Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK–p130Cas complex

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Tumor cell migration and the concomitant degradation of extracellular matrix (ECM) are two essential steps in the metastatic process. It is well established that focal adhesions (FAs) play an important role in regulating migration; however, whether these structures contribute to matrix degradation is not clear. In this study, we report that multiple cancer cell lines display degradation of ECM at FA sites that requires the targeted action of MT1-MMP. Importantly, we have found that this MT1-MMP targeting is dependent on an association with a FAK–p130Cas complex situated at FAs and is regulated by Src-mediated phosphorylation of Tyr 573 at the cytoplasmic tail of MT1. Disrupting the FAK–p130Cas–MT1 complex significantly impairs FA-mediated degradation and tumor cell invasion yet does not appear to affect invadopodia formation or function. These findings demonstrate a novel function for FAs and also provide molecular insights into MT1-MMP targeting and function.

Introduction

It is well known that invading tumor cells are surrounded by stroma and extracellular matrix (ECM) that is either displaced or degraded during the metastatic process. This degradative action is mediated by specialized organelles termed "invadopodia" that are centrally situated, dot-shaped actin-rich membrane protrusions that extend into the matrix-filled surroundings (Linder, 2007; Buccione et al., 2009; Caldieri et al., 2009). Active invadopodia are known to markedly potentiate invasion and are a hallmark of many types of cancer cells (Linder, 2007). Invadopodia perform both secretory and endocytic processes by mediating the targeted release of zinc-dependent matrix metalloproteinases (MMPs; Egeblad and Werb, 2002; Caldieri and Buccione, 2010) at the cell base and the subsequent internalization of the digested matrix for further processing by the lysosome (Coopman et al., 1996, 1998). Thus, cytoskeletal and membrane dynamics play an essential role in supporting invadopodia function (Gimona et al., 2008; Caldieri et al., 2009).

Both maturation and function of invadopodia are MMP dependent (Artym et al., 2006). To date, 25 MMPs have been identified in humans and are classified into either secreted MMPs or membrane-type MMPs (MT-MMPs). These two families share many structural elements except that MT-MMPs contain transmembrane or other membrane-tethering domains (Egeblad and Werb, 2002), and both have been identified at invadopodia. Among the known MMPs, MT1-MMP (MMP-14) is the best characterized and is believed to be the most prevalent form (Sabeh et al., 2009). MT1-MMP contains a single transmembrane domain, a 20-amino acid cytoplasmic tail, and is up-regulated in many types of cancer (Egeblad and Werb, 2002; Sato et al., 2005). Inhibition of MT1-MMP has been shown to significantly impair tumor cell invasion; however, the spatial and temporal regulation of MT1-MMP is not fully understood.

Although invadopodia are the primary site of action for MT1-MMP, it is also believed to function at other cellular locations such as lamellipodia where it is recruited via an interaction with CD44 (Mori et al., 2002). Moreover, Takino et al. (2006, 2007) have shown that in MT1-MMP–overexpressing Hela cells, ECM degradation takes place at the leading edge and in close proximity to focal adhesions (FAs). This is particularly interesting as FAs and invadopodia are related organelles that share many structural and regulatory components. In contrast to the dot-shaped invadopodia, FAs are streak-like structures at the basal membrane that bridge the actin cytoskeleton to the ECM,

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Abbreviations used in this paper: FA, focal adhesion; FAK, focal adhesion kinase; FAT, focal adhesion targeting; MMP, matrix metalloproteinase; PRR, proline-rich region.

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and the dynamic turnover of FAs is essential for the motility of both normal and tumor cells (Mitra et al., 2005; Gimona and Buccione, 2006). Despite distinct morphologies, FAs and invadopodia share many components including integrins, talin, paxillin, actin, cortactin, and dynamin, to name just a few (Linder and Aepfelbacher, 2003). A central component of FAs that provides both a scaffolding and signaling function is focal adhesion kinase (FAK). Although not observed at invadopodia (Bowden et al., 2006), FAK has been implicated in the regulation of invadopodia formation by modulating downstream signaling through Src and p130Cas (Hsia et al., 2003; Chan et al., 2009). Despite these molecular similarities, FAs are generally perceived to mediate cell migration but are not involved in matrix degradation.

Recently we have observed that a variety of cancer cell lines degrade matrix not only at invadopodia, but also at numerous peripheral sites that resemble FAs. Here we have confirmed that this peripheral ECM degradation represents bona fide FA sites, and have further revealed that this FA-type degradation is MT1-MMP dependent. Moreover, we find that this protease is targeted to FAs by a physical interaction between MT1-MMP and a FAK–p130Cas complex in response to the activation of Src. Formation of this FAK–p130Cas–MT1 complex is essential for the FA-centric ECM degradation as well as invasive migration of tumor cells through Matrigel.

Results

MT1-MMP mediates matrix degradation at focal adhesions

Matrix degradation by a wide variety of different tumor cell lines appears to occur at linear, peripherally located FA-like sites as well as the well-characterized, centrally situated and dot-shaped invadopodia. To determine if this pattern represents the action of FAs, human fibrosarcoma cells (HT-1080) were plated on fluorescent gelatin-coated coverslips for 6 h, and stained for the FA marker vinculin. As shown in Fig. 1 a, many of the peripheral, linear degradation sites colocalized with vinculin. The enlarged images reveal that mature FAs (Fig. 1 a') generated a solid, dark pattern of degradation whereas nascent FAs (Fig. 1 a'') appeared to be in the process of degrading matrix. A similar result was obtained from several cell lines (Fig. S1). As many actin stress fibers terminate in FAs, we colocalized actin with a FA marker, and sites of matrix degradation as just one feature of many used to distinguish FAs from other adhesive structures (Fig. S1, c and d). It is well known that cells exert physical tension on the ECM at FA sites that can result in matrix tearing. To distinguish enzymatic activity from physical effects, HT-1080 cells were treated with 10 µM of the MMP inhibitor BB94 (Davies et al., 1993) as the cells were plated on gelatin. As shown in Fig. S2 (a–a'), overall degradation including the ones at FAs were completely inhibited, while no significant change in FA morphology was observed. From these observations we conclude that ECM degradation at FAs occurs by the proteolytic activity of MMPs.

Because the FA-like degradation was sharply focused rather than diffuse in nature, we hypothesized that a membrane-type

