Rab27a plays a dual role in metastatic propensity of pancreatic cancer

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Pancreatic cancer is an aggressive malignancy, often diagnosed at metastatic stages. Several studies have implicated systemic factors, such as extracellular vesicle release and myeloid cell expansion, in the establishment of pre-metastatic niches in cancer. The Rab27a GTPase is overexpressed in advanced cancers, can regulate vesicle trafficking, and has been previously linked to non-cell autonomous control of tumor growth and metastasis, however, the role of Rab27a itself in the metastatic propensity of pancreatic cancer is not well understood. Here, we have established a model to study how Rab27a directs formation of the pre-metastatic niche. Loss of Rab27a in pancreatic cancer cells did not decrease tumor growth in vivo, but resulted in altered systemic myeloid cell expansion, both in the primary tumors and at the distant organ sites. In metastasis assays, loss of Rab27a expression in tumor cells injected into circulation compromised efficient outgrowth of metastatic lesions. However, Rab27a knockdown cells had an unexpected advantage at initial steps of metastatic seeding, suggesting that Rab27a may alter cell-autonomous invasive properties of the tumor cells. Gene expression analysis of gene expression revealed that downregulation of Rab27a increased expression of genes involved in epithelial-to-mesenchymal transition pathways, consistent with our findings that primary tumors arising from Rab27a knockdown cells were more invasive. Overall, these data reveal that Rab27a can play divergent roles in regulating pro-metastatic propensity of pancreatic cancer cells: by generating pro-metastatic environment at the distant organ sites, and by suppressing invasive properties of the cancer cells.

Over 90% of pancreatic intraepithelial neoplasia (PanIN) lesions contain oncogenic mutations in the Kras locus, making it the most common and likely the initiating transformation event in pancreatic ductal adenocarcinoma (PDA)1. The field of pancreatic cancer biology has benefited tremendously from the development of spontaneous mouse models of pancreatic carcinogenesis, which feature pancreas-specific endogenous expression of oncogenic Kras and the tumor suppressor p53, and faithfully recapitulate formation and histopathological progression of PDA to metastasis2,3. Importantly, both human and mouse PanINs feature extensive remodeling of surrounding microenvironment characterized by pronounced influx of immune cells, which has been shown to shape inflammatory and immunosuppressive pro-tumorigenic milieu4–6. While it is appreciated that host immunity plays a critical role in regulating tumorigenesis, the mechanisms behind immune response in pancreatic cancer are poorly understood. Furthermore, systemic immune changes have been documented in patients with metastatic pancreatic cancer, which most commonly metastasizes to liver, lung and diaphragm, however, our understanding of the role of the immune system in the process of pancreatic cancer metastasis remains scarce.

Systemic factors released by primary tumors have been shown to influence metastasis via a variety of mechanisms. For example, in models of lung cancer and melanoma it has been shown that secretion of VEGF and PIGF from the primary tumor increase levels of MMP9 in the lung and facilitate distant metastasis7. Accumulation of hematopoietic cells at distant sites have also been shown to facilitate metastasis, for example release of inflammatory proteins such as S100A8 results in accumulation of activated macrophages in the lung8. MicroRNAs have also been shown to promote metastasis. In a breast cancer model it was shown that miRNA 105 released from

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tumors contributed to the breakdown of vascular endothelial barriers by targeting ZO1\textsuperscript{9}. Extracellular vesicles (EV) can be released by cancer cells and have been postulated to influence a variety of pro-metastatic properties in part by altering immunological milieu of a variety of distant organ sites in systemic fashion\textsuperscript{10,11}. Extracellular vesicles have been implicated in many functions that regulate tumor progression, including immune escape, invasion and metastasis\textsuperscript{11–14}. In pancreatic cancer, ectopic transfer of EVs promoted the recruitment and activation of myeloid cells, potentiated establishment of pre-metastatic niche and experimental metastases\textsuperscript{11,15}. Rab27a is a small Ras-like GTPase that has been shown to be differentially expressed in cancer and is known to contribute to EV biogenesis\textsuperscript{10,16,17}. Overexpression of Rab27a has been linked to poor prognosis in cancer, presumably in the context of the contribution to the extensive remodeling by systemic EV release\textsuperscript{12,18}. However, the role of Rab27a itself in the metastatic propensity of pancreatic cancer \textit{in vivo} remains unclear. In this study, we aimed to address the role of Rab27a in modulating metastatic properties of pancreatic cancer \textit{in vivo}.

**Results**

**Primary pancreatic tumors promote expansion of myeloid cell subsets at distant organ sites.** To understand how the immunological milieu at distant organ sites is affected by the presence of primary pancreatic tumors, we used a well-established Kras-driven model of spontaneous pancreatic tumorigenesis, \textit{LSL-Kras\textsuperscript{G12D/+}}; \textit{p48Cre/+} (KC), which is not proficient at driving metastasis\textsuperscript{2}. To identify immune infiltrates in livers of mice with primary tumors, livers from WT, KC and KPC mice. Proportion of CD45\textsuperscript{+} cells is indicated. (C) Quantification of CD45\textsuperscript{+} CD11b\textsuperscript{-}/Gr1\textsuperscript{-} myeloid cell frequency in spleen and livers of WT, KC and KPC mice. Proportion of CD45\textsuperscript{+} cells is indicated. (D) Quantification of CD45\textsuperscript{+} CD11b\textsuperscript{+} Gr1\textsuperscript{-} myeloid cell frequency in spleen and livers of WT, KC and KPC mice. Proportion of CD45\textsuperscript{+} cells is indicated. Error bars indicate SEM; p value: *<0.05; **<0.01; ***<0.001. Data represents 3 independent experiments.
tumor sizes as well as the frequencies of myeloid cell populations in pancreatic tumors, spleens, livers, and lungs. 

