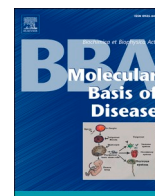




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BRD9 status is a major contributor for cysteine metabolic remodeling through MST and EAAT3 modulation in malignant melanoma

Ana Hipólito^{a,b}, Renato Xavier^b, Cheila Brito^b, Ana Tomás^{a,b}, Isabel Lemos^{b,c}, Luís C. Cabaço^a,
Fernanda Silva^{a,b}, Abel Oliva^c, Duarte C. Barral^a, João B. Vicente^c, Luís G. Gonçalves^c,
Marta Pojo^{b,1}, Jacinta Serpa^{a,b,*}

^a iNOVA4Health, NOVA Medical School, Faculdade de Ciências Médicas, NMS, FCM, Universidade NOVA de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal

^b Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG), Rua Prof Lima Basto, 1099-023 Lisboa, Portugal

^c Instituto de Tecnologia Química e Tecnológica (ITQB) António Xavier da Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

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ABSTRACT

Cutaneous melanoma (CM) is the most aggressive skin cancer, showing globally increasing incidence. Hereditary CM accounts for a significant percentage (5–15 %) of all CM cases. However, most familial cases remain without a known genetic cause. Even though, *BRD9* has been associated to CM as a susceptibility gene. The molecular events following *BRD9* mutagenesis are still not completely understood. In this study, we disclosed *BRD9* as a key regulator in cysteine metabolism and associated altered *BRD9* to increased cell proliferation, migration and invasiveness, as well as to altered melanin levels, inducing higher susceptibility to melanomagenesis. It is evident that *BRD9* WT and mutated *BRD9* (c.183G>C) have a different impact on cysteine metabolism, respectively by inhibiting and activating *MPST* expression in the metastatic A375 cell line. The effect of the mutated *BRD9* variant was more evident in A375 cells than in the less invasive WM115 line.

Our data point out novel molecular and metabolic mechanisms dependent on *BRD9* status that potentially account for the increased risk of developing CM and enhancing CM aggressiveness. Moreover, our findings emphasize the role of cysteine metabolism remodeling in melanoma progression and open new queues to follow to explore the role of *BRD9* as a melanoma susceptibility or cancer-related gene.

1. Introduction

Cutaneous malignant melanoma (CM) is the malignant transformation of skin melanocytes and the most hostile and invasive among skin cancers, accounting for most skin cancer-related deaths. Increased incidence of malignant CM is directly related to age, with median age of CM onset settled at 64 years [1–3]. Malignant CM aggressiveness is grounded on the higher invasive capacity of CM cells, causing metastatic colonization at early stages and frequent relapse events after primary tumor excision [1]. Importantly, metastasis is reported to decrease median overall survival (OS) of late stage CM patients. Nevertheless, recent studies reported an increase in OS numbers, which is probably a result of the implementation of more efficient therapies and higher clinical surveillance [1,4–8]. Since CM belongs to the group of cancers

that arise due to chronic mutagenic exposure, in this case to ultraviolet radiation (UVR), it is, as expected, one of the tumors that shows the highest mutational frequency [9]. In fact, exposure to UVR is the major known risk factor associated to CM, including natural exposure and tanning bed use, accounting for about 70 % of all CM cases due to extensive genome damage, in a wavelength-dependent manner [2,10–12]. This causes UVR-exposed melanocytes to undergo apoptosis or transformation, losing their initial morphology and function and becoming malignant [11,12]. Other risk factors for melanomagenesis are phenotypical characteristics as fair skin, as well as family history [2]. Hereditary CM covers 5 to 15 % of all CM cases, and is associated with germline mutations in a range of identified CM susceptibility genes [13–20]. However, among all familial CM cases, most remain without an identified genetic cause, which highlights the importance of identifying

* Corresponding author at: iNOVA4Health, NOVA Medical School, Faculdade de Ciências Médicas, NMS, FCM, Universidade NOVA de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal.

E-mail address: jacinta.serpa@fcm.unl.pt (J. Serpa).

¹ Equal contribution.

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novel susceptibility genes in hereditary CM [21]. We have previously identified the *Bromodomain-containing protein 9* (*BRD9*) as a promising susceptibility gene in the development of familial CM [21]. *BRD9* defines different sub-complexes of SWI/SNF [22], and it works as an epigenetic reader that can selectively identify and bind, through its bromodomain, acetylated lysine residues belonging to histone or non-histone proteins. *BRD9*, thus, functions as a gene expression modulator by remodeling chromatin and recruiting the transcriptional machinery for certain genes involved in cellular functions, such as proliferation, migration and invasion, as well as metabolic adaptation as the HIF target genes [23], through SWI/SNF(BAF) chromatin remodeling complexes [24–28]. Interestingly, *BRD9* was found to be overexpressed in several types of cancer [29–33].

In the skin, melanocytes produce the pigment melanin and transfer it to neighboring keratinocytes, where it protects the nuclei content of these cells against UVR-induced damage, acting in a photo protective way by forming supranuclear caps and scavenging absorbed radiation [34,35]. Melanin can be produced in two forms: brownish-black eumelanin and red/orange pheomelanin, the latter associated to the fair skin with a lower ability to tan, freckles and red hair phenotype [36]. The photolability of pheomelanin facilitates the generation of reactive oxygen species (ROS) [37,38], potentiating the mutational character of UVR. It is known that the incidence of skin cancer is directly correlated to skin pigmentation, thus individuals that produce higher levels of pheomelanin are more prone to develop CM [36,39,40]. Both forms of melanin derive, though, from a common precursor: dopaquinone, which is produced from the oxidation of tyrosine by tyrosinase to DOPA and then to dopaquinone [34,41–43]. Eumelanin is then formed by the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and oxidation of 5,6-dihydroxyindole (DHI) and DHICA, catalyzed by two other tyrosinase-related proteins (TYRP1 and 2) [34,41–44]. On the other hand, pheomelanin is spontaneously produced, requiring only cysteine to form 2, 5- or 5, 5-cysteinylDOPA that leads to the formation of cysteinylDOPA-quinones, and then benzo-thiazine and benzothiazole [34,41–46]. Besides cysteine, it is known that glutathione (GSH), a tripeptide formed by cysteine, glutamate, and glycine, can further combine with DOPAquinone to form pheomelanin [47,48]. Therefore, the metabolism of cysteine in melanocytes is deeply related to melanin synthesis and its UVR-protective role.

Interestingly, along with the high-rate mutational signature, CM is further described to display metabolic reprogramming features resultant from that mutational status [49]. Metabolic rewiring in crucial pathways such as glycolysis and amino acid metabolism are associated with increased CM malignancy and resistance [49]. However, little is known about cysteine metabolism in CM. In cancer, cysteine metabolic rewiring contributes to a better adaptation of cancer cells to hostile microenvironments, leading to tumor progression and resistance [50]. Rewired cysteine metabolism induces alterations in free radical scavenging and redox maintenance through its antioxidant properties by itself or as a glutathione constituent, altering the detoxifying potential in the microenvironment and constituting a severe chemoresistance mechanism [50–54].

Cysteine metabolic remodeling further interferes in the production of biomass as a carbon source, and in the production of energy as a key player in sulfur metabolism, but also through its reductive catabolism by specific enzymes (cystathionine β -synthase (CBS); cystathionine γ -lyase (CSE), and 3-mercapto-pyruvate sulfurtransferase (MST), the latter working together with cysteine aminotransferase (CAT)) leading to hydrogen sulfide (H_2S) production [55–58]. H_2S , besides working as an antioxidant, can also supply the mitochondrial electron transport chain (mETC), leading to ATP production and so, it is not surprising that H_2S levels are described to impact on cancer cell proliferation and bioenergetics [57,58]. Cysteine uptake occurs through specific transporters and exchangers, as the excitatory amino acid transporter 3 (EAAT3) or the cystine/glutamate antiporter xCT [50]. Both cysteine metabolic enzymes and transporters are described to be overexpressed in several

cancer types [57,59–66].

BRD9, as described above, is a key player in chromatin remodeling, and it can interfere with the action of several transcription factors. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor described to regulate the expression of antioxidant enzymes (AOEs) [67]. NRF2 binds to specific DNA regions designated by antioxidant responsive element (ARE) sequences, which are conserved genomic regions, involved in orchestrating cellular responses to oxidative stress, maintaining cellular redox balance [68]. Moreover, NRF2 has been described to regulate the *SCL1A1* expression encoding the excitatory amino acid transporter 3 (EAAT3), which is able to transport cysteine [69]. The role of SWI/SNF and *BRD9* in NRF2 action is controversial and it seems to be tightly dependent on the cellular and disease context. In NSCLC, the loss of function of SWI/SNF, is associated with the activation of NRF2/KEAP1 pathway [70]. However, some SWI/SNF sub complexes can also potentiate the expression of NRF2 target genes [71], in renal cell carcinoma. Furthermore, in chronic lymphocytic leukemia, the expression of NRF2 and downstream targets are significantly decreased upon *BRD9*-silencing [72].

Our previous *BRD9* study was dedicated to a familial melanoma cohort and no statistical association was found. However, this can be due to the relatively small number of individuals analyzed and the low representativeness of cancer stages. Nevertheless, *BRD9* can be targeted by somatic mutations in sporadic melanoma. Therefore, our study aims to analyze the putative regulatory role of *BRD9* in cysteine metabolism remodeling to disclose metabolic phenotypes contributing to CM aggressiveness. We verified that A375 cell line aggressiveness correlates with the overexpression of MST and EAAT3, and disclosed a putative mechanism linking mutated forms of *BRD9* to susceptibility to melanomagenesis.

2. Materials and methods

2.1. Biological samples and institutional approval

DNA samples from patients with multiple primary CM ($n = 25$), indexes with familial CM ($n = 42$) and relatives without CM ($n = 5$), diagnosed at IPOLFG were used in this study. The Familial Risk Clinic from IPOLFG monitored all participants that were subjected to genetic testing according to the current criteria used in Portugal [73]. The clinicopathological data of 72 Portuguese patients, which tested negative for pathogenic germline mutations in high/intermediate-risk CM susceptibility genes (*CDKN2A*, *CDK4* and *MITF*), is presented in Table S1. DNA was extracted from leukocytes and quantified using Qubit™ Fluorometer (ThermoFisher) as described elsewhere [21]. This study was approved by the IPOLFG Ethics Board Committee (UIC/829), and written informed consent was obtained from all patients.

2.2. In silico analysis

The potential impact of *BRD9* rare single nucleotide variants (SNVs) on protein function and/or splicing signals was assessed using 30 predictive programs. Since all identified SNVs were reported in public genome databases, most of pathogenicity prediction scores were available in distinct platforms, including Ensembl, Catalogue Of Somatic Mutation In Cancer (COSMIC), Varsome and Franklyn by Genoox. To complete the *in silico* analysis, MutationAssessor, Protein Variation Effect Analyzer (PROVEAN), Cancer Genome Interpreter, MutationTaster2021, Human Splicing Finder, IntSplice2, Functional Analysis through Hidden Markov Models (FATHMM) (including MKL- and XF-based algorithms) and Combined Annotation Dependent Depletion (CADD) were used [74–84].

2.3. Cell line culture

Human malignant melanoma cell lines A375 (American Type Culture

Collection (ATCC), CRL-1619) and WM115 (Rockland code WM115-01-0001, BRAF V600E mutated, USA) were cultured according to the supplier's instructions. We choose to use two cell lines with different levels of aggressiveness to test the impact of the expression of BRD9 WT and Mut variants, namely A375 (more invasive) and WM115 (less invasive). Both cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM) (41965-039, Life Technologies), supplemented with 10 % fetal bovine serum (FBS) (S 0615, Merck), 1 % Antibiotic-Antimycotic (AA; P06-07300, PAN Biotech) and 50 µg/mL Gentamicin (15750-060, Life Technologies). Cell cultures were maintained at 37 °C in a humidified environment of 5 % CO₂. Cells were detached with 0.05 % Trypsin-EDTA 1× (25300-054, Thermo Fisher Scientific) at 37 °C for approximately 5 min and split to new plates according to the experimental standard procedures. Cells were metabolically synchronized under starvation prior to the exposure to experimental conditions.

2.4. Primary cell culture

Human epidermal keratinocytes isolated from neonatal foreskin (HEKns) (C-0015C, ThermoFisher) were cultured in EpiLife medium (M-EPIcf-500, Gibco) supplemented with 1 % commercial mix of human keratinocyte growth factors (S-001-5, Gibco), 0.06 mM calcium chloride (J62905.K2, Gibco) and 1 % PenStrep (15070063, Gibco). Human dermal fibroblasts isolated from neonatal foreskin (HDFns) (C-004-5C, Gibco) were grown in DMEM (11960, Gibco) supplemented with 10 % FBS (10500064, Gibco), 2 mM L-alanyl-L-glutamine dipeptide (35050038, GlutaMAX, Gibco) and 1 % PenStrep (15070063, Gibco). HEKns were used until passage 4 and HDFn until passage 13. Both HEKns and HDFs were grown in a humidified incubator at 37 °C with 5 % CO₂.

