doi:10.1038/nature11183

Supplementary figure 1 - An Overview of Experimental Flow

Hypothesis: The tumor microenvironment has a significant impact on cancer cell chemoresistance.

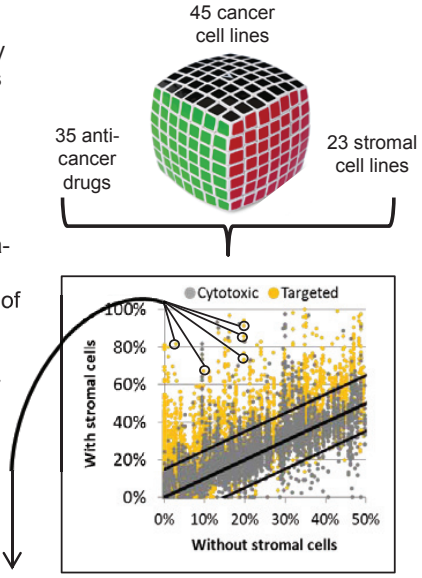
1 Screen

Co-culture assay for combinations that display rescue phenotype

↓

Identified stroma-mediated resistance to 16 of 35 drugs.

Follow fibroblast rescue from BRAFi in Melanoma BRAF V600E cell lines

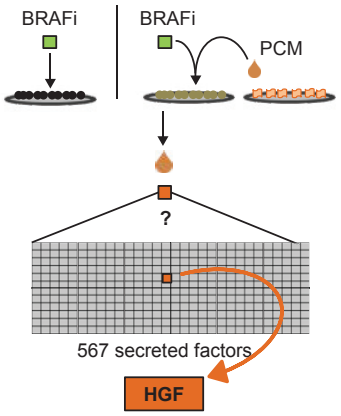


2 Secreted factor or cell-cell contact?

Stromal preconditioned media (PCM) sufficient for resistance
→ **secreted factor**

3 Identification of secreted factor

Screen 567 stromal factors
→ **HGF** responsible for rescue.

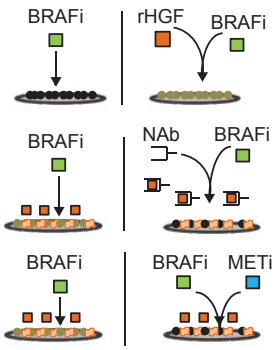


4 Validation

a. rHGF sufficient for rescue

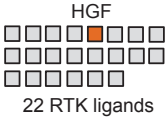
b. Neutralizing Ab abrogates stromal rescue

c. METi abrogates stromal rescue



5 Can other ligands confer resistance?

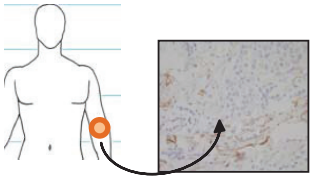
Screen 22 RTK ligands
→ No other tested ligand can rescue melanoma.



6 In vivo validation

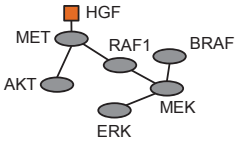
a. Stromal HGF, MET, and pMET detected in melanoma tumors.

b. Stromal HGF found to be correlated with max response to treatment



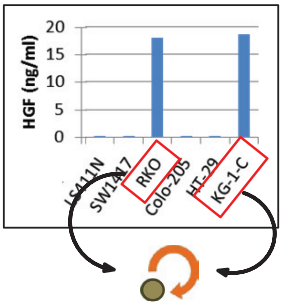
7 Mechanism

Western blots characterize mechanism of action of HGF/MET mediated rescue.



8 HGF secretion in other BRAF V600E cancers?

ELISA identifies autocrine HGF secretion in *BRAF* V600E CRC and Glioma cell lines

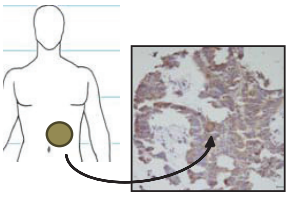


9 Validation

Co-treatment with BRAFi and METi abrogates resistance in HGF secreting cancer cell lines

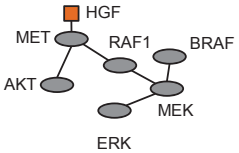
10 In vivo validation

IHC of CRC tumor samples confirm HGF expression in colorectal tumors



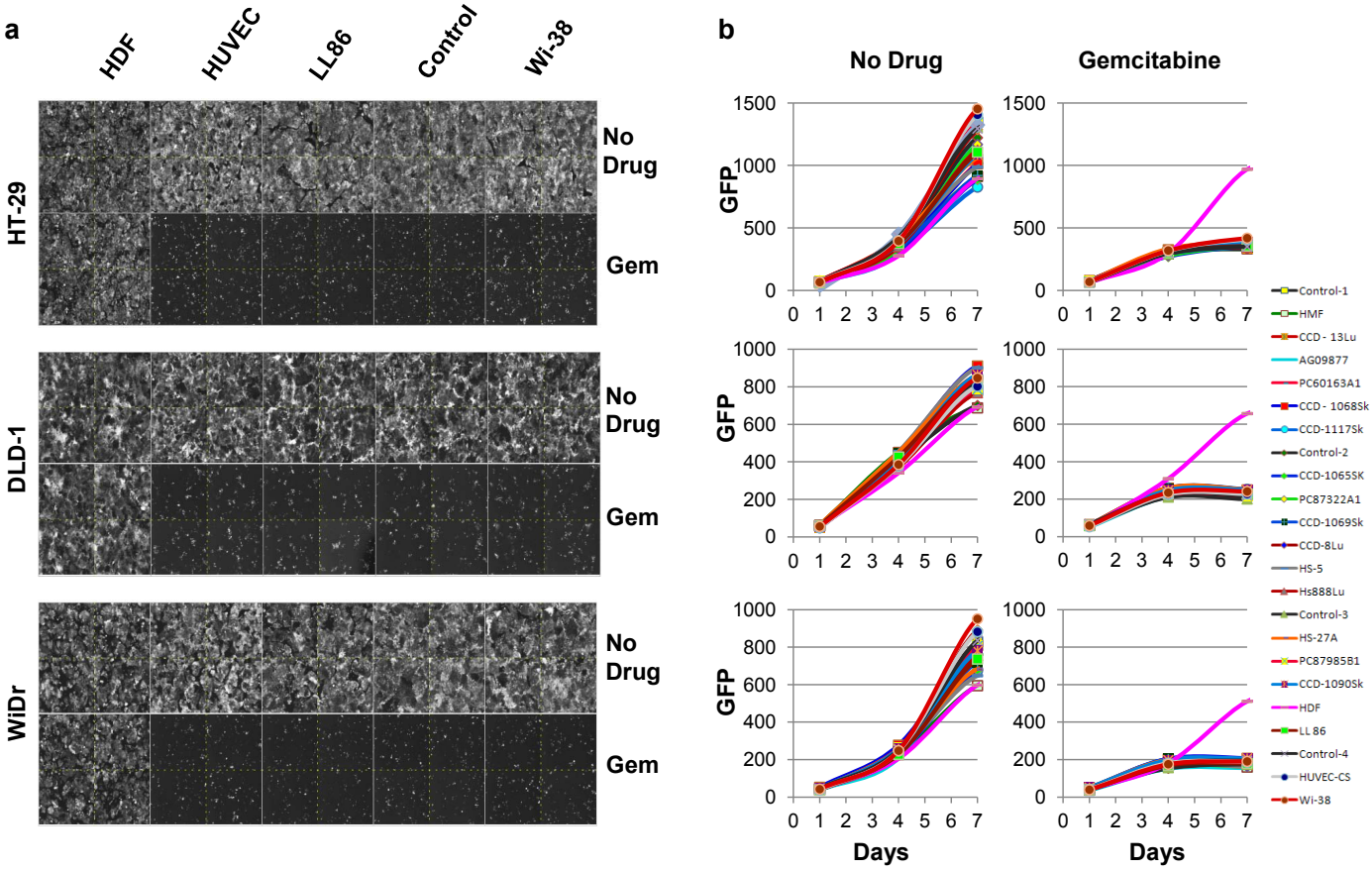
11 Mechanism

Characterization of mechanism of action of HGF/MET rescue found to be similar to melanoma.

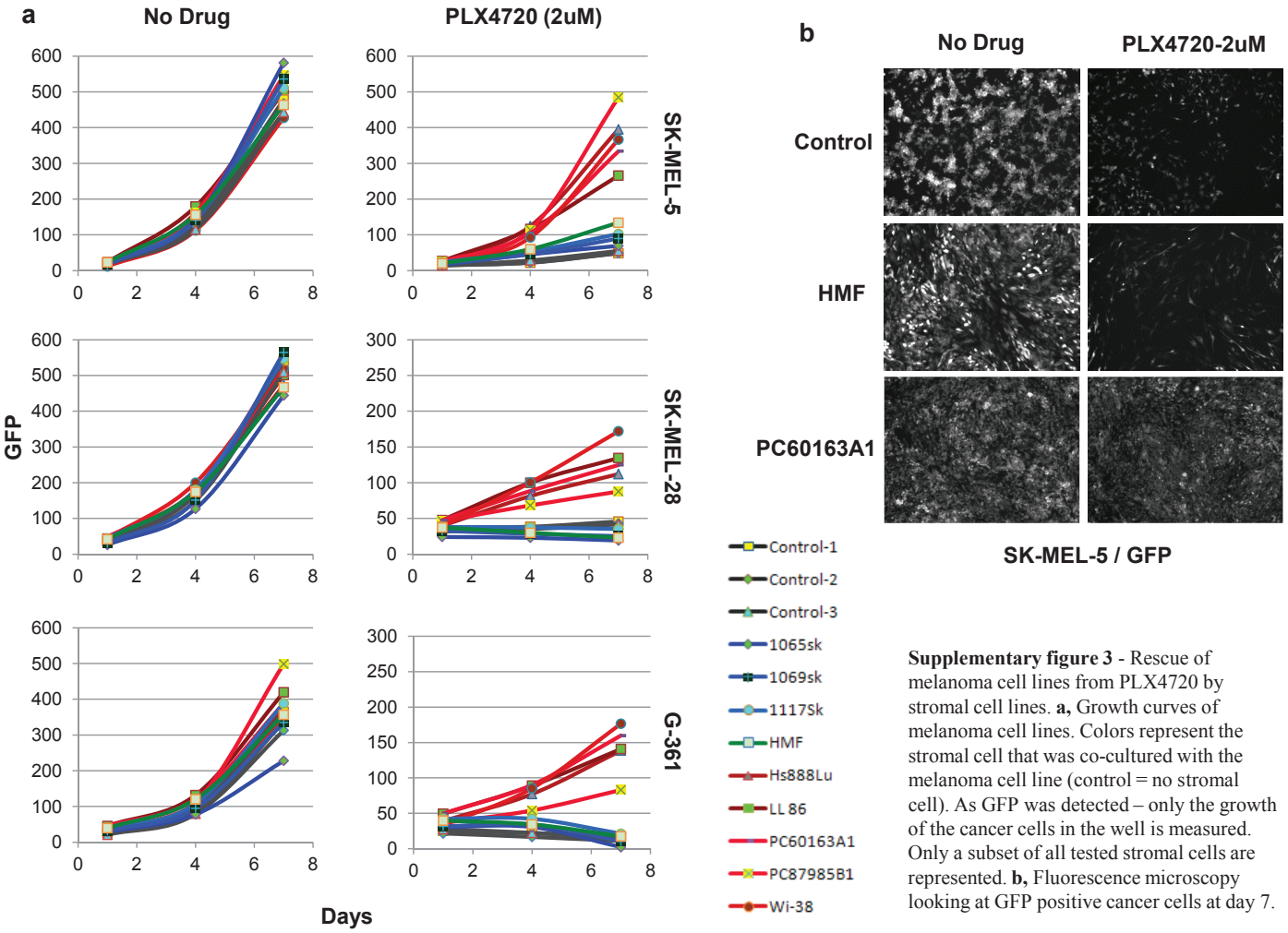


Legend:

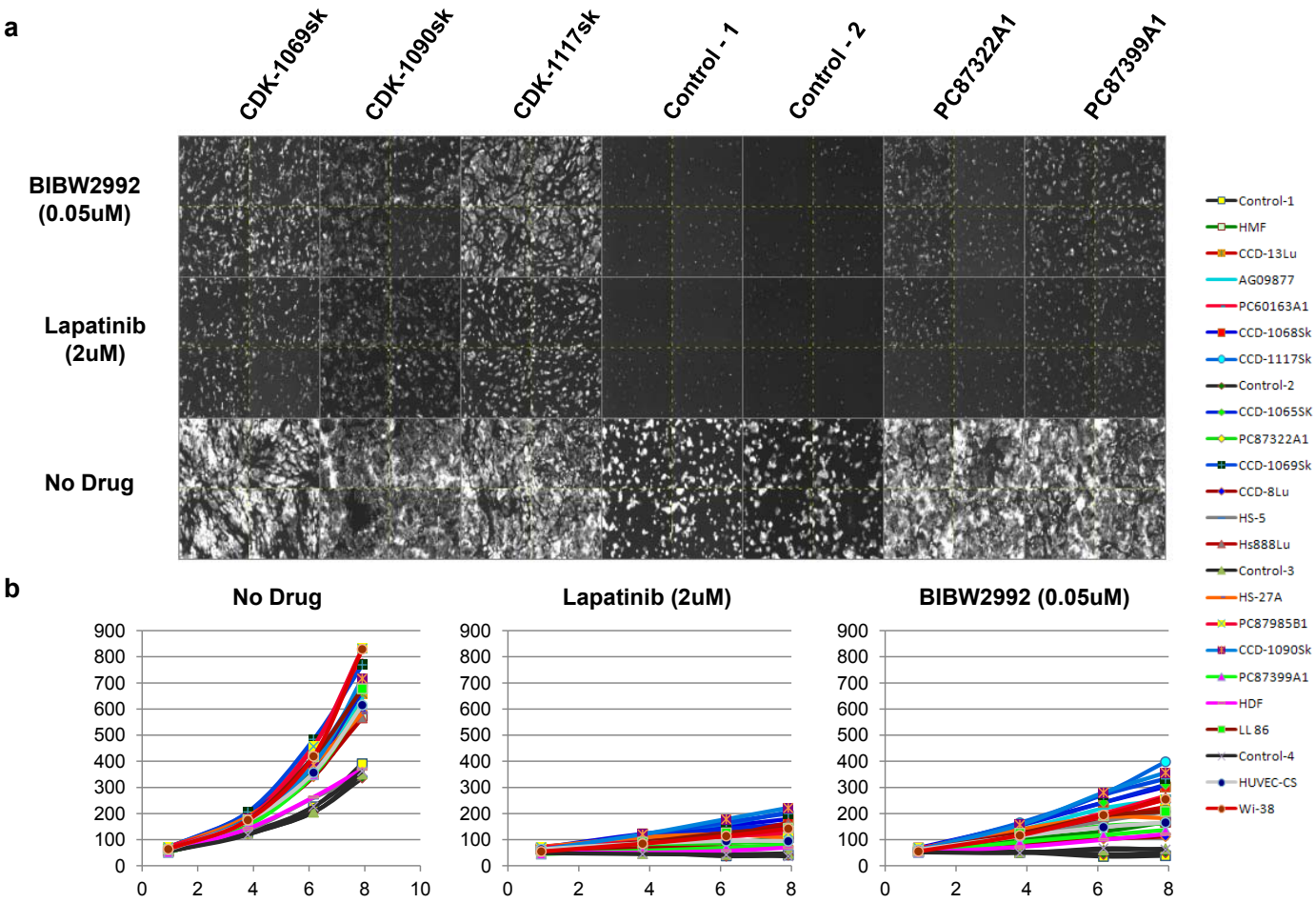
- Alive cancer cell
- Dead cancer cell
- Stromal cell
- HGF, rHGF
- BRAFi - PLX4720
- METi - Crizotinib
- Ligand, cytokine or other secreted factor



Supplementary figure 2 - Rescue of colorectal cancer cell lines from gemcitabine (Gem) by HDF stromal cells. HT-29 was treated with 0.02uM Gem while DLD-1 and RKO cells were treated with 0.1uM Gem. **a,** Fluorescence microscopy looking at GFP positive cancer cells at day 7. All 4 quadruplicate wells are shown. **b,** Growth curves of cancer cell lines. Color represent the stromal cell that was co-cultured with the cancer cell line (control = no stromal cell). As GFP was detected – only the growth of the cancer cells in the well is measured.

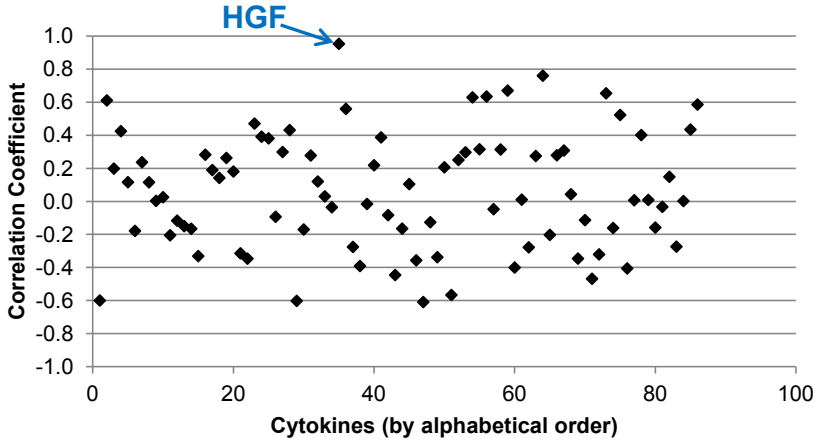


Supplementary figure 3 - Rescue of melanoma cell lines from PLX4720 by stromal cell lines. **a,** Growth curves of melanoma cell lines. Colors represent the stromal cell that was co-cultured with the melanoma cell line (control = no stromal cell). As GFP was detected – only the growth of the cancer cells in the well is measured. Only a subset of all tested stromal cells are represented. **b,** Fluorescence microscopy looking at GFP positive cancer cells at day 7.



Supplementary figure 4 - Rescue of the breast cancer cell line EFM192A from lapatinib and BIBW2992 by stromal cells. **a,** Fluorescence microscopy looking at GFP positive cancer cells at day 7. All 4 quadruplicate wells are shown. **b,** Growth curves of EFM192A. Color represent the stromal cell that was co-cultured with the cancer cell line (control = no stromal cell). As GFP was detected – only the growth of the cancer cells in the well is measured.

a



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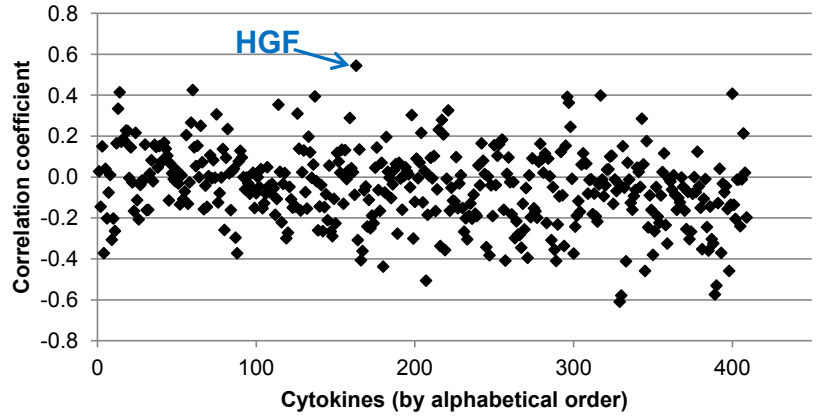
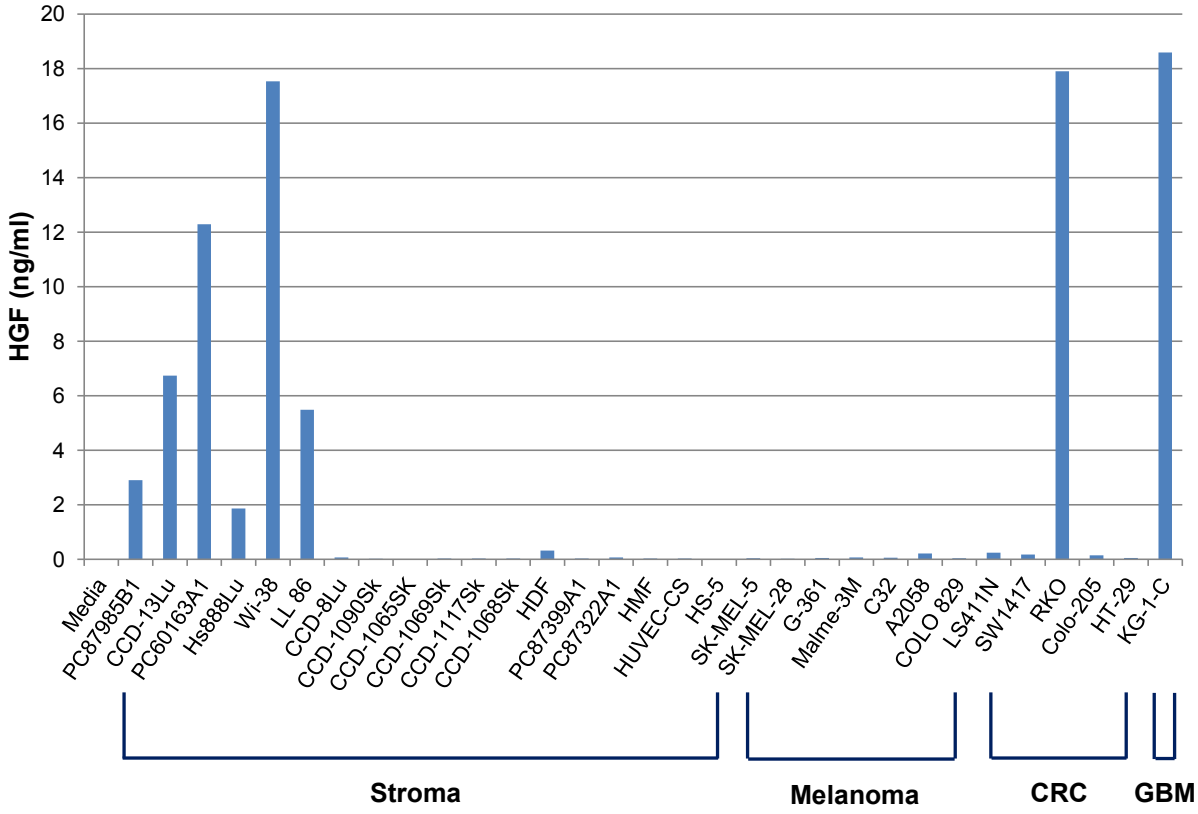


