Mechanistic insights into cancer cell killing through interaction of phosphodiesterase 3A and schlafen family member 12

Xiaoyun Wu1, Gavin R. Schnitzler1, Galen F. Gao1, Brett Diamond1, Andrew R. Baker1, Bethany Kaplan1, Kaylyn Williamson1, Lindsay Westlake1, Selena Lorrey1, Timothy A. Lewis2, Colin W. Garvie2, Martin Lange4, Sikander Hayat4, Henrik Seidel4, John Doench3, Andrew D. Cherniack1,5, Charlotte Kopitz4, Matthew Meyerson1,5, and Heidi Greulich1,5*

From the 1Cancer Program, 2Center for the Development of Therapeutics and 3Genetic Perturbation Platform, Broad Institute, 415 Main Street, Cambridge MA 02412; 4Research and Development, Pharmaceuticals, Bayer AG, 13342 Berlin, Germany; 5Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston MA 02215

Running title: Determinants of cancer cell response to PDE3A modulators

*To whom correspondence should be addressed: Heidi Greulich, Broad Institute, 415 Main Street, Cambridge MA 02142; heidig@broadinstitute.org; Tel: 617-714-7475.

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Abstract
Cytotoxic molecules can kill cancer cells by disrupting critical cellular processes or by inducing novel activities. 6-(4-(Diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one, or DNMDP, is a small molecule that kills cancer cells by generation of novel activity. DNMDP induces complex formation between phosphodiesterase 3A (PDE3A) and schlafen family member 12 (SLFN12) and specifically kills cancer cells expressing elevated levels of these two proteins. Here, we examined the characteristics and covariates of the cancer cell response to DNMDP. On average, the sensitivity of human cancer cell lines to DNMDP is correlated with PDE3A expression levels. However, DNMDP could also bind the related protein, PDE3B, and PDE3B supported DNMDP sensitivity in the absence of PDE3A expression. Although inhibition of PDE3A catalytic activity did not account for DNMDP sensitivity, we found that expression of the catalytic domain of PDE3A in cancer cells lacking PDE3A is sufficient to confer sensitivity to DNMDP, and substitutions in the PDE3A active site abolish compound binding. Moreover, a genome-wide CRISPR screen identified the aryl hydrocarbon receptor interacting protein (AIP), a co-chaperone protein, as required for response to DNMDP. We determined that AIP is also required for PDE3A-SLFN12 complex formation. Our results provide mechanistic insights into how DNMDP induces PDE3A-SLFN12 complex formation, thereby killing cancer cells with high levels of PDE3A and SLFN12 expression.

Introduction
Despite advances in targeted therapies and immunotherapies, cancer remains a leading cause of human mortality (1), indicating a need for new therapeutic modalities. We recently discovered a compound, DNMDP, which selectively kills cancer cells using a unique mechanism of action (2). DNMDP (6-(4-(diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one) is active against a subset of cancer cell lines across cell lineages, with nanomolar potency and no obvious toxicity in a panel of nontumorigenic cell lines. Elevated expression of phosphodiesterase 3A (PDE3A) is the strongest genomic correlate of DNMDP sensitivity (2). DNMDP binds PDE3A and inhibits its enzymatic activity; however, enzyme inhibition alone is not sufficient for cell death. Most PDE3A inhibitors instead prevent cell killing by DNMDP when introduced in combination, possibly by competing away the binding of DNMDP to PDE3A (2). PDE3A knockout moreover abrogates the sensitivity of HeLa cells to DNMDP (2). Taken together, these findings suggest that DNMDP has a gain-of-function or neomorphic effect on PDE3A (2).

Consistent with the hypothesized gain or change of function, DNMDP induces formation of a novel complex between PDE3A and the poorly-characterized protein, schlafen family member 12 (SLFN12) (2). Similar to PDE3A, elevated expression of SLFN12 correlates with, and is required for, DNMDP sensitivity (2). These observations suggest that DNMDP induces cancer cell death through a mechanism that is fundamentally different from the cancer cell dependencies exploited by typical targeted therapies. The cyclic nucleotide phosphodiesterase, PDE3A, is well-characterized with regards to its ability to hydrolyze the phosphodiester bonds of cAMP and cGMP in order to regulate and limit cellular responses to G-protein coupled receptor activation (3). More recently, evidence has also arisen for a role in hydrolysis of cUMP (4). Conversely, very little is known regarding SLFN12 function, although it may play a role in cell proliferation or differentiation (5-8). The molecular determinants of DNMDP response have not yet been explored.

Here, we define the determinants of cancer cell response to DNMDP. We characterize partial sensitivity at the single-cell level, investigate whether PDE3B can functionally substitute for PDE3A, and define the domains of PDE3A required for sensitivity. We furthermore use genome-wide CRISPR screening to identify additional genes required for DNMDP sensitivity. Results from these experiments indicate a central role for PDE3A protein expression levels in predicting degree of DNMDP response and uncover AIP as a critical player in DNMDP-induced cancer cell killing.
Results

PDE3A and SLFN12 expressing cell lines exhibit a gradient of sensitivity to DNMDP

We have shown that PDE3A and SLFN12 expression levels together serve as a predictive biomarker for DNMDP sensitivity (2). Our previous analysis of sensitivity data from 766 cancer cell lines defined the positive predictive value (PPV) of this combined biomarker to be about 50%, with “sensitive” defined by an AUC equivalent to 1.6 on a scale of 0-4 (2). In other words, among biomarker-positive cell lines, about half are sensitive to DNMDP. We took two measures to further optimize PDE3A and SLFN12 expression as a predictive biomarker. First, we quantified gene expression using newly available RNA sequencing data from the Cancer Cell Line Encyclopedia (9), which provided greater resolution in the low expression range. Second, we more rigorously defined the optimal biomarker thresholds by maximizing the geometric mean of the sensitivity and the PPV over all possible biomarker thresholds (Figure S1A). We found that the optimal expression thresholds for PDE3A and SLFN12 in this cell line panel were 2.65 and 1.47 log_2(RPKM+1), or 5.28 and 1.77 RPKM, respectively, resulting in a PPV of 62.5% and a sensitivity of 71.4% (Figure S1B).

