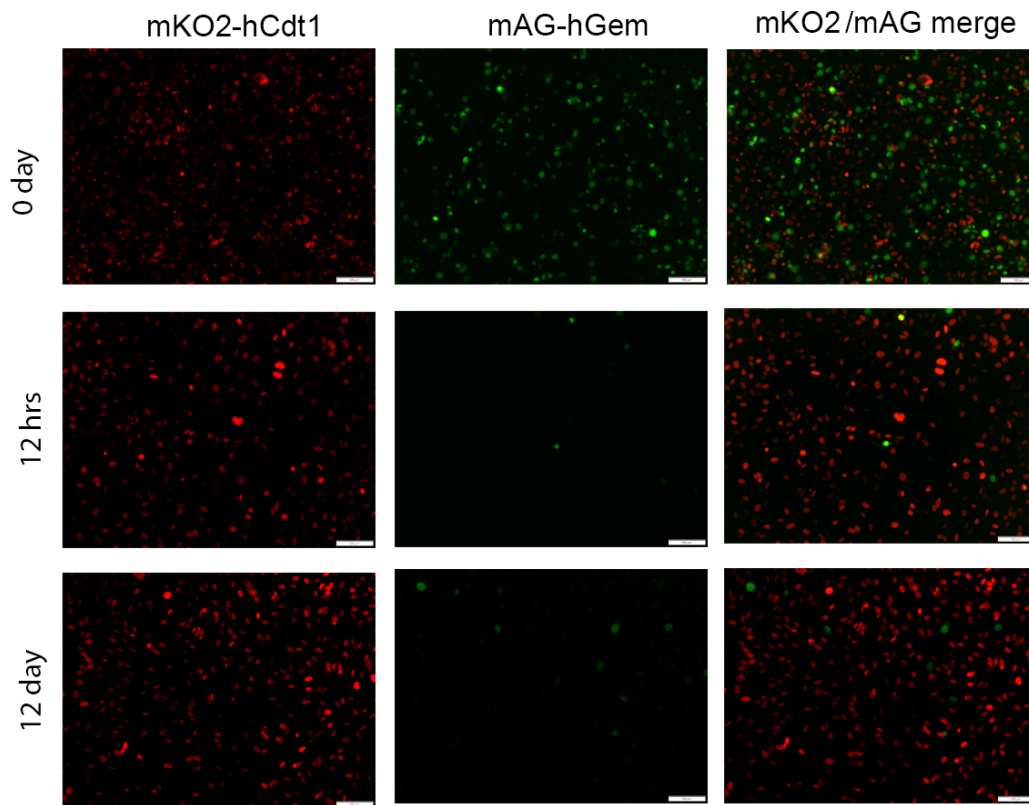


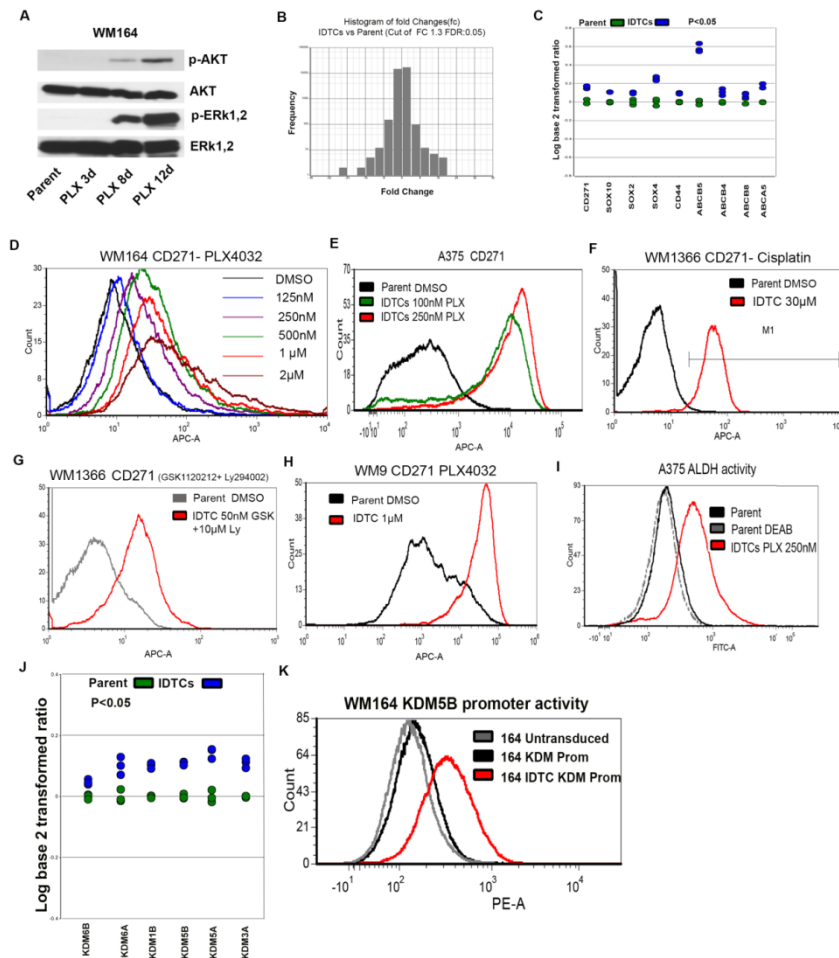
Supplemental Materials

Figure S1 – Figure S7

Supplemental Materials and Methods

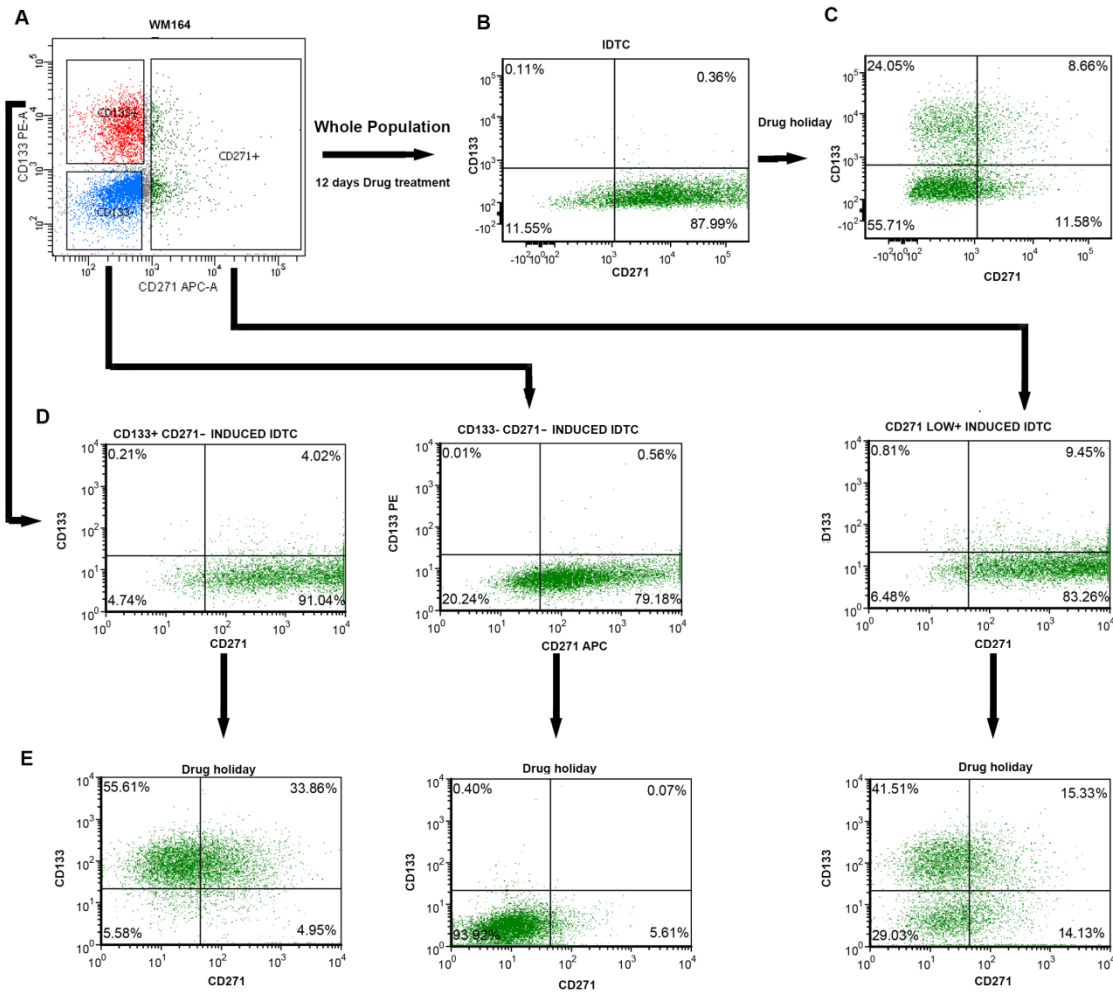


Supplemental Figure 1 (Related to Figure 1): Cell cycle analysis with the FUCCI system WM164 cells were transduced with the FUCCI system [1, 2] to analyse cell cycle progression when exposed to PLX4032 (500nM) at different time points. **mKO2-hCdt1 (red)** indicates G1 phase and **mAG-hGem (green)** S/G2/M phase. **mAG⁺mKO2⁺ double-positive (yellow)** indicates early S phase.'



