A

CONTROL vs M1

599 | 861

hypermethylated in M1 | hypomethylated in M1

CONTROL vs M2

351 | 2746

hypermethylated in M2 | hypomethylated in M2

CONTROL vs M3

660 | 3371

hypermethylated in M3 | hypomethylated in M3

B

M38-PL vs M38-NM

hypermethylated | hypomethylated

M21-PL vs M21-NM

hypermethylated | hypomethylated

M24-PL vs M24-NM

hypermethylated | hypomethylated

C

methylation
hypomethylation
hypermethylation

copy number variation
amplification
deletion

gene expression
up-regulation
down-regulation

D

Segmentation for 181531_532_181531_635 (181531_60000bp) - chr10; with 2 segments

M3

PTEN Δ

E

Segmentation for 180622_532_180622_635 (180622_60000bp) - chr9; with 3 segments

M4

p16 Δ

10

Figure S1

Supplemental Figure 1.

Volcano Plot showing difference of mean methylation (X Axis) and statistical significance of the difference (Y Axis) in epigenomic clusters M1, M2 and M3 compared to the controls, and displaying increasing hypomethylation. The number of differentially methylated loci are indicated above each Volcano Plot. Numbers to the left indicate hypermethylated loci and numbers to the right indicate hypomethylated loci in melanoma. The single dark blue point in all three Volcano Plots denote the *CSF1R*. A False Discovery Rate (FDR) of less than 5% is used as a marker of significant differences (**A**). DNA methylation in matched primary and metastatic samples. The normalized mean HpaII/MspI log ratios are plotted as density histograms for each serial primary lesion and metastatic sample pair, where higher log HpaII/MspI ratio means less methylation. It is apparent that for the metastatic samples (blue curve), the majority of loci falls into the more hypomethylated state as compared to the more hypermethylated matched primary melanoma samples (red plot) (**B**). Integrative analysis shows that differentially methylated regions in melanoma display specific functional and genomic characteristics: Circos plots showing differentially methylated (outer circle), copy number alterations (middle circle) and gene expression differences (inner circle) in melanoma samples (**C**). Deletions of *PTEN* (**D**) and *p16* (**E**) genes are shown for individual representative samples
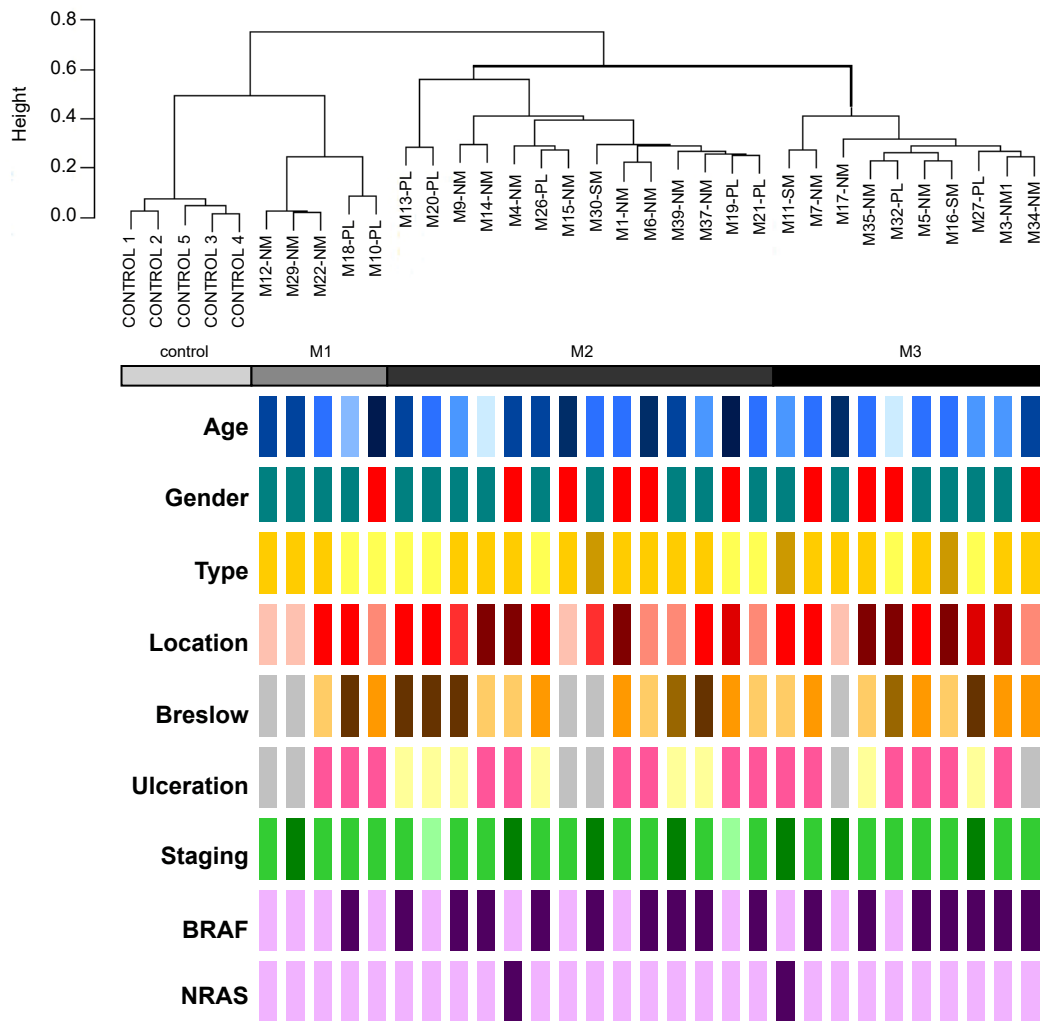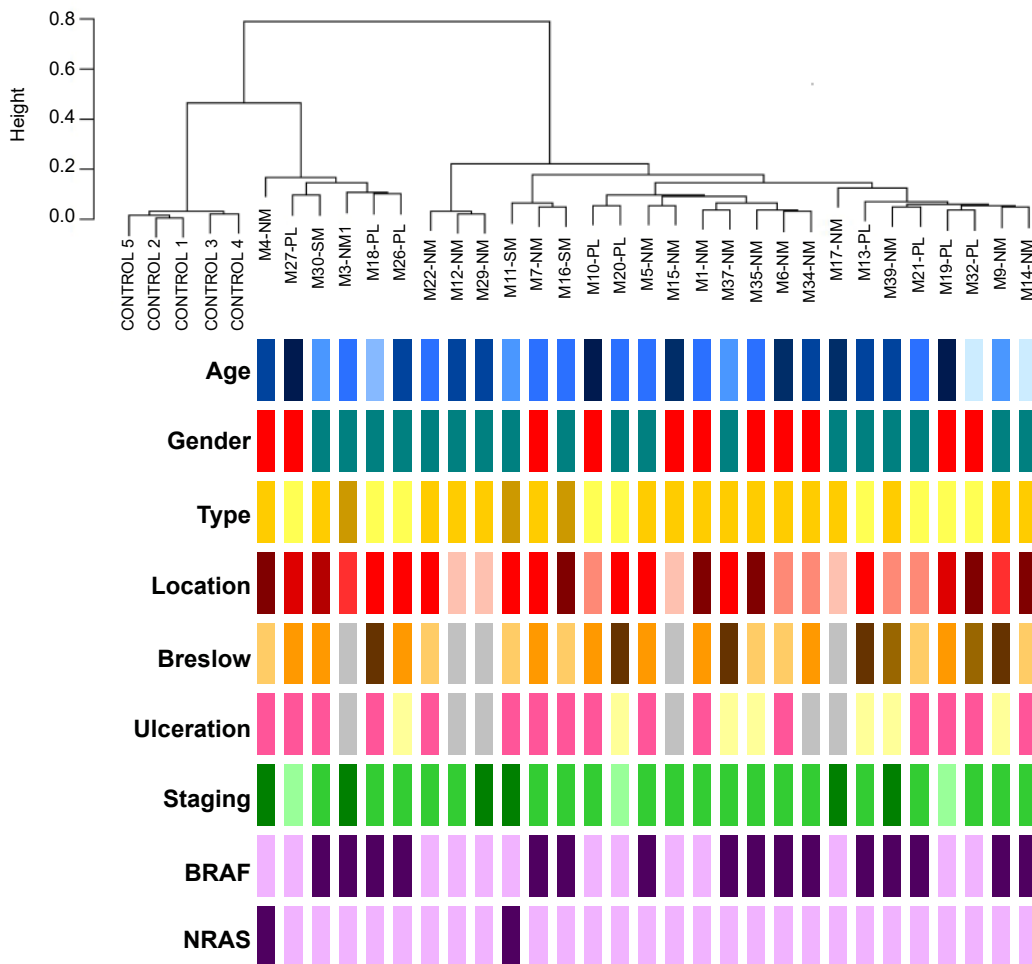
Figure S2