**b27a-KPC** shRa-**scr-KPC**

the presence of primary pancreatic lesions may facilitate metastatic outgrowth. To test this idea, we injected 

**KPC** alone (**KC-WT**) was not sufficient to alter immunological milieu (Fig. 2D–I). The metastatic events by 

such as VEGF, PIFG, MMP9, and extracellular vesicles among others 7,11. In this study, we wanted to under-

pre-metastatic niche microenvironment in pancreatic cancer has been linked to systemic production of factors,

stalked to facilitate myeloid cell accumulation, pre-metastatic niche formation, and/or metastatic outgrowth 

**b27a**-KPC tumors (Fig. 3F). To further validate that tumor cell growth was not affected 

in vivo growth of **KPC**-**shRab27a** cells and measured tumor growth 2 weeks after injection. Despite a change in 

**KPC** tumors by analyzing phospho-Histone H3 staining, and 

loss of Rab27a in primary tumors modulates distant immunological milieu. To test the ability 

of Rab27a-dependent changes in systemic immune composition, we orthotopically injected **scr-KPC** and **shRab27a-KPC** cells into the pancreata of WT mice. Mice were sacrificed 2 weeks after injections, and analyzed for 

tumor sizes as well as the frequencies of myeloid cell populations in pancreatic tumors, spleens, livers, and lungs.
Figure 2. Primary pancreatic tumors facilitate metastatic seeding. (A) Schematic of mouse models used in Fig. 2. (B) Representative histological images of livers of WT, KC or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection, H&E, GFP and CD45 as labeled. 20x objective, scale bar represents 100um. (C) Representative histological images of livers of WT mice intra-splenically injected with GFP-KPC cells at 2 weeks post-injection. H&E, GFP, and Gr1 at 10×. Final panel represents inset of Gr1 at 20×. Error bars represent 100um. (D) Representative flow cytometry plots of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the spleens of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. (E) Quantification of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the spleens of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. (F) Representative flow cytometry plots of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the livers of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. (G) Quantification of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the livers of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. (H) Representative flow cytometry plots of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the lungs of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. (I) Quantification of CD45+CD11b+F4/80+ and CD45+CD11b+Ly6G/Ly6C+ cells from the lungs of WT or KPC mice intra-splenically injected with GFP-KC cells at 2 weeks post-injection. Error bars indicate SEM; NS – not significant, p value: **< 0.01; ***< 0.001. Data represents 3 independent experiments.
We observed marked changes in the frequency of myeloid cells with Rab27a knockdown in the primary tumor (Fig. 4A). Prior studies in melanoma have linked Rab27a function with reduced metastatic burden, however, this effect was also associated with reduced primary tumor growth, complicating the analysis of metastatic outgrowth10. We, on the other hand, demonstrated that in pancreatic cancer, Rab27a has a role in facilitating systemic changes in immune milieu that are independent of its potential role in regulating primary tumor growth (Fig. 3F).

The most profound effect of Rab27a knockdown was observed in livers, where both CD11b+Ly6C-Ly6G- granulocytic cells; CD11b+Ly6C-Ly6G+ monocytic cells and CD11b+Ly6C-Ly6G+ macrophage populations were significantly decreased upon knockdown of Rab27a in the primary tumor (Fig. 4B). Among the myeloid populations in spleens and lungs, monocytic CD11b+Ly6C-Ly6G+ and granulocytic CD11b+Ly6C-Ly6G+ cells were decreased, but only reduction in CD11b+Ly6C-Ly6G+ macrophages was found to be significant (Fig. 4C). Among the myeloid populations in the pancreas, CD11b+Ly6C-Ly6G- granulocytic cells were significantly reduced,

