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RESEARCH ARTICLE

The CD44s splice isoform is a central mediator for invadopodia activity

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ABSTRACT

The ability for tumor cells to spread and metastasize to distant organs requires proteolytic degradation of extracellular matrix (ECM). This activity is mediated by invadopodia, actin-rich membrane protrusions that are enriched for proteases. However, the mechanisms underlying invadopodia activity are not fully understood. Here, we report that a specific CD44 splice isoform, CD44s, is an integral component in invadopodia. We show that CD44s, but not another splice isoform CD44v, is localized in invadopodia. Small hairpin (sh) RNA-mediated depletion of CD44s abolishes invadopodia activity, prevents matrix degradation and decreases tumor cell invasiveness. Our results suggest that CD44s promotes cortactin phosphorylation and recruits MT1-MMP (also known as MMP14) to sites of matrix degradation, which are important activities for invadopodia function. Importantly, we show that depletion of CD44s inhibits breast cancer cell metastasis to the lung in animals. These findings suggest a crucial mechanism underlying the role of the CD44s splice isoform in breast cancer metastasis.

KEY WORDS: CD44s, Splice isoform, Invadopodia, Breast cancer, Metastasis

INTRODUCTION

In the past few decades, significant advances have been made in the detection and treatment of breast cancer. However, tumor metastasis remains a major clinical obstacle for the survival of individuals diagnosed with breast cancer. The development of an effective therapy for such individuals thus relies on a better understanding of the mechanisms that drive metastatic tumor cells to disseminate from the primary site to distant organs.

In order for metastasis to occur, carcinoma cells must acquire the ability to degrade extracellular matrix (ECM), a process that allows tumor cells to intravasate from their original sites into the blood stream and to extravasate from circulation to distal organs (Chaffer and Weinberg, 2011). Accumulating evidence has shown that invadopodia are crucial structures for ECM degradation, and subsequent tumor cell migration and invasion (Courtneidge et al., 2005; Eckert et al., 2011; Li et al., 2013; Paz et al., 2013; Seals et al., 2005). Invadopodia are small dot-shaped actin-rich protrusions that are in close contact with ECM (Murphy and Courtneidge, 2011). Typical markers for invadopodia include cortactin and Tks5 (also known as SH3PXD2A), which accumulate at actin-rich dot-like structures that are characteristic of invadopodia. Cortactin is viewed as an integral molecule in invadopodia function that connects cellular signaling and the actin cytoskeleton (Murphy and Courtneidge, 2011; Weaver, 2008; Weed and Parsons, 2001). Cortactin is phosphorylated by Src and other tyrosine kinases (Boyle et al., 2007; Head et al., 2003; Huang et al., 1998; Kanner et al., 1990; Mader et al., 2011; Rajadurai et al., 2012), and this allows for cortactin to bind to N-WASP, promoting the Arp2/3-dependent actin polymerization that is required for invadopodia activity (DesMarais et al., 2004; Oser et al., 2009; Tehrani et al., 2007; Yamaguchi et al., 2005). The proteolytic activity of invadopodia for degradation of ECM is conferred by proteases, ultimately allowing metastatic tumor cells to migrate through the ECM and to localize to distant organs. The membrane-anchored metalloproteinase, MT1-MMP (also known as MMP14), is considered as a central player in mediating invadopodia-dependent ECM degradation (Artym et al., 2006; Hotary et al., 2006; Murphy and Courtneidge, 2011). However, how the metalloproteinases are recruited to sites of invadopodia has been unclear.

In addition to acquiring invadopodia-mediated ECM degradation activity, metastatic tumor cells are frequently associated with abnormal activation of a developmental process – the epithelial–mesenchymal transition (EMT) (Thiery and Sleeman, 2006; Yang and Weinberg, 2008). Interestingly, it has been recently reported that cells that have undergone EMT acquire invadopodia activity, suggesting that one of the key roles for tumor cells to undergo EMT during metastasis is perhaps to gain the ability to degrade ECM through invadopodia (Eckert et al., 2011). The aberrant activation of EMT can be driven by a family of transcription factors, such as the Snail- or Twist-family proteins, and by the cytokine TGFβ. Recent work from our lab has demonstrated that EMT is controlled at the level of alternative RNA splicing, a mechanism that is essential to generate protein diversity (Brown et al., 2011; Liu and Cheng, 2013; Xu et al., 2014). These studies relied on analysis of a crucial cell surface molecule, CD44. The CD44 gene comprises nine constitutive exons, and nine and ten variable exons in the human and mouse genome, respectively, residing between the constitutive exons (Ponta et al., 2003). Inclusion of one or more of the variable exons generates CD44 variant (CD44v), whereas skipping all of the variable exons produces CD44 standard (CD44s). We have previously demonstrated that CD44 isoform switching occurs during EMT, resulting in a shift in expression from CD44v in epithelial cells to CD44s in mesenchymal cells (Brown et al., 2011). Importantly, when CD44 isoform switching is perturbed, cells can no longer undergo EMT (Brown et al., 2011). Supporting the role of CD44s in EMT, recent work has also shown that CD44 depletion attenuates breast cancer bone metastasis (Hiraga et al., 2013).

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Received 16 April 2015; Accepted 4 February 2016

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Journal of Cell Science (2016) 129, 1355-1365 doi:10.1242/jcs.171959

Although these studies introduce CD44s as a crucial player in EMT and breast cancer, the mechanisms by which CD44, especially at the splice isoform level, functions have not been well understood.

Given the fact that CD44s plays a crucial role in EMT, and that its function has been implicated in tumor cell invasion and breast cancer progression, we questioned whether the CD44s isoform specifically plays an important role in invadopodia function, allowing for matrix degradation and tumor cell metastasis. The study presented here demonstrates that CD44s, but not CD44v, is a crucial component in invadopodia. Supporting these findings, we show that CD44s promotes breast tumor metastasis to the lung in a breast cancer metastasis model. Taken together, these results suggest that CD44s might serve as an effective therapeutic target for the treatment of metastatic breast cancer.

RESULTS

CD44 is localized in invadopodia in breast cancer cells and is required for invadopodia activity

Localization of actin, cortactin and Tks5 in dot-like structures that have the ability to degrade matrix is characteristic of invadopodia (Murphy and Courtneidge, 2011). Human MDA-MB-231 breast cancer cells were used to examine whether CD44 is a component of the invadopodia structure. Immunofluorescence analysis showed that CD44 colocalized together with actin at regions where matrix was degraded, suggesting that it is located in invadopodia (Fig. 1A, top row). Further analyses also revealed that CD44 colocalized with cortactin and Tks5 (Fig. 1A, middle and bottom rows, respectively) in areas of matrix degradation, supporting the notion that CD44 is a protein component in invadopodia. This observation was further confirmed in human metastatic breast cancer BT-549 cells (Fig. S1A).

To determine whether CD44 is functionally important in invadopodia activity in breast cancer cells, we knocked down CD44 by using small hairpin (sh)RNA in MDA-MB-231 cells (Fig. 1B). Co-staining of Tks5 and cortactin showed that approximately 40% of the control cells were positive for invadopodia (Fig. 1C,D). In stark contrast, less than 5% of cells in which CD44 had been knocked down exhibited invadopodia-like puncta. Furthermore, these CD44-knockdown MDA-MB-231 cells lost the ability to degrade matrix, as gauged by analyzing gelatin degradation (Fig. 1E,F), a phenotype that was also observed in HT1080 cells (Fig. S1B,C,D). In addition, depletion of CD44 caused a reduction in the invasion ability of cells, scored by using Boyden Chamber invasion assays (Fig. 1G). Taken together, these results demonstrate that CD44 is required for the activity of invadopodia and tumor cell invasion.

