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### ▶ To cite this version:

Alessia Castagnino, Antonio Castro-Castro, Marie Irondelle, Alan Guichard, Catalina Lodillinsky, et al.. Coronin 1C promotes triple-negative breast cancer invasiveness through regulation of MT1-MMP traffic and invadopodia function. Oncogene, 2018, 37 (50), pp.6425-6441. 10.1038/s41388-018-0422-x . hal-02377262

### HAL Id: hal-02377262 https://hal.science/hal-02377262

Submitted on 23 Nov 2019

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# Coronin 1C promotes triple-negative breast cancer invasiveness through regulation of MT1-MMP traffic and invadopodia function

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36	Conflict of interest disclosure statement: Authors have no conflict of interest to
37	declare.
38	
39	Running title: Coronin 1C regulates breast cancer invasion
40	Keywords: Coronin 1C, MT1-MMP traffic, invadopodia, breast cancer, invasion
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#### 44 Abstract

45 Membrane type 1-matrix metalloproteinase (MT1-MMP), a membrane-tethered 46 protease, is key for matrix breakdown during cancer invasion and metastasis. 47 Assembly of branched actin networks by the Arp2/3 complex is required for MT1-48 MMP traffic and formation of matrix-degradative invadopodia. Contrasting with the 49 well-established role of actin filament branching factor cortactin in invadopodia 50 function during cancer cell invasion, the contribution of coronin-family debranching 51 factors to invadopodia-based matrix remodeling is not known. Here, we investigated 52 the contribution of coronin 1C to the invasive potential of breast cancer cells. We 53 report that expression of coronin 1C is elevated in invasive human breast cancers, 54 correlates positively with MT1-MMP expression in relation with increased metastatic 55 risk and is a new independent prognostic factor in breast cancer. We provide 56 evidence that, akin to cortactin, coronin 1C is required for invadopodia formation and 57 matrix degradation by breast cancer cells lines and for 3D collagen invasion by 58 multicellular spheroids. Using intravital imaging of orthotopic human breast tumor 59 xenografts, we find that coronin 1C accumulates in structures forming in association 60 with collagen fibrils in the tumor microenvironment. Moreover, we establish the role of coronin 1C in the regulation of positioning and trafficking of MT1-MMP-positive 61 62 endolysosomes. These results identify coronin 1C as a novel player of the multi-63 faceted mechanism responsible for invadopodia formation, MT1-MMP surface 64 exposure and invasiveness in breast cancer cells.

#### 65 Introduction

66 Tumor cell escape and dissemination to distant sites are hallmarks of metastasis, the 67 leading cause of cancer-related death. During breast cancer progression, a key step is the transition from ductal carcinoma in situ (DCIS) - a mass of proliferative cells 68 inside the mammary duct surrounded by an intact myoepithelium and by the 69 70 basement membrane (BM) - to invasive breast cancers. DCIS-to-invasive breast cancer transition is associated with breakdown of the myoepithelium and the 71 72 underlying BM and increased metastasis risk. The capacity of cancer cells to remodel 73 extracellular matrix (ECM) barriers is essential for cancer progression and metastasis 74 <sup>1</sup>. Proteolytic remodeling of ECM components by cancer cells mobilizes a group of 75 transmembrane matrix metalloproteinases (MMPs), which includes membrane type 1 76 (MT1)-MMP (aka MMP14)<sup>1</sup>. We recently reported that MT1-MMP is up-regulated in 77 invasive hormone receptor- and epidermal growth factor receptor 2 (HER2)-negative 78 triple-negative breast cancers (TNBCs) as compared to DCIS lesions and normal 79 breast tissue and overexpression predicts the invasive potential of cancerous lesions 2. 80

Experimental data based on cancer cell models revealed that ECM remodeling by 81 carcinomatous cells is focused to the pericellular zone <sup>3, 4</sup>. Matrix gaps and tunnels 82 83 forming overtime support infiltrating cell passage through the BM and through the fibrous collagen microenvironment<sup>3</sup>. ECM breakdown is mediated by specialized 84 85 cellular structures of metastatic cells called invadopodia, which form at the plasma 86 membrane in association with ECM fibrils and require actin assembly for their formation <sup>5</sup>. Invadopodia are the sites of surface exposure and accumulation of MT1-87 MMP <sup>6-8</sup>. Due to difficulties inherent to the submicrometric size and transient nature 88 of invadopodia, fewer studies addressed their formation, dynamics and structure in 89

*vivo.* However, recent work based on emerging intravital microscopy technics,
 reported the existence of invadopodia-like protrusions in relation with matrix
 remodeling and metastasis in the natural tumor microenvironment <sup>9, 10</sup>.

93 Polymerization of invadopodial actin is a multistep process that requires activation of the Arp2/3 complex by nucleation promoting factor (NPF) Wiskott-Aldrich syndrome 94 95 like protein (N-WASP)<sup>11</sup>. Regulators of branched actin network dynamics including cortactin (CTTN) and cofilin are likewise essential for invadopodia formation <sup>6, 12</sup>. The 96 97 actin-binding protein CTTN promotes actin network assembly by facilitating N-WASP displacement from Arp2/3 complex and stabilizing Arp2/3 complex at branches <sup>13, 14</sup>. 98 99 Moreover, CTTN promotes branch stabilization by antagonizing members of the 100 WD40 domain-containing F-actin binding coronin family members, which control debranching and Arp2/3-nucleated filament network disassembly <sup>15-17</sup>. Additionally, 101 coronins bind to and inhibit nucleation by the Arp2/3 complex <sup>15, 18, 19</sup>. All together, 102 these data point to some coordinated functions of CTTN and coronin in the regulation 103 104 of branched actin network assembly and dynamics.

105 In carcinoma cells, altered phosphorylation and up-regulation of CTTN are associated with increased invadopodia density, promoting invasion and tumor 106 aggressiveness <sup>12, 20-24</sup>. Similarly, the coronin-family protein coronin 1C (CORO1C 107 108 aka coronin-3, CRN2) is up-regulated in human cancers including gastric cancers, 109 hepatocellular carcinomas and brain tumors and is correlated with increased invasiveness and metastasis <sup>24-28</sup>. In addition, experimental data have shown that 110 111 CORO1C is required for degradation of gelatin as a matrix mimic and for formation of invasive protrusions by human glioblastoma cells <sup>28, 29</sup>. 112

113 Convergent observations in carcinoma cell lines have identified a pathway involving

late endosome (LE)/lysosome compartments in returning internalized MT1-MMP to 114 invadopodia forming at plasma membrane/ECM contact sites <sup>7, 8, 23, 30-32</sup>. Docking of 115 MT1-MMP-positive LE/lysosomes to the invadopodial plasma membrane allows 116 117 surface exposure of the protease possibly through the formation of tubular connections between the limiting membrane of LE/lysosomes and the plasma 118 membrane<sup>8, 32, 33</sup>. The pathological relevance of this recycling circuitry has been 119 recently established in the context of the invasion program of TNBCs<sup>23, 31, 32</sup>. A 120 121 characteristic feature of this mechanism is the presence of F-actin/CTTN-enriched 122 puncta on MT1-MMP-positive LE/lysosomes, which depend on the Arp2/3 complex and its activator WASH complex for their formation <sup>8, 23, 34, 35</sup>. Perturbation of WASH 123 function affects endolysosomal F-actin/CTTN puncta, interferes with the trafficking of 124 125 MT1-MMP vesicles to the invadopodial plasma membrane and inhibits MT1-MMP surface exposure and invasion<sup>8, 32</sup>. Interestingly, a study in macrophages has 126 provided evidence for a role of coronin 1A (CORO1A) in controlling endolysosomal 127 actin assembly in the context of cholesterol trafficking and clearance <sup>36</sup>. In addition, 128 129 coordinated functions of CTTN and coronin 1B (CORO1B aka coronin-2) have been 130 implicated in trafficking and plasma membrane docking of multivesicular LEs and 131 regulation of exosome secretion from these compartments in conjunction with the small GTPase Rab27a function <sup>37, 38</sup>. All together, these data suggest some 132 133 cooperative functions of CTTN and coronins in the regulation of endolysosomal actin 134 dynamics and trafficking and exocytosis of LE/lysosomal cargo proteins including 135 MT1-MMP.