2.5. BRD9 overexpression and BRD9 c.183 G>C transfection

A375 and WM115 cell lines were transfected with three distinct expression plasmids: pCMV6-Empty (CAT# PS100001, Origene Technologies), pCMV6-BRD9 WT (CAT# RC229303, Origene Technologies; BRD9 - NM_023924 - Human Tagged ORF Clone) and pCMV6-BRD9 c.183G>C (CAT#CW305250, Origene Technologies). pCMV6-BRD9 c.183G>C contains the mutant open reading frame of RC229303 at nucleotide 183 from G to C leading to a E61D amino acid change. The transfection was performed with Lipofectamine 2000 Transfection Reagent (11668019, Thermo Fisher Scientific), following manufacturer's instructions. Stable transfected clones expressing neomycin resistance marker were selected using 1500 µg/mL of G-418 geneticin (10131035, Thermo Fisher Scientific). Validation of transfection was confirmed using Sanger sequencing, reverse transcription and quantitative real-time PCR and western blot, as described.

2.6. Sanger sequencing

The mutational status of all BRD9 exons in the IPOLFG cohort of familial CM was evaluated by Sanger sequencing, as described elsewhere [21], using specific pairs of primers for each exon, represented in Table 1.

Validation of transfection of A375 and WM115 cell lines, using cDNA, was performed upon amplification by PCR and Sanger sequencing, using forward 5' ACGAGGATTATGCCGACAAG 3' and reverse 5' CGTCCAGATGCTTCTCCTTC 3' primers, and the protocol proposed by Big Dye™ Terminator v1.1 Cycle Sequencing RR-100 Kit (4336768, Thermo Fisher Scientific). Afterwards, samples were purified using Exonuclease I 20 U/µl (EN0582, Thermo Fisher Scientific) and FastAP Thermosensitive Alkaline Phosphatase 1 U/µl (EF0654, Thermo Fisher Scientific), and analyzed in an automatic sequencer 3500 Genetic

Table 1

Primers used for BRD9 exons amplification. Detailed characterization of the primers used in Sanger sequencing for BRD9 gene analysis.

Exons	Primer designation	Primer sequence (5' → 3')	Size (nt)	Ta (°C)	G/C%	Fragment size (bp)
1	Forward	TAGCTTCGAATCTGCCGCGCA	22	60	59	500
	Reverse	TCTACCAGGAACGTAAAGCGCTA	23		52	
2	Forward	CATGCCGCCACTGTGGTCTTC	21	60	62	498
	Reverse	TACCAACGGAACACCCCT	19		58	
3	Forward	GGCAGACCTGTCCGGATC	18	60	67	262
	Reverse	GTGCCGACCCCTCATTTACA	20		55	
4	Forward	ACTTAAATGCTGTGAGACACAGA	25	60	40	272
	Reverse	GGTCTGTTTACAGCTGACGA	21		52	
5	Forward	CATGAACTCATTAGTGGCTCACTCT	25	60	44	526
	Reverse	CAGAAACACCTGTGAAAGCTCAA	23		44	
6	Forward	TCTTGGTAGTCATCTAGAGCTTTGC	25	60	44	274
	Reverse	GTTCACTGTGAGGCTCCCTT	20		55	
7	Forward	TGATTCTAGGAGCGGCTGTT	20	60	50	208
	Reverse	TGTGGTTTCCACAGAACCTTT	21		43	
8	Forward	CTCCAGGTGACTTGGTTGTCC	21	60	57	256
	Reverse	TGCTAGATCACACAGCACCAAC	22		50	
9	Forward	TGCTTTCACCCCCATTAAGCC	21	60	52	266
	Reverse	GCCATGGCTCAGCTTTTCATTAC	23		48	
10	Outer Forward	AATGAAAAGCTGAGCCATGGCCAT	24	64	46	1423
	Outer Reverse	TCCAACACGAGTTTCATTCTCTG	22		45	
	Inner Forward	CCTGCACCAATCACATCCACT	21		52	
	Inner Reverse	GCATTTGCCATCATCCACTAA	22		46	
11	Forward	TTCCACACAGCTCTTTTGTCTGT	23	60	44	496
	Reverse	GCACACATGTGGGACTCCACG	21		62	
12	Forward	GGCTCCTTTTACGCGTACCTT	20	60	55	226
	Reverse	GTCCAGGCTGCAGGCTCTT	18		61	
13	Forward	AGCCATTTTACGTGTGTGAATGTC	26	60	39	262
	Reverse	ATCTTACTGATCAGAAACGGACTCCA	26		42	
14	Forward	AGTGATTCAATAGCGAGTGTGAAGT	26	60	39	258
	Reverse	GGAGGCCGTGTGTTTCTTC	20		55	
15	Forward	TAGGTCTTGTCTGATGCTTTGCT	23	60	44	497
	Reverse	AATGCTGCCCACTACCTCGTCTA	23		52	
16	Forward	AGGTCAGGAAGCAGACCTTG	20	60	55	218
	Reverse	AATAAAGAGCTGAAGTGGTCT	23		39	

Analyzer (Thermo Fisher Scientific).

2.7. Reverse transcription and quantitative real-time PCR (RT-qPCR)

RNA from A375 and WM115 cell variants, cDNA synthesis and relative quantitative Real-Time PCR was performed as described elsewhere [85] in a LightCycler 480 instrument (Roche). Primers are presented in Table 2.

2.8. Western blotting

Western blotting was performed to confirm the expression of BRD9 protein variants and study the effect of PROTAC BRD9 degrader-1 (HY-103632, MedChemExpress). A375 or WM115 cells were seeded in T-25 cm² flasks (6.5 × 10⁵ cells/flask; 2.2 × 10⁵ cells/mL), collected by scraping and lysed for 1 h using ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (89900, Thermo Fisher Scientific) with protease and phosphatase inhibitor mixture (PPC1010, Sigma Aldrich). For BRD9 depletion assays, cells were exposed to the 13.5 nM of PROTAC BRD9 degrader-1 according to the manufacturer's instructions, for 24 h. Protein concentration in cell extracts was determined using Pierce BCA protein assay kit (23225, Thermo Fisher Scientific). Cell lysates (25 µg) were resuspended in Laemmli buffer. The protein extracts were separated in a 10 % SDS-polyacrylamide gel electrophoresis and transferred onto methanol-activated polyvinylidene difluoride membranes (1620177, Bio-Rad) using the Trans-Blot® Turbo Blotting System (Bio-Rad). The membrane processing, immunoblot and development were performed as described elsewhere [86]. The primary antibodies used were anti-BRD9 (1:7500, ab137245, Abcam), anti-MPST (1:250, HPA001240, Sigma-Aldrich), anti-EAAT3 (1:1000, 14501S, Cell

Signaling Technology), anti α-tubulin clone B-5-1-2 (1:4000; T5168, Merck KGaA) and anti-β-actin (1,5000, A5441, Sigma Aldrich).

2.9. Proliferation assay

To study cellular proliferative rate, A375 or WM115 cells were plated in 24-well plates (5 × 10⁴ cells/well; 1 × 10⁵ cells/mL) in supplemented DMEM and left to adhere for 24 h in normal culture conditions. Cells were exposed or not to 0.402 mM L-cysteine (102839, Merck). At each time-point, cells were detached as described (cells suspended in medium were also collected) and centrifuged for 5 min at 155 ×g. Supernatant was discarded and total cells were counted, using a Neubauer improved cell counting chamber.

2.10. Wound healing assay

The migratory capacity of A375 or WM115 cells was evaluated using the wound healing assay. Cells were plated in 12-well plates (1 × 10⁵ cells/well/mL) until the formation of a confluent monolayer. Cells were exposed or not to 0.402 mM L-cysteine (102839, Merck). Once confluent, cells were incubated for 3 h with 5 µg/mL mitomycin-C (M4287, Sigma Aldrich) and a linear scratch in each monolayer was made with a 20 µL pipette tip, creating a wound across the well diameter. The media was replaced to remove debris and cells in suspension. Bright-field images of each well were acquired on the Olympus IX53 Inverted Microscope at each time-point. The wound closure was quantified using the ImageJ software (imagej.nih.gov/ij/).

Table 2
Primers used in gene expression, MPST promoter constructs and relative occupancy quantification. Detailed characterization of the primers used in RT-qPCR assays, in pGL3-MPST promoter constructs and for chromatin immunoprecipitation (ChIP) assay.

Gene	Primer	Primer sequence (5' → 3')	Size (nt)	Ta (°C)	G/C %	Fragment size (bp)
RT-qPCR						
Bromodomain-containing protein 9 (BRD9)	Forward	GCGACTTGAAGTCGGACGAGAT	22	62	55	128
	Reverse	GTCACCACTTTCTTGCTGTAGC	23		52	
Cystathionine beta-synthase (CBS)	Forward	GAGCTCTGGCCAAGTGTG	19	60	58	232
	Reverse	GCACGTCCACCTTCTCGG	18		67	
Cystathionine Gamma-Lyase (CTH)	Forward	GCAGCCACTGTAACATTACCC	22	60	50	175
	Reverse	CTGGTGAATTGCTGCCTCTAG	22		50	
Mercaptopyruvate Sulfurtransferase (MPST)	Forward	CTTCATCAAGACCTACGAGGAC	22	60	50	134
	Reverse	GGTAGTGGCCAGGTTC AATG	20		55	
Glutamic-Oxaloacetic Transaminase 1 (GOT-1)	Forward	GAGAAGAGAGGATTGGACCTC	21	60	52	147
	Reverse	CATGACAGAAGCAATCTGCTTCC	23		48	
Glutamic-Oxaloacetic Transaminase 2 (GOT-2)	Forward	CCAGAGCCAGCTCCTGGT	18	61	67	171
	Reverse	CTGCCTTGGGACGCTAG	18		67	
Solute Carrier Family 7 Member 11 (SLC7A11)	Forward	GGTCCTGTCACTATTGGAGC	21	61	58	136
	Reverse	GAGGAGTTCCACCCAGACTC	20		59	
Solute Carrier Family 1 Member 1 (SLC1A1)	Forward	GTATCACGGCCACATCTGCC	20	61	60	121
	Reverse	GCAATGATCAGGGTGACATCC	21		52	
Hypoxanthine-guanine phosphoribosyltransferase (HPRT1)	Forward	TGACACTGGCAAAACAATGCA	21	58	43	94
	Reverse	GGTCCTTTTACCAGCAAGCT	21		52	
pGL3-MPST promoter constructs						
MPST 5'UTR (-870-28)	Forward	ATG <u>ACGCGT</u> CACCATTGTTGGCCAGACTGG	29	67	52	
	Mlu-I restriction					
	Reverse	ATG <u>AAGCTT</u> GCGACAGGGAGGATGTCAG	29		48	
ChIP assay						
MPST (a)	Forward	GCATACTCATGTGGGTAGGG	20	61	55	240
	Reverse	GAGGAACTGAGGCTCAGAGG	21		57	
MPST (b)	Forward	CTGCTCACAGATGCTAGG	20	61	55	396
	Reverse	GCCACCGGTGACATCCG	18		72	
SLC1A1	Forward	GCAAACTACCGGCTGG	18	60	61	202
	Reverse	GCTATTGTGCGCTGGGCTC	19		63	

2.11. Nuclear magnetic resonance analysis (^1H NMR)

Metabolic profiles were analyzed by ^1H NMR. Briefly, cells were seeded in 175 cm² culture flasks (6.5×10^6 cells/flask; 5×10^5 cells/mL) and cultured in control conditions or exposed to 0.402 mM L-cysteine (102839, Merck). Cell extracts were treated and samples prepared as described [87]. The ^1H NMR (noesypr1d) was obtained at 25 °C in an Avance 500 II+ (Bruker) spectrometer operating at 500.133 MHz, equipped with a 5 mm TCI(F)-z H-C/N Prodigy cryo-probe. The chemical shifts in aqueous sample were referred to the TSP. Topspin 4.0.7 (Bruker) was used for acquisition and spectra analysis. Compound identification was performed by resorting to the Human Metabolome database (HMDB) and Chenomx NMR suite software version 8.1 (Chenomx Inc.). Metabolites' concentrations were determined using Chenomx NMR suite software version 8.1 for ^1H NMR spectra (Chenomx Inc.).

2.12. Immunofluorescence

Cells were seeded in glass coverslips in 24-well plates coated with 0.2 % gelatin from porcine skin (G-1890, Sigma-Aldrich) (1×10^5 cells/well; 2×10^5 cells/mL), and cultured as previously described. Once adherent, cells were fixed with 4 % paraformaldehyde for 15 min at 4 °C and permeabilized with 0.1 % saponin in PBS-BSA (0.5 %, w/v, BSA in PBS) for 15 min at room temperature. Immunofluorescence analysis was performed to determine the basal expression of MST and EAAT3 and to study the effect of PROTAC BRD9 degrader-1 (HY-103632, MedChemExpress). Fixed cells were then incubated overnight at 4 °C with anti-MPST (1:100, HPA001240, Sigma-Aldrich), anti-EAAT3 (1:100, 14501S, Cell Signaling Technology) and anti-BRD9 (1:100, ab137245, Abcam), and then incubated with secondary antibody for 2 h at room temperature (Alexa Fluor® 488 anti-rabbit, A-11034, Thermo Fisher Scientific). Slides were mounted in VECTASHIELD media with 4'-6-diamidino-2-phenylindole (DAPI, H-1200-10, Vector Labs) and examined by standard fluorescence microscopy (Zeiss Imager.Z1 AX10 microscope). Images were acquired and processed with CytoVision software (Leica Biosystems).

