Metastasis and Immune Evasion from Extracellular cGAMP Hydrolysis

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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 also promotes tumor progression by inducing chronic inflammatory signaling, leading to increased cancer cell migration and invasion (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). A potent immune-stimulatory molecule, cGAMP 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 inflammatory signaling arising from cGAS sensing of cytosolic dsDNA. The activation of cGAS–STING has cell-autonomous and cell-nonautonomous consequences, and therefore cancer cells must mitigate the effects of this inflammatory pathway at multiple levels. One mechanism by which chromosomally unstable cancer cells have evolved to cope with chronic cGAS–STING activation is through silencing of downstream type I IFN signaling while selecting for NFκB-dependent activity (16). The switch from type I IFN to NFκB-predominant signaling downstream of STING has been proposed to enable cancer cells to simultaneously evade immune surveillance—arising from IFN signaling—while activating noncanonical NFκB-dependent migratory programs, culminating in metastatic progression (1, 6).

In addition to its cell-intrinsic effects, cGAMP is readily exported to the extracellular space where it can promote anti-tumor immune responses by activating STING in host cells present in the tumor microenvironment (17–19). Unlike cancer

ABSTRACT
Cytosolic DNA is characteristic of chromosomally unstable metastatic cancer cells, resulting in constitutive activation of the cGAS–STING innate immune pathway. How tumors 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 loss suppresses metastasis, restores tumor immune infiltration, and potentiates response to immune checkpoint blockade in a manner dependent on tumor cGAS and host STING. Conversely, overexpression of wild-type ENPP1, but not an enzymatically weakened mutant, promotes migration and metastasis, in part through the generation of extracellular adenosine, and renders otherwise sensitive tumors completely resistant to immunotherapy. In human cancers, ENPP1 expression correlates with reduced immune cell infiltration, increased metastasis, and resistance to anti–PD-1/PD-L1 treatment. Thus, cGAMP hydrolysis by ENPP1 enables chromosomally unstable tumors to transmute cGAS activation into an immune-suppressive pathway.

SIGNIFICANCE: Chromosomal instability promotes metastasis by generating chronic tumor inflammation. ENPP1 facilitates metastasis and enables tumor cells to tolerate inflammation by hydrolyzing the immunotransmitter cGAMP, preventing its transfer from cancer cells to immune cells.

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Given the pervasive nature of CIN in human cancer (4), tumor cells must cope with the presence of persistent inflammatory signaling arising from cGAS sensing of cytosolic dsDNA. The activation of cGAS–STING has cell-autonomous and cell-nonautonomous consequences, and therefore cancer cells must mitigate the effects of this inflammatory pathway at multiple levels. One mechanism by which chromosomally unstable cancer cells have evolved to cope with chronic cGAS–STING activation is through silencing of downstream type I IFN signaling while selecting for NFκB-dependent activity to spread to distant organs (1). In line with this, an analysis of STING (Tmem173) expression in The Cancer Genome Atlas (TCGA) database found that tumor STING primarily correlates with NFκB-dependent transcriptional programs, such as the senescence-associated secretory phenotype, rather than IFN-stimulated genes (16). The switch from type I IFN to NFκB-predominant signaling downstream of STING has been proposed to enable cancer cells to simultaneously evade immune surveillance—arising from IFN signaling—while activating noncanonical NFκB-dependent migratory programs, culminating in metastatic progression (1, 6).

