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Supplemental Information

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Edgardo Castro-Pérez, Carlos I. Rodríguez, Dareen Mikheil, Shakir Siddique, Alexandra McCarthy, Michael A. Newton, and Vijayasaradhi Setaluri

SUPPLEMENTAL INFORMATION

Melanoma Progression Inhibits Pluripotency and Differentiation of Melanoma-Derived iPSC Produces Cells with Neural-like Mixed Dysplastic Phenotype

Edgardo Castro-Pérez[1], Carlos I. Rodríguez[1,#], Dareen Mikheil[1], Shakir Siddique[1], Alexandra McCarthy[1], Michael A. Newton[3] and Vijayasaradhi Setaluri[1,2,*]

Running Title: *Melanoma plasticity and drug resistance*

[1]Department of Dermatology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA

[2]William S. Middleton Memorial Veterans Hospital, Madison, WI 53705, USA

[3]Department of Statistics, Department of Biostatistics and Medical Informatics, University of Wisconsin-Madison, Madison WI, 53706 USA

[*]Corresponding author:

Vijayasaradhi Setaluri, PhD
Department of Dermatology
1300 University Avenue, B25
University of Wisconsin-Madison
Madison, WI 53706

Tel: (608) 263-5362
E-mail: setaluri@wisc.edu

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Current address:
Department of Pathology, University of California-San Francisco, 513 Parnassus Ave, San Francisco CA, 94143 USA.

SUPPLEMENTAL FIGURES

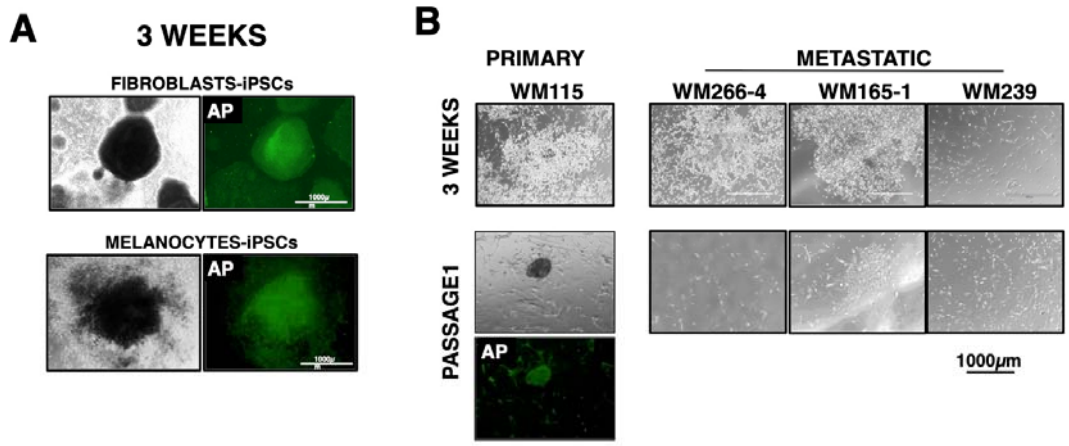

Figure S1 (Related to Figure 1)

Reprogramming of Fibroblasts and Melanocytes, and Matched Primary and Metastatic Melanoma Cells from the Same Patient.

(A) Morphology of fibroblast- and melanocyte-derived iPSC and AP staining after three weeks of reprogramming.

(B) Morphology of primary (WM115) and metastatic cells (WM266-4, WM165-1, WM239) from the same patient at 3 weeks (*top panels*) and passage 1 (*bottom panels*) of reprogramming. AP staining of WM115-miPSC colony.

