

# Actin-associated protein palladin promotes tumor cell invasion by linking extracellular matrix degradation to cell cytoskeleton

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**ABSTRACT** Basal-like breast carcinomas, characterized by unfavorable prognosis and frequent metastases, are associated with epithelial-to-mesenchymal transition. During this process, cancer cells undergo cytoskeletal reorganization and up-regulate membrane-type 1 matrix metalloproteinase (MT1-MMP; MMP14), which functions in actin-based pseudopods to drive invasion by extracellular matrix degradation. However, the mechanisms that couple matrix proteolysis to the actin cytoskeleton in cell invasion have remained unclear. On the basis of a yeast two-hybrid screen for the MT1-MMP cytoplasmic tail-binding proteins, we identify here a novel Src-regulated protein interaction between the dynamic cytoskeletal scaffold protein palladin and MT1-MMP. These proteins were coexpressed in invasive human basal-like breast carcinomas and corresponding cell lines, where they were associated in the same matrix contacting and degrading membrane complexes. The silencing and overexpression of the 90-kDa palladin isoform revealed the functional importance of the interaction with MT1-MMP in pericellular matrix degradation and mesenchymal tumor cell invasion, whereas in MT1-MMP-negative cells, palladin overexpression was insufficient for invasion. Moreover, this invasion was inhibited in a dominant-negative manner by an immunoglobulin domain-containing palladin fragment lacking the dynamic scaffold and Src-binding domains. These results identify a novel protein interaction that links matrix degradation to cytoskeletal dynamics and migration signaling in mesenchymal cell invasion.

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## INTRODUCTION

Metastasis of tumor cells to distant sites in the human body is the major cause of cancer mortality. One of the important mechanisms

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Abbreviations used: CAF, cancer-associated fibroblast; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; F-actin, filamentous actin; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, human influenza virus hemagglutinin; H&E, hematoxylin and eosin; HER-2, human epidermal growth factor receptor 2; Ig, immunoglobulin; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; pFAK, phosphorylated focal adhesion kinase; TNBC, triple-negative breast cancer.

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promoting metastasis is epithelial-to-mesenchymal transition (EMT), which is a common phenomenon in several types of epithelial cancers, including triple-negative breast cancers (TNBCs; Thiery *et al.*, 2009). In this process, neoplastic epithelium uses invasive programs, involving dissociation of epithelial cell junctions, loss of apical-basolateral polarization, cytoskeletal reorganization, and protein expression required for mesenchymal invasion (Thiery *et al.*, 2009; Yilmaz and Christofori, 2009). Mesenchymal invasion is defined by elongated cell morphology and dynamic membrane protrusions tailored for extracellular matrix (ECM) degradation (Friedl and Wolf, 2009; Poincloux *et al.*, 2009; Murphy and Courtneidge, 2011).

For the pericellular ECM degradation, many types of tumor and stromal cells use membrane-type matrix metalloproteinases (MT-MMP) to allow invasion and growth across basement membranes and dense interstitial or provisional matrices (Sabeh *et al.*, 2004; Rowe and Weiss, 2009; Kessenbrock *et al.*, 2010). MT1-MMP

(MMP14) is the most widely expressed member of the MT-MMP family and a major cellular collagenase, whose overexpression by tumor cells correlates with the transition of neoplastic epithelial cells to invasive mesenchymal cells (Rowe and Weiss, 2009; Sugiyama *et al.*, 2010a). MT1-MMP accumulates at tumor cell invasive fronts and invadopodia, which are actin-rich membrane protrusions specialized for ECM degradation in invasive cancer cells (Nakahara *et al.*, 1997; Lehti *et al.*, 2000; Poincloux *et al.*, 2009; Murphy and Courtneidge, 2011). The recruitment of the cytoskeletal protein cortactin, the nonreceptor tyrosine kinase Src, and MT1-MMP is essential for the maturation of functional invadopodia (Poincloux *et al.*, 2009; Murphy and Courtneidge, 2011). Although the trafficking of MT1-MMP has been extensively studied, the mechanisms by which the microtubular vesicular trafficking is linked to MT1-MMP localization and function in the actin-based invadopodia or related membrane protrusions remain incompletely understood (Jiang *et al.*, 2001; Uekita *et al.*, 2001; Galvez *et al.*, 2002; Remacle *et al.*, 2003; Mazzone *et al.*, 2004; Wang *et al.*, 2004; Bravo-Cordero *et al.*, 2007; Poincloux *et al.*, 2009).

Here we identify, based on an unbiased screen with a genome-wide mouse cDNA library, novel interactions between MT1-MMP and the cytoskeletal proteins myotilin and palladin. Palladin, belonging to the myotilin/palladin/myopalladin immunoglobulin (Ig) domain-containing protein family, is expressed ubiquitously as major 90- and 140-kDa isoforms (Otey *et al.*, 2009). Palladin functions as a dynamic scaffold protein by binding to actin and actin-associated proteins, such as  $\alpha$ -actinin, VASP, and profilin (Boukhelifa *et al.*, 2004, 2006; Ronty *et al.*, 2004; Endlich *et al.*, 2009). It is a Src substrate involved in the formation of ECM-contacting cytoskeletal structures (Mykkanen *et al.*, 2001; Ronty *et al.*, 2007; Goicoechea *et al.*, 2009, 2014; Brentnall, 2012). High palladin expression in cancer cells or cancer-associated fibroblasts (CAFs) has been associated with increased invasiveness of pancreatic, colorectal, and breast cancers (Ryu *et al.*, 2001; Ronty *et al.*, 2006; Otey *et al.*, 2009; Goicoechea *et al.*, 2009; Brentnall, 2012). However, antimigratory functions have also been reported, and precise mechanisms of palladin action in cancer progression remain unclear (Chin and Toker, 2010). Because palladin is a dynamic scaffold protein, with even 30-fold faster turnover than actin or  $\alpha$ -actinin in filamentous actin (F-actin)-containing membrane structures, the identified interaction provides cells with a unique mechanism to endorse mesenchymal tumor cell invasion by dynamic coordination between ECM proteolysis and the cytoskeleton (Endlich *et al.*, 2009; Gateva *et al.*, 2014).

## RESULTS

### Palladin and myotilin are novel MT-MMP-interacting proteins *in vitro*

The extracellular domains of MT1-MMP mediate pericellular protein interactions and ECM proteolysis, whereas the transmembrane and cytoplasmic domains regulate MT1-MMP localization and trafficking (Lehti *et al.*, 2000; Jiang *et al.*, 2001; Poincloux *et al.*, 2009; Sugiyama *et al.*, 2010b; Schroder *et al.*, 2013; Mori *et al.*, 2013). To search for intracellular binding partners that regulate the subcellular localization and proinvasive functions of MT1-MMP, we systematically screened an E17 mouse embryonic cDNA library by yeast two-hybrid using C-terminal MT1-MMP cytoplasmic tail (amino acids [aa] 563–582) as a bait. Two of the positive cDNA clones encoded C-terminal Ig domains of the cytoskeletal protein myotilin (Figure 1A).

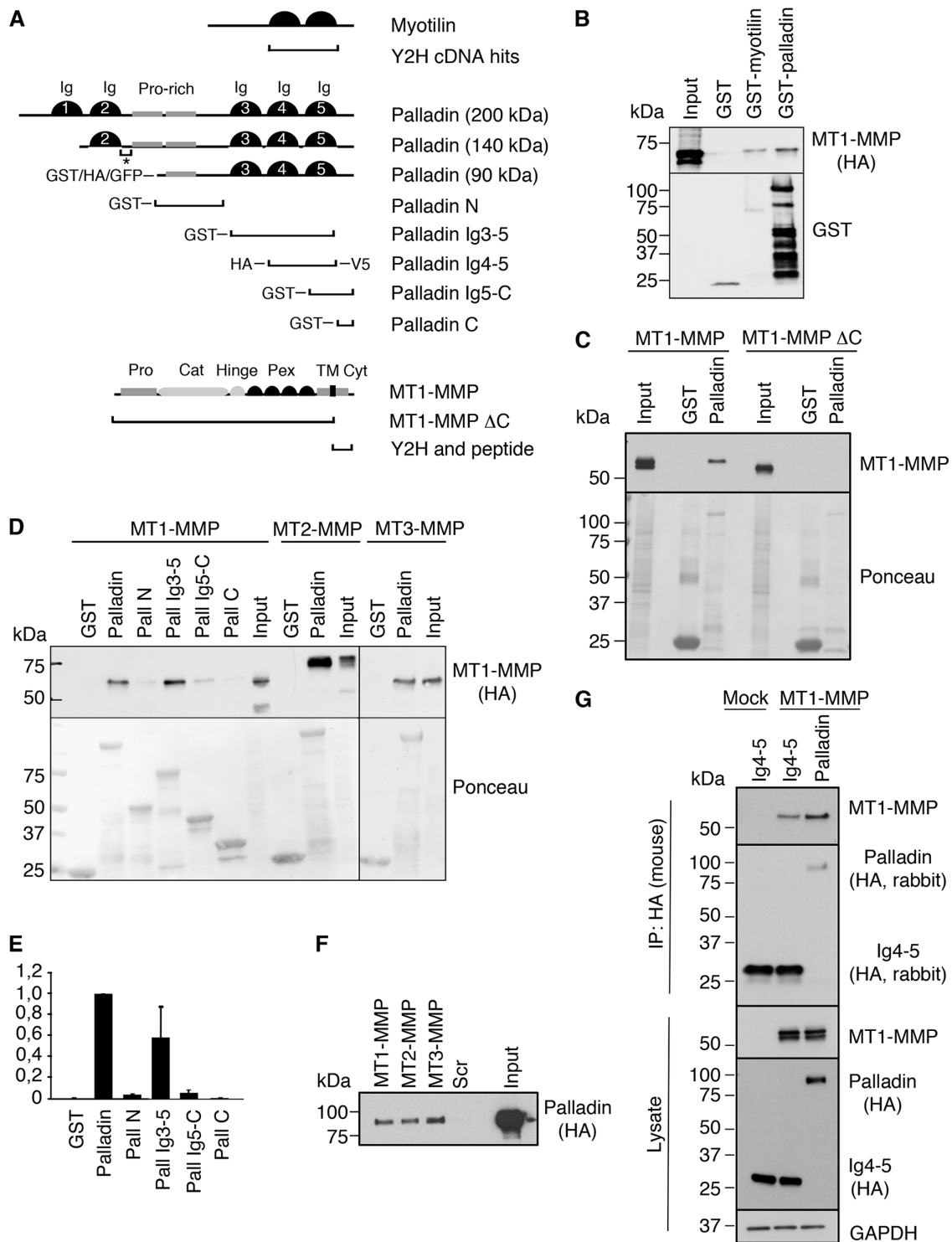
The interaction between MT1-MMP and myotilin, a protein mainly expressed in muscle, can be relevant for myogenesis or myoblast function (Salmikangas *et al.*, 2003; Ohtake *et al.*, 2006).