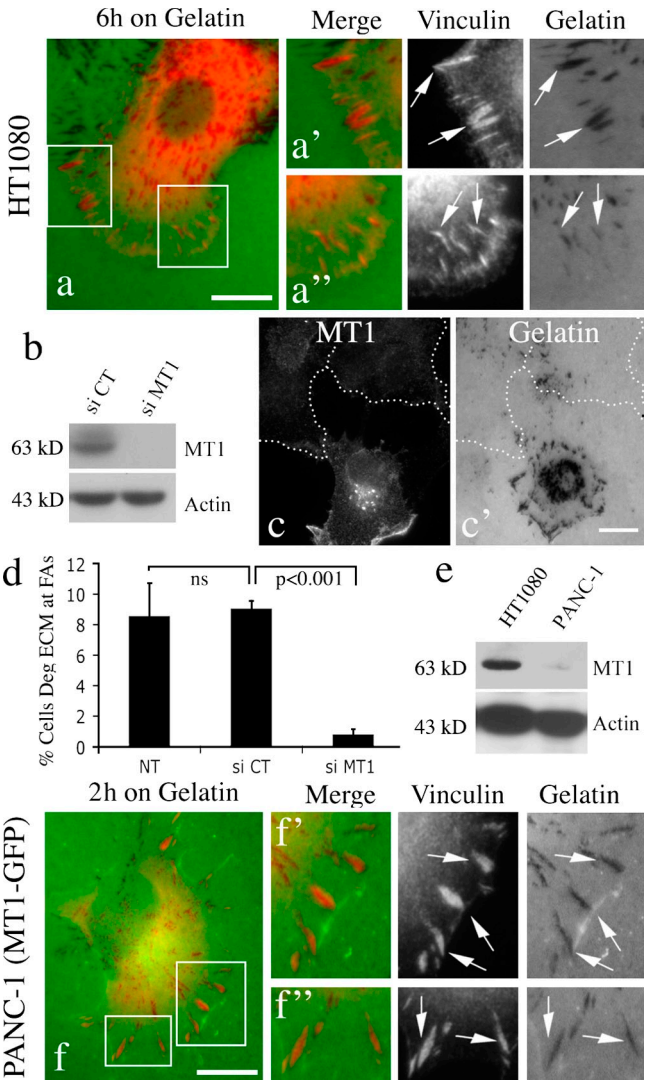

Figure 1. **MT1-MMP mediates ECM degradation at focal adhesions.** (a) To test if extracellular matrix degradation occurs at focal adhesions, HT-1080 cells were plated on fluorescent gelatin (green)-coated coverslips for 6 h before fixation and staining with a vinculin antibody (red). Matrix degradation occurred at vinculin-positive FA sites. Boxed regions provide a higher magnification showing the correlation between vinculin and matrix degradation in the gelatin matrix (dark spots in a' and a''). (b–c') To define if MT1-MMP is required for the FA-centered degradation, HT-1080 cells were treated with MT1 siRNA for 72 h to knock down endogenous MT1-MMP (b), and then were plated on gelatin-coated coverslips (c and c'). MT1 knockdown cells (dashed lines) exhibit a significant reduction in matrix degradation. (d) Quantification of the percentage of cells (in c) that degraded ECM at FAs indicates that MT1 is required for this type of degradation. Values represent the mean of three independent experiments ± SD. (e and f) In reciprocal experiments MT1-MMP was overexpressed in a human pancreatic tumor cell line (PANC-1) that does not express endogenous MTI-MMP (e) or degrade matrix (Fig. S2). Just 2 h after plating on gelatin the transfected PANC-1 cells exhibited substantial matrix degradation at FA sites (f–f''). Additional examples of pancreatic tumor cells degrading matrix at FAs are provided in Fig. S1. Bars, 10 µm.

MMP, not a secreted form, was responsible for degradation at FAs. MT1-MMP is the most prominent member of MT-MMPs and has been shown by many others to support ECM degradation (Sato et al., 2005; Itoh, 2006; Strongin, 2006). As shown in Fig. 1, c–c', knocking down MT1 in HT-1080 cells (dashed line) significantly impaired ECM degradation, including the

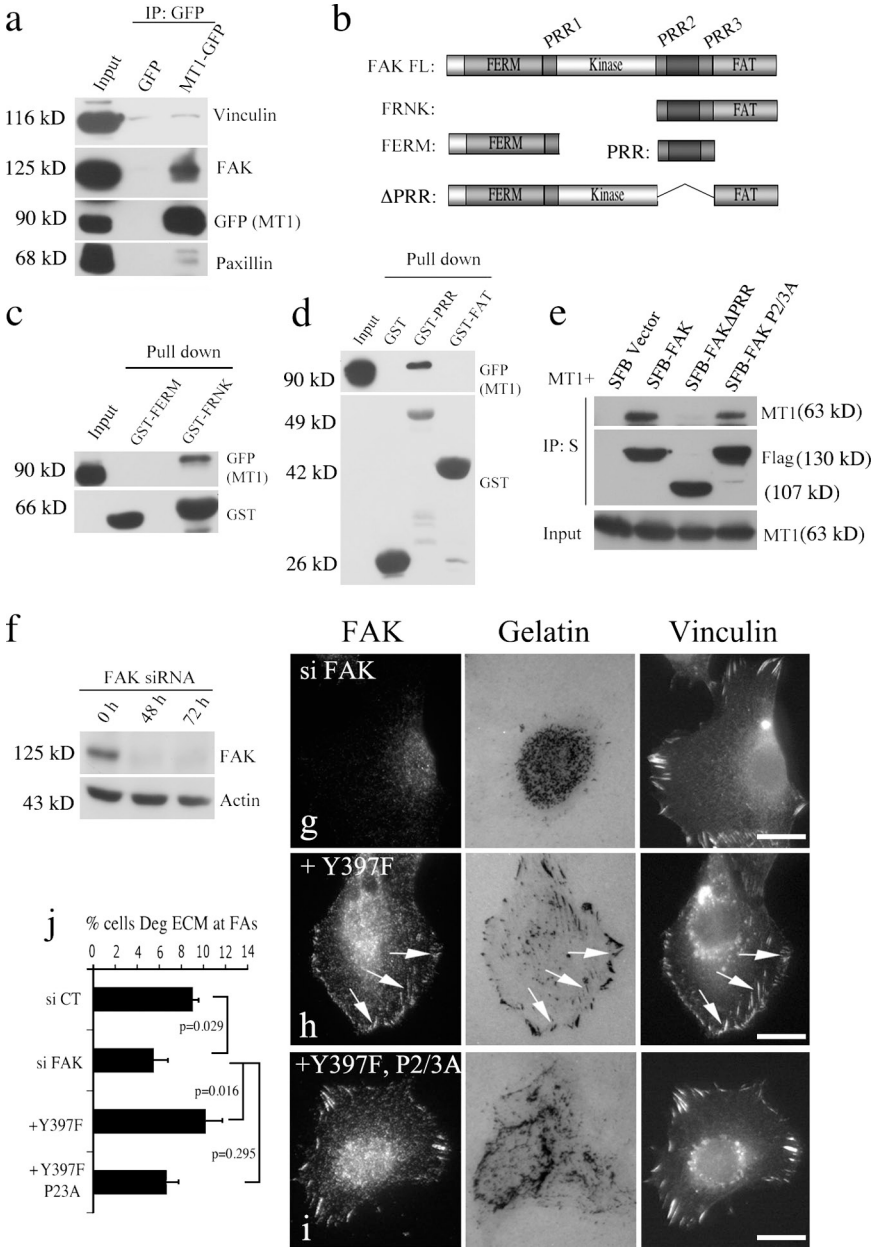

Figure 2. **An interaction between FAK and MT1 is required for matrix degradation at focal adhesions.** (a) To test for FA proteins that might interact with MT1, PANC-1 cells were transfected with vectors encoding either GFP alone or MT1-GFP then lysed and IP with a GFP antibody. The pellet was then subjected to Western blot analysis using antibodies against several major FA proteins. FAK is the most prominent MT1-interacting protein of those tested. (b) Illustration of FAK and the deletion constructs used to map FAK-MT1 binding. (c and d) Western blot analysis of homogenates from PANC-1 cells expressing MT1-GFP and incubated with different GST-FAK fragments for 2 h. Importantly, only portions of FAK that contain the PRR domain were able to interact with MT1. (e) To further define the importance of the PRR domain in MT1 binding, MT1-GFP was coexpressed with SFB-tagged FAK WT, the PRR domain deletion mutant (ΔPRR), or point mutations that disrupt the second and third PXXP motif (P2/3A), followed by IP with S-protein beads. Deletion or mutation of the PRR domain significantly impaired FAK–MT1 interactions. To test if FAK is required for degradation at FAs, HT-1080 cells were treated with FAK siRNA (f), then plated on gelatin for 16 h before IF analysis. FAK knockdown significantly reduced degradations at FAs (g). Re-expression of FAK Y397F rescued FA-type degradation (h), whereas FAK Y397F carrying P2/3A mutation (i) had only a minimal effect. (j) The percentages of cells (g–i) that degraded ECM at FAs were quantified. Values represent the mean of three independent experiments ± SD. Bars, 10 µm.

ones at FAs (Fig. 1 d). To further test the role of MT1-MMP in ECM degradation, we used a pancreatic cancer cell line (PANC-1) that does not express endogenous MT1 (Fig. 1 e). Although gelatin degradation was not observed by these cells even 24 h after plating (Fig. S2 b-b'), some FA-type degradations were observed in cells transfected with MT1-GFP just 2 h after plating on gelatin (Fig. 1 f). The same findings were obtained by cells expressing either an internal mCherry-tagged (see Fig. 5 h) or a nontagged MT1, indicating that this protease is essential for FA-mediated degradation.