This PPV is comparable to the originally-reported 50% objective response rate of HER2-positive metastatic breast cancer patients to the clinically-approved targeted therapy, trastuzumab, in combination with chemotherapy (10). However, we sought to better understand the less-than-perfect prediction power of these two expression markers alone, indicating the influence of additional factors. To distinguish between these two possibilities, we systematically assessed DNMDP response in 23 cell lines with PDE3A expression >5.28 RPKM and SLFN12 expression >1.77 RPKM with 18-point dose resolution, ranging from 0.26 nM to 3 µM (Table 1). We found good concordance between these results and AUCs from the published high-throughput data (2) (Figure S1C). However, there was no correlation between AUC values and the expression levels of other phosphodiesterase family members or basal cAMP levels in these cell lines (Supplementary Dataset 1).

These experiments revealed that sensitivity is not binary (Figure 1A); rather, dose response curves showed a continuous gradient of inhibition response across the retested lines. Based on maximum viability values, the tested cell lines could be split into strongly sensitive cell lines (13 cell lines with <25% maximum viability), partially sensitive lines (8 cell lines with 25-75% maximum viability), and insensitive cell lines (2 cell lines with 100% maximum viability). Individual cell fate analysis revealed a mix of single cell responses underlying this gradient of cellular response (Figure 1B). For example, in the highly sensitive glioblastoma cell line, GB1, most individual cells underwent apoptosis within 72 h, and the remaining surviving cells apotosed by about 96 h (Figure S2). In the partially sensitive cell line, TE4, many cells underwent apoptosis by about 96 h, but several cells survived beyond 96 h, exhibiting a cytostatic phenotype (Figure S2). In the more resistant cell line, NCI-H2172, many cells survived and continued to proliferate until the end of the experiment (Figure S2). Washout experiments furthermore revealed that, whereas the most sensitive cell lines underwent full apoptosis even after compound removal at 72 h, many partially sensitive cells recovered and continued to proliferate (Figure 1C).

Two cell lines, HCC15 and UACC257, expressing high levels of PDE3A and SLFN12 mRNA, were curiously completely insensitive to DNMDP (Table 1 and Figure 1A). We examined the expression of PDE3A in these two cell lines, and found that the HCC15 cells expressed very little PDE3A mRNA and no detectable PDE3A protein despite high PDE3A RPKM values in the Cancer Cell Line Encyclopedia dataset (9) (Figure 2A). Ectopic expression of PDE3A in the HCC15 cells conferred response to DNMDP, confirming that the lack of DNMDP response was due to a lack of PDE3A expression (Figure 2B). The UACC257 cells did express PDE3A protein (Figure 2A), indicating that there must be another explanation for their lack of response (see Figure 9, described below).
One additional cell line, CAL51, was similarly initially classified as biomarker-positive, based on mRNA expression, but was only slightly sensitive to DNMDP, with 75% viability at the bottom of the dose response curve (Table 1 and Figure 1A). Upon examination of genomic sequencing data from the Cancer Cell Line Encyclopedia (9), we found that the CAL51 cells harbor a heterozygous 1 bp deletion in SLFN12, shifting the reading frame after amino acid F185 and causing an early translation stop at amino acid 196 (Figure 3A, Figure 3B). We confirmed this finding by PCR-amplifying the relevant region from genomic DNA and Sanger sequencing the PCR products (Figure 3A, Figure 3C).

For the remaining biomarker-positive cells, the level of PDE3A protein expression (Figure 4A) correlated with RNA expression (Figure 4B) and DNMDP sensitivity, as demonstrated by the inverse correlation with DNMDP AUC (Figure 4C). There is no antibody available that recognizes endogenous SLFN12 protein, so it was not possible to perform a similar analysis for SLFN12. However, no continuous correlation was observed between DNMDP AUC and SLFN12 mRNA expression (data not shown).

**PDE3B can also mediate DNMDP sensitivity**

Two cancer cell lines, T-cell lymphoma HUT78 and melanoma cell line RVH421, were found to be sensitive to DNMDP in the absence of PDE3A expression (Table 1). We hypothesized that PDE3B, which is homologous to PDE3A in the catalytic domain, might substitute for PDE3A in these cells to support DNMDP cancer cell killing. Consistent with this idea, the cytotoxic response of HUT78 and RVH421 cells to DNMDP was competed away by trequinsin, suggesting a PDE3-mediated mechanism of response (Figure 5A). RNA sequencing data indicated that these two cell lines express PDE3B mRNA (Table 1), and immunoblotting analysis confirmed that both express high levels of PDE3B but not PDE3A protein (Figure 5B), suggesting that PDE3B could be the target for DNMDP in HUT78 and RVH421 cells.

To functionally investigate the role of PDE3B in the response to DNMDP, we knocked out PDE3B in RVH421 cells. Knockout of PDE3B in RVH421 cells abolished DNMDP sensitivity, confirming that PDE3B is necessary for sensitivity in these cells (Figure 5C). We then asked whether PDE3B can complement PDE3A function and restore sensitivity in PDE3A knockout cell lines. Ectopic expression of the PDE3B cDNA fully restored DNMDP sensitivity to the PDE3A-knockout melanoma cell line, A2058, and PDE3A-knockout cervical cancer cell line, HeLa (Figure 5D and Figure S3A). Taken together, these results indicate that PDE3B can function in place of PDE3A to mediate DNMDP response. However, inclusion of PDE3B expression level in the optimized biomarker of DNMDP response improved biomarker sensitivity but not PPV (Figures S3B, S3C) when compared to PDE3A alone with SLFN12 (Figure S1B). This indicates that there are cancer cell lines that express high levels of PDE3B and SLFN12 but do not respond to DNMDP (Figure S3C). Nonetheless, the ability of PDE3B to replace PDE3A in DNMDP response suggests that the catalytic domain, which is highly conserved between the two family members, may be the determinant for DNMDP sensitivity.

**The PDE3A catalytic domain is sufficient to confer DNMDP sensitivity**

The full-length PDE3A protein consists of two membrane association regions at the N-terminus followed by a catalytic domain that is conserved across phosphodiesterases (11-13). Interestingly, PDE3A undergoes alternative translation and exists as a set of protein isoforms that represent different N-terminal truncations. These naturally-occurring truncations affect one or both of the membrane-associated regions and multiple kinase phosphorylation sites (12,14,15), resulting in different subcellular localizations and modes of regulation (14-19).

