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# LAPTM4B is a PtdIns(4,5)$P_2$ effector that regulates EGFR signaling, lysosomal sorting, and degradation

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## Abstract

**Lysosomal degradation is essential for the termination of EGF-stimulated EGF receptor (EGFR) signaling. This requires EGFR sorting to the intraluminal vesicles (ILVs) of multi-vesicular endosomes (MVEs). Cytosolic proteins including the ESCRT machineries are key regulators of EGFR intraluminal sorting, but roles for endosomal transmembrane proteins in receptor sorting are poorly defined. Here, we show that LAPTM4B, an endosomal transmembrane oncoprotein, inhibits EGF-induced EGFR intraluminal sorting and lysosomal degradation, leading to enhanced and prolonged EGFR signaling. LAPTM4B blocks EGFR sorting by promoting ubiquitination of Hrs (an ESCRT-0 subunit), which inhibits the Hrs association with ubiquitinated EGFR. This is counteracted by the endosomal PIP kinase, PIPKIγi5, which directly binds LAPTM4B and neutralizes the inhibitory function of LAPTM4B in EGFR sorting by generating PtdIns(4,5)$P_2$ and recruiting SNX5. PtdIns(4,5)$P_2$ and SNX5 function together to protect Hrs from ubiquitination, thereby promoting EGFR intraluminal sorting. These results reveal an essential layer of EGFR trafficking regulated by LAPTM4B, PtdIns(4,5)$P_2$ signaling, and the ESCRT complex and define a mechanism by which the oncoprotein LAPTM4B can transform cells and promote tumor progression.**

**Keywords** EGFR; LAPTM4B; PIPKIγi5; PtdIns(4,5)$P_2$
**Subject Categories** Membrane & Intracellular Transport; Signal Transduction
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## Introduction

Epidermal growth factor receptor (EGFR) plays fundamental roles not only in physiological cellular processes, but also in diseases such as cardiovascular hypertrophy and cancers (Kagiyama *et al*, 2002; Eguchi *et al*, 2003; Mendelsohn & Baselga, 2006). Therefore, EGFR expression levels and signaling strength must be tightly controlled. One key mechanism to downregulate EGFR signaling is the lysosomal trafficking and degradation of the activated receptor. Upon ligand binding, activated EGFR is rapidly internalized to endosomes, where ligand-bound EGFR continues to signal until it is sorted to intraluminal vesicles (ILVs) in the multi-vesicular endosomes (MVEs) or late endosomes (Wiley, 2003; Sorkin & Goh, 2008). Finally, the MVE fuses with the lysosome, resulting in EGFR degradation (Eden *et al*, 2009).

Intraluminal sorting of EGFR is an essential step that terminates EGFR signaling, which is mediated by the endosomal sorting complex required for transport (ESCRT) machineries (Williams & Urbé, 2007; Raiborg & Stenmark, 2009; Henne *et al*, 2011). The ESCRT-mediated EGFR ILV sorting pathway requires ubiquitination of EGFR (Williams & Urbé, 2007). Upstream ESCRT subunits, including Hrs and TSG101, contain ubiquitin-interacting motifs (UIM) that recognize ubiquitinated EGFR, and cooperate with downstream ESCRT complexes for EGFR ILV sorting (Raiborg & Stenmark, 2009). Hrs, like other ESCRT subunits, is a cytosolic protein that is recruited to the endosome by phosphoinositides and protein–protein interactions (Di Paolo & De Camilli, 2006; Lindmo & Stenmark, 2006; Henne *et al*, 2013). The function of Hrs is also regulated by the E3 ubiquitin ligases Nedd4-1 and Nedd4-2 that ubiquitinate Hrs and trigger an intramolecular interaction between the Hrs-UIM and ubiquitin (Katz *et al*, 2002; Hoeller *et al*, 2006; Persaud *et al*, 2009). This interaction inhibits Hrs function by preventing it from binding to ubiquitinated cargos, like EGFR.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$) is a lipid messenger that regulates many cellular processes, including actin and focal adhesion dynamics, endocytosis, exocytosis, and gene expression (Anderson & Marchesi, 1985; Ling *et al*, 2002; Mellman *et al*, 2008; Thapa *et al*, 2012; Balla, 2013; Sun *et al*, 2013c). PtdIns(4,5)$P_2$ has been traditionally thought to be largely at the plasma membrane (Di Paolo & De Camilli, 2006), but a broader intracellular distribution and synthesis have been recently revealed (Sun *et al*, 2013c). Type I phosphatidylinositol 4 phosphate (PIP) 5-kinases (PIPKIα, β, and γ) are the major enzymes for PtdIns(4,5)$P_2$ generation in cells (Heck *et al*, 2007). PIPKI has critical functions in various protein trafficking processes, including endocytosis, exocytosis, and endosomal trafficking (Bairstow *et al*, 2006; Schramp *et al*, 2012; Thapa *et al*, 2012; Sun *et al*, 2013a,b). There are six splice variants of human PIPKIγ (i1-i6), each with distinct C-terminal extensions that mediate specific protein–protein interactions, leading to distinct intracellular targeting of each isoform (Schill & Anderson, 2009; Xia *et al*, 2011). PIPKIγi5 is targeted to endosomes and generates phosphoinositide signals that control EGFR intraluminal

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sorting (Sun *et al*, 2013b). This pathway requires an interaction between PIPKIγi5 and sorting nexin 5 (SNX5). PIPKIγi5 and its kinase activity regulate the interaction of SNX5 with Hrs to protect Hrs from ubiquitination and promote the Hrs association with EGFR. Thus, PIPKIγi5, its kinase activity, and SNX5 control Hrs function in EGFR intraluminal sorting and degradation (Sun *et al*, 2013b).

All the ESCRT subunits including Hrs are cytosolic proteins recruited to endosomal surface during ILV sorting. The roles for resident endosomal transmembrane proteins in the regulation of ESCRT complexes and ILV sorting are poorly defined. A family of the resident proteins is the mammalian lysosomal-associated protein transmembrane (LAPTM) that has three members, LAPTM4A, LAPTM4B, and LAPTM5, with ~36% sequence similarities. All LAPTMs are multi-transmembrane proteins primarily localized to the late endosome/lysosome (Adra *et al*, 1996; Hogue *et al*, 2002; Shao *et al*, 2003; Pak *et al*, 2006; Milkereit & Rotin, 2011). LAPTM4B has four transmembrane domains, with two cytoplasmic termini (Shao *et al*, 2003). LAPTM4B is upregulated in a wide variety of human cancers, including breast, liver, lung, colon, uterine, and ovarian cancers (Shao *et al*, 2003; Kasper *et al*, 2005; Li *et al*, 2010b). LAPTM4B overexpression in cancers correlates with poor prognosis (Yang *et al*, 2010b; Kang *et al*, 2012). Further, ectopic expression of LAPTM4B induces transformation and tumorigenesis of normal human cells (Li *et al*, 2011) and promotes proliferation and migration of cancer cells *in vitro* and *in vivo* (Yang *et al*, 2010a). The underlying mechanisms for the LAPTM4B oncogenesis are not defined, but LAPTM4B overexpression enhances AKT activation (Li *et al*, 2010a).

Here, we report that LAPTM4B blocks EGF-stimulated EGFR intraluminal sorting and degradation. In this pathway, LAPTM4B binds to PIPKIγi5 and its product PtdIns(4,5)$P_2$, and this neutralizes LAPTM4B inhibition of EGFR trafficking. These results reveal an essential layer of EGFR trafficking regulated by LAPTM4B and phosphoinositide signaling and may represent the underlying mechanism for LAPTM4B oncogenesis.

## Results

### PIPKIγi5 interacts with endosomal transmembrane protein LAPTM4B

The endosomal PIP kinase, PIPKIγi5, generates the lipid messenger PtdIns(4,5)$P_2$ and is required for EGFR intraluminal sorting and degradation (Sun *et al*, 2013b). Based on a yeast two-hybrid screen using the C-terminal 223 amino acids of PIPKIγi5 as bait (Sun *et al*, 2013b), the lysosomal-associated protein Transmembrane 4B (LAPTM4B) was identified as a PIPKIγi5 interactor. The interaction between endogenous LAPTM4B and PIPKIγi5 was confirmed by co-immunoprecipitation (co-IP) (Fig 1A). This is a specific interaction, as among the three LAPTM family members, PIPKIγi5 specifically associated with LAPTM4B (Fig 1B). The LAPTM4B interaction is also specific for PIPKIγi5, but not other PIPKIγ isoforms (Fig 1C). To test whether the kinase activity of PIPKIγi5 modulates its LAPTM4B interaction, a D316A kinase dead mutant (PIPKIγi5KD) was used in co-IP assays. As shown in Fig 1D, PIPKIγi5KD had diminished LAPTM4B association, indicating that phosphoinositide generation is required. PIPKIγi5 modulates EGF-stimulated EGFR lysosomal trafficking, but the PIPKIγi5–LAPTM4B interaction was not regulated by EGF stimulation (Fig 1D).

Ectopically expressed LAPTM4B localizes to late endosomes and lysosomes (Milkereit & Rotin, 2011; Vergarajauregui *et al*, 2011). Consistently, we observed that HA-tagged LAPTM4B was primarily colocalized with late endosome/lysosome markers CD63 and LAMP1 and a partial overlap with early endosome marker EEA1 (Supplementary Fig S1A). To ascertain the subcellular localization of the endogenous protein, rabbit polyclonal LAPTM4B anti-sera were used to stain cells. The anti-sera stained endogenous LAPTM4B with significant colocalization with both LAMP1 and EEA1 (Fig 1E and F), indicating a wide distribution of LAPTM4B through the endosomal system (Fig 1G). The specificity of the LAPTM4B anti-sera staining was validated by LAPTM4B knockdown that eliminated the endosomal but not the nuclear staining

**Figure 1. PIPKIγi5 Specifically Interacts with the Endosomal Transmembrane Protein LAPTM4B.**

A Endogenous PIPKIγi5 and LAPTM4B were immunoprecipitated from the whole-cell lysates of MDA-MB-231 cells followed by immunoblotting to examine the co-immunoprecipitated PIPKIγi5 and LAPTM4B.

B PIPKIγi5 specifically interacts with LAPTM4B, but not LAPTM4A or LAPTM5. Top: schematic diagram of all three LAPTM members. Bottom: each Flag-tagged LAPTM protein was immunoprecipitated from HEK293 cells cotransfected with Myc-tagged PIPKIγi5 and empty vector or Flag-tagged LAPTM, and the co-immunoprecipitated PIPKIγi5 was examined by immunoblotting.

C LAPTM4B selectively associates with PIPKIγi5. Top: schematic diagram of four PIPKI isoforms. Bottom: Myc-tagged PIPKIγi5 was immunoprecipitated from HEK293 cells expressing Flag-tagged LAPTM4B and each isoform of PIPKI, followed by immunoblotting to examine the co-immunoprecipitated LAPTM4B.

D Kinase activity of PIPKIγi5 is required for LAPTM4B association. HEK293 cells expressing LAPTM4B and wild-type (WT) or kinase dead (KD) PIPKIγi5 were starved overnight, stimulated or not with 100 ng/ml EGF for 15 min, and harvested for immunoprecipitation with anti-myc. The co-immunoprecipitated LAPTM4B was detected by immunoblotting.

E Endogenous LAPTM4B is targeted to both early and late endosomes. MDA-MB-231 cells were fixed and costained for endogenous LAPTM4B (red) and EEA1 or LAMP1 (green). Boxes are selected regions for magnified view. Note: non-specific nuclear staining by the LAPTM4B anti-sera. Scale bar: 10 μm.

F Quantification of LAPTM4B colocalization with EEA1 and LAMP1 (mean + SD; $n \geq 4$).

G Schematic diagram for LAPTM4B endosomal localization based on quantification in (F).

H LAPTM4B partially colocalizes with PIPKIγi5. MDA-MB-231 cells expressing Myc-tagged PIPKIγi5 were stained with LAPTM4B anti-sera (red), anti-Myc (green), and DAPI. Box is selected region for magnified view. Scale bar: 10 μm.

I MDA-MB-231 cells stably expressing Flag-LAPTM4B were stained with LAPTM4B anti-sera followed by silver-enhanced immuno-electron microscopy. The early and late MVEs were defined by the number of intraluminal vesicles. N, nucleus; M, mitochondria; MVE, multi-vesicular endosome; and PM, plasma membrane. Scale bars: 2 μm (left); 200 nm (middle and right).

Data information: Data are representative for at least four independent experiments. IP, immunoprecipitate; WCL, whole-cell lysates.
Source data are available online for this figure.

 

(Supplementary Fig S1B and C). LAPTM4B knockdown did not change LAPTM5 staining (Supplementary Fig S1B), indicating that both the LAPTM4B siRNA and anti-sera are specific. To determine whether LAPTM4B and PIPKIγi5 colocalize in cells, Myc-tagged PIPKIγi5 was expressed and costained with endogenous LAPTM4B. As shown in Fig 1H, PIPKIγi5 was localized to subdomains of

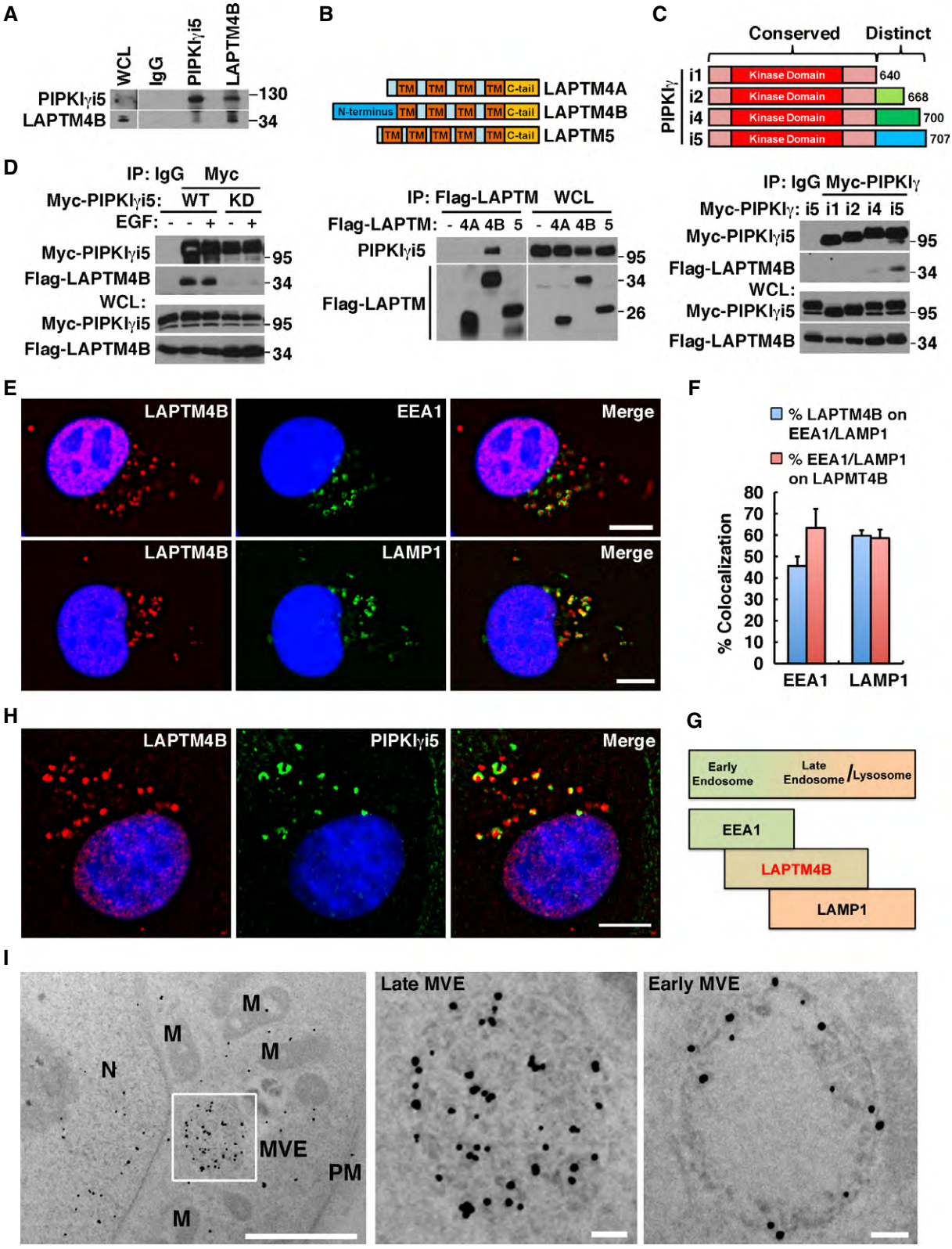


**Figure 1.**

LAPTM4B-positive endosomes. Loss of LAPTM4B did not prevent endosomal targeting of PIPKIγi5 (Supplementary Fig S1C), consistent with additional PIPKIγi5 targeting factors at endosomes.

As LAPTM4B is a transmembrane protein at endosomes, we examined whether LAPTM4B is targeted to both endosomal limiting membrane and intraluminal vesicles by silver-enhanced immuno-electron microscopy (immuno-EM) that detects the subendosomal localization of LAPTM4B. As shown in Fig 1I, LAPTM4B specifically accumulated at MVEs, on both the limiting membrane and intraluminal vesicles. Early MVEs have fewer ILVs, and LAPTM4B was primarily at the limiting membrane (Fig 1I, right). These data support that LAPTM4B is initially sorted to the limiting membrane of MVEs and then partially sorted onto ILVs as the MVE matures. This is consistent with a partial colocalization between LAPTM4B and PIPKIγi5 at endosome surfaces.

#### LAPTM4B inhibits EGF-stimulated EGFR degradation

PIPKIγi5 plays a key role in ESCRT-mediated EGFR ILV sorting and lysosomal degradation (Sun *et al*, 2013b). As LAPTM4B interacts with PIPKIγi5 (Fig 1), we explored whether LAPTM4B also regulates EGF-stimulated EGFR degradation. Endogenous LAPTM4B expression was knocked down by siRNA in MDA-MB-231 cells. Strikingly, the degradation rate of EGFR was significantly enhanced after LAPTM4B knockdown (Fig 2A and B). After 1 h of EGF stimulation, the EGFR levels in control cells were not significantly reduced, but half of the EGFR was degraded in LAPTM4B knockdown cells (Fig 2A and B). Accelerated EGFR degradation after LAPTM4B knockdown also reduced EGFR and AKT signaling (Fig 2A, C and D). Knockdown of LAPTM4B in A431 cells (Supplementary Fig S2A) resulted in even more dramatic acceleration of EGFR degradation (Supplementary Fig S2B, CQ- and C), indicating that this was not a cell-type-specific result. Pretreatment with the lysosomal inhibitor chloroquine fully blocked EGFR degradation in MDA-MB-231 and A431 cells pretreated with either control or LAPTM4B siRNA (Supplementary Fig S2B, CQ + and D), indicating that the EGFR degradation in LAPTM4B knockdown cells remains lysosomal mediated. A distinct siRNA (siLAPTM4B#2) also efficiently knocked down LAPTM4B expression (Supplementary Fig S2E) and accelerated EGFR degradation (Supplementary Fig S2F and G).

To further confirm that loss of LAPTM4B accelerates EGF-stimulated EGFR degradation, a pulse-chase experiment using Alexa-555-EGF was performed to analyze EGF degradation in control or LAPTM4B knockdown cells by fluorescence microscopy (Fig 2E). After a brief pulse with a lower concentration of Alexa-555-EGF, only a small pool (~10%) of total EGFR is EGF-bound and internalized (Fig 2F). Though similar amounts of EGF were initially internalized in control and LAPTM4B knockdown cells, the loss of EGF was more rapid in cells lacking LAPTM4B (Fig 2E and G). The combined results confirm that LAPTM4B inhibits EGF-stimulated EGFR degradation and enhances EGFR signaling.

LAPTM4B is overexpressed in many human cancers (Kasper *et al*, 2005; Li *et al*, 2010b). Therefore, we investigated whether ectopic expression of LAPTM4B could inhibit EGFR degradation. As shown in Fig 2H–K, overexpression of LAPTM4B strongly inhibited EGF-stimulated EGFR degradation, resulting in greatly enhanced and prolonged activation of EGFR and AKT.

#### LAPTM4B inhibits EGFR trafficking through late endosomes

LAPTM4B is an endosomal protein suggesting that it may inhibit EGFR degradation by modulating EGFR endosomal trafficking. Cells were stimulated with EGF, and EGFR was costained with EEA1 and LAMP1, respectively. Trafficking of EGFR through these compartments was analyzed by quantifying the colocalization of EGFR with EEA1 or LAMP1. After 15 min, the majority of EGFR accumulated at EEA1 compartments in both control and LAPTM4B knockdown cells (Fig 3A and B), signifying that internalization and trafficking to the early endosome were not affected. After 2 h, EGFR colocalized well with LAMP1 in control cells but surprisingly not in LAPTM4B knockdown cells where EGFR showed more colocalization with EEA1 [Fig 3A–D, chloroquine (−)]. It is important to note that EGFR degradation is more rapid in LAPTM4B knockdown cells compared to control cells (Fig 2); the decreased EGFR colocalization with LAMP1 may result from accelerated lysosomal delivery and degradation of EGFR in knockdown cells, but not a block of EGFR trafficking at the early endosome. To confirm this possibility, the EGFR trafficking assay was performed in cells pretreated with lysosomal inhibitor chloroquine to block EGFR degradation. As shown in Fig 3A–D, chloroquine pretreatment rescued EGFR colocalization with LAMP1 in LAPTM4B knockdown cells and decreased EGFR colocalization with EEA1. These combined results indicate that EGFR is delivered faster into lysosomes for degradation upon loss of LAPTM4B.

