

**a**

NCCs vs. melanocytes	Metastasis vs. Primary melanoma	Shared
Hypermethylated (n=1188)	Hypermethylated (n=784)	41
Hypomethylated (n=1373)	Hypomethylated (n=445)	14
Hypermethylated (n=1188)	Hypomethylated (n=445)	0
Hypomethylated (n=1373)	Hypermethylated (n=784)	1

β value



**b**

**41 hypermethylated CpGs in NCCs and melanoma metastasis**

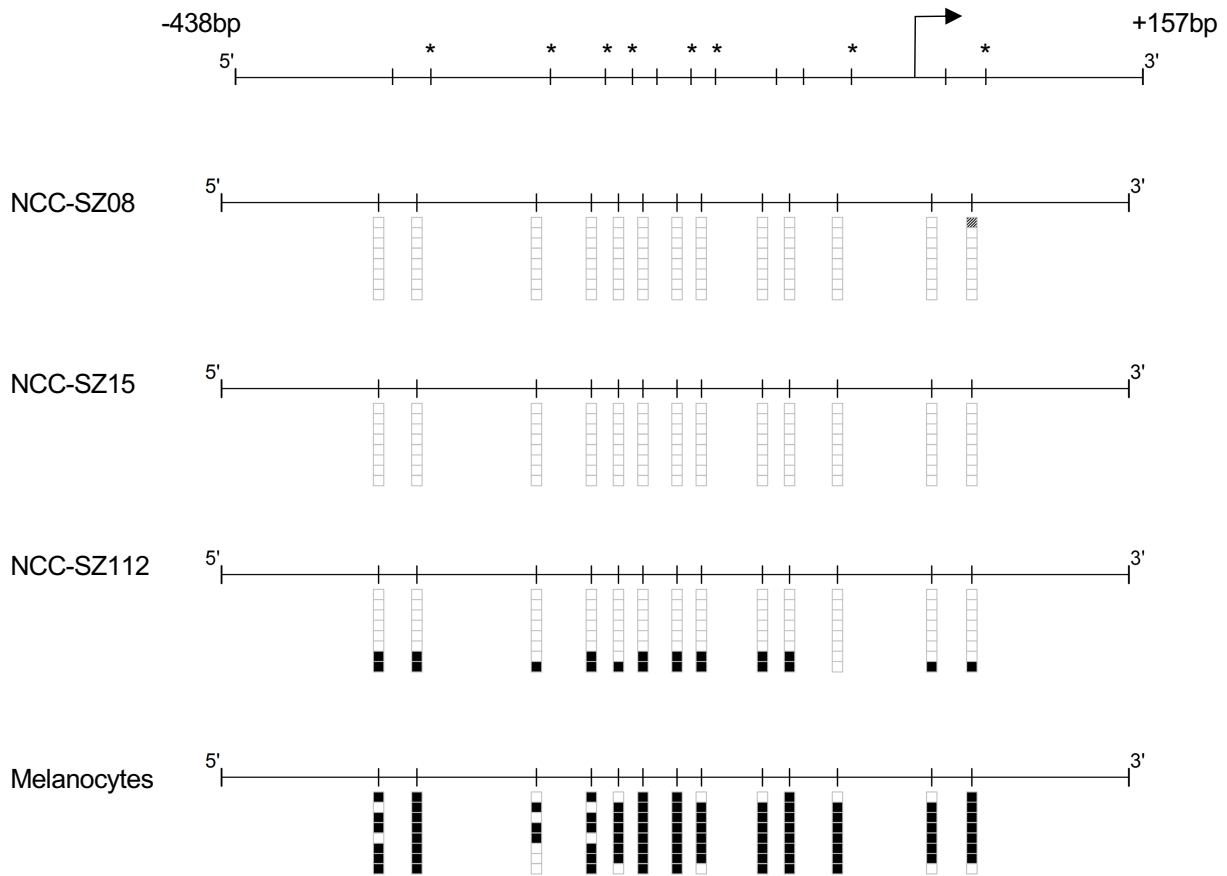
CpG ID	Chr	Coordinate	Genes	NCCs	Melanocytes	diff	p value	Primary	Metastasis	diff	p value
cg02025435	16	89986317	MC1R	1.0	0.2	-0.8	4.90416E-06	0.5	1.0	0.4	9.04653E-13
cg01511465	16	89986185	MC1R	1.0	0.1	-0.9	3.31277E-09	0.6	1.0	0.4	2.77595E-13
cg06475006	16	89985975	MC1R	1.0	0.2	-0.7	1.21266E-06	0.5	0.9	0.3	6.50386E-12
cg06297571	12	132270829	SFRS8	0.9	0.0	-0.9	9.89803E-11	0.4	0.7	0.3	8.3561E-11
cg07009334	6	1410489		0.9	0.2	-0.7	3.49868E-06	0.5	0.8	0.3	2.04064E-05
cg17044311	10	101542983	ABCC2	0.9	0.1	-0.7	1.70746E-07	0.5	0.8	0.3	4.45334E-12
cg01768446	16	89982419		1.0	0.1	-0.8	6.4127E-09	0.5	0.7	0.3	7.90363E-09
cg19458233	3	100148679	LNP1	0.9	0.0	-0.9	5.83777E-12	0.5	0.8	0.3	0.000116675
cg16613990	16	89982618		0.9	0.1	-0.8	2.16979E-09	0.6	0.8	0.2	2.09077E-09
cg02922150	16	1514587	CLCN7;CLCN7	0.9	0.2	-0.7	9.70742E-07	0.5	0.8	0.2	1.21004E-11
cg12198090	12	132270765	SFRS8	0.9	0.2	-0.7	1.81025E-06	0.6	0.8	0.2	1.29253E-08
cg15770585	7	4762742	FOXP1	1.0	0.0	-0.9	6.85955E-13	0.6	0.8	0.2	4.72437E-05
cg09867143	16	1514413	CLCN7;CLCN7	0.8	0.2	-0.7	2.15705E-07	0.6	0.8	0.2	5.45373E-12
cg14583973	4	3374767	RGS12;RGS12;RGS12	0.9	0.1	-0.8	2.18574E-05	0.5	0.8	0.2	7.1024E-05
cg22094306	2	131834952	FAM168B	0.8	0.1	-0.7	6.57535E-09	0.6	0.8	0.2	3.93509E-08
cg03699749	20	60869595	OSBPL2;OSBPL2	0.9	0.0	-0.9	9.40279E-12	0.4	0.6	0.2	8.64547E-07
cg20957542	22	43529321	MCAT;MCAT	0.8	0.1	-0.7	2.73133E-11	0.4	0.6	0.2	3.49224E-12
cg14414203	14	23315249	MMP14	0.9	0.2	-0.7	6.64945E-05	0.6	0.8	0.2	0.000183675
cg13496793	9	129645969	ZBTB34	0.9	0.1	-0.8	1.07848E-08	0.6	0.8	0.2	4.47707E-06
cg01198184	14	103433908	CDC42BPB	0.9	0.1	-0.8	3.50269E-11	0.4	0.6	0.2	1.54669E-07
cg09951047	9	97848306	C9orf3;MIR24-1	0.9	0.1	-0.7	1.17201E-07	0.5	0.7	0.2	3.65993E-08
cg03647436	10	134477441	INPP5A	1.0	0.1	-0.9	1.31383E-06	0.5	0.7	0.2	5.6155E-07
cg10914815	13	99148667	STK24;STK24	0.8	0.1	-0.7	4.59109E-08	0.3	0.5	0.2	2.18322E-06
cg10397930	11	109959298		0.9	0.1	-0.8	3.6357E-07	0.5	0.7	0.2	1.24517E-06
cg18773129	22	19938916	COMT;COMT;COMT	0.9	0.1	-0.9	1.82073E-10	0.4	0.6	0.2	0.002023505
cg11435167	19	1588449	MBD3	0.9	0.1	-0.9	6.77279E-13	0.5	0.6	0.2	1.18356E-09
cg07155331	12	125500511	BRI3BP	0.9	0.1	-0.8	1.34536E-09	0.5	0.7	0.2	0.000293968
cg09121485	7	6218888	CYTH3	0.9	0.2	-0.7	4.19519E-08	0.7	0.8	0.2	1.48573E-10
cg03562952	5	1499204	LPCAT1	0.9	0.2	-0.8	1.29539E-07	0.5	0.7	0.2	3.52919E-07
cg16871520	3	52442033	BAP1	0.9	0.2	-0.7	4.47327E-10	0.5	0.7	0.2	4.65478E-07
cg16283158	14	103433189	CDC42BPB	0.9	0.1	-0.8	3.08864E-07	0.7	0.8	0.2	8.59925E-13
cg05904317	4	120518737	PDE5A;PDE5A;PDE5A	0.9	0.1	-0.8	1.65271E-10	0.4	0.6	0.2	3.63482E-07
cg25030694	2	134329911		0.9	0.2	-0.7	9.16219E-08	0.6	0.7	0.2	4.35E-08
cg14873022	4	146841701	ZNF827	0.8	0.0	-0.8	4.91835E-08	0.3	0.5	0.2	1.96966E-07
cg24211657	17	76886742	TIMP2	0.9	0.1	-0.8	3.96996E-09	0.3	0.5	0.2	2.23063E-07
cg08240529	2	37571401	QPCT	0.9	0.2	-0.7	3.47555E-07	0.6	0.8	0.2	4.52068E-07
cg19612114	14	103433138	CDC42BPB	0.9	0.2	-0.7	3.12931E-07	0.7	0.8	0.2	1.40451E-11
cg25989745	9	12693326	TYRP1	0.8	0.1	-0.7	8.88522E-09	0.6	0.8	0.2	6.56294E-05
cg00163372	8	128752988	MYC	0.8	0.0	-0.8	1.13757E-08	0.3	0.5	0.2	0.016939887
cg24721056	18	11818701	GNAL;GNAL;GNAL	0.8	0.2	-0.7	6.59852E-06	0.4	0.5	0.2	1.33326E-06
cg01916088	6	41528531	FOXP4;FOXP4;FOXP4	0.8	0.0	-0.8	2.5837E-06	0.1	0.3	0.2	0.026830917

