Metastasis is the leading cause of cancer-related death. The interactions between circulating tumor cells and endothelial adhesion molecules in distant organs is a key step during extravasation in hematogenous metastasis. Surgery is a common intervention for most primary solid tumors. However, surgical trauma-related systemic inflammation facilitates distant tumor metastasis by increasing the spread and adhesion of tumor cells to vascular endothelial cells (ECs). Currently, there are no effective interventions to prevent distant metastasis. Here, we show that HECTD3 deficiency in ECs significantly reduces tumor metastasis in multiple mouse models. HECTD3 depletion downregulates expression of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, in mouse primary ECs and HUVECs stimulated by inflammatory factors and inhibits adhesion of tumor cells to ECs both in vitro and in vivo. We demonstrate that HECTD3 promotes stabilization, nuclear localization and kinase activity of IKKα by ubiquitinating IKKα with K27- and K63-linked polyubiquitin chains at K296, increasing phosphorylation of histone H3 to promote NF-κB target gene transcription. Knockout of HECTD3 in endothelium significantly inhibits tumor cells lung colonization, while conditional knockin promotes that. IKKα kinase inhibitors prevented LPS-induced pulmonary metastasis. These findings reveal the promotional role of the HECTD3-IKKα axis in tumor hematogenous metastasis and provide a potential strategy for tumor metastasis prevention.

INTRODUCTION
Metastasis accounts for 90% of deaths in cancer patients. Cancer patients without clinical symptoms after initial treatment frequently develop distant metastasis years later. Surgery is a common early intervention for most solid tumors. However, mechanical trauma and the subsequent wound healing process constitute favorable factors for metastasis through several mechanisms, including release of circulating tumor cells (CTCs) and triggering systemic inflammation. To avoid anoikis during metastasis, CTCs must attach to the vasculature of distant organs and extravasate into the perivascular tissue.

Accumulating data indicate that systemic inflammation potentiates the adhesion of CTCs to vascular endothelial cells (ECs) of distant organs. This is a key step of extravasation in hematogenous metastasis. CTC extravasation typically occurs in small capillaries, where cancer cells are arrested by the endothelium via interaction with a wide range of adhesion molecules of ECs, including E-selectin, ICAM-1 (intercellular-adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), through their cognate ligands. E-selectin is expressed exclusively by ECs in rapid response to inflammatory stimuli (e.g., TNF-α and IL-1β). E-selectin recognizes various glycoprotein ligands expressed on cancer cells, including a specific sialofucosylated glycoform of CD44, PSGL1, CD24, MUC1 and LGALS3BP. Cancer cell interaction with E-selectin seems to be the initial step for CTC extravasation and is essential for metastasis. It has been reported that bone vascular E-selectin directly captures breast cancer cells to promote bone metastasis. Consistently, atrial natriuretic peptide (ANP) prevents cancer metastasis by suppressing E-selectin expression by ECs. Subsequently, ICAM-1 and VCAM-1 on ECs allow adhesion of cancer cells to ECs. ICAM-1 forms Y-shaped covalent homodimers at the cell surface, which forceably bind to the abnormal glycoform of MUC1 associated with cancer cells. VCAM-1 is expressed on the luminal and lateral side of ECs in response to inflammatory factors. VCAM-1 also increases the adhesion of various subsets of leukocytes and tumor cells via interaction with a wide range of adhesion molecules of ECs.
Targeting HECTD3-IKKα axis inhibits inflammation-related metastasis

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recognition of integrins, such as VLA-4 (late activation antigen-4) or integrin α4. Pretreatment of inflammatory factors, such as TNFα, IL-1β and SDF-1, or exposure to surgical stress or sepsis increased VCAM-1 expression on pulmonary ECs of mice, leading to increased numbers of lung metastatic nodules after intravenous injection of tumor cells. E-selectin, ICAM-1 and VCAM-1 are NF-κB target genes in ECs. When endothelial cells receive inflammatory stimuli, such as TNFα or lipopolysaccharide (LPS), the IKK kinase complex, containing IKKα, IKKβ and NEMO (IKKγ), phosphorylates IκBα and targets IκBα for proteasomal degradation. Free from IκBα binding, the NF-κB dimer p50/65 accumulates in the nucleus and binds to specific promoters to activate transcription of downstream target genes. Although IKKα and IKKβ have similar structures, they exhibit differential regulatory patterns. IKKα is dispensable for IκBα degradation, but IKKα promotes processing of the p100 precursor into p52 in the noncanonical NF-κB pathway. Additionally, IKKα harbors a specific nuclear localization signaling and can directly regulate NF-κB-dependent gene transcription in the nucleus. Nuclear IKKα is recruited to NF-κB binding chromatin and phosphorylates histone H3 at Ser10 to activate NF-κB target gene transcription.

HECTD3 is a HECT-type E3 ubiquitin ligase with multiple substrates and functions. HECTD3 confers chemotherapy drug resistance by ubiquitinating MALT1. Caspase-8 and Caspase-9. HECTD3 promotes pathogenic Th17 cell generation by ubiquitinating MALT1 and STAT3 in an experimental autoimmune encephalomyelitis (EAE) mouse model. Our recent study suggested that Hectd3 promotes type I interferon production.

In most situations, HECTD3 ubiquitinates IKKα and IKKβ and NEMO (IKKγ), phosphorylates IκBα and targets IκBα for proteasomal degradation. Free from IκBα binding, the NF-κB dimer p50/65 accumulates in the nucleus and binds to specific promoters to activate transcription of downstream target genes. Although IKKα and IKKβ have similar structures, they exhibit differential regulatory patterns. IKKα is dispensable for IκBα degradation, but IKKα promotes processing of the p100 precursor into p52 in the noncanonical NF-κB pathway. Additionally, IKKα harbors a specific nuclear localization signaling and can directly regulate NF-κB-dependent gene transcription in the nucleus. Nuclear IKKα is recruited to NF-κB binding chromatin and phosphorylates histone H3 at Ser10 to activate NF-κB target gene transcription.

In this study, we utilized multiple mouse models to examine the function of HECTD3 in tumor metastasis and observed that HECTD3 promotes adhesion of tumor cells to the vascular endothelium by upregulating expression of adhesion molecules on ECs in response to inflammatory conditions, which promotes tumor hematogenous metastasis. The mechanism involves a process that HECTD3 ubiquitinates IKKα to promote its stability and nuclear kinase activity toward histone H3, eventually potentiating NF-κB-mediated gene transcription. We demonstrated that inhibition of the HECTD3-IKKα axis effectively inhibited tumor metastasis induced by systemic inflammation.

RESULTS

HECTD3 deficiency inhibits inflammation-induced tumor metastasis in mice

To determine the role of Hectd3 in tumor metastasis, we analyzed metastasis susceptibility in Hectd3-deficient mice utilizing two malignant mouse breast cancer metastasis models after surgery. We generated PyMT-induced mouse breast tumor cells by intraductal injection of lentivirus overexpressing PyMT into the mammary duct of wild type (WT) female FVB mice. Then, we transplanted PyMT-induced mouse breast tumor cells into WT and Hectd3−/− mice and examined the lungs 2 months after resection of orthotopic tumors. Compared to WT mice, Hectd3 deficiency significantly inhibited lung metastasis (Fig. 1a) and heart metastasis (Fig. 1b). 4T1-Luc2 can spontaneously metastasize to multiple organs in BALB/c mice from the breast. We orthotopically inoculated 4T1-Luc2 cells into the fourth mammary fat pad of WT and Hectd3−/− female BALB/c mice. Eleven days later, mice were imaged using a bioluminescent IVIS system, confirming that tumor size was consistent across animals between these two groups (Fig. 1c). On day 12, tumors were surgically removed. Actually, the volume of 4T1-Luc2 primary tumor was larger in Hectd3−/− mice than in WT mice (Data not shown). Tumor metastasis was monitored weekly by imaging. We found that 4T1-Luc2 tumor metastasis was markedly suppressed in Hectd3−/− mice (19%, 5/27), compared with that in WT mice (54%, 13/24) (Fig. 1c, d). In addition, Hectd3 deficiency significantly prolonged mouse survival (Fig. 1e), demonstrating that Hectd3 deficiency in the tumor microenvironment inhibits surgery-associated tumor metastasis.

