Distinct c-Met activation mechanisms induce cell rounding or invasion through pathways involving integrins, RhoA and HIP1

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ABSTRACT

Many carcinomas have acquired oncogenic mechanisms for activating c-Met, including c-Met overexpression and excessive autocrine or paracrine stimulation with hepatocyte growth factor (HGF). However, the biological outcome of c-Met activation through these distinct modes remains ambiguous. Here, we report that HGF-mediated c-Met stimulation triggers a mesenchymal-type collective cell invasion. By contrast, the overexpression of c-Met promotes cell rounding. Moreover, in a high-throughput siRNA screen that was performed using a library of siRNAs against putative regulators of integrin activity, we identified RhoA and the clathrin-adapter protein HIP1 as crucial c-Met effectors in these morphological changes. Transient RhoA activation was necessary for the HGF-induced invasion, whereas sustained RhoA activity regulated c-Met-induced cell rounding. In addition, c-Met-induced cell rounding correlated with the phosphorylation of filamin A and the downregulation of active cell-surface integrins. By contrast, a HIP1-mediated increase in β1-integrin turnover was required for the invasion triggered by HGF. Taken together, our results indicate that c-Met induces distinct cell morphology alterations depending on the stimulus that activates c-Met.

KEY WORDS: Cancer, Cell invasion, c-Met, HIP1, Integrin, RhoA

INTRODUCTION

The hepatocyte growth factor (HGF) and its transmembrane receptor c-Met have been implicated in the progression of many solid tumours to metastatic disease. The binding of HGF to c-Met induces the activation and auto-phosphorylation of the receptor. The phosphorylated receptor recruits a vast number of adapter and signalling proteins, which collectively trigger signalling cascades that result in the loss of cell–cell contacts, scattering and gain of motility, induction of angiogenesis, escape from apoptosis, and the ability to proliferate and settle in a new environment (Trusolino et al., 2010).

Oncogenic c-Met activation can be triggered by either HGF-dependent receptor activation, c-Met overexpression or activating point mutations in the kinase domain (Gentile et al., 2008; Graveel et al., 2004). The latter mechanism occurs most rarely and requires the active receptor to be trafficked from the plasma membrane to endosomes (Joffre et al., 2011). By contrast, the quantitative upregulation of c-Met is the most frequent mechanism of oncogenic c-Met activation. HGF-mediated c-Met stimulation, due to excessive autocrine or paracrine secretion of the ligand, also often occurs in human cancers and is associated with increased aggressiveness of the tumours (Stellrecht and Gandhi, 2009). In spite of the established role of c-Met in metastasis, the biological consequences of signalling originating from either c-Met overexpression or excessive HGF-mediated stimulation are not established. The most thoroughly investigated functional outcome of c-Met activation is the well-established HGF-induced scattering of polarised epithelial MDCK (Madin-Darby canine kidney) cells in two-dimensional (2D) models, and many of the molecular mechanisms involved are known (Birchmeier et al., 2003). By contrast, c-Met signalling in a three-dimensional (3D) environment is not as well understood, especially in cancer cells.

We therefore sought to investigate the cellular effects of c-Met signalling by using a 3D Matrigel model system. We find that different modes of c-Met activation trigger phenotypes that are distinct from the well understood HGF-induced scattering. c-Met overexpression induces a round and non-motile phenotype, whereas excessive HGF-mediated stimulation in the same cell line induces collective invasion. Moreover, using an RNAi-based cell spot microarray, we identified RhoA and HIP1 as important regulators of the c-Met-induced morphological switching.

RESULTS

Overexpression of c-Met triggers cell rounding in 3D Matrigel

With the aim of elucidating the c-Met-induced signalling pathways that contribute to the invasiveness of cancer cells, we used an epithelial cell model [human embryonic kidney (HEK) 293 cells] with a tetracycline (Tet)-inducible expression system for GFP-tagged c-Met (see Materials and Methods). In human carcinomas, the quantitative upregulation of c-Met has been found to increase the sensitivity of the carcinoma to HGF or to cause receptor oligomerisation and transactivation even in the absence of ligand (Gentile et al., 2008; Lutterbach et al., 2007). Consistently, following Tet-induced c-Met overexpression in our cell model, we detected constitutive activation of c-Met (supplementary material Fig. S1A). Interestingly, upon c-Met overexpression, cells cultured on a 2D surface underwent a striking change in cell shape and adopted a round morphology (Fig. 1A) with obvious cell blebbing, a result which was consistent with that reported in a previous publication (supplementary material Movie 1; Laser-Azogui et al., 2013). In order to investigate this morphological change in a more
Fig. 1. See next page for legend.
physiologically relevant environment, we embedded these cells into collagen gels or 50% Matrigel (which contains collagen IV, laminins and proteoglycans) and followed their behaviour by using time-lapse microscopy. For MDCK cells in 2D culture, it has been shown that the extracellular matrix (ECM) to which the cells adhere has a significant influence on the outcome of HGF treatment; collagen facilitates cell scattering, whereas laminin supports the epithelial phenotype (Sander et al., 1998). In 3D collagen, we found that the untreated HEK 293 cells spread and migrated predominantly as individual cells (supplementary material Movie 2), whereas in 3D Matrigel the cells spontaneously formed multicellular clusters with cell–cell contacts and a spherical shape (Fig. 1B; supplementary material Movie 3). Thus, the epithelial-like clusters formed in Matrigel were selected for further investigation of c-Met-induced signalling. In order to characterise the spheroids in more detail, we fixed HEK 293 cells in 3D culture and stained for β1-integrin and actin (Fig. 1C). Prior to induction with Tet, the cells formed densely packed spherical clusters, throughout which filamentous actin and plasma-membrane-associated β1-integrin were detectable.