In addition to its cell-intrinsic effects, cGAMP is readily exported to the extracellular space where it can promote anti-tumor immune responses by activating STING in host cells present in the tumor microenvironment (17–19). Unlike cancer
cells, host cells respond to STING activation by inducing a robust type I IFN signaling central to a productive cell-mediated immunity. How tumor cells with CIN evolve to 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, by unmasking them to the immune system, while sparing normal cells devoid of cytotoxic 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; refs. 1, 20). We have previously shown that in these otherwise isogenic cell lines, expression of dnMCAK promotes increased chromosome missegregation, leading to the formation of micronuclei, chronic activation of cGAS–STING signaling, and increased metastasis (1). In addition, we used three syngeneic metastasis-competent mouse models of TNBC (4T1 and E0771) and colorectal cancer (CT26). All three models exhibited evidence for CIN, including the presence of chromosome missegregation during anaphase and a preponderance of micronuclei with robust cGAS staining indicative of cytotoxic exposure of genomic dsDNA (Supplementary Fig. S1A and S1B). To test if cGAS localization to micronuclei also led to pathway activation, we measured cGAMP levels in total cell lysates of 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 in chromosomally unstable cells (Supplementary Fig. S1C and S1D). Furthermore, cGAMP levels were nearly 15-fold higher in conditioned media after 24 hours as compared with 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 directly test the role of ENPP1 in metastasis, we performed CRISPR/Cas9 KO of Enpp1 in 4T1 cells (Supplementary Fig. S2E). We also overexpressed wild-type (WT) ENPP1 or an enzymatically weakened mutant isoform containing a threonine-to-alanine substitution in the catalytic domain (T238A; ref. 24) in CT26 and E0771 cells which express low baseline levels of this enzyme (Supplementary Fig. S2C). As expected, loss of ENPP1 led to a significant increase in the extracellular-to-intracellular cGAMP ratio (Fig. 1D). Conversely, overexpression of WT ENPP1, but not the enzymatically weakened mutant, led to a reduction in the extracellular-to-intracellular cGAMP ratio in CT26 and E0771 cells (Fig. 1D). Enpp1 KO did not affect cellular proliferation in vitro or primary tumor growth in vivo when 4T1 cells were orthotopically transplanted in the mammary fat pad (Supplementary Fig. S2F and S2G).

We then transplanted parental and Enpp1-KO 4T1 cells into BALB/c hosts, either through tail-vein inoculation or orthotopic transplantation 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 and F; Supplementary Fig. S2K). Conversely, overexpression of WT ENPP1 led 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 ENPP1T238A in E0771 and quantified metastatic dissemination using bioluminescence
ENPP1, a Therapeutic Target in Chromosomally Unstable Tumors

Figure 1. ENPP1 promotes metastasis of chromosomally unstable tumors. A, Representative immunofluorescence images of control and ENPP1-depleted MDA-MB-231 CIN<sup>+</sup> cells stained with DAPI (DNA) and anti-ENPP1 antibody; scale bar, 5 μm. B, IHC 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 ± SEM. DTCs, disseminated tumor cells. D, Extracellular-to-intracellular cGAMP ratio in 4T1, CT26, and E0771 cells; bars represent median, n = 10 independent experiments; *, P < 0.05; **, P < 0.01, two-sided Mann–Whitney test. E, Overall survival of animals that were orthotopically transplanted by control and Enpp1-KO 4T1 tumors followed by tumor resection 7 days later (n = 15 animals per condition), and significance tested using log-rank test. F, Left, quantification of surface lung metastases after tail-vein injection of control and Enpp1-KO 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-KO 4T1 cells; scale bar, 3 mm. 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. FPKM, fragments per kilobase of transcript per million mapped reads.
ENPP1 promotes extracellular adenosine production. A, Left, total BLI 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 10⁷ cells after 16-hour incubation in serum-free media) in conditioned media of control, Cgas-KO, and Enpp1-KO 4T1 cells; bars represent mean ± SEM, n = 4 independent experiments; *, P < 0.05; **, P < 0.01 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 PSB1115. E, NT5e and Enpp1 mRNA expression in various stages of lung adenocarcinoma progression; bars, mean ± SEM. FPKM, fragments per kilobase of transcript per million mapped reads. F, Surface lung metastases after tail-vein injection of control, Enpp1-KO, NT5e-KO, and Enpp1/NT5e double KO 4T1 cells; bars, median, n = 15 animals per condition; ***, P < 0.001, two-sided Mann–Whitney test.

