Materials and methods

Cell lines

Human esophageal squamous cell carcinoma cell lines TE1, TE8, TE9, TE10, TE11, TE14, TE15, OE21, KYSE70, KYSE140, KYSE180, KYSE410, KYSE510 and head and neck squamous cell carcinoma cell line BICR6, lung squamous cell carcinoma cell line HARA, and gastric adenocarcinoma cell line GCIY were obtained from the Broad Institute. Breast cancer cell lines T47D and MCF7 were obtained from Dr Nikhil Wagle Lab from Dana-Farber Cancer Institute. Cell lines were tested negative for *Mycoplasma* and maintained in recommended medium: GCIY was cultured with 15% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin; T47D was cultured in Phenol Red-free RPMI-1640 (GIBCO #11835-030) with 10% FBS and 1% penicillin/streptomycin; MCF7 was cultured in Phenol Red-free MEM-α (GIBCO #41061-029) with 10% FBS and 1% penicillin/streptomycin; The rest of cell lines were all maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

Chemical Reagents

Small-molecule inhibitors such as afatinib (S1011) and palbociclib (S1116) were purchased from Selleck Chemicals (Houston, TX, USA). Both drugs were prepared as 5-10mM stock solutions in dimethylsulfoxide (DMSO) or water per manufacturer's instructions and stored at -20 °C until further use.

Subcutaneous implantation and animal treatment studies

All animal experiments were conducted in accordance with procedures approved by the institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute, in compliance with NIH guidelines. $2.5x10^6$ KYSE410 and TE11 cells (1:1 Matrigel: medium in 200ul) were implanted subcutaneously into both flanks of female nude mice (6-8 weeks old, Nu/Nu; Jackson Laboratory). Mice were then examined every 5-7 days for tumor growth. Caliper was used to measure the size of the tumor and tumor volume was calculated using the following formula: (Length x $Width^2$) x 0.5. Mice were then randomly separated into groups when their tumor reached 100-150mm^3 before drug administration.

For the optimization of inhibitor-dosage in mouse study, we started the initial dose assessment with the evaluation of afatinib and palbociclib at their full in vivo dose (afatinib 20mg/kg, gavage daily, . palbociclib 150mg/kg gavage daily,). We observed a dose-limiting toxicity (with mice losing weight >10% within 1 week) with combined afatinib 10-20 mg/kg and palbociclib 100 -150mg/kg. We then transitioned to a minimally effective dosing (both single agent and combination) to help decrease the dose-related adverse events.

Afatinib (in 0.5% hydroxypropyl methylcellulose with 0.4% Tween 80) was dosed at 5mg/kg and palbociclib (in 17% (2-Hydroxypropyl)- β-cyclodextrin) was dosed at 50mg/kg for KYSE410 and TE11

xenografts (monotherapy or combination, daily via oral gavage), and 100mg/kg (data not shown) and 50mg/kg for KYSE140 xenografts (daily via oral gavage). The KYSE140 experiment in the publication was performed after an earlier monotherapy experiment with 100mg/kg of palbociclib, an experiment which also documented the lack of single agent efficacy (not shown). Given that we subsequently utilized a 50mg/kg dose in combination studies (with a reduced dose to enable tolerability with combination with afatinib), we repeated the experiment in the KYSE140 cell line model using the palbociclib 50mg/kg for consistency in publication. Mice were sacrificed when the tumor reached endpoint. Dissected tumors were snap-frozen and stored in liquid nitrogen or were fixed in 10% buffered formalin for routine histopathologic processing.

Genetic constructs and lentiviral production

Lentiviral pLKO.1 vector containing shRNA targeting KLF5.

Transient transfections and virus preparation in 293T cells were performed using Xtreme-gene reagents (Roche) as per manufacturer's protocol. Lentivirus were prepared by transfecting two packaging plasmids into 293T cells using protocols from The RNAi Consortium (TRC; Broad institute).

