Necrosis- and apoptosis-related Met cleavages have divergent functional consequences

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Upon activation by its ligand hepatocyte growth factor/scatter factor, the receptor tyrosine kinase Met promotes survival, proliferation, and migration of epithelial cells during embryogenesis. Deregulated Met signaling can also promote cancer progression and metastasis. Met belongs to the functional family of dependence receptors whose activity switches from pro-survival to pro-apoptotic during apoptosis upon caspase cleavage. Although apoptosis resistance is a hallmark of cancer cells, some remain sensitive to other cell death processes, including necrosis induced by calcium stress. The role and fate of Met during necrotic cell death are unknown. Following treatment with calcium ionophores, cell lines and primary cells undergo necrosis, and the full-length Met receptor is efficiently degraded. This degradation is achieved by double cleavage of Met in its extracellular domain by a metalloprotease of the A disintegrin and metalloprotease (ADAM)-10,20 generating a soluble N-terminal fragment (Met-CTF). The latter is in turn efficiently degraded by the lysosome and by further γ-secretase cleavages.

Met is a receptor tyrosine kinase expressed predominantly by epithelial cells and activated by its stromal ligand, hepatocyte growth factor/scatter factor (HGF/SF). Met activation stimulates a biological program called invasive growth,1 involving survival, proliferation, invasion, and morphogenesis of epithelial cells. Ligand-stimulated Met acts, furthermore, as an angiogenic and neurotrophic factor.2,3 HGF/SF and Met are essential to several steps of embryogenesis, experiments on transgenic mice having shown that they are necessary for formation of the placenta, liver, limb muscle, neurons, and lung airspace.4–8 In adults, HGF/SF and Met promote regeneration of several organs, including the liver, kidneys, and thymus.9–13

Aberrant Met and HGF/SF signaling contributes to promoting tumorigenesis and metastasis (for review see Furlan et al.).14 A direct link between Met and cancer has been evidenced by observation of Met germinal mutations linked to hereditary papillary renal carcinoma.15 Met and/or HGF/SF are/is also overexpressed in several human cancers.16 Given its important oncogenic activity, Met is the target of many therapeutic agents currently under clinical investigation.17

Downregulation of Met following its activation by HGF/SF is an important negative regulatory mechanism preventing receptor overactivation. We have previously shown that Met expression and activity are also controlled by proteolytic cleavages. Under steady-state conditions, Met is processed by PS-RII (presenilin-regulated intramembrane proteolysis).18,19 This process involves cleavage of Met within its extracellular juxtamembrane domain by a disintegrin and metalloprotease (ADAM)-10,20 generating a soluble N-terminal fragment (Met-NTF), which is released into the extracellular space, and a membrane-anchored C-terminal Met fragment (Met-CTF). The latter is in turn efficiently degraded by the lysosome and by further γ-secretase cleavages.

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Abbreviations: ADAM, A disintegrin and metalloproteinase; ALLN, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal; ATP, adenosine triphosphate; Bak, Bcl-2 antagonist/killer Bax, Bcl-2 associated X-protein; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grb2, growth factor receptor-bound protein 2; HGF/SF, hepatocyte growth factor/scatter factor; IHC, immunohistochemistry; Met-CTF, Met C-terminal fragment; Met-NTF, Met N-terminal fragment; NSCLC, non-small-cell lung cancer; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PS-RII, presenilin-regulated intramembrane proteolysis QVD-OPh, N-(2-quinoxalyl)-l-cysteinyll-l-asparthly-(2,6-difluoro phenoxoxy) methylketone; RNA, ribonucleic acid; siRNA, small interfering RNA; TAPI, TNF-α processing inhibitor TRK, tropomyosine receptor kinase; Z-FA-FMK, Z-Phe-Ala fluoromethyl ketone
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Constitutive degradation of the Met receptor by PS-RIP contributes to regulating its half-life.