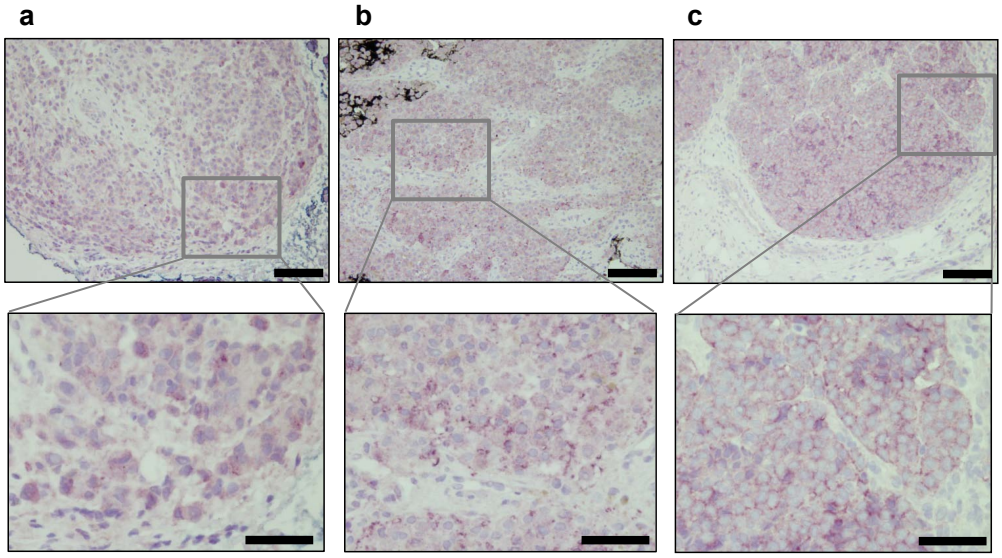
c

Stromal cell line	Rescue score
LL 86 *	0.35
Wi-38 *	0.33
PC60163A1 *	0.31
Hs888Lu *	0.24
CCD-13Lu *	0.23
PC87985B1 *	0.21
CCD-8Lu	0.07
HS-5	0.06
CCD-1068Sk	0.06
HDF	0.05
HUVEC-CS	0.05
PC87399A1	0.05
PC87322A1	0.05
CCD-1090Sk	0.04
CCD-1117Sk	0.04
CCD-1065Sk	0.04
HMF	0.03
CCD-1069Sk	0.02

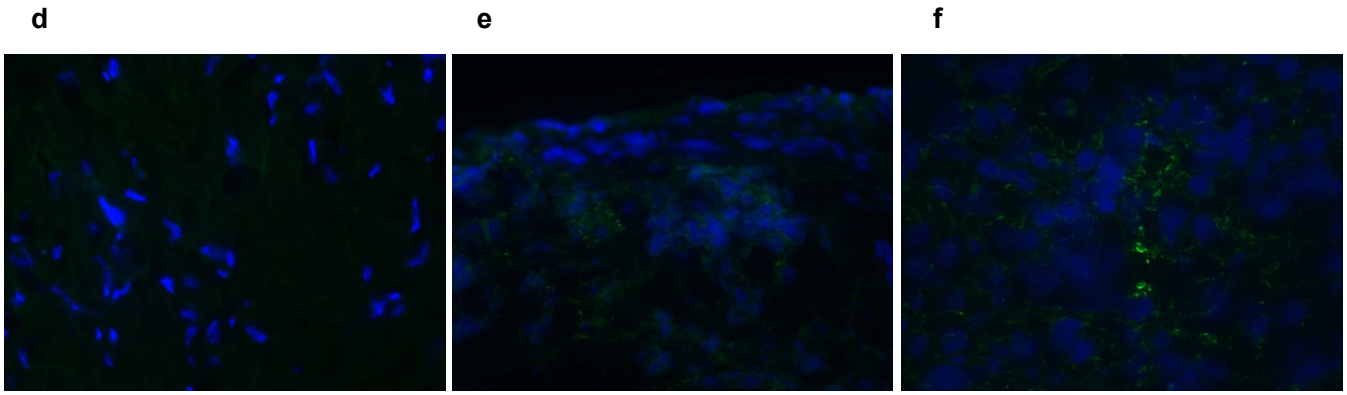
Supplementary figure 5 – Correlations between proteins secreted by stromal cells and stromal average melanoma rescue scores. Two types of antibody arrays were used to measure proteins secreted into the media by 18 stromal cells (Supplementary tables 4 and 5). The arrays measure 274 and 507 secreted proteins (214 proteins are measured by both) but only 86 (**a**) and 409 (**b**) cytokines, respectively, scored as positive (>50) in at least one stromal cell line. A correlation coefficient was calculated for each cytokine between the log2 of its secretion level by all 18 stromal cell lines (supplementary tables 4 and 5) and the average melanoma rescue score of each of these cell lines (see supplementary methods) (**c**). HGF had the highest correlation coefficient in both arrays. Cell lines that were found to secret HGF are labeled with asterisks (**c**).



Supplementary Figure 6 – HGF ELISA was done using media from stromal cell lines and *BRAF* V600E mutated cancer cell lines. CRC = Colorectal cancer. GBM = Glioblastoma multiforme.



Supplementary figure 7 – MET and pMET in melanomas. **a-c,** Melanoma sections analyzed for c-MET expression by immunohistochemistry (red staining). Sections represented are from patient #33 (pre-treatment) **(a)**, #25 (on treatment) **(b)**, and #27 (on treatment) **(c)**. Low magnification images are shown on top (scale bar - 100µm) while large magnification images are shown on the bottom (Scale bar - 50µm). **d-e,** Fresh frozen tissue sections were analyzed for phospho-MET by Immunofluorescence (green). DAPI staining in blue. Sections represented are from patient #1 (on treatment) **(d)**, #18 (on treatment) **(e)**, and #27 (on treatment) **(f)**. Note that patient #1 had a complete response to therapy and was negative for stromal HGF while patients #18 and #27 had partial responses and were positive for stromal HGF.

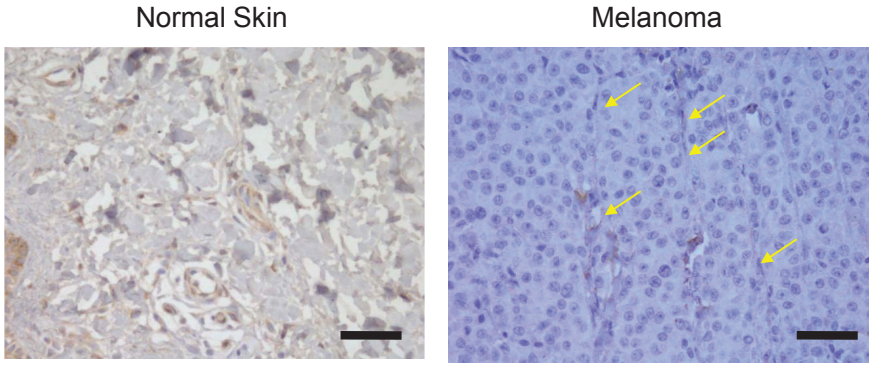


a

	BRAF status	Stromal HGF		
		Negative	Positive	% Positive
Normal skin	#N/A	23	17	43%
Nevi	#N/A	13	7	35%
Melanoma	#N/A	48	33	41%
Colorectal CA	All	547	46	7.8%
	WT	454	37	7.5%
	V600E	85	8	8.6%

Two sided P-value by Fisher's exact Test: 2.4×10^{-13}

b



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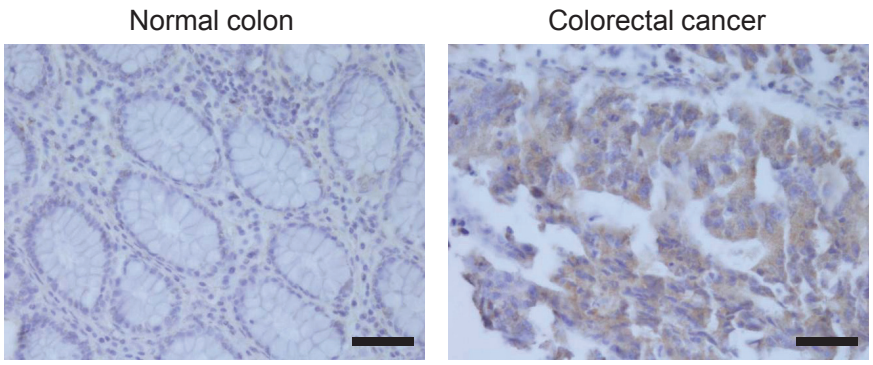
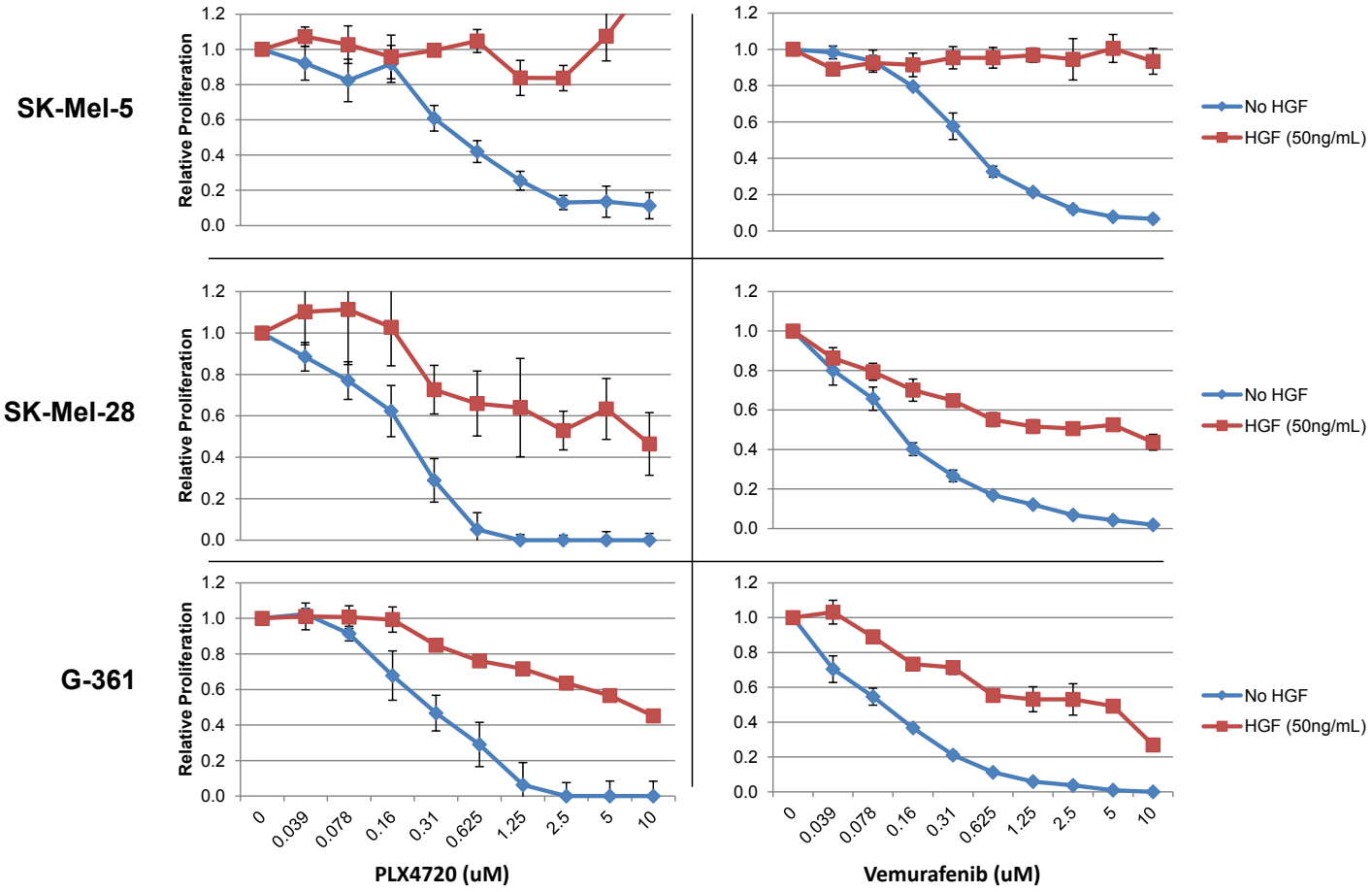
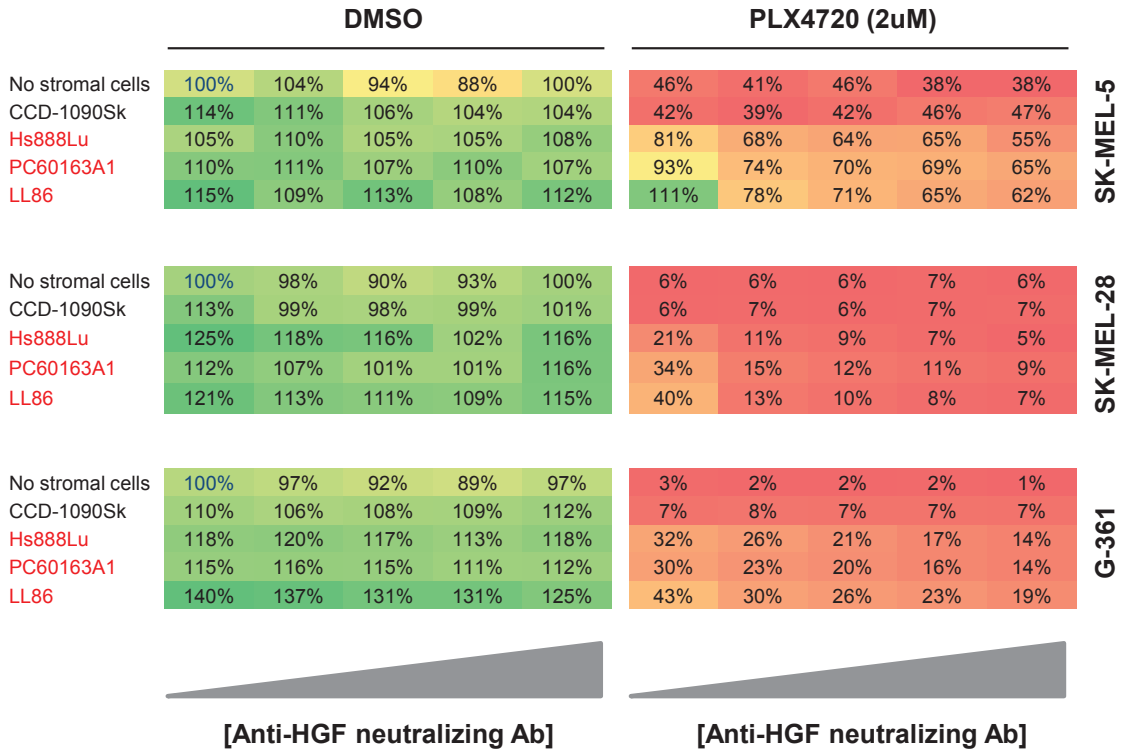


