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► **To cite this version:**

Yoshifumi Itoh, Ralf Palmisano, Narayanapanicker Anilkumar, Hideaki Nagase, Atsushi Miyawaki, et al.. Dimerization of MT1-MMP during cellular invasion detected by fluorescence resonance energy transfer. *Biochemical Journal*, 2011, 440 (3), pp.319-326. 10.1042/BJ20110424 . hal-00658151

HAL Id: hal-00658151

<https://hal.science/hal-00658151>

Submitted on 10 Jan 2012

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DIMERIZATION OF MT1-MMP DURING CELLULAR INVASION DETECTED BY FLUORESCENCE RESONANCE ENERGY TRANSFER*

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*This work was supported by Cancer Research UK project grant C1507/A5541, the Wellcome Trust Grant 075473, and Arthritis Research UK Core grant to the Kennedy Institute of Rheumatology.

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Short Title: MT1-MMP dimer detected by FRET imaging

Key Words: MT1-MMP, dimer, ECM, FRET, cell invasion, cytoskeleton

Abbreviations: MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; FRET, fluorescence resonance energy transfer; ECM, extracellular matrix; Sapph-GFP, sapphire green fluorescent protein; mRFP1, monomeric red fluorescent protein 1; Hpx, hemopexin; GSK3 β , glycogen synthase kinase-3 β .

ABSTRACT

Homo-dimerization of the membrane-bound collagenase MT1-MMP is crucial for its collagenolytic activity. However, it has not been clear if this dimerization is regulated during cellular invasion into 3D collagen matrices. To address this question, we established a fluorescence resonance energy transfer system to detect MT1-MMP dimerization and analysed the process in cells invading through 3D collagen. Our data indicates that dimerization occurs dynamically and constantly at the leading edge of migrating cells but not the trailing edge. We found that polarised dimerization was not due to ECM attachment, but was rather controlled by reorganization of the actin cytoskeleton by the small GTPases, Cdc42 and Rac1. Our data indicate that cell surface collagenolytic activity is regulated coordinately with cell migration events to enable penetration of the matrix physical barrier.

INTRODUCTION

Cell migration is a dynamic process that requires orchestration of many different molecules. Cells attach to the extracellular matrix (ECM) scaffold through receptors such as integrins, and then polarize towards the direction of migration by re-organizing the cytoskeleton by the action of small GTPases [1, 2]. The cell body is then pulled forward when integrins at trailing edge are disengaged from ECM. The coordination of these events occurs continuously to maintain the migratory phenotype [1, 2]. When cells migrate in a 3D environment, the ECM is not only a scaffold but also a physical barrier, especially in the case of fibrillar collagens [3]. In order to migrate through the matrix barrier, cells need to degrade matrix components using proteinases to make a path. Such ECM degradation needs to occur at the leading edge of the cells in a highly polarised manner during cell migration as indiscriminate degradation of ECM scaffold would be detrimental, compromising mechanotransduction. However, the regulatory mechanisms enabling polarised proteolytic activity on the cell surface during cell migration is not clearly understood.

One of the proteolytic enzymes that effectively promotes cell migration in a 3D ECM environment is membrane type 1 matrix metalloproteinase (MT1-MMP) [3-5]. MT1-MMP is a type I transmembrane proteinase that modifies the cellular microenvironment, cleaving a variety of pericellular ECM components including fibrillar collagens, laminins 1 and 5, fibronectin, vitronectin, fibrin and aggrecan core protein [5, 6]. It also cleaves cell adhesion molecules such as CD44, syndecan 1 and α V integrin, modifying ECM signalling [5, 6]. MT1-MMP activates proMMP-2 and proMMP-13 on the cell surface, expanding the repertoire of pericellular proteolytic activities [5, 6]. With these diverse biological activities, MT1-MMP has been shown to play important roles by promoting cellular migration in different physiological and pathological events including skeletal development, lung development, and wound healing, cancer invasion, cancer growth, tumour angiogenesis, chronic inflammation, atherosclerosis, aneurism and rheumatoid arthritis [7-10].

MT1-MMP is regulated at different levels including gene expression, activation of its zymogen by proprotein convertases, exocytosis, cell surface expression, homo-dimerization, proteolytic processing, ectodomain shedding, inhibition by endogenous inhibitors including tissue inhibitors of metalloproteinases (TIMPs), RECK, and Testicans, localisation to lamellipodia and invadopodia, endocytosis, lysosomal degradation and recycling [6]. These regulatory mechanisms are thought to be essential to coordinate MT1-MMP activity with dynamic cellular events. During cellular invasion through a 3D matrix, MT1-MMP activity needs to be present at the leading edge of cells in a polarized manner. It is thought that localization of MT1-MMP to the motility-associated membrane structures including lamellipodia and invadopodia is an important regulatory mechanism [11]. MT1-MMP on the cell surface has been shown to be distributed highly at the leading edge and trailing edge, and modestly over the cell body in 3D matrix [12]. Thus regulation of its localization alone may not be sufficient to achieve polarized MT1-MMP activity during invasion, and additional regulatory mechanisms may be necessary.

ProMMP-2 activation and fibrillar collagen degradation are thought to be important functions of MT1-MMP. Both of these activities uniquely require MT1-MMP to be in a dimeric form on the cell surface, and inhibition of dimerization effectively inhibits these activities [13-16]. Since these activities, especially collagen degradation, are closely related to cellular invasion, dimerization of MT1-MMP is also expected to associate with migration events. However, whether MT1-MMP dimerization is regulated during cell migration is an enigma. In this study, we established a fluorescence resonance energy transfer (FRET) system to detect dimerization of MT1-MMP in live cells. This study indicates that dimerization of the enzyme is dynamic, and that it occurs consistently at the leading edge, but not at the trailing edge, of cells migrating in a 3D collagen matrix. We also observed that dimerization is not

regulated by an outside-in signal from the ECM during migration, but by an inside-out signal involving actin cytoskeletal re-organization through the activity of Cdc42 and Rac1 small GTPases, events closely associated with cell migration.

