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## STING inhibits the reactivation of dormant metastasis in lung adenocarcinoma

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### Author contributions

JH and JM conceived studies, designed experiments, interpreted results, and wrote the manuscript; JH performed experiments and analyzed data; FJSR, YH and SWL designed and analyzed CRISPR screens; SG, SK and DP performed scRNA-seq data analysis; GJ and JPH provided technical assistance with experiments; ZW generated and characterized TGF- $\beta$  reporter, TGFBR2 knockout cell lines and performed IHC analysis; KG established the KPad1 cell line; KG, SU and CID procured patient-derived tumor samples and validated the STING IHC scoring; AM, RD and JS performed immune phenotyping and NK cell cytotoxicity experiments; HZ and EdS conducted drug treatment experiments in mice.

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Custom code are available at Zenodo under DOI [10.5281/zenodo.7618821](https://doi.org/10.5281/zenodo.7618821).

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SWL received funding and research support from Agilent Technologies for the purposes of massively parallel oligo synthesis to generate the sgRNA libraries described herein.

### Additional information

**Supplementary Information** is available for this paper.

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## Summary

Metastasis frequently develops from disseminated cancer cells that remain dormant after the apparently successful treatment of a primary tumor. These cells fluctuate between an immune evasive quiescent state and a proliferative state liable to immune-mediated elimination<sup>1–6</sup>. Little is known about the clearing of reawakened metastatic cells and how this process could be therapeutically activated to eliminate residual disease in patients. Here, we use models of indolent lung adenocarcinoma metastasis to identify cancer cell-intrinsic determinants of immune reactivity during exit from dormancy. Genetic screens of tumor-intrinsic immune regulators identified the STING (stimulator of interferon genes) pathway as a suppressor of metastatic outbreak. STING activity rises in metastatic progenitors that reenter the cell cycle and is dampened by *STING* promoter and enhancer hypermethylation in breakthrough metastases or by chromatin repression in cells reentering dormancy in response to TGF- $\beta$ . STING expression in cancer cells derived from spontaneous metastases suppresses their outgrowth. Systemic treatment of mice with STING agonists eliminates dormant metastasis and prevents spontaneous outbreaks in a T cell- and natural killer cell-dependent manner, these effects requiring cancer cell STING function. Thus, STING provides a checkpoint against the progression of dormant metastasis and a therapeutically actionable strategy for the prevention of disease relapse.

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Metastasis is the principal cause of death from cancer and frequently develops after a period of dormancy lasting for months up to decades depending on the type of tumor<sup>6</sup>. For example, nearly half of the cases with an early diagnosis (stage I or II) of lung adenocarcinoma (LUAD) develop metastasis months to years after treatment of the primary tumor<sup>7</sup>. This implies the existence of disseminated cancer cells that can reinitiate tumor growth after a period of dormancy<sup>8,9</sup>. Persistence of disseminated cancer cells in the bone marrow predicts increased risk for relapse<sup>10</sup>. TGF- $\beta$  and other growth inhibitory signals in the host tissue cause proliferative quiescence in disseminated cancer cells<sup>1,2,11</sup>, but this process is reversible. Immune-mediated elimination has emerged as an important barrier against the progression of dormant cancer cells that reenter the cell cycle<sup>2,3,5</sup>. During dormancy, quiescent cancer cells downregulate the expression of natural killer (NK) cell activating ligands and MHC class I molecules to evade recognition by NK cells and T cells, respectively<sup>2–4</sup>. Depletion of these immune cells in mice harboring dormant metastasis leads to macrometastatic outbreaks in multiple organs<sup>2,3</sup>. These observations suggest that disseminated cancer cell populations exist in an equilibrium between a predominant immune evasive quiescent state and a proliferative state that undergoes elimination by the immune system. In line with this hypothesis, immunosuppressed recipients of organ transplants from donors who had long been cured of melanoma develop donor-derived metastatic disease<sup>12</sup>. A better understanding of the immune mechanisms that suppress the progression of indolent metastasis could be harnessed for improved elimination of dormant cancer cells and the

prevention of aggressive disease relapse. Here, we report that cancer cell-autonomous signaling by STING, the innate immune sensor of cytosolic double-stranded DNA (dsDNA), is a powerful suppressor of reawakened LUAD metastatic cells.

## Models of dormancy under immune control

To identify immune suppressors of dormant metastatic outbreak, we employed models in which cancer cells derived from early-stage LUAD tumors are inoculated into the arterial circulation of mice to populate different organs where metastases remain dormant for months with few spontaneous outbreaks (Fig. 1a). We previously isolated and characterized one such model, H2087-LCC, derived from H2087 stage I human LUAD cells<sup>2</sup>. When inoculated into *Foxn1<sup>nu</sup>* athymic nude mice, which lack T cells, H2087-LCC cells populate the lungs, liver, bone marrow, and adrenal glands, remaining as quiescent single cells and small proliferative clusters that rarely form large tumors<sup>2</sup>. This phenotype is in stark contrast to that produced by the aggressive late-stage LUAD cell line H2030, which gives rise to aggressive multi-organ metastases (Extended Data Fig. 1a). Although only a small proportion of H2087-LCC harboring mice developed metastasis, nearly all mice developed multiorgan metastasis when treated with anti-asialo-GM1 antibody to deplete NK cells (Extended Data Fig. 1a–c). We previously showed that NK cells selectively eliminate proliferative H2087-LCC early-stage progenitors<sup>13</sup>. To develop a fully immunocompetent model of dormant metastasis, we initiated lung adenocarcinoma tumors in *Kras<sup>LSL-G12D/+</sup>;Ttp53<sup>flox/flox</sup>* (KP) mice by intranasal delivery of Cre recombinase<sup>14</sup> and derived a cell culture population from an early-stage LUAD lesion (KPad1 cells). When injected into syngeneic immunocompetent C57BL/6 mice or C57BL/6 derived B6-albino mice, KPad1 cells showed an indolent metastatic phenotype compared to cells derived from an aggressive KP LUAD tumor (KP-482T1 cells<sup>14</sup>) (Fig. 1b). Disseminated KPad1 cells remained as single cells or small clusters (<20 cells), a majority of which were quiescent as determined by Ki67 staining 25 or 75 days after inoculation (Fig. 1c). Notably, KPad1 cells rapidly formed multiorgan metastatic disease in B6-albino mice that were depleted of NK cells, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells by antibody treatment (Extended Data Fig. 1e–f) and in NOD/SCID/gamma-null (NSG) mice which lack NK cells and mature T and B cells (Fig. 1b; Extended Data Fig. 1d, 1f). Thus, immune surveillance restricted the awakening of dormant metastases both in the H2087-LCC and the KPad1 models.

## Screen to uncover metastasis suppressors

To identify cell-autonomous suppressors of exit from dormant metastasis, we conducted focused CRISPR screens *in vivo* using sgRNA libraries targeting various immunity activating factors in H2087-LCC and KPad1 cells (Fig. 1d). As NK cell activating ligands<sup>15</sup> and MHC class I molecules are implicated in the regulation of dormant metastasis, we designed and constructed libraries to target these genes. To identify new immune regulators of metastasis, we queried our previously published single-cell RNA sequencing (scRNA-seq) datasets comparing dormant H2087-LCC cells that were induced to proliferate in culture versus cultures of H2087-LCC that had grown *in vivo* as spontaneous outbreaks under immune surveillance<sup>13</sup>. Gene set enrichment analysis (GSEA) comparing reawakened cells with those from spontaneous outbreaks revealed a selective enrichment

for transcriptional signatures associated with immune regulation, including interferon  $\alpha$ , interferon  $\gamma$ , and complement pathways in the reawakened cells (Extended Data Fig. 1g; Supplemental Table 1). Genes from these pathways were included in the screen. As interferon  $\alpha/\beta$  expression lies downstream of pathways that sense dsDNA (stimulator of interferon genes, or STING pathway), RNA, and lipopolysaccharides<sup>16</sup>, we also included components from these immune-sensing pathways in the screen. In all, our libraries targeted 220 genes belonging to these classes of immune regulators (102 from H2087-LCC cells and 118 from KPad1 cells) (Supplemental Tables 2 and 3). To maximize library representation *in vivo*, we divided the libraries into sub-pools targeting 20 genes each (5 sgRNAs per gene) followed by library transduction and selection of sgRNA-expressing cancer cells. To mitigate against the high attrition that cancer cells suffer during dissemination<sup>17</sup>, we inoculated 10–12 athymic nude mice or 7–10 B6-albino mice per pool with  $2.5 \times 10^5$  cells to ensure high sgRNA representation *in vivo*. Library-transduced H2087-LCC and KPad1 cells were injected intracardially into the arterial circulation of recipient mice for dissemination to multiple organs. To assess lung colonization activity, library-transduced KPad1 cells were separately injected into the tail vein of recipient mice. Cells expressing a neutral safe-targeting sgRNA were inoculated as controls. Metastatic disease development was monitored longitudinally using luciferase bioluminescence imaging, and brain, lung, liver, bone, and adrenal gland tissues were individually harvested from mice showing overt metastases (Fig. 1d).

Several sgRNA pools enhanced the metastatic activity of H2087-LCC and KPad1 cells to multiple organs, suggesting that loss-of-function of one or more genes in these pathways is sufficient to allow the metastatic progression of reawakened dormant cancer cells (Fig. 1e–f; Extended Data Fig. 1h–j, Supplemental Table 4). To identify candidate sgRNAs that fuel this metastatic outbreak, we isolated genomic DNA from individual organs, followed by next-generation sequencing to quantify sgRNA enrichment. Consistent with previous studies showing that NK cells and T cells suppress the reawakening of dormant metastasis<sup>2,3</sup>, sgRNAs targeting specific NK activating ligands and MHC class I molecules were among the top enriched guides across all screens, supporting the robustness of our screening platform and results. Interestingly, multiple genes associated with the STING pathway (*STING*, *IFNB1*, *CCL5*) and interferon responsive genes downstream of STING (*IFI27*, *IFITM3*) were among the top-ranked sgRNA targets in the H2087-LCC and KPad1 screens (Fig. 1g, Extended Data Fig. 1k–l, Extended Data Fig. 2, Supplemental Table 5). These sgRNAs were enriched in liver metastases from H2087-LCC, brain and adrenal metastases from KPad1, and bone and lung metastases from both models (Extended Data Fig. 2). sgRNAs targeting the STING and interferon  $\alpha$  pathways also scored in the KPad1 lung colonization screen (Extended Data Fig. 1m), further pointing at these pathways as candidate suppressors of exit from dormant metastasis in LUAD.

## STING activation in reawakened cells

The STING pathway triggers innate immune responses to cytosolic dsDNA in cancer cells and cells infected with viruses or bacteria<sup>18</sup>. Cytosolic dsDNA binds to cyclic GMP-AMP synthase (cGAS), which produces the second messenger cGAMP. cGAMP binding induces STING to stimulate kinase-mediated activation of IRF3 and NF- $\kappa$ B transcriptional drivers

of type I interferon and pro-inflammatory chemokine expression<sup>19</sup>. We and others reported that cancer cells contain significant levels of cytosolic dsDNAs and cGAMP, likely due to genomic instability<sup>20,21</sup>. Notably, immunostaining of mouse lung or brain sections harboring disseminated H2087-LCC or KPad1 cells demonstrated higher levels of STING and the STING-induced chemokine CCL5 in single proliferative metastatic cells and small clusters compared to quiescent single cells, whereas the levels of STING and CCL5 in macrometastases were closer to those of dormant cells (Fig. 2a; Extended Data Fig. 3a–c), indicating heightened STING expression and pathway activity in recently reawakened metastatic cells.

We analyzed STING and CCL5 expression in lymph nodes from stage II and stage III LUAD patients harboring small clusters of disseminated cancer cells. STING and CCL5 immunofluorescence intensities were higher in proliferative (Ki67<sup>high</sup>) disseminated cancer cells than in quiescent cells (Fig. 2b, Extended Data Fig. 3d), whereas patient-derived macrometastases showed lower STING and CCL5 expression than their matched primary tumor (Fig. 2c–d; Extended Data Fig. 3e–g), all of which consistent with our findings in the dormant metastasis mouse model.

We previously showed that cancer cells in patient-derived LUAD metastasis samples and H2087-LCC-derived metastatic colonies comprise a developmental continuum spanning from stem-like lung epithelial regenerative progenitors expressing the transcription factor SOX2, through intermediate developmental stages expressing FOXA2 and NKX2-1, and late-stage alveolar progenitors expressing SOX9<sup>13</sup>. These distinct stages exhibit differential sensitivities to immune surveillance, with SOX2<sup>+</sup> progenitors being constrained by NK cell-mediated killing whereas SOX9<sup>+</sup> cells are more resistant to NK cell surveillance<sup>13</sup>. Disseminated H2087-LCC cells and small clusters predominantly include SOX2<sup>+</sup> cells both *in situ*<sup>2</sup> and upon isolation and placement in culture<sup>13</sup>, while spontaneous H2087-LCC metastases are enriched in SOX9<sup>+</sup> cells<sup>13</sup>. Thus, LUAD metastatic tumors include a heterogeneous continuum of developmental stages from SOX2<sup>+</sup> to SOX9<sup>+</sup> differentially recognized by the immune system.

To verify that STING upregulation selectively occurs in early-stage SOX2<sup>+</sup> metastatic progenitors, we compared scRNA-seq datasets derived from H2087-LCC cells that were isolated as dormant populations in mice and then placed in culture to reawaken their proliferation, versus H2087-LCC cell populations that had spontaneously grown as large outbreaks *in vivo* and were then placed in culture<sup>13</sup>. When stimulated to resume proliferation under culture conditions, dormancy-derived SOX2<sup>+</sup> cells expressed higher levels of *STING* compared to macrometastasis-derived SOX9<sup>+</sup> cells (Fig. 2e; Extended Data Fig. 3h–i). STING activation leads to expression of canonical immune-stimulatory genes, including type I interferons and their targets, which are anti-tumorigenic, and expression of non-canonical NF-κB target genes, which promote growth in tumors with chromosomal instability (CIN)<sup>22,23</sup>. Canonical STING target genes<sup>24–26</sup> and interferon α response genes (GSEA hallmark gene set) were more highly expressed in the reawakened dormant cells than in macrometastasis-derived populations (Extended Data Fig. 3h, Extended Data Fig. 4a, Supplemental Table 6). NF-κB targets were similarly expressed in both reawakened

STING-high cells and macrometastasis-derived STING-low cells, consistent with previous reports<sup>22,23</sup> (Extended Data Fig. 3h).

Analysis of scRNA-seq data from patient-derived LUAD macrometastases<sup>13</sup> showed that the SOX2<sup>+</sup> early-stage progenitors present in these lesions express high levels of *STING*, canonical STING targets, interferon  $\alpha$  response genes, and NF- $\kappa$ B target genes, compared to SOX9<sup>+</sup> cells from the same lesions (Extended Data 3j-k, Extended Data Fig. 4b). We observed no downregulation of *STING* in SOX9<sup>+</sup> progenitors in H2087-LCC metastases formed after NK cell depletion (Extended Data Fig. 3l), and H2087-LCC metastasis samples from spontaneous outbreaks showed lower levels of *STING* mRNA and protein compared to outbreaks after depletion of NK cells (Extended Data Fig. 3m-n), suggesting that the decline in *STING* expression is tied to development of the lesions under immune surveillance. Collectively, these data suggest that the transition of metastatic SOX2<sup>+</sup> progenitors from dormancy to a proliferative state is accompanied by heightened STING activity, and the more differentiated SOX9<sup>+</sup> progenitors downregulate STING as macrometastases develop under immune selective pressure.

## STING activation suppresses metastasis

To determine the effect of STING on metastasis progression, we performed necessity and sufficiency experiments in the H2078-LCC and KPad1 models, as well as their aggressive counterparts H2030 and KP-482T1, respectively. Knockout of *STING* in H2087-LCC cells using sgRNAs different from those used in the original screen accelerated the incidence of metastatic outbreaks and worsened metastasis-free survival in mice inoculated with these cells (Fig. 3a; Extended Data 5a). Cells derived from spontaneous metastatic outbreaks (H2087-LCC-SO cells) showed low *STING* expression and no induction of *IFNB1* expression by exogenous cGAMP compared to parental H2087-LCC cells (Extended Data Fig. 5b-d). Conversely, in H2087-LCC-SO cells with doxycycline-inducible overexpression of STING (Extended Data Fig. 5e), doxycycline administration 7 days after inoculation of these cells into athymic mice increased the metastasis free-survival (Fig. 3b; Extended Data Fig. 5f).

*STING* and cGAMP-induced *IFNB1* expression levels in H2087 human LUAD cells were comparable to those in human bronchial epithelial cells and higher than those in the aggressively metastatic H2030 and A549 LUAD cell lines<sup>27,28</sup> (Extended Data Fig. 5g-h), although H2030 and A549 cells expressed high levels of *IFNB1* in response to poly(I:C), a STING-independent agonist (Extended Data Fig. 5i). Since overexpression of wild-type STING in H2030 did not robustly activate the STING pathway, we transduced these cells with a constitutively active STING-V155M mutant<sup>29</sup>, which inhibited their metastatic activity in athymic mice (Extended Data Fig. 5j). *Sting* expression and cGAMP responsiveness were higher in KPad1 cells compared to the aggressive counterpart KP-482T1 (Extended Data Fig. 6a-b). *Sting* knockout in KPad1 cells increased their metastatic activity in immunocompetent mice (Fig. 3c; Extended Data Fig. 6c), whereas *STING* overexpression suppressed the strong metastatic activity of KP-482T1 cells (Fig. 3d). We labeled wild-type KPad1 cells with RFP and *Sting* knockout KPad1 cells with GFP, then mixed the cells at different ratios and intracardially inoculated them into mice.



Macrometastases largely consisted of *Sting* knockout cells even when these cells were only 10% of the inoculum (Extended Data Fig. 6d). *STING* knockout promoted metastasis in multiple organs including bone, brain, and lung in H2087-LCC, and bone, brain, lung, and adrenal glands in KPad1 (Fig. 3e). Of note, *STING* knockout or overexpression did not affect the proliferation of these models *in vitro*, as measured by an EdU uptake assay (Extended Data Fig. 6e–f, 7a–b), their initial survival in mice, as measured by quantitative bioluminescence imaging 1 to 7 days after intracardiac inoculation (Extended Data Fig. 6g–h, 7c–d), or expression of SOX2 or SOX9 (Extended Data Fig. 7e–f), as measured by immunofluorescence analysis of macrometastatic lesions.

The inhibitory effect of *STING* on KP-482T1 metastasis was dependent on both NK cells and CD8<sup>+</sup> T cells, as antibody-mediated depletion of these cell populations in mice eliminated the effect of *STING* (Fig. 3f). Analysis of cell culture supernatants confirmed that *STING* overexpression in KP-482T1 cells induced the secretion of CCL5, CCL20, CXCL10, and other pro-inflammatory factors<sup>26,30</sup> (Extended Data Fig. 6i), which are implicated in the recruitment and activation of innate and adaptive immune cells<sup>18,31–33</sup>.

We performed immune phenotyping of KP-482T1 metastasis-bearing bones after short-term (2-day or 7-day) induction of *STING* expression from a doxycycline-inducible vector (Extended Data Fig. 8a). *STING* overexpression increased the number of immune cells, including NK cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and conventional type 1 dendritic cells (cDC1), in metastasis-bearing femurs (Extended Data Fig. 8b, Supplemental Fig. 2), while not affecting degranulation or production of effector cytokines of NK and CD8<sup>+</sup> T cells (Extended Data Fig. 8c–f). Immunofluorescence analysis of NK cells (anti-NKp46 staining) and T cells (anti-CD3 staining) in KP-482T1 metastases showed an increased proportion of infiltrated NK cells and T cells in response to *STING* overexpression (Fig. 3g–h). In the H2087-LCC model, *STING* knockout did not affect the efficacy of NK cell-mediated direct killing (Extended Data Fig. 8g), but suppressed the migration ability of NK cells (Extended Data Fig. 8h). Collectively, these results suggest that cancer cell-intrinsic *STING* activation suppresses LUAD metastasis by promoting infiltration of NK cells and T cells.

## Basis for *STING* downregulation

Hypermethylation of the *STING* promoter has been reported in *KRAS-LKB1*-mutant lung cancer cell lines<sup>34</sup>. To examine if *STING* expression is regulated by DNA methylation during metastasis progression, we first conducted ChIP-seq analysis of histone marks in H2087-LCC cells, the spontaneous outbreak derivative line H2087-LCC-SO1, and the aggressive metastatic counterpart H2030, identifying the promoter region (marked by H3K4me3) and a 3' enhancer region (marked by H3K4me1) in *STING* (Extended Data Fig. 9a). We observed DNA hypermethylation on the *STING* promoter and 3' enhancer in two independent H2087-LCC spontaneous outbreak cell populations (SO1, SO2), as well as in H2030 and A549 cells compared to H2087-LCC cells, as determined by immunoprecipitation of 5-methylcytosine on the *STING* promoter and 3' enhancer (Fig. 4a). DNA methylation was associated with reduced or absent establishment of the 3' enhancer marked by H3K4me1 and reduced enhancer activity marked by H3K27ac in H2087-LCC-SO1 or H2030, respectively (Extended Data Fig. 9a). ChIP-PCR analysis of

DNA methyltransferase (DNMT) binding showed higher DNMT3B binding on the *STING* promoter and 3' enhancer in H2087-LCC-SO1 and SO2 as well as in H2030 and A549 (Extended Data Fig. 9b). Treatment of H2087-LCC-SO1 and -SO2, H2030, and A549 cells with the DNMT inhibitor 5-aza-2'-deoxycytidine (decitabine, 5-azadC) rescued *STING* expression in these cells (Fig. 4b, Extended Data Fig. 9c).