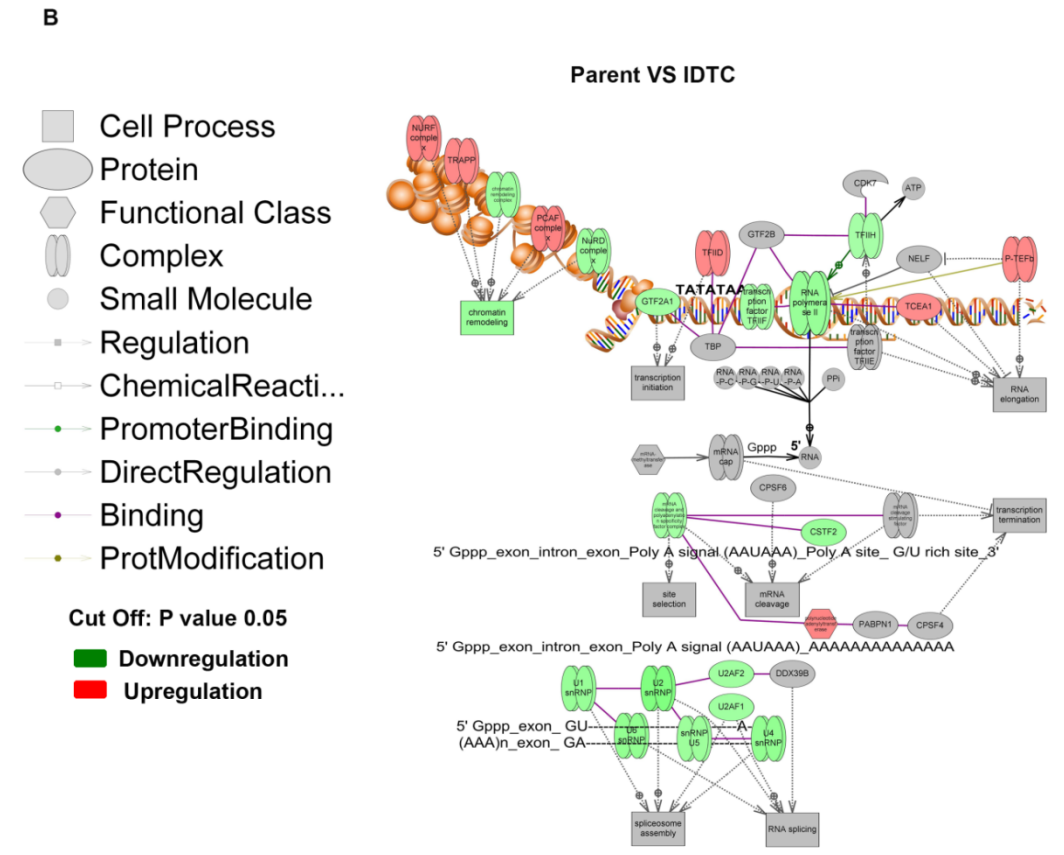
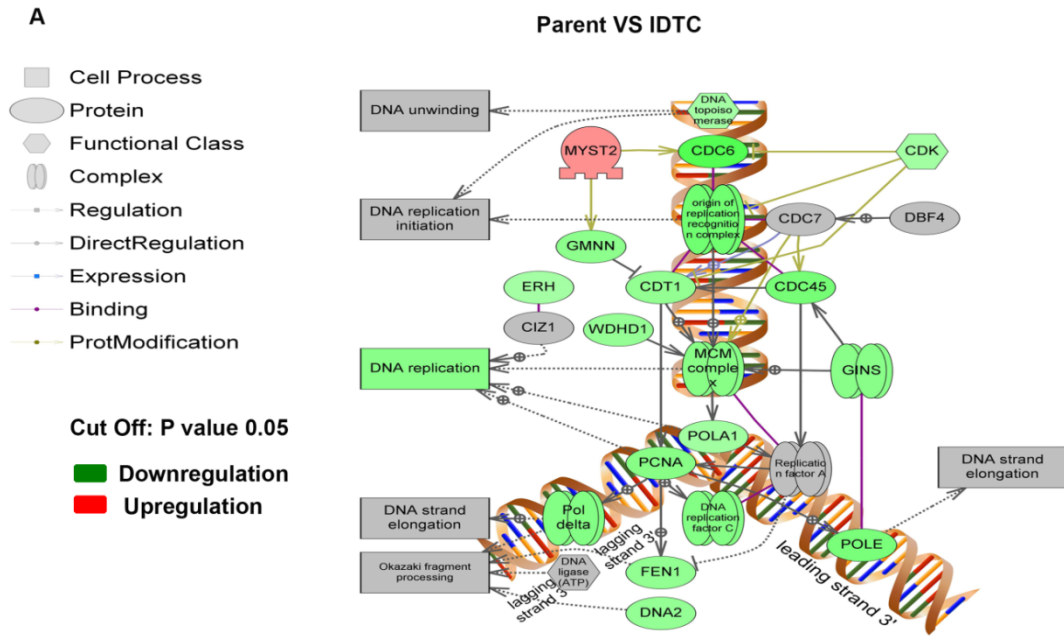
Supplemental Figure 2 (Related to Figure 2)