In cells, LAPTM4B is stochastically expressed with some cells having high and others low LAPTM4B levels (Fig 3E, see arrows). To determine whether EGF-stimulated EGFR trafficking is slowed in LAPTM4B-positive late endosomes, cells were stimulated with EGF for 15 min or 120 min and then fixed and costained for endogenous LAPTM4B and EGFR. After 15 min of EGF stimulation, all cells had similar amounts of EGFR staining at endosomes with partial colocalization with LAPTM4B (Fig 3E, top). After 120 min of EGF stimulation, significantly more EGFR was detected in cells with higher LAPTM4B expression (arrows), and the remaining EGFR colocalized with LAPTM4B (Fig 3E, bottom). These results indicate that EGF-stimulated EGFR trafficking is inhibited in LAPTM4B-positive endosomes. The scatter plot of the EGFR versus LAPTM4B levels in each cell at 15 min and 120 min, respectively, was shown in Fig 3F; no significant correlation between EGFR and LAPTM4B levels at 15 min was detected, but a positive correlation was observed at 120 min, consistent with the model that enhanced LAPTM4B expression inhibits EGFR degradation.

#### LAPTM4B inhibits EGF-stimulated EGFR intraluminal sorting

LAPTM4B may enhance EGFR signaling by inhibiting EGF-stimulated EGFR intraluminal sorting in LAPTM4B-positive endosomes. For this, intraluminal sorting of Alexa-555-EGF into enlarged endosomes induced by expression of constitutively active Rab5Q79L was quantified (Stenmark *et al*, 1994; Simonsen *et al*, 1998; Hanafusa *et al*, 2011). The results demonstrate diminished intraluminal sorting of EGF occurred at endosomes with higher LAPTM4B staining (Fig 4A). Quantification of EGF intraluminal sorting revealed a significant inverse relationship between the EGF intraluminal sorting and LAPTM4B levels at individual endosomes

 

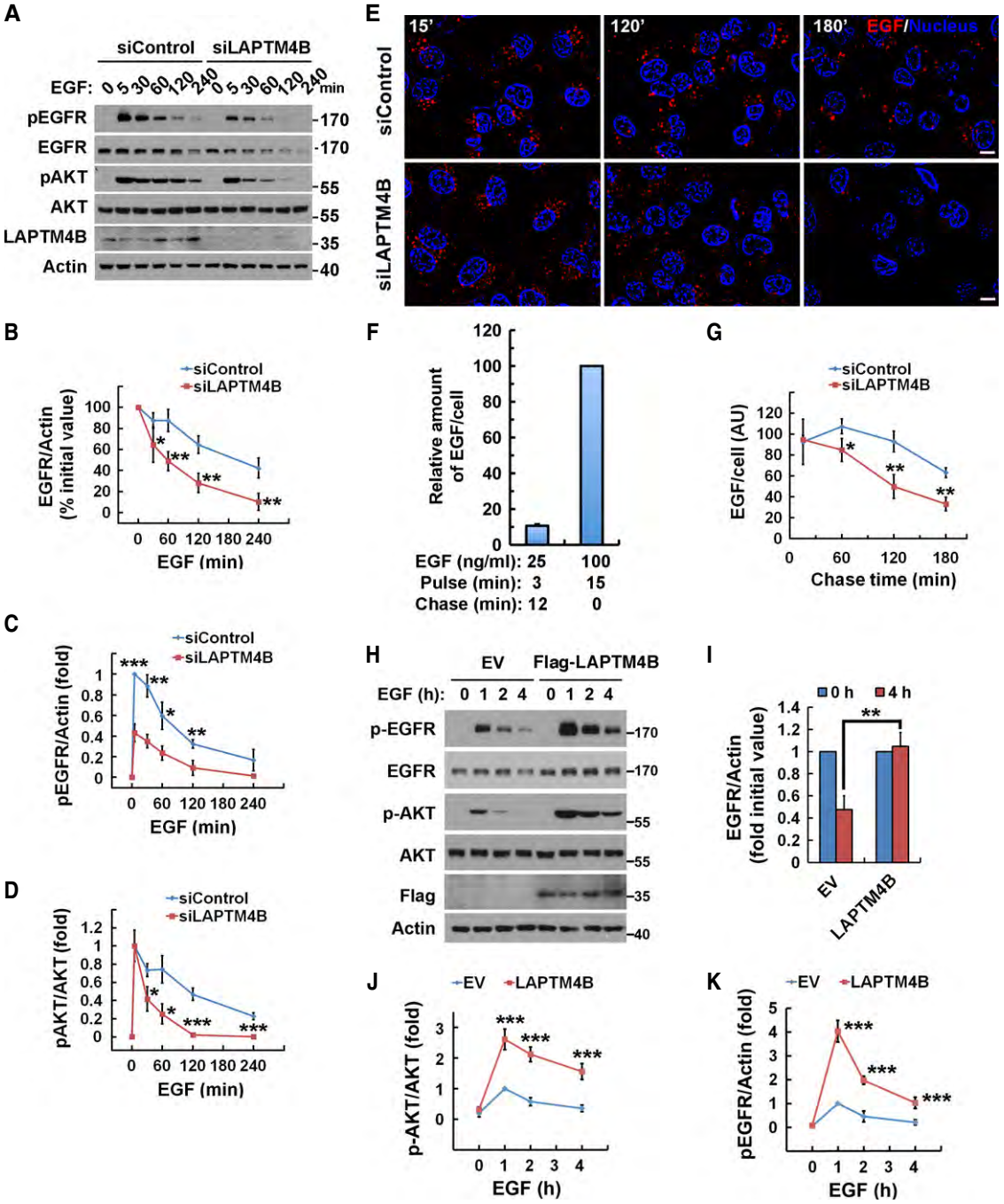


**Figure 2. LAPTM4B Knockdown Accelerates EGF-Stimulated EGFR Degradation.**

A MDA-MB-231 cells transfected with control or LAPTM4B siRNA were starved and stimulated with 100 ng/ml EGF for indicated time periods, followed by whole-cell lysate harvest for immunoblotting analysis of EGFR levels.

B–D Quantification of the levels of EGFR (B), pEGFR (Y1068)(C), and pAKT (S473)(D) from the analysis in (A) (mean ± SD; $n$ = 3).

E Control or LAPTM4B siRNA-transfected MDA-MB-231 cells were starved, pulsed with 25 ng/ml Alexa-555-EGF for 3 min, washed, and chased for indicated time periods followed by fixation, DAPI staining, and fluorescence microscopy. Scale bar: 10 μm.

F Quantification of the relative amounts of Alexa-555-EGF internalized in the indicated conditions (mean + SD, $n$ = 3).

G Quantification of the Alexa-555-EGF degradation in control and LAPTM4B knockdown cells in (E) (mean ± SD, $n$ = 3).

H Control or LAPTM4B-overexpressing MDA-MB-231 cells were starved and then stimulated with 100 ng/ml EGF for 1–4 h. EGFR degradation and signaling were analyzed by Western blot. Specific antibodies recognizing pEGFR (Y1068) and pAKT (S473) were used.

I–K Quantification for the levels of EGFR (I) and pEGFR (J) normalized to actin and pAKT (K) normalized to AKT in control or LAPTM4B-overexpressing cells (mean ± SD, $n$ = 3).

Data information: Data are representative for at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-tailed $t$-test.
Source data are available online for this figure.

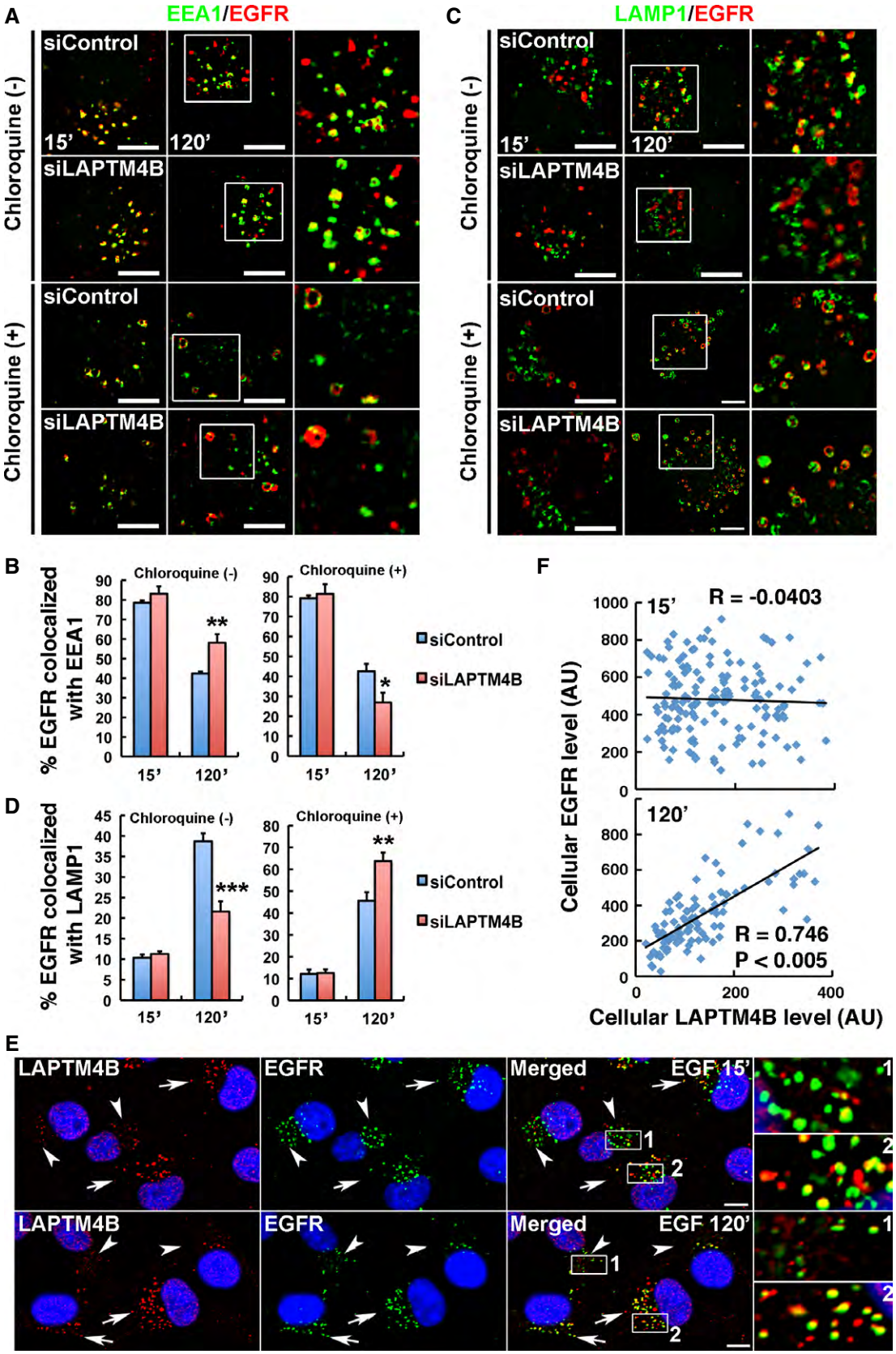


Figure 3.

**Figure 3. LAPTM4B Inhibits EGF-Stimulated EGFR Endosomal Sorting.**

A–D Control or LAPTM4B siRNA-transfected MDA-MB-231 cells were starved, pretreated or not with chloroquine for 2 h, stimulated with 100 ng/ml EGF for 15 min, washed, and chased for indicated time periods before fixation for costaining of EGFR (red) with EEA1 (A, green) or LAMP1 (C, green). Quantification of the average percentages of EGFR signals colocalized with EEA1 (B) and LAMP1 (D) at indicated time points; mean + SD; $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-tailed *t*-test.

E MDA-MB-231 cells were starved, stimulated with 100 ng/ml EGF for 15 or 120 min, fixed, and costained for EGFR and LAPTM4B, followed by fluorescence microscopy. Cells with higher and lower LAPTM4B expression were marked with arrows and arrowheads, respectively.

F The amounts of total EGFR staining in individual cells in (E) were plotted against LAPTM4B levels at 15 min and 120 min, respectively. Note: for LAPTM4B quantification, the non-specific nuclear staining was not included. Trend lines and Pearson's correlation coefficients are shown. AU, arbitrary unit.

Data information: Data are representative for three independent experiments. Boxes are selected regions for magnified view. Scale bars: 10 μm.

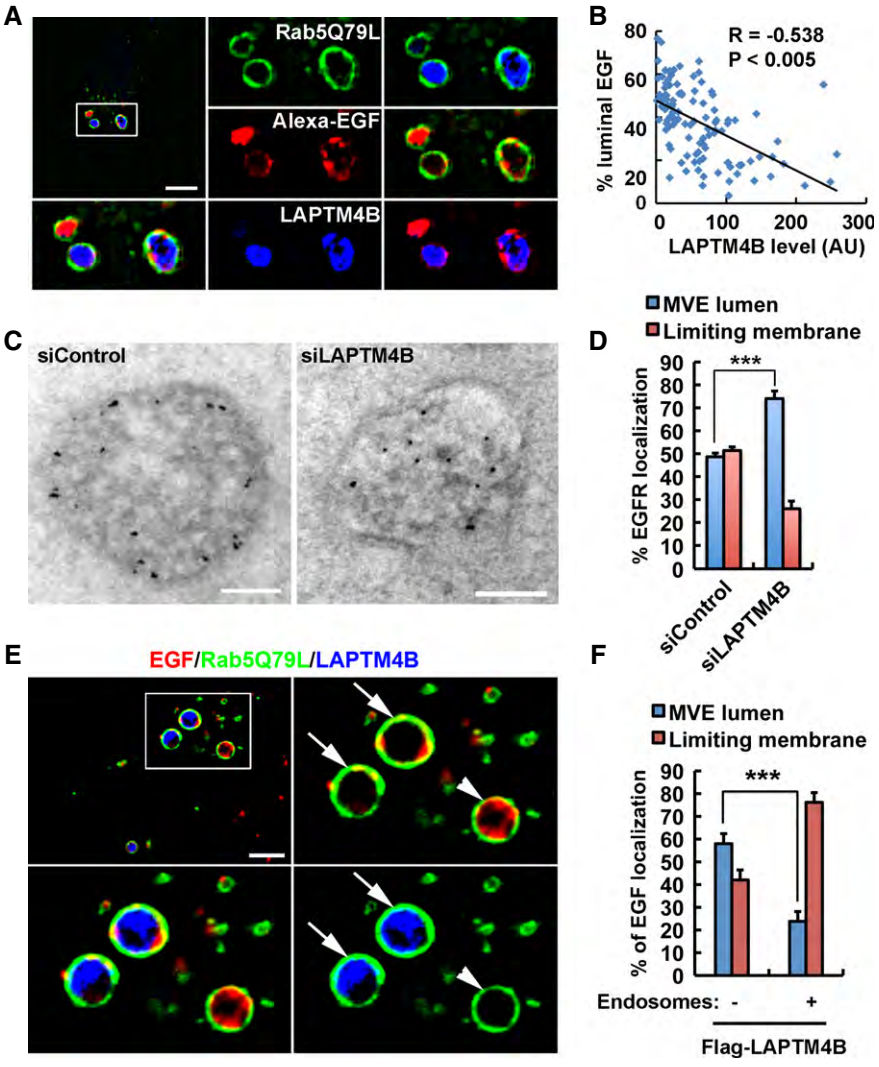


**Figure 4. LAPTM4B Inhibits EGF-Stimulated Intraluminal Sorting of EGFR.**

A MDA-MB-231 cells expressing GFP-Rab5Q79L were starved and stimulated with 100 ng/ml Alexa-555-EGF for 90 min followed by fixation and immunostaining for LAPTM4B (blue).

B The percentages of luminal Alexa-555-EGF in individual endosomes were plotted against endosomal LAPTM4B levels. Trend lines and Pearson's correlation coefficients are shown. Data are representative for three independent experiments.

C Control or LAPTM4B knockdown MDA-MB-231 cells were starved overnight, cell surface EGFR was labeled with immuno-gold on ice. Cells were then stimulated with EGF for 1 h at 37°C and fixed for the EM study. Scale bar, 200 nm. See Materials and Methods for details.

D Relative amounts of immuno-gold-labeled EGFR in the MVE lumen versus MVE limiting membrane were quantified. Over 80 endosomes for each siRNA treatment from three independent experiments were used for quantification (mean + SD; *** $P < 0.001$, one-tailed *t*-test).

E MDA-MB-231 cells were transfected with GFP-Rab5Q79L and Flag-LAPTM4B, starved, and stimulated with Alexa-555-EGF for 90 min and followed by intraluminal sorting analysis. See Materials and Methods for details.

F Quantification of EGF localization in LAPTM4B-positive and LAPTM4B-negative endosomes in (E) (mean + SD; $n = 3$; *** $P < 0.0007$, one-tailed *t*-test).

Data information: Boxes are selected regions for magnified view. Scale bars (A, E): 10 μm.

(Fig 4B), suggesting that LAPTM4B inhibits intraluminal sorting of EGF at LAPTM4B-positive endosomes.

To confirm the role for LAPTM4B in EGFR intraluminal sorting, an EM approach was used. Serum-starved cells were stimulated with 100 ng/ml EGF for 1 h, and the intraluminal sorting of immuno-gold labeled EGFR was quantified. Knockdown of LAPTM4B significantly increases EGF-stimulated intraluminal sorting of EGFR from ~50% in control cells to ~75% in knockdown cells (Fig 4C and D). When overexpressed, LAPTM4B displayed a non-uniform distribution among endosomes, and consistently, intraluminal sorting of Alexa-555-EGF was strongly inhibited in LAPTM4B-positive endosomes (Fig 4E and F). Together, these results demonstrate that LAPTM4B blocks EGF-stimulated EGFR intraluminal sorting in LAPTM4B-positive endosomes.

As LAPTM4B inhibits EGFR intraluminal sorting, the EGFR accumulated on the endosomal surface could be recycled (Sorkin *et al*, 1991). To assess this, EGFR recycling assay was performed in control and LAPTM4B knockdown cells (Sigismund *et al*, 2008). Surprisingly, knockdown of LAPTM4B did not change EGF-stimulated EGFR recycling (Supplementary Fig S3). These results indicate that LAPTM4B-promoted EGFR signaling comes from active EGFR at the endosome as LAPTM4B blocks EGFR intraluminal sorting at LAPTM4B-positive endosomes without enhancing EGFR recycling.

### PtdIns(4,5)$P_2$ regulates LAPTM4B interaction with PIPKIγi5

To examine how the LAPTM4B–PIPKIγi5 interaction may modulate EGFR trafficking and degradation, the interaction was further characterized. LAPTM4B is a unique member of the LAPTM family as it has an additional N-terminal extension (amino acids 1–91) (Shao *et al*, 2003). It has been shown that the pro-survival functions of human LAPTM4B require its N-terminal extension (Shao *et al*, 2003). Deletion of the LAPTM4B N-terminus abolished the interaction with PIPKIγi5 (Fig 5A), and the LAPTM4B N-terminus (LAPTM4B-N) directly interacted with PIPKIγi5 C-terminus in GST pull-down assay (Fig 5B). Further, co-IP experiments using LAPTM4B truncation mutants revealed that amino acids 1–40 were not critical for PIPKIγi5 interaction (Fig 5C), indicating that amino acids 41–91 were required. This region contains a polybasic motif (PBM) with a cluster of basic arginine residues (Fig 5D). The cytoplasmic PBMs in ion channels and transporters have been shown to bind PtdIns(4,5)$P_2$, which is essential for their functions (Suh & Hille, 2005; Huang, 2007). To analyze whether the LAPTM4B-PBM binds phosphoinositides, the LAPTM4B-N with or without PBM mutation (6RQ and 8RQ, Fig 5D) was expressed and purified from *E. coli* and assayed for phosphoinositide binding using PIP strips (Fig 5E). Wild-type LAPTM4B-N bound multiple phosphoinositides including PtdIns(4,5)$P_2$, while the 6RQ and 8RQ mutants lost all phosphoinositide binding ability (Fig 5F), indicating that the LAPTM4B-PBM is capable of binding to phosphoinositides.

In GST pull-down assays, wild-type LAPTM4B-N and the 6RQ mutant interacted with PIPKIγi5 equally well (Fig 5G), indicating that the mutated residues are not direct PIPKIγi5 binding sites. However, although the 6RQ mutant retained wild-type subcellular localization (Supplementary Fig S4A), it had reduced interaction with PIPKIγi5 in co-IP (Fig 5H, lanes 2 and 4), suggesting PtdIns(4,5)$P_2$ regulation of the interaction in cells. Consistently, PIPKIγi5KD that does not generate PtdIns(4,5)$P_2$ had diminished LAPTM4B association (Fig 1D). Additionally, while the wild-type PIPKIγi5 associates with wild-type LAPTM4B much more strongly than with PBM mutants (Fig 5H, lanes 2–4), the PIPKIγi5KD interacts equally with wild-type and PBM-mutated LAPTM4B (Fig 5H, lanes 5–7). These data signify that *in vivo* the LAPTM4B–PIPKIγi5 interaction is regulated by PtdIns(4,5)$P_2$ generated by PIPKIγi5.

To directly determine whether PtdIns(4,5)$P_2$ regulates the LAPTM4B–PIPKIγi5 interaction, a GST pull-down experiment with or without PtdIns(4,5)$P_2$ was performed. This revealed that PtdIns(4,5)$P_2$ dose-dependently enhanced LAPTM4B binding to PIPKIγi5 (Fig 5I). Although other phosphoinositides including PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)$P_2$ bound LAPTM4B-N in both PIP strips assays (Fig 5F) and liposome-binding assays (Fig 5J), yet regulation of the LAPTM4B–PIPKIγi5 interaction was PtdIns(4,5)$P_2$ specific (Fig 5K), consistent with regulation by PIPKIγi5 that generates PtdIns(4,5)$P_2$.

