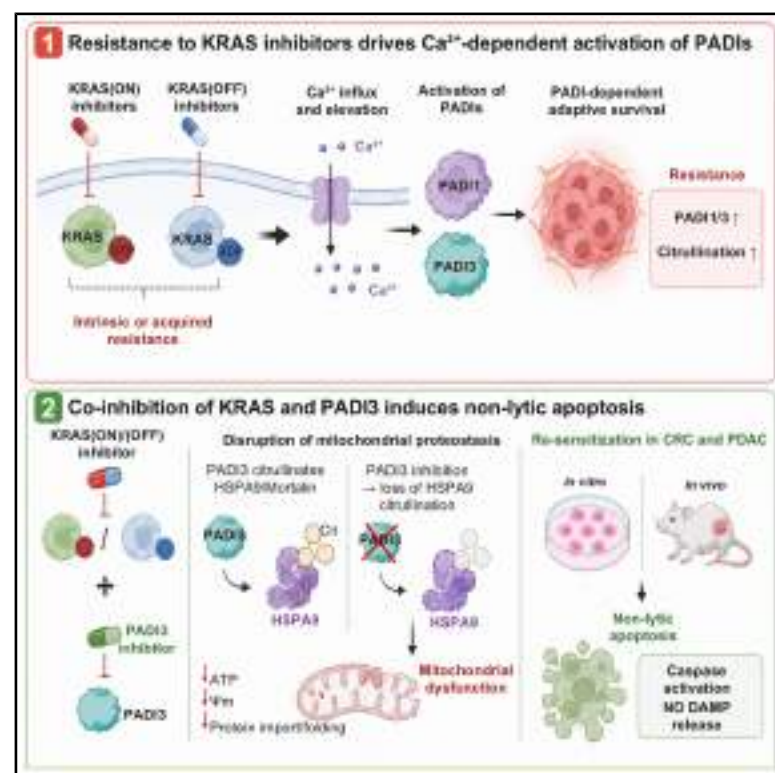


PADI-dependent mitochondrial adaptation drives resistance to KRAS inhibitors

Graphical abstract



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In brief

KRAS-mutant pancreatic and colorectal cancers frequently develop resistance to KRAS(ON) and KRAS(OFF) inhibitors. Here, Gani et al. identify a PADI-dependent adaptive circuit that promotes therapeutic escape through HSPA9/Mortalin citrullination. Pharmacological PADI inhibition restores KRAS inhibitor sensitivity and induces non-lytic apoptosis, revealing a therapeutic vulnerability in KRAS-driven tumors.

Highlights

- KRAS inhibitors resistance engages a Ca^{2+} -dependent PADI adaptive survival program
- PADI1/3 inhibition potentiates KRAS(ON) and KRAS(OFF) therapies and restores sensitivity
- PADI3 citrullinates HSPA9/Mortalin to sustain mitochondrial proteostasis
- Combined KRAS/PADI inhibition triggers mitochondrial dysfunction and non-lytic apoptosis



Article

PADI-dependent mitochondrial adaptation drives resistance to KRAS inhibitors

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<https://doi.org/10.1016/j.celrep.2026.118016>

SUMMARY

KRAS(ON) and KRAS(OFF) inhibitors have improved the treatment of KRAS-driven tumors, yet resistance remains a major challenge. Here, we identify PADI1 and PADI3 as negative prognostic markers in KRAS-mutant colorectal and pancreatic cancers. KRAS-driven metabolic rewiring sustains their expression through an enhancer within the PADI1 locus. Although KRAS inhibition suppresses PADI1/3 expression in sensitive cells, resistant models maintain elevated PADI1/3 expression and accumulate intracellular calcium, sustaining PADI-dependent adaptive survival. Pharmacological inhibition of PADIs synergizes with KRAS(ON) and KRAS(OFF) inhibitors in two- and three-dimensional cancer models, restores sensitivity in resistant cells, and enhances antitumor activity *in vivo*. Integrated transcriptomic and proteomic analyses identify HSPA9/Mortalin as a critical citrullinated effector. PADI3-mediated citrullination enhances Mortalin ATPase activity and ATP/ADP cycling, whereas loss of citrullination correlates with apoptosis. Disruption of this adaptive circuitry triggers mitochondrial dysfunction, caspase activation, and non-lytic apoptosis without detectable DAMP release, revealing a therapeutic vulnerability of KRAS-driven tumors.

INTRODUCTION

KRAS (Kirsten rat sarcoma viral oncogene homolog) is a member of the Ras family of small GTPases, which also includes HRAS and NRAS.^{1,2} These proteins act as molecular switches that regulate essential cellular processes such as growth, differentiation, and survival.^{1,2} Among them, KRAS stands out as the most frequently mutated oncogene in human cancers, representing a critical driver of tumorigenesis.² Activating KRAS mutations, occurring mainly at codons 12 (G12), 13 (G13), and 61 (Q61), make KRAS insensitive to GTPase-activating proteins (GAPs) and are a hallmark of several aggressive malignancies, including pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), and lung adenocarcinoma (LUAD).^{2,3} These mutations lead to constitutive activation of KRAS signaling via the MAPK and PI3K/AKT pathways, promoting uncontrolled cell proliferation and survival.^{1–3}

Recent breakthroughs in the development of KRAS-specific inhibitors (KRASi)—classified as KRAS(ON) and KRAS(OFF)

agents—have opened new therapeutic avenues by exploiting synthetic lethality and selectively targeting KRAS-mutant cancers.⁴ The discovery of a cryptic pocket (switch-II pocket) in KRAS^{G12C} gave rise to the development of first allele-specific KRAS(OFF) inhibitors.⁵ KRAS(OFF) inhibitors are allosteric effectors of KRAS^{G12C} which covalently bind the mutant cysteine residue and subvert the native nucleotide preference to favor GDP over GTP locking the protein in an inactive state.⁶ Currently, AMG510 (sotorasib) and MRTX849 (adagrasib) are the only two clinically approved KRAS^{G12C} inhibitors.⁶ Differently from G12C, the weak nucleophilicity of the carboxyl group of the aspartic acid residue of G12D mutation⁷ has made the development of covalent KRAS^{G12D}(OFF) inhibitors challenging.⁸ Based on the binding to switch pockets I and II observed with covalent KRAS^{G12C} inhibitors, non-covalent inhibitors targeting KRAS^{G12D} have been designed,⁹ many of which are currently in clinical trials (MRTX1133 and derivatives).⁶

KRAS(ON) inhibitors act through the opposite mechanism by binding KRAS in its GTP-bound state and locking it in an



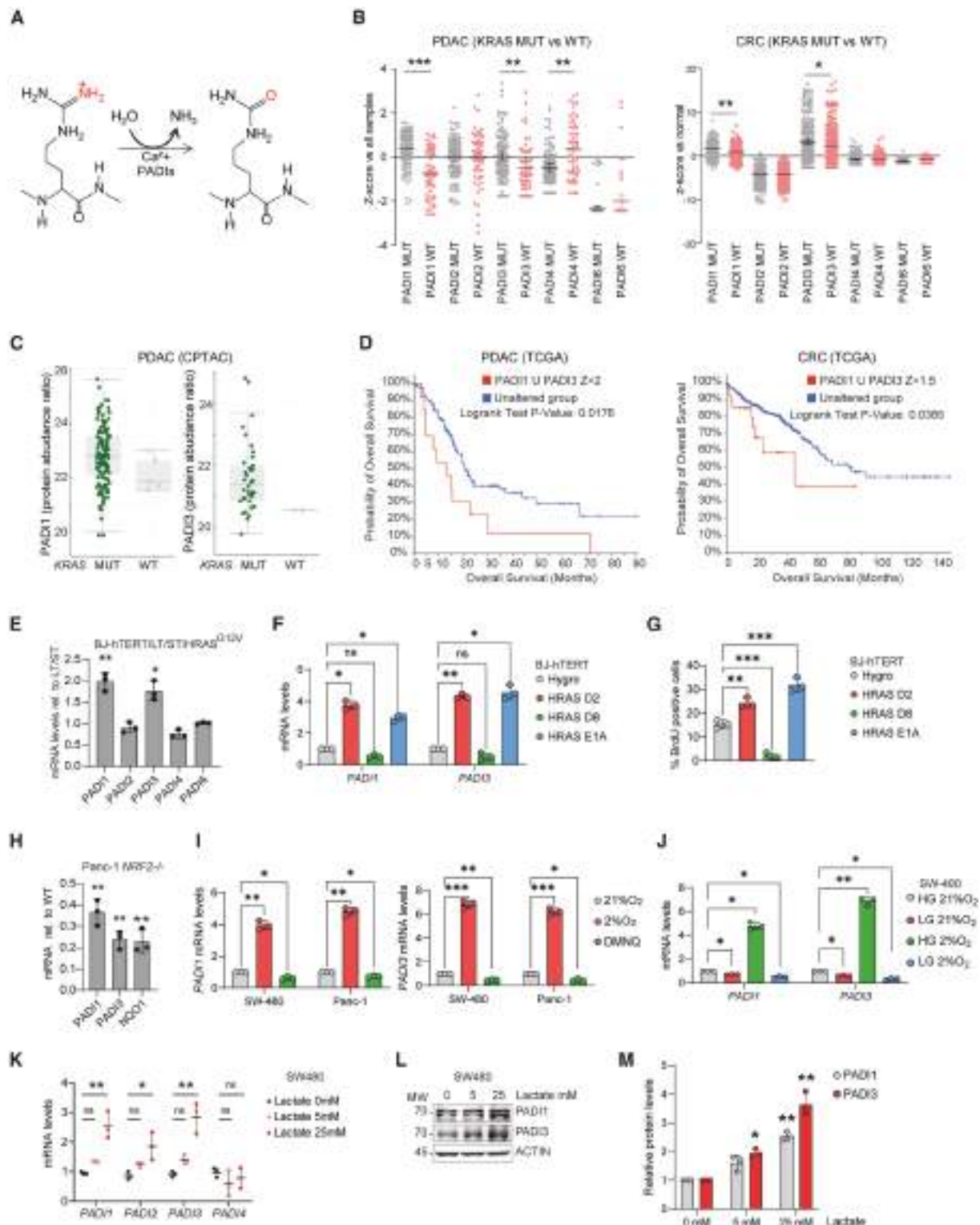


Figure 1. The expression of PADI1 and PADI3 is regulated by a complex metabolic network downstream of RAS signaling
(A) PAD-mediated deimination of peptidyl-arginine to peptidyl-citrulline at physiological pH, resulting in conversion of the guanidinium group into a ureido group and elimination of a positive charge.
(B) Relative expression levels of PADI1–6 in PDAC (wild-type, $n = 58$; KRAS gain-of-function, $n = 110$) and CRC (wild-type, $n = 307$; KRAS gain-of-function, $n = 217$). Data were obtained from the TCGA consortium through cBioPortal (RRID: SCR_014555).

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active conformation. RMC-4998, the first KRAS(ON) inhibitor, was designed toward KRAS^{G12C} and led to the formation of a CYPA:drug:KRAS^{G12C} tricomplex which inactivated KRAS signaling.^{10,11} Revolution Medicines has recently developed zoldonrasib (RMC-9805),¹² a KRAS^{G12D}(ON)-selective inhibitor that functions in a manner analogous to RMC-4998, and daraxonrasib (RMC-6236),¹³ a pan-RAS(ON) inhibitor that has demonstrated superior efficacy compared with chemotherapy as a single agent in patients with metastatic PDAC.¹⁴ Alongside this line of research, small-molecule pan-KRAS¹¹ inhibitors (BI-2865, BI-2852, BI 3706674) and noncovalent allele-specific KRAS degraders⁶ have emerged, leveraging proteolysis-targeting chimeras (PROTAC)-based technology.^{15,16}

Early clinical trials such as CodeBreak 100¹⁷ and KRYSTAL-1¹⁸ revealed the existence of primary,¹⁹ acquired^{20–22} or adaptive²³ resistance mechanisms in treated patients, which significantly limit the therapeutic efficacy of allele-specific KRAS inhibitors as monotherapies.^{1,6} Various combination therapies are being investigated to overcome resistance mechanisms to KRAS-targeted treatments, targeting downstream or convergent signaling pathways^{20–22} or boosting anti-cancer immunity.²⁴ Moreover, the combination of KRAS(OFF) and KRAS(ON) inhibitors proved effective in sotorasib-resistant patient-derived orthotopic xenografts characterized by elevated levels of active KRAS-GTP.²⁵

Protein arginine deiminases (PADI1–4 and PADI6) catalyze the calcium-dependent citrullination of proteins, which consists in the post-translational conversion of the positively charged guanidinium group of peptidyl-arginine into the neutral ureido group of peptidyl-citrulline²⁶ (Figure 1A). Protein citrullination has been shown to regulate several physiological and pathological states, including embryogenesis,²⁸ pluripotency,²⁹ tissue regeneration,³⁰ cell death,³¹ immune response modulation,³² neutrophil extracellular trap (NET) formation,³³ autoimmunity³⁴ and neurodegeneration.³⁵ However, apart from several extracellular proteins and few intracellular proteins such as histones (primarily citrullinated by PADI4 and PADI2) and RNA-binding proteins which were identified in various studies, there is still no consensus on defining the citrullinome.^{26,36} Despite these hurdles, PADIs and aberrant citrullination have been implicated in driving metabolic reprogramming, metastatic progression, and therapy resistance

across multiple cancer types.^{37–40} PADI1 and PADI3 act as metabolic and transcriptional modulators, controlling EMT-driven metastasis and therapy resistance.^{40–42} PADI2 and PADI4 play multifaceted roles in cancer progression, modulating cancer cell autonomous effects but also immune evasion.^{43–46} PADI4 was reported to citrullinate key regulators of cancer cell fitness such as p53 (enhancing tumor-suppressive gene binding)⁴⁷ and HIF-1 α (stabilizing hypoxia signaling),³⁹ and to modify chromatin to enable extracellular DNA release (CECNs,⁴⁸ TuNEPs^{31,49}), which promotes metastasis via RAGE/ERK activation.³¹ Emerging evidence indicates that protein citrullination contributes to mitochondrial fitness by regulating proteins involved in mitochondrial dynamics⁵⁰ and metabolic adaptation.⁵¹ However, whether PADI-dependent citrullination directly controls the mitochondrial proteostasis machinery and thereby influences the response to targeted therapies remains unknown.

Here, we identify PADI1 and PADI3 as metabolic effectors of adaptive resistance to KRAS-targeted therapies and demonstrate that calcium-dependent PADI activation promotes mitochondrial proteostasis through citrullination of HSPA9/Mortalin, thereby sustaining survival of KRAS-inhibited cancer cells.

RESULTS

The expression of PADI1 and PADI3 is governed by a complex metabolic regulatory network downstream of RAS signaling

First, we analyzed transcriptomic data from the TCGA PanCancer Atlas⁵² focusing on the two carcinoma types with the highest prevalence of KRAS mutations: PDAC and CRC. Patients were stratified based on KRAS mutation status (wild-type, WT vs. gain-of-function mutants, MUT), and the expression levels of PADI1–6 were examined. Analysis of TCGA PDAC and CRC cohorts revealed a significant association between activating KRAS mutations and increased PADI1/PADI3 expression (Figure 1B). This relationship was markedly stronger in PDAC (PADI1: $\rho = 0.52$, $p = 2.0 \times 10^{-7}$; PADI3: $\rho = 0.35$, $p = 2.0 \times 10^{-4}$) than in CRC (PADI1: $\rho = 0.20$, $p = 0.005$; PADI3: $\rho = 0.24$, $p = 9.0 \times 10^{-4}$), consistent with the dominant role of oncogenic KRAS in PDAC biology. This tendency was also reflected at the protein level. Reanalysis of data from the first PDAC proteogenomic study

(C) Protein levels of PADI1 and PADI3 in PDAC patient samples stratified by KRAS status (wild-type versus missense gain-of-function mutations). Data were obtained from the CPTAC consortium.²⁷

(D) Overall survival in patients with PDAC and CRC with increased expression (Z score) of *PADI1* or *PADI3* ($n = 14/177$ and $29/592$, respectively). Data were obtained from the TCGA consortium through cBioPortal (RRID: SCR_014555).

(E) mRNA expression levels in BJ-hTERT cells expressing LT + ST and *HRAS*^{G12V} compared to LT + ST alone (set to 1). Statistical analysis refers to this binary comparison for each gene using a paired *t* test.

(F) mRNA expression levels of *PADI1* and *PADI3* in BJ-hTERT cells expressing Hygro resistance (control), RAS+E1A, or *HRAS*^{G12V} for 2 (D2) or 8 (D8) days.

(G) Percentage of the indicated BJ-hTERT cells entering S-phase during a 3 h BrdU pulse.

(H) mRNA expression levels of the indicated genes in PANC-1 *NRF2*^{-/-} cells compared to wild-type cells (set to 1). Statistical significance was assessed using a paired *t* test for binary comparison.

(I) mRNA expression levels of the indicated genes in SW-480 and PANC-1 cells incubated for 30 h in 21% O₂ or 2% O₂, or treated under normoxia for 8 h with 20 μ M DMNQ.

(J) mRNA expression levels of the indicated genes in SW-480 cells incubated for 30 h in 21% O₂ or 2% O₂ with high glucose (HG, 4.5 g/L) or low glucose (LG, 1 g/L).

(K–M) mRNA (K) or protein (L and M) levels of PADI1/3 in SW480 cells treated under normoxia for 24 h with the indicated concentrations of lactate. Paired *t* test on the binary comparisons indicated (K) or with respect to the untreated condition (M). In C–M, data are presented as mean $\pm$ SD ($n = 3$). In (F, G, I, and J) Dunn's multiple comparison test was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

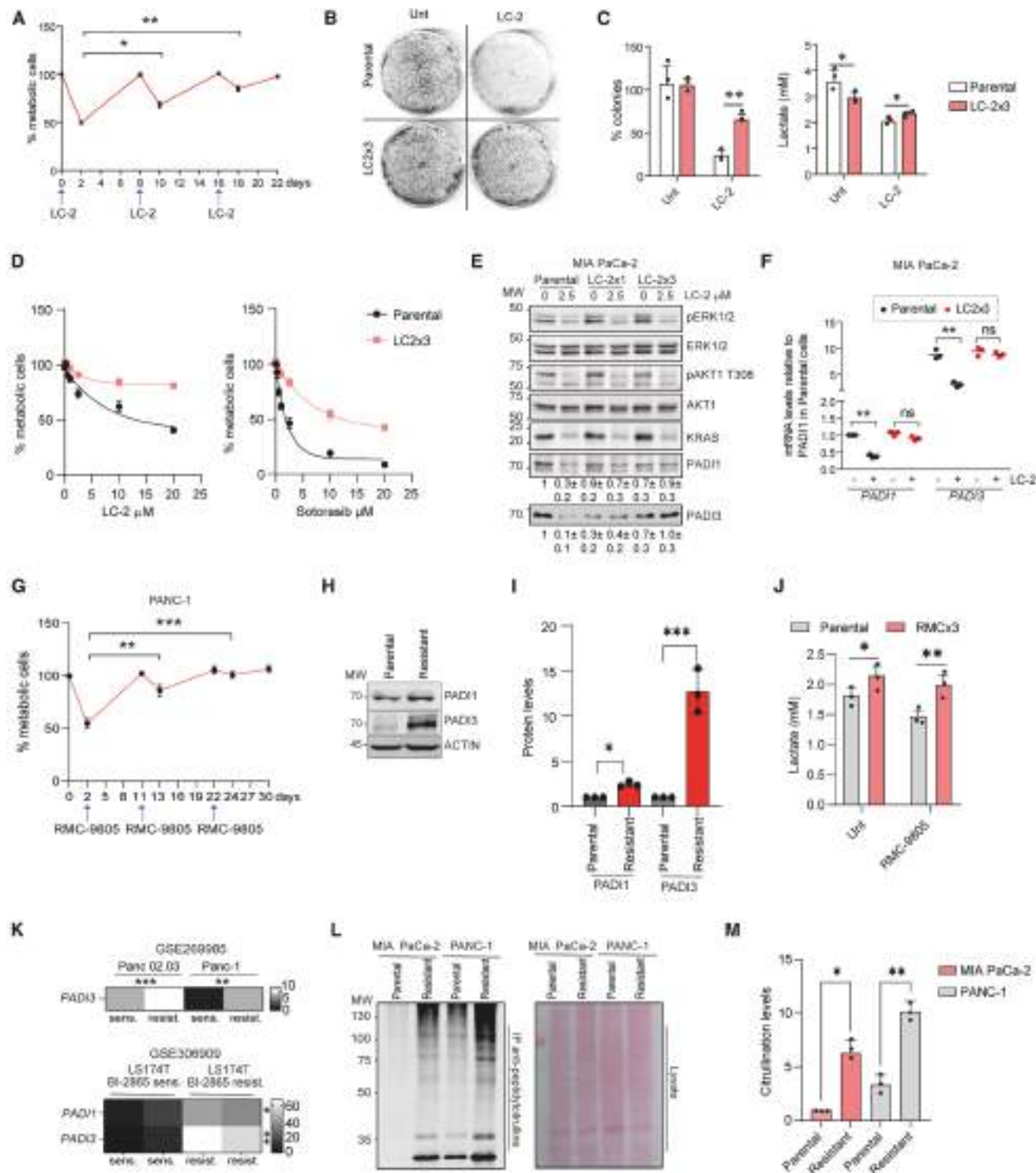


Figure 2. Acquired resistance to KRAS inhibition is associated with enhanced PADI1 and PADI3 expression and protein citrullination
(A) MIA PaCa-2 cells were treated three times with 2.5 μ M LC-2 for 48 h, followed by 6 days of recovery. Metabolic activity was assessed using a resazurin reduction assay.
(B) Clonogenic assay in MIA PaCa-2 cells (parental or treated three times with LC-2, referred to as LC-2x3) following the treatment schedule illustrated in Figure 2A. Cells were treated with 10.0 μ M LC-2 for 30 h and then allowed to grow for 7 days.
(C) Quantification of clonogenic assay and extracellular lactate levels in the indicated cells.