Under apoptotic conditions, Met is cleaved by caspases21 within its C-terminal tail and its intracellular juxtamembrane domain. These cleavages remove the C-terminal tail of Met and separate the extracellular ligand-binding domain from the intracellular kinase domain. The generated 40-kDa intracellular fragment, previously called p40Met and here called p40Met\textsuperscript{caspase}, can increase cell death by promoting mitochondrial permeabilization.\textsuperscript{22,23} Removal of the C-terminal tail of Met is required for the efficient pro-apoptotic action of the fragment. This pro-apoptotic function of Met makes it a member of the dependence receptor family.\textsuperscript{24} Met cleavages are illustrated in Figure 6a.

Although the mechanisms underlying apoptosis have been studied extensively, necrosis has only recently been described as a regulated cell death mechanism.\textsuperscript{25} Necrosis is an adenosine triphosphate (ATP)-independent cell death mechanism featuring early plasma membrane and organelle disruption. Many pathways can lead to cell necrosis, including calcium overload. This type of cell stress has been amply described in the nervous system, where an increase in intracellular calcium results in neuronal injury and neurodegenerative diseases. In many other cell types, calcium ionophores such as ionomycin can induce rapid necrosis. An increase in intracellular calcium triggers activation of several proteases, including calpains and cathepsins.\textsuperscript{26–28} Calpains are calcium-dependent proteases capable of cleaving multiple substrates and involved in regulating various cellular processes, including migration, autophagy, apoptosis, and necrosis. Interestingly, the effector role of calpains during necrosis is reminiscent of the function of caspases during apoptosis. Caspases are directly involved in morphological changes observed during apoptosis, while calpains can cleave cytoskeletal proteins such as spectrin and tubulin, thus favoring dismantling of cell structure during necrosis.\textsuperscript{29–31} Although apoptosis resistance is a hallmark of many cancer cells,\textsuperscript{32} some such cells remain sensitive to other cell death processes, including necrosis.\textsuperscript{33} Thus, a better understanding of the mechanisms underlying necrosis is important, as it could help to elaborate novel therapeutic strategies. Here we show that calcium stress induced by calcium ionophores triggers Met degradation during necrotic cell death. This loss of Met receptor occurs early during the process and is mediated by Met cleavages: by calpains in its intracellular part and by metalloproteases in its extracellular part. These cleavages generate an extracellular fragment and an intracellular fragment with a molecular weight close to that of p40Met\textsuperscript{caspase}.

Results

Calcium-stress-induced necrosis triggers Met degradation. In order to investigate the fate of Met during necrosis, we administered different pharmacological drugs known to induce either necrosis or apoptosis. Treatment of MCF-10A epithelial cells with staurosporine triggered cell death with the distinctive features of apoptosis, including cleavage of the caspase substrate poly(ADP-ribose) polymerase (PARP), Annexin V staining, along with degradation of full-length Met, and production of the pro-apoptotic fragment p40Met\textsuperscript{caspase} (Figures 1a and b). In contrast, treatment with the calcium ionophore ionomycin induced, after a few hours, cell death without PARP cleavage or any significant phosphatidylserine externalization. Instead, the cells displayed sustained propidium iodide (PI) staining, suggesting early plasma membrane disruption consistent with known necrotic cell death induced by calcium ionophores (Figure 1b). Ionomycin treatment also induced early degradation of Met and production of a fragment of ~40 kDa, only slightly different in molecular weight from p40Met\textsuperscript{caspase} (Figure 1a). Faint bands were also observed ~55 kDa, reminiscent of the Met-CTF fragments generated by PS-RIP.\textsuperscript{18,19} Ionomycin treatment was found to cause similar Met degradation and 40-kDa fragment generation in MDA-MB231 breast cancer cells and Met-overexpressing GTL-16 gastric cancer cells (Supplementary Figures S1A and B), and also in primary cultures of mouse mammary epithelial cells and in human primary hepatocytes (Figures 1c and d). Generation of the 40-kDa fragment was observed from 100 nM ionomycin upward, with maximal generation at 1 \( \mu \)M, associated with degradation of full-length Met (Supplementary Figure S1C). Ionomycin treatment in calcium-free medium failed to induce Met degradation (Supplementary Figure S1D), while treatment of MCF-10A cells with A23187, another calcium ionophore, caused a decrease in full-length Met and generation of the 40-kDa fragment (Supplementary Figure S1E).