Short hairpin sequences
Control shRNA GACTAGAAGGCACAGAGGGAG
KLF5 shRNA 1 GCGATTCACAACCCAAATTTA (human)
KLF5 shRNA 2 CCCTGAGTTCACCAGTATATT (human)

Proliferation assays

For palbociclib IC50 experiment, 1-2x10^5 cells per well were seeded in 6-well plates. Cells were treated with palbociclib in different concentrations and counted after 4-day (Beckman Coulter Counter). For Afatinib IC50 experiments and shRNA-doxycycline treatment experiments, 1000-2000 cells were plated in flat-bottomed 96-well pate. Cells were treated with either vehicle (DMSO) or different concentrations of afatinib or 1000 ug/ml doxycycline and measured using CellTiter-Glo for ATP amount after 3-5 days. Data were calculated as percentages of the viability of control (DMSO treated) cells.
For colony formation assay, 2x10^4-1x10^5 cells were plated in 6-well plates. Cells were then treated with DMSO or inhibitors and treatments were renewed every 3-4 days. After 7-10 days, cells were fixed in 1% paraformaldehyde for 15 minutes at room temperature, washed twice with PBS and stained with crystal violet solution for 15 minutes at room temperature.

Quantification of crystal violet

Crystal violet stainings were destained by acetic acid. We applied 500ul-1ml 10% acetic acid to each well, shake for 10 minutes in room temperature, and transfer 100ul to 96 well plate. The plates were read at 595 wavelength with plate reader.

Immunoblot analyses

Cells were lysed in RIPA buffer supplemented with protease inhibition cocktail (Roche) and phosphatase inhibitor cocktails (BD). Cell lysates were resolved on 4-12% or 8-16% Tris-Glycine SDS-polyacrylamide gel before transfer onto PVDF membrane (Invitrogen). Membrane was then probed with primary antibodies overnight at 4 °C, following by one hour incubation with appropriate HRP-conjugated secondary antibodies at room temperature. Super-Signal West Pico Chemiluminescent Substrate (34080, life Technologies) were used to detect signals. Following antibodies were used in this study: (all from Cell Signaling Technologies, Beverly, MA, USA, except where indicated): p-EGFR Tyr 1068 (3777, 1:1000), EGFR (4267, 1:1000), p-ERBB2 Tyr 1222 (2243, 1:1000), ERBB2 (2165, 1:1000), p-ERBB3 Y1289 (4791; 1:1000), ERBB3 (12708, 1:1000), p-ERK Thr-202/Tyr-204 (4370, 1:1000), ERK (4695, 1:1000), pAKT Ser-473 (4060, 1:1000), AKT (9272, 1:1000), p-S6 S235/236 (2211; 1:1000), S6 (2217, 1:1000) pRB S807/811 (9308, 1:1000), RB (9309, 1:1000), CCND1 (55506; 1:2000), CDK4 (12790; 1:1000), CDK6 (13331; 1:1000), β-Actin (12620; 1:2000), and KLF5 (Abcam, ab137676, 1:1000). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit 31460, Invitrogen; goat anti-mouse TG273230, Invitrogen).

Cell cycle and Apoptosis

For cell cycle analysis, cells were harvested after treating with DMSO or inhibitors for 24 or 48h and were then fixed with 70% ethanol at 4 °C for 30 minutes. Fixed cells were washed with PBS containing 1% FBS and stained with PI/RNase Staining Solution (CST 4087) for 30 minutes at room temperature. DNA content was measured by LSR II flow cytometer and analyzed using ModFIT LT software.

Analysis of cell apoptosis was performed using FITC annexin V (FITC annexin V Apoptosis Detection Kit I, Becton Dickinson, Cat. No. 556547). Briefly, cells were harvested after incubating with DMSO or inhibitors for 72 hours. $1x10^5$ cells were resuspended in 1x binding buffer and stained with FITC annexin V and propidium iodide for 15 minutes. Stained cells were then analyzed using LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJO software (TreeStar).

mRNA-seq analysis

ESCC cell line KYSE180 was treated with afatinib 20nM, palbociclib 500nM and combination for 72 hours and total RNA was extracted using Qiagen RNeasy kit and treated with on-column DNase I. RNA-seq libraries were prepared using the NEBNet Ultra Directional RNA library prep kit (NEB, E7420S) and sequenced on the Illumina next-seq instrument (150-bp single end reads for mRNA sequencing). Read

alignment, quality control and data analysis were performed using VIPER[1]. Sequencing reads were aligned and counted using STAR alinger[2], and expression matrix was generated by Cufflinks[3]. DESeq2 was used for differential gene expression analysis[4] and pre-ranked gene set enrichment analysis was performed to illustrate perturbed gene signatures[5].

