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Spatial Regulation of Signaling by the Coordinated Action of the Protein Tyrosine Kinases MET and FER

Jiali Zhang[1], **Zuo Wang**[1], **Siwei Zhang**[2,3], **Yanxun Chen**[4], **Xuexue Xiong**[1], **Xiaojuan Li**[1], **Nicholas K. Tonks**[2,*], and **Gaofeng Fan**[1,2,*]

[1]School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China;

[2]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA;

[3]Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, New York 11794, USA;

[4]First Affiliated Hospital, Fujian Medical University, Fuzhou 350005, China.

Abstract

A critical aspect of understanding the regulation of signal transduction is not only to identify the protein-protein interactions that govern assembly of signaling pathways, but also to understand how those pathways are regulated in time and space. In this report, we have applied both gain-of-function and loss-of-function analyses to assess the role of the non-receptor protein tyrosine kinase FER in activation of the HGF Receptor protein tyrosine kinase MET. Overexpression of FER led to direct phosphorylation of several signaling sites in MET, including Tyr1349, but not the activation loop residues Tyr1234/5; in contrast, suppression of FER by RNAi revealed that phosphorylation of both a C-terminal signaling site (Tyr1349) and the activation loop (Tyr1234/5) were influenced by the function of this kinase. Adaptin β, a component of the adaptor protein complex 2 (AP-2) that links clathrin to receptors in coated vesicles, was recruited to MET following FER-mediated phosphorylation. Furthermore, we provide evidence to support a role of FER in maintaining plasma membrane distribution of MET and thereby delaying protein-tyrosine phosphatase PTP1B-mediated inactivation of the receptor. Simultaneous up-regulation of FER and down-regulation of PTP1B observed in ovarian carcinoma-derived cell lines would be expected to contribute to persistent activation of HGF-MET signaling, suggesting that targeting of both FER and MET may be an effective strategy for therapeutic intervention in ovarian cancer.

Keywords

FER; MET; PTP1B; endocytosis; ovarian cancer

[*]To whom correspondence should be addressed. fangf@shanghaitech.edu.cn and tonks@cshl.edu.

Author contribution

GF and NKT designed the experiments. JZ, ZW, SZ, YC, XX, XL and GF performed the experiments. GF and NKT analyzed the data and wrote the paper.

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Introduction

Signal transduction is the process by which cells recognize stimuli in their environment and translate them into a response, often involving a change in gene expression in the nucleus. Extracellular stimuli, which include growth factors and hormones, engage receptors on the surface of the cell and trigger a series of reversible protein phosphorylation events that facilitate the formation of protein complexes that underlie signal transmission. For example, tyrosine phosphorylation creates binding sites for proteins with SH2 domains, which serve as protein interaction modules that drive complex formation. Although initially viewed as linear pathways, it is now clear that multiple signals are integrated into response networks, introducing considerable complexity [1]. Furthermore, it is now apparent also that there are spatial and temporal aspects to the regulation of a signaling response. Thus, where and when proteins interact is also an important determinant of an appropriate signaling outcome [1].

Since the discovery of epidermal growth factor receptor (EGFR) as the first receptor protein tyrosine kinase (RTK), the number of such receptor kinases has expanded [2]. In total, 59 out of the 90 protein tyrosine kinase genes in the human genome encode RTKs. These cell surface receptors, in concert with the spectrum of downstream signaling components, control proliferation, survival, differentiation and migration in response to environmental cues [3]. Considering that aberrant activation of RTK gene products has been established as an important component of the etiology of many major diseases, including cancer, there is an urgent need to understand fully the molecular basis by which they control signaling outcome.

The hepatocyte growth factor (HGF) and its cognate receptor, hepatocyte growth factor receptor HGFR/MET, represent an excellent exemplar through which to study the complexity of this regulation. MET is synthesized as a single-chain precursor (~170 kDa) that undergoes furin-mediated post-translational cleavage during maturation. The mature MET protein comprises an extracellular α subunit (45 kDa) and a transmembrane β subunit (140 kDa) linked by a disulphide bond [4]. Upon HGF stimulation, MET undergoes dimerization and autophosphorylation in its activation loop (Tyr1234 and Tyr1235), which leads to enhanced kinase activity and further autophosphorylation of Tyr1349 and 1356 in the C terminus of the receptor protein. This tyrosine phosphorylation creates docking sites for recruiting SH2 or PH domain-containing adaptor proteins, such as GRB2, GAB1 and SHC, and assembles downstream signaling components to activate RAS–MAPK, PI3K–AKT, and STAT3 signaling pathways [5, 6]. Recently, we identified an alternative, HGF-independent pathway for activation of MET, through a non-receptor protein tyrosine kinase (PTK), the feline sarcoma-related kinase FER [7]. We demonstrated that FER could phosphorylate Tyr1349 of MET directly and promote a kinase-independent scaffolding function of the receptor. This led to recruitment and phosphorylation of GAB1 and the specific activation of the SHP2–ERK signaling pathway in an ovarian cancer cell setting [7]. Overexpression of MET is a feature of several cancer cells [6, 8, 9]. These findings suggest that targeting MET alone in such a context may not be sufficient to abolish survival cues and may contribute to the lack of success in using MET inhibitors as single agents in clinical trials for treatment of ovarian and gastric cancers.

Following ligand stimulation, RTKs, including HGFR/MET, undergo internalization through clathrin-dependent or -independent mechanisms [5, 10–12]. After entering the cell, the receptor cargos are sorted in early endosomes; recycling to the plasma membrane facilitates further involvement in the signaling response, whereas those receptors destined for degradation are sorted into late endosomes/lysosomes [13]. Considerable progress has been made in elucidating molecular insights into the pathway of degradation utilized by HGFR/MET [14–16]; however, the mechanisms that coordinate recycling pathways of this RTK are less well understood.

In this current study, we have used ovarian carcinoma-derived cell lines as a model and illustrated a new tier of regulation of MET that is exerted through the function of the kinase FER. We have shown that adaptin β, one of the key components in the adaptor protein complex 2 (AP-2) that serves to link clathrin to receptors in coated vesicles, was recruited to MET upon FER-mediated phosphorylation. Furthermore, we demonstrated a novel function of FER in potentiating kinase activity of HGFR/MET by enhancing its membrane distribution, thereby avoiding protein-tyrosine phosphatase PTP1B mediated inactivation of the receptor. Collectively, these data illustrate a role of FER as a positive regulator of the RTK HGFR/MET in the context of ovarian cancer cells.

Methods

Cell Culture and Reagents

Conditions for culturing the ovarian cancer cell lines used in the present study are provided in [17]. Human recombinant HGF was from R&D systems. Cyclohexamide and Dynasore were from Sigma.

Plasmids and transfection

Mammalian expression plasmids used in this study were: FER WT, K592R and SH2 mutant (pMSCV-FER-Hygro), MET WT and mATP mutant (pXM), PTP1B WT and D181A (pMT2), PTP1B WT, D181A and C215S (pWZL-Hygro). Transient transfection followed the Mirus manufacturer protocol (Transit-2020, Mirus). In brief, 293T cells were seeded in a 6-well plate one day before transfection. Plasmid (2 μg) and Mirus transfection reagent (5 μL) were added to each well. Whole-cell lysates were collected 24 hr after transfection for either immunoprecipitation or immunoblotting.

Site-directed mutagenesis

Site-directed mutagenesis was performed following the Agilent protocol. Primers used in this study are summarized in Table S1.