Figure 3. Knockdown of Rab27a in KPC cells decreases EV release, but does not alter primary tumor growth in vivo. (A) Levels of Rab27a mRNA in KPC cells were assessed by quantitative RT-PCR. (B) Levels of Rab27a protein in KPC cells were assessed by Western blotting. (C) Nanosight profile of vesicle distribution in supernatants collected from KPC cells infected with scrambled vector or shRNA constructs directed against Rab27a. (D) Nanosight particle concentration of supernatants collected from KPC cells infected with scrambled vector or shRNA constructs directed against Rab27a. (E) Growth curve for Rab27a knockdown KPC cells as compared to Scrambled control KPC cells. “+” indicated statistical difference between sh1 and scrambled control; “**” indicates statistical difference between sh2 and scrambled control. (F) Tumor volume at two weeks post injection of KPC cells infected with scrambled vector or shRNA constructs directed against Rab27a. (G) Phospho-Histone 3 immunofluorescent staining of tumors generated from KPC cells infected with scrambled vector or shRNA constructs directed against Rab27a. (H) Quantification of Phospho-Histone 3 immunofluorescent staining from (G). Error bars indicate SEM; NS – not significant, p value: *<0.05; **<0.001. Data represents 3 independent experiments.
Figure 4. Knockdown of Rab27a in primary tumors has differential effect on systemic distribution of myeloid cell subsets. (A) Representative flow cytometry plots and quantification of CD45^+ CD11b^+Gr1^- and CD45^+ CD11b^+Ly6C/G^+ myeloid cells in pancreata of WT mice orthotopically injected with scr-KPC or shRab27a-KPC cells, at 2 weeks post-injection. Proportion of CD45^+ cells is indicated. (B) Representative flow cytometry plots and quantification of CD45^+ CD11b^+Gr1^- and CD45^+ CD11b^+Ly6C/G^+ myeloid cells in livers of WT mice orthotopically injected with scr-KPC or shRab27a-KPC cells, at 2 weeks post-injection. Proportion of CD45^+ cells is indicated. (C) Representative flow cytometry plots and quantification of CD45^+ CD11b^+Gr1^- and CD45^+ CD11b^+Ly6C/G^+ myeloid cells in lungs of WT mice orthotopically injected with scr-KPC or shRab27a-KPC cells, at 2 weeks post-injection. Proportion of CD45^+ cells is indicated. (D) Representative flow cytometry plots and quantification of CD45^+ CD11b^+Gr1^- and CD45^+ CD11b^+Ly6C/G^+ myeloid cells in spleens of WT mice orthotopically injected with scr-KPC or shRab27a-KPC cells, at 2 weeks post-injection. Proportion of CD45^+ cells is indicated. Error bars indicate SEM; NS – not significant, p value: *<0.05; **<0.01; ***<0.001. Data represents 3 independent experiments.
whereas there was no effect on CD11b+Ly6C-Ly6G- and CD11b+Ly6C-Ly6G+ subsets (Fig. 4C). Overall, expression of Rab27a in the primary tumor has distinct consequences for the composition of immune milieu at the primary tumor and distant organ sites with the most significant effects seen in the liver. In addition to these observations, we noticed that, although, there was reduction in certain myeloid compartments with Rab27a knockdown, a substantial proportion of myeloid cells remained, suggesting that a secondary mechanism, such as production of GM-CSF or similar factors by the cancer cells might compensate for recruitment and expansion of specific immune subsets.

We also tested if reduction in the myeloid compartment in livers upon Rab27a knock-down in primary pancreatic neoplasms is a consequence of deficiencies in myeloid compartment of the bone marrow. Expansion of myeloid compartments in cancer is often associated with expansion in bone marrow derived cells. However, consistent with previous findings in melanoma, we did not find significant differences in bone marrow-associated myeloid cells between scr-KPC and shRab27a-KPC cells. Anti-Gr1 treatment successfully depleted myeloid cells at the primary tumor site affects metastatic outgrowth, we have designed experiments, where GFP-scr-KPC or GFP-shRab27a-KPC cells were orthotopically injected into pancreata of WT mice, and then GFP-scr-KPC or GFP-shRab27a-KPC cells were injected intrasplenically, at the time when we know that the immune niche at the distant organ sites has been established (Fig. 5A,B). We then analyzed the extent of metastatic seeding at early stages (2 days post-splenectomy injection) or active outgrowth stages (2 weeks post-splenic injection) by counting the number of GFP+ cancer cells/lesions in liver sections. Contrary to our expectations, we observed an increase in the initial seeding capacity of GFP-shRab27a-KPC cells regardless of whether the primary tumor was proficient or deficient in Rab27a expression (Fig. 5B, C). This effect was lost at 2 weeks post-splenic injection, as we found that livers of mice that were pre-conditioned with either scr-KPC or shRab27a-KPC tumors had significantly fewer micrometastatic (micro≤200μm) lesions when GFP-shRab27a-KPC cells were injected into spleens (Fig. 5D) and no macrometastatic lesions (macro≥200μm) were present in any group. In addition, GFP-scr-KPC cells generated more metastases even in the context of Rab27a deficient primary tumors, suggesting that Rab27a-mediated primary tumor-induced myeloid expansion is not required for metastatic outgrowth. (Fig. 5D).

Myeloid cells, such as liver Kupffer macrophages and bone marrow-derived monocytes, have been shown to facilitate pancreatic metastasis formation. To follow up this observation, we attempted to deplete a subset of myeloid cells using anti-Gr1 antibody treatment. Anti-Gr1 treatment successfully depleted myeloid cells at the primary tumor site but was relatively ineffective in depleting the myeloid population in livers (Supplementary Figure 5A,B). This is consistent with studies by other groups, which suggest that difficulty with depletion of myeloid cells in livers of cancer-bearing mice could arise as a result of rapid repopulation by circulating cells.

