MT1-MMP targeting to endolysosomes is mediated by upregulation of flotillins


To cite this version:

Damien Planchon, Eduardo Rios Morris, Mallory Genest, Franck Comunale, Sophie Vacher, et al.. MT1-MMP targeting to endolysosomes is mediated by upregulation of flotillins. Journal of Cell Science, Company of Biologists, 2018, 131 (17), 10.1242/jcs.218925. hal-01871601
**MT1-MMP targeting to endolysosomes is mediated by upregulation of flotillins**

Damien Planchon¹, Eduardo Rios Moris⁴, Mallory Genest¹, Franck Comunale¹, Sophie Vacher², Ivan Bièche², Evgeny V. Denisov³,⁴, Lubov A. Tashireva³, Vladimir M. Perelmuter³, Stefan Linder⁵, Philippe Chavrier⁶, Stéphane Bodin¹,∗ and Cécile Gauthier-Rouvière¹,∗,‡

**ABSTRACT**

Tumor cell invasion and metastasis formation are the major cause of death in cancer patients. These processes rely on extracellular matrix (ECM) degradation mediated by organelles termed invadopodia, to which the transmembrane matrix metalloproteinase MT1-MMP (also known as MMP14) is delivered from its reservoir, the RAB7-containing endolysosomes. How MT1-MMP is targeted to endolysosomes remains to be elucidated. Flotillin-1 and -2 are upregulated in many invasive cancers. Here, we show that flotillin upregulation triggers a general mechanism, common to carcinoma and sarcoma, which promotes RAB5-dependent MT1-MMP endocytosis and its delivery to RAB7-positive endolysosomal reservoirs. Conversely, flotillin knockdown in invasive cancer cells greatly reduces MT1-MMP accumulation in endolysosomes, its subsequent exocytosis at invadopodia, ECM degradation and cell invasion. Our results demonstrate that flotillin upregulation is necessary and sufficient to promote epithelial and mesenchymal cancer cell invasion and ECM degradation by controlling MT1-MMP endocytosis and delivery to the endolysosomal recycling compartment.

**KEY WORDS:** Cancer cell invasion, Extracellular matrix degradation, Endolysosomal vesicular trafficking, Flotillin, MT1-MMP

**INTRODUCTION**

Metastasis formation, the major cause of death in patients with cancer, is a complex process that starts with the dissemination of cancer cells from the primary tumor. During this process, cancer cells interact with and remodel the extracellular matrix (ECM), notably through its degradation. Amongst all matrix metalloproteinases (MMPs), the transmembrane MT1-MMP (also known as MMP14) plays a pivotal role in tumor cell invasion. Once exposed on the cell surface, MT1-MMP can cleave multiple matrix components and also proteolytically activate other MMPs, thus constituting a master regulator of ECM degradation. Many studies demonstrate that tumor cells hijack MT1-MMP trafficking to increase its activity at degradation sites called invadopodia (reviewed in Castro-Castro et al., 2016; Frittoli et al., 2011; Poincloux et al., 2009). Data obtained using different carcinoma and sarcoma cell lines indicate that MT1-MMP endocytic/exocytic fluxes are crucial for its activity. Indeed, activated MT1-MMP has to be internalized and stored in intracellular compartments to be efficiently recycled to invadopodia. Particularly, in invasive cancer cells, a RAB7-positive endolysosomal compartment was identified as the MT1-MMP reservoir compartment, from which polarized trafficking of internalized MT1-MMP to invadopodia occurs (Chevalier et al., 2016; Hoshino et al., 2013; Macpherson et al., 2014; Monteiro et al., 2013; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012). The mechanisms of MT1-MMP-polarized exocytosis from the endolysosomal compartment to invadopodia have been recently identified. However, how MT1-MMP is localized from the plasma membrane (PM) to the endolysosomal reservoir compartment in tumor cells remains to be elucidated.

Flotillin-1 and flotillin-2 (hereafter referred to as ‘flotillins’) are two ubiquitous, highly conserved homologous proteins (Rivera-Milla et al., 2006). They preferentially assemble to form heterotetramers composed of two flotillin-1 and two flotillin-2 molecules localized at the membrane cytoplasmic face and associated with caveolin-independent membrane microdomains enriched in cholesterol and sphingolipids. Flotillin heterotetramers can assemble into large oligomers to form molecular scaffolds (Bickel et al., 1997; Bodin et al., 2014; Otto and Nichols, 2011). These properties confer to flotillins two major cellular roles: (1) at moderate physiological expression they promote or participate in membrane receptor clustering (Bodin et al., 2014; Guillaume et al., 2013), (2) when upregulated, they induce the formation of PM invaginations that are distinct from caveolae- and clathrin-coated pits (Frick et al., 2007; Glebov et al., 2006). Several cargo molecules, such as Niemann-Pick C1-like protein 1, CD59 (a glycosylphosphatidylinositol-anchor protein) and amyloid-β peptide precursor are endocytosed through a flotillin-dependent pathway (Ait-Slimane et al., 2009; Ge et al., 2011; Schneider et al., 2008). Recent studies reported that flotillin upregulation in many invasive carcinomas is associated with poor patient survival and high risk of metastasis formation. Upregulation of flotillin promotes cell invasion and metastasis formation, but the molecular mechanisms are not well understood (Berger et al., 2013; Cao et al., 2016; Koh et al., 2016; Pust et al., 2013).

We now demonstrate that flotillins are required for carcinoma and sarcoma cell invasion and that their upregulation, which occurs in many different solid tumor types, promotes cell invasion and ECM degradation through MT1-MMP. We show that flotillins are critical for MT1-MMP targeting to the endolysosomal compartment, which is the MT1-MMP reservoir in cancer cells before its delivery to invadopodia, the major ECM degradation sites. Flotillin upregulation induced RAB5-dependent MT1-MMP endocytosis from the PM to
this endolysosomal compartment, leading to its polarized secretion, thus favoring ECM degradation and invasion.

RESULTS

Upregulation of flotillins is associated with poor prognosis in patients with carcinoma or sarcoma and promotes cell invasiveness

In several carcinoma types, upregulation of flotillins is associated with poor prognosis (Liu et al., 2015a,b; Pust et al., 2013; Wang et al., 2013; Zhang et al., 2013; Zhu et al., 2013). Here, we confirmed that upregulation of flotillins was significantly associated with poor disease-free survival in a cohort of patients with breast cancer with known long-term outcome, and found that this was true also for sarcoma, using a cohort of patients with rhabdomyosarcoma (RMS) (Bièche et al., 1999; Williamson et al., 2010) (Fig. 1A,B).

Specifically, we found a trend toward poorer prognosis in flotillin-overexpressing breast tumors in the whole cohort of 527 breast tumors (Fig. S1A). This observation became significant when comparing in 415 samples the flotillin-1high, flotillin-2high with the flotillin-1low, flotillin-2low subgroups (Fig. 1A). In the 527 breast tumor samples, flotillin co-upregulation was significantly associated with the estrogen, progesterone and ERBB2 receptor status, molecular subtypes and KI67 expression (Table S1). FLOT1 and FLOT2 mRNA expression was positively correlated in breast tumors \( r(+0.498, P<10^{-7}) \) (Fig. S1B). Moreover, we confirmed flotillin-1 and -2 upregulation at the protein level in breast cancer cell lines (Lin et al., 2011; Wang et al., 2013), as previously reported in breast tumor specimens (Lin et al., 2011; Wang et al., 2013), and also in RMS-derived cell lines (Fig. S1C,D). Flotillin protein upregulation was more pronounced in the most invasive RMS cell lines; the alveolar RMS (ARMS) is more aggressive than embryonal RMS (ERMS) (Fig. S1D).

