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Role of Chitinase 3-like-1 and Semaphorin 7a in Pulmonary Melanoma Metastasis

Bing Ma[1,2], **Erica L. Herzog**[1,3], **Chun Geun Lee**[1,2], **Xueyan Peng**[3], **Chang-Min Lee**[2], **Xiaosong Chen**[3], **Sara Rockwell**[4], **Ja Seok Koo**[5], **Harriet Kluger**[5], **Roy S. Herbst**[5], **Mario Sznol**[5], and **Jack A. Elias**[2,3,6]

[2]Department of Molecular Microbiology and Immunology, Brown University, Providence R.I. 02912

[3]Yale University School of Medicine, Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, 300 Cedar Street (TAC S441), New Haven, CT 06520

[4]Department of Therapeutic Radiology Yale Cancer Center, PO Box 208040, 333 Cedar Street (213E HRT), New Haven, CT 06520-8040

[5]Yale University School of Medicine, Section of Medical Oncology, Department of Internal Medicine, Yale Cancer Center, PO Box 208028, 333 Cedar Street, New Haven, CT 06520-8028

Abstract

The prototypic chitinase-like protein Chi3l1 is induced in cancers and portends a poor prognosis, but whether it contributes to cancer progression is unknown. To address this gap in knowledge we investigated the production of Chi3l1 in melanoma lung metastases. We found that Chi3l1 was induced during pulmonary melanoma metastasis and that this induction was regulated by the semaphorin Sema7a, interacting in stimulatory or inhibitory ways with its β1 integrin or Plexin C1 receptors, respectively. In mouse strains with genetic deletions of Chi3l1 or Sema7a, there was a significant reduction in pulmonary metastasis. Notably, antiserum raised against Chi3l1 or Sema7a phenocopied the reduction produced by genetic deletions. Melanoma lung metastasis was also decreased in the absence of IL-13Rα2, a recently identified receptor for Chi3l1, consistent with a key role for Chi3l1 in melanoma spread. We confirmed roles for Sema7a and Chi3l1 in pulmonary metastasis of EMT6 breast cancer cells. Taken together, our studies establish a novel pathway through which Sem7a and its receptors regulate Chi3l1, revealing a host axis involving IL-13Rα2 that plays a critical role in generating a pulmonary microenvironment that is critical to license metastasis.

Keywords

Chi3l1; IL-13Rα2; melanoma; Lung metastasis; plexin C1; β1 integrin

[6]Corresponding author: Jack A. Elias, M.D., Program in Medicine and Biology, Warren Alpert Medical School, Brown University, Box A-1, 97 Waterman Street, Providence, RI 02912, jack_elias@brown.edu, 401-863-3336.

[1]equal contribution

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INTRODUCTION

The glycosyl hydrolase family 18 (GH 18) proteins are members of an ancient gene family that exists in species as diverse as plants, insects and man (1,2). This gene family contains true chitinases (Cs) which degrade chitin polysaccharides and chitinase-like proteins (CLPs) which bind to but do not degrade chitin. These 18 GH moieties have evolved during speciation with an impressive increase in CLP coinciding with the appearance of mammals (1). This retention over species and evolutionary time has led to the belief that these moieties play an essential role(s) in biology. However, the role(s) of these moieties in normal physiology and the ways they contribute to disease pathogenesis have not been adequately defined.

Chitinase 3-like-1 [Chi3l1, also called breast regression protein 39 (BRP-39) in the mouse and YKL-40 in human], is the prototypic mammalian CLP. It was originally discovered in mouse breast cancer cells (3). It is now known to be expressed by a variety of cells including macrophages, neutrophils, epithelial cells, smooth muscle cells and chondrocytes and is stimulated by a number of mediators including IL-13, IL-6, IL-1β, and IFN-γ (4–7). In keeping with these diverse sources and stimuli, elevated levels of Chi3l1 have been noted in a wide variety of diseases including infections, arthritis, inflammatory bowel disease, chronic obstructive lung disease, hepatitis, diabetes, atherosclerosis and giant cell arteritis (4–6,8,9). Studies over recent decades have also demonstrated that the levels of circulating Chi3l1 are increased in many malignancies including cancers involving the lung, prostate, colon, rectum, ovary, kidney, breast, glioblastomas and malignant melanoma (5,10,11). In these cases, the levels of Chi3l1 frequently correlate directly with disease progression and inversely with disease free interval and patient survival (10,12–14). Recently studies from our laboratory demonstrated that IL-13 receptor α2 (IL-13R2), a known decoy receptor for IL-13, functions as a receptor for Chi3l1 (15). Although, IL-13Rα2 can participate in inflammation, remodeling and tumor development (16–21), the roles that Chi3l1 and Chi3l1-IL-13Rα2 interactions play in the pathogenesis of these disorders have not been defined. This is particularly striking for the malignancies where the pathways that regulate Chi3l1 production have not been appropriately defined, the roles of Chi3l1 and IL-13Rα2 in disease progression have not been adequately addressed and the consequences of interventions that block Chi3l1 induction on metastatic spread have not been elucidated.

Malignant melanoma, a disease of transformed melanocytes, is one of the most aggressive forms of cancer. It accounts for only 4 % of skin cancers but causes 80% of skin cancer deaths (22). It is the 6th most common cancer in the USA and is increasing faster than any other malignancy (22,23). While there is a good chance of recovery for patients suffering from melanoma if the primary lesion is detected early, the 5 year survival of patients with distant melanoma metastases (stages III and IV) is less than 10% (22). Patients with malignant melanoma (MM) have increased levels of circulating Chi3l1 which has been shown to be a risk factor for disease progression (13,24,25). However, the mechanisms that stimulate Chi3l1 in this setting and the roles of host Chi3l1 in the pathogenesis of melanoma metastasis have not been defined. In addition, the consequences of interventions that alter this melanoma- Chi3l1 response have not been defined and the pathways that these interventions utilize to regulate Chi3l1 have not been described.

The semaphorins are a large family of phylogenetically conserved, secreted and membrane-bound proteins that are divided into 8 classes based on sequence similarities and distinct structural features (26). A variety of studies have documented the ability of members of this family to act as axon guidance molecules, and semaphorin abnormalities have been implicated in the pathogenesis of neurologic disorders such as Alzheimer's disease and motor neuron degeneration (26). Semaphorins are also expressed on myeloid and lymphoid cells including B cells, T cells, NK cells and macrophages and have been implicated in immune responses and the regulation of organogenesis, angiogenesis, apoptosis and neoplasia (26–28). Among these, semaphorin 7a (Sema7a) induces focal adhesion kinase (FAK) and MAPK activation via integrin β1 engagement. It also negatively regulates melanocyte dendricity via the receptor Plexin C1 (29). Interestingly, a loss of Plexin C1 expression is frequently observed during melanoma metastasis, and Plexin C1 expression correlates inversely with the depth of melanoma invasion (29,30). This suggests that Sema7a plays an important role in the development and progression of malignant melanoma. However, the pathways that melanoma cells use to regulate Sema7a and the contributions of Sema7a to melanoma metastasis have not been adequately defined.

We hypothesized that host Chi3l1 plays an essential role in the pathogenesis of malignant melanoma and that interventions that alter the induction of Chi3l1 decrease the metastatic spread of this tumor. To test this hypothesis we characterized the ability of B16-F10 melanoma cells to stimulate Chi3l1 in wild type mice and their ability to generate pulmonary metastasis in wild type (WT) mice and mice with null mutations of Chi3l1. These studies highlight a novel host pathway in which Sema7a stimulates the production of Chi3l1 by interacting with β1 integrin and inhibits Chi3l1 by interacting with Plexin C1. They also demonstrate that the host Sema7a-Chi3l1-IL-13Rα2 axis plays a critical role in the generation of a metastasis-permissive pulmonary microenvironment leading to melanoma lung metastasis.