During calcium-induced necrosis, consistently with the observed efficient degradation of Met, HGF/SF stimulation of ionomycin-pretreated cells failed to activate Met and its downstream signaling proteins ERK (extracellular signal-regulated kinase) and AKT (Figure 2a). It is worth noting that ionomycin treatment did cause ERK activation, but that HGF/SF was found to reduce Met degradation and generation of the 40-kDa fragment, concomitantly with a decrease in PI staining (Figures 2b and c).

Calpains perform intracellular Met cleavage during necrosis. To see whether the 40-kDa fragment observed during calcium stress is generated by proteolytic cleavage, we incubated epithelial cells with various protease inhibitors before ionomycin treatment. In contrast to caspase and cathepsin inhibitors (Figure 3a), the calpain inhibitor calpeptin was found to decrease production of the 40-kDa fragment in a dose-dependent manner (Figures 3a and b). Activation of calpains during calcium stress was confirmed by calpain 1 autolysis, detected by generation of a cleaved form (Figures 3c and d and Supplementary Figure S1C). Met-receptor-targeting ribonucleic acid (RNA) interference led to loss of the calcium stress fragment, confirming that it is a fragment of the Met receptor. RNA interference targeting calpains 1 and 2 (the two best-characterized ubiquitous calpain isoforms) decreased (but did not totally prevent) production of the calcium stress fragment, indicating an involvement of these two proteases (Figure 3d). Neither calpain inhibitor treatment nor RNA interference targeting calpains 1 and 2 was found to restore detection of full-length Met.
p40Met\textsuperscript{calpain} does not exert pro-apoptotic action. To confirm the involvement of calpains, we incubated purified calpain 1 with cell extracts or with the recombinant intracellular domain of Met. In the presence of calcium, cleavage of full-length Met in MCF-10A lysate by purified calpain 1 generated a Met-CTF fragment of the same size as the calcium stress fragment (Figure 4a). Similar proteolytic processing of the recombinant intracellular Met domain was observed in the presence of 2.5 nM calpain, with generation of a major 40-kDa fragment. Further cleavage to smaller fragments was observed with excess protease (Figure 4b).

By analogy to p40Met\textsuperscript{caspase}, this novel fragment was called p40Met\textsuperscript{calpain}.

Despite many attempts to predict consensual calpain cleavage sites, it seems likely that these proteases recognize secondary and ternary structures, making cleavage sites difficult to identify.\textsuperscript{34,35} To determine the calpain cleavage sequence, we analyzed the \emph{in vitro} Met cleavage product by mass spectrometry. AspN digestion followed by mass spectrometry revealed that the first N-terminal peptide begins at amino acid D1041, suggesting that cleavage occurs before this sequence (Supplementary Figures S2A and B).
Mass spectrometry also showed that p40Met calpain still includes the last amino acids of Met. A specific antibody targeting the C-terminal tail of Met detected p40Met calpain but failed to detect p40Met caspase, demonstrating that calpain processing of Met preserves its C-terminal end (Supplementary Figure S3). Analysis of the putative calpain cleavage region with the SitePrediction tool identified a potential cleavage site between residues T1036 and S1037 (Figure 4c). Therefore, we produced in transfected cells expressing an appropriate construct a version of Met starting at residue S1037 and ending at the natural stop codon. Western blot analysis showed that this fragment has the same molecular weight as endogenous p40Met calpain (Figure 4d).

We have previously demonstrated that loss of the C-terminal tail of Met is an important step in reshaping Met into a pro-apoptotic factor. Because the p40Metcalpain sequence is quite similar to p40Met caspase but retains the C-terminal tail, we wondered whether p40Metcalpain shares the ability of p40Met caspase to induce cell death. When epithelial cells were transfected with a construct encoding either Flag-p40Metcaspase, Flag-p40Met calpain, or a non-apoptotic version of p40Met caspase carrying the K1108A mutation, only Flag-p40Met caspase showed substantial pro-apoptotic activity, leading to 16% cleaved-caspase-3-positive cells. The respective percentages for p40Met caspase and the K1108A mutant were only ~5 and 2% (Figures 4e and f).