EXPERIMENTAL PROCEDURES

Cell culture and transfection

HT1080 human fibrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (BioWhittaker). Cells were transfected with expression plasmids using FUGENE6TM (Roche, Basel, Switzerland) according to the manufacturer's instructions. For 3D collagen cultures, we used acid-soluble collagen I without pepsin treatment (Nitta gelatin, Osaka, Japan), neutralized and final concentration adjusted to 2 mg/ml. Cell-collagen suspension was added to glass coverslip-bottomed 4-well chamber and incubated at 37 °C to set a gel. DMEM with 10 % FBS was added and cells cultured for live cell imaging.

Construction of expression plasmids

FLAG (DYKDDDDK)-tagged MT1-MMP (MT1F) was constructed as previously described [17] and subcloned into pSG5 (Stratagene, CA, USA). MT1F-ΔCat is a FLAG-tagged mutant MT1-MMP in which the region of Tyr112 to Pro312 was deleted. MT1F-mRFP1 and MT1F-Sapph are chimeric mutants of MT1F and mRFP1 and Sapph-GFP, respectively. The fluorescent protein was inserted between Cys574 and Gln575 in the cytoplasmic domain of MT1F with two glycines at the N-terminus of these fluorescent proteins as a linker. Constitutively active form of Rac1 (Rac1CA) is a G12V mutant, dominant negative form of Rac1 (Rac1DN) is a T17N mutant, constitutively active form of Cdc42 (Cdc42CA) is a G12V mutant, and dominant negative form of Cdc42 (Cdc42DN) is a T17N mutant [18], and subcloned into pSG5. All the mutants were generated by PCR extension methods and confirmed by DNA sequencing.

Western blotting and Zymography

Western blotting was carried out as described previously [19]. Total cell lysates were prepared by addition of 1 x SDS-PAGE loading buffer containing 2-mercaptoethanol to cells in the culture plate and subsequent boiling for 20 min. Zymography was carried out as described previously [19].

In situ collagen degradation assay

The experiments were done as described previously [15]. 6-well culture plates were coated with a thin-layer of chilled neutralised PureColTM collagen (Inamed Biomaterials, Fremont, CA, USA) at 2.7 mg/ml in 1 x RPMI medium (typically 100 ml /well), and incubated for 60 min at 37 °C for fibril formation, and COS7 cells (4 x 10⁵/well) were then seeded on the film. 18 hours later, cells were transfected with the expression plasmids in the growth medium (10 % FBS/DMEM) using FUGENE6TM according to the manufacturer's instructions. The following day, culture medium was changed to serum-free DMEM, and cells were cultured for a further 3 days at 37 °C. The remaining collagen film was exposed by removing cells using repeated treatment with PBS containing 0.5 mg/ml trypsin and 1 mM EDTA. The collagen film was then fixed with 3 % para-formaldehyde for 20 min at room temperature. Collagen was visualized by staining with Coomassie brilliant blue R250 and the images captured by CCD camera-equipped microscope (Nikon TE-2000) with 20x objective lens. Degraded areas were visualized as a white, unstained, non-collagen containing zone.

Microscopy and Normalized fluorescence resonance energy transfer (N_{FRET})

Cell images were captured on TE2000-E microscope (Nikon UK, Surrey, UK) equipped with Orca-ER cooled CCD camera (Hamamatsu Photonics, Hertfordshire, UK), excitation and emission filter wheels (Prior Scientific, Cambridge, UK), environmental chamber with heater, and Xenon lump (Nikon UK). Filters used to capture images of sapphire GFP, mRFP-1, and FRET are purchased from Chroma and the excitation filters used were 405/20 (60964) and 555/25 (86009), and the emission filters were 520/30 (86009) and 640/50 (55026). The microscope was operated by OpenLab software (Improvision/Perkin Elmer, Coventry, UK). N_{FRET} was calculated using the equation below according to Xia and Liu [20] in OpenLab software. I_{FRET} , I_{Sapph} and I_{RFP} are fluorescence intensity of FRET channel (405^{ex}/640^{em}), Sapphire GFP channel (405^{ex}/520^{em}), and RFP-1 channel (555^{ex}/640^{em}).

$$N_{\text{FRET}} = \frac{I_{\text{FRET}} - I_{\text{Sapph}} \times a - I_{\text{RFP}} \times b}{\sqrt{I_{\text{Sapph}} \times I_{\text{RFP}}}}$$

Images of Donor, FRET and Acceptor channels were obtained in identical conditions and images processed by pixel to pixel. Bleed through constant of Sapphire GFP into FRET channel is 0.05 (a) and mRFP1 0.08 (b) which are used to calculate N_{FRET} as above.

ECM protein-coated beads

Polybead carboxylated microspheres (4.5 μm) (Polysciences, Eppelheim, Germany) were coated with BSA(0.25 mg/ml) (Sigma Aldrich), collagen I (0.25 mg/ml) (Nitta gelatin), gelatin (0.25 mg/ml)(Sigma-Aldrich), fibronectin purified from human serum by gelatin Sepharose (0.25 mg/ml) (GE Health Care, Munich, Germany) according to the manufacture's instructions. Collagen I was neutralized before reacting with beads at 4 °C for 18h. Other proteins were reacted with beads at 23 °C for 18h. After blocking the beads with BSA, they were re-suspended in PBS at $5 \times 10^8/\text{ml}$ and kept at 4°C. A 20- μl portion of bead suspension was added to transfected cells on 19 mm coverslips for the experiments.

RESULTS

Construction of MT1-MMP chimeras with fluorescent protein for FRET analyses

Since homo-dimerization of MT1-MMP on the cell surface is essential for the enzyme to degrade a collagen matrix, we postulated that it also regulates MT1-MMP activity during cell invasion. Therefore, we aimed to detect dimerization of MT1-MMP in live cells using a FRET system. We constructed expression plasmids of MT1-MMP chimeras with sapphire green fluorescent protein (Sapph-GFP)[21] and with monomeric red fluorescent protein 1 (mRFP1)[22] to act as a FRET pair. Sapph-GFP is a GFP variant with a large Stokes' shift with the excitation peak at 395 nm and the emission peak at 517 nm [21]. mRFP1 has a broad excitation peak ranging from 480-580 nm and an emission peak at 607 nm [22]. This FRET pair is deemed to be efficient as the excitation for Sapph-GFP at 405 nm does not excite mRFP1; the emission peak of Sapph-GFP overlaps with the excitation peaks of mRFP1, that enabling effective resonance energy transfer; and finally, there is negligible emission of Sapph-GFP at 640 nm [23]. We inserted these fluorescence proteins into a specific site in the cytoplasmic domain of MT1-MMP so as to minimize potential effects on enzyme functions. We previously found that Cys574 in the cytoplasmic tail is palmitoylated, a process essential for MT1-MMP internalization through a clathrin-dependent pathway and promotion of cell migration [24]. It was also reported that Asp-Lys-Val582 in the cytoplasmic tail forms an essential motif for the recycling of MT1-MMP [25]. We therefore inserted Sapph-GFP or mRFP1 between Cys574 and Gln575 in the cytoplasmic tail to minimize effects on MT1-MMP function (Figure 1A). We confirmed that these chimera mutants activate proMMP-2 and degrade collagen on the cell surface in a similar manner to wild-type enzyme (Figure 1B