To investigate the role of flotillins in the invasive phenotype of both carcinoma and sarcoma cell lines, we used the invasive MDA-MB-231 (breast cancer) and Rh41 (ARMS) cell lines that exhibit high flotillin expression levels (Fig. S1C-E) (Lin et al., 2011; Wang et al., 2013). We generated stable MDA-MB-231 and Rh41 cell lines that express shRNAs against flotillin-2 (F2 shRNA) or luciferase (CTL shRNA, negative control). As expected, F2 shRNA expression led to a decrease in flotillin protein (Guillaume et al., 2013) (Fig. S1E). Using a three-dimensional (3D) spheroid cell invasion assay with fibrillar type I collagen, we then demonstrated that invasion of MDA-MB-231 and Rh41 F2 shRNA cells was significantly reduced compared with CTL shRNA cells, and that transfection of mCherry-tagged flotillin-2 rescued invasion (Fig. 1C,D). We next asked whether flotillin co-upregulation, as observed in tumors, was sufficient to promote invasive behavior. To this aim, we used non-invasive epithelial (MCF10A) and mesenchymal (C2C12) cell lines with low endogenous flotillin expression levels to generate stable cell lines (MCF10AF1F2 and C2C12F1F2), where flotillins are expressed at levels comparable to those observed in MDA-MB-231 and Rh41 cells (Fig. S1E). Flotillin upregulation increased the invasive potential of MCF10A and C2C12 cells in a 3D spheroid cell invasion assay compared with controls (expressing only mCherry; CTL) (Fig. 1E,F). To investigate the role of flotillins in vivo, we used a zebrafish xenograft model and quantified cell dissemination in the tail at day 4-5 after injection of Dil-labeled cells in the perivitelline cavity of fish embryos. Again, we found that compared with control cells, dissemination of MDA-MB-231 F2 shRNA cells was reduced (Fig. 1G), whereas that of C2C12F1F2 cells was increased compared with control cells (Fig. 1H). The number of cells at the time of injection (day 0) and at day 4-5 after injection was comparable in control and flotillin silenced/overexpressing cells (Fig. S2). These data show that flotillins, which are associated with poor prognosis, are required for and promote cancer cell invasion when upregulated.

Flotillin upregulation promotes cell invasion in a proteolytic MT1-MMP-dependent manner

Tumor invasion of collagen-rich ECM is often controlled by matrix metalloproteinases (MMPs) (Friedl and Wolf, 2010; Wolf et al., 2013). Here, we found that invasion of MFC10AF1F2 and C2C12F1F2 cells (3D spheroid cell invasion assay) was reduced by incubation with GM6001, a broad-spectrum MMP inhibitor (Fig. 1J). Then, to determine the role of MT1-MMP (MMP14), a key enzyme that degrades intact fibrillar collagen and is involved in tumor cell dissemination and cancer progression (Poincloux et al., 2009; Rowe and Weiss, 2009; Sabeh et al., 2004; Wolf et al., 2007), we analyzed whether flotillin upregulation was implicated in MT1-MMP-dependent gelatin degradation. Using an in situ matrix degradation assay, we showed that, as for cancer cell invasion (Fig. 1), flotillin knockdown in MDA-MB-231 and Rh41 cells decreased ECM degradation (Fig. 2A,B), whereas transfection of mCherry-tagged flotillin-2 rescued degradation. Conversely, flotillin upregulation in MCF10A and C2C12 cells increased matrix degradation (Fig. 2C,D), and this effect was impaired by MT1-MMP knockdown (Fig. 2F,G). Moreover, flotillin upregulation in NMuMG cells, which express very low levels of MT1-MMP (Fig. S3A), did not affect ECM degradation (Fig. 2E).

Finally, C2C12F1F2 cell dissemination in the zebrafish tail was impaired by knockdown of MT1-MMP expression (Fig. 2H).

However, the total protein and surface-bound levels of MT1-MMP were not affected by flotillin downregulation (F2 shRNA) and upregulation (F1F2) (Fig. S3A,B). Therefore, we assessed the impact of flotillin upregulation on MT1-MMP secretion because MT1-MMP exocytosis at invadopodia is important for local degradation of ECM at these sites in invasive tumor cells (Castro-Castro et al., 2016; Linder et al., 2011). To this aim, we used cells that express MT1-MMP-pHluorin, a protein that is fluorescent only at the extracellular pH of 7.4 (Monteiro et al., 2013), and total internal reflection fluorescence (TIRF) live-imaging to selectively illuminate the ventral PM. We found that flotillin expression level was correlated with MT1-MMP exocytosis. Specifically, the transient dot-like appearance of fluorescent MT1-MMP-pHfluorin spots at the ventral PM, reminiscent of exocytic events, was reduced in MDA-MB-231 F2 shRNA cells, and increased in MCF10AF1F2 cells. This effect of flotillin on MT1-MMP exocytosis was not correlated with major changes in the number of invadopodia (Fig. 2I,J). Similarly, we previously showed that flotillin knockdown in macrophages does not affect podosome numbers, but significantly impairs podosomal matrix degradation (Cornfine et al., 2011). Thus, (1) upregulation of flotillins mediates cancer cell invasion and ECM degradation, which strongly relies on MT1-MMP activity, and (2) the expression levels of flotillins influence MT1-MMP exocytosis.

In invasive cells, MT1-MMP endocytosis to the endolysosomal reservoir compartment requires flotillins

