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EGFR activation and signaling in cancer cells are enhanced by the membrane-bound metalloprotease MT4-MMP

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Abstract

MT4-MMP (MMP-17) is a GPI-anchored matrix metalloprotease expressed on the surface of cancer cells which promotes tumor growth and metastasis. In this report, we identify MT4-MMP as an important driver of cancer cell proliferation through CDK4 activation and retinoblastoma protein (Rb) inactivation. We also determine a functional link between MT4-MMP and the growth factor receptor EGFR. Mechanistic experiments revealed direct association of MT4-MMP and its positive effects on EGFR phosphorylation in response to TGF- α and EGF in cancer cells. Notably, the effects of MT4-MMP on proliferation and EGFR activation did not rely on metalloprotease activity. Clinically, MT4-MMP and EGFR expression were correlated in human triple negative breast cancer specimens. Altogether our results identify MT4-MMP as a positive modifier of EGFR outside-in signaling that acts to cooperatively drive cancer cell proliferation.

Introduction

Tumor growth relies on cancer cell properties dysregulation associated with an intense host tissue remodeling. Mitogenic signals consist mainly of growth factors and extracellular matrix (ECM) components acting through cell surface receptors (1). Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor (RTK) whose activation by ligand binding (such as EGF, amphiregulin, transforming growth factor alpha or TGF- α) contributes to cancer cell proliferation, invasion and metastasis. EGFR activation and signaling are leading causes of malignancy in many types of cancer such as breast, lung, metastatic colorectal, head and neck, ovarian and brain cancers (2-7). Notably, in breast cancers, EGFR is expressed by triple negative breast cancers (TNBC) or basal-like breast cancers (BLBC), which are highly aggressive cancers with a high rate of early-occurring lung and brain metastasis.

Several proteinases are able to control the bioavailability and activity of growth factors (8, 9). These effects rely on the release of active molecules from the ECM, the cleavage of growth factors binding proteins or the shedding of receptor ligands from the cell surface (9-12). Among proteinases that modulate growth factor/growth factor receptor activities or bioavailability are the zinc-binding endopeptidase family including matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs) (13-15). For instances, MMP9 and MMP2 are responsible for the release of Vascular Endothelial Growth Factor (VEGF) sequestered into the ECM and the activation of Transforming Growth Factor-beta (TGF- β) (16, 17), whereas ADAM10 and TACE (ADAM17) shed several EGFR ligands from the cell surface such as TGF- α , amphiregulin and proHB-EGF (18). The transmembrane MT1-MMP has been reported to induce intracellular signaling through Scr (19-21) and MAP kinase cascade (12, 22, 23). In addition, MT1-MMP has been identified as a modulator of tyrosine kinase receptors (TKR) including receptor of Platelet-Derived Growth Factor (PDGFR) (24) and Fibroblast Growth Factor (FGFR2) (25). Two other MT-MMPs (MT4- and MT6-MMP)

are linked to the cell membrane through a glycosylphosphatidyl inositol (GPI) anchor (26). In sharp contrast to the known role of MT1-MMP-mediated signaling, a putative outside-in cell signaling through the GPI anchored MT-MMPs (MT4 and MT6-MMPs) is unknown. Although, most MMPs are produced by stromal cells, MT4-MMP (MMP17) is produced by tumor cells in human breast cancer samples (27). MT4-MMP emerged recently as a key intrinsic feature of breast cancer cells that stimulates tumor growth and metastasis into the lung (27-29). This impact on metastatic dissemination is related to changes in blood vasculature characterized by pericyte detachment, vessel enlargement and destabilization (28). The aim of the present study is to determine whether this GPI-anchored cell surface proteinase could contribute to an outside-in signaling involved in tumor aggressiveness.

Herein, by using *in vivo* xenografts and *in vitro* 3D multicellular spheroids embedded in Matrigel, we show that MT4-MMP promotes tumor cell proliferation by inducing EGFR activation and signaling. Together these results not only identify an unexpected role of MT4-MMP in cancer proliferation, but also establish a functional link between MT4-MMP and EGFR.

Materials and Methods

Cell culture and plasmids

Human breast cancer (MDA-MB-231, ZR-75-1 and BT549) cells, epidermoid cancer A431 cells, Hela cells and monkey epithelial COS-1 cells were purchased from American Type Culture Collection (ATCC). MDA-MB-435 cells were kindly provided by Dr. Christine Gilles from the GIGA at University of Liège, Belgium. All cell lines described above were authenticated within 1 year before being used in experiments. Cell lines authentication for interspecies contamination was performed by Leibniz-Institute DSMZ, GmbH, Braunschweig, Germany. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-Glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a 5% CO₂ humid atmosphere. All culture reagents were purchased from Invitrogen (Paisley, UK). Cell transfection and plasmid construction are described in supplemental experimental procedures.

In vivo Tumorigenicity

For subcutaneous (s.c.) injection of breast cancer cells, subconfluent MDA-MB-231 cells were collected in serum-free medium (5×10^6 cells/ml) and mixed with an equal volume of cold Matrigel according to previous report (30). Cell suspension (10^6 cells/400 µl) was injected s.c. into *RAG-1* immunodeficient mice in both flanks (n=6). All assays were repeated at least three times. For orthotopic injection of cells in the mammary fat pad (MFP), 8 weeks old (n=6) *RAG1*^{-/-} female mice were anesthetized and 50 µl of cell suspension (10^6 cells) was injected in the mammary gland after a small incision in the skin (0.5 cm). Tumor volumes were estimated every 3 to 4 days as described (length x width² x 0.4). All animal procedures

were performed according to the Federation of European Laboratory Animal Sciences Associations (FELASA) within the accredited GIGA animal facility (University of Liège).

RT-PCR and Western blot analyses

RNA and proteins extractions, as well as RT-PCR and Western blot analyses are described in supplemental experimental procedures.

Co-immunoprecipitation and Immunofluorescence assay

Co-immunoprecipitation of EGFR with MT4-MMP in COS1 cells transfected with FLAG tagged MT4-MMP and their co-localization in MDA-MB-231, BT549 and A431 cells transfected with MT4-MMP cDNA or Hela, MDA-MB-435 and ZR-75-1 cells endogenously expressing MT4-MMP are described in supplemental experimental procedures.

Immunohistochemistry

Proliferating cells were visualized on tumor sections by using antibodies raised against Ki-67 and vimentin as described in supplemental experimental procedures.

In vitro 3-dimensional proliferation assay and DNA quantification:

The 3D culture model and quantification of cell proliferation methods are described in supplemental experimental procedures.

Statistical analysis

Differences between experimental groups were assessed using Mann-Whitney test. $P < 0.05$ (*) were considered as significant. Statistical analyses were carried out using the Prism 4.0 software (GraphPad, San Diego, CA).

Supplemental information

Supplemental information includes 2 figures and supplemental experimental procedures.

Results

MT4-MMP promotes tumor cell proliferation *in vivo*

MDA-MB-231 cells overexpressing MT4-MMP or control cells were sub-cutaneously (s.c.) injected into *RAG-1*^{-/-} mice as previously described (27, 29). In accordance with our previous reports, MT4-MMP expression resulted in increased tumor growth as indicated by the higher volumes of MT4-MMP expressing tumors (MT4 tumors) than those of control tumors (CTR tumors) (Figure 1A). A tumor promoting effect of MT4-MMP was also observed when cells were orthotopically injected in the mammary fat pad (MFP) of female *RAG1*^{-/-} mice (Figure 1A). To assess the proliferation index *in vivo*, s.c. and MFP tumors were double-immunostained with human Ki-67 for proliferating cells and human vimentin (Figure 1B). A computerized method based on image binarization was used to determine the density of proliferating cancer cells (Ki-67 and human vimentin positive) (Figure 1B). A significant enhancement of the proliferation index was observed in MT4-MMP expressing tumors, in both heterotopic (s.c.) and orthotopic (MFP) sites.