To test if cancer-educated myeloid cells were sufficient to rescue metastatic outgrowth of shRab27a-KPC primary tumors, we adoptively transferred splenic myeloid cells from tumor-bearing CD45.1 mice (Fig. 5E). The presence of CD45.1 cells was verified at 3 weeks in the spleen as well as in liver metastases by immunohistochemistry (Supplementary Figure 5C). We observed that supplementation with myeloid cells partially rescued metastatic outgrowth of shRab27a-KPC cells at 3 weeks post-injection by counting both micro and macrometastases (micro≤200μm, macro≥200μm) (Fig. 5F). This suggests that addition of cancer-educated myeloid cells can partially compensate for deficient metastatic outgrowth in the absence of Rab27a expression in cancer cells. Partial nature of such rescue could be due to either insufficient number of transferred myeloid cells, requirement for liver-resident myeloid cell expansion as opposed to spleen and/or the possibility that additional non-myeloid-cell dependent mechanisms contribute to the successful outgrowth of Rab27a-deficient cells at metastatic sites. To understand if EVs were sufficient to modulate myeloid cell frequency, we isolated EVs from supernatants of scr-KPC cells. EVs or PBS control were retro-orbitally injected into WT mice or mice bearing scr-KPC or shRab27a-KPC primary tumors (Fig. 5G). WT healthy mice treated with EVs demonstrated a significant increase in intrahepatic macrophages compared to control treated mice, however the increase in Gr1+ cells failed to reach statistical significance (Fig. 5H, I). Overall, we did not observe changes in MHC II or CD206 marker expression (data not shown). Supplementation with EVs, however, did not rescue intrahepatic CD11b+F4/80+ or CD11b+Gr1+ cell frequencies in mice with primary shRab27a-KPC tumors (Fig. 5H, I). These results indicate that EVs from pancreatic cancer cells can contribute to myeloid accumulation in the healthy liver, but also suggest that non-EV-dependent mechanisms may play a role in Rab27a-mediated myeloid modulation.

Knockdown of Rab27a results in increased expression of EMT related pathways and invasive morphology of primary tumors. To understand how expression of Rab27a at the primary tumor site affects metastatic outgrowth, we have designed experiments, where GFP-scr-KPC or GFP-shRab27a-KPC cells were orthotopically injected into pancreata of WT mice, and then GFP-scr-KPC or GFP-shRab27a-KPC cells were injected intrasplenically, at the time when we know that the immune niche at the distant organ sites has been established (Fig. 5A,B). This is consistent with studies by other groups, which suggest that difficulty with depletion of myeloid cells in livers of cancer-bearing mice could arise as a result of rapid repopulation by circulating cells. To understand if cancer-educated myeloid cells were sufficient to rescue metastatic outgrowth of shRab27a-KPC primary tumors, we adoptively transferred splenic myeloid cells from tumor-bearing CD45.1 mice (Fig. 5E). The presence of CD45.1 cells was verified at 3 weeks in the spleen as well as in liver metastases by immunohistochemistry (Supplementary Figure 5C). We observed that supplementation with myeloid cells partially rescued metastatic outgrowth of shRab27a-KPC cells at 3 weeks post-injection by counting both micro and macrometastases (micro≤200μm, macro≥200μm) (Fig. 5F). This suggests that addition of cancer-educated myeloid cells can partially compensate for deficient metastatic outgrowth in the absence of Rab27a expression in cancer cells. Partial nature of such rescue could be due to either insufficient number of transferred myeloid cells, requirement for liver-resident myeloid cell expansion as opposed to spleen and/or the possibility that additional non-myeloid-cell dependent mechanisms contribute to the successful outgrowth of Rab27a-deficient cells at metastatic sites. To understand if EVs were sufficient to modulate myeloid cell frequency, we isolated EVs from supernatants of scr-KPC cells. EVs or PBS control were retro-orbitally injected into WT mice or mice bearing scr-KPC or shRab27a-KPC primary tumors (Fig. 5G). WT healthy mice treated with EVs demonstrated a significant increase in intrahepatic macrophages compared to control treated mice, however the increase in Gr1+ cells failed to reach statistical significance (Fig. 5H, I). Overall, we did not observe changes in MHC II or CD206 marker expression (data not shown). Supplementation with EVs, however, did not rescue intrahepatic CD11b+F4/80+ or CD11b+Gr1+ cell frequencies in mice with primary shRab27a-KPC tumors (Fig. 5H, I). These results indicate that EVs from pancreatic cancer cells can contribute to myeloid accumulation in the healthy liver, but also suggest that non-EV-dependent mechanisms may play a role in Rab27a-mediated myeloid modulation.
Figure 5. Rab27a knockdown increases initial metastatic seeding. (A) Schematic of experimental design to test the role of Rab27a in metastasis. (B) Representative image of GFP-KPC cell seeding in the liver 2 days post splenic injection as detected by GFP immunohistochemistry. (C) Quantification of GFP-scr-KPC and GFP-shRab27a-KPC cell seeding at 2 days post splenic injection. First label indicates the genotype of KPC cells orthotopically injected into the pancreas, followed by the genotype of the KPC cells injected into the spleen. (D) Quantification of GFP-scr-KPC and GFP-shRab27a-KPC cell seeding at 2 weeks post splenic injection. First label indicates the genotype of KPC cells orthotopically injected into the pancreas, followed by the genotype of the KPC cells injected into the spleen. (E) Schematic of experimental design to test if tumor educated myeloid cells can rescue metastatic outgrowth of shRab27a KPCs. (F) Quantification of percent of liver tissues covered by metastatic lesions from mice treated with PBS or tumor educated myeloid cells. (G) Schematic of experimental design to test the role of EVs in myeloid cell expansion. (H) Quantification of CD11b+ Gr1+ cells in WT, scr KPC, shRab27a mice treated with PBS or EVs. Proportion of CD45+ cells is indicated. Error bars indicate SEM; statistical testing performed using one-way ANOVA with Sidak post-hoc correction for multiple comparisons where applicable: NS – not significant, p value: * < 0.05; ** < 0.01; *** < 0.001.
epithelial-to-mesenchymal transition (EMT) drivers such as Snail and Slug (Fig. 6B). We validated the significant overexpression of these genes using QPCR analysis on \[^{\text{scr-KPC}}\] and \[^{\text{shRab27a-KPC}}\] cells (Fig. 6C). Knockdown of Rab27a correlated with decrease in differentiation markers and downregulation of Trefoil Factors that are thought to inhibit EMT (Supplementary Table 4)\(^{24-29}\). Furthermore, we identified multiple metalloproteases as well as growth factors associated with remodeling of extracellular matrix and EMT conversion such as MMP13, Wnt7a, PGF and FGF18 that were significantly upregulated in our dataset, providing further support for the perturbed tumor phenotype of \[^{\text{shRab27a-KPC}}\] (Supplementary Table 4)\(^{30-38}\). These findings suggested that Rab27a may suppress cell-autonomous regulation of EMT-like process in pancreatic cancer cells. In support of this idea, our analysis of primary tumors revealed that while \[^{\text{scr-KPC}}\] derived tumors maintained a continuous border and did not invade surrounding pancreatic tissue, \[^{\text{shRab27a-KPC}}\] tumors exhibited invasive morphology and colonization normal pancreatic tissue (Fig. 6D,E). Examination of histological sections indicated consistent predominance of lymphocytes over neutrophils in the Rab27a knockdown samples (scrambled LNR at 3.3 ± 2.9; shRab27a LNR at 3.2 ± 3.1, \(p = 0.958; n = 4 \) samples/group). Additionally, shRab27a-KPC tumors also demonstrate

