Published in final edited form as:

Nat Cell Biol. 2018 August ; 20(8): 966–978. doi:10.1038/s41556-018-0138-8.

Pericyte-Like Spreading by Disseminated Cancer Cells Activates YAP and MRTF for Metastatic Colonization

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Abstract

Metastatic seeding by disseminated cancer cells principally occurs in perivascular niches. Here, we show that mechanotransduction signaling triggered by the pericyte-like spreading of disseminated cancer cells on host tissue capillaries is critical for metastatic colonization. Disseminated cancer cells employ cell adhesion molecule L1 (L1CAM) to spread on capillaries and activate the mechanotransduction effectors Yes-associated protein (YAP) and myocardin-related transcription factor (MRTF). This L1CAM-mediated spreading is robust enough to displace resident pericytes, which also use L1CAM for perivascular spreading. L1CAM activates YAP by engaging $\beta 1$ integrin and integrin linked kinase (ILK). L1CAM-YAP signaling enables

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AUTHOR CONTRIBUTIONS

E.E.E., M.V. and J.M. conceptualized the project. E.E.E., M.V., K.G. and S.M. designed and performed the experiments. Y.Z. performed bioinformatics analyses. S.A. and B.G. assisted with the experiments. F.G. contributed to experimental design. M.S. provided the *L1cam* flox mice. E.B., A.B. and M.R. provided clinical samples and interpretation. E.E.E., K.G., M.V.C and J.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

the outgrowth of metastasis-initiating cells both immediately upon their infiltration of target organs and after they exit from a period of dormancy. Our results identify an important step in the initiation of metastatic colonization, define its molecular constituents, and provide an explanation for the widespread association of L1CAM with metastatic relapse in the clinic.

Tumors abundantly release cancer cells into the circulation, but only a small proportion of these cells succeed at infiltrating and surviving in distant organs. Following a period of latency that can last from months to years, disseminated cancer cells may grow and form clinically overt metastatic lesions^{1–3}. Once metastasis is manifest, current treatment strategies often fail to eliminate it. Metastatic colonization involves a varied set of organ-specific interactions between the disseminated cancer cells and their surrounding stroma⁴. The diversity of these interactions and their underlying molecular mechanisms, together with the intrinsic heterogeneity of tumors, pose serious challenges to the development of treatments against disseminated cancer. Identification of common mediators of metastatic colonization in multiple organs is therefore of critical importance.

A common characteristic of cancer cells, visualized by intravital imaging during metastatic colonization, is their prevalent ability to intimately interact with the vasculature after they extravasate in secondary organs such as the brain, lungs and liver^{5–7}. Perivascular localization is thought to be advantageous primarily because it provides disseminated cancer cells with ready access to oxygen, nutrients and endothelium-derived paracrine factors that enhance cell self-renewal, proliferation and survival^{8, 9}. The perivascular niche also influences the latency and eventual outbreak of disseminated cancer cells^{10, 11}. Metastatic seeding within the perivascular space and subsequent interaction with the blood vessels, a process known as vascular cooption, precede macro-metastatic outgrowth and angiogenesis^{5, 12, 13}. But even though capillaries supply plenty of paracrine factors, oxygen and nutrients, recent observations in mouse models of brain metastasis by breast and lung cancer revealed that contact with brain capillaries was not sufficient for colony outgrowth of aggressive metastatic cells¹⁴. In addition to perivascular localization, cancer cells needed to spread over the abluminal surface of the vessels, which is encapsulated by a collagen- and laminin-rich basal lamina, in order to grow and form colonies. This spreading and subsequent outgrowth required the cell adhesion molecule L1CAM¹⁴, a molecule that is normally restricted to developing neurons and certain hematological and endothelial cells, but whose expression in many types of tumors is associated with an unfavorable clinical outcome^{15–17}.

Being a cell adhesion molecule, L1CAM has been implicated in cancer cell migration, an activity that has been demonstrated *in vitro*¹⁸. However, the precise role of L1CAM in cancer metastasis has remained unclear. Even though migration, proliferation and survival are important for metastasis, L1CAM deficient metastatic cells, which express many other pro-migratory genes, can reach the circulation and extravasate through brain capillaries, suggesting that L1CAM is not rate limiting for these steps of the metastatic cascade¹⁴. After extravasating in the brain parenchyma, L1CAM deficient cells fail to spread on the basal lamina of brain capillaries, proliferate and form macro-metastatic colonies, suggesting that L1CAM-mediated spreading is essential for initiation of metastatic colonization¹⁴.

In the present work, we have determined the mechanism and significance of L1CAM mediated metastatic outgrowth. We report that disseminated cancer cells use L1CAM to mimic pericytes, the perivascular mesenchymal cells that regulate blood vessel hemostasis, in their spreading on capillaries. This spreading results in activation of mechanotransduction effectors including YAP and MRTF, which we found to be necessary for colonization in multiple organs. At the molecular level, metastatic cells mimic a previously unknown reliance of pericytes on L1CAM for interacting with capillaries. We identify ILK, a core molecular component of pericyte spreading, as a downstream mediator of L1CAM-dependent YAP activation. These findings suggest a basis for L1CAM-dependent initiation of metastatic colonization in different organ sites by different tumor types, both by aggressive cancer cells and by latent metastatic cells that exit from a period of dormancy.

RESULTS

Pericyte-like spreading and pericyte competition during initiation of metastatic colonization

To define the significance of L1CAM-mediated vascular cooption in disseminated cancer cells, we initially used brain metastatic (BrM) derivatives of the KRAS-mutant human lung adenocarcinoma cell line H2030¹⁹ and the hormone receptor-negative human breast cancer cell line MDA231²⁰. H2030-BrM and MDA231-BrM cells breach the blood-brain barrier and adhere to brain capillaries within 3 to 6 days after hematogenous dissemination in mice¹⁴. The position and morphology of these cells were strikingly reminiscent of chondroitin sulfate proteoglycan 4 (Cspg4, also known as neural/glial antigen 2 – NG2) expressing pericytes (Fig. 1a). Pericytes are mesenchymal cells that wrap around capillaries and venules to control vascular wall permeability and homeostasis²¹. Pericytes and H2030-BrM cells also shared the ability to spread along tubules formed by endothelial cells on matrigel, whereas non-tumorigenic cells did not (Supplementary Fig. 1a). Because of these phenotypic similarities, we reasoned that studying metastatic cell-pericyte interactions and mechanisms of perivascular spreading would shed light on the basis for the initiation of metastatic colonization.

In order to study metastatic cell interactions within the perivascular niche, we imaged pericytes in the context of incipient metastatic lesions. Hematogenously disseminated H2030-BrM cells spreading on brain capillaries in mice *in vivo* appeared to wedge between pericytes and capillary surfaces (Fig. 1b). This could be explained either by an ability of metastatic cells to force pericytes out of position during extravasation, or by an active displacement of pericytes by cancer cells after extravasation. To better understand this process, we tracked it in an organotypic tissue culture model using 250 micron thick coronal sections of mouse brain cultured live for 2 days. Metastatic cells placed on top of these brain sections infiltrate the tissue, migrate towards capillaries, and then use L1CAM to spread and proliferate on capillary surfaces, which makes this model suitable for studying post-extravasation steps of brain metastasis seeding¹⁴. Approaching the endothelial cells and the resident pericytes from the abluminal side of the blood vessels, metastatic cells localized on top of pericytes or across from them on the same capillary (Supplementary Fig. 1b). Notably, we observed H2030-BrM and MDA231-BrM cells wedging between pericytes and

endothelial cells despite the initial abluminal orientation of the cancer cells (Supplementary Fig. 1b–c). Stromal cells closely interacting with cancer cells were positive for another pericyte maker, platelet-derived growth factor receptor β (PDGFR β), and not for oligodendrocyte progenitor marker O4, confirming these cells as pericytes (Supplementary Fig. 1d–e).

Next, we used genetically engineered mice expressing *Discosoma sp.* Red (DsRed) fluorescence reporter protein under the control of *Cspg4* promoter to identify pericytes during time-lapse confocal imaging of organotypic brain tissue cultures. Time-lapse imaging showed that H2030-BrM cells migrated along the vessels and dislodged pericytes that they consecutively encountered (Fig. 1c, Supplementary Fig. 1f, Supplementary Videos 1–2). Upon transit of metastatic cells between pericytes and endothelial cells, pericytes reattached to the capillaries suggesting that pericyte-cancer cell competition for the perivascular niche is more frequent than can be observed in still images. In contrast, when *L1CAM* knockdown cancer cells encountered pericytes they stalled or changed the direction of their migration on the capillary surfaces, and eventually adopted a round morphology (Fig. 1c, Supplementary Fig. 1f, Supplementary Videos 3–4). *L1CAM* knockdown cells did not detach from the capillaries suggesting that *L1CAM* was rate limiting specifically for the ability of cancer cells to spread on capillaries but not for adhesion or migration of these cells. Thus, metastatic cells expressing *L1CAM* can outcompete pericytes for position on capillaries. As these cells formed sheaths around the capillaries there was a reduction in pericyte coverage of involved capillary segments compared to adjacent vasculature devoid of cancer cells (Fig. 1d and Supplementary Fig. 1g).