and C), and are able to promote cell migration in a 3D collagen matrix [26] (see also supplement movie 1). We also confirmed that these chimera enzymes were palmitoylated and internalized in a clathrin-dependent manner (data not shown), regulatory mechanisms which are essential to promote cellular invasion [24, 27]. These data indicate that the insertion of fluorescent proteins did not affect the biological properties of MT1-MMP.

Validation of the FRET system

As shown in Figure 1D, when the Sapph-GFP chimera of MT1-MMP (MT1F-Sapph) was expressed in HT1080 cells, signals were detected only in the Sapph-GFP channel (405 nm excitation and 520 nm emission) with no signal detected in mRFP1 channel (555 nm excitation and 640 nm emission), and only very weak signals in the FRET channel (405 nm excitation and 640 nm emission). Similarly, in cells expressing only MT1-MMP chimera with mRFP1 (MT1F-mRFP1), signals detected in the mRFP1 channel, but not in the Sapph-GFP channel, and the FRET channel showed only negligible signals. Bleed-through of Sapph-GFP and mRFP1 into the FRET channel was 5 % and 8%, respectively, and this number was used to calculate normalized FRET (N_{FRET}) according to Xia and Liu [20]. When both MT1F-Sapph and MT1F-mRFP1 were expressed together, significant signals were detected in the FRET channel, and calculated N_{FRET} reveals that higher FRET signals were present at the edge of the cell. This FRET was decreased significantly when a dimerization-inhibitory mutant, MT1F- Δ Cat, was co-expressed, validating the specificity of this FRET system. We also confirmed that photo-bleaching of MT1F-mRFP1 resulted in a loss of the FRET signal and an increase in donor intensity (Figure 1E), confirming that transfer of the excited-state energy from Sapph-GFP to mRFP1. Thus, we concluded that this FRET system is a valid method to analyse homo-dimerization of MT1-MMP in cells.

Live cell FRET imaging in a cell migrating in 3D collagen gel

Next, we visualized dimerization of MT1-MMP in live cells migrating in 3D collagen gels. As shown in Figure 2 and supplement movie 2, higher FRET signal was constantly detected at the leading edge of cells where collagen degradation takes place. We could not detect any FRET signal in the trailing edge although fluorescent signals of both donor and acceptor were present. We captured images in the order of donor, FRET and acceptor channel, but the same results were obtained when the order of capture was changed to acceptor, FRET and donor (data not shown). This suggests that the above observation is not an artefact derived from potentially misregistered images. The data indicate that MT1-MMP dimerization is regulated in a spatiotemporal manner during cell migration in a 3D collagen gel.

ECM attachment of the cells does not affect MT1-MMP dimerization

It has been reported that attachment of cells to collagen-coated microsphere beads initiated accumulation of MT1-MMP around the beads by Rab8-mediated vesicle transport [28], implying that an ECM-driven outside-in signal regulates cell surface localization of MT1-MMP. We therefore investigated whether MT1-MMP dimerization can also be regulated by ECM attachment. Microsphere beads covalently bound to either collagen I, gelatin, fibronectin, or BSA were added to cells expressing the FRET pair. We found that collagen beads, fibronectin beads and gelatin beads attached to the membrane surface of HT1080 cells efficiently, but that BSA-coated beads did not attach to the cell surface. However, as shown in Figure 3 none of the beads attached to the cell surface increased the level of FRET. We observed apparent accumulation of MT1-MMP in the area of the plasma membrane where the beads coated with collagen, fibronectin and gelatin attached (appendix Figure A1), indicating that these accumulations are not specific to a particular matrix

molecule. We observed that cells re-organized the membrane structure to wrap around the beads upon attachment (data not shown), thus it is not clear whether this accumulation of MT1-MMP was directly mediated by specific integrin signalling or resulted from cytoskeletal re-organization. Nevertheless, these data suggest that direct outside-in signalling from the ECM is not sufficient to influence the local dimerization status of the enzyme.

Effects of cytoskeletal re-organization by small GTPases on MT1-MMP dimerization

Another potential regulatory mechanism of MT1-MMP dimerization could be cytoskeletal re-organization. To study this, we carried out live cell FRET imaging in 2D culture since this process does not involve matrix degradation. As shown in Figure 4A and supplement movie 3, FRET occurred at every membrane protrusion that extended from the cell body, suggesting that it indeed correlates with cytoskeletal re-organization. We then analyzed the effect of small GTPases on dimerization (Figure 4B). Over-expression of a constitutively active form of Rac1 (Rac1CA) enhanced the FRET signal, but it seems to enhance the level of FRET across the cell body including in ruffling membranes. In contrast, expression of the constitutively active form of Cdc42 (Cdc42CA) enhanced FRET signals almost exclusively in filopodial structures. Cells expressing the dominant negative form of Rac1, Rac1DN, do not possess lamellipodia, but showed filopodia-like structures, and high levels of FRET were observed in these structures, although the overall levels of FRET were similar to Mock transfected cells. Cells expressing the dominant negative form of Cdc42 (Cdc42DN) did not possess ruffling membranes but showed flat lamellipodia-like membrane structures. Interestingly the level of FRET was consistently lower than the Mock transfected cells, especially at the edge of the membrane. These data suggest that there are close correlations between Cdc42 and MT1-MMP dimerization. It is known that Cdc42 is responsible in formation of filopodial membrane structures [18] and in regulation of cell polarity in concert with glycogen synthase kinase-3 β (GSK3 β) and adenoma polyposis coli [29]. We therefore next examined which of regulatory pathways correlates with dimerization of MT1-MMP (Figure 4C). First we examined if Cdc42CA-induced FRET at filopodia is driven by cell polarity signals. Cdc42CA-expressing cells were treated with a GSK3 β inhibitor, lithium chloride (LiCl) or SB216763 that disturb cell polarity [29]. The results indicate that there are no effects of GSK3 β inhibition on Cdc42-induced MT1-MMP dimerization at filopodia, suggesting that the Cdc42-dependent polarity signal does not regulate MT1-MMP dimerization. We thus next examined the effect of disruption of the actin cytoskeleton by cytochalasin D (CyD). Upon CyD treatment for 60 min, filopodia regressed and the level of the FRET at the edge of membrane structures was significantly decreased, indicating that MT1-MMP dimerization is closely linked to re-organization of the actin cytoskeleton induced by Cdc42.