MT4-MMP mitogenic effect is associated with increased Rb inactivation *in vivo*

The increased proliferation index observed *in vivo* led us to analyze the phosphorylation status of Retinoblastoma protein (Rb), a signaling molecule involved in cancer cell proliferation (Figure 2). Inactivation of Rb upon hyperphosphorylation is known to stimulate cell proliferation through the transcription of early genes required at the G1/S-phase transition. A significant increase in Rb phosphorylation at S807/811 was found in MT4-MMP expressing s.c. tumors, as compared to control tumors. Rb hyperphosphorylation at S807/811 appeared specific for MT4-MMP expressing tumors, since other serine residues such as S780, S795 and Thr 821 were not affected (data not shown). Rb inactivation is known to be dependent on a concurrent increased expression or activation of the cyclin/CDK/E2F

pathway or on downregulation of endogenous cyclin inhibitors. The immunoblot analysis of these putative regulators (Figure 2) revealed a significant increase in the amounts of cyclin D1, cyclin D3, cyclin E and CDK4 in MT4-MMP expressing tumors (Figure 2, $P < 0.005$). P27 protein cyclin/CDK complex inhibitor was also increased in MT4-MMP expressing tumors. This protein has been reported to play a dual role in cell proliferation, either inhibiting cell proliferation or contributing to cyclin/CDK complex assembly leading to cell proliferation (31, 32). The other cell cycle regulators tested (cyclin A, cyclin D2, CDK2, CDK6, p15 and p21) were not affected by MT4-MMP expression. Finally, E2F protein, the central transcription factor downstream of Rb pathway was upregulated in MT4-MMP expressing tumors (Figure 2). Altogether, these data demonstrate that MT4-MMP expression promotes breast cancer cell proliferation and tumor growth *in vivo*, which is associated with Rb activation pathway.

MT4-MMP promotes tumor cell proliferation *in vitro* in a 3D culture model

The cell proliferation rate in 2D-cultures on plastic dishes was not affected by MT4-MMP expression (Figure 3A). In accordance with our previous study (27), this result confirms that MT4-MMP tumor promoting effect is microenvironment-dependent. This observation prompted us to embed cells in a 3D matrix (Matrigel) in order to mimic the *in vivo* situation of tumor microenvironment. After 7 days, MDA-MB-231 cells formed spheroids that were larger upon MT4-MMP expression (Figure 3B). In these culture conditions, a significant increase in the proliferation rate of MT4-MMP expressing cells was evidenced by DNA quantification (Figure 3B). Remarkably, the increase in cell proliferation induced by MT4-MMP production was inhibited upon treatment with CDK4 inhibitor PD0332991, confirming the key involvement of Rb pathway *in vitro* (Figure 3C). In order to extend our study to other cell types, breast carcinoma BT549 and epidermoid A431 cell lines expressing MT4-MMP

were generated by a stable transfection with MT4-MMP cDNA and spheroids were induced by 3D culture. (Figure 3 D and E). Again, a MT4-MMP-mediated mitogenic effect was observed with BT549 and A431 cells. Together, these observations highlight the suitability of this 3D-culture system to investigate the outside-in signaling pathway activated by MT4-MMP and resulting in enhanced tumor cell proliferation.

MT4-MMP exerts its mitogenic effect through EGFR signaling

A pharmacological approach using small molecule inhibitors was next applied to provide further insights into the mechanisms underlying MT4-MMP action (Figure 4). Neither the inhibitors of MAPK/P38 kinase pathway (SB203580), MAPK/JNK pathway (SP600125) adenylate cyclase pathway (MDL-12) nor protein kinase C pathway (GF109203x), affected the MT4-MMP-mediated mitogenic effect (Figure 4A). In contrast, U0126, a MAPK/MEK1/2 inhibitor slightly reduced the proliferative phenotype in MT4-MMP expressing cells without affecting the proliferative rate in control cells (Figure 4A). The PI3K inhibitor LY294002 induced a strong inhibition of cell proliferation in MT4-MMP expressing cells. Similarly, genistein, a broad spectrum tyrosine kinase (TK) inhibitor (TKIs), significantly inhibited MT4-MMP-mediated mitogenic effect (Figure 4B). These data pointed to a functional interplay between MT4-MMP and TKs which results in downstream MAPK and PI3K activation.

The implication of receptor or non receptor tyrosine kinases was then explored by treating cells with inhibitors of EGFR including AG1478 and Erlotinib, its analogue used in clinic (Figure 4B), Src inhibitor (PP2 or 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol[3,4-*d*]pyrimidine) or Insulin-like Growth Factor Receptor (IGFR) inhibitor (AG538) (supplemental Figure 1A and B). Only the inhibition of EGFR signaling with tyrosine kinase inhibitors (AG1478 and Erlotinib) led to a strong reduction of the MT4-MMP-mediated-

mitogenic effect (Figure 4B). Erlotinib anti-proliferative effect on MDA-MB-231 cells expressing or not MT4-MMP is more efficient than AG1478. Structurally, Erlotinib and AG1478 share the same structural quinazole backbone but AG1478 lacks the hydrophylic side chains that may confer different properties to Erlotinib (33). Erlotinib inhibited also the MT4-MMP mediated proliferation in BT549 cells and has a slight effect on control cells (Figure 4C). Altogether these data demonstrate that MT4-MMP exerts its mitogenic effect through EGFR signaling.

MT4-MMP promotes EGFR ligand expression and EGFR phosphorylation.

MT4-MMP could exert its mitogenic effect by releasing or activating growth factors embedded in the matrix. However, this mitogenic effect was still observed when cells were embedded in Matrigel depleted with growth factors (Figure 5A). Unexpectedly, a mutated inactive form (MT4-E249A) of the enzyme expressed by MDA-MB-231 cells promoted cell proliferation in a similar extent than the wild type active form (Figure 5B). Thus, MT4-MMP is acting on cell proliferation independently on its proteolytic activity. While MT4-MMP effect does not require matrix remodeling, it is dependent on cell-matrix interactions as assessed by the use of Focal Adhesion Kinase (FAK) inhibitor (Figure 5C).

We next hypothesized that MT4-MMP can regulate EGFR ligand expression. RT-PCR analyses revealed enhanced mRNA levels of EGFR ligands in MT4-MMP expressing xenografts (Figure 5D) and cells embedded in 3D matrix (Figure 5E). No modulation of ligand production was seen in 2D culture (Figure 5F). The anti-EGFR TKI reduced this modulation of EGF mRNA levels observed in 3D cultures (Figure 5G), suggesting the implication of an EGFR signaling loop that regulates growth factor production in the presence of MT4-MMP and matrix compounds. In 3D cultures, the blockade of ligand binding to the receptor with a neutralizing EGFR antibody reduced the proliferation of cells expressing or

not MT4-MMP (supplemental Figure 2A). This indicates that the binding of ligand to EGFR is important for cell proliferation, but is independent to MT4-MMP status. However, anti-EGFR TKI AG1478 and Erlotinib (Figure 4B and C) abrogated the MT4-MMP effect more efficiently than the blocking antibody.

Dose-dependent and time course EGFR ligand dependent EGFR phosphorylation was performed in 2D culture by comparing control and MT4-MMP expressing MDA-MB-231 cells treated or not with EGF and TGF- α (Figure 6 A and B). MT4-MMP increases the ligand-induced EGFR activation in a dose-dependent manner for both EGF and TGF- α . For a single concentration of ligand, MT4-MMP cells displayed a rapid response after 5 min of incubation with ligands. The EGFR activation was sustained for 40 min and decreased after 60 min of incubation.

The analysis of EGFR phosphorylation in 2D cultures was next extended to different cancer cell lines (MDA-MB-231, BT-549 and A431 cells), transfected or not with MT4-MMP (Figure 6C). Importantly, EGFR phosphorylation in response to both growth factors was significantly enhanced in cells expressing MT4-MMP as compared to their corresponding control cells. Similar results were obtained in COS-1 cells known to produce high amounts of EGFR receptor and transiently transfected with active and inactive (MT4-E249A) MT4-MMP cDNAs (Figure 6D). It is worth mentioning that similar increase of EGFR phosphorylation induced by MT4-MMP expression was detected when cell were embedded in 3D matrix (supplemental Figure 2B). Notably, Erlotinib and anti-EGFR blocking antibody blocked EGFR phosphorylation at a similar extend (supplemental Figure 2B). However, the phosphorylation of the key downstream EGFR signaling molecule ERK1/2 was inhibited by Erlotinib but not by neutralizing blocking antibody. Altogether, these findings support the increased EGFR activation upon ligand stimulation in the presence of MT4-MMP that is not dependent on its proteolytic activity.

MT4-MMP interacts with EGFR in cancer cells

COS-1 cells transiently transfected with FLAG-tagged MT4-MMP (MT4-FLAG) and whole cell extracts were subjected to immunoprecipitation (IP) with an anti-FLAG antibody to pull down MT4-MMP before immunodetection with an anti-EGFR antibody (Figure 6E). EGFR was co-immunoprecipitated with MT4-MMP in cells transfected with MT4-MMP cDNA, whereas a slight non specific signal was detected in cells transfected with the control vector. Furthermore, MT4-MMP co-immunoprecipitated with EGFR when IP was conducted with an anti-EGFR antibody prior immunodetection for FLAG-tag (Figure 6F). No MT4-MMP was detected in control cells. This reverse IP further supports our finding that MT4-MMP physically interacts with EGFR. The inactive form of MT4-MMP (MT4-E249A) was also found to immunoprecipitate with EGFR in COS1 cells (Figure 6E and F), demonstrating that the formation of a complex between EGFR and MT4-MMP is not dependent on its proteolytic activity.