A physical interaction between MT1-MMP and FAK is required for matrix degradation at focal adhesions

As MT1-MMP mediates ECM degradation at FAs, it is likely that a FA component acts to bind and recruit this protease to these sites. To test this possibility, MT1-GFP was overexpressed in PANC-1 cells, then immunoprecipitated (IP) with a GFP antibody and blotted for several core FA proteins, including FAK, paxillin, vinculin, talin, and β1-integrin. Of all these proteins, FAK was by far the most prominent interacting partner (Fig. 2 a). As FAK localizes to FAs and is generally not considered an invadopodial component (Bowden et al., 2006; Chan et al., 2009), this FAK–MT1 interaction was predicted to occur at FAs. To define this novel interaction, we generated a series of FAK truncation mutants as illustrated in Fig. 2 b. PANC-1 cells expressing MT1-GFP were lysed and incubated with different GST-tagged FAK domains to test for interaction. As shown in Fig. 2, c and d, the proline-rich region (PRR) of FAK is sufficient to pull down MT1. To confirm this finding, MT1-GFP and SFB-tagged (SFB: S protein, FLAG, and streptavidin-binding peptide triple tagged)

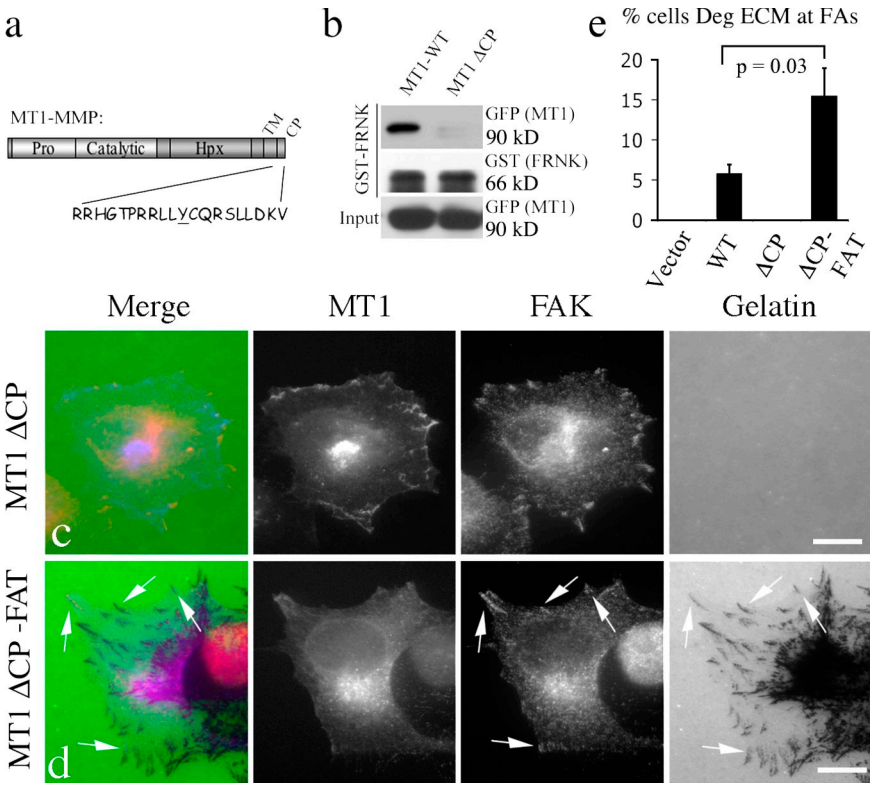

Figure 3. **The cytoplasmic tail of the MT1 associates with FAK.** (a) Cartoon illustrating the domain structures of MT1-MMP that includes the Pro peptide, catalytic domain, hemopexin domain (Hpx), transmembrane domain (TM), and a cytoplasmic tail (CP) of only 20 amino acids. (b) To test if FAK interacts with the MT1 cytoplasmic tail, PANC-1 cells were transfected with either full-length MT1 or MT1 missing the cytoplasmic tail (ΔCP), and subjected to a pull-down assay with GST-FRNK. Although a large amount of MT1 WT was pulled down by GST-FRNK, almost none of MT1ΔCP appeared to associate. To test if MT1ΔCP remains competent to degrade matrix at FAs, PANC-1 cells were plated and viewed as performed in Fig 1 f. The MT1ΔCP-expressing cells failed to degrade any matrix (c) whereas cells expressing an MT1 ΔCP fused with FAT domain, known to target proteins to FAs, exhibited excessive degradation at FA sites (d, arrow), suggesting that the MT1ΔCP protein has defective targeting but not enzymatic activity. (e) The percentages of cells that degrade ECM at FAs were quantified. Values represent the mean of three independent experiments ± SD. Bars, 10 µm.

FAK WT or a PRR mutant were expressed in 293T cells, followed by a FAK IP with S protein beads. As predicted, FAKΔPRR did not interact with MT1 (Fig. 2 e, lane 3). Within the FAK PRR domain the most characteristic features are two conserved PXXP motifs (second and third in the full-length protein; Mitra et al., 2005; Wu et al., 2005). Mutations of these two motifs (FAK P2/3A) did not fully abolish, but significantly impaired FAK's interaction with MT1-MMP (Fig. 2 e, lane 4). Taken together, these data suggest that two PXXP motifs within the PPR domain of FAK are essential to interact with MT1-MMP.

To test if FAK–MT1 association is important for matrix degradation at FA sites, HT-1080 cells were treated with FAK siRNA to knock down endogenous FAK (Fig. 2 f), then were plated on gelatin-coated coverslips for 16 h before IF analysis. Interestingly, degradation at FAs was significantly compromised in FAK knockdown cells (Fig. 2 g). To further confirm the role of FAK, FAK knockdown cells were rescued with siRNA-resistant rat-FAK. Surprisingly, WT FAK did not significantly increase degradation at FAs; however, expression of a FAK Y397F mutant rescued degradation to control levels (Fig. 2 h). As discussed later, this result is likely due to the fact that expression of WT FAK promotes FA turnover while the YF mutant is known to stabilize this structure (Hamadi et al., 2005; Wang et al., 2011). Accordingly, expression of a FAK double mutant, Y397F carrying the P2/3A mutation that reduces MT1 binding, did not rescue matrix degradation at FAs (Fig. 2 i). Taken together, we conclude that FAK interacts with MT1-MMP through the PRR domain and this interaction is important for FA-mediated degradation.

The cytoplasmic tail of MT1-MMP interacts with FAK indirectly through p130Cas

MT1-MMP is a transmembrane protein with a 20-amino acid cytoplasmic tail (CP; Fig. 3 a) that is known to interact with a variety of cellular proteins such as MTCBP-1 and AP2 (Uekita et al., 2004). To test if FAK also interacts with this domain, we generated a MT1 mutant missing the cytoplasmic tail (MT1ΔCP) and analyzed its interaction with FAK via GST pull-down assay. As predicted, removal of the CP domain completely abolished the FAK–MT1 interaction (Fig. 3 b), thus this mutant is likely defective in mediating degradation at FAs. Indeed, in contrast to MT1 WT (Fig. 1 f and Fig. S4 c), MT1 ΔCP expressing PANC-1 cells failed to generate any gelatin degradation at FAs (Fig. 3c). As this effect could result from defects in either MT1 targeting or enzymatic activity, we generated a chimeric protein by replacing the MT1 cytoplasmic tail with the focal adhesion targeting domain of FAK (FAT). Thus, the cytoplasmic tail truncated protease could be constitutively targeted to FAs. As shown in Fig. 3 d, PANC-1 cells expressing MT1ΔCP-FAT generated excessive degradation at FA sites (Fig. 3 e), indicating that MT1ΔCP remains enzymatically active but cannot be targeted to FAs.