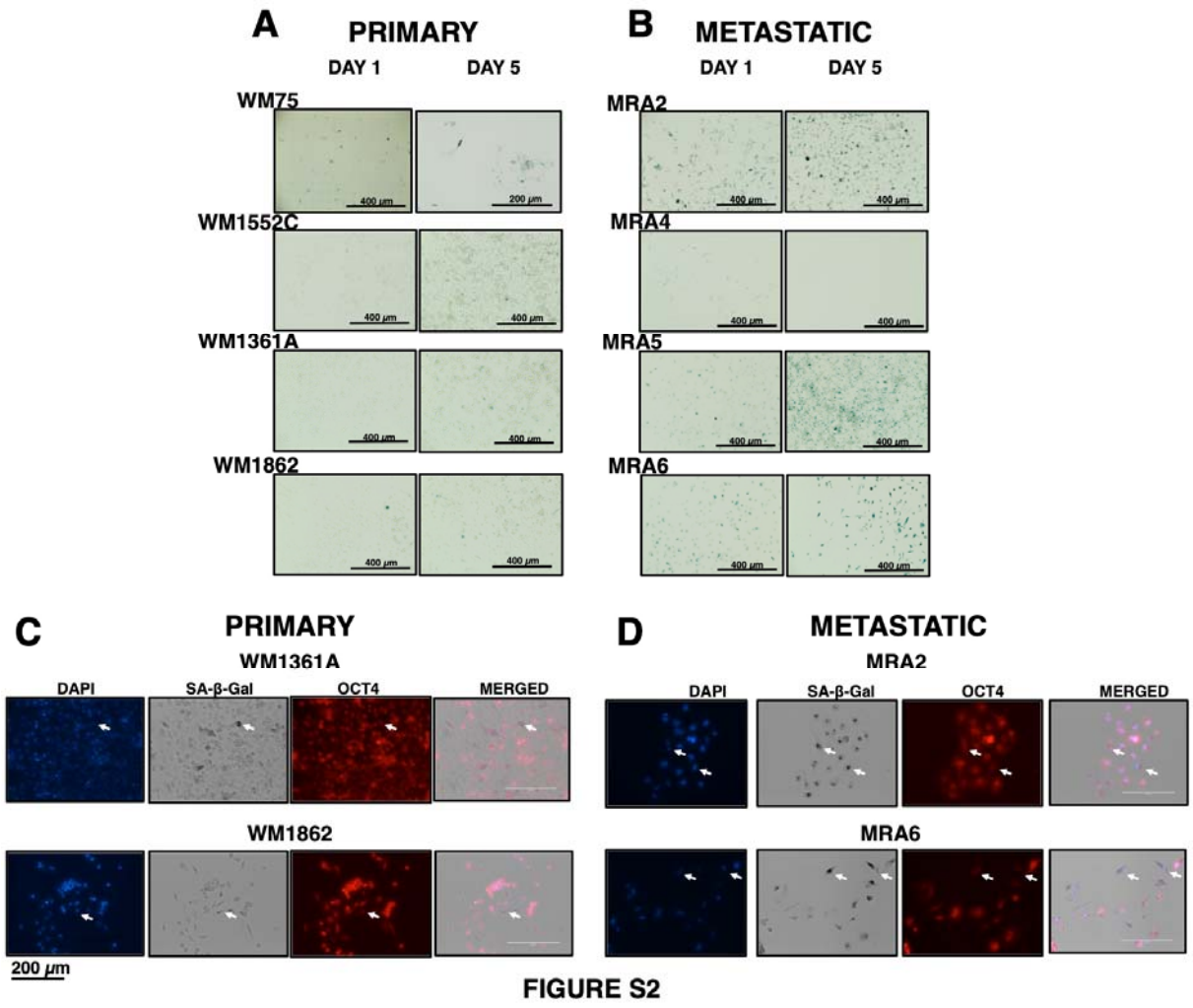

Figure S2 (Related to Figure 2)

Effect of Transduction with Reprogramming Factors on Senescence (SA-β-Gal Staining) and Co-staining with OCT4.

(A-B) SA-β-Gal staining of primary and metastatic cells at days 1 and 5 after transduction.

(C-D) SA-β-Gal co-staining with OCT4 determined by immunofluorescence at day 5 after transduction.

