

SUPPORTING INFORMATION

Title: Acquired Non-Thermal Plasma Resistance Mediates a Shift Towards Aerobic Glycolysis and Ferroptotic Cell Death in Melanoma.

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Cell Morphology of NTP-Resistant and Parental Cells

At the end of the 12-week cycle of NTP exposure and cell culture, the A375-NTP-R cell line did not appear to have any morphological changes compared to the parental A375 at the same passage number (**Fig. S1**).

Phase-images were taken on the Evos Fl (Thermo Fisher Scientific, USA) at 4x and 10x magnification.

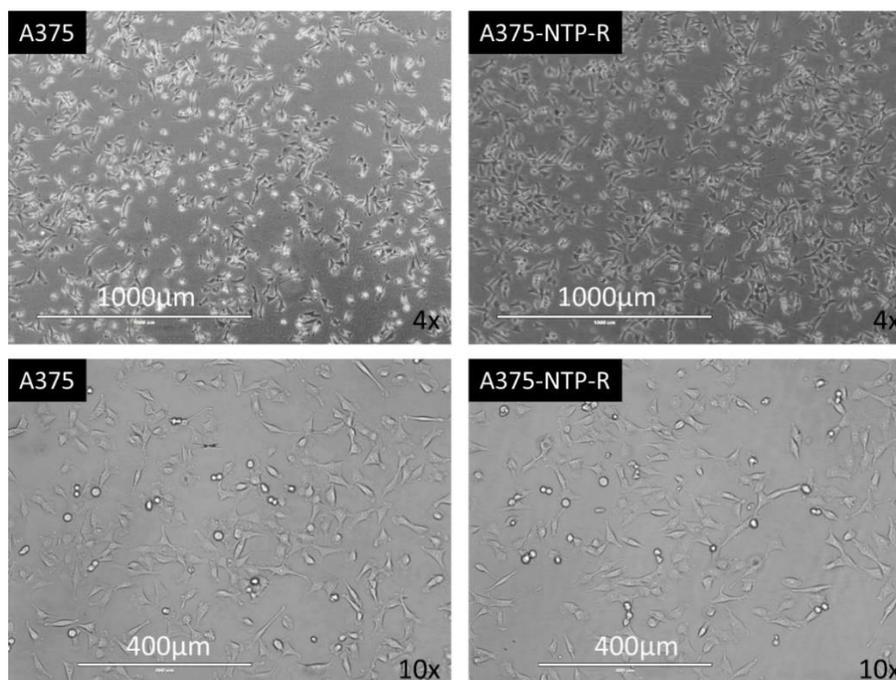


Fig. S1 NTP-resistant cells (A375-NTP-R) did not show visible morphological changes compared to the NTP-sensitive, parental cells (A375).

Gene Set Enrichment Analysis with Hallmark Gene Set

Gene set enrichment analysis (GSEA) of Hallmark gene sets showed significant enrichment of the hypoxia gene signature (**Fig. 2d**) when comparing NTP-resistant cells with the parental. Since hypoxic signatures are also associated with metabolic rewiring, where glycolysis is favored as the major energy process, we also looked at enrichment of glycolysis and oxidative phosphorylation signatures. While NTP-resistant cells had slightly higher enrichment scores for both signatures compared to the parental, neither was statistically significant (**Fig. S2**).