To determine whether MT4-MMP interacts with EGFR in cancer cells, MDA-MB-231 cells expressing Flag tagged MT4-MMP were co-immunostained with anti FLAG and anti-EGFR antibodies (Figure 7). Immunofluorescence analysis performed on stable transfectants of MDA-MB-231 cells expressing FLAG-tagged MT4-MMP revealed the presence of MT4-MMP and EGFR as yellow spots, at the cell surface and intracellularly (Figure 7A). The inactive form of MT4-MMP was also found to co-localize with EGFR in MDA-MB-231 cells (Figure 7A). MT4-MMP and EGFR were also co-localized in BT549 and A431 cells transfected with MT4-MMP (Figure 7B). Similar observation was done, although to a lesser extend in cells endogenously expressing MT4-MMP (Hela, MDA-MB-435 and ZR-75-1) (Figure 7C).

MDA-MB-231 cells being issued from TNBC, we extended our study to human samples of TNBC. MT4-MMP and EGFR stainings were found to be superimposed on the same tissue areas in 17/20 (85%) samples (Figure 7D). The 3 remaining samples were either double negative for MT4-MMP and EGFR or positive for a single marker. In sharp contrast, no MT4-MMP was found in normal breast tissues.

Discussion

The implication of the GPI-anchored MT4-MMP in breast cancer progression is supported by clinical data (27, 34, 35) and experimental observations (27-29). Until now, MT4-MMP functions have been ascribed to its capacity to regulate tumor vascularization. Herein, we describe a novel mechanism by which MT4-MMP promotes breast cancer cell proliferation *in vivo* and *in vitro* by promoting an outside-in signaling through the EGFR pathway in a non proteolytic manner. Our data highlight a central role for MT4-MMP in cancer cell proliferation and identify this enzyme as a partner of EGFR increasing tumor cell sensitivity to EGFR ligands. This mitogenic effect of MT4-MMP is supported by data generated using different cell lines.

The proliferative advantage conferred to tumors by MT4-MMP expression correlated with a strong inactivation of the tumor suppressor Rb protein that plays a pivotal role in the negative control of the cell cycle. The increase in Rb phosphorylation was associated with enhanced expression of cyclins D1, D3, E and CDK4 in MT4-MMP tumors. Rb hypophosphorylation represses gene transcription required for G1 to S phase transition, by binding to the promoter of targeted genes as a complex formed with E2F. Interestingly, E2F expression was increased in MT4-MMP tumors, supporting the implication of pRB pathway in MT4-MMP-mediated mitogenic effects *in vivo*. In line with this finding, the inhibition of CDK4 in the 3D *in vitro* culture model abolished the proliferation of MT4-MMP expressing cells but not that of control cells. These data provide evidence for a novel oncogenic function of MT4-MMP by suppressing Rb activity, thereby promoting cancer cell proliferation.

Through a pharmacological approach, we identify tyrosine kinases as targets of MT4-MMP-mediated effect and more specifically EGFR as a potential molecular partner. Accordingly, EGFR stimulates cell proliferation through Rb signaling pathway (36-38). Aberrant EGFR signaling is a major feature of breast cancers, particularly in highly

aggressive TNBC where EGFR pathway appears as a potential therapeutic target (39). Notably, both MT4-MMP and EGFR were immunodetected in human TNBC samples supporting the clinical significance of our finding. We found a significant increase in EGFR ligand (TGF- α , EGF and amphiregulin) expression, at the mRNA levels, in MT4-MMP tumors and in 3D cultures. Interestingly, EGF mRNA expression was partially reduced by anti-EGFR TKI. It is likely that the increased EGFR phosphorylation induced by the presence of MT4-MMP results in an EGFR signaling loop (40-42) that can reinforce the mitogenic effect of MT4-MMP. In addition, in these conditions, the anti-EGFR TKIs that inhibit EGFR phosphorylation abolished the proliferation of MT4-MMP expressing cells. In sharp contrast, EGFR neutralizing antibody that blocks EGFR ligand binding only partially inhibited the proliferation of MT4-MMP expressing cells and has similar effect on control cells. This difference observed between TKIs and neutralizing antibody could be explained by different capacity to induce sustained inhibition of EGFR downstream signaling molecule. Indeed, ERK1/2 activation was inhibited by Erlotinib but not by the antibody. Our data are in agreement with the report of Carrasco-Garcia et al. (33) in which they have compared the anti-proliferative effects of small molecule inhibitors of EGFR (AG1478, Erlotinib) and Cetuximab (the clinically used anti-EGFR antibody) on glioblastoma cell proliferation. This study demonstrated that EGFR TKIs are able to sustain the inactivation of ERK1/2, while Cetuximab did not. It is worth mentioning that TKI can also inhibit other members of the EGFR family such as erbB3 and erbB4, whereas Cetuximab only inactivates EGFR (erbB1). Thus, the differential effects of anti-EGFR drugs used herein might be related to the heterodimerization of EGFR with other members of the EGFR family such as erbB3 and erbB4 that could be affected by the presence of MT4-MMP. Altogether, our data provide clear evidence that MT4-MMP control EGFR phosphorylation and signaling.

Evidence for an interaction between EGFR and MT4-MMP was provided by immunoprecipitation and immunofluorescence experiments where both MT4-MMP and EGFR were found in immunoprecipitates obtained with anti-EGFR or MT4-MMP antibodies and co-localize at the cell surface and in the cytoplasm of breast cancer cells transfected or not with MT4-MMP. Due to the protein overexpression upon cell transfection, this co-localization was stronger in cells transfected with MT4-MMP than in cells endogenously producing MT4-MMP. Mechanistically, beside the capacity of MT4-MMP to interact with EGFR, we provide evidence that MT4-MMP is an activating partner for EGFR. Indeed, EGFR phosphorylation was observed in the presence of MT4-MMP upon stimulation with EGFR ligands in 4 different cell lines. Furthermore, these effects are not dependent on the catalytic function of MT4-MMP as demonstrated by the use of the mutated inactive form of the enzyme.

Our findings clearly establish a novel functional link between MT4-MMP and EGFR and give new insight into how a GPI-anchored MMP contributes to EGFR signaling in breast cancers. A proliferative advantage attributed to cancer cells by membrane-associated MMP expression has been previously reported. Indeed, MT1-MMP overexpression in cancer cells promotes tumor cell proliferation in 3D collagen matrix and tumor growth *in vivo* (43, 44). In addition, MT1-MMP expression in breast cancer confers protection against type I collagen-mediated apoptosis (45). In contrast to MT1-MMP, MT4-MMP endows cells with a proliferative advantage in Matrigel, but not in a collagen gel. Indeed, cell proliferation was not affected by MT4-MMP expression when cells were cultured in 3D type I collagen matrix or were first allowed to form spheroids in methyl cellulose and then embedded in type I collagen matrix (data not shown). The failure of MT4-MMP to induce proliferation when cells are mixed in 3D collagen matrix might be ascribed to its inability to cleave type I collagen in contrast to MT1-MMP (26). Alternatively, these observations combined with the

absence of a mitogenic effect of MT4-MMP in 2D-cultures (27, 29) suggest that MT4-MMP proliferating phenotype is dependent on matrix-induced cell aggregation, which could affect TK-mediated signal transduction. In addition, it is worth mentioning that 3D culture conditions can affect the homodimerization or heterodimerization of EGFR family member and downstream signaling pathway (46). Thus 3D matrix conformation could influence cell-cell interactions (47) and modulate MT4-MMP-EGFR interactions. FAK and EGFR are constitutively associated in promoting cancer cell survival (48, 49) and inhibition of FAK was shown to inhibit EGF-stimulated motility (50). In the context of our study, FAK inhibition in 3D matrix abolished the MT4-MMP/EGFR mediated mitogenic effect. This data emphasizes the importance of cell-matrix interaction in integrating proliferation signals for EGFR, a process that is not possible in 2D culture model.

