Figure S1 – see legend on next page
Figure S1

Heterogeneous ASS1 expression pattern in HNSCC cell lines does not correlate with their potential for compensating ADT-induced growth arrest and loss of regrowth capacity in the presence of citrulline (Cit).

(A) Representative Western blot and semi-quantitative densitometric analysis of relative intrinsic ASS protein level in the HNSCC cell line panel when grown as exponential monolayer culture under standard conditions. α-tubulin (α-tub) was used as loading control. Data represent means +SD of N=3 independent experiments.

(B) Representative RT-PCR analysis of ASS1 and ASL genes in HNSCC cell lines grown according to (A). ACTB mRNA level was recorded as reference.

(C) Correlation analysis of the expression of ASS1 gene and protein levels in the HNSCC cell lines determined by RT-PCR and Western blot analysis.

(D) Growth behavior of HNSCC cells under mono-ADT in the presence of Cit. Cells were exposed to Arg-free medium (-Arg) containing physiological Cit concentrations (0.04 mM) for up to 9 days. Selected samples were assessed for growth recovery by exchanging the supernatant to standard complete medium (+Arg) after 1d, 3d, 5d, 7d or 9d of exposure to the Arg-deprived condition; cell counts were measured 3 days later. Graphs show mean values +SD from N=3 independent experiments.
**Figure S2**

High level of ASS1 expression is a marker of poor prognosis for HNSCC patients and radioreponse.

(A) Correlation analysis of the expression of ASS1 gene and 0.1% surviving fraction (SF) doses of HNSCC lines determined *in vitro*.

(B) Correlation analysis of 0.1% SF doses determined *in vitro* and TCD<sub>50</sub> (dose required to control the disease in 50% of tumor-bearing animals) of the corresponding xenograft models of HNSCC lines published previously [20].

(C) Oncoprint of human HNSCC with upregulated expression of ASS1 gene based on the data in The Cancer Genome Atlas (TCGA; 517 non-randomized tumors; 1.5 z-score).

(D) Kaplan–Meier analysis to assess the correlation of ASS1 expression with overall and relapse-free survival of HNSCC patients from the TCGA (517 non-randomized tumors) database. Data were stratified by ASS1 expression level: ASS1<sub>median</sub> and ASS1<sub>high</sub> (≥3 f.c upregulation; correspondent 1.5 z-score) and comparative analysis by log-rank test was performed between these two different groups.
Figure S3

Cav at low concentrations is cytotoxic for HNSCC cells only in an Arg-free environment.

(A) Surviving fractions (±SD) of SAS and FaDu cells irradiated under standard culture conditions (+Arg) in the absence or presence of 0.04 mM Cit and 0.01-0.1 mM Cav (N=3, n≥3). Data were fitted with a linear-quadratic model as depicted in Materials and Methods.

(B) Colony forming capacity of SAS and FaDu cells treated by comb-ADT with 0.04 mM Cit and 0.05-0.1 mM Cav upon 0-6 Gy single dose irradiation.

(C) Representative RT-PCR analyses of spliced XBP1 expression in monolayer SAS and FaDu cells after defined times of Cav (0.1 mM) exposure. The spliced XBP1 mRNA (lower band) is apparent already after 4 h of comb-ADT; ACTB is shown as reference control and FaDu cells treated with tunicamycin (Tn; 3 µg/ml) served as positive control (right).
Figure S4

Single dose irradiation alone leads to spheroid growth delay and control; mono-ADT less effectively triggers ER stress in the 3-D environment than in 2-D culture (cf. Figs. 6 and 2).

(A) Spheroid volume growth is delayed after irradiation of 3-D cultures with single doses that do not yet abrogate growth recovery (up to 12.5 Gy for SAS and 10 Gy for FaDu spheroids); mean values (± SD) are shown for n≥28 spheroids.

(B) Representative data sets of ER stress response genes in SAS and FaDu spheroids analyzed by RT-PCR. Spheroids were incubated in Arg-free medium for up to 120 h or left untreated (0 h). ACTB mRNA level was determined as reference.

(C) Representative Western blots of proteins from SAS spheroids upon mono-ADT. Cell lysates were probed with specific antibodies against the indicated proteins; α-tubulin (α-tub) was used as loading control.
Figure S5 – see legend on next page
Figure S5

Mono-ADT induces specific metabolic alterations in HNSCC spheroids related to protein degradation and amino acid metabolism

(A) Intracellular arginine level detected via LC-MS in SAS and FaDu spheroids under control and mono-ADT conditions

(B) Effect of mono-ADT on intracellular levels of certain similarly regulated molecules (amino acids and glycerol) in SAS and FaDu spheroids

(C) Effect of mono-ADT on intracellular levels of certain differently regulated metabolites in SAS and FaDu spheroids

Data in (B) and (C) show the means of two independent experiments (n=3-6 technical repeats) measured using GCxGC-qMS (fold change ≥1.5; log2 value treated vs. untreated ≥0.584; *p≤0.01 significant in FaDu spheroids; # p≤0.01 significant in SAS spheroids
(A) (from top to bottom) Representative images of discoidal aggregates of retinal pigment epithelial cells under control conditions (+Arg) at days 5 and 9 in culture (± days 1 and 5 of exposure to treatment); mean number of membrane-intact ARPE-19 cells per “spheroid” (±SD) and the respective cell viabilities upon treatment relative to the corresponding (+Arg) control cultures (N≥3).

(B) Representative phase contrast images of fibroblast and HUVEC spheroid cultures exposed to various treatment conditions for 1 and 5 days.

(C) Mean number of membrane-intact fibroblasts per spheroid and relative cell viabilities (±SD) according to (A) (N≥3)

(D) Number of membrane-intact HUVEC per spheroid with intraexperimental variation (N=1, n=3 biological repeats) for two different HUVEC preparations and relative cell viabilities upon treatment (related to corresponding control cultures of the same preparation and day of exposure)

Figure S6
Neither mono-ADT nor comb-ADT induces cytotoxicity in 3-D cultures of normal cell types.