HGF-induced formation of the MET–AXL–ELMO2–DOCK180 complex promotes RAC1 activation, receptor clustering, and cancer cell migration and invasion

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The MET proto-oncogene–encoded receptor tyrosine kinase (MET) and AXL receptor tyrosine kinase (AXL) are independently operating receptor tyrosine kinases (RTKs) that are functionally associated with aggressive and invasive cancer cell growth. However, how MET and AXL regulate the migratory properties of cancer cells remains largely unclear. We report here that the addition of hepatocyte growth factor (HGF), the natural ligand of MET, to serum-starved human glioblastoma cells induces the rapid activation of both MET and AXL and formation of highly polarized MET–AXL clusters on the plasma membrane. HGF also promoted the formation of the MET and AXL protein complexes and phosphorylation of AXL, independent of AXL’s ligand, growth arrest–specific 6 (GAS6). The HGF-induced MET–AXL complex stimulated rapid and dynamic cytoskeleton reorganization by activating the small GTPase RAC1, a process requiring both MET and AXL kinase activities. We further found that HGF also promotes the recruitment of ELMO2 and DOCK180, a bipartite guanine nucleotide exchange factor for RAC1, to the MET–AXL complex and thereby stimulates the RAC1-dependent cytoskeleton reorganization. We also demonstrated that the MET–AXL–ELMO2–DOCK180 complex is critical for HGF-induced cell migration and invasion in glioblastoma or other cancer cells. Our findings uncover a critical HGF-dependent signaling pathway that involves the assembly of a large protein complex consisting of MET, AXL, ELMO2, and DOCK180 on the plasma membrane, leading to RAC1-dependent cell migration and invasion in various cancer cells.

The MET oncogene was originally identified as the oncogenic TPR-MET fusion gene due to a chromosomal translocation fusion event in an osteosarcoma cell line (1, 2). The TPR-MET fusion protein exhibits a constitutively active MET tyrosine kinase activity due to the dimerization of the leucine zipper domain in the translocated promoter region moiety (TPR)2 of the fusion protein. The MET (also called c-MET) proto-oncogene encodes a cell surface receptor tyrosine kinase (RTK) that is normally expressed in cells of epithelial origins during embryonic development for mitogenesis and morphogenesis of various tissues (1, 2). In embryogenesis or the wound-healing process in adult tissues, activation of the MET RTK by its cognate ligand, hepatocyte growth factor (HGF, also called scatter factor), initiates a morphogenic program of “invasive growth” that promotes disruption and remodeling of intercellular contacts, accompanied by cell proliferation, cell migration, and invasion (1, 2). The MET-dependent invasive growth signals are also present in many highly aggressive cancer cells. The abnormalities of MET in human malignancies are frequent and widely observed (1, 2). In glioblastoma multiforme (GBM), a highly aggressive brain tumor, the level of MET is often aberrantly up-regulated (3). Notably, abnormal activation of MET is responsible for resistance to targeted therapies against the vascular endothelial growth factor receptor in GBM and inhibitors of the epidermal growth factor receptor (EGFR) in lung cancers (4–6). Although it is well known that upon binding to HGF, MET is phosphorylated and activated on the plasma membrane, leading to the activation of downstream targets such as PI3K, AKT, and MAPK/ERKs (7, 8), the mechanism by which HGF and MET regulate the invasive cell growth and cell motility, however, remains less clear.

The AXL gene represents another pro-migratory and pro-proliferation gene, which was originally identified as a transforming gene in patients with chronic myelogenous leukemia (9). The AXL protein serves as the prototype of the TAM family of RTKs, consisting of TYRO3, AXL, and MERTK (9). The TAM family RTKs are unique among cell surface RTKs in that they all contain two Ig domains and two fibronectin type III domains in the extracellular region and a conserved KW(L/I)L(A/I)L ES motif in the kinase domain. Both Ig domains in AXL are required for the binding of its natural ligand, GAS6, which promotes the phosphorylation and activation of the AXL RTK.

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The abbreviations used are: TPR, translocated promoter region; RTK, receptor tyrosine kinase; HGF, hepatocyte growth factor; GBM, glioblastoma multiforme; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; PLA, proximity ligation assay; p-, phosphorylated; RLU, relative luminescence units; DAPI, 4,6-diamidino-2-phenylindole; NA, numerical aperture; mTOR, mechanistic target of rapamycin.
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The activation of AXL also leads to the activation of the MAPK/ERK signaling pathways for proliferation and the activation of PI3K, AKT, S6K, BAD, and NF-κB signaling pathways for cell survival (9). Whereas AXL is strongly expressed in human radial glia, brain capillaries, and microglia, it is dramatically overexpressed or activated in GBM (10, 11). Ectopic expression of a dominant negative mutant of AXL lacking the kinase domain caused reduced cell motility and suppressed invasion of glioblastoma cells (12). AXL was shown to act as the key regulator for the mesenchymal subtype of glioblastoma stemlike cells (13). The AXL signaling also negatively regulates the innate immune response, and activation of the TAM family RTK activities promotes phagocytic clearance of apoptotic cells (14). Overexpression of AXL also confers the resistance to anti-EGFR target therapies in non-small-cell lung carcinoma and in triple-negative breast cancers and, in the later case, through the EGFR-mediated transactivation of AXL (15–18).

RAC1, a small GTPase, is well known to be activated by many RTKs and play a critical role in cell migration and invasion (19). RAC1 activity and RAC1-dependent actin cytoskeleton reorganization have been shown to be critical for HGF/MET-stimulated epithelial cell scattering and cortical neuron migration (20–22). Activation of RAC1 requires the action of guanine nucleotide exchange factors (GEFs), which converts RAC1 from its GDP-bound form to GTP-bound form. There are about 20 GEFs that activate RAC1, which can be grouped into two distinct subfamilies, according to their catalytic domains. One group contains the DBL-homology domain, and the other group possesses the DOCK homology region-2 domain (23). MET has been reported to activate RAC1 GEFs, such as TIAM1 and VAV2, which belong to the DBL-related GEFs (24, 25). On the other hand, ELMO (engulfment and motility) proteins (ELMO1 and ELMO2), which are scaffold proteins, can interact the DOCK (dedicator of cytokinesis) proteins to form a bipartite RAC1 GEF, initially identified for their roles in phagocytosis of apoptotic cells (23, 26). Both ELMO and DOCK proteins have been reported to be involved in the invasive properties of the glioblastoma cells, although their upstream activators remain unclear (27).

We have previously shown that the presence of the MET RTK on the plasma membrane is regulated by ASM, an acidic sphingomyelinase that hydrolyzes sphingomyelins in the plasma membrane to produce ceramides (28). Here, we report that the binding of HGF to MET induces the clustering of multiple signaling molecules on the plasma membrane, RAC1 activation, and cell migration through a novel AXL-mediated signaling pathway.

Results

Clustering and activation of MET on the plasma membrane by ligand binding

It is known that upon ligand binding, the kinase activity of MET is turned on by the cross-phosphorylation of Tyr-1234 and -1235 within the transactivation loop of the MET RTK (1). To investigate how MET is activated on the plasma membrane, we have used specific anti-MET antibodies in immunostaining to examine the response of the MET protein to HGF in human U373-MG glioblastoma cells that express high levels of MET protein (28). In serum-starved cells, MET immunostaining was weak, and there were relatively evenly distributed MET proteins on the plasma membrane (Fig. 1A). Weak staining signals of MET were also present in the intracellular compartments. When immunostained with the antibodies for the Tyr-1234/1235–phosphorylated MET that recognize the active form of the kinase, no significant signals were detected in the serum-starved cells (Fig. 1A).