(A) Time course of ERK and AKT phosphorylation in IDTCs as to parental cells determined by immunoblotting to detect phosphorylated ERK1,2 (p-ERK1,2 (Thr202/Tyr204)), total ERK (T-ERK1,2), phosphorylated AKT (p-AKT (Ser473)), and total AKT (T-AKT). (B) Histogram representing frequency of genes based on microarray analyses showing the representative fold change difference in WM164 IDTCs vs parental cells microarray with a p-value of <0.0180382 (=passing multiple testing of FDR of 5%) and a minimum fold change between 1,3 to -1,3 within the dataset. (C) Selected genes which have been implicated in cancer stemness and drug efflux from microarray analyses, compared for the Log 2 transformed ratio of WM164 IDTCs (blue) to that of the parental population (green). The mean values of parental cells were equalized to zero, and each dot represents one of the triplicates. (D) WM164 cells exposed to various doses of PLX4032 analyzed for their CD271 expression by flow cytometry. (E) A375 Melanoma (BRAF mutant) parental cells and cells exposed to 100nM /250nM PLX4032 (IDTCs) analyzed for their CD271 expression by flow cytometry. (F, G) WM1366 melanoma cells (NRAS mutant) cells treated with either DMSO (Parental) or 30 μ M cisplatin or a combination of GSK1120212 (50nM) (MEK inhibitor) and Ly294002 (10 μ M) (Pi3K/AKT inhibitor) (IDTCs) for a period of 12 days analyzed for their CD271 expression. (H) WM9 melanoma cells (BRAF mutant) treated with either DMSO (parental) or 1 μ M PLX4032 (IDTCs) exposed for 12 days analyzed for their CD271 expression. (I) A375 250nM IDTCs analyzed for their ALDH activity in comparison to parental cells. (J) Selected histone demethylating genes from microarray analyses comparing the Log 2 transformed ratio of WM164 IDTCs (blue) to the parental cells (green). The mean value of parental samples was equalized to zero and each dot represents one of the triplicates. (K) WM164 cells transduced with the KDM5B promoter construct expressing DS red analyzed for their promoter activity in both parental (164 KDM prom) and IDTC (164 IDTC KDM prom) state in comparison to untransduced cells.



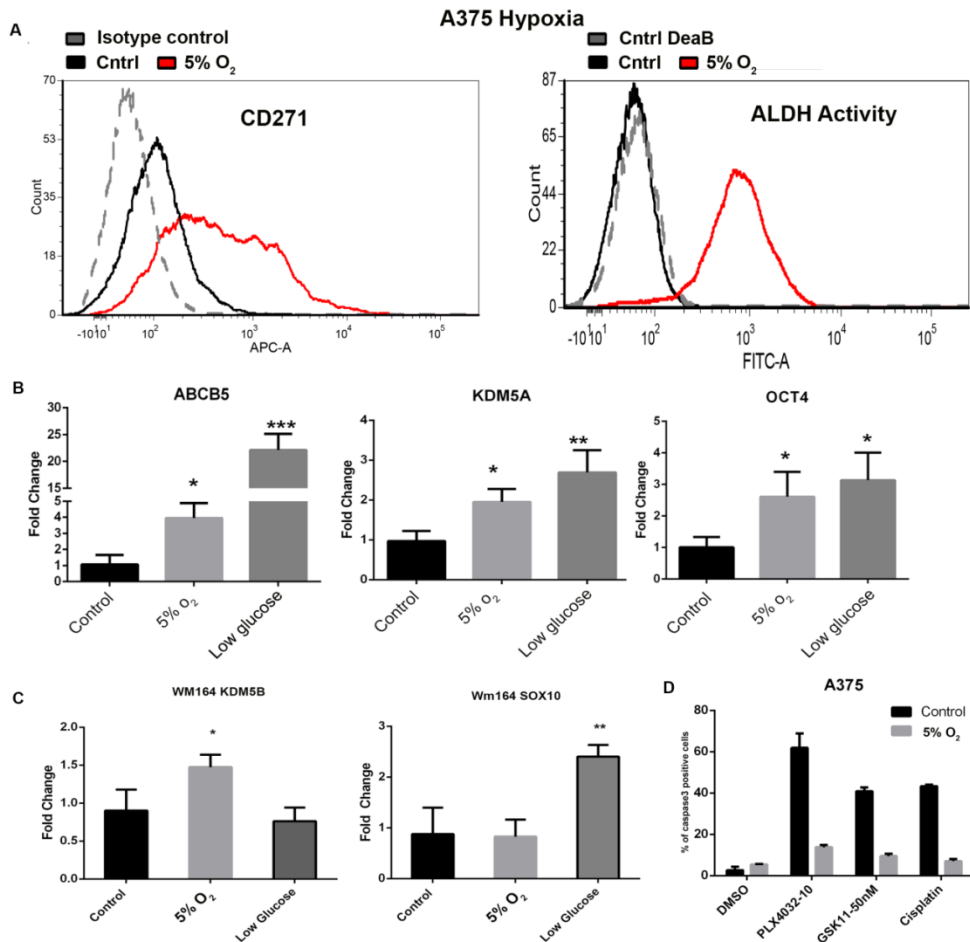
Supplemental Figure 3 (Related to Figure 2): Induction of IDTCs from various subpopulations and their reversal by drug holidays

WM164 parental cells analysed for putative stem cell markers CD271 and CD133 showed 2 distinct subpopulations either positive or negative for CD133. Both these subpopulations almost equally harboured low CD271+ve cells (A). Transition into the IDTC state led to a loss of CD133 and significant increase in CD271 expression (B) which could be reversed to the parental state by a 7 day drug holidays (C). Further each of this subpopulations (CD133-/CD271-, CD133+/ CD271-, CD271 low+/ CD133+/-) were sorted out and transformed into the IDTC state by PLX4032 exposure which again led to a similar phenotype independent of the origin of subpopulation (D). Drug holidays lead to a reversal of expression markers specific of that subpopulation in case of CD133-/ CD271-, CD133+/ CD271- sorted cells while all of them regained a population of cells expressing low levels of CD271(E-Left and Middle). CD271 low+/ CD133+/- cells after a drug holiday reverted into CD133+/- subpopulations including subpopulations expressing low levels of CD271 (E-Right).