Interestingly, upon Tet-induced overexpression of c-Met, the cell colonies in 3D culture lost their spheroid phenotype and converted into a loose assembly of individual round cells (Fig. 1B,C; supplementary material Movie 4). The cell rounding was not associated with cell death (supplementary material Fig. S1B) or with a switch in cell migration, because the cells were only marginally moving (Fig. 1D,E; supplementary material Movie 5), albeit occasional blebbing was observed in Matrigel (Fig. 1F) and also in collagen (supplementary material Movie 6). This was further confirmed by using an invasion assay in which cells invaded from a monolayer upwards into Matrigel. Tet induction further reduced the low basal invasiveness of the HEK 293 cells in this assay (Fig. 1G). This suggests that the cell rounding induced by c-Met was not linked to the induction of amoeboid invasion in these cells. The Tet-induced disruption of the spheroids could be prevented by treating cells with the c-Met kinase inhibitor SU11274, indicating a requirement for the kinase activity of c-Met in the process (Fig. 1B). Neither the level of cell-surface-associated E-cadherin nor total protein levels of E-cadherin were reduced following c-Met overexpression (supplementary material Fig. S1C,D), and no increase in the expression of vimentin was obvious (supplementary material Fig. S1E,F), suggesting that the cell rounding was not linked to an epithelial-to-mesenchymal-like switch. Taken together, these data indicate that c-Met overexpression induces cell rounding in 3D Matrigel. Consistently, gastric cancer cells that inherently carry amplified MET and express high levels of active c-Met [MKN-45 and GTL-16 (Bertotti et al., 2010); Fig. 2A; supplementary material Fig S1G] also adopted a round cell morphology in Matrigel (Fig. 2B). Importantly, inhibition of the kinase activity of c-Met (by the use of the inhibitor PHA665752) partially suppressed cell rounding in 3D culture and supported the spheroid-like clustering of these cells (Fig. 2B). Interestingly, with these c-Met-overexpressing cancer cell lines the rounded morphology was associated with invasiveness, and it was significantly reduced by treating cells with the c-Met inhibitor SU11274 (Fig. 2C). However, the inhibition was only 15%, suggesting that additional pathways also contribute to the invasive phenotype of these cells (Fig. 2C). By contrast, non-MET-amplified gastric cancer (AGS) cells spontaneously gathered in 3D Matrigel to form small epithelial-like spheroids. In these cells, transient overexpression of wild-type c-Met induced cell rounding and disruption of the spheroids, whereas overexpression of a kinase-dead c-Met failed to influence cell morphology (Fig. 2D). Taken together, these data show that high c-Met expression and activity correlates with cell rounding in 3D culture in different cell types.

**HGF stimulation promotes collective cell invasion**

We next sought to analyse the effects of ligand-dependent endogenous c-Met activation in HEK 293 cells. Interestingly, exogenous HGF stimulation of HEK 293 cells did not induce cell rounding or scattering but, instead, stimulated the progressive invasion of cell sheets into the surrounding matrix in a manner that was dependent upon c-Met activity (Fig. 3A; supplementary material Movie 7). The cells clearly retained junctional E-cadherin throughout the invasion, indicative of collective migration (supplementary material Fig. S1H). Consistently, HEK 293 cells strongly invaded towards a gradient of HGF but remained predominantly non-invasive in the absence of HGF (Fig. 3B; 1.8-fold±0.2 more invasion with HGF, **P<0.005**).

Constant endocytic and exocytic trafficking of integrins is necessary for invasion in many cell types (Bridgewater et al., 2012), and, recently, concomitant trafficking of integrins and c-Met has been shown to promote c-Met-dependent invasion (Muller et al., 2013). We also found integrin trafficking to be altered following HGF stimulation both in 2D and in 3D culture, such that the β1-integrin surface expression was rapidly reduced (Fig. 3C), most likely due to the induction of endocytosis. However, within one hour, the cell surface integrin levels were restored, and they remained elevated even at 15 hours of HGF treatment both in 2D and 3D environments (Fig. 3D). This could be indicative of increased integrin recycling back to the membrane in the cells treated with HGF, because no significant changes were observed in total cellular β1-integrin levels (Fig. 3E). Consistent with the HGF-induced elevation in the levels of integrin at the cell surface, we found that inhibition of β1-integrin prevented the HGF-triggered collective cell invasion
in 3D culture (Fig. 3F; supplementary material S1I) but had no effect on cell rounding in 3D Matrigel (supplementary material Fig. S1J). Collective cell invasion has been linked to matrix degradation, and c-Met signalling activates ECM-degrading matrix metalloproteinases (MMPs) in haematopoietic cells (Tesio et al., 2011). This mechanism might also allow HGF-stimulated HEK 293 cells to invade into Matrigel, because the inhibition of MMP function also inhibited HGF-induced invasion but not the c-Met-induced rounding (Fig. 3F; supplementary material Fig. S1J).

Thus, our results show that c-Met activation triggers differential cellular responses depending on the mechanism by which it is activated. In our model, HGF stimulation triggered MMP- and integrin-dependent collective migration, whereas c-Met upregulation induced cell rounding and disruption of the epithelial spheroids.

**Screen for novel c-Met effectors**

Based on the data reported above, c-Met-induced morphological changes appeared to involve alterations in integrin-mediated cell–matrix interactions. In order to identify effectors of c-Met activation, we screened for novel c-Met effectors.
oncogenic c-Met signalling, we performed an RNAi-based high-throughput cell spot microarray (Rantala et al., 2011) screen with the Tet-inducible HEK 293 cells, in which we specifically analysed for cell rounding on Matrigel spots. We selected 125 target genes encoding products that have been reported to function in the regulation of the activity of β1-integrin [as identified in a large-scale RNAi screen (Pellinen et al., 2012)], as well as some cytoskeleton-regulating genes, to investigate their impact on the c-Met-induced phenotypic alterations and on cell detachment from Matrigel. siRNAs against mRNAs encoding proteins that regulate the c-Met-induced cell rounding were identified based on their ability to prevent the phenotypic change (without affecting c-Met–GFP expression) (Fig. 4A). Interestingly, two ephrin receptor tyrosine kinases, EphA2 and EphA4, were implicated in the c-Met-induced rounding, consistent with a previous report demonstrating a role for ephrins in the rounding of HEK 293 and melanoma cells (Lawrenson et al., 2002). This suggests a possible crosstalk between c-Met and ephrin receptors in cell rounding, which would be an interesting topic for future research. Another hit in the screen was HIP1, which was of interest as it is a known clathrin-adapter protein implicated in endocytosis and...
tumorigenesis (Hyun and Ross, 2004). HGF-induced cell scattering has been linked to the dissolution of stress fibres and activation of Rac (Joffre et al., 2011; Ridley et al., 1995). However, somewhat unexpectedly among the most efficient inhibitors of c-Met-induced cell rounding were 14 genes that could be linked to RhoA signalling (Table 1), including RhoA itself. Interestingly, siRNAs targeting other Rho family members, RhoB and RhoC, did not inhibit rounding (Fig. 4A), suggesting a specific role for RhoA. This prompted us to further investigate the role of RhoA in c-Met-induced cell rounding (Fig. 4B).

