Supplemental Materials and Methods
Supplemental Figure Legends

Cell lines and culture conditions. We generated T-HEp3 cells expressing a Histone 2B (H2B)-GFP fusion protein under the control of the tetracycline-controlled transactivator (Tet-On) system (Tet-On H2B-GFP T-HEp3). These cells produce the nuclear H2B-GFP tag when in the presence of doxycycline, a more stable tetracycline analogue, and are unable to produce this tagged fusion protein in the absence of it. Due to the slow turnover of the Histone 2B protein, the GFP tag stays with cells for extended time, even if doxycycline is withdrawn; yet any cell division will quickly dilute the label down. We also generated MDA-MB-231 cell expressing both a permanently active GFP and, under the control of 5 hypoxic response elements and an oxygen-dependent degradation domain (5HRE-ODD)[1], a red mCherry fluorescent protein (MDA-MB-231-5HRE-ODD-mCherry-GFP)[2]. These cells always express the GFP and only in hypoxic conditions also express the red mCherry protein, which allows for detection of different oxygenation status. The MDA-MB-231 H2B-Dendra2 and ZR-75-1 H2B-Dendra2 cells express a fusion protein of Histone 2B and the photo-switchable fluorescent protein Dendra2, which can be irreversibly switched from green to a highly photostable red emission by near-UV light (400-405nm). We also utilized the Dendra2 tag in the polyoma middle t (PyMT) driven spontaneous breast cancer model in immunocompetent MMTV mice. These PyMT-Dendra2 tumors express the soluble Dendra2 tag in the cytoplasm. All plasmids also included the KanMX cassette that confers kanamycin resistance for clonal selection. Selection of successfully transfected clones through addition of G418 to culture medium was started 48 hours after transfection.

The T-HEp3 and MDA-MB-231 cells were cultured in DMEM medium (Corning Cellgro, US) with 10% FBS (Sigma, US, for T-HEp3 and Atlanta Biologicals, US, for MDA-MB-231) and 1% Pen/Strep (Gibco, US). ZR-75-1 cells were grown in RPMI medium (Corning Cellgro, US) with 10% FBS and 1% Pen/Strep (both same as for MDA-MB-231). The medium of Dendra2 transfected cells was supplemented with G418 (InvivoGen, US) for selection maintenance (MDA-MB-231: 1.0 mg/ml; ZR-75-1: 0.5 mg/ml; T-Hep3: 0.4 mg/ml).

For the plating efficiency assay, MDA-MB-231, ZR-75-1 and T-HEp3 cells were cultured 24h in normoxia or 24h, 48h or 72h in hypoxia, followed by plating (1000 cells/well) and culture in standard TC conditions. Colonies (clusters of >5cells) were counted at day 8, see **Fig S3E**. For the re-exposure to DFOM *in vitro*, T-HEp3 cells derived from the DFOM CAM tumors day 6 were plated on 8-well chamber slides (20,000 cells/well), let attach for 24h and then treated with PBS or DFOM (90 uM, Sigma Aldrich) for 24h before fixation and staining for GLUT1.

iNANIVID. The novel induction NANo IntraVital Device (iNANIVID) consists of two micro-fabricated glass halves with an etched chamber that can be loaded with hydrogel solutions containing different chemical compounds, which are released over time[2, 3]. The iNANIVIDs can be used in the in vivo chicken chorioallantoic membrane (CAM) model to influence the tumor microenvironment. We used iNANIVIDs loaded with 10mM doxycycline (Dox), 1mM desferrioxamine (DFOM, Hi-NANIVID) and phosphate buffered saline (PBS, PBS-NANIVID). DFOM is an iron-chelator and has previously been used extensively to mimic hypoxia. Iron is needed in the PHD mediated selective hydroxylation of HIF1α proline residues, which initiates proteosomal degradation. By blocking the degradation, DFOM leads to HIF1α accumulation within normoxic cells.

Chicken chorioallantoic membrane (CAM) and mouse models. In the MDA-MB-231 5HRE-ODD-mCherry-GFP xenograft experiments (**Fig 1A-C**), $2x10^6$ cells were subcutaneously injected in the inguinal mammary fat pad of athymic Foxn1 nu/nu mice and tumors were harvested at 1.5-2cm diameter and immediately snap frozen for IF staining.