DISCUSSION

When cells migrate in a 3D matrix, co-ordinated cytoskeletal organization, cell adhesion and proteinase activities are crucial [4, 30] and we consider that dimerization of MT1-MMP is one of the arms in this orchestration. Dimerization of MT1-MMP is required to promote cell invasion in a collagen-enriched environment. We recently identified the interface of dimerization in the Hpx domain of MT1-MMP, and mutations that weakened Hpx formation significantly decreased its dimer-dependent activities including proMMP-2 activation, cell surface collagen degradation, and collagen invasion [16]. Together with the findings in this report, we conclude that dimerization is indeed a regulatory step controlling MT1-MMP activity in a polarized manner across the cell surface during cell migration.

One of the potential mechanisms of polarized MT1-MMP dimerization is local activation of integrin signalling at the leading edge of the cells. We mimicked local ECM

signalling activation by incubating ECM-coated beads with the cells, but our data showed that there was no increase in FRET signal at the bead attachment sites on the plasma membrane in a time frame of up to 2h. We chose this time frame because while cells migrate in 3D collagen, attachment of the cell to a single ECM site is not maintained even for 20 min (see movie 2). Therefore, we concluded that local ECM signalling is not sufficient to regulate polarized MT1-MMP dimerization during cell migration.

We found a noticeable increase of the level of the FRET upon expression of Rac1CA, confirming our previous report [14]. Interestingly, high levels of FRET was observed not only at the ruffled membrane but across the cell body, suggesting that Rac1CA-driven MT1-MMP dimer formation may not only be attributed to cytoskeletal rearrangement. In contrast, in cells expressing Cdc42CA, a high level of the FRET was found almost exclusively at Cdc42-driven filopodia membrane structures, which are composed of parallel bundles of actin filaments [31s]. Since this FRET was not affected by GSK3 β inhibitors but by CyD, MT1-MMP dimer formation is likely to correlate with Cdc42-driven actin cytoskeletal rearrangement.

It has been shown that the GTP-bound form of both Rac1 and Cdc42 are present at the ruffling membrane structures of HT1080 cells [32]. It is possible that these ruffled membrane structures are the result of both of these small GTPase activities. When endogenous Rac1 activity was inhibited by overexpression of Rac1DN, it diminished ruffling membrane structures, and cells displayed filopodia-like structures, indicating that Cdc42 activity remained active under these conditions. Interestingly, the overall level of FRET was similar to that of Mock cells, but a high level of FRET was observed in these filopodia-like membrane structures. On the other hand when Cdc42DN were expressed in the cells, cells lost their ruffled membranes and formed flat lamellipodia-like structures, suggesting that endogenous Rac1 activity was maintained. Interestingly under these conditions the level of FRET especially at the edge of cell membrane was consistently lower. These data suggest that although ectopic expression of Rac1CA enhanced MT1-MMP dimerization, dimerization of MT1-MMP may be mainly regulated by Cdc42-driven cytoskeletal organization in HT1080 cells. It has been recently reported that MT1-MMP and Cdc42-dependent signalling regulates cell invasiveness in a 3D matrix by HT1080 cells [33]. It was also shown that cells in a 3D environment possess lower Rac activities than those in 2D culture conditions which links to directional persistence of migration [34]. Taken together, it is possible that a link between Cdc42 activity and MT1-MMP is a crucial determinant for the ability of cells to invade into a 3D matrix.

Filopodia are an initial membrane structures formed by cells when they attempt to migrate in a certain direction. The cell membrane at the tip of a filopodium then anchors to the substratum, and a Rac1-mediated lamellipodium will subsequently form [35]. Functionally active MT1-MMP dimers may thus need to be in these structures that enable cells to degrade the ECM barrier in the direction of movement. Cdc42 is also a key molecule that determines cellular polarity [29, 35, 36]. However, our data do not support the idea that MT1-MMP dimerization is regulated by Cdc42-driven cell polarity. Interestingly dimerization of MT1-MMP occurs at leading edge of migrating cells in 3D collagen that seems to correlate with cell polarity. Since cells in 3D collagen migrate with persistent directionality [34], it is likely that Cdc42-driven membrane protrusions actively occur only at the leading edge, and this may also explain why there was no MT1-MMP dimer detected at the trailing edge. In 2D, cells migrate randomly displaying new membrane protrusions to different directions potentially regulated by Cdc42, resulting in dimerization of MT1-MMP in these membrane structures. ECM signals are one of the upstream events for Rac1 and Cdc42 activation [37], but ECM signalling initiated by ECM-coated beads was not sufficient to

induce Cdc42-driven filopodia, and this may be the reason why ECM coated beads did not influence MT1-MMP dimerization.

From our analyses, it is still not clear how Cdc42-driven cytoskeletal re-organization affects dimerization of the enzyme. Since MT1-MMP dimerization determines MT1-MMP-dependent cellular invasiveness, understanding the detailed mechanisms of MT1-MMP dimerization may provide further insights into the mechanism of cellular invasion in a 3D matrix.

ACKNOWLEDGEMENT

We thank Prof. Roger Y. Tsien (University of California, San Diego) for donating mRFP1 cDNA and for helpful suggestions. This work was supported by Cancer Research UK project grant C1507/A5541, the Wellcome Trust Grant 075473, and Arthritis Research UK Core grant to the Kennedy Institute of Rheumatology.