**RhoA and ROCK are necessary effectors for the morphological switching downstream of c-Met**

In order to validate the requirement for RhoA in c-Met-induced cell rounding, we inhibited RhoA signalling either directly, with the Rho-specific inhibitor C3, or by inhibiting the RhoA effector kinase, ROCK, using Y-27632. Both inhibitors significantly impaired c-Met-induced cell rounding in 2D culture (supplementary material Fig. S2A). Interestingly, RhoA was activated upon c-Met overexpression in 2D culture, and the activation correlated with the level of c-Met induction (Fig. 5A). c-Met expression also induced myosin light chain (MLC) phosphorylation, which is indicative of increased ROCK function (Fig. 5A, lower panel). In 3D Matrigel, the cell rounding was first detected after 7 hours of induction with Tet (supplementary material Movie 4). Consistently, we detected increased RhoA activity after 7 hours of Tet treatment, and the RhoA activity was sustained for the duration of the c-Met induction (Fig. 5B). In 3D culture, both the siRNA-mediated loss of RhoA and the inhibition of ROCK by Y-27632 prevented c-Met-induced cell rounding (Fig. 5C–E). Because c-Met appeared to induce long-term rather than transient activation of RhoA, we wanted to test the effect of constitutive RhoA activity on the
Next we analysed the role of RhoA in HGF-induced cell invasion. HGF treatment induced a rapid and transient activation of RhoA in 2D culture (Fig. 5H, left panel). A similar transient HGF-induced RhoA activation was also observed in cells isolated from 3D Matrigel, (Fig. 5H, right panel). ROCK inhibition prevented the collective invasion of HGF-stimulated HEK 293 cells into Matrigel (Fig. 5I), suggesting that transient RhoA signalling, possibly in a subset of the cells, is required for the invasion but remains permissive for the maintenance of the cell–cell contacts in the group. Taken together, our data show that c-Met activates RhoA and suggest that sustained RhoA activation upon c-Met overexpression triggers cell rounding, whereas transient RhoA activation upon stimulation with HGF is necessary for invasive migration.

Table 1. Target genes related to RhoA signalling pathway

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>ARHGGEF18</td>
<td>p114RhoGEF</td>
<td>Cell junction-associated RhoA activator</td>
<td>Terry et al., 2011</td>
</tr>
<tr>
<td>CD14</td>
<td>CD14</td>
<td>Membrane receptor that stimulates Rho activity following monocyte adherence</td>
<td>Hmama et al., 1999</td>
</tr>
<tr>
<td>CD9</td>
<td>Tetranspanin CD9</td>
<td>Transmembrane protein, which contributes to RhoA-dependent actin cytoskeleton alterations</td>
<td>Imhof et al., 2008</td>
</tr>
<tr>
<td>CDC25C</td>
<td>cdc25C</td>
<td>Phosphatase, which regulates entry into mitosis; activated by RhoA</td>
<td>Misaki et al., 2001</td>
</tr>
<tr>
<td>EPHA3</td>
<td>Ephrin A3</td>
<td>Receptor tyrosine kinase implicated in RhoA inhibition</td>
<td>Clifford et al., 2008</td>
</tr>
<tr>
<td>EPHA4</td>
<td>Ephrin A4</td>
<td>Associated with RhoA activation</td>
<td>Ogita et al., 2003</td>
</tr>
<tr>
<td>GJA1</td>
<td>Gap-junction protein alpha1 (Connexin 43)</td>
<td>Correlated with RhoA activation and actin cytoskeletal rearrangements</td>
<td>Laing et al., 2005</td>
</tr>
<tr>
<td>IRS2</td>
<td>Insulin receptor substrate 2</td>
<td>IRS2 supports RhoA activation during migration of breast cancer cells</td>
<td>Zhang et al., 2004</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
<td>Activates the RhoA GEF ArhGGEF1 and has been correlated with metastasis</td>
<td>Guilluy et al., 2010; Abdulghani et al., 2008</td>
</tr>
<tr>
<td>LCK</td>
<td>Lymphotox-specific kinase</td>
<td>Activates the RhoA GEF Vav</td>
<td>Han et al., 1997</td>
</tr>
<tr>
<td>PTPN11</td>
<td>Protein tyrosine phosphatase SHP2</td>
<td>Activates RhoA and ROCK</td>
<td>Lee and Chang, 2008</td>
</tr>
<tr>
<td>RICS</td>
<td>p250GAP</td>
<td>Negative regulator of Cdc42 and Rac1</td>
<td>Nasu-Nishimura et al., 2006</td>
</tr>
<tr>
<td>YWHAG</td>
<td>14-3-3 gamma</td>
<td>Inhibits the Rho GAP DLC1, but also inhibits Rho GEF AKAP-Lbc</td>
<td>Scholz et al., 2009; D’ivi et al., 2004</td>
</tr>
</tbody>
</table>

To investigate the link between c-Met and integrins in more detail. In contrast to the HGF-induced elevation of cell-surface integrins during extended HGF exposure (Fig. 3D), we observed that Tet-induced c-Met overexpression resulted in a significant reduction of cell-surface β1-integrin levels in 2D culture. The same reduction also occurred (albeit with slower kinetics) in 3D culture (Fig. 6A). The total cellular integrin levels remained unaltered during Tet-induced c-Met expression (supplementary material Fig. S2G). In addition, β1-integrin activity at the cell surface (its affinity for ligand) was reduced following c-Met induction but was increased upon HGF stimulation (Fig. 6B). Recently, cell rounding induced by epidermal growth factor (EGF) has been linked to EGF receptor (EGFR)-mediated integrin inactivation and phosphorylation of filamin A, a well-established inhibitor of integrin activity (Vial and McKeown-Longo, 2012). This mechanism might be generally involved in cell rounding that is induced by receptor tyrosine kinases, because we also observed increased filamin A phosphorylation upon c-Met induction (Fig. 6C). Similarly, the gastric cancer cells MKN-45 and GTL-16, which inherently express high levels of active c-Met, exhibited increased phosphorylated filamin A levels that were lost upon inhibition of c-Met kinase activity with PHA (Fig. 6C, lower panel). Thus, c-Met overexpression induced morphological switching in cells by increasing RhoA activity and reducing integrin activity on the cell surface.