For the CAM xenograft modeling we used premium specific pathogen-free (SPF), fertile, E8 days incubated embryonated chicken eggs supplied by Charles River Laboratories, Inc. For the label chase experiments with pimonidazole (HP1-100Kit, Hypoxyprobe, US) staining (**Fig D-F**), Tet-On H2B-GFP T-HEp3 cells were cultured in the presence of doxycycline (dox) in standard conditions to label all cells with H2B-GFP. $5x10^5$ cells were then seeded on the CAM and allowed to grow a tumor for 5 days without dox. 4 hours before tumor collection, 50 µL pimonidazole (40mg/mL) was injected into the amniotic fluid. After collagenase digestion of the tumors, cells were cytospun on charged slides and stained for pimonidazole adducts (Ab 4.3.11.3, Hypoxyprobe, US). For the iNANIVID inoculation, a layer of minced T-HEp3 tumor was seeded inside of a sterile Teflon ring on the chorioallantoic membrane. In this ring, on the first layer of tumor cells, the iNANIVID was inoculated, as shown in **Figure 2B-C**. Then a second layer of minced T-Hep3 tumor was added to cover the iNANIVID completely. The ring kept the mass of minced tumor together. Following tumor implantation, the CAMs were incubated for 3 and 6 days at 37°C before the tumor was recovered and the upper left quadrant was harvested for further analysis (area of influence, **Fig 2B**). The stereoscopic images were taken with a Zeiss Lumar V12.0.

For the intravasation CAM experiment (**Fig 4B-C**), 10^6 T-HEp3 Tet-On-H2B-GFP or MDA-MB-231-5HRE-ODD-mCherry-GFP cells were inoculated on the CAM. The CAMs carrying T-HEp3 Tet-On-H2B-GFP cells were treated with 5µg/ml doxycycline daily to sustain the H2B-GFP expression. Prior to inoculation the MDA-MB-231 cells were incubated for 72h in either normoxic (only GFP expressed) or hypoxic conditions (to activate the 5HRE-ODD-mCherry red fluorescing protein). After

48h, the egg membranes perpendicular opposite to the implantation site (lower CAM) were recovered and intravasated cells in the vasculature were detected and quantified. For the Tet-On H2B-GFP T-HEp3 label chase experiment, the cells were incubated with 1ug/ml Dox in normal tissue culture conditions. $1x10^5$ cells were seeded on CAMs and tumors were grown for 5 days, without Dox treatment. At day 5, 50µl of pimonidazole (40mg/ml) was injected into the embryos and incubated for 4 hours. The tumors were excised and immediately collagenased. The cells were fixed in 4% formaldehyde and cytospins were prepared.

For the in vivo extravasation experiment (**Fig 4D**), stably GFP expressing T-HEp3 cells were grown in monolayer in normoxia (21%O_2) or hypoxia (1%O_2) for 72h. Then, $3x10^5$ cells in 50µl PBS were tail vein injected in Foxn1 nu/nu mice. 24h later mice were sacrificed and the lungs were flushed with 3ml ice cold PBS to exclude blood form the analysis and dissected from the animals. Then, one lung of each animal was minced and collagenased for 30 min at 37°C. The total lung cell suspension (1ml) was manually screened for GFP+ cells using a direct fluorescence microscope (Nikon Eclipse Ti-S® equipped with an EXFO X-Cite 120® fluorescence illumination system) and the total cell number per animal calculated. All Foxn1 nu/nu mice were acquired from Harlan Sprague Dawley, US. For additional tail vein injection experiments, the indicated amounts of cells suspended in 50µl PBS were injected in Foxn1 nu/nu mice and the animals were sacrificed using CO_2 at the designated time points (**Fig 5+6+7**). All Foxn1 nu/nu mice were acquired from Harlan Sprague Dawley, US. For the *in vivo* label retention assays, the lungs of the mice were flushed with 3ml PBS and one lung was immediately mounted in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA, Inc.), snap frozen and stored in -80°C until further use. The other lung was minced and digested using collagenase-IA (Sigma-Aldrich, US) for 30 min at 37°C. In case of the TET-ON-H2B-GFP T-HEp3 label retention experiment, the resulting cell suspension was diluted to 10ml volume and the amount of tagged tumor cells (H2B-GFP, Dendra2 green/red) in three 100µl samples of each animal was manually counted using a fluorescence microscope (see above). Using the total number of lung lodged T-HEp3 cells obtained by re-inducing the TET-ON-H2B-GFP by culturing the total lung cell suspension with doxycycline for 16h (**Fig 5C**), the percentage of label retaining cells was calculated. The results are displayed as individual data points (one for each 100µl sample) in **Fig 5D**. For the MDA-MB-231-H2B-Dendra2 tail-vein experiment, the lungs were treated as above. Following digestion of the lungs, the amount of Dendra2 green cells was counted manually as above (Tet-on H2B-GFP T-HEp3 cells), followed by assessment of Dedndra2 red expression. Using these results, the total number of lung lodged MDA-MB-231 H2B-Dendra2 cells (Dendra2 green, **Fig 5G**) and the percentage of red label retaining cells (Dendra2 red, **Fig 5H**) was calculated per mouse.

