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Supplemental information

L858R emerges as a potential biomarker predicting response of lung cancer models to anti-EGFR antibodies: Comparison of osimertinib vs. cetuximab

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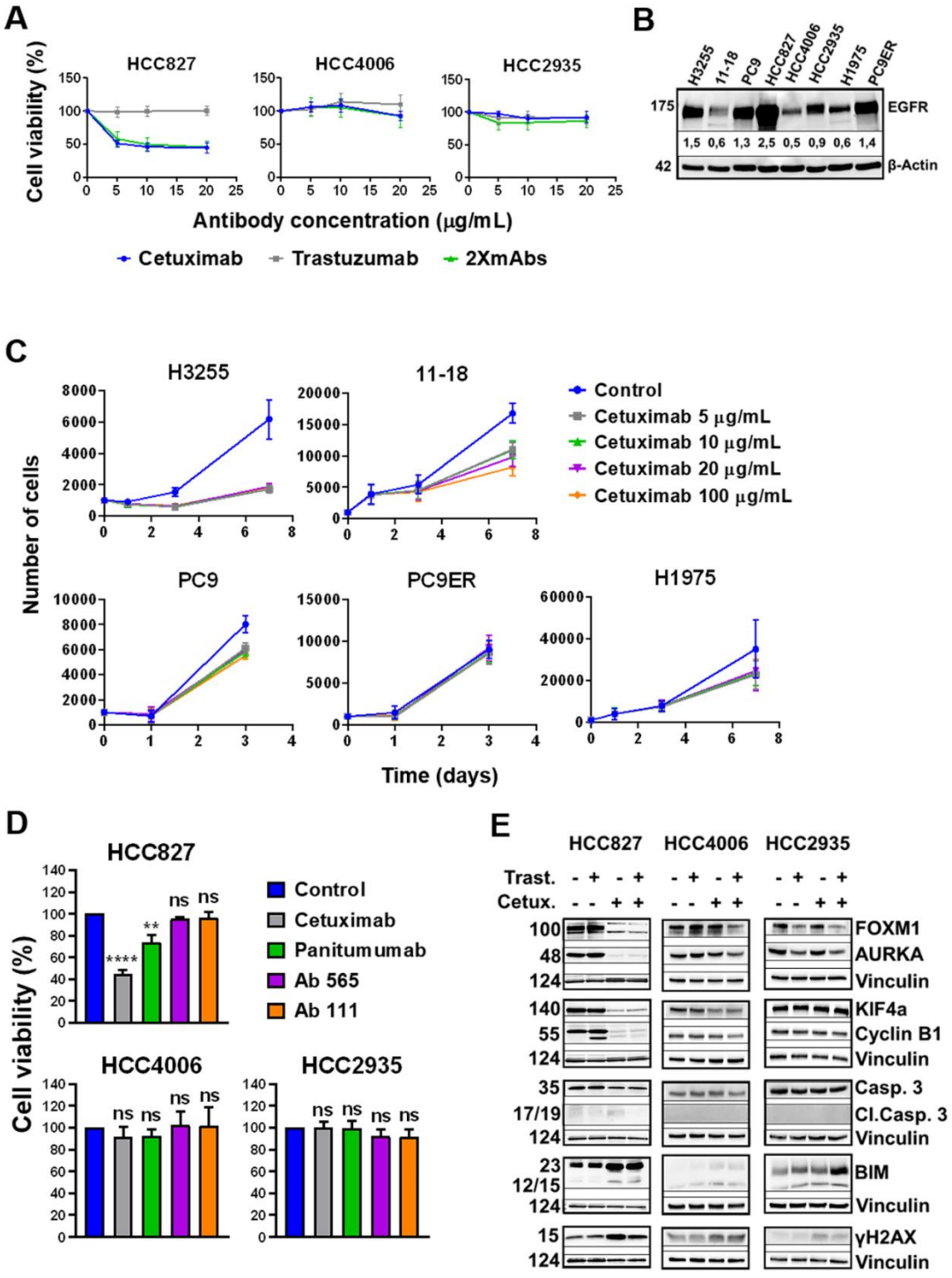