Interaction between MT1-MMP and FAK is essential for FA-centric matrix degradation; however, whether this interaction is direct was unclear. To test this, we purified His-MT1 protein from *Escherichia coli*, and performed direct binding analysis with GST-PRR of FAK. No direct interaction was detected (unpublished data), suggesting the participation of an adaptor. One candidate known to bind directly to both MT1

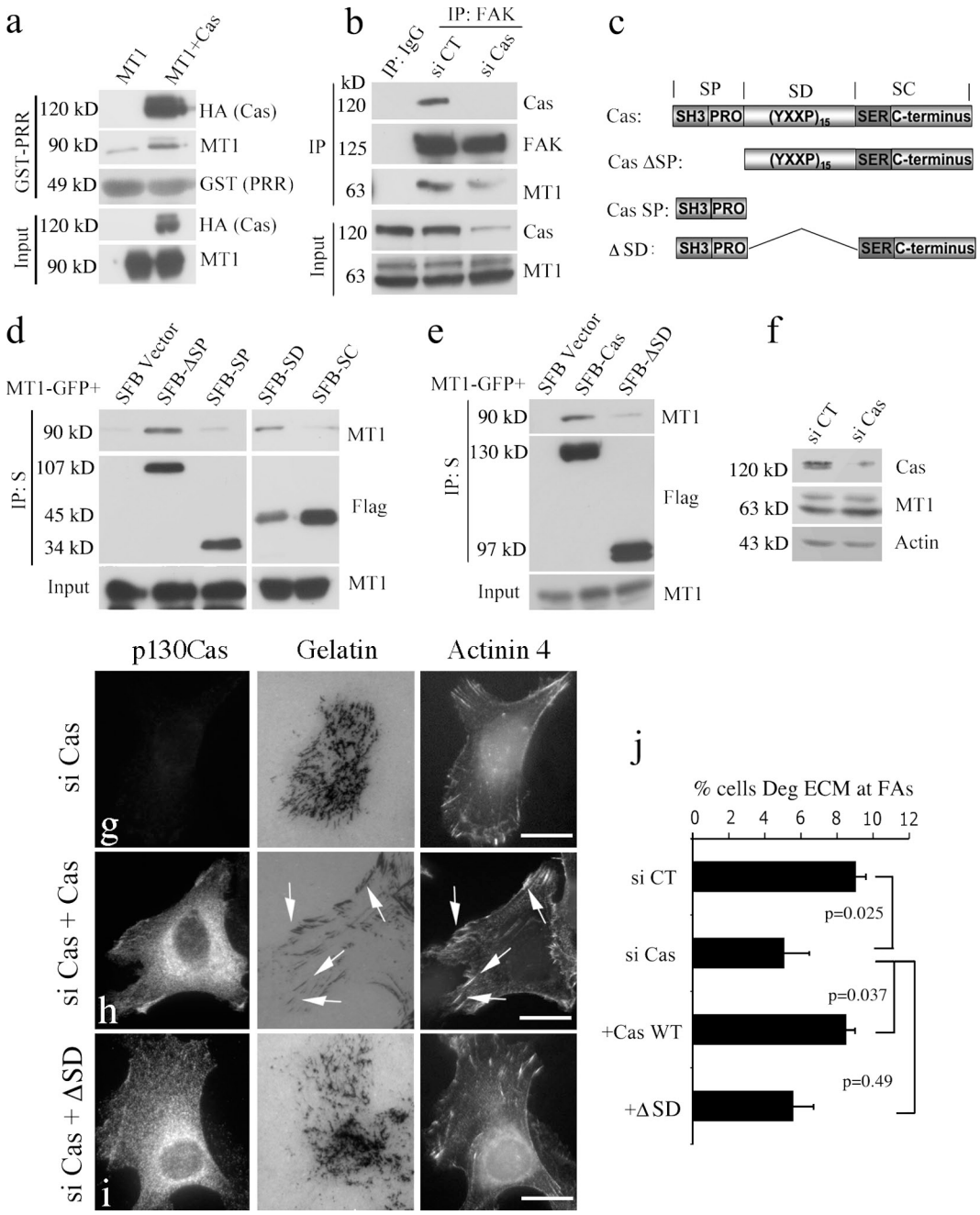

Figure 4. **Interaction between MT1 and FAK is mediated by p130Cas.** (a) To test if p130Cas adapts MT1 to FAK, 293T cells were transfected with MT1-GFP alone or together with HA-p130Cas, and the homogenates were incubated with the GST-PRR domain of FAK. Although GST-PRR exhibited modest interaction with MT1, this was markedly increased when p130Cas was coexpressed. (b) To further test p130Cas as an adaptor between MT1 and FAK, HT-1080 cells were treated with control or p130Cas siRNA, then lysed and IP with a FAK antibody. Although both p130Cas and MT1 were coprecipitated in control cells, MT1 coIP was significantly reduced in p130Cas knockdown cells, suggesting p130Cas links MT1 to FAK. (c) Illustration of the domains of full-length Cas and the truncation mutants used to study MT1 interaction. SP, SH3 and proline rich domain; SD, substrate domain; SC, serine-rich region and C terminus. (d and e) MT1-GFP was coexpressed with the indicated SFB-tagged Cas mutants in 293T cells, and IP with S-beads. The substrate domain alone is sufficient to interact with MT1 (d), whereas deletion of this domain abolished Cas–MT1 interaction (e). To test if p130Cas is required for ECM degradation at FAs, HT-1080 cells were treated with p130Cas siRNA (f) and plated on gelatin for 16 h. p130Cas knockdown significantly reduced degradation at FAs (g). Re-expression of Cas WT (h) but not the MT1-binding mutant (i) rescued degradation at FAs. (j) The percentages of cells (g–i) that degraded ECM at FAs were quantified. Values represent the mean of three independent experiments ± SD. Bars, 10 µm.

(Gingras et al., 2008; Gonzalo et al., 2010) and to the second and third PXXP motifs of FAK is p130Cas (Harte et al., 1996; Mitra et al., 2005). However, whether these three proteins form a functional complex is not clear. To test this possibility, we transfected 293T cells with either MT1 alone or together with p130Cas and then performed pull-down assays with the purified GST-PRR of FAK. As shown in Fig. 4 a, p130Cas strongly binds to GST-PRR, and overexpression of p130Cas significantly enhances the amount of MT1-MMP in the pulldown, suggesting that p130Cas facilitates MT1–FAK interaction.

