Dorsal ruffles are apical protrusions induced in response to many growth factors, yet their function is poorly understood. Here we report that downstream from the hepatocyte growth factor (HGF) receptor tyrosine kinase (RTK), Met, dorsal ruffles function as both a localized signaling microdomain as well as a platform from which the Met RTK internalizes and traffic to a degradative compartment. In response to HGF, colonies of epithelial Madin-Darby canine kidney cells form dorsal ruffles for up to 20 min. Met is transcytosed from the basolateral membrane on Rab4 endosomes, to the apical surface where Met, as well as a Met substrate and scaffold protein, Gab1, localize to the dorsal ruffle membrane. This results in activation of downstream signaling proteins, as evidenced by localization of phospho-ERK1/2 to dorsal ruffles. As dorsal ruffles collapse, Met is internalized into EEA1- and Rab5-positive endosomes and is targeted for degradation through delivery to an Hrs-positive sorting compartment. Enhancing HGF-dependent dorsal ruffle formation, through overexpression of Gab1 or activated Pak1 kinase, promotes more efficient degradation of the Met RTK. Conversely, the ablation of dorsal ruffle formation, by pre-treatment with SITS (4-acetamido-4′-isothiocyanato stilbene-2′,2′-disulfonic acid) or expression of a Gab1 mutant, impairs Met degradation. Taken together, these data support a function for dorsal ruffles as a biologically relevant signaling microenvironment and a mechanism for Met receptor internalization and degradation.

The polarized distribution and duration of signals downstream from receptor tyrosine kinases (RTKs) regulates fundamental cellular processes involved in cell migration, proliferation, and morphogenesis. This is achieved in part through the entry of RTKs into the endocytic pathway (1, 2). Following activation at the cell surface, RTKs are internalized onto endosomes where they initially remain signaling competent and may be recycled back to the cell surface to a specific membrane microdomain, or sorted for lysosomal degradation (2). Hence, the tenancy of an activated RTK at the plasma membrane and in the endocytic pathway, prior to degradation, will impact on specificity and duration of RTK signaling (2, 3). Mechanisms that abrogate the targeting of RTKs to the endocytic degradative pathway can lead to prolonged signaling and contribute to the deregulation of RTKs and tumorigenesis (4).

Multiple routes for ligand-dependent uptake of RTKs from the plasma membrane have been identified (5). The best characterized involves the formation of membrane invaginations coated with clathrin, known as clathrin-coated pits (6). Following activation, RTKs can be sorted into clathrin-coated pits by adaptor molecules. These are then internalized into the cell when the clathrin-coated pit buds off into the cytoplasm through a scission event catalyzed by dynamin (7), forming a clathrin-coated vesicle. Clathrin-coated vesicles fuse with early endosomes, delivering their contents, in general, to Rab5- and EEA1-positive early endosomes. RTKs may either enter the recycling compartment and be recycled back to the plasma membrane through Rab4 and Rab11-positive endosomes (8), or sorted for lysosomal degradation by components of the ESCRT complex (endosomal sorting complex required for transport) (9). Additional mechanisms also exist for internalization of RTKs. These involve caveolae (10), flotillin-rich microdomains (11), macropinocytosis (12–15), and more recently, dorsal ruffles have been proposed as a means for internalization of the epidermal growth factor receptor (EGFR) (16). Dorsal ruffles, also known as circular ruffles or waves, are ring-like, F-actin-rich, membrane protrusions, which extend from the apical cell surface in response to a variety of growth factors including, epidermal (EGF), hepatocyte (HGF), platelet-derived (PDGF) and vascular endothelial growth factors (17–19). Dorsal ruffles form only transiently in response to stimulation, making them dynamically distinct from peripheral ruffles and lamellipodia, which form continuously in response to growth factors (20, 21). Depending on the cell type, each dorsal ruffle protrusion persists from 2 to 8 min and then collapses back onto the cell surface generating a large pool of intracellular vesicles (17, 22–25) as well as tubular structures (16). Several physiological roles have been demonstrated for dorsal ruffles, including macropinocytosis and cell migration (17, 21, 25–27). Although, dorsal ruffles have also been observed as a site of internalization for the EGFR (16), the functional consequence...
of internalization of an RTK from this structure has yet to be determined.

Hepatocyte growth factor, the ligand for the Met RTK, is a potent inducer of dorsal ruffles in colonies of MDCK epithelial cells (17, 27) and can induce dorsal ruffles in tumor cell lines, such as HeLa cells (28). We have established that the Gab1 scaffold protein is essential for dorsal ruffle formation downstream from the Met, EGF, and PDGFβ RTKs (27). Moreover, the ability of Gab1 to coordinate dorsal ruffle formation is also required for HGF-induced cell migration and epithelial morphogenesis in polarized sheets of MDCK cells (27), supporting a role for dorsal ruffles as physiologically relevant signaling microdomains for the Met RTK.

