Supplementary material for:

An increased cell cycle gene network determines MEK and Akt inhibitor double resistance in triple-negative breast cancer.

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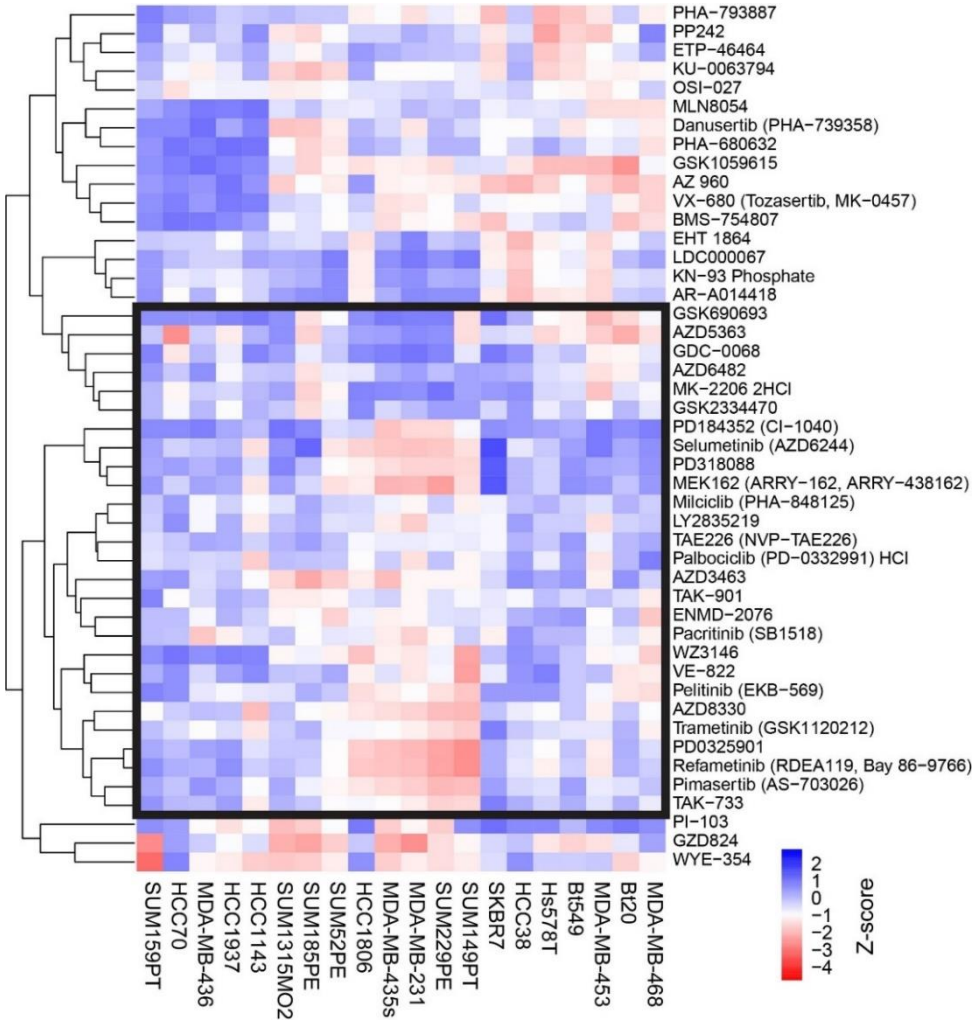
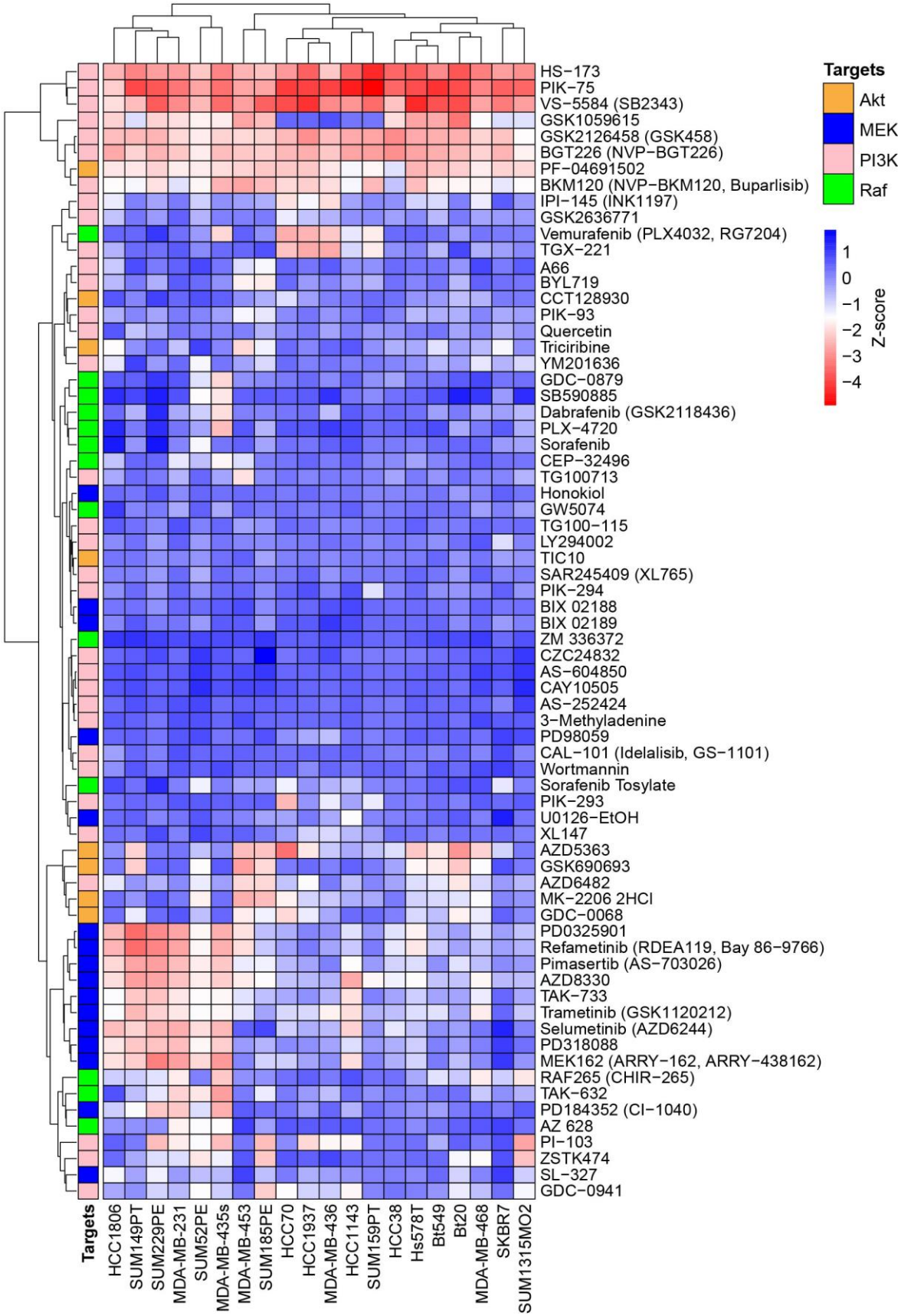
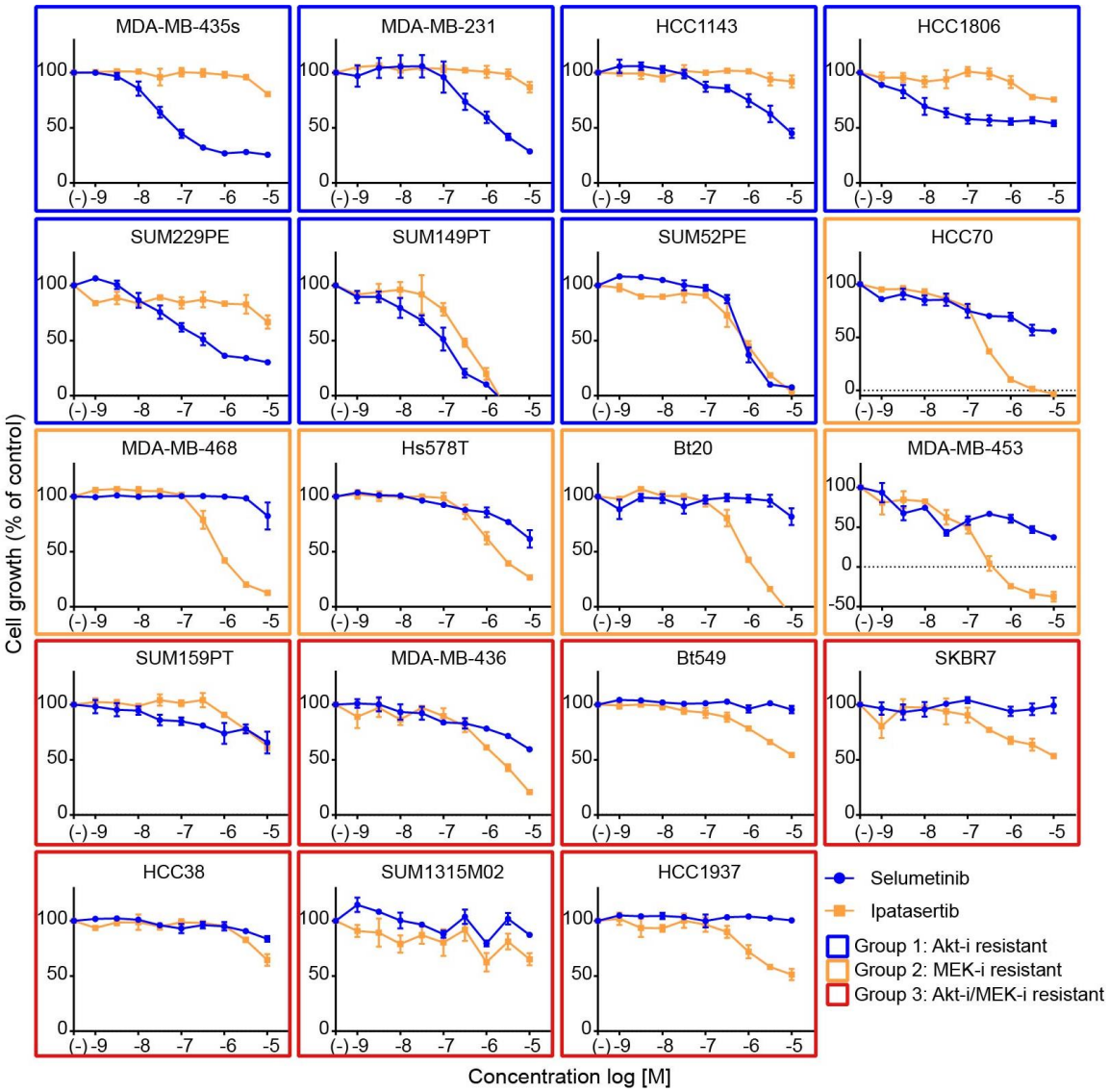