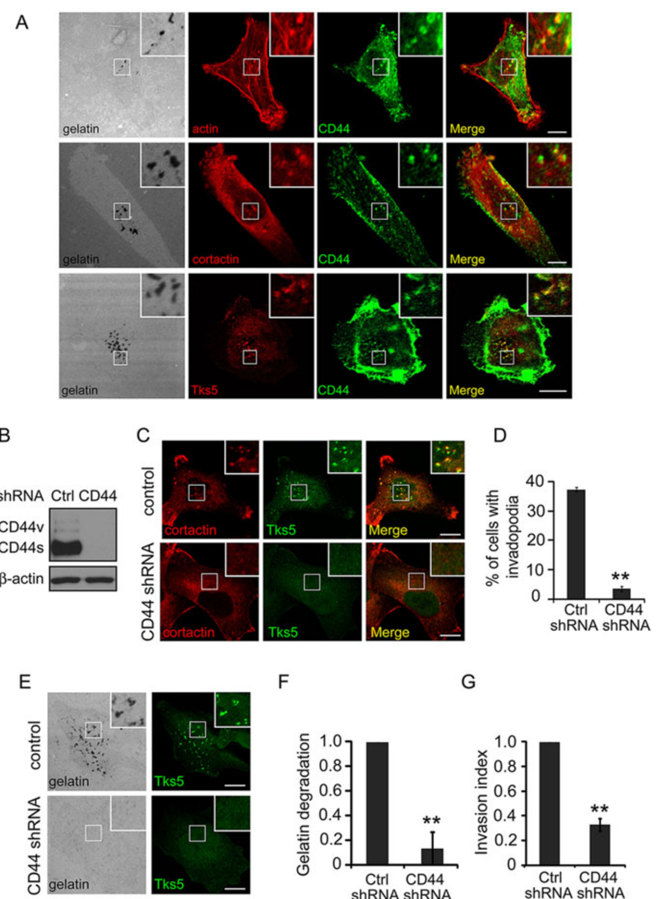

Fig. 1. CD44 is localized in invadopodia and is required for invadopodia activity. (A) Breast cancer cells MDA-MB-231 were grown on gelatin-coated coverslips (gray) and co-stained for actin, cortactin or Tks5, and CD44 (green). Immunofluorescence images (60× for actin and cortactin, and 100× for Tks5) are shown. CD44 colocalized with invadopodia components actin, cortactin and Tks5 in regions of gelatin degradation. (B) Immunoblot analysis showing knockdown efficiency of CD44 in MDA-MB-231 cells. Ctrl, control. (C,D) Immunofluorescence images (100×) (C) and quantification of invadopodia (D), showing decreased numbers of invadopodia, gauged by counting the cortactin (red) and Tks5 (green) co-stained dot-like structures in control (Ctrl shRNA) and CD44-knockdown (CD44 shRNA) cells. (E,F) Immunofluorescence images (100×) (E) and quantification of gelatin degradation (see Materials and Methods for explanation of the gelatin degradation index) (F), showing that CD44-knockdown inhibits invadopodia-mediated gelatin degradation. Gelatin is labeled in gray and Tks5 is stained in green. (G) Boyden Chamber assay showing that CD44-shRNA-expressing MDA-MB-231 cells exhibited impaired tumor cell invasion activity. The invasion index was calculated as the ratio of the invasion of CD44-knockdown cells (in percent) to the invasion of control cells (in percent). Data are mean±s.d.; n=3. **P<0.01 (Student's t-test). Scale bars: 10 µm.

CD44s is the major isoform associated with the invadopodia complex

An intriguing observation was that cells that formed invadopodia, including MDA-MB-231, BT-549, and HT-1080, mainly expressed the CD44s isoform (Fig. S2A). By contrast, cells that predominantly expressed CD44v did not form invadopodia structures (Fig. S2A,B). These results suggest that the isoform specificity of CD44 determines its role in the activity of invadopodia. To test this hypothesis, CD44s and CD44v cDNAs were ectopically expressed in the CD44-knockdown MDA-MB-231 cells to levels comparable to those in the parental cells (Fig. 2A). We found that reconstitution of CD44s in the CD44-depleted cells fully rescued the impaired phenotype of invadopodia, as evidenced by the colocalization of Tks5 with cortactin or actin at sites of matrix degradation (Fig. 2B; Fig. S2C). Quantification of the number of cells that exhibited the invadopodia puncta, as well as the extent of the area of gelatin degradation, also supported these findings (Fig. 2C, top and bottom panels, respectively). By contrast, forced expression of CD44v did not rescue the impaired invadopodia phenotype (Fig. 2B,C; Fig. S2C). These results were further confirmed by z-sectioning images, showing that although both CD44s and CD44v were localized on the basal surface of the cell, only CD44s protruded into sites of gelatin degradation (Fig. 2D). CD44v-expressing cells, by contrast, did not show any matrix degradation activity (Fig. 2D). Close examination of these CD44s- and CD44v-reconstituted cells showed that signals of actin and cortactin colocalization were noticeably lower at dot-like structures in CD44v-reexpressing cells (Fig. S2D,F), although the

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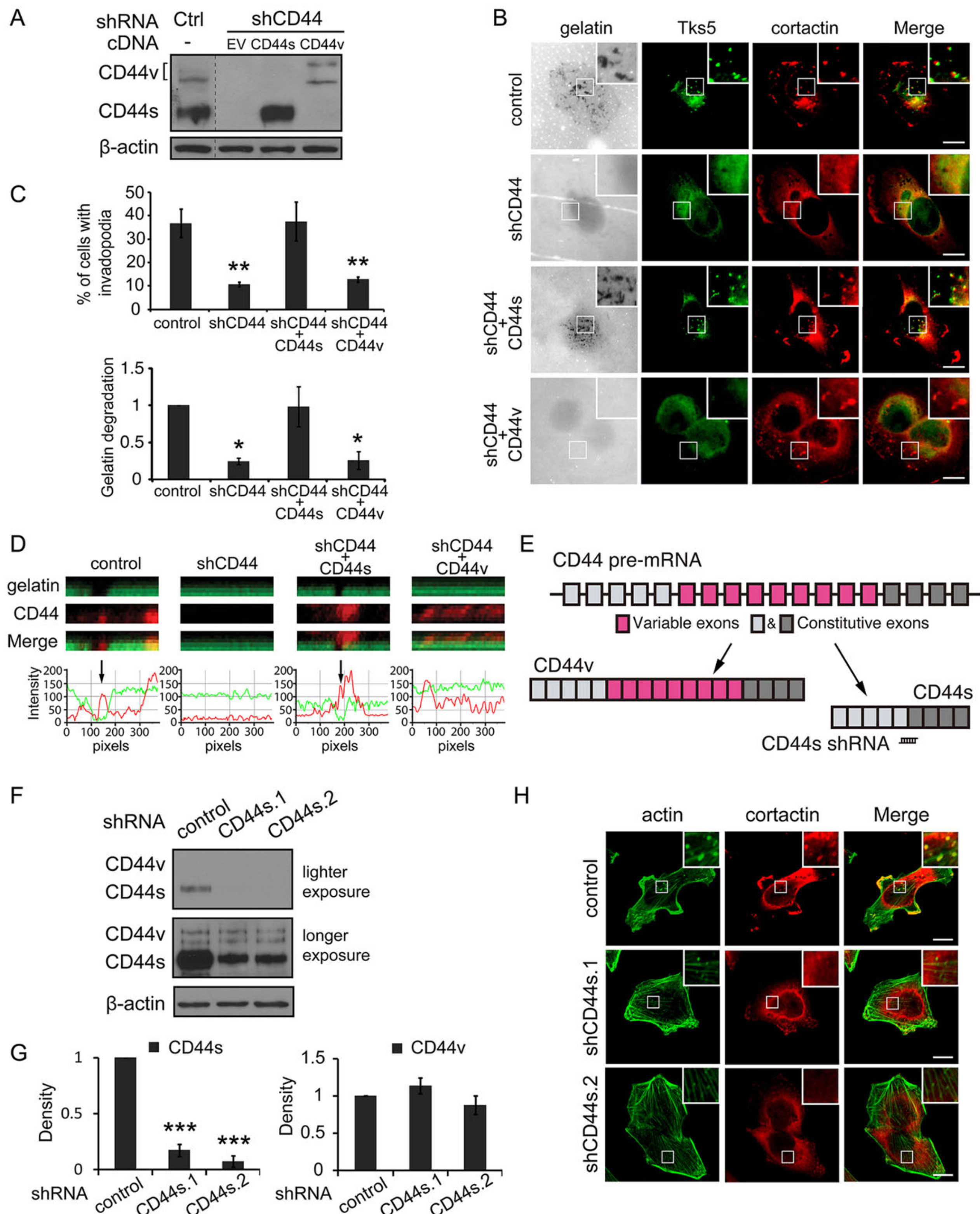

Fig. 2. See next page for legend.

overall signal of colocalization appeared to be of a similar level in both CD44s- and CD44v-reexpressing cells. Furthermore, immunostaining of cortactin and Tks5 indicated that CD44s-reconstituted cells contained a significantly higher number of cortactin–Tks5 aggregates as compared to CD44v-reconstituted cells, where Tks5 was not located at dot-like structures, suggesting that Tks5 recruitment is impaired in the absence of CD44s (Fig. S2E,F).