In the chemotherapy experiment (**Fig 7 C-F**) we used 3.5mg/kg body weight cis-platin (NDC 63323-103-65, Fresenius Kabi, US) or equal volume of sterile, injection grade 0.9% NaCl solution for i.p. injection. Mice were weighted every other day before injection to account for possible weight-loss due to therapy.

For the spontaneous lung DTC experiments we used MMTV-PyMT-Dendra2 mice. The spontaneously luminal-like mammary tumors carry the photoswitchable Dendra2 protein (**Fig 7**). When the tumors were palpable in the fat pad, they were excised, minced and injected in the inguinal mammary fat pad of non-tumor bearing FVB mice, to eliminate the effect of multiple simultaneous tumors. After ca. 8 weeks, when the tumors were 1.5-2cm in diameter, the whole tumor was photo converted *in vivo* for 5min using a custom built 405nm light emitting, low heat diode. This method caused no thermal damage to the tissue and the wound was closed over the tumor. Seven days later the animals were sacrificed and the lungs were snap frozen in O.C.T. Compound for further analysis.
For the DFOM diffusion experiments, $0.5x10^6$ MDA- 231 HIF reporter cells were seeded on recipient CAMs and resulting tumor nodules were harvested 7 days later, minced and re-seeded in Teflon rings and implanted with NANIVIDs (**Fig S2 A-D**). After 3 days, whole CAMs with tumors were removed from the eggs and mCherry and GFP signals were imaged using a LSM 880 Zeiss confocal microscope.

Photo conversion. A custom built 405nm LED-Array (3A/20V) was constructed for photo conversion of the Dendra2 expressing cells *in vitro* and *in vivo*. Photo conversion of plated cells was carried out for 90-120 seconds, depending on the cell density and visually controlled for >95% photo conversion (**Fig 5E-M**). For the spontaneously disseminated PyMT-Dendra2 experiment, the skin covering the tumor was incised and temporarily retracted to expose the tumor, which was photo converted for 5min *in vivo*. On the lung slides, the Dednra2-GREEN signal was used to identify the DTCs (**Fig 7**). Using a Leica DM 5500 B microscope (Leica, Germany), the cells were imaged before and after 60 seconds irradiation with the DAPI channel (405nm), to photo convert additional Dendra2-GREEN protein to Dednra2-RED. The change in pixel intensity was measured using the Metamorph software. For red label retaining cells, the red pixel intensity in arbitrary units after PC was normalized to the value before PC. For not label retaining cells the absolute value for red pixel intensity in arbitrary units is used.

Immunohistochemistry (IHC) and Immunofluorescence (IF) assays. Paraffin embedded tissue sections were stained using the Vectastain® ABC-kit (Vector Laboratories, US) according to the manufacturer's instructions. See **Table S2** for complete list of antibodies. Briefly, following slide

hydration in Xylene and a graded alcohol series, slides were microwaved in 10mM citrate buffer for 12 minutes for antigen retrieval. Endogenous peroxidase activity was quenched by 3% H_2O_2. Binding of the primary antibody was carried out at 4ºC overnight, detection by secondary antibodies at room temperature for 1 hour. Liquid DAB was used as chromogen (Vector Laboratories, US). For IF stainings, paraffin embedded sections were first de-paraffinized and rehydrated and antigen retrieval was conducted as describes above, frozen sections were thawed to room temperature (RT) while covered with PBS. Cell membranes were permeabilized with 0.5% Triton X in PBS for 5 minutes at RT. Sections were blocked with 3% normal goat serum (NGS) in PBS for 60 min at RT prior to the first antibody incubation. Antibody binding was carried out at 4ºC overnight, followed by washing with PBS (3x5 min) and blocking for 60 min at RT. A secondary, fluorescent antibody reacting to the primary was applied for 1 hour in the dark at RT (all: Invitrogen, US). Slides were mounted with Pro-Long® Gold with DAPI (Life Technologies, US). The IF and IHC stainings were evaluated using a Leica DM 5500 B microscope with DAPI, GFP, Cy-3 and Y5 filter-cubes or a Leica TCS SP5 II confocal Laser-microscope (Leica, Germany).

