Metastasis and immune evasion from extracellular cGAMP hydrolysis

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ABSTRACT

Cytosolic DNA is characteristic of metastatic and chromosomally unstable cancer cells and it results in constitutive activation of the cGAS-STING innate immune pathway. How cancer cells co-opt inflammatory signaling while evading immune surveillance remains unknown. Here we show that the ectonucleotidase ENPP1 promotes metastasis by selectively degrading extracellular cGAMP, an immune stimulatory metabolite whose breakdown products include the immune suppressor, adenosine. ENPP1 depletion restores tumor immune infiltration, suppresses metastasis, and potentiates tumor response to immune checkpoint blockade (ICB) therapy in tumor cell cGAS and host STING-dependent manners. Conversely, ENPP1 overexpression renders otherwise sensitive tumors completely resistant to ICB. In human cancers, ENPP1 expression correlates with reduced immune cell infiltration, increased metastasis, and resistance to anti-PD1/PD-L1 therapy. Thus, cGAMP hydrolysis by ENPP1 enables metastatic cancer cells to transmute an immune stimulatory pathway into an immune suppressive mechanism and it represents a therapeutic vulnerability in tumors with pervasive chromosomal instability.

SIGNIFICANCE

Chromosomal instability promotes metastasis by generating chronic tumor inflammation. ENPP1 enables tumor cells to tolerate inflammation by negatively regulating paracrine innate immune signaling

INTRODUCTION

Chromosomal instability (CIN) is a hallmark of human cancer and it is associated with metastasis, immune evasion, and therapeutic resistance (1-5). In addition to the generation of chromosome copy number heterogeneity, which serves as a substrate for natural selection, CIN can also promote tumor evolution by inducing chronic inflammatory signaling leading to increased cancer cell migration, invasion, as well as epithelial-to-mesenchymal transition (EMT) (1,6). Chromosome segregation errors lead to the formation of micronuclei (7,8). Micronuclear envelopes are highly rupture-prone, often exposing genomic double-stranded DNA (dsDNA) to the cytosol (1,9-12). Cytosolic dsDNA is sensed by cGAS, which upon binding to its substrate, catalyzes the formation of the cyclic dinucleotide, cGAMP (13). cGAMP is a potent immune-stimulatory molecule that promotes inflammatory signaling in a manner dependent on its downstream effector STING (14,15).

Given the pervasive nature of CIN in human cancer (4), tumor cells must cope with the presence of persistent innate inflammatory signaling arising from cGAS-sensing of cytosolic dsDNA to avoid immune-mediated clearance. The activation of cGAS-STING has both cell-autonomous and cell non-autonomous consequences and therefore cancer cells must modulate this signaling pathway at multiple levels. One mechanism by which chromosomally unstable cancer cells have evolved to cope with chronic cGAS-STING activation is through silencing downstream type I interferon signaling whilst co-opting NF-κB-dependent transcription to spread to distant organs (1). In line with this, human tumor STING expression was found to correlate primarily with NF-κB-dependent transcriptional programs rather than type I interferon (IFN) signaling (16). The switch from IFN to NF-κB-predominant signaling downstream of STING enables cancer cells to simultaneously evade immune surveillance in response to type I IFN and activate noncanonical NF-κB-dependent migratory programs leading to metastatic progression thereby co-opting CIN-induced inflammatory signaling as a vehicle for tumor progression.

In addition to its cell-intrinsic effects, cGAMP can be readily exported to the extracellular space where it can promote anti-tumor immune responses by activating STING in neighboring host cells (17-19). Unlike cancer cells, host cells in the tumor microenvironment respond to STING activation by activating type I IFN signaling central to robust cell-mediated immune responses. How tumor cells with CIN eschew the deleterious effects of paracrine cGAMP signaling remains poorly understood. Understanding the adaptive mechanisms employed by cancer cells to evade immune surveillance in response to chronic inflammatory signaling represents an attractive therapeutic opportunity to selectively target tumor cells with CIN while sparing normal cells devoid of cytosolic dsDNA.

RESULTS

ENPP1 is upregulated in cells with CIN

To investigate the status of cGAS-STING signaling in cancer cells with CIN, we used a human triple-negative breast cancer (TNBC) cell line, MDA-MB-231, that was engineered to exhibit different rates of CIN through overexpression of the kinesin-13 proteins, Kif2b or MCAK, or the dominant-negative mutant isoform of MCAK (dnMCAK) (1,20). We have previously shown that in these otherwise isogenic cell lines, expression of dnMCAK promotes increased chromosome missegregation leading to micronuclei formation, chronic activation of cGAS-STING signaling, and metastasis (1). In addition, we employed three syngeneic metastasis-competent mouse models of TNBC (4T1 and E0771) and colorectal cancer (CT26). In all three models we observed high levels of CIN, as evidenced by the presence of chromosome missegregation during anaphase and a preponderance of micronuclei exhibiting robust cGAS localization indicative of cytosolic exposure of genomic dsDNA (**Supplementary Fig. S1A-B**). To test if cGAS localization to micronuclei also led to pathway activation in mouse cell lines, we measured cGAMP levels in total cell lysates of control 4T1 cells and upon CRISPR-Cas9 mediated knockout (KO) of *Cgas*. Loss of cGAS resulted in a significant reduction in the levels of the cyclic dinucleotide, in line with constitutive activation of the pathway (**Supplementary Fig. S1C-D**). Furthermore, cGAMP levels were nearly 15-fold higher in conditioned media after 24hr, as compared to cell lysates, when both were normalized to cell counts (**Supplementary Fig. S1D**), suggesting that cGAMP is readily exported from cancer cells, as previously proposed (17-19).

To determine how chromosomally unstable cells cope with ongoing cGAMP production, we performed pairwise differential expression analysis of otherwise isogenic CIN^{high} (highly metastatic) and CIN^{low} (poorly metastatic) MDA-MB-231 cells. Among the large number of differentially-expressed genes, *ENPP1*, stood out because of its role as an ectonucleotidase that hydrolyses cGAMP (21). Through a single transmembrane domain, ENPP1 localizes to the plasma membrane with its catalytic domain facing the extracellular space where it has been proposed to selectively hydrolyze the extracellular pool of cGAMP (19). Both ENPP1 messenger and protein levels were markedly increased in CIN^{high} cells compared with their CIN^{low} counterparts (**Supplementary Fig. S1E-F**, Log_2 fold change = 1.23, FDRq = $8.4x10^{-4}$). Staining of MDA-MB-231 CIN^{high} cells using an anti-ENPP1 antibody revealed strong membrane localization in that was abolished upon shRNA-mediated depletion (**Fig. 1A**). A similar pattern of cell membrane staining was also seen in orthotopically transplanted tumors (**Fig. 1B** and **Supplementary Fig. S2A-B**).

We next surveyed *ENPP1* expression across the mouse cancer cell lines and found that 4T1 had the highest mRNA expression levels compared to CT26 and E0771. Interestingly, E0771.LMB, a more aggressive metastatic derivative (22) had significantly higher levels of ENPP1 expression compared to the parental line (**Supplementary Fig. S2C**), suggesting that ENPP1 might be highly expressed in metastatic cancer cells. In line with this, ENPP1 mRNA was significantly elevated in 4T1 cells derived from lung metastases compared with the parental cell line (**Supplementary Fig. S2D**). We next analyzed *ENPP1* expression in the various stages of tumorigenesis from a mouse model of lung adenocarcinoma (23). Strikingly, mRNA levels of *ENPP1* exhibited a stepwise increase from normal tissue and benign hyperplasias, to primary tumors to metastases with metastatic primary tumors expressing higher levels of this ectonucleotidase compared with non-metastatic primary tumors (**Fig. 1C**). ENPP1 protein expression mirrored this trend, with increased levels observed selectively in tumor cells invading nearby intra-mammary lymph nodes (**Fig. 1B**). Notably, we have previously shown that CIN levels increase during the progression from primary tumors to metastases, suggesting that ENPP1 might play a role in facilitating metastasis of chromosomally unstable cells.

ENPP1 promotes cancer metastasis

To directly test the role of ENPP1 in metastasis, we performed CRISPR-Cas9 KO of *Enpp1* in 4T1 cells (**Supplementary Fig. S2E**). Conversely, we overexpressed wildtype (WT) ENPP1 or an enzymatically weakened mutant isoform containing a threonine-to-alanine substitution in the catalytic domain (T238A) [R] in CT26 and E0771 cells which express lower levels of this enzyme at baseline. As expected, loss of ENPP1 led to a significant increase in the extracellular-to-intracellular cGAMP ratio (**Fig. 1D**). Conversely, overexpression of the wildtype but not the enzymatically weakened mutant of ENPP1 led to a reduction in the extracellular-to-intracellular cGAMP ratio in CT26 and E0771 cells (**Fig. 1D**). *Enpp1*-KO did not impact cellular proliferation *in vitro* or primary tumor growth *in vivo* when cells were orthotopically transplanted in the mammary fat pad (**Supplementary Fig. S2F-G**). We then transplanted parental and *Enpp1*-KO 4T1 cells into BALB/c hosts either through tail vein inoculation or orthotopic transplantation into the mammary fat pad followed by primary tumor excision. Loss of ENPP1 led to significantly longer overall survival and a marked reduction in local tumor recurrence and metastasis regardless of whether cells were introduced directly into the tail vein or orthotopically transplanted followed by surgical excision of the primary tumor (**Fig. 1E-F and Supplementary Fig. S2H-K**). Conversely, overexpression of WT ENPP1 to a significant increase in the number of surface lung metastases upon tail vein inoculation of CT26 cells (**Fig. 1G**).