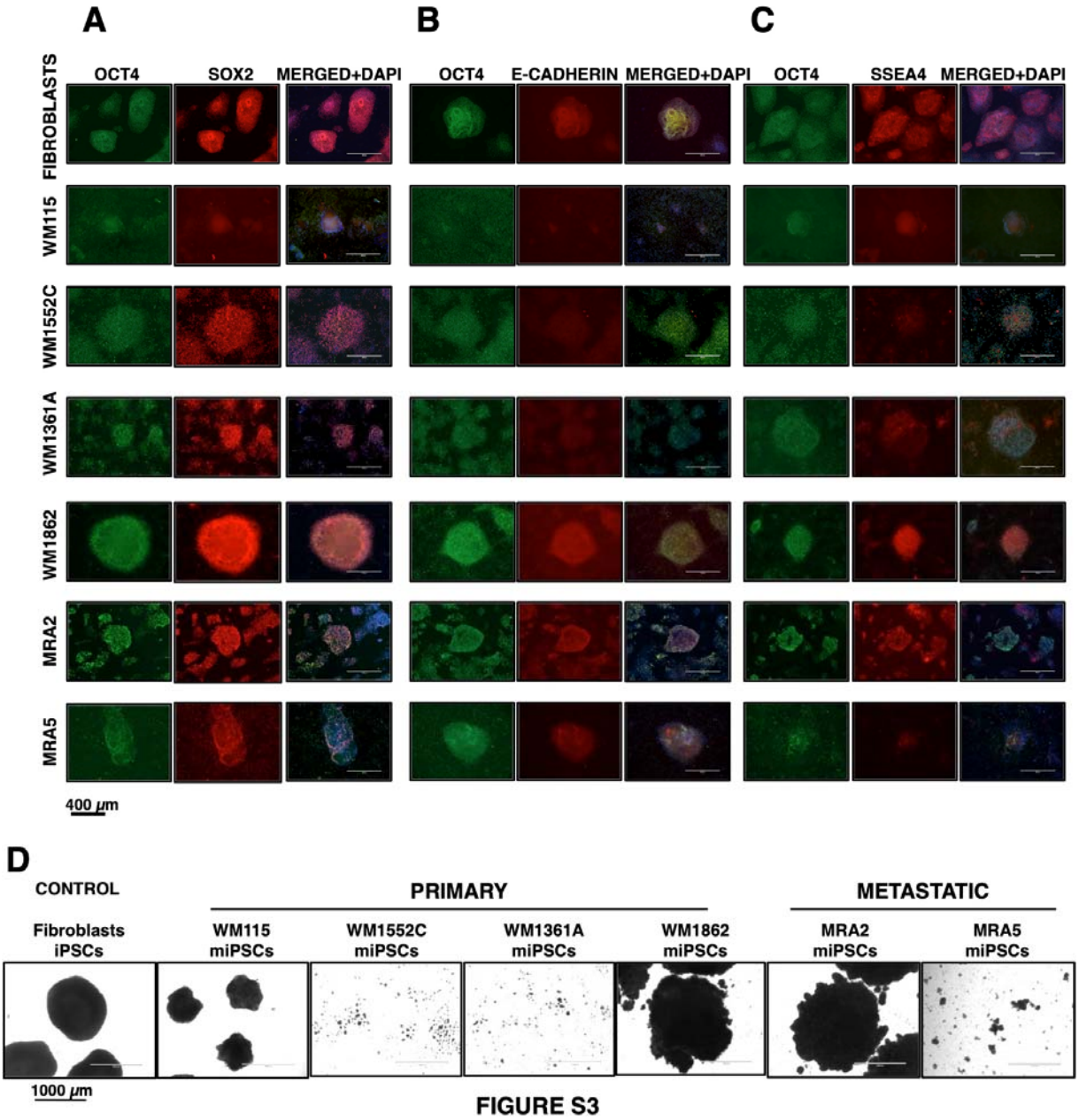

Figure S3 (Related to Figure 4)

Expression of Stem Cell Markers and EB formation.
Immunofluorescence staining of (A) reprogramming factors OCT4 and SOX2; (B) OCT4 and E-Cadherin; (C) OCT4 and SSEA4. (D) Embryoid body formation of fibroblasts iPSCs, and primary and metastatic melanoma miPSCs.

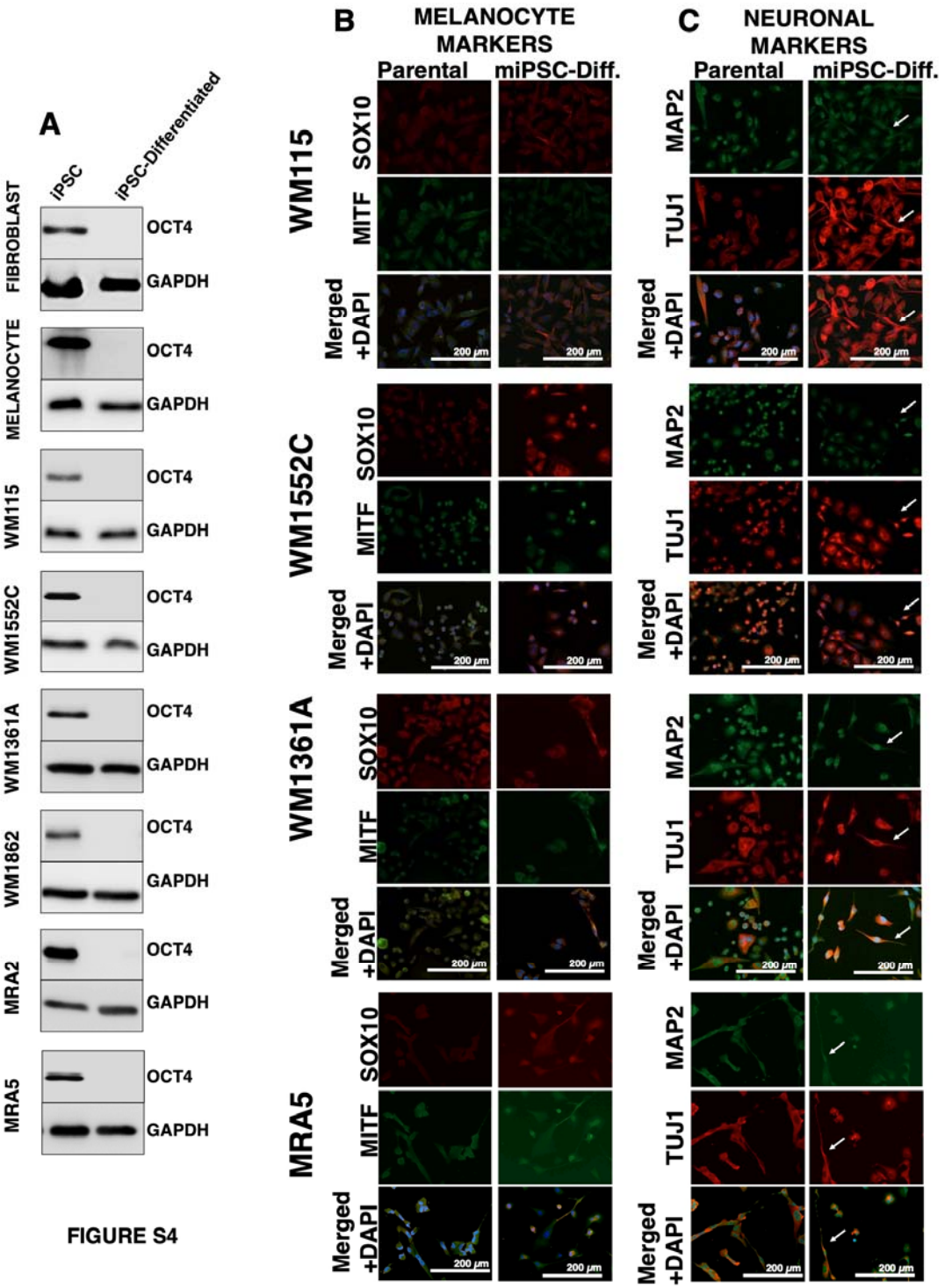
A
iPSC
iPSC-Differentiated
FIBROBLAST
MELANOCYTE
WM115
WM1552C
WM1361A
WM1862
MRA2
MRA5
OCT4
GAPDH
B
MELANOCYTE MARKERS
Parental
miPSC-Diff.
C
NEURONAL MARKERS
Parental
miPSC-Diff.
WM115
WM1552C
WM1361A
MRA5
SOX10
MITF
Merged +DAPI
MAP2
TUJ1
200 μm