Imaging (BLI). Only WT ENPP1—but not ENPP1T328A—led to increased metastatic dissemination (Fig. 2A). Importantly, the role of ENPP1 in metastasis was dependent on host STING, as both control and WT ENPP1-overexpressing cells had similar metastatic proclivity when transplanted into MPYS−/− (Tmem173−/−) hosts (Fig. 2A). Collectively, these results suggest that ENPP1 promotes metastatic progression through extracellular cGAMP hydrolysis, preventing protective 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 extracellular adenosine, an immune-suppressive and tumor-promoting metabolite (25). cGAMP hydrolysis by ENPP1 leads to the formation of AMP and GMP. AMP can be subsequently hydrolyzed into adenosine by NT5E (also known as CD73; 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-[4-(Nitrophenyl)methyl]-6-thioinosine (NBMPR), which prevent cellular reuptake of adenosine (Supplementary Fig. S3A; ref. 26). Extracellular adenosine levels—as assessed by LC/MS in conditioned media—were reduced by up to 40% upon KO 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 (H₂O₂) 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 H₂O₂ production and observed a concentration-dependent increase in H₂O₂ production after the addition of exogenous 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 imparts potent immune-suppressive effects (25, 27). Interestingly, KO of either Cgas or Enpp1 in 4T1 cells led to a significant reduction in cellular migration, whereas exogenous addition of cGAMP to the conditioned media rescued migration only in Cgas-KO—but not Enpp1-KO—tumor cells (Fig. 2D). The effect of cGAMP was dependent on activation of the extracellular adenosine receptors and was abolished upon the addition of PSB1115, an inhibitor of the adenosine A₂B receptor on cancer cells (Fig. 2D). Conversely, overexpression of WT ENPP1—but not ENPP1Δ328A— in E0771 or CT26 cells led to increased migration, an effect that was abolished upon treatment of the conditioned media with ADA (Supplementary Fig. S3C and S3D).

In addition to cGAMP hydrolysis by ENPP1, ATP hydrolysis by either ENPP1 or ENTPD1 (also known as CD39) is considered to be a major source of extracellular AMP. Interestingly, in the lung adenocarcinoma tumorigenesis model, expression of mouse Nt5e mirrored that of Enpp1 in that it progressively increased from normal tissues to primary tumors to metastases (Fig. 2E). On the contrary, Enpp1Δ expression followed the opposite trend with the lowest expression levels observed in metastatic lesions (Fig. 2E). These opposing trends suggest that although ATP hydrolysis might represent a major source of extracellular adenosine in primary tumors, the relative contribution from cGAMP hydrolysis as an adenosine source increases along with metastatic progression. In line with this finding, KO of either Enpp1 or Nt5e in 4T1 cells led to a significant reduction in the number of lung metastases in a manner commensurate with combined loss of both enzymes (Fig. 2F; Supplementary Fig. S3E).

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 contributions of tumor cell STING activation 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-dependent STING activation and extracellular cGAMP 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 KO in CIN™ MDA-MB-231 orthotopic xenografts and 4T1 metastatic 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 and S4B), in line with previous reports demonstrating a role for cGAMP transfer in activating NK cells (17). In the 4T1 model, metastatic lesions formed on Enpp1-KO 4T1 cells exhibited significant infiltration by CD45⁺ cells and an approximately 3- to 5-fold enrichment with CD8⁺ T cells compared with WT counterparts (Fig. 3A and B). Flow cytometry–based immune profiling of dissociated lungs revealed a significant increase in CD45⁺ cells, CD4⁺ T cells, and granulocytic CD11b⁺Ly6G⁺ cells as compared with controls (Fig. 3C; Supplementary Fig. S4C). Unlike our IHC-based results, we did not observe an absolute enrichment for CD8⁺ T cells in the injected lungs using flow cytometry; however, there was a significant increase in PD-1⁺ subpopulations of CD3⁺CD8⁺ and CD3⁺CD4⁺ cells (Fig. 3C). 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. 3D). Collectively, these findings suggest that in addition to lymphocytes, granulocytic cells may also play a role in restricting metastatic colonization of Enpp1-KO cells, in line with previous reports showing an antitumor and proinflammatory effect of CD11b⁺Ly6G⁺ cells (28–30).

We next assessed the impact of WT ENPP1 overexpression on subcutaneously transplanted CT26 tumors. Expectedly, exogenous expression of Enpp1 led to reduced CD8⁺ T cells and NK cells as well as the proportion of PD-1⁺ CD8⁺ and CD4⁺ T cells. In line with these findings, there was a decrease in the proportion of CD44⁺ T cells, suggesting reduced T-cell activation (Fig. 3E; Supplementary Fig. S3A). The fraction of FOXP3⁺ regulatory cells remained constant with a significant reduction in the CD8⁺:FOXP3⁺ ratio noted, consistent with an immunosuppressive response (Fig. 3E; Supplementary Fig. S5A). To determine whether the increased immune infiltration upon ENPP1 loss was dependent on tumor cell–derived cGAMP, we performed population-level depletion of Cgas using CRISPR KO and found a trend toward reduced CD45⁺ cell and CD8⁺ T-cell infiltration when cGAS was codepleted in Enpp1-KO 4T1 cells (Supplementary Fig. S5B–S5D). We posit that the lack of complete rescue might be due to the residual fraction of cells with functional cGAS or alternative sources of cGAMP in the tumor microenvironment. Nonetheless, these data suggest that ENPP1 dampens proinflammatory tumor immune infiltration through extracellular cGAMP hydrolysis.

