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Supplemental Information

PHD2 Targeting Overcomes Breast Cancer Cell Death upon Glucose Starvation in a PP2A/B55α-Mediated Manner

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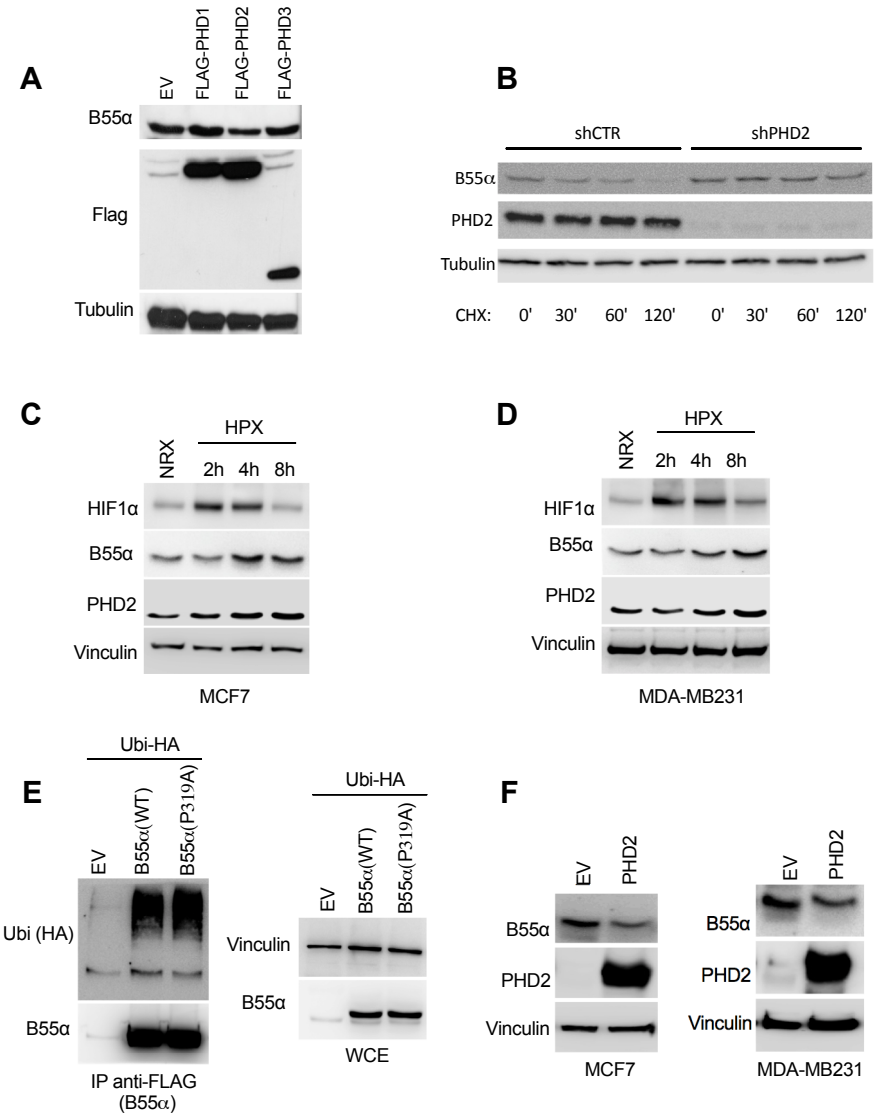
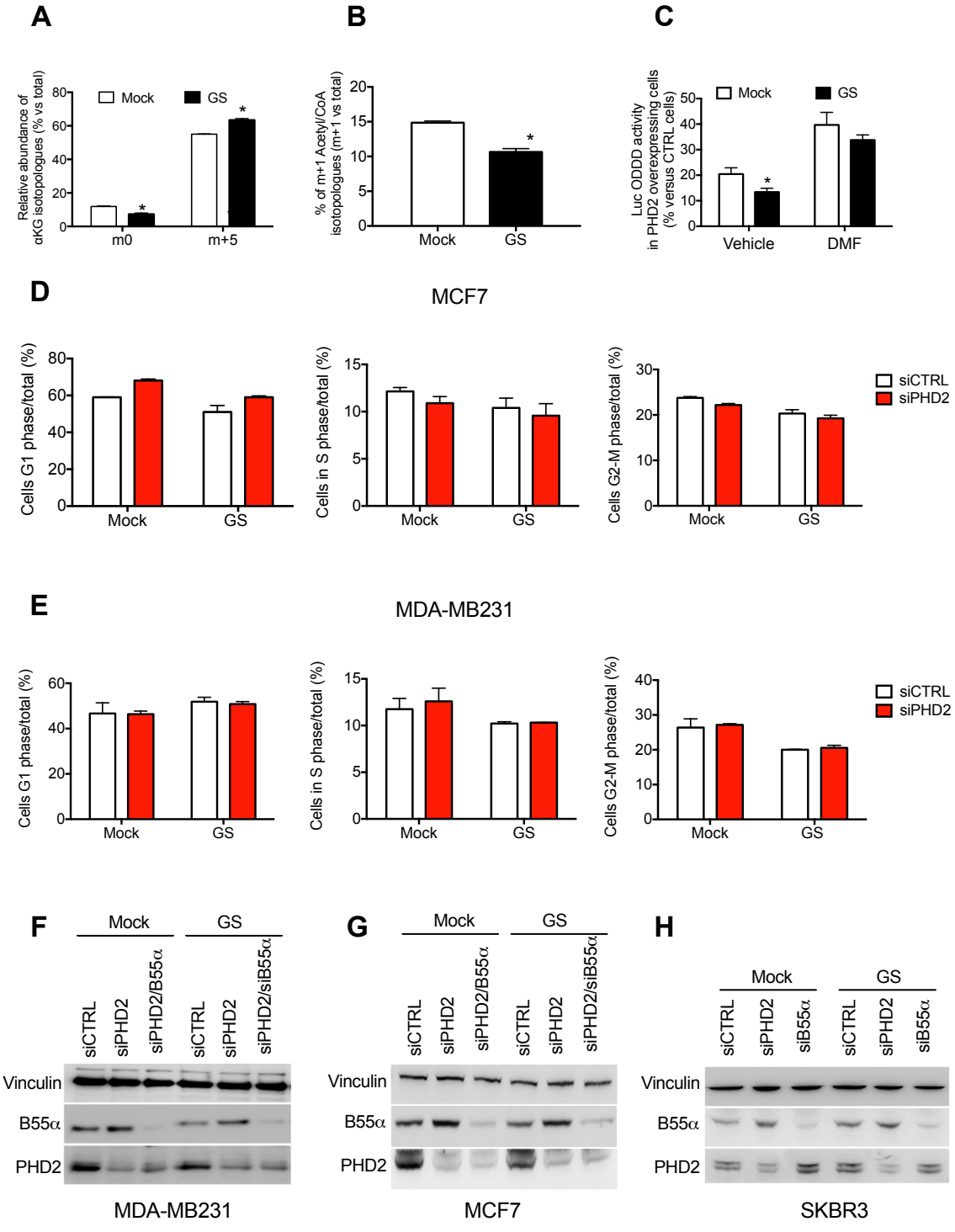