Figure S1: An anti-EGFR antibody inhibits viability and proliferation of lung cancer cell lines expressing L858R-EGFR but it does not affect the majority of cells expressing Del19-EGFR or T790M-EGFR. Related to Figure 1. (**A**) NSCLC cell lines harboring deletions in exon 19 of EGFR (HCC827, $8X10^3$ cells; HCC4006, $1X10^4$; and HCC2935, $1.5X10^4$) were seeded in 96-well plates and treated for 72 hours with different concentrations of the following antibodies: cetuximab, trastuzumab and the antibody combination (2XmAbs). Cell viability was assessed using the MTT assay. Data are shown as mean ± SEM of three experiments. (**B**) Protein extracted from the indicated NSCLC cell lines were blotted and probed with an EGFR-specific antibody. Signals were quantified and normalized to the signals corresponding to beta-actin. Normalized numerical signals are shown below each lane. (**C**) The indicated lung cancer cell lines were seeded on 96-well plates (1000 cells/well) and on the next day they were treated for 1, 3 or 7 days with increasing concentrations of cetuximab (5, 10, 20 or 100 μg/ml). Thereafter, cells were fixed and stained with crystal violet. Data are shown as mean ± SEM of three experiments. (**D**) HCC827 ($8X10^3$ cells), HCC4006 ($1X10^4$) and HCC2935 ($1.5X10^4$) cells were seeded in 96-well plates and later treated for 72 hours with anti-EGFR monoclonal antibodies (cetuximab, panitumumab, Ab 565 and Ab 111; each at 10 μg/ml). Cell viability was assessed using the MTT assay. Results are presented as means + SEM of three experiments. Significance was assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. $**p < 0.01$, $****p < 0.0001$, ns (no significance). (**E**) HCC827, HCC4006 and HCC2935 cells were treated for 48 hours with cetuximab (10 μg/ml), trastuzumab (10 μg/ml), or the combination of the two antibodies (each at 5 μg/ml; 2XmAbs). Protein extracts were blotted and probed with antibodies specific to the indicated apoptosis and cell cycle markers. Vinculin was used as the loading control protein.