The addition of HGF to the serum-starved cells caused a rapid and dramatic change of MET protein distribution on the plasma membrane (Fig. 1A). Within minutes, HGF stimulation induced a rapid and strong MET staining “patch” on nearly every cell (Fig. 1A). The strong patches of MET staining were highly polarized on the plasma membrane, with usually only one area of cell surface showing the bright MET patch staining, whereas other areas of the plasma membrane remained weakly stained on each cell (Fig. 1A). Examination of the phosphorylated and active form of the MET protein with the specific antibodies showed that only the strong and polarized MET staining patches contained the active MET RTK on the plasma membrane, whereas the phosphorylated MET protein was mostly absent from other parts of the cell (Fig. 1A). These anti-phosphorylated MET immunostainings were specific and dependent on the MET RTK activity, as treatment of cells with a specific MET kinase inhibitor, crizotinib, completely eliminated these staining signals, even in the presence of HGF (Fig. 1B). In cells treated with crizotinib, MET was present uniformly on the plasma membrane, irrespective of whether HGF was added (Fig. 1B). In addition, our examination of the temporal response to the HGF addition revealed that the mobilization and redistribution of the activated and phosphorylated MET protein on the plasma membrane occurred very rapidly and exhibited highly dynamic patterns (Fig. 1C). The addition of HGF, within 2 min, initiated the appearance of the phosphorylated and active MET protein on multiple areas of the plasma membrane in each cell (Fig. 1C), forming small and intermediate patches on the cell surface. The active and phosphorylated staining of the receptor then grew increasingly stronger and merged into one or two large and polarized patches on the plasma membrane in each cell within 5 min. By 7 min, the staining of the active form of MET further developed into a strong and often concentrated single bright spot on the plasma membrane. By about 10 min, the strong staining spots containing the active and phosphorylated MET protein started to disappear from the plasma membrane, with the concurrent appearance of the intracellular staining of the phosphorylated MET protein inside of the cell. The phosphorylated MET protein subsequently disappeared from intracellular compartment at about 15 min after the addition of HGF (Fig. 1C). Our characterization of the induction of the active and phosphorylated MET protein on the plasma membrane suggests the following scheme of MET activation. 1) HGF initially binds to the relatively evenly distributed, inactive MET protein, leading to the localized clustering and activation of MET on multiple areas of the plasma membrane. 2) The HGF-engaged MET proteins are further clustered and merged into one or two large and polar...
ized patches containing the activated MET RTK on the plasma membrane. 3) The clustered and active MET proteins on the plasma membrane become internalized via endocytosis at about 10 min and subsequently deactivated by 15 min, resulting in the termination of the HGF-MET signaling. It is well established that the T cell receptors can be activated by the binding of the antigen-presenting complex, which promotes the oligomerization of the receptors and subsequent assembly of multiprotein complexes, leading to the formation of a “patch” (“cap” or “cluster”) of receptor complexes on the T-cell plasma membrane, later known as the immune synapse (29). Our studies revealed that the activation of MET RTK is also associated with the receptor clustering or patch formation on the plasma membrane when binding to its cognate ligand, HGF.

**Clustering of the AXL RTK by HGF but not GAS6**

We wondered whether other RTKs could form the clusters/patches on the plasma membrane in response to their corresponding ligands. Our studies revealed that AXL, a highly expressed RTK in U373-MG cells, can be detected by immunostaining with specific anti-AXL antibodies (Fig. 2, A and B). However, the immunostaining of AXL was also weak on the plasma membrane in serum-starved cells (Fig. 2A). It is known that binding of GAS6, a cognate ligand, to AXL, can lead to the dimerization of AXL and activation of AXL RTK activities (9). Our studies, however, revealed that the addition of GAS6 to the serum-starved U373-MG cells did not induce AXL clustering (Fig. 2A), although AXL phosphorylation on Tyr-702, which is located in the activation loop of the AXL kinase domain (9), was strongly induced by GAS6 when detected by Western blotting analyses (Fig. S1A and Fig. 2E). Strikingly, however, we found that the addition of HGF to the serum-starved cells led to the rapid, strong, polarized and patched staining of AXL on the plasma membrane (Fig. 2A, second row). The AXL activation was reported to associate with the phosphorylation of Tyr-702 and -779 (9). Our immunostaining with anti-phosphorylated Tyr-779 antibodies revealed that the phosphorylation of AXL Tyr-779 is strongly induced by HGF, as there were patched regions on the plasma membrane intensively stained by the specific antibodies (Fig. 2A, third row).

**Figure 1. HGF binding to MET induces receptor clustering and patch formation on the plasma membrane.** A, U373-MG cells were serum-starved and then stimulated with or without HGF (50 ng/ml) for 7 min. Cells were immunostained with an antibody against p-MET (Tyr-1234/1235–phosphorylated), followed with a secondary antibody conjugated to Alexa Fluor 647 (red). Cells were further immunostained with an anti-total MET antibody preconjugated to Alexa Fluor 488 (green). Arrows indicate the clusters of receptors on the plasma membrane. B, the MET clustering is sensitive to the MET inhibitor crizotinib. Cells were treated similarly as in A, except that cells were serum-starved and treated with 1 μM crizotinib or control (DMSO) for 3 h before the addition of HGF or left untreated. C, time course for MET cluster formation on the plasma membrane. Serum-starved cells were stimulated with HGF for the indicated time, fixed, and immunostained with the anti-p-MET (Tyr-1234/1235–phosphorylated) antibody. Scale bars in A–C, 20 μm.
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**Co-clustering of MET and AXL RTKs in response to HGF**

Because HGF induced both MET and AXL clustering on the plasma membrane, we wondered whether the clusters formed by these two RTKs overlap with each other. Not surprisingly, our immunostaining analysis revealed that the phosphorylated AXL is indeed co-localized with the patched MET staining on the plasma membrane in response to HGF (Fig. 2, B and higher magnification images in C), suggesting that HGF induces the co-clustering of activated MET and AXL RTKs on the plasma membrane. To determine whether HGF induces the direct
interaction between endogenous MET and AXL RTK proteins, we conducted the proximity ligation assay (PLA), and our analysis indicated that these two RTK proteins indeed interact with each other at the single cell level in an HGF-dependent manner (Fig. 2D).

**HGF induced the phosphorylation of AXL primarily at Tyr-779 but not Tyr-702**

We further examined the effects of HGF and GAS6 on the MET and AXL signaling pathways in U373-MG cells by Western blotting (Fig. 2E). We found that compared with the serum-starved cell control, HGF induced rapid and strong phosphorylation of Tyr-1234/1235 in MET (Fig. 2E). HGF also activated the downstream signaling molecules of MET pathways, including the phosphorylated AKT and S6K (p-AKT and p-S6K). In addition, HGF promoted strong phosphorylation, primarily at Tyr-779, instead of Tyr-702, in AXL (Fig. 2E), a result consistent with our immunostaining studies (Fig. 2, A and B). In parallel, we found that GAS6 promoted the phosphorylation of Tyr-702 but not Tyr-779 in AXL (Fig. 2E and Fig. S1A). In AXL, Tyr-702 is likely to be the autophosphorylation site, whereas Tyr-779 can be phosphorylated by other kinases (9). AXL is also a highly modified protein (30), and our studies revealed that AXL is primarily present in two forms, p140 (upper form) and p120 (lower form), in U373-MG cells (Fig. 2E, longer exposure). We found that HGF-stimulated phosphorylation of Tyr-779 is primarily associated with the p140 form. In contrast, GAS6 promoted the phosphorylation of Tyr-702 in the p120 form (Fig. 2E, longer exposure). Thus, our studies revealed that HGF and GAS6 induced differential phosphorylation of two different tyrosine residues, Tyr-779 and Tyr-702, and in two different modified forms of AXL, respectively. These analyses indicate that HGF and GAS6 employ different mechanisms to activate the AXL RTK in U373-MG cells. Consistent with these observations, we found that HGF/MET-induced AXL phosphorylation is specific, as HGF did not induce the phosphorylation of EGFR under the same condition, whereas GAS6 caused a slight stimulation of EGFR phosphorylation at tyrosine 1068 (Fig. S1B).

To independently confirm that HGF can induce activation of both MET and AXL, we also performed immunoprecipitation with the anti-phosphorylated tyrosine antibodies and examined the presence of the phosphorylated MET and AXL proteins in the immunoprecipitates (Fig. 2F). Western blotting analyses showed that HGF greatly enhanced the MET and AXL proteins in the anti-phosphotyrosine antibody immunocomplexes (Fig. 2F). Among the phosphorylated MET and AXL RTKs, HGF promoted the significant increase of the levels of the phosphorylated Tyr-779 in AXL and the phosphorylated Tyr-1234 and Tyr-1235 in MET (Fig. 2F), demonstrating again that HGF activated both MET and AXL when added to the serum-starved U373-MG cells.

**MET and AXL form a protein complex in response to HGF**

The HGF-induced co-clustering of MET and AXL on the plasma membrane and PLA analyses indicate that these two receptors physically interact to form a protein complex (Fig. 2, A–D). To further determine whether MET and AXL indeed form protein complexes in the presence of HGF, we conducted immune co-precipitation analysis of these two RTKs under stringent conditions with the lysis buffer containing 150 mM NaCl, 1% Triton X-100, and 0.1% SDS. Our studies revealed that whereas there are some basal levels of association between these two RTKs, HGF greatly enhanced the interaction between MET and AXL proteins (Fig. 2G). In the immunoprecipitated MET complexes from the HGF-treated cells, AXL was found to be in the Tyr-779–phosphorylated form, which is presumably an activated form of AXL, whereas MET is also phosphorylated on Tyr-1234/1235 and thus activated. Our data therefore show that HGF induces the tyrosine phosphorylation of both RTKs and promotes the formation of the MET–AXL complex in U373-MG cells.

