# *Elucidate the underlying molecular mechanisms of the combination treatment effects of Afatinib (EGFR/HER2 inhibitor) and Crizotinib (MET inhibitor) in cutaneous malignant melanoma*

**Ishani Das [1], Huiqin Chen [2], Gianluca Maddalo [3], Margareta Wilhelm [4], Rainer Tuominen [1], Veronica Hoiöm [1], Johan Hansson [1], Michael Davies [2], Suzanne Egyházi Brage [1]**

[1] Department of Oncology, Karolinska Institutet, Stockholm, Sweden
[2] Department of Melanoma Medical Oncology, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, USA
[3] Science for Life Laboratory, School of Biotechnology, KTH Royal Institute of Technology, Stockholm, Sweden
[4] Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden

**Aim:** To unravel novel molecular targets that determine the efficacy behind combining afatinib and crizotinib in CMM

## Background

Melanoma is the main cause of skin cancer related deaths with approximately 230,000 new cases diagnosed yearly worldwide according to a 2016 World Health Organization report. Early detection of the disease is associated with a good prognosis, but patients with disseminated disease has generally poor clinical outcome due to therapy resistance. Modern therapies of disseminated melanoma with mutant BRAF inhibitors or immunotherapy have shown promising clinical results with longer progression free survival but relapses are common.
Overexpression of receptor tyrosine kinases like ERBB and MET have been identified before as resistance mechanisms to BRAFi. It has been shown that there is a crosstalk between MET and EGFR. Therefore, having a better understanding of this receptor crosstalk can enable us to design optimal drug therapy regimes to combat drug resistance.

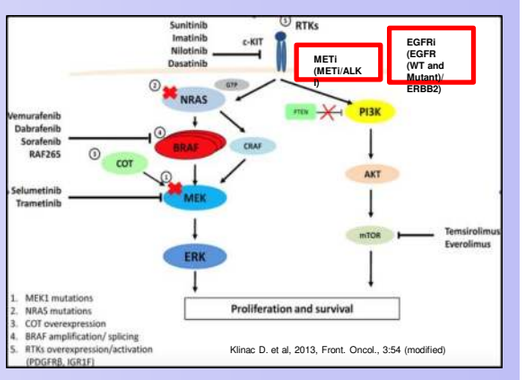


**Figure 1. Important pathways involved in melanoma and targeted therapies currently under study**

## Results

### Combining afatinib and crizotinib abrogates tumor growth significantly *in vivo*

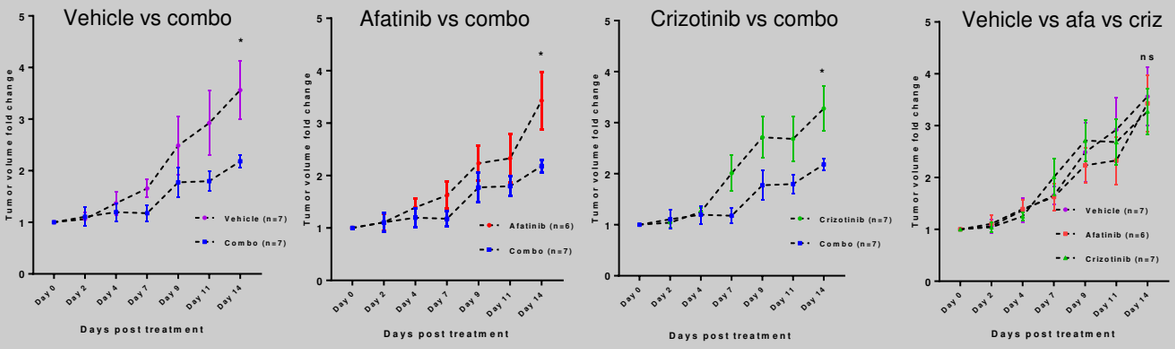


**Figure 2: Afatinib and crizotinib in combination was able to reduce tumor growth significantly in a A375 xenograft mouse model.** A375 cells ($3.6x10^6$) were mixed 1:1 with growth factor reduced matrigel matrix (VWR) and injected subcutaneously in the flank of 6week old CB-17/Icr-*Prkdc*$^{scid/scid}$ females (Janvier). Treatment commenced when tumors reached palpable size. Tumor size was measured thrice a week using calipers, tumor volume was calculated as vol= (D x $d^2$) x 0,52, D= largest diameter and d= smallest diameter. Animals for dosed with 20mg/Kg of afatinib and 15mg/Kg of crizotinib for 5 consecutive days, followed by two days drug off and this regime was repeated for a second round. Animals were sacrificied on day 14.. P< 0.05, **P<0.01, ***P<0.001, **** P<0.0001 (Student's t-test).