Calcium stress increases Met shedding, which participates in Met degradation. We next wondered whether the intracellular cleavage yielding p40Met calpain might also yield a membrane-anchored Met-NTF. Immunostaining with two distinct antibodies failed to reveal any Met-NTF at the membrane surface of MCF-10A cells undergoing necrosis (Figure 5a). Western blotting also failed to reveal the Met-NTF (Figures 5b and c). In contrast, analysis of conditioned medium revealed abundant accumulation of an N-terminal fragment of ~95 kDa (Figures 5b and c). These results suggest that, in addition to calpain processing, Met undergoes an extracellular cleavage releasing its N-terminal region into the medium. According to its apparent size, this N-terminal fragment could be Met-NTF, generated by Met shedding mediated by ADAM metalloproteases during PS-RIP.

The ionomycin-induced appearance of Met-NTF in the conditioned medium was efficiently inhibited by the metalloprotease inhibitor TAPI (TNF-α processing inhibitor), but not by the calpain inhibitor calpeptin (Figure 5d). TAPI treatment did not affect p40Met calpain generation, indicating that the two proteolytic processes are independent. Furthermore, in contrast to calpeptin treatment, TAPI treatment was found to rescue full-length Met, indicating that shedding is the major event involved in Met degradation during calcium stress (Figure 5d).

Shedding of the Met-NTF generates a C-terminal counter-part of ~55 kDa (Met-CTF), which is efficiently degraded by the lysosome or further cleaved by γ-secretases. Consistently with this, ionomycin treatment led to increased detection of Met-CTF when this fragment was stabilized by...
treatment with lysosome and γ-secretase inhibitors. This confirms that Met undergoes metalloprotease-mediated shedding during necrosis (Supplementary Figure S4). To assess whether Met-CTF is generated upon receptor cleavage within the extracellular juxtamembrane region, we looked for Met-CTF in MDCK cells expressing either an uncleavable chimeric receptor (uncleavable tropomyosine receptor kinase (TRK)-Met, in which the entire extracellular domain of Met is replaced with the extracellular domain of the TRKA receptor) or a cleavable chimera in which the first 50 juxtamembrane extracellular amino acids of Met are present (cleavable TRK-Met; Supplementary Figure S5A). As expected, cotreatment with γ-secretase and lysosome inhibitors stabilized Met-CTF and the full-length chimera only in cells expressing the cleavable TRK-Met. In these cells, calcium ionophore treatment was found to increase Met-CTF generation, with a concomitant decrease in full-length chimera (Supplementary Figure S5B). In contrast, cells expressing the uncleavable chimera showed no generation of Met-CTF and no degradation of the full-length chimera. Taken together, these data demonstrate that during calcium-stress-induced necrosis, Met is processed by both calpain and metalloproteases, participating in its efficient degradation over a few minutes.

Met proteolytic processing in lung tumors. In previous studies and the present one, we have shown that the Met receptor can be cleaved during various physiological processes (Figure 6a). Although many immunohistochemistry (IHC) studies have shown Met to be overexpressed in a variety of cancers, the Met cleavage state was never characterized in these studies. About half of all non-small-cell lung cancers (NSCLCs) are known to overexpress Met. We thus analyzed the amount and state of Met by both IHC and western blotting in a library of 13 surgically resected NSCLCs (Supplementary Figure S6A). Among the tumor samples, four displayed a score of 0, two a score of 1, five a score of 2, and two a score of 3 (Supplementary Figure S6B). Representative Met IHC images are shown (Figure 6b). The amount of full-length Met detected on western blots of tumor lysates was found to correlate,
globally, with the Met IHC score (Figure 6c and Supplementary Figure S7). In addition, several Met fragments were detected in tumors with two different antibodies directed against the intracellular region, and their levels were found to correlate with that of full-length receptor (Supplementary Figure S7). Immunoblotting with an antibody directed against the extracellular domain confirmed the full-length Met score and allowed detection of abundant Met-NTFs of ~95 kDa, the likely N-terminal counterparts of the C-terminal fragments.