TUNEL assay. Paraffin embedded tissue sections were stained using the *In Situ* Cell Death Detection kit (Roche) according to the manufacturer's instructions. Briefly, following slide dewaxation and rehydration in Xylene and a graded alcohol series, slides were microwaved in 10mM citrate buffer, pH 6.0 for 12 minutes. TUNEL reaction was performed at 37ºC fro 30 minutes and stopped by washing the slides with 0.3M NaCl 0.03M $Na_2C_6H_6O_7$. Vimentin staining was performed as described in the above section.

Scoring of IHC and IF assays using ImageJ and MetaMorph software. The positive area in the MDA-MB-231-5HRE-ODD-mCherry-GFP xenografts and the Glut1 staining (See table S2 for antibody information) of the T-HEp3 CAM tumors were evaluated using the open source processing software Fiji (ImageJ) (**Fig 1A-C, S1A-C, S2A-D, S3A**). The pictures were duplicated; one picture was thresholded to the staining appropriate intensities and a binary image was created. The area covered by positive staining was quantified in the original file using the binary image as a template. In the xenografts, the area covered by the specific antigen was normalized to the expression of mCherry for hypoxic areas and GFP for normoxic areas. The histograms were created using Fiji (ImageJ). For the diffusion analysis, pixel intensity of the red channel in >60 cell clusters was measured at indicated distances from the iNANIVID, as indicated above. For the xenografts, 20 high power fields were scored. For the Glut1 staining, 10 high power fields were scored per condition and time-point.

For the scoring of nuclear HIF1α and nuclear p27 in CAM tumor sections, the nucleus of T-HEp3 cells in 5-6 different fields per tumor were outlined and the integrated fluorescence intensity of the red channel (HIF1α) and from the green channel (p27) were quantified using MetaMorph software (Molecular devices). For HIF1α^{high} and p27^{high} nuclear levels we considered values >1.5X the negative control (**Fig S3B-D**).

Table S1: Stably transfected cell lines

Cell line	Plasmid	Base color	Color change (induced by)
T-HEp3	Tet-On-H2B-GFP	-	green (Dox)
	H2B-Dendra2	green	green & red (PC)
	GFP	green	-
MDA-MB-231	5HRE-ODD-mCherry-GFP	green	green & red (hypoxia)
	H2B-Dendra2	green	green & red (PC)
ZR-75-1	H2B-Dednra2	green	green & red (PC)

Dox = Doxycycline; PC = photo conversion by 405nm UV-light

Table S2: Antibodies, supplier and concentrations

Antigen	Species	Antibody	Supplier	Dilution
DEC2	Rabbit	sc-32853	Santa Cruz	1:75
Glut1	Rabbit	07-1401	Millipore	1:100
	Mouse	ab40084	abcam	1:200
NR2F1	Rabbit	27610002	Novus Biologicals	1:100
	Mouse	H8132	abcam	1:200
p27	Rabbit	D69C12	Cell Signaling	1:800
	Mouse	610241	BD Biosciences	1:100
pRb	Goat	sc-16671	Santa Cruz	1:75
HIF1 α	Rabbit	NB100-449	Novus Biologicals	1:100
Vimentin	Rat	MAB2105	R&D Systems	1:100
Cleaved C3	Rabbit	9664S	Cell Signaling	1:100
Pimonidazol	Mouse	4.3.11.3	Hypoxyprobe	1:50
murine CD45-PE	Rat	30-F11	eBioscience	1:100
H3K37me3	Rabbit	C36B11	Cell Signaling	1:500
H3K4me3	Rabbit	C42D8	Cell Signaling	1:500