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### RPPA and Mass-spectrometry data unravels genes in the mTOR pathway, DNA repair pathway and mitochondrial fusion to be most frequently altered upon combination treatment

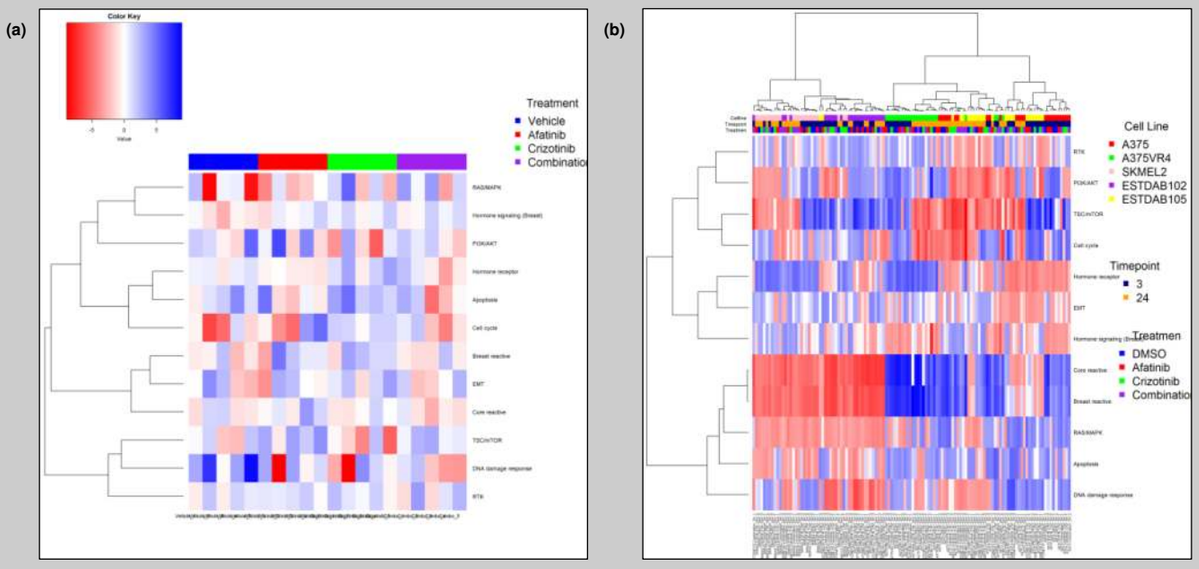
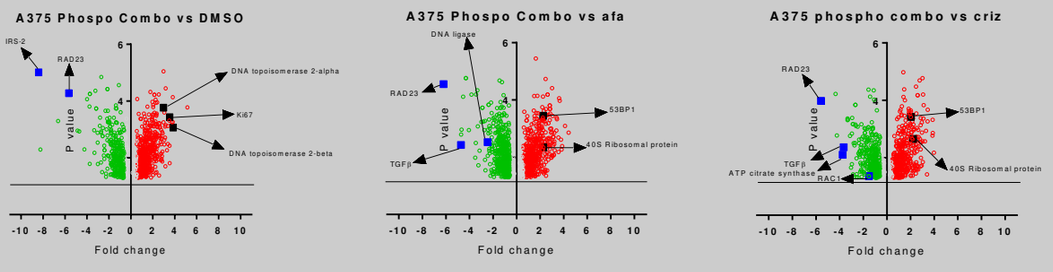