MATERIALS AND METHODS

Genetically modified mice

Chi3l1 null mutant ($Chi3l1^{-/-}$) and Chi3l1/YKL-40 transgenic (Tg) mice were generated and characterized in our laboratory as previously described (7,31). $Sema7a^{-/-}$ mice were provided by Dr. A.L. Kolodkin (Johns Hopkins University, Baltimore, MD). The IL-13Rα2 null and Plexin C1 null mice were previously described (26,32). All animals were anesthetized with Ketamine/Xylazine (100mg/10mg/kg) before any intervention was performed. All experiments were performed according to the NRC *Guide for the Care and Use of Laboratory Animals* and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) under the supervision of the Institutional Animal Care and Use Committee (IACUC) at Yale University.

Double-label IHC and Neutralization

Double-label IHC was undertaken using a modification of procedures described previously by our laboratory (7). Detailed information on the antibodies used are provided in Supplementary Materials.

Immunoblot analysis

Protein lysates were prepared from whole lungs using RIPA lysis buffers and subjected to immunoblotting using a modification of procedures described previously by our laboratory (7).

Administration of melanoma and breast cancer cells

The mouse melanoma cell line (B16-F10) established from C57BL6/J mouse melanoma was purchased from ATCC (Cat#: CRL-6475, Manassas, VA, STR verified). After being cultured to confluence in Dulbecco's Modified Eagles Medium (DMEM), the cells were collected, adjusted to a concentrations of 10^6 cells/ml and delivered to the mice by tail vein injection (2×10^5 cells/mouse in 200 μl of DMEM) (33). The EMT6 breast cancer cell line was purchased from ATCC (Cat# CRL:2755, STR verified) and was freshly prepared by Dr. S. Rockwell (Therapeutic Radiology, Yale School of Medicine) before *in vivo* injection. EMT6 cells (2×10^4 cells/moues in 200 μl of DMEM) were delivered via tail vein injection to Balb/c WT and Chi3l1$^{-/-}$ mice, because these cells only survive in Balb/c animals (34).

In vivo silencing of Sema7a

To characterize the role of Sema7a on EMT6 metastasis WT mice were employed that were treated with non-target control (Sigma-Aldrich, MISSION ShRNA, SHC002) or validated Sema7a-specific shRNA (Sigma-Aldrich, MISSION ShRNA, TRCN0000067540) via intratracheal (i.t.) inoculation (10^7 virion/mouse).

Assessment of melanoma and breast cancer metastasis in the lung

Melanoma lung metastasis was quantified by counting the number of melanoma colonies (which appear as black dots) on the surface of the lung. For the evaluation of EMT6 metastasis, the lungs were harvested, treated with Bouin's fixative overnight and the number of colonies on the surface of the lungs was counted under an operating microscope.

Assessments of Chi3l1, Sema7a mRNA and or protein in the lung

The levels of pulmonary Chi3l1/BRP-39 and Sema7a mRNA and protein were assessed using real-time RT-PCR (RT-PCR), Western blotting or ELISA as previously described by our laboratory (7,35).

Assessment of the effects of recombinant Sema7a on Chi3l1

Normal human volunteers were recruited from the Translational Lung Research Program at Yale. All studies were performed with HIC approval. CD14$^+$ monocytes were isolated from PBMC using the Miltenyi negative selection kit and these cells were stimulated for 48 hours with rSema7a as we have previously described (36). In selected experiments neutralizing antibodies against β1, β3, αv or α1 integrins and appropriate controls were employed as described by Kang et al (37). Murine peritoneal macrophages were collected after 5 days after thioglycollate treatment (3% thioglycollate, 3ml/mouse I.P.), stimulated with recombinant Sema7a-Fc and its FC control (R & D Systems, Minneapolis, MN) for 72 hours. In these evaluations, human and murine rSema7a was used at a dose of 5nM/ml. The

timing and dose of Sema7a were chosen based on *in vitro* dose response and kinetic evaluations (Supplementary Fig. 1 and data not shown)

Statistics

Statistical evaluations were undertaken with SPSS software. As appropriate, groups were compared with 2-tailed Student's *t* test or with nonparametric Mann-Whitney *U* test. Values are expressed as mean ± SEM. Statistical significance was defined as a level of $P < 0.05$.

RESULTS

Pulmonary Melanoma Metastasis and Chi3l1 Regulation in Wild Type Mice

To assess the mechanisms of pulmonary melanoma metastasis, wild type (WT) mice received B16-F10 malignant melanoma cells or vehicle via tail vein injection and melanoma metastasis were assessed at intervals thereafter. Ten to fourteen days after melanoma cell injection, metastatic melanoma colonies were readily appreciated in lungs from WT mice that received malignant cells (Fig. 1A and B). To address the possibility that Chi3l1 might contribute to these metastatic responses, we compared the expression of Chi3l1 in lungs from WT mice with and without melanoma metastasis. Injection of B16-F10 cells increased the expression of Chi3l1 mRNA and protein in lungs and BAL fluids from WT mice (Fig. 1C–E). In WT lungs with metastatic melanoma, double label immunohistochemistry (IHC) demonstrated that Chi3l1 protein was most prominent in macrophages and epithelial cells in the normal tissues surrounding the metastases (Fig. 1F). Additional IHC evaluations did not reveal significant numbers of CD4+, Cd11b(+), or Ly-6G(+) cells and the few that were seen did not express significant amounts of Chi3l1 (Supplementary Fig. 2 and data not shown). These studies demonstrate that intravenously administered malignant melanoma cells readily metastasize to the lung where they stimulate pulmonary Chi3l1 expression.

Role of Chi3l1 in Pulmonary Melanoma and Breast Cancer Metastasis

To define the role(s) of host Chi3l1 in melanoma metastasis, we compared the metastatic responses in WT mice and Chi3l1 null ($Chi3l1^{-/-}$) mice. These studies demonstrated that null mutations of Chi3l1 ameliorated melanoma metastasis (Fig. 2A and B). Diminished melanoma metastasis could also be seen in comparisons of WT mice that were treated with antibodies against Chi3l1 or appropriate controls (Fig. 2C and D). This effect was not melanoma-specific because the metastasis of EMT6 breast cancer cells to the lung was similarly decreased in the absence of Chi3l1 (Fig. 2E and F). It was also not lung-specific because the growth of subcutaneous melanoma cells was also blunted in Chi3l1 null mice (Supplementary Fig. 3). These studies demonstrate that host Chi3l1 plays a critical role in the generation of a metastasis permissive microenvironment in the murine lung.

Sema7a in Lungs With and Without Metastasis

We next characterized the expression of Sema7a in lungs from WT mice with and without melanoma metastasis. These studies demonstrated that mRNA encoding Sema7a and Sema7a protein could be appreciated in lungs from WT mice and were further induced in lungs from mice with metastatic tumor (Fig. 3A and B). They also highlighted the stimulation of soluble and tissue Sema7a in BAL fluids and lungs from mice with metastatic

melanoma (Fig. 3C). Importantly, metastatic EMT6 cells also stimulated pulmonary and BAL Sema7a accumulation (Supplementary Fig. 4A). These studies demonstrate that melanoma and breast cancer cell metastatic spread to the lung also induces Sema7a.