Supplementary Figure S1. Clustered kinase inhibitors differentially affecting TNBC cell proliferation. Enlarged and annotation version of the clustered branch of differentially effective kinase inhibitors from Figure 1A. The black box highlights mainly MEK and Akt inhibitors. Low Z-scores (red) indicate reduced cell proliferation, whereas high Z-scores indicate resistance (blue) upon treatment with the kinase inhibitors (1 µM).

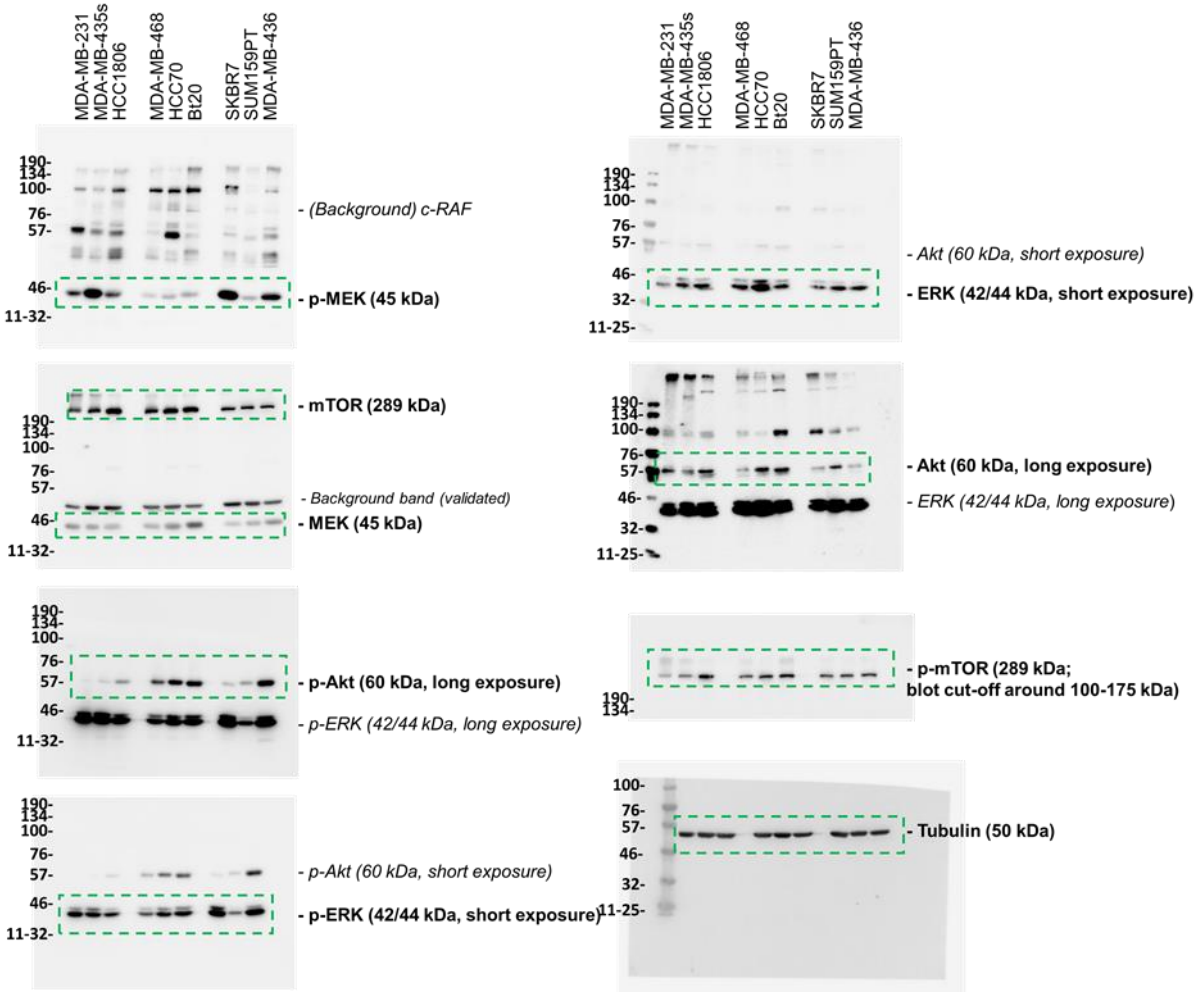


Supplementary Figure S2. Proliferative responses to MEK, Akt, Raf and PI3K inhibitors (1 μM) among TNBC cell lines. Low Z-scores (red) indicate reduced cell proliferation, whereas high Z-scores indicate resistance (blue) upon treatment with the kinase inhibitors.



Supplementary Figure S3. Sensitivity profiling in TNBC cell lines. Validation of Akt-i/MEK-i sensitivity classifications from primary screening (Figure 1B). Proliferative responses of cell lines to increasing concentrations (0.001-10 µM) of selumetinib (MEK-i) and ipatasertib (Akt-i) are shown.