Immunoblotting, Immunoprecipitation and GST Pull-down Assay

Cells were lysed in either 1% NP40 lysis buffer (20mM Hepes pH7.5, 150mM NaCl, 1%NP40, 50mM NaF, 1mM Na_3VO_4, 10% glycerol, protease inhibitor cocktail from Roche) or modified RIPA buffer (50mM Tris-HCl pH7.4, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 50mM NaF, 1mM Na_3VO_4, 10% glycerol, protease inhibitor cocktail from Roche) at 4 ºC for 30 minutes. Total protein concentration was determined by

the Bradford assay. The primary antibodies used were as follows: p-Tyr (4G10) and pY402 FER (Millipore); PTP1B (FG6); FER, pErk½, Erk½, pY1234/1235 and pY1349 MET, MET, SRC, PLCγ1, pY580 and total SHP2 (Cell Signaling Technology); Rab5 (BD Bioscience); PAG (R&D systems); BRK (Santa Cruz Biotechnology); β-tubulin and β-actin (Sigma).

Precleared cell extracts were incubated with the indicated antibody for 4 h in a cold room with rotation followed by 1 h of pull-down by 1:1 protein A/G agarose beads. Immunoprecipitates were washed with lysis buffer three times before electrophoresis.

Immunofluorescence

Cells were fixed for 15 minutes in 3.6% formaldehyde diluted in PBS. After washing with PBS, the cells were incubated in 100% ice-cold methanol at −20ºC for 10 minutes. Following fixation, cells were incubated in blocking solution (5% goat serum and 0.3% Triton-X100 in PBS) at room temperature for 1 hour and were then incubated with primary antibodies diluted in blocking solution at 4ºC overnight. Following washing with PBS, the cells were stained at room temperature for 1 hour with secondary antibodies diluted in blocking solution. The nucleus of the cells was highlighted with DAPI. The cells were mounted in Prolong Antifade (Molecular Probe, Invitrogen). The primary antibodies used were: MET (#8494, CST), FER (#4268, CST), RAB11 (#3539, CST), Adaptinβ (#610381, BD), EEA1 (#610456, BD), and GRB2 (#G16720, Transduction Lab)

RNAi

Lentiviral infection of shRNA, followed by puromycin selection, was used to suppress FER in CAOV4 cells. Specifically, lentivirus expressing targeted shRNA was generated in 293T cells by co-transfecting shRNA vector, deltaR8.91, and VSVG at a ratio of 3:2:1. Twenty-four hours after transfection, viral particles were harvested and passed through a 0.45-μm filter to remove cell debris. The cleared supernatants were then incubated with recipient cells in the presence of Polybrene (8 μg/ml). Twenty-four hours after infection, cells were placed under puromycin selection (2 microgram/ml), and the effectiveness of infection was further confirmed by immunoblotting with indicated antibody. FER shRNA#1 seq: 5’-GCAGAAAGTTTGCAAGTAATG-3’; FER shRNA#2 seq: 5’-GCCAAGGAACGATACGACAAA-3’

Membrane fractionation extraction

We used a Subcellular Protein Fractionation Kit for Cultured Cells (#78840, Pierce) to extract membrane compartments of CAOV4 shCon and shFER cells, following the Manufacturer’s protocol.

Sucrose gradient ultracentrifugation

Endocytic vesicles were isolated by density gradient centrifugation [18]. Briefly, the cell pellet was harvested, and suspended in three times the pellet volume of homogenization buffer (HB: 250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, ddH2O, protease and phosphatase inhibitors). The suspension was passed through a syringe (1ml syringe with a 22-G needle) 3–10 times. HB buffer (700 μl) was added to each 1 ml of homogenized cells, followed by centrifugation at 2,000 x g for 10 min at 4 °C. The post-nuclear supernatant

(PNS) was retrieved following fractionation. The sucrose concentration of the PNS was adjusted to 40.6% by adding 62% sucrose (usually 1:1.2 v/v). The diluted PNS was loaded on the bottom of an ultracentrifuge tube, then overlaid sequentially with 1.5 vol of 35% sucrose, 1 vol of 25% sucrose and, finally, the rest of the tube was filled with HB. The interphases were marked, and the samples were centrifuged at 210,000 x g at 4 °C for 1.5 h. After centrifugation, a milky band of membrane particles was detected at each interphase. These were collected, frozen in liquid nitrogen and stored at −80 °C.

Preparation of Lipid Raft Fraction

CAOV4 cells were lysed in homogenization buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM 2-mercaptoethanol) supplemented with 0.35% Triton X-100 and subjected to ultracentrifugation on a discontinuous sucrose density gradient (4 ml; 40% to 35% to 5%). The raft fraction was solubilized with ODG buffer (homogenization buffer plus 1.5% octyl-D-glucoside and 1% Nonidet P-40).

Results

FER-mediated phosphorylation of multiple tyrosine residues in MET/HGFR.

Our previous work established a novel ligand- and autophosphorylation-independent activation of MET, the hepatocyte growth factor receptor (HGFR), by the non-receptor PTK FER [7]. To define further this mechanism of activation, we examined the tyrosine phosphorylation of MET/HGFR that was promoted by FER following overexpression in HEK 293T cells. We used PhosphoSitePlus (https://www.phosphosite.org/home) to select key potential sites of tyrosine phosphorylation within the cytoplasmic segment of MET/HGFR (Fig 1A). To avoid interference from autophosphorylation, we used a kinase-dead form of MET (mATP-MET), in which the K1003R inactivating mutation was introduced into the ATP-binding pocket. Whereas ectopic expression of wild-type MET resulted in robust autophosphorylation (on Tyr1234/5 and 1349) in HEK 239T cells, essentially no phosphorylation signal was detected in cells in which the mATP mutant MET was overexpressed alone (Fig 1B). Upon co-expression of mATP-mutant MET with wild-type FER, we did not detect phosphorylation of the activation loop Tyr residues 1234 and 1235, however, phosphorylation of the C-terminal Tyr1349 in mATP-MET was observed. This was not detected following co-expression of kinase-dead (FER K592R) or SH2 mutant forms of FER.

In addition, we generated Tyr to Phe mutations for residues 1349 and 1356 within the mATP-MET backbone, and applied anti-MET immunoprecipitation followed by pTyr (4G10) blotting to assess the effects on tyrosine phosphorylation (Fig 1C). Compared to mATP-MET, the Tyr to Phe mutation at residue 1356 resulted in a decrease in tyrosine phosphorylation of the receptor, suggesting both Tyr 1349 and 1356 were substrates of FER kinase. Interestingly, the Tyr 1349/1356 to Phe double mutation did not ablate tyrosine phosphorylation of the mutant protein completely, indicating that there may be other tyrosine residue(s) that could be phosphorylated by FER.

We applied a similar strategy to establish a series of Tyr to Phe mutants of mATP-MET and test the effects of these mutations for other potential phosphorylation sites (Fig 1A and 1D, in particular Tyr 971, 1003, 1026, 1093). Interestingly, phosphorylation of the Y1026F MET mutant was also decreased in the presence of FER when compared to mATP-MET control (Fig 1D). In light of this, we generated a triple mutant (Tyr 1026, 1349, 1356 Phe) form of mATP-MET and observed that its phosphorylation by FER was profoundly diminished compared to single and double mutants (Fig 1E), suggesting that Tyr 1026, 1349 and 1356 are three major residues within MET subjected to FER phosphorylation.

FER knockdown in ovarian cancer cells led to decreased tyrosine phosphorylation of the activation loop of MET.