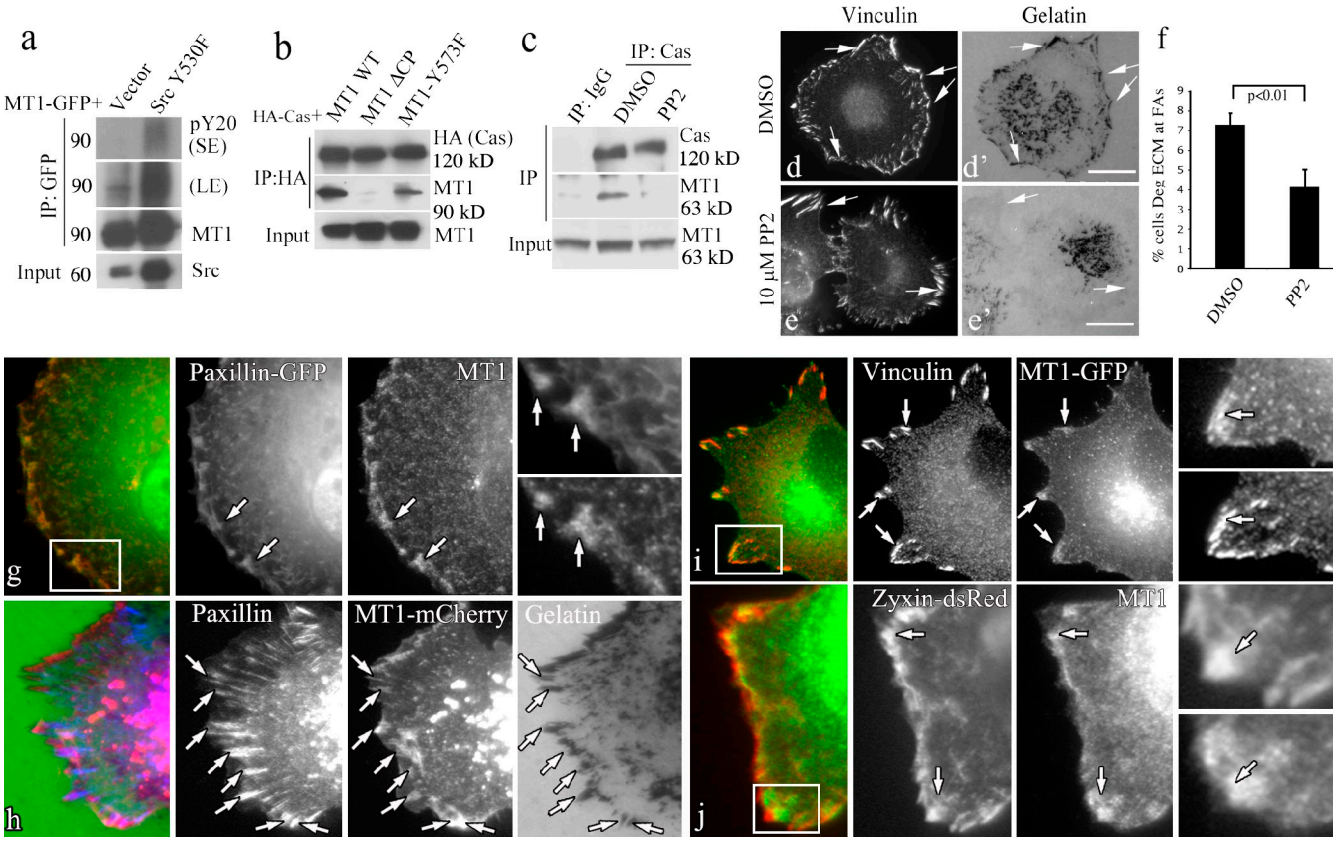

Figure 5. **Src regulates formation of a FAK–p130Cas–MT1 complex.** (a) To confirm MT1-MMP as a Src substrate, MT1-GFP was cotransfected with SrcY530F in 293T cells, IP with a GFP antibody, and blotted with a phosphor-Tyr antibody (pY20). MT1 was strongly phosphorylated by Src. (SE, short exposure; LE, long exposure). (b) To test if phosphorylation of Y573 at the MT1 cytoplasmic tail is important for binding to p130Cas, HA-Cas was cotransfected with MT1 WT, ΔCP, or Y573F, followed by IP with an HA antibody. MT1ΔCP did not interact with p130Cas, whereas MT1 Y573F significantly reduced the interaction. (c) To further test a role for Src in regulating MT1–p130Cas interactions, HT-1080 cells were treated with DMSO or 10 μM PP2 for 3 h before IP with p130Cas antibody, and PP2 treatment significantly reduced MT1–p130Cas binding. (d–e') HT-1080 cells were plated on gelatin in the presence of DMSO or PP2 for 6 h. PP2 significantly attenuated FA-mediated degradation compared with DMSO control. (f) Percentages of PP2- or DMSO-treated cells that degraded matrix at FAs. Data represent results from three independent experiments ± SD. MT1-MMP is recruited to FAs and increased upon elevated Src activity. HT1080 cells were transfected with paxillin-GFP and stained for endogenous MT1 (g–j), or transfected with MT1-mCherry and plated onto gelatin for 6 h and stained for paxillin (h). Certain colocalization between MT1 and FA marker (g and h, arrows and inserts) or sites of degradation (h) was observed. Same treatment was applied to cells coexpressing either MT1-GFP (i) or zyxin-dsRed (j) together with a constitutively active SrcY530F mutant. Importantly, SrcY530F markedly enhanced MT1's recruitment to FAs (I and j, arrows and inserts). Bars, 10 μm.

To test if these three proteins interact endogenously, FAK was immunoprecipitated from HT-1080 cells, and both p130Cas and MT1 were detected in the Co-IP (Fig. 4 b). Importantly, the FAK–MT1 interaction was attenuated in p130Cas knockdown cells (Fig. 4 b), suggesting that p130Cas mediates the interaction between MT1-MMP and FAK.

Although it is known that p130Cas binds to FAK via the N-terminal SH3 domain (Harte et al., 1996), and FAK recruits p130Cas to FAs (Fig. S3, a–b'), it remained unclear how p130Cas might interact with MT1. To address this question, a series of SFB-p130Cas truncation mutants were generated (Fig. 4 c) and coexpressed with MT1-GFP in 293T cells followed by Co-IP analysis. As shown in Fig. 4 d, the substrate domain (SD) of p130 Cas was sufficient to bind to MT1, and the deletion of this domain abolished the interaction (Fig. 4 e). Although p130Cas is an established signaling molecule at FA sites (O'Neill et al., 2000), its function during matrix degradation remains elusive. To test this premise, HT-1080 cells were treated with p130Cas siRNA for 72 h to knock down the endogenous protein (Fig. 4 f), and then were plated on gelatin-coated coverslips. As shown in Fig. 4 g, p130Cas knockdown significantly reduced FA-mediated degradation, while FA morphology remained unchanged (Fig. S3, c–c'). To further test p130Cas's effect, p130Cas WT or ΔSD (Fig. 4, h and i) were reexpressed and tested for matrix degradation. Importantly, p130Cas WT but not the MT1-binding mutant (ΔSD) rescued ECM degradation at FAs. These data suggest that ECM degradation at FAs is regulated by a novel mechanism involving a FAK–p130Cas–MT1 complex.

Src regulates FAK–p130Cas–MT1 complex formation and ECM degradation at focal adhesions

Src kinase is a central signaling molecule known to promote ECM degradation (Yeatman, 2004; Courtneidge et al., 2005; Kelley et al., 2010) by phosphorylating a variety of substrates including Tyr573 on the cytoplasmic tail of MT1-MMP (Nyalendo et al., 2007). Consistent with this premise, we observed a marked