The homologous protein palladin is expressed in multiple tissues and up-regulated in certain invasive tumors in a more similar pattern with MT1-MMP (73% similarity within the two most C-terminal Ig domains of palladin and myotilin; BLAST [<http://blast.ncbi.nlm.nih.gov>]; Will and Hinzmann, 1995; Kinoh *et al.*, 1996; Apte *et al.*, 1997; Ryu *et al.*, 2001; Wang and Moser, 2008; Otey *et al.*, 2009). To assess the binding of MT1-MMP to these proteins, we used recombinant glutathione *S*-transferase (GST) fusions of myotilin and palladin (90-kDa isoform) to pull down MT1-MMP from the lysate of COS1 cells expressing human influenza virus hemagglutinin (HA) epitope-tagged MT1-MMP. MT1-MMP bound to both myotilin and palladin, whereas no binding to GST alone was observed by immunoblotting (Figure 1B). To verify the involvement of MT1-MMP C-terminus in palladin binding, we used full-length and the cytoplasmic tail-deleted mutant of MT1-MMP in the GST pull down. Full-length MT1-MMP bound palladin, whereas no binding of the cytoplasmic tail-deleted mutant was observed by immunoblotting (Figure 1C). To further define the palladin domains involved in this interaction, we used GST-tagged palladin fragments (Figure 1, A and D). MT1-MMP bound most prominently to the C-terminal Ig domains of palladin (Ig3-5; Figure 1, D and E), whereas the binding with Ig5-C fragment was less efficient (Figure 1, D and E). The C-termini of MT1-MMP, MT2-MMP, and MT3-MMP are homologous, with 73 and 66% identity of MT1-MMP to MT2-MMP and MT3-MMP, respectively, and >50% identity between all the three sequences. Therefore we tested the binding of these other MT-MMPs to palladin. Similarly to MT1-MMP, MT2-MMP and MT3-MMP bound to GST-palladin (Figure 1D).

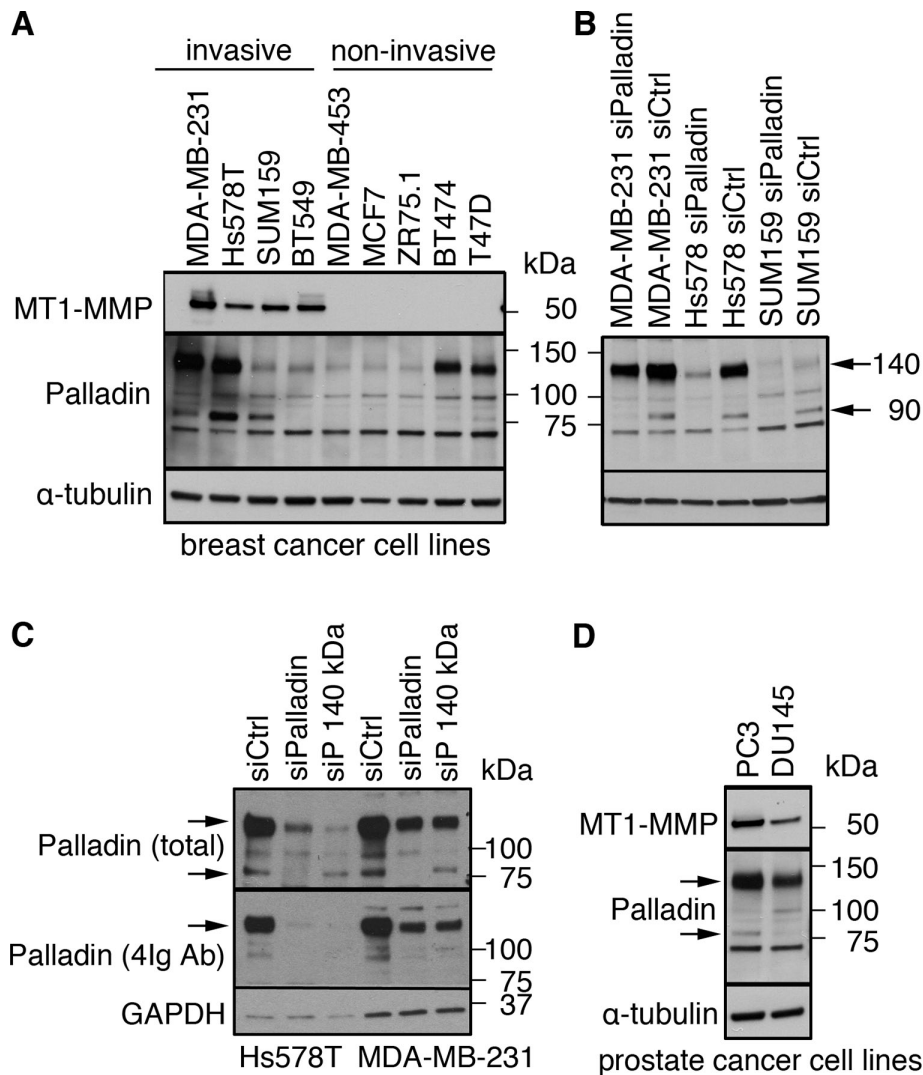
To verify the interaction of MT-MMP cytoplasmic tail and palladin, we used biotinylated synthetic peptides consisting of the intracellular C-terminal 20 amino acids of MT1-MMP, MT2-MMP, and MT3-MMP, as well as a peptide with scrambled sequence of the 20 amino acids of MT1-MMP. The peptides were incubated with lysate from COS1 cells expressing HA-tagged palladin, and the peptide-protein complexes were pulled down using streptavidin Sepharose. Palladin bound to all the peptides with MT-MMP sequence but not to the scrambled peptide (Figure 1F). For the binding to full-length MT1-MMP, the Ig4-5 domains, most homologous to the yeast two-hybrid hit domains of myotilin, were sufficient, as shown by coimmunoprecipitation (Figure 1G). Together these results indicate that the C-terminal Ig domains of 90-kDa palladin isoform bind to the intracellular C-terminus of the MT-MMPs.

### The 90-kDa isoform of palladin is coexpressed with MT1-MMP in mesenchymally invasive carcinoma cells

To investigate the potential function of the palladin–MT1-MMP interaction in tumor cell invasion, we first analyzed the expression of these proteins in cultured cancer cells (Figure 2, A–D). In the MDA-MB-231, Hs578T, SUM159, and BT549 invasive human breast cancer cells (TNBC cell lines), different levels of both MT1-MMP and palladin were expressed (Figure 2A; Sugiyama *et al.*, 2013). Palladin was detected as the main protein forms of 90 and 140 kDa in size (Figure 2A). The 90-kDa palladin was abolished, and the 140-kDa form reduced by a pool of small interfering RNAs (siRNAs) designed to target the different palladin isoforms (Figure 2, B and C, and Supplemental Figure S1, A and B). In contrast, siRNAs targeting the sequence close to the Ig2 domain (absent from 90-kDa isoform) decreased mostly 140-kDa palladin while having minor effects on the 90-kDa form (Figures 1A and 2, B and C, and Supplemental Figure S1, A and B). This indicates that these two palladin proteins are produced by separate transcripts.



**FIGURE 1:** Palladin binds MT-MMPs in vitro. (A) Schematic representation of myotilin, MT1-MMP bait region, myotilin yeast two-hybrid (Y2H) cDNA hit, three main palladin isoforms and the used palladin and MT1-MMP fragments. Cat, catalytic; Cyt, cytoplasmic; Ig, immunoglobulin-like; Pex, pexin-like; Pro-rich, proline-rich; TM, transmembrane. Respective protein tags are also indicated. Asterisk indicates the siRNA target sequence in the corresponding transcripts encoding 200- and 140-kDa palladin isoforms. (B–D) GST, GST-myotilin, GST-palladin, and GST-palladin fragments prebound to glutathione-Sepharose were allowed to interact in vitro with lysates from HA-tagged (B) or untagged (C, D) MT1-, MT1- $\Delta$ C-, MT2-, or MT3-MMP-expressing COS1 cells as indicated. The bound MT-MMPs were detected by immunoblotting ( $n = 3$ ). Immunoblotting or Ponceau staining visualized GST-tagged proteins (bottom). (E) Quantification of the MT1-MMP binding indicates the strongest binding of Ig3-5 fragments (full-length palladin set to 1; mean  $\pm$  SD,  $n = 3$ ). (F) Lysates from COS1 cells expressing HA-tagged palladin were allowed to interact with biotinylated synthetic peptides consisting of the intracellular C-terminal 20 amino acids of MT1-MMP, MT2-MMP, and MT3-MMP, as well as a peptide with scrambled sequence (Scr) of the MT1-MMP cytoplasmic amino acids. Peptide-



**FIGURE 2:** Palladin and MT1-MMP are coexpressed in breast and prostate carcinoma cell lines. (A) Immunoblotting of breast carcinoma cell lines as indicated. (B, C) The detection of 140- and 90-kDa palladin isoforms (arrows) was verified by transfection with scrambled (siCtrl), a pool of siRNAs designed to target the different palladin isoforms (siPalladin), and a specific siRNA targeting the mRNA sequence of the 140-kDa isoform (aa 625–629; UniProt ID Q8WX93), absent from the 90-kDa palladin. (D) Palladin (arrows) and MT1-MMP protein expression in PC3 and DU145 cells. Palladin was detected using an antibody recognizing all the major isoforms and MT1-MMP with an antibody against the catalytic domain recognizing both the latent and active protease. Where indicated, an antibody against the N-terminus of the 140-kDa isoform not present in the 90-kDa palladin (4Ig Ab) was used.  $\alpha$ -Tubulin or GAPDH served as loading control ( $n = 3$ ).