As mentioned earlier, surgery may promote metastasis by increasing tumor cell dissemination and inducing systemic inflammation. In some tumor surgeries, >40% of patients develop peritonitis, pneumonia, sepsis, or severe postoperative infection, all of which can lead to recurrence and metastasis. Firstly, we detected several inflammation factors, such as LPS, TNFα, IL-1β and IL-6, in the sera of WT and Hectd3−/− mice burdening primary tumor (0 h) or surgically removed primary tumor 6 h or 12 h later. Limulus amebocyte lysate assay and ELISA results showed that the serum LPS activity/level increased significantly in both WT and Hectd3−/− mice 12 h after surgery, but there was no difference between WT and Hectd3−/− mice (Supplementary Fig. 1a, b). ELISA results showed that the serum TNFα level increased 6 h and 12 h after surgery (Supplementary Fig. 1c), but the serum IL-1β and IL-6 levels had no significant change (Supplementary Fig. 1d, e). These results indicate that surgery induces inflammation by increasing LPS and TNFα levels in both WT and Hectd3−/− mice.

To further investigate whether Hectd3 deficiency inhibits the metastasis of cancer cells to inflamed organs, we intravenously injected WT and Hectd3−/− female FVB mice with LPS, which mimics systemic inflammation in response to surgical stress, followed by tail-vein injection of PyMT-induced mouse breast tumor cells. Hectd3 knockout (KO) had no effect on the lung colonization of tumor cell in the absence of LPS pretreatment. However, LPS increased the number of colonization nodules and the weight of the lung in both WT and Hectd3−/− mice. However, the increase of lung colonization of tumor cell induced by LPS was significantly compromised in Hectd3−/− mice (Fig. 1f–h). Consistently, Hectd3 KO significantly prolonged mouse survival (Fig. 1i). Similar results were observed when we used 4T1-Luc2 breast tumor cells and B16-F10 melanoma cells (Supplementary Fig. 1f–k). When we replaced inflammation factor LPS with TNFα, Hectd3 KO also significantly inhibited lung colonization of 4T1-Luc2 breast tumor cells (Supplementary Fig. 1l–n). Taken together, we conclude that Hectd3 deficiency suppresses the inflammation-induced lung colonization of tumor cell.

HECTD3 promotes adhesion of tumor cells to human umbilical vein endothelial cells (HUVECs) by upregulating E-selectin, ICAM-1 and VCAM-1 expression

Adhesion of cancer cells to ECs is a key step for metastasis. Systemic inflammation provoked by surgical trauma or LPS/TNFα stimulation increases the adhesion of CTCs to the vascular endothelium of distant organs by upregulating adhesion molecules in the endothelium. To determine the mechanism by which HECTD3 regulates inflammation-related metastasis, we isolated and validated primary HUVECs from the neonatal umbilical cord vein and performed genome-wide expression analysis to profile differentially expressed genes in control and HECTD3-KD HUVECs treated with or without TNFα for 2 h. Compared with control HUVECs, 59 genes showed lower expression in HECTD3-KD HUVECs in response to TNFα. Most of them are NF-κB target genes, including adhesion molecules, such as SELECT (E-selectin), ICAM-1, and VCAM-1, and inflammation factors, such as IL-6 and CXCL8 (Fig. 2a). To confirm the results of the RNA-seq, we knocked down HECTD3 in HUVECs with a siRNA pool, both protein and mRNA expression levels of adhesion molecules, including E-selectin, ICAM-1 and VCAM-1, were significantly downregulated in
response to LPS (Fig. 2b, c). Similar results were observed for TNFα stimulation (Supplementary Fig. 2a, b). As a positive control, knockdown of p65/RelA abolished the induction of adhesion molecules in response to these inflammatory factors (Fig. 2b, c and Supplementary Fig. 2a, b). Knockdown of HECTD3 with two different siRNAs showed similar results (Supplementary Fig. 2c, d). Knockdown of HECTD3 in HUVEC also significantly decreased LPS- and TNFα-induced transcription of inflammation factors, such as IL-6 and CXCL8 (Data not shown). These findings suggest that HECTD3 contributes to NF-κB signaling pathway.

Next, we assessed whether HECTD3 promotes the adhesion of tumor cells to HUVECs. We treated monolayer-cultured HUVECs with...
Fig. 1  Hectd3 knockout inhibits inflammation-induced tumor metastasis in mice. a A comparison of the incidence of lung metastases in WT (n = 9) versus Hectd3−/− (n = 11) mice with an FVB genetic background. PyMT-induced tumor cells were orthotopically injected into the fat pad of both groups of mice (5 × 10^6 cells per mouse). Primary tumors were removed 20 days later. Mice were sacrificed after 2 months, and the incidence of lung metastasis was recorded. b PyMT-induced breast tumor cells were inoculated as described above. The incidence of metastasis (left), representative heart metastasis nodule images and H&E staining (right) are shown. c 4T1-Luc2 cells were injected orthotopically into the fourth pair of fat pads of WT (n = 24) and Hectd3−/− (n = 27) BALB/c mice (bilateral, 1 × 10^6 cells per point). Eleven days after transplantation (Day 0), primary tumors were removed, then tumor metastasis was monitored weekly by imaging and representative bioluminescence images are shown (lower). d A comparison of the incidence of metastases in WT versus Hectd3−/− mice from panel c. e Kaplan–Meier survival curves of WT (n = 24) and Hectd3−/− (n = 27) mice which 4T1-Luc2 primary tumors were removed 12 days after transplantation. f WT and Hectd3−/− FVB mice were intravenously injected with or without LPS (1 mg/kg). 5 h later, PyMT-induced breast tumor cells were injected through the tail vein (2 × 10^6 cells per mouse). Each group contained 9–10 mice, and mice were sacrificed 20 days after injection of tumor cells. The graph shows the number of pulmonary tumor metastases in each group of mice. g The weight of the whole lung with metastatic nodules of WT and Hectd3−/− group of mice from panel f. h Representative lung metastasis nodule images and corresponding H&E staining of the lungs in different groups of mice from panel f. i Kaplan–Meier survival curves of WT (n = 6) mice and Hectd3−/− (n = 6) mice pretreated with LPS and transplanted with PyMT-induced breast tumor cells through tail vein. Data represent three independent experiments for all of the above experiments. Data are presented as the mean ± SEM, and statistics were calculated using the Chi-square test for b, d and f, two-way ANOVA for a, b, d, i, and log-rank test for c and e. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Scale bars are 500 μm for b and 2 mm for h.

LPS to induce adhesion molecule expression, added suspended GFP-labeled tumor cells to allow attachment, washed away the unattached tumor cells, and quantified tumor cells adhered to monolayer-cultured HUVECs (Fig. 2d). As expected, LPS and TNFα increased attachment of GFP-labeled breast cancer cells (HCC1937-GFP, MDA-MB-231-GFP and MDA-MB-468-GFP) and leukemia cells (Jurkat-GFP) to monolayer-cultured HUVECs (Fig. 2e, f and Supplementary Fig. 2e, f). Likewise, knockdown of HECTD3 or p65 significantly inhibited attachment of cancer cells to monolayer-cultured HUVECs (Fig. 2e, f and Supplementary Fig. 2e, f).