2.13. Promoter activity by luciferase reporter gene assay

A fragment of 5' UTR region (−870–28) of *MPST* gene were amplified by PCR (Table 2) and inserted in pGL3-Basic vector (E1751, Promega) using *MluI* and *HindIII* restriction sites and standard techniques [88]. Cells were transfected with 0.5 µg of each pGL3-*MPST* promoter construct, positive control (pGL3-Control; E1741; Promega) or negative control (pGL3-Basic), and co-transfected with 0.1 µg Renilla vector (E2810; Promega). Luciferase activity of constructs, Firefly and Renilla luciferases were measured using the Dual Luciferase Assay System (E1910, Promega) according to the manufacturer's protocol and results were acquired and analyzed as described [89].

2.14. H_2S quantification in cell homogenates

Cells were seeded in 6-well plates (5×10^5 cells/well, 2.5×10^5 cells/mL) and cultured in control conditions or exposed to 0.402 mM L-cysteine (102839, Merck) and/or 30 µM NaHS (161527, Sigma-Aldrich) for 16 h. Then, cells were scraped in PBS and centrifuged at 210 ×g for 5 min. The cell pellet was homogenized in NP40 lysis buffer (1 % NP40, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0) on ice for 30 min and centrifuged for 5 min at 20,000 ×g 4 °C. Cell homogenates (20 µL) were incubated in black 96-well plates with 10 µM 7-Azido-4-Methylcoumarin probe (AzMC, L511455, Sigma Aldrich) in the absence or presence of 1 mM o-(carboxymethyl)hydroxylamine hemihydrochloride (AOAA, C13408, Sigma Aldrich) and 3 mM DL-propargylglycine (PAG, P7888, Sigma Aldrich). H_2S production was monitored following the fluorescent signal of AzMC probe (λ_{exc} : 355 nm; λ_{em} : 460 nm) every 30 min for 2 h, in a

VICTOR3 instrument from PerkinElmer/Wallac 1420 v3.0 software. The protein concentration of each lysate was determined with the Bradford method using protein assay dye reagent concentrate (500–0006, Bio-Rad). The H_2S production activities were normalized to the total protein concentration and to a blank sample (cellular lysates without probe).

2.15. ATP quantification

Cells were seeded in 6-well plates (5×10^5 cells/well, 2.5×10^5 cells/mL) and cultured in control conditions or exposed to 0.402 mM L-cysteine (102839, Merck) and/or 30 µM NaHS (161527 Sigma Aldrich) for 16 h. Then, cells were scraped in PBS containing 2 mM EDTA, centrifuged at 210 ×g for 5 min and homogenized in 1 % NP40 lysis buffer (1 % NP40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) with 5 % protease inhibitor (S8830, Sigma) on ice for 30 min and centrifuged at 20,000 ×g for 5 min at 4 °C. Protein concentration was quantified using the Bradford method. ATP determination kit (A22066, Molecular probes) was used in accordance with the manufacturer's instructions, using an ATP calibration curve, within the 0–30 µM ATP range. The measurements were performed using the Luciferase protocol in a VICTOR3 (PerkinElmer), using the Wallac 1420 software.

2.16. Chromatin immunoprecipitation (ChIP) analysis

To analyze potential interactions between the transcription factor NRF2 and ARE sequences in *MPST* and *SLC1A1* promoters, ChIP was employed. We identified two putative ARE sequences recognized by NRF2 in the *MPST* promoter and one in the *SLC1A1* promoter, and designed primers for those regions to perform ChIP analysis. Cell preparation and ChIP assay were performed using the OneDay ChIP kit (kchonedIP-060, Diagenode) according to the manufacturer's protocol. The chromatin complexes were immunoprecipitated with 1 µg/mL of specific anti-human NRF2 antibody (ab31163; Abcam). The relative occupancy of the immunoprecipitated factors at a specific promoter region was performed by absolute qPCR, using primers for *MPST* and *SLC1A1* promoter (Table 2) and calculated using the following formula:

$$\text{Relative occupancy} = 2^{(\text{CtNegCtrl} - \text{CtTarget})}$$

2.17. Melanin quantification

Melanin content was measured through spectrophotometry as described elsewhere [90]. Cells were exposed or not to 0.402 mM L-cysteine (102839, Merck) for 16 h after 16 h of starvation. Briefly, A375 and WM115 cell variants were collected with no phenol red 2.5 % Trypsin 10× (15090046, Thermo Fisher Scientific) as described previously, and counted using a Neubauer improved cell counting chamber. Cells were resuspended in 1 M NaOH and the melanin content was measured at 490 nm. Melanin concentration was determined through a standard curve (0 to 200 µg/mL range) obtained with synthetic melanin (M0418, Sigma Aldrich).

2.18. Metabolic viability assays

All variants of A375 and WM115 were plated in 96-well clear plates (2×10^4 cells/well; 2×10^5 cells/mL) and treated with a commercial MST inhibitor (I3MT-3, HY-128206, CAS No.459420-09-8, MedChemExpress) at increasing concentrations (0, 30 µM, 100 µM, 300 µM, 600 µM and 900 µM). Upon 48 h of exposure, metabolic viability assays were performed by colorimetry using Cell Proliferation Reagent WST-1 (11644807001, Roche), according to the manufacturer's instructions.

2.19. Cell death assays

All variants of A375 and WM115 were seeded in 24-well plates ($1 \times$

10^5 cells/well; 2×10^5 cells/mL). After exposure to MST inhibitor (I3MT-3, HY-128206, CAS No. 459420-09-8, MedChemExpress), cells were collected and labeling with FITC-labeled Annexin V (640906, BioLegend) and propidium iodide (PI; P4170, Sigma Aldrich) was performed as described [86]. Results were analyzed by flow cytometry (BD FACSCanto II). FlowJo X v10.0.7 software (<https://www.flowjo.com/>) was used to analyze data.

2.20. Three-dimensional (3D) in vitro melanoma skin model

To establish the three-dimensional melanoma skin model used, we adapted the protocols published elsewhere [91,92]. Essentially, porous polystyrene scaffolds (Alvetex®, AVP005-48, REPROCELL Europe Ltd.) with an area of 1.13 cm^2 were inserted in 6-well plates and pretreated with 70 % ethanol for 30 min to render them hydrophilic properties. The scaffolds were washed twice with phosphate-buffered saline (PBS) and 2×10^6 HDFns were seeded onto the scaffolds in 100 μL of HDFn medium, followed by incubation in a humidified incubator at 37°C with 5 % CO_2 for 1.5 h to allow cell adhesion. Next, the scaffolds containing HDFns were submerged with 9 mL of HDFn medium supplemented with 100 $\mu\text{g/mL}$ L-ascorbic acid (11487487, Fisher Scientific) and maintained up to a further 15 days at 37°C in a 5 % CO_2 humidified incubator. The culture medium was renewed every 5 days to allow the formation of a dermal equivalent. After this, 5×10^4 A375 or 1×10^5 WM115 melanoma cells were seeded on the dermis equivalents in 2 mL of melanoma culture medium supplemented or not with 0,402 mM L-cysteine (102839, Merck). After incubating for 3 days at 37°C and 5 % CO_2 , 1×10^6 HEKns were added in 2 mL of HEKn medium containing the final concentration of 1.5 mM calcium chloride (Gibco) without L-cysteine (102839, Merck). Finally, after further 3 days of incubation, keratinocytes were exposed to air-liquid interface and the culture medium was changed to HEKn medium containing the final concentration of 1.5 mM calcium chloride (Gibco), 10 ng/ μL keratinocyte growth factor (K1757, Sigma), 50 $\mu\text{g/mL}$ L-ascorbic acid (11487487, Fisher Scientific) and 0,402 mM L-cysteine (102839, Merck) or not. The medium was changed every 2 days for 18 days to allow HEKn differentiation in the several epidermis strata, as well as melanoma cell invasion into the dermis. The tissues were fixed overnight in 10 % neutral buffered formalin (Formalin 10 %, Cat. number 9713.9010, VWR) and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and Eosin staining (H&E, Hematoxylin, Cat. Number CS700, Dako; and Eosin, Cat. Number CS701, Dako). Melanoma thickness was measured in 20 different spots in two biological replicates using Aperio ImageScope software (<https://www.leicabiosystems.com/en-pt/digital-pathology/manage/aperio-imagescope/>).

2.21. Immunohistochemistry

The invasion of melanoma cells, in 3D skin model, was evaluated upon immunodetection of S-100 protein, which is expressed in neural crest-derived cells, being considered an efficient melanoma marker [93,94]. Immunohistochemistry analysis was performed using anti-S100 (Cat. Number 760-2523, Roche Diagnostics, pre-diluted for 16 min; pretreatment CC1–48 min; Ventana Medical Systems) with appropriate positive and negative controls samples, on the BenchMark ULTRA IHC/ISH Automatic staining platform (Ventana Medical Systems) using OptiView DAB IHC Detection Kit with diaminobenzidine as the chromogen to detect antigen expression. Tissue sections were counterstained with Mayer's hematoxylin before mounting.

2.22. Statistical analysis

Statistical analyses and half maximal inhibitory concentration (EC_{50}) values were performed in GraphPad Prism 8.0 software (www.graphpad.com). Data are presented as mean $\pm$ SD. Assays were performed with at least three biological replicates. For comparisons of two groups, a

two-tailed unpaired *t*-test was used. For more than two groups, one-way and two-way analyses of variance (ANOVA) were used. Statistical significance was established as $p < 0.05$.

3. Results

3.1. Analysis of BRD9 mutational profile in a cohort of familial CM patients predicts identified BRD9 SNVs as benign

BRD9 physiological functions and role in CM are not completely understood. In order to further disclose the function of altered BRD9 in hereditary CM, we evaluated the BRD9 mutational profile in a cohort of familial CM patients ($n = 72$) from IPOLFG, by Sanger sequencing, to investigate the presence of variants in both coding and non-coding regions. Overall, we identified 21 BRD9 single nucleotide variants (SNVs), including 2 in 5'UTR, 13 intronic SNVs and 6 exonic SNVs (Table 3). However, most of them were considered common polymorphisms in the European population, presenting an allele frequency $> 1\%$ according to gnomAD genomes r3.0 and 1000 Genomes databases [95,96]. Eight BRD9 rare SNVs in the European population were identified by Sanger sequencing: c.-65G>A, 1.4 % (1/72) in 5'UTR; c.1043-19C>T, 1.4 % (1/72), c.1383 + 37C>G, 1.4 % (1/72), and c.1383 + 29G>A, 9.7 % (7/72) intronic variants; c.6C>G, 5.6 % (4/72), c.183G>C, 5.6 % (4/72), c.816G>A, 4.2 % (3/72) and c.1190T>C, 1.4 % (1/72) exonic variants (highlighted in blue in Table 3). Out of 72 patients, 16 were identified with at least one rare variant in BRD9. All these 16 familial CM patients, 8 indexes with familial CM and 8 MPM cases, were negative for pathogenic germline mutations in the current criteria genes for hereditary melanoma (CDKN2A, CDK4 and MITF (c.952G>A/p.E318K)). Among the rare exonic SNVs, c.183G>C and c.1190T>C are missense variants, leading to single amino acid changes, while the other ones are synonymous (c.6C>G, c.816G>A) (Table 3). Overall, BRD9 c.183G>C was identified in 5.6 % of this cohort (Table 3). Only 4 patients showed two or more BRD9 rare variants, 2 of them were selected for whole exome sequencing (WES) study performed by us [21]. Interestingly, the same BRD9 mutational profile was found in 2 patients, which includes the exonic variants c.183G>C (missense) and c.816G>A (synonymous) but also c.1383+29G>A, located in intron 13.

None of the identified BRD9 SNVs have their clinical significance reported according to the ClinVar database, except c.816G>A (rs74984870), described as benign. It is important to highlight that c.-74G>A and c.-65G>A BRD9 5'UTR variants were both found in homozygosity (the first in only one patient, Table 3), which is not consistent with the autosomal dominant inheritance pattern characteristic of CM susceptibility [21].