Following HGF stimulation, the Met RTK undergoes Cbl-dependent ubiquitination (29, 30). Met receptors are subsequently internalized in a clathrin-dependent manner (31–33) and are targeted to Rab5 endosomes where they may continue to signal (29, 32). Ubiquitinated Met receptors, localized on endosomes, are recognized by Hrs (HGF-regulated tyrosine kinase substrate) (29), which as part of the ESCRT-0 complex, sorts ubiquitinated cargo for internalization into the endosomal lumen (9). Uncoupling Met from ubiquitination abrogates this down-regulation, resulting in prolonged endocytic signaling and cellular transformation (29, 30). Importantly, this mechanism of deregulation contributes to the transforming potential of naturally occurring oncogenic Met mutants (34–37). Previous studies have focused on understanding Met internalization and trafficking in cell lines, such as HeLa cells where Met internalizes into clathrin-coated vesicles, but the ability of Met to internalize by other mechanisms has not been thoroughly addressed.

In this study, we sought to examine the role of dorsal ruffles in internalization of the Met RTK in sheets of polarized epithelial cells as well as in single cells. We show that following ligand stimulation, Met is transcytosed to dorsal ruffles on the apical membrane with Rab4. Activated Met receptors persist for the duration of dorsal ruffles, which also contain signaling complexes including Gab1 and phosphorylated ERK1/2. Following collapse of dorsal ruffles, Met is delivered to Rab5 and Hrs-positive endosomal structures, which results in efficient Met degradation, whereas disruption of dorsal ruffles promotes stabilization of the Met RTK. Taken together, these data demonstrate that dorsal ruffles represent a spatially restricted Met RTK signaling microdomain and paradoxically, also mediate efficient down-regulation of the Met RTK.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibody 147 raised against a carboxyl-terminal peptide of the human Met protein was used for Western blot in HeLa cells (38, 39). Commercial antibodies usws were canine Met AF4140 (used for immunofluorescence staining in MDCK cells) and human Met AF276 (used for immunofluorescence staining in HeLa cells) from R&D Systems (Minneapolis, MN); Met clone 14G9 (used for Western blot on MDCK cell lysates), NanoTools (Teningen, Germany); anti-ubiquitin (P4D1), actin, polyclonal EEA1, clathrin heavy chain, from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); GFP antibody, phalloidin-Alexa Fluor 488 and 546, Alexa Fluor 555-EGF, and Alexa 488- and 555-conjugated secondary antibodies from Molecular Probes (Eugene, OR). Total and phospho-specific ERK1/2 (pThr202/pTyr204), pY1234/35 Met, pY627 Gab1, and pY992 EGFR were from Cell Signaling Technology (Mississauga, ON); monoclonal EEA1, BD Biosciences (Mississauga, ON); HA.11 and Myc 9E10 monoclonal antibodies, Covance (Berkeley CA) and Babco (Richmond, CA); and polyclonal ZO-1 antibody from Zymed Laboratories Inc. Clathrin small interfering RNA was purchased from Qiagen. Cycloheximide, lactacystin, concanamycin, and SITS (4-acetamido-4'-isothiocyanostilbene-2',2'-disulfonic acid) were purchased from Sigma. The following constructs were described previously: pcDNA1.1 pcDNA1.1-HA-Gab1, pXM Met GFP Rab7, and GFP-Hrs (29, 39). PRK5-myc vector and PRK5-myc-Pak1 His83-Lue86 were a generous gift from Dr. G. Bokoch (40). GFP-Rab4, GFP-Rab5, and GFP-Hrs were generous gifts from Dr. S. Ferguson, Dr. L. Lodge, and Dr. S. Urbe, respectively. pcDNA3.1 HA-ERK1 and -ERK2 constructs were a generous gift from Dr. J. Blenis.

Cell Culture and Transfections—MDCK and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. MDCK cell lines expressing, GFP-Gab1 (M-GFP-Gab1, clone B1–3), HA-Gab1 (M-HA-Gab1, clone 7d6), and HA-Gab1ΔDR (M-HA-Gab1ΔDR, clones A, D, and F) were established as described previously (39, 41–43). Transient transfection of HeLa and MDCK cells was performed using Lipofectamine Plus (Invitrogen) reagent according to the manufacturer’s instructions.

Confocal Immunofluorescence Microscopy—MDCK (2.5 × 10⁴) and HeLa (4 × 10⁴) cells were seeded on glass coverslips (Belco Glass Inc., Vineland, NJ) in 24-well plates (Nalgene NUNC, Rochester, NY) and 48 h later, stimulated with 0.5 mM HGF or 5 mM EGF. To study Met trafficking, MDCK and HeLa cells were treated with cycloheximide for 2 h prior to HGF stimulation. Coverslips were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde (Fisher Scientific). Staining procedures have previously been described in Ref. 29. Staining for actin was always performed using an Alexa Fluor-labeled phalloidin. Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss, Canada Ltd., Toronto, ON, Canada) with ×100 or 40 objectives. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON, Canada).