To pursue further the role of the kinase FER in phosphorylating MET, we generated FER-deficient ovarian cancer cells by shRNA knockdown. Following lentivirus infection, stable CAOV4 cell lines were established successfully with either shControl or two distinct shRNAs targeting FER, in which the knockdown efficiency was ~ 90% and 70% (Fig 2A). We observed decreased tyrosine phosphorylation of the C-terminal docking site (Tyr1349) of MET (Fig 2B), which was consistent with our overexpression studies in HEK 293T cells. Surprisingly, in contrast to the HEK 293 cell study (Fig. 1), we observed also that suppression of FER led to an even more profound decrease in phosphorylation of Tyr1234 and 1235 in the activation loop of MET (Fig 2A). In addition, when we serum starved both shCon and shFER cells overnight, then re-stimulated them with HGF, the ligand for MET, we observed also that the tyrosine phosphorylation status of MET at Tyr1234/1235 remained suppressed (Fig 2B), suggesting a previously undefined role of the kinase FER in regulating activity status of the HGF receptor, MET.

FER mediated recruitment of GRB2 and Adaptin β to MET/HGFR.

The contrast between the effects of FER that were manifested by overexpression and those seen following RNAi-based suppression prompted us to test further mechanisms by which FER may modulate the activity of MET. Using immunofluorescence, we observed co-localization of wild-type FER and mATP-mutant form of MET (Fig 3A). Also, as expected, we observed that wild-type, but not kinase-dead (K592R), FER triggered phosphorylation-dependent recruitment of GRB2 to mATP-MET (Fig 3B). In addition, Adaptin β, one of the key components of the adaptor protein complex 2 (AP-2), which serves to link clathrin to receptors in coated vesicles, was recruited to mATP-MET in the presence of wild-type FER (Fig 3C). This result suggests that FER may play a role in regulating the endocytosis of MET.

Colocalization of FER and MET in endocytic vesicles.

Considering our observation that FER facilitated recruitment of Adaptin β to mATP-MET, together with the unique ability of FER to bind lipid [19], we investigated the function of this kinase in modulating endocytosis of MET. We performed ultracentrifugation on sucrose gradients to analyze the cellular distribution of FER kinase in CAOV4 ovarian cancer cells. In total, 11 fractions were collected from the top to the bottom of the ultracentrifuge tube, then analyzed by SDS-PAGE. It is known that sucrose gradient ultracentrifugation can resolve lipid raft and cytosol fractions. As expected, PAG, a marker protein for lipid rafts

[20, 21], was detected in the early fractions, whereas the cytosolic proteins BRK and PLCγ1 [22] were both detected in later fractions (Fig 4A). FER and MET, which displayed a similar distribution, were detected in later fractions. We performed another round of ultracentrifugation to collect early endosome, late endosome and lysosome fractions (Fig 4B). Among these three compartments, the majority of MET and FER was detected in Rab5-containing early endosomes, indicative of endocytosis. Interestingly, we observed that MET co-localized with EEA1, a marker for early endosomes in CAOV4 cells; in contrast, this co-localization was disrupted following suppression of FER (Fig 4C). Compared to FER-deficient cells, we detected also a greater proportion of MET co-localized with Rab11, a marker of recycling endosomes, in cells expressing FER (Fig 4C).

These changes in localization were not associated with a change in half-life of MET in the presence, compared to the absence of FER (Fig 4D). To examine this point further, we extracted the membrane fraction from CAOV4 cells expressing either shCon or shFER and compared the levels of MET in each. Consistent with our immunofluorescence data, we observed that in the absence of FER the levels of MET associated with the membrane fraction were dramatically decreased (Fig. 4E). Although there was no change of MET expression overall in whole cell lysates, cells with control shRNA had higher levels of MET within the membrane fraction, suggesting a heretofore-unknown role of FER in maintaining the membrane distribution of MET.

Dephosphorylation of MET and FER by the protein tyrosine phosphatase PTP1B.

It has been reported that the protein tyrosine phosphatase PTP1B could inactivate MET by direct dephosphorylation of Tyr 1234 and 1235 in its activation loop, and that this dephosphorylation takes place in peri-nuclear region of the cell [23]. Consistent with these previous findings, we observed that co-expression of PTP1B dramatically decreased total tyrosine phosphorylation of MET compared to HEK 293T cells expressing MET alone (Fig. 5A). These effects were only observed following co-expression with wild-type, but not the substrate trapping mutant form of PTP1B (Fig. 5B). Furthermore, the substrate-trapping mutant form of PTP1B, D181A, formed a stable complex with MET, suggesting direct binding and dephosphorylation (Fig. 5B). Finally, ectopic expression of wild-type PTP1B, but not the inactive C215S mutant, in CAOV4 cells decreased the activation of MET (Fig. 5C), indicative of negative regulation of MET by the phosphatase.

Interestingly, our data suggest that FER was also a substrate of PTP1B. We observed a decrease in total tyrosine phosphorylation of FER in HEK 293T cells following co-expression of wild-type PTP1B (Fig 5D). The substrate-trapping mutant PTP1B-D181A formed a stable complex with FER in HEK 293T cells and use of phosphosite-specific antibodies suggested that PTP1B exerted effects on Tyr 402 of FER (Fig 5E). Furthermore, expression of wild-type PTP1B led to suppression of FER phosphorylation and PTP1B-D181A mutant formed a complex with FER in CAOV4 ovarian cancer cells (Fig 5F). These results demonstrate both MET and FER were substrates of PTP1B, which, combined with the data from Fig 4, led us to hypothesize that FER may potentiate the duration of MET activation by maintaining its cell surface distribution, therefore delaying the termination of its signaling function by PTP1B.

Dynasore, an inhibitor of Dynamin, led to enhanced MET activation in ovarian cancer cells.

Given the essential role of endosomal signaling in modulating the oncogencity of MET [24], as well as the fact that the unique N-terminal F-BAR and FX domains of FER have been implicated in regulation of vesicular trafficking [19, 25], we investigated further a potential role of FER in regulating endocytosis of MET. We pre-treated CAOV4 cells with Dynasore, an inhibitor of Dynamin, to impair endocytosis [26], and assessed the activation status of MET following HGF stimulation. Loss of FER had an inhibitory effect on MET activation (Fig. 2) despite the fact that FER did not phosphorylate the tyrosine residues in the activation loop of MET (Fig. 1B). As shown in Figure 6, we observed that treatment with Dynasore, a cell-permeable inhibitor of dynamin, led to an increase in MET activation in a dose-dependent manner, supporting the notion that sustained membrane distribution of receptor kinase positively correlates with its activity. Collectively, these data suggest a signal promoting role for FER in maintaining plasma membrane distribution of MET.

Expression of PTP1B was attenuated in ovarian tumor-derived cancer cells.

Consistent with a role of PTP1B as a negative regulator of MET and FER, we previously noted that the levels of the phosphatase were decreased in ovarian cancer cells [17, 27]. This prompted us to evaluate further the expression of PTP1B in a panel of ovarian cancer cell lines. We extracted protein lysates from both human ovarian surface epithelial (HOSE) cell lines and eleven ovarian carcinoma-derived cell lines, and compared the expression of PTP1B by immunoblotting. We observed ubiquitously low expression of PTP1B in all eleven ovarian carcinoma-derived cell lines relative to HOSE controls (Fig 7A). Furthermore, we searched the Oncomine database to evaluate the mRNA expression pattern of PTP1B in samples from human patients and found that the expression of PTP1B was consistently down-regulated in multiple subgroups of ovarian cancer patients compared to normal controls (Fig 7B).