Given the well-documented impact of pathogenic SNVs on gene expression (at transcriptional and post-transcriptional levels) and protein function, but also their potential role in cancer susceptibility, we decided to analyze the effect of BRD9 rare SNVs at distinct levels of gene function, using predictive *in silico* tools (Fig. 1). Since the set of BRD9 rare SNVs includes coding and non-coding variants, the predictive programs were selected according not only to the effect of amino acid substitution on protein function, structure, and stability, but also according to regulatory changes and impact on splicing. We only considered an overall pathogenic variant when most available *in silico* tools suggested a deleterious effect. Thus, all BRD9 rare SNVs were considered likely benign according to the selected predictors and the mentioned criteria (Fig. 1). In addition, we sought to understand the potential effect of the missense mutations by analyzing the corresponding amino acid substitutions in the structural model of BRD9 obtained from AlphaFold. However, both substitutions are located in regions of the protein for which the model cannot reliably assign elements of secondary structure. Therefore, the structural and functional impact of these substitutions at the protein level remains undetermined.

Table 3

List of 5'UTR, intronic and exonic SNVs found by Sanger sequencing in the IPOLFG cohort of familial cutaneous melanoma (CM) patients (n = 72). The rare variants, presenting a European population frequency < 1 %, are highlighted in blue. Nucleotide change according to dbSNP HGVS (GRCh38.p13 chromosome 5, *BRD9* transcript variant 1, NM_023924.5). Their dbSNP reference (rs) number, genome location, gene location, amino acid change (when applicable), type (molecular consequence) and frequency (%) in IPOLFG cohort of familial CM patients are also presented.

dbSNP reference (rs) number	Genome location (Sp15.33)	Gene location	Nucleotide change	Amino acid change	Type	Frequency in CM (%)	European population frequency (%)*
rs111286164	892731	5'UTR region	c.-74G>A	–	5'UTR	2.8 (2/72)	5.0
rs542129307	892722	5'UTR region	c.-65G>A	–	5'UTR	1.4 (1/72)	0.04
rs72703159	889579	Intron 5	c.461+8G>T	–	Splice region variant	6.9 (5/72)	4.7
rs72703154	889212	Intron 5	c.462-47C>T	–	Intronic	6.9 (5/72)	3.2
rs72703155	889254	Intron 5	c.462-89G>A	–	Intronic	2.8 (2/72)	5.0
rs66859331	889382	Intron 5	c.461+205G>T	–	Intronic	2.8 (2/72)	5.0
rs10062549	889281	Intron 5	c.462-116C>T	–	Intronic	8.3 (6/72)	5.1
rs546947145	879908	Intron 10	c.1043-19C>T	–	Intronic	1.4 (1/72)	0.05
rs41283151	878632	Intron 11	c.1139-145T>C	–	Intronic	8.3 (6/72)	40.4
rs41283153	878645	Intron 11	c.1139-158G>T	–	Intronic	2.8 (2/72)	4.7
rs184819406	876064	Intron 13	c.1383+37C>G	–	Intronic	1.4 (1/72)	0.4
rs72703144	876046	Intron 13	c.1383+55C>T	–	Intronic	9.7 (7/72)	6.2
rs10039297	876072	Intron 13	c.1383+29G>A	–	Intronic	9.7 (7/72)	0.2
rs55744146	871483	Intron 14	c.1422+43T>C	–	Intronic	8.3 (6/72)	5.3
rs28497180	870440	Intron 14	c.1525+33T>C	–	Intronic	5.6 (4/72)	5.3
rs375328319	892652	Exon 1	c.6C>G	p.G2=	Synonymous	5.6 (4/72)	0.9
rs199607133	891724	Exon 2	c.183G>C	p.E61D	Missense	5.6 (4/72)	0.03
rs141678469	891195	Exon 3	c.360G>A	p.P120=	Synonymous	1.4 (1/72)	1.8
rs74984870	886609	Exon 7	c.816G>A	p.P272=	Synonymous	4.2 (3/72)	0.04
rs145107515	878436	Exon 11	c.1190T>C	p.V397A	Missense	1.4 (1/72)	0.4
rs1051630	870504	Exon 14	c.1494T>C	p.V498=	Synonymous	8.3 (6/72)	5.3

3.2. Overexpression of *BRD9* and *BRD9* c.183G>C induces a less proliferative but more migratory phenotype in A375 CM cells

Given that the c.183G>C SNV had been already identified in a smaller Portuguese cohort [21], we hypothesized that, although it was predicted to have benign effects by *in silico* analysis (Fig. 1), this variant could induce alterations in CM cells that could correlate with hereditary CM features. To test this hypothesis, we transfected A375 melanoma cells with a *BRD9*-encoding plasmid (A375 *BRD9* WT), as well as *BRD9* (c.183G>C)-encoding plasmid. In addition, we transfected the empty pCMV6 vector as a negative control. We then obtained A375 clones successfully overexpressing *wild-type* (WT) or c.183G>C *BRD9* at mRNA and protein levels (Fig. 2A, B and C). To disclose the cellular effects of both *BRD9* forms in A375 cells, we studied the proliferation rate of these clones over 48 h and found that both A375 *BRD9* WT or *BRD9* c.183 G>C showed a significant reduction in proliferation from 6 h, and substantial reduction of proliferative capacity at 48 h, when comparing to control even in the presence of cysteine (Fig. 2D and E). We then wondered if these *BRD9* alterations could also affect the migratory capacity of these cells. We assessed cell migration by wound healing over a period of 48 h, in the presence of mitomycin to exclude the interference of cell proliferation on the wound closure. Interestingly, we found that both A375 *BRD9* WT or *BRD9* c.183 G>C showed a significant increase in migration capacity, when comparing with control, starting at 10 h, in the absence and presence of cysteine (Fig. 2F and G). We can, then, conclude that overexpression of *BRD9* in its WT or c.183G>C variants promotes a shift in cellular features, inducing a less proliferative but more migratory phenotype in A375 cells.

3.3. *BRD9* wild-type and *BRD9* c.183G>C overexpression induces metabolic remodeling in A375 CM cells

Alterations in cellular features induced by overexpression of *BRD9* and *BRD9* c.183G>C in this cell line, led us to wonder if A375 altered phenotype could be due to metabolic shifts induced by these genetic alterations.

¹H NMR analysis showed that several amino acids show contrasting

trends, when comparing to control (A375 empty) levels (Fig. 2H). Glutamate tended to decrease in A375 *BRD9* WT and A375 *BRD9* c.183G>C. The same tendency was observed for glycine, which presents more prominently in A375 *BRD9* c.183G>C than in A375 *BRD9* WT. Glutamine levels appear to be low and are only noteworthy in A375 *BRD9* c.183G>C. Interestingly, methionine levels seemed to decrease only in A375 *BRD9* c.183G>C, which indicates that a downstream rewiring in cysteine production may be occurring. While cysteine was not detected in the analysis of any cell variants, the levels of reduced glutathione (GSH) were similar between them, which may indicate that cysteine is being rapidly consumed to maintain glutathione availability, since it is a rate-limiting amino acid for glutathione synthesis (Fig. 2H). Moreover, we also found different metabolic patterns concerning sugar and organic acids metabolism (Fig. 2I and J), as for acetate and choline, which seem to increase in A375 *BRD9* c.183G>C, and creatine, which decreases in A375 *BRD9* WT (Fig. 2I and J). Notably, upon cysteine exposure, all A375 cell variants presented a trend to decrease the availability of glutamate and glycine. Accordingly, the GSH levels also tended to decrease upon cysteine exposure in A375 cells overexpressing WT or mutated *BRD9*, which indicates that this metabolic remodeling may require GSH consumption in order to keep the metabolic rate upon accelerating glutathione turnover. Moreover, cysteine canalization as a supplier for other pathways can also be an explanation, since cysteine catabolism dependent on MST seem to be active. Cysteine exposure further induced alterations in sugar and organic acids levels, as well as in other relevant metabolites, as creatine and creatine phosphate (Figs. 3A, B and C). Metabolic profiling led to the conclusion that both A375 *BRD9* WT and A375 *BRD9* c.183G>C present metabolic variations.

3.4. *BRD9* wild-type and *BRD9* c.183G>C overexpression modulates cysteine metabolism in A375 CM cells

The impact of *BRD9* WT and *BRD9* c.183G>C overexpression on the modulation of cysteine synthesis and reductive degradation in A375 cells, was assessed by the quantification of mRNA expression of key enzymes and transporters, namely: CBS (*CBS* gene), CSE (*CTH* gene), MST (*MPST* gene), cytosolic CAT (*GOT-1* gene), mitochondrial CAT

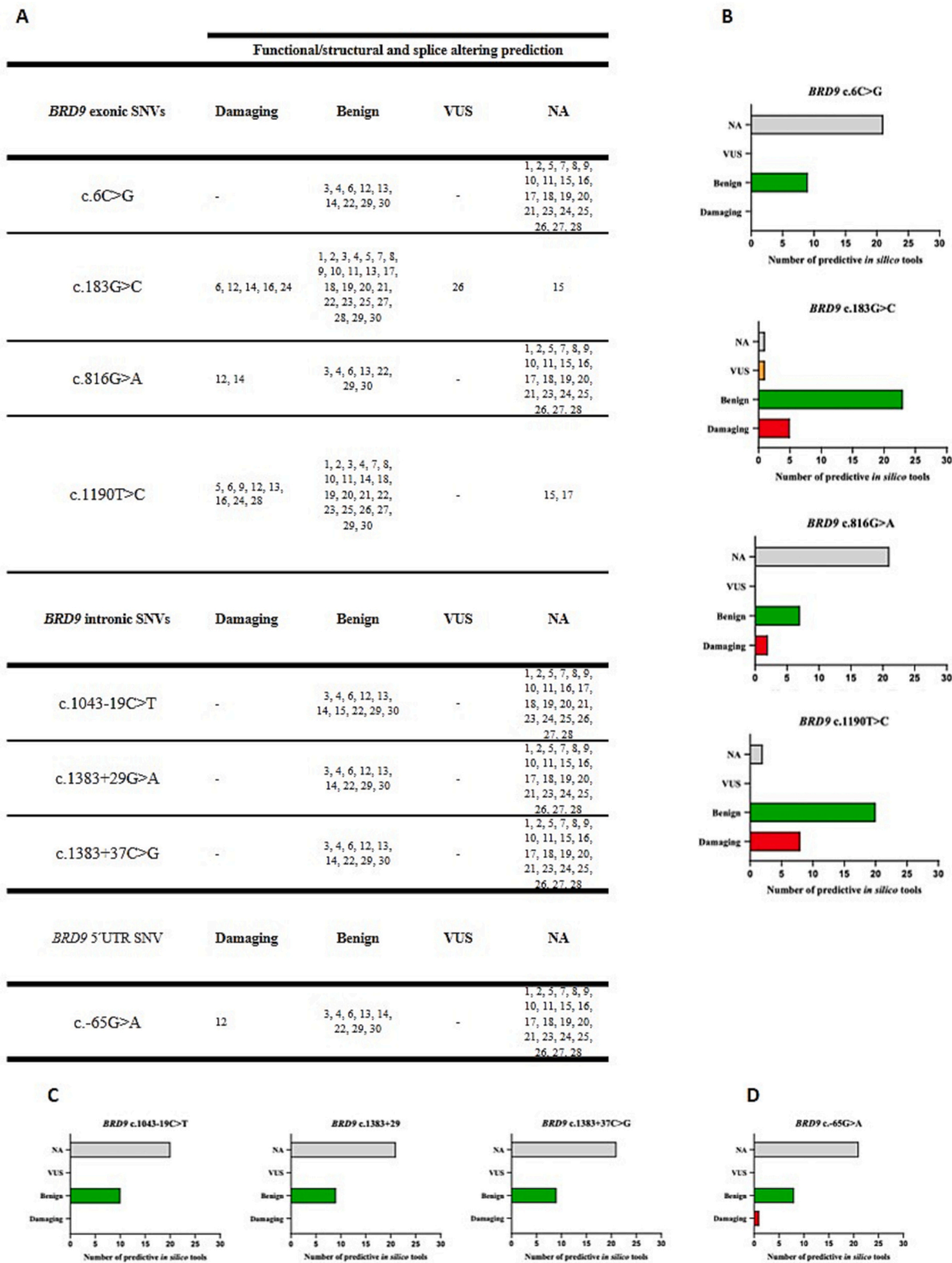
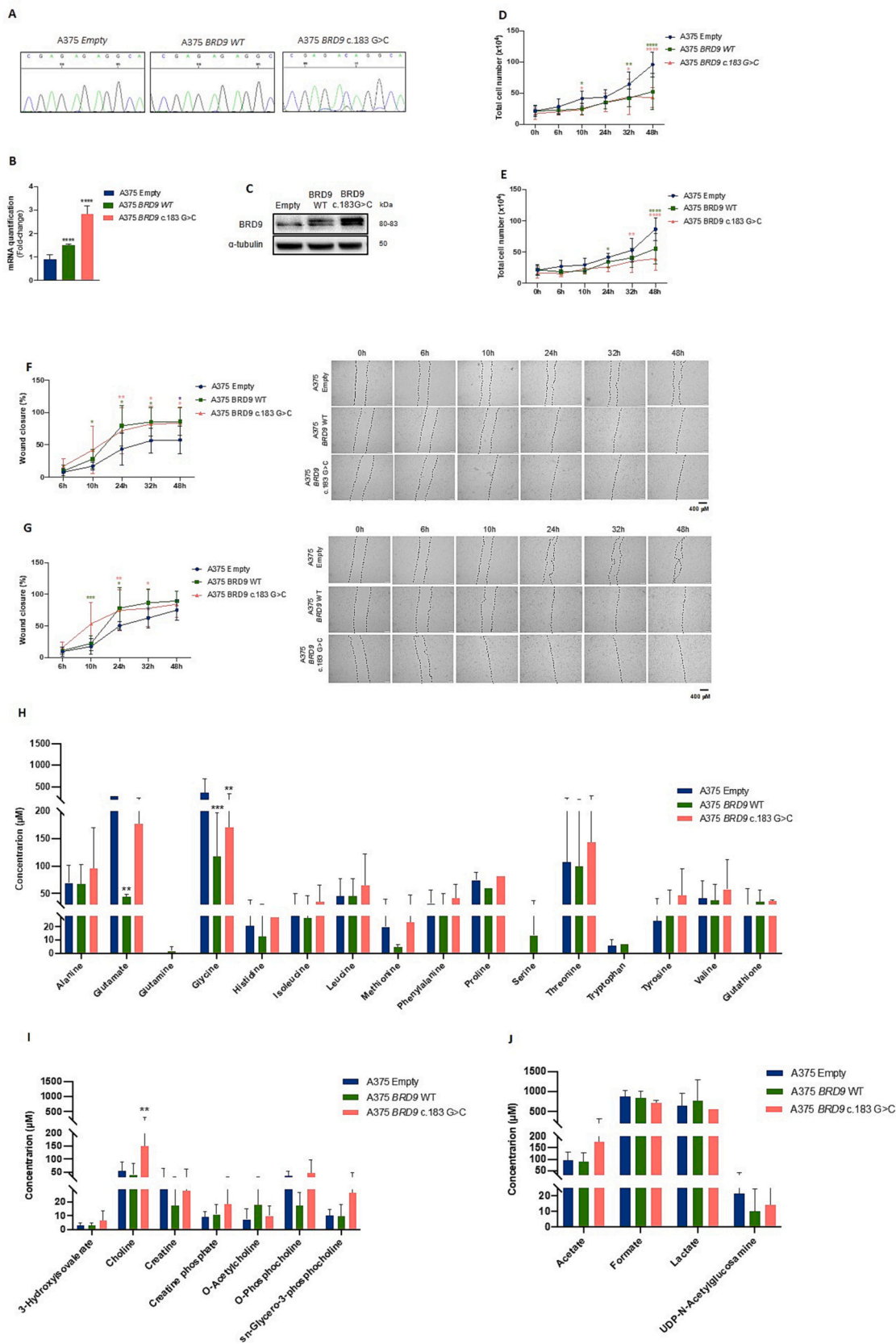


