Stem Cell Reports, Volume *8*

Supplemental Information

Melanoma-Derived iPCCs Show Differential Tumorigenicity and Therapy Response

Mathias Bernhardt, Daniel Novak, Yassen Assenov, Elias Orouji, Nathalie Knappe, Kasia Weina, Maike Reith, Lionel Larribere, Christoffer Gebhardt, Christoph Plass, Viktor Umansky, and Jochen Utikal

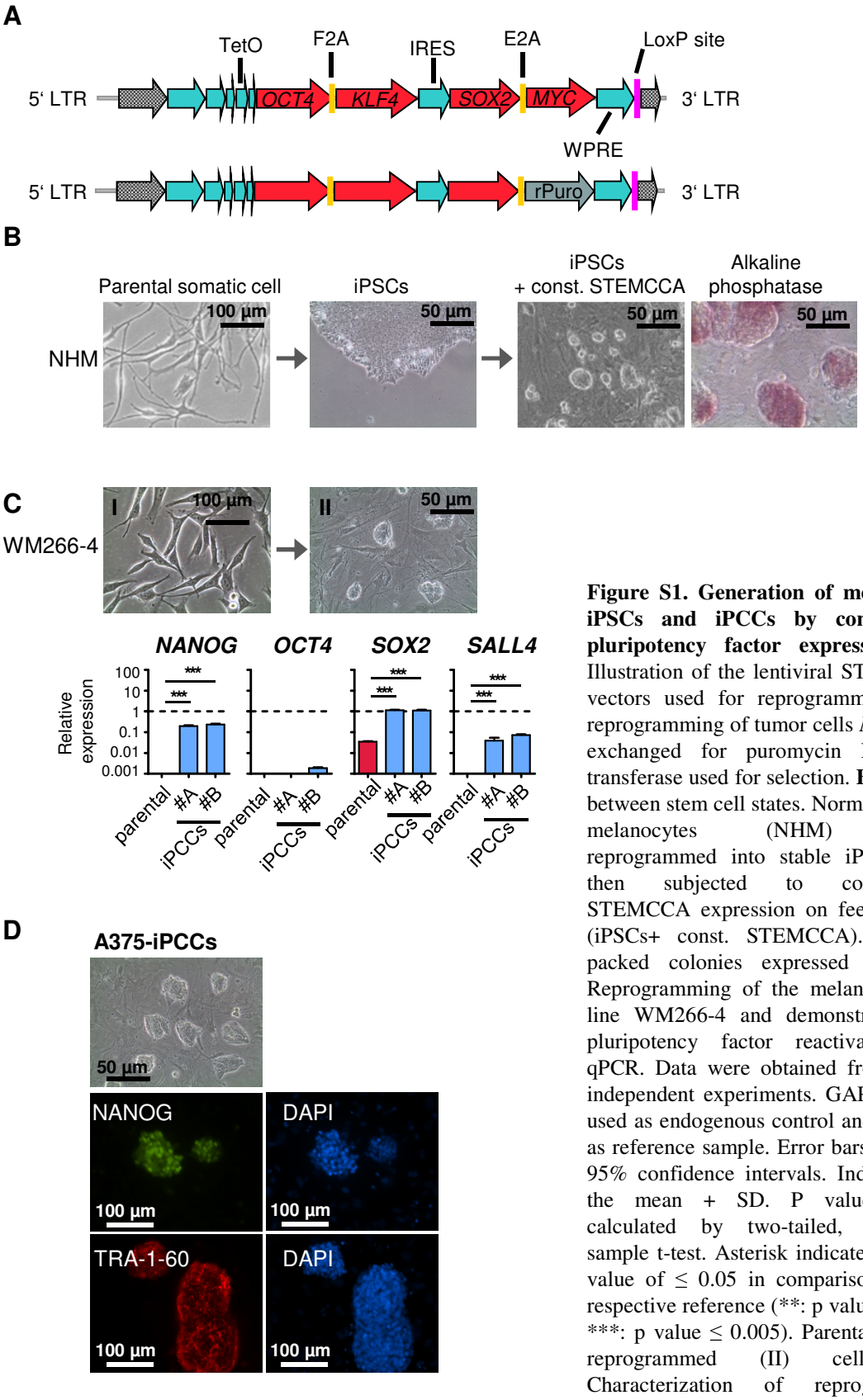

Figure S1. Generation of metastable iPSCs and iPCCs by constitutive pluripotency factor expression. A) Illustration of the lentiviral STEMCCA vectors used for reprogramming. For reprogramming of tumor cells *MYC* was exchanged for puromycin N-acetyl-transferase used for selection. **B)** Switch between stem cell states. Normal human melanocytes (NHM) were reprogrammed into stable iPSCs and then subjected to constitutive STEMCCA expression on feeder cells (iPSCs+ const. STEMCCA). Tightly packed colonies expressed AP. **C)** Reprogramming of the melanoma cell line WM266-4 and demonstration of pluripotency factor reactivation by qPCR. Data were obtained from three independent experiments. GAPDH was used as endogenous control and hiPSCs as reference sample. Error bars indicate 95% confidence intervals. Indicated is the mean + SD. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). Parental (I) and reprogrammed (II) cells. **D)** Characterization of reprogrammed A375 melanoma cells by morphological analysis and immunofluorescence staining of NANOG and TRA-1-60.