Because of the observation that AXL Tyr-779 phosphorylation was induced by HGF in the MET–AXL complex (Fig. 2, F and G), we wondered whether MET could directly trans-phosphorylate AXL. We have tested this possibility by examining whether recombinant MET protein can directly phosphorylate AXL in vitro. We initially assayed the activities of the recombinant MET protein (containing its C-terminal kinase domain) and showed that it can readily phosphorylate poly(Glu4, Tyr1), a common substrate used for tyrosine kinases in vitro, and this activity was potently inhibited by the MET kinase inhibitor crizotinib (Fig. 3A, left). We next examined whether the recombinant MET kinase could phosphorylate a peptide derived from the sequence surrounding the Tyr-779 residue in AXL (AXL Tyr-779 peptide). Indeed, MET kinase can also efficiently phosphorylate the AXL Tyr-779 peptide, and this AXL phosphorylation activity was also strongly inhibited by the MET inhibitor
crizotinib (Fig. 3A, right). These studies suggest that MET is likely to be the kinase that directly phosphorylates AXL on Tyr-779 in the MET–AXL complex induced by HGF.

In addition, we have tested whether the kinase activity of MET and AXL is required for the stable MET–AXL complex formation. We found that pretreatment of the serum-starved cells with MET kinase inhibitor crizotinib was sufficient to abolish the formation of the MET–AXL complex in response to HGF (Fig. 3B) and to completely prevent the HGF-induced phosphorylation of Tyr-1234/1235 in MET and the phosphorylation...
of Tyr-779 in AXL (Fig. 3B). We also tested whether the AXL RTK activity is required for the formation of the MET–AXL complex. Our studies revealed that pretreatment of serum-starved cells with AXL kinase inhibitor R428 (31) reduced the HGF-induced formation of the MET–AXL complex to the basal levels (Fig. 3C). R428 also partially reduced the phosphorylation of Tyr-1234/1235 in MET and phosphorylation of Tyr-779 in AXL in the HGF-stimulated cells (Fig. 3C).

The complex formation between MET and AXL in response to HGF is consistent with our observation that HGF induced co-clustering of MET and AXL proteins on the plasma membrane (Fig. 2, A–C). We further examined whether the MET clustering requires the kinase activity of AXL. Consistently, we found that the phosphorylated MET clustering on the plasma membrane is also sensitive to AXL inactivation mediated by AXL knockdown with two specific siRNAs (Fig. 3D and Fig. S1 (C and D)) or by R428 treatment (Fig. 3E). These studies indicate that MET can trans-phosphorylate and activate AXL, and both MET and AXL activities are required for the stable complex formation and for maintaining the activated states of these two RTKs.

**HGF induced clustering of MET and AXL in the presence of GAS6**

Under the serum starvation conditions, we found that the addition of HGF, but not GAS6, the natural ligand for the AXL RTK, induced the clustering and activation of MET (Fig. 4A, top row). However, pretreatment of starved cells with GAS6 for 7 min was sufficient to abolish the clustering and activation of MET during a subsequent addition of HGF to the GAS6-treated cells (Fig. 4A, bottom row). These studies indicate that the binding of GAS6 to AXL may deplete a pool of unengaged AXL receptor, which is required for the HGF-dependent clustering and activation of MET on the plasma membrane. Because under normal physiological conditions, HGF and GAS6 may both be present in the same environment, we further examined whether HGF could still promote the clustering of MET in the presence of GAS6. We found that the addition of HGF and GAS6 simultaneously to the serum-starved cells led to the formation of MET clustering and activation (Fig. 4A, top row, last column). A dose titration of HGF revealed that effective clustering of the MET and AXL RTKs occurred at around 50 ng/ml HGF (Fig. 4C), and the MET clusters can form in the presence of equal or even much higher concentrations of GAS6 (50–400 ng/ml) in the same medium. By immune co-precipitation studies, we found that the MET–AXL protein complex can be formed upon stimulation with HGF irrespective whether cells were co-stimulated with GAS6 or not (Fig. 4B). Consistently, Western blot analysis revealed that the phosphorylation of AXL at Tyr-779 still took place when cells were stimulated simultaneously with HGF and GAS6, as long as the HGF concentration was higher than 25 ng/ml. Above this threshold HGF level, the effect of HGF stimulation was relatively independent of the co-presence of various GAS6 concentrations in the medium (Fig. 4, D and E). However, pretreatment of the starved cells with GAS6 reduced the subsequent HGF-induced AXL phosphorylation at Tyr-779 (Fig. 4F). Our studies thus reveal that HGF-induced clustering of MET and AXL, the phosphorylation of Tyr-779 in AXL, and the MET–AXL complex formation occur in the presence of GAS6, suggesting that HGF-bound MET can effectively compete with GAS6-bound AXL to bind AXL if HGF and GAS6 are co-present. However, the formation of HGF-induced clustering of the MET and AXL complex and AXL phosphorylation at Tyr-779 require the existence of the unengaged AXL RTK pool in the serum-starved cells, which can be depleted by pretreatment of GAS6.

**HGF activated a strong and rapid actin cytoskeleton reorganization process**

Both MET and AXL are strongly associated with cell mobility and the invasive properties of cancer cells (1, 2, 9). In some experiments, we have noticed that the addition of HGF to serum-starved U373-MG cells caused rapid changes in cell shape (Figs. 1 and 2). We therefore examined the response of actin cytoskeleton organization to HGF by staining cells with phalloidin, a mushroom toxin that tightly andselectively binds to filamentous actin (F-actin). We found that within minutes of HGF addition to serum-starved cells, dramatic actin cytoskeleton reorganization occurred, with strong and highly dynamic cortical actin staining at the membrane ruffles and lamellipodia (Fig. 5A). Whereas the reorganization of actin cytoskeleton actin was extensively observed on the plasma membrane, the polarized patches of the activated MET staining also overlapped with parts of the intense actin staining on the lamellipodia (Fig. 5A). Our studies indicate that HGF can induce strong actin cytoskeleton reorganization in the serum-starved cells.

**HGF-MET activated the RAC1 small GTPase**

The extensive reorganization of actin cytoskeleton in response to HGF prompted us to examine whether the RAC1 small GTPase is activated, as RAC1 is known to be a critical molecule involved in actin cytoskeleton reorganization and membrane ruffle formation (19). Whereas HGF and activation of MET did not alter the total RAC1 protein levels, the GTP-binding activity of RAC1 was greatly and rapidly stimulated by HGF (Fig. 5C). Our studies revealed that the binding of HGF to MET not only activates the well-characterized MET downstream targets, such as the AKT and S6K signaling pathways (Figs. 2E and 5C (C and E)); HGF also promotes the activation of RAC1 and actin cytoskeleton reorganization (Fig. 5, A and C). The involvement of RAC1 in the HGF-stimulated dynamic reorganization of cytoskeleton is further verified by the siRNA-mediated knockdown of RAC1, which abolished the actin cytoskeleton reorganization and formation of membrane ruffles in response to HGF (Fig. 5B). In addition, we also found that loss of RAC1 eliminated the HGF-induced MET clustering on the plasma membrane (Fig. 5B), suggesting that the dynamic formation of MET clusters requires RAC1 activity and cytoskeleton reorganization.

**HGF-induced RAC1 activation requires the signaling of the MET–AXL complex**

The rapid mobilization of actin cytoskeleton reorganization in response to HGF indicates that RAC1 is another critical downstream target of MET signaling process (Fig. 5). Indeed, we found that the HGF-induced RAC1 activation requires

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**A**

| GAS6 | HGF | p-MET 1234/5 |
|------|-----|-------------|
| -    | 50 ng/ml | -           |
| 50 ng/ml | 400 ng/ml | -           |
| 50 ng/ml | 400 ng/ml | (stimulation) |

**B**

| IP: anti-MET |
|--------------|
| kDa | IgG | +PBS | +HGF | +GAS6 | +GAS6+HGF |
|------|-----|------|------|-------|-----------|
| 130- | AXL | p-MET | p-MET | MET |

**C**

| HGF | GAS6 | p-MET 1234/5 |
|-----|------|-------------|
| -   | 6 ng/ml | -           |
| 12 ng/ml | 400 ng/ml | -           |
| 25 ng/ml | 400 ng/ml | -           |
| 50 ng/ml | 400 ng/ml | (stimulation) |

**D**

| Simultaneous stimulation |
|--------------------------|
| HGF | GAS6 | 50 | 25 | 12 | 6 | 3 | 1 | 0 | (ng/ml) | (ng/ml) |
| 130- | p-MET | Y1234/S |
| 130- | MET |
| 130- | p-AXL (Y702) |
| 130- | p-AXL (Y779) |
| 130- | AXL |
| 130- | p-AKT |
| 120- | AKT |
| 70- | p-S6K |
| 70- | S6K |
| 35- | Actin |

**E**

| Simultaneous stimulation |
|--------------------------|
| HGF | GAS6 | 50 | 25 | 12 | 6 | 3 | 1 | 0 | (ng/ml) | (ng/ml) |
| 130- | p-AVL (Y1234/S) |
| 130- | MET |
| 130- | p-AXL (Y779) |
| 130- | p-MET | Y1234/S |
| 130- | AXL |
| 130- | p-AKT |
| 70- | AKT |
| 70- | p-S6K |
| 70- | S6K |
| 35- | Actin |

**F**

| Sequential stimulation (GAS6 1st, HGF 2nd) |
|-------------------------------------------|
| HGF | GAS6 | 50 | 25 | 12 | 6 | 3 | 1 | 0 | (ng/ml) | (ng/ml) |
| 130- | p-MET | Y1234/S |
| 130- | MET |
| 130- | p-AXL (Y779) |
| 130- | p-AXL (Y702) |
| 130- | AXL |
| 130- | p-AKT |
| 70- | AKT |
| 70- | p-S6K |
| 70- | S6K |
| 35- | Actin |
the kinase activity of MET (Fig. 5D). Inhibition of the MET activity by crizotinib abolished the GTP-binding activity of RAC1 in response to HGF (Fig. 5D). Consistent with this observation, inhibition of the MET RTK activity by crizotinib also blocked the HGF-induced cytoskeleton reorganization (Fig. 8A).