To further examine whether ENPP1 disrupts paracrine tumor-to-host cGAMP transfer during metastatic progression, we overexpressed WT ENPP1 or ENPP1-T328A in E0771 and quantified metastatic dissemination using bioluminescence imaging. Only WT ENPP1 – and not the enzymatically weakened mutant – led to increased metastatic dissemination (**Fig. 2A**). The role of ENPP1 on metastasis was dependent on host STING as both control and WT ENPP1-overexpressing cells had similar metastatic proclivity when transplanted into STING (*Tmem173*)-KO hosts (**Fig. 2A**). Collectively these results suggest that ENPP1 promotes metastatic progression in through extracellular cGAMP hydrolysis, preventing STING activation in host cells.

Extracellular cGAMP hydrolysis by ENPP1 generates adenosine

We next explored the fate of tumor-derived extracellular cGAMP and asked whether the breakdown products of this metabolite might contribute to the production of adenosine, an immune-suppressive and tumor-promoting molecule (**Fig. 2B**). Measuring adenosine in conditioned media is technically challenging given the presence of enzymes that either degrade this nucleoside (adenosine deaminase, ADA) or promote its cellular reuptake (**Supplementary Fig. S3A**). To overcome these challenges, we added serum-free media to 4T1 cells in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an ADA inhibitor, along with dipyridamole and 6-*S*-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR), which prevent cellular reuptake of adenosine (**Supplementary Fig. S3A**) (24). Extracellular adenosine levels – as assessed by liquid chromatography-mass spectrometry in conditioned media – were reduced by up to 40% upon knockout of either *Cgas* or *Enpp1* (**Fig. 2C**). Using an orthogonal approach, we added exogenous cGAMP to 4T1 cells and used a fluorescence-based method to detect hydrogen peroxide (H_2O_2) resulting from the oxidation of hypoxanthine, a breakdown product of adenosine (**Supplementary Fig. S3A**). By comparing fluorescence in the presence and absence of EHNA, we were able to assess relative contribution from adenosine degradation toward H_2O_2 production and observed a concentration-dependent increase in H_2O_2 production after the addition of exogenous addition of cGAMP (**Supplementary Fig. S3B**), suggesting that this cyclic dinucleotide can be readily converted into adenosine in the extracellular environment.

Through its ability to bind extracellular adenosine receptors in both tumor and immune cells, adenosine promotes cancer cell migration and is a potent immune suppressor, respectively (25,26). Interestingly, knockout of either *Cgas* or *Enpp1* in 4T1 cells led to a significant reduction in migration, whereas exogenous addition of cGAMP rescued migration only in *Cgas*-KO but not *Enpp1*-KO tumour cells (**Fig. 2D**). The effect of cGAMP was dependent on adenosine receptor activity and was abolished upon the addition of PSB115, an inhibitor of the adenosine A2B receptor on cancer cells (**Fig. 2D**). Conversely, overexpression of WT ENPP1 – but not ENPP1-T328A – in E0771 or CT26 cells led to increased migration, an effect that was abolished upon treatment with adenosine deaminase (ADA) (**Supplementary Fig. 3D-E**).

In addition to cGAMP hydrolysis, ATP hydrolysis by CD39 (also known as ENTPD1) is considered to be a major source of extracellular adenosine (**Fig. 2B**). Both produce AMP that is subsequently hydrolyzed into adenosine by CD73 (also known as NT5E). Interestingly, of NT5E mirrored that of ENPP1 progressively increasing from normal tissues, to primary tumors, to metastases (**Fig. 2E**). On the contrary, ENTPD1 expression followed the opposite trend with the lowest expression in metastases (**Fig. 2E**). These opposing trends suggest that while ATP hydrolysis might represent a primary source of adenosine in primary tumors, the relative contribution from cGAMP hydrolysis toward extracellular adenosine increases during metastatic progression. In line with this finding, KO of either *Enpp1* or *Nt5e* led to significant reduction in metastasis that was equivalent to cells lacking both enzymes (**Fig. 2F** and **Supplementary Fig. S3C**).

We had recently shown that tumor cell-intrinsic STING activation by intracellular cGAMP can also promote cellular migration and metastasis (1). To test the relative contribution of tumor cell STING and extracellular cGAMP hydrolysis by ENPP1, we assessed metastatic potential of control, *Enpp1*-KO, *Tmem173*-KO, and *Enpp1*/*Tmem173* double KO 4T1 cells by comparing animal survival after tail vein inoculation. Loss of either ENPP1 or STING in tumor cells led to reduced metastasis and

lifespan extension and their combined KO led to an additive effect (**Supplementary Fig. S3F**). Collectively, this suggests that intracellular cGAMP activation of STING and its extracellular hydrolysis by ENPP1 independently contribute to metastatic progression. Furthermore, these results also indicate that the impact of ENPP1 on metastasis is mediated through activation of host – but not tumor cell – STING.

ENPP1 promotes tumor immune evasion

We next examined the effect of ENPP1 loss on tumor immune infiltration using shRNA-mediated depletion or CRISPR-Cas9-mediated KO in CIN^{high} MDA-MB-231 orthotopic xenografts and 4T1 allografts, respectively. Loss of ENPP1 led to increased tumor necrosis and enhanced infiltration of natural killer (NK)-cells in MDA-MB-231 tumors (**Supplementary Fig. S4A-B**), in line with previous reports demonstrating a role for cGAMP transfer in activating NK-cells (17). In the 4T1 model, metastatic lesions formed from *Enpp1*-KO cells exhibited significant infiltration by CD45+ cells and a ~3-5-fold enrichment with CD8+ T-cells compared to wildtype counterparts (**Fig. 2A-B**). Flow cytometry-based immune profiling of dissociated lungs revealed a significant increase in CD45+ cells, CD4+ T-cells as well as granulocytic CD11b+Ly6G+ cells as compared to controls (**Fig. 2C and Supplementary Fig. S5**). There was no overall enrichment for CD8+ T-cells in the injected lungs observed by flow cytometry, however there was a significant increase in PD1+ subpopulations of CD3+CD8+ and CD3+CD4+ cells (**Fig. 2C**). The overall preponderance of granulocytic cells was notable, given that ENPP1-KO tumors had higher levels of GM-CSF as measured using ELISA-based assays (**Fig. 2D**). These findings suggest that granulocytic cells may play a role in restricting metastatic colonization of *Enpp1*-KO cells, in line with previous reports showing an anti-tumor and pro-inflammatory effect of CD11b+Ly6G+ cells (27-29).

We next assessed the impact of WT ENPP1 overexpression on subcutaneously transplanted CT26 tumors. Expectedly, increased ENPP1 levels led reduced CD8+ T-cells, NK-cells as well as the proportion of PD1+ CD8+ and CD4+ T-cells. In line with these findings, there was also a decrease in the proportion of CD44+ T-cells suggesting reduced T-cell activation (**Fig. 2E** and **Supplementary Figure S4C**). While we did not observe an absolute change in the overall fraction of FoxP3+ T-regulatory cells, there was a significant reduction in the CD8+ T-cell:T-regulatory cell ratio (**Fig. 2E** and **Supplementary Figure S4C**).

To determine whether increased immune infiltration upon ENPP1 loss was dependent on tumor cell-derived cGAMP, we performed population-level depletion of cGAS using CRISPR-knockout and found a trend towards reduced CD45+ cell and CD8+ T-cell infiltration when cGAS was co-depleted with ENPP1 (**Supplementary Fig. S4D-F**). We posit that lack of complete rescue might be due to the residual fraction of cells with functional cGAS or alternative sources of cGAMP. Nonetheless, this data suggests that ENPP1 dampens pro-inflammatory tumor immune infiltration through extracellular cGAMP hydrolysis.

ENPP1 inhibition potentiates response to immune checkpoint blockade therapy

We then asked whether ENPP1 depletion might be used as a therapeutic vulnerability to sensitize otherwise resistant chromosomally unstable tumors to immune checkpoint blockade (ICB) therapy. Interestingly, baseline ENPP1 mRNA expres-

sion levels in the three mouse cancer cell lines (**Supplementary Fig. S2C**) mirrored what is known about their sensitivity to ICB therapy with CT26 and E0771 being considered responsive to ICB in contrast to the highly resistant 4T1 model (18,30). We postulated that *Enpp1* knockout would render 4T1 tumors responsive to ICB therapy whereas its overexpression would confer resistance to otherwise sensitive CT26 and E0771 tumors (**Fig. 4A** and **Supplementary Fig. S6A**). Luciferase-expressing 4T1 cells were orthotopically transplanted into the mammary fat pad of BALB/c mice and primary tumor growth were assessed over the span of 25 days (**Fig. 4B and Supplementary Fig. S6B-C**). Animals were treated with combined ICB (anti-PD1 and anti-CTLA4) starting at day 6 after tumor cell inoculation for 4 doses followed by maintenance aCTLA4 treatment every 3 days for 4 additional doses. *Enpp1*-KO tumors exhibited a profound response to combined ICB when compared to wildtype counterparts, leading to significantly prolonged survival (**Fig. 4B-C**). Importantly, cGAS depletion in *Enpp1*-KO cells led to a reduction in tumor responsiveness as evidenced by significant shortening of animal survival after treatment (**Fig. 4C**).

We next asked whether overexpression of ENPP1 would confer ICB treatment resistance in otherwise sensitive CT26 and E0771 tumors (**Fig. 4A** and **Supplementary Fig. S6A**). CT26-bearing mice were treated with combined ICB starting at day 6 for a total of 5 doses. Strikingly, not only did eGFP-ENPP1 expression lead to increased metastasis and reduced survival of isotype control-treated mice, it also rendered this model completely resistant to combined ICB (**Fig. 4D**). Conversely, eGFP-expressing CT26 tumors were responsive to combined ICB with 60% of animals surviving for over 140 days. Similarly, overexpression of eGFP-ENPP1 in orthotopically transplanted E0771 tumors led to their resistance to aPD1 treatment, wherein 50% of treated animals underwent a durable complete response of their eGFP-expressing E0771 tumors compared to 0% of their cGFP-ENPP1 overexpressing counterparts (**Fig. 4E-F**). Interestingly, the difference in response between eGFP and eGFP-ENPP1 expressing tumors was abolished when transplanted in *Tmem173*$^{-/-}$ hosts (**Fig. 4E-F** and **Supplementary Fig. S6D**). Collectively, these results suggest that ENPP1 inhibition represents an attractive therapeutic strategy to potentiate the response of chromosomally unstable cancer cells to ICB therapy.