FIGURE S4

Figure S4 (Related to Figure 5)

Expression of OCT4, Melanocyte and Neural Markers in miPSC-differentiated cells

(A) Western blot analysis of melanoma-iPSCs (miPSC) and miPSC-Differentiated cells with OCT4 and GAPDH. Data show that miPSC-differentiated cells no longer expressed the reprogramming factors.

(B) Immunofluorescence staining of melanocyte markers in parental and miPSC-differentiated cells in melanocyte differentiation medium.

(C) Neuronal markers expression in parental and miPSC-differentiated cells in melanocyte differentiation medium.

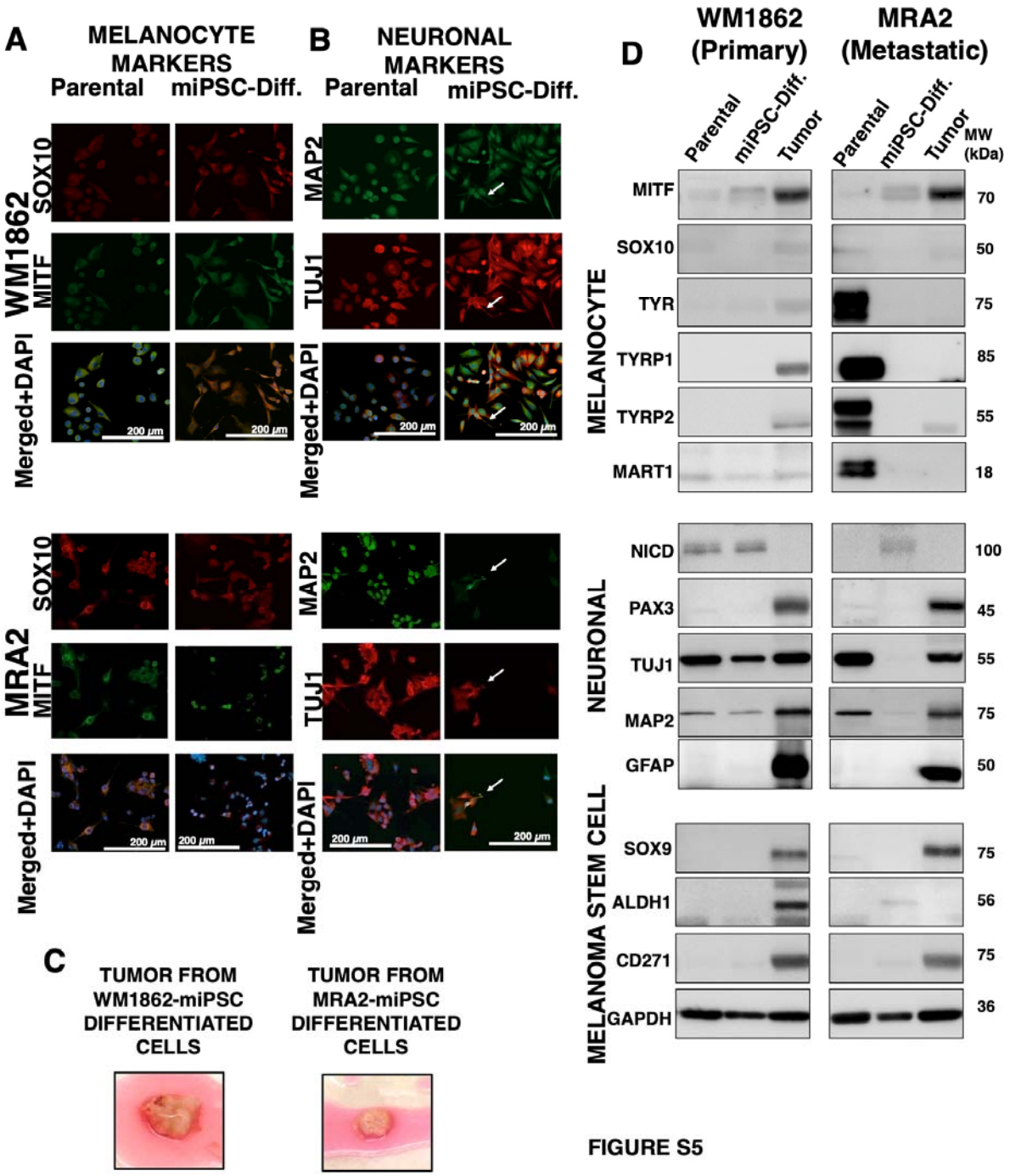

Figure S5 (Related to Figure 5)
Expression of Melanocyte and Neural Markers in miPSC-differentiated Cells and Tumors.