**Figure 3: RPPA analysis shows proteins in the mTOR pathway and DNA repair to be altered upon treatment.** (a) protein samples from the xenograft study shown in figure 1 were analyzed using RPPA (data represented here with supervised clustering) and (b) CMM cells treated for 3h or 24h with afatnib (2µM), crizotinib (2µM) or the combination (data representted here with unsupervised clustering) . Among the top candidates were pMTOR, pRPS6KB1, ATRX, MFN2, pAMPK



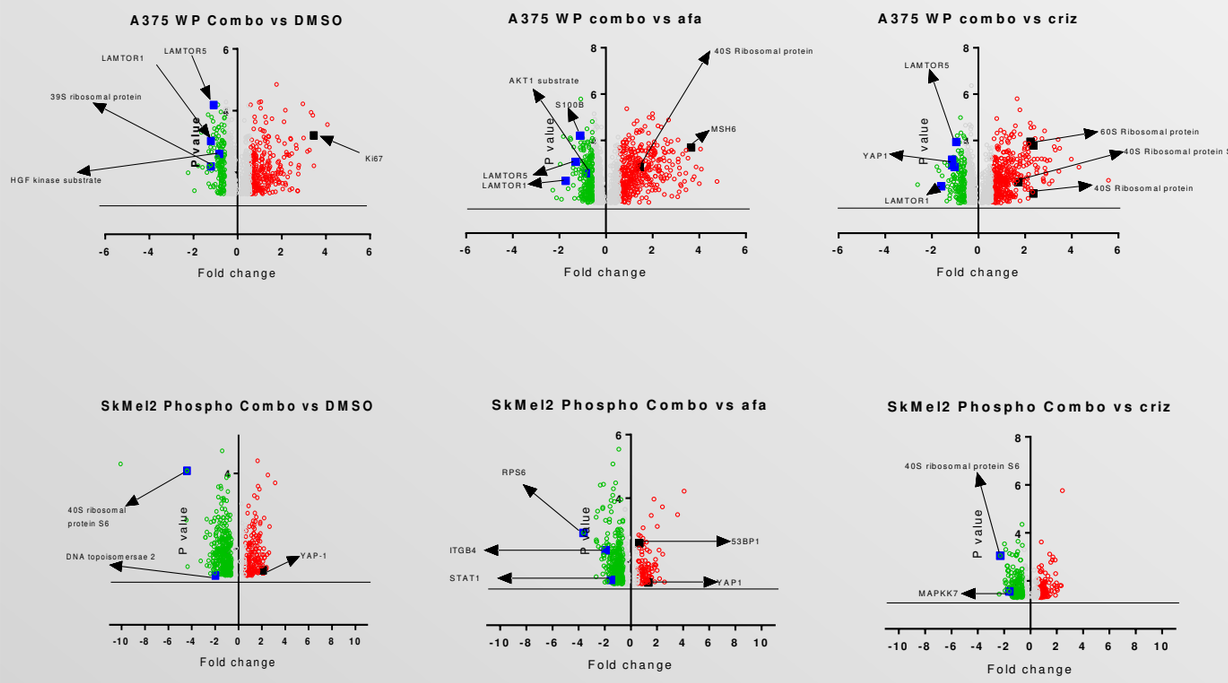




**Figure 4: Mass-spec data shows proteins involved in mTOR pathway, DNA damage pathway and ribosomal proteins to be affected after exposure to combination.** CMM cells (*BRAF* mutant and *NRAS* mutant) were exposed for 3h to either 4µM afatinib, 4µM crizotinib or the combination and lysates were analyzed using mass-spectrometry both for total and phospho proteins