ENPP1 is associated with metastasis in human cancer

We next sought to interrogate the role of ENPP1 in human cancers by analyzing *ENPP1* mRNA and protein expression in a large number of tumors from various tissues of origin. *ENPP1* mRNA was investigated in tumors found in the Tumor Cancer Genome Atlas (TCGA), an independent set of primary and metastatic tumors, two independent sarcoma cohorts, and in tumor-derived organoids. ENPP1 protein expression was performed in three independent breast cancer cohorts, including two estrogen-receptor-negative (ER-) cohorts (n = 223 and 91) and one estrogen receptor-positive (ER+) cohort (n = 115) as well as in mucosal melanoma primary and metastatic tumors (n = 24).

ENPP1 mRNA expression was highly variable across cancer types in the TCGA, with highest expression levels observed in sarcomas, liver, breast, and thyroid cancers (**Supplementary Fig. S7A**). Elevated *ENPP1* mRNA was associated with reduced overall survival in multiple tumor types including breast cancer, irrespective of the hormone receptor status (**Supplementary Fig. S7B-D**). We then asked if *ENPP1* expression was associated with metastatic progression. First, we compared ENPP1 expression levels in a large number of primary tumors and metastatic tumor samples as well as in a collection of tumor-

derived organoids. In both cases, *ENPP1* mRNA was higher in metastases compared to primary tumors (**Fig. 5A** and **Supplementary Fig. S8A**). When tumors were stratified by tissue site, we found liver and brain metastases to contain the highest expression levels of *ENPP1* (**Fig. 5A**). We next surveyed ENPP1 protein expression in primary and metastatic mucosal melanoma tumors. Unlike cutaneous melanoma, mucosal melanoma is characterized by elevated CIN, reduced tumor mutational burden, and increased resistance to immune checkpoint blockade (31,32). In these tumors, membrane ENPP expression was seen in both tumor cells and the stroma and this pattern was evenly distributed across primary tumors samples. Conversely, metastases displayed significantly increased cancer cell-specific ENPP1 staining (**Fig. 5B**). Tumor cell-intrinsic ENPP1 protein expression was most remarkable in lymph-node metastases where cancer cell clusters exhibited strong ENPP1 expression in an otherwise immune-cell replete microenvironment (**Fig. 5C-D**).

To investigate the impact of ENPP1 protein expression on metastasis, we analyzed a total of 429 primary breast tumors from three independent cohorts for which there were long-term clinical follow up data available. Similar to our findings in mucosal melanoma, we observed three distinct patterns of ENPP1 protein expression: tumor-cell-dominant, stroma-dominant, and negative (**Fig. 5E** and **Supplementary Fig. S8B**). Overall, 64% of primary TNBCs exhibited moderate or strong ENPP1 staining in either tumor cells or the stroma – a distribution that was consistent across the two ER- cohorts. On the other hand, 90% of ER+ tumors exhibited elevated ENPP1 protein expression. Notably, the tissue distribution and expression patterns varied between the two breast cancer subtypes, with ER- tumors exhibiting both stromal and tumor cell-specific expression compared with their ER+ counterparts, which displayed a proclivity for tumor cell-specific staining (**Supplementary Fig. S8B**). Irrespective of the expression pattern however, moderate-to-strong ENPP1 staining was associated with poor prognosis, as evidenced by reduced overall survival, distant metastasis-free survival, and recurrence-free survival (**Supplementary Fig. S8C-E**). We next reasoned that if the association between ENPP1 expression and prognosis was related to its function as a negative regulator of cGAS-STING signaling, then its expression levels should only be discriminatory in tumors with high cGAS activity. Staining using anti-cGAS antibodies revealed predominant staining at micronuclei (not shown). Indeed, ENPP1 protein expression was associated with worse prognosis and increased metastasis only in tumors with a preponderance of cGAS+ micronuclei and it had no significant association in those without micronuclear cGAS (**Fig. 5F**). Collectively, this data is in line with our *in vivo* experimental results and further support the role of ENPP1 as an important determinant of cancer progression through its suppression of CIN-induced inflammatory signaling.

ENPP1 is associated with immune suppression in human cancer

We next correlated ENPP1 protein levels with tumor-infiltrating lymphocytes (TILs) and CD8+ T-cell density across breast cancers and found an inverse correlation between ENPP1 IHC expression intensity and lymphocytic infiltration (**Fig. 6A-B** and **Supplementary Fig. S9A-B**). Similar patterns were seen across the TCGA breast tumor cohort. We segregated 1,079 breast tumors into four subsets based on their relative *CGAS* and *ENPP1* expression levels and used the CIBERSORT method to infer the prevalence of immune cell subsets from tissue expression profiles (33). Expectedly, *ENPP1* expression was minimally associated with the immune cell fraction in tumors with low *CGAS* expression, whereas in those with high *CGAS* mRNA, it was inversely correlated with the overall leukocyte fraction as well as with the proportion of CD8+ T-cells, CD4+ T-cells, and pro-inflammatory macrophages in tumors with elevated *CGAS* mRNA (**Fig. 6C**). Furthermore, PD-L1 expression

was highest in tumors with high *CGAS* and low *ENPP1* expression. Gene Set Enrichment Analysis (GSEA) comparing cGAS^{high}ENPP1^{high} to cGAS^{high}ENPP1^{low} breast tumors revealed upregulation of inflammatory pathways related to allograft rejection, type I interferon, and interferon-γ-associated responses in the latter subset of tumors (**Supplementary Fig. S9C**). These findings suggest that ENPP1-to-cGAS ratio might be more predictive of tumor immune infiltration compared to *ENPP1* expression levels alone. We orthogonally validated this assumption in sarcomas and mucosal melanoma tumors. In sarcomas, *ENPP1*-to-*CGAS* expression ratio was more strongly associated with the cytotoxic lymphocyte score compared with ENPP1 expression levels alone (**Supplementary Fig. S9D**). In mucosal melanomas, tumors with numerous cGAS-positive micronuclei and low ENPP1 expression exhibited increased CD8+ T-cell density, whereas those with elevated ENPP1 expression in the setting of widespread cGAS-positive micronuclei exhibited significantly reduced CD8+ T-cell infiltration (**Supplementary Fig. S10A-D**).

In line with its role modulating tumor immune responses, we found that ENPP1 expression within a given cancer type negatively correlates with its overall response rate to anti-PD1/PD-L1 therapy (34). This inverse association was again restricted to tumor types characterized by elevated overall levels of cGAS expression (**Fig. 6D** and **Supplementary Fig. S10E**). We next analyzed the mRNA expression levels of cGAS and ENPP1 in 228 bladder cancers treated with anti-PD-L1 (aPD-L1) therapy and a smaller cohort of 52 TNBC tumors treated with aPD1 (35,36). There was an overall positive correlation between *CGAS* and *ENPP1* expression and *ENPP1* levels were significantly lower in *CGAS*^{high} tumors that responded to aPD-L1 therapy. A low ENPP1-to-cGAS expression ratio was significantly correlated to tumor response across both the bladder cancer and TNBC cohorts (**Supplementary Fig. S9F-G**).

DISCUSSION

Our work reveals an adaptive mechanism by which chromosomally unstable tumours co-opt cancer cell-intrinsic cGAS-STING signaling, arising from chronic exposure to cytosolic dsDNA, without eliciting anti-tumor immune surveillance (**Fig. 6E**). By virtue of their constant exposure to cytosolic dsDNA, chromosomally unstable cancer cells must address the consequences of cGAMP leakage into the extracellular space and its potential uptake by cells in the tumor microenvironment. Through their ability to degrade cGAMP selectively in the extracellular environment, tumor cells can maintain relatively high levels of this metabolite in the intracellular compartment while minimizing paracrine STING activation in neighboring immune cells (**Fig. 6E**).

Previous work has implicated ENPP1 to the ability of tumor cells to disseminate especially in the context of bone metastasis (37), however the precise mechanisms underlying this relationship has been poorly understood. One possible mechanism is through its pyrophosphatase activity from ATP hydrolysis leading to bone remodeling and facilitating metastatic dissemination. Our work, however, indicates that the role of ENPP1 in tumor evolution extends beyond osseous metastases owing to its ability to hydrolyze cGAMP and therefore suppresses the host's ability to control metastatic progression through activation of STING signaling.

Extracellular cGAMP hydrolysis by ENPP1 generates the substrate for adenosine production converting an immune stimulatory pathway into an immune suppressive mechanism that promotes tumor progression (**Fig. 6E**). Our findings suggest that cGAMP represents a significant source for extracellular adenosine. Furthermore, the stepwise increase in ENPP1 levels – and concomitant decrease of CD39 – during the evolution from primary tumors to metastasis suggests dynamic changes in the extracellular sources of adenosine with ATP generating a significant proportion in the microenvironment of primary tumors with the relative contribution of cGAMP increasing as tumors progress and acquire additional genomic instability. The Inhibition of extracellular adenosine production and signaling is currently being investigated at the pre-clinical and clinical stages (25). ENPP1 inhibition would achieve the dual purpose of reducing extracellular adenosine while simultaneously increasing the extracellular levels of the immunotransmitter cGAMP. These findings highlight an important STING-independent function for tumor cGAS and suggests that in the presence of ENPP1, high tumor cGAS activity might in fact be paradoxically immune suppressive enabling tolerance for chromosomal instability and pervasive cytosolic dsDNA in advanced tumors.