**c**

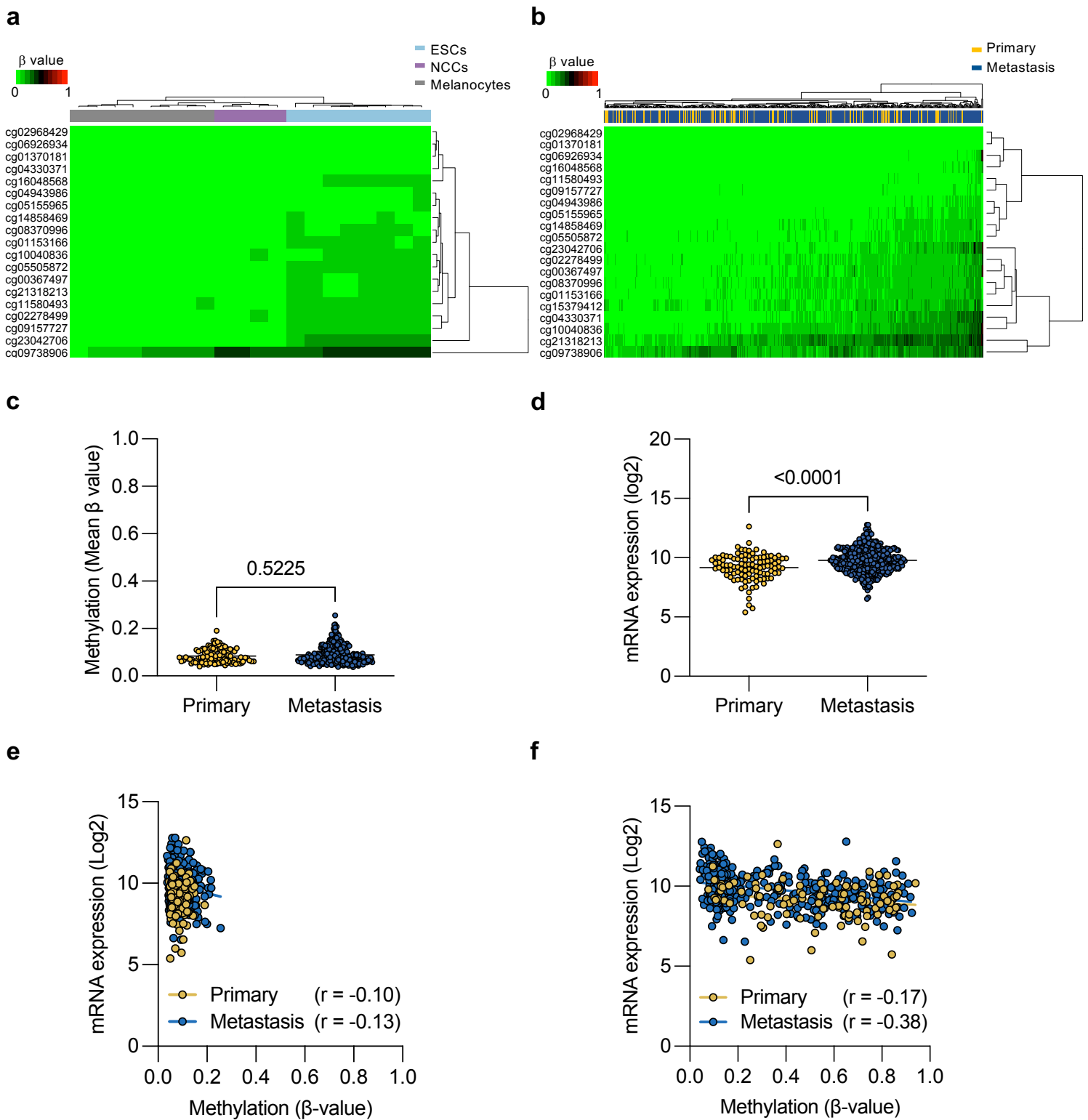
**14 hypomethylated CpGs in NCCs and melanoma metastasis**