To rigorously examine the importance of CD44s in invadopodia, isoform-specific shRNAs (Fig. 2E) that had been designed uniquely to target the CD44s isoform (Fig. 2F,G) were expressed in MDA-MB-231 cells. In agreement with our observations shown above,

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Fig. 2. CD44 splice isoform specificity controls invadopodia activity. (A) Immunoblot analysis showing CD44 isoform expression in CD44-knockdown MDA-MB-231 cell lines that reexpressed CD44s and CD44v cDNA. The presence of doublets of CD44v is thought to be due to post-translational modification. Ctrl, control; EV, empty vector. (B) Immunofluorescence images (63×) showing that CD44s, but not CD44v, is capable of fully rescuing the defects in invadopodia activity caused by CD44 shRNA-mediated (shCD44) knockdown in MDA-MB-231 cells. Tks5 is green, and cortactin is red. Black dots on the gray background indicate sites of gelatin degradation. (C) Quantification of cells that contained invadopodia (top panel) and of areas of gelatin degradation (bottom panel) showing that CD44s fully restored invadopodia structure and matrix degradation activity caused by expression of shRNA against CD44. (D) *z*-section images showing that CD44s (red) was located in the holes where gelatin (green) had been degraded, whereas CD44v (red) was also localized in the basement of the membrane but did not degrade gelatin. Top panel, representative images. Bottom panel, measurement of immunofluorescence intensity of CD44 (red) and gelatin (green) staining. Arrows indicate signals of CD44s coinciding with gelatin degradation. (E) Schematic diagram of CD44 pre-mRNA and the design of the shRNA against CD44s. Constitutive exons are shown in light and dark gray boxes separated by variable exons in magenta. The shRNAs against CD44s were designed to target the exon junction of CD44s that would otherwise be destroyed by insertion of the variable exons in CD44v. (F,G) Immunoblot analysis (F) and quantification (G) of CD44 isoform expression showing that the CD44s-targeting shRNA specifically depleted CD44s without affecting the expression levels of CD44v. (H) Immunofluorescence images (60×) showing that CD44s knockdown inhibited colocalization of actin (green) and cortactin (red) in dot-like structures. Data are mean±s.d.; n=3 replicates. *P<0.05, **P<0.01, ***P<0.001 (one-way Anova). Scale bars: 10 µm.

knockdown of CD44s by its isoform-specific shRNA inhibited invadopodia function, as indicated by the loss of colocalization of actin and cortactin at invadopodia structures in CD44s-depleted cells (Fig. 2H). Thus, these different sets of approaches demonstrate that the CD44s isoform, but not CD44v, is essential for mediating invadopodia activity.

We also tested whether CD44s is sufficient for invadopodia activity. We utilized a non-invasive breast cancer T47D cell line and generated T47D-SrcY527F cells, which express a constitutively active mutant of Src (SrcY527). However, no invadopodia activity was observed when CD44s was overexpressed in these cells (Fig. S2G,H). We suspect that this could be due to the lack of other crucial components in the T47D-SrcY527F cells. For instance, Tks5 has been shown to be insufficiently expressed in these cells (Seals et al., 2005).

Depletion of CD44 impairs cortactin phosphorylation

It has been well established that phosphorylation of the adaptor protein cortactin is a key event for invadopodia function through initiation of actin polymerization. In line with the importance of CD44s in invadopodia, CD44-depleted MDA-MB-231 cells showed reduced levels of phosphorylated cortactin, whereas the total levels of cortactin and the other invadopodia-associated proteins Tks5 and Src were unaffected (Fig. 3A, left panel; Fig. 3B). This observation was further confirmed in the HT-1080 cells (Fig. 3A, right panel; Fig. 3B).

To determine the relationship between CD44s and cortactin in invadopodia function, we utilized the CD44s-reexpressing MDA-MB-231 cells in which CD44 had been knocked down because, in these cells, invadopodia activity is dependent on CD44s. We found that knockdown of cortactin significantly decreased the number of invadopodia and impaired matrix degradation activity in these cells (Fig. 3C–E), suggesting that CD44s-mediated invadopodia activity also requires cortactin. Because cortactin phosphorylation is mediated chiefly by the Src kinase, we also treated the CD44s-reexpressing cells with the Src-family inhibitor PP2, which blocks Src activity and, thus, cortactin phosphorylation. Consistent with the above observations, treatment with PP2 abolished the ability of invadopodia to degrade gelatin (Fig. S3A–C).

It is worth mentioning that CD44 knockdown did not affect Src activity, at least under conditions where decreased cortactin phosphorylation was observed (Fig. S3D). This prompted us to test the possibility that CD44s interacts with cortactin, facilitating cortactin phosphorylation. An immunoprecipitation assay showed cortactin in the CD44-immunoprecipitated fraction (Fig. 3F, left panel). However, CD44s was also detected in the cortactin-immunoprecipitated fraction (Fig. 3F, right panel). To examine whether this CD44 and cortactin interaction was specific to the CD44s isoform, we next performed immunoprecipitation experiments in the CD44s-reexpressing MDA-MB-231 cells, as well as in MCF7 cells where CD44v is predominantly expressed (Fig. S2A). We found that both CD44 isoforms interacted with cortactin and that the major form of cortactin in the CD44s-interacting fraction was phosphorylated cortactin (Fig. S3E,F). Although CD44v exhibited a stronger binding affinity to cortactin (Fig. S3F), nearly undetectable levels of phosphorylated cortactin were associated with CD44v (Fig. S3F). Thus, these results suggest that the CD44s splice isoform preferentially interacts with phosphorylated cortactin.

Constitutive activation of Src restores cortactin phosphorylation but not invadopodia activity in CD44-knockdown cells

Given the above results that depletion of CD44 inhibits cortactin phosphorylation and invadopodia activity, we next examined whether restoring cortactin phosphorylation in the CD44-knockdown cells could rescue the impaired invadopodia phenotype. Forced expression of constitutively active oncogenic SrcY527F restored cortactin phosphorylation in CD44-knockdown MDA-MB-231 cells to levels comparable to those in control cells (Fig. 4A). Interestingly, however, expression of SrcY527F failed to rescue the impaired invadopodia activity in the CD44-knockdown cells (Fig. 4B, left panel). Roughly, 11% of CD44-knockdown cells contained invadopodia, whereas 65% of control cells were observed to contain invadopodia (Fig. 4B, right panel). Furthermore, a greater than tenfold reduction in matrix degradation activity was shown in CD44-depleted SrcY527F-expressing cells as compared to that of control cells (Fig. 4C).

Analysis of SrcY527F-expressing NIH3T3 (3T3-SrcY527F) cells further supported the above observations. Although cortactin phosphorylation was at the same level in control and CD44-knockdown cells (Fig. 4D), knockdown of CD44 impaired invadopodia activity (Fig. 4E) and reduced matrix degradation activity (Fig. 4F). Taken together, these results indicate that, in addition to affecting cortactin phosphorylation, CD44s impacts pathways downstream of cortactin that are crucial for invadopodia activity.

CD44s-dependent invadopodia activity requires MT1-MMP activity

Matrix degradation activity in invadopodia is mediated by metalloproteinases. In accordance with this, treatment of MDA-MB-231 parental cells with a pan-metalloproteinase inhibitor, GM6001, abolished gelatin degradation (Fig. 5A, top panel). Notably, GM6001 treatment also prevented gelatin degradation in the CD44s-reconstituted cells, in which the matrix degradation activity was mediated through CD44s (Fig. 5A,B).