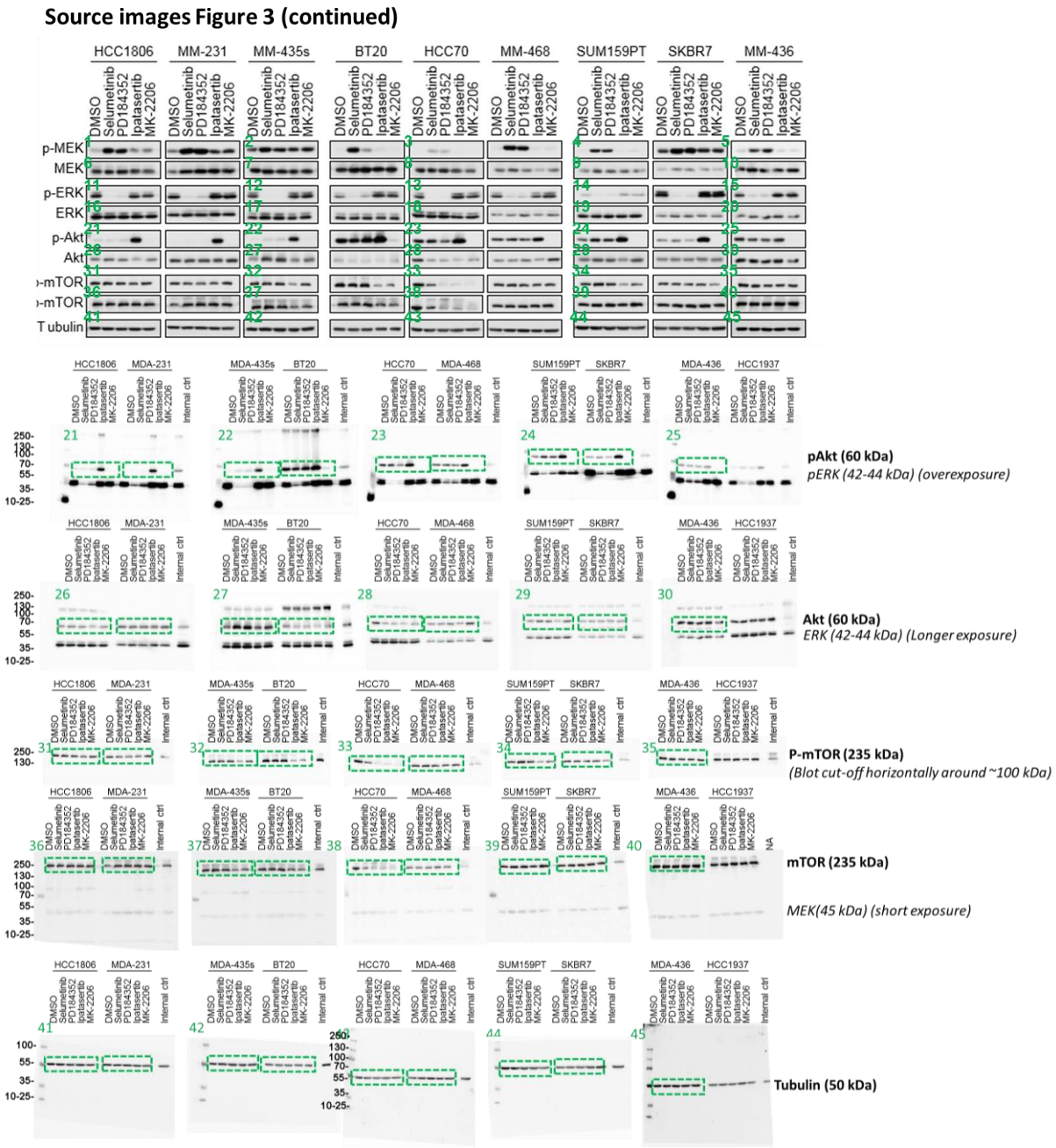
Source images Figure 2



Supplementary Figure S4. Uncropped full-length images of blots. Source images for the western blot bands presented in Figure 2, 3 and 6. Green dotted squares indicate the approximate cropping area. Antibody combinations were previously tested to not interfere with each others bands. In some cases, blots were cut horizontally to perform separate stainings and full-length blots are not depicted. Protein sizes are according to pre-stained protein ladders from Cell signalling (#13953) or Thermo Fisher (#26619).

Source images Figure 3

Supplementary Figure S4. Uncropped full-length images of blots (continued). Source images for the western blot bands presented in Figure 2, 3 and 6. Green dotted squares indicate the approximate cropping area. Antibody combinations were previously tested to not interfere with each others bands. In some cases, blots were cut horizontally to perform separate stainings and full-length blots are not depicted. Protein sizes are according to pre-stained protein ladders from Cell signalling (#13953) or Thermo Fisher (#26619).