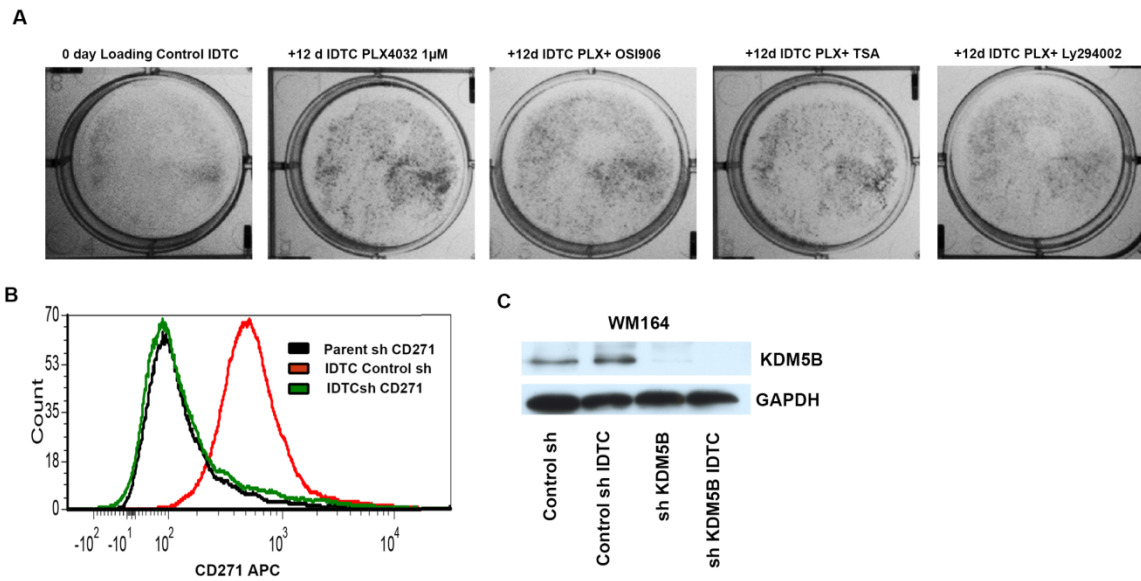
Supplemental Figure 4 (Related to Figure 2): pathway analyses of IDTCs

Ariadne[®] pathway analyses depicting proteins and enzymes involved in DNA replication (A) and transcription (B) in IDTC mRNAs compared to parental mRNAs from microarray data. Green represents a down regulation of the corresponding mRNAs and red an up regulation in IDTCs. Cut off P value 0.05



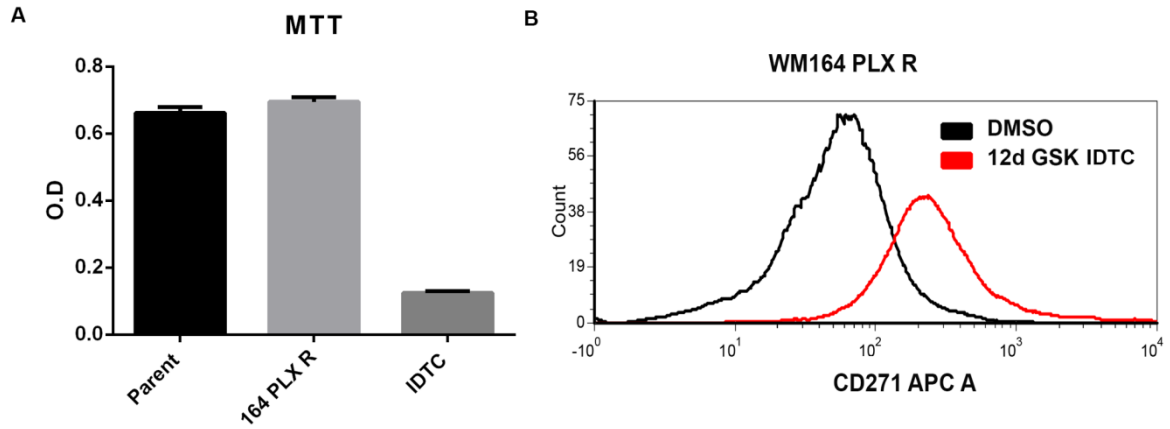
Supplemental Figure 5 (Related to Figure 4)