The binding between PIPKIγi5 and the 6RQ mutant of LAPTM4B is no longer stimulated by PtdIns(4,5)$P_2$ addition (Fig 5L),

**Figure 5. Phosphoinositide Regulates LAPTM4B Interaction with PIPKIγi5.**

A Co-immunoprecipitation (co-IP) of full-length or N-terminus-deleted LAPTM4B with PIPKIγi5 in HEK293 cells.
B GST-tagged LAPTM4B N-terminus (93 amino acids) and T7-tagged PIPKIγi5 C-terminus (223 amino acids) were purified from *E. coli* for *in vitro* GST pull-down assay.
C Co-IP of full-length or N-terminal deletion mutants of LAPTM4B with PIPKIγi5 in HEK293 cells. Δ25: amino acids 1–25 deleted.
D Schematic diagram of LAPTM4B with polybasic motif (PBM) magnified and PBM mutants specified.
E Coomassie Brilliant Blue staining of purified wild-type and mutated LAPTM4B N-termini.
F Purified LAPTM4B N-termini from (E) were used in PIP strips assay. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; PI3,4,5P3, PtdIns(3,4,5)$P_3$, phosphatidylinositol (3,4,5)-trisphosphate; PA, phosphatidic acid; PS, phosphatidylserine.
G GST pull-down assay of wild-type or 6RQ mutant of LAPTM4B N-termini with PIPKIγi5 C-terminus.
H Co-IP of wild-type or PMB-mutated LAPTM4B with wild-type or kinase dead PIPKIγi5 in HEK293 cells.
I Top: GST pull-down assay of LAPTM4B N-terminus and PIPKIγi5 C-terminus with increased concentration of PtdIns(4,5)$P_2$ addition. Bottom: Quantification of the relative amounts of PIPKIγi5 C-terminus bound to LAPTM4B N-terminus in top panel (mean ± SD; $n$ = 4).
J LAPTM4B N-terminus binds multiple phosphoinositides including PtdIns(4,5)$P_2$ in liposome-binding assay.
K Top: GST pull-down assay of LAPTM4B N-terminus and PIPKIγi5 C-terminus with 0.5 μM addition of different phosphoinositides. Bottom: Quantification of the relative amounts of PIPKIγi5 C-terminus bound to LAPTM4B N-terminus in top panel (mean + SD; $n$ = 4).
L Top: GST pull-down assay of wild-type or 6RQ mutant of LAPTM4B N-terminus and PIPKIγi5 C-terminus with 0.5 μM addition of PtdIns(4,5)$P_2$. Bottom: Quantification of the relative amounts of PIPKIγi5 C-terminus bound to LAPTM4B N-terminus in top panel (mean + SD; $n$ = 4).

Data information: Data are representative from at least three independent experiments; L4B, LAPTM4B; L4B-N, LAPTM4B N-terminus; Iγi5-CT, PIPKIγi5 C-terminus; PI, PtdIns, phosphatidylinositol; PI3P, PtdIns(3)P, phosphatidylinositol (3)-phosphate; PI4,5P2, PtdIns(4,5)$P_2$, phosphatidylinositol (4,5)-bisphosphate.
Source data are available online for this figure.

 

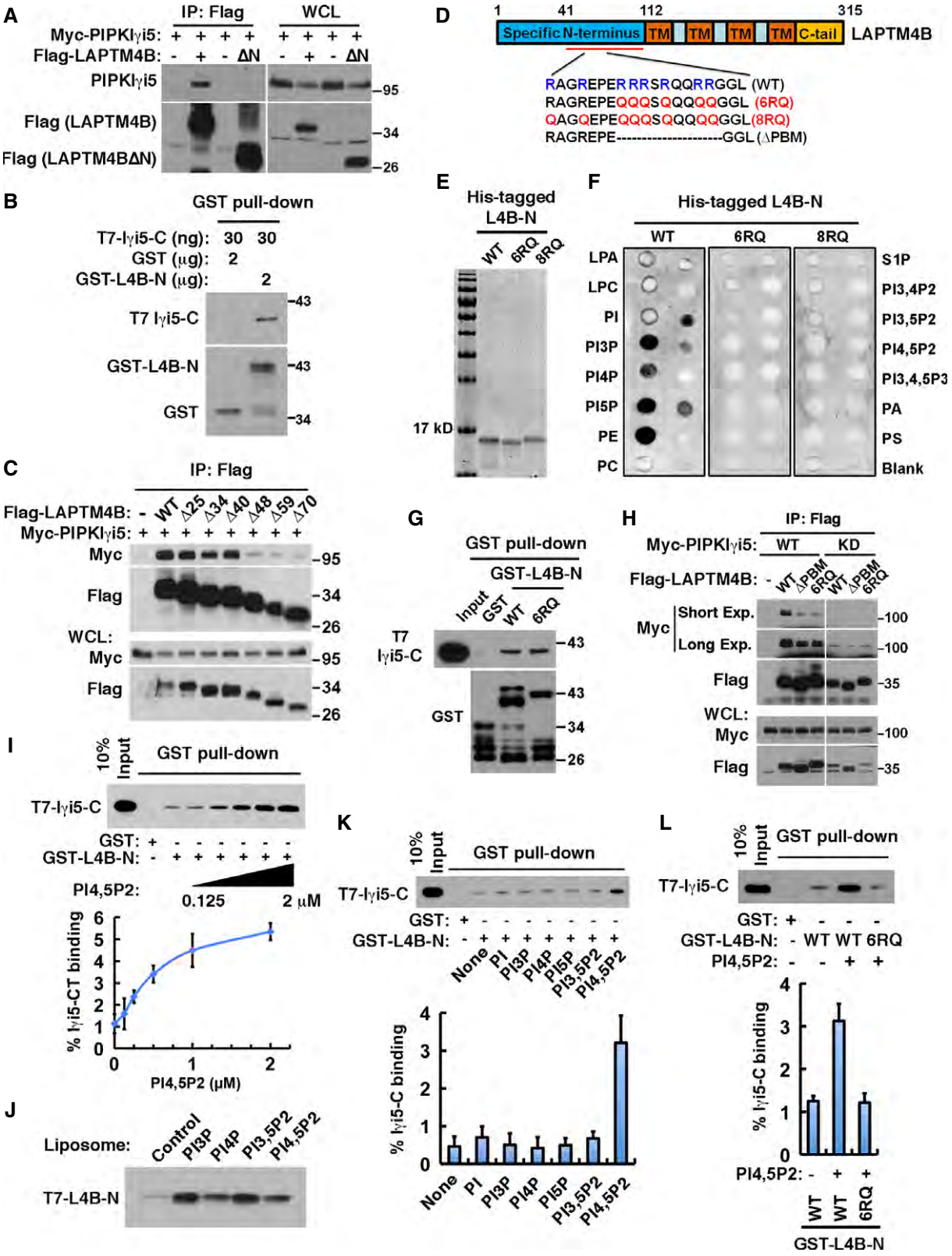
A
IP: Flag
WCL
Myc-PIPKIγi5:
Flag-LAPTM4B:
PIPKIγi5
Flag (LAPTM4B)
Flag (LAPTM4BΔN)
95
34
26
B
GST pull-down
T7-Iγi5-C (ng): 30 30
GST (μg): 2
GST-L4B-N (μg): 2
T7 Iγi5-C
GST-L4B-N
GST
43
34
C
IP: Flag
Flag-LAPTM4B: - WT Δ25 Δ34 Δ40 Δ48 Δ59 Δ70
Myc-PIPKIγi5:
Myc
Flag
WCL:
95
34
26
D
1 41 112 315
Specific N-terminus TM TM TM TM C-tail
LAPTM4B
RAGREPERRRSRQQRRGGL (WT)
RAGREPEQQQSQQQQGGL (6RQ)
QAGQEPEQQQSQQQQGGL (8RQ)
RAGREPE----------GGL (ΔPBM)
E
His-tagged L4B-N
WT 6RQ 8RQ
17 kD
F
His-tagged L4B-N
WT 6RQ 8RQ
LPA
LPC
PI
PI3P
PI4P
PI5P
PE
PC
S1P
PI3,4P2
PI3,5P2
PI4,5P2
PI3,4,5P3
PA
PS
Blank
G
GST pull-down
GST-L4B-N
Input GST WT 6RQ
T7 Iγi5-C
GST
43
34
26
H
IP: Flag
Myc-PIPKIγi5: WT KD
Flag-LAPTM4B: - WT ΔPBM 6RQ WT ΔPBM 6RQ
Myc Short Exp.
Long Exp.
Flag
WCL:
Myc
Flag
100
35
I
10% Input
GST pull-down
T7-Iγi5-C
GST: + - - - - - -
GST-L4B-N: - + + + + + +
PI4,5P2: 0.125 2 μM
% Iγi5-CT binding
PI4,5P2 (μM)
J
Liposome: Control PI3P PI4P PI3,5P2 PI4,5P2
T7-L4B-N
K
10% Input
GST pull-down
T7-Iγi5-C
GST: + - - - - - - -
GST-L4B-N: - + + + + + + +
None PI PI3P PI4P PI5P PI3,5P2 PI4,5P2
% Iγi5-C binding
L
10% Input
GST pull-down
T7-Iγi5-C
GST: + - - -
GST-L4B-N: - WT WT 6RQ
PI4,5P2: - - + +
% Iγi5-C binding
PI4,5P2: - + +
WT WT 6RQ
GST-L4B-N

**Figure 5.**

consistent with the loss of phosphoinositide binding for the 6RQ mutant (Fig 5F). The GST pull-down experiments were performed in the presence of PtdIns(4,5)$P_2$ in incubation buffer, but not washing buffer. To assess whether washing the pull-down complex with detergent removed PtdIns(4,5)$P_2$ from the complex, PtdIns (4,5)$P_2$ was added to the washing buffer to protect the protein–lipid complex. This addition did not further enhance the PtdIns(4,5)$P_2$-stimulated interaction between LAPTM4B and PIPKIγi5 (Supplementary Fig S4B), indicating that detergent wash did not disrupt the pull-down complex. Together, these results indicate that PtdIns(4,5) $P_2$, the product of PIPKIγi5, specifically regulates the interaction between LAPTM4B and PIPKIγi5.

### Phosphoinositide binding inhibits the role of LAPTM4B in Hrs ubiquitination and EGFR degradation

For intraluminal sorting, activated EGFR is ubiquitinated and recognized by the ESCRT-0 subunit Hrs that sequesters EGFR and recruits downstream ESCRT complexes (Raiborg *et al*, 2002; Raiborg & Stenmark, 2009; Sorkin & von Zastrow, 2009). To explore the mechanism by which LAPTM4B inhibits EGFR intraluminal sorting, we examined how LAPTM4B impacts the association between EGFR and Hrs. Loss of LAPTM4B enhanced the EGFR interaction with Hrs (Fig 6A), consistent with the enhanced EGFR intraluminal sorting and degradation in knockdown cells. This suggests that LAPTM4B may inhibit EGFR ILV sorting by blocking EGFR interaction with Hrs.

The interaction of EGFR with Hrs is dependent on the ubiquitination of EGFR (Eden *et al*, 2012), but loss of LAPTM4B did not affect EGFR ubiquitination (Supplementary Fig S5A). Ubiquitination of Hrs induces an intramolecular interaction between the Hrs-UIM and ubiquitin that inhibits Hrs interaction with ubiquitinated EGFR (Hoeller *et al*, 2006). Therefore, whether LAPTM4B regulates the ubiquitination of Hrs was determined. Loss of LAPTM4B diminished Hrs ubiquitination (Fig 6B), while overexpression of LAPTM4B enhanced Hrs ubiquitination (Fig 6C and D). Consistently, LAPTM4B interacts with Hrs in co-IP (Fig 6E) and colocalizes with Hrs at endosomes (Supplementary Fig S5B). Overexpression of the 6RQ mutant that lacks phosphoinositide binding further enhanced Hrs ubiquitination (Fig 6C and D). These results indicate that LAPTM4B interacts with Hrs, promoting Hrs ubiquitination, and that this role is inhibited by LAPTM4B phosphoinositide binding. Consistently, loss of PIPKIγi5 also promotes Hrs ubiquitination and diminished the Hrs interaction with EGFR (Sun *et al*, 2013b).

Nedd4, an E3 ubiquitin ligase that ubiquitinates Hrs, directly interacts with LAPTM4B (Katz *et al*, 2002; Persaud *et al*, 2009; Milkereit & Rotin, 2011). LAPTM4B and Nedd4 interact in both the endogenous and overexpressed conditions (Supplementary Fig S5C). We then examined whether LAPTM4B regulates Nedd4 interaction with Hrs. As shown in Fig 6F, overexpression of LAPTM4B enhanced Hrs association with Nedd4. Consistently, the 6RQ LAPTM4B mutant had increased interaction with Hrs compared to wild-type LAPTM4B (Fig 6E), and it also further enhanced the Nedd4-Hrs interaction (Fig 6F).

To investigate how phosphoinositide binding regulates the role of LAPTM4B in EGFR degradation, we used a lentivirus-based system to modestly overexpress LAPTM4B or the 6RQ mutant in cells. As shown in Fig 6G, the wild-type and 6RQ mutant of LAPTM4B were expressed at comparable levels. Wild-type LAPTM4B overexpression modestly inhibited EGFR degradation and enhanced AKT activation. The LAPTM4B 6RQ mutant overexpression showed enhanced inhibition of EGFR degradation and this resulted in enhanced AKT activation (Fig 6G and H). These data indicate that phosphoinositide binding relieves LAPTM4B inhibition of EGFR degradation.

LAPTM4B interacts with Nedd4 through two PY motifs (L/PPXY) at the LAPTM4B C-tail (Milkereit & Rotin, 2011). To confirm that Nedd4 interaction is required for LAPTM4B to inhibit EGFR degradation, the Nedd4-non-interacting LAPTM4B mutant (2PA) was generated in which the second conserved proline residues in both PY motifs were mutated into alanines. The 2PA mutant lost Nedd4 interaction but retained association with Hrs and had

**Figure 6. Phosphoinositide Binding Inhibits LAPTM4B Interaction with Hrs and Compromise the Inhibitory Effects of LAPTM4B in EGF-Stimulated EGFR Degradation.**

A Control or LAPTM4B knockdown MDA-MB-231 cells were starved and stimulated with 100 ng/ml EGF for 30 min, and whole-cell lysates were subject to co-immunoprecipitation (co-IP) assay.

B Control or LAPTM4B knockdown MDA-MB-231 cells were transfected with His-tagged ubiquitin, starved, and stimulated with 100 ng/ml EGF for 30 min. Total ubiquitinated proteins were purified from whole-cell lysates by Ni-NTA agarose and analyzed by Western blot.

C Control or LAPTM4B-WT/6RQ-overexpressing MDA-MB-231 cells were transfected with His-tagged ubiquitin and myc-tagged Hrs, starved, and stimulated with 100 ng/ml EGF for 30 min before whole-cell lysate harvest. Total ubiquitinated proteins were purified by Ni-NTA agarose and analyzed by Western blot.

D Quantification of Hrs ubiquitination levels from the Western blot in (C) (mean + SD; $n$ = 5).

E The co-IP of Myc-Hrs with Flag-LAPTM4B-WT or 6RQ mutant in HEK293 cells.

F The co-IP between Hrs and Nedd4 with Flag-LAPTM4B-WT or 6RQ mutant overexpression in HEK293 cells.

G The effects of LAPTM4B-6RQ mutant overexpression on EGF-stimulated EGFR degradation and signaling. Flag-LAPTM4B-WT or 6RQ mutant overexpression was accomplished by lentivirus-mediated infection approach. Cells with low levels of expression were selected as polyclonal pools for comparison. Control or overexpressing cells were starved and stimulated with 100 ng/ml EGF for indicated time periods and whole-cell lysates were analyzed by Western blot.

H Quantification of EGFR and pAKT levels from the Western blot in (G) (mean ± SD; $n$ = 5).

I The PY motif mutant LAPTM4B-2PA loses interaction with Nedd4 but keeps interaction with Hrs in HEK293 cells cotransfected with the indicated proteins.

J The effects of LAPTM4B-2PA mutant overexpression on EGF-stimulated EGFR degradation and signaling. Overexpression of Flag-LAPTM4B-WT or 2PA mutant overexpression was accomplished by lentivirus-mediated infection approach. Cells with high expression of Flag-LAPTM4B were selected as polyclonal pools for comparison. Cells were starved and stimulated with 100 ng/ml EGF for the indicated time periods, and whole-cell lysates were analyzed by Western blot.

K Quantification of EGFR degradation from Western blot in (J) (mean ± SD; $n$ = 3).

Data information: Data are representative from at least three independent experiments. L4B, LAPTM4B; Ub, ubiquitin; EV, empty vector.
Source data are available online for this figure.

 

enhanced PIPKIγi5 interaction (Fig 6I). When comparing two pools of cells overexpressing high levels of LAPTM4B-WT or LAPTM4B-2PA, EGFR degradation was strongly inhibited in LAPTM4B-WT-expressing cells, but not in LAPTM4B-2PA cells (Fig 6J and K). This supports a role for the LAPTM4B–Nedd4 interaction in mediating inhibition of EGFR degradation.

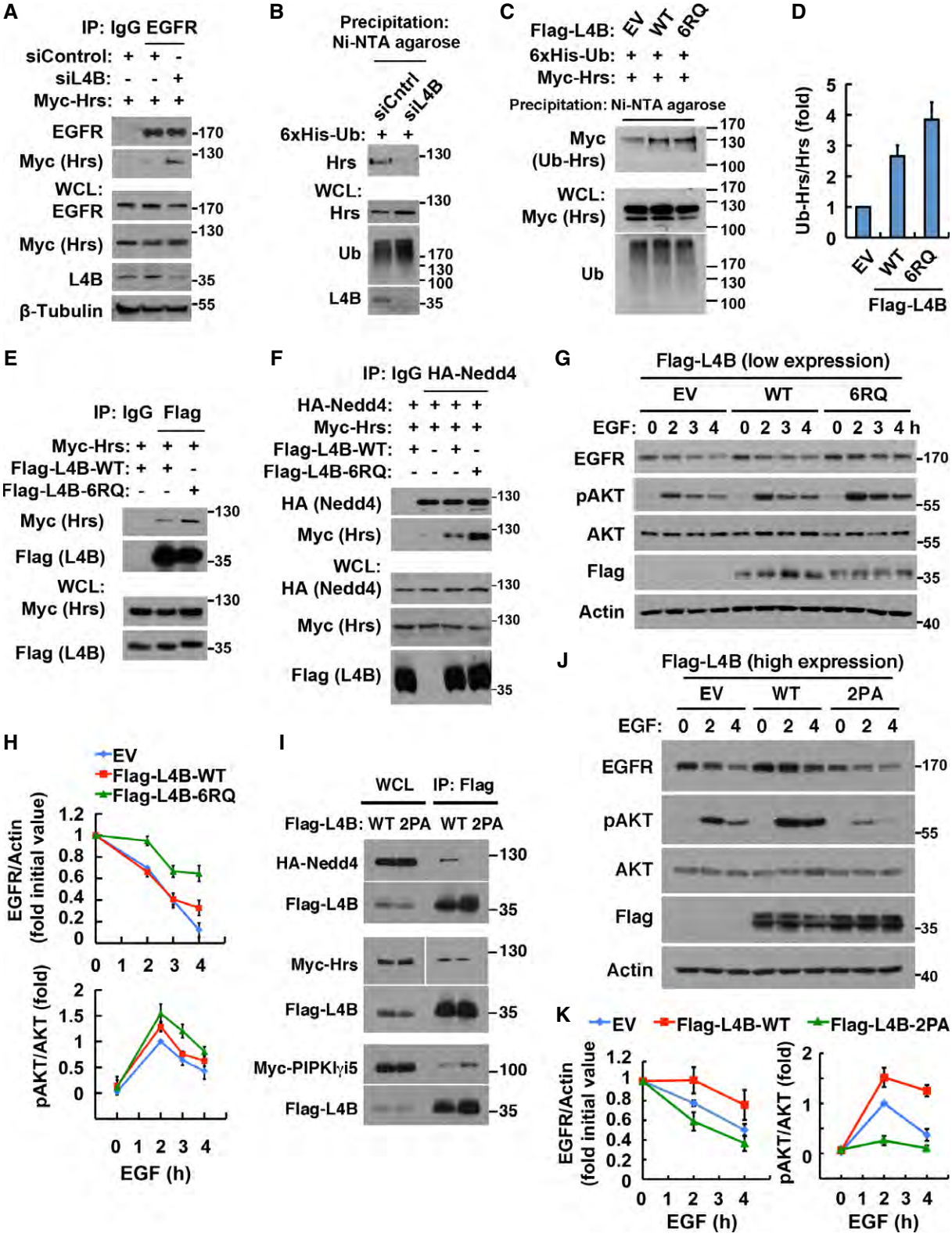


**Figure 6.**

### PIPKIγi5 recruits SNX5 to inhibit LAPTM4B association with Hrs

We have previously shown that PIPKIγi5 C-tail also specifically interacts with SNX5 and controls SNX5 function in protecting Hrs from ubiquitination by Nedd4 (Sun *et al*, 2013b). As LAPTM4B and SNX5 both bind the PIPKIγi5 C-tail, we investigated whether they bind the same or distinct regions of the C-tail. A series of PIPKIγi5 C-terminal deletion mutants were generated and used for co-IP assays with LAPTM4B and SNX5. This revealed that the most N-terminus of the C-tail (amino acids 640-652) was required for SNX5 interaction, while the remaining C-terminus was not required (Fig 7A). In contrast, the LAPTM4B interaction with PIPKIγi5 required the full-length C-tail of PIPKIγi5 and deletion of 6 amino acids at the C-terminus diminished LAPTM4B interaction (Fig 7B and C). These data indicate that LAPTM4B and SNX5 may associate together with PIPKIγi5 on the C-tail. To test whether the PIPKIγi5 interactions integrate LAPTM4B and SNX5 into the same complex, a co-IP experiment was performed with or without PIPKIγi5 overexpression. This indicted that PIPKIγi5 strongly enhanced SNX5 association with LAPTM4B (Fig 7D). As SNX5 blocks Nedd4 interaction with Hrs (Sun *et al*, 2013b), we assessed whether SNX5 also inhibits LAPTM4B interaction with Nedd4 or Hrs. Overexpression of SNX5 suppressed LAPTM4B association with Hrs (Fig 7E), but not Nedd4 (Supplementary Fig S6A).