Of note, the mesenchymally invasive Hs578T and SUM159 cells, as well as MDA-MB-231 cells, contained 90-kDa palladin at markedly higher levels than BT549 cells, which express MT1-MMP but display a more rounded morphology during invasion in three-dimensional (3D) collagen, or the MT1-MMP-negative, noninvasive breast carcinoma cells (T47D, BT474, ZR75.1, MCF7, and MDA-MB-453; Figure 2A; Sugiyama *et al.*, 2013). The levels of the 140-kDa palladin

90-kDa palladin in Hs578T cells (Figure 3D).

### Palladin regulates ECM degradation

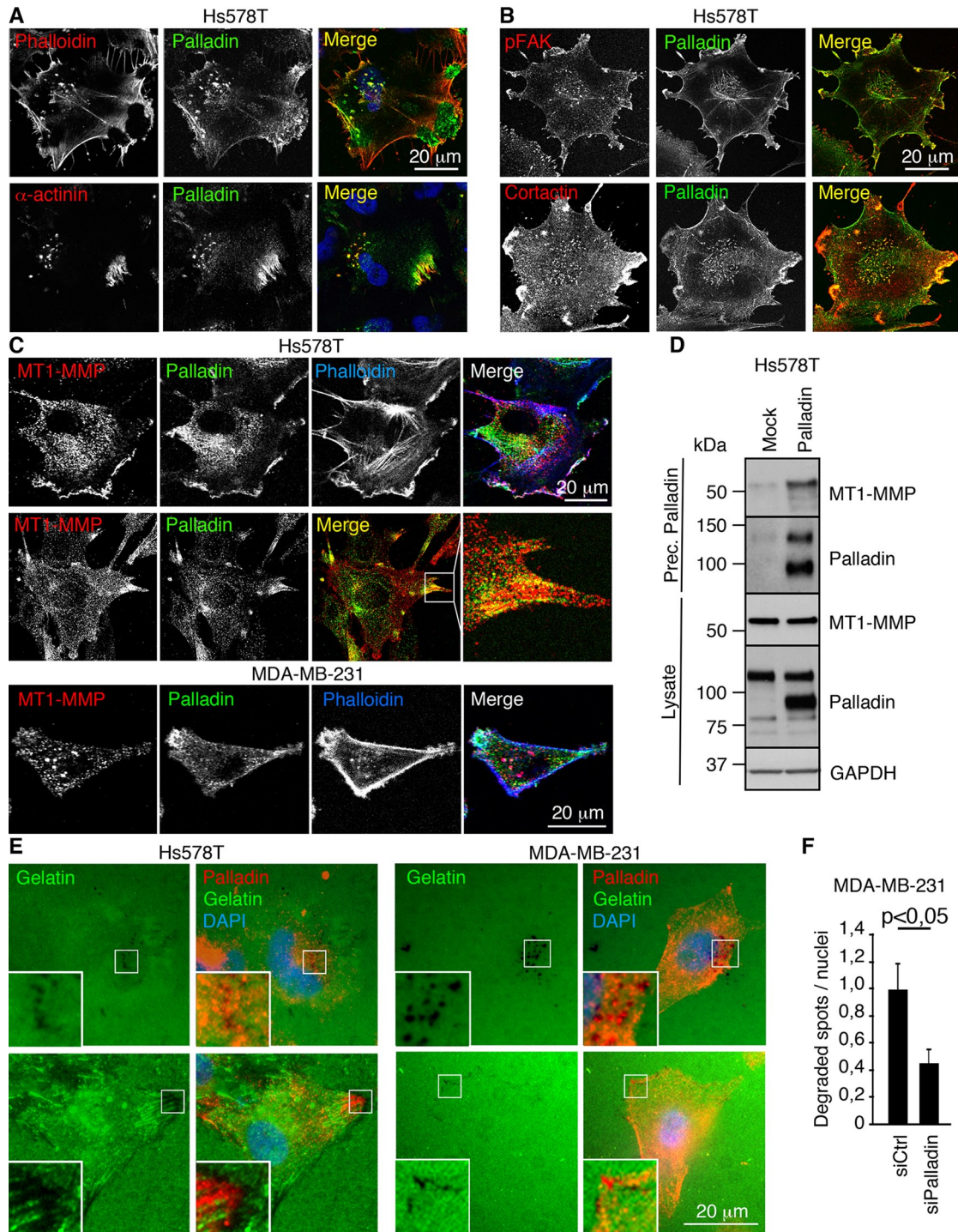
Previously, palladin was to function in the assembly of actin-based membrane protrusions linked to ECM degradation (Goicoechea *et al.*, 2006, 2009, 2014; Dixon *et al.*, 2008; Otey *et al.*, 2009; Brentnall, 2012). However, the physical interaction of palladin and

instead varied across the different types of breast carcinoma cells (Figure 2A). Both PC3 and DU145 prostate cancer cells also coexpressed MT1-MMP and palladin (Figure 2D). Consistent with the coexpression in breast cancer cells, the more mesenchymally invasive PC3 cells expressed 90-kDa palladin, whereas this isoform was negligible in the collectively invasive DU145 cells (Figure 2D; Sugiyama *et al.*, 2010a). Therefore the major palladin isoforms were widely expressed in different cancer cells, but the 90-kDa isoform was mainly coexpressed with MT1-MMP in the mesenchymally invasive cells.

### Palladin and MT1-MMP interact in cell-ECM adhesions and invadopodia

To characterize the palladin–MT1-MMP interaction, we first assessed the localization of the endogenous proteins in mesenchymally invasive breast carcinoma cells. As expected, palladin colocalized with its known interaction partners, F-actin and  $\alpha$ -actinin, in Hs578T breast carcinoma cells (Figure 3A; Ronty *et al.*, 2007; Dixon *et al.*, 2008). Moreover, palladin colocalized with both phosphorylated focal adhesion kinase (pFAK) and the invadopodia marker cortactin, indicating that palladin specifically localizes to ECM contacting and degrading structures in these invasive breast cancer cells (Figure 3B). Of importance, palladin and MT1-MMP were colocalized in actin-rich structures such as focal adhesions (FAs) or membrane ruffles at the cell edge and dot-like invadopodia at the ventral side of Hs578T, MDA-MB-231, and SUM159 cells, as well as in PC3 prostate carcinoma cells (Figure 3C and Supplemental Figure S1C). Consistent with the *in vitro* interaction results and colocalization, the activated form of MT1-MMP was coprecipitated in the same protein complexes with palladin and coenriched after overexpression of the

bound palladin pulled down using streptavidin Sepharose was detected by immunoblotting. (G) The HA-tagged Ig domains 4 and 5 or full-length palladin expressed in COS-1 cells was bound to anti-HA-conjugated agarose beads and allowed to interact with lysates from COS1 cells transfected with control (Mock) or MT1-MMP vectors. Bead-bound proteins were detected by immunoblotting as indicated. GAPDH served as loading control. MT1-MMP precipitates with both Ig domains 4 and 5 and full-length palladin.



**FIGURE 3:** Palladin localizes to cell–ECM contact structures of invasive breast carcinoma cells. Hs578T breast carcinoma cells were seeded on monomeric collagen I. Representative confocal micrographs show palladin. (A) Palladin colocalizes with F-actin (phalloidin) and  $\alpha$ -actinin. DAPI (blue) visualizes nuclei. (B) Palladin colocalizes with pFAK and the invadopodia marker cortactin. (C) Colocalization of MT1-MMP and palladin with or without F-actin staining in Hs578T (top) and MDA-MB-231 cells (bottom) is visualized in merged images (see also Supplemental Figure S1C). (D) Lysates of control or HA-palladin–expressing Hs578T cells were subjected to coimmunoprecipitation and immunoblotting as indicated. (E) Hs578T and MDA-MB-231 cells were seeded on fluorescein isothiocyanate–gelatin. Representative epifluorescence micrographs show palladin enriched at matrix-free spots, displaying sites of gelatin degradation. (F) Relative gelatin degradation by MDA-MB-231 cells after palladin silencing, quantified as number of gelatin-free spots/nuclei. Value of control cells (scrambled siRNA) was set to 1.

MT1-MMP identified here raises the additional possibility of more-direct involvement of palladin in ECM degradation. To assess whether palladin regulates ECM degradation in invadopodia or focal

adhesions, we analyzed cell-subjacent gelatin degradation. Hs578T cells displayed matrix degradation at palladin-containing FA-like edge structures and to some extent at more centrally located

invadopodia (Figure 3E). In MDA-MB-231 cells with less abundant stress fibers and FAs, palladin localized more prominently to the matrix-degrading invadopodia underneath the cells (Figure 3E). Significantly, silencing of palladin reduced the number of these ECM degradation spots (Figure 3F). This was not associated with dramatic changes in the actin cytoskeleton, although the overall F-actin was slightly decreased in conjunction with reduced accumulation of MT1-MMP at the cell edge structures (Supplemental Figure S1, D and E).