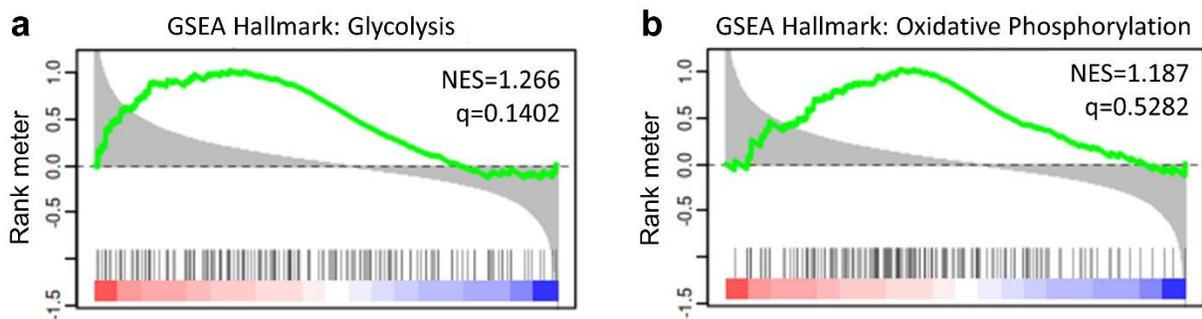


Fig. S2 NTP-resistant cells did not have significantly higher enrichment scores for the glycolysis and oxidative phosphorylation signatures when compared to the parental cells.

Flow Cytometry Gating for Glucose Uptake Assay

The fluorescent glucose analog, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), was added to NTP-resistant and parental cells in glucose-free media and cultured for 30 minutes. Afterwards, cells were detached and measured using flow cytometry. Population gating and flow cytometry analysis was performed using FlowJo v10.7.1 (**Fig. S3**). Unstained cells were used to set the gates for the negative, 2-NBDG (−), and positive, 2-NBDG (+), populations.

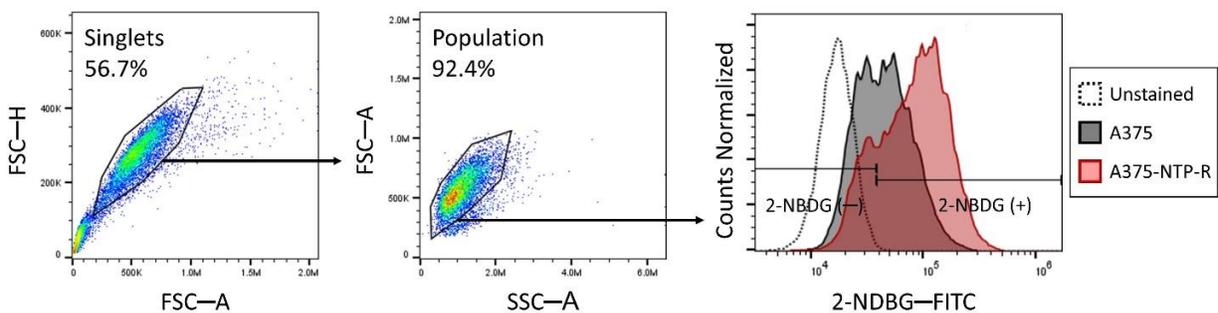


Fig. S3 The gating strategy for measuring cellular uptake of 2-NBDG.

Baseline Lactate Concentrations and pH for Cell Culture Media Without Cells

The lactate concentrations and pH of the media in the absence of cells (both in normoxia and hypoxia) were measured in order to establish baseline levels. Measurements were performed at 37°C (corresponding to samples from the cell incubator) and 4°C (corresponding to refrigerated samples). Significant differences were not measured for both extracellular lactate and media acidification (**Fig. S4**).