We generated a series of V5-tagged, N-terminally truncated cDNA expression constructs representing two of the shorter PDE3A isoforms, as well as truncations that more closely isolate the catalytic domain (Figure 6A). The ability of the PDE3A deletion constructs to restore DNMDP sensitivity of PDE3A-knockout A2058 cells was tested. All truncation constructs fully restored DNMDP sensitivity in these cells, including the...
shortest construct, comprised of amino acids 668-1106 (Figure 6B). This suggests that the isolated catalytic domain of PDE3A is sufficient to mediate DNMDP sensitivity.

Since the cytotoxic effects of DNMDP can be competed away by trequinsin, a potent PDE3A/PDE3B inhibitor that does not have cancer cell killing activity (2), and because the catalytic domain is the only region of PDE3A required for the activity of DNMDP, we hypothesized that DNMDP binds to the active site of PDE3A, within the catalytic domain. To test this hypothesis, we generated PDE3A active site mutants H840A, D950A, and F1004A, which are impaired for substrate binding, and H752A, affecting Mg\(^2+\) coordination, homologous to residues reported for PDE3B (20). We found that all four mutants exhibited impaired binding of and response to DNMDP (Figure 7A, Figure 7B, Figure 7C). In contrast, three point mutations, Y807A, Y814F, and C816A, in the 44-amino acid catalytic domain insert unique to the PDE3 family (21), had no effect on the ability of PDE3A to bind DNMDP and support cancer cell killing (Figure 7A, Figure 7B, Figure 7C). Consistent with the survival assay results, a non-mutated C-terminal construct of PDE3A encoding primarily the catalytic domain retained the ability to bind DNMDP (Figure 7C).

Taken together, these data indicate that the catalytic domain of PDE3A is necessary and sufficient for mediating DNMDP sensitivity, and the active site of the PDE3A catalytic domain is required for DNMDP binding, although inhibition of enzymatic activity is likely not the mechanism of cancer cell killing by DNMDP (2).

**A genome-wide CRISPR knockout screen for resistance to DNMDP uncovers AIP**

In order to identify additional genes required for DNMDP cancer cell killing, we performed a genome-wide CRISPR loss-of-function screen in HeLa cells treated with DNMDP or DMSO. We identified sgRNAs that were consistently enriched under DNMDP treatment, compared to DMSO treatment, suggesting cell survival in the presence of DNMDP. The sgRNAs best supporting survival in the presence of DNMDP were those targeting the Aryl Hydrocarbon Receptor Interacting Protein (AIP) (Figure 8A, Supplementary Dataset 2). As expected, SLFN12 and PDE3A knockout also strongly supported HeLa cell survival in the presence of DNMDP, ranking second and third behind AIP, respectively. Knockout of the histone acetyltransferase complex protein, transformation/transcription domain associated protein (TRRAP), exhibited a significant but much weaker resistance phenotype (Figure 8A, Supplementary Dataset 2).

AIP is an HSP90 co-chaperone that is known to interact with several other proteins, regulating protein stability and subcellular localization (22). Interestingly, AIP is also a tumor suppressor gene in familial isolated pituitary adenoma (FIPA) (23). We confirmed that AIP knockout eliminates HeLa cell response to DNMDP by validating with independent sgRNAs (Figure 8B). Similar results were observed upon knockout of AIP from the melanoma cell line, A2058 (Figure 8B).

To determine whether AIP knockout impacts DNMDP response upstream or downstream of PDE3A-SLFN12 complex formation, we tested the effects of AIP knockout directly on complex formation. We ectopically expressed V5-tagged SLFN12 in parental or AIP-knockout HeLa cells, immunoprecipitated endogenous PDE3A, and assessed whether we could detect V5-SLFN12 in the PDE3A immunoprecipitates. AIP knockout completely abolished PDE3A-SLFN12 complex formation in response to DNMDP (Figure 8C), confirming that AIP functions upstream of DNMDP-induced complex formation.

Among the biomarker-positive cell lines, the melanoma cell line, UACC257, was conspicuously insensitive to DNMDP (Table 1). When we examined AIP expression across 1019 cancer cell lines (9), we found that UACC257 was the only cell line that did not express AIP (Figure 9A). Ectopic expression of AIP sensitized the UACC257 cells to DNMDP (Figure 9B), indicating that loss of AIP was a major determinant of resistance of these cells. Interestingly, ectopic expression of PDE3A also increased sensitivity of the UACC257 cells to DNMDP (Figure 9B), suggesting that loss of AIP can be compensated for by an increase in PDE3A levels. Taken together, our results demonstrate an essential role for DNMDP-induced and AIP
facilitated PDE3A-SLFN12 complex formation in cancer cell death in response to treatment of cancer cells with DNMDP (Figure 9C).

Discussion

Predictive biomarkers have become an essential component of cancer treatment, enabling identification of the patients most likely to respond to a given therapy. We have identified a new method of cancer cell killing involving complex formation between PDE3A and SLFN12. Because sensitive tumor cells are found across cancer lineages, predictive biomarkers will play an especially important role in patient identification if DNMDP-like molecules that induce PDE3A-SLFN12 complex formation are evaluated in the clinic.

We previously showed that DNMDP response is dependent upon expression of PDE3A and SLFN12 (2). Optimization of the mRNA expression thresholds for these two proteins resulted in a positive predictive value (PPV) of 62.5% (Figure S1), with expression level of PDE3A protein being a major determinant in degree of response (Figure 4). It is likely that establishment of a protein-based biomarker assay would further improve PPV, as mRNA expression may not precisely reflect protein levels. For example, we used genomic techniques to uncover a frameshift mutation of one SLFN12 allele in the CAL51 cell line that is likely to result in decreased overall expression of SLFN12 protein, explaining the cryptic resistance in this seemingly biomarker-positive cell line (Figure 3). However, a protein-based biomarker assay awaits development of a suitable antibody for SLFN12.

Other factors could also contribute to a biomarker with increased PPV. Although PDE3B was found to support DNMDP sensitivity in the absence of PDE3A expression (Figure 5), and PDE3B expression was essential for response of two biomarker-negative but DNMDP-sensitive cell lines, inclusion of PDE3B did not improve biomarker PPV (Figure S3). We identified another gene, AIP, more broadly required for DNMDP sensitivity (Figure 8). AIP acts upstream of DNMDP-induced PDE3A-SLFN12 complex formation. A second biomarker-positive but DNMDP-insensitive cell line, UACC257, was found to express no AIP, and ectopic expression of AIP conferred sensitivity (Figure 9). Thus, although it appears to be only rarely deleted, AIP expression could be considered as a component of a further improved biomarker.