Through extensive assessment of ENPP1 mRNA and protein expression levels across human cancer, our work positions ENPP1 into a broader clinical context and makes the case for the development of ENPP1 inhibitors for the treatment of advanced and chromosomally unstable cancers. Interestingly, cancer types with elevated ENPP1 expression are generally thought to be less responsive to immune checkpoint blockade therapy raising the possibility that extracellular purine metabolism might represent an important innate immune checkpoint that must be overcome for full activation of the adaptive immune response against cancer. Indeed, our work suggests that ENPP1 inhibition is a viable mechanism to sensitive otherwise resistant tumors to immune checkpoint blockade therapy. Interestingly, the widespread stromal staining patterns of ENPP1 in human cancers – reminiscent of fibroblast expression – suggests that this mechanism of immune evasion might not only arise from tumor cells but also cells in the tumor microenvironment. Given the low expression levels in normal tissues, it will be important to dissect tumor-derived factors that promote paracrine induction of ENPP1 in the stroma. Interestingly, our data suggest that in metastatic cancers, ENPP1 staining is biased towards cancer cell-intrinsic pattern raising the possibility that chromosomally unstable tumor cells that acquire the ability to eschew cGAMP-mediated immune activation have a selective advantage to spread to distant sites.

Our work has important implications for the clinical development of therapies directed against tumor cGAS-STING signaling. STING agonists have been the focus of intense investigation given their ability to elicit anti-tumor immunity through type I interferon signaling (38). Inhibition of ENPP1 is distinct from direct pharmacologic activation of STING in a number of important ways. First, ENPP1 tilts the relative balance of STING activation away from cancer cells, where it promotes metastatic progression (1), and towards host cells where it potentiates anti-tumor immunity. STING agonists indiscriminately activate STING in both cancer cells and the host promoting dichotomous outcomes. Second, inhibition of cGAMP hydrolysis by ENPP1 would primarily impact cGAMP concentrations at the microscopic scales relevant to paracrine tumor cell-host cell interactions. This, along with the short half-life of cGAMP in circulation (21), is likely to minimize systemic side effects of ENPP1 inhibition, offering a larger therapeutic window. Third, ENPP1 is selectively upregulated in metastatic and chromosomally unstable tumor cells and thus a systemic ENPP1 inhibitor would interfere with the ability of disseminated tumor

cells to evade immune surveillance arising from CIN. This work highlights the therapeutic utility of selectively targeting cancer cell dependencies on CIN and the mechanism by which they have evolved to tolerate it.

METHODS

Cell culture: 4T1, CT26, and B16F10 cells lines were purchased from the American Type Culture Collection (ATCC) and E0771 was a gift from Alexander Rudensky. Cells were cultured in DMEM (B16F10 and E0771) or RPMI (4T1 and CT26) supplemented with 10% FBS and 2 mM L-glutamine in the presence of penicillin (50 U ml^{-1}) and streptomycin (50 µg ml^{-1}). All cells were found to be negative for mycoplasma upon repeated testing.

The generation of knockout cell lines: Murine cancer cells deficient in *Cgas*, or *Enpp1* were generated by Cas9 ribonucleoprotein nucleofection using a Lonza 4D-Nucleofector and SF Cell line Kit. crRNA (IDT) sequences is listed in Supplementary Table 1. Four guides were screened per target and knockout cell lines were confirmed using immunoblotting. Antibody information used in immunoblotting experiments is listed in Supplementary Table 2. Stable knockdown of ENPP1 in MDA-MB-231 cells was achieved using shRNAs in pRRL (SGEP or SGEN) plasmids obtained from the MSKCC RNA Interference Core. Four distinct shRNA hairpins were screened per target. Targeted shRNA sequences are listed in Supplementary Table 1.

cGAMP quantification: For intracellular and extracellular cGAMP quantification in cancer cell lines, cancer cells were seeded in 15 cm culture dishes. When culture plates were 80-90% confluent, media was changed to serum free phenol red free RPMI (Corning). Sixteen hours following media exchange, the conditioned media was removed and centrifuged at ≥ 600 x g at 4°C for 15 minutes. Supernatant was assayed directly. All the steps were performed on ice. Cells were washed with PBS twice then trypsinzed for 5 min at 37°C and cells counts were measured. Cells were then centrifuged at ≥ 600 x g at 4°C for 15 minutes. Whole cell lysates were generated by lysing the cell pellet in LP2 lysis buffer (Tris HCl pH 7.7 20 mM, NaCl 100 mM, NaF10 mM, beta-glycerophosphate 20 mM, MgCl2 5 mM, Triton X-100 0.1% (v/v), Glycerol 5% (v/v)). The homogenate was then subjected to centrifugation at 10,000 g for 15 min. cGAMP ELISA was performed according to manufacturer's protocol using DetectX® Direct 2',3'-Cyclic GAMP Enzyme Immunoassay Kit (Arbo Assay).

Immunofluorescence microscopy: Cells were fixed with ice-cold (−30 °C) methanol for 15 min (when staining for centromeres and cGAS) or 4% paraformaldehyde (when staining for GFP). Subsequently, cells were permeabilized using 1% triton for 4 min. See Supplementary Table 3 for antibody information. TBS–BSA was used as a blocking agent during antibody staining. DAPI was added together with secondary antibodies. Cells were mounted with Prolong Diamond Antifade Mountant (Life Technologies, P36961).

Immunoblotting: Cells were pelleted and lysed using RIPA buffer. Protein concentration was determined using BCA protein assay and 20–30 µg total protein were loaded in each lane. Proteins were separated by gradient SDS–PAGE and transferred to PVDF or nitrocellulose membranes. See Supplementary Table 2 for antibody information. Membranes were imaged using the LI-COR Odyssey software.

ENPP1 staining of human xenografts: Immunohistochemistry for ENPP1 in human breast cancer xenografts was performed on the automated Discovery XT processor (Ventana Medical Systems) by the Molecular Cytology Core Facility at MSKCC. Briefly, after deparaffinized and tumor tissue conditioning, the antigen was retrieved using sodium citrate pH6 buffer for 30 min. Following blockage with Background Buster (Innovex), the slides were incubated with 2.5 μg/ml anti-ENPP1 antibody (Abcam ab4003 at 1:200, Supplementary Table 4) for 4 hr, and then incubated with the biotinylated secondary antibody for 30 minutes. The Streptavidin-HRP D (DABMap kit, Ventana Medical Systems) and the DAB detection kit (Ventana Medical Systems) were used to detect the signal according to the manufacturer instructions. Then the slides were counterstained with hematoxylin and were mounted with Permount mounting medium. Tumor necrosis was assessed semi quantitatively by a certified pathologist based on the cross-sectional area containing necrosis. The pathologist was blinded to tumor group allocation.

H&E staining and Immune phenotyping of lung metastases: Lungs were excised from euthanized mice and submerged in 4% PFA overnight at 4 ^{o}C and then were transferred to 70% ethanol. Tissue embedding, slide sectioning, and H&E staining were performed by the Molecular Cytology Core Facility at MSKCC. Immunohistochemistry for CD8 and CD45 staining were performed using anti-CD8 (Cell Signaling Technology #98941) and anti-CD45 (Biosciences 550539) by the Laboratory of Comparative Pathology at MSKCC. For immune profiling using flow cytometry, animals were sacrificed 18 days after tail vein injection with control and ENPP1 KO 4T1 cells. Lungs were perfused through the right ventricle with 10-15 ml of PBS. The lungs were removed, and the large airways, thymus, lymph nodes were dissected from the peripheral lung tissue. The peripheral lung tissue was minced and transferred into 50 ml falcon tubes and processed in digestion buffer by mouse tumor dissociation kit (Miltenyi), according to the manufacturer's instructions. Homogenized lungs were passed through 40-μm nylon mesh to obtain a single-cell suspension. The remaining red blood cells were lysed using BD Pharm Lyse (BD Biosciences, San Jose, CA). Cells were stained with viability dye LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Invitrogen), followed by incubation with FcBlock (Invitrogen), and stained with a mixture of fluorochrome-conjugated antibodies (see Supplementary Table 5 for a list of antibodies, clones, fluorochromes, and manufacturers). Data were acquired on a BD LSR II flow cytometer using BD FACS Diva software (BD Biosciences); compensation and data analysis were performed using FCS express 7 software. Unstained biological controls and single-color controls were used. Cell populations were identified using sequential gating strategy (**Supplementary Fig. S5**).

Quantitative PCR: RNA was extracted from cells with Trizol (Invitrogen #15596026). cDNA was synthesized using the RNA to cDNA EcoDry™ Premix (Double Primed) kit (Takara #639549). Real-time PCR was performed to measure the relative mRNA expression levels of ENPP1 and the control GAPDH using Luna® Universal qPCR Master Mix (NEB M3003L). The qPCR reaction and analysis were performed on a QuantStudio 6 platform (Life technology). Two sets of primers for Enpp1 were used. The sequence for one set of primers is 5'-CTGGTTTTGTCAGTATGTGTGCT-3' and 5'-CTCACCGCACCTGAATTTGTT-3' and the sequence for another set of primers is 5'-CTTTGAAAGGACGTTCAGCAAC-3' and 5'-AGGAGCACACGAACCTGGA=3'. The sequence for primers for GAPDH is 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Adenosine measurements: 4T1 cells were seeded in 10 cm culture dishes in quadruplicates. When culture plates reached

80-90% confluence, 7 ml serum free phenol red free RPMI (Corning) with and without inhibitors (EHNA 100 μmol/L, NBMPR 100 μmol/L, Dipyridamole 40 μmol/L) was added to plates. Conditioned media was collected after 16 h incubation. Conditioned media was centrifuged at 10,000 g for 10 min at 4ºC. Cells were harvested and cell counts were recorded for back calculations. Direct quantification of adenosine in flash-frozen conditioned media was performed by Charles River Laboratories Inc. (San Francisco). Adenosine concentrations were determined by high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection in multiple-reaction-monitoring mode (MRM). In brief, 4 μL of internal standard solution containing 10nM Adenosine-13C5 was added to 10 μL of undiluted experimental sample. 10 μL was injected into an Infinity 1290 LC system (Agilent, USA) by an automated sample injector (SIL-20AD, Shimadzu, Japan). Analytes were separated by liquid chromatography using a linear gradient of mobile phase B at a flow rate of 0.200 mL/min on a reversed phase Atlantis T3 C18 column (2.1*150 mm, 3.0 μm particle size; Waters, USA) held at a temperature of 40 °C. Mobile phase A consisted of 5mM ammonium formate in ultrapure water. Mobile phase B was Methanol. Acquisitions were achieved in the positive ionization mode using a QTrap 5500 (Applied Biosystems, USA) equipped with a Turbo Ion Spray interface. The ion spray voltage is set at 5.0 kV and the probe temperature is 500°C. The collision gas (nitrogen) pressure was kept at the Medium setting level. The following MRM transitions were used for quantification: *m/z* 268.2/136.1 for Adenosine. Data were calibrated and quantified using the Analyst™ data system (Applied Biosystems, version 1.5.2). For indirect adenosine measurements in conditioned media after cGAMP addition were performed using the adenosine assay kit (Cell Biolabs) according to a modified manufacturer's protocol: for each sample, we measured fluorescence intensity at 600nm with and without the adenosine deaminase inhibitor, EHNA (**Supplementary Fig. S3A-B**).