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indicates that PADI3 and PADI1 protein levels were on average higher in patients with KRAS mutations, although the analysis is constrained by the limited sample size (Figure 1C). Moreover, higher mRNA expression levels of PADI1 and PADI3 were associated with poor prognosis in patients with PDAC and CRC (Figure 1D), confirming previous evidence.^{53,54} The correlation between KRAS and PADI1/PADI3 expression was not exclusive to KRAS, as HRAS mutations also showed a positive association with PADI1 and PADI3 expression. Specifically, overexpression of *HRAS*^{G12V} in BJ-hTERT/LT/ST cells⁵⁵ induced PADI1 and PADI3 expression, but not that of other PADI family members (Figure 1E). Notably, *HRAS*^{G12V} expression in BJ-hTERT cells initially triggered a proliferative burst (day 2), followed by premature cell-cycle arrest and senescence induction by day 8 (Figures 1F and 1G). The increase in PADI1 and PADI3 mRNA levels was observed only during the proliferative phase, but not in senescent cells, where a feedback mechanism suppresses MAPK signaling^{56,57} (Figures 1F and 1G). Interestingly, bypassing senescence through co-expression of E1A restored the expression of *PADI1* and *PADI3* (Figure 1F). Furthermore, we recently demonstrated that NRF2 knockout in *KRAS*^{G12D}-mutant PDAC cells (PANC-1) leads to KRAS pathway suppression.⁵⁸ RNA-seq analysis revealed that *PADI1* and *PADI3* were among the most significantly downregulated genes following NRF2 knockout,⁵⁸ a finding confirmed by qRT-PCR (Figure 1H).

Overall, these findings demonstrate that PADI expression is under the control of the HRAS/KRAS-MAPK signaling pathway. Previous studies reported that PADI1 and PADI3 expression is modulated by hypoxia.⁵¹ Consistent with this, incubation of KRAS-mutant CRC and PDAC cells (SW-480: *KRAS*^{G12V;wt}; PANC-1: *KRAS*^{G12D;wt}) under hypoxic conditions led to an upregulation of *PADI1* and *PADI3* transcripts (Figure 1I). However, HIF-1 α is not a direct inducer of *PADI1* and *PADI3* expression, as hypoxic incubation in low-glucose medium (LG: 1 g/L) failed to increase the expression of either gene (Figure 1J). Moreover, stabilization of HIF-1 α achieved by exposing normoxic cells to cobalt chloride⁵⁹ did not lead to any increase in either PADI1 or PADI3 mRNAs (Figure S1A) or protein levels (Figure S1B), in contrast to the upregulation observed under hypoxic conditions (Figure S1B). This suggested that PADI1 and PADI3 regulation is mediated by metabolic reprogramming rather than hypoxia/HIF-1 α per se. Supporting this hypothesis, exposure to increasing concentrations of lactate led to a significant dose-dependent upregulation of PADI1 and PADI3 mRNA levels (Figure 1K) and pro-

tein expression (Figures 1L and 1M), with a partial effect on PADI2 (Figure 1K). Taken together, these findings identify HRAS/KRAS-MAPK signaling, hypoxia, and lactate as regulators of PADI1 and PADI3 expression. Given the established role of oncogenic RAS in driving aerobic glycolysis and Warburg-like metabolic reprogramming,⁶⁰ our data suggest that PADI1 and PADI3 expression is linked to the broader metabolic state imposed by RAS activation, rather than to hypoxia per se.

Acquired resistance to KRAS inhibition is associated with enhanced PADI1 and PADI3 expression and protein citrullination

Given that PADI expression is regulated downstream of RAS signaling, we investigated whether acquired resistance to KRAS-targeted therapies is associated with altered PADI expression. To this end, MIA PaCa-2 cells harboring biallelic *KRAS*^{G12C} mutations were treated with LC-2, a *KRAS*^{G12C}-selective PROTAC derived from the adagrasib binding scaffold and designed to induce mutant KRAS degradation (Figure 2A). Repeated exposure to LC-2 at 2.5 μ M, previously identified as the optimal concentration for efficient *KRAS*^{G12C} degradation,¹⁶ led to resistance after the third treatment cycle. Post-treatment, the surviving cell population exhibited complete refractoriness to LC-2 (Figures 2A–2C) and underwent partial metabolic reprogramming, as evidenced by increased lactate secretion following LC-2 treatment (Figure 2C).

Notably, LC-2-resistant cells (LC-2x3; three treatment cycles) displayed cross-resistance not only to LC-2 but also to the *KRAS*^{G12C}(OFF) inhibitor sotorasib¹² (Figure 2D). Moreover, repeated LC-2 exposure did not impair target engagement, as KRAS degradation and suppression of ERK1/2 and PI3K/AKT signaling remained comparable between LC-2x3 cells, parental cells, and cells exposed to a single treatment cycle (LC-2x1) (Figure 2E). Importantly, whereas LC-2 treatment in parental cells resulted in a marked reduction of PADI1 and PADI3 mRNA and protein expression, this response was substantially attenuated in LC-2x3 cells, which maintained significantly higher levels of both PADI paralogues than their treatment-sensitive counterparts (Figures 2E and 2F).

To determine whether this phenomenon extended beyond *KRAS*^{G12C}-targeted degradation, we generated an independent resistance model by repeatedly treating *KRAS*^{G12D}-mutant PANC-1 cells with the *KRAS*^{G12D}-selective inhibitor RMC-9805.¹² Although resistant populations emerged with

(D) Parental and LC2x3-resistant MIA PaCa-2 cells were treated with the indicated concentrations of LC-2 or sotorasib. Polynomial regression curves are shown to illustrate the dose-response relationship.

(E) Immunoblot analysis of the indicated proteins in MIA PaCa-2 cells (parental or treated once or three times with LC-2) after 24 h of treatment. We labeled ERK1/2 as pERK1/2 when phosphorylated at Thr202/Tyr204. Densitometric quantification of PADI1 and PADI3 protein levels is provided.

(F) mRNA levels of *PADI1* and *PADI3* in MIA PaCa-2 cells treated with 2.5 μ M LC-2 for 24 h. Data are expressed as absolute mRNA levels relative to PADI1.

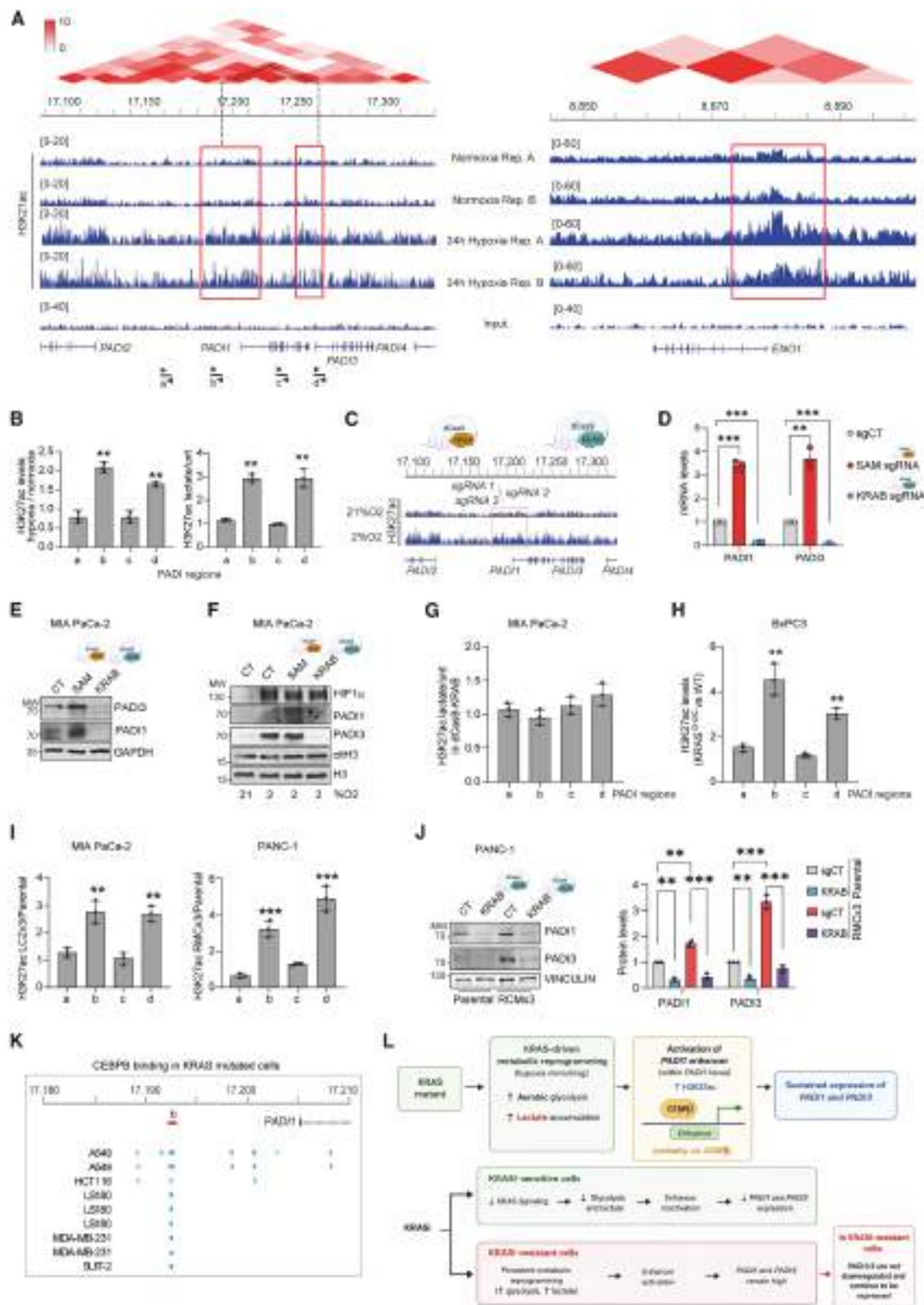
(G) PANC-1 cells were treated three times with 2.0 μ M RMC-9805 for 36 h, followed by 9 days of recovery. Metabolic activity was assessed using a resazurin reduction assay.

(H and I) Immunoblot analysis and quantification of PADI1 and PADI3 protein levels in parental and resistant (RMCx3) PANC-1 cells treated for 36 h with 2.0 μ M RMC-9805.

(J) Quantification of extracellular lactate levels in the indicated cells, treated or not for 36 h with 2.0 μ M RMC-9805.

(K) mRNA levels (TPM) of PADI3 in the indicated parental and MRTX1133-resistant PDAC cells (GSE269985) and of PADI1 and PADI3 in the indicated parental and BI-2865 resistant CRC cells (GSE306909). Statistical significance was assessed using a paired *t* test.

(L and M) Global peptidyl-citrulline levels in the indicated cell lines after treatment with 2.5 μ M LC-2 (MIA PaCa-2) or 2.0 μ M RMC-9805 (PANC-1) for 16 h. In (A, C, D, F, G, I, J, and M), mean $\pm$ SD are shown (*n* = 3). Pairwise *t* test was used. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.



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slower recovery kinetics than those observed in MIA PaCa-2 cells, repeated treatment similarly led to cells refractory to KRAS inhibition. Consistent with the LC-2-resistant model, resistant PANC-1 cells exhibited increased expression of both PADI1 and, more prominently, PADI3 following treatment (Figures 2H and 2I), accompanied by elevated lactate secretion into the culture medium (Figure 2J). Supporting these findings, reanalysis of published RNA-seq datasets⁶¹ revealed increased PADI expression in independent models of acquired resistance, with PADI3 upregulated in PDAC cells resistant to the KRAS^{G12D} inhibitor MRTX1133 and both PADI1 and PADI3 upregulated in CRC cells resistant to the pan-KRAS inhibitor BI-2865 (Figure 2K).

Importantly, the increased expression of PADI1 and, particularly, PADI3 observed in both resistant models (MIA PaCa-2 LC-2x3 and PANC-1 RMCx3) was accompanied by elevated levels of protein citrullination relative to the corresponding parental cells (Figures 2L and 2M). Collectively, these findings demonstrate that distinct models of acquired resistance to KRAS-targeted therapies converge on a common phenotype characterized by enhanced PADI1 and PADI3 expression, increased protein citrullination, and metabolic rewiring.

A metabolically responsive enhancer coordinates PADI1 and PADI3 expression in KRAS-inhibited and KRAS-resistant cells

A putative enhancer located upstream of the *PADI1* gene has been proposed to regulate the expression of PADI family members.⁴² These genes are clustered on chromosome 1 (1p36.13), a locus known to exhibit chromatin looping.⁶² To investigate the regulatory dynamics of this region, we performed ChIP-seq for H3K27ac—a marker of active enhancers—in MIA PaCa-2 cells cultured for 24 h under normoxic (21% O₂) or hypoxic (2% O₂) conditions. As shown in Figure 3A, hypoxia induced a moderate increase in H3K27 acetylation within a genomic region located 10–20 kb upstream of the *PADI1* locus. As a positive control, a much more pronounced chromatin opening was observed at the promoter of *ENO1*, a well-established HIF-1α

target⁶³ (Figure 3A). Hi-C data⁶⁴ revealed that this upstream region is capable of looping to the *PADI3* locus, contacting a regulatory element spanning the promoter and first intron of *PADI3* (Figure 3A). Notably, we also observed increased H3K27ac at this *PADI3*-associated region under hypoxia (Figure 3A). To validate the functional relevance of these regions, we performed ChIP-qPCR targeting four regions (a–d) identified in Figure 3A. As shown in Figure 3B (Figure 3B, left), hypoxia significantly increased H3K27ac levels at region **b** (the upstream enhancer of PADI1) and region **d** (a regulatory region of PADI3). Having previously demonstrated that HIF-1α stabilization is not directly involved in PADI regulation (Figures S1A and S1B) and that lactate induces PADI expression (Figure 1M), we investigated whether lactate treatment under normoxic conditions could promote H3K27 acetylation at the identified regulatory regions **b** and **d**. Confirming this hypothesis, lactate treatment induced H3K27 acetylation at both the PADI1 and PADI3 regulatory regions (Figure 3B, right), while LDHA inhibition with sodium oxamate decreased PADI1 and PADI3 mRNA levels (Figure S1C). This effect of lactate was not due to enhanced transcript stability but rather to increased transcription, as demonstrated by Actinomycin D-mediated transcriptional inhibition (Figure S1D). These findings suggest that hypoxia and the associated metabolic resetting—particularly lactate accumulation—act as priming events for the transcriptional activation of PADI1 and PADI3.

To directly demonstrate that the upstream enhancer of PADI1 (region **b**) regulates both PADI1 and PADI3, we designed three sgRNAs targeting this region to recruit either dCas9-VP64 (to activate transcription) or dCas9-KRAB (to repress transcription) (Figure 3C). In 293T cells, sgRNA2 proved to be the most effective in recruiting dCas9-VP64 (SAM system), resulting in robust activation of PADI1 and, to a lesser extent, PADI3 under normoxic conditions (Figure S1E). This sgRNA was therefore selected for further experiments in MIA PaCa-2 cells. As shown in Figures 3D and 3E, MIA PaCa-2 cells engineered to express sgRNA2 together with either dCas9-VP64 or dCas9-KRAB exhibited strong transcriptional activation or repression, respectively, of both PADI1 and PADI3 under normoxia (Figures 3D

Figure 3. Epigenetic remodeling of an enhancer regulating PADI1 and PADI3 expression modulates sensitivity to BICI-based co-treatments

(A) ChIP-seq signal of H3K27ac at the PADI locus and the HIF-1α target *ENO1* in MIA PaCa-2 cells grown under normoxia or hypoxia for 48 h, along with Hi-C data. (B) qChIP-PCR analysis of H3K27ac enrichment at the four regulatory regions of *PADI1* and *PADI3* (regions a–d, as indicated in Figure 3A). Data are presented as fold enrichment relative to normoxic cells and compared with cells cultured under hypoxia or treated with lactate for 24 h under normoxic conditions, as indicated. (C) Genomic position relative to the *PADI1*-*PADI3* locus of the three sgRNAs designed to activate (dCas9-VP64) or repress (dCas9-KRAB) the expression of PADI1 and PADI3. (D–F) mRNA and protein levels of PADI1 and PADI3 in cells infected with lentiviral particles expressing dCas9-VP64 and sgRNA2 under normoxia (D and E) and hypoxia (F). (G and H) qChIP-PCR analysis of H3K27ac enrichment at the four regulatory regions of PADI1 and PADI3 (regions a–d, as indicated in Figure 3A). (G) MIA PaCa-2 cells treated with lactate for 24 h, expressed relative to untreated cells. (H) BxPC3 cells expressing KRAS^{G12C}, expressed relative to parental BxPC3 cells. (I) qChIP-PCR analysis of H3K27ac enrichment at the four regulatory regions of PADI1 and PADI3 (regions a–d, as indicated in Figure 3A) in parental and LC-2 resistant MIA PaCa-2 cells following treatment with 2.5 μM LC-2 for 16 h, and in parental and RMC-9805 resistant PANC-1 cells following treatment with 2 μM RMC-9805 for 16 h. (J) PADI1 and PADI3 protein levels in parental and resistant PANC-1 cells expressing dCas9-KRAB with or without the sgRNA targeting regulatory region b, following treatment with 2 μM RMC-9805 for 16 h. (K) Genome browser view of C/EBPβ ChIP-seq peaks across the 1p36.13 locus in the indicated KRAS-mutant cell lines. The b region, identified in this study as a shared regulatory element for PADI1 and PADI3, is indicated. (L) Schematic model illustrates the epigenetic regulation of the shared PADI1/PADI3 enhancer. KRAS-driven metabolic rewiring and lactate accumulation promote H3K27 acetylation at enhancer region b, which, through chromatin looping, coordinates activation of both PADI1 and PADI3. This enhancer-dependent transcriptional program is maintained in KRAS inhibitor-resistant cells and is predicted to be mediated by C/EBPβ. In (B, D, G, H, I, and J), mean ± SD are shown (n = 3). Dunn's multiple comparison test was used. In (B, H, and I), statistics are relative to the control condition. **p < 0.01 and ***p < 0.001.