Supplementary Figure S1. Related to Figure 1. PHD2 degrades B55α. A, HEK293T cells were transfected with B55α alone or in combination with FLAG-PHD1, FLAG-PHD2 or FLAG-PHD3. After 24 hours, WCEs were collected and analyzed by WB. **B,** HEK293T cells stably expressing a shRNA specifically targeting PHD2 (shPHD2) or a scramble control (shCTR) were transfected with B55α. After 16 hours, cells were treated with 100 μg/ml cycloheximide for the indicated time points; WCEs were then collected and analyzed by WB. **C and D,** MCF7 (C) and MDA-MB231 (D) were incubated in hypoxia (HPX) or normoxia (NRX) for the indicated time points. WCE were collected and analyzed by WB. **E,** HEK293T cells were transfected with Ubiquitin-HA alone (EV) or in combination with FLAG-B55α(WT) or FLAG-B55α(P319A). After 16h transfection, cells were lysed and immunoprecipitated using anti-flag M2 beads to detect Ubiquitin-HA. WB on WCEs is shown on the right. **F,** MCF7 and MDA-MB231 were transfected with either an empty vector (EV) or PHD2. After 24 hours, cells were lysed and analyzed by WB.


A
Relative abundance of αKG isotopologues (% vs total)
Mock
GS
m0
m+5
B
% of m+1 Acetyl/CoA isotopologues (m+1 vs total)
Mock
GS
C
Luc ODDD activity in PHD2 overexpressing cells (% versus CTRL cells)
Mock
GS
Vehicle
DMF
D
MCF7
Cells G1 phase/total (%)
Cells in S phase/total (%)
Cells G2-M phase/total (%)
Mock
GS
siCTRL
siPHD2
E
MDA-MB231
Cells G1 phase/total (%)
Cells in S phase/total (%)
Cells G2-M phase/total (%)
Mock
GS
siCTRL
siPHD2
F
Mock
GS
siCTRL
siPHD2
siPHD2/B55α
siCTRL
siPHD2
siPHD2/siB55α
Vinculin
B55α
PHD2
MDA-MB231
G
Mock
GS
siCTRL
siPHD2
siPHD2/B55α
siCTRL
siPHD2
siPHD2/siB55α
Vinculin
B55α
PHD2
MCF7
H
Mock
GS
siCTRL
siPHD2
siB55α
siCTRL
siPHD2
siB55α
Vinculin
B55α
PHD2
SKBR3

Supplementary Figure S2. Related to Figure 2. In glucose starvation, PHD2 activity is increased and promotes B55α degradation. A and B, MCF7 cells were cultured in complete (Mock) or glucose deprived (GS) medium for 16h. In both growth media, ^{12}C-glutamine (*i.e.* regular glutamine) was absent and replaced with either U-^{13}C-glutamine (in A) or 5-^{13}C-glutamine (in B). LC-MS was then performed on biological triplicates as described in the Experimental procedures in order to measure in A the levels of unlabeled α-KG (m0, derived from carbon sources other than U-^{13}C-glutamine) or labeled α-KG (m+5, derived from U-^{13}C-glutamine) and in B the levels of labeled Acetyl-CoA (m+1, derived from 5-^{13}C-glutamine). **C,** MCF7 were transfected with plasmids expressing ODDD (oxygen degradation domain) alone (CTR) or together with PHD2 (PHD2). After 8 hours, cells were refreshed with complete (Mock) or glucose deprived (GS) medium in presence of vehicle or dymethil-fumarate (DMF). After 16 hours, cells were lysed and luciferase activity was measured and normalized for protein concentration. **D and E,** MCF7 (D) and MDA-MB231 (E) cells were transfected with siRNA targeting PHD2 (siPHD2) or a scramble control (siCTR). After 16 hours, cells were cultured in complete (Mock) or glucose deprived (GS) medium for other 24 hours. Cell cycle phases were assessed by propidium iodide staining and FACS analysis. **F-H,** WB control of MDA-MB231 (F), MCF7 (G) and SKBR3 (H) used for FACS analysis in main figure 3D, 3E, 3I,J respectively. *$p<0.05$ versus all other conditions in **C**. All graphs show mean ± SEM.

Supplemental Experimental Procedures

Plasmids, siRNA and antibodies. In the overexpression experiments the following plasmids were used: pcDNA3-PHD2-FLAG, pcDNA3-PHD1-FLAG, pcDNA3-PHD3-FLAG, pcDNA3 empty vector, pLA-B55α-FLAG, pcDNA3-B55α-HA, pLAB55α(P136A), pLA-B55α(P259A) and pLA-B55α(P319A), pcDNA-UbiquitinHA. Commercially available siRNAs were purchased from Life Technologies and their sequences or assay IDs are listed below:

For B55α: PPP2R2AHSS108370, PPP2R2AHSS108371 (used in combination);

For PHD2: EGLN1HSS123076, EGLN1HSS182577 (used in combination);