The main C-terminal fragments observed in tumors, ~55, 45, and 40 kDa in size, are similar in size to the fragments identified in previous studies and the present one. To assess this, we carried out co-migration of three high-Met tumor lysates with cell extracts containing generated or stabilized Met fragments. Met-CTF was stabilized by treatment with γ-secretase and lysosome inhibitors, p45 Met was generated in highly confluent cells (manuscript under revision), and p40Metcaspase and p40Met calpain were generated by inducing...
apoptosis or necrosis, respectively, (Figure 6c). The apparent molecular weights of these four fragments perfectly matched those of the Met fragments observed in tumors. It is worth noting that 11 of the 13 tumors had not been subjected to any treatment, suggesting that the Met fragments were not the result of stress induced by therapeutic agents. Unlike the antibodies targeting the Met receptor, an antibody directed against the intracellular domain of epidermal growth factor receptor failed to detect any intracellular fragments. Altogether, these results suggest that proteolytic degradation of the Met receptor is drastically increased in lung cancers overexpressing Met.

Discussion

Necrosis is a caspase- and ATP-independent cell death process characterized by a loss of plasma membrane and organelle integrity. It lacks features of apoptosis, such as phosphatidyserine externalization and apoptotic body release. Although first defined as accidental cell death, necrosis is currently regarded as a programmed cell death mechanism, as pharmacological inhibitors or gene deletions can protect cells from this demise. Some necrotic cell death can be induced by calcium overload, for example, in neural cells in the case of neurodegenerative disorders or following

Figure 5  Ionomycin (iono) treatment increases Met shedding. (a) MCF-10A cells were grown on glass coverslips, serum starved overnight, and treated with vehicle or 1 μM ionomycin for 4 h. Immunofluorescence staining was performed with two different antibodies directed against the Met extracellular region and the nuclei were stained with Hoechst. (b) MCF-10 A and (c) GTL-16 cells were grown for 24 h, serum starved overnight, and treated with 1 μM ionomycin for the indicated time. Both cell lysates and an equal volume of conditioned medium were analyzed by western blotting with an antibody against the Met extracellular region. (d) MCF-10A and GTL-16 cells were grown for 24 h, serum starved, and pretreated overnight with TAPI-1 and/or calpeptin, and treated for 1 h with 1 μM ionomycin. Cell lysates and conditioned medium were analyzed by western blotting with an antibody against the Met extracellular region, the Met kinase domain (intracellular Met), and GAPDH to assess loading. Arrows indicate positions of full-length Met, Met-NTF, and p40Metcalpain.
ischemia. In the case of cancer cells, cells deficient in Bak (Bcl-2 antagonist/killer) and Bax (Bcl-2-associated X-protein), which are resistant to many apoptosis inducers, have been found to die from necrosis following calcic stress. Yet the mechanisms underlying calcium-stress-induced necrosis are still poorly understood. As Met is actively involved in both survival and apoptosis, we have sought to determine its fate and potential role during calcium-stress-induced necrosis.

We demonstrate here that calcium-stress-induced necrosis causes a substantial decrease in Met within minutes following treatment with a calcium ionophore. Consistently with this decrease, HGF stimulation can no longer induce Met phosphorylation or activation of downstream signaling pathways under these conditions. This Met degradation might prevent cell protection triggered by ligand-dependent activation of Met. Rapid Met degradation, associated with detection of fragments, suggests that Met undergoes proteolytic cleavages during this process. Accordingly, we have found that Met is cleaved by both calpains and membrane metalloproteases. We show that p40Metcalpain generation can be inhibited efficiently by treatment with a pharmacological calpain inhibitor and partially by means of interfering RNAs targeting calpains 1 and 2. This demonstrates that these two proteases, and likely other calpains, are involved in Met cleavage. We demonstrate as well that calcium stress induces autocatalytic cleavage of calpain 1, consistent with activation of calpain by the intracellular calcium level.