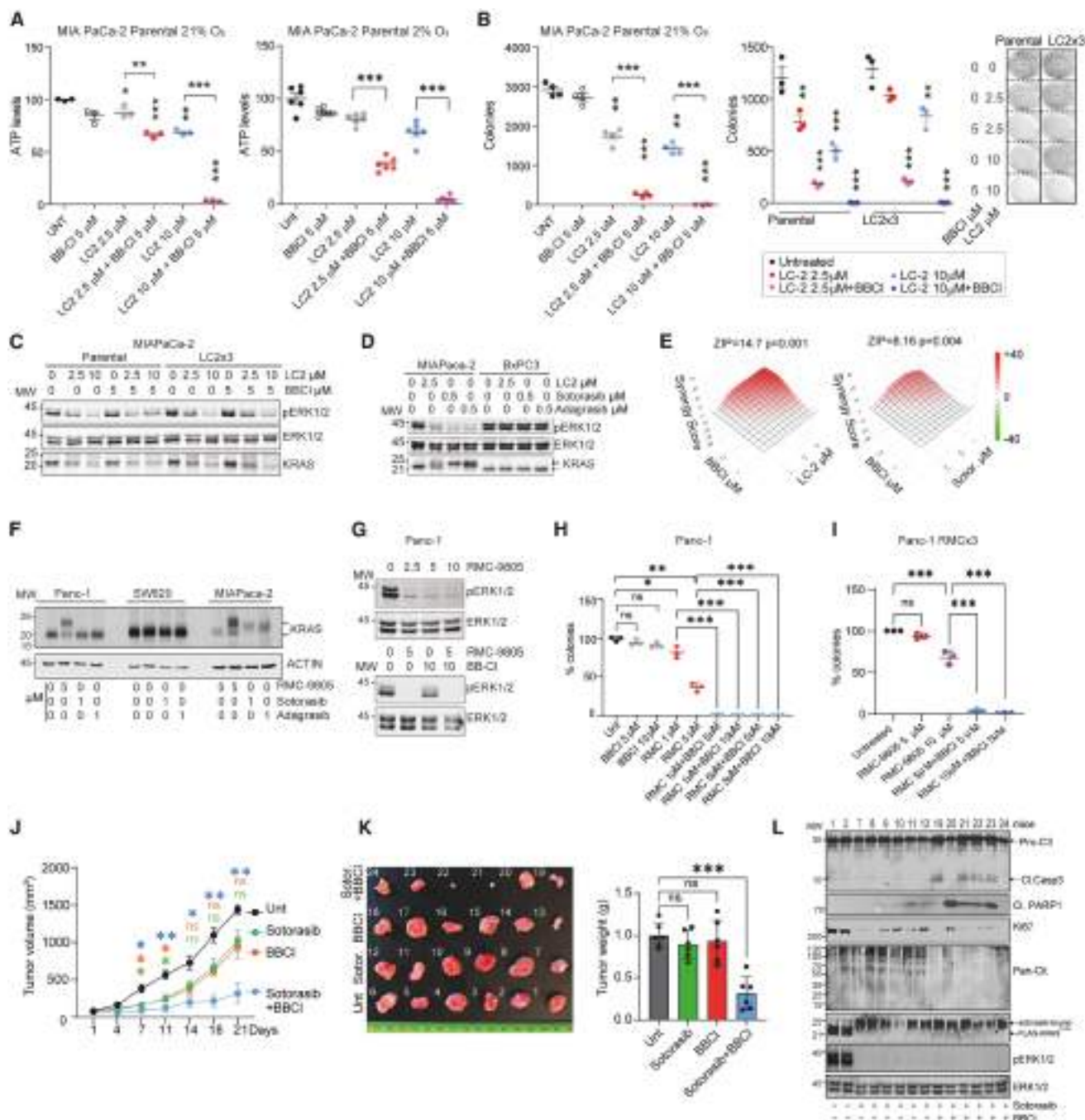


Figure 4. PADI inhibition potentiates KRAS-targeted therapies and overcomes acquired resistance

(A) ATP levels in MIA PaCa-2 cells treated as indicated for 36 h under normoxia or hypoxia (2% O₂).
 (B) Clonogenic assay in the indicated MIA PaCa-2 cells treated as indicated for 30 h and then grown in 60-mm plates for 7 days. Colonies were stained with methylene blue.
 (C) Immunoblot analysis of the indicated proteins in MIA PaCa-2 cells (parental or treated once or three times with LC-2) and BxPC3, after 24 h of the indicated treatment. We labeled ERK1/2 as pERK1/2 when phosphorylated at Thr202/Tyr204.
 (D) Immunoblot analysis of the indicated proteins in MIA PaCa-2 (biallelic KRAS^{G12C}) and BxPC-3 (wild-type KRAS) cell lines treated as indicated for 24 h. Arrows indicate band shifts of KRAS molecules covalently bound by adagrasib or sotorasib.
 (E) Synergy plot in MIA PaCa-2 cells after 48 h in the presence of the indicated concentrations of sotorasib, LC-2, and BBCI. ATP levels were used as readouts of cell viability and metabolic activity.
 (F) Immunoblot analysis of the indicated proteins in MIA PaCa-2 (KRAS^{G12C/G12C}), PANC-1 (KRAS^{wt/G12D}), and SW620 (KRAS^{G12V}) cells treated for 48 h with the indicated KRAS(OFF) and KRAS(ON) inhibitors. Arrows indicate band shifts of KRAS molecules covalently bound by RMC-9805.
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and 3E). These effects were further enhanced under hypoxic conditions (Figure 3F), supporting the hypothesis that the identified enhancer plays a central role in regulating PADI gene expression. Notably, modulation of PADI1 and PADI3 expression levels did not affect global histone H3 citrullination (Figure 3F). Importantly, recruitment of dCas9-KRAB to region **b**—which forms a chromatin loop with region **d**—abolished the lactate-induced increase in H3K27 acetylation at both regulatory regions (Figure 3G). Moreover, regions **b** and **d** were more acetylated in BxPC3 cells engineered to express KRAS^{G12C} compared to BxPC3 wild-type (KRAS WT) cells (Figure 3H), and this difference was abolished upon treatment with sodium oxamate (Figure S1F). Notably, LC-2x3 MIA PaCa-2 and RMC-9805-resistant PANC-1 cells (Figures 2A–2G) exhibited increased H3K27ac enrichment at these enhancer regions following 16 h treatment with 2.5 μ M LC-2 and 2 μ M RMC-9805, respectively, compared with their parental counterparts (Figure 3I). These findings suggest that this regulatory element contributes to the sustained expression of PADI1 and PADI3 upon KRAS inhibition in resistant cells. Consistent with this interpretation, epigenetic silencing of enhancer region **b** using dCas9-KRAB in PANC-1 RMCx3 cells abolished the elevated expression in resistant cells of both PADI1 and PADI3 following 16 h treatment with 2 μ M RMC-9805 (Figure 3J). Finally, *in silico* interrogation of 33,368 publicly available ChIP-seq datasets using ChIP-Atlas identified C/EBP β as the only transcription factor recurrently associated with the PADI1/PADI3 enhancer region across multiple KRAS-mutant cellular models (Table S1; Figure 3K). Together with its established roles downstream of KRAS signaling and in enhancer activation during metabolic stress,⁶⁵ these findings implicate C/EBP β as a potential mediator of the metabolic-to-epigenetic signaling axis controlling PADI1 and PADI3 expression.

Collectively, these findings support a model in which KRAS-driven metabolic reprogramming promotes activation of a shared PADI1/PADI3 enhancer, thereby sustaining PADI expression during adaptation to KRAS inhibition and acquired drug resistance (Figure 3L).

PADI inhibition potentiates KRAS-Targeted Therapies and Overcomes Acquired Resistance

We hypothesized that restoring PADI downregulation would enhance sensitivity to KRAS inhibitors by preventing the emergence of resistant clones and by re-sensitizing tumors with acquired resistance. To test this, we treated MIA PaCa-2 cells with the pan-PADI inhibitor BB-CI-amidine⁶⁶ (BBCI; 5 μ M), corresponding to its reported IC₅₀ for PADI enzymatic inhibition. BBCI or LC-2 alone had minimal effects on ATP production, whereas

the combination produced a marked synergistic reduction in metabolic activity that was further enhanced under hypoxia (Figure 4A). This effect reflected increased cell death, as demonstrated by activation of effector caspases (Figure S2A), and translated into a profound suppression of clonogenic growth (Figure 4B). Notably, BBCI completely restored LC-2 sensitivity in resistant MIA PaCa-2 cells (LC-2x3), abolishing colony formation without further affecting KRAS degradation or ERK1/2 inhibition (Figures 4B and 4C). Thus, PADI inhibition enhances the efficacy of KRAS degradation, prevents the emergence of resistant clones under the tested conditions, and restores sensitivity following acquired resistance.

To evaluate whether this effect extended to clinically relevant KRAS-targeted therapies, we compared MIA PaCa-2 (KRAS^{G12C/G12C}) and BxPC-3 (KRAS^{wt/wt}) cells treated with LC-2, adagrasib, or sotorasib. LC-2 selectively degraded KRAS in MIA PaCa-2 cells, whereas adagrasib and sotorasib induced the expected electrophoretic mobility shift of covalently modified KRAS^{G12C} only in mutant cells (Figure 4D). In all cases, BBCI synergized with KRAS inhibition exclusively in KRAS-mutant MIA PaCa-2 cells, markedly reducing ATP production, activating apoptosis, and completely suppressing clonogenic growth, while producing no significant effects in KRAS wild-type BxPC-3 cells (Figures 4E and S2C–S2F). Similar results were obtained in SW837 colorectal cancer cells harboring a monoallelic KRAS^{G12C} mutation, where combined treatment significantly reduced viability and clonogenic potential despite the intrinsic resistance of CRC cells to KRAS(OFF) inhibitors (Figures S2G–S2I).

We next investigated whether PADI inhibition also potentiates KRAS(ON) inhibitors. RMC-9805 selectively bound KRAS^{G12D} in PANC-1 cells, exhibited partial activity toward KRAS^{G12C} in MIA PaCa-2 cells, and showed no detectable binding to KRAS^{G12V} or KRAS wild-type alleles (Figure 4F). In PANC-1 cells, RMC-9805 effectively inhibited ERK signaling (Figure 4G), and co-treatment with BBCI markedly enhanced caspase activation and suppressed clonogenic growth, whereas BBCI alone remained largely inactive (Figures 4H, S2J, and S2K). Importantly, BBCI also restored sensitivity in RMC-9805-resistant PANC-1 cells (RMCx3), completely abolishing their clonogenic capacity (Figure 4I).

The therapeutic benefit of PADI inhibition extended across additional KRAS-driven models. BBCI synergized with RMC-9805 in murine KRAS^{G12D} PDAC cells (Figures S3A–S3C), potentiated sotorasib in BxPC-3 cells engineered to express KRAS^{G12C} (Figures S3D and S3E) and enhanced the activity of the pan-KRAS inhibitor BI-2865 in both SW620 (KRAS^{G12V}) and MIA PaCa-2 cells (Figures S3F and S3G), demonstrating

(G) Immunoblot analysis of the indicated proteins in PANC-1 cells treated for 48 h with the indicated μ M concentrations of BBCI and/or RMC-9805.

(H) Clonogenic assay in PANC-1 cells treated as indicated for 30 h and then grown in 35-mm plates for 7 days.

(I) Clonogenic assay in PANC-1 RMCx3 cells treated as indicated for 30 h and then grown in 35-mm plates for 7 days.

(J and K) Quantification of tumor volumes (J) and weight (K) in the BxPC-3 FLAG-KRAS^{G12C} xenograft model. Nude mice were subcutaneously injected with 2×10^6 cells. When tumors reached a mean volume of ~ 100 mm³, mice were randomized into four groups and treated intraperitoneally (i.p.) twice per week as indicated in Figure S4D. After the indicated number of days, mice were sacrificed, and tumors were excised (K). Data are expressed as mean $\pm$ st.error, $n = 6$. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test.

(L) Immunoblot analysis of the indicated proteins and global peptidyl-citrullination levels in tumors excised from the indicated mice, corresponding to the numerical identifiers shown in K. Cleaved caspase-3 and covalent KRAS engagement by sotorasib are indicated. In (A, B, H, I, J, and K) data are presented as mean $\pm$ SD. In (A, B, H, I, and K), Dunn's multiple comparison test was used. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

that PADI inhibition can potentiate multiple classes of KRAS-targeted therapies.

Given the reported off-target activity of BBCI toward STING, we next asked whether STING contributed to the observed therapeutic response. MIA PaCa-2 cells were STING-null (Figure S3H), BBCI did not perturb STING signaling in PANC-1 cells, and pharmacological STING degradation using the PROTAC PSD-1⁶⁷ failed to phenocopy the synergistic effects observed with BBCI (Figures S3I and S3J), indicating that the anti-neoplastic activity of the combination is independent of STING inhibition.

To validate these findings in more physiologically relevant models, we treated KRAS^{G12C} patient-derived PDAC organoids (PDM-197) with LC-2 or sotorasib in the presence or absence of BBCI. Combination treatment markedly increased apoptotic nuclei formation and reduced organoid viability to levels comparable to staurosporine (STS) (Figures S4A and S4B). Similarly, BBCI enhanced RMC-9805-induced cell death in KRAS^{G12D} CRC organoids (PDM-96) (Figure S4C).

Finally, we evaluated the therapeutic efficacy of the combination *in vivo*. BxPC-3 cells engineered to express KRAS^{G12C} were implanted subcutaneously into nude mice and treated twice weekly with sotorasib (10 mg/kg) and BBCI (5 mg/kg). Combined treatment produced significantly greater tumor growth inhibition than either monotherapy without detectable systemic toxicity, as assessed by body weight and examination of the liver and spleen (Figures 4J, 4K, and S4D). Tumor regression strongly correlated with caspase-3 activation and was accompanied by PARP1 cleavage in all but one tumor, together with reduced Ki67 staining, consistent with enhanced apoptotic cell death and suppression of tumor proliferation (Figure 4L). Importantly, while sotorasib efficiently engaged KRAS^{G12C} and inhibited ERK signaling, BBCI treatment markedly reduced the abundance of global peptidyl-citrullinated proteins within the tumors, demonstrating effective *in vivo* target engagement (Figure 4L). Similarly, co-treatment with sotorasib and BBCI produced a marked anti-tumor response in the MIA PaCa-2 xenograft model, significantly outperforming either monotherapy (Figure S4E).

Collectively, these findings demonstrate that PADI inhibition potentiates KRAS(OFF), KRAS(ON), and pan-KRAS inhibitors across human and murine PDAC and CRC models, suppresses the emergence of resistant clones, and restores sensitivity following acquired resistance, supporting PADI inhibition as a broadly applicable strategy to enhance KRAS-targeted therapies.

Genetic modulation of PADI1/3 expression validates their role in KRAS inhibitor sensitivity

To genetically validate the specificity of the pharmacological effects of BB-CI-amidine, we generated multiple gain- and loss-of-function models for PADI1 and PADI3. At first, rather than targeting each gene individually, we exploited the shared enhancer identified upstream of the PADI1 locus using CRISPR/dCas9-based epigenome editing to coordinately modulate the expression of both enzymes (as characterized in Figures 3E and 3F). Activation of the enhancer using dCas9-VP64 (SAM) increased PADI1 and PADI3 expression (Figure 3E) and significantly reduced the sensitivity of MIA PaCa-2 cells to both LC-2 and sotorasib under normoxic and hypoxic conditions (Figure S5A). Conversely, enhancer repression using dCas9-KRAB markedly reduced

PADI1 and PADI3 expression (Figure 3E) and re-sensitized MIA PaCa-2 LC-2x3 and PANC-1 RMCx3 cells to both KRAS inhibitors (Figure S5B). Importantly, dCas9-KRAB-expressing cells no longer displayed synergistic responses to LC-2 or sotorasib in combination with BBCI (Figures S5A and S5B), indicating that the therapeutic activity of BBCI requires functional PADI expression and is not mediated by off-target effects.

To further corroborate our findings, we used lentiviral vectors to overexpress *PADI1-V5* and *PADI3-V5* in MIA PaCa-2 cells at supraphysiological levels. As shown in Figure S5C, overexpression of *PADI1* and *PADI3* did not alter the efficacy of LC-2, sotorasib, or adagrasib in degrading or covalently binding KRAS^{G12C}, nor did it affect downstream inhibition of the ERK1/2 signaling pathway. Importantly, the overexpression of either PADI1 or PADI3 partially saturated the inhibitory capacity of BBCI, leading to a measurable reduction in its anti-neoplastic efficacy when used in combination with KRAS^{G12C}-targeting agents (Figure S5D).

As shown above (Figure 2F), MIA PaCa-2 cells express substantially higher levels of *PADI3* than *PADI1* mRNA. This difference is explained, at least in part, by the greater stability of the PADI3 transcript, although both mRNAs are intrinsically long-lived (Figure S5E). Based on these observations, we hypothesized that the synergistic effects of BBCI in combination with KRAS inhibition might be primarily mediated by PADI3. To test this hypothesis, we transiently depleted PADI3 using two independent siRNAs (siRNA1 and siRNA3) in both parental and LC-2-resistant (LC-2x3) MIA PaCa-2 cells. Both siRNAs efficiently reduced PADI3 expression (Figure S5F). Interestingly, siRNA3, but not siRNA1, elicited a compensatory increase in PADI1 expression, suggesting the existence of a feedback mechanism between the two PADI family members (Figure S5F). Despite this difference, PADI3 silencing, particularly with siRNA1, significantly enhanced the anti-proliferative activity of LC-2 and sotorasib while markedly reducing the additional benefit conferred by BBCI co-treatment (Figure S5G), indicating that a substantial fraction of BBCI activity is mediated through PADI3 inhibition.

To exclude transient knockdown-specific effects, we generated stable PADI3 knockdown cells using two lentiviral shRNAs (Figure S5H). In contrast to the acute siRNA-mediated depletion, stable PADI3 silencing induced only a modest, non-significant increase in PADI1 expression, consistent with long-term cellular adaptation (Figure S5H). Importantly, stable PADI3 depletion sensitized MIA PaCa-2 cells to LC-2, sotorasib, and adagrasib (Figure S5I). Moreover, PADI3 knockdown alone significantly impaired clonogenic growth (Figure S5J), demonstrating that PADI3 contributes to the maintenance of the malignant phenotype independently of KRAS inhibition.

Collectively, these complementary gain- and loss-of-function approaches establish PADI1 and PADI3 as key mediators of the adaptive response to KRAS inhibition and support the conclusion that the anti-neoplastic activity of BBCI is largely mediated through on-target inhibition of PADI1 and, predominantly, PADI3.