We found that the catalytic domain of PDE3A was sufficient for complex formation and DNMDP sensitivity, and that mutations in the active site of PDE3A abolish DNMDP response (Figure 6, Figure 7). However, although DNMDP is a PDE3A inhibitor, inhibition of phosphodiesterase activity does not correlate with complex formation or cancer cell killing (2). It is possible that some early and more recent reports of PDE3A dependency in cancer cells may in fact also involve PDE3A-SLFN12 complex formation (24-28), as zardaverine and anagrelide phenocopy DNMDP (2,29). Others have subsequently also observed sensitivity of cancer cells to DNMDP (30) and induction of PDE3A-SLFN12 complex formation (31).

Unlike traditional targeted inhibitors that leverage dependencies in cancer cells created by genomic alterations, DNMDP instead initiates induced cell death in cancer cells expressing elevated levels of PDE3A and SLFN12 by a likely gain-of-function or change-of-function mechanism. DNMDP initiates these processes by binding to PDE3A and inducing PDE3A-SLFN12 complex formation. Here, we defined the factors that influence PDE3A-SLFN12 complex formation and response to DNMDP; however, elucidation of the downstream mechanism of action of DNMDP requires further study. Despite this open question, DNMDP-like molecules may represent a new therapeutic opportunity for cancer patients with tumors expressing elevated levels of PDE3A and SLFN12.

Experimental Procedures

Cell culture and viability assays
To measure cancer cell death in response to DNMDP treatment, cells were plated in 384-well assay plates at the following cell density per well: 500 cells of HeLa (DMEM), A2058 (DMEM), HMCB (EMEM), IGR37 (DMEM), NCIH1734 (RPMI), 750 cells of CAL51 (DMEM), COLO741 (RPMI), DKMG (RPMI), GB1 (EMEM), HEL (RPMI), HEL9217 (RPMI),
JHUEM1 (DMEM+F12), L3.3 (RPMI) and TE4 (RPMI), HCC15 (RPMI), UACC257 (RPMI), 1000 cells for HUT78 (IMEM), NCIH1563 (RPMI), NCIH2122 (RPMI), NCIH2172 (RPMI), RVH421 (RPMI) and SKMEL3 (McCoy’s 5A), 1500 cells for C32 (EMEM), HS578T (DMEM) and JHOM1 (DMEM+F12). Cells were incubated at 37°C overnight and then treated with a DNMDP dose dilution series using an HP D300 digital dispenser. For experiments involving trequinsin competition, 100nM trequinsin was also added to each well at the same time of DNMDP addition. After 72 h, the viability of cells in each well were measured by Cell Titer Glo (Promega G755B and G756B). Percent viability values were determined using the values from untreated wells and AUC values were calculated using a 4-parameter fit. X-axis values are reported in log 10 M. IC50s and maximal killing values for all dose response curves are listed in Supplemental Table S2. DNMDP was purchased from Life Chemicals (F1638-0042) and trequinsin was purchased from Sigma-Aldrich.

**Single cell fate mapping**

1400 cells per well were seeded in a 96 well plate in media that had been centrifuged at 500 x g for 5 min to remove particulates. The next day, the red fluorescent DNA-staining dye, Incucyte Nuclite Rapid Red, and green fluorescent apoptosis dye, Incucyte Caspase-3/7 Green Apoptosis Reagent (Essen Biosciences), were added in 2 µl FBS to a final concentration of 1:1000 and 1:1500, respectively. Two hours later, [2 µM DNMDP + 0.2% DMSO] or 0.2% DMSO was added. Because even sensitive cells sometimes divided before 24 hours, cells were tracked starting at 24 h, although cells that apoptosed before 24 h were also counted. For the washout study, the media was removed from DNMDP-treated cells at 72 h, the cells were rinsed with media, and incubation was continued in the absence of DNMDP. Cells were tracked starting at 72 h. Images were taken every 1 h up to 96 h, and every 2 h thereafter, with an Incucyte S3 machine (Essen Biosciences). Three channels were recorded: phase contrast, red fluorescence (DNA), and green fluorescence (apoptosis). For cell tracking, a movie superimposing all three channels was analyzed. To avoid effects due to depletion of media components over time, cells were followed up to the last hour before DMSO control cells started to show slowed division or increased apoptosis (136 h for HeLa, 194 h for SKMEL3, 160 h for GB1, 130 h for TE4, 130 h for A2058, 144 h for DKMG, 106 h for HS578T, 186 h for H2172, 220 h for C32).

**Cell lysis and immunoblotting**

Cells were plated in 10 cm petri dishes and collected at 50-90% confluence. For PDE3A immunoblotting in biomarker positive cells and in AIP KO cells, cells were seeded in 15cm plates at similar density as in viability assays with a vessel scaling factor of 5000, e.g., 500 cells per well was scaled to 10^6 cells per 10cm plate or 2.5x10^6 cells per 15cm plate, and then cultured for 72 hours before collection. Cell pellets were lysed at 4°C for 20 minutes in modified RIPA buffer (150 mM NaCl, 10% glycerol, 50 mM Tris-Cl pH 8.0, 50 mM MgCl2, 1% NP-40) supplemented with EDTA-free protease inhibitors (Sigma-Aldrich 4693159001) and PhosSTOP phosphatase inhibitors (Sigma-Aldrich 4906837001). Lysates were clarified by centrifugation at 13,000 rpm x 10 min at 4°C and quantified using BCA protein assays (Thermo Fisher Scientific 23225). Clarified lysates were resolved on 4–12% Bis-Tris PAGE gels, transferred to nitrocellulose membranes (Thermo Fisher Scientific IB23001) and immunoblotted with primary antibodies against PDE3A (Bethyl A302-740A, 1:2000), PDE3B (Bethyl A302-743A), V5 (Life Technologies R96205 at 1:5,000), AIP (Thermo Fisher Scientific MA3-16515 at 1:2000), Vinculin (Sigma-Aldrich V9264 at 1:5,000), GAPDH (Cell Signaling Technology 2118 at 1:2000) and secondary antibodies from LiCOR Biosciences (92632210 and 926068021, each at 1:10,000). Blots were washed and imaged using a LiCOR Odyssey infrared imager, and fluorescent signals quantified using the Image Studio software provided by the LiCOR manufacturer. See Figure S1D for full-length PDE3A and PDE3B immunoblots indicating sufficient antibody specificity.

