1 **Supplementary Methods**

2 **Landscape of immune-related signatures induced by targeting of**
3 **different epigenetic regulators in melanoma: implications for immunotherapy.**

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28 **Supplementary Methods.**

29 **NGS analysis.** DNA was extracted from 0.5-1x10^6 melanoma cell lines using the Purelink Genomic
30 DNA Mini Kit (Invitrogen). RNAse treatment was performed using RNAse cocktail (Invitrogen).
31 Quantitative assessment of DNA was performed using a Nanodrop 2000 spectrophotometer and
32 DNA integrity was confirmed by gel electrophoresis. NGS assays on melanoma cell lines DNA were
33 performed using Ion GeneStudio S5 System and carried out on Ion AmpliSeq™ Comprehensive
34 Cancer Panel, which provides highly multiplexed target selection of 409 genes implicated in cancer
35 pathogenesis. Starting DNA and libraries were accurately quantified using a fluorescence-based
36 method, such as Qubit dsDNA HS. Data analysis workflow was performed by automated data

37 transfer, from the Ion Torrent™ Server to the Ion Reporter Server for variant analysis; it includes
38 result filtering, annotation, and data analysis results. To get a total amount of at least 10 mutated
39 alleles for each candidate amplicon, the following mutation selection criteria were adopted:
40 coverage of >200 reads and frequency of mutated alleles >5% for gene amplicon. The copy
41 number variation (CNV) determination was obtained by adding a custom control copy number
42 baseline to the comprehensive cancer profile analysis workflow. Results of NGS analysis of 14
43 melanoma cell lines used in this study are shown in Supplementary Table S1D**.**

44 **Gene expression analysis.** The total RNA (20 ng to 50 ng) was reverse transcribed using
45 GeneChip® WT Pico Reagent Kit (Affymetrix; Thermo Fisher Scientific, Inc.). The resulting cDNA
46 was used as a template for in vitro transcription using the same kit. The obtained antisense cRNA
47 was purified using Nucleic Acid Binding Beads (GeneChip® WT Pico Reagent Kit, Affymetrix) and
48 used as a template for reverse transcription to produce single-stranded DNA in the sense
49 orientation. During this step, dUTP was incorporated. The DNA was then fragmented using uracyl
50 DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) and labeled with DNA
51 reagent covalently linked to biotin using terminal deoxynucleotidyl transferase (TdT, GeneChip®
52 WT Pico Reagent Kit, Affymetrix). Hybridization of each fragmented and labeled target was
53 performed using the GeneChip® Hybridization, Wash and Stain Kit (Affymetrix; Thermo Fisher
54 Scientific, Inc). A single GeneChip® Human Clariom S was then hybridized with each biotin-labeled
55 sense target. GeneChip arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G
56 using default parameters. Affymetrix GeneChip® Command Console software (AGCC) was used to
57 acquire GeneChip® images and generate .DAT and .CEL files. Gene expression data were analyzed
58 by Transcriptomic Analysis Console (TAC) software (Applied Biosystems, Thermo Fisher Scientific).
59 Modulation of immune-related genes by epigenetic drugs in ten melanoma cells was assessed by
60 the NanoString nCounter PanCancer Immune profiling panel enabling determination of 731 genes

61 (NanoString Technologies, Seattle, USA). The manufacturer's gene classification associated with
62 the PanCancer Immune profiling panel was revised by retrieving information on gene function at
63 http://genecards.org and then by grouping genes into 21 functional classes. For NanoString
64 experiments panel probes (capture and report) and 200 ng of RNA were hybridized overnight at 65
65 °C for 16 h. Samples were scanned at maximum scan resolution capabilities (555 FOV) using the
66 nCounter Digital Analyzer. Quality control of samples, data normalization and data analysis were
67 performed using nSolver software 4.0 (NanoString Technologies).

68 Whole gene expression profile of treated and untreated mesothelioma cell lines was
69 performed by Agilent whole human genome oligo microarray kits. The quantity and the quality of
70 RNA, extracted as previous described, was assessed with NanoDrop® ND-1000 UV-Vis
71 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100
72 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). *In vitro* transcription, labeling and
73 purification of dye-labeled cRNA were performed using the Quick Amp Labeling Kit, one-color
74 (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's guidelines. Gene expression
75 profiling was performed by a One-Color strategy using Cy3-labeled aRNA from guadecitabine-
76 treated and untreated cells (Quick Amp Labeling, Agilent Technologies, Santa Clara, CA, USA). A
77 mixture of 1650 ng of Cy3-labeled reference cRNA, Blocking Agent and Fragmentation Agent was
78 hybridized to Whole Human Genome (1x44K) oligo microarray platform (Agilent Technologies,
79 Santa Clara, CA, USA). Hybridization was performed for 17 hours at 65°C in 2x GEx Hybridation
80 Buffer HI-RPM (Agilent Technologies, Santa Clara, CA, USA), using Agilent's Hybridization Oven at
81 10 rpm. Following washing, slides were analyzed by Agilent Microarray Scanner. Feature
82 Extraction Software provided by Agilent (version 9.5.3) was used to quantify the intensity of
83 fluorescence images and to normalize results by subtracting local background fluorescence

84 according to the manufacturer's instruction. Genes modulated with a FC ≥ 2 or ≤ -2 in treated vs
85 untreated cells were used for upstream regulator analyses.