Fig. 1. Effect of BRD9 rare SNVs on protein function and/or splicing signals according to distinct *in silico* tools. (A) Pathogenicity prediction of 8 BRD9 rare SNVs (4 exonic, 3 intronic and 1 in 5'UTR) according to 30 *in silico* bioinformatic tools. Predictive programs: 1, BayesDel_addAF; 2, BayesDel_noAF; 3, CADD; 4, Cancer Genome Interpreter; 5, Condel; 6, DANN; 7, DEOGEN2; 8, EIGEN; 9, EIGEN-PC; 10, FATHMM cancer (coding SNVs); 11, FATHMM inherited disease (coding SNVs); 12, FATHMM-MKL; 13, FATHMM-XF; 14, Human splicing finder; 15, IntSplice2; 16, LIST-S2; 17, M-CAP; 18, MetaLR; 19, MetaRNN; 20, MetaSVM; 21, Mutation Assessor; 22, MutationTaster2021; 23, MVP; 24, Polyphen-2; 25, PROVEAN; 26, PrimateAI; 27, REVEL; 28, SIFT; 29, SpliceAI; 30, TraP. The number of *in silico* tools out of the total available used for each variant analysis is represented in brackets. (B-D) Simplified output representation of pathogenicity predictive programs for (B) panel of BRD9 rare exonic SNVs (C) panel of BRD9 rare intronic SNVs and (D) 5'UTR BRD9 rare SNV. Graphic representation refers to the number of *in silico* tools corresponding to each predictive category (Damaging (red), Benign (green), VUS (orange) and NA (grey)). NA, not available; UTR, Untranslated region; VUS, Variant of unknown significance.



(caption on next page)

Fig. 2. *BRD9* wild-type and *BRD9* c.183G>C overexpression induces a less proliferative but more migratory phenotype and rewires amino acid metabolism in A375 melanoma cells. By Sanger sequencing the sequence mRNA was confirmed (A) and by qPCR (B) and western blotting (C) the transfection of A375 melanoma cells and the expression of the *BRD9* variants was confirmed. A375 cells transfection with *wild-type* overexpression and *BRD9* c.183G>C (over)expression vectors induced increased mRNA and protein levels of *BRD9*, comparing to empty vector. Overexpression of *BRD9* WT or *BRD9* c.183 G>C induced a decreased proliferation rate in A375 cells, comparing to empty vector, in the absence (D) and presence (E) of cysteine. (F) Overexpression of *BRD9* WT or *BRD9* c.183 G>C induced an increased migration capacity in A375 cells, by wound healing, comparing to empty vector, in the absence of cysteine. (G) Overexpression of *BRD9* WT or *BRD9* c.183 G>C induced an increased migration capacity in A375 cells, by wound healing, comparing to empty vector, in the presence of cysteine. (H-J) Nuclear magnetic resonance spectroscopy of transfected A375 melanoma cells extracts in the absence of cysteine indicates that *BRD9* WT and *BRD9* c.183G>C overexpression induces a metabolic remodeling in several key metabolic pathways. Data is represented as mean $\pm$ SD. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments were performed with biological triplicates.

(*GOT-2* gene), xCT (*SLC7A11*), and EAAT3 (*SLC1A1*). Interestingly, the overexpression of *BRD9* WT strongly repressed the expression of the entire panel of studied genes, comparing to control. Moreover, overexpression of c.183G>C *BRD9* exhibited a similarly repressive, yet highly attenuated, pattern regarding *CBS*, *CTH*, *GOT-1*, *GOT-2* and *SLC7A11* expression. Surprisingly, A375 *BRD9* c.183G>C appeared to induce *MPST* and *SLC1A1* expression, comparing to control (Fig. 3D and E). These results were confirmed at the protein level (MST and EAAT3, Fig. 3F and G). To confirm the differential effect of *BRD9* WT and mutated variants on *MPST* expression, we exposed A375 empty, *BRD9* WT and *BRD9* c.183 G>C to *BRD9* degrader-1 and analyzed the expression of *BRD9* and MST proteins. Our results indicate that PROTAC *BRD9* degrader-1 depleted *BRD9* WT but not mutated *BRD9* (Fig. 3H and J). Therefore, the effect of *BRD9* WT on MST expression was abrogated but mutated *BRD9* still upregulated the expression of MST (Fig. 3I and K), as previously observed. These results indicate that *BRD9* degrader-1 does not deplete the mutated variant.

We then studied the effect of these expression shifts in cysteine related enzymes and transporters on the production of H_2S , which, as stated above, is produced through cysteine reductive degradation by CBS, CSE and/or CAT/MST. In these assays, we analyzed H_2S levels in the absence or presence of AOAA and PAG, inhibitors of the reverse transulfuration enzymes CBS and CSE, to estimate their relative roles with respect to MST (or other H_2S sources). A375 cells overexpressing either *BRD9* WT or *BRD9* c.183G>C presented an increase in H_2S levels, which were not altered by CBS/CSE inhibitors (Fig. 4A). While the increase in H_2S levels in *BRD9* c.183G>C-overexpressing cells matched the increase in MST expression, the same correlation was not observed with *BRD9* WT-overexpressing cells (Fig. 4A). It cannot be excluded that the capacity of cells to disposing of H_2S may have also been affected.

Pre-incubation of the cells with cysteine did not induce any change in steady-state H_2S levels between the three cell lines. Moreover, cysteine seems to have abrogated the *BRD9*-induced increase in H_2S levels, as compared to the control conditions (Fig. 4A). Pre-incubation with the polysulfides-containing H_2S donor NaHS increased H_2S levels in every cell line, irrespectively of co-incubation with cysteine, in the absence of inhibitors (Fig. 4A). The extent of H_2S levels decrease in the presence of AOAA+PAG was more evident in NaHS-incubated cells.

Since H_2S can serve as a source of electron equivalents to the mETC, we checked the effect of *BRD9* overexpression on the ATP levels. In control conditions, overexpression of *BRD9* WT or c.183G>C did not significantly change ATP levels. Incubation with NaHS in the absence or presence of cysteine resulted in a mild increase in ATP levels in all cell lines, whereas co-incubation with cysteine alone showed no significant effect comparing to control (Fig. 4B). In the presence of AOAA+PAG, in comparison with control cells bearing the empty vector and with control conditions, ATP levels were significantly lower for *BRD9* c.183G>C expressing A375 cells pre-incubated with cysteine and for cells expressing either *BRD9* variant pre-incubated with cysteine (Fig. 4B).

3.5. *BRD9* status modulates *MPST* but not *SLC1A1* through *NRF2*, inducing a remodeling in the melanin production pathway

Since *BRD9* can modulate the action of *NRF2*, which is described to modulate *SLC1A1* expression [69], and MST is an H_2S -producing AOE,

we hypothesized that *NRF2* regulates not only *SLC1A1* but also *MPST* expression. ChIP analysis revealed that *NRF2* does not seem to bind to *SLC1A1* promoter not even in the presence of cysteine nor among the different variants of *BRD9* expressed in A375. We found, however, that *NRF2* binding to *MPST* promoter in A375 *BRD9* c.183 G>C is increased and stimulated by cysteine (Fig. 4C). Luciferase activity studies of *MPST* promoter in all A375 cell variants were performed, in the presence or absence of cysteine. Our results indicate that cysteine significantly decreased *MPST* promoter activity in A375 *BRD9* WT cells (Fig. 4D). No significant changes were seen in A375 empty nor in A375 *BRD9* c.183 G>C (Fig. 4D).

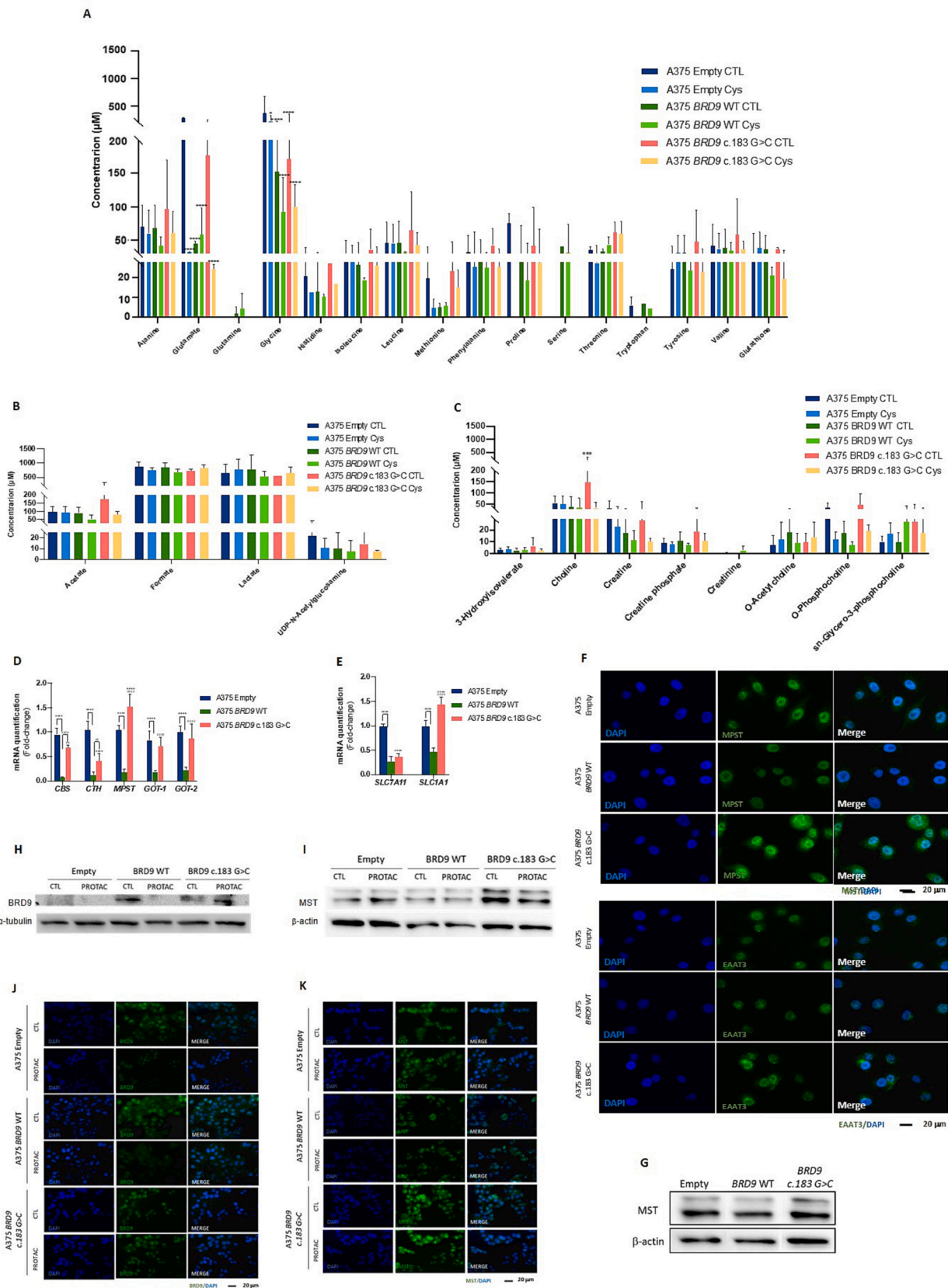
We further evaluated the effect of overexpressing WT or c.183 G>C *BRD9* in A375 melanin production. In control culture conditions, A375 *BRD9* WT presented the lowest level of melanin production, while cysteine prompted the decrease of melanin levels in A375 *BRD9* c.183G>C (Fig. 4E and F).