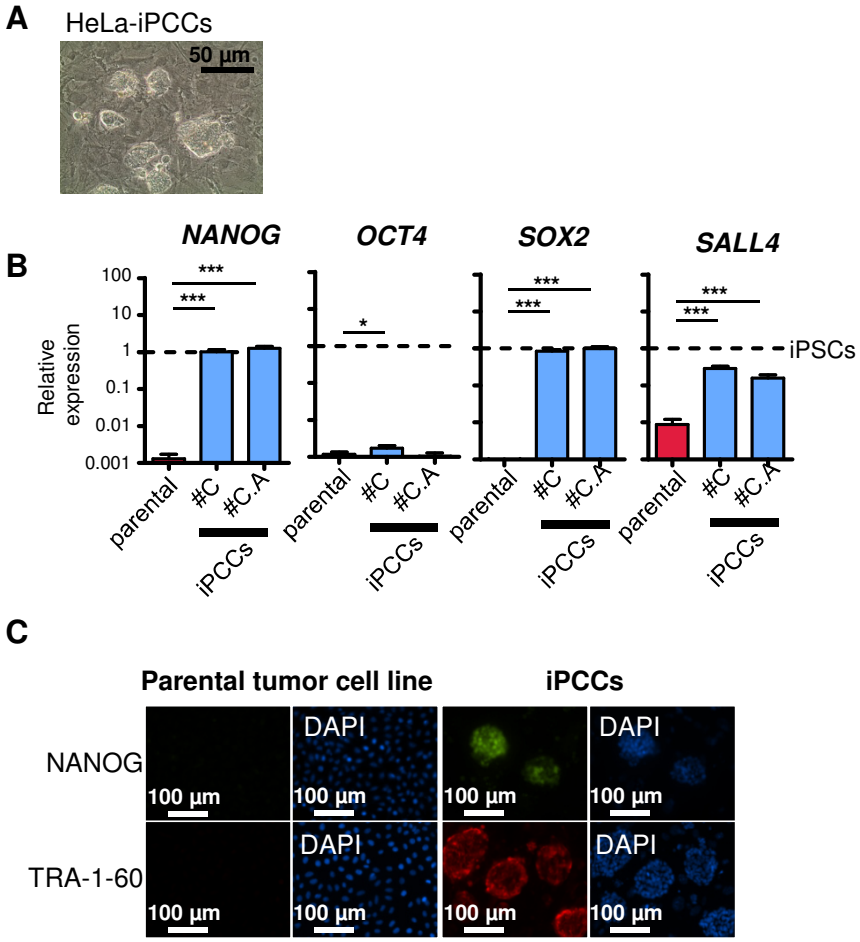

Figure S2. Generation of iPCCs from the cervical carcinoma cell line HeLa. A) Morphological characterization of HeLa-iPCCs. **B)** QPCR validation of the endogenous expression of the pluripotency markers *NANOG*, *OCT4*, *SOX2* and *SALL4*. Data were pooled from three independent experiments. GAPDH was used as endogenous control and hiPSCs as reference sample. Error bars indicate 95% confidence intervals. Indicated is the mean + SD. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). **C)** Immunofluorescence staining of NANOG and TRA-1-60 in HeLa cells and HeLa-iPCCs. Nuclear counterstaining with DAPI as indicated.

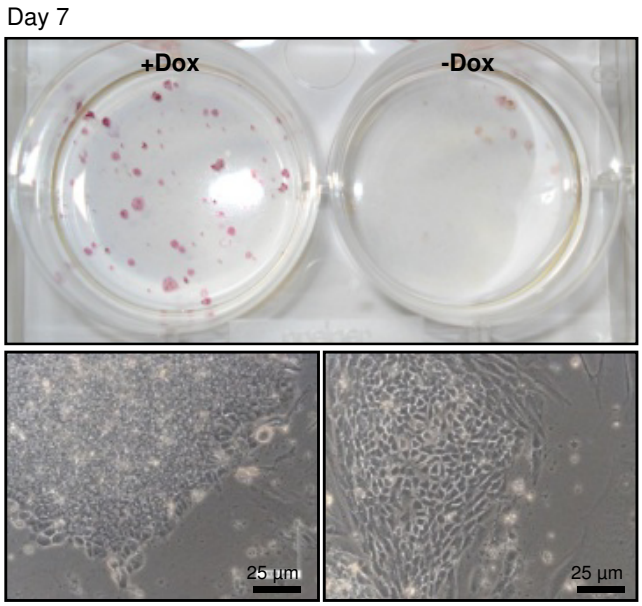

Figure S3. Transgene-dependence of metastable iPCCs. Doxycycline was withdrawn for 7 days followed by staining for AP activity.