Table S3: Primer sequences

Primer	Sequence
DEC2 for	5'-CTG-ATG-CTG-TTG-CTC-GGT-TA-3'
DEC2 rev	5'-TGC-AGA-CTC-TGG-GAC-ATC-TG-3'
GAPDH for	5'-CCC-CTG-GCC-AAG-GTC-ATC-CA-3'
GAPDH rev	5'-ACA-GCC-TTG-GCA-GCG-CCA-GT-3'
Glut1 for	5'-TTG-GCT-CCC-TGC-AGT-TTG-GC-3'
Glut1 rev	5'-CCC-CAT-AGC-GGT-GGA-CCC-AT-3'
HIF1α for	5'-CCT-CAG-TCG-ACA-CAG-CCT-GGA-3'
HIF1α rev	5'-CGG-CCT-AAA-AGT-TCT-TCT-GGC-TCA-3'
NR2F1 for	5'-GCC-TCA-AAG-CCA-TCG-TGC-TG-3'
NR2F1 rev	5'-CCT-CAC-GTA-CTC-CTC-CAG-TG-3'
p27 for	5'-GGT-TAG-CGG-AGC-AAT-GCG-CA-3'
p27 rev	5'-AAC-CGG-CAT-TTG-GGG-AAC-CGT-C-3'

Supplemental Figure Legends

Supplemental Figure 1: Quantification of the hypoxia-biosensor response, hypoxic adducts and influence of different oxygen tensions *in vitro*. A-C. Representative IF images and fluorescence histograms of MDA-231-HIF reporter cell xenografts in nude mice. Cells are GFP-tagged (green) and express mCherry (red) when hypoxic (5HR-ODD-mCherry). Sections were stained for (**A**) Glut1, (**B**) DEC2 and (**C**) NR2F1. Histograms show the percent fluorescence intensity normalized to maximum fluorescence for each channel over the boxed area (x-axis: distance, in arbitrary units). **D.** IF staining and quantification for pimonidazole adducts in T-HEp3 cell cytospins. Cells were *in vitro* cultured 72h in hypoxia (1% O_2) or normoxia (21% O_2) and then treated with 50µM pimonidazole for 3h. Pimonidazole adducts are exclusively detectable in hypoxic cells. Bars show mean±SD, n=4 independent experiments. Scale bar 25µm. **E.** Fold change of DEC2, GLUT1 and NR2F1 mRNA in T-HEp3, MDA-MB-231 and ZR-75-1 cells grown in different oxygen tensions (21%, 10%, 5%, 1% O_2) for 72 hours. Whole RNA was isolated from triple rinsed monolayers using TRIzol reagent (Invitrogen, US) according to the manufacturer's instructions. Following reverse transcription using MMuLV RT (NEB), quantitative qPCR for GLUT1, DEC2, NR2F1 and GAPDH was performed. GAPDH was used as a housekeeping gene. Primers were purchased from IDT. For the specific primer sequences see **Table S3**. Data points represent mean±SEM of n=3 independent experiments, each qPCR in triplicate; *p<0.05, **p<0.01; two tailed Student's t test. **F**. Images showing a 3-day-old Tet-On H2B-GFP T-HEp3 tumor on CAM with implanted PBS-iNANIVID (control). Top panel: stereoscopic image of the tumor in situ (4x). Panel **a:** merged image (bright field and fluorescence) of a representative tumor area showing no GFP activation. Box indicates area in panel b. **b:** Detail of GFP negative tumor area. Scale bar: 250µm; n=3 tumors.

Supplemental Figure 2: iNANIVID microenvironments and hypoxia responses in vitro and in vivo. A. Representative intra-vital images of mCherry and GFP signal of MDA-231 HIF reporter cells grown on the CAM for 3 days in indicated distances of PBS- or Hi-NANIVIDs. n=3 tumors; scale bar 10µm. **B.** Illustration of the placement of the NANIVID and the areas the fluorescence was measured. D=distal, M=middle distance, P=proximal. **C.** Quantification of overall (all distances) mCherry fluorescence in PBS- and Hi-NANIVID influenced MDA-231 HIF reporter tumors. Bars show mean±SEM of n=3 tumors per group, >180 cell cluster; *p<0.05, one tailed Student's t test. **D.** Red mCherry signal at different distances from Hi-NANIVID after 3 days *in vivo*. D=distal, M=middle distance, P=proximal to iNANIVID (see **Fig S2B**). Bars show mean±SD of n=3 tumors, per group, >60 cell clusters each distance. **E**. T-HEp3 tumors in CAMs implanted with DFOM or PBS iNANAVIDs were harvested at day 3 (D3) and day 6 (D6) and then fixed, sectioned (n=2 per group) and stained