ChIP-seq Analysis

ChIP-seq assays were performed as previously described[55]. Briefly, cells were cross-linked with 1% formaldehyde and lysed. The chromatin extract was sonicated by a Diagenode bioruptor and immunoprecipitated with antibodies that were co-incubated with mixed Dynabeads A and G (Thermo Scientific). Antibodies that were include H3K27ac (2ug per ChIP; Abcam, ab 4729) and KLF5 (4ug per ChIP; Abcam, ab137676). The sequencing libraries were prepared using the NEB ChIP-seq library prep kit (NEB, E6200L) and sequenced on the Illumina next-seq instrument (75 bp single-end reads). Sequencing reads were aligned to the hg19 human genome. ChiLin pipeline 2.0.0 was used for QC and preprocess of the ChIP-seq. We used Burrows-Wheeler Aligner (BWA) as a read mapping tool, and Model-based Analysis of ChIP-Seq (MACS2) as a peak caller. Based on a dynamic Poisson distribution, MACS2 identifies local biases in the genome sequence, allowing for more sensitive and robust prediction of binding sites. We called the narrow peaks with FDR=0.01 as cut off and removed redundancy. DEseq2 were used to identify differential peaks. The output bedgraph files from MACS2 are converted to bigwig files which were loaded into IGV for visualization.

Quantitative PCR

Quantitative PCR (qPCR) was performed using Power SYBR green PCR master mix on StepOnePlus Cycler (Applied Biosystems). The primers used in qPCR were either obtained from previously published sequences or designed by PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), as listed in Supplementary Table 1. mRNA expression was quantified in technical triplicates with 10ng cDNA in a 20ul reaction volume and relative mRNA expression was calculated from Cq values using ΔΔCq method.

Datasets and Omics Data Analysis

CRISPR dependency data were taken from the 20Q2 Avana dataset (https://figshare.com/articles/DEMETER2_data/6025238/6) (Achiles_gene_effect file) downloaded from depmap.org. These gene essentiality scores were processed with the CERES algorithm[6]. RNA interference (RNAi) dependency data were taken from the DEMETER2 combined dataset (https://figshare.com/articles/DEMETER2_data/6025238/6), which integrates data from several large-scale RNAi screens including the Broad Institute Project Achilles and Novartis Project DRIVE datasets[7]. Both gene dependency datasets can be downloaded from Broad Institute DepMap web portal (https://depmap.org/portal/download/). Cancer cell line genomic data used in this study, including gene expression, gene-level relative copy number data were retrieved from the DepMap 20Q2 data release.

These multi-omics data can be downloaded from Broad Institute DepMap web portal (https://depmap.org/portal/download/). Drug sensitivity AUC data were retrieved from Genomics of Drug Sensitivity in Cancer dataset(GDSC- https://www.cancerrxgene.org/), Cancer Target Discovery and Development dataset (CTD2- https://ocg.cancer.gov/programs/ctd2/data-portal), and PRISM repurposing screen 20Q2 release (PRISM). All drug sensitivity data can be downloaded from Broad Institute DepMap web portal (https://depmap.org/portal/download/).

Two group comparisons using gene dependency data or drug sensitivity data were performed with limma-based linear model. Unpaired two-samples Wilcoxon test was used to compare gene expression and drug sensitivity data between squamous cancer cell lines and non-squamous cancer cell lines. Pearson correlation analysis was performed on variables extracted from gene dependency data, gene expression data, gene relative copy number data and drug sensitivity data. To investigate the gene whose expression was more associated with ERBB gene dependencies in squamous cancers than non-squamous cancers, low-expression genes (gene that has transcript per million value greater than 1 in less than 25% samples) and genes with low-expression variance (bottom 25% variable genes) were filtered before performing correlation analysis. Correlation co-efficient difference was calculated by subtracting Pearson coefficient of squamous cancer cell line group from Pearson coefficient of non-squamous cancer cell line group.

Statistical analysis

Statistical analysis was performed using Microsoft Office statistical tools or in Prism 8.0 (GraphPad). Data were represented as mean± S.D. or S.E.M. as indicated in the Figure legends. For each experiment, the number of independent biological experiments are as noted in the Figure legends and p value was calculated for biological replicates. Pairwise comparisons between experimental and control groups were performed using an unpaired two-tailed Student's *t*-tests, Mann-Whitney test, one-way ANOVA, or two-way ANOVA where appropriate. $P<0.05$ is considered to be statistically significant. P-value are denoted by *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

References

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