To capture these close interactions between pericytes and cancer cells during metastasis initiation in human cancer, we turned to clinical tissues. As these interactions represent early metastatic events, we sought tissue from patients with clinically silent, untreated micrometastatic disease. We examined brain tissue obtained at autopsy of a treatment-naïve ER+ breast cancer patient and found occult brain metastases as small as 50 microns in diameter. Immunostaining of pericytes and vascular mural cells with alpha smooth muscle actin (aSMA) revealed that blood vessels harboring metastatic cells were incompletely covered by pericytes (Fig. 1e, Supplementary Fig. 1h). Within these areas of discontinuous aSMA staining, cancer cells were lodged on and around the vessel wall and stained positive for *L1CAM*. Using immunofluorescence we confirmed this close interaction between cancer cells, endothelial cells and mesenchymal pericytes (Supplementary Fig. 1i). Together with data obtained from mouse models of experimental metastasis, these results suggest that *L1CAM*+ metastatic cancer cells gain access to the perivascular niche and can displace pericytes at the initiation of metastatic colonization.

L1CAM is necessary for metastatic colonization in multiple organs

Pericytes cover capillaries in many tissues, and *L1CAM* expression in primary tumors is associated with metastatic relapse in multiple organs, not only the brain¹⁵. Therefore we investigated whether the requirement of *L1CAM* for metastasis, first observed in the brain, was a general phenomenon and applied in multiple organs. Whereas MDA231-BrM cells infiltrating the brain were elongated over the web-like cerebral capillaries for pericyte like

spreading, the lung metastatic derivative MDA231-LM²² adopted a stellate morphology with multiple protrusions upon infiltrating the pulmonary parenchyma (Fig. 2a, Supplementary Fig. 2a–b). These protrusions extended over the adjacent multi-branched capillary network of pulmonary alveoli, and were eliminated by RNAi-mediated knockdown of L1CAM expression in the cancer cells (Fig. 2a). Conversely, over-expression of L1CAM in a breast cancer model with moderate L1CAM expression, MDA-MB-468, increased spreading on brain tissue culture slices (Supplementary Fig. 2c–e). Metastatic cell protrusions resembled filopodia-like structures previously described in lung metastasis^{23, 24} and lung pericytes²⁵ suggesting that the pericyte-like spreading phenotype was manifested in different forms depending on the architecture of the capillary network in the host organ.

The involvement of L1CAM in lung colonization by MDA231-LM cells was also observed in metastasis from orthotopic tumors. *L1CAM* knockdown in MDA231-LM cells reduced their tumor growth potential by two-fold in mouse mammary fat pads (Fig. 2b), and also decreased spontaneous lung metastasis in these mice by more than 50-fold (Fig. 2c–d). L1CAM knockdown also inhibited vascular spreading of the disseminated cancer cells in the liver and lungs (Fig. 2e–f). To determine the effect of *L1CAM* knockdown in metastatic colonization of the lungs and other organs without its confounding effect on a source orthotopic tumor, we inoculated cancer cells directly into the circulation. *L1CAM* knockdown strongly inhibited lung colonization by MDA231-LM cells (Supplementary Fig. 2f–g) and bone colonization by MDA231-BoM cells (Supplementary Fig. 2h). We validated the role of L1CAM in metastasis in a syngeneic mouse lung cancer model. The 393N1 cell line derived from a genetically engineered *Kras*^{G12D}; *p53*^{−/−} mouse lung tumor²⁶ displays high frequency of brain metastasis from the circulation¹⁴ and spreads along brain capillaries (Supplementary Fig. 2i). We found that brain metastatic activity of 393N1 cells was strongly inhibited upon *L1cam* knockdown when cells were injected into the intracardiac circulation of immunocompetent mice (Supplementary Fig. 2j). L1CAM knockdown also significantly inhibited multi-organ and lung colonization by 786-M1A renal cancer cells and liver colonization by HCT116 colon cancer cells (Supplementary Fig. 2k–2m). Since L1CAM has been implicated in cell proliferation and survival¹⁸, we determined whether L1CAM regulation of proliferation and survival could explain the observed defect in metastatic colonization in cancer cells with *L1CAM* knockdown. L1CAM depletion did not affect the growth rate of the cancer cell lines in monolayer culture or their viability in suspension culture (Supplementary Fig. 2n–p).

These experiments left open the question of whether the role of L1CAM was limited to the steps of cancer cell extravasation and initial positioning on capillaries. To address this question, we determined whether L1CAM was required after the extravasation step by using a doxycycline-inducible L1CAM shRNA vector (Supplementary Fig. 3a–d). In the MDA231-LM, MDA231-BoM and H2030-BrM models, extravasation in target organs is complete in 2 days in lung and 7 days in brain after cell inoculation into the circulation^{5, 6, 14}. Dosing of doxycycline starting 9 days after cell injections inhibited the subsequent growth of macrometastases in lungs, bones and brain (Fig. 3a–d), demonstrating a post-extravasation role of L1CAM in all these sites.

Role of L1CAM during emergence from metastatic latency

The emergence of clinically manifest metastases is often preceded by a period of latency^{1–3}. During latency, metastasis-initiating cells exist in a dynamic equilibrium, in which intrinsic and extrinsic factors regulate cell entry into an immune-evasive, slow-cycling state¹⁰. To probe the role of L1CAM in this important stage of metastasis, we used HCC1954-LCC1 cells, a latency competent cancer cell model isolated from an early-stage HER2+ breast tumor¹⁰. Upon extravasating in the brain of athymic mice, HCC1954-LCC1 cells initially spread on brain capillaries¹⁰. *L1CAM* knockdown reduced this initial spreading (Supplementary Fig. 4a). Within 14 days, most of the disseminated cells cease to spread, become round and enter a slow proliferative state to remain latent for extended periods¹⁰. Periodic re-entry of these cells into the proliferative state triggers their expression of NKG2D ligands for cancer cell recognition and elimination by natural killer (NK) cells. Accordingly, NK interactions with HCC1954-LCC1 metastatic foci in brains of mice cells were readily observable even 30 days after intracardiac injections (Supplementary Fig. 4b). Depletion of NK cells with anti-asialo-GM1 ganglioside (anti-GM1) antibody in mice harboring latent HCC1954-LCC1 populations allows the progressive outgrowth of metastatic lesions¹⁰. Cancer cells that entered the proliferative state under these conditions formed colonies wrapping the vasculature, providing a model in which to test the role of L1CAM in metastatic outgrowth after elimination of innate immune surveillance (Supplementary Fig. 4c).

We engineered HCC1954-LCC1 with a doxycycline-inducible sh*L1CAM* vector and subjected the cells to this latency outbreak protocol (Fig. 4a). Doxycycline administration to mice starting on day 14 after cancer cell inoculation did not have a significant effect on metastatic foci in the presence of NK cells. However, induction of L1CAM knockdown two days prior to initiation of NK cell depletion, prevented HCC1954-LCC1 from spreading on capillaries and proliferating to form metastatic colonies, and inhibited the exit of these disseminated cancer cells from metastatic latency (Fig. 4b–c and Supplementary Fig. 4d–g). In sum, L1CAM was required for the outgrowth of aggressive metastasis-initiating cells immediately upon extravasation and of dormant metastatic cells upon exiting from quiescence (Fig. 4d).

L1CAM driven transcriptional programs for metastasis initiation

The perivascular environment is rich in oxygen, nutrients, and growth factors^{8,9}, yet metastatic cells that lose L1CAM expression fail to grow in this environment (Fig. 2–4 and Ref. 14). We postulated that L1CAM removes a proliferation checkpoint and licenses metastasis-initiating cells for growth in this context. To identify downstream events triggered by L1CAM mediated pericyte-like spreading, we investigated the gene expression pattern of H2030-BrM cells as a function of L1CAM-mediated interactions on brain capillaries. We engineered H2030-BrM cells with a vector encoding ribosomal protein L10a fused to EGFP, and performed translating ribosome affinity purification and mRNA sequencing (TRAP-seq)²⁷ of these cells after spreading on capillaries in brain tissue slices (Fig. 5a). The results of TRAP-seq showed that L1CAM-dependent spreading was associated with a distinct gene expression profile (Supplementary Fig. 5a). Gene set enrichment analysis of the data with classifiers that denote activation of specific pathways revealed a selective, L1CAM-

dependent increase in YAP and MRTF-A transcriptional signatures (Fig. 5b). Less pronounced gains occurred in Ras, hedgehog and nuclear factor κ B (NF- κ B) transcriptional signatures (Supplementary Fig. 5b). YAP signaling integrates cell-cell contact cues that regulate proliferation and actin cytoskeleton by controlling the activity of the YAP and TAZ transcriptional coactivators²⁸. MRTF-A (encoded by *MKL1*) is a coactivator that binds to serum response factor (SRF) in response to actin polymerization, and activates cell motility and adhesion programs²⁹. YAP and MRTF transcriptional programs are active in experimental metastasis^{30, 31}. Using qRT-PCR, we confirmed that L1CAM knockdown reduced the expression of YAP and MRTF-A target genes (Supplementary Fig. 5c–d).