(A) Immunofluorescence staining of melanocyte markers in parental and miPSC-differentiated cells in melanocyte differentiation medium.

(B) Neuronal markers expression in parental and miPSC-differentiated cells in melanocyte differentiation medium.

(C) Tumors from miPSC-differentiated cells in melanocyte differentiation medium.

(D) Western blot analysis of melanocyte (*top panels*), neural (*middle panels*) and melanoma/stem cell markers (*bottom panels*) in parental melanoma and miPSC-differentiated cells in melanocyte medium and tumors from miPSC-differentiated cells. WM1862 (*left panels*) and metastatic MRA2 (*right panels*).

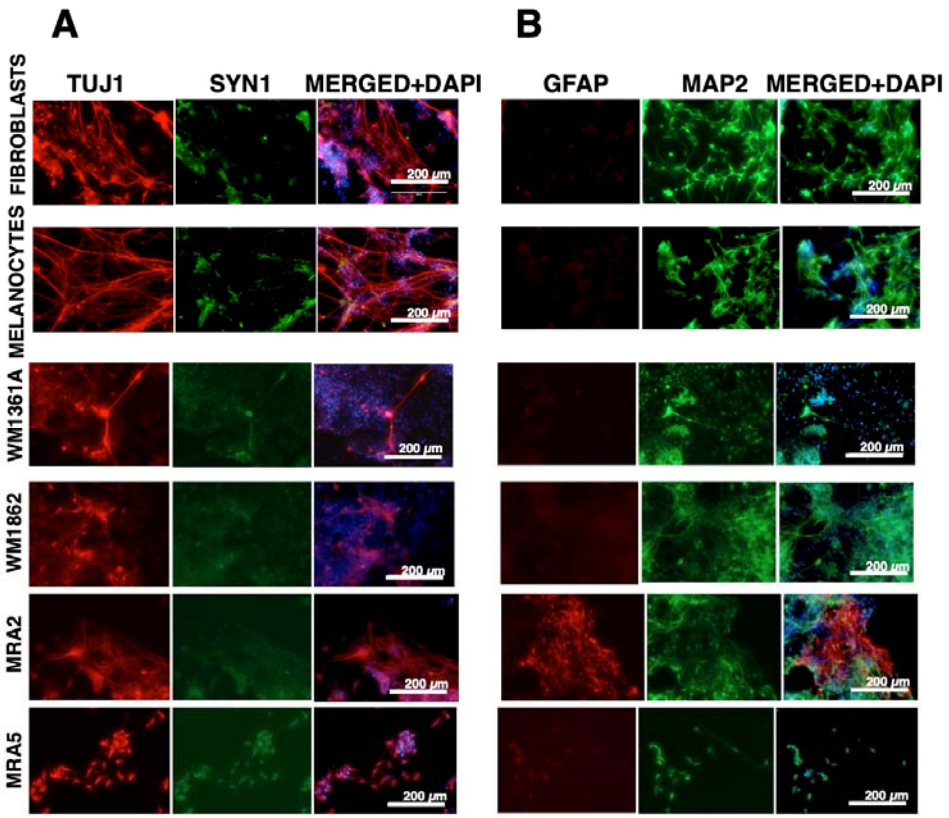

FIGURE S6

Figure S6 (Related to Figure 5)

Neuronal Differentiation of Fibroblast-, Melanocyte- and miPSCs.

(A) Immunofluorescence staining of neuronal markers neuronal tubulin (TUJ1) and SYNAPSIN1 (SYN1) from neuronal differentiation of fibroblast- and melanocyte-iPSCs and melanoma-miPSCs.

(B) Immunofluorescence staining of glial (GFAP) and neuronal (MAP2) markers from neuronal differentiation of fibroblast- and melanocyte-iPSCs and miPSCs.

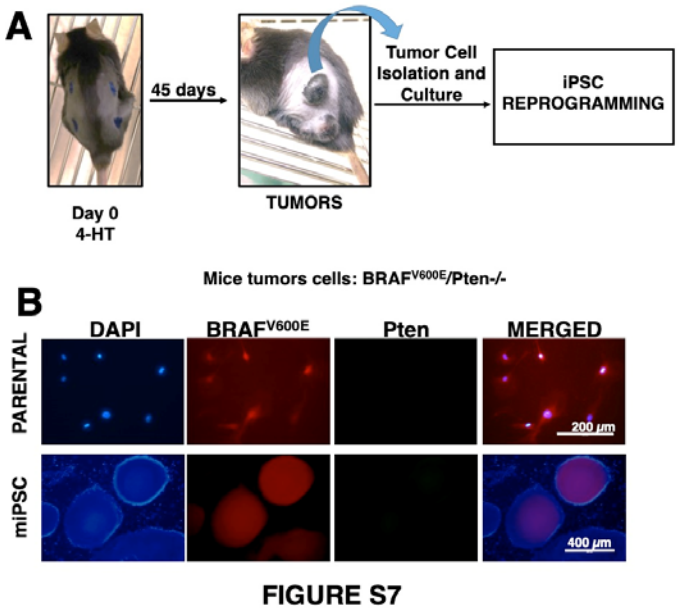

Figure S7 (Related to Figure 5)

Isolation and Reprogramming of $BRAF^{V600E}/Pten^{-/-}$ Mouse Tumor Cells.