### **Palladin–MT1-MMP interaction promotes tumor cell invasion in 3D collagen**

To investigate whether palladin regulates MT1-MMP-driven cell invasion, we stably expressed green fluorescent protein (GFP) or GFP-palladin (90 kDa; Figure 4, A and B) in WM852 melanoma cells, which were essentially devoid of endogenous palladin but expressed MT1-MMP (Figure 4B). Whereas all the MT1-MMP-expressing breast cancer cell lines show invasive activities in collagen, the control WM852 cells display an MT1-MMP-dependent expansive growth phenotype with limited collagen invasive ability as single cells or multicellular sprouts (Figure 4, C and D; Tatti *et al.*, 2011; Sugiyama *et al.* 2013). To assess the effects of the 90-kDa palladin on cell-invasive growth and dissemination, we implanted control and GFP-palladin-expressing cells as single-cell suspension or preformed spheroids of 500 or 3000 cells in 3D matrix. Cross-linked collagen that typifies tumor adjacent ECM was used as the cell-surrounding matrix, for which MT1-MMP-dependent pericellular proteolysis is required for cell invasion (Rowe and Weiss, 2009; Sugiyama *et al.*, 2010a; Tatti *et al.*, 2011). During a 7-d culture, the singly embedded control cells grew to form cohesive, noninvasive colonies (Figure 4C). Of importance, palladin changed this growth pattern to efficient invasion as single elongated cells with a mesenchymal morphology in 3D collagen (Figure 4C). Similarly, the palladin-expressing cells invaded out from the preformed spheroids as single cells with prominent cellular protrusions projecting into collagen (Figure 4D and Supplemental Figure S2A). This invasion, as well as the interaction of overexpressed MT1-MMP and palladin, was exacerbated in these cells by platelet-derived growth factor (PDGF)-AB (see *Materials and Methods*; Figure 4, E and F, and Supplemental Figure S2A). Moreover, control cells seeded atop collagen displayed only limited invasive ability even in the presence of the growth factor as a chemoattractant, whereas palladin overexpression specifically increased the number of invasive cells by greater than fivefold (Figure 4, G and H). Of note, palladin did not alter cell growth or disrupt the overall integrity of the actin cytoskeleton in 2D culture (Figure 4A and Supplemental Figure S2B).

To further examine whether the 90-kDa palladin increased cell invasion via MT1-MMP, we silenced endogenous MT1-MMP in the palladin-expressing cells and followed colony growth in 3D collagen (Figure 4, I and J). Whereas the control siRNA-transfected cells invaded efficiently, MT1-MMP silencing specifically decreased the palladin-induced cell invasion (Figure 4, I and J). Because both MT1-MMP and palladin are Src substrates and closely associated with Src signaling, which is also critical for invadopodia and FA function, we tested the effect of Src inhibition on the palladin-induced invasion (Nyalendo *et al.*, 2007; Ronty *et al.*, 2007; Ouyang *et al.*, 2010; Sugiyama *et al.*, 2010a). Of note, PP2, the inhibitor of Src family kinases, similarly prevented invasion, as well as the PDGF-AB-induced tyrosine phosphorylation of GFP-palladin (Figure 3, K and L). These results indicate that the overexpressed 90-kDa palladin was sufficient to induce mesenchymal-type invasion of the PDGF-AB-stimulated WM852 cells from a cohesive cell mass into surrounding 3D collagen in an MT1-MMP and Src dependent manner.

### **Palladin is essential for efficient mesenchymal breast carcinoma cell invasion**

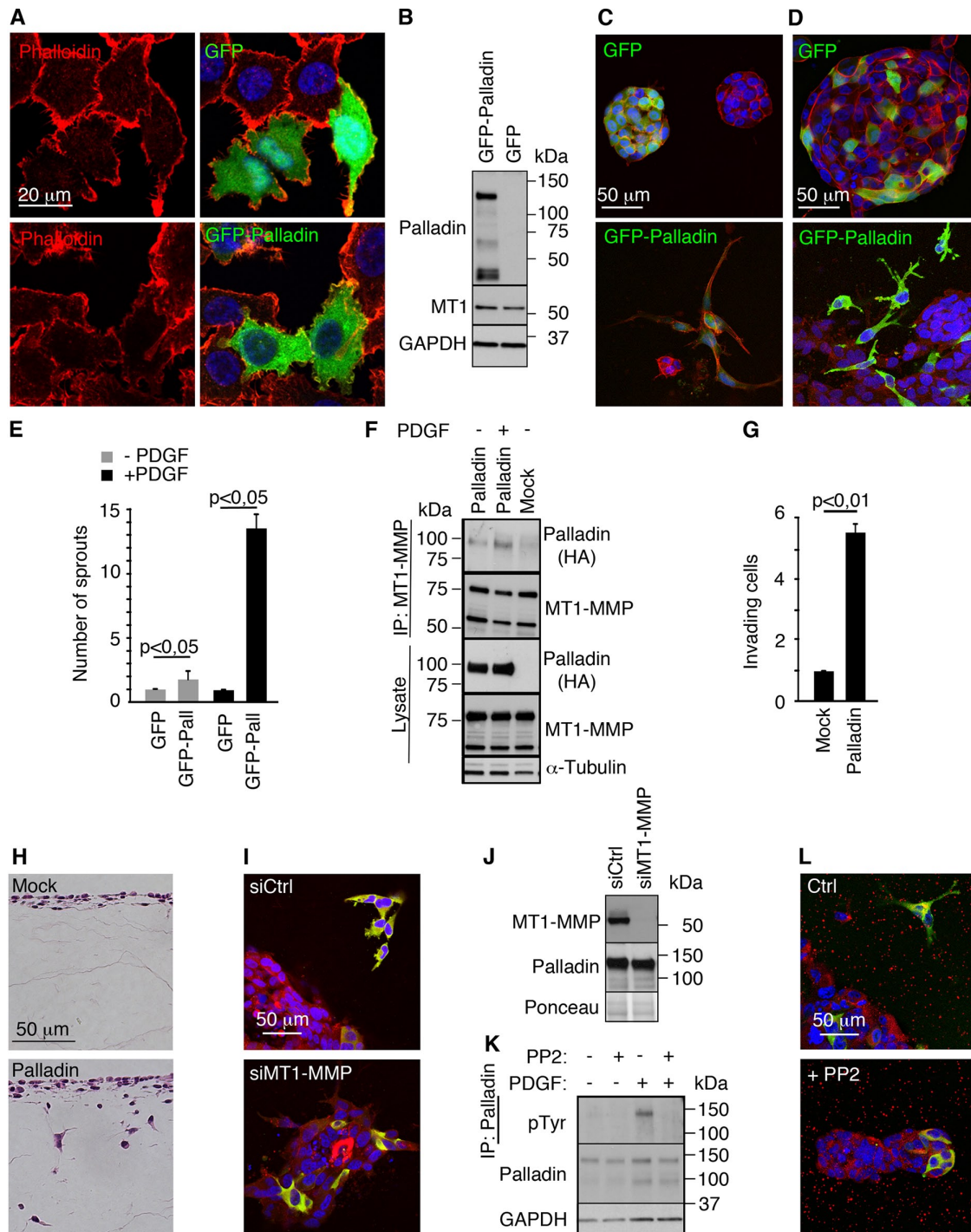
To examine the function of endogenous palladin in the MT1-MMP-dependent collagen invasion, we used Hs578T and MDA-MB-231 cells, where palladin was constitutively tyrosine phosphorylated in a Src-dependent manner (Supplemental Figure S3, A and B). The cells were embedded as single-cell suspension in 3D collagen after transfection with specific siRNA pools. Of interest, MT1-MMP and palladin colocalized at the tips of invading pseudopods in control Hs578T cells that invade with an elongated, front-rear-polarized, mesenchymal morphology with strong stress fibers (Figure 5A; Sugiyama *et al.*, 2013). In the invading MDA-MB-231 cells, these proteins colocalized at small spot-like structures behind the protruding tips (Figure 5B). Of note, the silencing of palladin diminished MT1-MMP localization to the Hs578T pseudopods in conjunction with impaired directional cell movement and extensions, as well as cortactin localization in protrusions in 3D collagen (Figure 5, C–E, and Supplemental Figure S3B).

During a 6-d culture in 3D collagen, Hs578T and MDA-MB-231 control cells grew to form invasive colonies with different types of multicellular outgrowths and singly invading cells (Figure 6A). Knockdown of MT1-MMP reduced collagen invasion, as reported previously (Figure 6, A–C; Sugiyama *et al.*, 2013). Significantly, palladin depletion also reduced collagen invasion without markedly altering the levels of cortactin or the EMT markers vimentin, E-cadherin, N-cadherin, and cadherin11 (Figure 6, A–C). The 90-kDa palladin remained efficiently depleted during the assay, whereas the 140-kDa isoform was only partially silenced, as assessed by immunoblotting of the cell extracts 3 and 6 d after siRNA transfection (Supplemental Figure S3B). The growth of the cells in 2D remained unaltered after the knockdown of palladin or MT1-MMP (Supplemental Figure S3C).