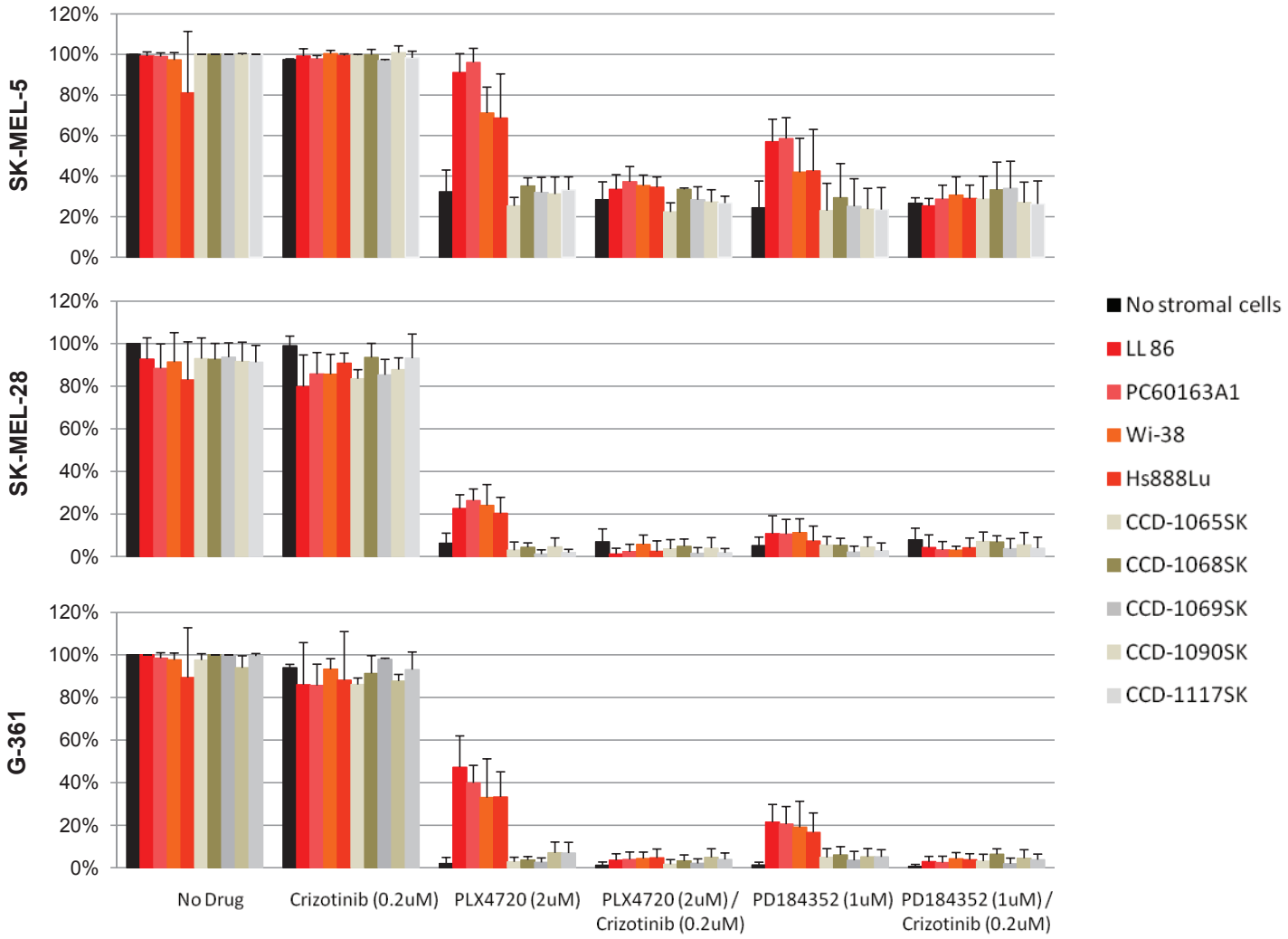
Supplementary Figure 8 – Tissue micro array (TMA) analysis for stromal HGF. **a**, Summary table of TMA sections analyzed for stromal HGF expression by immunohistochemistry (IHC) **b**, HGF expression was detected in normal skin and melanoma stromal cell. Yellow arrows point to stromal HGF. (Scale bar - 50μm). **c**, HGF expression was negative in normal colon and positive in colorectal cancer (*BRAF* V600E) sections. (Scale bar - 50μm).



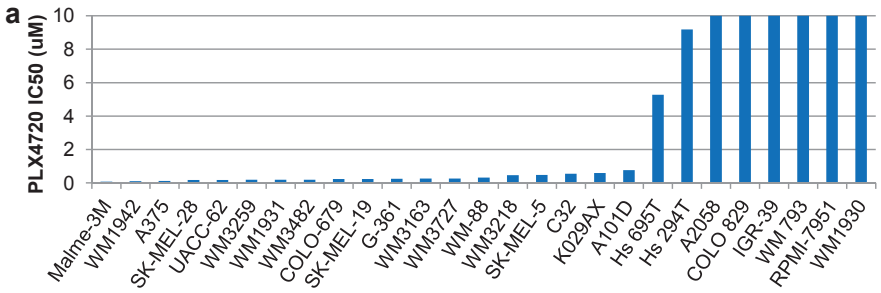
Supplementary Figure 9 – The effect of 50ng/ml HGF on the sensitivity of melanoma cell lines to the BRAF inhibitors PLX4720 and vemurafinib. Results were normalized to no drug and averaged. Bars represent standard error between replicates (n = 3).

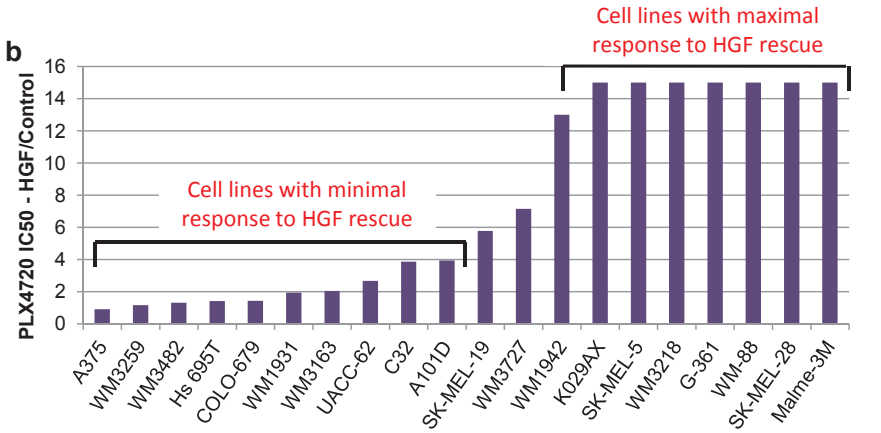


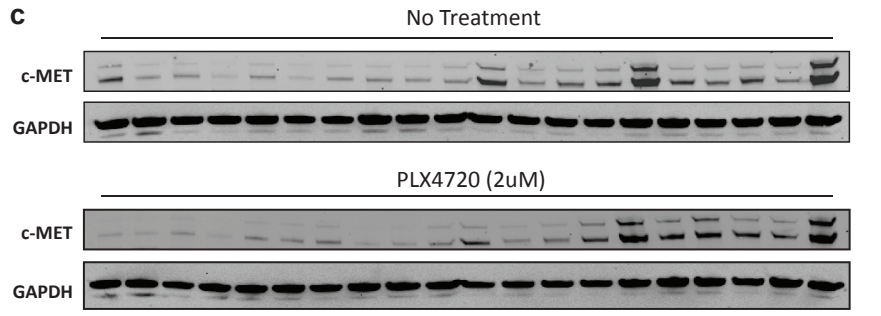
Supplementary Figure 10 - The effect of anti-HGF neutralizing antibodies on stromal cells rescue. Melanoma cell lines were co-cultured with stromal cell lines or control media and treated with PLX4720 (2uM) or DMSO control. Stromal cells that secrete HGF are marked in red. Neutralizing anti-HGF antibodies (0, 0.44, 1.33, 4 or 12 ug/ml) were added on days 0 and 4. Proliferation rate was normalized to samples with no stromal cells, no drug and no antibody (marked in blue). Average results from 3 experiments are presented.