(A) $BRAF^{V600E}/Pten^{-/-}$ mice tumors were induced by topical application of tamoxifen (4-HT) at day 0 and tumors were excised on day 45. Tumor cells were isolated and cultured for iPSC reprogramming.

(B) $BRAF^{V600E}/Pten^{-/-}$ mouse tumor cells and miPSC derived colonies stained for $BRAF^{V600E}$ and Pten. iPSC colonies generated from $BRAF^{V600E}/Pten^{-/-}$ tumor cells expressed $BRAF^{V600E}$ but not Pten similar to parental cells.

SUPPLEMENTAL TABLES

Table S1: Cell lines used in the study, tumor stage and mutations (Related to Figure 1 and Figure S1)

Cell Line	Tumor Stage	Mutations	WT
WM115	Primary	BRAFV600D, PTEN loss	NRAS, c-KIT, CDK4
WM239A	Metastatic	BRAFV600D, PTEN loss	NRAS, c-KIT, CDK4
WM165-1	Metastatic	BRAFV600D, PTEN loss	NRAS, c-KIT, CDK4
WM266-4	Metastatic	BRAFV600D, PTEN loss	NRAS, c-KIT, CDK4
WM1361A	Primary	NRASQ61R, PTEN loss	NRAS, c-KIT, CDK4
WM1552C	Primary	BRAFV600E, PTEN loss	NRAS, c-KIT, CDK4
WM1862	Primary	BRAFV600E	NRAS, c-KIT, CDK4
WM75	Primary	BRAFV600E	PTEN, NRAS, c-KIT, CDK4
MRA2	Metastatic	PTEN loss	BRAF
MRA4	Metastatic	PTEN loss	BRAF
MRA5	Metastatic	BRAFV600E, PTEN loss	ND
MRA6	Metastatic	BRAFV600E, PTEN loss	ND
MRA9	Metastatic	PTEN loss	BRAF

Table S2: List of antibodies

			Dilution	
Marker	**Company**	**Cat. #**	**WB**	**IF**
OCT4	Cell Signaling	2840	1:1000	1:50
SOX2	R&D Systems	MAB2018	N.A.	1:50
NANOG	Cell Signaling	4903	1:1000	N.A.
c-MYC	Cell Signaling	13987	1:1000	N.A.
p21	Cell Signaling	2946S	1:1000	N.A.
MITF	Thermo Fisher	MA5-14146	1:500	1:50
MITF	R&D Systems	AF5769	N.A.	1:50
SOX10	R&D Systems	AF2864	1:1000	1:50
SYN1	Cell Signaling	5297	N.A.	1:50
TUJ1	R&D Systems	MAB1195	1:1000	1:50
MAP2	Cell Signaling	8707S	1:1000	1:50
GFAP	Cell Signaling	3670S	1:1000	1:50
ERK	Cell Signaling	9122S	1:1000	N.A.
pERK	Cell Signaling	4370S or 4696S	1:1000	N.A.
$BRAF^{V600E}$	Spring Biosciences	E19290	N.A.	1:50
PTEN	Cell Signaling	9559S	N.A.	1:50
TYR	Abcam	ab170905	1:1000	N.A.
TYRP1	Santa Cruz	sc-166857	1:1000	N.A.
TYRP2	Santa Cruz	sc-74439	1:1000	N.A.
MART1	Bio Care Medical	CM077A	1:1000	N.A.
NICD	Cell Signaling	4147S	1:1000	N.A.
PAX3	R&D Systems	MAB2457	1:1000	N.A.
SOX9	R&D Systems	AF3075	1:1000	N.A.
ALDH1	R&D Systems	AF5869	1:1000	N.A.
CD271	R&D Systems	MAB367	1:1000	N.A.
GAPDH	Proteintech	60004-1-Ig	1:3000	N.A.

Table S3: Media, supplements and recombinant proteins used

Media/Supplement	Company	Cat. #
Knockout(tm) Sr;	Thermo Fisher	10828028
DMEM	Thermo Fisher	11995065
DMEM/F-12	Thermo Fisher	11320033
Medium 254	Thermo Fisher	M254500
HMGS-2	Thermo Fisher	S0165
Neurobasal	Thermo Fisher	A1371201
Glutamax	Thermo Fisher	35050061
Knockout(tm) D-mem;	Thermo Fisher	10829018
B-27	Thermo Fisher	17504044
N-2	Thermo Fisher	17502048
Ham's F-10	Corning	10-070-CV
Matrigel	Corning	356231
ITS	Sigma	I3146
Linoleic Acid	Sigma	L9530
Endothelin 3	Tocris	1162
Fibronectin	R&D Systems	1030-FN-01M
SCF	R&D Systems	7466-SC-010
Wnt-3a	R&D Systems	5036-WN-010
bFGF	R&D Systems	233-FB-025
Noggin	R&D Systems	6057-NG-025
LIF	Thermo Fisher	PHC9484
NEAA	Thermo Fisher	11140050
Antibiotics	Thermo Fisher	15140122
Versene	Thermo Fisher	15040066