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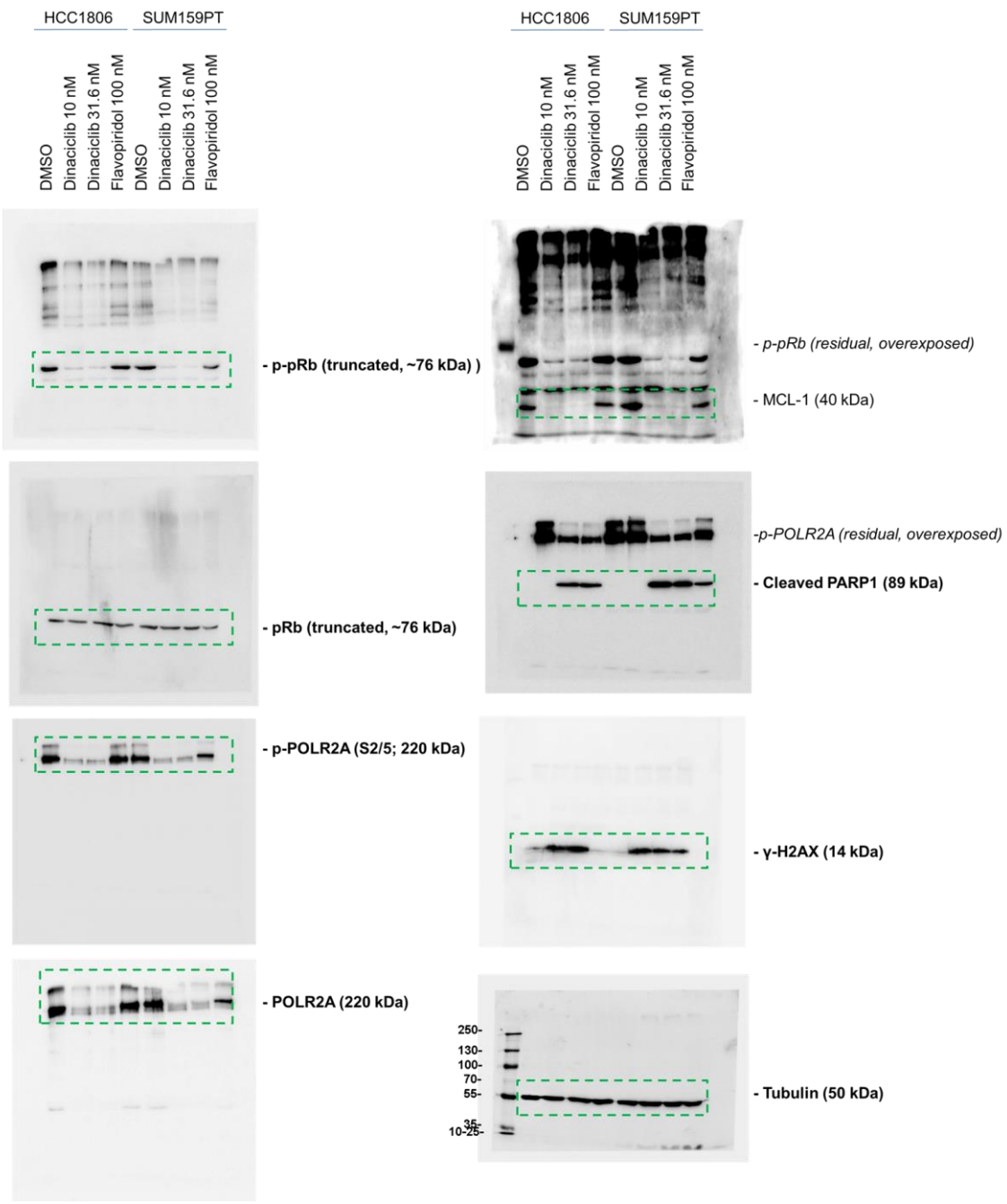