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FIGURE LEGENDS

Figure 1. A FRET system to detect MT1-MMP homo-dimerization.

- A. Schematic representation of the MT1-MMP constructs. S, signal peptide; Pro, propeptide; Cat, catalytic domain; L1, linker-1; Hpx, hemopexin domain; L2, linker-2; TM, transmembrane domain; CP, cytoplasmic tail; FLAG, FLAG epitope; Sapph-GFP, Sapphire GFP. Note that Sapphire GFP or mRFP-1 was inserted between Cys⁵⁷⁴ and Gln⁵⁷⁵ in the cytoplasmic tail.
- B. COS-7 cells expressing MT1F, MT1F-mRFP1 or MT1F-Sapph were incubated with recombinant proMMP-2 in serum free culture medium for 18 h. Activation of proMMP-2 in the media and expression of MT1-MMPs were analyzed by gelatin zymography and Western Blotting using anti-FLAG M2 antibody, respectively.
- C. COS-7 cells expressing MT1F, MT1F-mRFP1 or MT1F-Sapph were subjected to collagen film degradation assay as described in the Methods section. After 3 days, the remaining collagen film were visualized as described in the Methods section.
- D. HT1080 cells were transfected with the expression plasmids as indicated and fixed with 3% paraformaldehyde for 10 min at room temperature. Fluorescent images of cells were taken in Sapph-GFP (405/520), mRFP1(555/640), and FRET (405/640) channels, and these images were used to calculate N_{FRET} . FRET values (0.0 - 0.7 in this experiment) are expressed in pseudo colors where the highest (0.7) is in red and the lowest (0.0) in blue.
- E. Effect of acceptor (mRFP-1) bleaching was assessed in HT1080 cells expressing both MT1F-Sapph and MT1F-mRFP1. The fixed cell was exposed to 555 nm light for 30 min to bleach mRFP-1. The images before and after bleaching were analyzed for fluorescence intensity of Sapph-GFP and mRFP1 at 4 different areas of the cells where fluorescent signals were not saturated. Note that Sapphire GFP fluorescence intensity was increased by 20 % upon bleaching of mRFP-1.

Figure 2. Live Cell FRET imaging of cells migrating in a 3-D collagen gel.

HT1080 cells were transfected with MT1F-Sapph and MT1F-mRFP1. Cells were then embedded in a 3-D collagen gel (2 mg/ml) and subjected to time-lapse FRET imaging at 37 °C. Images were taken every 5 min, and the selected time points are shown. These images are parts of supplement Movie 1. Bar indicates 50 μ m.

Figure 3. Effect of attachment to ECM-coated beads on MT1-MMP dimerization.

HT1080 cells were transfected with MT1F-Sapph and MT1F-mRFP1 and cultured on gelatin-coated cover slips. The ECM-coated beads (1×10^7) were then added to each well and allowed to attach to the cells for 2 h. Cells were then fixed and FRET was measured. Top images are FRET images and bottom images are merged images with bright field images to locate the position of the beads shown with white arrows. Bar indicates 20 μ m.

Figure 4. Effect of cytoskeletal re-organization on MT1-MMP dimerization.