Supplemental Figure 2

Melanoma is characterized by aberrant DNA hypomethylation**:** unsupervised hierarchical clustering of methylation profiles generated by the HELP assay can distinguish melanoma samples from healthy melanocyte controls. The melanoma samples are separated in three major clusters with increasing dissimilarity from normal controls. Unsupervised clustering reveals that methylation profiles are independent of patient demographics (Age/Sex), mutational status, stage and extent of tumor. (PL: Primary Lesion; NM: Nodal Metastasis; SM: Soft tissue Metastasis) (**A**). Unsupervised hierarchical clustering of gene expression profiles can distinguish melanoma samples from healthy melanocyte controls (**B**).
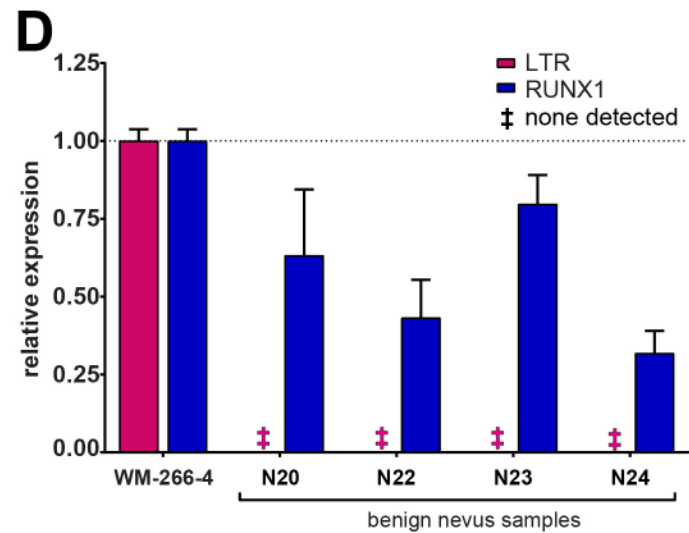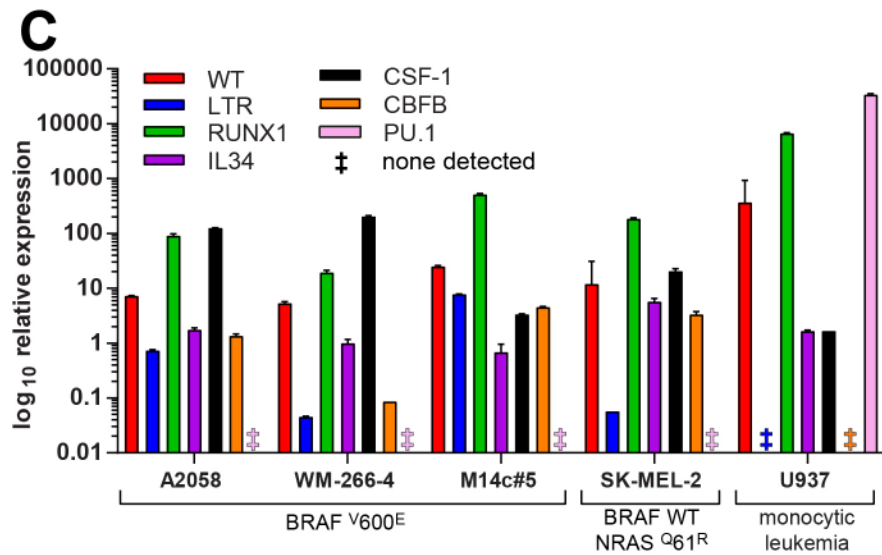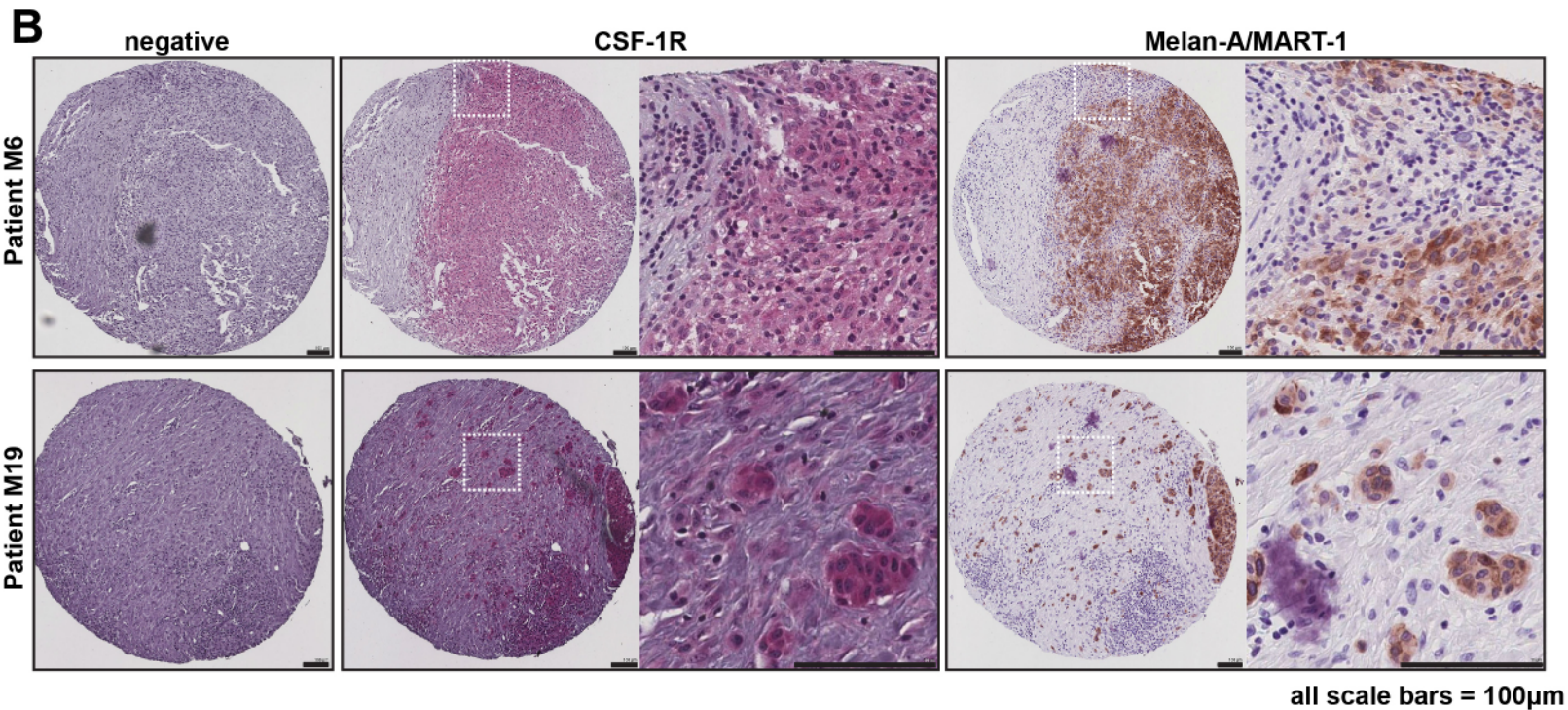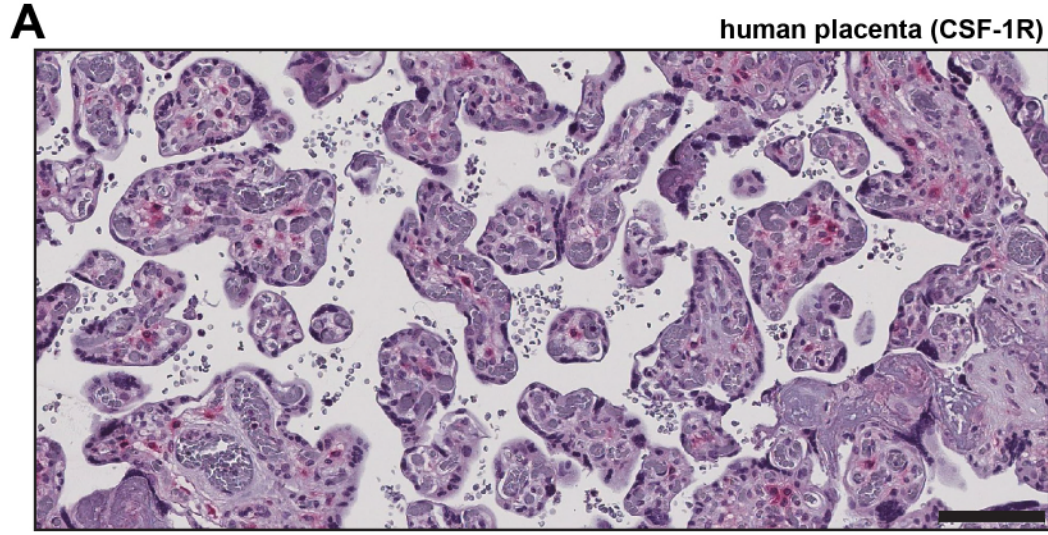
A human placenta (CSF-1R)