Tumor cells hijack MT1-MMP trafficking and develop a recycling circuitry via the endolysosomal compartment that is crucial for MT1-MMP activity (reviewed in Castro-Castro et al., 2016). In invasive MDA-MB-231 and Rh41 cells, we found that flotillin-1 and -2 always colocalized (Fig. S5B), and accumulated with MT1-MMP in RAB7- and CD63-positive vesicles (Fig. 3A and 3B)
Fig. 1. High flotillin-1 and -2 expression level is correlated with cancer cell invasiveness. (A,B) Kaplan-Meier curves showing metastasis-free survival and overall survival in 415 patients with breast cancer and 101 patients with rhabdomyosarcoma subdivided according to flotillin expression levels in the tumor (high versus low F1 and F2 mRNA levels; optimal cut-offs: F1>1.83 and F2>1.42 for high expression in breast tumors; high>median flotillin expression level in RMS; Spearman rank). (C-F) The invasive breast cancer MDA-MB-231 (C) and invasive RMS Rh41 (D) cell lines that express shRNAs against luciferase (CTL shRNA) or flotillin-2 (F2 shRNA), and the epithelial MCF10A (E) and mesenchymal C2C12 (F) cell lines in which flotillins are upregulated (F1F2) or not (CTL; empty vector) were used for 3D spheroid cell invasion assay. Images taken at the indicated times after embedding in type I collagen are representative of at least 20 spheroids per condition. The insets (top right) show spheroids at day 0 just after embedding. The invasive potential is represented as the mean±s.e.m. of four independent experiments (n=20 spheroids). The histogram also includes analysis of invasion in MDA-MB-231 and Rh41 F2 shRNA cells after rescue of flotillin-2 expression by transfection of a FLOT2-mCherry plasmid (F2mChry). Control cells were used as reference and set at 100%. (G,H) After injection in the zebrafish perivitelline cavity, dissemination of DiI-labeled MDA-MB-231 CTL shRNA and F2 shRNA cells (G) and C2C12 CTL and F1F2 cells (H) in the tail was monitored for 5 days. Confocal images taken at the indicated times after injection are representative of at least 30 injected embryos. Micro-tumor foci are indicated by arrows. Insets show the whole embryos and the yellow box the selected area shown at higher magnification. The histograms (number of disseminated foci per embryo) display the mean±s.e.m. of five independent experiments (n=30 fish). (I,J) 3D collagen invasion assay using MCF10A/F1F2 (I) or C2C12/F1F2 (J) cells incubated with vehicle alone (DMSO) or with GM6001. The histograms show the mean±s.e.m. of four independent experiments (n=20 spheroids). *P<0.05; ***P<0.001; ns, non-significant (Mann-Whitney test, two-tailed P-value). Scale bars: 150 μm (C-F), 100 μm (G,H).
Fig. S4A). We confirmed that these flotillin- and MT1-MMP-containing vesicles were late/multivesicular endosomes (LE/MVEs), which are described as the endolysosomal MT1-MMP reservoir compartment in cancer cells (Hoshino et al., 2013; Rosse et al., 2014). Indeed, they were positive for RAB7a, LAMP1 and CD63 but not for RAB4, RAB8, RAB11, CD9 and CD81 markers (Fig. 3A and Fig. S4A-D). Moreover, other MMPs such as MMP2, MMP7 and MMP9, did not colocalize with flotillin-2 in intracellular vesicles in MDA-MB-231 cells (Fig. S4E). In addition, confocal time-lapse and TIRF live-imaging analysis of MDA-MB-231 cells confirmed that flotillins and MT1-MMP colocalized in intracellular vesicles that emerge from the PM (Fig. 3B, see white arrow in Movie 1), and also in vesicles addressed to ECM degradation sites (invadopodia) (Fig. 3C and Fig. S4F, Movie 2). We also demonstrated that in MDA-MB-231 cells, MT1-MMP was exocytosed from flotillin-rich intracellular vesicles (Fig. 3D, Movie 3), that we characterized as LE/MVEs/endolysosomes (Fig. 3A and Fig. S4A-D). Patching of the ganglioside GM1 with the CTX-B subunit and using antibodies against CTX-B resulted in co-patching of flotillins and MT1-MMP, supporting the finding that flotillins and MT1-MMP are in the same PM microdomains (Fig. 3E). In addition to their colocalization at vesicles and at the PM in GM1-containing membrane
microdomains, flotillins and MT1-MMP were also co-immunoprecipitated from MDA-MB-231 cell extracts (Fig. 3F), confirming their close association.

As we observed flotillin and MT1-MMP emerging together from the PM into intracellular vesicles (Fig. 3B), we then investigated whether flotillin upregulation influenced MT1-MMP internalization and trafficking to the endolysosomal compartment in MDA-MB-231 cells using an MT1-MMP uptake assay (Remacle et al., 2003). The cell surface level of MT1-MMP was similar in MDA-MB-231 F2 shRNA and control shRNA cells (Fig. 4A,B and Fig. 4C).

Fig. 3. Flotillins colocalize with MT1-MMP from the PM to the endolysosomal compartment. (A) Confocal micrographs of MDA-MB-231 and Rh41 cells transfected with FLOT2-mCherry (F2-mChry) and GFP-tagged Rab7a or CD63 and incubated with an anti-MT1-MMP antibody to detect endogenous MT1-MMP. The zooms of boxed regions appear on the left side of each image. (B) Still images of a representative time-lapse series (see Movie 1) of one MDA-MB-231 cell that expresses MT1-MMP-GFP and F2-mChry to show F2/MT1-MMP colocalization at the PM (a), at endocytic vesicles forming at the PM (a; arrow) and in intracellular vesicles (b). (C) Still images of one MDA-MB-231 cell that expresses MT1-MMP-GFP and F2-mCherry on Alexa Fluor 633-conjugated fluorescent gelatin (see Movie 2). Kymograph analysis shows the fluorescence intensities in a line scan through the boxed area. MT1-MMP and flotillin-2-containing vesicles are delivered at the degradation area. (D) Spinning disk images of a representative movie (Movie 3) of one MDA-MB-231 cell that expresses MT1-MMP-pHluorin (MT1-ph) and F2-mCherry to visualize MT1-MMP exocytosis at F2-mCherry-positive vesicles (arrowhead). (E) GM1 molecules in MDA-MB-231 cells were labeled with Alexa Fluor 488-conjugated CTX-B subunit and then patched by addition of a secondary antibody. Endogenous MT1-MMP and flotillin-2 were labeled using specific antibodies. Merge images of GM1 and MT1-MMP or GM1 and flotillin-2 are shown. (F) Flotillin-1 was immunoprecipitated from MDA-MB-231 cell lysates. Co-immunoprecipitation of MT1-MMP and flotillin-2 was assessed by immunoblotting. Scale bars: 5 μm.
Conversely, MT1-MMP endocytosis was decreased in F2 shRNA cells (Fig. 4A,B). Specifically, MT1-MMP accumulation in endolysosomes was decreased in MDA-MB-231 F2 shRNA cells, without apparent modification of the RAB7 compartment (Fig. 4C). Moreover, in parental MDA-MB-231 cells, MT1-MMP was mainly endocytosed in flotillin-positive vesicles, with almost no colocalization with caveolin (Fig. 4D). We also observed some MT1-MMP-containing vesicles that colocalized with the clathrin adaptor protein 2 (AP2, a clathrin marker), as expected (Fig. 4D) (Remacle et al., 2003). Altogether, these observations strongly suggested that in invasive cancer cells, flotillin upregulation is required for MT1-MMP endocytic traffic from the PM towards endolysosomes.

**Flotillin upregulation is associated with its accumulation with MT1-MMP in non-degradative lysosomes**

As we observed that the cellular distribution of flotillins strongly differed according to their level of expression, we generated flotillin-knockout C2C12 and MCF10A cells using the CRISPR/Cas9 method to completely abolish their expression (Fig. S1G), and then re-expressed flotillins at low to moderate levels, which corresponds to the endogenous level in non-tumor cells (KO F1/F2 cells). In these cells, flotillins localized to the PM and in few intracellular vesicles (Fig. 5A). Upon re-expression at high levels, flotillins accumulated in intracellular vesicles, as observed in MDA-MB-231 and Rh41 invasive cancer cells where they are endogenously overexpressed (Fig. 5A,B). Flotillin-1 always fully colocalized with flotillin-2 either at the PM or in vesicles (Fig. S5A,B) that we characterized as endolysosomes (Fig. 3A, Fig. S4A-C and Fig. S5C-F). Similarly, analysis of flotillin distribution in invasive breast tumor samples (see Table S2 for description) revealed that flotillin-2 accumulated in intracellular vesicles in malignant cells, whereas it was mainly at the PM in normal cells (acini) (Fig. 5C and Table S2).