CpG ID	Chr	Coordinate	Genes	NCCs	Melanocytes	diff	p value	Primary	Metastasis	diff	p value
cg05108467	15	96869132	NR2F2	0.1	0.8	0.7	1.45523E-06	0.6	0.2	-0.3	7.33737E-08
cg03018496	15	96868989	NR2F2	0.1	0.9	0.7	1.07305E-05	0.7	0.4	-0.3	1.8228E-07
cg26593946	15	96869043	NR2F2	0.1	0.9	0.8	3.7246E-07	0.6	0.3	-0.3	7.30956E-07
cg09969043	15	96868971	NR2F2	0.1	0.8	0.7	1.02828E-05	0.5	0.2	-0.3	5.76819E-08
cg17826834	15	96868857	NR2F2	0.2	0.9	0.7	5.98012E-06	0.6	0.3	-0.3	3.61954E-07
cg20245757	17	46663759		0.0	0.9	0.8	1.65151E-07	0.6	0.4	-0.2	0.000182355
cg17719317	4	6909606		0.1	0.9	0.8	9.89475E-09	0.5	0.3	-0.2	0.003165727
cg24811290	4	159092553	FAM198B	0.2	0.9	0.7	7.69637E-07	0.5	0.3	-0.2	1.30675E-06
cg04883026	11	66487938	SPTBN2	0.2	0.9	0.7	1.03279E-06	0.5	0.4	-0.2	0.008358493
cg22957135	14	33409651	NPAS3	0.1	0.8	0.7	2.46678E-08	0.3	0.1	-0.2	2.04732E-07
cg08325519	12	130571246		0.2	0.9	0.7	6.5131E-10	0.4	0.2	-0.2	0.000171311
cg27164797	6	57038231	BAG2	0.1	0.9	0.8	5.67013E-08	0.7	0.6	-0.2	9.75422E-06
cg00436496	15	96885119		0.2	0.9	0.7	1.51868E-05	0.6	0.5	-0.2	0.000105765
cg03159396	6	50808813	TFAP2B	0.1	0.8	0.7	3.90715E-09	0.7	0.5	-0.2	0.008125247

**Suppl. Fig. 1. Differentially methylated CpGs between Neural crest cells (NCCs) vs. melanocytes, or in TCGA metastatic vs. primary melanoma samples. a.** Integration analysis was performed to identify hypermethylated or hypomethylated CpGs between NCCs vs. melanocytes and metastasis vs. primary melanoma samples. Forty-one hypermethylated (**b**) and fourteen hypomethylated (**c**) CpGs shared by NCCs and metastasis were identified. Top 5 hypomethylated CpGs were located in the Nuclear Receptor Subfamily 2 Group F Member 2 (*NR2F2*) promoter region. Methylation scores (Beta values) ranging from 0 (unmethylated, green) to 1 (complete methylated, red) were obtained from HumanMethylation450K arrays. Differential methylation between groups and p values (unpaired t-test) are depicted. Of note, only one hypomethylated CpG (cg08426157, *HDAC9* gene body) and no hypermethylated CpGs were shared between NCCs and primary melanoma samples (**a**).