B

	negative	CSF-1R	Melan-A/MART-1
Patient M6			
Patient M19			

all scale bars = 100μm

C

Legend:
- WT
- LTR
- RUNX1
- IL34
- CSF-1
- CBFB
- PU.1
- ‡ none detected

log₁₀ relative expression

A2058, WM-266-4, M14c#5 — BRAF V600E
SK-MEL-2 — BRAF WT NRAS Q61R
U937 — monocytic leukemia

D

Legend:
- LTR
- RUNX1
- ‡ none detected

relative expression

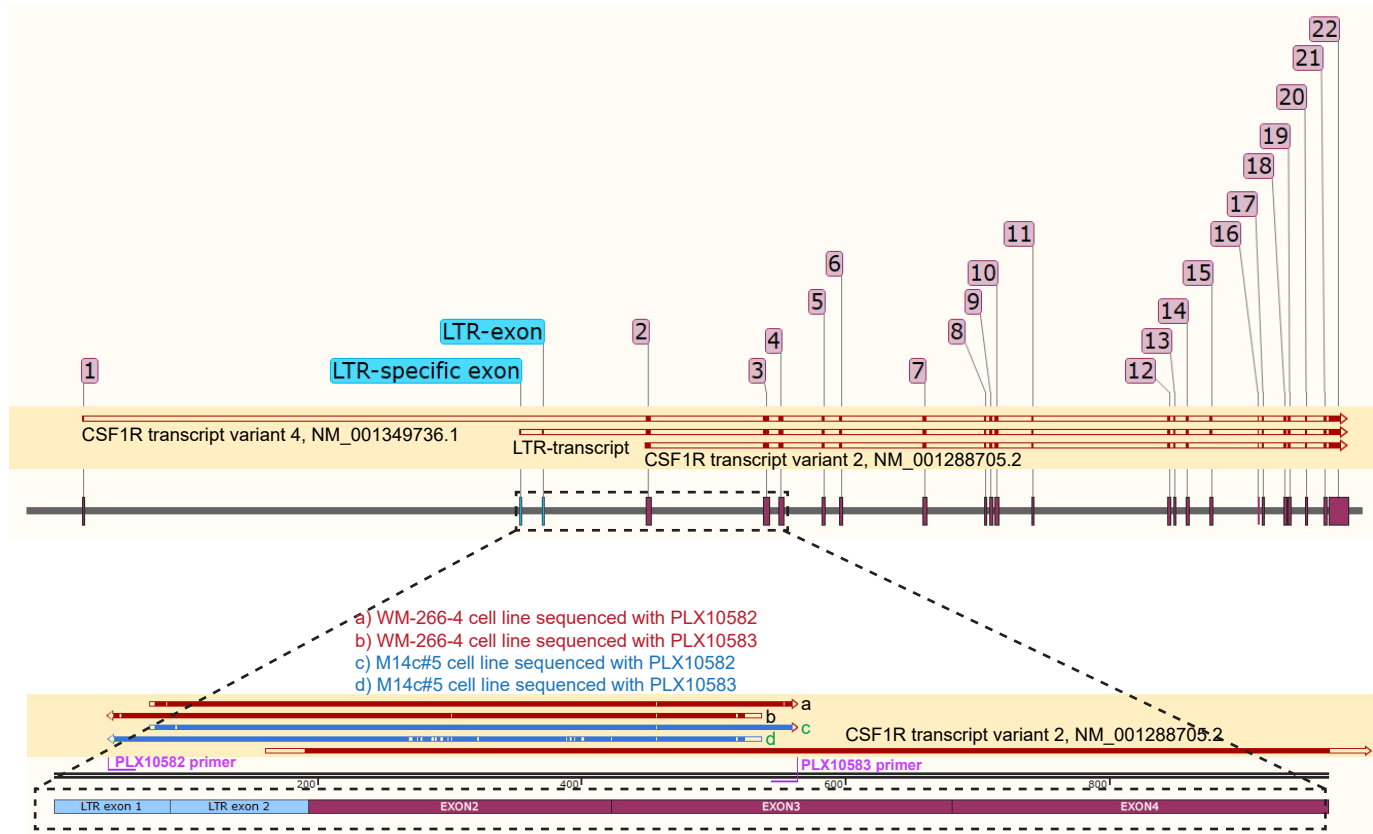WM-266-4, N20, N22, N23, N24 — benign nevus samples