Here we show that CORO1C is up-regulated in invasive breast cancers and correlates positively with MT1-MMP expression in TNBCs in relation with poor prognosis. Additionally, our results indicate that CORO1C plays a dual function in

- 139 MT1-MMP-dependent pericellular matrix degradation by regulating invadopodia
- 140 formation and by controlling the positioning of MT1-MMP storage endolysosomes.

141 **Results** 

142 **CORO1C** is associated with poor outcome in breast cancer. Expression of 143 CORO1A, -B and -C type I coronin family members and pro-invasive MT1-MMP was analyzed by immunoblotting in a panel of 30 breast cancer cell lines. These cell lines 144 145 were stratified in Normal-like, Luminal, TNBC and HER2<sup>+</sup> subtypes based on the expression patterns of estrogen and progesterone receptors (ER and PR) and HER2 146 amplification <sup>39</sup>. Expression of hematopoietic-specific CORO1A was detected in 147 148 Jurkat leukemic T-cells while levels were low to barely detectable in the breast 149 cancer cell lines (Fig. 1AB). In contrast, CORO1B expression was detected in most breast cancer cell lines irrespective of subtypes (Fig. 1A and C). Expression of 150 CORO1C was mostly restricted to TNBC cells similar to MT1-MMP expression 151 152 pattern (Fig. 1A and DE). Expression of CORO1B and CORO1C mRNAs was analyzed by quantitative RT-PCR in invasive breast cancers using a retrospective 153 154 cohort of 446 patients with long-term follow-up. It allowed us to examine if variations 155 in CORO1B and -C mRNA expression had a value for patient prognosis (Table S3). 156 We found that 12.1% of breast tumors overexpressed CORO1B mRNA (>3 relative to 157 normal tissues, not shown). Highest CORO1B expression levels were observed in 158 HER2 tumors and positively correlated with Ki-67 proliferation marker (Table S4). However, we did not see an impact of CORO1B expression on the metastasis-free 159 160 survival (MFS) of breast cancer patients (not shown).

161 Tumors expressing high levels of CORO1C showed more often ER and PR 162 negativity, correlated with high Ki-67 and epidermal growth factor receptor (EGFR) 163 levels and were more frequently of an advanced grade indicative of an association 164 with bad prognosis (Table S5). Most importantly, CORO1C up-regulation was 165 associated with significantly shorter MFS (Fig. 1F). Multivariate analysis using a Cox

proportional hazard model revealed independent predictive value for MFS of lymph node status, tumor size and grade and CORO1C mRNA expression parameters, indicating that CORO1C expression is a new independent prognostic factor in breast cancer (Table S6, *P*=0.0029).

Based on available RT-gPCR data in the same cohort<sup>2</sup>, we found a strong positive 170 correlation of CORO1C and MT1-MMP mRNAs (Fig. 1G). Correlation of CORO1C 171 172 and MT1-MMP expression was similarly observed at the protein level by 173 immunoblotting analysis of a subset of tumor samples selected based on low and 174 high mRNA levels; while there was no obvious correlation with CORO1B or CTTN 175 expression in these tumor samples (Fig. S1AB). Importantly, we analyzed the 176 association of CORO1C and MT1-MMP transcript levels with clinical outcome and 177 found significantly increased metastatic risk in patients with both high MT1-MMP and CORO1C mRNA levels (Fig. 1H). The prognostic significance of 'MT1-MMP and 178 179 CORO1C expression level' persisted in Cox multivariate regression analysis (Table 180 S7, P=0.00007).

181 Next, we focused our study on CORO1C showing strongest correlation with proinvasive MT1-MMP and previously implicated in cancer progression and metastasis 182 <sup>25, 26, 28</sup>. Changes in CORO1C protein levels in epithelial cancer cells were 183 184 investigated by immunohistochemistry (IHC) analysis of a tissue microarray (TMA) of 185 invasive breast cancers and adjacent peritumoral tissues from an independent cohort 186 of 136 patients (Table S8). Specificity of CORO1C IHC staining was established by 187 analysis of human MCF10DCIS.com breast cancer-derived cells knocked down for 188 CORO1C expression (Fig. S1C). In peritumoral epithelial tissues, we detected low 189 cytoplasmic levels of CORO1C in luminal cells and strong expression in 190 myoepithelial cells (Fig. 2AB). CORO1C staining was observed in the cytoplasm of

191 breast carcinoma cells (Fig. 2AB). Quantification and analysis of CORO1C H-score 192 (intensity multiplied by percentage of positively stained cells) revealed significantly 193 higher levels of CORO1C in invasive cancer relative to normal breast tissues (Fig. 194 2C), in agreement with RNA expression data (Table S5) and protein analysis in 195 breast cancer cell lines (Fig. 1A and D). CORO1C levels were highest in TNBC 196 subtype although difference with other subtypes was not statistically significant (Fig. 197 2D). Therefore we concluded that CORO1C expression was up-regulated in invasive 198 breast cancers at the mRNA and protein levels, particularly in TNBCs and correlated 199 with worse prognosis and increased metastasis risk in association with MT1-MMP 200 up-regulation. At the mechanistic level, these data suggested some involvement of 201 CORO1C in MT1-MMP-dependent pericellular ECM proteolysis activity during breast 202 tumor progression and dissemination.

CORO1C is required for invadopodia function and tumor cell invasion. 203 <sup>GFP</sup>CORO1C was stably overexpressed in MDA-MB-231 cells used as a TNBC model 204 205 (Fig. S2A). These cells invaded through the 3D fibrous type I collagen network with 206 an elongated morphology typical of this highly invasive mesenchymal cell line (Fig. 3A, low magnification inset and Supplementary Movie S1). CTTN and GFPCORO1C 207 208 co-localized in lamellipodia at the edge of invasive protrusions extending within the collagen gel (Fig. 3A, low magnification inset). In addition, arc-shape structures 209 210 enriched for CTTN and CORO1C were visible in association with collagen fibrils in front of the nucleus, which was located at the rear of the cell (Fig. 3A, inset 1). Strong 211 212 pericellular collagenolysis was observed in association with the bulbous nuclear cell 213 region as revealed by staining for neoepitope of MMP-cleaved type I collagen (Fig. 214 3B, yellow). When cells were plated on top of a 2D layer of collagen fibrils, CORO1C was similarly found at the cell edge as well as in curvilinear structures forming in 215

association with the underlying collagen fibrils, which were positive for the invadopodia protein TKS5 (Fig. 3C)<sup>8, 40</sup>. Collectively, our observations indicated that CORO1C accumulated at proteolytically active invadopodia forming in association with confining collagen fibrils.

The effect of GFPCORO1C overexpression on the invasive capacity of MDA-MB-231 220 cells in 3D collagen was assessed. Multicellular spheroids of MDA-MB-231 cells 221 overexpressing GFP or  $^{\rm GFP} \rm CORO1C$  were formed and embedded in the type I 222 collagen gel and invasion was monitored after two days. Overexpression of 223 <sup>GFP</sup>CORO1C significantly increased the invasive potential of MDA-MB-231 cells in 224 the 3D collagen gel (Fig. 3DE). CORO1C-dependent invasion required MMP activity 225 as shown by inhibition in the presence of generic GM6001 MMP inhibitor (Fig. 3E). 226 227 The proinvasive potential of CORO1C was generalized using multicellular spheroids of human MCF10DCIS.com cells overexpressing <sup>GFP</sup>CORO1C or YFP as a control 228 229 (Fig. S3AB).