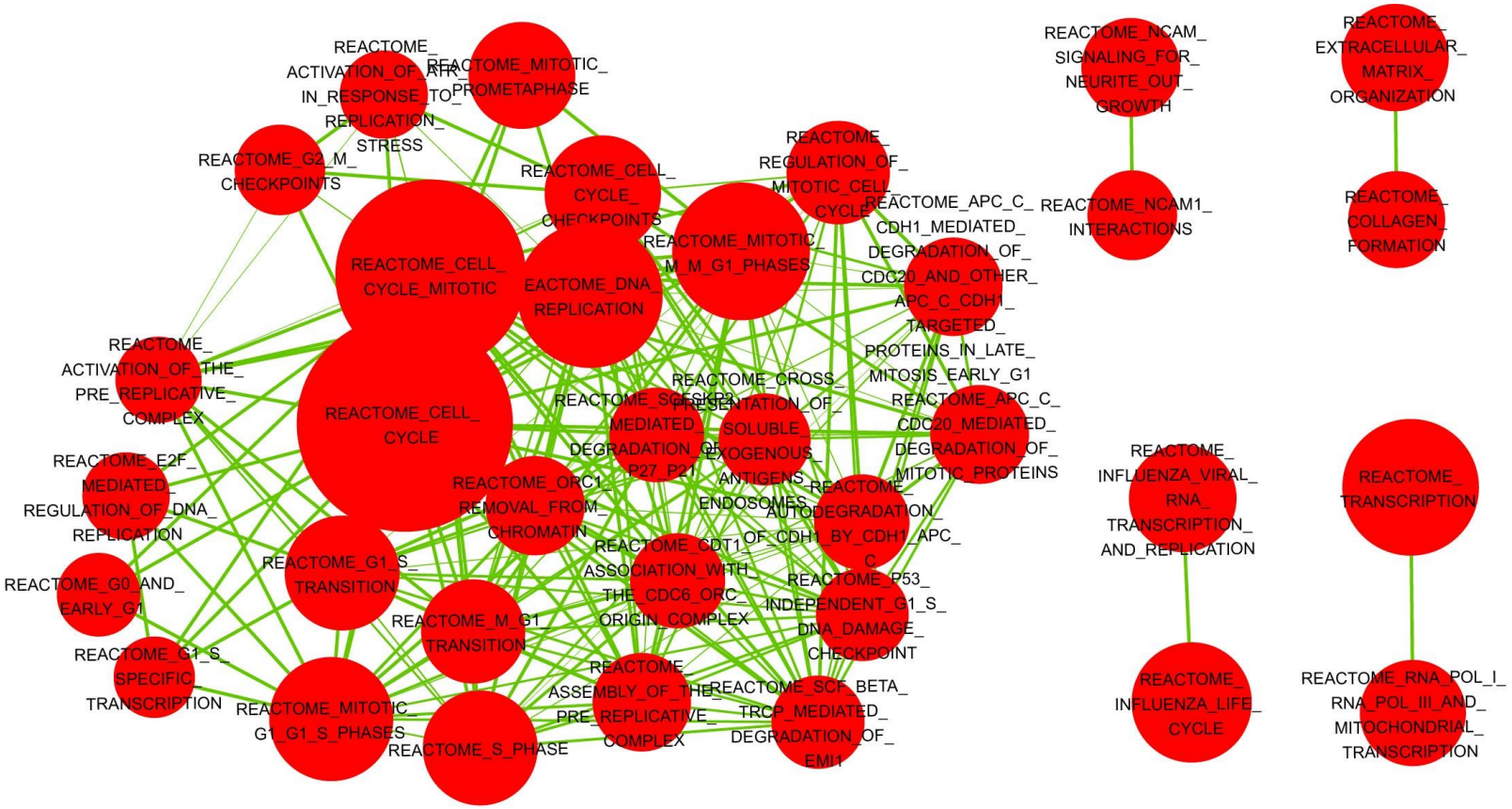
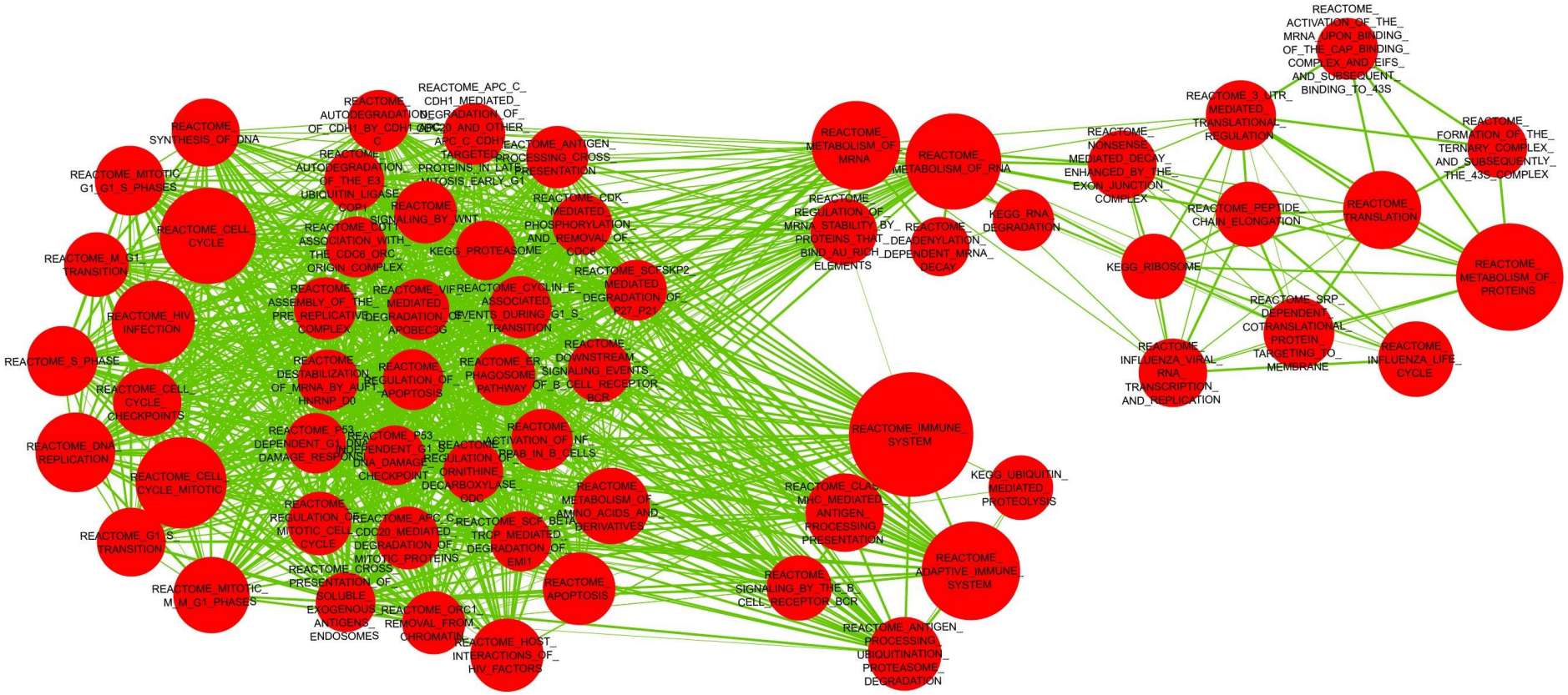
Source images Figure 6:



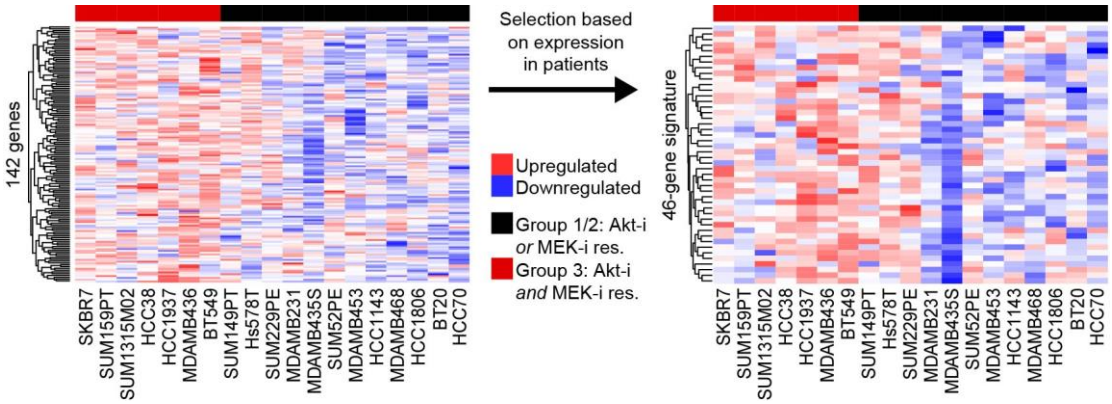
Supplementary Figure S4. Uncropped full-length images of blots (continued). Source images for the western blot bands presented in Figure 2, 3 and 6. Green dotted squares indicate the approximate cropping area. Antibody combinations were previously tested to not interfere with each others bands. In some cases, blots were cut horizontally to perform separate stainings and full-length blots are not depicted. Protein sizes are according to pre-stained protein ladders from Cell signalling (#13953) or Thermo Fisher (#26619).



Supplementary Figure S5. Gene set enrichment map for sets enriched in mRNA expression for Akt-i/MEK-i-resistant Group 3 cell lines. This figure represents an enlarged and annotated version of Figure 4A (left part). mRNA expression was compared between MEK and Akt inhibitor-resistant Group 3 versus MEK or Akt inhibitor-resistant Group 1 and 2 cell lines using gene set enrichment analysis (GSEA). The enrichment map visualises enriched gene sets (P<0.005, FDR Q-value<0.1, overlap>0.7), among which cell cycle gene sets were most abundant. Every node (red) represents a single overexpressed gene set and the size of the node indicates the size of the gene set. Every line (green) represents overlap between gene sets.



Supplementary Figure S6. Gene set enrichment map for sets enriched in protein expression for Akt-i/MEK-i-resistant Group 3 cell lines. This figure represents an enlarged and annotated version of Figure 4A (right part). Protein expression was compared between MEK and Akt inhibitor-resistant Group 3 versus MEK or Akt inhibitor-resistant Group 1 and 2 cell lines using gene set enrichment analysis (GSEA). The enrichment map visualises enriched gene sets (P<0.005, FDR Q-value<0.1, overlap>0.7), among which cell cycle gene sets were most abundant. Every node (red) represents a single overexpressed gene set and the size of the node indicates the size of the gene set. Every line (green) represents overlap between gene sets.



Supplementary Figure S7. Expression of the 142 cell cycle genes and the selected 46 clinically differentially expressed genes in TNBC cell lines. The mRNA expression of the 142 cell cycle genes, as given in Figure 4B, and of the later selected 46 clinically differentially expressed genes, as shown in Figure 5, in the TNBC cell lines is shown.

Supplementary Table S1. IC50's for Akt and MEK inhibitors. To validate the resistance to Akt and/or MEK inhibitors, cells were treated with a dose range (0.01 – 3.16 µM) of inhibitors in three technical replicates in two independent experiments. Proliferation was evaluated using an SRB assay and IC50's were calculated using GraphPad Prism. If 50% of proliferation was not reached, IC50's are noted as >3.16. For the case of more than 50% reduction of growth at the lowest concentration, IC50's were noted as <0.01.

		Akt-i resistant			**MEK-i resistant**			**Akt-i & MEK-i double resistant**		
	IC50 (µM)	**MDA-MB-231**	**MDA-MB-435s**	**HCC1806**	**MDA-MB-468**	**BT20**	**HCC70**	**SKBR7**	**SUM159PT**	**MDA-MB-436**
Akt inhibitors	**MK-2206**	>3.16	>3.16	>3.16	0.30	>3.16	0.26	>3.16	1.35	>3.16
	GDC-0068	>3.16	>3.16	>3.16	0.16	0.38	0.21	>3.16	>3.16	>3.16
	AZD5363	>3.16	>3.16	>3.16	0.15	0.34	0.17	>3.16	~3.16	>3.16
	GSK690693	>3.16	>3.16	>3.16	>3.16	0.15	1.31	>3.16	>3.16	>3.16
MEK inhibitors	**Selumetinib**	0.05	0.03	>3.16	>3.16	>3.16	>3.16	>3.16	2.74	>3.16
	MEK-i162	<0.01	<0.01	7.617	>3.16	>3.16	>3.16	>3.16	~3.16	>3.16
	Trametinib	<0.01	<0.01	<0.01	0.03	>3.16	>3.16	>3.16	NA	NA
	PD318088	0.15	<0.01	<0.01	0.32	>3.16	>3.16	>3.16	>3.16	>3.16
	PD184352	0.79	0.12	<0.01	>3.16	>3.16	>3.16	>3.16	>3.16	>3.16
	PD032590	<0.01	<0.01	0.48	0.53	>3.16	>3.16	>3.16	>3.16	>3.16
	Pimasertib	<0.01	<0.01	2.27	2.39	>3.16	>3.16	>3.16	>3.16	>3.16
	Refametinib	<0.01	<0.01	<0.01	>3.16	>3.16	>3.16	>3.16	~3.16	>3.16
	TAK-733	<0.01	<0.01	<0.01	0.27	>3.16	>3.16	>3.16	~3.16	>3.16
	AZD8330	<0.01	<0.01	<0.01	>3.16	>3.16	>3.16	>3.16	>3.16	>3.16

Supplementary Table S2. Common mutations in cell lines in components of the PI3K, MAPK, cell cycle and DNA damage repair pathways. Mutations in Cancer Gene Census genes were deducted from the COSMIC database (http://cancer.sanger.ac.uk/cosmic).

