



SARM1-dependent antimetabolic cancer therapy

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1. Introduction

Great effort has been focused on identifying molecules targeting cancer synthesis of NAD⁺ [1], the latter being a cofactor whose increased neofunction is exploited by neoplasms to boost energetic metabolisms and growth [2]. Various enzymatic inhibitors of NAD⁺ synthesis have been developed as anticancer drugs, but failed in clinical trials because of safety reasons and the redundancy of the biochemical pathways contributing to NAD⁺ synthesis [3,4]. The antitumor potential of extensive collapse of NAD⁺ pool within cancer cells still waits to be determined. Notably, whether NAD⁺-dependent growth advantage of cancer cells can be better targeted by promoting NAD⁺ hydrolysis rather than inhibiting its formation remains unexplored. Among the cellular NAD⁺ hydrolases, sterile alpha and TIR motif-containing protein 1 (SARM1) recently emerged as a key determinant of axonal degeneration [5]. Specifically, while under homeostatic conditions SARM1-dependent NAD⁺ hydrolysis is prevented by NAD⁺ itself bound to the autoregulatory ARM domain [6], upon different types of bioenergetic insults SARM1 activates and prompts collapse of the NAD⁺ pool, metabolic failure and neurodegeneration [7]. Notably, a very recently study identifies a novel mechanism of SARM1 activation by caspase-3-dependent cleavage, occurring during cell death of neurons as well as macrophages and T lymphocytes [8].

2. Results and discussion

This knowledge prompted us to investigate the cytotoxic potential of SARM1 activation in cancer cells. To this end, we developed different SARM1 constructs expressing the wild type protein (S1-wt), an inactive form lacking the NAD⁺-hydrolyzing TIR domain (S1-ΔTIR), or two constitutively active mutants either lacking the ARM domain (S1-ΔARM), or the aa 226-232 fragment (S1-Δ226-232), the latter being a mutation found in amyotrophic lateral sclerosis patients [9]. Although all SARM1 constructs prompted time-dependent transcript and protein accumulation (Fig. 1A–C and S1), intracellular NAD⁺ and NADH depletion occurred only with S1-Δ226-232 and, more robustly, S1-ΔARM (Fig. 1D and E). Importantly, cell autofluorescence, a prototypical index of mitochondrial NADH contents [10], dropped in S1-ΔARM-transfected cells (Fig. 1F), indicating depletion of the organelle dinucleotide pool. Collapse of NAD⁺ contents was accompanied by loss of mitochondrial membrane potential, ATP depletion, intracellular Ca²⁺ accumulation and, eventually, cell death (Fig. 1G–J). The latter was counteracted by NAD⁺ or its metabolic precursors, as well as by the SARM1 enzymatic inhibitor 5-iodo-isoquinoline (Fig. 1K and L).

Episomal plasmid dilution in the rapidly growing cell population may cause underestimation of S1-ΔARM anticancer potential. To circumvent this bias, by means of lentiviral vector transduction we developed cancer cell lines of different origins expressing S1-ΔARM under a TET-ON promoter (TET-S1-ΔARM) together with puromycin

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resistance. We found that all the tumor cell lines, irrespective of their solid or hematological origin, underwent massive cell death after doxycycline exposure (Fig. 2A). The TET-S1- Δ ARM-transduced human melanoma A375 cell line (TET-S1- Δ ARM-A375 cells) was then adopted for further analysis in light of the key relevance of NAD⁺ metabolism in development and growth of melanoma [11]. Exposure of these cells to doxycycline confirmed rapid accumulation of transgene transcripts and protein (Fig. 2B–D), along with progressive NAD⁺ and ATP depletion and cell death (Fig. 2E). Efficient cytoprotection was prompted by S1- Δ ARM inhibitor 5-iodo-isoquinoline (Fig. 2F), but not by NAD⁺ and its metabolic precursors (data not shown). This is probably due to the fact that S1- Δ ARM increases by 1000- and 200.000-fold in transfected and transduced cells (Fig. S1A with Fig. 2D).