To validate these observations in vivo, we freshly isolated H2087-LCC tumor cells from spontaneous outbreaks and conducted targeted next-generation bisulfite sequencing analysis on the *STING* promoter and 3' enhancer. Approximately half of the CpG sites in these regions showed higher levels of DNA methylation in cells sorted from spontaneous outbreaks in athymic nude mice compared to outbreaks in NSG mice or to H2087-LCC cells in culture (Extended Data Fig. 9d, Fig. 4c–d, Supplemental Table 7).

To investigate the basis for *STING* downregulation in dormant cells, we examined the response of H2087-LCC cells to TGF- $\beta$ , an inducer of metastatic dormancy in breast, and head and neck carcinomas<sup>1,11,35,36</sup>. TGF- $\beta$  causes growth inhibition as a part of broader response programs<sup>37,38</sup>. We treated H2087-LCC cells with TGF- $\beta$  or palbociclib, a CDK4/6 inhibitor which specifically induces cell cycle arrest. Both TGF- $\beta$  and palbociclib inhibited cell proliferation (Extended Data Fig. 10a), but only TGF- $\beta$  caused a decrease in *STING* expression and *STING*-dependent *IFNB1* and *CCL5* expression (Fig. 4e, Extended Data Fig. 10b–c). TGF- $\beta$  had limited effects on *cGAS* or cGAMP levels (Extended Data Fig. 10d–e). ChIP-seq analysis of histone modification marks showed that TGF- $\beta$  causes a loss of the H3K27ac activation mark in the *STING* 3' enhancer while not affecting enhancer establishment as marked by H3K4me1 (Extended Data Fig. 10f) or enhancer DNA methylation (Extended Data Fig. 10g).

We next examined whether TGF- $\beta$  also regulates expression of *STING* and *CCL5* in H2087-LCC *in vivo*. H2087-LCC cells engineered with a TGF- $\beta$  and SMAD-dependent mCherry transcriptional reporter (Extended Data Fig. 10h) exhibited TGF- $\beta$  signaling activity as dormant metastatic cells in the lungs after inoculation into athymic mice (Extended Data Fig. 10i). TGF- $\beta$  receptor 2 (*TGFBR2*) knockout in H2087-LCC cells prevented canonical gene responses (induction of *SMAD7* and *SNAI1*) and *STING* downregulation by TGF- $\beta$  (Extended Data Fig. 10j). When inoculated via the tail vein into NSG mice, used here to avert the interference of immune surveillance, *TGFBR2* knockout H2087-LCC cells showed accelerated lung metastasis (Extended Data Fig. 10k) and elevated expression of *STING* and *CCL5* compared to control (Fig. 4f, Extended Data Fig. 10l). Collectively, these results are consistent with a role of TGF- $\beta$  in downregulating *STING* expression by enhancer inhibition during dormancy, and a role of promoter and enhancer DNA methylation in the downregulation of *STING* expression in metastatic outbreaks (Fig. 4g).

## Leveraging *STING* to prevent metastasis

We tested the hypothesis that pharmacologic activation of *STING* in mice harboring dormant metastasis would deplete disseminated cancer cells by enhancing *STING*-dependent elimination of metastatic cells reentering the cell cycle (Extended Data Fig. 11a). To this end, mice harboring disseminated KPad1 or H2087-LCC cells were treated with a



STING agonist followed by either harvesting the brains and lungs to quantify disseminated tumor entities (DTE) which include single DTCs, small cell clusters and metastases or allowing the mice to proceed until spontaneous metastasis emerged (Fig. 5a). We used MSA-2 (benzothiothiophene oxobutanoic acid), a non-nucleotide STING agonist with anti-tumor effects<sup>39</sup>. MSA-2 treatment *in vitro* increased the expression of *STING* target genes *CXCL10* and *CCL5* in wild-type (but not *STING* knockout) KPad1 and H2087-LCC cells (Extended Data Fig. 11b–c).

We intracardially inoculated *STING* wild-type or knockout KPad1 cells into syngeneic immunocompetent mice and H2087-LCC cells into athymic nude mice, allowed one week (KPad1) or two weeks (H2087-LCC) for disseminated cells to settle in distant organs, and then started weekly treatments with MSA-2 for two weeks (KPad1) or four weeks (H2087-LCC) during the dormancy period in each model (Fig. 5a). Treatment with MSA-2 led to a STING-dependent depletion of disseminated KPad1 cancer cells, with *Sting* knockout KPad1 cells accumulating at higher levels compared to *Sting* proficient cells (Fig. 5b). Moreover, MSA-2 increased metastasis-free and overall survival of mice inoculated with *Sting* proficient (but not knockout) KPad1 cells (Fig. 5c–d). The incidence of metastasis was proportional to the number of disseminated KPad1 cells in the various conditions (compare Fig. 5b with Fig. 5c–d).

The metastasis suppression effect of MSA-2 required not only STING activity in the cancer cells but also the presence of NK, CD4<sup>+</sup> T, and CD8<sup>+</sup> T cells, as depletion of these cells in the mice abolished the effect (Extended Data Fig. 11f–j). Pre-treatment of KPad1 cells with MSA-2 *in vitro* before inoculation in mice did not affect metastatic progression (Extended Data Fig. 11k). Moreover, treatment with MSA-2 led to depletion of disseminated H2087-LCC cells, and this effect was absent in *STING* knockout H2087-LCC cells (Fig. 5e). Since spontaneous outbreaks are infrequent in the H2087-LCC model, we depleted NK cells one week after the last MSA-2 dose to allow metastasis formation by cancer cells remaining after MSA-2 treatment, which serves as another readout of the residual burden of metastasis-initiating H2087-LCC cells. MSA-2 treatment decreased the incidence of metastasis, an effect that was also dependent on *STING* in the cancer cells (Fig. 5f–g).

We also tested ADU-S100 (also known as ML-RR-S2 CDA)<sup>40</sup>, an earlier generation STING-activating cyclic di-adenosine mimic of cGAMP. ADU-S100 treatment of KPad1 and H2087-LCC cells *in vitro* increased the expression of *CXCL10* and *CCL5* in a STING-dependent manner (Extended Data Fig. 11d–e). As ADU-S100 is unstable after systemic administration<sup>41</sup>, we delivered the drug locally through intratracheal instillation in mice harboring KPad1 or H2087-LCC cells in the lungs. Consistent with our findings using MSA-2, ADU-S100 treatment inhibited the formation of metastatic outbreaks in mice harboring *Sting* wild-type (but not knockout) KPad1 cells (Extended Data Fig. 11l). In the H2087-LCC model, ADU-S100 treatment reduced the number of disseminated cancer cells in the lungs (Extended Data Fig. 11m).

## Discussion

Our results reveal a previously unappreciated role of STING signaling in the interaction between dormant disseminated LUAD cells and anti-tumor immunity, preventing the progression of these cancer cells from an indolent state to aggressive metastasis. The ability of STING agonists to eliminate disseminated cancer cells and suppress relapse when administered during the indolent phase of metastasis is in line with our genetic screens identifying cancer cell-intrinsic STING as a target of interest. This effect of STING agonists requires cancer cell STING and depends on NK, CD4<sup>+</sup> T, and CD8<sup>+</sup> T cells. The precise mechanism leading to STING activation in dormant SOX2<sup>+</sup> LUAD cells reentering the cell cycle, and in proliferative SOX2<sup>+</sup> subpopulations in patient-derived metastasis samples, remains to be fully elucidated. It is worth noting that micronuclei arising from chromosomal instability in cancer cells accumulate through mitosis and activate the cGAS/STING pathway<sup>21,42,43</sup>.

Current clinical trials with STING agonists are in the setting of advanced tumors, where this pathway plays a complex role, promoting antitumor immune responses<sup>40,44,45</sup>, cancer cell senescence and apoptosis<sup>46–48</sup>, but also inducing pro-tumorigenic effects<sup>20,49</sup> through NF-κB signaling in some models<sup>22,23</sup> though not in others<sup>50</sup>. These disparate roles may complicate the outcome of STING agonist trials in established tumors. However, the dormant metastasis microenvironment is very different from that of the advanced metastases. Targeting specific vulnerabilities of dormant metastasis in the adjuvant setting may provide unique opportunities to prevent metastasis. Our observations warrant further investigation towards rationally harnessing the immune system in the adjuvant setting.

## METHODS

### Clinical samples

Human specimens were obtained and approved under Memorial Sloan Kettering Cancer Center Institutional Review Board biospecimen research protocol 17–239. Lung cancer primary tumor and metastatic samples were obtained from patients confirmed Stage II, III, or IV lung cancer. Patients were both male and female, with an age range of 56–81 at recruitment. Four pairs of tissues from primary tumor and metastases were collected at autopsy (non-human subject by NIH definition). All patients provided pre-procedure informed consent.

### Animal studies

All animal experiments were performed in accordance with protocols approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee (IACUC). Athymic nude (*Foxn1<sup>nu</sup>*, stock 069) mice were obtained from Envigo. NSG (NOD.Cg-Prkdc<sup>scid</sup>IL2rg<sup>tm1Wjl</sup>/SzJ, stock 005557), C57BL/6J (stock 000664), and B6(Cg)-*Tyr<sup>c-2J</sup>/J* (B6-albino, stock 000058) mouse strains were obtained from The Jackson Laboratory. Female mice from 6 to 8 weeks of age were used for these studies. Animals were housed under the following conditions: temperatures of 21.1–22.2 °C (70–72 °F), 30%

–70% humidity, 10–15 fresh air exchanges hourly, and a 12–12 hour light-dark cycle (lights were on from 0600-1800).

For multi-organ metastasis assays,  $0.2\text{--}2.5\times 10^5$  cells were resuspended in 100  $\mu\text{L}$  phosphate-buffered saline solution (PBS) and inoculated into the right cardiac ventricle of mice with a 26-gauge needle syringe. Lung colonization assays were performed by injecting  $1.0\text{--}2.5\times 10^5$  cells in the lateral tail vein of mice with a 28-gauge needle syringe<sup>2</sup>. Metastatic burden of luciferase-transduced cancer cells was monitored by bioluminescence imaging (BLI) of luciferase activity using retro-orbital injection of D-luciferin (150 mg/kg) and an IVIS Spectrum Xenogen instrument (PerkinElmer). Data were analyzed using Living Image software (PerkinElmer, v 4.5.0). The black-colored fur of the C57BL/6J strain interferes with bioluminescence imaging. Therefore, metastasis formation experiments with KPad1 and KP-482T1 cells were conducted in B6(Cg)-*Tyr<sup>c-2J</sup>*/J (B6-albino) mice, which are C57BL/6J mice that carry a mutation in the tyrosinase gene (*Tyr<sup>c-2J</sup>*), resulting in loss of pigment from skin, hair, and eyes. Human cancer cells were inoculated in athymic nude mice. For *ex vivo* imaging of metastases-bearing organs at experimental end points, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and retro-orbitally injected with D-luciferin prior to organ isolation. Isolated organs were analyzed using the IVIS Spectrum Xenogen instrument and Living Image software. Cell clusters or metastatic lesions were classified according to the size of the lesion and the number of cells contained. Cell clusters were defined as clusters containing fewer than 20 cells ( $<10^3 \mu\text{m}^2$ ) from BLI negative organs; micrometastases were defined as lesions containing approximately between 500 and 2500 cells ( $>10^3 \mu\text{m}^2 - 5\times 10^4 \mu\text{m}^2$ ) from organs with weak BLI signal (intensity  $<10$ -fold over background); macrometastases were defined as lesions containing more than 2500 cells ( $>5\times 10^4 \mu\text{m}^2$ ) from organs with strong BLI signals (intensity  $>10$  fold of signal from background, refer to Extended Data Fig. 10k). To score metastasis-free survival, metastasis onset was defined when whole-body BLI signal intensity reaches to 10-fold over background. In accordance with protocols approved by the IACUC, animals were sacrificed when tumor burden exceeded 1000  $\text{mm}^3$  (or 1.5cm in diameter), or if the mass of the tumor exceeded more than 10% of the animal's mass. If animals showed any signs of respiratory distress or other signs of illness, such as hunched posture, reduced and/or erratic movement, greater than 10–15% weight loss, diminished appetite, or failure to groom, the animals were euthanized prior to the endpoint. These limits were not exceeded in any of the experiments.

For NK cell depletion assays in athymic nude mice, 33  $\mu\text{g}$  anti-asialo-GM1 antibody (Wako Chemical, Cat# 986-10001) was injected intraperitoneally once every 5 days<sup>2</sup>. For NK, CD4, or CD8 T cell depletion in C57BL/6J and B6(Cg)-*Tyr<sup>c-2J</sup>*/J mice, 200  $\mu\text{g}$  *In Vivo*Mab anti-mouse NK1.1 antibody (clone PK136, BioXCell, Cat# BE0036), CD4 antibody (clone GK1.5, BioXCell, Cat# BE0003-1), CD8 $\alpha$  antibody (clone 53–6.7, BioXCell, Cat# BE0004-1), or IgG2a control (clone 2A3, BioXCell, Cat# BE0089) was injected intraperitoneally once weekly<sup>51</sup>. Immune cell depletion started on the same day as cell inoculation unless otherwise indicated.

For inducible STING overexpression experiments, mice were maintained on a diet of doxycycline food pellets (2500 mg/kg, Envigo). For experiments with H2087-LCC cells, STING induction started 7 days after cell inoculation. For experiments with KP-482T1 cells,

STING induction started on the same day or one day after cell inoculation, unless otherwise indicated.

For experiments with *Sting* knockout KPad1 cells, cells were mixed 1:1 from two cell lines in which STING was knocked out by two different sgRNAs. For STING agonist treatment assays, MSA-2 (MedChemExpress, Cat# HY-136927) was dissolved in PBS with mild pH adjustment and injected subcutaneously (50 mg/kg) once weekly<sup>39</sup>. ADU-S100 ammonium salt (MedChemExpress, Cat#HY-12885B)<sup>40</sup> was intratracheally delivered (25–125 µg/mouse in 50 µL water) once weekly.

## Cell culture

For establishment of the KPad1 cell line,  $2.5 \times 10^6$  adenovirus expressing Cre recombinase (Ad5CMVCre-mCherry, University of Iowa, Cat# VVC-U of Iowa-649) was delivered to *Kras<sup>LSL-G12D/+</sup>;Tp53<sup>flox/flox</sup>* mice through intranasal inhalation, as described<sup>52</sup>. Lung tumors were isolated 18 weeks later. Tissues were washed in PBS, chopped into small pieces using a sterile razor blade, and resuspended in 30 mL digestion buffer containing 5% fetal bovine serum (FBS), 2 mM L-glutamine (Glu), 100 IU/mL penicillin/streptomycin (P/S), 40 µg/mL gentamicin, 250 µg/mL type III collagenase (Worthington Biochemical, Cat# LS004208), 0.0625 U/mL dispase solution, and 0.05 mg/mL DNase, in DMEM media. After incubation at 37°C for 30 min, tissue was passed through a 70 µm strainer, spun down, and plated in DMEM supplemented with 10% FBS for cell culture.

Mouse lung cancer cell line KP-482T1 was a gift from T. Jacks (MIT)<sup>14</sup>. Human bronchial/tracheal epithelial cells (NHBE) were purchased from Lonza (Cat# CC-2540). A549 cells were purchased from ATCC (Cat# CCL-185). H2087 (ATCC, Cat# CRL-5922)-LCC derivatives and the H2030 (ATCC, Cat# CRL-5914)-BrM derivative were isolated as described in previous reports<sup>2,27</sup>. H2087-LCC derived spontaneous outbreak cell lines (SO) were generated from spontaneous metastases in mice inoculated intracardially with H2087-LCC cells. KPad1 and KP-482T1 cell lines were genotyped to verify the presence of *Kras<sup>G12D</sup>* and *Tp53* mutations using PCR amplification. A549, H2087-LCC, and H2030-BrM cells were authenticated with STR profiling. Human bronchial/tracheal epithelial cells (NHBE) was not authenticated.

H2030-BrM, KP-482T1, and KPad1 were cultured in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin/streptomycin, and 1 µg/mL amphotericin B. A549 cells were cultured in DMEM with the same supplements. 293T cells were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine. Normal human bronchial epithelial cells were cultured in BEGM<sup>TM</sup> bronchial epithelial cell growth basal medium supplemented with SingleQuots<sup>TM</sup> (Lonza, Cat# CC-3170). H2087-LCC cells and outbreak derivatives were cultured in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin/streptomycin, 1 µg/mL amphotericin B, 0.5 mM sodium pyruvate, 10 mM HEPES, 50 nM hydrocortisone, 25 nM sodium selenite, 20 µg/mL insulin, 10 µg/mL transferrin, 0.5% bovine serum albumin (BSA), and 1 ng/mL recombinant human epidermal growth factor. All cell lines tested negative for mycoplasma prior to use in experiments.

## Cell culture treatments

Treatment of cell cultures with 2'3'-cyclic GMP AMP (cGAMP) or polyinosinic-polycytidylic acid [poly(I:C)] was performed as previously described<sup>53,54</sup>. For cGAMP treatment, cells were incubated in digitonin permeabilization buffer (50 mM HEPES pH 7.3, 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM DTT, 85 mM sucrose, 0.2% BSA, and 1 µg/mL digitonin) for 30 min, then fresh media was added with 0.5 µg/mL or 4 µg/mL cGAMP (Invivogen, Cat# tlr1-nacga23) or no additions. For poly(I:C) treatment, cells were incubated with control or 0.5 µg/mL poly(I:C) (Invivogen, Cat# tlr1-picw) and Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Cat#11-668-027). Cell lysates were harvested 4 h after treatment and processed for RNA isolation.

For other treatments, 50 µM ADU-S100 (MedChemExpress, Cat# HY-12885B), 33 µM MSA-2 (MedChemExpress, Cat# HY-136927) or vehicle was added to the media for 4 h to 6 h. 100 nM 5-aza-2'-deoxycytidine (Millipore Sigma, Cat# A3656-5MG) or DMSO vehicle was added to the media for 3 days. TGF-β1 (100 nM, R&D Systems) or TGF-β receptor inhibitor SB-505124 (2.5 µM, Millipore Sigma, Cat# S4696-5MG) was added in 2% FBS supplemented media for the indicated time. 2 µM CDK4/6 inhibitor palbociclib (PD-0332991) (Selleckchem, Cat# S1116) was added to the media for 5–8 days. 20 ng/mL or 1 µg/mL Doxycycline was added to the media for 1–2 days. RNA extraction followed all these incubations.

## CRISPR screen

**Plasmids and sgRNA cloning**—To generate stable Cas9-expressing cell lines, lentiCas9-Blast (Addgene, Cat# 52962) was used. To express sgRNAs, pUSEPR (U6-sgRNA-EFS-Puro-P2A-TurboRFP) was used<sup>55</sup>. Briefly, pUSEPR vector was linearized with BsmBI (NEB) or Esp3I (NEB) and ligated with BsmBI/Esp3I-compatible annealed and phosphorylated oligos encoding sgRNAs, using high concentration T4 DNA ligase (NEB). All sgRNA sequences used are listed in Supplemental Tables 2 and 3.

**Design and cloning of focused CRISPR libraries**—sgRNA sequences (5 per gene) targeting genes of interest were designed using a combination of the Broad Institute sgRNA Designer tool<sup>56</sup> and the Vienna Bioactivity CRISPR score<sup>57</sup> (Supplemental Tables 2 and 3). sgRNAs were divided into small pools of libraries, and oligo pools were synthesized by Agilent Technologies. Libraries were cloned into pUSEPR using a modified version of the protocol published by Doench *et al.*<sup>56</sup> to ensure a library representation of > 10,000-fold<sup>58</sup>. Briefly, each library was selectively amplified using uniquely barcoded forward and reverse primers that append cloning adapters at the 5' and 3' ends of the sgRNA insert (Supplemental Table 8A), purified using the QIAquick PCR Purification Kit (Qiagen), and ligated into BsmBI/Esp3I-digested and dephosphorylated pUSEPR, using high-concentration T4 DNA ligase (NEB). A total of 1.2 µg of ligated pUSEPR-CRISPR Library plasmid DNA was then electroporated into Endura electrocompetent cells (Lucigen). Competent cells were recovered for 1 h at 37°C, plated across four 15 cm LB-Carbenicillin plates (Teknova), and incubated at 37°C for 16 h. The total number of bacterial colonies per sub-pool was quantified using serial dilution plates, to ensure a library representation of > 10,000-fold. The next morning, bacterial colonies were scraped and briefly expanded for

4 h at 37°C in 500 mL of LB-Carbenicillin. Plasmid DNA was isolated using the Plasmid Plus Maxi Kit (Qiagen). A validated control sgRNA targeting a neutral region in mouse chromosome 8<sup>59</sup> was cloned into the pUSEPR backbone and spiked into each of these libraries at a defined fraction to achieve equimolarity between sgRNAs in the library and the control. To assess sgRNA distribution, the sgRNA target region was amplified using primers that append Illumina sequencing adapters on the 5' and 3' ends of the amplicon, as well as a random nucleotide stagger and unique demultiplexing barcode on the 5' end (Supplemental Table 8B). Library amplicons were size-selected on a 2.5% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen), and sequenced on an Illumina NextSeq instrument (75 nt single-end reads).