HIP1 mediates β1-integrin turnover following c-Met activation

Integrins are endocytosed either by clathrin-dependent or -independent routes depending on the cell type and migration stimulus. In HEK 293 cells, the inhibition of clathrin, by using the clathrin antagonist Pitstop, fully inhibited both HGF- and Tet-induced integrin endocytosis from the plasma membrane, suggesting that clathrin-dependent endocytosis is the predominant route of integrin internalisation in these cells (supplementary material Fig. S3A). One of the genes that was identified in our screen as a regulator of c-Met-induced morphological changes was the clathrin-adapter protein HIP1, a protein involved in the recruitment and selection of clathrin-endocytosed cargo (Gottfried et al., 2009). Therefore, we wanted to investigate whether HIP1 was
Fig. 5. See next page for legend.
Fig. 5. RhoA is necessary for c-Met-induced cell rounding and invasion. (A) Results of a RhoA activation assay from 2D-cultured HEK 293 cells that were induced with Tet as indicated. Representative blots are shown. Quantification of the amount of active RhoA is shown beneath the upper panel, and quantification of the amount of phosphorylated MLC (p-MLC, indicative of ROCK function) is shown beneath the lower panel. Results are from three experiments (mean ± s.d., *P<0.05). The same cell lysates were used to blot for total and active (phosphorylated) c-Met (p-c-Met), p-MLC, total MLC and tubulin levels. (B) Results of a RhoA activation assay from Tet-induced cells embedded into 50% Matrigel. Shown is a representative blot from one of three experiments (mean ± s.d., **P<0.01, ***P<0.005). (C) Western blot showing the siRNA silencing of RhoA in cells used in experiment D. Scr, scrambled control siRNA. (D) RhoA-silenced HEK 293 cells cultured in 50% Matrigel were either uninduced or induced with Tet (n=3, two different siRNAs were used). (E) Matrigel-embedded HEK 293 cells were stimulated with Tet in the absence or presence of 10 μM Y-27632 (ROCK inhibitor). (F–G) At 24 h post-transfection cells transfected with (F) GFP or GFP-tagged dominant-negative (DN) or constitutively active (QL) RhoA mutants or (G) GFP-tagged constitutively active (V14) RhoA mutant were embedded into Matrigel, and their ability to form spheroids was monitored by using time-lapse microscopy. (H) Results of a RhoA activation assay from HGF-treated cells from 2D culture (left panel) or from culture in 50% Matrigel (right panel). Shown are representative results from one of three experiments (mean ± s.d., **P<0.01, ***P<0.005). (I) HEK 293 cells cultured in 50% Matrigel were stimulated with HGF in the presence or absence of Y-27632. The images shown in D–G and I are representative images from time-lapse movies.

necessary for the c-Met-induced alterations in integrin traffic and cell shape. We found that β1-integrin and HIP1 associated in HEK 293 cells, based on reciprocal co-immunoprecipitations (supplementary material Fig. S3B), and that β1-integrin was recruited to HIP1 upon c-Met induction in intact cells, as indicated by increased proximity-ligation signal between the two (Fig. 7A). In addition, in HEK 293 cells, HIP1-silencing prevented the loss of integrins from the plasma membrane that was induced by either HGF treatment (Fig. 7B,E) or c-Met–GFP overexpression (Fig. 7C; supplementary material Fig. S3C). Interestingly, the silencing of HIP1 attenuated the Tet- and HGF-stimulated endocytosis rate of biotinylated cell-surface β1-integrin without influencing the basal rate of integrin endocytosis in non-induced cells (Fig. 7D,E; supplementary material Fig. S3D), suggesting that HIP1 specifically regulates c-Met-induced integrin uptake. Taken together, these data show that c-Met induces integrin internalisation in these cells through a previously unknown pathway involving clathrin and HIP1.

Consistent with these data, HIP1 was also involved in the HGF-induced invasion of these cells from spheroids, because HIP1 depletion completely prevented HGF-induced invasion of cells from the cluster (Fig. 7F,G). This is most likely due to impaired HGF-stimulated integrin trafficking in the HIP1-silenced cells (Fig. 7E), because integrin traffic regulates cell migration, and the HGF-induced invasion in this model is integrin-dependent. In addition, the loss of HIP1 (supplementary material Fig. S3D) had no effect on the phosphorylation of c-Met following HGF stimulation (supplementary material Fig. S3E) nor on c-Met internalisation (supplementary material Fig. S3F,G), suggesting that HIP1 might regulate integrin traffic in HGF-induced cells without influencing c-Met signalling.

By contrast, the integrin-independent rapid cell rounding induced by c-Met was not sensitive to the depletion of HIP1, whereas the addition of either c-Met kinase inhibitor or ROCK inhibitor prevented the phenotypic change (Fig. 7H). This suggests that HIP1 regulates c-Met-induced integrin turnover from the cell surface and is thus not required for cell rounding, which is independent of β1-integrin. In our siRNA screen on 2D Matrigel, the loss of HIP1 prevented the Tet-induced rounding of HEK 293 cells (Fig. 3A). The reason for this apparent discrepancy is that, on 2D surfaces, cell adhesion is regulated by integrins and small GTPases (Ridley, 2011), and the c-Met-induced reduction of cell-surface integrins is therefore predicted to induce cell detachment and rounding. Thus, the inhibition of integrin trafficking by siRNA against HIP1 has an impact on the morphology of the cells in 2D culture. However, as the cell rounding in 3D culture was integrin independent and was linked to constitutive RhoA activation, HIP1 was not crucial for the rounding of c-Met-overexpressing HEK 293 cells in 3D Matrigel.
Fig. 7. See next page for legend.
Fig. 7. HIP1 mediates β1-integrin endocytosis downstream of c-Met. (A) myc–HIP1-expressing HEK 293 cells at 24 h post-transfection were embedded into Matrigel for 24 h, induced with Tet for 7 h and thereafter were treated for 15 min with a dynamin inhibitor (dynasore) to halt endocytosis. The presence of proximity ligation assay (PLA) signal (grey dots) indicates close proximity between HIP1 (detected using the anti-myc antibody) and IgG (detected using the anti-GST antibody) in the upper panel, or between HIP1 and β1-integrin (detected using the EP1041Y β1-integrin-tail antibody) in the lower panel. Shown are representative images from >100 cells. The average number of PLA signals per cell is indicated (mean±s.d., ****P<0.0005). (B) FACs analysis of cell-surface β1-integrin from HGF-stimulated and HIP1-silenced HEK 293 cells cultured on a 2D surface. Non-transfected control cells are from the experiment shown in Fig. 3C (mean±s.d., n=3 using two different siRNAs against HIP1, *P<0.05). (C) FACs analysis of cell-surface β1-integrins from Tet-induced HIP1-silenced and scrambled-control (Scr)-treated HEK 293 cells cultured on a 2D surface (mean±s.d., n=3 using two different siRNAs against HIP1, ***P<0.005). (D–E) Biochemical integron endocytosis assay performed on either Scr-treated or HIP1-silenced HEK 293 cells upon (D) Tet induction or (E) stimulation with HGF. The mean values from three independent experiments are shown (±s.d., *P<0.05, ***P<0.005). (F) Representative images from time-lapse movies. Scr-treated or HIP1-silenced HEK 293 cells were cultured in Matrigel with or without stimulation with HGF. (G) Western blot analysis of the cells used in B and F. (H) Representative images from time-lapse movies. HIP1-silenced HEK 293 cells were embedded in 50% Matrigel and stimulated with Tet in the presence or absence of 2 μM SU11274 (c-Met kinase inhibitor) or 10 μM Y-27632 (ROCK inhibitor).

