**SUPPLEMTARY FIGURE LEGENDS**

**Supplementary Figure 1 Determination of the AKT pathway downstream effector activity in HNSCC models of acquired trametinib resistance.** (A) Lysates from CAL27-C, CAL27-TR, HSC3-C, and HSC3-TR, treated with either trametinib (100nM) or DMSO for 24 hours were resolved by SDS-PAGE for p-4E-BP1, total 4E-BP1, p-S6, total S6, YAP, p-Erk1/2 and total Erk1/2. Vinculin was used as a loading control.

**Supplementary Figure 2.**

Isobolograms from verteporfin (V) and trametinib (T) treatment combinations of HSC3-C (left panel) and CAL27-C (right panel) from Calcusyn analysis of Cell Titer Glo data. Cells were treated for 96 hours with vehicle, verteporfin alone, trametinib alone or combinations of the two drugs at a fixed ratio for each cell line. Combination Index (CI) values and interpretation at effective dose (ED) 50, ED75 and ED90 are provided.

**Supplementary Figure 3. Intrinsically trametinib resistant Detroit562 cells display high YAP1 protein expression.** (A) Western blot showing YAP protein expression levels for various HNSCC lines at base line.(B) SNU899, HSC2, BICR56 and Detroit562 cells were treated with various concentrations of trametinib for 96 hours. IC50, cell viability and maximal inhibition results are shown (n=3). (C) Western blot showing MAPK pathway, STAT3 and AKT activities in Detroit562 under trametinib treatment.

**Supplementary Figure 4. YAP knock-down in HSC3-TR cells does not alter growth kinetics while moderately resensitizing HSC3-TR cells to trametinib.** (A) Cell doubling times, determined using the xCelligence platform, did not differ between HSC3-TR sgNT and HSC3-TR YAP knockout lines generated using CRISPR targeting. (B) YAP1 knockdown with all five YAP-targeting sgRNAs increased HSC3-TR sensitivity to trametinib compared to the NT control. Cells were treated for 96 hours with varying trametinib concentrations followed by CellTiterGlo assay. Data are representative of two independent experiments. (C) Western blot of HSC3-TR cells stably transduced with a lentivirus encoding YAP shRNA (or a scrambled control). (D) HSC3-TR-shScrambled and HSC3-TR-shYAP #3 cells were treated with various concentrations of trametinib for 96 hours. IC50 and maximal inhibition results are shown (n=3). (A and B) are representative of at least three independent experiments.

**Supplementary Figure 5. CAL27-TR cells did not have altered trametinib sensitivity following YAP1 knockdown despite reduced YAP and YAP target gene expression.** (A) Immunoblot demonstrating knockdown of YAP1 in CAL27-TR cells using three independent YAP1 targeting sgRNAs compared with CAL27-TR cells transduced with NT control. (B) Following 96 hours of treatment with varying doses of trametinib, CAL27-TR and CAL27-TR transduced with YAP1 targeting sgRNAs or NT control RNA were assayed using Cell Titer Glo. IC50s were determined using PRISM software. (C) CTGF and ANKRD1 expression levels were assessed by qPCR in CAL27-C, CAL27-TR and CAL27-TR cells transduced with YAP1 targeting sgRNAs or NT control RNA. Expression was normalized to CAL27-C cells. Reductions in YAP1 target gene expression in YAP1 targeting sgRNA CAL27-TR transduced cells were tested by comparing to NT control. (\*\*\*\*, p<0.0001).

**Supplementary Figure 6. Targeting YAP1 in combination with trametinib did not resensitize HSC3-TR cells to trametinib killing.** (A) Percent viability of HSC3-C and HSC3-TR cells determined by Celltox Green assay following 96 hours of treatment with trametinib at varying concentrations. IC50 values provided were determined using PRISM software. (B) Viability of HSC3-TR CRISPR/Cas9 mediated YAP1 knockouts, HSC3-TR YAP#2 and HSC3-TR YAP#4, or the non-targeting control, HSC3-TR sgNT, were determined using Celltox Green following 96 hours of treatment with varying concentrations of trametinib. (C-D) HSC3-TR cell viability determined by Celltox Green following 96 hours of combination treatment with trametinib alone or trametinib in combination with a fixed verteporfin dose. All data are representative of 3 independent experiments.

**Supplementary Figure 7.**  (A) p-Erk1/2 biomarker response in patient 2233-western blot showing attenuation of p-Erk1/2 in lysates generated from patient’s tumor. Baseline is prior to and post-drug is after 2 weeks of trametinib. (B) Western blot of YAP1 levels in select patients total tumor tissue where matched tissue samples were available.