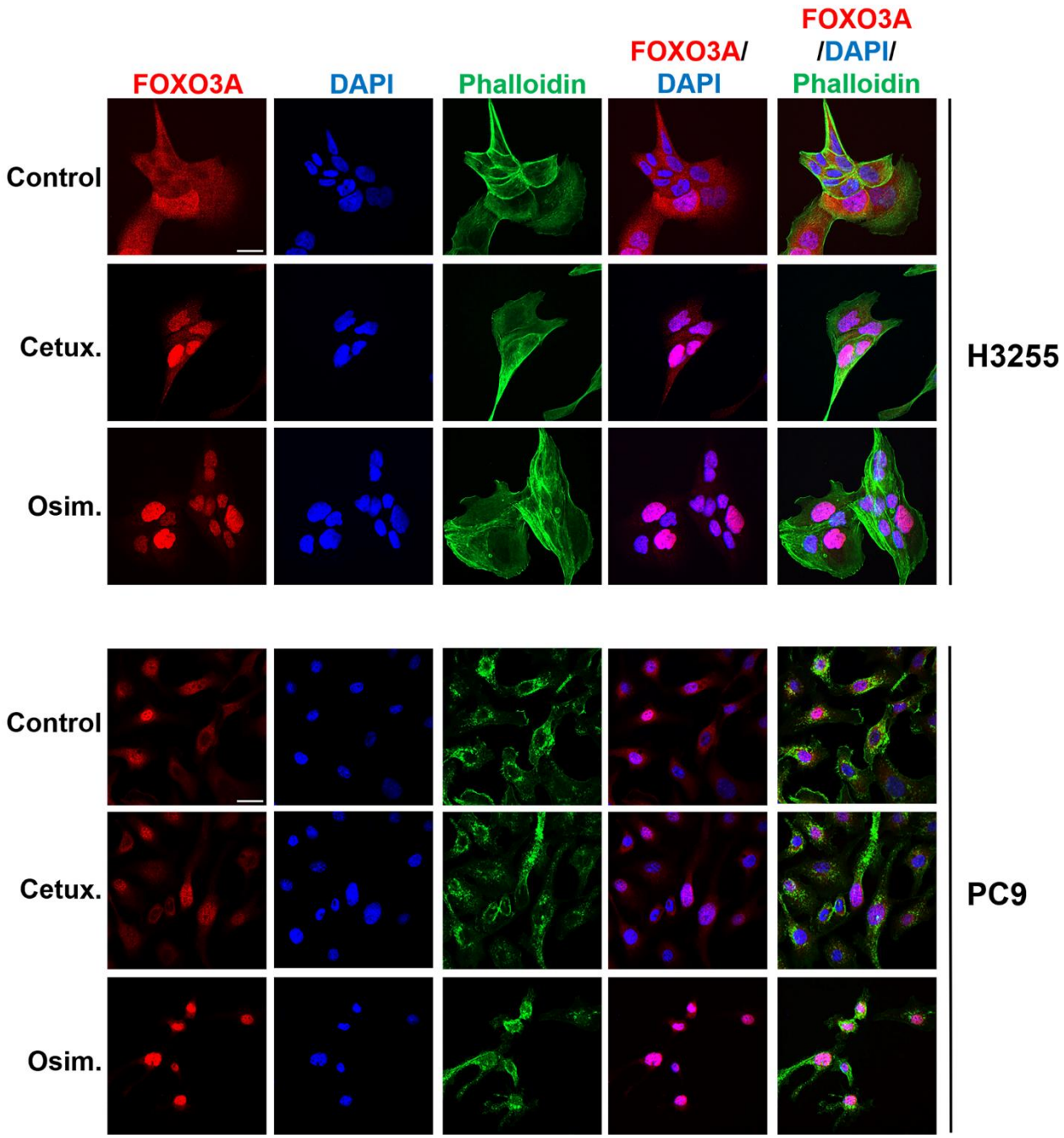

Figure S2: Both cetuximab and osimertinib induce translocation of FOXO3A to the nucleus of H3255 cells (L858R-EGFR), but only osimertinib induces the translocation in PC9 cells (Del19-EGFR). Related to Figure 1. H3255 and PC9 cells were seeded on coverslips and treated for 24 hours with cetuximab (*Cetux.*; 10 µg/ml) or with osimertinib (*Osim.*; 50 nM). Thereafter, cells were fixed in paraformaldehyde (4%) and incubated with an anti-FOXO3A primary antibody, followed by an Alexa Fluor 555-conjugated secondary antibody (red). DAPI (blue) was used to stain nuclei. FITC-conjugated phalloidin was used to stain actin filaments (green). Images were captured using a confocal microscope (40x magnification). Scale bar, 20 µm.