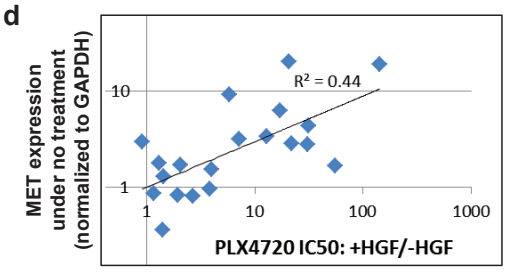
Supplementary Figure 11 - Melanoma cell lines were co-cultured with stromal cell lines and treated with PLX4720 (2uM) or PD184352 (1um) with or without 0.2uM of crizotinib. Proliferation was quantified after 7 days and normalized to non-treated cells. Bars represent standard error between replicates (n = 3).

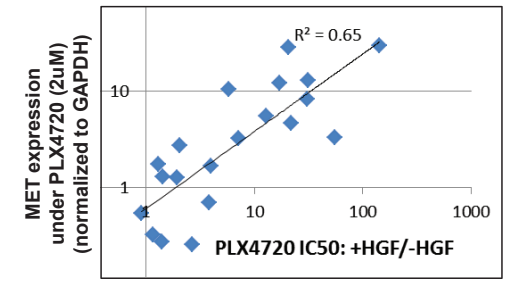




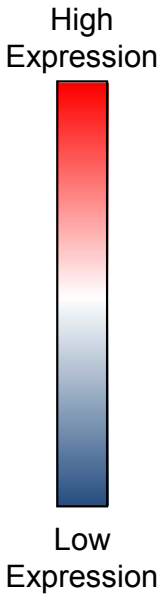


Supplementary figure 12 – Correlation between MET expression and HGF-mediated resistance to PLX4720. **a,** The IC50s of 27 V600E *BRAF* melanoma cell lines were generated from a 10-point PLX4720 concentration range using CellTiter Glo readout after 72 hours of treatment. IC50s > 10 are represented as 10 **b,** 20 cell lines with IC50 < 6uM (**a**) were selected for further evaluation. The IC50s of these 20 cell lines were generated as above with or without the presence of 50ng/ml HGF. The calculated IC50s +HGF divided by the IC50s -HGF are represented. Ratios > 15 are represented as 15. **c**, Levels of total MET were evaluated in the 20 selected cell lines before or 24h' after treating the cells with 2uM of PLX4720. **d,** The correlation between PLX4720 IC50 (+HGF/-HGF) (**b**) and c-MET expression (**c**) is represented.

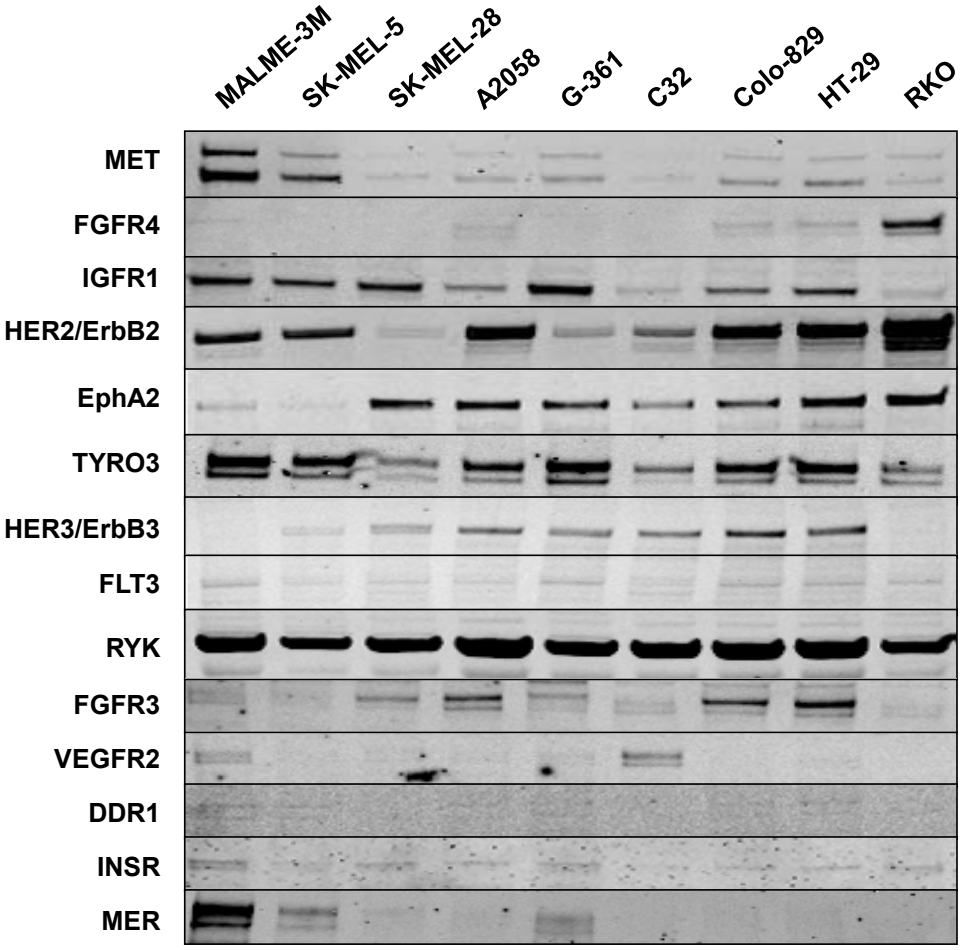




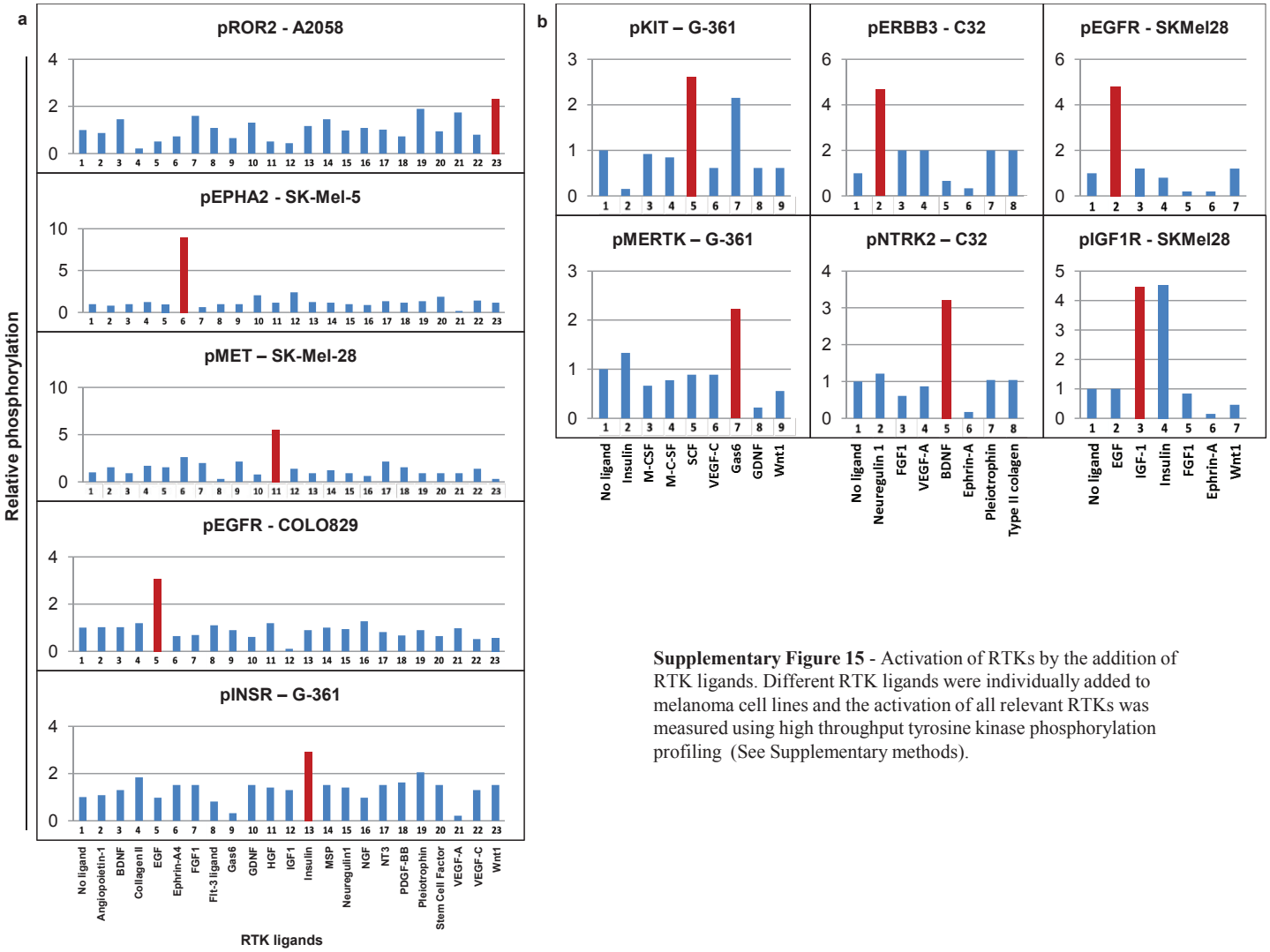
RTK	RTK Class	A2058	C32	COLO829	G-361	SKMEL28	SKMEL5	MALME3M	Ligand
EGFR	1								EGF
ERBB3	1	4688.4	3895.4	3065.6	1689.2	1382.5	3194.5	5694.7	Neuroregulin
ERBB4	1		30.8				13.0	15.1	Neuroregulin
IGF1R	2	585.2	1167.3	1248.6	1580.1	1302.6	689.2	1016.8	IGF-1
INSR	2		215.3	318.3	113.8	152.0	152.8	107.5	Insulin
KIT	3				51.0			988.8	SCF
CSF1R	3	19.5			17.4			18.3	M-CSF
PDGFRA	3	17.6							PDGF-BB
PDGFRB	3								PDGF-BB
FGFR1	4	97.1	140.9	129.1	103.9	134.6	224.1	245.5	FGF-1
FGFR2	4	55.7	56.1	42.4	85.3	53.2	42.1	43.1	FGF-1
FGFR3	4	77.1	206.3			38.9			FGF-1
FLT1	5	87.2	114.0	180.8	76.3	100.4	98.2	74.4	VEGF-A
KDR	5	24.1	297.2	897.5	44.7	17.3	64.4	54.8	VEGF-A
FLT4	5								VEGF-C
MET	6	1193.7	1458.6	4325.5	1686.0	686.6	2309.2	1721.2	HGF
MST1R	6								MSP
NTRK1	7	18.2							NGF
NTRK2	7	29.3	40.3	31.9				18.8	BDNF
NTRK3	7			13.8				25.4	NT3
EPHA2	8	268.4		135.5				136.0	Ephrin-A
EPHA3	8	713.8	291.7	193.4	389.3	34.5	583.4	83.3	Ephrin-A
EPHA4	8	337.2		14.4	518.9	377.0	219.6	291.8	Ephrin-A
AXL	9	1643.7	196.5	1629.6	44.5	46.7		1264.3	Gas6
MERTK	9	38.0	20.4	110.9	1116.4	83.3	1803.9	544.4	Gas6
TYRO3	9	248.8	157.8	195.1	375.8	181.6	574.3	307.7	Gas6
ALK	10	31.5	54.3	30.0		51.3	23.3	59.6	pleiotrophin
ROR1	12	203.4	52.5	85.5	105.7	96.6	74.2	85.3	Wnt1
ROR2	12								Wnt1
DDR1	13	1264.7	1161.7	1128.8	590.1	126.0	665.7	1153.4	Type II colagen
DDR2	13	612.3	939.5	393.9	71.3	400.5	249.3	183.2	Type II colagen
RET	14	14.1							GDNF
RYK	16	670.7	915.4	892.3	860.5	1495.4	634.5	699.6	Wnt1