**Focused CRISPR-Cas9 genetic screening**—To ensure that most cells harbor a single sgRNA integration event, the volume of viral supernatant that would achieve an MOI of ~0.3 upon spinfection of a population of Cas9-expressing cancer cells was determined. Briefly, cells were plated at  $1 \times 10^6$  per well in 12-well plates along with increasing volumes of master pool viral supernatant and 10 µg/mL polybrene (EMD Millipore). Cells were then centrifuged at 1,500 rpm for 2 h at 37°C and incubated at 37°C overnight. Viral infection efficiency was determined by the percentage of TurboRFP+ cells assessed by flow cytometry on an LSRFortessa (BD Biosciences) instrument 72 h post infection. The volume of viral supernatant that achieved 30% infection rate was utilized in the screen. To ensure a representation of 1000X at the transduction step, the appropriate number of cells in 12-well plates was spininfected with viral supernatant. 24 h after infection, cells were pooled into two 150 mm tissue culture plates (Corning) per infection replicate and selected with 2.5 µg/mL puromycin (Gibco) for 4 days. Subsequently,  $5 \times 10^5$  puromycin-selected cells were pelleted and stored at 20°C (cumulative population doubling T0, input population). For H2087-LCC,  $2.5 \times 10^5$  cells expressing individual pool of library were intracardially injected into 10–12 athymic nude mice (cumulative population doubling T3, input population). For KPad1,  $2.5 \times 10^5$  cells per mouse were intracardially or intravenously injected into 7–10 B6-albino mice (cumulative population doubling T9, input population). This ensured high representation of sgRNAs (500x) in disseminated cancer cells, as a majority of circulating cancer cells perish and only a small percentage of cells (<10%) survive during colonization<sup>17,60</sup>. Metastasis progression was monitored weekly through BLI.

### Genomic DNA isolation

**Isolation of gDNA from cells:** Genomic DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Cell pellets containing  $5 \times 10^5$  cells were processed in parallel and resulting gDNA was resuspended in 100 µL of 10 mM Tris-Cl with 0.5 mM EDTA, pH 9.0.

**Isolation of gDNA from tumor tissues:** At the endpoint, mice were sacrificed and tissues containing metastases were harvested. Genomic DNA was extracted from tissues using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's instructions. Tissues were either processed immediately by finely mincing the tissue and incubating overnight in a lysis buffer containing proteinase K following the manufacturer's instructions, or snap-frozen in liquid nitrogen and stored at -80°C until the day of processing. Resulting



gDNA was resuspended in 100–200  $\mu\text{L}$  of 10 mM Tris-Cl with 0.5 mM EDTA, pH 9.0, and samples from common tissue or tumor fragments were pooled at the gDNA level, measured using a NanoDrop 2000 (ThermoFisher), and normalized before performing sequencing deconvolution.

**Deconvolution of CRISPR screens:** The library was amplified from gDNA by a modified 2-step PCR version of the protocol published by Doench *et al.*<sup>56</sup> All *in vivo* gDNA was sampled over 2-step PCR reactions. Briefly, an initial “enrichment” PCR was performed, whereby the integrated sgRNA cassettes were amplified from gDNA (PCR#1), followed by a second PCR to append Illumina sequencing adapters on the 5’ and 3’ ends of the amplicon, as well as a random nucleotide stagger and unique demultiplexing barcode on the 5’ end (PCR#2). Each “PCR#1” reaction contained 25  $\mu\text{L}$  of Q5 High-Fidelity 2X Master Mix (NEB), 2.5  $\mu\text{L}$  of Nuc PCR#1 Fwd Primer (10  $\mu\text{M}$ ), 2.5  $\mu\text{L}$  of Nuc PCR#1 Rev Primer (10  $\mu\text{M}$ ), and a maximum of 5  $\mu\text{g}$  of gDNA in 20  $\mu\text{L}$  of water. PCR#1 amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and used as template for “PCR#2” reactions. Each PCR#2 reaction contained 25  $\mu\text{L}$  of Q5 High-Fidelity 2X Master Mix (NEB), 2.5  $\mu\text{L}$  of a unique Nuc PCR#2 Fwd Primer (10 $\mu\text{M}$ ), 2.5  $\mu\text{L}$  of Nuc PCR#2 Rev Primer (10  $\mu\text{M}$ ), and 300 ng of PCR#1 product in 20  $\mu\text{L}$  of water. Two PCR#2 reactions were run per PCR#1 product. Library amplicons were size-selected on a 2.5% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen) followed by normalization, pooling, purification using AMPure XP beads (Beckman Coulter), and sequencing on an Illumina NextSeq500 instrument (75 nt single end reads). All primer sequences are available in Supplemental Table 8B. PCR settings for PCR#1 and PCR#2 were: initial denaturation at 98°C for 30 s; then 98°C for 10 s, 65°C for 30 s, 72°C for 30 s for 24 cycles; followed by extension at 72°C for 2 min.

**Analysis of CRISPR-Cas9 genetic screen data**—FASTQ files were processed and trimmed to retrieve sgRNA target sequences followed by alignment to the reference sgRNA library file. Raw sequencing read counts were quantified for each sgRNA, and samples were pooled at the organ level for downstream analyses (reads from the same organ across multiple mice were pooled). Reads were normalized to the total read counts per sample, and input samples were used as references to calculate log<sub>2</sub> fold change values per sgRNA using a combination of MAGeCK (v 0.5.9.4) and custom R (v 4.0.5) scripts. Enriched fold change (log<sub>2</sub>FC>0) values were used to average across all samples to generate average fold changes. For each library, the most enriched sgRNA for each gene was identified, and the corresponding genes were rank-ordered based on average fold change enrichments, as previously described<sup>61</sup>. See Supplemental Table 5 for all screening data.

### Knockout and overexpression constructs

CRISPR-mediated knockouts were generated by cloning sgRNAs into the Guide-it CRISPR/Cas9 vector (Red, TaKaRa, Cat# 632602), transfecting the construct into cells, then isolating and expanding the cells with knockouts from single cell colonies. Sequences of sgRNA oligos are: *hSTING.sg1* (forward CCGGGCTGGGACTGCTGTAAACG; reverse AAACCGTTTAAACAGCA GTCCCAGC), *mSTING.sg1* (forward CCGGTTGATCCCCGAAATCGGG;

reverse AAACCCC GATTTCCGGGGGATCAA), *mSTING.sg2* (forward CCGGTTAGAGGAATTCGGAGTGCG; reverse AAACCGCACTCCGAATTCCTCTAA), *hTGFB2* (forward CCGGAACGTGCGGTGGG ATCGTGC; reverse AAACGCACGATCCCACCGCACGTT).

To overexpress wild-type STING or STING-V155M, human wild-type *STING* was amplified by PCR from pUNO1-hSTING-HA3x (Invivogen, Cat# puno1ha-hsting). The V155M mutation was introduced in *STING* by PCR. Mouse wild-type *Sting* was amplified by PCR from pUNO1-mSTINGwt-HA3x (Invivogen, Cat# puno1ha-mstingwt). PCR amplified products were ligated to pLVX-Tight-Puro (Takara, Cat# 632162), a tetracycline (Tet)-inducible lentiviral expression vector.

To generate a TGF- $\beta$  reporter system, DNA sequences containing twelve SMAD-binding elements (SBE, 5'-AGCCAGACA-3') and a minimal CMV promoter<sup>62</sup> were subcloned into pLenti CMV rtTA3 Hygro (w785-1) vector (Addgene, Plasmid# 26730) replacing the original CMV promoter. The mCherry coding sequence was subcloned into pLenti CMVtight eGFP Puro (w771-1) vector (Addgene, Plasmid# 26431) replacing eGFP, to generate a Tet-inducible expression vector expressing mCherry. Both pLenti CMV rtTA3 Hygro and pLenti CMVtight eGFP Puro vectors were gifts from Eric Campeau.

### Single-cell RNA sequencing data analysis

We used our previously reported single-cell RNA sequencing (scRNA-seq) dataset from human LUAD specimens in Laughney *et al* (GSE123904).<sup>13</sup> The normalized, imputed gene expression generated by Laughney *et al*.<sup>13</sup> was used as the input. Metastatic tumor cells were ranked by average lung epithelial development score and assigned to different stages<sup>13</sup>. A gene signature of STING pathway targets (*IFNA2*, *IFNB1*, *CCL5*, *CXCL10*, *TNF*, *OASL*, *IRF1*, *IRF6*, *IRF7*, *IRF9*, *ADAR*, *CCL2*, *CCL26*, *IL33*) was assembled by integrating STING regulated genes in human or mouse cells (Extended Data Fig. 3h, j) from previous reports<sup>24–26,63</sup>. Z-normalized, imputed expression of genes or gene signatures was plotted on heatmaps using the `seaborn.heatmap` (v 0.11.2) function in Python (v 3.8.8). Violin plots were generated using the `seaborn.violinplot` (v 0.11.2) function. The gene signature “STING targets” in the violin plots represents average Z-scores of the constituent genes in the signature.

We reprocessed the scRNA-seq dataset of H2087 cells derived from Laughney *et al*.<sup>13</sup>, consisting of 3 samples and 2245 cells in total, including: 351 cells and 892 cells grown in culture from the dormant metastasis stage of mice M4585 and M4587, respectively; and 1002 cells grown in culture from multiple macrometastases of mouse M4770. The raw sequencing results were aligned and pre-processed by SEQC (v 0.2.1) to human reference genome build GRCh38. The SEQC output was filtered to remove apoptotic cells (i.e., cells with more than 20% of mitochondrial gene transcripts), and further filtered to exclude empty droplets (i.e., with small library size or lacking gene-gene correlation structure) as well as mitochondrial genes, ribosomal genes, and genes detected in fewer than 10 cells. The filtered counts of 3 samples were then merged and normalized by the library size. Phenograph clustering was conducted on 258 principal components accounting for a total of

70% variance of the merged normalized expression. The expression was further imputed by running MAGIC (v 0.1.1)<sup>64</sup> with  $t=3$ .

For the mouse data heatmap (Extended Data Fig. 3h), cells were first sorted by their pseudotime as computed by Palantir (v 1.0.0)<sup>64</sup>. The z-scored expression of the displayed genes and/or gene signatures was then plotted using `seaborn.heatmap`, without hierarchical clustering or moving average smoothing. The z-scored expression was also visualized by violin plots using `seaborn.violinplot`. Clustered heatmap (Extended Data Fig. 3i) was computed in Python (v 3.8.8) using the normalized dataset from Laughney *et al.*<sup>13</sup> without any further processing, and using the `seaborn.clustermap` function, with the method parameter set to “Ward” and the metric parameter kept as the default “euclidean” distance. UMAP plots were generated using `scanpy` (v 1.8.1)<sup>65</sup>. Genes differentially expressed between the Phenograph (v 1.5.2) clusters were determined by running MAST (v 1.0.1)<sup>66</sup> using default parameters, with normalized counts as the input. Genes with FDR (false discovery rate)-corrected p values < 0.01 and absolute  $\log_2FC > 1.2$  were considered significantly different. GSEA<sup>67,68</sup> was run to identify significantly enriched gene signatures among differentially expressed genes using hallmark gene sets<sup>69</sup>. Additional open-source algorithms used were Pandas (v 1.4.3), Numpy (v 1.21.5), Scipy (v 1.7.3), Scikit-learn (v 1.1.1), and Matplotlib (v 3.5.1).

### Flow cytometry analysis of cell surface and intracellular protein expression

For immune phenotyping of metastases-bearing femurs, tissues were washed in PBS, then chopped into small pieces using a sterile razor blade and resuspended in 5 mL of digestion buffer containing 1 mg/mL collagenase D (Millipore Sigma, Cat# 11088866001) and 0.05 mg/mL DNase in RPMI media. After incubation at 37°C for 40 min, the tissue sample was passed through a 70  $\mu$ m strainer, spun down, and processed in 1x eBioscience™ 10X red blood cell lysis buffer (Thermo Fisher Scientific, Cat# 00-4300-54).

Cells were washed and resuspended with stain media (PBS supplemented with 2% FBS) that contained fixable viability dye eFluor 506 (Thermo Fisher Scientific, Cat# 65-0866-14, 1:400), anti-mouse CD16/32 antibodies (clone 2.4G2, BioXCell, Cat# BE0307, 1:1000) and antibodies specific for proteins of interest. Cells were stained for surface proteins for 25 min at 4°C, washed, and stained for intracellular proteins according to the FoxP3/Transcription Factor Staining Buffer Set instructions (Thermo Fisher Scientific, Cat# 00-5523-00). Stained cells were washed, resuspended in stain media that contained a known number of 123count eBeads (Thermo Fisher Scientific, Cat# 01-1234-42) to calculate absolute cell number, and analyzed on a Cytex Aurora (Cytex Biosciences). Flow cytometry data was further analyzed using FlowJo software (BD Biosciences, v 10.6.2).

The following antibodies were from Biolegend: anti-mouse NKp46 (clone 29A1.4, Cat#137612, 1:50), anti-mouse CD44 (clone IM7, Cat# 103057, 1:400), anti-mouse XCR1 (clone ZET, Cat# 148220, 1:100), anti-mouse TCR $\beta$  (clone H57-597, Cat# 109220, 1:400), anti-mouse CD11c (clone N418, Cat# 117318, 1:400), anti-mouse CD19 (clone 6D5, Cat# 115530, 1:400), anti-mouse TNF $\alpha$  (clone MP6-XT22, Cat# 506308, 1:100). The following antibodies were from BD Biosciences: anti-mouse CD49b (clone HMa2, Cat# 741523, 1:200), anti-mouse NK1.1 (PK136, Cat# 741926, 1:200), anti-mouse CD8 $\alpha$  (53-6.7, Cat#

748535, 1:200), anti-mouse CD4 (GK1.5, Cat# 565974, 1:200). The following antibodies were from Thermo Fisher Scientific: anti-mouse IFN- $\gamma$  (clone XMG1.2, Cat# 48-7311-82, 1:100). Anti-mouse TCR $\beta$  (clone H57-597, Cat# 1785-16, 1:200) was from Southern Biotech and anti-mouse CD45 (30-F11, Cat# 80-0451-U100, 1:200) was from Tonbo.

### Ex vivo cytokine stimulation assay

Approximately  $1.5 \times 10^6$  cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 1% penicillin/streptomycin, 2mM L-glutamine, 55 $\mu$ M 2-mercaptoethanol, 1 mM sodium pyruvate, and 1x non-essential amino acids. Cells were cultured with 10 ng/mL phorbol 12-myristate 13-acetate (Millipore Sigma), 1  $\mu$ g/mL ionomycin (Millipore Sigma), 10  $\mu$ g/mL brefeldin A (Millipore Sigma), BD GolgiStop containing monensin (BD Biosciences) according to vendor instructions, and anti-mouse CD107a (clone 1D4B, Biolegend, Cat# 121624, 1:100) for 4 h at 37°C. Cells were washed and processed for staining and flow cytometric analysis.

### NK cell sorting and expansion

To purify NK cells prior to sorting, spleens from athymic nude mice were harvested and dissociated through a 100  $\mu$ m strainer. Single-cell suspensions were then incubated with rat antibodies against mouse Ly6G, CD19, CD3e, CD8 $\alpha$ , CD4, and Ter-119 antibodies (Bio X Cell, clones 1A8, 1D3, 17A2, 2.43, GK1.5 and TER-119, respectively, 1:100), washed twice in PBS supplemented with 2% FBS, then incubated with BioMag goat anti-rat IgG beads (Qiagen, Cat# 310107, 5mg per spleen) to remove antibody-bound cells. Purified NK cells were stained with antibodies against NK1.1 (PK136, Tonbo, Cat# 65-5941, 1:100), CD49b (DX5, BioLegend, Cat# 108918, 1:100), and NKp46 (29A1.4, BioLegend, Cat# 137604, 1:100) in the presence of purified rat anti-mouse CD16/CD32 (BD Biosciences, Cat# 553142, 1:1000) to block non-specific Fc binding and Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific, Cat# 65-0866-14, 1:400) to distinguish dead cells. Cell sorting was performed on an Aria III cytometer (BD Biosciences). Sorted NK cells were expanded by culturing in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 2mM L-glutamine, 55 $\mu$ M 2-mercaptoethanol, 1% penicillin/streptomycin, and recombinant human IL-15 (50 ng/ml; Miltenyi Biotec, Cat# 130-095-765).

### NK cell cytotoxicity assay

Cytotoxicity assays were performed as described previously<sup>70</sup>. Briefly, sorted NK cells were resuspended in NK cell medium (phenol-red free RPMI 1640 containing 10% FBS, 1x non-essential amino acids, 2mM L-glutamine and 1mM sodium pyruvate). Indicated target cells were labelled with 15  $\mu$ M calcein-AM (Thermo Fisher Scientific, Cat# C1430) for 30 min at 37°C, washed twice and resuspended in NK cell medium. Effector and target cells were combined at indicated ratios in triplicate wells of a round-bottom 96-well plate. Maximum and spontaneous lysis controls were generated by combining target cells with 2% Triton X-100 or media alone in place of effector cells, respectively. Cells were incubated at 37°C for 4 h. Calcein release was quantified by carefully transferring 100  $\mu$ L of cell-free supernatant to opaque 96-well plates and measuring fluorescent emission using the EnVision Robot Plate Reader (Perkin Elmer) set to excitation  $485 \pm 9$  nm and emission  $525 \pm 15$  nm.

### ***In vitro* transwell NK migration assay**

NK migration assays were performed as previously described<sup>71</sup>. To prepare conditioned media from cancer cells, cells were cultured in 6-well plates, washed twice with PBS and switched to serum-free media 16 h before medium collection. On the day of experiment, NK cells were washed twice and resuspended in serum-free media.  $2.5 \times 10^5$  NK cells in 100  $\mu$ L media were plated on the upper compartment of a transwell permeable chamber (6.5 mm diameter insert and 5  $\mu$ m pore size, Corning, Cat# 3421). 600  $\mu$ L serum-free medium or conditioned medium from cancer cells were plated in the bottom chamber. After incubation at 37 °C for 4 h, non-adherent and migrated NK cells from the bottom chamber were collected, mixed with 123count eBeads™ Counting beads and counted using flow cytometry.

### **Immunohistochemistry (IHC) and Immunofluorescence (IF)**

For staining of 5  $\mu$ m sections, human tissue samples were fixed in formalin and mouse metastatic tissue samples were fixed in 4% paraformaldehyde for 24 h prior to paraffin embedding. Sections were deparaffinized using Histo-Clear (National Diagnostics, Cat# HS-200), rehydrated, and incubated with hydrogen peroxide to quench endogenous peroxidase activity, followed by antigen retrieval in antigen unmasking solution, citrate-based (Vector Laboratories, Cat# H-3300) in a steamer for 30 min. For vimentin IHC, after antigen retrieval, sections were incubated with 2.5% normal horse serum (Vector Labs) and then anti-vimentin antibody (Abcam, Cat# ab8069, 1:200) overnight at 4°C. ImmPRESS horseradish peroxidase (HRP) anti-mouse IgG and ImmPACT DAB Peroxidase (Vector Labs) were used for detection. For IF staining, sections were incubated with 10% normal goat serum (Life Technologies, Cat# 50062Z) or 10% normal donkey serum (Millipore Sigma, Cat# D9663-10ML) supplemented with 2% BSA (Fisher Scientific, Cat# BP9706100) and 0.25% Triton X-100. Sections were then incubated in primary antibodies overnight at 4°C. After washing in PBS with 0.25% Triton X-100 (PBS-Tr), sections were incubated in fluorophore-conjugated secondary antibodies for 2 h, followed by washing and staining with nuclear dye Hoechst 33342 (Thermo Fisher Scientific, Cat# H3570, 1:5000). Tissue sections were then dehydrated in 70–100% ethanol and Histo-Clear, followed by mounting in Vectashield mounting medium (IHC) or ProLong diamond antifade mountant (IF) (Life Technology, Cat# P36970). For STING IHC, sections were stained with anti-STING antibody (D2P2F, Cell signaling technology, Cat# 13647, 1:500) via standardized automated protocols, on a Ventana Discovery XT instrument.