Figure 6  Met fragments are produced in NSCLC tumors overexpressing Met receptor. (a) Schematic representation of the different cleavages of the Met receptor. Under steady-state conditions, membrane metalloproteases cause shedding of an N-terminal fragment (Met-NTF) into the extracellular space, with formation of an unstable membrane-anchored C-terminal fragment (Met-CTF). During apoptosis, cleavage of Met by caspases generates p40Metcaspase, a pro-apoptotic fragment of ~40 kDa. During calcium-stress-induced necrosis, Met is cleaved by both metalloproteases (to Met-NTF and Met-CTF) and calpains (to p40Metcalpain). (b) Tumor samples were analyzed by IHC to determine the level of Met. (c) Three tumor samples overexpressing Met were analyzed by western blotting, by comparison with MCF-10A cell lysates treated for 5 h with 1 μM compound E and 5 nM bafilomycin (CpdE+baf), for 6 h with 1 μM staurosporine (stauro), for 1 h with 1 μM ionomycin (iono), or cultured to high density (confluence).
p40Met\textsuperscript{calpain}. Thus, the p40Met\textsuperscript{caspase} and p40Met\textsuperscript{calpain} fragments differ at both their N- and C-terminal ends. Consistently with the fact that p40Met\textsuperscript{calpain} retains the C-terminal tail of Met, known to prevent its pro-apoptotic action,\textsuperscript{72} we show here that it does not induce increased caspase 3 activity and is thus not a pro-apoptotic fragment.

In addition to intracellular p40Met\textsuperscript{calpain}, calcium overload induces an increase in two other Met fragments: Met-NTF (in conditioned medium) and the labile Met-CTF, both known to induce an increase in two other Met fragments: Met-NTF and Met-CTF (p55 Met) in tumor samples suggests that lysosomal degradation is altered in these tumors or that the amount of generated fragment exceeds the capacity of the cells to degrade it. We have demonstrated in a cell model that most of the Met fragments are associated with degradation of the full-length Met receptor. Thus, in tumor samples overexpressing Met, several proteolytic degradation mechanisms might compensate for receptor overexpression, thus limiting the amount of receptor.

At first glance, the mechanism of Met degradation appears similar during apoptosis and calcium-stress-induced necrosis: in both cases, it involves generation of an intracellular fragment of \sim 40 kDa. In both processes, the extra cellular ligand-binding domain of Met is separated from the intracellular kinase domain, preventing survival. Calcium-stress-induced necrosis, however, triggers a more complex proteolytic process, also involving cleavage by membrane metalloproteases. Consequently, the cleavages occurring during necrosis are quicker than caspase cleavages, leading to Met degradation within minutes of stress induction. In addition, while p40Met\textsuperscript{caspase} somewhat resembles p40Met\textsuperscript{calpain}, these two fragments appear to be functionally different: the former can favor cell death while the latter is unable to promote such a response.

Materials and Methods

Cytokines, drugs, and cell cultures. Ionomycin and A23187 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Staurosporine was obtained from Sigma-Aldrich (St Louis, MO, USA). Purified calpain 1 was obtained from Calbiochem (San Diego, CA, USA). Recombinant human HGF was obtained from Peprotech (Rocky Hill, CT, USA). The caspase inhibitor QVD-OPh (N\textsuperscript{(2}-quinolyl)-L-valyl-L-aspartyl-(2,6-difluorophenoxy) methylketone), the calpain inhibitors ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal) and calpeptin, the metalloprotease inhibitor TAPI-1, and the H\textsuperscript{+}-pump inhibitor bafilomycin A1 were purchased from Calbiochem. The cathepsin inhibitor Z-FA-FMK (Z-Phe-Ala fluoromethyl ketone) was obtained from Bachem (Bubendorf, Switzerland). The proteasome inhibitor lactacystin was from Sigma-Aldrich. The γ-secretase inhibitor compound E was from Alexis/Coger (Lausen, Switzerland).