The data indicate a model where LAPTM4B inhibits Hrs-mediated EGFR degradation by promoting Hrs ubiquitination by Nedd4 (Fig 7F). To assess whether EGFR is selectively regulated by LAPTM4B, the consequence of LAPTM4B overexpression on the degradation of other receptors was examined. Neither hepatocyte growth factor (HGF)-induced degradation of c-Met nor SFLLRN-induced degradation of the G protein-coupled receptor PAR1 was inhibited by LAPTM4B overexpression (Supplementary Fig S6B–E). PAR1 degradation was accelerated in LAPTM4B-overexpressing cells. This indicates that LAPTM4B overexpression does not cause a general dysfunction of the lysosome or regulate the degradation of all receptors in the lysosome. These results are consistent with reports that Hrs is not essential for the degradation of c-Met or PAR1 (Gullapalli *et al*, 2006; Dores *et al*, 2012; Sun *et al*, 2013b), and suggest that LAPTM4B is selective for a subset of receptors sorted through Hrs.

## Discussion

Agonist-activated cell surface receptors continue to signal through the endosomal pathways after internalization, and sorting to ILVs is required for signal termination of many receptors (Katzmann *et al*, 2002; Raiborg *et al*, 2003; McCullough *et al*, 2013). Intraluminal sorting followed by degradation in the lysosome downregulates EGFR. LAPTM4B is an endosomal tetra-transmembrane protein that is overexpressed in most epithelial cancers, induces transformation and tumorigenesis of normal human cells, correlates with poor prognosis in a number of cancers, and is linked to chemotherapy resistance and recurrence of breast cancer (Kasper *et al*, 2005; Li *et al*, 2010b, 2011; Yang *et al*, 2010b). Here, we show that LAPTM4B enhances EGFR signaling by blocking EGF-stimulated EGFR intraluminal sorting and degradation. In this pathway, LAPTM4B promotes Hrs ubiquitination, which blocks the recognition of ubiquitinated EGFR by Hrs (Fig 7). These results establish a key mechanism by which the known oncoprotein LAPTM4B facilitates pro-survival signaling in cancers. Consistently, cells with higher LAPTM4B expression are more proliferative and migratory (Yang *et al*, 2010a; Li *et al*, 2011), and this may explain why overexpression of LAPTM4B in cancers correlates with poor prognosis.

Intraluminal sorting is a highly conserved biological process that has been extensively studied over the past decade. The crystal structures of multiple ESCRT subunits have been solved resulting in detailed models of how ESCRT subunits could be recruited to endosomes and sort receptor cargos onto the ILVs (Williams & Urbé, 2007; Raiborg & Stenmark, 2009; Hurley, 2010). The ESCRT-related ILV sorting components so far identified are all cytosolic proteins, the functions of which require their recruitment to endosomes (Henne *et al*, 2011). LAPTM4B is an example of an endosomal resident transmembrane protein with a role in the intraluminal sorting of EGFR. While other studies reported a block of EGFR trafficking at early endosomes upon dysfunction of early ESCRT subunits, we observed that LAPTM4B regulates EGFR sorting at LAPTM4B-positive endosomes, primarily MVEs/late endosomes (Figs 3 and 4). This is due to inhibition of Hrs function at LAPTM4B-positive MVEs (Supplementary Fig S5B) and explains why LAPTM4B-expressing cells show enhanced EGFR–LAMP1 colocalization in the absence of lysosome inhibitor as this reflects a block at MVEs. The results demonstrate that LAPTM4B binds PIPKIγi5 and is a PtdIns(4,5)$P_2$ effector. PIPKIγi5 and its product PtdIns(4,5)$P_2$ are required for EGFR intraluminal sorting (Sun *et al*, 2013b), but LAPTM4B inhibits this pathway, indicating that LAPTM4B adds a layer of control to EGFR signaling by controlling sorting to ILVs and EGFR degradation. Our results demonstrate an additional layer of control regulated by the unexpected role of PtdIns(4,5)$P_2$ in endosome sorting (Sun *et al*, 2013b). LAPTM4B is a phosphoinositide-modulated

**Figure 7. PIPKIγi5 Recruits SNX5 to Inhibit LAPTM4B Association with Hrs.**

A–C Co-immunoprecipitation (co-IP) of wild-type (WT) or C-terminal deletion mutants of HA-PIPKIγi5 with SNX5 (A) or LAPTM4B (B and C) in HEK293 cells cotransfected with indicated proteins. Δ675 indicates a deletion from amino acid 675 to the C-terminus.

D PIPKIγi5 promotes SNX5 association with LAPTM4B. HEK293 cells cotransfected with indicated proteins were harvested for co-IP to assay the interaction between Myc-SNX5 and Flag-LAPTM4B.

E SNX5 inhibits Hrs association with LAPTM4B. HEK293 cells cotransfected with indicated proteins were harvested for co-IP to assay the interaction between Myc-Hrs and Flag-LAPTM4B.

F A model for the PIPKIγi5–LAPTM4B pathway in EGFR trafficking. Hrs is an established key regulator of EGFR intraluminal sorting. LAPTM4B, by promoting Nedd4-mediated Hrs ubiquitination, inhibits the recognition of ubiquitinated EGFR by Hrs and therefore inhibits EGFR intraluminal sorting and lysosomal degradation. PIPKIγi5 directly binds to LAPTM4B and antagonizes the function of LAPTM4B in EGFR sorting by generating PtdIns(4,5)$P_2$ signals and recruiting SNX5.

Data information: Data are representative from at least three independent experiments.
Source data are available online for this figure.

 

resident endosomal transmembrane protein that may represent a protein family that functions to regulate receptor sorting by integrating with the ESCRT complexes that are recruited to endosomal surfaces.

The current understanding of ILV sorting is largely based on studies in yeast as most ESCRT subunits are conserved throughout evolution (Henne *et al*, 2011). Mammalian cells have evolved more complex ILV sorting systems that are less well defined. Our results expanded the understanding of the layer of regulation for EGFR ILV sorting in mammalian cells controlled by the PIPKIγi5–LAPTM4B interaction that is not conserved to yeast. The pro-survival role for human LAPTM4B depends on its N-tail (Shao *et al*, 2003), further emphasizing the importance of the LAPTM4B–PIPKIγi5 nexus in controlling EGFR signaling.

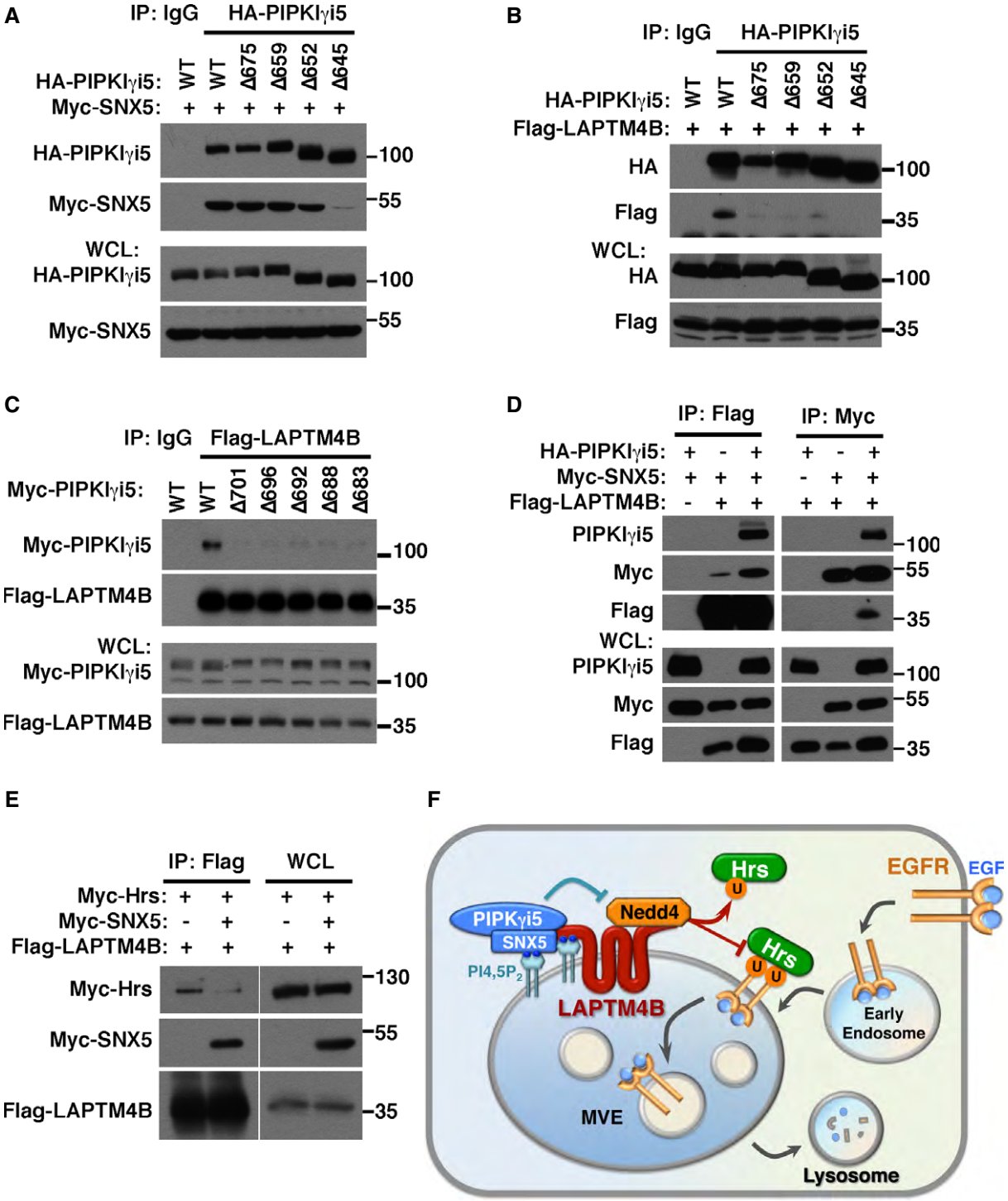


**Figure 7.**

Though the majority of PtdIns(4,5)$P_2$ is localized at the plasma membrane (Watt *et al*, 2002; Sun *et al*, 2013c), PtdIns(4,5)$P_2$ is also observed at the endosomal limiting membrane, ILVs, and lysosomes (Arneson *et al*, 1999; Watt *et al*, 2002; Vicinanza *et al*, 2011; Rong *et al*, 2012; Shi *et al*, 2012). A number of PtdIns(4,5)$P_2$ binding proteins including SNX5, SNX9, adaptor protein 3 (AP3), and a group of Arf-GAPs were found to regulate endosomal trafficking (Vicinanza *et al*, 2008). Endosomal PIPKIγi5 and its kinase activity modulate the function of SNX5, a PtdIns(4,5)$P_2$ and PI3P effector, in EGFR endosomal sorting (Sun *et al*, 2013b). Consistently, depletion or accumulation of PtdIns(4,5)$P_2$ at endosomes have been reported to impact EGFR sorting and degradation (Ramel *et al*, 2011; Vicinanza *et al*, 2011). Although LAPTM4B binds multiple phosphoinositides *in vitro*, yet its interaction with PIPKIγi5 combined with its membrane topography indicates that it is an effector of PtdIns(4,5)$P_2$ (Fig 7F). In contrast to SNX5, also a PtdIns(4,5)$P_2$ effector, LAPTM4B acts as an inhibitor of the ESCRT-mediated EGFR degradation, and PtdIns(4,5)$P_2$ binding diminishes its inhibitory role. Interestingly, while endogenous LAPTM4B is localized to both early and late endosomes, LAPTM4B regulates EGFR trafficking primarily at the late endosome, possibly due to inhibition of LAPTM4B functions by PIPKIγi5, PtdIns(4,5)$P_2$, and SNX5 at early endosomes. The results indicate that generation of PtdIns(4,5)$P_2$ modulates the interactions between SNX5, LAPTM4B, the ESCRT-0 subunit Hrs, and the E3 ubiquitin ligase Nedd4. This regulates the interaction of Nedd4 with its substrate Hrs. The ubiquitination of Hrs in turn controls its interaction with EGFR and subsequent ILV sorting of EGFR. LAPTM4B has two PY motifs, while Hrs has only one. LAPTM4B may interact with Nedd4 and orient it with Hrs so that Hrs is a better Nedd4 substrate, suggesting that LAPTM4B has a scaffold function that regulates Nedd4 activity toward Hrs. The LAPTM4B complex with PIPKIγi5 also suggests that it may regulate Nedd4 specificity toward other substrates in addition to Hrs.

Epidermal growth factor receptor and hundreds of other receptors are downregulated by ILV sorting and lysosomal degradation (Raiborg & Stenmark, 2009; Sorkin & von Zastrow, 2009), suggesting that there is receptor specificity for sorting and degradation. LAPTM4B is a selective regulator for EGFR trafficking, as it does not inhibit degradation of c-Met or PAR1 (Supplementary Fig S6B–E). PIPKIγi5 and SNX5 also selectively regulate the degradation of EGFR (Sun *et al*, 2013b). The role of PIPKIγi5, PtdIns(4,5)$P_2$, SNX5, and LAPTM4B putatively defines a pathway for selective and regulated sorting and destruction of receptors. The receptor preference for different ESCRT subunits and the cross talks between ESCRT complexes and LAPTM proteins as well as other endosomal transmembrane proteins including the tetraspanin family members may modulate this specificity.

Overexpression of LAPTM4B has been shown to enhance chemotherapy resistance in cancer cells, and this was associated with AKT activation (Li *et al*, 2010a,b). Phosphorylated AKT is a known locus for cancer multi-drug resistance (Radisavljevic, 2013). Our study indicates that LAPTM4B promotes AKT signaling by blocking EGFR degradation and this would be one mechanism for the role of LAPTM4B in drug resistance. The combined information suggests that LAPTM4B could be a potential therapeutic target for cancers that are addicted to EGFR signaling or in circumstances where chemotherapy resistance occurs.

## Materials and Methods

### Reagents, constructs, and cells

A detailed description of reagents, constructs, and cell treatment is included in Supplementary Materials and Methods. MDA-MB-231 and HEK293 cells were cultured in DMEM supplemented with 10% FBS. Transfection of plasmids and siRNA oligonucleotides was carried out using Lipofectamine 2000 and Oligofectamine (Invitrogen, Carlsbad, CA, USA), respectively, according to manufacturer's instructions.

### Immunofluorescence microscopy

Cells on glass coverslips were washed with PBS, fixed in 4% paraformaldehyde (PFA), and permeabilized in 0.5% Triton X-100 in PBS for 10 min, followed by blocking in 3% BSA for 1 h at room temperature. Incubation with primary antibodies was performed at 37°C for 2 h or 4°C over night. Then cells were washed twice in washing buffer (0.1% Triton X-100 in PBS) and incubated with secondary antibodies at room temperature for 30–60 min, followed by washing three times with washing buffer. Fluorescence images were obtained using MetaMorph with Nikon Eclipse TE2000-U microscope and further processed in MetaMorph. For colocalization quantification, images were background-subtracted and split into individual channels (e.g. channel 1 for EGFR; channel 2 for EEA1), and the colocalization quantification of signals from two individual channels was performed using the Coloc 2 plugin of Fiji (ImageJ). The thresholded Manders M1 coefficient was expressed as percentages (e.g. M1 = 0.3 was expressed as 30%) to show the fraction of intensities in channel 1 above threshold that is colocalized with intensities in channel 2 above threshold.

### Electron microscopy

For EGFR intraluminal sorting, control or LAPTM4B knockdown cells were starved, labeled with anti-EGFR, followed by protein A-gold labeling. Cells were then stimulated with EGF for 1 h at 37°C, fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), and processed for EM examination in UW Medical School EM facility. See Supplementary Materials and Methods for more details.

The specific localizations of LAPTM4B at endosomes were detected by silver-enhanced immuno-EM. Briefly, cells were fixed with 0.1% glutaraldehyde and 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 30 min, blocked in Aurion blocking solution for 1 h, followed by another 1 h of blocking with incubation buffer. Cells were then stained with LAPTM4B anti-sera, washed and incubated with ultra-small gold-conjugated goat anti-rabbit IgG overnight at 4°C. Cell were washed and post-fixed with 2% glutaraldehyde and then silver-enhanced. Ultra-thin sections were examined as described above. See Supplementary Materials and Methods for more details.

### Alexa-555-EGF degradation assays

MDA-MB-231 cells transfected with control or LAPTM4B siRNA were re-plated on to cover slips 48 h after transfection. Cells were

 

starved for 3 h and pulsed with 25 ng/ml Alexa-555-EGF for 3 min, and unbound EGF washed out by changing medium twice. Cells were fixed at indicated time points after Alexa-555-EGF stimulation. Fixed cells were incubated with DAPI for 2 min and washed three times with washing buffer. The total amount of Alexa-555-EGF fluorescence was quantified in Fiji, and the cell number on each image was counted using the nucleus (DAPI) staining as a reference. The average amount of Alexa-555-EGF per cell was calculated by dividing the total amount of Alexa-555-EGF fluorescence by the total cell number.

#### Intraluminal sorting of EGF

MDA-MB-231 cells on coverslips were transfected with EGFP-Rab5Q79L, starved for 4 h, and stimulated with 100 ng/mL Alexa-555-EGF for indicated time periods, followed by fixation in 4% PFA before fluorescence microscopy. Quantification of EGF on the limiting membrane and within the endosomal lumen was done as described (Trajkovic *et al*, 2008). Briefly, central images of endosomes (diameter $> 2$ μm) were taken with the GFP-Rab5Q79L outline as a reference. For quantification, the GFP-Rab5Q79L outline was also used as a reference to determine the EGF localization on the limiting membrane. EGF localized inside the GFP-Rab5Q79L outline was considered as intraluminal EGF. The total intensities of endosomal EGF fluorescence and the intensities inside the GFP-Rab5Q79L outline were quantified using Fiji. Calculation of significance is made by Student's *t*-test.

**Supplementary information** for this article is available online: http://emboj.embopress.org


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### Author contributions

XT and RAA conceived the study. XT, YS, ACH, and RAA designed experiments. XT, YS, YL, and NT performed experiments. XT, YS, ACH, and RAA analyzed data. XT and RAA wrote the manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest.

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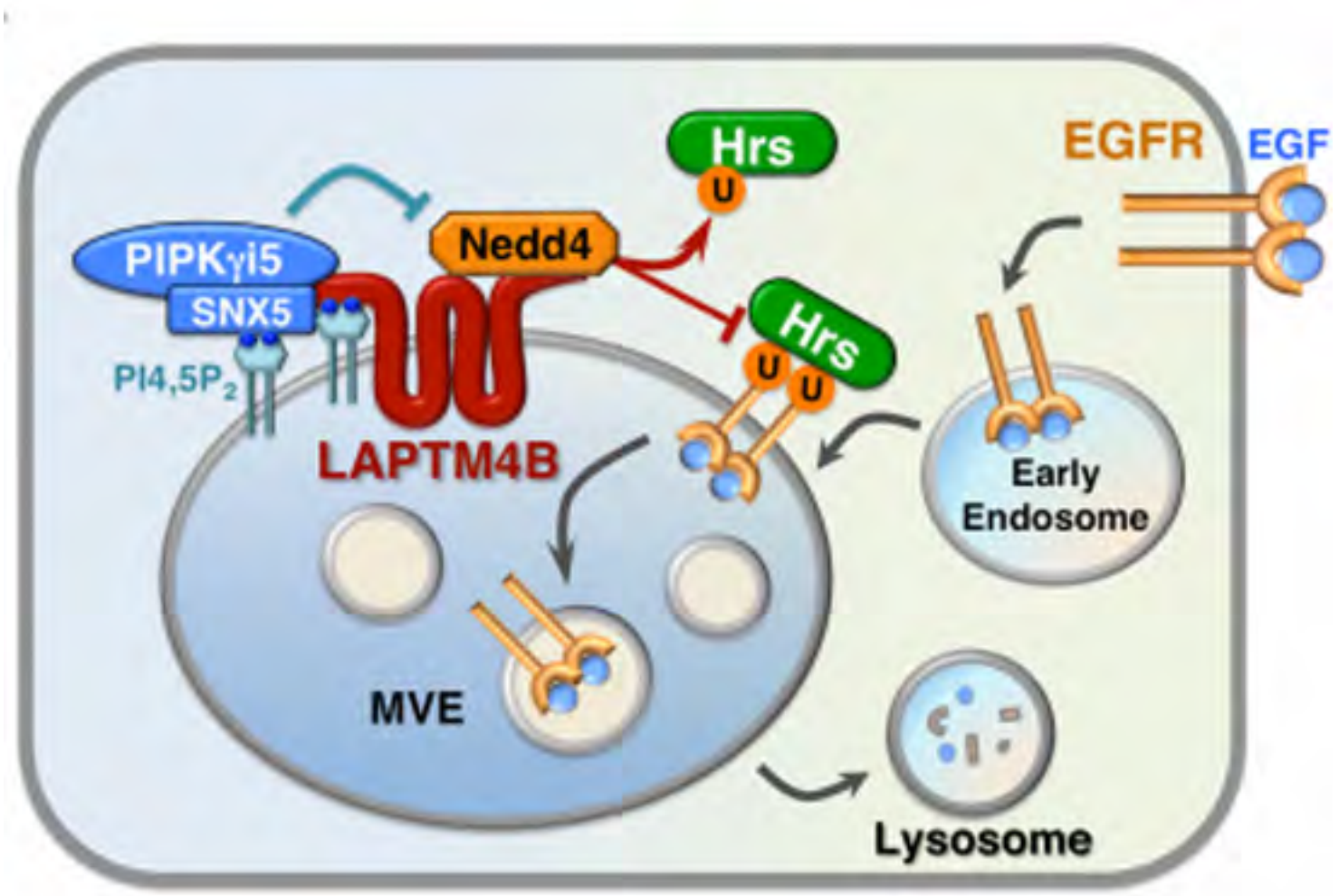
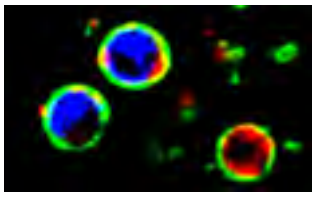
## Synopsis Abstract




The oncogene *LAPTM4B* prolongs EGFR signalling. LAPTM4B promotes ubiquitination of ESCRT component Hrs, preventing endosomal EGFR sorting, which is counteracted by phosphoinositide PtdIns(4,5)P2.