**ENPP1 Inhibition Potentiates Response to Immune Checkpoint Blockade Therapy**

We then asked whether targeting ENPP1 might represent a selective therapeutic vulnerability to sensitize otherwise resistant chromosomally unstable tumors to immune checkpoint blockade (ICB) therapy. Interestingly, baseline Enpp1 mRNA expression levels in the three mouse cancer cell lines (Supplementary Fig. S2C) mirrored their previously reported sensitivities to ICB therapy, with CT26 and E0771 being considered responsive to ICB treatment in stark contrast to the highly resistant 4T1 model (18, 31). We postulated...
that Enpp1 KO would render 4T1 tumors responsive to ICB therapy, whereas its overexpression would confer resistance to otherwise sensitive CT26 and E0771 tumors (Fig. 4A; Supplementary Fig. S6A). Luciferase-expressing 4T1 cells were orthotypically transplanted into the mammary fat pad of BALB/c mice, and primary tumor growth was assessed over the span of 25 days (Fig. 4B; Supplementary Fig. S6B and S6C). Animals were treated with combined ICB [anti-PD-1 (aPD-1) and anti-CTLA4 (aCTLA4)] starting at day 6 after tumor cell inoculation for four doses followed by maintenance aCTLA4 treatment every 3 days for four additional doses. Enpp1-KO tumors, derived from two independent KO lines, exhibited reduced tumor growth rates compared with their WT counterparts when both were treated with combined ICB therapy, leading to significantly prolonged survival of the former (Fig. 4B and C; Supplementary Fig. S6C). Importantly, Cgas KO in Enpp1-KO cells diminished the responsiveness of 4T1 tumors, leading to significantly shorter survival (Fig. 4C). Notably, loss of cGAS did not lead to a full rescue of tumor response seen upon Enpp1 KO, suggesting that the hydrolysis of either
non–tumor-derived cGAMP or ATP might contribute to the immune evasion phenotype mediated by ENPP1.