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**Figure 6.** Knockdown of Rab27a results in increased expression of EMT related genes. (A) 10 representative diseases and functions of the 10 identified using Ingenuity Pathway Analysis of genes 2 fold up or down regulated between \[^{\text{shRab27a}}\] and scrambled infected KPC cells. The full list can be found in Supplementary Table 1. (B) Example network showing altered expression of EMT related genes. A full list of identified networks can be found in Supplementary Table 2. (C) Table of the number of tumors with invading tumor edge for \[^{\text{scr-KPC}}\] and \[^{\text{shRab27a-KPC}}\] at 2 and 4 weeks. Represented as the number tumors with an invading tumor edge out of the total number evaluated in that group. (D) Quantitative RT-PCR of SNAI1 and SNAI2 genes identified as upregulated by the microarray analysis. (E) Representative H&E of \[^{\text{scr-KPC}}\] and \[^{\text{shRab27a-KPC}}\] tumors demonstrating the margin between tumor and normal tissue in \[^{\text{scr-KPC}}\] and \[^{\text{shRab27a-KPC}}\] at 4x and 20x. (F) Quantification of Transwell invasion assay of \[^{\text{scr-KPC}}\] and \[^{\text{shRab27a-KPC}}\] cells in vitro. Error bars indicate SEM; \(p \) value: *<0.05; ***<0.001. Data represents 3 independent experiments.
disorganization of smooth muscle actin (SMA) positive regions, and loss of E-cadherin organization at the tumor margins (Supplementary Figure 6A,B). We also observed that shRab27a-KPC cells had elevated basal levels of phospho-Erk activation and were significantly more invasive in vitro (Fig. 6f and Supplementary Figure 6C).

Overall, these observations suggest that Rab27a may be important for suppressing some of the intrinsic abilities of cancer cells to invade tissue at early seeding stages. Furthermore, Rab27a-proficiency by cancer cells is important for their ability to colonize distant organs independent of pre-metastatic niche conditioning, potentially through alterations in EMT and/or mesenchymal-to-epithelial (MET) properties.

Discussion
Rab27a is a Rab GTPase that functions to facilitate extracellular vesicle release from the cells17. Function of Rab27a in cancer has been previously linked to non-cell autonomous effects of downregulating EV production in controlling tumor growth and metastasis40–42. Rab27a is only one component of protein machinery that is important for EV biogenesis. The selection of Rab27a is motivated by several factors: it has been shown to modulate EV biogenesis in the setting of cancer17 and has been indirectly implicated in facilitating pre-metastatic niche formation10. Rab27a is overexpressed in pancreatic cancer and predicts poor survival38. Furthermore, high-throughput efforts aimed at finding inhibitors of EV biogenesis for therapeutic purposes are underway, and understanding the consequences of targeting such pathway(s) on a genetic level, such as by decreasing Rab27a function would provide valuable information44. Thus, emerging data suggests, that Rab27a could in fact have both cell-autonomous as well as non-cell-autonomous effects on tumor progression45, however, this has not been yet studied in a fully immune-competent system in order to address both aspects.