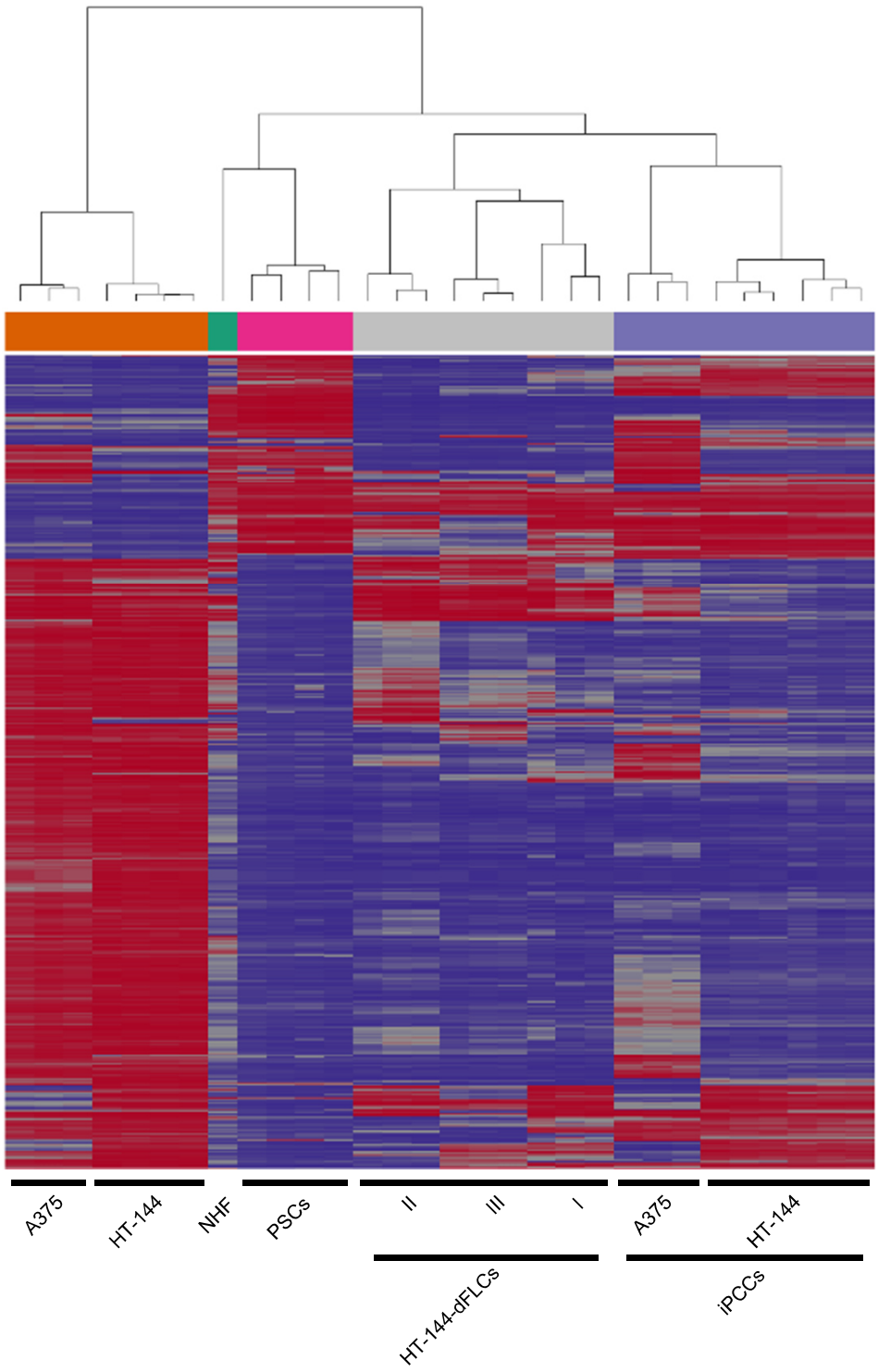

Figure S4. Dendrogram of melanoma cells and iPCCs. Heatmap showing the methylation profiles of melanoma cell lines, melanoma-iPCCs and iPCC-derived fibroblast-like cells compared to hiPSCs and hESCs. Rows depict promoters of Ensembl genes, and columns: individual samples. Methylation values are color-coded using a continuous scale from red (0, unmethylated) through grey (0.5, hemi-methylated) to blue (1, fully methylated). Only the 500 promoters with highest variance for methylation values across all samples are displayed. The dendrogram shows the result of hierarchical clustering based on Euclidean distance and complete linkage.