- LAPTM4B inhibits EGF stimulated EGFR intraluminal sorting and degradation and enhances EGFR signaling
- LAPTM4B promotes Hrs ubiquitination by the E3 ubiquitin ligase Nedd4
- LAPTM4B interacts with PIPKIγi5, an enzyme generating PtdIns(4,5)$P_2$ at endosomes
- LAPTM4B is a PtdIns(4,5)$P_2$ effector, and PtdIns(4,5)$P_2$ binding alleviates the inhibitory effect of LAPTM4B on EGFR degradation

# eToc



The oncogene *LAPTM4B* prolongs EGFR signalling. LAPTM4B promotes ubiquitination of ESCRT component Hrs, preventing endosomal EGFR sorting, which is counteracted by phosphoinositide PtdIns(4,5)P2.

**Subject Categories:** Membrane & Intracellular Transport; Signal Transduction

# SUPPLEMENTARY INFORMATION

## SUPPLEMENTARY MATERIALS AND METHODS

### Cell Culture, Transfection, Starvation, and EGF treatment

Cells were cultured in DMEM supplemented with 10% FBS at 37 °C and 5% $CO_2$. Transfection of plasmids was carried out using Lipofectamine 2000, and siRNA oligonucleotides were transfected using Oligofectamine or Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. For serum starvation, cells were incubated in starvation medium (0.1% BSA in DMEM) for indicated time periods. For EGF stimulation, cells were firstly starved, and then EGF was diluted in starvation medium and added to cell cultures.

### Reagents

EGF and Alexa-555-EGF were purchased from Invitrogen. The following antibodies were used: EEA1 (610457, BD Transduction Laboratories), LAMP1 (H4A3, Abcam), EGFR (WB: 610016, BD Transduction Laboratories; IP: LA1, Millipore; IF: E235, Millipore), pEGFR (pY1068, Abcam), AKT and pAKT (pY473, Cell Signaling), LAPTM4B (18895-1-AP, Proteintech), LAPTM4B anti-sera (Proteintech), LAPTM5 (3266.00.02, Strategic Diagnostics Inc.), Myc (4A6, Millipore), Flag (M2 and F7425, Sigma), HA (HA1.1, Abcam), Hrs (31-Q, Santa Cruz), Nedd4-1 (H-135, Santa Cruz), PAR1 (Thrombin R/ATAP2, sc-13503, Santa Cruz), Met (25H2, Cell signaling), GFP (Clones 7.1 and 13.1, Roche), β-Tubulin (H-235, Santa Cruz), Actin (C4, MP Biomedicals), Myc-HRP (9E11, Santa Cruz), T7-HRP (69048, Novagen), GST-HRP (RPN1236V, GE Healthcare). PIPKIγi5 antibody was homemade. DAPI (Sigma) was used for nuclear staining.

### Constructs

LAPTM4A, LAPTM4B, and LAPTM5 constructs were described previously. The sequence encoding LAPTM4B N-terminus was subcloned into pET28 and pET42 for expression in *E. coli*. PIPKIγ constructs were described previously (Schill and Anderson, 2009). LAPTM4B mutants were generated by PCR with primers containing the desired mutations. For lentivirus mediated expression of LAPTM4B, the cDNA of LAPTM4B was subcloned into pWPT vector. Polyclonal pools of infected cells were used for experimental analysis.

**siRNA**

siControl, 5'- AGG UAG UGU AAU CGC CUU G -3';

siPIPKIγi5, 5'- GGA UGG GAG GUA CUG GAU U -3';

siLAPTM4B#1 was a commercial pool from Santa Cruz (sc-77665) which was used in all experiments except in Figure S2D-F.

siLAPTM4B#2, 5'-GCG UCU GGU AUC UGA UCA U-3'.

**Immunoprecipitation and Immunoblotting**

Cells were lysed with lysing buffer containing 25 mM HEPES, pH 7.2, 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, and protease inhibitor cocktail. The lysates were centrifuged at 15,000 rpm for 15 min and supernatants were used for immunoprecipitation or immunoblotting. For immunoprecipitation, the lysates were incubated with indicated antibodies and protein-G-conjugated beads at 4 °C for 2 h. The beads were precipitated and washed with lysing buffer twice. The bound proteins were eluted in loading buffer containing 1% SDS, 1% 2-Mercaptoethanol and separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), and analyzed as indicated.

**Electron Microscopy**

EGFR intraluminal sorting was examined by EM as described (Hanafusa et al., 2011; Sun et al., 2013). Control or LAPTM4B siRNA treated cells were starved over night, incubated with anti-EGFR (LA22, Millipore) at 4 °C for 30 min, washed and followed by incubation at 4 °C for another 30 min with 10 nm protein A-gold (Electron Microscopy Sciences). Cells were then washed, stimulated with 100 ng/ml EGF for 1 h at 37 °C, fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB) and processed for EM examination in UW Medical School EM facility. The ultrasections were viewed using a Philips CM120 transmission electron microscope (FEI Company, Hillsboro, OR).

The specific localizations of LAPTM4B at endosomes were detected by silver-enhanced immuno-EM. Cells on coverslips with 90% confluency were fixed with 0.1 M PB containing 0.1% glutaraldehyde and 2% paraformaldehyde for 30 min. Cells were washed and residual aldehyde groups were inactivated with 0.1% $NaBH_4$ in PB for 10 minutes. Cells were then washed and permeabilized with 0.1% Triton-X 100 in PBS for 30 min, washed with PSB, blocked in Aurion blocking solution (Aurion, Wageningen, Netherlands) for 1 h, followed by another 1 h of blocking with incubation buffer (IB, 3% BSA, 0.1% Aurion BSA-c (Aurion) in PBS). Cells were then incubated with LAPTM4B anti-sera (1:2000 in IB, Proteintech) at room temperature for 2 hours, washed 6 $\times$ 10 min with IB, and incubated with ultra small gold conjugated goat anti-rabbit IgG (1:100 in IB, Aurion) overnight at 4°C. Cell samples were washed and post-fixed with 2% gluteraldehyde in PB for 30 minutes, washed and silver-enhanced for 2.5 h. Ultra-thin sections were examined as described above.

**FACS-based EGFR Recycling Assay**

EGFR recycling assay was performed as described by Raiborg (Raiborg et al., 2008) but the signal was quantified by FACS (Sigismund et al., 2008). MDA-MB-231 cells were transfected with control or LAPTM4B siRNA. 48 h after transfection cells were starved over night and then pretreated with 200 µg/ml cycloheximide for 1 h to inhibit new receptor synthesis and all the following steps were performed in the presence of 200 µg/ml cycloheximide. To measure the amount of total internalized EGFR, cycloheximide-pretreated cells were stimulated with 100 ng/ml Alexa-488-EGF for 15 min, washed, detached with FACS buffer (2% FBS in BSA) and fixed by adding an equal volume of 4% paraformaldehyde (PFA). The total amount of internalized Alexa-488-EGF represents the amount of total initially internalized EGFR. To measure EGFR recycling, cycloheximide-pretreated cells were firstly stimulated with 100 ng/ml non-labeled EGF for 15 min, washed and chased for 0 min, 30 min and 60 min to allow for EGFR recycling. Then cells were incubated with 100 ng/ml Alexa-488-EGF for 15 min, washed, detached and fixed as described above. The amount of Alexa-488-EGF taken up in these cells represents the amount of EGFR recycled after the initial stimulation. The geometric mean of EGF signals in each condition was analyzed by FACS. The recycling ratio was calculated as [ I(t) - I(0)] / [ I(total) - I(0)].

**GST Pull-Down Assay**

GST tagged LAPTM4B N-terminus purified from *E. coli* was immobilized on glutathione-agarose beads and incubated with T7-tagged PIPKIγi5 C-terminus (30 ng) in 1 ml binding buffer (1% Triton-X100 in PBS) at 4 °C for 1 h. The beads were washed five times with binding buffer. For GST pull-down with $PIP_2$ addition, the incubation was performed in PBS containing 0.04% of Triton-X100 and beads were then washed with 0.5% Triton-X100 in PBS four times. The bound protein complexes were eluted with loading buffer and analyzed by immunoblotting.

**PIP Strips Assay**

T7-tagged wild type or mutated LAPTM4B N-tail was purified from E. coli. For PIP strips assay. Briefly, the PIP strips membrane was blocked with 3% BSA in PBS-T (0.1% v/v Tween-20) at room temperature for 1 h and then incubated with 0.2 μg/ml purified LAPTM4B N-tail in PBS-T with 3% BSA for1 hours, followed by three washes 5 min each with PBS-T. The LAPTM4B N-tail bound on the membrane was detected by blotting with HRP-conjugated anti-T7 tag antibody.

**Liposome Binding Assay**

PolyPIPosomes (Echelon) containing 65 mol % phosphatidylcholine (PC), 29 mol % Phosphatidylethanolamine (PE), 1 mol % biotin-PE, and 5 mol % phosphatidylinositol (PI) were used for the liposome binding assay. The PIs used were Phosphatidylinositol (3) phosphate, Phosphatidylinositol (4) phosphate, Phosphatidylinositol (3,5) bisphosphate, and Phosphatidylinositol (4,5) bisphosphate. Control PolyPIPosomes containing 70% of PC, 29% PE, and 1% biotin-PE were used as a negative control. 10 ul of PolyPIPosomes were incubated with 10 ul of EZview™ Red Streptavidin Affinity Gel (Sigma-Aldrich) in 0.5 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2mM $MgCl_2$, 0.05% NP-40) with rotation for 1 h at room temperature (25 °C) to allow conjugation of PolyPIPosomes to the gel. Then 1 ug of purified T7-LAPTM4B-N-terminus was added with 0.1% of BSA and rotated for 10 min at room temperature. Samples were centrifuged at 6000 rpm for 10 s to precipitate the gel with bound PolyPIPosomes and T7-LAPTM4B-N-terminus, and supernatants were discarded. The gel was washed with 1 ml of binding buffer, centrifuged, and supernatants discarded. The wash step was repeated once more and the protein bound to gel was finally eluted with 100 ul of loading buffer and 20 ul of each sample was analyzed by Western blot with HRP-conjugated anti-T7 tag antibody.

**Ubiquitination Assay**

The in vivo ubiquitination of Hrs and EGFR was examined as described (Pan and Chen, 2003). Cells were transfected with His-tagged Ubiquitin DNA and the total ubiquitinated proteins were purified by $Ni^{2+}$-nitrilotriacetic acid (NTA) agarose. Cells were lysed in binding buffer (25 mM HEPES, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, and protease inhibitor cocktail) and incubated with $Ni^{2+}$-NTA agarose (Qiagen) for 2 hours at 4 °C. The agarose was washed with binding buffer, wash buffer A (8 M urea, 0.1 M $Na_2PO_4/NaH_2PO_4$, 0.01 M Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol), and wash buffer B (8 M urea, 0.1 M $Na_2PO_4/NaH_2PO_4$, 0.01 M Tris-HCl, pH 6.3, 10 mM β-mercaptoethanol), and proteins bound were eluted with elution buffer (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS). The eluted proteins were analyzed by Western blotting for Ubiquitinated Hrs or EGFR.

**Statistics**

Statistical significance was determined by Student *t*-test. Data were expressed as mean ± SD. Pearson correlation coefficient R was calculated in excel and the significance was determined by comparing the R values with the critical value table.

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## SUPPLEMENTARY FIGURE LEGENDS

### Supplementary Figure S1

### Endosomal Localization of LAPTM4B and PIPKIγi5.

A. Ectopically expressed LAPTM4B is primarily localized at the late endosome/lysosome. MDA-MB-231 cells transfected with HA-tagged LAPTM4B, and HA-LAPTM4B (red) was co-stained with EEA1, CD63, or LAMP1 (green), respectively.

B. The LAPTM4B siRNA specifically knocks down LAPTM4B but not LAPTM5. This also indicates that the LAPTM4B anti-sera specifically stain LAPTM4B. Control or LAPTM4B-siRNA transfected MDA-MB-231 cells were stained for endogenous LAPTM4B or LAPTM5.

C. Loss of LAPTM4B does not affect endosomal targeting of PIPKIγi5. Control or LAPTM4B siRNA transfected MDA-MB-231 cells were transfected with Myc-tagged PIPKIγi5. Cells were then co-stained for endogenous LAPTM4B (red) and Myc-PIPKIγi5 (green).

DAPI was used to stain the nuclei. Bar: 10 μm.

### Supplementary Figure S2

### LAPTM4B Inhibits EGF Stimulated EGFR Degradation.

A. Immunofluorescence images showing efficient knockdown of LAPTM4B in A431 cells. DAPI was used to stain the nuclei. Note: non-specific staining of the nuclei by LAPTM4B anti-sera. Boxes are selected regions for magnified view; Bar: 10 μm.

B. Knockdown of LAPTM4B accelerates EGFR degradation in A431 cells. Control or knockdown cells were starved, pretreated or not with 100 μM chloroquine (CQ), and stimulated with 20 ng/ml EGF for indicated time periods, and EGFR degradation were analyzed by Western blot.

C. Quantification of EGFR degradation in A431 cells without chloroquine pretreatment (mean ± SD, n = 3).

D. Chloroquine (CQ) treatment blocks EGF stimulated EGFR degradation in control and LAPTM4B-knockdown MDA-MB-231 cells.

E. Two different LAPTM4B siRNAs have similar efficiencies in knocking down LAPTM4B protein expression in MDA-MB-231 cells.

F. LAPTM4B knockdown by the second LAPTM4B siRNA (siLAPTM4B#2) also accelerates EGF stimulated EGFR degradation.

G. Quantification of EGFR levels normalized to Actin in Panel E (mean ± SD, n = 3).

**Supplementary Figure S3**

**LAPTM4B Knockdown does not affect EGF Stimulated EGFR Recycling.**

Control or LAPTM4B siRNA transfected cells were starved and processed for recycling assay measured by FACS. The “Total” Panel: total Alexa-488-EGF binding to cells before (blue) or after (red) 15 min of 100 ng/ml unlabeled EGF stimulation (pulse). Panels “30 min” and “60 min”: the amounts of Alexa-488-EGF binding to cells after 30 or 60 min of chase following the pulse. Data are representative from three independent experiments (see Supplementary Experimental Procedures).

**Supplementary Figure S4**

**The LAPTM4B-PIPKIγi5 Interaction.**

A. Co-staining of myc-LAPTM4B-6RQ (green) with EEA1 or LAMP1 (red) MDA-MB-231 cells. Boxes are selected regions for magnified view; Bar: 10 μm.

B. Washing the GST-pull down complex with 0.5% Triton-X100 in PBS in the presence or absence of $PI4,5P_2$ does not affect the $PI4,5P_2$ stimulation of the LAPTM4B-PIPKIγi5 interaction (mean ± SD, n = 3). L4B-N, LAPTM4B N-terminus; Iγi5-CT, PIPKIγi5 C-terminus; PI4,5P2, $PtdIns(4,5)P_2$, Phosphatidylinositol (4,5) bisphosphate.

**Supplementary Figure S5**

**LAPTM4B Interaction with Nedd4 and Colocalization with Hrs.**

A. Western blot showing that LAPTM4B knockdown does not affect ubiquitination levels of EGFR. Control or LAPTM4B knockdown MDA-MB-231 cells were transfected with 6xHis-tagged ubiquitin, starved and stimulated with 100 ng/ml EGF for 30 min. The ubiquitinated proteins were purified by Ni-NTA agarose and analyzed by Western blot. Ub: ubiquitin.

B. MDA-MB-231 cells were co-stained for endogenous LAPTM4B (red) and Hrs (green). Box is selected region for magnified view; Bar: 10 μm.

C. Co-immunoprecipitation of endogenous LAPTM4B and Nedd4 in MDA-MB-231 cells (left) or overexpressed Flag-LAPTM4B and HA-Nedd4 in HEK 293 cells (right).

D. Endosomal localization of the LAPTM4B-2PA mutant. MDA-MB-231 cells were stained for Myc-LAPTM4B-2PA (red) with EEA1 or LAMP1 (green). Bar: 10 μm.

**Supplementary Figure S6**

**LAPTM4B Overexpression Does not Inhibit Ligand Stimulated Degaradtion of c-Met or PAR1.**

A. SNX5 overexpression does not inhibit LAPTM4B interaction with Nedd4 in HEK 293 cells co-transfected with indicated proteins.

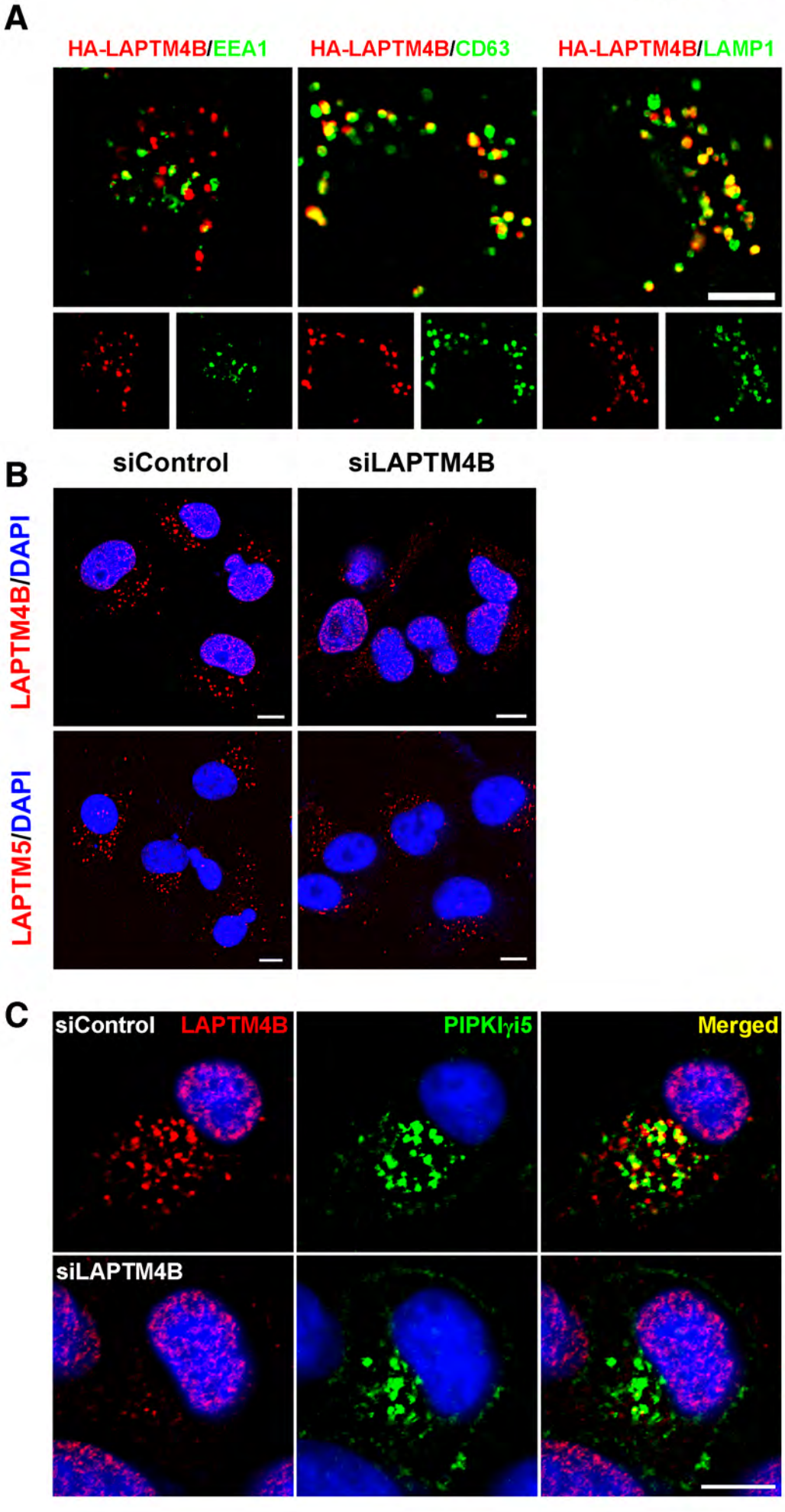
B. Western blot showing that LAPTM4B over-expression does not affect 50 ng/ml HGF-induced degradation of c-Met in MDA-MB-231 cells.