Subsequently, we examined whether HECTD3 functions through its E3 ligase activity. We knocked down endogenous HECTD3 and then transfected siRNA-resistant HECTD3 WT or HECTD3 C823A (a catalytically inactive mutant) constructs, which contained noncoding changes resistant to knockdown via RNAi, in HUVECs using the pcDH lentivirus overexpression system. Immunoblotting results showed that overexpression of WT HECTD3 in HUVECs significantly increased both protein and mRNA levels of E-selectin, ICAM-1 and VCAM-1 induced by LPS (Fig. 2g, h) and TNFα (Supplementary Fig. 2g, h). Interestingly, overexpressing HECTD3, but not C823A, rescued the downregulation of adhesion molecules induced by knockdown of endogenous HECTD3 with siRNA (Fig. 2g, h and Supplementary Fig. 2g, h). These results suggest that the E3 ligase activity of HECTD3 is essential for induction of adhesion molecule expression by inflammatory factors. Consistently, overexpression of WT HECTD3 but not HECTD3 C823A mutant in HUVECs significantly increased the adhesion of cancer cells to LPS-treated HUVECs (Supplementary Fig. 2i, j). Finally, we inhibited expression of E-selectin, ICAM-1 and VCAM-1 using a siRNA mixture of siE-selectin, siCAM-1 and siVCAM-1, which blocked upregulation of expression of these adhesion molecules (Supplementary Fig. 2k), as well as the increase in tumor cell adhesion (Fig. 2i, j) induced by HECTD3 overexpression in HUVECs. These results suggest that HECTD3 promotes the adhesion of tumor cells to HUVECs by upregulating the expression of E-selectin, ICAM-1 and VCAM-1 in HUVECs, which based on its E3 ligase activity.

HECTD3 inhibits inflammation-induced metastasis by regulating the E-selectin, ICAM-1 and VCAM-1 gene transcriptional upregulation in the endothelium in response to inflammatory stimuli. Although LPS and TNFα activate the NF-kB pathway through different receptors and adapters, the extracellular signals converge on recruitment and activation of the IKK complex, which contains IKKa, IKKB and IKKγ (NEMO). We suspected that HECTD3 regulates a key component of the NF-kB pathway. We first knocked down HECTD3 in HUVECs, and then stimulated cells with LPS and TNFα, and examined the expression of the IKK complex and the activation of the NF-kB signaling pathway. We found that protein levels of total IKKα, but not IKKB or NEMO, decreased significantly (Fig. 3a and Supplementary Fig. 3a). Interestingly, the changes in p-IKKα/β, p-kBα, and total IkBa were only slightly inhibited by HECTD3 knockdown. Thus, we subsequently focused on IKKα.

We firstly investigated whether IKKα is essential for inducing the expression of adhesion molecules by inflammatory factors. When IKKα was knocked down, the mRNA and protein expression levels of E-selectin, ICAM-1 and VCAM-1 induced by the inflammatory factors LPS (Fig. 3b, c) and TNFα (Supplementary Fig. 3b, c) in HUVECs were significantly decreased. IKKα overexpression largely rescued the reduction of E-selectin and ICAM-1, and partially rescued the reduction of VCAM-1 caused by HECTD3 KD (Fig. 3d). IKKα overexpression promoted the attachment of cancer cells to monolayer-cultured HUVECs and largely rescued the reduction of adhesion phenotype caused by HECTD3 KD (Fig. 3e, f). Furthermore, knockdown of IKKα blocked the increase of adhesion molecule expression induced by HECTD3 overexpression (Fig. 3g) and TNFα (Supplementary Fig. 3d). Expectedly, IKKβ depletion almost completely abolished these increases (Fig. 3g). These results showed that HECTD3 regulates the adhesion molecules expression and adhesion phenotype through IKKα.

Since HECTD3 knockdown decreased E-selectin protein levels but did not affect IkBα phosphorylation or degradation in response to inflammation (Fig. 3a and Supplementary Fig. 3a), we hypothesized that HECTD3 promoted adhesion molecule gene transcription in an IKK complex-independent manner. Actually, IKKa translocates into the nucleus and phosphorylates histone H3 at Ser10 and histone H3.3 at Ser31 to facilitate NF-kB-dependent transcription and it is crucial for p65 binding to the ICAM-1 promoter. Indeed, we demonstrated that HECTD3 knockdown significantly decreased IKKa-mediated phosphorylation of histone H3 at Ser10 (H3S10ph) and histone H3.3 at Ser31(H3.3S31ph), while HECTD3 overexpression increased H3S10ph and H3.3S31ph (Fig. 3h and Supplementary Fig. 3e, f). Consistently, we demonstrated that HECTD3 knockdown obviously decreased nuclear localization of IKKa in HUVECs under LPS stimulation by immunoblotting (Supplementary Fig. 3e, f) and immunofluorescence staining (Supplementary Fig. 3h). To determine whether IKKa was recruited to E-selectin, ICAM-1 and VCAM-1 gene promoters to increase transcription through epigenetic modifications, the chromatin immunoprecipitation (ChIP) assays were performed using the IKKa antibody. Nuclear IKKa does not bind to DNA sequence directly, but it can interact with p65 through CBP and is recruited to NF-kB binding chromatin to activate NF-kB.
target gene transcription. We designed the ChIP-PCR primers for E-selectin, ICAM-1 and VCAM-1 promoters containing a p65 binding site. As anticipated, recruitment of IKKα to these loci was obviously increased in response to LPS, while HECTD3 knockdown significantly inhibited this process (Fig. 3i, j). These results indicate that HECTD3 promotes IKKα stabilization, nuclear localization, and specific recruitment in HUVECs in response to inflammatory stimuli.

To characterize the mechanism by which HECTD3 stabilizes IKKα, we first examined IKKα mRNA levels after HECTD3
Next, we demonstrated that Flag-HECTD3 immunoprecipitated and performed GST pulldown assays with glutathione sepharose fused HECTD3 truncated mutants with Flag-IKK α. Fig. 3m, both lysosome inhibitors, but not proteasome inhibitor, to test whether IKK α was well-known that IKK α is an important member of the NF-κB pathway.23 We wondered whether HECTD3 also involved in the exception of IKK α. Therefore, we constructed a series of IKK α truncated mutants fused with GST and transfected them with Flag-HECTD3 in HEK293T cells, performing a GST pulldown experiment. We demonstrated that IKK α interacts with HECTD3 through its SDD domain (amino acids 408-665) (Fig. 4f and Supplementary Fig. 4b).

HECTD3 increases IKKα protein stability, nuclear localization and kinase activity by promoting the K27- and K63-linked polyubiquitinations of IKKα at K296. Next, we investigated whether HECTD3 ubiquitinates IKKα. As expected, HECTD3, but not the HECTD3 C823A inactive mutant, significantly increased polyubiquitination of IKKα in HEK293T cells (Fig. 5a). Additionally, we showed that knockdown of HECTD3 decreased endogenous IKKα polyubiquitination in HUVECs (Fig. 5b). Moreover, we performed an in vitro ubiquitination assay using purified components, including E1, E2 (Ubch5b), E3 (HECTD3 or HECTD3 C823A) (Fig. 5c), Flag-IKKα, HA-Ub, and ATP. As shown in Fig. 5d, HECTD3 dramatically increased Flag-IKKα polyubiquitination in an E3 ligase activity-dependent manner. The IKKα protein contains 51 lysine (K) residues. To identify the lysine residues responsible for HECTD3-mediated polyubiquitination, we artificially divided IKKα into 9 regions and constructed a series of mutants termed IKKα R1-9 in which we replaced all lysine residues with arginine (R) residues. We found that HECTD3 induced polyubiquitination of IKKα (WT) and its mutants, with the exception of IKKα R4 (Supplementary Fig. 5a). These results implied that region 4 of IKKα, which includes K296, K311, and K322, contains potential ubiquitination sites for HECTD3. Following these experiments, we further investigated the linkage of IKKα polyubiquitination mediated by HECTD3. Using a series of Ub mutants (K only), we found that K27- and K63—only Ub supported HECTD3-catalyzed IKKα polyubiquitination, similar to WT Ub (Fig. 5f) and Supplementary Fig. 5b). We further confirmed this result using linkage-specific anti-Ub antibodies. HECTD3-mediated IKKα polyubiquitination was recognized by specific antibodies against K27-polyUb and K63-polyUb but not K48-polyUb (Fig. 5g). Consistently, knockdown of endogenous HECTD3 specifically decreased both K27-linked and K63-linked, but not K48-linked, polyubiquitination of IKKα in HEK293T cells (Supplementary Fig. 5c). These findings suggest that HECTD3 ubiquitinates IKKα with a mixture of K27-linked and K63-linked polyubiquitin chains.