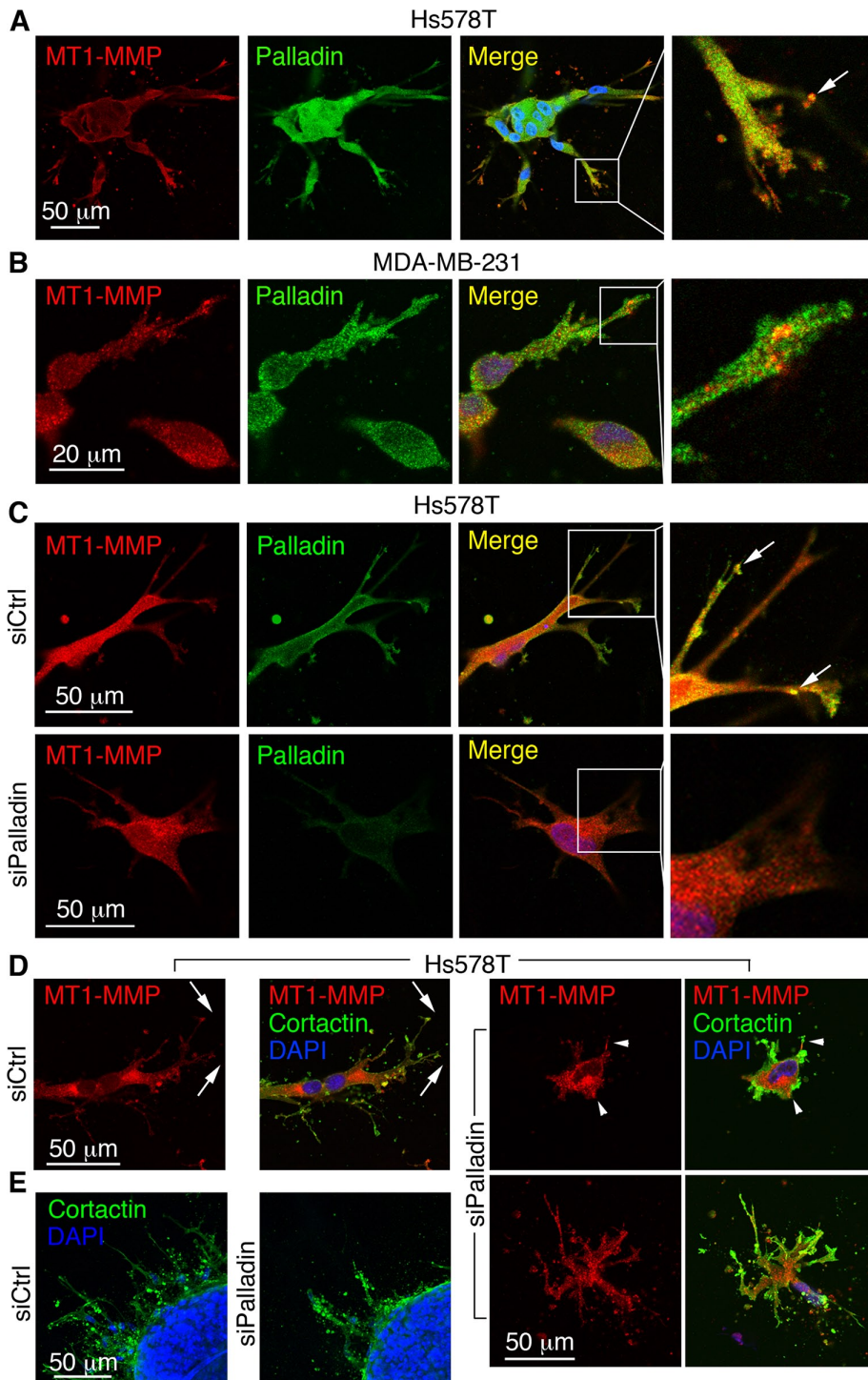
To study the effect of palladin isoforms on cell invasion, we cultured preformed spheroids containing Hs578T or MDA-MB-231 cells, transfected with either total palladin siRNA or siRNA targeting the 140- but not the 90-kDa isoform, in 3D collagen for 3 d (Figures 2C and 6D). Palladin depletion reduced significantly the number of invasive sprouts compared with control cells, whereas silencing of the 140-kDa palladin only slightly reduced invasion (Figures 2C and 6, D and E). Of importance, in the MT1-MMP and 90-kDa palladin-negative MCF7 cells, GFP-palladin expression did not confer invasive abilities or alter the 3D cell phenotype (Figure 6F). Together these results indicate that the 90-kDa palladin isoform cooperates with MT1-MMP in cancer cell invasion in collagen.

### **Palladin Ig4-5 fragment inhibits cell invasion**

Because palladin binds MT1-MMP through the Ig domains (Figure 1, D and G), Ig4-5 domains being sufficient for the binding, we expressed a palladin fragment containing these domains in Hs578T cells. This fragment, which lacks most of the protein binding sites for dynamic scaffold functions, acquired stable localization in strong peripheral membrane clusters, where it partially colocalized with the endogenous palladin (Figure 7, A and B). To assess the effects of the Ig4-5 fragment on the functional interaction of endogenous palladin and MT1-MMP in invasion, the cells overexpressing this fragment were seeded atop 3D collagen. During a 6-d culture, Ig4-5 significantly inhibited cell invasion (Figure 7, C and D). In 3D, this fragment localized throughout the cell and at the pseudopods, where it efficiently replaced the endogenous palladin (Figure 7E), suggesting a competitive/dominant-negative effect toward the endogenous palladin/MT1-MMP complexes in cell invasion.



**FIGURE 4:** Palladin promotes MT1-MMP and Src family kinase–dependent tumor cell invasion. (A) Stable pools of GFP-tagged palladin or GFP-expressing WM852 melanoma cells were stained with phalloidin and DAPI (blue;  $n = 3$ ). (B) The cells were also subjected to immunoblotting as indicated ( $n = 3$ ). (C, D) The cells were embedded as single-cell suspension (C) or preformed spheroids of 500 cells (D) within cross-linked 3D collagen and cultured for 7 d with PDGF-AB. Confocal micrographs show F-actin (phalloidin; red) in representative colonies (six collagen preparations/cell; see also Supplemental Figure S2A). (E) The cells were embedded within 3D collagen as spheroids containing 3000 cells, and sprouting was quantified after 7 d (sprouts/spheroid; mean  $\pm$  SEM; four collagen preparations/cell). (F) HA-tagged palladin was transfected in WM852 cells stably expressing GFP-tagged MT1-MMP, followed by immunoprecipitation using anti-HA-agarose beads and immunoblotting as indicated. (G) The cells expressing GFP or GFP-palladin were plated atop 3D cross-linked collagen. Invasion was quantified as number of cells that invaded  $>10 \mu\text{m}$ /cross section. Invasion of control cells (Mock) was set to 1 (mean  $\pm$  SEM; three collagen preparations/cell). (H) Light micrographs of collagen cross sections visualize the H&E-stained invasive cells. (I) The cells were treated with control (top) or MT1-MMP siRNA (bottom) before embedding preformed spheroids containing 500 cells within collagen. Silencing MT1-MMP reduced invasion (three collagen preparations/cell). (J) Western blot shows efficient knockdown of MT1-MMP 72 h after



**FIGURE 5:** Palladin and MT1-MMP colocalize in invasive pseudopods. Confocal micrographs show MT1-MMP and palladin in representative colonies of Hs578T and MDA-MB-231 cells cultured within 3D collagen. (A, B) The proteins colocalize in invasive, dot-like structures (arrow). (C) Palladin silencing inhibits the formation of the MT1-MMP-containing invasive structures (arrows; D, E) and directional cell elongation and extensions, visualized by cortactin staining (arrowheads).

### Palladin and MT1-MMP are codistributed in invasive human breast cancer

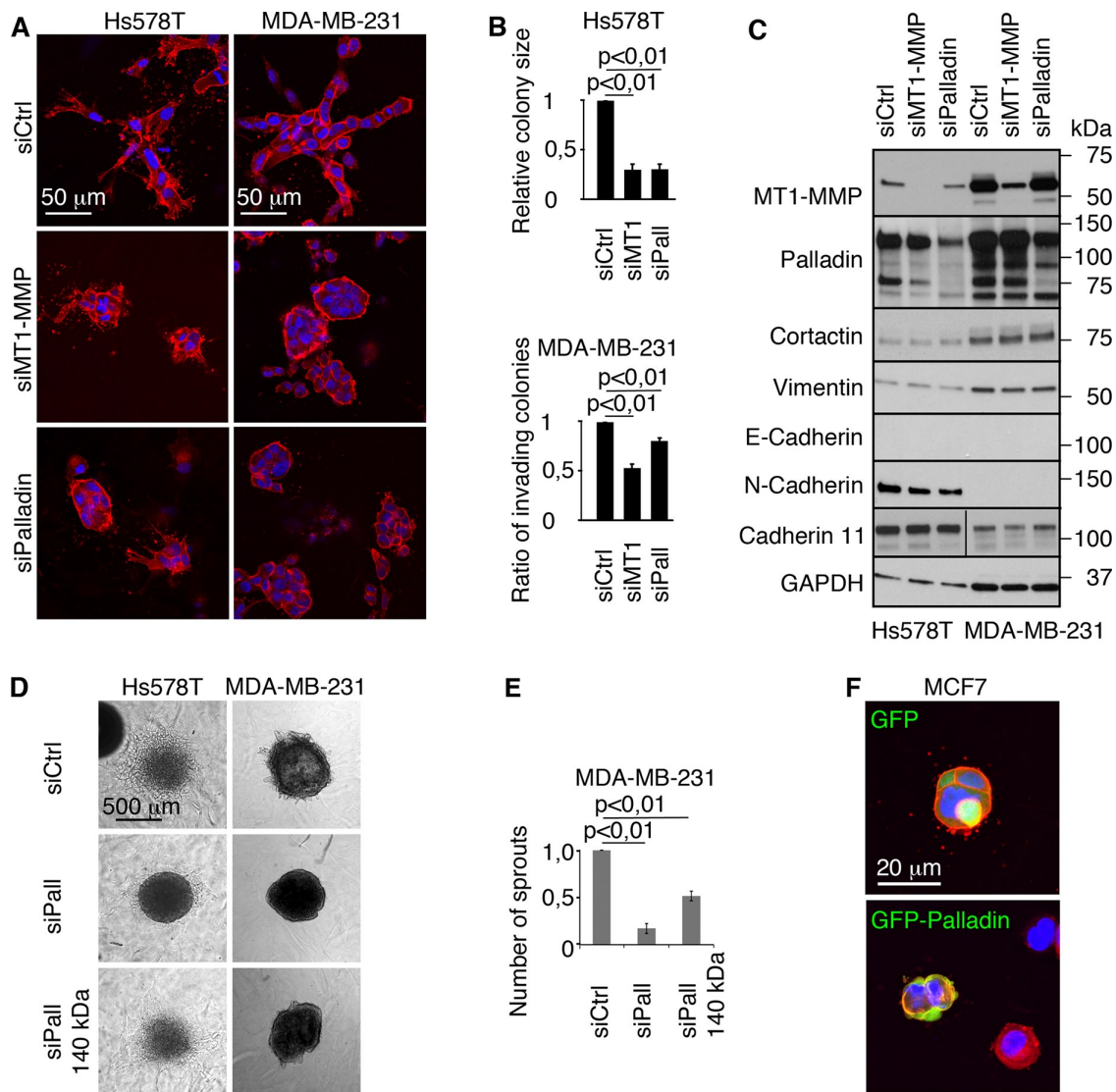
To investigate the potential biological relevance of the identified interaction, we analyzed the expression and codistribution of palladin and MT1-MMP in human breast carcinoma tissue biopsies. Given our expression results that showed specific coexpression of palladin and MT1-MMP in the TNBC cell lines, as well as the functional interaction in mesenchymal cell invasion typical in TNBC, tissue sections of 12 human TNBCs characterized by EMT and an unfavorable prognosis were subjected to immunohistochemistry (Otey *et al.*, 2009; Kessenbrock *et al.*, 2010). The cells in the stained TNBC tissues lacking the prognostic factors oncogene ErbB-2/human epidermal growth factor receptor 2 (HER-2), estrogen receptor (ER), and progesterone receptor (PR) expressed basal cytokeratin CK5/6 and were thus considered as aggressive, basal-like carcinomas (Cheang *et al.*, 2008). Variable levels of palladin and MT1-MMP coexpression were detected in the main tumor mass or tumor-invasive fronts in each TNBC tissue ( $n = 12$ ; Figure 8A and Supplemental Figure S4A), as well as in the tumor or stromal cells of invasive ductal breast carcinomas analyzed ( $n = 2$ ; Her-2-/ER+/PR+), whereas HER-2+ tumors displayed stronger codistribution of MT1-MMP and palladin in CAFs ( $n = 3$ ; Figure 8B and Supplemental Figure S4A). Of importance, both proteins were expressed at significantly higher levels at the tumor perimeter than the inside tumor masses of each TNBC (Figure 8C and Supplemental Figure S4B; palladin,  $p < 0.01$ ; MT1-MMP,  $p < 0.05$ ). As such, the coexpression was strongest at tumor-stroma borders in mesenchymal-like TNBC, suggesting that the palladin–MT1-MMP interaction can operate in the invasion of breast carcinoma cells *in vivo*.