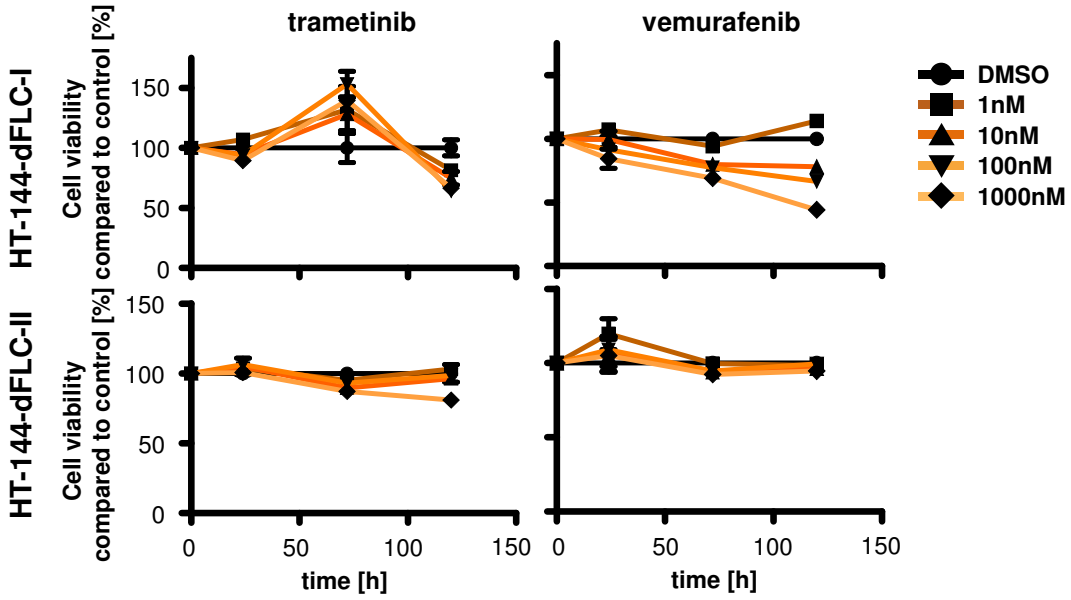

Figure S5. Reduced response to targeted melanoma therapy in fibroblast-like differentiated melanoma cells. Two independent differentiations of HT-144-iPCCs into fibroblast-like cells (I) & (II) were treated with increasing concentrations of the MEK inhibitor trametinib and the $BRAF^{V600E}$ inhibitor vemurafenib followed by cell metabolism analysis by AlamarBlue assay at indicated time points.

Supplemental Experimental Procedures

Plasmid constructs

The reverse tetracycline-controlled transactivator FUdeltaGW-rtTA was a gift from Konrad Hochedlinger (Addgene plasmid # 19780) (Maherali et al., 2008). FUdeltaGW-rtTA-zeocin was generated by cloning a zeocin resistance gene for the selection of eukaryotic cells into FUdeltaGW-rtTA. The doxycycline-inducible lentiviral human stem cell cassette (tetO-hSTEMCCA-loxP) was generated by exchanging the EF1a promoter from hSTEMCCA-loxP (Somers et al., 2010) for a minimal CMV promoter under the control of a tetracycline responsive element (TRE). The puromycin-selectable tetO-hSTEMCCA-puro-loxP was constructed by exchanging *MYC* from tetO-hSTEMCCA-loxP for the gene encoding the puromycin-N-acetyltransferase.

Cell culture of primary adult human melanocytes

Melanocytes were isolated from foreskins. Excised foreskins were incubated for 15 min at room temperature in 10% Braunol solution (Braun) and then washed with PBS (Sigma-Aldrich). Afterwards, the subcutaneous fat was removed and the foreskin cut into pieces of 10 x 4 mm in size. These pieces were digested in dispase (Stemcell Technologies) (1 mg/ml) at 4°C overnight. The next day, the epidermis was peeled off the skin with a forceps followed by 15 min digestion at 37°C in trypsin solution mixed 1:1 with PBS. After digestion samples were gently vortexed and trypsin activity stopped by adding FBS. Next, cells were filtered through a 70 µm-cell strainer (BD Falcon) and centrifuged at 2500 rpm for 5 min. Primary melanocytes were washed with PBS and again spun down at 2500 rpm for 5 min, resuspended in medium 254 (Thermo Fisher Scientific) with 20 µg/ml G418 (Sigma-Aldrich) and transferred to 10 cm dishes. Medium 254 (Thermo Fisher Scientific) is supplemented with 1% (v/v) 100x human melanocyte growth supplement (HMGS) (Thermo Fisher Scientific) resulting in a final concentration of 0.2% (v/v) bovine pituitary extract, 0.5% v/v fetal bovine serum, 1 µg/ml recombinant human insulin-like growth factor-I, 5 µg/ml bovine transferrin, 3 ng/ml basic fibroblast growth factor (bFGF), 0.18 µg/ml hydrocortisone, 3 µg/ml heparin and 10 ng/ml phorbol 12-myristate 13-acetate.