Remarkably, monitoring of extracellular acidification rate (ECAR, an indirect index of the glycolytic flux) of TET-S1- Δ ARM-A375 cells showed that as early as 2hrs after the doxycycline challenge glycolysis was impaired in both resting cells and in those undergoing oligomycin-dependent aerobic glycolysis. These findings revealed an exquisite sensitivity of glycolysis to S1- Δ ARM-dependent NAD⁺ shortage. ECAR progressively increased in cells exposed for 18 and 24hrs to doxycycline, a likely index of ongoing cell death (Fig. 2G). We also found that 6hrs after doxycycline exposure TET-S1- Δ ARM-A375 cells showed a time-dependent reduction of oxygen consumption both under basal conditions and upon FCCP-induced OXPHOS decoupling (i.e. maximal respiration) (Fig. 2H). Plotting ECAR against OCR (i.e. glycolytic activity vs oxidative capacity) revealed that glycolysis was fully compromised 4hrs

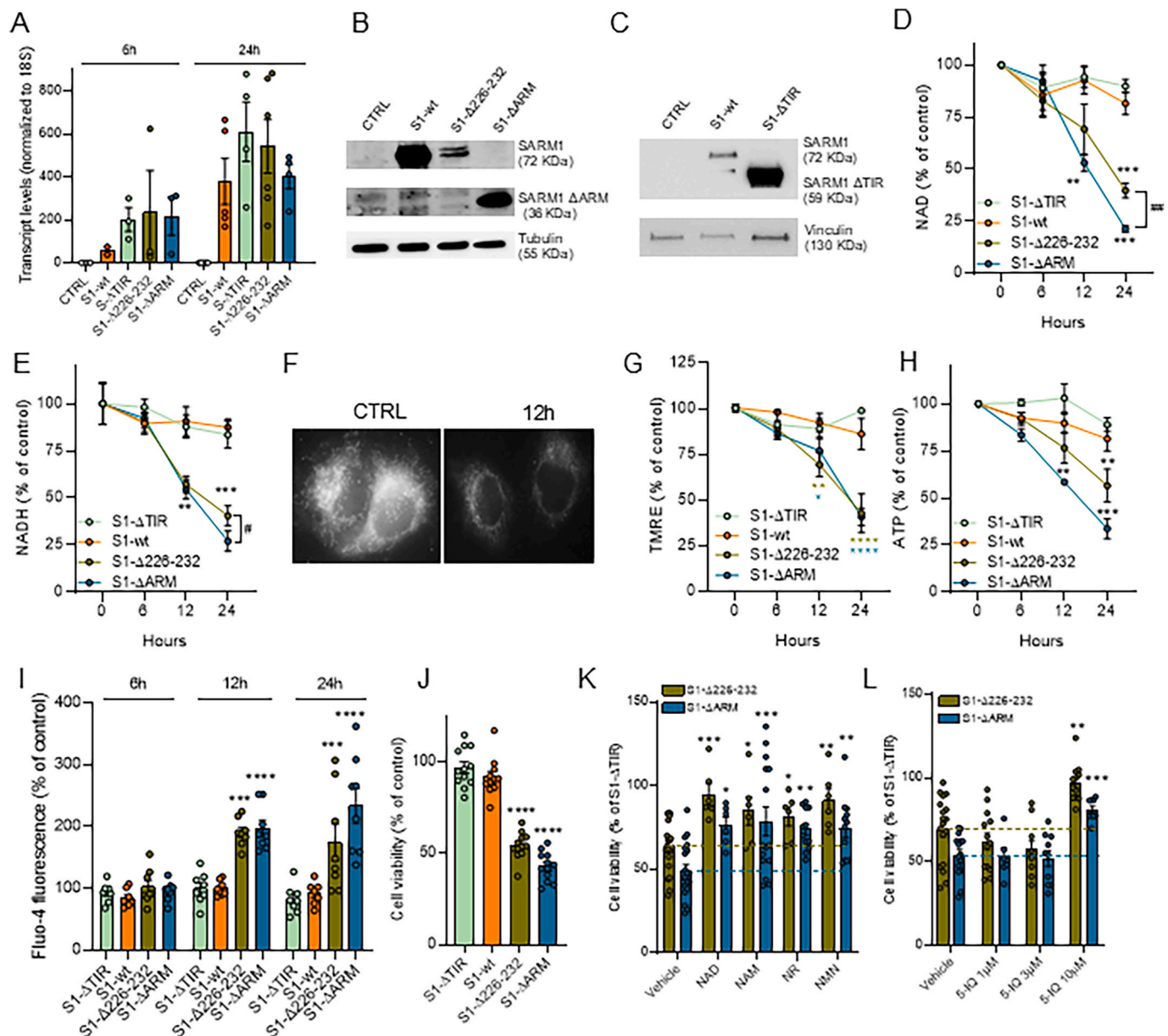
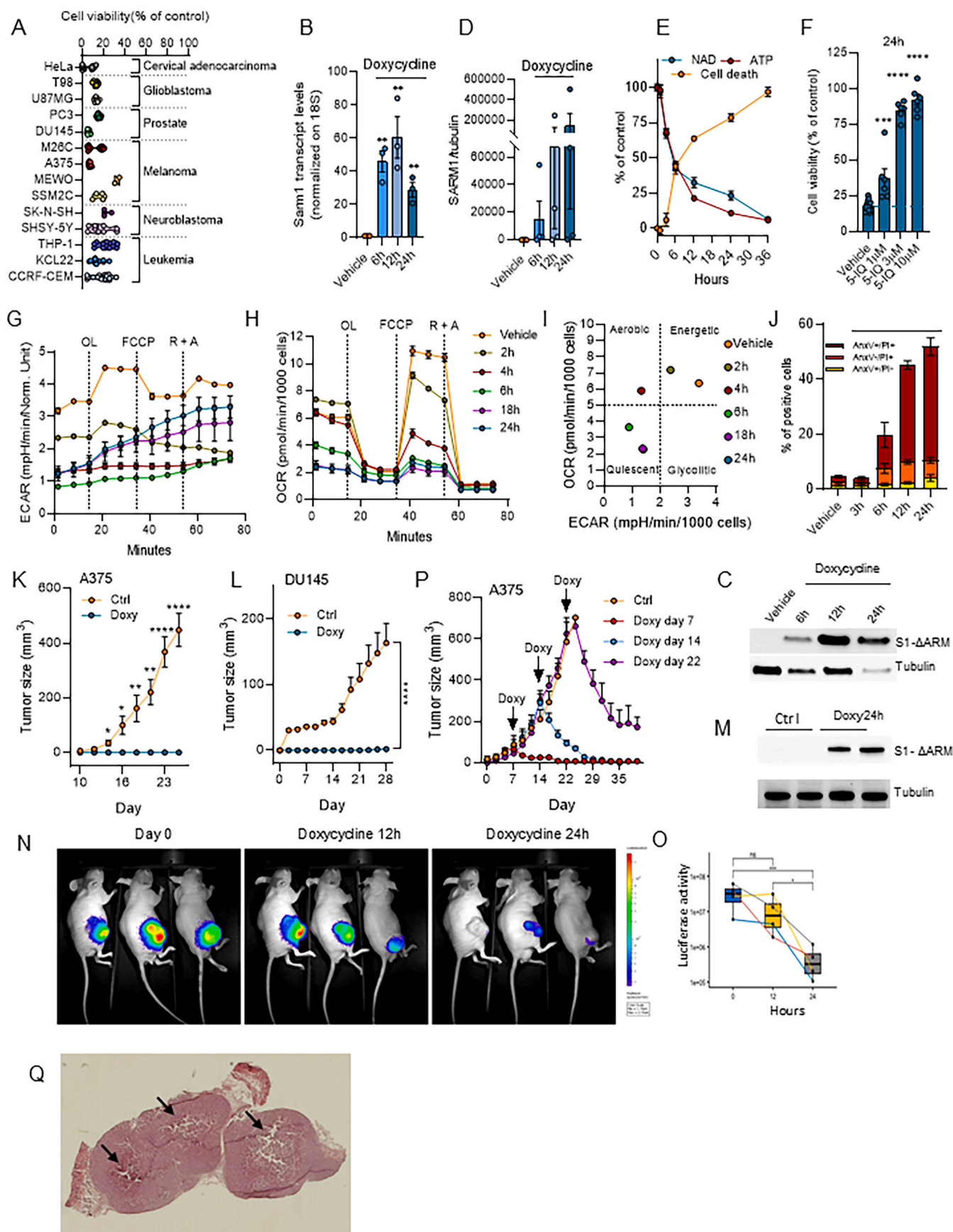


Fig. 1. Effects of SARM1 overexpression on cancer HeLa cell bioenergetics and viability.