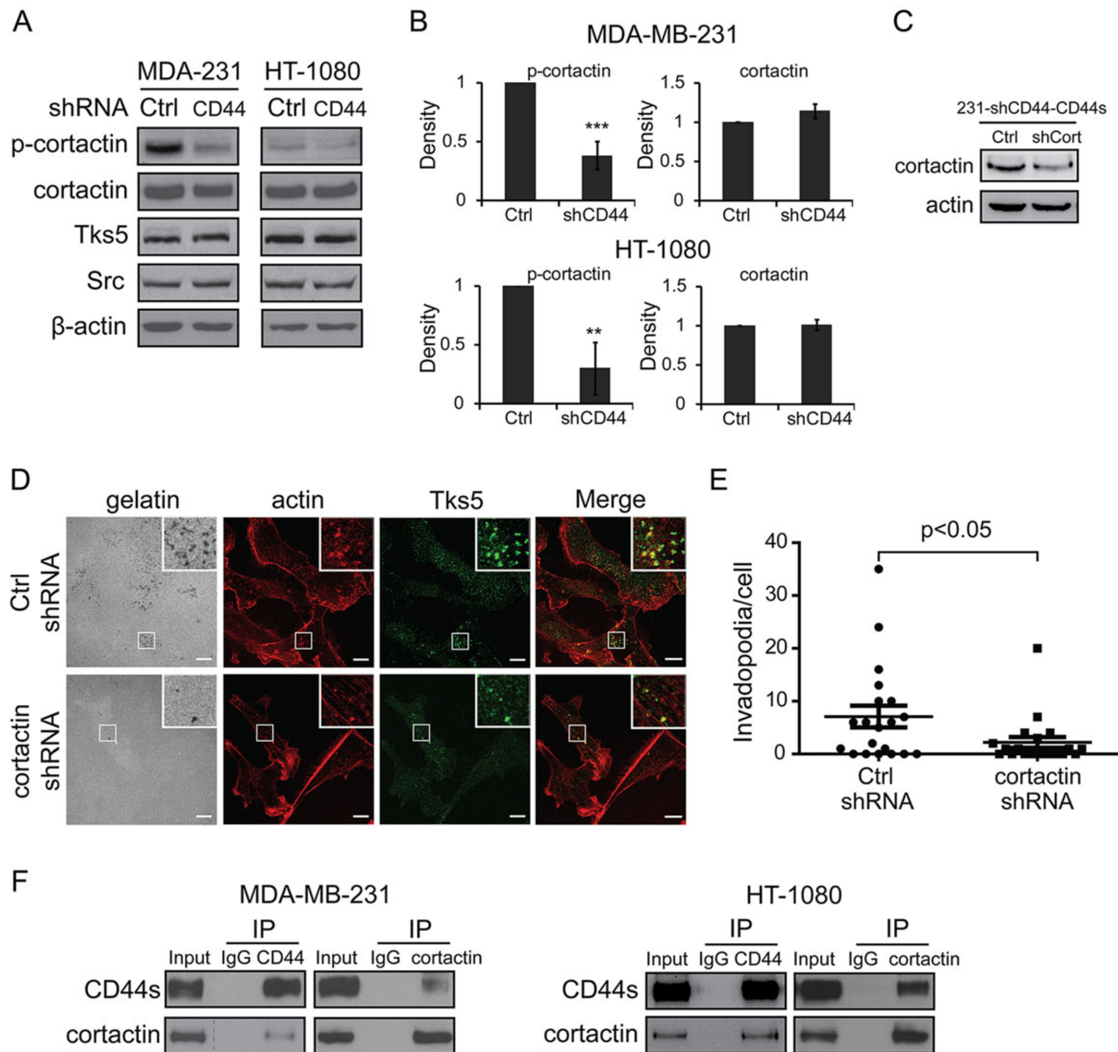

Fig. 3. CD44s interacts with cortactin and promotes cortactin phosphorylation. (A,B) Immunoblot images (A) and quantifications (B) showing that phosphorylated cortactin levels were decreased in MDA-MB-231 and HT-1080 cells that expressed CD44-targeting shRNA, whereas cortactin, Tks5 and Src levels remained the same. Data are mean±s.d.; *n*=3. ***P*<0.01, ****P*<0.001 (Student's *t*-test). (C) Immunoblot images showing cortactin-knockdown efficiency in CD44-knockdown CD44s-rescued MDA-MB231 cells (231-shCD44-CD44s cells) expressing cortactin-targeting shRNA. (D,E) Immunofluorescence images (60×, D) and quantification (E) showing that cortactin knockdown inhibits CD44-mediated invadopodia function. Scale bars: 10 µm. Black lines indicate the mean with 95% confidence intervals indicated. *n*=20 cells. *P* value was calculated by using unpaired *t*-test with Welch's correction. (F) Co-immunoprecipitation assays indicating that an antibody against CD44 immunoprecipitates cortactin (left) and that an antibody against cortactin immunoprecipitates CD44 (right) in both MDA-MB-231 and HT-1080 cells. A quarter of the immunoprecipitated fraction was loaded and analyzed by SDS-PAGE for detection of target proteins, and three-quarters were used for the detection of protein of interaction. Ctrl, control; IP, immunoprecipitation.

MT1-MMP is considered as the main protease that is responsible for the degradation activity in invadopodia (Artym et al., 2006; Hotary et al., 2006; Murphy and Courtneidge, 2011). We found that CD44s-mediated matrix degradation required MT1-MMP protease activity because elimination of MT1-MMP by using small interfering (si)RNA showed a 12-fold inhibition in gelatin degradation in the CD44s-reconstituted cells (Fig. 5C–E).

CD44s and MT1-MMP interact at sites of invadopodia

It has been previously reported that CD44 interacts with MT1-MMP, resulting in colocalization in lamellipodia, a two-dimensional structure that mediates cell movement (Mori et al., 2002). This observation led us to suspect that CD44 and MT1-MMP interact in invadopodia. We expressed a DsRed-tagged MT1-MMP cDNA in MDA-MB-231 cells, and found that CD44s and MT1-MMP–DsRed colocalized in the actin-rich dot-like invadopodia structures (Fig. 6A). Furthermore, immunostaining of endogenous CD44 and MT1-MMP in 3T3-SrcY527F cells showed that they colocalized at sites of gelatin degradation, resembling CD44 and cortactin colocalization (Fig. 6B). Additionally, reciprocal immunoprecipitation experiments revealed that endogenous CD44s and MT1-MMP interacted in both MDA-MB-231 and HT-1080 cells (Fig. 6C).

To further understand the relationship between CD44s and MT1-MMP, total internal reflection fluorescence (TIRF) microscopy time-lapse experiments were performed using MDA-MB-231 cells expressing MT1-MMP–DsRed and CD44–ZsGreen. As illustrated in Fig. 6D,E, two types of relationship between CD44 and MT1-MMP were detected at the cell membrane. In the first senario (Fig. 6D), CD44 initially appeared in a dot-like structure in the absence of MT1-MMP. MT1-MMP migrated to the dot-like CD44-marked structure and colocalized with CD44. Once MT1-MMP

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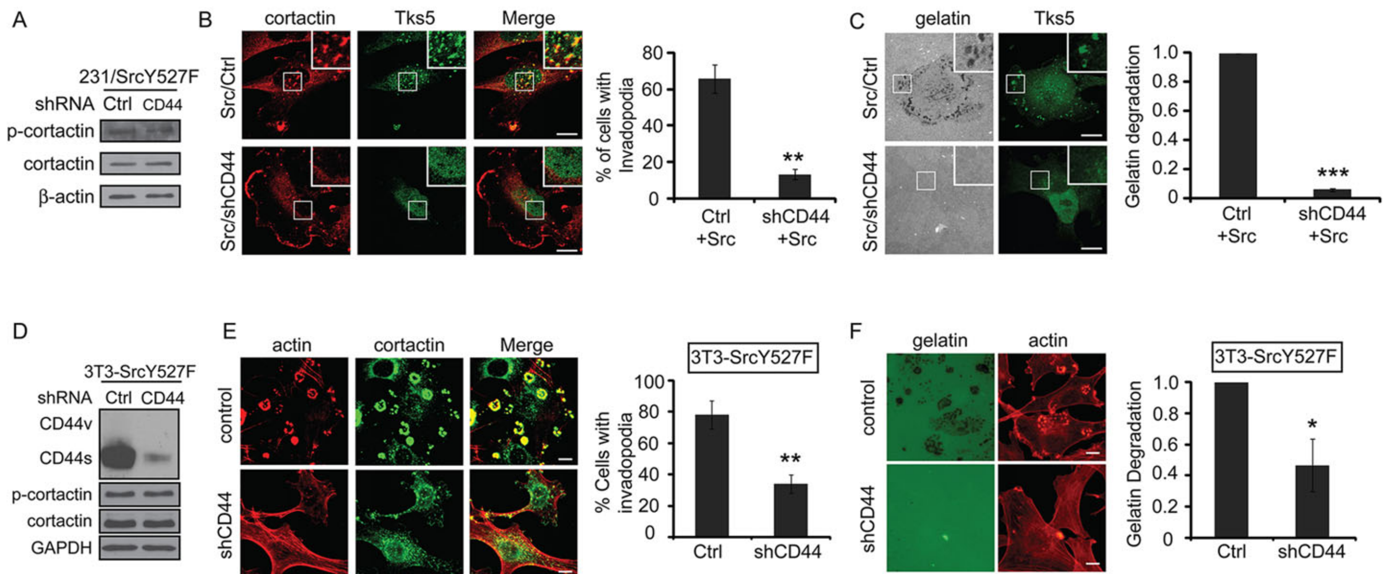

Fig. 4. Restoration of cortactin phosphorylation does not rescue invadopodia defects caused by CD44 knockdown. (A) Immunoblot analysis showing similar levels of phosphorylated (p-)cortactin in control and CD44-knockdown MDA-MB-231 cells that express SrcY527F (231/SrcY527F cells). (B) Immunofluorescence images (100×, left panel) and quantification (right panel) showing that ectopically expressing SrcY527F (Src) did not restore invadopodia structure in CD44-knockdown (shCD44) MDA-MB-231 cells. '/' denotes co-expression. Cortactin is in red and Tks5 is in green. (C) Immunofluorescence images (100×, left panel) and quantification (right panel) showing that SrcY527F expression does not restore invadopodia-mediated gelatin degradation activity in the CD44-knockdown MDA-MB-231 cells. Gelatin is labeled in gray, and Tks5 is shown in green. (D) Immunoblot analysis showing levels of CD44 and p-cortactin in control-shRNA-expressing (Ctrl) and shCD44-expressing NIH3T3 cells that overexpressed SrcY527F (3T3-SrcY527F cells). (E) Immunofluorescence images (63×, left panel) and quantification (right panel) showing that 3T3-SrcY527F cells that have had CD44 knocked down retained invadopodia defects, as gauged by co-staining of actin (red) and cortactin (green). (F) The cells shown in E also had impaired gelatin degradation activity. Data are mean±s.d.; n=3 replicates. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test). Scale bars: 10 µm.

colocalized with CD44, they remained colocalized for the entire duration of the time lapse. In the second scenario (Fig. 6E), CD44 and MT1-MMP colocalized with each other for an extended period of time without apparent separation. In contrast to the above findings, we did not observe a single case in which MT1-MMP appeared first and recruited CD44. These results in Fig. 6D,E suggest that CD44s recruits and colocalizes with MT1-MMP at the cell membrane.

