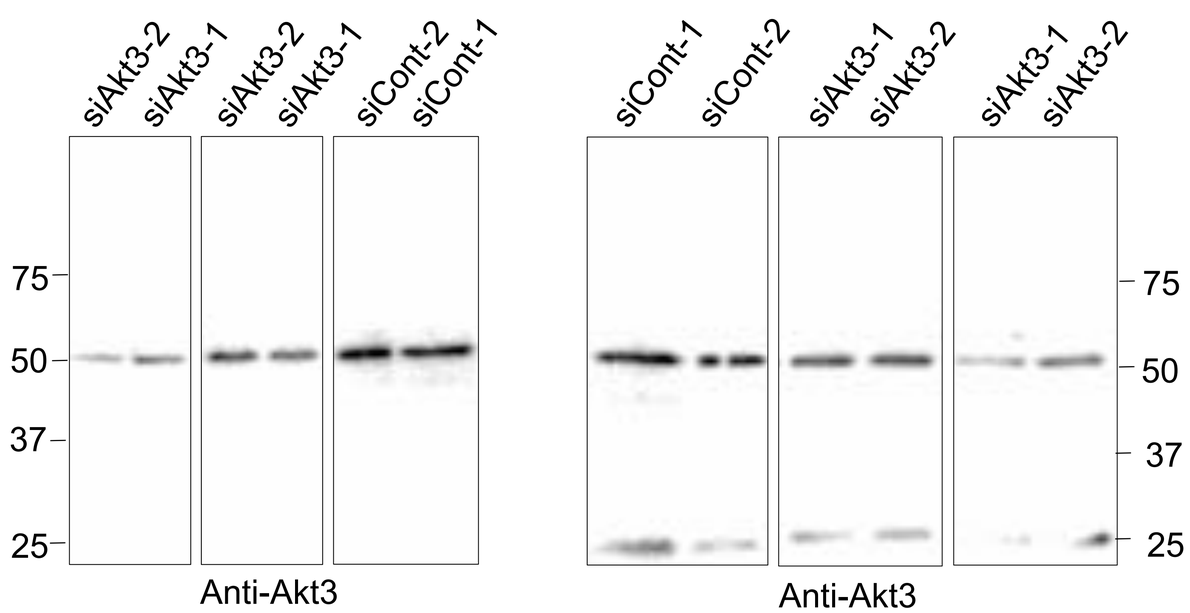


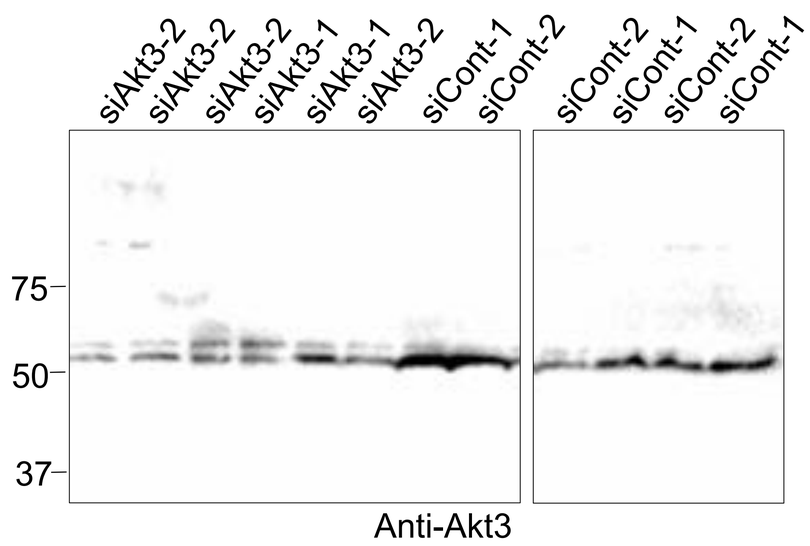


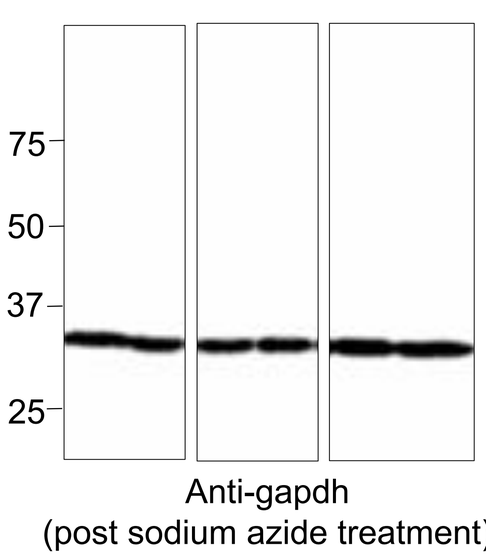