Additionally, similar mitogenic effect of MT4-MMP observed in complete Matrigel as well as in growth factor-depleted Matrigel demonstrates that this effect does not rely on the release of growth factors sequestered in the matrix, but rather suggests that MT4-MMP can directly affect cell proliferation by enhancing the transduction of mitogenic signals through EGFR activation. Surprisingly, this novel function of MT4-MMP is independent of its catalytic activity as assessed by the use of a mutant proteolytically inactive form. These data point to unprecedented non catalytic function of MT4-MMP on cell proliferation. This differs from its angiogenic effect requiring its proteolytic activity (29). Indeed, we previously demonstrated that the inactive MT4-MMP E249A form failed to induce an early angiogenic switch leading to tumor growth. This is in line with data reported with MT1-MMP, which induces intracellular signaling and promotes cancer cell migration in a non proteolytic manner (22, 23) . These novel findings shed light on the catalytic dependent effect of MT4-MMP during the angiogenic switch (29) and the non proteolytic mitogenic effect leading to tumor growth and metastatic dissemination.

Altogether our data establish an unexpected link between MT4-MMP and EGFR pathway and highlight the importance of the GPI-anchored MMP in breast cancer etiopathology. Although the targeted anti-EGFR strategy has shown considerable clinical promise in colorectal and lung cancers, minimal activity of anti-EGFR drugs is seen in TNBC that are very aggressive cancers. In addition, most patients do not respond or become resistant to targeted strategies. Our data demonstrate a novel interplay between a membrane-associated proteinase (MT4-MMP) and EGFR produced by TNBC cells. These findings open new opportunities for designing novel therapies targeting the non-proteolytic functions of MT4-MMP. The combination of such anti-MT4-MMP agents and anti-EGFR drugs might improve the treatment of EGFR dependent cancers and/or to overcome acquired resistance to anti-EGFR strategies used currently in clinics.

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Figure legends

Figure 1: MT4-MMP expression promotes cell proliferation in tumors. Control cells (CTR) and MT4-MMP expressing cells (MT4-MMP) were subcutaneously (s.c.) or orthotopically injected into mammary fat pad (MFP) of *Rag1* ^{-/-} mice. Mice were sacrificed at day 21 for the s.c. injection and day 45 for the MFP injection (n=6). (A) Tumor volume of the s.c. (left panel) and MFP tumors (right panel). (B) Representative images illustrating the immunohistochemistry detection of proliferating cells in s.c. tumor sections by double immunostaining using human Ki-67 antibody (brown staining) and human vimentin antibody (red staining) (upper panels, primary image). Binarized images were used to calculate the percentage of Ki-67⁺ cells (brown color) among cells positive for vimentin (red color, lower panels, binary image). Results are expressed as the ratio of Ki-67⁺ cells to vimentin⁺ cells in s.c. (left) and MFP (right) tumors. * $p < 0,05$; ** $p < 0,01$.

Figure 2: MT4-MMP-dependent cell proliferation is associated with Rb phosphorylation and cyclin-dependent kinase in tumors. Tumors collected at day 21 were analyzed for production of cell proliferation regulator proteins: Rb and its phosphorylated form at (S807-811), cyclins (-A, -D1, -D2, -D3 and -E), cyclin-dependent kinases (CDK2, CDK4 and CDK6) and inhibitors (P15, P21 and P27). MT4-MMP tumors showed hyperphosphorylation of Rb and higher levels of cyclin D1, D3, E, CDK4 and P27. Representative western blots are shown for 3 different tumors out of 4 (n=4) and densitometry quantifications (Right panels) are those from 4 different tumors. Actin is used as a loading control except for pRb (S807-811) where total Rb is used. * $p < 0,05$; ** $p < 0,01$.

Figure 3: MT4-MMP induces cell proliferation in 3D culture matrix. MDA-MB-231 cells transfected with MT4-MMP (MT4) or control vector (CTR) were incubated in 2D plastic dishes (A) or 3D Matrigel (B). Representative images of cell culture are shown on the left (original magnification 20x). Cell proliferation was assessed by DNA measurement ($\mu\text{g/ml}$) (graphs). Cells were incubated in 3D Matrigel with CDK4 inhibitor (PD0332991) (C). Proliferation of BT549 (D) and A431 (E) cells transfected with MT4-MMP cDNA (MT4) or control vector (CTR) in 3D Matrigel matrix. * $p < 0,05$; ** $p < 0,01$.

Figure 4: MT4-MMP-dependent mitogenic effect is dependent on tyrosine kinase activity and EGFR signaling. Cells expressing MT4-MMP (MT4) and control (CTR) cells were incubated in 3D Matrigel with or without inhibitors. (A) MDA-MB-231 cell proliferation in the presence or absence of MEK1/2 (U0126), JNK (SP600125), PI3K (Ly-294002), adenylate cyclase (MDL-12) and PKC (GF109203x) inhibitors. (B) MDA-MB-231 cell proliferation with or without genistein, EGFR (AG1478 and Erlotinib) inhibitors. (C) BT549 cell proliferation with or without Erlotinib. Cell proliferation was assessed after 7 days of incubation. Results are expressed as DNA content ($\mu\text{g/ml}$). * $p < 0,05$; ** $p < 0,01$.

Figure 5: MT4-MMP promotes EGFR ligand expression and EGFR phosphorylation in tumors. (A) MT4-MMP promotes cell proliferation growth factor-depleted Matrigel. (B) Cell proliferation in 3D culture of MDA-MB-231 cells transfected with active (MT4), inactive (MT4-E249A) forms of MT4-MMP or control vector (CTR). (C) Cell proliferation of MDA-MB-231 cells expressing MT4-MMP (MT4) and control (CTR) cells incubated in Matrigel with or without Focal Adhesion Kinase (FAK) inhibitor PF573228. (D-F) RT-PCR analysis of EGFR ligands in MT4-MMP expressing tumors (MT4), control (CTR) tumors *in vivo* (D) or *in vitro* in 3D culture (E) and in 2D culture (F). Graphs correspond to the densitometric

quantification of RT-PCR analysis showing a significant increase of EGFR ligand mRNA in MT4-MMP tumors and spheroids. (G) RT-PCR analysis of EGF expression in cells expressing (MT4) or not (CTR) MT4-MMP incubated in 3D matrix and in the presence or not of Erlotinib. * $p < 0,05$; ** $p < 0,01$.

Figure 6: EGFR phosphorylation is enhanced in the presence of MT4-MMP. (A-B) Ligand dependent EGFR phosphorylation in MDA-MB-231 cells expressing or not MT4-MMP cultured in 2D. Cells were serum-starved for 1 h and incubated for 40 min with increasing concentrations of EGF (left panel) or at different incubation time with EGF (20 ng/ml) (right panel) (A). Cells were incubated with for 40 min with increasing concentrations of TGF α (left panel) or at different incubation time with TGF- α (10 ng/ml) (right panel) (B). EGFR phosphorylation status was analyzed in total cell extracts by western blot using antibodies against phospho-EGFR (Tyr 1068) and total EGFR. Membranes were stripped and re-incubated with total EGFR and actin for loading control. (C) MDA-MB-231, BT549, A431 cells expressing or not MT4-MMP were serum-starved for 1 h and incubated with or without TGF- α (10 ng/ml) or EGF (20 ng/ml) for 40 min, and EGFR phosphorylation status was analyzed in total cell extracts by western blot using antibodies against phospho-EGFR (Tyr 1068) and total EGFR. (D) COS-1 cells transiently transfected with MT4-MMP (MT4) or its inactive form (MT4-E249A) cDNA or control vector (CTR) were serum-starved for 1 h and incubated with TGF- α (10 ng/ml) or EGF (20 ng/ml) for 20 min and EGFR phosphorylation was analyzed by western blot for phospho-EGFR (Tyr 1171) and total EGFR. Cell incubation with EGFR ligands induced a more pronounced EGFR phosphorylation in the presence of MT4-MMP or its inactive form MT4-E249A. Graphs correspond to the ratio of pEGFR/EGFR after densitometry quantification using Quantity-One software. (E-F) Co-immunoprecipitation of EGFR and MT4-MMP. (E) Total cell lysates from COS-1 cells transfected with an empty

vector (control vector) or a vector containing MT4-MMP cDNA with a FLAG-tag insertion (MT4-MMP-FLAG) (left panel) or inactive form of MT4-MMP (MT4-E249A-FLAG) cDNA (right panel) were immunoprecipitated with FLAG antibody prior to western blots for EGFR. For loading control (lower panels), membranes were stripped and reincubated with the FLAG antibody. (F) Total cell lysates from COS-1 cells were immunoprecipitated with EGFR antibody prior western blot for FLAG (right panel) or EGFR for loading control (left panel). Mouse or rabbit IgG controls were used for immunoprecipitation for FLAG and EGFR after incubation with cell lysate from COS1 cells transfected with MT4-MMP-FLAG cDNA.