Discussion

A major focus of research on the signaling function of RTKs, including the HGF receptor MET, has been to characterize their kinase activity, which is induced following ligand binding. In this way, the direct effects of the RTK on substrate phosphorylation can be assessed and their functional significance determined. In this report, we have characterized an indirect effect of FER in potentiating the kinase activity of HGFR/MET by enhancing its membrane distribution (Fig 4), thereby avoiding protein-tyrosine phosphatase PTP1B-mediated dephosphorylation and inactivation (Fig 5). This illustrates a new aspect of the control of HGFR/MET function. It is interesting to note that both up-regulation of FER and down-regulation of PTP1B (Fig 7) has been detected in ovarian carcinoma-derived cell lines [7]. Therefore, one would anticipate that this mechanism may underlie persistent activation of HGF-MET signaling in this disease, and perhaps may present new opportunities for therapeutic intervention.

FER, as well as FES, the other member of this kinase family, distinguish themselves from other protein tyrosine kinases by their N-terminal phospholipid-binding and membrane targeting FER/CIP4 homology/Bin1/Amphiphysin/RVS (F-BAR) and F-BAR extension

(FX) domains, which are thought to function in vesicular trafficking and endocytosis [19, 25]. Consistent with this, FER preferentially binds to phosphatidic acid (PA), and lipid binding leads to enhanced kinase activity [28]. Using immunofluorescence staining, we observed recruitment of Adaptin β to the mATP mutant of MET in the presence of FER kinase (Figure 3C). Furthermore, in the absence of FER, the route for endocytosis of MET, in terms of internalization and membrane recycling, was altered (Fig 4C and 4E). This is consistent with a critical function of FER in trafficking of MET within cells. Similarly, Greer's lab demonstrated that FER deficiency was associated with increased rates of epidermal growth factor (EGF)-induced epidermal growth factor receptor (EGFR) internalization [29].

The precise control of RTK endocytosis is key to regulating the activity of the receptor. In the case of MET, Tyr1003 represents a CBL-binding site, phosphorylation of which leads to its recognition by the ESCRT-0 subunit HRS, increasing entry of MET into Multi-Vesicular Bodies, thereby decreasing MET stability and activation [30]. Interestingly in lung and gastric cancers, an alternatively spliced variant of MET has been reported, in which exon 14, which encodes the segment of the protein containing Tyr1003, has been deleted [31, 32]. These MET mutants display elevated stability and oncogenic capacity, emphasizing the potential importance of MET ubiquitination *in vivo* to suppress transforming activity. In our studies, we did not observe a change in the stability of MET in the presence and absence of FER (Fig 4D) and the phosphorylation of Tyr1003 in MET was not altered by FER (Fig 1). In addition, two oncogenic mutant forms of MET, which displayed increased internalization and recycling activity, were identified in human papillary carcinomas [24]. These mutants, D1246N and M1268T, are distinct from those that are defective in CBL binding. Although we also observed alteration to the entry and recycling steps of MET endocytosis in ovarian cancer cells that lack FER, further study will be needed to determine whether there is a common regulatory mechanism.

Our studies emphasize the potential importance of sequestering MET from the effects of PTP1B as a mechanism for activation of the RTK. Interestingly, it was reported from a SILAC (Stable Isotope Labeling by Amino acids in Cell culture) quantitative mass spectrometry screen that of 13 proteins that were hyperphosphorylated in PTP1B-deficient fibroblasts under basal cell culture conditions, one was FER [33]. Using our substrate trapping strategy, we verified both MET (Tyr1234/5) and FER (Tyr402) as bona fide substrates of PTP1B (Fig 5). Furthermore, ectopic expression of PTP1B in ovarian carcinoma-derived cells inhibited the activation of both kinases (Fig 5), consistent with a negative regulatory role of the phosphatase. Similar negative regulation of MET by PTP1B has been reported in glioblastoma multiforme (GBM) [34].

Overexpression of HGFR/MET has been detected in ~ 60% of ovarian cancer patients [35, 36], and this up-regulation correlates with poor prognosis [37, 38]. Targeting c-MET by RNAi in ovarian cancer cell lines inhibited adhesion, invasion, peritoneal dissemination and tumor growth, which supported the idea that HGFR/MET inhibitors may have utility as anticancer therapeutics [38]. Small molecule inhibitors of MET, including tivantinib (ARQ197) and cabozantinib (XL184), as well as the HGF-blockade monoclonal antibody rilotumumab (AMG-102), have entered clinical trials in ovarian cancer patients.

Combinations of tivantinib and either gemcitabine [39] or temsirolimus (an mTOR inhibitor) [40] showed a promising partial response and/or stable disease in ovarian patients with advanced disease. Nevertheless, in several phase II trials rilotumumab showed no, or limited, efficacy in patients with ovarian cancer, metastatic renal cell carcinoma (RCC), castration-resistant prostate cancer (CRPC) or recurrent glioblastoma [41]. These data emphasize the importance of a better understanding of the regulation of HGF-MET signaling, with the possibility of harnessing that knowledge to develop/optimize a more effective strategy for treating ovarian cancer. Combined with our previous findings, we have proposed a dual function of FER in positive regulation of MET signaling in ovarian cancer cells (Fig 7C). When HGF is limited, FER associates with MET, and directly phosphorylates MET, GAB1, and possibly SHP2. This results in the activation of SHP2–MAPK and RAC1–PAK1 signaling downstream from MET, which contributes to the motility and invasiveness of ovarian cancer cells [7]. Now our data illustrate that in the presence of HGF, FER may influence the route of endocytosis of MET. FER may facilitate the recycling and re-activation of MET in plasma membrane, protecting it from dephosphorylation and inactivation by ER-anchored PTP1B. Consequently, simultaneous inhibition of both FER and MET may suppress kinase activity more effectively and offer an improved strategy for therapeutic intervention in ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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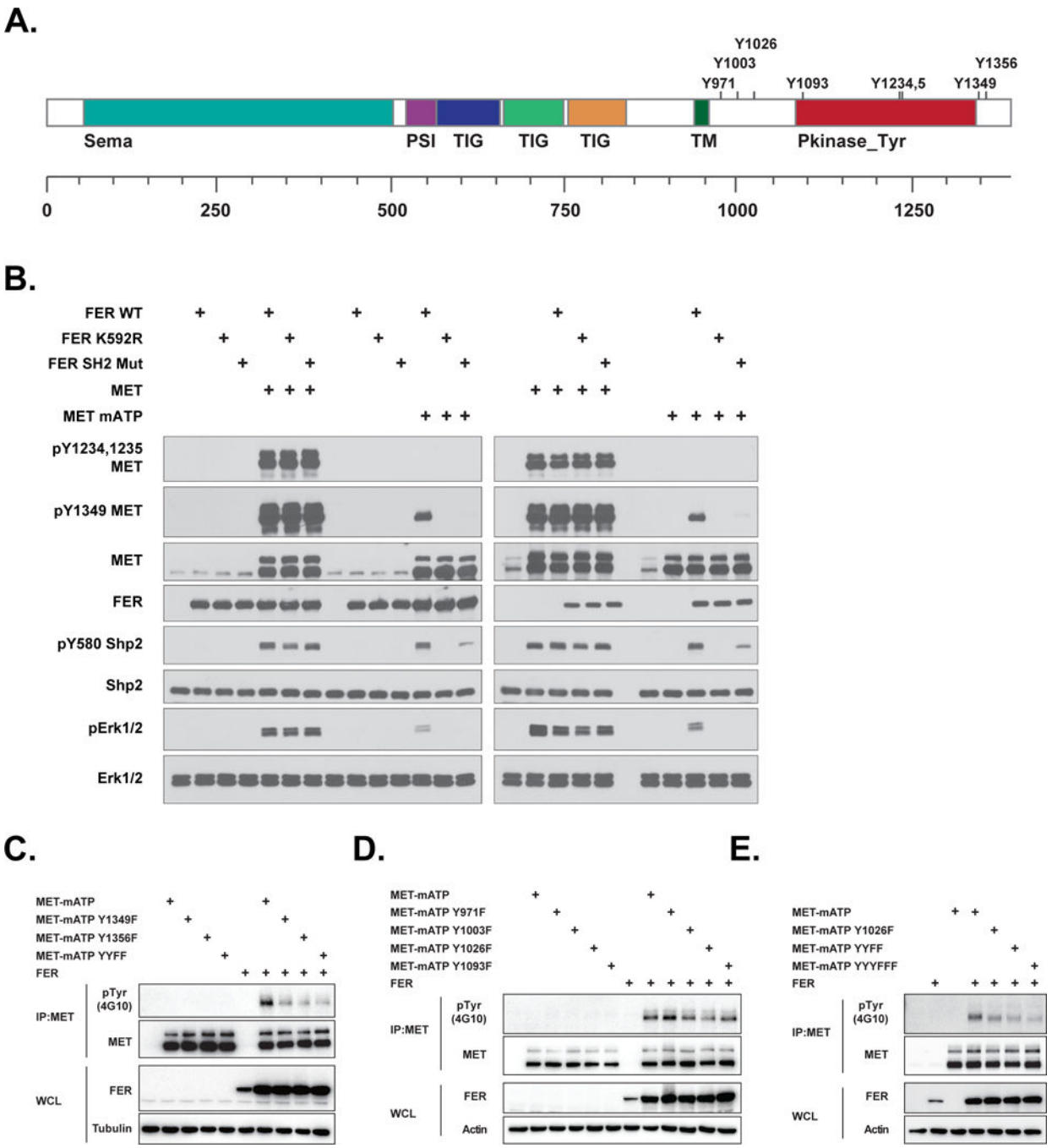