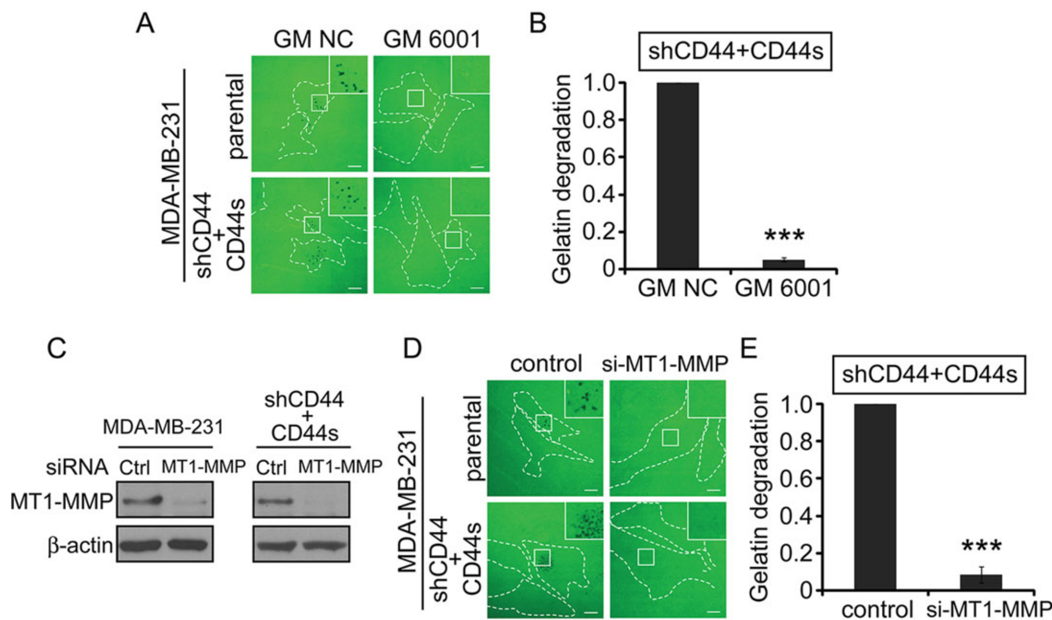

Fig. 5. CD44s-dependent invadopodia activity is mediated by MT1-MMP. (A,B) Fluorescence images (100×) of gelatin degradation (A) and quantification (B) showing that treatment with the protease inhibitor GM6001 abolishes CD44s-dependent matrix degradation activity. MDA-MB-231 parental cells and cells co-expressing shRNA against CD44 (shCD44) with cDNA encoding CD44s were used. (C) Immunoblot analysis showing the efficiency of MT1-MMP knockdown with siRNA. (D,E) Fluorescence images (100×) of gelatin degradation (D) and quantification (E) showing that CD44s-dependent matrix degradation activity is mediated by MT1-MMP. Data are mean±s.d.; n=3 replicates. ***P<0.001 (Student's t-test). Scale bars: 10 µm. Ctrl, control; GM NC; negative control for GM6001; si-MT1-MMP, siRNA against MT1-MMP.

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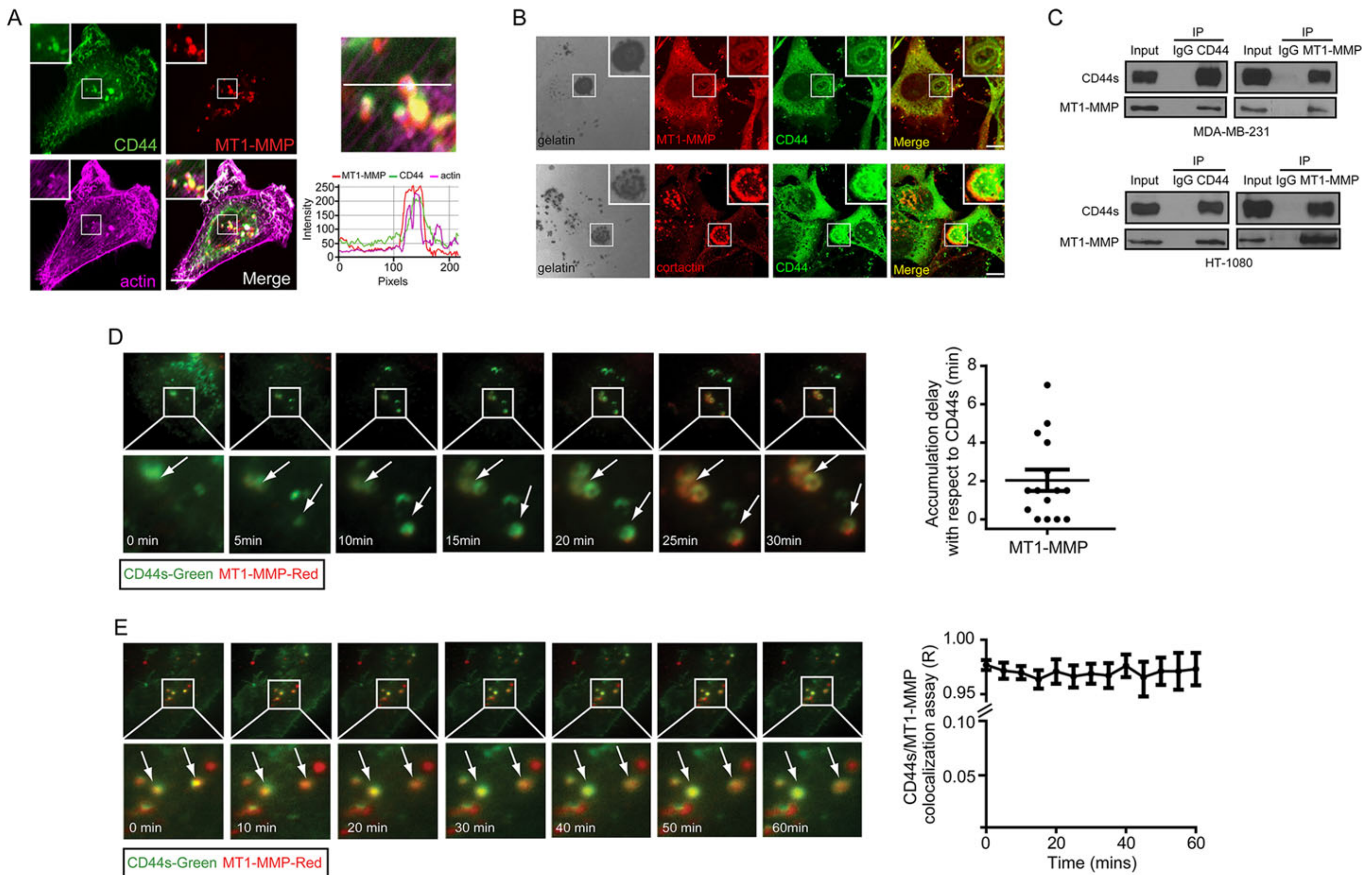

Fig. 6. CD44s and MT1-MMP interact at sites of invadopodia. (A, left panel) Immunofluorescence images (100×) showing colocalization of CD44s (green) and MT1-MMP (red) at actin-enriched dot-like structures (magenta) in MDA-MB-231 cells that expressed MT1-MMP–DsRed. (Right panel) Measurement of immunofluorescence intensity of CD44s (green), MT1-MMP (red) and actin (magenta) in the section indicated with a white line. (B) Immunofluorescence images in 3T3-SrcY527F cells showing colocalization of CD44 (green) and MT1-MMP (red, top panel), and of CD44 (green) and cortactin (red, bottom panel) at sites of gelatin degradation (black hole). (C) Co-immunoprecipitation analysis showing that CD44s and MT1-MMP interact when they are present at endogenous levels in both MDA-MB-231 and HT-1080 cells. For immunoprecipitation experiments in MDA-MB-231 cells, a quarter of the immunoprecipitated fraction was analyzed by SDS-PAGE for detection of target proteins, and three-quarters were used for detection of protein interactions. For HT-1080 cells, one-tenth of the immunoprecipitated fraction was analyzed by SDS-PAGE for detection of target proteins, and nine-tenths were used for detection of protein interactions. These results suggest that close to 10% of CD44s interacts with MT1-MMP and that ∼15–30% of MT1-MMP interacts with CD44s. (D) Time-lapse TIRF imaging showed that MT1-MMP localizes to dots where CD44s is present (left panel) and that MT1-MMP arrival is delayed with respect to that of CD44s (right panel). Black lines indicate means with 95% confidence intervals shown. *n*=15 CD44s and MT1-MMP colocalization dots. Arrows indicate the recruitment of MT1-MMP by CD44s. (E) Time-lapse TIRF imaging (left panel) and quantification (right panel) showing that CD44s and MT1-MMP colocalize at the cell membrane in MDA-MB-231 cells for an extended period of time. Data are mean±s.d.; *n*=3 replicates. CD44s was expressed with a C-terminal green tag and MT1-MMP was expressed with a C-terminal red tag. Arrows indicate colocalization of MT1-MMP with CD44s. Scale bars: 10 µm.

CD44 is required for breast tumor cells metastasizing to distant organs

The above data demonstrate that CD44s acts as a crucial component in mediating invadopodia activity. Given the functional link between invadopodia and tumor metastasis, the requirement for CD44s in metastasis was assessed using an LM2 lung metastatic cell line that had been derived from the parental MDA-MB-231 cells and that predominantly expresses the CD44s isoform (Fig. 7A). In agreement with our results in Fig. 1, CD44 depletion (Fig. 7A) resulted in a significant decrease in invadopodia activity in LM2 cells (Fig. S4A,B).