MDA-MB-231 and GTL-16 cells were cultured in Dulbecco’s Modified Eagle’s Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). HEK-293T cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids (Life Technologies), 1% penicillin (10 000 U/ml)–streptomycin (10 000 μg/ml); Life Technologies). HEK-293T cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids (Life Technologies), and antibiotics. MCF-10 A cells were cultured in DMEM and HAM’s F12 (vol/vol, Life Technologies) supplemented with 10% FBS.
Antibodies. Rabbit polyclonal antibodies directed against phosphorylated (Y1234/1235) Met (no. 3126), phosphorylated (S473) Akt (no. 9271), and active caspase 3 (no. 9661) and mouse monoclonal antibody directed against phosphorylated (T202/Y204) Erk were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against the kinase domain of Met (3D4) was purchased from Life Technologies. Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC5-32233), rabbit polyclonal antibodies against the C-terminal domain of human PARP-1 (H2505), against Akt (H136), and against Erk2 (C14), and goat polyclonal antibodies against the calpain 1 large subunit (C-20) and the calpain 2 large subunit (C-19) were purchased from Santa Cruz Biotechnology. Rabbit monoclonal antibody directed against the C-terminal tail of Met (SP44) was purchased from Roche (Schlieren, Switzerland). Mouse monoclonal antibody directed against the extracellular domain of Met (MAB3582) and goat polyclonal antibody directed against the extracellular domain of Met (AF276) were purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal antibody directed against the C-terminal domain of Met (DL-21) was kindly provided by Dr. Sylvia Giordano (University of Torino Medical School, Italy). Regions recognized by the anti-Met antibodies are shown in Supplementary Figure S8. Mouse monoclonal antibody directed against Gb2 (growth factor receptor-bond protein 2; 81/GRB2) was purchased from BD Transduction Laboratories (San Jose, CA, USA). Green-fluorescent Alexa Fluor 488-conjugated anti-mouse IgG (H+L) and red-fluorescent Alexa Fluor 594-conjugated anti-rabbit IgG (H+L) were purchased from Life Technologies. Rabbit polyclonal antibodies against the Flag epitope were purchased from Sigma-Aldrich. Rabbit polyclonal antibody directed against tubulin (PM054) was purchased from Medical and Biological Laboratories (Nagoya, Japan).

Plasmid constructs. The vector expressing Flag-p40Met

\[\text{Flag-p40Met}\]

was constructed as described previously.\textsuperscript{22} The vector expressing p40Met\textsuperscript{GRABAN} (Met S1037–S1390) was constructed as follows. The portion of Met between D1030 and S1390 was amplified by PCR from plasmid by kindly provided by Dr. G Vande Woude, Van Andel Research Institute, MI, USA) used as template, with the following primers: 5′-AGGSATCGATGACCTGGATCGCCCCCATCTTAATAG-3′ containing a BamH1 restriction site and 5′-AGCTGGACGCTGATCTCCTGAGGAGACC-3′ containing a XhoI restriction site. The PCR product was subcloned into pDNA3 between the BamH1 and XhoI restriction sites. The amino acids D1030–S1037 were removed with the QuickChange site-directed mutagenesis system of Stratagene (Santa Clara, CA, USA), with the following primers: 5′-GTAGCCAGGCTCGAGATGACCTGGATCGCCCCCATCTTAATAG-3′ and 5′-GGATCAGGATCCCTGAGGAGACC-3′. The vector expressing Flag-p40Met\textsuperscript{S1037A} was constructed as follows. pDNA3-p40Met\textsuperscript{GRABAN} was subcloned into pDNA3 FLAG between the BamH1 and XhoI restriction sites.

Transfections and RNA interference. Transfections of HEK-293T and MCF-10 A cells with the reagents polyethyleneamine Exgen 500 (Euromedex, Italy) and Modugene (Santa Clara, CA, USA) were performed using the FuGENE 6 reagent (Roche, Basel, Switzerland). For gene silencing, a suspension of 600 000 cells was incubated with 60 nM small interfering RNA (siRNA), and then plated in a six-well plate in complete medium. The Met-targeting siRNAs were a pool of three stealth siRNAs (Invitrogen) 60 nM small interfering RNA (siRNA), and then plated in a six-well plate in complete medium. The Met-targeting siRNAs were a pool of three stealth siRNAs (Invitrogen) designed to silence the expression of Met (AF276) were purchased from R&D Systems (Minneapolis, MN, USA). Regions recognized by the anti-Met antibodies are shown in Supplementary Figure S8. Mouse monoclonal antibody directed against Gb2 (growth factor receptor-bond protein 2; 81/GRB2) was purchased from BD Transduction Laboratories (San Jose, CA, USA). Green-fluorescent Alexa Fluor 488-conjugated anti-mouse IgG (H+L) and red-fluorescent Alexa Fluor 594-conjugated anti-rabbit IgG (H+L) were purchased from Life Technologies. Rabbit polyclonal antibodies against the Flag epitope were purchased from Sigma-Aldrich. Rabbit polyclonal antibody directed against tubulin (PM054) was purchased from Medical and Biological Laboratories (Nagoya, Japan).