PADI3 inhibition recapitulates the synergistic effects of BBCI in combination with KRAS inhibitors

To further investigate the role of PADI3 in modulating the response to KRAS-targeted therapies, we compared the

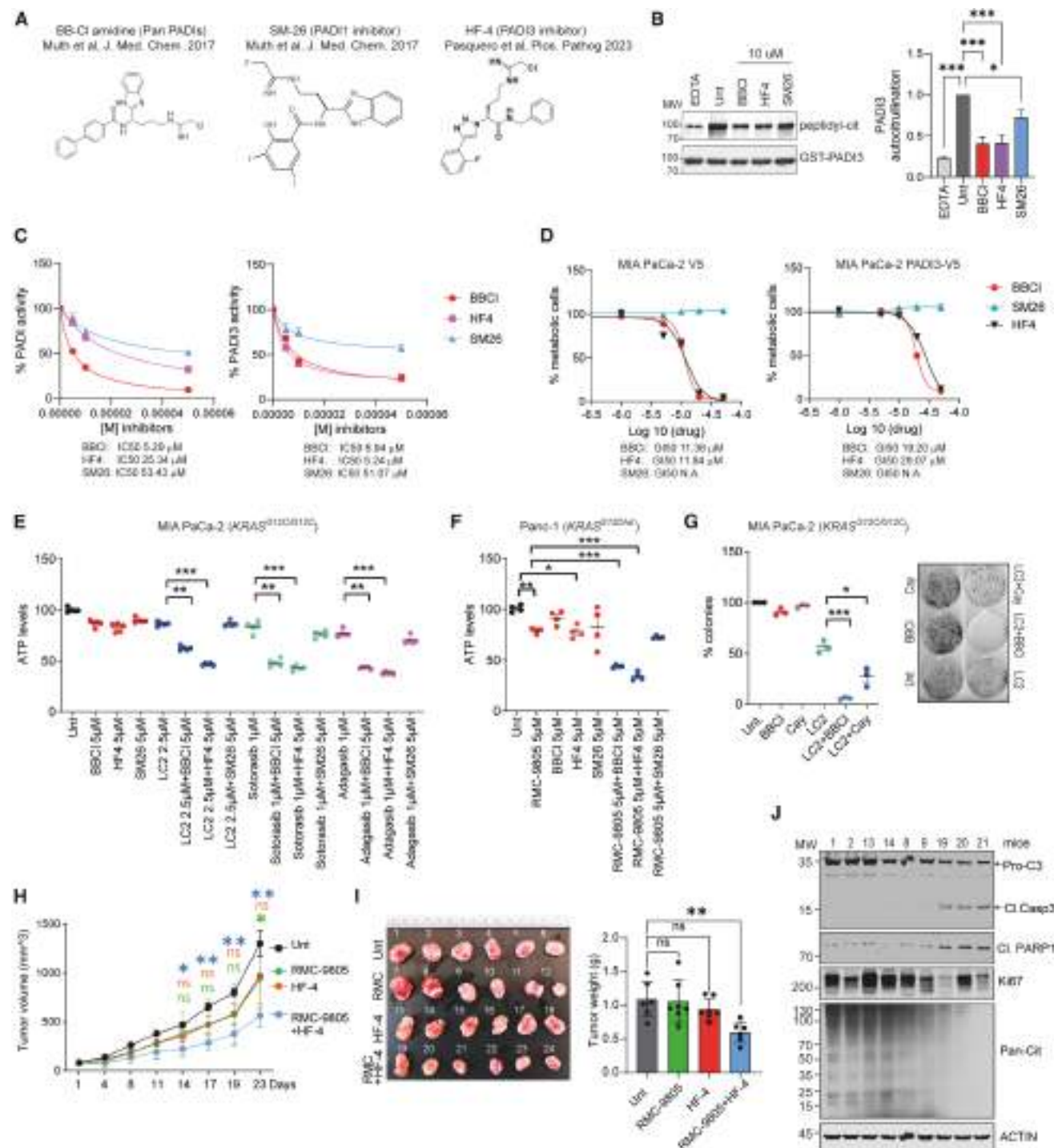


Figure 5. PADI3 inhibition recapitulates the synergistic effects of BBCI in combination with KRAS inhibitors

(A) Chemical structures of the three PADI inhibitors used in this study and their bibliographic reference.
 (B) PADI3 auto-citrullination assay performed in the presence of 10 μ M of the indicated PADI inhibitors.
 (C) *In vitro* citrullination assay using 4MEBz-FluMe as a probe for citrullination activity and either a cocktail of PADIs (PADI1, 2, 3, 4, 6; left) or recombinant PADI3 (right) in the presence of increasing concentrations of PADI inhibitors. IC₅₀ values were determined by non-linear regression analysis using a variable-slope model.
 (D) Growth inhibition 50 (GI₅₀) of the indicated PADI inhibitors in MIA PaCa-2 V5 or PADI3-V5 cells treated for 72 h. GI₅₀ values were determined by non-linear regression analysis using a variable-slope model.

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biochemical and biological effects of three inhibitors: the pan-PADI inhibitor BBCI, a PADI1-selective inhibitor (SM-26),⁶⁶ and a PADI3-selective inhibitor (HF-4)⁶⁸ (Figure 5A). Using catalytically active recombinant GST-PADI3, we confirmed its ability to undergo auto-citrullination in a calcium-containing citrullination buffer (Figure 5B). At 10 μ M, both BBCI and HF-4 effectively inhibited PADI3 auto-citrullination, whereas SM-26 showed lower activity against PADI3 (Figure 5B).

To further validate these findings, we employed the 4MEBz-FluME probe⁶⁹ to quantify citrullination activity *in vitro* (Figure 5C). We tested dose-response curves of the three inhibitors using either a cocktail of all five human PADIs (PADI1, 2, 3, 4, and 6) or PADI3 alone, and calculated IC₅₀ values. In this assay, BBCI exhibited IC₅₀ values of 5.3 μ M (pan-PADI) and 6.9 μ M (PADI3), HF-4 showed 25.3 μ M and 5.2 μ M, respectively, while SM-26 was significantly less potent (53.4 μ M with pan-PADI and 51.07 μ M with PADI3) (Figure 5C). These results confirm a certain degree of selectivity and efficacy of HF-4 as a PADI3 inhibitor at 5 μ M. To test the biological effect of PADI inhibitors and their specificity, we used MIA PaCa-2 cells overexpressing or not PADI3-V5. As expected, we obtained a decreased antiproliferative effect (expressed as GI₅₀) for BBCI and HF-4, but not SM-26, in cells overexpressing PADI3 (Figure 5D).

We next evaluated the cytotoxic effects, measured by ATP levels, following co-treatment with KRAS inhibitors and either BBCI, HF-4, or SM-26 in MIA PaCa-2 (biallelic *KRAS*^{G12C}) and PANC-1 (monoallelic *KRAS*^{G12D}) cells. As shown in Figures 5E and 5F, both BBCI and HF-4 exhibit effective synergy with KRAS(OFF) (LC-2, sotorasib) and KRAS(ON) (RMC-9805) inhibitors, while SM-26 had only marginal effects.

To further support the role of PADI3, we tested a second PADI3-selective inhibitor (CAY10727⁶⁸ referred to as “Cay” in Figure 5G). Co-treatment with CAY10727 and LC-2 in MIA PaCa-2 cells produced a moderate anti-neoplastic effect, although less pronounced than the strong synergy observed with BB-CI-amidine (Figure 5G). Although we cannot fully exclude the possibility of shared off-target effects among BBCI, HF-4, and CAY10727, the consistent results across multiple PADI3-targeting compounds strongly suggest that PADI3 inhibition is a key driver of the synergistic response observed when combining PADI inhibitors with KRAS-targeted therapies in KRAS-addicted cancer cells.

Finally, we evaluated the *in vivo* efficacy of the selective PADI3 inhibitor HF-4 using a murine xenograft model generated by subcutaneous implantation of FC1199-P3 cells harboring the *KRAS*^{G12D} mutation. Mice were treated twice weekly with the KRAS(ON) inhibitor RMC-9805, either alone or in combination

with HF-4 (Figure S6A). Whereas RMC-9805 displayed only limited antitumor activity as a single agent, consistent with the intrinsic resistance of FC1199-P3 cells to KRAS(ON) inhibition (Figure S3C), co-treatment with HF-4 potentiated its efficacy, resulting in a significant suppression of tumor growth (Figures 5H and 5I) without detectable systemic toxicity, as assessed by body weight (Figure S6B) and examination of the status of liver and spleen (Figure S6C). Importantly, the *in vivo* response closely correlated with increased caspase-3 activation, PARP1 cleavage, and a pronounced reduction in the overall burden of citrullinated proteins within the tumor tissue (Figure 5J), demonstrating effective target engagement.

KRAS inhibition induces a calcium-dependent PADI survival program

To gain mechanistic insight into how BBCI potentiates KRAS inhibition, we performed transcriptomic profiling of parental and LC-2-resistant (LC-2x3) MIA PaCa-2 cells treated for 30 h with DMSO, LC-2 (2.5 μ M), BBCI (5 μ M), or the combination. LC-2 alone significantly modulated 396 and 238 genes in parental and resistant cells, respectively ($|FC| > 2$, $p_{adj} < 0.005$), whereas BBCI alone produced only limited transcriptional changes (41 and 40 genes, respectively), with extensive overlap between the two cell types (Figure S7A; Table S2). In contrast, the combination treatment elicited a markedly broader transcriptional response, affecting 1,277 genes in parental cells and 661 genes in resistant cells (Figure S7A).

Gene Set Enrichment Analysis confirmed that LC-2 efficiently suppressed KRAS signaling in both parental and resistant cells (Figures S7B and S7C). However, whereas KRAS inhibition activated a pro-apoptotic transcriptional program in parental cells, LC-2x3 cells preferentially induced inflammatory and senescence-associated secretory phenotype (SASP) signatures (Figure S7B). Addition of BBCI shifted both parental and resistant cells toward a common transcriptional response characterized by disruption of glutathione metabolism and oxidative stress pathways (Figures S7D–S7G). Consistently, the combination markedly downregulated *GGT5*, *GPX1*, *GCLM*, and *RRM2*, genes required for glutathione homeostasis and deoxyribonucleotide synthesis, thereby coupling oxidative stress to impaired proliferation and cell death (Figures S7D–S7G). These findings are consistent with previous reports demonstrating that oncogenic KRAS sustains pancreatic tumor growth by maintaining glutathione metabolism and redox homeostasis through metabolic rewiring.^{70,71}

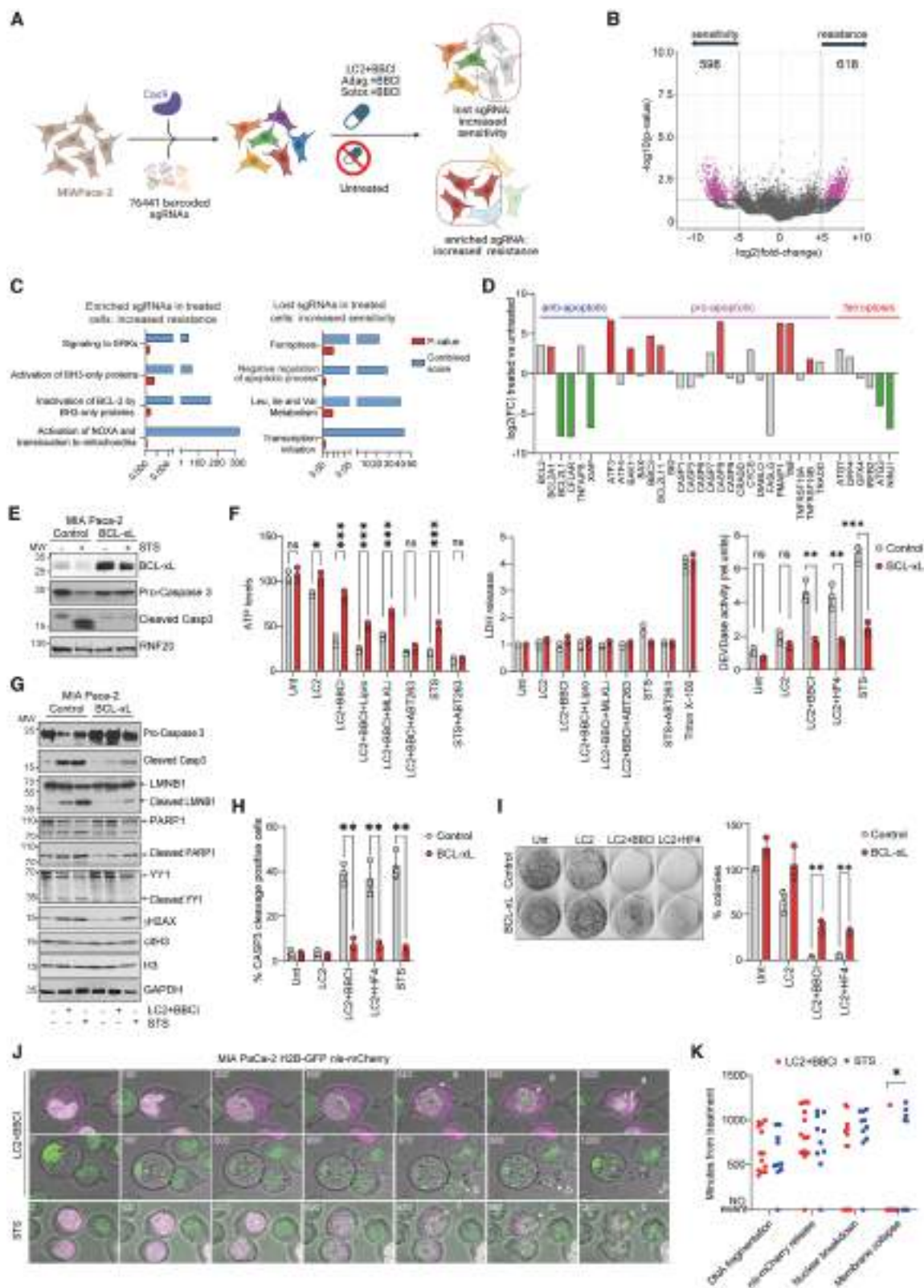
The relatively modest transcriptional effects of BBCI alone further suggested that basal PADI activity is limited under resting conditions. Indeed, PADI enzymes require high micromolar-to-millimolar intracellular calcium concentrations for catalytic

(E and F) ATP levels in the indicated PDAC cells treated for 36 h with the indicated inhibitors.

(G) Clonogenic assay in MIA PaCa-2 cells treated with LC-2 (2.5 μ M), BBCI (5 μ M), and the PADI3 inhibitor (CAY10727 “Cay”, 5 μ M).

(H and I) Quantification of tumor volumes (H) and weight (I) in the FC1199-P3 xenograft model. Nude mice were subcutaneously injected with 2×10^6 cells. When tumors reached a mean volume of ~ 100 mm³, mice were randomized into four groups and treated intraperitoneally (i.p.) twice per week with RMC-9805 (10 mg/kg) and/or HF-4 (5 mg/kg), as indicated. After the indicated number of days, mice were sacrificed, and tumors were excised (I). Data are expressed as mean $\pm$ st.error, $n = 6$.

(J) Immunoblot analysis of the indicated proteins and global peptidyl-citrullination levels in tumors excised from the indicated mice, corresponding to the numerical identifiers shown in I. Cleaved caspase-3 is indicated. In B–I, data are presented as mean $\pm$ SD. Dunn’s multiple comparison test was used in B–G. One-way ANOVA followed by a Bonferroni post hoc test was used in (H and I). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



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activation through positive allosteric regulation.⁷² Consistent with previous reports,⁷³ inhibition of KRAS signaling increased the expression of multiple components of the Store-Operated Calcium Entry (SOCE) machinery, including STIM1 and ORAI1, the principal mediators of SOCE in PDAC and CRC cells^{73,74} (Figure S7H). Based on these observations, we hypothesized that KRAS inhibition promotes a calcium-dependent adaptive survival program mediated by PADI activation. Consistent with this model, untreated MIA PaCa-2 cells exhibited basal intracellular calcium concentrations of 88 ± 3 nM (Figure S7I). As expected, treatment with the calcium ionophore A23187 increased intracellular calcium into the micromolar range. Remarkably, both LC-2 and sotorasib also induced a significant increase in intracellular calcium, which was completely prevented when cells were cultured for 16 h in calcium-free medium before treatment (Figure S7I), indicating that KRAS inhibition promotes extracellular calcium influx rather than intracellular calcium release. Importantly, pharmacological inhibition of SOCE using BTP2 markedly suppressed KRAS inhibitor-induced calcium influx, identifying SOCE as the primary route of calcium entry following KRAS blockade (Figure S7I).

Collectively, these findings identify calcium mobilization as the mechanistic link between KRAS inhibition and PADI dependency. By promoting SOCE-mediated calcium influx, KRAS inhibition converts otherwise poorly active PADIs into catalytically competent enzymes, thereby establishing a transient PADI-dependent adaptive survival program. We propose that, during the acquisition of resistance, this program becomes constitutively engaged through sustained activation of the PADI1/PADI3 regulatory enhancer, allowing resistant cells to maintain elevated PADI expression and activity despite continued KRAS inhibition.

Combined KRAS and PADI inhibition engages mitochondrial apoptosis while preserving plasma membrane integrity

To elucidate the mechanism of cell death induced by KRAS inhibition in combination with BBCI or HF-4, we performed a genome-wide loss-of-function screen using the Brunello lentiviral library,⁷⁵ which encodes Cas9 and 76,441 sgRNAs. MIA PaCa-2 cells were infected and selected, then divided into four treatment groups: DMSO (untreated control), LC-2 + BBCI, adagrasib + BBCI, and sotorasib + BBCI. After 30 h of treatment, surviving clones were expanded for five days, genomic DNA was extracted, and sgRNA barcodes were amplified, sequenced, and quantified (Figure 6A). Positive enrichment of sgRNAs indicates that knockout of the corresponding gene confers resistance to the treatment, while depletion suggests a pro-death role. Genes were considered significantly enriched or depleted if they met the following criteria: sgRNA enrichment $>10^5$ -fold compared to control, p value < 0.05 , and at least two concordant sgRNAs per gene. Using these parameters, we identified 618 positively enriched genes and 598 negatively enriched genes (Figures 6B; Table S3). Functional annotation of these gene sets revealed that positively enriched genes were associated with intrinsic and extrinsic apoptotic pathways and ERK signaling (Figure 6C). In contrast, negatively enriched genes included anti-apoptotic regulators, ferroptosis-related genes, and genes involved in basal cellular functions such as transcription and branched-chain amino acid metabolism (Leu, Ile, Val) (Figure 6C). Overall, the data indicated that the disruption of apoptotic signaling or activation of anti-apoptotic pathways enables resistance to KRAS inhibition combined with BBCI (Figures 6C and 6D). Interestingly, depletion of ferroptosis-associated genes was negatively enriched in cells that survived the dual treatment, suggesting that loss of these

Figure 6. Combined KRAS and PADI inhibition engages mitochondrial apoptosis while preserving plasma membrane integrity