**Supplementary Figure 8. CRISPR/Cas 9 mediated YAP1 knock-out impeded PDX2233-TR growth *in vivo.*** (A) Schematic representing genetic manipulation of acquired trametinib resistant HNSCC PDX model, PDX2233-TR. (B) Western blot showing p-Erk1/2 inhibition in PDX2233-TR after four hours of trametinib treatment *in vitro*. Western blot confirming decreased YAP1 protein levels before injection (C) and after injection into NSG mice (D) in PDX2233-TR transduced with lentivirus encoding sgYAP #2. (E) Equal numbers of PDX2233-TR sgNT and PDX2233-TR sgYAP#2 were implanted into NSG mice (n=6/ group). Tumors were measured at least twice weekly and tumor volumes plotted (\*\* p< 0.005, unpaired t-test).

**SUPPLEMENTARY MATERIALS AND METHODS**

**Cell line derived from PDX2233-TR**

Tumors from PDX2233-TR were harvested in IMDM/F12 (2:1) media containing 10% heat-inactivated fetal bovine serum (Hyclone), penicillin/streptomycin (Lonza), 5 ng/mL EGF (Millipore), 400 ng/mL hydrocortisone (Sigma Aldrich), 100 ng/mL cholera toxin (Sigma Aldrich) and 5 ug/mL insulin. Single-cell populations from PDX2233-TR were obtained through a combination of collagenase 1 (Life Technologies) digestion and mechanical dissociation using the GentleMACS (Miltenyi Biotec). The resulting single-cell populations were briefly washed with sterile PBS (Corning) and seeded at high confluency on collagen-IV (Sigma Aldrich) coated plates in the same media used to harvest the tumors. The isolated cells derived from PDX2233-TR were maintained in culture for a maximum of 7 days before being transplanted to a secondary host or used directly for experiments.

Lentiviral infection of pLenti-CRISPRv2 containing sgNT and sgYAP1 #2 for cells derived from 2233-TR PDX was performed as described in materials and methods. For cells derived from 2233-TR PDX, the optimal puromycin concentration to achieve more than 95% cell killing in 72 hours was determined by measuring cell viability with a Cell Titer Glo assay for a ten-point dose response ranging from 0 to 8 µg of puromycin. After 72 hours of puromycin selection, 2233-TR sgYAP#2 and 2233-TR sgNT cells were collected and injected (1 x 106 in 1:1 Matrigel and PBS) to NSG mice for further experiments.

**Primary Antibodies for immunoblotting**

YAP (#12395S), P-Erk (#4370S), Erk (#9102), P-AKT (#4060 S), AKT (#9272 S), P-STAT3 (#9145 S), STAT3 (#4904 S), P-4EBP1 (#2855 S), 4EBP1 (#9644 S), P-S6 (#4858 S), S6 (#2217 S), V5 (#13202 S) primary antibodies were purchased from Cell Signaling Technologies. Vinculin (#9131) primary antibody was purchased from Sigma Aldrich.

**Supplementary Table 1: Small guide RNA oligonucleotides sequences**

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| --- | --- |
| sgNT | 5'-TATCTTGTGGAAGGACGAAA-3' |
| sgYAP #1 | 5'-GATGAACCTTTACCAAAACG-3' |
| sgYAP #2 | 5'-GTGCACGATCTGATGCCCGG-3' |
| sgYAP #3 | 5'-CGATCAGACAACAACATGGC-3' |
| sgYAP #4 | 5'-CGAGGTTACCTGTCGGGAGT-3' |
| sgYAP #5 | 5'-TGTCGAAGATGCTGAGCTGT-3' |

**Supplementary Table 2: SNVs and Indels detected across all PT2233 patient samples.** Each row corresponds to an annotated mutation detected by WES in any of the PT2233 tumor or PDX DNA samples.

**Supplementary Table 3: Gene expression analysis in PTX2233.** Differential expression analysis was performed using the DESeq2 R package, comparing PTX2233-TR (n=3) to PTX2233-C (n=3). Genes were labeled “significant” if the p-value was less than 0.05 and the Benjamini-Hochberg-corrected p-value was less than 0.20.

**Supplementary Table 4: Pathway analysis in PTX2233.** Differential expression analysis (in Supplementary Table 3) was used as an input for gene set enrichment analysis (GSEA) across the Hallmark, KEGG, and REACTOME gene sets. Gene sets were labeled “significant” if the p-value was less than 0.05 and the FDR was less than 0.20.

**Supplementary Table 5: SNVs and Indels detected across all clinical trial samples.** Each row corresponds to an annotated mutation detected by WES in any of the baseline tumor DNA samples.

**Supplementary Table 6: Gene expression analysis in clinical trial samples.** Differential expression analysis was performed using the DESeq2 R package, comparing either post- vs. pre-treatment tumor RNA in responsive patients, post- vs. pre-treatment tumor RNA in non-responsive patients, or baseline tumor RNA in responsive vs. non-responsive patients. Genes were labeled “significant” if the p-value was less than 0.05 and the Benjamini-Hochberg-corrected p-value was less than 0.20.Only genes that were significant by at least one of these comparisons are shown.