Next, we engineered a lentiviral YAP promoter reporter construct to assess YAP activity *in situ*. By fusing a destabilization domain (DD)³² to the N-terminus of red fluorescence protein (RFP) under the control of YAP responsive promoter (8 $\times$ GTIIC)³³, we measured spatio-temporal information on YAP activity in each cell. In the absence of a stabilizer compound (trimethoprim), YAP-driven RFP expression is undetectable because RFP is quickly degraded. Adding the stabilizer to the culture media leads to accumulation of RFP. Conversely, in the absence of YAP activity, RFP is not transcribed due to lack of promoter activity (Figure 5c). YAP knockdown and latrunculin A treatment to prevent YAP activity in 2D culture resulted in reduction in RFP signal, validating our approach (Supplementary Figure 5e–f). L1CAM knockdown in MDA231-BrM cells and H2030-BrM cells significantly reduced RFP signal in brain tissue cultures, suggesting that L1CAM regulates YAP activity during pericyte-like spreading *in situ* (Figure 5d–e). However, L1CAM knockdown did not regulate phosphorylation of Hippo pathway kinases that inhibit YAP activity in response to cell-cell contact²⁸ (Supplementary Figure 5g). This suggests that L1CAM regulation of YAP is similar to YAP regulation by cytoskeleton and may be parallel to the Hippo pathway^{33–36}.

The potential involvement of YAP and MRTF-A as downstream effectors suggested an intriguing link between mechanotransduction signaling and L1CAM-dependent initiation of metastatic outgrowth. shRNA-mediated knockdown of YAP or TAZ showed a requirement for these transcription factors in brain metastasis, both in the H2030-BrM and MDA231-BrM models (Fig. 5f). Knockdown of MRTF-A/MKL1 inhibited metastasis in H2030-BrM but not MDA231-BrM cells (Fig. 5g; and data not shown), suggesting cell type specific differences in the requirement for MRTF-A in these models. Without substantially affecting the growth of the cells in monolayer culture, YAP knockdown reduced pericyte-like spreading of H2030-BrM lung cancer cells in organotypic brain slice cultures (Supplementary Fig. 5h–i). YAP knockdown also inhibited lung and bone colonization by breast cancer cells with these tropisms (Fig. 5h–i). Doxycycline-inducible knockdown of YAP after cancer cell dissemination to lungs and bones reduced the subsequent development of metastases (Fig. 5j–k and Supplementary Fig. 5j). YAP knockdown also hindered the outbreak of latent HCC1954-LCC1 cells following NK cell depletion in mice (Fig. 5l), suggesting that YAP loss of function phenocopies L1CAM loss of function during the initiation of metastatic colonization. Taken together with the data showing L1CAM regulation of YAP mediated transcription, these results pointed at YAP as a key effector of L1CAM driven metastasis.

L1CAM Regulates YAP Nuclear Localization During Metastatic Colonization

Since YAP signaling scored as the most salient mediator of L1CAM-dependent metastasis in these models, we focused on further analysis of this pathway. YAP is active when localized to the nucleus³⁷. Using metastasis tissue samples from breast cancer patients with disseminated disease, we focused on cancer cell clusters located on or near CD31-positive blood capillaries. Intense L1CAM immunohistochemical staining was present in the boundaries of cancer cells with the vasculature and with each other (Fig. 6a). A substantial proportion of metastatic cells also showed nuclear YAP immunostaining (Supplementary Fig. 6a–b). This was similar to the pattern of L1CAM and YAP staining in our experimental models, in which L1CAM staining was observed both on the cancer-vasculature and cancer cell-cancer cell boundaries (Fig. 6b). YAP was predominantly nuclear in H2030-BrM, MDA231-BrM and MDA231-LM cells spreading on brain or lung capillaries, whereas *L1CAM* knockdown decreased the nuclear localization of YAP. This effect was comparable to that of latrunculin A, a pharmacologic inhibitor of actin polymerization and of YAP nuclear localization³³ (Fig. 6c–d, Supplementary Fig. 6c–e). Collectively, these results suggested a role for L1CAM upstream of YAP.

To determine whether L1CAM-mediated YAP activation was sufficient to promote metastatic colonization, we performed rescue experiments. Expression of the constitutively nuclear mutant YAP(5SA) rescued the expression of YAP target genes (Supplementary Fig. 5c), and metastatic activity in L1CAM-depleted cells (Fig. 6e), while mutant YAP(S94A), which is deficient in binding the pro-growth TEA domain containing (TEAD) transcription factor³⁸ failed to do so. Expression of these YAP mutants did not affect the growth of the cells in culture (Supplementary Fig. 6d). Furthermore, YAP(5SA) overexpression did not augment the metastatic activity of H2030-BrM and MDA231-BrM cells, indicating that endogenous YAP is not rate limiting when L1CAM is present (Fig. 6f). Taken together, these results suggest that L1CAM in metastatic cells activates YAP signaling to initiate metastatic colonization.

Role of L1CAM in pericyte spreading

Next, we investigated the mechanistic link between L1CAM, pericyte-like spreading and subsequent YAP activation. Because of the extensive interactions of cancer cells with pericytes and similarities in their morphologies and positioning, we postulated that L1CAM + metastatic cells and pericytes share molecular machinery for interaction with the basal lamina of capillaries. L1CAM consists of six immunoglobulin-like domains, five fibronectin-like domains, a transmembrane region, and an intracellular domain. Two alternatively spliced exons encode segments that are included in neuronal L1CAM but absent in tumor-associated L1CAM³⁹ (Fig. 7a). We found that human primary pericytes express L1CAM at the mRNA and protein levels. Moreover, pericytes, like cancer cells, predominantly express the non-neuronal L1CAM isoforms (Fig. 7b).

Because *L1cam* null mice do not present major vascular defects⁴⁰, we sought evidence for a role of pericyte L1CAM during vascular stress and neoangiogenesis. Lipopolysaccharide (LPS) administration in mice causes systemic inflammation, which results in loosening of the vascular junctions allowing for increased vessel permeability and leakage⁴¹. LPS

induced vascular stress is coupled to reduction in pericyte coverage therefore perturbing pericyte investment and function during LPS treatment augments vascular leakage⁴². To determine if L1CAM has a role in pericyte function, we crossed *L1cam*^{fl/fl} mice⁴³ with pericyte specific tamoxifen inducible Cre expressing *Cspg4*-CreERT2 mice⁴⁴. Indeed, pericyte-specific knockout of *L1cam* in *Cspg4*-CreERT2/*L1cam*^{fl/y} mice augmented the induction of LPS mediated vascular permeability, as visualized by Evans blue dye extravasation from the vasculature in the skin (Fig. 7c), suggesting a role for L1cam in pericyte function during vascular stress. We also tested the role of pericyte L1cam in regulation of neo-angiogenesis *in vivo*. Sub-cutaneous implantation of matrigel in mice attracts endothelial cells and mural cells to invade and form capillary structures that ultimately lead to blood flow and erythrocyte accumulation within these matrigel plugs⁴⁵. In this neoangiogenesis assay, pericyte-specific *L1cam* knockout inhibited the accumulation erythrocytes into the plugs, suggesting that maximal neoangiogenic response required L1CAM (Fig. 7d). These loss-of-function phenotypes in *L1cam* knockout mouse pericytes correlated with reduced spreading of *L1CAM* knockdown human pericytes along the endothelial tubules generated on matrigel *in vitro* (Fig. 7e–f). These results suggested that pericytes use L1CAM for vascular homeostasis under conditions of vascular stress and neoangiogenesis, and metastasis-initiating cells adopt this machinery for perivascular spreading and outgrowth.

L1CAM increases β 1 Integrin-ILK signaling for YAP nuclear localization

Because both metastatic cells and pericytes use L1CAM for spreading on the vasculature, we reasoned that metastatic cells might activate YAP by employing the molecular machinery that pericytes use for vessel investment. During mouse development, β 1 Integrin and the scaffold protein integrin-linked kinase (ILK) constitute the core machinery that regulates pericyte adhesion to the developing vasculature^{46–48}. Pericyte specific *Itgb1* and *Ilk* knockout in mice results in pericyte rounding, reduced pericyte association with underlying endothelium, decreased pericyte investment and eventual embryonic lethality with severe defects in vessel formation^{47, 48}. Indeed, we found that *ILK* knockdown in primary human pericytes inhibited their spreading on endothelial tubules (Fig. 8a), thus phenocopying the effect of *L1CAM* knockdown in pericytes in this assay (Fig. 7f). β 1 Integrin and ILK are necessary for breast cancer metastasis to lungs^{23, 24}. ILK has also been linked to YAP nuclear localization in cancer cell lines⁴⁹. Therefore, we postulated that ILK provides a common link between these various observations.