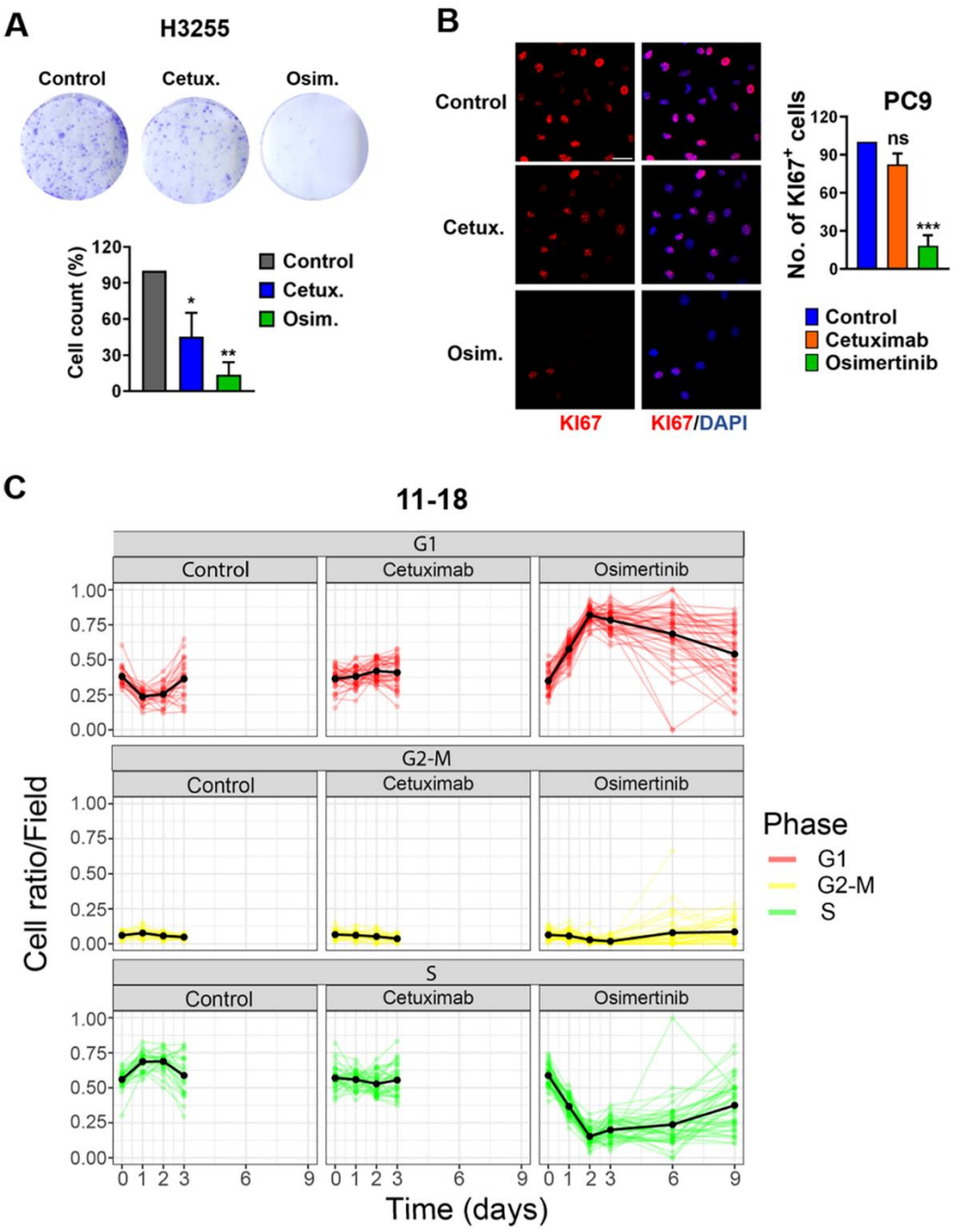

Figure S3: Cetuximab decreases colony formation by H3255 cells and it does not alter the S-phase fraction of 11-18 cells (both express L858R-EGFR), but it cannot reduce the KI67-positive fraction of PC9 cells (Del19-EGFR). Related to Figure 2. (**A**) H3255 cells were seeded on 6-well plates and on the next day they were treated for 48 hours with cetuximab (*Cetux.*; 10 μg/ml) or with osimertinib (*Osim.*; 50 nM). Thereafter, the cells were treated with trypsin and 5000 cells were seeded in 6-well plates to allow colony formation. Media (without drugs) were replaced once every three days. After 14 days, cells were fixed and stained with crystal violet. For quantification, images corresponding to five non-overlapping fields per sample were captured and quantified using ImageJ. Signals were normalized and the values are presented as means + SEM of three experiments. Significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. ∗, $p < 0.05$; ∗∗, $p < 0.01$. (**B**) PC9 cells were seeded on coverslips and treated for 48 hours with cetuximab (*Cetux.*; 10 μg/ml) or with osimertinib (*Osim.*; 50 nM). Cells were fixed in paraformaldehyde (4%) and incubated with an anti-KI67 antibody, followed by an Alexa Fluor 555-conjugated secondary antibody. DAPI (blue) was used to stain nuclei. Images were captured using confocal microscopy (40x magnification). KI67 staining was quantified using ImageJ and normalized to the number of nuclei. Values represent mean + SEM of three experiments. Significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison test. ∗∗∗, $p < 0.001$; ns, no significance. Bar, 20 μm. (**C**) 11-18 cells were labeled using the Fucci reagent. Following 1-9 days in culture, in presence or absence of drugs (cetuximab, 10 μg/ml, or osimertinib, 2 μM), we determined the number of intact nuclei by imaging Fucci fluorescence (nuclear labeling) and eliminating the non-circularly stained debris. In the next step, we measured fluorescence intensity of green and red signals to determine the proliferating fraction of cells (S-phase), arrested cells (G1-phase) and other fractions (G2-M). Fluorescence fractions corresponding to individual cells are presented, along with the population average (black lines).