Using a combination model of experimental liver metastasis and primary tumor seeding, we demonstrated that Rab27a has both a cell-autonomous and non-cell autonomous role in modulation of the immune niche and metastatic outgrowth. Our data reinforces the notion that immune cells accumulate not only in the cancerous lesions of the pancreas but also at the sites of future metastasis, such as the liver. EVs are utilized by numerous cell types, including cancer cells. We utilized an in vivo mouse model in which we could alter EV release by knocking down Rab27a. Additionally, we added EVs isolated in culture back into mice with shRab27a-KPC tumors. By utilizing an in vivo model of pancreatic cancer, we show that primary tumors lead to expansion of myeloid subsets in distant sites such as the spleen, liver, and lung. This systemic regulation can promote seeding of metastasis-incompetent pancreatic cancer cell lines in the liver. Expression of Rab27a at the site of the primary tumor mediates not only changes in EV production, but regulates expression of multiple secreted factors associated with EMT processes and tumor remodeling. Consistent with this idea, downregulation of Rab27a led to decreases in myeloid subsets both at the primary tumor site and distant sites such as spleen, lung, and liver. We noticed that depending on the organ site, the nature of immune regulation by Rab27a was distinct: although the type of cells differed – both macrophages and MDSC were downregulated in liver, while MDSCs alone were decreased in the primary pancreatic tumor and macrophages alone were decreased in the spleen and lung. This suggests that additional systemic sources, such as remaining GM-CSF may potentially contribute to recruitment of these cells. In addition to previously reported ability of primary pancreatic cancer to increase macrophage frequencies in livers, we report that Rab27a may also regulate different subtypes of myeloid cells in livers. However, in lungs and spleens, only macrophages are affected, whereas we also noticed that knockdown of Rab27a affected recruitment of Gr1+ cells to primary pancreatic lesions. Therefore, it seems that either different populations of immune cells at targeted organ sites are regulated by Rab27a proficient cancer cells; or there might be distinct types of systemic cues that are regulated by Rab27a that confer organ specificity.

To understand how expression of Rab27a affects tumor progression in an immune-competent model, we pre-conditioned mice by implanting primary tumors for two weeks. Surprisingly, direct delivery of Rab27a knockdown cells to livers of pre-conditioned mice revealed an increase in the short-term seeding ability. We found that downregulation of Rab27a perturbed expression of genes related to cell motility and increased an EMT gene signature in the cancer cells. This was consistent with results reported in hepatocellular carcinoma cells and could be a result of enhanced internal signaling by the components of unreleased EVs45. Concordant with this, primary tumors were much more invasive with downregulation of Rab27a. In contrast, primary tumors with downregulated Rab27a expression could not support long-term seeding of metastatic nodules. This could reflect overall changes in inflammatory modulators produced by Rab27a knockdown cells. Since knockdown of Rab27a resulted in increased EMT, one possibility is that these cells may have impaired ability to undergo mesenchymal-to-epithelial transition (MET), which is characterized by reversion to the epithelial phenotype and has been implicated in potentiating successful metastatic outgrowth46.

Overall, our work identified that the GTPase Rab27a functions in both a cell-autonomous as well as a non-cell-autonomous fashion to affect tumor progression. Disrupted EV biogenesis in cancer cells by virtue of downregulation of Rab27a may have a cell-autonomous role in promoting EMT processes that promote primary tumor invasion and increase the initial seeding rate of metastases, whereas systemic factors regulated by Rab27a may be necessary to re-program pre-metastatic niche and facilitate long-term survival of metastatic lesions.

Materials and Methods
All experiments were performed in accordance with relevant guidelines and regulations.

Mice. All mouse protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. All animals were maintained in specific pathogen-free facilities. Six to eight week-old wild-type C57Bl/6 mice were purchased from the Charles River Laboratories (stock #027). Both males and female mice were used for orthotopic and splenic injections of PDA cells. For orthotopic injection of cancer cells into the pancreas, mice were anesthetized using a Ketamine (100 mg/kg)/Xylazine (10 mg/kg) cocktail administered via intraportal injection. Depth of anesthesia was confirmed.
by verifying the absence of a toe pinch response. A small incision was made in the left flank and 100,000 KPC cells in ice-cold PBS mixed at a 1:1 dilution with Matrigel (#354234, Corning) in a volume of 50ul was injected into the tail of the pancreas using a 28-gauge needle. The incision was closed in two layers, with running 5-0 Vicryl Rapidite sutures (Ethicon) for the body wall, and interrupted 5-0 Prolene sutures (Ethicon) for the skin. Pain was managed using subcutaneous administration of Buprenorphine (0.1 mg/kg). For spleen injections, 500,000 KPC cells in 100ul of ice cold PBS were injected into the spleen using a 28-gauge needle. Mice receiving a splenic injection after a primary orthotopic injection were injected with 200,000 KPC cells. Mice were euthanized by carbon dioxide-induced narcosis at two weeks or four weeks post injection of cancer cells. Tumors were measured using digital calipers and processed for histology and flow cytometry. Livers, lungs, and spleens were collected and processed for histology and flow cytometry.

**Pancreatic Cancer Cell lines.** The murine PDA cell line 4662 was derived from primary pancreatic tumors LSL-KrasG12D/+; LSL-Trp53R172H/+; p48Cre/+ (KPC) mice on C57Bl6/J background, as reported in Bayne et al., and was a kind gift from Dr. Vonderheide.

**Extracellular vesicle collection.** KPC cells were seeded in 10 cm dishes and allowed to reach 70% confluence and the media was changed to serum-free DMEM and incubated for an additional 24–48 hours. Supernatant was collected, spun briefly, filtered through a 0.22μm filter to remove cell debris, and incubated with 40% PEG overnight at a ratio of 1:4 (PEG to supernatant). Supernatant and PEG solutions were then spun at 3500 rpm for 30 minutes. Supernatant was discarded and EVs were resuspended in PBS and quantified on the NanoSight. For mice treated with extracellular vesicles, the vesicles were quantitated by a Pierce BCA assay (Thermo Fisher). Mice were treated with 5μg of extracellular vesicles diluted in PBS or PBS alone as a control by retro-orbital injection. Mice were treated with extracellular vesicles two days prior to orthotopic implantation, the day of implantation, and then 3 times per week until harvest.