Tail vein injection of LM2 cells into mice results in lung metastasis within seven weeks. Depletion of CD44s in LM2 cells by using shRNA caused a significant decrease in lung metastatic tumor formation, as monitored by using bioluminescence imaging (BLI) (Fig. 7B,C). This decrease occurred even at week 1 after injection, suggesting a role for CD44 in the initial seeding stage of metastasis development. This observation is in line with the role for CD44s in invadopodia and supports the idea that CD44 is indispensable in tumor metastasis.

DISCUSSION

Tumor metastasis remains a major clinical obstacle for the treatment of breast cancer. Invadopodia serve as crucial cellular structures that enable tumor cells to degrade extracellular matrix, allowing them to disseminate from primary sites and metastasize to distant organs. In this study, we provide multiple lines of evidence demonstrating that the CD44s splice isoform specifically plays a crucial role in invadopodia activity, leading to breast tumor metastasis. We show that CD44s interacts with cortactin and promotes its phosphorylation, a key step for actin polymerization in invadopodia. We also find that CD44s interacts with MT1-MMP at sites of invadopodia, promoting proteolytic activity. Using a mouse model of metastatic breast cancer, our data reveal that depletion of CD44s prevents breast cancer cells from disseminating to the lung. Hence, these results demonstrate that the CD44s splice

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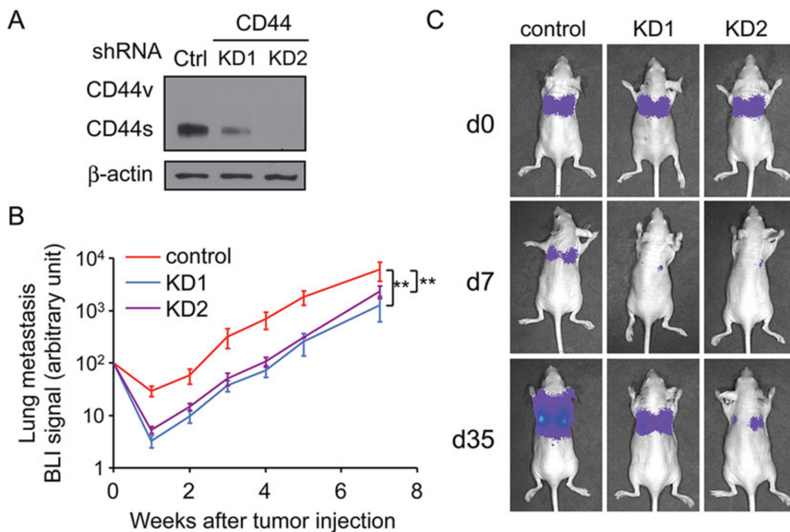

Fig. 7. CD44s is required for breast cancer lung metastasis. (A) Immunoblot analysis showing knockdown efficiency of CD44 in human breast cancer LM2 cells with two different shRNAs (called KD1 and KD2). Ctrl, control shRNA. (B) BLI curves of lung metastasis development in female Athymic nude mice injected through the lateral tail vein with control and CD44-knockdown cells. Data represent mean±s.d. n=9. **P<0.01 (Mann–Whitney test). (C) Representative BLI images of mice, for which the data are shown in B, at 0, 7 and 35 days post injection.

isoform plays a crucial role in invadopodia function and breast tumor metastasis.

The CD44 gene encodes a family of cell surface proteins that comprises the short CD44s isoform and the long variable-exon-containing CD44v proteins, generated by alternative splicing. Previous studies have suggested a role for CD44 in invadopodia (Chabadel et al., 2007; Grass et al., 2013; Vikesaa et al., 2006). However, whether specific isoforms of CD44 are functionally crucial in invadopodia in cancer cells has not been determined. By performing isoform reconstitution assays and by undertaking isoform-specific shRNA approaches, our data demonstrate that the CD44s isoform, but not CD44v, constitutes the function of CD44 in the activity of invadopodia, emphasizing splice isoform specificity in the activity of invadopodia. These results are in agreement with our previous findings that isoform switching of CD44 to the production of CD44s is essential for EMT (Brown et al., 2011), a process that allows for the acquisition of active invadopodia (Eckert et al., 2011). We suspect that the CD44 isoform-specific activities are controlled, at least in part, by their different interacting proteins, resulting in distinguished phenotypes of cells, an area of research that warrants further investigation.

Cortactin phosphorylation is a key step for the recruitment of actin-regulating proteins, such as Nck, N-WASP and Arp2/3, which initiate actin polymerization (DesMarais et al., 2004; Murphy and Courtneidge, 2011; Oser et al., 2009; Paz et al., 2013; Tehrani et al., 2007; Yamaguchi et al., 2005), resulting in invadopodia activity. Our study shows that CD44s is required for cortactin phosphorylation, which is a downstream substrate of Src. It has been previously shown that CD44 interacts with Src and that treatment with hyaluronic acid, the CD44 ligand, promotes Src activity, cortactin phosphorylation and tumor cell migration (Bourguignon et al., 2001). In our system, however, we did not find differences in the levels of Src phosphorylation in control and CD44-knockdown cells, suggesting that Src activity as a whole remains consistent in these cells. We suspect that local Src activity is elevated in the invadopodia loci and that CD44s brings cortactin to sites of phosphorylation. Supporting this view, we found that CD44s interacts with cortactin and that they colocalize at invadopodia. This is also in line with our observation that CD44s is crucial for Tks5 and cortactin colocalization, and to a lesser extent, cortactin and actin colocalization. We suspect that recruitment of Tks5, the scaffold protein for Src, might facilitate Src-mediated cortactin phosphorylation. An alternative mechanism could be that CD44 promotes cortactin phosphorylation through a CD44-dependent complex that contains receptor tyrosine kinases (RTKs) because CD44–RTK interactions have been previously reported (Ponta et al., 2003). This idea is supported by recent studies showing that MET activation causes increased cortactin phosphorylation through a cortactin–Gab1 scaffold complex, resulting in invadopodia formation and matrix degradation (Rajadurai et al., 2012). Although our results, together with previous findings, infer that CD44s promotes cortactin phosphorylation that is important for invadopodia activity, it would be of great interest to investigate the precise mechanisms by which CD44s affects cortactin phosphorylation, as well as the relationship of these two proteins to the stages of invadopodia formation, in future studies.

What is surprising is that upon restoring cortactin phosphorylation by ectopically expressing a constitutively active SrcY527F mutant, CD44-knockdown cells continued to show impaired invadopodia activity. This observation led to the finding that CD44s plays an essential role in MT1-MMP-mediated matrix degradation activity. The relationship between CD44 and MT1-MMP has been previously reported in the context of tumor cell invasion. For instance, MT1-MMP interacts with CD44, resulting in the shedding of CD44 and cell migration (Kajita et al., 2001; Suenaga et al., 2005; Wiesner et al., 2010; Zarrabi et al., 2011). Whether the shedding of CD44s is involved in the activity of invadopodia will be of interest to investigate. Moreover, it has been previously shown that vesicle trafficking mediated by the vesicle (v)-SNARE VAMP7 is involved in the degradation activity of MT1-MMP in invadopodia (Steffen et al., 2008) and that MT1-MMP exocytosis is required for matrix degradation (Monteiro et al., 2013). Considering CD44s as a cell surface molecule that facilitates MT1-MMP localization at sites crucial for matrix degradation, it will be particularly interesting to investigate the involvement of CD44s in vesicle-mediated MT1-MMP transportation and exocytosis in invasive cells.

The expression of CD44 and its isoforms has been analyzed in different types of tumors, and results have been controversial. Data from our and other studies have shown that CD44s expression correlates with higher-grade breast tumors, but it has also been shown that CD44s expression correlates with increased survival (Brown et al., 2011; Diaz et al., 2005; Liu et al., 2005). Furthermore, DNA methylation at the CD44 promoter has been observed in prostate

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cancers (Lou et al., 1999; Woodson et al., 2006). These seemingly ununified results point to the complexity of CD44 isoforms in cancer, perhaps due to context, stage or differences in tumor type, as well as the lack of a CD44s-isoform-specific antibody. These results suggest that isoform specificity must be considered when dissecting the role of CD44 in tumor invasion and metastasis.

In summary, the study presented here demonstrates the importance of a specific splice isoform, CD44s, in the activity of invadopodia. CD44s is required for cortactin phosphorylation and interacts with MT1-MMP at sites of invadopodia. In addition to our findings on the requirement of splice isoforms in the activity of invadopodia, a recent study has shown that a specific Tks5-long isoform, generated by the use of an alternative transcription start site, promotes invadopodia-mediated metastasis, whereas the Tks5-short isoform inhibits it (Li et al., 2013). That and our studies indicate that biological processes are, in many cases, regulated at the level of protein isoforms encoded by the same gene. Given the fact that greater than 90% of human genes undergo alternative splicing (Pan et al., 2008; Wang et al., 2008), investigating the role of splice isoforms in cancer processes could lead to a better understanding of molecular mechanisms and to the development of novel therapeutic interventions that target specific splice isoforms for the treatment of advanced cancers.