230 Actin polymerization is essential for invadopodia formation and function and CORO1C is known to regulate branched actin dynamics. We therefore investigated 231 the contribution of CORO1C to the formation of TKS5-positive invadopodia in MDA-232 233 MB-231 cells plated on top of a collagen fibril layer as in Fig. 3C. Overexpression of CORO1C increased significantly the formation of TKS5-positive invadopodia (Fig. 234 3F). <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub> harboring the R28D, K418E, K419E, K427E, and K428E 235 mutations, which is defective for F-actin binding <sup>41</sup>, was diffusely distributed indicating 236 237 that CORO1C association to invadopodia required binding to F-actin similar to CORO1B and -C recruitment to the lamellipodia <sup>41, 42</sup> (Fig. S3C). Remarkably, actin-238 binding deficient CORO1C<sub>R28D/2KE</sub> exerted a dominant inhibitory effect on 239 240 invadopodia formation (Fig. 3F). An equivalent mutation in CORO1B has been shown

to affect F-actin binding but no other molecular interactions <sup>15, 42</sup>, which may explain 241 242 the observed dominant inhibitory potential by titration of important functional partners. 243 In addition, CORO1C was knocked-down by treatment with two independent siRNAs leading to >95% silencing of the protein with no effect on MT1-MMP or TKS5 levels 244 245 (Fig. S2C). Loss of CORO1C expression reduced by 50-60% the capacity of MDA-246 MB-231 cells to form TKS5-positive invadopodia in association with the collagen fibrils (Fig. 3G and Fig. S3DE). CORO1B knockdown similarly decreased the 247 formation of TKS5-positive invadopodia by ~50% (Fig. 3G and Fig. S2D). Loss of 248 249 both CORO1B and -1C did not further reduce invadopodia formation, suggesting that the two isoforms may function together as recently reported <sup>17</sup> (Fig. 3G). Along this 250 line, we found that overexpressed CORO1B<sup>GFP</sup> accumulated together with MT1-251 MMP<sup>mCh</sup> in invadopodia forming in association with collagen fibers (Fig. 3H, inset 1 252 253 and Fig. S2B).

Consequences of modulation of CORO1C expression on pericellular matrix degradation in 3D collagen were analyzed by staining of cleaved collagen. Collagen proteolysis mediated by MT1-MMP was strongly up-regulated upon CORO1C overexpression, while ECM degradation was inhibited by  $CORO1C_{R28D/2KE}$  or CORO1C silencing (Fig. 3I-K). Collectively, these data indicated that CORO1C had a significant contribution to invadopodia formation and to the invasive program of TNBC cells.

We found that CORO1C co-localized with CTTN at invadopodia (Fig. 3A). Using the same assays, we observed that CTTN knockdown strongly decreased 3D collagen invasion of MDA-MB-231 multicellular spheroids similar to the silencing of MT1-MMP and CORO1C (Fig. S4A-C). In addition, knockdown of CTTN also correlated with a strong reduction of pericellular collagen degradation (Fig. S4DE). Thus we concluded

that up-regulation of CORO1C expression promoted the invasive potential of TNBC
 by favoring the formation of proteolytically active invadopodia and that CORO1B/1C
 and CTTN contributed to invadopodia activity suggesting some cooperative function.

CORO1C puncta are observed in association with collagen fibers in breast 269 270 tumor cells in vivo. Our data identified CORO1C as a key invadopodia component of invasive MDA-MB-231 cells. In agreement with CORO1C requirement during 271 272 collective invasion by epithelial MCF10DCIS.com breast cancer cells (Fig. S3AB), we also observed a strong association of <sup>GFP</sup>CORO1C with TKS5-positive proteolytically 273 274 active invadopodia forming in association with collagen fibrils in this cell line (Fig. S5A-C). Thus, the distribution of GFPCORO1C, which localized to invadopodia in 275 breast cancer cells was investigated in vivo. We used intravital imaging of mammary 276 MDA-MB-231 and MCF10DCIS.com cell tumor xenografts to visualize GFPCORO1C 277 in relation with the tumor ECM microenvironment. MDA-MB-231/GFPCORO1C tumor 278 279 xenografts growing in the mammary gland of SCID mice upon fat pad injection were 280 imaged using 2-photon laser scanning intravital microscopy combined with second 281 harmonic generation (SHG) to visualize collagen fibrils (Fig. 4A). Accumulations of <sup>GFP</sup>CORO1C were visible in tumor cells adjacent to collagen fibers (Fig. 4A-C, arrows 282 283 and insets 1-3). These structures were morphologically heterogeneous ranging from small (submicrometric) puncta (Fig. 4B and insets 1-2) to several µm-long linear 284 285 structures similar to curvilinear invadopodia observed in tumor cell lines in vitro (Fig. 286 4C and inset 3). Contrastingly, intravital imaging of fat pad tumors of MDA-MB-231 cells expressing <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub> revealed a diffuse cytosolic distribution of the 287 actin-binding defective CORO1C variant in tumor xenografts (Fig. 4D and inset 4). 288 289 Therefore, similar to our observations in cultured cells, accumulation of CORO1C in association with collagen fibers in vivo required F-actin binding capacity of CORO1C. 290

291 The distribution of CORO1C was similarly analyzed during the growth and invasion of 292 MCF10DCIS.com tumor xenograft generated using the intraductal (nipple-) injection model <sup>43</sup>. We and others reported that injection of this cell line into the duct lumen in 293 294 SCID mice leads to the formation of DCIS tumor xenografts, which can further progress into invasive lesions upon BM breaching depending on MT1-MMP activity<sup>2</sup>, 295 <sup>43</sup>. Stable overexpression of <sup>GFP</sup>CORO1C did not affect MCF10DCIS.com cell's 296 297 growth in culture (Fig. S5D). Whole-mount and tissue section staining of tumor 298 xenografts analyzed 4-5 weeks after intraductal injection revealed the presence of large epithelial tumors growing within the mammary duct system of SCID mice 299 injected with control or <sup>GFP</sup>CORO1C-overexpressing MCF10DCIS.com cells (Fig. 300 301 S5E, F and H). Tumors presented a necrotic center and some invasive foci of 302 epithelial cells were visible within the mammary gland stroma (Fig. S5E, arrowhead). 303 Tumor burden (foci number and size) was increased in mammary glands injected with GFPCORO1C-overexpressing cells as compared to control MCF10DCIS.com 304 305 cells, although differences were not statistically significant 4 to 5-weeks after injection (Fig. S5F-I). These data suggested that <sup>GFP</sup>CORO1C-overexpression conferred some 306 growth advantage to MCF10DCIS.com epithelial tumors. Ductal GFPCORO1C-307 308 expressing tumor xenografts were analyzed by intravital 2-photon imaging of GFP 309 and SHG signals. Intraductal tumors were detected as tumor mass surrounded by a 310 thick bed of collagen bundles tangential to the duct (Fig. 4EF). Some microinvasive buds were visible consisting of few <sup>GFP</sup>CORO1C-overexpressing epithelial cells that 311 312 migrated in collective manner within the type I collagen bed (Fig. 4EF and insets 5 and 6). Invasion of GFPCORO1C cells progressed overtime in the collagen-enriched 313 314 stroma (compare inset 5 and 6 acquired at 1-week time interval). Intravital imaging at later time point (7-weeks post injection) revealed frankly invasive tumor lesions (Fig. 315

316 4G-I). The presence of <sup>GFP</sup>CORO1C accumulations forming at the surface of tumor 317 MCF10DCIS.com cells in contact with intratumoral collagen fibers was visible similar 318 to MDA-MB-231 tumors (Fig. 4G-I and inset 7). Collectively, these data indicated that 319 CORO1C accumulated in structures reminiscent of ECM degradative invadopodia, 320 which formed in association with collagen fibrils in the invasive breast tumor 321 xenografts *in vivo*.