HECTD3 interacts with IKKa

To test whether IKKa is a HECTD3 substrate, we firstly tested protein interaction between these two factors. We performed co-immunoprecipitation (co-IP) experiments and demonstrated that Flag-HECTD3 immunoprecipitated endogenous IKKa and Flag-IKKα immunoprecipitated exogenous HECTD3 in HEK293T cells (Fig. 4a). Next, we demonstrated that Flag-HECTD3 immunoprecipitated the endogenous IKKa protein in HUVECs (Fig. 4b). More importantly, endogenous IKKa and HECTD3 proteins also interact with each other, as shown using an anti-IKKα antibody in normal HUVECs (Fig. 4c). Consistently, IKKa colocalized with HECTD3 in HUVECs (Fig. 4d). However, the interaction was not increased by inflammatory factor stimulation (Supplementary Fig. 4a). Furthermore, we mapped the interaction domains of HECTD3 and IKKa. Our previous studies showed that the HECTD3 DOC domain (amino acids 216-393) is responsible for recruiting substrates, including MALT1 and Caspase-8.36,27 We transfected several GST-fused HECTD3 truncated mutants with Flag-IKKα in HEK293T cells and performed GST pulldown assays with glutathione sepharose beads. We found that the DOC domain also mediates the interaction between HECTD3 and IKKa (Fig. 4e). Similarly, we constructed a series of IKKa truncated mutants fused with GST and transfected them with Flag-HECTD3 in HEK293T cells, performing a GST pulldown experiment. We demonstrated that IKKa interacted with HECTD3 through its SDD domain (amino acids 408-665) (Fig. 4f) and Supplementary Fig. 4b).
To test the consequence of IKKα ubiquitination by HECTD3, we first compared the IKKα protein stabilities of K296R and WT in HEK293T cells. Compared to WT, K296R exhibited a shorter protein half-life, as measured by CHX chase experiments (Fig. 5h). Unlike WT IKKα, K296R failed to promote expression of E-selectin, ICAM-1 and VCAM-1 in response to TNFα (Supplementary Fig. 5d). Additionally, overexpression of WT IKKα rescued HECTD3 knockdown-induced downregulation of adhesion molecule expression in HUVECs under TNFα stimulation, but K296R failed to do so (Supplementary Fig. 5d). To eliminate the impact of endogenous IKKα, we generated IKKα KO HUVECs using CRISPR/Cas9 technology. IKKα depletion inhibited but did not abolish the...
expression levels of H3S10ph and E-selectin, ICAM-1 and VCAM-1 in response to LPS and TNFα stimulation (Fig. 5i and Supplementary Fig. 5e). As expected, WT IKKa, but not IKKa K296R, overexpression in IKKa KO HUVECs rescued the expression levels of H3S10ph and the three adhesion molecules (Fig. 5i and Supplementary Fig. 5e) and recovered the adhesion to cancer cells under inflammatory stimuli (Supplementary Fig. 5f, g). These results showed that HECTD3 promoted the expression of adhesion molecules and cancer cell adhesion through the ubiquitination of IKKa at K296.

To test whether HECTD3-mediated IKKa ubiquitination promotes IKKa nuclear translocation, we compared the subcellular distribution of IKKa WT and IKKa K296R in HUVECs treated with TNFα by collecting cytoplasmic and nuclear fractions for immunoblotting. TNFα stimulation increased nuclear localization of IKKa WT, but not K296R, in HUVECs (Supplementary Fig. 5h). This result implied that the polyubiquitination of IKKa mediated by HECTD3 is required for nuclear localization of IKKa under inflammatory conditions.

IKKa is a kinase for histone H3 and other substrates, and it is reasonable to deduce that HECTD3-mediated IKKa ubiquitination may promote its kinase activity. We next performed in vitro kinase assays to measure the kinase activities of IKKa WT, K296R, and S176/180 A proteins purified from HEK293T cells. IKKa S176/180 A is a well-known kinase dead mutant. Given that IKKa phosphorylates histone H3 at Ser10, we purified GST-fused histone H3 and IkBa from E. coli as substrates of IKKa. Notably, WT IKKa robustly phosphorylated GST-H3, while IKKa K296R and IKKa S176/180 A showed only weak kinase activities toward GST-H3 (Fig. 5j). Consistently, knockdown of endogenous HECTD3 in HEK293T cells decreased the kinase activity of IKKa (Fig. 5k), and overexpression of HECTD3, but not HECTD3 C233A, in HEK293T cells significantly increased the kinase activity of WT IKKa but not IKKa-K296R (Fig. 5l). Furthermore, similar results were obtained using GST-IκBα as the substrate in the kinase assay (Supplementary Fig. 5i, j). These results suggest that IKKa ubiquitination mediated by HECTD3 promotes IKKa kinase activity.

Dimerization is necessary for IKKα activation. Therefore, we tested whether HECTD3-mediated IKKa ubiquitination promotes its dimerization using co-IP experiments in HEK293T cells cotransfected with GST-IKKα and Flag-IKKα (WT, K296R, S176/180 A) or IKKβ. We found that the K296R mutation did not affect dimerization of IKKα (Supplementary Fig. 5k). Likewise, K296R mutation also did not influence the protein-protein interaction between HECTD3 and IKKα (Supplementary Fig. 5l). Interestingly, we found that purified GST-H3, but not GST-IκBα, pulled down more Flag-IKKα than Flag-IKKα K296R in HEK293T cell lysates (Fig. 5m and Supplementary Fig. 5m). Therefore, it is plausible that HECTD3-mediated IKKa ubiquitination increases the interaction between IKKa and histone H3 so that IKKa can efficiently phosphorylate it. However, this mechanism did not hold up for IkBα.

In order to determine whether the ubiquitination of IKKa by HECTD3 promotes its recruitment to the promoters of adhesion molecules, we knocked out the endogenous IKKa in HUVECs and restored Flag-IKKα WT or K296R mutant (Supplementary Fig. 5n). ChIP assays showed that WT, but not K296R, could be efficiently recruited to E-selectin, ICAM-1 and VCAM-1 promoters under treatment of LPS (Supplementary Fig. 5o). To test whether IKKa binds to the promoter of adhesion molecules through p65, we knocked down p65 in HUVECs and performed ChIP assays with IKKa antibody. The result showed that p65 KD significantly inhibited the recruitment of IKKa to the promoters of adhesion molecules under treatment of LPS (Supplementary Fig. 5p).

The recruitment of IKKa should promote phosphorylation of H3 at Ser10 at the promoters of adhesion molecules, thus it should promote chromatin open. In order to test this, we performed ChIP assays with H3S10ph antibody and found that HECTD3 KD in HUVECs decreased the H3S10ph level in the promoters of E-selectin, ICAM-1 and VCAM-1 under treatment of LPS (Supplementary Fig. 5q). Consistently, ChIP assays with H3K4me3 and H3K27ac antibodies showed that K296R could not efficiently promote the chromatin open (Supplementary Fig. 5r).

Hect3 promotes adhesion of tumor cells in the lung under inflammatory conditions.