For immunofluorescence of thick metastases samples, organs were fixed in 4% paraformaldehyde overnight at 4°C and then washed twice with 1x PBS. Organs were cryo-protected by sequential immersion in 15% w/v and 30% w/v sucrose, then mounted using Tissue-Tek Optimal Cutting Temperature Compound (Sakura, Cat# 4583) on a sliding microtome with a platform freezing unit (Thermo Scientific, Cat# Microm KS-34 and Microm HM-450). 80  $\mu$ m sections were cut and stored in anti-freezing solution (40% w/v ethylene glycol and 30% v/v glycerol in PBS) at –20°C. Floating sections representative of the entire organ were permeabilized by washing with PBS-Tr three times, followed by incubation for 1 h in blocking buffer containing 10% normal goat or donkey serum, supplemented with 2% BSA and 0.25% Triton X-100. Sections were then incubated in primary antibodies diluted in blocking buffer, overnight at 4°C. After washing with PBS-Tr



six times, sections were incubated in fluorophore-conjugated secondary antibodies for 2 h. Sections were washed with PBS-Tr and then PBS three times each, followed by staining with nuclear dye Hoechst 33342 for 5 min, and three additional washes in PBS. Sections were transferred onto slides and mounted using ProLong diamond antifade mountant.

For immunofluorescence of NK and T cells in bone metastases samples, bone tissues were fixed in 4% paraformaldehyde overnight at 4°C and washed twice with 1x PBS. Bone decalcification was achieved by incubating the bone tissue in EDTA-based decalcification solution containing 140 g EDTA/L (pH 7.4, Millipore Sigma) for 1 to 2 weeks at 4°C with agitation, changing decalcification solution every other day. Bone tissues were then washed with water for 1 h to remove EDTA, re-fixed in 4% PFA overnight at 4°C, washed twice in PBS, and incubated in 70% ethanol overnight at 4°C. After paraffin embedding, tissue blocks were cut into 5 µm sections. Sections were deparaffinized, rehydrated, incubated with hydrogen peroxide, followed by antigen retrieval in antigen unmasking solution, citrate-based. For NK cell staining, the Alexa Fluor 594 Tyramide SuperBoost kit, streptavidin (Thermo Fisher Scientific, Cat# B40935) was applied. Sections were blocked with normal donkey serum, then incubated with the avidin/biotin blocking kit (Vector Laboratories, Cat# SP-2001), followed by incubation in goat anti-mouse NKp46/NCR1 (R&D systems, Cat# AF2225, 1:250) overnight at 4°C. Sections were then washed with PBS-Tr and incubated with donkey anti-goat IgG, biotin (Thermo Fisher Scientific, Cat# A16009, 1:500) at room temperature for 2 h. After washes with PBS-Tr and PBS, sections were incubated with HRP-conjugated streptavidin for 1 h at room temperature, followed by three washes with PBS. Sections were then incubated with tyramide reagent for 5 min and stop reagent for 5 min. After NK cell staining, sections were stained for T cells and cancer cells by incubating with primary antibodies against CD3e (D4V8L, Cell Signaling Technologies, Cat# 99940S, 1:200) or cytokeratin 8 (TROMA-1, Millipore Sigma, Cat# MABT329, 1:100), followed by washes and incubation in fluorophore conjugated secondary antibodies (Thermo Fisher Scientific) and nuclear staining in Hoechst 33342. Sections were mounted using ProLong diamond antifade mountant.

For quantification of IHC, slides were scanned by the Mirax Scanner (Carl Zeiss). A STING IHC intensity score was assigned to each tumor lesion based on the scoring levels depicted in Extended Data Fig. 3e, and the tumor area was measured by ImageJ (v 2.1.0). H-score for metastases from each organ was calculated using the following formula:  $[1 \times (\% \text{ tumor area } 1+) + 2 \times (\% \text{ tumor area } 2+) + 3 \times (\% \text{ tumor area } 3+)]$  where 1+, 2+, or 3+ refers to the IHC staining intensity level. For quantification of IF staining, images were acquired with an SP5 confocal microscope (Leica Microsystems) or a Zeiss Imager.Z1 fluorescence microscope (Carl Zeiss). Fluorescent intensity was analyzed with ImageJ software (v 2.1.0/1.53c). The following commercial antibodies were used: rat anti-Ki67 (Invitrogen, Cat# 14-5698-80, 1:100), mouse anti-Vimentin (Abcam, Cat# ab8069, 1:500 (IF), 1:200 (IHC)), chicken anti-GFP (Aves Labs, Cat# GFP-1010, 1:250), mouse anti-human cytokeratin (AE1/AE3) (Agilent Technologies, Cat# M351529-2, 1:100), rabbit anti-STING (D2P2F) (Cell Signaling Technologies, Cat# 13647, 1:250), goat anti-CCL5/RANTES (Novus Biologicals, Cat# AF278(human), AF478 (mouse), 1:40), rabbit anti-CD3e (D4V8L) (Cell Signaling Technologies Cat# 99940S, 1:200), goat anti-mouse NKp46/NCR1 (R&D Systems, Cat# AF2225, 1:250), Rat anti-Cytokeratin 8 (TROMA-1)



(Millipore Sigma, Cat# MABT329, 1:100), rat anti-SOX2 (Btjce) (Thermo Fisher Scientific, Cat# 14-9811-82, 1:200), rabbit anti-SOX9 (Millipore Sigma, Cat# AB5535, 1:300), rabbit anti-mCherry (Abcam, Cat# ab167453, 1:500), goat anti-chicken 488 (Thermo Fisher Scientific, Cat# A-11039, 1:500), goat anti-rat 647 (Thermo Fisher Scientific, Cat# A-21247, 1:500), goat anti-rabbit 594 (Thermo Fisher Scientific, Cat# A-11012, 1:500), goat anti-mouse 488 (Thermo Fisher Scientific, Cat# A-11001, 1:500), donkey anti-mouse 488 (Thermo Fisher Scientific, Cat# A-21202, 1:500), donkey anti-rat 647 (Abcam, Cat# ab150155, 1:500), donkey anti-goat 594 (Thermo Fisher Scientific, Cat# A-11058, 1:500), donkey anti-chicken 488 (Biotium, Cat# 20166, 1:500).

### qRT-PCR analysis

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Cat# 74106). cDNA was generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche, 04379012001). Relative gene expression was determined using Taqman or SYBR green assays (Life Technologies). Quantitative PCR was performed on the ViiA 7 Real-Time PCR System (Life Technologies). The housekeeping gene *ACTB* encoding  $\beta$ -actin was used as the internal control for calculating relative gene expression. Taqman assays used for human genes were: *STING* (Hs00736955\_g1), *IFNB* (Hs01077958\_s1), *SMAD7* (Hs00998193\_m1), *SNAI1* (Hs00195591\_m1), *cGAS* (Hs00403553\_m1) and *ACTB* (Hs01060665\_g1). Taqman assays used for mouse genes were: *Sting* (Mm01158117\_m1), *Ifnb1* (Mm00439552\_s1), *Ccl5* (Mm01302427\_m1), *Cxcl10* (Mm00445235\_m1), *Actb* (Mm01205647\_g1). Primers used for SYBR green assays (human genes) are: *ACTB* (forward GTTGTCGACGACGAGCG; reverse GCACAGAGCCTCGCCTT), *IFNB* (forward CAGGAGAGCAATTTGGAGGA; reverse CTTTCGAAGCCTTTGCTCTG), *CCL5* (forward TGTACTCCCGAACCCATTTTC; reverse TACACCAGTGGCAAGTGCTC), *CXCL10* (forward GCTGATGCAGGTACAGCGT; reverse GAGCCTACAGCAGAGGAACC).

### Targeted next-generation bisulfite sequencing analysis

**Sample preparation**—H2087 cell pellets freshly isolated and sorted from metastases in athymic nude mice or NSG mice were frozen in  $-80^{\circ}\text{C}$  and analyzed at EpigenDx. The promoter (H3K4me3 containing region) and 3' enhancer (H3K4me1 and H3K27ac containing region) sequences of *STING* were acquired from the Ensembl genome browser and annotated. The target sequences were re-evaluated against the UCSC genome browser for repeat sequences including LINE, SINE, and LTR elements. Sequences containing repetitive elements, low sequence complexity, high thymidine content, and high CpG density were excluded from the *in silico* design process. Cell pellets were lysed using M-digestion buffer (ZymoResearch, Cat# D5021-9) and 5–10 $\mu\text{L}$  of protease K (20mg/mL), with a final M-digestion concentration of 2X. The samples were incubated at  $65^{\circ}\text{C}$  for a minimum of 2 h. 20  $\mu\text{L}$  of the supernatant from the sample extracts were bisulfite modified using the EZ-96 DNA Methylation-Direct Kit<sup>TM</sup> (ZymoResearch, Cat# D5023) as per the manufacturer's protocol with minor modification. The bisulfite modified DNA samples were eluted using M-elution buffer in 46  $\mu\text{L}$ .

**Multiplex PCR**—All bisulfite modified DNA samples were amplified using separate multiplex or simplex PCR reactions. PCR reactions included 0.5 units of HotStarTaq (Qiagen, Cat# 203205), 0.2  $\mu$ M primers, and 3  $\mu$ L of bisulfite-treated DNA in a 20  $\mu$ L reaction. All PCR products were verified using the Qiagen QIAxcel Advanced System (v 1.0.6). Prior to library preparation, PCR products from the same sample were pooled and then purified using the QIAquick PCR Purification Kit columns or plates (Qiagen, Cat# 28106 or 28183). PCR settings included initial denaturation at 95°C for 15 min; then 95°C for 30 s, specific annealing temperature for each primer for 30 s, 68°C for 30 s for 45 cycles; and extension at 65°C for 5 min. Samples were run alongside established reference DNA samples created by mixing high- and low-methylated DNA to obtain samples with 0%, 5%, 10%, 25%, 50%, 75%, and 100% methylation. The high-methylated DNA was enzymatically methylated genomic DNA with >85% methylation. The low-methylated DNA was chemically and enzymatically treated to contain <5% methylation. Both were tested on numerous gene-specific and global methylation assays using pyrosequencing.

**Library preparation and sequencing**—Libraries were prepared using a custom library preparation method created by EpigenDx and purified using Agencourt AMPure XP beads (Beckman Coulter, Cat# A63882). Barcoded samples were then pooled in an equimolar fashion before template preparation and enrichment were performed on the Ion Chef™ system using Ion 520™ and Ion 530™ ExT Chef reagents (Thermo Fisher Scientific, Cat# A30670). Following this, enriched, template-positive library molecules were sequenced on the Ion S5™ sequencer using an Ion 530™ sequencing chip (Cat# A27764).

**Data analysis**—FASTQ files from the Ion Torrent S5 server were aligned to a local reference database using the open-source Bismark Bisulfite Read Mapper program (v 0.12.2) with the Bowtie2 alignment algorithm (v 2.2.3). Methylation levels were calculated in Bismark by dividing the number of methylated reads by the total number of reads. An R-squared value (RSQ) was calculated from the controls set at known methylation levels to test for PCR bias.

### Chromatin immunoprecipitation analysis

For chromatin immunoprecipitation analysis (ChIP-seq and ChIP-PCR), cells were crosslinked with 1% formaldehyde (Millipore Sigma, Cat# F8775) for 10 min and quenched with 0.125 M glycine for 5 min at room temperature. ChIP was performed using the ChIP assay kit (Millipore Sigma, Cat# 17-295) according to the manufacturer's instructions. Antibodies against H3K4me3 (Millipore Sigma, Cat# 05-745, 5  $\mu$ g per sample), H3K27me3 (Millipore Sigma, Cat# 07-449, 10  $\mu$ g per sample), H3K4me1 (Abcam, Cat# ab8895, 5  $\mu$ g per sample), H3K27Ac (Active Motif, Cat# 39133, 5  $\mu$ g per sample), DNMT1 (Abcam, Cat# ab13537, 5  $\mu$ g per sample), DNMT3B (Cell Signaling Technologies, Cat# 57868S, 5  $\mu$ g per sample) were used. Immunoprecipitated DNA was purified using the QIAquick PCR Purification Kit (Qiagen).

For ChIP-seq, immunoprecipitated DNA was quantified by PicoGreen and the size was evaluated by Agilent BioAnalyzer. When possible, fragments between 100 and 600 bp were size selected using AMPure XP beads (Beckman Coulter, Cat# A63882) and Illumina

sequencing libraries were prepared using the KAPA HTP Library Preparation Kit (Kapa Biosystems KK8234) according to the manufacturer's instructions, with an undetectable mass to 10 ng input DNA and 10 cycles of PCR. Barcoded libraries were run on the NovaSeq 6000 in a PE100 run, using the NovaSeq 6000 S4 Reagent Kit (200 Cycles) (Illumina). An average of 45 million paired reads were generated per sample. For mapping and visualization, paired-end (50/50 bp) FASTQ reads were mapped to human genome hg19 using Bowtie2 (v 2.1.0) with default filtering criteria<sup>72</sup>. Resulted SAM files were converted to BAM files through Samtools (v 1.9)<sup>73</sup>. BAM files were sorted and indexed with Samtools (v 1.9)<sup>73</sup>. BAM files were normalized, converted to bigwig files using bamCoverage-deepTools (v 3.3.2)<sup>23</sup> and displayed with IGV browser (v 2.14.1). Peak calling from BAM files was performed with MACS (v 2.2.7.1).

For ChIP-PCR, DNA samples were amplified by real-time PCR using specific primers. The amplification product was calculated as the percentage of the input, then normalized to the control experiment for each condition. ChIP-PCR primers for the *STING* promoter are forward AGAAACACTCACCGCAGTC and reverse GGAGTCCTGCTCAGGTGTTA. ChIP-PCR primers for the *STING* 3' enhancer are forward CATGGCATCATTTGTGTGCTG and reverse GTCCTTTCAGTGCCCTTCTCT.

### Methylated DNA immunoprecipitation analysis

Methylated DNA immunoprecipitation (MeDIP) was performed as described in Karpova *et al.*<sup>74</sup> Genomic DNA was extracted from cells using the QIAamp DNA Mini Kit (Qiagen, Cat# 51304), following the manufacturer's instructions. 9 µg DNA in 1 mL of immunoprecipitation (IP) buffer (10 mM Na-phosphate pH 7.0, 0.14 M NaCl, 0.05% v/v Triton X-100) was sonicated (the range of DNA fragment is 200–500 bp), then denatured at 99°C for 10 min. 4 µg of DNA was then incubated at 4°C overnight shaking in IP buffer containing either mouse (G3A1) IgG1 isotype control (Cell Signaling Technologies, Cat# 5415, 3 µg per sample) or 5-methylcytosine, clone 33D3 (Diagenode, C15200081-100, 3 µg per sample) antibody. The following day, the DNA-antibody complex was incubated with Dynabeads™ Protein G for Immunoprecipitation (Thermo Fisher Scientific, Cat# 10003D) at 4°C for 2 h. DNA combined with beads was washed three times in IP buffer, and DNA was eluted from beads in proteinase K digestion buffer (60 µg proteinase K, 50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS) at 56°C while shaking for 2 h. DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Cat# 28106), followed by qPCR analysis. 20% input was used as control. The amplification product was calculated as the percentage of the input, then normalized to the H2087-LCC control. PCR primers for the *STING* promoter (forward ACCAGTAAAGCTGCGGTTTG; reverse CTGCTCTGGATGATGACGAG) were obtained from a previous study<sup>34</sup>. PCR primers for the *STING* 3' enhancer were CATGGCATCATTTGTGTGCTG (forward) and GTCCTTTCAGTGCCCTTCTCT (reverse).

### Immunoblotting

Cells were lysed using RIPA cell lysis buffer (Cell Signaling Technology, Cat# 9806S) supplemented with protease inhibitors (Roche, cOmplete, mini, EDTA-free protease inhibitor tablets, Cat# 11836170001) and phosphatase inhibitor (Thermo Scientific, Halt

Phosphatase Inhibitor Cocktail, Cat# 78427, 1:1000). Protein concentration was measured using the BCA Protein Assay Kit (Pierce). Protein was then mixed with NuPage LDS sample buffer (Thermo Fisher Scientific, Cat# NP0007) supplemented with NuPAGE Sample Reducing Agent (Thermo Fisher Scientific, Cat# NP0009), heated at 95°C for 10 min, briefly sonicated, then loaded in NuPAGE Novex 4–12% Bis-Tris gels (Thermo Fisher Scientific) using 1x MOPS SDS running buffer (Thermo Fisher Scientific, Cat# NP0001) and transferred to nitrocellulose membranes after electrophoresis. Membranes were blocked with Odyssey blocking buffer (Tris-buffered saline) (LI-COR Biosciences, Cat# 927-60001) and incubated overnight with primary antibodies against STING (Cell Signaling Technology, Cat# 13647, 1:1000) or  $\beta$ -actin (Cell Signaling Technology, Cat# 3700, 1:5000). After washing in PBS + 0.1% Tween (PBST) three times, membranes were incubated with IRDye 680RD goat anti-mouse (LI-COR Biosciences, Cat# 926-68070, 1:10000) or IRDye 800CW goat anti-rabbit (LI-COR Biosciences, Cat# 926-32211, 1:10000) secondary antibodies. Signal was detected using an Odyssey CLx imager (LI-COR Biosciences) and analyzed using ImageStudioLite (LI-COR Biosciences, v3.1.4).

### Cytokine array

Cells were plated in triplicate in 6-well plates and treated for 3 days with or without 1  $\mu$ g/mL doxycycline hydrochloride (Thermo Fisher Scientific, Cat# BP2653-5) in 2 mL media. Conditioned media was collected and aliquots (75  $\mu$ L) were then analyzed using the Mouse Cytokine/Chemokine 44-Plex Discovery Assay<sup>®</sup> Array from Eve Technologies.

### Other assays

EdU incorporation assay was performed with Click-iT<sup>™</sup> Plus EdU Alexa Fluor<sup>™</sup> 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific, Cat# C10634), following the manufacturer's instructions. Cells were plated in triplicates in 6-well plates and incubated with 10  $\mu$ M EdU for 1.5–2 h, followed by flow cytometric analysis.

For cell viability assays, H2087-LCC cells were plated in four replicates in 96-well plates and incubated with 100 pM TGF- $\beta$ 1 or 2  $\mu$ M CDK4/6 inhibitor palbociclib (Selleckchem, Cat# S1116) in culture media. Cell proliferation was quantified at the indicated time points using the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega, Cat# G7572), following the manufacturer's instructions. Samples were analyzed using the Synergy H1 Hybrid microplate reader (Agilent).

For determinations of 2'3'-cGAMP,  $5 \times 10^5$  cells were lysed using M-PER<sup>™</sup> mammalian protein extraction reagent (Thermo Fisher Scientific, Cat# 78503). 2'3'-cGAMP content was measured with a 2'3'-cGAMP ELISA kit (Cayman Chemical Company, Cat# 501700) following the manufacturer's instructions.

### Statistics and reproducibility

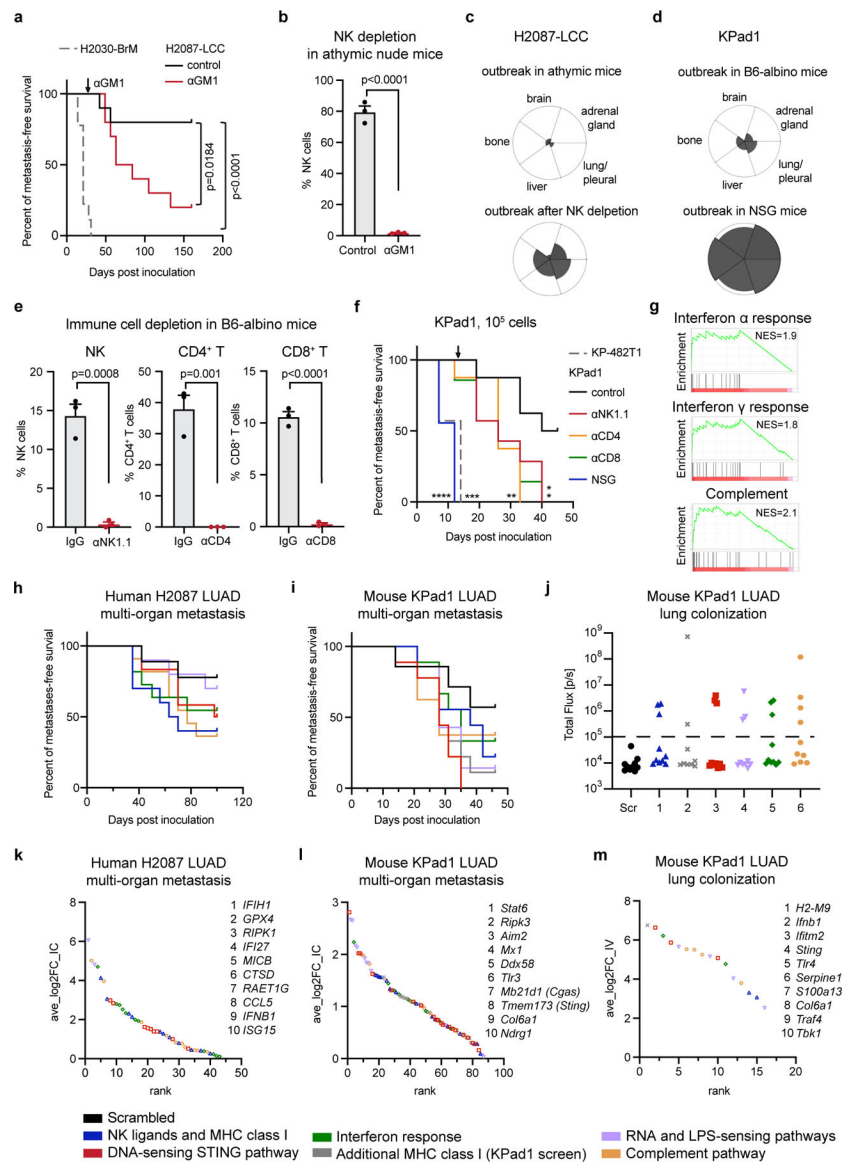
Statistical analyses and number of samples ( $n$ ) are given in each figure panel. Mann-Whitney U-tests, t-tests, and log-rank tests were performed in Graphpad Prism (v 9.0.0.). No statistical method was used to predetermine the sample size for *in vivo* experiments. Sample sizes were chosen based on prior experience and pilot experiments for detecting

statistically significant differences between conditions. For *in vivo* treatment assays with STING agonists, doxycycline and immune cell depleting antibodies, mice were distributed into treatment groups with approximately equal bioluminescent intensities. For other experiments, samples were randomly assigned to each group. Analysis of protein expression from IHC or IF images were blindly conducted using scripts of ImageJ batch analysis. IHC staining of STING expression in patient specimens was confirmed by a pathologist as a blinded reviewer. Investigators were blinded to conduct and analyze CRISPR screen assays. Investigators were not blinded to treatment groups for other *in vivo* or *in vitro* studies, as knowledge of this information was essential to conduct the studies.