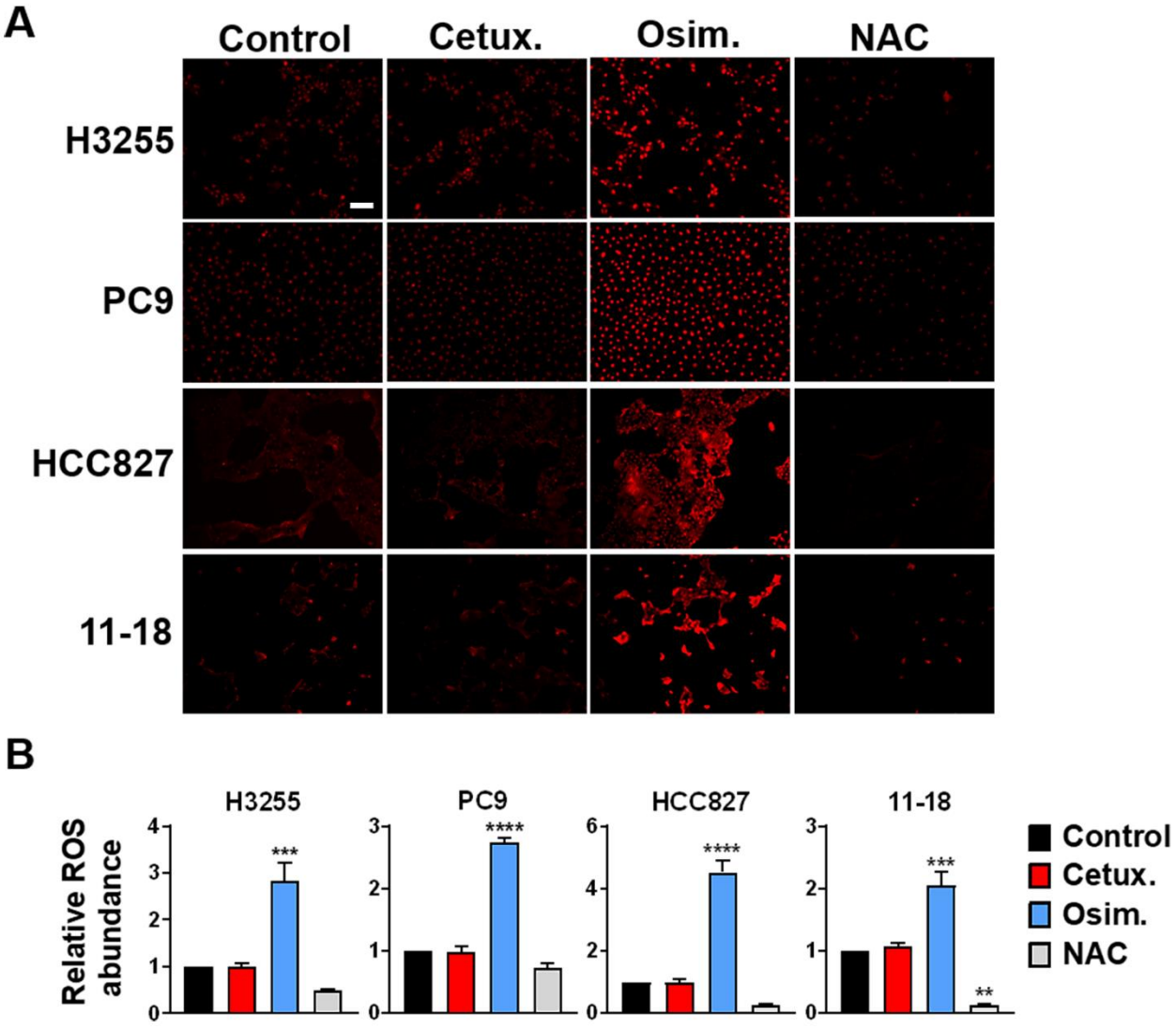

Figure S4: Independent of the identity of the expressed mutant form of EGFR, cetuximab induces no ROS production, but osimertinib strongly increases ROS abundance. Related to Figure 4. (**A**) H3255, PC9, HCC827 and 11-18 cells were treated with cetuximab (*Cetux.,* 10 µg/ml) or osimertinib (*Osim*., 500 nM for 11-18 cells or 50 nM for all other cell lines) for 8 hours. NAC (N-acetyl-l-cysteine; 10 mM) was used as a ROS scavenger. DHE (dihydroethidium) was used as a superoxide detector. Representative epifluorescence microscopy images are presented (original magnification, 100x). Scale bar, 200 µm. (**B**) Shown is the quantification of anion superoxide fluorescence (mean + SEM) from three independent experiments. Significance was assessed by means of one-way ANOVA followed by Dunnett's multiple comparison test. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