Cell culture of primary human fibroblasts

Fibroblasts were isolated from skin biopsies obtained from the department of Dermatology at the University Medical Center Mannheim. Biopsies were rinsed with 70 % ethanol and then with PBS. Adipose tissue was removed and the remaining tissue was put into a small petri dish and digested with dispase overnight at 4°C. The next day, the epidermis was removed, the dermis cut into little pieces and digested with collagenase mix (Gibco® Life Technologies) (2.5 mg/ml collagenase II & 2.5 mg/ml collagenase IV) for 1 h at 37°C with constant agitation. Centrifuge 5 min at 1200 rpm. Resuspend the tissue in fully supplemented DMEM (see above) and seed it out in small petri dishes or 6-well plates. Carefully change medium every few days. Fibroblasts will grow out from the tissue pieces. Split the cells as soon as they reach confluence.

Generation of mitotically-inactivated feeder cells

MEFs were expanded either in T175 cell culture flasks or 150 mm cell culture dishes until passage three. Confluent MEFs were incubated with 10 µg/ml mitomycin C (Carl Roth) for 4 h and washed with PBS containing Ca^{2+} and Mg^{2+} for three times followed by rinsing the cells with PBS without Ca^{2+} and Mg^{2+} for three times. The now postmitotic cells were trypsinized for 5-7 min at 37°C until they detached followed by neutralization of the enzymatic activity with complete medium. After centrifugation these feeder cells were resuspended in 80% FBS with 20% (v/v) DMSO and aliquoted at a concentration of 1 x 10^6 cells per vial. Until thawing the vials were stored in liquid nitrogen.

RNA isolation and cDNA transcription

RNA extraction was performed using the RNeasy kit (Qiagen) or PicoPure RNA isolation kit (Life Technologies) according to the manufacturer's instructions. Briefly, pelleted cells were lysed and RNA extracted using a column-based purification. Every sample was DNase I treated for 15 min at room temperature on the purification column followed by two washing steps and elution with RNase-free H_2O. RNA concentration and quality were measured using a NanoDrop ND-1000 Spectrophotometer. Only samples fulfilling the quality recommendations were further analyzed. From each sample 500 ng RNA were incubated with oligo (dT)18 primers in a volume of 12 µl for five min at 65°C. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's advice. Before use cDNA was diluted 1:5 in nuclease-free H_2O.

Quantitative real-time polymerase chain reaction (qPCR)

QPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and an Applied Biosystems 7500 Real-Time PCR System. Each primer pair was evaluated and amplification efficiency confirmed to lie in a range from 85-110%. Gene quantification was calculated using the Pfaffl method (Bustin et al., 2009) calculating the delta-delta Ct in three technical replicates. Results were normalized to *GAPDH* and *18S* RNA or *GAPDH* and B-actin if not otherwise stated, serving as endogenous controls. Statistical analysis was carried out in Excel and visualization of graphs in GraphPad Prism 5. Following primers were used in the study:

	Forward primer	Reverse primer
*SOX2*_endo	*GCTAGTCTCCAAGCGACGAA*	*GCAAGAAGCCTCTCCTTGAA*
*OCT4*_endo	*GACAGGGGGAGGGGAGGAGCTAGG*	*CTTCCCTCCAACCAGTTGCCCCAAAC*
*NANOG*_endo	*CAGTCTGGACACTGGCTGAA*	*CTCGCTGATTAGGCTCCAAC*
SALL4	*ATTCCCTGGGTGGTTCACT*	*AGCACATCAACTCGGAGGAG*
TET1	*CGCTACGAAGCACCTCTCTTA*	*CTTGCATTGGAACCGAATCATTT*
DPPA4	*GACCTCCACAGAGAAGTCGAG*	*TGCCTTTTTCTTAGGGCAGAG*
NODAL	*CAGTACAACGCCTATCGCTGT*	*TGCATGGTTGGTCGGATGAAA*
LEFTY1	*AGGAGCTGGTCATCCCCAC*	*GCCACCTCTCGGAAGCTCT*
LEFTY2	*TGGACCTCAGGGACTATGGAG*	*CCGAGGCGATACACTGTCG*
DNMT3L	*TGAACAAGGAAGACCTGGACG*	*CAGTGCCTGCTCCTTATGGCT*
PAX6	*AACGATAACATACCAAGCGTGT*	*GGTCTGCCCGTTCAACATC*
MAP2	*CGAAGCGCCAATGGATTCC*	*TGAACTATCCTTGCAGACACCT*
RBFOX3	*TCGTAGAGGGACGGAAAATTGA*	*GCCGTTGGTGTAGGGGTTC*
MITF-M	*AGAGGGAGGGATAGTCTACCG*	*ACTTGGTGGGGTTTTCGAGG*
AP2	*GGAGACGTAAAGCTGCCAAC*	*GGTCGGTGAACTCTTTGCAT*
SOX10	*AGCCCAGGTGAAGACAGAGA*	*ATAGGGTCCTGAGGGCTGAT*
TRP1	*AGCAGTAGTTGGCGCTTTGT*	*TCAGTGAGGAGAGGCTGGTT*
E-cadherin	*AGCCAACCTTAACTGAGGAGT*	*GGCAAGTTGATTGGAGGGATG*
GAPDH	*GAAGGTGAAGGTCGGAGTC*	*GAAGATGGTGATGGGATTTC*
B-actin	*GGATGCCACAGGATTCCATACCCA*	*TCACCCACACTGTGCCGATCTACGA*
18S	*GAGGATGAGGTGGAACGTGT*	*TCTTCAGTCGCTCCAGGTCT*

Genomic DNA isolation and cell authentication

Genomic DNA was isolated using the QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer's instructions. Briefly, cells were lysed and digested with proteinase K for 10 min at 56°C. Genomic DNA was isolated from the mixture through column purification and eluted with water. DNA concentration and quality were measured using a NanoDrop ND-1000 Spectrophotometer. A 24-plex single nucleotide polymorphism profiling assay was performed by MULTIPLEXION as described in Castro et al. (2013) to confirm their common identity. Additionally, cell authentication was performed by DMSZ applying short tandem repeat DNA typing and comparison to the DNA reference database of human cell lines.

Fluorescence *in situ* hybridization (FISH)

Dual-color fluorescent *in situ* hybridization (FISH) with locus specific FISH probes for *BRAF* (Chr. 7), *MAP2K1* (*MEK1*) (Chr. 15), *MAP2K2* (*MEK2*) (Chr. 19), *MAPK3* (*ERK1*) (Chr. 16) and *MAPK1* (*ERK2*) (Chr. 22) on parental melanoma cells as well on melanoma-derived iPCCs was performed as recently described (Orouji et al., 2016).