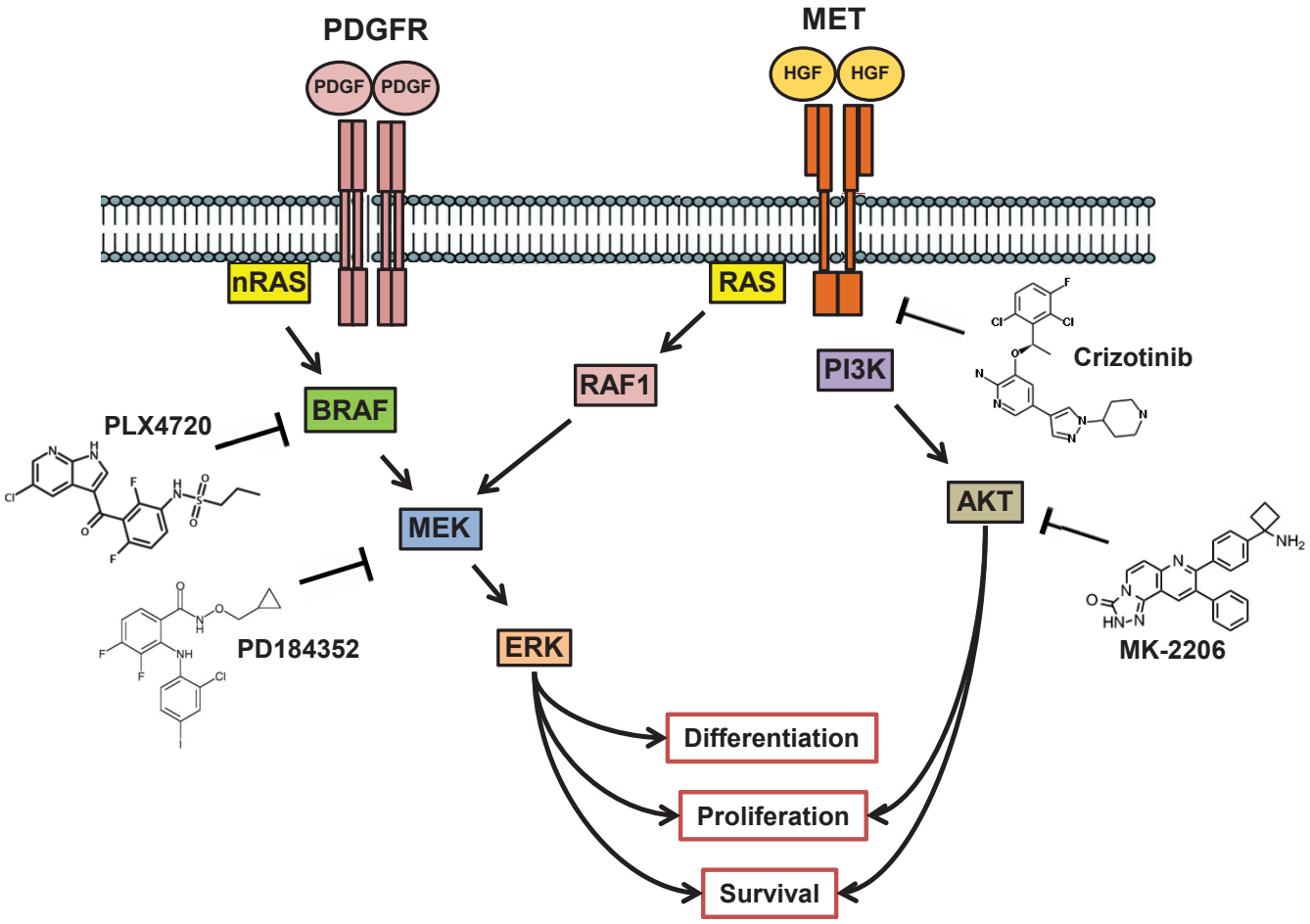
Supplementary Figure 13 – Expression of RTKs in 7 melanoma cell lines. Data from expression arrays was provided by the Cancer cell line encyclopedia (http://www.broadinstitute.org/ccle/home).



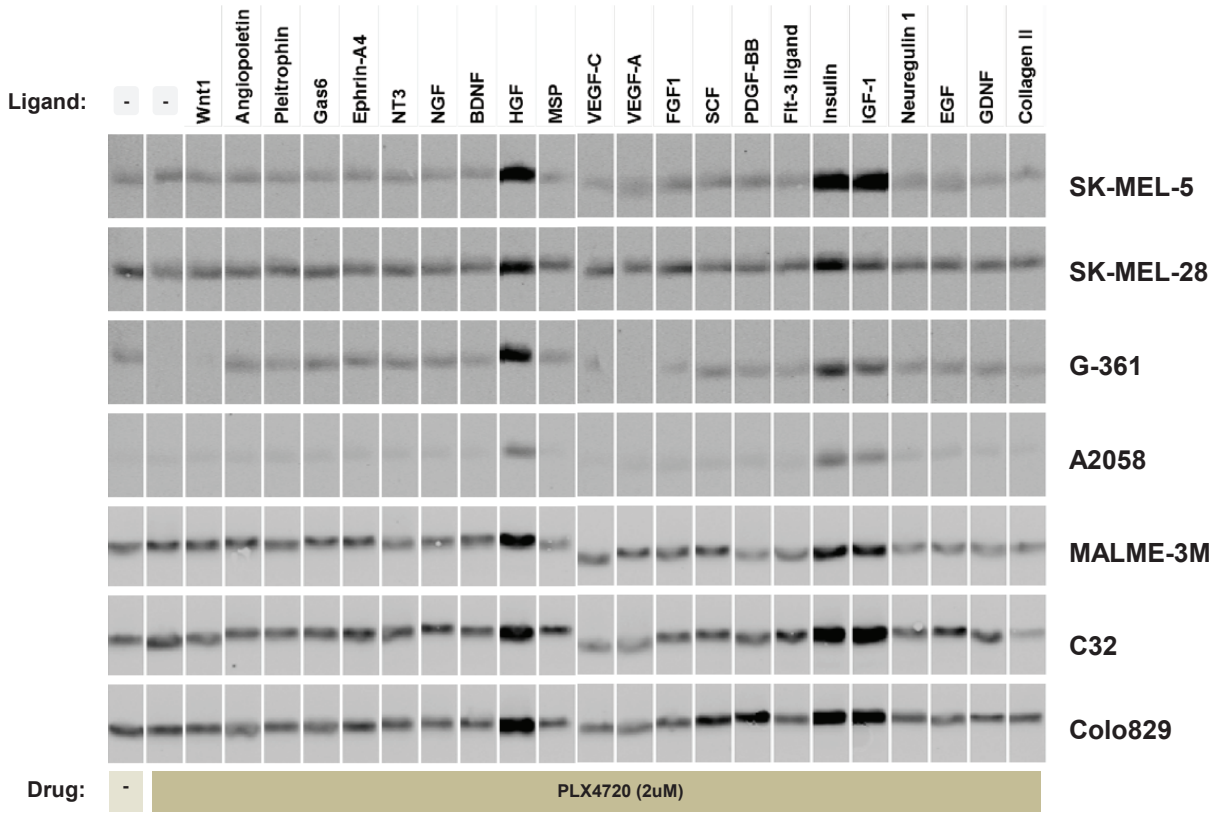
Supplementary Figure 14 - Expression of receptor tyrosine kinases (RTKs) in melanoma and colorectal cancer cell lines by Western blot analysis.



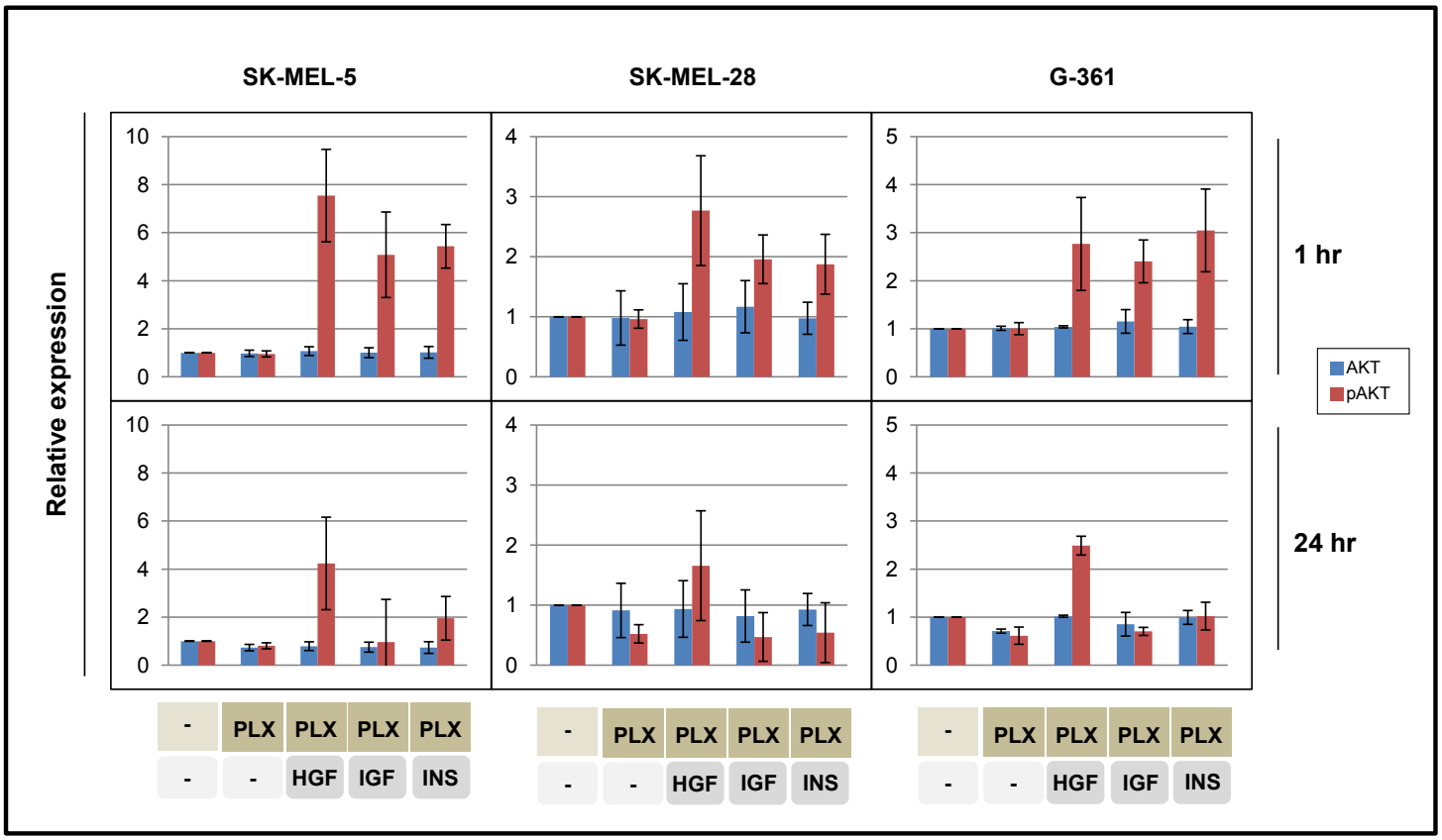
Supplementary Figure 15 - Activation of RTKs by the addition of RTK ligands. Different RTK ligands were individually added to melanoma cell lines and the activation of all relevant RTKs was measured using high throughput tyrosine kinase phosphorylation profiling (See Supplementary methods).