Role of Sema7a in Melanoma and Breast Cancer Metastasis

To define the *in vivo* role(s) of host Sema7a in melanoma metastasis, B16-F10 melanoma cells were injected intravenously into mice with wild type (+/+) and null (−/−) Sema7a loci and the metastatic response was assessed at intervals thereafter. As shown in Fig. 3D and E, melanoma metastasis was significantly decreased in lungs from Sema7a null mice compared to WT controls. Similar results were obtained with antibodies that neutralize Sema7a (Fig. 3F and G) and in comparisons of Balb/c WT mice treated with Sema7a shRNA versus scrambled controls (Fig. 3H and I). These studies demonstrate that Sema7a plays an important role in the pathogenesis of melanoma and breast cancer metastasis in the lung.

Relationship Between Chi3l1 and Sema7a

Because null mutations of Chi3l1 or Sema7a both reduced pulmonary melanoma metastasis, studies were undertaken to determine if they were part of the same or different regulatory pathways. To do this, we evaluated the expression of Chi3l1 in the lungs from WT and Sema7a null mice and the expression of Sema7a in lungs from WT and Chi3l1 null mice. Interestingly, null mutations of Sema7a significantly decreased the expression of Chi3l1 in melanoma-challenged mice (Fig. 4A). Similarly, the silencing of Sema7a also decreased the levels of Chi3l1 in mice challenged with EMT6 cells (Supplementary Fig. 4B and data not shown). In contrast, the levels of Sema7a protein were not altered in mice that lacked Chi3l1 (Fig. 4B). These studies demonstrate that Sema7a is a critical stimulator of Chi3l1 in the murine lung.

Role of Sema7a Receptors

The studies noted above demonstrate that Sema7a is an important regulator of Chi3l1. To further understand this process we used *in vitro* approaches to define the roles of the known Sema7a receptors (β1 integrin and Plexin C1) in this regulation. In these experiments we isolated human $CD14^+$ monocytes from PBMCs and stimulated them for 48 hr with a recombinant human (rh)Sema7a or a heat-inactivated control. As shown in Fig. 4C, Chi3l1 production was significantly higher in the rSema7a-stimulated monocytes compared to controls ($p=0.013$, $n=10$ in each group). In another group of *in vitro* experiments we isolated WT mouse peritoneal macrophages and stimulated them with a recombinant murine(rm) Sema7a-Fc fusion protein or its Fc control for 72 hr. Sema7a had a similarly impressive ability to stimulate Chi3l1 in these assays (Fig. 4D). However, these stimulatory effects of Sema7a were markedly decreased when neutralizing anti-β1 integrin antibodies were added with the Sema7a (Fig. 4D). This result was at least partially β1 specific because antibody neutralization of β3 integrin did not have a similar effect (Supplementary Fig. 5). This β1 integrin appeared to be complexed with αv integrin because a neutralizing antibody against αv but not α1 had a similar inhibitory effect on Sema7a stimulation of Chi3l1 (Fig. 4E). In marked contrast, the null mutations of Plexin C1 and antibody neutralization of Plexin C1 caused a marked increase in the ability of Sema7a to stimulate Chi3l1 (Fig. 4F and G).

These studies demonstrate that Sema7a stimulates Chi3l1 via interacting with its β1 integrin receptor in complex with αv integrin and that interactions with Plexin C1 inhibit this response.

To further understand the importance of β1 integrin and Plexin C1 *in vivo* experiments were also undertaken. In these experiments we compared the levels of melanoma metastasis in mice treated with anti-β1 integrin or controls and WT mice and mice with null mutations of Plexin C1. As can be seen in figure 5, neutralization of β1 integrin caused a significant decrease in B16 cell metastasis which was associated with a significant decrease in the levels of BAL Chi3l1 (Fig. 5, A–C). In contrast, melanoma metastasis and the levels of BAL Chi3l1 were significantly increased in mice that contained null mutations of Plexin C1 (Fig. 5, D–F). Thus, in accord with our *in vitro* studies, these experiments demonstrate that β1 integrin and Plexin C1 play important roles in the stimulation and inhibition of melanoma metastasis and Chi3l1 elaboration respectively.

To further understand the mechanisms that might play a role in the noted β1 integrin-mediated cellular responses, studies were undertaken to determine if Sema7a-stimualted macrophage expression of IL-1β and IL-6 and activated PBK/Akt and MAPK-Erk signaling. These studies demonstrated that rSema7a stimulated the expression of IL-1β and IL-6 and activated the Akt and Erk signaling pathways (Fig. 5, G and H). Studies with anti-β1 integrin antibody also demonstrated that these events were mediated by a β1 integrin-dependent mechanism(s) (Fig. 5, G and H).

Role of IL-13Rα2 in Melanoma and Breast Cancer Lung Metastasis

Recent studies from our laboratory have demonstrated that Chi3l1 binds, signals and modulates cellular and tissue responses via IL-13Rα2 (15). Thus, studies were undertaken to define the role of IL-13Rα2 in the effects of Chi3l1 in pulmonary metastasis. Comparisons of WT and IL-13R2 null mice demonstrated that the spread of melanoma or EMT6 cells to the lung is markedly decreased in the absence of IL-13Rα2 (Fig. 6, A–D). When viewed in combination these studies highlight the important role(s) that IL-13Rα2 plays in pulmonary metastasis.

Regulation of LIM Kinase and Cofilin

Previous studies demonstrated that melanoma metastasis and invasion are associated with decreased expression of LIM kinase (LIMK) and reduced levels of phosphorylated Cofilin (P-Cofilin) (30). Because Sema7a inactivates (phosphorylates) Cofilin via Plexin C1 and LIM kinase 2 (LIMK2) (which phosphorylates Cofilin) is upregulated by Sema7a via Plexin C1 (30), we evaluated the activation status of LIMK2 and Cofilin in the lungs from WT, Chi3l1 null, and Plexin C1 null mice challenged with vehicle (B16−) or tumor (B16+). As shown in Fig 7A and 7B, LIMK2 and P-Cofilin were readily apparent in lungs from WT mice without metastatic disease and the expression of both were decreased by melanoma or EMT6 cell metastasis. Interestingly, null mutations of Chi3l1 partially abrogated these tumor-induced suppressive effects suggesting that they are mediated, in part by Chi3l1 (Fig. 7, A and B). As can be seen in new Fig. 7C, melanoma challenge significantly reduced the levels of LIMK2 and P-Cofilin in WT mice and these inhibitory events were also further

augmented by null mutations of Plexin C1. These findings confirm the important role that Plexin C1 plays in the regulation of LIMK2 and inactivation of Cofilin. They also demonstrate that LIMK2, Cofilin phosphorylation and Chi3l1 elaboration track together in this setting and further support the possibility that Chi3l1 suppresses Cofilin phosphorylation.

DISCUSSION

To characterize the biologic and pathobiologic functions of Chi3l1 and its role in cancer metastasis we defined the processes that regulate the production of Chi3l1 and its role in the pathogenesis of pulmonary melanoma metastasis. These studies reveal a novel relationship between Chi3l1 and Sema7a and its receptors with Sema7a interacting with integrin β1 to stimulate and with Plexin C1 to inhibit Chi3l1. They also define the importance of this pathway in the pathogenesis of pulmonary metastasis by demonstrating that host Chi3l1 and Sema7a both play critical roles in the generation of a metastasis-permissive pulmonary microenvironment. These studies also demonstrate that the Chi3l1 receptor IL-13Rα2 also plays a significant role in lung melanoma metastasis.