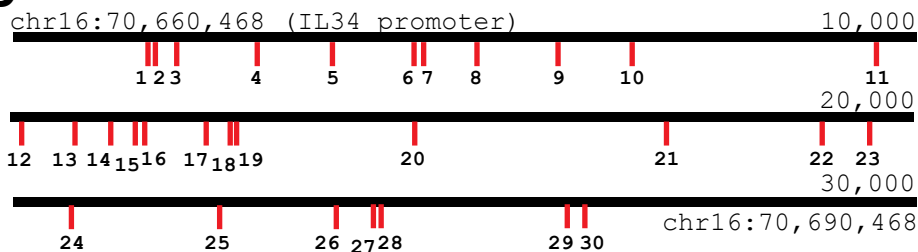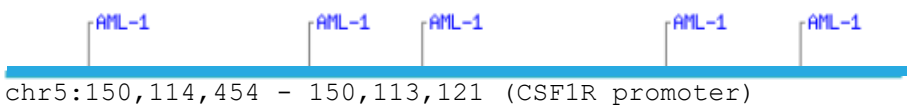**Figure S3.**

Supplemental Figure 3

Immunohistochemistry staining of CSF-1R: placenta control (**A**) and representative images of primary samples along with MelanA-counterstain (**B**). Gene expression analysis (quantitative RT-PCR, mean±SEM) in cell lines of the canonical *CSF1R* (WT) and of the viral (LTR) transcripts, as well as of the two ligands (CSF-1, IL-34), the transcription factor RUNX1, its co-factor CBFβ (CBFB) and of PU.1. Cell-lines used: three BRAF V600E mutant melanoma cell lines, one BRAF WT cell line, and a monocytic leukemia cell-line as control (**C**). Gene expression analysis (quantitative RT-PCR, mean±SEM) in benign nevus (birthmark) samples demonstrates no detection of the LTR form of the *CSF1R* mRAN (**D**).

Figure S4

Panel D table:

	sequence	from	-to	score
1	TTTTTGGTC	70662010	70662018	8.372
2	ATTGTGGTC	70662063	70662071	12.295
3	TTTGTTGTT	70662321	70662329	7.944
4	TCTGAGGTT	70663224	70663232	8.077
5	CATGTGGTT	70664057	70664065	9.467
6	TTTGTGGTT	70664961	70664969	12.626
7	TCTTTGGTT	70665078	70665086	7.426
8	CCAGTGGTT	70665664	70665672	7.468
9	TTTGTGTTT	70666564	70666572	7.944
10	TTTGAGGTC	70667397	70667405	9.023
11	TTTGTGTTC	70670112	70670120	7.614
12	CCTGTGGTC	70670560	70670568	10.065
13	TTTGTGCTT	70671153	70671161	7.944
14	TATGTGGTA	70671562	70671570	8.842
15	TCTGTGGTT	70671822	70671830	11.349
16	TTTGTTGTC	70671928	70671936	7.614
17	ACTGTGGGT	70672621	70672629	7.503
18	GTTGTGGTA	70672883	70672891	8.344
19	TCTGTGGCT	70672948	70672956	7.503
20	CTTGAGGTC	70674957	70674965	8.07
21	ATTGTGATT	70677754	70677762	7.944
22	TGTGTGGTT	70679482	70679490	9.296
23	ACTGAGGTC	70680000	70680008	7.747
24	CTTGTGGGT	70681175	70681183	7.826
25	CTTGTGGCC	70682823	70682831	7.496
26	CTTGAGGTT	70684121	70684129	8.4
27	CTTGTGGAC	70684545	70684553	7.496
28	TCTGTGGTT	70684598	70684606	11.349
29	TTAGTGGTT	70686689	70686697	9.698
30	CTTGAGGTC	70686890	70686898	8.07

Panel F table:

	sequence	from	-to	score
1	TACCCCAG	148904731	148904739	6.100
2	TACCAAAGA	148905059	148905067	5.847
3	TTTGTGGTC	148905225	148905233	12.295
4	GCCCACAGG	148905589	148905597	6.219
5	ACTGCGGCC	148905788	148905796	6.513

Supplemental Figure 4

Detailed sequencing showing of the presence of the LTR-form of *CSF1R* transcript in two melanoma cell lines: WM-266-4 and A2058. Alignment performed with SnapGene® software (from GSL Biotech; available at snapgene.com). (**A**). Genomic sequence of the *CSF1R* LTR (chr5:148,904,955-148,904,605): the 5′ UTR of the melanoma cell lines from the transcription start site (marked as +1) to the first splice site is underlined. GATA, Sp1, E-Box, AP-1, NF-κB and RUNX1 indicate putative transcription factor binding sites; TATA indicates a TATA box (**B**). Schematic (**C**) and tabular representation (**D**) of the putative RUNX1 binding sites in the *IL-34* promoter (chr16:70,660,468-70,690,468). Schematic (**E**) and tabular representation (**F**) of the putative RUNX1 binding sites in the *CSF1R* promoter (chr5: 150,114,454-150,113,121). ConSite TF cutoff score: 85%.

A

	24h		48h	
		p		p
A2058-shCSF1R#1	**	**0.0031**	*	**0.012**
A2058-shCSF1R#5	ns	0.0717	**	**0.002**
A2058-shRUNX1#1	*	**0.0423**	**	**0.0037**
A2058-shRUNX1#4	*	**0.0123**	***	**0.0001**
M14-shCSF1R#1	ns	0.1468	**	**0.002**
M14-shCSF1R#5	*	**0.0221**	**	**0.0017**
M14-shRUNX1#1	*	**0.0246**	ns	0.0537
M14-shRUNX1#4	*	**0.0368**	**	**0.0059**