Cellular growth and migration assays: Cellular proliferation rates were assessed by seeding $5x10^4$ control or *Enpp1*-KO 4T1 cells in 6-well plates (3-4 replicates per condition). For migration assays, cells were seeded in the regular RPMI medium with 10% Fetal bovine serum (FBS). About 48 hours before cells growing to form a 90% confluency monolayer, regular media were replaced with media containing indicated drugs. The working concentration of cGAMP, adenosine, and the A2B antagonist PSB115 was 5.5 μM, 5.5 μM, and 1 μM, respectively. Fresh medium was changed every 12 hours. When reaching ~ 90% confluency, cells were treated with RPMI medium containing 10 μM Mitomycin C for 1 hour. Wounds were formed using sterile P200 pipette tips for experiments using 4T1 and CT26 cells and directly using wound-healing inserts (IBIDI) for experiments using E0771 cells. Adenosine deaminase was used at concentration of (1 IU/ml) and added daily. Images of the wounds were captured every 8 hours and were analyzed with a wound healing tool macro in ImageJ (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool).

Animal metastasis studies: Animal experiments were performed in accordance with protocols approved by the MSKCC Institutional Animal Care and Use Committee. For survival experiments in 4T1 experiments, power analysis indicated that 15 mice per group would be sufficient to detect a difference at relative hazard ratios of <0.25 or >4.0 with 80% power and 95% confidence, given a median survival of 58 days in the control group and a total follow up period of 180 days also accounting for accidental animal death during procedures. There was no need to randomize animals. Investigators were not blinded to group allocation. For tail vein injections, 2.5×10^4 4T1 or 10^5 CT26 cells were injected into the tail vein of 6-7-week old BALB/c mice. Metastasis was primarily assessed through overall survival. Overall survival endpoint was met when the mice

died or met the criteria for euthanasia under the IACUC protocol. Surface lung metastases were assessed at endpoint by direct visual examination after euthanasia at which points lungs were perfused and fixed in 4% paraformaldehyde (4T1 experiments) or stained using india-ink (CT26 experiments). Furthermore, lung metastasis after injection of 4T1 cells was qualitatively assessed using routine hematoxylin and eosin (H&E) staining as shown in **Fig. 5E**. Metastatic dissemination in **Supplementary Fig. S2J** was determined using bioluminescence imaging. Mice were injected with d-luciferin (150 mg kg^{-1}) and subjected to bioluminescence imaging (BLI) using tan IVIS Spectrum Xenogen instrument (Caliper Life Sciences) to image locoregional recurrence as well as distant metastases. BLI images were analyzed using Living Image Software v.2.50. For orthotopic tumor implantation, 5×10^5 4T1 cells in 50 μl PBS were mixed 1:1 with Matrigel (BD Biosciences) and injected into the fourth mammary fat pad. Only one tumor was implanted per animal. Primary tumors were surgically excised on day 7 after implantation and metastatic dissemination was assessed by monitoring overall survival or on day 30 through quantification of surface lung metastases upon euthanasia. In the E0771 metastasis model, 5×10^5 tdTomato-Luciferase expressing E0771 cells were injected into the tail vein of 7-12-week old C56BL/6 or MPYS-/- (STING KO) mice. Metastatic dissemination were accessed by BLI.

Analysis of ENPP1 protein expression and tumor infiltrating lymphocytes in breast tumor samples: Primary analysis of ENPP1 protein expression was performed on a tissue microarray (TMA) of comprising 226 TNBC FFPE tumor samples of which 223 had sufficient material. Samples and follow up data for cohort 1 were collected MSKCC IRB approval. There were 3 cores per tumor sample. Detailed clinical characteristics and clinical follow-up data were previously reported (39). Immunohistochemistry for ENPP1 in breast cancer cohort 1 was performed on the automated Discovery XT processor (Ventana Medical Systems) by the Molecular Cytology Core Facility at MSKCC. Briefly, after deparaffinized and tumor tissue conditioning, the antigen was retrieved using standard CC1 (Ventana Medical Systems). Following blockage with Background Buster (Innovex), the slides were incubated with 2.5 μg/ml anti-ENPP1 antibody for 5 hr, and then incubated with the biotinylated secondary antibody for 60 minutes. The Streptavidin-HRP D (DABMap kit, Ventana Medical Systems) and the DAB detection kit (Ventana Medical Systems) were used to detect the signal according to the manufacturer instructions. Then the slides were counterstained with hematoxylin and were mounted with Permount mounting medium. Slides of immunofluorescence and immunohistochemistry were scanned with Pannoramic Flash 250 (3DHistech, Budapest, Hungary) with 20x/0.8 NA air objective by the Molecular Cytology Core Facility at MSKCC. ENPP1 protein expression levels were performed by a board-certified breast pathologist who was blinded to other clinicopathological characteristics and outcome. ENPP1 protein expression levels were assessed manually using scores of 0 (absent), 1 (weak), 2 (moderate) and 3 (strong) for both stromal and tumor compartments. Given this analysis was performed on small core material, ENPP1 expression was considered when >1% of cells showed a given staining pattern. Distant metastasis-free survival data were collected by reviewing medical records available at MSKCC. TILs were scored according to the recommendations of the international TILs working group (40) based on the original hematoxylin and eosin-stained sections corresponding to each of the tumors present in the TMA. Tumors were stratified as having low (negative or weak) or high (moderate or strong) ENPP1 expression. Independent validation studies were performed on a tissue microarray of n = 91 estrogen receptor (ER) negative (Cohort 2) and n= 115 ER positive (Cohort 3) FFPE breast tumors identified by the Northern Ireland Biobank (NIB), previously described elsewhere (41,42). Resected tumors were available between 1998 and 2008, with long-term follow-up data (relapse-free and overall

survival) collated via the Northern Ireland Cancer Registry. Immunohistochemistry (IHC) was performed on 4 μm sections for CD8 (NIB15-0168, Office for Research Ethics Committees Northern Ireland (ORECNI) 13-NI-0149) using C8/144B, M7103, Dako at 1:50 dilution after an ER2 20 minutes retrieval, and for ENPP1 (NIB19-0301, ORECNI 13-NI-0149) using EPR22262-72, ab24538, Abcam at 1:1000 dilution. Slides were scanned on an Aperio AT2 Digital scanner at 40x. CD8+ T cell infiltration was reported as CD8+ cell density per mm^2 based on the total number of cells in each core and determined using the open-source digital pathological analysis software QuPath *v0.1.2* (43,44). Cores with < 100 tumor cells were removed from analysis and multiple core data were averaged. Rigorous quality control steps were taken to remove necrosis or keratin, tissue folds and entrapped normal structures; this was confirmed by a second reviewer with frequent consultation following an established method. ENPP1 protein expression levels were assessed manually using scores of 0 (absent), 1 (weak), 2 (moderate) and 3 (strong) for both stromal and tumor compartments as described above. Both analyses were performed blinded to other clinicopathological characteristics and outcome. Survival analysis was restricted to tumors with low nodal disease burden (N0-1). For OS analysis, ER- tumors were stratified as either positive (n= 59) or negative (n = 15) for ENPP1 staining. Given increased expression of ENPP1 in ER+ tumors in general, tumors were stratified as either having low (negative, weak, or moderate, n = 41) or high (strong, n = 42) ENPP1 staining.

ENPP1 staining and immune profiling of mucosal melanoma samples: Immunofluorescence for ENPP1 and cGAS was performed on the automated Discovery XT processor (Ventana Medical Systems) by the Molecular Cytology Core Facility at MSKCC (45). The procedure of deparaffinization, cell condition, antigen retrieval, and nonspecific blockages was similar as described in the immunohistochemistry section above. Instead of DAB detection kit, Tyramide-Alexa Fluor 488 (Invitrogen B40932) and Tyramide-Alexa Fluor 594 (Invitrogen B40957) were used for signal detection. cGAS and ENPP1 staining were sequentially performed with 1:200 diluted anti-cGAS and 1 ug/ml of anti-ENPP1 antibodies as the primary antibody. DNA was stained with 5 μg/ml of DAPI in PBS for 10 minutes. Then the slides were mounted with Mowiol mounting medium.