for HIF1α detection via IF. Bottom Graph, quantification of HIF1α levels was done by taking images at 100x. Then HIF1α tumor sections (S2E) were scored in 10-12 different fields per condition. The nuclei of T-HEp3 cells were outlined and average integrated fluorescence intensity of the red channel (HIF1α) was quantified using MetaMorph software. Each dot indicated a scored cell in the sections. Note that at Day 6 there is no difference in HIF1α levels in tumor cells in PBS and DFOM influenced microenvironments. **** p<0.0001 Mann-Whitney test, n.s. non significant. **F.** Representative images of IHC staining for DEC2, NR2F1 and Glut1 in 6 days PBS- or Hi-NANIVID treated T-HEp3 CAM tumors. White arrows: negative, black arrows: positive cells. **G.** Quantification: fold change in positive cells from PBS- to Hi-NANIVID treated CAM tumors; bars indicate mean±SEM. Each point one HPF. Scale bar 10μm, n=2; Mann-Whitney test. **H.** T-HEp3 tumors grown for 6 days CAMs, when HIF1α and GLUT1 are downregulated, in the presence of DFOM iNANAVIDs were harvested (see schematic) digested and the single cell suspensions cultured for 24 hours with either PBS (n=4) or re-90 μM DFOM (n=3) in complete media. Cells on slides were fixed and stained for GLUT1 via IF. Images were taken at 40x and 100x. For staining of GLUT1 in culture slides, and 9-12 total fields were scored per condition. Images were thresholded to a baseline and T-HEp3 cells were outlined to quantify average integrated fluorescence intensity per cell in the green channel (GLUT1) using MetaMorph software. Each dot indicated a scored cell in the plates. Note that at DFOM treatment re-induced GLUT1 expression in cells that had downregulated the response to DFOM in vivo. **** p<0.0001 Mann-Whitney test. **I.** Representative images of IF staining for phospho-Rb (pRb) and cleaved Caspase3 (Cl-C3) of 3 days PBS- or Hi-NANIVID treated T-HEp3 CAM tumors. Scale bar 50μm. For quantification see **Fig 2G+H**. **J.** Representative low-magnification images of H&E stained T-HEp3 CAM tumors treated for 6 days with PBS- or Hi-NANIVID or no iNANIVID at all. n=3. Scale bar 50μm.

Supplemental Figure 3: Knock-down controls, plating efficiencies in hypoxia, staining controls and PC controls using the DENDRA2 system. A. Images and quantification of 3 and 6 days PBS- or Hi-NANIVID treated T-HEp3 CAM tumors stained for Glut1. Glut1 expression was measured in a binary image of the staining and quantifying the area covered by Glut1 signal (ImageJ). Scale bar 50μm. Mean±SD; n=5 low power images (10x) per tumor; **p<0.01, Mann-Whitney test. **B.** Representative images of HIF1α and p27 levels in PBS and DFOM CAM sections (day 3) used as a key to score the high and low levels of p27 and HIF1α in the nuclear region**. C.** Quantification (integrated intensity) of nuclear levels of p27 and HIF1α in CAM sections of PBS (blue symbols) and DFOM-treated (red symbols) tumors (day 3); black symbols negative control – background signal.