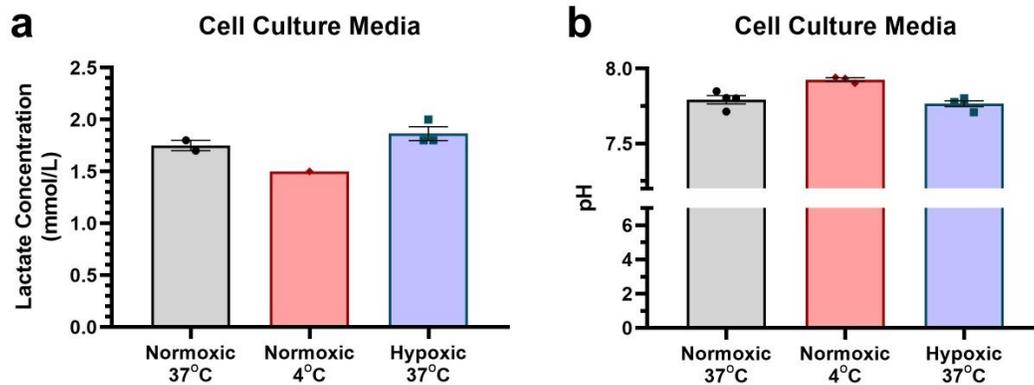


Fig. S4 Quantification of lactate concentration and pH in cell culture media without cells. No significant differences were observed at different temperatures and oxygen concentrations. For the hypoxic condition, 1% oxygen was used. Data are represented as mean \pm SEM and individual data points are shown.

Gating Strategy for Flow Cytometry Analysis

Flow cytometry was performed on cells triple-stained with CD47 (PE), CD73 (APC), and PD-L1 (BV787). Cells were also stained with Live-Dead Aqua as an indicator of live cells and analyzed using FlowJo v10.7.1 (**Fig. S5**). Parental cells cultured under hypoxic conditions (1% oxygen) were used as a positive control (A375-Hypoxia).

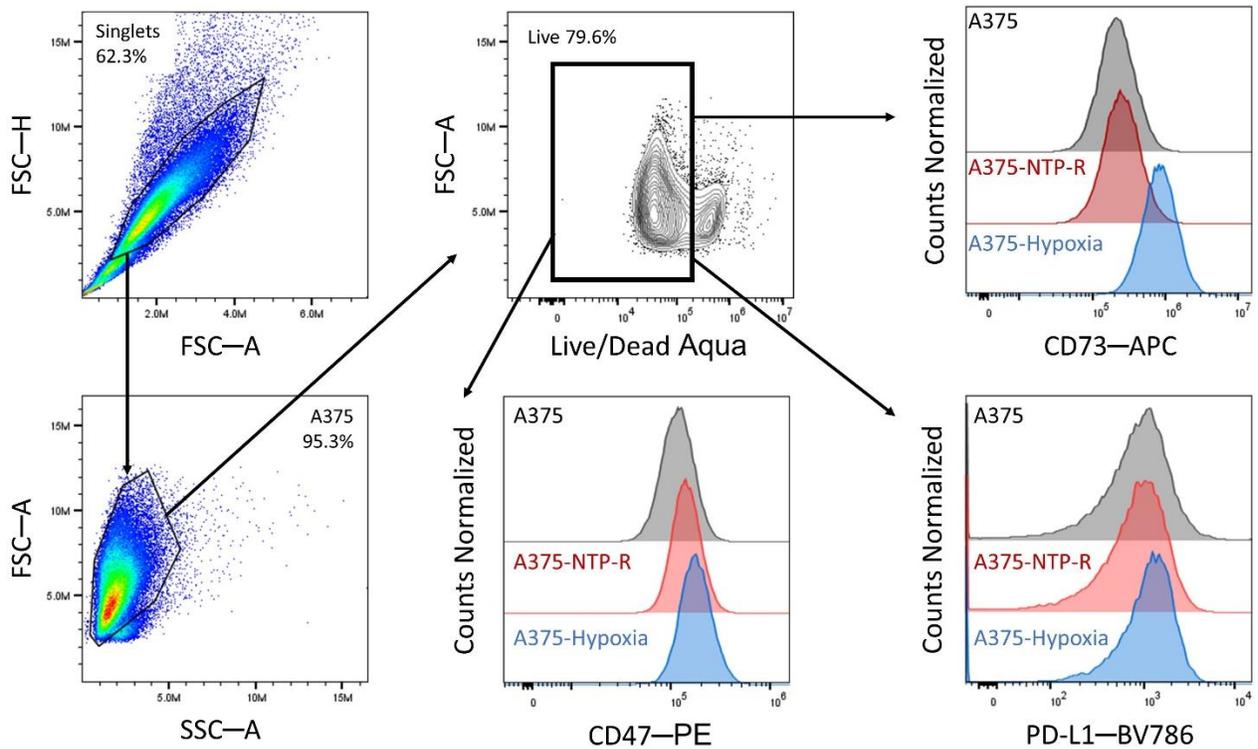


Fig. S5 The gating strategy for measuring cellular expression of CD47, CD73, and PD-L1.