- (A) MIA PaCa-2 cells were transduced with the Brunello CRISPR library targeting coding genes with 76,441 barcoded sgRNAs. After puromycin selection, cells were treated for 48 h with DMSO, LC-2+BBCI, adagrasib+BBCI, or sotorasib+BBCI. Resistant clones were then expanded for 120 h, genomic DNA was extracted, libraries were prepared, and barcodes were quantified and analyzed for abundance relative to the untreated (DMSO) condition. Enriched sgRNAs correlate with resistance and therefore target genes that regulate sensitivity to the proposed treatments.
- (B) Volcano plot of sgRNAs enriched or depleted across the four conditions tested (LC-2+BBCI, adagrasib+BBCI, sotorasib+BBCI vs. untreated).
- (C) Pathway analysis with Enrichr of sgRNAs enriched or depleted in the four conditions tested (LC-2+BBCI, adagrasib+BBCI, sotorasib+BBCI vs. untreated). For each functional category, p value and combined enrichment score are reported.
- (D) Positive or negative enrichment expressed as the mean fold change of sgRNAs targeting genes belonging to functional categories associated with anti-apoptotic, apoptotic, and ferroptotic responses.
- (E–G) Immunoblot of the indicated proteins in MIA PaCa-2 cells expressing Control (Hygro) or BCL-xL as indicated and treated with STS (1 μ M, 24 h) or LC-2+BBCI (2.5 and 5 μ M, respectively) for 72 h. Caspase processing (cleavage) is shown. For the detection of CASP3 and PARP1 cleavage, specific antibodies were used as indicated. (F) Bar graphs show ATP levels, extracellular LDH release, and caspase activity in MIA PaCa-2 cells expressing control (hygromycin resistance) or BCL-xL, as indicated, and treated for 48 h with STS (1 μ M), LC-2+BBCI (2.5 and 5 μ M), or LC-2+BBCI combined with Liproxstatin-1 (Liprox, 5 μ M), ABT263 (1 μ M), or MLKL1 (5 μ M).
- (H) Bar graph shows the percentage of MIA PaCa-2 cells expressing Control (Hygromycin resistance) or BCL-xL that become fluorescent in the presence of a probe monitoring Caspase-3 processing during 40 h of time-lapse microscopy under the indicated treatments. BBCI and HF4 were used at 5 μ M.
- (I) Clonogenic assay in the indicated cells treated as described for 48 h and then allowed to grow for 10 days in 60-mm dishes. Colonies were stained with methylene blue at the end of the experiment.
- (J) Representative time-lapse images of MIA PaCa-2 cells expressing H2B-GFP (in green) and nls-mCherry (in magenta); for each image, the time in minutes of each frame from the start of the indicated treatment is shown (LC-2 2.5 μ M, BBCI 5 μ M, STS 1 μ M). The arrows indicate: a. an event characterized by DNA fragmentation, release of nls-mCherry into the cytoplasm, preservation of overall nuclear structure, and maintenance of plasma membrane integrity; b. an event characterized by DNA fragmentation, cytoplasmic release of nls-mCherry, dismantling of nuclear structure, and maintenance of plasma membrane integrity; c. an event characterized by DNA fragmentation, cytoplasmic release of nls-mCherry, dismantling of nuclear structure, formation of apoptotic bodies, and subsequent loss of plasma membrane integrity. Scale bars, 25 μ m.
- (K) Dot plot shows the minute after which one of the four indicated events occurs (the frame marking the start of the event is considered) or the “non-event”, meaning an event not identified during 1200 min of video recording. In (F, H, I, and K), data are shown as mean $\pm$ st.dev.; $n = 3$. Pairwise t test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

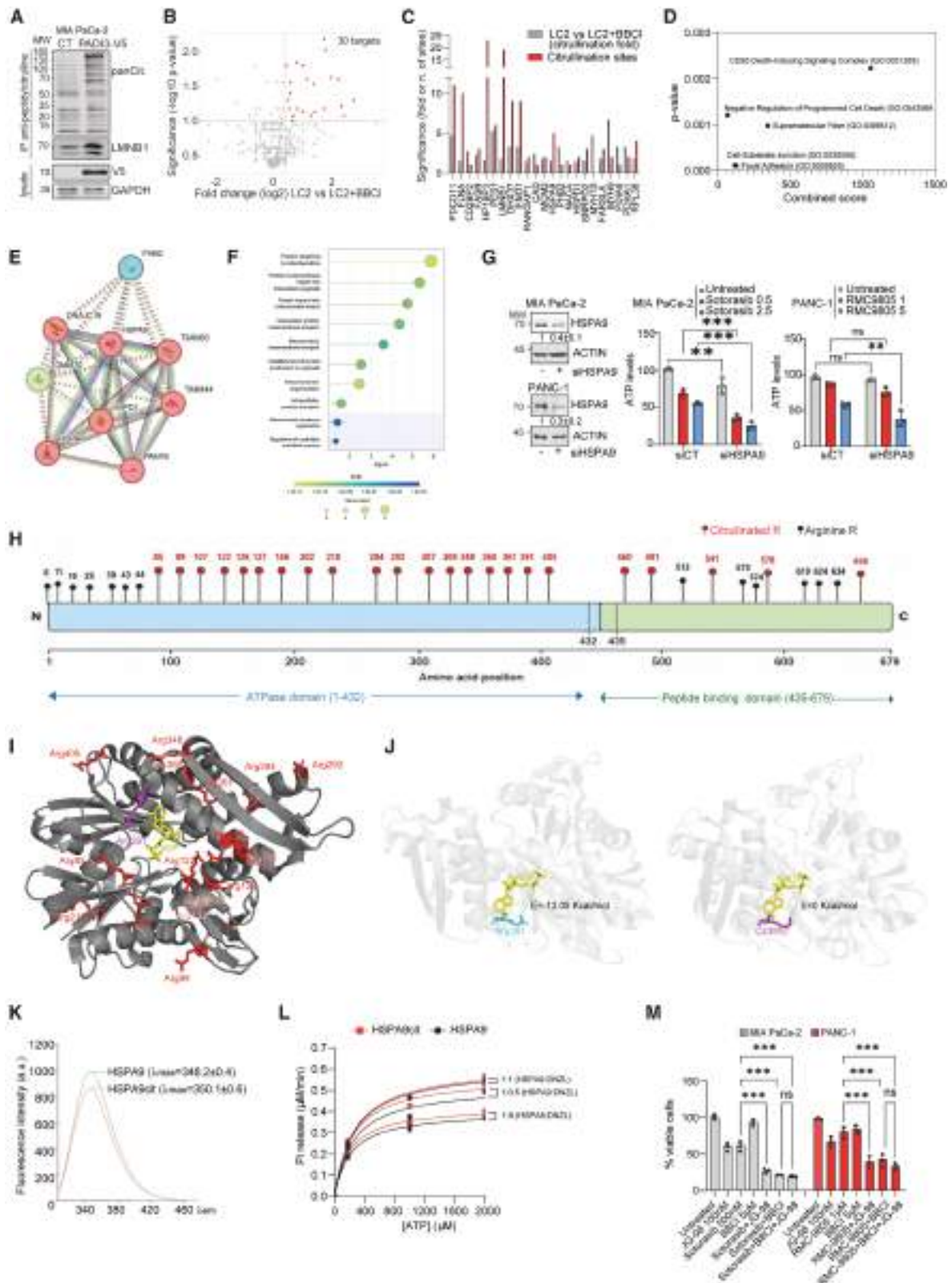
genes may confer a survival advantage under these conditions (Figure 6D). Supporting the specificity of the screen, perturbation of KRAS signaling rendered cells refractory to the combination treatment (Figure 6C). Consistent with our pharmacological and genetic data, sgRNAs targeting PADI1 were among the most significantly enriched in surviving cells across all co-treatment conditions (Figure S8A), indicating that loss of PADI1 confers resistance to BBCI. In contrast, sgRNAs targeting PADI3 were consistently depleted in both treated and untreated populations, reflecting the intrinsic requirement of PADI3 for cell fitness and proliferation, in agreement with our previous observation that stable PADI3 depletion markedly impairs clonogenic growth (Figure S5J).

To confirm the apoptotic nature of cell death induced by KRAS inhibition in combination with BBCI or HF-4, we overexpressed BCL-xL in MIA PaCa-2 cells using a lentiviral vector, with empty vector-transduced cells serving as controls. Overexpression of BCL-xL was sufficient to block caspase-3 activation following treatment with staurosporine (STS, 1 μ M, Figure 6E), a known inducer of intrinsic apoptosis.⁷⁶ Remarkably, co-treatment with LC-2 + BBCI was almost completely ineffective in BCL-XL-overexpressing cells, showing even greater resistance than STS-treated cells (Figure 6F). Pharmacological inhibition of BCL-xL using ABT-263⁷⁷ restored sensitivity to the co-treatment, confirming the specificity of the pro-survival effect (Figure 6F). Interestingly, co-treatment with the radical-trapping antioxidant and ferroptosis inhibitor lipoxstatin-1⁷⁸ enhanced cell death in both control and BCL-xL-overexpressing cells, whereas inhibition of necroptosis via MLKL blockade⁷⁹ had no significant effect (Figure 6F). This apoptotic response of the co-treatment was observed in both parental MIA PaCa-2 cells and LC-2-resistant (LC-2X3) cells (Figure S8B). Co-treatment with LC-2 + BBCI in the presence of ferroptosis inhibitors—ferrostatin-1 (Fer-1) and lipoxstatin-1, but not with a DPP4 inhibitor⁸⁰—consistently resulted in reduced cell survival, further enhancing the cytotoxic effect of LC-2 + BBCI (Figure S8B). Given that MIA PaCa-2 cells are partially resistant to Erastin treatment⁸¹ (Figure S8B), we repeated the experiments in PANC-1 cells. Similar to MIA PaCa-2, co-treatment of PANC-1 cells with RMC-9805 and BBCI resulted in increased cell death upon co-treatment with Fer-1 or lipoxstatin-1, whereas these compounds were protective against erastin-induced ferroptosis, as expected (Figure S8C). This increase in cell death following ferroptosis inhibition correlated with pronounced membrane damage and elevated lactate dehydrogenase release, as shown in Figure S8C. Supporting this, stable knockdown of NINJIN-1 (NINJ1), a recently identified executor of pyroptosis- and ferroptosis-associated plasma membrane rupture,⁸² enhanced the induction of cell death achieved with KRAS inhibitors plus BBCI (Figure S8D), further confirming the observations from our LOF screening (Figure 6A). The precise level at which ferroptosis signaling interferes with cell death activation in the context of KRAS inhibition combined with PADI3 targeting remains to be clarified.

Consistent with apoptosis induction, BCL-xL overexpression (Figure 6E) blocked effector caspase activation following co-treatment with LC-2 + BBCI or LC-2 + HF-4 (Figure 6F), similar to the effect observed with STS (Figure 6E). Importantly, no

LDH release was detected in the culture medium after 48 h of treatment, in contrast to STS-treated cells,⁷⁶ indicating that plasma membrane integrity was preserved (Figure 6F). Immunoblot analysis revealed caspase-3 activation in LC-2+BBCI- and STS-treated cells after 48 h of treatment, which was completely rescued in BCL-xL-overexpressing cells (Figure 6G). LMNB1 cleavage, a caspase-6 substrate,⁸³ was detected in LC-2+BBCI-treated cells but to a lesser extent than in STS-treated cells (Figure 6G). PARP1 and YY1, two substrates of caspases 3 and 7,⁸⁴ were equally cleaved in LC-2+BBCI- and STS-treated cells and fully rescued in BCL-xL-overexpressing cells (Figure 6G). Importantly, γ H2AX phosphorylation was completely rescued in BCL-xL-overexpressing cells following LC-2+BBCI treatment, indicating that DNA damage in this context was a secondary event downstream of caspase activation (Figure 6G). Notably, no differences in H3 citrullination were observed between samples (Figure 6G). Overall, these data indicate predominantly apoptotic signaling in LC-2+BBCI-treated cells, with caspase-3 and -7 activation comparable to STS and reduced caspase-6 activity (Figure 6G). Using a fluorescent caspase-3 activity probe in time-lapse microscopy experiments, we observed a progressive accumulation of caspase-3-positive cells following co-treatment with KRAS inhibitors and BBCI (Figures 6H and S8E). This supports the conclusion that the induced cell death is apoptotic and occurs in a gradual, non-lytic manner at the single-cell level. To further validate the apoptotic mechanism underlying cell death induced by KRAS inhibition combined with BBCI or HF-4, we treated MIA PaCa-2 cells with 100 μ M zVAD-fmk, an inhibitor of effector caspases⁸⁵ effective in blocking STS-induced apoptosis at this concentration (Figure S8F). zVAD-fmk provided complete protection against cell death induced by LC-2 + BBCI or sotorasib + BBCI co-treatment (Figure S8G), confirming the caspase-dependent nature of the response. Long-term survival assays revealed that BCL-xL overexpression conferred sustained protection against apoptosis. After two weeks, resistant colonies were observed only in BCL-XL-overexpressing cells following co-treatment with LC-2 + BBCI or LC-2 + HF-4, further supporting the role of mitochondrial apoptosis in mediating cell death (Figure 6I).

To investigate how cells undergo apoptosis while maintaining plasma membrane integrity, we engineered MIA PaCa-2 cells to express nuclear-localized mCherry (nls-mCherry), a reporter of nuclear envelope integrity, together with H2B-GFP to monitor chromatin condensation, fragmentation, and nuclear disassembly.¹⁰ Cells were subjected to live-cell confocal microscopy following treatment with LC-2 plus BBCI or, as a reference, staurosporine (STS). Both treatments induced comparable kinetics and efficiency of chromatin fragmentation, cytoplasmic release of nls-mCherry, and nuclear dismantling (Figures 6J and 6K). However, plasma membrane blebbing followed by membrane rupture was rarely observed following LC-2 plus BBCI treatment (<5% of recorded events), whereas approximately 40% of STS-treated cells underwent secondary membrane rupture (Figures 6J and 6K; Videos S1 and S2). Importantly, in cells receiving the combination treatment, mitochondrial membrane potential ($\Delta\Psi$ m) dissipated within 16 h (Figure S8H), consistent with mitochondrial outer membrane permeabilization and activation of the intrinsic apoptotic pathway, a



(legend on next page)

hallmark event preceding caspase activation and apoptotic execution.⁸⁶ Despite extensive mitochondrial dysfunction, plasma membrane integrity remained largely preserved throughout the execution phase of apoptosis.

To determine whether the preservation of plasma membrane integrity resulted from active membrane repair, we generated MIA PaCa-2 H2B-GFP cells expressing CHMP4B-mCherry, a core component of the ESCRT-III membrane repair machinery,⁸⁷ and monitored its localization by live-cell time-lapse microscopy following co-treatment with sotorasib (1 μ M) and BBCI (5 μ M). Under these conditions, we did not observe persistent CHMP4B-positive membrane puncta, arguing against sustained activation of ESCRT-mediated membrane repair (Videos S3 and S4). The relative contribution of membrane repair versus intrinsic membrane preservation remains to be established.

Finally, cells undergoing lytic cell death release damage-associated molecular patterns (DAMPs), which activate pattern recognition (PRRs) and non-PRR receptors on both immune and non-immune cells, functioning as either immunostimulatory or immunosuppressive signals.⁸⁸ In PDAC, DAMPs release was reported to activate pancreatic stellate cells boosting pancreatic cancer proliferation and metastasis.⁸⁹ Consistent with the non-lytic nature of the cell death program, transcriptomic analysis of LC-2+BBCI-treated MIA PaCa-2 cells showed no evidence of PRR activation or sterile inflammatory signaling (Figure S8I), and only minimal HMGB1 release into the extracellular medium compared with STS-treated cells (Figure S8J).

Collectively, these findings identify a previously unrecognized non-lytic mitochondrial apoptotic program elicited by combined KRAS/PADI inhibition, in which efficient caspase-dependent cell

death proceeds without DAMP release or activation of sterile inflammatory signaling, thereby uncoupling apoptotic execution from inflammatory cell lysis.

PADI-mediated citrullination remodels a mitochondrial proteostasis network centered on HSPA9/Mortalin

To identify the functional substrates responsible for the adaptive response to KRAS inhibition, we characterized the citrullinome of LC-2-resistant MIA PaCa-2 cells and determined how its remodeling by PADI inhibition correlates with restoration of apoptosis. Citrullinated proteins were immunopurified using the monoclonal anti-peptidyl-citrulline antibody (F95),⁹⁰ which efficiently recognizes citrullinated proteins,⁹¹ but displays limited cross-reactivity toward homocitrullinated species.⁹² As a technical validation, ectopic expression of PADI3-V5 markedly increased the abundance of F95-reactive proteins, confirming the robustness of the immunopurification strategy (Figure 7A).

To identify functionally relevant PADI substrates, we compared untreated, LC-2-treated, and LC-2+BBCI-treated LC-2-resistant cells. We reasoned that proteins selectively citrullinated following KRAS inhibition but losing this modification upon BBCI treatment would represent candidate mediators of the adaptive survival program. Among 598 immunopurified proteins identified by LC-MS/MS, 30 displayed significantly reduced citrullination following BBCI treatment (fold change > 1.3, $p < 0.01$), and 23 of these also exhibited increased citrullination following LC-2 treatment compared with untreated cells (Figures 7B and 7C; Table S4). With the exception of MYH10, all identified proteins had previously been reported as bona fide citrullination substrates, supporting the specificity of the analysis.

Figure 7. PADI-mediated citrullination remodels a mitochondrial proteostasis network centered on HSPA9/Mortalin

(A) Citrullinated proteins were detected in lysates from MIA PaCa-2 LC2x3 cells overexpressing V5 or PADI3-V5. Pan-citrulline antibody was used to detect peptidyl-citrulline following the protocol established by the Asaga group⁹³ in lysates subjected to citrulline immunopurification with F95 antibody. Anti-pan-citrulline and anti-LMN1 antibodies were employed to detect peptidylcitrulline and citrullinated LMN1 in F95-based peptidylcitrulline immunopurifications, respectively.

(B) Volcano plot of proteins significantly citrullinated in MIA PaCa-2 LC2x3 cells treated with LC-2 compared to co-treatment with LC-2 + BBCI after immunopurification with the F95 antibody.

(C) Histogram shows the fold change in citrullination of the 23 proteins identified as significantly citrullinated in our comparisons and the number of arginine residues identified as citrullinated in a previous study.⁹⁴

(D) Gene Ontology analysis of the 23 proteins significantly citrullinated in the LC-2 vs. LC-2 + BBCI condition and not significantly citrullinated in the control condition; for each category, the p value and combined score are reported.

(E) STRING protein-protein interaction network of proteins identified as significantly citrullinated in the analysis shown in Figure 7C. The highly interconnected mitochondrial module centered on HSPA9, HSPD1, and PHB2 is highlighted.

(F) Gene Ontology (Biological Process) enrichment analysis of proteins identified in Figure 7C, showing the significantly enriched functional categories associated with the STRING interaction network.

(G) Immunoblot validation of HSPA9 knockdown (48 h) in MIA PaCa-2 and PANC-1 cells. Intracellular ATP levels were quantified in cells transfected with control or HSPA9 siRNA and treated with the indicated KRAS inhibitors for 42 h following a 6 h recovery after transfection.

(H) Lollipop diagram shows the distribution of arginine residues identified as citrullinated following *in vitro* incubation of recombinant HSPA9 with recombinant PADI3. Citrullinated residues are mapped onto the ATPase (nucleotide-binding) and peptide-binding domains.

(I) Cartoon representation of the ADP-bound ATPase domain of HSPA9 (PDB: 6NHK) highlighting all citrullinated arginine residues (red). Arg391, the residue located closest to the ADP phosphate group (~ 6 Å), is highlighted in magenta, whereas ADP is shown in yellow.

(J) Structural model of the HSPA9 ATPase domain (PDB: 6NHK) compares the predicted interaction of Arg391 or *in silico*-citrullinated Arg391 (Cit391) with the ADP phosphate group. Electrostatic interaction energies were estimated from the shortest residue-phosphate distance using a Coulomb-based electrostatic approximation, yielding a predicted interaction energy of approximately -13.05 kcal/mol for Arg391 and ~ 0 kcal/mol following citrullination.

(K) Intrinsic tryptophan fluorescence spectra of recombinant HSPA9 and *in vitro*-citrullinated HSPA9 (2.25 μ M). The wavelength of maximum fluorescence emission (λ_{max}) is indicated.

(L) ATPase activity of recombinant HSPA9 or *in vitro*-citrullinated HSPA9 measured as inorganic phosphate (P_i) release over increasing ATP concentrations in the absence or presence of increasing stoichiometric amounts of the J-domain co-chaperone DNZL.

(M) Cell viability was measured by the resazurin assay in the indicated cell lines treated as indicated for 48 h. Data in G and M are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined using Dunn's multiple-comparison test. ** $p < 0.01$ and *** $p < 0.001$.