Using 3D structured illumination microscopy (3D-SIM), we demonstrated that flotillins and MT1-MMP are in the same PM domains (Fig. 5D). In C2C12F1F2 and MCF10A F1F2 cells, flotillins strongly colocalized with MT1-MMP intracellular vesicles (Fig. 5E), and trafficked together (see Movie 4 for C2C12F1F2 cells), as in invasive cancer cell lines. Moreover, immunohistochemistry analysis of breast tumor sections (Table S2) showed that flotillins and MT1-MMP colocalized in intracellular vesicles of malignant cells in invasive cancer specimens (shown for flotillin-2 in Fig. 5H). In agreement, MT1-MMP and flotillins were co-immunoprecipitated from MCF10A F1F2 cell extracts (Fig. 5F).

The perinuclear vesicles containing flotillins and MT1-MMP were positive for RAB7a and CD63, but not for RAB4, RAB5,
RAB8, RAB11, CD9 and CD81, showing that they co-accumulated in the endolysosomal compartment (Fig. 5G and Fig. S5C-F). Time-lapse analysis revealed that flotillin-rich endocytic vesicles forming at dynamic PM ruffles (red arrow in Movie 5) were rapidly enriched in RAB7, an LE marker (Fig. 5I, and green arrow in Movie 5). Moreover, RAB7 knockdown in MCF10AF1F2 cells led to a strong decrease in ECM degradation, indicating the functional requirement of this compartment for upregulated flotillin-induced ECM degradation (Fig. 5J).

It is known that proteins can be transported to endolysosomes not only for degradation but also for efficient targeting to the cell surface to drive cell invasion (Rainero and Norman, 2013). To exclude the
Fig. 5. Upregulated flotillins colocalize with MT1-MMP-containing endocytic carriers trafficking toward the endolysosomal compartment. (A) C2C12 or MCF10A cells in which flotillin-1 and -2 (F1 and F2) were knocked down were transfected with 1× (0.5 µg/ml=low expression) or 5× (2.5 µg/ml=high expression) F1-GFP and F2-mCherry expression vectors (KO F1/F2). They were then fixed and flotillin distribution analyzed by confocal microscopy. The inverted contrast image shows the F1-GFP signal. Insets illustrate flotillin-1 and -2 colocalization in the boxed areas. (B) Rh41 and MDA-MB-231 cells were incubated with anti-flotillin-1 and -2 antibodies. The inverted contrast image shows the F1-GFP signal. Insets illustrate flotillin-1 and -2 colocalization. (C) Distribution of flotillin-2 analyzed by immunohistochemistry in breast tumor sections. Flotillin-2 localized mainly to intracellular vesicles in invasive breast cancer specimens (arrows), and to the PM in the adjacent peritumoral breast epithelium tissue (normal acini). Magnification ×400. (D) Analysis of MT1-MMP and flotillin-2-mCherry at the PM in MCF10A/F1F2 cells using 3D-SIM. (E) Still images of a representative time-lapse series (see Movie 4) of one C2C12/F1F2 cell (GFP-tagged F2) that expresses MT1-MMP-mCherry to illustrate F2/MT1-MMP colocalization in intracellular vesicles. (F) Flotillin-1 was immunoprecipitated from MCF10A/F1F2 cell lysates and co-immunoprecipitation of MT1-MMP was assessed by immunoblotting. (G) Detection of CD63 in C2C12 or MCF10A cells that express F1-GFP, F2-GFP and MT1-MMP-mCherry. (H) Immunofluorescence analysis of cytokeratin-7, flotillin-2 and MT1-MMP expression in invasive breast tumor sections (Table S2). Nuclei were stained with Hoechst. Arrows indicate flotillin-2 and MT1-MMP colocalization in intracellular vesicles. (I) C2C12/F1F2 cells that express also RAB7A-GFP were monitored by time-lapse confocal imaging (Movie 5). Arrowheads indicate the position of the same vesicle on each frame. (J) FITC-gelatin degradation assay with MCF10A/F1F2 cells transfected with anti-RAB7A siRNAs. The histogram shows the mean±s.e.m. of four independent experiments (n=240 cells per condition). ***P<0.001 (Student’s t-test, two-tailed P-value). Western blot analysis confirmed the shRNA efficiency. Actin was used as loading control. (K) MDA-MB-231 CTL shRNA, C2C12/F1F2 and Rh41 CTL shRNA cells were incubated with DQ-BSA (20 µg/ml) for 4, 3 and 4 h, respectively. Cells were fixed, permeabilized and incubated with an anti-flotillin-1 antibody, as indicated. Images are representative of at least five independent experiments. Scale bars: 5 µm (A, C,G,I,K), 2 µm (D,E,H).

Flotillin upregulation is sufficient to promote MT1-MMP endocytosis from the PM to its reserve compartment

As flotillin downregulation in cancer cells affected MT1-MMP endocytosis and impaired trafficking towards endolysosomes, we next asked whether flotillin upregulation was sufficient to directly influence MT1-MMP endocytosis. Using the MT1-MMP uptake assay and analysis of MT1-MMP internalization by immunocytochemistry (Fig. 6A) or flow cytometry (Fig. 6B), we found that flotillin upregulation in MCF10A cells promoted MT1-MMP internalization and also its accumulation in endolysosomes (Fig. 6D). As observed in MDA-MB-231 cells, MT1-MMP colocalized with flotillins at the PM (Fig. 6C, top) and was endocytosed in flotillin-positive vesicles (Fig. 6C, bottom). These data showed that flotillin upregulation is sufficient to induce MT1-MMP endocytosis to endolysosomes, its reserve compartment.

Flotillin upregulation-mediated MT1-MMP endocytosis and endolysosomal accumulation requires RAB5

As some MT1-MMP-containing vesicles detected outside the perinuclear region were positive for flotillins and also for RAB5 (Fig. S4D), a Rab family protein involved in the control of the proteolytic invasive program (Frittoli et al., 2014), we analysed the role of RAB5 in flotillin upregulation-mediated MT1-MMP endocytosis. Time-lapse analysis of C2C12/F1F2 cells revealed that flotillin-rich endocytic vesicles forming at dynamic PM ruffles were RAB5-positive early endosomes that were fusing together (Fig. 7A and yellow arrow in Movie 6). Once fused and enlarged, flotillin-positive vesicles were morphologically similar to the RAB7-positive vesicles shown in Movie 5, and RAB5 was no longer detected (red arrow in Movie 6).

Moreover, RAB5 knockdown in MCF10A/F1F2 (ectopic flotillin upregulation) and MDA-MB-231 cells (endogenous flotillin overexpression) using specific siRNAs (Fig. 7B) strongly decreased MT1-MMP endocytosis (Fig. 7C) and its accumulation in the endolysosomal compartment (Fig. 7D), and also decreased ECM degradation (Fig. 7E). Altogether, these data show that RAB5 is required for flotillin-induced MT1-MMP endocytosis towards the endolysosomal compartment and for ECM degradation.