Taken together, our data suggest that c-Met activation regulates the switching of cell morphology through HIP1 and RhoA. c-Met overexpression triggers a round single-cell morphology through sustained RhoA activation, whereas HGF-induced receptor activation results in collective cell invasion and HIP1-dependent integrin turnover.

DISCUSSION

Our results suggest that c-Met activation triggers cells to adopt distinct morphologies depending on the c-Met-activating mechanism (Fig. 8). By using the same epithelial cell line and the same 3D matrix environment combined with a high-throughput cell spot microarray screen, we found that ligand-dependent c-Met stimulation with exogenous HGF caused integrin-mediated, HIP1- and MMP-dependent collective migration of cells with mesenchymal characteristics. By contrast, the overexpression of c-Met induced sustained RhoA activation, loss of active cell-surface integrins and cell rounding. To the best of our knowledge, these data are the first to analyse comprehensively the functional outcomes of the two clinically relevant modes of oncogenic c-Met activation.

Our data are consistent with the possibility that distinct c-Met-activating mechanisms allow cancer cells to switch from a proliferative to an invasive phenotype during cancer progression (‘go or grow’ hypothesis (Hatzikirou et al., 2012)). In accordance with this hypothesis, c-Met is differentially expressed in various locations of head and neck cancer, with the highest expression of c-Met at the primary tumour location (Choe et al., 2012). We found that c-Met upregulation induced cell rounding and disruption of the epithelial spheroids. This could be linked to the loss of contact inhibition that is commonly observed during tumour progression in vivo, because quantitative upregulation of c-Met has been found to occur frequently in tumours and to generally contribute to cancer growth and development (Gherardi et al., 2012; Graveel et al., 2004). In addition, gastric tumours, which frequently overexpress endogenous MET, have been shown to be ‘addicted’ to c-Met for their proliferation and retention (Corso et al., 2008). Furthermore, in gastric carcinomas (Graziano et al., 2011) and hepatocellular carcinomas (Ogunwobi et al., 2013), c-Met overexpression correlates with increased expression of the HGF ligand. It is thus possible that, as the cancer progresses, increased HGF stimulation of c-Met might trigger a switch to an invasive phenotype. The HGF-triggered, MMP- and integrin-dependent collective cell migration observed here upon HGF stimulation might be linked to the observed increased aggressiveness of tumours with excessive autocrine or paracrine HGF secretion (Gentile et al., 2008).

The activation of c-Met through different pathways might allow cancer cells to switch between different migration modes. We observed collective mesenchymal-type cell invasion following HGF stimulation. However, as seen from the time-lapse imaging performed on the cells overexpressing c-Met, the cell rounding in 3D culture was associated with the loss of cell–cell contacts and modest blebbing, but not with increased cell invasion. This is in contrast to a recent report where, on 2D plastic, c-Met overexpression was suggested to induce amoeboid migration (Laser-Azogui et al., 2013). Consistent with many previous reports, we show here that epithelial cells adopt different morphologies in response to matrix cues in conditions that are more similar to those encountered in vivo (tight spheroids in Matrigel and mesenchymal-like single cells in collagen). The fact that the outcome of c-Met activation is determined both by the mechanism of activation (stimulation with the ligand or overexpression of the receptor) and the matrix environment is most likely linked to the apparent crosstalk between c-Met and integrins in cells. Here, we find that HGF induces integrin turnover and eventually increases cell-surface integrin levels, whereas c-Met overexpression drives integrin endocytosis and the loss of cell-surface receptors. In addition, overexpression of c-Met triggered filamin A phosphorylation, which correlated with cell rounding and reduced integrin activity. The ability of c-Met to regulate filamin A might be important in metastasis, because filamin has recently been shown to function as a suppressor of metastasis in breast cancer (Xu et al., 2010). However, this new link has yet to be investigated in detail.

Increased actomyosin contractility through Rho-kinase-dependent signalling increases amoeboid migration in cancer cells as well as increasing stroma stiffness, which facilitates the collective migration of certain carcinomas (Sanz-Moreno et al., 2011). In keratinocytes, the inhibition of RhoA by injection of C3 attenuates HGF-induced cell motility in 2D culture (Takaishi et al., 1994), and in MDCK cells RhoA signalling was found to counteract ephrin-A1-induced loss of stress fibres in HGF-treated cells (Miao et al., 2003). However, the role of RhoA in c-Met-induced rounding or 3D invasion has not been clearly demonstrated. We found that RhoA was required for collective migration and for the c-Met-induced spheroid dissociation and cell rounding. However, RhoA activation during collective migration was transient compared with the sustained high RhoA–GTP levels that correlated with the round morphology. This is consistent with the notion that ROCK-dependent local matrix remodelling and deformation crucially contributes to the migration of fibroblasts in 3D culture (Calvo et al., 2013; Gadea et al., 2007). Thus, HGF-stimulated cells might employ RhoA signalling in order to locally remodel and invade the microenvironment.