Dorsal Ruffle Assays—Cells, plated on coverslips, were stimulated with 0.5 mM HGF for 5 min, fixed in 4% paraformaldehyde, and stained with phallolidin Alexa Fluor 488 or 545. Using a confocal microscope with a ×40 objective, the number of cells that formed dorsal ruffles were counted and are represented as percent of the total number of cells counted. At least 8 fields of view were counted for each experiment.

Immunoprecipitation and Western Blotting—HeLa and MDCK cells were harvested in TGH lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM...
sodium vanadate, 10 μg/ml of aprotinin, and 10 μg/ml of leupeptin). To detect Met receptor ubiquitination in HeLa cells, cells were lysed 24 h post-transfection in RIPA lysis buffer (0.05% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.05% sodium deoxycholate) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM sodium fluoride, 10 μg/ml of aprotinin, and 10 μg/ml of leupeptin. Cells harvested under boiling lysis conditions were lysed in 200 μl of boiling buffer (2% SDS, 1 mM EDTA). Lysates were boiled for 10 min and diluted to 1 ml with a buffer containing 2.5% Triton, 12.5 mM Tris, pH 7.5, 187.5 mM NaCl. For immunoprecipitation, lysates were incubated with the indicated antibody for 2 h at 4 °C with gentle rotation. Proteins collected on either protein A- or G-Sepharose were washed three times in their respective lysis buffers, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked in 3% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween 20) for 1 h, incubated with primary and secondary antibodies in TBST for 2 and 1 h, respectively. Met 147 antibody was used to detect Met in HeLa cells and antibody 14G9 was used to detect Met in MDCK cells. After four washes with TBST, bound proteins were visualized with an ECL detection kit (Amersham Biosciences). Densitometric analysis of Western blots was performed using NIH Image J software. Where indicated, data from densitometric analysis was processed using Prism 4.9 to generate a one-phase exponential decay best fit curve to determine the receptor half-life (t½) with an error using a 95% confidence interval.

Inhibitor Treatment—MDCK cells were plated in 60-mm dishes. 48 h later, cells were treated with lactacystin (10 mM), concanamycin (100 nM), or vehicle control (DMSO) for 4 h, then stimulated with HGF in the presence of cycloheximide plus inhibitors for 2 h before being lysed. For immunoﬂuorescence, coverslips were treated as above then stimulated and fixed after a 5-min HGF treatment. Where indicated, M-GFP-Gab1 cells were pre-treated with DMSO or SITS (0.5 mM) for 20 min prior to growth factor stimulation in the presence of SITS.

Deconvolution and Three-dimensional Visualization—Live cell imaging of HGF-stimulated M-GFP-Gab1 cells was performed with a WaveFX spinning disk confocal microscope (Quorum Technologies Inc., Guelph, ON, Canada) mounted on a motorized microscope, ×63/1.4NA objective (Leica, Wetzlar, Germany), using an EM-CCD camera (Hamamatsu, Shizuoka, Japan). AutoQuant X (MediaCybernetics, version 2.1.4) was used to perform z-plane cropping (to correct for a slight stage drift over the time course) and to apply blind three-dimensional deconvolution prior to region of interest cropping, volume, and isosurface visualization using Imaris (Bitplane, version 6.3.1).

RESULTS

Activated Met RTK and Complexes Are Recruited into HGF-induced Dorsal Ruffles—In response to HGF stimulation of the Met RTK, colonies of MDCK epithelial cells form dorsal ruffles (17) (Fig. 1A, arrowheads). To establish if the Met RTK is recruited into dorsal ruffles, as demonstrated in single cells for the EGFR (16), MDCK cells were fixed post-HGF stimulation and the localization of Met determined following indirect immunofluorescence. In unstimulated MDCK cells, Met localizes to basolateral membranes near sites of cell-cell adhesion and is absent from the apical membranes (Fig. 1A, and B, and supplemental Fig. S1) (44). Following HGF stimulation, Met localizes to dorsal ruffles, as identified by co-staining for actin (Fig. 1A).

Because MDCK cells in colonies form both adherens as well as tight junctions (45), the localization of Met from the basolateral membrane to dorsal ruffles could require either transcytosis of Met from the basal surface or the rapid delivery of newly synthesized Met to the apical dorsal ruffle membranes. Depletion of clathrin by small interfering RNA in MDCK cells inhibited HGF-induced dorsal ruffle formation (supplemental Fig. S1B), consistent with a requirement for clathrin-dependent internalization of Met from the basolateral membranes. As Rab4, an early recycling endosomal marker, has previously been demonstrated to mediate recycling of proteins to apical membranes in MDCK cells (46), we examined whether Rab4 could localize with Met and dorsal ruffles. In response to HGF, Met co-localized with Rab4 prior to as well as within dorsal ruffles (2–5 min; supplemental Fig. S1, C and D). Pre-treatment of MDCK cells with cycloheximide did not diminish the formation nor localization of Met to dorsal ruffles indicating that dorsal ruffle formation does not require targeting of newly synthesized Met (Figs. 1, 2, 4, and 5, see “Experimental Procedures”). Taken together, these data support that Met receptors internalize from the basolateral membrane, transcytose to the apical membrane at least in part with Rab4 recycling endosomes, and lead to dorsal ruffle formation.