322 Loss of CORO1C causes MT1-MMP-positive LE/lysosome mispositioning and collapse. We have shown that MT1-MMP trafficking to invadopodia depends on 323 actin dynamics on endolysosomes <sup>8, 23</sup>. The distribution of <sup>GFP</sup>CORO1C was analyzed 324 by live cell imaging by confocal microscopy in MDA-MB-231 cells expressing MT1-325 MMP<sup>mCh</sup>. As previously described, MT1-MMP<sup>mCh</sup> accumulated in vesicles that we 326 previously identified as LE/lysosomes <sup>7, 32</sup>. In addition to <sup>GFP</sup>CORO1C localization to 327 lamellipodia, we observed the accumulation of <sup>GFP</sup>CORO1C in dynamic and discrete 328 puncta on a majority of MT1-MMP<sup>mCh</sup>-positive vesicles (Fig. 5A and Supplementary 329 Movie S2). In contrast, F-actin-binding-deficient GFPCORO1C<sub>R28D/2KF</sub> was diffuse and 330 331 cytosolic (Fig. 5B). Endosomal CORO1C-enriched puncta were also positive for CTTN, although a slight shift was visible between the localization of the two proteins 332 in line with the distinct roles played by these proteins in the actin branching cycle <sup>15, 17</sup> 333 (Fig. 5C, inset). Similarly, CORO1B<sup>GFP</sup>-positive puncta were visible on MT1-MMP 334 335 LE/lysosomes (Fig. 3H, inset 2). Cryoimmunoelectron microscopy confirmed the association of <sup>GFP</sup>CORO1C-positive puncta on the cytosolic face of LE/lysosomes 336 337 (Fig. 5D). All together, these data revealed that CORO1C and CORO1B localized to 338 endosomal actin-, CTTN-rich puncta, which depend on WASH and Arp2/3 complexes for their formation and are implicated in MT1-MMP delivery to invadopodia in breast 339 cancer cells <sup>8, 23, 32, 34, 35</sup>. 340

We went on analyzing the consequences of CORO1C knockdown on the distribution 341 and morphology of MT1-MMP-positive LE/lysosomes. In control RNAi treated cells, 342 343 MT1-MMP-positive LE/lysosomes were distributed throughout the cell, with a concentration in the central region of the cell (Fig. 5E and IJ). In cells silenced for 344 345 CORO1C, MT1-MMP-positive LE/lysosomes were also predominantly localized to 346 the cell center (Fig. 5F and I). In addition, we observed that reduced expression of CORO1C resulted in a tight clustering of MT1-MMP-positive LE/lysosomes in ~70% 347 of the cells (Fig. 5J). We recently identified the scaffolding proteins JIP4 and JIP3 as 348 key players in MT1-MMP endosome positioning by linking LE/lysosomes to the 349 microtubule motors kinesin-1 and dynein/dynactin <sup>32</sup>. As previously described <sup>32</sup>, 350 351 depletion of JIP3 and JIP4 resulted in MT1-MMP endosome dispersion toward the 352 cell periphery (Fig. 5G and IJ and Fig. S6A). Remarkably, triple knockdown of 353 JIP3/JIP4 and CORO1C partially restored some level of scattering of MT1-MMP-354 positive endosomes (Fig. 5H and IJ). All together, these data suggested that tight 355 clustering of MT1-MMP-positive LE/lysosomes induced upon loss of CORO1C 356 function required retrograde endosomal trafficking in a JIP3/JIP4-dependent manner.

Cells expressing MT1-MMP<sup>mCh</sup> were fixed and stained for JIP4 and CTTN, and 357 fluorescence signal associated with MT1-MMP<sup>mCh</sup>-positive compartments was 358 quantified. As previously reported <sup>32</sup>, JIP4, which is predominantly cytosolic, was 359 360 detected throughout the cytoplasm and in close proximity to CTTN-positive puncta on the cytosolic face of MT1-MMP endosomes (Fig. 6A and guantification in panels DE, 361 see also Fig. S6C-C" for lower magnification images). Quantification of fluorescence 362 signal associated with clustered, enlarged MT1-MMP<sup>mCh</sup>-positive endosomal 363 compartments in CORO1C-depleted cells revealed opposite effects on CTTN and 364 JIP4 association, i.e. CTTN association was diminished, while JIP4 accumulated on 365

MT1-MMP compartments as compared to siNT-treated cells (Fig. 6B and DE and Fig. S6D-E'). Additionally, CTTN knockdown did not alter significantly the association of JIP4 with endosomal membranes (Fig. 6C and DE and Fig. S6B and FF').

369 We noticed that morphological changes observed in CORO1C-depleted cells 370 including enlargement and perinuclear clustering of LE/Lysosomal compartments were reminiscent of cellular alterations typical of Niemann-Pick disease type C 371 (NPC). Indeed, cholesterol-enriched LEs cluster in the perinuclear area, as a 372 373 consequence of defective LE/Lysosome traffic and cholesterol clearance in cells of lysosomal storage disease patients including NPC cells <sup>44</sup>. After fixation, MDA-MB-374 231 cells treated with control siRNA were stained with the fluorescent cholesterol-375 376 binding probe filipin and scattered cholesterol-positive LE structures were observed 377 (Fig. S6G). Treatment of the cells with U18666A, a widely-used amphipathic steroid, which blocks exit of cholesterol from LE/lysosomes and recapitulates the NPC 378 phenotype <sup>45</sup>, induced tight clustering of cholesterol-laden LE/Lysosomes in the 379 380 perinuclear cell region (Fig. S6H). Similarly, LE/Lysosome clusters forming in 381 CORO1C-depleted cells were strongly enriched for cholesterol indicative of defective 382 cholesterol exit from the clustered endosomes (Fig. S6IJ).

383 In order to get further insight at phenotypic alterations of MT1-MMP-positive 384 compartments induced by loss of CORO1C function, MDA-MB-231 cells knocked 385 down for CORO1C were analyzed by transmission electron microscopy. Figure 6F 386 shows the distribution of LE/lysosomes in control cells. CORO1C depletion caused 387 various morphological alterations of LE/lysosomal compartments, which were 388 enlarged, aggregated and fused together and accumulated osmiophilic materials 389 including lamellar inclusions reminiscent of the NPC phenotype (Fig. 6G-I). 390 Collectively, these data indicated that CORO1C played a key role in MT1-MMP

LE/lysosome positioning. Loss of CORO1C expression altered the association of JIP4 (and CTTN) on endosomal membranes resulting in LE/lysosome clumps in the perinuclear area possibly responsible for deficient recycling and surface-exposure of MT1-MMP.

#### 395 Discussion

Previous findings indicated that CORO1C, a member of the coronin family of highly conserved F-actin-binding proteins and regulators of branched actin networks, contributes to invasiveness and metastasis in several cancer types including glioblastoma, primary effusion lymphoma and TNBC <sup>24, 26-29, 46</sup>. In addition, CORO1C is a biomarker for invasive progression of hepatocellular carcinoma <sup>25</sup>. However, our understanding of the cellular and molecular mechanisms underlying the role of CORO1C in tumor invasion has remained limited.