It is becoming increasingly evident that tumour cells harbour great plasticity and can thus alter their morphology and migration modes in response to different ECM conditions and different
levels of adhesion receptor expression. Here, we describe how the mode of receptor activation, the duration and the intensity of the signalling, and the matrix environment together dictate the functional outcome of c-Met activation. Therefore, full understanding of the phenotype switching of malignant cells during cancer progression and metastasis requires careful consideration of the interplay between soluble factors, the properties of cancer cells and their matrix environment.

MATERIALS AND METHODS

Time-lapse microscopy and morphology analysis

HEK 293, MKN-45, GTL-16 and AGS cells were embedded into either 2 mg/ml collagen or into 50% Matrigel, treated with Tet or HGF (100 ng/ml), and 80 μg/ml of Mab13 (an antibody that blocks integrin function), 80 μg/ml anti-GST antibody, DMSO or 10 μM MMP inhibitor III (Calbiochem) as indicated. The live-cell time-lapse imaging was performed by using an inverted widefield microscope [AxioCam MRm camera, EL Plan-Neofluar 20× or 10×/0.5 NA objective (Carl Zeiss)] equipped with a heated chamber (37°C) and CO2 controller (4.8%). Images were taken every 10–15 min for the indicated lengths of time. Image processing was performed by using MetaMorph and ImageJ software.

The granularity of rounded cell clusters was quantified as follows. First, the whole cell cluster was detected by trainable segmentation using the Advanced Weka ImageJ plugin. Segmentation was further fine-tuned by masking the original images with the preliminary segmentation result and then performing an additional thresholding step. All image analysis was performed with the same parameters for all images. Following image segmentation, all cell clusters were recognised as individual objects. Second, inner edges were detected as a measure of cluster granularity. Therefore, the difference of Gaussians (DoG) algorithm was applied to the original images. Images with edges made salient by DoG were masked using the cell-cluster area in order to eliminate any irrelevant artefacts outside the cluster. Edges that were deemed to be artefacts (too small or too circular) were filtered out. Third, both inner edges and the cell-cluster area were skeletonised for accurate length detection. Images with skeletonised edges were masked, using slightly shrunk cell clusters as the mask, in order to obtain only inner edges. Finally, the total area occupied by masked skeletonised edges was divided by the total area occupied by skeletonised cell-cluster outlines.

3D Immunofluorescence

HEK 293 cells were incorporated into 50% Matrigel and then either induced with Tet for 24 h or left untreated. The cells were then simultaneously fixed and permeabilised by using 2% paraformaldehyde (PFA) in PBS supplemented with 0.5% Triton X-100 for 1.5 h at room temperature. After three consecutive washing steps (10–15 min per wash at room temperature) with glycine buffer (130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4 and 100 mM glycine in PBS), blocking was performed for 2 h at room temperature in buffer containing 130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 7.7 mM NaN3, 0.1% bovine serum albumin (BSA), 0.2% Triton X-100, 0.05% Tween 20 and 10% horse serum in PBS. Primary antibodies were used at 5–10 mg/ml in blocking buffer and incubated overnight at 4°C. The cells were then washed three times (for 20 min each) with blocking buffer at room temperature with gentle rocking. Alexa-conjugated secondary antibodies were added at a concentration of 5 μg/ml, followed by incubation at room temperature for 1 h and another round of washing. The spheroids were mounted with Mowiol containing anti-fading reagent (Vectashield, Vector Labs) for 1 h at 37°C. Immunofluorescent samples were analysed by using an inverted wide-field microscope (Carl
Zeiss) with a confocal unit, Orca-ER camera (Hamamatsu Photonics), Plan-Neofluar 40× or 63× oil/1.4 NA objective (Carl Zeiss) and SlideBook 5.0 imaging software (Intelligent Imaging Innovations).

**FACS analysis**

HEK 293 cells were plated onto 2D plastic or embedded in 3D 50% Matrigel and treated with 100 ng/ml HGF, 100 ng/ml Tet or 15 μM Pitstop (Abcam), as indicated. The cells were then placed on ice, and the cells from 2D culture were collected into fixation solution (2% PFA in PBS) by using a cell scraper, incubated for 15 min at 4°C, and washed once with PBS. The cells from 3D Matrigel cultures were isolated at 4°C by using Cell Recovery solution (BD). Blocking was performed in a solution of 1% FBS in PBS for 20 min on ice, and cell-surface integrins were stained with K20 anti-β1 antibody, 9EG7 anti-active-β1 antibody or anti-c-Met antibody at 4°C for 1 h, washed with PBS and stained with Alexa-Fluor-conjugated secondary antibody for 1 h at 4°C. Samples were analysed on a FACSArray (Becton Dickinson). The mean fluorescence intensity was determined from 10,000 counted events.

**Rho activation assay**

Rho activation in Tet-induced or HGF-stimulated HEK 293 cells was measured in pull-downs from cell lysates according to the manufacturer’s instructions (Rho activation assay; Cytoskeleton). For 3D cultures, cells were isolated at 4°C with Cell Recovery solution (BD) prior to lysis and pull-down.

**Cell spot microarray**

The preparation of cell spot microarrays and seeding of the cells was performed as described previously (Pellinen et al., 2012). Briefly, Tet-inducible HEK 293 (T-Rex-GFP-Met neo) cells were cultured to 80% confluence and dissociated by using HyQtase (ThermoFishes Scientific). The cells were then placed on ice, washed off using PBS. Cells remaining on the array were gently washed off using PBS. Cells remaining on the array were fixed with 4% PFA in PBS for 15 min, and analysed by microarray scanning the arrays for GFP expression with a Tecan LS400 and 25× objective. The cells were allowed to adhere at 37°C for 30 min. Subsequently, non-adherent cells were washed off, and a second layer of 50% Matrigel was added on top of the cells. After polymerisation, a feeder layer of complete medium with the indicated supplements was added. The cells were maintained at 37°C.

**Antibodies and DNA constructs**

Commercial antibodies against the following antigens were used: c-Met (R&D systems); β1-integrin [PSD2 (Developmental Studies Hybridoma Bank), K20 (Beckman Coulter), MabT3 (BD Pharmingen) and EP1041Y β1-integrin tail (Abcam)]; vimentin and RhoB (Santa Cruz Biotechnology); biotin, phospho-c-Met Y1234/5, E-cadherin, filamin A, phospho-filamin A, RhoA and RhoC (Cell Signaling Technology); HIP1 (Chemicon); and GFP, GST and fluorescently conjugated secondary antibodies (Invitrogen).