**RNA extraction and real-time qRT-PCR**

To measure PDE3A mRNA expression levels in cell lines, cells were collected and total RNA was
made using Monarch total RNA miniprep kit (New England Biolabs T2010S), reverse transcribed using the SuperScript III First-Strand synthesis system (Thermo Fisher Scientific 18080051). Parallel negative control reactions were carried out by leaving out the reverse transcriptase. TaqMan real-time PCR was performed on the cDNA products for PDE3A (Hs01012698_m1) and GAPDH (Hs02758991_g1) and relative gene expression levels were calculated as negative delta CT values between sample and respective no reverse transcriptase control.

cDNA ectopic expression
ORF overexpression constructs were obtained from TRC consortium: PDE3A (ccsbBroad304_06701), PDE3B (ccsbBroad304_06702), GFP (ccsbBroad304_99997) and AIP (BRDN0000465180), for which the K228 snp was corrected to Q228 to match UniprotKB protein sequence (Accession O00170). For lentivirus-based gene delivery, 293T cells were transfected with ORF overexpression constructs and packaging plasmids psPAX2 and pMD2.G. Virus was collected 48 h after transfection and added to target cells for spin infection with 4 or 8 µg/ml of polybrene. Transduced target cells were selected using 15 µg/ml blasticidin and then expanded for 3 days before plating into 384 well plates for DNMDP sensitivity testing.

Assay for SLFN12 F185 frameshift mutation status in cell lines
Genomic DNA was isolated from cells using QIAamp DNA mini kit (Qiagen 51304) and SLFN12 genomic region was amplified by PCR using Q5 High-Fidelity 2X Master Mix (New England Biolabs M0492) and primers SLFN12_2_F or SLFN12_428_F and SLFN12_858_R. PCR products were purified using QIAquick PCR Purification Kit (Qiagen 28104) and send for sequencing using Forward or Reverse primers used for PCR. Sequencing reads were aligned to reference sequence using Benchling alignment tools.

CRISPR knockout
PDE3A CRISPR KO cells (sgRNA#2) were generated according to de Waal et al, 2016 (2). CRISPR target sites for PDE3B and AIP were identified using the CHOPCHOP CRISPR Design Tool (chopchop.cbu.uib.no). Oligo sequences are listed in Supplementary Table S3. For cloning of sgRNAs, forward and reverse ologs were annealed, phosphorylated and ligated into a BsmBI-digested lentiCRISPRv2 vector. Lentivirus carrying each guide construct was packaged as described above and used to infect target cells. Transduced target cells were selected using 1 µg/ml puromycin and passaged for 7 days before use.

Site-directed mutagenesis
A pDONR-PDE3A plasmid from the TRC consortium (ccsbBroadEn_06701) was used to generate mutated PDE3A ORFs. Primers used for mutagenesis are listed in Supplementary Table S3. PDE3A deletion mutant and H752A point mutant expression constructs were generated using GeneArt Site-Directed Mutagenesis PLUS system (Thermo Fisher Scientific A14604) while point mutants Y807A, C816A, Y814A, H840A, D950A, and F1004A were generated by recombination of PCR products (32). Mutated ORFs were then shuttled into the pLX304 lentiviral expression vector using Gateway LR Clonase (Thermo Fisher Scientific 11791020). To generate the SNP-corrected AIP expression construct, the AIP ORF was shuttled into pDONR223 using Gateway BP Clonase (Thermo Fisher Scientific 11789020), mutagenized using the GeneArt Site- Directed Mutagenesis PLUS system and then shuttled back into pLX304 using Gateway LR Clonase. All plasmids were sequence-verified along the entire ORF length for the presence of desired mutation and the absence of additional unwanted mutations.

Linker resin pulldown
To measure the binding of DNMDP to wild type and mutant PDE3A proteins, A2058 PDE3A KO cells stably transduced with lentiviral ORF overexpression constructs were harvested. Linker resin pulldown experiments were performed as described previously (2). Briefly, cells were lysed in modified RIPA lysis buffer.
(150 mM NaCl, 10% glycerol, 50 mM Tris-Cl pH 8.0, 50 mM MgCl2, 1% NP-40) supplemented with EDTA-free protease inhibitors (Sigma- Aldrich 4693159001) and PhosSTOP phosphatase inhibitors (Sigma-Aldrich 4906837001). 200 µg of total protein (0.5 mg/ml, 400 µl) was incubated with 3 µl DNMDP-2L affinity linker resin (2) with or without 10 uM trequinsin for four hours at 4°C. Beads were then washed three times with lysis buffer and proteins bound were eluted with 50 µl LDS sample loading buffer with reducing agent added (Thermo Fisher Scientific NP0007 and NP0009). 20 µl of each eluted sample and 50 µg of corresponding matched input lysate (50% input) were separated on 4–12% Bis-Tris PAGE gels (Thermo Fisher Scientific) and immunoblotted with anti-PDE3A antibody (1:1,000, Bethyl A302-740) as described above.

Genome-wide CRISPR screen
The Brunello CRISPR KO library (33) was used for the DNMDP resistance screen. Lentiviral infection was carried out in duplicate and for each replicate with enough HeLa cells to achieve >1000 infected cells per library member (80000 sgRNAs, >8x10⁷ cells total) and at low MOI to achieve transduction of a single sgRNA per cell. Infection efficiencies for the two replicates were 24% and 31% respectively, corresponding to an MOI of about 0.3, meaning about 85% of infected cells would be predicted to have single sgRNA integration (34). At the time of infection, HeLa cells were resuspended in media and mixed with Brunello library virus in the presence of 8 µg/ml polybrene (library lentivirus provided by the Genetic Perturbation Platform at the Broad Institute), plated in 12 well dishes at 3x10⁶ cells per well, and spun at 931 x g for 2 h at 30°C. 2 h after the spin infection, virus-containing media was removed and fresh media was added for incubation overnight. The day after the infection, cells were trypsinized and pooled into T225 flasks at 50% confluence (8x10⁶ cells per flask) for three additional days to allow CRISPR KO to complete. Cells were collected at 8 days after infection, and 8x10⁷ cells each were split into DMSO control arm (plating at 8x10⁶ cells per T225 flask) or 25 nM DNMDP treatment arm (plating at 2x10⁷ cells per T225 flask). Cells were passaged every 3 to 4 days at 25% confluence for the next 14 days. For the DMSO arm, 8x10⁷ cells were maintained at every passage, whereas all surviving cells were passaged for the DNMDP arm. After 14 days of compound treatment, cells were harvested, washed with cold PBS and flash frozen at 2x10⁷ cells (DMSO arm) or less portions for genomic DNA isolation. Genomic DNA was isolated using the Nucleospin Blood XL kit (DMSO samples, 4 preps to cover 8x10⁷ cells, Machere-Nagel 740950.50) or the QIAamp DNA Blood Mini kit (DNMDP-treated samples, Qiagen 51104). PCR amplification of sgRNA tags and pooled library sequencing were carried out as described in Sanson, et al. (33).