Full List of Differentially Expressed Genes and Functional Terms from Over-Representation Analysis

Differentially Expressed Genes (DEGs) were determined when contrasting NTP-treated (500Hz: 9.5 J) cells with untreated cells (0 J). Upregulated (**Table S1**) and downregulated (**Table S2**) DEGs for both parental (A375) and NTP-resistant (A375-NTP-R) cell lines are shown. Common DEGs between NTP-Sensitive (A375) and NTP-Resistant (A375-NTP-R) DEGs are highlighted in green. The upregulated DEGs were used for over-representation analysis (ORA) using g:GOSt (g:Profiler, Estonia) and genes were mapped to the gene ontology database. The full list of functional terms is provided in **Table S3**.

Table S1 Table of upregulated DEGs between NTP-treated (9.5 J) and untreated cells (0 J) for both NTP-Sensitive (A375) and NTP-Resistant (A375-NTP-R) cells

Upregulated							
A375 (9.5 J vs 0 J)				A375-NTP-R (9.5 J vs 0 J)			
Gene	Gene Title	logFC	q value	Gene	Gene Title	logFC	q value
NFATC2	nuclear factor of activated T cells 2	1.436	0.000143	HMOX1	heme oxygenase 1	1.656	0.000965

CRTC3	CREB regulated transcription coactivator 3	1.386	0.028251	NFATC2	nuclear factor of activated T cells 2	1.232	0.00406
THBS1	thrombospondin 1	1.255	8.04E-05	FST	follistatin	0.960	0.00059
HMOX1	heme oxygenase 1	1.222	0.014152	DIP2B	disco interacting protein 2 homolog B	0.923	0.033994
CDKN1A	cyclin dependent kinase inhibitor 1A	1.048	1.15E-10	GCLM	glutamate-cysteine ligase modifier subunit	0.906	0.000417
CIITA	class II major histocompatibility complex transact...	1.024	0.022464	SHROOM2	shroom family member 2	0.902	0.01558
AREG	amphiregulin	0.973	0.000211	TFAP2C	transcription factor AP-2 gamma	0.896	8.26E-11
PRSS23	serine protease 23	0.903	1.93E-05	IGFBP4	insulin like growth factor binding protein 4	0.892	2.79E-07
ALDH1A3	aldehyde dehydrogenase 1 family member A3	0.889	1.86E-09	MCAT	malonyl-CoA-acyl carrier protein transacylase	0.877	0.045861
GCLM	glutamate-cysteine ligase modifier subunit	0.868	0.000418	MOK	MOK protein kinase	0.867	0.000278
HAPLN1	hyaluronan and proteoglycan link protein 1	0.848	0.020763	CDKN1A	cyclin dependent kinase inhibitor 1A	0.866	2.79E-07
IGFBP4	insulin like growth factor binding protein 4	0.792	4.19E-06	ALDH1A3	aldehyde dehydrogenase 1 family member A3	0.866	1.41E-08
PLAT	plasminogen activator, tissue type	0.786	0.001234	EFNB2	ephrin B2	0.812	0.038034
NQO1	NAD(P)H quinone dehydrogenase 1	0.691	1.77E-10	RNF6	ring finger protein 6	0.807	0.034109
SLC25A44	solute carrier family 25 member 44	0.665	0.007979	AKAP8	A-kinase anchoring protein 8	0.732	0.000176
SLC7A5	solute carrier family 7 member 5	0.582	1.57E-05	MFSD12	major facilitator superfamily domain containing 12	0.728	7.95E-08
TIMP3	TIMP metalloproteinase inhibitor 3	0.552	4.76E-07	SLC5A6	solute carrier family 5 member 6	0.670	0.004177
MDM2	MDM2 proto-oncogene	0.522	0.016693	EHD1	EH domain containing 1	0.648	0.001276
TFAP2C	transcription factor AP-2 gamma	0.502	0.000973	GPT2	glutamic--pyruvic transaminase 2	0.601	0.032759
LMNA	lamin A/C	0.498	0.038947	TUBB4B	tubulin beta 4B class IVb	0.598	0.008865
GPAT3	glycerol-3-phosphate acyltransferase 3	0.469	0.017802	LRRC8A	leucine rich repeat containing 8 VRAC subunit A	0.587	0.039785
TXNRD1	thioredoxin reductase 1	0.441	0.009903	ARL13B	ADP ribosylation factor like GTPase 13B	0.557	0.031981
RPS27L	ribosomal protein S27 like	0.428	0.000607	LSG1	large 60S subunit nuclear export GTPase 1	0.552	0.023182
MFSD12	major facilitator superfamily domain containing 12	0.394	0.031622	SLC25A44	solute carrier family 25 member 44	0.543	0.039785