On the other hand, we often found that inactivation of AXL by R428 or siRNA-mediated knockdown of AXL was not sufficient to completely block the phosphorylated Tyr-1234/1235 of MET proteins in the cells (Figs. 3C and 5E). There is always some residual phosphorylated Tyr-1234/1235 of MET in the AXL-inactivated cells. Similarly, inactivation of AXL often appeared to only partially reduce the HGF-induced phosphorylation of AKT, a downstream target of MET signaling (Fig. 5E). These observations suggest that not all MET proteins are complexed with AXL, and inactivation of AXL might only affect the MET activity in the MET–AXL complex, which might represent a fraction of total MET activities in response to HGF. Importantly, however, we repeatedly found that inactivation of AXL by R428 or siRNA-mediated knockdown can effectively blunt the HGF-stimulated increase of RAC1-GTP levels (Fig. 5, D and E), although in serum-starved cells without HGF addition, R428 in fact slightly increased the basal levels of RAC1-GTP (Fig. 5D). These lines of evidence suggest that nearly all HGF-induced RAC1 activation requires the participation of AXL, and the MET–AXL complex transduces the HGF-induced signaling to RAC1 for actin cytoskeleton reorganization.

**ELMO2 co-clusters with MET in response to HGF**

Based on the observation that the HGF-induced MET–AXL complex is required for RAC1 activation, we tried to identify the potential signaling components of this pathway. RTK signaling–mediated increase of the RAC1-GTP form requires an active participation of a GEF for RAC1. MET has also been reported to activate RAC1 GEFs, such as TIAM1 and VAV2, which belong to the DBL-related GEFs, and both TIAM1 and VAV2 have been reported to activate RAC1 on the endosomes (24, 25). However, treatment of cells with EHT1864, a chemical inhibitor that blocks RAC1 activation by TIAM1 (32), did not block the HGF-induced MET clustering in U373-MG cells (data not shown). In searching for RAC1 GEF that functions on the plasma membrane, we found that EHT1864 might play a critical role in relaying the signals from the MET–AXL complex to RAC1. ELMO2 and its closely related protein ELMO1 were recently reported to be phosphorylated by AXL in response to GAS6 (33). Our initial immunostaining using specific anti-ELMO2 antibodies revealed that in the serum-starved cells, whereas there was virtually no plasma membrane staining of phosphorylated MET, there was also little or very weak but evenly distributed staining of ELMO2 on the plasma membrane (Fig. 6, A and B). However, the addition of HGF induced ELMO2 to form strongly stained patched/clustered regions on the plasma membrane (Fig. 6, A and B). The patched plasma membrane staining of ELMO2 is specific, as siRNA-mediated knockdown ELMO2 abolished this staining (Fig. S2, A and B). These HGF-induced patched regions of ELMO2 overlapped with that of the phosphorylated MET (Fig. 6, A and B), indicating that ELMO2 and the activated MET proteins co-clustered on the plasma membrane. Because AXL also co-clustered with MET on the plasma membrane in response to HGF (Fig. 2), we examined whether AXL and ELMO2 also co-clustered during the HGF stimulation. Our studies showed that the patched regions of ELMO2 and AXL indeed overlapped with each other on the plasma membrane (Fig. 6, F and H). Importantly, treatment of cells with either MET inhibitor crizotinib or AXL inhibitor R428 or knockdown of AXL by specific siRNAs abolished the HGF-induced formation of the patched regions of ELMO2, phosphorylated MET, and AXL on the plasma membrane (Fig. 6, B and F–H). Our studies indicate that ELMO2 is recruited into the clusters with the MET–AXL RTK complex on the plasma membrane in response to HGF, and this recruitment of ELMO2 requires the RTK activities of both MET and AXL.

**ELMO2 forms a protein complex with MET and AXL in response to HGF**

The co-clustering of ELMO2 with both MET and AXL proteins suggests that ELMO2 may also physically interact with MET and AXL in response to HGF. To investigate this possibility, we conducted immune co-precipitation of the MET or AXL protein complexes and examined the presence of ELMO2 in the complexes by Western blot analysis (Fig. 6, C and D). Our studies revealed that HGF indeed induced the association of ELMO2 with both MET and AXL protein complexes using either anti-AXL or anti-MET antibodies for immunoprecipitation (Fig. 6, C and D). Conversely, the anti-ELMO2 antibodies also reciprocally immune co-precipitated both MET and AXL (Fig. 6F). In all of these cases, the association of MET or AXL with ELMO2 was greatly stimulated by HGF (Fig. 6, C–E). These studies are consistent with the notion that HGF promotes the recruitment of ELMO2 into the MET–AXL complex for their co-clustering on the plasma membrane (Fig. 6, A and B).
HGF-induced MET–AXL–RAC1 signaling for cell migration

A

- HGF  + HGF

p-MET Y1234/S
Phalloidin
Merge

B

Luc siRNA  RAC1 siRNA

- HGF  + HGF  - HGF  + HGF

MET
Phalloidin
Merge

C

kDa

17-

RAC1-GTP total RAC1
Pull down assay

Relative
RAC1 activity

- HGF  + HGF

D

DMSO  R428  Crizotinib

- HGF  + HGF  - HGF  + HGF

RAC1-GTP total RAC1
Pull down assay

Relative
RAC1 activity

E

Luc siRNA  AXL siRNA

- HGF  + HGF  - HGF  + HGF

Rac1-GTP total RAC1
Pull down assay

Relative
RAC1 activity

- HGF  + HGF  - HGF  + HGF
HGF activates the MET–AXL–ELMO pathway in multiple glioblastoma cell lines

Because the addition of HGF to the serum-starved U373-MG cells can activate both MET and AXL, we wondered whether this pathway also exists in other glioblastoma cell lines. We tested three additional glioblastoma cell lines, T98G, LN229, and LN18 cells, and found that the addition of HGF to the serum-starved cells also induced the phosphorylation of Tyr-1234/1235 in MET, the phosphorylation Tyr-779 in AXL, and the phosphorylation of AKT and S6 kinase in all three cell lines (Fig. S3A). Furthermore, we found that AXL and ELMO2 also form a protein complex with MET, which was enhanced by HGF in T98G cells, as detected by reciprocal immune co-precipitation (Fig. S3, B and C). Our studies thus indicate that the activation of the MET–AXL–ELMO2 pathway by HGF is present in multiple glioblastoma cell lines.

DOCK180 is also recruited into the MET–AXL–ELMO2 complex to regulate RAC1 activation

Because ELMO2 is known to be complexed with DOCK family proteins to form bipartite GEFs for RAC1 (23, 26), we further investigated the potential involvement of the DOCK family proteins in the MET–AXL–ELMO2 complexes. To identify the specific DOCK protein(s) that regulates the clustering of the activated MET protein in response to HGF, we conducted an siRNA-based candidate screen of various DOCK proteins, including DOCK180 (DOCK1), DOCK2, DOCK4, DOCK5, and DOCK7. Our analysis revealed that only down-regulation of DOCK180, and not other DOCK members, caused a significant reduction of MET clusters on the plasma membrane (Fig. S2C). In addition, our biochemical analysis revealed that HGF induced an increased association of DOCK180 with the ELMO2 protein complex, which also contained MET and AXL (Fig. 7, A, B, and E). Reciprocal immunoprecipitation of endogenous DOCK180 protein complexes also revealed that HGF stimulated the association between DOCK180, AXL, and ELMO2 (Fig. 7, D, F, and H). Notably, the recruitment of DOCK180 into the MET–AXL–ELMO2 complex is reduced if the activity of either MET or AXL is inhibited by their respective inhibitors or by the AXL siRNA-mediated knockdown (Fig. 7, B and D–H), suggesting that MET and AXL RTK activities are required for their interactions. However, we have not been able to determine whether DOCK180 is co-clustered with MET–AXL–ELMO on the plasma membrane in response to HGF, because several anti-DOCK180 antibodies that we have tried did not work well in the immunostaining experiments.