### Schematics

Schematics were prepared using Office 365 (Microsoft, v 16.57), Adobe Illustrator (v 25.0.1), and BioRender ([www.biorender.com](http://www.biorender.com)) with publication permissions.

## Extended Data

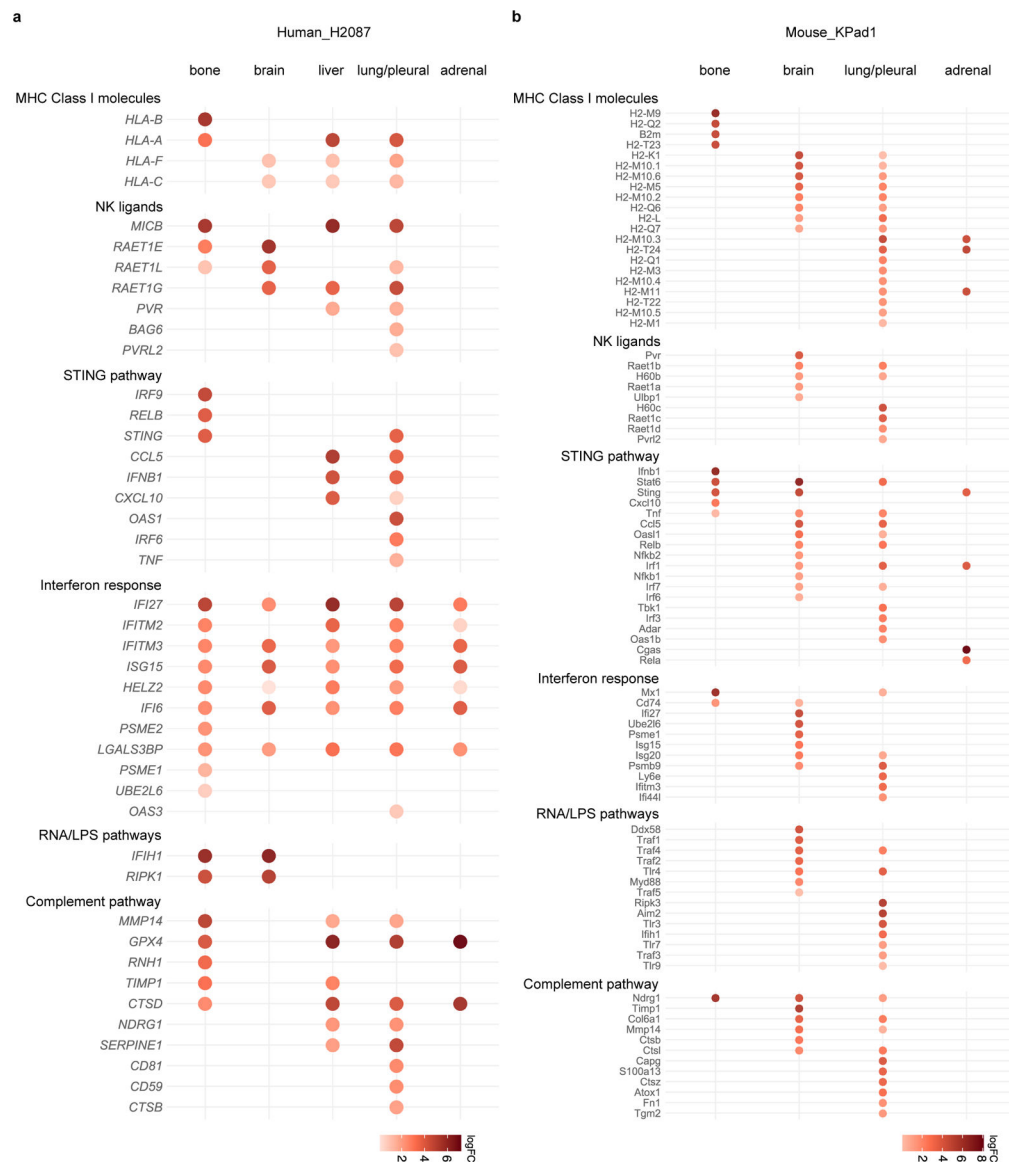


**Extended Data Figure 1. *In vivo* CRISPR screen for cell-autonomous immune regulators of dormant metastasis**

(a) Metastasis-free survival Kaplan-Meier plots of athymic mice intracardially inoculated with  $1 \times 10^5$  H2030-BrM or H2087-LCC cells. One set of mice inoculated with H2087-LCC cells was treated with anti-asialo-GM1 antibody to deplete NK cells 30 days after cell inoculation.  $n=9$  (H2030-BrM) or 10 (H2087-LCC) mice per group, log-rank test. (b) Flow cytometry analysis of Lineage<sup>-</sup>CD45<sup>+</sup>NKP46<sup>+</sup> NK cells in blood from athymic mice that were treated with IgG control or anti-asialo-GM1 antibody for 4 days. Lineage<sup>-</sup>: TCR $\beta$ -CD3<sup>-</sup>CD19<sup>-</sup>B220<sup>-</sup>CD11c<sup>-</sup>Ly6G<sup>-</sup>F4/80<sup>-</sup>.  $n=3$  mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. (c) Petal charts of metastasis incidence in specific organs of 10 athymic mice intracardially inoculated with  $1 \times 10^5$  H2087-LCC cells, treated with or without anti-asialo-GM1 antibody. Metastasis progression was monitored by BLI for 160 days

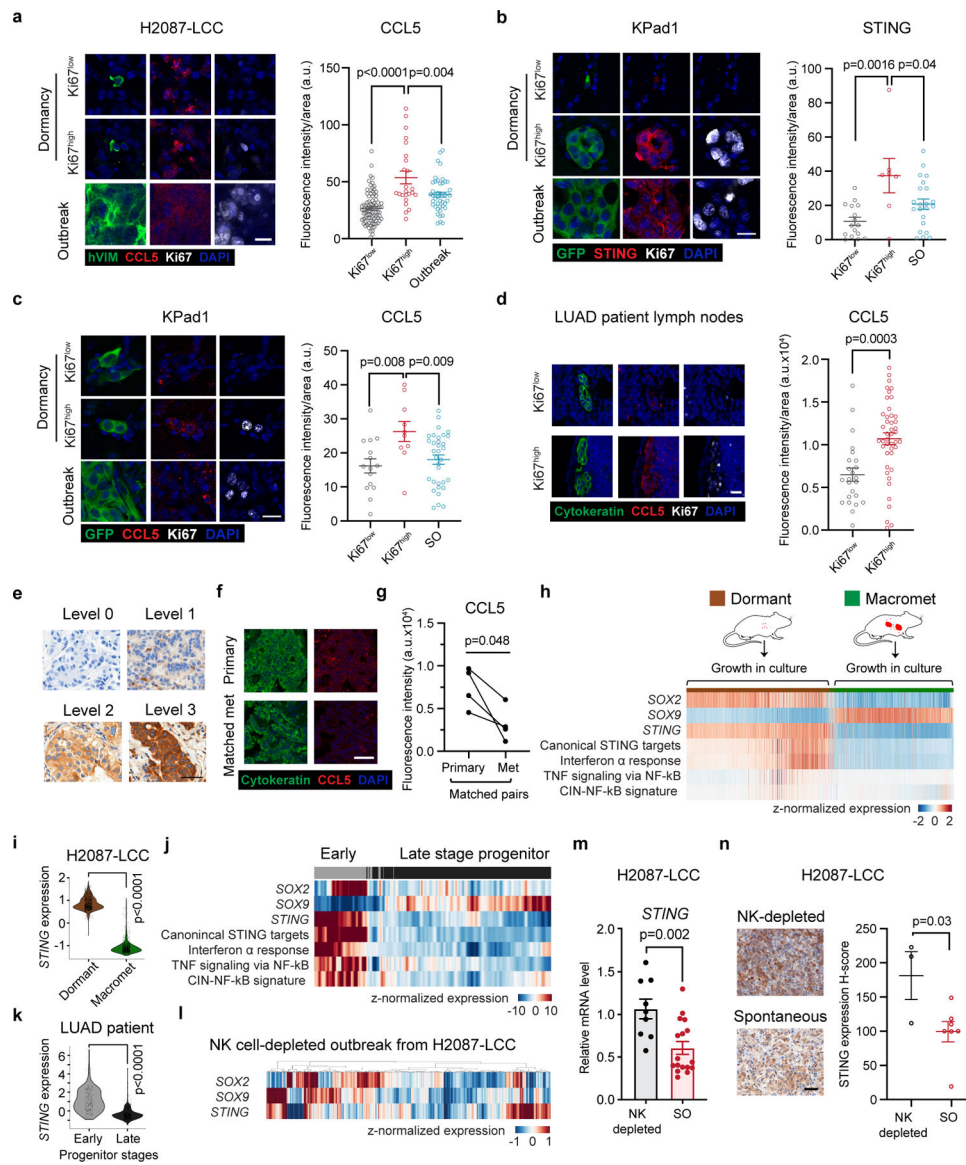


after cell inoculation. **(d)** Petal charts of metastasis incidence in specific organs after intracardiac inoculation of  $2 \times 10^4$  KPad1 cells into 8–9 B6-albino mice or NSG mice. Metastasis progression was monitored by BLI for 60 days (B6-albino mice) or 28 days (NSG mice) after cell inoculation. **(e)** Flow cytometry analysis of Lineage<sup>-</sup>CD45<sup>+</sup>NKP46<sup>+</sup> NK cells, CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>, and CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> T cells in blood from B6-albino mice that were treated for 3 days with IgG control, anti-NK1.1, anti-CD4, or anti-CD8 antibodies.  $n=3$  mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(f)** Metastasis-free survival Kaplan-Meier plots of NSG mice inoculated intracardially with KPad1 cells, or B6-albino mice inoculated intracardially with KPad1 cells or KP-482T1 cells, and treated with the indicated antibodies to deplete specific types of immune cells.  $1 \times 10^5$  cells were inoculated per mouse. Antibody treatments started 12 days after inoculation.  $n=8$  (KPad1/control, KPad1/ $\alpha$ CD4), 7 (KPad1/ $\alpha$ NK1.1, KPad1/ $\alpha$ CD8, KP-482T1), or 9 (NSG) mice per group, log-rank test. \*,  $p=0.03$  (KPad1/ $\alpha$ NK1.1 vs. KPad1/control), \*,  $p=0.0146$  (KPad1/ $\alpha$ CD8 vs. KPad1/control), \*\*,  $p=0.0094$  (KPad1/ $\alpha$ CD4 vs. KPad1/control), \*\*\*,  $p=0.0002$  (KP-482T1 vs. KPad1/control), \*\*\*\*,  $p<0.0001$  (KPad1/NSG vs. KPad1/control in B6-albino). **(g)** Gene set enrichment analysis (GSEA) showing pathways enriched in dormant H2087-LCC cells that were induced to proliferate in culture compared to cells from spontaneous metastatic outbreaks, analyzed from previous scRNA-seq data sets. **(h-i)** Metastasis-free survival of athymic mice (h) or B6-albino mice (i) intracardially inoculated with  $2.5 \times 10^5$  H2087-LCC (h) or KPad1 cells (i), expressing a scrambled RNA control or various sgRNA pools.  $n=9$  (Scrambled), 10 (NK ligands and MHC class I), 12 (STING pathway), 10 (LPS and RNA-sensing pathways), or 11 (Interferon response, Complement pathway) mice per group (h);  $n=7$  (Scrambled, LPS and RNA-sensing pathways), 8 (Complement pathway), or 9 (other groups) mice per group (i). **(j)** BLI quantification of lung metastatic colonies after intravenous inoculation of  $2.5 \times 10^5$  KPad1 cells expressing a scrambled control or different sgRNA pools in B6-albino mice. Tissues above the dotted line were harvested 57 days after cell inoculation, then subjected to sgRNA recovery and analysis.  $n=9$  (Additional MHC class I) or 10 (other groups) mice per group. **(k-l)** Gene rank based on the average fold enrichment of sgRNAs in multi-organ metastases from H2087-LCC (k) or KPad1 (l) cells. **(m)** Gene rank based on average fold change of sgRNAs enriched in the lung colonies formed after tail-vein inoculation of  $2.5 \times 10^5$  KPad1 cells into B6-albino mice. For k-m, genes with average  $\log_2FC > 0$  are plotted and genes targeted by the top 10 enriched sgRNAs are listed.



**Extended Data Figure 2. sgRNAs enriched in metastases of specific organs**

**(a-b)** Dot plots showing genes targeted by the enriched sgRNAs in metastases of the indicated organs after intracardiac inoculation of  $2.5 \times 10^5$  H2087-LCC cells into athymic mice (a) or  $2.5 \times 10^5$  KPad1 cells into B6-albino mice (b). Genes with average  $\log_2\text{FC} > 0$  are plotted.

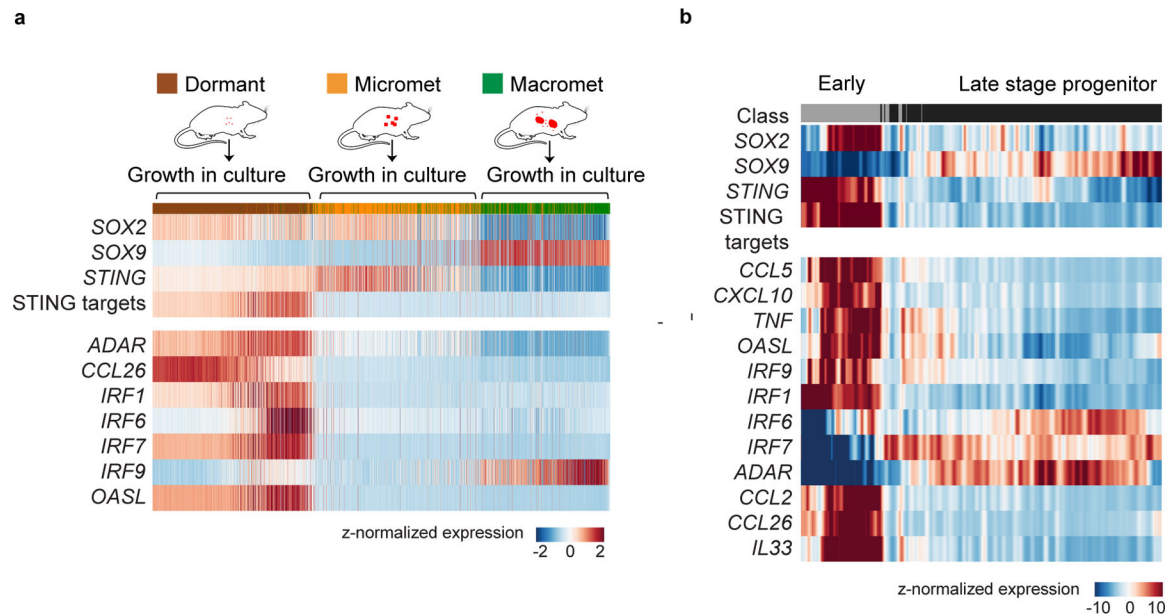


### Extended Data Figure 3. STING in dormant and reactivated metastatic progenitors and progenies

(a) As in Fig. 2a, representative IF images of single quiescent ( $Ki67^{low}$ ) and proliferative ( $Ki67^{high}$ ) cells during dormancy, and spontaneous outbreaks (SO) from H2087-LCC, stained for CCL5 IF (red), human vimentin (green), Ki67 IF (white), and DAPI (blue). The staining signal intensity of CCL5 in vimentin-positive cells was quantified and plotted (a.u., arbitrary units). Scale bar: 10  $\mu$ m.  $n=89$  cells ( $Ki67^{low}$ ), 24 cells ( $Ki67^{high}$ ), or 45 regions (outbreaks), from 5 mice per group. Mean  $\pm$  s.e.m, two-sided unpaired t-test. (b-c) Representative IF staining images of KPad1 cells (GFP, green) in brains of B6-albino mice that were intracardially inoculated with  $1 \times 10^5$  KPad1 cells. Organs were harvested 25 days after inoculation to capture the dormant state, and 43–61 days after inoculation to capture spontaneous outbreaks. Tissue sections were stained for STING (b, red), CCL5 (c, red), Ki67 (b and c, white), and DAPI (b and c, blue), and the staining signal intensity of STING (b) and CCL5 (c) in GFP-positive cells was quantified and plotted (a.u., arbitrary units).

Scale bar: 20  $\mu\text{m}$ .  $n=16$  cells (Ki67<sup>low</sup>), 7 cells (Ki67<sup>high</sup>), or 22 lesions (outbreaks), from 4 mice per group in (b);  $n=15$  cells (Ki67<sup>low</sup>), 10 cells (Ki67<sup>high</sup>), or 33 lesions (outbreaks), from 4 mice per group in (c). Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(d)** Representative images of micrometastatic cells (cytokeratin AE1/AE3, *green*) in lymph nodes from stage II and stage III LUAD patients, stained for CCL5 IF (*red*), Ki67 (*white*), and DAPI (*blue*). The signal intensity of CCL5 in cytokeratin-positive cells was quantified and plotted. Scale bar: 20  $\mu\text{m}$ .  $n=24$  (Ki67<sup>low</sup>) or 44 (Ki67<sup>high</sup>) regions, from 14 lymph nodes of 9 patients. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(e)** Representative images of STING IHC intensity levels (0, none; 1, weak, 2, moderate; 3, strong) in patient-derived lung adenocarcinoma tissue samples. STING expression H-scores were calculated based on the intensity levels, representative of 2 independent experiments. Scale bar: 50  $\mu\text{m}$ . **(f-g)** IF staining (f) and quantification (g) of CCL5 expression in patient-derived LUAD metastases compared to matched primary tumors. Tissue sections were stained for cytokeratin AE1/AE3 IF (*green*), CCL5 IF (*red*), and DAPI (*blue*), and the staining signal intensity of CCL5 in cytokeratin-positive areas was quantified and plotted (a.u., arbitrary units). Scale bar: 50  $\mu\text{m}$ .  $n=4$  matched pairs, two-tailed paired t-test. **(h)** Expression of *SOX* transcription factors specifying early (*SOX2*) and late (*SOX9*) lung epithelial progenitor states, *STING*, canonical *STING* pathway targets, hallmark\_interferon  $\alpha$  response genes (GSEA), canonical and non-canonical NF-kB target genes (rows) across H2087-LCC single cells isolated from dormant metastases or spontaneous macrometastases in athymic mice, and cultured under growth-promoting conditions. *STING* pathway targets refers to the mean expression of the genes shown in Extended Data Fig. 4. For each gene, imputed expression was z-normalized across all cells. In the top row, cells (columns) are colored and labeled by source.  $n=2245$  cells isolated from 3 mice. **(i)** Violin plots showing imputed and z-normalized expression of *STING* in H2087-LCC cells from the data set used in (h).  $n=2245$  cells isolated from 3 mice. Two-sided Mann-Whitney U-test. **(j)** Expression of *SOX* transcription factors, *STING*, canonical *STING* pathway targets, hallmark interferon  $\alpha$  response genes (GSEA), canonical and non-canonical NF-kB target genes (rows) across patient-derived metastatic tumor cells assigned to early or late-stage progenitor states. *STING* pathway targets refers to the mean expression of the genes shown in Extended Data Fig. 4. For each gene, imputed expression was z-normalized across all cells and smoothed using a 20-cell moving average window. Individual cells (columns) are ranked left to right by average lung epithelial development score.  $n=991$  cells isolated from 5 patients. **(k)** Violin plots showing imputed and z-normalized expression of *STING* across patient-derived tumor cells assigned to early or late-stage progenitor states from the data set used in (j).  $n=991$  cells isolated from 5 patients. Two-sided Mann-Whitney U-test. **(l)** Clustered heatmap showing expression of *SOX2*, *SOX9*, and *STING* in H2087-LCC cells isolated from metastases formed after NK cell depletion by anti-asialo-GM1 antibody treatment in athymic mice. For each gene, imputed expression was z-normalized across all cells.  $n=6073$  cells isolated from 5 mice. **(m)** qRT-PCR analysis of *STING* mRNA levels in H2087-LCC cell cultures derived from spontaneous outbreaks (SO) compared to those derived from outbreaks following depletion of NK cells. NK cells were depleted by treating mice with anti-asialo-GM1 antibody 30 days after cancer cell inoculation.  $n=9$  H2087-LCC cell lines from 4 mice after NK cell depletion or  $n=17$  cell lines from 8 mice with spontaneous outbreaks. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(n)** IHC staining and quantification of *STING* expression (H-score) in

H2087-LCC spontaneous outbreaks and outbreaks following NK cell depletion in multiple organs from athymic mice. Scale bar: 50  $\mu$ m.  $n=3$  organs from 3 mice (NK depletion) or 7 organs from 3 mice (spontaneous). Mean  $\pm$  s.e.m., two-sided unpaired t-test.