C. Quantification of c-Met levels normalized to Actin in control or LAPTM4B over-expressing cells (mean ± SD, n = 5).

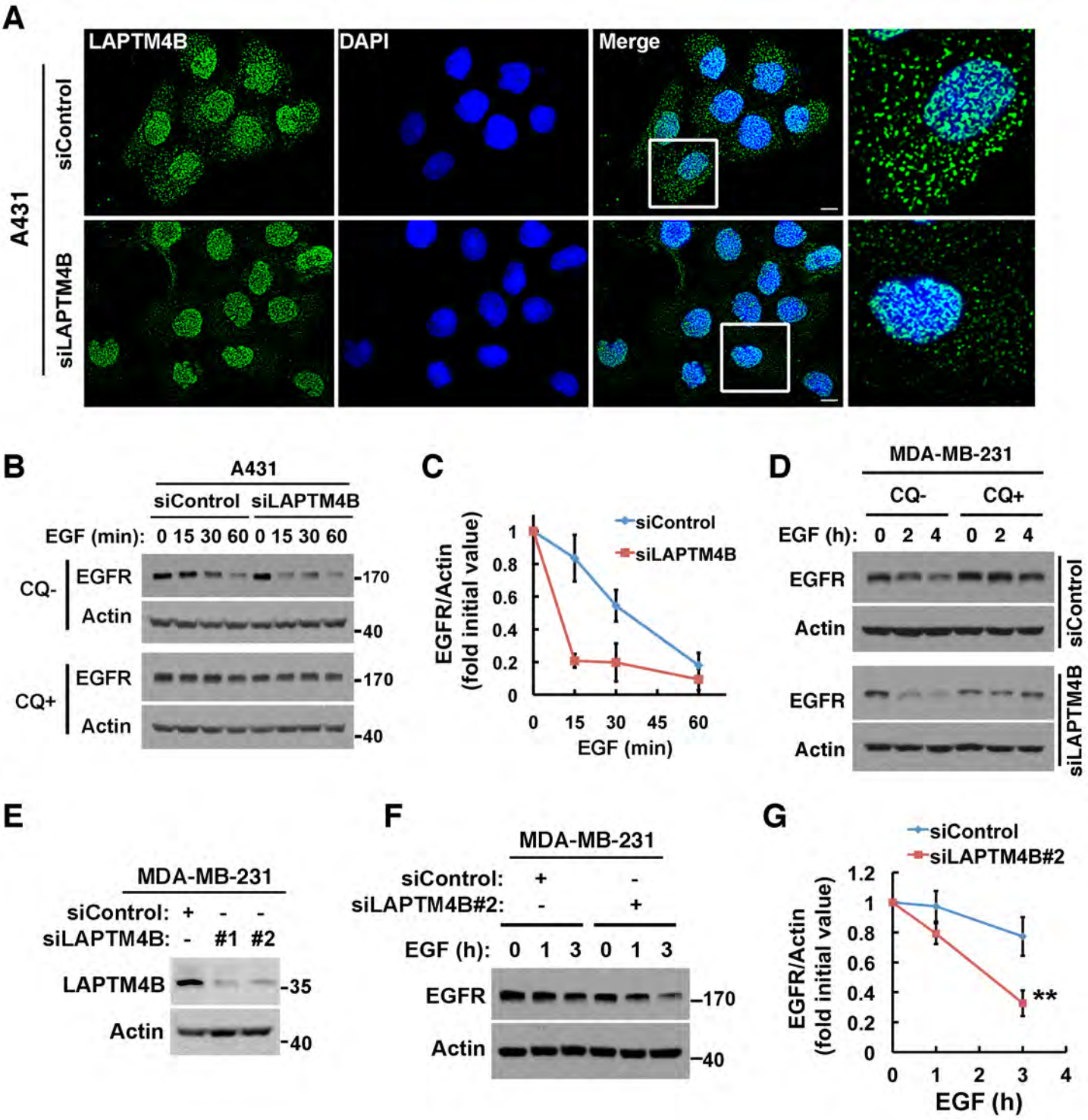
D. LAPTM4B over-expression does not inhibit 100 μM SFLLRN-induced degradation of PAR1 in MDA-MB-231 cells.

E. Quantification of PAR1 levels normalized to Actin in control or LAPTM4B over-expressing cells (mean ± SD, n = 5).

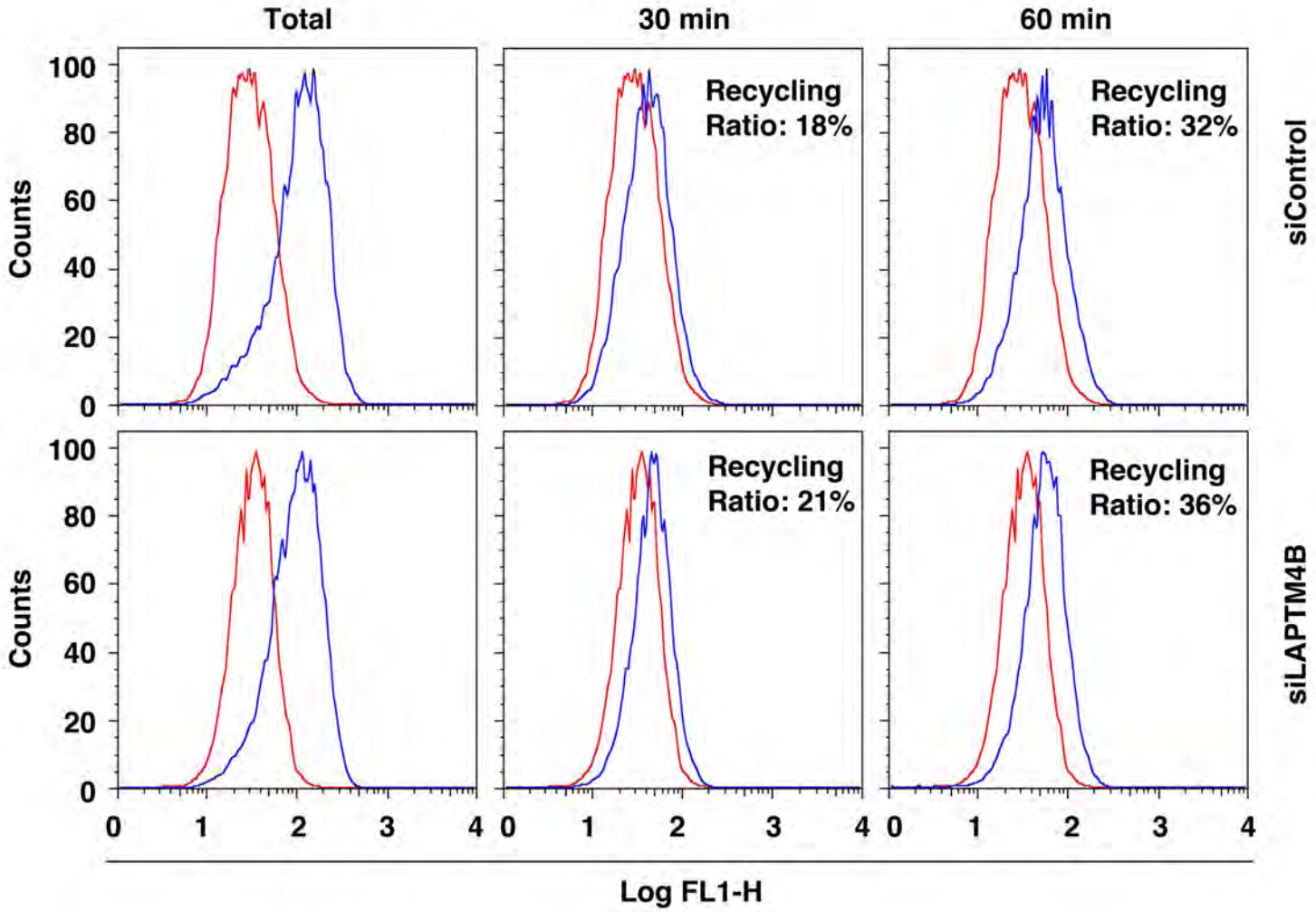
Supplementary Figure S1



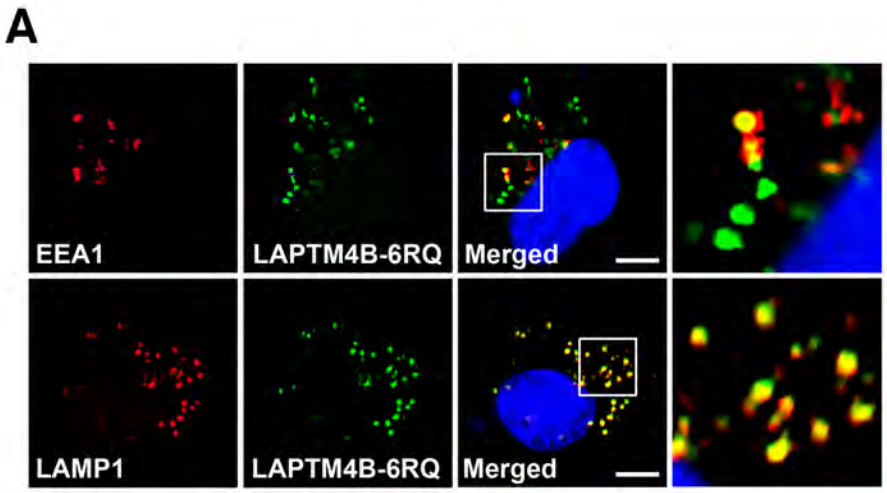
# Supplementary Figure S2

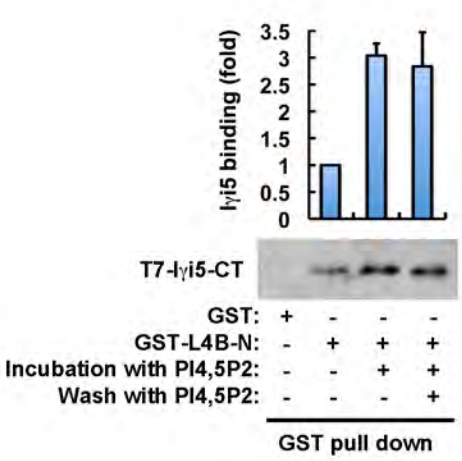


**Supplementary Figure S3**

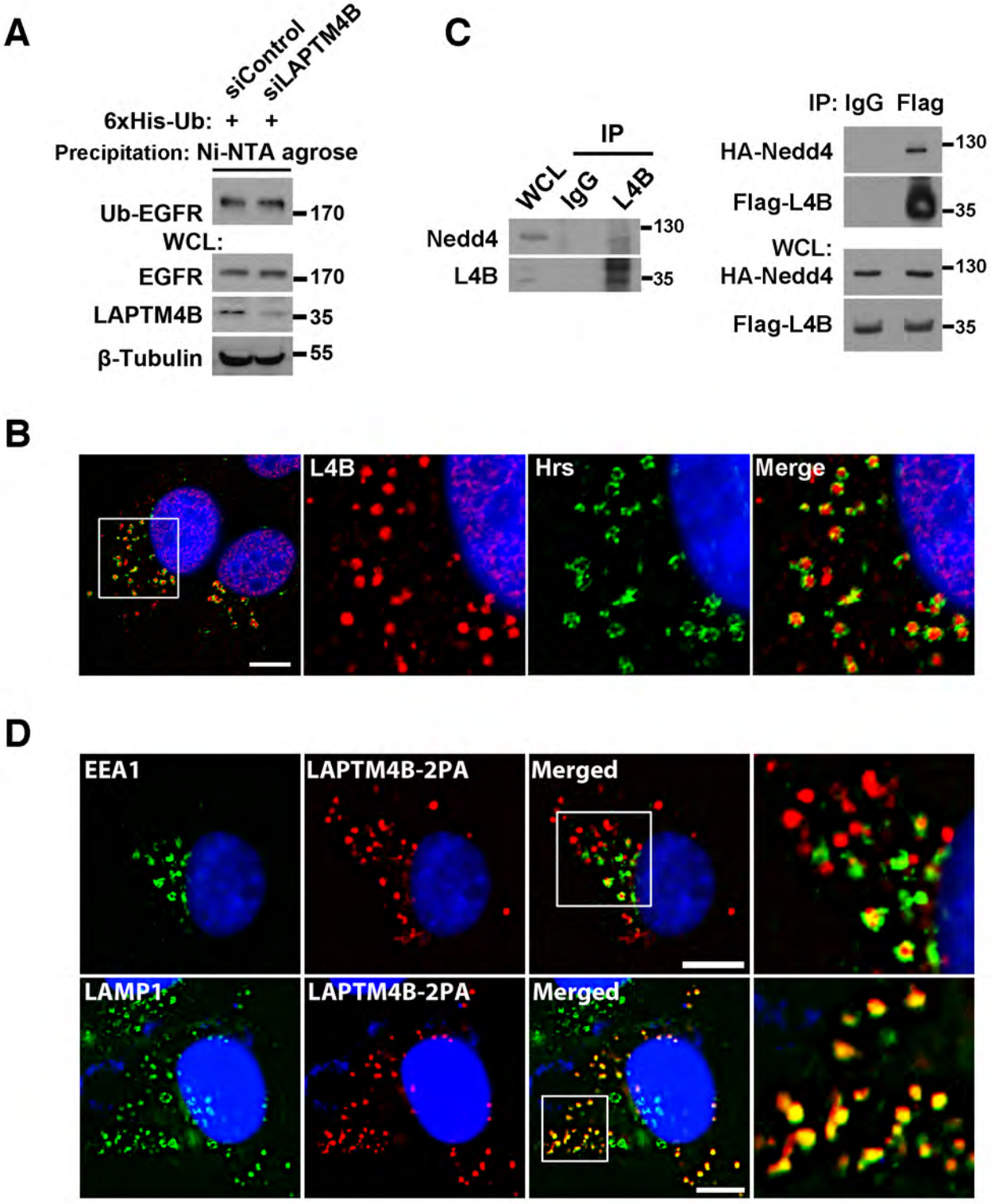


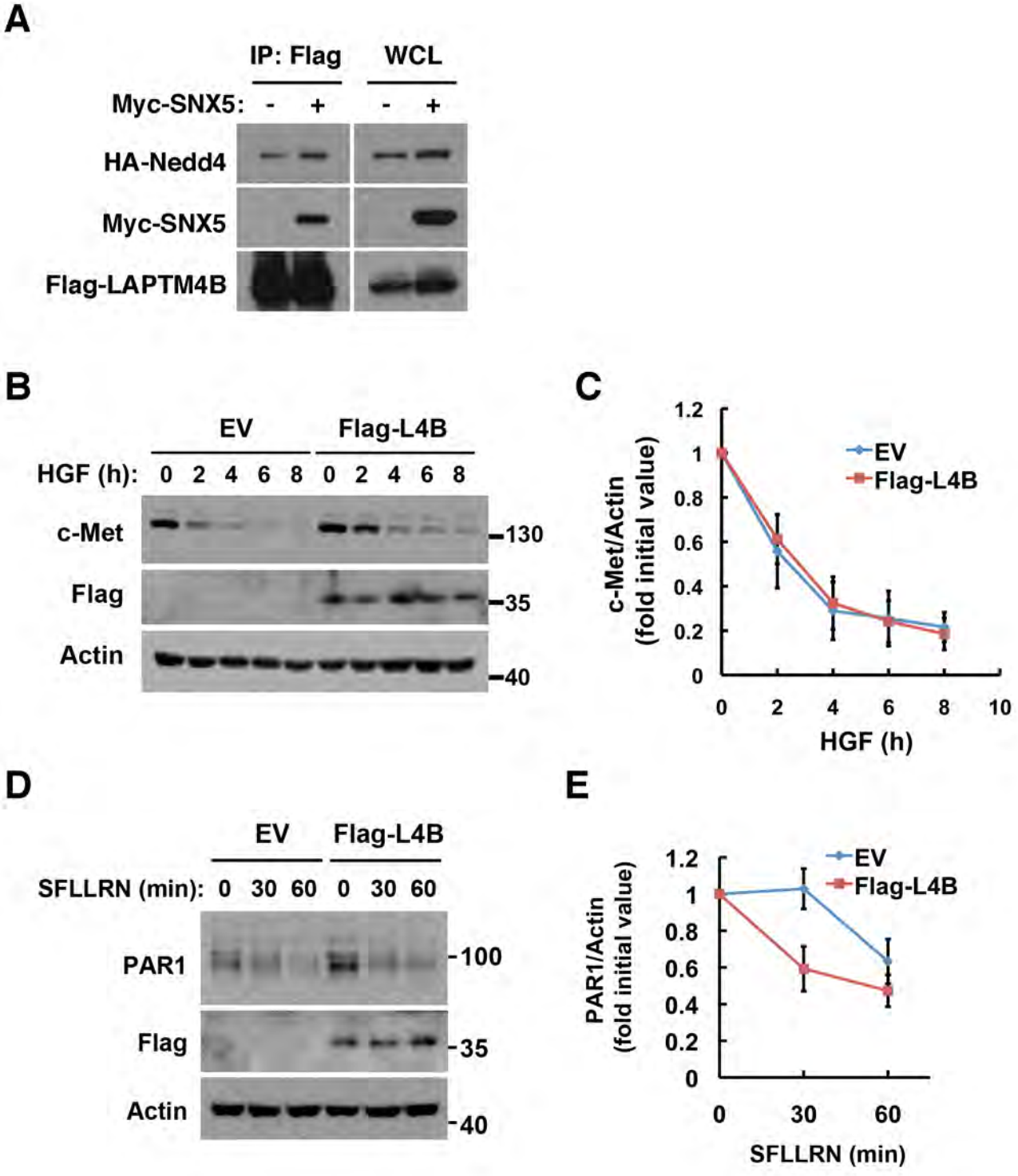
# Supplementary Figure S4



# Supplementary Figure S5




A
IP: Flag
WCL
Myc-SNX5: - + - +
HA-Nedd4
Myc-SNX5
Flag-LAPTM4B
B
EV
Flag-L4B
HGF (h): 0 2 4 6 8 0 2 4 6 8
c-Met
Flag
Actin
130
35
40
C
c-Met/Actin (fold initial value)
EV
Flag-L4B
HGF (h)
D
EV
Flag-L4B
SFLLRN (min): 0 30 60 0 30 60
PAR1
Flag
Actin
100
35
40
E
PAR1/Actin (fold initial value)
EV
Flag-L4B
SFLLRN (min)

THE EMBO JOURNAL

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# LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation

Xiaojun Tan, Yue Sun, Andrew C. Hedman, Narendra Thapa and Richard A. Anderson

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**Review timeline:**

| | |
|---|---|
| Submission date: | 10 July 2014 |
| Editorial Decision: | 07 August 2014 |
| Revision received: | 23 November 2014 |
| Editorial Decision: | 10 December 2014 |
| Revision received: | 11 December 2014 |
| Accepted: | 12 December 2014 |

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

*Editor: Andrea Leibfried*

1st Editorial Decision 07 August 2014

Thank you for submitting your manuscript entitled 'LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation'. I have now received the reports from all referees.

As you can see below, all referees find your manuscript potentially interesting. However, they raise various concerns and find that your conclusions are currently not sufficiently supported by the data provided. More specifically, the PIP2 binding to LAPTM4B has to be better supported and more insight into the LAPTM4B-Nedd4-Hrs interaction and the role of SNX5 in this context are needed. Referee #2 and #3 also raise various concerns regarding the quantitative imaging analysis of both EGFR trafficking and co-localization assays, which need to be resolved to allow further consideration here.

Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. Please contact me in case of further questions. I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address all concerns raised at this stage.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

 

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REFEREE COMMENTS

Referee #1:

This manuscript describes a novel function for the cancer biomarker LAPTM4B as negative regulator of endosomal sorting of EGFRs. The authors identified LAPTM4B as an interactor of the endosomal PIP kinase PIPKIgi5. They found that its overexpression inhibited EGF-induced endosomal sorting and lysosomal degradation of EGFRs whereas its depletion had the opposite effects. Mechanistically, it was found that LAPTM4B promotes ubiquitination of the ESCRT-0 subunit Hrs, a condition previously reported to attenuate the activity of Hrs, apparently by promoting interaction between Hrs and the E3 ubiquitin ligase Nedd4. The authors also found that the basic N-terminus of LAPTMB interacts with various phospholipids and that this was abolished by mutation of 6 arginine residues to glutamine. Interestingly, the 6RQ mutant was found to inhibit EGFR degradation even stronger than wild-type LAPTM4B, and the authors suggest that PI(4,5)P2, generated by PIPKIgi5, serves as negative regulator of LAPTM4B.

Overall this study contains excellent imaging and biochemical data, which are well supported by quantifications. Given the interest for LAPTM4B and EGFR in cancer, the present story should be of significant interest.

Major point:

The idea that LAPTM4B binds PI(4,5)P2, and that this is of functional importance, is not well supported by the lipid blot in Fig. 5F. Here, the strongest binding of LQPTM4B-N is to PE, PI3P and PI5P whereas binding to PI(4,5)P2 is barely detectable. The ability of PI(4,5)P2 to stimulate the LAPTM4B-PIKIgi5 interaction could therefore be different from the one proposed. Because PI(4,5)P2 binding is central in the proposed model, the authors should investigate further the binding between LAPTM4B and phospholipids using alternative assays.

Minor point:

Since Nedd4 is thought to bind directly to Hrs, it is not evident how LAPTM4B would enhance this interaction. Any further insight on this would be helpful.