We have previously demonstrated that the Gab1 scaffold protein, a key signaling molecule for Met, localizes to dorsal ruffles (27). Using MDCK cells stably expressing Gab1 (M-GFP-Gab1), we established that Met was also recruited into Gab1-containing dorsal ruffles (Fig. 1B). Importantly, by using phosphospeciﬁc antibodies that reﬂect Met catalytic activation (pY1234/35), we demonstrate that Met within dorsal ruffles is activated (Fig. 1B, bottom panel). Each HGF-induced dorsal ruffle persists for up to 8 min (27), and cells retain the ability to form these structures for up to 20 min post-HGF stimulation (supplemental Fig. S2A). Hence, the prolonged localization of both active Met and Gab1 in dorsal ruffles supports that these structures are an active signaling microenvironment, in which Gab1 can couple an activated Met with downstream signaling molecules. In agreement with this, both tyrosine-phosphorylated Gab1 and phospho-ERK1/2 are observed in dorsal ruffles in response to HGF (Fig. 1, C and D), which contain both ERK1 and ERK2 isoforms (supplemental Fig. S2B).

Met RTKs Internalize from Dorsal Ruffles—The formation of HGF-induced dorsal ruffles in colonies of MDCK cells is initiated by protrusions from apical membranes that coalesce to form a tube or chimney-like structure (circular ruffle; Fig. 2A). This structure subsequently constricts, sealing the most apical point of the tube (ruffle constriction) and eventually collapses back onto the cell surface (ruffle collapse; Fig. 2A). To determine the time dependence and consequence of Met localization to dorsal ruffles, we examined Met localization in newly formed circular ruffles (between 2 and 5 min post-HGF stimulation) and those undergoing constriction, as visualized by
Dorsal Ruffles Mediate Met RTK Degradation

GFP-Gab1 (between 5 and 10 min post-stimulation) (Fig. 2B). Met and GFP-Gab1 were observed throughout the wall of the newly forming ruffle (Fig. 2B(i), Z-stacks a, c, and b). Staining for the early endosomal marker EEA1 and Rab5 were restricted mainly to the base of the ruffle (Fig. 2B(i) and supplemental Fig. S3A). Upon constriction of the dorsal ruffle, punctate Met staining was observed within the ruffle and localization of Met and EEA1 to punctate structures, indicative of endocytic internalization into the endocytic pathway, leading to lysosomal degradation of the receptor (47). Concentration of the EGF RTK in dorsal ruffles was proposed to allow bulk internalization of activated receptors following ligand stimulation (16). However, this has not been addressed biochemically. To determine whether dorsal ruffles act as a microdomain involved in trafficking of the Met RTK for degradation, we established if the steady state levels of Met are altered in MDCK cells with an
Dorsal Ruffles Mediate Met RTK Degradation

A

apical protrusion  circular ruffle  ruffle constriction  ruffle collapse

B

(i) circular ruffle  (ii) ruffle constriction  (iii) ruffle collapse

C

Met  GFP  Merge

D

30°  Hrs  Merge

E

15°  30°  Gab1  Met  EEA1  Gab1  Met  EEA1
enhanced capacity to form dorsal ruffles. To this end we used MDCK cells stably overexpressing HA-Gab1 (M-HA Gab1 cells), which form 2-fold more dorsal ruffles in response to HGF when compared with control MDCK cells (Fig. 3, A and B) (27). Notably, loss of the Met RTK is more rapid in M-HA-Gab1 cells when compared with control cells, where the half-life of Met is 24.3 ± 2.1 min versus 71.5 ± 5.8 min, respectively (Fig. 3, C and D). This supports the interpretation that the Met RTK enters the endocytic pathway from the dorsal ruffle microenvironment and is subsequently targeted for degradation. Moreover, Gab1, which fails to traffic with the Met RTK (Fig. 2 E), does not undergo HGF-dependent degradation (Fig. 3 C). Significantly, 4 h post-HGF stimulation, the steady state levels of Met in both MDCK cell populations were similar, indicating that internalization of Met from the dorsal ruffle microenvironment enhances the initial rate of degradation.

**FIGURE 3. Dorsal ruffles mediate efficient Met receptor down-regulation.** A, MDCK and M-HA-Gab1 cells were pre-treated with cycloheximide for 2 h, then stimulated or not with 0.5 nM HGF for 5 min. Cells were then fixed and stained for actin to visualize dorsal ruffles. Scale bar represents 10 μm. B, 10 fields of view of cells from A taken with a ×63 objective were scored for dorsal ruffle formation and the data expressed as the mean percentage of total cells forming dorsal ruffles ± S.E. of three independent experiments. C, cells were treated as in A and stimulated with HGF for the indicated time points after which cells were lysed and cell extracts were separated by SDS-PAGE and immunoblotted for Met (14G9), HA-Gab1, and actin. D, densitometric analysis of Met degradation as percentage of initial receptor remaining after HGF stimulation ± S.E. from three independent experiments were used to generate a best fit one-phase decay curve to determine the half-life (t1/2) of the receptor. E, MDCK and M-HA-Gab1 cells were pre-treated with DMSO, 10 μM lactacystin, or 0.1 μM concanamycin for 2 h, then stimulated or not with 0.5 nM HGF for 2 h. Met protein was immunoprecipitated, separated by SDS-PAGE, and immunoblotted for Met (14G9) and actin. Densitometric analysis of the remaining Met levels compared with unstimulated DMSO control are shown below.