Immunofluorescence and alkaline phosphatase staining

Tumor and feeder cells were seeded on gelatin-coated coverslips while neuronal differentiated cells were seeded on Matrigel-coated coverslips. For nuclear staining cells were fixed in methanol for 7 min at -20°C and subsequently rinsed with -20°C cold acetone. Samples for surface marker staining were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 5 min at room temperature and permeabilized with 0.1% Tween 20 (AppliChem) in PBS for additional 5 min. Blocking was performed with PBS containing 0.5% (w/v) BSA (Carl Roth), 1% (v/v) FCS and 0.1% (v/v) Triton X-100 (Carl Roth) for 30 min at room temperature. Then, samples were incubated with primary antibodies overnight at 4°C in blocking solution with the indicated dilutions: rabbit anti-NANOG (Abcam, cat. no. ab80892) 1:150, mouse anti-TRA-1-60 (Cell Signaling Technology, cat. no. 4746) 1:250, mouse anti-TRA-1-81 (Cell Signaling Technology, cat. no. 4745) 1:250 and rabbit anti-B3-tubulin (TUJ-1) (Synaptic Systems, cat. no. 302302) 1:250. Samples were washed twice with blocking solution and incubated with either Atto 488 goat anti-rabbit IgG (Sigma-Aldrich, cat. no. 18772) 1:500, Atto 647 goat anti-mouse IgG (Sigma-Aldrich, cat. no. 50815) 1:500 or Atto 647 goat anti-rabbit IgG (Sigma-Aldrich, cat. no. 40839) 1:500 in blocking solution for 4 h at 4°C. Afterwards, samples were washed twice, counterstained with 100 ng/ml DAPI (Roche diagnostics) in PBS and mounted with Dako Fluorescent Mounting Medium (Dako, S3023). The next day samples were analyzed with a Nikon ECLIPSE Ti fluorescent microscope. Staining for alkaline phosphatase activity was performed using the Stemgent Alkaline Phosphatase Staining Kit II according to the manufacturer's protocol. Following fixation, cells were washed with PBS and incubated with AP staining solution for 15 min at room temperature. The reaction was stopped by washing with PBS and pictures were taken using a Nikon ECLIPSE TS 100 light microscope.