Functional enrichment analysis revealed that these proteins clustered into pathways controlling extracellular matrix organization, cell adhesion, apoptosis, and mitochondrial homeostasis (Figure 7D). Notably, protein-protein interaction analysis (STRING) identified a highly interconnected mitochondrial network centered on HSPA9/Mortalin, HSPD1 and PHB2, proteins that cooperate to maintain mitochondrial proteostasis and fitness (Figures 7E and 7F). Among these, HSPA9/Mortalin was prioritized for further investigation because of its hub position within the network and its established role in mitochondrial protein import, protein folding and stress adaptation.⁹⁵ HSPA9 is the mitochondrial member of the Hsp70 family of molecular chaperones, whose ATPase cycle is controlled by J-domain co-chaperones that stimulate ATP hydrolysis and coordinate substrate binding and release.⁹⁵ Recent functional genomic studies have identified HSPA9 as a critical dependency in KRAS-driven cancers.⁹⁶ Consistent with this notion, siRNA-mediated depletion of HSPA9 significantly enhanced the cytotoxic activity of both KRAS(OFF) and KRAS(ON) inhibitors in cells harboring either KRAS^{G12C} or KRAS^{G12D} mutations (Figure 7G).

Having established its functional relevance, we next asked whether HSPA9 represents a direct substrate of PADI3. Recombinant Mortalin was therefore incubated with recombinant PADI3 *in vitro* and analyzed by mass spectrometry (Figures S9A–S9G; Table S5 and S6), identifying 23 citrullinated arginine residues, the majority of which mapped to the nucleotide-binding (ATPase) domain (Figures 7H and 7I). Structural modeling identified Arg391 as a residue located within electrostatic interaction distance of the ADP phosphate group (Figure 7J). *In silico* citrullination of Arg391 abolished this interaction without altering the overall architecture of the nucleotide-binding pocket (Figure 7J). Since ADP release represents the rate-limiting step of the Hsp70 ATPase cycle,⁹⁷ these observations raised the possibility that citrullination could accelerate nucleotide turnover by destabilizing ADP retention rather than by altering nucleotide binding itself.

Consistent with this prediction, citrullinated Mortalin displayed increased intrinsic tryptophan fluorescence, indicative of altered conformational dynamics (Figure 7K). Biochemical assays further demonstrated that citrullination significantly increased ATP hydrolysis both in the absence of co-chaperones and under submaximal stimulation by the J-domain co-chaperone DNZL, whereas this difference largely disappeared under saturating co-chaperone conditions (Figure 7L; Table S7). These findings are consistent with citrullination enhancing the intrinsic ATPase cycle of HSPA9 by increasing nucleotide turnover.

To determine whether HSPA9 citrullination occurs before or after mitochondrial import, we first examined the subcellular localization of HSPA9 and PADI3 by confocal microscopy. HSPA9 localized predominantly to mitochondria, with a smaller cytoplasmic pool, whereas PADI3 was largely cytoplasmic. Partial colocalization between HSPA9 and PADI3 was observed mainly within the cytoplasm and was not altered by sotorasib treatment, although PADI3 fluorescence was markedly reduced (Figures S10A and S10B), consistent with its decreased protein abundance (Figure 2E). These observations suggested that HSPA9 is citrullinated before mitochondrial import. To directly test this hypothesis, we expressed C-terminally FLAG-tagged

HSPA9 either as the full-length precursor or as a variant lacking the mitochondrial targeting sequence (Δ MTS; residues 1–48, Figure S10C). Following PADI3 activation with the calcium ionophore A23187, both proteins were immunopurified and analyzed for citrullination. As expected, the full-length protein was detected as both precursor and mature processed species, reflecting mitochondrial processing of the targeting sequence (Figure S10D). Remarkably, both HSPA9 variants were robustly citrullinated, demonstrating that mitochondrial targeting is not required for HSPA9 modification (Figure S10D). Collectively, these findings indicate that PADI3-mediated citrullination occurs predominantly before TOM/TIM23-dependent mitochondrial import, during the cytoplasmic phase of HSPA9 biogenesis.^{95–97}

To determine whether HSPA9 represents a relevant downstream effector of PADI activity, we pharmacologically inhibited Mortalin using JG-98, a second-generation allosteric Hsp70 inhibitor that binds the nucleotide-binding domain of HSPA9 and disrupts its interaction with BAG-family nucleotide exchange factors, thereby impairing ATP/ADP cycling and chaperone function.⁹⁸ JG-98 markedly sensitized cells to both KRAS(OFF) and KRAS(ON) inhibitors, closely phenocopying the effects of BBCI (Figure 7M). Importantly, addition of JG-98 to KRAS inhibitor plus BBCI failed to produce further enhancement of cell death, indicating that both compounds converge on the same adaptive pathway (Figure 7M).

Collectively, our data identify the PADI3–HSPA9/Mortalin axis as a mechanism linking protein citrullination to mitochondrial proteostasis and adaptive resistance to KRAS-targeted therapies.

DISCUSSION

In this study, we have identified PADI1 and PADI3 as critical and previously underappreciated modulators of KRAS-driven tumor fitness and therapeutic response. Our data demonstrated that pharmacological inhibition of PADI1 and, most prominently, PADI3 strongly synergized with both KRAS(OFF) and KRAS(ON) inhibitors¹ across multiple KRAS-addicted models, including CRC and PDAC systems in two- and three-dimensional settings as well as *in vivo*. This synergy is particularly relevant in the context of acquired^{20–22} or adaptive^{22,23} resistance to KRAS-targeted therapies, where resistant models retain basal PADI1/3 expression despite efficient KRAS degradation or inhibition. These findings identify PADI1/3 as a calcium-dependent adaptive module that enables KRAS-mutant cells to tolerate pharmacological KRAS inhibition, thereby revealing a previously unrecognized non-oncogene vulnerability.

A striking feature of combined KRAS-PADI inhibition was the induction of a non-lytic cell death⁹⁹ program characterized by sustained plasma membrane integrity. Unlike classical necrosis or immunogenic cell death, this phenotype lacks DAMP release and does not trigger overt inflammatory signaling.^{88,99} Multi-omics analyses converge on an apoptotic-like program without membrane rupture, which may offer therapeutic advantages in solid tumors such as PDAC, where excessive inflammation can paradoxically promote tumor progression and therapy resistance.¹⁰⁰ Interestingly, this phenotype resembles the absence of chromatin extracellular release previously reported upon genetic ablation of PADI4,^{31,48,49} suggesting that PADIs broadly

regulate non-lytic death programs through isozyme-specific mechanisms. However, unlike PADI4 knockout, combined KRAS and PADI1/3 inhibition did not alter chromatin citrullination—a hallmark of PADI4 activity^{94,101} linked to chromatin decondensation¹⁰² and nuclear expulsion.³¹ Instead, our data indicate that PADI1/3 primarily sustain mitochondrial adaptation through selective citrullination of proteins involved in proteostasis and apoptotic control.

Our citrullinome analysis identified HSPA9/Mortalin as the principal downstream effector of the PADI-dependent adaptive response to KRAS inhibition. HSPA9 is the mitochondrial member of the Hsp70 family and plays a central role in maintaining mitochondrial proteostasis by coordinating protein import and folding through an ATP-dependent chaperone cycle.⁹⁷ Functional genomic studies have independently identified HSPA9 as an essential dependency in KRAS-driven tumors, highlighting the requirement for mitochondrial proteostasis to sustain the high metabolic demands imposed by oncogenic KRAS.⁹⁶

Here, we demonstrate that HSPA9 is directly citrullinated by PADIs and that this modification enhances its intrinsic ATPase activity. Pharmacological inhibition of HSPA9 using the allosteric Hsp70 inhibitor JG-98 phenocopied the effects of PADI inhibition and failed to further enhance the activity of BBCI, strongly suggesting that both interventions converge on the same adaptive pathway.⁹⁸ These findings identify the PADI3-HSPA9 axis as a previously unrecognized mechanism that preserves mitochondrial proteostasis during pharmacological KRAS inhibition.

Our findings considerably broaden the emerging concept that protein citrullination regulates mitochondrial fitness. Whereas recent work identified DRP1 citrullination as a regulator of mitochondrial dynamics,⁵⁰ our study demonstrates that citrullination also controls mitochondrial proteostasis by directly modulating the ATPase cycle of HSPA9. Together, these observations suggest that PADIs coordinate multiple layers of mitochondrial adaptation, including organelle dynamics and proteostasis, by modifying distinct mitochondrial effectors.

Finally, our data provide a mechanistic framework linking KRAS inhibition to this adaptive response. KRAS blockade induces SOCE-dependent calcium influx,⁷⁴ transiently activating PADI enzymes. In resistant cells, sustained activation of the PADI1/PADI3 enhancer maintains elevated PADI expression, enabling persistent citrullination of HSPA9 and continued mitochondrial adaptation despite KRAS inhibition. More broadly, our findings establish protein citrullination as a dynamic regulator of mitochondrial adaptation to targeted therapy and identify the calcium-PADI3-HSPA9 axis as a tractable therapeutic vulnerability in KRAS-driven cancers.

An important next step will be to determine whether the therapeutic benefits of combined KRAS/PADI inhibition extend to immunocompetent settings. Because protein citrullination is increasingly recognized as a regulator of anti-tumor immunity and NETosis,³⁷ future studies in syngeneic models will be required to establish how disruption of the calcium-PADI3-HSPA9 axis shapes the tumor immune microenvironment and whether modulation of neutrophil extracellular trap formation further contributes to treatment response.

Limitations of the study

Although our findings support PADI inhibition as a strategy to potentiate the therapeutic effects of KRAS-targeted therapies, some limitations should be acknowledged. Future studies should compare the efficacy of PADI inhibition across a broader range of clinically relevant KRAS(OFF) and, particularly, KRAS(ON) inhibitors to identify combinations capable of achieving more durable responses. In addition, validation in orthotopic PDAC models and genetically engineered mouse models will be important to confirm the therapeutic potential of this strategy in more physiologically relevant settings and to establish the preclinical framework required for successful clinical translation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eros Di Giorgio (eros.digiorgio@uniud.it).

Materials availability

All unique reagents generated as part of this study are available upon request from the [lead contact](#) with a completed Materials Transfer Agreement.

Data and code availability

- RNA-seq data have been deposited at Gene Expression Omnibus under accession GEO: GSE314343 and are publicly available.
- The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD073019 and are publicly available.
- This paper does not report original code. All software packages used are listed in the [key resources table](#).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

We are grateful to Claudio Brancolini (Department of Medicine, University of Udine) for helpful discussions and for providing reagents. We are grateful to Vincenzo Bronte and Francesco De Angelis (Department of Medicine, University of Verona) for providing FC1199-P3 and FC1243. We also thank all members of our labs for helpful discussions. This work was supported by AIRC (Associazione Italiana Ricerca Cancro, grant no. MFAG ID25000) to E.D.G. NextGenerationEU and Italian Ministry of University and Research (grant no. PRIN_2022) to E.D.G.; Departmental Strategic Plan (PSD) of the University of Udine-Interdepartmental Project on Healthy Ageing (2020-25); Worldwide Cancer Research grant (25-0202) to E.D.G. FA and VT were PhD student financed by Italian Ministry of Education.

AUTHOR CONTRIBUTIONS

Conceptualization, E.D.G.; methodology, F.D.E., R.P., L.D.P., M.G., F.A., L.S., R.S., D.L., and E.D.G.; investigation, M.G., F.A., L.S., L.D.P., R.S., F.D.E., A.F., and H.C.; writing – original draft, E.D.G. and L.E.X.; writing – review and editing, E.D.G., L.E.X., V.R., L.S., and R.S.; funding acquisition, E.D.G.; resources, L.B., Y.O., H.N., V.T., and P.R.T.; supervision, E.D.G., L.E.X., and V.R.

DECLARATION OF INTERESTS

E.D.G., M.G., F.A., and P.R. T. are inventors on the patent application I0214513, entitled “A combined preparation for use in the treatment of tumors characterized by an activating KRAS gene mutation” with respect to the combined use of KRAS inhibitors (KRASi) and PADI inhibitors (PADIi) in KRAS-mutant cancers.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplementary data related to this article can be found online at <https://doi.org/10.1016/j.celrep.2026.118016>.

Received: January 16, 2026

Revised: July 2, 2026

Accepted: September 1, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Actin	Merck	RRID: AB_476693
Tubulin	Santa Cruz Biotechnology	RRID: AB_2010699
GAPDH	Merck	RRID: AB_1078991
KRAS	Merck	clone 3B10-2F2
PADI1	Cayman Chemical	22997
PADI3	Cayman Chemical	24377
ERK1/2	Cell Signaling Technology	RRID: AB_390779
pERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	RRID: AB_2315112
AKT1	Cell Signaling Technology	RRID: AB_1147620
anti-pAKT1 (Ser473)	Cell Signaling Technology	RRID: AB_2315049
pAKT1 (Thr308)	Cell Signaling Technology	RRID: AB_2629447
STING	Merck	RRID: AB_2924096
HIF-1 α	R&D Systems	RRID: AB_2126514
BCL-XL	Cell Signaling Technology	RRID: AB_10695899
Histone H3	Abcam	RRID: AB_2823497
Histone H3 citrullinated (R2, R8, R17)	AbboMax	630-180
Monoclonal anti-peptidyl citrulline (F-95)	Merck	RRID: AB_11212557
Caspase-3	Abcam	RRID: AB_725745
RNF-20	Cell Signaling Technology	RRID: AB_2797708
FLAG	Merck	RRID: AB_259529
PARP-1	Cell Signaling Technology	RRID: AB_2160739
Cleaved Caspase-3	Cell Signaling Technology	RRID: AB_2341188
YY-1	Cell Signaling Technology	RRID: AB_2799676
LMNB1	Proteintech	RRID: AB_2136298
Cleaved PARP1	Proteintech	RRID: AB_331426
HSPA9/Mortalin	Sigma-Aldrich	SAB5700947
Vinculin (clone V11F9)	Merck	MAB3574
Biological samples		
BxPC-3 KRAS ^{G12C} tumors in mice	This paper	N/A
MIA PaCa-2 tumors in mice	This paper	N/A
Fc1199-P3 tumors in mice	This paper	N/A
Chemicals, peptides, and recombinant proteins		
BB-CI-amidine	MedChemExpress	HY-111347A
LC-2	MedChemExpress	HY-137516
Sotorasib	MedChemExpress	HY-114277
Adagrasib	MedChemExpress	HY-130149
RMC-9805	MedChemExpress	HY-156819
BI-2865	MedChemExpress	HY-153724
CAY10727	Cayman Chemicals	26543
Recombinant DNLZ	Cusabio	CSB-EP729194HU
Recombinant Human GRP75/HSPA9B/Mortalin	Bio-Techne	NBC1-18380
ON-TARGETplus SMARTpool	Dharmacon	NM_004134

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
CellTiter-Glo 2.0 Cell Viability Assay	Promega	RRID: SCR_017302
Apo-ONE Homogeneous Caspase-3/7 Assay	Promega	RRID: SCR_017302
CellEvent Caspase-3/7 Detection Reagent	Thermo Fischer Scientific	RRID: SCR_014441
LDH Cytotoxicity Assay Kit	Abcam	ab197004
L-Lactate Assay Kit	Merck	RRID: SCR_022429
Calcium Flux Assay Kit (Fluo-8)	Abcam	RRID: SCR_022415
GSH+GSSG/GSH Assay Kit	Abcam	ab239709
Phosphate Assay Kit - PiColorLock™	Abcam	ab270004
Anti-Citrulline Detection Kit	MilliporeSigma	RRID:AB_10097769
Deposited data		
RNA-seq	GEO Dataset	GSE314343
MS	ProteomeXchange Consortium	PXD073019
Experimental models: Cell lines and PDOs		
BJ-HTERT	ATCC	RRID: CVCL_C8QN
MIA PaCa-2	ATCC	RRID: CRL-1420
PANC-1	ATCC	RRID: CRL-1469
HEK-293	ATCC	RRID: CVCL_9867
Phoenix-Ampho	ATCC	RRID: CVCL_H716
SW480	ATCC	RRID: CCL-228
SW-620	ATCC	RRID: CCL-227
SW-837	ATCC	RRID: CCL-235
BxPC-3	ATCC	RRID: CRL-1687
FC1199P3	N/A	De Sanctis et al.
FC1243	N/A	De Sanctis et al.
PDM-96	ATCC	HCM-CSHL-0143-C20
PDM-197	ATCC	HCM-CSHL-0176-C25
Experimental models: mice	N/A	N/A
Athymic Nude-Foxn1nu female mice	Envigo	RRID:IMSR_ENVIGO:Hsd:Athymic Nude-Foxn1nu
Oligonucleotides		
HPRT_qPCR_FW:AGACTTTGCTTTCCTTGGTCAGG	This paper	N/A
HPRT_qPCR_RV:GTCTGGCTTATATCCAACACTTCG	This paper	N/A
GAPDH_qPCR_FW:CCCTTCATTGACCTCAACTACATG	This paper	N/A
GAPDH_qPCR_RV:TGGGATTTCATTGATGACAAGC	This paper	N/A
GFP_qPCR_FW:GCAAGCTGACCCTGAAGTTC	This paper	N/A
GFP_qPCR_REV:AAGTCGTGCTGCTTCATGTG	This paper	N/A
PADI1_qPCR_FW:AGGTCTTCATGGTCTACAACCG	This paper	N/A
PADI1_qPCR_RV:CATCAGTGTCTAGCGGCCAA	This paper	N/A
PADI2_qPCR_FW:ACAAAGTGGGCGTGTCTACG	This paper	N/A
PADI2_qPCR_RV:CCACCCGTGTAAGTACCA	This paper	N/A
PADI3_qPCR_FW:CCTATGCGGTGCTCTACCTC	This paper	N/A
PADI3_qPCR_RV:CGCTTGTCTACAAAGTTCCTGT	This paper	N/A
PADI4_qPCR_FW:CAGGGGACATTGATCCGTGTG	This paper	N/A
PADI4_qPCR_RV:GGGAGGCGTTGATGCTGAA	This paper	N/A
NQO1_qPCR_FW:GGTTTGGAGTCCCTGCCATT	This paper	N/A
NQO1_qPCR_RV:CCTTCTTACTCCGAAGGGTC	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
ENO1_qPCR_FW:AAAGCTGGTGCCGTTGAGAA	This paper	N/A
ENO1_qPCR_RV:GGTTGTGGTAAACCTCTGCTC	This paper	N/A
a_qPCR_FW:GGGGAGCAGGACTGAGTTAG	This paper	N/A
a_qPCR_RV:AGTTTCCACGGTCAGAGAG	This paper	N/A
b_qPCR_FW:ATGTTGGCCAGGCTAGTCTT	This paper	N/A
b_qPCR_RV:GACAAAGAGCTCGCATCCAG	This paper	N/A
c_qPCR_FW:AGGTCATGAAGTTCCAGGG	This paper	N/A
c_qPCR_RV:CCACTCAGCAGGACCTAACA	This paper	N/A
d_qPCR_FW:CAATGCCTGGTGAAGTCTCG	This paper	N/A
d_qPCR_RV:ACCCACATATCTGCAGACGT	This paper	N/A
P5_0nt_stagger: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTTTGTGAAAGGACGAAACACCG	This paper	N/A
P5_1nt_stagger: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCTT CCGATCTCTTGTGAAAGGACGAAACACCG	This paper	N/A
P5_2nt_stagger: AATGATACGGCGACCACCGA GATCTACACTC TTTCCCTACACGACGCTCTTC CGATCTGCTT GTGAAAGGACGAAACACCG	This paper	N/A
P5_3nt_stagger: AATGATACGGCGACCACCGA GATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTAGCTTGTGAAAGGACGAAACACCG	This paper	N/A
P5_4nt_stagger: AATGATACGGCGACCACCG AGATCTACACTCTTTCCCTACACGACGCTCTTC CGATCTCAACTTGTGAAAGGACGAAACACCG	This paper	N/A
P5_6nt_stagger: AATGATACGGCGACCACCGAGA TCTACACTCTTTCCCTACACGACGCTCTTC CGATCTTGACCTTGTGAAAGGACGAAACACCG	This paper	N/A
P5_7nt_stagger: AATGATACGGCGACCACCGAGA TCTACACTCTTTCCCTACACGACGCTCTTCGA TCTACGCAACTTGTGAAAGGACGAAACACCG	This paper	N/A
P7_A01: CAAGCAGAAGACGGCATACTGA GATCGGTTCAAGTGACTGGAGTTGACGCTGTG CTCTCCGATCTCCAATCCCCTCTTCAAGACCT	This paper	N/A

Software and algorithms

GraphPad Prism	GraphPad Prism	RRID: SCR_002798
FlowJo	FlowJo	RRID: SCR_008520
Excel Statistics	Microsoft	RRID: SCR_017294
Trimmomatic software (v 0.39)	Trimmomatic software (v 0.39)	RRID: SCR_011848

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines and reagents

Phoenix Ampho (CRL-3213), 293T (CRL-3216), MIA PaCa-2 (CRL-1420), PANC-1 (CRL-1469), SW480 (CCL-228), SW620 (CCL-227), SW837 (CCL-235), and BxPC-3 (CRL-1687) were obtained from ATCC (Virginia, USA). BJ-hTERT cells overexpressing HRAS^{G12V} in a 4-OHT-dependent manner, and BJ-hTERT overexpressing both HRAS^{G12V} and adenovirus E1A in a constitutive manner were previously generated and characterized.¹⁰³ Kras^{LSL-G12D/+}, Trp53^{LSL-R172H/+}, Pdx1-Cre (KPC) cells (FC1199P3 and FC1243) were provided by the laboratory of prof. Vincenzo Bronte (University of Verona, Italy). KPC cells were established from a KPC mouse, a PDAC mouse model engineered to express mutated Trp53 (Trp53^{R172H}) and Kras (Kras^{G12D}) genes selectively in pancreatic tissues.¹⁰⁴

BJ-hTERT cells were grown in Minimum Essential Medium supplemented with non-essential amino acids, 10% fetal bovine serum (FBS), penicillin/streptomycin, and 2 mM L-glutamine (all from Euroclone, Milan, Italy). The other cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine (all from Euroclone, Milan, Italy). Cells were grown at 37°C and 5% CO₂. For the experiments performed in hypoxia, cells were grown in InvivoO₂ hypoxic chamber

(Baker Ruskin, Bridgend, UK) at 37°C, 5% CO₂, and 2% O₂. Cell lines were authenticated by short tandem repeat (STR) profiling and were routinely tested for mycoplasma contamination.