We first tested the role of ILK in pericytic spreading of metastatic cells. *ILK* knockdown suppressed vascular cooption in H2030-BrM cells in brain organotypic culture (Fig. 8b) and *in vivo* (Supplementary Fig. 7a). *ILK* knockdown also inhibited the metastatic colonization of brain, lungs and bones by H2030 and MDA231 organotropic derivatives (Supplementary Fig. 7b–d). Similar to L1CAM and YAP, inducible ILK knockdown after metastatic seeding also downregulated metastatic colonization in bones and lungs (Supplementary Fig. 7e–f). Together these results suggested that ILK is necessary for both metastatic cell spreading and outgrowth, as is L1CAM. Next, we determined whether L1CAM functions upstream of β 1 integrin and ILK. L1CAM and the interacting protein ankyrin 2 are implicated in β 1 integrin activation^{50,51}. To test the role of L1CAM in β 1 integrin activation in our model, we plated

H2030-BrM cells either on type I collagen to activate $\beta 1$ integrin or on poly-L-lysine to support cell adhesion without robust integrin activation. Immunolabeling cells with an active confirmation specific $\beta 1$ integrin antibody (12G10) (Ref. 52) revealed that *L1CAM* knockdown reduced $\beta 1$ integrin activation in H2030-BrM cells plated on type I collagen down to a basal level measured when cells are plated on poly-L-lysine (Fig. 8c and Supplementary Fig. 7g). Additionally, ankyrin 2 could be co-immunoprecipitated with L1CAM (Supplementary Fig. 7h) and its knockdown reduced $\beta 1$ integrin activation (Supplementary Fig. 7i).

$\beta 1$ integrin signaling through ILK triggers phosphorylation of the p21-activated kinases 1 and 2 (PAK1/2), which leads to formation of filamentous actin (F-actin)-rich filopodia-like cellular protrusions that precede metastatic colonization of breast cancer cells in the lung microenvironment^{23, 24, 53}. In line with a role of L1CAM in regulating signaling downstream of ILK, *L1CAM* knockdown inhibited PAK1/2 phosphorylation in H2030-BrM cells that were plated on type I collagen or treated with a $\beta 1$ integrin-activating antibody (Fig. 8d–e). *L1CAM* knockdown also reduced the generation of ILK dependent F-actin-rich protrusions in these cells in matrigel (Fig. 8f). Then we tested whether $\beta 1$ integrin and ILK are required for L1CAM regulation of YAP. ILK knockdown suppressed YAP nuclear localization (Fig. 8g). Expression of the constitutively active $\beta 1$ integrin mutant T188I (Ref. 54) rescued PAK phosphorylation in L1CAM depleted cells, and rescued YAP nuclear localization in these cells in organotypic brain slice cultures (Fig. 8h–i). $\beta 1$ integrin T188I mutant also provided a partial rescue of metastatic colonization in the brain in L1CAM knockdown cells while wild-type $\beta 1$ integrin failed to do so (Supplementary Figure 7j–k). These results indicated that L1CAM is epistatic to Integrin-ILK signaling. Collectively, the evidence suggests that $\beta 1$ integrins and ILK link L1CAM to YAP activation during pericytic spreading for the initiation of metastatic outgrowth, while L1CAM mediated activation of $\beta 1$ integrin independent signaling pathways, potentially via homotypic L1CAM interactions between cancer cells, may contribute to metastatic outgrowth during later stages of colonization.

DISCUSSION

Two significant insights emerge from the present work. First, whereas vascular cooption is conventionally viewed as a means for cancer cell access the blood supply for oxygen and nutrients¹³, our data show that L1CAM-dependent vascular cooption in disseminated cancer cells can be a critical a source of mechanotransduction inputs, without which blood supply and capillary contact alone are not sufficient for metastatic outgrowth (Fig. 8j). Second, whereas prior studies have highlighted the existence of diverse mechanisms for organ-specific colonization⁴, the present work provides a common pathway that underlies metastatic colonization by disseminated cancer cells in multiple organs. Moreover, our evidence suggests that this mechanism is required for the growth of aggressive metastatic cells immediately after extravasation as well as the growth of disseminated cancer cells upon exiting from a period of dormancy.

Perivascular localization of tumor cells was described over twenty years ago^{55, 56}. In clinical pathology this phenomenon has been termed “extravascular migratory metastasis”, “non-

angiogenic growth pattern”, “vascular cooption” or “pericytic mimicry”, depending on context and authors¹³. Extravascular migratory metastasis usually refers to the ability of melanoma cells to travel from the primary tumor to the metastatic site without the need for intravasation into the circulation⁵⁷. Non-angiogenic growth, typically seen in liver and lung tumor specimens, involves secondary tumor growth in these organs with cancer cells adhering to existing capillaries and no evidence of neo-vascularization^{58–60}. The term vascular cooption classically refers to invasive tumor cells forming sheaths on capillaries to grow along the vasculature in brain tumors treated with anti-angiogenic therapy⁵⁶. Pericytic mimicry was used to describe the differentiation of glioblastoma cells into pericytes in order to support tumor angiogenesis⁶¹. Here we use pericyte-like spreading to refer to a distinct phenomenon in which metastatic cells spread on capillaries and compete with pericytes for position by adopting the molecular machinery that mediates pericyte adhesion and spreading along the basement membrane of the vasculature.

We show that L1CAM-mediated pericyte-like spreading is a general requirement for the initiation of metastatic colonization in multiple organs by different cancer cell types after initial seeding. Disseminated cancer cells use L1CAM to engage the basal lamina of the endothelium, which is readily available upon extravasation in secondary organs. The fact that L1CAM is expressed in primary tumors suggests that L1CAM may also play a role in primary tumor growth by engaging vascular or non-vascular extracellular matrix in that context. L1CAM is also present in the interface between cancer cells, both in experimental models of metastasis and clinical samples, suggesting that L1CAM homotypic interactions may also contribute to metastatic growth. It will be interesting to identify the function of L1CAM engagement during primary tumor growth and signaling pathways activated upon L1CAM homotypic interactions in cancer.

In the course of elucidating the molecular mechanism of metastatic cell spreading on blood capillaries, we discovered that pericytes also express and use L1CAM (Fig. 8j). Pericytes require L1CAM particularly in the context of vascular stress or neovascularization. *L1cam* knockout mice do not present with gross vascular defects, suggesting that L1CAM is not essential for vascular homeostasis in the absence of stress⁶². This may reflect the abundance of growth factors such as endothelial cell expressed PDGF that guide pericyte recruitment and investment during development and homeostasis. Notably, PDGF receptor β , which regulates pericyte investment under pathological and physiological conditions^{63, 64}, is not expressed in our models of metastasis.

Whereas pericytes use L1CAM and ILK for spreading and downstream regulation of vascular function, in metastatic cells L1CAM-ILK signaling results in YAP nuclear translocation and transcriptional activity, which licenses these cells for growth in the nutrient-rich environment of the perivascular niche (Fig. 8j). YAP/TAZ and the associated TEAD are well-established promoters of tumor growth and metastasis, and several of the inhibitory components of the Hippo pathway act as tumor suppressors⁶⁵. However, aberrant YAP activation by deletion of the inhibitory LATS kinase has been shown to impede tumor growth by increasing immune infiltration in the tumor⁶⁶. These observations suggest that balanced activation of YAP by regulatory inputs including L1CAM-ILK engagement is important for cancer cells that are proceeding through the metastatic cascade. Our data

showing heterogeneity in the degree of YAP nuclear localization in L1CAM+ patient samples is consistent with the idea that moderate activation of YAP is optimal for metastatic progression. By identifying a vital L1CAM-ILK-YAP signaling node for metastatic progression in multiple organs, our work highlights strategic vulnerabilities of metastatic cancer that may be amenable to future therapeutic targeting.