**FIGURE 2. Met localizes to the canonical endosomal network upon internalization from dorsal ruffles.** A, three-dimensional images were generated from a live cell movie of M-GFP-Gab1 cells stimulated with HGF (1-min time intervals shown) using IMARIS software. Z-X (top panel) and Y-X (bottom panel) projections of the various stages of dorsal ruffle formation and collapse are shown. B, M-GFP-Gab1 cells were pre-treated with cycloheximide for 2 h and then stimulated with 0.5 nM HGF for 5 and 10 min, fixed, and stained for Met (red) and EEA1 (purple) and GFP (green). Two representative images of the different stages of dorsal ruffle formation are shown (top 2 images, individual cells are outlined) with the confocal Z-stack images of these fields (lower panels) of (i) circular dorsal ruffle (a, b, and c), (ii) constricting dorsal ruffle (c’, b’, and c’), and (iii) a collapsed dorsal ruffle (d, e, and f). M-HA-Gab1 cells were transfected with GFP-Rab5 (C) or GFP-Hrs (D), pre-treated with cycloheximide for 2 h, and then stimulated with 0.5 nM HGF for 15 and 30 min, respectively. E, cells were treated as in B and stimulated with 0.5 nM HGF for 15 and 30 min. Samples fixed at the indicated time points were stained for Met (red) and 4′,6-diamidino-2-phenylindole (blue). Arrows indicate re-localization of Gab1 to the plasma membrane. Confocal images were acquired with ×100 objective. All scale bars represent 10 μm.
Dorsal Ruffles Mediate Met RTK Degradation

FIGURE 4. Gab1 induced dorsal ruffle formation in HeLa cells promotes more rapid Met RTK degradation. A, HeLa cells, plated on glass coverslips were transiently transfected with vector or HA-Gab1 and 48 h later, pre-treated with cycloheximide for 2 h, stimulated or not with 0.5 nM HGF for 5 min, fixed, and stained for HA (red) and actin (green). B, cells treated as in A were scored for dorsal ruffle formation, the data are expressed as the mean percentage of total cells forming dorsal ruffles ± S.E. of three independent experiments. C, HeLa cells transfected with vector or HA-Gab1 were stimulated or not with 0.5 nM HGF, fixed, and stained for Met (red) and HA (green). D, HeLa cells transfected as in C were stimulated with 0.5 nM HGF for the indicated time points. Total cell extracts were separated by SDS-PAGE and immunoblotted for Met (147), HA, and actin. E, densitometric analysis of Met degradation from D presented as percentage of the initial receptor remaining after HGF stimulation, ± S.E. from three independent experiments. F, HeLa cells transiently transfected with vector or increasing amounts of HA-Gab1 were stimulated or not with 0.5 nM HGF for 5 min and lysed in RIPA buffer. Met protein was immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted for ubiquitin, stripped, and reprobed for Met (147). Total cell extracts were immunoblotted for HA. Scale bars represent 10 μm.

Met RTK degradation, but does not lead to degradation of a larger pool of Met (Fig. 3D).

Rapid Met RTK Degradation Is Dependent on Lysosomal and Proteasomal Functions—Previous studies in HeLa cells have demonstrated a requirement for both the lysosome and the proteasome for degradation of Met (32). To determine whether these functions were required for Met degradation downstream from dorsal ruffles, MDCK and M-HA-Gab1 cells were pre-treated with either an inhibitor for the lysosome (100 μM concanamycin) or the proteasome (10 μM lactacystin) and stimulated with HGF for 2 h. As previously shown in HeLa cells (32), the degradation of Met was dependent on both activity of the lysosome and the proteasome in both MDCK and M-HA-Gab1 cells (Fig. 3E). Importantly, pre-treatment with either inhibitor did not affect the formation of HGF-induced dorsal ruffles (supplemental Fig. S4). However, we did observe that in Gab1 overexpressing cells, the proteasomal inhibitor was less efficient at rescuing Met stability (to only 82% of initial Met levels; Fig. 3E), suggesting that the lysosome may play a more important role in the enhanced receptor degradation observed. Overall, these data demonstrate that in MDCK cells that form dorsal ruffles in response to HGF, Met degradation, following internalization, appears to proceed through mechanisms similar to those observed in HeLa cells (32).