To investigate whether Hect3 functions similarly in mouse vascular endothelial cells, we purified mouse pulmonary vascular endothelial cells (mECs) using Dynabeads coated with anti-CD31 antibody and cultured them. Hect3 KO inhibited the induction of E-selectin, ICAM-1 and VCAM-1 in mECs in response to LPS and TNFα stimuli, as examined by qRT-PCR (Fig. 6a and Supplementary Fig. 6a) and immunoblotting (Fig. 6b and Supplementary Fig. 6b). Consistently, Hect3 KO decreased the protein levels of IKKa and H3S10ph in mECs (Fig. 6a and Supplementary Fig. 6a). Immunofluorescence analysis showed that Hect3-/-indeed inhibited LPS-induced E-selectin protein expression in the mouse pulmonary CD31 vascular endothelium (Supplementary Fig. 6c). We also examined the expression level changes of IKKa, E-selectin, VCAM-1, and ICAM-1 protein in the endothelial cells of the mice before and after surgical resection of the primary tumors by...
immunofluorescence staining. *Hectd3−−* dramatically decreased IKKα nuclear translocation after surgery (Supplementary Fig. 6d) and slightly inhibited IKKα expression (Supplementary Fig. 6h) in CD31+ endothelia. The expression levels of E-selection, VCAM-1 and ICAM-1 were significantly induced by surgery and *Hectd3−−* could significantly inhibit the induction (Supplementary Fig. 6e–g, i–k). These results suggest that Hectd3 activates IKKα and promotes adhesion molecule expression in mECs under inflammatory conditions.

As shown at the beginning of this study, *Hectd3−−* mice exhibited significantly decreased lung colonization of tumor cells which were intravenously injected into mice treated with LPS in advance. We next examined whether Hectd3 deficiency suppresses metastasis by downregulating adhesion molecule expression to inhibit tumor cell colonization in the lung. To address this question, we conducted in vivo tumor cell adhesion assays (Supplementary Fig. 6l) by injecting GFP-labeled PyMT-induced mouse breast tumor cells into WT or *Hectd3−−* mice through the tail vein. Mice were treated with LPS stimulation for 5 h before tumor cell injection and sacrificed and perfused with PBS 20 h after tumor cell injection. Immunofluorescence was performed to detect GFP-positive tumor cells in the lung. As a result, the number of tumor cells infiltrated into WT mouse lung tissues is more than that into *Hectd3−−* mouse lung tissues (Fig. 6c).
confirmed this result by digesting the lung tissues of WT and Hectd3\(^{-/-}\) mice and performing flow cytometry analyses. We detected the number of GFP-labeled tumor cells in the lungs in a time course experiment and found that, at 10 min after injection, the number of GFP-positive tumor cells in the lungs of Hectd3\(^{-/-}\) mice was almost equal to that in WT mice, but significantly decreased at 10 h and 20 h after injection (Fig. 6d, e). To test whether the increased lung colonization of GFP-positive tumor cells was caused by differential survival of tumor cells in WT and Hectd3\(^{-/-}\) mice under LPS stimulation, we detected the apoptosis
of GFP-labeled tumor cells in the lung and found that, at 10 min, 10 h, and 20 h after intravenous injection of GFP+ tumor cells, the percentage of apoptotic GFP+ tumor cells had no significant difference between Hectd3−/− mice and WT mice pretreated with LPS (Supplementary Fig. 6m, n). These results clearly indicated that, compared to WT, Hectd3 deficiency did not affect the survival of the foreign tumor cells in the lung, but decreases lung adhesion of tumor cells under inflammatory conditions.

To further confirm that Hectd3 deficiency inhibited tumor metastasis specifically through endothelial cells, we generated C57BL/6 strain Hectd3 floxed mice (Supplementary Fig. 6o–q) and crossed these mice with Tie2-Cre mice to obtain Tie2-Cre × Hectd3fl/fl mice in which Hectd3 was specifically deleted in the endothelium (Fig. 6f). We detected the Hectd3 protein expression in mECs, muscle, liver, spleen and kidney of Tie2-Cre × Hectd3fl/fl (WT) and Tie2-Cre × Hectd3fl/fl (cKO) mice. The results confirmed that mECs isolated from cKO mice lost the expression of Hectd3, but the tissues from muscle, liver, spleen and kidney of cKO mice still expressed Hectd3 protein (Supplementary Fig. 6r). We transplanted subcutaneously B16-F10 mouse melanoma cells into WT and cKO mice, surgically removed primary tumor 12 days after transplantation and examined the lungs 45 days after resection of primary tumors. Compared to WT mice, Hectd3 cKO significantly inhibited lung metastasis (Fig. 6g, h). Furthermore, we pretreated mice with LPS and TNFα for 5 h and injected B16-F10 melanoma cells into the tail vein of WT and cKO. Hectd3-specific KO in the endothelium significantly inhibited LPS- and TNFα-induced lung metastasis (Fig. 6i–k). On the other hand, we created Hectd3 conditional knockin (cKO) mice in endothelial cells. We generated loxP-Stop-loxP-Hectd3fl/floxP-C57BL/6 mice by inserting the targeting sequence of CAG pr-loxP-Stop-loxP-Hectd3 CDS-P2A-eGFP-WPRE-pA into the Rosa26 site using the EGE system based on CRISPR/Cas9 developed by Beijing Biocytogen Co., Ltd (Supplementary Fig. 6s–u). We crossed our loxP-Stop-loxP-Hectd3fl/floxP mice with a background of Cre recombinase expression driven by the Tie2 promoter, which allows specific deletion of the Stop sequence to overexpress Hectd3 and GFP in the endothelium (Supplementary Fig. 6v). We isolated pulmonary vascular endothelial cells from Tie2-Cre × Hectd3fl/floxP and Tie2-Cre × Hectd3fl/floxP mice and assessed the expression of Hectd3 and GFP. As expected, Hectd3 and GFP protein expression levels were robustly increased in mECs from Tie2-Cre × Hectd3fl/floxP mice (Supplementary Fig. 6w). Importantly, IKKa protein levels were also increased in the endothelium of Tie2-Cre × Hectd3fl/floxP mice (Supplementary Fig. 6x). Subsequently, we treated mice with LPS for 5 h and injected B16-F10 melanoma cells into the tail vein. Twenty days later, Tie2-Cre × Hectd3fl/floxP mice showed significantly increased pulmonary metastasis compared to Tie2-Cre × Hectd3fl/fl control mice (Supplementary Fig. 6x–z). These animal experimental data confirm that Hectd3 promotes tumor metastasis by enhancing tumor cell colonization mediated by vascular endothelial cells in response to inflammation.

IKKa kinase inhibitor BAY 32-5915 suppresses lung metastasis HECTD3 promotes adhesion molecule expression by ubiquitinating IKKa, and Hectd3 KO inhibits tumor metastasis. Currently, there are no effective HECTD3 inhibitors available, so we examined whether an IKKa inhibitor could suppress tumor metastasis. The small molecule 8-hydroxynipecotic acid (BAY 32-5915) is a reported IKKa-specific kinase inhibitor.38 We demonstrated that BAY 32-5915 significantly inhibited induction of H3S10ph and adhesion molecules in HUVECs in response to LPS or TNFα (Supplementary Fig. 7a, b). To our surprise, levels of p-IKKα/β and p-pp65 in HUVECs treated with BAY 32-5915 were significantly increased over time under the stimulation of LPS and TNFα, while there were no significant changes in the phosphorylation or degradation of IkBα (Supplementary Fig. 7a, b). This was likely caused by a negative feedback mechanism of IKKa inhibition by BAY 32-5915, which somehow activated a compensatory pathway. Next, we pretreated HUVECs with different concentrations of BAY 32-5915 for 12 h and then added either LPS or TNFα for 4 h. BAY 11-7082, a classic inhibitor of the NF-κB pathway, was used as a positive control. Results showed that BAY 32-5915 inhibited expression of H3S10ph, E-selectin, ICAM-1, and VCAM-1 in HUVECs in a concentration-dependent manner (Supplementary Fig. 7c). Consistently, immunofluorescence analysis showed that BAY 32-5915 actually inhibited LPS-induced E-selectin expression in the lung CD31+ vascular endothelium of mouse (Supplementary Fig. 7d).