Table 4: List of Chemicals and reagents used

Protein/Chemical	Company	Cat. #
Y-27632	AdipoGen	AG-CR1-3564
Valproic Acid	Selleckchem	S1168
CHIR99021	Selleckchem	S1263
RepSox	Selleckchem	S7223
Tranylcypromine	Selleckchem	S4246
Forskolin	Selleckchem	S2449
PD0325901	Selleckchem	S1036
Ascorbic acid	Fisher Scientific	PHR1008-2G
Dexamethasone	Sigma	D4902
SB431542	Fisher Scientific	NC1173633
LDN-193189	Fisher Scientific	NC1117386
Choleratoxin	Sigma	C8052
TPA	Sigma	736414
G418 (Geneticin)	Mirus	MIR 5920
IBMX	Sigma	I5879
2-Mercaptoethanol	Sigma	M7522
PLX4032	Selleckchem	S1267
AZD6244	Selleckchem	S1008
4HT	Sigma	06734
MTT dye solution	Sigma	M5655

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Lentivirus Production and Cell Transduction

HEK293 cells were plated on 10 cm plates in DMEM and 10% FBS without antibiotics and allowed to reach 70 – 90 % confluence within 24-48 hours. Following the Lipofectamine 2000 (Thermo Fisher, #11668019) protocol cells were then triple co-transfected with plasmids containing packaging (psPAX2, Addgene #12260) and VSV G envelope (pMD2.G, Addgene #12259) and target genes [(OCT4-SOX2, pSIN4-EF2-O2S, Addgene #21162) or KLF4-cMYC (pSIN4-CMV-K2M, Addgene #21164) or NANOG-LIN28 (pSIN4-EF2-N2L, Addgene #21163) or BRAFV600E-GFP [a gift from Dr. Raabe (Raabe et al., 2011)] or NICD-GFP or Empty vector-GFP [gifts from Dr. Herlyn (Zabierowski et al., 2011)].

For reprogramming, cells were plated in 6-well plates at a density of $5x10^4$ cells per well in 2mL of corresponding medium. The next day cells were transduced with three lentiviruses containing the reprogramming factors (200μL each) and polybrene (8 μg/mL). Approximately 18 - 20 hours after transduction, the medium was removed and replaced with fresh medium. Cells were then allowed to grow for 5 days before reprogramming. For $BRAF^{V600E}$, NICD and Empty vector lentiviruses we used a similar procedure for transduction.

Stem cell markers expression and pluripotency assays
Expression of stem cell markers was performed using the Human Pluripotent Stem Cell Marker Antibody Panel Plus (R&D Systems #SC009). Differentiation of miPSCs into three germ layer was evaluated using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems #SC027B). Images were taken using an EVOS™ FL Auto Imaging System (Thermo Fisher).

Embryoid Body Formation and Melanocyte Differentiation
Embryoid bodies (EBs) and melanocyte differentiation were performed as reported by (Ohta et al., 2011; Yang et al., 2011) with some modifications. Briefly, EBs were formed from hanging drop culture in EB medium consisting of DMEM/F12 containing 20% KOSR, 1X glutamax, 1X nonessential amino acids (NEAA), $2x10^{-4}$ M 2-mercaptoethanol, and 1% antibiotics in a cell culture dish with sterile PBS in the bottom for 5 days. EBs were then plated on a fibronectin-coated (FN) dish in Melanocyte differentiation medium consisting of 1:1 DMEM/254 melanocyte medium +HMGS2, 10% FBS, and supplemented with 50 ng/ml WNT3a, 50 ng/ml stem cell factor (SCF), 100 mM endothelin-3 (ET-3), 20 pM cholera toxin (CT), 50 nM TPA, 4 ng/ml bFGF, 100 mM L-ascorbic acid, 0.05 mM dexamethasone, 1X linoleic acid and 1X insulin-transferrin-selenium (ITS). Medium was changed every 3-4 days until the appearance of pigmented cells (35 - 40 days). After differentiation, melanocyte differentiation medium was replaced with F-10 melanocyte medium [Ham's F10, 5% FBS, 1% antibiotics, choleratoxin (0.02µg/mL), TPA (0.5 µg/mL), IBMX (0.1mM)] containing geneticin (G418, 100µg/ml) for 2 days and kept in F-10 melanocyte medium for expansion and long-term maintenance. G418 treatment was repeated as needed. For MTT assays, F-10 melanocyte medium was replaced by DMEM/10% FBS/1% antibiotic for two weeks and passed at least 5 times.