We next asked whether overexpression of ENPP1 would confer ICB therapy resistance in otherwise sensitive CT26 and E0771 tumors (Fig. 4A; Supplementary Fig. S6A). CT26-bearing mice were treated with combined ICB starting at day 6 for a total of five 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 more than 140 days. Similarly, overexpression of eGFP-ENPP1 in orthotopically transplanted E0771 tumors led to their resistance upon three treatments of
aPD-1 antibody, wherein 50% of animals bearing eGFP-expressing E0771 tumors underwent a durable complete response compared with 0% of their eGFP-ENPP1–expressing tumor-bearing counterparts (Fig. 4E; Supplementary Fig. S6D). Importantly, the difference in response between eGFP- and eGFP-ENPP1–expressing tumors was abolished when they were transplanted in MOPS−/− (Timen173−/−) hosts (Fig. 4E; Supplementary Fig. S6D). Collectively, these results suggest that ENPP1 inhibition represents an attractive therapeutic strategy to potentiate the response of chromosomally unstable cancers 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 TCGA, an independent set of primary and metastatic tumors, two separate sarcoma cohorts, and in tumor-derived organoids. ENPP1 protein expression was also analyzed in three independent breast cancer cohorts, including two estrogen receptor-negative (ER−) cohorts (n = 223 and 91) and one ER-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 found in the TCGA, with the highest expression levels observed in sarcomas and liver and 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 its hormone receptor status (Supplementary Fig. S7B–S7D). To determine if Enpp1 expression was associated with metastatic progression, we first compared Enpp1 expression levels in a large number of primary and metastatic tumor samples as well as in a collection of tumor-derived organoids. In both cases, Enpp1 mRNA was higher in metastases compared with primary tumors (Fig. 5A; Supplementary Fig. S8A). When metastatic 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 ICB (32, 33). In these tumors, membrane ENPP1 expression was seen in both tumor cells and the stroma, and this pattern was evenly distributed across primary tumor 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 displayed strong ENPP1 expression in an otherwise immune cell replete microenvironment (Fig. 5C and 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). 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 (Supplementary Fig. S8B). 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 displaying both stromal and tumor cell–specific expression compared with their ER− counterparts, which had a predilection for tumor cell–specific staining (Supplementary Fig. S8B). Irrespective of the expression patterns, however, moderate-to-strong ENPP1 staining in the tumor was associated with poor prognosis, as evidenced by reduced overall survival, distant metastasis-free survival, and recurrence-free survival (Supplementary Fig. S8C–S8E). 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 be discriminatory only in tumors with high cGAS expression and activity in micronuclei. Staining using anti-cGAS antibodies revealed predominant staining at micronuclei in human tumors (example shown in Supplementary Fig. S9A and S9B). Indeed, ENPP1 protein expression was associated with reduced distant metastasis-free survival only in tumors with a preponderance of cGAS-positive micronuclei, and it had no significant association with metastasis in tumors with sparse cGAS-positive micronuclei (Fig. 5F). Collectively, these data are in agreement 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 (TIL) and CD8+ T-cell density across breast cancers and found an inverse correlation between ENPP1 IHC expression intensity and lymphocytic infiltration (Fig. 6A and B; Supplementary Fig. S9C and S9D). 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 (34). 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 proinflammatory macrophages (Fig. 6C). Furthermore, PD-L1 expression was highest in tumors with high Cgas and low Enpp1 expression. Gene set enrichment analysis (GSEA) comparing Cgashi-Enpp1hi with Cgaslo-Enpp1lo breast tumors revealed upregulation of inflammatory pathways related to allograft rejection, type I IFN, and IFN-γ-associated responses in the latter subset of tumors (Supplementary Fig. S9E). These findings suggest that Enpp1-to-Cgas ratio might be more predictive of tumor immune infiltration compared with 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. S9F). In mucosal...
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**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, median; *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, Percentage of patients with mucosal melanoma with tumor-specific or stromal-specific ENPP1 staining patterns in primary as well as metastatic mucosal melanoma human tumor samples. *, P < 0.05, χ² test. C and 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, 1 mm (C) and 50 μm (D). E, Representative images of human TNBCs stained using anti-ENPP1 antibody; scale bar, 100 μm. F, Distant metastasis-free survival (DMFS) in patients with TNBC stratified based on their Enpp1 and Cgas expression; n = 159, significance tested using log-rank test.

In line with its role modulating tumor immune responses, we found that Enpp1 expression negatively correlates with its overall response rate to aPD-1/anti-PD-L1 (aPD-L1) therapy (35). This inverse association was again restricted to tumor types characterized by elevated overall levels of Cgas expression (Fig. 6D; Supplementary Fig. S10C). We next analyzed the mRNA expression levels of Cgas and Enpp1 in 228 bladder cancers treated with aPD-L1 therapy and a smaller cohort of 52 TNBC tumors treated with aPD-1 (36, 37). Based on our TCGA analysis, these 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 and S10B).
two cancer types exhibit relatively distinct Enpp1 expression levels, representing opposite end of the spectrum. Nonetheless, there was an overall positive correlation between CGAS and Enpp1 expression in bladder tumors where Enpp1 levels were significantly lower in the CGAS hi subset of tumors that responded to aPD-L1 therapy. Expectedly, a low Enpp1-to-Cgas expression ratio was significantly associated with tumor response across both the bladder cancer and TNBC cohorts (Supplementary Fig. S10D and S10E).