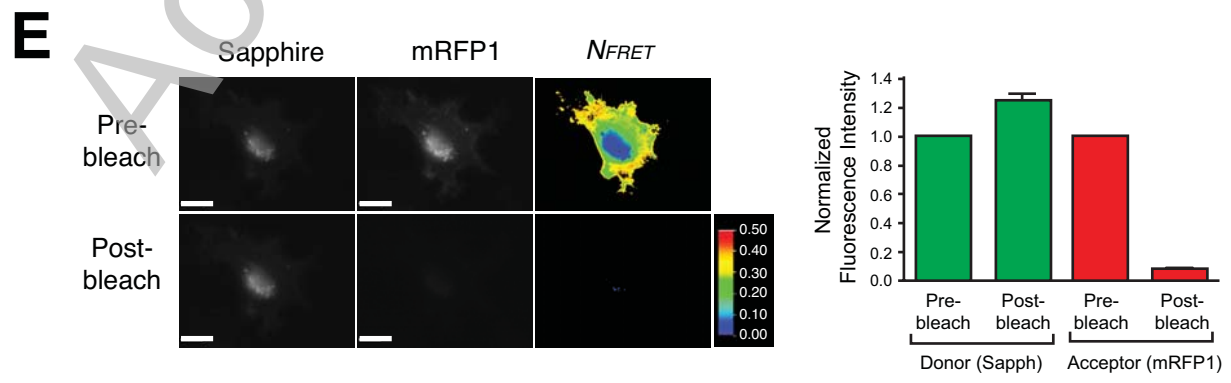
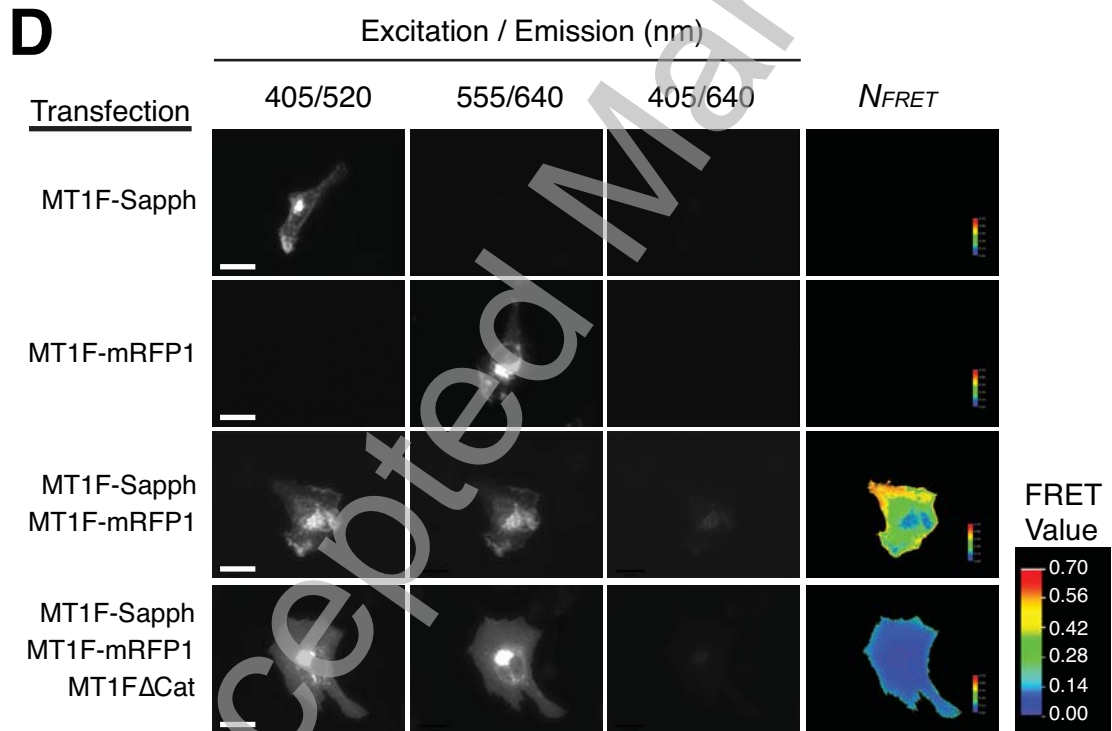
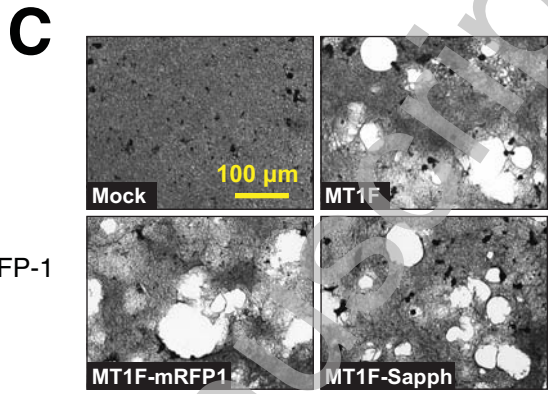
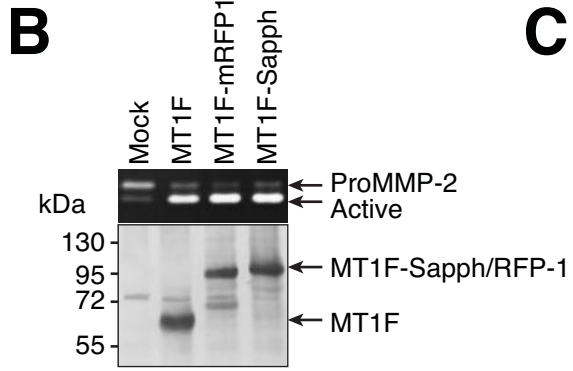
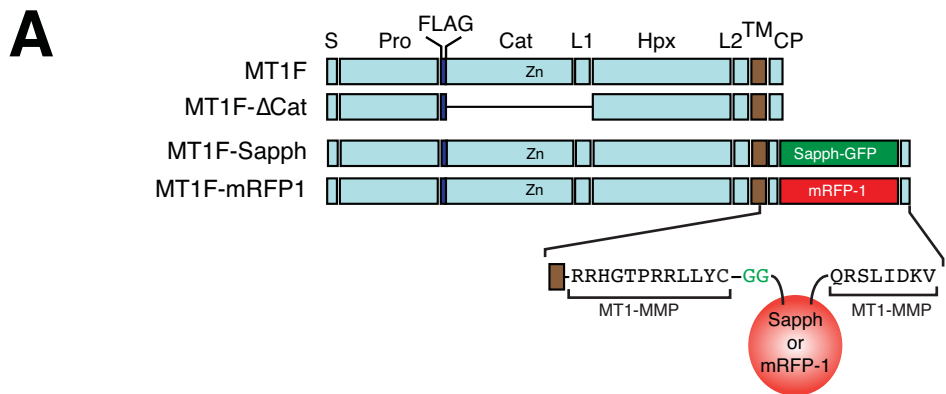
A. HT1080 cells expressing MT1F-Sapph and MT1F-mRFP1 were seeded on gelatin-coated slide chambers. Time-lapse FRET imaging of the the cells was performed in every 5 min, at time points as indicated. Bar indicates 40 μ m.

B. HT1080 cells were co-transfected with expression plasmids for MT1F-Sapph, MT1F-mRFP1 and Rac1CA with or without Cdc42CA, Rac1DN or cdc42DN. Cells were fixed and FRET was measured. Donor (Sapph) and acceptor (mRFP1) images are shown below the FRET images. Bar indicates 20 μ m.