(A) Transcript levels of the four SARM1 isoforms at different time points upon transfection. Representative (B and C) levels of the wild type isoform (S1-wt) and those of the S1- Δ ARM, S1- Δ 226-232 and SARM1 Δ TIR variants are shown. Effects of the four SARM1 constructs on NAD (D), NADH (E-F), mitochondrial membrane potential (G) and ATP (H) levels in transfected cells. Intracellular Ca²⁺ accumulation (I) and cell death (J) occurs 12 and 24hrs after transfection, respectively. Cell death is counteracted by NAD⁺ and its precursors nicotinamide (NAM), nicotinamide riboside (NR), nicotinamide mononucleotide (NMN) (K), as well as by the SARM1 inhibitor 5-iodo-isoquinoline (5-IQ) (L). Each point/column is the mean $\pm$ SEM of at least three experiments conducted in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Tukey's post hoc test.



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Fig. 2. Cytotoxic effects of S1-ΔARM transduction. (A) Lentiviral dependent transduction of a TET-inducible S1-ΔARM prompts cellular demise of multiple cancer cell lines 48hrs after doxycycline exposure. Doxycycline added to A375 cells transduced with TET-S1-ΔARM prompts accumulation of S1-ΔARM transcripts (B) and protein (C and D). (E) A375 cells stably expressing the TET-S1-ΔARM construct and exposed to doxycycline undergo NAD and ATP depletion along with progressive loss of cell viability. Effects of the SARM1 inhibitor 5-iodo-isoquinoline (5-IQ) on cell death occurring in TET-S1-ΔARM-transduced A375 cells exposed to doxycycline for 24hrs (F). S1-ΔARM expression reduces basal and oligomycin (OL)-induced extracellular acidification rate (ECAR) (G) as well as basal and protonophore (FCCP)-induced mitochondrial oxygen consumption rate (OCR) (H) in a time dependent manner upon doxycycline exposure. (I) Plotting extracellular acidification rate (ECAR) against oxygen consumption rate (OCR) shows that TET-S1-ΔARM-transduced A375 cells shift from an energetic to an aerobic state within 4hrs after doxycycline exposure, entering an energetically quiescent state at later time points. (J) Quantification of the cytofluorimetric analysis of TET-S1-ΔARM-transduced A375 cells and incubated at different time points after doxycycline exposure with Annexin V (AnxV) and propidium iodide (PI). Xenograft growth in nude mice injected with TET-S1-ΔARM-transduced A375 (K) or TET-S1-ΔARM-transduced DU145 cells (L), exposed or not to doxycycline soon after tumor cell injection. (M) S1-ΔARM levels in xenografts of mice injected with TET-S1-ΔARM-transduced A375 cells and exposed or not to doxycycline. Representative (N) and quantification (O) of bioluminescence in mice bearing tumor xenografts of TET-S1-ΔARM-transduced A375 cells and luciferase, after 12 or 24hrs doxycycline exposure. (P) Mice bearing tumor xenografts of TET-S1-ΔARM-transduced A375 cells have been exposed to doxycycline at different stages of xenograft growth. (Q) Representative tumor section showing necrotic foci upon 72 h doxycycline exposure. Each point/column represents the mean ± SEM of at least three experiments conducted in duplicate. In (K), (L) and (P) 19, 10 and 5 mice per group have been used, respectively. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, one-way ANOVA with Tukey's post hoc test. In (K) and (L) Mann–Whitney test (according to Kolmogorov–Smirnov test for normality and Bartlett's test for variance equality) was used. In (O) one-way ANOVA test, followed by pairwise T-Test with Benjamini–Hochberg (BH) correction was conducted.

after S1-ΔARM induction, and cells entered an exclusively aerobic state. At later time points, the progressive impairment of OXPHOS shifted the cells into a bioenergetically quiescent state (Fig. 2I). In keeping with the rapid and severe energy failure prompted by S1-ΔARM, FACS analysis of propidium iodide and annexin V-positive cells showed that S1-ΔARM mostly triggered necrotic cell death, with a negligible component of apoptosis (Fig. 2J).