METHODS

Cell Culture

Metastatic derivatives of the MDA-MB-231 human triple-negative breast cancer cell line, including the brain metastatic MDA-MB-231-BrM2a (MDA231-BrM)²⁰, the bone metastatic MDA-MB-231-1833 (MDA231-BoM) (Ref. ⁶⁷), and the lung metastatic MDA-MB-231-LM2-4175 (MDA231-LM) (Ref. 22), HCT116 human colon cancer and metastatic 393N1 Kras/p53-mutant mouse lung adenocarcinoma cell lines were cultured in DMEM with 10% FBS, 2mM L-glutamine, 100 IU penicillin and 100µg mL⁻¹ streptomycin. 393N1 cells were a gift from Dr. Tyler Jacks²⁶. The BrM3 brain metastatic derivative of the H2030 KRAS-mutant human lung adenocarcinoma cell line¹⁹, (abbreviated H2030-BrM), a multi-organ metastatic derivative of the 786-O human clear cell renal cell carcinoma cell line (786-M1A) (Ref. ⁶⁸) and a latency competent derivative of the HCC1954 human HER2+ breast carcinoma cell line (HCC1954-LCC1) (Ref. 10) were cultured in RPMI 1640 media with 10% FBS, 2mM L-glutamine, 100 IU penicillin and 100µg mL⁻¹ streptomycin. Primary human brain pericytes, astrocytes and brain microvascular endothelial cells were cultured in media supplied by the provider (ScienCell). The MCF10A human mammary breast epithelial cell line and primary normal mammary epithelial cells were cultured in Mammary Epithelial Cell Growth Medium (MEGM) Bulletkit™ (Lonza) according to manufacturer's instructions. Human umbilical vein endothelial cells were cultured according to the supplier's specifications (Angiocrine Bioscience). 293T human embryonic kidney cells were cultured in DMEM with 10% FBS, 2mM L-glutamine. All cells were free of mycoplasma.

Animal Studies

All animal experiments were conducted in accordance with protocols approved by the MSKCC Institutional Animal Care and Use Committee. Athymic NCR nu/nu mice (Envigo, 069) and NOD.CB17- Prkdcscid/NCrHsd mice (Envigo, 170), C57BL/6J (Jackson Labs, 000664) were used between ages 4–7 weeks of age. For mammary fat pad injections and intracardiac injections 1×10⁵ cells were delivered in 100µL in PBS. For tail vein and intrasplenic injections 1.5×10⁵ cells, 50–100,000 cells were delivered in 100µL of PBS, respectively²⁰. Metastatic colonization was monitored weekly as previously described by BLI using retro-orbital injection of D-luciferin (150mg kg⁻¹) and IVIS Spectrum Xenogen instrument (Caliper Life Sciences)¹⁴. Data was analyzed using Living Image software v. 2.50. For brain metastasis experiments, at the end point, animals were anesthetized with 100mg kg⁻¹ ketamine and 10mg kg⁻¹ xylazine, retro-orbitally injected with D-luciferin. Isolated brains were analyzed using IVIS Spectrum Xenogen and Living Image software. Blinding of the BLI readings was not possible. 5–10 animals were used per experimental condition. Sample sizes were determined using Mead's resource equation with 8 degrees of freedom and based on prior experience with metastatic animal models effect size. For

doxycycline inducible knockdowns, mice were given doxycycline hyclate in the diet (Envigo) and water (Sigma). Animals were randomly assigned to doxycycline or control groups. For experiments with HCC1954-LCC1 cells, mice were treated with doxycycline in the diet 2 days prior to NK cell depletion with anti-asialo-GM1 antibody⁶⁹ (Wako Chemical, 33ug of antibody injected into the peritoneum every 5 days). All animal experiments were statistically analyzed using Mann-Whitney rank sum test. No assumptions were made on the normal distribution of the data. For angiogenic plug and Evans blue dye extravasation experiments, *Cspg4*-CreERT2 mice (Jackson Laboratory, no: 008538) were crossed with *L1cam*^{flox/flox} mice⁴³. 8–10 week old *Cspg4*-CreERT2 *L1cam*^{flox/y} male mice were injected with either 100μL of corn oil or 100μL of 1mg mL⁻¹ tamoxifen dissolved in corn oil, twice a day for 5 consecutive days and let to recover for 2 days. For angiogenic plug⁴⁵ experiments 500μL of growth factor reduced phenol red-free matrigel, containing 200ng mL⁻¹ recombinant murine vascular endothelial growth factor 165 (PeproTech), 500ng mL⁻¹ recombinant murine fibroblast growth factor-basic (PeproTech) and 100μg mL⁻¹ Heparin (Sigma) was injected subcutaneously on each side of the groin on the ventral side of the mice. 100μL of 1mg mL⁻¹ tamoxifen injected once a day for 5 consecutive days and angiogenic plugs were removed on the 7th day embedded in paraffin and sectioned for H & E staining. Erythrocyte area was measured by staining the paraffin embedded matrigel tissue with TER119 antibody (BD biosciences, 550565). Slides were scanned using Mirax scanner equipped with a 20X air objective. Area of the matrigel was manually annotated to separate the surrounding fat and skin tissue. TER119 positive area was measured using Fiji (code available upon request). For Evans blue dye extravasation assay, mice were injected with 2mg/kg lipopolysaccharide to induce systemic blood vessel leakage⁴¹. 24 h later, 1% (weight/volume) Evans blue dye was injected into the peritoneum. Two hours later, abdominal skin of the mice were collected, weighted, and treated with formamide (Sigma-Aldrich) for 48 h at 50°C. Optical density of Evans blue dye absorbance released from tissue was measured at 600nm using a spectrophotometer.

Tissue processing and imaging

Isolated mouse brains were fixed in 4% PFA overnight at 4°C. Mouse lungs were perfused with PBS and 4% PFA and post-fixed for at least 4 h at 4°C. Fixed tissues were washed three times with PBS and incubated with 15% and 30% sucrose for 24 h at 4°C. Dehydrated tissues were frozen in optimal cutting temperature (O.C.T., Tissue-Tek) solution on a Microm KS34 freezer unit at -35°C and cut into 80μm sections using Microm HM 450 microtome (Thermo Scientific). Lungs were aliquoted into 5 consecutive sections and brains into 10. Sections were blocked and stained free floating in 10% normal goat serum, 2% BSA and 0.25% Triton-X. All sections were mounted on a glass slide with ProLong Gold Antifade mounting media (Thermo Scientific) and stored in -20°C until imaging. Sections were scanned with 3DHISTECH Panoramic confocal scanner using a 20X air objective. Representative images were acquired using Leica SP5 using a 63X oil objective. Images were analyzed using Metamorph, Fiji and CaseViewer softwares. 3 dimensional reconstruction of confocal images were done using Imaris software. Protrusions in the lungs were defined as cell projections that extended more than 2 microns from the cell body and were manually counted. Cell spreading was measured using Fiji software by drawing an outline around each cell and calculating roundness using the formula $4 \times \text{Area} \times (\pi \times$

(Major axis)²)⁻¹. YAP localization was quantified using one circular selection with a 0.52-micron diameter to sample YAP staining intensity within the nucleus and cytoplasm. The ratio of median intensities of each selection area was calculated using Fiji (Code available upon request).

***In vitro* experiments**

For integrin activation, 2×10^6 cells were plated in a 10cm tissue culture dish, deprived of serum for 24 h. The next day cells were detached using 5mM EDTA in PBS and collected with 0.01% (weight/volume) bovine serum albumin. Cells were resuspended in serum free media and counted. 5×10^5 cells were allowed to adhere for 1 h on 6-well dishes coated either with on collagen I ($5 \mu\text{g cm}^{-2}$) (BD Biosciences) or with poly-L-lysine ($4 \mu\text{g cm}^{-2}$). Cells were then lysed with 60 mM Tris.HCl, 1% SDS, 10% glycerol and 0.2% 2-mercaptoethanol. For antibody mediated integrin activation, 2.5×10^5 cells were plated on 6-well dishes, deprived of serum for 24h and stimulated with $5 \mu\text{g mL}^{-1}$ $\beta 1$ integrin antibody clone: TS2/16 (Ref. ⁷⁰) (Santa Cruz Biotechnology) for 15 min and lysed as above. Lysates were ran 5 times through a 21G syringe and incubated at 90°C for 7 min. For measuring active $\beta 1$ integrin staining using immunofluorescence, 2×10^4 cells were plated on 8 well Lab-Tek dishes coated with collagen I or poly-L-lysine as above. Cells allowed to adhere for 1 h and fixed with 4% paraformaldehyde for 20 min at room temperature and stained with active confirmation specific $\beta 1$ integrin antibody clone 12G10 ($0.5 \mu\text{g/mL}$) (Abcam) in 10% normal goat serum, 2% BSA and 0.25% Triton-X and 0.05% Tween-20. After the staining, 50 fields of view from each well were imaged using Zeiss Axio Imager M1 equipped with a $40\times$ air objective. Integrated intensity values from each cell were calculated by Fiji (code available upon request). For co-immunoprecipitation experiments, cells were lysed with a lysis buffer containing 0.3% NP40, 150mM NaCl, 50mM Tris-Cl (pH 7.4), 2mM EDTA (pH 8.0) and 10mM NaF. Antibodies for L1CAM (5G3 Thermo Fisher Scientific, 14-1719-82) and Ankyrin 2 ($1 \mu\text{g/mL}$) (Santa Cruz Biotechnology, sc-28560) or IgG isotype controls (Cell Signaling Technology) were incubated with cell lysates overnight followed by a 2-h incubation with A/G Plus Agarose Beads (Thermo Fisher Scientific). Measurement of actin-rich protrusions were done by plating serum-deprived cells on $80 \mu\text{L}$ growth factor reduced Matrigel (Corning Biosciences) in 4-well Lab-Tek dishes (Electron Microscopy Sciences) in serum-free media containing 2% matrigel. Cells were fixed 24 h later and stained with Alexa 568 conjugated phalloidin (1:40) (Cell Signaling Technologies). Images were taken using Zeiss Axio Imager Z1 microscope with $20\times$ air objective and a Zeiss AxioCam HRc camera. Representative cells were imaged using Leica SP5 confocal microscope with Leica HyD Camera with $63\times$ oil objective. For endothelial cell co-culture experiments, pericytes, H2030-BrM cells, primary normal mammary epithelial cells, MCF10A cells, and 293T cells were labeled with CellTracker Green CMFDA (Thermo Fisher Scientific, C7025) according to manufacturer's instructions. 4×10^3 labeled test cells were plated together with non-labeled 1×10^5 endothelial cells in endothelial cell media on $100 \mu\text{L}$ of growth factor reduced matrigel to allow for endothelial cell tubule formation⁷¹ in 4-well Lab-Tek dishes. 24 h later cell spreading was imaged using EVOS imaging (Thermo Fisher Scientific) at $20\times$ magnification. All *in vitro* and *ex vivo* experiments were statistically analyzed using Mann-Whitney rank sum test. No assumptions were made on the normal distribution of the data.