**DISCUSSION**

Our work reveals an adaptive mechanism by which chromosomally unstable tumors co-opt cancer cell-intrinsic cGAS-STING signaling without eliciting antitumor immune surveillance (Fig. 6E). By virtue of their constant exposure to cytosolic dsDNA in micronuclei, cancer cells with CIN must address the consequences of cGAMP leakage into the extracellular space and its potential uptake by cells in the

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Figure 6. Enpp1 expression is associated with reduced lymphocytic infiltration in human cancer. A, Percentage of TILs in breast tumors stratified based on their ENPP1 expression; bars, mean ± SEM; ***, P < 0.001, two-tailed t test. B, Representative images of human breast cancers stained using anti-ENPP1 or anti-CD8 antibodies. Scale bar, 100 μm. 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 = 1,079 tumors; ****, P < 0.0001, two-sided Mann–Whitney test. D, Percent objective response rate to anti–PD-1/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.
tumor microenvironment. By acquiring the ability to degrade cGAMP selectively in the extracellular environment, tumor cells can maintain relatively high levels of this metabolite in the intracellular compartment where it promotes metastatic progression (1), while minimizing antitumor paracrine STING activation in neighboring immune cells (Fig. 6E).

Previous work has linked ENPP1 to the ability of tumor cells to disseminate especially in the context of bone metastasis (38), yet the precise mechanisms underlying this relationship had remained poorly understood. One possible mechanism by which ENPP1 would facilitate tumor spread to the bone is through its contribution to pyrophosphate metabolism, promoting bone remodeling (39). Our data, however, indicate that the role of ENPP1 in tumor progression 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 protective STING signaling in the tumor microenvironment.

Extracellular cGAMP hydrolysis by ENPP1 generates AMP, a substrate for adenosine production, thereby transforming an immune-stimulatory pathway into an immune-suppressive mechanism that promotes tumor progression (Fig. 6E). Our findings suggest that cGAMP represents a significant source of 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 representing a significant source in primary tumors and the fractional contribution of cGAMP as an adenosine source increasing during tumor progression. Targeting extracellular adenosine production and signaling is currently being investigated at the preclinical and clinical stages (25). ENPP1 inhibition would achieve the dual purpose of reducing the extracellular levels of an immune suppressor while simultaneously increasing extracellular levels of the immunostimulatory metabolite cGAMP. These findings highlight an important STING-independent function for tumor cGAS and suggest that, in the presence of ENPP1, high tumor cGAS activity might in fact be paradoxically immune-suppressive, enabling tolerance for CIN and pervasive cytotoxic dsDNA in advanced cancers.

Through extensive assessment of ENPP1 mRNA and protein expression levels across human tumors, our work positions ENPP1 into the broader clinical context and makes the case for the development of ENPP1 inhibitors for the treatment of advanced and chromosomally unstable cancers (19, 40, 41). Interestingly, cancer types with elevated ENPP1 expression are generally thought to be less responsive to ICB therapy, raising the possibility that extracellular purine metabolism might represent an important innate immune checkpoint that must be overcome for the full activation of the adaptive immune response against cancer. Indeed, our work suggests that ENPP1 inhibition is a viable mechanism to sensitize otherwise resistant tumors to ICB therapy. Interestingly, the widespread stromal staining patterns of ENPP1 in human cancers—reminiscent of fibroblast expression—suggest that this mechanism of immune evasion might arise not only from tumor cells but also from cells in the tumor microenvironment. Given its low expression levels in normal tissues, it will be important to dissect tumor-derived factors that promote induction of ENPP1 in the stroma. Nonetheless, our data suggest that in metastatic cancers, ENPP1 staining is biased toward a cancer cell–intrinsic pattern, raising the possibility that tumor cells that acquire the ability to transmute cGAMP-mediated immune activation into immune suppression have a selective advantage to spread to distant organs.

Therapies that activate STING (also known as STING agonists) have been the focus of intense investigation given their ability to elicit antitumor immunity through type I IFN signaling (42). 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 toward host cells where it potentiates antitumor 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 affect cGAMP concentrations at the microscopic scales relevant to paracrine tumor cell–host cell interactions. This is particularly relevant given the short half-lives of extracellular cellular cGAMP and adenosine (21). Furthermore, this critical spatial consideration is likely to minimize any potential side effects that might be observed during the systemic administration of STING agonists, thus offering a larger therapeutic window. Third, ENPP1 is selectively upregulated in metastatic and chromosomally unstable tumor cells, and a systemic ENPP1 inhibitor would interfere with the ability of disseminated tumor cells to evade immune surveillance arising from CIN, bypassing the need for technically challenging intratumoral administration that is typical of STING agonists.