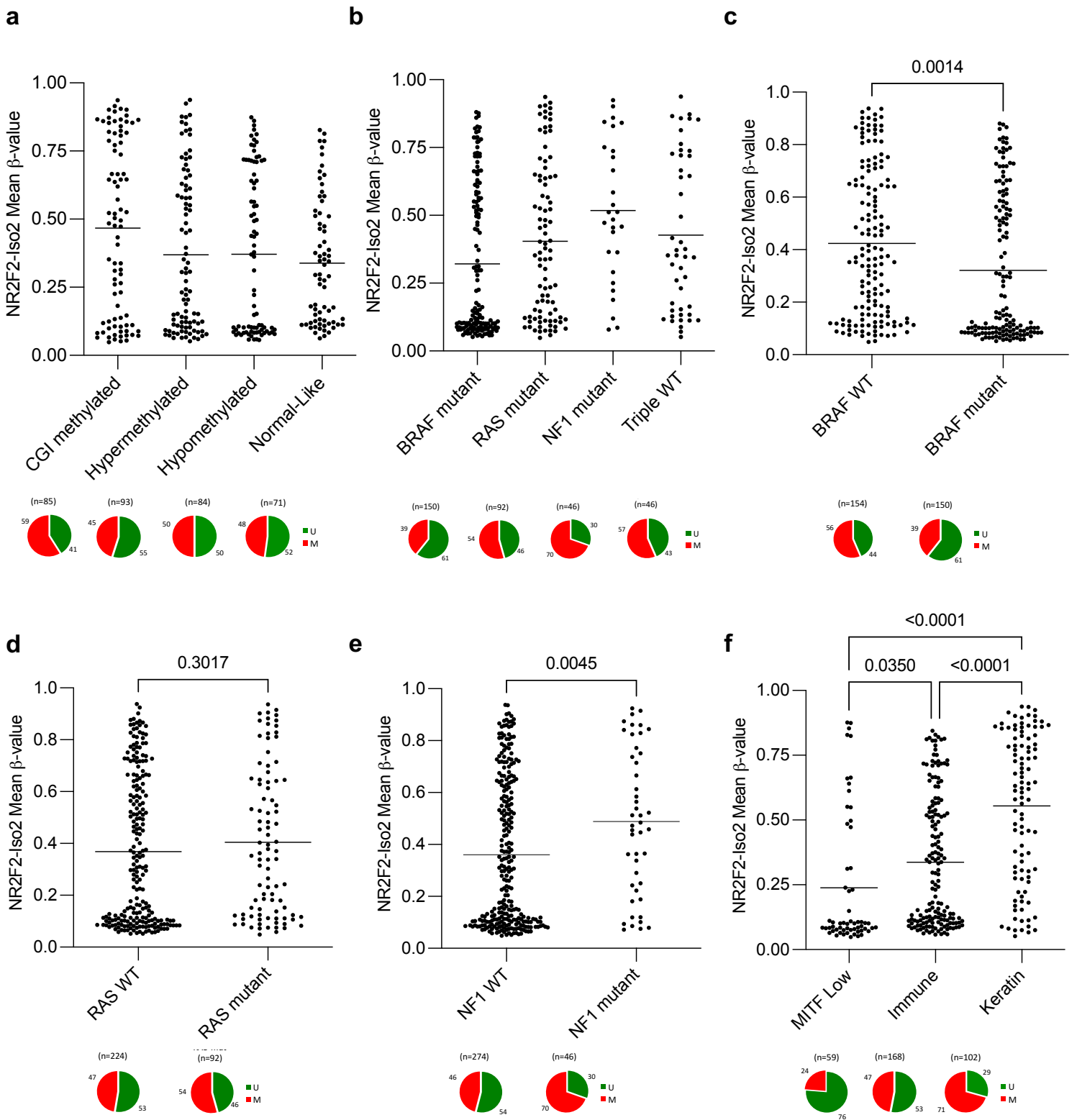


**Suppl. Fig. 2. *NR2F2*-Isoform 2 methylation profiles in neural crest cells and melanocytes.** Bisulfite sequencing of the region around the *NR2F2*-Iso2 transcription start site (-438bp to +157bp) in neural crest cells and melanocytes, validating the data generated by DNA methylation arrays. CpGs are represented as white (unmethylated) or black (methylated) squares. \*: CpGs interrogated in the 450K methylation array; arrow: Transcription start site (TSS).



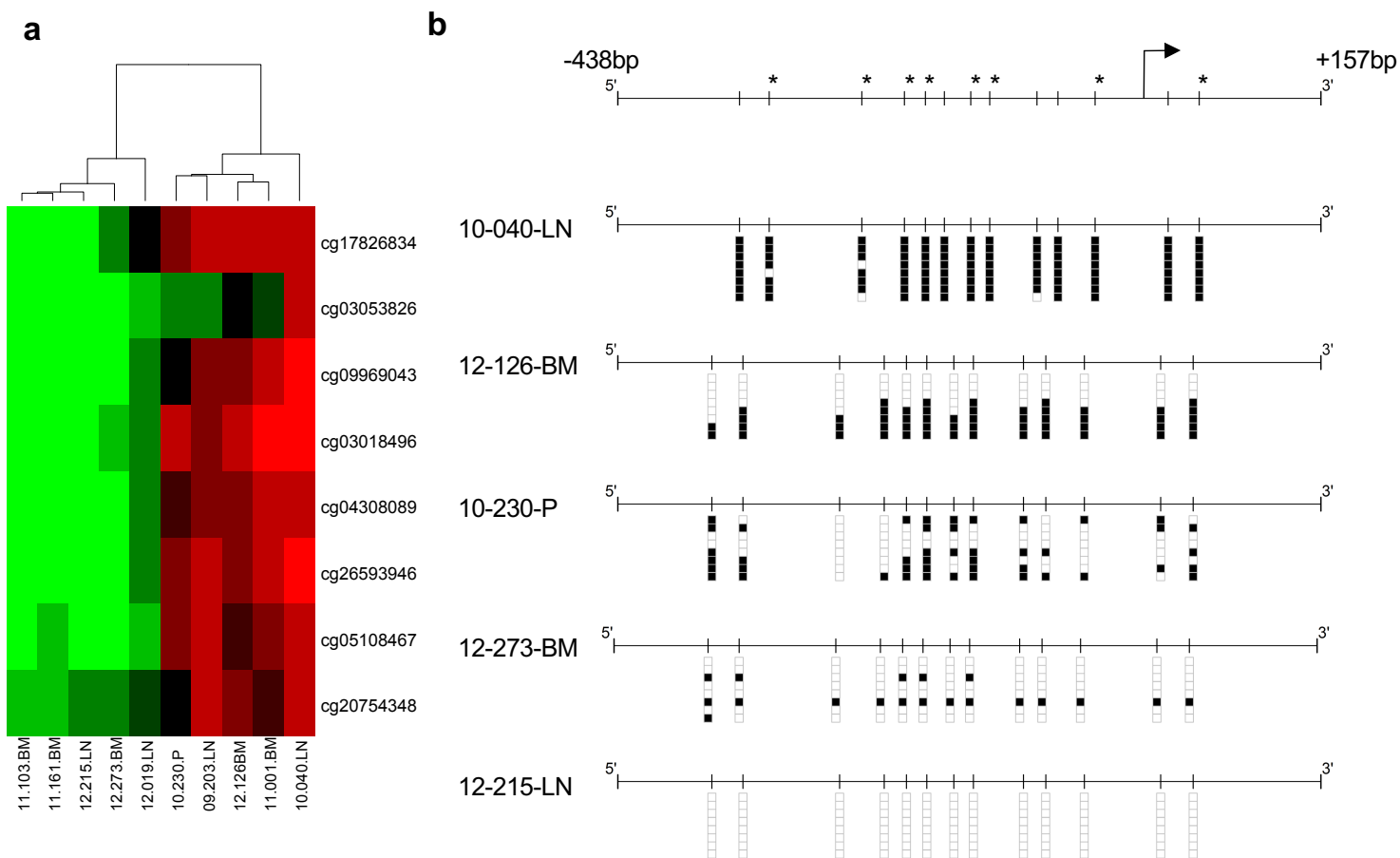
**Suppl. Fig. 3. *NR2F2*-Isoform 1 methylation and expression in ESCs, NCC, melanocytes and during melanoma progression.** Absence of methylation in the CpG island around the transcription start site (TSS) of isoform 1 in embryonic stem cell lines (ESCs), neural crest cells (NCCs) and melanocytes (**a**); and in primary and metastatic melanoma (**b**, **c**). Methylation scores (Beta values) ranging from 0 (unmethylated, green) to 1 (completely methylated, red) were obtained from HumanMethylation450K arrays. The depicted CpGs are located from -261 to 1719pb to the *NR2F2*-Iso1 TSS. *NR2F2*-Iso1 mRNA expression of primary and metastatic TCGA melanoma samples (**d**, two tailed Mann-Whitney test, bars represent min. and max.). Correlation between *NR2F2*-Iso1 expression and methylation around the TSS of isoform 1 (**e**, Spearman's rank correlation) and of isoform 2 (**f**, Spearman's rank correlation) in TCGA melanoma samples. Source data are provided as a Source Data file.