Chi3l1 is located in chromosome 1 and is produced by a variety of cells including macrophages and epithelial cells. A striking feature of this CLP is its impressive regulatability with the levels of circulating and or tissue Chi3l1 being increased in many diseases and models of these disorders (4–6,8,15). A number of cytokines including IL-13, IFN-γ, IL-1β and IL-6 are known to stimulate Chi3l1 production (4–7). The degree to which other pathways stimulate these important CLPs, however, has not been defined. In addition, although the levels of Chi3l1 can be decreased in oxidant injury states(38), the pathways that decrease the production of these moieties have not been defined. Our studies add to our understanding of the biology of Chi3l1 by demonstrating, for the first time, that Sema7a regulates Chi3l1 by interacting with stimulatory β1 integrin and inhibitory Plexin C1 receptors. Sema7a provides axon guidance signals, enhances axonal growth and has been implicated in immune responses and the regulation of organogenesis, angiogenesis, apoptosis and neoplasia (27,39). It is tempting to speculate that some of these effects are mediated, at least in part, by this Sema7a-Chi3l1 pathway.

Metastasis is an ominous feature of malignant solid tumors and an ultimate reason for the death of cancer patients (40). It occurs as the result of a multistep process in which cancer cells acquire the ability to enter the blood or lymph, disseminate in the circulation, extravasate through the endothelium, invade local connective tissues and withstand local anti-tumor defenses (41). This is readily apparent in malignant melanoma which wreaks havoc mostly via its propensity to spread and establish metastatic lesions at distant sites (42). Studies of the metastatic process have highlighted the importance of the seed (the tumor cells) and the soil (the host) in metastatic spread. Our studies add new information to the current understanding of the metastasis-regulating effects of the host by demonstrating that Sema7a and Chi3l1 play critical roles in the generation of tumor permissive pulmonary “soil”. The finding that Sema7a and Chi3l1 play similar roles in melanoma and breast cancer metastasis demonstrates that these roles are not melanoma-specific and instead may be a general property of pulmonary metastatic spread. The demonstration that Chi3l1 plays a

critical role in melanoma growth in subcutaneous tissues also suggests that the role of Chi3l1 in metastasis is not lung-specific. Previous studies demonstrated that the levels of circulating Chi3l1 are increased in patients with advanced stage melanoma (13,24,25). When these and our present studies are viewed in combination, they suggest that Chi3l1 is a biomarker that associates with advanced melanoma and that the Sema7a-Chi3l1 pathway is a therapeutic target that can be manipulated to regulate the development or progression of melanoma and breast cancer metastasis.

Our studies demonstrate that null mutations of host Chi3l1, interventions that decrease its production or accumulation and anti-Chi3l1 antibodies markedly decrease melanoma metastasis. Although the mechanisms that underlie the contributions of Chi3l1 to melanoma metastasis have not been defined, knowledge of the biology of metastasis and the effector functions of Chi3l1 raise a number of interesting possibilities. Studies of melanoma and other metastasis have highlighted the importance of tumor cell survival, inflammation, a type 2 dominant cytokine microenvironment, and the presence of specific cytokines like TGF-β1 in distant tumor progression (43–48). In accord with these findings, studies from our laboratory have demonstrated that Chi3l1 inhibits inflammatory and epithelial cells apoptosis, augments type 2 immune responses and stimulates M2 macrophage differentiation and TGF-β1 elaboration (7,38). Importantly, our studies also demonstrate that β1 integrin interacts with Sema7a to induce Chi3l1 and that antibodies against β1 also decrease Chi3l1 and tumor metastasis. These observations suggest that the effects of anti-β1 are mediated, at least in part, by its effects on Chi3l1. However, it is also important to point out that β1 integrin also stimulates melanoma adherence to extracellular matrix and motility (49) and, as shown in this manuscript, play a critical role in Sema7a stimulation of inflammatory cytokine elaboration, and Akt and MAPK/Erk signaling. They also demonstrate that Plexin C1 plays an important role in Sema7a-Chi3l1 regulation of the tumor regulating LIMK2/Cofilin pathway. When viewed in combination the Sema7a, β1 integrin and Plexin C1 studies allow one to envision the pathway hypothetically laid out in figure 7D. However, additional experimentation will be required to determine if these moieties interact in other important ways in the regulation of pulmonary metastasis.

IL-13Rα2 was originally identified as a decoy receptor for IL-13. Recent studies from our laboratory demonstrated that IL-13Rα2 is also a receptor for Chi3l1 that mediates a variety of effects of Chi3l1 including its ability to inhibit cellular apoptosis (15). Interestingly, the expression of IL-13Rα2 is frequently dysregulated in the development or progression of tumors and vaccination against IL-13Rα2 was shown to prevent or decrease of tumor development or progression (16,17). In combination, these studies suggest that IL-13Rα2 plays an important role in tumor biology. However, the mechanism(s) underlying these roles of IL-13Rα2 have not been clearly defined. The current studies address this issue by suggesting that IL-13Rα2, contributes to the lung melanoma or breast cancer spread by mediating the cellular and tissue effects of Chi3l1 that promote metastasis.

In conclusion, our studies characterize the Sema7a-Chi3l1-IL-13Rα2 axis, describe its induction at sites of pulmonary metastasis, and define its critical role in the generation of a tumor permissive host microenvironment. Additional investigations of the Sema7a-Chi3l1-

IL-13Rα2 axis, and its roles as a disease biomarker and or therapeutic target in pulmonary metastasis are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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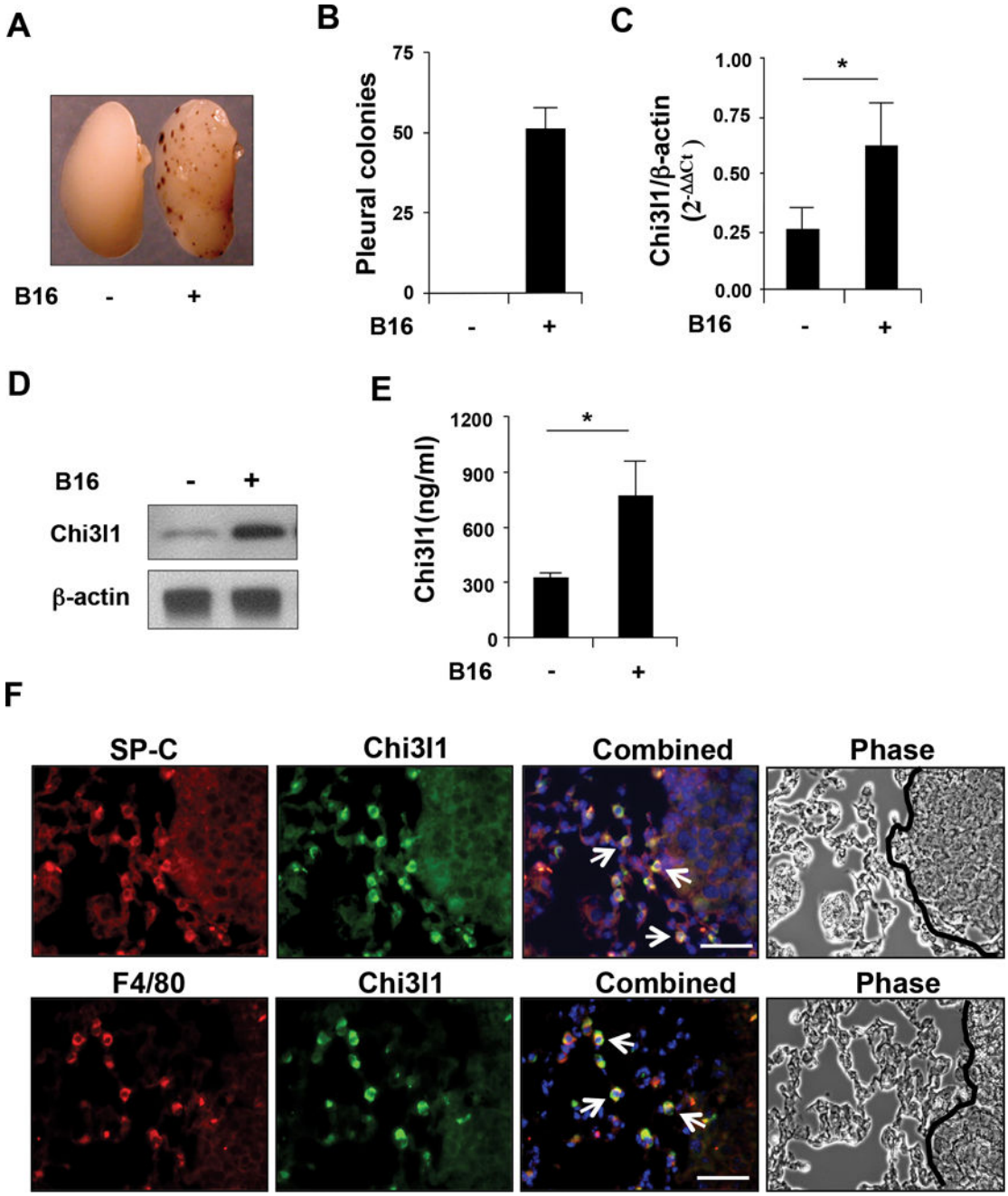