B

control — A2058 in 3D

shCSF1R #1 shCSF1R #5

shRUNX1 #1 shRUNX1 #4

C

A2058 in 3D

phase

fluorescein

DAPI

Apoptosis Proliferation

DMSO 0.1 μM 1.0 μM 10 μM PLX3397

D

M14c#5
WM-266-4

% invasion

p=0.0169
p=0.0014
p=0.0015 p=0.0007

μM PLX3397
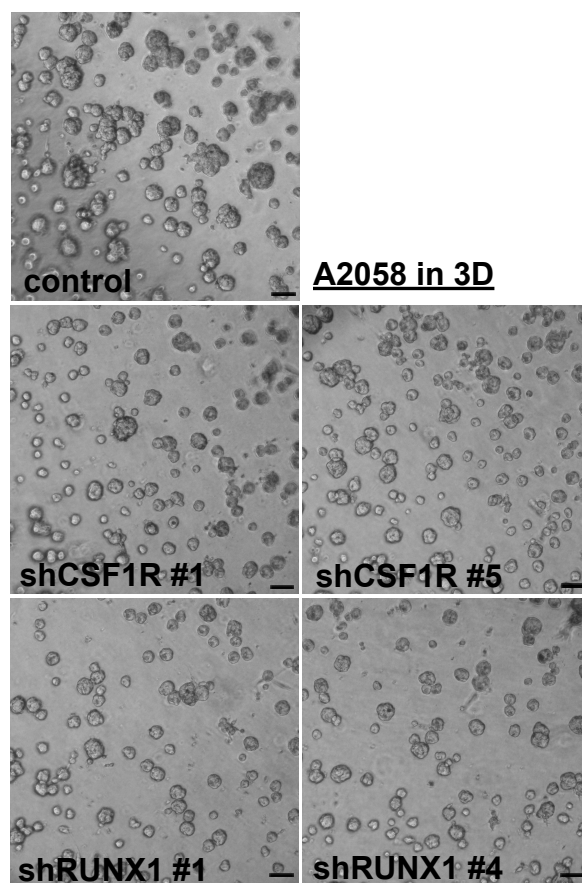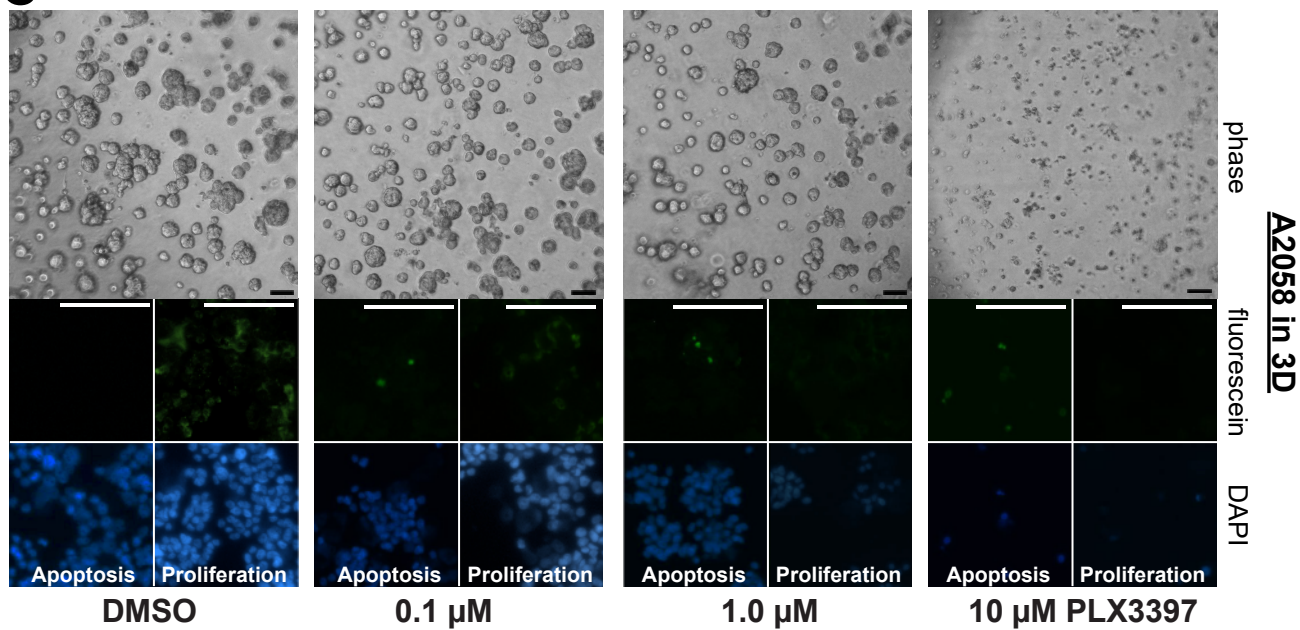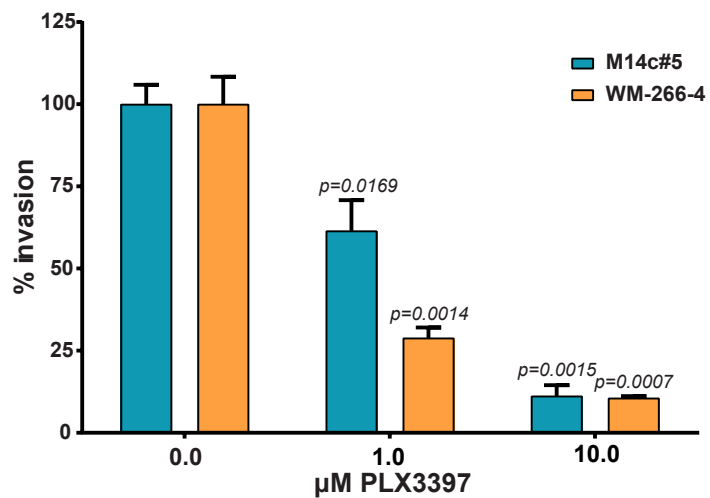
Figure S5