Referee #2:

In this work the impact of LAPTM4B on EGFR endosomal sorting and degradation is investigated. A number of solid and straightforward experiments are conducted that cogently demonstrate that LAPTM4B is able to inhibit EGFR sorting into intraluminal vesicles (ILVs) of late endosomes/MVBs. Importantly, this function is inhibited by binding of LAPTM4B to PtdIns(4,5)P2 and PIPKIγi5, unveiling a fine-tuned regulation of the process. Interestingly, within cells population the authors were able to correlate high levels of LAPTM4B to the inhibition of EGFR sorting into ILVs in normal growing conditions, in absence of perturbations. Molecularly, the authors showed that LAPTM4B could facilitate Nedd4-Hrs binding, thus increasing Hrs ubiquitination. As a consequence, they suggest that Hrs is inhibited (via an intramolecular interaction between Ub and ubiquitin-interacting motif within the Hrs moiety), thus not any more able to recruit the EGFR for sorting and degradation into the late endosomes/MVBs.
Although this final model is not completely conclusive, the work is straightforward and the experiments are very well controlled and of technical high quality. The mechanism of LAPTM4B regulation by PtdIns(4,5)P2 and PIPKIγi5 is deeply dissected. Finally, the findings are novel and clarify how EGFR fate and signaling is regulated by LAPTM4B in physiological conditions; they may also help to better understand the basis for LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO after minor revision.

There are two points that need to be strengthened:

 

1) One critical issue is what is the fate of the EGFR that is not targeted to the intraluminal vesicles of MVBs due to inhibition by LAPTM4B. Authors measured EGFR recycling in a very indirect and not quantitative way, which actually cannot discriminate between alterations in recycling from differences in internalization rates. Authors need to provide a more quantitative and reliable assay to measure EGFR recycling, e.g. based on radioactive EGF ligand and/or FACS-based methods using EGFR antibodies to follow recycling of the receptor (see, for instance, Sorkin and Duex 2010; Sigismund et al., 2008).
2) A second issue concerns the mechanism of action of LAPTM4B on Nedd4 and Hrs ubiquitination. First, authors should provide more controls for the Hrs ubiquitination assay. Indeed, authors overexpress His-Ubiquitin but they did not show any western blot (WB) to control for equal level of transfection in the different samples. This control is mandatory. In addition, authors should confirm ubiquitination of Hrs in the opposite way, by immunoprecipitating Myc-Hrs and blotting anti-Ub (anti-His might be very dirty in WB, while for Ub different commercially available antibodies exist that work well in WB, e.g. P4D1 from Santa Cruz or FK2 from BIOMOL). Finally, in previous reports it was shown that LAPTM4B possesses PY motifs through which it can bind to Nedd4 (Milkereit and Rotin, 2011). This mechanism is not contemplated by the authors. However, it would be important to show that LAPTM4B mutant in PY motifs abolishes its binding to Nedd4 and, as a consequence, revert LAPTM4B phenotypes (i.e. increased Hrs ubiquitination and decreased EGFR degradation). This experiment would provide a final prove that LAPTM4B action on EGFR is via Hrs ubiquitination. Since Hrs has no PY motifs, one intriguing possibility -that can be discussed - might be that LAPTM4B serves as an adaptor/scaffold between Hrs and Nedd4, as shown for ARTs adaptors in Yeast and, more recently, for ARRDC3 in mammals (Nabhan et al., 2010).

Minor points:

Figure 2F. It is not clear what is required for. Authors compared two doses after different pulse and chase times. What are the conclusions? I would remove this panel (or put in the supplementary) since it does not add any valuable information.

Supplementary Figure S5 C-F. Authors try to show that LAPTM4B-dependent mechanism is specific for EGFR, while not applying to other receptors. However, data do not support this conclusion. In addition authors cite literature in support of the idea that degradation of c-Met does not require Hrs. However there are reports show the opposite (e.g. Hammond et al., 2003; Row et al., 2005; Abella et al., 2005). I strongly suggest to remove this part, which in my opinion does not add anything to the final message of the paper.

Referee #3:

In this manuscript Tan et al. describe a novel function to the lysosomal protein, LAMTM4B, namely, the regulation of intraluminal sorting of the activated EGF receptor (EGFR).
The authors show that LAMTM4B overexpression delays the degradation of EGF receptors and prolongs its signaling activity. Conversely, LAMTM4B depletion accelerated the termination of EGF signaling and EGFR degradation. The authors also demonstrated a direct interaction between PIPKIγ isoform 5 and LAMTM4B N-terminus that appears to be regulated by PI(4,5)P2. They showed that the membrane-adjacent polybasic domain of the LAMTM4B at the N-terminus associates with endosomal PtdIns(4,5)P2, which stabilizes the PIPKIγi5-LAMTM4B interaction.
To explain the mechanism of the LAMTM4B effect on EGF sorting, the authors showed that LAMTM4B acts as a negative regulator of Hrs-driven EGFR intraluminal sorting at the MVB. This is mediated by the interaction of LAMTM4B with Nedd4 ubiquitin ligase with enhanced ubiquitination of Hrs, thus inhibiting its interaction with the ubiquitinated EGFR.
This is an important study, which describes a novel mechanism by which LAPTM4B inhibits EGFR sorting, possibly explaining the role of LAPTM4B as a tumorigenic protein upregulated in various cancers.
The strength of this study is its solid biochemical data. However, its weakness is the microscopy analysis in which many important conclusions are based on heavily processed and difficult to interpret images. In particular, most micrographs showing co-localization of various molecules

 

appear to be overinterpreted (most critical of them listed below). Moreover, some of the findings seem to be inconsistent with the authors' general conclusions.
There is also a need for clarification of some methods used for quantification and statistical analysis of experiments throughout the manuscript (most significant ones listed below).
Major points:

1. Figure 1 E and F panels are of poor quality and show only marginal co-localization of LAPTM4B with either LAMP1 or the PIP5KIγi5 in F. These panels clearly do not support the authors' statement that "LAPTM4B is initially sorted to the limiting membrane of MVEs and then partially sorted onto ILVs as the MVE matures". This statement may be supported by the EM pictures, but without some sort of additional markers it is difficult to judge whether the two panels indeed represent late or early MVEs. The number of internal vesicles may not be enough to make this distinction.
2. The localization of the endosomal markers to one side of the nucleus is very peculiar. Endosomes usually populate the whole cell except the recycling compartment, which is pericentrosomal.
3. PIP5KIγi5 was previously shown by the authors to interact with sorting nexin 5 (SNX5) and regulate Hrs binding to EGFR and sorting into intralumenal vesicles during MVB maturation. Given that both LAPTM4B and SNX5 bind PIP5KIγi5 and PtdIns(4,5)P2 but exert opposing effects on EGFR sorting and degradation, how does the availability of LAPTM4B for PIP5KIγi5 affect SNX5 function? Do the two proteins compete for PIP5KIγi5, such that overexpression of LAPTM4B and its knockdown affect SNX5 binding to PIP5KIγi5? While this issue was briefly mentioned in the Discussion, this Reviewer finds it important to address this issue experimentally (under the LAPTM4B or SNX5 overexpression and/or knockdown conditions)
4. The promiscuity of lipid binding by the N-terminus of LAPTM4B does not show a particularly strong PI(4,5)P2 preference, in fact mono-PIs are stronger binders and the PI3P binding may suggest that PI3P would occupy this site in the cells. In light of this finding it is really curious how the PI(4,5)P2 specificity is achieved at the level of PIP5Kgi5-CT interaction.
5. Fig. 2E shows enhanced rate of disappearance of EGF-Alexa555 fluorescence in LAPTM4B-depleted cells. This is interpreted by the authors as a result of enhanced degradation of EGFR that complements their biochemical data. However, in a similar experiment, a higher concentration of EGF-Alexa555 was added to cells preincubated with EGF. Here, LAPTM4B depleted cells have less EGF-Alexa555 signal (same as in 2F), but in this case the authors interpret this as an EGFR recycling defect (based on the fact that cells were pre-incubated with EGF). It is necessary to support these conclusions with the use of proper markers of recycling endosomes and/or recycling cargos such as transferrin. Furthermore, the authors show and quantify "recycled EGFR" although these samples only show EGF (without staining against the receptor). Why we never see any EGFR staining in the plasma membrane? The recycling experiments are really confusing in the way they are presented.
6. The arguments built around data shown in Fig. 3 are quite unconvincing. There is an increased co-localization of EGFR with EEA1 after LPTM4B knock-down and a decrease in co-localization with LAMP1. The authors argue that this is due to an accelerated degradation. Why should enhanced degradation increase the retention of the receptor in the EEA1 compartment? In control si cells there is no effect of chloroquine treatment on EEA1 retention of the receptor (120 min), so lysosomal degradation does not seem to have an impact on EGFR trafficking through the early endosomal compartment.
7. How was the EGFR co-localization with EEA1 and LAMP1 in Fig. 3A-D quantified? How did the authors arrive to the number "% EGFR colocalized with EEA1"? There is no mention of the co-localization analysis used and what does this scale represent. This is especially relevant as none of the picture show a full cell only a subset next to the nucleus.
8. Does the quantification shown in Fig. 3F represent analysis of many cells from a single experiment?
9. The effects of chloroquine on EGFR degradation should be also demonstrated biochemically.
10. In Fig. 4C and D how did the authors distinguish early and late MVEs for their quantification? Is it again based on the number of internal vesicles?
11. In experiments with constitutively active Rab5-what was the criteria used to determine the "% luminal EGF"?
12. It is strange that Hrs shows almost perfect co-localization with LAPTM4B (Fig. 6E) when it shows no co-localization with EEA1. Hrs and EEA1 are known to be in largely overlapping compartments. How can this apparent discrepancy be explained?
13. Figure 6I does not show what the authors describe in the text: wild-type L4B does not have an effect on EGFR degradation contrary to what the authors state. Also, these effects are smaller and in

 

the case of pAKT are questionable. These experiments are supposed to be mimicking more "physiological" overexpressions, yet the effects are marginal at best.
14. Similarly, the authors state that there is no effect of L4B overexpression on PAR1 degradation (Fig. S5E and F). The Figure shows otherwise.
15. I am not sure the model accounts for all the findings. For example, how would the 6RQ mutant be more potent in its biological effects when it cannot interact with the lipid and cannot recruit the PIP5K? Would the PIP5K recruitment induce a positive feed-back loop that does increase recruitment, more PIP2, more recruitment etc? What would break this cycle?
Minor points:
1. LAPTM4B localization is interchangeably referred to as lysosomal, endosomal and late-endosomal throughout the manuscript.
2. Nedd4 should be added to the model shown in Fig. 7 as it was shown in this study to play an integral role in LAPTM4B regulation of Hrs ubiquitination and EGFR degradation.

1st Revision - authors' response 23 November 2014

Response to the Referees' Comments

*Referee #1:*

*This manuscript describes a novel function for the cancer biomarker LAPTM4B as negative regulator of endosomal sorting of EGFRs. The authors identified LAPTM4B as an interactor of the endosomal PIP kinase PIPKIgi5. They found that its overexpression inhibited EGF-induced endosomal sorting and lysosomal degradation of EGFRs whereas its depletion had the opposite effects. Mechanistically, it was found that LAPTM4B promotes ubiquitination of the ESCRT-0 subunit Hrs, a condition previously reported to attenuate the activity of Hrs, apparently by promoting interaction between Hrs and the E3 ubiquitin ligase Nedd4. The authors also found that the basic N-terminus of LAPTMB interacts with various phospholipids and that this was abolished by mutation of 6 arginine residues to glutamine. Interestingly, the 6RQ mutant was found to inhibit EGFR degradation even stronger than wild-type LAPTM4B, and the authors suggest that PI(4,5)P2, generated by PIPKIgi5, serves as negative regulator of LAPTM4B. Overall this study contains excellent imaging and biochemical data, which are well supported by quantifications. Given the interest for LAPTM4B and EGFR in cancer, the present story should be of significant interest.*

We thank the reviewer for the supportive comments and have addressed the specific comments below.

*Major point:*

*The idea that LAPTM4B binds PI(4,5)P2, and that this is of functional importance, is not well supported by the lipid blot in Fig. 5F. Here, the strongest binding of LAPTM4B-N is to PE, PI3P and PI5P whereas binding to PI(4,5)P2 is barely detectable. The ability of PI(4,5)P2 to stimulate the LAPTM4B-PIKIgi5 interaction could therefore be different from the one proposed. Because PI(4,5)P2 binding is central in the proposed model, the authors should investigate further the binding between LAPTM4B and phospholipids using alternative assays.*

We have now added liposome-binding assay to test phosphoinositide binding of the LAPTM4BN-terminus (L4B-N). Consistent with the PIP strips data (Figure 5F), L4B-N also binds to multiple phosphoinositides including PtdIns(4,5)P2 in liposome-binding assay (Figure 5J). It is important to note that *in vitro* binding affinity does not necessarily correlate with functional relevance. For example, sorting nexin 5 (SNX5) binds all the phosphoinositides including PI3P with higher affinity than with PtdIns(4,5)P2 in liposome-binding assay, but both PI3P and PtdIns(4,5)P2 equally promote the SNX5 interaction with Hrs in vitro (Sun et al, 2013). Another example is the IQGAP1-C-terminus that binds most of other phosphoinositides with much higher affinity than with PtdIns(4,5)P2, but only PtdIns(4,5)P2 specifically inhibited the intramolecular interaction within the IQGAP1-C-terminus (Choi et al, 2013), likely due to a specific conformational change induced by PtdIns(4,5)P2 binding. Both SNX5 and IQGAP1 interact with

 

PIP 5-kinases that generate PtdIns(4,5)P2 that would be spatially generated in close proximity. Thus, it is not surprising that the L4B-N interaction with PIPKIγi5-C tail is specifically stimulated by PtdIns(4,5)P2, as PIPKIγi5 generates PtdIns(4,5)P2. It is also important to note that the Nterminus
in the full length LAMPT4B is tethered to the membrane interface and this may not only change the specificity of the interaction with phosphoinositides but would constrain and orient the association of the PtdIns(4,5)P2 generated by the associated PIPKIγi5 (see Figure 7F).

*Minor point:*

*Since Nedd4 is thought to bind directly to Hrs, it is not evident how LAPTM4B would enhance this interaction. Any further insight on this would be helpful.*

This is an important point that we have now discussed in the revised manuscript. It is likely that without LAPTM4B the direct interaction between Nedd4 and Hrs is weak, and LAPTM4B may interacts with both of them changing their structures so that they bind with higher affinity. Or alternatively, LAPTM4B may function as a scaffold to link Nedd4 and Hrs, as LAPTM4B has two PY motifs (L/PPXY) while Hrs has only one (Figure R1). See also comment 2 from Reviewer 2.

MGRGSGTFERLLDKATSQLLLETDWESILQICDLIRQGDTQAKYAVNSIKKKVNDKNPHVALYALEVMES
VVKNCGQTVHDEVANKQTMEELKDLLKRQVEVNVRNKILYLIQAWAHAFRNEPKYKVVQDTYQIMKVEGH
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SVSMGYQPYNMQNLMTTLPSQDASLPPQQPYIAGQQPMYQQMAPSGGPPQQQPPVAQQPQAQGPPAQGSE
AQLISFD

Figure R1. Human Hrs sequence (GenBank: BAA23366.1) withthe PY motif highlighted.

*Referee #2:*

*In this work the impact of LAPTM4B on EGFR endosomal sorting and degradation is investigated. A number of solid and straightforward experiments are conducted that cogently demonstrate that LAPTM4B is able to inhibit EGFR sorting into intraluminal vesicles (ILVs) of late endosomes/MVBs. Importantly, this function is inhibited by binding of LAPTM4B to PtdIns(4,5)P2 and PIPKIγi5, unveiling a fine-tuned regulation of the process. Interestingly, within cells population the authors were able to correlate high levels of LAPTM4B to the inhibition of EGFR sorting into ILVs in normal growing conditions, in absence of perturbations. Molecularly, the authors showed that LAPTM4B could facilitate Nedd4-Hrs binding, thus increasing Hrs ubiquitination. As a consequence, they suggest that Hrs is inhibited (via an intramolecular interaction between Ub and ubiquitin-interacting motif within the Hrs moiety), thus not any more able to recruit the EGFR for sorting and degradation into the late endosomes/MVBs.*
*Although this final model is not completely conclusive, the work is straightforward and the experiments are very well controlled and of technical high quality. The mechanism of LAPTM4B regulation by PtdIns(4,5)P2 and PIPKIγi5 is deeply dissected. Finally, the findings are novel and clarify how EGFR fate and signaling is regulated by LAPTM4B in physiological conditions; they may also help to better understand the basis for LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO after minor revision.*

We thank this reviewer for the positive comments.

*There are two points that need to be strengthened:*
*1) One critical issue is what is the fate of the EGFR that is not targeted to the intraluminal vesicles of MVBs due to inhibition by LAPTM4B. Authors measured EGFR recycling in a very indirect and not quantitative way, which actually cannot discriminate between alterations in recycling from differences in internalization rates. Authors need to provide a more quantitative and reliable assay to measure EGFR recycling, e.g. based on radioactive EGF ligand and/or*

 

*FACS-based methods using EGFR antibodies to follow recycling of the receptor (see, for instance, Sorkin and Duex 2010; Sigismund et al., 2008).*

This is an important point. We now have performed FACS-based EGFR recycling assay in control and LAPTM4B knockdown cells. The data reveal that EGFR recycling was not affected upon loss of LAPTM4B (Figure S3), indicating that LAPTM4B inhibition of EGFR intraluminal sorting does not promote EGFR recycling, consistent with prolonged EGFR retention at LAPTM4B positive endosomes in LAPTM4B expressing cells (Figures 3E and 3F). These combined data support that the LAPTM4B-promoted EGFR signaling comes from endosomes instead of the plasma membrane. In fact, endosomes are emerging as an essential site for receptor tyrosine kinase signaling (Murphy et al, 2009).

*2) A second issue concerns the mechanism of action of LAPTM4B on Nedd4 and Hrs ubiquitination. First, authors should provide more controls for the Hrs ubiquitination assay. Indeed, authors overexpress His-Ubiquitin but they did not show any western blot (WB) to control for equal level of transfection in the different samples. This control is mandatory. In addition, authors should confirm ubiquitination of Hrs in the opposite way, by immunoprecipitating Myc-Hrs and blotting anti-Ub (anti-His might be very dirty in WB, while for Ub different commercially available antibodies exist that work well in WB, e.g. P4D1 from Santa Cruz or FK2 from BIOMOL). Finally, in previous reports it was shown that LAPTM4B possesses PY motifs through which it can bind to Nedd4 (Milkereit and Rotin, 2011). This mechanism is not contemplated by the authors. However, it would be important to show that LAPTM4B mutant in PY motifs abolishes its binding to Nedd4 and, as a consequence, revert LAPTM4B phenotypes (i.e. increased Hrs ubiquitination and decreased EGFR degradation). This experiment would provide a final prove that LAPTM4B action on EGFR is via Hrs ubiquitination. Since Hrs has no PY motifs, one intriguing possibility -that can be discussed - might be that LAPTM4B serves as an adaptor/scaffold between Hrs and Nedd4, as shown for ARTs adaptors in Yeast and, more recently, for ARRDC3 in mammals (Nabhan et al., 2010).*

These are important and constructive comments. We have now added blots for HA-Ub levels in the whole cell lysates (Figures 6B and 6C). We believe assaying Hrs ubiquitination by immunoprecipitating (IP) Hrs has its intrinsic difficulties, because other ubiquitinated proteins would be co-IP'ed with Hrs interfering with the detection of Hrs ubiquitination, and denatured IP would on the other hand compromise the IP efficiency. Additionally, while by purifying ubiquitinated proteins the mono-ubiquitinated Hrs (mono-Ub-Hrs) was clearly detected as the major band of Ub-Hrs (Figures 6B and 6C), by IP Hrs and blotting with anti-Ubiquitin (or anti-HA) the band corresponding to mono-Ub-Hrs was not detectable, possibly because the mono-Ub-Hrs was not IP'ed by the antibody.
We have generated the PY motif mutant for LAPTM4B (P296/312A, or 2PA) and assessed the effects of this mutant on EGFR degradation and signaling. As shown in Figure 6I, LAPTM4B-2PA lost Nedd4 interaction, but showed normal Hrs interaction, with enhanced interaction with PIPKIγi5. In support of a requirement for the LAPTM4B-Nedd4 interaction in LAPTM4B function, we observed inhibition of EGFR degradation in LAPTM4B-WT overexpressing cells but no inhibition in LAPTM4B-2PA mutant overexpressing cells (Figures 6J and 6K).

*Minor points:*

*Figure 2F. It is not clear what is required for. Authors compared two doses after different pulse and chase times. What are the conclusions? I would remove this panel (or put in the supplementary) since it does not add any valuable information.*

This panel shows how much of total EGFR is internalized to endosomes and tracked for degradation in Figure 2E. We compared the two EGF stimulation conditions to get a sense of what percentage of EGFR is internalized after a 3 min pulse of 25 ng/ml Alexa-555-EGF in Figure 2E, assuming that continuous stimulation with 100 ng/ml EGF for 15 min induces accumulation of most EGFR at endosomes. The result indicates that under the condition used in Figure 2E, only around 10% of receptor is internalized. Thus, the data in Figures 2E and 2G represent the degradation of only a very small pool of EGF stimulated EGFR. We have clarified

 

our interpretations of these experiments in the revised manuscript.