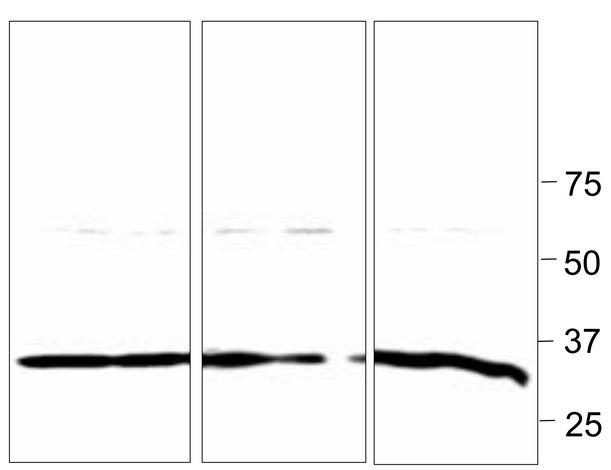


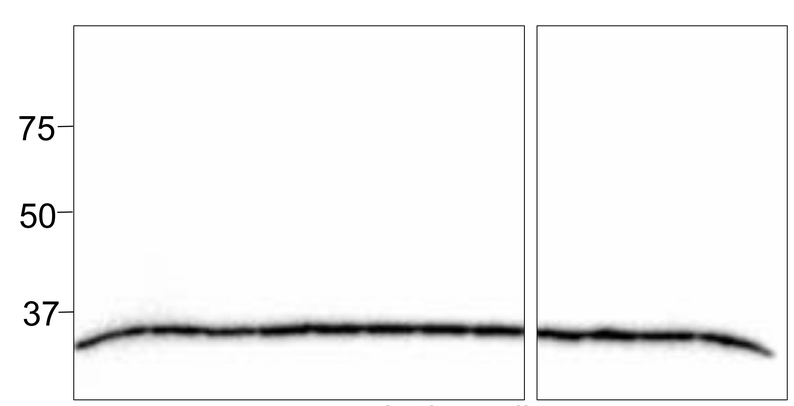
Representative replicates related to data in Supplemental Figure S11 - continued