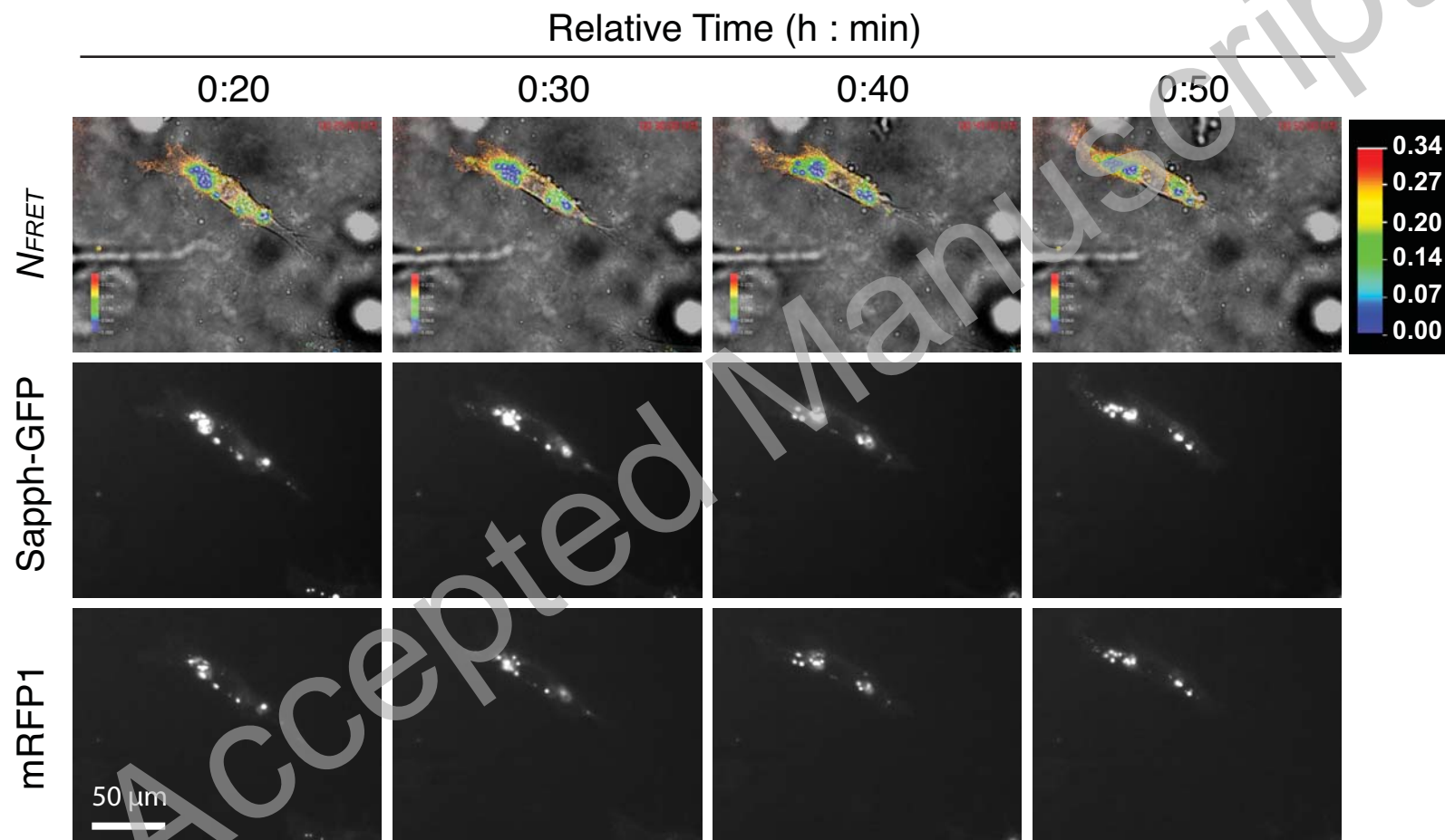
C. HT1080 cells expressing MT1F-Sapph, MT1F-RFP and Cdc42CA were treated with LiCl (20 mM), SB216763 (20 μ M) or CyD (1 μ g/ml) for 1h before fixation. Cells were then subjected to FRET measurement. Bar indicates 20 μ m.