MATERIALS AND METHODS

Cell lines

Cell lines MDA-MD-231, MCF7 and HT-1080 were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. BT-549 cells were grown in RPMI 1640 supplemented with 10% FBS and antibiotics, NIH3T3 cells were cultured with DMEM supplemented with 10% calf serum and antibiotics. Retroviral or lentiviral infections were performed for generation of stable cell lines.

Plasmids and shRNA

The pBRIT-HA/Flag vector (AddGene) was used for the generation of pBRIT-CD44-HA and pBRIT-MT1-MMP-HA plasmids. Subsequently, ZsGreen1 and DsRed sequences were cloned into a *SalI* restriction site of pBRIT-CD44-HA and pBRIT-MT1-MMP-HA, respectively. Plasmids expressing CD44s, CD44v (comprising variable exons v3–v10) and shRNA against CD44 that targeted all CD44 isoforms have been described previously (Brown et al., 2011). shRNAs targeting the CD44s isoform specifically were PCR amplified by using the following oligonucleotides and cloned into LMP vector. CD44s.1: 5′-TGCTGTTGACAGTGAGCG-TCTACCAGAGACCAAGACACATTAGTGAAGCCACAGATGTAAT-GTGTCTTGGTCTCTGGTAGCTGCCTACTGCCTCGGA-3′. CD44s.2: 5′-TGCTGTTGACAGTGAGCGATACCAGAGACCAAGACACATTTA-GTGAAGCCACAGATGTAAATGTGTCTTGGTCTCTGGTAGTGCCT-ACTGCCTCGGA-3′. The shRNA oligonucleotides targeting cortactin were as follows. Forward: 5′-CCGGTGTAACATCAGAGCTAACTTTG-CTCGAGCAAAGTTAGCTCTGATGTTACTTTTTG-3′; Reverse: 5′-A-ATTCAAAAAGTAACATCAGAGCTAACTTTGCTCGAGCAAAGTT-AGCTCTGATGTTACA-3′. They were cloned into the pLKO.1 vector. The siRNA targeting MT1-MMP was purchased from Invitrogen.

In vitro invasion assay

MDA-MB-231 and NIH3T3 cells were plated onto a 6-cm plate to reach 30–50% confluence the next day. At 24 h after plating, cells were starved in 3 ml of 0.5% serum in DMEM. At 24 h after starvation, cells were trypsinized, washed with PBS twice and resuspended in serum-free DMEM. A total of 2.5×10^4 cells in 500 µl of DMEM were plated into a BD BioCoat™ Matrigel™ Invasion Chamber insert. The inserts were placed in the lower chamber that contained 700 µl of DMEM with 10% FBS. After 24 h of incubation, non-invading cells were removed with a cotton swab. The remaining invaded cells were stained with 0.1% Crystal Violet in 75% ethanol for 10 min, followed by a wash with H_2O. The membranes of the inserts were fixed with Permount mounting solution. Triplicate biological repeats were performed for all invasion assays.

Immunoprecipitation

MDA-MD-231 or HT-1080 cells were lysed in lysis buffer (20 mM Tris-HCL, pH 7.5, 100 mM NaCl, 0.5% NP-40, 5 mM EDTA, 20 mM β-glycerophosphate, 16% glycerol, 25 mM NaF, 2 mM Na_3VO_4) containing a 1:20 dilution of a protease inhibitor cocktail tablet (Roche). For the immunoprecipitation assay, 2 µg of primary antibodies were conjugated with Protein G beads and incubated with cell lysates overnight at 4°C. Beads were washed in lysis buffer four times. Proteins were eluted from beads using SDS sample buffer and analyzed on a 10% SDS-PAGE gel.

Gelatin matrix degradation assay

Glass coverslips were incubated in 1 M HCl at 55°C overnight and washed with distilled H_2O for 4 h, then coverslips were incubated with 50 µg/ml poly-L-lysine in PBS for 15 min and washed with PBS three times. After incubating with 0.15% glutaraldehyde in PBS for 10 min, followed by three washes with PBS, coverslips were inverted onto 20-µl droplets that contained 0.1% of gelatin conjugated with Oregon Green® 488 (Molecular Probes) and 0.2% porcine gelatin in a 1:9 ratio for 10 min. Coverslips were subsequently incubated in 5 mg/ml $NaBH_4$ for 15 min, rinsed in PBS and incubated at 37°C in growth medium for 2 h. 2.5×10^4 cells were seeded on each coverslip, incubated for 8 h and processed for immunofluorescence analysis. Each experiment was performed in triplicate. Images were taken for a total of approximately 100 cells per sample. Gelatin degradation was quantified using Image J software. The percentage degradation area was normalized to the total cell number (counted by DAPI staining for nuclei) in each sample. The final gel degradation index is the average percentage area of degradation per cell and normalized to control. Each experiment was repeated three times.

Immunofluorescence

Cells were fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 2 min and blocked with 1% BSA in PBS. Samples were incubated with primary antibodies overnight at 4°C and with secondary antibodies and/or phalloidin for 1.5 h. A negative control using an appropriate IgG antibody was performed in each experiment. No signals were detected from negative controls in the settings used for capturing images. Coverslips were mounted with ProLong® Gold Antifade Reagent (Molecular Probes). Images were taken using a Zeiss UV LSM 510 Meta laser scanning confocal microscope with 100× or 63× objectives, or a Nikon C1 confocal microscope with a 60× objective.

Antibodies

Antibodies used in this study were as follows: CD44H (R&D), CD44 IM7 (Santa Cruz), CD44 (DF1485, Santa Cruz), Tks5 (Santa Cruz), cortactin (Millipore), cortactin phosphorylated at Y421 (Sigma-Aldrich), Src (Cell Signaling), Src phosphorylated at Y416 (Cell Signaling), MT1-MMP (Millipore), β-actin (Sigma-Aldrich) and GAPDH (Millipore). Antibody information is listed in Table S1.

Tumor xenografts and metastasis analysis

All animal experiments were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee (IACUC) of Princeton University. Female Athymic nude mice (NCI) at age 4–6 weeks were used to test lung metastasis development with LM2 and corresponding sublines. Lateral tail vein intravenous injection of tumor cells was performed as described (Chakrabarti et al., 2012). For luciferase-labeled LM2 cells, the development of metastases was monitored by using BLI with the IVIS Imaging System (Caliper Life Sciences) and analyzed with Living Image software (Caliper Life Sciences) as described previously (Chakrabarti et al., 2012).

Statistical analysis

All raw measurements are presented as mean±s.d. (standard error) as indicated. *P*-values were calculated using Student's *t*-test for two-group comparison or one-way ANOVA for comparison of three or more groups.

Acknowledgements
We thank members in the Cheng lab for discussion and critical reading of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
P.Z. and C.C. designed the study. P.Z., with the help of Y.X., performed most of the experiments. Y.W and Q.Q. conducted animal studies. T.-L.C. assisted with TIRF experiments. P.Z., Y.K. and C.C. wrote the paper.

Funding
This research was supported in part by grants from the USA National Institutes of Health [grant numbers R01CA182467 to C.C. and R01CA141062 to Y.K.], the American Cancer Society [grant number RSG-09-252], the A Sister's Hope Foundation, and the Lynn Sage Breast Cancer Research Foundation and Scholar Award (to C.C.), the Breast Cancer Research Foundation, the Brewster Foundation, the Department of Defense [grant number BC123187 to Y.K.] and the Natural Science Foundation of China [grant number 31301108 to P.Z.]. Deposited in PMC for release after 12 months.

Supplementary information
Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.171959/-/DC1