86 **Quantitative Real-Time Methylation Specific PCR (qMSP) Analysis**. Genomic DNA was extracted
87 from 9 melanoma cell lines untreated and treated with 1 µM of guadecitabine, using QIAmp DNA
88 Blood mini-Kit (Qiagen, Hilden, Germany), and modified (500 ng) with sodium bisulfite using the EZ
89 DNA Methylation-Gold Kit (Zymo Research, CA, United States). SYBR green qMSP reactions were
90 performed with methylated- or unmethylated-specific primer pairs on 2 µl of bisulfite-modified
91 genomic DNA. Primers for the analysis of the methylation status of LINE-1, MAGE-A1 and NY-ESO-
92 1 were designed using the free on-line software MethPrimer, and are reported in the following
93 table.

		Forward	*Reverse*
LINE-1	*Unmetilated*	TGTGTGTGAGTTGAAGTAGGGT	ACCCAATTTTCCAAATACAACCATCA
	Methylated	CGCGAGTCGAAGTAGGGC	ACCCGATTTTCCAAATACGACCG
MAGE-A1	*Unmetilated*	GTTAGGTTTTTTTGGTTGAATTTTAT	CCCAAATATAACACCACTAACTTACA
	Methylated	TAGGTTTTTTCGGTCGAATTTTAC	CGAATATAACGCGACTAACTTACG
NY-ESO1	*Unmetilated*	CTACACACAAAAACCCTACTTCCA	TGGTATTGTGGTTATTTTTGGTTT
	Methylated	GCACAAAAACCCTACTTCCG	TTGCGGTTATTTTTCGGTTC

94 The copy number of methylated or unmethylated sequences for LINE-1, MAGE-A1 and NY-ESO-1
95 genes was established by extrapolation from the standard curves. The percentage (%) of
96 methylation was defined as ratio between methylated molecules and the sum of methylated and
97 unmethylated molecules and data were reported as % of LINE-1, MAGE-A1 and NY-ESO-1
98 methylation in treated and untreated cells.

99 **Quantitative western blot analysis.** Primary antibodies were diluted in milk 5% or BSA 5%
100 in TBST and incubated overnight. Development was performed with the ECL normal western blot
101 detection system by the chemiluminescence method. Images were acquired with the Alliance

102 Imaging System (Uvitec). Densitometric analysis was carried out by Quantity One software (Bio-
103 Rad Laboratories Inc.). Normalized treated/control ratios were computed on the basis of
104 background-adjusted density values and then visualized by a color code.

105 **Data Analysis.** The Transcriptomics Analysis Console (TAC) software (Applied Biosystems)
106 was used to identify significantly modulated genes by treatment of two melanoma cell lines
107 (VRG100 and CST30) with guadecitabine, givinostat, JQ1, OTX-015, GSK126 or abemaciclib, and of
108 melanoma nodules removed from immunodeficient mice treated with Guadecitabine. Analysis
109 settings were as follows: gene level fold change >|1.2|, gene-level p value: <0.05; gene-level FDR:
110 <0.05. Ingenuity Pathway Analysis (IPA 8.5, www.ingenuity.com) was used to carry out Upstream
111 Regulator (UR) analysis on: a) significantly modulated genes by different treatments; b)
112 differentially expressed genes in pre-therapy and on-treatment lesions from responding vs non
113 responding patients from the NIBIT-M4 trial (ref. 24 in the manuscript). Upstream regulator
114 analysis allows to identify upstream transcriptional regulators that can explain the observed gene
115 expression changes in the dataset. This computational tool returns results based on p-values and Z
116 score statistics. P values indicate the likelihood of the association between a set of genes and
117 related function, or the likelihood of the overlap between the genes in the dataset and those that
118 are regulated by a predicted upstream regulator. The meaning of the Z score statistics is to infer
119 the activation states ("increased" or "decreased") of the identified biological functions and of the
120 predicted transcription factors. Only Z scores greater than 2 or smaller than -2 were considered
121 significant. IPA was also used for canonical pathway analysis. Canonical pathway analysis is a
122 computational tool allowing to determine if canonical pathways are activated or inhibited on the
123 basis of gene expression in the dataset. Activation or inhibition states of canonical pathways are
124 predicted based on the Z-score algorithm. The significance values (p-value of overlap)

125 are calculated by the right-tailed Fisher's Exact Test and indicate the probability of association of

126 molecules in the dataset with the canonical pathway by random chance alone.