To test whether BAY 32-5915 could inhibit tumor metastasis in vivo, we pretreated BALB/c mice with BAY 32-5915 (12.5 or 25 mg/kg) for 24 h and LPS (1 mg/kg) for 5 h by intravenous injection, and then injected 4T1-Luc2 cells through the tail vein. We found that the number of pulmonary metastatic nodules and the lung weight gradually decreased with increasing BAY 32-5915 concentrations (Fig. 6l, m). Pretreatment with BAY 32-5915 prolonged the survival of mice with tumor metastasis (Supplementary Fig. 7e). These results indicate that the IKKa kinase inhibitor BAY 32-5915 suppresses tumor lung metastasis induced by inflammation.
DISCUSSION
Cancer metastasis is a multistep process by which tumor cells disseminate from their primary site, circulate in the vessels, and eventually form secondary tumors at a distant site. Most CTCs expire in the bloodstream due to shear stress and attack of the immune system, but a small proportion of tumor cells infiltrate distant organs and survive. Surgery, including biopsy, is a double-edged sword that removes the primary tumor but induces tumor-dormancy escape and subsequent metastatic outgrowth by impairing tumor-specific immunity or by producing a transient...
immunosuppressive state associated with wound healing. In other words, surgery triggers abundant detachment of tumor cells readily drilling into the vasculature and induces systemic inflammation that assists adhesion of tumor cells to distant ECs. A retrospective study examining the incidence of cancer recurrence in patients with lung cancer surgery showed that perioperative treatment with ANP, which inhibits the expression of E-selectin on ECs, improved relapse-free survival after surgery compared to surgery alone. Another retrospective analysis of tumor recurrence in patients undergoing breast cancer surgery revealed that perioperative treatment with ketorolac was related to a significant decrease in recurrence and mortality after surgery. Surprisingly, perioperative stimulation with ketorolac and resolvins (RvD2, RvD3, or RvD4) for resolution of inflammation dramatically reduced lung metastasis in mice by primary tumor removal or multiple tumor nodules. Surgery triggers and mediates the recruitment of myeloid-derived suppressor cells (MDSCs) into the lung to form premetastatic niches, and blockade of this recruitment with 5-azacytidine and entinostat effectively inhibits lung metastasis in a mouse model. Therefore, it is important to identify additional therapeutic targets and drugs to prevent cancer metastasis caused by inflammation.

In this study, we provided evidence to support the notion that HECTD3 promotes tumor cell adhesion to ECs and metastasis by ubiquitinating IKKα in response to inflammation. First, we demonstrated that HECTD3 KO inhibited distant tumor relapse in spontaneous metastasis models in response to surgery or systemic inflammation. Second, HECTD3 depletion in HUVECs and mouse ECs blocked inflammation-induced adhesion molecule expression and tumor cell adhesion to ECs in vitro and in vivo. In addition, HECTD3 conditional depletion in mECs inhibited tumor cell lung colonization in vivo, while HECTD3-specific overexpression in mECs increased that. Moreover, we characterized the molecular mechanism by which HECTD3 promotes metastasis. HECTD3 ubiquitinates IKKα with K63- and K27-linked polyubiquitin chains at K296, which prevents IKKα degradation by lysosomes and increases nuclear IKKα kinase activity. Activated IKKα is recruited to the promoters of adhesion molecules and phosphorylates histone H3 to facilitate transcription. Finally, we showed that an IKKα kinase inhibitor significantly suppressed inflammation-induced adhesion molecule expression and cancer metastasis in vivo. Taken together, the HECTD3-IKKα axis may serve as an effective prevention target for inflammation-induced cancer metastasis (Fig. 6n). However, HECTD3 conditional depletion in endothelium efficiently inhibited metastasis, but these results did not rule out that Hectd3 promotes metastasis through other mechanisms.

In our previous study, Hectd3 deletion decreased type I interferon production in macrophages. Macrophages have an important regulatory role in metastasis, like induction of cancer cell EMT and promotion of premetastatic niche formation. Therefore, it is remained to explore whether Hectd3 also promotes metastasis through macrophages.

Histone H3 phosphorylates histone H3 at Ser10 to increase the transcription of adhesion molecules. These adhesion molecules on the EC plasma membrane promote the adhesion of tumor cells, such as neutrophils, monocytes, and macrophages, as well as inflammatory conditions. It is warranted to develop therapeutics that block inflammation-related metastasis. Historically, inflammation-induced metastasis has long been recognized that metastasis can be enhanced by active inflammation, such as in response to IL-1β or LPS, which induces endothelial adhesion molecules that facilitate adhesion of cancer cells to ECs. Adhesion molecules, such as E-selectin, ICAM-1 and VCAM-1, have fundamental functions in leukocytes and hemostasis by mediating the rolling of leukocytes on activated ECs and transmigration through endothelial cell junctions. Unfortunately, tumor cells can utilize the same route to achieve distant metastasis, especially when the vascular endothelium undergoes short inflammatory stimulation, such as in response to surgery. In the bloodstream, CTCs also aberrantly express adhesion molecules to interact with platelets and immune cells, such as neutrophils, monocytes, and macrophages, as well as endothelial cells. This provides a potential approach to suppress metastasis by interrupting adhesive interactions. E-selectin + mice show bone metastasis blockade. Anti-VCAM-1 or anti-integrin α4 mAbs also dramatically reduced bone metastasis in breast cancer. Here, we showed that HECTD3 and IKKα control inflammatory adhesion molecule expression and that inhibition of HECTD3 genetically or IKKα pharmacologically suppresses metastasis induced by acute inflammation. It is warranted to develop small molecule inhibitors for HECTD3 and IKKα for tumor suppression in inflammatory conditions.
metastasis prevention, especially in patients who undergo surgical removal of the primary tumor. To date, a number of small molecules like ACHP, BMS-345541, selenium-based compounds and heterocyclic adamantyl artonoids, show inhibition to IKKα activity, but some of them also inhibit IKKβ. High selectivity remains an essential issue for IKKα inhibitor screening. E3 ligases are promising potential therapeutic targets due to their high substrate specificity. As an E3 ligase, HECTD3 can autoubiquitinate and ubiquitinate substrates in vitro, which is a good experimental basis for inhibitor screening of HECTD3.

IKKα is dispensable for IkBa phosphorylation and degradation but remains essential for NF-κB-dependent transcription because of its nuclear kinase activity. In the nucleus, IKKα is recruited to the NF-κB transcription complex to phosphorylate multiple substrates, such as CBP at Ser1382/1386, p65 at Ser536, and SMRT at Ser2410, to promote NF-κB-dependent gene transcription. Yumi Yamamoto and Vasiliki Anest independently found that nuclear IKKα was recruited to NF-κB binding chromatin and phosphorylates histone H3 at Ser10 to activate NF-κB target gene transcription after stimulation. It is well-known that H3S10ph plays a crucial role in activation of transcription. H3S10ph at promoters may lead to chromatin remodeling by recruiting 14-3-3 proteins, MSK1, and BRG1, the ATPase subunit of the SWI/SNF remodeler, to promote transcription. H3S10ph also ejects heterochromatin factors, such as HP1, HDAC1, 2 and 3, or prevents deposition of H3K9me2 associated with transcriptional repression to facilitate gene expression. Additionally, A recent study showed that IKKα phosphorylated histone variant H3.3 at Ser31, which deposited to associated with transcriptional repression to facilitate gene transcription initiation and elongation through H3S10ph and H3S351ph, respectively. Herein, we show that the ubiquitination of IKKα mediated by HECTD3 increased its kinase activity toward H3. Whether there is crosstalk between S10ph and S31ph remains unknown. Additionally, nuclear IKKα has been shown to regulate DNA damage response, radioresistance, apoptosis, and cell cycle. Whether HECTD3 regulates other functions of IKKα remains to be investigated.