3.6. *BRD9* wild-type and *BRD9* c.183G>C overexpression in WM115 cell line does not modulate *MPST*/*MST* and *SLC1A1*/*EAAT3* expression

To study if our results could be replicated in another melanoma cell line and if *BRD9* status was the only factor inducing these metabolic and expression alterations, we chose to transfect WM115 cells with the *BRD9*-encoding plasmids. We then successfully obtained WM115 clones overexpressing *BRD9* WT or *BRD9* c.183G>C (Fig. 5A). This was confirmed at mRNA (Fig. 5B) and protein levels (Fig. 5C). We then studied the expression of both *MPST* and *SLC1A1* in the transfected WM115 control, WM115 *BRD9* WT or WM115 *BRD9* c.183G>C variants. WM115 *BRD9* WT presented decreased *MPST* and *SLC1A1* mRNA, comparing to WM115 transfected with the empty vector. However, WM115 *BRD9* c.183G>C also showed a significant decrease in *MPST* and *SLC1A1* expression, comparing to control (Fig. 5D). The same trend was found in protein expression of both MST and EAAT3 (Fig. 5E and F). Notably, the WM115 cell line expresses high basal levels of *MPST*/*MST* and *SLC1A1*/*EAAT3*, but no alteration was seen concerning the effect of *BRD9* variants. WM115 *BRD9* WT or WM115 *BRD9* c.183G>C did not present significant differences in cell migration capacity in the absence or presence of cysteine (Fig. 5G and H). We also analyzed melanin production in these variants, and found that, although no differences were found in the absence of cysteine, in the presence of cysteine, WM115 *BRD9* c.183G>C significantly increased melanin production (Fig. 5I and J). Moreover, ChIP analysis showed no binding of *NRF2* to *SLC1A1* or *MPST* promoters (Fig. 5K).

3.7. *BRD9* c.183 G>C increases invasiveness of A375 CM cell line in a 3D *in vitro* skin model

To address the role of *BRD9* (WT and c.183 G>C) in melanoma aggressiveness, the capacity of invasion of A375 and WM115 cell line variants was tested in a 3D *in vitro* skin model. As observed in Fig. 6A and B, a higher invasive capacity of A375 cells, comparing to WM115 cells, was verified, since WM115, although presenting primary tumors, does not invade. However, upon cysteine exposure, WM115 control and *BRD9* WT formed statistically significant thicker tumors than in the absence of cysteine. Considering the A375 variants, the exposure to



(caption on next page)

Fig. 3. *BRD9* wild-type and *BRD9* c.183G>C overexpression alters the metabolic response of A375 cells to cysteine availability and modulates cysteine metabolic enzymes and transporters mRNA and protein expression. (A-C) Nuclear magnetic resonance spectroscopy of transfected A375 melanoma cells extracts in the presence (Cys) or absence (CTL) of cysteine indicates that *BRD9* wild-type and *BRD9* c.183G>C overexpression modulates the metabolic response to cysteine exposure, regarding intracellular amino acid, sugars and organic acids metabolism and other metabolic compounds. (D) By qPCR, mRNA levels of genes encoding enzymes *CBS*, *CTH*, *MPST*, *GOT-1* and *GOT-2*, as well as (E) genes encoding cyst(e)ine transporters *SLC7A11* and *SLC1A1* in A375 transfected cells were quantified and it was observed that induction of *BRD9* WT and c.183G>C variants overexpression regulates the expression of cysteine enzymes and transporters. A375 *BRD9* c.183 G>C induces an upregulation of both *MPST* and *SLC1A1*, analyzed by qPCR. (F) Protein expression of *MST* and *EAAT3* show upregulation of both proteins induced by *BRD9* c.183 G>C induction, evaluated by immunofluorescence for *Mst* and *EAAT3*, and (G) western blotting for *MST*. Western blotting of *BRD9* (H) and *MST* (I) in A375 cells exposed to PROTAC *BRD9* degrader-1. Immunofluorescence to *BRD9* (J) and *MST* (K) in A375 cells exposed to PROTAC *BRD9* degrader-1. Data is represented as mean $\pm$ SD. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (* in relation to Empty; # in relation to *BRD9* WT). Experiments were performed with biological triplicates.

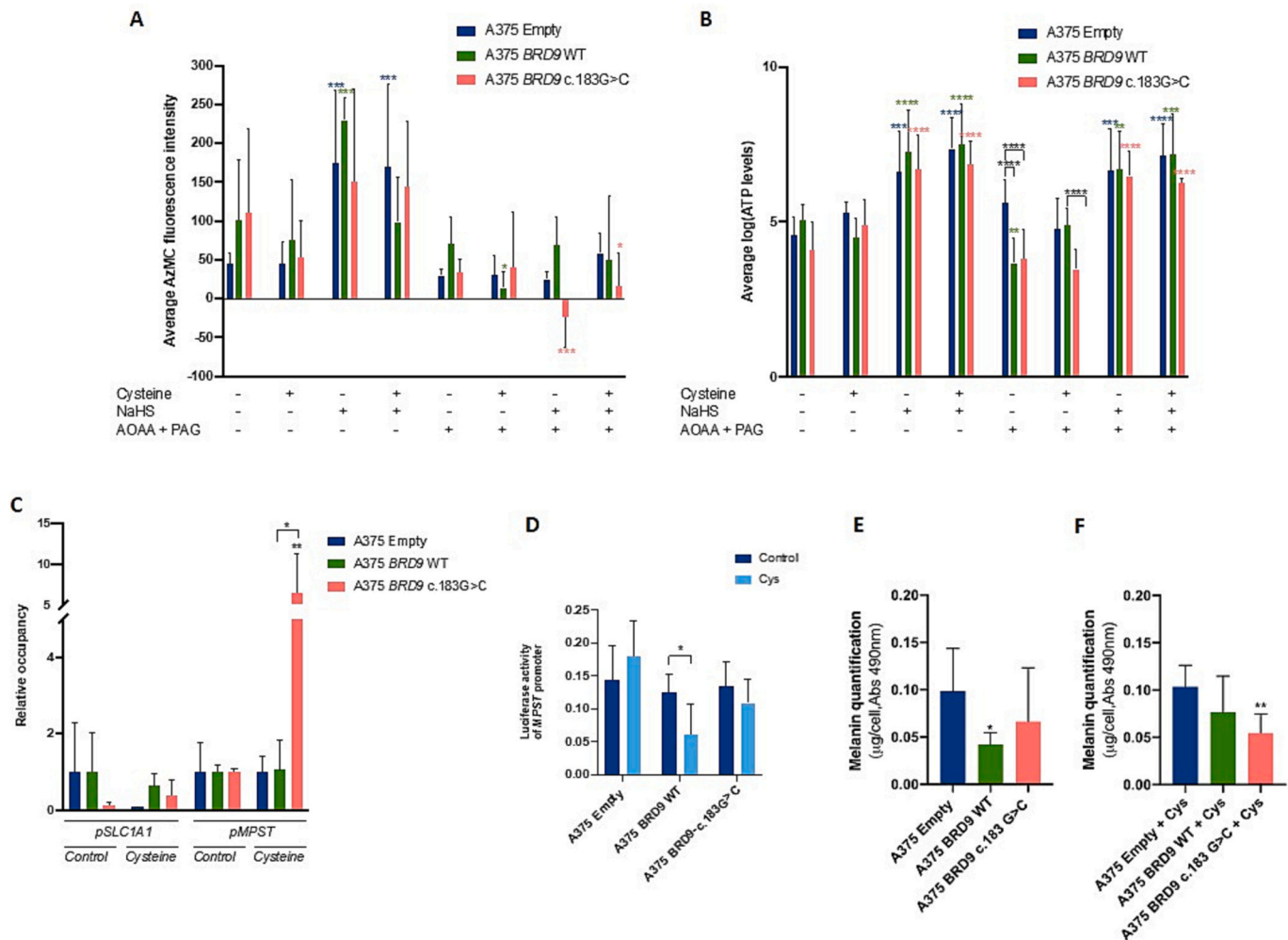
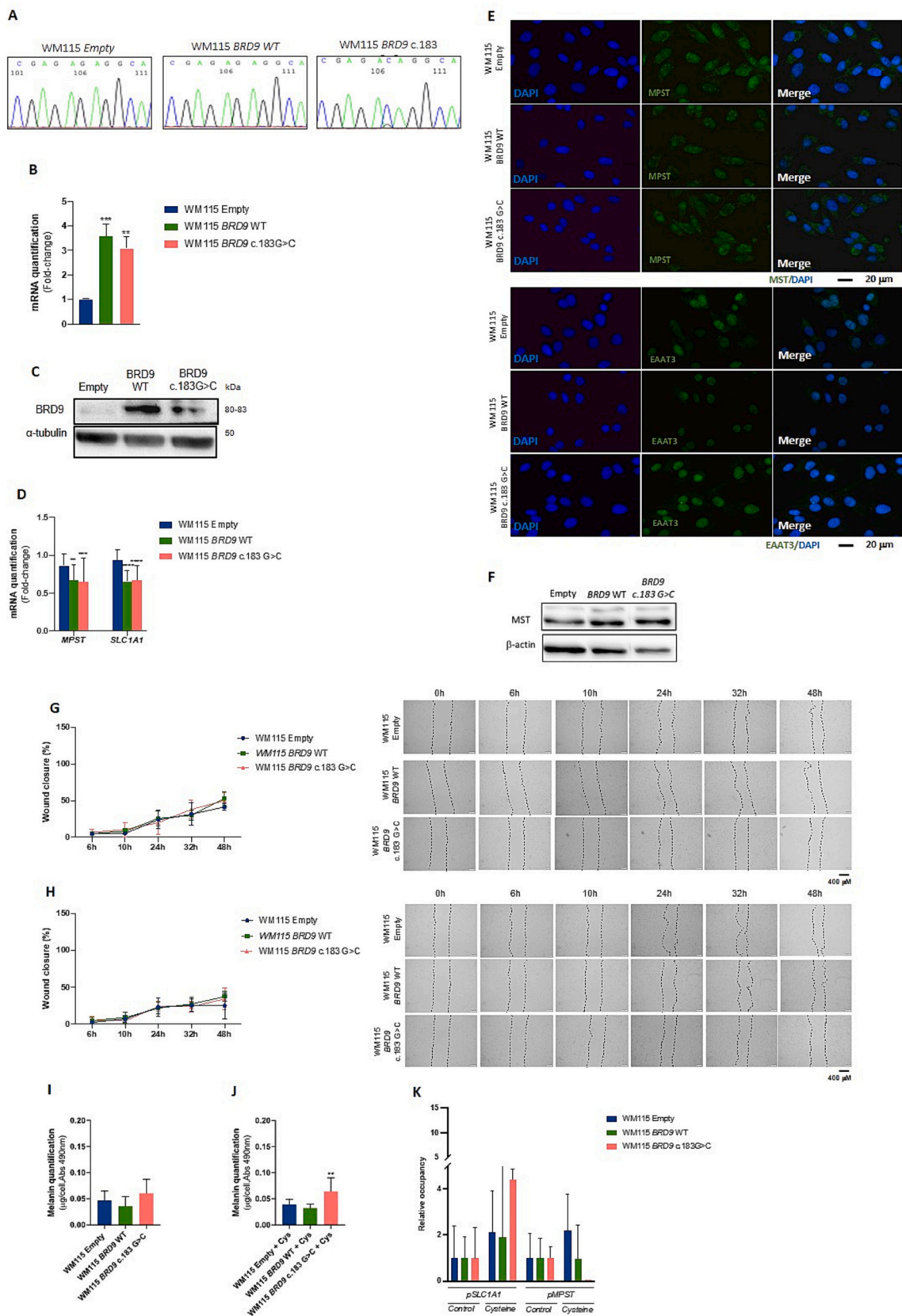