Organotypic Brain Slice Culture

Brains were isolated in 1X HBSS buffer as previously described⁷². Isolated brains were sectioned into 250 μm slices using Leica VT100S systems. Brain tissue slices were cultured on 0.8 μm pore size membranes (Millipore) floating on brain slice media. 1×10^4 cancer cells were introduced onto the brain slice and incubated for 48 h. Sections were fixed in 4% PFA in PBS overnight at 4°C and labeled with primary and secondary antibodies in 10% Normal Goat Serum, 2% BSA and 0.25% Triton-X. For time-lapse confocal imaging of cancer cell displacement of pericytes, Cspg4-DsRed mice (The Jackson Laboratory, Stock No: 008241) were anesthetized using 100mg kg⁻¹ ketamine and 10mg kg⁻¹ xylazine. 0.5mg mL⁻¹ DiD (DiIC₁₈ (5)) solid dye in ethanol was further diluted to 0.05mg mL⁻¹ in 30% sucrose-PBS. 1mL of this dye was injected into the left ventricle of the anesthetized mouse to label the endothelial cells. After 1 min of incubation, excess dye was washed with 5mL of PBS injection to the left ventricle of the mouse. Brain was collected in 1× HBSS and sectioned as above, in the dark and delivered to culture. 3×10^4 CellTracker green labeled H2030-BrM cells added on top of the brain slice. After 24 h of incubation under slice culture conditions, brain slices were placed on hydrophilic Millicell® cell culture inserts (Millipore, PICM0RG50) and transferred over to a glass bottom Fluorodish (World Precision of Instruments). Time lapse images were taken using SP5 or SP8 inverted confocal microscopes (Leica) using a long distance 20X oil objective every 20 min for 14 – 20 h. Pericyte displacement was determined by the ability of cancer cell to wedge between a pericyte and DiD labeled endothelium after a side-by-side encounter of a pericyte and a cancer cell on the same blood vessel. For YAP promoter report assays, RFP expression was driven by YAP responsive promoter in a lenti-virus construct. RFP was tagged at the C-terminal to a mutant (R12H/N18T/V19A/G67S) *E. coli* folA dihydrofolate reductase (Addgene #29236), which targets the fused protein for proteasomal degradation in the absence of its small molecule stabilizer trimethoprim (TMP)³². H2030-BrM and MDA231-BrM cells were seeded on brain slice cultures for 24 hours and were treated with 10uM final TMP for another 24 hours. Fixed brain slices were immunostained for GFP, RFP and CD31. Sections were scanned with 3DHISTECH Panoramic confocal scanner. GFP positive cancer cells associated with CD31 positive blood vessels were analyzed for RFP expression using Fiji (Code available upon request). For translating ribosome affinity purification 3×10^4 cancer cells transduced with EGFP-L10a were cultured as above. Lysates for 7 brain slices were combined and processed with TRAP protocol as previously described⁷³. Brain slices were lysed with TRAP lysis buffer (20mM HEPES, pH7.3, 150mM KCl, 5mM MgCl₂, 1% NP-40, with protease inhibitor cocktail, 0.5mM DTT and RNasin added fresh before use) and EGFP-L10a was immunoprecipitated with GFP antibodies (clone number 19F7 & 19C8, MSKCC Monoclonal Antibody Core Facility)^{73–75}. The quality and quantities of RNA samples purified from anti-EGFP immunoprecipitation products were determined using Agilent BioAnalyzer 2100. RNA-Seq libraries were prepared with TruSeq RNA Sample Prep kit v2 (Illumina) following manufacturer's instructions. RNA-Seq was performed on a HiSeq2000 platform using TruSeq SBS Kit v3 (Illumina). Sequencing reads were mapped to human genome hg19 with STAR 2.3.0. Mapped reads were counted with HTSeq v5.4.0. Raw and normalized counts were analyzed in R-studio with DESeq2 package⁷⁶ (Bioconductor) and gene set enrichment analysis was performed using previously established signatures^{77–79}. The datasets generated and analysed during the current study are

available in GEO datasets under accession number GSE82281:<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gjgkcykbpadzgz&acc=GSE82281>.

Clinical Samples and Immunohistochemistry

Formalin fixed paraffin embedded tissue sections from 10 cases of L1CAM⁺ invasive breast and lung cancer patients and brain tissue obtained at an autopsy from an ER⁺ breast cancer patient were obtained under an MSKCC Institutional Review Board (IRB) approved biospecimen protocol from the MSK Department of Pathology. Slides were sequentially immunostained using the Ventana Discovery XT: L1CAM, CD31 (0.83 µg/mL), aSMA (1 µg/mL), panKeratin (4.63 µg/mL) or Leica Bond XT (YAP) by Pathology Core Facility using standard automated techniques. Metastatic cluster comprising with fewer than 20 cells were scored for YAP nuclear staining. For mouse tissue, immunohistochemistry was performed as for human samples except that L1CAM staining was performed using a Leica BOND RX platform using a mouse-on-mouse kit (Stat Labs). Antibodies used were L1CAM (CD171 Clone 14.10, Biolegend), YAP (Cell Signaling Technology, D24E4), CD31 (Dianova, SZ31) and aSMA Clone A14 (Millipore Sigma, CBL171-I), panKeratin (Roche, 760–2135). For image alignment studies, each of the L1CAM IHC and YAP IHC images was segmented independently. Regions encapsulating the nuclei of L1CAM positive cells were dilated to calculate an L1CAM intensity value for each cell. These regions of interest were then assigned a YAP nuclear signal intensity based on nearest neighbor analysis (code available upon request).

Reagents

Alexa-568 conjugated phalloidin (1:40), phospho-PAK1/2 Ser199/Ser202 (1:500), total PAK, GAPDH (1:5000) antibodies were from Cell Signaling Technologies. L1CAM (UJ127.11) (1 µg/mL), Ankyrin 2 (1 µg/mL) and activating β1 integrin antibody (TS2/16) (5 µg/mL) antibodies were from Santa Cruz Biotechnology. L1CAM antibody 5G3 for immunoprecipitation was from eBioscience. Antibodies for immunofluorescence used were GFP (10 µg/mL) (Avis Biosciences), CD31 MEC13.3 (2 µg/mL), Collagen IV (4 µg/mL), and active conformation specific β1 integrin (12G10) (0.5 µg/mL) (Abcam), Alexa 647 conjugated Isolectin B4 (4 µg/mL) (Thermo Fisher Scientific). TaqMan probes were all predesigned from Thermo Fisher Scientific. Latrunculin A was from Tocris. pQCXIH-Myc-YAP (Addgene: 33091), pQCXIH-Myc-YAPS5A (Addgene: 33093), pQCXIH-Myc-YAP S94A (Addgene: 33094) were kind gifts from K. Guan (University of California, San Diego). All viruses were infected at MOI of 1–1.6. All constitutive knockdown plasmids were based on pLKO.1 (Dharmacon): *L1CAM* (TRCN0000063913, TRCN0000063916, TRCN0000063917), *L1cam* (TRCN0000094553), *ANK2* (TRCN0000064911), *ILK* (TRCN0000000970, TRCN0000000971), *YAP* (TRCN0000107265, TRCN0000107268), *WWTR1* (TRCN0000019471, TRCN0000019473). Inducible knockdown plasmids were based on mir30 for HCC1954 cells and on mirE⁸⁰ for H2030 BrM, MDA231-BoM and MDA231-LM cells with the following target sequences:

1. CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAAGGGATGGTGTCCA
CTTCAAATAGTGAAGCCACAGATGTATTTGAAGTGGACACCATCCCTCT
GCCTACTGCCTCGGACTTCAAGGGGCTAGAATTC

2. CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCAGCAAGATCTTGC
ACATCAATAGTGAAGCCACAGAtGTATTGATGTGCAAGATCTtGCTGTTG
CCTACTGCCTCGGACTTCAAGGGGCTAGAATTC

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We acknowledge members of the MSKCC Molecular Cytology Core and Pathology Core Facilities for their assistance with staining, tissue processing, image acquisition and analysis and Young-Mi Kim for help with YAP activity assays. This work was supported by NIH grants P01-CA094060, P01-CA129243 (JM) and P30-CA008748 (MSKCC), T32-CA009207 (KG), DOD Innovator award W81XWH-12-0074 (JM), and the Alan and Sandra Gerry Metastasis Research Initiative (J.M.), Shulamit Katzman Endowed Postdoctoral Research Fellowships (E.E.E. and K.G.), an AACR Basic Cancer Research Fellowship and Conquer Cancer Foundation of ASCO Young Investigator Award (K.G.). Authors declare no financial interests in connection to this work.