Figure 1. Pulmonary melanoma metastasis and expression of Chi3l1 in lungs from WT Mice

WT mice were given melanoma cells or vehicle control and evaluated 2 weeks later. A, representative lungs. B, the number of pleural melanoma colonies. C, the levels of Chi3l1 mRNA in lung lysates. D and E, the expression of BAL Chi3l1 protein was evaluated using Western Blot and ELISA evaluations on the lung samples from the mice treated with vehicle (−) and B16-F10 tumor cell challenge (+). F, the colocalization of the expression of Chi3l1 with the macrophage marker F4/80 and alveolar epithelial cell marker SP-C in melanoma colonized lungs. Arrows indicate the colocalized cells. The line in the phase view was used to demarcate the tumor and parenchymal areas of the lung. Blue dots in the combined view

indicate DAPI positive nuclei. Panels A, D, F are representative of a minimum of 5 similar experiments. The values in panels B, C, E represent the means± SEM of evaluations in a minimum of 4 mice. Bar in Panel F = 50μm. *P<0.05.

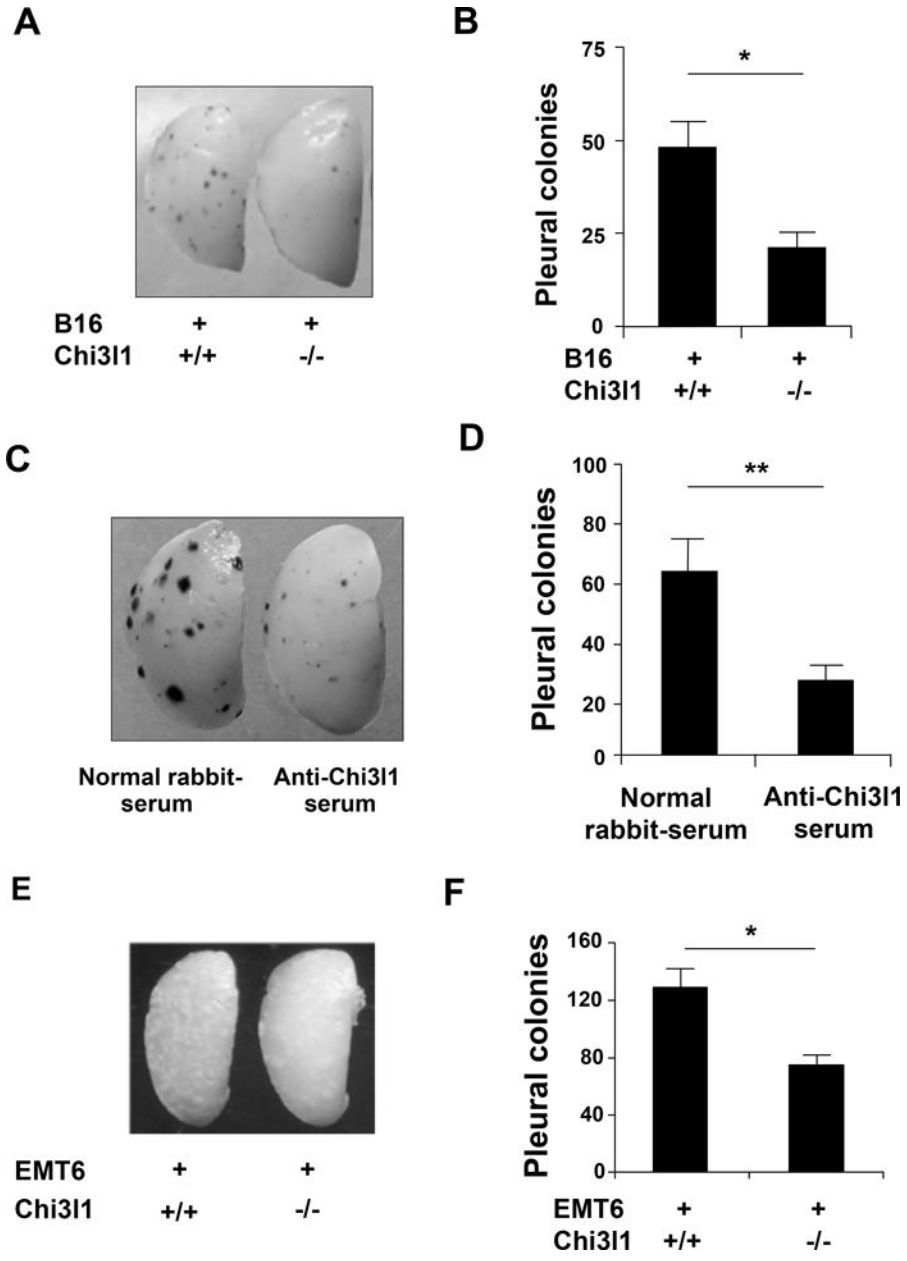

Figure 2. Role of Chi3l1 in pulmonary melanoma or EMT6 breast cancer cell metastasis

WT and Chi3l1 null mice ($Chi3l1^{-/-}$) were given melanoma or EMT6 breast cancer cells and lung metastasis were evaluated 2 weeks later. A and B, the levels of melanoma metastasis in WT mice and Chi3l1 null mice. C and D, the levels of melanoma metastasis in WT mice treated with rabbit anti-Chi3l1 or control serum. E and F, EMT6 breast cancer metastasis in WT mice and Chi3l1 null mice. Panels A, C, E are representative of a minimum of 5 similar experiments. The values in panels B, D, F represent the means± SEM of evaluations in a minimum of 4 mice. *P<0.05; **P<0.01.