Induction of Dorsal Ruffles in HeLa Cells Promotes More Rapid Met RTK Degradation—The internalization of Met in HeLa cells is predominantly mediated through the canonical clathrin-dependent pathway (31, 32). To determine whether HGF-induced degradation of Met is enhanced dependently on dorsal ruffle formation, we compared Met degradation in HeLa cell lines, which do not readily form dorsal ruffles in response to HGF (vector control, Fig. 4, A and B) to cells overexpressing HA-Gab1, which show an 8-fold enhanced capacity to form dorsal ruffles in response to HGF (Fig. 4, A and B)(27). Expression of HA-Gab1 in HeLa cells promoted the formation of HGF-induced dorsal ruffles, which contained endogenous Met in a similar manner to MDCK cells (Fig. 4C). Notably, 1 h post-HGF stimulation, the steady state level of Met in HA-Gab1 expressing HeLa cells was reduced by 40%, compared with 10% in vector control cells (Fig. 4, D and E). As observed in MDCK cells, Met levels 4 h post-HGF stimulation were similar in both Gab1 expressing and control HeLa cell lines (Fig. 4E). To ensure that recruitment of Met into dorsal ruffles does not target it to a Triton-insoluble compartment, the HGF-dependent loss of Met was compared in cells expressing vector or HA-Gab1, under boiling lysis conditions (48). Consistently, enhanced loss of Met was observed in cells that form elevated levels of dorsal ruffles through expression of HA-Gab1 (supplemental Fig. S5A), demonstrating that differences in Met levels were not due to Met entering an insoluble compartment. Moreover, the enhanced degradation of Met observed following expression of HA-Gab1 requires HGF stimulation, as increasing levels of Gab1 alone is not sufficient to induce Met degradation (supplemental Fig. S5B). Because degradation of the Met RTK is dependent on its ubiquitination (29) the more rapid degradation of Met in cells that form enhanced dorsal ruffles may be the result of enhanced Met receptor ubiquitination. However, using antibodies that recognize ubiquitin, no increase in overall ubiquitination of Met was observed in Gab1 overexpressing HeLa cells when compared with control cells, indicating that the enhanced rate of degradation is not a result...
Disruption of Dorsal Ruffle Formation Delays Met Receptor Degradation—To establish if disruption of dorsal ruffle formation would alter HGF-induced Met degradation, we sought to inhibit their formation without altering the levels of Gab1 or Pak1. SITS, which inhibits the Na+ independent Cl−/HCO− ion exchanger, also inhibits dorsal ruffle formation in MDCK cells through an unknown mechanism (17). Pretreatment of M-GFP-Gab1 cells with 0.5 mM SITS reduced HGF-induced dorsal ruffle formation by 7-fold (Fig. 6, A and B). Significantly, in the presence of SITS, a delay in HGF-induced degradation of Met was observed when compared with control DMSO-treated cells (Fig. 6, C and D). This also correlated with a change in Met localization at 5 min post-stimulation, where Met localized to peripheral ruffles in SITS-treated cells (Fig. 6E, arrowheads). Importantly, SITS pre-treatment does not interfere with HGF-induced activation and tyrosine phosphorylation of Met, hence the defect in Met degradation is not due to reduced activation of Met (Fig. 6C, second row). Moreover, SITS did not interfere with Met internalization in MDCK cells, because by 15 min post-HGF stimulation, Met was observed on EEA1-positive endosomes in both DMSO- and SITS-treated cells (Fig. 6E, inset). Similarly, SITS pre-treatment did not alter Met degradation or internalization in HeLa cells, which show low levels of dorsal ruffles and where Met internalization utilizes the canonical clathrin-mediated pathway (supplemental Fig. S6, A and B) (32, 33). To further establish that SITS treatment did not induce a general block on receptor internalization in MDCK cells, we examined EGFR-induced down-regulation of the EGFR, which internalizes via the clathrin-mediated pathway (49), but does not promote dorsal ruffle formation in MDCK cells (supplemental Fig. S6C). EGFR internalization and degradation in response to EGF was unaffected in M-GFP-Gab1 cells pre-treated with SITS (supplemental Fig. S6, D and E). Hence, the inhibitory effect of SITS on dorsal ruffle formation and Met degradation is not as a result of a block in clathrin-dependent receptor internalization.

To confirm that efficient Met receptor degradation is coupled to the formation of dorsal ruffles, we evaluated Met degradation in MDCK cells where dorsal ruffle formation was inhibited independently of SITS treatment, through expression of a Gab1 mutant. For this we made use of MDCK cells lines stably expressing a Gab1 mutant uncoupled from the Nck and Crk adaptor molecules, through six tyrosine to phenylalanine substitutions, which is unable to promote dorsal ruffles in of increased Met ubiquitination (Fig. 4F). Collectively, these data support a more rapid degradation of the Met RTK in cells that form elevated levels of dorsal ruffles in response to HGF.