RNAseq analysis of TCGA tumors: RNA-seq data for human tumor samples from TCGA patients were obtained from (https://gdc.cancer.gov/about-data/publications/pancanatlas) (46). The data is upper-quartile normalized RSEM for batch-corrected mRNA gene expression and is from 33 different cancer types. Overall leukocyte fractions and CIBERSORT immune fractions for the TCGA Breast Cancer (BRCA) patients were obtained from (https://gdc.cancer.gov/node/998) (47). The absolute abundance of the CIBERSORT immune cell types was obtained by multiplying the leukocyte fraction by the CIBERSORT immune fractions. The expression values for ENNP1 and CGAS from the TCGA RNA-seq data were utilized to categorize tumors into the four groups $ENPP1^{low}CGAS^{low}$, $ENPP1^{high}CGAS^{low}$, $ENPP1^{low}CGAS^{high}$, and $ENPP1^{high}CGAS^{high}$. The median expression value per cancer type was used to categorize tumors into $ENPP1^{low}$ and $ENPP1^{high}$ groups. Tumors with expression values less than or equal to the median for a given cancer type were considered $ENNP1^{low}$, while tumors with expression values above the median were considered $ENPP1^{high}$. The bottom tertile expression value per cancer type was used to categorize tumors into $CGAS^{low}$ and $CGAS^{high}$ groups. Tumors with expression values less than or equal to the bottom tertile (<33%) of *CGAS* expression in a given cancer type were categorized as $CGAS^{low}$, while tumors with expression values greater than the bottom tertile (>33%) were categorized as $CGAS^{high}$. The Wilcoxon Rank-Sum test was used to compare the relative abundance of CIBERSORT immune cell types between different *CGAS*/*ENPP1* expression subgroups. For pathway enrich-

ment analysis, the DESeq2 R package (48) was used to identify differentially expressed genes between the *ENPP1*^{low}*CGAS*^{high} and *ENPP1*^{high}*CGAS*^{high} groups within the TCGA BRCA cohort. The Gene Set Enrichment Assay (GSEA) method (49) was used to perform a pathway enrichment analysis between the *ENPP1*^{low}*CGAS*^{high} and *ENPP1*^{high}*CGAS*^{high} groups. A pre-ranked gene list from DESeq2 was created and sorted by the following: sign of the log fold change * -log(adjusted p-value). The sorted pre-ranked list was run in GSEA with the Hallmark gene set database that was downloaded from the Molecular Signatures Database (MSigDB) (49). Survival analysis across TCGA tumor types were performed using KMPlot (http://www.kmplot.com) using auto-selection for best cutoff between the 25th and 75th percentiles.

RNAseq analysis of human sarcomas: Matched clinicopathological and RNA sequencing data for samples annotated as undifferentiated pleomorphic sarcoma (UPS, also known as malignant fibrous histiocytoma) were obtained from The Cancer Genome Atlas (TCGA) Genomic Data Commons Data Portal repository in May 2018. Raw read counts were utilized for our analysis. Two additional publicly available RNA sequencing datasets of UPS tumors were obtained for validation (50,51). For analysis of the Steele et *al*. dataset (EGAD00001004439), we utilized previously processed data (transcripts per million). For analysis of the Lesluyes et *al*. dataset, FASTQ files (SRA accession ID SRP057793) were preprocessed with Kallisto (52) using the human genome reference GRCh38 and transcript level abundances were computed using the Bioconductor package tximport (53). The abundance of tissue-infiltrating immune cells was estimated using transcriptome-based methods. The Microenvironment Cell Populations-counter (MCP-counter) method (54) was used to determine relative abundance of various tumor immune microenvironment constituents. Specifically, MCP-counter cytotoxic T-lymphocyte (CTL) scores were calculated from expression of seven transcripts including *CD8A* and log2-normalized. CTL scores were validated using an orthogonal transcriptome-based method, cytolytic activity (CYT) scores (55), calculated as the geometric mean of granzyme A (*GZMA*) and perforin (*PRF1*) transcript counts.

Bladder cancer response data to anti-PD-L1 treatment: RNA sequencing data was obtained from Mariathasan et *al*. (32), a metastatic urothelial cancer anti-PD-L1 treated cohort in SRA format, and reverted back to FASTQ using bam2fastq (v1.1.0). FASTQ reads were aligned to the hg19 genome using STAR (56). Transcript quantification was performed using RSEM with default parameters (57). Response was defined based on radiological response as per the RECIST criteria, with "CR/PR" being classified as a responder and "SD/PD" being a non-responder. The *CGAS*^{high} group was defined as the upper two tertiles, and *CGAS*^{low} as the bottom tertile, of *CGAS* expression.

Animal immunotherapy experiments: To assess the role of ENPP1 in the primary tumor growth upon the immune checkpoint blockade (ICB), we adopted the 4T1 orthotopic mammary fat pad implantation model. First, 4T1 cells (4T1-Luc) cells and 4T1-Luc *Enpp1* knockout (KO) cells were generated by stably integrating the Lentivirus pLVX vector expressing the tdTomato-Luciferase fusion gene in the 4T1 and 4T1 *Enpp1*-KO cells, respectively. Fifteen ~7-week-old mice were used for each of the arm, including four combinations of two cell lines (4T1-Luc and 4T1-Luc ENPP1 KO) and two conditions (ICB and the isotype control treatment). 2.5 x 10^5 4T1-Luc cells or 4T1-Luc *Enpp1*-KO cells in PBS:Matrigel (1:1) mix were injected into the mammary fat pad of Balb/c mice. 200 μg rat anti-mouse PD1 IgG2a antibody (aPD1) and 100 μg mouse anti-mouse CTLA4 IgG2b antibody (aCTLA4) or their corresponding isotype control antibodies were delivered intraperitoneally in 100 ml

of PBS to mice every 3 days starting at day 6 post implantation. After 4 doses of combined ICB, maintenance aCTLA4 treatment and the corresponding isotype control were given every 3 days. The length (L) and width (W) of the tumor were measured using calipers. The tumor size was calculated according to the following formula: $L*W^2/2$. For experiment in **Fig. 4C and 4F**, endpoint was determined when primary tumor size of 2000 mm^3. For the CT26 model, 100,000 eGFP or eGFP-ENPP1 expressing CT26 cells were delivered intravenously to 7 week-old Balb/c mice. Treatment with aPD1/aCTLA4 antibodies and their corresponding isotype control antibodies was initiated intraperitoneally starting on day 6 and given every 3 days for 5 total doses. Animals were monitored for overall survival. For the E0771 model, 1 x 10^6 eGFP or eGFP-ENPP1 expressing E0771-Luc cells in PBS:Matrgel (1:1) mix were injected into the mammary fat pad of C57BL/6 WT mice or MPYS-/- (STING KO) at the age of 7-weeks. Treatment with 200 μg of aPD1 or its corresponding isotype control antiboidy were given on day 6, 10, and 13.

Data Availability: Tumor DNA and RNA sequence data used in this manuscript is publicly available and cited as appropriate in the text and methods section. No new code was used in this manuscript

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

SFB holds a patent related to some of the work described targeting CIN and the cGAS-STING pathway in advanced cancer. He owns equity in, receives compensation from, and serves as a consultant and the Scientific Advisory Board and Board of Directors of Volastra Therapeutics Inc. He has also consulted for Sanofi, received sponsored travel from the Prostate Cancer Foundation, and both travel and compensation from Cancer Research UK. JDW served as a consultant for Adaptive Biotech, Advaxis, Amgen, Apricity, Array BioPharma, Ascentage Pharma, Astellas, Bayer, Beigene, Bristol Myers Squibb, Celgene, Chugai, Elucida, Eli Lilly, F Star, Genentech, Imvaq, Janssen, Kleo Pharma, Kyowa Hakko Kirin, Linneaus, MedImmune, Merck, Neon Therapuetics, Northern Biologics, Ono, Polaris Pharma, Polynoma, Psioxus, Puretech, Recepta, Takara Bio, Trieza, Sellas Life Sciences, Serametrix, Surface Oncology, Syndax, and Syntalogic. He also receives research support from Bristol Myers Squibb, Medimmune, Merck Pharmaceuticals, and Genentech. He owns equity in Potenza Therapeutics, Tizona Pharmaceuticals, Adaptive Biotechnologies, Elucida, Imvaq, Beigene, Trieza, and Linneaus. He has received honoraium from Esanex. TM is a consultant for Immunos Therapeutics and Pfizer; is a co-founder with equity in IMVAQ therapeutics; receives research funding from Bristol-Myers Squibb, Surface Oncology, Kyn Therapeutics, Infinity Pharmaceuticals Inc., Peregrine Pharmaceuticals Inc., Adaptive Biotechnologies, Leap Therapeutics Inc., and Aprea; is an inventor on patent applications related to work on Oncolytic Viral therapy, Alpha Virus Based Vaccine, Neo Antigen Modeling, CD40, GITR, OX40, PD-1 and CTLA-4. KL reports speaker fees from Roche Tissue Diagnostics, travel compensation from BMS and grant income for Genetech. JSR-F has received fees for consulting for Goldman Sachs, REPARE Therapeutics and Paige.AI, and serves as an advisory board member for Roche Diagnostics, InVicro, Genentech, Paige.AI, Volition RX, REPARE Therapeutics and GRAIL. C.S. receives grant support from Pfizer, AstraZeneca, BMS, Roche-Ventana and Boehringer-Ingelheim. C.S. has consulted for Pfizer, Novartis, GlaxoSmithKline, MSD, BMS, Celgene, AstraZeneca, Illumina, Genentech, Roche-Ventana, GRAIL, Medicxi, the Sarah Cannon Research Institute and is an Advisor for Dynamo Therapeutics. C.S. is a shareholder of Apogen Biotechnologies, Epic Bioscience, GRAIL, and has stock options in and is co-founder of Achilles Therapeutics. Outside of the submitted work, K.L. and C.S. have a patent on indel burden and checkpoint inhibitor response pending and a

patent on targeting of frameshift neoantigens for personalized immunotherapy pending. BI is a consultant for Merck & Co. Remaining authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

SFB conceived of this project and provided overall supervision. JL, MAD, ND, RKS, JK, JCM, SV, JAC, BI, EEP performed animal and in vitro cell-based experiments, PL provided critical experimental design input, WC, KL, MLN, PD, NR performed bioinformatics analysis, MH, CP, LG, TH, TM, HW, JRF, JDW, AS, MPH, MST, JAJ, EEP, performed and/or supervised pathologic analysis of tumor samples, OE, CS, BI, EEP, and SB supervised experimental work, performed data analysis and interpretation, and contributed to the writing of this manuscript.