Two different siRNAs targeting HIP1 (sense, 5′-GGGACCCGCACAGGCCAAAT-3′ and 5′-GGCCUGAUAGCAGCAAGATT-3′; Qiagen), RhoA (sense, 5′-GGGACUAGGAGCAAGCAAT-3′ and 5′-AGCUAGGACUGGAAAAAGAT-3′; Qiagen) or RhoC (sense, 5′-CCGAGGUGGCGAGAAATA-3′; Qiagen) or scramble control siRNA (Ambion) were transfected at a concentration of 100 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

The plasmid encoding myc-tagged HIP1 was an Addgene plasmid (ID 31250), and was described previously (Rao et al., 2002). Constructs for the expression of wild-type c-Met and kinase-dead c-Met were kind gifts from D. Tulasne (Foveau et al., 2009). Plasmids encoding myc-tagged RhoC and GFP–RhoA-V14 were kindly provided by R. Grosse (University of Marburg), and the plasmid encoding myc-tagged RhoB was a generous gift from H. Mellor (University of Bristol).

**Cell lines and 3D cell culture**

HEK 293 cells and the human gastric cell line GTL-16 were cultivated in DMEM 4500 supplemented with 2 mM glutamine and 10% FBS. Tetracycline-inducible HEK 293 (T-Rex-GFP-Met neo) cells were grown in HEK 293 medium supplemented with 5 μg/ml blasticidin (InvivoGen) and 25 μg/ml zeomycin (Invitrogen) and were generated as follows. The c-Met sequence was kindly provided by Clive Dickson (Cancer Research UK). The c-Met open reading frame (ORF) was first introduced into pEGFP-N1 (BD Clontech) and amplified by PCR, using the flanking primers 5′-CCGCTCGAGATGAAGGCCCCCGCTGTGC-3′ (containing a XhoI site) and 5′-CCCTCAAGCTTCAATGATGTCTCCCCAGAA-GGAGGC-3′ (containing a HindIII site). The plasmid containing the c-Met-EGFP coding sequence was then digested with EcoRI and NotI restriction enzymes and was introduced into pcDNA™ 4/TO (Invitrogen) containing full-length wild-type c-Met digested with both restriction enzymes. The construct was then verified by sequencing. The Tet-inducible stable T-Rex-GFP-Met neo HEK 293 cell line was generated using the Invitrogen T-Rex™-293 cells according to the manufacturer’s instructions. The human gastric carcinoma cell lines MKN-45 and AGS were maintained in RPMI 1640 supplemented with 1% l-glutamine, 10% FBS and 1% penicillin-streptomycin.

For 3D cell culture, the cells were incorporated into 3D collagen (2 mg/ml) or into 50% growth-factor-reduced Matrigel (BD Biosciences). The matrix was diluted 1:1 in ice-cold serum-free medium and plated onto tissue culture wells. After polymerisation at 37°C for 20 min, the cells were collected and added onto the first Matrigel layer. The cells were allowed to adhere at 37°C for 30 min. Subsequently, non-adherent cells were washed off, and a second layer of 50% Matrigel was added on top of the cells. After polymerisation, a feeder layer of complete medium with the indicated supplements was added. The cells were maintained at 37°C.

**In situ proximity ligation assay**

Tet-inducible HEK 293 cells were transfected with a plasmid encoding myc-tagged HIP1. At 24 h post-transfection, the cells were allowed to form spheroids in 50% Matrigel for 24 h. Following induction with Tet for 7 h, endocytosis was halted by the addition of 100 μM dynasore in serum-free medium for 15 min at 37°C and the cells were subsequently fixed and permeabilised as described above. Parallel samples were incubated with either anti-myc and EP1041Y anti-β1-integrin-tail antibodies or with anti-myc and anti-GST antibody [as the negative (IgG) control]. Proximity ligation was performed according to the manufacturer’s instructions (Duolink In Situ Proximity Ligation Assay, Olink Bioscience). Proximity ligation assay signals (which appear as bright fluorescent dots) are produced when two antibodies bind their antigens in close proximity to each other (~20–100 nm), and they were detected by using a Zeiss wide-field microscope.

**Invasion assay**

The cells were seeded equably onto eight-well Ibidi μ-slides and allowed to grow overnight to a confluency of ~70%. Growth-factor-reduced Matrigel was diluted to 25% with ice-cold DMEM 4500 and added on top of the cells. After polymerisation of the Matrigel at 37°C for ~3 h, medium was added containing 2% FBS with or without 10 ng/ml HGF. The cells were allowed to invade for 4 days, during which time the concentration of HGF was progressively increased up to 30 ng/ml by exchanging the medium every day. The cells were then fixed with 4% PFA in PBS for 20 min at 37°C, washed with buffer (1% BSA, 2 mM MgCl₂ and 5 mM EGTA in PBS), permeabilised with wash buffer containing 0.3% Triton X-100 for 10 min at room temperature, and stained with either Alexa-Fluor-488–phalloidin or Syto®60 (Invitrogen) overnight at 4°C. Visualisation of the cells was performed with a confocal microscope, and z intervals of 1.8 mm were taken from randomly chosen optical fields using a 20× objective.

**Biotinylation-based endocytosis assay**

Briefly, the cells were placed on ice, washed once with ice-cold PBS, and surface proteins were then labelled with cleavable NHS-SS–biotin (Pierce) in PBS at 4°C for 30 min. Labelled cells were transferred to pre-warmed serum-free medium, and internalisation was allowed to occur at 37°C for 30 min. Biotin that remained on the cell surface was removed by cleavage with Mesna at 4°C for 30 min, followed by...
quenching with iodoacetamide for 15 min on ice. Cells were lysed and subjected to an enzyme-linked immunosorbent assay (ELISA). For this assay, 96-well plates were coated overnight at 4°C with 3 μg/ml A12R2 anti-fib-1-integrin antibody (Fig. 7D) or K20 anti-fib-1-integrin antibody (Fig. 7E), and were blocked with 0.05% Tween 20 and 5% BSA in PBS for 1 h at room temperature. For each cell lysate, 100 μl was added to three parallel anti-integrin-coated wells and incubated at 4°C overnight. After washing with PBS containing 0.1% Tween 20, horseradish peroxidase (HRP)-linked anti-biotin antibody was added, and incubated for 2 h at room temperature, and was detected by using an ELISA kit according to the manufacturer’s instructions (Thermo Scientific).