Pak1-enhanced Dorsal Ruffle Formation Leads to Enhanced Met RTK Degradation—Our data supports the hypothesis that dorsal ruffles provide a mechanism for bulk internalization and subsequently efficient degradation of the Met RTK. To test if the more efficient early degradation of Met is coupled to the formation of dorsal ruffles and not a result of overexpression of Gab1, we evaluated Met degradation in HeLa cells, where dorsal ruffle formation was enhanced by Pak1 overexpression. A Pak1 mutant, which is impaired in its autoinhibitory ability (His83-Leu86), promotes robust dorsal ruffle formation in response to PDGF (40). When expressed in HeLa cells, Pak1 His83-Leu86 enhances dorsal ruffle formation in response to HGF (Fig. 5A). Immunostaining for endogenous Met revealed that Met was present on the dorsal ruffle membrane (Fig. 5B). Consistent with dorsal ruffle formation contributing to enhanced Met internalization, Met was more rapidly degraded in Pak1 His83-Leu86 expressing cells, which form enhanced dorsal ruffles in response to HGF, when compared with vector control cells (Fig. 5, C and D). Taken together, these data support that enhanced induction of dorsal ruffles per se, in an HGF-dependent manner, promotes efficient Met receptor degradation.
FIGURE 6. Disruption of dorsal ruffles delays Met receptor down-regulation. A, M-GFP-Gab1 cells were pre-treated with cycloheximide for 2 h and then 20 min prior to stimulation with 0.5 nM HGF, were also incubated with DMSO or 0.5 mM SITS. Cells were then fixed and stained for 4’,6-diamidino-2-phenylindole (blue). B, the number of cells forming dorsal ruffles was scored from 10 fields of view from A and represented as the percentage of cells forming dorsal ruffles in the presence of DMSO or SITS treatment ± S.E. from three independent experiments. C, M-GFP-Gab1 cells were pre-treated with DMSO or 0.5 mM SITS and stimulated with 0.5 nM HGF for the indicated times. Proteins from cell extracts were separated by SDS-PAGE and immunoblotted for endogenous Met receptor, pY1234/35 Met, and actin. D, densitometric analysis of Met degradation from C presented as percentage of initial receptor remaining after HGF stimulation, ± S.E. from three independent experiments. E, M-GFP-Gab1 cells were pre-treated with DMSO or 0.5 mM SITS and stimulated with 0.5 nM HGF for the indicated time points, fixed, and stained for Met (red) and EEA1 (green). Dorsal ruffles are indicated by arrows and peripheral ruffles by arrowheads. F, M-HA-Gab1 and M-HA-Gab1ΔDR cell lines were pre-treated with cycloheximide for 2 h, stimulated or not with 0.5 nM HGF for 3 min, fixed, and stained for HA. Representative images of M-HA-Gab1 and M-HA-Gab1ΔDR clone D are shown. G, the percentage of cells from F forming dorsal ruffles was calculated and represented as the mean ± S.E. of three independent experiments. H, cells from F were stimulated with 0.5 nM HGF in the presence of cycloheximide for the indicated time points. Densitometric analysis of Met levels (see immunoblots in supplemental Fig. S6F) are presented as percentage of initial receptor remaining after HGF stimulation ± S.E. from three independent experiments. All scale bars represent 10 μm.
response to HGF, (MDCK-ΔDR, 3 cell lines; Fig. 6, F and G) (27). Examination of three independent clones of cells expressing MDCK-ΔDR demonstrated that the initial rate of Met receptor degradation is significantly delayed when compared with MDCK cells overexpressing wild type Gab1 (M-HA-Gab1 cells) (Fig. 6H and supplemental Fig. S6F). Under steady state conditions, whereas Met levels decreased by 60% within the first 30 min of HGF stimulation in M-HA-Gab1 cells, Met levels decreased by only 30% in MDCK-ΔDR cells (Fig. 6H and supplemental Fig. S6F). Altogether, these data support that Met down-regulation in MDCK cells is more efficient following bulk internalization from dorsal ruffles.

**DISCUSSION**

In response to multiple growth factors, cells remodel their plasma membrane through formation of dorsal and peripheral ruffles. Dorsal ruffles have been well documented, yet their function is still poorly understood. In this study, we demonstrate that dorsal ruffles act as a spatially restricted microdomain involved in Met receptor signaling, and serve as a platform for bulk Met receptor internalization and subsequent degradation.