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Figures and Figure legends

Figure 1. **ENPP1 promotes metastasis of chromosomally unstable tumors**. (A) Representative immunofluorescence images of control and ENPP1-depleted MDA-MB-231 CIN^{high} cells stained with DAPI (DNA) and anti-ENPP1 antibody, scale bar 5μm. (B) Immunohistochemistry of an orthotopically transplanted MBA-MB-231 tumor using anti-ENPP1 antibody. (C) *ENPP1* mRNA expression in various stages of lung adenocarcinoma progression, bars represent mean ± s.e.m. (D) Extracellular-to-intracellular cGAMP ratio in 4T1, CT26, and E0771 cells, bars represent median, n = 10 independent experiments, ** p<0.01, two-sided Mann-Whitney test. (E) Overall survival of animals that were orthotopically transplanted by control and *Enpp1*-knockout 4T1 tumors followed by tumor resection 7 days later, n = 15 animals per condition, significance tested using log-rank test. (F) *Left,* Quantification of surface lung metastases after tail vein injection of control and *Enpp1*-knockout 4T1 cells, bars represent median, n = 13-15 animals per condition, **** p<0.0001, two-sided Mann-Whitney test. *Right*, Representative hematoxylin and eosin-stained lungs from animals injected with control and ENPP1-knockout 4T1 cells, scale bar 3mm. (G) Surface lung metastases after tail vein injection of eGFP and eGFP-ENPP1-expressing CT26 cells, bars represent median, n = 15 animals per condition, **** p < 0.0001, two-sided Mann-Whitney test.

Figure 2. **ENPP1 promotes extracellular adenosine production.** (A) *Left*, total bioluminescence imaging of WT or *Tmem173*$^{-/-}$ animals inoculated with E0771 cells expressing WT or enzymatically weakened ENPP1 (T328A), bars represent median, n = 13-15 mice per group for the WT animals and 11-12 for the *Tmem173*$^{-/-}$ animals, * p < 0.05, Welch t-test. (B) Schematic showing the generation of adenosine from extracellular cGAMP and ATP hydrolysis. (C) Normalized adenosine concentration (per 10^7 cells after 16 hours incubation in serum-free media) in conditioned media of control, *Cgas*-KO, *Enpp1*-KO 4T1 cells, bars represent mean ± s.e.m., n = 4 independent experiments, *p<0.05, two-sided t-test. (D) Percent wound remaining after 24 hours in control, *Cgas*-KO, and *Enpp1*-KO 4T1 cells treated with cGAMP or cGAMP and the adenosine receptor blocker, PSB115. (E) *NT5E* and *ENTPD1* mRNA expression in various stages of lung adenocarcinoma progression, bars represent mean ± s.e.m. (F) Surface lung metastases after tail vein injection of control, *Enpp1*-KO, *Nt5e*-KO, and *Enpp1*/*Nt5e* double KO 4T1 cells, bars represent median, n = 15 animals per condition, **** p < 0.001, two-sided Mann-Whitney test.

Figure 3. **ENPP1 reduces tumor immune infiltration.** (A) Representative immunohistochemistry (IHC) of control and ENPP1-knockout TNBC lung metastases stained using an anti-CD45 antibody. (B) The number of metastasis-infiltrating CD8+ T-cells (left) and representative IHC of control ENPP1-knockout TNBC lung metastases stained using anti-CD8 antibody (right), bars represent median, n = 13-31 metastases, **** p<0.0001, two-sided Mann-Whitney test. (C) Percentage of CD45+, CD11b+Ly6G+, CD4+, and CD8+ cells out of the total cells as well as the percentage of PD1+ cells out of the CD3+CD4+ and CD3+CD8+ cells obtained from dissociated lungs after injection with control or ENPP1-knockout 4T1 cells, n = 5 animals per group. (D) GM-CSF levels measured in orthotopically transplanted control and ENPP1-knockout tumors, bars represent median, n = 15 tumors per condition, ** p<0.01, two-sided Mann-Whitney test. (E) Percentage of CD8+ T-cells, CD4+ T-cells (and the PD1+ and CD44+ fractions of thereof), and NK-cells obtained from dissociated subcutaneously transplanted control and ENPP1 expressing CT26 tumors, n = 5 animals per group, bars represent median, * p < 0.05.

Figure 4. **ENPP1 promotes resistance to immune checkpoint blockade therapy**. (A) Schematic diagram of immunotherapy experiments. (B) Growth curves of control and *Enpp1*-KO orthotopically transplanted tumors 4T1 upon treatment with combined ICB or corresponding isotype controls, data points represent mean ± s.e.m., n = 15 animals per group, ****p<0.0001, two-sided t-test. (C) Survival of animals after orthotopic transplantation with control, *Enpp1*-KO, *Cgas*-KO, or *Enpp1*/*Cgas* double-KO 4T1 cells treated with combined ICB or corresponding isotype controls, significance tested using log-rank test, *** p < 0.001, n = 15 animals per group. (D) Survival of BALB/c mice injected with eGFP or eGFP-ENPP1 expressing CT26 cells, treated with combined ICB or isotype controls, n = 15 animals per group, significance tested using log-rank test, ***p<0.001. (E) Spider plots of eGFP or eGFP-ENPP1 expressing E0771 orthotopic tumors inoculated in WT or *Tmem173*$^{-/-}$ C57BL/6 hosts. (F) Survival of WT C57BL/6 mice with eGFP or eGFP-ENPP1 expressing E0771 orthotopic tumors, treated with combined ICB or isotype controls, n = 10 animals per group, significance tested using log-rank test, *p<0.05.

Figure 5. **ENPP1 expression is associated with metastasis in human cancer**. (A) ENPP1 expression across primary and metastatic tumors, stratified by the site of metastasis, n = 180 tumors for primary tumors and 331 tumors for metastases, bars represent median, * p < 0.05, ** p < 0.01, *** p < 0.001. (B) Percentage of mucosal melanoma patients with tumor-specific or stromal specific ENPP1 staining patterns in primary as well as metastatic mucosal melanoma human tumor samples, *p < 0.05, χ^2-test. (C-D) Representative immunofluorescence images of low (C) and high (D) magnification images of lymph node metastases from mucosal melanoma stained using DAPI (DNA) and anti-ENPP1 antibody showing selective membrane staining of ENPP1 on metastatic cancer cells. Scale bar 1mm (C) and 50μm (D). (E) Representative images of human TNBCs stained using anti-ENPP1 antibody, scale bar 100μm. (F) Distant-metastasis-free survival in patients with TNBC stratified based on their ENPP1 and cGAS expression n = 159, significance tested using log-rank test.

Figure 6. **ENPP1 expression is associated with reduced lymphocytic infiltration in human cancer**. (A) Percentage of tumor-infiltrating lymphocytes (TILs) in breast tumors stratified based on their ENPP1 expression, bars represent mean ± s.e.m., *** p < 0.001, two-tailed t-test. (B) Representative images of human breast cancers stained using anti-ENPP1 or anti-CD8 antibodies. Scale bar 100μm. (C) Tumor immune infiltration inferred using the CIBERSORT method on breast tumors found in the TCGA, box plots represent median, lower and upper quartiles, error bars represent 10th and 90th percentiles, n = 1079 tumors, **** p<0.0001, two-sided Mann-Whitney test. (D) Percent objective response rate (ORR) to anti-PD1/PD-L1 therapy as a function of ENPP1 expression by cancer type for tumor histologies with high levels of *CGAS* expression. (E) Schematic illustrating the consequence of ENPP1 activity (right) or its absence (left) on cancer metastasis and immune evasion.

Supplementary Figures and Figure legends:

Supplementary Figure S1. (A) Representative images of 4T1 cells undergoing error-free anaphase or anaphase with evidence of chromosome missegregation, scale bar 2μm. (B) Representative image of a 4T1, CT26, and E0771 cells with micronuclei stained using DAPI and anti-cGAS antibody, scale bar 2μm. (C) Immunoblots of control, *Cgas*-KO, and STING(*Tmem173*)-KO 4T1 cell lysates stained using anti-STING, anti-cGAS, α-tubulin and β-actin antibodies. (D) *Left,* cGAMP levels in cell lysates of 4T1 cells incubated in serum-free media for 24 hours. cAGMP levels were normalized for cell number. *Right*, Relative intracellular and extracellular cGAMP production in 4T1 cells. Bars represent mean ± s.e.m. n = 6 inde-

pendent experiments ** p<0.01, two-sided t-test. (E) Volcano plot showing differentially expressed genes between MDA-MB-231 cells expressing MCAK or Kif2b (CIN^{low}) or dominant-negative MCAK (CIN^{high}). (F) Immunoblots of CIN^{low} and CIN^{high} cell lysates stained with anti-ENPP1 and anti-β-actin antibodies.

Supplementary Figure S2. (A) Representative immunohistochemistry (IHC) images of control and ENPP1-depleted orthotopically transplanted human TNBCs stained using anti-ENPP1 antibody, scale bar 200μm. (B) Immunoblots of control and ENPP1-depleted CIN^{high} MDA-MB-231 cell lysates stained using anti-ENPP1 and anti-β-actin antibody. (C) Relative ENPP1 mRNA levels in 4T1, CT26, E0771, E0771.LMB cells. (D) ENPP1 mRNA levels in 4T1 cells as well as cells derived from lung metastases. ***p < 0.001, two-tailed t-test. (E) Sequence of 4T1 single-cell derived clones showing successful ENPP1 knockout and absence of wildtype allele. (F) Proliferation of control and *Enpp1*-knockout 4T1 cells over time. (G) Volume of orthotopically transplanted control and ENPP1-knockout tumors over time. Data points represent average ± s.e.m. (H-I) Recurrent primary tumor weight (H) and surface lung metastases (I) after resection of control or *Enpp1*-knockout primary tumor resection, bars represent median, * p<0.05, ** p<0.01, two-sided Mann-Whitney test. (J) Representative bioluminescence images of BALB/c mice 35 days after orthotopic transplantation with control and *Enpp1*-KO 4T1 tumors followed by tumor resection on day 7. (K) Overall survival of animals injected by control or *Enpp1* knockout 4T1 cells, n = 15 animals per condition, significance tested using log-rank test.