Note the higher levels of both antigens in DFOM exposed tumor cells. Lower graph, percentage of $p27^{high}$/$HIF1a^{high}$ cells (high signal was determined as signal 1.5X higher than neg.cont.) in CAM tumors (n=4). p by Mann-Whitney. **D**. Pearson's correlation between nuclear p27 and nuclear HIF1α in CAM tumors. Note that there is a strong correlation between HIF1α induction and the growth arrest of tumors cells marked by high p27 levels. **E.** Quantification of H3K4me3 staining in normoxic (GLUT1 negative) and hypoxic (GLUT1 positive) areas of n=3 T-HEp3 CAM tumors (see **Fig 3D-E**). Bars show mean ± SEM; two tailed Student's t test. **F.** Relative NR2F1 mRNA expression of T-HEp3 cells transfected 24h with 50nM NR2F1 siRNA compared to siControl (siCtr) transfected cells. Cells were cultured 24h in normoxic (21% O_2) or hypoxic (1% O_2) tissue culture conditions. Cells from this pool were seeded on CAMs, see **Fig 3G**. PCR in triplicate, bars show mean ± SD, n=4; *p<0.05, **p<0.01, two tailed Student's t test. **G.** Relative HIF1α mRNA expression of T-HEp3 cells transfected 24h with 20nM NR2F1 siRNA compared to siControl (siCtr) transfected cells. Cells were cultured 24h in normoxic (21% O_2) or hypoxic (1% O_2) tissue culture conditions. Cells from this pool were seeded on CAMs, see **Fig 3H**. PCR in triplicate, bars show mean ± SD, n=3; ****p<0.0001, two tailed Student's t test. **H.** Graphs show plating efficiency (PE) for 24h normoxic (N), 24h hypoxic (24h), 48h hypoxic (48h) and 72h hypoxic (72h) pre-treated cells. Bars show mean ± SEM of n=4 independent experiments; *p<0.05, two tailed Student's t test. **I.** Relative NR2F1 mRNA expression of ZR-75-1 H2B-Dendra2 cells transfected 24h with 50nM NR2F1 or control siRNA and cultured in normoxia (21% O_2) or hypoxia (1% O_2) for 72h. Cells were then photo converted and seeded in 3D Matrigel (see **Fig 5K-M**). Bars show mean NR2F1 level ± SD; n=4; *p<0.05, one tailed Student's-t test.

Supplemental Figure 4: Controls for Vimentin specific detection, P-Rb-detection in DTCs, Controls for Dendra2 photoconversion and NR2F1 expression in PyM tumors,

A. Gray scale image of mock tail vein injected (no T-HEp3 cells, only PBS) Foxn1 nu/nu mouse lung stained for human vimentin and DAPI. Scale bar 25µm (40x) and 10µm (100x). Vimentin antibody showed no reaction with mouse lung tissue. Boxed are in 40x indicates higher magnification shown in 100x. **B.** Representative images and quantification of T-HEp3 DTCs in mouse lungs at day 10, treated with cis-platin, stained for p-Rb (green), human vimentin (red) and DAPI (blue). graph on the right shows mean ± SEM, n=3 mice, >50 cells per animal; *p=<0.05, one tailed Student's t test. **C.** Frozen section of PyMT-Dendra2 PT photo converted with UV channel for 15sec (see dashed line in merged image). Scale bar 250µm. **D.** Representative images of a spontaneous MMTV-PyMT-Dendra2 (green) lung micro-metastasis stained for macrophage marker CD45 (red) and DAPI (blue). No significant green Dendra2 signal was detected in CD45 positive macrophages. Asterisks indicate cytoplasmic aggregates of Dendra2 protein observed in MMTV-PyMT-Dendra2 cells, as in **Fig 7H-I**.

n=3 mice; scale bar 10µm. **E.** Increase in red pixel intensity of 34 label retaining (LRC) and 34 not label retaining (NLRC) PyMT-Dendra2 lung DTCs measured using MetaMorph. Graph Label Retaining Cells: fold increase normalized to red pixel intensity before photo conversion of slide. Graph Not Label Retaining Cells: absolute red pixel intensity in arbitrary units, no red pixel intensity before PC. See **Figure 7I** for cells marked with #. **F.** Quantification of all cells positive for NR2F1 in PyMT-Dednra2 primary tumor (PT) and spontaneous lung DTCs (see **Fig 7G-K**). Bars show mean ± SD. 10 HPF per PT, 187 lung DTCs, n=3 mice.

References

1. Harada, H. *et al.* The combination of hypoxia-response enhancers and an oxygen-dependent proteolytic motif enables real-time imaging of absolute HIF-1 activity in tumor xenografts. *Biochem Biophys Res Commun* **360**, 791-796 (2007).
2. Wang, Y. *et al.* Direct visualization of the phenotype of hypoxic tumor cells at single cell resolution in vivo using a new hypoxia probe. *Intravital*, 00 (2016).
3. Raja, W.K. *et al.* Development path and current status of the NANIVID: a new device for cancer cell studies. *Journal of micro/nanolithography, MEMS, and MOEMS : JM3* **11** (2012).