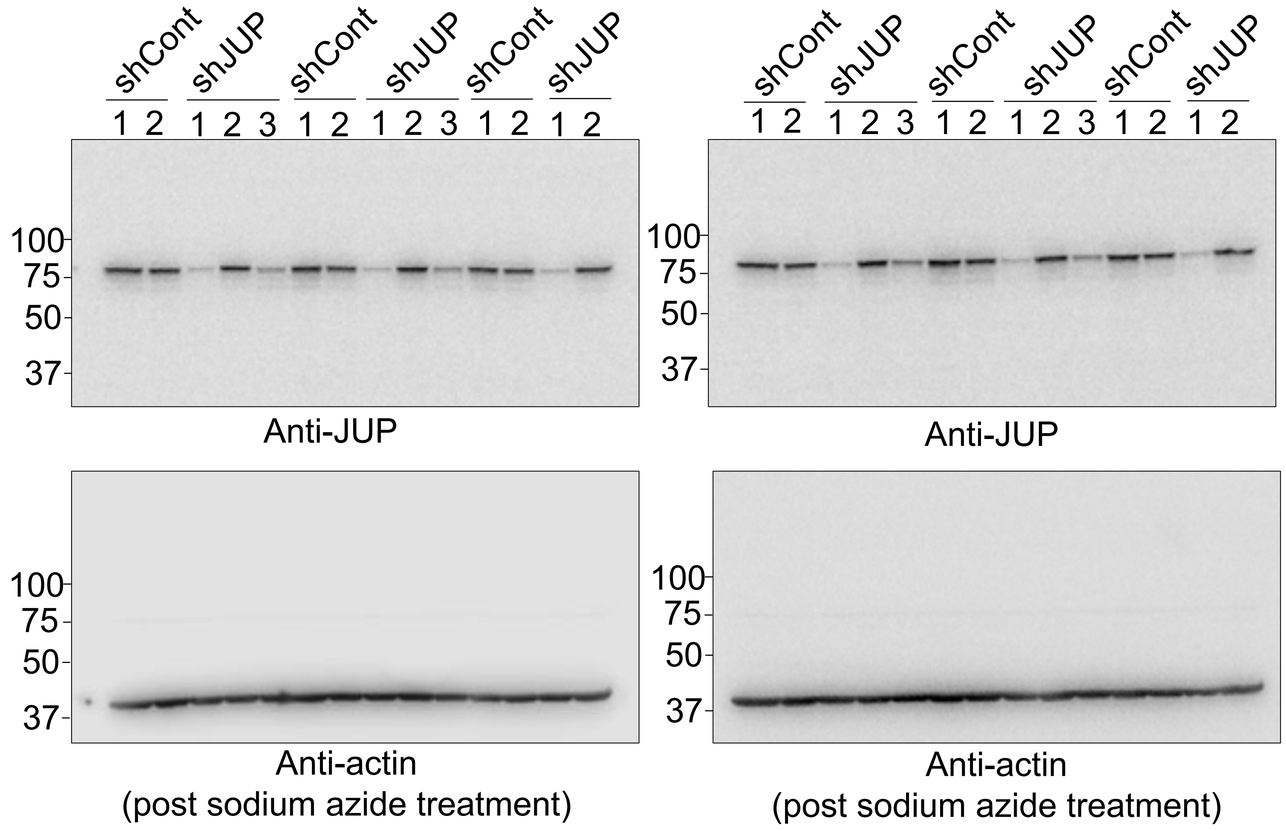




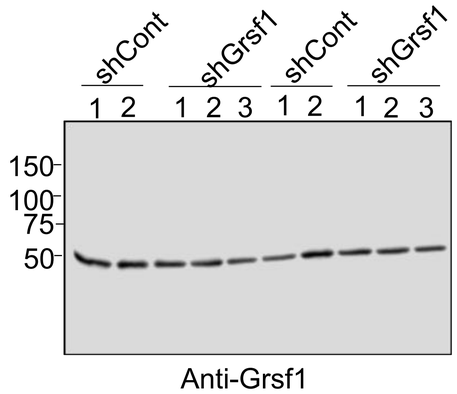


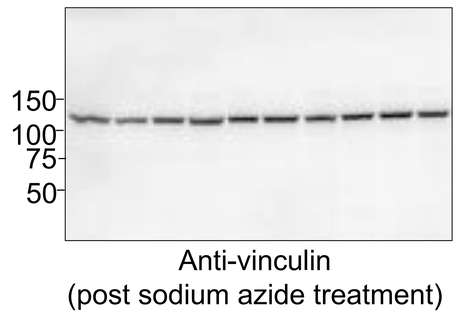


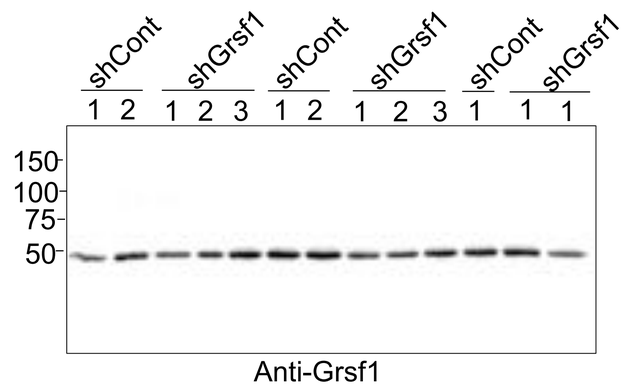
Representative replicates related to data in Supplemental Figure S16