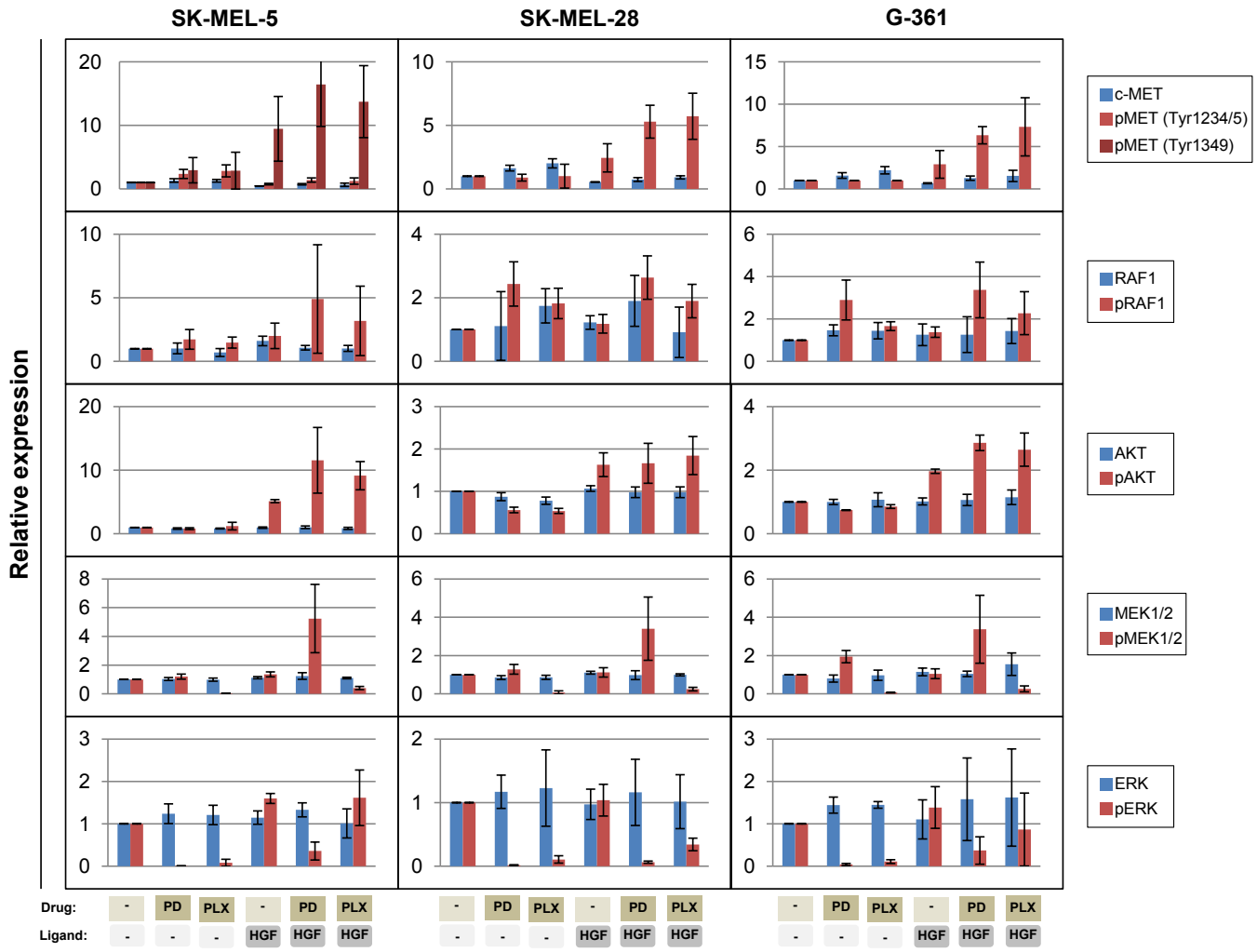
Supplementary Figure 16 - Model diagram



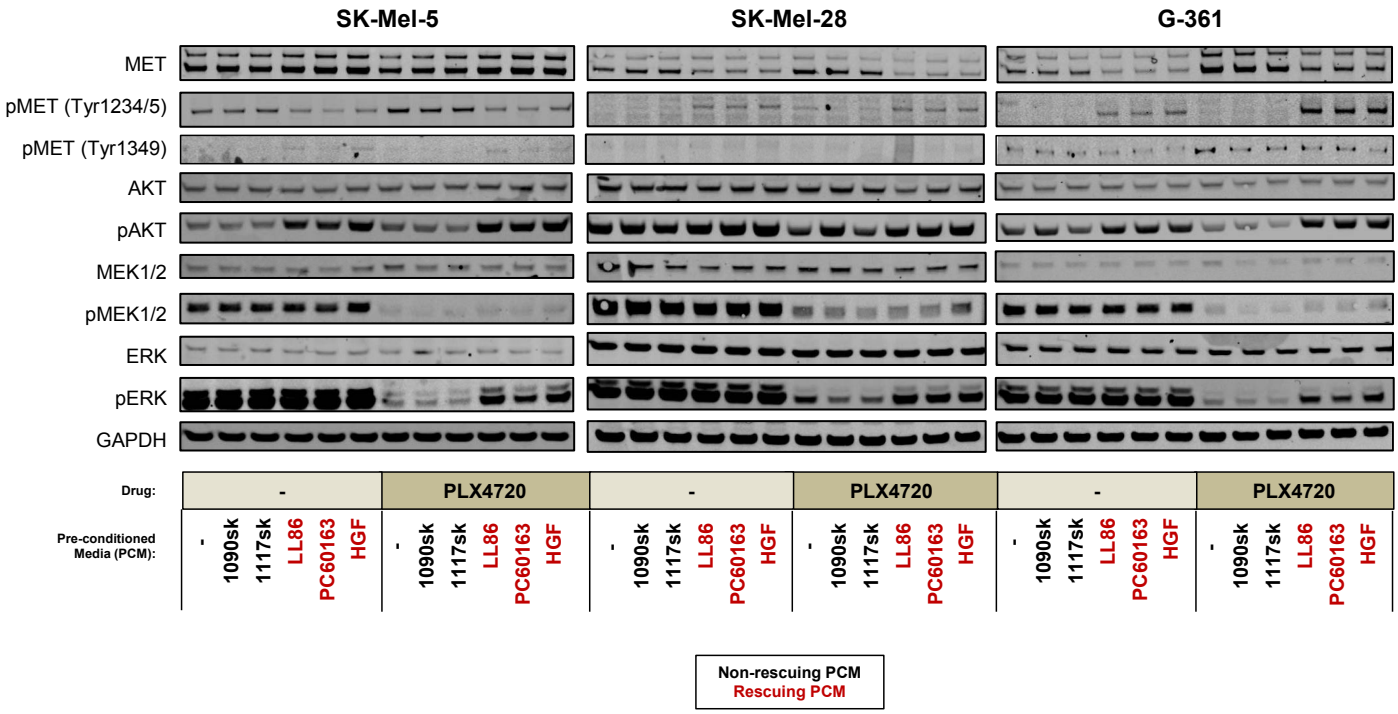
Supplementary figure 17 – Activation of AKT by cytokines. Levels of phosphorylated AKT (Ser473) were assayed 1 hour after treatment with media (-) or with 22 cytokines in the presence of 2uM PLX4720 or DMSO as a negative control.



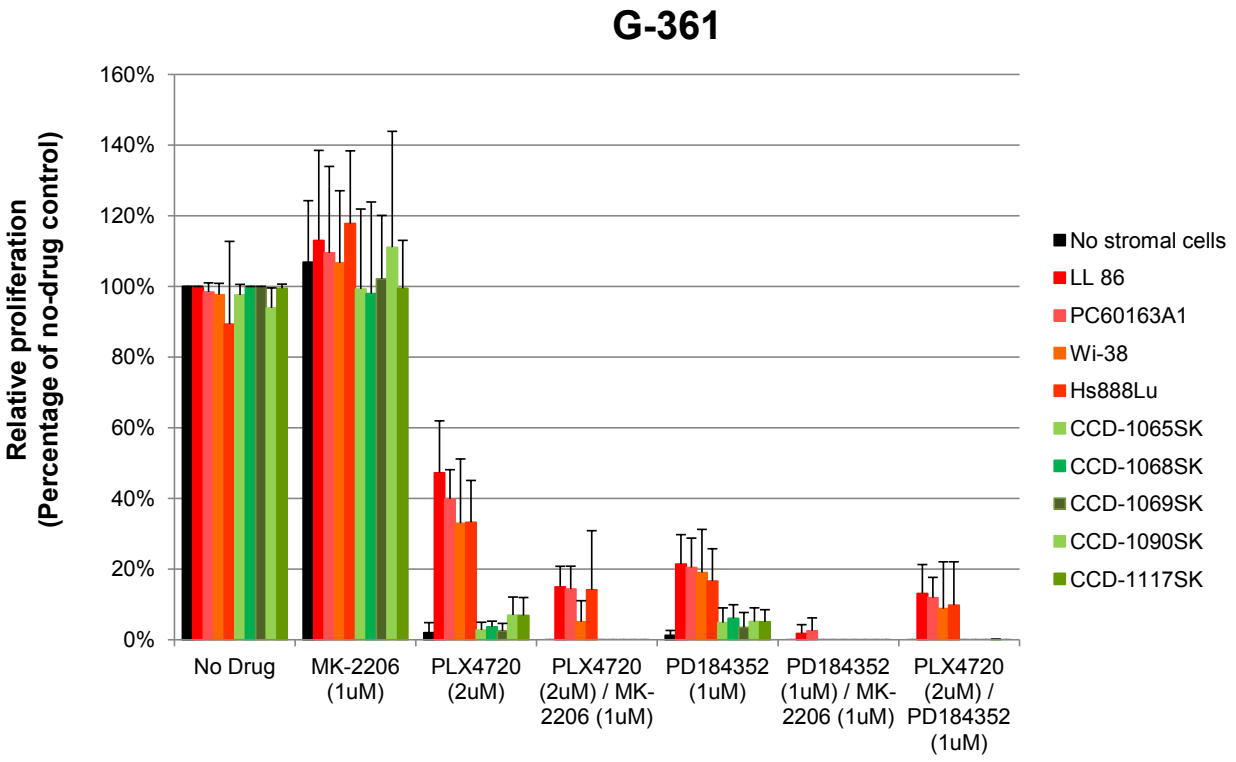
Supplementary Figure 18 – The activation of AKT by HGF, IGF-1 (IGF), and Insulin (INS). Levels of phosphorylated AKT were assayed by immunoblot analysis 1 hour and 24 hours after treatment with HGF, IGF-1, or Insulin in the presence of PLX4720 (2uM) (Figure 4b). Bars represent standard error between replicates (n = 3).