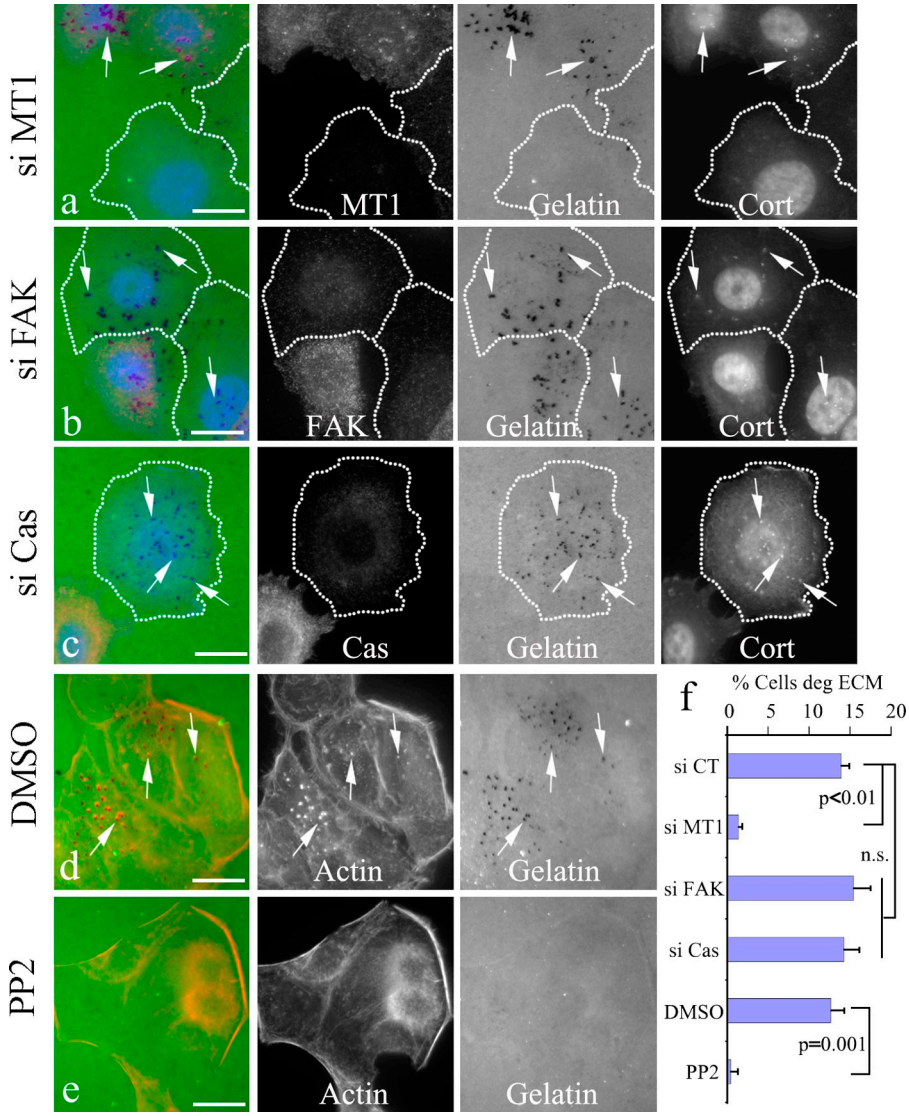

Figure 6. **ECM degradation at FAs and invadopodia are differentially regulated.** (a–c) To test if MT1-MMP, FAK, and p130Cas are required for invadopodia formation and function, BxPC-3 cells were treated with siRNA against these proteins for 72 h, followed by plating on gelatin for 16 h. Cells were stained for cortactin as an invadopodia marker. (a) MT1-MMP knockdown (dashed line) significantly impaired invadopodia formation and ECM degradation. Importantly, in contrast to FA-based degradation, FAK (b) or p130Cas (c) knockdown had no effect on invadopodia function. (d and e) To confirm that Src kinase regulates invadopodia formation and function, BxPC-3 cells were plated on gelatin in the presence of DMSO or 10 µM PP2 for 16 h. Cells were then stained for actin as an invadopodia marker. PP2 completely abolished invadopodia formation and degradation. (f) The percentages of cells that degrade ECM at invadopodia were quantified. Data represent results from three independent experiments ± SD. Bars, 10 µm.

tyrosine phosphorylation of MT1-MMP when coexpressed with active Src (Y530F; Fig. 5 a). As expression of an MT1-Y573F mutant did not eliminate the phospho-tyrosine signal (unpublished data), we assume that other tyrosine substrates exist on MT1-MMP. Importantly, however, phosphorylation of Y573 appears to facilitate the MT1–p130Cas interaction as the MT1 Y573F mutant exhibited a significantly reduced affinity to p130Cas compared with the MT1 WT in a Co-IP assay (Fig. 5 b). To confirm a role for Src, HT-1080 cells were treated with the Src family kinase inhibitor PP2 (10 µM, 3 h) before IP with p130Cas antibody. As shown in Fig. 5 c, PP2 treatment significantly reduced p130Cas–MT1 interaction compared with the DMSO control. Because p130Cas adapts MT1-MMP to FAK, we predicted that a Y573F mutant should also reduce the MT1–FAK interaction. This was indeed the case, as confirmed by both Co-IP and GST pull-down assay (Fig. S4, a and b). To test if Src activity is important for ECM degradation at FAs, HT-1080 cells were plated on gelatin in the presence of 10 µM PP2. As expected, PP2 treatment significantly inhibited ECM degradation at FAs (Fig. 5, e–e'). Consistent with this result, PANC-1 cells expressing MT1 Y573F showed a reduced FA-type degradation compared with MT1 WT–expressing cells (Fig. S4, c–d'). Taken together, these data suggest that Src regulates matrix degradation at FAs by phosphorylating MT1-MMP on Y573 that promotes MT1's interaction with the FAK–p130Cas complex.

ECM degradation at FAs and invadopodia are differentially regulated

The observations described above suggest that Src-mediated formation of a FAK–p130Cas–MT1 complex regulates ECM degradation at FAs. It is intriguing to test if invadopodia are regulated by the same mechanism. It is well established that Src kinase is a central regulator of invadopodia (Courtneidge et al., 2005). Surprisingly the numerous, centrally located degradation sites generated by the HT-1080 cells were not attenuated by PP2 treatment (Fig. 5 e). Importantly, these central sites did not co-stain for invadopodial markers such as actin and cortactin (Fig. S5, a and c), suggesting that these are distinct structures. In contrast, BxPC-3 cells, which do form numerous classical

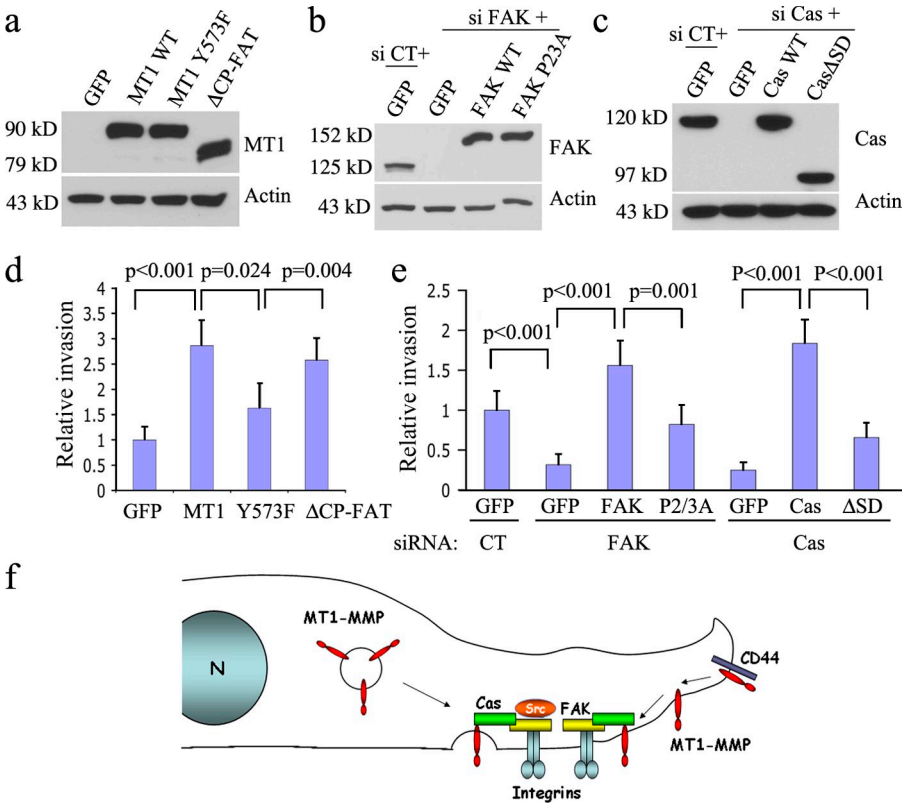

Figure 7. **FA-mediated matrix degradation promotes tumor cell invasion.** PANC-1 cells were transfected with MT1-MMP WT or mutants (a), and invasive potential was analyzed by the Boyden chamber assay in which filters were coated with 1 mg/ml Matrigel. Constructs that facilitate FA-type degradation (MT1-WT, ΔCP-FAT) significantly promoted invasion compared with the GFP vector or the MT1-Y573F mutant that cannot bind p130Cas (d). To test if a reduction of FAK and p130Cas exerts an inhibitory effect, HT-1080 cells were transfected with siRNA twice, and then rescued with either WT or mutant proteins (b and c). Although knockdown of either FAK or p130Cas significantly impaired invasion (e), this could be reversed by expression of WT proteins. Mutant proteins that cannot reconstitute the FAK–p130Cas–MT1 complex did not provide a functional rescue (e). The graphed data represent results from three independent experiments ± SD, and were normalized to the average of control cells (as 1). (f) Illustration depicting the central theme of this study: a FAK–p130Cas complex mediates the docking of MT1-MMP at FAs. MT1-MMP is trafficked to the surface via exocytotic vesicles for fusion with the membrane to release MT1 that can be recruited by the FAK–p130Cas complex at FAs. In addition, MT1 is recruited to lamellipodia by CD44 that may diffuse along the membrane for recruitment to FAs. Src kinase activity at FAs mediates MT1 phosphorylation at Y573 that facilitates an interaction with the FAK–p130Cas complex at FAs.