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Figure 1: Pericytic spreading of cancer cells during metastasis initiation.

(a) Top left panel: 3D reconstructed image of a CSPG4⁺ microvascular pericyte (Cspg4 staining, *green*) on the brain vasculature of a mouse (lectin staining, *red*). Bottom left panel: cross sectional view of a pericyte encircling an endothelial cell. Right panel: GFP⁺ H2030-BrM lung cancer cell (*green*) spreading over the vasculature after infiltrating the brain parenchyma of a mouse via the arterial circulation (scale bars, 5 μ m). (b) 3D reconstruction of confocal images of a metastatic outgrowth in an 80-micron thick mouse brain tissue following intracardiac injection of cancer cells for hematogenous dissemination. GFP⁺

H2030-BrM cells (*white*) that infiltrated mouse brain via the arterial circulation are wedged between CSPG4⁺ pericytes (*green*) and CD31⁺ endothelium (*red*). Nuclei are labeled with Hoechst (*blue*). Bottom panel is a duplication of the top panel rotated 180° along the Z-axis (scale bars, 10 μm). (c) Time lapse imaging of H2030-BrM cancer cells (*Ca*; *white*) consecutively displacing three CSPG4-DsRed⁺ pericytes (*p1*, *p2* and *p3*; *green*) along the endothelium (DiD staining, *red*) in cultured brain tissue. t: time in hours and minutes (hh:mm). Refer to Supplementary Videos 1–4. Bar graph to the right, average number of pericytes that has been displaced (red bars and grey circles) or resisted displacement (black bars and orange circles) per cancer cell after a side-by-side pericyte – cancer cell encounter on the same blood vessel during the time lapse confocal imaging exemplified in Supplementary Videos 1–4. Error bars, S.E.M (*n* = 20 cancer cells from 3 independent experiments per group) *P* values are calculated using Mann-Whitney test. (d) 3D reconstruction of confocal images from an 80-micron thick mouse brain tissue bearing a metastatic outgrowth and adjacent vasculature (Cd31 staining, *red*) 4 weeks after intracardiac injection of GFP⁺ H2030-BrM and MDA231-BrM cells (*white*). Dotted yellow line outlines the tumor area (scale bars, 20 μm). (e) Top panel: immunohistochemistry images of metastatic cells (*white arrowheads*) interacting with pericytes and vascular smooth muscle cells (alpha-smooth muscle actin staining, *dark brown*) along the vascular wall (*red dotted line*) in clinically silent micrometastases in brain tissue obtained during autopsy of a treatment-naïve ER⁺ breast cancer patient. Adjacent blood vessel is devoid of metastatic cells. Bottom panel: L1CAM staining (*brown*) of perivascular metastatic cells in brain tissue (scale bars, 50 μm).

Figure 2: L1CAM is necessary for metastatic colonization in multiple organs

(a) Representative images of GFP⁺ MDA231-LM control and *L1CAM* knockdown cells (green) showing protrusions (arrowheads) associated with CD31⁺ alveolar blood capillaries (red) after extravasation from the venous circulation into the lungs of a mouse, 2 d after tail vein injection. Dotted lines outline alveolar spaces (as). Images are from 80-micron thick lung sections. Data on the right are mean vascular coopting protrusions per metastatic foci. Error bars, S.E.M. ($n = 36$ per group). P value is calculated using Mann-Whitney test (b) Mammary tumor volume in female mice injected with MDA231-LM cells orthotopically into the fourth mammary fat pad. Error bars, S.E.M ($n = 6$ mice with two mammary tumors each). P value is calculated using Mann-Whitney test. (c) *Ex vivo* lung BLI (bioluminescence) of the mice in panel B at the endpoint of the experiment (9 weeks post orthotopic injection). Whiskers, minimum and maximum. P value is calculated using Mann-Whitney test. (d) Representative BLI images of lungs and livers from mice in panel B. (e-f) Representative images of GFP⁺ MDA231-LM (green) control and *L1CAM* knockdown cells disseminated to the (e) livers and (f) lungs of mice carrying orthotopic mammary tumors in (b) CD31⁺ capillaries (red). Images are from 80-micron thick tissue sections.

Figure 3: *LICAM* is necessary for colonization post metastatic seeding

(a) Schematic of the protocol for *LICAM* knockdown with a doxycycline inducible sh*LICAM* vector in latent metastatic cells. Doxycycline treatments were given on Day 9 and animals were sacrificed on Day 42 post injection. (b) BLI quantitation of metastatic burden 6 weeks after mice were injected with cells expressing a doxycycline-inducible shRNA targeting *LICAM* (TetON-sh*LICAM*). Mice were treated with doxycycline starting 9 days post injection. Data are individual mice. Red line, median ($n = 4$ per group). P values are calculated using Mann-Whitney test. (c) Representative images of H&E staining of lungs from mice in panel B. Bar graph, mean tumor area. Error bars, S.E.M. ($n > 25$ lesions per group in 3 mice). P value is calculated using Mann-Whitney test. (d) Representative BLI images of mice injected with MDA231-LM, H2030-BrM and MDA231-BoM cells expressing the doxycycline inducible *LICAM* shRNA as in (b).

Figure 4: L1CAM regulates emergence from metastatic latency

(a) Schematic of the protocol for *L1CAM* knockdown with a doxycycline inducible sh*L1CAM* vector in latent metastatic cells, concurrently with NK cell depletion using anti-asialo-GM1 antibody. 14 d after intracardiac injections of HCC1954 LCC cells, cohorts of mice were separated and given doxycycline diet. On day 16, anti-asialo-GM1 injections were initiated. Each downward arrow represents an intraperitoneal anti-asialo-GM1 antibody injection 5 d apart. (b) Representative images of GFP⁺ HCC1954 LCC cells (green) near endothelial cells (lectin staining, red), 4 days (day 20) and 14 days (day 30) post NK cell depletion (scale bar, 20 μm). (c) Bar graph showing number of lesions larger than (red bars and grey circles) and smaller than (black bars and open circles) 1500 μm^2 per each brain. Error bars, S.E.M. ($n = 5$ mice per group). P value is calculated using Mann-Whitney test. (d) Schematic model showing L1CAM mediated pericyte like spreading leading to metastatic outgrowth both immediately after metastatic seeding and after a period of natural killer (NK) cell enforced metastatic latency. MIC: metastasis initiating cell.

Figure 5: L1CAM driven transcriptional programs necessary for metastasis initiation

(a) Schematic representation of translating ribosome affinity purification experiments. H2030-BrM cells expressing ribosomal protein L10a fused to EGFP (EGFP-L10a) were allowed to migrate into brain tissue in culture and coopt capillaries for 48 h. Cancer cell mRNAs associated with actively translating ribosomes were immunoprecipitated using an anti-GFP antibody and processed for RNA sequencing. (b) Gene signature enrichment analysis (GSEA) of YAP and MRTF-A transcriptional signatures. NES, normalized enrichment score. FDR, false discovery rate. (c) Design and working principal for YAP