SRRM2	serine/arginine repetitive matrix 2	0.380	0.014451
PTP4A1	protein tyrosine phosphatase 4A1	0.378	0.04451
UBN1	ubiquitin 1	0.362	0.042446
RBM25	RNA binding motif protein 25	0.362	0.012137

MEPCE	methylphosphate capping enzyme	0.538	0.045861
NOP2	NOP2 nucleolar protein	0.538	0.012302
NQO1	NAD(P)H quinone dehydrogenase 1	0.536	4.67E-07
GPATCH4	G-patch domain containing 4	0.532	1.18E-06
SBF1	SET binding factor 1	0.528	0.040476
MDM2	MDM2 proto-oncogene	0.515	0.011009
USP36	ubiquitin specific peptidase 36	0.510	0.046106
ATP6V0B	ATPase H+ transporting V0 subunit b	0.477	0.008099
ZMPSTE24	zinc metallopeptidase STE24	0.476	0.048174
RBM25	RNA binding motif protein 25	0.475	4.82E-05
ISG20L2	interferon stimulated exonuclease gene 20 like 2	0.471	0.016285
SAAL1	serum amyloid A like 1	0.469	0.04895
TRIAP1	TP53 regulated inhibitor of apoptosis 1	0.467	0.015618
PTP4A1	protein tyrosine phosphatase 4A1	0.465	0.001542
PLK2	polo like kinase 2	0.463	0.038221
COPRS	coordinator of PRMT5 and differentiation stimulat...	0.462	0.008174
TIMP3	TIMP metallopeptidase inhibitor 3	0.456	2.39E-05
GRWD1	glutamate rich WD repeat containing 1	0.455	0.03288
UBAP2L	ubiquitin associated protein 2 like	0.448	0.008865
CLSPN	claspin	0.443	0.005725
ISY1	ISY1 splicing factor homolog	0.442	0.048024
SRSF6	serine and arginine rich splicing factor 6	0.437	0.012421
NCDN	neurochondrin	0.427	0.045861
CDK12	cyclin dependent kinase 12	0.419	0.031981
MEN1	menin 1	0.419	0.042048
YKT6	YKT6 v-SNARE homolog	0.416	0.008865
PRPF38B	pre-mRNA processing factor 38B	0.408	0.00406
PYGB	glycogen phosphorylase B	0.397	0.021492
RPS27L	ribosomal protein S27 like	0.396	0.000802
SREK1	splicing regulatory glutamic acid and lysine rich ...	0.391	0.009489