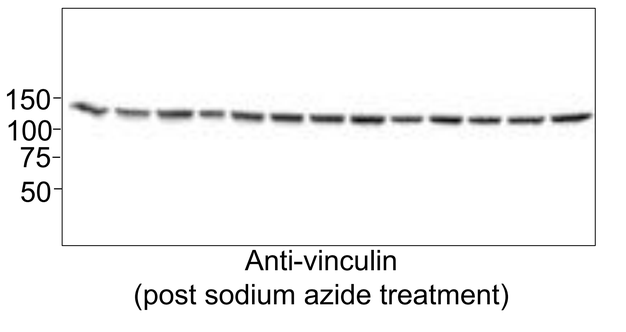


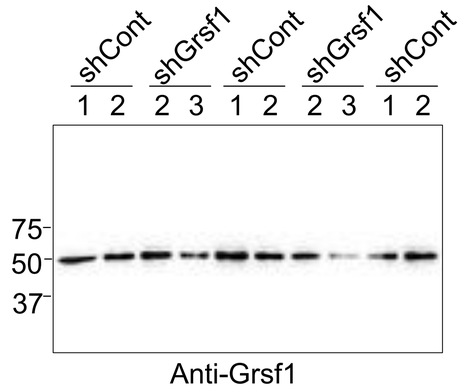
Representative replicates related to data in Supplemental Figure S16 - continued