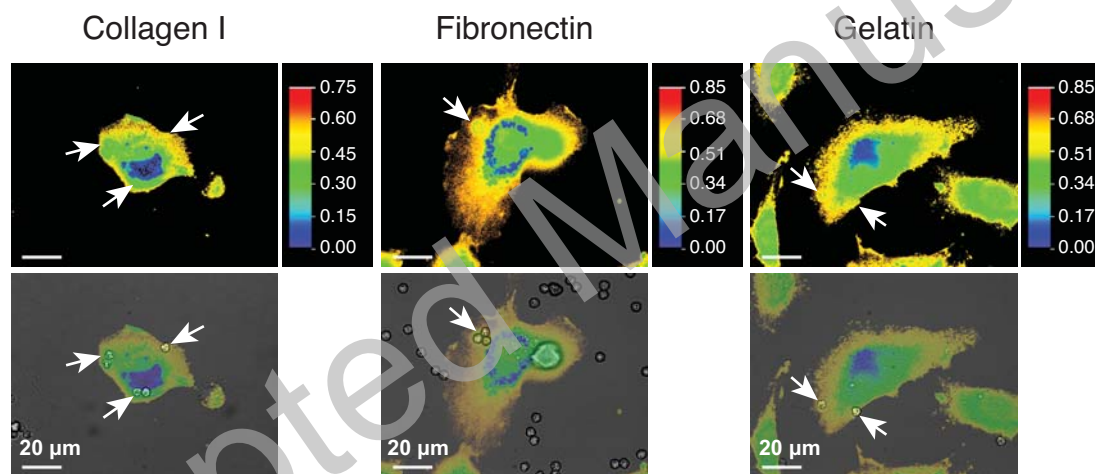
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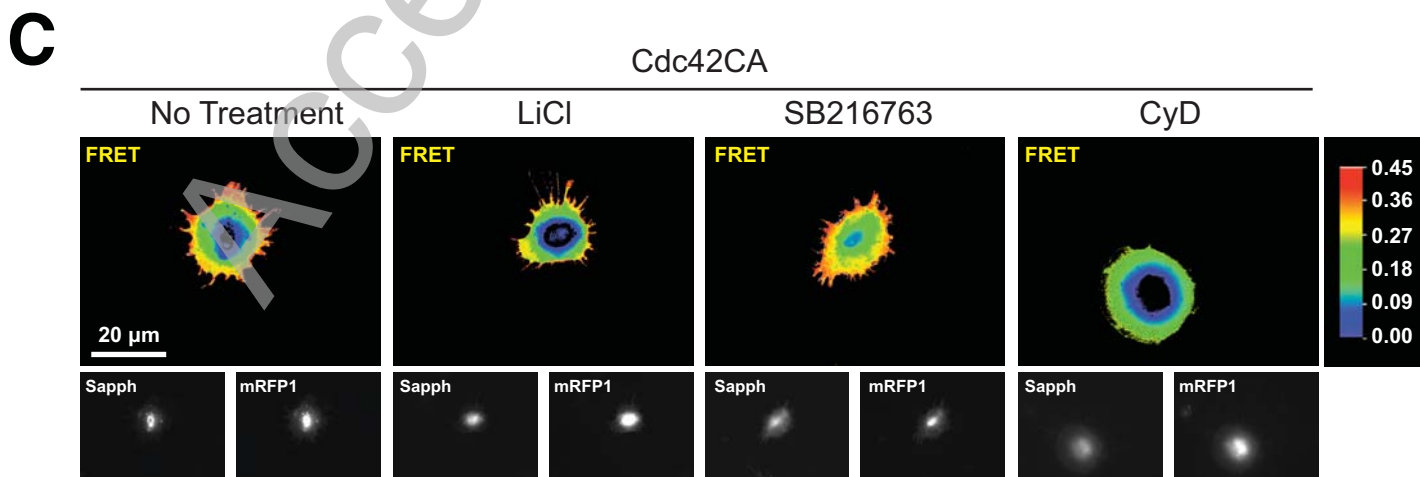
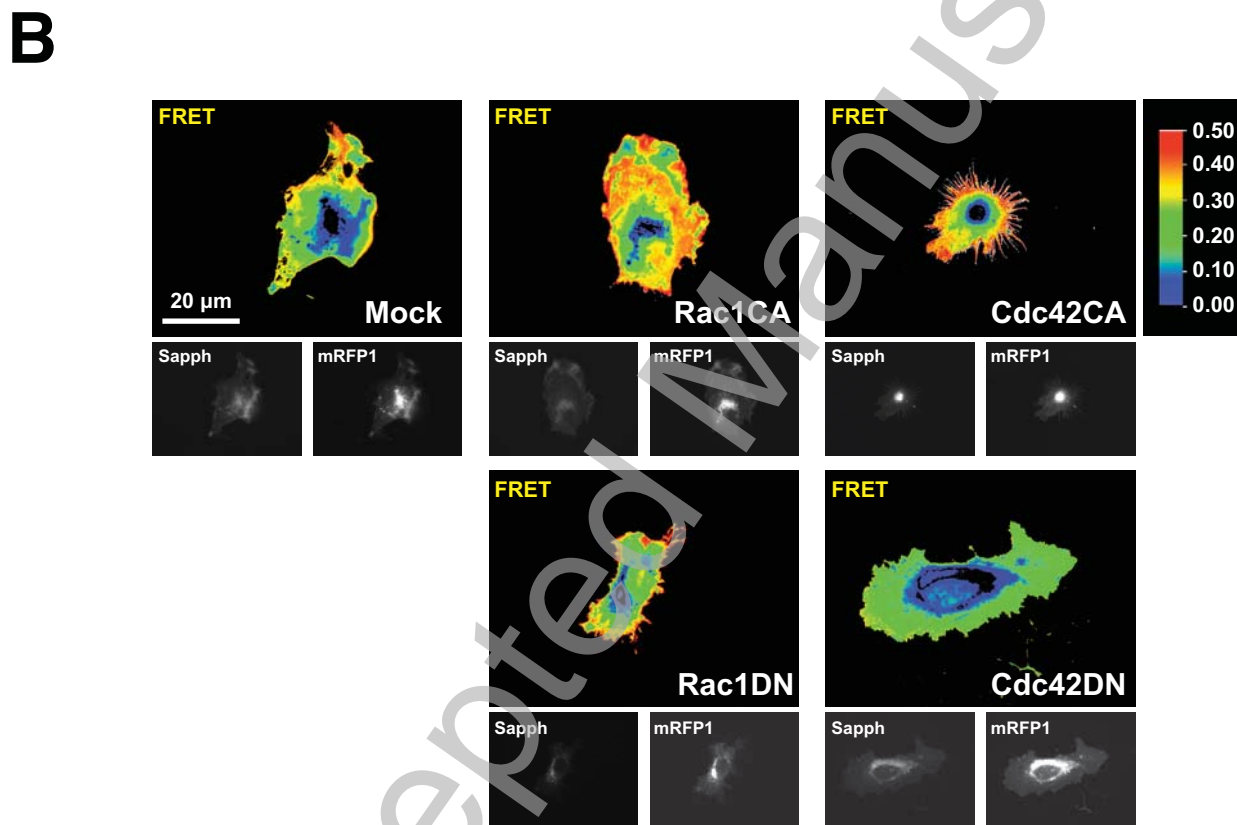
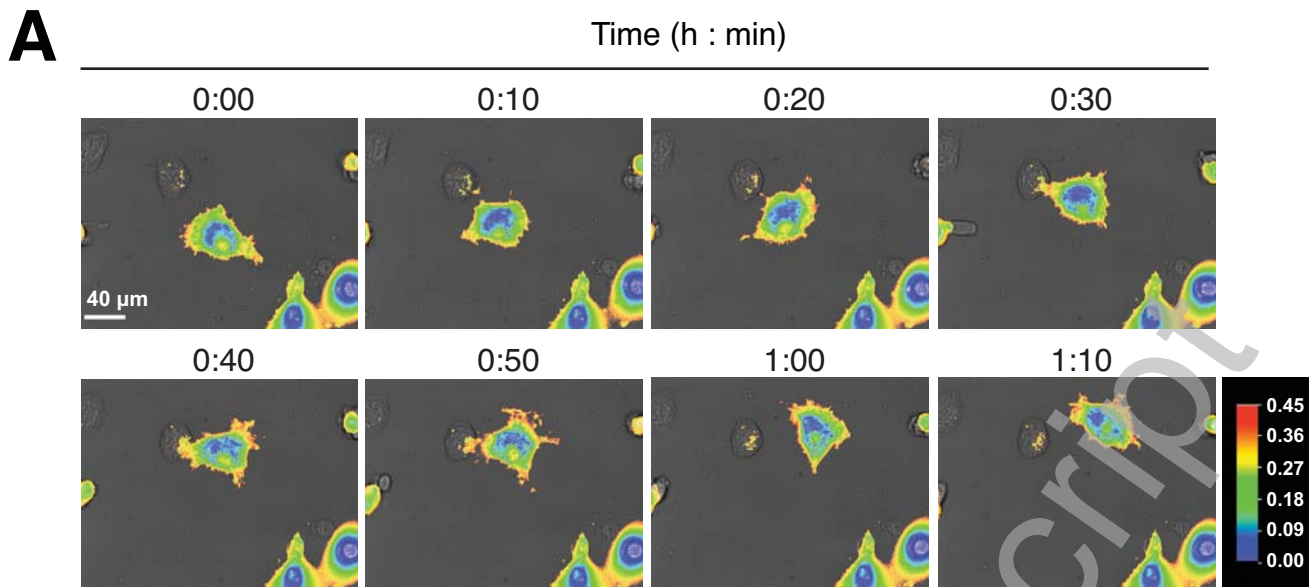
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