		PI3K	MAPK	PI3K/ MAPK	Cell cycle	DNA damage repair
1): Akt-i resistant	**HCC1143**			FGFR2	TP53	
	MDA-MB-435s		BRAF		TP53, CDKN2A, EP300	
	MDA-MB-231		BRAF,NF1	PDGFRA, KRAS	TP53, CDKN2A	
	HCC1806		NFATC2, ROS1	PDGFRB	TP53, CDKN2A	
	SUM229PE			KRAS	CDKN2A	
	SUM52PE				TP53, CDKN2A	
	SUM149PT				TP53, CDKN2A, EP300	BRCA1
2) MEK-i resistant	**HCC70**	PTEN			TP53, RB1	
	MDA-MB-453	PIK3CA, PTEN	ROS1		CREBBP, ATM, STAG2	CREBBP, ATM
	SUM185PE	PIK3CA			TP53	
	BT20	PIK3CA			ATM, CDKN2C, CDKN2A, RB1, TP53	MLH1
	Hs578T	PIK3R1	NF1	HRAS	TP53, CDKN2A, POLE	POLE
	MDA-MB-468	PTEN	CACNA1D, MAP3K13		TP53, CDKN2A, RB1	
3) Akt/MEK-i resistant	**BT549**	PTEN		FGFR1	TP53, RB1, BUB1B, (DCTN1), PCM1	MUTYH
	SKBR7			KRAS, NRAS	CDKN2A	
	HCC1937	PTEN	MAPK13		TP53, RB1	BRCA1
	MDA-MB-436	PIK3R1			TP53, RB1	BRCA1, POLE
	HCC38		IKBKB		TP53, CDKN2A	PMS2, BRCA1*
	SUM159PT	PIK3CA		HRAS	TP53	
	SUM1315MO2	PIK3CA			TP53	BRCA1

* methylation

Supplementary Table S3. Enriched cell cycle genes in Akt-i/MEK-i resistant group 3 cell lines. From the significantly enriched, cell cycle associated, gene sets, enriched genes or proteins (signal-to-noise < 0.1) were used for further analysis.

mRNA expression		Protein expression		Shared		
PROBE	Signal-to-noise	PROBE	Signal-to-noise	PROBE	Signal-to-noise (mRNA)	Signal-to-noise (protein)
1. SNAP25	0.710	1. SEC13	0.797	1. PSMD11	0.447	0.631
2. STX11	0.640	2. RUVBL1	0.765	2. PSMC2	0.379	0.632
3. KAT2B	0.530	3. PSMC4	0.737	3. SEC13	0.124	0.797
4. PPP2R3B	0.528	4. PSMD3	0.737	4. PSMD3	0.107	0.737
5. SGOL1	0.499	5. PSMA3	0.693	5. PSMD1	0.106	0.678
6. PTRF	0.489	6. PSMD1	0.678	6. PCM1	0.409	0.336
7. TUBB4A	0.448	7. PSMA4	0.670	7. PSMD12	0.103	0.611
8. PSMD11	0.447	8. CKAP5	0.666	8. PSMA7	0.139	0.574
9. CDC25A	0.425	9. YWHAG	0.647	9. PSMB7	0.117	0.571
10. STX2	0.423	10. PSMC2	0.632	10. ORC5	0.240	0.424
11. PCM1	0.409	11. PSMD11	0.631	11. RPS27	0.108	0.545
12. CENPI	0.393	12. PSMD12	0.611	12. PSMD9	0.375	0.239
13. PSMC2	0.379	13. RUVBL2	0.604	13. CCNB1	0.170	0.415
14. PSMD9	0.375	14. PSMD4	0.589	14. CDC23	0.267	0.308
15. NINL	0.360	15. PSMA7	0.574	15. GINS4	0.143	0.414
16. POLR3D	0.332	16. PSMB7	0.571	16. ACTR1A	0.150	0.375
17. RFC3	0.316	17. PSMA1	0.569	17. CUL1	0.164	0.338
18. HIST1H2AC	0.308	18. SKP1	0.557	18. ANAPC7	0.141	0.338
19. CENPO	0.298	19. RPS27	0.545	19. NDC80	0.104	0.360
20. PPP2R1B	0.293	20. CDK4	0.533	20. CDK2	0.138	0.316
21. RPA4	0.289	21. PSME2	0.526	21. CDK5RAP2	0.180	0.255
22. GTF3C3	0.284	22. PSMC5	0.514	22. TYMS	0.228	0.198
23. GTF2H2	0.283	23. PSMD8	0.504	23. SPC25	0.209	0.147
24. SNAPC1	0.277	24. RBX1	0.498	24. PPP2CB	0.107	0.223
25. TUBA1A	0.274	25. NUDC	0.487	25. ZWILCH	0.154	0.176
26. POLR1A	0.274	26. PSMC1	0.478	26. ERCC6L	0.175	0.127
27. CDK6	0.269	27. PSME1	0.478	27. KIF2A	0.117	0.143
28. CDC23	0.267	28. PSMB4	0.472	28. GINS1	0.115	0.141
29. LIN54	0.250	29. PSMD13	0.466	29. SYNE1	0.112	0.120
30. STX4	0.245	30. DCTN2	0.445	30. MAX	0.116	0.116
31. CEP250	0.244	31. RPS27A	0.443			
32. ORC5	0.240	32. CLASP1	0.442			
33. CDC6	0.235	33. UBE2E1	0.440			
34. TFDP1	0.232	34. MAGED1	0.438			
35. MCM6	0.232	35. DCTN3	0.435			
36. ATRIP	0.229	36. ORC5	0.424			
37. TYMS	0.228	37. UBE2I	0.423			
38. WRAP53	0.223	38. CUL5	0.422			
39. NUP43	0.222	39. CCNB1	0.415			
40. BUB1	0.220	40. GINS4	0.414			