Figure 7: MT4-MMP is detected with EGFR in cancer cells and in human TNBC tumors.

Detection of MT4-MMP and EGFR in breast cancer cells by immunofluorescence and confocal microscopy. (A) Flag tagged MT4-MMP or its inert form MT4-249A expressing MDA-MB-231 cells were cultured on cover slips and stained with anti-FLAG (green) and rabbit anti-EGFR antibodies (red). (B) BT549 and A431 cells transfected with MT4-MMP cDNA or (C) Hela, MDA-MB-435 and ZR-75-1 cells expressing MT4-MMP endogenously were stained with MT4-MMP (green) or EGFR (red). Co-localization of MT4-MMP (left panels) and EGFR (middle panels) are shown as yellow spots (arrows in merged images, right panels). Data are those of three independent experiments. (D) Immunohistochemical staining of MT4-MMP and EGFR on human breast samples. MT4-MMP is not immunodetected in normal breast tissues (top panel). Both EGFR and MT4-MMP are expressed by cancer cells in the same areas in serial sections of TNBC (left panels and right panels, respectively). Two representative samples are shown with a higher magnification of the area corresponding to the dashed square. Scale bar = 100 μ m.

Figure 1

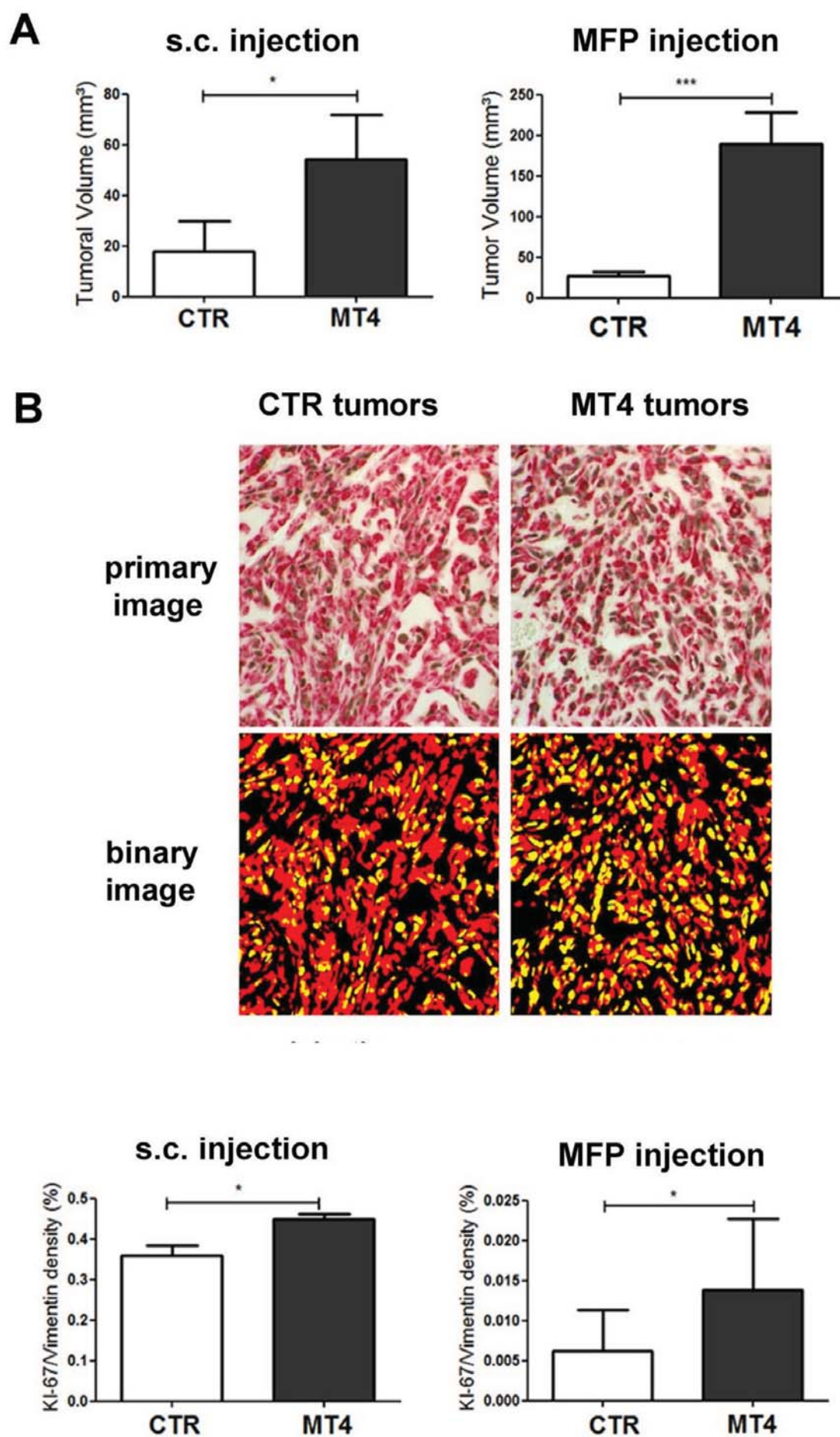


Figure 2

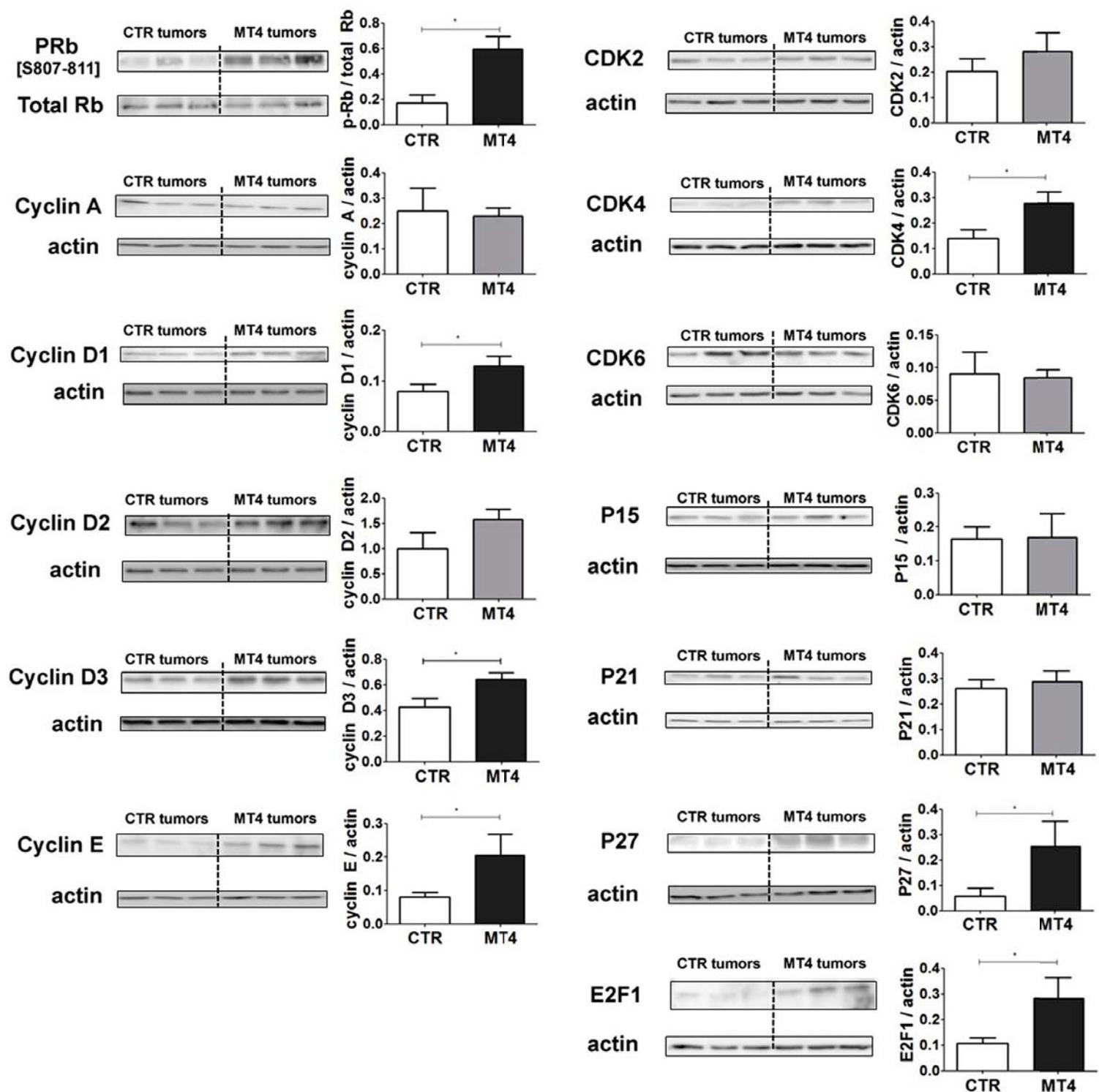


Figure 3

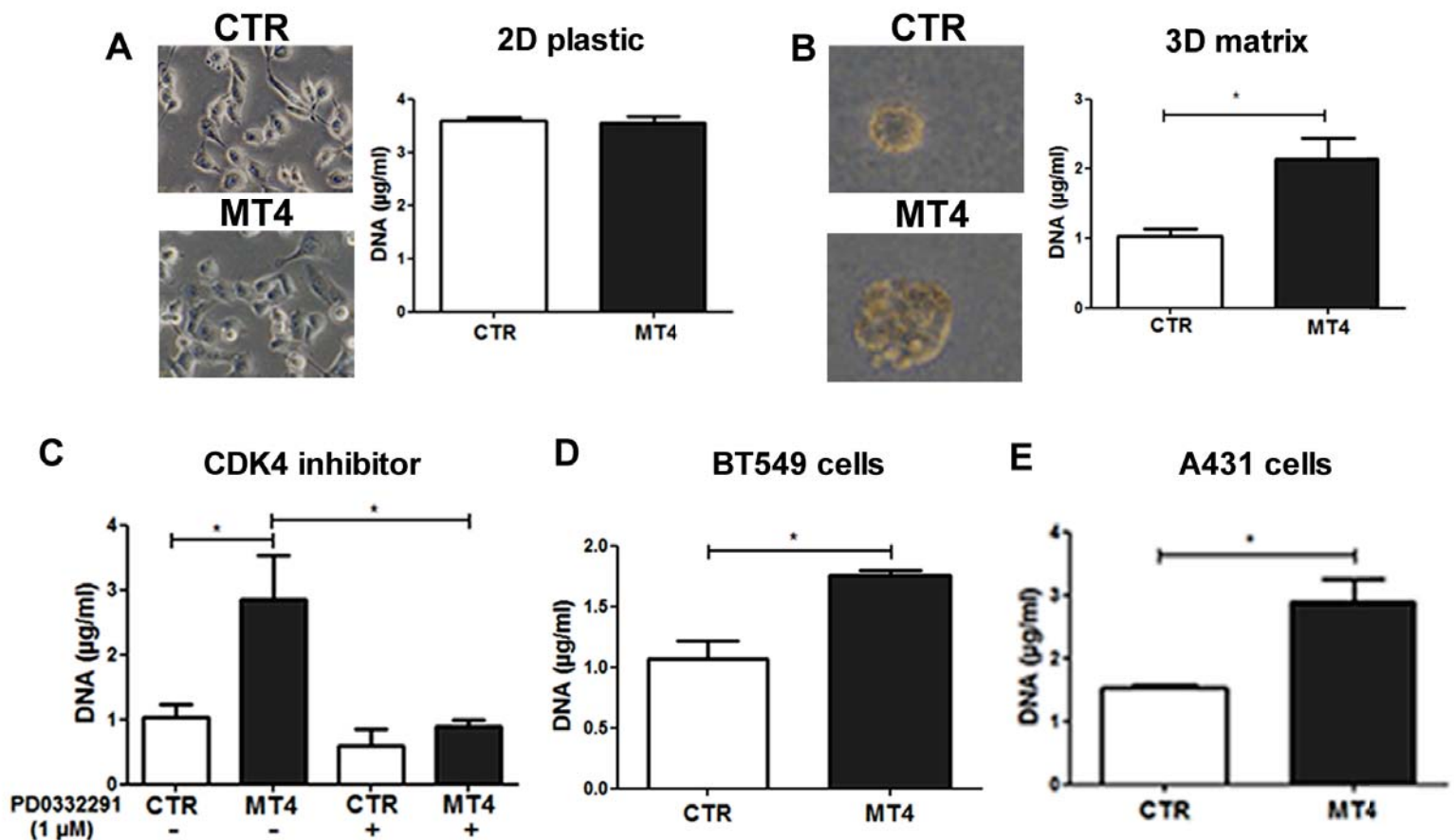


Figure 4

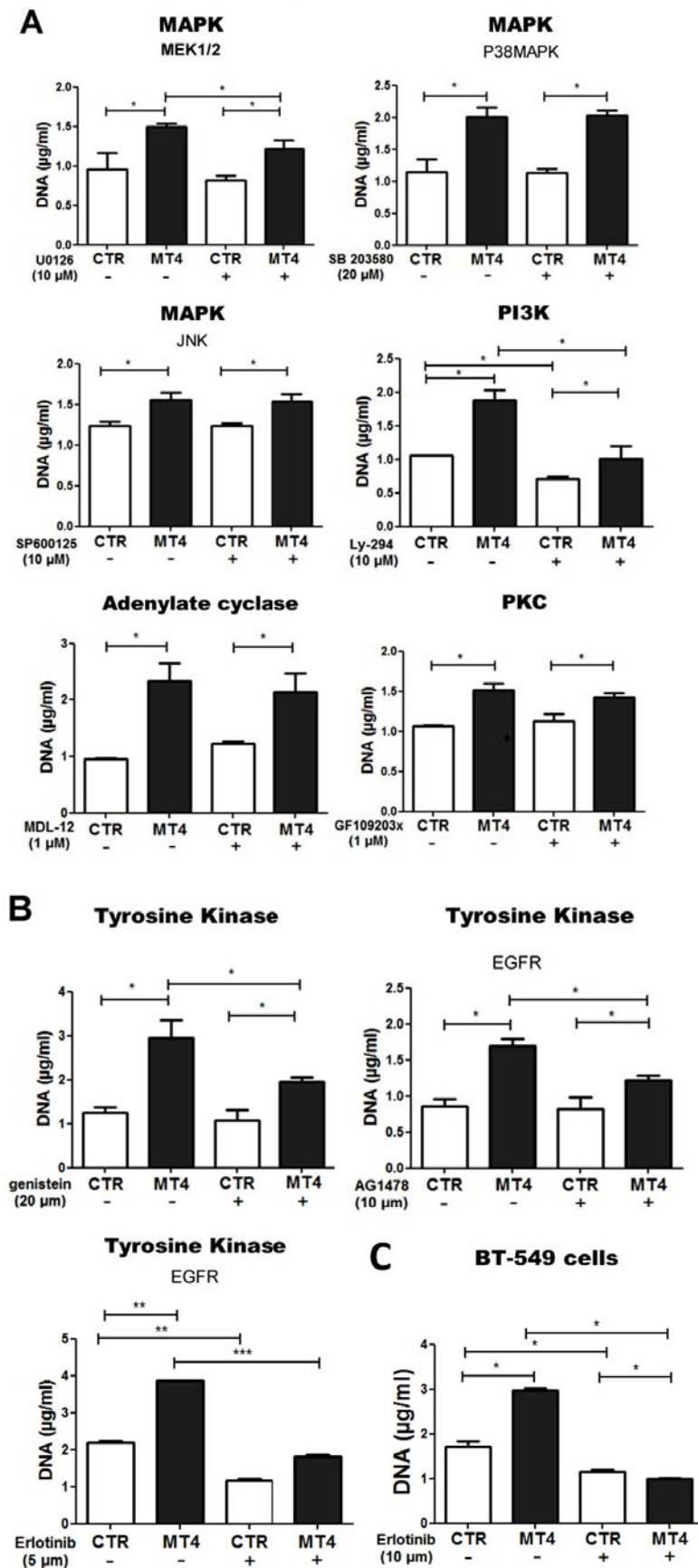
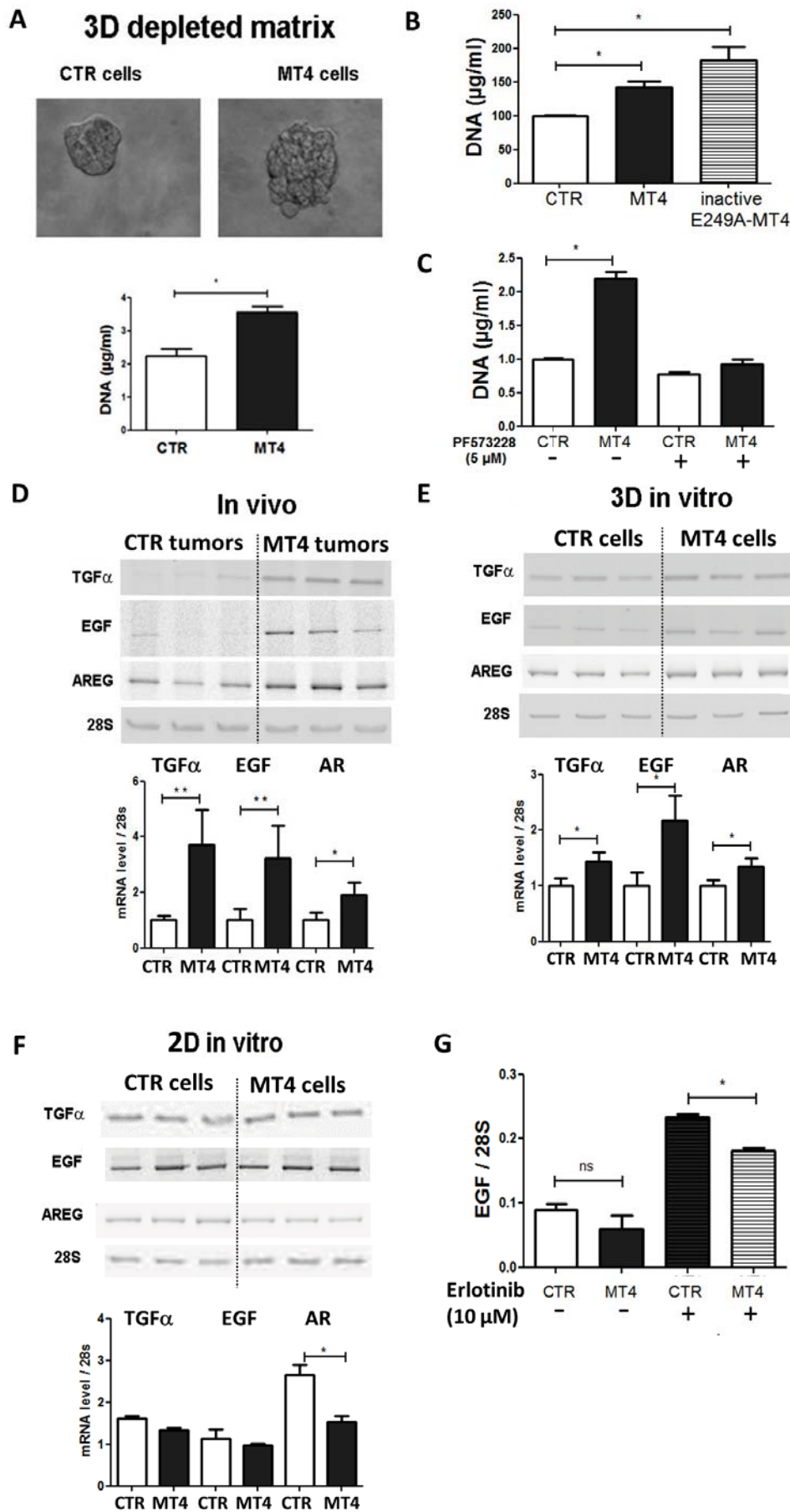
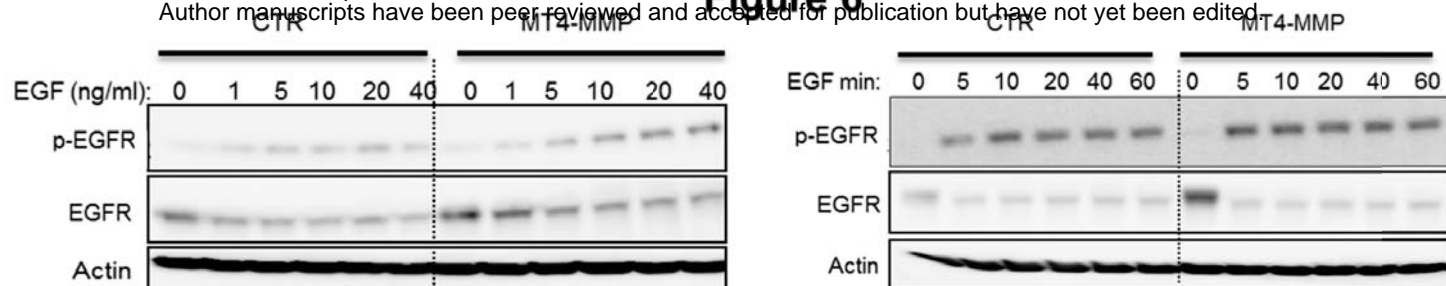