Here, we analyzed the expression profile of CORO1C in breast cancer in relation 403 404 with MT1-MMP, the protease responsible for pericellular matrix degradation by 405 carcinoma cells with major implications in the invasive potential and progression of breast tumors <sup>2, 47, 48</sup>. In a panel of cell lines representative of the breast cancer 406 molecular subtypes <sup>39</sup>, CORO1C protein expression was detected mainly in TNBC 407 cells similar to MT1-MMP<sup>2</sup>. CORO1B was broadly expressed irrespective of the 408 molecular subtypes, while expression of hematopoietic-specific CORO1A was low to 409 undetectable <sup>16</sup>. Based on RT-qPCR analysis of a retrospective cohort of invasive 410 411 breast cancers, we found that CORO1C transcript was up-regulated breast cancers 412 and was associated with hormone receptor-negativity, high grade and metastasis risk. In addition, co-up-regulation of CORO1C and MT1-MMP also correlated with 413 414 significantly higher metastases risk. 'High CORO1C expression' was an independent 415 prognostic factor of breast cancer in multivariate analysis and prognostic significance 416 of 'MT1-MMP and CORO1C expression level' persisted in Cox multivariate regression analysis. Likewise, IHC analysis of invasive breast cancer TMAs 417 418 confirmed the up-regulation of CORO1C in breast carcinoma at the protein level as compared to normal epithelial tissue. CORO1B mRNA was also up-regulated in a 419

breast tumor subset, although with no impact on metastasis-free survival. Similar to our findings in breast cancer, published data have correlated CORO1C expression with high-grade gliomas and liver cancers and poor prognosis in stomach cancers <sup>25,</sup> while no such correlation has been reported between CORO1B and cancer progression. In conclusion, our data, which extend a previous analysis of publicly accessible databases <sup>46</sup>, indicate that CORO1C is up-regulated in hormone receptornegative breast tumors and associates with poor outcome.

427 Several studies reported that coronins, including CORO1C, regulate lamellipodial branched actin network and CORO1C activity impinges on cell migration and 428 invasion by cancer cells including MDA-MB-231 TNBC cells in vitro 28, 29, 46, 49. 429 430 Moreover, CORO1C was associated with invasive cell protrusions and has been 431 implicated in the degradation of the matrix mimic gelatin by human U373 glioblastoma-derived cells <sup>28, 29</sup>. In the present study, we observed that CORO1C co-432 localized with CTTN and TKS5 in matrix-degradative actin-based invadopodia 433 434 forming in association with type I collagen fibrils in TNBC cells. Importantly, 435 knockdown of CORO1C inhibited invadopodia formation, pericellular collagenolysis 436 and invasive migration in 3D collagen in two TNBC cell models with distinct mesenchymal (MDA-MB-231) or epithelial (MCF10DCIS.com) features. Thus, our 437 work indicates that CORO1C is required both for single-cell and collective invasion 438 439 patterns. Association of CORO1B with invadopodia was also observed and CORO1B 440 was required for invadopodia formation. Moreover, loss of both CORO1C and 1B did 441 not further inhibited invadopodia formation, suggesting that both isoforms may function as a complex as previously shown <sup>17</sup>. 442

Actin polymerization is a key component of invadopodia-based invasion program by driving invasive cell protrusions through the matrix and maintaining tight apposition of

surface-exposed MT1-MMP with confining ECM fibrils <sup>4, 8</sup>. In addition, binding of 445 446 MT1-MMP cytosolic tail to the invadopodial actin network is thought to anchor MT1-MMP to these structures <sup>50</sup>. Assembly of invadopodial actin requires activation of the 447 Arp2/3 complex by N-WASP<sup>8, 11, 50</sup>. An antagonism of CTTN and coronin functions 448 has been proposed in the control of a cycle of Arp2/3 branch stabilization and 449 destabilization (15-17, 51 and references herein). In addition, coronin and CTTN have 450 been implicated in the regulation of cofilin activity in actin filament turnover, which is 451 crucial during invadopodia assembly <sup>11, 51</sup>. We observed that CORO1B/1C and CTTN 452 co-localized at invadopodia. Alike CORO1B/1C, CTTN has been identified as a key 453 454 regulator of invadopodia formation and its overexpression in various cancer types is associated with tumor progression and metastasis <sup>6, 12</sup>. Here, we found that 455 unbalanced CTTN and CORO1B/1C activity upon individual silencing (or inhibition) of 456 457 either proteins impairs invadopodia formation and function, emphasizing complex and cooperative functions of CTTN and CORO1B/1C in the branching/debranching 458 459 cycle to replenish Arp2/3 complex and actin pools to promote invadopodia actin 460 dynamics and cancer cell invasion. Overall, our data suggest that CORO1B and 461 CORO1C function together in the mechanism of invadopodia formation and in matrix 462 remodeling. Yet, the reason for the specific association of CORO1C up-regulation -463 and not CORO1B - with cancer aggressiveness is unclear, we cannot rule out some 464 additional function of CORO1C related to its association with cancer aggressiveness.

Invadopodia's role in matrix degradation has been experimentally established, however the existence and physiological relevance of invadopodia in cancer is questionable because of a lack of direct evidence in vivo. Yet, recent studies provided convincing evidence that invadopodia formation is essential for extravasation and intravasation of tumor cells and promotes lung metastasis in mice

<sup>9, 10, 52</sup>. Reports based on intravital imaging of tumor cells expressing fluorescently-470 471 tagged markers documented the existence of cytoplasmic protrusions identified as invadopodia based on CTTN, N-WASP or TKS5 enrichment and that correlated with 472 cancer cell intra- or extravasation in vivo <sup>9, 10</sup>. Importantly, intravital imaging of 473 GFPCORO1C-expressing tumor xenografts of MDA-MB-231 474 mammary or MCF10DCIS.com cells revealed the presence of <sup>GFP</sup>CORO1C accumulations 475 adjacent to collagen fibers in the tumor microenvironment. However, evidence that 476 477 these structures are proteolytically active is lacking and we cannot formally conclude that <sup>GFP</sup>CORO1C accumulations represent *bona fide* invadopodia *in vivo*. Noticeably, 478 <sup>GFP</sup>CORO1C-positive structures were observed both in mesenchymal MDA-MB-231 479 480 cells and in strands of invasive epithelial MCF10DCIS.com cells supporting the implication of CORO1C in mesenchymal and collective invasion programs. 481

Branched actin networks also exist on intracellular compartments including 482 endolysosomes where their main function is to sculpt and stabilize endosomal 483 membrane microdomains involved in cargo sorting and recycling <sup>34, 35, 53</sup>. Endosomal 484 485 actin patches are nucleated by the Arp2/3-complex upon WASH-mediated activation <sup>34, 35</sup>. Earlier on, we found that the WASH complex localizes to MT1-MMP-containing 486 LE/lysosomes and its function is required for MT1-MMP trafficking to invadopodia<sup>8</sup>. 487 In addition, several groups reported that depletion of WASH led to enlargement and 488 489 massive tubulation of the endolysosomal system supporting the idea that WASHdependent actin assembly may regulate the fission of endosomal recycling tubules <sup>34,</sup> 490 <sup>35, 54</sup>. CTTN was also found to associate with and regulate endosomal actin puncta on 491 MT1-MMP-positive LE/Lysosomes, and it was reported that CTTN loss or 492 493 deregulation led to the accumulation of enlarged LE/Lysosomes and defective endosomal traffic <sup>8, 23, 37, 55</sup>. The present study provides corroborating evidence that in 494

495 the absence of CORO1C, endolysosomal compartments dramatically enlarged and collapsed in the perinuclear cell area. It has been argued that deficient cargo exit 496 497 exemplified by massive cholesterol accumulation and organelle obstruction may lead to endosome enlargement <sup>54</sup>. Alteration of the endosomal actin coat may induce 498 endosomal collapse possibly through uncontrolled fusion of individual endosomes. 499 500 This assumption is supported by findings that interfering with endosomal actin 501 regulatory components such as WASH, CTTN, CORO1A or -C lead to perinuclear redistribution and collapse of endolysosomal compartments <sup>34-37, 54</sup> (this study). 502