SRRT	serrate, RNA effector molecule	0.374	0.014408
UFC1	ubiquitin-fold modifier conjugating enzyme 1	0.359	0.042533
ZC3H15	zinc finger CCCH-type containing 15	0.350	0.035909
SLC7A5	solute carrier family 7 member 5	0.347	0.03214
PURA	purine rich element binding protein A	0.344	0.031981
POP7	POP7 homolog, ribonuclease P/MRP subunit	0.340	0.045861
PRPF4B	pre-mRNA processing factor 4B	0.336	0.032513
PNISR	PNN interacting serine and arginine rich protein	0.325	0.038976
GSTP1	glutathione S-transferase pi 1	0.314	0.042772
SRRM2	serine/arginine repetitive matrix 2	0.309	0.045861
CALM1	calmodulin 1	0.299	0.032513
WDR43	WD repeat domain 43	0.292	0.035909
SRSF11	serine and arginine rich splicing factor 11	0.275	0.036558
HSPH1	heat shock protein family H (Hsp110) member 1	0.261	0.04769

Table S2 Table of downregulated DEGs between NTP-treated (9.5 J) and untreated cells (0 J) for both NTP-Sensitive (A375) and NTP-Resistant (A375-NTP-R) cells

Downregulated							
A375 (9.5 J vs 0 J)				A375-NTP-R (9.5 J vs 0 J)			
Gene	Gene Title	logFC	q value	Gene	Gene Title	logFC	q value
LRRC17	leucine rich repeat containing 17	-1.412	0.006228	CA9	carbonic anhydrase 9	-2.854	0.002283
TOX2	TOX high mobility group box family member 2	-1.052	0.000168	NPTX2	neuronal pentraxin 2	-1.867	0.013713
NR2F1	nuclear receptor subfamily 2 group F member 1	-0.941	0.000296	PBXIP1	PBX homeobox interacting protein 1	-1.586	0.004769
TEAD2	TEA domain transcription factor 2	-0.926	0.02274	PNRC1	proline rich nuclear receptor coactivator 1	-1.404	0.039785
FOXP1	forkhead box P1	-0.919	4.85E-06	LRRC17	leucine rich repeat containing 17	-1.358	0.016725
TMEM158	transmembrane protein 158	-0.894	1.77E-10	TANK	TRAF family member associated NFKB activator	-1.335	0.039785
GMDS	GDP-mannose 4,6-dehydratase	-0.872	0.046356	NEGR1	neuronal growth regulator 1	-1.208	0.043286
CITED4	Cbp/p300 interacting transactivator with Glu/Asp r...	-0.827	0.001114	SYT1	synaptotagmin 1	-1.207	0.018979