**Proliferation and viability assays.** For KPC growth assays, cells were seeded at a density of 1,000 cells/well in a 96-well tissue culture plate. At indicated time points, the cell culture medium was aspirated and the wells were washed with RPMI (without phenol red, BioWhittaker). 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) was added to the wells for 2 hours at 37 °C. The reagent was aspirated and 100μl of DMSO was added to each well for 20 minutes at room temperature. Plates were read at an absorbance of 570 nm with background subtraction at 690 nm using a Synergy 2 microplate reader (BioTek). For the day 0 time point, the cells were treated with the MTT reagent 12-18hrs after plating.

**Invasion assay.** Transwells (8 μm) (VWR) were coated with 2μg of Matrigel per well and allowed to dry overnight. 100,000 KPC cells were seeded on top of the coated-chambers in 300ul serum free DMEM ( Gibco). The lower chambers contained 800ul of complete media. Inserts were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells remaining in the top chamber were scraped by cotton swabs, while the invading cells were stained with crystal violet (Sigma) for 5 minutes at room temperature. Each Transwell was imaged at 10x magnification with 3 individual images counted per well. The assay was run in triplicate.

**Lymphocyte isolation.** Single cell suspensions were prepared from tumors, spleens, livers, and lungs. Spleens were mechanically disrupted and resuspended in 1% FBS/PBS. Pancreatic tumors were minced and digested with 1.25 mg/ml collagenase IV (Cat. No.LS004188, Worthington), 1 mg/ml Hyaluronidase (Cat. No. LS 002592, Worthington), 0.1% Trypsin Inhibitor from soybean (Cat. No. T9128, Sigma), and 100μg/ml DNase I (Cat. No. LS002007, Worthington) in RPMI for 30 minutes at 37 °C. Livers were minced and digested in 1mg/ml collagenase IV (Cat. No.LS004188, Worthington) and 100μg/ml DNase I (Cat. No. LS002007, Worthington) in RPMI for 30 minutes at 37 °C. Lungs were perfused, minced, and digested in 0.5 mg/ml Collagenase IV (Cat. No.LS004188, Worthington) and 100μg/ml DNase I (Cat. No.LS002007, Worthington) in RPMI for 45 minutes at 37 °C. Cell suspensions were passed through a 70μm strainer and resuspended in 1%FBS/PBS. 1x RBC lysis buffer (eBioscience, #00-4333-57) was used according to manufacturers instructions. Lymphocytes by OptiPrep (Sigma) density gradient centrifugation. Tumor-infiltrating leukocytes were enriched using CD45-microbeads (Miltenyi, #130-052-301). MDSCs were isolated using the MDSC isolation kit (Miltenyi, #130-094-538). Once isolated, cells were washed and blocked with an α-CD16/CD32 antibody (BD Biosciences, Clone 2.4G2) for 5 minutes on ice and then stained with labeled antibodies against surface markers for 30 minutes in 1% FBS/PBS. Cells were also stained with Live/Dead Aqua (Thermo Fisher, #L34966) in PBS to determine viability and fixed in 2% PFA. The following mAbs directed against mouse antigens were used for flow cytometry: α-Gr1-FITC (Clone: RB6-8C5, Biolegend), or α-Ly6C-AF488 (Clone: HK1.4, Biolegend) and α-Ly6G-AF700 (Clone: 1A8, Biolegend), α-F4/80-PerCP-Cy5.5 (Clone: BM8, Biolegend), α-CD45-Pacific Blue (Clone: 104,
Biolegend), α-CD11b-APC (Clone: M1/70, Biolegend). All samples were acquired on LSR II or LSRII Fortessa (BD Biosciences) at the UNC Flow Cytometry Core Facility and analyzed using FlowJo (Treestar, Inc.).

**Immunohistochemistry.** Mouse tumors, spleens, livers and lungs were fixed in 10% phosphate-buffered formalin (Fisher Scientific) for 24–48 hours and embedded in paraffin at the UNC Animal Histopathology and Laboratory Medicine Core. Six micron sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched using a solution of 1% hydrogen peroxide (30% hydrogen peroxide stock, Sigma) in methanol at room temperature for 10 minutes. Antigen retrieval was done in 10 mM sodium citrate/0.05% Tween-20 (pH 6) for 15 minutes in a microwave oven for all antibodies other than Gr1. For antigen retrieval for Gr1 slides were treated with pronase (0.05 mg/ml) for 10 minutes at 37 °C. Blocking was performed for 1 hour at room temperature, in 10% goat serum/10 mM Tris-HCl/0.1 M magnesium chloride/1% BSA/0.5% Tween-20. Sections were incubated with primary antibodies overnight at 4 °C in humidified chambers. Secondary biotinylated goat-anti-rabbit or goat-anti-rat antibody (Vector Laboratories) was diluted in 2% BSA/PBS and incubated for 1 hour at room temperature. Tertiary ABC solution was prepared according to manufacturer’s instructions (Vectastain ABC kit, Vector Laboratories) and was incubated for 45 minutes at room temperature. Stain was developed using 3,3′-diaminobenzidine tetrahydrochloride kit (DAB peroxidase substrate kit, Vector Laboratories). Slides were counterstained with Harris hematoxylin (Sigma), dehydrated and mounted with DPX mounting media (Sigma). The following antibodies were used: α-GFP (Cell Signaling Technologies, 1:200), α-CD45 (BD Biosciences, 1:200), and α-Gr1 (Biolegend 1:100). Livers were cut at sequential depths. Specifically, sections were collected by advancing the block by 25 microns and before collecting another set of sections. This was done for at least 4 steps for each liver. Multiple liver sections were stained and counted for each time point. Early liver seeding was counted by scanning each of the liver sections at 20x magnification and counting the total number of GFP-positive cells for the section. Metastatic out growth was counted by scanning the H&E stained sections at multiple depths at 10×.