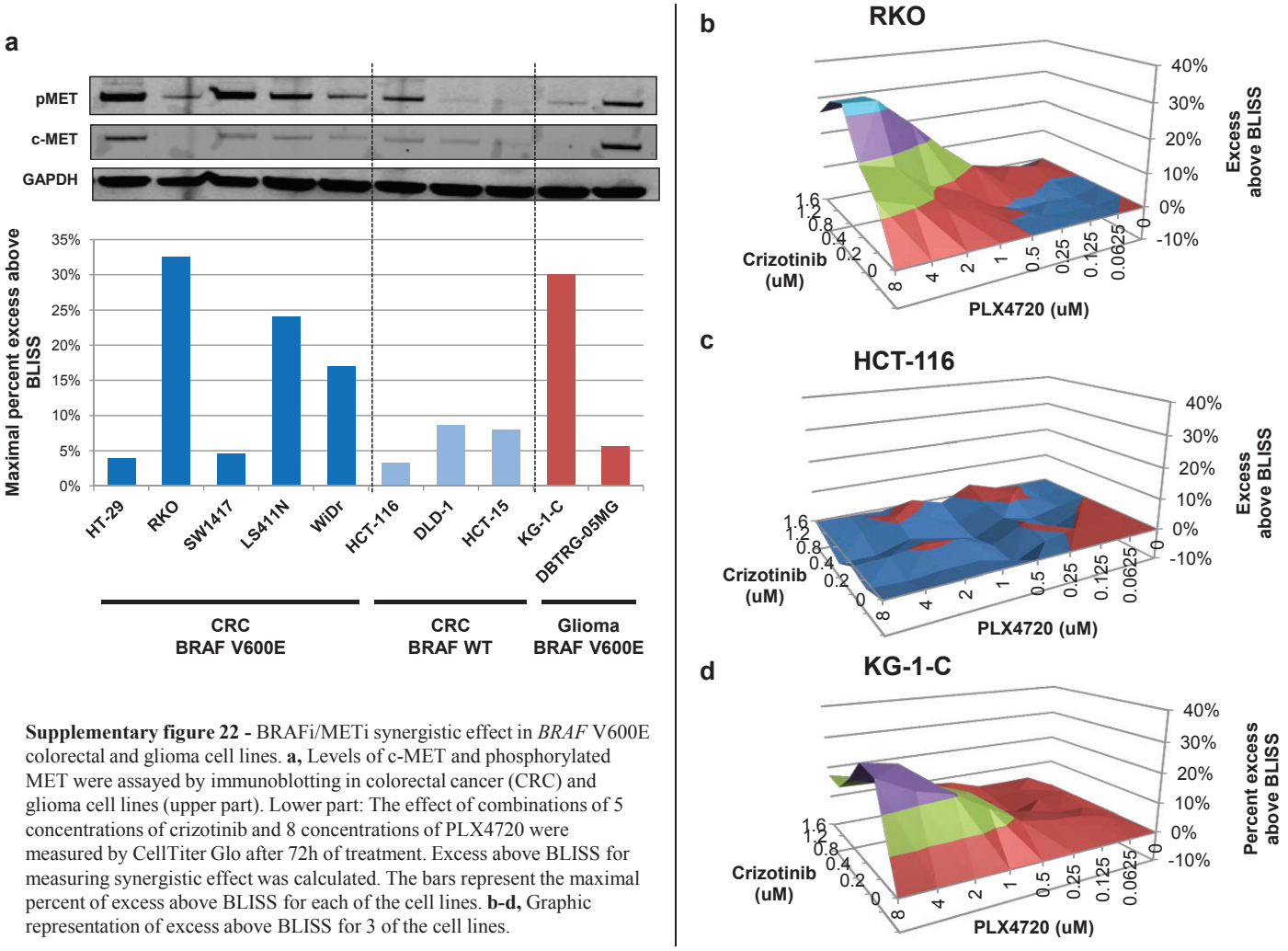
Supplementary Figure 19 - Effect of HGF (25ng/ml) on the treatment of melanoma cell lines with 2uM PLX4720 or 2uM PD184352. MAPK and PI3K/AKT pathways activation was assessed after 24 hours of treatment by immunoblot analysis of pRAF1, pMEK, pERK and pAKT. pMET levels were also assessed. Bars represent standard error between replicates (n = 3).



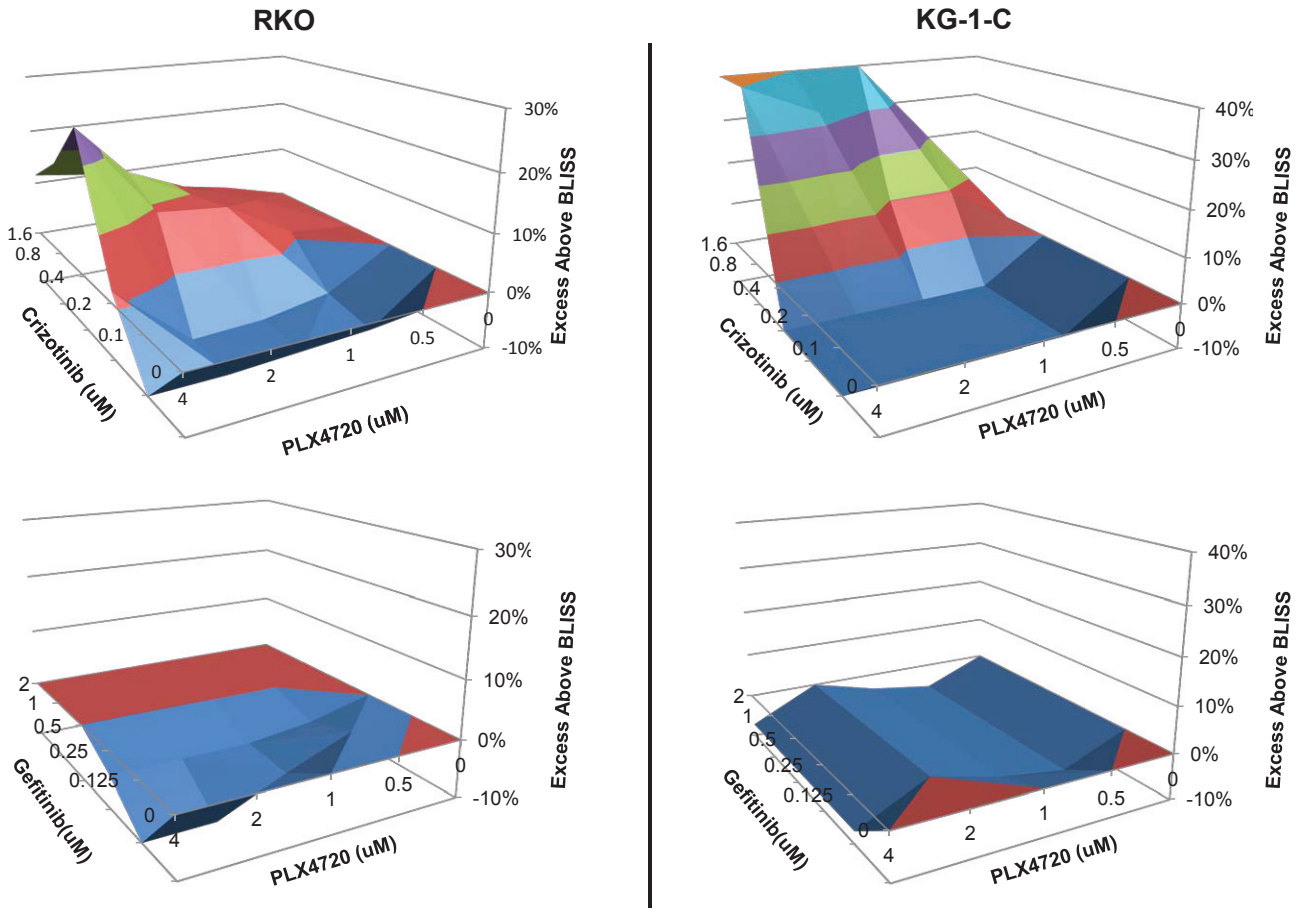
Supplementary Figure 20 - The effect of stromal PCM on MAPK and PI3K pathway activation under 2uM PLX4720 treatment. MAPK and PI3K/AKT pathway activation was assessed after 24 hours of treatment by immunoblot analysis of MET, AKT, MEK, ERK and their respective phosphorylation status. PCM from stromal cells that do not rescue the melanoma cell lines from PLX4720 is labeled in black while PCM from stromal cells that can rescue is labeled in red.



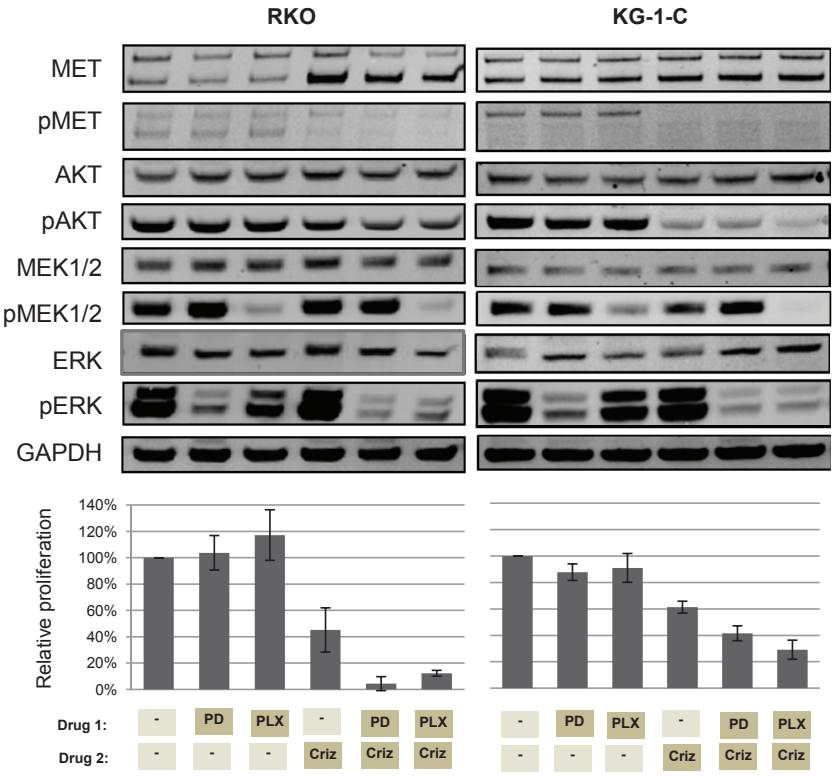
Supplementary figure 21 – Synergistic effect of AKT inhibition with BRAF or MEK inhibitors. Melanoma cell lines were co-cultured with stromal cell lines and treated with PLX4720 (2uM) or PD184352 (1uM) with or without 1uM of AKT inhibitor MK-2206. Proliferation was quantified after 7 days and normalized to non-treated cells. Bars represent standard error between replicates (n = 3).



Supplementary figure 22 - BRAFi/METi synergistic effect in *BRAF* V600E colorectal and glioma cell lines. **a,** Levels of c-MET and phosphorylated MET were assayed by immunoblotting in colorectal cancer (CRC) and glioma cell lines (upper part). Lower part: The effect of combinations of 5 concentrations of crizotinib and 8 concentrations of PLX4720 were measured by CellTiter Glo after 72h of treatment. Excess above BLISS for measuring synergistic effect was calculated. The bars represent the maximal percent of excess above BLISS for each of the cell lines. **b-d,** Graphic representation of excess above BLISS for 3 of the cell lines.



Supplementary figure 23 - BRAFi/METi synergistic effect in *BRAF* V600E compared to BRAFi/EGFRi synergistic effect in RKO (CRC) and KG-1-C (glioma) cell lines. The effect of combinations of 5 concentrations of crizotinib/gefitinib and 4 concentrations of PLX4720 were measured by GFP at day 7. Excess above BLISS for measuring synergistic effect was calculated.



Supplementary Figure 24 - The effect of crizotinib on the treatment of the colorectal cancer cell line RKO (0.8uM) and the glioblastoma cell line KG-1-C (1.6uM) with 2uM PLX4720 or 2uM PD184352. MAPK and PI3K/AKT pathways activation was assessed after 24 hours of treatment by immunoblot analysis of pMEK, pERK and pAKT. pMET levels were also assessed. The normalized proliferation of the cell lines under the same conditions is shown below. Bars represent standard error between replicates (n = 4).