invadopodia (Fig. S5, b and d), exhibited a marked inhibition in the formation of centrally located ECM degradation sites by PP2 treatment (Fig. 6, d and e). Therefore, we tested if a disruption of the FAK–p130Cas–MT1 complex in BxPC-3 cells affects ECM degradation at invadopodia as it did at FAs (Figs. 2 and 4). BxPC3 cells were treated with siRNAs against MT1, FAK, or p130Cas, then plated on gelatin for 16 h, and stained for the invadopodia marker cortactin (Fig. 6, a–c). As expected, the MT1 knockdown affected invadopodia-type degradation due to an inhibition of invadopodia formation (Fig. 6 a). In contrast, FAK or p130Cas knockdown did not affect invadopodia function (Fig. 6, b and c), suggesting that, unlike that observed at FAs, MT1-MMP's function at invadopodia is independent of the FAK–p130Cas complex.

Disruption of the FAK–p130Cas–MT1 complex impairs tumor cell invasion in vitro

The observations described above have implicated a novel role for a FAK–p130Cas–MT1 complex in the degradation of ECM at FAs. To test if this FA-centric protein complex contributes to tumor cell invasion, an in vitro Boyden chamber invasion assay was implemented. As PANC-1 cells do not express endogenous MT1-MMP, cells were transfected to express MT1 WT, Y573F, or MT1ΔCP-FAT (Fig. 7 a). Cells were suspended in serum-free medium and plated in the upper chamber 24 h after transfection, then challenged to invade through a Matrigel-coated filter into the lower chamber containing full serum (10% FBS) medium. After a 36-h period, cells were fixed and the number of invaded cells per field was quantified. Importantly, the protein components that facilitate ECM degradation at FAs (MT1 WT and MT1ΔCP-FAT, Fig. 3 e) also significantly enhanced invasion through the filter compared with MT1 Y573F or GFP vector (Fig. 7 d). As a reciprocal approach, HT-1080 cells were transfected twice with siRNA against FAK or p130Cas, and subsequently rescued with either WT or mutant constructs (Fig. 7, b and c). Interestingly, cells with reduced levels of FAK or p130Cas exhibited significantly reduced invasion compared with siRNA control cells, which is consistent with previous reports (Ueda et al., 2003; Chan et al., 2009; Cunningham-Edmondson and Hanks, 2009). Moreover, reexpression of the respective WT proteins rescued invasion whereas expression of the mutants FAK P2/3A and CasΔSD that perturb formation of the trimeric complex did not (Fig. 7 e). Taken together, these data suggest that the targeting of MT1-MMP to FAs by the FAK–p130Cas complex is important for cancer cell invasion.

Discussion

In this study we provide evidence for a novel, unconventional function of focal adhesions that entails the regulated degradation of the surrounding matrix (Fig. 1 and Fig. S1). This process is observed in a wide variety of human tumor cell types such as the fibrosarcoma cell line HT1080 and several different pancreatic tumor cell lines including BxPC3, HuPT3, CFPAC-1, and HPAF. It is mediated by, and dependent upon, MT1-MMP as cells without this protease do not degrade matrix at these sites (Fig. 1 and Fig. S2). In addition, we have found that the MT1-MMP is targeted to FAs through a novel interaction with a FAK–p130Cas complex. Disruption of these interactions results in a significant reduction of ECM degradation at FAs (Figs. 2–4), but not at

invadopodia (Fig. 6), suggesting a FA-specific mechanism. Finally, the formation of this trimeric complex is regulated by Src, and is essential for tumor cell invasion in vitro.

FAK as an essential adaptor for the recruitment of MT1-MMP to focal adhesions

Two of the interesting central concepts addressed in this study focus on the mechanics of MT1-MMP recruitment to FAs and the functional equilibrium of matrix degradation occurring at FAs and invadopodia during cancer invasion. Although degradation predominates at invadopodia in most tumor cells, FA-based degradation does occur and is not from mechanical tearing of the ECM, as inhibiting MMP activity with the drug BB94 abolished FA-centric degradation. This is further supported by the fact that MT1-MMP–deficient cells (PANC-1) or HT-1080 cells with MT1 knockdown failed to degrade ECM at FAs. These data suggest that matrix degradation at FAs, like that at invadopodia, is a proteolytic event, and the essential role of a membrane-type MMP (MT1-MMP) is consistent with the confined degradation pattern at FAs.

Key evidence supporting MT1-MMP's function at FAs is an interaction with FAK. Although FAs and invadopodia share a substantial number of components, FAK is generally believed to be FA specific. Accordingly, we hypothesized that MT1-MMP may be selectively recruited to FAs by FAK. Further analysis revealed that FAK–MT1 interaction is mediated by the PRR domain of FAK and the cytoplasmic tail of MT1-MMP (Figs. 2 and 3). Functionally, FAK knockdown in HT-1080 cells resulted in a reduction of FA-mediated degradation, and consistent with a previous report (Chan et al., 2009) invadopodia-type degradation was not affected (Fig. 6 b), again supporting FAK's distinct roles between these two structures. However, this defect could not be rescued by reexpression of WT FAK. We believe this is due to the fact that FAK overexpression promotes FA turnover (Ezratty et al., 2005; Mitra et al., 2005) and thus reduces the time allotted for matrix degradation to occur. This concept is supported by a subsequent finding that FAK Y397F, a mutant known to inhibit FA turnover (Hamadi et al., 2005), exhibited a significant rescue of FA-based degradation (Fig. 2), and importantly, no rescue was observed by FAK Y397F carrying the two PXXP mutations (P2/3A) that reduce MT1 binding. Moreover, cells expressing a MT1-MMP mutant that cannot interact with FAK (MT1ΔCP) were defective in degrading ECM at FAs (Fig. 3). These data strongly suggest that the FAK–MT1 interaction is important for FA-based degradation, but is dispensable for degradation by invadopodia. As a direct interaction between FAK and MT1 was not detected, we had searched for an adaptor protein that could act as a functional linker. Based on previous reports (Harte et al., 1996; Gingras et al., 2008) we pursued, and subsequently found, that the FAK–MT1 connection is largely mediated by p130Cas (Fig. 4).