CRC patient-derived organoids (PDOs) PDM-96 (HCM-CSHL-0143-C20) and PDAC PDOs PDM-197 (HCM-CSHL-0176-C25) were purchased from ATCC (Manassas, VA, USA). A total of two independent PDO models were included in the study, comprising one CRC PDO model (PDM-96) and one PDAC PDO model (PDM-197). As commercially available, pre-established PDO models were used, no human subjects or samples were prospectively recruited or allocated to experimental groups in this study; each PDO model was assigned to the experimental conditions described for the respective experiments. Organoids were embedded in Cultrex (R&D Systems, MS, USA). CRC organoids were grown in Advanced DMEM/F12 (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine (Euroclone, Milan, Italy), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 1X B-27 supplement (Gibco, Waltham, MA, USA), 10 mM Nicotinamide (Sigma-Aldrich, Milan, Italy), 1.25 mM N-acetylcysteine (Sigma-Aldrich, Milan, Italy), 50 ng/mL EGF, 100 ng/mL Noggin and 500 ng/mL R-spondin recombinant proteins (all from Pepro Tech), 3 μ M SB202190 (Sigma-Aldrich, Milan, Italy). PDAC organoids were grown in Advanced DMEM/F12 (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine (Euroclone, Milan, Italy), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 1X B-27 supplement (Gibco, Waltham, MA, USA), 10 mM Nicotinamide (Sigma-Aldrich, Milan, Italy), 1.25 mM N-acetylcysteine (Sigma-Aldrich, Milan, Italy), 50 ng/mL EGF, 100 ng/mL Noggin and 500 ng/mL R-spondin recombinant proteins (all from PeproTech, London, UK), 10 nM Gastrin (MedChemExpress, Sollentuna, Sweden), 100 ng/mL FGF-10 (SinoBiological, Beijing, China), and 50% Wnt-3A conditioned medium (produced from L Wnt-3A cells, ATCC CRL-2647). Y27632 analogue Pro survivin (Sigma-Aldrich, Milan, Italy) was added for the first days of organoids culture after thawing. Organoids were passaged 1:2/1:4 1–2 times/week.

The following chemicals were used: 1 μ M 4-hydroxytamoxifen (H6278, Sigma-Aldrich, Milan, Italy), 20 μ M 2,3-dimethoxy-1,4-naphthoquinone (ALX-420-027, Enzo Life Sciences, Farmingdale, USA), 1 μ M staurosporine (S6942, Sigma-Aldrich, Milan, Italy), 5 μ M liprostatin-1 (SML1414, Sigma-Aldrich, Milan, Italy), 5 μ M MLKL inhibitor (480073, Sigma-Aldrich, Milan, Italy), 1 μ M ABT263 (285063, Clinisciences, Guidonia Montecelio, Italy), 100 μ M cobalt chloride (202185, Sigma-Aldrich, Milan, Italy), 5 and 25 mM lactate (252476, Sigma-Aldrich, Milan, Italy), 25 mM sodium oxamate (O2751, Sigma-Aldrich, Milan, Italy). BB-Cl-amidine (HY-111347A, MedChemExpress, Sollentuna, Sweden), LC-2 (HY-137516, MedChemExpress, Sollentuna, Sweden), sotorasib (HY-114277, MedChemExpress, Sollentuna, Sweden), adagrasib (HY-130149, MedChemExpress, Sollentuna, Sweden), RMC-9805 (HY-156819, MedChemExpress, Sollentuna, Sweden), STING PROTAC (PSD-1), Erastin, BI-2865, JG-98, Ferostatin-1 (Fer-1, 1 μ M), BTP2 and z-VAD-FMK (all from MedChemExpress, Sollentuna, Sweden), CAY10727 (Cayman Chemical, Ann Arbor, MI, USA), Actinomycin D and DDP4 inhibitor (DPP4i, 10 μ M) (Sigma-Aldrich, St. Louis, MO, USA) were used at the indicated concentrations. SM-26 and HF-4 were provided by Paul Thompson (Department of Biochemistry and Molecular Biotechnology, University of Massachusetts Chan Medical School). For simplicity, in the manuscript we referred to as “untreated” the control condition, which in each experiment consisted of the vehicle used to dissolve the drugs (DMSO, ethanol, or PBS:DMSO).

Animals

Six-week-old female athymic Nude-Foxn1^{nu} mice (*Mus musculus*; Envigo, USA) were used for the *in vivo* studies. Mice were housed in individually ventilated cages (IVC system; Sealsafe Plus GM500 Cage, Green Line; Tecniplast) under controlled environmental conditions (24°C, 50–60% relative humidity, and a 12-h light/12-h dark cycle). All animals were maintained under the same housing and environmental conditions throughout the study. All *in vivo* experiments were conducted at MTTLab (Trieste, Italy) in accordance with applicable institutional and national regulations for the care and use of laboratory animals and were performed under authorization from the Italian Ministry of Health (Ministerial Authorization No. 695/2025-PR). Only female mice were used in this study. Therefore, the influence of biological sex on the experimental outcomes could not be assessed, and the potential sex-specific effects of the investigated treatments represent a limitation of the study.

METHOD DETAILS

Plasmid construction

Lenti-(BB)-EF1a-KRAB-dCas9-P2A-BlastR (#118154), lentiSAM v2 (Puro) (#92062), pLX-sgRNA (#50662) were obtained from Addgene (Watertown, MA, USA). pLenti6.3/V5-DEST PADI1 (HsCD00939893) and pLenti6.3/V5-DEST PADI3 (HsCD00940447) were obtained from DNASU (Arizona State University's Biodesign Institute, USA). pLKO Puro shCT (SHC001), pLKO Puro shPADI3_1 (TRCN0000051633), pLKO Puro shPADI3_2 (TRCN0000051634) were purchased from Sigma-Aldrich (Milan, Italy). siCT, siPADI3_1, and siPADI3_3 (PADI3 Human Pre-designed siRNA Set A, HY-RS09962) were obtained from MedChemExpress (MedChemExpress, Sollentuna, Sweden).

pBABE Puro H2B-GFP, pWZL Hygro BCL-XL, and pWZL Neo nls-mCherry were previously generated by subcloning H2B-GFP, BCL-XL and nls-mCherry in pBABE, pWZL Hygro and pWZL Neo backbones, respectively.^{105,106} pLenti-PGK-FLAG-KRAS^{G12C}, pLenti-Hygro-HSPA9-FLAG and pLenti-Hygro-HSPA9 Δ MTS(1–48)-FLAG were generated by PCR followed by restriction enzyme-based cloning into the pLenti-backbone.

Cell transfection and retroviral infection

For transient plasmid expression, transfection of 293T cells was performed with linear polyethylenimine (L-PEI, Sigma-Aldrich, Milan, Italy) (PEI:DNA 4:1), while PANC-1 and MIA PaCa-2 cells were transfected with Lipofectamine 2000 (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. For siRNA-mediated silencing, cells seeded in 6-well plate were transfected for with 148 pmol of siRNA and 5.5 μ L of Lipofectamine 2000 per well.

For lentivirus-based infection, 293T cells seeded in 60 mm plates were transfected with 10 μ g plasmid of interest, 5 μ g psPAX2, and 1.8 μ g pMD2.G using L-PEI. The virions were harvested 48 and 96 h after transfection and used to infect the cells of interest at 0.3 MOI. For retrovirus-based infection, Phoenix Amphi cells seeded in 60 mm plates were transfected with 10 μ g plasmid of interest using L-PEI, and then grown at 32°C. The virions were harvested 48 and 96 h after transfection and used to infect the cells of interest at 0.3 MOI. Infected cells were centrifuged at 1800 rpm for 1 h and incubated for 24 h at 32°C. After washing, infected cells were grown at 37°C and selected with the appropriate selectable marker.

MIA PaCa-2 cells overexpressing PADI1-V5 and PADI3-V5 were generated by lentiviral infection with pLX-sgRNA (control), pLenti6.3/V5-DEST PADI1 and pLenti6.3/V5-DEST PADI3. MIA PaCa-2 stably silenced for PADI3 were generated by lentiviral infection with pLKO Puro shCT (control), pLKO Puro shPADI3_1, and pLKO Puro shPADI3_2. MIA PaCa-2 cells stably transduced to overexpress BCL-XL were generated by retroviral infection with pWZL Hygro BCL-XL. MIA PaCa-2 cells stably transduced to overexpress H2B-GFP and nls-mCherry were generated by two consecutive retroviral infections with the plasmids pBABE Puro H2B-GFP and pWZL Neo nls-mCherry. BxPC3 FLAG-KRAS^{G12C} were generated by lentiviral infection with the plasmid pLenti-PGK-FLAG-KRAS^{G12C}-PURO. The knockdown of HSPA9 was achieved using two independent shRNAs, TRCN0000029453 and TRCN0000029449 (Merck, Darmstadt, Germany). The knockdown of NINJ1 was achieved using two independent shRNAs, TRCN0000063768 and TRCN0000063770 (Merck, Darmstadt, Germany). The transient silencing of HSPA9 was achieved using siRNAs (HY-RS06437; MedChemExpress).

Uninfected cells were subjected to negative selection using Puromycin (1.0 μ g/mL), Hygromycin B (300 μ g/mL), Blasticidin (8 μ g/mL) or G418 (500 μ g/mL).

CRISPR/Cas9-mediated transcriptional regulation of PADI1/3 locus

To modulate transcriptional activity at the PADI1/3 locus, three sgRNAs were designed using the CRISPOR tool (RRID:SCR_015935): sgRNA1: 5'-GAACATAGAGAGGTCCCCC-3'; sgRNA2: 5'-AAACCCGGAATCAGCCTACG-3'; sgRNA3: 5'-GCACATGCCTTCGCTCGCCG-3'.

These sgRNAs were cloned via oligo annealing into the lentiviral vectors lentiSAM v2 (Puro) (Addgene #92062) for transcriptional activation and Lenti-(BB)-EF1a-KRAB-dCas9-P2A-BlastR (Addgene #118154) for transcriptional repression. Lentiviral particles were produced using standard packaging protocols and used to infect MIA PaCa-2 cells. Post-infection, cells were selected and harvested for downstream expression analysis. The efficiency of CRISPR-mediated activation or repression of *PADI1/3* was evaluated by quantitative reverse transcription PCR (qRT-PCR).

RNA extraction and quantitative PCR

Total RNA was extracted by a phenol/chloroform extraction method using Tri-reagent (Sigma-Aldrich, Milan, Italy). 2.0 μ g of total RNA was retro-transcribed by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). qRT-PCRs were performed using SYBR Green chemistry (KAPA Biosystems, Wilmington, MA, USA) with transcript-specific primers (listed in Key resource table). Expression levels were normalized to housekeeping genes GAPDH and HPRT, and relative quantification was performed using the $\Delta\Delta$ Ct method.

Immunoblot analysis

Cells were lysed with 2 $\times$ Laemmli sample buffer [4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-Cl (pH 6.8), 10% 2-mercaptoethanol]. The proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted on nitrocellulose (Whatman, UK). The films were incubated with the primary antibodies indicated in Key Resource Table. Horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (Danvers, USA) and the blots were developed using Super Signal West Dura (Pierce, Waltham, MA, USA). AF660 or AF760 secondary antibodies were used for fluorescence detection (Sigma-Aldrich, Milan, Italy), and images were acquired using the Odyssey M Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Raw, unprocessed immunoblot images are presented in [Figures S11](#) and [S12](#).

Immunofluorescence and time-lapse videomicroscopy

For 3D culture immunofluorescence, CRC organoids were seeded into 8-well chamber slides (Ibidi) and treated as indicated after 3 days from seeding. Organoids were fixed with 3% paraformaldehyde (PFA) for 20 min and permeabilized with 0.3% Triton X-100 for 5 min. Actin was labeled with phalloidin-AF546 (Molecular Probes), incubated 30 min at 37°C in a humidified chamber, and nuclei were stained with Hoechst 33342 (Sigma-Aldrich, Milan, Italy). Images were taken with a Leica TCS SP8 X confocal microscope.

Time-lapse microscopy was performed at 37°C with controlled humidity and CO₂, using Leica TCS SP8 X confocal microscope. MIA PaCa-2 cells stably expressing nls-Cherry and H2B-GFP were seeded into IBIDI chamber slides (ibidi GmbH, Gräfelfing,

Germany). Twenty-four hours post-seeding, cells were subjected to the indicated treatments and immediately placed under live-cell imaging conditions. Time-lapse imaging was performed using Leica TCS SP8 X confocal microscope with a pinhole size of 1.3 Airy units. Images were acquired every 5 min, capturing z-stacks (5 slices per time point) to monitor dynamic cellular changes over time. The following excitation/emission (Ex/Em) settings were used: H2B-GFP: Ex 488 nm/Em 510–550 nm; nls-Cherry (mCherry): Ex 587 nm/Em 610–650 nm. Post-acquisition, image sequences were analyzed frame by frame using ImageJ (RRID: SCR_003070) to assess: Plasma membrane integrity loss, nuclear envelope breakdown, chromatin condensation, release of H2B-GFP from the nucleus into the cytoplasm. This analysis allowed for the temporal characterization of cell death events and nuclear dynamics in response to the applied treatments.

Flow cytometry

For flow cytometry analysis, cells were collected by trypsinization, resuspended in PBS, filtered through 70 μ m cell strainers and collected in FACS tubes. For propidium iodide (PI) incorporation, 10 μ g/mL PI was added to the single cell suspension after filtration. After incubation for 5 min, samples were acquired on BD FACSCalibur flow cytometer and data were analyzed with FlowJO software (RRID:SCR_008520). Mitochondrial membrane potential ($\Delta\Psi$ m) was assessed by staining cells with tetramethylrhodamine methyl ester (TMRM), whereas intracellular reactive oxygen species (ROS) levels were measured using DCFDA. Cells were incubated with the respective fluorescent probes according to the manufacturers' instructions, washed, and immediately analyzed.

Cell viability assay, clonogenic assay, caspase activation assay and LDH release

For resazurin reduction assays, the cells were seeded in 96-well plate and treated as indicated. Then, resazurin solution (Sigma-Aldrich, freshly diluted in DMEM at 0.15 mg/mL) was incubated for 120 min at 37°C, the fluorescence of the reduced product (resorufin) was quantified on Synergy H1 (Biotek, Winooski, VT, USA).

For ATP quantification assay, the cells were seeded in a black 96-well plate and treated as indicated. To measure ATP levels, supernatant was removed and 50 μ L of CellTiter-Glo reagent (CellTiter-Glo 2.0 Cell Viability Assay, Promega) was added on top of the cells. After mixing by pipetting and incubation for 5 min at RT, the luminescence of each culture well was measured with Synergy H1 (Biotek, Winooski, VT, USA). Synergism between the two drugs was quantified using the ZIP score, calculated with the SynergyFinder R package.¹⁰⁷

For clonogenic assay, cells were seeded in a 96-well plate and treated as indicated. After 24h treatment, cells were diluted 1:9 and seeded in a 60 mm plate; cells were left to grow for 7 days. Colonies were stained with Methylene blue (1% in 50% Ethanol) and rinsed with water. The ImageJ Colony Counter.jar plugin was used for colonies quantification.

The caspase activity was evaluated with Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA), following manufacturer's instruction. Cells were seeded in a black 96-well plate and treated as indicated. 100 μ L of Apo-ONE Caspase-3/7 Reagent (freshly prepared by mixing the substrate Z-DEVD-R110 with the provided buffer) was added into each well and the fluorescence intensity of the substrate cleaved by caspase 3/7 was recorded every 5 min for 1 h with Synergy H1 (Biotek, Winooski, VT, USA). Caspase 3/7 activity reported in the graphs corresponds to the point of v_{max} of fluorescence increase.

For *in vivo* monitoring of caspase 3/7 cleavage, MIA PaCa-2 cells were seeded in 12-well plates and allowed to adhere for 24 h. Following this incubation period, cells were treated as indicated in the manuscript. To monitor apoptosis, cells were incubated with CellEvent Caspase-3/7 Detection Reagent (Thermo Fisher Scientific, USA) at a dilution of 1:400 (excitation: 502 nm; emission: 530 nm), which selectively fluoresces upon activation of caspase-3/7, indicating apoptotic activity.

Live-cell imaging was performed using the Leica AF6000 LX fluorescence microscope. Images were acquired every 10 min over a continuous period of 48 h, allowing for real-time tracking of apoptotic progression and cellular responses to the treatments.

Plasma membrane lysis was quantified by measuring the lactate dehydrogenase (LDH) amount in cell supernatant using the LDH Cytotoxicity Assay Kit (ab197004, Abcam, Cambridge, UK), following manufacturer's instruction. Cells were seeded in a 96-well plate and treated as indicated for 72 h. 5 μ L of culture medium was added to 95 μ L of freshly prepared reaction mix in a well of a black 96-well plate, and the fluorescence intensity was acquired with Synergy H1 (Biotek, VT, USA). Cell treatment with Lysis Buffer for 30 min was used as positive control.

Lactate quantification assay and intracellular calcium quantification assay

Lactate production was evaluated with L-Lactate Assay Kit (MAK329, Sigma-Aldrich, Milan, Italy), following manufacturer's instruction. Cells were seeded in 24-well plates and treated as indicated. 20 μ L of culture medium were added to 80 μ L of freshly prepared mix, into a well of a 96-well plate. 20 μ L of lactate standards were used to generate a calibration curve. Absorbance was acquired with Synergy H1 (Biotek, USA).