Whole genome expression analysis

Whole genome expression profiling was performed at the DKFZ Core Facility for Genomics and Proteomics. Samples were analyzed on a HumanHT-12 v4 Expression BeadChip (Illumina) that is able to quantify expression levels of 48107 human genes. 1 µg total RNA from three (HT-144) or two independent experiments, respectively, was isolated as described and sent to microarray analysis using Illumina HumanHT-12v4

Expression BeadChip at the DKFZ Genomics and Proteomics Core Facility. Resulting raw data were exported to Chipster software. First, array data were $\log_2$ transformed and quantile normalized. For assessing differentially regulated genes two group test or several group test using empirical Bayes method were performed with a p value adjustment according to Benjamini-Hochberg (BH). P value threshold was set to 0.05 to filter for significantly deregulated genes. Differentially expressed genes were clustered using Spearman correlation as a distance measure visualizing up-regulated genes in red and downregulated genes in green. As result of the hierarchical clustering dendrograms were constructed by the average linkage method. Gene sets were analyzed for enrichment of distinct pathways using DAVID software from the Bioinformatics Resources from the National Institute of Allergy and Infectious Diseases (NIAID), NIH (http://david.abcc.ncifcrf.gov/) and pathway analysis was performed by MetaCore, THOMSON REUTERS (https://portal.genego.com/). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE95281 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95281).

Immunohistochemical staining

Tissue samples were isolated, washed and fixed in 4% PFA overnight at room temperature. Paraffin-embedded samples were cut and sections were deparaffinized in xylene followed by rehydration. After antigen retrieval the following targets were stained: S100B (Santa Cruz, cat. no. sc-7851), KI67 (Abcam, cat. no. ab15580), PanCK (Dako, cat. no. Z062201-2), $BRAF^{V600E}$ (ZytoMed Systems, cat. no. E19290), CK20 (Dako, cat. no. M701929-2), MelanA (Vector laboratories, cat. no. VP-M646), SOX2 (Abcam, cat. no. ab97959), p44/42 (Cell Signaling Technologies, cat. no. 4695), phospho-p44/42 (Cell Signaling Technologies, cat. no. 9106). Afterwards, samples were counterstained with haematoxylin. For histological analysis of tumors and evaluation of teratomas, sections were stained with haematoxylin and eosin (H&E). All samples were analyzed under a Leica DM LS light microscope.

Supplemental References

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. *55*(4):611-22.

Castro, F., Dirks, W.G., Fähnrich, S., Hotz-Wagenblatt, A., Pawlita, M., and Schmitt, M. (2013). High-throughput SNP-based authentication of human cell lines. Int J Cancer *132*(2):308-14.

Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell 3(3):340-5.

Orouji, E., Orouji, A., Gaiser, T., Larribère, L., Gebhardt, C., and Utikal, J. (2016). MAP kinase pathway gene copy alterations in NRAS/BRAF wild-type advanced melanoma. Int J Cancer *138*(9):2257-62.

Somers, A., Jean, J.C., Sommer, C.A., Omari, A., Ford, C.C., Mills, J.A., Ying, L., Sommer, A.G., Jean, J.M., Smith, B.W. et al. (2010). Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. Stem Cells *28*(10):1728-40.