503 Moreover, we previously found that the related JIP3 and JIP4 scaffolding proteins 504 regulate the recruitment of minus-end and plus-end microtubule motor complexes to WASH-positive domains <sup>32</sup>. Loss of JIP3 and JIP4 proteins led to the accumulation of 505 506 MT1-MMP compartments at the cell periphery indicating unbalanced minus/plus-end motor activities (at minus-end dynein expense) <sup>32</sup>. Here we found that loss of 507 CORO1C moderately increases JIP3/JIP4 recruitment on MT1-MMP-positive 508 endosomes although the underlying mechanism remains unknown. Interestingly, 509 510 enhanced recruitment of JIP4 to endolysosomal compartments was recently shown to induce endolysome collapse into the cell center <sup>56</sup>. Excess JIP3/JIP4 on MT1-MMP 511 512 endosomes is expected to promote their perinuclear accumulation due to increased dynein activity, possibly favoring endosome collision and collapse. In agreement with 513 514 this assumption, we found that formation of endosomal clusters upon loss of 515 CORO1C was abrogated when JIP3/JIP4 were silenced. Also to be considered, 516 coordinated functions of coronins and CTTN were also implicated in the regulation of endolysosome docking with the plasma membrane and exocytosis <sup>37, 38</sup>. All together, 517 518 our work highlights a molecular framework based on interplay of WASH, CTTN,

- 519 CORO1C and JIP3/JIP4 function in the regulation of endosome positioning and
- 520 dynamics as an essential component of MT1-MMP-based metastatic program.

#### 521 Materials and Methods

522 **Cell culture**. MDA-MB-231 cells (ATCC HTB-26) were grown in L15 medium 523 supplemented with 15% fetal calf serum and 2 mM glutamine at 37°C in 1% CO<sub>2</sub>. 524 MCF10DCIS.com cell line was purchased from Asterand and maintained in DMEM-525 F12 medium with 5% horse serum. MDA-MB-231 and MCF10DCIS.com cell lines 526 were checked monthly for mycoplasma contamination using a real-time PCR method 527 (MYCOPLASMACHECK, GATC Biotech AG).

528 Stable and transient transfection and siRNA treatment. MDA-MB-231 cells stably expressing MT1-MMP<sup>mCh</sup> have been previously described <sup>57</sup>. Lentiviral constructs 529 encoding <sup>GFP</sup>CORO1C or <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub> <sup>41</sup> cloned in pLKO.1 vector have been 530 described <sup>29</sup>. MDA-MB-231 cells expressing <sup>GFP</sup>CORO1C, <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub>, 531 <sup>GFP</sup>CORO1C/MT1-MMP<sup>mCh</sup>, <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub>/MT1-MMP<sup>mCh</sup> and MCF10DCIS.com 532 cells stably expressing <sup>GFP</sup>Coro1C were generated by lentiviral transduction as 533 described <sup>58</sup>. pLL7.0-m1B-EGFP construct encoding CORO1B<sup>GFP</sup> was provided by 534 535 J.E. Bear. For transient expression, MDA-MB-231 cells were transfected with plasmid constructs using AMAXA nucleofection (Lonza). Cells were analyzed by live cell 536 imaging 24-48 hr after transfection. For knockdown, MDA-MB-231 cells were treated 537 with the indicated siRNA (50 nM, see Table S1) using Lullaby (OZ Biosciences) and 538 539 analyzed 72 hours after treatment.

540 **Antibodies and reagents**. Antibodies used for this study are listed in Table S2. 541 GM6001 (Millipore) was diluted in ethanol and used at a concentration of 40  $\mu$ M. 542 Filipin III (stock in DMSO, 0.1 mg/ml  $\mu$ M working concentration) and U18666A (stock 543 in H<sub>2</sub>0, 10  $\mu$ M working concentration) were purchased from Sigma.

Indirect immunofluorescence microscopy. Samples were fixed with 4% 544 paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with 545 specific antibodies (see Table S2). For quantification of CTTN or JIP4 on MT1-546 MMP<sup>mCh</sup>-containing endosomes, cells were stained with specific antibodies and z-547 dimension series of images (z-stacks) were acquired with 0.2 µm interval with a wide-548 549 field Eclipse 90i Upright Microscope (Nikon) by mean of a piezoelectric motor (Physik Instrument), a 100x Plan Apo VC 1.4 oil immersion objective and a cooled interlined 550 551 charge-coupled device (CCD) camera (CoolSnap HQ2, Roper Scientific). Images were deconvolved <sup>59</sup>, and detection of CTTN or JIP4 on MT1-MMP<sup>mCh</sup>-containing 552 553 endosomes from the median plane of the z-stack was performed using a CellProfiler pipeline as previously described <sup>23, 60</sup>. Briefly, MT1-MMPmCh-containing endosomes 554 were identified by thresholding and intensity-based watershed; CTTN or JIP4 spots in 555 a 3-pixel wide neighborhood around each MT1-MMP<sup>mCh</sup>-positive vesicle were 556 identified using a Laplacian of Gaussian filter followed by a watershed on the 557 automatically thresholded image. Finally, the total area of CTTN or JIP4 spots was 558 559 normalized over the total of MT1-MMPmCh-positive vesicles.

Live cell spinning disk confocal microscopy. For live cell confocal spinning disk 560 microscopy, MDA-MB-231 cells stably expressing GFPCoro1C and MT1-MMP<sup>mCh</sup> or 561 <sup>GFP</sup>CORO1C<sub>R28D/2KE</sub> and MT1-MMPmCh were plated on glass-bottom dishes (MatTek 562 Corporation) coated with cross-linked gelatin and kept in a humidified atmosphere at 563 564 37°C in 1% CO2. Time-series were acquired using a spinning disk microscope 565 (Roper Scientific) based on a CSU X1 head (Yokogawa) mounted on the lateral port of an inverted microscope (TE-2000U-Nikon) steered by MetaMorph software and 566 567 equipped with a 60x 1.45 NA oil-immersion objective, a Piezzo Z stage (Mat-City-

Lab), a dual output laser launch that included 491- and 561-nm 50-mW DPSS lasers (Roper Scientific) and a CCD camera (CoolSNAP HQ2).

570 **Analysis of endosome distribution and clustering.** For the analysis of the 571 distribution of MT1-MMP<sup>mCh</sup>-positive endosomes, a MATLAB software has been 572 developed to determine the relative position of each endosome to the cell centroid as 573 previously described <sup>61</sup>.

574 **Invadopodia formation assay.** Coverslips were layered with 100 µl of a 2.2 mg/ml 575 solution of acid-extracted type I rat tail collagen (Corning) mixed with AlexaFluor 647-576 conjugated type I collagen (5% final). After gelling for 3 min at 37°C, the collagen layer was washed gently in PBS and 1 ml of cell suspension (10<sup>5</sup> cells/ml) was 577 added. Cells were incubated for 90 min at 37°C in 1% CO<sub>2</sub> incubator, then pre-578 579 extracted with 0.5% Triton X-100 in 4% paraformaldehyde in PBS during 90 s and 580 fixed in 4% paraformaldehyde in PBS for 20 min. Cells were analyzed by 581 immunofluorescence staining with TKS5 and CTTN antibodies (Table S2), and zstacks of images were acquired as described above. For quantification of TKS5 582 583 associated with curvilinear invadopodia, 5 consecutive planes corresponding to the plasma membrane in contact with collagen fibers were projected and surface 584 585 covered by TKS5 signal was determined using the thresholding command of ImageJ excluding regions <8 pixels (*i.e.* <1 µm) to avoid non-invadopodial structures. Surface 586 587 covered by TKS5 was normalized to the total cell surface and values normalized to 588 control cells.