Neuronal Differentiation
Neuronal differentiation was performed essentially as reported (Bernhardt et al., 2017). Briefly, cells were plated on 24 well plates coated with matrigel at 2 x 10^4 cells per cm^2 in DMEM/F12 with 20% KOSR, 2 mM glutamax, 1% NEAA, 1% antibiotic, 0.1 mM 2-mercaptoethanol, 10 ng/mL human LIF and 1 µM of ROCK inhibitor Y-27632. After 2 days, medium was changed to 1:1 DMEM/F12 and Neurobasal media with 1% B-27 and 0.5% N-2 supplements, 100 ng/mL Noggin, 0.5 µM LDN-193189, 10 µM SB-431542, 2 µM CHIR99021, 10 µM FSK, and 10 ng/mL bFGF for 1-2 weeks. Then, cells were cultured for an additional week without small chemical compounds with 10 ng/mL bFGF.

BRAF-Pten Mice Tumor Cells Induction and Isolation
Transgenic *Tyr::CreER;*$Braf^{CA}$*;*$Pten^{lox4\text{-}5/lox4\text{-}5}$ mice (Dankort et al. 2009) were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice (females and males 4-weeks old). Tumor cell induction was performed as previously described (Rodríguez et al., 2017). Briefly, mice were hair trimmed and a single 2µL topical dose of 5mM 4-Hydroxytamoxifen (4-HT) was applied on the skin. Mice were monitored every 3 days and between days 40-45, mice were euthanized and each tumor excised. BRAF mutation status was confirmed by DNA sequencing and western blot. $Braf^{CA}/Pten^{-/-}$ mouse melanoma cell lines were established and cultured as previously described (Jenkins et al., 2014; Rodríguez et al., 2017). Briefly, 4-HT-induced tumors were digested in 4 mg/mL collagenase type I (Thermo Fisher, #17018029) for 1 hr. Following digestion, the tumor pieces were manually dissociated with a serological pipette and filtered through a 70µm cell strainer. Cells were cultured in DMEM/F-12 advanced (Thermo Fisher, #12634010) with 5% FBS and 1%

antibiotics for up to two weeks. Cells from tumors were plated in DMEM with 10% FBS, 1% antibiotics for expansion and maintenance. Animal experiments were approved by the institutional animal care and use committee.

Xenograft of miPSC-Differentiated Cells
For tumoriginenicity analysis, $1x10^6$ cells from miPSC-differentiated cells in melanocyte differentiation medium, were resuspended in 50% Matrigel and subcutaneously injected in NOD/SCID mice (4 weeks old male or female, ENVIGO). Mice were euthanized 6 weeks after cells were injected or when developing tumors reached ~1 cm size diameter.

Western Blotting
Cells were harvested and lysed using RIPA buffer containing Halt protease inhibitor cocktail (Thermo Fisher, #78410) and phosphatase inhibitor cocktail (Bimake.com, #B15001). Samples were then sonicated, centrifuged for 30 min at 4ºC and the supernatants were collected. Protein concentration was estimated using Pierce BCA Protein Assay Kit (Thermo Fisher, #23227). SDS-PAGE was performed using 20µg of protein and proteins were transferred to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk prepared in TBST buffer. After incubation overnight with primary antibodies at optimized dilutions (Table S2, WB), membranes were washed and incubated with HRP conjugated secondary antibodies. Protein bands were detected using ECL Start Western Blotting Detection Reagent (Thermo Fisher, #32106), imaged on ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA).

Immunofluorescence
Cells were plated on 8-well slides (Ibidi, USA, #80826), fixed in 4% PFA/PBS for 20 min and then incubated in permeabilization/blocking buffer consisting of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS for 45 minutes (0.5 mL/well of a 24-well plate). Primary antibodies (Table S2, IF) were diluted in permeabilization/blocking buffer and incubated overnight at 4 degrees. Next day, cells were washed 3 times with 1% BSA in PBS for 5 minutes and incubated for 1h with secondary antibody (1:200) at RT. After three washes with 1% BSA/PBS, ProLong Gold Antifade with DAPI (Thermo Fisher, #P36935) was added to the cells and cells were visualized using the EVOS microscope (Thermo Fisher).

Senescence-Associated β-Gal Staining and Cell Quantification
Cellular senescence was determined using the Senescence β-Galactosidase Staining Kit (Cell Signaling #9860S) following the manufacturer's protocol. Briefly, five thousand cells were plated on 24-well plates and next day transduced with equivalent reprogramming factors. Senescence associated-β-galactosidase staining was performed at days 1 and 5 after transduction. Wells were then scanned using the EVOS microscope and images were processed using Adobe PhotoShop and ImageJ for cell quantifiation.

RNA-Seq analysis
Quality control was carried out using FastQC (Andrews 2010, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); no samples were omitted from the analysis. Sequence reads were mapped against the Human genome (Hg19 Refseq reference) using Bowtie 0.12.8 (Langmead et al., 2009) allowing up to two mismatches and up to 20 multiple hits. Transcripts per million (TPMs) expected were estimated via RSEM 1.2.3 (Li et al., 2010).

Differential gene expression analysis, clustering, and gene set analysis were performed in R (R-Development-Core-Team, 2007). Differentially expressed (DE) genes between MPKi-resistant (MRA5, MRA6BR, MRA6MR) and MAPKi-sensitive (MRA6) cell lines were identified using EBSeq (Leng et al., 2013) and fold-change cut off. We selected genes with differential expression probability at least 0.99 (i.e. 1% FDR) and at least a two-fold change. We retained only genes that also were annotated to several relevant Gene Ontology categories.

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