41. BRCA1	0.218	**41. PSMD7**	0.382
42. VAMP1	0.218	**42. ACTR1A**	0.375
43. E2F1	0.211	**43. PAFAH1B1**	0.365
44. STX1B	0.210	**44. PSMB1**	0.364
45. SPC25	0.209	**45. PSMC3**	0.361
46. TUBG1	0.205	**46. NDC80**	0.360
47. ERCC6	0.205	**47. PSMD6**	0.355
48. SKA1	0.201	**48. CLIP1**	0.352
49. CCNE1	0.199	**49. XPO1**	0.347
50. ORC1	0.199	**50. ANAPC2**	0.338
51. TAOK1	0.192	**51. CUL1**	0.338
52. CCNE2	0.190	**52. PCM1**	0.336
53. HIST1H4J	0.187	**53. ANAPC7**	0.333
54. GTF2H2B	0.187	**54. LMNA**	0.331
55. CCNA2	0.184	**55. KIF2C**	0.328
56. CDKN2C	0.181	**56. CDK2**	0.316
57. CDK5RAP2	0.180	**57. TUBGCP2**	0.314
58. TUBGCP5	0.179	**58. TCEB2**	0.309
59. BTRC	0.178	**59. CDC23**	0.308
60. PSMB2	0.177	**60. PSMB5**	0.288
61. ERCC6L	0.175	**61. DYNLL1**	0.279
62. CENPK	0.172	**62. PSMB8**	0.277
63. CCNB1	0.170	**63. CDK5RAP2**	0.255
64. POLE2	0.169	**64. PSMD2**	0.251
65. CEP135	0.167	**65. NUF2**	0.248
66. CUL1	0.164	**66. PSMD9**	0.239
67. RBBP4	0.163	**67. CETN2**	0.233
68. DNA2	0.161	**68. PSMC6**	0.232
69. DIDO1	0.160	**69. DYNC1I2**	0.232
70. PPP2R2A	0.156	**70. KIF20A**	0.226
71. CEP72	0.156	**71. RPA1**	0.226
72. ZWILCH	0.154	**72. PPP2CB**	0.223
73. HAUS2	0.152	**73. HSPA2**	0.222
74. CENPH	0.151	**74. PSMD10**	0.217
75. CDC25C	0.150	**75. PPP2R1A**	0.206
76. ACTR1A	0.150	**76. FEN1**	0.200
77. TAF1B	0.147	**77. TYMS**	0.198
78. MCM10	0.147	**78. MNAT1**	0.178
79. UBTF	0.145	**79. PPP2R5D**	0.177
80. MCM4	0.144	**80. ZWILCH**	0.176
81. CDC27	0.144	**81. ANAPC5**	0.176
82. ANAPC10	0.143	**82. PRKACA**	0.166
83. LIN52	0.143	**83. ORC2**	0.166
84. GINS4	0.143	**84. SEH1L**	0.155
85. SNAPC3	0.142	**85. YWHAE**	0.153
86. SNAPC4	0.142	**86. DCTN1**	0.150
87. SGOL2	0.142	**87. PSMA2**	0.150
88. PLK4	0.141	**88. HSP90AA1**	0.149

89.	**ANAPC7**	0.141
90.	**MIS18A**	0.139
91.	**PSMA7**	0.139
92.	**CDK2**	0.138
93.	**ZW10**	0.138
94.	**KIF18A**	0.138
95.	**CDCA8**	0.136
96.	**CCDC99**	0.135
97.	**PRIM2**	0.134
98.	**CEP41**	0.134
99.	**TFAM**	0.134
100.	**CDC20**	0.132
101.	**POLR3H**	0.129
102.	**CEP76**	0.126
103.	**POLA1**	0.126
104.	**AURKA**	0.125
105.	**BIRC5**	0.124
106.	**SEC13**	0.124
107.	**CEP63**	0.123
108.	**SMC1A**	0.123
109.	**POLR1D**	0.122
110.	**BRF2**	0.119
111.	**KIF2A**	0.117
112.	**PSMB7**	0.117
113.	**B9D2**	0.117
114.	**SPC24**	0.117
115.	**MAX**	0.116
116.	**STX1A**	0.116
117.	**CEP164**	0.115
118.	**SMARCA5**	0.115
119.	**CDKN2A**	0.115
120.	**PLK1**	0.115
121.	**ORC3**	0.115
122.	**GINS1**	0.115
123.	**CDC26**	0.113
124.	**SYNE1**	0.112
125.	**RAD17**	0.112
126.	**DSN1**	0.111
127.	**HIST1H2A**	0.110
128.	**POLR3GL**	0.110
129.	**CDK7**	0.109
130.	**RPS27**	0.108
131.	**PSMD3**	0.107
132.	**PPP2CB**	0.107
133.	**CASC5**	0.107
134.	**PSMD1**	0.106
135.	**MYBL2**	0.106
136.	**ORC6**	0.105

89.	**NUP37**	0.148
90.	**SPC25**	0.147
91.	**KIF2A**	0.143
92.	**TERF2**	0.141
93.	**GINS1**	0.141
94.	**PAK2**	0.132
95.	**ERCC6L**	0.127
96.	**SYNE1**	0.120
97.	**MAX**	0.116
98.	**PSMD5**	0.112
99.	**CDC16**	0.106
100.	**PSMA5**	0.104
101.	**DYNC1H1**	0.103
102.	**MAPRE1**	0.101

137.NDC80	0.104
138.SSNA1	0.104
139.PSMD12	0.103
140.SSB	0.101
141.MCM2	0.100
142.MCM8	0.100

Supplementary Table S4. Differential sensitivity of group 3 cell lines to CDK inhibitors dinaciclib and flavopiridol. IC50 values for selected cell lines from Group 1 (Akt-i-resistant), Group 2 (MEK-i-resistant) and Group 3 (Akt-i/MEK-i double-resistant) for pan-CDK inhibitors dinaciclib and flavopiridol. IC50 values were calculated using non-linear regression fitting in GraphPad Prism.

	Group 1 (Akt-i-resistant)		**Group 2 (MEK-i-resistant)**		**Group 3 (Akt-i/MEK-i-resistant)**		
IC50 (μM)	HCC1806	MDA-MB-435s	BT20	HCC70	SKBR7	MDA-MB-436	SUM159PT
Dinaciclib	0.2973	0.3121	0.2069	0.1371	0.1465	0.1095	0.1543
Flavopiridol	0.4887	0.2758	0.149	0.1246	0.09521	0.1775	0.1768