**Immunofluorescence.** Mouse tumors, spleens, livers, and lungs were fixed in 10% phosphate-buffered formalin (Fisher Scientific) for 24–48 hours and embedded in paraffin at the UNC Animal Histopathology and Laboratory Medicine Core. Six micron sections were deparaffinized and rehydrated. Antigen retrieval was done in 10 mM sodium citrate/0.05% Tween-20 (pH 6.1) for 15 minutes in a microwave oven. Blocking was performed for 1 hour at room temperature, in 10% chicken serum/10 mM Tris-HCl/0.1 M magnesium chloride/1% BSA/0.5% Tween-20. Sections were incubated with primary antibodies overnight at 4 °C in humidified chambers. Sections were stained with chicken anti-rabbit alexa fluor 488 and chicken anti-rat alexa fluor 594 (Life Technologies) secondary antibodies diluted in 2% BSA/PBS and incubated for 1 hour at room temperature. Additional staining antibodies were incubated for 1 hr followed by relevant secondary for one hour at room temperature. Nuclei were stained with DAPI (Molecular Probes) and slides were mounted with Prolong Gold Anti-fade mountant (Life Technologies) and coveredslipped. The following primary antibodies were used for immunofluorescence staining: α-phospho-histone 3 (Millipore, 1:150), α-smooth muscle actin (Abcam, 1:200), α-CK8 (DHB, trona-1, 1:100), and α-E-cadherin (Abcam, 1:200).

**Adoptive transfer of CD11b+ myeloid cells.** CD45.1 mice were orthotopically implanted with 150,000 KPC cells. Approximately 2 weeks post-implantation, the spleens from the CD45.1 mice were collected and CD11b+ cells were isolated using a CD11b positive-selection kit (StemCell Technologies, #18103). Isolated CD11b+ cells were counted and resuspended in PBS. Approximately 4 × 10⁶ CD11b+ cells or equal volumes of PBS were injected via the tail vein into CD45.2 C57Bl/6 mice. The following day the CD45.2 mice underwent a splenic injection of 500,000 scr-KPC or shRab27a-KPC cells. Mice were collected at 3 weeks post-splenic injection.

**Microarray methods.** Microarray samples were prepared and run by the UNC Functional Genomics Core. In short, Total RNA (250 ng) was used to synthesize fragmented and labeled sense-strand cDNA and hybridized onto Affymetrix arrays. The Affymetrix GeneChip® WT PLUS Reagent Kit Manual was followed to prepare the samples. Briefly, the GeneChip® WT PLUS Reagent Kit (Affymetrix) was used to generate sense-strand cDNA from total RNA. Following synthesis of sense-strand cDNA, the cDNA was fragmented and labeled with the kit. Fragmented and labeled cDNA was used to prepare a hybridization cocktail with the Affymetrix GeneTitan Hybridization Wash and Stain Kit for WT Arrays. Hybridization, washing, staining and scanning of the Affymetrix peg plate arrays was carried out using the Affymetrix GeneTitan MC Instrument. GeneChip Command Console Software (AGCC) was used for GeneTitan Instrument control. Affymetrix Expression Console Software was used for basic data analysis and quality control. Microarray gene expression data can be found at NIH Gene Expression Omnibus (GEO) repository under accession #GSE148439.

**Quantitative PCR (qPCR) analysis.** RNA was purified from cells using the RNaseasy mini kit (Qiagen, 74134). RNA was normalized to 1 μg of total RNA and cDNA was generated using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, K1671). qPCR analysis was performed using PerfectTaq SYBR Green SuperMix (Quanta Biosciences) and the Applied Biosystems (ABS) 7500 real-time PCR system. Results were normalized to the expression of GAPDH and then calculated using ΔΔCT. All samples were run in triplicate. SNAI1 (forward 5′ CTGGTGAGAAAGCACCATTCTCCT 3′ and reverse 5′ CCTGGGACCTGTATCTCCTCA 3′) SNAI2 (forward 5′ TGGTCAAGAAACATTTCAACGCC 3′ and reverse 5′ GGTGAGGATCTGCTGTTGTTGGA 3′) primers were used for PCR amplification.

**Statistical analysis.** To obtain sufficient statistical power, at least 5-12 mice were used in each group, and the experiments were repeated a minimum of 3 times to validate reproducibility. Group means were compared with student’s t-test or ANOVA with a Sidak post-hoc test. Statistical analysis were performed using GraphPad Prism software. Data are presented as mean ± s.e.m. A p-value of less than 0.05 was considered statistically significant.
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Author contributions
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Competing interests
The authors declare no competing interests.

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