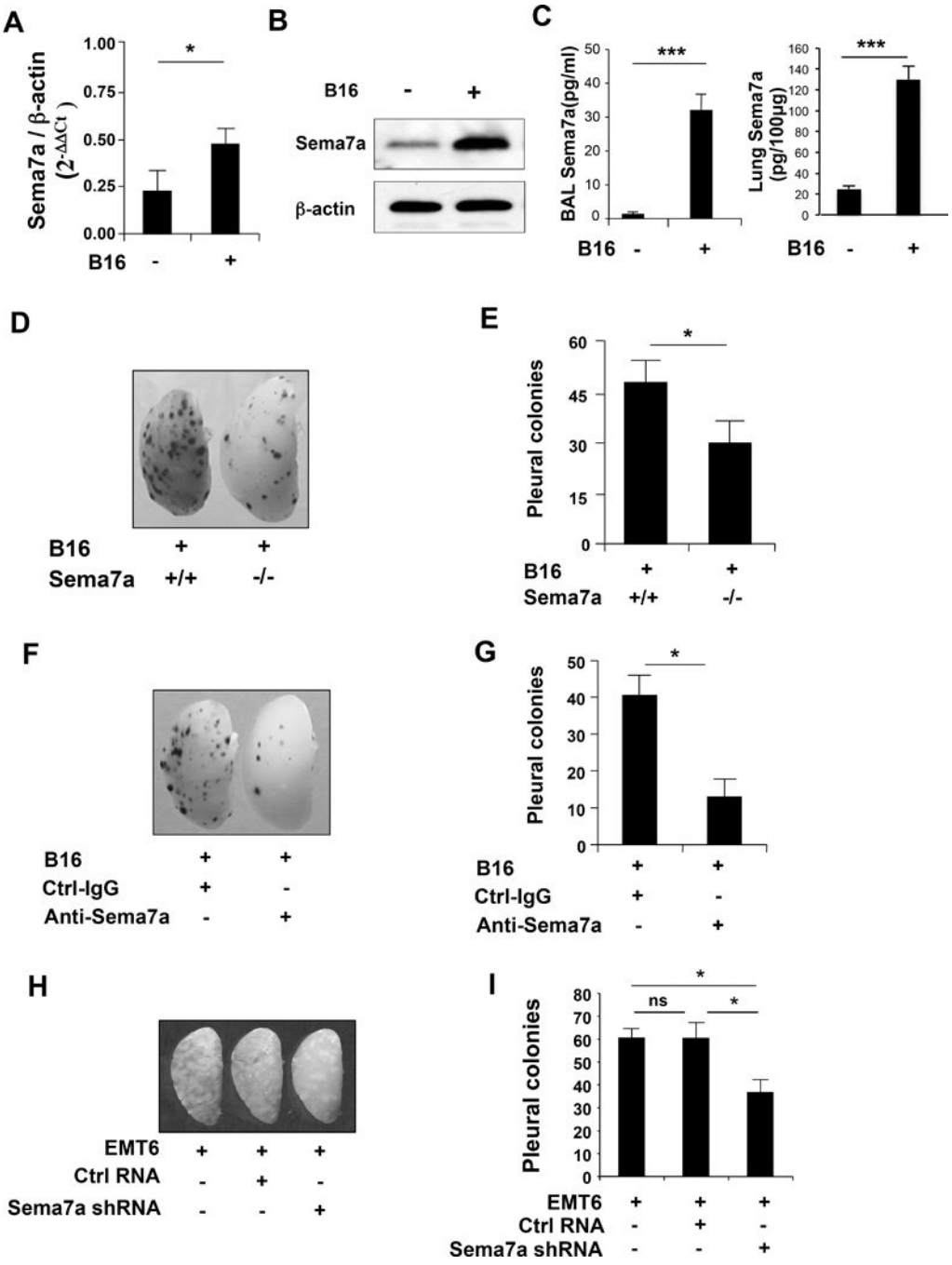

Figure 3. Role of Sema7a in pulmonary melanoma or breast cancer metastasis

A–C. WT mice were given melanoma cells or vehicle control and the levels of mRNA encoding Sema7a, Sema7a protein in lung lysates and Sema7a in BAL and lung tissues were evaluated 2 weeks later by qRT-PCR, Western blot and ELISA, respectively. In the ELISA evaluations of lung lysates the levels of sema7a are normalized to lung protein. D–E, WT and Sema7a null (Sema7a$^{-/-}$) mice were challenged with melanoma cells and metastasis were evaluated 2 weeks later. F and G, WT mice were challenged with melanoma cells and metastasis were evaluated 2 weeks later with control (Ctrl) IgG or anti-Sema7a antibody

treatment (35μg/mouse/every other day). H and J, WT mice were challenged with EMT-6 breast cancer cells with and without silencing of Sema7a using control (Ctrl) or Sema7a-specific shRNA. Panels B, D F and H are representative of a minimum of 5 similar experiments. The values in the remaining panels represent the means± SEM of evaluations in a minimum of 4 mice. *P<0.05; ***P<0.001