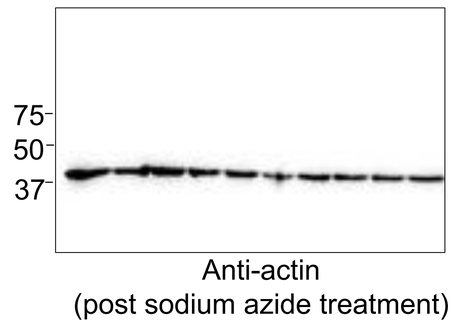


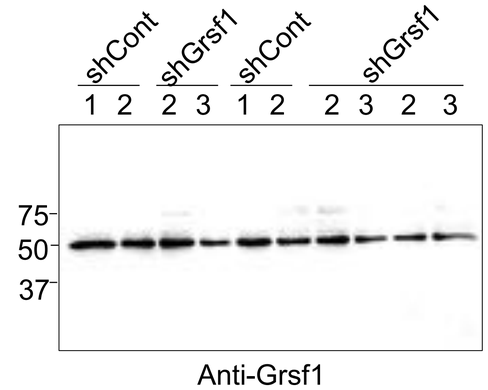


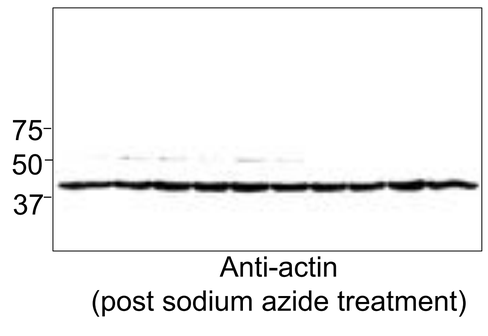






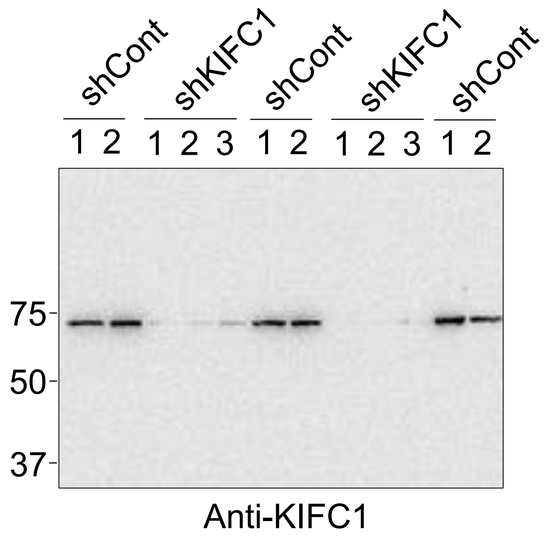




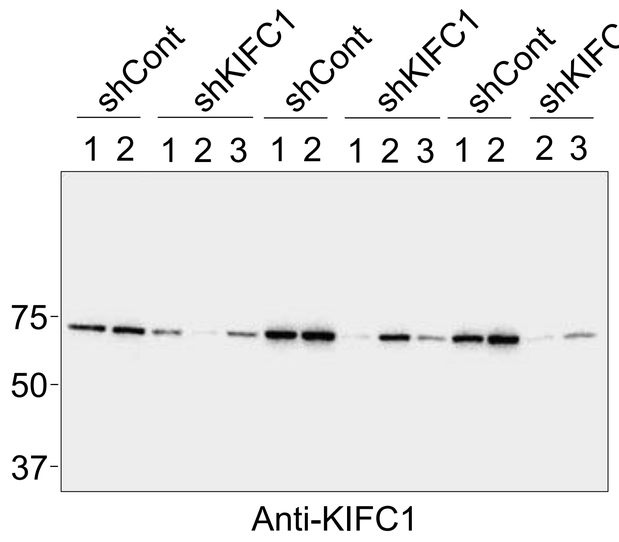


Actin protein was inefficiently transferred in these two blots, therefore, *vinculin was used as loading control*