### Resistance developed to the combination can be reverted by drug holiday

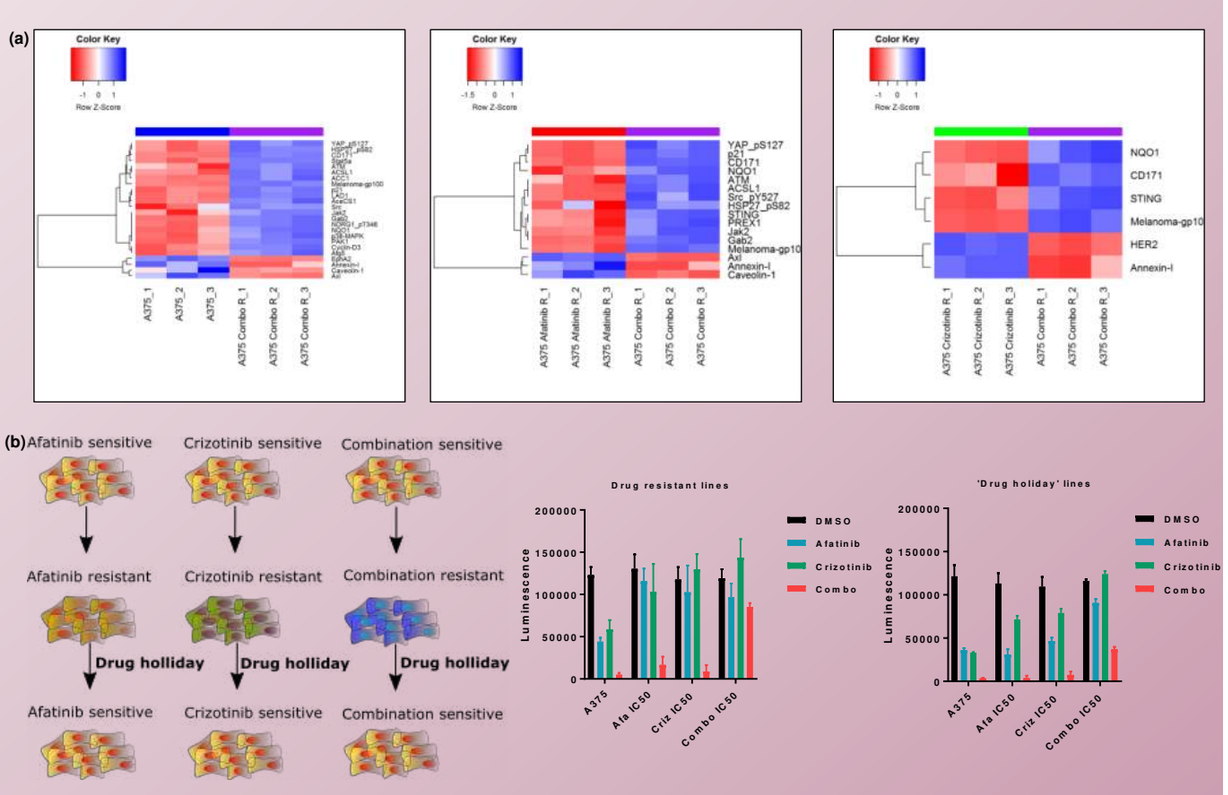



**Figure 5: RPPA unravels induction of p-YAP1, Melanoma gp100, ACSS1, CD171 as resistance factors for the combination. The induced combination is reversible.** A375 cells were grown in presence of afatinib, crizotinib or the combination at IC50 doses and resistance was induced after ~2 months. (a) Lysates from resistant cells were analyzed for alterations at the molecular level using RPPA. (b) Resistant cells were then cultured for 2 week 'drug-off' period and checked if they could be resensitized to the single and combination drugs

## Conclusions

- **Combination treatment of afatinib and crizotinib significantly abrogates tumor growth in a A375 xenograft mouse model**
- **RPPA analysis on cells treated for 3h and 24h show pRPS6KB1, pRPS6, pmTOR, pAMPK, TRIM25 as proteins that are significantly altered, whilst RPPA analysis conducted on in vivo samples reveals pYAP1, MFN2, ACSS1, ATRX and RIP being significantly altered**
- **Mass-spec data also indicate that mTOR family members, DNA damage related proteins and ribosomal proteins to be affected with the combination treatment**
- **Resistance developed towards the combination can be reverted back ensuing a 'drug holiday' phase**

**Contact:**
Suzanne Egyházi Brage, PhD
Email: suzanne.egyhazi.brage@ki.se
Phone: +46-851770365
Department of Oncology-Pathology
Cancer Center Karolinska
Karolinska University Hospital
171 76 Stockholm, Sweden

Ishani Das
Email: ishani.das@ki.se
Phone: +46-851770365
Department of Oncology-Pathology
Cancer Center Karolinska
Karolinska University Hospital
171 76 Stockholm, Sweden

Karolinska Institutet