DISCUSSION

In this study, we identified an unprecedented role of flotillins in MT1-MMP trafficking. MT1-MMP is a crucial player in cancer cell invasion because this transmembrane metalloprotease, which can be found at the invasive tumor front, is involved in the breaching of the basement membrane by tumor cells and also in cell invasion through type-I collagen-rich interstitial tissues (Hofmann et al., 2003; Leong et al., 2014; Sabeh et al., 2004; Ueno et al., 1997). MT1-MMP activity is highly regulated, and the control of its intracellular trafficking in cancer cells is of central importance (Castro-Castro et al., 2016). Indeed, alteration of the endocytic machinery can induce cell transformation. Moreover, endocytosis and trafficking of many cargos are misregulated during cancer progression (Sigismund et al., 2012). In cancer cells, MT1-MMP is accumulated in RAB7-positive endolysosomes from which it is transported to invadopodia at the PM (Chevalier et al., 2016; Hoshino et al., 2013; Macpherson et al., 2014; Monteiro et al., 2013; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012). However, what drives its accumulation in this endosomal compartment was unknown. We report here that flotillin upregulation plays a major role in MT1-MMP trafficking from the PM to this non-degradative endolysosomal compartment. Flotillin-1 and -2 are upregulated in many invasive carcinoma types, such as melanoma, breast, nasopharyngeal, gastric, cervical, lung, renal and hepatocellular cancer, and this is considered a poor prognostic marker (Bodin et al., 2014; Hazarika et al., 2004; Li et al., 2014; Liu et al., 2015a; Wang et al., 2013; Yan et al., 2014; Zhang et al., 2013, 2014; Zhao et al., 2015; Zhu et al., 2013). We confirmed that flotillin expression is associated with poor prognosis in a large cohort of patients with breast cancer. Moreover, we provided the first demonstration of this correlation also in RMS, a sarcoma of skeletal muscle origin. The only previous study on sarcomas revealed increased FLOT1 mRNA expression in synovial sarcoma, fibrous histiocytoma and malignant schwannoma (Arkhipova et al., 2014). Using RMS cell lines, we demonstrated higher flotillin-1 and -2 expression in the cell lines derived from ARMS (the most invasive type) than in those derived from ERMS. Altogether, these data suggest that flotillin co-upregulation could be a general mechanism during epithelial and mesenchymal cell invasion.
transformation. We then demonstrated for the first time that upregulation of flotillin-1 and -2 is necessary and sufficient to promote breast and RMS cancer cell invasion in 3D collagen matrices and also in the zebrafish tail. This is in agreement with data obtained in a well-established mouse model of mammary tumorigenesis and metastasis showing the crucial role of flotillins in metastasis formation (Berger et al., 2013).

Moreover, flotillin upregulation promoted ECM degradation through the regulation of MT1-MMP endocytosis, an essential process during tissue invasion by tumor cells (Hotary et al., 2006; Leong et al., 2014; Lodillinsky et al., 2016; Rowe and Weiss, 2009). Flotillin localization in cells depends on their expression levels. In invasive cancer cells, where they are upregulated, they accumulate in endolysosomes with MT1-MMP. Progression of carcinoma in situ to invasive carcinoma is associated with flotillin translocation from the PM to intracellular vesicles, where they colocalize with MT1-MMP. We then confirmed that this endolysosomal compartment allows preferentially MT1-MMP recycling and not its degradation, thus acting as the reservoir compartment for this metalloprotease in invasive cancer cells (see model in Fig. 8) as previously suggested (Hoshino et al., 2013; Monteiro et al., 2013). We also found that flotillin upregulation allowed the formation of endocytic carriers that contain flotillins and MT1-MMP and that address MT1-MMP to its storage compartment. This is consistent with earlier studies showing that flotillins co-assemble to form membrane microdomains that can induce the formation of GM1-containing PM invaginations and of intracellular vesicles (Frick et al., 2007; Glebov et al., 2006; Lang et al., 1998). Using 3D-SIM, we found that flotillins and MT1-MMP localize in the same PM microdomains, thus explaining MT1-MMP endocytosis into flotillin-rich PM invaginations. Moreover, GM1 patching with the CTX-B subunit resulted in co-patching of flotillins and MT1-MMP, again supporting the finding that flotillins and MT1-MMP are in the same PM microdomains (Fig. 3E). It was previously demonstrated that these cholesterol-rich PM microdomains contain MT1-MMP (Annabi et al., 2001; Galvez, 2003). It was proposed that in HT1080 fibrosarcoma cells, MT1-MMP endocytosis is controlled by several clathrin-dependent and -independent endocytic pathways (Baldassare et al., 2015; Remacle et al., 2003). Although caveolae might interfere with MT1-MMP-dependent ECM degradation in MDA-MB-231 cells (Yamaguchi et al., 2009), their direct role in MT1-MMP uptake is unlikely, and they might be involved in membrane and invadopodia organization and in sensing the mechanical stress encountered by tumor cells during invasion (Parton and del Pozo, 2013; Yamaguchi et al., 2009). Indeed, we never observed caveolin and MT1-MMP colocalization in MDA-MB-231 cells during MT1-MMP endocytosis from the PM to endolysosomes. Conversely, we demonstrated that flotillin upregulation-induced endocytosis is the major clathrin-independent pathway for MT1-MMP internalization from the cell surface to the endolysosomal compartment.

Some members of the Rab family of small GTPases, key regulators of membrane trafficking, are involved in the regulation of MT1-MMP

---

**Fig. 6. Upregulated flotillins promote the formation of MT1-MMP-containing endocytic carriers and their movement to the endolysosomal compartment.** (A,B) MCF10A CTL and F1F2 cells were incubated with an anti-MT1-MMP antibody at 4°C followed by incubation at 37°C for 5 min. Cells were fixed to allow MT1-MMP detection by immunocytochemistry (A) or by FACS (B) (n=50,000 events per cell line). The histogram shows the mean±s.e.m. of five independent experiments. **P<0.01 (Student’s t-test, two-tailed P-value). (C) Confocal micrographs of MCF10A F1F2 cells processed as in A showing flotillin-2 and MT1-MMP colocalization at the PM and in endocytic vesicles. (D) MCF10A CTL and F1F2 cells were processed as in A, fixed for 15 min after incubation at 37°C and incubated with anti-MT1-MMP and anti-RAB7 antibodies. Images are representative of at least five independent experiments. The histogram shows the mean±s.e.m. of four independent experiments (n=20 cells). ***P<0.001 (Mann-Whitney test, two-tailed P-value). Scale bars: 5 μm.
endo-exocytic recycling pathways (Bravo-Cordero et al., 2007; Frittoli et al., 2014; Kajiho et al., 2016; Macpherson et al., 2014; Remacle et al., 2003; Steffen et al., 2008; Williams and Coppolino, 2011). Our data highlight a role for upregulated flotillins in MT1-MMP endocytosis from early to endolysosomes before PM delivery at degradation sites, which corresponds to the main trafficking pathway in cancer cells. Consistently, we demonstrated that flotillin upregulation-induced matrix degradation in normal cells is abrogated upon knockdown of RAB5 or RAB7, two Rab proteins with major function during tumorigenesis (Alonso-Curbelo et al., 2014; Frittoli et al., 2014). In invasive MDA-MB-231 cancer cells, in which flotillins are upregulated, RAB5 knockdown impaired matrix