References
Artym, V. V., Zhang, Y., Seillier-Moiseiwitsch, F., Yamada, K. M. and Mueller, S. C. (2006). Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. *Cancer Res.* **66**, 3034-3043.
Bourguignon, L. Y. W., Zhu, H., Shao, L. and Chen, Y.-W. (2001). CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration. *J. Biol. Chem.* **276**, 7327-7336.
Boyle, S. N., Michaud, G. A., Schweitzer, B., Predki, P. F. and Koleske, A. J. (2007). A critical role for cortactin phosphorylation by Abl-family kinases in PDGF-induced dorsal-wave formation. *Curr. Biol.* **17**, 445-451.
Brown, R. L., Reinke, L. M., Damerow, M. S., Perez, D., Chodosh, L. A., Yang, J. and Cheng, C. (2011). CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. *J. Clin. Invest.* **121**, 1064-1074.
Chabadel, A., Banon-Rodriguez, I., Cluet, D., Rudkin, B. B., Wehrle-Haller, B., Genot, E., Jurdic, P., Anton, I. M. and Saltel, F. (2007). CD44 and beta3 integrin organize two functionally distinct actin-based domains in osteoclasts. *Mol. Biol. Cell* **18**, 4899-4910.
Chaffer, C. L. and Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science* **331**, 1559-1564.
Chakrabarti, R., Hwang, J., Andres Blanco, M., Wei, Y., Lukačišin, M., Romano, R.-A., Smalley, K., Liu, S., Yang, Q., Ibrahim, T. et al. (2012). Elf5 inhibits the epithelial–mesenchymal transition in mammary gland development and breast cancer metastasis by transcriptionally repressing Snail2. *Nat. Cell Biol.* **14**, 1212-1222.
Courtneidge, S. A., Azucena, E. F., Pass, I., Seals, D. F. and Tesfay, L. (2005). The SRC substrate Tks5, podosomes (invadopodia), and cancer cell invasion. *Cold Spring Harb. Symp. Quant. Biol.* **70**, 167-171.
DesMarais, V., Macaluso, F., Condeelis, J. and Bailly, M. (2004). Synergistic interaction between the Arp2/3 complex and cofilin drives stimulated lamellipod extension. *J. Cell Sci.* **117**, 3499-3510.
Diaz, L. K., Zhou, X., Wright, E. T., Cristofanilli, M., Smith, T., Yang, Y., Sneige, N., Sahin, A. and Gilcrease, M. Z. (2005). CD44 expression is associated with increased survival in node-negative invasive breast carcinoma. *Clin. Cancer Res.* **11**, 3309-3314.
Eckert, M. A., Lwin, T. M., Chang, A. T., Kim, J., Danis, E., Ohno-Machado, L. and Yang, J. (2011). Twist1-induced invadopodia formation promotes tumor metastasis. *Cancer Cell* **19**, 372-386.
Grass, G. D., Tolliver, L. B., Bratoeva, M. and Toole, B. P. (2013). CD147, CD44, and the epidermal growth factor receptor (EGFR) signaling pathway cooperate to regulate breast epithelial cell invasiveness. *J. Biol. Chem.* **288**, 26089-26104.
Head, J. A., Jiang, D., Li, M., Zorn, L. J., Schaefer, E. M., Parsons, J. T. and Weed, S. A. (2003). Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. *Mol. Biol. Cell* **14**, 3216-3229.
Hiraga, T., Ito, S. and Nakamura, H. (2013). Cancer stem-like cell marker CD44 promotes bone metastases by enhancing tumorigenicity, cell motility, and hyaluronan production. *Cancer Res.* **73**, 4112-4122.
Hotary, K., Li, X.-Y., Allen, E., Stevens, S. L. and Weiss, S. J. (2006). A cancer cell metalloprotease triad regulates the basement membrane transmigration program. *Genes Dev.* **20**, 2673-2686.
Huang, C., Liu, J., Haudenschild, C. C. and Zhan, X. (1998). The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. *J. Biol. Chem.* **273**, 25770-25776.
Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H. and Seiki, M. (2001). Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J. Cell Biol.* **153**, 893-904.
Kanner, S. B., Reynolds, A. B., Vines, R. R. and Parsons, J. T. (1990). Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **87**, 3328-3332.
Li, C.-M., Chen, G., Dayton, T. L., Kim-Kiselak, C., Hoersch, S., Whittaker, C. A., Bronson, R. T., Beer, D. G., Winslow, M. M. and Jacks, T. (2013). Differential Tks5 isoform expression contributes to metastatic invasion of lung adenocarcinoma. *Genes Dev.* **27**, 1557-1567.
Liu, S. and Cheng, C. (2013). Alternative RNA splicing and cancer. *Wiley Interdiscip. Rev. RNA* **4**, 547-566.
Liu, Y.-J., Yan, P.-S., Li, J. and Jia, J.-F. (2005). Expression and significance of CD44s, CD44v6, and nm23 mRNA in human cancer. *World J. Gastroenterol.* **11**, 6601-6606.
Lou, W., Krill, D., Dhir, R., Becich, M. J., Dong, J. T., Frierson, H. F., Jr, Isaacs, W. B., Isaacs, J. T. and Gao, A. C. (1999). Methylation of the CD44 metastasis suppressor gene in human prostate cancer. *Cancer Res.* **59**, 2329-2331.
Mader, C. C., Oser, M., Magalhaes, M. A. O., Bravo-Cordero, J. J., Condeelis, J., Koleske, A. J. and Gil-Henn, H. (2011). An EGFR-Src-Arg-cortactin pathway mediates functional maturation of invadopodia and breast cancer cell invasion. *Cancer Res.* **71**, 1730-1741.
Monteiro, P., Rosse, C., Castro-Castro, A., Irondelle, M., Lagoutte, E., Paul-Gilloteaux, P., Desnos, C., Formstecher, E., Darchen, F., Perrais, D. et al. (2013). Endosomal WASH and exocyst complexes control exocytosis of MT1-MMP at invadopodia. *J. Cell Biol.* **203**, 1063-1079.
Mori, H., Tomari, T., Koshikawa, N., Kajita, M., Itoh, Y., Sato, H., Tojo, H., Yana, I. and Seiki, M. (2002). CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *EMBO J.* **21**, 3949-3959.
Murphy, D. A. and Courtneidge, S. A. (2011). The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. *Nat. Rev. Mol. Cell Biol.* **12**, 413-426.
Oser, M., Yamaguchi, H., Mader, C. C., Bravo-Cordero, J. J., Arias, M., Chen, X., DesMarais, V., van Rheenen, J., Koleske, A. J. and Condeelis, J. (2009). Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly and maturation. *J. Cell Biol.* **186**, 571-587.
Pan, Q., Shai, O., Lee, L. J., Frey, B. J. and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40**, 1413-1415.
Paz, H., Pathak, N. and Yang, J. (2013). Invading one step at a time: the role of invadopodia in tumor metastasis. *Oncogene.* **33**, 4193-4202.
Ponta, H., Sherman, L. and Herrlich, P. A. (2003). CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.* **4**, 33-45.
Rajadurai, C. V., Havrylov, S., Zaoui, K., Vaillancourt, R., Stuible, M., Naujokas, M., Zuo, D., Tremblay, M. L. and Park, M. (2012). Met receptor tyrosine kinase signals through a cortactin-Gab1 scaffold complex, to mediate invadopodia. *J. Cell Sci.* **125**, 2940-2953.
Seals, D. F., Azucena, E. F., Jr., Pass, I., Tesfay, L., Gordon, R., Woodrow, M., Resau, J. H. and Courtneidge, S. A. (2005). The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. *Cancer Cell* **7**, 155-165.
Steffen, A., Le Dez, G., Poincloux, R., Recchi, C., Nassoy, P., Rottner, K., Galli, T. and Chavrier, P. (2008). MT1-MMP-dependent invasion is regulated by TI-VAMP/VAMP7. *Curr. Biol.* **18**, 926-931.
Suenaga, N., Mori, H., Itoh, Y. and Seiki, M. (2005). CD44 binding through the hemopexin-like domain is critical for its shedding by membrane-type 1 matrix metalloproteinase. *Oncogene* **24**, 859-868.
Tehrani, S., Tomasevic, N., Weed, S., Sakowicz, R. and Cooper, J. A. (2007). Src phosphorylation of cortactin enhances actin assembly. *Proc. Natl. Acad. Sci. USA* **104**, 11933-11938.
Thiery, J. P. and Sleeman, J. P. (2006). Complex networks orchestrate epithelial–mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* **7**, 131-142.
Vikesaa, J., Hansen, T. V., Jønson, L., Borup, R., Wewer, U. M., Christiansen, J. and Nielsen, F. C. (2006). RNA-binding IMPs promote cell adhesion and invadopodia formation. *EMBO J.* **25**, 1456-1468.
Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S. F., Schroth, G. P. and Burge, C. B. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**, 470-476.
Weaver, A. M. (2008). Cortactin in tumor invasiveness. *Cancer Lett.* **265**, 157-166.

Journal of Cell Science (2016) 129, 1355-1365 doi:10.1242/jcs.171959

Weed, S. A. and Parsons, J. T. (2001). Cortactin: coupling membrane dynamics to cortical actin assembly. *Oncogene* **20**, 6418-6434.

Wiesner, C., Faix, J., Himmel, M., Bentzien, F. and Linder, S. (2010). KIF5B and KIF3A/KIF3B kinesins drive MT1-MMP surface exposure, CD44 shedding, and extracellular matrix degradation in primary macrophages. *Blood* **116**, 1559-1569.

Woodson, K., O'Reilly, K. J., Ward, D. E., Walter, J., Hanson, J., Walk, E. L. and Tangrea, J. A. (2006). CD44 and PTGS2 methylation are independent prognostic markers for biochemical recurrence among prostate cancer patients with clinically localized disease. *Epigenetics* **1**, 183-186.

Xu, Y., Gao, X. D., Lee, J.-H., Huang, H., Tan, H., Ahn, J., Reinke, L. M., Peter, M. E., Feng, Y., Gius, D. et al. (2014). Cell type-restricted activity of hnRNPM promotes breast cancer metastasis via regulating alternative splicing. *Genes Dev.* **28**, 1191-1203.

Yamaguchi, H., Lorenz, M., Kempiak, S., Sarmiento, C., Coniglio, S., Symons, M., Segall, J., Eddy, R., Miki, H., Takenawa, T. et al. (2005). Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *J. Cell Biol.* **168**, 441-452.

Yang, J. and Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev. Cell* **14**, 818-829.

Zarrabi, K., Dufour, A., Li, J., Kuscu, C., Pulkoski-Gross, A., Zhi, J., Hu, Y., Sampson, N. S., Zucker, S. and Cao, J. (2011). Inhibition of matrix metalloproteinase 14 (MMP-14)-mediated cancer cell migration. *J. Biol. Chem.* **286**, 33167-33177.