CAPS	calcyphosine	-0.811	0.023849	TXNIP	thioredoxin interacting protein	-1.166	0.04816
IL13RA1	interleukin 13 receptor subunit alpha 1	-0.806	0.016307	ITM2C	integral membrane protein 2C	-1.142	0.036507
IGFBP5	insulin like growth factor binding protein 5	-0.750	2.16E-06	OSMR	oncostatin M receptor	-1.085	0.025371
PBX3	PBX homeobox 3	-0.725	0.000973	ZNF654	zinc finger protein 654	-1.076	0.038984
RGS2	regulator of G protein signaling 2	-0.719	1.87E-05	SMIM14	small integral membrane protein 14	-1.072	0.032513
MEOX2	mesenchyme homeobox 2	-0.718	0.001114	ANTXR2	ANTXR cell adhesion molecule 2	-1.007	0.039785
SERPINA3	serpin family A member 3	-0.672	0.001114	DERA	deoxyribose-phosphate aldolase	-1.002	0.021091
VGF	VGF nerve growth factor inducible	-0.669	1.87E-05	ID1	inhibitor of DNA binding 1, HLH protein	-0.859	0.001908
BNIP3L	BCL2 interacting protein 3 like	-0.659	0.030385	SEMA3C	semaphorin 3C	-0.828	0.004769
SCD	stearoyl-CoA desaturase	-0.657	5.20E-07	MARCKS	myristoylated alanine rich protein kinase C substr...	-0.816	0.000201
MARCKS	myristoylated alanine rich protein kinase C substr...	-0.655	0.009949	MARCKSL1	MARCKS like 1	-0.774	0.037995
ID1	inhibitor of DNA binding 1, HLH protein	-0.653	0.022229	IGFBP5	insulin like growth factor binding protein 5	-0.769	5.46E-06
ITGA1	integrin subunit alpha 1	-0.650	0.000308	RGS2	regulator of G protein signaling 2	-0.768	2.21E-06
SEMA3C	semaphorin 3C	-0.636	0.049088	VGF	VGF nerve growth factor inducible	-0.762	7.70E-07
RHOBTB3	Rho related BTB domain containing 3	-0.624	0.00261	MEOX2	mesenchyme homeobox 2	-0.754	0.000687
TFPI2	tissue factor pathway inhibitor 2	-0.610	0.010966	NIPSNAP1	nipsnap homolog 1	-0.747	0.021492
CHKB	choline kinase beta	-0.563	0.04451	ANKH	ANKH inorganic pyrophosphate transport regulator	-0.712	0.006657
CXXC5	CXXC finger protein 5	-0.550	0.006129	ERRFI1	ERBB receptor feedback inhibitor 1	-0.687	4.65E-06
ERRFI1	ERBB receptor feedback inhibitor 1	-0.545	0.000218	TOX2	TOX high mobility group box family member 2	-0.680	0.045861
DHRS3	dehydrogenase/reductase 3	-0.535	0.018008	RAD23A	RAD23 homolog A, nucleotide excision repair protei...	-0.675	0.045861
MBTPS1	membrane bound transcription factor peptidase, sit...	-0.452	0.04451	UBXN7	UBX domain protein 7	-0.636	0.036507
NRIP1	nuclear receptor interacting protein 1	-0.429	0.047324	ZNF395	zinc finger protein 395	-0.629	0.008099
PRRX1	paired related homeobox 1	-0.408	0.014451	BNIP3	BCL2 interacting protein 3	-0.629	0.01258
STMN1	stathmin 1	-0.399	0.001114	SCD	stearoyl-CoA desaturase	-0.616	1.31E-05

HSPB1	heat shock protein family B (small) member 1	-0.390	0.001902	HCFC1R1	host cell factor C1 regulator 1	-0.608	0.010963
MT2A	metallothionein 2A	-0.359	0.001104	NDUFB7	NADH:ubiquinone oxidoreductase subunit B7	-0.551	0.038447
PGK1	phosphoglycerate kinase 1	-0.334	0.041725	PRRX1	paired related homeobox 1	-0.550	0.000269
DCBLD2	discoidin, CUB and LCCL domain containing 2	-0.306	0.037462	PJA2	praja ring finger ubiquitin ligase 2	-0.549	0.03214
				TMEM158	transmembrane protein 158	-0.535	0.001276
				NRIP1	nuclear receptor interacting protein 1	-0.534	0.011021
				MTAP	methylthioadenosine phosphorylase	-0.522	0.003933
				KDM1A	lysine demethylase 1A	-0.519	0.031981
				RPL22	ribosomal protein L22	-0.508	0.008865
				ITGA1	integrin subunit alpha 1	-0.492	0.031981
				AHR	aryl hydrocarbon receptor	-0.460	0.018616
				APP	amyloid beta precursor protein	-0.439	0.032759
				PGK1	phosphoglycerate kinase 1	-0.430	0.003933
				IFI16	interferon gamma inducible protein 16	-0.424	0.031981
				BEX3	brain expressed X-linked 3	-0.416	0.018616
				UACA	uveal autoantigen with coiled-coil domains and ank...	-0.381	0.039785
				SPARC	secreted protein acidic and cysteine rich	-0.365	0.030788
				PSIP1	PC4 and SFRS1 interacting protein 1	-0.332	0.040877
				RASSF8	Ras association domain family member 8	-0.321	0.046106

Table S3 Table of functional terms from ORA for both parental and NTP-resistant cells