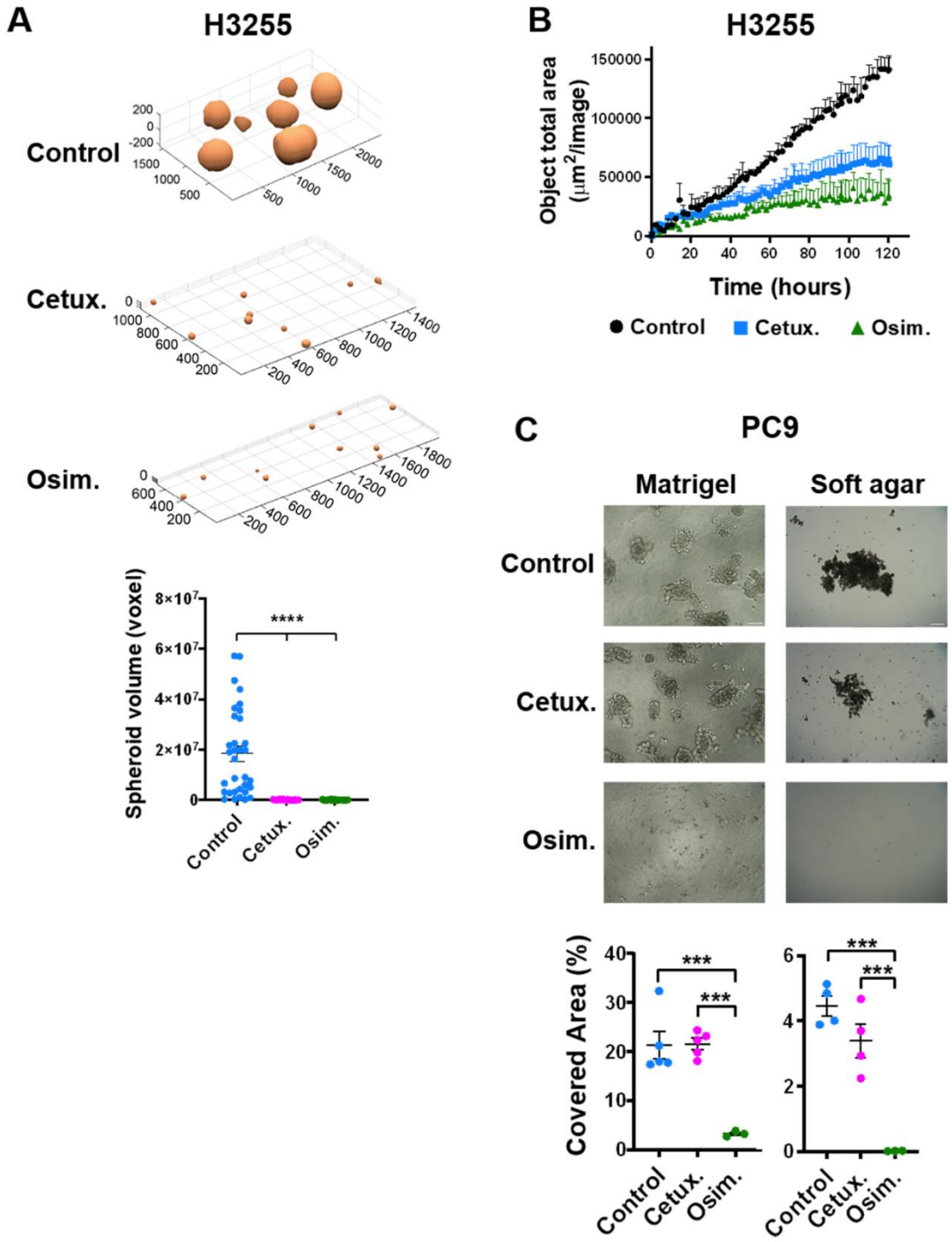

Figure S5: Cetuximab inhibits growth of H3255 spheroids, but this antibody cannot affect 3D growth of PC9 cells. Related to Figure 5. (**A**) H3255 cells were grown under low attachment conditions, which allow spheroid formation. $3x10^4$ cells were seeded in 6-well plates, pre-coated with agar (0.6%), in full medium containing the following agents: cetuximab (Cetux., 10 µg/mL) or osimertinib (Osim., 20 nM). After 10 days, several photos were captured from non-overlapping fields using an OpTech IB4 microscope. For each condition (control, cetuximab or osimertinib), spheroid volume was estimated from a single 2D projection, using the ReViSP software. Significance was assessed using one-way ANOVA followed by Dunnetts' multiple comparisons test. ****, $p < 0.0001$. The experiment was repeated twice. (**B**) H3255 cells ($5x10^4$) were seeded in 96-well plates pre-coated with 80% BME (basement membrane extract) in 5% BME medium containing the indicated drugs. Spheroid growth was followed for 5 days using the Incucyte® Live-Cell Analysis Instrument. Visualization and quantification of total area were determined once every 2 hours. The experiment was performed twice. (**C**) PC9 cells ($1x10^4$) were seeded in 96-well plates pre-coated with BME (80%) and embedded in BME medium (5%) containing either cetuximab (Cetux., 10 µg/ml) or osimertinib (Osim., 20 nM). After 10 days, the cells formed flat layers both when grown in BME (Matrigel) or when cultured under low attachment conditions (i.e., soft agar). The experiments were stopped after 10 days. Brightfield photos are presented (10X magnification) along with the percentages of covered area (assessed using ImageJ). Scale bars, 100 µm. Significance was calculated using one-way ANOVA followed by Tukey's multiple comparison test. ***, $p < 0.001$.