Figure 1. Tyrosine kinase FER phosphorylated HGF receptor MET at multiple tyrosine residues.

(A) Schematic illustration of domain structure of HGF receptor MET. Multiple tyrosine residues within cytosolic segment of the receptor are listed. The following domains are highlighted: SEMA, semaphoring structural domain; PSI, plexin repeat domain; TIG, IPT/TIG Ig-like fold; TM, transmembrane domain.

(B) WT, kinase-dead (K592R) or SH2 mutant of FER were expressed alone, or co-expressed with WT or kinase-dead mutant (mATP) of MET in 293T cells, as indicated.

Phosphorylation levels of MET, SHP2 and ERK, as well as their expression levels, were examined by immunoblotting.

(C) mATP MET, or its Y1349F, Y1356F or YY1349/1356FF mutants, were expressed alone, or co-expressed with FER in 293T cells, as indicated. Lysates were harvested and MET immunoprecipitated. Tyrosine phosphorylation of MET was examined with pTyr antibody 4G10. Expression levels of FER were also probed, with tubulin as loading control.

(D) mATP MET, or its Y971F, Y1003F, Y1026F or Y1093F mutants, were expressed alone, or co-expressed with FER in 293T cells, as indicated. Lysates were harvested and MET immunoprecipitated. Tyrosine phosphorylation of MET was examined with pTyr antibody 4G10. Expression levels of FER were also probed, with actin as loading control.

(E) mATP MET, or its Y1026F, YY1349/1356FF or YYY1026/1349/1356FFF mutants, were co-expressed with FER in 293T cells, as indicated. Lysates were harvested and MET immunoprecipitated. Tyrosine phosphorylation of MET was examined with pTyr antibody 4G10. Expression levels of FER were also probed, with actin as loading control.

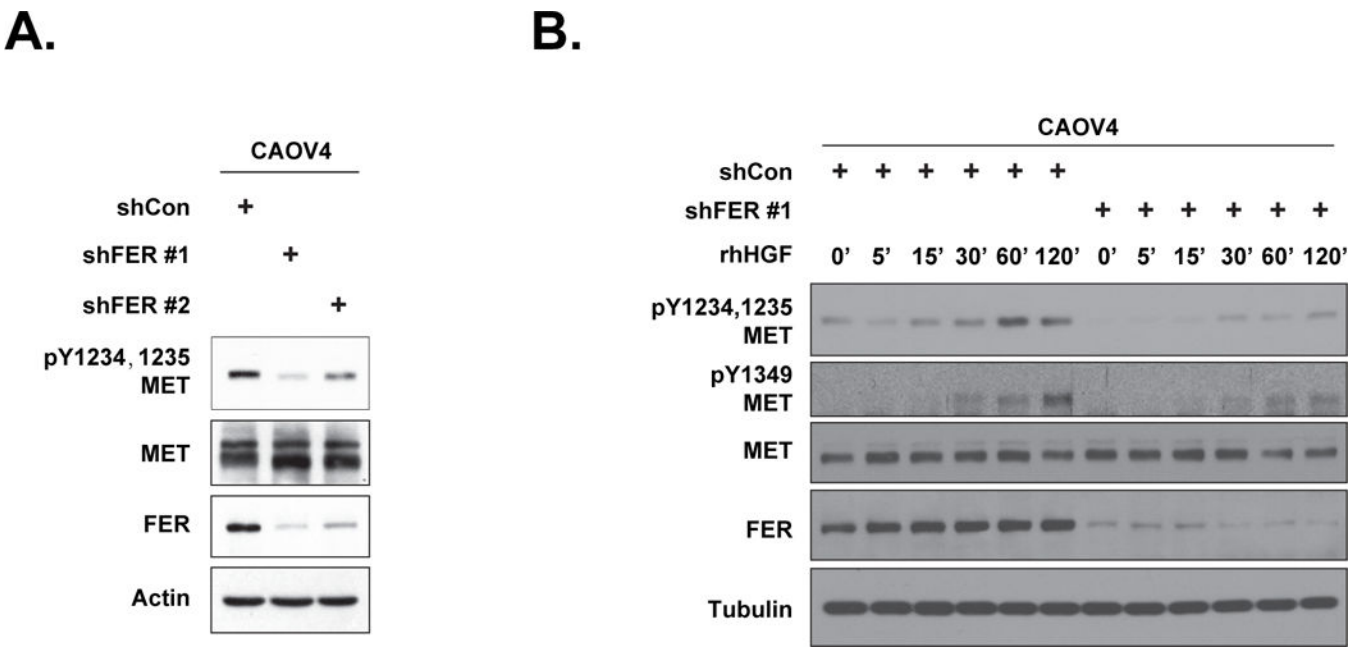

Figure 2. Tyrosine phosphorylation of the activation loop of MET was decreased in FER-deficient ovarian cancer cells.

(A) CAOV4 cells, expressing either control or FER shRNA, were lysed, and immunoblotted as indicated to measure the activation of MET and ERK signaling pathways.

(B) CAOV4 cells, expressing either control or FER shRNA, were serum-starved and stimulated with recombinant human HGF for the indicated times, lysed, and immunoblotted with the designated antibodies to illustrate the impact of FER deficiency on HGF-induced phosphorylation.