589 **Quantification of pericellular collagenolysis**. Cells were trypsinized and 590 resuspended in 0.2 ml of 2.2 mg/ml ice-cold collagen I solution in 1x MEM, pH 7.5 591 buffer ( $2.5 \times 10^5$  cells/ml). A 40 µl-drop of the cell suspension in collagen was added

592 on a 18-mm diameter glass coverslip and collagen polymerization was induced by 593 incubation at 37°C for 30 min. Complete medium was added, and the collagenembedded cells were incubated for 12 hrs at 37°C. Samples were fixed in 4% 594 paraformaldehyde in PBS at 37°C for 30 min, and incubated with Col1-<sup>3/4</sup>C antibody 595 (2.5 µg/ml) for 2 hrs at 4°C, washed extensively with PBS and counterstained with 596 597 Cy3-conjugated anti-rabbit IgG antibodies and with DAPI. Image acquisition was performed with an A1R Nikon confocal microscope with a 40× NA 1.3 oil objective 598 599 using high-sensitivity GaASP PMT detector and a 595 ± 50 nm band-pass filter for 600 red fluorescence detection. Quantification of the degradation spots was performed as previously described <sup>8</sup>. 601

Electron microscopy. Cells were fixed with 2,5% glutaraldehyde in 0,1M cacodylate 602 buffer and processed for EPON embedding and ultrathin sections as described <sup>62</sup>. 603 For cryoimmunolabeling, cells were fixed in a mixture of 2% paraformaldehyde and 604 0.125% glutaraldehyde in a 0.1M phosphate buffer pH7.4 during 48 hrs <sup>62</sup>. Ultrathin 605 606 sections were prepared with an ultracryomicrotome Leica EM UC7 and underwent 607 single immunogold labeling with protein A conjugated to 10-nm diameter gold 608 particles (Cell Microscopy Center, Department of Cell Biology, Utrecht University). 609 Samples were analyzed with a Tecnai Spirit electron microscope (FEI Company, 610 Eindhoven Netherlands) and digital acquisitions were made with a QUEMESA CCD 611 camera (EMSIS GmbH, Münster, Germany).

512 **Statistics and reproducibility.** GraphPad Prism (GraphPad Software) was used for 513 statistical analysis. Sample size was chosen based on estimates from pilot 514 experiments and our published results such that appropriate statistical tests could 515 yield significant results. Data were tested for normal distribution using the

D'Agostino-Pearson normality test and nonparametric tests were applied otherwise 616 617 as indicated in the figure legends. Statistical significance was defined as \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001; \*\*\*\*, P<0.0001; ns, not significant. Relationships between 618 protein expression and distribution in tumors vs. normal adjacent tissues were 619 620 estimated using Two tailed Student's t-test and Kruskal-Wallis test (for links between 621 qualitative and quantitative parameters). Metastasis-free survival was determined as 622 the interval between initial diagnosis and detection of the first metastasis. Survival 623 distributions were estimated by the Kaplan-Meier method, and the significance of 624 differences between survival rates were ascertained with the log-rank test. The Cox 625 proportional hazards regression model was used to assess prognostic significance, 626 and the results are presented as hazard ratio (HR) and 95% confidence interval (CI). 627 The following variables were included in the analysis: SBR grade, lymph node status, 628 macroscopic tumor size, PR status (all the classical variables with a P value under 629 0.10 in univariate analysis) and CORO1C or combined CORO1C and MT1-MMP 630 mRNA expression. For animal studies, no animals were excluded from analyses and 631 no blinding procedure was used.

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See Supplementary Materials and Methods for additional cell lines, western blot analysis, patient cohort for mRNA analysis, RT-qPCR analysis, patient biopsies for western blot analysis, Immunohistochemistry analysis of human breast biopsies, Mammary fat pad and intraductal injections, Mammary imaging window surgical implantation and near-infrared multiphoton microscopy of mammary tumor xenografts.

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883 Acknowledgments. The authors greatly acknowledge the Breast Cancer Study Group and patients of Institut Curie for breast tumor samples. They thank the Cell 884 885 and Tissue Imaging facility (PICT-IBiSA) and Nikon Imaging Centre, Institut Curie, 886 member of the French National Research Infrastructure France-BioImaging (ANR10-887 INBS-04) for help with image acquisition and Dr Jakko van Rheenen for help with the 888 mammary window and intravital imaging. A.C. was supported by a grant from Worldwide Cancer Research (Grant 16-1235 to P.C.), A.C.C. by an EMBO Long 889 890 Term postdoctoral fellowship and a grant from 'Institut National du Cancer' 891 (INCA 6521 to P.C.), A.G. by INVADE grant from ITMO Cancer (Call Systems 892 Biology 2012) to P.C., S.A.G. by a grant from INCa (INCa 2014-11/474/AB-SD to 893 P.C.). and A.A.N. and C.S.C by a grant from the German Research Foundation (DFG 894 NO 113/22-2). The intravital imaging was supported by the Fondation pour la 895 Recherche Médicale (FRM N° DGE20111123020), the Cancerople-IdF (n°2012-2-EML-04-IC-1), InCA (Cancer National Institute, n° 2011-1-LABEL-IC-4) and SIRIC 896 897 (INCa-DGOS- 4654). This work was supported by grants from 'Institut National du 898 Cancer' (INCa 2014-11/474/AB-SD and INCA 6521), Worldwide Cancer Research 899 (Grant 16-1235) and 'Equipe labellisée 2015' from 'Lique Nationale contre le Cancer' 900 to P.C.

#### 902 Author contributions

903 AC carried out invadopodia formation and collagen degradation assays with the help 904 of AZM and characterized the effects of CORO1C depletion on endolysosomal 905 morphology and position. ACC who initiated the study, generated the cell lines stably 906 expressing GFPCORO1C constructs and analyzed CORO1B and -1C expression in 907 lysates of breast cancer cell lines provided by TD. LF and AVS generated the breast 908 cancer TMA, performed CORO1C IHC labeling and scored the breast tumor TMA for 909 CORO1C expression with the help of CEK. SV and IB generated the invasive breast 910 cancer cohort and performed RT-qPCR and multivariate analyses. MI performed 911 multicellular spheroid invasion assays, analyzed the phenotype of cells silenced for 912 cortactin and performed intravital imaging. AG, CL and SAG carried out mammary 913 gland injections and analyzed tumor xenografts. MR and GR performed electron 914 microscopy analyses. JEB, AAN and CSC provided essential reagents. PC 915 supervised the study and wrote the manuscript with contribution of all authors.

#### 917 Figure Legends

918 Figure 1. Patients displaying high CORO1C and MT1-MMP mRNA expression 919 have poor outcomes in breast cancer. (A) Whole-cell lysates from human breast 920 carcinoma cell lines and Jurkat T cells were normalized for protein concentration and 921 immunoblotted with indicated antibodies. Stratification of breast cancer cell lines in Normal-like, TNBC, Luminal and HER2 subtypes was according to <sup>39</sup>. (B-E) 922 923 Expression of the indicated proteins in the different cell lines divided in four subtypes 924 was normalized to GAPDH level. Protein levels were analyzed in 2 to 3 experiments 925 for each protein in each cell line and plots correspond to average expression levels ± 926 SEM in each subtype. (F) Expression of the indicated genes was analyzed by RT-927 qPCR in 446 mRNA tumor samples. Patients exhibiting high CORO1C mRNA have 928 reduced MFS (log-rank test using optimal cutoff of 1.52; p=0.00082). Number of 929 patients in each subgroup is indicated in parenthesis. (G) Correlation between 930 indicated gene transcripts was analyzed using the Spearman's rank correlation test 931 (in bold type). P values are in italics. (H) Patients exhibiting both high CORO1C and 932 MT1-MMP mRNAs have reduced MFS (log-rank test using optimal cut-off of 1.52 for CORO1C and 1.30 for MT1-MMP; P=0.00048). 933

Figure 2. Increased CORO1C expression in invasive breast cancers. (A, B) CORO1C immunohistochemistry staining on sections of human invasive breast cancer (IBC) tissue microarray showing adjacent non-neoplastic tissue (Normal) and representative TNBC and HER2+ breast cancers. (C, D) Semi-quantitative analysis of CORO1C protein expression by the H-score method comparing adjacent breast epithelial tissues and carcinoma tissues (C) or in the different subtypes (D). Two tailed t-test (C); Kruskal-Wallis test (D).