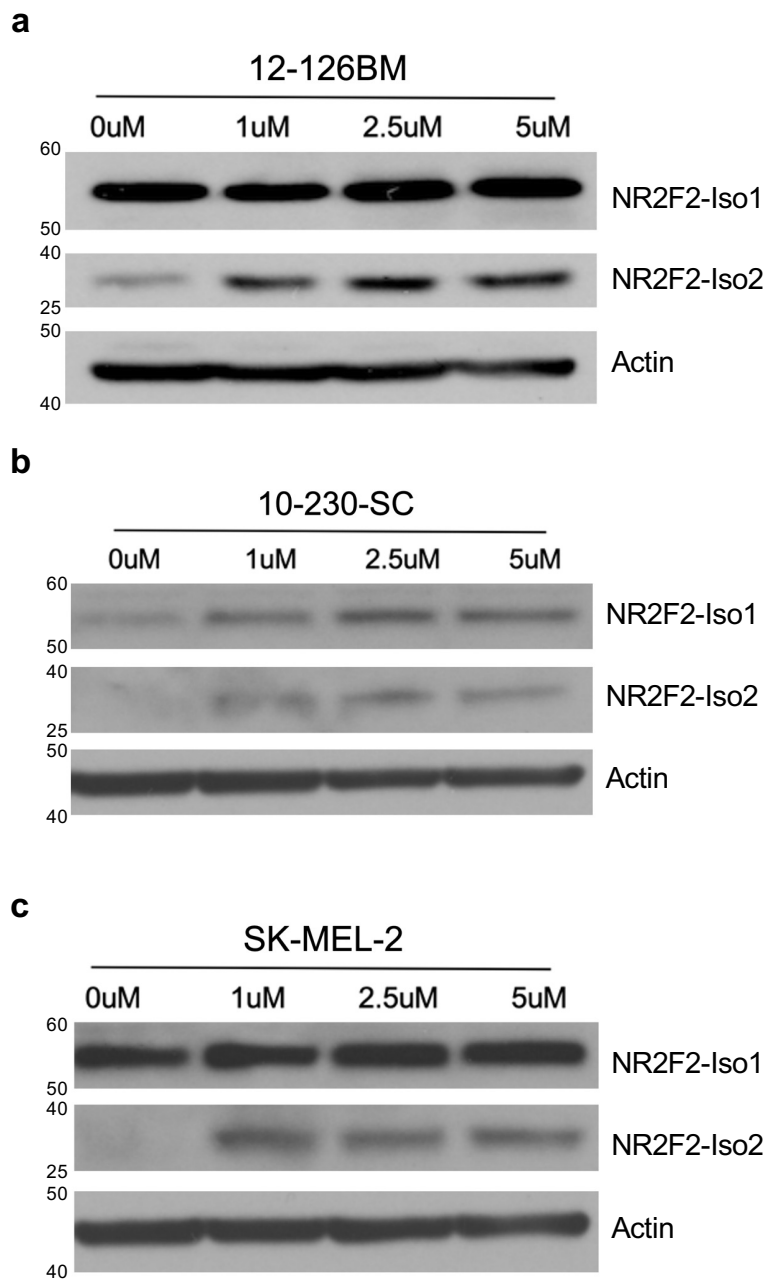


**Suppl. Fig. 4. NR2F2-Iso2 methylation in different melanoma subtypes. (a-f).** NR2F2 isoform 2 methylation status was evaluated across the distinct subtypes defined by The Cancer Genome Atlas Network (Cell, 2015). NR2F2-Iso2 methylation (mean Beta-values) in patients classified by DNA methylation clusters (a); patients harboring BRAF, RAS (NRAS, KRAS or HRAS) or NF1 hot-spot mutations, and triple wild-type (b); additional comparisons of NR2F2-Iso2 methylation levels between BRAF mutant and wt (c, unpaired two-tailed T-test), RAS (NRAS, KRAS or HRAS) mutant and wild-type (d, unpaired two-tailed T-test) and NF1 mutant and wt (e, unpaired two-tailed T-test); Comparison of NR2F2-Iso2 methylation levels among TCGA-defined transcriptomic subclasses named “MITF low”, “immune” and “keratin” (f, Two-way ANOVA). U: unmethylated, green; M: methylated, red. Bars represent the mean. Source data are provided as a Source Data file.

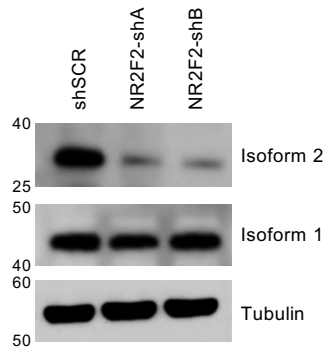
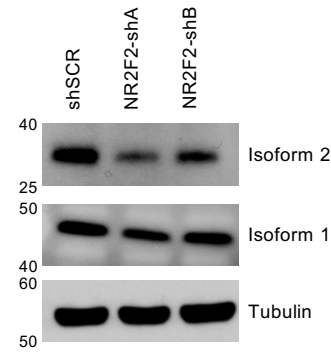
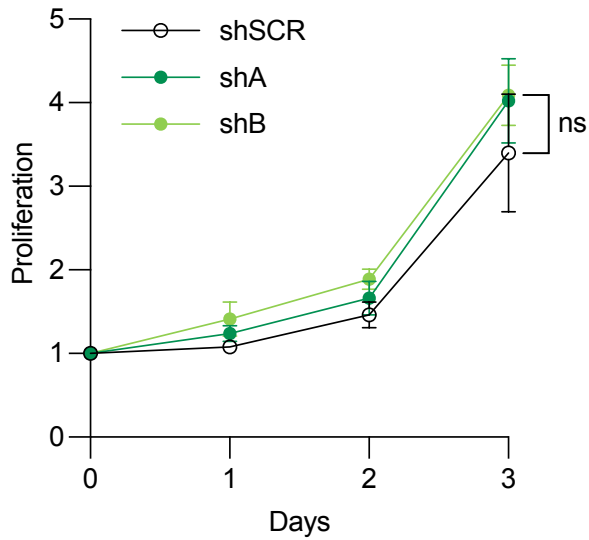
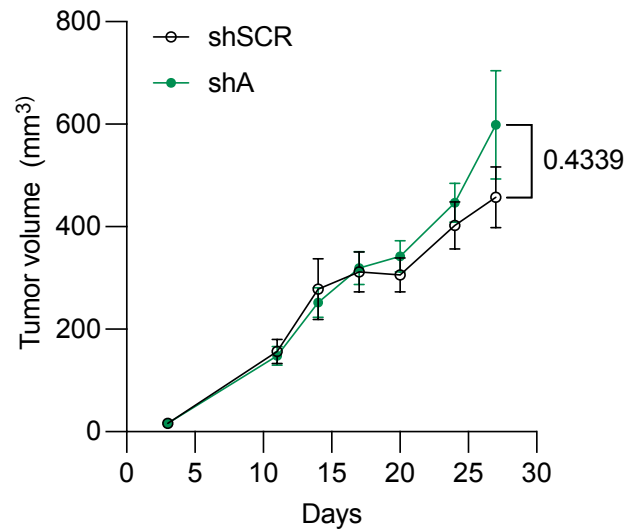