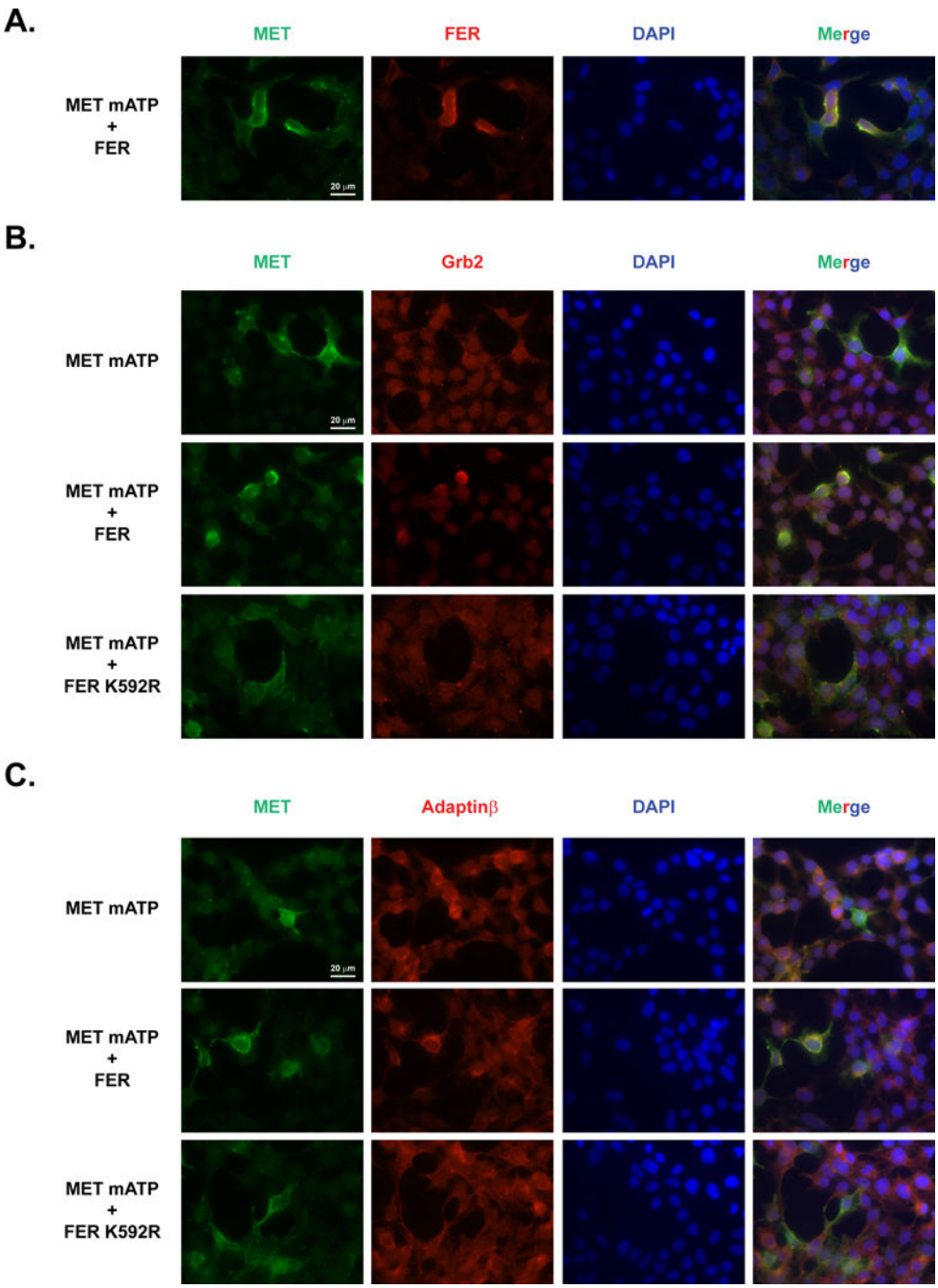

Figure 3. FER co-localized with MET, and promoted recruitment of GRB2 and Adaptinβ to the receptor.

(A) MET mATP mutant was co-transfected with FER in 293T cells, and the co-localization of MET (Green) and FER (Red) was assessed by immunofluorescence. Nuclei were stained with DAPI (Blue).

(B) MET mATP mutant was transfected alone, or together with wt or kinase-dead FER, in 293T cells, and the co-localization of MET (Green) and GRB2 (Red) was assessed by immunofluorescence. Nuclei were stained with DAPI (Blue).

(C) MET mATP mutant was transfected alone, or together with wt or kinase-dead FER, in 293T cells, and the co-localization of MET (Green) and Adaptinβ (Red) was assessed by immunofluorescence. Nuclei were stained with DAPI (Blue). The illustrated images are representative of recordings in at least 3 replicates.

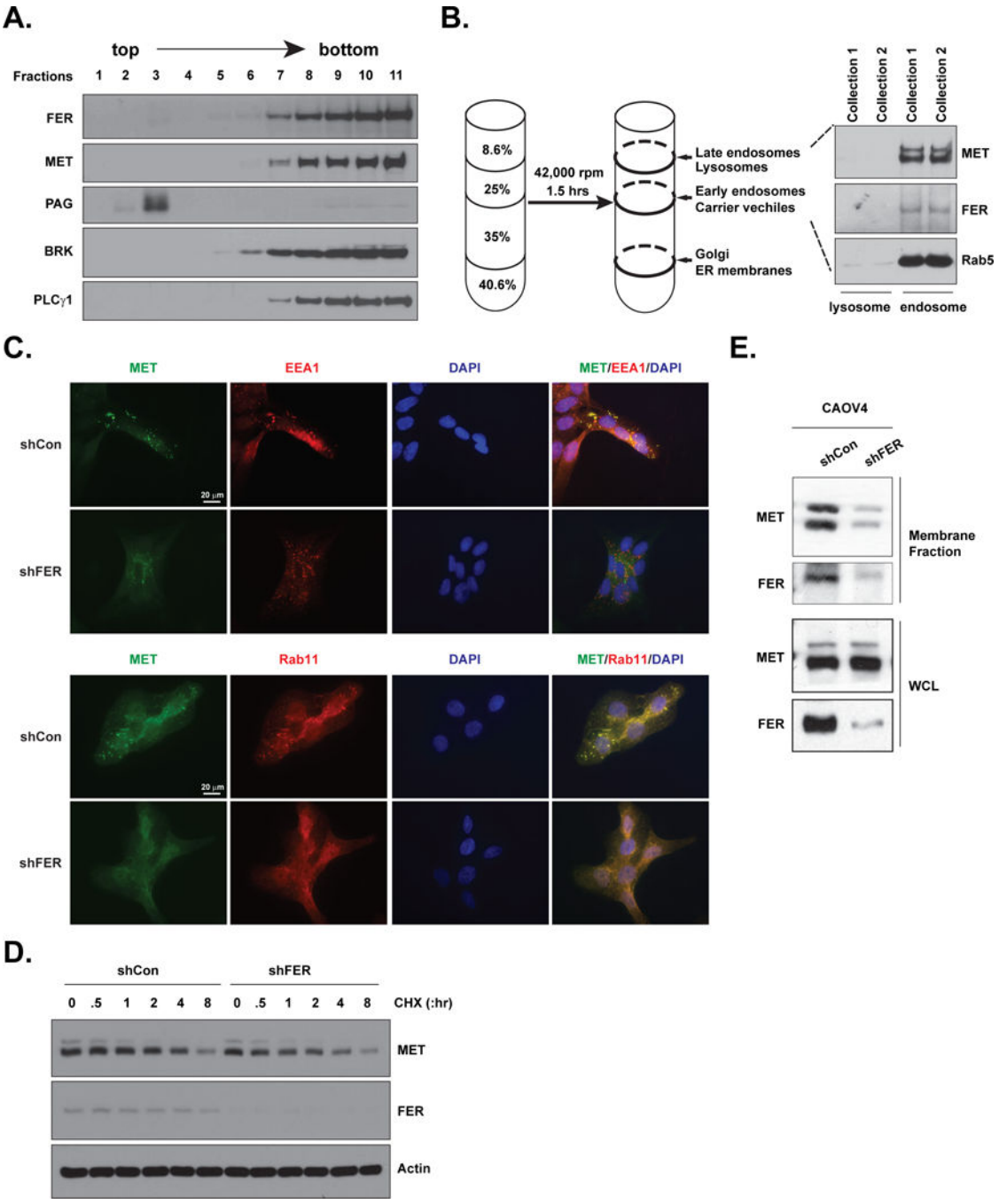

Figure 4. Regulation of endocytosis of the HGF receptor MET by FER.