Although it has been reported that IKKα ubiquitination promotes its nuclear translocation in hepatoma cells, its E3 ligase and modification details have not been fully elucidated. For the first time, we identified HECTD3 as an IKKα E3 ligase that promotes K63- and K27-linked polyubiquitination at K296. This ubiquitination on the target kinase promotes IKKα protein stability, nuclear localization and kinase activity. Furthermore, we found that blocking ubiquitination of IKKα inhibited the interaction of IKKα with the target protein histone H3 but not IkBα. However, how ubiquitination promotes the kinase activity of IKKα needs further investigation.

In summary, our data characterize the function of the HECTD3-IKKα axis in the adhesion of tumor cells to the endothelium through the NF-κB signaling pathway, which provides a potential strategy for tumor hematogenous metastasis prevention and treatment.

**MATERIALS AND METHODS**

**Mouse strains**

*Hectd3*<sup>−/−</sup> mice and *Hectd3*<sup>+/+</sup> mice for the similar tumor metastasis experiments using 4T1-Luc2 breast cancer cells or B16-F10 melanoma cells. The *loxP*-Hectd3-LoxP C57BL/6 strain mice (project number: EGE-SSH-021-B) were generated using the EGE system based on CRISPR/Cas9 developed by Beijing Biocytogen Co., Ltd, which were crossed with Tie2-Cre mice to generate mice with Hectd3-specific deficiency in the endothelium. These conditional KO (cKO) mice were confirmed for analysis of lung metastasis after tail vein injection of B16-F10 melanoma cells. The *loxP*-Stop-*loxP*-Hectd3<sup>ko</sup>-GFP C57BL/6 strain mice (project number: EGE-ZLY-004 KO) were generated by inserting the targeting sequence of *Cag* Pr-*loxP*-Stop-*loxP*-Hectd3 CDS-P2A-eGFP-WPRE-pA into the Rosa26 site by using the EGE system based on CRISPR/Cas9 developed by Beijing Biocytogen Co., Ltd. The Stop sequence is flanked by two *loxP* sites. Followed the second *loxP* site is a Hectd3 CDS-P2A-eGFP-WPRE-pA expression element. The targeted allele was driven by chicken β-actin promoter and inserted into the Rosa26 allele in C57BL/6 background. The *loxP*-Stop-*loxP*-Hectd3<sup>ko</sup>-GFP mice were crossed with Tie2-Cre to generate the conditional knockin (ckI) mice, which allows specific overexpression of Hectd3 and GFP in the endothelium. These ckI mice were confirmed for analysis of lung metastasis after tail vein injection of B16-F10 melanoma cells. All littermate used for tumor analysis above were virgin females. All mice were kept in specific pathogen-free (SPF) conditions at the Animal Resource Center of Kunming Institute of Zoology, Chinese Academy of Sciences. All animal experiments were conducted in accordance with the guidelines and were approved by the Kunming Institute of Zoology, Chinese Academy of Sciences Animal Care and Use Committee.

Intraductal injection of PyMT lentivirus to induce breast tumor Wild type FVB female mice were anesthetized, mammary ducts were exposed by cutting nipple ends, and a 50 μl microsyringe was used to inject 10 μl viral concentrate of lentivirus overexpressing PyMT and GFP (FUGCW-PyMT-GFP lentivirus) into the ductal lumen of glands #4. Two weeks later, the PyMT-induced breast tumors were examined by palpation of mammary glands if the injection was successful. Resected the tumor and digested it to single-cell suspension using collagenase type III (Worthington-biochemical, NJ, USA) and hyaluronidase (Sigma-Aldrich, MO, USA), which was applied to tail vein injection or orthotopic allograft transplantation.

Spontaneous metastasis assay

WT and *Hectd3*<sup>−/−</sup> mice with FVB or BALB/c genetic background received mammary fat pad transplantation of 1 x 10<sup>6</sup> PyMT-induced breast tumor cells or 4T1-Luc2 cells suspended in 75 μl mixture of PBS and Matrigel (1:1; BD Biosciences, CA, USA). Twenty days (PyMT-induced tumor) or 12 days after transplantation (4T1-Luc2 tumor), surgical resection was operated to remove the tumors. In PyMT-induced tumor cell experiments, the lung and heart metastasis were record when mice being natural mortality or sacrificed 2 months after removal of tumor. In 4T1-Luc2 experiment, bioluminescence imaging 4T1-Luc2 tumor burden on day 1 before the tumor resection and tumor metastatic burden was monitored weekly by IVIS imaging after tumor removal.

**Immunoblotting analysis and antibodies**

Protein samples were separated by 11% SDS-PAGE and followed by electrophoretic transfer onto PVDF membranes and blocked with 5% non-fat milk and further incubated overnight in primary antibody (dilute 1:500-5000 in 3% BSA) at 4 °C as described previously. The following primary antibodies were used: anti-E-selectin (sc-137054), anti-ICAM-1 (sc-8439), anti-VCAM-1 (sc-8304), anti-α-IκB (SC-805) and anti-GAPDH (sc-25778) Antibodies were purchased from Santa Cruz Biotechnology, California. The anti-p-κB (8242), anti-p-p65(3033), anti-κBα (9242), anti-p-κBα...
digest was biochemically at 37 °C for 45 min with occasional agitation. The cellular supernatant at 4 °C overnight and then washed three times with PBS gently to remove non-adherent tumor cells, then fixed with 4% (wt/vol) paraformaldehyde. The number of adhering GFP-positive cells in the fixed plate was counted by using images obtained with a fluorescence microscope (Nikon).

In vivo tumor cell adhesion assay
Lentiviruses overexpressing PyMT and GFP were injected intraductally to induce breast tumors and tumor cells were digested into a single-cell suspension as described above. The single-cell suspension (5 × 10^6 cells per mouse) was injected through the tail vein into WT or Hectd3−/− mice pretreated with LPS (1 mg/kg) stimulation for 5 h. Twenty hour after tumor cell injection, the mice were sacrificed and perfused to analyze tumor cell colonization in the lung.

RNA-sequencing analysis
Total RNA was extracted from HUVECs transfected with control siRNA or siHectd3 treated with TNFa for 2 h or without using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to commercial RNA-Seq analysis (LC-Bio Technology Co., Ltd., Hangzhou, China). Poly (A) RNA was purified from 1 μg total RNA per sample using Dynabeads Oligo (dT) 25-61005 (Thermo Fisher, CA, USA) for the final CDNA library with average insert size 300 ± 50 bp to perform the 2 × 150 bp paired-end sequencing (PE150) on an illumina Novaseq™ 6000 following the recommended protocol. Fastp software (https://github.com/OpenGene/fastp) was used to verify sequence quality and HISAT2 (https://ccb.jhu.edu/software/seq) was used to map reads to the reference genome of Homo sapiens GRCh38. StringTie (https://ccb.jhu.edu/software/stringtie) was used to assemble the mapped reads of each sample. Gffcompare (https://github.com/gperalta/gffcompare/) was used to reconstruct a comprehensive transcriptome. Then, we used StringTie to perform expression level for mRNAs by calculating FPKM. The significantly differential expressions were selected with fold change < 0.5 or fold change > 2 and with parametric F-test comparing nested linear models (p-value < 0.05) by R package edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html).