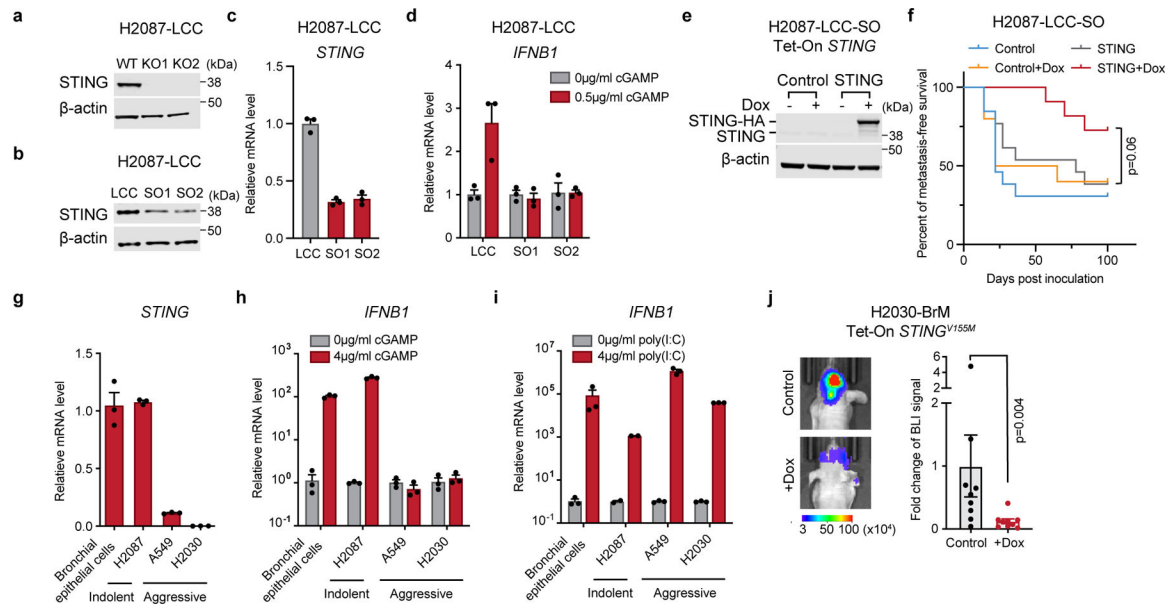


**Extended Data Figure 4. Expression of canonical STING pathway targets from scRNA-seq datasets**

**(a)** Expression of SOX transcription factors specifying early (SOX2) and late (SOX9) lung epithelial progenitor states, STING and canonical STING pathway targets (rows) across H2087-LCC single cells isolated from dormant metastases, or spontaneous macrometastases in athymic nude mice, and cultured under growth-promoting conditions. STING pathway targets refers to the mean expression of the genes shown in the bottom heatmap panel. For each gene, imputed expression was z-normalized across all cells. In the top row, cells (columns) are colored and labeled by source.  $n=2245$  cells isolated from 3 mice.

**(b)** Expression of SOX transcription factors, STING and canonical STING pathway targets (rows) across patient-derived metastatic tumor cells assigned to early or late-stage progenitor states. STING pathway targets refers to the mean expression of the genes shown in the bottom heatmap panel. For each gene, imputed expression was z-normalized across all cells and smoothed using a 20-cell moving average window. Individual cells (columns) are ranked left to right by average lung epithelial development score, as in Laughney et al.  $n=991$  cells isolated from 5 patients.

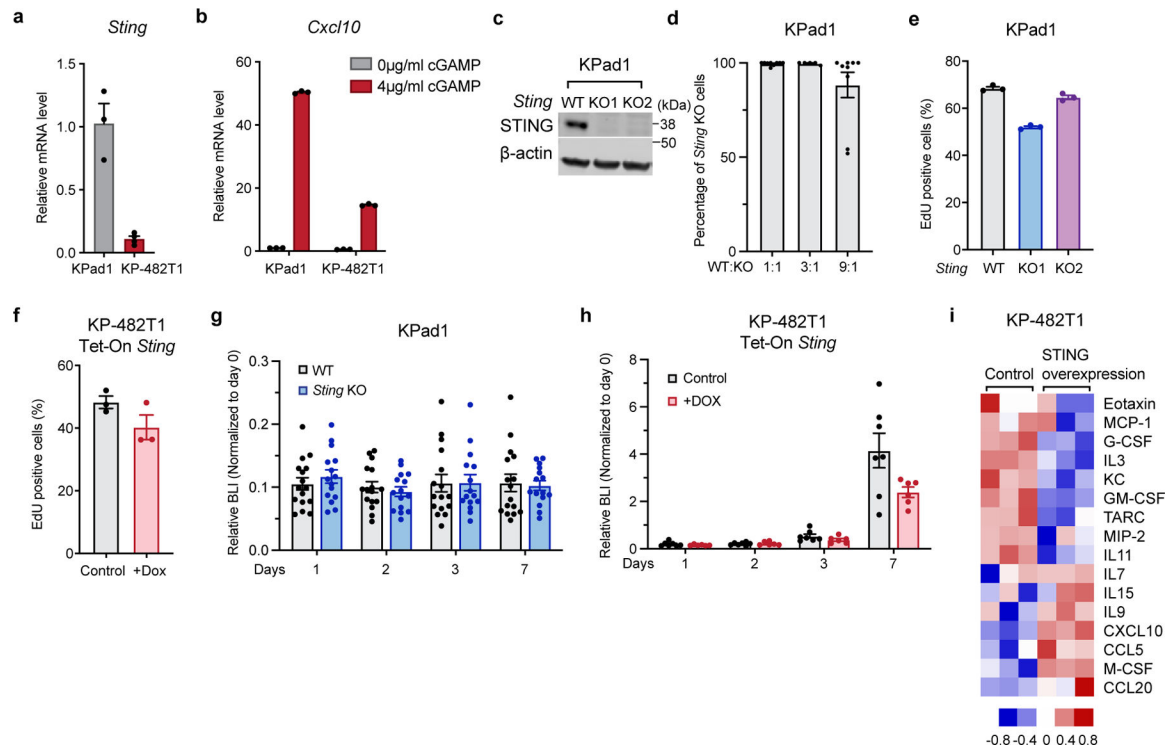




### Extended Data Figure 5. Perturbation of STING activity in human LUAD models

(a) Western immunoblot analysis of STING and  $\beta$ -actin in WT and *STING* knockout H2087-LCC cells, representative of 2 independent experiments. (b-c) Immunoblotting (b) and qRT-PCR analyses (c) of STING expression in parental H2087-LCC cells and the spontaneous outbreak derivatives SO1 and SO2. Mean  $\pm$  s.e.m, representative of 2 independent experiments. In (c), each dot represents a technical replicate of the assay. (d) qRT-PCR analysis of *IFNB1* expression after cGAMP treatment of H2087-LCC, SO1, and SO2 cells. Mean  $\pm$  s.e.m, representative of 2 independent experiments. Each dot represents a technical replicate of the assay. (e) Western immunoblot analysis of STING (endogenous STING and HA-tagged STING) and  $\beta$ -actin in H2087-LCC-SO cells inducibly expressing STING-HA under doxycycline (Dox) treatment for 24 h. Representative of 2 independent experiments. (f) Metastasis-free survival plots of athymic mice inoculated intracardially with  $1 \times 10^5$  H2087-LCC-SO cells. Cells were transduced with a vector control or a Dox-inducible STING expression vector. Dox treatment started 7 days after cell inoculation.  $n=13$  (Control, STING), 10 (Control+Dox), 11 (STING+Dox) mice per group, log-rank test. (g) qRT-PCR analysis of *STING* mRNA levels in human primary bronchial epithelial cells, H2087, A549, and H2030 LUAD cell lines. Mean  $\pm$  s.e.m, representative of 3 independent experiments. Each dot represents a technical replicate of the assay. (h-i) qRT-PCR analysis of *IFNB1* mRNA expression in human primary bronchial epithelial cells and lung adenocarcinoma cell lines treated with cGAMP (h) or poly(I:C) (i). Mean  $\pm$  s.e.m, representative of 3 independent experiments. Each dot represents a technical replicate of the assay. (j) BLI and quantification of metastases formed by H2030-BrM cells inducibly expressing activated STING-V155M, starting the day after intracardiac inoculation of  $1 \times 10^5$  cells in athymic mice. Metastases were quantified 21 days after inoculation.  $n=9$  mice per group. Mean  $\pm$  s.e.m., two-sided Mann-Whitney U-test. For gel source data, see Supplemental Figure 1.

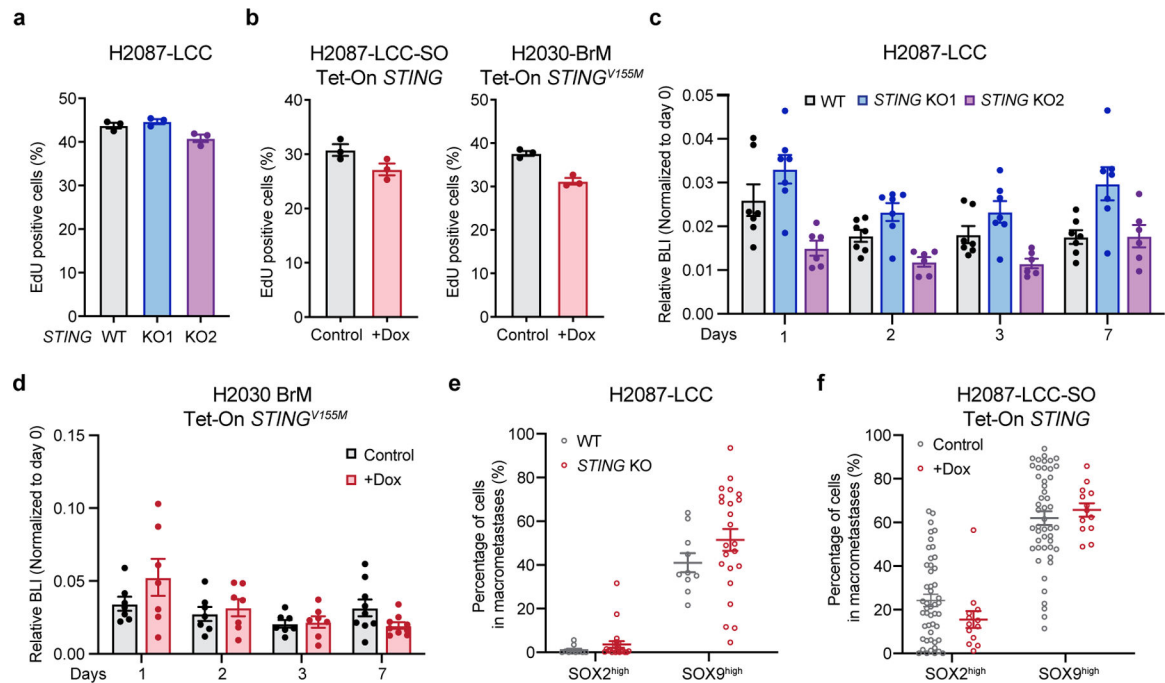




### Extended Data Figure 6. STING activity in mouse lung adenocarcinoma cells

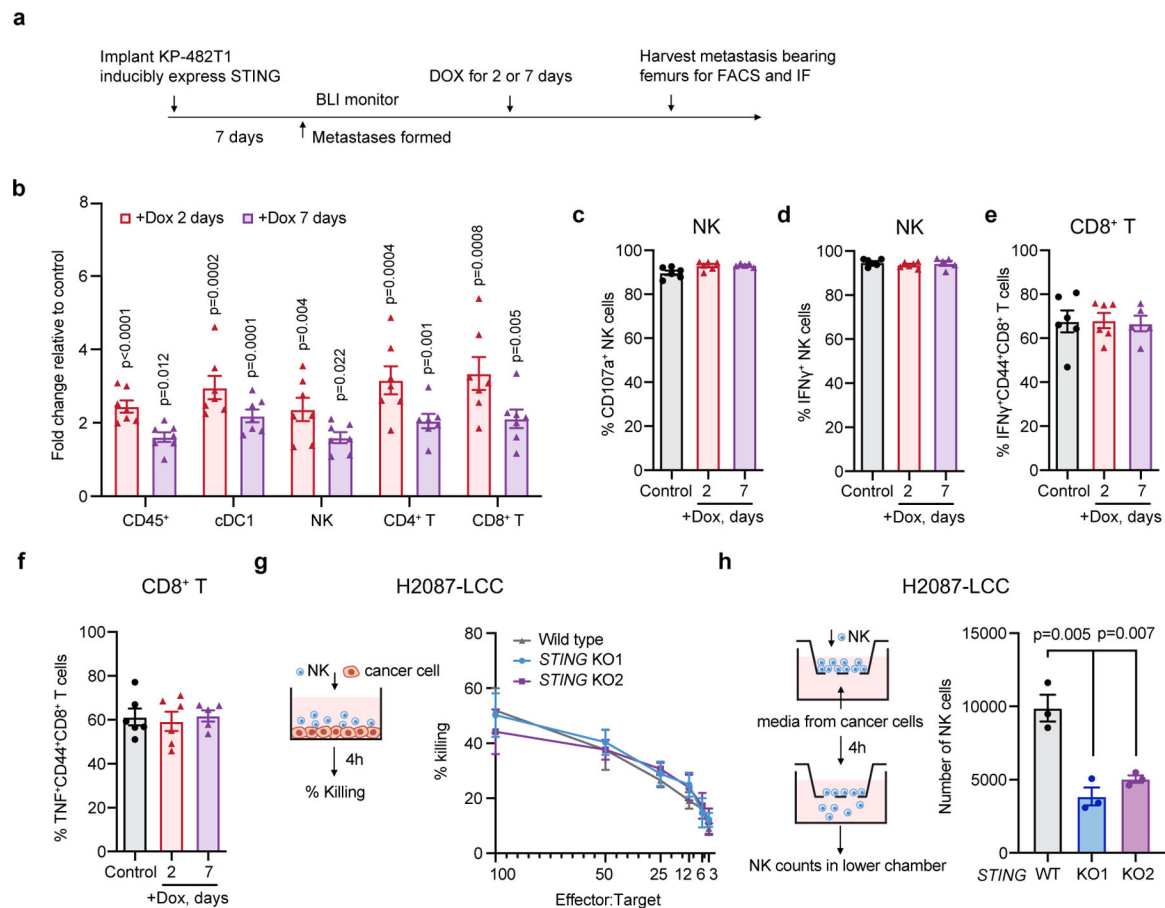
(a-b) qRT-PCR analysis of *Sting* (a) and *Cxcl10* (b) mRNA levels after cGAMP treatment in KPad1 and KP-482T1 cells. Mean  $\pm$  s.e.m, representative of 2 independent experiments.

Each dot represents a technical replicate of the assay. (c) Western immunoblotting of STING and  $\beta$ -actin in parental and *Sting* knockout KPad1 cells. Representative of 2 independent experiments. For gel source data, see Supplemental Figure 1. (d) RFP-positive WT KPad1 cells and GFP-positive *Sting* KO KPad1 cells were mixed at the indicated ratios and intracardially inoculated ( $1 \times 10^5$  cells) in B6-albino mice. Metastases-bearing organs were harvested 4–17 weeks after inoculation and the percentage of GFP-positive cells and RFP-positive cells was determined by flow cytometry.  $n=9$  (1:1, 9:1) or 5 (3:1) lesions from 3–5 mice per group. Mean  $\pm$  s.e.m. (e) Percentage of EdU positive cells of WT or *Sting* knockout KPad1 cells incubated with EdU for 2h.  $n=3$  per group. Mean  $\pm$  s.e.m. (f) Percentage of EdU positive cells in KP-482T1 Tet-On *Sting* cultures treated with Dox for 24h and then incubated with EdU for 2h.  $n=3$  per group. Mean  $\pm$  s.e.m. (g-h) BLI signal intensity at the indicated time points after intracardiac inoculation of  $1 \times 10^5$  WT or *Sting* knockout KPad1 cells (g) or KP-482T1 cells with induced overexpression of STING (h) in B6-albino mice.  $n=16$  (WT) or 15 (KO) mice (g);  $n=7$  (control) or 6 (Dox) mice (h). Mean  $\pm$  s.e.m. (i) Heatmap representation of cytokine array analysis of culture supernatant from KP-482T1 cells transduced with a vector control or a *Sting* overexpression vector.  $n=3$  for each condition.



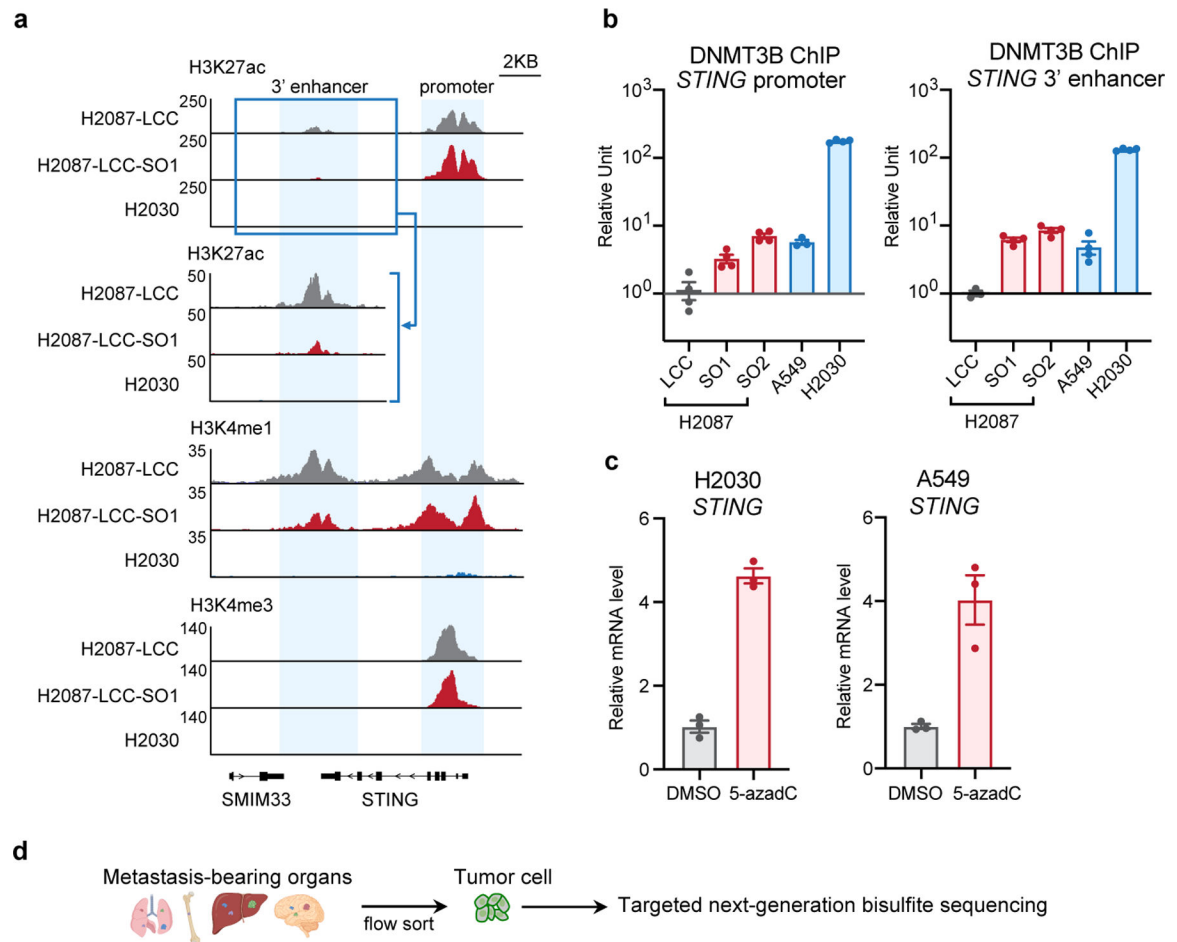
**Extended Data Figure 7. STING knockout and overexpression effects on growth and differentiation of metastatic progenitors**

(a) Percentage of EdU positive cells of WT or *STING* knockout H2087-LCC cells incubated with EdU for 2h.  $n=3$  per group. Mean  $\pm$  s.e.m. (b) Percentage of EdU positive cells in H2087-LCC-SO Tet-On *STING* or H2030-BrM Tet-On *STING*<sup>V155M</sup> cultures treated with Dox for 24h, and then incubated with EdU for 2h.  $n=3$  per group. Mean  $\pm$  s.e.m. (c-d) BLI signal intensity at the indicated time points after intracardiac inoculation of  $1 \times 10^5$  WT or *STING* knockout H2087-LCC cells (c) or H2030-BrM cells with Dox-induced overexpression of *STING*<sup>V155M</sup> (d) in athymic mice.  $n=7$  (WT, KO1) or 6 (KO2) mice per group (c);  $n=9$  (7 days) or 7 (other groups) mice per group (d). Mean  $\pm$  s.e.m. (e) IF staining analysis showing percentage of SOX2<sup>high</sup> and SOX9<sup>high</sup> cells in spontaneous metastases formed after intracardiac inoculation of  $1 \times 10^5$  WT or *STING* knockout (KO) H2087-LCC cells in athymic mice. Organs were harvested 12 weeks after inoculation.  $n=10$  lesions (WT) or 23 lesions (*STING* KO). Mean  $\pm$  s.e.m. (f) IF staining analysis showing percentage of SOX2<sup>high</sup> and SOX9<sup>high</sup> cells in metastases formed after intracardiac inoculation of  $1 \times 10^5$  H2087-LCC-SO cells inducibly overexpressing *STING* in athymic mice. Organs were harvested 7–13 weeks after inoculation for the control cells and 18 weeks after inoculation for the *STING* overexpressing cells.  $n=48$  images from 5 mice (control) or 13 images from 3 mice (Dox). Mean  $\pm$  s.e.m.