Representative replicates related to data in Main Figure 5



Anti-KIFC1

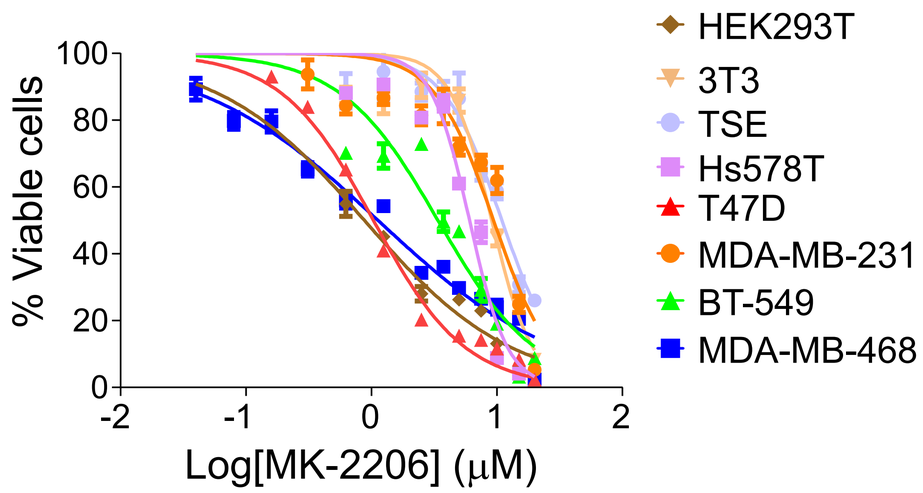


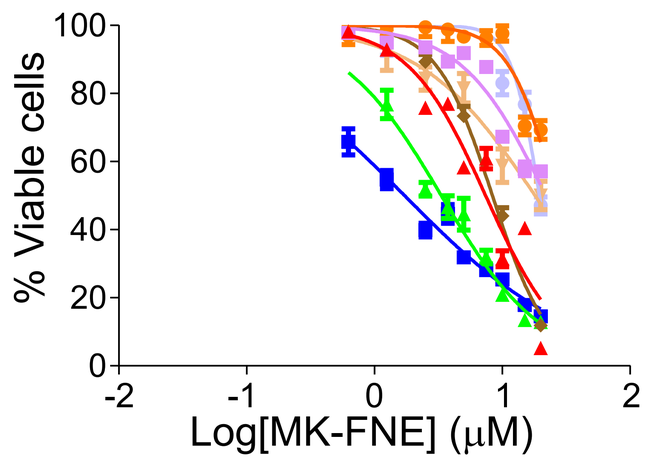
Anti-KIFC1

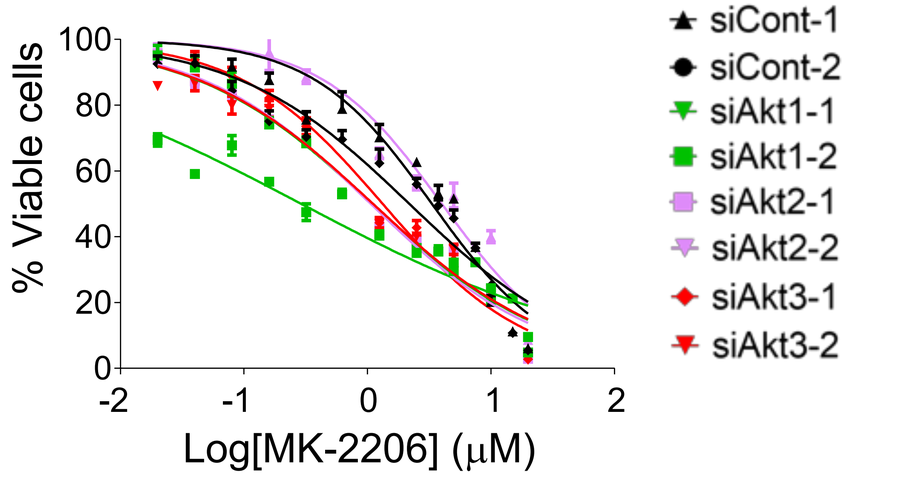
Anti-actin
(post anti-KIFC1 probing)

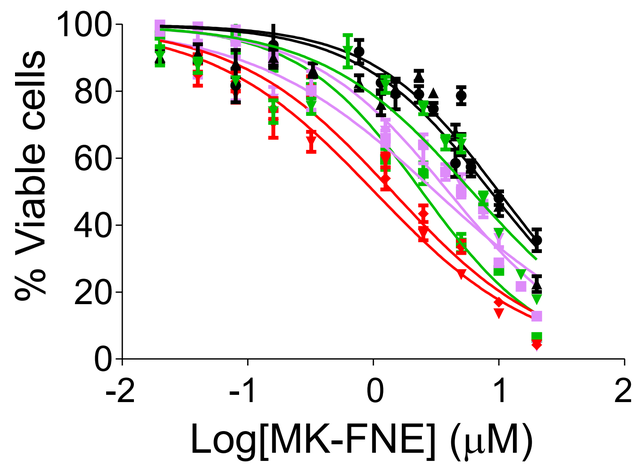
Anti-actin
(post anti-KIFC1 probing)