(A) CD271 expression of A375 parental cells (black), and 5%O₂ IDTCs (red) subjected to flow cytometry. Grey line represents the isotype control. ALDH activity of A375 parental (black) and 5%O₂ IDTCs (red) compared to their respective DEAB negative control. (B,C) mRNAs isolated from WM164 parental cells exposed to 5% O₂ or low glucose for 12 days analyzed for ABCB5, KDM5A, OCT4, KDM5B and SOX10 expression by RT –PCR. Error bars represent the standard deviation from the mean. Statistical analysis was done using the T test, P value is represented by (*) where P<= 0.0001 (****), P<= 0.001 (***), P<= 0.01 (**) and P<= 0.05 (*). (D) A375 cells exposed for 12 days to hypoxic conditions were challenged with PLX4032 at 10μM, GSK1120212 at 50nM and Cisplatin at 10μM for 48hrs and the percentage of caspase 3 positive cells was analyzed by flow cytometry and compared to the parental population. Error bars represent the standard deviation from the mean.



Supplemental Figure 6 (Related to Figure 5)

(A) Representative pictures of 1×10^5 WM164 IDTCs per well exposed over a period of 12 days to $1 \mu\text{M}$ PLX4032 alone or in combination with OSI906 ($5\mu\text{M}$), Ly294002 ($10\mu\text{M}$), or TSA (100nM). The surviving cells were fixed and stained with crystal violet. (B) WM164 shCD271 RNA transduced parental, shCD271 RNA transduced IDTCs, and control sh IDTCs subjected to CD271 expression analysis by flow cytometry. (C) Immunoblotting of protein lysates from transduced WM164 with control shRNA (Control sh), control sh RNA transduced IDTCs (Control sh IDTC), sh KDM5B RNA transduced cells (sh KDM5B) and sh KDM5B transduced IDTCs (sh KDM5B IDTC).



Supplemental Figure 7 (Related to figure 7)

(A) MTT assay of WM164 parental cells (parental), WM164 PLX4032 resistant (164 PLX-R) and WM164 IDTCs showing readings of the optical density (O.D) relative to growth rate. (B) CD271 expression in WM164 PLX R control (DMSO, black) and WM164 PLX R exposed to GSK1120212 at 50nM for 15 days (12d GSK, red) analyzed by flow cytometry.

Supplemental Materials and Methods

Gene expression analysis

Gene expression analysis were carried out as previously reported [3] by the Core Facility for Molecular Biology at the Centre of Medical Research at the Medical University of Graz, Graz, Austria. Briefly, the Ambion WT Expression Kit for Affymetrix GeneChip whole transcript (WT) Expression Arrays[®] (Life Technologies; Carlsbad, CA, USA) was used to label total RNA. GeneChip Human 1.0 ST arrays with 250ng of total RNA per array were used for hybridization according to the manufacturer's instruction. The Affymetrix GeneChip scanner GCS3000 was used for reading. Genomic Suite v6.5 (Partek Inc, St Louis, MO, USA) was used for the analyses of the results. All samples passed the quality control check. Further the data generated were loaded into an Ariadne[®] pathway studio (Elsevier) for pathway analyses.

RPPA analysis

Cell lysates were prepared in lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na₃VO₄, 10% glycerol), containing freshly added protease and phosphatase inhibitors from Roche Applied Science (Cat. # 05056489001 and 04906837001). The lysates were mixed with SDS buffer without bromophenol blue after calorimetric protein determination and boiled for five minutes and kept frozen at -80°C. Cell lysates were two-fold-serial diluted for five dilutions (from undiluted to 1:16 dilutions) and spotted on a nitrocellulose-coated slide. Samples were probed with antibodies by CSA amplification approach and visualized by DAB colorimetric reaction. Slides were scanned on a flatbed scanner to produce 16-bit tiff images. Spots from tiff images were identified and the density was quantified by an Array-Pro Analyzer. Relative protein levels for each sample were determined by interpolation of each dilution curve from the standard curve of the slide (antibody). All data points were normalized for protein loading and transformed to a linear value which is represented as bar graphs.

Angiogenesis array

Expression profiles of angiogenesis related cytokines and growth factors were analyzed with a Proteome Profiler Human Angiogenesis Array Kit[®] (ARY007-R&D systems) according to the manufacturer's protocol. Briefly, parental cells and IDTCs in 6 well plates were incubated with 1ml

FBS free RPMI media for 16hrs, isolated, and spun down to remove any cell debris. The total protein content of the samples was analyzed by the Bradford method and protein equalization was carried out before loading onto the provided antibody strips. In order to be able to compare the results the assays were carried out simultaneously and were developed simultaneously on the same x-ray film to ensure the same exposure time. The x-ray films were scanned using a Canon 8400F scanner. The pixel density was evaluated using the image J software.