promoter activity responsive lentiviral construct. RFP expression is driven by synthetic YAP promoter (8×GTIIC) and protein product is quickly degraded due to an N-terminal fused destabilization domain (DD). Upon addition of a small molecule stabilizer, RFP is stabilized giving spatio-temporal information on YAP activity. **(d)** Representative images of MDA231-BrM and H2030-BrM (GFP) cells showing reduced YAP activity (RFP signal accumulation) with L1CAM knockdown in brain slice *cultures in situ*. **(e)** Quantitation of integrated RFP signal intensity per cell in brain slice cultures in (d). **(f)** Brain BLI intensities in mice, 28 d after injection of the indicated H2030-BrM and MDA231-BrM cell lines into the circulation. Red lines, median ($n = 5$ per group). P values are calculated using Mann-Whitney test. **(g)** Brain BLI intensities in mice, 28 d after injection of H2030-BrM control or *MKL1* (MRTF-A) knockdown cells. Red lines, median ($n > 7$ mice per group). P values are calculated using Mann-Whitney test. **(h)** Weekly BLI measurements of mice injected with MDA231-LM control or *YAP* knockdown cells. P values are calculated using two-tailed unpaired t-tests without assuming equal standard deviations. Error bars, S.E.M. ($n = 6$ in each group). **: $P < 0.01$, ***: $P < 0.001$ **(i)** Weekly BLI measurements of mice injected with MDA231-BoM control or *YAP* knockdown cells. P values are calculated using two-tailed unpaired t-tests without assuming equal standard deviations. Error bars, S.E.M. ($n = 6$ in each group). **: $P < 0.01$. **(j-k)** BLI intensities of mice 4 weeks post injection of MDA231-LM (j) and MDA231-BoM (k) cells expressing doxycycline inducible sh *YAP* vector (TetON-sh *YAP*) ($n > 10$ mice per group). **(l)** Brain BLI intensities of mice 30 days after HCC1954-LCC1 TetON-sh *YAP* cells were injected into the arterial circulation of mice. Doxycycline was administered starting on day 14 and NK cells were depleted starting on day 16 using the anti-asialo-GM1 antibody treatment regimen as described in Figure 4a.

Figure 6: L1CAM regulates YAP nuclear localization during metastatic colonization

(a) Immunohistochemical staining of serially sectioned dermal metastasis tissue from a breast cancer patient after surgical resection. Arrowheads point to the limits of the capillary segments and red arrows point to L1CAM staining between vasculature and cancer cells and between cancer cells (scale bar, 25 μ m). (b) Immunohistochemical staining of MDA231-BrM cells that infiltrated the brain of mice 4 weeks after arterial injection. Serial sections of cells in the process of colonization are stained with CD31, L1CAM and YAP antibodies. To the right of the blue dotted line is endogenous L1CAM staining in the brain tissue as

expected (scale bar, 25 μ m). **(c)** Representative images of YAP immunofluorescence (*red*), nuclear staining (Hoechst, *blue*) in GFP⁺ H2030-BrM cells (*green*) on capillaries (collagen IV staining, *magenta*) in cultured brain tissue. Graphs show the ratio of nuclear to cytoplasmic YAP immunostaining in the indicated cell lines. Each dot represents one cell; red lines, median. Cells were transduced with a control shRNA or different L1CAM shRNAs (*shL1* #1 and #2). LatA, latrunculin A ($n = 37$ cells). *P* values are calculated using Mann-Whitney test **(d)** Representative images of YAP immunostaining (*magenta*) and nuclei (Hoechst, *blue*) in GFP⁺ MDA231-LM cells (*green*) extravasated from lung capillaries (CD31 staining, *red*) 48 h after tail vein injection. Right panel is the nuclear to cytoplasmic YAP ratio in each condition. ($n = 50$ cells, 2 mice per group). *P* values are calculated using Mann-Whitney test **(e)** Brain BLI intensities in mice, 28 d after mice were injected with H2030-BrM and MDA231-BrM cells transduced with the indicated vectors. YAP^{5SA}, constitutively nuclear YAP; YAP^{S94A}, TEAD binding deficient YAP ($n = 10$ per group). *P* values are calculated using Mann-Whitney test, *n.s.*, not significant, $P > 0.05$. **(f)** Brain BLI intensities of mice 4 weeks after injection of MDA231-BrM and H2030-BrM cells expressing either YAP^{5SA} or YAP^{S94A} mutants ($n = 4$ per group). *P* values are calculated using Mann-Whitney test, *n.s.*, not significant, $P > 0.05$.

Figure 7: Role of L1CAM in pericyte spreading

(a) Schematic representation of neuronal and non-neuronal L1CAM isoforms. Red ovals, neuronal isoform specific domains. Ig 1–6, immunoglobulin-like domains 1–6. FN 1–5, fibronectin type 3 domains 1–5. Gray horizontal line represents the cell membrane. (b) qPCR data of *L1CAM* isoform expression normalized to B2M levels in the indicated cell lines relative to the level of total *L1CAM* mRNA in H2030-BrM cells. Error bars, S.D. ($n = 3$ replicates). (c) Amount of Evans blue dye extravasated from the vasculature per milligram of skin tissue in *Cspg4*CreERT2 *L1cam*^{fl/y} mice following a 24 h treatment with LPS. Error

bars, S.E.M [Control, $n = 7$; Tamoxifen (*L1cam* knockout), $n = 7$]. *P* value is calculated using Mann-Whitney test. **(d)** Representative H&E images of abdominal subcutaneous matrigel plugs from *Cspg4* CreERT2 *L1cam*^{fl/y} mice treated with vehicle (control) or tamoxifen (*L1cam* knockout). Erythrocytes (*red*) and vascular cells (*purple*) invaded the matrigel mass (*pink*). Right panel, erythrocyte area measurements using TER-119 immunofluorescence. Error bars, S.E.M ($n = 12$ plugs per group, 2 independent experiments). *P* value is calculated using Mann-Whitney test. **(e)** Immunoblot analysis of L1CAM protein in H2030-BrM and primary human pericytes with or without *L1CAM* knockdown. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is loading control. **(f)** Representative images of CellTracker-labeled pericytes (*green*) in co-culture with endothelial cell (EC) tubular structures (bright field) on matrigel. Bar graph, percentage of pericytes with spread, intermediate and round morphology ($n = 500$ cells from 2 independent experiments; scale bar, 40 μ m).

Figure 8: L1CAM increases $\beta 1$ Integrin-ILK signaling for YAP nuclear localization

(a) Representative images of CellTracker labeled pericytes (green) in co-culture with endothelial tubes (bright field) on matrigel. Bar graph, percentage of spread, round and intermediate pericytes (Scale bar, 40 μ m, $n > 141$ cells from two independent experiments). (b) Representative images of control and *ILK* knockdown GFP⁺ H2030-BrM cells (green) spreading along the vasculature (collagen IV staining, red) in cultured brain tissue. Panel to the right is roundness index of individual H2030-BrM cells on the vasculature. Lower roundness index indicates a higher degree of cell spreading ($n = 180$ cells, 2 independent experiments).

experiments). Red bars, median. *P* value is calculated using Mann-Whitney test. **(c)** Normalized to the mean integrated fluorescence intensity values of cells labeled with active confirmation specific $\beta 1$ integrin antibody, Clone 12G10. H2030-BrM control and *L1CAM* knockdown cells were labeled with 12G10 after cells were plated on collagen I to promote integrin activation or on poly-L-lysine to promote attachment without integrin activation for 1 hour ($n = 150$ cells from 2 independent experiments). Red bars, median. *P* value is calculated using Mann-Whitney test. **(d)** Immunoblot analysis of phospho-PAK1 (Ser199/Ser204) and phospho-PAK2 (Ser192/Ser197) levels in H2030-BrM cells adhering to poly-L-Lysine or collagen I coated plates for 1 hour ($n = 3$ independent experiments). **(e)** Immunoblot analysis of phospho-PAK1 (Ser199/Ser204) and phospho-PAK2 (Ser192/Ser197) levels in H2030-BrM cells treated with activating anti- $\beta 1$ integrin antibody (TS2/16) for 15 min on tissue culture dishes after serum starvation ($n = 3$ independent experiments). **(f)** Representative images of filamentous actin (phalloidin staining, *green*) in H2030-BrM cells plated in matrigel (scale bar, 10 μ m). Data on the right are number of control or *L1CAM*-depleted cells scored based on their content of protrusions (1–4) as shown in the images. Data are normalized to the total number of cells counted per group ($n = 63$ cells). **(g)** Ratio of nuclear to cytoplasmic YAP immunostaining in control and *ILK* knockdown H2030-BrM cells ($n = 100$ cells from 2 independent experiments). *P* value is calculated using Mann-Whitney test. **(h)** Immunoblot analysis of phospho-PAK1 (Ser199/Ser204) and phospho-PAK2 (Ser192/Ser197) in control and *L1CAM* knockdown H2030-BrM cells expressing control, $\beta 1$ integrin ($\beta 1^{WT}$) or constitutively active $\beta 1$ integrin mutant ($\beta 1^{T188I}$) vectors, after cells were allowed to adhere to collagen I for 1 hour ($n = 3$ independent experiments). **(i)** Nuclear to cytoplasmic YAP immunofluorescence ratio in the indicated H2030-BrM cells spreading on capillaries in cultured brain tissue ($n > 100$ cells from 2 independent experiments). Red lines, median. *P* value is calculated using Mann-Whitney test. **(j)** Model for *L1CAM* mediated pericyte-like spreading and subsequent, $\beta 1$ integrin, ILK and YAP activation adapted by metastatic cells for colonization in target organs both immediately after seeding or after a period of NK cell induced dormancy.