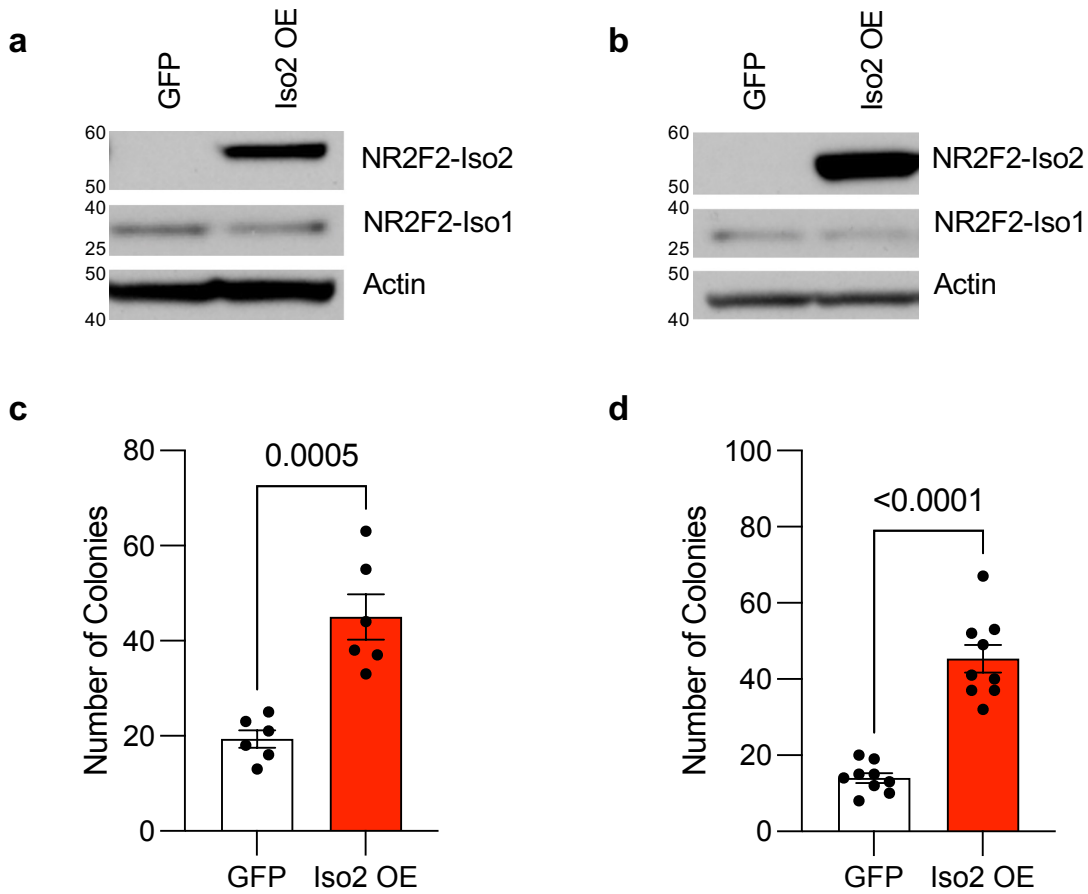
**Suppl. Fig 6. *NR2F2*-Isoform 2 methylation profiles in melanoma patient-derived short term cultures (STC).** **a.** Methylation scores (Beta values) ranging from 0 (unmethylated, green) to 1 (completely methylated, red) were obtained from HumanMethylation450K arrays. The depicted CpGs are located from -300 to +63bp to the *NR2F2*-Iso2 TSS. **b.** Bisulfite sequencing of the region around the *NR2F2*-Iso2 TSS (-438bp to +157bp) in STCs, validating the data generated by HumanMethylation450K arrays. CpGs are represented as white (unmethylated) or black (methylated) squares. \*: CpGs interrogated in the HumanMethylation450K arrays; arrow: Transcription start site (TSS).



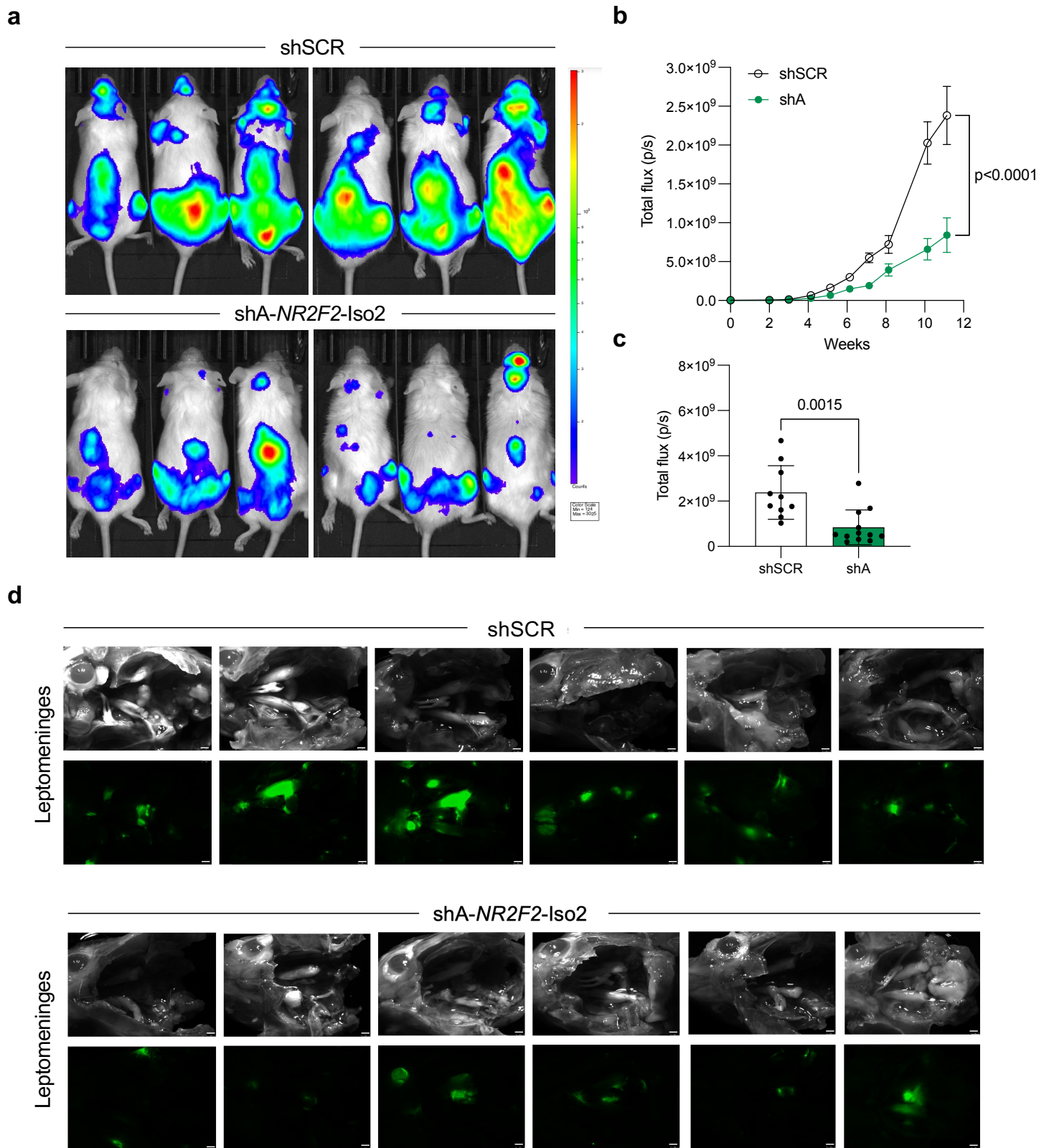
**Suppl. Fig 7. Global demethylation of hypermethylated *NR2F2-Iso2* melanoma cells restores *NR2F2-Iso2* expression.** Treatment of 12-126BM (a), 10-230SC (b), and SK-MEL-2 (c) hypermethylated cells with DNA demethylating agent 5'-aza-2'-deoxycytidine (1, 2.5, and 5uM aza, 72h) restores *NR2F2-Iso2* protein expression evaluated by immunoblot. Experiments have been conducted twice. Source data are provided as a Source Data file.