Both ELMO2 and DOCK180 are required for the HGF-induced RAC1 activation and cytoskeleton reorganization

We have shown that the addition of HGF to the serum-starved cells led to the rapid and dynamic cytoskeleton reorganization, and this process is RAC1-dependent (Fig. 5, A and B). We have also found that this process requires the activities of MET and AXL, as both MET inhibitor crizotinib and AXL inhibitor R428 blocked the HGF-induced actin cytoskeleton reorganization response (Fig. 8A). We further examined whether the HGF-induced actin cytoskeleton reorganization requires ELMO2 and DOCK180. We found that siRNA-mediated knockdown of either ELMO2 or DOCK180 abolished the dynamic actin cytoskeleton reorganization induced by HGF (Fig. 8, B and C). In addition, this effect on the cytoskeleton is specific for ELMO2 because loss of ELMO1, a related protein, did not significantly affect the HGF-induced phalloidin staining pattern (Fig. 8B). Consistently, knockdown of ELMO1 did not affect the MET receptor clustering (Fig. 8B). Western blot analysis has shown that both ELMO1 and ELMO2 were efficiently ablated in these experiments (Fig. S2B). Our studies indicate that ELMO2 and DOCK180 are indeed required for the HGF-induced actin cytoskeleton reorganization. We have also directly analyzed the effects of ELMO2 and DOCK180 knockdown on RAC1 activation. We found that knockdown of either ELMO2 or DOCK180 reduced the HGF-induced elevation of RAC1-GTP levels (Fig. 7C). Taken together, our studies indicate that in response to HGF, MET recruits AXL, ELMO2, and DOCK180 to form a large protein complex, which can be detected as protein clusters on the plasma membrane by immunostaining or by immune co-precipitation. This MET–AXL–ELMO2–DOCK180 protein complex promotes the activation RAC1, thereby controlling the dynamic receptor clustering and actin cytoskeleton reorganization.

On the other hand, we found that siRNA-mediated knockdown of either ELMO2 or DOCK180 abolished the HGF-induced clustering of the Tyr-1234/1235–phosphorylated MET on the plasma membrane (Fig. 8, B and C), whereas knockdown of DOCK180 also greatly reduced the HGF-induced co-clustering of the activated MET with ELMO2 on the plasma membrane (Fig. 8C). Our studies indicate that DOCK180 and ELMO2 are recruited into the MET–AXL RTK protein complex in response to HGF, and this recruitment depends on the kinase activity of both MET and AXL RTKs. Conversely, the presence of ELMO and DOCK180 in the MET–AXL complex in turn mediates the MET–AXL receptor clustering on the...
The MET–AXL–ELMO–DOCK180 complex is required for HGF-induced cell migration and invasion

Because MET and AXL are each implicated in the invasiveness of many cancer cells, we wondered whether the assembly...
HGF-induced MET–AXL–RAC1 signaling for cell migration

Although we initially showed that HGF induced the activation of the MET–AXL RTK complex in human glioblastoma cells, we subsequently found that the HGF-dependent formation and activation of the MET–AXL complexes exist in various types of cancer cells, such as A375, a human malignant melanoma cell line, or MDA-MB-231, a human breast cancer cell line (Fig. 10); both cell lines were known to highly express MET (28, 34, 35). Our studies on these different types of cancer cells revealed that HGF also induced AXL Tyr-779 phosphorylation in these cells (Fig. 10A) and that the HGF-induced assembly of MET and AXL complex also involves ELMO2 (Fig. 10, B and C). Additionally, HGF-dependent cell migration (Fig. 10, D and E) and cell invasion (Fig. 10, F and G) in A375 and MDA-MB-231 cells were inhibited either by the MET inhibitor crizotinib or by the AXL inhibitor R428. Our studies thus reveal that the HGF-dependent activation of the MET–AXL–ELMO–RAC1 RTK signaling pathway is a widespread mechanism employed by various cancer cells.

Discussion

In this report, we showed that HGF, a high-affinity ligand for MET, induces MET to trans-phosphorylate AXL and form a stable complex with AXL, leading to the formation of receptor clusters on the plasma membrane. We further showed that HGF-activated MET–AXL complex recruits ELMO2 and DOCK180 to form a large protein complex, which is critical for the HGF-dependent RAC1 activation, cytoskeleton reorganization, and subsequent cell migration and invasion. The HGF-induced MET–AXL signaling complex can also be efficiently formed even in the presence of the high concentration of GAS6, which induces formation of AXL–AXL homodimers. In addition, such complexes are detected in a variety of cancer cells, including glioblastoma, breast cancer, and melanoma. As both MET and AXL RTKs are well established for their critical roles in cancer cell migration and invasion, and both RTKs are highly expressed and activated in various cancer cells, we believe that our studies revealed a novel mechanism by which MET and AXL act in concert to regulate these important cellular processes.

We have found that the HGF-dependent phosphorylation and activation of AXL is very different from the GAS6-induced AXL activation in several aspects. One aspect is that whereas activation of AXL by its cognate ligand GAS6 is mediated by...

Figure 6. ELMO2 is co-clustered with the activated MET in response to HGF. A, serum-starved U373-MG cells were treated with or without HGF for 7 min, and the cells were processed for double-immunostaining with antibodies for p-MET (Tyr-1234/1235–phosphorylated) (red) and ELMO2 (green) as indicated. B, serum-starved U373-MG cells were pretreated with DMSO or 1 μM MET kinase inhibitor crizotinib for 3 h and then treated with or without HGF. Cells were immunostained as in A. C, association of ELMO2 with AXL in the HGF-stimulated cells. U373-MG cells were treated as in A. C, except that cell lysates were immunoprecipitated with anti-MET antibody and then blotted with anti-ELMO2 or anti-p-AXL (Tyr-779–phosphorylated) antibodies. The blots were then stripped and reblotted with anti-AXL antibodies. D, same as in C except that cell lysates were immunoprecipitated with anti-AXL antibody. E, same as in C except cell lysates were immunoprecipitated with anti-ELMO2 antibody and then blotted with anti-MET and anti-ELMO2 antibodies as indicated. F, serum-starved U373-MG cells were pretreated with DMSO or 1 μM MET kinase inhibitor crizotinib as in B. Cells were co-immunostained with anti-AXL (total AXL) (red) and anti-ELMO2 (green) antibodies. G, cells were treated as in F except for pretreatment with 1 μM AXL kinase inhibitor R428 and immunostained with anti-p-MET (Tyr-1234/1235–phosphorylated) (red) and anti-ELMO2 (green) antibodies as indicated. H, cells were transfected with control (luciferase; Luc) or AXL siRNAs for 72 h. Cells were serum-starved and then treated with or without HGF for 7 min. Cells were immunostained with anti-AXL (total AXL) (red) and anti-ELMO2 (green) antibodies as indicated.
autophosphorylation on Tyr-702 in AXL (9) (Figs. 2E and 4 (D–F)), the HGF-induced AXL phosphorylation primarily occurred on Tyr-779 (Figs. 2E and 4 (D–F)). The second aspect is that HGF-induced tyrosine phosphorylation of AXL occurred only in the higher-molecular weight form of AXL (p140), but not the lower-molecular weight form of AXL (p120), which is phosphorylated on Tyr-702 in response to GAS6 (Fig. 2E). These observations suggest that the mechanisms of phosphorylation and activation of AXL in the MET–AXL heterodimer complex is different from that of the AXL–AXL homodimer complex activated by GAS6 (model in Fig. 10H).