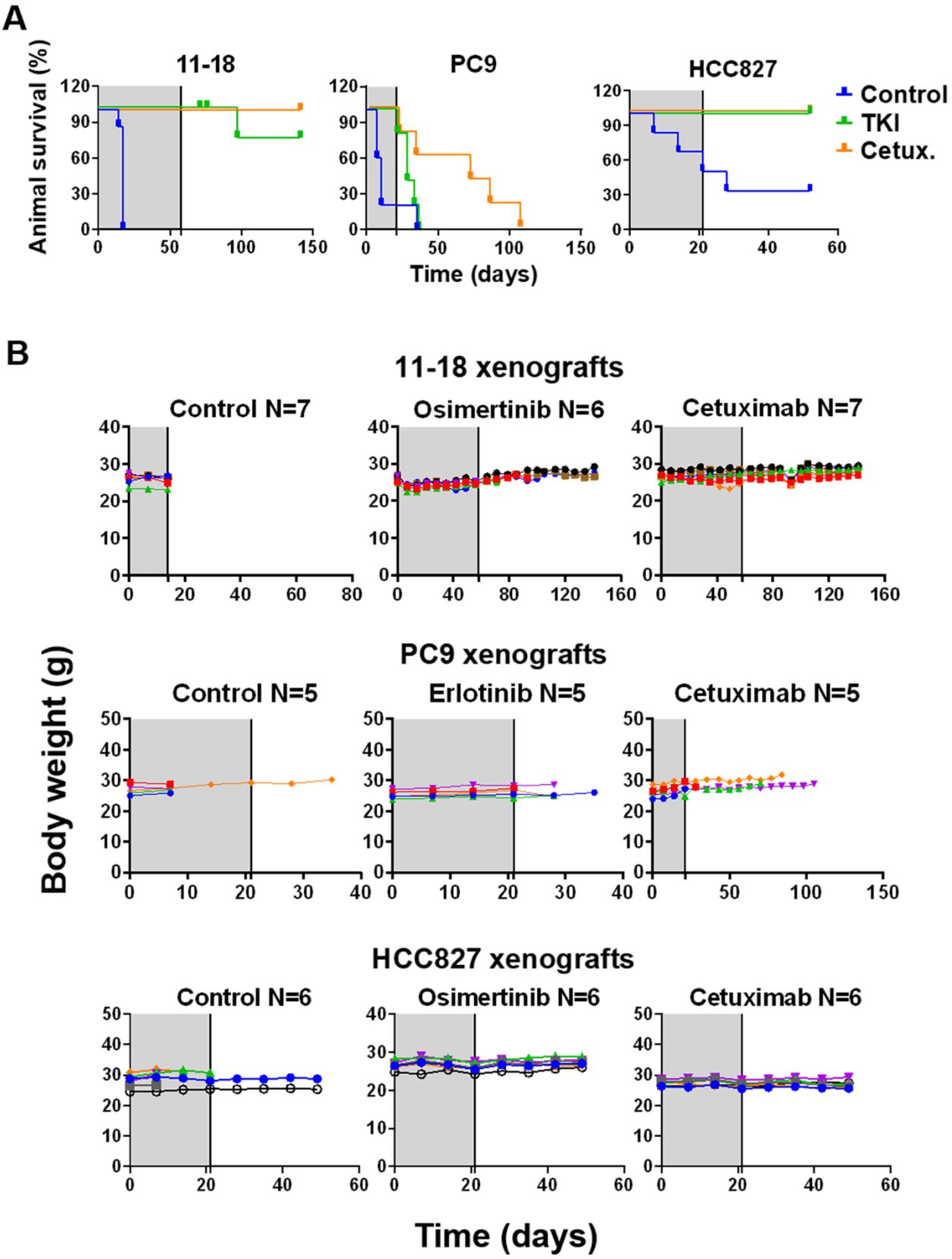

Figure S6: Survival curves and analyses of body weights of mice treated with either cetuximab or an EGFR-specific TKI. Related to Figure 5. All results correspond to the tumor growth curves presented in Figure 5. (**A**) Survival curves of CD1-nu/nu mice bearing 11-18, PC9 or HCC827 xenografts. Mice were treated for 58 days (11-18) or 21 days (PC9 and HCC827, grey areas) with cetuximab (*Cetux.*; 0.2 mg/injection) twice a week, or daily with an EGFR TKI, either erlotinib (*Erlot.*; 50 mg/kg; PC9) or osimertinib (*Osim.*; 10 mg/kg for 11-18 or 5 mg/kg for HCC827). (**B**) Analyses of animal body weights corresponding to the respective animal model experiments. Note that each color represents one animal.