Supplementary Figure S3. (A) Schematic of extracellular adenosine metabolism illustrating an indirect fluorescence-based method of quantifying extracellular adenosine production. By subtracting fluorescence measurements obtained from media containing adenosine deaminase inhibitor, PSB115, from media without the inhibitor, we are able to quantify the amounts of downstream products arising from extracellular adenosine degradation. (B) Relative fluorescence intensity at 600 nm with and without the addition of PSB115 in the presence of increasing amounts of exogenous cGAMP. (C) Immunoblots of control, *Enpp1*-KO, *Nt5E*-KO, and *Enpp1*/*Nt5e* double-KO 4T1 cell lysates stained using anti-NT5E and β-actin antibodies. (D) Percent wound remaining after 48 hours in CT26 and E0771 cells expressing eGFP, eGFP-ENPP1, or eGFP-ENPP1-T328A treated with or without Adenosine deaminase inhibitor (ADA), bars represent mean ± s.e.m., n = 5-6 biological replicates (E0771) and 15 replicate (CT26), significance tested using ANOVA. (E) Representative images of wounds at 0hr and 48 hr with or without ENPP1 expression and ADA treatment. (F) Overall survival of animals injected by control, *Enpp1*-KO, STING-KO, or Enpp1/*STING* double-KO 4T1 cells, n = 15 animals per condition, significance tested using log-rank test.

Supplementary Figure S4. (A) Semi-quantitative measurement of tumor necrosis in control and ENPP1-depleted human TNBC xenografts. (B) Representative IHC images of control and ENPP1-depleted TNBC xenografts stained using NK1.1 (to stain NK-cells), scale bar 200μm. (C) Percentage of live cells, CD45+ cells, and FoxP3+ CD4+ T-cells obtained from dissociated subcutaneously transplanted control and ENPP1 expressing CT26 tumors, n = 5 animals per group, bars represent median, * p < 0.05. (D) Immunoblots of control, *Enpp1*-KO, *Cgas*-KO, and *Enpp1*/*Cgas* double-KO 4T1 cell lysates stained using anti-cGAS and β-actin antibodies. (E) Representative immunohistochemistry (IHC) of control, *Enpp1*-KO, *Cgas*-KO, and *Enpp1*/*Cgas* double-KO 4T1 lung metastases stained using an anti-CD45 antibody. (F) The number of CD8+ T-cells per field

(4000μm^2) in control, *Enpp1*-KO, *Cgas*-KO, and *Enpp1*/*Cgas* double-KO 4T1 lung metastases, bars represent median, n = 17-26 fields, **** p<0.01, two-sided Mann-Whitney test.

Supplementary Figure S5. FACS gating scheme for immune profiling experiments of dissociated lungs containing control and *Enpp1*-KO lung metastasis from 4T1 tumors.

Supplementary Figure S6. (A) Representative immunofluorescence images of control, eGFP-expressing, and eGFP-ENPP1 expressing CT26 cells stained using DAPI (DNA), scale bar 10μm. (B) Immunoblots of control and luciferase expressing wildtype or *Enpp1*-KO 4T1 cells stained using anti-tdTomato-Luciferase and Lamin B1 antibodies (C) Spider plots showing growth of orthotopically transplanted control and *Enpp1*-KO 4T1 tumors (derived from two independent knockout populations) as well as *Cgas*-KO or *Enpp1*/*Cgas*-double KO tumors treated with combined ICB or isotype control antibodies. (D) Survival of *Tmem173*$^{-/-}$ C57BL/6 mice with eGFP or eGFP-ENPP1 expressing E0771 orthotopic tumors, treated with combined ICB or isotype controls, n = 10 animals per group, significance tested using log-rank test, *p<0.05, n = 4-5 animals per group.

Supplementary Figure S7. (A) ENPP1 mRNA levels across human cancer types found in the TCGA database. (B) Hazard ratio for death of patients stratified by tumor ENPP1 median expression values. Data points represent HR ± 95% CI, red data points represent p < 0.05. (C) *CGAS* and *ENPP1* mRNA expression levels across breast cancer subtypes found in the TCGA, bars represent median ± interquartile range, ** p<0.01, **** p<0.0001, two-sided Mann-Whitney test. (D) Overall survival of breast cancer patients stratified by tumor receptor status and ENPP1 expression levels, significance tested using log-rank test.

Supplementary Figure S8. (A) ENPP1 mRNA expression levels across human tumor-derived organoids. Bars represent median values, * p < 0.05, two-sided t-test. (B) Distribution tumor samples exhibiting stroma-specific and cancer cell-specific staining patterns of ENPP1 in three independent cohorts of human breast cancer. (C-E) Distant metastasis-free survival (DMFS, C), Overall survival (OS, D), and relapse-free survival (RFS, E) in patients with TNBC (C-D) or ER+ breast cancer (E) stratified based on their ENPP1 expression n = 69 (C), 73 (D), and 78 (E) patients, significance tested using log-rank test.

Supplementary Figure S9. (A-B) Percentage of tumor or stromal CD8+ T-cells two independent human breast cancer cohorts stratified based on their tumor and stromal ENPP1 expression. (C) Gene-set enrichment plots comparing cGAS^{high}-ENPP1-^{high} and cGAS-^{high}ENPP1-^{low} human breast tumors showing upregulation of inflammation related gene sets in ENPP1-low tumors. (D) Correlation between cytotoxic lymphocyte score and either ENPP1 levels or the ratio of ENPP1-to-cGAS mRNA levels in 3 independent sarcoma datasets.

Supplementary Figure S10. (A) Representative immunofluorescence images of mucosal melanoma samples stained for using DAPI (DNA), anti-cGAS antibody, and anti-ENPP1 antibody, sale bar 100□m. (B) A representative high-resolution immunofluorescence image of a mucosal melanoma sample stained using DAPI (DNA) or anti-cGAS antibody showing cGAS locali-

zation to micronuclei. Scale bar 2μm. (C) Representative multispectral immunofluorescence images of mucosal melanoma samples stained using DAPI (DNA), anti-CD8, and anti-Melan A antibodies. (D) CD8+ T-cell density as a function of combined cGAS and ENPP1 staining intensity in mucosal melanoma samples. Scale bar 100 μm. Bars represent median, *p<0.05 two-sided Mann-Whitney test. (E) Percent objective response rate (ORR) to anti-PD1/PD-L1 therapy by cancer type in tumor histologies with low levels of *CGAS* expression. (F-G) ENPP1 and cGAS mRNA expression levels of in bladder tumors (F) or TNBC (G) stratified by response to ICB. Bars represent median ± interquartile range, * p<0.05, **** p<0.0001, two-sided Mann-Whitney test.

Supplementary Table 1. crRNA guide sequences

Gene target	crRNA vs. shRNA	Catalog number
Enpp1	crRNA	TACAACGCAAGTTGCCACTG
Enpp1	crRNA	GATTCCGGATAAAGTCCCTA
Enpp1	crRNA	GGTGACCGCTAATCATCAGG
Enpp1	crRNA	GATTACCGTGATCTGAAATG
Enpp1	crRNA	GAAGTCTATAACTTAATGTG
Cgas	crRNA	ACGCAAAGATATCTCGGAGG
Cgas	crRNA	GCGAGGGTCCAGGAAGGAAC
ENPP1	shRNA	TTAATAATCTTCTCTTCTGCCA
ENPP1	shRNA	TTTCAATAAAAAATCATTCCAC
ENPP1	shRNA	TTAGAGACAATTATATTCCGTA
ENPP1	shRNA	TATTAAATAATTTTGAGTTGTA

Supplementary Table 2. Antibodies used in immunoblots

Antibodies against	Company	Catalog number
mouse cGAS	Cell Signaling Technology	31659
β-actin	Abcam	ab6276
STING	Cell Signaling Technology	13647
α-tubulin	Sigma-Aldrich	T9026
Lamin B1	Abcam	ab16048
human ENPP1	Abcam	ab223268
human ENPP1	Abcam	ab40003

Supplementary Table 3. Antibodies used in immunofluorescence

Antibodies against	Company	Catalog number
human ENPP1	Abcam	ab223268
human centromere proteins	Antibodies Incorporated	15-234-0001
mouse cGAS	Cell Signaling Technology	31659
GFP	Sigma-Aldrich	11814460001

Supplementary Table 4. Antibodies used in immunohistochemistry

Antibodies against	Company	Catalog number
human ENPP1	Abcam	ab40003
human ENPP1	Abcam	ab223268
CD45	BD Pharmingen	550539
CD8α	Cell Signaling Technology	98941
NK1.1	Thermo Fisher Scientific	MA1-70100
human cGAS	LifeSpan BioSciences	LS-C757990
Melan-A	Santa Cruz Biotechnology	sc-20032

Supplementary Table 5. Antibodies used in flow cytometry

Antibodies against	Company	Catalog number
CD45, APC-eFluor 780	Thermo Fisher Scientific	47-0451-82
Ly6G, APC	Thermo Fisher Scientific	17-9668-82
CD4, PE-Texas Red	Thermo Fisher Scientific	MCD0417
F4/80, PE/Cy5	BioLegend	123112
CD8, PE	Tonbo Biosciences	50-0081-U500
CD11b, PE/Cy7	Thermo Fisher Scientific	25-0112-82
CD3ε, BV785	BioLegend	100355
PD1, APC/Cy7	BioLegend	135224