It is reported that the SRC family kinases can bind to the juxtamembrane domain of AXL and promote AXL phosphorylation in the corresponding Tyr-779 residue and activate AXL in mouse endothelial cells stimulated with VEGF (36). We have...
**Figure 8. Regulation of actin cytoskeleton by ELMO2 and DOCK180.** A, MET and AXL kinase activities are required for the HGF-induced cytoskeleton reorganization. Serum-starved U373-MG cells were pretreated with DMSO, 1 μM crizotinib or 1 μM R428 for 3 h and then treated with or without HGF for 7 min. Cells were processed for co-immunostaining with anti-p-MET (Tyr-1234/1235–phosphorylated) (red) and anti-ELMO2 (green) antibodies as indicated. Cells were also counterstained with phalloidin (magenta). B, ELMO2, but not ELMO1, regulates cytoskeleton reorganization in response to HGF. U373-MG cells were transfected with control (luciferase; Luc), ELMO2, or ELMO1 siRNAs for 72 h. Cells were then serum-starved and stimulated with HGF as in A. Cells were co-immunostained with antibodies for p-MET (Tyr-1234/1235–phosphorylated) (red) and MET (total Met) (green) and then counterstained with phalloidin (magenta) as indicated. C, cells were transfected with control (luciferase) or DOCK180 siRNAs for 72 h and processed as in B. Cells were co-immunostained with antibodies for p-MET (Tyr-1234/1235–phosphorylated) (red) and ELMO2 (green) and then counterstained with phalloidin (magenta) as indicated.
HGF-induced MET–AXL–RAC1 signaling for cell migration

A

DMSO  Crizotinib  R428

0 hr

24 hr - HGF

24 hr + HGF

Relative cell numbers

***

B

Relative cell numbers

***

C

kDa  Luc  siMET

130  MET  Actin

35

kDa  Luc  siAXL

130  AXL  Actin

35

kDa  Luc  siELMO2

70  ELMO2  Actin

35

kDa  Luc  siDOCK180

25  DOCK180  Actin

35

kDa  Luc  siRAC1

17  RAC1  Actin

35

D

Relative cell numbers

***

E

Relative cell numbers

***

DMSO  Crizotinib  R428  Crizotinib+R428

HGF  GAS6  HGF+GAS6

DMSO  Crizotinib  R428  Crizotinib+R428

HGF  GAS6  HGF+GAS6
found that recombinant MET enzyme can efficiently phosphor-
ylate an AXL peptide derived from sequence surrounding the
Tyr-779 site in vitro, suggesting that the HGF/MET may acti-
vate AXL through MET-mediated trans-phosphorylation of
Tyr-779 in AXL. Interestingly, AXL Tyr-779 phosphorylation is
reported to be strongly elevated in glioblastoma tissue samples,
often without GAS6 overexpression, suggesting the existence
of a GAS6-independent mechanism that phosphorylates AXL
in these cancer tissues (11). Consistent with our observation, it
was reported that phosphorylation level of AXL at Tyr-779 can
be reduced by a neutralizing antibody against HGF in a three-
dimensional spheroid culture of breast cancer cells, suggesting
the potential involvement of HGF/MET in mediating AXL
transphosphorylation (37). However, our results differed from
the reported cross-talk between MET and AXL in GnRh
(gonadotropin-releasing hormone) neuron cells, which showed
that whereas HGF did not induce AXN phosphorylation and
activation, the kinase-dead mutant of AXL was able to block the
activation of MET and downstream signaling to AKT and
p38MAPK (38).

What is the functional role of AXL in the MET–AXL com-
pex? In contrast to Tyr-702, Tyr-779 in AXL is localized out-
side the activation loop of the kinase (39, 40). Phosphoryla-
tion of Tyr-779, which is situated in the sequence motif of YALM,
is known to serve as a docking site for the Src homology 2
domains of the p85 regulatory subunit of PI3K, for the subse-
quent PI3K activation and downstream AKT phosphorylation
(36, 39). Although one possible role of AXL in the MET–AXL
complex is to provide a new tyrosine phosphorylation site to
recruit signaling molecules, our studies showed that the HGF-
dependent MET–AXL RTK clustering, RAC1 activation, and
subsequent cell migration and invasion are all potently sup-
pressed by R428, an AXL kinase inhibitor (Figs. 5D, 6G, 8A, and
9A and C). Our observations suggest that the kinase activity
of AXL is required for the signaling of the MET–AXL complex.
Recent crystal structural studies of AXL revealed that the AXL
kinase domain possesses an unusual dynamic nature (40). It is
possible that formation of the MET–AXL complex triggers a
conformational change in AXL that leads to the activation of
AXL kinase activity, a process accompanied by phosphor-
ylation of Tyr-779 in the absence of Tyr-702 phosphorylation.
Alternatively, phosphorylation of AXN at Tyr-779 may repre-
sent a critical step of a novel mechanism to activate AXL.
Future studies are needed to address these questions.

Substantial evidence indicates that AXL is heavily modified,
and different isoforms may have different functions (30).
Recent studies showed that a low-molecular weight form of
AXL (p120 or p114) activates the PI3K/AKT/mTOR pathway and
promotes survival in low-glucose culture medium, whereas
the higher-molecular weight form of AXL (p140) leads to the
increased cell migration in high glucose culture medium (30).
In our system, we found that only the high molecular weight
species, p140, and not the GAS6-dependent lower molecular
species, p120, of the AXN proteins is phosphorylated by HGF/
MET on Tyr-779 (Fig. 2F). Our data are consistent with the data
showing activation of the p140 form of AXN by EGFR, which
was very recently reported to occur through the formation of an
EGFR–AXL protein complex and the EGFR-mediated trans-
phosphorylation of AXN at Tyr-779 (41). The p140 form, which
likely represents a more glycosylated form of AXN than p120
(30), may facilitate AXN to interact with other RTKs in the
extracellular domains. On the other hand, the extracellular
domain of AXN may also influence the signaling output from
the intracellular kinase domain of the receptor. It has been
reported that a chimeric receptor composed of EGFR extracel-
ular domain fused to the intracellular kinase domain of AXN
has signaling output that is different from that of natural AXN
activated by GAS6 (42). In our system, we have observed that
whereas GAS6 can induce p120 AXN activation through phos-
phorylation of Tyr-702, there is no RAC1 activation on the
plasma membrane, as indicated by the lack of AXN receptor
cluster formation in the GAS6-stimulated cells (Fig. 2A). These
observations suggest that the signaling capabilities from the
HGF-induced MET–AXL complex and the GAS6-induced
AXL–AXL complex may be distinct (Fig. 10H). Consistently,
whereas there are reports indicating that AXN can form a het-
eromeric complex with EGFR to signal through AKT and
p38MAPK (17), our studies revealed that the MET–AXL com-
plex signals through the recruitment of ELMO2 and DOCK180
to regulate the activation of RAC1.

Figure 9. AXL, ELMO2, DOCK180, and RAC1 are each required for HGF-induced cells migration and invasion. A, cell migration assay. Serum-starved U373-MG cells were pretreated with 1 μM crizotinib or 1 μM R428 for 3 h. An equal number of cells were then seeded in the Oris cell migration apparatus for 16 h before removal of the stopper. Cells were then treated with HGF (50 ng/ml) or left untreated for another 24 h. Cells were subsequently fixed, stained with DAPI, and imaged with a Nikon fluorescence microscope, and the numbers of cells that migrated into the central circular areas were quantified using the ImageJ software. HGF-dependent cell migration was quantified (see “Experimental procedures” for details) and shown on the right (shown are the means with S.D. (error bars)). Statistical analysis was conducted for each of the drug-treated samples versus the DMSO control (***, p < 0.001). B, cells were transfected with siRNAs against MET, AXL, ELMO2, DOCK180, and RAC1 for 24 h and then used for a cell migration assay as described in A. HGF-dependent cell migration was quantified and shown. Statistical analysis was conducted for each condition versus luciferase siRNA (Luc) control (***, p < 0.001). Knockdown efficiency for each gene was verified by Western blot analysis with the corresponding antibodies. C, invasion assay using a Boyden chamber precoated with Matrigel. Cells were pretreated with either 1 μM Crizotinib or 1 μM R428 or specific siRNAs as in A and B. An equal number of cells were then seeded to the top chamber of the Boyden chamber, and the bottom chamber contained HGF (50 ng/ml). After 24 h, cells that migrated to the bottom chamber (invaded through Matrigel) were stained, imaged by an EVOS light microscope, and quantified using the ImageJ software. No cells invaded into the bottom chamber in the absence of HGF (data not shown). Assays were performed in triplicates, and the means (with S.D.) are shown on the right. Statistical analysis was conducted (compared with the corresponding control in each group) (***, p < 0.001). D, serum-starved U373-MG cells were pretreated with DMSO, 1 μM crizotinib, 1 μM R428, or 1 μM crizotinib together with 1 μM R428 for 3 h and then assayed for cell migration in the presence of either HGF (50 ng/ml), GAS6 (400 ng/ml), or HGF (50 ng/ml) and GAS6 (400 ng/ml) together. Cell migration assays were conducted as in A. Quantitation of the growth factor-stimulated cell migration is shown, after normalization to the DMSO control in the presence of HGF (shown are the means with S.D.). Statistical analyses were conducted for each condition, as compared with the DMSO control within each subgroup treated with the same growth factor(s) (***, p < 0.001; * p < 0.05). E, cells were pretreated as in D and then subjected to the invasion assay as in C. Growth factors, HGF (50 ng/ml), GAS6 (400 ng/ml), or HGF (50 ng/ml) and GAS6 (400 ng/ml) together, were added to the lower chamber of the Boyden chamber. Quantitation of the cells that had invaded through the Matrigel is shown, after normalization to the DMSO control in the presence of HGF (shown are the means with S.D.). Statistical analysis was conducted (compared with DMSO control within each subgroup with the indicated growth factor(s) (***, p < 0.001; * p < 0.05).
HGF-induced MET–AXL–RAC1 signaling for cell migration