***In vivo* tumorigenicity assay**

NOD.CB17-Prkdcscid/J were separated into groups receiving injections of 50, 500 or 5000 cells of WM164 parental cells (neck), PLX4032 500nM IDTCs (left flank) and resistant cells (right flank) in a suspension of Matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) with complete media but without FCS in a 1:1 ratio. Tumor growth was monitored in each group once every week for the entire period of the experiment. Tumor volume was measured using a caliper by applying the formula: $(width \times length \times height)/2$. Mice were sacrificed after the experiment or on the onset of tumor erosion. Tumor tissue was formalin fixed and paraffin embedded and tissue slides were stained for CD31 expression. The work was approved and carried out under the guidelines of the Bundesministerium für Wissenschaft und Forschung (B.M.W_F:GZ66.010/0003-11/3b/2011), Vienna, Austria.

Sphere forming assay

50, 500 or 5000 WM164 melanoma cells were suspended in 500µl serum free Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham media (Invitrogen) supplemented with 10ng/ml FGF2 (Cell Signaling #8910) and 10ng/ml EGF (Cell Signaling #8916). The assay has been modified to a previously reported protocol to produce more stringent conditions, whereupon sphere formation of IDTCs and parental cells could be compared in the absence of substantial external stimuli from B27 like stem cell supplements [4], which aids melanoma sphere formation. The cells were plated in ultra-low attachment 24 well cell culture plates and media was replenished every third day with additional 500µl of media and sphere formation was assessed after a one week period.

Histone Isolation

The Epiquick™ total histone extraction kit (OP-0006, Epigentek) was used according to the manufacturer's protocol. IDTCs and parental cell pellets were resuspended in a pre-lysis buffer and after incubation the supernatant was removed. Pellets again were re-suspended in lysis buffer, followed by the extraction of lysates. The pH of the lysates was compensated with a balancing buffer. The total protein content was determined and immunoblotting performed.

Antibodies

Antibodies directed against KDM5B, pIGF1R, pAKT, pan-AKT, p44/42 ERK1/2, total ERK1/2 and p217/221MEK1/2 were purchased from Cell Signaling Technology. The CD271 antibody used for immunohistochemistry of paraffin-fixed slides was purchased from Abcam. Flow cytometry antibody CD271 APC was obtained from Mitenyi Biotech and CD31-PE from BD Bioscience. H3K4me3, H3k27me3, H3k9me3 and H3 antibodies were purchased from ACTIVE MOTIF®.

Inhibitors

PLX4032, GSK1120212, Linsitinib (OSI-906), Cisplatin, Aducril, Docetaxel and TSA were purchased from Selleckchem. Ly294002 was purchased from Cell Signaling Technology.

Cell lines

The BRAF mutant melanoma cell lines WM164, A375 and 451Lu were provided by MH. WM164-FUCCI was provided by NKH [2].

Fluorescent ubiquitination-based cell cycle indicator (FUCCI)

To generate stable melanoma cell lines expressing the FUCCI constructs, mKO2-hCdt1 (30-120) and mAG-hGem (1-110) [1] were subcloned into a replication-defective, self-inactivating lentiviral expression vector system as previously described [5]. The lentivirus was produced by co-transfection of human embryonic kidney 293T cells, high-titer viral solutions for mKO2-hCdt1 (30/120) and mAG-hGem (1/110) were prepared and used for co-transduction into WM164 and subclones were generated by single cell sorting [2].

Long term cell survival crystal violet staining

Cells, which have been exposed to drugs, hypoxic conditions or low glucose for various time points, were fixed with 4% paraformaldehyde, followed by 30min incubation with 0.5% crystal violet solution. The plates were washed and pictures were taken using a microscope and gel documentation unit after drying.

Flow cytometry of cell surface markers

The expression of cell surface markers was determined after incubating the respective antibodies with the MACS Miltenyi staining solution (rinsing solution with 0.5% BSA stock) for 20 min followed by a wash. In case of double staining the procedure was repeated with the secondary antibody. CD31 and CD271 antibodies were used in concentrations according to the user manual. The samples were analyzed with a BD LSR II Flow Cytometer from the Center for Medical Research (ZMF) FACS core facility. CD31-PE was measured using the 488/Red fluorescent channel. CD271 APC was measured using the 633/APC fluorescent channel. Individual compensations were done prior to analyses. KDM5B promoter activity was analyzed in the 488/Red fluorescent channel.

ALDH activity measurements

ALDH activity was analyzed using the ALDEFLUOR™ Stem Cell Identification & Isolation Kit from Stem Cell Technologies according to the user manual. Briefly, cells were split into two sets and incubated with the ALDEFLUOR™ assay buffer containing efflux inhibitors. One set was preincubated with 20µl of DEAB for two minutes as a negative control and both sets were subjected to incubation with the ALDH-substrate BAAA (BODIPY®-aminoacetaldehyde) for 20mins. The cells were washed afterwards with the ALDEFLUOR™ assay buffer and analyzed with the BD LSR II Flow Cytometer in the 488/Green fluorescent channel.

Caspase 3 apoptosis assay

The Caspase 3 apoptosis assay was carried out using a PE Active Caspase-3 Apoptosis Kit™ from BD Pharmingen, according to the manufacturer's protocol. The cells were washed and permeabilized

with cytofix/cytoperm solution followed by washing and re-incubation with the caspase 3 antibody further analyzed by flow cytometry. All experiments were done in triplicate.