Fig. 7. See next page for legend.
Fig. 7. MT1-MMP endocytosis promoted by flotillin upregulation is dependent on RabB5. (A) C2C12F1F2 myoblasts were transfected with GFP-RAB5 and monitored by time-lapse confocal imaging (Movie 6). Arrowheads indicate the position of the same vesicle on each frame. (B) MCF10AF1F2 and MDA-MB-231 cells were transfected with siRNAs against luciferase (CTL siRNA) or RAB5a/b/c and luciferase efficiency assessed by RT-qPCR. The histogram shows the mean±s.e.m. of four independent experiments, **P<0.01, ***P<0.001 determined with the Mann-Whitney test, two-tailed P-values (B-D) and the Student’s t-test, two-tailed P-values (E). Scale bars: 5 µm (insets in A, 2 µm).

degradation, as previously shown upon RabB7 knockdown (Kajihc et al., 2016).

Flotillin-mediated MT1-MMP targeting to endolysosomal compartments is crucial for its subsequent efficient recycling and exocytosis at degradation sites from this storage compartment. This endolysosomal recycling route has emerged as a key process in the delivery at the cell surface of cargos such as integrins or their ligands, to regulate cell migration and tumorigenesis (Dozynekiewicz et al., 2012; Rainero and Norman, 2013; Sung et al., 2015). This flotillin-containing endolysosomal compartment participates in MT1-MMP recycling, but not in its degradation. Indeed, the flotillin- and MT1-MMP-containing vesicles were not associated with major proteolytic activity, measured with the DQ-BSA probe, and MT1-MMP protein level was not modified in cells where flotillins were knocked down or upregulated. This shows that MT1-MMP is sorted in this recycling non-degradative endolysosomal compartment, named LE/MVEs by Hoshino et al. (2013) and Rosse et al. (2014), to allow its exocytosis. This is in agreement with data showing MT1-MMP-polarized trafficking from the RabB7-positive endolysosomal vesicles to sites of cell-matrix adhesion (Macpherson et al., 2014; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012), and with the release of internalized MT1-MMP in the extracellular space (Uekita et al., 2001). The absence of a detectable change in MT1-MMP cell surface level upon flotillin upregulation or knockdown suggests the endocytosis of a small, but crucial fraction of MT1-MMP and/or the existence of a very fast recycling process. This trafficking process allows the efficient concentration of MT1-MMP from its homogenous PM distribution to degradation sites, thus promoting its activity. This is achieved by MT1-MMP targeting from the PM to endolysosomes that allows its polarized delivery and concentration at degradation sites. This study provides evidence for a crucial gain-of-function role of flotillins, which are upregulated in many carcinomas and sarcomas, during cancer cell invasion and ECM degradation and reveals a new opportunity for diagnostic and/or therapeutic interventions.

MATERIALS AND METHODS

Cell lines

All cell lines were authenticated and tested for contamination. The MCF10A non-tumorigenic mammary epithelial cell line (American Type Culture Collection, ATCC CRL-10317) was grown in DMEM/HAM F-12 medium supplemented with 5% horse serum (Gibco, Thermo), 10 µg/ml insulin, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone and 100 ng/ml cholera toxin. C2C12 mouse myoblasts (ATCC CRL-1772) and Rhb1 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) (Biowest, Eurobio). The NMuMG mouse mammary epithelial cell line (ATCC CRL-1636) was grown in DMEM supplemented with 10% FCS (Biowest, Eurobio) and 10 µg/ml insulin. The human breast adenocarcinoma cell line MDA-MB-231 (ATCC HTB-26) and all ERMS and ARMS cell lines (Thuault et al., 2013) were grown in DMEM with 10% FCS (Biowest, Eurobio). Retrovirus production in Phoenix cells (Garry Nolan, Stanford University, CA, USA), infection and selection were performed as described (Fortier et al., 2008). Cells were grown continuously in 1 µg/ml puromycin and 200 µg/ml hygromycin.

Plasmid constructs

The retroviral plasmid containing flotillin-2 tagged with mCherry was made by subcloning the human FLOT2 sequence in the pBabe-puro-mCherry vector using the XhoI and EcoRI restriction sites. The retroviral plasmid encoding HA-tagged flotillin-1 was obtained by subcloning the FLOT1-HA sequence from the previously described pHCM3-Flot1-HA plasmid (Guillaume et al., 2013) into pMSCV-hygro between the XhoI and Hpal sites. The retroviral plasmid encoding GFP-tagged flotillin-2 was made by subcloning the FLOT2-GFP sequence from the previously used pEGF-P-NF1-Flot2 construct (Guillaume et al., 2013) into pMSCV-puro between the Ncol and NotI sites. The pBabe-puro-mCherry vector was obtained by subcloning mCherry from pcDNA3-NF1 into pBabe-puro between the AgeI and BglII sites. The pEGF-P-NF1-MT1-MMP plasmid was a gift from Maria Montoya (CNIC, Madrid, Spain). The pcDNA3.1 MT1-MMP-mCherry and MT1-MMP-pHbluorin plasmids were gifts from Philippe Chavrier (Curie Institute, Paris, France) (Rosse et al., 2014). MMP2-GFP was a gift from Santiago Rivera (Université de Marseille, France). The pcDNA3-MMP7-GFP plasmid was purchased from Addgene, and MMP9-GFP was a gift from Rene Harrison (University of Toronto, Canada). GFP-RAB8 was a gift from Mariane Martin (Université de Montpellier, France) (Chevalier et al., 2016). The CD63-GFP encoding plasmid was a gift from Alissa Weaver (Vanderbilt University, Nashville, USA) (Sung et al., 2015).

RNA interference

The pSiRENA-RetroQ-Flot2-shRNA (targeting both human and marine Flot2) and pSiRENA-RetroQ-Luciferase shRNA vectors were previously described (Guillaume et al., 2013). For RNA-mediated interference experiments, cells were transfected using Interferin (Polyplus Transfection) with siRNA oligonucleotide duplexes (Eurogentec, Belgium). The sequences of the siRNA duplexes are listed in Table S3. Luciferase shRNA was used as a control (Fortier et al., 2008).

CRISPR/Cas9 knockout of flotillin-1 and flotillin-2

The guide RNAs were cloned as previously described (Ran et al., 2013) into the pSpcas9(BB)-2A-GFP (PX458) vector (Addgene plasmid #48138 deposited by Feng Zhang; Ran et al., 2013). The guide RNA plasmids were transiently transfected into C2C12 or MCF10A cells to knockout mouse Flot1 and Flot2, and human FLOT1 and FLOT2.