A

A375 MDA-MB-231

kDa

130-
P-AXL (Y779)

130-
AXL

130-
P-MET (Y1234/5)

130-
MET

130-
P-AKT

130-
AKT

130-
P-S6K

130-
S6K

55-
Actin

B

A375

IP: anti-Met

kDa

IgG

HGF

HGF

P-AXL (Y779)

AXL

P-MET (Y1234/5)

MET

P-AKT

AKT

P-S6K

S6K

ELMO2

C

MDA-MB-231

IP: anti-Met

kDa

IgG

HGF

HGF

P-AXL (Y779)

AXL

P-MET (Y1234/5)

MET

P-AKT

AKT

P-S6K

S6K

ELMO2

D

MDA-MB-231

DMSO Crizotinib R428

0 hr

24 hr - HGF

24 hr + HGF

F

A375

DMSO Crizotinib R428

MDA-MB-231

DMSO Crizotinib R428

G

A375

Relative cell numbers

DMSO Crizotinib R428

MDA-MB-231

Relative cell numbers

DMSO Crizotinib R428

H

RAC1 activation

Cell migration and invasion
Our studies also suggest that the MET–AXL complex utilizes a very specific ELMO family member, ELMO2, but not ELMO1, and DOCK180, but not other DOCK family members, for activating RAC1 in U373-MG cells (Figs. 7C and 8B and C) and Fig. S2C). In addition, whereas cell migration induced by HGF requires ELMO2, the GAS6-induced cell migration requires ELMO1 (Fig. S4B). It has been reported that AXL can phosphorylate ELMO1 and ELMO2 in vitro, and ELMO1 and ELMO2 can functionally substitute each other for the GAS6/AXL-dependent cell migration in breast cancer cells (33). It is possible that the HGF-activated MET–AXL heterodimer may act differently from the GAS6-activated AXL–AXL homodimer in mediating phosphorylation and activation of ELMO2, which lead to the specific recruitment of DOCK180 as a distinct GEF for RAC1 (model in Fig. 10H). MET has been reported to activate other RAC1 GEFs, such as TIAM1 and VAV2, which are known to activate RAC1 on the endosomes (24, 25). Our studies showed that the MET–AXL–ELMO2–DOCK180 complex activates RAC1 on the plasma membrane. The plasma membrane localization of this MET–AXL–associated GEF activity is consistent with the role of ELMO2/DOCK180 in mediating phagocytosis and engulfment of dying cells, a process known to involve extensive plasma membrane activities (23). In addition, our studies also revealed that the formation and maintenance of the activated state of the MET–AXL RTK complex not only depends on both MET and AXL activities, but also involves ELMO2 and DOCK180, suggesting that a potential positive feedback mechanism may be present. In summary, our studies suggest a novel mechanism in which HGF can activate the assembly of the MET–AXL–ELMO2–DOCK180 signaling complex on the plasma membrane to regulate dynamic actin cytoskeleton reorganization, cell migration, and invasion, a highly orchestrated process used by cancer cells for their invasive growth.

**Experimental procedures**

**Cells, antibodies, and immunological methods**

Human glioblastoma cell lines U373-MG, T98G, LN229 and LN18, human malignant melanoma cell line A375, and human breast cancer cell line MDA-MB-231 were obtained from American Type Culture Collection (ATCC). Recombinant human HGF (PHG0254) and Alexa Fluor 568–phalloidin (A12380) were from Life Technologies, Inc. Recombinant human GAS6 (885-GSB) was from R&D Systems. Antibodies against MET (goat, AF276), phosphorylated AXL (p-779-AXL, rabbit, AF2228), and AXL (mouse, MAB-154) were from R&D Systems. Rabbit antibodies against MET (D1C2), phosphorylated MET (pTyr-1234/1235, D26), phosphorylated AXL (pTyr-702-AXL, D12B2), AXL (C89E7), phosphorylated S6K (Thr-389, 108D2), phosphorylated AKT (at Ser-473, recognizing all AKT isoforms, D9E), S6K (9D7), AKT (recognizing all isoforms, 867E7), and the Alexa-Fluor–488–conjugated rabbit anti-mouse IgG,Alexa Fluor 488–conjugated goat anti-rabbit IgG, and Alexa Fluor 647–conjugated goat anti-mouse or anti-rabbit IgG were from Jackson ImmunoResearch Laboratories. Rabbit antibodies against MET (goat, AF276), phosphorylated AXL (p-779-AXL, D12B2), AXL (C89E7), phosphorylated S6K (Thr-389, 108D2), phosphorylated AKT (at Ser-473, recognizing all AKT isoforms, D9E), S6K (9D7), AKT (recognizing all isoforms, 867E7), and the Alexa-Fluor–488–conjugated rabbit anti-MET antibody (clone D1C2) were from Cell Signaling Technology. Anti-MET (3D4) mouse mAb was from Thermo Fisher. Monoclonal mouse antibodies against ELMO2 (C-12), ELMO1 (B-7), or DOCK180 (H-4) and polyclonal goat antibody against DOCK180 (C-19) were from Santa Cruz Biotechnology, Inc. Rabbit antibody against DOCK 180 (A301-287A) was from Bethyl Laboratories. Rabbit antibody against ELMO2 (EPR13567) was from Abcam. Alexa Fluor 488–conjugated goat anti-mouse IgG, Alexa Fluor 488–conjugated goat anti-rabbit or anti-mouse IgG, and Alexa Fluor 647–conjugated goat anti-mouse or anti-rabbit IgG were from Jackson Immunological Sciences. Mouse antibodies against RAC1 and the RAC1 Pull-down Activation Assay Biochem Kit were from Cytoskeleton, Inc. The Dylight® In Situ Orange Starter Kit Mouse/Rabbit was from Sigma. The CellTiter 96® AQueous One Solution Kit was from Promega. Tyrosine kinase inhibitors for MET and AXL, crizotinib and R428, were obtained from Cell Signaling Technology and Selleck Chemicals, respectively.

**Gene silencing by siRNAs**

The siRNAs specific for AXL (siAXL#1, sc-29769), ELMO2 (sc-40527), ELMO1 (sc-40525), or DOCK180 (sc-44294) were from Santa Cruz Biotechnology, and these silencing siRNAs generally consisted of pools of 3–5 target-specific sequences, each 19–25 nucleotides in length. Alternatively, siRNAs against specific gene were synthesized by Dharmacon: siAXL#2, ACAUAGGCUAAGGCAAGA; RAC1, ACCGGT-GAATCTGGGCTTA; MET, GAGACAUCAUGCGCUAGU; luciferase control, CGUACCGGAUACUUCGCA. Transfection of siRNAs (50 nM) was conducted using Oligofectamine (Life Technologies) according to the protocol recommended by the manufacturer.

**Figure 10. HGF-dependent interaction and activation of MET and AXL in melanoma and breast cancer cells.** A, serum-starved A375 or MDA-MB-231 cells were treated with or without HGF (50 ng/ml) for 7 min. Western blotting analyses were conducted using antibodies specific for the indicated proteins with actin as a loading control. B, serum-starved A375 cells were treated with or without HGF for 7 min. Cell lysates were used for immunoprecipitation (IP) with anti-MET antibodies and then blotted with anti-p-AXL (Tyr-779–phosphorylated), AXL, p-MET (Tyr-1234/1235–phosphorylated), MET, and ELMO2 antibodies as indicated. C, same as in B except that MDA-MB-231 cells were used for immunoprecipitation and Western blotting analyses. D, serum-starved MDA-MB-231 cells were pretreated with 1 µM crizotinib or 1 µM R428 for 3 h. Cells were assayed for cell migration using the Oris cell migration chamber as described in the legend to Fig. 9A, either in the absence or presence of HGF (50 ng/ml). After 24 h, cells were fixed, stained with DAPI, and imaged with a Nikon fluorescence microscope. Quantification of the HGF-dependent cell migration was conducted similarly as in Fig. 9A and shown on the right (shown are the means with S.D.). Statistical analysis was conducted for each of the drug-treated samples versus the DMSO control (***, p < 0.001). E, quantitation of the HGF-dependent cell migration of A375 cells. Cell migration assay of A375 cells was conducted similarly to that described for MDA-MB-231 cells in D and quantified. F and G, A375 and MDA-MB-231 cells were subjected to the cell invasion assays using a Boyden chamber precoated with Matrigel like that described as in the legend to Fig. 9C. Serum-starved A375 cells (top of F) or MDA-MB-231 cells (bottom of F) were pretreated with 1 µM crizotinib or 1 µM R428 for 3 h. Cells were then seeded to the top chamber of the Boyden chamber, and the bottom chamber contained HGF (50 ng/ml). After 24 h, cells that migrated to the bottom chamber (invaded through Matrigel) were stained and imaged. Assays were performed in triplicates, and quantification results are shown in G (means with S.D.). Statistical analysis was conducted (compared with the control) (***, p < 0.001). H, schematic model summarizing our results. HGF binds to the MET RTK to form the MET–AXL heterodimer on the plasma membrane, promoting the phosphorylation of Tyr-779 on the p120 isoform of AXL. The MET–AXL RTK signaling complex recruits ELMO2 and DOCK180 to activate the RAC1-dependent cell migration and invasion. This pathway is different from the HGF-dependent activation of MET homodimer, which leads to the activation of AKT and S6 kinases. It is also distinct from the GAS6-mediated AXL homodimerization that leads to the phosphorylation of Tyr-702 on the p120 isoform of the AXL RTK, leading to the ELMO1/RAC1-dependent cell migration.
**HGF-induced MET–AXL–RAC1 signaling for cell migration**