Fig. 4. *BRD9* wild-type and *BRD9* c.183G>C overexpression induces a more efficient H₂S-producing phenotype, which does not always correspond to accumulated ATP levels in A375 melanoma cells, and modulates *MST* and *EAAT3* activity through *NRF2*, inducing a remodeling in the melanin production pathway. (A) H₂S quantification following 7-Azido-4-Methylcoumarin probe (AzMC) signal indicates that A375 cells overexpressing *BRD9* WT and *BRD9* c.183 G>C, despite cysteine availability, show higher H₂S production, comparing to A375 Empty, independently of o-(carboxymethyl)hydroxylamine hemihydrochloride (AOAA) and dl-propargylglycine (PAG) treatment. NaHS treatment further increased H₂S levels in every cell line tested, irrespectively of co-incubation with cysteine. (B) ATP measurement in transfected A375 cells did not show a modulation of energy production by neither *BRD9* WT nor *BRD9* c.183 G>C overexpression, despite increased H₂S levels. NaHS alone or in combination with cysteine but not cysteine alone led to increased ATP levels across the three cell lines. (C) ChIP analysis show a high binding of *NRF2* to *MPST* promoter in the presence of cysteine. (D) Transfection of A375 melanoma cells with *BRD9* WT induces decreased *MPST* promoter activity in the presence of cysteine. Melanin quantification in the absence (E) and presence (F) of cysteine was performed and cysteine exposure. Melanin quantification show decreased levels in A375 overexpressing-*BRD9* WT in the absence of cysteine, but also decrease A375 *BRD9* c.183 G>C melanin production in the presence of cysteine. Data is represented as mean $\pm$ SD. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments were performed with biological triplicates.

cysteine did not affect the development of tumors but the overexpression of *BRD9* c.183 G>C induced tumors with statistically significant higher thickness and marked invasion edge comparing to A375 control and *BRD9* WT.

3.8. Inhibition of *MST* induces a significant decrease in A375 and WM115 cell viability

Since our results pointed to *MST* as a crucial player in the rewiring of metabolic and cellular features of A375 overexpressing *BRD9* WT and



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Fig. 5. Overexpression of *BRD9* WT and *BRD9* c.183G>C was successfully induced in WM115 cells and modulates the expression of *MPST*/*MST* and *SLC1A1*/*EAAT3*, leading to deregulated melanin production in the presence of cysteine. By Sanger sequencing the sequence mRNA was confirmed (A) and by qPCR (B) and western blotting (C) the transfection of WM115 melanoma cells and the expression of the *BRD9* variants was confirmed. WM115 cells transfection with *wild-type* overexpression and *BRD9* c.183G>C (over)expression vectors induced increased mRNA and protein levels of *BRD9*, comparing to empty vector. (D-E) qPCR and immunofluorescence analysis indicate that *BRD9* WT and c.183G>C overexpression induces decreased *MPST*/*MST* and *SLC1A1*/*EAAT3* expression in WM115. (F) Western blotting for *MST* expression. (G) *BRD9* WT/c.183G>C overexpression do not induce effects in WM115 migration, by wound healing, in the absence of cysteine. (H) *BRD9* WT/c.183G>C overexpression does not induce effects in WM115 migration, by wound healing, in the presence of cysteine. Melanin quantification in the absence (I) and presence (J) of cysteine was performed and cysteine exposure increased melanin levels in *BRD9* c.183G>C WM115 cells. (K) ChIP analysis show that *NRF2* does not bind to *MPST* or *SLC1A1* promoter in the absence nor presence of cysteine in WM115 cell variants. Data is represented as mean $\pm$ SD. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments were performed with biological triplicates.

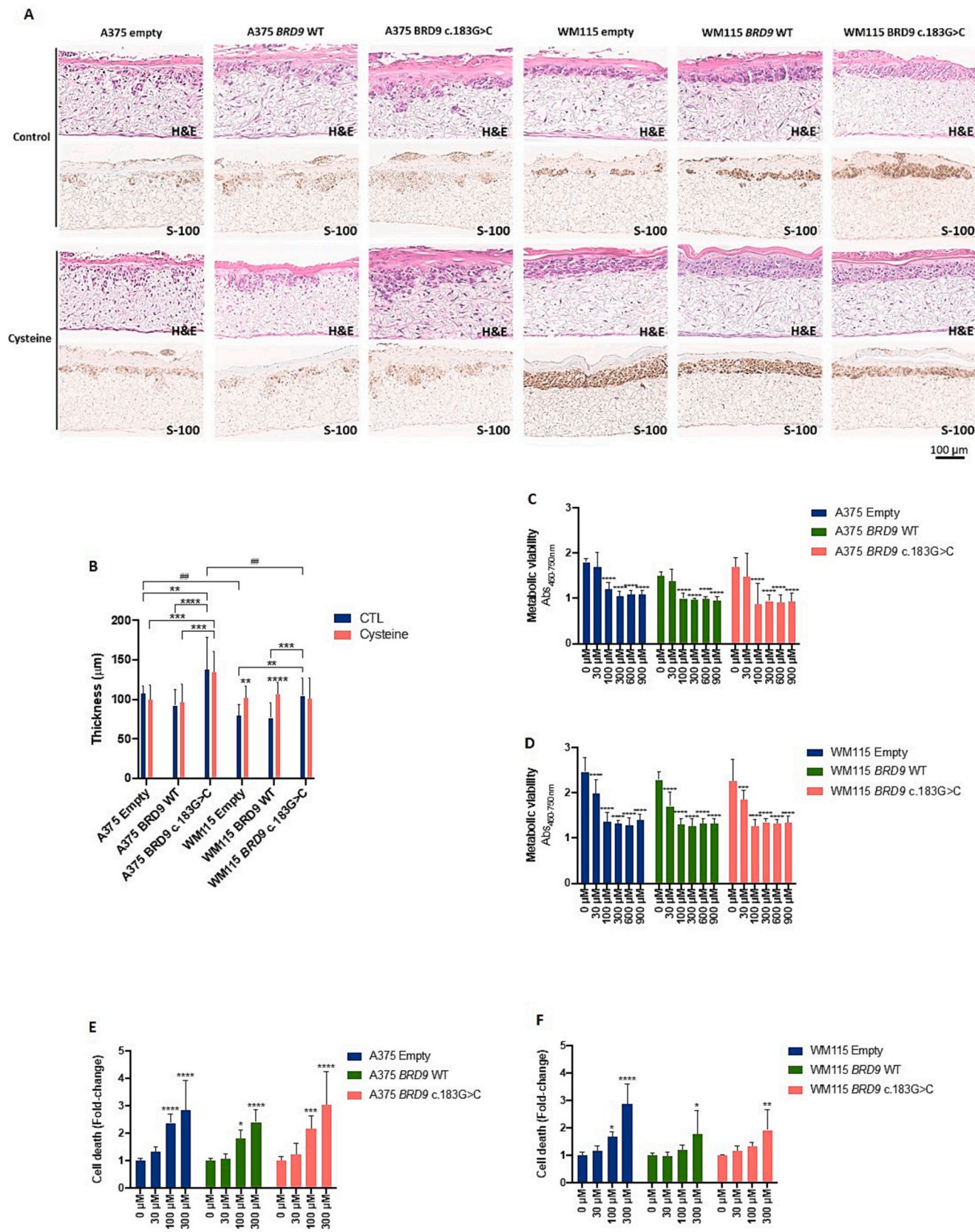
BRD9 c.183 G>C; and also WM115 express high basal levels of *MST* independently of *BRD9* status, we supposed that *MST* can be a target in these melanoma models. Therefore, Empty, *BRD9* WT and *BRD9* c.183 G>C variants of A375 and WM115 were exposed to increasing concentrations of an *MST* chemical inhibitor for 48 h. A375 *BRD9* WT/c.183 G>C and WM115 *BRD9* WT/c.183 G>C showed similar responses to the inhibitor, with a more significant viability decrease between the concentrations of 30 to 100 μ M of the inhibitor (Fig. 6C, D, E and F). Furthermore, upon exposure to the *MST* inhibitor, A375 presents a higher EC_{50} comparing to WM115, corroborating a different reliance of both cell lines on *MST* (Supplementary Fig. S1). Both cell lines expressing *BRD9* (WT and c.183 G>C) displayed lower EC_{50} with respect to the 'empty' parent lines, confirming the *BRD9* modulation of *MPST* expression.

4. Discussion

Hereditary CM constitutes a significant part of all CM cases, in which most of the diagnoses remain without a known genetic familial cause. It is, thus, crucial to explore and acknowledge novel genetic targets that can be associated with CM familial history and that can explain increased susceptibility. To our knowledge, this is the first study exploring the molecular effects of the *BRD9* c.183G>C variant in melanoma. Our study indicates that this variant, even though predicted to be benign rather than disease-causing (Fig. 1), induces different alterations in the CM cell lines tested, A375 and WM115. According to Sanger COSMIC database [97] A375 cells present a different genetic background from WM115, presenting mutated *BRAF* (c.1799T>A, p. V600E) and *CDKN2A* (c.181G>T, p.E61*; c.205G>T, p.E69*), while WM115 presents mutated *BRAF* (c.1799_1800TG>AT, p.V600D), *CDKN2A* (c.1_150del150, p.?) and *PTEN* (c.493_634del142, p.?), presenting deletions in both *CDKN2A* and *PTEN*. The significantly different genetic background of A375 and WM115 cells can explain the distinct effect of overexpressing *BRD9* WT and mutated variants. In fact, it is already described that coexisting *BRAF* and *PTEN* mutations are directly linked to metastatic melanoma [98]. Importantly, in this study we disclosed *BRD9* role as a regulator of cysteine metabolism. The involvement of *NRF2* in the increased expression of *MPST* gene, as we verified that *NRF2* binding to *MPST* promoter in A375 *BRD9* c.183 G>C is increased and stimulated by cysteine (Fig. 4C), is in agreement with *BRD9* and *SWI/SNF* loss of function, already described in NSCLC [70]. Importantly, new studies are needed to determine the impact of c.183 G>C in *BRD9* structure and function, in order to understand the way this *BRD9* mutated variant can interfere with *SWI/SNF* complex function and somehow differentially modulate the expression of genes. Despite the structurally and chemically conservative substitution of glutamate by aspartate resulting from this mutation, the impact on its structure is reinforced by the inability of PROTAC *BRD9* degrader-1 to deplete the mutated variant in A375 cells (Fig. 3H and J). Indeed, both *BRD9* WT and c.183G>C induced distinct metabolic profiles, with emphasis on amino acid metabolism (Fig. 2H, I and J; Fig. 3A, B and C). Different genetic backgrounds induce cellular diversity, and our *in vitro* models can represent two distinct melanoma profiles: a cysteine remodeled metabolic profile by *BRD9* with increased *MST* expression leading to a more invasive phenotype, and a molecular profile with decreased

expression of *MST* induced by *BRD9* altered status, with no influence in cell migration. Therefore, the *BRD9* c.183G>C variant associates to a significantly more migratory phenotype in A375 cells (Fig. 2G), but not in WM115 cells, which maintained their migratory capacity independently of the *BRD9* status (Fig. 5G). The role of mutant in melanoma was confirmed in the increased invasive capacity exhibited by A375 *BRD9* c.183G>C variant (Fig. 6A and B). This highlights the possibility that *BRD9* may not be so relevant in terms of migration in WM115 cells comparing to A375 cells. A detail that likely accounts for this observation is the fact that WM115 cells have the deletion of the E-cadherin encoding gene. It was previously shown that E-cadherin re-expression in melanoma cells decreases the migratory and invasive capacity of cells and rescues the ability of cell-to-cell binding [99]. Despite the fact that A375 cells show higher migration rates in their basal state than WM115 cells (Figs. 2G, 5G), WM115 cells have loss of E-cadherin function, hampering finding differences between the migratory capacities of these cell line variants. Furthermore, and looking at the interference of cysteine metabolic adaptation in cell migration, all WM115 cell variants migrate at a lower rate in the presence of cysteine comparing to control conditions (Fig. 5G and H). The opposite was observed in A375 cell line, in which A375 *BRD9* c.183G>C cell variant exposed to cysteine migrates at a higher rate than in control conditions (Fig. 2F and G). The 3D skin model revealed that A375 cell line is more invasive than WM115 cell line, which besides presenting tumors, cells do not invade (Fig. 6A and B). However, upon cysteine exposure, WM115 control and *BRD9* WT formed statistically significant thicker tumors than in the absence of cysteine. By comparing the metabolic viability of all cell variants, it was verified that WM115 cells present higher values than A375 variants, in control conditions. This possibly indicates that WM115 cells proliferate at a higher rate than A375 cells (Fig. 6D and E), and this will contribute for the increased thickness of WM115 melanomas in 3D skin model, suggesting that cysteine exposure may stimulate WM115 proliferation. Considering the A375 variants, the exposure to cysteine did not affect the development of tumors but the overexpression of *BRD9* c.183 G>C induced tumors with statistically significant higher thickness and marked invasion edge comparing to A375 control and *BRD9* WT (Fig. 6A and B).