Zebrafish tumor model

Zebrafish (Danio rerio) AB lines were raised and kept in standard laboratory conditions, according to the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). Animal studies were approved by the Direction Sanitaire et Vétérinaire de l’Hérault and the Comité d’Ethique for l’Expérimentation Animale under reference B-24-172-39. At 24 h post-fertilization, fish embryos were incubated with water containing phenylthiourea to prevent pigmentation. At 48 h post-fertilization, zebrafish embryos were dechorionated and anesthetized with 0.04 mg/ml tricaine (Sigma). 500 Dil-labeled tumor cells were injected into the perivitelline space using an Eppendorf microinjector. After injection, embryos were transferred into housing water, and tumor cell invasion was monitored using a fluorescent microscope (Leica SPS) for 5 days. Cell invasion was measured by automated
(48x56)(30 µM) was added to the coating, the embedding solution and the medium. Spheroids were prepared as described (Thuault et al., 2013). GM6001 3D spheroid cell invasion assays

Immunofluorescence and image acquisition section. Secondary antibody. Samples were then processed as described in the primary antibody molecules were detected using the appropriate fluorescent points. Cells were then fixed and permeabilized, and the internalized by incubating cells at 37°C in serum-free medium for the indicated time 10 min incubation at 4°C in serum-free medium, cells were incubated at 4°C 70% confluence. They were then washed twice with PBS at 4°C. After 10 min incubation at 37°C in serum-free medium before use. Cells were plated on fluorescent matrix was then quenched with 5 mg/ml sodium borohydride and rehydrated Oregon Green 488-conjugated gelatin (Life Technologies). The gelatin glutaraldehyde, and then inverted on a 20 µl drop of 10:1 mixture of gelatin/layered on top of the collagen. After embedding, spheroids were monitored by time-lapse imaging, and phase-contrast photographs were taken over 5 days. The invaded area was measured using ImageJ (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Phase_Contrast_Cell_Analysis_Tool_%28Trainable_WEKA_Segmentation%29). Control conditions were set at 100%. Data are the mean±s.e.m. of at least four independent experiments in which at least five spheroids were embedded per experimental condition.

Degradation assays
Coverslips were coated with 50 µg ml−1 poly-D-lysine followed by 0.5% glutaraldehyde, and then inverted on a 20 µl drop of 10:1 mixture of gelatin/Oregon Green 488-conjugated gelatin (Life Technologies). The gelatin matrix was then quenched with 5 mg/ml sodium borohydride and rehydrated in complete growth medium before use. Cells were plated on fluorescent gelatin for 12 h (MDA-MB-231 and Rh41 cells) or 24 h (C2C12 and MCF10A cells), and then fixed in 3.2% paraformaldehyde (in phosphate-buffered saline, PBS) for 15 min, followed by a 2 min permeabilization with 0.1% Triton X-100 in PBS and saturation with 2% BSA (in PBS). For CD9, CD63 and CD81 detection, cells were permeabilized using 0.1% saponin. Cells were incubated with primary and secondary antibodies in PBS containing 2% BSA. Ganglioside GM1 patching was performed as described (Causeret et al., 2005) using cholera toxin subunit-β conjugated to Alexa Fluor 488 (1:1000, Thermofisher, C34735) and an anti-cholera toxin subunit-β antibody (1:1000, Abcam, ab34992).

Confocal images were acquired using a Confocal Leica SP5-SMD microscope and a Leica 63×/1.4 oil HCX PL APO CS objective controlled using the Leica LAS AF software. Co-localization data originated from multichannel fluorescence stacks collected using a Leica SP5 microscope. For each stack, a single value of the Pearson’s coefficient (ranging between −1 and 1) quantification of the number of Dil-labeled cells in the zebrafish tail using ImageJ. Embryos were dissociated and fluorescent cells counted by FACS.

Antibodies and reagents
Mouse antibodies used were against actin (1:5000, A5441, Sigma), flotillin-1 (1:1000, 610820, BD Biosciences), flotillin-2 (1:100, 610383, BD Biosciences), MT1-MMP (1:400, 3328, Millipore), Rab7 (1:400, 50533, Abcam), LAMP1 (1:500, 555798, BD Biosciences), caveolin 2 (1:100, 610684, BD Biosciences), AP2 (1:100, 610501, BD Biosciences), ER (IR657 clone 1D5, ready-to-use, Dako), PR (IR068 clone PgR636, ready-to-use, Dako), Ki-67 (M306 clone SP6, ready-to-use, Spring Bioscience), CD63 (1:50, clone R5G2, MBL), CD81 (1:50, D131-4, clone R5G2, MBL) and CD9 (1:50, D131-3, MBL).

Rabbit antibodies against MT1-MMP (1:1000 WB, 1:50 Uptake, ab51074, Abcam), flotillin-1 (1:100 IF, 1:50 IHC, F1180, Sigma), flotillin-2 (1:100 IF, 1:1000 WB, 3436, Cell Signaling Technology), Rab7 (1:1000, 9367, Cell Signaling Technology), Tks5 (1:100, sc30122, Santa Cruz), HER2 (1:800, A0485, Dako), HA (1:100, 71-5500, Thermo), HER2 (1:800, A0485, Dako) and actin (1:5000, A5441, Sigma) were also used. Alexa Fluor 350-, 405-, 488-, 546-, 633- and Dylight 680/800-conjugated secondary antibodies were from Thermo Scientific. Alexa Fluor 350-conjugated phallolidin (A-22281), DQ-Green BSA (D12050) and Cell Tracker CM-Dil Dye (C7000) were from Thermo Scientific and GM6001 conjugated phalloidin (A-2066, Sigma) were also used. Mouse antibodies used were against actin (1:5000, A5441, Sigma), flotillin-1 (1:1000, 610820, BD Biosciences), flotillin-2 (1:100, 610383, BD Biosciences), MT1-MMP (1:400, 3328, Millipore), Rab7 (1:400, 50533, Abcam), LAMP1 (1:500, 555798, BD Biosciences), caveolin 2 (1:100, 610684, BD Biosciences), AP2 (1:100, 610501, BD Biosciences), ER (IR657 clone 1D5, ready-to-use, Dako), PR (IR068 clone PgR636, ready-to-use, Dako), Ki-67 (M306 clone SP6, ready-to-use, Spring Bioscience), CD63 (1:50, clone R5G2, MBL), CD81 (1:50, D131-4, clone R5G2, MBL) and CD9 (1:50, D131-3, MBL).

Fig. 8. Model for the role of flotillins in MT1-MMP endolysosomal recycling. Flotillin-1 and -2 upregulation (in invasive tumor cells or after ectopic expression) promotes the formation of MT1-MMP-containing RAB5-positive early endosomes that fuse together and quickly undergo early to late endosomal maturation and become enriched in RabR7. This MT1-MMP-containing endolysosomal compartment promoted by flotillin upregulation does not show major degradation activity and corresponds to the MT1-MMP reservoir compartment from which MT1-MMP is subsequently efficiently recycled and exocytosed at degradation sites.
1) was measured at the cell-cell junction imposing a threshold value calculated based on the algorithms by Costes et al. (2004) for green and red channels using the ‘co-localization analysis’ section of Imaris (Bitplane). For 3D-SIM, cells were treated and imaged as described (Guillaume et al., 2013).

**Time-lapse imaging**

Images were acquired using a confocal microscope (Confoa Leica SP5–SMD) equipped with thermal, CO2 and humidity controllers and a Leica 63×/1.4 oil HCX PL APO CS objective and captured with a hybrid detector (Leica HyD) controlled using the Leica LAS AF software. Images were taken every 5 s.