Supplemental Figure 5

Statistical analysis (one-way ANOVA) of the proliferation of the shCSF1R and shRUNX1 stable A2058 and M14c#5 cell lines as compared to the non-targeting control (**A**). Magnified images of the *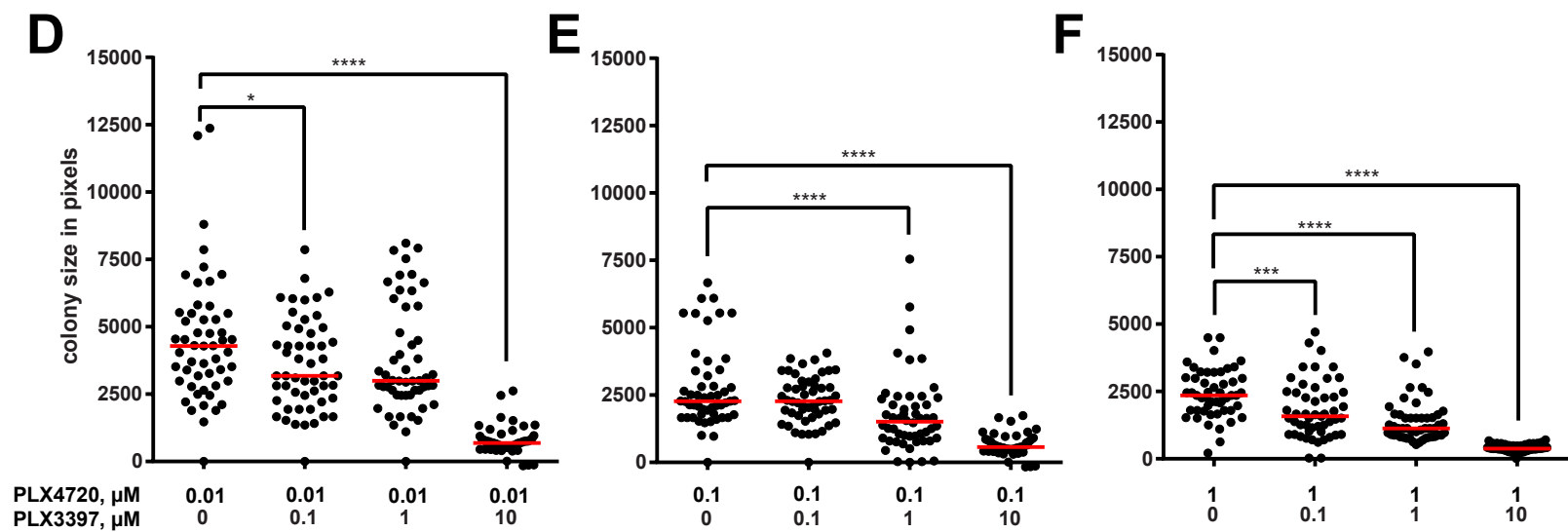CSF1R* and *RUNX1* stable A2058 clones in 3D culture on day 5 (**B**). Magnified images of A2058 treated with three different doses of PLX3397 in 3D, showing green for proliferation (BrdU-assay, fluorescein) or apoptosis (TUNEL, fluorescein) observed in these colonies. Nuclei counterstained with DAPI. All 3D images taken at 10X magnification. Scale bar (black bar in phase contrast images, white bar in fluorescent images): 100µm (**C**). Transwell invasion assay (mean±SEM) of the M15c#5 and the WM-266-4 melanoma cell lines treated with PLX3397 (One-way ANOVA) (**D**).

A M14C#5 PLX4720 dose response

BRAFi IC$_{45}$
- IC$_{45}$=0.005μM (blue triangle)
- IC$_{45}$=0.037μM (red square)
- IC$_{45}$=0.085μM (black circle)

y-axis: % viability (0–125)
x-axis: [PLX4720] μM (10^{-4} to 10^{2})

B WM-266-4 PLX4720 dose response

BRAFi IC$_{50}$
- IC$_{50}$=0.032μM (15 μM Ci) (blue triangle)
- IC$_{50}$=0.121μM (10 μM Ci) (red square)
- IC$_{50}$=0.294μM (0 μM Ci) (black circle)

y-axis: % viability (0–125)
x-axis: [PLX4720] μM (10^{-4} to 10^{2})
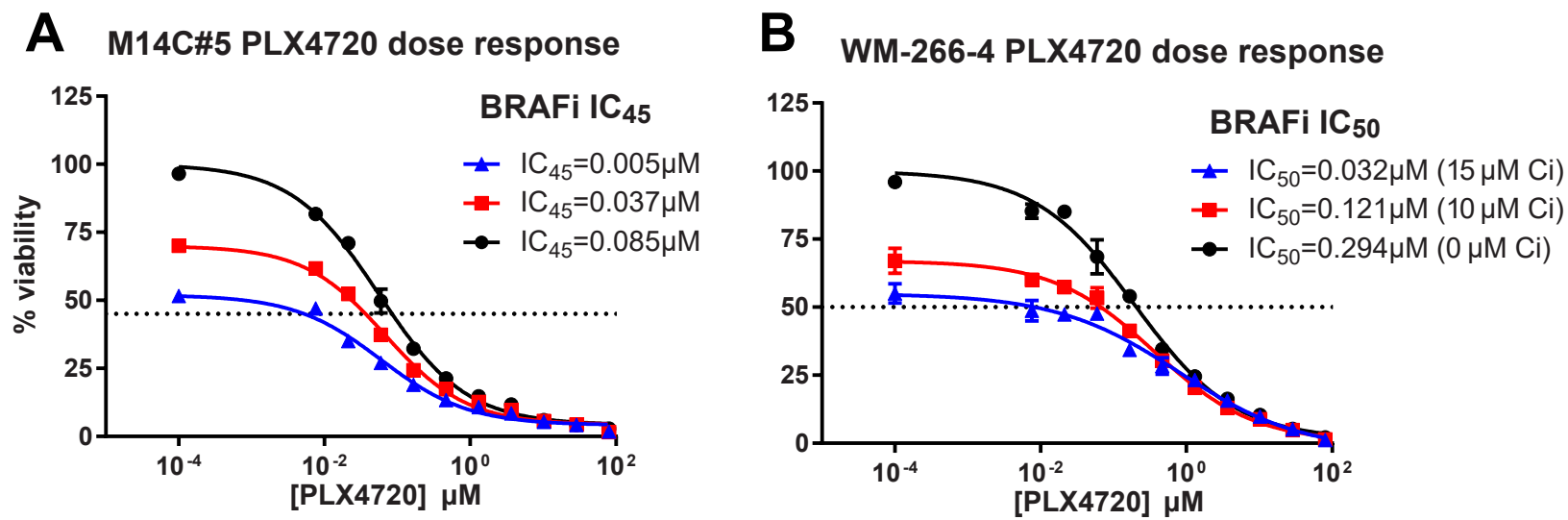
C