MTT assay

2×10^4 cells were seeded in 96-well culture plates for allocated times as mentioned in the experiments. They were subsequently incubated with MTT (3-(4, 5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) reagent (10 μ l) at 37°C for 3hrs. 100 μ l detergent reagent was then added to the wells and incubated in the dark at room temperature for 3hrs. Absorbance was measured at 570nm using a microtiter plate reader. All experiments were done in triplicate or duplicate.

Lentiviral vectors

shCD271 (p75NTR), shKDM5B (PLU1) and shRNA control scrambled pools were purchased from Santa Cruz Biotechnology and transduction were carried out according to the manufacturer's protocol. Cells were incubated with 8ng/ml polybrene complete media and incubated overnight with the lentiviral particles and selected with 10 μ g/ml puromycin for a one week period.

RT-PCR

Total RNAs were isolated using a QIAGEN kit according to the manufacturer's protocol and 1 μ g of RNA was used to generate cDNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Life Sciences). The SYBR Green Master Mix from Applied Biosystems was used for PCR amplification and products were detected by an AB7900 Standard real-time PCR system (Applied Biosystems). 18s RNA was used as an internal control and relative gene expression levels were calculated as delta CT.

RT-PCR Primers

Forward

Reverse

RT-PCR Primers	Forward	Reverse
KDM5A	CCAGCCTGATCTTCTGCATC	CCAGCACACTGATTGGTCCT
KDM5B	TCATTTTACGCCACGTATCCA	CTTTGCAATCTGGTCCAAGAA
SOX10	ACACCTTGGGACACGGTTTT	GGTCTCGCAAAGAGTCCA
ABC5	AGGGAAGCAAATGCGTATGA	GCTCCTTTTTCCCCTACCAA
OCT4	CTTCTGCTTCAGGAGCTTGG	GAAGGAGAAGCTGGAGCAAA
18S	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG

***In vivo* low dose CD271 induction**

NOD SCID IL2 receptor gamma chain knockout (NSG) mice were injected subcutaneously with 1×10^6 451Lu human melanoma cells in a suspension of Matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) / complete media at a ratio of 1:1. After tumors had reached a volume of 200-300 mm³, mice were randomized into groups of five. The groups were then treated with either vehicle (5% DMSO, 1% methylcellulose in dH₂O) alone or with PLX4720 at a dose of 15 mg/kg BID per oral gavage. The tumors were measured twice weekly using calipers and volumes were calculated as (width x length x height)/2. Mice were sacrificed after 2 weeks of treatment and tumors were harvested 4 hours after the last dose. Tumor tissues were formalin fixed and paraffin embedded and the slides were further stained for CD271 expression. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by The Wistar Institute Animal Care and Use Committee (Protocol Number: 111954).

Immunohistochemistry

Samples of formalin-fixed paraffin embedded tissues were retrieved from the archives of the Department of Dermatology, Medical University of Graz according to ethical guidelines. Briefly, slides were de-paraffinized and antigen retrieval was done using 0.01 M sodium-citrate buffer (pH 6.0) and the specimens were then blocked with 1% H₂O₂ in methanol. The slides were further incubated with the CD271 (Abcam mouse monoclonal ab 3125) or CD31 (Thermo Scientific, USA) followed by an incubation with a biotinylated anti-mouse antibody (Dako 5001, ready to use). Slides were incubated with the streptavidin peroxidase detection system (Dako 5001, ready to use) and developed with Dako Real DAB+ chromogen (Dako 5001, 1:50).

Immunocytochemistry

WM164 control and ADTC cells were grown in poly D lysine BD biocoat chamber slides (BD Bioscience). A monoclonal antibody to Mart-1/Melan A Cocktail (Biocare Medical CM077B) was used as the primary antibody (1:50 with antibody diluent Dako S202230). Slides were pretreated for 40min in a water bath (98.5°C) with an epitope retrieval kit (Dako K500111), and then left for 20min at room

temperature. Blocking was performed with a peroxidase blocking solution (Dako S202386). Staining was detected by a Dako Real Detection Kit (Dako K500111) and AEC (Dako 346430) as a chromogen. Tyrosinase staining was carried out similarly with a tyrosinase antibody (Novocastra, NCL-L-TYROS) and epitope retrieval was done with Target Retrieval Solution, Dako S236784 with a DakoEnVision Detection Kit (Dako K500711).

Immunoblotting

Protein concentrations were measured using the Bradford protein assay (BioRad, Hercules, CA). Bio Rad wet tank blotting systems were used to perform immunoblotting. Samples were loaded and separated on SDS-polyacrylamide gels (10%), transferred to polyvinylidene difluoride (PVDF) membranes and probed with specific primary antibodies overnight at 4°C. To detect the signal, peroxidase-conjugated secondary antibody was added and incubated for 4 hours in room temperature. Proteins were visualized using ECL reagents (Amersham, Piscataway, NJ) and exposed to X-ray film (Kodak). The quantification of bands was done with the Bio Rad quantity one software by background subtraction method, or with Image J analysis.

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