For scramble: Stealth RNAi™ siRNA Negative Control Lo GC, 12935-200. All the B55α mutants (B55α ^{P159A}, B55α^{P236A}, B55α^{P319A}) were generated by using QuikChange II XL Site-Directed Mutagenesis (Promega), according to manufacturer's instructions. The following forward (Fw) and reverse (Rv) primers were respectively used:

P159A Fw: 5'GAGTGCCAGTCTTTAGGGCTATGGATCTAATGGTTG3', Rv: 5'CAACCATTAGATCCATAGCCCTAAAGACTGGCACTC3';

P236A Fw: 5'CAGCAGCAGAATTTCATGCAAACAGCTGTAACAC3' , Rv: 5'GTGTTACAGCTGTTTGCATGAAATTCTGCTGCTG3';

P319A Fw: 5'CTTAAATATGGAAAACAGGGCTGTGGAAACATACCAGGTG3',

Rv: 5'CACCTGGTATGTTTCCACAGCCCTGTTTTCCATATTTAAG3'.

To generate stable knockdown MDA-MB231 cells, mir-155 miRNA/microRNA lentiviral vectors (shPHD2 and respective scramble (SIMA)) carrying the following shRNA were used:

For PHD2:

TGCTGTCAACATGACGTACATAACCCGTTTTGGCCACTGACTGACGGGTTATG
CGTCATGTTGA

For the SIMA:

TGCTGCATGAATATCTCTGTCTCCTTGTTTTGGCCACTGACTGACAAGGAGAC
AGATATTCATG.

For western blot analysis, the following antibodies were used: PPP2R2A (B55α) (Clone 2G9, Cell Signaling); PP2A, Aβ subunit (Santa Cruz Technology); PP2A, Cα subunit (Santa Cruz Technology); EGLN1 (human PHD2) (NB-100-137, Novus Biological); Vinculin (Monoclonal anti-Vinculin, V9131, SIGMA Aldrich); Tubulin (HRP-conjugated anti-beta-tubulin, Abcam); FLAG (Monoclonal anti-FLAG, SIGMA Aldrich).

Western Blot analysis. Protein extraction of both cell or tumor samples was performed by using RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche). Lysates were incubated on ice for 30 minutes before centrifuging 15 minutes at 4°C to remove cellular debris. Supernatants were subsequently collected. Protein concentration of cell extracts was determined by using bicinchoninic acid (BCA) reagent (Pierce) according to the manufacturer's instructions. Protein samples were denaturated by adding loading buffer 6X (β-mercaptoetanolo 0,6 M; SDS 8%; Tris-HCl 0,25 M pH 6,8; glycerol 40%; Bromophenol Blue 0,2%), incubated at 95°C for 5 minutes. After electrophoresis, proteins were transferred onto a nitrocellulose membrane using the iBlot® Dry Blotting System (Invitrogen) according to manufacturer's instructions. The membranes were blocked for non-specific binding in 5% non-fatty dry milk in Tris Buffered Saline-Tween

0.1 % (50 mM Tris HCl ph 7.6, 150 mM NaCl, 0.1% Tween; TBS-T) for 1h at room temperature (RT) and incubated with primary antibody for 2h at RT or overnight (ON) at 4°C. After incubation with the indicated primary antibodies, the membranes were washed for 15 minutes in TBS-T and incubated with secondary antibody (1/5000 in 5% non fatty dry milk in TBS-T) for 50 minutes at RT. The following secondary antibodies were used: goat anti-mouse and goat anti-rabbit (Santa Cruz biotechnology). The signal was visualized with Enhanced Chemiluminescent Reagents (ECL; Invitrogen) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) with a digital imager (ImageQuant LAS 4000, GE Health Care Life Science Technologies).