In summary, our 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 (ATCC; catalog no. CRL-2539), CT26 (ATCC; catalog no. CRL-2638), and B16F10 (ATCC; catalog no. CRL-6475) cell lines were purchased from the 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) and streptomycin (50 μg/mL). All cells were found to be negative for Mycoplasma upon repeated testing every 2 months using the MycoAlert Mycoplasma Detection Kit (Lonza; catalog no. LT07-318). Cells were used within three to five passages. Details of cell line generation using CRISPR/Cas9 KO and shRNA knockdowns are included in the Supplementary Methods section, and gRNA and shRNA sequences are listed in Supplementary Table S1.

**Immunofluorescence and Immunoblotting**

Detailed protocols for immunoblotting and immunofluorescence are described in the Supplementary Methods, and antibodies used in these protocols are listed in Supplementary Tables S2 and S3, respectively.

**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% to 90% confluent, media were changed to serum-free phenol red-free RPMI (Corning). Sixteen hours following media
exchange, the conditioned media were removed and centrifuged at ≥ 600 × g at 4°C for 15 minutes. Supernatant was assayed directly. All the steps were performed on ice. Cells were washed with PBS twice and then trypsinized for 5 minutes at 37°C, and cell counts were measured. Cells were then centrifuged at ≥ 600 × 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, NaF 10 mM, betaglycerophosphate 20 mmol/L, 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 minutes. cGAMP ELISA was performed according to the manufacturer’s protocol using DetectX Direct 2’,3’-Cyclic GAMP Enzyme Immunoassay Kit (Arbo Assay).

**Hematoxylin and Eosin (H&E) Staining and Immune Phenotyping of Lung Metastases**

All antibodies used in IHC are listed in Supplementary Table S4. Lungs were excised from euthanized mice and submerged in 4% paraformaldehyde (PFA) overnight at 4°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 Memorial Sloan Kettering Cancer Center (MSKCC). IHC for CD8 and CD45 staining was performed using anti-CD8 (Cell Signaling Technology no. 98941) and anti-CD45 (Biosciences 550359) 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 to 15 mL of PBS. The lungs were removed, and the large airways, thymus, and 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). 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 S5 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. S4C).

**Adenosine Measurements**

4T1 cells were seeded in 10 cm culture dishes in triplicates. When culture plates reached 80% to 90% confluence, 7 mL serum-free phenol red-free RPMI (Corning) with and without inhibitors (EHNA 100 μM, NBMPR 100 μM, and dipyridamole 40 μM) was added to plates. Conditioned media were collected after 16-hour incubation. Conditioned media were centrifuged at 10,000 × g for 10 minutes at 4°C. 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. Adenosine concentrations were determined by high-performance liquid chromatography with MS-MS detection in multiple reaction monitoring (MRM) mode. In brief, 4 μL of internal standard solution containing 10 nM Adenosine-13CS was added to 10 μL of undiluted experimental sample. Ten microliters was injected into an Infinity 1290 LC system (Agilent) by an automated sample injector (SIL-20AD, Shimadzu). Analyses were separated by LC 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) held at a temperature of 40°C. Mobile phase A consisted of 5 mM ammonium formate in ultrapure water. Mobile phase B was methanol. Acquisitions were achieved in the positive ionization mode using a QTrap 5500 (Applied Biosystems) equipped with a Turbo Ion Spray interface. The ion spray voltage was set at 5.0 kV, and the probe temperature was 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). 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 600 nm with and without the ADA inhibitor EHNA (Supplementary Fig. S3A and S3B).

**Animal Metastasis Studies**

Animal experiments were performed in accordance with protocols approved by the MSKCC Institutional Animal Care and Use Committee (IACUC). For survival experiments in 4T1 experiments, power analysis indicated that 15 mice per group would be sufficient to detect a difference at relative HRs 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 × 105 4T1 or 5 × 105 CT26 cells were injected into the tail vein of 6- to 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 using the IACUC protocol. Surface lung metastases were assessed at endpoint by direct visual examination after euthanasia at which point lungs were perfused and fixed in 4% PFA (4T1 experiments) or stained using India ink (CT26 experiments). Furthermore, lung metastasis after injection of 4T1 cells was qualitatively assessed using routine H&E staining as shown in Fig. 5E. Metastatic dissemination in Supplementary Fig. S2J was determined using BLI. Mice were injected with d-luciferin (150 mg/kg) and subjected to BLI using the 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, 1.25 × 105 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, 2.5 × 104 tdTomato-Luciferase–expressing E0771 cells were injected into the tail veins of 7- to 12-week-old C56BL/6 or MIPY3+/− (Tnem173−/−, The Jackson Laboratory stock number 025085) mice. Metastatic dissemination was assessed by BLI.