Log-plot of cytotoxic plots in Figure 4





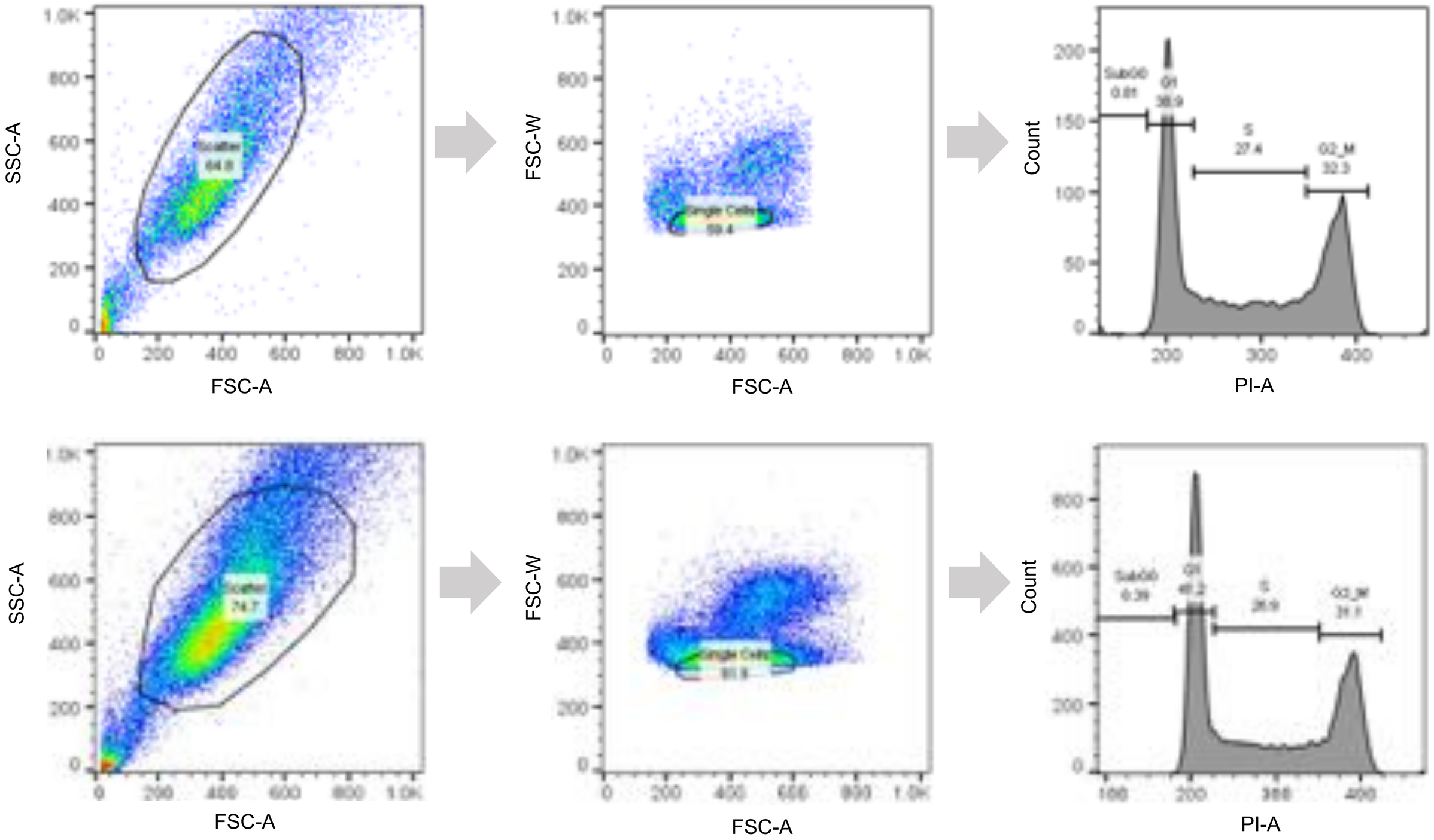




Flow-cytometry analysis (related to **Supplementary Table S2**)

Cells were analyzed based on cellular DNA content using PI dye (excited at 488 nm) to quantitate the percentage of cells in the respective phases (G_1, S, G_2/M and sub-G_0) of the cell cycle. Flow experiments were carried out on separate days using MDA-MB-468 in different passage numbers. Each treatment group was normalized relative to the respective DMSO control group on the same day. The relative mean percentages of cell cycle arrest by the tested compounds are summarized in Supplementary Table S2.

Gating demonstration: control samples



Representative FACS analysis of cell cycle arrest