CRISPR screen data analysis was done largely as described in Sanson et al. (33). Briefly, deconvolution of sequencing reads yielded read counts for each sgRNA under each replicate treatment condition. Log2-Normalized-Reads for each guide per condition was calculated using the formula log2 (guide/total*1000000+1) and averaged across the two replicates. Subtracting DMSO values from those for 25 nM DNMDP generated Log-Fold-Change values for each sgRNA, which were then averaged across all sgRNAs targeting the same gene to generate gene-level Average-Log-Fold-Change score. To statistically evaluate gene-level enrichment in DNMDP treatment relative to DMSO, sgRNAs were rank ordered based on Average-Log-Fold-Change, and p-values for each sgRNA relative to the rank order were determined by running a hypergeometric distribution without replacement, equivalent to a one-sided Fisher’s exact test. The average of the negative log10 p values for each sgRNA targeting the same gene was calculated to generate the average negative log10 p-value for each gene. A volcano plot was generated using the average-log2-fold-change and the average negative log10 p-value for all genes with 3 to 8 sgRNAs per gene to visualize gene enrichments.
after the positive selection of 25 nM DNMDP treatment.

**Transient transfection and immunoprecipitation**

HeLa or HeLa AIP KO cells were transfected with ORF overexpression constructs expressing V5-tagged SLFN12 (TRC consortium, TRCN0000476272) using the Fugene transfection reagent (VWR E2691). At 72 hours post transfection, cells were treated with 10 μM DNMDP or DMSO for 8 h and collected and snap frozen until lysis. Cell pellets were lysed as described above. 3 mg of total protein was incubated with 4 μg of anti-PDE3A antibody (Bethyl 302-740A) at 4°C overnight, followed by addition of 20 μl each of Protein A- and Protein G-Dynabeads (Thermo Fisher Scientific 10001D and 10003D) and additional incubation for 2 h. Beads were washed and bound proteins were eluted with 20 μl of LDS sample loading buffer (Thermo Fisher Scientific NP0007) with reducing agent added (Thermo Fisher Scientific NP0009) and subject to SDS-PAGE separation and anti-PDE3A and anti-V5 immunoblotting as described above.

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**Conflicts of Interest**

Several authors on this study received funding from Bayer AG (XW, GRS, GFG, ARB, BK, LW, TAL, CWG, ADC, MM, HG) and are co-inventors on patent applications submitted with Bayer AG (XW, TAL, MM, HG).
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| Cell line  | Lineage    | Biomarker positive | log2(PDE3A RPKM+1) | log2(SLFN12 RPKM+1) | log2(PDE3B RPKM+1) | AUC1 | AUC2 | % Maximal viability |
|-----------|------------|--------------------|--------------------|--------------------|--------------------|------|------|---------------------|
| HeLa      | CERVICAL   | yes                | 5.65               | 2.85               | 1.68               | 0.36 | 0.79 | 1                   |
| IGR37     | SKIN       | yes                | 5.29               | 2.50               | 1.18               | 1.16 | 1.17 | 2                   |
| COLO741   | SKIN       | yes                | 4.30               | 2.41               | 1.20               | 2.29 | 1.19 | 4                   |
| NCIH1563  | LUNG       | yes                | 4.31               | 1.84               | 0.88               | 1.98 | 1.34 | 2                   |
| NCIH2122  | LUNG       | yes                | 4.06               | 1.62               | 2.15               | 1.91 | 1.56 | 11                  |
| SKME13    | SKIN       | yes                | 2.69               | 2.96               | 1.28               | 1.37 | 1.74 | 13                  |
| JHUEM1    | ENDOMETRIUM| yes                | 3.69               | 2.39               | 0.03               | 1.03 | 1.62 | 6                   |
| HEL92.1.7 | HEMATOPOIETIC| yes              | 4.17               | 2.51               | 0.79               | 1.42 | 1.64 | 6                   |
| GB1       | CNS        | yes                | 2.90               | 3.49               | 0.01               | 2.07 | 1.81 | 19                  |
| HMCB      | SKIN       | yes                | 3.82               | 2.09               | 1.72               | 1.78 | 1.85 | 12                  |
| JHOM1     | OVARY      | yes                | 4.29               | 3.38               | 1.13               | 2.38 | 1.94 | 18                  |
| HEL       | HEMATOPOIETIC| yes              | 3.74               | 1.85               | 0.46               | 2.13 | 2.11 | 19                  |
| A2058     | SKIN       | yes                | 4.64               | 2.02               | 1.32               | 2.03 | 2.11 | 19                  |
| L3.3      | PANCREAS   | yes                | 4.25               | 2.04               | 2.47               | 2.84 | 2.82 | 37                  |
| NCIH1734  | LUNG       | yes                | 2.72               | 2.65               | 2.37               | 2.41 | 2.63 | 44                  |
| TE4       | ESOPHAGUS  | yes                | 3.09               | 2.58               | 1.32               | 3.86 | 2.56 | 34                  |
| DKMG      | CNS        | yes                | 3.43               | 1.95               | 0.04               | 1.83 | 2.71 | 41                  |
| HS578T    | BREAST     | yes                | 2.78               | 2.22               | 0.00               | 2.52 | 2.91 | 42                  |
| C32       | SKIN       | yes                | 2.97               | 3.37               | 1.32               | 3.69 | 3.13 | 61                  |
| NCIH2172  | LUNG       | yes                | 3.35               | 2.33               | 0.03               | 3.45 | 3.17 | 56                  |
| CAL51     | BREAST     | yes                | 4.74               | 2.88               | 0.55               | 3.22 | 3.50 | 75                  |
| UACC257   | SKIN       | yes                | 4.90               | 2.39               | 1.95               | 3.86 | 4.00 | 103                 |
| HCC15     | LUNG       | yes                | 4.12               | 2.90               | 0.35               | 3.96 | 3.71 | 100                 |
| HUT78     | HEMATOPOIETIC| no               | 0.08               | 5.48               | 3.84               | 2.02 | 1.85 | 7                   |
| RVH421    | SKIN       | no                 | 0.16               | 2.16               | 2.66               | 2.35 | 3.02 | 45                  |