Figure 3. CORO1C is required for 3D invasion and invadopodia function. (A) 941 MDA-MB-231 cells expressing <sup>GFP</sup>CORO1C (green) were embedded in 2.2 mg/ml 942 943 fluorescent type I collagen (magenta) and polymerization was induced at neutral pH 944 at 37°C. Cells were fixed after 12-16 h and stained for cortactin (red). The nucleus is 945 stained with DAPI (blue). The image is a single optical plane from a z-stack 946 (Supplementary Movie S1). The low magnification inset is a projection of the z-stack 947 of images (collagen is omitted). Inset 1 shows separated channels corresponding to 948 the boxed region. (B) Cells in 3D collagen (magenta) stained for cleaved collagen neoepitope (Col1-<sup>34</sup>C antibody, yellow) and F-actin (blue). DAPI-stained nuclei are 949 shown in cyan. (C) MDA-MB-231 cells expressing GFPCORO1C (green) were 950 951 incubated on a thick layer of fibrous type I collagen (magenta). Curvilinear 952 invadopodia forming in association with collagen fibrils (cyan) were labeled for TKS5 953 (red). Insets show separated channels corresponding to the boxed region. (D) 954 Phalloidin-labeled multicellular spheroids of MDA-MB-231 cells expressing GFP or <sup>GFP</sup>CORO1C after 2 days in 3D collagen I (T2). Insets show spheroids immediately 955 956 after embedding in collagen (T0). (E) Mean invasion area of multicellular spheroids at 957 T2 in the presence or absence of GM6001 normalized to mean spheroid area at T0  $\pm$ SEM. n, spheroid number. Two-way ANOVA. (F) Quantification of invadopodia-958 associated TKS5 signal in MDA-MB-231 cells expressing GFP, GFPCORO1C or 959 960 mutant GFPCORO1C<sub>R28D/2KE</sub> plated on a thick layer of type I collagen as in panel C. Y-961 axis indicates TKS5 area normalized to total cell area and to mean value in MDA-962 MB-231 cells (as percentage) ± SEM. n, number of cells analyzed from three 963 independent experiments. Kruskal-Wallis test. (G) TKS5-positive invadopodia in 964 MDA-MB-231 cells knocked down for CORO1C, CORO1B or both as in panel F. n, number of cells analyzed from five (siNT), three (siCORO1C<sup>#04</sup> and <sup>#08</sup>) or two 965

(siCORO1B and siCORO1B+C<sup>#04</sup>) independent experiments. (H) MDA-MB-231 cells 966 expressing CORO1B<sup>GFP</sup> (green) and MT1-MMP<sup>mCh</sup> (red) plated on fibrous type I 967 collagen (magenta). CORO1B and MT1-MMP colocalize in invadopodia forming in 968 969 association with the collagen fibers (inset 1). CORO1B-positive puncta are also visible on MT1-MMP endolysosomes (inset 2). (I) Representative images of 970 pericellular collagenolysis detected with Col1-<sup>3/4</sup>C antibody (black signal in the 971 972 inverted images). Nuclei were stained with DAPI (red). (J, K) Collagen degradation measured by Col1-<sup>3/4</sup>C necepitope staining by indicated cell populations normalized 973 to GFP-expressing (I) or siNT-treated (J) control cells ± SEM; n, number of cells 974 975 analyzed from three independent experiments. Data were transformed using the log 976 transformation y = log(y) to make data conform to normality and analyzed using one-977 way ANOVA.

#### 978 Figure 4. In vivo association of CORO1C-positive invadopodia with ECM fibers.

(A-D) Near-infrared multiphoton microscopy images of tumor sections of 8-week-old 979 fat pad tumor xenografts from MDA-MB-231/GFPCORO1C (panels A to C) or MDA-980 MB-231/<sup>GFP</sup>CORO1C<sub>R28D/2KF</sub> cells (panel D). Collagen fibers detected by second 981 982 harmonic generation (SHG, magenta). (G-I) Time-course of the growth and invasion of MCF10DCIS.com/GFPCORO1C intraductal tumor xenograft monitored by near-983 infrared multiphoton microscopy 5 to 7-week post injection. All images are single 984 985 optical sections from z-stack except in panel G and H, which show maximum 986 intensity projections of indicated optical planes. Insets are higher magnification of boxed regions. White arrows point to accumulations of <sup>GFP</sup>CORO1C in association 987 with collagen fibers. 988

Figure 5. Altered MT1-MMP endosome positioning and morphology upon
 CORO1C knockdown. (A) Still image from live cell confocal microscopy sequence

MDA-MB-231 cells expressing MT1-MMP<sup>mCh</sup> and <sup>GFP</sup>CORO1C 991 of (see Supplementary Movie S2). Inset is a high magnification of the boxed region. Bottom 992 row shows the fluorescence intensity profiles of MT1-MMP<sup>mCh</sup> and <sup>GFP</sup>CORO1C 993 along the dotted line. (B) High magnification of MDA-MB-231 cell expressing MT1-994 MMP<sup>mCh</sup> and <sup>GFP</sup>CORO1C<sub>R28D/2KF</sub> as in panel A. Fluorescence intensity profiles are 995 shown. (C) MDA-MB-231 cells expressing MT1-MMP<sup>mCh</sup> (red) and <sup>GFP</sup>CORO1C 996 997 (green) stained for CTTN (blue). (D) Cryoimmunoelectron microscopy of MDA-MB-231 cells expressing <sup>GFP</sup>CORO1C. The micrograph shows the localization of 998 <sup>GFP</sup>CORO1C on LE/lysosomes (asterisk) visualized with anti-GFP antibodies and 999 1000 secondary antibodies coupled to 10-nm diameter protein A-gold (arrows). Arrowheads point to <sup>GFP</sup>CORO1C associated with the cytosolic face of the plasma 1001 1002 membrane. (E-H). Confocal fluorescence microscopy images of MDA-MB-231 cells expressing MT1-MMP<sup>mCh</sup> (red) superimposed with the phase-contrast image (grey). 1003 1004 Cells are treated with indicated siRNAs. Cell contour is shown with dashed line. (I) Distribution of MT1-MMP<sup>mCh</sup>-positive endosomes in MDA-MB-231 cells treated with 1005 1006 indicated siRNAs. Mean percentage of MT1-MMP-positive endosomes according to 1007 their cell center-to-cell periphery position ± SEM from three independent experiments. (J) Proportion of MDA-MB-231 cells (%) with juxtanuclear clustered or 1008 1009 scattered MT1-MMPmCh endosome distribution from four to five independent 1010 experiments (Fig. S6C-E).

1011 Figure 6. Knockdown of CORO1C affects the distribution of MT1-MMP-positive 1012 endolysosomes and JIP4 and **CTTN** association. (A-C) Indirect immunofluorescence analysis of MDA-MB-231 cells expressing MT1-MMP<sup>mCh</sup> treated 1013 1014 with indicated siRNAs with CTTN (blue) and JIP4 (green) antibodies. DAPI-stained nuclei are shown in cyan. (D, E) CTTN (D) and JIP4 (E) association with the cytosolic 1015

1016 face of MT1-MMPmCh-positive endosomes analyzed based on images such as in 1017 panel A-C, compared with siNT-treated cells ± SEM from three independent 1018 experiments. n, number of cells analyzed for each population. **(F-I)** Electron 1019 microscopy of ultrathin sections of epon-embedded cells treated with indicated 1020 siRNAs. Notice the large aggregated endolysosomes in CORO1C-depleted cells 1021 (asterisks). N, nucleus; LE, endolysosome; m, mitochondria.







# Fat pad injection - MDA-MB-231/GFPCoro1C (8-week pi)



# Intraductal injection - MCF10DCIS.com/GFPCoro1C