### DISCUSSION

To facilitate metastasis and cell invasion through tissue microenvironments, many types of tumor and stromal cells up-regulate MT1-MMP for efficient ECM degradation and remodeling (Seiki, 2003; Rowe and Weiss, 2009). Although several reports describe MT1-MMP proteolysis at the invadopodia or invasive pseudopods, the mechanisms directing this membrane protease to

siRNA transfection. (K) PDGF-AB induces tyrosine phosphorylation of palladin. The cells were incubated with PP2 (5  $\mu$ M), PDGF (20 ng/ $\mu$ l), or both for 16 h, followed by immunoprecipitation and immunoblotting as indicated. Ponceau or GAPDH served as loading control ( $n = 3$ ). (L) The invasion of GFP-palladin-expressing cells embedded within collagen was reduced by the Src family kinase inhibitor PP2 (10  $\mu$ M; three collagen preparations/cell). Representative confocal micrographs show MT1-MMP in red.



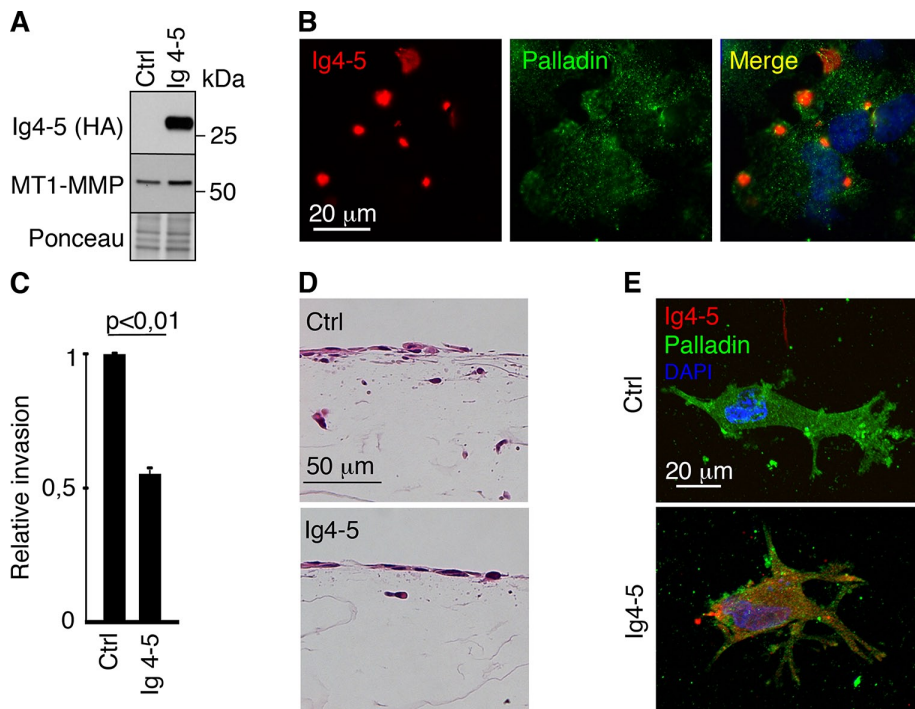


**FIGURE 6:** Palladin is essential for MT1-MMP–dependent breast cancer cell invasion in collagen. (A) Hs578T and MDA-MB-231 breast cancer cells were embedded in 3D collagen after palladin or MT1-MMP silencing and cultured for 5 d (see also Supplemental Figure S3B). Confocal micrographs show F-actin (phalloidin, red) and nuclei (DAPI, blue) in representative colonies. (B) Quantification of the invasion is expressed as relative colony size (Hs578T) or ratio of invading colonies (MDA-MB-231; mean  $\pm$  SEM,  $p < 0.01$ ; three collagen preparations/cell). (C) The cells were subjected to immunoblotting for EMT markers, as indicated. (D) The cells were treated with scrambled siRNA (siCtrl), a pool of siRNAs designed to target the different palladin isoforms (siPall), and a siRNAs targeting the sequence close to the Ig2 domain (absent from 90-kDa isoform). The cells were then embedded as preformed spheroids containing 4500 cells into 3D collagen and cultured for 3 d. Representative phase-contrast images show reduced invasion after palladin depletion (siPall). Treatment with siPalladin 140 kDa reduced invasion less efficiently. (E) Quantification of the invasion is expressed as relative number of sprouts longer than 50  $\mu$ m; mean  $\pm$  SEM,  $p < 0.01$ ; at least four collagen preparations/cell). (F) Noninvasive MCF7 breast cancer cells were embedded in 3D collagen after GFP-palladin transfection. Confocal micrographs show no induction of invasion after 7 d.

the actin-rich core of these invasive structures have remained poorly understood (Poincloux *et al.*, 2009; Murphy and Courtneidge, 2011). We identified here the Ig domain–containing, actin-associated scaffold protein palladin as a unique linker between the actin cytoskeleton and the pericellular ECM-degrading protease MT1-MMP in invasive cancer cells. This was based on our yeast two-hybrid screen, with the two Ig domains of myotilin identified as the MT1-MMP cytoplasmic tail-interacting hit. Such common interaction properties of myotilin and palladin were anticipated by high overall homology of the palladin and myotilin C-terminal Ig domains. However, protein interaction interfaces can also be composed of short structural

segments that consist of minimal motifs for binding separated by varying number of amino acids in primary sequence (Jones and Thornton, 1996; Calderwood *et al.*, 2003; Solmaz *et al.*, 2008). Although the coexpression of MT2-MMP or MT3-MMP prerequisite for the palladin interaction will need to be examined, the 20–amino acid cytoplasmic tails of MT1-MMP, MT2-MMP, and MT3-MMP can share a common palladin-binding motif, as they all bound palladin *in vitro*.

An exocyst complex coordinates MT1-MMP delivery from the microtubular network to the actin-rich invadopodia, a localization also regulated by cortactin, Tks4, and neural Wiskott–Aldrich



**FIGURE 7:** Palladin Ig domains 4 and 5 inhibit cell invasion. (A) Immunoblotting for HA-tagged Ig4-5 palladin fragment and MT1-MMP in Hs578T cells. Ponceau red served as loading control. (B) Representative epifluorescence micrographs show expression of the Ig4-5 fragment in strong peripheral membrane clusters partially colocalizing with endogenous palladin. (C) Relative invasion of the cells indicates reduced collagen invasion after Ig4-5 fragment expression (mean  $\pm$  SEM,  $n = 5$ ). (D) Light micrographs of collagen cross sections visualize invasion of the H&E stained cells ( $n = 6$ ). (E) Epifluorescence micrographs show invading cells after 2-d culture. Palladin Ig4-5 fragment expression inhibits the formation of palladin-containing invasive structures and front-rear cell polarity.