Table 1. Biomarker expression and DNMDP response data. RNA-Seq expression values are from the Cancer Cell Line Encyclopedia (9). Maximal viability is defined as the bottom of the dose response curve. Red, biomarker-positive but DNMDP-insensitive cell lines. Green, biomarker-negative but DNMDP sensitive cell lines. AUC, area under the dose-response curve for DNMDP. CNS, central nervous system. 1AUC1: DNMDP sensitivity data from high throughput assay, normalized to an AUC scale of 0-4 (ref. #2). Originally-reported DNMDP AUC values were normalized on a scale of 0-4 by capping values at 7 and dividing by 4/7, in order to facilitate direct comparison to validation AUC values. 2AUC2: Manual validation DNMDP sensitivity data.
Figure legends

Figure 1. Cancer cell lines exhibit a range of sensitivities to DNMDP. (A) Gradient of DNMDP sensitivity among biomarker-positive cancer cell lines in a 72-hour CellTiter-Glo assay. (B) Single cell analysis of cell fates upon DNMDP treatment of cancer cell lines with a range of DNMDP sensitivities. Cells were treated continuously with 2 mM DNMDP, stained with an activated caspase substrate-conjugated dye, and imaged by Incucyte live cell analysis. Cells were tracked starting at 24 h. Limited division, cells divided at most twice between 24 h and the end of the observation period. (C) Cell fate tracking after 72 h treatment with 2 mM DNMDP, followed by a washout with control media. Cells were tracked starting at 72 h. No HeLa cells survived beyond 72 h. Limited division, cells divided at most once between 72 h and the end of the observation period.

Figure 2. The insensitive cell line, HCC15, does not express PDE3A protein. (A) Left panel, PDE3A protein expression was analyzed by immunoblotting in cancer cell lines. Right panel, PDE3A or GAPDH mRNA expression was analyzed by quantitative PCR. mRNA expression displayed as log2(relative gene expression) values. (B) Ectopic expression of PDE3A confers DNMDP sensitivity in the HCC15 cells, assayed by a 72-hour CellTiter-Glo assay. Ectopic PDE3A expression was confirmed by immunoblotting. NT, no transduction. GFP, green fluorescent protein.

Figure 3. The CAL51 breast cancer cell line harbors a heterozygous SLFN12 F185 frameshift mutation. (A) SLFN12 gene diagram showing the position of the F185fs mutation. The locations of the primers, located within a single exon, used for genomic DNA PCR and sequencing are indicated underneath. (B) Heterozygous 1 bp deletion as reported by CCLE (8). (C) Sequence alignment of PCR products to the reference sequence. Note that clean sequence peaks align perfectly to reference sequence before the stretch of Ts (marked by an asterisk) and overlapping peaks appear after the stretch of Ts. GB1 glioma cells, used as a wild type control, showed perfect alignment across the entire region. fs, frameshift. WES, whole exome sequencing. WGS, whole genome sequencing. alt, alternate allele. ref, reference allele.

Figure 4. (A) Immunoblot measuring PDE3A protein expression across biomarker-positive cell lines. (B) Correlation between PDE3A RNA expression, as measured by CCLE RNA sequencing, and PDE3A protein expression, as measured by Licor quantification of the immunoblot in (A). The 95% confidence interval of the best-fit line is indicated. RFU, relative fluorescence units. (C) Correlation between PDE3A protein expression and DNMDP response (AUC2 from Supplementary Table 1). The 95% confidence interval of the best-fit line is indicated. RFU, relative fluorescence units.

Figure 5. PDE3B can mediate DNMDP sensitivity. (A) DNMDP sensitivity of HUT78 and RVH421, which lack PDE3A mRNA expression, can be competed away by co-treatment with 100 nM trequinsin in a 72-hour CellTiter-Glo assay. Treq, trequinsin. (B) Immunoblotting reveals that HUT78 and RVH421 do not express any PDE3A protein, but do express high levels of PDE3B protein. (C) Left panel, CRISPR knockout of PDE3B in the partially-sensitive cell line, RVH421, abolished DNMDP sensitivity in a 72-hour CellTiter-Glo assay. Right panel, immunoblot showing loss of PDE3B protein expression in knockout cells made with two PDE3B-specific CRISPR guide RNAs, sg2 and sg5. (D) Left panel, ectopic expression of PDE3B in PDE3A knockout A2058 cells restores sensitivity to DNMDP in a 72-hour CellTiter-Glo assay. Right panel, immunoblot showing loss of PDE3A protein and ectopic expression of PDE3B protein in the PDE3A knockout A2058 cells. Treq, trequinsin. Vinculin or GAPDH were used as loading controls.

Figure 6. The PDE3A catalytic domain is sufficient to confer DNMDP sensitivity. (A) V5-tagged PDE3A deletion mutants tested for ability to support DNMDP cancer cell killing. Amino acid numbers are shown. TM (blue), membrane-associated region. (B) Top panel, DNMDP sensitivity of PDE3A CRISPR knockout A2058 cells can be restored by ectopic expression full-length, wild-type PDE3A or any of the truncation constructs tested in a 72-hour CellTiter-Glo assay. Bottom panel, truncation construct expression was
confirmed by immunoblotting with anti-PDE3A (N-terminal epitope) or anti-V5. Note that constructs with larger N-terminal deletions are not recognized by anti-PDE3A. GFP, green fluorescent protein.

**Figure 7.** PDE3A active site mutants impair DNMDP sensitivity. (A) A2058 cells. Top panel, 72-hour CellTiter-Glo assay. Bottom panel, anti-PDE3A immunoblot confirming construct expression. (B) HeLa cells. Top panel, 72-hour CellTiter-Glo assay. Bottom panel, anti-PDE3A immunoblot confirming construct expression. (C) Left panel, active site mutations (purple), but not 44-amino acid catalytic domain insert mutations (blue) abolish binding of PDE3A in 200 mg cell lysate from transduced A2058 cells to resin-linked DNMDP. Right panel, the isolated V5-tagged PDE3A catalytic domain fragment (amino acids 668-1141) in 200 mg cell lysate from transduced A2058 cells binds resin-linked DNMDP. Treq or T, trequinsin. i, input. p, pellet.