**a****b****c****d**

**Suppl. Fig 8. NR2F2-Iso2 silencing does not impact proliferation in vitro or in vivo. a.** NR2F2 isoform 2 knock-down in 12-273BM (**a**) and WM278 (**b**) melanoma cells confirmed by immunoblot. Tubulin is used as loading control. **c.** Proliferation curve of 12-273BM control (SCR) and cells with silenced Iso2 expression (shA and shB); Two-way ANOVA. **d.** NR2F2-Iso2 depletion in 4L cell line did not affect tumor growth by subcutaneous injection in mouse flanks; Two-way ANOVA. Bars represent SEM. Experiments have been conducted twice each. Source data are provided as a Source Data file.

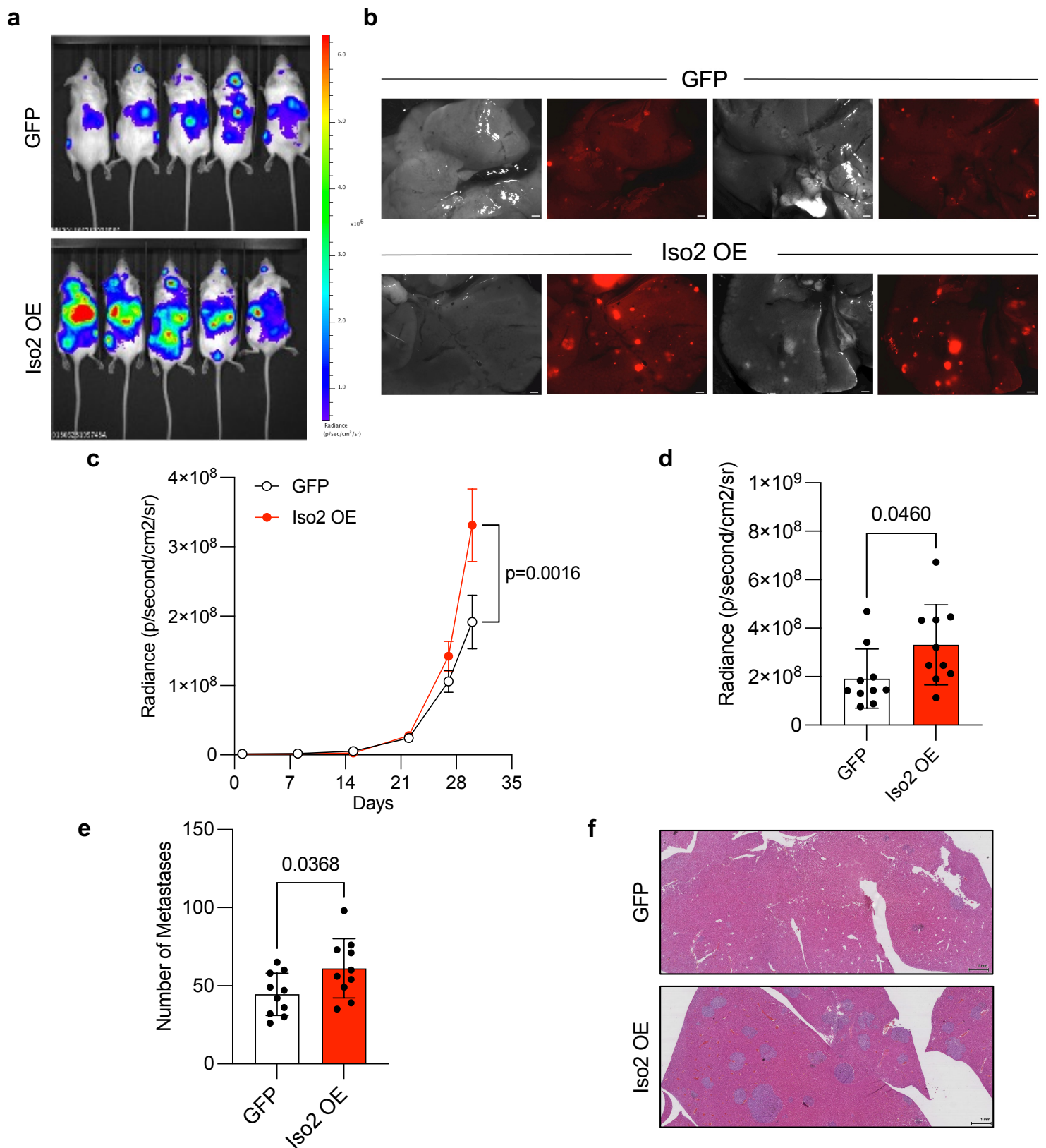


**Suppl. Fig 9. Colony formation assays in melanoma cells with *NR2F2*-Iso2 hypermethylation ectopically expressing *NR2F2*-Iso2 or control GFP.** *NR2F2*-isoform 2 overexpression in IGR-1 (a) and SK-MEL-197 (b) confirmed by immunoblot. Increased colony formation ability upon *NR2F2*-Iso2 ectopic expression found in MeWo cells (Fig. 2I) was confirmed in two additional melanoma cell lines: IGR-1 (c) and SK-MEL-197, after 21 days (d). Unpaired two-tailed T-test. Error bars represent SEM. Experiments have been conducted three times. Source data are provided as a Source Data file.