Cysteine exposure induced decreased levels of glutamate in both A375 Empty and *BRD9* c.183G>C, which was accompanied by undetectable glutamine levels. However, in A375 *BRD9* WT, cysteine led to an increasing tendency of glutamate and glutamine levels (Fig. 3A). Decreased levels of glutamate and glutamine can be explained by increased synthesis of glutathione, as described in ovarian cancer [53]. Glutathione's antioxidant role depends on cysteine's reactive thiol group [100]. Indeed, we found increased GSH levels in A375 Empty but not in A375 *BRD9* c.183G>C. Instead, this variant showed a tendency towards decreased GSH levels in the presence of cysteine, as observed in cells expressing the A375 *BRD9* WT variant (Fig. 3A). Decreased glutathione levels can derive from a slow glutathione synthesis and/or fast GSH consumption. However, the results point towards increased metabolic demands of *BRD9* expressing cell variants, presenting a more demanding redox turnover, prompting the consumption of reduced glutathione, in contrast to a lower metabolic flow of A375 Empty cells. This is also supported by the evident signs of oxidative stress presented by A375 *BRD9* c.183G>C cells, such as increased expression of the antioxidant



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Fig. 6. *BRD9* c.183G>C increased the invasiveness of A375 in a 3D *in vitro* skin model, and MST inhibition impacts on metabolic viability in all variants of A375 and WM115 cells. The effect of *BRD9* WT and *BRD9* c.183G>C overexpression in melanoma cell lines was evaluated in a 3D *in vitro* skin model. (A) Representative images of *in vitro* tumors in hematoxylin and eosin (H&E) staining and S-100 protein immunohistochemistry to specifically detect melanoma cells, and (B) the quantification of tumor thickness are presented, considering 20 imaging spots *per* slide of the 2 biological replicates. The impact of MST inhibition was assessed in A375 and WM115 *BRD9* WT and *BRD9* c.183G>C variants. Statistical significance between different conditions within each cell line (*) and between cell lines (#) were considered. By WST-1 assay (subtracting the absorbance measured at 480 nm and 760 nm), it was verified that MST inhibitor (I3MT-3) induces a significant decrease in metabolic viability of A375 (C) and WM115 (D) *BRD9* WT and *BRD9* c.183G>C variants, upon 48 h of exposure, with more significant viability decrease between the concentrations of 30 to 100 μ M of the inhibitor. The cell death, measured by flow cytometry (Annexin V-FITC and PT labeling), induced by I3MT-3 in all A375 (E) and WM115 (F) variants upon 48 h of exposure show differences in resistance to this treatment between A375 and WM115 empty variants *versus* variants overexpressing *BRD9* WT and *BRD9* c.183G>C: empty variants of both A375 and WM115 showed more resistance to the inhibitor and presented higher EC_{50} values comparing to *BRD9* WT/c.183 G>C variants. Data is represented as mean $\pm$ SD. * p < 0.5, **/# p < 0.01, *** p < 0.001, **** p < 0.0001. Experiments were performed with biological triplicates.

enzyme MST [101] and EAAT3 transporter (Fig. 3D, E and F). This is in line with our results regarding the enzymatic activity study in all three variants of A375. In fact, we found that A375 *BRD9* c.183G>C cells exhibit increased steady-state levels of H_2S comparing to A375 Empty, in control conditions. Interestingly, A375 *BRD9* WT, even though showing significant decreased expression of antioxidant enzymes and transporters, show similarly increased H_2S levels, comparing to A375 *BRD9* c.183G>C (Fig. 4A), which can either indicate an increased H_2S production or a lower detoxifying ability. H_2S is described to act in two equally important ways: either as a potent antioxidant or as source of electron equivalents to the mETC, leading to ATP production [57,102]. In our results, increased H_2S levels do not translate in increased ATP levels (Fig. 4B). This may mean that *BRD9* overexpression whether in its WT or mutated form can further induce imbalances in H_2S production as a distinct way to overcome oxidative stress.

Since complete culture medium contains glutamine, we expected to detect this amino acid in our metabolomics study. Low (A375 *BRD9* WT) or inexistent (A375 Empty and A375 *BRD9* c.183 G>C) levels of glutamine, independently of cysteine availability, further indicate high uptake and consumption of this amino acid (Fig. 3A). Decreased glutamine is associated with a necessity for redox control within the cell, and is usually found during catabolic stress and antioxidant imbalance [103]. Glutamine can act either as a positive or negative redox status modulator, depending on the activation of specific transcription factors triggered by stressful microenvironments [103]. In this case, high consumption of glutamine may act as an attempt by the cell to compensate the decreased GSH production due to cysteine diversion to other pathways, since these cells probably show increased ROS levels. Decreased glutathione levels are also described to be associated with decreased proliferation rates [103], which is in accordance with our results (Fig. 2D and E). Glycine levels drastically decrease in the presence of cysteine (Fig. 3A). Again, decreased availability of glycine may indicate a cellular response to oxidative stress, since glycine is also a component of glutathione. Moreover, the decreased levels of serine may indicate increased glycine synthesis and altogether highlight that glycine turnover is in operation in A375 *BRD9* c.183 G>C cell variant.

We further observed a decrease in methionine levels in A375 Empty and *BRD9* c.183 G>C when exposed to cysteine and in A375 *BRD9* WT, independently of cysteine presence (Fig. 3A). Decreased methionine levels in the presence of cysteine suggest that exogenous cysteine stimulates methionine demethylation, with the resulting homocysteine either being remethylated back to methionine or entering the reverse transsulfuration pathway (RTP, comprising CBS and CSE). The observation that both A375 Empty and *BRD9* c.183 G> variants express high levels of EAAT3, CBS and CSE-encoding genes favors the latter fate involving the RTP and leading to cysteine production (Fig. 3D, E and F). A375 *BRD9* WT show low levels of methionine, unaffected by cysteine availability (Fig. 3A), probably related to low CBS expression by this variant. This A375 variant likely relies on a different source of cysteine, since H_2S steady-state levels are comparable to A375 *BRD9* c.183 G>C in control conditions (Fig. 4A). Decreased levels of acetate (Fig. 3B) are in agreement with decreased proliferation rate [104,105] in cells overexpressing *BRD9* (Fig. 2D and E), independently of the mutational

status. Indeed, the average number of cells between time point 10 h and 16 h is significantly lower in A375 *BRD9* c.183 G>C comparing to A375 *BRD9* WT. Decreased levels of choline and phosphocholine can also be indicative of a decreased proliferation rate [106,107] (Fig. 3 C; Fig. 2D and E). Additionally, high levels of formate and lactate may be related to increased invasion, independently of cysteine presence [108,109] (Figs. 3B; 2F and G).

Cysteine metabolism is deeply associated with increased tumor aggressiveness as described before, and so, we hypothesize that *BRD9*-induced cysteine metabolic remodeling may be an extremely relevant feature to further disclose in malignant CM. In fact, our results suggest that *BRD9* WT has an inhibitory effect on *MPST* expression and the mutated *BRD9* variant exhibits the opposite effect by upregulating *MPST*, as verified at the RNA as protein level (Fig. 3). Cysteine, besides being able to supply energy to cancer cells, can also provide them with the resistance mechanisms to overlap standard therapy regimens applied to cancer patients. Moreover, it is a pivotal intervenient in pheomelanin production. Thus, increased cysteine metabolism and uptake can intervene in different ways, all of them contributing to tumorigenesis and/or tumor progression. We postulate that overexpression of WT or c.183G>C *BRD9* can contribute to imbalances in melanin production, deregulating the eumelanin/pheomelanin ratio, thus leading to a higher susceptibility to CM development. In fact, while eumelanin exerts a photo/radioprotection role, pheomelanin can be a source of mutagenic events upon radiation exposure [34,110]. Several studies have uncovered that, indeed, pheomelanin can be associated with increased production of ROS upon UVR exposure [110–113]. Moreover, imbalances in this ratio are already described in uveal melanoma cells, which show much higher levels of pheomelanin than eumelanin [114].

Metabolic rewiring has become clear to have a central role in tumor development, progression and resistance and has, in fact, been considered a core hallmark of cancer [115]. Thus, it is essential to further explore the role of *BRD9* as a CM susceptibility gene but also as a metabolic regulator. The present work opens a new door towards understanding hereditary melanoma as a complex entity, in which tumor, not only genetic but also metabolic profiling may be a central piece to disentangle familial history and cancer susceptibility, as well as an important therapy response predictor. Additionally, this study supports a broad role of MST enzyme as a crucial player in CM, not only in tumors presenting alterations in *BRD9*. In fact, our results show that inhibition of MST leads to a decrease in cell viability in both A375 and WM115 cell lines, with a significantly more pronounced effect in A375 and WM115 variants overexpressing *BRD9* WT or c.183 G>C, presenting lower EC_{50} values for the latter (Fig. 6C, D, E and F). Indeed, increasing evidence points towards MST as a player in cancer metabolic remodeling and its encoding gene, *MPST*, though constitutively expressed in normal differentiated cells, is also often found overexpressed in several different cancer cell lines as well as across a wide range of tumors [55,116]. Moreover, studies have correlated increased expression of MST with chemoresistance in different cancer types [117,118] and have revealed the crucial role of MST in cancer cell proliferation [119–121]. We have previously suggested a critical role for MST expression and activity in cancer according to cancer type and metabolic context, with emphasis

on its role in carcinogenesis and/or cancer progression [55]. In this study, we disclosed a putative role of MST in the metabolic reliance of *BRD9*-mutated CM. MST elevated expression can define a parameter regarding CM aggressiveness, being associated with more migratory and invasive but less proliferative phenotypes, with low melanin production, thus characterizing a specific subset of CM. More experiments to characterize the mechanism underlying the effect of mutated *BRD9* should be performed, to better understand the function of the mutated protein.

5. Conclusion

BRD9 is a CM-associated gene with yet much potential to be further explored, namely in larger cohorts of CM patients in familial and sporadic contexts. This study highlights that not only the mutational status of *BRD9* but also its expression level can be used as a marker for CM aggressiveness. Here, *BRD9* was disclosed as a regulator of cysteine metabolism in a CM *in vitro* model. Overexpression of *BRD9* in its WT or c.183G>C variants can induce metabolic remodeling, leading to altered cellular features. *BRD9* c.183G>C, thus, introduces cysteine metabolism as a putative central player in CM susceptibility, as well as in CM progression. Overall, we demonstrated that A375 cell line aggressiveness correlates with the overexpression of MST and EAAT3, in a mutated *BRD9*-dependent way. Because cysteine also conditions the production of melanin forms, the increased expression of EAAT3 may contribute for more pheomelanin production, which has a lower protective effect against the mutagenic effect of UV radiation. On the one hand, cysteine catabolism through MST allows the use of cysteine as an energetic and synthetic source; on the other hand, the increased intracellular bioavailability favors the production of pheomelanin, resulting in lower protection against UV damage. Moreover, we disclosed a putative mechanism linking mutated forms of *BRD9* to susceptibility to melanomagenesis. This study opens new perspectives to reinforce the need to explore the impact of c.183 G>C mutation in the conformation and function of *BRD9* protein, and ultimately in the transcriptional complexes to which *BRD9* belong that will greatly influence gene modulation and control cancer behavior. Disclosing these alterations will help defining the role of *BRD9* in cutaneous melanoma. Furthermore, cysteine metabolism is a hub for innovative strategies to fight cancer, as it is a core net of metabolic pathways essential to sustain cancer cells survival and the mechanisms supporting aggressiveness and disease progression. Therefore, MST can be a suitable marker for CM aggressiveness and a potential target, since it correlates with increased migratory and invasive potential and decreased production of melanin, accounting for an increased risk of accumulating mutations due to decreased DNA protection against radiation.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2023.166983>.

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CRedit authorship contribution statement

AH- wrote the 1st draft of the paper, planned and performed the most experiments, revised and discussed the paper; RX- performed NGS analysis of melanoma samples and analysis of mutated variants, revised and discussed the paper; CB and AT- established stable transduced cell lines, revised and discussed the paper; IL- performed NMR and immunofluorescence analysis, revised and discussed the paper; LCC- performed 3D skin model experiments, revised and discussed the paper; FS- responsible for the 3D skin models processing and IHC analysis, revised and discussed the paper; AO- guide the optimization procedures of the 3D skin models, revised and discussed the paper; DCB- coordinated and supervised the 3D skin models, revised and discussed the paper; JBV- supervised hydrogen sulfide measurement and enzymatic experiments, revised and discussed the paper; LGG- coordinated NMR spectroscopy analysis, revised and discussed the paper; MP- coordinated and supervised the patients samples analysis and cell line variants establishment, responsible for funding, revised and discussed the paper; JS- coordinated and supervised the cancer biology and metabolism project components, responsible for funding, revised and discussed the paper.

Declaration of competing interest

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Data availability

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7230562/>

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