**Figure 8.** AIP is required for DNMDP sensitivity. (A) A genome-wide CRISPR knockout screen in HeLa cells reveals that AIP expression is required for DNMDP sensitivity. Volcano plot of average log fold change (LFC) across guide RNAs and average –log p value across two replicates for each gene following 14 day exposure with 25 nM DNMDP. Only genes with 3-8 guide RNAs are plotted to eliminate guides with likely off-target effects. Grey dots represent nontargeting control guide RNAs, randomly grouped into symbolic genes at 4 guides per gene. Full data are available in the Supplementary Dataset. (B) Left panel, 72-hour CellTiter-Glo assay with independent AIP CRISPR gRNAs (sg) confirming that AIP expression is required for sensitivity of HeLa cells to DNMDP. Right panel, 72-hour CellTiter-Glo assay validating a requirement of AIP for DNMDP sensitivity in the melanoma cell line, A2058. (C) AIP knockout prevents DNMDP-induced complex formation. PDE3A immunoprecipitates from HeLa cells transiently transfected with V5-tagged SLFN12 and treated with 10 mM DNMDP were immunoblotted with anti-V5 to assess DNMDP-induced PDE3A-SLFN12 complex formation.

**Figure 9.** Melanoma cell line UACC257 does not respond to DNMDP due to lack of AIP expression. (A) Of 973 CCLE cancer cell lines, only UACC257 cells have an AIP deletion and express no AIP mRNA. (B) Left panel, ectopic expression of AIP in UACC257 cells confers DNMDP sensitivity in a 72-hour CellTiter-Glo assay. Increased expression of PDE3A similarly confers DNMDP sensitivity. NT, no genes transduced. Right panel, immunoblots confirm expression of PDE3A, AIP, and V5-tagged GFP. (C) Model of DNMDP-induced complex formation between PDE3A and SLFN12. Complex formation is facilitated by AIP.
A. Strongly sensitive

![Graph showing percent survival against Log M DNMDP for strongly sensitive cells.]

- HEL
- JHOM1
- GB1
- A2058
- HMCB
- SKMEL3
- NCIH2122
- JHU1M1
- HEL92.1.7
- COLO741
- IGR37
- GS578
- HS578T
- NCIH1734
- DKMG
- L3.3
- HE

B. Partially sensitive or insensitive

![Graph showing percent survival against Log M DNMDP for partially sensitive or insensitive cells.]

- HCC15
- UACC257
- CAL51
- C32
- NCIH2172
- HS578T
- NCIH1734
- DKMG
- L3.3
- TE4

C. Continuous incubation

![Bar graph showing percent survival for continuous incubation.]

- Apoptosis
- Arrest
- Limited Division
- Resistance

C. Compound washout

![Bar graph showing percent survival for compound washout.]

- Apoptosis
- Arrest
- Limited Division
- Recovery
Figure 2
A. SLFN12

- **PCR #1**: Reverse
- **PCR #2**: Forward

| Exonic Change | Protein Change | Reference Transcript | Reference Allele | Tumor Seq Allele | Annotation Transcript | Tumor Sample Barcode | Tumor Sample Barcode | Reference Sample Barcode |
|---------------|---------------|----------------------|------------------|-----------------|-----------------------|-----------------------|------------------------|-------------------------|
| SLFN12        | p.F185fs      | ENST00000394562.1    | A                | -               | p.F185fs              | CAL51_BREAST          | CAL51_BREAST          | GB1                     |

B. Hugo Symbol | Tumor Sample Barcode | Reference Allele | Tumor Seq Allele | Annotation Transcript | Protein Change | CCLE WES alt.ref | CCLE WGS alt.ref | Sanger WES alt.ref |
|---------------|---------------------|-----------------|-----------------|-----------------------|----------------|----------------|----------------|-------------------|
| SLFN12        | CAL51_BREAST        | A               | -               | ENST00000394562.1    | p.F185fs      | NA             | 17:20          | 54:98             |

C. Reference sequence

**Sequencing primer**

**PCR #1** Reverse

**PCR #2** Forward

**PCR #1** Reverse

**PCR #2** Forward

Figure 3
Figure 4

A. TE4 L3.3 H1563 DKMG H2122 COLO741 H2172 HeLa A2058 IGR37 SKMEL3 JHUEM1 HMCB G1 JHOM1 C32 Hs578T HEL HEL92.1.7

B. PDE3A mRNA log$_2$(RPKM+1) vs PDE3A protein log$_2$(RFU)

C. DNMDP AUC2 vs PDE3A protein log$_2$(RFU)
Figure 5
Figure 6
Figure 7

A. PDE3A CRISPR KO A2058

B. PDE3A CRISPR KO HeLa

C. Treq competition:

Input or pellet: i p p---- T i p p---- T i p p---- T i p p---- T i p p---- T i p p---- T

668-1141

GFP

PDE3A wild-type
PDE3A Y807A
PDE3A Y814F
PDE3A C816A
PDE3A H752A
PDE3A H840A
PDE3A D950A
PDE3A F1004A

44aa insert mutants

active site mutants

V5

GAPDH

Percent survival

Log M DNMDP

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin

75 kDa

100 kDa

PDE3A

Vinculin
Figure 8

A. 

Average log-fold change vs. Average log(p-value), DNMDP

B. 

AIP CRISPR KO HeLa

AIP CRISPR KO A2058

C. 

Cell line: HeLa AIP KO sg2

DNMDP: + - + - 

WB

PDE3A

V5 (SLFN12)
A. AIP mRNA expression (RNAseq log2 RPKM) versus AIP Copy Number (log2 relative copy number).

B. Percent survival of UACC257 cells treated with various concentrations of DNMDP.

C. Diagram illustrating the regulation of PDE3A/B by SLFN12 and the role of DNMDP in cancer cell death.
Mechanistic insights into cancer cell killing through interaction of phosphodiesterase 3A and schlafen family member 12

Xiaoyun Wu, Gavin R. Schnitzler, Galen F. Gao, Brett Diamond, Andrew R. Baker, Bethany Kaplan, Kaylyn Williamson, Lindsay Westlake, Selena Lorrey, Timothy A. Lewis, Colin W Garvie, Martin Lange, Sikander Hayat, Henrik Seidel, John Doench, Andrew D. Cherniack, Charlotte Kopitz, Matthew Meyerson and Heidi Greulich

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