(A) CAOV4 ovarian cancer cells were homogenized in a buffer containing 0.35% Triton X-100, and the sub-cellular fractions were separated on a discontinuous sucrose gradient. The fractions were collected from the top of the gradient (total volume: ~3 ml; each fraction: ~300 μl) and subjected to immunoblotting with antibodies against indicated proteins. (B) (Left) Schematic diagram for sucrose gradient ultracentrifugation. (Right) Fractions from both endosome and lysosome were collected, lysed and immunoblotted for MET and FER. Rab5 was used as an endosome marker. CAOV4 ovarian cancer cells, expressing either

control or FER shRNA, were serum-starved and stimulated with HGF for 30 mins. (Upper) The co-localization of MET (Green) and EEA1 (Red, upper panels) or Rab11 (Red, lower panels) was assessed by immunofluorescence. Nuclei were stained with DAPI (Blue). The illustrated images are representative of recordings in at least 3 replicates.

(C) CAOV4 ovarian cancer cells, expressing either control or FER shRNA, were treated with cycloheximide for the indicated times, lysed, and immunoblotted with antibodies against MET and FER. Actin was probed as loading control.

(D) CAOV4 ovarian cancer cells, expressing either control or FER shRNA, were subjected to cell fractionation and the distribution pattern of HGFR/MET and FER was assessed by immunoblotting.

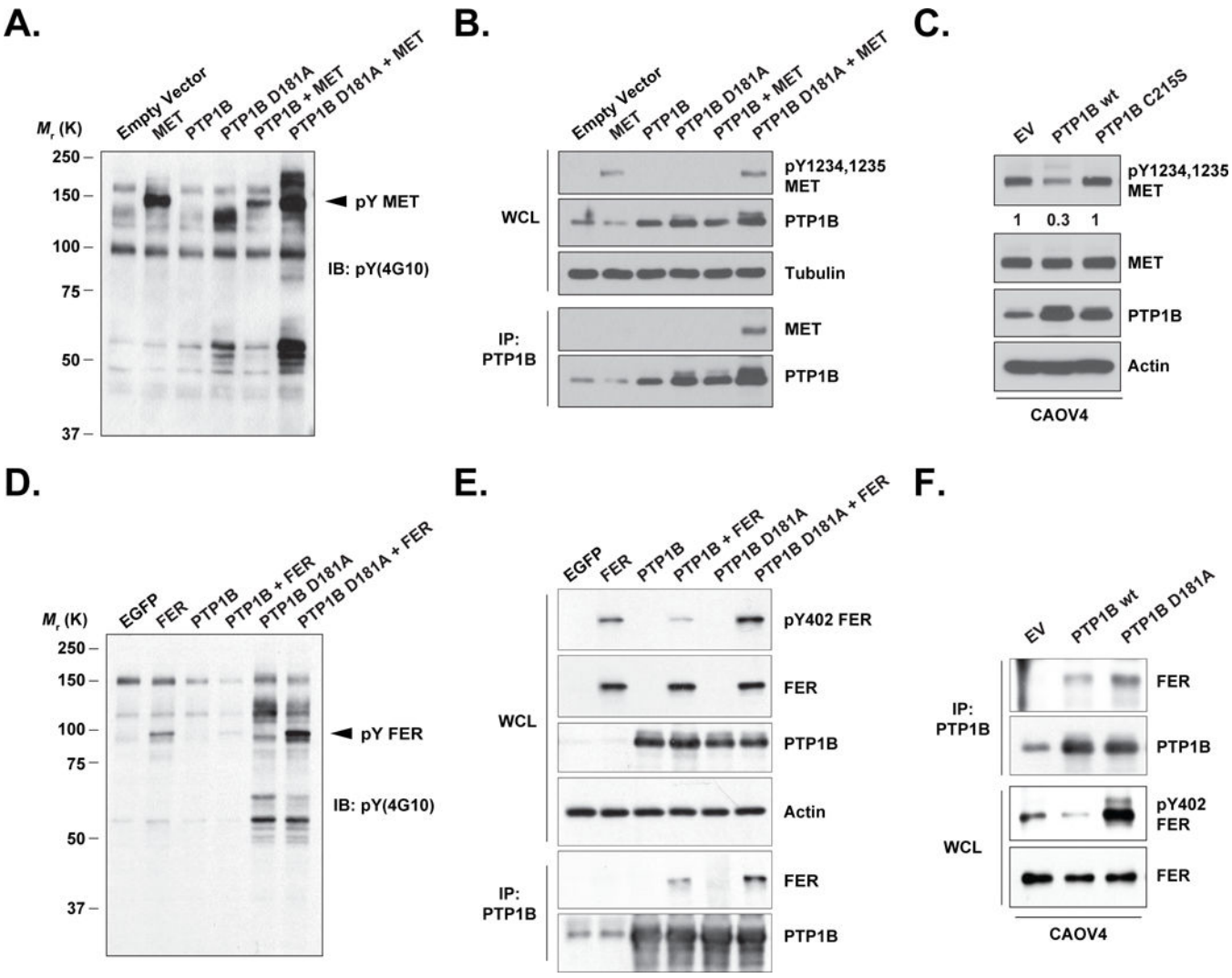

Figure 5. Dephosphorylation of MET and FER by PTP1B.

(A) MET was expressed alone, or co-expressed with wild-type or D181A (substrate trapping mutant) forms of PTP1B in 293T cells, as indicated. Lysates were harvested and immunoblotted with pTyr antibody 4G10.

(B) MET was expressed alone, or co-expressed with wild-type or D181A mutant forms of PTP1B in 293T cells, as indicated. (Upper) Lysates were harvested and immunoblotted for pTyr 1234/1235 MET and PTP1B. Tubulin was probed as loading control. (Bottom) PTP1B was immunoprecipitated from cell lysates, and the co-immunuoprecipitation of MET was examined with MET antibody.

(C) Immunoblot to demonstrate ectopic expression of PTP1B, both wild-type and D181A mutant, in ovarian carcinoma-derived cell line CAOV4. Actin was probed as loading control. CAOV4 lysates were also subjected to immunoblotting for pTyr 1234/1235 MET.

(D) FER was expressed alone, or co-expressed with wild-type or D181A mutant forms of PTP1B in 293T cells, as indicated. Lysates were harvested and immunoblotted with pTyr antibody 4G10.

(E) FER was expressed alone, or co-expressed with wild-type or D181A mutant forms PTP1B in 293T cells, as indicated. (Upper) Lysates were harvested and immunoblotted for pTyr 402 FER and PTP1B. Actin was probed as a loading control. (Bottom) PTP1B was immunoprecipitated from cell lysates, and the co-immunuoprecipitation of FER was examined with FER antibody.