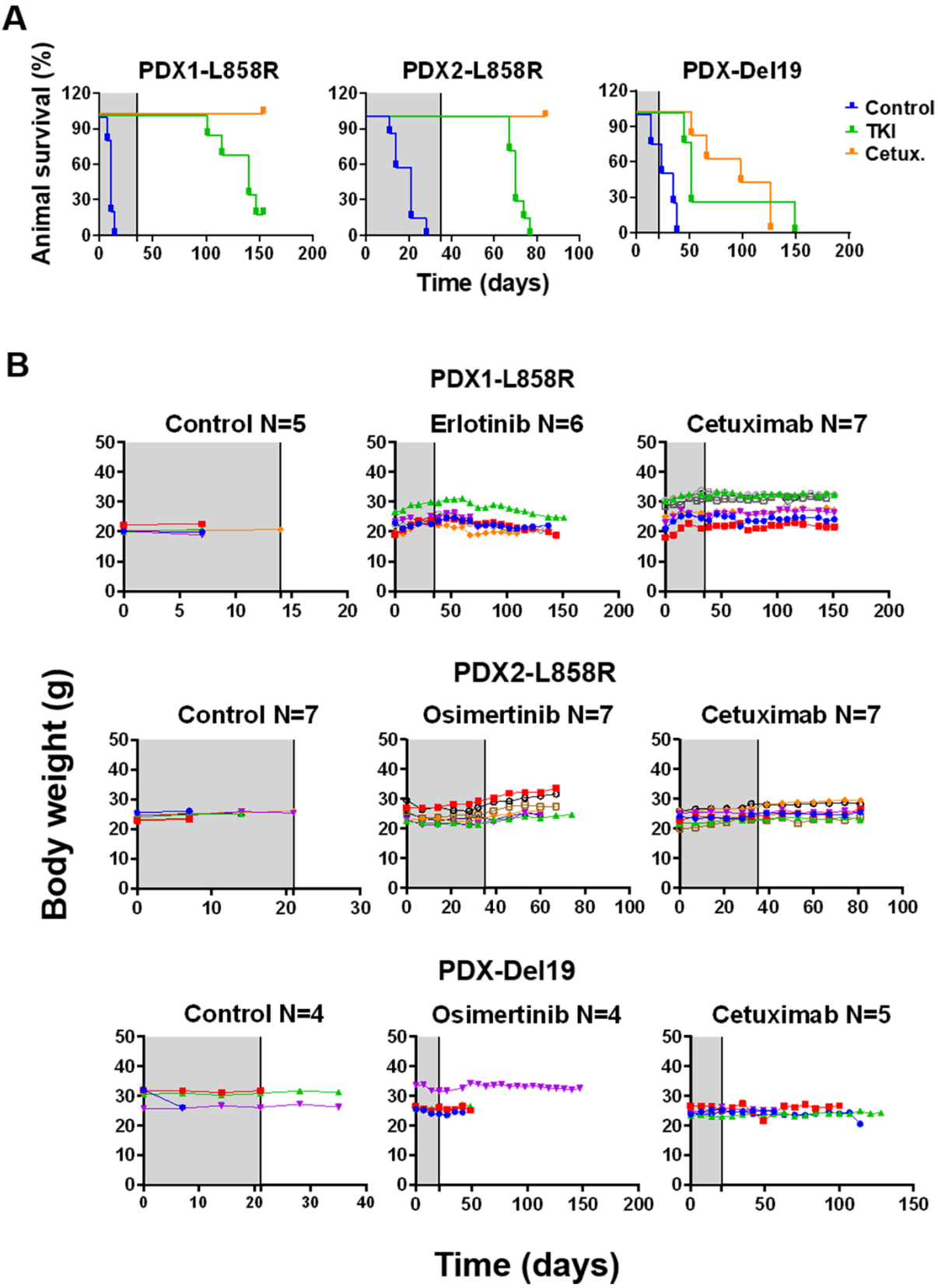

Figure S7: Survival curves and analyses of body weights of mice treated with either cetuximab or an EGFR TKI. Related to Figure 6. (**A**) Survival analyses of NSG mice bearing the PDX models TM00199 (PDX1, L858R-EGFR; left panel), TM00253 (PDX2, L858R; middle panel) or TM00193 (Del19; right panel). Mice were treated (grey areas) twice a week for either 5 weeks (TM00199 and TM00253) or 3 weeks (TM00193) with cetuximab (*Cetux.*; 0.2 mg/injection), or daily with an EGFR TKI, either erlotinib (50 mg/kg; TM00199) or osimertinib (10 mg/kg; TM00253 and TM00193). (**B**) Analyses of animal body weights corresponding to the animal model experiments shown in Figure 6. Note that each color represents one animal.