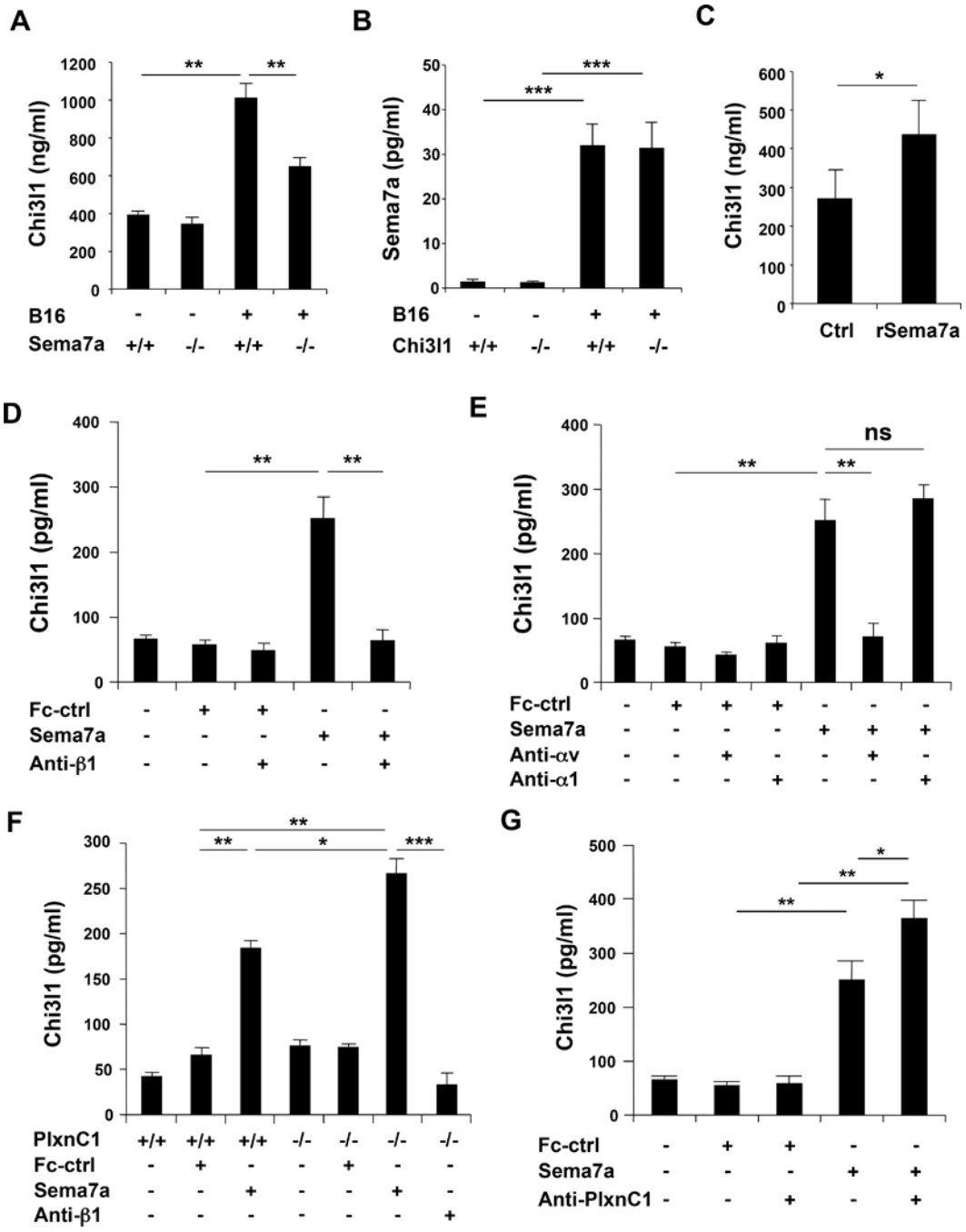

Figure 4. Relationships between Chi3l1 and Sema7a and its receptors

WT mice were bred with mice with wild type (+/+) or null (−/−) Chi3l1or Sema7a loci. A, the levels of BAL Chi3l1 were evaluated by ELISA. B the levels of Sema7a in BAL fluid were evaluated by ELISA. C, human monocytes isolated from PBMC were stimulated with recombinant (r)Sema7a for 48 hrs and the levels of Chi3l1/YKL-40 protein were evaluated by ELISA (n=10 in each group). D and E, murine peritoneal macrophages were stimulated with Sema7a-Fc or Fc control(Fc-crtl) in the presence of or absence of antibodies that block β1 integrin, αv integrin, or α1 integrin and the levels of Chi3l1/YKL-40 protein were

evaluated by ELISA. F, murine peritoneal macrophages were obtained from WT (+/+) and Plexin C1 null (−/−) mice and their ability to produce Chi3l1 in response to Sema7a was evaluated by ELISA in the presence and absence of anti-β1 integrin antibody. G, murine peritoneal macrophages were stimulated with Sema7a-Fc or Fc control (Fc-crtl) in the presence of absence of Plexin C1 antibody. The noted values represent the means ± SEM of evaluations in a minimum of 4 mice each. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

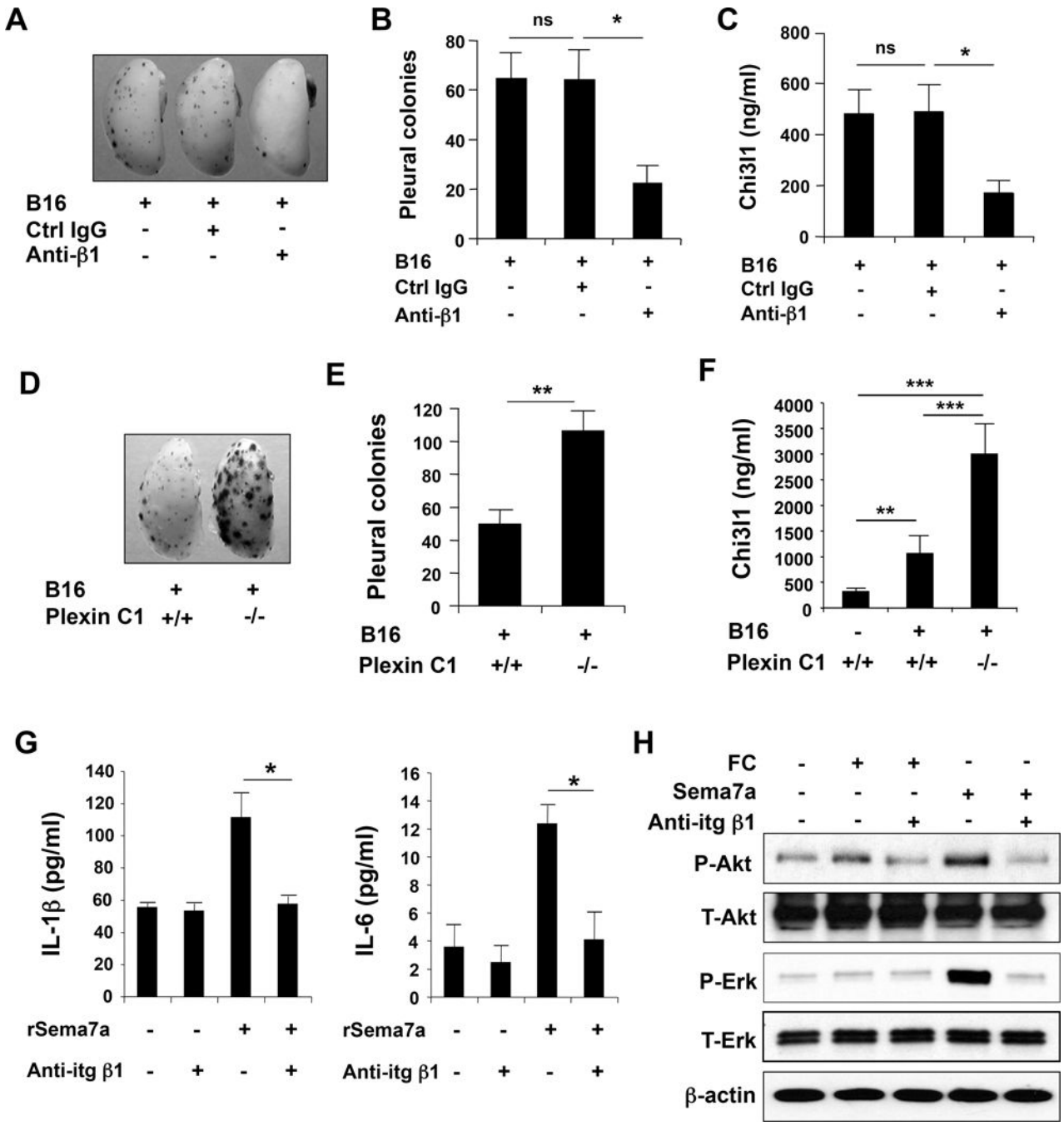

Figure 5. Inter-regulation between Chi3l1 and Sema7a and its receptors

A–C, WT mice were challenged with B16 melanoma cells and the effects of anti-β1 integrin on melanoma lung metastasis and Chi3l1 expression. Representative lungs(A), the number of metastatic colonies(B) are illustrated and the levels of BAL Chi3l1 were assessed by ELISA(C). D–F, WT(+/+) and Plexin C1 null(−/−) mice were challenged with melanoma cells and the levels of metastatic spread were evaluated. Representative lungs (D), the number of pleural colonies, and BAL Chi3l1 assessed by ELISA (E) were illustrated. G and H, murine peritoneal macrophages were stimulated recombinant (r)Sema7a (5nM/ml) for 48

hours with and without anti-integrin β1 neutralizing antibody and the levels of IL-1β and IL-6 in culture supernatant were measured by ELISA. H, the levels of activated (phosphorylated) and total Akt or Erk were evaluated by Western blots. Panels A, D, and H are representative of a minimum of 5 similar experiments. The values in the remaining panels represent the means± SEM of evaluations in a minimum of 4 mice. *P<0.05; **P<0.01, ***P<0.001; ns, not significant.