**Immunoprecipitation, SDS-PAGE, and Rac-GTP assays**

U373-MG cells were serum-starved for 3 h, and the starved cells were then stimulated with or without HGF (50 ng/ml) for 7 min unless otherwise noted. Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, supplemented with 5 mM NaF, 1 mM Na$_3$VO$_4$, and 1× complete protease inhibitor (Pierce). For immunoprecipitation, clarified cell lysates were incubated with the indicated antibodies, and the immune complexes were allowed to form for 16 h at 4°C. Protein A or G-Sepharose beads were added for 2 h to recover the immune complex. The beads were washed four times with lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting as described (43). For the RAC1 activation assay, U373-MG cells were treated and lysed as described in the immunoprecipitation assays. The GTP loading status of RAC1 was analyzed by affinity binding and pulldown with the purified p21-binding domain of PAK protein kinase expressed as a GST fusion protein (GST-PAK-PBD), using the kit from Cytoskeleton Inc. Equal amounts of protein lysates from different conditions were used for pull-downs and then analyzed by Western blot analysis, along with aliquots of straight lysates, and RAC1 was detected by immunoblots. Levels of RAC1 activation were quantified by densitometry analysis of the blots using the Fiji (ImageJ) software (44).

**PLAs**

MET and AXL interaction assays were performed using the Duolink® In Situ Orange Starter Kit Mouse/Rabbit (Sigma). All of the steps were performed according to the manufacturer's protocol. Serum-starved U373-MG cells were treated with HGF (50 ng/ml) for 7 min, fixed, and processed for staining according to the protocol. The anti-MET (3D4) mouse mAb and the anti-AXL (C89E7) rabbit mAb were used. The assayed cells were then examined and recorded using the Nikon confocal microscope in the same way as immunostaining. An average of 50 cells were counted, and quantitation was conducted using the GraphPad software for each condition.

**Cell proliferation assays**

Cell proliferation assays were performed using the CellTiter 96® AQueous One Solution kit from Promega, following the company’s protocol. Briefly, equal numbers of U373-MG cells were seeded in the 96-well plate and were then starved overnight. Cells were subsequently treated with HGF (50 ng/ml) or PBS for different times (0, 24, 48, or 72 h). At each time point, 20 μl of CellTiter 96® AQueous One solution were added to each well of the 96-well plate containing the cells in 100 μl of cell culture medium. The plate was further incubated at 37°C for 1–4 h in a humidified, 5% CO$_2$ atmosphere. The absorbance of cells in each well was recorded at 490 nm using a 96-well plate reader and quantified using the GraphPad software.

**In vitro kinase activity assay**

The MET (M1250T) Kinase Assay Kit and ADP-Glo Assay Kit were obtained from Promega and used according to the manufacturer’s recommendations. Briefly, a reaction volume of 25 μl was set to contain recombinant MET kinase (10 ng/μl), ATP (25 μM), peptide substrates (poly(Glu$_9$, Tyr$_1$) (0.1 μg/μl), or AXL Tyr-779 peptide (0.2 μg/μl), in the presence of DMSO (vehicle control) or the MET inhibitor crizotinib (2 μM). An enzyme-only (no substrate) reaction was also included and used as a control. The reaction was allowed to proceed for 60 min at room temperature, and ADP produced during the reaction was subsequently measured using the ADP-Glo reagents. Luminescence (RLU) was recorded using a Tecan Spark M10 multimode microplate reader and processed with the on-board Magellan data analysis software. Levels of substrate phosphorylation were calculated from the RLU values for each reaction after subtracting the values obtained from the no substrate control reaction. Reactions were carried out in duplicates, and the means (with S.D.) were calculated. The AXL Tyr-779 peptide (CKQPADLDGLY{$\text{Tyr}^779$}ALMSRCWELNPQD) was derived from the sequence of human AXL surrounding the Tyr-779 site and synthesized by ABI Scientific Inc.

**Immunostaining and fluorescence microscopy**

U373-MG cells were serum-starved for 3 h, and the starved cells were then stimulated with or without HGF (50 ng/ml) or GAS6 (400 ng/ml) for 7 min before fixation. Immunofluorescence experiments were conducted as described (28). Briefly, cells were fixed and permeabilized with Fixation and Permeabilization Solution (BD Biosciences), blocked with goat serum, and incubated with primary antibodies and then secondary antibodies in PBS containing 0.05% Tween 20 and 7% goat serum. Cells were counterstained with phalloidin or 4’,6-diamidine-2’-phenylindole dihydrochloride (DAPI) when needed. Images were acquired using a Nikon A1R confocal laser-scanning microscopy system equipped with the NIS-Elements Advance Research software with a ×20 air lens (NA 0.8) or ×40 oil immersion lens (NA 1.3). For double immunostaining, images were captured using channels for Alexa Fluor 488 and Alexa Fluor 647, which have minimal fluorescence bleed-throughs. Exposure settings were kept constant for all conditions within each set of experiments. When necessary, images were also captured using channels for phalloidin staining (phalloidin-Alexa Fluor 568 conjugate) or DAPI staining. The captured images were further analyzed and quantified by using the Fiji (ImageJ) software (44).

**Cell migration and invasion assays**

Cell migration assay were performed using Oris™ assembly kits (Platypus Technologies). Serum-starved U373-MG cells treated with c-MET inhibitor; AXL inhibitor; siRNAs for MET, AXL, ELMO2, DOCK180 and RAC1; or DMSO or Luc siRNA controls were detached and placed in the 96-well plate with a stopper and then starved overnight. The stoppers were then removed, and cells were exposed to HGF (50 ng/ml) for another 24 h, fixed, and stained with DAPI. Cells were imaged with a Nikon confocal microscope, and Fiji (ImageJ) software (44) was used to count the number of the cells that migrated into the circular “open” field. HGF-dependent cell migration was quantified by subtracting the number of cells that had migrated in the absence of HGF from that in the presence of HGF. Assays were performed in triplicates. Cell invasion assays were per-
formed using Boyden chambers with 8-μm pores (Costar, Cambridge, MA), coated with the growth factor-reduced Matrigel (BD Biosciences) diluted in Dulbecco’s modified Eagle’s medium. Cells were detached by trypsin and washed once with starvation medium (Dulbecco’s modified Eagle’s medium, 0.5% fetal bovine serum). Twenty thousand cells were seeded in the upper chamber for each condition in starvation medium, and cells were allowed to invade for 24 h toward the lower chamber containing starvation medium supplemented with HGF (50 ng/ml), GAS6 (400 ng/ml), or HGF (50 ng/ml) together with GAS6 (400 ng/ml). Cells were then fixed with 4% paraformaldehyde. Cells in the upper chambers were mechanically removed using cotton swabs. Cells invaded into the lower chambers were fixed and stained with the Differential Quik Stain Kit (modified Giemsa stain) (from Electron Microscopy Sciences). Cells were imaged using an EVOS XL Core system with >10 objective and quantified using the ImageJ software.

For each condition, cells were counted from eight independent experiments. H. S. analyzed data and wrote the manuscript. In the absence of growth factors, no cells had invaded through the Matrigel.

**Statistics and bioinformatics**

Quantitative data are expressed as the mean ± S.D., normalized to the control condition, and plots were prepared using the GraphPad Prism version 5 software. Statistically significant differences between means were determined using a one-tailed equal-variance Student’s t test (45). Different data sets were considered to be statistically significant when the p value was <0.001 (****), <0.01 (**), or <0.05 (*).

Author contributions—H. S. conceived the idea, and H. S. and W. L. designed and organized the approach. W. L. performed most of the experimental work; X. X., A. A., S. A., L. Z., and F. L. assisted in some experiments. H. S. analyzed data and wrote the manuscript.

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