**Immunoprecipitation**

The monoclonal anti-flotillin-1 (1 µg, BD Biosciences, 610820) antibody was incubated with Protein-G Sepharose beads at room temperature for 1 h. After washing, 750 µg of protein extract was added at 4°C for 2 h. Immunoprecipitates were analyzed by immunoblotting.

**Cell surface biotinylation**

The presence of MT1-MMP at the cell surface was analyzed as described previously (Charrasse et al., 2006). Quantification of at least three independent experiments was performed using the Odyssey system (LI-COR Biosciences).

**Microarray analysis**

The described HG-U133plus2.0 Affymetrix microarray data for RMS tumor samples were used for gene expression analyses (Williamson et al., 2010). Low and high expression were lower and higher than the median expression level, respectively.

**RT-qPCR analysis of breast tumor samples**

Flotillin mRNA level in tumor samples from the cohort of patients with breast cancer were analyzed as described (Rosse et al., 2014). Values for FLOT1 >1.83 and FLOT2 >1.42 were considered to represent high mRNA expression levels in tumor samples.

**Patients and specimens**

Patients (n=10) with breast carcinoma treated at the Cancer Research Institute, Tomsk NRMC (Tomsk, Russia) were included (Table S2). The histological type was defined according to the World Health Organization recommendations (Lakhani et al., 2012). Tumor grade was determined using the Bloom and Richardson system (Bloom and Richardson, 1957). Estrogen receptor (ER) and progesterone receptor (PR) immunostaining were scored using the HSCORE method (Kinsel et al., 1989). HER2 expression was analyzed by immunohistochemistry and calculated on a scale 0–3+, according to the ASCO/CAP guidelines (Wolff et al., 2007). Ki-67 expression was calculated as the percentage of Ki-67-positive cells relative to all cells. Molecular subtypes were categorized using the ER, PR, HER2 and Ki-67 status of the primary tumor according to the St Gallen recommendations (Coates et al., 2015): luminal A (ER⁺ and/or PR⁺, HER2⁻ and Ki-67<20%), luminal B (ER⁺ and/or PR⁺, HER2⁻/c and Ki-67≥20%), HER2⁺ (ER⁻ and PR⁻, HER2⁺), and triple-negative (ER⁻, PR⁻, HER2⁻).

Frozen and formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens obtained during surgery were used for immunohistochemical and immunofluorescence analyses. The procedures followed in this study were in accordance with the Helsinki Declaration (1964, amended in 1975 and 1983). This study was approved by the institutional review board; all patients signed an informed consent for voluntary participation.

Clinicopathological characteristics were noted of the patients who gave the breast cancer samples used for the expression analysis in Table S2. #7425: 55-year-old woman, multifocal invasive carcinoma (right side), T2N1M0 (T1N0M0 after surgery), grade 2, ER+ (8), PR+ (7), HER2 (2+), Ki-67-positive cells (40%), luminal B. Six courses of neoadjuvant chemotherapy, effect – intermediate residual disease (RCB-II). Lympm node-negative (0 metastasis in 12 lymph nodes); #20265: 47-year-old woman, invasive carcinoma (right side), T2N1M0, grade 2, ER+ (7), PR+ (7), HER2 (2+), Ki-67-positive cells (25%), luminal B. No neoadjuvant chemotherapy. Lympm node-positive (14 metastases in 21 lymph nodes); #4384: 50-year-old woman, multifocal invasive carcinoma (right side) with micropapillary component, T2N1M0, grade 2, ER+ (7), PR+ (7), HER2 (1+), Ki-67-positive cells (45%), luminal B. Two courses of neoadjuvant chemotherapy without effect (extensive residual disease, RCB-III).

**Immunohistochemical staining of tumor samples**

Immunohistochemical staining was used for the standard scorings (ER, PR, HER2 and Ki-67) and to assess flotillin-1 and -2, MT1-MMP and cytokeratin 7 expression. Primary mouse antibodies were against ER (AbD067, clone 1D5, ready-to-use, Dako, Denmark), PR (IR068, clone PgR636, ready-to-use, Dako, Denmark), Ki-67 (M306, clone SP6, ready-to-use, Spring Bioscience). Primary rabbit antibodies against HER2 (A0485, 1:800, Dako, Denmark), flotillin-1 (F1180, 1:50, Sigma), and flotillin-2 (3436, 1:100, Cell Signaling Technology) were also used. Immunohistochemical staining was performed as previously described (Zavyalova et al., 2013).

**Immunofluorescence staining of tumor samples**

Seven-nm-thick FFPE tumor sections were deparaffinized, rehydrated, processed for heat-induced epitope retrieval in PT Link (Dako, Denmark) with EDTA buffer (pH 8.0), and blocked with 3% bovine serum albumin (Amresco, USA) in PBS. Sections were incubated with primary antibodies against cytokeratin 7 (CK7, clone N-20, 1:50, Santa Cruz), flotillin-1 (F1180, 1:100, Sigma), flotillin-2 (3436, 1:100, Cell Signaling Technology) and MT1-MMP (MAB3328, 1:400, Millipore). The following secondary antibodies were used: donkey anti-mouse Alexa Fluor 488 (Abcam, USA), donkey anti-rabbit Cy 3 (Abcam, USA), and donkey anti-goat Alexa Fluor 647 (Abcam, USA). Vectashield mounting medium (Vector Laboratories, USA) containing the DAPI nuclear staining was used to mount the sections. Samples were analyzed using a LSM 780 NLO confocal microscope (Carl Zeiss, Germany).

**Statistical analysis**

All statistical analyses were performed using SPSS version 22, and graphs were generated with Prism GraphPad version 5. All data were first tested for normality using the Kolmogorov-Smirnov normality test. For experiments with n>30, the Student’s t-test was used to identify significant differences between experimental conditions. For experiments with n<30, the non-parametric Mann-Whitney U-test was used. The n value and number of independent experiments are listed in the figure legends. At least three independent experiments were performed.

**Acknowledgements**

We thank Clément Chevalier, Christine Bénistant, Eric Rubinstein and Clothilde Théry for discussions, Marie Morille for Nanosight experiments, Nabla Elarouchi and Aurélien De Reynies from the Ligue Nationale contre le Cancer (Carte d’identité des tumeurs) for transcriptomic analysis and Anaïs Bellanger for model drawing. We acknowledge Virginie Georget and Sylvain de Rossi from the Montpellier Imaging Facility MIP, member of the national infrastructure France-Biomaging infrastructure and the Tomsk Regional Common Use Center.

**Competing interests**

The authors declare no competing or financial interests.

Funding
This work was supported by the Institut National du Cancer (INCa, 9483) and the Russian Science Foundation (14-15-00318). Montpellier Imaging Facility is supported by the Agence Nationale de la Recherche (ANR-10-NBS-04, ‘Investments for the future’). The Torsmo-Reggio Calabria Common Use Center was supported by the Institut National de la Santé et de la Recherche Médicale and D.P. by the Fondation pour la Recherche Médicale.

Supplementary information
Supplementary information available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.218925.supplemental

References