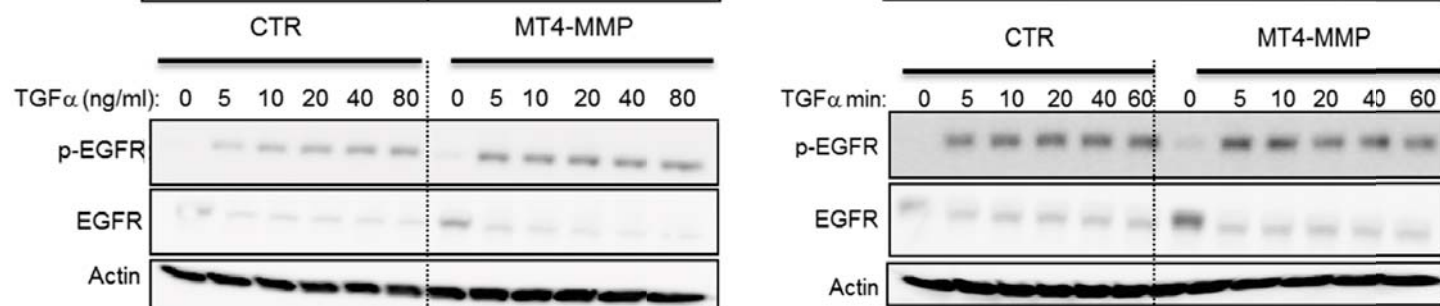
Figure 5



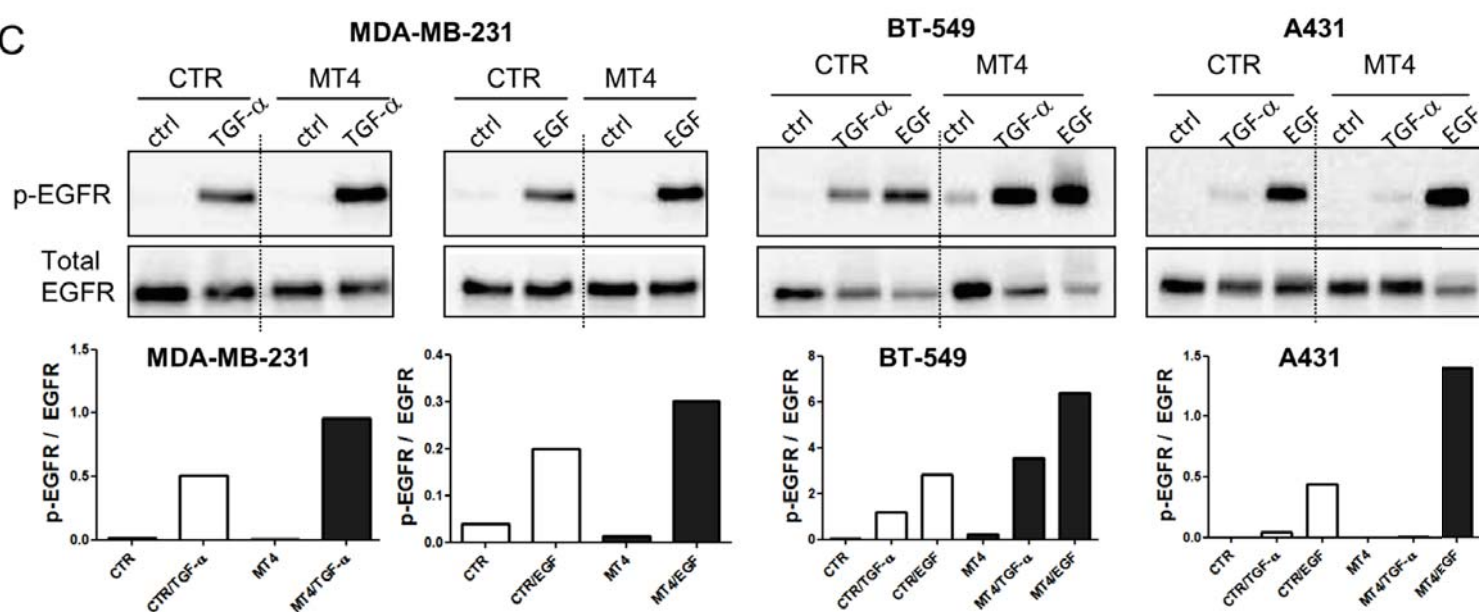
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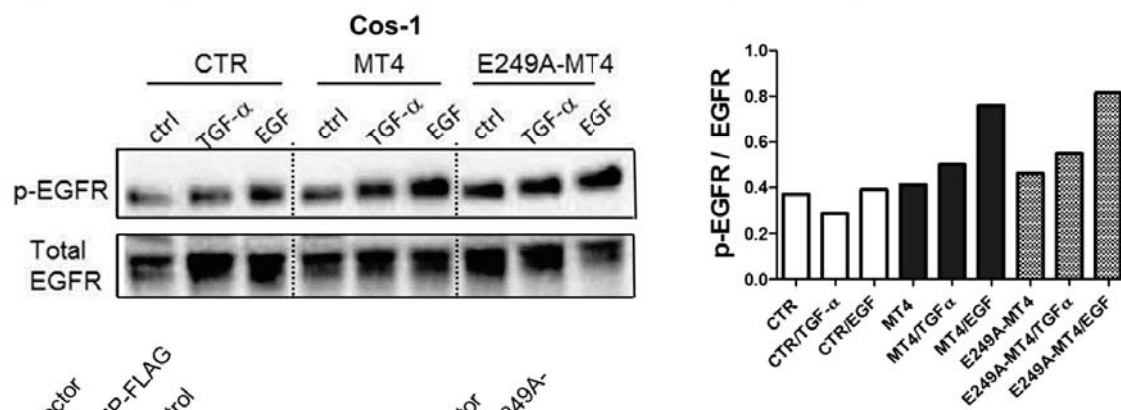
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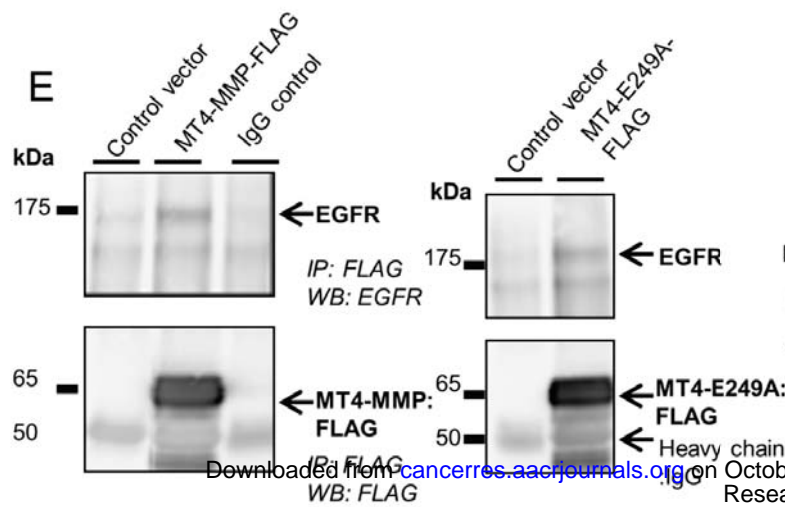
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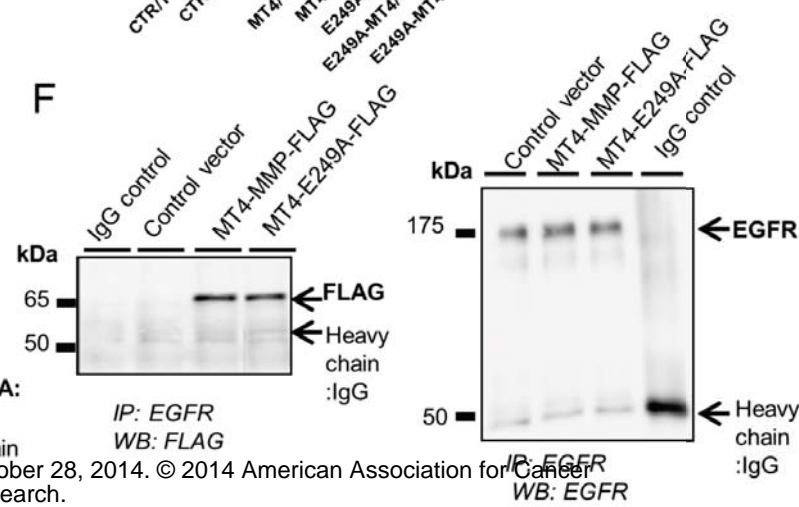
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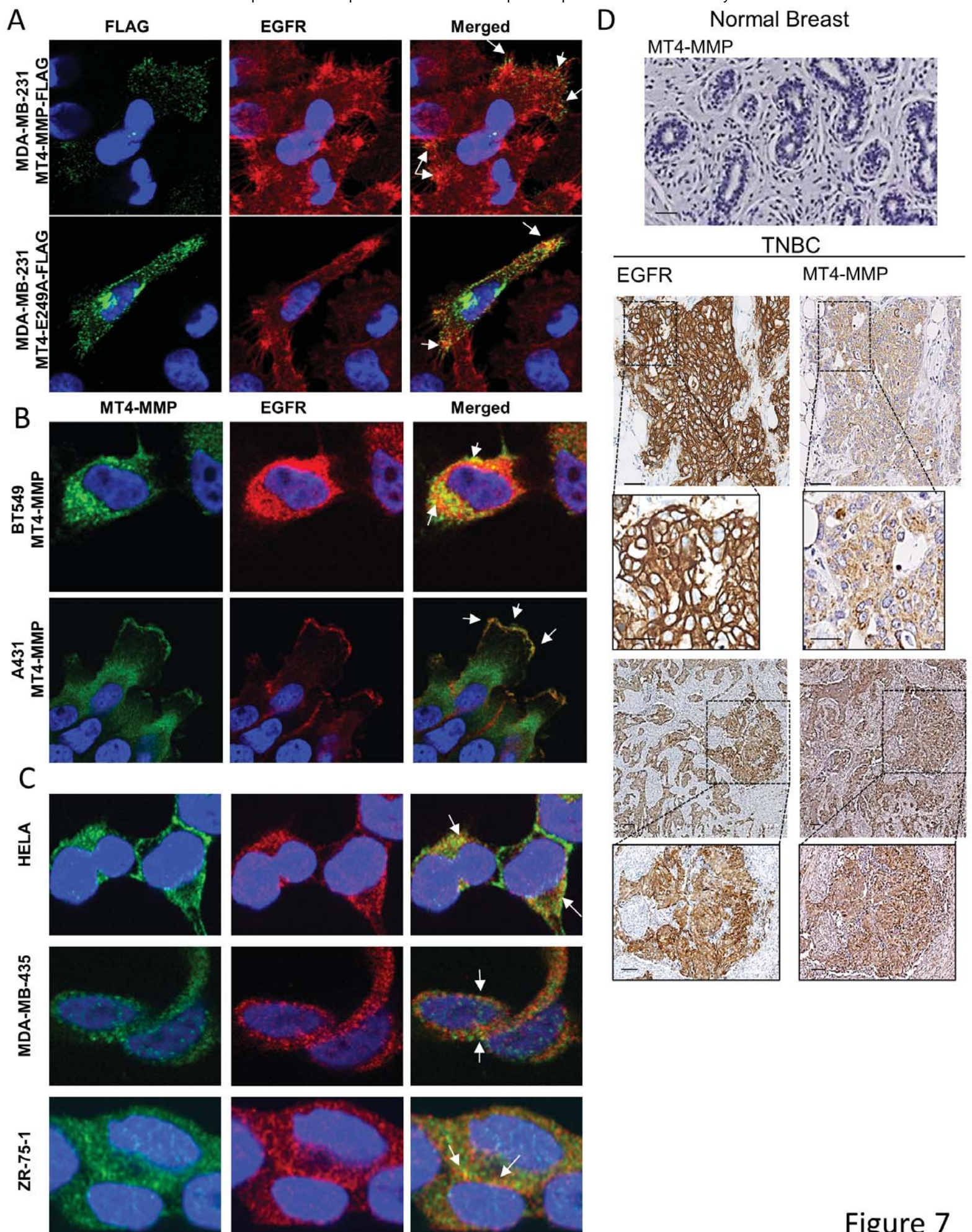


Figure 7