	Single agent	Combination			
	PLX4720	10μM PLX3397		15uM PLX3397	
	(μM)	(μM)	**CI**	(μM)	**CI**
M14	0.085	0.037	**0.91**	0.005	**0.78**
WM-266-4	0.294	0.121	**0.91**	0.032	**0.86**

D

y-axis: colony size in pixels (0–15000)

| PLX4720, μM | 0.01 | 0.01 | 0.01 | 0.01 |
| PLX3397, μM | 0 | 0.1 | 1 | 10 |

(* , ****)

E

| PLX4720, μM | 0.1 | 0.1 | 0.1 | 0.1 |
| PLX3397, μM | 0 | 0.1 | 1 | 10 |

(**** , ****)

F

| PLX4720, μM | 1 | 1 | 1 | 1 |
| PLX3397, μM | 0 | 0.1 | 1 | 10 |

(*** , **** , ****)

G

	p-value
control vs. PLX3397	*0.0003*
control vs. PLX4720	*0.0012*
control vs. PLX/PLX	*<0.0001*
PLX3397 vs. PLX/PLX	*0.0007*
PLX4720 vs. PLX/PLX	*<0.0001*

Figure S6.

Supplemental Figure 6

72 hour dose response curves for PLX4720 monotherapy or PLX4720 with fixed concentrations (10 or 15µM) of PLX3397 (Ci) for the M14c#5 (**A**) and the WM-266-4 (**B**) cell lines. Combination indices at IC_{45} (M14c#5) or at IC_{50} (WM-266-4) shown in table (**C**). Statistical analysis (one-way ANOVA, statistical significance levels are noted with asterisks, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$) of the effect of combining the BRAF and the CSF1R inhibitors in 3D cell culture. Average colony size in pixels plotted for 0.01uM (**D**), 0.1uM (**E**) or 1uM PLX4720 (**F**) combined with 3 different doses of PLX3397. Tumor size (mean ±SEM) in A2058 xenografted mice treated with PLX3397 and PLX4720 or with combination, 10 mice in each group (**G**) and statistical analysis of tumor size, one-way ANOVA (**I**). Statistical analysis of survival of xenografted mice treated with BRAF- or CSF1R-monotherapy or combination therapy (**H**).

A

Timeline of 96h time course experiment

🟥	cells incubated with serum free medium
🟦	cells incubated with 3µM PLX4720
⬇️ (cyan)	serum-free medium with 3µM PLX4720 fully replaced
⬇️ (brown)	serum-free medium fully replaced
⬇️ (green)	PLX4720 added for a final concentration of 3µM
⬇️ (red dashed)	optional: PLX3397 added for a final concentration of 30µM

cells counted

cell lysate collected
(RNA, protein work)

48h supernatants collected for all (ELISA)

B

PLX4720
(no PLX3397)

0 2 4 8 16 24 48 72 96h

pERK
ERK
Actin

M14c#5

PLX4720
(1h PLX3397)

0 2 4 8 16 24 48 72 96h

pERK
ERK
Actin

M14c#5

C

PLX4720 (h)	-	-	-	1	2	1	2	1	2
PLX3397 (h)	-	1	2	-	-	-	-	1	1
CSF-1 (15min)	-	-	-	-	-	+	+	+	+

pERK
ERK
pAKT
AKT

WM-266-4

Figure S7.

Supplemental Figure 7

Schematic representation of the 96h BRAF-V600E inhibition experiments. Cells are seeded in full growth medium. On day one, all wells are replaced with either serum-free medium (SFM) or 3uM PLX4720 in SFM ("96h" condition). Cell culture media are replaced with either SFM or SFM with the drug according to the timeline. In the last 48h, all medium is refreshed and from this point, drug is added to the media already present in order to ensure that the supernatants are conditioned by the cells for exactly 48 hours. For the combination experiment, 30uM PLX3397 is added to the cultures in the final hour of the 96h time course (**A**). The effect of combined PLX3397/PLX4720 treatment on signaling in M14c#5. The levels of pERK1/2, total ERK1/2 and actin are shown after a 96h 3μM PLX4720 time course treatment with or without 30μM PLX3397 added in the last hour (**B**). The effect of CSF-1 on the rebound of ERK phosphorylation in WM-266-4 cells. The levels of pERK1/2, total ERK1/2, pAKT, total AKT and actin are shown after a 1 or 2h treatment with 3μM PLX4720 with or without 30μM PLX3397 with 100ng/μL rCSF-1 of added in the last 15 min (**C**).