Electron microscopy
Samples were fixed with 5% glutaraldehyde in 0.16 M s-collidine buffer pH 7.4, postfixed with 2% osmium tetroxide solution containing 3% potassium ferrocyanide for 2 h, dehydrated with a series of increasing ethanol concentrations (70%, 96% and twice at 100%) and embedded using an Epoxy Embedding Medium kit. Thin sections were cut to a thickness of 70 nm by using an ultramicrotome, and were stained with 1% uranyl acetate and 0.3% lead citrate. The sections were examined with a JEOL-JEM-1400 Plus transmission electron microscope operated at 80 kV acceleration voltage.

Apopotis assay
Caspase-3 and -7 activities were measured in adherent HEK 293 cells (with or without Tet-induction for 24 h) with the Caspase-Glo® 3/7 assay kit from Promega, used according to the manufacturer’s instructions. Treatment with 1 μM staurosporine for 4.5 h was used as a positive control.

Statistics
In all experiments, statistical significance was determined by using Student’s t-test.

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Figure S1.
(A) Western blots from lysates of HEK 293 cell that have been stimulated with tetracycline (Tet) for the times indicated.
(B) Apoptosis assay with cells induced or not with Tet. Incubation with 1 µM staurosporine for 4.5 h was used to create a positive control (mean±SD, n=3, ***, p<0.005).
(C) Western blot analysis from lysates of HEK 293 cells that have been stimulated or not with Tet.
(D) Representative image of Tet-induced (24 h) HEK 293 cells in 3D Matrigel stained for E-cadherin.
(E) Western blot analysis from lysates of HEK 293 cells that have been stimulated or not with Tet.
(F) Immunofluorescence-stainings of Matrigel-embedded HEK 293 cells with or without Tet-induction and subsequent staining for vimentin. The inserts show higher magnification, single channel images of Tet-induced cells.
(G) Western blot analysis from lysates of HEK 293 cells that have been stimulated or not with Tet or HGF as well as GTL-16 and MKN-45 cells.
(H) Representative image of HGF stimulated (48 h) HEK 293 cells in 3D Matrigel stained for E-cadherin.
(I) HEK 293 cells were embedded into 3D Matrigel and thereafter stimulated or not with HGF in the presence or absence of either 80 µg/ml anti-GST-antibody (IgG control) or DMSO.
(J) Tet-inducible HEK 293 cells were allowed to form spheroids in 3D Matrigel and subsequently induced with Tet in the presence or absence of either 80 µg/ml Mab13 or anti-GST-antibody (IgG control), DMSO or 10 µM MMP inhibitor III.
Figure S2.
(A) HEK 293 cells growing in 2D were induced or not with Tet for 24 h in the presence or absence of either C3 or Y-27632 and subsequently thoroughly washed. Cells that remained attached onto the cell culture plate were counted mean±SD, n=3, **, p<0.01; ***, p<0.005; t-test).
(B) Western blot analysis from HEK 293 cells transiently expressing myc-tagged RhoA, RhoB, or RhoC. Actin and tubulin were blotted to control for loading.
(C-D) Biochemical Rho activation assay from HEK 293 on 2D using cells (C) stimulated with HGF or (D) induced with Tet as indicated. Representative blots are shown, as well as the quantification of active GTP-RhoC from 3 independent experiments (mean±SD).
(E) Representative still images from time-lapse videos of control (Scr)- or RhoC-silenced HEK 293 cells, which were embedded into 50 % Matrigel and stimulated or not with Tet. On the right shown is a western blot analysis verifying siRNA silencing efficiency in the cells.
(F) Biochemical Rho activation assay from HEK 293 on 2D using cells transfected with control (Scr)- or RhoA-siRNA. Representative blots are shown, as well as the quantification of active GTP-RhoC from 3 independent experiments (mean±SD).
(G) Western blots from lysates of HEK 293 cell that have been induced with Tet for the times indicated.
Figure S3.
(A) FACS analysis of cell surface β1-integrins from cells treated as indicated with Tet (6 h) or HGF (30 min) in the presence or absence of 15 µM pitstop (PS, 30 min prior to FACS) (mean±SD, n=3, *, p<0.05, t-test).
(B) Myc-tagged Hip1-expressing HEK 293 cells were induced with Tet (5 h) and thereafter subjected to immunoprecipitation with the antibodies indicated.
(C+D) siRNA silencing efficiency in cells used in experiments (C) Fig 7C-D and (D) Fig 7E and Fig S3E-G.
(E) Control or Hip1-silenced cells were stimulated with HGF for the indicated times. The cell lysates were then subjected to western blot analysis. The graph illustrates the quantitation of phospho–c-Met levels in the cells (3 independent experiments, mean±SD).
(F) FACS analysis of the c-Met cell surface level following HGF stimulation of control (Scr), and Hip1-silenced HEK 293 cells (mean±SD, n=4).
(G) The basal c-Met cell surface level of non-stimulated cells used in S3F is the same.
**Movie 1.** Representative time-lapse video of Tet-inducible HEK 293 cells on 2D Poly-L-lysine coated coverslip following Tet induction for 16 h. Images were taken every 30 sec for 1h.

**Movie 2 and 3.** Representative time-lapse videos of Tet-inducible HEK 293 cells (video S2) in 3D collagen and (video S3) 3D Matrigel. Images were taken every 10 min for the times indicated.

**Movie 4.** Tet-inducible HEK 293 cells were allowed to form spheroids in 3D Matrigel for 24 h and subsequently induced with Tet for 24 h. Images were taken every 10 min following Tet induction for the times indicated.
**Movie 5.** Tet-induced HEK 293 cells of experiment shown in video S4 were imaged for another 24 h (48 h of Tet induction). Images were taken every 15 min for the times indicated.

**Movie 6.** Representative time-lapse video of HEK 293 cells in 3D collagen following Tet induction for 24 h. Images were taken every 15 min for the times indicated.

**Movie 7.** Representative time-lapse video of HEK 293 cells in 3D Matrigel following stimulation with HGF. Images were taken every 10 min for the times indicated.