A375			
Term Name	P_{adj} value	$-\log_{10}P_{adj}$ value	
apoptotic signaling pathway	0.001333184	2.875	
regulation of extrinsic apoptotic signaling pathway	0.003101393	2.508	
regulation of apoptotic signaling pathway	0.012089292	1.918	
negative regulation of extrinsic apoptotic signaling pathway	0.015182055	1.819	
regulation of programmed cell death	0.018047631	1.744	
mitotic G1 DNA damage checkpoint signaling	0.01945328	1.711	
extrinsic apoptotic signaling pathway	0.019542471	1.709	
negative regulation of apoptotic signaling pathway	0.019970429	1.700	

mitotic G1/S transition checkpoint signaling	0.021379602	1.670
apoptotic process	0.024968593	1.603
programmed cell death	0.032606692	1.487
negative regulation of signal transduction	0.039719194	1.401
negative regulation of cellular process	0.03972745	1.401
regulation of cell death	0.0415013	1.382
negative regulation of response to stimulus	0.043118887	1.365

A375-NTP-R

Term Name	P _{adj} value	-log ₁₀ P _{adj} value
mitotic G1 DNA damage checkpoint signaling	8.35E-05	4.079
mitotic G1/S transition checkpoint signaling	9.81E-05	4.008
DNA damage response, signal transduction by p53 class mediator	0.000235717	3.628
negative regulation of cell cycle G1/S phase transition	0.000372062	3.429
mitotic DNA damage checkpoint signaling	0.000429802	3.367
mitotic DNA integrity checkpoint signaling	0.00056746	3.246
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	0.000702244	3.154
signal transduction by p53 class mediator	0.002128727	2.672
signal transduction in response to DNA damage	0.002569939	2.590
DNA damage checkpoint signaling	0.003369029	2.472
DNA integrity checkpoint signaling	0.004921872	2.308
negative regulation of G1/S transition of mitotic cell cycle	0.006033533	2.219
mitotic cell cycle checkpoint signaling	0.006718676	2.173
regulation of cell cycle G1/S phase transition	0.024581875	1.609
negative regulation of cell cycle phase transition	0.029466863	1.531
cell cycle checkpoint signaling	0.030986073	1.509
response to gamma radiation	0.045531173	1.342

Proliferation Rate of NTP-Treated Parental and Resistant Cells

Cell confluence at 24 hours, was normalized to the starting confluence to calculate the proliferation rate for the parental A375 and the NTP-resistant A375-NTP-R cells exposed to low (NTP_L) and high (NTP_H) energy NTP treatment (**Fig. S6**).

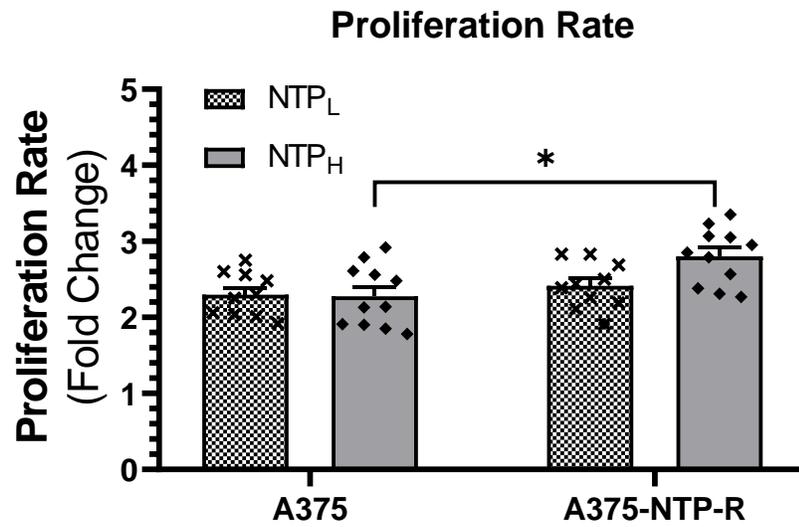


Fig. S6 NTP-resistant cells demonstrated a significantly higher proliferation rate compared to the parental cells following NTP_H-treatment at 24 hours. All biological replicates are shown and data are represented as mean \pm SEM. Statistical significance was calculated using the generalized mixed model and the Tukey's multiple comparisons test.
* $p \leq 0.05$