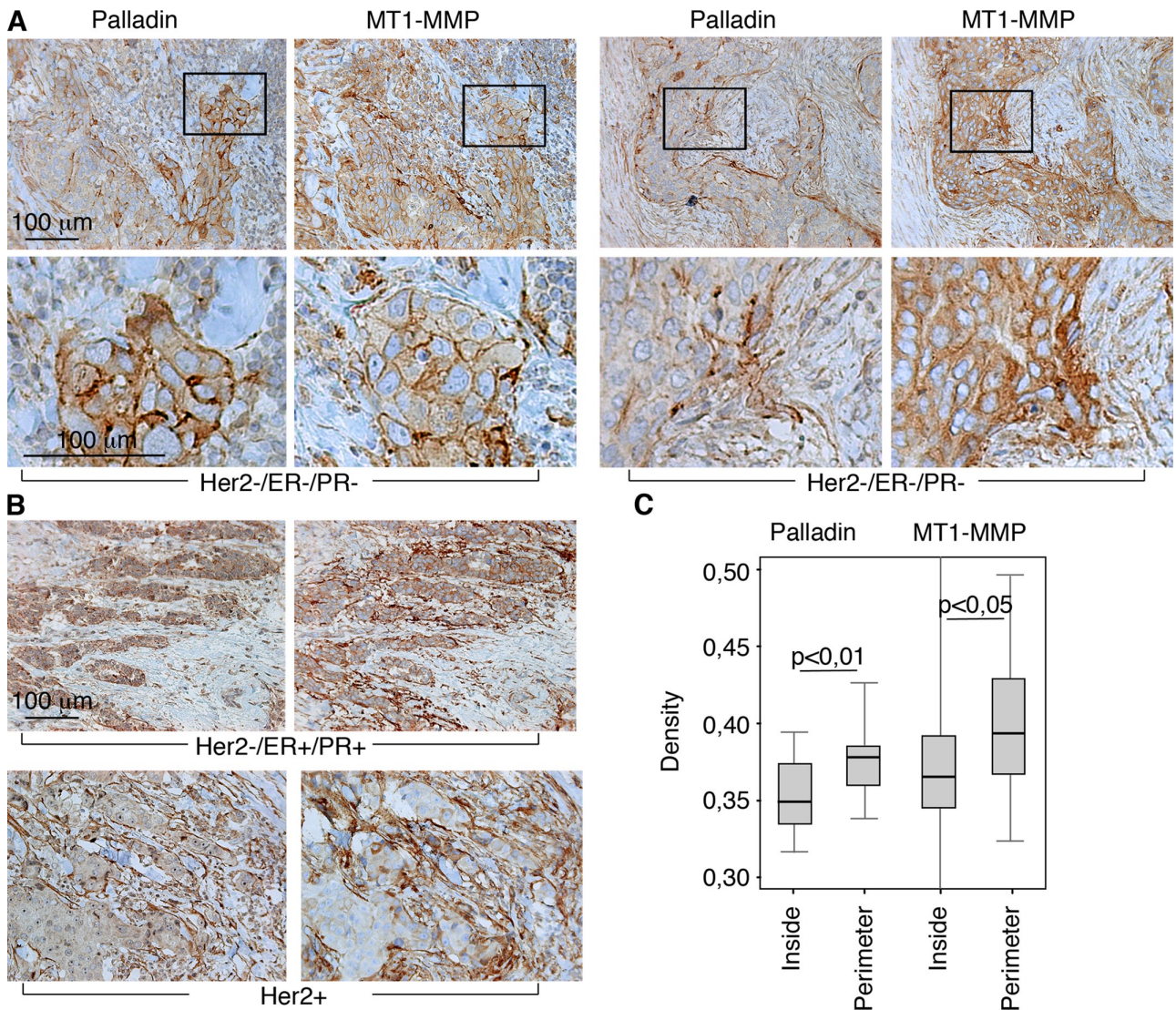
syndrome protein (Buschman *et al.*, 2009; Poincloux *et al.*, 2009; Yu *et al.*, 2012). However, direct binding of these proteins to MT1-MMP has not been described (Buschman *et al.*, 2009; Poincloux *et al.*, 2009; Yu *et al.*, 2012). Based on precipitation with isolated rabbit muscle actin, direct F-actin-mediated capture of MT1-MMP has instead been suggested to stably target MT1-MMP to invasive pseudopods (Yu *et al.*, 2012). In contrast to the relatively stable F-actin assembly in invasive structures, dynamic MT1-MMP turnover is considered essential for efficient or sustained proteolysis within inhibitor-rich tissue microenvironments (Artym *et al.*, 2006; Watanabe *et al.*, 2013). Because palladin is a cancer-associated scaffolding protein with considerably faster turnover than actin or  $\alpha$ -actinin in membrane ruffles and FAs, the interaction identified here provides cells with a more dynamic mechanism for invadopodial MT1-MMP targeting (Endlich *et al.*, 2009; Gateva *et al.*, 2014).

Palladin is ubiquitously expressed in several isoforms of three to five Ig domains flanked by one or two proline-rich regions (Figure 1A; Rachlin and Otey, 2006; Ronty *et al.*, 2006; Wang and Moser, 2008; Goicoechea *et al.*, 2010). Whereas differential expression and actin-organizing properties of the isoforms have been reported, their specific functions have remained unclear in diverse cell types (Rachlin and Otey, 2006; Goicoechea *et al.*, 2009; Otey *et al.*, 2009). In cancer, the overall palladin expression has been mainly linked to increased invasion, but antimigratory or antitumorigenic functions have also been reported, which could be related to the expression of different isoforms in specific cancers (Rachlin and Otey, 2006; Otey *et al.*, 2009; Goicoechea *et al.*, 2009; Chin and Toker, 2010; Tay *et al.*, 2010; Asano *et al.*, 2011; Brentnall, 2012). The isoforms expressed in

breast and prostate carcinoma cells have not been previously defined. Here we found that the carcinoma cell lines contained mainly 90 and 140-kDa palladin. In the mesenchymal breast carcinoma cell lines, the 90-kDa palladin was specifically coexpressed with MT1-MMP, where its efficient knockdown, coupled with only partial reduction of the 140-kDa palladin, inhibited ECM degradation and invasion. Moreover, specific knockdown of the 140-kDa palladin reduced invasion less efficiently. Of importance, ectopic expression of the 90-kDa palladin isoform was sufficient for induction of a dramatic mesenchymal-type cell invasion of MT1-MMP-expressing, but not of MT1-MMP-negative, tumor cells. Therefore our results strongly suggest a unique function for the 90-kDa palladin in the regulation of MT1-MMP-driven ECM degradation and cell invasion.

The 90-kDa palladin contains an N-terminal proline-rich region, followed by the identified C-terminal MT1-MMP-binding Ig domains (Ig3-5). The Ig3 domain being sufficient for the actin binding, whereas Ig4-5 domains bind cortical actin-associated ezrin (Mykkanen *et al.*, 2001; Dixon *et al.*, 2008; Beck *et al.*, 2013). Several adaptor, actin regulatory, and signaling proteins, such as VASP, profilin, SPIN-90, eps8, and Src, instead interact with palladin through the SH3 domain-binding proline-rich region, establishing palladin as

a signaling scaffold important for cytoskeletal dynamics (Boukhelifa *et al.*, 2004, 2006; Goicoechea *et al.*, 2006; Ronty *et al.*, 2007). Moreover, interactions through the C-terminal PDZ domain-binding motif target palladin to stress fibers (Maeda *et al.*, 2009; von Nandelstath *et al.*, 2009). We found that the invasion induced by 90-kDa palladin was sensitive to Src inhibition and associated with Src family kinase-mediated palladin phosphorylation coincident with the palladin-MT1-MMP interaction. Previously, Src was shown to induce relocalization of palladin to Src- and actin-containing membrane extensions in conjunction with actin remodeling in fibroblasts (Ronty *et al.*, 2007). This is consistent with a key activity of Src in connecting actin cytoskeleton and MT1-MMP through the identified palladin interaction. However, the Src-binding, proline-rich region and the other sites for the foregoing interactions are also included in the 140-kDa palladin, which differs from the 90-kDa isoform by containing an additional N-terminal Ig domain and a second proline-rich region (Rachlin and Otey, 2006). As such, a potential inhibitory effect of these 140-kDa, isoform-specific domain repeats on the scaffold functions and cross-talk with Src by, for example, conformational means or alternative interactions will be of interest (Rachlin and Otey, 2006). Consistently, the palladin Ig4-5 fragment, which lacks most of the scaffold sites, accumulated at cell edges similarly to what was reported for 140-kDa palladin. Moreover, the Ig4-5 fragment localized to the tips of invasive pseudopods, where it efficiently replaced endogenous palladin. The observed dominant-negative effect of this Ig4-5 fragment on collagen invasion further emphasizes the importance of palladin in the MT1-MMP-dependent cell invasion.



**FIGURE 8:** (A, B) Palladin and MT1-MMP immunohistochemistry of tissue biopsies from basal-like triple-negative (A) and other human breast cancer subtypes (B). Palladin expression was visualized using an antibody recognizing the major, 90-, 140-, and 200-kDa, isoforms and MT1-MMP using an antibody against the catalytic domain recognizing both the latent and active protease. Palladin and MT1-MMP colocalize in the invasive carcinoma cells and reactive stroma. Bottom, magnifications of the framed areas ( $n = 17$ ; see also Supplemental Figure S4, including control staining). (C) Quantitative image analysis of palladin and MT1-MMP staining in two fields per specimen quantified by Anduril (<http://csbi.ltdk.helsinki.fi/anduril/site/>). The mean densities at the interior and perimeter were compared using the paired two-tailed Student's  $t$  test. The  $p$  values were corrected with the Bonferroni correction. Error bars indicate SD. Palladin and MT1-MMP were expressed at significantly higher levels at the tumor perimeter than inside of the tumor masses (see also Supplemental Figure S4).

Taken together, the present results identify a unique palladin–MT1-MMP interaction that helps to explain 1) palladin function in mesenchymal cancer cell invasion and 2) the dynamic targeting and turnover of MT1-MMP in invasive pseudopods. In addition to the proinvasive MT1-MMP function on tumor cells, substantial evidence supports a critical role of this protease in the invasive and tissue-remodeling functions of mesenchymal stromal cells such as CAFs. Palladin expression has also been associated with myofibroblast phenotype and CAF invasion (Ronty *et al.*, 2006; Brentnall, 2012; Goicoechea *et al.*, 2014). We found extensive MT1-MMP and palladin coexpression in both spindle-shaped tumor cells, in which cancer-related EMT likely occurs, and tumor-adjacent CAFs. Because the mesenchymal invasion of cancer cells recapitulates the invasive activities of

CAF, we conceive that the palladin–MT1-MMP interaction identified here likely affects the invasion and function of different cell types with the actin-based contractile cytoskeleton, such as mesenchymal cancer cells, CAFs, or myofibroblasts and smooth muscle cells.

## MATERIALS AND METHODS

### Yeast two-hybrid screening

The MT1-MMP cytoplasmic tail encoding amino acids 563–582 was used to screen a mouse 17-d embryo matchmaker cDNA library in pACT2 (ML4013AH; Clontech Laboratories, Palo Alto, CA), essentially as described (Gyuris *et al.*, 1993). Positive clones were subjected to sequence analysis or analyzed by digestion.

## Cell lines

Human ZR75-1, MCF7, BT-474, T47D, MDA-MB-453, Hs578T, BT-549, MDA-MB-231 (American Type Culture Collection [ATCC, Manassas, VA]), and SUM159 breast carcinoma cells (from O. Kallioniemi, Institute for Molecular Medicine Finland, Helsinki, Finland; Neve *et al.*, 2006), DU145 and PC3 prostate adenocarcinoma cells (ATCC), and WM852 melanoma cells (established at the Wistar Institute, Philadelphia, PA; Airola *et al.*, 1999), and COS-1 cells (ATCC) were cultured according to manufacturer's instructions and as described (Tatti *et al.*, 2011). Stable WM852 cell pools were generated by G418 (400 µg/ml; Calbiochem, Merck KGaA, Darmstadt, Germany) selection.