### Extended Data Figure 8. Cancer cell STING increases NK and T cell levels in metastases

**(a)** Schematic of flow cytometry or immunofluorescence analysis of immune cells in bone metastasis. **(b)** Flow cytometry analysis and quantification of CD45<sup>+</sup> leukocytes, CD45<sup>+</sup>CD19<sup>-</sup>TCR $\beta$ <sup>-</sup>XCR1<sup>+</sup>CD11c<sup>+</sup> cDC1 cells, CD45<sup>+</sup>NK1.1<sup>+</sup>TCR $\beta$ <sup>-</sup>CD49b<sup>+</sup>NKp46<sup>+</sup> NK cells, CD45<sup>+</sup>TCR $\beta$ <sup>+</sup>NK1.1<sup>-</sup>CD8<sup>+</sup> T cells, and CD45<sup>+</sup>TCR $\beta$ <sup>+</sup>NK1.1<sup>-</sup>CD4<sup>+</sup> T cells in KP-482T1 metastasis-bearing femurs after Dox-induced expression of STING for 2 days or 7 days. Cell numbers per gram of femur tissue were counted and normalized to control (-Dox). Metastasis-bearing femurs were collected 2 weeks after intracardiac inoculation of  $2 \times 10^4$  KP-482T1 cells in B6-albino mice.  $n=7$  mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. p values comparing each Dox treatment group with control (-Dox). **(c-f)** Percentage of NK cells degranulating (CD107a<sup>+</sup>) (c) or producing IFN $\gamma$  (d), and percentage of CD44<sup>+</sup>CD8<sup>+</sup> T cells producing IFN $\gamma$  (e) or TNF (f), isolated from KP-482T1 metastasis-bearing femurs after Dox-induced expression of STING for 2 days or 7 days, and then cultured *ex vivo* with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4h.  $n=5$  (+Dox 7 days) or 6 (other groups) mice per group. Mean  $\pm$  s.e.m. **(g)** Schematic of the NK cell-mediated killing assay, and the percentage of WT or *STING* KO H2087-LCC cells killed by incubation with naive NK cells for 4h at the indicated effector:target ratios.  $n=3$  per group. Mean  $\pm$  s.e.m. **(h)** Schematic of the trans-well migration assay, and the number of NK cells migrated into cell culture media conditioned by WT or *STING* knockout H2087-LCC cells.  $n=3$  per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test.



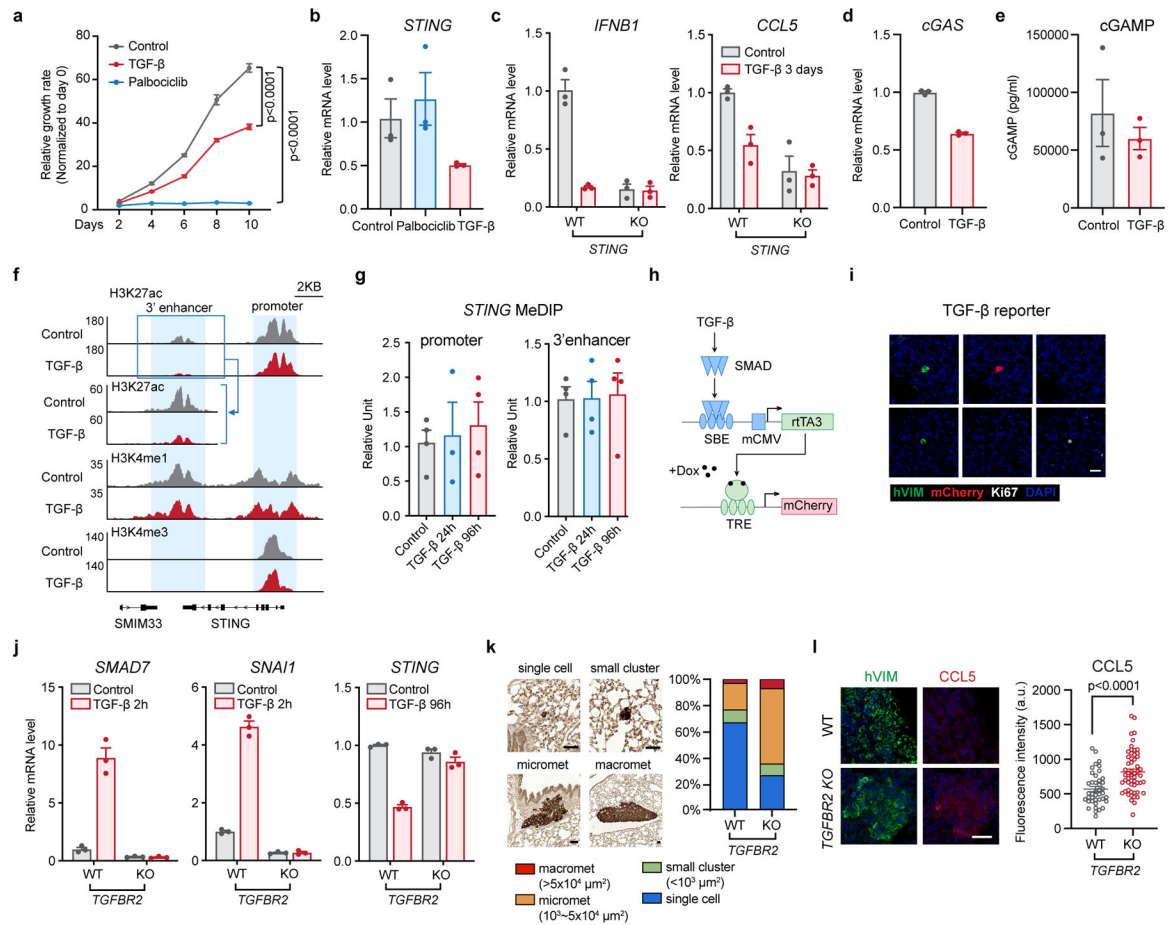
### Extended Data Figure 9. *STING* locus regulation during metastatic progression

**(a)** Gene track view for H3K27ac, H3K4me1, and H3K4me3 ChIP-Seq tags at the *STING* locus in H2087-LCC, spontaneous outbreak derivative H2087-LCC-SO1, and H2030 cells.

**(b)** ChIP-PCR analysis of DNMT3B binding to the *STING* promoter or 3' enhancer regions in parental H2087-LCC, H2087-LCC-SO1, H2087-LCC-SO2, and in A549 and H2030 cells. Mean  $\pm$  s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay.

**(c)** qRT-PCR analysis of *STING* mRNA levels in H2030 and A549 cells after a 3-day treatment with 100nM 5-aza-2'-deoxycytidine (5-azadC) in culture. Mean  $\pm$  s.e.m., representative of 3 independent experiments. Each dot represents a technical replicate of the assay.

**(d)** Schematic of targeted bisulfite sequencing analysis of the *STING* locus in metastatic cells of interest.

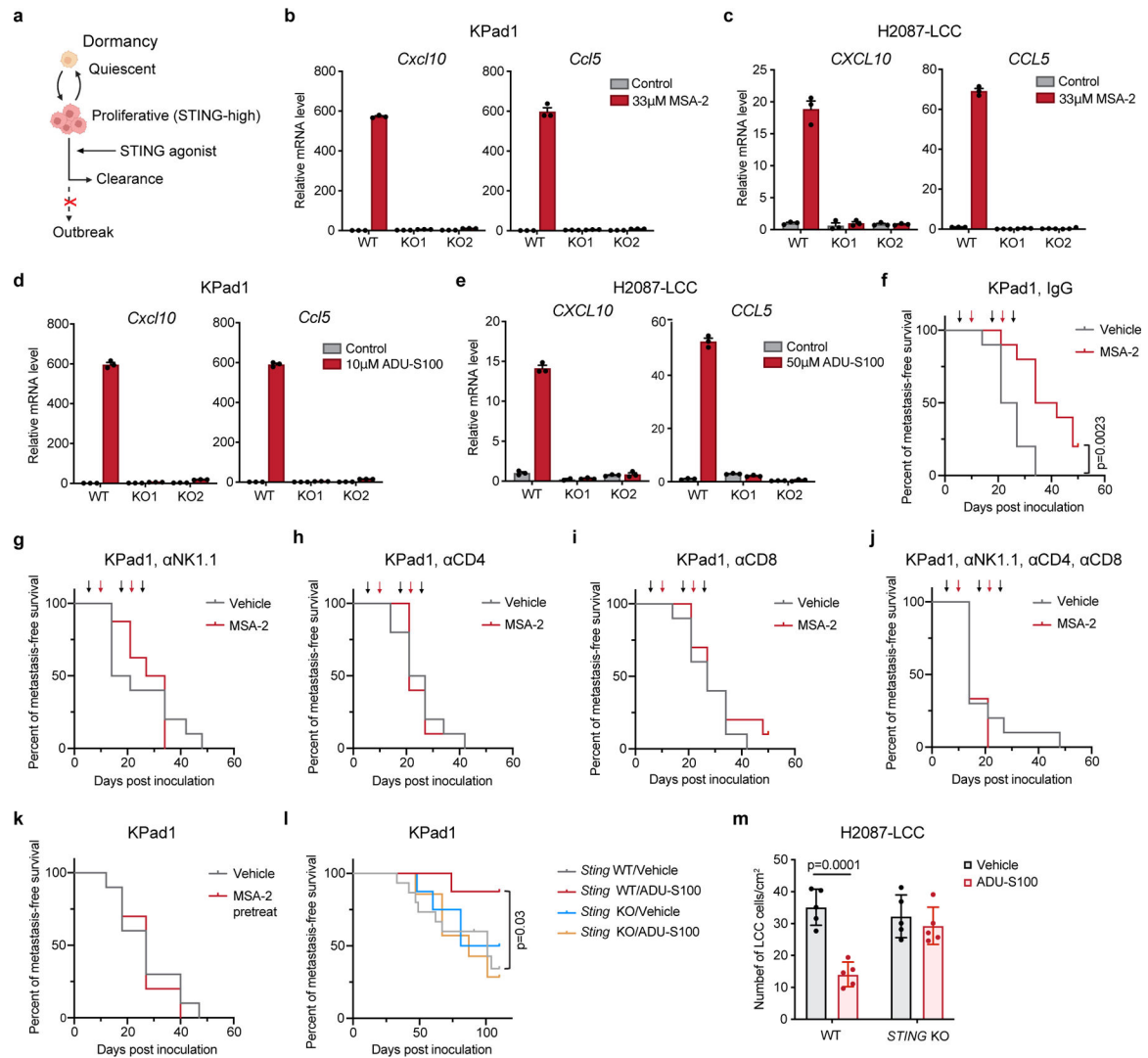


### Extended Data Figure 10. TGF- $\beta$ mediated suppression of *STING* expression

(a) Growth rate of H2087-LCC cells treated with TGF- $\beta$  or CDK4/6 inhibitor palbociclib for the indicated times.  $n=4$ . Mean  $\pm$  s.e.m., two-way ANOVA analysis. (b) qRT-PCR analysis of *STING* mRNA levels in H2087-LCC cells treated with TGF- $\beta$  or palbociclib for 7 days. Mean  $\pm$  s.e.m., representative of 3 independent experiments. Each dot represents a technical replicate of the assay. (c) qRT-PCR analysis of *IFNB1* and *CCL5* expression in WT or *STING* knockout H2087-LCC cells treated with TGF- $\beta$  for 3 days. Mean  $\pm$  s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. (d) qRT-PCR analysis of *cGAS* mRNA levels in H2087-LCC cells treated with TGF- $\beta$  for 7 days. Mean  $\pm$  s.e.m., representative of 3 independent experiments. Each dot represents a technical replicate of the assay. (e) ELISA analysis of cGAMP levels in H2087-LCC cells treated with TGF- $\beta$  for 5 days.  $n=3$  per group. Mean  $\pm$  s.e.m. (f) Gene track view of H3K27ac, H3K4me1, and H3K4me3 ChIP-Seq tags at the *STING* locus in H2087-LCC cells treated with TGF- $\beta$  for 4 days. (g) PCR based quantitation of methylated *STING* promoter and 3' enhancer sequences in MeDIP samples from H2087-LCC cells that were treated with TGF- $\beta$  for the indicated times. Mean  $\pm$  s.e.m., representative of 3 independent experiments. Each dot represents a technical replicate of the assay. (h) Schematic of the TGF- $\beta$  and SMAD-dependent transcriptional reporter. SBE: Smad Binding Element. mCMV: minimal CMV promoter. TRE: Tetracycline-Responsive promoter Element. (i) Representative IF staining images of H2087-LCC cells (human vimentin, *green*) expressing

SMAD-responsive mCherry reporter in the lungs of athymic mice intravenously inoculated with  $1 \times 10^5$  cells. Organs were harvested 8 weeks after inoculation. Disseminated cancer cells were present as single quiescent (Ki67<sup>low</sup>) or proliferative cells (Ki67<sup>high</sup>, *white*). Representative of 2 independent experiments. Scale bar: 20  $\mu\text{m}$ . **(j)** qRT-PCR analysis of *SMAD7*, *SNAI1* and *STING* expression in H2087-LCC cells treated with TGF- $\beta$  or no additions for 2 h or 96 h, respectively. Mean  $\pm$  s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. **(k)** Representative IHC images (left) and quantification (right) of the proportion of H2087-LCC single cells, small clusters, micrometastases, and macrometastases in the lungs of NSG mice 7 weeks after intravenous injection of  $1 \times 10^5$  cells. Scale bar: 50  $\mu\text{m}$ .  $n=102$  (WT) or 33 (KO) single cells, 17 (WT) or 12 (KO) small clusters, 31 (WT) or 71 (KO) micrometastases, and 5 (WT) or 9 (KO) macrometastases. **(l)** IF staining of human vimentin (*green*), DAPI (*blue*), and CCL5 (*red*), in lung metastases generated 7 weeks after intravenous inoculation of  $1 \times 10^5$  WT or *TGFBR2* knockout H2087-LCC cells in NSG mice. The IF staining intensity in vimentin-positive areas was quantified. Scale bar: 50  $\mu\text{m}$ .  $n=41$  (WT) or 55 (KO) lesions from 5 mice. Mean  $\pm$  s.e.m., two-sided unpaired t-test.





### Extended Data Figure 11. Immune cell dependence of STING agonist-mediated suppression of metastasis reactivation

**(a)** Rationale for experimental design. **(b-c)** qRT-PCR analysis of *CXCL10* and *CCL5* mRNA levels in WT or *STING* knockout KPad1 (b) or H2087-LCC (c) cells treated with 33 μM MSA-2 for 4h. Mean ± s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. **(d-e)** Treatment was performed as in (b, c) but with 10 μM (KPad1) or 50 μM (H2087-LCC) ADU-S100. Mean ± s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. **(f-j)** Metastasis-free survival plots of B6-albino mice intracardially inoculated with  $2.5 \times 10^5$  KPad1 cells, treated with both MSA-2 and IgG control (f,  $n=10$  mice) or individual antibodies to deplete NK cells (g,  $n=10$  mice for vehicle or 8 mice for MSA-2), CD4<sup>+</sup> T cells (h,  $n=10$  mice), CD8<sup>+</sup> T cells (i,  $n=10$  mice), or a combination of these antibodies (j,  $n=10$  mice for vehicle or 9 mice for MSA-2). Mice were administered antibodies (200 μg/mouse) once weekly for 3 weeks starting 6 days after cell inoculation, and vehicle or MSA-2 (50 mg/kg of body weight) once weekly for 2 weeks starting 9 days after cell inoculation. Log-rank test. **(k)** Metastasis-free survival plots of B6-albino mice intracardially inoculated

with  $2.5 \times 10^5$  KPad1 cells pre-treated with 33  $\mu\text{M}$  MSA-2 for 24h before inoculation.  $n=10$  mice per group. **(l)** Metastasis-free survival plots of C57BL/6J mice intravenously inoculated with  $2.5 \times 10^5$  WT or *Sting* knockout KPad1 cells and treated with vehicle or ADU-S100 (1.25 mg/kg of body weight) once weekly by intratracheal delivery until the study endpoint. ADU-S100 treatment started 5 days after cell inoculation.  $n=15$  mice (control) or 8 mice (ADU-S100). Log-rank test. **(m)** Quantification of WT or *STING* knockout H2087-LCC cell numbers in lungs from athymic mice treated with vehicle or ADU-S100 (6.25 mg/kg of body weight) once weekly for 4 weeks by intratracheal delivery. ADU-S100 treatment started 1 week after intravenous inoculation of  $1 \times 10^5$  cells. Lungs were harvested 5 weeks after inoculation.  $n=5$  mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Data Availability Statement

The raw sequencing data corresponding to CRISPR screens, and targeted bisulfite sequencing have been deposited in the NCBI SRA under accession number PRJNA786579 and PRJNA867723, respectively. ChIP-seq data have been deposited in the GEO under accession number GSE210946. scRNA-seq data of human LUAD specimens and mouse H2087-LCC model were reanalyzed from Laughney *et al* (GSE123904). Source data are provided with this paper.