A FAK–p130Cas–MT1-MMP complex regulates FA-based tumor cell invasion

An interesting component of this study is the Src-dependent regulation of protease–adaptor interactions. Consistent with the findings of others (Gingras et al., 2008; Gonzalo et al., 2010), we observed that phosphorylation of a specific tyrosine (Tyr 573) on the cytoplasmic tail of MT1 by Src facilitates interactions with p130Cas (Fig. 5) and FAK (Fig. S4). It is well known that Src signaling promotes ECM degradation (Yeatman, 2004; Courtneidge et al., 2005; Kelley et al., 2010), yet it appears that Src regulates FAs and invadopodia through distinct mechanisms. For example, Src-mediated phosphorylation of cortactin, AMAP1, paxillin, and p190 RhoGAP (Stylli et al., 2008) are essential for invadopodia-mediated degradation, presumably by regulating the assembly of this organelle. In contrast, Src phosphorylation of MT1-MMP showed no obvious effects on FA structure but did enhance MT1-MMP's recruitment to FAs (Fig. 5, I and j). This is both significant and consistent with our biochemical observations that Src facilitates MT1 interactions with FAK–p130Cas (Fig. 5 and Fig. S4). Moreover, FAK or p130Cas knockdown (Figs. 2 and 4) significantly compromised degradation at FAs, but not at invadopodia (Fig. 6, b and c). From these contrasting findings we believe that matrix degradations at these two structures are related yet distinct.

Despite the fact that FAs and invadopodia share many structural components, the relative contributions of these two organelles to cell migration and invasion remain unclear. One interesting study (Oikawa et al., 2008) has suggested that FAs could be transformed into podosomes, an invadopodia homologue, in a Src-dependent manner. As many signaling molecules, including Src, are elevated or activated in cancer cells, it is attractive to predict that FAs could acquire some invadopodia-like properties during cancer progression, while largely maintaining its FA morphology and function. The Boyden chamber–based invasion assay (Fig. 7) suggests that FA-centric degradation is relevant for cancer invasion. As demonstrated in Figs. 2–4, disruption of the FAK–p130Cas–MT1 complex significantly impaired ECM degradation at FAs, as well as invasion through Matrigel (Fig. 7). Admittedly, due to the multifunctional nature of these proteins, we cannot rule out the possibility that the mutant FAK, p130Cas, and MT1-MMP proteins affect invasion through other mechanisms.

Finally, it is attractive to speculate that FA-based degradation may play a central role during cancer invasion in vivo, in which cells acquire a compact spindle-like morphology (Cukierman et al., 2001, 2002) while FAs become smaller and predominate at the pseudopodia–ECM contact at the leading edge (Friedl and Wolf, 2009; Wolf and Friedl, 2009; Kubow and Horwitz, 2011). From this organization it is likely that in situ FAs act to macerate, degrade, and reorganize matrix at the front edge and allow the cell body to migrate through. Although the biology of FAs in situ remains elusive and a topic of debate (Fraley et al., 2010; Kubow and Horwitz, 2011), it has been reported that the leading edge localization of FAK–p130Cas is important for invasion (Hsia et al., 2003), and proteolytic activity has been observed at the pseudopodia–ECM contacts (Wolf and Friedl, 2009). Moreover, specific targeting of an enzymatic defective chimeric MT1 to FAs significantly hinders cancer invasion (Takino et al., 2007). All of these related findings suggest that FA-mediated ECM degradation

could play a significant role in the invasive process. It will be of great interest to define how FAs maintain a balance between adhesive and degradative properties.

Materials and methods

Antibodies and reagents

MT1-MMP antibody (MAB3328) was purchased from Millipore. The antibodies against FAK, paxillin, and p130Cas were from BD. Anti-Src antibody (sc-18) and anti-GST (sc-138) were purchased from Santa Cruz Biotechnology, Inc. The phospho-Src family antibody pY416 was from Cell Signaling Technology, the monoclonal vinculin antibody was from Sigma-Aldrich, anti-Flag antibody from Cell Signaling Technology, and anti-GFP antibody from Roche. Goat anti–rabbit or goat anti–mouse secondary antibodies conjugated to either Alexa 488 or 594 were from Invitrogen. BB-94 was purchased from Tocris Bioscience. All the other chemicals and reagents, unless otherwise stated, were from Sigma-Aldrich.

Cell culture

Pancreatic cancer cell lines BxPC3, Panc 04.03 and PANC-1 cells were purchased from American Type Culture Collection (Manassas, VA). HT1080 cells were a gift from Dr. Zhenkun Lou (Mayo Clinic, Rochester, MN). BxPC-3 and Panc04.03 cells were maintained in RPMI plus 10% fetal bovine serum, and PANC-1 and HT1080 cells were maintained in DME plus 10% fetal bovine serum (Invitrogen), and all cell lines had 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO_2 at 37°C. Cells were cultured in T-75 flasks (Thermo Fisher Scientific).

Plasmid, siRNA, and transfection

MT1-MMP-GFP and MT1-mCherry constructs were gifts from Drs. Roberto Buccione (Consorzio Mario Negri Sud, Chieti, Italy) and Philippe Chavrier (Institut Curie, Paris, France). FAK was amplified from a rat brain cDNA library and inserted into pEGFP-C1 or SFB vector (pIRES2-EGFP with S peptide, Flag tag, and streptavidin-binding peptide) as described previously (Wang et al., 2011). p130Cas constructs were subcloned from pEBG-p130cas (Addgene). Constructs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs targeting MT1-MMP (D-004145-02), FAK (M-003146-02), and p130Cas (M-020465-01) were purchased from Thermo Fisher Scientific and transfected with Lipofectamine RNAiMAX (Invitrogen) following the standard protocol. Knockdown and reexpression experiments were performed as follows: two rounds of siRNA transfection in a 24-h interval, and 24 h after the second transfection, cells were rescued with indicated constructs.

Immunoprecipitation and Western blotting

Cells were lysed with NETN buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris, and 1 mM EDTA) at 4°C. Cell debris was removed by centrifugation, and the supernatant was incubated with 5 µg of the appropriate antibody and protein A beads at 4°C for 4 h. For the S protein IP, cell lysate was incubated with S protein Agarose (EMD) at 4°C for 2 h. The pellet was washed with NETN buffer three times, eluted in Laemmli sample buffer, and analyzed by Western blot as described previously (Wang et al., 2011).

Fluorescence microscopy

Cells were grown on 22-mm coverslips for transfections and immunocytochemistry. Cells were fixed in formaldehyde and processed as described previously (Wang et al., 2011). Images were taken with an AcioObserver microscope (Carl Zeiss) and Orca II camera (Hamamatsu Photonics), analyzed with iVision software (BioVision Technologies) and processed with Adobe Photoshop (Wang et al., 2011).

Gelatin coating and degradation assay

Acid-washed coverslips were first coated with 50 mg/ml poly-L-lysine for 20 min at room temperature, and then fixed with 0.5% glutaraldehyde for 15 min. Gelatin matrix was prepared by mixing 0.2% gelatin and Oregon Green gelatin (Invitrogen) at an 8:1 ratio. After coating for 10 min, coverslips were washed with PBS and quenched with 5 mg/ml sodium borohydride for 15 min followed by extensive wash. For degradation assay, cell suspensions were plated on top of these coated coverslips for designated amounts of time, and subjected to IF analysis as described above. Cells that degraded ECM at FA sites were scored as positive, and more than 600 random selected cells were quantified (n > 200, three independent experiments). The percentage of cells with FA-mediated degradation was plotted and statistical significance was analyzed by two-sample t test.

Invasion assay

This assay was performed in a Boyden chamber (#BW200L; Neuro Probe) with #PFA 8 filter (Neuro Probe). The filters were coated with 1 mg/ml growth factor–reduced Matrigel as described previously (Hauck et al., 2002). The lower chamber was filled with full serum medium, and cells suspended in serum-free medium were plated on the upper chamber. Cells were allowed to invade for 24 or 36 h at 37°C before fixing and IF analysis.

Online supplemental material

Fig. S1 shows ECM degradation at FAs in multiple cell lines. Fig. S2 shows that deficiency or inhibition of MT1-MMP abolishes ECM degradation at FAs. Fig. S3 shows that FA localization of p130Cas is FAK dependent, and that p130Cas knockdown does not affect FA integrity. Fig. S4 shows that phosphorylation on MT1 Y573 facilitates MT1–FAK interaction, as well as ECM degradation at FAs. Fig. S5 shows cell model–specific FA and invadopodia matrix degradation patterns. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201105153/DC1.

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