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.

INTRODUCTION Metastasis of tumor cells to distant sites in the human body is the major cause of cancer mortality. One of the important mechanisms 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
Volume 25 September 1, 2014 Palladin-MT1-MMP axis in cell invasion I 2557

(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 Courtenide, 2011). The recruitment of the cytoskeletal protein contactin, the nonreceptor tyrosine kinase Src, and MT1-MMP is essential for the maturation of functional invadopodia (Poincloux et al., 2009; Murphy and Courtenide, 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 isofoms (Otey et al., 2009). Palladin functions as a dynamic scaffold protein by binding to actin and actin-associated proteins, such as α-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 (Myykanen 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, immunotagory 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 α-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, 2014; 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 communoprecipitation (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). 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-ΔC-, 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 ± 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-
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 α-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). Of interest, in Hs578T cells with strong stress fibers, the colocalization was most prominent at the edges, whereas the other cells displayed predominant colocalization at more central invadopodia-like structures (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 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 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.
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 α-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 (Nylund 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 contactin 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 contactin 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 ± 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 μm/cross section. Invasion of control cells (Mock) was set to 1 (mean ± 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
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 μM), PDGF (20 ng/μl), 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 μM; three collagen preparations/cell). Representative confocal micrographs show MT1-MMP in red.
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 syndrome protein (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.

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.

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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 Ig3-5 domains contain the actin-binding activity of palladin, the Ig3 domain being sufficient for the actin binding, whereas Ig4-5 domains bind cortical actin—associated ezrin (Mykkänen 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 domain, 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 Nandelstädth 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.

 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 α-actinin in membrane ruffles and FAs, the interaction identified here provides cells with a more dynamic mechanism for infodapodial 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
CAFs, 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.

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**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.ttk.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).
**Cell lines**

Human ZR75-1, MCF7, BT-474, T47D, MDA-MB-435, 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 WMB82 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 WMB82 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 41g 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), α-actinin, α-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, Temecula, CA), HA (Cell Signaling Technology, Danvers, MA), and N-cadherin (BD Transduction Laboratories, Franklin Lakes, NJ). Antibodies and reagents were generated by G418 (400 μg/ml; Calbiochem, Merck KGaA, Darmstadt, Germany) selection.

**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), and 715–772 (von Nandelstadh et al., 2009) in pHPl (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 ΔC (1–772) in pEGFP-C2 (Clontech; Gateva et al., 2014) was used for expression and to amplify palladin Ig4-5 fragment (primers: 5′-GGGAATTCCTAGCTACATCTGCTCTTG-3′ and 5′-CCGTCGACGTGGGTGTAAACGTCCAGCCG-3′). A second amplification (primers: 5′-GGGAGAAGTTGAAATGAGCGCTTGGGAATTTCTC-3′; ACCATGCTCCATCTGCTCTTG-3′ and 5′-GGGGGACACATTGTGACAAAGACGTGTTGATAGCAAGCCAGCCG-3′) introduced attB sites for Gateway cloning into pcDNA-DEST40 (V5-tag; Invitrogen) or pTO-HA-StrpIll-GW-FRT (HA tag; M. Varjosalo, University of Helsinki, Helsinki, Finland). The constructs were verified by sequencing. Human MT1-MMP, MT1-MMP ΔC, 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-RRHGGTPRRLRYCQRCSLLDKV (MT1-MMP C-terminus), -Ttds-QRKGAPRLYLCKKRLQEYW (MT2-MMP C-terminus), -Ttds-KRGGTPRHILYCKRSMQEYW (MT3-MMP C-terminus), and -Ttds-RQLSLPLDILKYRTGVHRR (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; Dharmaco, 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; isom1-200 kDa; UniProt ID Q8WX93), absent from the 90-kDa palladin isoform CDS (GGCAACGCUACAGUAUAUAdTdT, dTdTCCGGUUGAUGUGUCA-UUAU; Dharmaco, 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 40×/1.3 numerical aperture (NA) oil objective and with Plan-Apochromat 10×/0.45 NA objective or using an LSM 780 confocal microscope with Plan-Neofluor 40×, 1.3 NA objective. In addition, an Axiosmager.Z1 upright epifluorescence microscope with Apotome and Plan-Apochromat 40×/1.4 NA objective and an Axiosplan 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 × 10³ 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 sprots per colony or number of invading colonies was manually counted. Relative colony size (area) and length of sprots were quantified using ImageJ software from random epifiuorescence 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 Axiosmager.Z1 epifiuorescence microscope), and the cells that invaded >10 μM (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 20× 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 ± SD or SEM. Statistical significance was determined using the Mann–Whitney or Student’s t test.

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Palladin-MT1-MMP axis in cell invasion

Volume 25 September 1, 2014 Palladin-MT1-MMP axis in cell invasion

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