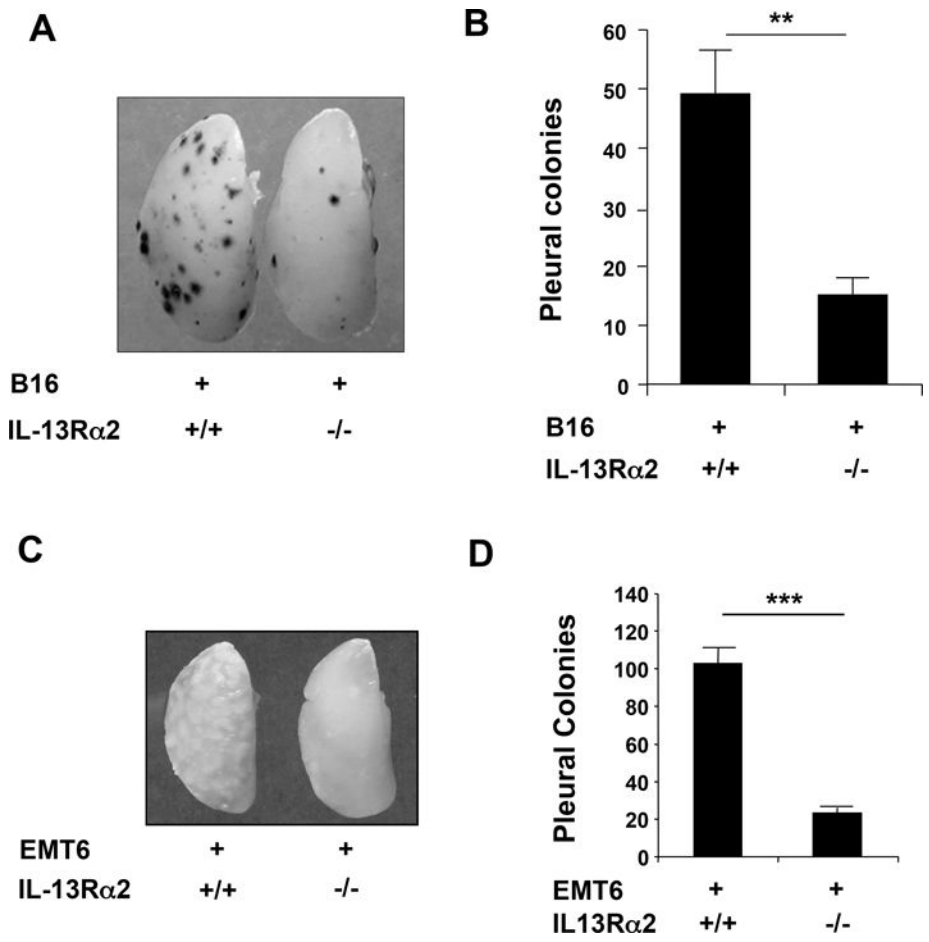

Figure 6. Roles of IL-13Rα2 in melanoma or breast cancer lung metastasis

WT mice with WT (+/+) and null (−/−) IL-13Rα2 loci were injected with melanoma or breast cancer cells or vehicle control and lung metastases were evaluated 2 weeks later. A and C, melanoma metastasis in representative lungs. B and D, the number of metastatic pleural colonies. Panels A and C are a representative of a minimum of 5 similar experiments. The values in panel B and D represent the means± SEM of evaluations in a minimum of 4 mice. **P<0.01.

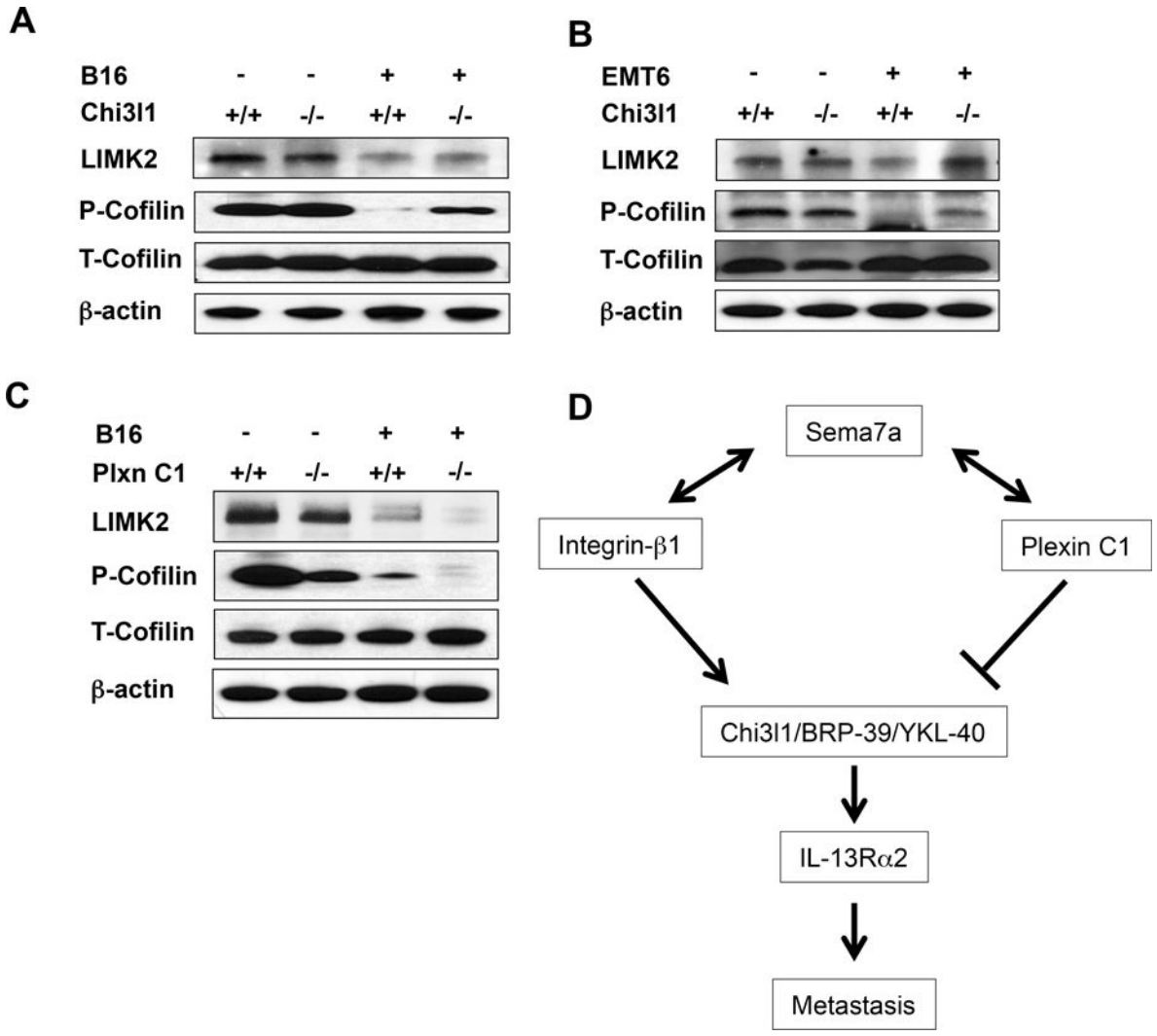

Figure 7. Chi3l1 regulation of LIMK2/P-Cofilin and Schematic illustration of the Sema7a-Chi3l1-IL-13Rα2 axis in pulmonary melanoma metastasis

WT mice with WT (+/+) and null (−/−) Chi3l1 or Plexin C1(−/−) loci were injected with melanoma or breast cancer cells or vehicle control. A–C, After 2 weeks later, lung lysates from these mice were subjected to Western blot evaluations for LIM kinase 2 (LIMK2), phosphorylated (P) and total (T) Cofilin with β-actin internal control. D, Sema7a stimulates Chi3l1 by interacting with β1 integrin (associated with αv integrin). This process is inhibited by Sema7a interaction with Plexin C1. The Chi3l1 that is produced contributes to the establishment of a metastasis permissive microenvironment, at least in part, by interacting with IL-13Rα2.