(F) Wild-type or D181A mutant PTP1B was stably expressed in CAOV4 ovarian carcinoma cells. (Upper) Lysates were harvested, PTP1B was immunoprecipitated, and the co-immunuoprecipitation of FER was examined with FER antibody. (Bottom) Lysates were also immunoblotted with antibody against pTyr 402 FER.

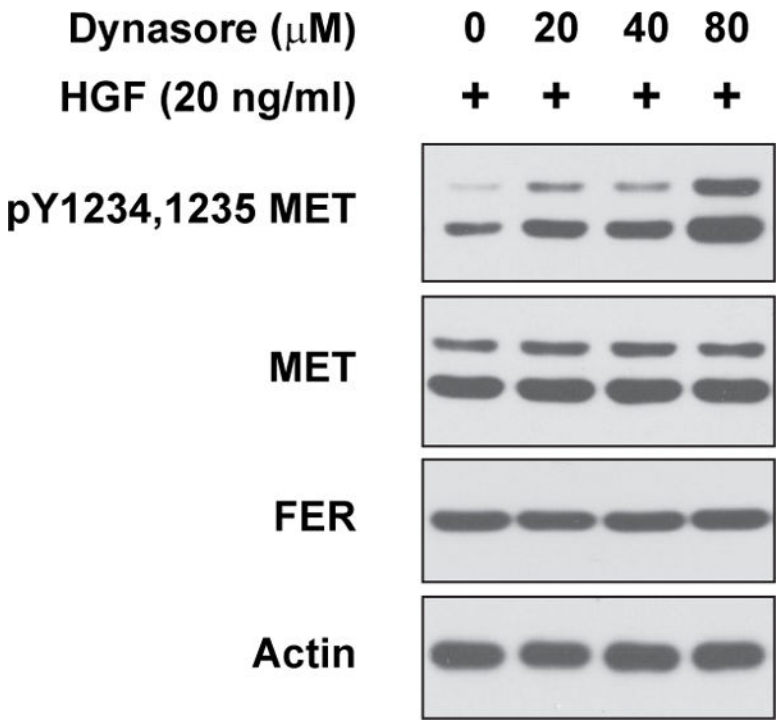

Figure 6. Treatment of Dynasore, an inhibitor of dynamin, enhanced MET activation in ovarian cancer cells.

CAOV4 cells were serum-starved for 16hrs, followed by treatment with Dynasore at indicated concentrations for 30 mins. Cells were then stimulated with HGF for 30 mins, and the activation of MET was examined with pTyr1234/1235 MET antibody, with actin as loading control.

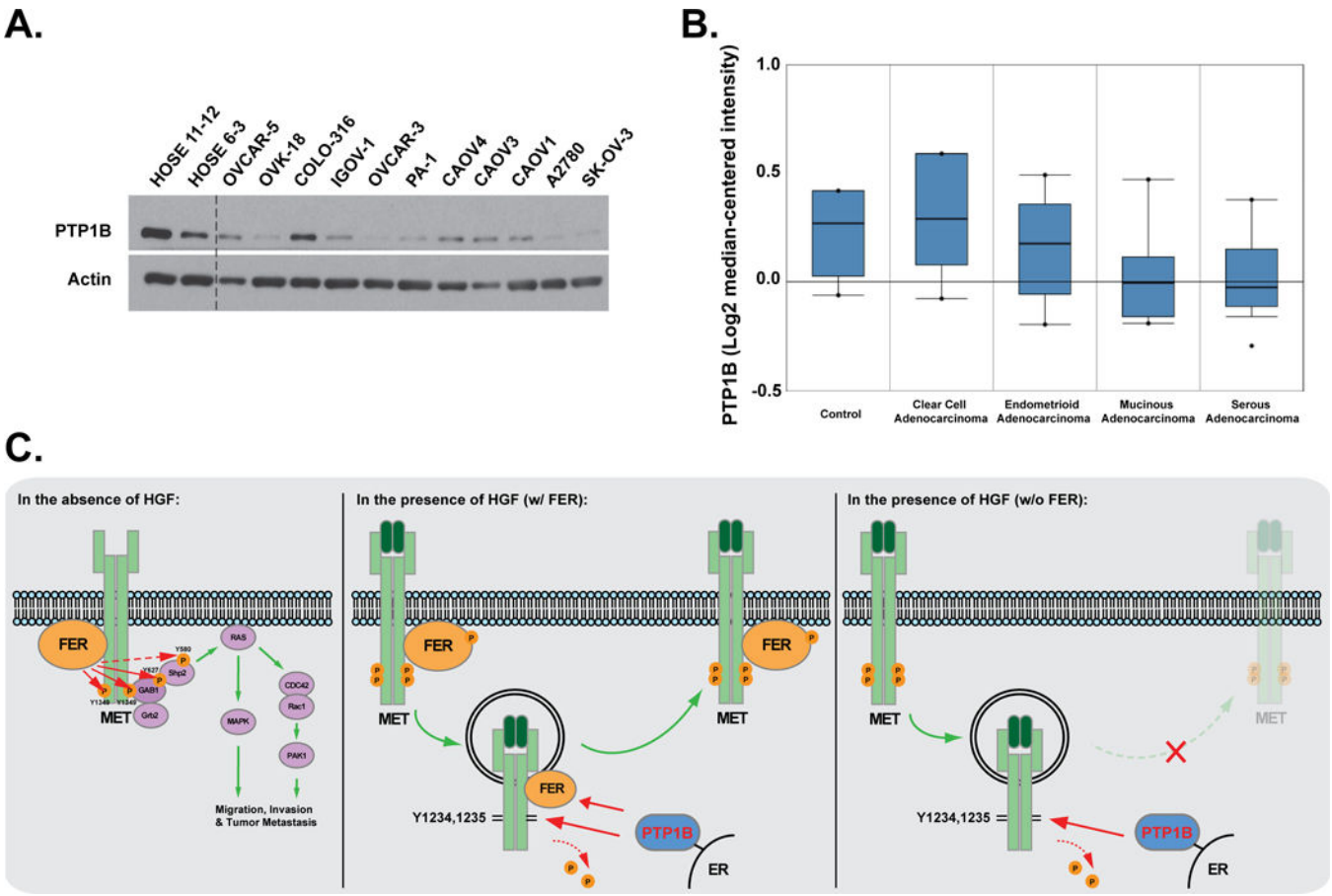

Figure 7. PTP1B was underexpressed in ovarian tumor derived cancer cells.

(A) Expression of PTP1B protein in both normal human ovarian surface epithelial cells (HOSE) and ovarian carcinoma-derived cell lines was assessed by immunoblotting.

(B) Box plots to illustrate down-regulation of *PTP1B* mRNA in primary specimens from human clear cell adenocarcinoma, endometrioid adenocarcinoma, mucinous adenocarcinoma and serous adenocarcinoma compared with normal ovarian surface epithelium (Lu dataset), as extracted from the Oncomine database.

(C) Working model. In the absence of HGF: FER associated with MET, and directly phosphorylated MET, GAB1, and possibly SHP2. This resulted in the activation of SHP2–MAPK and RAC1–PAK1 signaling downstream from MET which contributed to the motility and invasiveness of ovarian cancer cells [7]. In the presence of HGF: FER influenced the endocytosis of the HGF receptor MET. Upon endocytosis, both MET and FER were exposed to the ER-anchored protein tyrosine phosphatase PTP1B with dephosphorylation and inactivation. However, the presence of FER facilitates the recycling of MET to the plasma membrane and its re-activation by HGF.