**Suppl. Fig. 10. NR2F2 isoform 2 silencing inhibits melanoma metastasis.** Loss-of-function (LOF) cellular model was generated in WM278 (melanoma cells, hypo-methylated that express NR2F2-Iso2). Cells were stably transduced with luciferase/mCherry and shA-NR2F2-Iso2 or shSCR and GFP to generate NR2F2 isoform 2 loss-of-function cellular systems. **a-d.** WM278 NR2F2-Iso2 depleted (shNR2F2-Iso2) or control (SCR) cells were injected intracardially into NSG mice (n=10 shSCR, 12 shA). A significant reduction in metastatic ability was detected in shA-NR2F2-Iso2 group (**a-c**). Six representative mice (**a**) and corresponding leptomeningeal metastases (*ex vivo* GFP fluorescence microscopy images, right panel) from one independent experiment is shown (**d**). Two-way ANOVA, error bars represent standard error of the mean (**b**). Unpaired two-tailed T-test, error bars represent SEM (**c**). Scale bars=1cm in macroscopic images of leptomeninges. Source data are provided as a Source Data file.

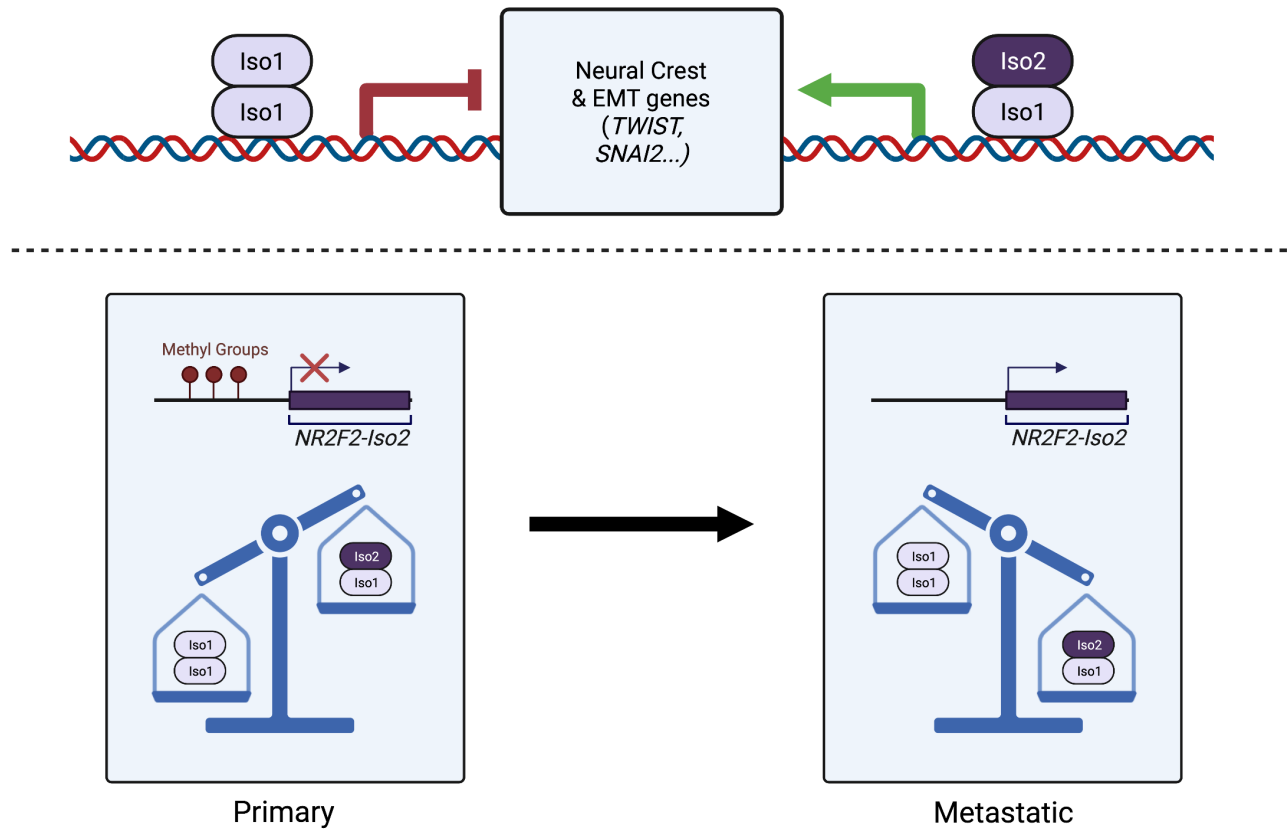




**Suppl. Fig. 11. NR2F2 isoform 2 promotes melanoma metastasis.** Gain-of-function (GOF) cellular model was generated in MeWo (melanoma cells with hypermethylated and silenced NR2F2-Iso2) ectopically expressing isoform 2 (Iso2). Cells were intracardiac injected into NSG mice (n=10 per group). **(a,b)**. Representative luciferase images (a) and *ex vivo* fluorescence microscopy of dissected livers (b) showing a significant increase in melanoma metastatic potential upon Iso2 ectopic expression from one independent experiment is shown. Radiance levels, monitored over time (**c**, two-way ANOVA, bars represent SEM) and at the end of the experiment (**d**, unpaired two-tailed T-test, bars represent SD), show significant differences between groups. Number of metastatic foci per liver section (**e**, unpaired two-tailed T-test, bars represent SEM) with representative images of H&E stained sections (**f**). Scale bars=1cm in macroscopic images of livers, 1mm in histological sections. Source data are provided as a Source Data file.







**Suppl. Fig. 13. Proposed model of NR2F2-mediated melanoma pathogenesis.** Hypermethylation of the *NR2F2-iso2* locus prevents iso2 transcription and translation, yielding a greater level of iso1 homodimers in primary melanoma cells (*bottom*), inhibiting the transcription of neural crest and EMT genes (*top*). Hypomethylation of the *NR2F2-iso2* locus allows for iso2 transcription and subsequent translation, yielding a greater level of iso1-iso2 heterodimers in metastatic melanoma cells (*bottom*), promoting the transcription of neural crest and EMT genes (*top*). Figure was created with Biorender.com.