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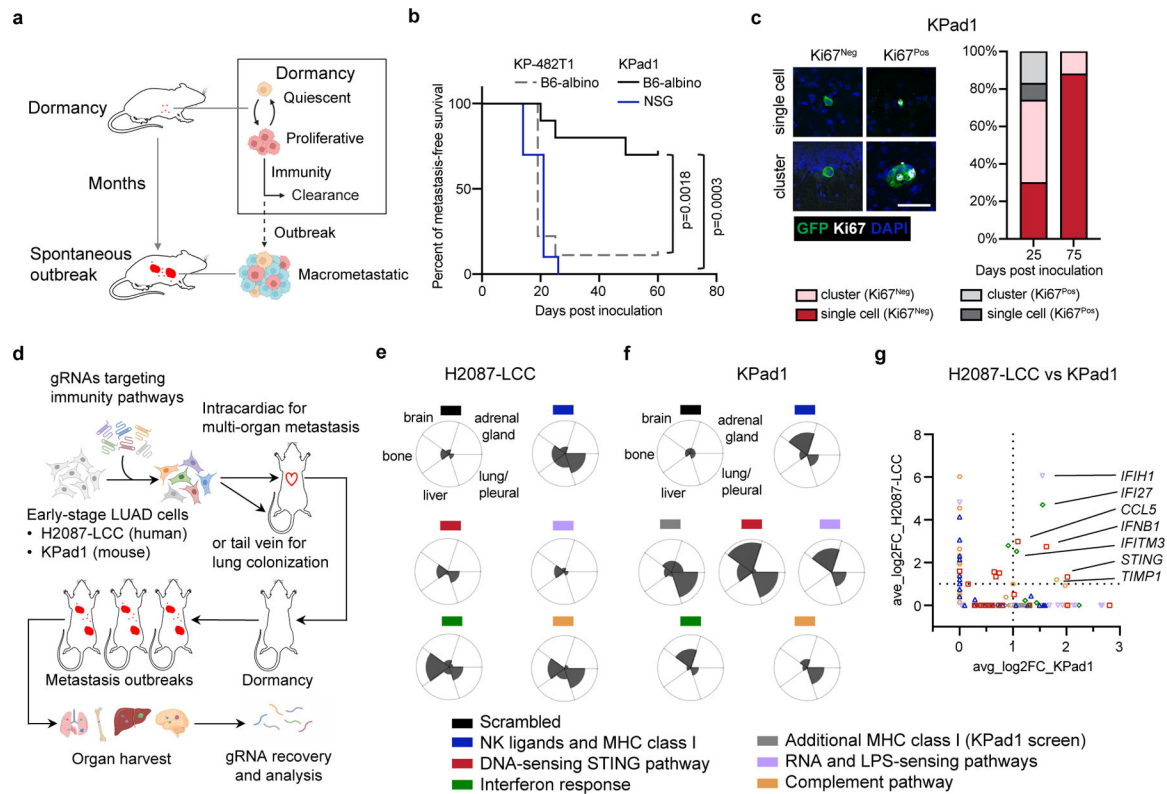
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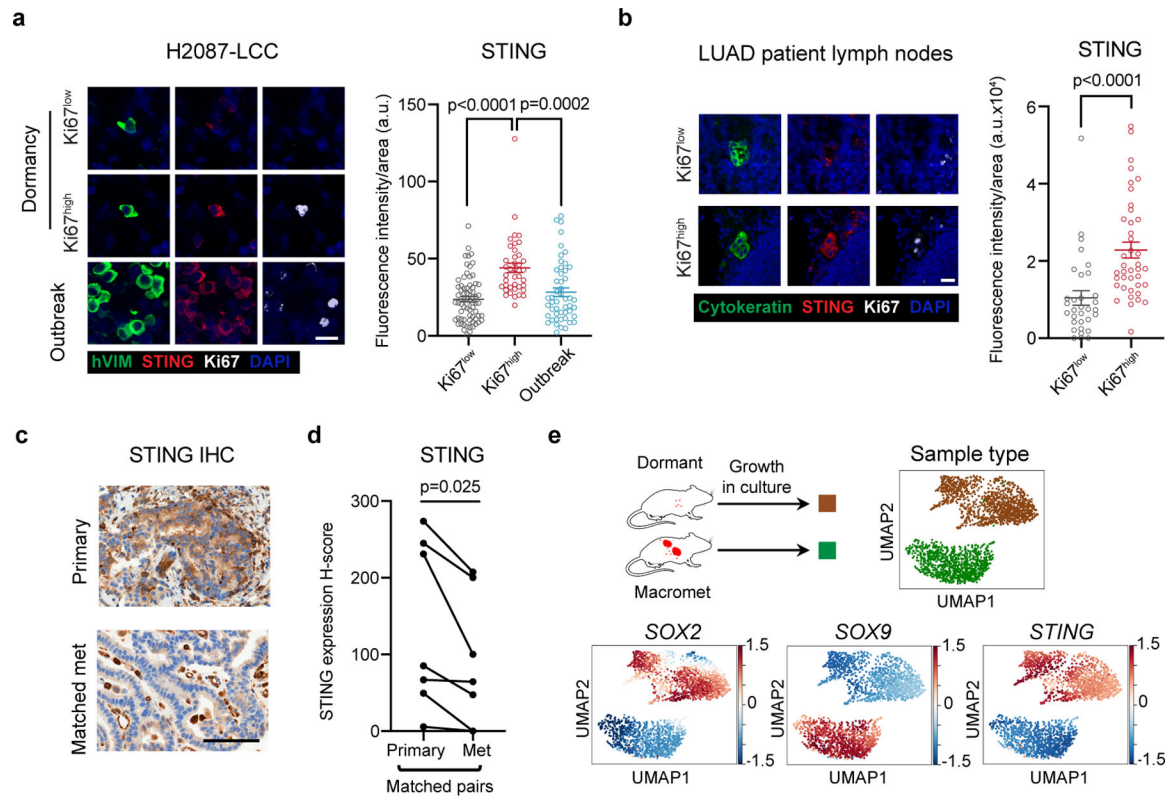
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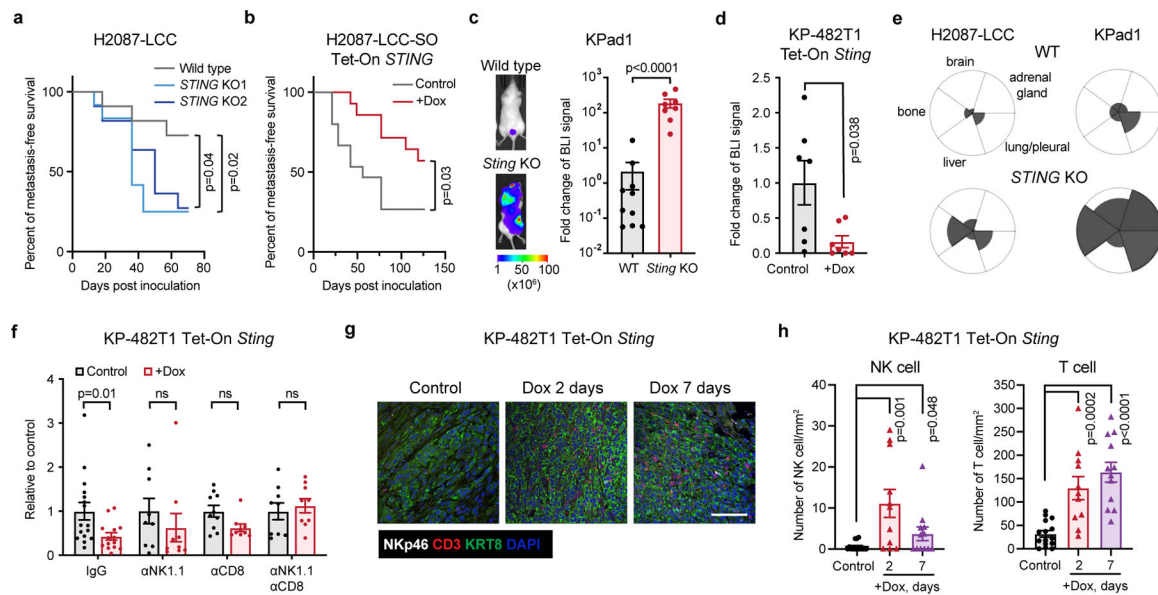
**Figure 1. Genetic screens identify cell-autonomous immune regulators of dormant metastasis**

**(a)** Schematic of experimental models of metastatic dormancy in which disseminated cancer cells fluctuate between an immune evasive quiescent state and a proliferative state eliminated by the immune system. **(b)** Metastasis-free survival Kaplan-Meier plots of immunocompetent B6-albino or immunodeficient NSG mice intracardially inoculated with  $2 \times 10^4$  KPAd1 or KP-482T1 cells.  $n=9$  (KP-482T1) or 10 (KPAd1) mice per group, log-rank test. **(c)** Representative immunofluorescence (IF) images (left) and quantification (right) of the proportion of KPAd1 single cells and clusters in the brains of B6-albino mice 25 or 75 days after intracardiac inoculation of  $2 \times 10^4$  cells. Scale bar, 50  $\mu$ m.  $n=23$  (25 days) or 17 (75 days) single cells or clusters. **(d)** Schematic of CRISPR screen design. **(e-f)** Petal plots of metastasis incidence in specific organs after intracardiac inoculation of  $2.5 \times 10^5$  H2087-LCC cells in athymic mice (e) or KPAd1 cells in B6-albino mice (f). Cells were transduced with the indicated sgRNA pools. Metastasis progression was monitored by bioluminescence imaging (BLI) for 106 (e) or 62 days (f) after inoculation. The radius of each black petal represents the proportion of mice developing metastases in the indicated organ from 8–12 mice per pool (e) or 7–9 mice per pool (f). **(g)** Enrichment of sgRNAs in multi-organ metastases from H2087-LCC and KPAd1 cells after intracardiac inoculation into athymic mice or B6-albino mice, respectively. Genes with average  $\log_2$  fold change ( $\log_2FC$ )  $>0$  are plotted. Genes targeted by the top enriched sgRNAs in both screens are listed. Due to non-homology of genes encoding NK activating ligands and MHC class I in human and mouse, these genes scored in the H2087-LCC screen or the KPAd1 screen but not both. See also Extended Data Figures 1–2.



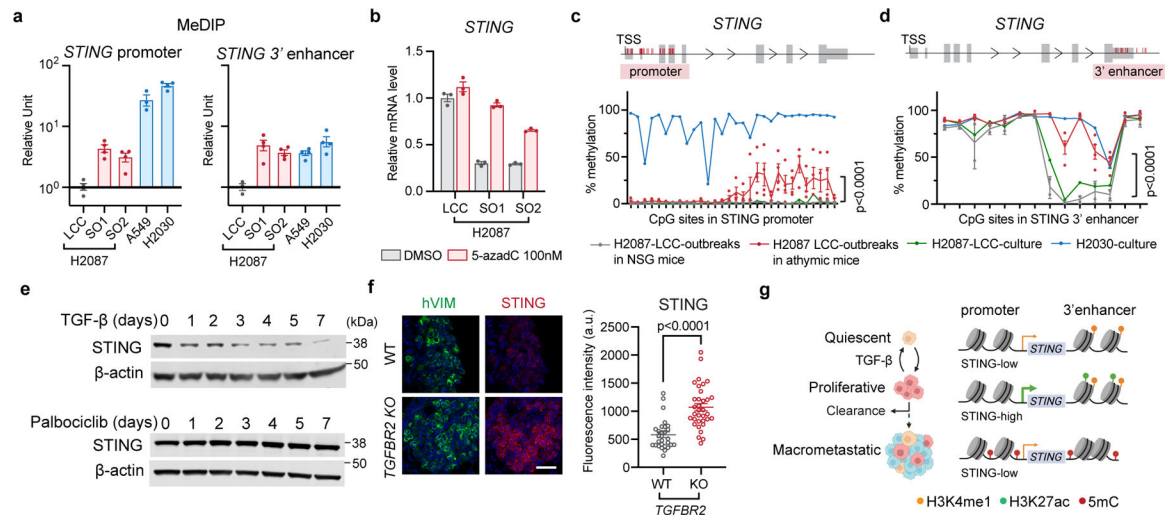
**Figure 2. STING activation in metastatic progenitors reentering the cell cycle**

(a) Representative IF staining images of H2087-LCC cells (human vimentin, *green*) in lungs of athymic mice inoculated with  $1 \times 10^5$  cells. Organs were harvested 4 weeks after inoculation to capture the dormant state, and 10–13 weeks after inoculation to capture spontaneous outbreaks (SO). Tissue sections were stained for STING IF (*red*), Ki67 IF (*white*), and DAPI (*blue*). Disseminated cancer cells were present as single quiescent ( $Ki67^{low}$ ) and proliferative cells ( $Ki67^{high}$ ) during dormancy. The signal intensity of STING in vimentin-positive cells was quantified and plotted (a.u., arbitrary units). Scale bar: 10  $\mu$ m.  $n=70$  cells ( $Ki67^{low}$ ), 40 cells ( $Ki67^{high}$ ), or 50 regions (outbreaks), from 5 mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. (b) Representative images of micrometastatic cells (cytokeratin AE1/AE3, *green*) in lymph nodes from stage II and stage III LUAD patients, stained for STING IF (*red*), Ki67 (*white*), and DAPI (*blue*). The signal intensity of STING in cytokeratin-positive cells was quantified and plotted. Scale bar: 20  $\mu$ m.  $n=32$  ( $Ki67^{low}$ ) or 40 ( $Ki67^{high}$ ) regions from 14 lymph nodes of 9 patients. Mean  $\pm$  s.e.m., two-sided unpaired t-test. (c-d) Immunohistochemistry (IHC) staining (c) and quantification (d) of STING expression (H-score) in patient-derived LUAD metastases compared to matched primary tumors. Scale bar: 50  $\mu$ m.  $n=7$  matched pairs, two-tailed paired t-test. (e) UMAP plots showing imputed and z-normalized expression of *STING*, *SOX2*, and *SOX9* in H2087-LCC cells isolated from dormant metastases or spontaneous macrometastases in athymic mice and placed in growth-promoting culture conditions.  $n=2245$  cells isolated from 3 mice. See also Extended Data Figure 3–4.



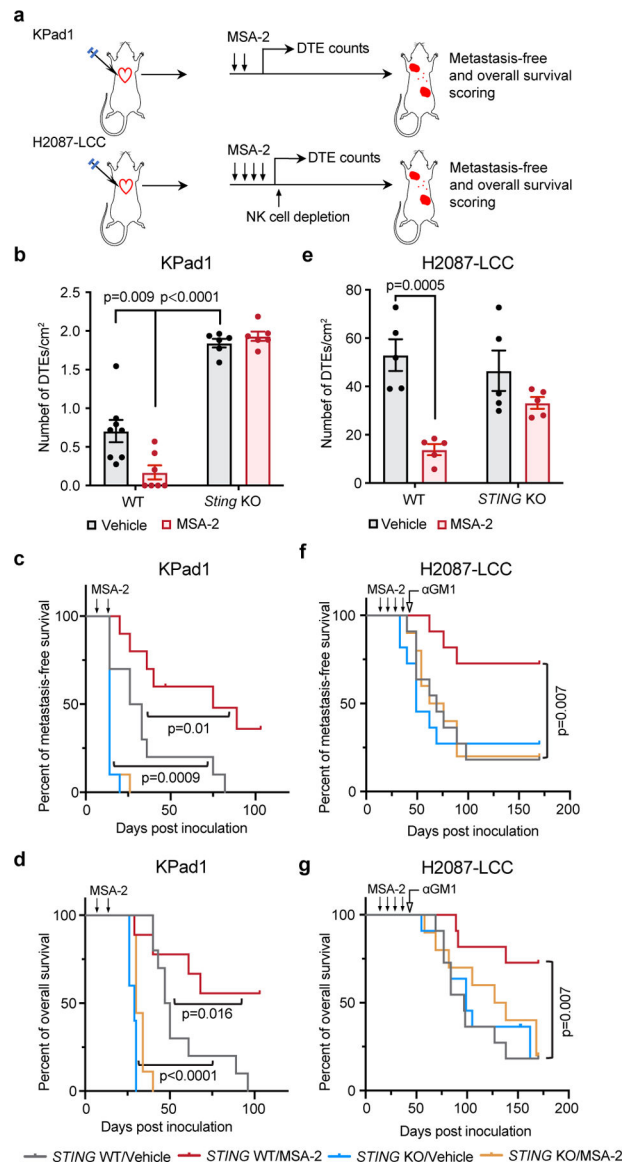
**Figure 3. Metastasis suppression by tumor-intrinsic STING**

(a) Metastasis-free survival plots of athymic mice intracardially inoculated with  $1 \times 10^5$  wild-type (WT) or *STING* knockout H2087-LCC cells.  $n=11$  (WT, KO2) or 12 (KO1) mice per group, log-rank test. (b) Metastasis-free survival of athymic mice intracardially inoculated with  $1 \times 10^5$  H2087-LCC cells derived from spontaneous outbreaks and inducibly overexpressing *STING* by doxycycline (Dox).  $n=15$  (Control) or 14 (Dox) mice, log-rank test. (c) Metastases 26 days after intracardiac inoculation of  $2.5 \times 10^5$  WT or *Sting* knockout KPad1 in B6-albino mice. Mean  $\pm$  s.e.m.  $n=10$  (WT) or 8 (KO) mice. Two-sided Mann-Whitney U-test. (d) Metastases 21 days after inoculation of  $5 \times 10^4$  KP-482T1 cells with Dox-inducible *STING* expression in C57BL/6J mice.  $n=7$  mice per group. Mean  $\pm$  s.e.m., two-sided Mann-Whitney U-test. (e) Metastasis incidence in specific organs after intracardiac inoculation of  $1 \times 10^5$  WT or *STING* knockout H2087-LCC cells into 11 athymic mice (left), or  $2.5 \times 10^5$  KPad1 cells into 8–10 B6-albino mice (right). Metastasis monitored for 12 (H2087-LCC) or 7 (KPad1) weeks after inoculation. (f) Metastases by KP-482T1 cells with Dox-inducible expression of *STING* after inoculation of  $2 \times 10^4$  cells into B6-albino mice. Mice were treated with the indicated antibodies. BLI results are plotted relative to values without Dox per treatment group.  $n=16$  (control/IgG), 14 (Dox/IgG), 10 (control/ $\alpha$ CD8) or 9 (other groups) mice per group. Mean  $\pm$  s.e.m., two-sided Mann-Whitney U-test. (g-h) Representative IF images (g) and quantification (h) of NKp46+ NK cells (white) or CD3+ T cells (red) in femoral metastases in C57BL/6J mice. Mice were intracardially inoculated with  $1 \times 10^5$  KP-482T1-Tet-On *Sting* cells and placed on Dox diet one week later for 2 days or 7 days. Femurs were harvested 14 days after cell inoculation. Scale bar: 100  $\mu$ m.  $n=15$  (control), 11 (2 days), 12 (7 days) lesions, from 4–8 mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. See also Extended Data Figure 5–8.



**Figure 4. STING downregulation during dormancy and metastatic progression**

**(a)** PCR based quantitation of *STING* promoter (left) or 3' enhancer (right) reads in methylated DNA immunoprecipitation (MeDIP) samples from parental H2087-LCC, two spontaneous metastasis outbreaks (SO1 and SO2), and the aggressive cell lines A549 and H2030. Mean  $\pm$  s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. **(b)** qRT-PCR analysis of *STING* mRNA levels after 3-day treatment of the indicated cell lines with 100nM 5-aza-2'deoxyctidine (5-azadC). Mean  $\pm$  s.e.m., representative of 2 independent experiments. Each dot represents a technical replicate of the assay. **(c-d)** Schematic of the *STING* locus and the locations of 30 CpG sites analyzed in the *STING* promoter (c) and 14 sites in the *STING* 3' enhancer (d), and percentage of methylation on each CpG site as determined by bisulfite sequencing of H2087-LCC cells freshly sorted from 4 metastatic outbreaks in 2 athymic mice or 2 outbreaks in 2 NSG mice, and H2087-LCC and H2030 cells in culture. Organs were harvested 5–7 weeks after intracardiac inoculation of  $1 \times 10^5$  H2087-LCC cells.  $n=4$  (athymic) or 2 (NSG) outbreaks. Mean  $\pm$  s.e.m., two-way ANOVA analysis. **(e)** Western immunoblot analysis of STING in H2087-LCC cells treated with TGF- $\beta$  or CDK4/6 inhibitor palbociclib for the indicated time periods, representative of 2 independent experiments. For gel source data, see Supplemental Figure 1. **(f)** IF staining of human vimentin (green), DAPI (blue), and STING (red), in lung metastases generated 7 weeks after intravenous inoculation of  $1 \times 10^5$  wild-type (WT) or *TGFBR2* knockout H2087-LCC cells in NSG mice. The IF staining intensity in vimentin-positive areas was quantified. Scale bar: 50  $\mu$ m.  $n=28$  (WT) or 34 (KO) lesions from 6 mice. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(g)** Schematic summary of epigenetic mechanisms regulating *STING* expression during dormancy and metastasis progression. See also Extended Data Figure 9–10.



**Figure 5. STING agonist depletion of disseminated cancer cells to prevent metastasis**  
**(a)** Schematic of experimental design. DTE, disseminated tumor entities. **(b)** B6-albino mice were intracardially inoculated with  $1 \times 10^5$  wild-type (WT) or *Sting* knockout KPad1 cells and treated with vehicle or MSA-2 (50 mg/kg of body weight) once weekly at weeks 2 and 3. Brains were harvested 5 weeks after inoculation and the number of DTEs were counted. DTEs included single cells and small cell clusters, except in the case of *Sting* knockout KPad1 cells, which included mostly micro and macrometastases.  $n=8$  (WT/Vehicle), 7 (WT/MSA-2), or 6 (KO/Vehicle, KO/MSA-2) mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(c-d)** Kaplan-Meier plots of metastasis-free survival (c) or overall survival (d) of B6-albino mice that were intracardially inoculated with  $2.5 \times 10^5$  WT or *Sting* knockout KPad1 cells, treated as in (b) and tracked for metastasis formation and for survival.  $n=10$  mice per group. Log-rank test. **(e)** Athymic mice were inoculated with  $1 \times 10^5$  WT or *STING* knockout H2087-LCC cells via the tail vein and treated with vehicle or MSA-2 (50 mg/kg)



once weekly for 4 weeks starting two weeks after inoculation. Lungs were harvested 6 weeks after inoculation and the number of DTEs were counted. DTEs included single cells and small cell clusters.  $n=5$  mice per group. Mean  $\pm$  s.e.m., two-sided unpaired t-test. **(f-g)** Kaplan-Meier plots of metastasis-free survival (f) and overall survival (g) of athymic mice intracardially inoculated with  $1 \times 10^5$  WT or *STING* knockout H2087-LCC cell and treated as in (e). Anti-asialo-GM1 treatment started 6 weeks after cell inoculation.  $n=11$  (WT/Vehicle, WT/MSA-2, KO/Vehicle), or 10 (KO/MSA-2) mice per group. Log-rank test. See also Extended Data Figure 11.

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