Immunofluorescence staining and microscopy
For IKKa and Flag immunostaining, LPS treated and untreated HUVECs were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were washed with 1×PBS, blocked in 5% BSA buffer with 0.1% saponin for 1 h. The cells were stained with anti-IKKa (CST, 2682) at 1:300 dilution or anti-Flag (Sigma-Aldrich, F7425) at 1:500 dilution, overnight at 4 °C. The cells were washed, stained with fluorescence conjugated secondary antibody for 1 h at room temperature, nuclear was staining by Hoechst (Invitrogen), and mounted using mounting medium (Vector Laboratories, H-1200). For lung tissues frozen immunofluorescence assay, anti-GFP (Abcam, ab13970), anti-CD31 (BD, 553766), anti-p100/52 (4882) and HRP-labeled anti-rabbit, anti-mouse or anti-goat secondary antibodies were purchased from Cell Signaling Technology (CST, MA, USA). The anti-GST (G7781) and anti-FLAG (F3165) antibodies were from Sigma-Aldrich. The anti-UB (04–263) Ab was from Millipore(MA, USA). The anti-K27 Ub Ab (ab181537) was from Abcam (MA, USA). The anti-tubulin Ab (11224-1-AP) was from Proteintech (IL, USA). The anti-GFP Ab (11814460001) was from Roche (Basel, Switzerland). The anti-H3.3531pH (39637) was from Active motif. Then anti-HECTD3 antibody was previously described.26

siRNAs
Small interfering RNA (siRNA) for human genes was synthesized by Guangzhou RiboBio Co., LTD as follows: HECTD3-specific siRNA (1#: sense, GCC GGA ACU AGG GUU GAA Utt; 2#: sense, GGU AUU UCA CUU CUU AAG Att), IKKβ-specific siRNA (sense, GCA GCC UCU UUC AGG GA Catt), IKKβ-specific siRNA (sense, CAGGGAGCAGAUUAGCCAU), p65-specific siRNA (sense, GCC CUU UCC CUU UAC GUC Att), E-selectin-specific siRNA (sense, CAA CAA UAG GCA AAA AGA Ut), ICAM-1-specific siRNA (sense, AGU CAA CAG CUA AAA CUU UCC Ut), VCAM-1-specific siRNA (sense, GGA GUU AAU UUG AUU GGG GTt), siRNA oligonucleotides were transfected in HUVEC cells with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions.

Mouse ECs isolation and culture
Lung tissues from WT and Hectd3−/− mice were removed aseptically, rinsed in 1× PBS, minced into ~1 × 1 mm squares, and digested in 10 ml of collagenase A (1 mg/ml, Worthington-biochem) at 37 °C for 45 min with occasional agitation. The cellular suspenstion (5 × 10^6 cells per mouse) was injected through the tail vein into WT or Hectd3−/− mice pretreated with LPS (1 mg/kg) stimulation for 5 h. Twenty hour after tumor cell injection, the mice were sacrificed and perfused to analyze tumor cell colonization in the lung.
Chromatin immunoprecipitation assay

The crosslinking ChIP assay was performed using HUVECs following the manufacturer's procedure (Abcam, Cambridge, MA, USA) with slight modifications. HUVECs were fixed with 1% formaldehyde for 10 min. Glycine (125 mM) was added to terminate the crosslinking reaction. Fixed HUVECs were collected and resuspended in cytoplasmic lysis buffer (85 mM KCl, 0.5% NP-40, 5 mM PIPES, pH 8.0) with protease inhibitors for 10 min. The pellet nuclei was collected by centrifuge and resuspended in nuclear lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris-Cl, pH 8.1) with protease inhibitors on ice for 10 min. Then, the pellet nuclei were broken by ultrasonic crusher for release of the DNA-protein complex. The DNA-protein complex derived from HUVECs was incubated with antibodies and Protein A/G beads at 4 °C for 10 h. The chromosomal DNA was purified and analyzed by normal or quantitative PCR. Anti-p-H3S10ph (CST, 53348), anti-H3K4me3 (Abcam, ab58580), anti-H3K27ac (Abcam, ab47299) and anti-IKKα (CST, 2682) antibodies were used for Chip assays. Primers for Chip as followed: (human) E-selectin: forward 5'- CGG GAA AGT TTT TGG ATG C-3', reverse 5'- GAG GGA TTG CCT GTG AA-3'; ICAM-1: forward 5'- GGG GGC GGA GATT CAC AAG-3'; reverse 5'- GCC ATC CAG AGA GGC ATA TT-3'; VCAM-1: forward 5'- TTG GCT GGG TGT CTT AAA-3'; reverse 5'- TAA AGG GTC TTT CAG AGG-3'.

Co-immunoprecipitation and GST Pull-down experiment

HEK293T and IKKα were cloned into pCDH-CMV-MCS-EF1-puro-3×Flag or pLenti6 vector. Truncated mutants of HECTD3 and IKKα were cloned into pEBG-GST vector. Lipofectamine 2000 reagents (Invitrogen) were used for transient transfection of plasmids into HEK293T cells. For immunoprecipitation (IP), whole HEK293T cells were cloned into pCDH-CMV-MCS-EF1-puro-3×Flag vector. The plasmids were transfected in HEK293T cells with Lipofectamine 2000. After 36 h, cell extracts were immunoprecipitated with anti-Flag M2 beads. Histone H3 and kxBα were cloned into pGEX-6p-1 vector and transfected into DE3 (BL21) E. coli. GST- H3 and GST-kxBα proteins were purified with glutathione sepharose beads. Briefly, the immunoprecipitates were incubated with GST-H3 or GST-kxBα in the kinase reaction buffer which contains 20 mM HEPES at pH 7.5, 10 mM MgCl2, 20 mM b-glycerophosphate, 10 mM PNPP, 50 mM Na3VO4, 1 mM DTT, 20 mM ATP, and at 30 °C for 30 min. The products were subjected to SDS-PAGE and immunoblotting with anti-H3S10ph antibody or anti-p-kxBα (Ser32/36) antibody.

Real-time quantitative PCR

Total RNA was isolated from tissues or cells using Trizol (Invitrogen) and purified by RNeasy Mini Kit (QIAGEN), cDNA was reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). Primers were designed according to the published sequences and listed as follows: (human) E-selectin: forward 5'- CTG GCT TCG GAA ATG TTC ATC AC-3', reverse 5'- CCA GAG ACC CGA GGA GAG TT-3'; ICAM-1: forward 5'- GAG CAC TCA AGG GGA GGT C-3', reverse 5'- CAT TAT GAC TGG TGC TGA TA-3'; VCAM-1: forward 5'- GAA CCC AAA CAA AGG CAG AG-3', reverse 5'- GGA TTT TCG GAG CAG GAA AG-3'; 18 S: forward 5'- CGC CGC TAG AGG TGA AAT TCT-3', reverse 5'- GCA ACC TCC TCC GTG TTA GC-3'; GAPDH: forward 5'- AAA CAC TCC TCC GGA CTT GGC TTA C-3', reverse 5'- CTT CAC AAT CAG TCC TGG TTC T-3'.
Statistical analysis
Data are given as mean ± SEM. Statistical analyses were performed using two-tailed t-test, two-way ANOVA, Log-rank test or Chi-square test. P-values ≤ 0.05 were considered significant.

DATA AVAILABILITY
The data that support the findings of this study are available from the authors upon reasonable request. The RNA-seq data are submitted at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under record number GSE201759. These data can be accessed with the following link. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201759.

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AUTHOR CONTRIBUTIONS
F.L. performed most experiments with crucial help from H.L., H.Y., J.X., X.C., M.H., Z.C., C.Y., W.L., H.Z., L.Z., Y.W., F.G., Z.L., W.Z., Z.Z., R.L., D.J., N.X., B.L., Y.W., Y.K. and Z.L., F.L. and C.C. wrote the manuscript, and C.C. conceived and designed the study. All authors discussed the results and commented on the manuscript. All authors read and approved the article.

ADDITIONAL INFORMATION
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