## Antibodies and reagents

Antibodies used were goat anti-GST antibody (GE Healthcare, Piscataway, NJ), palladin (reacts with the 90-, 140-, and 200-kDa isoforms), or a 4Ig isoform-selective palladin (reacts with the 140- and 200-kDa isoforms; Ronty *et al.*, 2006), HA and MT1-MMP rabbit polyclonal antibodies (Abcam, Cambridge, United Kingdom), and mouse monoclonal antibodies against MT1-MMP (antibody against the catalytic domain recognizing both the latent and active protease), cortactin, phospho-tyrosine (EMD Millipore Corporation, Temecula, CA), HA (Cell Signaling Technology, Danvers, MA),  $\alpha$ -actinin,  $\alpha$ -tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich, St. Louis, MO), phospho-FAK, E-cadherin, N-cadherin (BD Transduction Laboratories, Franklin Lakes, NJ), vimentin (Santa Cruz Biotechnology, Dallas, TX), and cadherin 11 (Invitrogen), as well as Alexa Fluor 647-phalloidin secondary antibodies (Invitrogen), and horseradish peroxidase-conjugated secondary antibodies (Dako, Agilent Technologies, Santa Clara, CA). We also used GM6001, and PP2 inhibitors (Millipore), PDGF-AB (R&D Systems, Minneapolis, MN), enhanced chemiluminescence (GE Healthcare), phalloidin-tetramethylrhodamine isothiocyanate, and collagen type I from rat tail (Sigma-Aldrich).

## cDNAs and peptides

Full-length human myotilin (aa 1–498; von Nandelstadh *et al.*, 2005) or human palladin variants encoding aa 8–772 (palladin), 8x228 (N), 387–772 (Ig1-3; Mykkanen *et al.*, 2001), 619–772; Ig3-tail), and 715–772 (von Nandelstadh *et al.*, 2009) in pAHP (Ronty *et al.*, 2005) for eukaryotic expression of HA-tagged proteins or pGEX-4T1 (Amersham Pharmacia Biotech, Piscataway, NJ) for bacterial expression of GST fusions have been described. Full-length GFP-palladin (1–772) in pEGFP-C2 (Clontech; Gateva *et al.*, 2014) was used for expression and to amplify palladin Ig4-5 fragment (primers: 5'-GGAATTCTCACCATGCCTCACTTCTTG-3' and 5'-CCGCTCGA-GTGGGTGTAACGTCACGCC-3'). A second amplification (primers: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGAAT-TCTC ACCATGCCTCACTTCTTG-3' and 5'-GGGGGACCACTTT-GTACAAGAAAAGC TGGGTTCCGCTCGAGTGGGTGTAACGTC-AGC-3') introduced attB sites for Gateway cloning into pcDNA-DEST40 (V5-tag; Invitrogen) or pTO-HA-StrepIII-GW-FRT (HA tag; M. Varjosalo, University of Helsinki, Helsinki, Finland). The constructs were verified by sequencing. Human MT1-MMP, MT1-MMP  $\Delta$ C, MT2-MMP, and MT3-MMP cDNAs and MT1-MMP-GFP fusion in a pMX-GFP retroviral vector were prepared and produced as described (Lehti *et al.*, 2000; Hotary *et al.*, 2002; Li *et al.*, 2008; Cheng *et al.*, 2011).

Custom N-terminally biotinylated peptides used were -Ttds-RRHGTPRRLLYCQRSLLDKV (MT1-MMP C-terminus), -Ttds-QRK-GAPRVLLYCKRSLQEWV (MT2-MMP C-terminus), -Ttds-KRK-GTPRHILYCKRSMQEWV (MT3-MMP C-terminus), and -Ttds-RCR-

QLSLPLDLKYRTGVHRR (scrambled; JPT Peptide Technologies, GmbH, Berlin, Germany).

## Cell transfections and treatments

The cells were transfected with expression vectors using FuGENE HD (Promega, Madison, WI). siRNAs (siGENOME SMARTpool; Dharmacon, Thermo Scientific, Waltham, MA) against human *PALLD* (M-016891-01-0005) and *MMP14* (MT1-MMP; M-004145-00-0005) and nontargeting control siRNA, as well as a siRNA duplex targeting the mRNA sequence in the corresponding transcripts encoding 200- and 140-kDa palladin isoforms (aa 625–629; isoform1-200 kDa; UniProt ID Q8WX93), absent from the 90-kDa palladin isoform CDS (GGCAAAGCUAACAGUAAUAdTdT, dTdTCCGUUUCGAUUGUCA-UUAU; Dharmacon, Thermo Scientific; Rachlin *et al.*, 2006) were transfected using Lipofectamine 2000 (Invitrogen). Western blotting after 72 or/and 96 h monitored knockdown efficiency. For immunofluorescence, cells were seeded on monomeric collagen-coated (100 µg/ml) coverslips, fixed, and stained as previously described (von Nandelstadh *et al.*, 2005) and mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Fluorescence images were obtained using an LSM 5 DUO confocal microscope with a Plan-Apochromat 40x/1.3 numerical aperture (NA) oil objective and with Plan-Apochromat 10x/0.45 NA objective or using an LSM 780 confocal microscope with Plan-Neofluar 40x, 1.3 NA oil objective. In addition, an Axiolmager.Z1 upright epifluorescence microscope with Apotome and Plan-Apochromat 40x/1.4 NA oil objective and an Axioplan 2 upright epifluorescence microscope were used (all from Carl Zeiss, Oberkochen, Germany). Brightness and contrast were linearly adjusted using Photoshop CS5.1 (Adobe, San Jose, CA). Single optical sections or a combination of two serial optical sections were used for image display.

## In vitro binding assays

GST, GST-myotilin, GST-palladin, and GST-palladin fragments were produced as described (von Nandelstadh *et al.*, 2005). COS1 cells were transfected to express HA-tagged MT1-, MT2-, or MT3-MMP and lysed with 140 mM NaCl, 1 mM Tris, pH 7.5, 1% Triton X-100, 10 µM GM6001, and Complete protease inhibitors (Roche, Indianapolis, IN). Cleared supernatants were incubated with GST fusion proteins bound to glutathione-Sepharose overnight at 4°C. Alternatively, cleared lysates from COS1 cells expressing HA-tagged palladin were allowed to interact with synthesized biotinylated peptides representing the intracellular C-terminal 20 amino acids of MT1-MMP, MT2-MMP, and MT3-MMP as well as a scrambled peptide for 2 h at 4°C. The peptide-protein complexes were pulled down by rotation with Streptavidin Sepharose Bead Conjugate (Cell Signaling Technology) overnight at 4°C. After washing, the bound proteins were detected by SDS-PAGE and immunoblotting. Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

## Gelatin degradation

Coverslips were coated with 50 µg/ml poly-L-lysine (Sigma-Aldrich), fixed with 0.5% glutaraldehyde (Sigma-Aldrich), coated with 1 mg/ml Oregon green Gelatin (Molecular Probes, Eugene, OR)/2 mg/ml gelatin 1:3 and incubated with 5 mg/ml sodium borohydride and complete media. Cells were fixed and immunostained 16 h after plating.

## Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously (Lehti *et al.*, 2000). Palladin was

immunoprecipitated from soluble lysates by specific rabbit polyclonal antibodies and GammaBind G Sepharose (GE Healthcare). Alternatively, HA-tagged proteins were immunoprecipitated with anti-HA-agarose beads (Sigma-Aldrich). See Supplemental Materials and Methods for immunoprecipitation from COS1 cells, real-time qPCR, cell proliferation assay, and gelatin zymography.

### Three-dimensional type I collagen growth and invasion assays

Type I collagen (2.2 mg/ml) was prepared; invasion and growth were assessed essentially as described (Sugiyama *et al.*, 2013). For invasive growth assay, a single-cell suspension ( $3 \times 10^3$  cells/ml) or spheroids of 500 or 3000 cells preformed under nonadherent conditions were mixed in collagen and cultured for 5 d. Based on initial growth factor testing (epidermal growth factor, hepatocyte growth factor, fibroblast growth factor, PDGF-AA, PDGF-AB, PDGF-BB), PDGF-AB (20 ng/ml) was used to stimulate WM852 cell invasion. Cultures were fixed and immunostained. The number of sprouts per colony or number of invading colonies was manually counted. Relative colony size (area) and length of sprouts were quantified using ImageJ software from random epifluorescence or phase contrast images of three to six gels.

PDGF was used as a chemoattractant (WM852 cells) in the invasion assay. After 5 (Hs578T) or 15 d (WM852), cells were fixed, and paraffin sections were stained with hematoxylin and eosin (H&E staining). Sections were photographed (Leica DM LB; Leica Microsystems, Wetzlar, Germany; or Zeiss AxioImager.Z1 epifluorescence microscope), and the cells that invaded  $>10 \mu\text{m}$  (WM852) or total number of invading cells (Hs578T) were counted from at least three random sections of each sample.

### Immunohistochemistry

Breast carcinoma tissue biopsies were collected, fixed, and immunohistochemically stained as described (Gardberg *et al.*, 2010). As specificity controls, the sections were also stained with mouse and rabbit preimmune serum. Each specimen was used with the approval of the Hospital District of Southwest Finland Ethics Committee.

### Quantitative image analyses

To score the stainings, palladin and MT1-MMP positivity was evaluated from two random microscopic fields of each TNBC with the Leica DM LB bright-field microscope with an N Plan 20x/0.4 NA objective. Quantitative image analyses were performed using Anduril (Ovaska *et al.*, 2010). The colors of each image were categorized into four expected representative color classes: brown, light brown, blue, and white. Only the brown and light brown classes were considered for staining density measurement. The color classes were selected by pointing at seven example colors for each class. The MT1-MMP and palladin staining colors were selected separately. All of the 24 images within the same staining were subjugated to use the same class specification. The regions of interest (ROIs), tumor interiors, and perimeters were drawn on the images. The density within each ROI was measured only in the brown and light brown color classes. The mean densities at the interior and perimeter were compared using the paired two-sided Student's *t* test. The *p* values were corrected with the Bonferroni correction.

### Statistical analysis

All numerical values represent mean  $\pm$  SD or SEM. Statistical significance was determined using the Mann-Whitney or Student's *t* test.

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