**RNA-sequencing Analysis of TCGA Tumors**

RNA-sequencing (RNA-seq) data for human tumor samples from TCGA patients were obtained from https://gdc.cancer.gov/about-data/publications/pancanatlas (43). The data are upper-quartile–normalized RNA-seq by expectation minimization for batch-corrected mRN gene expression and are 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 (44). 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 ENPP1 and CGAS from the TCGA RNA-seq data were utilized to categorize tumors into the four groups: Enpp1hi-Cgaslo, Enpp1hi-Cgashi, Enpp1lo-Cgashi, and Enpp1lo-Cgaslo. The median expression value per cancer type was used to categorize tumors into Enpp1hi and...
**ENPP1, a Therapeutic Target in Chromosomally Unstable Tumors**

Tumors with expression values less than or equal to the median for a given cancer type were considered **Enpp1**-, whereas tumors with expression values above the median were considered **Enpp1**. The bottom tertile expression value per cancer type was used to categorize tumors into **Cgas** and **Cgas** 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**, whereas tumors with expression values greater than the bottom tertile (>33%) were categorized as **Cgas**. 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 enrichment analysis, the DESeq2 R package (45) was used to identify differentially expressed genes between the **Enpp1** and **Cgas** groups and **Enpp1** and **Cgas** groups within the TCGA BRCA cohort. The GSEA method (46) was used to perform a pathway enrichment analysis between the **Enpp1** and **Cgas** groups. A prerranked gene list from DESeq2 was created and sorted by the following: sign of the log fold change × log-adjusted (P value). The sorted prerranked list was run in GSEA with the Hallmark gene set database that was downloaded from the Molecular Signatures Database (46). Survival analysis across TCGA tumor types was performed using KMPlot (http://www.kmplot.com) using autoselection for best cutoff between the 25th and 75th percentiles.

**Animal Immunotherapy Experiments**

To assess the role of **ENPP1** in the primary tumor growth upon the ICB, we adopted the 4T1 orthotopic mammary fat pad implantation model. First, 4T1 (4T1-Luc) cells and 4T1-Luc **Enpp1**-KO cells were generated by stably integrating the Lentivirus plLVX 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 arms, including four combinations of two cell lines (4T1-Luc and 4T1-Luc **ENPP1** KO) and two conditions (ICB and the isotype control treatment). 4T1-Luc cells or 4T1-Luc **Enpp1**-KO cells (1.25 × 10^5) in PBS:Matrigel (1:1) mix were injected into the mammary fat pad of BALB/c mice. Two hundred microgram rat anti-mouse PD-1 IgG2a antibody (aPD-1) and 100 μg mouse anti-mouse CTLA4 IgG2b antibody (aCTLA4) or their corresponding isotype control antibodies were delivered intraperitoneally in 100 μL of PBS to mice every 3 days starting at day 6 after implantation. After combination 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-E, endpoint was determined when primary tumor reached the size of 2,000 mm^3. For the CT26 model, 5 × 10^6 eGFP or eGFP-ENPP1-expressing CT26 cells were delivered intravenously to 7-week-old BALB/c mice. Treatment with aPD-1/aCTLA4 antibodies and their corresponding isotype control antibodies was initiated intraperitoneally starting on day 6 and given every 3 days for five total doses. Animals were monitored for overall survival. For the E0771 model, 5 × 10^5 eGFP or eGFP-ENPP1-expressing E0771-Luc cells in PBS:Matrigel (1:1) mix were injected into the mammary fat pad of 5×C57BL/6 WT mice or MPYS (Tmenv173/-, The Jackson Laboratory stock number 025805) at the age of 7 weeks. Treatment with 200 μg of aPD-1 or its corresponding isotype control antibody was given on days 6, 10, and 13.

**Data Availability**

Tumor DNA and RNA-seq data used in this article are publicly available and cited as appropriate in the text and Methods section. No new code was used in this article.

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Authors’ Contributions


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