*Supplementary Figure S5 C-F. Authors try to show that LAPTM4B-dependent mechanism is specific for EGFR, while not applying to other receptors. However, data do not support this conclusion. In addition authors cite literature in support of the idea that degradation of c-Met does not require Hrs. However there are reports show the opposite (e.g. Hammond et al., 2003; Row et al., 2005; Abella et al., 2005). I strongly suggest to remove this part, which in my opinion does not add anything to the final message of the paper.*

We thank the reviewer for bringing these papers into our attention. We noticed that the initial work by Hammond et al., 2003 reported a "modest retardation" of c-Met degradation in Hrs knockdown cells, and the other two papers in 2005 only suggested an involvement of Hrs in c-Met signaling. However, we did not observe a change of c-Met degradation upon knockdown of Hrs or PIPKIγi5, or upon LAPTM4B overexpression in MDA-MB-231 cells. We think both our data and others' are consistent and we would like to retain these data in the supplementary figures, but we have changed the wording from "not required" into "not essential", the latter of which we believe is more appropriate.
The PAR1 data is an important control. First, though EGFR degradation is blocked upon LAPTM4B overexpression, PAR1 degradation is actually accelerated in LAPTM4B overexpressing cells, which rules out the possibility that LAPTM4B overexpression causes a general dysfunction of the lysosome or blocks the broad degradation of receptors in the lysosome. Second, it is known that PAR1 degradation does not require PAR1 ubiquitination or Hrs binding. In fact, PAR1 directly associates with an ESCRT-3 subunit for lysosomal targeting (Dores et al, 2012; Gullapalli et al, 2006). Thus, these data provide an additional control that LAPTM4B inhibits EGFR degradation by inhibiting Hrs but not downstream ESCRT subunits.

*Referee #3:*

*In this manuscript Tan et al. describe a novel function to the lysosomal protein, LAMTM4B, namely, the regulation of intraluminal sorting of the activated EGF receptor (EGFR). The authors show that LAMTM4B overexpression delays the degradation of EGF receptors and prolongs its signaling activity. Conversely, LAMTM4B depletion accelerated the termination of EGF signaling and EGFR degradation. The authors also demonstrated a direct interaction between PIPKIγ isoform 5 and LAMTM4B N-terminus that appears to be regulated by PI(4,5)P2. They showed that the membrane-adjacent polybasic domain of the LAMTM4B at the N-terminus associates with endosomal PtdIns(4,5)P2, which stabilizes the PIPKIγi5-LAMTM4B interaction.To explain the mechanism of the LAMTM4B effect on EGF sorting, the authors showed that LAMTM4B acts as a negative regulator of Hrs-driven EGFR intraluminal sorting at the MVB. This is mediated by the interaction of LAMTM4B with Nedd4 ubiquitin ligase with enhanced ubiquitination of Hrs, thus inhibiting its interaction with the ubiquitinated EGFR. This is an important study, which describes a novel mechanism by which LAPTM4B inhibits EGFR sorting, possibly explaining the role of LAPTM4B as a tumorigenic protein upregulated in various cancers. The strength of this study is its solid biochemical data. However, its weakness is the microscopy analysis in which many important conclusions are based on heavily processed and difficult to interpret images. In particular, most micrographs showing colocalization of various molecules appear to be overinterpreted (most critical of them listed below). Moreover, some of the findings seem to be inconsistent with the authors' general conclusions. There is also a need for clarification of some methods used for quantification and statistical analysis of experiments throughout the manuscript (most significant ones listed below).*

We thank the reviewer for recognizing the importance and strength of this study. We have addressed the specific comments below with additional experiments and editing clarifications.

*Major points:*

*1. Figure 1 E and F panels are of poor quality and show only marginal co-localization of LAPTM4B with either LAMP1 or the PIP5KIγi5 in F. These panels clearly do not support the authors' statement that "LAPTM4B is initially sorted to the limiting membrane of MVEs and then partially sorted onto ILVs as the MVE matures". This statement may be supported by the EM*

*pictures, but without some sort of additional markers it is difficult to judge whether the two panels indeed represent late or early MVEs. The number of internal vesicles may not be enough to make this distinction.*

The comments regarding LAPTM4B localizations are important (see also comment 12). We have put much effort into re-examining the localization of endogenous LAPTM4B and have quantified the colocalization. This reveals that ~45% of LAPTM4B is colocalized with EEA1 and ~60% with LAMP1. On the other hand, ~60% of either EEA1 or LAMP1 is colocalized with LAPTM4B (Figures 1 E and 1F). These data indicate that although less LAPTM4B is colocalized with EEA1, the majority of either EEA1 or LAMP1 compartments are LAPTM4B positive. This is consistent with partial LAPTM4B colocalization with PIPKIγi5 and Hrs. We agree that the immunofluorescence (IF) data alone do not support the sentence pointed out by the reviewer due to resolution limit of the IF method. However, the IF data provided important controls for the EM, as they clearly showed LAPTM4B localization to both EEA1 and LAMP1 compartments. It is also important to note that multivesicular endosomes (MVEs) are defined on the morphological observation of small intraluminal vesicles (ILVs) (50-80 nm diameter) and the morphological differences between early and late MVEs are the number of ILVs. Thus, we believe additional markers for EM are not necessary.

*2. The localization of the endosomal markers to one side of the nucleus is very peculiar. Endosomes usually populate the whole cell except the recycling compartment, which is pericentrosomal.*

In MDA-MB-231 cells the endosomes are always well polarized on one side of the nucleus (see Figure R2).

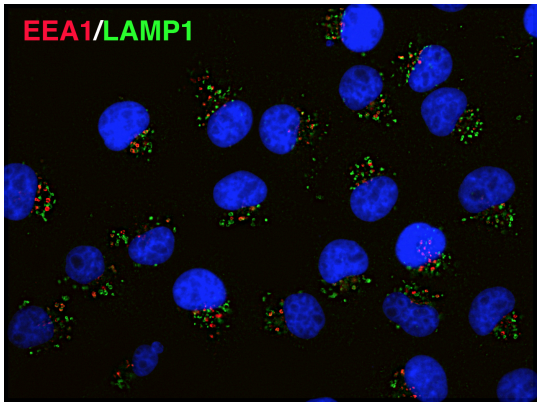


Figure R2. Co-staining of EEA1 and LAMP1 in MDA-MB-231 cells.

*3. PIP5KIγi5 was previously shown by the authors to interact with sorting nexin 5 (SNX5) and regulate Hrs*
*binding to EGFR and sorting into intraluminal vesicles during MVB maturation. Given that both LAPTM4B and SNX5 bind PIP5KIγi5 and PtdIns(4,5)P2 but exert opposing effects on EGFR sorting and degradation, how does the availability of LAPTM4B for PIP5KIγi5 affect SNX5 function? Do*
*the two proteins compete for PIP5KIγi5, such that overexpression of LAPTM4B and its knockdown affect SNX5 binding to PIP5KIγi5? While this issue was briefly mentioned in the Discussion, this Reviewer finds it important to address this issue experimentally (under the LAPTM4B or SNX5 overexpression and/or knockdown conditions)*

We have explored the interaction of PIPKIγi5 with LAPTM4B and SNX5. To narrow down the interaction regions for LAPTM4B and SNX5 on the C-tail of PIPKIγi5, a series of C-terminal deletion mutants of PIPKIγi5 were generated and used in co-immunoprecipitation experiments to characterize interactions with LAPTM4B or SNX5. The data indicate that LAPTM4B interaction required the very C-terminal part of the PIPKIγi5 C-tail, while SNX5 interaction required the Nterminal
part of the C-tail (Figures 7A-C), suggesting that LAPTM4B and SNX5 may not

 

compete for PIPKIγi5 interaction. In fact, overexpression of PIPKIγi5 promoted the interaction between LAPTM4B and SNX5 (Figure 7D). We previously showed that SNX5 inhibits Hrs ubiquitination by excluding Nedd4 recruitment to Hrs (Sun et al, 2013). As LAPTM4B facilitates the Hrs-Nedd4 association, it is likely that SNX5 interacts with LAPTM4B to inhibit LAPTM4B function. To test this, we examined if SNX5 inhibits LAPTM4B interaction with Nedd4 or Hrs. And we found that SNX5 overexpression strongly suppressed LAPTM4B interaction with Hrs (Figure 7E) but not Nedd4 (Figure S6A). Our combined data support that PIPKIγi5 inhibits the function of LAPTM4B by both generating PtdIns(4,5)P2 and recruiting SNX5 (Figure 7F).

*4. The promiscuity of lipid binding by the N-terminus of LAPTM4B does not show a particularly strong PI(4,5)P2 preference, in fact mono-PIs are stronger binders and the PI3P binding may suggest that PI3P would occupy this site in the cells. In light of this finding it is really curious how the PI(4,5)P2 specificity is achieved at the level of PIP5Kgi5-CT interaction.*

See also discussion above for Reviewer 1. We have performed liposome-binding assay to test lipid binding of the LAPTM4B N-terminus. This reveals that, similar to PIP strips assay (Figure 5F), LAPTM4B N-terminus also binds multiple phosphoinositides including PtdIns(4,5)P2 in liposome binding assay (Figure 5J). Binding affinity does not always correlate with functional relevance. SNX5 and IQGAP1 are another two examples that are functionally regulated by PtdIns(4,5)P2 but bind all phosphoinositides in liposome binding assays (Choi et al, 2013; Sun et al, 2013). In addition, as PtdIns(4,5)P2 is the product of PIPKIγi5 (Schill & Anderson, 2009), this also explains the specificity of PtdIns(4,5)P2 in regulating the LAPTM4B-PIPKIγi5 interaction.

*5. Fig. 2E shows enhanced rate of disappearance of EGF-Alexa555 fluorescence in LAPTM4B depleted cells. This is interpreted by the authors as a result of enhanced degradation of EGFR that complements their biochemical data. However, in a similar experiment, a higher concentration of EGF-Alexa555 was added to cells preincubated with EGF. Here, LAPTM4B depleted cells have less EGF-Alexa555 signal (same as in 2F), but in this case the authors interpret this as an EGFR recycling defect (based on the fact that cells were pre-incubated with EGF). It is necessary to support these conclusions with the use of proper markers of recycling endosomes and/or recycling cargos such as transferrin. Furthermore, the authors show and quantify "recycled EGFR" although these samples only show EGF (without staining against the receptor). Why we never see any EGFR staining in the plasma membrane? The recycling experiments are really confusing in the way they are presented.*

The EGF-Alexa555 degradation assay monitored EGF signal after 1-3 h. In the original EGFR recycling assay cells were pre-incubated with EGF to internalize EGFR and the new synthesis of EGFR was inhibited by cycloheximide. After allowing for recycling for 1 h, the binding of EGFAlexa555 (15 min treatment) to the cells reflects the amount of EGFR recycling (Raiborg et al, 2008). However, the original quantification method has its limitation, and we have re-examined EGFR recycling more quantitatively by FACS, as raised by reviewer 2. The new data indicate that loss of LAPTM4B does not affect EGFR Recycling rate (Figure S3), supporting that LAPTM4B enhances endosomal EGFR signaling, consistent with EGFR stabilization at LAPTM4B positive endosomes (Figure 3E).

*6. The arguments built around data shown in Fig. 3 are quite unconvincing. There is an increased co-localization of EGFR with EEA1 after LPTM4B knock-down and a decrease in colocalization*
*with LAMP1. The authors argue that this is due to an accelerated degradation. Why should enhanced degradation increase the retention of the receptor in the EEA1 compartment? In control si cells there is no effect of chloroquine treatment on EEA1 retention of the receptor (120 min), so lysosomal degradation does not seem to have an impact on EGFR trafficking through the early endosomal compartment.*

We realized that the LAPTM4B knockdown phenotype here is quite unique, as most previous studies showed enhanced EGFR-EEA1 colocalization after a block of EGFR at early endosomes. However, it is important to note that the increased colocalization of EGFR with EEA1 does not always mean enhanced EGFR retention in early endosomes; in the case of LAPTM4B knockdown, it in fact reflects less EGFR in LAMP1 compartment due to accelerated

 

lysosomal delivery and subsequent degradation.
At 120 min, in control cells, only 37% of EGFR is degraded, but in siLAPTM4B cells, ~75% of EGFR is lost (Figure 2). This may explain why chloroquine treatment had a more evident effect on the EGFR-EEA1 colocalization in LAPTM4B-knockdown cells than in control cells.

*7. How was the EGFR co-localization with EEA1 and LAMP1 in Fig. 3A-D quantified? How did the authors arrive to the number "% EGFR colocalized with EEA1"? There is no mention of the co-localization analysis used and what does this scale represent. This is especially relevant as none of the picture show a full cell only a subset next to the nucleus.*

We have now clarified the methods for colocalization analysis in the revised manuscript. Images were background subtracted and splitted into individual channels (eg. channel 1 for EGFR; channel 2 for EEA1), and the colocalization quantification of signals from two individual channels was performed using the Coloc 2 plugin of Fiji (ImageJ). The threshoulded Manders M1 coefficient was expressed as percentages (eg. M1 = 0.3 was expressed as 30%) to show the fraction of intensities in channel 1 above threshold that is colocalized with intensities in channel 2 above threshold.
We did not intentionally show only a subset of a cell instead of a full cell in Figures 3A and 3C. Because the EGFR (and EEA1/LAMP1) signals are highly concentrated at endosomes in all of these conditions, we could not see additional signals outside of the endosome-rich region, as exemplified in Figure 3E. For images of each condition in Figures 3A and 3C, we have shown all the visible signals of one representative cell. Showing more background region (purely black) does not add any further information.

*8. Does the quantification shown in Fig. 3F represent analysis of many cells from a single experiment?*

Yes, this is one representative analysis out of three independent experiments. We have now clarified this in the figure legends.

*9. The effects of chloroquine on EGFR degradation should be also demonstrated biochemically.*
We initially had a control that chloroquine blocks EGFR degradation in A431 cells (Figure S2B, bottom). We have now added the same control for MDA-MB-231 cells in Figure S2D.

*10. In Fig. 4C and D how did the authors distinguish early and late MVEs for their quantification? Is it again based on the number of internal vesicles?*

The quantification is based on all MVEs since LAPTM4B is targeted to both early and late MVEs. All endosomes with gold particles and internal vesicles are included.

*11. In experiments with constitutively active Rab5-what was the criteria used to determine the "% luminal EGF"?*

Quantification of EGF on the limiting membrane and within the endosomal lumen was done as described (Trajkovic et al, 2008). Central images of endosomes (diameter > 2 μm) were taken with the GFP-Rab5Q79L outline as a reference. For quantification, the GFP-Rab5Q79L outline was also used as a reference to determine the EGF localization on the limiting membrane. EGF localized inside the GFP-Rab5Q79L outline was considered as intraluminal EGF. The total intensities of endosomal EGF fluorescence and the intensities inside the GFP-Rab5Q79L outline were quantified in ImageJ.

*12. It is strange that Hrs shows almost perfect co-localization with LAPTM4B (Fig. 6E) when it shows no co-localization with EEA1. Hrs and EEA1 are known to be in largely overlapping compartments. How can this apparent discrepancy be explained?*

This is a very important point that we have now fully addressed. Clearly, Hrs and EEA1 are largely overlapping, which has also been confirmed on our hands. The observation that most Hrs also co-localizes with LAPTM4B encouraged us to re-consider the localization of LAPTM4B. We have now re-examined and quantified LAPTM4B colocalization with EEA1 and

 

LAMP1 (Figures 1E and 1F). Both our previous and current data indicate that while only part of LAPTM4B colocalizes with EEA1, most EEA1 endosomes are positive for LAPTM4B, indicating that LAPTM4B targets to both early and late endosomes. This is consistent with partial LAPTM4B colocalization with PIPKIγi5 and Hrs. See also Comment 1.

*13. Figure 6I does not show what the authors describe in the text: wild-type L4B does not have an effect on EGFR degradation contrary to what the authors state. Also, these effects are smaller and in the case of pAKT are questionable. These experiments are supposed to be mimicking more "physiological" overexpressions, yet the effects are marginal at best.*

The effects of LAPTM4B overexpression on EGFR degradation and signaling were shown in Figures 2H-K. However, robust overexpression of either WT or 6RQ LAPTM4B had too strong effects on EGFR degradation, which totally masked the differences between WT and the mutant. Thus, we compared two pools of cells that overexpress more physiological levels of LAPTM4B-WT or -6RQ for their effects on EGFR degradation and signaling. For this, single clones were selected and the expression levels of ectopic LAPMT4B were examined by staining with anti-Flag. Low expression clones were selected and combined into a polyclonal pool for either LAPTM4B-WT or 6RQ. We have now clarified this in the updated figure legends.

*14. Similarly, the authors state that there is no effect of L4B overexpression on PAR1 degradation (Fig. S5E and F). The Figure shows otherwise.*

We clarified it as “did not inhibit” but not that there was “no effect”. In fact, LAPTM4B overexpression accelerated PAR1 degradation. We have now described this result more clearly in the revised manuscript.

*15. I am not sure the model accounts for all the findings. For example, how would the 6RQ mutant be more potent in its biological effects when it cannot interact with the lipid and cannot recruit the PIP5K? Would the PIP5K recruitment induce a positive feed-back loop that does increase recruitment, more PIP2, more recruitment etc? What would break this cycle?*

The current study and our previously work indicate that PIPKIγi5 and PIP2 are negative regulators of LAPTM4B. In this scenario, the 6RQ mutant, lacking PIPKIγi5 and PIP2 binding, showed enhanced activity in promoting Hrs-Nedd4 association, increasing Hrs ubiquitination and inhibiting EGFR degradation. We have now improved the description of our model with additional clarifications, including the explanation of Nedd4 in this pathway (as suggested by this reviewer in Minor point 2).
We appreciate the comment regarding the positive feedback loop for PIPKIγi5 recruitment to LAPTM4B. However, we do not have a clear answer to this question now. Both PIPKIγi5 and LAPTM4B are posttranslational modified. Possibly, upstream signals may lead to posttranslational modifications of PIPKIγi5 and/or LAPTM4B that would accelerate or block this positive feedback loop. This is one of our future directions that are being explored for another manuscript.

*Minor points:*

*1. LAPTM4B localization is interchangeably referred to as lysosomal, endosomal and late*10 *endosomal throughout the manuscript.*

Based on the quantification of LAPTM4B localization (see comment 1), we have now consistently used “endosomal” throughout the manuscript.

*2. Nedd4 should be added to the model shown in Fig. 7 as it was shown in this study to play an integral role in LAPTM4B regulation of Hrs ubiquitination and EGFR degradation.*

We have now added this.

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Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brugger B, Simons M (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319(5867): 1244-1247

2nd Editorial Decision 10 December 2014

Thank you for submitting the revised version of your manuscript entitled 'LAPTM4B is a PtdIns(4,5)P2 Effector that Regulates EGFR Signaling, Lysosomal Sorting and Degradation'. I have now received the reports from all referees and I am happy to inform you that they support publication pending minor amendments to the manuscript.

As you will see below, referee #3 has two remaining concerns that I would like to ask you to address:
1) Referring to a luminal localization of LAMPTM4B in lysosomes should be restricted to Figure 1I.
2) To address the second point of this referee regarding figure 3, I suggest that you amend the text of your manuscript and/or figure legend to better explain and outline what the observed data suggest. For this, it might be good to explain how the analysis was performed for Figures 3B and D - I assume that you analyzed all EGFR punctae present under each condition (= 100%) for co-localization with EEA1/Lamp1 (= depicted %). Maybe you could mention at this point again that the total EGFR levels (and punctae?) are reduced upon LAPTM4B knock-down.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we have received the final version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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REFEREE COMMENTS

Referee #1:

The authors have successfully addressed the concerns I had. In my opinion, this revised manuscript is well suited for publication in EMBO Journal.

 

Referee #2:

The authors have addressed all major concerns I have raised in the first round of revision. As a minor point, I still think that data in supplementary Figure S6 on Met and Par1 are not totally convincing and I suggest to remove them. Nonetheless, I believe that the work is straightforward and the molecular mechanism is deeply dissected, helping to clarify how EGFR fate and signaling are regulated at the sorting station by LAPTM4B/Nedd4/Hrs interplay. Importantly, this may represent also the basis to rationalize LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO J.

Referee #3:

The authors have addressed most of my critical comments and provided extra explanations and clarifications. I still found a few issues that were not properly addressed in my opinion. However, these may not reach a level of dissatisfaction that would warrant holding the paper back.

-P7, Luminal localization of LAPTM4B cannot be judged in panels E and H, so this statement should be referred to Fig.1I.

-This reviewer still does not understand the explanation of the results shown in Fig. 3B and D and the authors response to this comment did not make it more understandable.

2nd Revision - authors' response 11 December 2014

*Referee #1:*

*The authors have successfully addressed the concerns I had. In my opinion, this revised manuscript is well suited for publication in EMBO Journal.*

We thank this reviewer for support of publication.

*Referee #2:*

*The authors have addressed all major concerns I have raised in the first round of revision. As a minor point, I still think that data in supplementary Figure S6 on Met and Par1 are not totally convincing and I suggest to remove them. Nonetheless, I believe that the work is straightforward and the molecular mechanism is deeply dissected, helping to clarify how EGFR fate and signaling are regulated at the sorting station by LAPTM4B/Nedd4/Hrs interplay. Importantly, this may represent also the basis to rationalize LAPTM4B overexpression in cancer. For this reasons I suggest publication in EMBO J.*

We thank this reviewer for support of publication. We have agreed with all of this reviewer's excellent comments and suggestions, except that we respectfully disagree with the reviewer on the removal of the data in Figure S6. We have replicated this data many times and clearly LAPTM4B or the PIPKIγi5 pathway do not regulate sorting of these two receptors. This shows a degree of receptor specificity and that the endosomal pathway is not disrupted generally and is important to be presented here.

*Referee #3:*

*The authors have addressed most of my critical comments and provided extra explanations and clarifications. I still found a few issues that were not properly addressed in my opinion. However, these may not reach a level of dissatisfaction that would warrant holding the paper back.*

We thank this reviewer for support of publication.

 

*-P7, Luminal localization of LAPTM4B cannot be judged in panels E and H, so this statement should be referred to Fig.1I.*

We have now only referenced the luminal localization of LAPTM4B to Fig. 1I.

*-This reviewer still does not understand the explanation of the results shown in Fig. 3B and D and the authors response to this comment did not make it more understandable.*

We have added more details to the figure legends and result description to improve our explanations of these data. Details regarding co-localization quantification are included in the Materials and Methods section of the main text.

 