In colonies of MDCK cells stimulated with HGF, an active, tyrosine-phosphorylated Met RTK localizes to newly forming dorsal ruffles and remains within the dorsal ruffle for its duration (8–10 min; see Figs. 1 and 2). During this time period, dorsal ruffles are also enriched in the Met substrate, the Gab1 scaffold protein (Fig. 2) (27), which is essential for Met signaling and biological responses (50) as well as downstream signaling protein ERK1/2 (Fig. 1D and supplemental Fig. S2B). Hence, dorsal ruffles provide a prolonged but localized plasma membrane microdomain for the Met RTK. This prolonged signaling microdomain in MDCK cells is in contrast to cells where the Met RTK is rapidly endocytosed (32) and where sustained Met signaling is associated with endosome-localized complexes (29, 31). Notably, in contrast to phosphatidylinositol 1,4,5-trisphosphate-enriched endosomal signaling microenvironment (2), dorsal ruffles represent a large domain rich in phosphatidylinositol 1,4,5-trisphosphate within the apical plasma membrane (51). Hence, dorsal ruffles have potential to concentrate a distinct subset of signaling complexes. Indeed, the Gab1 scaffold predominantly localizes with Met in dorsal ruffles (Figs. 1B and 2B), reflecting the affinity of the Gab1-PH domain for phosphatidylinositol 1,4,5-trisphosphate (52). In addition to Gab1 and ERK1/2, localization of other signaling molecules to dorsal ruffles, such as Abl, Abi, Eps8, and EGF RTK in response to EGF, as well as Src, Pak1, and Cbl in response to PDGFbb, has been reported (18). Moreover, we have previously shown that inhibition of dorsal ruffle formation in MDCK cells correlates with an inability of HGF to induce a morphogenic program, suggesting that signals emanating from this compartment are critical (27). Altogether, these data support that dorsal ruffles reflect a specialized signaling microdomain downstream from multiple extracellular signals.

In apparent contradiction to positively regulating Met signaling, dorsal ruffles also represent a mechanism for efficient Met RTK down-regulation. In MDCK or HeLa cells, when dorsal ruffle formation is enhanced by expression of Gab1 or Pak1, Met is more rapidly degraded (see Figs. 3–5). Moreover, disruption of dorsal ruffle formation through treatment with SITS or expression of a Gab1 mutant that blocks dorsal ruffle formation (27) resulted in a delay in Met degradation (Fig. 6). Hence, dorsal ruffles constitute a membrane microdomain where the Met RTKs accumulate and signal, but also represent a platform from
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which receptors subsequently internalize and are targeted for degradation. The EGFR is also recruited into dorsal ruffles in response to EGF in the pancreatic tumor cell line, Panc1, and is subsequently internalized into the cell upon collapse of the ruffle (16). Significant amounts of EGFR have been reported to be lost from the plasma membrane upon dorsal ruffle formation, in agreement with our findings that the Met receptor is efficiently cleared through this mechanism.

As previously observed in HeLa cells, where Met traffics to a degradative compartment (32), the ligand-dependent loss of Met from dorsal ruffles in MDCK cells and in Gab1 overexpressing cells was dependent on both the lysosome and the proteasome (Fig. 3E). This implicates the canonical pathway where sorting of ubiquitinated RTKs for degradation in the lysosome is dependent on both lysosomal and proteasomal functions (53). Ligand-dependent ubiquitination of Met is an important signal recognized by the ESCRT protein Hrs (29, 31), targeting Met for internalization into multivesicular bodies and subsequent degradation in the lysosome (54). No increase in Met ubiquitination was observed in cells overexpressing Gab1, where enhanced bulk degradation of Met correlated with enhanced formation of dorsal ruffles. Hence, ubiquitination of Met was not a signal for the more rapid degradation of Met (Fig. 4F). Instead, these data support bulk internalization of Met as the dorsal ruffle collapses, and entry into the canonical degradative pathway (Rab5 and Hrs, Figs. 2 and 7) leads to a more synchronous degradation of the Met RTK.

Because Met is normally localized to basolateral membranes in polarized monolayers of MDCK cells (44) (supplemental Fig. S1), recruitment of Met into dorsal ruffles within minutes of HGF stimulation requires that Met is internalized and recycled to the apical membrane (Fig. 7). In support of this hypothesis, small interfering RNA depletion of clathrin, blocking Met internalization, prevents HGF-induced dorsal ruffle formation (supplemental Fig. S1B). In addition, Rab4, which can mediate recycling to apical membranes from early endosomes in MDCK cells (46), localizes with Met prior to and within dorsal ruffles (supplemental Fig. S1, C and D). These data are consistent with a previous report demonstrating that HGF-dependent dorsal ruffle formation requires transferrin-positive, Rab5 endosomes, for delivery of activated Rac to the plasma membrane (28). Although we did not observe Rab5 localization to dorsal ruffles in MDCK cells (supplemental Fig. S3A), our work would support a requirement for the early recycling pathway, to transcytose Met from basolateral membranes (Fig. 7).

Based on our findings with Met, the promotion of dorsal ruffles downstream from EGF would also be predicted to enhance EGFR degradation, although this has yet to be demonstrated. Internalization of the TrkB RTK and interleukin-2 receptors also occurs through membrane protrusions morphologically similar to dorsal ruffles (13, 55). Hence, dorsal ruffles increasingly represent a common mode for internalization and subsequent entry into the endosomal compartment for several types of membrane receptors.

As the balance of RTK activation and degradation is critical for normal physiology, a full understanding of the molecular events that control these processes is essential. We have demonstrated that dorsal ruffles function as both positive regulators of RTK signaling and paradoxically act as an internalization portal that leads to efficient Met RTK degradation. Future studies to determine how dorsal ruffles regulate signal specificity and whether these structures bear a specific receptor sorting function, akin to that which occurs on sorting endosomes, will be required to determine physiological roles of these polarized membrane protrusions.

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