Mass Spectrometry. To identify B55α hydroxylation sites, overexpression of the FLAG-tagged protein of interest has been performed in HEK293T cells. Cells were harvested, lysed in extraction buffer and 3 mg of total protein extract was used for immunoprecipitation using anti-FLAG M2 affinity beads, according to protocol (Sigma-Aldrich). Immunoprecipitated proteins were separated by SDS-PAGE and stained using Coomassie. Gel bands of interest were excised and washed several times with water and acetonitrile, and completely dried in a SpeedVac. Subsequently, an in-gel trypsin digest was performed using sequence-grade modified trypsin, porcine (Promega, Madison, Wi USA) and samples were incubated overnight at 37°C. The supernatants containing the peptides were then isolated, transferred to MS-compatible vials and acidified with trifluoroacetic acid (TFA) (pH < 3). The obtained peptide mixtures were introduced into an LC-MS/MS system; the Ultimate 3000 RSLC nano (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) for analysis. Peptides were first loaded on a trapping column (made

in-house, 100 mm internal diameter (I.D.) x 20 mm, 5 mm C18 Reprosil-HD beads, Dr. Maisch, Ammerbuch-Entringen, Germany). After back-flushing from the trapping column, the sample was loaded on a reverse-phase column (made in-house, 75 μm I.D. x 150 mm, 3 μm C18 Reprosil-HD beads, Dr. Maisch). Peptides were loaded with solvent A (0.1% TFA, 2% acetonitrile) and separated with a linear gradient from 98% solvent A' (0.1% formic acid in water) to 55% solvent B' (0.1% TFA, 80% ACN) at a flow rate of 300 nl/min followed by a wash reaching 100% solvent B'. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the ten most abundant peaks in a given MS spectrum. In the LTQ-Orbitrap Velos, full scan MS spectra were acquired in the Orbitrap at a target value of 1E6 with a resolution of 60,000. The ten most intense ions were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 40 s. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. From the MS/MS data in each LC run, Mascot Generic Files were created using Distiller software (version 2.4.3.3, Matrix Science, www.matrixscience.com/Distiller). While generating these peak lists, grouping of spectra was allowed in Distiller with a maximum intermediate retention time of 30 s and a maximum intermediate scan count of 5 was used where possible. Grouping was done with 0.005 Da precursor tolerance. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks. There was no deisotoping and the relative signal to noise limit was set at 2. These peak lists were then searched with Mascot search engine (MatrixScience) using the Mascot Daemon interface (version 2.3.01, Matrix Science). Spectra were searched against the Swiss-Prot database restricted to *Homo sapiens* (SwissProt 2012_04, 20.324 protein sequences). Variable modifications were set to pyro-

glutamate formation of amino-terminal glutamine, acetylation of the protein N-terminus, oxidation of methionine, hydroxylation of proline, propionamidation of cysteine. Tolerance on precursor ions was set to ± 10 ppm (with Mascot's C13 option set to 1) and on fragment ions to ± 0.5 Da. The peptide charge was set to 2+, 3+, and the instrument setting was put on ESI-TRAP. Enzyme was set to trypsin, allowing for one missed cleavage, also cleavage was allowed when arginine or lysine is followed by proline. Only peptides that were ranked one and scored above the threshold score, set at 99% confidence, were withheld. All data management was done by ms_lims (PMID: 20058248).

Liquid chromatography mass spectrometry. Polar metabolites were measured by using a Dionex UltiMate 3000 LC System (Thermo Scientific) in-line connected to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). 20 ul of sample was injected and loaded onto a Hilicon iHILIC-Fusion(P) column (Achrom). A linear gradient was carried out starting with 90% solvent A (LC-MS grade acetonitrile) and 10% solvent B (10 mM ammoniumacetate pH 9.3). From 2 to 20 minutes the gradient changed to 80% B and was kept at 80% until 23 min. Next a decrease to 40% B was carried out to 25 min, further decreasing to 10% B at 27 min. Finally 10% B was maintained until 35 min. The solvent was used at a flow rate of 200 υl/min, the columns temperature was kept constant at 25 degrees Celsius. The mass spectrometer operated in negative ion mode, settings of the HESI probe were as follows: sheath gas flow rate at 35, auxiliary gas flow rate at 5 (at a temperature of 260 degrees Celsius). Spray voltage was set at 4.8 kV, temperature of the capillary at 300 degrees Celsius and S-lens RF level at 50. A full scan (resolution of 140.000 and scan range of m/z 50-1050) was applied. Data was processed using Thermo

Quan software (XCalibur, Thermo Scientific). Abundance of metabolites is expressed as extracted ion chromatogram (EIC).