We adopted tumor xenograft models to investigate the ability of S1-ΔARM to counteract tumor growth. We found that addition of doxycycline in drinking water of mice soon after transplantation of TET-S1-ΔARM-A375 melanoma (Fig. 2K) or DU145 prostate carcinoma (Fig. 2L) cells prevented tumor development. By adopting a post-treatment protocol, we found that 24hrs after addition of doxycycline to drinking water, S1-ΔARM accumulated in neoplasms of mice bearing TET-S1-ΔARM-A375 tumor xenografts (Fig. 2M). Strikingly, bioluminescence imaging showed that A375 melanoma xenografts transduced with a TET-S1-ΔARM construct also expressing luciferase underwent time-dependent, massive loss of ATP contents (Fig. 2N and O). Accordingly, exposure of mice bearing TET-S1-ΔARM-A375 xenografts to doxycycline at different stages of xenograft growth invariably prompted robust necrotic tumor regression (Fig. 2P and Q), without evidence of animal distress or malaise.

Our findings reveal that the metabolic machinery synthesizing NAD⁺ within the subcellular compartments of cancer cells along with its mitochondrial-NAD⁺ rheostat [12] cannot keep pace with the NAD⁺ hydrolytic rate of S1-ΔARM. The present study, therefore, suggests that strategies aimed at increasing cancer NAD⁺-hydrolysis tackle the redundancy of the metabolic routes of NAD⁺ formation and their ability to sustain tumor growth. Still, cancer cell type specific rewiring of NAD homeostasis upon S1-ΔARM transduction can differently affect this antitumor strategy. Results are consistent with our previous observations linking NAD⁺ metabolism, Vacor-dependent SARM1 activation, and antitumor effects [13,14]. In the future, it will be relevant to overcome the safety issues of transducing S1-ΔARM driven by tumor-specific gene promoters leaving unaffected metabolic NAD homeostasis in healthy tissues. It will also be instrumental to evaluate the possible synergism with strategies overexpressing additional NAD-ases such as CD38, as well as vulnerability of non-transduced cells.

3. Materials and methods

We evaluated NAD homeostasis in cancer cell lines transfected with plasmids expressing active SARM1 mutants. A lentiviral vector expressing TET-ON S1-ΔARM was developed to evaluate the effects of NAD depletion during tumor growth (SI Appendix).

CRediT authorship contribution statement

Alice Molli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Daniela Buonvicino:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration. **Andrea Lapucci:** Data curation, Formal analysis, Investigation, Methodology. **Luisa Maresca:** Formal analysis, Methodology. **Jose Maria Lanza-Arnaiz:** Formal analysis, Investigation, Methodology. **Simone Polvani:** Data curation, Formal analysis, Investigation. **Francesca Cialdai:** Data curation, Formal analysis, Methodology. **Giacomo Inero:** Data curation, Formal analysis, Methodology. **Tommaso Mello:** Data curation, Formal analysis, Methodology. **Barbara Stecca:** Data curation, Formal analysis, Investigation. **Laura Formentini:** Data curation, Formal analysis, Investigation, Methodology. **Alberto Chiarugi:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft.

Declaration of competing interest

The authors Alice Molli, Daniela Buonvicino, Andrea Lapucci, Luisa Maresca, Jose Maria Lanza-Arnaiz, Simone Polvani, Francesca Cialdai, Giacomo Inero, Tommaso Mello, Barbara Stecca, Laura Formentini, and Alberto Chiarugi of the manuscript entitled “SARM1-Dependent Anti-metabolic Cancer Gene Therapy” declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2026.218762>.

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