Plasmid constructs. The vector expressing Flag-p40Met\textsuperscript{GRABAN} (Met S1037–S1390) was constructed as follows. The portion of Met between D1030 and S1390 was amplified by PCR from plasmid by kindly provided by Dr. G Vande Woude, Van Andel Research Institute, MI, USA) used as template, with the following primers: 5′-AGGSATCGATGACCTGGATCGCCCCCATCTTAATAG-3′ containing a BamH1 restriction site and 5′-AGCTGGACGCTGATCTCCTGAGGAGACC-3′ containing a XhoI restriction site. The PCR product was subcloned into pDNA3 between the BamH1 and XhoI restriction sites. The amino acids D1030–S1037 were removed with the QuickChange site-directed mutagenesis system of Stratagene (Santa Clara, CA, USA), with the following primers: 5′-GTAGCCAGGCTCGAGATGACCTGGATCGCCCCCATCTTAATAG-3′ and 5′-GGATCAGGATCCCTGAGGAGACC-3′. The vector expressing Flag-p40Met\textsuperscript{S1037A} was constructed as follows. pDNA3-p40Met\textsuperscript{GRABAN} was subcloned into pDNA3 FLAG between the BamH1 and XhoI restriction sites.

Transfections and RNA interference. Transfections of HEK-293T and MCF-10 A cells with the reagents polyethyleneamine Exgen 500 (Euromedex, France), and Jet Prime (Polyplus Transfection, Illkirch, FRANCE) were performed as previously described.\textsuperscript{22,23} For gene silencing, a suspension of 600 000 cells was incubated with 60 nM small interfering RNA (siRNA), and then plated in a six-well plate in complete medium. The Met-targeting siRNAs were pools of three peptide Stealth siRNAs (Invitrogen) (5′-CUAUAUGUGUGCUUCGCGGCUAdTdT-3′ and 5′-GGACAAGAUGUGGCAUCUAAdTdT-3′) and calpain 2 (5′-GCCGATGUGUGUGUGUGUGCAUCUAAdTdT-3′). The reaction was stopped by adding 3 x Laemmli buffer and heating the sample at 95 °C for 4 min. After electrophoresis, the polyacrylamide gel was fixed in 40% methanol, 7% acetic acid solution and stained with Coomassie blue. Stained band corresponding to Met was excised from the gel, desalted, and spotted onto a MALDI plate with freshly dissolved 1-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% CH\textsubscript{3}CN, TFA 1/1000). Mass spectrometry was performed by MALDI-TOF-TOF Autoflex Speed (Brucker Daltonics, Fremon, CA, USA). MS and MS/MS data were analyzed by BioTools software (Fremont, CA, USA). Peptides were identified with Mascot, http://www.matrixscience.com/.

Tumor sample preparation. After surgery, tumor samples were divided into two parts. One was frozen in a Snapfrost fast freezing system (Excilon, Apero, CA, USA) and stored at −80 °C, and the other was formaldehyde fixed and paraffin embedded (FFPE). For western blot analysis, frozen tissue samples were sliced into pieces ~ 1.2 mm in diameter and transferred into Lysis Matrix type D tubes containing ceramic beads (MP Biomedical, Santa Ana, CA, USA) in the presence of RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 2.5 mM EDTA, 50 mM NaF, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40). The samples were lysed with a FastPrep homogenizer (MP Biomedicals; four cycles of 40 s at 6 m/s, each followed by a 5-min pause on ice). The samples were then centrifuged at 20 000 x g for 30 min and proteins in the supernatant were quantified by the BCA Protein Assay (Pierce). Equal amounts of protein were analyzed by western blotting. For IHC, FFPE tissue sections were stained with hematoxylin/eosin/safran and IHC was performed with an antibody against the intracellular domain of Met (SP44 CONFIRM, Ventana Medical Systems). Met expression was scored according to the study of Spigel et al.\textsuperscript{50} (score 3: high-intensity staining of at least 50% of the tumor and high-intensity staining of at least 50% of the tumor and moderate or strong staining of < 50%; score 0: no staining or staining at any intensity of < 50% of the tumor).

Conflict of Interest

The authors declare no conflict of interest.

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