The levels of intracellular calcium were evaluated with Calcium Flux Assay Kit (Fluo-8) (ab112129, Abcam, Cambridge, UK), following manufacturer's instruction. Cells were seeded in black 96-well plates and treated as indicated. To measure calcium levels, supernatant was removed and 100 μ L of Fluo-8 dye-loading solution (freshly prepared by mixing Fluo-8, HHBS and 10x Pluronic F127 Plus, and 2.5 μ g/mL of the calcium ionophore A23187 (C7522, Sigma-Aldrich) only in the indicated samples) was added on top of the cells. The fluorescence intensity of Fluo-8 loaded with calcium was recorded every 2 min for 1 h with Synergy H1 (Biotek, USA).

Calcium standards were used to generate a calibration curve. Calcium concentrations reported in the graphs correspond to the point of v_{max} of fluorescence increase.

Detection of citrullinated proteins by immunoblot and mass spectrometry

Proteins modified by citrullination were immunopurified from cell lysates prepared in a freshly supplemented hypotonic low-salt buffer (200 mM Tris-HCl, pH 7.5; 1% Triton X-100; 10 mM MgCl₂; 10 mM KCl; 20 mM CaCl₂; 1% glycerol) containing a protease inhibitor cocktail, 1 mM PMSF, 1 mM Na₃VO₄, and 1 mM NaF. Cells were lysed on ice for 10 min, after which 150 mM NaCl was added to terminate lysis. Nuclei were then sheared using an insulin syringe and clarified by centrifugation. For immunoprecipitation, 1 μg of monoclonal anti-peptidyl-citrulline antibody (F95, MABN328, Merck, Darmstadt, Germany) was used per 10 μg of total lysate. 30 μL Streptavidin-coated magnetic beads (Pierce, 88817) were employed to capture the antibody-citrullinated protein complexes. After thorough washing, proteins were eluted in 2× Laemmli sample buffer.

For immunoblot detection, a modified version of the Asada group's protocol⁹³ was used, employing the Anti-Citrulline Detection Kit (MilliporeSigma, RRID:AB_10097769). Citrulline residues were chemically derivatized using 2,3-butanedione monoxime and antipyrine under strong acidic conditions. Detection was conducted via standard Western blotting using the Anti-Modified Citrulline, clone C4 primary antibody and a goat anti-human IgG-HRP conjugate.

For mass spectrometry, 400 μg lysates were employed for each experimental condition. After in-gel trypsin digestion, peptides were resuspended in 20 μL of 0.1% formic acid and then subjected to Nano UPLC (Ultimate 3000 nano UHPLC system, Thermo Fisher Scientific, USA) MS/MS analysis. The full scan was performed between 300 and 1650 m/z at a resolution of 60,000 at 200 m/z, with the automatic gain control (AGC) target for the full scan set to 3e6. The MS/MS scan was operated in Top 20 mode using these settings: resolution 15,000 at 200 m/z; AGC target 1e5; maximum injection time 19 ms; normalized collision energy 28%; isolation window 1.4 Th; charge state exclusion: unassigned, 1, >6; dynamic exclusion 30 s. Raw MS files were analyzed and searched against the Homo sapiens protein database based on the species of the samples using MaxQuant (version 1.6.2.6). The parameters were set as follows: protein modifications were carbamidomethylation (C) (fixed) and oxidation (M) (variable); enzyme specificity was set to trypsin; maximum missed cleavages set to 2; precursor ion mass tolerance 10 ppm; MS/MS tolerance 0.5 Da. MS/MS analysis was performed by Creative Proteomics (Shirley, USA). To identify significantly citrullinated proteins and rule out potential interference from homocitrullination, we applied the following approach: i) identification of immunopurified citrullinated proteins across the three experimental conditions; ii) selection of proteins showing significant citrullination in LC-2 compared to LC-2 + BBCL (fold-change >1.3; *p* < 0.1).

In vitro citrullination assay and detection with 4MEBz-FluME probe

The assay consists of the citrullination of BAEE to BCEE by recombinant PAD enzymes, and detection is mediated by the 4MEBz-FluME probe, which exhibits a quantum fluorescence increase at 520 nm in the presence of BCEE.⁶⁹

In vitro citrullination was performed in 100 μL reactions containing: 10 μL 4MEBz-FluME probe (1 mM), 20 μL BAEE (100 mM, Merck), 20 μL TFA (Merck), 40 μL PAD buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM CaCl₂, 2 mM DTT), 10 μL BB-Cl-amidine analogues at scalar concentrations, and 1.5 μL recombinant PADI enzymes [2.2 μmol of each recombinant PADI (Merck, SRP0707), or PADI3 (Cayman Chemical, 10786)]. The reaction was incubated at 50°C for 1 h and then transferred to a Biotek plate reader for fluorescence measurement (excitation at 498 nm, emission at 520 nm). L-citrulline (stock solution 100 mM, Merck, C7629) was used as a standard and serially diluted to generate the calibration curve.

For the PADI auto-citrullination assay, 5 μmol of recombinant PADI enzymes were incubated in 50 μL of deimination buffer (50 mM HEPES, pH 7.5; 2 mM DTT) with or without 10 mM CaCl₂ at 37°C for 1 h. After incubation, samples were resuspended in sample buffer and analyzed by immunoblotting using an anti-pan-citrulline antibody (F-95, Merck).

In vitro citrullination of HSPA9 and LC-MS/MS analysis

Recombinant His-tagged HSPA9 (25 μg) was citrullinated *in vitro* in a final reaction volume of 100 μL containing 40 μL PAD reaction buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM CaCl₂, 2 mM DTT), 50 μL recombinant HSPA9, and 3 μmol recombinant GST-PADI3. Reactions were incubated at 37°C for 1 h. His-HSPA9 was subsequently purified using His-tag magnetic beads (Thermo Fisher Scientific), washed three times with PAD reaction buffer, and eluted in 2× Laemmli sample buffer. Citrullinated arginine residues were identified by LC-MS/MS following the workflow described below.

Filter-aided sample preparation (FASP)

Protein digestion was performed using the FASP protocol with minor modifications, as previously described.¹⁰⁸ Briefly, 5 μg of purified HSPA9 were loaded onto Ultracel 30-kDa MWCO centrifugal filters (Millipore), and buffer exchange was performed with three washes using 200 μL of urea buffer (8 M urea, 100 mM Tris-HCl, pH 8.5). Proteins were reduced with 50 mM DTT in urea buffer at 55°C for 30 min, followed by alkylation with 50 mM iodoacetamide for 20 min at room temperature in the dark. Samples were washed twice with urea buffer, followed by sequential exchanges with 100 mM and 50 mM ammonium bicarbonate. Proteins were digested overnight at 37°C using sequencing-grade modified trypsin (Promega) at an enzyme-to-substrate ratio of 1:50. Peptides were recovered by centrifugation, washed twice with 50 mM ammonium bicarbonate, dried under vacuum, and resuspended in 3% acetonitrile/0.1% formic acid at a final concentration of 0.1 μg/μL.

LC-MS/MS analysis

Peptides (2 μ g) were analyzed using a Vanquish™ Neo UHPLC system coupled to an Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific) equipped with an EASY-Spray ion source. Peptides were first trapped on a PepMap™ Neo C18 trap column (300 μ m $\times$ 5 mm, 5 μ m particles) and subsequently separated on an EASY-Spray PepMap™ Neo C18 analytical column (75 μ m $\times$ 150 mm, 2 μ m particles). Elution was performed at 250 nL/min using a 16-min gradient from 3% to 55% solvent B (0.1% formic acid in acetonitrile) over 11 min, followed by an increase to 99% solvent B over 5 min. Data were acquired in data-dependent acquisition (DDA) mode. Full MS scans were collected over an m/z range of 300–2,000 at 120,000 resolution, and the five most abundant precursor ions were selected for higher-energy collisional dissociation (HCD; normalized collision energy 30). MS/MS spectra were acquired at 15,000 resolution (Top5 method).

Protein identification and citrullination analysis

Raw files were processed using Proteome Discoverer™ 3.1 SP1 (Thermo Fisher Scientific) with the Sequest HT search engine. Spectra were searched against the UniProt human HSPA9 sequence (Accession P38646). Trypsin was specified as the proteolytic enzyme, allowing up to five missed cleavages to account for the inability of trypsin to cleave citrullinated arginine residues. Precursor and fragment mass tolerances were set to 10 ppm and 0.02 Da, respectively. Carbamidomethylation of cysteine residues was specified as a fixed modification, whereas methionine oxidation and arginine citrullination (+0.984 Da) were included as variable modifications.

ATPase kinetics of HSPA9

ATP hydrolysis by recombinant HSPA9 was quantified by measuring inorganic phosphate (Pi) production using the EnzChek Phosphate Assay Kit (Invitrogen). Native HSPA9, in vitro-citrullinated HSPA9, and HSPA9 supplemented with the J-domain co-chaperone DNZL were analyzed under identical experimental conditions. Reactions were performed in 50 μ L volumes in clear-bottom 96-well microplates (Thermo Scientific Nunc). Each reaction contained 2.25 μ M HSPA9 (native or citrullinated) and ATP (Merck) at concentrations ranging from 0 to 2.0 mM in ATPase assay buffer. Where indicated, DNZL was added at the appropriate molar ratio. The EnzChek detection mixture, containing MESG and purine nucleoside phosphorylase (PNP), was prepared according to the manufacturer's instructions and added immediately before data acquisition. Following a 10-min equilibration at 25°C, Pi production was monitored continuously for 90 min by measuring absorbance at 360 nm with a BioTek Synergy II plate reader. All reactions were performed in triplicate. Blank reactions lacking enzyme were included to determine background phosphate levels, and phosphate standards were used to convert absorbance values into absolute Pi concentrations. Initial reaction rates were calculated from the linear portion of the progress curves after subtraction of the corresponding blank values. Michaelis–Menten kinetic parameters were obtained by nonlinear regression using GraphPad Prism. Maximum velocity (Vmax) and the Michaelis constant (Km) were estimated directly from curve fitting, whereas the catalytic constant (kcat) was calculated by normalizing Vmax to the concentration of catalytically active HSPA9 present in each reaction.

Xenograft mouse model

Athymic Nude-Foxn1nu female mice (Envigo, USA), 6 weeks old, were housed in appropriate cages (IVC system, Sealsafe Plus GM500 cage Green line; Techniplast, Canada) and were kept in a controlled environment with controlled temperature (24°C) and 50–60% humidity, with a light/dark cycle of 12 h. All animals were subjected to the same environmental conditions. Animals were delivered to the animal facility 14 days prior to tumor cell inoculation to ensure acclimatization of the animals for the study. Mice were injected subcutaneously in the right flank with 1×10^6 BxPC-3 FLAG-KRAS^{G12C}, 2.5×10^6 MIA PaCa-2 and 2.0×10^6 FC1199-P3 cells. Tumors were allowed to grow until they reached ~ 100 mm³ and mice were then randomized to the following treatment groups: vehicle (65% PBS, 35% H₂O, 1.25% DMSO), sotorasib 10 mg/kg + vehicle, sotorasib 10 mg/kg + BBCL5 mg/kg, BBCL+vehicle, RMC-9805 10 mg/kg + vehicle, RMC-9805 10 mg/kg + HF-4 5 mg/kg, HF-4 +vehicle. Cages were clearly labeled with an ID card indicating study number, group, gender and treatment schedule. Treatments were administered intraperitoneally in a volume of 200 μ L twice per week. Tumor volumes (TV) were estimated by measuring the width (W) and length (L) of the tumor using a digital caliper and calculated based on the following formula: $TV = W^2L/2$. Mouse body weights and tumor volumes were measured twice per week over the course of each study. At the end of the experimental protocol, mice were euthanized, the tumors explanted and processed to evaluate the effectiveness of the treatment with molecular biology experiments. Furthermore, all internal organs were evaluated and inspected for any toxicities that were not obvious. *In vivo* experiments were carried out at MTTLab (Trieste, Italy) (Ministerial Authorization n. 695/2025-PR).

RNA-sequencing

For RNA-seq, MIA PaCa-2 cells were seeded in 35-mm plates and treated 24 h after seeding, as indicated, for 24 h. Total RNA was extracted by a phenol/chloroform extraction method using Tri-reagent (Sigma-Aldrich, Milan, Italy), treated with DNase I (NEB) and purified with the RNA cleanup protocol of RNeasy Mini Kit (QIAGEN). RNA-seq poly A enriched transcripts library preparation and sequencing were performed at Genewiz (Germany) following Illumina specifications. Quality control for raw sequencing reads was performed with FastQC (v0.11.9) and MultiQC (v1.09). Raw reads were clipped with Trimmomatic software (v 0.39). Reads with an overall sequence mean Phred quality lower than 28 and length <36 bp were discarded. The selected reads were mapped to the Human reference genome downloaded from Ensembl (version 107) using STAR (v

2.7.3a). Transcript assembly and quantification were done with StringTie (v 2.1.5). A Python script (prepDE.py) was used to extract all the read count information directly from the files generated in the last step. Differentially expressed genes (DEGs) were identified using DESeq2 library (v 1.46.0). The thresholds applied were log2 fold change ($|\log_2 \text{FC}| > 1$) and FDR < 0.05 . Principal component analysis was carried out with the plotPCA function from the DESeq2 package (v1.28.1). Genes with a mean raw count < 64 across replicates of each condition were removed from the analysis. Functional annotation was performed using Enrichr¹⁰⁹ (RRID:SCR_001575). Gene set enrichment analysis (GSEA, RRID:SCR_003199) and the MSigDB database (RRID:SCR_016863) were used to investigate statistically relevant biological associations. Heatmaps were generated for each gene set and the expression of each gene was expressed as log2 (fold change) of the indicated samples. The transcriptomic profiles of FC1199 and FC1242 cells were previously characterized.¹¹⁰

Chromatin immunoprecipitation and sequencing

Chromatin was extracted from MIA PaCa-2 cells grown in normoxia or hypoxia for 48 h. The DNA-protein complexes were crosslinked with 1% formaldehyde (Sigma-Aldrich, Milan, Italy) in PBS for 15 min at RT. After quenching with PBS/glycine 125 mM and washing twice in PBS, cells were collected and then lysed for 10 min with hypotonic lysis buffer [5 mM Pipes, 85 mM KCl, 0.5% NP-40] containing a protease inhibitor cocktail and 1 mM PMSF. Samples were centrifuged and pellets were resuspended in Nuclei lysis buffer [10 mM Tris-HCl pH 7.5, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS] and sonicated using Bioruptor Plus (Diagenode, Belgium) with pulses of 30 s for 30 min, resulting in an average size of ~ 300 bp for genomic DNA fragments. Samples were centrifuged and the supernatants were precleared with magnetic Dynabeads protein G (Thermo Fisher Scientific, Waltham, MA, USA). Inputs were taken and samples were then immunoprecipitated O/N at 4°C in a rotating wheel with 3 μg of anti-H3K27ac (Abcam, UK) in the presence of 8 μL of magnetic Dynabeads protein G (Thermo Fisher Scientific, Waltham, MA, USA) and ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 16.7 mM Tris-HCl pH 8, 167 mM NaCl] up to 750 μL . Immunoprecipitated complexes were washed four times with Low salt immune complex wash buffer [0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 150 mM NaCl], High salt immune complex wash buffer [0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA, 500 mM NaCl], LiCl immune complex wash buffer [250 mM LiCl, 1% NP-40, 0.2% Na deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8] and TE [10 mM Tris-HCl pH 8, 1 mM EDTA]. 100 μL of TE/RNase A (0.2 mg/mL, Ambion) were added to immunocomplexes and inputs and samples were incubated 30 min at 37°C. Beads and inputs were added in 200 μL of a 1:1 mixture of TES [50 mM Tris-HCl pH 8, 10 mM EDTA, 2% SDS] and Reverse crosslinking [250 mM Tris-HCl pH 7.5, 62.5 mM EDTA, 1.25 M NaCl] with 0.27 mg/mL Proteinase K (Euroclone, Milan, Italy) to degrade proteins, and the crosslinking was reversed with the following protocol in a thermocycler: 5 h 37°C, 10 h 68°C, 1 h 10°C. Genomic DNA was finally purified with ChIP DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA) and eluted in 30 μL water. Two biological replicates were taken from each sample and subjected to library preparation and sequencing. PE150 with at least 30 million reads per sample were obtained after NGS for each library at Genewiz (Germany). The ChIP-seq libraries were sequenced on the Illumina HiSeq 2000 platform to generate high-throughput paired-end (PE) reads. Quality assessment of the raw sequencing data was performed using the ShortRead package from the R/Bioconductor suite (RRID:SCR_005824), which allowed assessment of read quality scores, base composition and potential adapter contamination. Reads that passed quality control were aligned to the human reference genome (GRCh38, NCBI build) using Bowtie 2 (RRID:SCR_016368), allowing sensitive and efficient mapping of ChIP-enriched fragments. Peak calling was performed with MACS2 in “sharp” mode to identify regions of significant enrichment compared to the input controls. Genomic loci of interest and ChIP-seq signal traces were visualized using the Integrative Genomics Viewer (IGV, RRID:SCR_011793) to facilitate inspection of peak profiles and chromatin features. ChIP-Atlas (RRID:SCR_018900) was used to identify transcription factors binding the PADI1/PADI3 regulatory enhancer (chr1:17192615–17192926). The analysis was restricted to KRAS-mutant cell lines, and the resulting ChIP-seq tracks were exported and visualized using IGV.

CRISPR loss-of-function (LOF) screening

The genome-wide loss-of-function screening was performed using the Brunello lentiviral library (Lentiviral Prep #73179-LV, Addgene), which encodes SpCas9 and 76,441 sgRNAs. 30×10^6 MIA PaCa-2 cells were infected with lentiviral particles. After selection with puromycin, cells were divided into four treatment groups: DMSO (untreated control), 2.5 μM LC-2 + 2 μM BB-Cl, 0.5 μM adagrasib + 2 μM BB-Cl, and 0.5 μM sotorasib + 2 μM BB-Cl. After 30 h of treatment, surviving clones were expanded for five days and genomic DNA (gDNA) was collected. gDNAs were extracted with DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MA, USA), and sgRNA barcodes were PCR amplified with a mix of 8 P5 primers (forward) and P7 primer (reverse) (Key resource table). PCR reactions were purified with QIAquick PCR Purification Kit (QIAGEN, Germantown, MA, USA) and subjected to sequencing (Genewiz, Germany). Enrichment analysis of sequencing data was performed using a custom script based on the MAGeCK (Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout) algorithm (RRID:SCR_025016). The analysis aimed to identify significantly enriched sgRNAs following treatment conditions. Hits were defined according to the following criteria: Log2 fold change > 5 , indicating strong enrichment; adjusted p -value < 0.05 , ensuring statistical significance; concordance of at least 2 sgRNAs per gene, to reduce false positives and increase confidence in gene-level effects. The MAGeCK pipeline was executed in “count” and “test” modes to quantify sgRNA abundance and perform differential analysis. Normalization was applied to account for library size and sequencing depth.

QUANTIFICATION AND STATISTICAL ANALYSIS

The Student's t test was applied to compare experimental data between two groups, with a significance threshold set at $p < 0.05$. For comparisons involving more than two groups, one-way ANOVA was performed followed by Dunn's or Bonferroni post hoc multiple-comparison tests, as indicated in the corresponding figure legends. Correlations between two variables were assessed using Pearson's correlation for normally distributed data, and Spearman's correlation or the Wilcoxon signed-rank test for non-normal distributions.

Routine analyses were conducted using Microsoft Excel and GraphPad Prism, while large-scale data processing and heatmap generation utilized R/Bioconductor packages. Statistical significance was indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Unless otherwise specified, all figure data are presented as arithmetic means $\pm$ standard deviations (SD) from at least three independent experiments.