Gas chromatography mass spectrometry. To the dried fractions 20 µl of Mox solution (2%, methoxyamine hydrochloride) was added, the reaction was carried out for 90 min at 37°C. Next, 50 uL of TBDMS (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane) was added for 60 min at 60°C. The reaction mixtures were centrifuged for 15 min at 20.000 x g and the supernatant was transferred to Agilent glass vials with conical inserts. GC-MS analyses were performed using an Agilent 7890A GC equipped with a HP-5 ms 5% Phenyl Methyl Silox (30 m - 0.25 mm i.d. - 0.25 µm; Agilent Technologies, Santa Clara, California, USA) capillary column, interfaced with a triple quadruple tandem mass spectrometer (Agilent 7000B, Agilent Technologies) operating under ionization by electron impact at 70 eV. The injection port, interface and ion source temperatures were kept at 230 °C. Temperature of the quadrupoles was maintained at 150°C. The injection volume was 1 µl, and samples were injected at 1:5 split ratio. Helium flow was kept constant at 1 ml/min. The temperature of the column started at 90 °C for 5 min and increased to 150 °C at 10 °C/min, to 270 °C at 20 °C/min, and to 300°C at 30 °C/min. Next, column regeneration was carried out for 3 min at 325°C. The GC/MS/MS analyses were performed in Multiple Reaction Monitoring (MRM) mode. Data analysis was performed using the Agilent Masshunter Quan software. Abundances of metabolites are expressed as area under the curve.

Data Analysis 13C tracer experiments. Correction for natural isotope abundances was carried out as described by Fernandez et al., 1996 (Fernandez et al., 1996) and fractional

labeling was calculated by using an *in-house* software tool as previously described (Buescher et al., 2015).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR). To assess gene expression, RNA from MCD-MB231 seeded in triplicate in 12-well plates was extracted with a RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Reverse transcription to cDNA was performed by using Quantitect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. cDNA, primer/probe mix and TaqMan Fast Universal PCR Master Mix were prepared in a volume of 10 ml according to manufacturer's instructions (Applied Biosystems). qRT-PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR system. Pre-made assays were purchased and their assay IDs are: PPP2R2A (B55α), Hs.PT.58.25465949 from IDT; bActin, Hs.PT.39a.22214847 from IDT; EGLN1 (PHD2): Hs00254393_m1 from Applied Biosystem.

FACS. Supernatants from MCF7, MDA-MB231 and SKBR3 cells cultured in complete or glucose-free DMEM were collected in order to keep the floating dead cells. Adherent cells were subsequently trypsinized, added to the supernatant previously collected and centrifuged at 300g for 5 minutes. After one wash with PBS, cells were fixed with 1 ml of 70% ethanol. Cells were incubated 2h or overnight at 4°C, prior to another centrifugation at 300g for 5 minutes. Supernatant was removed and the pellet was resuspended in 200 µl of PBS, containing 500 µg of RNase (10 mg/ml). 200 ml of Propidium Iodide (0,1mg/ml) was added to a final volume of 400 µl. Samples were incubated for 1-2 h at 37°C and subsequently analyzed by Fluorescence-Activated Cell Sorting by using FACS Verse (BD Bioscience).

Histology. To obtain serial 7-µm-thick sections, tissue samples fixed in 2% PFA overnight at 4°C, dehydrated and embedded in paraffin. Paraffin slides were first rehydrated to further proceed with antigen retrieval in citrate solution (DAKO). Apoptotic cells were detected by the TUNEL method, using the ApopTag peroxidase in situ apoptosis detection kit (Millipore) according to the manufacturer's instructions. Microscopic analysis was done with an Olympus BX41 microscope and CellSense imaging software. The analysis was performed by acquiring 4-6 fields per sections on 5 independent sections from the same biological tissue sample. The values in the graphs represent the average of the means of, at least, 5 samples and the standard error indicates the variability among the different samples.

Supplemental References

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